Oncogene (2002) 21, 3978–3987

www.nature.com/onc

CK2-dependent phosphorylation of the E2 ubiquitin conjugating enzyme UBC3B induces its interaction with β-TrCP and enhances β-catenin degradation

Francesca Semplici[1], Flavio Meggio[2], Lorenzo A Pinna[2] and Salvatore Oliviero*[,1]

[1]*Dipartimento di Biologia Molecolare, Università degli Studi di Siena, Italy;* [2]*Dipartimento di Chimica Biologica and Centro del CNR per lo Studio delle Biomembrane, Università degli Studi di Padova, Italy*

Protein kinase CK2 is a ubiquitous and pleiotropic Ser/Thr protein kinase involved in cell growth and transformation. Here we report the identification by yeast interaction trap of a CK2 interacting protein, UBC3B, which is highly homologous to the E2 ubiquitin conjugating enzyme UBC3/CDC34. *UBC3B* complements the yeast *cdc34-2* cell cycle arrest mutant in *S. cerevisiae* and transfers ubiquitin to a target substrate *in vitro*. UBC3B is specifically phosphorylated by CK2 *in vitro* and *in vivo*. We mapped by deletions and site directed mutagenesis the phosphorylation site to a serine residue within the C-terminal domain in position 233 of UBC3B and in the corresponding serine residue of UBC3. Following CK2-dependent phosphorylation both UBC3B and UBC3 bind to the F-box protein β-TrCP, the substrate recognition subunit of an SCF (Skp1, Cul1, F-box) ubiquitin ligase. Furthermore, we observed that co-transfection of CK2α′ together with UBC3B, but not with UBC3ΔC, enhances the degradation of β-catenin. Taken together these data suggest that CK2-dependent phosphorylation of UBC3 and UBC3B functions by regulating β-TrCP substrate recognition.
Oncogene (2002) **21,** 3978–3987. doi:10.1038/sj.onc.1205574

Keywords: CK2; UBC3; β-TrCP; phosphorylation

Introduction

Cell cycle progression is triggered by a signalling cascade which involves a series of protein phosphorylations, as well as induction and degradation of positive regulators of the cell cycle. The deregulated activation of molecules involved in this signalling might lead to uncontrolled cell cycle progression and cell transformation. Protein kinase CK2 is a ubiquitous Ser/Thr kinase (for review see Allende and Allende, 1995; Issinger, 1993; Pinna, 1990; Pinna and Meggio, 1997; Tuazon and Traugh, 1991). Studies from several laboratories have shown a deregulated expression of this kinase in haematopoietic and solid tumours. In particular, in tumours of the prostate, kidney, colon and squamous cell carcinoma of the head and neck (Ahmed, 1994; Gapany *et al*., 1995; Munstermann *et al*., 1990; Rayan *et al*., 1985; Seitz *et al*., 1989; Stalter *et al*., 1994; Yenice *et al*., 1994). CK2 over-expression leads to tumorigenesis *in vivo* and transformation of primary embryo fibroblasts together with Ras (Landesman-Bollag *et al*., 2001; Orlandini *et al*., 1998; Seldin and Leder, 1995). CK2 holoenzyme consists of two catalytic (α and/or α′) and two regulatory (β) subunits assembled as stable heterotetramers whose site specificity is determined by multiple acidic and/or previously phosphorylated residues, the one at position $n+3$ relative to the phosphoacceptor amino acid being of crucial relevance (minimum consensus S/TXXE/D/Yp/Sp) (Meggio *et al*., 1994b). Several proteins phosphorylated by CK2 are implicated in signal transduction, transcriptional activation, cell cycle progression, and cell differentiation (Allende and Allende, 1998; Blanquet, 2000; Guerra and Issinger, 1999; Guerra *et al*., 1999; Pinna and Meggio, 1997).

To identify CK2 interacting proteins we performed a genetic screen in yeast. A positive clone in our screen codes for a new member of the ubiquitin conjugating enzyme family that we named UBC3B for its high similarity with the E2 ubiquitin conjugating enzyme UBC3/CDC34.

The ubiquitin-dependent proteolytic pathway plays a pivotal role in regulating the abundance of regulatory proteins involved in several cell regulatory pathways. Ubiquitin-proteasome-mediated protein degradation involves the covalent binding of the 76 amino acid ubiquitin polypeptide to substrate proteins that will be recognized by the proteasome. Ubiquitination reaction is initiated by the ubiquitin-activating enzyme (E1) which forms a thioester bond with ubiquitin, which is then transferred to a reactive cysteine residue of a ubiquitin-conjugating enzyme (E2 or UBC) (Hershko and Ciechanover, 1998; Hershko *et al*., 2000; Hochstrasser, 1996). Ubiquitin is then transferred to the ε-group of the reactive lysine residue of the target protein from E2 generally with the help of the ubiquitin ligase (E3) enzymes (reviewed in Jackson *et*

*Correspondence: S Oliviero, Dipartimento di Biologia Molecolare, Università degli Studi di Siena, via Fiorentina 1 53100 Siena, Italy; E-mail: oliviero@unisi.it
Received 19 December 2001; revised 12 March 2002; accepted 15 April 2002

al., 2000; Weissman, 2001). E2 proteins are a family of proteins with a conserved core domain containing a cysteine residue that is the active site for the thioester bond with ubiquitin. The specificity of the substrate recognition is achieved by the combinatorial interactions among different E2 and E3 factors (DeSalle and Pagano, 2001). In *S. cerevisiae* Cdc34 is required for the G1 to S transition (Goebl *et al*., 1988). Many proteins involved in cell cycle progression are degraded in a cdc34-dependent manner including Sic1p, Far1, CDC6, G1 cyclins, and E2F-1 (Deshaies *et al*., 1995; Drury *et al*., 1997; Feldman *et al*., 1997; Henchoz *et al*., 1997; Marti *et al*., 1999; Schwob *et al*., 1994). In eukaryotes the E2 CDC34 (also called UBC3), together with SCF (Skp1, Cullin, F-box protein) ligases are required for the G1 to S phase progression of the cell cycle (DeSalle and Pagano, 2001). UBC3 participates in the G1 to S phase transition targeting the mammalian cyclin-dependent kinase inhibitor p27 for proteolysis (Pagano *et al*., 1995; Sutterluty *et al*., 1999).

We first tested in yeast whether UBC3B would behave as bona fide CDC34 performing a complementation test using the cell cycle defective mutant *cdc34-2*. CK2 phosphorylates UBC3B in its C-terminal domain at a serine residue both *in vitro* and *in vivo* in transfected cells. We mapped the phosphorylation site to a serine which is conserved in UBC3B and UBC3. Importantly, the CK2-dependent phosphorylation site lies within a sequence highly similar to the consensus site for β-TrCP binding. *In vitro* binding experiments demonstrated that phosphorylated UBC3 and UBC3B bind specifically to β-TrCP. The UBC3 and UBC3B binding to β-TrCP determines an increase in the β-catenin degradation. Thus, our results suggest that CK2-dependent phosphorylation of UBC3 and UBC3B controlling their physical interaction with β-TrCP might regulate the activity of this F-box protein in its substrate recognition.

Results

CK2 interacting protein UBC3B codes for a new member of the CDC34 family

Using CK2 catalytic subunit α' in a two hybrid screen we have identified among others, and in addition to several clones, coding for the β-subunit, as expected, the cDNA coding for a ubiquitin conjugating enzyme (UBC) gene highly similar to the human CDC34/UBC3 (Plon *et al*., 1993). Analogous results were obtained using the other catalytic subunit CK2α. By screening mouse and human cDNA libraries we cloned both the full length mRNAs and named the new gene UBC3B.

