Roles of DNA repair factors in the regulation of the gene expression

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Roles of DNA repair Factors in the regulation of the gene expression

Mémoire présenté par

Nicolas Le May

En vue d'obtenir l'habilitation à diriger des recherches

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Contents

Curriculum Vitae

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PhD dissertation :
« Pathogenesis mechanisms of Rift Valley Fever Virus Non structural NSs protein »

2001-2000 **DEA Microbiology, Université Paris VII, France.**
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Publications

Peer reviewed journals

1- Anastasina M., **Le May N**. *****, Ohman T., Tynell J., Nyman T.A., Julkunen I., Butcher S.J., Egly J.M. and Kainov D.E. « Non-structural protein NS1 of influenza A virus binds cellular double-stranded DNA and inhibits transcription of antiviral genes ». In revision

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Grants and Fellowships

Post-doctoral training	-INSERM Young investigator Award (5 years) -ARC Fellowship (1 year)
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2012	Pasteur Institute, Paris, France
2011	Pasteur Institute, CIIL, Lille, France
2011	CMBN, Oslo, Norway
2011	Seminar, Institut de Génétique et de dévelopement , Rennes, France
2011	Seminar, Centre de recherche en Cancérologie, Nantes, France
2011	Sir William Dunn School of Pathology, Oxford, UK
2010	Internal seminar, IGBMC, Strasbourg, FRANCE
2009	Stowers Institute, Kansas City, USA
2006	Pasteur Institute internal seminar, Paris, FRANCE
2005	European RNA viruses zoonosis conference, St Raphael, FRANCE
2004	Pasteur Institute departmental seminar, Paris, FRANCE
2003	Negative Strand RNA Viruses congres, Pise, ITALY
2002	IUMS Virology international congres, Paris, FRANCE

Teaching and mentoring experience

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Introduction

1 Introduction

In the following, I briefly describe my scientific journey, which started with haemorrhagic fever viruses and brings me today to the field of the regulation of transcription by DNA repair factors. My initial scientific fascination was the viral world; I was wondering how such absolute parasite with this small but condensed genome was able to replicate efficiently, evade the host anti-viral defences and sometimes become highly lethal. Quickly, I understood that the answers of these questions were multiple, complex and specific for each virus. However, my doctoral work made me realized that whatever the viral strategy, the control of the host transcription process by the virus was a crucial step. To be able to deeply dissect these viral mechanisms, my next goal was therefore to investigate the transcription field and understand its different regulation steps. Finally, my journey leads today to initially unexpected crossroads between two disparate processes i.e the transcription and the DNA repair pathways. Building upon my previous experiences, my future projects will attempt to conciliate my different backgrounds. Viruses will be used as tools to understand host nuclear processes in healthy and pathological contexts. Reciprocally, our better knowledge of gene expression regulation will be useful to determine better strategies to fight viral infections.

In this manuscript, I will present my past, current works and my future projects. A very brief description of my doctoral work will be given in Chapter 2 focusing on the main results. In Chapters 3 and 4, I will present the main projects that I undertook until today. Finally, in Chapters 5 and 6, I will introduce my current and future projects that consist to finish my on going work and start a new line of research that will combine virology and transcription fields.

I performed my PhD thesis at the Pasteur Institute of Paris under the supervision of Dr. Michèle Bouloy. The aim of my doctoral research was to determine the pathogenesis mechanisms of a viral protein of the Rift Valley Fever Virus (RVFV). I studied especially a small viral protein called non-structural protein from small segment (NSs) that has the particularity to form filaments in the nucleus of infected cells. We first showed that this protein was able to shut off the transcriptional activation of the innate immune response and later during the viral cycle the global RNA synthesis. To understand such inhibitions, we then identified the host partners of NSs. Among the different candidates, we focused on two proteins and highlighted, using different RVFV strains expressing deleted NSs and several experimental including molecular, biochemical and imaging approaches, two different transcriptional strategies to overcome the induction of the anti-viral defences and the initiation step of the gene expression (*1, 2*). Besides this project, we also developed a reverse genetic system to produce recombinant RVFV viruses. Using this system, we combined two attenuated strains and produce a

recombinant RVFV expressing a deleted NSs protein unable to block the innate immune response and inhibit the RNA synthesis (*3*). Such recombinant virus became the basis for the development of vaccine that has been validated and used in some African countries.

For my postdoctoral research, I joined the Jean-Marc Egly/Frédéric Coin's team at the Institute of Genetics and Molecular and Cellular Biology to develop skills in the transcription field. My goal was to strengthen my background in this topic for later projects involving viruses. My initial project should follow my doctoral research by studying the different transcriptional functions of TFIIH and especially its kinase activity on the family of nuclear receptors (NR). Thereby, we demonstrated that the phosphorylation of NRs by TFIIH regulated their turnover through the recruitment of specific ubiquitin E3 ligase leading to their ubiquitination and subsequently their degradation by the proteasome machinery at the promoter of target genes ensuring efficient cyclic transactivation. Interestingly, in cells bearing mutations of TFIIH subunits, such cascade of events was impaired leading to deregulation of NR-target genes transactivation (*4*). In parallel, we surprisingly observed that transactivation of NR-target genes was correlated with the presence of DNA repair factors belonging the Nucleotide Excision Repair (NER) pathway. Initially, these NER factors XPC, CSB, TFIIH, XPA, XPG and XPF-ERCC1 were only known to eliminate DNA lesions induced by genotoxic attacks like UV irradiation except for TFIIH that possesses dual activities in DNA repair and transcription. This observation was particularly intriguing since mutations on NER factors originate severe genetic disorders such as *Xeroderma Pigmentosum* (XP), Trichothiodystrophy (TTD), and Cockayne syndrome (CS) that were primarily defined as DNA repair syndromes. Using Chromatin-IP (ChIP)-based technics, we observed that the NER factors are sequentially recruited together with the RNA pol II transcription machinery at the promoter of the activated *RARβ2* in absence of genotoxic attack. Moreover, the presence of these DNA repair factors was defective in XP, XP/CS and TTD patient-derived cells correlating with a lower transactivation of *RARβ2*. Finally, we could discriminate the recruitment of NER factors at active promoter from the NER pathway and correlate their presence upon transcription with chromatin remodelling including histone post-translational modifications (PTMs), active DNA demethylation (*5, 6*). Altogether these data strongly suggested that the pathologies related to NER factors mutations developed phenotypes that stem from DNA repair deficiencies AND transcriptional deregulations. Our next goal was to investigate the roles of these NER factors in the chromatin remodelling upon transcription. Subsequent experiments demonstrated that the endonucleases XPG and XPF are required for DNA breaks induction and DNA demethylation at the promoter and terminator of the activated *RARβ2* respectively upon transactivation. These two steps are crucial for the formation of gene looping between promoter and terminator (*7*). Our next step was to establish whether TFIIH could influence the functions and the recruitment of NER factors at the promoter of activated genes for chromatin remodelling. By using several cells bearing mutations in genes encoding TFIIH subunits, we observed that TFIIH participated in the recruitment of the NER factors during transcription in a different way to that observed during NER, and participated on histone PTMs, DNA break induction, DNA demethylation and gene loop formation (*8*).

Such dual activity in DNA repair and transcription is not limited to NER factors. Several studies have documented similar duality for other DNA repair factors like the Base Excision Repair (BER) factors or Poly-(ADP-Ribose) polymerase 1 (PARP1) (*9*). In

collaboration with the team of Valérie Schreiber, we studied a new candidate called Poly –(ADP-Ribose) glycohydrolase (PARG) that cleaves ADP-ribose polymers synthetized by PARPs. PARG was primarily involved in the repair of single- and double-strand breaks. We demonstrated the requirement of PARG for NR-target genes. Mechanistically, we observed that PARG accumulated at promoters upon transactivation and promoted the formation of an appropriate chromatin environment suitable for transcription. Our results defined PARG as a co-activator regulating chromatin remodelling (*10*).

Recently, it has been demonstrated that XPF assembles on active promoters *in vivo* and facilitates chromatin modifications for transcription during mammalian development via an interaction with TFIID (*11*). Moreover, upon RNA pol I transcription, recruitment of the NER machinery at the ribosomal DNA promoter is also observed and mediated by Gadd45a to trigger demethylation of promoter-proximal DNA (*12*). Finally, a DNA repair complex containing XPC has been recently isolated in stem cells working as co-activator of Oct4/Sox2 transcription factors crucial for the pluripotency (*13, 14*). The potential roles in transcription of NER factors and more globally DNA repair factors are now well accepted but have to be further investigated in light of our previous results as well as the ones of others (*9*). Our most recent data suggested that XPC could regulate several histone PTMs through direct interactions with related histone modifying enzymes (HME) and histone variants upon transcription. Moreover, we showed that XPG and TFIIH are involved together in active DNA demethylation around promoter and we suspected that they could be involved in the formation/regulation of specific DNA topology for this process like R-loops (*15*). The current projects chapter aim to improve our understanding on (i) the functions of XPC and TFIIH/XPG on histone PTMs and active DNA demethylation respectively upon transcription, (ii) their recruitment modes at promoters and (iii) the biochemical defects of genetic disorders arising from mutations in these NER factors. In addition to contribute to a better knowledge of mechanisms controlling gene expression, these projects will hopefully allow the development of diagnostic tools such as biochemical assays and subsequently treat patients for different transcription disorders.

My next goal would be to conciliate my two different backgrounds in virology and transcription/chromatin remodelling in a project that will aim to identify and dissect the various viral strategies developed to overcome the host transcriptional and DNA repair reprogramming. Indeed, up to now, most of the studies focused on the viral functions antagonizing the innate immune response which is an immediate and the first line of defence. However, viruses have evolved other strategies to target cellular functions like basal transcription or DNA Damage repair, which collectively participate to the viral pathogenesis and will deserve to be studied in the near future. Like we showed for NER factors, the interconnection between the induction of DDR pathways and inhibition of host transcriptional reprogramming will constitute further important perspectives and investigations in the viral field. To start such project, I would like to first analyse more carefully the impact of RVFV NSs protein on the different novel functions played by TFIIH and the other NER factors that we and other have recently highlighted. Thereby, we will be able to evaluate the benefits for RVFV for its own replication. I strongly believe that the fundamental research on the viral signalling cascades triggered to subvert the cellular machineries in mammalian hosts are important for the later development of efficient vaccines and/or antiviral agents.

Part I: Past work

2 Brief description of doctoral research

2.1 Introduction

I performed my PhD between 2001-2005 at the Pasteur Institute in the laboratory of Dr. Bouloy that worked on the *Bunyaviridae* family and especially the human pathogen, *Rift Valley Fever Virus* (RVFV) (Figure 1)(*16*). This arthropod-borne virus (arbovirus) is a

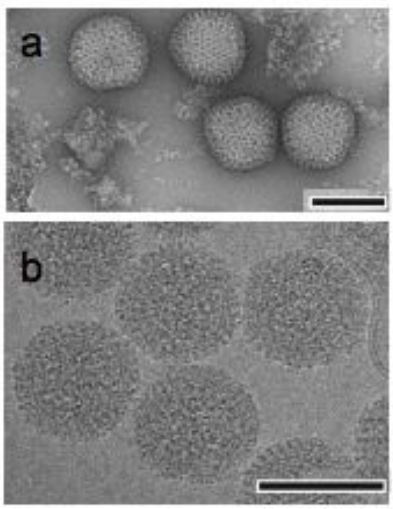


Figure 1. RVFV particles visualized by electronic microscopy

mosquito-borne zoonotic pathogen that has caused large outbreaks in sub-Saharan countries, Yemen, Saudi Arabia, South Africa and Madagascar. RVFV infection is especially lethal for newborn animals, causes febrile illness and a high rate of abortion in adult ruminants. Humans infected with RVFV usually develop an acute febrile myalgic syndrome and sometimes a hepatic damage, hemorrhagic fever-like illness, encephalitis and/or retinal vasculitis that result in a lethal illness. This enveloped virus possesses a single-stranded segmented RNA genome of negative or ambisense polarity composed of a large (L), a medium (M), and a small (S) segment (*17*). The L and M segments code for the L RNA-dependent polymerase and the glycoproteins precursor, respectively. S codes, in an ambisense strategy, for the nucleoprotein N that oligomerizes with viral genome and polymerase to form ribonucleoparticles (*18*) and NSs protein (*19*) (Figure 2).

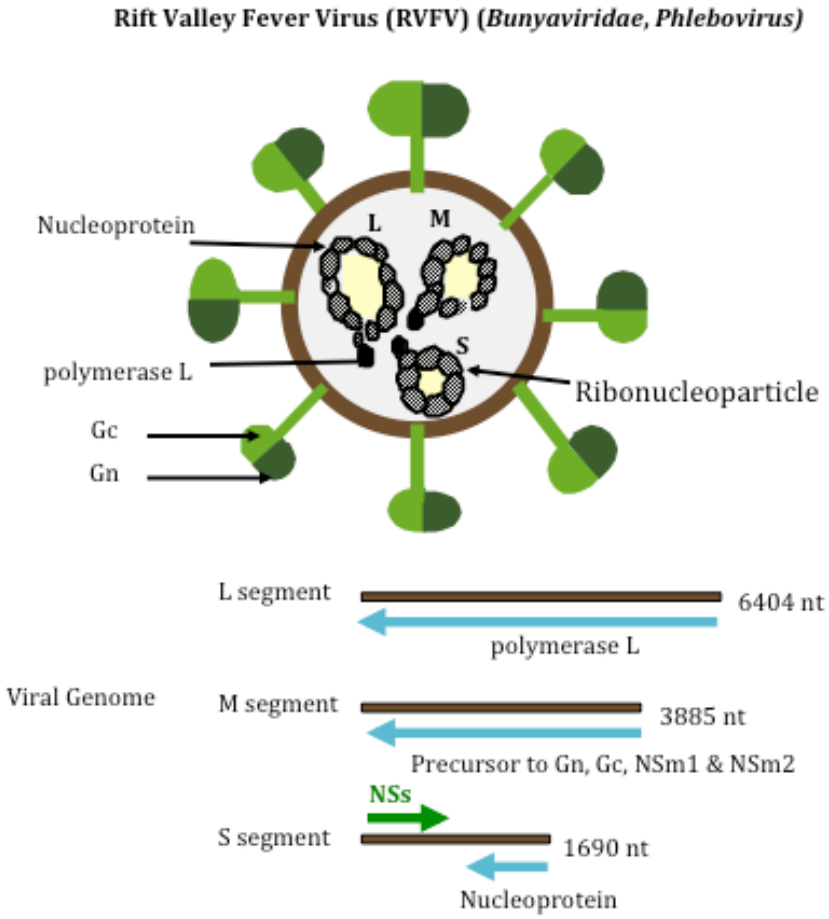
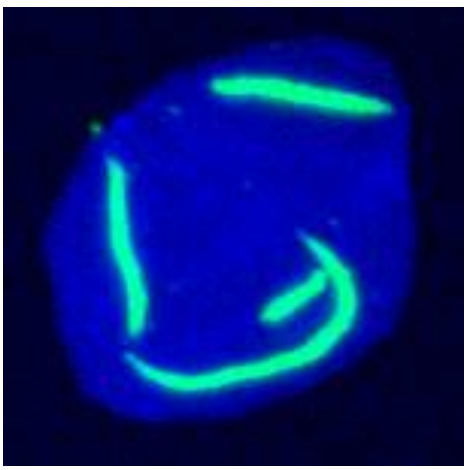


Figure 2. Schematic representation of RVFV particle and genome

This last viral protein is not essential for the RVFV cytoplasmic replication and is located in the nucleus of infected cells forming a filamentous structure (*20*) (Figure 3). It was also demonstrated that the virulence was linked to an absence of interferon (IFN) production due to the blockage by NSs of the transcription of the *IFNβ* implicated in the establishment of an antiviral state (*21, 22*). To understand the inhibition of innate immunity by RVFV, my project thus consisted of identifying the host partners of NSs.



From Le May et al., 2004

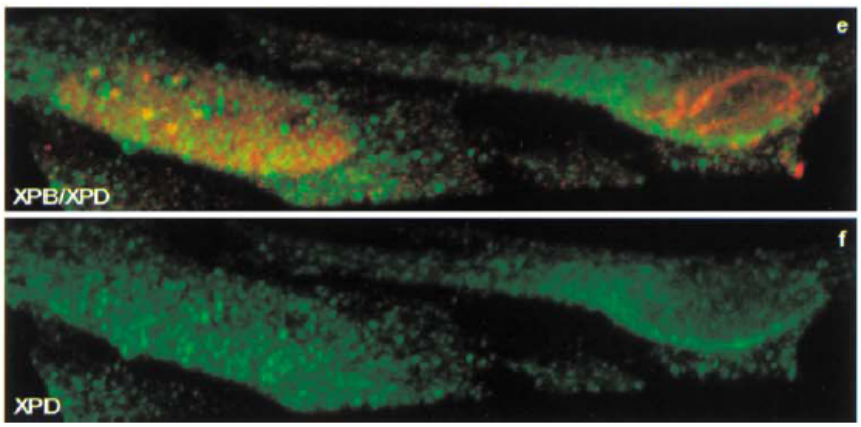
Figure 3. NSs nuclear filament from RVFV infected visualized by Immunofluorescence

2.2 Methodology

To identify the host partners of NSs, I designed and performed in collaboration with Dr. Jacob (Pasteur Institute) a Yeast Two Hybrid screening. Among the numerous candidates, I focused my analysis on proteins associated with the transcription. I later characterized these interactions and the important domains of each protein by biochemical approaches (Yeast two hybrid, GST pull-down or IP using purified proteins). The *ex vivo* validation was performed through co-immunoprecipition (co-IP) approaches and immunofluorescence visualized by confocal microscopy. Two RVFV strains were used, a virulent one (ZH 548) and another non-virulent expressing a deleted and inactive NSs (Clone 13). Recombinant RVFV expressing mutated NSs were also generated via a reverse genetic system and allowed the *in vivo* characterization. The mechanistic analysis was carried out in collaboration with the Egly/Coin and Bonnefoy teams. We used either *in vitro* approaches such as reconstituted transcription system with nuclear extracts from RVFV infected cells or *ex vivo* models using Chromatin Immunoprecipitation (ChIP) methodology on reporter or endogenous genes in RVFV infected cells.

2.3 RVFV inhibits host transcriptional reprogramming by targeting the TFIIH Transcription Factor (*1*)

My first published doctoral work has demonstrated that NSs interacts with TFIIH subunit p44. TFIIH is one of the basal transcription factors that can be resolved in two sub-complexes: the core that contains XPB, p62, p52, p44, p34 and TTD-A/p8 is bridged by the XPD subunit to the CAK (CDK- activating kinase) composed of CDK7, cyclin H, and MAT1 proteins. TFIIH possesses several enzymatic activities during transcription: (1) the XPB participates in promoter opening through a translocase activity; (2) CDK7 phosphorylates RNA polymerase II and numerous transcription factors to control gene expression. We showed that NSs suppresses the synthesis of host RNA by interacting and sequestrating p44 and XPB into the NSs nuclear filament.



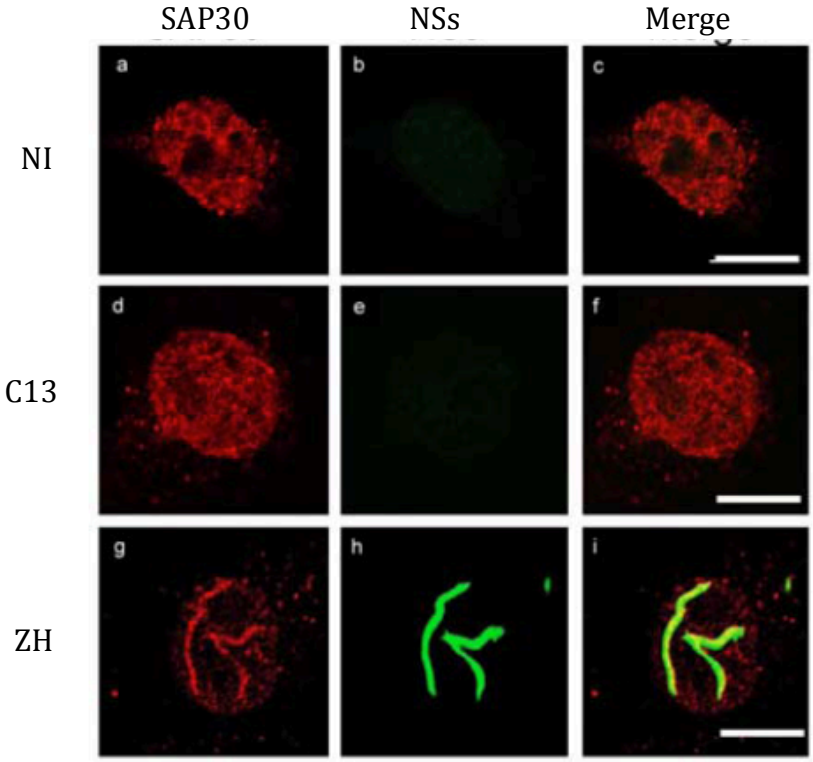
From Le May et al., 2004

Figure 4. Exclusion of XPD TFIIH subunit from nucleus containing NSs filament sequestrating XPB TFIIH subunit visualized by Immunofluorescence

Through this interaction, NSs competes also with the usual partner of p44, XPD unable to enter into the nucleus, inhibiting consequently the neo-formation of TFIIH (Figure 4). Moreover, we have demonstrated that NSs is able to induce the proteolysis, through the ubiquitin/proteasome pathway, of several other TFIIH subunits including CDK7 or p62 destabilizing the nuclear TFIIH. Consequently, its concentration strongly decreases in RVFV infected cells leading to the inhibition of the cellular transcription.

2.4 RVFV through NSs maintains a SAP30 co-repressor on IFNβ promoter inhibiting the gene expression (*2*)

We have first observed that the inhibition of IFNβ induction by NSs is earlier compared to its effect on TFIIH. We demonstrated that the viral-related maintained transcriptional repressed state of the *IFNβ* promoter is correlated to an interaction and co-localisation into the nuclear filaments between NSs and SAP30 (Sin3A Associated Protein 30) (Figure 5). This protein is a subunit of Sin3A co-repressor complexes (such as NcoR) as well as a partner of YY1 (the activator/repressor of *IFNβ* transcription). This work has shown that SAP30 through its binding to NSs on one hand and to YY1 on the other hand, forms a complex that represses the recruitment of CBP, the acetylation of histone H3 and consequently the transcriptional activation at the *IFNβ* promoter. We have generated through a reverse genetic system recombinant RVFV that expresses a mutated NSs protein unable to interact with SAP30. Such recombinant virus cannot inhibit the IFN production and is not virulent in infected mice.



From Le May et al., 2008

Figure 5. Colocalisation between NSs and SAP30 visualized by immunofluorescence only observed in RVFV infected with virulent ZH strain but not the attenuated one without NSs (C13) and mock (NI)

2.5 Conclusions

My doctoral research allows the identification of two RVFV pathogenesis mechanisms. We showed how the RVFV NSs protein hijacks the host machinery through its interaction with TFIIH p44 subunit and the co-repressor SAP30 subunit (Figure 6A and B).

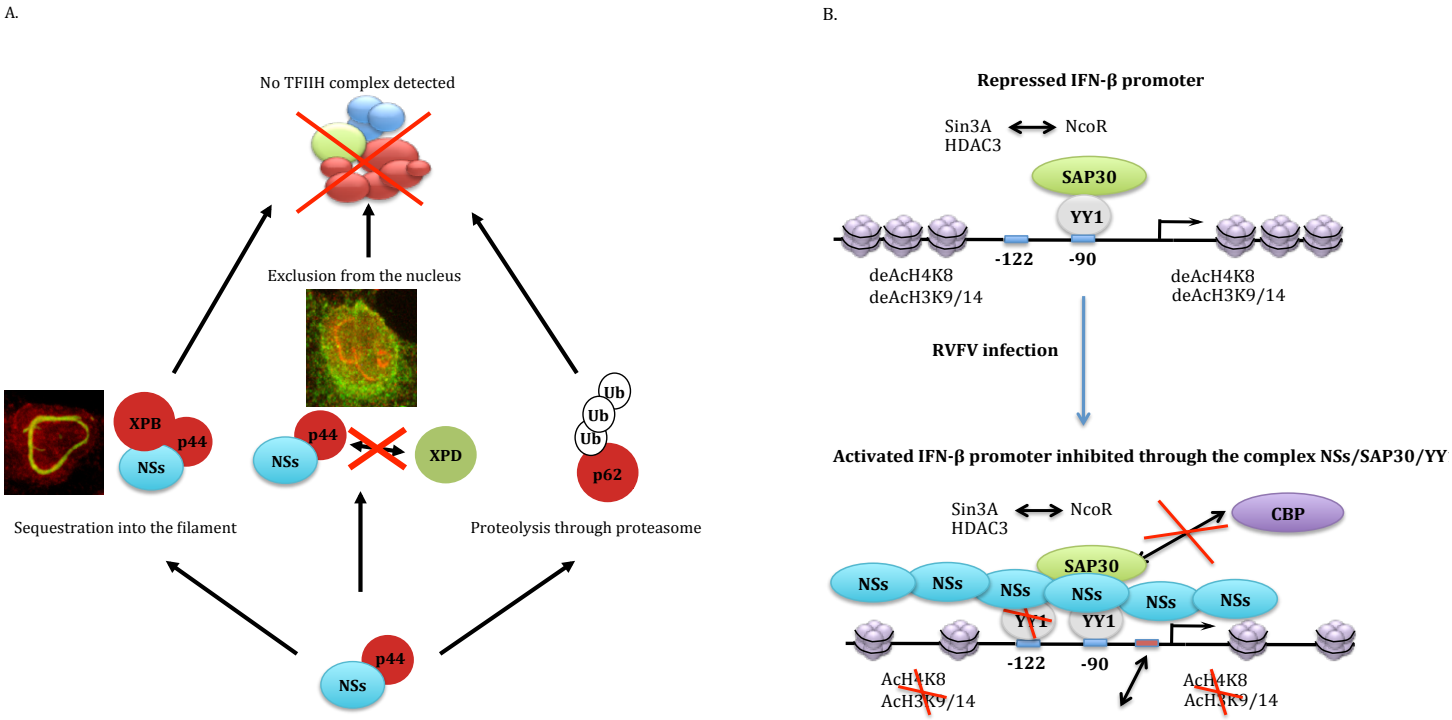


Figure 6. Schematic representation of two RVFV NSs pathogenesis mechanisms: (A) Mechanism showing how RVFV NSs through its interaction with TFIIH p44 subunit inhibits TFIIH formation by sequestration of XPB and p44 into the filament, nuclear exclusion of XPD and proteolysis of p62 leading RNA synthesis shut off. (B) Representation illustrating the maintenance of repressed IFNB promoter through the interaction between NSs and SAP30 with YY1.

These projects and the obtained results were possible through an important technical breakthrough. Indeed, the laboratory of M. Bouloy pioneered the development of reverse genetic system to recover infectious RVFV from transfected plasmids based on the use of the cellular RNA polymerase I promoter to synthesize viral transcripts. Such approach allowed the production of recombinant RVFV with the possibility to manipulate and change the genetic information (*3*). Thereby, we generated chimera recombinant RVFV corresponding to different attenuated strains and also recombinant viruses expressing deleted NSs containing additional reporter. Using this reverse genetic system, we could validate the important NSs interaction domains for p44 and SAP30 and test recombinant RVFV in the mouse model. We demonstrated that the

absence of an active NSs rendered RVFV non lethal after infection (*2*). Moreover, we demonstrated that the secondary effects observed with the current RVFV vaccines available during my PhD were correlated to NSs protein that was still able to counteract the innate immune response and the TFIIH formation (*3*). Finally, based on these results, M. Bouloy elaborated an attenuated RVFV strain that completely abolished the virulent effects of NSs. Such RVFV strain has been approved as veterinary vaccine available now in certain African countries upon important outbreaks. Since these studies, an additional mechanism for RVFV NSs protein has been demonstrated involving the degradation of the cytoplasmic PKR protein leading to the translational inhibition (*23, 24*).

However, RVFV NSs protein did not reveal all its secrets. This nuclear filament is still mysterious. No data are available explaining its formation, functions or accurate position into the nucleus. We still don't understand the goal for the RVFV to form such nuclear structure since NSs is not necessary for the viral replication. Moreover, knowing the multiple activities of TFIIH, it would be interesting to investigate the NSs involvement in the other nuclear processes. These questions of fundamental virology remain important since neither antiviral agents nor protective human vaccines are currently available.

3 TFIIH and DNA repair factors in Transcription

3.1 Introduction

I next started my postdoctoral training in the laboratory of J.M. Egly and F. Coin at IGBMC. My goal was to improve my background in the transcription field for later projects concerning the effects of viral infection on host transcriptional reprograming.
I wanted particularly to develop projects about TFIIH because I believed that the inhibitory mechanism induced by RVFV NSs protein in the pathological context of an infectious disease could really improve the understanding of the roles of such complex in the regulation of the gene regulation. Therefore I chose the team of J.M Egly and F. Coin knowing their important expertise in such field and because they share my approach by using pathologies to perform fundamental research on TFIIH. Indeed, their studies during the last decades on TFIIH and NER factors functions in both transcription and DNA repair (*25-27*) greatly benefited from the existence of human genetic disorders. Mutations in TFIIH (in XPB, XPD and p8/TTDA subunits) and NER factors lead to the rare autosomal recessive disorders Trichothiodystrophy (TTD) or *Xeroderma Pigmentosum* (XP) that can be combined with Cockayne syndrome (XP/CS) (*28*). The patients have severe clinical features, such as mental retardation, immature sexual development, skeletal abnormalities, dwarfism, premature ageing and photosensitivity that can induce skin cancers. Although these diseases have been primarily characterized as DNA repair syndromes due to the inability of patient's cells to eliminate DNA lesions, the results obtained from team of J.M Egly and F. Coin suggest that many of the phenotypes stem from transcriptional impairments (*29, 30*).
In this third chapter, I will introduce and present my different publications from my work on TFIIH and NER factors to elucidate their transcriptional roles. I won't completely follow the chronological publications of these projects. I'd rather present the chronology of the topics starting from the role of TFIIH on the regulation of nuclear receptor upon transcription to the involvement of NER factors in gene expression in absence of genotoxic attacks. Finally, I will describe our work on PARG as a new example of protein being involved in both DNA repair and transcription as co-activator.

3.2 Methodology

As genic models, we employed systems where the NRs (such as the retinoic acid receptor [RAR], peroxisome proliferator-activated receptor [PPAR] or androgen receptor [AR]) were the key regulatory elements that control the transactivation. A chromatin Immunoprecipitation (ChIP)-based methodology was used to study the dynamic recruitment of RNA polymerase II machinery, TFIIH subunits, NER factors and

players involved in the different projects during transcription in the absence or presence of exogenous genotoxic attack. The formation of repair complexes was carried out by, either ChIP coupled to immunoblot or immunofluorescence. As cellular models, we used either primary fibroblasts derived from XP and TTD patients or cells stably transfected by sh-RNA that silenced the different NER factors. We performed genome-wide analysis of gene expression through micro-arrays. To study the transcriptional functions upon chromatin remodelling of NER factors and TFIIH, we performing ChIP analysis of histones PTMs, Methylated DNA Immunoprecipitation (MeDIP) and pyrosequencing for DNA methylation analysis or metabolic labelling for the TFIIH kinase activity. I also developed a new technic called Bio-ChIP that allowed the analysis of DNA breaks and their location when this approach is combined with pyrosequencing in collaboration with Pr. P. Bougnères and Dr. D. Fradin. Finally, we also investigated the chromatin rearrangements by performing chromatin conformation capture (3C) in collaboration with Dr. D. Vernimen. These results were complemented by *in vitro* tests such as kinase, ubiquitination, histone modifications or interactions assays using purified recombinant proteins.

3.3 Phosphorylation of nuclear receptor by TFIIH and turnover

3.3.1 Context

TFIIH is a general RNA pol II-dependent transcription factor (*31, 32*) also involved in RNA pol I-dependent transcription (*33*) and nucleotide excision repair pathway (*29, 34, 35*). In RNA Pol II-dependent transcription, following the assembly of the pre-initiation machinery (including TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH and RNA pol II), TFIIH opens the DNA around the proximal promoter through its XPB translocase subunit (*36*) and phosphorylates the carboxyl-terminal (CTD) domain of the largest subunit of RNA pol II via its CDK7 kinase (*37, 38*), allowing promoter escape and RNA elongation (*39*). TFIIH also phosphorylates several transcription factors including nuclear receptors (*40, 41*), making it a key factor in the transactivation process.
Wen I started this project, our laboratory pioneered studies showing that ligand-dependent transactivation mediated by several nuclear hormonal receptors, such as the Retinoic Acid Receptors (RAR) (*6, 40*), the Vitamin D Receptor (VDR) (*42*), the Peroxisome Proliferator Activated Receptors (PPAR)(*43*), the Thyroid hormone Receptors (TR)(*44*) was defective in cells derived from XP, TTD, XP/CS patients-derived cells with mutations in either TFIIH or XPG (*45*), explaining some hormonal deregulation (*46, 47*). However, the role of such phosphorylation by TFIIH on so many different NRs remained elusive.
Pierre Chymkowitch that was PhD student in 2005 initiated this study described below. His initial goal was to investigate if another NR, the androgen receptor (AR), involved in development and maintenance of male reproductive organs could be also phosphorylated by TFIIH. Our second purpose was to analyse the AR phosphorylation and the AR-target genes in XP and TTD-derived cells bearing mutations on TFIIH subunits. As we previously demonstrated for other NRs, the hypothesis was that the sexual retardation observed in patients could be linked to transcriptional impairments of AR-related transcription program. Finally, we aimed to understand the role of such phosphorylation proposing a crosstalk with ubiquitination. Indeed, interplay between phosphorylation and ubiquitination was shown for the recycling of other nuclear

receptors, such as the glucocorticoid receptor GR or the RARg2, which is crucial for the control of their transactivation activity (*48, 49*).

3.3.2 Publication: Regulation of NR turnover by TFIIH phosphorylation

Chymkowitch P., **Le May N.***, Compe E., Charneau P. and Egly J.M. « The phophorylation of the Androgen Receptor by TFIIH directs the ubiquitin/proteasome process ». **EMBO J**. 30, 468-479. 2011. (*4*)

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The phosphorylation of the androgen receptor by TFIIH directs the ubiquitin/proteasome process

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In response to hormonal stimuli, a cascade of hierarchical post-translational modifications of nuclear receptors are required for the correct expression of target genes. Here, we show that the transcription factor TFIIH, via its cdk7 kinase, phosphorylates the androgen receptor (AR) at position AR/S515. Strikingly, this phosphorylation is a key step for an accurate transactivation that includes the cyclic recruitment of the transcription machinery, the MDM2 E3 ligase, the subsequent ubiquitination of AR at the promoter of target genes and its degradation by the proteasome machinery. Impaired phosphorylation disrupts the transactivation, as observed in cells either overexpressing the non-phosphorylated AR/S515A, isolated from xeroderma pigmentosum patient (bearing a mutation in XPD subunit of TFIIH), or in which cdk7 kinase was silenced. Indeed, besides affecting the cyclic recruitment of the transcription machinery, the AR phosphorylation defect favourizes to the recruitment of the E3 ligase CHIP instead of MDM2, at the *PSA* promoter, that will further attract the proteasome machinery. These observations illustrate how the TFIIH phosphorylation might participate to the transactivation by regulating the nuclear receptors turnover.

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Introduction

Following induction by ligand, nuclear hormone receptors (NR) target their responsive elements to initiate the formation of a large preinitiation complex, including co-activators, histone-modifying enzymes, mediator, RNA polymerase II (RNA pol II) and the general transcription factors TFII-A, -B, -D, -E, -F and -H (Lemon and Tjian, 2000). TFIIH, also known as a DNA repair factor, is a multiprotein complex composed of two subcomplexes: the core (containing the helicase XPB, p62, p52, p44, p34 and p8) and the cdk-activating kinase complex CAK (containing MAT1, cyclin H and the cdk7 kinase). Whereas the XPB helicase is devoted to the promoter opening (Holstege *et al*, 1996; Coin *et al*, 1999), cdk7 phosphorylates the C-terminal domain of the largest subunit of RNA pol II (Lu *et al*, 1992) thereby facilitating the promoter clearance, as well as NRs, which is an essential step for transactivation of target genes (Rochette-Egly *et al*, 1997).

Mutations in some of the transcriptional components disrupt protein/protein interaction and/or reversible post-translational modifications (PTMs) that are essential for complex maintenance and its further activation. This situation is well illustrated for TFIIH. Indeed, mutations in the C-terminal domain of the XPD subunit of TFIIH disturbs the architecture of TFIIH and its molecular communication with the retinoic acid receptor α (RARα) (Keriel *et al*, 2002), the peroxysome proliferator-activated receptors (PPARs) (Compe *et al*, 2005), the estrogen receptor (Chen *et al*, 2000) or the thyroid hormone receptors (Compe *et al*, 2007), leading to the dysregulation of cdk7-related phosphorylation. Defects in the expression of these NRs responsive genes might explain part of the broad range of clinical features of the human genetic disorders such as xeroderma pigmentosum (XP) or trichothiodystrophy (TTD) (Lehmann, 2001). Some of the symptoms such as hypogonadism, sterility, growth retardation, hypoplasy of the adipose tissue and bone abnormalities could be related to an androgen response deficiency.

Androgen steroid hormones that induce the androgen receptor (AR) activity are involved in the development and maintenance of male reproductive organs but also in adipose tissue, skeletal muscle and the bone homeostasis (Mooradian *et al*, 1987; Brinkmann, 2001; Matsumoto *et al*, 2008; Vanderschueren *et al*, 2008). AR and its co-regulators exert their action during the development of normal prostate and in the initiation and progression of prostate cancer, the most common cancer in men in western countries (Heinlein and Chang, 2004; Balk and Knudsen, 2008). As most of the steroid hormone receptors, AR is characterized by a conserved structural and functional organization (Mangelsdorf *et al*, 1995; Kumar *et al*, 2004): a heterogeneous N-terminal A/B domain that contains a ligand-independent transactivation domain (AF-1 domain), a highly conserved DNA-binding domain, a homo- and heterodimerization domain, and a large C-terminal ligand-binding domain that harbours a ligand-inducible transactivation function (AF-2 domain). All these domains are subjected to PTMs, such as phosphorylation, acetylation, sumoylation and ubiquitination (Faus and Haendler, 2006).

The main function of these PTMs is to regulate either positively or negatively the interaction between the various biomolecules present in the cell thus providing signals to initiate a given mechanism that is essential for transactivation. Besides being targeted by several protein kinases, NRs

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are also substrates for E3 ubiquitin ligases (Gaughan *et al*, 2005; Bour *et al*, 2007) that engages the ubiquitin–proteasome process during the waves of transcription complex formation (Kodadek *et al*, 2006). Such process is initiated by protein ubiquitination involving an activating enzyme (E1) and a conjugating enzyme (E2), which relays ubiquitin to the substrate in the presence of an E3 ligase (Hochstrasser, 2009). Polyubiquitinated proteins next bind to the 19S regulatory particle of the proteasome and are then unfolded in an ATP-dependent manner through the action of the ATPases that sit atop the opening to the 20S core particle cavity part of the 26S proteasome (Baumeister *et al*, 1998). One prominent intersection between phosphorylation and ubiquitination is likely to regulate the turnover of NRs. Whether a crosstalk between these two PTMs occurs and how it might regulate the AR transcriptional activity remains unclear.

In this study, we show how the phosphorylation of AR (at position S515), by the cdk7 kinase of TFIIH, influences the specific recruitment of the mouse homologue of double minute 2 protein (MDM2) E3 ligase and the proteasome to the prostate-specific antigene PSA promoter, one of the most studied AR responsive genes and participates in the regulation of its turnover. Impairing AR/S515 phosphorylation, results in transactivation defect due to an abnormal recruitment of the transcription machinery. In this case, we found that the recruitment of CHIP, another E3 ligase, at the promoter of AR responsive gene is favourized. This results in the dysregulation of the turnover of AR and the cyclic transactivation of its target gene PSA, explaining at least partially some of the mechanistic defects leading to XP or TTD patients.

Results

Specificity in the TFIIH/AR interaction during transactivation

We first investigated the potential effect of XPD mutations on AR transactivation using human primary fibroblasts isolated from XP and TTD patients. The PSA promoter including its androgen responsive elements (AREs) was cloned upstream the luciferase reporter gene (pGL3. PSA-Luc) and transfected together with a vector expressing AR (pSV.AR), and the β-galactosidase encoding vector as an internal control. Following 5α-dihydrotestosterone (DHT) induction, we observed that AR transactivation was largely reduced in XPD/R683W cells in which XPD is mutated at position R683W but not in cells carrying mutations at position XPD/R722W and XPD/R112H. In the latter cell lines, the androgen response reached the same level as in XPD/WT cells (Figure 1A, left panel). We also analysed the effect of these mutations on the expression of a luciferase reporter gene under the control of RARα (Keriel *et al*, 2002). Here, XPD/R683W as well as XPD/R722W mutations affected RARα transactivation (Figure 1A, right panel), suggesting that XPD mutations differentially disrupted the transactivation mediated by a given nuclear receptor.

We then investigated the phosphorylation status of AR during transactivation (Figure 1B). AR-transfected WT and XPD/R683W (named XPD) cells were incubated with [^{32}P]-orthophosphate and collected over time after DHT treatment. AR was then immunoprecipitated (IP) from the corresponding cell extracts, resolved by SDS–PAGE and analysed by autoradiography and western blot. We repeatedly observed that AR phosphorylation was weaker in XPD cells compared with WT cells (Figure 1B, lanes 1–6; see also the histogram depicting the ratio ([^{32}P]-AR/AR). This defect was compensated in XPD cells, by overexpressing XPD/WT (Figure 1B, compare lane 9 with lanes 6 and 3), indicating a role for XPD and by extension of TFIIH in the phosphorylation of AR.

We then asked whether AR could functionally and physically interact with TFIIH in a relevant physiological context (Figure 1C). After immunoprecipitation of AR from human prostate adenocarcinoma LNCaP cells (which endogenously express AR), we clearly detected the presence of TFIIH (Figure 1C, left panel, lane 5). Non-specific antibodies were unable to immunoprecipitate AR/TFIIH (Figure 1C, left panel, lane 4). In parallel, we also found that AR co-immunoprecipitates in a ligand-independent manner with recombinant TFIIH, as detected by antibodies directed against XPD, p62, p44 or cdk7 subunits of TFIIH (Figure 1C, right panel). We further investigated which TFIIH subunits interact with AR. Each of the 10 subunits of TFIIH was separately overexpressed in insect cells and incubated with the recombinant AR. We found that XPB, XPD and p44 interacted with AR (Figure 1D); the interaction between AR and the other TFIIH subunits was either much weaker (as observed for cdk7) or absent (data not shown). Interestingly, the pattern of interaction between AR and the TFIIH subunits differs from that observed for other NRs, underlying how specific was the interaction between an NR and TFIIH (Supplementary Table I).

The above results prompted us to ask whether AR could be a substrate for the cdk7 kinase of TFIIH. *In vitro* kinase assays showed that TFIIH, via its cdk7 kinase subunit, phosphorylated AR (Figure 1E, lanes 1–4), an event that was also observed for another NR such as PPARα (lanes 12 and 13). TFIIH phosphorylated A/B domain of AR (A/B.AR) but not the truncated form of AR lacking the A/B domain (ARΔA/B, compare lanes 8–11 with lanes 6–7). A careful screening of the 560 residues of the A/B domain followed by systematic mutagenesis (data not shown) suggested that among the various serine/threonine candidates, serine 515 (S515) is a potential phosphorylation site for cdk7, a proline-directed kinase (Morgan, 1997). Accordingly, when we mutated the S515 into alanine (AR/S515A), the phosphorylation of A/B.AR was largely reduced (compare lanes 16 and 18).

To determine the role of the S515 residue in AR-mediated transcription, the pGL3.PSA-Luc luciferase reporter plasmid together with either pSV.AR/S515A or pSV.AR/S515E (in which S515 was mutated into an alanine or a glutamic acid that mimics a non-phosphorylated or a constitutive phosphorylated AR, respectively), was co-transfected in both WT and XPD cells (Figure 1F). Following DHT induction, AR/S515A did not accurately transactivate in either WT or XPD cells. On the contrary, we observed in XPD cells that AR/S515E compensated the transactivation defect observed with AR/WT.

In addition to demonstrating that the cdk7 kinase of TFIIH specifically phosphorylates AR at position S515 that promote transactivation, the above data also show that the interaction between the NRs and TFIIH is receptor specific, rather than 'universal' and that the effect of a given mutation in TFIIH during NRs transactivation depends on both the nature of the mutation and the nature of the NRs.

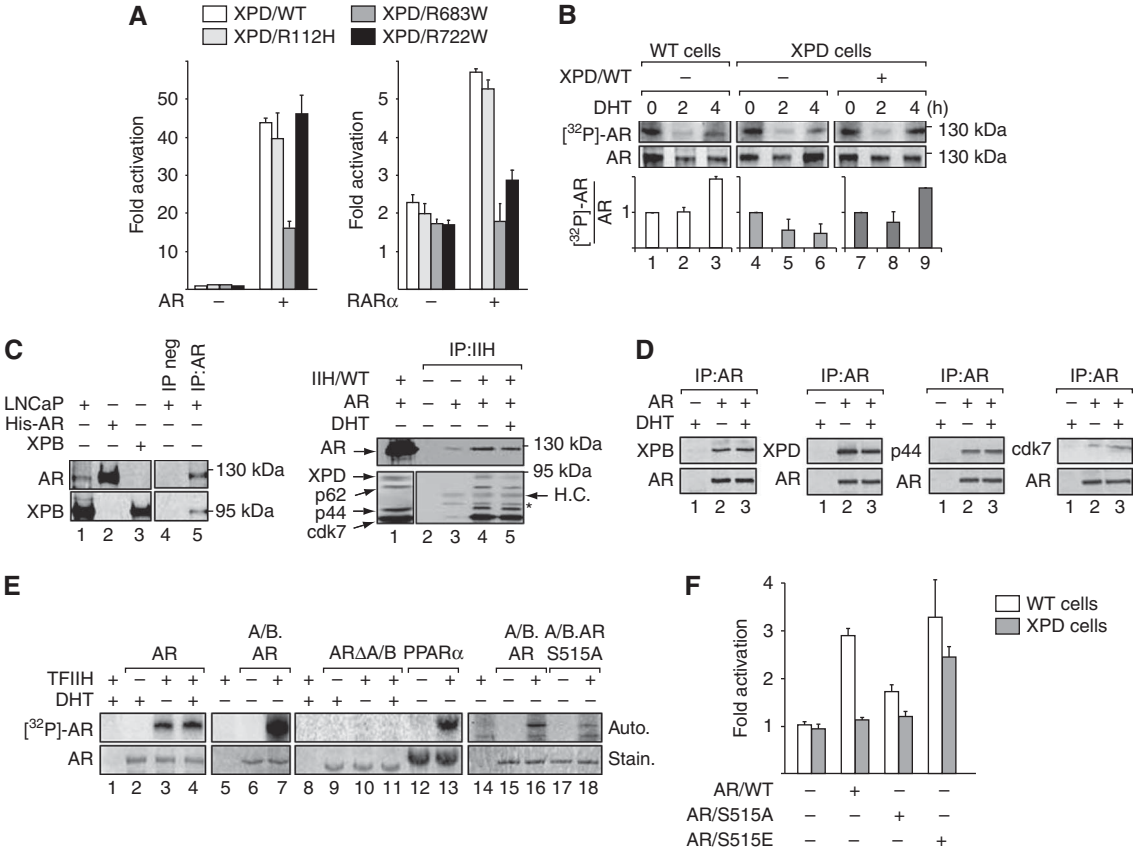

Figure 1 The transactivation and the phosphorylation of AR are disrupted by the XPD/R683W mutation. (**A**) GM03348D (XPD/WT), TTD12PV (XPD/R112H), XPJCLO (XPD/R683W) and TTD8PV (XPD/R722W) fibroblasts were transiently co-transfected with 100 ng of pGL3.PSA-Luc, pCMV.β-gal and either pSG5.AR (AR) or pSG5.RARα (RARα) or the corresponding empty vector. The cells were then treated with a specific ligand for AR (DHT, 10^{-7} M) or RARα (t-RA, 10^{-8} M). Luciferase activity was measured 24 h later and normalized to β-galactosidase activity. The results are the mean of three different experiments. (**B**) *In vivo* phosphorylation of AR was investigated in HeLa (WT), HD2 (XPD) and XPD cells overexpressing XPD/WT upon AR immunoprecipitation, autoradiography ([^{32}P]-AR) and western blotting (AR). Quantitative analysis of AR phosphorylation in WT and XPD cells represents the ratio autoradiography/western blot signals ([^{32}P]-AR/AR). (**C**) AR interacts with TFIIH. (Left panel) Endogenous AR was immunoprecipitated (IP:AR, lane 5) from LNCaP cells and after washes (150 mM NaCl), co-precipitated TFIIH was detected using an anti-XPB antibody. The control IP (IP neg, lane 4) was performed with a non-specific antibody. The input (lane 1) represents 10% of the total volume of LNCaP extract used for the immunoprecipitation. His-AR (lane 2) and purified XPB (lane 3) were used as size markers for the immunodetection of the endogeneous AR and XPB in LNCaP cells. (Right panel) Highly purified-recombinant AR was incubated with *Sf9* cell extracts overexpressing TFIIH complex containing a FLAG-tagged p34, in the presence or absence of DHT (10^{-6} M). Immunoprecipitation was performed using an anti-FLAG antibody (IP:IIH) and after extensive washes (300 mM NaCl) and SDS–PAGE, bound proteins were detected with an anti-AR, XPD, p62, p44 and cdk7 antibodies. (**D**) Equal amounts of highly purified-recombinant AR was incubated with *Sf9* cell extracts overexpressing separately the subunit XPB, XPD, p44 or cdk7 of TFIIH in the presence or absence of DHT (10^{-6} M). After AR immunoprecipitation, co-precipitated TFIIH subunits were detected by western blot. The control IP (lane 1) was performed without AR. (**E**) AR, A/B.AR, ARΔA/B, PPARα or A/B.AR S515A were incubated with purified-recombinant TFIIH in the presence or absence of DHT (10^{-6} M) as indicated. After SDS–PAGE, each protein was resolved by Coomassie blue staining (Stain.) and radioactive labelling was analysed by autoradiography (Auto.). The complete Coomassie blue stainings and western blots of the different purified-recombinant proteins are shown in Supplementary Figure S1A. (**F**) WT (HeLa) and XPD (HD2) cells were transiently co-transfected with 100 ng of pGL3.PSA-Luc, pCMV.β-gal and pSG5 AR/WT, /S515A, or /S515E before DHT (10^{-7} M) treatment. The results were obtained as described in **A**.

Phosphorylation of AR regulates its turnover

Phosphorylation has a major role in the regulation of steroid receptor stability (Weigel and Moore, 2007). We were therefore wondering whether a defect in the phosphorylation of the activation domain of AR would affect its stability. We examined the turnover of AR protein by conventional pulse chase at different times following ligand exposure. Twenty-four hours after transfection with the various AR expression vectors, WT- and XPD-deficient cells were metabollically labelled with ^{35}S-methionine for 1 h and then treated with the AR ligand. Cells were collected at different times of treatment and AR was IP before being resolved by SDS–PAGE and autoradiographied. Newly synthesized ^{35}S-AR/WT was detected until the first hour in WT cells while in XPD cells, AR labelling was visible until 2/4 h post-DHT induction (Figure 2A and B, lower panels). To localize ^{35}S-AR/WT, western blots were performed in parallel (Supplementary Figure S2). A control pulse chase realized in WT and XPD cells, which have not been transfected, is also presented (Figure 2A and B, upper panels). Interestingly, the transfection in XPD cells of either AR/S515E or XPD/WT (together with AR/WT), that reestablished both the phosphorylation status and the transactivation process of AR (Figure 1B and F), restored the half-life of AR to a similar level of that observed in normal cells for AR/WT (compare Figure 2D and F with Figure 2A). Strikingly, we repeatedly observed

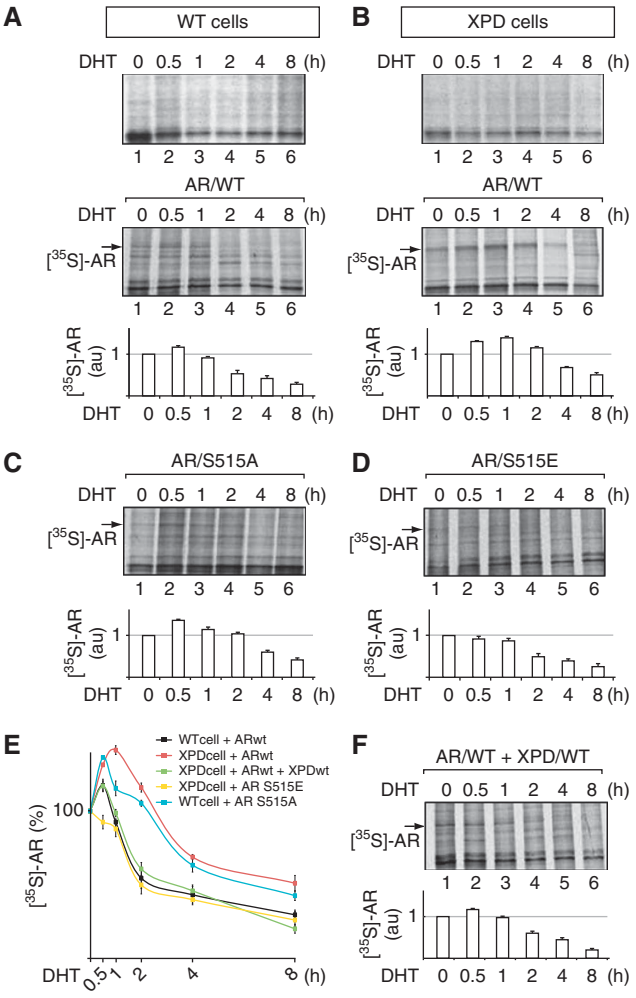

Figure 2 Pulse chase of AR protein in WT and XPD cells. (**A**–**F**) WT (**A**, **C**) and XPD cells (**B**, **D**) were transiently transfected in order to overexpress AR/WT (**A**, **B**, lower panels), AR/S515A (**C**) or AR/S515E (**D**). XPD cells were also co-transfected in order to simultaneously overexpress AR/WT and XPD/WT (**F**). Control experiments were performed with cells transfected with empty vectors (**A**, **B**, upper panels). Following a [^{35}S] pulse, cells were maintained in the presence of DHT (10^{-7} M) for the indicated time points (0, 0.5, 1, 2, 4 and 8 h). After immunoprecipitation, AR was resolved by SDS–PAGE and [^{35}S] labelling quantified with a phosphoimager. Western blots were performed in parallel to localize AR (see Supplementary Figure S2). Arrows indicate the position of the radiolabelled [^{35}S]-AR. Graphs depict AR protein levels normalized to that observed in absence of DHT treatment (arbitrary units, au). The values are the mean ± s.e.m. of three independent experiments. (**E**) Summary of AR turnover following DHT treatment in WT cells overexpressing either AR/WT (black curve) or AR/S515A (blue curve), and in XPD cells overexpressing either AR/S515E (yellow curve) or AR/WT in the absence (red curve) or presence of XPD/WT (green curve). The [^{35}S]-AR levels are presented as percentages, 100% being the [^{35}S]-AR levels in absence of DHT treatment. Data are the mean ± s.e.m. of three independent experiments.

that in WT cells, the labelling of ^{35}S-AR/S515A (in which the phosphorylated serine site was abrogated) was visible past 2 h post-DHT treatment (Figure 2C).

Altogether, our results suggest that a deficiency in the AR phosphorylation, resulting from either mutation in the XPD subunit of TFIIH or abrogation of the AR/S515 phosphorylation site, prolongs the turnover of AR following ligand induction (Figure 2E).

Selective recruitment of the ubiquitin ligase at the PSA promoter

The above results prompted us to analyse the expression of endogenous gene known to be under the control of AR in WT and XPD cells. Following DHT treatment, PSA mRNA cyclically peaked at 2 and 16 h in WT cells (Figure 3A), while in XPD cells, the PSA mRNA synthesis only peaked at 1 h and then slowly decreased (Figure 3B). Strikingly, the transfection in XPD cells of either XPD/WT (Figure 3C) or AR/S515E (Figure 3D) restored the profile of the mRNA synthesis observed in WT cells (Figure 3A).

The transactivation of NR-target genes involves phosphorylation by specific kinases, ubiquitination by E3 ligases and degradation by the proteasome (Kodadek *et al*, 2006; Bour *et al*, 2007). We thus studied the dynamic recruitment of the various components of the transcriptional complex at the PSA promoter over time by chromatin immunoprecipitation (ChIP) assays analysed by quantitative PCR. In WT cells, AR, TFIIH (as visualized by the presence of its XPB and cdk7 subunits) as well as RNA pol II, were recruited to the PSA promoter at 2 and 16 h post-ligand induction (Figure 3E); their concomitant recruitments paralleled the PSA mRNA synthesis (Figure 3A). Conversely, in XPD cells, AR strongly accumulated after 2 h of DHT treatment to progressively decrease during the following 20 h (Figure 3F). Here, the recruitment of TFIIH paralleled the one observed for AR. It is worthwhile to mention that the recruitment pattern of RNA pol II (Figure 3F) as well as of p300 (a co-activator of AR, data not shown) (Popov *et al*, 2007) were strongly disturbed in XPD cells when compared with that observed in WT cells (compare Figure 3F and E). Interestingly, when overexpressing either XPD/WT (together with AR/WT) or AR/S515E in XPD cells, we observed the restoration of the biphasic recruitment of AR, RNA pol II and TFIIH (compare Figure 3G and H with Figure 3E).

We also investigated whether defects in the AR phosphorylation impact its targeting by the ubiquitin–proteasome pathway. We particularly focused our attention on the MDM2 (the human homologue of the 'Murine double minute') and CHIP (C-terminus of Hsc70-interacting protein) E3 ligases, which are known to interact with AR in a phosphorylation-dependent manner (Lin *et al*, 2002; Rees *et al*, 2006) and to be associated with active PSA promoter (Kang *et al*, 2002; Gaughan *et al*, 2005). We found that MDM2 was mainly recruited with AR after DHT induction in WT cells (Figure 3I). Surprisingly, in XPD cells, in addition to the MDM2 recruitment, we also observed the recruitment of CHIP, which was hardly detected in WT cells (Figure 3J and I). It should be noticed that in both WT and XPD cells, the cellular concentration of MDM2 and CHIP was similar (unpublished results). Furthermore, upon expression in XPD cells, of either AR/WT (+XPD/WT) or the constitutive phosphorylated AR/S515E, the preferential MDM2 cyclic recruitment was restored (Figure 3K and L). ChIP/re-ChIP analysis using first an anti-ubiquitin antibody and second an anti-AR antibody showed that the AR-containing fraction that was ubiquitinated, paralleled the recruitment of the E3 ligases (Figure 3I–L).

ChIP assays next revealed that the presence of S1 and SUG1, two regulatory subunits of the 19S proteasome and the β5 catalytic subunit of the 20S proteasome (Baumeister *et al*, 1998; Gianni *et al*, 2002; Kang *et al*, 2002) was concomitant to the presence of the MDM2 or CHIP E3 ligases (Figure 3M–P), when AR is at the PSA promoter.

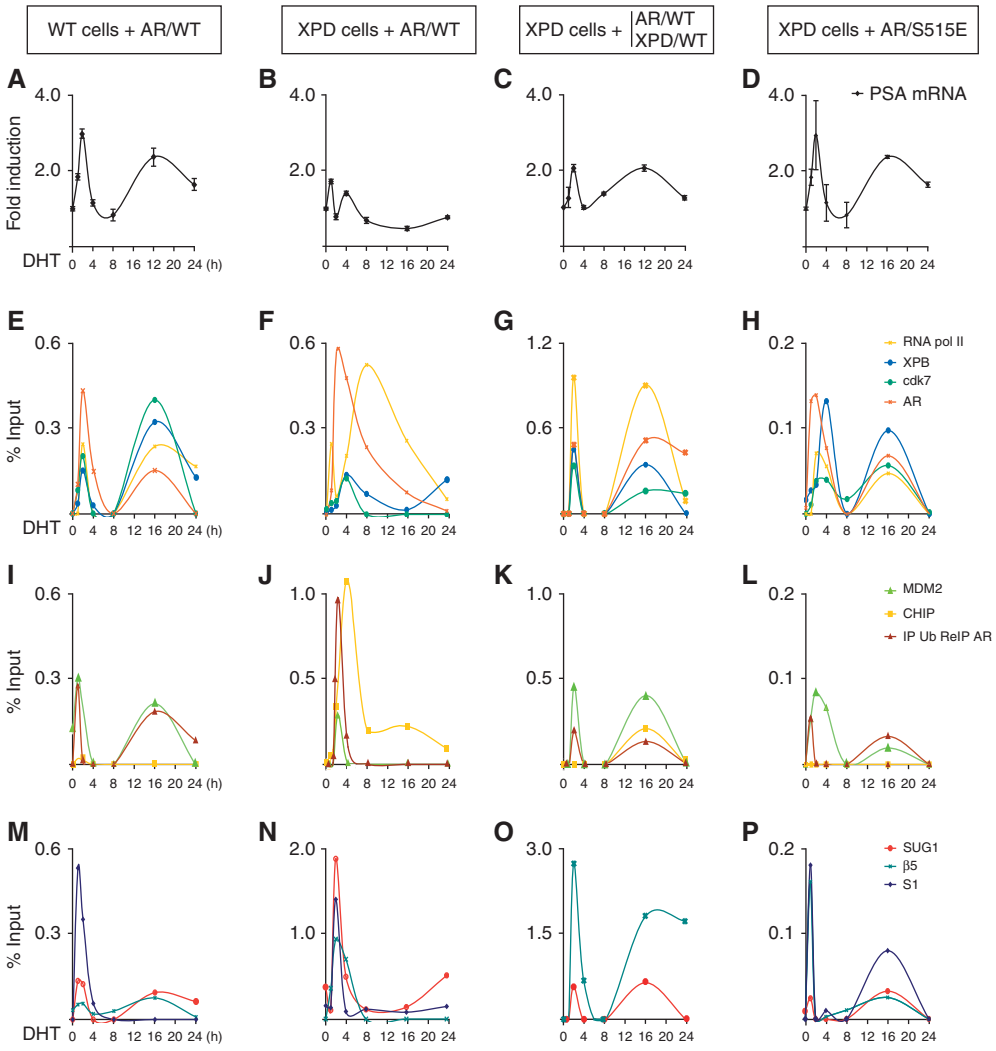

Figure 3 AR phosphorylation status selectively promotes the recruitment of ubiquitin–proteasome components at the PSA promoter. WT and XPD cells were transiently transfected to overexpress either AR/WT, AR/WT together with XPD/WT or AR/S515E (as indicated at the top of each panel). (**A**–**D**) Expression of the PSA gene: RT–qPCR analysis was performed at indicated times after DHT (10^{-7} M) treatment. The values were normalized relative to the GAPDH mRNA expression. The results of three independent experiments are presented as *n*-fold induction relative to non-treated cells. (**E**–**H**) After DHT treatment, the recruitment of RNA pol II (yellow curve), TFIIH (via its XPB and cdk7 subunits, blue and green curve, respectively) and AR (red curve) were analysed by ChIP assays at the PSA proximal promoter. The results are presented as percentage of DNA immunoprecipitated relative to the input (% input). (**I**–**L**) Recruitment of the MDM2 (green curve) and CHIP (yellow curve) E3 ligases on the PSA promoter. The recruitment of the ubiquitinated AR-containing fraction (brown curve) was also analysed by ChIP/re-ChIP assays using first an anti-ubiquitin antibody and second with an anti-AR antibody. (**M**–**P**) Recruitment of SUG1 (red curve), β5 (light blue) and S1 (dark blue) subunits of the proteasome at the PSA promoter was analysed by ChIP assays. Note that the recruitment of S1 has not been analysed in XPD cells transiently co-transfected with plasmids encoding AR/WT and XPD/WT (**O**).

The AR/E3 ligases relationship

The above data underline a link between the phosphorylation status of AR and the selective recruitment of E3 ligases that direct the ubiquitination and the recruitment of the proteasome machinery at the PSA promoter. To address the effect of protein interactions in these settings, we first overexpressed AR/WT, /S515A or /515E in DHT-treated HeLa cells. After immunoprecipitation and extensive washes (300 mM KCl), the different forms of AR were incubated with either MDM2 or CHIP E3 ligases (see Materials and methods). We found that MDM2 exhibits a higher affinity for AR/WT and AR/S515E contrary to what we observed for AR/S515A (Figure 4A, compare lanes 2–3 and 10–11 with lanes 6–7). Conversely, CHIP is able to interact with the three forms of AR although to a lower extent with AR/S515E (compare lanes 8–9 with lanes 4–5 and 12–13). In parallel, incubations of AR and E3 ligases were also performed in the presence of HSP90α. We observed that HSP90α specifically co-IP with AR in the presence of CHIP (lanes 5, 9 and 13), which is in accordance with previous observations, suggesting that HSP90α is a molecular chaperone preferentially associated to U-box-type ubiquitin protein ligases such as CHIP (Murata *et al*, 2003; Hatakeyama *et al*, 2004; Yan *et al*, 2010).

We next performed an *in vitro* ubiquitination assay in which AR/WT was incubated with either MDM2 or CHIP, the suitable E1/E2 enzymes and His-Ubwt. AR was equally polyubiquitinated with both E3 ligases (Figure 4B, lanes 3 and 7). Knowing that MDM2 and CHIP can distinctly mono- or polyubiquitinate their substrates (Li *et al*, 2003, 2007), we also analysed the AR monoubiquitination by replacing His-Ub_{WT} by His-Ub_{K0} (a mutated Ubiquitin, with all the lysine residues replaced by alanine). Interestingly, contrary

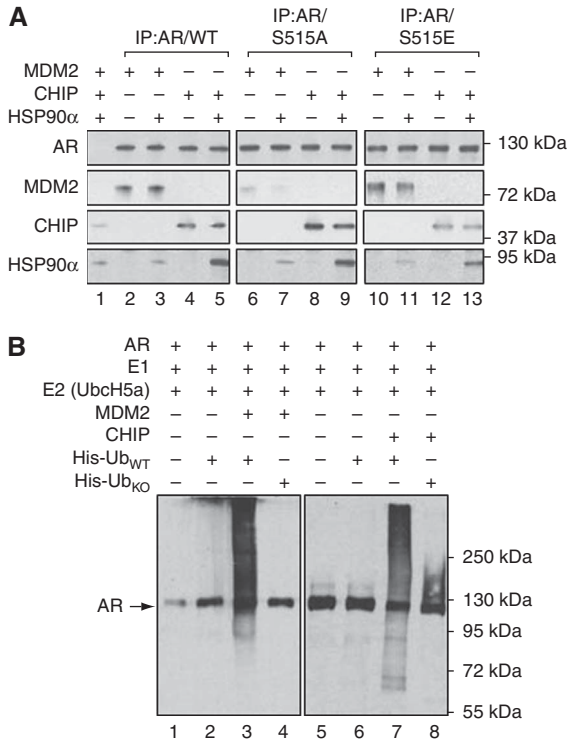

Figure 4 AR interacts with MDM2 and CHIP and is ubiquitinated by both E3 ligases. (**A**) AR/WT, /S515A or /S515E, immunoprecipitated (IP:AR/) from DHT (10^{-7} M) treated HeLa cells, were incubated with either purified-recombinant MDM2 or CHIP E3 ligases, in the presence or absence of HSP90α, as indicated. After extensive washes (300 mM KCl), western blot analyses were performed with AR-, MDM2-, CHIP- and HSP90α-specific antibodies. The control IP (lane 1) was performed using a non-specific antibody. Inputs are shown in the Supplementary Figure S3. (**B**) Recombinant purified AR was incubated with the ubiquitin-activating enzyme E1 (UBE1) and E2 (UbcH5a) in the presence of either MDM2 (1 μM, lanes 3 and 4) or CHIP (1 μM, lanes 7 and 8) E3 ligase as indicated on top of the panel. His-Ub$_{WT}$ (wild type) or His-Ub$_{K0}$ (in which all seven lysine residues critical for polyubiquitination are replaced with alanine) have been added to the reaction, when indicated. Western blot analyses were then performed with Ubiquitin and AR-specific antibodies.

to that observed with MDM2, AR monoubiquitination was clearly detected in the presence of CHIP (compare lanes 8 and 4), which might be explained by our *in vitro* experimental conditions (see Discussion).

The above data indicate that MDM2 and CHIP are able to equally polyubiquitinate AR. Strikingly, these results demonstrate that AR phosphorylation status contributes to a selective recruitment of E3 ligases.

CHIP is preferred to MDM2 when the AR phosphorylation is impaired

To further investigate the role of TFIIH in the recruitment of the ubiquitin–proteasome machinery at the PSA promoter upon AR phosphorylation, we have generated stable HeLa cell lines by using a lentiviral system producing AR and have performed silencing experiments. In si-cdk7-treated cells, in which the cdk7 protein level was abrogated (Supplementary Figure S4A), ChIP assays showed the co-presence of AR as well as RNA pol II at the PSA promoter during the first 4 h post-DHT ligand treatment (Figure 5E and F). Strikingly, in si-cdk7-treated cells, in which the phosphorylation of AR was deficient (Supplementary Figure S4B) and its turnover much longer (compare Figure 5R with Q), we noticed that the CHIP recruitment was prominent while MDM2 was hardly detected (compare Figure 5J with I). The preference for CHIP recruitment in the si-cdk7 cells did not affect the recruitment of the proteasome, as clearly illustrated by the presence of the S1, SUG1 and β5 subunits at the PSA promoter (Figure 5N and M). However, contrary to that was observed in si-ctl, SUG1 was highly detected in si-cdk7-treated cells; this was likely due to a differential accessibility of the SUG1 antibody (Figure 5N).

We also analysed whether silencing MDM2 might favourize the CHIP recruitment. In si-MDM2 cells, in which AR and RNA pol II were recruited to the PSA promoter (Figure 5G), CHIP (Figure 5K) as well as the proteasome machinery (Figure 5O) were present. These data prompted us to investigate whether the absence of CHIP might affect the transactivation process. Whereas silencing CHIP impaired PSA induction, the recruitment of RNA pol II and AR was conserved at the promoter (Figure 5H). In such case, MDM2 is not found, suggesting a co-requirement of both CHIP and MDM2 E3 ligases during the normal transactivation process (Figure 5L). We also failed to detect a recruitment of either SUG1 or β5 proteasome subunits upon DHT treatment (Figure 5P).

Altogether, the above data show that in case of impaired AR phosphorylation and/or MDM2 gene expression abrogation, CHIP replaces MDM2, to further allow the recruitment of the proteasome/ubiquitin machinery at the promoter of activated genes. Regardless, the absence of cdk7, MDM2 or CHIP leads to a defect in the transactivation machinery, which impedes the DHT-induced PSA gene expression (Figure 5A–D).

Discussion

The basic mechanism of cyclic transactivation of nuclear receptors following ligand induction relies on their translocation into the nucleus, their binding to their cognate sequences, and the recruitment of the transcription machinery to the promoter of a given gene. The transactivation process mediated by the nuclear receptors may require PTMs, such as acetylation, sumoylation, ubiquitination or phosphorylation. Although it became clear that the TFIIH-dependent phosphorylation of NRs controls their transactivation capacity (Keriel *et al*, 2002; Compe *et al*, 2005), many questions remain related to other potential consequences of these PTMs.

