E2F-1, Skp2 and cyclin E oncoproteins are upregulated and directly correlated in high grade neuroendocrine lung tumors.

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Abstract

The transcription factor E2F-1 plays a crucial role in the control of cellular growth. We previously reported its differential pattern of expression in human lung tumors. In this study, we have investigated the relationships linking the status of E2F-1 and a mediator of its proteasomal degradation, the Skp2 F-box protein. Using immunohistochemistry in a series of 129 lung tumors of all histological types, we demonstrate that Skp2 accumulates preferentially in high grade neuroendocrine (HGNE) lung carcinomas (86%, p<0.0001), and show that Skp2 overexpression is associated with advanced stages (p<0.0001) and nodal metastasis (p<0.0001) in NE lung tumors. Unexpectedly, we observe that Skp2 and E2F-1 expression directly correlates in NE lung tumors (p<0.0001). Moreover, using cellular models, we identify Skp2 as a new E2F-1 transcriptional target. Furthermore, we also provide evidence that Skp2 interacts physiologically with E2F-1 and stimulates its transcriptional activity towards the cyclin E promoter. Consistently, we demonstrate that cyclin E expression directly correlates with Skp2 (p<0.0001) and E2F-1 (p=0.0001) status in NE lung tumors. Overall, our data provide the first evidence of a direct and functional interconnection between the E2F-1, Skp2 and cyclin E oncoproteins in HGNE lung carcinomas.

Introduction

Bronchogenic carcinomas arise from the sequential accumulation of genetic and epigenetic abnormalities that affect various oncogene and tumor suppressor gene networks. In this respect, we have previously reported the constant alteration of p53/Rb signaling pathways in lung tumors (Brambilla et al., 1993; Brambilla et al., 1999; Gouyer et al., 1998). E2F-1, the first identified member of a family of transcription factors generically referred as E2F (DeGregori, 2002), is located at the interface of both p53/Rb pathways. E2F-1 is best-known for its role in driving cell cycle progression, a process that is tightly regulated by its interaction with Rb (Harbour & Dean, 2000). Moreover, E2F-1 can also trigger p53-dependent or -independent apoptosis (Ginsberg, 2002). Aiming at identifying some components of the p53/Rb network that could contribute to lung carcinogenesis, we previously observed that E2F-1 is overexpressed in high grade neuroendocrine (HGNE) lung tumors, whereas it is undetectable in a majority of non small cell lung carcinoma (NSCLC) (Eymin et al., 2001a). These results indicate that a deregulated E2F-1 activity contributes to lung tumorigenesis.

The ubiquitin/proteasome pathway plays a crucial role in the selective and temporally controlled elimination of key regulatory proteins. The conjugation of a polyubiquitin chain on substrates requires the sequential activity of three distinct enzymes including an ubiquitin-protein ligase E3 involved in substrate recognition. The SKP1-CUL1-F-box-protein (SCF) complexes belong to a large family of multi-subunit E3 ligases that consist of three core components: the adaptator protein SKP1, the scaffold protein cullin-1 (CUL1) and the RING-domain protein Rbx1/Roc (Zheng et al., 2002). Their association with a variable F-box-containing protein determines the substrate specificity. Initially identified as a cyclin A/CDK2-interacting protein in transformed cells (Bai et al., 1996; Zhang et al., 1995), Skp2

(S-phase kinase-associated protein 2) is the substrate-recognition subunit of the SCF^{skp2} E3 ligase complex. Through its capacity to target the proteasomal degradation of key regulators of the cell cycle such as the cyclin-cdk inhibitor $p27^{Kip1}$ (Carrano et al., 1999; Nakayama et al., 2004), the cyclin E (Yeh et al., 2001) and the E2F-1 (Marti et al., 1999) proteins, Skp2 is required for cell cycle progression at multiple stages (Carrano et al., 1999; Deshaies, 1999; Nakayama et al., 2004; Zhang et al., 1995). Owing to this central role in the control of cell cycle regulatory proteins proteolysis, aberrant Skp2 expression is thought to exert oncogenic properties. Consistently, it has been shown that Skp2 cooperates with H-Ras in transforming primary rodent fibroblasts and in inducing tumor formation in nude mice (Gstaiger et al., 2001). Furthermore, Skp2 expression is greatly enhanced in a broad spectrum of human tumors (Nakayama & Nakayama, 2006, for review). In this setting, it is currently thought that the oncogenic phenotype resulting from high Skp2 levels is mainly related to the proteasomal degradation of various Skp2 substrates.