Human and mouse UBC3B showed only one substitution while in UBC3B are present 47 substitutions with respect to UBC3. Both human and murine UBC3B contain the conserved cysteine and leucine residues which are part of the active site in the E2 enzyme (Hochstrasser, 1996). UBC3B is identical to the rabbit cdc34 (Sun *et al*., 1997) suggesting that this latter is the orthologue of UBC3B rather than of CDC34/UBC3. UBC3B shows 35% identity to *S. cerevisiae* cdc34 over the first 240 amino acids. In addition, the yeast protein contains a long C-terminal tail of 94 residues rich in aspartic and glutamic acid residues (Figure 1). Human *UBC3* complements a mutant strain containing *cdc34-2* t.s. mutant gene for growth at the restrictive temperature (Goebl *et al*., 1988). To test whether *UBC3B* complements the cell cycle arrest *cdc34-2* mutant strain we transformed this *S. cerevisiae* mutant strain with *UBC3B* under the control of *GAL* 1-10 promoter. As shown in Figure 2, we observed complementation only on galactose at the non-permissive temperature therefore demonstrating that *UBC3B* complements the yeast *cdc34* t.s. to overcome the cell cycle arrest.

Ubiquitin conjugating enzymes form thioesters with ubiquitin and transfer the ubiquitin to a target substrate. As shown in Figure 3, *in vitro* UBC3B, similarly to UBC3, forms a thioester bond with ubiquitin. We also generated a mutant in the active site substituting both residues cysteine 93 and leucine 97 with serine residues. The double mutant is no longer charged with ubiquitin.

```
          1                                                  48
 UBC3B    ~MAQQQMTSS QKALMLELKS LQEEPVEGFR ITLVDESDLY NWEVAIFG.P
rcdc34    ~MAQQQMTSS QKALMLELKS LQEEPVEGFR ITLVDESDLY NWEVAIFG.P
mUbc3B    ~MAQQQMTSS QKALMLELKS LQEEPVEGFR ITLVDESDLY NWEVAIFG.P
  UBC3    ~MARPLVPSS QKALLLELKG LQEEPVEGFR VTLVDEGDLY NWEVAIFG.P
Cdc34p    MSSRKSTASS LLLRQYRELT DPKKAIPSFH IELEDDSNIF TWNIGVMVLN

          49                                                 98
 UBC3B    PNTLYEGGYF KAHIKFPIDY PYSPPTFRFL TKMWHPNIYE NGDVCISILH
rcdc34    PNTLYEGGYF KAHIKFPIDY PYSPPTFRFL TKMWHPNIYE NGDVCISILH
mUbc3B    PNTLYEGGYF KAHIKFPIDY PYSPPTFRFL TKMWHPNIYE NGDVCISILH
  UBC3    PNTYYEGGYF KARLKFPIDY PYSPPAFRFL TKMWHPNIYE TGDVCISILH
Cdc34p    EDSIYHGGFF KAQMRFPEDF PFSPPQFRFT PAIYHPNVYR DGRLCISILH
          99                                                148
 UBC3B    PPVDDPQSGE LPSERWNPTQ NVRTILLSVI SLLNEPNTFS PANVDASVMF
rcdc34    PPVDDPQSGE LPSERWNPTQ NVRTILLSVI SLLNEPNTFS PANVDASVMF
mUbc3B    PPVDDPQSGE LPSERWNPTQ NVRTILLSVI SLLNEPNTFS PANVDASVMF
  UBC3    PPVDDPQSGE LPSERWNPTQ NVRTILLSVI SLLNEPNTFS PANVDASVMY
Cdc34p    QS.GDPMTDE PDAETWSPVQ TVESVLISIV SLLEDPNINS PANVDAAVDY

          149                                               198
 UBC3B    RKWRDSKGKD KEYAEIIRKQ VSATKAEAEK DGVKVPTTLA EYCIKTKVPS
rcdc34    RKWRDSKGKD KEYAEIIRKQ VSATKAEAEK DGVKVPTTLA EYCIKTKVPS
mUbc3B    TKWRDSKGKD KEYAEIIRKQ VSATKAEAEK DGVKVPTTLA EYCIKTKVPS
  UBC3    RKWKESKGKD REYTDIIRKQ VLGTKVDAER DGVKVPTTLA EYCVKTKAPA
Cdc34p    RK......NP EQYKQRVKME VERSKQDIPK .GFIMPTSES AYISQSKLDE

          199                                  238
 UBC3B    NDNSSDLLYD DLYDDDIDDE DEEEEDADCY DDDDSGNEES
rcdc34    NDNSSDLLYD DLYDDDIDDE DEEEEDADCY DDDDSGNEES
mUbc3B    NDNSSDLLYD DLYDDDIDDE DEEEEDADCY DDDDSGNEES
  UBC3    PDEGSDLFYD DYYED..GEV EEEADSCFGD DEDDSGTEES          240
Cdc34p    PESNKDMADN FWYDSDLDD. DENGSVILQD DDYDDGNNHI PFEDDDVYNY

                                                               290
Cdc34p    NDNDDDDERI EFEDDDDDDD DSIDNDSVMD RKQPHKAEDE SEDVEDVERV

          294
Cdc34p    SKKI
```

Figure 1 Deduced amino acid sequence of UBC3B and alignment with other UBCs belonging to the UBC3/CDC34 family. The active site is indicated in bold. The human UBC3/CDC34 homologue B (UBC3B) is identical at the amino acid level to its rabbit (rUBC3) homologue and shows one amino acid substitution with respect to the mouse protein (mUBC3B). Yeast protein (cdc34p) shows high identities. In addition the yeast protein contains a long acidic C-terminal domain. The sequences were aligned using the PILEUP algorithm

UBC3B is phosphorylated by CK2

The interaction trap screen suggested protein–protein interaction between CK2 and UBC3B. We confirmed this interaction *in vitro* using recombinant GST-UBC3B fusion and *in vitro* synthesized CK2α and α' subunits. Both subunits did bind to UBC3B (Figure 4). Most importantly, CK2 binding suggested that UBC3B could be a CK2 substrate. We therefore performed *in vitro* incubation of the recombinant GST-UBC3B with CK2 in the presence of γ-[^{32}P]ATP. As shown in Figure 5a, CK2 phosphorylated UBC3B in standard conditions. CK2 phosphorylation of UBC3B appeared to be quite specific since the kinases CK1, cdc2, MAPK and PKA were all unable to phosphorylate UBC3B *in vitro* to any appreciable extent while they were able to phosphorylate their specific substrate in the same condition (Figure 5a, compare lanes 2–5 with 6–9). CK2 preferentially phosphorylates Ser/Thr residues in regions rich in acidic residues (Meggio *et al*., 1994b). Based on the consensus S/T-X-X-D/E in UBC3B two putative CK2-phosphorylation sites are present: namely the serine residues 19 and 233. To test which residue(s) is the main target we generated two deletion mutants UBC3BΔN20 and UBC3BΔC232 which were missing one or the other serine residues. *In vitro*, mutant UBC3BΔC232 was no longer phosphorylated by CK2 while mutant UBC3BΔN20 was phosphorylated as efficiently as wild type (Figure 5b) indicating that serine 233 of UBC3B is the main CK2 target. Site directed mutagenesis of serine residue 233 demonstrated that this serine residue is phosphorylated by CK2 (Figure 5c). Mutagenesis of the corresponding serine residue at position 231 of UBC3 showed that this is the major CK2-phosphorylation site in this protein. However, some residual CK2-dependent