The present work proposes a model in which the phosphorylation of AR by TFIIH can induce its polyubiquitination, the recruitment of the proteasome and therefore the regulation of the expression of the AR responsive gene (Figure 6). We identified the serine S515 of AR as a phosphorylation substrate site for the cdk7 kinase of TFIIH as a key step of the transactivation process. Contrary to other phosphorylation of AR that modulate either the expression of its responsive genes (Weigel and Moore, 2007) or its cellular localization (Lin *et al*, 2002; Shank *et al*, 2008), the phosphorylation of AR/S515 is not required for its translocation into the nucleus (see Supplementary Figure S5A) and its subsequent binding to its responsive elements (Figure 3) and therefore seems

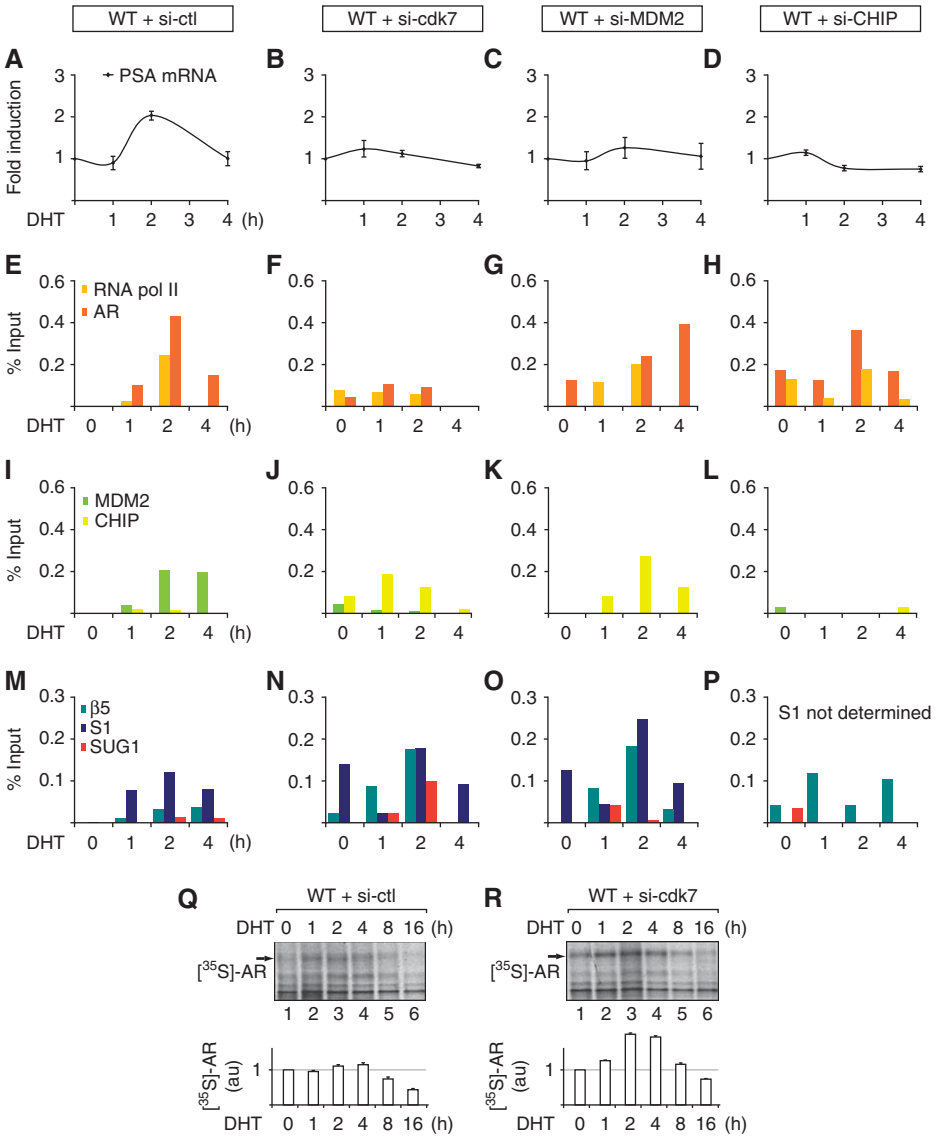

Figure 5 Silencing either cdk7 or MDM2 promotes CHIP recruitment at the PSA promoter. WT cells stably expressing AR/WT were transfected with either a pool of non-targeting si-RNAs (used as control, si-ctl, panels **A**, **E**, **I**, **M**, **Q**), si-cdk7 (panels **B**, **F**, **J**, **N**, **R**), si-MDM2 (panels **C**, **G**, **K**, **O**) or si-CHIP (panels **D**, **H**, **L**, **P**). (**A**–**D**) PSA gene expression was analysed by RT–qPCR at indicated times after DHT (10^{-7} M) treatment in cells transfected with si-ctl (**A**), si-cdk7 (**B**), si-MDM2 (**C**) or si-CHIP (**D**). The values were normalized relative to the GAPDH mRNA expression. The results of three independent experiments are presented as *n*-fold induction relative to non-treated cells. (**E**–**H**) The recruitment of RNA pol II (yellow bars) and AR (orange bars) at the PSA promoter was analysed by ChIP assays. The results are presented as percentage of DNA immunoprecipitated relative to the input (% input). (**I**–**L**) Recruitment of the E3 ligases MDM2 (green bars) and CHIP (yellow bars) at the PSA promoter was next investigated. (**M**–**P**) ChIP assays analysed the recruitment of the proteasomal subunits β5 (green bars), S1 (blue bars) and SUG1 (red bars). (**Q**–**R**) [^{35}S] Pulse chase of AR protein in cells stably expressing AR/WT and transfected with either si-ctl (**Q**) or si-cdk7 (**R**). Experiments were performed as described in Figure 2. The values are the mean ± s.e.m. of three independent experiments.

to exhibit a specificity towards the transactivation process *per se*.

Following ligand induction, we observed that the AR/S515 phosphorylation contributes to the accurate enrolment of components required for the active and cyclic transactivation of target gene such as the PSA gene (Figure 3). Even more interestingly, we here show that the S515 phosphorylation status of AR dictates its turnover via an ubiquitin/proteasome pathway, which implies either MDM2 or CHIP E3 ligases. Indeed, once bound to its responsive element, the phosphorylated AR/S515 provides a specific recognition signal for the recruitment of the MDM2 E3 ligase (Figures 3I and 4A) that might use AR as a substrate (Figure 4B). Conversely, in the absence of AR/S515 phosphorylation, as noticed in both si-cdk7-treated and XPD cells or upon transfection of AR/S515A (unpublished results), we observed a greater and sustained accumulation of AR at the PSA promoter. In this situation, the cyclic recruitment of the transcription machinery cannot take place properly and CHIP E3 ligase is preferentially recruited at the PSA promoter (Figures 3J and 5J). Here, the recruitment of CHIP does result from both the absence of the AR/S515 phosphorylation and the MDM2 recruitment defect. Indeed when MDM2 is silenced, CHIP that was showed to physically and functionally interact with AR (Cardozo *et al*, 2003; He *et al*, 2004; Rees *et al*, 2006; Adachi *et al*, 2007; DaSilva *et al*, 2009) is enrolled at the PSA promoter (Figure 5K). It is noteworthy that the *in vitro* association of AR with MDM2 is more sensitive than with

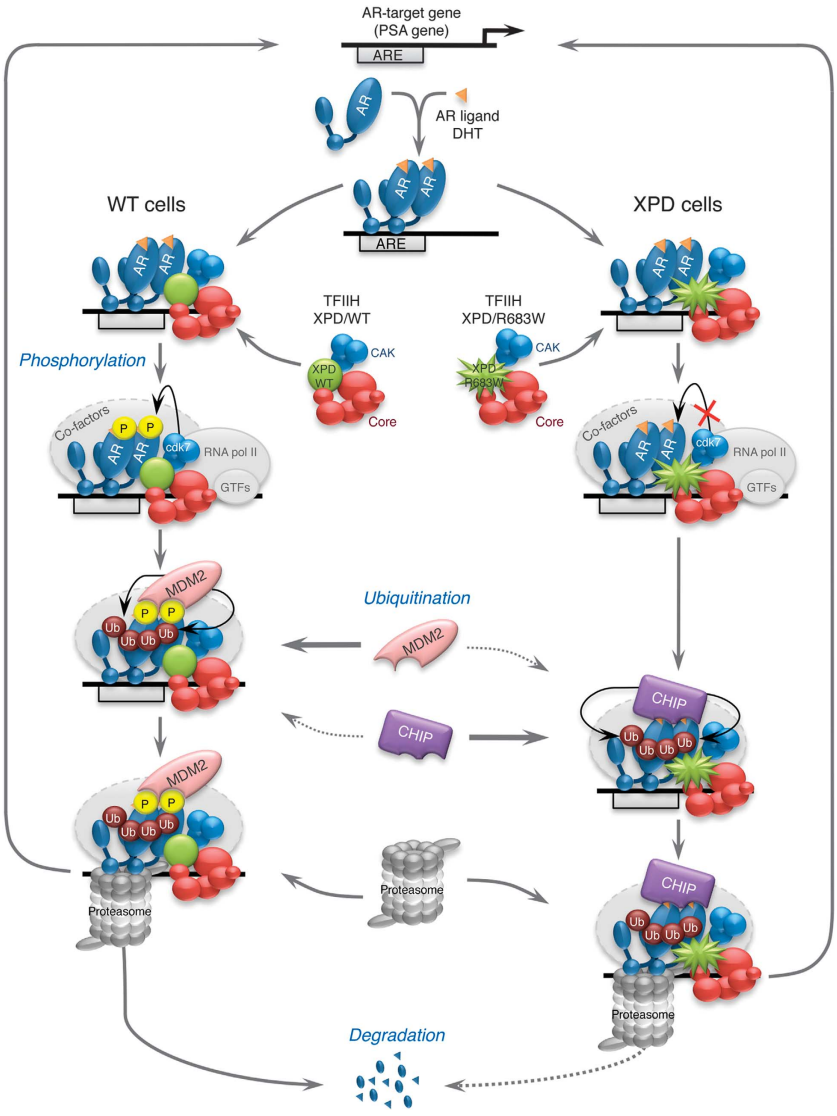

Figure 6 TFIIH-mediated phosphorylation of AR regulates its turnover by triggering its degradation by the ubiquitin/proteasome pathway. Upon the DHT ligand induction, the transactivation complex is formed once AR homodimer has targeted its responsive element (ARE) at the PSA promoter; co-factors are assembled at the promoter together with RNA pol II and the general transcription factors (GTFs) including TFIIH. In WT cells, the AR/S515 phosphorylation by TFIIH (via its cdk7 subunit) promotes the recruitment of both MDM2 E3 ligase that helps for AR polyubiquitination and the proteasome. In XPD cells (bearing the XPD/R683W mutation), AR/S515 phosphorylation is strongly inhibited, preventing the recruitment of MDM2. The E3 ligase CHIP is thus preferentially recruited, which allows with a lesser efficiency the AR polyubiquitin/proteasome process resulting in a much slower turnover.

CHIP to the phosphorylation status of AR (Figure 4A). In a cellular context, when the AR/S515 phosphorylation is disrupted, we cannot exclude that other PTMs would contribute to attract CHIP E3 ligase. However, providing a 'phosphorylated AR' by transfecting either AR/S515E or AR/WT together with XPD/WT into XPD cells restored the recruitment and the transactivation of AR similarly to that observed in WT cells (Figure 3).

Overall, our observations illustrate the crosstalk between two PTMs for example phosphorylation and ubiquitination during AR transactivation. The interplay between these two processes was shown for the recycling of other nuclear receptors, such as the glucocorticoid receptor GR or the RARγ2, which is crucial for the control of their transactivation activity (Wallace and Cidlowski, 2001; Bour *et al*, 2007). Here, we show that in the absence of an efficient phosphorylation by TFIIH, the recruited CHIP E3 ligase could still polyubiquitinate AR although the ubiquitin/proteasome process seems to be less efficient (Figure 6). This is illustrated by the abnormal accumulation of polyubiquitinated AR, which is observed in MG132-treated XPD cell extracts compared with WT cell extracts (Supplementary Figure S5B). Our *in vitro* ubiquitination assays showed that AR is polyubiquitinated by either MDM2 or CHIP (Figure 4B). Interestingly, whereas these E3 ligases can distinctly mono- or polyubiquitinate their substrates (Li *et al*, 2003, 2007), AR monoubiquitination has been only observed in the presence of CHIP (Figure 4B). As illustrated for p53 (Li *et al*, 2003), it is likely that AR monoubiquitination might be observed depending on the MDM2 concentration. Investigations should be undertaken to further define the selective role (if any) of both E3 ligases, in the AR ubiquitination process. It could not be excluded that both MDM2 and CHIP might work together to recruit the proteasome machinery for an optimal AR degradation

process (Figures 3 and 5). Such cooperation is illustrated by the absence of MDM2 on the active PSA promoter when CHIP is silenced, which leads to a recruitment defect of the proteasome (Figure 5L and P).

In addition to the E3 ligases located at the promoter of activated genes, we also detected subunits of the 26S proteasome associated to the transcription machinery. This suggests that both the ubiquitination and the degradation of the nuclear receptor could already be engaged at the PSA promoter. We should mention that non-proteolytic activities of the ubiquitin–proteasome pathway could have a role in AR-mediated transcription. On many occasions, such non-proteolytic activities of the proteasome was observed (Kodadek *et al*, 2006; Kodadek, 2010), as illustrated by our previous results showing that the SUG1 subunit of the 26S proteasome interacted with the XPB subunit of TFIIH without being proteolysed (Fraser *et al*, 1997; Weeda *et al*, 1997).

Disruption of the AR phosphorylation by mutations into TFIIH might have consequences at a physiological level. However, it is difficult to establish a precise genotype/phenotype relationship for XP and TTD patients because they develop a broad range of clinical features (Lehmann, 2003; Kraemer *et al*, 2007), resulting from the combination of defects in both DNA repair and transcription. Recent work has revealed how certain mutations in XPD (Keriel *et al*, 2002) or XPG, a partner of TFIIH (Ito *et al*, 2007), disturb hormonal responses in a way that the phosphorylation of NRs by TFIIH was hampered and resulted in an impaired expression of their responsive genes. Clinical features such as hypogonadism, cachexia, growth retardation, kyphosis or bone loss, which are related to an AR deficiency (Mooradian *et al*, 1987; Matsumoto *et al*, 2008; Vanderschueren *et al*, 2008) are only developed by certain XP and TTD patients (Lehmann, 2001; Cleaver *et al*, 2009). Interestingly, some TFIIH mutations do not disrupt the transactivation mediated by AR (Figure 1A). Moreover, AR as well as other NRs has a specific and unique pattern of interaction with TFIIH (Supplementary Table I), suggesting that each TFIIH mutation might differently affect the transactivation process according to the NRs. Taken together, these observations suggest that the pleiotropic nature of the XPD and TTD phenotypes might result from various defects of NRs (Keriel *et al*, 2002; Compe *et al*, 2007), which have different consequences at a transcriptional level depending on the nature of the target genes since the organization of the transactivation process for each gene is unique (Brivanlou and Darnell, 2002). Among the effect of TFIIH mutations, we demonstrated here that disruption of the AR/S515 phosphorylation affects the turnover of AR. It is likely that mutations close to the AR/S515 site (Takahashi *et al*, 1995), as found in several prostate cancer can also modify the half-life of AR thereby preventing it to properly transactivate its responsive genes (Heinlein and Chang, 2004).

By investigating the defects leading to XP and TTD phenotypes, the present work contributes to explain the complex and peculiar mechanism that regulates the nuclear receptor transactivation. We demonstrate how a single PTM of a nuclear receptor, for example the phosphorylation of AR at serine 515, regulates its ubiquitination and subsequently its proteolysis as part of the transactivation process. In addition, the simultaneous presence of the transcription and the ubiquitination/proteasome machineries on the promoter of a given gene indicates how this later process has an impact on the transactivation. Taken together, the present study furthers our understanding of how the AR activity is regulated.

Materials and methods

Plasmids and construction of AR mutants

For luciferase reporter assays, the full-length PSA promoter (−5025 to +25) was amplified from human placental genomic DNA and cloned into pGL3.basic (Promega) using the Gateway technology (Invitrogen) giving rise to pGL3.PSA-Luc.

For recombinant his-AR expression in *Sf*9 cells, PCR product for the entire coding sequence of human AR (aa 1–919) was cloned into pTriEx vector (Novagen) using the appropriated restriction endonucleases. For recombinant protein expression in bacteria, PCR product for the AR truncated form without the A/B domain (his-ARDA/B; aa 559–919) or the A/B domain alone (GST-A/B.AR-his; aa 1 to 558) were, respectively, cloned into pET15b (Novagen) and pGEX.4T3 (Pharmacia) vectors using appropriated restriction endonucleases. Serine residue changes to alanine or glutamic acid were introduced using site-directed mutagenesis kit (Stratagen).

Antibodies

Monoclonal antibodies against the TFIIH subunits XPB (1B3), XPD (2F6), p62 (3C9), p44 (1H5), RNA polymerase II (7C2), SUG1 (2SU 1B8) were produced at the IGBMC facility. Polyclonal antibodies against TFIIH subunit cdk7 (C-19), AR (C-19), CHIP (H-231), Ubiquitin (FL-76), MDM2 (SMP14), β-tubulin and 20S proteasome β5 (C-19) were purchased from Santa Cruz Biotechnology. Rabbit antibodies against proteasome S1 (Rpn2) subunit were purchased from Abcam.

Stable expression of AR in HeLa cells

HeLa lenti-AR cell line was produced through infection with a recombinant lentivirus. The human AR gene was cloned and inserted into the vector plasmid pTripgatewayCMV using the Gateway technology (Invitrogen). Vector particles were produced as previously described (Zennou *et al*, 2000). Infection efficiency was measured by immunofluorescence using a specific antibody for AR and positive cells were clonally selected and amplified. Finally, stabilization of infection was measured after several passages by western blot.

Cell culture and transfection

HeLa (WT), HeLa lenti-AR and HD2 (XPD), which results from the fusion between human fibroblasts (harbouring the XPD/R683W mutation) and HeLa cells (Johnson *et al*, 1985), were grown in Dulbecco's modified Eagle medium (DMEM; 1 g glucose/l; GIBCO-BRL) containing 10% foetal calf serum (FCS) and 40 μg/ml gentamicine. Normal fibroblasts (GM03348D; Coriel Cell Repository), XPD-mutated fibroblasts (XPJCLO, XPD/R683W) (Taylor *et al*, 1997), TTD8PV (XPD/R112H) and TTD12PV (XPD/R722W) (Botta *et al*, 1998) were grown in DMEM supplemented with 10% FCS and 40 μg/ml gentamicine. Human prostate adenocarcinoma LNCaP cells were grown in RPMI Media 1640 (Invitrogen) supplemented with 10 mM Hepes, 10% FCS, 1 mM sodium pyruvate and 40 μg/ml gentamicine. All cell types were plated at ∼60% confluency and pGL3.PSA-Luc, pSG5.AR, ARS515A, ARS515E, pCDNA3.XPD/WT and pCMV.β-gal, as indicated, were transiently transfected in WT or XPD cells using JetPei reagent (Polyplus transfection). After 6 h of transfection, cells were treated with phenol red-free medium containing 5% charcoal-treated FCS and 40 μg/ml gentamicine. Following 16 h of incubation, appropriate ligand for RARα or AR (respectively, t-RA and DHT) was added and the mixture was incubated for 24 h. Cell extracts were analysed for luciferase and β-galactosidase activities as previously described (Keriel *et al*, 2002). The results are the mean of three different experiments done in duplicate.

Short interfering RNA

The short interfering RNA (si-RNA) corresponding to human cdk7, MDM2, CHIP and the non-targeting si-RNA (si-ctl) were purchased from Dharmacon and transiently transfected at a final concentration of 100 nM using Lipofectamine 2000 reagent (Invitrogen). After 24 h of transfection, the cells were treated with DHT (10^{-7} M) and

prepared for analysis of RNA, chromatin or protein. si-RNA sequences are available on the Dharmacon web site (http://www.dharmacon.com).

Purification of recombinant proteins

Full-length recombinant his-AR was produced in *Sf9* cells grown in medium containing 10^{-7} M DHT. Cells were harvested in a lysis buffer (20 mM Tris–HCl (pH 6.8), 20% glycerol, 150 mM NaCl, 0.1% NP40, 5 mM β-mercaptoethanol, 500 mM 1-(3-sulphopropyl) pyridinium betain, 10 mM imidazol) before ultracentrifugation. His-AR was then purified using Ni-NTA agarose (Qiagen) according to the manufacturer's instructions. GST-A/B.AR-his was produced in *Escherichia coli* BL21.RARE strain and purified using first Glutathione Sepharose 4B (Amersham Biosciences) and second Ni-NTA agarose. His-ARΔAB was produced in *E. coli* BL21.RARE strain grown in medium containing DHT (10^{-7} M) and purified using Ni-NTA agarose.

ChIP assays

Cells were transiently transfected with each indicated vector or si-RNA. After DHT (10^{-7} M) treatment, ChIP experiments were carried out as previously described (Compe *et al*, 2005). Chromatin was prepared and sonicated on ice 20 min using a Bioruptor (Diagenode) in 10 s pulse followed by 20 s cooling. Samples were IP with antibodies at 4°C overnight and Protein G-Sepharose beads (Upstate) were added, incubated 4 h at 4°C and sequentially washed. For ChIP/re-ChIP experiments, after the first immunoprecipitation and washes, protein–DNA complexes were eluted with a 10-mM DTT solution and diluted before addition of antibodies and protein G-sepharose beads for the immunoprecipitation. The complexes were eluted and the crosslinking was heat reversed. DNA fragments were purified using QIAquick PCR purification kit (Qiagen) and analysed by real-time quantitative PCR using sets of primers, available upon request, amplifying the region of interest in the PSA promoter. Results are expressed relative to the amount of input DNA per ChIP.

In vivo and in vitro phosphorylation of AR

Equal amounts of cells were transiently transfected with indicated expression vectors or si-RNA. Next, DHT (10^{-7} M) treatment, [^{32}P]-orthophosphate labelling and immunoprecipitation were done as previously described (Keriel *et al*, 2002). After washes, AR was resolved by SDS–PAGE and transferred to a nitrocellulose filter. AR radioactive labelling was then evaluated by exposing an autoradiographic film to the filter. The western blot analysis of AR was then performed on the same filter.

Equal amounts (1 μg) of recombinant purified AR, ARΔAB, A/B.AR, A/B.AR.S515A and PPARα were incubated with highly purified HeLa TFIIH, [γ-^{32}P] ATP (0.14 μM) in the presence or absence of DHT (10^{-7} M) and reaction was carried out as described (Rossignol *et al*, 1997).

Co-immunoprecipitation assays

For *in vivo* co-IPs, total extracts of LNCaP cells treated with DHT (10^{-7} M) were prepared. After AR immunoprecipitation, followed by extensive washes (150 mM NaCl), TFIIH co-precipitation was detected using an antibody specific for XPB.

For *in vitro* co-IPs, *Sf9* cells were infected with a virus expressing all TFIIH subunits with a FLAG-tagged version of p34 (Tirode *et al*, 1999). Whole-cell extracts were then incubated with recombinant purified AR in the presence or absence of DHT (10^{-6} M) before a FLAG immunoprecipitation was carried out. After washes, bound proteins were resolved by SDS–PAGE and detected by western blot. Alternatively, *Sf9* cells were infected with each subunits of TFIIH separately (Tirode *et al*, 1999) and whole-cell extracts were incubated with recombinant purified AR in the presence or absence of DHT (10^{-6} M). Whole-cell extracts were also prepared from DHT-treated (10^{-7} M) HeLa cells overexpressing AR/WT, AR/S515A or AR/S515E. AR were IP (using Dynabeads protein A/G, Invitrogen) and incubated in the presence of DHT (10^{-6} M) with purified MDM2, CHIP (Boston Biochem) or HSP90α (Assay Designs) in an interaction buffer (20 mM Hepes, 100 mM KCl, 0.2 mM EDTA, 10% glycerol, 0.05% NP40, 0.2 mM DTT). After extensive washes (300 mM KCl), bound proteins were resolved by SDS–PAGE and detected by western blot.

Reverse transcription and real-time quantitative PCR

Total RNA was isolated using a GenElute Mammalian Total RNA Miniprep kit (Sigma) and reverse transcribed with SuperScript II reverse transcriptase (Invitrogen). The quantitative PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen) and the Lightcycler 480 (Roche). The primer sequences for *PSA* and *GAPDH* genes used in real-time qPCR are available upon request. *PSA* mRNA levels were normalized against the *GAPDH* mRNA.

Half-life measurements

Pulse-chase analysis were carried out in cells transfected with expression vectors or si-RNA, as indicated, that were pretreated during 2 h in DMEM cys$^-$/met$^-$ medium and metabolically labelled with 100 μCi/ml ^{35}S-methionine for 1 h in DMEM cys$^-$/met$^-$ medium. Cells were then washed with growth medium consisting of phenol red-free DMEM with 5% charcoal-treated FCS. Cells were then treated with DHT (10^{-7} M) for the indicated times. Whole-cell extracts were prepared using RIPA buffer (0.01 M Tris–HCl pH 8.0, 0.14 M NaCl, 1% Triton X-100, 0.1% Na-Deoxycholate, 0.1% SDS) and ^{35}S-AR protein was IP using an anti-AR antibody and resolved by SDS–PAGE. ^{35}S-AR bands were quantified by phosphoimager analysis using ImageJ software.

In vitro AR ubiquitination

His-AR was purified using Ni-NTA beads (Qiagen) that were extensively washed before ubiquitination reactions. Beads were then mixed with 100 nM of E1, 500 nM of E2 (GST-UbcH5a), 100 mM of His-Ubiquitin wild type (His-Ub_{WT}) or with all the lysines mutated to arginine (His-Ub_{K0}), 2 mM of ATP and Ub buffer 5 × (Tris–HCl 250 mM, $MgCl_2$ 25 mM, DTT 1 mM). All recombinant proteins were purchased from Boston Biochem. Recombinant E3 ligase, either MDM2 (1 μM, Boston Biochem) or CHIP (1 μM, Upstate), was also added to the mix that was incubated 1 h at 37°C. The reaction was stopped with 10 mM of EDTA and bound proteins were resolved by SDS–PAGE and revealed by western blot using antibodies against Ub (FK2, Enzo Life Sciences) and AR.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: Pchy, NLM, EC and JME conceived and designed the experiments. PCha produced the lentiviral particles to establish the stable cell lines. Pchy, NLM and EC carried out the experiments. Pchy, NLM, EC and JME analysed the data. Pchy, NLM, EC and JME wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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3.3.3 Highlights and perspectives:

The obtained results were important in the TFIIH field because it was one the first study that demonstrated the function of the CDK7-related phosphorylation of NR. In addition to identify a new TFIIH target i.e the androgen receptor, we showed a crosstalk between phosphorylation and ubiquitination that remains important to regulate the AR-turnover upon the transactivation of the target genes. However, we did not investigate whether this crosstalk was conserved for the other NR targeted by TFIIH although the turnover of RAR, GR or PPAR though kinase-related ubiquitination and proteasome-dependent proteolysis is known (*49, 99*). Such investigations would be important to characterize the aetiology and better understand the transcriptional defect observed in patients bearing mutations on TFIIH.

Moreover, it was interesting to notice, in the XP and TTD derived-patients cells bearing mutations on XPD, that the defect of phosphorylation by CDK7 did not impair the turnover of AR but rather led to an alternative pathway less efficient. The consequences were not the inhibition of transcription but a lower RNA synthesis coupled to a temporal deregulation with the lost of the cyclic waves of transactivation. Surprisingly, the analysis of longer time-course showed that the deficient expression of AR-target genes in XP/TTD cells disappeared and became comparable to the wild type. This could indicate that either the alternative pathway needs more time to regulate efficiently the AR turnover or there is another TFIIH-independent mechanism that is induced later in the AR-transcriptional program.

Finally, we used fibroblasts to demonstrate this mechanism differently deregulated following the mutations on TFIIH. Therefore it was impossible for us to connect our conclusions to the clinical features including hypogonadism, cachexia, growth retardation, kyphosis or bone loss, which could be related to an AR deficiency. However, it is interesting to notice that AR mutations found in prostate cancer often co-localized with the AR domain phosphorylated by TFIIH (*67*).

3.4 NER factors in Transcription

3.4.1 Context

In 2006-2007, I unexpectedly started my work on the involvement of NER factors in transcription. Indeed, I was searching, for ChIP experiments targeting several promoters and enhancers of NR-responsive genes for TFIIH subunits, negative controls and I decided to use NER factors. Of course, my ChIP data did not validate these controls as negative but indicated the presence of all NER factors with RNA pol II at active promoters. Retrospectively, my choice was not so wise because several previous studies already suggested an important orchestration between transcription and NER factors.
Such connection between transcription and DNA repair was firstly identified when it was shown that upon genotoxic attack, the transcribed genes were preferentially repaired (*100*). Several years later, our group demonstrated how TFIIH firstly identified as a general transcription factor was also involved in NER pathway (*29*). Later in the 90's, a RNA pol II complex containing NER factors was purified (*101*). During the last

decade, several reports rendered the involvement of NER factors in the regulation of gene expression even more obvious. In 2001, the yeast homolog of XPG was implicated in transcription (*102*). Other and we demonstrated that XPG was also able to interact, stabilize and participate to the transactivation function of TFIIH. Indeed, it has been recognized that TFIIH and the endonuclease XPG can bind relatively stably to one another (*45, 103*). The TFIIH-XPG complex can function both in DNA repair to remove the DNA damage and in transcription to phosphorylate RNA pol II (*45*). Another NER Factor, XPA-binding protein 2 (XAB2), identified by virtue of its ability to interact with XPA,(*104*) was also associated to RNA pol II participating in transcription and pre mRNA splicing (*105*).
Finally, NER factors have been rapidly associated to different steps of chromatin remodelling upon transcription. In addition to its role in transcription-coupled repair (TCR), CSB was shown to participate in several steps of the transcription process including elongation especially on chromatin remodelling through its ATPase activity (*106-108*). Moreover, a study from Niehrs showed that XPG was associated to transcription by participating with Gadd45α in active DNA demethylation process. Interestingly, such relationship between NER factors (including XPG, XPF and TFIIH) and DNA demethylation in the DNA repair pathway have been suggested even earlier in 1996 by Chu and Mayne (*109*).
My contribution in this field, simultaneously or later strengthened by studies from other laboratories (*9, 11, 12, 14, 110*), opened a new field largely not restricted to NER factors but involving many other DNA repair factors (*9*).

3.4.2 Publication: NER factors and chromatin remodelling upon transcription: the first step

Le May N., Mota-Fernandes D., Vélez-Cruz R., Iltis I., Biard D. and Egly J.M**.** "NER factors are recruited to active promoters and facilitate chromatin modification for transcription in the absence of exogenous genotoxic attack". **Molecular Cell.** 2010, 38:54-66. (*6*)

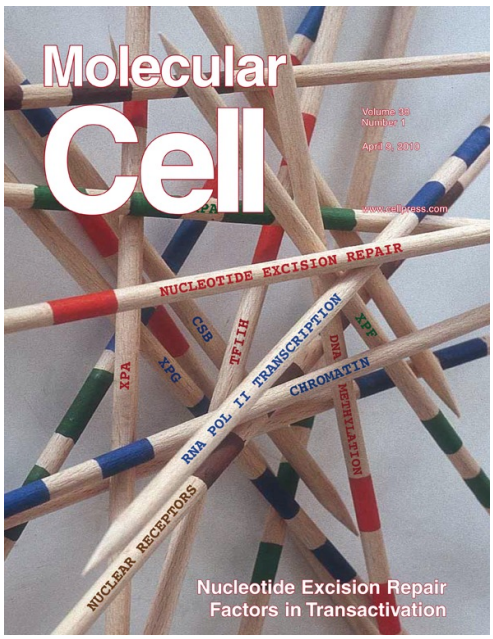

Figure 7. Molecular Cell cover in 2010 related to our article

Molecular Cell
Article

NER Factors Are Recruited to Active Promoters and Facilitate Chromatin Modification for Transcription in the Absence of Exogenous Genotoxic Attack

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SUMMARY

Upon gene activation, we found that RNA polymerase II transcription machinery assembles sequentially with the nucleotide excision repair (NER) factors at the promoter. This recruitment occurs in absence of exogenous genotoxic attack, is sensitive to transcription inhibitors, and depends on the XPC protein. The presence of these repair proteins at the promoter of activated genes is necessary in order to achieve optimal DNA demethylation and histone posttranslational modifications (H3K4/H3K9 methylation, H3K9/14 acetylation) and thus efficient RNA synthesis. Deficiencies in some NER factors impede the recruitment of others and affect nuclear receptor transactivation. Our data suggest that there is a functional difference between the presence of the NER factors at the promoters (which requires XPC) and the NER factors at the distal regions of the gene (which requires CSB). While the latter may be a repair function, the former is a function with respect to transcription unveiled in the current study.

INTRODUCTION

Protein-coding gene expression requires the presence of a battery of proteins, which includes RNA polymerase II (RNA pol II), the transcription factors (such as nuclear receptors, NR), coactivators, and mediator and histone-modifying enzymes at the promoter of activated genes. The formation of the transcription preinitiation complex (PIC) is accompanied by an important chromatin-remodeling phase including histones posttranslational modifications (PTMs) and active DNA demethylation of the promoter region. These processes produce an euchromatin environment necessary to begin the synthesis of the primary transcript (Cedar and Bergman, 2009; Li et al., 2007). If the DNA is not properly demethylated or is damaged by genotoxic agents, or if one of the members of the transcriptional machinery is missing, RNA synthesis becomes defective (Barreto et al., 2007; Gramantieri et al., 2005).

To maintain genome integrity and ensure the continuation of transcription, DNA lesions originated by UV irradiation, antitumor drugs, and other environmental products that modify the DNA structure, are eliminated through two subpathways of NER (Lindahl and Wood, 1999). Global genome repair (GGR) removes DNA damage from the entire genome, and the transcription-coupled repair (TCR) corrects DNA lesions located on the actively transcribed genes (Fousteri and Mullenders, 2008; Hanawalt and Spivak, 2008). In GGR, the removal of lesions requires their recognition by the repair factor XPC/HR23b and the subsequent opening of the DNA duplex by TFIIH (Riedl et al., 2003; Sugasawa et al., 2001). The resulting single-stranded structure is stabilized by XPA and RPA. XPG is recruited through its interaction with TFIIH on the 3′ side of the lesion and its positioning on the cut site requires RPA. The interaction between XPA and ERCC1 stimulates the recruitment of ERCC1-XPF on the 5′ side of the DNA lesion. In such condition the damaged oligonucleotide can be removed following the double incision by XPG and ERCC1-XPF endonucleases, thus allowing the DNA resynthesis (Wakasugi et al., 1997; Constantinou et al., 1999). In TCR, these factors (except XPC/HR23B) are recruited by the stalled RNA pol II in front of the damage with the help of the CSB and CSA proteins (Laine and Egly, 2006).

Mutations in 11 genes (XPA-G, ERCC1, TTD-A, CSA, and CSB), among the 30 genes involved in NER, have been associated with the human genetic disorders xeroderma pigmentosum (XP), trichothiodystrophy (TTD), cockayne syndrome (CS), and cerebro-oculo-facio-skeletal syndrome (COFS). Patients of these syndromes display a wide variety of clinical features (Lehmann, 2003; Jaspers et al., 2007**)**. XP is characterized by sun sensitivity and greater than 1000-fold increased risk of cutaneous neoplasms. Approximately 30% of affected individuals have neurologic symptoms, including acquired microcephaly, diminished deep tendon stretch reflexes, and progressive cognitive impairment. TTD is characterized by abnormally sulfur-deficient brittle hair and accompanied by ichthyosis, in addition to neurological abnormalities. CS patients are sensitive to sunlight, have short stature, and display traits of premature aging. The majority of these clinical features cannot be explained by a DNA-repair deficiency and argue for these repair factors performing others functions beyond their role in the repair process. Besides TFIIH, whose role, in transcription and DNA repair, is well accepted (Schaeffer et al., 1993), others factors

involved in NER are also linked to the transcription process. For instance, mutations in XPG disturb the architecture of TFIIH and as a consequence affect NR-dependent transactivation (Ito et al., 2007). A similar scenario was described for XPD mutations (Compe et al., 2005, 2007; Keriel et al., 2002).

We therefore questioned whether others NER factors play a role in the transcriptional process, which can be distinguished from their functional role in DNA repair. In the present study, we found that PIC formation preceded the sequential recruitment of the NER factors XPC, XPA, RPA, XPG, and XPF/ERCC1 at the promoters of inducible genes, in the absence of exogenous genotoxic attack. All these NER factors (except CSB) were required to allow histone PTMs and active DNA demethylation necessary for efficient transcription. Finally, a transcriptional dysregulation was detected in cells with silenced NER factors and cells derived from XP patients.

RESULTS

NER Factors Are Recruited on Active Promoters

Employing systems where the nuclear receptors (such as the retinoic acid receptor [RAR] and peroxisome proliferator-activated receptor [PPAR]) were the key regulatory elements, and a chromatin immunoprecipitation (ChIP)-based methodology, we studied the dynamic recruitment of RNA pol II known partners and NER factors during transcription in the absence of exogenous genotoxic attack. Eight hours posttreatment of HeLa cells with all-trans retinoic acid (t-RA), we observed the accumulation of *RARβ2* mRNA (Figure 1A). ChIP followed by quantitative PCR showed at 8 hr a concomitant recruitment of RAR, the retinoid X receptor (RXR) partner, and RNA pol II (Figures 1F and 1G), that correlates with the peak of mRNA synthesis. At this time, TFIIH was also recruited as visualized by the presence of its subunits XPB, XPD and p44 (Figure 1G). While t-RA treatment resulted in a cyclical recruitment of various components of the transactivation complex at the target *RARβ2* promoter, productive RNA synthesis only occurred at 8 hr, which coincided with the concomitant arrival of the transcription intermediary factor 2 (TIF2) coactivator, the mediator subunit Med6 (Figure 1F), Med1 and the p300 coactivator (data not shown), all involved in the formation of RAR transactivation complex. On the *RARβ2* promoter, we also detected the presence of the XPG endonuclease, which interacts with TFIIH and plays a role in transactivation (Ito et al., 2007). Unexpectedly, we also detected XPF, the other NER endonuclease (Figure 1H). Moreover, we observed the presence of XPA and RPA, two NER factors with no known function in the transcription process (Figure 1H). Surprisingly, we also detected on the promoter, the CSB protein, required for TCR, and XPC, the damage-sensing protein of GGR (Figure 1I).

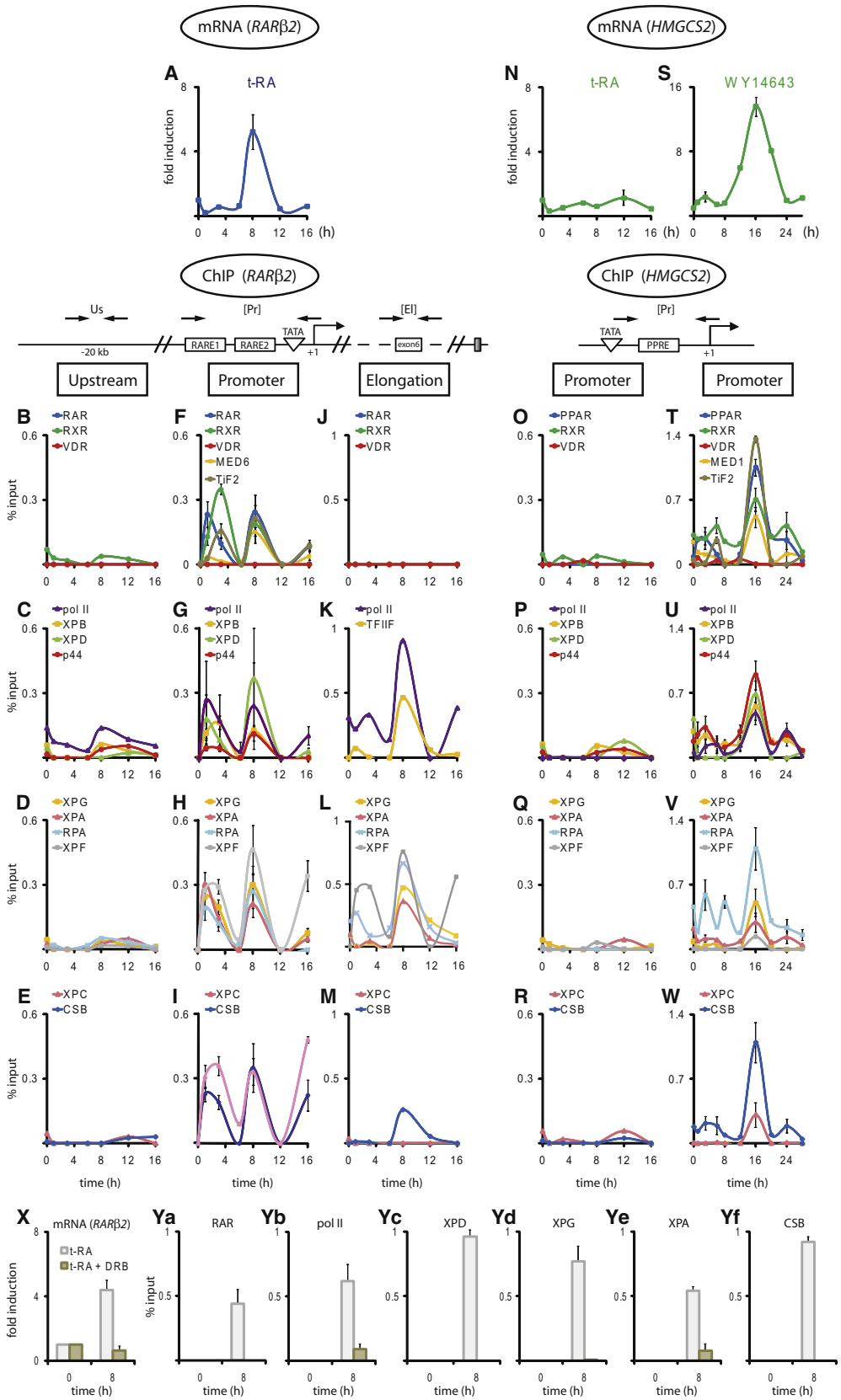
We next questioned whether the NER factors that we observed on the *RARβ2* promoter were also part of the elongating transcription machinery. ChIP analysis showed the presence of XPA, RPA, XPG, XPF, and CSB together with the elongating RNA pol II and the transcription elongation factor TFIIF at 8 hr after t-RA treatment, on exon 3, 4 (Figures S1B–S1G), and exon 6 (Figures 1K–1M). Neither RAR nor RXR were detected at the elongation regions of the *RARβ2* gene (Figure 1J). It must be noticed that under our experimental conditions, we only detected background levels of XPC at the elongation regions (Figure 1M, S1D, and S1G). To ensure that the recruitment of the repair factors was specific to the transactivation process, we analyzed the promoter of an inactive gene. In t-RA-treated HeLa cells (in which PPARα is not expressed), neither the transcription machinery nor the NER factors were found associated to the promoter of 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 *(HMGCS2)*, a PPAR-responsive gene (Figures 1O–1R), and no accumulation of mRNA synthesis was observed (Figure 1N). On the contrary, when cells were transfected with a PPARα-expressing vector and treated with WY14643 (PPARα-specific ligand), *HMGCS2* mRNA was highly expressed at 16 hr posttreatment (Figure 1S). Concomitantly, we observed the recruitment of the transcription and all NER factors, together with PPAR/RXR on the promoter (Figures 1T–1W) and on the elongation regions (Figures S1I–S1L). To further demonstrate that the presence of the NER factors at the promoter did reflect a real association with the transcription machinery, we designed several control experiments. To test our ChIP protocol, we analyzed the presence of two unrelated proteins on the promoters of *RARβ2*/*HMGCS2*. Neither cyclin A (data not shown) nor the vitamin D receptor (VDR) (endogeneously expressed in HeLa cells) were present either at the promoter or elongating regions (Figures 1F, 1J, 1O, 1T, S1B, S1E, and S1I). Second, although we detected low amounts of RNA pol II far upstream from the *RARβ2* initiation site (−20 kb), none of the factors required either for the formation of the RAR/RXR-dependent transactivation complex or for the NER reaction were detected at the *RARβ2* upstream promoter region (Figures 1B–1E). We cannot exclude the possibility that the low amounts of RNA pol II detected upstream the *RARβ2* gene represent another transcription complex of a gene not currently identified.

Simultaneous treatments of HeLa cells with the transcription inhibitor 5,6-Dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) and t-RA, resulted in the inhibition of the *RARβ2* mRNA synthesis (Figure 1X). Under those conditions, neither RNA pol II nor NER factors were detected on *RARβ2* promoter (Figures 1Ya–1Yf) and exon 6 (Figure S1N). Similar results were obtained when HeLa cells were treated with α-amanitin, another transcription inhibitor, and simultaneously the t-RA ligand (Figures S1O and S1P).

Altogether, our data show that NER factors were recruited with RNA pol II at the promoters of activated genes and that this recruitment is specific to the transactivation process and sensitive to transcription inhibitors. In addition to *RARβ2* and *HMGCS2* genes, it is important to note that all these repair factors together with the transcription apparatus were observed upon activation of all the genes so far tested such as *RARα2*, the Vitamin D responsive *Cyp24* as well as the PPAR responsive liver fatty acid binding protein *LFABP* (data not shown).

The NER Factors Associated to RNA Pol II Are Discriminated from a Repair Complex

We next questioned whether the NER factors that assembled on promoters of inducible genes could be distinguished from a "DNA repair complex" formed upon genotoxic attack. First,


mRNA (RARβ2)
mRNA (HMGCS2)
A
t-RA
fold induction
N
t-RA
S
WY14643
ChIP (RARβ2)
ChIP (HMGCS2)
Us
[Pr]
[El]
-20 kb
RARE1
RARE2
TATA
+1
exon6
PPRE
Upstream
Promoter
Elongation
Promoter
Promoter
B
RAR
RXR
VDR
F
MED6
TiF2
J
O
PPAR
T
MED1
% input
C
pol II
XPB
XPD
p44
G
K
TFIIF
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XPG
XPA
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XPF
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XPC
CSB
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time (h)
X
mRNA (RARβ2)
t-RA
t-RA + DRB
Ya
RAR
Yb
pol II
Yc
XPD
Yd
XPG
Ye
XPA
Yf
CSB

HeLa cells were UV irradiated and treated with t-RA simultaneously. Under these conditions, expression of the growth arrest DNA damage-inducible 45 alpha *(GADD45α)*, as well as the human double minute 2 (*hDM2*), two UV-inducible genes (Adimoolam and Ford, 2002), was elevated at 4 hr posttreatment (Figure 2A) (data not shown). The *RARβ2* gene expression peak, originally found at 8 hr post-t-RA treatment, was delayed to 16 hr (Figure 2E, compared blue dotted line with blue line respectively; see also Figure 1A). This delay may be due (at least in part) to the priority of the DNA repair process (Smith and Hanawalt, 1978; Friedberg et al., 1995). ChIP analysis performed over time further showed that RNA pol II, TFIIH (XPB, XPD, and cdk7), as well as all XPC, XPA, RPA, XPG, and CSB were recruited at 4 hr at the *GADD45α* promoter together with p53 (Figures 2B–2D), and at 16 hr to the *RARβ2* gene promoter together with RAR (Figures 2F–2H).

At this stage of our work, we could speculate that NER factors recruitment reflected repair event at the analyzed promoters. However, it seems hard to conceive that *GADD45α* as well as all the promoters so far tested were damaged under our experimental conditions and consequently subjected to either a GGR and/or a TCR process. Indeed, we have also observed the corecruitment of NER factors with RNA pol II machinery at the dihydrofolate reductase (*DHFR*) housekeeping gene (data not shown). Additionally, it is worthwhile to notice that none of the PPAR, TFIIH, RNA pol II transcriptional components and NER factors was detected at the promoter of the nonactivated *HMGCS2* gene (Figures 2I–2L).

ChIP/reChIP analysis using specific antibodies showed that TFIIH and RNA pol II (ChIP-cdk7/reChIP-pol II), XPA and RNA pol II (ChIP-XPA/reChIP-pol II), as well as TFIIH and XPA (ChIP-XPB/reChIP-XPA and ChIP-cdk7/reChIP-XPA) co-occupied both the *GADD45α* and *RARβ2* promoters at 4 hr and 16 hr post-UV irradiation and ligand treatment (Figures 2M and 2O). To validate our ChIP and ChIP/reChIP data, we used a combination of antibodies against an unrelated protein (VDR, see also Figures 2B, 2F, and 2J) and either XPA, RNA pol II, XPB, or cdk7. We were not able to detect any co-occupancy between VDR and either XPA, TFIIH (XPB and cdk7) or RNA pol II (Figures 2N and 2P). It is worthwhile to notice that the presence of XPA together with CAK/cdk7 (which is crucial for transcription) and the transcriptional machinery at the activated RARβ2 promoter, argue for a complex unrelated to a GGR complex (see also below). Indeed, in GGR, the presence of XPA and cdk7 is mutually exclusive: the arrival of XPA at the damage sites leads to the removal of CAK from TFIIH core (Coin et al., 2008).

To further test the idea that NER factors containing transcription complex is different to the "repair complexes," we designed a second set of experiments. MRC5 cells were UV irradiated and treated with the transcription inhibitor DRB. Under these conditions, and as previously observed for the *RARβ2* gene (Figures 1X–1Yf), transcription of *GADD45α* gene was inhibited contrary to what occurred in cells only treated with UV in which the *GADD45α* mRNA synthesis peaks at 2 hr (Figures 3A and 3B, blue dotted histograms). At that time, in the UV-irradiated cells, our ChIP and ChIP/reChIP analysis demonstrated that RNA pol II, NER factors as well as p53 transcription factor, were corecruited and co-occupied *GADD45α* promoter (Figure 3A, colored histograms; Figures S2C–S2F) as similarly observed in UV-irradiated and t-RA treated HeLa cells (Figures 2B–2D). This corecruitment of RNA pol II/NER factors and RNA pol II/TFIIH was lost in UV-irradiated MRC5 simultaneously treated with DRB (Figures 3B and S2G–S2J), thus explaining the absence of mRNA synthesis. ChIP using antibodies against XPB, coupled to Western blot (Fousteri et al., 2006; Coin et al., 2008), allowed us to observe the formation and the composition of the NER repair complexes between 0.25 and 2/4 hr post UV irradiation on the chromatin extract (Figure 3C, lanes 2–6 and curves). These repair complexes were observed even when the cells were simultaneously treated with the transcription inhibitor DRB and UV (Figure 3D, lanes 2–6 and curves). Both in the presence and absence of DRB, we clearly observed the arrival of the NER factors such as RPA and XPF concomitantly to the release of the CAK subcomplex from TFIIH (Figures 3C and 3D compared with Figures S2K and S2L for the specificity of the immunoprecipitation). To further support our results suggesting that NER complexes at promoter are different then "repair complexes," we used local UV irradiation technology combined with fluorescent immunostaining (Volker et al., 2001). Analysis of locally UV-irradiated MRC5 cells by confocal microscopy showed that XPC, XPB/TFIIH, and XPA colocalized at sites of UV damage even in the presence of DRB (Figures 3E and 3F).

Altogether, our data demonstrate that a GGR complex is resistant to transcription inhibitors whereas the transcription complex with NER factors we describe here is sensitive to DRB. Our results suggest that the NER factors-containing transcription complex is not a NER repair complex.

Deficiencies in Some NER Factors Impede the Recruitment of Others

We next investigated how either the silencing of XPC, XPA, ERCC1 or XPG (using the corresponding "silencix HeLa" cells

Figure 1. NER Factors Form Part of the Initiating and Elongating Transcription Machinery

(A–M) Relative mRNA expression of *RARβ2* gene from HeLa cells treated with t-RA (1 μM) (A). Error bars represent the standard deviation of three independent experiments. Schematic representation of the *RARβ2* gene with the indicated amplicons designed at the upstream (Us), promoter (Pr), and elongation (El, exon 6) regions. ChIP monitoring the t-RA-dependent occupancy of: RAR, RXR, VDR, TIF2, and Med 6 (B, F, and J); RNA pol II, TFIIH subunits (XPD, XPB, and p44) and TFIIF (C, G, and K); XPG, XPA, RPA and XPF (D, H, and L); XPC and CSB (E, I, and M), on the different amplicons of RARβ2 gene.

(N–Y) Relative mRNA expression of *HMGCS2* gene from HeLa cells treated either with t-RA (N) or with WY14643 (1 μM) and transfected with the pSG5-PPARα (S). Error bars represent the standard deviation of three independent experiments. ChIP monitoring the t-RA- and WY1464 -dependent occupancy of PPAR, RXR, VDR, TIF2, and Med1 (O and T); RNA pol II and TFIIH subunits (XPD, XPB, and p44) (P and U); XPG, XPA, RPA, and XPF (Q and V); XPC and CSB (R and W), on the *HMGCS2* promoter (Pr). Each series of ChIP is representative of at least two independent experiments. Values are expressed as percent input, which are the average of at least two qPCR reactions and error is within 10%. Relative RARβ2 mRNA expression (X) and ChIP monitoring the occupancy of RAR, RNA pol II, XPD, XPG, XPA, and CSB (Ya–Yf) on the RARβ2 promoter in t-RA treated HeLa cells in absence (gray histogram) or presence (green histogram) of DRB (100 μM).

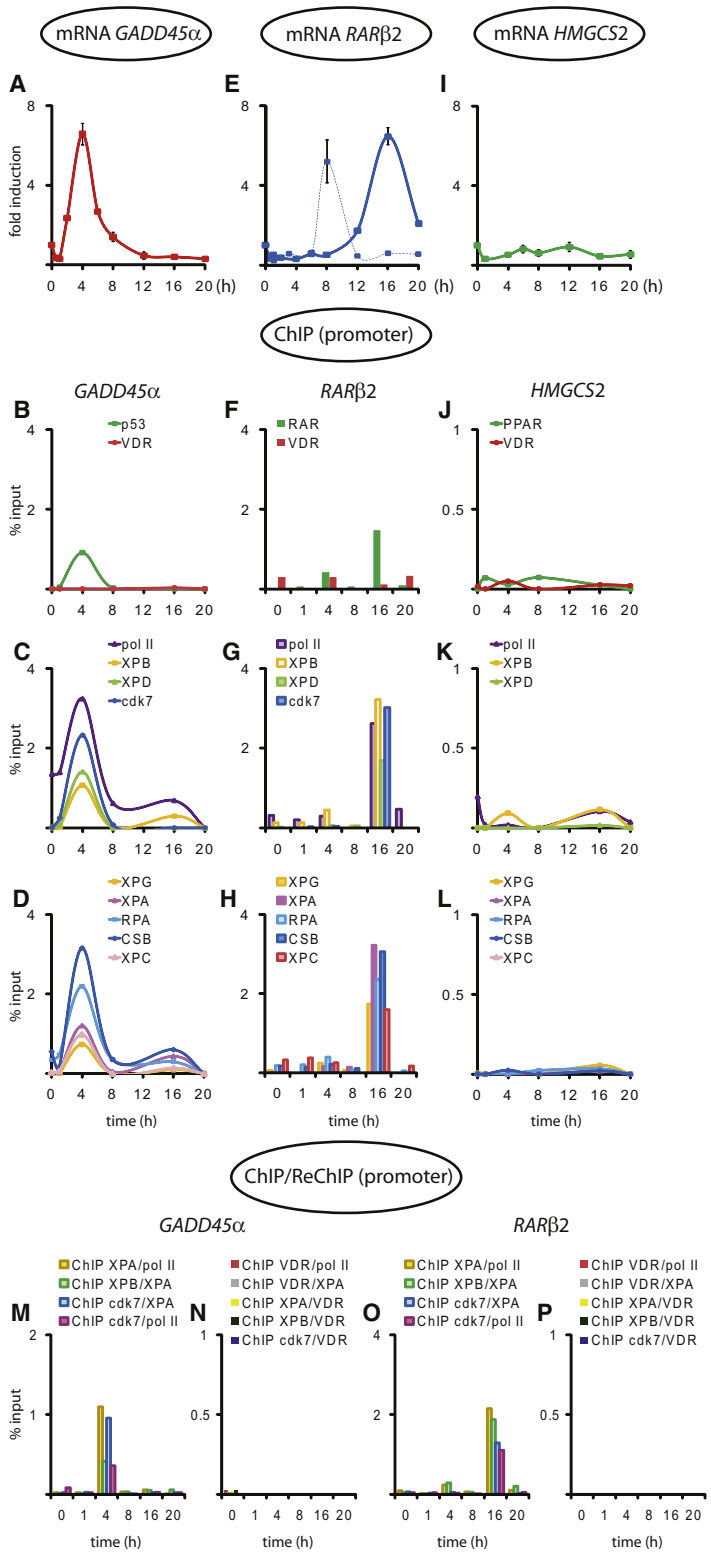

Figure 2. NER Factors Are Corecruited with RNA pol II Machinery after UV Irradiation

(A–L) Relative mRNA expression of GADD45α (A), RARβ2 (E), and HMGCS2 (I) genes monitored by qPCR from UV-irradiated (20 J/m^2) HeLa cells treated with t-RA (1 μM). Relative mRNA expression of RARβ2 gene measured by qPCR from t-RA-treated cells (E, dotted blue line). A dose of 20 J/m^2 generates around two photolesions per 10 kb of genomic DNA (van Hoffen et al., 1995). ChIP monitoring the UV- and t-RA-dependent recruitment of p53, RAR, PPAR, and VDR (B, F, and J) as indicated; RNA pol II and TFIIH subunits (XPB, XPD, cdk7) (C, G, and K); XPG, XPA, RPA, CSB and XPC (D, H, and L), on the *GADD45α* (curves), *RARβ2* (histograms), and *HMGCS2* (curves) promoters. Each series of ChIPs is representative of two independent experiments. Values are expressed as percent input, as previously.

(M–P) For ChIP/ReChIP experiments on *GADD45α* and *RARβ2* promoters, samples were subjected to either (M and O) a first IP against XPA, XPB, or cdk7 and then purified complexes were further subjected to a second IP using antibodies against either pol II or XPA or (N and P) a first IP using an antibody against an unrelated protein (VDR) and then purified complexes were subjected to a second antibody against either XPA, RNA pol II, XPB, or cdk7 or vice versa as indicated.

that stably express siRNA and abolish their expression, see Figure S3), or mutations in XPC, XPA, XPG, and CSB (using fibroblasts derived from XP or CS patients) would affect the t-RA dependent *RARβ2* transactivation.

First, in SiXPC, -XPA, and -ERCC1 cells (Figure 4A) as well as in XPC/579st and XPA/R207st cells (Figures 5Aa and 5Ba), we observed a significant defect in the *RARβ2* mRNA expression compared to either the SiControl or the rescued cells, respectively. ChIP analysis from each of the defective cells, treated with t-RA, showed that, similarly to what was observed in SiControl, RAR, RXR, TFIIH (XPB and cdk7), and RNA pol II were concomitantly recruited to the *RARβ2* promoter (Figures 4B–4I, S4B, and S4C).

Contrary to the coordinated recruitment of XPC, XPA, XPG, XPF, and DDB1 (known to facilitate the recruitment of XPC on damaged chromatin [Fitch et al., 2003]), with the RNA pol II machinery observed in the SiControl cells, none of the NER factors were observed at the promoter in SiXPC cells (compare Figures 4J, 4N, and 4R with 4K, 4O, and 4S).

In absence of XPA (SiXPA), only XPC and CSB were detected at the promoter but neither XPF nor XPG (Figures 4L and 4P; see also Figures 4D and 4H). Remarkably, in patients fibroblasts bearing mutations on either XPC or XPA compared to XPA rescued cells, we observed a similar defect in the recruitment of NER factors on the *RARβ2* promoter while the transcriptional machinery was present (Figures 5Ab–5Ae, 5Bb–5Be, and 5Cb–5Ce). Interestingly, in SiERCC1 cells, where expression of ERCC1 and its partner XPF were silenced (Figure S3) (Gaillard and Wood, 2001), we detected XPC, XPA, and XPG together with RAR, RXR, RNA pol II, and TFIIH (Figures 4E, 4I, 4M, and 4Q). On both SiXPG and XP-G fibroblasts derived from XPCS1RO patient, all the NER factors (except XPG) were recruited to the corresponding activated promoters (Figures S4F–S4N). We also analyzed the consequences of CSB mutations in CS1AN fibroblasts in which the Q336st mutation abolished CSB expression (Figure S3). When compared to the CSB rescued cells, CSB-deficient cells

displayed a similar RARβ2 mRNA expression pattern (Figure 5Da). Moreover, the absence of CSB protein did not prevent the recruitment of XPC, XPA, XPG, and XPF together with the transcriptional machinery on the *RARβ2* promoter (compare Figures 5Db–5De with Figures 5Eb–5Ee).

Altogether, our data showed first that the recruitment of the basal transcription machinery occurred even in the absence of the NER factors and second that the NER factors were assembled on the promoter in the following order: XPC, XPA, followed by XPG and XPF. Moreover, it seems that the absence of CSB (required for TCR) was not required for the recruitment of the others NER factors and the *RARβ2* mRNA synthesis. This suggests, once again, that the transcription complex associated to NER factors at the promoter of activated gene can be discriminated from a DNA repair complex, including a TCR complex.

NER Factors and Gadd45α Facilitate DNA Demethylation and Histones PTMs

Recent studies revealed that the Gadd45α protein required XPG during active DNA demethylation, an essential step for efficient transcription (Barreto et al., 2007; Gramantieri et al., 2005). We thus investigated whether a defect in the recruitment of the NER factors on the *RARβ2* promoter could affect the Gadd45α recruitment. ChIP analysis showed a recruitment of Gadd45α concomitant with the NER factors on *RARβ2* promoter in SiControl cells (Figures 4N and S4E). In SiXPC, SiXPA, as well as in XPC/579st and XPA/R207st cells, Gadd45α was not detected together with the other components at the promoter (Figures 4O, 4P, 5Ae, 5Be). Surprisingly, we observed its presence together either with XPG in SiERCC1 cells or with XPF in SiXPG and in XPG-deficient cells (Figures 4Q, S4J, and S4N). In CSB-deficient as well in CSB-rescued cells, Gadd45α was recruited together with all the NER factors on the active *RARβ2* promoter (Figures 5De and 5Ee).

We next investigated whether the simultaneous presence of Gadd45α together with the NER factors was necessary to replace the methylated cytosines by unmethylated ones required for efficient transcription initiation (Appanah et al., 2007). Since hypo- and hypermethylation of CpG dinucleotides across the genome contribute to changes in gene expression, we used the Imprint Methylated DNA quantification technique to measure global DNA methylation shifts. We first found that the global methylation level is much higher in genomic DNA from SiXPC, SiXPA, SiERCC1, and SiXPG HeLa cells compared to the SiControl cells, whereas no differences were observed between CSB-mutated patient fibroblasts and the corresponding rescued cells (Figures S5A and S5B, respectively).

We then used the methylation-sensitive enzymes HpaI*I* and *Dpn I* to evaluate the methylated status of CpGs islands localized in the *RARβ2* promoter surroundings (Figure 4, lower panel) (Appanah et al., 2007; Santoro and Grummt, 2001). In wild-type cells (SiControl) as well in CS1AN, CS1AN/CSB, and XP12RO/XPA-rescued cells, the presence of all NER factors together with the RNA pol II machinery and Gadd45α, at 6/8 hr post t-RA induction, was accompanied by a significant increase in the *Dpn I* sensitivity (thus DNA demethylation), while no variation was obtained with Hpa II (Figures 4V, 5Cg, 5Dg, and 5Eg). This reflected a decrease of methylation in CpG islands located in the 3′-proximal promoter region. On the contrary in SiXPC, SiXPA, as well as in XPC/579st and XPA/R207st cells, in which we observed a defect in the recruitment of XPG, XPF and also the Gadd45α protein, the CpG islands were not digested by *Dpn I* (Figures 4W, 4X, 5Ag, and 5Bg). Interestingly, in SiERCC1, in which XPG together with Gadd45α were recruited, no significant changes in the CpG islands methylation were detected (Figure 4Y). This also occurs in SiXPG, where XPF together with Gadd45α were recruited at the *RARβ2* promoter (data not shown).

DNA methylation and histones PTMs are dependent on one another. Hypermethylated CpG islands have been associated with a heterochromatin landmark such as di-/trimethylation of histone H3K9 (H3K9me); conversely, di-/trimethylation of histone H3K4 (H3K4me), acetylation of H3K9/14 (H3K9/14ac), related to active transcription, are accompanied by DNA hypomethylation (Cedar and Bergman, 2009; Hashimshony et al., 2003). We thus questioned whether a defect in the active demethylation (in absence of recruitment of NER factors) would also disturb histones PTMs. ChIP analysis revealed that in WT (SiControl) cells, the recruitment of the NER factors, Gadd45α, and RNA pol II paralleled an increase of H3K4me, H3K9/14ac concomitantly to a decrease of H3K9me (Figure 4R). This profile was repeatedly observed at 8 hr in both CSB, XPA rescued, and CSB-deficient cells (Figures 5Cf, 5Df, and 5Ef). These "signatures" in histone H3 modifications were not observed in SiXPC, SiXPA, and SiERCC1 cells or in XPC/579st and XPA/R207st cells (Figures 4S, 4T, 4U, 5Af, and Bf). In those cells, we observed an increase of H3K9me and a decrease of H3K4me that paralleled the recruitment of transcriptional machinery, contrary to what occurs in wild-type cells.

The above results strongly suggest that in addition to XPG, other NER factors (XPC, XPA, XPF/ERCC1, except CSB) are necessary for the Gadd45α recruitment to the promoter of activated genes and the concomitant occurrence of DNA demethylation and histones PTMs.

DISCUSSION

Synthesis of mRNA is the result of a cascade of events that requires more than ~200 proteins that constitute the transcriptional machinery (Brivanlou and Darnell, 2002; Kornberg, 2007). How are each of these proteins connected in this intricate network to initiate transcription, at the right time and in the proper cell, would help to further our understanding of gene expression regulation.

The NER Factors Are Sequentially Recruited to the Activated Genes

In the present study we demonstrate that all the NER factors are associated with the transcription machinery on the promoters of active genes (in the absence of exogenous genotoxic attack) and escort the elongating RNA polymerase (Figure 1). The recruitment of the NER factors and their association with RNA pol II is abolished in the presence of the transcription inhibitor DRB (Figure 1). The presence of these factors is specific to factors that are involved in NER, since the base excision repair and mismatch repair proteins Ogg1 glycosylase and MutS homolog 2 factor (MSH2), respectively, were not detected (Figure S1Q).

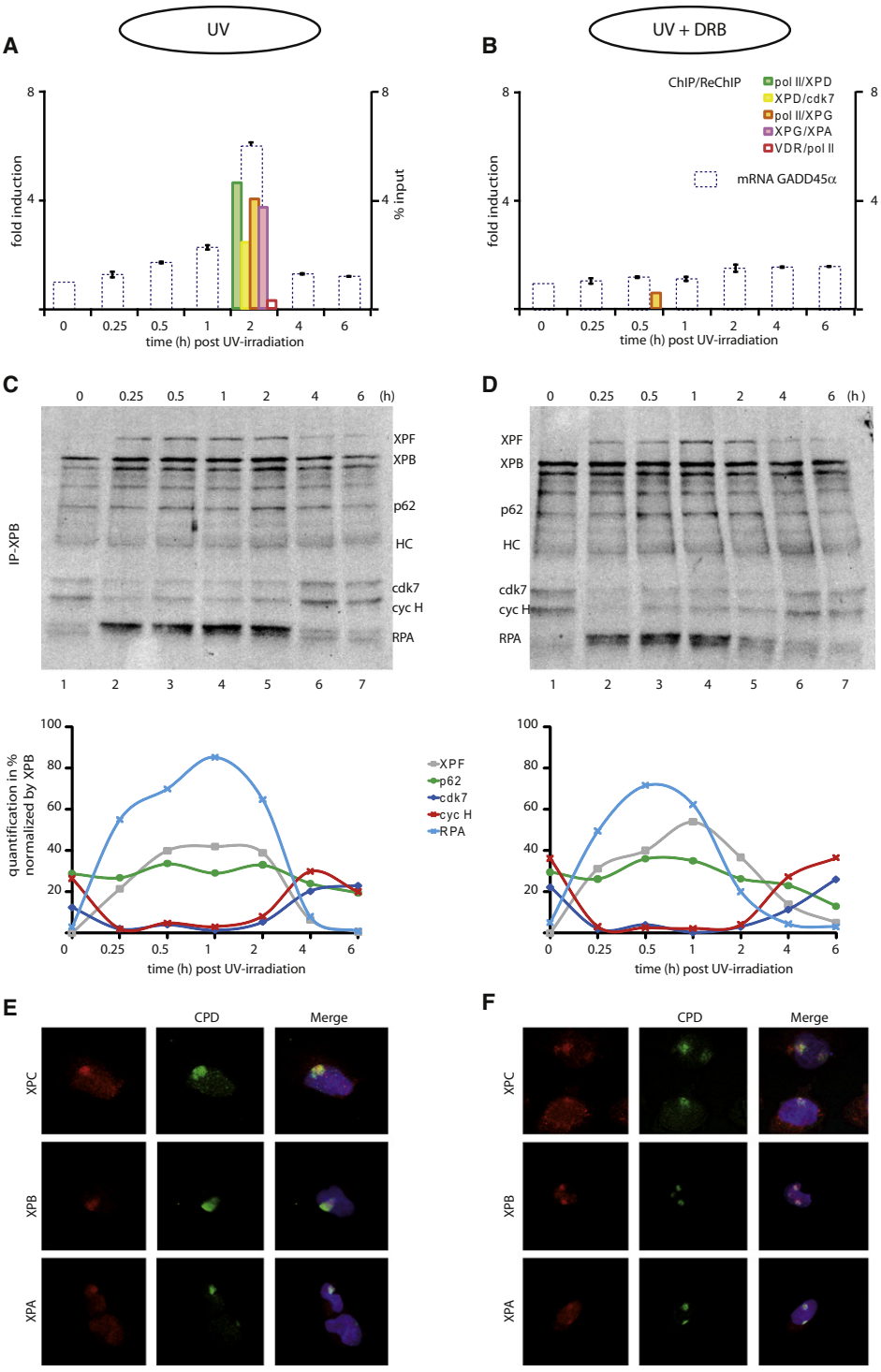

Figure 3. NER Factors-Containing Transcriptional Complex Can Be Discriminated from a DNA Repair Complex

(A and B) Relative GADD45α mRNA expression (blue dotted histograms) and ChIP/ReChIP (colored histograms) monitoring the coimmunoprecipitation of the corresponding promoter using antibodies combinations against RNA pol II/ XPD, XPD/cdk7, RNA pol II/XPG, XPG/XPA, and VDR/pol II from UV-irradiated (20 J/m^2) MRC5 fibroblasts in absence (A) or presence (B) of DRB (100 μM), harvested at indicated times.

(C and D) Western-blotting analysis of Ab-XPB ChIP samples from chromatin extracts of MRC5 fibroblasts incubated overtime after UV irradiation, in absence (C) or pretreated with DRB (100 μM) during 6 hr (D). The WB signals for XPF, XPB, p62, cdk7, cyc H, and RPA were quantified using Genetool and plotted on the graphs. For each single lane, XPB was used as reference. HC indicates the heavy chain of the antibody.

Moreover, silencing or mutations in these factors revealed that the NER factors are sequentially recruited to these promoters in the following order: XPC, XPA, and XPG, XPF/ERCC1/Gadd45α, once the transcription machinery is positioned into place (Figures 4, and 5; see also model Figure 6).

One thus can wonder whether this "entourage" of the RNA pol II would render the transcription machinery equipped to deal with potential threats (such as DNA lesions) or whether these factors participate in a mechanism that renders these promoters ready for efficient RNA synthesis. Importantly, this transcriptional complex equipped with NER factors is distinguishable from a repair complex, whether it would be TCR or GGR, in several ways. First, this transcription complex is sensitive to the transcription inhibitor DRB, whereas GGR is not (Figure 3). Second, this transcription complex does not require the CSB protein, whereas this protein is absolutely required for TCR (Figure 5). Third, this transcription complex requires XPC, whereas this protein is dispensable for TCR (Figures 4 and 5). Therefore, our data indicates that this transcription complex loaded with NER factors is different to a repair complex based on its composition and its sensitivity to transcription inhibitors.

Despite these results, two lines of evidence are surprising; in the absence of the CSB protein, we did not observe the presence of the NER factors at the distal regions of the genes (i.e., escorting the elongating RNA pol II) and in the absence of XPC, we did not observe the recruitment of the NER factors at the promoters of activated genes (Figure S6). Although these results may suggest that we are observing a TCR complex, analysis of the transactivation capacity of XP-C cells showed disrupted transcription, whereas the same analysis of CS-B cells (TCR deficient) did not show a transcriptional deficiency. Altogether, these data suggest that there is a functional difference between the presence of the NER factors at the promoters (which requires XPC) and the NER factors at the distal regions of the gene (which requires CSB). While the latter may be a repair function, the former is a function with respect to transcription unveiled in the current study.

The NER Factors Are Part of the Transcription Process

We then raised the question of the potential role of the NER factors in transcription at the promoters of active genes. The formation of the PIC is accompanied by important chromatin remodeling resulting from histone PTMs and DNA demethylation (Li et al., 2007). In the present study we observed the concomitant recruitment of the transcription machinery and the NER factors, Gadd45α with the 3′-proximal promoter DNA demethylation, histone H3K4 di-/trimethylation, H3K9 demethylation, and H3K9/K14 acetylation (Figures 4 and 5) as well as H4K16 acetylation and H2B monoubiquitination (Figure S7) at active promoters. These chromatin "signatures" were not observed when one of the NER factors was absent (Figures 4 and 5). Indeed, in NER-deficient cells we observed unchanged DNA methylation levels and histones PTMs representative of a heterochromatin environment surrounding the promoters of activated genes, which could explain the dysregulation in mRNA synthesis despite the presence of the transcriptional machinery. It is possible that the various other enzymatic activities of the NER factors, such as the ATPase, helicase, kinase, E3-ubiquitin ligase, and/or endonuclease may participate in some local chromatin modifications as well.

The Transcriptional Phenotype of the XP Patients

This study demonstrates that others NER factors play a role in the transcription process. Although these factors may not be essential for the PIC formation, they clearly fine-tune the transactivation to an optimal level. This optimization may be very important during development and can potentially explain the clinical symptoms observed in XP patients that cannot be explained by a DNA-repair deficiency. For instance, a significant fraction of XP-A patients display neurological abnormalities and developmental problems. Why do these patients not display a more severe phenotype, like CS or XP/CS patients? We know XPB and XPD (thus TFIIH) are essential for the transcription process (Coin et al., 1999). TFIIH phosphorylates transcriptional activators and RNA pol II (Keriel et al., 2002; Lu et al., 1992), all of which are crucial for the formation of the PIC, and thus for RNA synthesis. On the other hand, we observed in the current work that XPA, XPC, and XPF are involved in the transcription process in a more indirect way and are thus less critical for this process (since the PIC formation is not affected). The stage at which these factors function during transcription may explain the differences in spectrum and severity between patients from different XP groups. There is also the possibility that others factors can perform redundant functions to optimize the transcription process. Finally, we should not ignore the fact that the phenotypes of XP patients are broad and although we categorize patients for a single mutation, recent work has shown that the secondary allele may also have an effect in the clinical symptoms (Ueda et al., 2009).

