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scientific report

An intact NEDD8 pathway is required for Cullin-dependent ubiquitylation in mammalian cells

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Received June 6, 2001; revised December 5, 2001; accepted December 13, 2001

Skp1-Cdc53/Cul1-F-box (SCF) complexes constitute a class of E3 ubiquitin ligases. Recently, a multiprotein complex containing pVHL, elongin C and Cul2 (VEC) was shown to structurally and functionally resemble SCF complexes. Cdc53 and the Cullins can become covalently linked to the ubiquitin-like molecule Rub1/NEDD8. Inhibition of neddylation inhibits SCF function *in vitro* and in yeast and plants. Here we show that ongoing neddylation is likewise required for VEC function *in vitro* and for the degradation of SCF and VEC targets in mammalian cells. Thus, neddylation regulates the activity of two specific subclasses of mammalian ubiquitin ligases.

INTRODUCTION

Cellular homeostasis requires that certain proteins are degraded in a spatially and temporally controlled manner. One means of achieving this involves the regulated addition of a polyubiquitin tail, which marks the recipient protein for proteasomal destruction. Polyubiquitylation involves the action of a ubiquitin activating enzyme (E1 or Uba), a ubiquitin conjugating enzyme (E2 or Ubc) and a ubiquitin ligase (E3). Seminal studies in yeast have identified a class of E3 enzymes known as SCF complexes that contain Skp1, Cdc53, an F-box protein and Rbx1/ROC1/Hrt1 (for reviews, see Deshaies, 1999; Ciechanover *et al.*, 2000). SCF substrate specificity is conferred by the choice of F-box protein, whereas Cdc53 and Rbx1 recruit an E2 enzyme. Skp1 nucleates the complex by bridging the F-box protein and Cdc53. For example, SCF^{Cdc4} selectively ubiquitylates the cdk inhibitor Sic1. Analogous complexes containing the Cdc53 ortholog Cul1 and specific F-box proteins have been identified in mammalian cells.

The product of the von Hippel-Lindau gene, pVHL, contains a region called the α domain, which loosely resembles an F-box, and forms a multimeric complex (VEC) that contains elongin B (a ubiquitin-like protein), elongin C (a paralog of hSkp1), Cul2 (a paralog of Cul1) and Rbx1 (for a review, see Kondo and Kaelin, 2001). This complex ubiquitylates the α subunits of the hypoxia-inducible transcription factor HIF. Recently, HIF1α was shown to be hydroxylated at a conserved proline residue at position 564 in an oxygen-dependent manner (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001; Yu *et al.*, 2001). This modification is critical for recognition by pVHL, allowing subsequent ubiquitylation by the VEC complex (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001; Yu *et al.*, 2001). Replacement of pVHL with SOCS1, which contains a region that is similar to the pVHL α domain, generates an E3 ligase implicated in the ubiquitylation of VAV and TEL-JAK2 (De Sepulveda *et al.*, 2000; Frantsve *et al.*, 2001; Kamizono *et al.*, 2001). In summary, complexes containing elongins B/C, Cul2 and an α domain protein such as pVHL or SOCS1 structurally and functionally resemble SCF complexes.

Cdc53 becomes covalently linked to the small ubiquitin-like (Ubl) molecule Rub1, and genetic experiments in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Arabidopsis thaliana* are consistent with Rub1 modulating SCF activity (Lammer *et al.*, 1998; Liakopoulos *et al.*, 1998; del Pozo and Estelle, 1999; Osaka *et al.*, 2000). NEDD8, the mammalian homolog of Rub1, can likewise become covalently linked to members of the Cullin family (which are Cdc53 orthologs) *in vitro*, as well as in mammalian cells that have been engineered to overproduce a specific Cullin (Osaka *et al.*, 1998; Hori *et al.*, 1999; Kamura *et al.*, 1999; Liakopoulos *et al.*, 1999;

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Wada *et al.*, 1999a,b; Furukawa *et al.*, 2000). Intriguingly, Cul1-dependent ubiquitylation of p27 and IκBα is impaired when neddylation of Cul1 is blocked *in vitro* (Morimoto *et al.*, 2000; Podust *et al.*, 2000; Read *et al.*, 2000; Wu *et al.*, 2000). Here we report that Cul2 is neddylated in mammalian cells under physiological conditions and that Cul2-dependent ubiquitylation likewise requires ongoing neddylation *in vitro*. More importantly, we show for the first time that SCF and VEC function in mammalian cells requires an intact NEDD8 conjugation system.