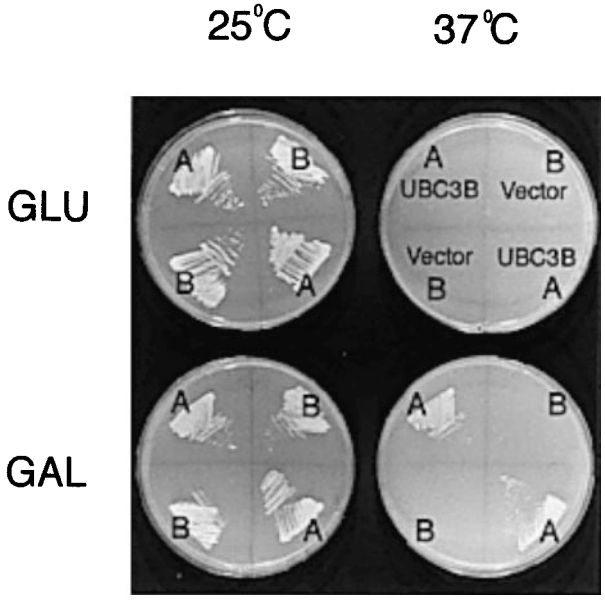

Figure 2 UBC3B complements a *cdc34* temperature-sensitive mutant. The expression of UBC3B in yeast allows a t.s. strain to regain viability under selective conditions. SP259 cells, containing the *cdc34-2* temperature sensitive mutation, were transformed with a plasmid that allowed the expression of UBC3B in the presence of galactose (A) or with the plasmid alone (B). Replica patches were then made and cells incubated for 3 days either at the permissive temperature (25°C) or at the restrictive temperature (37°C) in the presence of glucose (GLU) or galactose + raffinose (GAL) as the sole carbon source

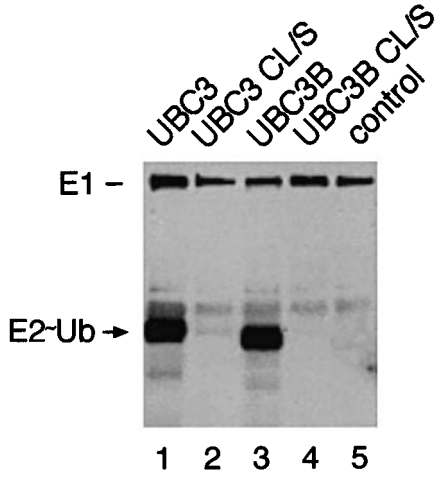

Figure 3 Thioester bond assay: a comparison between the two members of the CDC34 family and their respective mutants. Purified wild type UBC3, double mutant inactive C93S, L97S (UBC3 CL/S), UBC3B wild type, UBC3B CL/S were tested for the ability to be charged with biotinylated ubiquitin in the presence of purified ubiquitin-activating-enzyme E1. Samples were analysed by immunoblotting with HRP-streptavidin. The arrowhead marks the position of the UBC3-ubiquitin thioester

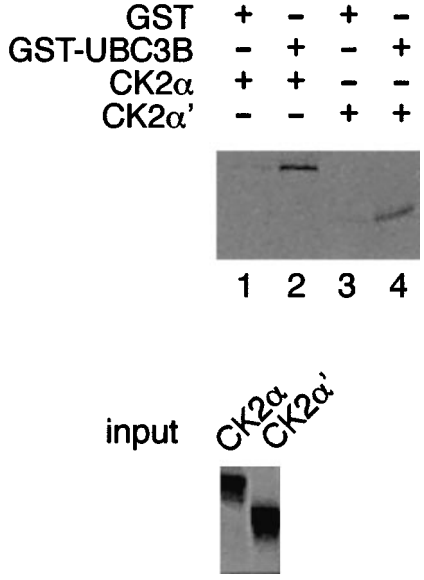

Figure 4 GST-pulldown assay between GST or GST-UBC3B and *in vitro* translated CK2α' or CK2α. Four micrograms of GST (lane 1 and 3) or GST-UBC3B (lanes 2 and 4) fusion protein were incubated with 5 μl of ([^{35}S]methionine and cysteine) *in vitro* translated CK2α (lanes 1 and 2) or CK2α' (lanes 3 and 4) and then bound to reduced Glutathione (GSH)-Sepharose beads. CK2α and CK2α' proteins eluted from the beads were detected by autoradiography

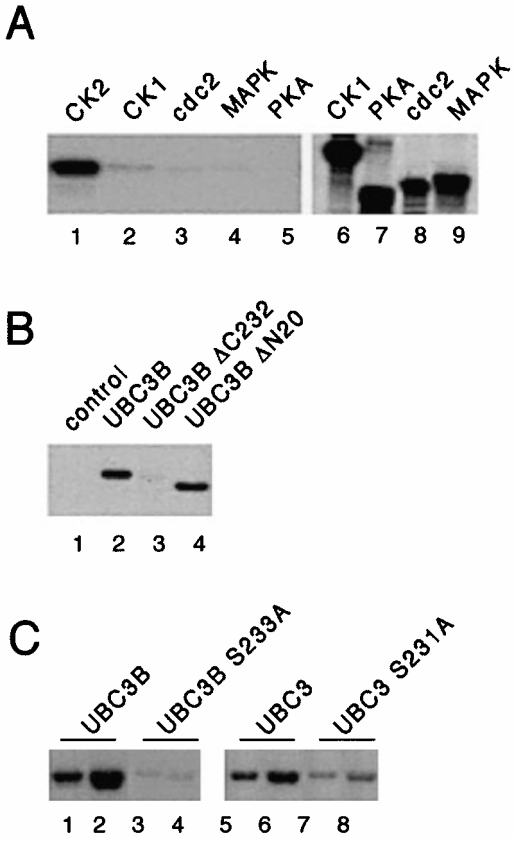

Figure 5 UBC3B is specifically phosphorylated *in vitro* by protein kinase CK2 at serine 233. (**a**) Recombinant UBC3B wild type protein was tested *in vitro* for phosphorylation by various Ser/Thr protein kinases: casein kinases CK1 and CK2, cyclin-dependent kinase 2 (Cdc2), Mitogen-Activated Protein Kinase p42 (MAPK), Protein Kinase A (PKA). Positive controls for each kinase used are β-casein (lane 6) histone IIAS (lane 7), histone H1 (lane 8) and myelin (lane 9). Recombinant UBC3B was incubated in a reaction mixture at 37°C for 10 min with the indicated purified kinases in the presence of γ-[^{32}P]ATP. (**b**) The amino acidic sequence of UBC3B contains two major serine-residues responding to the consensus CK2 phosphorylation site (S/TXXE/D/Sp/Tp): Ser-19 and Ser-233. The deletion mutants lacking respectively the serine-residue in position 19 (UBC3BΔN20) and the one in position 233 (UBC3BΔC232) were tested for *in vitro* phosphorylation by purified CK2. (**c**) 1 and 2 μg of recombinant wild type UBC3B and a mutant containing the serine 233 substituted with alanine (UBC3B S233A); wild type UBC3 and serine to alanine mutant were tested for *in vitro* phosphorylation by purified CK2

phosphorylation can still be observed with UBC3 S231A (Figure 5c, lanes 7 and 8). This is possibly due to the phosphorylation of threonine 233 and/or serine 203 at the C-terminal domain of UBC3. Although none of these residues display the canonical consensus for CK2 phosphorylation (Meggio *et al*., 1994b), they are nevertheless located in an acidic context which could give rise to 'atypical' CK2 phosphoacceptor sites.

To investigate whether UBC3B was phosphorylated *in vivo* we performed metabolic ^{32}P-labelling of HeLa cells co-transfected with UBC3B or the UBC3BΔC mutant with or without an expression vector for CK2α'. Equal amounts of UBC3B or the mutant were immunoprecipitated with anti-FLAG antibodies. In this assay only the w.t. UBC3B was phosphorylated (Figure 6) while its deletion mutant was not. Moreover, UBC3B phosphorylation was increased by the co-transfection of CK2α' and totally abolished by the CK2 specific inhibitor TBB (Sarno *et al*., 2001).