To summarize, our work allows us to propose a model in which, upon gene activation and in the absence of exogeneous genotoxic attack, RNA pol II machinery, including TFIIH (XPB/XPD), is recruited at the promoter followed by the sequential arrival of the NER factors, concomitant histone modifications, and promoter DNA demethylation required for optimal RNA synthesis (Figure 6). Interestingly, this work points out once more the similarities between RNA pol II and RNA pol I transcription, which share in addition to some basal transcription factors, NER factors such as CSB, TFIIH, and XPG as well as Gadd45α (Cavallini et al., 1988; Eberhard et al., 1993; Iben et al., 2002). This may force us to question whether the role of XPC, XPA, XPG, and XPF/ERCC1 would be first transcriptional and then upon genotoxic attack could also be required for elimination of DNA lesions. Further studies have to be engaged to dissect the specific function of the previously known "repair factors" in the various steps of the transcriptional process.

(E and F) MRC5 cells were treated or not by DRB (100 μM), as indicated (E and F), during 6 hr and UV irradiated with 70 J/m^2 through a 6 mm pore filter and fixed 30 min later. Immunofluorescent labeling was performed using rabbit polyclonal anti-XPC, anti-XPB, anti-XPA, and mouse monoclonal anti-cyclobutane pyrimidine dimers (CPD) antibodies. Nuclei were counterstained with DAPI and slides were merged.

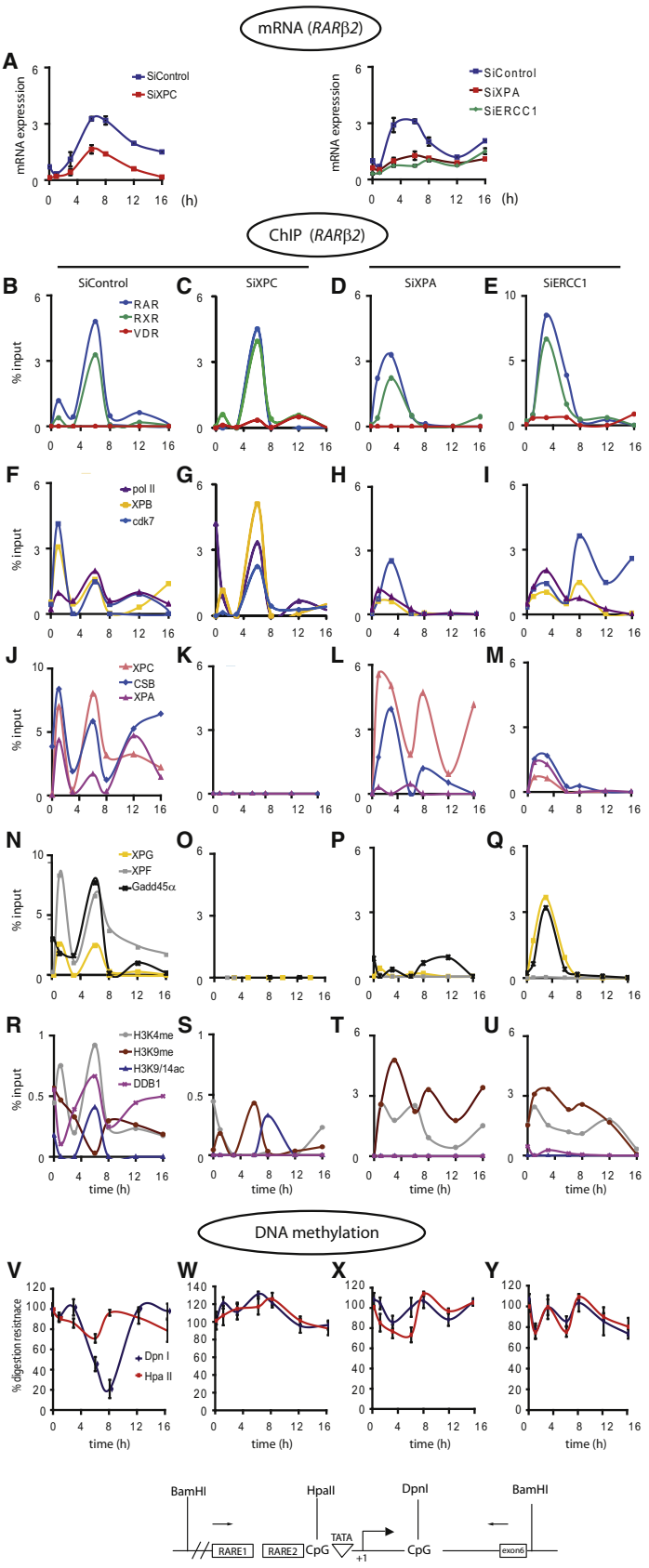

Figure 4. Sequential Recruitment of NER Factors on RARβ2 Promoter Necessary for Histones PTMs and Active DNA Demethylation

(A–Y) Relative RARβ2 mRNA expression monitored by qPCR from stable SiControl, SiXPC, SiXPA, and SiERCC1 HeLa cell lines treated with t-RA (1 μM). ChIP monitoring the t-RA-dependent occupancy of RAR, RXR, VDR (B–E), RNA pol II, XPB, cdk7 (F–I), XPC, CSB, XPA (J–M), XPG, XPF, Gadd45α (N–Q) and di-/trimethylated histones H3K9, H3K4, acetylated H3K9/14, and DDB1 (R–U) on RARβ2 promoter from stable SiControl, SiXPC, SiXPA, and SiERCC1 HeLa chromatin extracts as indicated. Schematic representation of the *RARβ2* promoter with the indicated CpG islands, restriction enzymes, and primers used to evaluate the methylation status (lower panel). Methylation of CpG islands of RARβ2 promoter was determined by measuring the ratio of PCR products obtained after digestion by BamHI/Hpa II, BamHI/ *DpnI*, or just BamHI of genomic DNA of t-RA treated stable SiControl (V), SiXPC (W), SiXPA (X), and SiERCC1 (Y) HeLa cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents

HeLa, HeLa SiXPG (gift from R. Tanaka), XPC HeLa Silencix (SiXPC), XPA HeLa Silencix (SiXPA), ERCC1 HeLa Silencix (SiERCC1), Control HeLa Silencix (SiControl), XP12RO (XPA/R207st) (Satokata et al., 1992), GM14867 (XP-C/R579st) (Chavanne et al., 2000), XPCS1RO (XP-G/frameshift 925) (Hamel et al., 1996), CS1AN (CSB/Q336st) (Troelstra et al., 1992) fibroblasts derived from XP and CS patients and the corresponding rescued cells were cultured in the appropriate medium.

Cells, treated with 1 or 10 μM all-trans retinoic acid (Biomol), were incubated with red phenol-free medium containing 10% charcoal treated Fetal Calf Serum (FCS) and 40 μg/ml gentamycin during 12 hr prior to the treatment.

HeLa cells were rinsed with PBS, UV-irradiated (20 J/m^2) and treated with t-RA (1 μM). HeLa cells were transfected by pSG5-PPARα with JetPei (Poly-Plus) and treated 24 hr later with 1 μM of WY-14643 ligand (Calbiochem) in a red phenol-free medium containing 10% delipidated FCS, 40 μg/ml gentamycin. To inhibit transcription, 5,6-Dichloro-1-beta-D-Ribofuranosyl-benzimidazole (DRB) (100 μM) or α-amanitin (10 μg/μl) (Calbiochem) were added to media during 6 hr in addition to t-RA treatment.

Antibodies

Monoclonal antibodies against the TFIIH subunits XPB (1B3), XPD (2F6), p62 (3C9), p44 (1H5), cdk7 (2F8), cyclin H (2D4), RNA polymerase II (7C2), TBP (3G3), TFIIF (2A3), RXR (3A2), RAR (9A6), XPG (1B5), CSB (1A11/3H8), XPA (1E11), RPA (1E9), MED1 (1A10), TIF2 (1D12), and methylated H3K4 (2A12) acetylated H3K9 (IGBMC). Polyclonal antibodies against TFIIH subunit cdk7 (C-19), XPB (S-19), PPARα (H-98), VDR (H-81), MED6 (C-16), Gadd45α (C-20), and DDB1 (V-17), XPF (H-300), XPC (D-18), MSH2 (N-20), Ogg1 (N-20) were from Santa-Cruz Biotechnology. Antibodies against XPF (H5), monoubiquitinated H2B (uH2B), di-methyl H3K9, acetylated H3K9/14 and acetylated H4K16 were obtained from ThermoScientific, Medimabs, Cell Signaling Technology and Epigentek, respectively.

Reverse Transcription and Quantitative PCR

Total RNA was isolated using a GenElute Mammalian Total RNA Miniprep kit (Sigma) and reverse transcribed with SuperScript II reverse transcriptase (Invitrogen). The quantitative PCR was done using the QuantiTect SYBR Green PCR kit (QIAGEN) and the Lightcycler 480 (Roche). The primer sequences for *RARβ2*, *GADD45α*, *HMGCS2*, and Glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)* genes used in real-time PCR are available upon request. *RARβ2*, *GADD45α*, and *HMGCS2* mRNA levels represent the ratio between values obtained from treated and untreated cells normalized against the housekeeping *GAPDH* mRNA.

Chromatin Immunoprecipitation (ChIP)

Cells were crosslinked at room temperature for 10 min with 1% formaldehyde. Chromatin was prepared (Drane et al., 2004) and sonicated on ice 20 min using a Bioruptor (Diagenode, Liège, Belgium). Samples were immunoprecipitated (IP) with antibodies at 4°C overnight and protein G-Sepharose beads (Upstate,Bellerica, MA) were added, incubated 4 hr at 4°C and sequentially washed. For ChIP/ReChIP experiments, after the first immunoprecipitation and washes, protein-DNA complexes were eluted with a 10 mM DTT solution and diluted before addition of antibodies and protein G-sepharose beads for the second immunoprecipitation. The complexes were eluted and the cross-linking was heat reversed. DNA fragments were purified using QIAquick PCR purification kit (QIAGEN) and analyzed by real-time PCR using sets of primers, available upon request, targeting different regions of *RARβ2*, *HMGCS2*, and *GADD45α* genes.

ChIP/Western Blot on UV-Irradiated Cells

MRC5 treated with or without DRB (100 μM) during 6 hr were rinsed with PBS, UV irradiated (20 J/m^2). Cells were crosslinked at room temperature for 30 min with 1% formaldehyde at indicated times post-UV irradiation and chromatin was prepared (Fousteri et al., 2006). ChIP/Western blot, using XPB antibodies, was performed as previously described (Coin et al., 2008). Briefly, the chromatin suspension was sonicated in buffer S (10 mM Tris-HCl [pH 8.0], 140 mM NaCl, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS). Samples were spun down (13,000 rpm, 15 min). In each assay, 600 μg of protein from crosslinked chromatin were immunoprecipitated with 1 μg of antibody in buffer S, 4 hr at 4°C. The immunocomplexes were collected by adsorption to protein G-Sepharose beads overnight at 4°C. The beads were next washed and resuspended in 1 X Laemlli SDS Buffer. Samples were incubated at 95°C for 90 min for crosslinking reversal prior electrophoresis. After Western blotting following XPB-immunoprecipitation, the intensity of XPB, XPF, p62, RPA, cyclin H, and cdk7 corresponding bands was quantified by densitometry using Genetool (Syngene).

DNA Methylation Assay and Global Methylation Level Measurement

Genomic DNA was extracted using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma). Methylation of CpG islands localized in and downstream *RARβ2* promoter was investigated by digesting 1 μg genomic DNA with 5 units of Hpa*II* or BamHI followed or not by a second digestion with 5 units of *DpnI* (Fermentas). Methylation status was evaluated by measuring the ratio between BamHI/*DpnI* or BamHI/*HpaII* and BamHI PCR amplified products using specific primers for the RARβ2 promoter (sequences are available upon request). The quantification of PCR products was performed using Genetool.

Local UV Irradiation, Fluorescence, and Confocal Microscopy

The cells were rinsed with PBS and were covered with an isopore polycarbonate filter with pores of 6 mm diameter (Millipore, Bedford, MA). Cells were then exposed to UV irradiation with a Philips TUV lamp (predominantly 254 nm) at a dose of 70 J/m^2. Subsequently, the filter was removed, the medium was added back to the cells, and cells were returned to culture conditions for 30 min.

Fibroblasts were fixed in 3% paraformaldehyde for 10 min at room temperature and permeabilized with PBS/0.5% Triton for 5 min. After washing with PBS-Tween (0.05%), the slides were incubated for 1 hr with the indicated antibodies. After extensive washing with PBS-Tween, they were incubated for 1 hr with Cy3-conjugated goat anti-rabbit IgG (Jackson Laboratory) or with anti-mouse Alexa 488 IgG (Jackson Laboratories) diluted 1:400 in PBS-Tween (0.5%). The slides were counterstained for DNA with DAPI prepared in Vectashield mounting medium (Vector lab). All images were collected using a Leica Confocal TCS 4D microscope equipped with both UV laser and an Argon/Kripton laser and standard filters to allow collection of the data at 488 and 568 nm. The software TCSTK was used for three-color reconstructions, and figures were generated using the ImageJ software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.molcel.2010.03.004.

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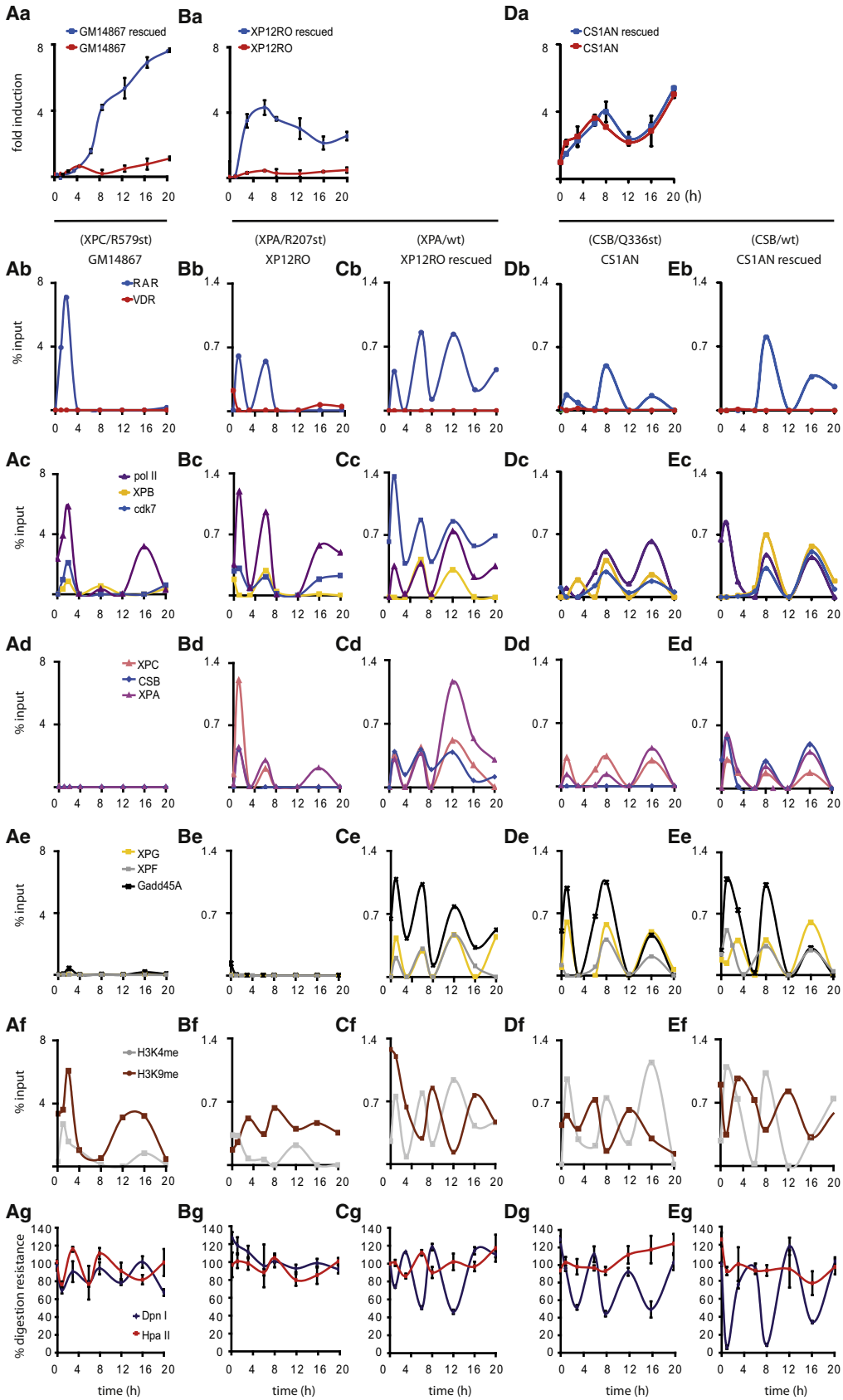
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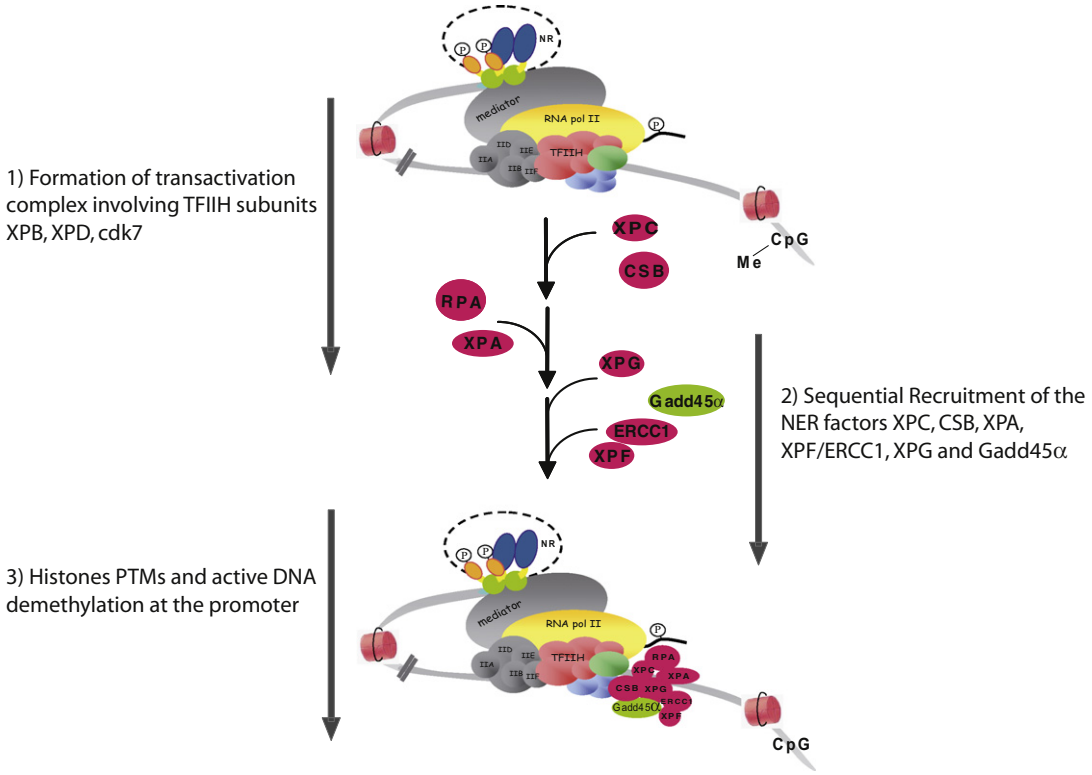

Figure 6. Sequential Recruitment of the NER Factors at the Promoter of Activated Genes Facilitates Chromatin Remodeling

Upon the t-RA ligand induction, the transactivation complex is formed once RAR/RXR has targeted its responsive element; coactivators and mediator are assembled at RARβ2 promoter together with the transcriptional machinery. XPC is next recruited and allows the sequential arrival of the others NER factors in the following order CSB, XPA/RPA, XPG/XPF/Gadd45α. This association of NER factors and Gadd45α with transcription machinery leads to a cascade of histones PTMs. Concomitantly, an active demethylation of 5′CpG islands of RARβ2 gene occurs. Altogether, these remodeling chromatin events are crucial for accurate RNA synthesis. We can mention that XPG has, at least, a double action of stabilization of TFIIH and chromatin remodeling in transcription process.

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Figure 5. RARβ2 Induction, NER Factors Recruitment, Histones PTMs, and DNA Demethylation on Active Promoter in Fibroblasts Derived from XP and CS Patients

(A–E) Relative RARβ2 mRNA expression monitored by qPCR from GM14867 (XPC/R579st) (Aa), XP12RO (XPA/R207st) (Ba), and CS1AN (CSB/Q336st) (Da) fibroblasts treated with t-RA (10 μM) and compared to the corresponding rescued fibroblasts as indicated. Error bars represent the standard deviation of three independent experiments. ChIP monitoring the t-RA-dependent occupancy of RAR, VDR (Ab, Bb, Cb, Db, and Eb), RNA pol II, XPB, cdk7 (Ac, Bc, Cc, Dc, and Ec), XPC, CSB, XPA (Ad, Bd, Cd, Dd, and Ed), XPG, XPF, Gadd45α (Ae, Be, Ce, De, and Ee) and di-/trimethylated histones H3K9, H3K4 (Af, Bf, Cf, Df, and Ef) on RARβ2 promoter from GM14867, XP12RO, XP12RO rescued, CS1AN and CS1AN rescued chromatin extracts as indicated. Methylation of CpG islands of RARβ2 promoter determined by the ratio of PCR products obtained after digestion by HpaII, BamHI/ *DpnI* or just BamHI of genomic DNA from t-RA treated, GM14867 (Ag), XP12RO (Bg), XP12RO rescued (Cg) CS1AN (Dg), and CS1AN rescued (Eg) fibroblasts. Error bars represent the standard deviation of three independent experiments.

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3.4.3 Publication: True lies; the double life of the nucleotide excision repair factors in transcription and DNA repair

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Review Article

True Lies: The Double Life of the Nucleotide Excision Repair Factors in Transcription and DNA Repair

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Nucleotide excision repair (NER) is a major DNA repair pathway in eukaryotic cells. NER removes structurally diverse lesions such as pyrimidine dimers, arising upon UV irradiation or bulky chemical adducts, arising upon exposure to carcinogens and some chemotherapeutic drugs. NER defects lead to three genetic disorders that result in predisposition to cancers, accelerated aging, neurological and developmental defects. During NER, more than 30 polypeptides cooperate to recognize, incise, and excise a damaged oligonucleotide from the genomic DNA. Recent papers reveal an additional and unexpected role for the NER factors. In the absence of a genotoxic attack, the promoters of RNA polymerases I- and II-dependent genes recruit XPA, XPC, XPG, and XPF to initiate gene expression. A model that includes the growth arrest and DNA damage 45α protein (Gadd45α) and the NER factors, in order to maintain the promoter of active genes under a hypomethylated state, has been proposed but remains controversial. This paper focuses on the double life of the NER factors in DNA repair and transcription and describes the possible roles of these factors in the RNA synthesis process.

1. Introduction

A number of DNA repair pathways protect us from the deleterious effects of DNA damage. The importance of these mechanisms is highlighted by the existence of genetic disorders in which impaired DNA repair mechanisms predispose patients to cancer and early onset of aging. A major advance in our understanding of these DNA repair mechanisms has been to uncover the tangled connection existing between these systems and other fundamental cellular processes such as DNA replication and transcription. These cellular processes are not only highly connected with DNA repair pathways but they also share common factors with them. This complexity leads to new hypothesis about the cause of the phenotypes displayed by patients suffering from DNA repair disorders and may even force us to re-evaluate the place of the repair factors in cellular homeostasis.

2. The NER Pathway: The Fountain of Youth of Our Genome

We do not live forever young. We all have to experience aging, a functional decline coupled to an increased mortality risk from diseases such as cancer. The molecular origins of aging can be sought, at least in part, in an alteration of the expression of our genes that results from the physicochemical constitution of DNA, which does not guarantee life-long stability (for reviews see [1, 2]). Over time, DNA accumulates a tremendous diversity of lesions that, if unrepaired, lead to mutations that dysregulate the function of proteins. DNA lesions originate from environmental agents such as the ultraviolet (UV) component of sunlight, ionizing radiation, and numerous genotoxic chemicals, and also from the products of normal cellular metabolism. Aging is a relatively slow process for most of us, but unfortunately premature appearance of multiple symptoms of aging can be observed in a growing family of human syndromes [3, 4]. Among

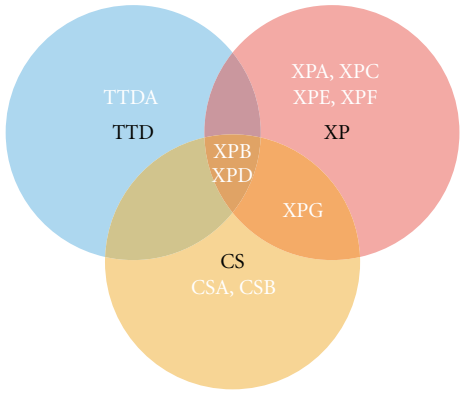

Figure 1: Three disorders for nine genes. Mutations in ten genes are responsible for the *xeroderma pigmentosum* (XP), the *trichothyodystrophy* (TTD) or the Cockayne syndrome (CS). XPA, XPC, XPE and XPF are only involved in XP; CSA and CSB are only involved in CS; XPG is involved in pure XP or in an combined XP/CS syndrome; XPB and XPD are involved in TTD, XP, or in a combined XP/CS syndrome. TTDA is only involved in TTD.

them, the *xeroderma pigmentosum* (XP), *trichothyodystrophy (TTD)*, and Cockayne syndrome (CS) are remarkable as they all include two distinct phenotypes: either a 1000-fold elevated suninduced skin cancer risk, for XP patients, or a segmental progeria without an increase in cancer incidence, for CS and TTD [5]. These syndromes uncover what our lives would be if a "fountain of youth" was not protecting our genome day after day from endogenous and exogenous assaults. Indeed, the main molecular defect of the XP, CS, and TTD patients resides in a defect in the nucleotide excision repair (NER) pathway [6]. NER is an evolutionarily conserved DNA repair caretaker pathway involving about 30 proteins, ten of which (designated XPA to G; TTDA, CSA, and CSB) are differentially associated with XP, CS, or TTD disorders in an intricate network (Figure 1). NER is divided into two subpathways, which differentially remove damages from our genome depending on their location [7]. In the Global Genome NER (GGNER), the XPChHR23B complex recognizes damages. The DNA is then opened by the XPD and XPB helicasecontaining transcription/repair complex TFIIH together with XPA and RPA to generate the damaged single stranded DNA ready for incision by the specific endonucleases XPG and ERCC1-XPF. DNA gap filling is done by the replicative DNA polymerases δ and ε or the translesional polymerase k, in the presence of PCNA, RFC, and RPA [8] (Figure 2). In transcription-coupled NER (TC-NER), blockage of transcribing RNA Polymerase II (RNA-Pol II) on the damaged DNA template is thought to initiate the repair reaction in a process that requires, in addition to TFIIH, XPA, XPG, and ERCC1-XPF, the TCR-specific proteins CSB and CSA [9] (Figure 2). Although CSB is required to recruit NER factors to the stalled RNA-Pol II, CSA is coming later and is not needed for the formation of the TCR complex [10] (Figure 2).

Next to the basal NER machinery, additional factors modulate the efficiency of the NER reaction but are not required to incise a damaged oligonucleotide *in vitro*. The GG-NER damage recognition factor, XPC, forms *in vivo* a heterotrimeric complex involving one of the two human homologs of *S.cerevisiae* Rad23p (hHR23B) and centrin 2, a centrosomal protein [11]. The role of centrin 2 and hHR23B in NER has been elusive but they seem to increase the damage recognition capacity of XPC [12]. The XPE complex, mutated in XP-E patients, is another accessory NER factor composed of DDB1 and DDB2. The role of the XPE protein remains unclear, but it could participate in the recognition of lesions together with XPC [13]. Another NER factor, XPA-binding protein 2 (XAB2), was identified by virtue of its ability to interact with XPA [14]. XAB2 also associates with the TC-NER specific proteins CSA and CSB, as well as with RNA-Pol II, after UV irradiation and is specifically involved in the TC-NER subpathway [15]. Finally, the DDB2 and CSA polypeptides can be found integrated into nearly identical complexes containing cullin 4A, Roc1, and COP9 that seem to favor NER [16]. Although limited today, the list of proteins that modulate the NER reaction should increase in a near future and benefit from high throughput technologies. The study of these cofactors will constitute an important challenge, as the modulation of the efficiency of NER to eliminate DNA lesions may explain some cancer predispositions in healthy people. Moreover, identifying the complete set of proteins that participate in NER is a crucial aspect of cancer therapy since the resistance to chemotherapy treatment could partially rely on the capacities of the cell to eliminate drug-induced DNA lesions.

3. The NER Pathway in a Chromatin Context: Take Old Factors to Make Them New

New DNA repair players have also emerged from the study of NER in the chromatin context. Reorganisation of nucleosome structure following NER was observed over 30 years ago [17], and many studies demonstrate that chromatin acts as a barrier for the recognition of the lesions by NER factors [18]. Not surprisingly, chromatin remodelers identified in NER were already known to promote accessibility to the DNA for the transcription machinery. The ATP-dependent chromatin remodelling complexes SWI/SNF or ISW2 have been shown to act on UV-damaged nucleosomes and to stimulate repair *in vitro* [19]. In yeast, UV irradiation increases contacts between SWI/SNF and the homologs of XPC-hHR23B, and inactivation of SWI-SNF leads to a slow removal of CPD lesions [20]. Finally, the ATP-dependent chromatin assembly factor-1 (CAF-1) is required to restore the chromatin conformation after the removal of the lesions [21].

Apart from ATP-dependent nucleosome remodelling, many forms of histones modifications have been unveiled after UV irradiation. Histone acetylation was the first modification to be shown to play a role in NER. Treatment of nonreplicating human cells with the histone deacetylase inhibitor sodium butyrate enhances NER [22]. PCNA, the replicative protein involved in the DNA resynthesis step of NER, interacts with the p300 histone acetyltransferase

following UV irradiation [23]. On the other hand, a complex containing the damaged DNA-binding protein DDB1, the CREB-binding protein CBP, and p300 has been isolated *in vivo* [24]. Another complex, TBP-free-TAFII complex (TFTC), directs histone H3 acetylation by hGCN5 after UV irradiation and facilitates access of DNA repair machinery to lesions within chromatin [25]. In addition to histone acetylation, UV damage also induces histone H2A monoubiquitination in the vicinity of DNA lesions [26]. Monoubiquitination of H2A depends on functional NER and occurs after incision [27].

Overall, these data show that histone modifications form part of the cellular response to UV damage and clearly play a role in chromatin remodelling during DNA repair. However, the exact nature of the modified histones and residues as well as the role of these modifications in the facilitation of DNA damage access or in the DNA damage response not clear. Much remains to be done to define a histone code in NER, comparable to that acquired in other fundamental cellular processes like transcription or double-strand break repair.

4. The Unveiled Side of the XP, CS, and TTD Syndromes

Although the UV sensitivity and/or cancer predisposition of XP, CS, and TTD patients can be explained by defects in NER, some other of their phenotypes (including neurological and developmental defects) are more difficult to rationalize. For instance, some group A patients show the most severe progressive neurological disorders while the XPA protein is only known for its role in the verification of the damages [5]. Thus, several studies have aimed to discover additional processes that may be disrupted in these pathologies and at a first glance have found evidence for transcription defect in TFIIH-, XPG-, and CSB-mutated cells.

TFIIH is a ten-subunit complex composed of a core (XPB, p62, p52, p44, p34, and TTDA) coupled to the Cdk-activating kinase complex (CAK) through the XPD subunit [28]. A recent study showed that CAK does not participate to NER and is released from the core TFIIH during the formation of the preincision complex following the recruitment of XPA [29] (see also Figure 2). As a component of TFIIH, CAK phosphorylates both the carboxyl terminal domain of RNA-Pol II and some nuclear receptors (NRs) including the retinoic acid receptors (RARα and γ) [30], the thyroid hormone receptor (TR) [31], and the peroxysome proliferator-activated receptor (PPAR) [32]. Phosphorylation of these NRs is required for the transactivation of specific genes. Cdk7 also activates the vitamin D receptor indirectly, by phosphorylating the Ets1 coactivator [33] (Table 1).

Patients with mutations in XPB and XPD display a transcriptional defect in specific genes, which may help clarifying the origin of their developmental or neurological problems. In TTD-XPD cells, mutations in XPD destabilize the CAK complex from TFIIH leading to defects in the phosphorylation of RAR, ER, and PPAR. In XP-B patients, two mutations in XPB (F99S and fs740) lead to the combined XP/CS defect with a very low level of residual NER activity [34]. However, only the fs740 mutation is cancer prone [35]. It was shown that this mutation specifically blocks transcription activation by the FUSE-Binding Protein (FBP), a regulator of c-myc expression, and inhibition by the FBP-Interacting Repressor (FIR) [36]. The fact that the regulation by FBP and FIR is impaired could directly affect proper regulation of c-myc expression and explain the development of malignancy in the corresponding patient. The XPB and XPD subunits of TFIIH are not the only NER polypeptides to be involved in transcription.

The first evidence for an involvement of XPG in transcription came from a study in yeast. RAD2, the *S.cerevisiae* counterpart of XPG, was shown to be required in promoting efficient RNA-Pol II transcription [37]. Later, it was demonstrated that mutations in human XPG, as found in XP-G/CS patient cells, prevent the association of XPG with TFIIH, resulting in the dissociation of the CAK and XPD from the core TFIIH [38]. This dissociation leads to an impair transactivation of the NR-dependent responsive genes.

The TCR-specific CSB protein belongs to the ATP-dependent SWI2/SNF2 family of chromatin remodeling proteins and has been shown to play a role in both remodeling the chromatin structure and disrupting protein–DNA interactions [39]. Besides its role in TCR, CSB is involved in the transcription recovery of housekeeping genes after UV irradiation [40]. CSB is specifically recruited to the promoters of these genes and helps in the recruitment of both the RNA-Pol II and the associated basal transcription factors, probably through its chromatin remodeling activity (Table 1).

Altogether, these data show that the transcription defect in XP/CS, CS, or TTD is subtle and more difficult to evaluate than the NER defect because this defect targets specific genes, under specific conditions, and probably in a cell-specific manner. However, the involvement of transcription dysregulation in aging and cancer makes these studies very important for the understanding of these diseases. Interestingly, a picture emerges from these studies, which shows that mutations in XP factors lead to a modification of the expression of specific genes by possibly two means; either through the accumulation of unrepaired lesions that will lead to mutations or through a direct involvement of repair factors in gene expression. However, a piece of the puzzle is missing. Even though a clear involvement of XPB, XPD, XPG, or CSB in transcription was documented, it has been more difficult to assign a transcriptional role to XPC, XPA, or ERCC1-XPF until the recent works discussed below.

5. Behind the Evidence: A Transcriptional Role for the NER Factors

Protein coding genes expression is the result of an acute process that starts at the promoter of a given gene and involves, in the addition to the RNA-Pol II and the basal transcription factors, a cocktail of proteins such as the NR, coactivators, mediator, and histone-modifying enzymes. A study from our group [41] shows that some NER factors are associated with the transcription machinery at the promoter

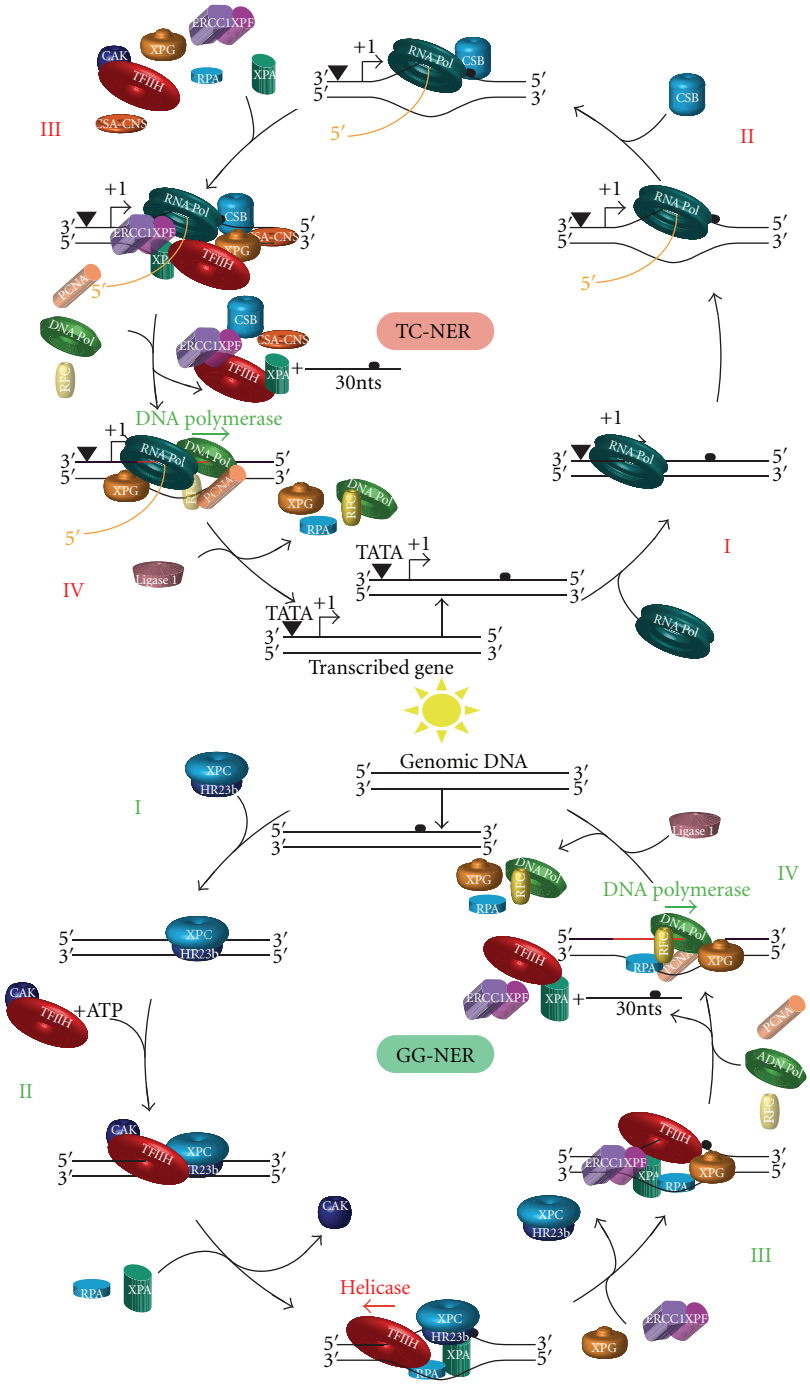

FIGURE 2: The two subpathways of mammalian NER. Physical or chemical agents like UV, cis-platin, or benzopyrene can damage DNA and induce damage-mediated helix distortions anywhere in the genome (GG-NER in green, bottom panel) or on the transcribed strand of a gene (TC-NER in red, top panel). Bottom panel: (I) XPC-RAD23B recognizes and binds to DNA damage-mediated helix distortion to initiate GG-NER. (II) TFIIH is recruited in an ATP-dependent manner, followed by XPA and RPA, which verify the presence of the lesion. During this step, the CAK module of TFIIH is released from the preincision complex [29]. (III) Within the preincision complex, ERCC1-XPF and XPG structure-specific endonucleases incise the damaged strand on the 5′ and 3′ sides of the lesion, respectively. Following incision, NER factors are released from the DNA, except XPG and RPA that favour the recruitment of the replication machinery composed of PCNA, RFC, and the DNA Polymerases $\delta, \varepsilon,$ or κ (ref). (IV) Following replication of the gap, the DNA is sealed by the ligase 1 (or the ligase III-XRCC1 complex in nondividing cells). Top Panel: (I) TC-NER is triggered by DNA damage-mediated blockage of RNA-Pol II (Top panel). (II) CSB is then recruited to the stalled RNA-Pol II enzyme and triggers the recruitment of the NER factors TFIIH, XPA, RPA, ERCC1-XPF, and XPG together with the CSA-CNS complex (III). (IV) Following the excision of the damaged oligonucleotide, the same DNA replication machinery of the GG-NER subpathway fills the gap created by the incision/excision step.

Table 1: Repair/transcription factors and their functions.

Factors	Role in NER	Role in transcription
TFIIH	Opens DNA around the damage. Favors 5′ incision by ERCC1-XPF.	Opens DNA around the promoter. Phosphorylates the CTD of RNA-Pol II. Phosphorylates NR and coactivators.
XPG	Incises DNA in 3′ from the lesion	Involved in NR-dependent transcription. Stabilizes the interaction of CAK to the core TFIIH.
CSB	TC-NER-specific factor. Recruits NER factors to stalled RNA-Pol II.	Chromatin-remodeling factor (SWI-SNF family). Reinitiates transcription after DNA damage removal.
XPC	**Recognition of lesions**	**Involved in NR**-dependent **transcription**. **Removal of 5meC** **Chromatin modification**?
XPA	**Verification of lesions**	**Involved in NR**-dependent **transcription**. **Removal of 5meC** **Chromatin modification**?
XPF	**Incises DNA in 3′from the lesion**	**Involved in NR**-dependent **transcription**. **Removal of 5meC**, **Chromatin modification**? **Incision**?

In bold, the new NER factors involved in transcription.

of several activated NR-dependent genes. The recruitment occurs in a sequential order after the formation of the preinitiation complex (PIC) and induces XPC, CSB, XPA, and the XPG and ERCC1-XPF endonucleases. The transcriptional complex equipped with NER factors is formed in the absence of any exogenous genotoxic attack and is distinct from a repair complex, since it is specifically sensitive to transcription inhibitors and can be formed in the absence of the TCR specific-CSB protein (Figure 3). Following transcription initiation, NER factors escort the RNA-Pol II during the elongation step to form a complex that does not include XPC but requires CSB (Figure 3). These observations suggest a different function for the NER factors located at promoters in respect to those located at distal regions of the gene; while the latter may represent a pre-TCR complex ready to remove lesions on transcribed genes, the former may play an active role in transcription. In line with this hypothesis, patient cell lines mutated in XPC, XPA, or XPG show a dysregulation of the NR-dependent genes that results from a defect in the association of the NER factors with the transcription machinery. Although the corresponding XPC, XPA, XPG, and ERCC1-XPF repair factors are not essential for PIC formation, it remains that they optimize the efficiency of transcription.

6. Insight into the Function of the NER Factor in Transcription

How do NER factors favor NR-dependent genes transcription? Several studies have reported a controversial role for Gadd45α in association with the endonuclease activity of XPG in transcription: the active demethylation of CpGs islands localized at proximal promoters [42–44]. Recent works support these findings and demonstrate that the recruitment of XPC, XPA, XPG, and ERCC1-XPF on the promoter of active RNA-Pol I- and II-dependent genes allows the association of Gadd45α to the PIC and induce the demethylation of promoters [41, 44]. Mutations in XPC, XPA and XPG found in XP patients dysregulate the corecruitment of the NER factors and Gadd45α to active promoters, thereby abolishing the active demethylation step and thus affecting transcription.

How can the NER factors demethylate DNA? Similar to a classical NER lesion, 5′-methylcytosine (meC) combined to the specific chromatin environment during transcription initiation could be recognized and eliminated by the NER machinery [45] (Figure 4(a)). Indeed, a previous study demonstrated a faster repair rates near the transcription initiation site linked to increased local concentrations of DNA repair factors associated with basal transcription factors [46]. The sequential recruitment of NER factors could help the incision and the replacement of meC with unmethylated nucleotides. Even if incision by XPG on the promoter of RNA Pol I-dependent genes has been reported [44], this hypothesis is highly controversial, and several groups propose other alternatives to explain the demethylation of meC. Recent studies have supported a model involving at least two steps [43, 47] (Figure 4(b)). The model predicts the conversion of meC to cytosine by the direct removal of the methyl group or by the hydrolytic deamination of meC to thymine further excised by a DNA repair enzyme. The first step concerns the deamination reaction and implies apolipoprotein B mRNA editing enzyme (APOBECS) proteins such as activation-induced cytidine deaminase (AID) and APOBEC1, which function in sequence specific context. Alternatively, it has also been suggested that enzymes called DNA methyltransferases (DNMTs) exhibit dual and opposite actions, not only to methylate CpG islands but also to deaminate them [48]. The second step is related to the action of a DNA glycosylase such as Mdb4 or TDG that remove thymine from G/T mispairs to generate abasic sites rapidly cleaved through the activity of apurinic/apyrimidinic

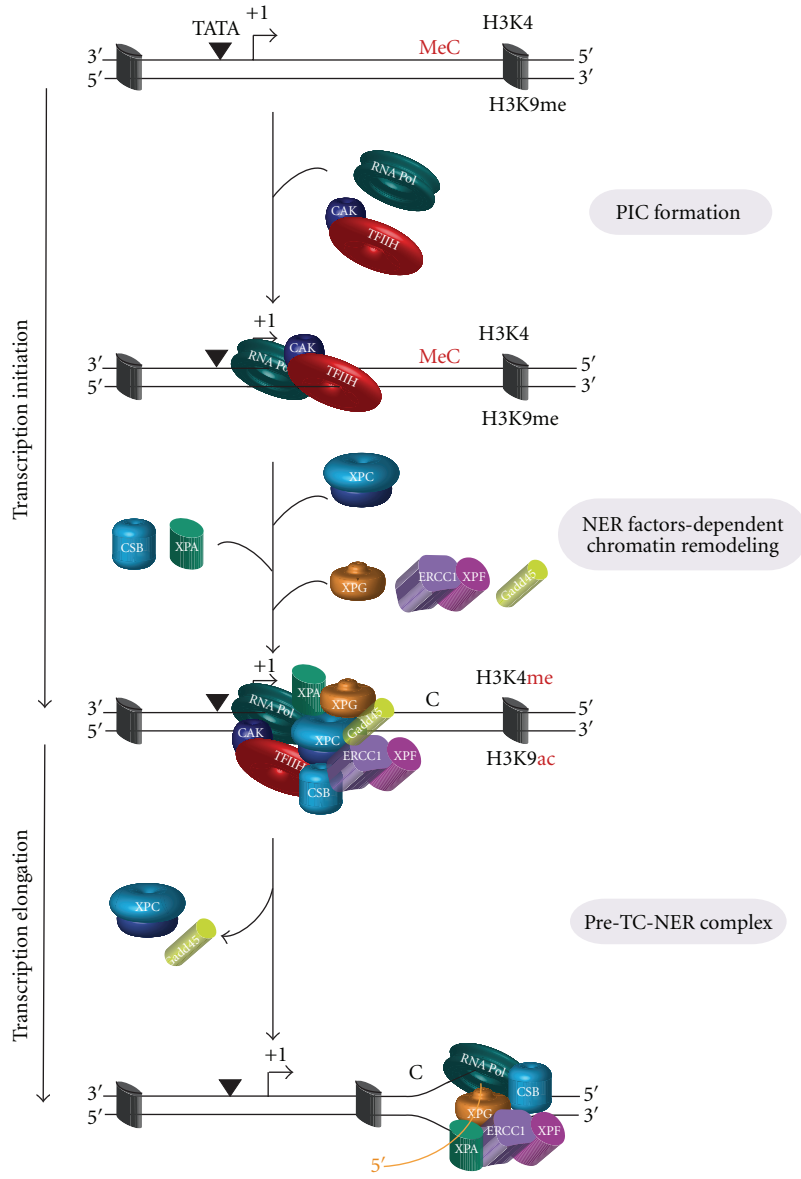

Figure 3: NER factors and gene transcription. Upon gene activation, the formation of preinitiation complex (PIC) precedes the recruitment of XPC, which allows the sequential arrival of the other NER factors in the following order CSB, XPA/RPA, XPG/ERCC1-XPF, and Gadd45α. The association of NER factors and Gadd45α with the transcription machinery leads to a cascade of histone PTMs. Concomitantly, an active demethylation of 5′CpG islands occurs.

endonuclease (APE) (Figure 4(b)) [43]. It was recently shown that Mdb4 is corecruited to active promoters with proteins from base-excision-repair (BER) process such as APE-1, DNA ligase I, or polymerase δ [48]. Even though the role of Gadd45α is controversial, it clearly increases the efficiency of the demethylation process.

The results obtained recently by several groups lead us to propose another hypothesis that could account for the active demethylation of promoters during transcription and involves both the NER and the BER factors. Active DNA demethylation at promoters is intimately linked with histones posttranslational modifications (PTMs) [49]. Di/tri methylation of H3K4 (H3K4me) and di/trimethylation of H3K9 (H3K9me) correlate with active transcription and heterochromatin, respectively. In a repressed status, the methyltransferase G9a catalyzes the methylation of H3K9, which allows the binding of the heterochromatin protein 1 (HP1) to facilitate the local formation of heterochromatin. The G9a-containing complex also recruits the DNA methyltransferases DNMT3A and DNMT3B that catalyze the *de novo* methylation of DNA at promoters. Conversely, during active transcription and concomitantly to demethylation/acetylation of H3K9, methylation of H3K4 inhibits contacts between nucleosome and DNMT3 to facilitate active

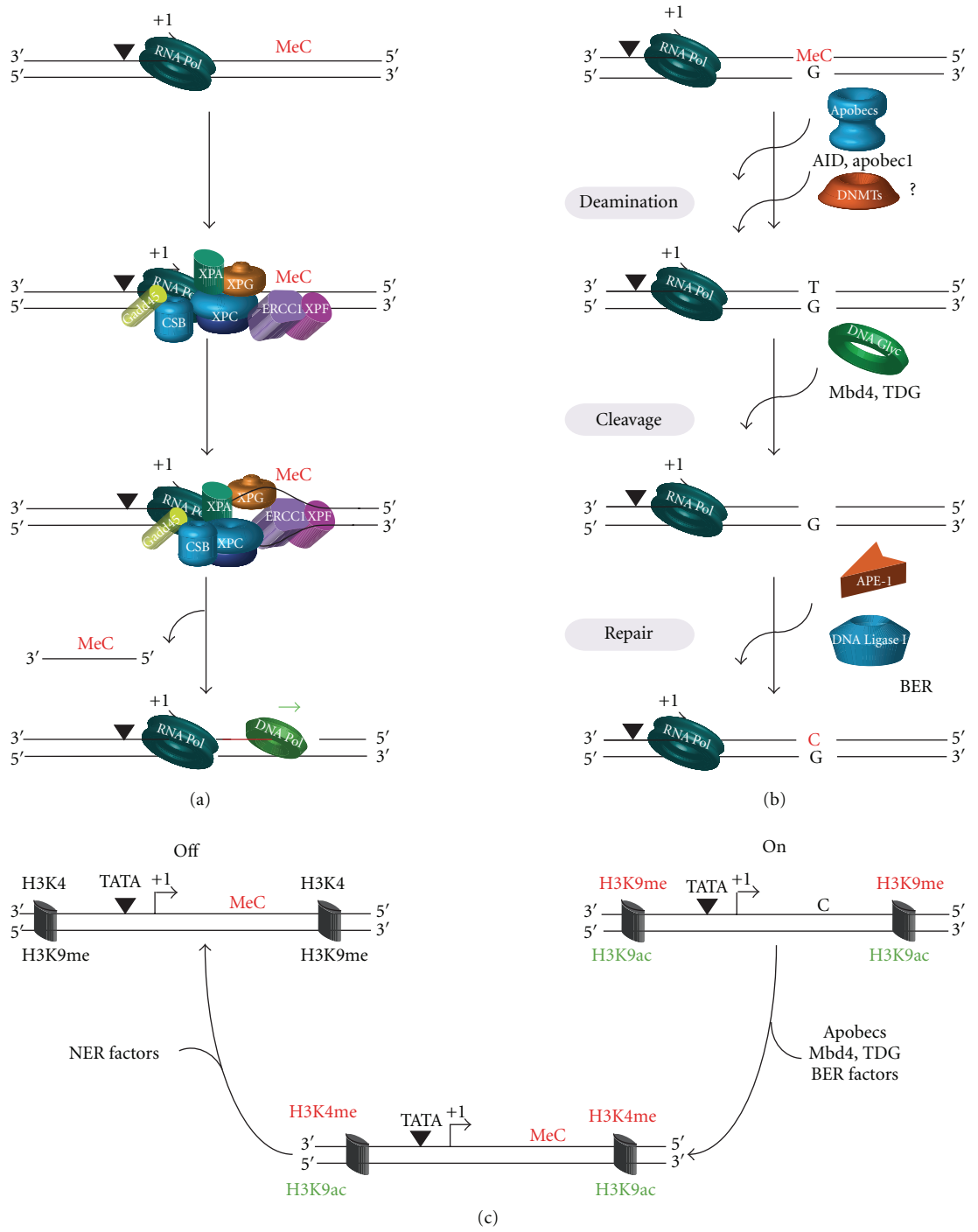

Figure 4: Potential mechanisms of cytosine demethylation. (a) The NER machinery, recruited to the preinitiation complex, can recognize the 5-methyl cytosine (meC) as a NER-specific substrate, in the presence of Gadd45α, and eliminate it in a process closely related to the canonical NER process with the incision/excision of the oligonucleotide containing the meC. (b) Another pathway involves two steps; first, the deamination of 5-methylcytosine (meC) to thymine, which involves proteins from Apobec family such as AID or APOBEC1. A role in deamination has been also suggested for DNMTs proteins. Consequently, the impairment of the thymine with the guanine in the opposite strand induces the recruitment of DNA glycosylases such as Mbd4 or TDG that remove thymine through cleavage of the glycosidic bond. Following the action of DNA glycosylases, it remains an apyrimidinic site, which is cleaved by an AP endonuclease such as APE1 and repaired through the polymerase β and DNA ligases. NER factors and Gadd45α are involved in this mechanism but their roles are not determined. (c) We propose that NER factors control the epigenetic environment of the promoter favouring the demethylation of H3K9 and the methylation of H3K4. Following the action of the NER factors, Apobec proteins and BER factors demethylate the meC in a process similar to (b).

DNA demethylation [50]. NR-dependent altered transcription observed in XP-C, XP-A, or XP-G cells is accompanied by dysregulation of PTMs of histones. The hypermethylated status of these promoters in these cells is associated with absence of H3K4me and maintenance of the H3K9me marks. These observations imply that the promoter of NR-dependent genes persists in a heterochromatin environment, despite the formation of the RNA-Pol II machinery, thereby impairing optimal transcription. There is no evidence of a direct role of repair factors in the regulation of histones PTMs or histone-modifying enzymes; it can be hypothesised that NER factors function upstream of the BER factors to help to maintain a euchromatin environment characterised by a demethylation of H3K9 and a methylation of H3K4 (Figure 4(c)).

7. Conclusion

Almost twenty years after the discovery that the basal transcription factor TFIIH was also involved in NER [51], a new age arises from the discovery that basal NER factors are involved in activated transcription. The emergence of repair factors in transcription forces us to modify our approach for the understanding of the broad clinical features described for the so-called XP, TTD, and CS "repair syndromes", but it also represents a breakthrough in gene expression studies. Indeed, the effects of DNA methylation variations on gene expression have been largely studied, but the mechanisms that promote active demethylation combined to histones modifications are just appearing with the finding that DNA repair factors may participate to this process.

Besides the 2D space organisation of a gene, one has also to consider the 3D space organisation of the nucleus. Transcription is deeply associated to genome organization; the location of a gene within the chromosome territories influences its ability to be reached by the suitable machinery [52]. Since the PTMs of histone and the methylated status of genomic DNA are connected to the dynamic topological regulation of chromatin, we have to consider that NER factors could contribute to transcription through a role in the nonrandom organization of the nucleus. Surprisingly, besides the DNA repair disorders, a second group of diseases that are characterized by accelerated aging comprises the Hutchinson-Gilford syndrome (or Progeria) that is due to a point mutation in Lamin A [53, 54]. This protein is a structural component of the nuclear matrix that plays a role in the 3D organization of the genome. It is then tempting to propose that changes in the nuclear architecture in these disorders participate in the modification of the transcription program and possibly to the impairment of the repair of some lesions, that altogether lead to accelerated aging and cancer.

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3.4.4 Publication: XPG and XPF: Break, DNA demethylation and gene looping

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XPG and XPF Endonucleases Trigger Chromatin Looping and DNA Demethylation for Accurate Expression of Activated Genes

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SUMMARY

Nucleotide excision repair factors, initially characterized as part of DNA repair, have been shown to participate in the transcriptional process in the absence of genotoxic attack. However, their molecular function when recruited at the promoters of activated genes together with the transcription machinery remained obscure. Here we show that the NER factors XPG and XPF are essential for establishing CTCF-dependent chromatin looping between the promoter and terminator of the activated *RARβ2* gene. Silencing XPG and/or XPF endonucleases, or mutations in their catalytic sites, prevents CTCF recruitment, chromatin loop formation, and optimal transcription of *RARβ2*. We demonstrated that XPG endonuclease promotes DNA breaks and DNA demethylation at promoters allowing the recruitment of CTCF and gene looping, which is further stabilized by XPF. Our results highlight a timely orchestrated activity of the NER factors XPG and XPF in the formation of the active chromatin hub that controls gene expression.

INTRODUCTION

RNA synthesis is the result of a cascade of chronologically orchestrated events that requires several hundreds of proteins. Upon gene activation, a host of proteins including RNA polymerase II (pol II), the general transcription factors (GTFs), coactivators, corepressors, and chromatin remodelers are assembled around the promoter, and their enzymatic activities contribute to protein coding gene expression (Brivanlou and Darnell, 2002; Kornberg, 2007). Among these proteins necessary to initiate RNA synthesis are the nucleotide excision repair (NER) factors. These factors are sequentially recruited and required for optimal chromatin remodeling including histone posttranslational modifications (PTMs) as well as DNA demethylation at the activated genes (Le May et al., 2010; Schmitz et al., 2009; Barreto et al., 2007). These NER factors (XPC, CSB, TFIIH, XPA, XPG, XPF-ERCC1) were first characterized as part of the DNA repair machinery to eliminate lesions originated by exogenous or endogenous genotoxic attacks (for review, see Nouspikel, 2009). Mutations in the genes coding for NER factors have been associated with the human genetic disorders xeroderma pigmentosum (XP), trichothiodystrophy (TTD), Cockayne syndrome (CS), XFE progeroid syndrome, and cerebro-oculo-facio-skeletal syndrome (COFS) characterized by a combined dysregulation of DNA repair and transcription processes (Jaspers et al., 2007; Kraemer et al., 2007; Niedernhofer et al., 2006).

In the present study, we investigated the implication of XPG and XPF endonucleases in the transactivation of nuclear receptor (NR) target genes. We found that these two factors, detected at the promoter and terminator of the activated *RARβ2* gene, were required for DNA breaks and DNA demethylation. These two steps are crucial for the recruitment of the CCCTC-binding factor (CTCF) chromatin organizer and consequently the formation of gene looping between promoter and terminator. Altogether, these events optimize RNA synthesis.

RESULTS

XPG and XPF Are Recruited at the Promoter and Terminator of *RARβ2* upon Activation

We first attempted to investigate the presence of the transcription and NER factors along the *RARβ2*-activated gene. Analysis of the promoter region (Pro), including two RAR responsive elements (RAREs) and the TATA box as well as a region defined as the terminator (Ter) including the Poly(A) site, was performed using chromatin immunoprecipitation (ChIP) followed by quantitative PCR at various times as indicated (Figure 1). Cells that either stably express targeted shRNA silencing XPG (ShXPG) and ERCC1 (named ShXPF), XPA (ShXPA), or transiently express siRNA against CTCF (SiCTCF) as well as their respective corresponding controls (named Ctrl1-3, see the Experimental Procedures) were treated with all-trans retinoic acid (t-RA). Silencing ERCC1 also silenced XPF (Figure 1, left panel; see also Gaillard and Wood, 2001).

In each silenced cell line, we observed a significant defect in *RARβ2* mRNA expression compared to the Ctrl1-3 cells (Figure 1 and Figure 2, left panels). We noticed that as a function of the cell line and the set of experiments, *RARβ2* mRNA synthesis peaks either at 3 or at 6 hr. Therefore ChIPs were performed either at 0, at 3, or at 6 hr posttreatment. In Ctrl1 cells, we observed at 6 hr posttreatment the concomitant recruitment of RAR, pol II, and TFIIB together with the NER factors indicated by the

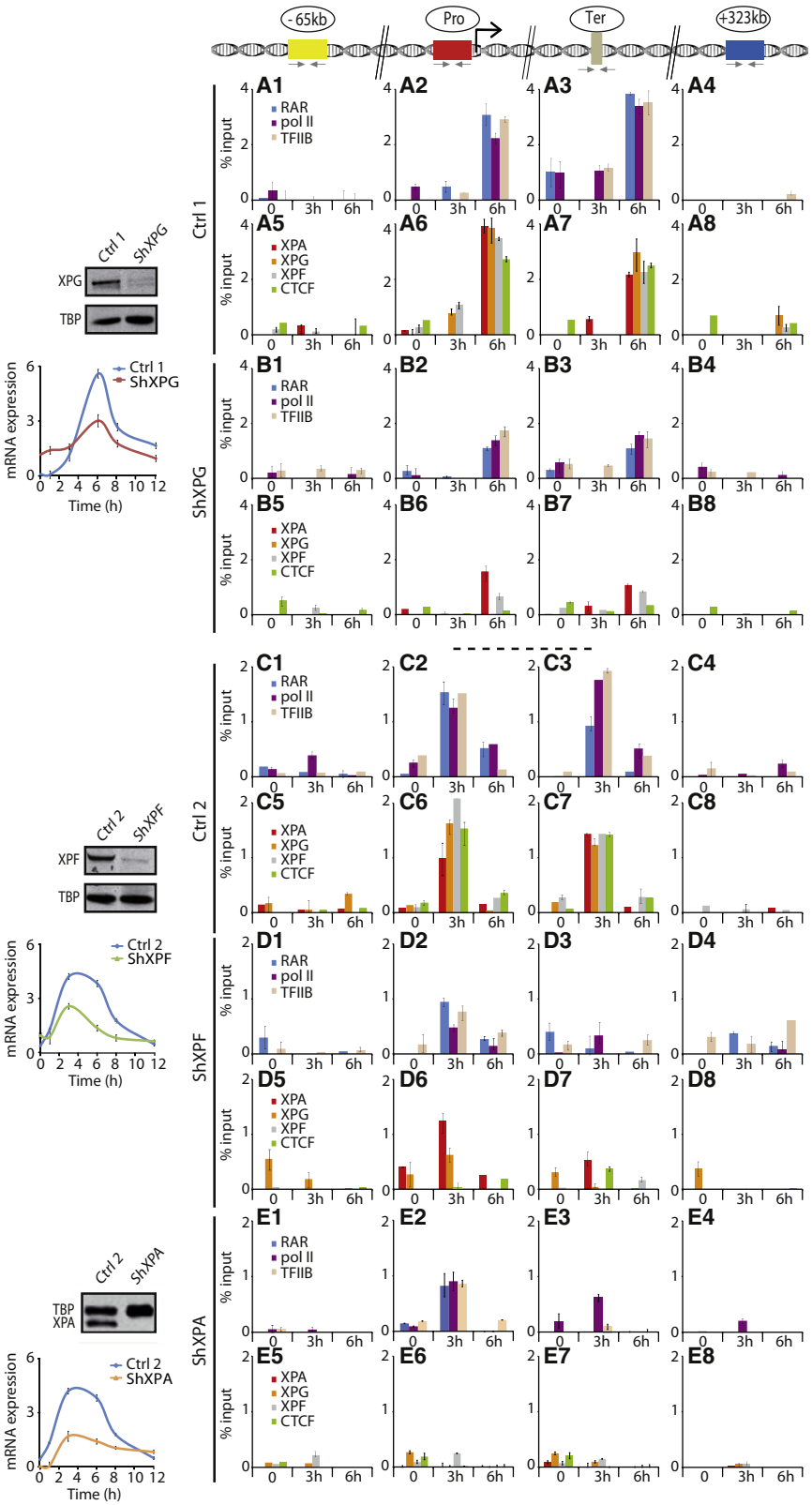

Figure 1. XPG and XPF Are Necessary for Concomitant Recruitment of Transcription Machinery at Promoter and Terminator upon *RARβ2* Induction

Schematic representation of the *RARβ2*; the amplicons at the −65 kb, Pro, Ter, and +323 kb regions are indicated (upper panel). Relative *RARβ2* mRNA expression monitored over time by qPCR from t-RA-treated ShXPG, ShXPF, ShXPA HeLa cells, and the corresponding Ctrl1 and Ctrl2 control cells (left panels). Error bars represent the standard deviation of three independent experiments. Western blotting analyses of XPG, XPF, XPA, and TBP were performed from chromatin extracts of ShXPG, ShXPF, ShXPA HeLa cells and their corresponding controls (left panels). TBP is used as a positive nuclear marker. ChIP monitoring the t-RA-dependent occupancy of RAR, pol II, TFIIB (A1-4, B1-4, C1-4, D1-4, E1-4), XPA, XPG, XPF, and CTCF (A5-8, B5-8, C5-8, D5-8, E5-8) on *RARβ2* locus from ShXPG (B1-8), ShXPF (D1-8), ShXPA (E1-8), and the corresponding control (Ctrl1, Ctrl2) chromatin extracts at 0, 3, and 6 hr upon t-RA induction as indicated. A1-4, A5-8—E5-E8 are aligned under the corresponding region of the *RARβ2* gene being probed. Each series of ChIP is representative of at least two independent experiments as indicated by standard deviation, and values are expressed as percentage of the input.

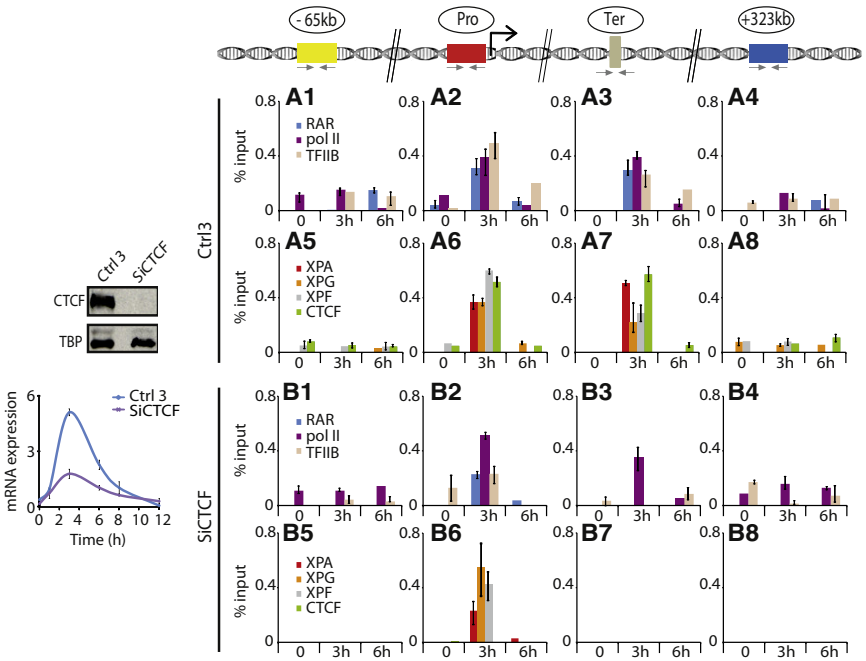

Figure 2. CTCF Is Necessary for Concomitant Recruitment of Transcription Machinery at Promoter and Terminator upon *RARβ2* Induction

Schematic representation of the *RARβ2*; the amplicons at the −65 kb, Pro, Ter, and +323 kb regions are indicated (upper panel). Relative *RARβ2* mRNA expression monitored over time by qPCR from t-RA-treated SiCTCF HeLa cells, and the corresponding Ctrl3 control cells (left panel). Error bars represent the standard deviation of three independent experiments. Western blotting analyses of CTCF and TBP were performed from chromatin extracts of SiCTCF HeLa cells and the corresponding control (left panel). TBP is used as a positive nuclear marker. The t-RA-dependent occupancy of RAR, pol II, TFIIB (A1-4, B1-4), XPA, XPG, XPF, and CTCF (A5-8, B5-8) was monitored by ChIP on RARβ2 locus using SiCTCF (B1-8) and Ctrl3 (A1–A8) chromatin extracts at 0, 3, and 6 hrs upon t-RA induction. A1-4, A5-8—B5-B8 are aligned under the corresponding region of the *RARβ2* gene being probed. Each series of ChIP is representative of at least two independent experiments as indicated by standard deviation, and values are expressed as percentage of the input.

presence of XPA, XPG, and XPF (Figure 1, panels A2 and A6) at Pro, paralleling *RARβ2* mRNA synthesis. All of these proteins were also observed at Ter (panels A3 and A7). Such a recruitment pattern was not detected at position 65 kb (−65 kb) and 323 kb (+323 kb), upstream and downstream to the transcription start site (TSS), respectively (panels A1, A5 and A4, A8). The recruitment pattern at Pro/Ter is different from the one found at exon 6 that lacks the RAR as well as the TFIIB transcription initiation factor (see Figure S1 available online). In Ctrl2 and Ctrl3, the transcription and the NER factors were similarly detected at Pro and Ter 3 hr post-t-RA treatment (Figure 1, panels C1–C8, and Figure 2, panels A1–A8). Moreover, we also noticed the absence of these proteins at the −65 kb and +323 kb regions (Figure 1 and Figure 2).

We demonstrated that upon gene activation there is formation of a large transcription complex containing the transcription and NER machineries, using as a target both the promoter and the terminator of the *RARβ2* gene. We next wondered whether the absence of NER factors would prevent complex formation.

In ShXPG cells, XPA together with XPF, and in ShXPF cells, XPA together with XPG were detected at Pro, although to a lower extent compared with their respective Ctrl1-2 controls (Figure 1, compare panels B2 and B6, with panels A2 and A6, and panels D2 and D6 with panels C2 and C6). In ShXPG cells, transcription and NER factors were still recruited at Ter, but to a lower extent in comparison to Ctrl1 (compare panels B3 and B7 with panels A3 and A7). However, in ShXPF cells, the corecruitment of transcription machinery with XPA and XPG, obvious at Pro, was less significant at Ter (compare panels D2, D6 and D3, D7 with panels B2, B6 and B3, B7).

Previous work has described the sequential arrival of the NER factors following the preinitiation complex (PIC) formation, in which XPC, RPA, and XPA precede XPG and XPF at the *RARβ2* promoter (Le May et al., 2010). As expected, in ShXPA cells, neither XPG nor XPF was detected at Pro and Ter (Figure 1, compare panels E6, E7 and C6, C7). Moreover, as in ShXPG and shXPF cells, the transcriptional machinery was recruited to a lower extent at Ter than at Pro in ShXPA cells (panels E3 and E2).

Our data indicated that the presence of the later-recruited NER factors XPG and XPF was crucial in the formation of a transactivation complex that encompassed both the promoter and the terminator regions of the *RARβ2*-activated gene, suggesting a long-range chromatin rearrangement.

XPG, XPF, and CTCF Are Required for Gene Looping

Several studies have underlined the influence of DNA binding proteins in specific loop formation and distinct gene expression as demonstrated for the imprinted *Igf2/H19* and the role of the chromatin organizer CTCF (Murrell et al., 2004). In Ctrl1-3 cells, CTCF was recruited at Pro and Ter upon t-RA induction (Figure 1, panels A6, A7, C6, C7, and Figure 2, panels A6, A7). However in ShXPG, ShXPF, and ShXPA cells in which the concomitant presence of XPG and XPF was abolished, CTCF was not detected at Pro and Ter (Figure 1, panels B6, B7, D6, D7, and E6, E7, respectively) when compared with Ctrl1-2 (panels A6, A7 and C6, C7).

In SiCTCF cells, *RARβ2* expression was significantly reduced compared to Ctrl3 but not totally abolished (Figure 2, left panel). *RARβ2* mRNA synthesis still occurred, as indicated by the presence of pol II at exon 6 and at Ter (Figure S1 and Figure 2, panel B3). The ChIP assay showed a concomitant detection of the NER factors with the transcriptional machinery at Pro, but not at Ter (Figure 2, compare panels B2, B6 with panels A2, A6 and panels B3, B7 with panels A3, A7).

