SCF^{Fbl12} increases $p21^{Waf1/Cip1}$ expression level through atypical ubiquitin chains synthesis

Fuminori Tsuruta[1, 2, 3], Ai Takebe[1], Kousuke Haratake[1], Yoshinori Kanemori[1, 2], Jaehyun Kim[1], Tomoyuki Endo[1], Yu Kigoshi[1], Tomomi Fukuda[1], Hatsumi Miyahara[1], Manato Ebina[1], Tadashi Baba[1, 2], Tomoki Chiba[1, 2, 3]

1. Graduate School of Life and Environmental Sciences, University of Tsukuba,
2. Ph.D. Program in Human Biology, School of Integrative and Global Majors, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan

*Running title: SCF^{Fbl12} regulates p21 expression level

3. To whom correspondence should be addressed: Fuminori Tsuruta, Tomoki Chiba, Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan, Tel.: +81-29-853-4662 or 6887; Fax: +81-29-853-6887; E-mail: ftsuruta@biol.tsukuba.ac.jp, tchiba@biol.tsukuba.ac.jp

Key words: proteasome; ubiquitin; SCF ubiquitin ligase; p21; Fbl12; PA28γ

Abstract

The cyclin-dependent kinase inhibitor p21 is an unstructured protein regulated by multiple turnover pathways. The p21 abundance is tightly regulated and its defect causes tumor development. However, the mechanisms that underlie the control of p21 level are not fully understood. Here, we report a novel mechanism by which a component of the SCF ubiquitin ligase, Fbl12, augments p21 via the formation of atypical ubiquitin chains. We found that Fbl12 binds and ubiquitinates p21. Unexpectedly, Fbl12 increases an expression level of p21 by enhancing the mixed-type ubiquitination including not only K48- but also K63-linked ubiquitin chains, followed by promotion of a binding between p21 and CDK2. We also found that proteasome activator, PA28γ, attenuates p21 ubiquitination by interacting with Fbl12. In addition, UV irradiation induces a dissociation of p21 from Fbl12 and decreases K63-linked ubiquitination, leading to p21 degradation. These data suggest that Fbl12 is a key factor that maintains adequate intracellular concentration of p21 at the normal condition. Our finding may provide a novel possibility that p21 fate is governed by diverse ubiquitin chains.

Introduction

The inhibitor of cyclin-dependent protein kinase (CDK), $p21^{Waf1/Cip1}$ (hereafter referred to as p21), plays important roles in the regulation of cellular functions such as cell cycle, DNA repair and apoptosis. The relationship between p21 functions and cellular progression has been extensively studied (1). Normally, p21 inhibits CDKs through direct binding, leading to the suppression of cellular progression. p21 also suppresses Proliferating cell nuclear antigen (PCNA), which is a crucial factor for DNA replication and repair. Thus, p21 acts as a master modulator that governs cell cycle, and defects in p21 increase the risk of developing cancer.

p21 abundance is tightly controlled at all stages from transcription, translation to posttranslational regulation, via such processes as mRNA clearance, translational rate and protein degradation. Defects in this mechanisms that result in aberrant p21 level can cause cancer development (1, 2). This suggests that intracellular p21 abundance is intrinsically determined through the regulation of transcriptional and posttranslational modification. The transcriptional regulation of p21 is mediated by a variety of factors, such as p53, E2F1, Klf6, Myc and AP4 (3-7), indicating that transcriptionally-regulated p21 expression is governed by several pathways

51 in a context-dependent manner. In addition, posttranslational modification is also associated with
52 cellular p21 protein levels. Recent studies have determined that p21 phosphorylation at several
53 sites is involved in its stability. For instance, Akt phosphorylates p21 at the Thr145 residue,
54 resulting in p21 accumulation in the cytoplasm (8-10), and JNK1 and p38α stabilize p21 through
55 phosphorylation of Ser130 (11, 12). Therefore, it is clear that p21 is controlled not only by
56 transcriptional regulation but also by posttranslational modification.

