CDK11(p58) is required for centriole duplication and Plk4 recruitment to mitotic centrosomes.
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CDK11p58 Is Required for Centriole Duplication and Plk4 Recruitment to Mitotic Centrosomes

Nathalie Franck*, Emilie Montembault*, Pierre Romé, Aude Pascal, Jean-Yves Cremet, Régis Giet*

UMR6061-CNRS, Institut de Génétique et Développement de Rennes, Université de Rennes I, Rennes, France

Abstract

Background: CDK11p58 is a mitotic protein kinase, which has been shown to be required for different mitotic events such as centrosome maturation, chromatid cohesion and cytokinesis.

Methodology/Principal Findings: In addition to these previously described roles, our study shows that CDK11p58 inhibition induces a failure in the centriole duplication process in different human cell lines. We propose that this effect is mediated by the defective centrosomal recruitment of proteins at the onset of mitosis. Indeed, Plk4 protein kinase and the centrosomal protein Cep192, which are key components of the centriole duplication machinery, showed reduced levels at centrosomes of mitotic CDK11-depleted cells. CDK11p58, which accumulates only in the vicinity of mitotic centrosomes, directly interacts with the centriole-associated protein kinase Plk4 that regulates centriole number in cells. In addition, we show that centriole from CDK11 defective cells are not able to be over duplicated following Plk4 overexpression.

Conclusion/Significance: We thus propose that CDK11 is required for centriole duplication by two non-mutually-exclusive mechanisms. On one hand, the observed duplication defect could be caused indirectly by a failure of the centrosome to fully mature during mitosis. On the other hand, CDK11p58 could also directly regulate key centriole components such as Plk4 during mitosis to trigger essential mitotic centriole modifications, required for centriole duplication during subsequent interphase.

Introduction

The centrosome of somatic cells is the main microtubule organising center [1]. It is required to organise the cytoplasmic microtubule network during interphase and the mitotic spindle during mitosis. This organelle consists of two centrioles embedded in an amorphous pericentriolar material (PCM). In proliferating cells before division, the centrosome needs to be duplicated precisely once so that the mitotic cell harbours two centrosomes, each comprising two centrioles. These two centrosomes will be used to nucleate the microtubules required to assemble the mitotic bipolar spindle during mitosis [2]. The centrosome duplication cycle is divided into several key steps. First, during mitosis, the two paired-centrioles inherited by the daughter cell lose their orthogonal configuration, a process called disengagement. Then, procentrioles (daughter centrioles) nucleate in G1/S phase at an orthogonal angle next to each mother centriole. The two newly synthesised centrioles elongate during the S and G2 phases. In late G2, as cells prepare for mitosis, centrosomes increase in size and recruit additional PCM to enhance their ability to nucleate microtubules. This process is referred to as centrosome maturation. At the end of the G2 phase, the two newly duplicated centrosomes separate to organise a bipolar mitotic spindle, enabling each daughter cell to inherit one centrosome after cell division.

In contrast to normal cells, tumour cells frequently show centrosome number defects that are thought to be the consequence of abnormal regulation of the centriole duplication machinery. Thus, the restriction to a single round of centriole duplication per cell division cycle contributes to the prevention of aberrant centrosome numbers, multipolar spindles and chromosomal instability [3,4].

Several proteins are critical for the canonical centriole assembly. Based on genome-wide RNAi and genetic screens in C. elegans, a molecular hierarchy for the recruitment of five essential proteins for centrosome assembly has been established. SPD-2 is necessary to recruit ZYG-1 at the centriole. Then, both proteins in turn recruit SAS-5 and SAS-6, followed by SAS-4 [5,6,7]. Most of these proteins have conserved roles in centriole duplication in other systems. Cep192, the human homolog of C. elegans SPD-2 protein, is a pericentriolar protein required for both centriole duplication and centrosome maturation [8,9]. Plk4, related to C. elegans ZYG-
Figure 1. CDK11p58 depletion leads to a reduction in centriole number in mitotic HeLa cells. Mitotic HeLa cells stably expressing a GFP-tagged centrin were subjected to control or CDK11 RNAi and analysed after 72 hours. A) Western blots showing CDK11p110 and CDK11p58 (with 10 and 600 seconds exposure times respectively) and actin protein levels are shown. The positions of CDK11p110 and CDK11p58 are indicated. B) Mitotic cells stained for DNA (blue) and centrin (red) following control or CDK11 RNAi. Some of the observed defects are shown here. The insets show a 3× magnification view of the centriole region in monochrome. The treatment is displayed at the bottom of each panel. Scale bar is 5µm. C) Quantitative analysis of the centriole distribution following control, CDK11, Plk4 or double RNAi in mitotic cells. See the reduced number of centriolpe following CDK11 RNAi (also shown in Table S1). D) Example of the mitotic figures observed in control, CDK11, Plk4 and double (CDK11 and Plk4) siRNA-transfected cells. See also detailed analysed in Table S1. Centrioles are green, microtubules are red and chromosomes are blue. The insets show a 3× magnification view of the centriole region in monochrome (indicated by a white triangle in the merge panels). Bar is 5µm.