Based on our data demonstrating a differential pattern of E2F-1 protein expression in human lung tumors (Eymin et al., 2001a), we wondered whether some of the proteins involved in the control of E2F-1 proteolysis could be abnormally expressed in these tumors. In this context, the aim of this study was to investigate the relationships linking E2F-1 and its regulator Skp2 in lung cancer.

Results

Skp2 is overexpressed and associated with tumor progression in HGNE lung tumors.

Skp2 expression was studied by immunohistochemistry on a panel of 50 NSCLCs, 25 lung carcinoids and 54 HGNE lung carcinomas. Because Skp2 immunostaining was heterogeneous among lung tumors, differential scores were ascribed in each case according to

the intensity of staining and the percentage of stained cells. Tumor samples were grouped in three classes as described in the material and method section. According to this, Skp2 was undetectable (class 0) in normal lung epithelium adjacent to tumor cells on sections, as well as in normal lung tissues localized at distance from lung cancer. As compared to these normal tissues, we observed an heterogeneous pattern of Skp2 expression with tumors exhibiting either undetectable (class 0), moderate (class 1) or strong (class 2) immunostaining (Table 1, $p<0.0001$). In this setting, overexpression of Skp2 was observed in 48% (62/129) of all tumors tested, predominantly in high grade NE lung tumors (46/54, 85%) as compared to NSCLC (16/50, 32%) (Table 1 and Figure 1). Consistent with previous reports (Yokoi et al., 2004; Zhu et al., 2004), high levels of Skp2 were more frequent in squamous carcinomas (15/29, 51%) than in adenocarcinomas (1/21, 4%) ($p<0.0001$). Moreover, Skp2 was undetectable in 25/25 (100%) atypical and typical lung carcinoids, whereas it was overexpressed in the majority of high grade NE lung tumors, with SCLCs displaying higher levels (34/35, 97%) than LCNECs (12/19, 63%) ($p=0.003$). Therefore, these data indicate that Skp2 overexpression is associated with a high grade phenotype in NE lung tumors. To validate these IHC data, 24 of the 104 tumoral samples were analyzed for Skp2 expression by western blotting and a good concordance (88%) was observed between both techniques (data not shown).

Then, to further characterize the role of Skp2, we analyzed the relationships between its IHC status and some clinicopathological parameters (Table 2). When all histological subtypes were considered, we observed that Skp2 overexpression was associated with advanced stages III-IV ($p< 0.0001$) and nodal metastasis ($p<0.0001$). Similar correlations were observed in NE lung tumors ($p<0.0001$) but not in NSCLCs.

The expression of Skp2 and E2F-1 is directly correlated in NE lung tumors.

We previously reported a differential pattern of E2F-1 protein expression in a series of 60 lung tumors (Eymin et al., 2001a). In this study, we have extended these results to a larger series and included atypical and typical carcinoids (Table 3 and Figure 2). Our data confirmed our previous results with E2F-1 protein being predominantly overexpressed in HGNE lung tumors (46/54, 86%) whereas it was undetectable or faintly expressed in 90% (45/50) of NSCLC (Table 3). Importantly, high amounts of E2F-1 protein were associated with a high grade NE phenotype as compared to carcinoids (Table 3; $p<0.0001$ and Figure 2B).

When we analyzed the status of both Skp2 and E2F-1 proteins, we found an unexpected direct correlation in all tumors tested (Table 4; $p<0.0001$), as well as in NE lung tumors ($p<0.0001$). In contrast, E2F-1 and Skp2 status was not linked in NSCLCs, but we noticed that 64% (32/50) of the cases did not express both proteins.