CK2-dependent UBC3B phosphorylation induces its binding to β-TrCP

The F-box protein β-TrCP recruits to the ubiquitin degradation pathway proteins once they are phosphorylated in serine residues within the highly similar consensus sites DSGXXS (IkB, β-catenin, Vpu) or DSGXXXS (ATF4) (Laney and Hochstrasser, 1999; Lassot *et al*., 2001; Margottin *et al*., 1998). As the CK2-dependent phosphorylation of UBC3B sequence DSGNEES generates a potential β-TrCP binding site, we tested direct interaction of UBC3B wild type and mutants with β-TrCP, by incubating *in vitro* synthesized ^{35}S labelled β-TrCP with GST-fusion proteins prephosphorylated by CK2α' *in vitro*. Both phosphorylated UBC3 and UBC3B bound β-TrCP *in vitro* (Figure 7a). No binding was observed with UBC3B and UBC3 serine mutants. To confirm this interaction *in vivo*, β-TrCP expression vector was co-transfected either with UBC3B, the UBC3B C-terminal mutant, or the UBC3B mutant in which the serine residue 233 was substituted with alanine in 293 cells. UBC3B proteins were then immunoprecipitated with anti-Flag affinity matrix (Sigma). β-TrCP co-immunoprecipitated with UBC3B, but not with the mutants (Figure 7b). Thus, strongly suggesting that CK2-dependent phosphoryla-

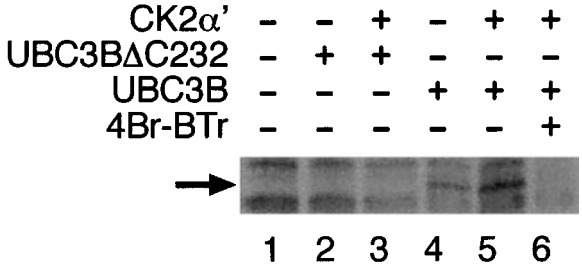

Figure 6 UBC3B is phosphorylated by protein kinase CK2 *in vivo*. Wild type protein UBC3B expressed in eukaryotic cells is phosphorylated *in vivo*; co-expression of protein kinase CK2α' catalytic subunit greatly enhances UBC3B phosphorylation. UBC3B phosphorylation is completely abrogated either by expressing the mutant protein UBC3B ΔC232 or by adding to the culture medium the specific CK2 inhibitor TBB as indicated. Murine NIH3T3 fibroblasts were transiently cotransfected with pCDNA3 alone (lane 1) or with pCDNAs expressing protein FLAG-UBC3B wild type (lanes 4, 5 and 6), protein FLAG-UBC3B ΔC232 deletion mutant lacking the CK2 phosphorylation site Ser-233 (lanes 2 and 3), in presence (lanes 3 and 5) or absence (lanes 2 and 4) of the CK2α' catalytic subunit (CMV promoter controlled). The CK2 specific inhibitor TBB (lane 6) was added after transfection to the culture media to final concentration of 50 μM. Transfected cells were ^{32}P-labelled, lysed and immunoprecipitated with a monoclonal anti-FLAG M2 antibody. Equal amounts of wild type or mutant UBC3B were loaded

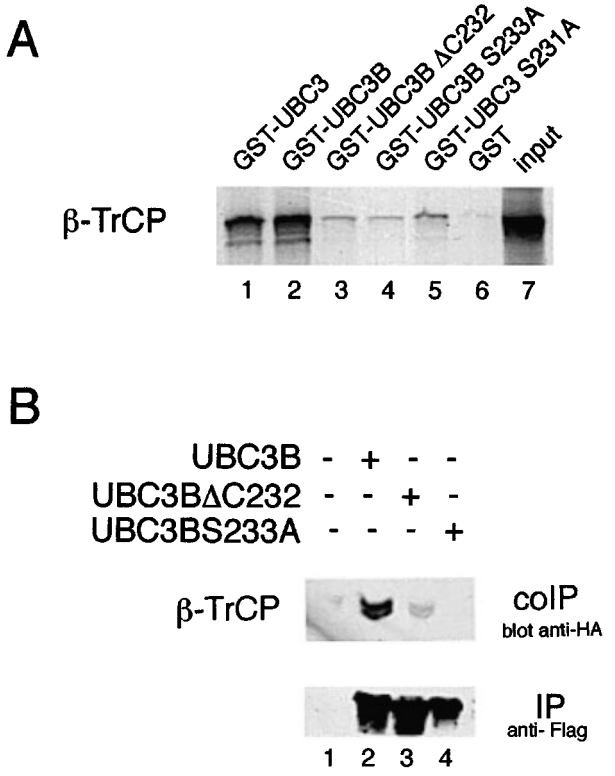

Figure 7 Phosphorylated UBC3B and UBC3 interact with β-TrCP. (**a**) *In vitro* translated β-TrCP was incubated with glutathione-sepharose beads in the presence of GST fusion proteins as indicated. The pull down products were boiled in sample buffer and analysed by gel electrophoresis. (**b**) 293 cells were transfected with expression vectors for FLAG-tagged UBC3B, UBC3B C-terminal deletion and UBC3B serine to alanine substitution together with HA tagged β-TrCP construct as described. Cell extracts were immunoprecipitated for UBC3B with anti-FLAG affinity gel. Co-immunoprecipitated proteins were analysed by immunoblotting using antibodies against HA

tion of UBC3 and UBC3B regulates their interaction with the F-box β-TrCP factor.

CK2-dependent UBC3B phosphorylation induces β-catenin degradation

Taken together the above results imply that CK2-dependent phosphorylation of UBC3 and UBC3B might be relevant for protein degradation. Interestingly, the HIV-1 Vpu protein upon phosphorylation by CK2 interacts with β-TrCP and this interaction influences positively or negatively the degradation of the β-TrCP substrates CD4 and IkB (Bour *et al*., 2001; Margottin *et al*., 1998; Schubert *et al*., 1994). Therefore, it is possible that, upon binding to β-TrCP, UBC3 and UBC3B could modulate the ubiquitin conjugation of β-TrCP substrate specificity. As β-catenin is a known substrate recognized by β-TrCP we tested whether CK2-dependent phosphorylation of UBC3B could affect β-catenin stability. We therefore co-transfected 293 cells with constructs expressing β-catenin and UBC3B with or without CK2. As shown in Figure 8a, lower levels of β-catenin were observed when both CK2 and UBC3B were present. No β-catenin degradation was observed co-transfecting UBC3BΔC which is not phosphorylated by CK2 and does not bind β-TrCP.

In another set of experiments we tested the levels of transcriptional activation mediated by a reporter containing multimeric responsive TCF-binding sites (TOP-CAT) or mutated TCF-binding sites (FOP-CAT) (Korinek *et al*., 1997). Transfection of β-catenin induced strong TOP-CAT expression while no response was obtained with the control promoter. Significant reduction of the TOP-CAT activity was observed when β-catenin was co-transfected together with CK2 and UBC3B expression vectors. On the contrary, transfection of UBC3BΔC significantly induced CAT activation suggesting that this mutant functions as dominant negative over the endogenous UBC3B.