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1, as well as CPAP and HsSAS-6, human homologs of C. elegans SAS-4 and SAS-6, have been identified as key regulators of centrosome duplication [8,10,11,12]. For example, overexpression of Plk4 leads to centriole amplification; conversely, mutations or inhibition of the DmSAK/PLK4 gene dramatically impairs centriole duplication [10,11]. Of these proteins, Plk4 is the only enzyme. It is a short-lived protein kinase that localises at centrioles throughout the cell cycle. Its degradation and activity are tightly regulated during the cell cycle, these two processes being crucial for the centriole biogenesis process [13,14].

SAS-6, Clik2, CDC25c and Hand1 remain the only identified substrate of Plk4/SAK/ZYG-1 [15,16,17,18,19,20]. The physiological relevance of CDC25c and Chk2 phosphorylation events by Plk4 is unknown. SAS-6 phosphorylation by ZYG-1 in C. elegans triggers its targeting to the newborn centriole. The Plk4-mediated phosphorylation of the Hand1 transcription factor inhibits its sequestration in the nucleolus. Hand1 is then released in the nucleus where it activates the transcription of genes required for cell differentiation. Interestingly, a growing list of studies in different models shows that Plk4 centrosomal levels and activity are maximal during mitosis, suggesting the kinase also plays a key role during mitosis, possibly to licence the centriole for subsequent duplication in S phase. However, this possible role remains speculative and difficult to challenge experimentally [14,21].

Cyclin-dependant kinase 11 (CDK11) mRNA produces a 110 kDa isoform protein expressed throughout the cell cycle and a 58 kDa isoform protein which is specifically translated from an internal ribosomal entry site sequence during G2/M [22]. The small isoform has been shown to be an essential regulator of mitosis. Indeed, CDK11p58 is required for centrosome maturation, bipolar spindle assembly, maintenance of sister chromatid cohesion and cytokinesis [23,24,25].

In this article we describe a new role for CDK11p58 in the duplication of centrioles. Our data reveal that CDK11 is necessary to ensure proper centriole duplication. Furthermore, we identify Plk4 as a novel partner of CDK11p58 and show that CDK11p58 depletion impairs the proper recruitment of Plk4 to mitotic centrosomes.

Results

We have shown previously that CDK11p58 is required for centrosome maturation (Petretti et al., 2006). A growing list of studies reports that failure to properly orchestrate centrosome maturation can trigger centriole duplication defects. As an example, Cep192 is a major regulator of both centrosome maturation and centriole duplication in C. elegans and in mammalian cells [7,8,26]. These data prompted us to investigate whether CDK11p58 was also required for centriole duplication. To do so, we first looked at the effects of a three-day siRNA-mediated depletion of CDK11 on centriole numbers in asynchronously growing mitotic cells (Figure 1 A). HeLa cells stably expressing GFP-centrin protein [27] were transfected with control or CDK11 specific siRNAs [24] and centrioles were quantified on mitotic figures after fixation of the cells (Figure 1 D). As expected, 90.6% of control metaphase cells showed four centrioles with two centrin dots at each pole of the mitotic spindle (Figure 1 and Table S1). By contrast, after depletion of CDK11, as shown previously, only 37.9% of the spindles were bipolar [24]. Amongst these, only 10.8% of the cells looked normal with two centrin dots at each pole whereas 26.9% of the cells showed spindles with less than two centrioles at one pole. The remaining mitotic cells appeared to form very short or monopolar spindles (54.4%) and all of them showed a reduction in centriole numbers (i.e. less than four centrioles in total). Plk4 RNAi treatment, used as a positive control (Figure 1 C, D and Table S1), yielded highly similar phenotypes with only 14.4% of the cells showing bipolar mitotic spindles with four centrioles, two at each pole. The vast majority of the mitotic cells displayed monopolar and disorganised spindles and showed less than four centrioles. Interestingly, co-depletion of Plk4 and CDK11p58 proteins resulted in an 8- to 9-fold decrease of the number of cells showing bipolar spindles with four centrioles (10.8% and 14.4% respectively in CDK11- and Plk4-depleted cells vs. 1.5% in co-depleted cells). We observed a dramatic increase in the number of cells with only one centriole when both CDK11 and Plk4 were knocked down (13.9% and 17.8% respectively in CDK11 and Plk4-depleted cells vs. 67.8% in co-depleted cells) (Figure 1 C, D and Table S1).