Skp2 is a transcriptional target of E2F-1

To further investigate the functional relationships between Skp2 and E2F-1 proteins, we undertook a series of experiments in cell lines. Firstly, we postulated that Skp2 could be a new downstream target of E2F-1. To answer, we neutralized E2F-1 using siRNA in the small cell lung carcinoma cell line H69 and analyzed Skp2 expression by immunoblotting and RT-PCR. As shown in figure 3A, the knock-down of E2F-1 was associated with a significant down-regulation of both Skp2 protein (left panel) and mRNA levels (right panel). Furthermore, using a stable E2F-1 tetracycline-inducible clone (H358/Tet-On) that we previously established in the human lung adenocarcinoma cell line H358 (Salon et al., 2006), we observed an increase in Skp2 mRNA (Figure 3B, right panel) and protein (Figure 3B, left panel) expression upon doxycyclin induction. Altogether, these data indicate that Skp2 expression is positively regulated by E2F-1. Then, we investigated whether E2F-1 was able to

control *Skp2* promoter activity. A549 cells were transfected with a plasmid encoding the luciferase protein under the control of the human *Skp2* promoter, together with increasing amounts of an E2F-1 expression vector. Because of their low transfection efficiency, H69 cells could not be used here. As reflected by the increase in luciferase activity, E2F-1 was able to activate in a dose dependent manner the *Skp2* promoter in these cells (Figure 3C, left panel). Similar results were obtained in the H1299 cell line (Figure 3C, right panel). Therefore, these data demonstrate that Skp2 is a transcriptional target of E2F-1. Interestingly, we noticed that the Skp2 promoter response to E2F-1 increasing levels was stronger in H1299 cells deriving from a large cell carcinoma with neuroendocrine features than in A549 adenocarcinoma cells. These data were consistent with our findings of a direct correlation between E2F-1 and Skp2 in NE lung tumors.

Skp2 interacts with and stimulates the transcriptional activity of E2F-1

It was previously demonstrated that Skp2 interacts with and acts as a transcriptional cofactor of c-Myc (Kim et al., 2003; Von der Lehr et al., 2003). These data prompted us to investigate whether Skp2 could also regulate E2F-1 transcriptional activity. Firstly, we tested the possibility that E2F-1 and Skp2 could physiologically interact in our cellular models. As illustrated, E2F-1 was clearly recovered from Skp2 immunoprecipitates obtained from H69 (Figure 4A) or H810 (data not shown) neuroendocrine lung carcinoma cell lines. More importantly, an E2F-1/Skp2 complex was also detected in cellular extracts obtained from three small cell lung carcinomas with high levels of both proteins (Figure 4B). Altogether, these data demonstrate that Skp2 interacts with E2F-1 in HGNE human lung cell lines as well as in primary tumors.

Then, we asked whether Skp2 could regulate E2F-1 transcriptional activity. To answer, A549 cells were transfected with plasmids encoding the luciferase under the control

of the cyclin E promoter, a well-known E2F-1 target gene (Soucek et al., 1997), E2F-1 and increasing amounts of Skp2. As reflected by the increase in luciferase levels, the cyclin E promoter was activated upon E2F-1 expression (Figure 4C). Importantly, the addition of Skp2 stimulated in a dose-dependent manner the transcriptional activity of E2F-1 in this setting (Figure 4C). In contrast, Skp2 alone was unable to promote cyclin E activation, nor to stimulate the activity of a control pGLuc vector (Figure 4C). Taken together, these results indicate that Skp2 stimulates the transcriptional activity of E2F-1. Similar results were obtained in H1299 cells as well as in another cellular model, the human osteosarcoma cell line SAOS2 (Figure 3D).

Relationships between Skp2, E2F-1 and cyclin E status in human lung tumors.