Discussion

In this study we have identified and characterized a novel protein that interacts with and is phosphorylated by CK2. Sequence analysis revealed that this protein codes for a new member of the E2 family of ubiquitin conjugating enzymes that is highly similar to UBC3/CDC34. UBC3B differs from UBC3 in 47 amino acids. It retains the catalytical site for the ubiquitin charging. Both *in vitro* and *in vivo* experiments demonstrated that UBC3B is a functional member of the E2 family as, like UBC3, it is able to be charged by ubiquitin and resulted positive in the complementation test for cell cycle progression in *cdc34* t.s. mutant in yeast. UBC3B is therefore a novel member of the E2 family (Haas and Siepmann, 1997; Jentsch, 1992; Matuschewski *et al*., 1996). Its high similarity with UBC3 suggests that UBC3B represents another example of redundancy within the human genome and for the moment it is difficult to tell whether UBC3B has specific functions within the cells. UBC3 maps on chromosome 19p13.3 (Plon *et al*., 1993) while UBC3B with the symbol name FLJ20419 has been mapped to chromosome 9p22.1 (NCBI Annotation Project Direct Submission). In our experiments both genes behaved similarly although with some subtle differences. For instance, while UBC3B point mutation of serine residue 233 completely abrogated CK2-dependent phosphorylation, the corresponding mutant of UBC3 showed significant residual phosphorylation most likely due to some extra serine and a threonine residue present on the C-terminal domain of this protein.

Our genetic screen identified UBC3B as a protein interacting with CK2. This could reflect that UBC3B is a substrate of CK2 or CK2 is a substrate of UBC3B. Although CK2 is a very stable protein we cannot exclude that CK2 could be an UBC3B physiological substrate. On the other hand, we have demonstrated here that UBC3B is a CK2 substrate. UBC3B is phosphorylated by CK2 both *in vitro* and *in vivo* since in HeLa cells UBC3B phosphorylation is increased by CK2 co-transfection while inhibition of its phosphor-

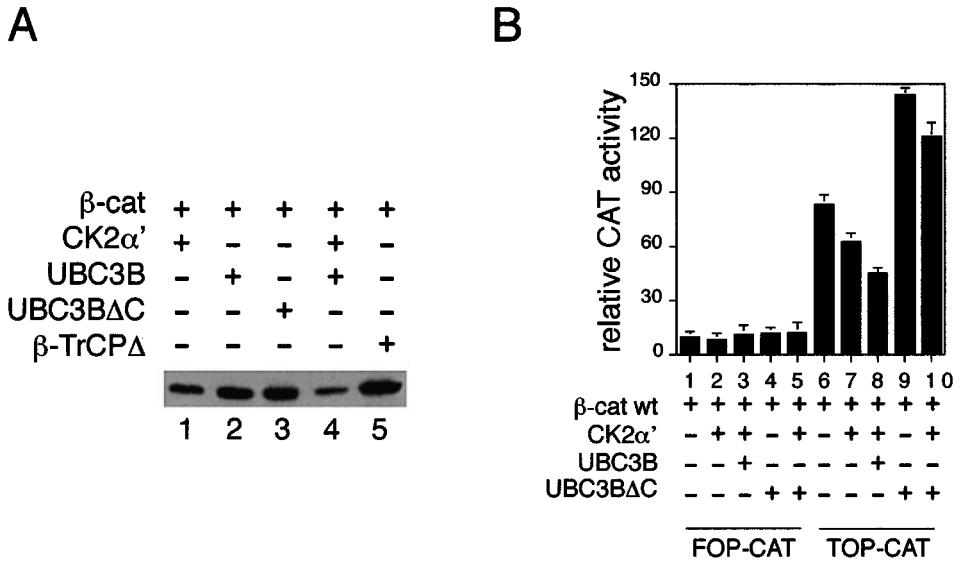

Figure 8 CK2-dependent UBC3B phosphorylation leads to β-catenin degradation. (**a**) Wild type β-catenin was co-transfected into 293 cells either with UBC3BΔC (lane 3) or with UBC3B in presence (lane 4) or absence of CK2α′ (lane 2). β-catenin myc tagged was revealed with anti-myc antibodies. As positive control dominant negative β-TrCPΔ was used (lane 5). (**b**) 293 cells were co-transfected with the TCF responsive promoter TOP-CAT or the promoter with mutated sites FOP-CAT together with the wild type β-catenin with UBC3B (lanes 3 and 8) or UBC3ΔC (lanes 4, 5, 9 and 10) in the presence or absence of CK2α′ as indicated. The amount of transfected DNA was held constant by addition of pcDNA3 DNA. The mean of four independent transfections is shown

ylation was obtained either with a specific CK2 inhibitor or by deletion of the CK2 target site in UBC3B.

The biological role of UBC3B and UBC3 phosphorylation needs further investigation. For instance, it is not possible to exclude that UBC3B phosphorylation is required for regulating its turnover. However, the level of UBC3 protein does not appear to vary with the cell cycle (Reymond *et al*., 2000) and we did not observe a decrease of UBC3 and UBC3B in their stability upon CK2 phosphorylation (unpublished observations). Recently it has been proposed that CK2-dependent phosphorylation of UBC3 plays a role for protein localization (Block *et al*., 2001). In this respect UBC3 and UBC3B might differ considerably as we did not observe differences in UBC3B localization upon phosphorylation (not shown). Instead, our experiments are fully consistent with the view that CK2-dependent phosphorylation of UBC3B (but also of UBC3) induces the formation of β-TrCP docking site which was observed both *in vitro* with GST fusion proteins and *in vivo* by co-immunoprecipitations. The binding of UBC3B to β-TrCP appears to have a functional role in substrate recognition as we observed a significant decrease of the β-TrCP target substrate β-catenin in cells over-expressing both CK2 and UBC3B. In addition, UBC3BΔC, which cannot bind β-TrCP, behaved as a dominant negative with respect to β-catenin degradation. Thus suggesting that UBC3BΔC, which could still bind ROC1 and CUL1 (Wu *et al*., 2002) might form either inactive complexes or complexes with different substrate specificity. In this light CK2-dependent phosphorylation of UBC3 and UBC3B might be important to redirect the protein degradation substrates in response to specific cellular conditions. Our finding that CK2-dependent phosphorylation leads to increased β-catenin degradation is intriguing. However, as β-TrCP recognizes several different substrates it is likely that its binding to UBC3 and UBC3B has an effect on several other substrates with a profound impact on cell cycle progression. As CK2 over-expression is able to contribute to cell transformation *in vitro* and tumour formation in transgenic animals, CK2 could exert its transforming effect, at least in part, via its regulatory activity on the proteasome-dependent protein degradation.

Materials and methods

Yeast interactor screening

The screening procedure for isolation of proteins interacting with the protein kinase CK2α′ catalytic subunit was essentially performed as described (Gyuris *et al*., 1993). The pEG202-CK2α′ expression vector was transformed into yeast strain EGY48 bearing the pSH18-34 lacZ reporter plasmid and used for screening of a HeLa cell derived cDNA library.

Cloning of full-length UBC3B from cDNA libraries

The UBC3B cDNA fragment from the yeast vector plasmid pJG-45 was used to screen a mouse and a human fibroblast cDNA libraries (Orlandini *et al*., 1996). The positive clones, inserted into the pBluescript SK vector, were sequenced on both strands. The positive clones from the human library did not contain the complete 5′ end of the UBC3B cDNA, therefore we performed a 5′-rapid amplification of cDNA ends (5′-RACE). The 5′-end of clone#10/UBC3B was

obtained by nested RACE–PCR using a placenta-derived cDNA template (CLONTECH). Primers used were AP1 and AP2 (CLONTECH) for the 5′-end; G301: 5′-CTGGGGA-GAAGGTGTTGGGCT-3′ and G302 5′-CTCCATCCTTTT-CTGCTTCGGCC-3′ for the 3′-end. In two independent experiments, the nested PCR resulted in a single cDNA fragment of identical nucleotide sequence.