RESULTS AND DISCUSSION

To determine whether neddylation of Cullins occurs in mammalian cells under physiological conditions, PC-3 cells were immunoprecipitated with anti-pVHL antibody. As expected, Cul2 co-immunoprecipitated with pVHL, as determined by immunoblot analysis, and migrated as a doublet (Figure 1). The upper band of the doublet reacted with a polyclonal anti-NEDD8 antibody (Figure 1). Similar results were obtained in other cell lines (data not shown). In control experiments, we confirmed that this antibody recognized NEDD8, but not other (Ubl) molecules such as SUMO, elongin B and ubiquitin itself (see Supplementary figure 1 available at *EMBO reports* Online).

Ubiquitylation of proteins requires the action of the E1 ubiquitin activating enzyme (UBE1) and an E2 ubiquitin conjugating enzyme. Neddylation involves analogous enzymes. NEDD8 activation is carried out by APP-BP1 and UBA3, which are homologous to the N-terminus and C-terminus, respectively, of UBE1 (Liakopoulos *et al.*, 1998; Osaka *et al.*, 1998; Gong and Yeh, 1999; Kamura *et al.*, 1999). hUbc12 is the known NEDD8 conjugating enzyme (Liakopoulos *et al.*, 1998; Osaka *et al.*, 1998; Gong and Yeh, 1999; Kamura *et al.*, 1999). We developed an *in vitro* Cul2 neddylation assay in which unmodified, ^{35}S-labeled Cul2 was incubated with a HeLa cell fraction enriched for APP-BP1/UBA3 (FII fraction) and recombinant NEDD8. The addition of recombinant hUbc12 led to the appearance of neddylated Cul2, as expected, whereas the addition of other E2-like molecules such as hUbc10 or hUbc17 did not (Figure 2A). Use of this assay, in conjunction with specific Cul2 deletion and point mutants, allowed us to map the Cul2 neddylation site to lysine 689, in keeping with an earlier report (Wada *et al.*, 1999b) (Figure 2A and data not shown). Thus, Cul2 is neddylated on K689 by hUbc12. Notably, the primary sequence surrounding the Cul2 neddylation site is well conserved in the various Cullin family members, and the corresponding lysine residue is neddylated in the Cullins analyzed to date (Wada *et al.*, 1999b; Furukawa *et al.*, 2000; Morimoto *et al.*, 2000; Osaka *et al.*, 2000; Read *et al.*, 2000; Wu *et al.*, 2000).

Conjugation to Ubl may affect protein stability, subcellular localization and transport, as well as function (Hochstrasser, 2000). In pilot experiments, we transiently transfected cells to produce epitope-tagged wild-type Cul2 or Cul2 K689R. The two Cul2 species were both primarily nuclear and comparatively stable (data not shown). Similar findings have been reported by others (Furukawa *et al.*, 2000). Recently, it was shown that inhibition of Cul1 neddylation by a dominant-negative version of hUbc12 (hUbc12OH) leads to a decrease in SCF-dependent ubiquitylation of targets such as p27 and IκBα *in vitro*

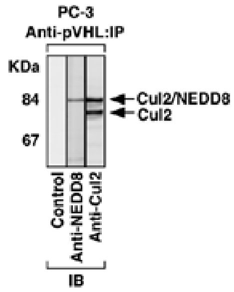

Fig. 1. Conjugation of NEDD8 to Cul2. PC-3 cells were immunoprecipitated (IP) with anti-pVHL antibody. Bound proteins were loaded in a wide well, resolved by electrophoresis and transferred to a membrane. The membrane was cut into strips and immunoblotted (IB) with control, anti-NEDD8 or anti-Cul2 antibodies.

(Morimoto *et al.*, 2000; Podust *et al.*, 2000; Read *et al.*, 2000; Wu *et al.*, 2000). In analogous experiments using S100 extracts that did (WT) or did not (RC) contain wild-type pVHL, we found that dominant-negative hUbc12 blocked the ubiquitylation of HIF by the VEC complex (Figure 2B). Importantly, the addition of wild-type hUbc12 restored HIF ubiquitylation in the face of hUbc12OH (Figure 2B).