The above data indicate that the presence of both XPG and XPF is required for the recruitment of CTCF. The correlated

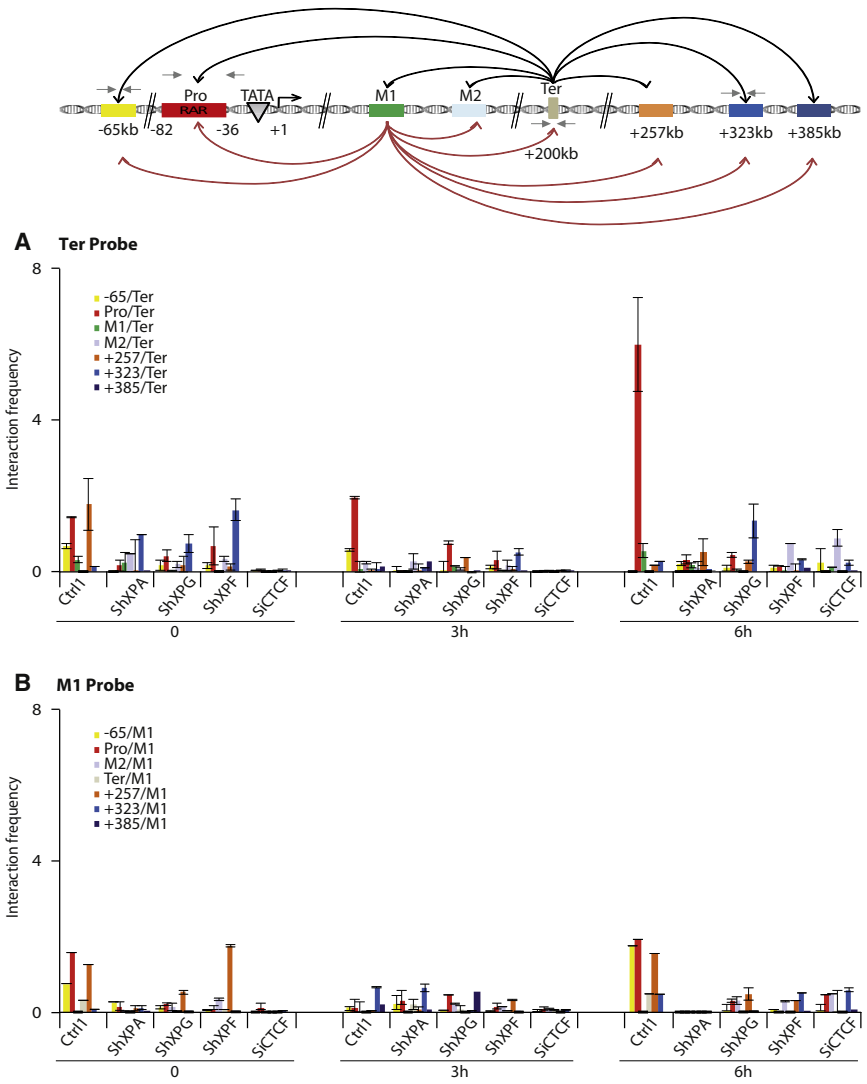

Figure 3. XPG and XPF Are Involved in CTCF-Induced *RARβ2* Gene Looping

Schematic representation of the quantitative chromatin conformation capture (q3C) (upper panel). Two probes were designed at Ter (gray square) and M1 (green square) of *RARβ2* gene. These probes were used to investigate the associations between the different elements including upstream (−65 kb, Pro), intronic (M1, M2), and downstream (Ter, +257 kb, +323 kb, +385 kb) regions as indicated by the black (Ter probe) and red (M1 probe) arrows. q3C assays were performed using crosslinked and HindIII-digested chromatin from Ctrl1, ShXPG, ShXPA, ShXPF, and siCTCF HeLa cells at 0, 3, and 6 hr post-t-RA treatment (10 μM). The bar chart (y axis) shows the enrichment of PCR product (%) normalized to the enrichment within the human *xpb* gene (=100%), as illustrated in Figure S2G. Each PCR was performed at least three times and averaged as indicated by standard deviation. Signals were normalized to the total amount of DNA used, estimated with an amplicon located within a HindIII fragment in *RARβ2* gene (see the Experimental Procedures).

recruitment to distant regions along the *RARβ2* locus suggested the formation of gene looping. We thus performed quantitative chromatin conformation capture assays (q3C) and analyzed interactions between several regions along the *RARβ2* gene (Vernimmen et al., 2007). Digestion of crosslinked chromatin by HindIII resulted in restriction fragments equidistantly separated by 60 kb and containing either −65 kb, Pro, Ter, or +323 kb as well as an intronic region (M1) of the *RARβ2* locus (Figure 3, upper scheme). Ter and M1 of *RARβ2* gene were used as baits. In t-RA-treated Ctrl1 cells, we observed that Pro could specifically and significantly interact with Ter at 6 hr (Figure 3A), paralleling the *RARβ2* mRNA synthesis (Figure 1, left panel) and the recruitment of the transcriptional apparatus (Figure 1, panels A2, A3, A6, A7). Similar observations were made at 3 hr in Ctrl2-3 (data not shown). By contrast, in ShXPG, ShXPF, and ShXPA cells as well as in SiCTCF, no spatial proximity between Ter and Pro was revealed. As controls, no specific interactions were observed between the intronic M1 bait and Pro or between all the other analyzed fragments upon t-RA treatment (Figure 3B).

Altogether our data showed that the t-RA induction could initiate a CTCF-related long-range interaction between Pro and Ter of *RARβ2* gene that was abolished in the absence of either XPG or XPF, underlining the role of these NER factors in chromatin remodeling.

The Catalytic Activity of Both XPG and XPF Is Required for Gene Looping

Consistent with the role of XPG and XPF-ERCC1 in NER (Gillet and Scharer, 2006; O'Donnovan et al., 1994; Sijbers et al., 1996), we wondered about the requirement of their endonuclease activity in chromatin looping upon transactivation. The SV40-immortalized XP-G (XPCS1RO) and XP-F (XP2YO) fibroblasts that were derived from a XP/CS and XP patient, respectively (Ellison et al., 1998; Yagi and Takebe, 1983), were stably transfected either with XPG/WT, XPG/E791A or with XPF/WT, XPF/D676A (Figure S2A). XPG/E791A and XPF/676A mutations were found to abolish the catalytic activity of these two endonucleases and consequently their ability to eliminate DNA damages when added in an in vitro NER assay (Lalle et al., 2002; Staresincic et al., 2009; and Figure S2B). *RARβ2* mRNA synthesis was significantly inhibited in XPG/E791A and XPF/D676A cells as well as in the corresponding parental cells compared to the cells expressing either XPG/WT or XPF/WT, respectively (Figure 4, panels A1, B1, and Figures S2C and S2D). In XPG/WT and XPG/E791A cells, we observed a conserved recruitment of RAR, pol II, XPA, XPG, and XPF both at Pro and Ter at 3 and 8 hr, respectively (Figure 4, compare panels A2, A3 with panels A4, A5). In XPG/E791A cells, the optimal ChIP recruitment

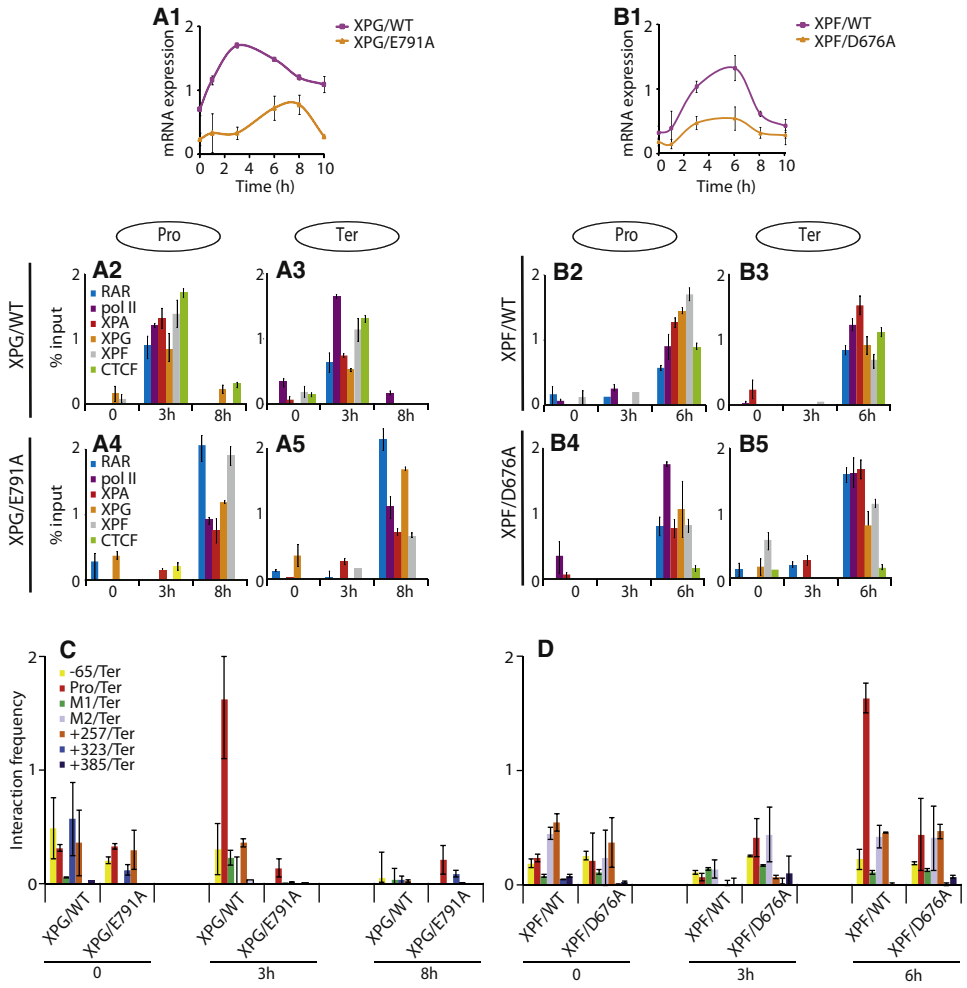

Figure 4. Endonuclease Activity of XPG and XPF Is Required for Gene Looping

Relative *RARβ2* mRNA expression monitored by quantitative PCR from XPG/WT (A1), XPG/E791A (A1), XPF/WT (B1), XPF/D676A (B1) cells at 0, 1, 3, 6, 8, and 10 hr post-t-RA treatment. Error bars represent the standard deviation of three independent experiments. ChIP monitoring of the t-RA-dependent occupancy of RAR, pol II, XPA, XPG, XPF, and CTCF at Pro and Ter regions of *RARβ2* gene from XPG/WT (A2-3), XPG/E791A (A4-5), and XPF/WT (B2-3), XPF/D676A (B4-5) chromatin extracts as indicated. Each series of ChIP is representative of two independent experiments. Intrachromosomal interactions between Ter and upstream/downstream elements of *RARβ2* gene are as follows: q3C assays were performed using crosslinked, HindIII-digested chromatin from t-Ra-treated XPG/WT or XPG/E791A (C) and XPF/WT or XPF/D676A (D) fibroblasts at 0, 3, and 6 hr. These independent graphs represent a measure of the association between the upstream −65 kb; Pro; the intronic M1 and M2 elements; and the downstream +257 kb, +323 kb, and +385 kb regions to either the terminal region (Ter Probe) or the intronic region (M1 Probe, see Figures S2E and S2F) as a negative control. Each PCR was performed at least three times and averaged as indicated by standard deviation. Signals were normalized to the total amount of DNA used, estimated with an amplicon located within a HindIII fragment in *RARβ2* gene.

reflected the *RARβ2* mRNA synthesis peak at 8 hr (panel A1). In XPF/D676A and XPF/WT cells, the recruitment patterns of transcription and NER factors were similar both at Pro and Ter (compare panels B4, B5 with panels B2, B3), contrary to what was observed at Ter in ShXPF cells (Figure 1, panels D3, D7). We also noticed that both XPG/E791A and XPF/D676A proteins were recruited with the other NER factors. However, in spite of the presence of both endonucleases in XPG/E791A and XPF/D676 cells, the q3C assay did not show any stable interaction between Pro and Ter, while in both XPG/WT and XPF/WT cells, such interaction was observed at 3 and 6 hr upon t-RA induction, respectively (Figures 4C and 4D). In XPG/WT and XPF/WT cells, CTCF was detected at both Pro and Ter concomitantly with gene looping (Figure 4, panels A2, A3 and B2, B3). In XPG/E791A or XPF/D676 cells, the inhibition of gene looping was coincident with the absence of CTCF (Figure 4, panels A4, A5 and B4, B5).

These data indicated that the endonuclease activities of XPG and XPF were necessary to recruit CTCF and to allow stable chromatin looping between Pro and Ter of the *RARβ2*-activated gene.

XPG Endonuclease Induces DNA Breaks and DNA Demethylation at the Promoter Region

Previous works have revealed the formation of transient DNA breaks upon gene activation (Ju et al., 2006). This raises

questions regarding the interplay between XPG and XPF endonucleases and the transactivation process. Using a Bio-ChIP assay which measures the incorporation of biotinylated dUTP within broken DNA, we observed a concomitant increase of DNA cleavage specifically both at Pro and Ter in all five of the t-RA-treated Ctrl1, Ctrl2, XPG/WT, XPF/WT, and Ctrl3 cells (Figure 5, panels A1, B1, C1, and D1, respectively). In ShXPG and XPG/E791A cells, DNA breaks were hardly detected at Pro (Figure 5, panels A1, C1), whereas in ShXPF and XPF/D676 cells, DNA cleavage could only be observed at Pro (panels B1 and C1). Nevertheless, we noticed that in XPG-deficient cells there was a slight incorporation of bio-UTP at Ter (panel A1).

Several studies have documented a relationship between XPG and DNA demethylation upon transcription (Barreto et al., 2007; Jin et al., 2008). We thus investigated whether or not the endonuclease activity XPG and XPF was linked to DNA demethylation upon *RARβ2* gene activation. Using an unmethylated DNA immunoprecipitation (unMeDIP) approach, we measured the removal of the 5′-methylcytosine (5mC) along the different regions of activated *RARβ2*. In all of the five control cells, Pro and Ter were found unmethylated (Figure 5, panels A2, B2, C2, and D2). In ShXPG and XPG/E791A cells, Pro remained methylated, while a slight DNA demethylation was observed at Ter when compared to their corresponding Ctrl1 and XPG/WT cells (panels A2, C2). In ShXPF and XPF/D676 cells, Pro but not Ter was unmethylated (panels B2 and C2). Using the methylated DNA immunoprecipitation (MeDIP) approach, no significant decrease was observed in DNA methylation at either Pro or Ter in t-RA-treated XPG/E791A and XPF/D767A cells (Figure S3), which was found to be consistent with the UnMEDIP (see above) assays and pyrosequencing data (see below, panel E); in XPG/WT and XPF/WT, we observed a weak but significant decrease of DNA methylation. Interestingly, in ShXPA, neither DNA breaks nor DNA demethylation was detected at Pro and Ter (panels B1 and B2, respectively), likely due to the absence of XPG and XPF (Figure 1, panels E6 and E7).

Having demonstrated the connection between CTCF and the XPG and XPF endonucleases for chromatin looping, we then investigated whether the presence of CTCF was a prerequisite for DNA modifications. In SiCTCF, we repeatedly observed both DNA breaks and DNA demethylation at Pro but not at Ter when compared to Ctrl3 (Figure 5, panels D1 and D2). These data strongly suggest a role for CTCF in gene looping and Ter modification following DNA breaks and DNA demethylation at Pro. However, our results showed that these DNA modifications followed the dynamic recruitment of NER factors and the transcriptional machinery upon transactivation (Figure 5). To further localize these DNA modifications, we thus pyrosequenced the promoter of *RARβ2* from t-RA-treated cells.

Pyrosequencing analysis of Bio-ChIP DNA samples from t-RA-treated XPG/WT and XPF/WT cells indicated that a high frequency of DNA breaks on both strands localized downstream the *RARβ2* TSS (Figure 5E, red arrows); most of them were detected on the transcribed strand (TS). In XGP/E791A cells, the frequency of DNA cleavage strongly decreased and the detected breaks were differently localized (Figure 5E, light blue arrows). Interestingly, in XPF/D676A, the cleavage pattern observed around the *RARβ2* promoter was similar (Figure 5E, dark blue arrow), demonstrating that XPF endonuclease inhibition did not prevent Bio-UTP incorporation and thus DNA breaks at Pro (see also panels B1 and C1).

Genomic DNA was next bisulfate converted and pyrosequenced to localize the demethylated 5mC at Pro in t-RA-treated cells. Seventeen CpG dinucleotides, localized inside the proximal promoter and downstream of the TSS, were analyzed (Figure 5E). In XPG/WT, XPF/WT, and XPF/D676A cells, CG1, CG3, CG15, and CG16 (closed dark blue circles) were significantly demethylated, whereas only CG1 remained demethylated in XPG/E791A (Figure 5E). The above data underlined the role of XPG and XPF endonucleases in both the formation of DNA breaks and active DNA demethylation at Pro and Ter, respectively. Our data also showed that XPG-dependent DNA breaks and DNA demethylation at Pro preceded the recruitment of CTCF.

DISCUSSION

Discovering the components of the eukaryotic transcription apparatus has been a major theme of research over the past decades. In the present study, we describe how XPG and XPF endonucleases, which are sequentially recruited following the arrival of the transcription machinery at the promoter of activated genes, are involved in their optimal expression.

We especially found that upon induction by t-RA, RAR as well as GTFs and pol II were found at both the promoter and the terminator of *RARβ2* gene. NER factors, where XPA recruitment precedes XPG and XPF, are established once RNA pol II and transcription factor are recruited (Figure 1, see also Le May et al., 2010). We next demonstrate that both XPG and XPF endonuclease activity are crucial to recruit the chromatin organizer CTCF and to promote gene looping between the promoter and the terminator of the activated *RARβ2* gene.

The formation of such an "active chromatin hub" resulting from inter- and intrachromosomal interactions between specific subsets of transcription units of activated genes as described for NR-target genes (Hu et al., 2008), *β-Globin* (de Laat and Grosveld, 2003), *T helper type 2* (Spilianakis and Flavell, 2004), *BRCA1* (Tan-Wong et al., 2008), *GATA-4* loci (Tiwari et al., 2008), and HIV-1 provirus (Perkins et al., 2008) might provide additional control mechanisms for regulated gene expression (Deng and Blobel, 2010). We here show that the presence of XPG and XPF together with CTCF in the long-range interactions can be considered as part of the regulatory biological program of the *RARβ2* gene. Indeed, in ShXPG, ShXPF, and ShXPA cells, in which the concomitant presence of XPG and XPF is abolished, CTCF is absent, and, as well as in as in SiCTCF cells, there is no gene looping and *RARβ2* RNA synthesis is downregulated (Figure 1, Figure 2, and Figure 3). Moreover, it is interesting to notice the crucial role of XPA in recruiting XPG and XPF, knowing that XPA recruitment occurs before XPG and XPF (Le May et al., 2010). It is not surprising to notice the presence of XPA together with RNA pol II at exon 6 and Ter (although at a very low level) in ShXPG and ShXPF cells (Figure S1 and Figure 1).

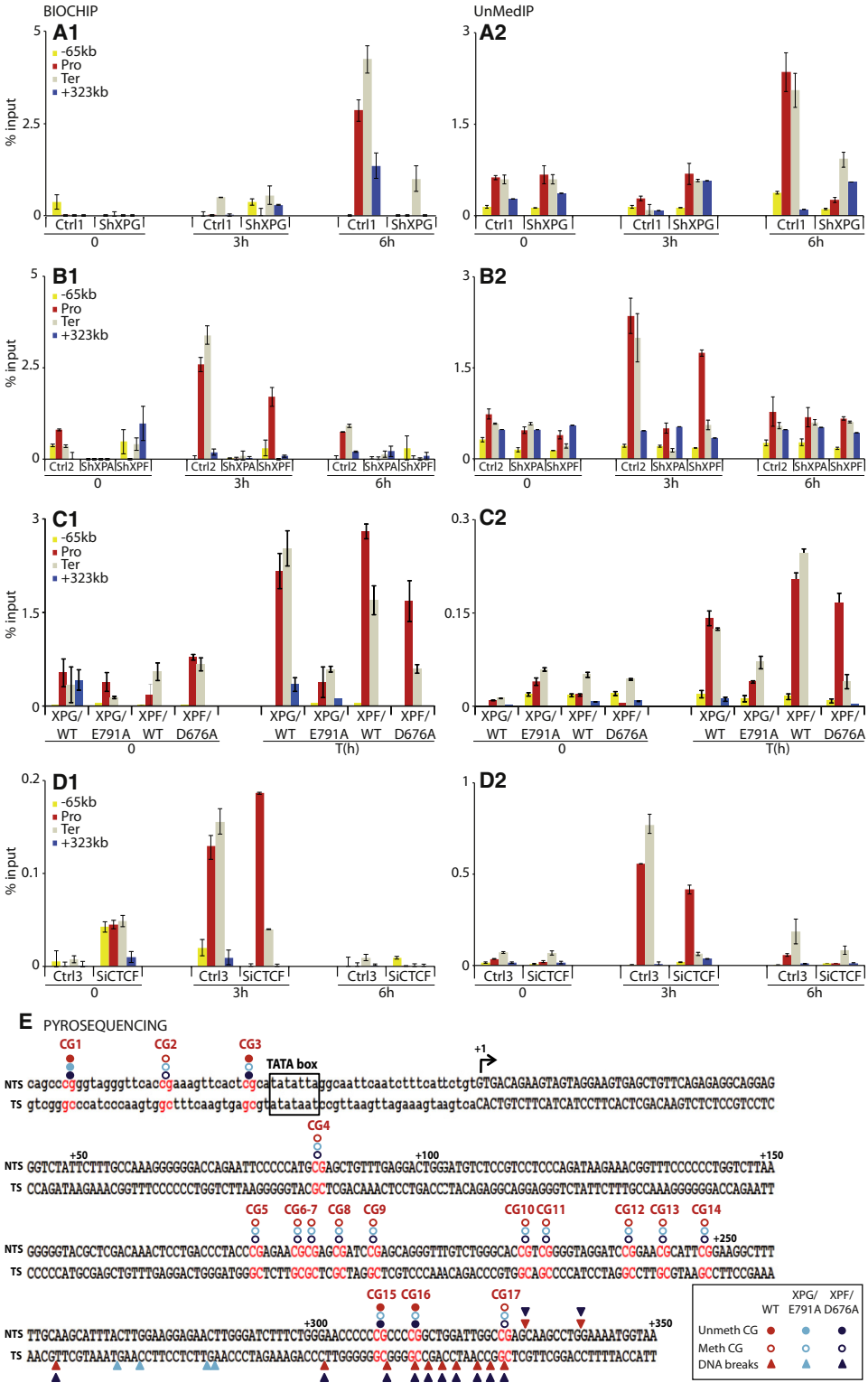

Figure 5. XPG and XPF Are Required for DNA Breaks and DNA Demethylation at Pro and Ter, Respectively

Detection of DNA break at −65 kb, Pro, Ter, and +323 kb of the *RARβ2* locus from Ctrl1, ShXPG (A1), Ctrl2, ShXPA, ShXPF (B1), XPG/WT, XPG/E791A and XPF/WT, XPF/D676A (C1), Ctrl3, SiCTCF (D1) t-RA-treated cells. DNA breaks are detected first through the incorporation of Biotin-dUTP via a terminal deoxynucleotidyl transferase (TdT) reaction and second via a ChIP approach. DNA fragments containing DNA breaks are immunoselected by Biotin antibodies and analyzed by qPCR. Each series of Bio-ChIP is representative of three independent experiments as indicated by standard deviation, and values are expressed as

In addition, mutations that affect the catalytic activity of XPG and XPF (XPG/E791A and XPF/D676A, respectively) were found to prevent CTCF recruitment. More interestingly, we discover that both gene looping and CTCF recruitment occur in parallel with the formation of DNA breaks (Figure 5). Having demonstrated (1) that XPG/E791A mutation prevented DNA breaks formation at Pro while XPF catalytic mutation does not (Figure 4) and (2) that in SiCTCF there are DNA breaks at Pro but not at Ter around which no NER factors were detected (Figure 2 and Figure 5), it is tempting to propose the following model: upon t-RA transactivation and transcription and NER factors recruitment (Figure 6, step 1), XPG induces some DNA breaks at Pro (step 2) independently of both the status of XPF and the presence of CTCF. Once XPG has fulfilled its role at Pro, CTCF recruitment is initiated with the help of XPF (step 3).

Such a scenario raises questions regarding the interplay between molecular machineries that are involved in the formation of DNA breaks, DNA demethylation, the recruitment of CTCF, and the activation of the gene transcription. Using different approaches (UnMEDIP, MEDIP, and pyrosequencing), we observed a relationship between the DNA demethylation at Pro and Ter and the presence of CTCF necessary for the loop formation of the *RARβ2*-activated gene. In particular, we have shown that demethylation at Pro is not sufficient to attract CTCF when Ter is not demethylated (Figure 5). Indeed, XPG and XPF defects abolish DNA demethylation at Pro and Ter, respectively, and consequently CTCF recruitment (Figure 5). Accordingly, DNA breaks and DNA demethylation at Pro are not sufficient to attract CTCF in the absence of Ter domain modification. We thus can speculate that the DNA breaks induced by XPG and the consequent recruitment of CTCF at demethylated promoter could be responsible for important torsional tensions of DNA that allow the promoter-bound XPF to be brought in proximity to the terminator (Figure 6, step 3).

Mechanisms underlying the removal of methyl groups from genomic DNA are very controversial (Barreto et al., 2007; Jin et al., 2008) and have implicated DNA repair processes either through deamination followed by excision of the 5mC or by direct removal of the methyl moiety from the base (Gehring et al., 2009; Ma et al., 2009). Our results do not allow the identification of the precise causal relationships between DNA demethylation and the effects of XPG and XPF endonucleases. However, our data indicated that the DNA breaks induced by XPG were located near the demethylated CG dinucleotides in the vicinity of *RARβ2* TSS and almost exclusively on the TS (Figure 5E). Whether or not DNA cuts are a prerequisite for DNA demethylation remains to be shown. However, the pattern of DNA cuts and demethylated 5mC was strongly disturbed when XPG and XPF endonucleases were inactivated. Such localized roles of XPG at Pro and XPF at Ter, as well as their cooperation in promoting loop formation and consequently optimal *RARβ2* gene expression, are reminiscent to the NER process in which XPF and XPG excise at the 3′ and 5′ of the DNA lesion, respectively, in a cooperative manner (Tapias et al., 2004b; Araujo et al., 2000; Staresincic et al., 2009). In NER, XPG mutations completely abolished the incision, while XPF mutations still allow XPG cuts at the 5′ side of the DNA damage (Lalle et al., 2002).

The present study underlines the essential role of XPG, XPF, and CTCF recruitment in the chromatin loop organization required for optimal expression of activated genes. Further genome-wide investigations are needed to question the general role of these endonucleases. Any events as presently documented that distort the interaction network involving both protein factors and the subsets of transcription units upon gene activation might result in inaccurate transactivation complex formation and altered gene expression. It would thus be legitimate to raise the possibility of some other interconnecting links in this cascade of events followed by the action of XPG, XPF, and CTCF. For example, defects in either the basal transcription factor TFIIB (Singh and Hampsey, 2007), the cdk7 kinase of TFIIH that abolishes RNA pol II (O'Sullivan et al., 2004), and/or NR phosphorylation (Compe et al., 2007), as well as mutations in the mediator that weaken its interaction with activator (Hashimoto et al., 2011; Wang et al., 2005), might indirectly disturb the formation of the transactivation complex by preventing, in the end, the recruitment and/or the function of XPG/XPF endonucleases and/or the recruitment of CTCF that is essential in chromatin rearrangement and, further, in optimal gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture

HeLa Silencix cells (Tebu-Bio, provided by D. Biard) were used, including Ctrl (Ctrl2, BD690), ShXPF, and ShXPA cells. ShXPG and the corresponding control (Ctrl1, SiLuc) cells were provided by K. Tanaka. SiCTCF and Ctrl3 cells were obtained by respectively transfecting HeLa cells with SMART pool siRNA targeting *CTCF* or scrambled siRNA (Dharmacon) at a final concentration of 100 nM. XPG/WT and XPG/E791A cells, as well as XPF/WT and XPF/D676A cells (gift from O. Scharer and W. Vermeulen), were obtained by the stable transfection of the corresponding wild-type or mutated XPG and XPF constructs (Staresincic et al., 2009) in SV40-immortalized XP-G (XPCS1RO) and XP-F (XP2YO) (Ellison et al., 1998) (Yagi and Takebe, 1983).

All cells were cultured in appropriate medium. Cells were incubated with red phenol-free medium containing 10% charcoal-treated fetal calf serum (FCS)

percentage of the input. Analysis of the unmethylation of DNA (UnMedIP) at −65 kb, Pro, Ter, and +323 kb of the *RARβ2* locus from Ctrl1, ShXPG (A2), Ctrl2, ShXPA, ShXPF (B2), XPG/WT, XPG/E791A XPF/WT, XPF/D676A (C2), Ctrl3, SiCTCF (D2) t-RA-treated cells. UnMedIP was performed using the UnMethylCollector kit (Active Motif). MseI-digested genomic DNA was selected using magnetic beads conjugated with CXXC domains, and the resulted samples containing unmethylated DNA were analyzed by qPCR. Each series of UnMedIP is representative of two independent experiments as indicated by standard deviation, and values are expressed as percentage of the input. BioChIP and UnMedip were performed at the indicated times except for XPG/WT, XPG/E791A, XPF/WT, and XPF/D676A (T[h] corresponds to the peaks of factors recruitment in Figures 3A2–3B5). (E) Schematic representation of the methylated (open circle) or unmethylated (closed circle) CpG dinucleotides (in red) and DNA breaks (closed triangle) along the *RARβ2* promoter for WT (red), XPG/E791A (light blue), and XPF/D676A (dark blue) cells. DNA breaks were localized at Pro by pyrosequencing the samples generated by Bio-ChIP. The methylated status of CpG dinucleotides at Pro was measured using bisulfite converted genomic DNA from XPG/WT, XPG/E791A and XPF/WT, XPF/D676A fibroblasts and pyrosequencing.

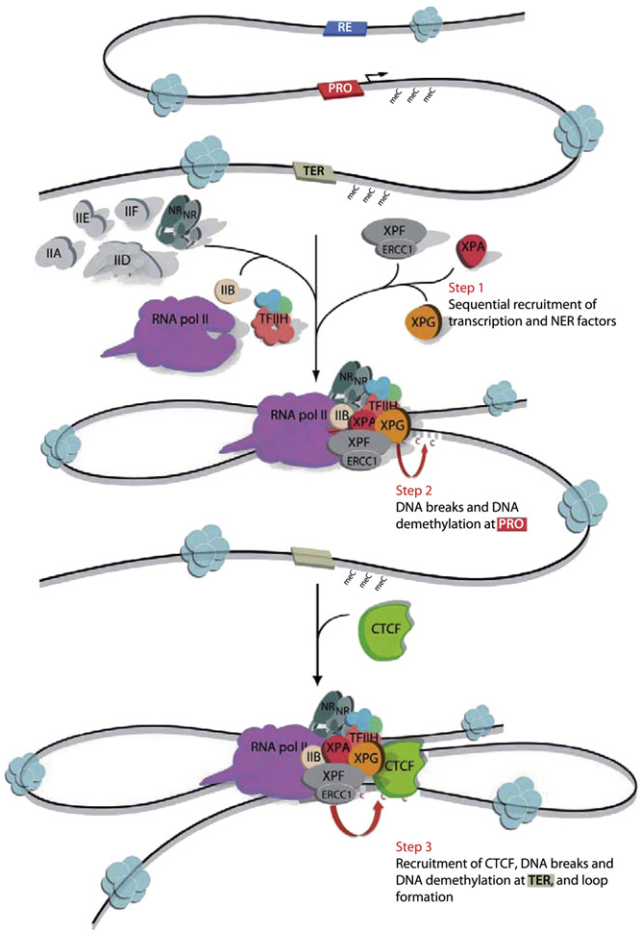

Figure 6. A Role for XPG and XPF in the CTCF-Dependent Gene Looping for Optimal Gene Expression

Upon ligand induction, nuclear receptor (NR) targets its responsive element (RE). The transactivation complex is then formed at the promoter (PRO) including the arrival of GTFs (IIA, IIB, IIE, IIF, IIH) and pol II. After the formation of the PIC, NER factors (XPA, XPG, and XPF/ERCC1) are sequentially recruited (step 1). XPG (red arrow) initiates DNA break (dotted DNA) and demethylation (meC-C) at PRO (step 2), thus favoring the CTCF recruitment and gene looping between PRO and the terminator TER (step 3); concomitantly, XPF (red arrow) induces DNA break (dotted DNA) and DNA demethylation at TER.

and 40 μg/ml gentamycin. Cells were treated with 10 μM t-RA into the same medium.

Antibodies

RNA pol II (7C2), RAR (9A6), XPA (1E9), TBP (3G3), and XPG (1B5) antibodies were from IGBMC antibody facilities. TFIIB (C-18), XPF (H-300), and Biotin (33) antibodies were obtained from Santa-Cruz Biotechnology, β-tubulin from Millipore, and CTCF (ab70303) from Abcam.

Reverse Transcription and Quantitative PCR

Total RNA was isolated from several cell lines using a GenElute Mammalian Total RNA Miniprep kit (Sigma) and reverse transcribed with SuperScript II reverse transcriptase (Invitrogen). The quantitative PCR was done using the Lightcycler 480 (Roche). The primer sequences for *RARβ2* and glyceraldehyde 3-phosphate deshydrogenase (*GAPDH*) genes used in qPCR are available upon request. The *RARβ2* mRNA expression represents the ratio between values obtained from treated and untreated cells normalized against the housekeeping *GAPDH* mRNA.

Chromatin Immunoprecipitation

Cells were crosslinked at room temperature (RT) for 10 min with 1% formaldehyde. Chromatin was prepared and sonicated on ice for 30 min using a Bioruptor (Diagenode) as previously described (Le May et al., 2010). Samples were immunoprecipitated with antibodies at 4°C overnight, and protein G Sepharose beads (Upstate) were added, incubated for 4 hr at 4°C, and sequentially washed. Protein-DNA complexes were eluted, and DNA fragments were purified using QIAquick PCR purification kit (QIAGEN) and analyzed by quantitative PCR.

Biotin-ChIP

Crosslinked cells following 1% formaldehyde treatment for 10 min at RT were permeabilized with cytonin (Active Motif) for 30 min at RT. After extensive washes with phosphate buffer salt (PBS), terminal deoxynucleotidyl transferase (TdT) reaction was performed using Biotin-16-dUTP (Roche) and 60 units of recombinant enzyme rTdT (Promega). TdT reaction was stopped with specific stop buffer (Chemicon International) for 15 min at RT. After extensive washes with PBS, the resulting samples were sonicated on ice for 20 min (40 cycles, pulse 10 s, pause 20 s) using a Bioruptor (Diagenode) and immunoprecipitated using anti-Biotin antibodies and treated as described in the ChIP protocol. DNA fragments were purified using QIAquick PCR purification kit (QIAGEN) and analyzed by quantitative PCR using sets of primers.

Quantitative Chromosome Conformation Capture

The quantitative chromosome conformation capture (q3C) assay was performed as previously described (Vernimmen et al., 2007). Briefly, cells were crosslinked at RT for 10 min with 2% formaldehyde. Crosslinked chromatin was digested in the appropriate restriction buffer by 400 units of enzyme HindIII. The restriction enzyme mixture was stopped, diluted in ligation buffer, and incubated with the highly concentrated T4 DNA ligase (Roche) for 4 hr at 16°C. The crosslinking was heat reversed, and DNA fragments were purified. Undigested DNA or digested DNA without ligation step was used as negative control. Moreover, we used the endogenous *xpb* locus, which has been reported to adopt the same spatial conformation in different tissues (Vernimmen et al., 2007), as an internal positive control. All q3C results were normalized by data from *xpb* analysis (see Figure S2G), controlling for changes in nuclear size, chromatin density, and crosslinking efficiency. Primers and probes were designed as follows: a universal sequence-specific Taqman probe and corresponding reverse primer on a fixed restriction fragment (Ter or M1) were used in combination with different forward primers specific for the other restriction fragments (see upper panel, Figure 3). Quantitative 3C templates (200 ng) were used for Taqman/PCR reaction using the universal PCR Master Mix and the Lightcycler 480 from Roche.

Unmethylated DNA Immunoprecipitation

Genomic DNA was extracted using GenElute Mammalian Genomic DNA Mini-prep Kit (Sigma). Unmethylation of 5mC on the *RARβ2* locus was measured by digesting 2 μg genomic DNA with 10 units of MseI (Fermentas) and by using the UnMethylcollector kit (Active Motif). Unmethylated DNA Immunoprecipitation Kit (UnMeDIP) is based on the affinity of the three zinc-coordinating CXXC domains, localized in chromatin-associated proteins such as DNA methyltransferase 1 (DNMT1) or mixed lineage leukemia (MLL), that specifically bind nonmethylated CpG sites. The resulting samples were selected using magnetic beads conjugated with CXXC domains, extensively washed, and analyzed by quantitative PCR.

Pyrosequencing

Sequencing Analyses

Pyrosequencing was performed on a Pyromark Q96 ID Platform using the PSQ Gold SQA reagent kit (QIAGEN). Briefly, 20 μl amplified DNA products were mixed with 2 μl streptavidin Sepharose beads (Amersham Biosciences AB), 38 μl binding buffer (10 mM Tris-HCl [pH 7.6], 2 M NaCl, 1 mM EDTA, and 0.1% Tween 20), and 20 μl H2O, followed by shaking for 10 min. Then

the immobilized biotinylated PCR products-streptavidin Sepharose beads complex was captured using the QIAGEN Pyromark Q96 Work Station. Single-strand DNA purification was achieved by sequential washes with 75% ethanol, 0.2 M NaOH, and 10 mM Tris-acetate, pH 7.6 buffer. The unbiotinylated strand was dissociated and discarded. The immobilized single biotinylated strands were released to a 96-well microtiter plate, which was preadded with 38 μl annealing buffer and 2 μl complementary sequencing primer. The plate was incubated at 80°C for 2 min, followed by slow cooling to RT. The processed mixture was loaded onto the PyroMark ID system equipped with PyroMark ID software. The resulting pyrograms and associated sequences were generated and analyzed automatically using PSQ 96 SQA software (QIAGEN).

Methylation Analyses

Pyrosequencing was performed using a PyroMark Q96 ID Pyrosequencing instrument (QIAGEN). Pyrosequencing assays were designed using MethPrimer (http://www.urogene.org/methprimer/index1.html). Genomic DNA (200 ng) was treated with EZ DNA Methylation-Gold Kit (Zymo Research Corporation) and amplified using 1.25 U Platinum Taq DNA Polymerase (Invitrogen) and 1 μM each forward and reverse primers in a 50 μl reaction volume with 0.2 mM dNTP and 1.5 mM $MgCl_2$. PCR conditions were 94°C for 3 min, then 40 cycles of 94°C for 30 s, Tm for 30 s, and 72°C for 30 s, followed by a 9 min extension at 72°C. Biotin-labeled single-stranded amplicons were isolated using the QIAGEN Pyromark Q96 Work Station and underwent pyrosequencing with 0.5 μM primer. The percent methylation for each of the CpGs within the target sequence was calculated using PyroQ cpG Software (QIAGEN). All methylation analyses were performed in duplicate.

All the sets of primers and probes targeting different regions of *RARβ2* gene and used for the qPCR analysis are available upon request.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at http://dx.doi.org/10.1016/j.molcel.2012.05.050.

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3.4.5 Publication: TFIIH the orchestra chief for NER factors in Transcription?

Singh A., Compe E., **Le May N.** and Egly J.M. « Mutations in genes encoding TFIIH subunits causing xeroderma pigmentosum and trichothiodystrophy disorders specifically disturb several steps during transcription ». **AJHG**. 2014, 96. 1-14. (*8*)

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ARTICLE

TFIIH Subunit Alterations Causing Xeroderma Pigmentosum and Trichothiodystrophy Specifically Disturb Several Steps during Transcription

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Mutations in genes encoding the ERCC3 (XPB), ERCC2 (XPD), and GTF2H5 (p8 or TTD-A) subunits of the transcription and DNA-repair factor TFIIH lead to three autosomal-recessive disorders: xeroderma pigmentosum (XP), XP associated with Cockayne syndrome (XP/CS), and trichothiodystrophy (TTD). Although these diseases were originally associated with defects in DNA repair, transcription deficiencies might be also implicated. By using retinoic acid receptor beta isoform 2 (*RARB2*) as a model in several cells bearing mutations in genes encoding TFIIH subunits, we observed that (1) the recruitment of the TFIIH complex was altered at the activated *RARB2* promoter, (2) TFIIH participated in the recruitment of nucleotide excision repair (NER) factors during transcription in a manner different from that observed during NER, and (3) the different TFIIH variants disturbed transcription by having distinct consequences on post-translational modifications of histones, DNA-break induction, DNA demethylation, and gene-loop formation. The transition from heterochromatin to euchromatin was disrupted depending on the variant, illustrating the fact that TFIIH, by contributing to NER factor recruitment, orchestrates chromatin remodeling. The subtle transcriptional differences found between various TFIIH variants thus participate in the phenotypic variability observed among XP, XP/CS, and TTD individuals.

Introduction

The evolutionarily conserved general transcription factor IIH (TFIIH) plays a key role in maintaining genome stability.[1,2] Mammalian TFIIH comprises a core (containing the six subunits ERCC3 [XPB], GTF2H1 [p62], GTF2H4 [p52], GTF2H2 [p44], GTF2H3 [p34], and GTF2H5 [p8 or TTD-A]) bridged by ERCC2 (XPD) to the CDK-activating kinase (CAK) module (composed of the three subunits CDK7, CCNH [cyclin H], and MNAT1 [MAT1]; Figure 1A). In addition to having a function in transcription, TFIIH is also involved in the nucleotide excision repair (NER) pathway, thus illustrating the important interplay between these distinct processes.[3,4] NER is responsible for the removal of a variety of bulky DNA adducts, such as those induced by UV irradiation, and is subdivided into two sub-pathways: global genome repair (GGR) is responsible for the removal of DNA lesions from the whole genome, and transcription-coupled repair (TCR) is responsible for the accelerated removal of lesions arising on the transcribed strand of active genes.[5,6] In GGR, the XPC-RAD23B complex recognizes the damage-induced DNA distortion, whereas in TCR, RNA polymerase II (pol II) stalled in front of a lesion promotes the recruitment of the TCR-specific proteins ERCC6 (CSB) and ERCC8 (CSA). Both NER sub-pathways then funnel through the TFIIH action that unwinds the DNA via the ATPase and helicase activities of ERCC3 and ERCC2, which are regulated by the GTF2H4-GTF2H5 and GTF2H2 subunits, respectively. XPA and RPA are then recruited to assist the expansion of the DNA bubble around the damage and the arrival of endonucleases ERCC5 (XPG) and ERCC4 (XPF). ERCC5 and ERCC4 then generate cuts in the 3′ and 5′ sides of the lesion, respectively, thereby removing the damaged oligonucleotide before the re-synthesis machinery fills the DNA gap. During transcription of protein-coding genes, TFIIH is involved via its ERCC3 subunit in the opening of the promoter,[7,8] whereas its CDK7 kinase phosphorylates serines 5 and 7 of the C-terminal domain (CTD) of pol II, as well as others activators.[9,10] Interestingly, NER factors (XPC, ERCC6, XPA, ERCC5, and ERCC4) have been found to also be involved in the regulation of gene expression.[11–13] However, it remains to be established whether TFIIH influences the recruitment and the function of NER factors at the promoter of activated genes for chromatin remodeling and accurate transcription.

The importance of TFIIH and its relationship with the other NER factors are highlighted by the existence of human genetic disorders with a broad range of clinical features (Table 1).[14–20] Indeed, mutations in excision repair complementation group 3 (*ERCC3* [MIM 133510]), excision repair complementation group 2 (*ERCC2* [MIM 126340]), and general transcription factor IIH polypeptide 5 (*GTF2H5* [MIM 608780]) cause three distinct autosomal-recessive disorders: xeroderma pigmentosum (XP [MIM 278730]), XP associated with Cockayne syndrome (XP/CS [MIM 610651]), and trichothiodystrophy (TTD [MIM 610675]), respectively.[21,22] XP is characterized by numerous skin abnormalities ranging from excessive freckling and ichthyosis to multiple skin cancers, the frequency

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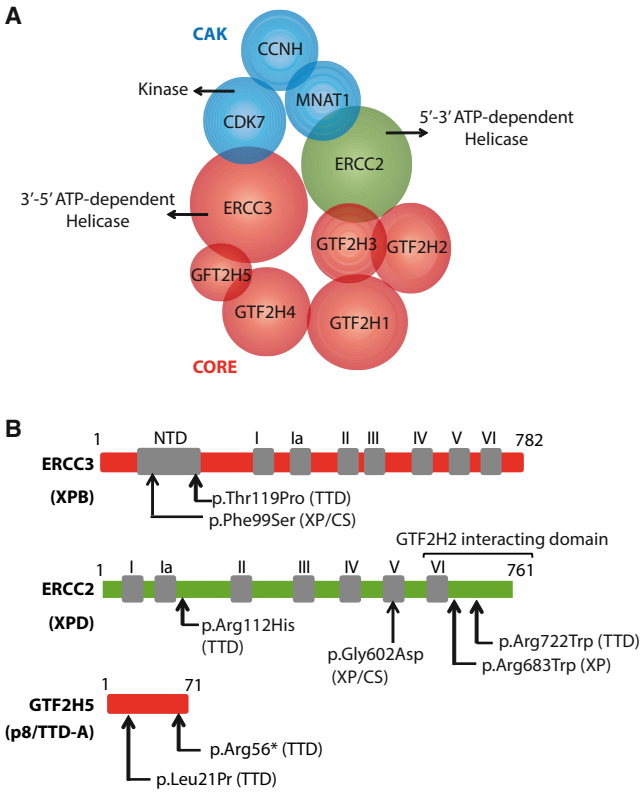

Figure 1. Schematic Representation of TFIIH
(A) The CAK sub-complex (in blue) is bridged to the core TFIIH (in red) by the ERCC2 (XPD) helicase (in green).
(B) Variants of the ERCC3 (XPB), ERCC2 (XPD), and GTF2H5 (TTD-A) subunits are indicated. Black squares indicate the helicase motifs (I–VI). NTD stands for N-terminal domain.

of which is about 2,000-fold greater than that in normal individuals. In addition to having hyper-photosensitivity, XP individuals display a progressive neurological degeneration.[23] XP/CS individuals display a combination of the cutaneous abnormalities found in XP and the severe neonatal later onset of neurological and developmental anomalies typical of CS. The typical hallmark of TTD is sulfur-deficient brittle hair, caused by a greatly reduced content of cysteine-rich matrix proteins in the hair shafts. Intellectual disability and ichthyosis also characterize TTD individuals,[24] and some of them are sensitive to sunlight without any unusual pigmentation changes and severe skin lesions or cancer.

Accumulating evidence suggests that the clinical features of these three disorders cannot be exclusively explained by defects in DNA repair and might also involve transcription deficiencies[25] (Table 1).[14–20] In this study, we intend to understand how mutations in *ERCC3*, *ERCC2*, and *GTF2H5* result in impairment of gene expression in order to provide explanations for the large and diverse range of clinical features associated with these mutations. Knowing the close connections between TFIIH and NER factors in the removal of DNA damage,[26] we have investigated the consequences of ERCC3, ERCC2, and GTF2H5 variants on the recruitment of the NER factors and their roles in the various steps that lead to accurate RNA synthesis, including histone post-translational modifications (PTMs), DNA breaks, DNA demethylation, and gene-loop formation. In addition to improving our understanding of the cascade of events that drive RNA synthesis, such a systematic approach could help to determine transcriptional default hallmarks that molecularly define different genetic disorders.

Material and Methods

Cell Lines, Culture Conditions, and Transfection

Wild-type (WT)-ERCC3 (XPB) cells, ERCC3-p.Phe99Ser cells, and ERCC3-p.Thr119Pro cells are SV40-transformed human fibroblasts (XPCS2BASV) expressing WT *His-ERCC3-HA*,[27] *ERCC3* c.296T>C (RefSeq accession number NM_000122.1), and *ERCC3* c.355A>C, respectively.[28] GTF2H5 (p8 or TTD-A) cells are SV40-transformed human fibroblasts (TTD1Br-SV) expressing *GTF2H5* c.[62T>C]; [116C>T], p.[Leu21Pro];[Arg56*] (RefSeq NM_207118.2).[16] WT-GTF2H5 cells (TTD1Br-SV + TTDA-GFP and TTD1Br-SV + TTDA-HA) are rescued TTDBr1-SV cells with *pEGFP-N1-TTDA* and *pCDNA3-HA-TFB5* vectors that stably express *GTF2H5-GFP* and *GTF2H5-HA*, respectively.[29,30] WT-ERCC2 (XPD) cells (GM637) are SV40-transformed human fibroblasts from a normal 18-year-old female. ERCC2-p.Gly602Asp cells (XPCS2),[18] ERCC2-p.Arg112His cells (TTD8PV),[16] ERCC2-p.Arg683Trp cells (XP135LO),[18] and ERCC2-p.Arg722Trp cells (TTD1BEL)[19] are human primary fibroblasts expressing *ERCC2* c.1805G>A (RefSeq NM_000400.3), *ERCC2* c.335G>A, *ERCC2* c.2047C>T, and *ERCC2* c.2164C>T, respectively. Cells were incubated with phenol-red-free medium containing charcoal-treated fetal calf serum and 40 μg/ml gentamicin. Cells were treated with 10 μM of all-trans retinoic acid (t-RA, MP Biomedicals). ERCC2-p.Arg112His, ERCC2-p.Gly602Asp, ERCC2-p.Arg683Trp, and ERCC2-p.Arg722Trp cells were transiently transfected with the X-tremeGENE HP DNA transfection reagent (Roche) 48 hr before the t-RA treatment with WT *pEGFP-ERCC2*.

Antibodies

Mouse monoclonal antibodies toward ERCC3 (1B3), ERCC2 (2F6), GTF2H2 (p44, 1H5), retinoic acid receptor alpha (RARα, 9A6), XPA (1E11), ERCC5 (XPG, 1B5), and pol II (7C2) were produced by the Institut de Génétique et de Biologie Moléculaire et Cellulaire. CDK7 (C-19), general transcription factor IIB (GTF2B [TFIIB], C-18), ERCC4 (XPF, H-300), and BIOTIN (33) antibodies were purchased from Santa-Cruz Biotechnology. CTCF (ab70303) and RNA pol II ser5P (61085) antibodies were purchased from Abcam and Active Motif, respectively. H3K4me2 (9726), H3K9me2 (9753), and H3K9Ac (9671) antibodies were purchased from Cell Signaling Technology.

Reverse Transcriptase and qPCR

Total RNAs were isolated with the GenElute Mammalian Total RNA Miniprep Kit (Sigma) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen). qPCRs were performed with the QuantiTect SYBR Green PCR Kit (QIAGEN) and the LightCycler 480 apparatus (Roche). The primer sequences for retinoic acid receptor beta isoform 2 (*RARB2* [MIM 180220]) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH* [MIM 138400])

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Table 1. *ERCC3*, *ERCC2*, and *GTF2H5* Mutations and the Related Clinical XP, XP/CS, and TTD Phenotypes

Gene Mutation	Protein Variant	Syndrome	Individual	Clinical Features	Reference
ERCC3 c.296T>C	ERCC3 p.Phe99Ser	XP/CS	male	severe sunburn at 6 weeks of age and later hyper-pigmentation, but no evidence of any malignancy; CS neurological anomalies including cerebellar atrophy, sclerosis of sutures, neuron demyelination, and some hearing difficulties; development of sexual anomalies with age	Scott et al.[14]
ERCC3 c.355A>C	ERCC3 p.Thr119Pro	TTD	male	congenital ichthyosis (collodion baby); hair with tiger-tail pattern; moderate skin photosensitivity; mild learning disability	Weeda et al.[15]
GTF2H5 c.[62T>C]; [116C>T]	GTF2H5 p.[Leu21Pro]; [Arg56*]	TTD	male	congenital ichthyosis (collodion baby); moderate skin photosensitivity, but no skin cancer; developmental delay, asthmatic attacks, and short stature; severe mental retardation	Stefanini et al.[16]
ERCC2 c.335G>A	ERCC2 p.Arg112His	TTD	male	moderate skin photosensitivity, but no skin cancer; sulfur-deficient brittle hair and nails; delayed puberty and short stature; neurological anomalies including axial hypotonia and reduced motor coordination	Stefanini et al.[17]
ERCC2 c.1805G>A	ERCC2 p.Gly602Asp	XP/CS	male	high skin photosensitivity and skin cancer at 2 years of age; CS neurological anomalies; progeroid features	Takayama et al.[18]
ERCC2 c.2047C>T	ERCC2 p.Arg683Trp	XP	male	high skin photosensitivity and skin cancers; moderate mental retardation	Broughton et al.[19]
ERCC2 c.2164C>T	ERCC2 p.Arg722Trp	TTD	male	high skin photosensitivity, but no skin cancer; sulfur-deficient brittle hair and nails; severe physical and mental retardation	Taylor et al.[20]

are listed in Table S1. *RARB2* mRNA levels were normalized to *GAPDH*.

Chromatin Immunoprecipitation and BIOTIN-ChIP Assays

Cells were cross-linked at room temperature (RT) for 10 min with 1% formaldehyde. Chromatin was prepared and sonicated on ice for 30 min with a Bioruptor (Diagenode) as previously described.[12] Samples were immunoprecipitated with antibodies at 4°C overnight, and protein G Sepharose beads (Upstate) were added, incubated for 4 hr at 4°C, and sequentially washed. Protein-DNA complexes were eluted and de-cross-linked. DNA fragments were purified with the QIAquick PCR Purification Kit (QIAGEN) and analyzed by qPCR with a set of primers targeting the promoter and terminator regions of *RARB2* (Table S1).

In parallel, cross-linked cells were permeabilized with cytonin (Trevigen) for 30 min at RT. After extensive washes with PBS, a terminal deoxynucleotidyl transferase (TdT) reaction was performed with Biotin-16-dUTP (Roche) and 60 units of recombinant enzyme rTdT (Promega). The resulting samples were next sonicated and immunoprecipitated with anti-Biotin antibodies and treated as described in the chromatin immunoprecipitation (ChIP) assay. The purified DNA fragments were analyzed by qPCR with the previously described primers (Table S1).

Unmethylated DNA Immunoprecipitation Assays

Genomic DNA was extracted with the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma). Loss of 5-methylcytosine (5mC) at the promoter and terminator regions of *RARB2* was measured by digestion of genomic DNA (2 μg) with 10 units of MseI (Fermentas) and with the UnMethylcollector Kit (Active Motif). The unmethylated DNA immunoprecipitation (unMeDIP) kit is based on the affinity of the three zinc-coordinating CXXC domains that specifically bind nonmethylated CpG sites. The resulting samples were selected with magnetic beads conjugated with CXXC domains, extensively washed, and analyzed by qPCR.

Quantitative Chromosome Conformation Capture

The quantitative chromosome conformation capture (q3C) assay was performed as previously described.[31] Cells were cross-linked at RT for 10 min with 2% formaldehyde. Cross-linked chromatin was digested in the appropriate restriction buffer with 400 units of HindIII. The digestion was stopped after overnight incubation at 37°C, diluted in ligation buffer, and incubated with the highly concentrated T4 DNA ligase (Roche) for 4 hr at 16°C. The cross-linking was heat reversed, and DNA fragments were purified. Undigested DNA and digested DNA without the ligation step were used as negative controls. Moreover, as an internal positive control, we used the endogenous *ERCC3*, which has been reported to adopt the same spatial conformation in different tissues.[31] All q3C results were normalized by data from *ERCC3* analysis, which controlled for changes in nuclear size, chromatin density, and cross-linking efficiency. Primers and probes were designed as follows: a universal sequence-specific Taqman probe and corresponding reverse primer on a fixed restriction fragment (Ter or M1) were used in combination with different forward primers specific to the other restriction fragments (see Figure 4, upper panel). q3C templates (200 ng) were used for the Taqman PCR reaction with the universal PCR Master Mix and the Lightcycler 480 apparatus from Roche.

Construction of Baculoviruses and Purification of Complexes

Baculoviruses overexpressing the FLAG-ERCC3, FLAG-ERCC2, GTF2H1 (p62), GTF2H4 (p52), GTF2H2, GTF2H3 (p34), FLAG-CDK7, CCNH (cyclin H), MNAT1 (MAT1), and GTF2H5 subunits of TFIIH were produced as previously described.[32] The cDNAs encoding altered FLAG-ERCC3 and FLAG-ERCC2 were obtained by PCR-site-directed mutagenesis. The resulting vectors were recombined with baculovirus DNA (BD BaculoGold, Pharmingen). The recombinant viruses were purified from isolated plaques, and viral stocks were prepared by three-step growth amplification.

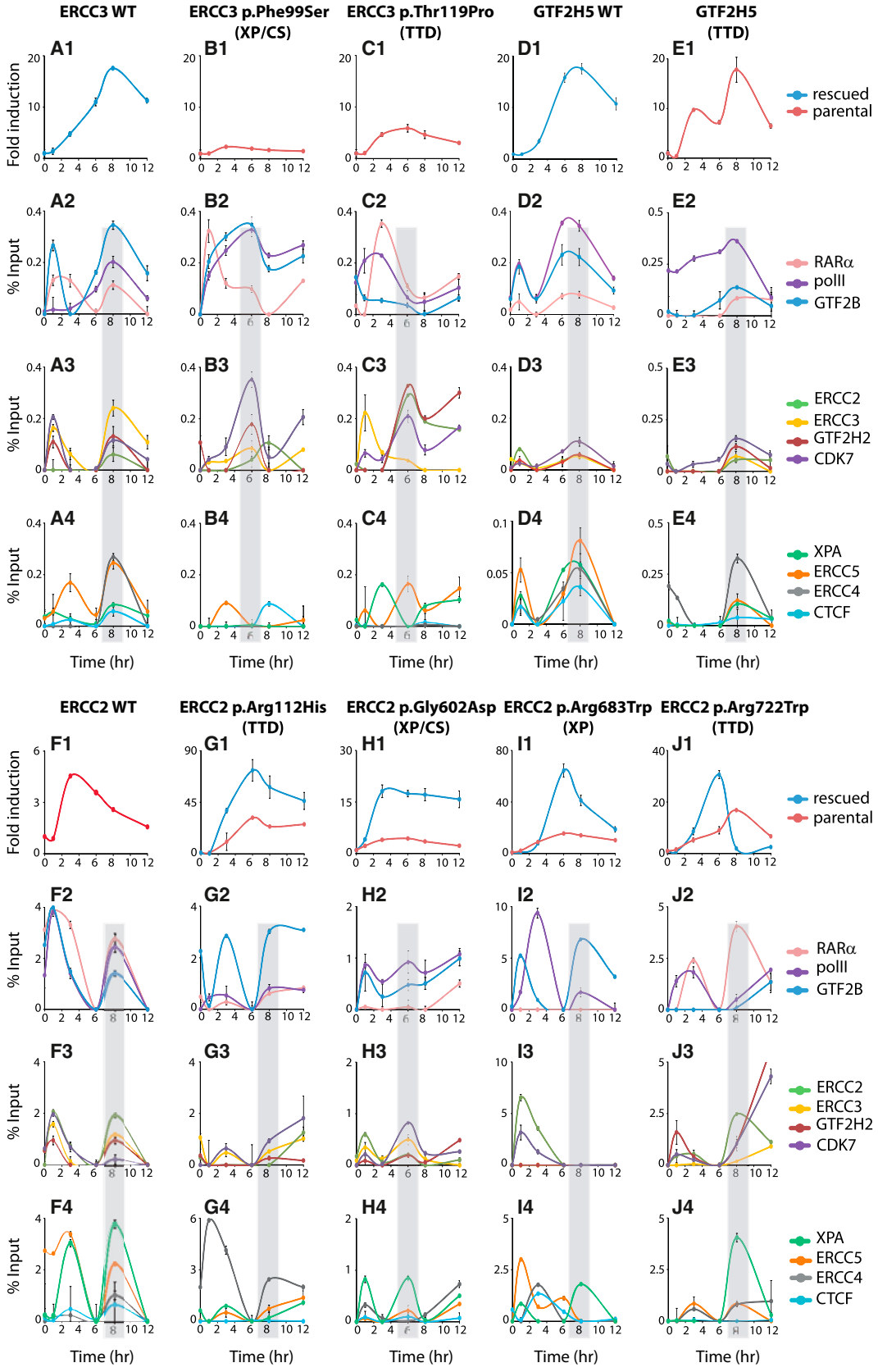

Figure 2. *RARB2* mRNA Expression and Transcriptional-Machinery Recruitment on the *RARB2* Promoter Are Disturbed in Cells Bearing Mutations in Genes Encoding TFIIH Subunits

(A1–J1) Relative *RARB2* mRNA expression monitored by qPCR over time from t-RA-treated (1) WT-ERCC3, ERCC3-p.Phe99Ser, and ERCC3-p.Thr119Pro cells, which are SV40-transformed human fibroblasts (XPCS2BASV) expressing WT *His-ERCC3-HA*,[27] *ERCC3*

(legend continued on next page)

We infected Sf21 insect cells with the different baculoviruses in order to separately obtain core-IIH (with or without an ERCC3 variant), CAK, and ERCC2 (with or without a variant). The different whole-cell extracts were incubated for 4 hr at 4°C with anti-M2-FLAG antibody bound to agarose beads. After extensive washings, the immunoprecipitated fractions were eluted. The recombinant TFIIH was made by a mixture of purified core-IIH, CAK, and ERCC2, allowing the preparation of the different TFIIH complexes containing either ERCC2 or ERCC3 variants.

In Vitro Transcription Assays

Run-off transcription assays were performed with recombinant GTF2B, TFIIE, TFIIF, TBP, endogenous pol II, and the different TFIIH complexes (recombinant IIHs) as previously described.[32] Pol II phosphorylation was carried out as a classical run-off transcription assay as previously described.[33] Hypo-phosphorylated (IIA) and hyper-phosphorylated (IIO) forms of pol II were resolved by SDS-PAGE and detected with a monoclonal antibody (7C2).

Results

Mutations in *ERCC2* and *ERCC3* Compromise the Formation of the Transactivation Complex

To determine the transcriptional defects due to the mutations in *ERCC3*, *ERCC2*, and *GTF2H5*, we analyzed cells derived from XP, XP/CS, and TTD individuals bearing different mutations as indicated (Table 1; Figure 1B). We used *RARB2* as a model to investigate the transcription process. A few hours after t-RA treatment of these mutated cells, we observed that their patterns of *RARB2* mRNA synthesis were different from those of the respective WT-ERCC3 (XPB), WT-ERCC2 (XPD), and WT-GTF2H5 (p8 or TTD-A) cells (Figures 2A1–2J1). The amount of *RARB2* mRNA was significantly lower for ERCC3-p.Phe99Ser and ERCC3-p.Thr119Pro cells than for WT-ERCC3 cells, which peaked at 8 hr post-treatment (Figures 2A1–2C1). Compared to the cells rescued with an overexpressing WT ERCC2, the four cell lines bearing mutations in *ERCC2* showed similarly reduced *RARB2* induction (Figures 2F1–2J1). Compared to rescued cells, GTF2H5 cells did not show a reduction of the *RARB2* mRNA level (Figures 2D1 and 2E1).

We next performed ChIP assays to evaluate the dynamic recruitment of pol II partners at the *RARB2* promoter over time. We observed a concomitant recruitment of RARα, pol II, and GTF2B at 8 hr after t-RA treatment in WT-ERCC3, WT-ERCC2, and WT-GTF2H5 cells (Figures 2A2, D2, and F2, respectively). At this time, TFIIH was also recruited, as seen by the presence of its ERCC3, ERCC2, GTF2H2 (p44), and CDK7 subunits (Figures 2A3, 2D3, and 2F3). We also detected the simultaneous presence of XPA, ERCC5 (XPG), and ERCC4 (XPF) (Figures 2A4, 2D4, and 2F4).

Each mutation in *ERCC3*, *ERCC2*, and *GTF2H5* led to different and specific deregulation of recruitment of the components of the transactivation complex at both the promoter and the terminator (Figure 2; Figure S1). Whereas RARα was detected at early time points in ERCC3-p.Phe99Ser cells, pol II and GTF2B only accumulated at the promoter after 1 hr of t-RA induction (Figure 2B2). We also noticed a non-concomitant recruitment of the TFIIH subunits and NER factors. Whereas ERCC5 was recruited at 3 hr in ERCC3-p.Phe99Ser cells, XPA and ERCC4 were not detected over a 12-hr period (Figure 2B4). In ERCC3-p.Thr119Pro cells, RARα, pol II, and GTF2B were detected at the promoter around the 3-hr mark (Figure 2C2). The ERCC3 subunit was found at the promoter at 1 hr, whereas the ERCC2, GTF2H2, and CDK7 subunits were detected later (Figure 2C3). Similarly, XPA arrival (1 hr) preceded ERCC5 (6 hr), whereas ERCC4 was not detected (Figure 2C4). In cells bearing mutations in *GTF2H5*, we observed a concomitant recruitment of TFIIH subunits and NER factors with the transcriptional machinery at 8 hr after t-RA treatment; a similar result was observed with the corresponding rescued cell types (Figures 2D2–2D4 and 2E2–2E4).

We next focused on the cells bearing mutations in *ERCC2* and resulting in three different phenotypes (Figure 1B; Table 1). In ERCC2-p.Arg112His cells, the recruitment of transcription and NER factors was temporarily correlated. All these factors were found in a second recruitment cycle that peaked at 8 hr, remained present until 12 hr after t-RA treatment, and paralleled *RARB2* mRNA induction (Figures 2G1 and 2G2–2G4). In ERCC2-p.Gly602Asp cells, the recruitment pattern at 1 hr and 6 hr after t-RA treatment of pol II, GTF2B, TFIIH, and NER factors at the promoter was comparable to that in WT-ERCC2 cells (Figures 2H2–2H4). The ERCC2 p.Gly602Asp variant, which affects the helicase motif V, does not seem to disturb TFIIH architecture.[32] In ERCC2-p.Arg683Trp cells, the recruitment of TFIIH and NER factors was highly deregulated at early time points (Figures

c.296T>C, and *ERCC3* c.355A>C, respectively; (2) GTF2H5 (p8 or TTD-A) cells, which are SV40-transformed human fibroblasts (TTD1Br-SV) expressing *GTF2H5* c.[62T>C];[116C>T], p.[Leu21Pro];[Arg56*]; (3) WT-GTF2H5 cells (TTD1Br-SV + TTDA-HA), which are rescued TTD1Br-SV cells stably expressing *GTF2H5-HA*; and (4) WT-ERCC2 (XPD) cells (GM637), ERCC2-p.Gly602Asp cells (XPCS2), ERCC2-p.Arg112His cells (TTD8PV), ERCC2-p.Arg683Trp cells (XP135LO),[18] and ERCC2-p.Arg722Trp cells (TTD1BEL), which are human primary fibroblasts expressing WT *ERCC2*, *ERCC2* c.1805G>A, *ERCC2* c.335G>A, *ERCC2* c.2047C>T, and *ERCC2* c.2164C>T, respectively. Red curves show the mRNA expression of *RARB2* in the parental cells, including cells bearing mutations in *ERCC2* and *ERCC3* and the WT cell line (GM637). Blue curves show mRNA expression of *RARB2* in the rescued cells (expressing WT ERCC3 and WT GTF2H5) and cells overexpressing WT *GFP-ERCC2* (ERCC2 p.Arg112His, ERCC2 p.Gly602Asp, ERCC2 p.Arg683Trp, and ERCC2 p.Arg722Trp). Error bars represent the SD of three independent experiments.

(A2–J4) ChIP monitoring of the t-RA-dependent recruitment of RARα, pol II, GTF2B (A2–J2), ERCC3, ERCC2, GTF2H2, and CDK7 subunits of TFIIH (A3–J3) and XPA, ERCC5, ERCC4, and CTCF (A4–J4) on the *RARB2* promoter. Each series of ChIP is representative of at least two independent experiments. Values are expressed as the percentage of the input. Error bars represent the SD.

2I2–2I4). At 8 hr, only pol II, GTF2B, and XPA were detected. In ERCC2-p.Arg722Trp cells, in which the aberration is located in the C-terminal unfolded domain of ERCC2, we observed the recruitment of both transcription and NER factors at 8 hr (Figures 2J2–2J4); a much higher and continuous accumulation of pol II, GTF2B, and some of the TFIIH subunits also occurred at 12 hr. In these cells, the ERCC2 p.Arg683Trp and p.Arg722Trp variants weaken the interaction with the GTF2H2 subunit and consequently destabilize the architecture of TFIIH.[32]

All together, our data suggest that mutations in *ERCC3* and *ERCC2* disturb *RARB2* activation by impeding the formation of the pre-initiation complex at the promoter. Although the dysregulation was different depending on the mutation, we noticed a compromised integrity of the TFIIH complex and, in some cases, the absence of NER factors at the activated promoter.

Mutations in Genes Encoding TFIIH Subunits Affect Chromatin Remodeling

Previous works have underlined the sequential recruitment of the transcription and NER components at the promoter of activated genes and their role in bringing about chromatin modifications, e.g., histone PTMs, DNA breaks, and DNA demethylation.[11,13] Among the histone hallmarks characterizing the chromatin status, we have focused and compared euchromatin histone PTMs (acetylated lysine 9 histone H3 [H3K9Ac] and dimethylated lysine 4 histone H3 [H3K4me2]) to the heterochromatin mark (dimethylated lysine 9 histone H3 [H3K9me2]) previously observed at the *RARB2* promoter.[12,34]

In WT-ERCC3 and WT-ERCC2 cells treated with t-RA, ChIP showed the characteristic histone PTM signature of gene activation (increased H3K9ac and H3K4me2 and decreased H3K9me2; Figures 3A1 and 3F1), correlating with the *RARB2* mRNA induction peak at 8 hr. In all mutated cells tested so far (Figures 3B1, 3C1, and 3G1–3J1), we observed a persistence of active histone PTMs around the *RARB2* promoter. As an example, in ERCC2-p.Arg112His cells, in which the transcription and NER factors were shown to be recruited at the promoter by 8 hr post-treatment, H3K9me3 remained low; however, we noticed high acetylation and methylation of H3K9 and H3K4, respectively (Figure 2G1). In both GTF2H5 and rescued cells, H3K9me2 was hardly detectable, whereas H3K9ac and H3K4me2 were visible (Figure 3D1 and E1).

The formation of transient DNA breaks upon gene activation is linked to endonucleases ERCC5 and ERCC4 and has previously been reported at the *RARB2* promoter.[35,36] We thus evaluated the formation of such DNA breaks in the cells bearing mutations in *ERCC3*, *ERCC2*, and *GTF2H5* by performing a BioChIP assay that measured the incorporation of biotinylated dUTP within broken DNA. We observed a concomitant increase in DNA cleavage at both the promoter and the terminator of all WT cells upon t-RA activation (Figures 3A2, 3D2, and 3F2; Figure S2). In these cells, we noticed a parallel among the presence of ERCC5, ERCC4, and DNA breaks at both promoters and terminators. Except for in GTF2H5, ERCC2-p.Arg112His, ERCC2-p.Arg722Trp, and ERCC3-p.Thr119Pro cells (although to a much lower extent) in which ERCC5 and ERCC4 were still detected (Figures 3C2, 3E2, 3G2, and 3J2), a significant induction of DNA breaks near the *RARB2* promoter was not detected in mutated cell lines (Figures 3B2, 3H2, and 3I2). It should be noted that the DNA breaks were observed around the *RARB2* terminator in WT and GTF2H5 cells, whereas in all the other mutated cell lines, no DNA breaks were detected (Figure S2).

Studies have documented a relationship between ERCC5 and DNA demethylation upon transcription.[11] Using an unMeDIP approach, we measured the removal of 5mC at the *RARB2* promoter. We found that DNA demethylation occurred at the promoter by 8 hr after t-RA treatment and perfectly paralleled the recruitment of the entire transcription machinery in the three WT cell lines (Figures 3A3, 3D3, and 3F3). On the contrary, there was a complete lack of DNA demethylation in all cells bearing mutations in *ERCC2* and *ERCC3* (Figures 3B3, 3C3, and 3G3–3J3), but not in GTF2H5 cells (Figure 3E3).

Taken together, the above data strongly support an involvement of TFIIH in chromatin remodeling, including the histone PTMs, the formation of DNA breaks, and active DNA demethylation.