Most of the proteins required for centrosome duplication localise to this structure. To localise CDK11p58 protein kinase within the cell, we decided to generate a HeLa “Tet-ON” cell line which allows the temporally controlled expression of Myc-tagged CDK11p58 (Figure 1 S1). The Myc antibody readily recognised one specific band in non-induced or control cells validating our Tet-inducible expression system. Immunofluorescence experiments were performed after a very short period of induction (1 to 2 hours), when the protein was expressed at low level. We found that Myc-tagged CDK11p58 protein kinase was cytoplasmic in interphase (Figure S1 A and B). During early prophase, CDK11p58 was principally detected around the separating centrosome and on microtubules by cold treatment, no obvious localisation of CDK11p58 was observed at the mitotic centrosome suggesting that the centrosomal localisation is microtubule dependent (data not shown) and also in transiently transfected cells (Figure 2 A) but did not reveal any specific bands in non-induced or control cells validating our Tet-inducible expression system. Immunofluorescence experiments were performed after a very short period of induction (1 to 2 hours), when the protein was expressed at low level. We found that Myc-tagged CDK11p58 protein kinase was cytoplasmic in interphase (Figure S1 A and B). During early prophase, CDK11p58 was principally detected around the separating centrosome and on microtubules by cold treatment, no obvious localisation of CDK11p58 was observed at the mitotic centrosome suggesting that the centrosomal localisation is microtubule dependent (data not shown). CDK11p58 persisted on microtubules emanating from the poles and on the central spindle during telophase and was finally enriched on both sides of the midbody during cytokinesis (Figure S1). Interestingly enough, longer induction to express high levels of Myc-tagged protein increased the cytoplasmic pool resulting in the masking of the signal on mitotic microtubules. This latter result might suggest the existence of a limited number of CDK11p58-binding sites on the mitotic spindle.

In this article we describe a new role for CDK11p58 in the duplication of centrioles. Our data reveal that CDK11 is necessary to ensure proper centriole duplication. Furthermore, we identify Plk4 as a novel partner of CDK11p58 and show that CDK11p58 depletion impairs the proper recruitment of Plk4 to mitotic centrosomes.
A