The HIF ubiquitylation assays were performed with S100 extracts that contained endogenous, neddylated Cul2 (Figure 2C) and yet the dominant-negative hUbc12 experiments implied that ubiquitylation of HIF by pVHL required neddylation of one or more proteins present in the reaction. Thus, either Cul2 was not the relevant target of hUbc12OH and/or Cul2 undergoes cyclical deneddylation and reneddylation under these assay conditions. To begin to resolve this issue, aliquots of the ubiquitylation reactions were removed at various time points following the start of the reaction. The VEC complex was recovered by immunoprecipitation and the status of Cul2 was assessed by immunoblot analysis (Figure 2C). The endogenous Cul2 present in the VEC complex was present in both neddylated and unneddylated forms during the reaction. However, in reactions supplemented with wild-type hUbc12, there was a significant shift towards the neddylated form of Cul2 in the VEC complex, whereas hUbc12OH promoted the accumulation of the unneddylated form of Cul2. The ratio of neddylated/unneddylated Cul2 reached equilibrium within the first 15 min (Figure 2C), well within the 60–90 min incubation period used for the *in vitro* ubiquitylation assays. One interpretation of these data is that the efficient ubiquitylation of HIF by VEC is coupled to ongoing deneddylation and reneddylation of Cul2. This idea is consistent with the recent finding that a non-neddylatable Cul1 mutant cannot support robust SCF-dependent ubiquitylation *in vitro* (Furukawa *et al.*, 2000; Morimoto *et al.*, 2000; Wu *et al.*, 2000). Furthermore, two recent studies showed that the COP9 signalosome was required for deneddylation of Cul1 and that the absence of this activity led to impaired SCF function (Lyapina *et al.*, 2001; Schwechheimer *et al.*, 2001). A role for deneddylation, in addition to reneddylation, could account for the slightly diminished HIF ubiquitylation observed in the presence of excess wild-type hUbc12 (Figure 2B, lane 10).