57 p21 is an unstructured protein that is easily degraded by the proteasome under basal
58 conditions (13). In order to protect itself from this default degradation, newly synthesized p21
59 associates with target proteins to prevent from this default degradation (14-16). It has also been
60 shown that carcinogenic factors, including an exposure to UV, changes expression of p21.
61 Interestingly, substantial studies have shown that increased p21 expression significantly
62 associates with metastasis, recurrence and survival in human (17, 18). Therefore, the
63 maintenance of p21 abundance at an adequate level is critically important, and abnormal
64 expression results in an increase of risk of various disorders (1). So far, it has been reported that
65 several E3 ubiquitin ligases, including SCF^{skp2}, $Cul2^{LRR1}$, $Cul4^{CDT2}$, APC/C^{CDC20} and MKRN1,
66 enhance p21 ubiquitination, leading to proteasome degradation (14, 19-23). Interestingly, p21

67 mutant with all lysines mutated to arginines is still ubiquitinated. This suggests that p21
68 ubiquitination occurs not only on intramolecular lysines but also on the N-terminal methionine
69 (24). Moreover, p21 has been reported to interact with the proteasome α7 subunit of 20S
70 proteasome and proteasome activator PA28γ (25-27) to promote proteasomal degradation of p21,
71 independently of ubiquitination. Despite the topic of p21 degradation and ubiquitination, the
72 physiological significance of p21 ubiquitination associated with its degradation remains unclear.
73 In this study, we report that Fbl12 and PA28γ regulate the p21 expression level. We
74 found that Fbl12 associates with both p21 and PA28γ, resulting in a complex formation. In
75 addition, mixed-type ubiquitination of p21 induced by Fbl12 is positively associated with the
76 amount of p21 via attenuation of degradation rate. In addition, this effect was suppressed by
77 PA28γ, resulting in a decrease in p21 expression level. Furthermore, UV irradiation promotes
78 p21 degradation irrespective of Fbl12 expression. Thus, our findings provide the novel
79 mechanisms by which both Fbl12 and PA28γ mediate p21 turnover via the control of mixed-type
80 ubiquitin chain.
81

Materials and Methods

Materials - The following antibodies were used for immunoblot analyses: Flag (M2, SIGMA), Myc (9E10, Santa Cruz), HA (Y-11, Santa Cruz), GFP (Cat.#598, MBL), GST (B-14, Santa Cruz), Tubulin (DM1A, SIGMA), His (Cat.#27-4710-01, GE Healthcare) (Cat#.PM032, MBL), p21 (F-5, Santa Cruz), PA28γ (47/Psme3, BD Transduction Lab.), β5 (ab3330, abcam), Fbl12 (ab96831, abcam), Ubiquitin Lys48-specific (Apu2, Merck Millipore), Ubiquitin Lys63-specific (Apu3, Merck Millipore). The following antibodies were used for immunocytochemistry: GFP (Cat.#598, MBL), p21 (F-5, Santa Cruz) and PA28γ (47/Psme3, BD Transduction Lab.). Lipofectamine 2000 was purchased from Invitrogen. The siFbl12 (EHU054981) and anti-Flag M2-agarose beads were purchased from SIGMA. Hoechst 33342 was purchased from Life Technologies. TALON metal affinity resin was purchased from Clontech. Glutathione Sepharose was purchased from GE Healthcare. MG132 was purchased from Peptide Institute. Polyethyleneimine was purchased from Polyscience. Cyclohexamide was purchased from Wako.

Cell culture, transfection and stimulation - HEK 293, HEK 293T, HeLa, and HCT116 cells

were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Wako) containing 5-10% fetal bovine serum, penicillin (100 units) and streptomycin (100 mg; P/S). To generate *Fbxl12* deficient cells, HEK293 cells were transfected with pSpCas9(BB)-2A-Puro-Fbl12 plasmid and incubated in the presence of 1.0 μg/ml of puromycin. Cells were transfected using Lipofectamine 2000, Lipofectamine RNAi MAX Reagent (Life Technologies), and polyethyleneimine (Polyscience) according to the manufacturer's instructions. HEK 293 and HeLa cells were irradiated by 12 μW/cm^2 UV and then subjected to each analysis.

Plasmid construction - pCAGEN-His-Ub, pCAGEN-His-Ub K48R, pCAGEN-His Ub K63R, pCAGEN-His-Ub 48K, and pCAGEN-His-Ub 63K constructs were provided by Y. Gotoh (University of Tokyo, Japan). pcDNA3-Flag-Fbl12, pcDNA3-Flag-Fbl12ΔF and pcDNA3-Flag-Skp1, constructs were described previously (28). p21 and Skp2 cDNA were amplified by PCR and subcloned into pcDNA-Flag. The Fbl12 and Skp1 cDNA were subcloned into pRSFDuet-1. The Fbl12 cDNA was subcloned into the EcoRI and XhoI sites of pCS4-Myc and pCS4-EGFP. The PA28γ and CDK2 cDNAs were amplified from the human HCT116 cDNA library and subcloned into the BglII sites of pCS4-Myc. The p21 cDNA was amplified

