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Altered pattern of Cul-1 protein expression and neddylation in human lung tumors: relationships with CAND1 and cyclin E protein levels.

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Abstract

The Cul-1 protein is the scaffold element of SCF complexes that are involved in the proteasomal degradation of numerous proteins regulating cell cycle progression. Owing to this central role in cell growth control, aberrant expression of the components of SCF is supposed to play a role during tumorigenesis. Nothing is known about Cul-1 expression in human tumors. In this study, we have analyzed its status in a series of 128 human lung carcinomas comprising 50 NSCLC (29 squamous cell carcinoma and 21 adenocarcinoma) and 78 neuroendocrine (NE) lung tumors (24 typical and atypical carcinoids, 19 large cell NE carcinoma and 35 small cell lung carcinoma). By using immunohistochemistry, we report for the first time an altered pattern of Cul-1 expression in human tumors. Indeed, we show that Cul-1 expression is upregulated in 40% (51/128) of all lung tumors as compared to normal lung tissues, including 34% (17/50), 75% (18/24) and 30% (16/54) of NSCLC, carcinoids and high grade neuroendocrine lung carcinoma, respectively. Furthermore, we demonstrate that high levels of Cul-1 protein are associated with a low KI67 proliferative index (p=0.005) as well as with the decrease of the cyclin E oncoprotein (p=0.0003), one of the major target of SCF complexes. These data suggest that upregulation of Cul-1 could protect cells from hyperproliferative signals through cyclin E downregulation. Cul-1 is modified by neddylation, a post-translational modification that grafts ubiquitin-like Nedd8/ Rub1 residues and controls Cul-1 activity. We also provide evidence that neddylated forms of Cul-1 are specifically expressed in high grade NE lung tumors and are associated with a downregulation of the Cul-1 inhibitor CAND1 (p=0.03) and a high level of cyclin E (p=0.0002). These data support the notion that alterations in the Cul-1 neddylation/ deneddylation pathway could contribute to the tumorigenesis of these highly aggressive lung tumors.

Key words: Cul-1, cyclin E, lung tumors, neddylation, Skp2.

Introduction

The ubiquitin/proteasome pathway plays an essential and broad role in the stability and activity of many proteins involved in various physiological processes. The conjugation of a polyubiquitin chain on substrates requires the sequential activity of three distinct enzymes: E1, E2 and an ubiquitin-protein ligase, E3, the latter playing an important role in substrate recognition. To date, two major families of E3 ligases have been described, namely the HECT-domain family and the RING family. One of the best-characterized RING E3 ligase is the Roc1-Skp1-Cul-1/Cdc53-F box polypeptide (SCF). This complex consists of three invariable subunits, a Rbx1/Roc1 RING-finger protein, a Skp1 adaptator and a Cul-1 scaffold element, together with a variable component known as the F-box protein that determines the substrate specificity (1). One of the best-characterized F-box protein is Skp2 (S-phase kinase-associated protein 2), which is the substrate-recognition subunit of the SCF^{SKP2} E3 ligase complex and a critical binding partner of Cul-1 as such (2). Primary targets of SCF^{SKP2} include cell cycle regulators such as G_1-phase cyclins, Cdk inhibitors, DNA replication and transcription factors, as well as non-cell cycle-specific substrates (2). To date, six *cul* genes have been identified in humans (3). While all of the Cul products are able to bind to Roc1, only Cul-1 interacts with Skp1 to form the SCF complex (4, 5). Most, if not all, cullin proteins are post-translationally modified by the ubiquitin-like protein Nedd8/Rub1 (6, 7, 8, Wu, 2000 #67), a phenomenom called neddylation, that is currently thought to enhance the activity of SCF ligases (9).

Bronchogenic carcinoma is the most frequent fatal malignancy in males in Europe and in both sexes in United States. According to the WHO classification of lung cancer (10), lung tumors comprise non small cell lung carcinoma (NSCLC) that include squamous carcinoma, adenocarcinoma and large cell carcinoma, and tumors with neuroendocrine (NE) features that

are presented in four different entities, small cell lung carcinoma (SCLC), large cell NE carcinoma (LCNEC), a variant of large cell carcinoma, and typical and atypical carcinoids. LCNEC and SCLC can be grouped as high grade neuroendocrine lung cases (HGNE) based on their indistinguishable clinical outcome after stratification per stage (11), their gene expression profiling analysis using cDNA microarrays (12), and their similar patterns of protein expression (13 , 14 , 15 , 16). By contrast, typical and atypical carcinoids are considered as low and intermediate grade NE tumors. Despite the central role of Cul-1 in the proteolytic control of cell-cycle regulators, its status has never been investigated in human tumors. Therefore, the aim of this study was to investigate the expression pattern of the Cul-1 protein in a series of 128 human lung tumors of all histological types, by using immunohistochemistry and immunoblotting.