Mutations in Genes Encoding TFIIH Subunits Affect Gene Loop Formation

The detection of the basal transcription machinery together with the NER factors at both the promoter and the terminator of *RARB2* has previously been correlated with a chromatin-loop formation mediated by the CCCTC-binding factor (CTCF) chromatin organizer.[35] Such loop formation was shown to parallel DNA demethylation and DNA breaks at both regions. We performed q3C assays to analyze the interactions between the promoter and the terminator, as well as intronic (M1), upstream (−65 kb), and downstream (+323 kb) regions of *RARB2* (Figure 4, upper scheme). Using the terminator and M1 as bait, we observed that the promoter specifically and significantly interacted with the terminator by 8 hr in t-RA-treated WT cells (Figures 4A, 4D, and 4F), paralleling the recruitment of the entire transcriptional apparatus to both regions (Figure 2; Figure S1). By contrast, in all cells bearing mutations in genes encoding TFIIH subunits, including GTF2H5 cells, no significant increase in the frequency of terminator-promoter interactions occurred upon *RARB2* transactivation (Figures 4B–4J). In addition, we also observed that the absence of loop formation correlated with the absence or delayed recruitment of CTCF at both the promoter and the terminator of *RARB2* (Figures 2B4, 2C4, 2G4, 2H4, and 2J4; Figure S1). In GTF2H5 cells, we did not observe an increase in the interaction frequency between the terminator and the promoter upon transcription, even though there were no perturbations in

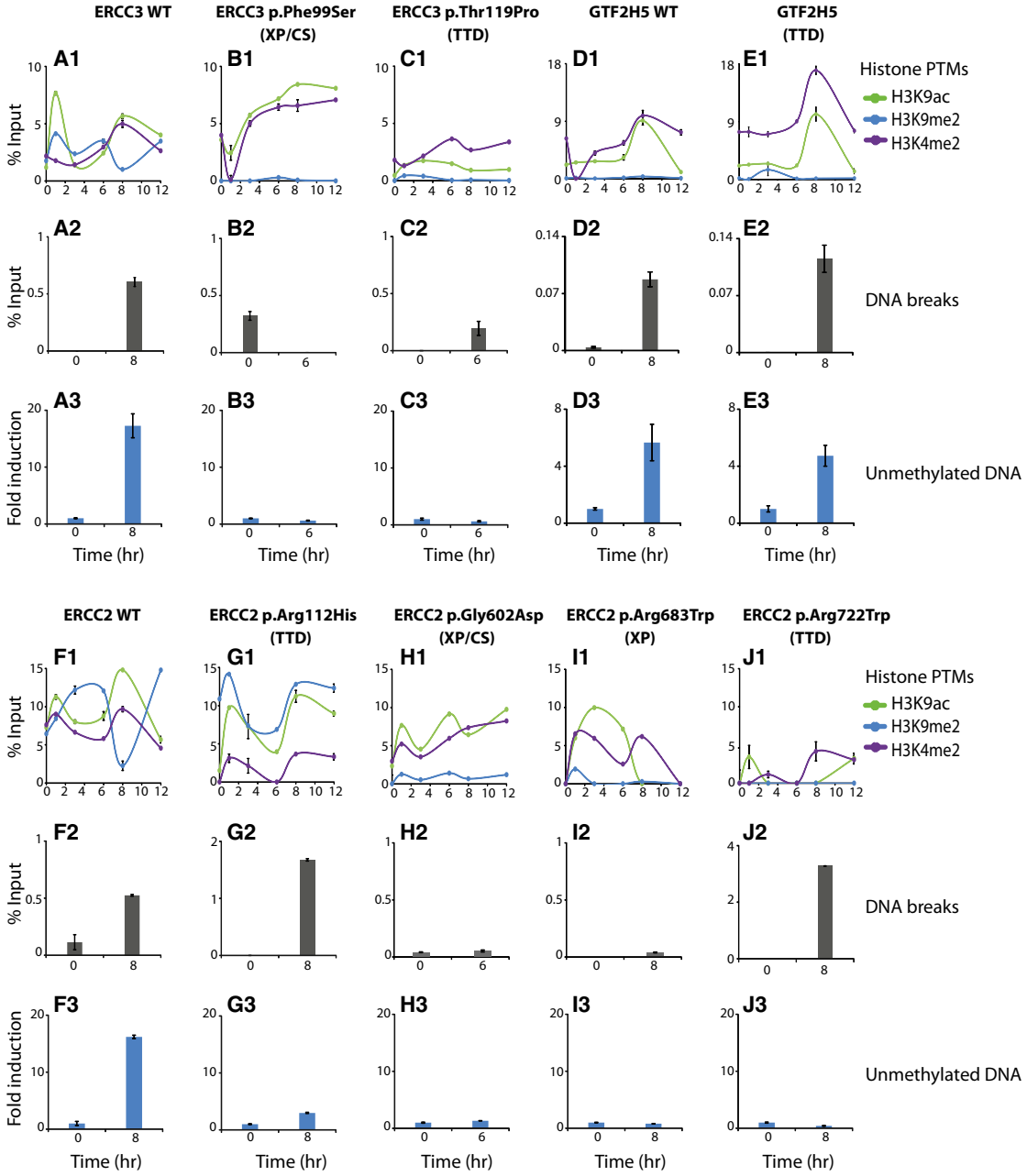

Figure 3. Mutations in Genes Encoding TFIIH Subunits Disrupt Histone PTMs, DNA Breaks, and Active DNA Demethylation on the *RARB2* Promoter

(A1–J1) ChIP monitoring of the t-RA-dependent presence of H3K4me2, H3K9me2, and H3K9ac on the *RARB2* promoter over time in all the mentioned cell lines. Each series of ChIP is representative of at least two independent experiments.

(A2–J2) Detection of DNA breaks at the *RARB2* promoter at 0 hr and at either 6 or 8 hr after t-RA treatment (depending on the timing of the RNA expression peak; see shadowed areas in Figure 2). Each series of BioChIP is representative of three independent experiments, and values are expressed as the percentage of the input. Error bars represent the SD.

(A3–J3) UnMeDIP experiments. Samples containing unmethylated DNA on the *RARB2* promoter were analyzed by qPCR. Each series of unMeDIP is representative of two independent experiments, and values are expressed as the percentage of the input. Error bars represent the SD.

recruitment of transcriptional or NER machinery, nor of other tested chromatin-remodeling events (Figure 4E). No specific interactions were observed between the intronic M1 bait and the promoter or between all the other analyzed fragments upon t-RA treatment (Figure S3).

By impeding the recruitment of NER factors at the activated *RARB2* promoter, TFIIH variants further disturbed the chromatin-loop formation required for optimal gene expression.

Mutations in Genes Encoding TFIIH Subunits Impair Some of Its Enzymatic Activities

We next addressed the contribution of ERCC3 and ERCC2 activities to the formation of an accurate

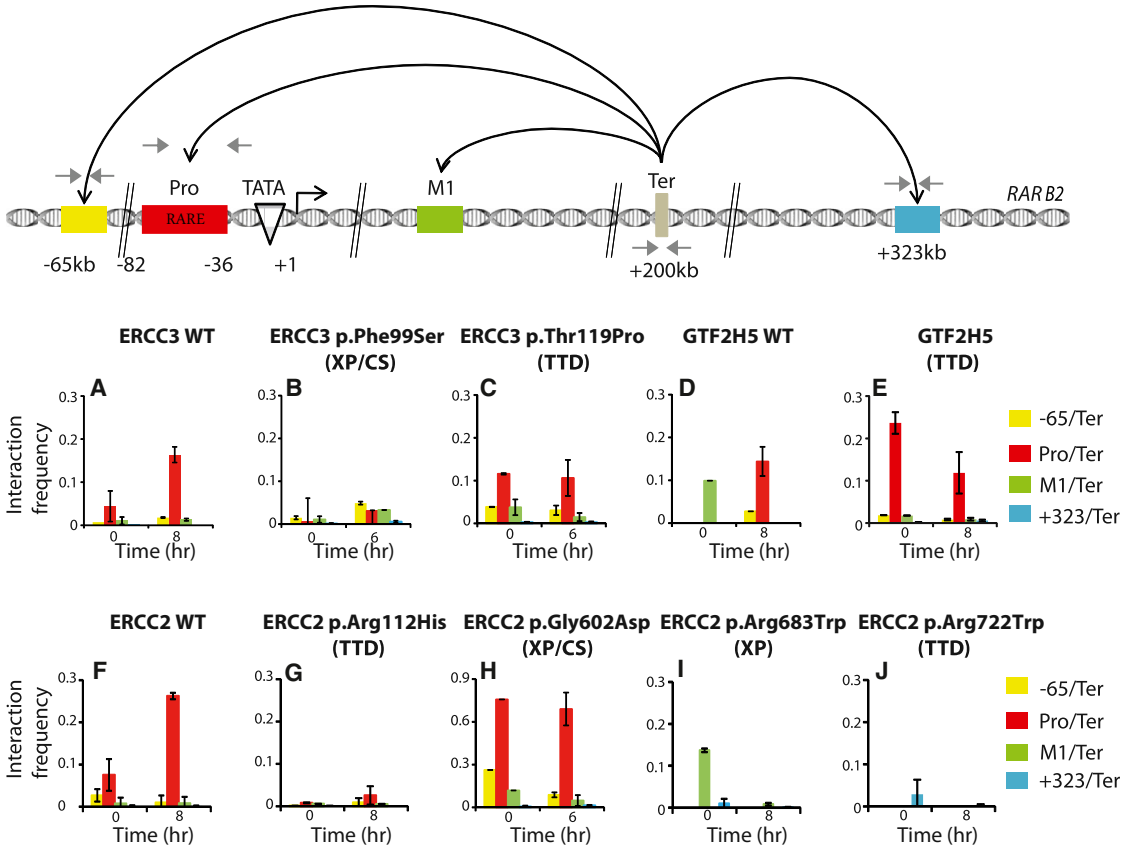

Figure 4. TFIIH Is Involved in *RARB2*-Loop Formation

(Upper panel) Schematic representation of the primers used in q3C. One primer was designed at the *RARB2* termination region (Ter) for investigating the associations between the different elements, including upstream (−65 kb), promoter (Pro), intronic (M1), and downstream (+323 kb) regions as indicated by the black arrows.

(A–J) q3C assays were performed with cross-linked and HindIII-digested chromatin from all cells as indicated at 0 hr and at either 6 or 8 hr after t-RA treatment depending on the timing of the RNA expression peak (see shadowed areas in Figure 2). The bar chart (y axis) shows the PCR product enrichment (%) normalized to the enrichment within human *ERCC3*. Each PCR was performed at least three times. Signals were normalized to the total amount of DNA used and were estimated with an amplicon located within a HindIII fragment in *RARB2*. Error bars represent the SD.

transcription-initiation complex, a prerequisite for optimal RNA synthesis. We first generated recombinant IIH6 (rIIH6, the core TFIIH containing GTF2H1, GTF2H4, GTF2H2, GTF2H3, GTF2H5, and either WT or altered ERCC3), WT or altered ERCC2, and CAK. These rIIH6 sub-complexes were added to an in vitro transcription assay containing the adenoviral major late promoter (run-off of 309 nt), all the basal transcription factors, and pol II,[32] along with CAK and ERCC2, either alone or in combination. When added to the transcription assay containing all the factors (including the ERCC2-CAK sub-complex), ERCC3-p.Phe99Ser rIIH6 exhibited a much weaker basal transcription activity than did WT-ERCC3 rIIH6 and ERCC3-p.Thr119Pro rIIH6 (Figure 5A). The addition of CAK together with WT ERCC2 and ERCC3-p.Phe99Ser rIIH6 did not improve RNA synthesis (lanes 5–7), contrary to what occurred with WT-ERCC3 rIIH6 and ERCC3-p.Thr119Pro rIIH6, which absolutely required the CAK sub-complex for optimal RNA synthesis (lanes 1–3 and 8–10). Because the variant weakens the contact with the GTF2H4 regulatory subunit within TFIIH,[37] it results in a defect in the unwinding of the *RARB2* promoter by ERCC3 and a defect in RNA synthesis. Interestingly, we also noticed that the absence of WT ERCC2 resulted in very weak RNA synthesis (lane 4 in Figure 5A and lanes 1 and 2 in Figure 5B). Because the ERCC2-GTF2H2 interaction allows the anchoring of CAK to the core TFIIH,[38] we next investigated the transcription activity of TFIIHs containing ERCC2 variants. The addition of CAK and increasing amounts of WT ERCC2 to our transcription assay, which already contained WT rIIH6, stimulated RNA synthesis (lanes 3 and 4 in Figure 5B); this was also observed in the presence of ERCC2 p.Arg112His or ERCC2 p.Gly602Asp (lanes 5–8). On the contrary, when either ERCC2 p.Arg683Trp or ERCC2 p.Arg722Trp was added, no significant increase in RNA synthesis was observed (lanes 9–12).

We then investigated the impact of CAK on the phosphorylation status of pol II during a classical run-off transcription experiment (see Material and Methods; Figure 5C). We observed that the hyper-phosphorylated form of pol II (IIO) was prevalent in the presence of WT

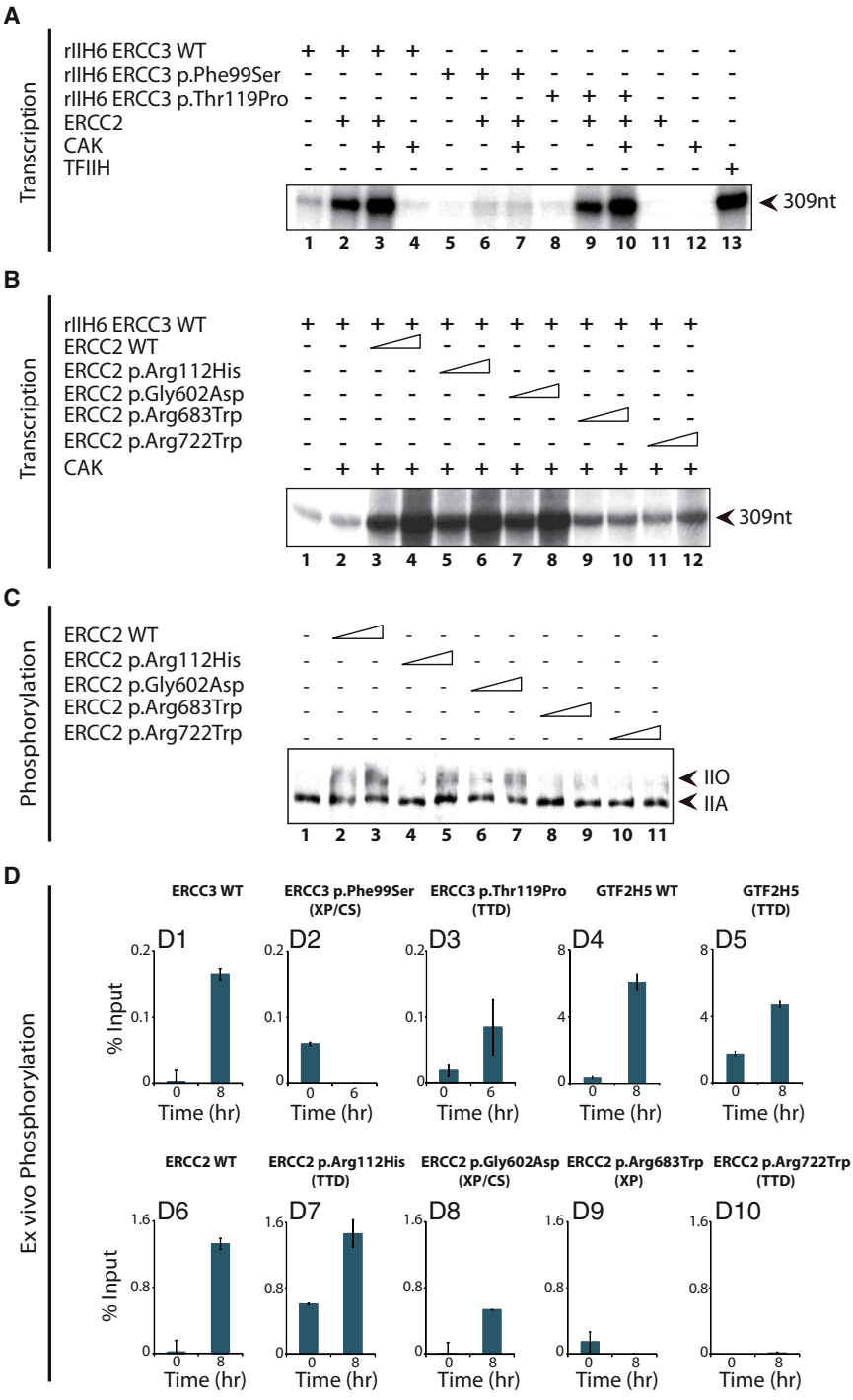

Figure 5. Effect of the TFIIH Alterations during In Vitro RNA Synthesis and Pol II Phosphorylation

(A) In vitro transcription assays with rIIH6 including WT ERCC3, ERCC3 p.Phe99Ser, or ERCC3 p.Thr119Pro. When indicated (+), these rIIH6 were incubated either alone or in combination with ERCC2 and/or CAK.

(B) WT rIIH6 was incubated either alone or in association with CAK and WT ERCC2, ERCC2 p.Arg112His, ERCC2 p.Gly602Asp, ERCC2 p.Arg683Trp, or ERCC2 p.Arg722Trp as indicated. The size (309 nt) of the transcript is indicated on the right side of each panel.

(C) Phosphorylation of pol II during in vitro transcription assays in the presence of WT rIIH6, CAK, and increasing amounts of ERCC2 p.Arg112His, ERCC2 p.Gly602Asp, ERCC2 p.Arg683Trp, and ERCC2 p.Arg722Trp as indicated. Arrows indicate hypo-phosphorylated (IIA) and hyper-phosphorylated (IIO) forms of pol II.

(D) ChIP monitoring the t-RA-dependent occupancy of the serine 5 phosphorylated pol II on the *RARB2* promoter from the different indicated cells. Each series of ChIP is representative of at least two independent experiments. Values are expressed as the percentage of the input. Error bars represent the SD.

ERCC2, ERCC2 p.Arg112His, or ERCC2 p.Gly602Asp (lanes 2–7) and paralleled the increase in RNA synthesis (Figure 5B, lanes 3–8). On the contrary, in the presence of ERCC2 p.Arg683Trp or ERCC2 p.Arg722Trp, which is deficient in stimulating RNA synthesis (Figure 5B, lanes 9–12), pol II was not hyper-phosphorylated (Figure 5C, lanes 8–11). Moreover, ChIP experiments demonstrated that in WT cells—as well as in GTF2H5, ERCC3-p.Thr119Pro, ERCC2-p.Arg112His, and ERCC2-p.Gly602Asp cells—phosphorylated pol II was detected at the *RARB2* promoter (Figures 5D1 and 5D3–5D8). On the contrary, in ERCC3-p.Phe99Ser, ERCC2-p.Arg683Trp, and ERCC2-p.Arg722Trp cells, phosphorylated pol II was not detected (Figure 5D2, 5D9, and 5D10). The effects of the ERCC2 variants on pol II phosphorylation were thus similar in both in vitro and ex vivo contexts.

All together, the above data suggest that the pol II phosphorylation defects might contribute to the gene-expression deregulation observed in some cells bearing mutations in *ERCC3* and *ERCC2*.

Discussion

After the assembly of the pre-initiation machinery (including TFIIA, GTF2B, TFIID, TFIIE, TFIIF, and RNA pol II), TFIIH unwinds the DNA around the proximal promoter through its ERCC3 (XPB) helicase subunit[7] and phosphorylates the CTD of the largest subunit of pol II via its CDK7 kinase,[9,39] allowing promoter escape and RNA elongation.[40] In addition to regulating pol II, TFIIH regulates other components of the transcription machinery (such as nuclear receptors).[41–43] Conversely, some of them—including TFIIE, ERCC5 (XPG), and the Mediator complex—regulate the activity of TFIIH, highlighting the pivotal role played by this complex in transcription.[44–47]

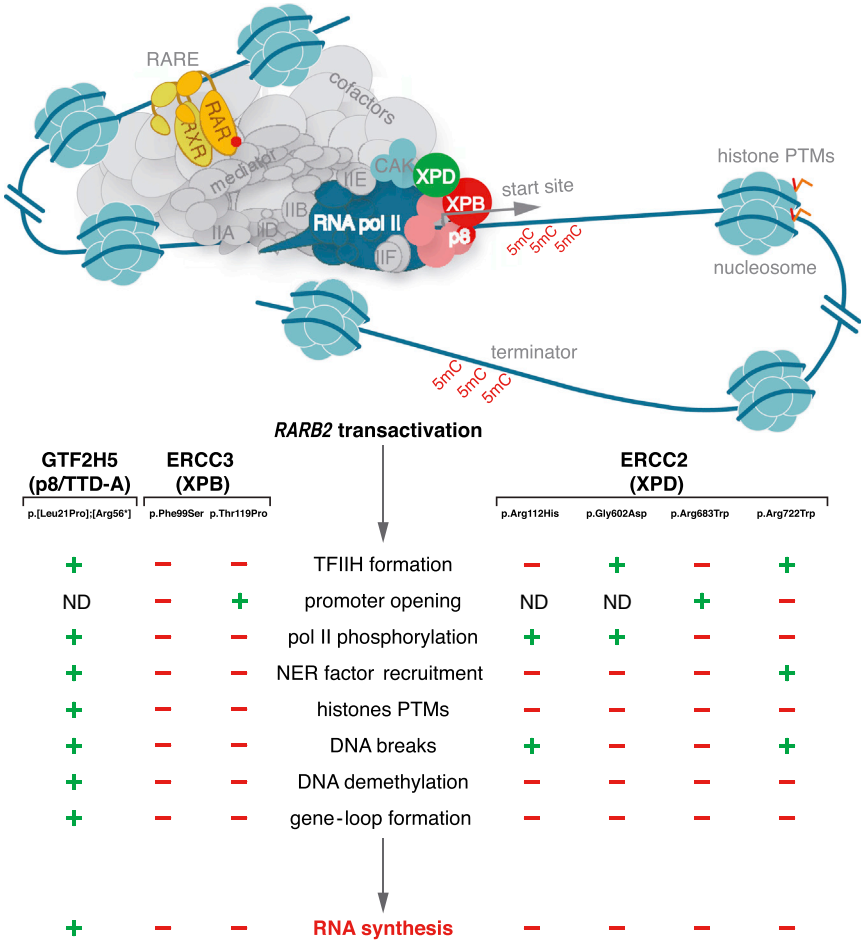

Figure 6. Mutations in Genes Encoding TFIIH Subunits Specifically Disrupt Transcription

(Upper panel) Upon t-RA ligand induction, the transactivation complex is formed once RARα and RXR targets its responsive element; co-activators and the Mediator complex are assembled at the *RARB2* promoter together with the pre-initiation complex. NER factors (XPC, ERCC6 [CSB], XPA, ERCC5 [XPG], and ERCC4 [XPF]) are then sequentially recruited and regulate RNA synthesis by participating in chromatin remodeling. These include histone PTMs, induction of DNA breaks, and active demethylation of 5mC, all together necessary for the gene-loop formation between the promoter and the terminator of *RARB2*. (Lower panel) Cells bearing mutations in genes encoding GTF2H5 p.[Leu21Pro];[Arg56*], ERCC3 p.Phe99Ser, ERCC3 p.Thr119Pro, ERCC2 p.Arg112His, ERCC2 p.Gly602Asp, ERCC2 p.Arg683Trp, and ERCC2.Arg722Trp differently disrupt the *RARB2* mRNA synthesis by deregulating several transcriptional steps. When indicated (−), each variant specifically affects (1) the formation of TFIIH, (2) its enzymatic activities (such as promoter opening and pol II phosphorylation), (3) the recruitment of NER factors, and (4) chromatin remodeling. ND stands for not determined.

By investigating the mechanistic defects resulting from mutations in genes encoding TFIIH subunits, the present study intends to go deeper in the understanding of the transcriptional role played by TFIIH at the cellular level. Here, we document the involvement of TFIIH in pol II phosphorylation, as well as in promoter unwinding, by showing that TFIIH influences (1) the concomitant recruitment of NER factors, (2) histone PTMs, (3) DNA breaks, (4) DNA demethylation, and (5) gene-loop formation, all of which are essential for accurate RNA synthesis (Figure 6).

Plasticity of TFIIH Recruitment at Activated Promoter

In both WT and GTF2H5 (p8 or TTD-A) cells, we observed a coordinated recruitment of all TFIIH subunits at the *RARB2* promoter upon t-RA induction (Figure 2). Conversely, the TFIIH subunits were no longer recruited together in cells bearing mutations in *ERCC3* and *ERCC2*. In particular, the recruitment of the TFIIH subunits appeared disorganized and took place over a longer period of time in ERCC2 (XPD)-p.Arg683Trp and ERCC2-p.Arg722Trp cells (Figures 2I2–2I4 and 2J2–2J4). These observations suggest that TFIIH is not recruited as a whole in the presence of subunit variants that affect its integrity. Interestingly, this observation illustrates the plasticity existing within TFIIH. Immunofluorescence experiments have previously shown that GTF2H5 is present in two distinct kinetic pools: one bound to TFIIH and a free fraction that shuttles between the cytoplasm and nucleus.[29] Distinct movements of TFIIH subunits were also observed in Rift-Valley-fever-virus-infected cells, in which the GTF2H2 (p44) and ERCC3 subunits were sequestered in nuclear filaments formed by the viral protein NSs, whereas ERCC2 and GTF2H1 (p62) were maintained in the cytoplasm and proteolyzed in the nucleus, respectively.[48,49] Moreover, it has been shown that a dynamic composition of TFIIH occurs during NER, in which the arrival of XPA at damaged DNA is subsequent to the removal of the CAK sub-complex.[50] This might explain at least partially why CAK is also found free in cellular extracts. The ERCC2 subunit that can be found associated with CAK[51,52] might also be implicated in a complex other than TFIIH, such as MMXD, a TFIIH-independent ERCC2-MMS19 protein complex involved in chromosome segregation.[53] Although each subunit and/or sub-complex can act independently, our data suggest that the activity of TFIIH in pol-II-dependent transcription results from the coordinated assembly of all its subunits.

TFIIH: A Platform to Recruit NER Factors during Transcription

In some cases, failure in the integrity and/or formation of TFIIH affects the enzymatic activities of this complex. In particular, the ERCC3 p.Phe99Ser variant weakens the

interaction between ERCC3 and its regulatory GTF2HH4 (p52) subunit,[37] disrupting the unwinding of activated promoters, which is a key point during transcription initiation. Similarly, the ERCC2 p.Arg683Trp and p.Arg722Trp variants are known to weaken the binding between ERCC2 and GTF2H2 (p44)[32] and thus affect the ability of CAK to phosphorylate pol II (Figure 5), a key step for promoter escape. Accordingly, the transcriptional defects associated with these *ERCC2* mutations might be more related to disruption of pol II phosphorylation than to ERCC2 helicase deficiencies, reinforcing the idea that ERCC2 helicase activity is not crucial for transcription.

The gene-expression defects observed in cells bearing mutations in *ERCC2* and *ERCC3* could not, however, be solely explained by deficiencies in the enzymatic activities of TFIIH. This is well illustrated by the fact that ERCC3 p.Thr119Pro, ERCC2 p.Arg112His, and ERCC2 p.Gly602Asp did not affect basal transcription in an in vitro context, whereas the expression of *RARB2* was defective in cells expressing the same variants (Figures 5A and 5B and Figures 2C1, 2G1, and 2H1). It is likely that TFIIH variants might disturb the accurate recruitment and/or positioning of components required for RNA synthesis. Our results suggest that such recruitment of NER factors is either incomplete or unsynchronized with the general transcription machinery (Figure 2). In particular, although ERCC5 stabilizes TFIIH and contributes to its transactivation function, no simultaneous recruitment of ERCC5, TFIIH, or the transcription machinery was observed in ERCC2-p.Arg683Trp cells (Figures 2I2–2I4); this might be related to the inability of ERCC5 to interact with this aberrant form of ERCC2.[46] Furthermore, our results showed that the recruitment of NER factors during transcription was differently affected depending on the nature of the TFIIH variants. For instance, in ERCC3-p.Thr119Pro cells, ERCC4 (XPF) was not detected at the *RARB2* promoter, on which the recruitment of all basal transcription machinery, including TFIIH, was deregulated (Figures 2C2–2C4). However, in ERCC2-p.Gly602Asp cells, TFIIH subunits and NER factors XPA and ERCC5, but not ERCC4, were correctly recruited (Figures 2H2–2H4).

All together, our results show that TFIIH promotes the recruitment of NER factors during transcription in a manner different from that observed during NER. This was well observed in GTF2H5 cells, in which the recruitment of NER factors was normal during transcription (Figures 2E2–2E4), whereas the GTF2H5 variant prevented the recruitment of these factors during the NER pathway.[54] In parallel, the recruitment of the NER factors during transcription occurred normally in cells expressing ERCC2 p.Gly602Asp (Figures 2H2–2H4), which is known to prevent damaged DNA from opening and the recruitment of XPA in NER.[32] The molecular aspects that differently influence the NER factors during transcription and DNA repair are currently unclear.

TFIIH Orchestrates Chromatin Remodeling

According to the chromatin signatures observed in WT cells, it seems that TFIIH variants alter chromatin remodeling upon transcription by disturbing histone PTMs, DNA demethylation, and gene-loop formation. These variants prevent the establishment of permissive chromatin by causing an intermediate environment that includes euchromatic histone PTMs (H3K4me and H3K9ac) and heterochromatin hallmarks (methylated DNA and impaired gene-loop formation). In cells bearing mutations in genes encoding TFIIH subunits, we observed that H3K4me and H3K9ac occurred all along the time course and that these modifications did not follow the pattern of RNA synthesis (Figures 2 and 3). In most cases, H3K9me was strongly reduced, suggesting that the deficiency might particularly concern the methylation or demethylation process of the lysine residue. Furthermore, mutations in *ERCC3* and *ERCC2* seem to profoundly alter the tight connection that exists between histone PTMs and the methylated status of DNA,[55] which is well illustrated by the concomitant presence of H3K4me2 and H3K9ac and the methylated DNA at the promoter (Figure 3). Indeed, in addition to the histone PTM disturbance, the active DNA demethylation that occurs upon transcription was abolished for all of cells bearing mutations in *ERCC2* and *ERCC3*. Several recent studies have documented the involvement of DNA-repair factors, including the endonuclease ERCC5, in the regulation of DNA demethylation.[11,56] We have notably correlated DNA breaks involving ERCC5 with the 5mC sites that were demethylated.[35] Interestingly, ERCC3 p.Phe99Ser, ERCC2 p.Gly602Asp, and ERCC2 p.Arg683Trp, which affect ERCC5 recruitment, might contribute to the absence of DNA breaks surrounding the *RARB2* promoter (Figures 2B4, 2H4, and 2I4 and Figures 3B2, 3H2, and 3I2). Conversely, DNA breaks were maintained in GTF2H5, ERCC2-p.Arg112His, and ERCC2-p.Arg722Trp cells, in which ERCC5 was normally recruited but without the expected co-detection of unmethylated DNA (Figures 3D2, 3D3, 3G2, 3G3, 3J2, and 3J3). DNA breaks therefore occur even in the absence of DNA demethylation, indicating that the presence of ERCC5 and the related cuts are necessary but not sufficient to achieve such a process. It should be noted that unmethylated DNA influences long-range chromosomal interactions by being targeted by chromatin organizers such as CTCF, as previously observed for the imprinted *Igf2-H19*.[57,58] Consequently, the defect of DNA demethylation observed in cells bearing mutations in genes encoding TFIIH subunits might contribute in part to the absence of an inducible CTCF-dependent chromatin loop between the promoter and the terminator of *RARB2* (Figure 4).

Taken together, our data underline the key role of TFIIH in the transcription process, in which its primary enzymatic activities are combined with the recruitment of NER factors to further orchestrate events such as histone PTMs, DNA breaks, DNA methylation, and loop formation.

Although each mutation in genes coding for TFIIH subunits specifically affects transcription, it seems that, in addition to causing DNA-repair deficiencies, subtle differences in the transcription defects might contribute to the phenotypic heterogeneity observed among XP, XP/CS, and TTD individuals.

Supplemental Data

Supplemental Data include three figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2014.12.012.

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Web Resources

The URLs for data presented herein are as follows:

OMIM, http://www.omim.org/
RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq
UCSC Genome Browser, http://genome.ucsc.edu

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3.4.6 Highlights and perspectives

The results accumulated in these different publications clearly demonstrated that finally the NER factors like TFIIH possess dual activities in transcription and DNA repair. Their sequential recruitment at promoters is different compared to the one described upon the DNA repair process. Although some chromatin remodelling events are common both in transcription and DNA repair, we can expect also some specific functions for each NER factors and each process. Our next goal will be therefore to dissect these different and specific roles in chromatin remodelling and show how the different NER factors collaborate each other, especially TFIIH and XPG in the active DNA demethylation (see also part II).

One of the most concerns in these studies is the absence of overview for the actions of NER factors. We limited our analysis to the *RARβ2* model and we still don't know whether the involvement of NER factors is widespread or restrained to a low number of genes. A first hint came from Grummt's laboratory showing that NER factors could be also recruited to the promoter of rRNA genes (*12*). Moreover, a co-activator complex containing XPC has been identified to regulate through OCT4 and SOX2 transcription factors the transcriptional program of embryonic stem cells (*14*). Our next big effort will be therefore the investigation of the genome-wide distribution of these NER factors to determine their specific location. Preliminary ChIP-seq data sets suggest that XPC for example can be localized at promoters as expected but also at intergenic regions like enhancers. Of course, I also aim to define the genome-wide location of XPG and XPF related to DNA nicks and finally analyse their impact on chromatin rearrangements between the different DNA regulatory regions.

Whereas the recruitment of NER factors at DNA lesions is already highly documented, we don't have information explaining their recruitment upon transcription. The determination of the promoters targeted by NER factors will hopefully help us to characterize them (TATA box vs TATA less box or High vs low CpG density…etc) and eventually identify what could be the mode of recruitment. Our preliminary results and those from colleagues in previous reports made us speculate a putative association between NER factors and TAFs in collaboration with specific histones PTMs and variants. Indeed, it has been showed that (i) NER factors are recruited at promoter of rRNA genes through TAF12 and (ii) XPF-ERCC1 interacts with TFIID assembling with the basal transcription machinery on promoters *in vivo* (*11, 12*).

The analysis of XP, XP/CS and TTD-derived fibroblasts are precious tools to unveil the roles of NER factors and characterize the transcriptional defect previously observed in these pathologies. However, these cellular models are too far from the pathological context. What is the relevance to perform transcriptomic studies in fibroblasts and extrapolate the obtained results to understand the deficiencies leading to skin cancers or mental retardation? We have to develop new approaches and models to molecularly define the phenotypes and my goal is to work in closer collaboration with clinicians and patients.

3.5 PARG another example of factors involved in DNA repair and transcription

3.5.1 Context

These last years, many publications have reported a growing list of proteins and complexes that were long though to function exclusively in DNA repair are revealing to be involved in transcription as well (Table 1).

Protein (Official Name)	Repair Pathway	Enzymatic Activity	Function in DNA Repair	Function in Transcription
CSB (ERCC6)	TCR (NER)	DNA-dependent ATPase	Initiates TC-NER at stalled Pol II	• Functions as an ATP-dependent chromatin remodeler • Stimulates transcription by Pol I and II
DNA-PKcs	NHEJ BER	Protein kinase	• Facilitates DNA end processing and resealing in NHEJ by autophosphorylation • May stimulate BER of oxidative DNA damage	• Facilitates gene activation by chromatin remodeling • Modulates the activity of transcription factors
FANCD2	ICL	ND	Initiates ICL repair	Activates transcription of *TAp63* and promotes senescence of tumorigenic cells
FANCP (SLX4)	ICL	ND	Acts as a scaffold for multiple structure-specific endonucleases	Cooperates with FANCD2 in transcriptional activation of *TAp63*
TFIIH	TCR (NER) GGR (NER)	• ATP-dependent DNA helicase • Protein kinase	• Unwinds the DNA at damaged sites • Facilitates XPF incision	• Unwinds the DNA at gene promoters • Phosphorylates Pol II carboxy-terminal domain • Phosphorylates NRs and coactivators
PARP-1	NHEJ HR BER NER	DNA-dependent poly(ADP-ribosyl)transferase	Interacts physically and functionally with components in NHEJ, HR, BER, and NER pathways	• Modulates chromatin structure • Functions as activator/coactivator or repressor • Poly(ADP-ribosyl)ates chromatin remodeling factors
TDG	BER	DNA glycosylase	Excises damaged nitrogenous bases	• Regulates DNA demethylation at gene regulatory regions • Bridges CBP/p300 to transcription factors and NRs
XPC-RAD23B-CETN2	GGR (NER) BER	ND	• Initiates GGR at bulky DNA lesions • Stimulates TDG-mediated BER	• Activates transcription at NR target genes • Functions as a coactivator for OCT4 and SOX2 in embryonic stem cells
XPF (ERCC4)	TC-NER GG-NER ICL	Structure-specific endonuclease	Incises the damaged strand 5′ to the DNA lesion	• Promotes active DNA demethylation at terminators of NR targets • Recruits CTCF and facilitates DNA looping at NR targets • Stimulates transcription initiation
XPG (ERCC5)	TCR (NER) GGR (NER)	Structure-specific endonuclease	Incises the damaged strand 3′ to the DNA lesion	• Stabilizes TFIIH • Promotes active DNA demethylation at promoters of NR targets • Recruits CTCF and facilitates DNA looping at NR targets

Definitions are as follows: BER, base excision repair; GGR, global genome repair; HR, homologous recombination; ICL, interstrand crosslink repair; NER, nucleotide excision repair; NHEJ, nonhomologous end-joining; NR, nuclear receptor; TCR, transcription-coupled repair; and ND, not determined.

From Fong et al., 2013

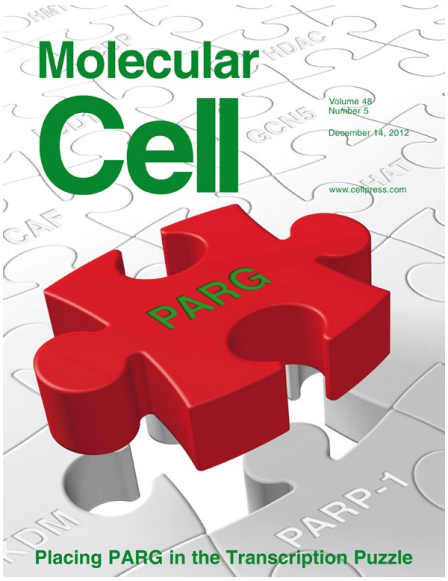
Table 1. DNA repair factors in Repair and Transcription

Among these different proteins, we have been interested in Poly-(ADP-ribose) Polymerase I (PARP1) that catalyses 90% of the total NAD(+)-dependent PTM Poly-(ADP-ribosyl)ation (PARsylation) (*239*). PARP-1 participates in various cellular processes including several DNA repair pathways and transcription ensuring different functions (Table 1). Interestingly, it has been demonstrated that PARP-1 is required for NR-mediated transactivation including those of RAR and especially the *RARβ2* model that we used for the study of NER factors (*186, 240*). It has been proposed that the bound liganded-NR interacting with cofactors will generate 8-oxo-guanines repaired by DNA glycosylases and consequently generating DNA nicks. These breaks act as entry points for DNA endonucleases such as Topoisomerase IIβ leading to double strand breaks (DSBs). It has been suggested that these DSBs relax DNA strands and facilitate the recruitment of other DNA repair enzymes including PARP-1 that induce a permissive chromatin environment suitable for transactivation (*186, 241*).
The involvement of PARP-1 enzymatic activity for such NR-mediated transcription was controversial although the importance of PAR was established (*242*). The metabolism of PAR is highly regulated not only though PARP but also through PARG that dePARsylates the PARP-substrates via exo- and endo-glycosylase activities generating free ADP-ribose (*243*). Like PARP-1 and to counteract it, PARG has been involved in the DNA damage response (DDR) and some studies have also suggested roles in transcription (*244*). In collaboration with the team of V. Schreiber, we thus decided to employ the methodology that we previously used for NER factors to investigate the transcriptional roles of PARG and understand its relationship with PARP-1 to regulate the chromatin remodelling upon NR-mediated transactivation.

3.5.2 Publication: PARG a new player in the transcriptional game

Le May N., Iltis I., Amé J.C., Biard D., Egly J.M., Schreiber V. and Coin F. « Poly (ADP-Ribose) Glycohydrolase Regulates Retinoic Acid Receptor-Mediated Gene ». **Molecular Cell**. 2012, 48. 785-798. (*10*)

Figure 8. Molecular cell cover for the related article published in 2012



Molecular Cell
Article

Poly (ADP-Ribose) Glycohydrolase Regulates Retinoic Acid Receptor-Mediated Gene Expression

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SUMMARY

Poly-(ADP-ribose) glycohydrolase (PARG) is a catabolic enzyme that cleaves ADP-ribose polymers synthesized by poly-(ADP-ribose) polymerases. Here, transcriptome profiling and differentiation assay revealed a requirement of PARG for retinoic acid receptor (RAR)-mediated transcription. Mechanistically, PARG accumulates early at promoters of RAR-responsive genes upon retinoic acid treatment to promote the formation of an appropriate chromatin environment suitable for transcription. Silencing of PARG or knockout of its enzymatic activity maintains the H3K9me2 mark at the promoter of the RAR-dependent genes, leading to the absence of preinitiation complex formation. In the absence of PARG, we found that the H3K9 demethylase KDM4D/JMJD2D became PARsylated. Mutation of two glutamic acids located in the Jumonji N domain of KDM4D inhibited PARsylation. PARG becomes dispensable for ligand-dependent transcription when either a PARP inhibitor or a non-PARsylable KDM4D/JMJD2D mutant is used. Our results define PARG as a coactivator regulating chromatin remodeling during RA-dependent gene expression.

INTRODUCTION

Poly-(ADP-ribosyl)ation (PARsylation) of proteins is a NAD(+)-dependent posttranslational modification catalyzed by poly-(ADP-ribose) polymerases (PARPs) (Krishnakumar and Kraus, 2010b; Rouleau et al., 2010). PARP-1 is responsible for about 90% of the total PARsylation activity in the cell (Amé et al., 2004) and is involved in various cellular processes such as single-strand/base excision repair, alternative end-joining during immunoglobulin class switch recombination and mitotic segregation (Robert et al., 2009; Schreiber et al., 2006).

Increasing evidence supports the active involvement of PARP-1 in gene expression and repression (Ji and Tulin, 2010; Kraus, 2008). Studies of the *Parp-1*$^{-/-}$ mouse and PARP-1-silenced human cells have shown that an absence of PARP-1 alters the expression of many genes involved in both cell-cycle control and the stress response (Frizzell et al., 2009; Krishnakumar and Kraus, 2010a; Ogino et al., 2007; Simbulan-Rosenthal et al., 2000). In addition, it has been demonstrated that PARP-1 is also required for nuclear receptor (NR)-mediated transcription processes, including those of the retinoic acid (RAR) and estrogen (ER) receptors (Ju et al., 2006; Pavri et al., 2005). The involvement of PARP-1 enzymatic activity in transcription depends on the nature of the gene and stimuli studied, for reasons that are not yet fully understood (Hassa et al., 2003; Ju et al., 2006; Pavri et al., 2005; Simbulan-Rosenthal et al., 2003; Tulin and Spradling, 2003).

The metabolism of PAR is tightly regulated not only through the regulation of PARP activity, but also through the activity of poly(ADP-ribose) glycohydrolase (PARG), an enzyme with both exo- and endoglycosidase activities that generates large amounts of free ADP-ribose (Heeres and Hergenrother, 2007). PARG is encoded by a single gene in humans, which produces several isoforms with various subcellular localizations (Meyer et al., 2007; Meyer-Ficca et al., 2004; Niere et al., 2008). The existence of these multiple PARG isoforms and the embryonic lethality of mice deficient in all of them (Koh et al., 2004) complicate the study of this enzyme in the context of fundamental cellular processes. However, the use of human knockdown cells deficient in all PARG isoforms revealed that PARG is involved in the repair of single- and double-strand breaks and in the mitotic spindle checkpoint (Amé et al., 2009; Erdélyi et al., 2009; Fisher et al., 2007). The structure of a bacterial PARG has been recently solved and has revealed that the PARG catalytic domain is a distant member of the ubiquitous ADP-ribose binding macrodomain family (Slade et al., 2011). However, our understanding of the cellular function of PARG remains poor compared to the PARP family members.

Given the importance of PAR in NR-dependent transcription and the increasing evidence that inhibitors of PARG are potent anticancer drug candidates (Miwa and Masutani, 2007), we sought to investigate the potential function of PARG in NR-dependent gene transcription. The NR superfamily consists of a variety of DNA binding transcription factors that control the expression of genes involved in many cellular processes

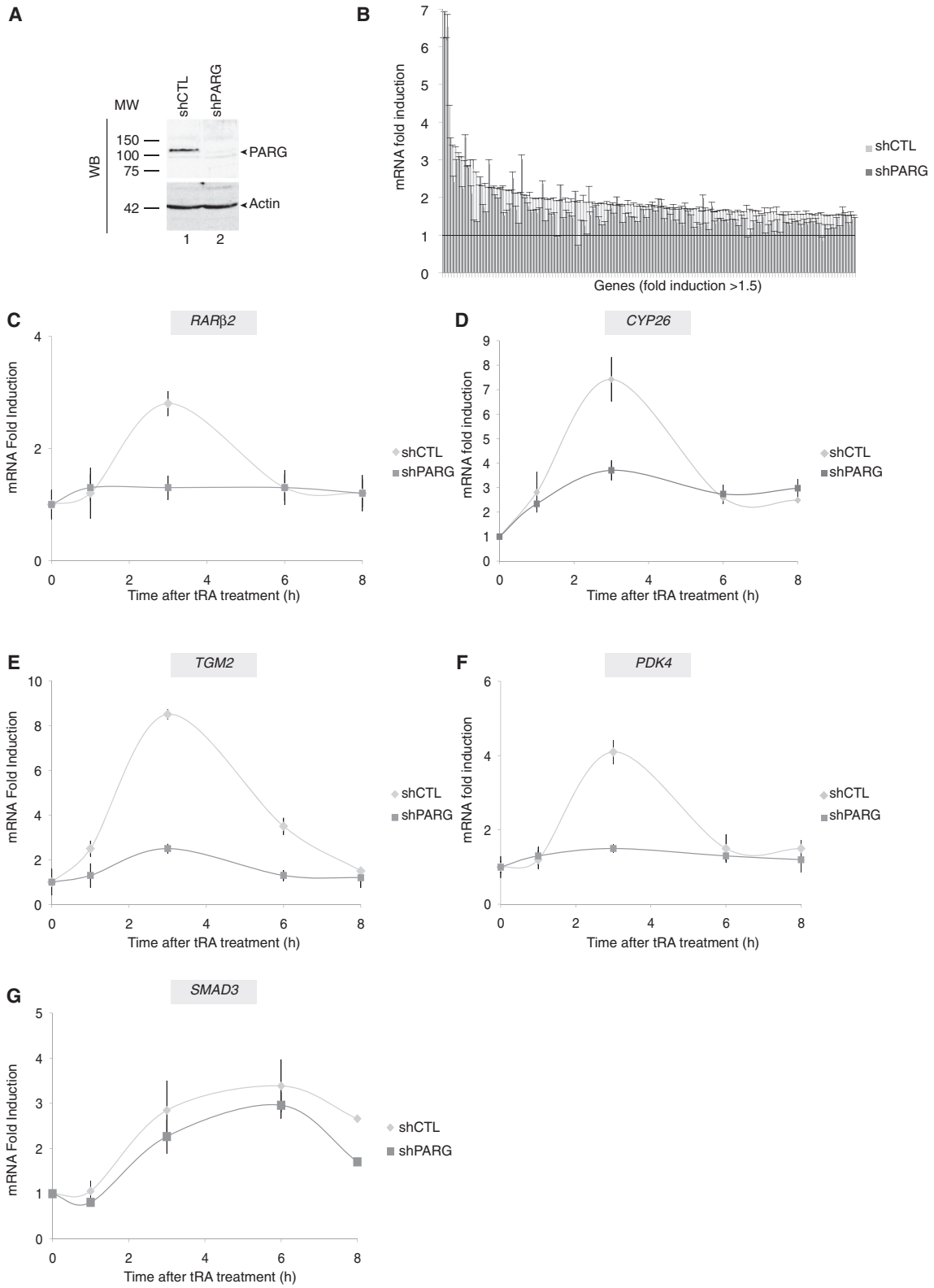

Figure 1. PARG Stimulates RAR-Dependent Gene Expression

(A) Proteins from whole-cell extracts (50 μg) of either shCTL or shPARG cells were resolved by SDS-PAGE followed by western blotting using anti-PARG and anti-actin antibodies. Molecular weights are indicated.

(Kastner et al., 1995). NRs are mainly classified as either steroid receptors, such as ER, or nonsteroid receptors, such as RAR. These receptors target promoters by binding specific recognition sequences and activate transcription by recruiting coactivators (Glass and Rosenfeld, 2000). The all-trans retinoic acid (t-RA), the ligand of RAR, is a pleiotropic signaling molecule derived from vitamin A that regulates critical genetic programs, thereby controlling cell proliferation and differentiation, as well as cell death or survival (Clagett-Dame and Knutson, 2011).

In the present study, we demonstrate the participation of the PAR-degrading activity of PARG in the transactivation of genes mediated by RAR. We have shown that t-RA-induced F9 embryonic cell differentiation depends on PARG. Global transcriptome analysis revealed that the induction of a significant number of RA-induced genes was stimulated by PARG. Mechanistically, we show that PARG is recruited to the promoters of RAR-dependent genes in a ligand-dependent manner to promote the formation of a permissive chromatin environment suitable for transcription. In particular, PARG targeted the demethylase KDM4D/JMJD2D to favor the removal of the repressive H3K9me2 mark around the promoters of RAR-dependent genes and the subsequent formation of the transcription preinitiation complex (PIC).

RESULTS

Absence of PARG Impairs RAR-Dependent Gene Transactivation

To evaluate the influence of PARG in transcription, we assayed RAR-mediated transcriptional activity of a HeLa cell line constitutively expressing shRNA directed against the catalytic domain of all PARG isoforms (shPARG) (Amé et al., 2009). The HeLa control cell line (shCTL) expresses a nonfunctional shRNA. A robust knockdown of PARG (Figure 1A) and an absence of PAR-degrading activity (see Figure S1A online) were observed in shPARG cells.

We analyzed the transcriptome profiles of shCTL and shPARG cells 3 hr after t-RA treatment (10 μM). Treatment of shCTL cells with t-RA resulted in the induction of several immediate-early responsive genes, such as *RARβ2*, *CYP26*, or *TGM2* (Table S1 and Table S2). Interestingly, ~70% of genes showing an induction ≥1.5 in shCTL cells had a lower induction level in shPARG cells (Table S3 and Figure 1B). Using RT-qPCR, we confirmed that *RARβ2* (Figure 1C), *CYP26* (Figure 1D), *TGM2* (Figure 1E), *PDK4* (Figure 1F), *NRIP1* (Figure S1B), and *TNFAIP2* (Figure S1B) were less induced in shPARG cells over time after t-RA treatment. In contrast, another group of genes including *SMAD3* was induced similarly in shCTL and shPARG cells (Table S1 and Figure 1G). Note that no dramatic changes in expression were observed in the absence of t-RA between shCTL and shPARG cells (Table S4).

Since PAR activity has been implicated in mRNA splicing (Malanga et al., 2008), we measured the accumulation of pre-mRNA to determine if the defect in transactivation could be explained by a deficiency in splicing (Figure S1C). We observed a defect in *RARβ2* expression in shPARG cells whether we measured pre-mRNA or total mRNA (Figures S1D and S1E), consistent with the participation of PARG in an early step of the RAR-dependent transcription process, before mRNA splicing.

RAR-Dependent Gene Transactivation Depends on the PAR-Degrading Activity of PARG

We next explored whether the PAR-degrading activity of PARG was required for RAR-dependent gene transcription. We used GFP-tagged shRNA-resistant constructs expressing either a wild-type ($PARG^{WT}$-GFP) or a catalytically inactive version of PARG, targeting the two consecutive glutamates of the active site ($PARG^{E755/756A}$-GFP) (Patel et al., 2005; Mortusewicz et al., 2011; Slade et al., 2011). Transfection of $PARG^{WT}$-GFP into shPARG cells treated with H_2O_2 resulted in the depletion of the nuclear PAR (Amé et al., 2009), which was not achieved with $PARG^{E755/756A}$-GFP (compare Figures 2Aa–2Ac with Figures 2Ad–2Af). Furthermore, expression of $PARG^{WT}$-GFP reduced the genotoxic-stress-independent PARsylation of PARP-1 observed in shPARG cells (Amé et al., 2009) that was not achieved with GFP-$PARG^{E755/756A}$ (Figure 2B). These results indicate that the E755/756A mutations abolished the PAR-degrading activity of PARG.

When expressed in shPARG cells, $PARG^{WT}$-GFP rescued the ligand-dependent expression of *RARβ2* (Figure 2C), *CYP26*, *PDK4*, *NRIP1*, and *TNFAIP2* (Figure S2A). The rescue was greatly compromised following expression of $PARG^{E755/756A}$-GFP (Figure 2C and Figure S2A) despite producing similar protein amounts as $PARG^{WT}$-GFP (Figure 2B). These data indicate that the PAR-degrading activity of PARG stimulates RAR-dependent gene transcription and imply that PAR polymerase activity may thwart gene activation in the absence of an active PARG. Consequently, we pretreated shPARG cells with the PARP-specific inhibitor KU0058948 (K948) (McCabe et al., 2005) for 12 hr prior to t-RA addition and observed a rescue of the transactivation of *RARβ2* (Figure 2D), *CYP26*, *PDK4*, *NRIP1*, and *TNFAIP2* (Figure S2B). The transactivation of *RARβ2* was not affected by the inhibition of PARP in shCTL cells. Altogether, these results suggest that the catalytic activity of PARG is involved in NR-dependent gene transactivation as a countermeasure to PAR polymerase activity.

Absence of PARG Inhibits RA-Induced F9 Embryonic Cell Differentiation

Treatment with t-RA induces proliferation arrest, differentiation, and apoptosis in many cells (Strickland and Mahdavi, 1978). To study the role of PARG in a physiological context, we transfected F9 mouse embryonic carcinoma cells with siRNA against

(B) Transcriptome profiling of shCTL (light gray) or shPARG (dark gray) cells treated with t-RA (10 μM; 3 hr) using the whole transcript coverage Affymetrix Human Gene 1.0 ST arrays. Based on two independent experiments, these genes show an upregulation >1.5 (±SEM) compared to t = 0 hr (F test, $p < 0.005$).
(C–G) Relative mRNA expression of *RARβ2* (C), *CYP26* (D), *TGM2* (E), *PDK4* (F), and *SMAD3* (G) in either shCTL or shPARG cells measured at different time points after treatment with t-RA (10 μM). Error bars represent the standard deviation (SD) of three independent experiments. The values are plotted relative to the expression level of the no treatment control that is set to 1 in all experiments.

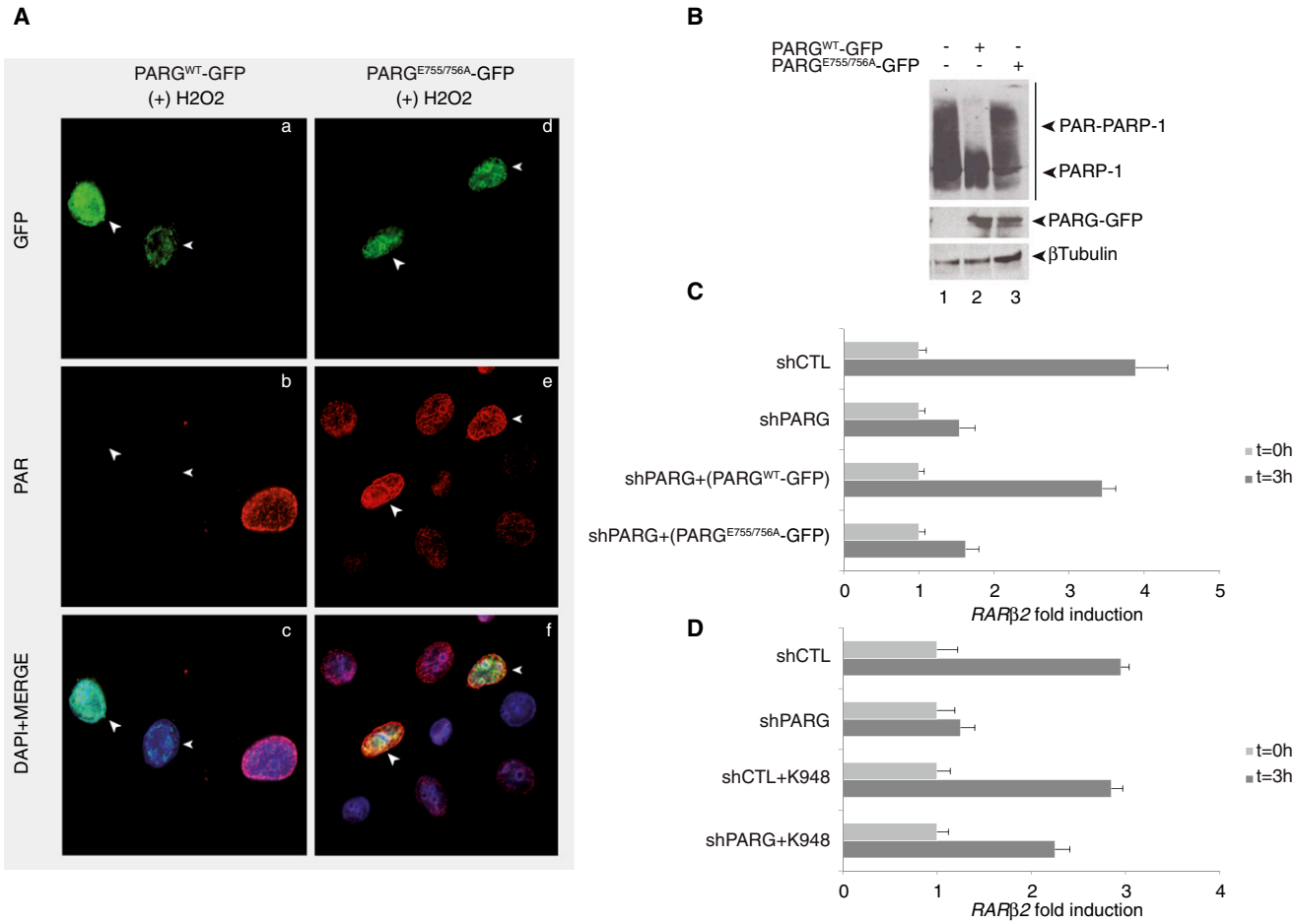

Figure 2. The PAR-Degrading Activity of PARG Is Required for RAR-Dependent Gene Expression

(A) Immunodetection of PAR in shPARG cells treated with 1 mM H_2O_2, during 10 min (to stimulate PAR synthesis). Cells were transfected 36 hr before treatment with either $PARG^{WT}$-GFP (Aa–Ac) or $PARG^{E755/756A}$-GFP (Ad–Af). Transfected cells (indicated with an arrow) were detected with GFP. PAR was detected with a mouse monoclonal anti-PAR antibody (10H). DNA was counterstained with DAPI. Immunofluorescence was performed as described (Amé et al., 2009).

(B) Proteins from whole-cell extracts (50 μg) of shPARG cells expressing either $PARG^{WT}$-GFP or $PARG^{E755/756A}$-GFP were resolved by SDS-PAGE followed by western blotting using anti-GFP, anti-PARP-1, and anti-β-tubulin antibodies.

(C) Relative mRNA expression (±SD, three independent experiments) of *RARβ2* measured at the transactivation peak, 3 hr post-t-RA treatment, in the indicated cell lines. The values are expressed relative to the expression level of the no treatment control that is set to 1 in all experiments.

(D) Relative mRNA expression (±SD, three independent experiments) of *RARβ2* measured 3 hr after t-RA treatment in shCTL and shPARG cells incubated with K948 (100 nM) for 12 hr before addition of t-RA. The values are plotted relative to the expression level of the no treatment control that is set to 1 in all experiments.

PARG (siPARG versus siCTL; Figure S3) and followed the process of differentiation after t-RA treatment based on the morphological features characteristic of pre-endoderm-like cells. Seventy-two hours after t-RA treatment, siCTL-transfected F9 cells became more adhesive and grew separately (compare Figures 3Aa and 3Ab). At the same time point, the morphology of the siPARG-transfected F9 cells treated with t-RA remained unchanged, revealing a weak adhesive capacity and the formation of clusters (compare Figures 3Ae and 3Af). In the presence of the PARP inhibitor K948, the morphology and individual growth capacities of the siPARG-transfected F9 cells were similar to those of the siCTL-transfected cells treated with t-RA alone (compare Figures 3Ac and 3Ad with Figures 3Ag and 3Ah). The treatment of siCTL-transfected cells with K948 only slightly interfered with the differentiation process (compare Figures 3Aa and 3Ab with Figures 3Ac and 3Ad), in agreement with previous data (Quénet et al., 2008).

In parallel to the morphological study, we followed the relative expression of genes associated with t-RA-induced differentiation of F9 cells in pre-endoderm-like cells (Eifert et al., 2006; Lalevée et al., 2011). *CYP26*, *HOXA1*, *HOXB1*, and *RARβ2* showed increased expression in siCTL-transfected F9 cells 48 hr after t-RA treatment (Figure 3B). The expression of all of these genes was reduced in siPARG-transfected F9 cells treated under the same conditions (Figure 3B), while the maintenance of K948 treatment during differentiation restored their expression (Figure 3B). Altogether, these studies suggest a physiological role for PARG in RA-induced F9 embryonic cell differentiation.

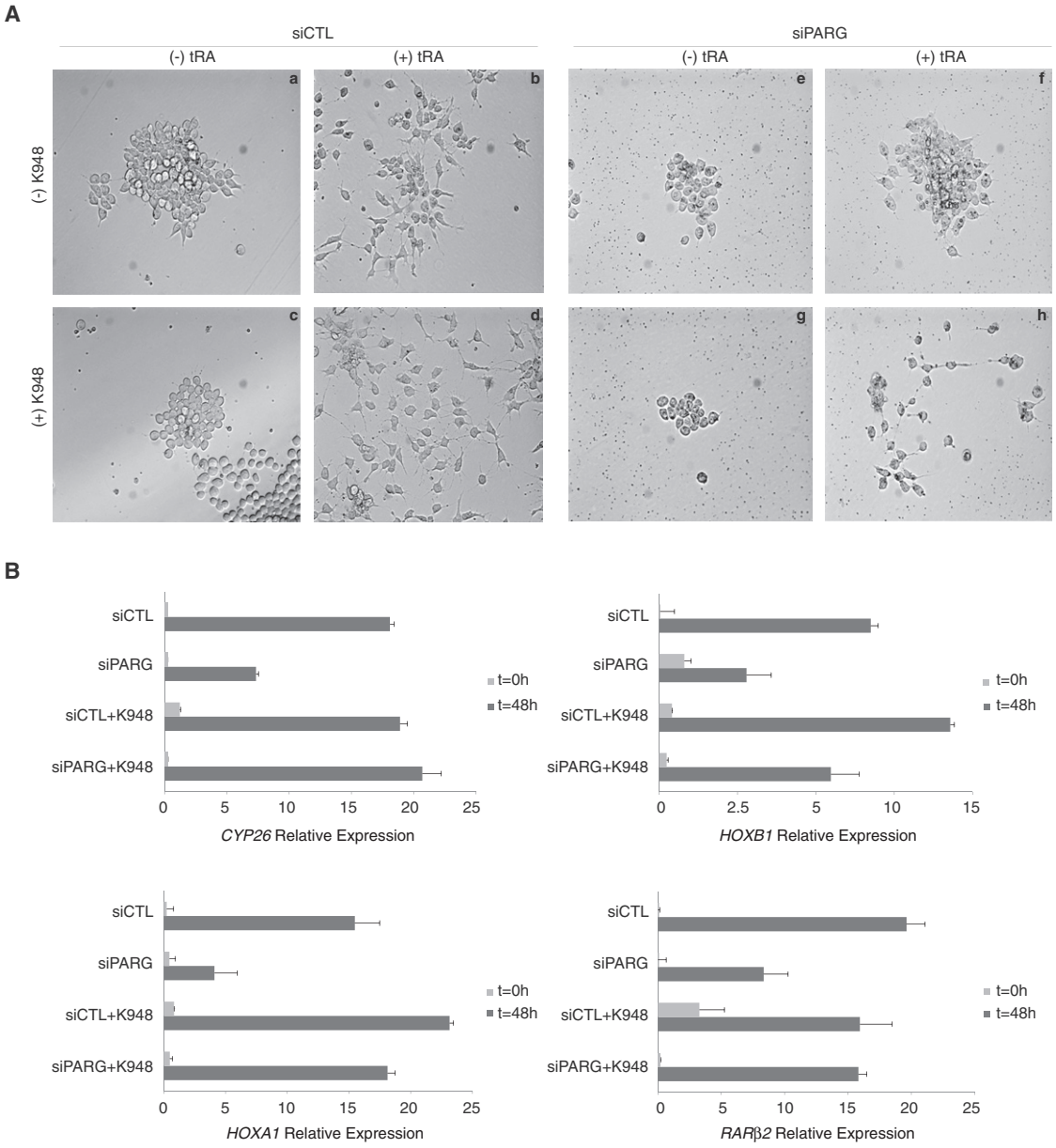

Figure 3. PARG Is Required for RA-Induced Differentiation of Pluripotent F9 Cells

(A) Effects of PARG silencing on the differentiation of F9 embryonic carcinoma cells. Thirty-six hours after siRNA transfection, cells were treated with t-RA (1 μM) in the presence or absence of the PARP inhibitor K948 (100 nM). The morphology of the F9 cells was analyzed 72 hr later by phase-contrast microscopy.

(B) mRNA level (±SD, three independent experiments) of upregulated RAR target genes in differentiated F9 cells, measured 48 hr after t-RA treatment. F9 cells were incubated with the PARP-1 inhibitor K948 during differentiation when indicated. Values are expressed as the percentage of mRNA level compared with *GAPDH*.

Absence of PARG Leads to Defective Preinitiation Complex Formation

To address the precise role of PARG in RAR-dependent transcription, we used chromatin immunoprecipitation (ChIP) assay and monitored the recruitment of transcription factors to several *RAR*-dependent promoters in shCTL versus shPARG HeLa cells. In shCTL cells, we observed the ligand-dependent recruitment of RNA Pol II and RARα to the *RARβ2* (Figure 4A and Figure S4A) and *CYP26* promoters (Figure S5A), which was optimal at the mRNA transactivation peak, 3 hr after t-RA treatment. In contrast, PIC formation was deficient in PARG-depleted cells throughout the time course (Figure 4A and Figures S4B and S5B) and partially restored following the expression of $PARG^{WT}$-GFP (Figure 4A and Figure S4G). The recruitment of PARP-1 parallels that of the PIC in shCTL cells (Figures S4A and S5A). In shPARG cells, the recruitment of PARP-1 was still

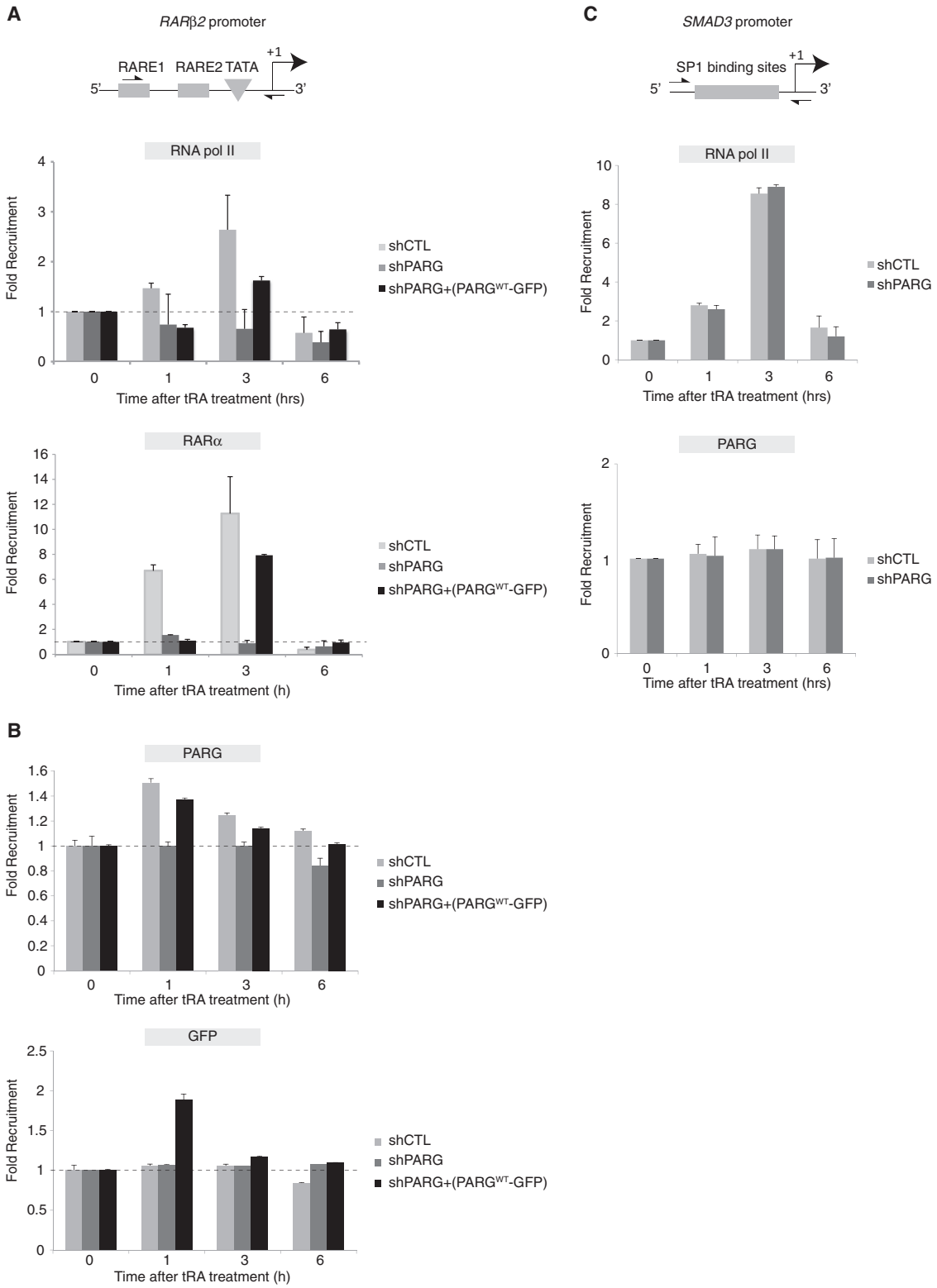

Figure 4. Defect in PIC Assembly at the *RARβ2* Promoter in the Absence of PARG

(A) (Upper panel) Diagram of the endogenous human *RARβ2* promoter. Positions of the primers for amplification of promoter region in ChIP are indicated with arrows. (Lower panels) ChIP monitoring the t-RA-dependent occupancy (±SEM) of RNA Pol II and RARα on the *RARβ2* promoter in chromatin extracts from either shCTL (light gray), shPARG (dark gray), or shPARG cells transfected with shRNA-resistant $PARG^{WT}$-GFP construct (black).

detected, although to a lesser extent than in shCTL cells (Figures S4B and S5B).

We also observed the recruitment of PARG to the *RAR*-dependent promoters in a ligand-dependent manner either in shCTL cells or in shPARG cells transiently expressing shRNA resistant PARG^{WT}-GFP (Figure 4B and Figures S4A, S4G, and S5A). In contrast to PIC, the optimal accumulation of both endogenous and transiently expressed PARG occurred before the transactivation peak, 1 hr after t-RA treatment. The formation of the PIC on the *SMAD3* promoter was not altered in shPARG cells, and PARG was not recruited to this promoter in shCTL cells (Figure 4C and Figures S6A and S6B).

PARG Controls Chromatin Derepression at RAR-Dependent Gene Promoters

We next assayed chromatin remodeling around the *RAR*-dependent promoters. We found a parallel between the recruitment of the PIC and the increase in both dimethylation of histone H3 lysine 4 (H3K4me2) and acetylation of histone H3 lysine 9 (H3K9ac), two marks of active transcription, on the promoters of *RARβ2* (Figures 5A and 5B and Figure S4C), *CYP26* (Figure S5C), and *SMAD3* (Figure S6C) in shCTL cells. A decrease in the dimethylation of histone H3 lysine 9 (H3K9me2), a mark of repressive transcription, took place before PIC formation, 1 hr after t-RA treatment, on both *RARβ2* (Figure 5C, Figure S4E) and *CYP26* promoters (Figure S5E). In addition, the exclusion of the repressive histone H1 from these promoters was observed soon after t-RA treatment, along with H3K9me2 demethylation (Figure 5D and Figures S4E and S5E).

In shPARG cells, H3K4me2 and H3K9ac appeared on *RARβ2*, *CYP26*, and *SMAD3* promoters, 3 hr after treatment, similarly to shCTL cells (Figures 5A and 5B and Figures S4D, S5D, and S6D). In contrast to shCTL cells, a dramatic increase in H3K9me2 and Histone H1 (for RARβ2) occurred at the *RARβ2* and *CYP26* promoters, 1 hr posttreatment (Figures 5C and 5D, Figures S4F and S5F). Transfection of PARG^{WT}-GFP in shPARG restored a profile of H3K9me2 and H1 occupancy at the promoter of *RARβ2* similar to that of shCTL cells (Figures 5C and 5D and Figure S4I). Note that H3K9me2 and H1 were released similarly from the promoter of *SMAD3* in both cell lines (Figures S6E and S6F).

Recent finding demonstrated that PARP-1 plays a role in the regulation of histone methylation (Krishnakumar and Kraus, 2010a). Since the absence of PARG led to an increase in the repressive H3K9me2 histone mark, we next focused on a histone methyltransferase and a demethylase whose activities regulate the methylation state of H3K9. The methyltransferase KMT1C (also called G9a) (Allis et al., 2007) is responsible for the majority of H3K9me2 marks in cells (Patnaik et al., 2004). The level of KMT1C decreased at the *RARβ2* promoter soon after t-RA-treatment in shCTL cells, while it remained stable in shPARG cells (Figure 5E). The H3K9me3/2 demethylase KDM4D (Klose et al., 2006; Whetstine et al., 2006; Shin and Janknecht, 2007) accumulated at the *RARβ2* promoter in shCTL cells soon after t-RA treatment but was not recruited throughout the time course in shPARG cells (Figure 5F). However, transfection of PARG^{WT}-GFP construct restored the accumulation of KDM4D and the release of KMT1C (Figures 5E and 5F).