The above results led us to examine whether Skp2, E2F-1 and cyclin E expression was linked in human lung tumors. First, we analyzed the status of the cyclin E protein in our series of lung tumors (Table 5 and Figure 5). As compared to normal lung tissues in which immunostaining was not detectable (Figure 5A, a), we observed an heterogeneous pattern of cyclin E expression with tumors exhibiting either undetectable (class 0), moderate (class 1) or strong (class 2) immunostaining (Table 5, p<0.0001). Interestingly, we noticed that cyclin E was undetectable in 100% of carcinoids whereas high levels were observed in 21% (4/19) of LCNEC and 71% of SCLC (25/35) (Table 5, p=0.001). In addition, a strong cyclin E immunostaining was associated with advanced stages III-IV (p< 0.0001) and nodal metastasis (p<0.0001) in these tumors (data not shown). Thus, as for Skp2 and E2F-1, accumulation of cyclin E appears to be a hallmark of a high grade phenotype in NE lung tumors.

Then, we studied the relationships linking Skp2, E2F-1 and cyclin E proteins in our series of lung tumors. As shown in table 6, we found a direct correlation between cyclin E and Skp2 (p<0.0001) or E2F-1 (p=0.0001) expression in NE lung tumors. In contrast, no

significant correlation was found in NSCLC. Furthermore, the expression profile of the 3 proteins was associated (either undetectable or overexpressed) in 56% (71/126) of all tumors tested, more frequently in NE tumors (66%) as compared to NSCLC (40%) (Supplementary Table 1, p=0.006).

Discussion

The Skp2 protein, one of the F-box protein of the E3-ubiquitin ligase SCF complexes, targets a variety of cell cycle regulators for proteasomal degradation (Nakayama & Nakayama, 2006, for review). In this respect, Skp2 acts as a growth promoter which deregulation is thought to exert oncogenic functions. The *Skp2* gene is located at 5p13, a region that is commonly amplified in lung cancer. Consequently, *Skp2* amplification and overexpression have been reported in NSCLC cell lines and primary tumors (Takanami, 2005; Yokoi et al., 2004; Zhu et al., 2004). In contrast, Skp2 status has been poorly investigated in NE lung tumors (Yokoi et al., 2002). In agreement with previous studies, we report that Skp2 is overexpressed in 32% of NSCLC, especially in squamous carcinomas. More importantly, we demonstrate for the first time that HGNE lung tumors display a more frequent Skp2 upregulation (46/54; 86%) than NSCLC (p <0.0001), and that Skp2 protein expression is undetectable in low grade NE typical and atypical carcinoids. Elevated expression of Skp2 was previously linked to positive lymph node metastasis, advanced stages and poor or moderate differentiation in NSCLC (Yokoi et al., 2004). In addition, a high level of Skp2 protein was also reported as an independent poor prognostic marker (Osoegawa et al., 2004; Zhu et al., 2004). In this study, we did not find any correlation between Skp2 overexpression and either of these clinicopathological parameters in NSCLC. In contrast, we observe that high levels of Skp2 expression are associated with advanced stages (III-IV) and nodal

metastasis in NE lung tumors. Overall, our data support the notion that overexpressed Skp2 acts as an oncogenic protein in HGNE lung tumors.

It is currently thought that the oncogenic properties of Skp2 are related to its ability to induce the ubiquitination and proteasomal degradation of its cell cycle targets (Nakayama & Nakayama, 2006, for review). Therefore, based on our previous demonstration of an altered pattern of E2F-1 expression in human lung cancer (Eymin et al., 2001a), we had hypothesized that the status of E2F-1 and Skp2 proteins could be inversely related in these tumors. In this study, we did not observe any significant correlation between both proteins in NSCLC. In contrast and unexpectedly, Skp2 and E2F-1 status was directly correlated in NE lung tumors, with a predominant upregulation of both proteins in HGNE lung tumors (78% of the cases). In addition, a direct correlation between Skp2 and cyclin E (p<0.0001, this study) or p27^{KIP1} (p=0.006; data not shown), another target of Skp2-mediated proteolysis (Carrano et al., 1999; Nakayama et al., 2004), was also observed in these carcinomas. Taken together, these results clearly demonstrate that overexpression of Skp2 does not always correlate with the downregulation of its main targets in tumors. What might be the potential explanations for such observation? One plausible hypothesis is that the activity of SCF^{Skp2} complexes is altered in HGNE lung tumors, maybe due to mutations or loss of expression of either SCF members. This could explain the concomittant expression of Skp2, E2F-1 and cyclin E proteins in these tumors. It remains to determine whether alterations of other SCF components exist in human lung tumors. In addition, our results suggest that Skp2 overexpression might have proteasomal-independent functions. Another but not exclusive explanation is the existence of a positive loop linking E2F-1 and Skp2 protein expression. In agreement with such hypothesis, we provide evidence that Skp2 is a transcriptional target of E2F-1. Interestingly, in a gene expression profiling analysis, Vernell and co-workers had observed that Skp2 expression is upregulated in response to E2F-1 (Vernell et al., 2003). Furthermore, during the