Methods

Tissue samples

One hundred and twenty eight human lung tumors were included in this study. Tissue samples were taken at surgical resection of lung tumors in 102 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 taken at distance from tumor bulk 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 WHO Classification (10). They consisted in 29 squamous cell carcinomas (7 stages I, 6 stages II, 15 stages III and 1 stage IV), 21 adenocarcinomas (14 stages I, 1 stages II, 5 stages III), 14 typical carcinoids (14 stages I) and 10 atypical carcinoids (4 stages I, 4 stages III and 2 stages IV), 19 LCNECs (9 stages I, 2 stages II, 7 stages III and 1 stage IV) and 35 SCLCs (1 stage I, 2 stages II, 28 stages III and 2 stages IV). Sixty three patients were suffering from nodal metastasis at the time of diagnosis. We used tumors according to the ethical laws of our country.

Antibodies

The antibodies used in this study were anti-actin (Sigma, L'Isle d'Abeau), anti-CAND1 (MO1, clone 5D7, Abnova), anti-Cul-1 (Ab1, Neomarkers, Fremont, CA), anti-human Ki67 antigen (DAKO, Glostrup, Denmark), anti-Skp2 (Skp2-2C8D9, Zymed, San Francisco, CA), anti-cyclin E (13A3, Novocastra Newcastle UK) and anti-NEDD8 (Zymed; Alexis).

Immunohistochemistry (IHC)

Cul-1 and CAND1 immunostainings were performed on 5 μm thick frozen sections that were air-dried and fixed in 10% paraformaldehyde for 10 minutes at 37°C or formalin fixed paraffin-embedded sections, respectively. Endogenous peroxydase activity was inhibited using an H_2O_2 solution. Then, a three step immunohistochemical method was applied, with an antigen retrieving step for CAND1. Non specific binding sites were blocked by incubating the sections for 1 hour in donkey normal serum (diluted at 1:50 in phosphate-buffer saline [PBS] containing 0.03% bovine serum albumine [BSA]). The sections were subsequently incubated overnight at 4°C with the anti-Cul-1 (Ab-1) or anti-CAND1 antibody at 1:300 or 1:500 dilution, respectively. After four ten minutes washes with PBS/0.03% BSA, the biotinylated donkey anti-rabbit or anti-mouse F(ab')2 secondary antibody (1:1000; The Jackson Laboratory, West Grove, PA) was added for 1 hour at room temperature. The detection of bound antibody was accomplished using the streptavidin-biotin-peroxidase complex (1:200; Strept-AB complex; DAKO) for 1 hour at room temperature with diaminobenzidine as a chromogene. The slides were then counterstained with Harris' hematoxylin. Slides incubation with normal rabbit or mouse IgG at the same concentration as the primary antibody served as a negative control. Immunohistochemical analyses of KI67, Skp2 and cyclin E were carried-out as previously described (16)

Immunohistochemical staining evaluation

CAND1, Cul-1, Skp2 and cyclin E immunohistochemical analyses were evaluated by two independent observers (CS and EB), in distinct areas of the slide sections for correlation and confirmation of tissues analysis. Immunostaining was 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+). For Cul-1 and CAND1

immunodetection analyses, normal bronchi and alveolar epithelial cells were used as internal control (intensity 2+, score 80 to 120 for Cul-1 ; intensity 2+, score 150 to 200 for CAND1). For Cul-1, as compared to internal controls, tumor samples were graded into two classes according to their score.(class 1: ≤120, class 2: > 120). Because Cul-1 protein was clearly detected in normal lung tissues, tumors exhibiting a final score > 120 (class 2) were considered as cases overexpressing Cul-1. For CAND1, tumors exhibiting a final score <100 were considered as tumors having lost CAND1. Skp2 and cyclin E scores were assessed as previously described (16).