Using siRNA against KDM4D, we next demonstrated the involvement of KDM4D in *RARβ2*, *CYP26*, and *PDK4* gene activation in HeLa cells (Figure 5G and Figure S7). Interestingly, KDM4D was dispensable for the transactivation of the PARG-independent gene *SMAD3* (Figure S7). Taken together, these results suggest that PARG controls chromatin derepression at RAR-dependent gene promoters.

Mutations of Two Residues Located in the JmjN Domain of KDM4D Prevent PARsylation after t-RA Treatment

The above data suggest that the interplay between PARP and PARG may control the activity of KDM4D or KMT1C. Since any substrate of PARG is first a substrate of PARP enzymes, we asked whether KDM4D or KMT1C was modified by PARP-1. Recombinant KDM4D (His-KDM4D[1–350] [Weiss et al., 2010]) was modified following the addition of recombinant affinity-purified human PARP-1, in vitro (Figure 6A, compare lanes 6 and 7). By comparison, KMT1C was only weakly modified by PARP-1 (Figure 6A, compare lanes 4 and 5). Histone H1, a well-known PARP target, was used as a positive control (Figure 6A, lanes 2 and 3). Furthermore, a polymer blot assay (Quénet et al., 2008) showed that KDM4D was not able to bind PAR noncovalently (data not shown).

The catalytic Jumonji C domain (JmjC) characterizes the KDM4/JMJD2 family members (Tsukada et al., 2006). In addition, a conserved N-terminal domain called JmjN, rich in amino acids (aa) that can be substrates for PARP-1, is present in KDM4 proteins including KDM4D (from aa 15 to 58) (Balciunas and Ronne, 2000; Chen et al., 2006; Katoh and Katoh, 2004). After deletion of its JmjN domain, GST-KDM4D$^{Del(15-58)}$ was no longer modified by PARP-1 (Figure 6B, compare lanes 5 and 6 with lanes 3 and 4). Two potential substrates for PARP-1 (E26 and E27) present in the JmjN domain and conserved in the KDM4 demethylase family (Figure 6C, upper panel) were mutated to alanine, and the resulting GST-KDM4D$^{E26/27A}$ was assayed for PARsylation. The double mutant showed a complete lack of modification by PARP-1 (Figure 6C, compare lanes 8 and 9 with lanes 2–7), demonstrating that mutation of these two residues prevents PARsylation. In a Histone H3K9me2 demethylation assay, the mutations E26A/E27A did not affect the activity of the demethylase (Figure 6D, compare lanes 5–7 with lanes 2–4), suggesting that they do not alter the structure of KDM4D.

We next analyzed the PARsylation of KDM4D in vivo. We expressed GFP-KDM4D^{WT} and GFP-KDM4D$^{E26/27A}$ in shPARG

(B) ChIP monitoring the t-RA-dependent occupancy (±SEM, two independent experiments) of PARG on the *RARβ2* promoter in chromatin extracts from either shCTL (light gray), shPARG (dark gray), or shPARG cells transfected with shRNA-resistant PARG^{WT}-GFP construct (black). Chromatin extracts were immunoprecipitated with an anti-PARG in the upper panel and with an anti-GFP in the lower panel.

(C) (Upper panel) Diagram of the endogenous human *SMAD3* promoter. Positions of the primers for amplification of promoter region in ChIP are indicated with arrows. (Lower panels) ChIP monitoring the t-RA-dependent occupancy (±SEM, two independent experiments) of RNA Pol II and PARG on the *SMAD3* promoter in chromatin extracts from either shCTL or shPARG cells.

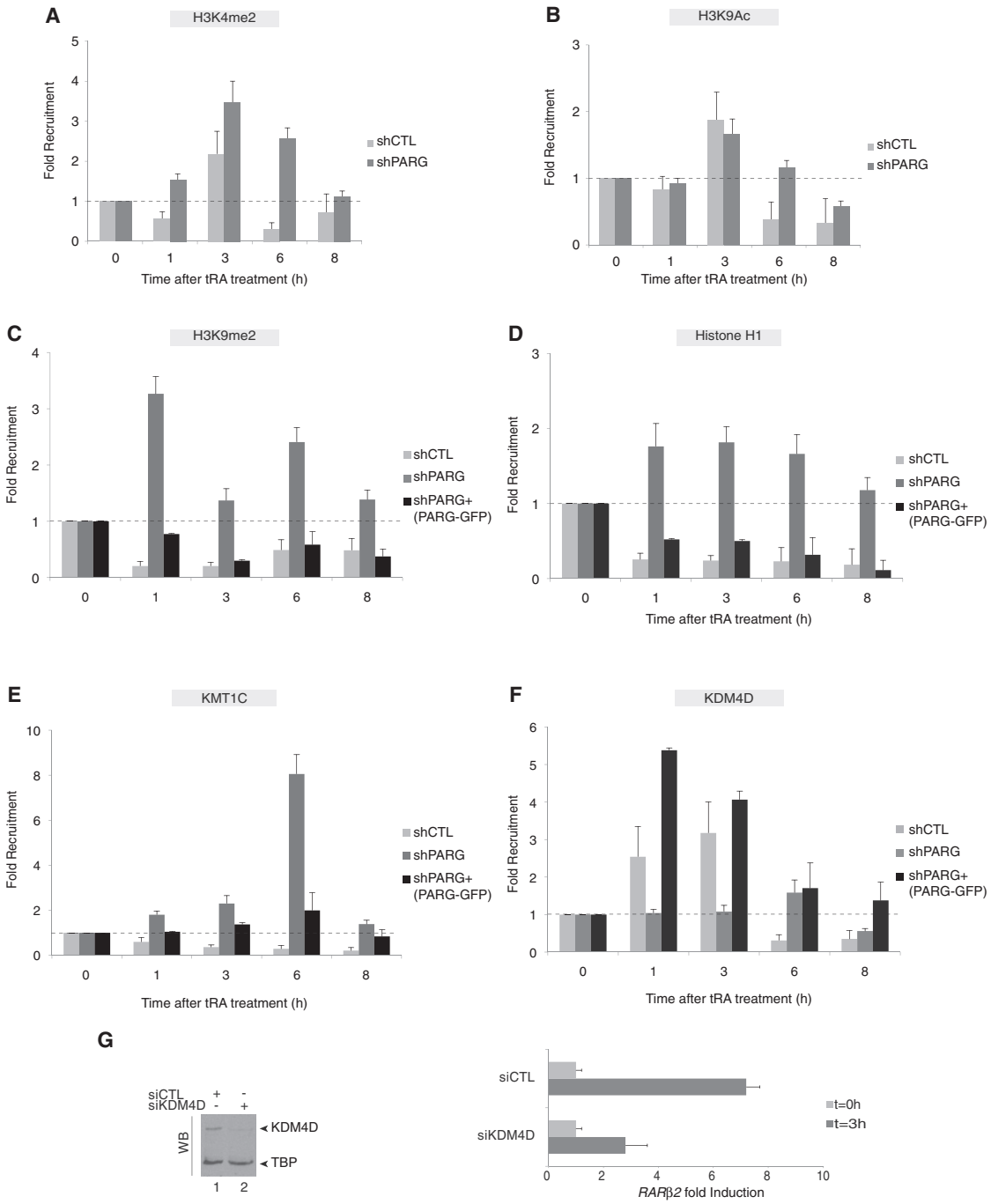

Figure 5. Chromatin Remodeling at the *RARβ2* Promoter

(A and B) ChIP monitoring the t-RA-dependent occupancy (±SEM, two independent experiments) of H3K4me2 (A), H3K9Ac (B) on the promoter of *RARβ2* gene in chromatin extracts from either shCTL cells or shPARG cells.

(C–F) ChIP monitoring the t-RA-dependent occupancy (±SEM, two independent experiments) of H3K9me2 (C), Histone H1 (D), KMT1C (E), KDM4D (F) on the promoter of *RARβ2* gene in chromatin extracts from either shCTL cells, shPARG cells, or shPARG cells transfected with ($PARG^{WT}$-GFP).

(G) (Left panel) HeLa cells were transfected either with siRNA control or with siRNA against KDM4D. Forty-eight hours later, proteins from whole-cell extracts (50 μg) were resolved by SDS-PAGE followed by western blotting using anti-KDM4D and anti-TBP antibodies. (Right panel) Relative mRNA expression (±SD, three independent experiments) of *RARβ2* in HeLa cells transfected either with siRNA control or with siRNA against KDM4D, measured 3 hr after treatment with t-RA (10 μM). The values are expressed relative to the expression level of the no treatment control that is set to 1 in all experiments.

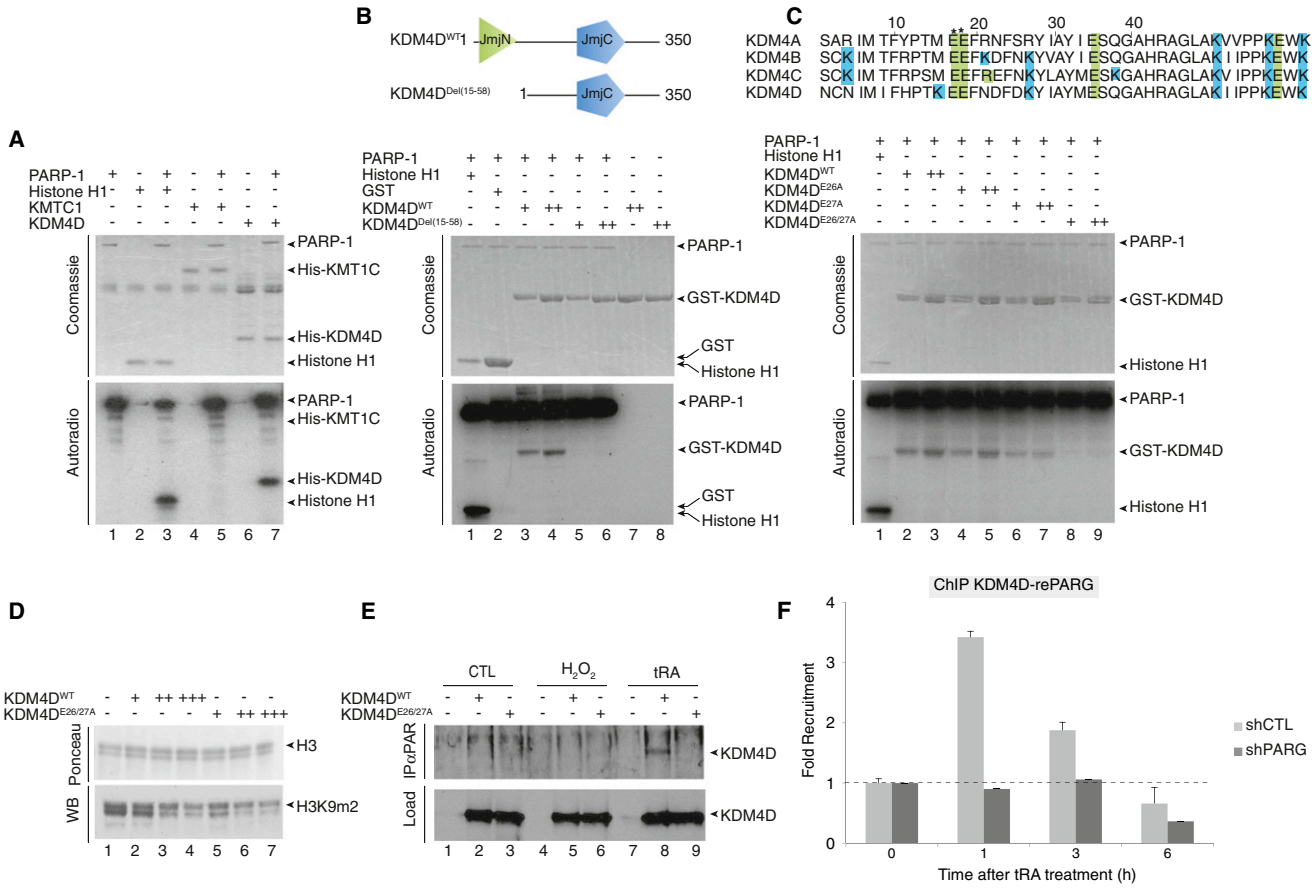

Figure 6. Two Glutamic Acid Residues in the JmJN Domain of KDM4D Are Required for PARsylation

(A) Two hundred and fifty nanograms of recombinant Histone H1 (lanes 2 and 3), His-KMT1C (lanes 4 and 5), and His-KDM4D(1–350) (lanes 6 and 7) were mixed with 100 ng of recombinant PARP-1 (lanes 3, 5, and 7) in the presence of ^{32}P-NAD$^+$ and 100 nM of cold NAD+. These assay conditions used favor synthesis of short polymers due to limiting amounts of NAD. Samples were run on a SDS-PAGE gel followed by Coomassie staining (Coomassie) and autoradiography (Autoradio).

(B) (Upper panel) Schematic representation of KDM4D(1–350). JmJN and JmJC domains are represented in green and blue, respectively. (Lower panel) One hundred and fifty nanograms of recombinant Histone H1 (lane 1), 300 ng of GST (lane 2), 150 and 300 ng of GST-KDM4D^{WT} (lanes 3 and 4), or GST-KDM4D$^{Del(15-58)}$ (lanes 5 and 6) was mixed with 100 ng of recombinant PARP-1 and ^{32}P-NAD$^+$. Samples were run on a SDS-PAGE gel followed by Coomassie staining (Coomassie) and autoradiography (Autoradio). Three hundred and fifty nanograms of GST-KDM4D^{WT} (lane 7) or GST-KDM4D$^{Del(15-58)}$ (lane 8) was incubated alone as controls.

(C) (Top panel) Sequence alignment of the JmJN domains of the KDM4 family members performed with the ClustalX multiple sequence alignment software. PARP-1 potential targets (lysine and glutamic acid residues) are indicated in blue and green, respectively. Lysine and glutamic acid residues are particularly enriched in the JmjN domain of KDM4D in which they represent ~30% of the total amino acids content compared with 6% in the total protein. The two conserved glutamic acids present in all KDM4 family members are marked with a star. (Lower panel) GST-KDM4D^{WT} (lanes 2 and 3), GST-KDM4D^{E26A} (lanes 4 and 5), GST-KDM4D^{E27A} (lanes 6 and 7), and GST-KDM4D$^{E26/27A}$ (lanes 8 and 9) were treated as in (B).

(D) GST-KDM4D^{WT} and GST-KDM4D$^{E26/27A}$ were used in a demethylation assay containing core histones from HeLa. The reactions were then subjected to either western blotting using anti-Histone H3K9me2 antibody (WB) or Red Ponceau staining (Ponceau).

(E) GFP-KDM4D^{WT} or GFP-KDM4D$^{E26/27A}$ was transiently expressed in shPARG cells. Immunoprecipitation was performed from 200 μg of whole-cell extracts 10 min after treatment with H_2O_2 (1 mM) or 1 hr after treatment with t-RA (10 μM), using anti-PAR antibody. Following SDS-PAGE, western blotting analysis was performed with an anti-GFP antibody on the immunoprecipitated material (upper panel) or on 20 μg of whole-cell extracts (lower panel).

(F) ChIP-reChIP monitoring the t-RA-dependent coaccumulation (±SEM, three independent experiments) of PARG and KDM4D on the *RARβ2* promoter in chromatin extracts of shCTL and shPARG cells. A first IP performed against KDM4D was followed by a second IP using anti-PARG antibody on the eluted complexes.

cells, treated the cells with t-RA for 1 hr, immunoprecipitated the material with an anti-PAR antibody, and subjected the precipitate to western blotting with an anti-GFP antibody. GFP-KDM4D^{WT} was pulled down by anti-PAR antibody after t-RA treatment (Figure 6E, compare lanes 2 and 8), while GFP-KDM4D$^{E26/27A}$ was not (Figure 6E, compare lanes 3 and 9). We also noticed that GFP-KDM4D^{WT} was not pulled down following H_2O_2 treatment (Figure 6E, compare lanes 2, 5, and 8), suggesting that PARsylation of KDM4D(1–350) is specific to t-RA treatment.

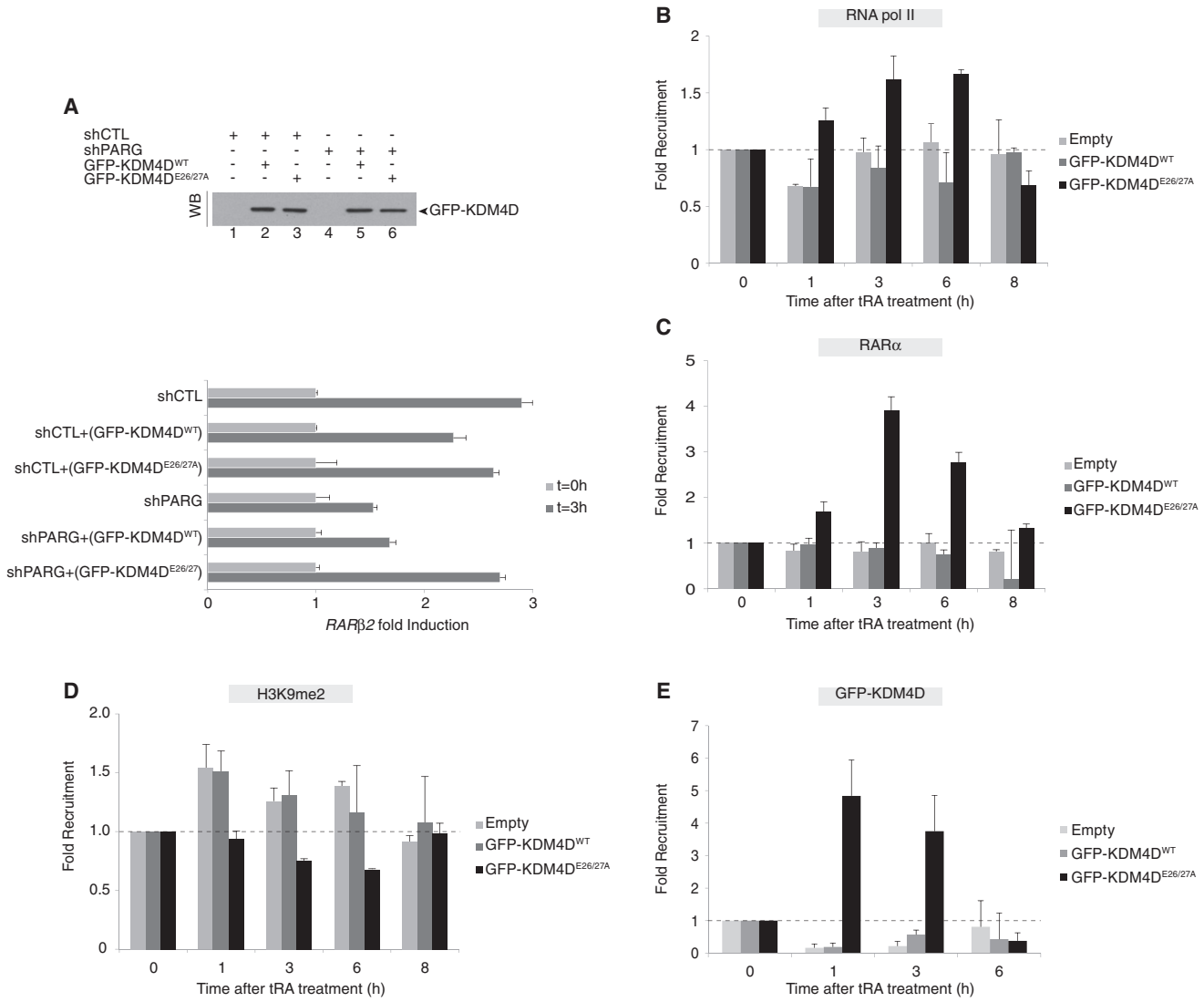

Figure 7. KDM4D$^{E26/27A}$ Restores RAR-Dependent Transactivation in the Absence of PARG

(A) (Upper panel) shCTL (lanes 1–3) or shPARG (lanes 4–6) cells were transfected either with GFP-KDM4D^{WT} (lanes 2 and 5) or GFP-KDM4D$^{E26/27A}$ (lanes 3 and 6). Forty hours later, proteins from whole-cell extracts (50 μg) were resolved by SDS-PAGE followed by western blot using an anti-GFP antibody. (Lower panel) Relative mRNA expression of *RARβ2* in the indicated cells (±SD, three independent experiments), measured 3 hr after treatment with t-RA (10 μM). The values are expressed relative to the expression level of the no treatment control that is set to 1 in all experiments.

(B–D) ChIP monitoring the occupancy (±SEM, two independent experiments) of RNA Pol II (B), RARα (C), and H3K9me2 (D) on the *RARβ2* promoter in chromatin extracts from shPARG cells transfected either with an empty vector (light gray), with GFP-KDM4D^{WT} (dark gray), or with GFP-KDM4D$^{E26/27A}$ (black) expression vectors.

(E) ChIP monitoring the occupancy (±SEM, two independent experiments) of either GFP-KDM4D^{WT} or GFP-KDM4D$^{E26/27A}$ on the *RARβ2* promoter in chromatin extracts from shPARG cells after t-RA treatment.

Next, we used a ChIP-reChIP assay to test whether PARG and KDM4D were in close contact at the *RARβ2* promoter. Our results revealed a coaccumulation of PARG and KDM4D, which occurred 1 hr after t-RA treatment in shCTL cells (Figure 6F and Figure S8). This coaccumulation was absent in shPARG cells (Figure 6F and Figure S8). No signal was detected in the ChIP-reChIP experiment with KMT1C (data not shown). Altogether, these data suggest that KDM4D is PARsylated following t-RA treatment and that PARG counteracts this modification.

KDM4D$^{E26/27A}$ Restores RAR-Dependent Transactivation in the Absence of PARG

To test the above hypothesis, we sought to assess the need of KDM4D PARsylation for RAR-mediated gene expression. Expression of the unPARsylable GFP-KDM4D$^{E26/27A}$ in shPARG cells rescued *RARβ2*, *CYP26*, *PDK4*, and *NRIP1* expression, which was not achieved with GFP-KDM4D^{WT} (Figure 7A and Figure S9). In agreement with the rescue of mRNA transactivation, expression of GFP-KDM4D$^{E26/27A}$, but not GFP-KDM4D^{WT},

partially restored the recruitment of both RNA Pol II and RARα to the *RARβ2* promoter (Figures 7B and 7C and Figures S10A and S10B). This recruitment was accompanied by a decrease in H3K9me2 modification until 6 hr posttreatment (Figure 7D and Figure S10C). We also monitored the presence of KDM4D at the *RARβ2* and *CYP26* promoters in shPARG and found a recruitment of GFP-KDM4D$^{E26/27A}$ early after t-RA treatment that did not take place with GFP-KDM4D^{WT} (Figures 7E, Figures S10D and S10E), suggesting that PARsylation regulates the binding of the histone demethylase to the chromatin (Krishnakumar and Kraus, 2010a). These results demonstrate that an unPARsylable KDM4D mutant can compensate for the absence of PARG and restores a permissive chromatin environment suitable for RAR-dependent gene transcription.

DISCUSSION

PARG Is a Coactivator of RAR-Mediated Gene Expression

PARG has been extensively studied for its role in the DNA damage response and in cell death, but some studies have also suggested a role in transcription (Frizzell et al., 2009; Tulin et al., 2006). Here, we demonstrate that PARG functions as a transcriptional coactivator that modulates RAR-mediated gene expression. Global transcription profiling and individual gene studies show that the transactivation of several RAR-dependent genes was impaired in the absence of PARG, which localized to the promoter of these genes in a ligand-dependent manner. The recruitment of PARG to these promoters occurred 1 hr posttreatment and preceded the recruitment of the transcription machinery. This observation suggests that PARG may be required for the formation of the PIC. Indeed, we observed that the recruitment of RNA Pol II or RAR to the promoters of RAR-dependent genes was dependent on PARG. An shRNA-resistant PARG^{WT} construct rescued the NR-dependent transactivation in shPARG cells, excluding any off-target effects of the shRNA. We also identified genes whose transactivation by t-RA was independent of PARG. In agreement with this observation, PARG does not localize to the promoter of these genes, indicating that transactivation by t-RA can occur following different molecular pathways.

PAR-Degrading Activity Is Required for RAR-Mediated Transcription Process

The role of the PAR-degrading activity of PARG in transcription has been examined but has led to contradictory results (Frizzell et al., 2009; Rapizzi et al., 2004). In our well-defined system, we observed that RAR-mediated transcription required the PARG catalytic activity. Our data imply that PARG targets a substrate that must be dePARsylated before transactivation takes place. In agreement with this hypothesis, a rescue in the transactivation of the RAR-dependent genes in PARG-depleted cells was obtained by treatment of these cells with a PARP-specific inhibitor. We noticed that treatment of shCTL cells with this inhibitor had no effect on RAR-dependent gene expression, in agreement with previous results indicating that RAR-mediated gene transcription was dependent on the presence of the PARP-1 protein but not on its activity (Pavri et al., 2005). Our data support these observations but further demonstrate that PARP activity may even have a repressive effect on RAR-dependent gene expression, in the absence of PARG.

PARG Promotes a Permissive Chromatin Environment around the Promoter of NR-Dependent Genes

The absence of PIC formation in PARG-depleted cells and the involvement of PAR in the regulation of chromatin structure (Krishnakumar and Kraus, 2010b) led us to analyze histone modifications around the promoters of RAR-dependent genes. H3K9ac or H3K4me2, active marks of transcription, were present after t-RA treatment even in the absence of PARG. Surprisingly, we detected a peak of H3K9me2 and the persistence of Histone H1, two strong marks of transcriptional repression, soon after t-RA treatment of shPARG cells. The peak of H3K9me2 appeared at the promoter of the RAR-dependent genes in shPARG between 1 and 3 hr after t-RA-treatment, a time when PARG was recruited to these promoters in shCTL cells. This indicates that PARG may work to alleviate the transcriptional repression induced by H3K9me2, thereby triggering the formation of the PIC.

In line with this hypothesis, the H3K9me2 demethylase KDM4D was efficiently PARsylated by PARP-1 and coaccumulates with PARG on these promoters after t-RA treatment. Besides, KDM4D was required for the transactivation of PARG-dependent genes but dispensable for PARG-independent ones. What exactly dictates a gene's PARG dependency remains to be further investigated. However, it appears that *SMAD3*, a PARG-independent gene, contains a promoter with multiple SP1 binding sites that are supposed to recruit RAR. On the other hand, *RARβ2*, *CYP26*, *PDK4*, and *TNFAIP2* show the classical RXR/RAR binding sites (Cheng et al., 2011). Therefore, the nature of the NR binding element may induce a variation in the cofactors required to transactivate various genes with the same NR.

Interplay between PARP-1 and PARG Controls the Activity of KDM4D on the Promoter of NR-Dependent Genes

The JmjC domain-containing histone demethylases (JHDM proteins) consist of several subfamilies that can remove methyl groups from the modified H3K4, H3K9, H3K27, or H3K36 residues (Hou and Yu, 2010; Varier and Timmers, 2011). KDM2A is the founding member of this large family, which is characterized by the presence of a JmjC catalytic domain (Tsukada et al., 2006). Some JHDM proteins also possess a conserved N-terminal motif (JmjN domain), strictly associated with the JmjC domain (Balciunas and Ronne, 2000). The function of the JmjN domain is still unclear, but it has been shown to have extensive interactions with JmjC through the formation of hydrogen bonds and hydrophobic contacts (Chen et al., 2006). Furthermore, removal of the JmjN domain impairs the stability and activity of JmjC (Chen et al., 2006). The JmjN domain typically spans 40 amino acids, as depicted in Figure 6. Our results show that two glutamic residues belonging to the JmjN domain are required for the modification of KDM4D by PARP-1.

The fact that unPARsylable mutant can compensate for the absence of PARG and rescued RAR-dependent transactivation

in shPARG cells, while overexpression of KDM4D^{WT} did not, suggests that PARsylation of KDM4D was responsible for the inhibition of RAR-dependent gene transactivation. These data suggest that PARsylation of KDM4D negatively regulates its activity at the promoter of RAR-dependent genes and, thereby, the transactivation of these genes. PARsylation may lead to the destabilization of the JmjN/JmjC interaction that is required for KDM4D activity. Alternatively, the PARsylation of KDM4D may destabilize the interaction between KDM4D and the chromatin. ChIP results show a higher occupancy of the GFP-KDM4D$^{E26/27A}$ mutant at RAR-dependant promoters following t-RA treatment that may suggest that PARsylation of KDM4D regulates its binding to chromatin, as demonstrated for the H3K4 demethylase KDM5B (Krishnakumar and Kraus, 2010a). Due to the low level of PARsylation of KDM4D that we were able to achieve in vitro, it was not possible to test the consequences of PARsylation on KDM4D activity in a demethylase assay. Altogether, our data allow us to propose a model in which the activity of PARP-1 regulates RAR-dependent gene transcription through the depletion of an active KDM4D from the chromatin, thereby establishing a heterochromatin landscape. In this model, PARG counteracts the action of PARP-1 to induce an open chromatin structure and an active transcription process. Whether or not the amount of PARG, PARP-1, or their subcellular localization in different cell types would lead to a cell-specific modulation of RAR-dependant gene transcription remains to be established.

As stated above, it is also likely that other KDMs are regulated in a similar manner. Krishnakumar and Kraus demonstrated recently that the PARsylation of KDM5B regulates the genome-wide methylation of histone H3K4 in basal transcription by impacting its interaction with the nucleosomes (Krishnakumar and Kraus, 2010a). It is interesting to note that KDM5B contains a JmjN domain (Yamane et al., 2007) with two conserved successive glutamic acid residues that, in light of our results, may be crucial for its PARsylation. Future work in this area will broaden our knowledge of the role of PARG in transcription, which could prove as important as that of PARP-1.

EXPERIMENTAL PROCEDURES

Cell Lines

shCTL (BD650) and shPARG (PARG^{KD}) cells were characterized previously (Amé et al., 2009). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Invitrogen) supplemented with 10% fetal calf serum (FCS), 40 μg/ml gentamicine, and 125 μg/ml hygromycin B. NR-dependent gene activation was performed as described (Keriel et al., 2002) (also see the Supplemental Information). F9 mouse embryonic carcinoma cells were cultured in DMEM supplemented with 10% FCS and 40 μg/ml gentamycin. ON-TARGET plus smart pool siRNA control or targeting the mouse PARG was transiently transfected in F9 cells with Lipofectamine 2000 (Invitrogen). After 24 hr of transfection, the cells were first treated with KU0058948 (100 nM) to avoid the lethal accumulation of PAR and 24 hr later with t-RA (10^{-7}M) for differentiation.

^{32}P-NAD+ PARsylation Assay

PAR reaction was carried out as described (Amé et al., 1999). Briefly, 100 ng of recombinant PARP-1 (Enzo Life Sciences) was incubated with 250 ng of recombinant histone H1.2 (Enzo Life Sciences), KMT1C (active motif), or KDM4D (Weiss et al., 2010) or purified GST-tagged proteins as indicated in the Figure 6 legend, in PAR reaction buffer (50 mM Tris-HCl [pH 8.0], 4 mM $MgCl_2$, 300 mM NaCl, 1 mM DTT) supplemented with 0.1 μg/ml BSA, 6 pmol of DNase I-activated DNA, 1 μC^{32P}-NAD+, and 100 nM cold NAD+. Reaction was allowed for 20 min at 25°C, stopped by addition of SDS-PAGE loading buffer and boiling for 5 min. Samples were subjected to SDS-PAGE. Detection of automodification was carried out by autoradiography and detection of proteins by Coomassie staining.

Demethylation Assay

Demethylation of core histones by KDM4D was performed in 20 mM Tris (pH 7.3), 150 mM NaCl, 50 μM [NH4]2Fe[SO4]2·6H2O, 1 mM α-ketoglutarate, 2 mM ascorbic acid using 5 μg of purified HeLa histones. The demethylation reaction was incubated for 12 hr at 37°C, and the samples were subsequently analyzed by western blotting.

Antibodies

Mouse monoclonal antibodies against the RNA polymerase II (7C2), RARα (9A6), and TBP (3G3) were produced at the IGBMC facility.

Rabbit polyclonal anti-Nter-PARG, mouse monoclonal anti-PARP1 (C2-10), and anti poly(ADP-ribose) (10H) were previously described (Amé et al., 2009).

Rabbit polyclonal anti-GFP (TP401) was produced at Torrey Pines Biolabs. Rabbit polyclonal anti-KMT1C/G9a (3306), anti-dimethyl histone H3K9 (9753), anti-dimethyl histone H3K4 (9726), and anti-acetylhistone H3K9 (9671) were produced at Cell Signaling. Rabbit polyclonal anti-KDM4D (AB93694) was produced at Abcam. Mouse monoclonal anti-H1 antibody (clone AE4) was produced at Millipore. Mouse monoclonal anti-β-tubulin (MAB3408) was produced at Chemicon.

ACCESSION NUMBERS

The data in this paper have been deposited in the GEO Database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE40883.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four tables, ten figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at http://dx.doi.org/10.1016/j.molcel.2012.09.021.

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3.5.3 Highlights and perspectives

This project demonstrated how the regulation of PARsylation of KDM4D by PARP-1 and PARG is crucial to ensure NR-mediated transcription. Interestingly, our data showed that the enzymatic activity of PARP-1 have to be fine tuning to avoid detrimental effects on transcription like the maintained PARsylation of KDM4D leading to absence of chromatin remodelling. I can once again make the same comment about the lack of genome-wide appreciation of such mechanisms. Although we showed that the involvement of PARG was not detected for all analysed RAR-target genes, it could be interesting to define and characterize the DNA regulatory regions that support this relationship PARP/PARG.

As it has been mentioned in the discussion, PARsylation regulates KM4D and KDM5B. It is an important question to know whether this mechanism can be observed for the other enzymes possessing the conserved JmjN domain. In addition to the KDMs, it would be also interesting to analyse the impact of PARG on other PARP-1 substrates. I would particularly focus on NER factors XPC and XPA known to be targeted and regulated by PARP-1 upon DNA repair process (*293, 294*). Although we demonstrated the recruitment of PARG at *RARβ2* promoter, no data are available for the conservation of these physical and functional associations between NER factors and PARP-1 upon transcription.

4 Still a step in Virology Collaborations

4.1 Introduction

Meantime I developed the different projects described above, I kept an intact interest for my doctoral subject and I continued to document myself on the strategies induced by bunyaviruses to escape the innate immune response. Therefore, when I had the opportunity in 2011 to write a review about this topic, I embraced it and proposed to my former PhD supervisor M. Bouloy to work on it together.
Surprisingly, the publication of this review and probably also my doctoral research on RVFV gave me another opportunity to come back transiently in virology. A former postdoc and colleague from J-M Egly/F. Coin's laboratory, Denis Kainov, started his own group in Helsinki by working on Influenza virus. He collected interesting results concerning an accessory protein from H5N1 influenza A virus called NS1 that could, like RVFV NSs protein, inhibit the innate immune response in infected cells at the transcriptional level.
I was very enthusiastic when he asked me to participate to the investigation of the NS1 related mechanisms explaining this transcriptional shut down. Our collaboration allowed the identification of a new viral process to escape the antiviral response. A previous report already described how H3N2 Influenza A NS1 by mimicking the histone H3 tail could trap elongation factor PAF1 complex and inhibit cellular transcription (*295*). We showed here how H5N1 NS1 protein could bind DNA and disturb histones composition on chromatin impeding RNA pol II recruitment at promoters normally activated upon a viral infection.
This story strengthens my fascination for viruses and their brilliant strategies for survival and their constant battle against the hosts. Through different accessory proteins, viruses can target and deregulate every possible checkpoint that fines tune cellular transcription making them precious tools to understand the complexity of the gene expression.

4.2 Review: Back to bunyaviruses

Le May N. & Bouloy M. "Antiviral escape strategies developed by bunyaviruses pathogenic for humans". **Frontiers in Bioscience**. *2012*.

Antiviral escape strategies developed by bunyaviruses pathogenic for humans

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1. ABSTRACT

New or re-emerging pathogens for humans have emerged outside of their usual endemic range during the last decade originating severe public health concern and economical losses. Climate changes have played a significant role in the emergence or re-emergence of arboviruses. Among these pathogens, several viruses belong to the *Bunyaviridae* family. This family is composed of RNA viruses grouped into five genera–*Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus* characterized by their antigenic, genetic and ecological properties. These viruses use cellular proteins to promote their own replication/transcription and reciprocally the host induces, in response, an important transcriptional reprogramming to activate antiviral defences including the interferon type I pathways. The virulence of the pathogenic bunyaviruses is directly linked to the roles of viral virulence factors and their capacity to counteract the host pathways. This review summarizes the various strategies developed by the different genera of the *Bunyaviridae* family to overcome and escape the innate immune response and eventually other cellular functions.

2. INTRODUCTION

During the past decade, the emergence of either emerging or re-emerging virus diseases in new areas of the world occurred with increasing frequency and became a serious public health concern and economical losses. Arboviruses are distributed worldwide and represent approximately 30% of all emerging infectious diseases during the last decade (1). Viruses such as West Nile (WNV), Chikungunya (CHIKV), Dengue (DENV), Yellow Fever (YFV), Crimea-Congo Hemorrhagic Fever (CCHF) and Rift Valley Fever (RVFV) viruses, which are pathogenic for humans and/or animals have emerged outside of their usual endemic range and caused epidemics in North America, Europe and the Arabian Peninsula. Their emergence may be related to the climate fluctuations due to the global warming and/or human activities (human travel increasing, deforestation, political and military activities) that facilitate the dispersion of the arthropods beyond their current geographic boundaries (2, 3). As an example of new emerging pathogens, one should recall the newly identified phlebovirus isolated in China. It is transmitted by ticks and closely related to the tick borne Uukuniemi (UUKV) virus. However, in contrast with UUKV, which is not pathogenic for humans, this novel phlebovirus designated severe fever with thrombocytopenia syndrome virus or Huaiyangshan virus depending on the laboratory where it was isolated, is responsible for severe thrombocytosis and multi-organ dysfunction with high morbidity and mortality (initial fatality rate 30%) (4, 5). Studies on these arboviruses thus become a major issue since neither safe vaccine for protection nor antiviral treatments for therapy is currently available. Among these emerging pathogens, several viruses belong to the large family of RNA viruses, the *Bunyaviridae* family (Table 1).

The *Bunyaviridae* family comprises more than 350 members and is subdivided in five genera (i.e. *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus*) characterized by their antigenic, genetic and ecological properties. Orthobunyaviruses, nairoviruses and phleboviruses infect vertebrates and are vectored by haematophagous arthropods including mosquitoes, ticks, midges and sandflies whereas tospoviruses are plant pathogens and are vectored by different thrips (6). In

contrast, hantaviruses are not transmitted by arthropods but by rodents and insectivores which act as reservoirs. Infections by hantaviruses are persistent in their reservoir hosts and humans become infected through contamination by excretions or carcasses of infected reservoirs (7).

All the members of this family, referred as bunyaviruses, are enveloped, spherical virions between 80-120 nm in diameter with its replicative cycle in the cytoplasm and maturation and budding of the newly formed particles in the Golgi apparatus (8, 9). These viruses possess single-stranded RNA genomes that consist of three segments L (Large), M (Medium) and S (Small) having a negative- or ambi-sense polarity (6). The L and M segments code respectively for the viral RNA-dependent RNA polymerase (L-RdRp) and a precursor to the envelope glycoproteins (Gn and Gc). The S segment codes for the internal protein N or nucleocapsid protein that is able to oligomerize and associate with the viral polymerase and the three different segments of the viral genome to form viral ribonucleoparticles (RNPs) packaged into virions. Depending on the genus, other proteins are encoded by the M and S segments, namely the non-structural proteins NSm1, NSm2 and NSs harbouring multiple roles. The virulence of the pathogenic bunyaviruses illustrates the permanent co-evolution between the viruses and their hosts. The virus utilizes cellular proteins or functions to promote their own replication/transcription. This is particularly well illustrated in the case of bunyaviruses which utilize capped oligonucleotides from host cell mRNAs to prime transcription through the cap-snatching mechanism mediated by the L-RdRp which possesses an endonuclease activity to cleave the capped oligonucleotides (10). In response to viral infection, the host induces an important transcriptional reprogramming to activate various defences including the innate immunity. However, viruses have developed strategies to overcome the numerous host responses; this will be described in the following sections of this review.

3. THE INNATE IMMUNITY

In many cases, arboviruses infect vertebrates through mosquito or tick bites, which inoculate the virus in the skin, making Dendritic Cells (DCs) such as skin Langerhans cells the primary cell targets (11, 12). Infected Langerhans cells migrate to the draining lymph node, allowing the virus to enter the bloodstream and disseminate. After pathogen intrusion, the first response of the cell or the organism is an inflammatory reaction including secretion of soluble mediators such as cytokines and chemokines. Among these mediators, Interferons (IFNs) and especially type I IFNs (IFNα/β) are the most efficient to mount a rapid host response with the aim to block the viral replication (13, 14). The initial response to infection involves the production and secretion of IFNα1 and IFNβ followed by a subsequent amplification phase linked to induction of other IFNα subtypes. The secreted IFNs act in autocrine and paracrine manner to stimulate the expression of antiviral, antiproliferative and immunomodulatory genes.

The induction of type I IFNs occurs mainly by an intracellular pathway and necessitates the initial recognition between specific cellular Pattern Recognition Receptors (PRR) and viral inducers so-called Pathogen-Associated Molecular Patterns (PAMP). Double-stranded (ds) RNA or 5'-triphosphorylated single-stranded (ss) RNA from RNA viruses trigger several cytosolic PAMPs including two RNA helicases, RIG-I and MDA-5, and the dsRNA-dependent Protein Kinase R (PKR). The binding of viral RNA to RIG-I and MDA-5 induces a signaling chain, which leads to the activation and nuclear translocation of several transcription factors including the constitutive Interferon Regulatory Factor-3 (IRF3) and NF-κB. The RIG-I and MDA-5 dependent pathways result in the phosphorylation of IRF-3 by TBK1, the homodimerisation and nuclear translocation of IRF-3 being a prerequisite to initiate the messenger RNA (mRNA) IFNβ and human IFNα1 synthesis (15, 16). The kinase PKR is a constitutive protein that is activated by dsRNA inducing its auto-phosphorylation. The activated PKR can therefore phosphorylate several substrates including IκB and eIF-2α (eukaryotic Initiation Factor 2 alpha). The phosphorylated IκB is degraded through the proteasome activating the transcription factor NF-κB whereas its effect on eIF-2α leads to the inhibition of translation of cellular and viral mRNA.

The secreted IFNα1 and IFNβ, triggered through the IRF-3-dependent induction, are able to bind and activate IFNAR1 and IFNAR2 present on all host cells. These IFNARs dimerize and induce the phosphorylation of members of Janus Kinase (JAK), TYK-2 and JAK1 that are pre-associated with their cytoplasmic tails. The latent cytoplasmic transcription factors from the Signal Transducer and Activator of Transcription (STAT) family are therefore phosphorylated by JAK1 and TYK-2 leading to their activation (17, 18). Phosphorylated STAT1 and STAT2 heterodimerize and recruit IRF-9 to form a complex called IFN stimulated gene factor 3 (ISGF-3) that translocates to the nucleus, binds to IFN-stimulated response elements (ISRE) localized in numerous IFN-induced gene promoters such as IFN-Stimulated Genes (ISGs) and IRF-7 and activates the transcription of these genes (Figure 1). IRF-7 is not constitutively expressed in most cells (except in plasmacytoid Dendritic Cells, pDCs) and is an IFN-inducible and virus-inducible protein similar to IRF-3. Active IRF-7 homodimers or heterodimers with IRF-3, bind to promoter of all IFNα genes, and are therefore responsible for the induction of delayed type I IFNs and amplification of the IFN response (16) (Figure 1). IFNs also induce more than 350 ISGs, which have antiviral, antimicrobial, antiproliferative and immunomodulatory functions (19). The proteins induced by IFNs include enzymes, signalling proteins, chemokines, cytokines, transcription factors, heat shock proteins, surface glycoproteins or pro-apoptotic proteins. Among this list, only a few ones have been characterized with antiviral activities such as the Mx GTPases, PKR, the 2',5' Oligoadenylate Synthetases (2-5 OAS)/RNAse L system, the RNA-specific adenosine deaminase 1 (ADAR1), viperin, ISG20 or p56 which are largely described in more detailed in other reviews (20). In response to the type I IFNs action, each virus targets specific cellular proteins and

usually possesses unique strategy to counteract these pathways. This review illustrates the diversity and originality in the strategies developed by different pathogenic bunyaviruses.

4. ORTHOBUNYAVIRUSES

This genus consists of more than 150 viruses; it is subdivided into 19 different serogroups: among which the California, Bunyamwera and Simbu serogroups comprise respectively, three serious human pathogens: La Crosse, Ngari and Oropouche viruses. Bunyamwera virus is the prototype for the *Orthobunyavirus* genus and the *Bunyaviridae* family. The orthobunyaviruses are able to replicate both in mammalians and in insects. The infection in mammalian cells is lytic and causes host cell shut off and cell death whereas it is noncytolytic and leads to viral persistent in invertebrate cells. Besides the classical organization, the M segment encodes a polyprotein precursor post-translationally cleaved into the envelope glycoproteins Gn and Gc and a third protein NSm of unknown function. In addition to the nucleocapsid, the S segment also encodes the NSs protein in an overlapping reading frame. The NSs protein has been reported to act as an interferon antagonist playing an important role in viral pathogenesis (21-23). The innate immune escape mediated by NSs protein was more precisely studied for two orthobunyaviruses Bunyamwera (BUNV) and La Crosse (LACV) viruses. The NSs protein impairs the antiviral response by preventing cellular transcription through the proteolysis of RNA polymerase II (RNAPII) (Figure 2).

4.1. Bunyamwera virus

The BUNV NSs protein is predominantly cytoplasmic although a significant fraction was also observed in the nucleus of cells transfected with a Flag-NSs expressing plasmid (22). Upon infection with wild type (wt) BUNV, the induction of IFN or IRF-3 mediated pro-apoptosis genes is abolished although the activation of IRF-3 and PKR is maintained indicating the NSs effect is downstream the primary signaling chain of innate immunity (24, 25). In BUNV-infected mammalian cells, NSs inhibits the phosphorylation of serine 2 in heptapeptide repeat of the CTD of RNA polymerase II and induces also its degradation (26, 27). Such a dysregulation of the host transcription machinery results from the interaction between the C-terminal region of NSs and Med8, a subunit of mediator complex (27). Mediator regulates RNAPII phosphorylation CTD-ser-5 residue and contacts the CTD-ser-2 kinase P-TEFb (28, 29). Moreover, Med8 can form an ubiquitin E3 ligase with Elongin B/C, Cul2 and Rbx1 targeting putatively RNAPII. Thus, NSs can evade the innate immune response by a general block of transcription of all cellular protein-coding genes including type I IFNs. This inhibition of the host transcription machinery caused by NSs is at least partially responsible for the shut off of cell protein synthesis (23, 26). Indeed, a recent study demonstrated the interaction with Med8 alone is not sufficient to impair the cellular gene expression and that other host partners requiring the N-terminal region of NSs are involved in this process (30).

4.2. La Crosse virus

LACV causes severe encephalitis and meningitis in children in the Midwestern United States including 300,000 cases per year with more than 10% developing long-lasting neurological defaults. LACV NSs protein was identified as a type I IFNs antagonist without affecting the PKR activity and the RIG-I pathway that is normally induced in infected cells (21, 31, 32). Two studies have ascribed the LCAV NSs protein as RNAi suppressor and pro-apoptotic protein since it has similarities with the Reaper protein from Drosophila involved in translation inhibition and apoptosis in insects (33-35). However, these two putative functions seem to be side effects of the NSs molecular mechanisms to inhibit IFN induction. Indeed, expression of NSs has no apparent pro-apoptotic activity in insects and no advantage conferred by NSs was detected for the RNAi inhibition in insects and mammalian cells (21). A recent study indicates that NSs acts downstream the canonical RIG-I/IRF-3 signaling pathway and its effect is based on the shutdown of RNAPII-dependent transcription. LACV NSs exploits the response of cells to damaged DNA to induce the degradation of the elongating RNAPII. Indeed, DNA damage causes the arrest of elongating RNAPII, which in turn triggers the proteolysis through the ubiquitin/proteasome pathway. In LACV-infected cells, NSs activates part of the DDR machinery such as the DDR pak6 gene and the phosphorylation of H2A.X (32). The authors suggest that such an effect can lead to a general mRNA shut off. However the precise mechanism inhibiting and degrading the elongating RNAPII through LACV NSs remains to be characterized.

5. HANTAVIRUSES

Contrary to other bunyaviruses, hantaviruses are not transmitted by arthropods. They are rodent-borne viruses, present throughout the world. They are classified into New World or Old World based on their geographic location. Pathogenic New World hantaviruses (Andes, ANDV, Sin Nombre, SNV and New York1, NY-1V viruses) cause a Hantavirus Cardiopulmonary Syndrome (HCPS) in Americas whereas pathogenic Old World hantaviruses (Hantaan, HNTV, Puumala, PUUV and Seoul, SEOV viruses) cause a Hemorrhagic Fever with Renal Syndrome (HFRS) in Europe and Asia. The New World hantaviruses Andes and Sin Nombre are responsible for mortality rate of 50% while the Old World hantavirus Hantaan can reach a mortality rate of 15% (7). Hantaviruses developed species-specific mechanisms to escape the innate immune responses. However, even non-pathogenic viruses (Tula TULV, Prospect Hill PHV viruses) antagonize some IFN pathways indicating that the IFN dysregulation alone is insufficient for hantaviruses to cause disease (36, 37). The different hantaviral strategies may involve several viral proteins including the glycoproteins, the nucleocapsid and the NSs proteins that can collectively block different steps in the type I IFNs pathways (Figure 3).

5.1. Glycoproteins Gn and Gc

Two reports have demonstrated that the ectopic expression of the glycoprotein Gn cytoplasmic tail from NY-1V was sufficient to inhibit the NF-κB and IRF-3-directed transcriptional responses. The TBK1 and RIG-I-directed steps were involved in such dysregulation and especially the components of TBK1 complexes (38, 39). TBK1 plays a crucial role in the IFN regulation since it directs the activation of both IRF-3 and NF-κB (15, 40). TRAF3 forms a complex with TBK1 and links upstream IFN signaling responses of the RIG-I/MDA-5 induced MAVS (IPS-1/Cardiff/VISA) to the TBK-1-directed phosphorylation of IRF-3 (41). TBK1 also activates NF-κB through interactions with TRAF2 (40). The NY-1V Gn cytoplasmic tail interacts with the TRAF3 N-terminus and consequently impairs cellular TBK-1-TRAF3 complex formation (39) (Figure 3). It was also suggested that such viral glycoprotein could block, through a similar mechanism, the TRAF2-directed NF-κB activation. For other pathogenic hantaviruses, both glycoproteins Gn and Gc could be necessary to overcome the IFN response. Indeed, the expression of the glycoprotein precursor (GPC) of SNV and ANDV is sufficient to inhibit the IFNβ induction, IRF-3 activation and the JAK/STAT signaling through the blockage of the phosphorylation and the nuclear translocation of STAT1/STAT2 (36, 42).

5.2. Nucleocapsid

The nucleocapsid is the most abundant viral protein in bunyavirus-infected cells. It is essential for the replicative cycle playing multiple functions. In the case of hantaviruses, it was shown to be involved in the formation of RNP through its homotrimerization and its interaction with viral RNA (43, 44). The nucleocapsid participates to the translation initiation mechanism through its interaction with Ribosomal Protein S19 (RPS19) (45). The IFN antagonism was also associated to this viral protein. Indeed, N proteins from PUUV, HTNV and TULV have been shown to interact respectively with the apoptotic protein Daxx, the Ubiquitin-conjugating enzyme 9 (Ubc9) and the Small Ubiquitin-like Modifier 1 (SUMO-1) (46-48). However the link between these interactions and the IFN escape remains to be determined. HTNV can also block the activation of NF-κB via the TNF-α through its nucleocapsid. It was demonstrated that the N protein interacts with importin α proteins, impairing the nuclear translocation of activated NF-κB that remains into the cytoplasm (49, 50). Another role linked to the nucleocapsid of ANDV is the inhibition of STAT1 phosphorylation, nuclear translocation and IFNβ-induced ISRE activity. However, the ANDV N protein alone is not sufficient but rather functions in synergy with the glycoprotein precursor (GPC) to inhibit significantly these different steps of IFN signaling (36).

5.3. NSs proteins

The S segment of some hantaviruses possesses an additional open reading frame (ORF) coding for a non-structural protein NSs and overlapping the N ORF. This is a coding strategy resembling to the one observed for the orthobunyaviruses. Such an ORF is found in the S segments of hantaviruses transmitted by Arvicolinae and Sigmodontinae rodents (voles and lemmings of the north hemisphere and New world mice and rats) whereas it is absent for hantaviruses associated with Old World mice and rats (51). Recent studies have demonstrated that the NSs of TULV could accumulate in perinuclear area and was necessary for the viral survival in IFN-competent cells but not in IFN-deficient cells (52, 53). Moreover, the NSs protein of TULV and PUUV expressed via recombinant plasmids inhibited the induction of IFNβ and the activation of IRF-3 and NF-κB responsive promoters but to a weaker extent compared to the NSs protein from BUNV or RVFV (54). The host protein synthesis is not importantly impaired in TULV and PUUV-infected cells arguing for a weak effect of the NSs protein on cellular transcription. Contrary to the other bunyaviruses, hantaviruses have developed a different strategy to avoid more gently the IFN response. Indeed the hantaviruses are the only bunyaviruses causing an asymptomatic persistent infection in their rodent hosts (55). Such viruses encode several proteins antagonizing multiple cellular targets collectively inhibiting different steps of the antiviral response.

6. NAIROVIRUSES: CRIMEA-CONGO HEMORRHAGIC FEVER VIRUS

The *Nairovirus* genus includes 34 viruses grouped into seven different serotypes. All of the members are transmitted by argasid or ixodid ticks, but only three have been implicated as causes of human disease: the Dugbe (DUGV) and Nairobi sheep disease (NSD) and Crimean Congo hemorrhagic fever (CCHF) viruses, which is the most important human pathogen amongst them. CCHF is the causative agent of severe hemorrhagic fever in human transmitted either through bites by infected ticks or by nosocomial contamination (56). The average mortality rate is 30% but can reach 70% in some outbreaks. Mice lacking IFNAR or STAT1 are very sensitive to the CCHF infection, indicating the strong antiviral effect of type I IFNs (57, 58). Moreover, pre-treatment with type I IFNs inhibits CCHF replication, involving the activity of ISGs like MxA, whereas an established CCHF infection is insensitive to subsequent treatment (59, 60). CCHF can counteract the IFN signaling through at least three independent mechanisms involving different viral elements. Firstly, the RIG-I dependent pathway is not activated since CCHFV does not produce significant amounts of the well-known inducers, which include the dsRNA and the 5’ triphosphate ssRNA (61). It was proposed that CCHF cleaves the 5’ triphosphate group during the replication to evade the recognition by this PRR. Secondly, it was demonstrated that the virus possesses at least one factor of virulence as an IFN antagonist that can delay the activation of IRF-3 (62). However, the factor is not identified yet. The existence of a coding capacity for a NSs-like gene in the S segment has been suggested but the protein remains to be determined. Thirdly, an ovarian tumor (OTU) domain has been identified on the L-RdRp of CCHF, DUGV and NSD (63). Proteins with an OTU domain belong to proteases super family deconjugating a broad spectrum of proteins modified with Ubiquitin (Ub) or ubiquitin-like proteins like IFN-Stimulated Gene

product 15 (ISG15). The expression of a mutant L-RdRp defective in OTU protease function did not impair the replication and the transcription of a CCHF minigenome system indicating that the L-OTU activity is not required for these functions of L-RdRp (64). However, a role in the innate immunity escape has been suggested. The L-OTU activity would deconjugate ubiquitin and ISG15 from different cellular targets dysregulating their activities notably related to the antiviral responses (63).

7. PHLEBOVIRUSES: RIFT VALLEY FEVER VIRUS

Rift Valley Fever Virus (RVFV) is a mosquito-borne zoonotic pathogen that has caused large outbreaks in sub-Saharan countries, Yemen, Saudi Arabia, South Africa and Madagascar. RVFV infection is lethal for newborn animals and causes febrile illness and a high rate of abortion in adult ruminants. Humans infected with RVFV usually develop an acute febrile myalgic syndrome. However, in a small proportion of patients, RVFV infection leads to hepatic damage, hemorrhagic fever-like illness, encephalitis and/or retinal vasculitis that result in a lethal illness. The S segment utilizes an ambisense strategy and codes for the nucleoprotein N and the non-structural NSs protein in opposite polarities (65). This later protein is not essential for the viral replication. In addition, while the replication cycle occurs in the cytoplasm as it is the case for all bunyaviruses, NSs is located in the nucleus of infected cells, forming a filamentous structure that is unique among bunyaviruses (66). It was also demonstrated that the virulence was linked to the absence of IFN production due to NSs, which was shown to block transcription of the IFNβ (67, 68).

7.1. The multifunctionality of the RVFV NSs protein

At least, three complementary mechanisms linked to the interaction of NSs with three different cellular partners, enable RVFV to evade the innate antiviral host responses (Figure 4). These mechanisms target the expression of IFN-ß, the activity of cellular transcription and translation.

The specific inhibition of IFNβ mRNA induction by NSs occurs as early as 3-4 h post-infection (p.i.). The molecular mechanism sustaining the transcriptional repressed state of the IFNβ promoter is correlated to the interaction between YY1 (the activator/repressor of IFNβ transcription Ying Yang 1), NSs and SAP30 (Sin3A Associated Protein 30) that is a subunit of Sin3A co-repressor complexes (such as NcoR/SMRT). It has been shown that SAP30 binds both NSs on and YY1 proteins, forming a complex that contains NcoR, HDAC1 and HDAC3, repressing the recruitment of CBP, the acetylation of histone H3 and consequently the transcriptional activation at the IFNβ promoter. To ascertain the role of this interaction, we created through a reverse genetic system, a recombinant ZH548 RVFV (ZH548-NSsΔ210-230) that expresses a mutated NSs protein unable to interact with SAP30. Such recombinant virus cannot inhibit the IFN production and is avirulent in mice (69).

Later during the viral cycle, after 8 h p.i., a second mechanism leads to the inhibition of the cellular RNA synthesis; it involves the interaction between NSs and the TFIIH factor. This complex is one of the basal transcription factors that can be resolved in two sub-complexes: the core that contains XPB, p62, p52, p44, p34 and TTD-A p8 is bridged by the XPD subunit to the CAK (Cdk- Activating Kinase) composed of cdk7, cyclin H, and MAT1 proteins. TFIIH possesses several enzymatic activities during transcription: (1) the XPB participates in promoter opening through a helicase/ATPase activity; (2) cdk7 phosphorylates RNA polymerase II (CTD-ser-5 residue) and numerous transcription factors controlling gene expression (70). The RVFV NSs protein suppresses the synthesis of host RNA by interacting and sequestrating p44 and XPB into the NSs nuclear filament. Through this interaction, NSs competes also with the usual partner of p44, XPD, unabling it to enter into the nucleus. The RVFV NSs protein also promotes the proteolysis of the TFIIH subunit p62 through the proteasome (71). Altogether, the effects of the NSs/p44 and NSs/p62 interactions inhibit the formation of TFIIH. Consequently, its concentration strongly decreases in RVFV infected cells leading to the inhibition of the cellular transcription (72).

A third function for NSs was recently described preventing the inhibition of host and viral translation (31, 73). RVFV is resistant to the PKR-mediated virus inhibition early in the course of the infection. Such a role is linked to the post-transcriptional down-regulation of PKR degraded through the proteasome and consequently the impairment of the phosphorylation of eIF2α.

In addition, it was shown that the formation of NSs filament strongly disturbs the architecture of nucleus of RVFV-infected cells and affects chromosome cohesion leading to segregation defects in murine and ovine cells. The genomic DNA is largely excluded from the NSs filament. However, specific DNA regions associated with heterochromatin can interact with NSs such as pericentromeric gamma-satellite sequences (74). Such a nuclear reorganization may be involved in the foetal deformities, necrosis of the placenta and abortions observed in infected ruminants. Although it seems likely that NSs proteins of different bunyaviruses have their specific partners (31, 69), the NSs proteins from other phleboviruses like Toscana (TOSV) and Punta Toro (PTV) viruses are also involved in the inhibition of IFNβ-induction (75, 76), strongly indicating that NSs proteins have conserved functions through unique mode of actions.

7.2. Anti-apoptotic role of RFVF NSm proteins

A recent analysis of RVFV infection in cells of the lung epithelium indicated a viral strategy that aims at controlling cellular apoptosis (77). Indeed, a recombinant RVFV lacking the expression of NSm proteins, encoded by the M segment, can

induce extensive cell death. The expression of NSm, dispensable for viral replication, suppresses apoptosis triggered through the cleavage of caspase-8 and -9 induced by staurospine (78). However, the control of apoptosis by RVFV seems complex since significant changes in PI3K-AKT, caspase, MAPK and p53 pathways, involved in survival and death of cells, were also observed (77).

8. PERSPECTIVE

Altogether the data on how bunyaviruses evade the cellular response indicate that each virus has developed its own strategy. For orthobunyaviruses and phleboviruses, NSs and to a lesser extend NSm proteins play a crucial role but in the case of hantaviruses, also other proteins such as the nucleocapsid and the glycoproteins appear to have IFN antagonist functions. For nairoviruses, less is known on the IFN antagonistic activities but the L polymerase may be the virulent factor. Such studies were important for the production of rationally designed attenuated vaccines, which have been developed so far, for RVFV. The naturally avirulent Clone 13 carrying a large deletion in the NSs ORF appears as a good vaccine candidate to protect against virulent RVFV (79). Other viruses obtained by reverse genetics were also produced with the deletion of the complete NSs ORF and /or the preglycoprotein region corresponding to NSm (33, 78, 80). Creation of mutated viruses by reverse genetics has been a valuable tool to decipher the function of the non-structural proteins (21, 23, 69) but the methodology is not yet available for hantaviruses and nairoviruses. Other investigations are needed for the development of antivirals targeting the different steps of the viral cycle, which would be of great value for the treatment of these infections. Up to now, most of the studies focused on the viral functions antagonizing the innate immune response which is an immediate and the first line of defence. However, viruses have evolved other strategies to target cellular functions, which participate to the viral pathogenesis and will deserve to be studied in the near future.

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Abbreviations: CHIKV: Chikungunya Virus, DENV: Dengue Virus, YFV: Yellow Fever Virus, CCHF: Crimea-Congo Hemorrhagic Fever Virus, RVFV: Rift Valley Fever Virus, UUKV: Uukuniemi Virus, L: Large, M: Medium, S: Small, L-RdRP: viral RNA-dependent RNA polymerase, RNPs: ribonucleoparticles, IFN: Interferon, DC: Dendritic Cell, pDC: plasmacytoid Dendritic Cell, PRR: Pattern Recognition Receptors, PAMP: Pathogen-Associated Molecular Patterns, ds: double-stranded, ss: single-stranded, PKR: Protein Kinase R, IRF-3: Interferon Regulatory Factor 3, mRNA: messenger RNA, eIF-2α: eukaryotic Initiation Factor 2 alpha, IFNAR: Type I IFN receptor, JAK: Janus Kinase, STAT: Signal Transducer and Activator of Transcription, ISGF-3: IFN stimulated gene factor 3, ISRE: IFN-stimulated response elements, ISGs: IFN-stimulated genes, ADAR1: adenosine deaminase 1, BUNV: Bunyamwera Virus, LACV: La Crosse Virus, RNAPII: RNA polymerase II, ANDV: Andes Virus, SNV: Sin Nombre Virus, NY-1V: New York Virus 1, HNTV: Hantaan Virus, PUUV: Puumala Virus, SEOV: Seoul Virus, HCPS: Hantavirus Cardiopulmonary Syndrome, HFRS: Hemorrhagic Fever with Renal Syndrome, GPC: Glycoprotein Precursor, RPS19: Ribosomal Protein S19, Ubc9: Ubiquitin-conjugating enzyme 9, SUMO-1: the Small Ubiquitin-like Modifier 1, ORF: Open Reading Frame, OTU: ovarian tumor, ISG15: IFN-stimulated gene product 15, Ub: Ubiquitin, TULV: Tula Virus, PHV: Prospect Hill Virus, DUGV: Dugbe Virus, NSD: Nairobi Sheep Disease, SAP30: Sin3A associated protein, CAK: Cdk-Activating Kinase, TOSV: Toscana Virus, PTV: Punta Toro Virus, YY1: Ying Yang 1.

Key Words bunyaviruses, innate immunity, escape, virulence factors

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Table 1. Human pathogens in the different genera of the *Bunyaviridae* family

Genus	Virus	Vector	Human Disease
Orthobunyavirus			
	La Crosse	Mosquito	Severe encephalitis, meningitis
	Ngari	Mosquito	Haemorrhagic Fever
	Oropouche	Midge	Acute febrile illness
	Tahyna	Mosquito	Non fatal flu-like illness
Hantavirus			
	Andes	Mouse	Hantavirus cardiopulmonary syndrome Fatality 40-50%
	Hantaan	Field Mouse	Severe haemorrhagic fever with renal syndrome Fatality 5-15%
	Puumala	Bank Vole	Mild haemorrhagic fever with renal syndrome Fatality 0.1%
	Seoul	Rat	Moderate haemorrhagic fever with renal syndrome Fatality 1%
	Sin Nombre	Deer Mouse	Hantavirus cardiopulmonary syndrome Fatality 50%
Nairovirus			
	Crimean Congo hemorrhagic Fever	Tick	Haemorrhagic Fever Fatality 20-80%
Phlebovirus			
	Naples sandfly Fever	Sandfly	"three day fever" with Influenza-like symptoms
	Punta Toro	Sandfly	Acute febrile illness
	Rift Valley Fever	Mosquito	Hepatitis, encephalitis, haemorrhagic fever Fatality 1-10%
	Toscana	Sandfly	Meningitis, meningoencephalitis, encephalitis
	Sicilian sandfly Fever	Sandfly	"three day fever" with Influenza-like symptoms
	Huaiyangshan	Tick	severe thrombocytosis and multiorgan dysfunction Fatality 30%

Figure 1. Induction and signaling pathways of the innate immunity. The viral replication of bunyaviruses can produce 5' triphosphate ssRNA and dsRNA that activate the cytoplasmic PAMPs RIG-I, MDA-5 and PKR. The RIG-I/MDA-5 dependent pathways induce the mitochondrial MAVS (IPS-1/Cardiff/VISA) that is necessary for the subsequent TBK-1-directed phosphorylation of IRF-3 and NF-κB that translocate into the nucleus and transactivate collectively the IFNβ and IFNα1 promoters. In parallel, activated PKR can phosphorylate eIF2α and inhibit the host and viral translation. The secreted IFNβ and IFNα1 acting in autocrine and paracrine manner bind and activate the IFNARs allowing the expression of numerous ISGs and IRF-7 through the JAK/STAT pathways. In these cells, bunyaviruses can be blocked through the products of these ISGs. Moreover, 5' triphosphate ssRNA and dsRNA produced during the viral replication activate the cytoplasmic RIG-I, MDA-5 and consequently IRF-7/IRF-3 leading to a positive-feedback loop that initiates the synthesis of IFNβ and several IFNα subtypes.

Figure 2. Mechanisms of immune escape targeting the cellular transcriptional machinery for BUNV, LACV and RVFV. NSs proteins from BUNV, LACV and RVFV block the type I IFN response and shut off the mRNA expression by targeting the cellular transcriptional machinery through different strategies. RVFV NSs protein inhibit the formation of the basal transcription TFIIH complex that is crucial for the initiation step by phosphorylating notably the CTD repeat serine 5 residue of RNAPII. BUNV and LACV NSs proteins induce the proteolysis of RNAPII engaged in elongation (characterized by the CTD repeat serine

2 phosphorylation) through their interactions respectively with the Mediator subunit Med8 and the DNA Damage Repair pathway.

Figure 3. Hantaviral strategies played by Gn glycoprotein, nucleocapsid and NSs protein to evade the type I IFN response. The NY-1V Gn cytoplasmic tail can inhibit the activation of the transcription factors IRF-3 and putatively NF-κB by blocking their phosphorylation through TBK-1 that necessitates the formation of complexes with respectively TRAF3 and TRAF2. The viral glycoprotein interacts with the TRAF3 N-terminus and consequently impairs cellular TBK-1-TRAF3 complex formation. The HTNV nucleocapsid block the NF-κB-directed transcriptional response by interacting with importin α proteins impairing the nuclear translocation of activated NF-κB that remains into the cytoplasm. Finally, the NSs protein of TULV and PUUV localized in perinuclear area inhibits the induction of IFNβ and the activation of IRF-3 and NF-κB responsive promoters.

Figure 4. Multifunctional RVFV NSs protein overcomes the host response. The RVFV NSs protein has multiple activities targeting several cellular partners and inhibiting different steps of the type I IFN response. Through an interaction with SAP30, NSs can maintain via YY1 a co-repressor complex containing NcoR and HDACs at the IFNβ promoter although IRF-3 and NF-κB are recruited. Such repressive environment specifically blocks the formation of transcriptional machinery and consequently the induction of IFNβ mRNA. Later in the viral replicative cycle, NSs can also inhibit the global cellular mRNA synthesis through its interaction with TFIIH subunit that disrupts the entire complex (see also Figure 2). The RVFV NSs protein can also induce the proteolysis of PKR blocking the inhibition of viral and host translation linked to the PKR-dependent phosphorylation of eIF2α.