Myc-CDK11P58  -  -  +  
Myc-CDK11-P58DN  -  +  -  

Myc-Plk4  -  +  -

Myc
-150
-100
-75
-37
Actin
-37

B

Transfection of Plasmodium CDK11 or CDK11-DN
Aphidicolin
Fixation/counting

0  24  72

C

Control  Myc-CDK11P58  Myc-CDK11P58DN  Myc-Plk4

DNA-Centrin-GFP

Centrin-GFP

71%
69%
58%
83%

4 centrioles  4 centrioles  2 centrioles  >4 centrioles

D

% of cells

<4 centrioles  4 centrioles  >4 centrioles

Cont.  CDK11P58  CDK11P58DN  Plk4
The RNA interference of known regulators of centriole duplication leads to a decrease of the centriole number. By contrast, only a very low number of these molecules, including the Plk4 protein kinase, triggers centriole amplification via their overexpression (reviewed in Bettancourt 2009). To continue the investigation on the function of CDK11p58 in centriole biogenesis, we decided to check whether the overexpression of a Myc-tagged wild-type CDK11p58 protein, a kinase-dead variant (CDK11p58DN) and Plk4 (as a positive control) could modify centriole duplication (see Figure 2 A); CDK11p58, CDK11p58DN, Plk4 were transiently expressed in HeLa cells stably expressing the GFP-centrin protein. A day after transfection, aphidicolin was added for 48 hours on these cells to arrest them in S phase (Figure 2 B). Cells were then immuno-stained with Myc antibody and the centrioles of Myc-positive arrested cells were counted (Figure 2 C). As expected, the vast majority (71%) of control cells had duplicated their centrioles during this arrest and thus exhibited four centrioles per cell (two pairs of two closely-linked centrioles). When compared to controls, cells overexpressing CDK11p58 did not show significant differences in their centriole numbers (∼70%, Figure 2 D). By contrast, the overexpression of the catalytically inactive CDK11p58DN caused a decrease in the number of cells with four centrioles. Indeed, only 38% of these cells contained four centrioles, while 58% failed to duplicate their centrioles, resulting in the presence of cells with either two or three centrioles (Figure 2 C and D). In a parallel assay, the ability of these cells to amplify their centrioles after Plk4 overexpression was assessed. As published before [11], most of the Myc-Plk4 positive cells amplified their centrioles after Plk4 overexpression was added for 48 hours on these cells to arrest them in S phase by aphidicolin treatment for 24 hours and the centrioles were counted after 48 more hours. C) The transfected cells expressing Myc-tagged proteins were visualised using an anti-Myc antibody (red), DNA is blue. The centriole (green), indicated by arrows, (also displayed in monochrome in the lower panels) were counted in control or transfected cells. In cells expressing CDK11p58 as well as in control cells, the vast majority of the cells (∼70%) displayed four centrioles. This percentage dropped to 38.2% when CDK11p58DN was expressed. Therefore, the dead variant of CDK11p58 has the ability to inhibit cells amplified their centrioles (Figure 2 C, right panels, and D).

Previous experiments showed that CDK11-depletion diminished γ-tubulin and Plk1 recruitment at the centrosomes during centrosome maturation [24]. Interestingly, Plk1 depletion abolishes Cep192 recruitment at centrosomes [8,26]. We first decided to analyse the levels of Cep192 protein at mitotic centrosomes (containing 2 centrioles) following control or CDK11 RNAi (Figure S3 A). Quantification of the signal revealed that centrosomal Cep192 was reduced by 3-fold at centrosomes in mitotic CDK11-depleted cells (Figure S3 B) but the interphase centrosome level was unchanged (data not shown). SPD-2, the worm counterpart of Cep192 is also required for ZYG-1, the related orthologue of Plk4, to be recruited at centrosomes [5,7]. Plk4 is a centriole-associated protein kinase. The protein kinase levels in the whole cell and at the centrosome are elevated during mitosis, by contrast to interphase. We next monitored whether CDK11p58 was also implicated in Plk4 recruitment to the centrosomes in mitosis. Control and CDK11-RNAi treated cells were stained with Plk4, centrosome (Figure 3 A) or centrioles probes antibodies (Figure 3 B). Strikingly, CDK11 depletion also impaired Plk4 accumulation at mitotic centrosomes (Figure 3 A-C) whereas staining of Plk4 at centrosomes in interphase cells was not reduced (Figure 3 D and E). In parallel, we also checked whether the Plk4 protein was stable in CDK11 knock down cells to rule out the possibility that the low amount of centrosomal Plk4 after CDK11 RNAi was caused by an overall Plk4 protein disappearance. As endogenous Plk4 protein cannot be detected by Western blot, we examined the stability of full length Plk4 or a shorter Plk4 fragment also containing its phosphodegron motif [13]. For this purpose, we co-transfected control or CDK11 siRNA together with the Myc-tagged Plk4 constructs and examined by Western blot exogenous Plk4 protein levels. Exogenous Plk4 proteins were very unstable and difficult to detect in the control cells. However, we found that Myc-Plk4 proteins were more stable in CDK11 knock down cells (Figure 3 F, suggesting that more Plk4 protein was present in this case but not targeted to the mitotic centrosomes. Taken together, the above results point out that CDK11p58 is essential for the correct recruitment of multiple centrosomal proteins including Plk1 and γ-tubulin, but also Cep192 and Plk4.