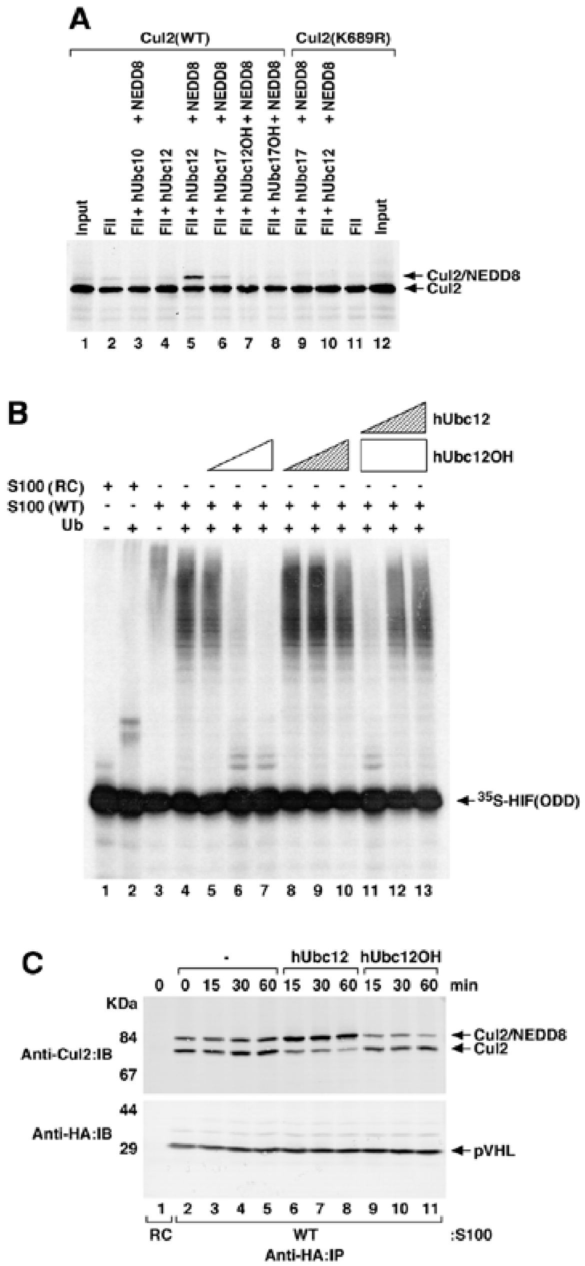

Fig. 2. Dominant-negative hUbc12OH blocks Cul2-dependent ubiquitylation of HIF *in vitro*. (**A**) ^{35}S-labeled Cul2 *in vitro* translate was incubated with a HeLa FII extract and, where indicated, recombinant NEDD8 and a recombinant hUbc. Modified and unmodified Cul2 were immunoprecipitated with an anti-Cul2 antibody, resolved by SDS–PAGE and detected by fluorography. The background level of Cul2/NEDD8 observed in lane 1 likely reflects the presence of rabbit NEDD8 and Ubc12 in the reticulocyte lysate used for *in vitro* translation. (**B**) ^{35}S-labeled Gal4-HIF *in vitro* translate containing the HIF oxygen-dependent degradation domain (ODD) was incubated with S100 extracts that did (WT) or did not (RC) contain pVHL under conditions permissive for *in vitro* ubiquitylation. Where indicated, recombinant wild-type hUbc12 or dominant-negative hUbc12OH was added (1, 2 or 4 μM as indicated by triangles). Modified and unmodified Gal4-HIF were immunoprecipitated with anti-Gal4 antibody, resolved by SDS–PAGE and detected by fluorography. (**C**) hUbc12 or hUbc12OH was added to a WT S100 extract as in (B) (4 μM). At the indicated times the status of Cul2 and HA-pVHL in anti-HA immunoprecipitates (IP) was determined by immunoblot analysis (IB).

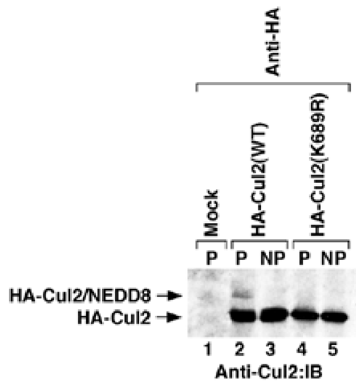

Fig. 3. *In vivo* neddylation of Cul2. CHO cells with temperature-sensitive APP-BP1 (ts41) were stably transfected with empty expression plasmid (Mock) or plasmids encoding HA-tagged wild-type (WT) or Cul2 K689R. Clones were grown at permissive (P) or non-permissive (NP) temperature for 15 h and immunoprecipitated with anti-HA antibody. Bound proteins were immunoblotted with anti-Cul2 antibody.

Collectively, our results and those of others suggest that SCF- and VEC-dependent ubiquitylation is linked to neddylation *in vitro*. Furthermore, disruption of Rub1 in yeast and plants causes phenotypes that are likely due to altered SCF function (Lammer *et al.*, 1998; del Pozo and Estelle, 1999; Osaka *et al.*, 2000). To determine whether an intact NEDD8 pathway affects SCF and VEC function in mammalian cells, we made use of two Chinese hamster ovary (CHO) cell lines, v79 and ts41. The ts41 cell line contains a temperature-sensitive mutation in APP-BP1 (Hirschberg and Marcus, 1982; Handeli and Weintraub, 1992; Chen *et al.*, 2000) and failed to neddylate ectopically expressed HA-Cul2 (wild type) at the non-permissive temperature (Figure 3). The endogenous Cul2 in ts41 cells likewise did not conjugate to NEDD8 at the non-permissive temperature (see Supplementary figure 2). As expected, HA-Cul2 (K689R) did not become neddylated at either temperature (Figure 3). In addition, ts41 cells, like mouse fibroblasts with a temperature-sensitive mutation in the ubiquitin UBE1 enzyme (ts20) (Chowdary *et al.*, 1994), accumulated HIF1α at the non-permissive temperature (Figure 4A and data not shown). The observed differences in HIF1α mobility between ts41 and ts20 might be due to differences in post-translational modification and species of origin. Notably, the accumulation of HIF1α observed at the non-permissive temperature in ts41 cells was comparable to that observed under hypoxic conditions (Figure 4B). As expected, the accumulation of HIF1α observed at the non-permissive temperature was due to increased half-life, as shown by a cyclohexamide-chase assay (Figure 4C). The increase in HIF1α was associated with increased levels of the GLUT1 glucose transporter, which is encoded by a HIF target gene (Figure 4D). No such increase was observed in v79 cells (data not shown).