114 from Flag-p21 and subcloned into the BglII sites of pCS4 and pCS4-EGFP. The p21ΔNLS
115 cDNA was amplified from Flag-p21 and subcloned into the BglII sites of pCS4-EGFP. The p21
116 NLS oligonucleotides were annealed and inserted into the EcoRI and XbaI sites of pCS4-EGFP.
117 The p21 and PA28γ cDNA were subcloned into the BamHI sites of pGEX-6p-1.
118 pSpCas9(BB)-2A-Puro was purchased from Addgene. The oligonucleotides of Fbl12 were
119 designed and inserted into pSpCas9(BB)-2A-Puro by ligation into the BbsI sites. The primers
120 used were as follows:
121 PA28γ forward, 5'- aggatccgccaccatggcctcgttgctgaaggtg-3'; and reverse, 5'-
122 gggatcctcagtacagagtctctgcattgctgctccg-3'; p21 forward, 5'-taggatccgccaccatgtccaatcctggtgatg-3';
123 and reverse, 5'- gcggatcctcagggttttctcttgcagaag acc-3'; p21ΔNLS forward,
124 5'-taggatccgccaccatgtccaatcctggtgatg-3'; and reverse, 5'-taggatcctc atcggccctg agatgttccg g-3';
125 CDK2 forward, 5'- gggatccgccaccatggagaacttccaaaag g-3'; and reverse, 5'-
126 aggatcctcagagtcgaagatggggtactggcttgg -3'; the oligonucleotides of p21 NLS were used as
127 follows: sense,
128 5'-taattcaaacggaggcagaccagcctgacagatttctatcactccaagcgcagattggtcttctgcaagagaaaaccctgat-3'; and
129 antisense, 5'-

ctagatcagggttttctcttgcagaagaccaatctgcgcttggagtgatagaaatctgtcaggctggtctgcctccgtttg-3'; the oligonucleotides of Fbl12 for CRISPR/Cas9 were used as follows: sense, 5'-caccgacctgacgctctacacgatg-3'; and antisense 5'- aaaccatcgtgtagagcgtcaggtc-3'

RT-qPCR - Total RNAs were prepared by Isogen II (NIPPON GENE). The cDNA were synthesized by SuperScript III Reverse Transcriptase (Life Technologies). Quantitative RT-PCR was performed with THUNDERBIRD SYBR qPCR Mix (TOYOBO) and appropriated primers, then analyzed by using Thermal Cycler Dice Real Time System (TAKARA). The primers used were as follows:

Fbl12 forward, 5'-cggtggctgtggcgacatgtc-3'; and reverse, 5'-caggtagccacccatccgca-3';

p21 forward, 5'-tacccttgtgcctcgctcag-3'; and reverse, 5'-ggagaagatcagccggcgtt-3'; Actin forward, 5'- tggacatccgcaaagacctg-3'; and reverse, 5'-ggaggagcaatgatcttgatcttc-3'

Immunoblot analysis - Cells were lysed in extraction buffer (0.5% NP-40, 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM, EDTA, 1 mM DTT) and centrifuged at 14,000 rpm for 5 minutes. The cleared lysates were separated by SDS-PAGE, transferred to PVDF membrane, probed with

primary antibodies, and detected with HRP-conjugated secondary antibodies and chemiluminescence reagent (ECL Plus Western Blotting Detection Reagents, GE Healthcare). Immunoblotting data were quantified using Image J software.

Co-immunoprecipitation and mass spectrometory analysis – The cell lysates (see immunoblot analysis) were mixed with either anti-Flag agarose beads (SIGMA) or Protein G agarose beads (Thermo Scientific) containing either anti-Myc (9E10, Santa Cruz) or anti-GFP (Cat.#598, MBL) antibodies for 3 hours at 4°C. The immunoprecipitants were washed and subjected to immunoblot analysis with the indicated antibodies. All experiments have been performed more than twice and data are reproducible. The mass spectrometry analysis has been described previously (29). Briefly, Flag-tagged Fbl12 was expressed in HEK293 cells, immunoprecipitated using anti-Flag antibody, and subjected to LC-MS/MS analysis. These analyses were performed four times. All proteins identified by MS analyses in every experiment were shown in Table 1.

GST pull down assay - The recombinant GST, GST-p21, GST-PA28γ, His-Fbl12/Skp1 were purified from *Eschericha coli.* BL21-Gold (DE3). The recombinant proteins were mixed with

Glutathione Sepharose beads (GE Healthcare) in buffer (0.5% NP-40, 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM, EDTA, 1 mM DTT) and incubated for 3 hours at 4°C. The precipitants were washed and subjected to immunoblot analysis with the indicated antibodies.