Running title: Antiviral escape by bunyaviruses pathogenic for humans

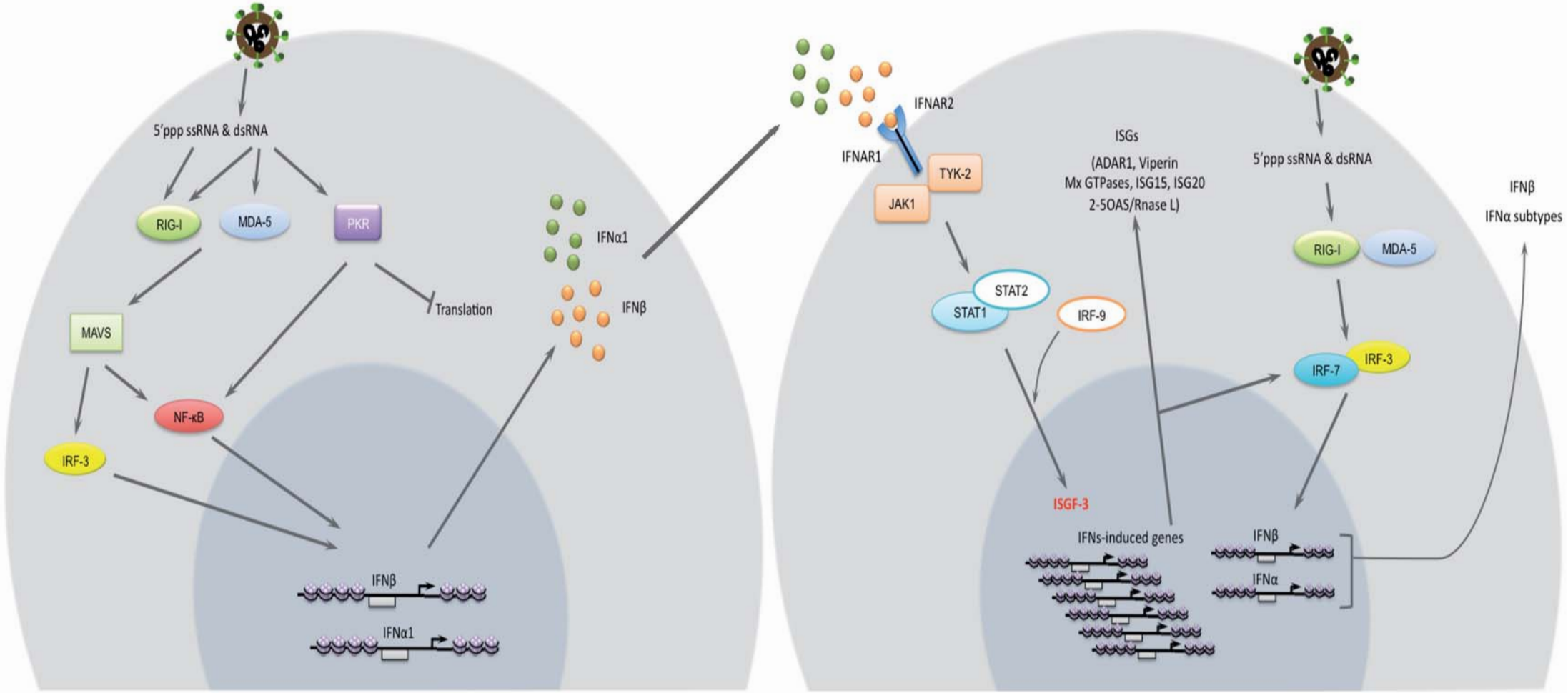
5'ppp ssRNA & dsRNA
RIG-I
MDA-5
PKR
MAVS
NF-κB
IRF-3
Translation
IFNα1
IFNβ
IFNβ
IFNα1
IFNAR2
IFNAR1
TYK-2
JAK1
STAT2
STAT1
IRF-9
ISGF-3
ISGs
(ADAR1, Viperin
Mx GTPases, ISG15, ISG20
2-5OAS/Rnase L)
IFNs-induced genes
5'ppp ssRNA & dsRNA
RIG-I
MDA-5
IRF-7
IRF-3
IFNβ
IFNα
IFNβ
IFNα subtypes

Figure 1

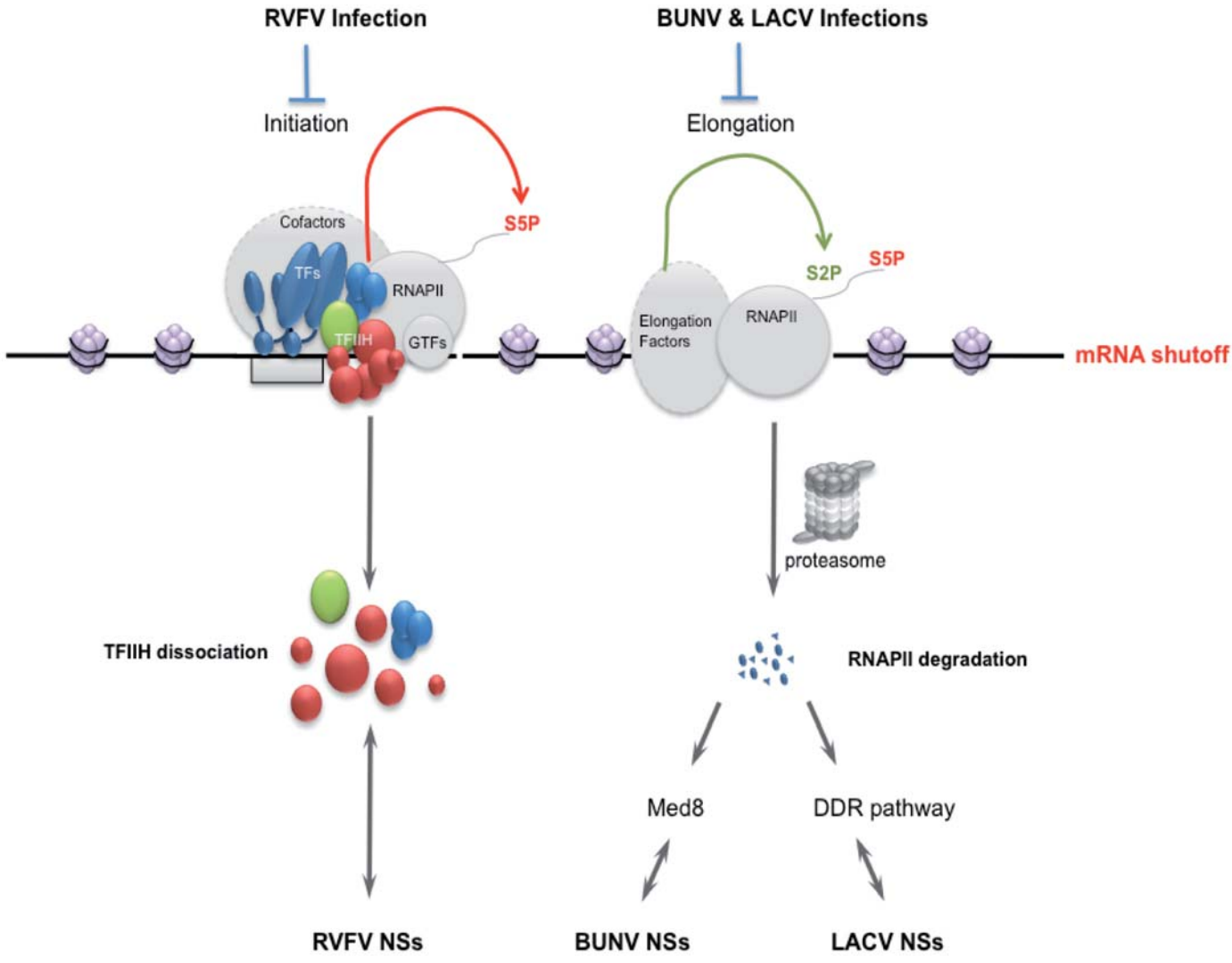
RVFV Infection
Initiation
Cofactors
TFs
TFIIH
RNAPII
GTFs
S5P
BUNV & LACV Infections
Elongation
Elongation Factors
RNAPII
S2P
S5P
mRNA shutoff
proteasome
TFIIH dissociation
RNAPII degradation
Med8
DDR pathway
RVFV NSs
BUNV NSs
LACV NSs

Figure 2

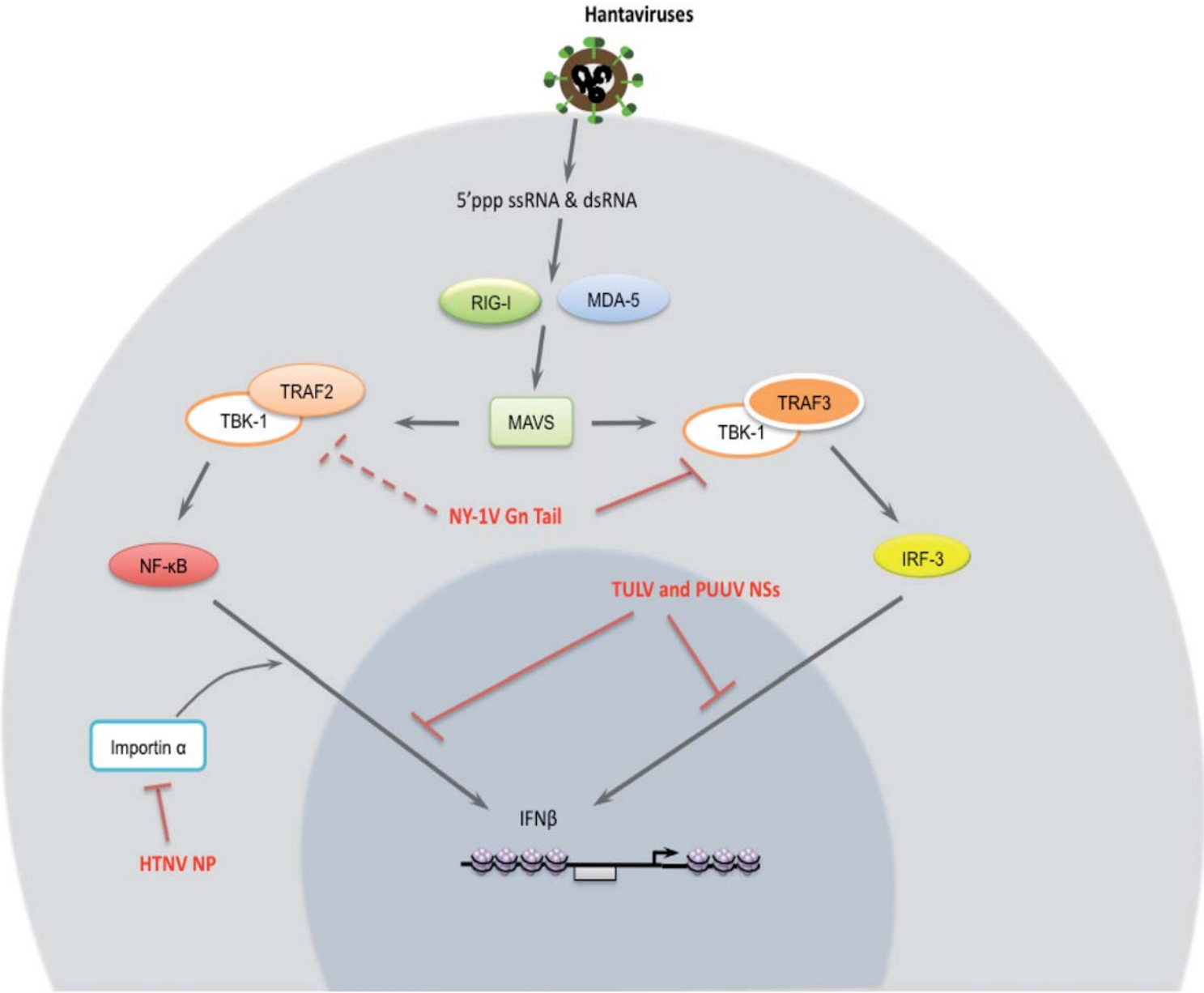
Hantaviruses
5'ppp ssRNA & dsRNA
RIG-I
MDA-5
MAVS
TRAF2
TBK-1
TRAF3
TBK-1
NY-1V Gn Tail
NF-κB
IRF-3
TULV and PUUV NSs
Importin α
HTNV NP
IFNβ

Figure 3

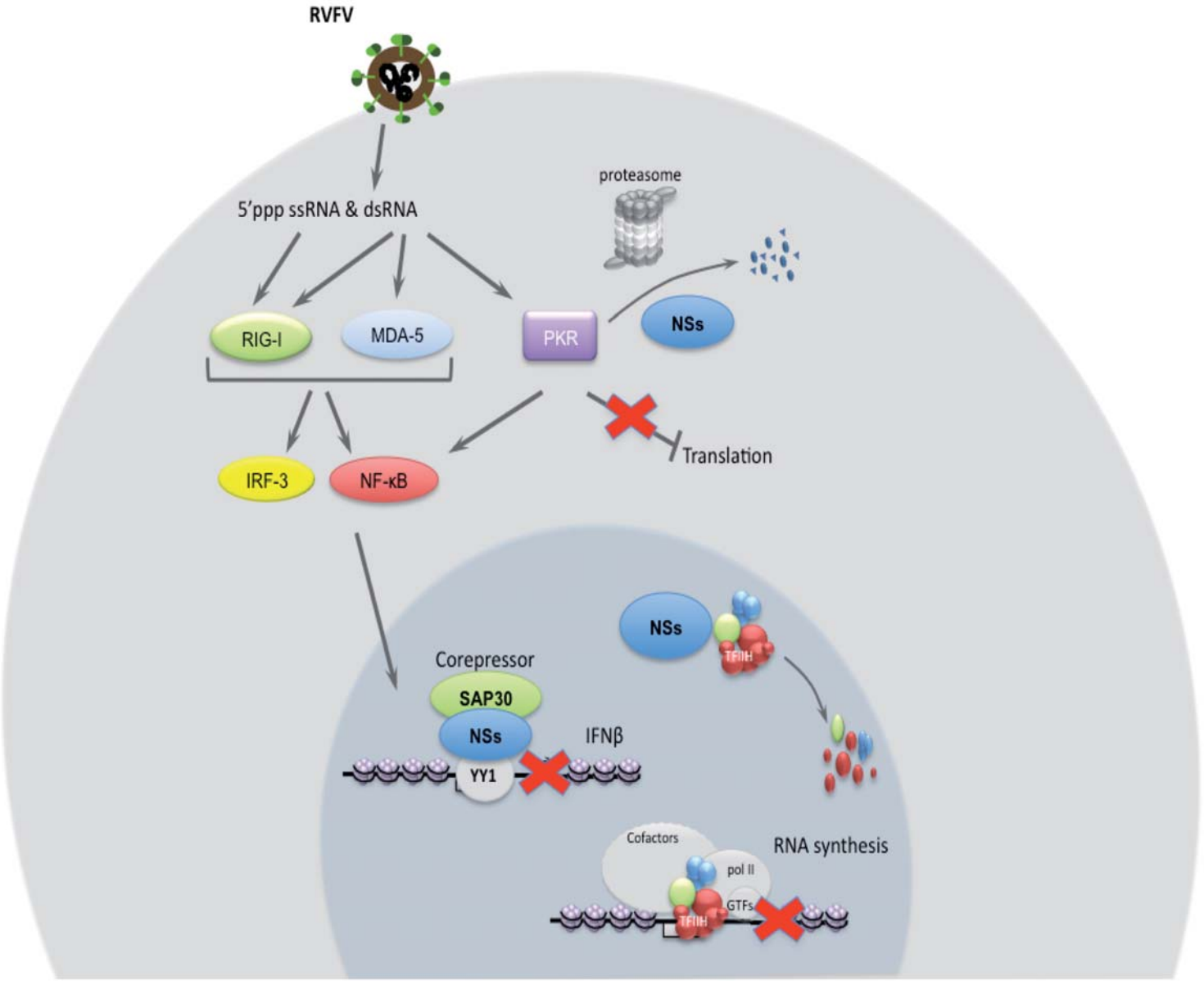
RVFV
5'ppp ssRNA & dsRNA
proteasome
RIG-I
MDA-5
PKR
NSs
Translation
IRF-3
NF-κB
NSs
TFIIH
Corepressor
SAP30
NSs
IFNβ
YY1
Cofactors
RNA synthesis
pol II
GTFs
TFIIH

Figure 4

4.3 H5N1 NS1 protein: an enemy hidden into the chromatin

Anastasina M.*, **Le May N**. *****, Ohman T., Tynell J., Nyman T.A., Julkunen I., Butcher S.J., Egly J.M. and Kainov D.E. « Non-structural protein NS1 of influenza A virus binds cellular double-stranded DNA and inhibits transcription of antiviral genes ». In revision

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Non-structural protein NS1 of influenza A virus binds cellular double-stranded DNA and inhibits transcription of antiviral genes

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ABSTRACT

Influenza A virus non-structural protein NS1 antagonizes antiviral responses in infected cells. In particular, NS1 interacts with viral RNA species during virus replication and sequesters them away from recognition by cellular pattern recognition receptors, which trigger cascades of antiviral responses. Two residues R38 and K41 of NS1 are essential for this process. Here we showed that R38 and K41 also mediate the interaction of NS1 with cellular double-stranded (ds) DNA to inhibit transcription of antiviral genes. In particular, we demonstrated that interaction between NS1 and dsDNA is sufficient to prevent transcription initiation step in reconstituted *in vitro* transcription assays. In infected cells NS1 via R38 and K41 associates with chromatin and impedes RNA polymerase II recruitment on immune-related genes. In addition NS1 alters the composition of chromatin. Thus, we identified a previously undescribed strategy by which influenza A virus escapes the innate immune response and secures its replication.

INTRODUCTION

Influenza A viruses are important human pathogens that cause global epidemics and pandemics. It is estimated that influenza A viruses are responsible for up to 500,000 deaths a year (1). The successful recovery from viral infection largely depends on development of innate immune responses which are triggered by cellular pattern recognition receptors (PRRs) in infected cells. PRRs, such as RIG-I, TLR3, NLRP3 and MDA5, activate expression of antiviral genes which limit viral replication and promote the development of adaptive immune responses (2,3).

Upon infection, influenza A viruses express the non-structural protein NS1 which inhibits antiviral response and thereby secures virus replication (4-6). In particular, NS1 inhibits PRRs

activation and IRF3-, NFκB- and ATF2/cJun-driven transcription of antiviral genes by interacting with host and viral RNAs (7-9). NS1 also alters host RNA processing by binding to CPSF30 and PABPII (10,11). In addition, it inhibits host mRNA export from the nucleus to the cytoplasm by targeting the mRNA export machinery (12). In the cytoplasm, NS1 targets the translation machinery and other cellular factors to secure viral replication (13).

NS1 has an RNA-binding domain (RBD), an effector domain (ED) and a flexible C-terminal region that are required for its interactions with host and viral RNA and proteins (14). It has been shown that the RBD of NS1 of A/Udorn/72(H3N2) and A/WSN/33(H1N1) strains bind viral minus-sense RNA and U6 snRNA, as well as random double-stranded (ds) and polyadenylated RNA (15-20). Substitutions of R38A or/and K41A abrogated RNA binding by the H1N1 and H3N2 NS1 (21,22). Moreover, viruses encoding NS1 R38A or/and K41A are attenuated in immune-competent systems (21,23,24). Interestingly, NS1 is also able to oligomerize and form tubular filaments with a channel that was proposed to accommodate dsRNA, hindering it from recognition by cellular PRRs (25). However, the central channel of the NS1 filament has a diameter of 20Å that could accommodate dsDNA, but not dsRNA.

Here we hypothesised that NS1 can inhibit transcription of antiviral genes by binding to cellular dsDNA. We found that the conserved R38 and K41 residues mediate NS1 interaction with dsDNA. This interaction antagonized RNA polymerase II (pol II) recruitment and consequently inhibited the transcriptional activation of immune-related genes upon infection. Moreover, our results demonstrated that the NS1 interaction with dsDNA via R38 and K41 is accompanied by an alteration of the chromatin composition in infected cells. Using reconstituted *in vitro* transcription assays on naked DNA, we showed that the dsDNA-binding of NS1 was sufficient to explain the inhibition of pol II transcription. Thus, we present here a previously undescribed mechanism by which influenza A virus can manipulate basic cellular process to escape the antiviral response.

MATERIAL AND METHODS

Proteins, viruses, plasmids, and cells

Wild type ($NS1^{WT}$) and dsDNA/RNA-binding deficient mutant ($NS1^{RK/AA}$) of NS1 of avian highly pathogenic influenza A/chicken/Nigeria/OG10/2007(H5N1) virus were produced in E. coli and purified to homogeneity as described previously (26,27).

Influenza A/WSN/33(H1N1) viruses expressing wild type (WSN^{WT}) or R38A, K41A mutant NS1 ($WSN^{RK/AA}$) were generated using WSN eight-plasmid-based reverse genetics system in HEK and Vero cells as described previously (28,29). Viruses were titrated in MDCK cells using plaque assay as described previously (30).

Wild type and mutant NS1 (R38A, K41A) genes of influenza A/WSN/33(H1N1) virus were amplified by PCR using 5'GGCCGAGCTCGCCACCATGGATCCAAACACTGTGT and 5'-GGCCCTCGAGAACTTCTGACCTAATTGTCCCGCCAT primers from plasmids used for generation of

WSN^{WT} and WSN$^{RK/AA}$ viruses. The PCR fragments were cloned into pCMV-Tag4A. The resulted pNS1^{WT} and pNS1$^{RK/AA}$ plasmids encoded C-terminally Flag-tagged wild type and mutant NS1 proteins.

Madin-Darby canine kidney epithelial (MDCK), Human embryonic epithelial cells (HEK293T) and African green monkey kidney epithelial cells (Vero) were grown in Dulbecco modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2mM L-glutamine (Lonza; Basel, Switzerland), 50 U/ml penicillin-streptomycin mix (PenStrep, Lonza) and 10% fetal bovine serum (FBS; Gibco, Paisley, UK). Human retinal pigment epithelial cell line (RPE) was grown in DMEM-F12 medium supplemented with 50 U/ml PenStrep, 2mM L-glutamine, 10% FBS, and 0,25% sodium bicarbonate (Sigma-Aldrich). The cells and viruses were propagated at 37 °C in 5% CO_2.

Transfection of HEK293T cells with pNS1^{WT} and pNS1$^{RK/AA}$ plasmids

HEK293T cells were transiently transfected with pNS1^{WT}, pNS1$^{RK/AA}$ or pCMV-Tag4A vector using X-tremeGENE-9 DNA Transfection Reagent (Roche) according to the manufacturer's instructions. Twenty four hours post-transfection, immune responses in transfected HEK cells were induced by addition to culture medium of 0.025mg/ml of poly(I:C) sodium salt (Sigma-Aldrich).

Fractionation of cells

Cytoplasmic, soluble nuclear and chromatin fractions of HEK293T cells transfected with pNS1^{WT} and pNS1$^{RK/AA}$ plasmids or empty vector were prepared using the Nuclear extract Kit from Active Motif.

Infection of RPE cells with WSN^{WT} and WSN$^{RK/AA}$ viruses

The growth medium of RPE cells was changed to the virus growth medium (VGM) containing 0.2% BSA (Sigma-Aldrich), 2mm l-glutamine, 0.348% $NaHCO_3$ and 1 µg/ml l-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (TPCK)-trypsin (Sigma-Aldrich) in DMEM-F12. The RPE cells were then infected with WSN^{WT}, WSN$^{RK/AA}$ viruses at moi 1 or mock.

Electrophoretic mobility shift assay (EMSA)

The synthetic 199bp-long dsDNA was produced by PCR using two oligonucleotides (forward 5'-ATGGATCCAAACACTGTGTCA, reverse 5'-CTCCACTATTTGCTTTCCA) and pHW188-NS plasmid as a template (29). 100 ng of dsDNA was incubated with 2-fold dilutions of NS1^{WT} or NS1$^{RK/AA}$ purified recombinant proteins (starting from 40 µM) for 15 min on ice. 10x loading buffer (20 mM Tris-HCl pH 8.6, 50 mM NaCl, 10% glycerol) was added to the samples and the samples were resolved in 1 % agarose gel containing ethidium bromide in a TAE buffer (40 mM Tris-base, 20 mM acetic acid, 1 mM EDTA).

Microscale thermophoresis assay

The fluorescently labelled synthetic 199bp-long dsDNA was produced by PCR using two oligonucleotides (forward 5'- ATGGATCCAAACACTGTGTCA, reverse 5'-

CTCCACTATTTGCTTTCCA) containing a cyanine 5 fluorophore covalently linked to the 5' ends and pHW188-NS plasmid as a template. 10 pM of Cy5-labeled synthetic dsDNA was incubated with indicated concentration of purified $NS1^{WT}$ or $NS1^{RK/AA}$ recombinant proteins for 5 minutes on ice. Differences in thermophoretic properties of free and NS1-bound dsDNA were determined using Monolith NT.115 instrument (NanoTemper Technologies, Munich, Germany).

In vitro transcription assay

Run-off transcription assays were performed using recombinant $NS1^{WT}$ or $NS1^{RK/AA}$, TFIIB, TFIIE, TFIIF, TBP, RNA polymerase II and TFIIH as described previously (31).

Immuno-fluorescence analysis

HEK293T cells transfected with $pNS1^{WT}$ and $pNS1^{RK/AA}$ plasmids or empty vector were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), then permeabilized and blocked in the BP buffer (10% Bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS) supplied with 5% goat serum (Life Technologies, USA). Primary rabbit anti-NS1 antibodies (32) were added followed by secondary goat anti-rabbit antibodies with an Alexa488 fluorophore (Life Technologies, USA) in BP buffer, nuclei were counterstained with DAPI, and the slides were mounted with Prolong Gold antifade reagent (Life Technologies, USA). Images were captured with Nikon 90i microscope and processed with NIS elements AR software.

Protein electrophoresis and immunoblotting

Proteins were mixed with a 2x Laemmli loading buffer (4% sodium dodecyl sulphate, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH 6.8) and resolved in 4-20% gradient SDS-polyacrylamide gel (Biorad, Hercules, USA) at 150 V for 50 minutes. The gels were stained using Coomassie blue or immunoblotted.

Immunoblotting was carried out as described in (33). NS1 was detected with a rabbit anti-NS1 antibody and with rabbit anti-Flag antibody (Sigma-Aldrich). Histones H1 and H3 were detected with primary rabbit anti-histone H1 antibody (Sigma-Aldrich) and primary rabbit anti-histone H3 antibody (Cell Signalling), respectively.

Chromatin immunoprecipitation

ChIP experiments were performed on HEK293T cells transiently expressing $NS1^{WT}$, $NS1^{RK/AA}$ or none, as well as RPE cells infected with WSN^{WT}, $WSN^{RK/AA}$ viruses or mock as previously described (34). Briefly, after cross-linking with formaldehyde at room temperature, chromatin was prepared, sonicated on ice using Bioruptor (Diagenode, Philadelphia, PA, USA) and pre-cleared. Samples were incubated with the antibody and then with protein G Sepharose beads. After extensive washes the protein-DNA complexes were eluted, the cross-linking was heat-reverted. DNA was purified with QIAquick PCR purification kit (Qiagen) and quantified by PCR with specific primers.

Cytokine profiling

For cytokine profiling the media from WSN^{WT}-, $WSN^{RK/AA}$- or mock-infected RPE cells was collected at 24 h post infection and clarified by centrifugation for 5 min at 14000 rpm. 26 cytokines were analyzed using Proteome Profiler Human Cytokine Array panel A kit (R&D Systems) according to manufacturer's recommendations. The results were analyzed using DotBlot analyzer macro for ImageJ software.

Enzyme-linked immunosorbent assay (ELISA)

The levels of CXCL10, TNFα, IFNβ and IFNλ in the cell supernatants were assayed with ELISA (PBL Interferon Source) as described previously (35).

Gene expression profiling

RNA was extracted from WSN^{WT} -, $WSN^{RK/AA}$ - or mock-infected RPE cells at 8 h.p.i. using RNeasy Plus mini kit (Qiagen). Gene expression profiling was done using Illumina Human HT-12 v4 Expression BeadChip Kit according to manufacturer's recommendation as described in (28,33). Raw microarray data were normalized using the BeadArray and Limma packages from Bioconductor suite for R. Normalized data were further processed using a variance and intensity filter. Genes differentially expressed between samples and controls were determined using the Limma package. Benjamini-Hocberg multiple testing correction testing method was used to filter out differentially expressed genes based on a q-value threshold ($q<0.05$). Filtered data were sorted by logarithmic fold change ($\log_2 Fc$).

Quantitative PCR

Quantitative PCR was done on the Lightcycler 480 (Roche) using Fast SYBR® Green Master Mix (Roche). The following sets of primers were used for detection of specific genes or cDNA: EML4 promoter (forward: 5'-CCTCATATTAGGTGTATATCAAATCAGC, reverse: 5'-CCAGTTAATAACATCCCATTTCTCATC), EML4 exon (forward: 5'-TGGCTTCAGTGCAACTCTT, reverse: 5'-AATCTCCATCACTGCCCATC), IFNB1 (forward: 5'-GCCGCATTGACCACTATGA, reverse: 5'-GCCAGGAGGTTCTCAACAATAG), IFNA1 (forward: 5'-ATGGCAACCAGTTCCAGAAG, reverse: 5'-CATCCCAAGCAGCAGATGAA), IFNA16 (forward: 5'-GACTCACTTCTATAACCACCACAA, reverse: 5'-TAGTGCCTGCACAGGTAAAC), IL6 (forward: 5'-TCATCACTGGTCTTTTGG, reverse: 5'-CTCTGGCTTGTTCCTCAC), CXCL1 (forward: 5'- TGAGCATCGCTTAGGAGA, reverse: 5'-AGGACAGTGTGCAGGTAG), IL29 (forward: 5'- AGGCTGAGCTGGCCCTGA, reverse: 5'-GGTGTGAAGGGGCTGGTC).

Mass spectrometry

Chromatin fractions were extracted from WSN^{WT}-, $WSN^{RK/AA}$ or mock -infected RPE cells at 8h post infection as described (36). Protein identification and quantification was done using four-plex iTRAQ (isobaric tag for relative and absolute quantitation) labelling combined with liquid chromatography-

tandem mass spectrometry (LC-MS/MS) analysis as described in (37). In brief, protein alkylation, trypsin digestion and labelling of the resulting peptides were done according to manufacturer's instructions (AB Sciex). Labelled peptides were fractionated by strong cation exchange chromatography and each fraction containing labelled peptides was analysed twice with nano-LC-ESI-MS/MS using Ultimate 3000 nano-LC (Dionex) and QSTAR Elite hybrid quadrupole time-of-flight-MS (AB Sciex). MS data were acquired automatically using Analyst QS 2.0 software. Protein identification and relative quantitation was performed using ProteinPilot 4.0 software (AB Sciex). Data files from both technical replicates of an iTRAQ sample set were processed together. The search database was a self-built combination of Uniprot Human protein sequences and Uniprot ssRNA negative-strand virus sequences (both form the release 55.0, 02/08). The search criteria were: cysteine alkylation with MMTS, trypsin digestion, biological modifications allowed, thorough search and detected protein threshold of 95% confidence (Unused ProtScore >1,3). Additionally, automatic bias correction was used. False discovery rates were calculated using a concatenated normal and reversed sequence database.

RESULTS

Purified recombinant NS1 binds synthetic dsDNA in vitro

It has been shown that NS1 binds the phosphate backbone of dsRNA via its conserved R38 and K41 residues *in vitro*, and therefore NS1-dsRNA interaction was proposed to be sequence-unspecific (15,25,27,38). We studied whether NS1 is able to interact also with random dsDNA *in vitro* by producing linear synthetic dsDNA and highly purified recombinant wild-type NS1 protein ($NS1^{WT}$) and its R38A and K41A double mutant ($NS1^{RK/AA}$) (Fig. 1A). Electrophoretic mobility shift assay (EMSA) showed that only $NS1^{WT}$ and not $NS1^{RK/AA}$ retarded migration of dsDNA in a concentration-dependent manner (Fig. 1B). We then used microscale thermophoresis to determine the dissociation constants (K_d) of dsDNA interaction with $NS1^{WT}$ or $NS1^{RK/AA}$. The K_d for $NS1^{WT}$ was 11.1±0.7μM and the K_d for $NS1^{RK/AA}$ was >100μM (Fig. 1C). Interestingly, the K_d for $NS1^{WT}$-dsDNA complex was comparable to that of $NS1^{WT}$-dsRNA complex which is also in the micromolar range (22,27,39,40). These results suggested that NS1 could bind dsDNA non-specifically with micromolar affinity, and that its residues R38 and K41 are essential for the binding.

NS1 alters histone content of chromatin in human cells

To study possible NS1-dsDNA interaction in human cells, $NS1^{WT}$ and $NS1^{RK/AA}$ were transiently and similarly expressed in HEK293T cells (Fig. 2A). Although immunofluorescence experiments indicated that both $NS1^{WT}$ and $NS1^{RK/AA}$ were efficiently expressed, $NS1^{RK/AA}$ concentration was significantly lower in the nucleus of transfected cells (Fig. 2B).

We next prepared cytoplasmic, soluble nuclear and chromatin fractions from the transfected HEK293T cells (Fig. 2C). Although both NS1 proteins were equally present in the cytoplasm, we found that $NS1^{WT}$ was abundant in the chromatin fraction, in contrast to its mutant variant. Moreover,

this difference was also observed in the histone fraction where NS1^{WT}, but not NS1$^{RK/AA}$, was found (Fig. 2D).

We next addressed whether NS1 association with chromatin occurs in an infection context. We infected human telomerase reverse transcriptase (hTERT)-immortalized retinal pigment epithelial (RPE) cells with influenza A/WSN/33 viruses expressing NS1^{WT} (WSN^{WT}) or NS1$^{RK/AA}$ (WSN$^{RK/AA}$) and compared them to the non-infected control. Chromatin fractions were prepared and their compositions were analysed using quantitative mass spectrometry (iTRAQ LC-MS/MS). At 8h post-infection, NS1 was detected in the chromatin fractions of the cells infected with WSN^{WT} but not with WSN$^{RK/AA}$ virus (Fig. 2E). Interestingly, the composition of the chromatin fractions isolated from WSN^{WT}- and WSN$^{RK/AA}$-infected cells were different in comparison to the mock infection: in both WSN^{WT}- and WSN$^{RK/AA}$-infected cells concentrations of histones H1.2, H1.5, H2A.1D were significantly increased, but the abundance of histone H3.2 was significantly decreased only in WSN^{WT}-infection (Fig. 2F). Importantly, the increase in H1.2, H1.5 and H2A.1D histone concentrations was more pronounced in WSN$^{RK/AA}$ than in WSN^{WT} infection. Altogether these data suggested that the chromatin location of NS1 through its residues R38 and K41 is correlated with an alteration of the histone composition.

NS1 inhibits transcription of immune-related genes in human cells

We next investigated the consequences of NS1 chromatin location and the altered histone composition on the inhibition of the antiviral response upon influenza A virus infection. We firstly determined the gene expression profiles of RPE cells infected with either WSN^{WT} or WSN$^{RK/AA}$ viruses compared to a mock infection and identified genes transcriptionally up-regulated over 8 fold (Fig. 3A, upper panel; GEO accessory number: GSE65699). At 8h post infection WSN$^{RK/AA}$ virus induced expression of 88 genes, whereas infection with WSN^{WT} virus activated expression of 33 genes (Fig. 3A, lower panel). Interestingly, 31 genes were highly expressed in both WSN$^{RK/AA}$- and WSN^{WT}-infected cells. However, these genes showed a significantly higher expression in WSN$^{RK/AA}$-infected cells. These results indicate that influenza A virus infection induces expression of dozens of innate-immune genes in human cells, that NS1 upon expression is able to suppress transcription of many of these genes, and DNA/RNA-binding residues R38 and K41 are important for NS1 function.

We next performed RT-qPCR targeting transcripts of selected immune-related genes identified by the microarrays and a control gene *EML4* (Fig. 3B). In agreement with our transcriptomics results, the virus encoding NS1$^{RK/AA}$ was unable to suppress transcription of antiviral *NFKB1*, *OAS1*, *CCL5*, *IL6*, and *IFNA16* genes in comparison with virus expressing NS1^{WT}. Meanwhile, WSN$^{RK/AA}$ infection had no effect on the expression of a control gene *EML4*.

We next tested whether infection with WSN$^{RK/AA}$ may increase production of cytokines in RPE cells in comparison with WSN^{WT}-infected cells. In agreement with our transcriptomics results, cytokine profiling and ELISA experiments revealed that WSN$^{RK/AA}$ was in general a stronger inducer of cytokine production than WSN^{WT} (Fig. S1). However, some cytokines, such as IFN□, did not follow the transcriptional differences observed in WSN^{WT}- and WSN$^{RK/AA}$-infected cells. This difference could

illustrate the multi-level mechanisms of inhibition of antiviral responses related to the different features of NS1 and other viral proteins.

The observed effects of NS1 on antiviral gene expression and cytokine production could originate from the direct regulation of gene expression by NS1 binding to dsDNA. Alternatively the regulation could occur indirectly by the NS1 binding to dsRNA and inhibition of PRRs signalling. To discriminate between these possibilities and directly study the transcriptional control of antiviral genes by NS1 alone, we analysed the transcriptional activation of the immune response in poly(I:C)-stimulated HEK293T cells transiently expressing either $NS1^{WT}$ or $NS1^{RK/AA}$. Poly(I:C) is a dsRNA mimic which is recognized by TLR- and RIG-I-like receptors followed by the expression of innate immune-related genes (41,42). Total RNAs harvested at 0, 3 and 6h post stimulation were analysed by RT-qPCRs. In particular, expression of interferon-apha and -beta genes was analysed, because it is activated in response to poly(I:C) stimulation (43,44). Expression of *IFNB1, IFNA1* and *IFNA16* genes was suppressed in poly(I:C)-stimulated cells transfected with $pNS1^{WT}$, but not with $pNS1^{RK/AA}$ or empty vector (Fig. 3C). These results suggested that $NS1^{WT}$, but not $NS1^{RK/AA}$, directly inhibited transcription of immune-related genes in poly(I:C)-stimulated HEK293T cells similarly to the viral infection, and that R38 and K41 of NS1 are critical for this process.

NS1 impedes pol II recruitment at immune-related genes in influenza A virus infected human cells

Our results indicated that the dsDNA/RNA-binding residues of NS1 have a direct effect on cellular gene expression. Our next goal was to understand how NS1 controls the transcription of immune-related genes through dsDNA binding. We first investigated whether NS1 could target specific gene regions using chromatin immunoprecipitation (ChIP). Promoters and exons of immune-related *IFNB1* and housekeeping genes were analysed using chromatin extracts from RPE cells infected for 8h with either WSN^{WT} or $WSN^{RK/AA}$ viruses. Interestingly, we found NS1 on promoters and exons for these genes from WSN^{WT} infected cells whereas the detection remained significantly weaker and comparable to the mock infection for the $WSN^{RK/AA}$ infected RPE cells (Fig. 4, left panels). These results indicated the preferential association of $NS1^{WT}$ versus $NS1^{RK/AA}$ with chromatin and suggested an unspecific distribution of functional NS1 on actively transcribed human genes.

We next compared NS1 ChIP data with the ChIP data for pol II (Fig. 4A-C, right panels). As expected, the recruitment of pol II paralleled the previously determined mRNA induction of *IFNB1* (see Fig 3). Indeed, pol II was detected at promoter and exon of this gene only in the $WSN^{RK/AA}$ infected RPE cells. Therefore, the presence of $NS1^{WT}$ on DNA of *IFNB1* would antagonize pol II recruitment and transcriptional activation. However, NS1 could be also detected along the *EML4* locus without any significant differences in the presence of pol II and gene expression (Fig. 4C and 3B). These results suggest that NS1 does not allow pol II to associate with immune-related genes during virus infection and that the residues R38 and K41 of NS1 are critical for this process.

NS1 binding to DNA is sufficient to inhibit in vitro transcription reaction

To address whether direct binding of NS1 to dsDNA could be sufficient for transcription inhibition, we performed *in vitro* run-off transcription assays containing naked AdMLP DNA template and purified recombinant $NS1^{WT}$ or $NS1^{RK/AA}$. We mixed simultaneously recombinant $NS1^{WT}$ or $NS1^{RK/AA}$ proteins, transcription factors, pol II and the DNA template, and found that $NS1^{WT}$ but not $NS1^{RK/AA}$ inhibited RNA synthesis in a concentration-dependent manner (Fig. 5A). This result suggested that NS1 binding to dsDNA via its R38 and K41 inhibited pol II-dependent transcription.

To further understand how NS1 was able to block *in vitro* transcription, we set up three different experimental conditions. We first pre-incubated NS1 with dsDNA, and then added transcription pre-initiation complex (PIC) and pol II before the reaction started (Fig. 5B, incubation 1). In another setting, we pre-incubated NS1 with PIC and pol II, and then added dsDNA (Fig. 5B, incubation 2). In both experimental settings, $NS1^{WT}$ but not $NS1^{RK/AA}$ inhibited *in vitro* RNA synthesis. Finally, in a third series of experiments, the transcriptional machinery was pre-formed and then the recombinant viral proteins were added at different time points. RNA synthesis was initiated with NTPs pre-, post or during NS1 addition. The reactions were stopped at 10, 20 or 45 min (Fig. 5C). Surprisingly, we detected the inhibitory effect of $NS1^{WT}$ only when it was added concomitantly with the components of the transcriptional machinery. In other words, the recombinant $NS1^{WT}$ did not affect the RNA synthesis once transcription complexes were formed or already engaged in the transcription reaction. We concluded that the binding of NS1 on the DNA template prevented loading of PIC and RNA pol II and thus inhibited initiation of transcription.

DISCUSSION

The RNA binding function of NS1 is very important for influenza A virus replication. It has been shown that mutations R38A and K41A of NS1 prevent its binding to RNA *in vitro*, increase IFN production by infected cells and attenuate viral replication *in vivo* (23,24). Here we demonstrated that NS1 via its R38 and K41 binds dsDNA. Furthermore, we showed that NS1 via R38 and K41 inhibited pre-initiation complex formation and pol II loading during an *in vitro* transcription reaction, thereby, preventing efficient transcription initiation. Based on available structural information which indicates that NS1 can form hollow filaments with a tunnel diameter of 20Å (25), we propose that NS1 can potentially oligomerize around B-form dsDNA, which also has a diameter of 20Å, in contrast to dsRNA, for which the diameter is 26Å. Thus, physical and biochemical properties of NS1 allow NS1-dsDNA interaction and dsDNA sequestration from transcriptional machinery.

Functional versatility of NS1 complicates studying the role of its dsDNA binding in infected cells, because residues R38 and K41 can also indirectly prevent transcription of antiviral genes via interaction with dsRNA (45,46). In our study we took advantage of cells overexpressing wild type NS1 or its R38A and K41A mutant form, and showed that NS1-dsDNA binding has a direct effect on poly(I:C)-stimulated transcription of immune-related genes. We further showed that influenza A virus expressing wild type NS1, but not its R38A and K41A mutant form, was able to suppress transcription of antiviral genes in influenza A virus infected cells. We also found that NS1 associated with immune-related genes, and that this interaction altered the composition of chromatin and prevented pol II

interaction with these genes. NS1 interaction with different regions of genes further illustrated the sequence-unspecific mode of NS1-dsDNA binding. Additional events, such as the chromatin remodelling around immune-related genes upon infection would likely determine the spectrum of the genes whose transcription is inhibited by NS1. Altogether, these results allowed us to conclude that NS1 binds host dsDNA non-specifically via its RNA-/dsDNA-binding domain (R/DBD) and attenuates transcription initiation of antiviral genes during influenza A virus replication.

Mechanistically, after influenza A virus uncoating, viral RNA is released into the perinuclear cytoplasm, and imported into the nucleus, where transcription and replication by viral RNA-dependent RNA polymerase occurs. Cellular PRRs, such as TLR3, RIG-I, MDA5, and PKR recognize vRNA or its intermediates and activate signaling pathways and specific transcription factors such as IRF3 and NF□B, thus triggering transcription of immune related genes in infected cells (3). NS1 which is expressed from newly transcribed viral mRNAs is imported into the nucleus where it interacts with cellular DNA of PRR-activated genes and suppresses their transcription. Thus, NS1 allows influenza A virus to escape the innate immune response at the transcriptional level and thereby to secure virus replication (Fig. 6).

It was shown that influenza A virus NS1 can attenuate antiviral responses at transcriptional, post-transcriptional, translational and post-translational levels (5). For example, it was shown that NS1 can inhibit cellular transcription by targeting histone H3-interacting transcription elongation complex PAF1. However, such an inhibition required histone-mimic sequence 226-ARSK-229 in NS1 C-terminus, which is present only in some influenza A strains of H3N2 subtype (47). Other functions of NS1, such as interaction with CPSF4, eIF4G, and PABPII, also depend on the strain-specific motifs within NS1 (14). By contrast, two residues R38 and K41 that are essential for interaction with RNA/dsDNA and suppression of host antiviral gene transcription are highly conserved across influenza A viruses of different subtypes. Thus, we identified here a general strategy by which influenza A viruses antagonize antiviral responses in infected cells.

Several RNA viruses are able to inhibit cellular transcription. For example, bunyamwera virus NS-S protein counteracts phosphorylation of pol II C-terminal domain, whereas Rift Valley Fever Virus NSs protein targets TFIIH to inhibit transcription (48,49). However, to our knowledge, there are no data available except this study, that negative- or positive-sense RNA viruses inhibit cellular transcription by direct binding to cellular DNA. Thus, our study provides a first example of such a mechanism that may be potentially exploited by other virus families.

The NS1-dsDNA interaction can be potentially exploited for treatment of influenza A virus infection. Our results indicate that available small-molecular inhibitors of NS1-dsRNA interaction (50) can also abolish NS1-dsDNA binding, and therefore, restore innate immune responses and inhibit virus replication. Finally, viruses expressing NS1 which lacks dsDNA-binding function may display characteristics desirable for live-attenuated immunogenic vaccines and hold potential as vaccine candidates (51-53).

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TABLE AND FIGURES LEGENDS

Figure 1. Purified recombinant NS1 binds synthetic dsDNA *in vitro*. (A) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of purified recombinant $NS1^{WT}$ and $NS1^{RK/AA}$ proteins. Serial dilutions of purified NS1 were resolved in SDS-PAGE. The gels were stained using Coomassie blue or immunoblotted using anti-NS1 antibodies. M – protein markers, kDa. (B) Electrophoretic mobility shift assay (EMSA) monitoring binding of recombinant purified NS1 ($NS1^{WT}$) and its R38A/K41A mutant ($NS1^{KK/AA}$) to synthetic dsDNA fragment. Two-fold dilutions of $NS1^{WT}$ and $NS1^{RK/AA}$ (starting from 40μM) were pre-incubated with dsDNA for 15 min at room temperature prior to analysis on agarose gel. M – dsDNA markers, bp. (C) Microscale thermophoresis assay monitoring thermophoretic mobility of dsDNA upon its binding to $NS1^{WT}$ or $NS1^{RK/AA}$ mutant. Normalized fluorescence of Cy5-labeled synthetic dsDNA after its incubation with indicated concentrations of $NS1^{WT}$ or $NS1^{RK/AA}$ is shown. An increase in normalized fluorescence is observed upon dsDNA interaction with NS1. The data points are mean values and error bars represent the SD from three independent experiments.

Figure 2. Recombinant NS1 associates with promoter and exon regions of host genes in HEK293T cells. (A) HEK293T cells were transfected with empty vector or plasmids encoding $NS1^{WT}$ or $NS1^{KK/AA}$. Expression of $NS1^{WT}$ and $NS1^{KK/AA}$ was analysed in whole cell extracts 20 hours post transfection by immunoblotting using anti-NS1 antibodies. (B) Immunostaining of NS1 in HEK293T cells transfected with 5 μg of empty vector or plasmids encoding $NS1^{WT}$ or $NS1^{KK/AA}$. Cells were fixed at 20 h post-transfection, and stained with anti-NS1 antibody and DAPI. Scale bars, 10 μm. (C) Immunoblot analysis of NS1 in cytoplasm, soluble nucleus and chromatin fractions of transfected HEK293T cells. (D) Immunoblot analysis of NS1, histone H1 and histone H3 in histone extract of the transfected HEK293T cells. (E) RPE cells were mock-, WSN^{WT}-, or $WSN^{RK/AA}$- infected. Cells were collected at 8 hours post infection, and chromatin fractions were prepared. SDS-PAGE with chromatin fractions (left panel) and whole cell lysates (right panel) are shown. (F) Chromatin fractions were prepared as in panel C. Quantitative mass-spectrometry analysis of proteins was performed. The table with fold change in concentrations of selected histones in infected versus non-infected cells are shown. Asterisks mark the statistically significant values ($p < 0.05$) and hashtags mark values obtained from

detection of a single peptide.

Figure 3. Functional NS1 inhibits transcription of immune-related genes in human cells. (A) RPE cells were mock-, WSN^{WT-}, or $WSN^{RK/AA}$- infected. Ten hours post infection cells were collected, total RNA was isolated and subjected to genome-wide gene expression profiling. A heat map of transcripts affected by influenza A virus infection is shown (upper panel). The heat map represents normalized expression data on the logarithmic scale (log_2 fold change > 3 and <-3) as compared to mock-infected cells. A Venn diagram of gene sets transcriptionally up-regulated (> 8 fold) with different viruses is also shown (lower panel). (B) RPE cells were treated as for panel A, total RNA was isolated, and the expression of 5 antiviral and 1 housekeeping genes was analysed using RT-qPCRs. The data points are mean values and error bars represent the SD. (C) HEK293T cells were transfected with plasmids expressing $NS1^{WT}$ or $NS1^{RK/AA}$ or with empty vector. Cells were stimulated with poly(I:C) at 20 hours post transfection. Cells were collected at 3 and 6 hours post stimulation and total RNA was extracted. The expression of *IFNB1*, *IFNA1* and *IFNA16* genes was monitored by RT-qPCR. The data points are mean values and error bars represent the SD.

Figure 4. NS1 co-precipitates with promoter and exon regions of cellular genes. RPE cells were mock-, WSN^{WT}-, or $WSN^{RK/AA}$- infected. Cells were collected at 8h post infection, and chromatin was prepared for immuno-precipitation. ChIP was performed with anti-NS1 and anti-pol II antibodies. qPCRs were performed with primers targeting different regions of cellular genes. The percentage of input DNA associated with NS1 or with pol II was quantified. The data points are mean values, and error bars represent the SD.

Figure 5. NS1 inhibits pre-initiation complex formation and pol II loading on DNA in an *in vitro* transcription reaction. (A) Purified recombinant $NS1^{WT}$ or $NS1^{KK/AA}$ were tested in a transcription assay. Increasing amounts of the proteins were incubated with transcription factors (TFx), pol II, nucleotides (NTPs), ^{32}P-labeled ATP and DNA for 45 min. Reactions were stopped and synthesized ^{32}P-labeled RNAs were resolved on denaturing PAGE. (B) $NS1^{WT}$ or $NS1^{RK/AA}$ was pre-incubated with DNA, or TFx and pol II. The remaining components of the transcription reaction were added and transcription was initiated with NTPs. Reactions were stopped at the indicated times and the products were analysed as in panel A. Schematic representations of the experiments and the radioautographs (RA) are shown. (C) $NS1^{WT}$ or $NS1^{KK/AA}$ were added to the transcription reaction before, during or after transcription initiation with NTPs. Reactions were stopped at indicated time points and transcription products were analyzed as in panel A.

Figure 6. Schematic representation of NS1 inhibition of host cell gene transcription during influenza A virus infection. Influenza A virus binds sialic acid-containing cell surface glycoproteins and the virus is endocytosed. Acidification of the virus particles in endosomes triggers HA-mediated fusion of viral and endosomal membranes and the release of viral protein-RNA complexes into the perinuclear cytoplasm. The viral protein-RNA complexes are recognised by PRRs which trigger chromatin remodelling and transcription of antiviral genes. Some vRNPs are transported to the nucleus through the nuclear pores. In the nucleus, viral polymerase transcribes eight vRNAs and processes the

resulting mRNAs. Then viral mRNAs are transported into the cytoplasm where they are translated into 10-12 viral proteins (depending on virus strain). Subsequently, eight of these proteins (including NS1) are transported to the nucleus. In the nucleus, PB1, PB2, PA and NP regulate viral RNA replication via cRNA intermediates. Transcription and replication intermediates of vRNA can further enhance transcription of antiviral genes. However, NS1 inhibits the transcription, as well as pre-mRNA processing and mRNA nuclear export of antiviral genes. Newly synthetized vRNAs are packed into ribonucleoprotein complexes and they are transported to the plasma membranes. The new virus particles bud from the cell and are released to infect other cells.

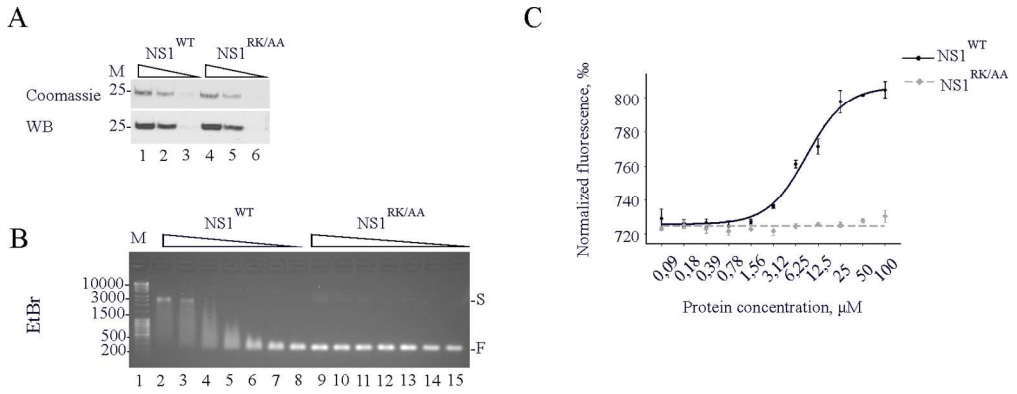

Figure 1
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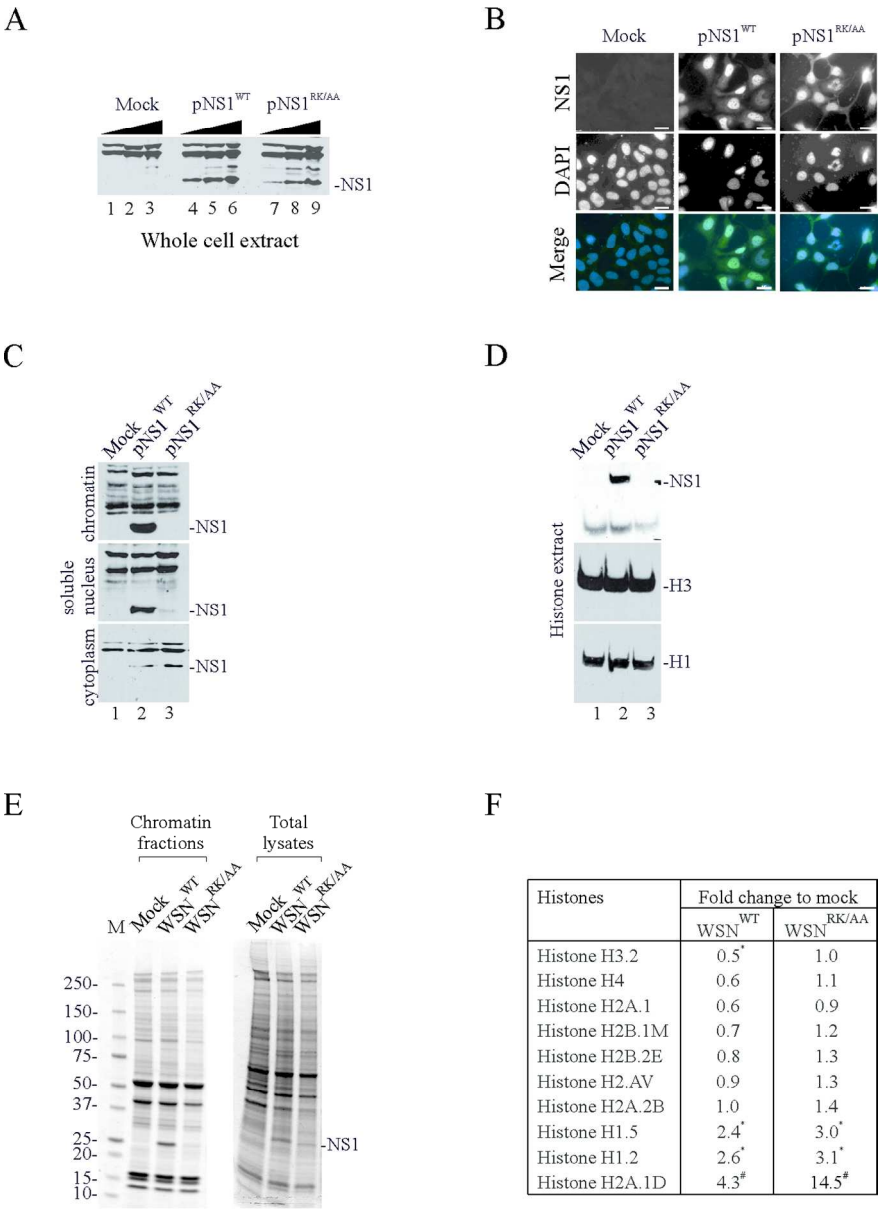

Histones	Fold change to mock	
	WSN^{WT}	$WSN^{RK/AA}$
Histone H3.2	0.5*	1.0
Histone H4	0.6	1.1
Histone H2A.1	0.6	0.9
Histone H2B.1M	0.7	1.2
Histone H2B.2E	0.8	1.3
Histone H2.AV	0.9	1.3
Histone H2A.2B	1.0	1.4
Histone H1.5	2.4*	3.0*
Histone H1.2	2.6*	3.1*
Histone H2A.1D	4.3#	14.5#

Figure 2
113x156mm (300 x 300 DPI)

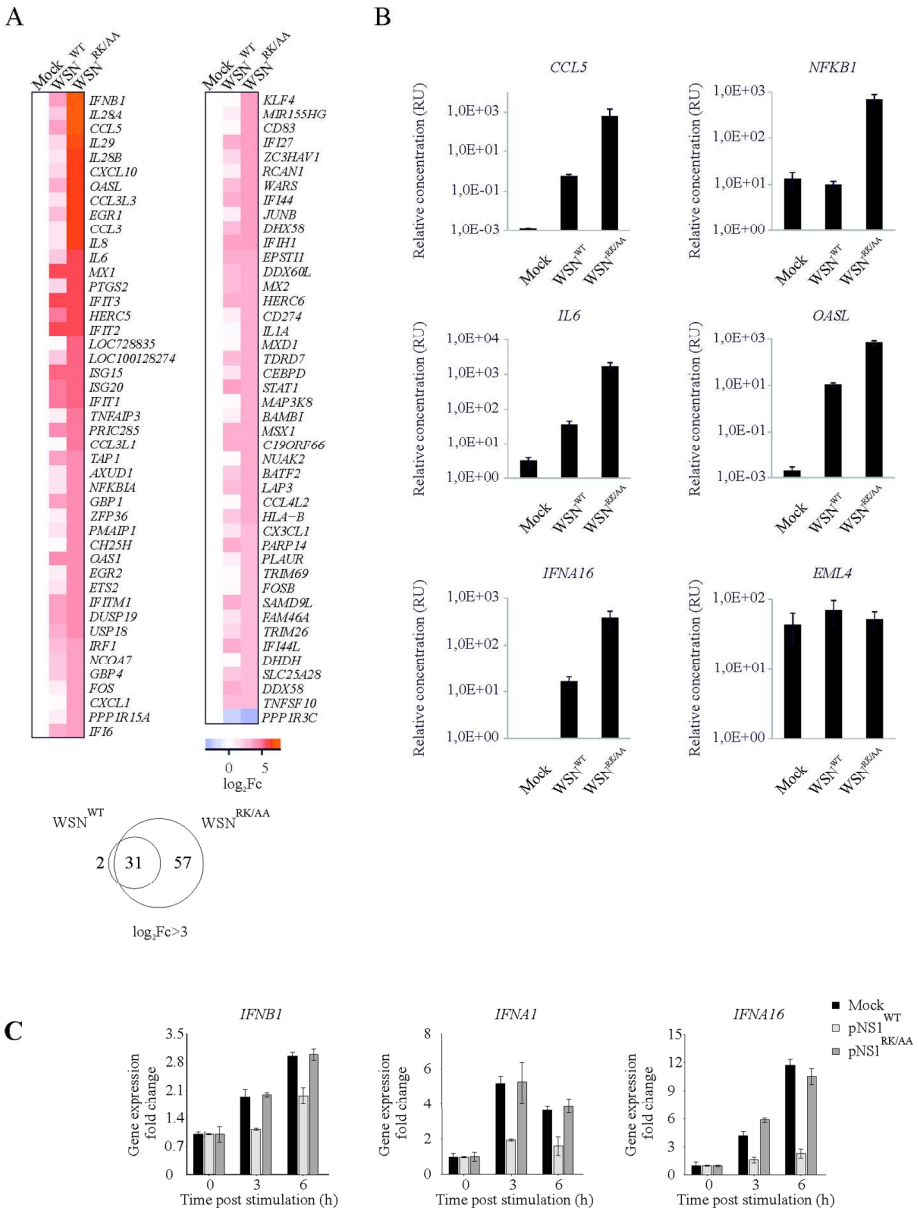

Figure 3
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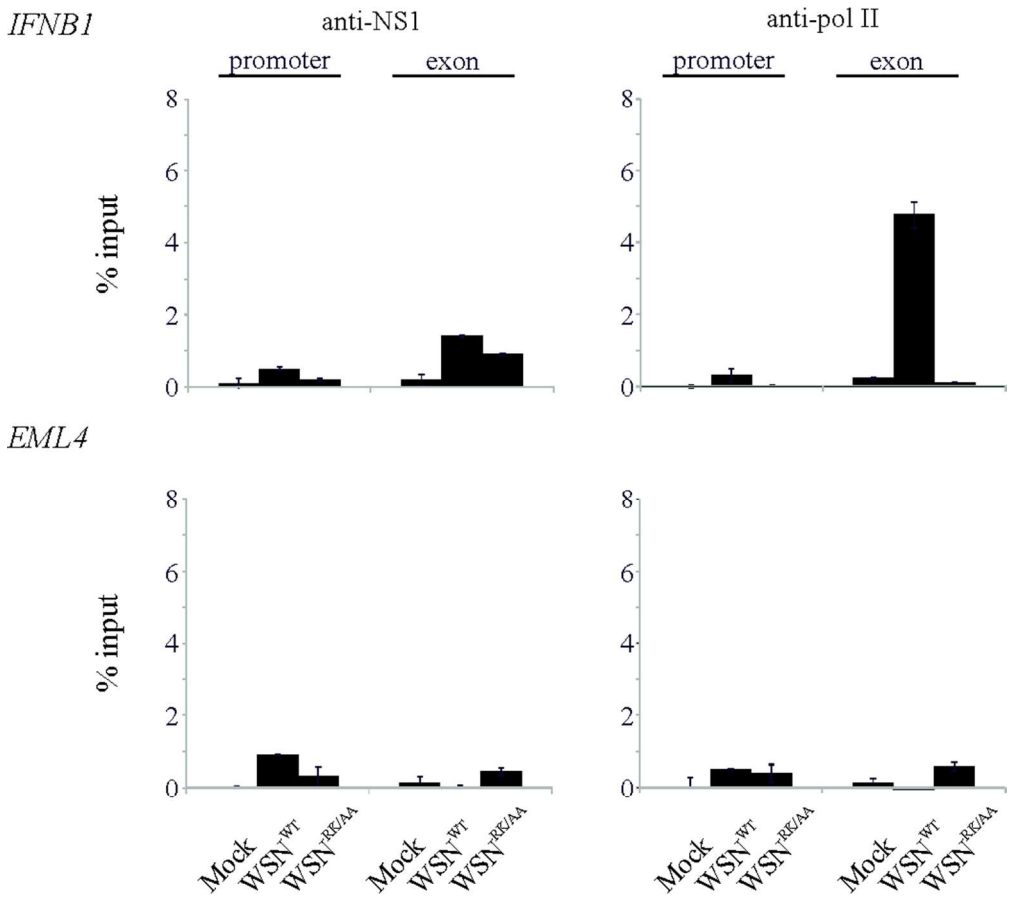

Figure 4
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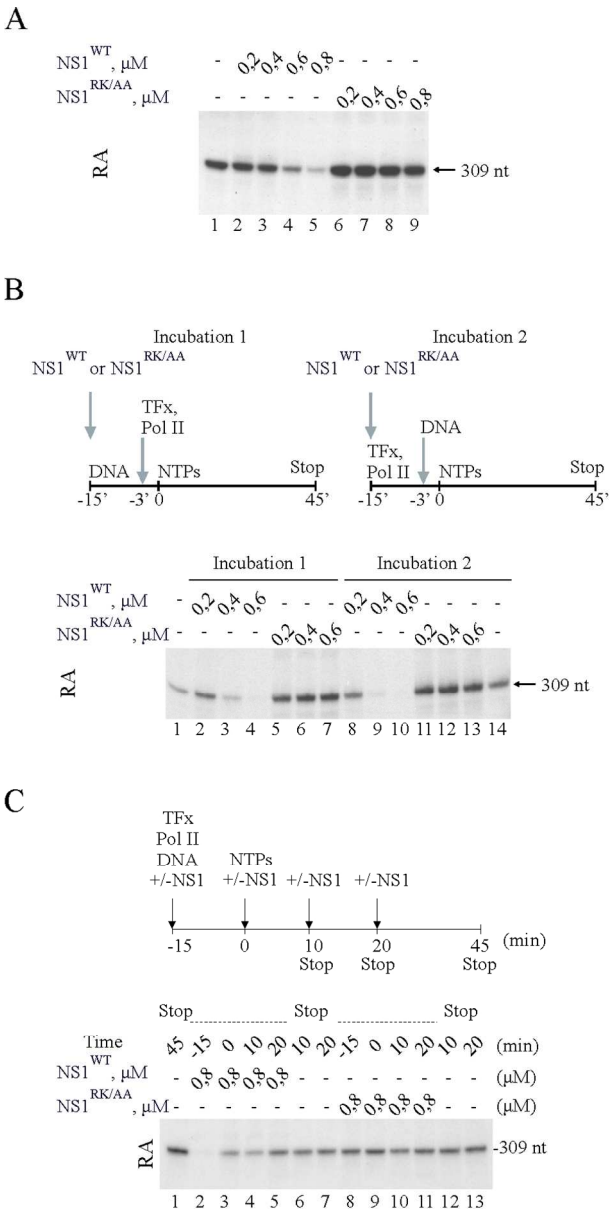

Figure 5
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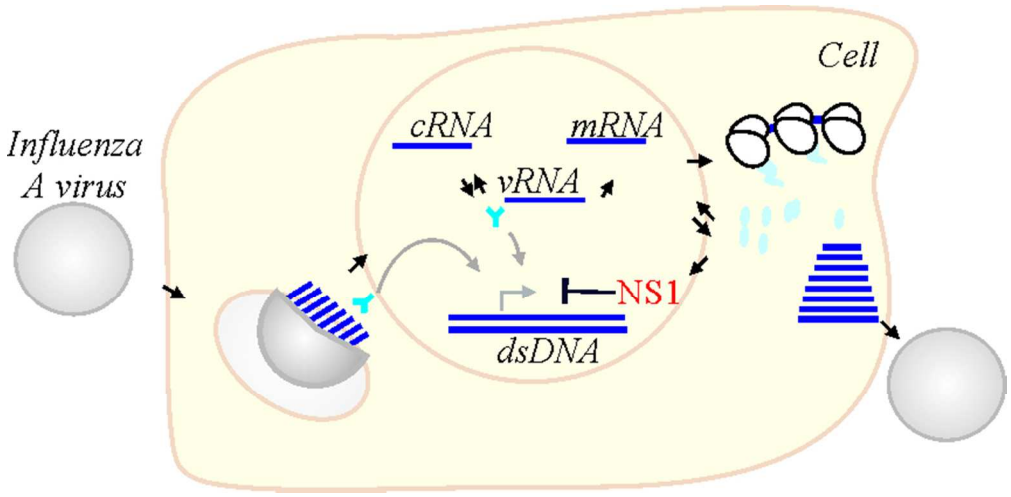

Figure 6
72x35mm (300 x 300 DPI)

Supplementary data

Non-structural protein NS1 of influenza A virus binds cellular double-stranded DNA and inhibits transcription of antiviral genes

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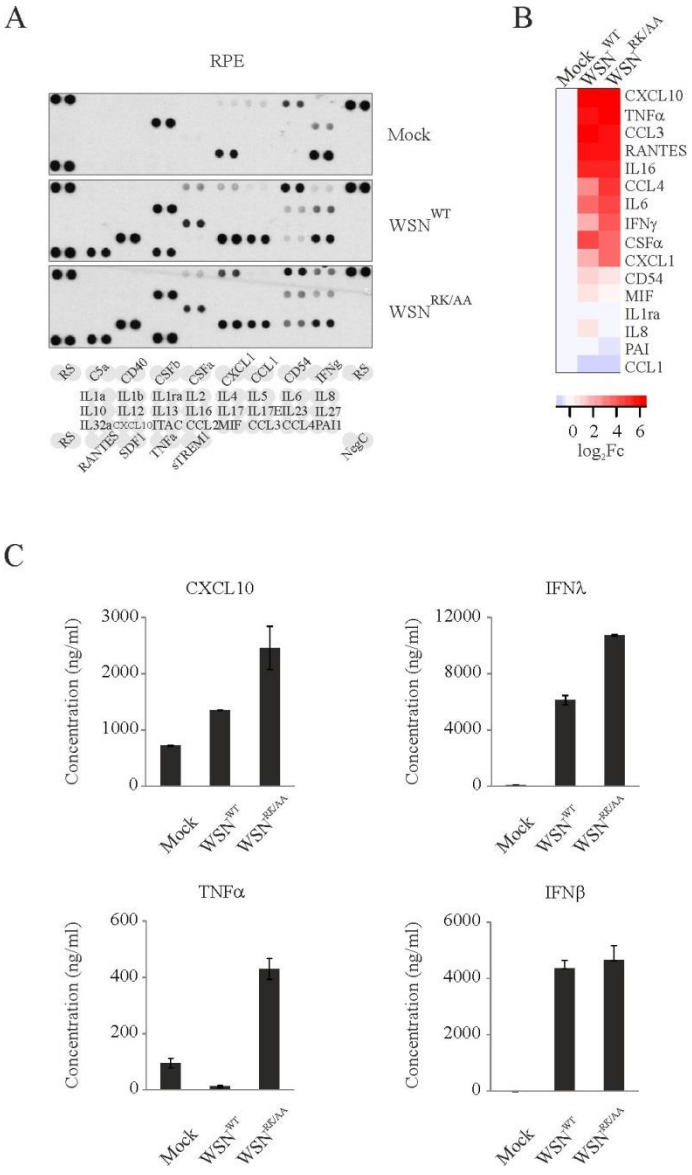


Fig. S1. NS1 inhibits production of cytokines by infected Human RPE cells. (A) RPE cells were mock-, WSN^{WT}-, or $WSN^{RK/AA}$- infected, cell culture supernatants were collected at 24 h post-infection, and cytokine levels were determined using Human cytokine array panel A. (B) The relative intensities of spots from panel A were calculated using ImageJ software. A heat map of cytokines affected by infection is shown. The heat map represents normalized expression data on the logarithmic scale as compared to mock-infected cells. (C) Cells were treated as for panel A, cell culture supernatants were collected at 24 h post-infection, and cytokine levels were determined using ELISA.

Table S1. Quantitative mass-spectrometry analysis of proteins in chromatin fractions of mock, WSN^{WT}-, or $WSN^{RK/AA}$-infected infected cells. RPE cells were mock, WSN^{WT}, or $WSN^{RK/AA}$-infected. Cells were collected at 8 h post infection and chromatin fractions were prepared. Quantitative mass-spectrometry analysis of proteins was performed. The table with fold change (FC) in concentrations of detected proteins in infected versus non-infected cells are shown along with the corresponding p-values. Missing p-values indicate that the comparison was done based on detection of single distinct peptide. Access no. - SwissProt accession number, Peptides - number of detected peptides, FC - fold change in infected cells compared to mock samples. IAV - Influenza A virus.