Treatment of v79 and ts41 cells with TNFα, which promotes the phosphorylation of IκBα on residues Ser32 and Ser36 required for the recognition and subsequent ubiquitylation by SCFβ^{TrCP} (Karin and Ben-Neriah, 2000), caused the rapid degradation of IκBα at the permissive temperature (Figure 5A). It should be noted that there was a consistent recovery of IκBα at 60 min, likely due to depletion of TNFα from the media.

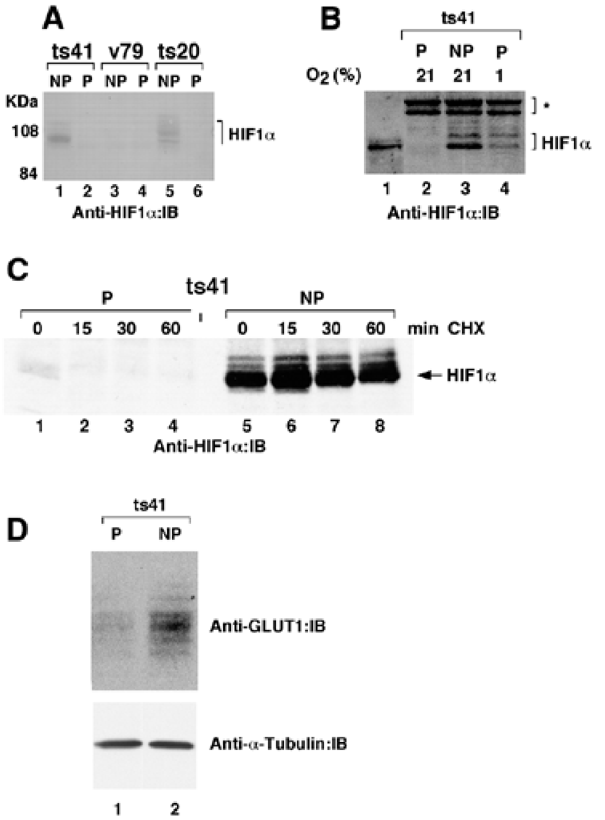

Fig. 4. Neddylation linked to VEC-dependent ubiquitylation in mammalian cells. (**A**) CHO cells with wild-type (v79) or temperature-sensitive APP-BP1 (ts41) or mouse fibroblasts with temperature-sensitive UBE1 (ts20) were grown at permissive (P) or non-permissive (NP) temperature for 15 h and immunoblotted with anti-HIF1α antibody. (**B**) ts41 CHO cells were grown at permissive or non-permissive temperature in 1 or 21% O_2 for 15 h and immunoblotted with anti-HIF1α antibody. Lane 1 contained recombinant HIF1α. The asterisk indicates unidentified protein. (**C**) ts41 cells grown at permissive or non-permissive temperature for 15 h were treated with cyclohexamide (CHX; 10 μg/ml) for the indicated times and immunoblotted with anti-HIF1α antibody. (**D**) ts41 cells grown at permissive or non-permissive temperature for 15 h were immunoblotted with anti-GLUT1 antibody (upper panel) and anti-α-tubulin antibody (lower panel).