Yeast complementation assay

To express *UBC3B* in a *cdc34-2* mutant yeast strain the murine cDNA complete sequence was amplified by PCR from the construct FS409 using the oligonucleotide G305: 5′-GAGAGAGAATTCGAGCTCATGGCCCAGCAGCAG-3′ for the 5′ and the oligonucleotide G306: 5′-GAGAGACTC-GAGGATCCTCACGACTCCTCATTTCC-3′ for the 3′. Both this fragment and the expression vector Yepsec1 were digested with the enzymes *Bam*HI and *Sac*I and sequently ligated. For the cloning, at the 5′-end *Sac*I had to be used necessarily to eliminate the DNA sequence encoding the Kluyveromyces lactis toxin leader peptide (Baldari *et al*., 1987). The protein was expressed in the *cdc34-2* mutant (temperature sensitive) *S. cerevisiae* strain SP259 (MATa, *cdc34-2*; *Omns*; *ade2-1*; *trp-1*; *leu2-3,112*; *his3-11,15*; *ura3*, *can1-100*) by adding galactose to the yeast culture medium, because Yepsec1 construct contains a galactose upstream activation sequence and the 5′ non-translated leader of the yeast CYC1 gene, up to position 4 from the ATG translation initiation codon (Baldari *et al*., 1987). The expression is conversely repressed by adding glucose to the medium. SP259 strain was a generous gift from P Plevani.

Ubiquitin thioester formation, phosphorylation assay

Purified recombinant UBC3 proteins were incubated at 37°C for 30 min in 30 μl of ubiquitination mix as described (Pagano *et al*., 1995). *In vitro* phosphorylation assay were performed as described (Meggio *et al*., 1994a). The incorporation of ^{32}P into UBC3 proteins was evaluated by subjecting samples to SDS–PAGE, staining and autoradiography, or by direct scanning of the gel on an Instant Imager Apparatus (Canberra Packard). Protein kinases CK1 and CK2 were partially purified from rat liver cytosol as described (Meggio *et al*., 1981). p34/cyclinB protein kinase (starfish) was kindly provided by Dr L Meijer (Roscoff, France). PKA and MAP kinase p42 were purchased from Sigma and from Santa Cruz Biotechnology, respectively.

Metabolic ^{32}P labelling for in vivo *phosphorylation assay*

NIH3T3 cells were transfected by Lipofectamine reagent according to the manufacturer's instructions (Life Technologies/Gibco BRL). Plasmid DNA (5 μg/60-mm dish) was transiently transfected for 3 h in the complete medium. Immediately post-transfection, cells were washed twice in PBS and twice in phosphate-free Dulbecco's modified Eagle's medium supplemented with pyruvate. Cells were then incubated for about 16 h in the same medium containing 0.38 mCi/ml [^{32}P]orthophosphate. Where indicated, the specific protein kinase CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) (Sarno *et al*., 2001) was added to the labelling medium at the final 50 μM concentration. At the end of the labelling incubation cells were set on ice and washed three times with ice-cold PBS. Lysate preparation and anti-FLAG immunoprecipitation were immediately performed. The resultant cell lysates were clarified at 4°C at 10 000 *g* for 10 min. Immunoprecipitations were performed by incubating cell lysate with anti-FLAG antibodies for 1 h at 4°C. The samples were then incubated with protein G-Sepharose for 1 h at 4°C. The resulting immunoprecipitates were washed four times with the same lysis buffer and then subjected to 10% SDS–PAGE. The gel was then dried and the presence of phosphorylated UBC3B proteins was analysed by autoradiography.

Constructs

To yield GST-UBC3B fusion protein, human UBC3B open reading frame was amplified by PCR from FS410 with a 5′ primer (G317) (5′-GAGAGACCATGGCCCAGCAGCA-GATGACCAGCTCGCAGA-3′) and a 3′ primer (G306) (5′-GAGAGACTCGAGGATCCTCACGACTCCTCATTT-CC-3′). The PRC product was digested with *Nco*I and *Xho*I and cloned into a modified pGEX-4T-1 (Pharmacia) expression vector, in which an *Nco*I site has been inserted at the 5′-end (FG333, kindly provided by F Galvagni).

To generate GST-UBC3 fusion protein, human UBC3 open reading frame was amplified by PCR from pQE30-hCDC34 (Pagano *et al*., 1995) with a 5′-primer (H242, 5′-GAGAGAGGATCCCCCATGGCTCGGCCGCTAGTG-3′) and a 3′- primer (H243, 5′-GAGAGATCTAGATCAG-GACTCCTCCGTGCC-3′). The amplified fragment was digested with *Bam*HI and *Xba*I and cloned into the GST-binding domain) of FG333.

GST-UBC3BΔN20 (from L-20 to S-238), lacking the first 19 amino acids at the N-terminal respect to UBC3 B wild type, was amplified by PCR from FS410 with a 5′-primer containing a *Bam*HI restriction site (G360: 5′-GAGAGAG-GATTCCTGCAGGAGGAACCGGTG-3′) and a 3′-primer containing an *Eco*RI restriction site (G351: 5′-ATATATCTC-GAGTCACGACTCCTCATTCCC-3′). The PCR product was digested with *Bam*HI and *Eco*RI and cloned into FG333.

GST-UBC3BΔC232 was amplified by PCR from FS410 as well, with a 5′-primer (G361: 5′-GAGAGAGAATTCAAT-CATCATCATCATAACA-3′) and the 3′-primer (G348: 5′-GAGAGAGGATCCATGGCCCAGCAGCAGATG-3′). After digestion with the restriction enzymes *Bam*HI and *Eco*RI, the amplified fragments were cloned into the FG333.

The S233A mutant GST-UBC3B S233A was generated amplifying by PCR from FS410 with G348 as the 5′-primer and the 3′-primer (H240: 5′-GAGAGATCTAGATCACGA-CTCCTCATTCCCAGCATCATCATCATCATAACA-3′). The amplified fragment was digested with the restriction enzymes *Eco*RI and *Xba*I and ligated into the vector FG333.

The GST-UBC3S231A mutant was obtained by PCR from pQE30-hCDC34 with the 5′-primer H242 containing a *Bam*HI restriction site and the 3′-primer (H241, 5′-GAGA-GATCTAGATCAGGACTCCTCCGTGCCAGCGTCATC-CTCATCGTCCCC-3′). After digestion with *Bam*HI and *Xba*I the amplified product was inserted into the vector FG333.

The GST-fusion proteins were generated by culturing the transformed *E. coli* under isopropyl–D-thiogalactopyranoside (IPTG)-inducing conditions (0.1 mM IPTG for 2–3 h at 30°C). The fusion protein and/or GST control protein was then purified and thrombin-cleaved (when necessary) from the GST-binding domain moiety, using glutathione-Sepharose 4B beads according to the manufacturer's protocol (Pharmacia).

The constructs expressing the N-terminally FLAG-tagged UBC3B wild type, UBC3BΔC232 and UBC3BS233A phosphorylation mutants, under the control of the CMV promoter, were made by subcloning the respective fragments from the GST-fusion constructs into a modified pcDNA3.1C

6Xhis (Invitrogen), in which the N-terminal sequence coding for six Histidine residues had been eliminated by cleavage with the restriction enzymes *Hind*III and *Kpn*I, and substituted with a sequence encoding the highly immunogenic FLAG peptide (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C), maintaining the original pCDNA3.1C polylinker sequence (kindly provided by L D'Adamio).

To generate pCDNA3-HA-β-TrCP encoding an N-terminally HA-tagged βTrCP, βTrCP(Fbw1a) was amplified by PCR from a plasmid (Cenciarelli *et al*., 1999) (H325 5′-GAGAGATATCGCCGCCATGTATCCTTATGATGTTCCTGATTATGCTATGGACCCGGCCGAGGCG-3′) which contains the HA epitope sequence, and the reverse primer (H327 5′-TCTCTCTCTAGATTATCTGGAGATGTAGGTGTATGTTCG).