Immunoblotting experiments

Fourty six representative tumoral samples were analyzed and were taken at the immediate vicinity of those studied by immunohistochemistry. Immunoblotting experiments were performed as previously described (14).

Statistical Analyses

Statistical analysis were performed with a Stat View Program Package (Abacus Concepts, Berkeley, CA, USA). The staining scores were compared through different categories using the Mann-Whitney *U*-Test or Kruskal-Wallis test and correlations were based on the chi-square (X^2) test with a p value < 0.05 considered significant.

Results

Cul-1 protein expression is altered in human lung carcinoma

To investigate the Cul-1 protein expression in bronchogenic carcinoma, we first performed immunohistochemical analysis on a panel of 50 NSCLC (21 adenocarcinoma and 29 squamous cell lung carcinoma), 19 LCNEC (Large Cell Neuroendocrine Carcinoma), 35 SCLC (Small Cell Lung Carcinoma) and 24 typical and atypical carcinoids (Table 1 and Figure 1). As Cul-1 immunostaining was heterogeneous among lung tumors, differential scores were ascribed to each case according to the intensity of staining and percentage of stained cells. A mean score was then calculated and tumor samples were grouped in two classes as follow: class 1: no staining or moderate staining, class 2: high staining. Cul-1 was expressed at a moderate level (class 1) in the normal lung parenchyma adjacent to tumor cells on sections, as well as in normal lung epithelium distant from tumor (Figure 1A). As compared to these normal lung tissues, we observed a high level of Cul-1 protein expression in 40% (51/128) of all tumors tested (Table 1). Of the 50 NSCLC analyzed, 34% (17/50) exhibited an upregulation of Cul-1 protein level (Table 1). No significative difference was found between adenocarcinoma and squamous carcinoma. In contrast, Cul-1 was differentially expressed in NE lung tumors. Indeed, carcinoids predominantly overexpressed Cul-1 (75%, 18/24), whereas 70% (28/54) of HGNE tumors displayed a low Cul-1 level (Table 1, $p<0.0001$ and Figure 1B). The distinction between atypical carcinoids (AC) and LCNEC is sometimes difficult based on morphological criteria only. Interestingly, the Cul-1 scores were strongly divergent between these two entities (Figure 1C, $p=0.0002$), indicating that Cul-1 IHC status could help to distinguish between AC and LCNEC. Altogether, these results provide the first evidence of an altered pattern of Cul-1 protein expression in human

lung carcinoma and demonstrate that high levels of Cul-1 are associated with a low grade NE phenotype.

Cul-1 is neddylated in high grade neuroendocrine lung tumors

To validate our IHC data, we performed western blot analyses in 46 representative tumor samples of the IHC series. Figure 2A illustrates an example of the results. As compared to normal lung tissues in which Cul-1 expression was clearly detected (lanes 1-2 and 9-10), tumor samples exhibited either similar (lanes 14-15), decreased (lanes 4-7, 13) or upregulated (lanes 3, 8, 11-12, 16) Cul-1 expression (Figure 2A). Overall, the immunoblotting results were consistent with those of the IHC study in 80% of the cases, reflecting a good concordance between both technics (data not shown).

Interestingly, when performing immunoblotting, we repeatedly noticed the appearance of higher migrating forms of Cul-1 in some tumors (Figure 2A, arrows). As these additional bands are reminiscent of neddylated Cul-1 protein (6 , 17), the immunoblots were repeated using an anti-Nedd8 antibody. As shown in Figure 2A, a neddylated fragment that co-migrates at the same electrophoretic size as Cul-1 was detected in the samples with higher Cul-1 migrating bands. Overall, neddylated forms of Cul-1 were detected in 11 of 24 (46%) HGNE lung tumors (Table 2), especially in SCLC (9/15, 60%) (data not shown). In contrast, neddylation was not observed in normal lung tissues as well as in NSCLC and carcinoids (Figure 2B). Moreover, we did not observe a relationship between the expression level and the neddylated pattern of Cul-1 (Table 2).

The CAND1 protein is an inhibitor of SCF ligases activity that binds unneddylated forms of Cul-1 (18 , 19). It was previously shown that CAND1 enhances deneddylation of Cul-1 by COP9 signalosome (20). Therefore, we asked whether neddylation of Cul-1 was related to CAND1 status in lung tumors. As shown in figures 3A & 3B, CAND1 was

expressed at a moderate level in normal lung tissues, NSCLC and carcinoids. In contrast, it was strongly downregulated in HGNE lung tumors, mostly in SCLC (Figures 3A&B, p<0.0001). Importantly, when investigating relationships between CAND1 status and Cul-1 neddylation, we observed an inverse correlation (Figure 3C, p=0.03), suggesting that downregulation of CAND1 contributes to Cul-1 neddylation in HGNE lung tumors.