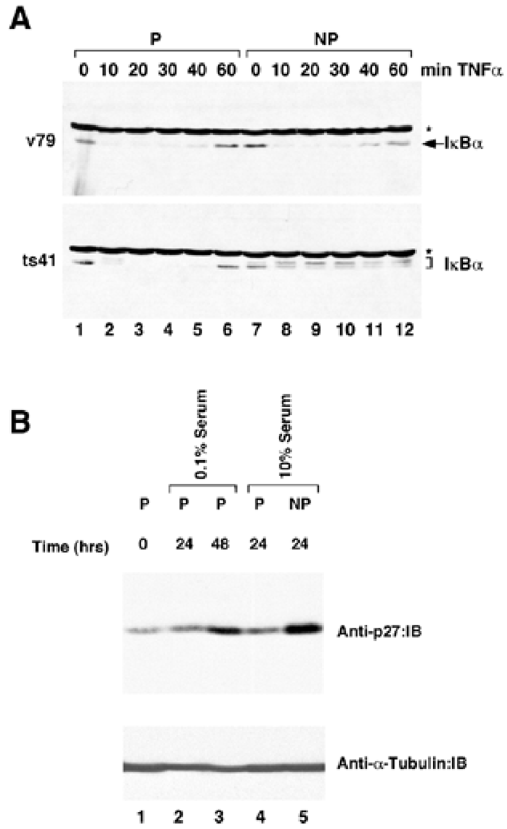

Fig. 5. Neddylation linked to SCF-dependent ubiquitylation in mammalian cells. (**A**) v79 and ts41 CHO cells were exposed to rat TNFα for the indicated time at the permissive (P) or non-permissive (NP) temperature and immunoblotted with anti-IκBα antibody. Note that IκBα migrates as a doublet due to phosphorylation. The asterisk indicates unidentified protein. (**B**) ts41 CHO cells were grown in the presence of 0.1 or 10% serum for the indicated time at the permissive or non-permissive temperature and immunoblotted with anti-p27 (upper panel) and anti-α-tubulin antibody (lower panel). Following 48 h of serum starvation, 92% of the cells were in G_0/G_1, as determined by fluorescence-activated cell sorter analysis of propidium iodide stained cells (data not shown).

However, at the non-permissive temperature, IκBα was markedly stabilized in the ts41 cells (Figure 5A). While these experiments were in progress, Furukawa *et al.* (2000) reported that a non-neddylatable form of Cul1 likewise failed to support efficient ubiquitylation of IκBα in transient transfection experiments. Similarly, ts41, but not v79, cells accumulated the SCF^{SKP2} target p27 at the non-permissive temperature to a level comparable to that observed following serum starvation, which is known to inhibit p27 degradation (Figure 5B). Collectively, these findings suggest that Cullin-dependent ubiquitylation in cells requires an intact NEDD8 pathway.

Finally, ts41 and ts20 cells were grown at the permissive or non-permissive temperature in the absence or presence of a proteasome inhibitor and immunoblotted with an anti-ubiquitin antibody (Figure 6). As expected, ts20 cells displayed a decrease in polyubiquitylated proteins at the non-permissive temperature (compare lane 5 with 6 or lane 7 with 8). In contrast, ts41 cells did not. Therefore, NEDD8 has a specific, rather than a global, effect on protein polyubiquitylation.

Collectively, these results suggest that NEDD8 influences SCF and VEC function *in vitro* and *in vivo*. Currently, there is no clear biochemical explanation for this finding, since neddylation does not appear to affect the assembly of the core SCF (Furukawa *et al.*, 2000; Osaka *et al.*, 2000; Read *et al.*, 2000) and VEC complexes (Pause *et al.*, 1997, 1999; Lonergan *et al.*, 1998). Likewise, complexes containing non-neddylatable Cul2 and Cul1 mutants retain the ability to bind to substrates such as HIF1α (data not shown) and IκBα (Furukawa *et al.*, 2000), respectively. Given the available biochemical data, neddylation may affect the specific activity of SCF and VEC ubiquitin ligases. For example, neddylation may affect the conformation of the SCF and VEC complexes in a way that influences whether they productively engage their respective substrates and ubiquitin conjugating enzymes. In this regard, Kawakami *et al.* (2001)

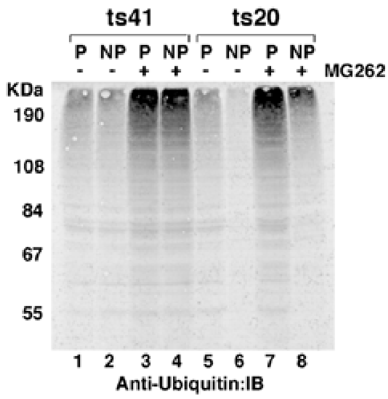

Fig. 6. Impaired neddylation does not globally affect protein polyubiquitylation. ts20 and ts41 cells were grown at permissive (P) or non-permissive (NP) temperature for 15 h with or without proteasome inhibitor MG262 (5 μM). Cells were then lysed, resolved by SDS–PAGE and immunoblotted with anti-ubiquitin antibody.

have recently reported that neddylation promotes E2 recruitment by Cul1-containing SCF complexes.