course of our study, it was demonstrated that E2F-1 directly interacts with the human *Skp2* promoter and positively regulates its activity in various cellular models (Zhang & Wang, 2006). Therefore, our demonstration of a direct correlation between E2F-1 and Skp2 in HGNE lung tumors provides a physiological relevance to the observations made in cell lines.

Finally, how could Skp2 and E2F-1 overexpression contribute to the development of HGNE lung tumors ? We demonstrate that endogenous Skp2 and E2F-1 proteins interact in HGNE lung carcinoma cell lines and identify for the first time a Skp2/E2F-1 complex in small cell lung carcinomas. Moreover, using various cellular models, we show that Skp2 stimulates the transcriptional activity of E2F-1 towards the cyclin E promoter and identify a direct relationship linking cyclin E with Skp2 (p<0.0001) and E2F-1 (p=0.0001) proteins in NE lung tumors. Interestingly, 93% (27/29) of the tumors overexpressing the 3 proteins were HGNE lung tumors, indicating that their concomittant upregulation is highly predictive of a high grade NE phenotype. Therefore, besides its ability to induce cyclin E proteasomal degradation (Nakayama et al., 2000; Yeh et al., 2001), Skp2 could also induce cyclin E expression, notably through E2F-1 activation. Such differential Skp2-mediated cyclin E control could depend on the cellular context (normal versus tumoral tissues) and/or on the co-activated signals (E2F-1 upregulation).

To conclude, our data provide the first evidence of a direct correlation linking the E2F-1, Skp2 and cyclin E oncoproteins in high grade neuroendocrine lung tumors and suggest that their cooperation might contribute to the tumorigenesis of these highly aggressive lung carcinomas.

Materials and Methods

Tissue samples

One hundred and twenty nine human lung tumors were included in this study. Tissue samples were taken at surgical resection of lung tumors in 103 cases or at mediastinoscopy of node metastases in 5 cases of Large Cell Neuroendocrine Carcinomas (LCNECs) and 21 cases of Small Cell Lung Carcinomas (SCLCs). Tumor tissues and normal lung parenchyma were immediately frozen and stored at -80°C until use. For histological classification, tumor samples were fixed in formalin and the diagnoses were made on paraffin-embedded material using the current World Health Organization International Histological Classification of Lung Tumors criteria (Travis et al., 1999). They consisted of 29 squamous cell carcinomas, 21 adenocarcinomas, 19 LCNECs and 35 SCLCs, 15 typical carcinoids and 10 atypical carcinoids . Sixty three patients were suffering from nodal metastasis at the time of diagnosis.

Immunohistochemistry (IHC)

Skp2 and cyclin E immunohistochemical analyses were carried-out on formalin-fixed and paraffin-embedded tissue sections. Four-micrometer sections were dehydrated in xylene and rehydrated in graded ethanol solutions. Endogenous peroxydase activity was blocked by immersion in H_2O_2 solution. For Skp2, the antigen immunoreactivity was enhanced by microwaving the sections for 3 x 5 minutes in EDTA buffer, pH 8. Then, a classical three step immunohistochemical method was applied. The primary antibody (clone 2C8D9) was used at a 1:100 dilution. Cyclin E immunodetection was performed using the Ventana Discovery Autostainer (Ventana Medical International Inc). Cut sections were boiled for 60 minutes in citrate buffer pH 6 for heat-induced epitope retrieval. The primary antibody (clone 13A3) was applied at a 1:10 dilution for one hour. E2F-1 protein expression was detected on frozen

sections using the KH95 monoclonal antibody diluted at 1:100, as previously described (Eymin et al., 2001a). Slides incubation with normal rabbit or mouse IgG at the same concentration as the primary antibodies served as negative controls.