To date, very few proteins have been found to associate with Plk4. We further tempted to investigate whether CDK11p58 could physically interact with this master regulator of centriole duplication. As endogenous CDK11p58 and Plk4 proteins are difficult to detect in cell lysates, we decided to co-express these two proteins with different tags in COS-7 cells and we carried out immunoprecipitation experiments (Figure 4 A, B and C). We were able to detect Myc-Plk4 in GFP-CDK11p58 pull down (IP GFP, Figure 4 B) and GFP-CDK11p58 was reciprocally found in Myc-Plk4 pull down (IP MYC, Figure 4 B). We then performed the same experiment using truncated Myc-Plk4 proteins containing either the N-terminal or the C-terminal end of the protein (Figure 4 C). Interestingly enough, the interaction was only observed between CDK11p58 and the N-terminal domain of Plk4 (NT).
The second hypothesis would be that CDK11p58 might also mediate its role via its direct interaction with Plk4. However, CDK11p58 associates with mitotic microtubules but is not localised at the centrosomes itself and was not, so far, characterised as a core component of purified human or Drosophila centrosomes [31,32]. This might suggest that the interaction with Plk4 occurs mostly in the cytoplasm of the cell or on spindle microtubules. Interestingly, the level of Plk4 protein kinase is tightly regulated throughout the cell cycle by ubiquitin-mediated proteolysis via SCF^{Mad} in Drosophila [13,14] and human cell lines [33]. Plk4 degradation and maintenance of appropriate protein levels in the cell requires phosphorylation of a phosphodegron motif present in the N-
Figure 4. Plk4 and CDK11<sup>ps8</sup> can interact in vitro and in vivo. A) Scheme of the different Plk4 constructs used for the co-transfection and immunoprecipitation experiments. Myc-Plk4-FL: Myc-tagged Plk4 full length; Myc-Plk4-NT: Myc-tagged Plk4 N-terminus domain; Myc-Plk4-CT: Myc-tagged Plk4 C-terminus domain. The catalytic domain (CD) is displayed in red, the polo box (PB) in green and the phosphodegron (PD) in yellow. B) GFP or GFP-CDK11<sup>ps8</sup> were co-transfected with Myc-Plk4 in COS7 cells and the proteins were immunoprecipitated using GFP (left) or Myc antibodies (right). Myc-Plk4 was co-immunoprecipitated with GFP-CDK11<sup>ps8</sup> but not with GFP (left). GFP-CDK11<sup>ps8</sup> (but not GFP) was also found in the Myc-Plk4 immunoprecipitates (right). C) Myc-Plk4-FL, Myc-Plk4-NT, Myc-Plk4-CT were co-transfected with GFP and/or GFP-CDK11<sup>ps8</sup> and the proteins were immunoprecipitated with GFP and/or Myc antibodies. GFP-CDK11<sup>ps8</sup> is detected in Myc-Plk4-FL and NT immunoprecipitates whereas it is absent from Myc-Plk4-CT pull down (bottom). HC: immunoglobulin heavy chains; LC: immunoglobulin light chains. D) Maltose Binding proteins (MBP) or MBP-CDK11<sup>ps8</sup> proteins were immobilised on amylose beads and incubated with recombinant Plk4-V5-(His)6. After washes, the
terminal fragment of the protein (Figure 4 A). Plk4 itself but possibly other kinases are required for phosphorylation of this important motif [33]. This phosphorylation of the phosphodegron is crucial to allow the binding of SCFSlimb and in turn for the degradation of the protein because defective Plk4 degradation triggers Plk4 stabilization and centriole amplification [13,14,33,34]. We first hypothesised that Plk4 was a substrate of CDK11p58 on this phosphodegron motif. Indeed, we found elevated levels of exogenous tagged-Plk4 proteins following CDK11p58 depletion reflecting a possible stabilisation of endogenous Plk4 following CDK11 RNAi. However, the global effect following CDK11 RNAi was a centriole duplication defect and not an over duplication as expected in case of Plk4 overexpression. In addition, centrioles from CDK11-depleted cells were not able to over duplicate even if forced Plk4 overexpression was induced. This result suggests that, in CDK11-depleted cells, the centrioles lacked essential mitotic modifications allowing their subsequent duplication in S phase (Figure S5). To conclude, if Plk4 does play a role during mitosis, this possible Plk4 mitotic function cannot be compensated by elevation of Plk4 protein levels during interphase after CDK11 depletion.