METHODS

Cell culture. 786-O renal carcinoma and PC-3 prostate carcinoma cell lines (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% or, where indicated, 0.1% heat-inactivated defined/supplemented bovine calf serum (Hyclone) at 37°C in the presence of 10% CO_2. The v79 and ts41 CHO cell lines (Hirschberg and Marcus, 1982) and the ts20 mouse fibroblast line (Chowdary *et al.*, 1994) were grown in DMEM containing 10% Hyclone at 34 or 39°C, as indicated. The 786-O subclones stably transfected to produce HA-tagged wild-type pVHL (Iliopoulos *et al.*, 1995) and ts41 CHO cells stably transfected to produce HA-Cul2 were maintained in media supplemented with G418 (1 mg/ml). Where indicated, rat TNFα (R and D Systems, Minneapolis, MN) was added to the media (20 ng/ml). Growth in 1% oxygen was carried out in an ESPEC BNP-210 Incubator.

Antibodies. Anti-HA polyclonal (Y11) and anti-Gal4 monoclonal antibodies were obtained from Santa Cruz Biotechnology. Anti-pVHL monoclonal antibody (IG32) was described previously (Kibel *et al.*, 1995). Anti-Cul2 polyclonal antibody was obtained from Zymed, anti-HIF1α monoclonal antibody from Novus Biologicals, anti-NEDD8 polyclonal antibody from Alexis Corporation, anti-GLUT1 polyclonal antibody from Alpha Diagnostics, anti-p27 polyclonal antibody from Transduction Laboratories, anti-α-tubulin monoclonal antibody from Sigma and anti-ubiquitin polyclonal antibody from Dako Corporation.

Immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblotting were performed as described previously (Ohh *et al.*, 2000). Cells were lysed in EBC buffer (50 mM Tris pH 8.0, 120 mM NaCl, 0.5% NP-40) supplemented with protease and phosphatase inhibitors and 5 mM iodoacetamide. Immunoprecipitates were washed five times with NETN (20 mM Tris pH 8.0, 120 mM NaCl, 1 mM EDTA, 0.5% NP-40) prior to SDS–PAGE.

***In vitro* neddylation assay.** ^{35}S-labeled Cul2 *in vitro* translates were incubated with 1.6 μg of NEDD8, 20 pmol of the indicated Ubc, an energy-regenerating system (ERS) (20 mM Tris pH 7.4, 2 mM ATP, 5 mM $MgCl_2$, 40 mM creatine phosphate, 0.5 μg/μl creatine kinase) and 20 μg of HeLa FII (Boston Biochem). Production and purification of wild-type or dominant-negative hUbc12 (hUbc12 Cys111Ser) were as described previously (Read *et al.*, 2000). hUbc10 (Townsley *et al.*, 1997) and hUbc17 (GenBank accession No. AF310723) were prepared in the same way. Reactions were adjusted to 50 mM Tris–HCl pH 7.5 in a total volume of 20 μl and incubated at 30°C for 30 min. Reactions were stopped by the addition of SDS sample buffer.

***In vitro* ubiquitylation assay.** Preparation of S100 extracts and *in vitro* ubiquitylation assays were conducted as described previously (Ohh *et al.*, 2000). Briefly, ^{35}S-labeled *in vitro* translates (4 μl) were incubated in the presence of S100 extracts (100–200 μg) supplemented with 8 μg/μl ubiquitin (Sigma), 100 ng/μl ubiquitin aldehyde (Boston Biochem, Cambridge, MA), ERS and 2.5 μM MG262 (Boston Biochem) in a reaction volume of 20–30 μl for 1.5–2 h at 30°C. Where indicated, recombinant wild-type or dominant-negative hUbc12 (Read *et al.*, 2000) were added.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

ACKNOWLEDGEMENTS

We thank Harvey Ozer for the ts20 cells, Rachael Neve for the ts41 cells and members of the Kaelin Laboratory for useful discussions. M.O. is a fellow of the National Cancer Institute of Canada and W.G.K. is a Howard Hughes Medical Institute Assistant Investigator.

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DOI: 10.1093/embo-reports/kvf028