Immunohistochemical staining evaluation

Skp2, E2F-1 and cyclin E immunostainings were evaluated by two independent observers (CS and EB) in distinct areas of the slide sections for correlation and confirmation of tissues analysis and scored by taking into account the tumor heterogeneity. A final score (0-300) was established by multiplying the percentage of labeled cells (0-100%) with the intensity of staining (1+, 2+, 3+). According to the final scores, tumoral samples were divided in three classes as follow : class 0, no staining, class 1, score < 60 for Skp2 or < 40 for cyclin E and E2F-1, class 2, score ≥ 60 for Skp2 or ≥ 40 for cyclin E and E2F-1. Samples with a final score ≥ 60 were considered as tumors overexpressing Skp2. Cyclin E and E2F-1 overexpressing cases displayed a final score ≥ 40. Stromal cells and background normal lung were negative for all the three tested proteins. Correlations between the expression of various factors and clinicopathological parameters were based on the chi-square (X^2) test, with a p value ≤ 0.05 considered significative.

Cell lines , plasmids, siRNA and transfection

The A549 human lung adenocarcinoma, H1299 human large cell lung carcinoma with neuroendocrine features and SAOS2 human osteosarcoma cell lines were cultured as previously described (Eymin et al., 2001b). The H69 small cell lung carcinoma cell line was cultured in 5% CO_2 at 37°C in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FCS, 2.5 mg/ml glucose, 10 mM HEPES and 1 mM sodium pyruvate. The H358/Tet-On/E2F-1 inducible clones were obtained as previously described (Salon et al.,

2006). Plasmids used were pcDNA3.1, pCMV-E2F-1, pGL2-cyclin E encoding the luciferase protein under the control of the cyclin E promoter, pcDNA3-Myc-Skp2 and Skp2-luc encoding the luciferase under the control of the human *Skp2* promoter region spanning from – 272 to +244 residues. Luciferase assays were performed as previously described (Eymin et al., 2001b). The sequences specifically targeting human *e2f-1* RNA were as follow: 5'-GUCACGCUAUGAGACCUCATT-3' and 5'-ACAAGGCCCGAUCGAUGUUTT-3'. The scrambled siRNA oligonucleotides were as follow: 5'-AAAGGUGACGCUGACGAAGTT-3'; 5'-CAAGAAAGGCCAGUCCAAGTT-3'. Cells were transiently transfected either with Fugene 6 (Roche Diagnostic) or JetSI™ (Polyplus transfection).

Immunoblotting and immunoprecipitation experiments

Immunoblotting and immunoprecipitation experiments were performed as previously described (Eymin et al., 2001a). The antibodies were anti-actin (Sigma), anti-E2F-1 (C-20, Santa Cruz; KH-95, Pharmingen), anti-cyclin E (13A3, Novocastra) and anti-Skp2 antibodies (Skp2-2B12 and 2C8D9, Zymed; H-435, Santa-Cruz).

RT/PCR analysis of Skp2 mRNA level

PCR was carried out for 30 cycles using the following conditions : 94°C for 30 sec ; 57°C for 30 sec and 72°C for 30 sec. The primers used were : Skp2 forward (sense) 5'-TCAACTACCTCCAACACCTATCAC-3' ; Skp2 reverse (antisense) 5'-GACAACTGGGCTTTTGCAGT-3'. Amplification of a fragment of the cDNA of G3PDH (Invitrogen) was performed in the same PCR reaction as an internal control.