GST pulldown affinity assay

For UBC3B and CK2α'/α *in vitro* binding assay, the expression of GST and GST-UBC3B fusion protein was induced in *E. coli* (TG1) cells by 100 μM isopropyl-l-thio-D-galactopyranoside for 5–6 h at 30°C. The cells were collected by centrifugation (5000 *g*, 10 min), resuspended in 50 ml of PBS-T1 (1 × PBS with 1% Triton X-100) and lysed by sonication. The lysate was centrifuged at 10 000 *g* for 10 min. The supernatant was incubated with 1 ml (50% slurry) reduced Glutathione(GSH)-Sepharose (Amersham Pharmacia). After washing the beads with cold PBS at 4°C, the binding proteins were eluted at room temperature with 1 ml 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0) for three times. The eluted proteins were dialyzed against PBS overnight at 4°C.

For the *in vitro* binding assay between the GST-fused UBC3B protein and protein kinase CK2α and α' catalytic subunits, 4 μg of purified GST or GST-UBC3B were mixed (in a 20-μl reaction) in RIPA buffer with sodium deoxycholate and containing protease inhibitors (SIGMA) (100 mM Tris-HCl pH 8.0, 10 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 1% Na DOC) with 5 μl of [^{35}S]methionine and cysteine (PRO-MIX^{tm}- Amersham Pharmacia) *in vitro* translated CK2α' or α (TNT Quick Coupled Transcription Translation Kit, Promega). The binding reactions were incubated at 30°C for 1 h, then diluted to 0.5 ml with RIPA buffer and clarified from insoluble precipitates by centrifugation for 10 min at 20 000 *g* at 4°C. The clear supernatants were transferred to a new tube, added with 50 μl (50% slurry) reduced Glutathione (GSH)-Sepharose (Amersham Pharmacia) equilibrated in RIPA buffer, and gently rotated on wheel at 4°C for 2 h. Then the beads were washed four times at 4°C with RIPA buffer and resuspended in 1 × Laemmli buffer. After boiling samples 5 min at 95°C bound proteins were resolved using SDS–PAGE and analysed by autoradiography.

TCF chloramphenicol acetyltransferase (CAT) assay

The reporter plasmids TOP-CAT and FOP-CAT (Korinek *et al*., 1997) were generously provided by Claudio Schneider. In brief, human transformed primary embryonic kidney 293 cells were transfected using the Polyfect Transfection Reagent (Quiagen) according to the manufacturer's instructions. The day before transfection cells were seeded 1.2×10^6 per 6-cm-diameter dish. Typically a transfection experiment included 0.6 μg of reporter plasmid (TOP-CAT or FOP-CAT) and 0.1 μg of cytomegalovirus-LacZ plasmid for the transfection efficiency control (Cherrington and Mocarski, 1989). The expression plasmids were added to the reporter as indicated in the legend of Figure 8 and the total amount of transfected plasmids was held constant at 4.0 μg by the addition of empty pCDNA3 as a DNA carrier. Plasmid pCS2+MMBCmyctag encoding myc tagged mouse β-catenin (Aberle *et al*., 1997) was kindly provided by Rolf Kemler. The CMV-driven expression construct for β-TrCP(ΔF), the F-box deleted mutant of βtrCP, which acts *in vivo* as a dominant negative mutant, being unable to bind Skp1-Cul1 complex (Latres *et al*., 1999) was generously provided by Michele Pagano (New York University Medical Center, New York, USA). CAT activity was determined as previously described (Gorman *et al*., 1982). Acetylated forms of chloramphenicol were quantified by scanning the thin-layer chromatography plates with the Image Quant radio analytic system from Molecular Dynamics. A minimum of four independent transfection experiments were performed with independent DNA preparations. The average variation of CAT activity observed for each construct was less than 10% in different transfections.

β-TrCP and UBC3B co-immunoprecipitation

Human embryonic kidney 293 cells (HEK293) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. Transfections were performed using LipofectAMINE reagent according to the manufacturer's instructions (LIFE Technologies/Gibco BRL). For co-immunoprecipitation experiments with NH2-terminally HA-tagged β-TrCP protein and FLAG-tagged UBC3B protein (wild type and C-terminally truncated), HEK293 cells were seeded in 100-mm plates, transfected at about 50% confluency with a total amount of 12 μg DNA per plate, held constant by the addition of empty pCDNA3 expression vector, and collected 48 h post-transfection. Cells were rinsed on ice twice with cold PBS, scraped in 5 ml of cold PBS supplemented with 1 mM EDTA, the pellet deriving from three plates was lysed in 0.5 ml of Triton lysis buffer containing 20 mM Tris-HCl pH 8.0, 1% Triton, 140 mM NaCl, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM EDTA, (2 μg/μl) 50 mM NaF, (1.8 μg/μl) 1 mM Na_3VO_4, 1 mM PMSF, supplemented with protease inhibitor cocktail (Sigma). The lysis was fulfilled at 4°C with gentle rotation on a wheel for half an hour. Cell debris was removed and immunoprecipitation was performed as recommended by the manufacturer's instructions (Sigma) adding to the cleared lysate (100 μl (50% suspension) of ANTI-FLAG® M2 Affinity Gel, (Sigma) and rotating the samples on a wheel at 4°C for at least 2 h. The immunoprecipitates were washed four times in the same buffer of lysis, resolved by SDS–PAGE and examined by immunoblotting experiment using anti-HA High Affinity (rat monoclonal antibody, clone 3F10) (Roche) and anti-FLAG®M2 (Sigma).

In vitro *binding*

In vitro binding of purified and CK2α' phosphorylated GST-fusion UBC3 proteins to *in vitro* translated human β-TrCP was carried out in 300 μl of binding buffer (10 mM Tris-HCl pH 7.6, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.1% NP40, 10% glycerol, 500 μg/ml BSA) containing about 2 μg purified and CK2α' phosphorylated GST fusion UBC3 proteins and reticulocyte lysate in which radiolabelled β-TrCP was produced by *in vitro* transcription and translation according to the manufacturer's instructions (TNT Quick Coupled Transcription/Translation systems Promega). Glutathione-Sepharose 4B (Pharmacia) equilibrated in binding buffer was added to the mix and the reactions were gently

rotated on a wheel at 4°C for 5 h. Then the matrix was sedimented by centrifugating at 500 *g* for 5 min and washed five times in washing buffer (10 mM Tris-HCl pH 7.6, 150 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.4% NP40, 10% glycerol). The matrix was resuspended in Laemmli buffer, boiled and analysed by SDS–PAGE and autoradiography. GST fusion UBC3 proteins were phosphorylated as described (Meggio *et al*., 1994a) using 2 μg of recombinant CK2α' subunit (Orlandini *et al*., 1998).

Acknowledgments
We are in debt to Michele Pagano, Andrea Carrano and Alessia Montagnoli for their help with the *in vitro* ubiquitination experiments; to Federico Galvagni and Maurizio Orlandini for their precious advice; to Beatrice Grandi for her technical support; Paolo Plevani, Luciano D'Adamio, Rolf Kemler, and Claudio Schneider for reagents. The work was supported by grants from AIRC and MURST (PRIN 2000) to S Oliviero and by grants from MURST (PRIN 2000), Armenise-Harvard Foundation, AIRC, Italian Ministry of Health (Project AIDS) and CNR (00.00369.ST74 and T.P. on Biotechnology) to F Meggio and LA Pinna.

Accession numbers
The nucleotide sequences reported in this article were submitted to the Gene bank with accession numbers AJ240086 (mouse) and AJ240087 (human).