High levels of Cul-1 correlate with a low KI67 proliferative index and downregulation of Skp2 and cyclin E protein expression in human lung tumors

To further investigate the role of Cul-1 in human lung carcinogenesis, we studied the relationships between its IHC status and the KI67 proliferative index. As Figure 4 illustrates, a high Cul-1 protein level was associated with a low KI67 index in all tumors tested (p=0.005). These results suggest that high levels of Cul-1 are predictive of a low proliferative status.

The Skp2 F-box protein is one of the most critical Cul-1 binding protein required for efficient and specific selection of SCF substrates. Interestingly, it was also reported that Cul-1 negatively regulates the expression of Skp2 in cell lines (21). Therefore, our demonstration of an altered pattern of Cul-1 expression in lung tumors led us to investigate the relationships between Cul-1 and Skp2 status. We previously analyzed Skp2 expression by IHC in the same series of lung tumors and showed its overexpression in 32% (16/50) of NSCLC and 59% (46/78) of NE lung tumors (16). When we integrated the Cul-1 status to these results, we observed that a high level of Cul-1 expression was associated with a low Skp2 score in all tumors (p=0.01, Figure 5A). Therefore, these results suggest a negative effect of Cul-1 on Skp2 protein expression.

The crucial function of Cul-1 inside SCF complexes is to promote the proteasomal degradation of key regulators of the cell cycle. Thus, we finally wondered whether alteration

of Cul-1 profile could be associated with a deregulated expression of some of its target genes. The cyclin E oncoprotein is one of the major target of SCF complexes (22). A previous IHC analysis of cyclin E in our series of tumor samples revealed its elevated expression level in 53% (26/49) and 37% (29/78) of NSCLC and NE lung tumors respectively (16). When investigating the relationships between Cul-1 and cyclin E IHC status, we observed that high levels of Cul-1 strongly correlated with low levels of cyclin E (p=0.0003, Figure 5B). Interestingly, we also found that cyclin E specifically accumulated in tumors displaying neddylated forms of Cul-1 (Table 3; p=0.0002). Overall, these data suggest that both Cul-1 protein level and neddylation pattern might be involved in the control of cyclin E status in lung tumors.

Discussion

Cul-1 is the scaffold component of SCF (Skp1-Cul-1-F-box protein) complexes that control the proteolysis of a large number of proteins predominantly involved in cell cycle progression (23, for review). Numerous studies have already reported the abnormal expression of F-box proteins in human cancers. In contrast, and despite its core position inside SCF complexes, the Cul-1 status remains largely unknown in human malignancies. In this study, we provide the first evidence of an altered pattern of Cul-1 expression in human lung carcinoma with 40% of tumors displaying high levels as compared to normal lung. Controversial data exist as regard to the role played by Cul-1 in cellular growth control. Some studies have suggested that Cul-1 positively regulates cell cycle progression (24 , 25, 26). In contrast, other reports implicate Cul-1 as an inhibitor of the cell cycle. Indeed, Cul-1 loss-of-function mutations in *C. elegans* lead to hyperplasia with a shortened G1 phase of the cell cycle (3). In this study, we demonstrate that high Cul-1 protein levels correlate with a low KI67 proliferative index, suggesting that upregulation of Cul-1 could counteract hyperproliferative signals generated in tumor cells. Furthermore, as inactivation of Cul-1 functions leads to genetic instability and neoplastic transformation in mice (27, 28), our results suggest that high levels of Cul-1 might also protect cells from genetic instability. This could explain why the majority of carcinoids (75%) which are characterized by low proliferative index and low genetic instability, display high levels of Cul-1.