Aknowledgements

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Titles and legends to figures

Figure 1. (A) Immunostaining of normal lung parenchyma and lung cancer tissue on paraffin embedded sections using an anti-Skp2 monoclonal antibody (2C8D9, Zymed). (**a**) Absence of immunostaining in the columnar epithelium lining a normal bronchial structure (less than 1% of positive cells). (**b**) Negative immunostaining (class 0) in a typical carcinoid. (**c,d**) Strong positive nuclear immunostaining (class 2) on (**c**) most or (**d**) 50% of tumor cells in small cell lung carcinomas. (**e**) Weak nuclear immunostaining (class 1) in a squamous cell carcinoma. (**f**) Negative immunostaining (class 0) in an adenocarcinoma. **(B)** Mean scores ± standard deviation of Skp2 protein expression according to the histological sub-types.

Figure 2. (A) Immunostaining of normal lung parenchyma and lung cancer tissue on paraffin embedded sections using an anti-E2F-1 monoclonal antibody (KH95, Pharmingen). (**a**) Absence of immunostaining in hyperplastic bronchiolar cells. (**b**) Weak positive immunostaining (class 1) in an atypical carcinoid consisting in few scattered positive cells (arrow). (**c**) Strong positive nuclear staining (class 2) detected on at least 50% of tumor cells in a small cell lung carcinoma. (**d**) Negative immunostaining (class 0) in an adenocarcinoma. **(B)** Mean scores ± standard deviation of E2F-1 protein expression according to the histological sub-types.

Figure 3. Skp2 is a transcriptional target of E2F-1. **(A)** The small cell lung carcinoma cell line H69 was transfected with either *mismatch* or *E2F-1* siRNA as indicated. Skp2 protein and mRNA levels were detected by immunoblotting (left panel) or RT-PCR (right panel), respectively. **(B)** H358 stable E2F-1-inducible clones were incubated in the presence (+) or absence (-) of 1μg/ml doxycyclin for 72 hours as indicated. E2F-1 and Skp2 protein

expression were detected by immunoblotting (left panel). Skp2 mRNA level was analyzed by RT-PCR (right panel). **(C)** A549 lung adenocarcinoma and H1299 large cell lung carcinoma cell lines were co-transfected for 48 hours with 1μg of a Skp2-luc plasmid encoding the luciferase under the control of the Skp2 promoter and increasing amounts of a pCMV-E2F-1 vector. The luciferase activity obtained in cells transfected with Skp2-luc alone was normalized to 1 and a relative luciferase activity was then calculated for each condition.

Figure 4. Skp2 interacts with E2F-1 and stimulates its transcriptional function towards the cyclin E promoter. **(A, B)** Endogenous Skp2 protein was immunoprecipitated from **(A)** H69 or **(B)** small cell lung carcinoma extracts (T1, T2, T3) with an anti-Skp2 antibody.. Immunoblotting was carried-out with an anti-E2F-1 antibody. An irrelevant IgG was used as a negative control for immunoprecipitation. **(C, D)** Luciferase experiments were performed in A549, H1299 and SAOS2 cell lines co-transfected for 48 hours with 1μg pGL2-cyclin E encoding the luciferase under the control of the cyclin E promoter or 1μg pGL2 as a control, in the presence or absence of 50ng pCMV-E2F-1 and increasing amounts of pcDNA3-Myc-p45^{SKP2} (100 and 250ng), as indicated. The luciferase activity obtained in cells transfected with pGL2-cyclin E or pGL2 was normalized to 1 and a relative luciferase activity was then calculated for each condition.

Figure 5. (A) Immunostaining of lung cancer tissues on paraffin embedded sections using a monoclonal anti-cyclin E antibody. (**a**) Absence of staining in epithelial cells lining a bronchiolar structure and in pneumonocytes. (**b**) Negative immunostaining (class 0) in a typical carcinoid. (**c**) Strong positive nuclear staining (class 2) on a majority of tumor cells in a small cell lung carcinoma. (**d**) Strong positive nuclear staining (class 2) detected on most

tumor cells in a squamous cell carcinoma. **(B)** Mean scores ± standard deviation of cyclin E

protein expression according to the histological sub-types.