To date, CDK11p58 was suggested to play key roles at various stages of the cell division, during centrosome maturation, chromatid cohesion, and cytokinesis. Our data show for the first time a new role for CDK11p58 during centriole duplication, probably mediated to some extent by the centrosomal direct or indirect recruitment of Plk1 and Plk4 protein kinases at centrosomes during mitosis. The emerging dual function of the Cep192, Plk1 and CDK11p58 proteins during centrosome maturation and centriole duplication suggests that these two mechanisms and the machinery involved in these processes are closely connected [8,26,28]. Further studies will be required to understand the mechanism of action of CDK11p58 to potentially validate key centrosomal proteins as substrates of this protein kinase.

Materials and Methods

Plasmid and constructs

CDK1p58 and CDK11p58N were amplified by PCR using the oligonucleotides 5'-TAGAATTCGGAATGATGAAAGCGCCGAGG3' and 5'-TAGGATCCGTAAGATGAAAGACGAG3' and sub cloned into the EcoRI and BamHI sites of the COM263 pcdNA3.1/3xmycA/TO vector [kind gift of Pr Erich Nigg, University of Basel]. Myc-tagged expression constructs were described before and were kindly given to us by Erich Nigg [11]. Myc-tagged expression constructs were described before and were kindly given to us by Erich Nigg [11]. Plk4-V5-(His)6 expression vector. pCMV-GFP-CDK11 p58 was expressed in the cells after g tubulin and/or centrin staining respectively, obviously visible as 2 dots at each pole of mitotic control cells. In U2OS interphase cells, centrosomes and centrioles were counted in the cells after g tubulin and/or centrin staining respectively, following a method described previously [6]. Each experiment was repeated 3 times and >100 cells were scored by experiment.

Cell culture, transfections, siRNA-mediated protein depletion and chemicals

The HeLa, HeLa TRex, and COS-7 cell lines were grown at 37°C in DMEM containing 10% FCS and 100U/ml of streptomycin and penicillin [Invitrogen]. The U2OS TRex cell line was grown in DMEM containing 10% tetracycline negative FCS (PA, A15–109), supplemented with 50µg/ml Hygromycine B [Invitrogen, 10687–010]. Tetracycline inducible cell lines expressing Myc-tagged wild type CDK11p58 was generated by transfection of U2OS-TRex cells kindly given to us by Pr Erich Nigg [11]. Stable transformants were established by selection for two weeks with 1mg/ml G418 [Invitrogen] and 50µg/ml G418 [27]. DNA transfections were performed using Lipofectamine 2000 following the manufacturer instructions [Invitrogen]. CDK11 and Plk4 proteins were depleted as already described [11,35]. For control RNAi, a luciferase siRNA was used [Eurogentec]. Cells were arrested in S-phase using aphidicolin (4µg/ml) from Nigrospora sphaerica [Sigma, A0701].

Antibodies

The following antibodies were used in this study: mouse anti-GFP (0,4mg/ml, Roche), mouse anti-myc (clone 9E10, dilution 1µg/ml, Invitrogen), rabbit anti-centrin antibody (dilution 1/1000, Abcam), rabbit anti-pericentrin (dilution 1/10 000, Abcam), mouse anti-γ-tubulin (dilution 1/1000, clone GTU-488, Sigma), rat anti-tubulin (clone YL1/2, dilution 1/1000, Millipore), rabbit anti-Cep192 (dilution 1/500, kind gift of Laurence Pelletier, Toronto, Canada), affinity-purified rabbit anti-Plk4 and affinity-purified rabbit anti-CDK11 antibodies [11,21,24]. All secondary antibodies used in immunofluorescence microscopy were from Molecular Probes, and the peroxidase-coupled secondary antibodies used for Western blotting were from Jackson Immunoresearch Laboratories.

Centrosome and centriole countings in fixed cells

Centriole countings in mitotic HeLa cells were performed using a cell line carrying a GFP-centrin transgene. Centrioles were obviously visible as 2 dots at each pole of mitotic control cells. In U2OS interphase cells, centrosomes and centrioles were counted in the cells after g tubulin and/or centrin staining respectively, following a method described previously [6]. Each experiment was repeated 3 times and >100 cells were scored by experiment.