His-tag pull down assay - Cells were transfected with the indicated constructs and lysed in extraction buffer (6M guanidinium-HCl, 50 mM sodium phosphate buffer [pH 8.0], 300 mM NaCl and 5 mM imidazole). Cell lysates were sonicated briefly and were then incubated with TALON metal affinity resin (Clontech) for 4 hours at 4 Cº. The precipitants were washed with buffer (50 mM sodium phosphate buffer [pH 8.0], 300 mM NaCl and 5 mM imidazole) and then subjected to immunoblot analysis. All experiments have been performed more than twice and data are reproducible.

Immunocytochemistry - HeLa cells plated on 15 mm coverslips and grown in 12-well plates were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes at room temperature. The coverslips were washed in PBS, blocked with 5% bovine serum albumin (BSA) in PBS with 0.4% Triton X-100, then incubated with the indicated primary antibodies for

1 hour at room temperature or overnight at 4°C. Following PBS wash, samples were incubated with secondary antibodies (Alexa Fluor 594 anti-mouse IgG [1:500], Alexa Fluor 488 anti-rabbit IgG [1:500]) for 30 minutes at room temperature in blocking solution. Cells were imaged using a fluorescence microscope (BIOREVO BZ-9000, Keyence). The images were quantified by using Image J software and GraphPad Prism (GraphPad software).

Cell proliferation assay - HEK 293 cells were transfected with either plasmids. The transfected cells were plated on 96 well plates and incubated for 4 days. Cellular proliferation was analyzed using microplate reader Model 680 (BIO-RAD) and Cell Counting kit-8 (DOJINDO) according to the manufacturer's instructions.

Results

Fbl12 promotes p21 ubiquitination

Previous study has shown that Fbl12 binds and ubiquitinates $p57^{Kip2}$ during TGFβ1-mediated inhibition of osteoblastic cell differentiation (28). It has also been reported that SCF^{Fbl12} enhances ubiquitination of target proteins implicated in cell cycle, DNA repair and embryonic differentiation (28, 30-32). In addition, recent large-scale screening identified a somatic mutation of Fbl12 in renal carcinoma (33). Thus, it is likely that SCF^{Fbl12} regulates cellular proliferation and differentiation; however, it has been unclear whether SCF^{Fbl12} mediates ubiquitination of another Cip/Kip protein, p21. To examine whether Fbl12 interacts with p21, we first introduced Flag-p21 and Myc-Fbl12 plasmids into HEK 293T cells. Immunoprecipitation of Flag-p21 resulted in co-immunoprecipitation of Myc-Fbl12 (Fig. 1A). We then tested if this interaction is direct. To investigate this, we purified the recombinant proteins and performed the GST pull down assay. As it is difficult to solubilize the recombinant Fbl12, we co-purified Fbl12 with Skp1, which is reported to stabilize the conformation of F-box proteins (34). GST-p21 binds to His-Fbl12/Skp1 proteins efficiently *in vitro* (Fig. 1B). This data cannot rule out the possibility

that p21 associates with Skp1; however, we speculate that p21 binds to Fbl12 directly since p21 is capable of interaction with Fbl12ΔF (see below). To further examine which region of Fbl12 is responsible for this interaction, we used the deletion mutants of Fbl12. Unexpectedly, both deletion mutants that lacked the leucine-rich repeat and F-box region were capable of interacting with p21 (Fig.1C), suggesting that p21 associates with both F-box and leucine-rich repeat regions. Next, to map the binding site of p21, we subdivided p21 into two fragments. p21ΔNLS interacted with Fbl12; on the other hand, NLS region of p21 could not associate with Fbl12. These data suggest that the CDK inhibitor domain (CDI) and linker region are responsible for this interaction (Fig. 1D). As Fbl12 forms the SCF ubiquitin ligase complex, we next sought to determine if expression of Fbl12 promotes p21 ubiquitination. To examine this, we transfected cells with histidine-tagged ubiquitin (His-Ub) and precipitated ubiquitinated proteins using TALON metal affinity resins under denatured conditions. In consequence, the amount of ubiquitinated p21 was markedly increased when Fbl12 was expressed (Fig. 1E), suggesting that SCF^{Fbl12} promotes p21 ubiquitination in cells.