Most, if not all cullin proteins are post-translationally modified by the ubiquitin-like protein Nedd8/Rub1 (6, 7, 8, Wu, 2000 #67). By using immunoblotting, we observed that neddylated forms of Cul-1 accumulate in HGNE lung tumors whereas we were unable to detect Cul-1 neddylation in NSCLC and carcinoids as well as in normal lung tissues. Neddylation was recently reported to control the stability of the Cul-1 protein (29) but we did

not observe a correlation between Cul-1 neddylation and total expression level in NE lung tumors. In contrast, we found that the Cul-1 inhibitor CAND1 is strongly downregulated in HGNE lung carcinoma as compared to normal lung tissues, carcinoids and NSCLC. Moreover, we also show that CAND1 protein level is inversely related with expression of neddylated forms of Cul-1 (Figure 3C, p=0.03). Altogether, these results suggest that a low level of CAND1 could contribute to the maintenance of neddylated Cul-1 in HGNE lung tumors. The conjugation of Nedd8 to the arginine residue at position 720 of Cul-1 is currently thought to enhance the activity of SCF ligases, likely by increasing their affinity for some E2 enzymes (9). However, it has also been reported that accumulation of hyperneddylated Cul-1 correlates with a decreased rather than an enhanced SCF activity (18, 30, 31). In this study, we provide evidence that neddylation of Cul-1 is associated with high levels of the cyclin E protein (p=0.0002), one of the major target of SCF complexes. These data suggest that accumulation of neddylated forms of Cul-1 in HGNE lung tumors could inactivate rather than activate SCF complexes. It was recently demonstrated that supplementation of cellular extracts with substrate could prevent the deneddylation of cullins (32). Therefore, we cannot exclude that high levels of cyclin E could cooperate with CAND1 downregulation for the maintenance of neddylated Cul-1 in HGNE lung tumors.

Knock-out mice models have previously shown that both Cul-1 and Cul-3 proteins negatively control cyclin E protein level (26, 27, 33). Here, we show that high Cul-1 levels correlate with a decrease of cyclin E protein in lung tumors (p=0.0003, Figure 5B). These data are in favor of cyclin E being an important target of Cul-1 in these tumors. Such effect was not linked to a general activation of SCF complexes, since we did not found a correlation between Cul-1 and $p27^{Kip1}$, another major target of SCF (data not shown). Therefore, as Cul-1 neddylation was unrelated to its total expression level in this study, both phenomena could contribute to the regulation of cyclin E expression, at least in HGNE lung tumors. So far, two

SCF complexes, namely SCF^{SKP2} and SCF^{FBW7}, have been involved in the proteasomal degradation of cyclin E (23). Since accumulation of Cul-1 strongly correlates with low levels of the Skp2 F-box protein in lung tumors (p=0.0117, Figure 4A), it is tempting to speculate that SCF^{FBW7} complexes could play a role in cyclin E downregulation. Interestingly, a predominant role of FBW7 in regulating cyclin E turnover was recently reported (34). It remains to be determined whether the status of FBW7 is altered and correlates with that of Cul-1 in human lung tumors.

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Table 1. Immunohistochemical analysis of Cul1 protein expression in human lung carcinoma according to histological sub-types.

Cul-1 expression level				
Histological sub-type	nb	class 1 [a]	class 2 [a]	p [e]
Adenocarcinoma	21	13 (62%)	8 (38%)	NS
Squamous carcinoma	29	20 (69%)	9 (31%)	
Total NSCLC [b]	**50**	**33 (66%)**	**17 (34%)**	
Carcinoids	24	6 (25%)	18 (75%)	<0.0001
LCNEC [c]	19	12 (63%)	7 (37%)	
Small Cell Carcinoma	35	25 (72%)	10 (28%)	
Total HGNE carcinoma [d]	**54**	**38 (70%)**	**16 (30%)**	
Total tumors	**128**	**77 (60%)**	**51 (40%)**	**0.0009**

Immunostaining scores were calculated by multiplying the number of labeled cells (0 to 100%) by the level of intensity (1 to 3). According to this, the samples were grouped in two classes as described in the material and methods section and as follow[a]: class 1: low level of Cul1 protein expression (score ≤120), class 2: high level of Cul1 protein expression (score >120). nb: number of cases in each histological type. [b] Non small cell lung carcinoma. [c] Large cell neuroendocrine carcinoma. [d] High grade neuroendocrine lung tumors. [e] $\chi 2$ test. NS, non significant.