Immunoprecipitations

5.10^6 cells expressing the tagged constructs were lysed into Lysis Buffer (LB: 50mM HEPES, 100mM KCl, 1mM MgCl2, 1mM EGTA, 0,5% NP40, 1mM β mercapto-ethanol, 20mM β glyceroxophate, anti-protease inhibitor cocktail (Roche)). The cell extracts were centrifuged for 10min at 10 000g and the soluble proteins were incubated with 3g of anti-GFP or anti-Myc antibodies adsorbed on 50µl of magentic Dynalbeads protein A beads [Invitrogen] for 3 hours. The immunoprecipitated proteins were washed 5 times in LB during 3min. The proteins were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blotting analysis.

Kinase assays

For Plk4 kinase assays, 100ng of GST-Plk4 (Ozyme) was incubated with b casein or Maltose Binding Protein (MBP) or MBP-tagged CDK11p58 [24] in kinase buffer (20mM Tris/HCl, 10mM MgCl2, 25mM NaCl) containing 100µM ATP and 1µCi of
Figure 5. CDK11-depleted cells cannot amplify their centrioles following Plk4 overexpression. A) Control or CDK11-depleted cells were transfected with Myc-Plk4 construct and their ability to amplify their centrioles was analysed after S phase arrest. B) Example of a control siRNA treated cell (left) over expressing Plk4 (red). See the presence of additional centrin (green) dots around each mother centriole. By contrast, most of Myc-Plk4 over expressing cells can not amplify their centrioles following CDK11 siRNA transfection although the over expressed Plk4 protein kinase is strongly recruited to the centriolar region. The insets shows a 5× magnification of the centrosomal region (centrin staining in displayed in monochrome). Bar is 10 μm.

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radio-labelled ATP. The kinase reactions were performed during 15min at 25°C, stopped by addition of one volume of 2× Laemlli buffer and boiled 2min. The samples were analysed by SDS-PAGE. The gels were stained by Coomassie dye, dried, and the phosphorylated proteins were revealed using a Phosphorimager (Molecular Dynamics).

Immunofluorescence

HeLa and U2OS cells grown on coverslips were fixed in methanol at -20°C and processed for immunofluorescence following standard protocols (Montembault et al., 2007). Cell preparations were visualised with a Leica DMIRE2 microscope (objective 63×, N.A. 1.4). Measurements of fluorescence intensities and image acquisition was performed using a CoolSnap ES camera with Metamorph software (Roper Scientific), and pictures were prepared as single sections or maximum intensity projections. Alternatively, slides were viewed with a Leica DMIRE2 (SP2) inverted confocal microscope (objective 63×, N.A. 1.4) using the LCS 3D software (Leica). Images were processed with Adobe Photoshop (v7.0).

Purification of recombinant proteins

MBP and MBP-CDK11p58 protein immobilisation on amylose column (Biolabs) was described before [35]. Plk4-V5-(His)6 expression was induced in E. coli for 4 hours at 25°C and the recombinant protein was purified following standard procedures [36] using Ni-NTA agarose beads (Qiagen). The Plk4-V5-(His)6 protein was stored at -20°C in 50% glycerol before using for the in vitro MBP-CDK11p58 binding assay.

In vitro binding assay

10µg of Maltose Binding Protein (MBP) or MBP-CDK11p58 were immobilised on amylose column (stored at 4°C in column buffer: 20mM Tris-HCL pH 7.4; 200mM NaCl; 1mM EDTA and 1mM Sodium Azide) and incubated with 5µg of purified Plk4-V5-(His)6 protein kinase in binding buffer (20mM Tris-HCL pH 7.4; 200mM NaCl; 1mM EDTA and 1mM sodium azide) for 1h at 4°C. After 3 washes for 5min at 4°C, the samples were analysed by anti-V5 Western blot.