Access no.	Description	Species	Peptides	WSN-WT		WSN-RK/AA	
				FC	p-value	FC	p-value
P05787	Keratin, type II cytoskeletal 8	Human	22	0.2666	0	0.529	0
P08670	Vimentin	Human	147	0.1399	0	0.3737	0
P13645	Keratin, type I cytoskeletal 10	Human	13	5.0023	0.0001	0.6067	0.0002
P15682	Nucleoprotein	IAV	14	5.7085	0.0003	13.044	0
Q13838	Spliceosome RNA helicase DDX39B	Human	5	0.2568	0.0003	0.7093	0.0389
P05783	Keratin, type I cytoskeletal 18	Human	22	0.1971	0.0008	0.3858	0
P06748	Nucleophosmin	Human	7	1.7804	0.0008	1.7333	0.001
A4GCM5	NS1	IAV	7	24.7667	0.0011	21.418	0.0002
P63261	Actin, cytoplasmic 2	Human	43	0.357	0.0011	0.6348	0.021
P03454	HA	IAV	15	4.3369	0.0013	6.8722	0.0002
Q15149	Plectin	Human	10	0.4728	0.0021	0.739	0.0724
P02545	Prelamin-A/C	Human	44	0.8046	0.008	0.6725	0.0001
P09651	Heterogeneous nuclear ribonucleoprotein A1	Human	13	2.455	0.0081	1.741	0.0472
P09382	Galectin-1	Human	27	1.8309	0.0083	1.6589	0.0603
P20700	Lamin-B1	Human	12	0.7359	0.009	0.9995	0.9961
Q0A2D5	M1	IAV	6	16.8559	0.009	5.8583	0.0854
P16403	Histone H1.2	Human	14	2.6039	0.01	3.077	0.0166
P38159	RNA-binding motif protein, X chromosome	Human	5	1.4404	0.0159	1.1638	0.3685
Q71DI3	Histone H3.2	Human	8	0.4674	0.0163	1.0222	0.8973
Q9UKM9	RNA-binding protein Raly	Human	4	0.5454	0.0174	0.6621	0.0364
P04264	Keratin, type II cytoskeletal 1	Human	14	2.7694	0.0189	0.5102	0.0193
P19338	Nucleolin	Human	5	2.0739	0.0202	1.7386	0.0371
P35908	Keratin, type II cytoskeletal 2 epidermal	Human	11	6.2622	0.0215	0.6725	0.3758
Q15424	Scaffold attachment factor B1	Human	3	1.7258	0.022	1.4374	0.048
P62987	Ubiquitin-60S ribosomal protein L40	Human	14	0.7366	0.023	0.7622	0.0226
Q07955	Serine/arginine-rich splicing factor 1	Human	6	2.0134	0.0345	1.2249	0.4124
P62750	60S ribosomal protein L23a	Human	4	1.5202	0.0455	1.3907	0.357
P16401	Histone H1.5	Human	11	2.3641	0.0468	3.0151	0.0115
Q96L21	60S ribosomal protein L10-like	Human	2	0.2119	0.0573	0.5622	0.1089
P62805	Histone H4	Human	52	0.6321	0.0582	1.1336	0.1905
Q03252	Lamin-B2	Human	8	0.7697	0.0631	0.8087	0.0567
Q16629	Serine/arginine-rich splicing factor 7	Human	7	1.4344	0.0839	1.3726	0.06
Q9Y3U8	60S ribosomal protein L36	Human	4	1.3302	0.0854	1.1908	0.1638

P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	Human	21	1.3625	0.0902	1.0433	0.5284
P11142	Heat shock cognate 71 kDa protein	Human	5	0.4067	0.0903	0.5609	0.0696
P23284	Peptidyl-prolyl cis-trans isomerase B	Human	2	1.5117	0.0965	1.1143	0.4685
P07951	Tropomyosin beta chain	Human	4	2.1299	0.0993	1.0155	0.962
P62277	40S ribosomal protein S13	Human	3	0.5554	0.1061	0.9077	0.5067
Q03135	Caveolin-1	Human	6	0.8396	0.1176	1.0244	0.8041
Q13242	Serine/arginine-rich splicing factor 9	Human	3	1.914	0.1213	1.291	0.5255
P31943	Heterogeneous nuclear ribonucleoprotein H	Human	2	0.3368	0.1249	0.6224	0.316
Q9UHB6	LIM domain and actin-binding protein 1	Human	2	0.6156	0.1302	0.7513	0.3454
A6NMY6	Putative annexin A2-like protein	Human	3	0.4477	0.1352	0.6859	0.0483
Q13247	Serine/arginine-rich splicing factor 6	Human	7	1.3258	0.1415	1.2744	0.2295
P36578	60S ribosomal protein L4	Human	2	0.2814	0.1462	0.7955	0.2615
P19105	Myosin regulatory light chain 12A	Human	6	0.8245	0.149	0.4556	0.0601
P39019	40S ribosomal protein S19	Human	2	2.0856	0.1615	1.1754	0.5975
O43707	Alpha-actinin-4	Human	4	0.5518	0.186	0.8163	0.1913
Q9Y3Y2	Chromatin target of PRMT1 protein	Human	3	0.8014	0.2105	1.0952	0.3851
Q99879	Histone H2B type 1-M	Human	48	0.744	0.2146	1.2037	0.3266
P82979	SAP domain-containing ribonucleoprotein	Human	5	1.3323	0.2338	1.265	0.5388
Q99848	Probable rRNA-processing protein EBP2	Human	1	1.2819	0.2413	0.9751	0.9364
P62269	40S ribosomal protein S18	Human	2	0.6739	0.2442	0.7688	0.2289
P05387	60S acidic ribosomal protein P2	Human	4	2.297	0.2511	1.0005	0.9996
P21589	5'-nucleotidase	Human	4	0.3873	0.2793	0.5207	0.0856
Q5VTE0	Putative elongation factor 1-alpha-like 3	Human	2	0.5396	0.2869	0.6171	0.1291
Q16778	Histone H2B type 2-E	Human	47	0.7516	0.3074	1.299	0.236
Q9UMY1	Nucleolar protein 7	Human	2	0.7896	0.3107	0.8968	0.7047
Q9BRL6	Serine/arginine-rich splicing factor 8	Human	2	1.7809	0.3537	1.1499	0.5112
O75367	Core histone macro-H2A.1	Human	6	0.6358	0.3587	0.8797	0.2572
Q0ZGT2	Nexilin	Human	6	1.1067	0.3595	0.7479	0.0486
Q05682	Caldesmon	Human	2	1.7515	0.3746	0.9416	0.8854
P31942	Heterogeneous nuclear ribonucleoprotein H3	Human	3	0.6403	0.3759	0.7066	0.2867
Q71UI9	Histone H2A.V	Human	16	0.8753	0.4087	1.2849	0.2504
P55769	NHP2-like protein 1	Human	2	0.8922	0.4576	0.992	0.949
Q92804	TATA-binding protein-associated factor 2N	Human	5	1.3398	0.4674	1.1398	0.2501
P45973	Chromobox protein homolog 5	Human	2	1.1148	0.4744	0.9206	0.5601
P22087	rRNA 2'-O-methyltransferase fibrillarin	Human	1	0.331	0.4785	0.5993	0.1403
P08579	U2 small nuclear ribonucleoprotein B	Human	2	1.1148	0.4816	1.1876	0.5836
P51991	Heterogeneous nuclear ribonucleoprotein A3	Human	7	1.2806	0.4915	1.0433	0.7437
Q7RTV0	PHD finger-like domain-containing protein 5A	Human	1	1.0869	0.5552	0.9923	0.9502
P02751	Fibronectin	Human	2	0.6672	0.5795	0.4202	0.2237
Q13435	Splicing factor 3B subunit 2	Human	8	0.9207	0.5816	0.988	0.9099
Q09666	Neuroblast differentiation-associated protein AHNAK	Human	31	1.0623	0.6128	0.9776	0.8291

P84103	Serine/arginine-rich splicing factor 3	Human	5	1.9397	0.6221	1.7634	0.6466
P16070	CD44 antigen	Human	7	1.0861	0.6326	1.1658	0.2084
P07910	Heterogeneous nuclear ribonucleoproteins C1/C2	Human	19	0.9102	0.6716	0.876	0.4875
P84090	Enhancer of rudimentary homolog	Human	4	1.1756	0.7089	1.2338	0.1364
Q13573	SNW domain-containing protein 1	Human	3	1.0631	0.7423	0.8283	0.52
P61978	Heterogeneous nuclear ribonucleoprotein K	Human	5	1.07	0.7532	0.8304	0.1969
O14979	Heterogeneous nuclear ribonucleoprotein D-like	Human	4	1.3273	0.7938	1.006	0.9945
Q15427	Splicing factor 3B subunit 4	Human	1	1.0469	0.8215	1.1236	0.4608
P83916	Chromobox protein homolog 1	Human	3	1.1338	0.841	1.4861	0.6036
Q9Y5S9	RNA-binding protein 8A	Human	4	0.9786	0.87	1.0734	0.6964
O00566	U3 small nucleolar ribonucleoprotein protein MPP10	Human	2	0.9849	0.9775	1.2855	0.3748
P42766	60S ribosomal protein L35	Human	3	0.9954	0.9917	0.9662	0.8037
P60660	Myosin light polypeptide 6	Human	5	0.9996	0.9988	0.5237	0.1368
O00161	Synaptosomal-associated protein 23	Human	1				
O00422	Histone deacetylase complex subunit SAP18	Human	1	1.2026		1.4939	
O00567	Nucleolar protein 56	Human	2	0.3184		0.7257	
O15511	Actin-related protein 2/3 complex subunit 5	Human	1	0.9487		1.0774	
O60216	Double-strand-break repair protein rad21 homolog	Human	1				
O75494	Serine/arginine-rich splicing factor 10	Human	6	1.1011		1.5505	
O75531	Barrier-to-autointegration factor	Human	2	0.8155		0.8924	
O75533	Splicing factor 3B subunit 1	Human	1	0.5017		0.9166	
P07477	Trypsin-1	Human	7				
P08174	Complement decay-accelerating factor	Human	1	0.7901		0.8432	
P08621	U1 small nuclear ribonucleoprotein 70 kDa	Human	3	0.8924		1.2103	
P09661	U2 small nuclear ribonucleoprotein A	Human	1	0.7114		0.8409	
P11021	78 kDa glucose-regulated protein	Human	1	0.5019		0.4601	
P13987	CD59 glycoprotein	Human	4	0.8886		0.6119	
P16989	DNA-binding protein A	Human	5				
P17096	High mobility group protein HMG-I/HMG-Y	Human	2	2.2812		1.5438	
P20671	Histone H2A type 1-D	Human	30	4.2616		14.55	
P21333	Filamin-A	Human	3	0.2952		0.9724	
P23246	Splicing factor, proline- and glutamine-rich	Human	1				
P23497	Nuclear autoantigen Sp-100	Human	2	1.158		0.7625	
P25398	40S ribosomal protein S12	Human	1				
P29590	Protein PML	Human	2	0.3536		0.8548	
P29966	Myristoylated alanine-rich C-kinase substrate	Human	2				
P30050	60S ribosomal protein L12	Human	1	1.3368		1.193	
P35527	Keratin, type I cytoskeletal 9	Human	2	4.4996		0.9573	
P38919	Eukaryotic initiation factor 4A-III	Human	2				
P42166	Lamina-associated polypeptide 2, isoform alpha	Human	2	1.9053		2.9033	
P46087	Putative ribosomal RNA	Human	1	0.8079		0.511	

	methyltransferase NOP2						
P47914	60S ribosomal protein L29	Human	2				
P50402	Emerin	Human	2				
P51608	Methyl-CpG-binding protein 2	Human	1				
P52272	Heterogeneous nuclear ribonucleoprotein M	Human	1	1.234		0.934	
P54289	Voltage-dependent calcium channel subunit alpha-2/delta-1	Human	1	0.2272		0.4879	
P55081	Microfibrillar-associated protein 1	Human	2				
P55145	Mesencephalic astrocyte-derived neurotrophic factor	Human	1				
P61313	60S ribosomal protein L15	Human	1	0.203		0.8363	
P61353	60S ribosomal protein L27	Human	1	0.2619		0.3738	
P62306	Small nuclear ribonucleoprotein F	Human	1				
P62316	Small nuclear ribonucleoprotein Sm D2	Human	1	1.0004		0.9157	
P62851	40S ribosomal protein S25	Human	1	1.6336		0.8114	
P62861	40S ribosomal protein S30	Human	1	3.3038		3.8569	
P62906	60S ribosomal protein L10a	Human	1	0.8581		1.5005	
P62995	Transformer-2 protein homolog beta	Human	2	1.815		1.2447	
P63104	14-3-3 protein zeta/delta	Human	1				
P67809	Nuclease-sensitive element-binding protein 1	Human	7	0.4752		1.5583	
P80723	Brain acid soluble protein 1	Human	4				
P81605	Dermcidin	Human	1				
Q00059	Transcription factor A, mitochondrial	Human	1	1.7125		1.4871	
Q12788	Transducin beta-like protein 3	Human	1				
Q13813	Spectrin alpha chain, brain	Human	1				
Q14103	Heterogeneous nuclear ribonucleoprotein D0	Human	2	1.3682		1.2729	
Q16643	Drebrin	Human	3	0.7073		0.4556	
Q6EEV6	Small ubiquitin-related modifier 4	Human	1	2.815		1.4309	
Q6NZI2	Polymerase I and transcript release factor	Human	1				
Q7Z406	Myosin-14	Human	1	0.4347		0.437	
Q7Z7B0	Filamin-A-interacting protein 1	Human	1				
Q86UP2	Kinectin	Human	1				
Q86V81	THO complex subunit 4	Human	1				
Q8IUE6	Histone H2A type 2-B	Human	25	0.9991		1.374	
Q8TAD7	Overexpressed in colon carcinoma 1 protein	Human	1	0.9542		0.6818	
Q8TER5	Rho guanine nucleotide exchange factor 40	Human	1				
Q8WXI9	Transcriptional repressor p66-beta	Human	1				
Q92945	Far upstream element-binding protein 2	Human	1				
Q9BZ81	Melanoma-associated antigen B5	Human	1	0.9465		5.295	
Q9H307	Pinin	Human	2				
Q9H361	Polyadenylate-binding protein 3	Human	1	2.1523		2.3146	
Q9NQC3	Reticulon-4	Human	1				
Q9NX63	Coiled-coil-helix-coiled-coil-helix domain-containing protein 3, mitochondrial	Human	1	0.8076		1.0481	

Q9NY12	H/ACA ribonucleoprotein complex subunit 1	Human	1	0.4044		0.5597	
Q9NYF8	Bcl-2-associated transcription factor 1	Human	1	1.6849		2.1136	
Q9UKV3	Apoptotic chromatin condensation inducer in the nucleus	Human	1				
Q9Y2W1	Thyroid hormone receptor-associated protein 3	Human	3	1.3648		1.2734	
Q9Y3C1	Nucleolar protein 16	Human	1	1.3602		1.1244	

Part II: Current and future projects

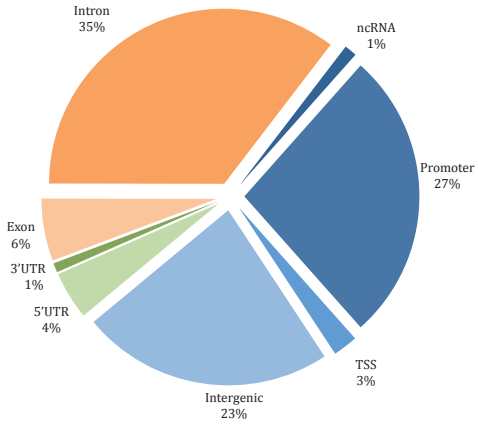
5 Current projects

5.1 Roles of XPC in the regulation of Histone PTMs and Histone variants upon transcription

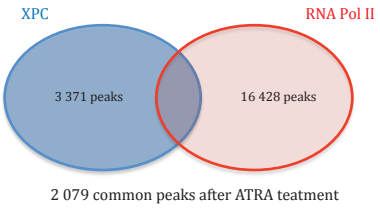
5.1.1 Context and preliminary data

During the sequential recruitment of NER factors upon transcription, XPC as the first one triggered the presence of the other at *RARβ2* promoter (*6*). We observed that the absence of XPC, using either silenced XPC HeLa cells or XP-C patients-derived fibroblasts impaired the recruitment of XPG and XPF at *RARβ2* promoter consequently preventing the induction of DNA nicks, the DNA demethylation, the presence of CTCF and the gene looping formation with the terminator. We also noticed the absence of specific transcriptional active marks including acetylation of H3K9 (H3K9ac) and tri-methylation of H3K4 (H3K4me3). These histone PTMs could be detected by only restoring the recruitment of XPC at promoter (*6*).
To better understand the function of XPC during transcription, we next sough to identify its genomic location. Two ChIP-seq series on XP-C patient-derived fibroblast restored by GFP-XPC wt yielded 14 750 and 21 300 XPC-binding events including 3,371 common peaks compared with input. Interestingly, the majority of XPC genomic distribution events (27% i.e 905 peaks) appeared to be enriched at promoters (Figure 9A). In parallel, we also determined the pol II genomic location and compared it to the XPC-binding events. Among the 25 387 and 41 458 pol II genomic distribution events, we identified around 2,079 common binding events with XPC (Figure 9B). 91% of the XPC-bound promoters also showed an enrichment of pol II (Figure 9B). To study the effect of XPC on pol II localization *in vivo*, we performed a comparative ChIP-seq analysis using XP-C derived patient XPC/R579Stp cells. Our data showed similar distribution of pol II around TSS compared to the rescue but the absence of XPC resulted in the significant decrease of promoter-associated pol II as illustrated for *C8orf76* (Figure 9C and D). The functional annotation of the XPC-bound promoters using DAVID indicated that 16% of the genes are involved in transcription, 12% are related to zinc-finger proteins and 4% are linked to cell cycle or mRNA processing (Figure 9E). We performed parallel RNA-seq and analysis is under progress to determine the impact of XPC on the expression of its targeted genes.

A. XPC binding sites on Ensembl protein coding genes

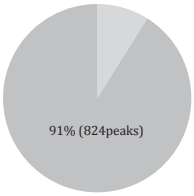


B. Overlapping between XPC and pol II binding sites

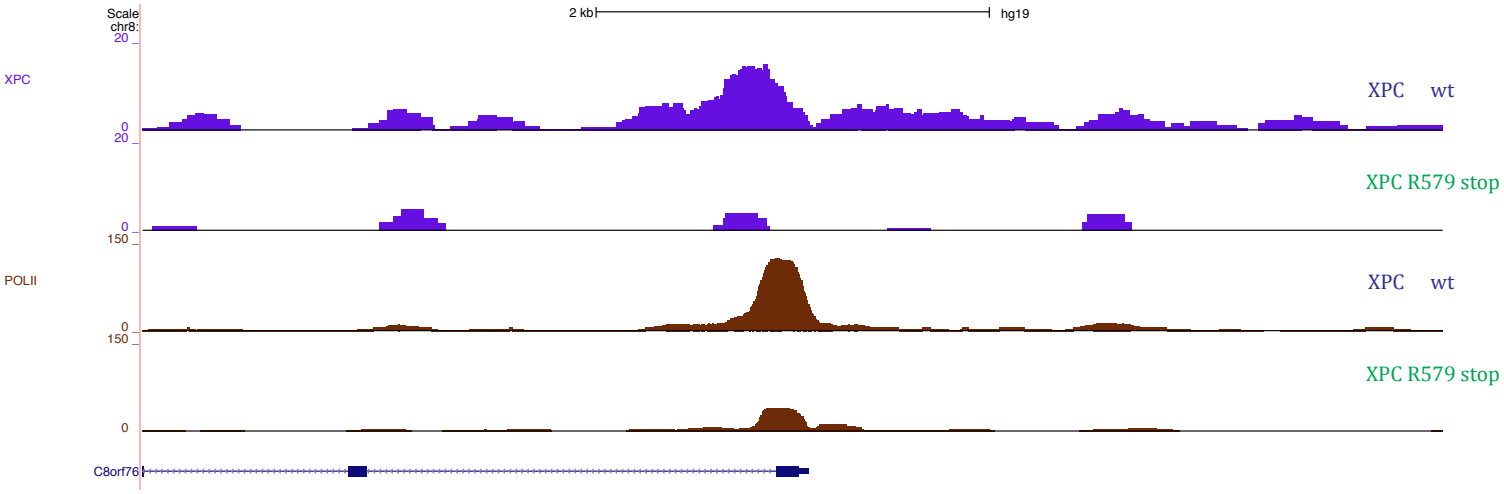


2 079 common peaks after ATRA teatment

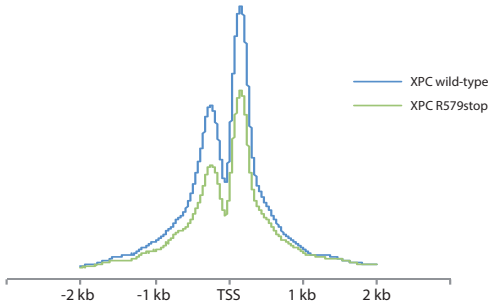
XPC promoters (905 peaks) containing RNA pol II peaks :



C. C8orf76 example using UCSC Browser



D. Average of Pol II recruitment on XPC promoters



E. Functional annotation of XPC-bound promoters

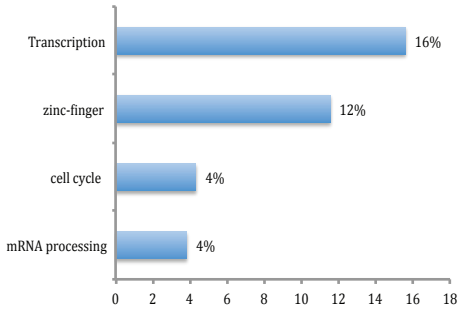


Figure 9. Genome-wide analysis of XPC

A) Homer annotation of XPC binding events determined by ChIP-seq.
B) XPC and pol II ChIP-seq comparison and determination of overlapping peaks including the XPC-bound promoters
C) representation using UCSC browser of C8orf76 targeted by XPC at its promoter
D) Comparison of Pol II ChIP-seq from XPC WT or XPC R579Stop chromatin and average pol II recruitment for XPC-bound promoters
D) DAVID functional annotation of XPC-target promoters.

We next analysed by ChIP-seq several active histone PTMs including H3K9ac, H3K4me3 located around the promoter and H3K36me3 along the gene body. The transcriptionally active genes bound by XPC at their promoters were characterized by the different histone PTMs as expected (Figure 10B). In contrast, in absence of XPC, we defined one cluster constituted by 215 XPC-bound promoters presented lower histone marks H3K9ac and H3K36me3 but unchanged H3K4me3 as it is shown for *c8orf76* (Figure 10A and B). To elucidate the XPC-related mechanisms controlling the histone PTMs upon transcription, we sought to identify the XPC partners by isolating the complexes containing this NER factor and analysing their composition by mass spectrometry. The collected data allow the identification of two candidates particularly interesting. My current project initiated by my former PhD student Izarn Iltis is to characterize these new interactions and functions for the histone PTMs regulation.

5.1.2 XPC and regulation of specific Histones PTMs

5.1.2.1 XPC and NSD3: regulation of H3K36me3?

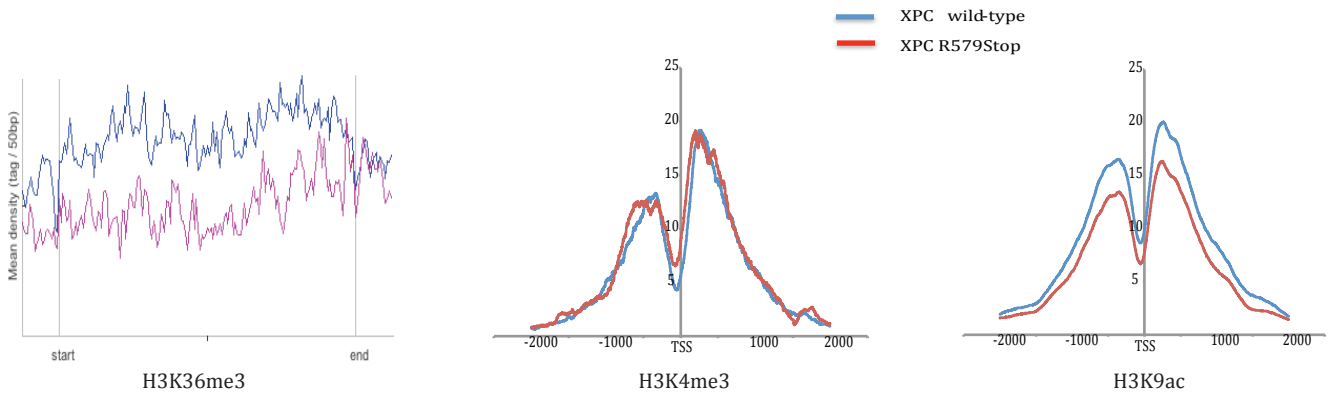
Among the identified proteins specifically co-precipitating with XPC, we found known XPC partners, Centrin 2 (*173*), hR23B (*413*), proteins previously implicated in NER and transcription in ES cells (*13*) and proteins with no known function in such pathways, like the Nuclear Receptor Binding SET Domain protein 3 (NSD3). This protein also known as WHSC1L1 was selected because it has been previously implicated in the H3K36 trimethylation (H3K36me3) at the promoter and 5'end of genes necessary to the subsequent H3K36me3 along the gene body (*414*). NSD3 is a histone methyltransferase that belongs to the mammalian NSD family of SET domain-containing methyltransferases, which also includes NSD1 and NSD2 (WHSC1/MMSET). The NSD family of proteins is essential in development and is mutated in human acute myeloid leukemia, Sotos syndrome, myeloma and lung cancer. The SET domain of NSD proteins is homologous to the *Saccharomyces cerevisiae* H3K36-specific methyltransferase SET2. The association between XPC and NSD3 was confirmed by co-immunoprecipitation using whole cell extracts prepared from HEK 293T cells over-expressing XPC tagged by B10 and NSD3 fused to GFP (data not shown).

We next determined NSD3 genomic location. ChIP-seq of NSD3 yielded 10 080 binding events compared with input. Interestingly, we observed that 6% of NSD3 binding events appeared to be enriched at promoters. Among the 14 750 XPC genomic distribution events previously determined, we identified 1 038 common binding events with NSD3 that were located to regions surrounding TSS. Bioinformatic analysis has to be run to combine the genome-wide data of NSD3, XPC and H3K36me3 with the RNA-seq.

We next wondered whether its association with NSD3 was also necessary in GG-NER by investigating the recruitment of NSD3 to damaged chromatin using local UVC irradiation combined with fluorescent immunostaining. As previously demonstrated (*415*), XPC and XPB accumulated at regions of local UVC irradiation in U2OS treated transfected with scrambled siRNA (siCtrl) but not in cells with siRNA targeting XPC (siXPC) (Figure 11A). Interestingly, XPC and XPB still co-localized with CPD in UVC-irradiated U2OS silenced for NSD3. Finally, DNA repair synthesis, revealed by 5-ethynyl 2'-deoxyuridine (EdU) incorporation at damage sites, was visualized in siCtrl and siNSD3 U2OS cells but lost in siXPC cells (Figure 11A upper panels). Altogether, these data strongly suggested that NSD3 was not recruited through its association with XPC at damage sites and was not needed for the DNA repair through the GG-NER pathway.

To further test the idea that the association between XPC and NSD3 is specific to the

A. Average enrichment of H3K4me3 and H3K9ac for the XPC-target promoters specific cluster



B. C8orf76 example using UCSC Browser

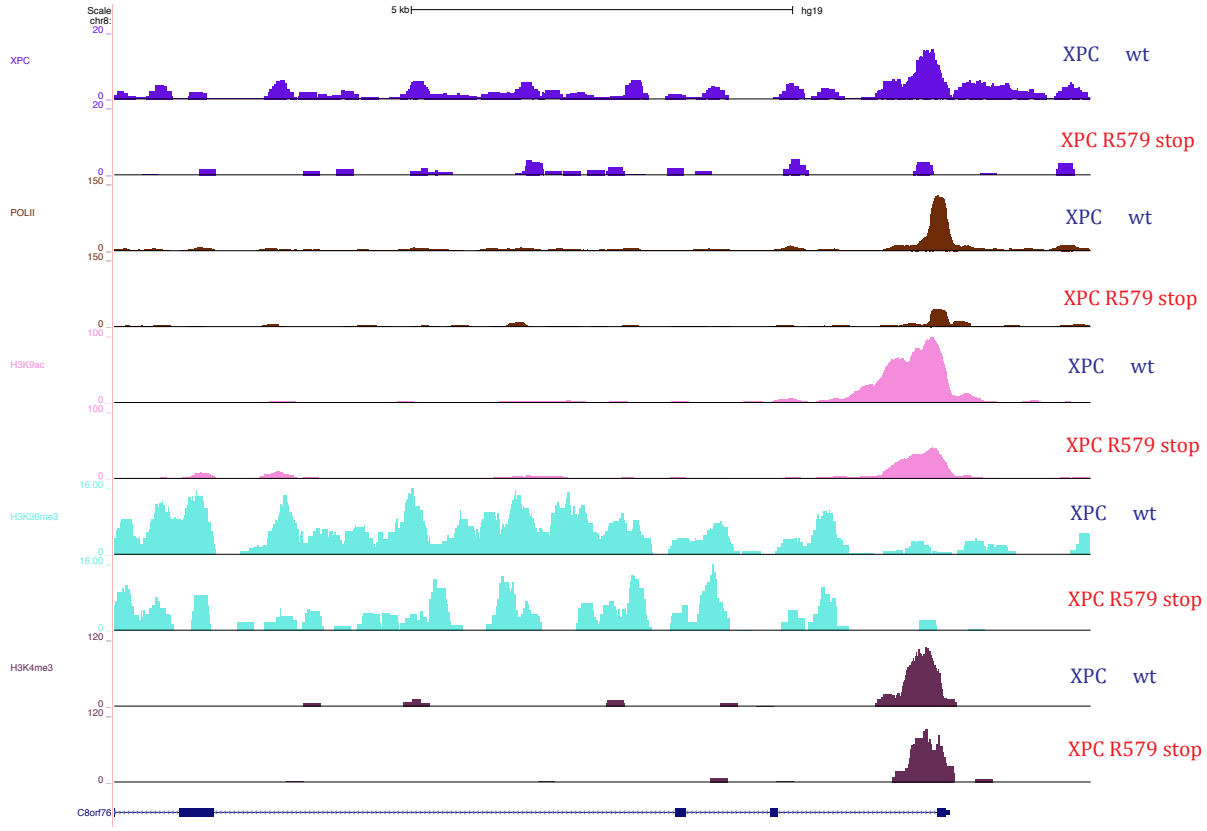


Figure 10. Impact of XPC on active Histone PTMs upon transcription

A) Comparison of H3K36me3 H3K4me3 and H3K9ac ChIP-seq data from XPC WT or XPC R579Stop chromatin and average enrichment for one specific cluster of XPC-bound promoters
B) Representation using UCSC browser of C8orf76 targeted by XPC at its promoter.

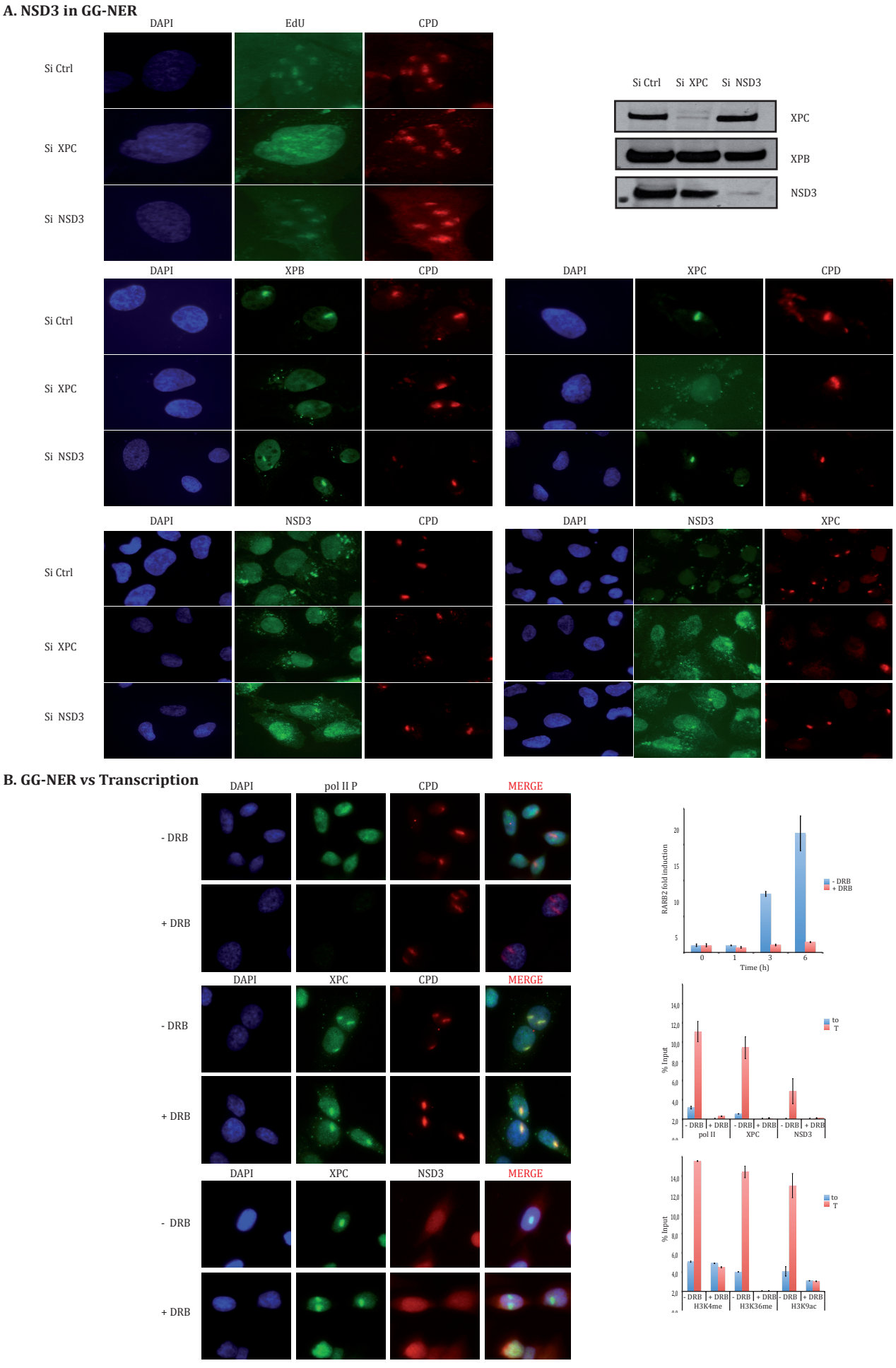


Figure 11. XPC/NSD3 interaction is specifically involved in transcription

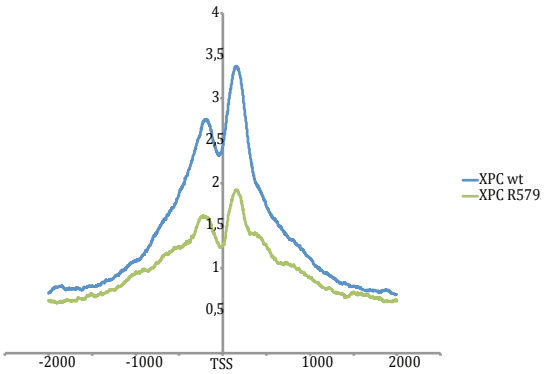
A) Visualization of DNA repair synthesis, revealed by 5-ethynyl 2'-deoxyuridine (EdU) incorporation at damage sites (upper panels) and recruitment of NSD3 to damaged chromatin using local UVC irradiation combined with fluorescent immunostaining (lower panels) in U2OS cells silenced for either XPC or NSD3. B) Transcriptional inhibtion using DRB did not impede recruitment of NER factors around the damage but abolished the synthesis of RARB2 mRNA correlated with the absence of NSD3 and XPC.

transcription process, we designed a second set of experiments. HeLa cells were UVC irradiated and treated with the transcription inhibitor DRB. Under these conditions, we observed the expected decrease of phosphorylated pol II while the accumulation of XPC and XPB at damage sites was still visualized (Figure 11B). However, we did not detect co-localization between XPC and NSD3 after UVC irradiation in cells untreated and DRB-treated. In parallel, cells were also co-treated with t-RA and DRB to analyse the *RARB2* transactivation including the induction of mRNA and the presence of pol II, XPC, NSD3 and histone PTMs at the promoter. Interestingly, the decreased *RARB2* mRNA expression in presence of DRB correlated with the absence of pol II, XPC, NSD3 and the absence of H3K4me3, H3K36me3 at promoter. Our results thus indicated that the inhibition of transcription abolished the recruitment and involvement of XPC in the regulation of histone PTMs notably through its association with NSD3 while its functions upon GG-NER were not disturbed (Figure 11B).
To complete this story, we now aim to identify the important domains for the interaction using *in vitro* assays with the purified recombinant proteins. We will produce several deleted mutants based on mutations found in XP-C patients. Secondly we will perform *in vitro* Histone PTMs assays to determine whether XPC through its association with NSD3 can regulate its enzymatic activity. Finally, we plan to analyse and characterize the transcriptional defect of XP-C patients-derived fibroblasts focusing on NSD3 and H3K36me3 for the genes targeted by XPC.

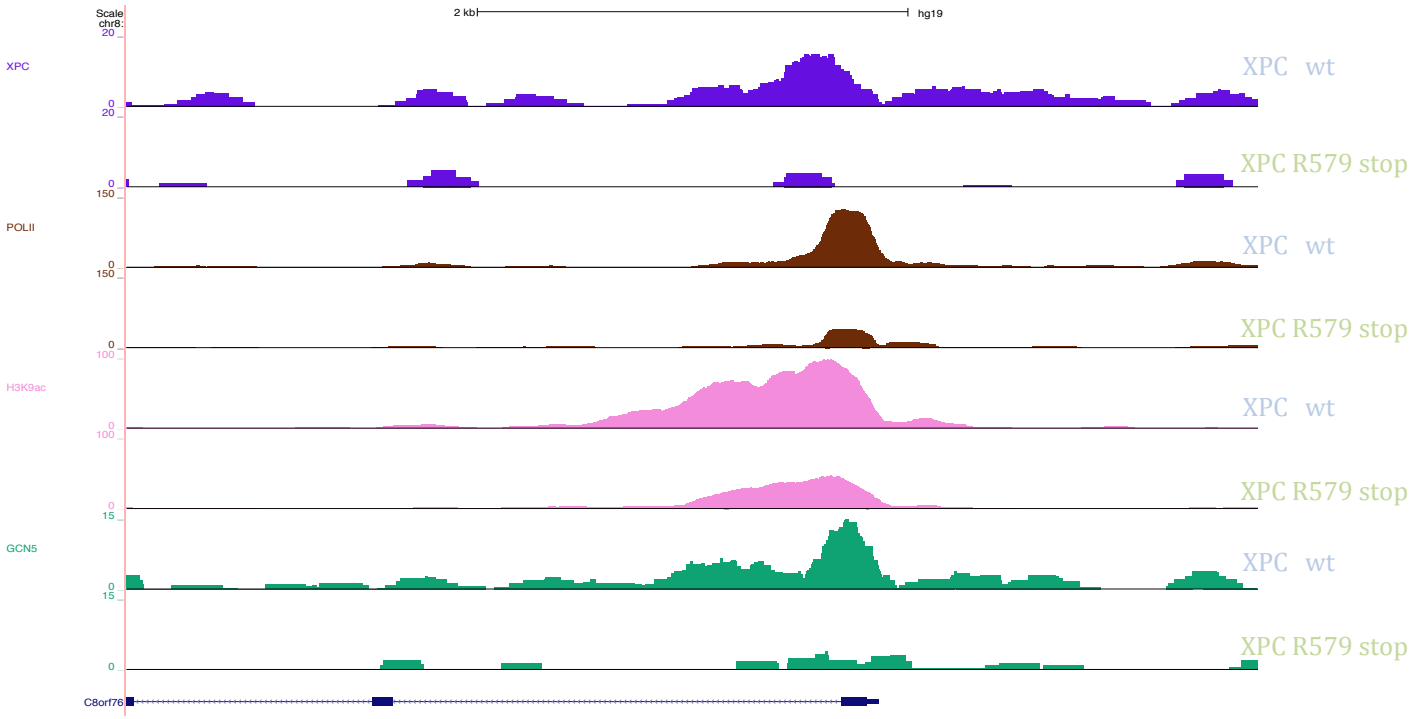
5.1.2.2 XPC and GCN5: regulation of H3K9ac?

It has been previously demonstrated that upon the NER pathway XPC can be stimulated by GCN5, allowing acetylation of H3K9 around DNA damage (*416*). We thus investigated whether the decrease of H3K9ac observed by ChIP-seq in XP-C cells at promoters bound by XPC could reflect physical and functional relationships between this NER factor and GCN5.
ChIP-seq of GCN5 yielded 19 763 binding events mainly enriched at promoters as expected. Among the XPC and pol II co-occupied promoters previously determined, we identified 1 109 common binding events with GCN5 including around 500 promoters. Similar ChIP-seq performed on XP-C derived patient cells indicated an important default of GCN5 recruitment around TSS of XPC-bound genes that correlated with the significant decrease of the amount of promoter-associated H3K9ac (Figure 12A and B). Interestingly, the silencing of GCN5 did not disturb the recruitment of pol II and XPC whereas H3K9ac remained low indicating that the NER factor might help the recruitment of GCN5 at active promoters (data not shown).
Mass spectrometry analysis of GCN5 contained-complexes suggested a putative association with XPC (Laslo Tora personal communication). We next wondered whether there was a direct interaction between XPC and GCN5. We observed that XPC co-immunoprecipitated with GCN5 when either the two protagonists were ectopically expressed in 293T cells by transient transfection or by using recombinant purified proteins (Figure 12C). Using XPC deletion mutants corresponding to mutations found in XP-C patients, we identified by co-IP with purified recombinant proteins a central domain important for the interaction with GCN5 encompassing the region necessary for the hR23B association (Figure 12D).
GCN5 belongs to several complexes including SAGA and ATAC. These complexes are co-activators recruited to DNA-bound activators to regulate the expression of a subset of genes. Recently it has been demonstrated that SAGA plays a critical role for pol II

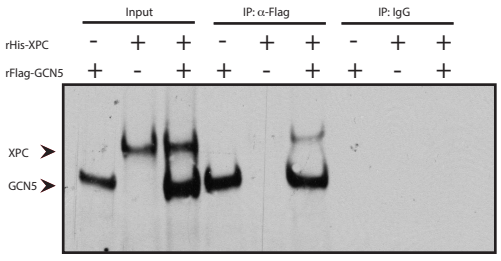
A. Average enrichment of GNC5 on XPC target-promoters from XPC wt and XPC R579Stop chromatins



B. C8orf76 example using UCSC Browser



C. Direct interaction between XPC and GCN5



D. Interaction domain determination on XPC

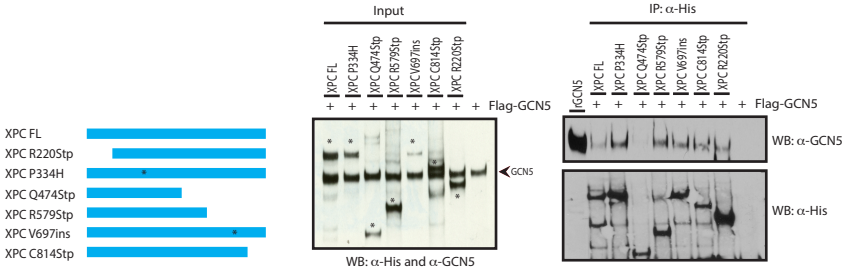


Figure 12. XPC regulates H3K9ac upon transcription through its association with GCN5

A) Comparison of GCN5 ChIP-seq data from XPC WT or XPC R579Stop chromatin and average enrichment for XPC-bound promoters B) Representation using UCSC browser of C8orf76 targeted by XPC at its promoter C) Co-IP between purified XPC and GCN5 expressed Sf9 cells infected with the corresponding baculoviruses D) Co-IP between purified GCN5 and deletion XPC mutants represented schematically on the left panel.

recruitment at all expressed genes and acts on the whole transcribed genome by acetylating the promoters (H3K9ac through GCN5) and deubiquitinates the transcribed regions (H2Bub) (*417*). We thus investigated whether XPC through GCN5 can be co-recruited with SAGA or ATAC. We observed by ChIP at *RARB2* promoter targeted by XPC only subunits of ATAC with GCN5. ChIP-seq will be performed to compare the distribution of ATAC and SAGA complexes with our data sets for XPC and GCN5.
To functionally define this new interaction, we recently started *in vitro* histone acetyltransferase assay using H3 tail peptide, the full histone H3, the octamer (H3/H4, H2A/H2B) and nucleosome with purified GCN5, SAGA or ATAC complexes in presence of XPC full length or deleted from the identified GCN5-interaction domain. Preliminary data did not indicate an effect of XPC on the enzymatic activity of GCN5 suggesting a model in which XPC would be a platform facilitating the recruitment of the HAT at promoters. Further experiments are needed to strengthen our conclusions.
Finally, we correlated the transcriptional defect of several XP-C patients-derived cells with the absence of GCN5 and H3K9ac at XPC-regulated genes. We now aim to induce the transient ectopic expression of full-length in XP-C cells to show the restored transcription of XPC-target genes with the recruitment of XPC and GCN5 at promoter concomitantly to the detection of H3K9ac. In contrast, we expect that the expression of mutated XPC invalidating the interaction with GCN5 will still fail to restore the transcriptional default.

5.1.3 XPC and Histone variant H2A.Z

The Ali Hamiche's team recently published the proteomic analysis of H2A.Z and among the identified partners we could notice the presence of XPC (*418*). Interestingly, our proper proteomic analysis of XPC revealed also H2A.Z as putative associate. The PhD student Maryssa Semer thus started to test the relevance of such association.
H2A.Z is an evolutionary conserved variant of the canonical histone H2A, which shares only 60% sequence identity with major H2A. This variant crucial for the viability of metazoans has been involved in various functions including both gene activation (*419*) and gene silencing (*420*), nucleosome turnover (*421*), DNA repair (*422*), heterochromatin silencing (*423*), chromosome segregation (*424*), progression though the cell cycle (*425*), suppression of antisens RNAs (*426*) or ES cells differentiation (*427*). H2A.Z is enriched in a bimodal distribution at nucleosomes surrounding the TSS of both active and poised genes promoters (*428*). The exchanges between H2A and H2A.Z are tightly regulated involving ATP-dependent SRCAP and p400 complexes for the incorporation of H2A.Z and INO80 or ANP32E complexes for the reciprocal action.
Recently a role for H2A.Z in gene deregulation in cancer has been suggested. *H2A.Z1* and *H2A.Z2* mRNA expression and protein levels are increased in several human malignancies including colorectal cancer, breast cancer or melanomas (*429*). XP patients present important risks to develop skin and also colorectal, lung or breast cancers. We thus decided to firstly investigate the mRNA and protein levels of H2A.Z in XP-C patients-derived fibroblasts. Interestingly, we observed a higher expression for both mRNA and protein (data not shown).
We next compared the H2A.Z genomic distribution revealed by ChIP-seq between XP-C cells and the corresponding rescue. ChIP-seq of H2A.Z yielded 39 044 binding events mainly enriched at promoters as expected. Among the XPC, GCN5 and pol II co-occupied promoters previously determined, we identified 1 106 common binding events with H2A.Z in the rescue cells. Similar ChIP-seq performed on XP-C derived patient cells

indicated a higher presence of H2A.Z around TSS of XPC-bound genes (Figure 13). Interestingly, such deregulated pattern was similarly observed in cells ANP32E-/-(*418*).

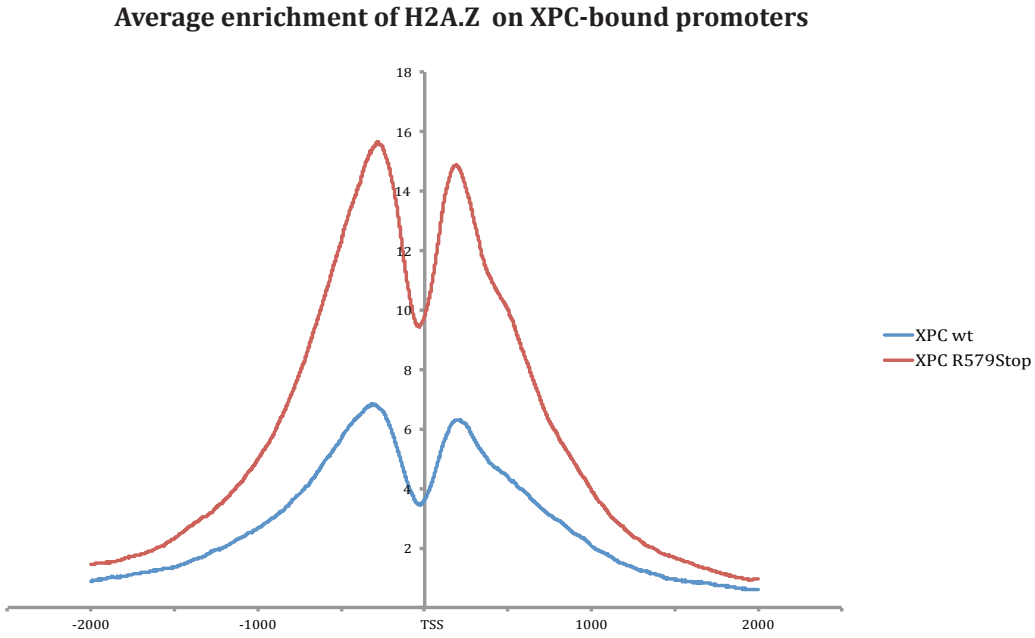


Figure 13. XPC absence led to higher enrichment of H2A.Z around XPC-bound promoters.
Comparison of H2A.Z ChIP-seq data from XPC WT or XPC R579Stop chromatin and average enrichment for the XPC-bound promoters

We next investigated the direct interaction between XPC and H2A.Z by performing i*n vitro* assay using purified recombinant XPC, H2A/H2B or H2A.Z/H2B. Preliminary results indicated a direct interaction through this method (data not shown). By using the different mutated XPC that we already produced, we expect to localize rapidly the interaction domain. We next aim to purify the XPC/H2A.Z complex from chromatin extracts and determine its composition by mass spectrometry. Our proteomic analysis of XPC notably revealed the presence of DMAP1 known to be a subunit of SRCAP. We thus speculated that XPC could belong to a complex participating to the exchange between H2A and H2A.Z. We plan to test this XPC complex in *in vitro* assays for the removal or the incorporation of H2A.Z as previously described (*418*).
H2A.Z is also subject to PTM such as acetylation of its N-terminal region. Recently, it has been demonstrated that acetylated H2A.Z (acH2A.Z) is only localized at the TSSs of active genes and anti-correlates with promoter showing transcriptional negative marks like H3K27me3 and DNA methylation (*428*). Regarding the roles of XPC at promoters in H3K9ac through GCN5 and the XPG/XPF related DNA demethylation, we will investigate a putative involvement of XPC in the acetylation of H2A.Z.
Finally, we will attempt to evaluate the incidence of the deregulation of such functional and physical association between XPC and H2A.Z in the transcriptional and DNA repair defaults observed in XP-C patients-derived cells.

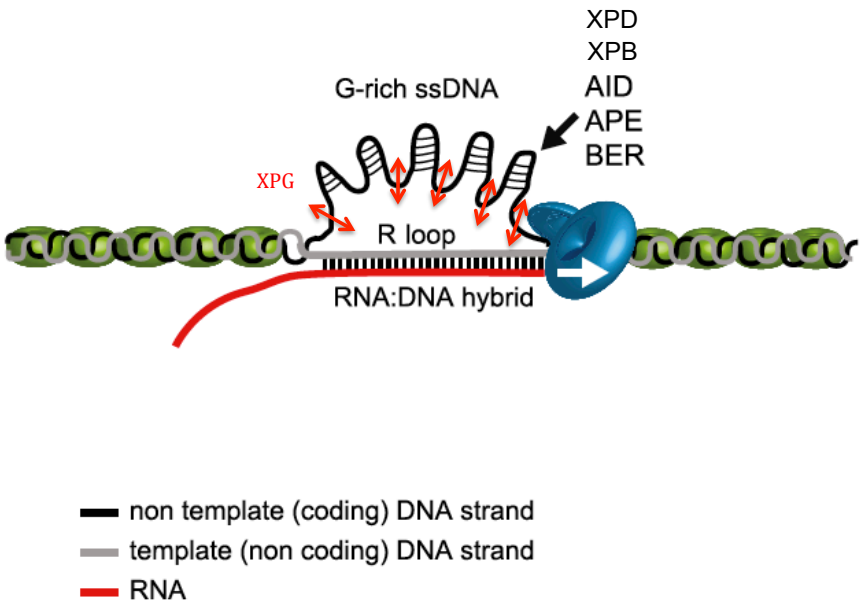
5.2 Roles of XPG and TFIIH in the active DNA demethylation upon transcription

5.2.1 Context

We have recently observed that certain mutations in TFIIH disrupting its interaction with XPG also impaired the recruitment of the endonuclease to active promoters, having in consequence to affect the DNA breaks formation and DNA demethylation. Conversely, other TFIIH mutations disturb DNA demethylation without affecting XPG recruitment and DNA breaks formation. These results suggested that (i) XPG is necessary but not sufficient to achieve DNA demethylation and (ii) TFIIH could be also involved in this process (*8*).

The regulation of gene-specific methyl-cytosine (meC) results from the balance between *de novo* methylation and demethylation of CpG islands. Upon gene activation, promoters are often hypomethylated resulting from the inhibition of *de novo* methylation and activation of the demethylation. Targeted *de novo* methylation is catalyzed by the DNA methyltransferases (DNMT). Reciprocally, demethylation is an active coordinated event involving the ten-eleven translocation factors (TeT) that can convert methyl-cytosine to hydroxymethyl-cytosine (HmeC) as well as other intermediates including formylcytosine (fC) and carboxylcytosine (cC). In this active DNA demethylation model, the methyl-cytosine has to be modified before it can be removed by glycosylation and excision repair through the BER pathway (*237*). It has been shown that active DNA demethylation is associated to G-rich sequences forming RNA/DNA hybrids called R-loops (*430*). This topology localized to GC skew regions can be stabilized by G-quadruplex structure on the non-template DNA strand (*431-433*). Strikingly, TFIIH XPB and XPD helicases are recruited and open G-quadruplex structures (*434*) and XPG is able to cleave R-loops, at least *in vitro* (*435*). The role of R-loops in DNA demethylation is not well understood although it has been demonstrated that such DNA/RNA hybrids protect CpG from the recruitment of DNMTs and can improve through the non-template DNA strand the recruitment of AID, APE or BER factors (Figure 9). Interestingly, R-loops peaks along the human gene are mainly located around TSS and Transcription Terminating Site (TTS) correlating with the XPG/XPF-related DNA nicks and demethylation.

Nothing is known about the putative roles of XPG and TFIIH in the regulation of the DNA demethylation. However, the different observations described above make us speculate that TFIIH and XPG could participate in this process by regulating the formation, stabilization or resolution of R-loops. The PhD student Federico Costanzo is the main investigator of this project and the most recent data are presented in the next part.



From Skourti-Stathaki & Proudfoot, 2014

Figure 14. Schematic representation of DNA/RNA hybrids called R-loop on G-rich sequences that can form G-quadruplex structures on the non-template DNA strand and recognized by the indicated proteins. This R-loop can be also cut by the XPG endonuclease.

5.2.2 XPG and TFIIH: a role in R-loop formation?

Our first goal is to localize XPG and TFIIH subunits along the genome and characterize these regions especially those showing unmethylated CpG associated to transcriptional activity. For instance, we compared the XPG genomic distribution revealed by ChIP-seq in XP-G cells rescued with GFP-XPG wt. ChIP-seq of XPG yielded 23 129 binding events. Interestingly, 22% of the XPG genomic distribution events appeared to be enriched at promoters (Figure 10A and B). In parallel, we also determined the XPB genomic location and compared it to the XPG-binding events. Among the 15 599 XPB genomic distribution events, we identified around 9 629 common binding events with XPG and especially 3 572 peaks that overlapped regions surrounding TSS (Figure 10C). The analysis of the promoters bound by XPG and XPB showed a higher content of CG motif (Figure 10D). We thus performed Methylated DNA Capture coupled to deep sequencing to evaluate the methylated status of these XPG-bound regions by comparing XP-G cells with the corresponding rescue. We observed 43% of the XPG-target promoters presented a differential methylation of CG motif in XP-G cells. These data are still analysed to identify these promoters and investigate the correlation between the presence of XPG and absence of methylation of CG motif. Finally, we also started RNA-seq for these fibroblasts to measure the transcriptional activity of the genes targeted by XPG and XPB. We will next investigate the formation of R-loops and G-quadruplex for several of these newly identified regions/promoters by performing DNA/RNA-IP (DRIP) and DNA IP (DIP) respectively. Federico Costanzo is producing and characterizing antibodies that recognize specifically such DNA topologies and work for IP as it was previously described (*15*). Preliminary DRIP experiments have been performed using our *RARβ2* model. We have observed R-loop formation around promoter upon transactivation in our control cells but not in XPG silenced cells.

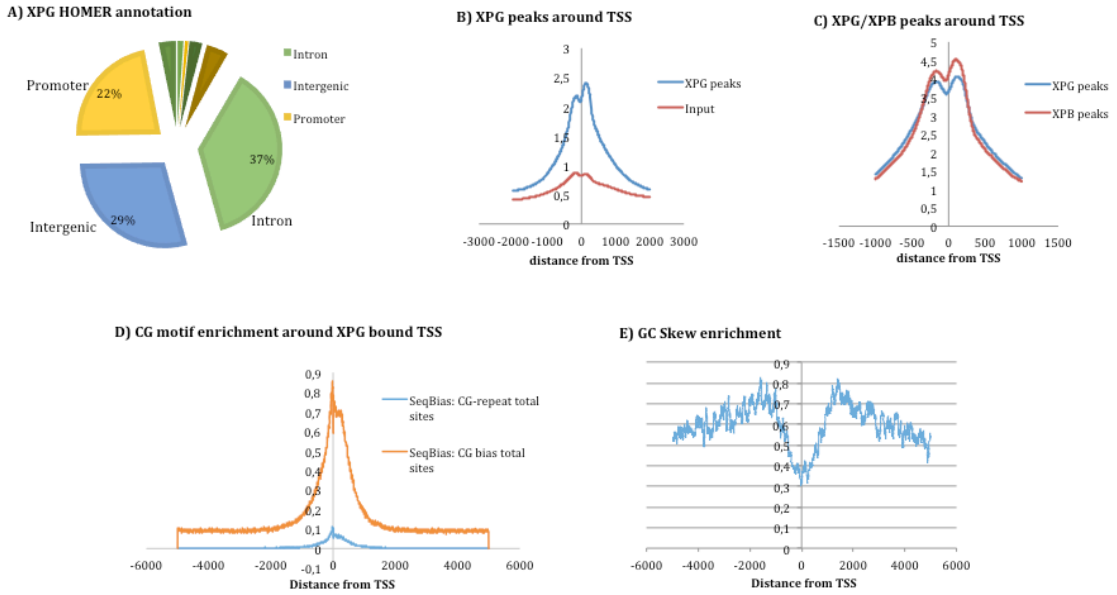


Figure 15. Genome-wide analysis of XPG.
A) Homer annotation of XPG binding events determined by ChIP-seq and B) distribution of these XPG peaks around the TSS. C) Comparison of XPG and XPB peaks that overlapped at promoters. E) CG motif enrichment analysis of XPB/XPG overlapped promoters. D) GC skew enrichment of XPG/XPB enriched promoters.

Meantime the optimization of the DRIP and DIP, we plan to cross our data genome-wide with available DRIP-seq data and hopefully identify some of our XPG-target genes (*15*). Interestingly, we observed using a program predicting GC skew that the XPB/XPG targeted promoters present profiles that matched perfectly with previously determined reverse GC rich promoters (Figure 10E) (*436*).
According to the model of active DNA demethylation, we will next analyse cells bearing mutations on XPG and TFIIH subunits for the presence of TET or BER factors and the different oxidative forms of cytosine including hydroxymethyl-cytosine as well as formylcytosine and carboxylcytosine at several selected regions identified by our genome-wide approaches. We already analysed these intermediate cytosines at *RARβ2* promoter in XPG silenced cells upon transactivation. Compared to the control cells showing unmethylation of cytosine, we observed accumulation of formylcytosine in absence of XPG.
We will also investigate the roles of XPG-related DNA nicks in the active DNA demethylation process by comparing XP-G cells rescued by either XPG wt or the endonuclease dead mutant XPG E791A (*7*). We will first measure the induction of XPG-dependent DNA breaks by Bio-ChIP at several XPG-bound regions showing DNA demethylation and R-loop formation. We will attempt to localize these breaks at the R-loop by pyrosequencing with a particular attention for the non-template DNA strand. Finally, to measure the impact of the endonuclease activity of XPG, we will repeat the different experiments presented here in the cells rescued by XPG E791A. To evaluate the putative roles of TFIIH in this process, similar approaches will be performed using TFIIH mutated cells or cells treated with specific inhibitors like THZ1 for CDK7 (*437*) or the spironolactone for XPB (*438*).

Diseases such as cancer and premature ageing have been genetically characterized by their transcriptional deregulations leading to a defect in the genome integrity. It is well documented that tumor cells are characterized by an abnormal pattern of DNA methylation including high degree of either *de novo* methylation or demethylation of CpG islands. By defining the roles of TFIIH and XPG in the active DNA demethylation process, we expect to establish a strong correlation between the abnormal pattern of DNA methylation and XP phenotypes.

5.3 How NER factors are recruited to promoters?

5.3.1 Context

Beside the elucidation of the functions of NER factors in the chromatin remodelling upon transcription, we also aim to investigate their recruitment modes at promoters. *In vitro* and *in vivo* approaches gave an important background concerning the recruitment of NER factors at DNA lesions. Upon transcription, it has been demonstrated that NER factors are recruited at promoter of rRNA genes through TAF12 and (ii) XPF-ERCC1 interacts with TFIID assembling with the basal transcription machinery on promoters *in vivo* (*11, 12*). Our proteomic analysis of XPC and XPG also allowed the identification of TAF4/TAF12 and TAF6 as putative partners respectively. These TAFs belong to the TFIID complex crucial for PIC formation at core promoter and the initiation of transcription.
The core promoter is a minimal stretch of DNA sequences (TATA boxes, initiator and downstream core promoter element) surrounding the TSS that directly interacts with the components of the pol II transcriptional machinery driving accurate transcription initiation. However, recent data have strongly suggested that the chromatin environment around the core promoter including Histones PTMs like acetylated H3 or variants such H2A.Z also collaborates with the DNA motifs to drive the formation of PIC. Thereby, some TAFs possess specific domains, like bromodomain or YEATS domain that make them interact with histone PTMs consequently directing their recruitment to the core promoter.
Based on these observations, our results on the roles of NER factors on histone PTMs regulation and their interactions with TAFs, we have speculated that the recruitment of NER factors to promoters could necessitate a combination between association with TAFs and Histones PTMs. This project has been initiated and is performed by the PhD student Baptiste Bidon.

5.3.2 XPG and XPC need TAFs to be recruited at promoters

We identified by mass spectrometry TAF4/TAF12 and TAF6 as putative partners of XPC and XPG respectively. These interactions have been confirmed by coIP in cells transiently expressing tagged- NER factors and TAFs. Moreover, we observed by ChIP-reChIP experiments, that XPC co-occupied *RARβ2* promoter with TAF4 and TAF12. We are also analysing these associations in *in vitro* assays using the corresponding purified recombinant proteins.
Our previous ChIP-seq on XPC and XPG combined to RNA-seq in XP-C, XP-G cells and their corresponding rescues defined the list of promoters and genes co-regulated by these NER factors. A bioinformatic analysis will start soon to characterize the identified core promoters and determine eventual specificities on the different DNA motifs.

Another serie of ChIP-seq will begin by including the different TAFs. However, the classical ChIP technology does not allow the precise location of a targeted protein at specific DNA regions like promoter. To overcome this limitation and accurately position NER factors and the TAFs along the genome, we will employ a modified-ChIP approach named lambda exonuclease coupled to Chromatin Immunoprecipitation (ChIP-exo) (*439*). We will measure the relevance of these TAF-NER factors interactions by silencing TAFs and investigating the presence of XPC and XPG on their targeted promoters by ChIP.
By identifying the important domains of NER factors for the interactions with the TAFs, we will expect to be able to discriminate these associations from their roles in the chromatin remodelling. It will be therefore possible to measure the impact of each domain or functions for the recruitment of NER factors at promoters.

5.4 Perspectives

Altogether my past and current projects will reconstitute the cascade of the different events involving the NER factors in the regulation of gene expression. We expect to decipher their recruitment modes, roles for chromatin remodelling at promoters and the reciprocal or mutually exclusive relationships between these different mechanisms. However, our ChIP-seq data indicated that the location of NER factors was not restricted to promoters since we could detect them also at regions presenting enhancers hallmarks. According to the NER factors-related gene looping formation between promoter and terminator *RARβ2* and the importance of enhancer/promoter rearrangements in transcription, it would be interesting to investigate the roles of these DNA repair factors in chromatin looping and architecture using a genome-wide approach like Chia-PET.
These projects deepen our knowledge of gene expression and demonstrate the connections with the initially unrelated DNA repair pathway. However, it would be important to define the common functions in the both pathways from the specific ones because behind fundamental research, our results have also a medical stake. Indeed, these projects have been based on cells derived from XP, TTD or XP/CS patients bearing mutations on NER factors. The molecular characterization of the transcriptional and DNA repair defects for each mutation can facilitate the diagnosis by identifying relevant markers for future patients. Nevertheless, our cellular model still limits us for a better explanation of the different phenotypes and the diverse clinical features.
To overcome this problem, I would like to develop projects more medically translational. I recently started to follow, in collaboration with clinicians, two undiagnosed patients presenting strong evidences for XP. We obtained first set of data confirming an NER defect for the both patients. We also showed that one patient possessed two mutations on *XPC* (including a new one) correlating with a strong decrease of XPC at mRNA and protein levels. Our next goal is to identify the mutations for the second patient. According to our experience in this field, we will characterize precisely the transcriptional and DNA repair defects. By developing a close collaboration with clinician and families' patients, we expect to follow the evolution of some clinical features by elaborating protocols to collect samples from healthy skin or skin cancers for example. We expect therefore to better understand the aetiology of skin cancers in patients bearing mutations on NER factors.
However, the involvement of NER factors in transcription still remains controversial. Indeed, it is very difficult to conciliate the fact that XP patients can live normally by

protecting them from UV lights and exogenous genotoxic attacks whereas these NER factors mutated in this genetic disorder are supposed to be crucial for the gene expression. Our ChIP-seq data from XP-patients derived rescued fibroblast indicated that NER factors could target a small amount of genes related to transcription. Studies from Tjian's laboratory recently described XPC-containing complex as a co-activator with also transcription factors SOX2 and OCT4 that controlled the transcriptional program for the pluripotency in ES cells. In XPC $^{-/-}$ ES cells, the pluripotency could not be maintained whereas the differentiation was engaged (*13, 14*). We should therefore expect that XP-C patients bearing XPC mutations leading to the absence of protein showed early developmental defaults. However, XP-C patients got born normally without clinical features that appeared later during the childhood. A spatio-temporal regulation of NER factors and consequently their functions exist, as demonstrated by Tjian's reports with a decreased expression of NER factors upon differentiation of ES cells (*110*). As we showed for TFIIH for the AR turnover (*440*), we have also to consider and identify alternative pathways that can replace and compensate the regular one through a different dynamic in a pathological context.

Finally it would be a mistake to limit NER factors and their related genetic disorders in the transcription/DNA repair duality. Indeed, it is already known that TFIIH subunit XPD has been found either with CAK subcomplex to regulate the mitosis entry or in another complex called MMXD involved in the chromosomal segregation (*29, 235*). In the same line, CSB protein can be also associated to mitochondrial functions that are deregulated in a CS context (*441, 442*). So, we have to keep an opened mind about the functions of NER factors.

6 Future project

6 .1 International context

During the past decade, the emergence of either emerging or re-emerging virus diseases in new areas of the world occurred with increasing frequency and originated a serious public health concern and economical losses. Arboviruses are distributed worldwide and represent approximately 30% of all emerging infectious diseases during the last decade (*296*). Viruses such as West Nile (WNV), Chikungunya (CHIKV), Dengue (DENV), Yellow Fever (YFV), Crimea-Congo Hemorrhagic Fever (CCHF) and Rift Valley Fever (RVFV) viruses, which are pathogenic for humans and/or animals have emerged outside of their usual endemic range and caused epidemics in North America, Europe and the Arabian Peninsula. Their emergence may be related to the climate fluctuations due to the global warming and/or human activities that facilitate the dispersion of the arthropods beyond their current geographic boundaries (*297, 298*). Studies on these arboviruses thus become a major issue since neither safe vaccine for protection nor antiviral treatments for therapy is currently available.

Among these pathogens, several viruses belong to the *Bunyaviridae* family. This family is composed of RNA viruses grouped into five genera—*Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus* characterized by their antigenic, genetic and ecological properties. Orthobunyaviruses, nairoviruses and phleboviruses infect vertebrates and are vectored by haematophagous arthropods including mosquitoes, ticks, midges and sandflies whereas tospoviruses are plant pathogens and are vectored by different thrips (*301*). In contrast, hantaviruses are not transmitted by arthropods but by rodents and insectivores which act as reservoirs. Infections by hantaviruses are persistent in their reservoir hosts and humans become infected through contamination by excretions or carcasses of infected reservoirs (*302*). All the members of this family are enveloped, spherical virions between 80-120 nm in diameter with its replicative cycle in the cytoplasm and maturation/budding of the newly formed particles in the Golgi apparatus (*303, 304*). These viruses possess single-stranded RNA genomes that consist of three segments L (Large), M (Medium) and S (Small) having a negative- or ambi-sense polarity (*301*). The L and M segments code respectively for the viral RNA-dependent RNA polymerase (L-RdRp) and a precursor to the envelope glycoproteins (Gn and Gc). The S segment codes for the internal protein N or nucleocapsid protein that is able to oligomerize and associate with the viral polymerase and the three different segments of the viral genome to form viral ribonucleoparticles (RNPs) packaged into virions. Depending on the genus, other proteins are encoded by the M and S segments, namely the non-structural proteins NSm1, NSm2 and NSs harbouring multiple roles. The virulence of the pathogenic bunyaviruses illustrates the permanent co-evolution between the viruses and their hosts. The virus utilizes cellular proteins or functions to promote their own replication/transcription. This is particularly well illustrated in the case of bunyaviruses which utilize capped oligonucleotides from host mRNAs to prime transcription through the cap-snatching mechanism mediated by the L-RdRp which possesses an endonuclease activity to cleave the capped oligonucleotides (*305*). In response to viral infections, the host induces an important transcriptional

reprograming to activate various defences including the innate immunity. The virulence of the pathogenic bunyaviruses is directly linked to the roles of viral virulence factors and their capacity to counteract the host pathways. The initial response to infection involves the production and secretion of type I interferons (IFNs) followed by a subsequent amplification phase linked to induction of other IFNs subtypes. In response to the type I IFNs action, each virus targets specific cellular proteins and usually possesses unique strategies to counteract directly or indirectly such transcriptional reprograming.

6.2 Bunyaviruses and escape of the host transcriptional reprograming

The diversity and originality in the strategies to hijack the host transcriptional reprograming are particularly well illustrated for the different pathogenic bunyaviruses as you could already read above in this manuscript (*23*).
Finally, the different proteins expressed by these bunyaviruses induce differently the deregulation of the host transcription reprograming. Such viral proteins, like NSs, have often several cellular partners reflecting their multi and overlapping functions. However to fully understand their roles, new investigations are necessary to:

- Describe the mechanisms explaining how these interactions can lead to an inhibition of the host RNA synthesis,
- Measure the impact of one interaction on the genome-wide transcription, on the organization of chromatin and more globally the nucleus organization,
- Integrate these different viral mechanisms and understand their spatial and temporal orchestration during the viral cycle,
- Evaluate the consequences and the benefits for the viral cycle.

My future project aims to identify and dissect the various strategies developed by different genera of the *Bunyaviridae* family to overcome the host transcriptional reprograming. The signalling cascades triggered to subvert the cellular machineries in mammalian hosts are important themes for the later development of efficient vaccines or/and antiviral agents.
To fully understand the viral deregulation of this nuclear process, my project aims therefore (i) to characterize the complexes containing the viral proteins with their host partners and (ii) to determine the transcription profile and chromatin landscape at genomic AND gene levels upon infection.

6.2.1 RVFV and BUNV models

This project starts with the study of RVFV and Bunyamwera virus (BUNV) that induce pathogenesis mechanisms that are clearly associated to the global host transcriptional shut off due to, at least, the relation between NSs/TFIIH for RVFV and NSs/Mediator for BUNV. TFIIH and the Mediator are crucial for transcription playing numerous functions. They can control the phosphorylation of RNA polymerase II, the recruitment and the activity of GTFs, proximal pausing, elongation factors and histone modifying enzymes (*443, 444*). TFIIH and the Mediator through the NER factors and the Cohesin complex respectively also regulate chromatin rearrangements (*6, 445*). Finally these complexes are associated with proteolysis via their relationships with Ubiquitin E3 ligases (*440, 444*).

The interactions between RVFV NSs and TFIIH p44 subunit or BUNV NSs and Med8 are well defined but the effects on the multiple functions of these cellular complexes and the consequences on transcription profile of the infected cells are not known. The first part of the project aims to study these interactions, their mechanisms and investigate their impact on the host transcriptional silencing.

6.2.2 Pathogenesis mechanisms for other phleboviruses or hantaviruses

Although it seems likely that NSs proteins of different bunyaviruses have their specific partners and unique mode of actions, such viral proteins may have conserved functions to inhibit the host response. Indeed, the NSs proteins from other human pathogenic phleboviruses like Toscana (TOSV) and Punta Toro (PTV) viruses are also involved in the inhibition of IFNβ-induction (*361, 362*). In addition, the S segment of some hantaviruses also possesses an additional open reading frame (ORF) coding for a non-structural protein NSs. The NSs protein of Tula (TULV) and Puumala (PUUV) hantaviruses expressed via recombinant plasmids weakly inhibited the induction of IFNβ and the activation of IRF-3 and NF-κB responsive (*346*).
However, the cellular partners and the precise pathogenesis mechanisms disturbing the host transcription linked to the NSs proteins of these phleboviruses and hantaviruses are totally unknown. This part of the project first aims to identify the host complexes associated to these viral proteins. Using the similar methodology developed in the RVFV and BUNV models, the impact of the newly identified NSs-containing complexes on the different parameters controlling the host transcription will be investigated.

6.3 Predictable consequences and perspectives

6.3.1 Vaccines, antiviral and virulence test

Altogether the data on how bunyaviruses counteract the host transcriptional reprograming to evade the cellular response will also improve our knowledge of fundamental process like transcription. For orthobunyaviruses and phleboviruses, NSs proteins play a crucial role but in the case of hantaviruses, less is known on the antagonistic activities against host transcription although also other proteins such as the nucleocapsid, the glycoproteins and the L polymerase appear to have IFN antagonist functions. Such studies are important for the production of rationally designed attenuated vaccines. In the case of RVFV, the naturally avirulent Clone 13 carrying a large deletion in the NSs ORF appeared as a good vaccine candidate to protect against virulent RVFV (*365*). The creation of mutated viruses by reverse genetics that has been a valuable tool to decipher the function of the non-structural proteins (*2, 316, 318*), as proposed here, could help for the development of antivirals targeting the different steps of the viral cycle, which would be of great value for the treatment of these infections.

In addition to the classical tools used to identify and test the bunyaviruses, the description of such pathogenesis mechanisms can lead to the identification of new virulence markers to characterize unknown strains. In RVFV vaccine strains MP12 or Smithburn presenting important secondary effects, the effect of NSs on TFIIH and consequently the host transcription remained strong whereas the good vaccine candidate Clone 13 expressing inactive and deleted NSs did not inhibit the host RNA

synthesis (*3*). Although it seems likely that NSs proteins have their specific partners, such viral proteins may have conserved functions to inhibit the host response. It could be therefore very interesting to determine common transcriptional marks for the phleboviruses, for example, that will indicate the putative virulence of the infection. To show the relevance of such tool, one should recall the newly identified phlebovirus isolated in China. It is transmitted by ticks and closely related to the tick borne Uukuniemi (UUKV) virus. However, in contrast with UUKV, which is not pathogenic for humans, this novel phlebovirus designated severe fever with thrombocytopenia syndrome virus or Huaiyangshan virus depending on the laboratory where it was isolated, is responsible for severe thrombocytosis and multi-organ dysfunction with high morbidity and mortality (initial fatality rate 30%) (*299, 300*).

6.3.2 Bunyavirus and DNA damage repair

Up to now, most of the studies focused on the viral functions antagonizing the innate immune response which is an immediate and the first line of defence. However, viruses have evolved other strategies to target cellular functions like DNA Damage repair (DDR), which also participates to the antiviral response and will deserve to be studied in the near future. It was recently demonstrated that RVFV via NSs was able to activate DDR through ATM, Chk.2 and p53 inducing S-phase arrest and consequently creating a favourable environment for viral replication. The interconnection between the induction of DDR pathways and inhibition of host transcriptional reprograming will constitute further important perspectives and investigations.

6.3.3 Comparison of the subverting mechanisms by viral proteins between vector and mammalian host

Orthobunyaviruses and phleboviruses infect vertebrates and are vectored by arthropods. Once the mechanisms in bunyaviruses-infected mammalian cells that block the host transcription will be identified, one perspective would be to investigate the conservation of these pathways in the vector. In arthropods, the innate immune system is different and the chromatin remodelling upon transcription also involves distinct modifications although the basal machinery is quite conserved. The next question is to know if it is possible to link the absence of virulence of these bunyaviruses in their vectors to either the absence of conservation of the pathogenesis mechanisms or the existence of other immune pathways that counteract the viral proteins.

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