Table 2. *Relationships between Cul-1 protein level and its neddylated status in human lung carcinomas.*

Histological types	*nb*	*Class 1* [a]		*Class 2* [b]		*P* [e]
		Nedd 8 + [c]	*Nedd 8 -* [d]	*Nedd 8 +* [c]	*Nedd 8 -* [d]	
NSCLC	*15*	*0*	*11*	*0*	*4*	*NS*
Carcinoids	*7*	*0*	*1*	*0*	*6*	*NS*
HGNE tumors	*24*	*4*	*10*	*7*	*3*	*NS*
Total tumors	***46***	***4***	***22***	***7***	***13***	*NS*

[a,b]Tumors displaying a low[a] or high[b] Cul-1 protein level as detected by immunoblotting using an anti-cullin-1 antibody. [c] Nedd 8 + : Tumor samples with neddylated Cul-1 as detected by immunoblotting using an anti-Nedd8 antibody. [d] Nedd 8 - : Tumor samples without neddylated Cul-1 as detected by immunoblotting. [e] $\chi 2$ test. nb: number of tumoral samples. NS : non significant.

Table 3. Relationships between Cul-1 neddylation and cyclin E protein level in HGNE lung carcinomas.

	Nedd 8 - [c]	Nedd 8 + [d]	P [e]
Cyclin E low level[a]	8	0	
Cyclin E high level[b]	1	8	0.0002

[a] Tumors displaying a score of cyclin E < 40. [b] Tumors displaying a score of cyclin E ≥ 40 and considered as overexpressing cases. [c,d] Nedd 8 -: tumor samples without neddylated Cul-1 as detected by immunoblotting using an anti-Nedd8 antibody ; Nedd 8 +: tumor samples with neddylated Cul-1. [e] $\chi 2$ test. HGNE: High Grade Neuroendocrine Lung tumors.

Legends

Figure 1. **(A)** Cul-1 immunostaining of normal lung parenchyma and lung cancer tissues on frozen sections using an anti-Cul-1 rabbit polyclonal antibody (Neomarkers). Moderate staining in (**a**) bronchiolar or (**b**) alveolar normal epithelium. **(c)** Strong Cul-1 immunostaining in a typical carcinoid. **(d)**. Cul-1 loss in a small cell lung carcinoma. **(e)** Moderate and **(f)** strong Cul-1 immunostaining in two squamous cell carcinoma. **(B)** Cul-1 scores in carcinoids, HGNE lung carcinoma and NSCLC. **(C)** Comparison of Cul-1 scores between atypical carcinoids and LCNEC. Statistical analyses were performed using a Mann-Whitney *U*-test. NS: not significant.

Figure 2. (A) Cul-1 expression was analyzed by immunoblotting in normal (N) and tumoral (T) lung samples. Cul-1 expression was upregulated (T3, T8, T11-12, T16) or downregulated (T4-7, T13) according to the samples. T14-15 expressed similar levels of Cul-1 as normal lung. Arrows in the upper blot represent higher migrating forms of neddylated Cul-1 protein. Neddylation was confirmed by using an anti-Nedd8 antibody. Actin was used as a loading control. **(B)** Cul-1 expression and neddylation in 7 lung carcinoids were analyzed as described in (A).

Figure 3. (A) CAND1 immunostaining of normal lung parenchyma and lung cancer tissues on paraffin-embedded sections using an anti-CAND1 mouse monoclonal antibody (5D7, Abnova). (**a**) Moderate staining in bronchiolar normal epithelium. **(b)** Strong CAND-1 immunostaining in a squamous cell carcinoma. **(c)** CAND-1 loss in a small cell lung carcinoma. **(d)** Moderate CAND-1 immunostaining in a typical carcinoid (only nuclear reactivity was assessed). **(B)** Comparison of CAND1 scores between carcinoids, HGNE

carcinoma and NSCLC. Statistical analyses were performed using a Kruskal-Wallis test. (C) CAND1 scores according to the presence (NEDD8 +) or absence (NEDD8 -) of Cul-1 neddylation. Statistical analyses were performed using a Mann-Whitney *U*-test.

Figure 4. Percentage of tumoral cells positive for KI67 immunostaining in lung tumors according to high (white box) or low (black hatched box) Cul-1 protein levels. Statistical analyses were performed using a Mann-Whitney *U*-test. NS: not significant.

Figure 5: Skp2 **(A)** and cyclin E **(B)** protein scores in lung tumors according to high (white box) or low (black hatched box) Cul-1 protein levels. Statistical analyses were performed using a Mann-Whitney *U*-test. NS: not significant.