Supporting Information

Figure S1 Localisation of Myc-tagged CDK11p58 in HeLa cells. A) HeLa stable cell line was induced to express Myc-CDK11p58 for approximately 2 hours, fixed and stained for α-tubulin (red in the left panels and monochrome in the middle panels) and Myc (green in the left panels and monochrome in the right panels). DNA is blue. Each phase of the cell cycle is indicated at the top of each panel. During interphase (top panels), CDK11 remained cytoplasmic and started to accumulate on the astral microtubules during prophase. The tagged protein remained associated with spindle microtubules throughout the duration of cell division. During cytokinesis a pool of the protein was found at the midbody. The mitotic phases are displayed on the merge panels. Scale bar is 10 µm. B) Interphase HeLa cell expressing Myc-CDK11p58. The lower panels show a 10 times magnification of the centrosomal region. C) Mitotic HeLa cell expressing Myc-CDK11p58. The lower panels show a 5 times enlargement of one of the spindle pole region. In panels B and C, Myc-CDK11p58 is green and monochrome in the right panel; pericentrin is red and monochrome in the middle panel; and DNA is blue. Scale bars are 10 µm.

Figure S2 CDK11p58 depletion interferes with centrosome overduplication in S phase-arrested U2OS cells. A) U2OS cells were transfected with control, Plk4, or CDK11 siRNAs. 24 hours post-transfection (duration of one cell cycle), the cells were treated with aphidicolin for 48 hours to be arrested in S phase. Cells were then fixed and the centrosomes were counted. B) The top panel shows an S phase-arrested cell with more than two centrosomes (white arrowhead). The bottom panel shows a cell with two centrosomes (white arrowhead). DNA is blue and γ tubulin, as a centrosomal marker, is red and also displayed in monochrome on the right panels. Scale bar is 10µm. C) Graph (±SD) showing the percentage of cells with more than two centrosomes, the siRNA treatment is indicated at the bottom. Note the 3-fold decrease of centriole overduplication following Plk4 and CDK11 RNAi.

Figure S3 The centrosomal recruitment of Cep192 in mitosis is compromised in CDK11-depleted cells. A) Control (top) or CDK11 siRNA-transfected (middle and lower panels) mitotic cells were fixed and stained for DNA (blue), a tubulin (red) and Cep192 (green and right panels in monochrome). Scale bar is 10 µm. B) Graph showing Cep192 signal intensity (±SD) at the mitotic centrosomes of control (green) and CDK11-depleted cells (red). Found at: doi:10.1371/journal.pone.0014600.s003 (1.05 MB TIF)

Figure S4 CDK11p58 is not a Plk4 substrate in vitro. β casein, Maltose binding protein (MBP) and MBP-CDK11p58 were incubated without (lanes 2, 3 and 4 respectively) or with active GST-Plk4 (lanes 5, 6 and 7) in the presence of radiolabelled ATP. β casein is phosphorylated but not MBP or MBP-CDK11p58. The dark arrowheads indicate the positions of the recombinant proteins. Auto-phosphorylation of GST-Plk4 (protein not visible by Coomassie staining) is detected on the autoradiography (lanes 1, 5 and 7) and is indicated by an arrow. Found at: doi:10.1371/journal.pone.0014600.s004 (0.66 MB TIF)

Figure S5 Possible model of centriole duplication. Centriole disengagement (licensing) is controlled by Plk1 and separase to allow subsequent centriole duplication in interphase. Plk1 protein kinase controls centrosome maturation during early mitosis (by recruitment of PCM proteins including SPD-2/Cep192) and centriole disengagement. Separase participates to this process in late mitosis. Plk1 recruitment is under the control of CDK11p58, which is only expressed during mitosis. Plk4 plays a key role for centriole duplication during interphase and enhances this process when overexpressed. Plk4 protein accumulation to the mitotic centrosome, is controlled by CDK11p58. Both proteins interacts directly with each other indicating a possible involvement of the Plk4 protein kinase during mitosis in the licensing process. Found at: doi:10.1371/journal.pone.0014600.s005 (0.66 MB TIF)

Table S1 Analysis of mitotic figures following CDK11, Plk4, and double RNAi in HeLa cells. The mitotic cells were fixed and analysed for their mitotic spindle shape and their centriole numbers (a scheme of each mitotic figure is displayed at the top of each column). The percentage of each mitotic figure is indicated (±SD). Found at: doi:10.1371/journal.pone.0014600.s006 (0.05 MB DOC)

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References


Author Contributions

Conceived and designed the experiments: NF EM RG. Performed the experiments: NF EM AP JYC RG. Analyzed the data: NF EM PR RG. Contributed reagents/materials/analysis tools: NF EM RG. Wrote the paper: NF EM PR RG.