References

Aberle H, Bauer A, Stappert J, Kispert A and Kemler R. (1997). *EMBO J.,* **16,** 3797–3804.
Ahmed K. (1994). *Cell. Mol. Biol. Res.,* **40,** 1–11.
Allende CC and Allende JE. (1998). *J. Cell. Biochem.* **30–31** (Suppl): 129–136.
Allende JE and Allende CC. (1995). *FASEB J.,* **9,** 313–323.
Baldari C, Murray JA, Ghiara P, Cesareni G and Galeotti C. (1987). *EMBO J.,* **6,** 229–234.
Blanquet PR. (2000). *Prog. Neurobiol.,* **60,** 211–246.
Block K, Boyer TG and Yew PR. (2001). *J. Biol. Chem.,* **276,** 41049–41058.
Bour S, Perrin C, Akari H and Strebel K. (2001). *J. Biol. Chem.,* **276,** 15920–15928.
Cenciarelli C, Chiaur DS, Guardavaccaro D, Parks W, Vidal M and Pagano M. (1999). *Curr. Biol.,* **9,** 1177–1179.
Cherrington JM and Mocarski ES. (1989). *J. Virol.,* **63,** 1435–1440.
DeSalle LM and Pagano M. (2001). *FEBS Lett.,* **490,** 179–189.
Deshaies RJ, Chau V and Kirshner M. (1995). *EMBO J.,* **14,** 303–312.
Drury LS, Perkins G and Diffley JF. (1997). *EMBO J.,* **16,** 5966–5976.
Feldman RM, Correll CC, Kaplan KB and Deshaies RJ. (1997). *Cell,* **91,** 221–230.
Gapany M, Faust RA, Tawfic S, Davis A, Adams GL and Ahmed K. (1995). *Mol. Med.,* **1,** 659–666.
Goebl MG, Yochem J, Jentsch S, McGrath JP, Varshavsky A and Byers B. (1988). *Science,* **241,** 1331–1335.
Gorman CM, Moffat LF and Howard BH. (1982). *Mol. Cell. Biol.,* **2,** 1044–1051.
Guerra B and Issinger OG. (1999). *Electrophoresis,* **20,** 391–408.
Guerra B, Siemer S, Boldyreff B and Issinger OG. (1999). *FEBS Lett.,* **462,** 353–357.
Gyuris J, Golemis E, Chertkov H and Brent R. (1993). *Cell,* **75,** 791–803.
Haas AL and Siepmann TJ. (1997). *FASEB J.,* **11,** 1257–1268.
Henchoz S, Chi Y, Catarin B, Herskowitz I, Deshaies RJ and Peter M. (1997). *Genes Dev.,* **11,** 3046–3060.
Hershko A and Ciechanover A. (1998). *Annu. Rev. Biochem.,* **67,** 425–479.
Hershko A, Ciechanover A and Varshavsky A. (2000). *Nat. Med.,* **6,** 1073–1081.
Hochstrasser M. (1996). *Annu. Rev. Genet.,* **30,** 405–439.
Issinger O-G. (1993). *Pharmacol. Ther.,* **59,** 1–30.
Jackson PK, Eldridge AG, Freed E, Furstenthal L, Hsu JY, Kaiser BK and Reimann JD. (2000). *Trends Cell. Biol.,* **10,** 429–439.
Jentsch S. (1992). *Annu. Rev. Genet.,* **26,** 179–207.
Korinek V, Barker N, Morin PJ, van Wiken D, de Weger R, Kinzler KW, Vogelstein B and Clevers H. (1997). *Science,* **275,** 1784–1787.
Landesman-Bollag E, Romieu-Mourez R, Song DH, Sonenshein GE, Cardiff RD and Seldin DC. (2001). *Oncogene,* **20,** 3247–3257.
Laney JD and Hochstrasser M. (1999). *Cell,* **97,** 427–430.
Lassot I, Segeral E, Berlioz-Torrent C, Durand H, Groussin L, Hai T, Benarous R and Margottin-Goguet F. (2001). *Mol. Cell. Biol.,* **21,** 2192–2202.
Latres E, Chiaur DS and Pagano M. (1999). *Oncogene,* **18,** 849–854.
Margottin F, Bour SP, Durand H, Selig L, Benichou S, Richard V, Thomas D, Strebel K and Benarous R. (1998). *Mol. Cell.,* **1,** 565–574.
Marti A, Wirbelauer C, Scheffner M and Krek W. (1999). *Nat. Cell. Biol.,* **1,** 14–19.
Matuschewski K, Hauser HP, Treier M and Jentsch S. (1996). *J. Biol. Chem.,* **271,** 2789–2794.
Meggio F, Boldyreff B, Issinger O-G and Pinna LA. (1994a). *Biochemistry,* **33,** 4336–4342.
Meggio F, Deana AD and Pinna LA. (1981). *J. Biol. Chem.,* **256,** 11958–11961.
Meggio F, Marin O and Pinna LA. (1994b). *Cell. Mol. Biol. Res.,* **40,** 401–409.
Munstermann U, Fritz G, Seitz G, Lu YP, Schneider HR and Issinger OG. (1990). *Eur. J. Biochem.,* **189,** 251–257.
Orlandini M, Marconcini L, Ferruzzi R and Oliviero S. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 11675–11680.
Orlandini M, Semplici F, Ferruzzi R, Meggio F, Pinna LA and Oliviero S. (1998). *J. Biol. Chem.,* **273,** 21291–21297.
Pagano M, Tam SW, Theodoras AM, Beer-Romero P, Del Sal G, Chau V, Yew PR, Draetta GF and Rolfe M. (1995). *Science,* **269,** 682–685.
Pinna LA. (1990). *Biochim. Biophys. Acta,* **1054,** 267–284.
Pinna LA and Meggio F. (1997). *Prog. Cell Cycle Res.,* **3,** 77–97.
Plon C, Leppig KA, Do HN and Groudine M. (1993). *Proc. Natl. Acad. Sci. USA,* **90,** 10484–10488.
Rayan A, Goueli SA, Lange P and Ahmed K. (1985). *Cancer Res.,* **45,** 2277–2282.
Reymond F, Wirbelauer C and Krek W. (2000). *J. Cell Sci.,* **113,** 1687–1694.

Sarno S, Reddy H, Meggio F, Ruzzene M, Davies SP, Donella-Deana A, Shugar D and Pinna LA. (2001). *FEBS Lett.,* **496,** 44–48.

Schubert U, Henklein P, Boldyreff B, Wingender E, Strebel K and Porstmann T. (1994). *J. Mol. Biol.,* **236,** 16–25.

Schwob E, Böhm T, Mendenhal MD and Nasmyth K. (1994). *Cell,* **79,** 233–244.

Seitz G, Munstermann U, Schneider HR and Issinger OG. (1989). *Biochem. Biophys. Res. Commun.,* **163,** 635–641.

Seldin DC and Leder P. (1995). *Science,* **267,** 894–897.

Stalter G, Siemer S, Becht E, Ziegler M, Remberger K and Issinger OG. (1994). *Biochem. Biophys. Res. Commun.,* **202,** 141–147.

Sun B, Jeyaseelan K, Chung MC, Tan TW, Chock PB and Teo TS. (1997). *Biochim. Biophys. Acta,* **1351,** 231–238.

Sutterluty H, Chatelain E, Marti A, Wirbelauer C, Senften M, Muller U and Krek W. (1999). *Nat. Cell. Biol.,* **1,** 207–214.

Tuazon PT and Traugh JA. (1991). *Adv. Second Messenger Phosphoprotein Res.,* **23,** 123–164.

Weissman AM. (2001). *Nat. Rev. Mol. Cell. Biol.,* **2,** 169–178.

Wu K, Chen A, Tan P and Pan ZQ. (2002). *J. Biol. Chem.,* **277,** 516–527.

Yenice S, Davis AT, Goueli SA, Akdas A, Limas C and Ahmed K. (1994). *Prostate,* **24,** 11–16.