Fbl12 increases p21 expression levels associated with mixed-type ubiquitination

Since it is known that the ubiquitination plays important roles in the selective protein degradation, we examined whether Fbl12 controls p21 expression level via the protein degradation. Unexpectedly, overexpression of Fbl12 in HEK293, HeLa, and HCT116 cells increased the amount of endogenous p21. These data imply that Fbl12 positively regulates p21 abundance in cells (Fig. 2A). We next investigated whether Fbl12 is necessary for an upregulation of p21 under normal condition. To investigate this, we developed a CRISPR/Cas9 construct, which produces single-guide Fbl12 RNA (sgFbl12) (35), and disrupted *Fbxl12* gene by sgFbl12 construct. The endogenous p21 is slightly reduced in *Fbxl12*-deficient cells, suggesting that Fbl12 affects the amount of p21 in cells (Fig. 2B). To further confirm whether Fbl12 is involved in intracellular p21 level, we used endoribonuclease-prepared siRNA pool (siFbl12). The knockdown of endogenous Fbl12 by siFbl12 seemed to decrease the amount of p21 (Fig. 2C). We next sought to determine whether Fbl12 regulates either p21 transcription or mRNA stability in cells. To test this, we quantified the p21 mRNA levels using qRT-PCR. The amount of p21 mRNA was not significantly affected by either overexpression or knockdown of Fbl12, implying that Fbl12 has little effect on the regulation of mRNA levels (Fig. 2D). Next, to investigate whether the increase of p21 level was dependent on the SCF^{Fbl12} ubiquitin ligase activity, we

237 used Flag-Fbl12ΔF mutant, which is unable to form a functional SCF complex. Expression of
238 Fbl12 clearly increased p21 expression level. On the other hand, Fbl12ΔF did not substantially
239 increase p21 compared to full length of Fbl12 (Fig. 2E), although Fbl12ΔF is capable of
240 interacting with p21 (Fig. 1C). Therefore, it is likely that upregulation of p21 by Fbl12 is
241 dependent on its ubiquitination. Our data raise the question of why the p21 expression level was
242 increased despite its ubiquitination. To answer this question, we analyzed the linkage-mode of
243 the polyubiquitin chain using linkage-specific antibodies. We transfected His-p21 into HEK 293
244 cells, precipitated by affinity resins, and followed by the detection of ubiquitin-linkage by
245 immunoblot analysis. Interestingly, expression of Fbl12 enhanced not only K48-linked but also
246 K63-linked ubiquitination, suggesting that SCF^{Fbl12} promotes mixed-type of ubiquitination. To
247 further confirm this, we used mutant ubiquitin. Consistent with Fig. 2F, expression of His-Ub
248 K48R, of which Lys48 residue is mutated to arginine, promoted p21 ubiquitination as well as
249 His-Ub WT (Fig. 2G). This data suggest that Fbl12 has an effect on the formation of not only the
250 K48-linked ubiquitin chains but also other linkage modes. Interestingly, His-Ub K63R slightly
251 but significantly attenuated this ubiquitination (Fig. 2G). In addition, expression of Ub 48K and
252 63K, of which all lysine residues are mutated to arginine except at Lys48 and Lys63 respectively,

253 formed polyubiquitin chain on p21 by expression of Fbl12 (Fig. 2H). Furthermore, both 48K and
254 63K-liked ubiquitinations of p21 are slightly decreased in *Fbxl12*-deficient cells (Fig. 2I). These
255 results suggest that expression of Fbl12 promotes the formation of mixed-type of polyubiquitin
256 chain containing both K48- and K63-linkage modes, leading to an increase of p21 expression
257 level.

258

259 **Fbl12 suppresses default degradation of p21.**

260 As p21 is known to be a CDKs inhibitor, we investigated whether Fbl12 impedes the cellular
261 proliferation. As we expected, Fbl12 expression slightly but significantly delayed the
262 proliferation (Fig. 3A), suggesting that Fbl12-induced p21 upregulation is involved in cell
263 growth. Next, to examine the mechanisms of how Fbl12 augments the expression level, we
264 analyze the synthesis rate using proteasome inhibitor. Interestingly, the amount of endogenous
265 p21 was clearly increased after treatment with MG132. Moreover, Fbl12 expression had little
266 effect on the synthesis rate (Fig. 3B), suggesting that p21 synthesis rate is rapid under basal
267 condition independently of Fbl12. We then examined the degradation rate of endogenous p21.
268 The half-life of p21 in Fbl12-expressing cells was clearly extended than control cells (Fig. 3C).

In addition, p21 degradation was completely blocked by treatment with proteasome inhibitor, MG132, regardless of Fbl12 expression (Fig. 3D), demonstrating that Fbl12 attenuates the proteasome-dependent p21 degradation. We further confirmed whether Fbl12 affects the half-life of overexpressed p21. Overexpression of Fbl12 attenuates a degradation rate of exogenous p21. Moreover, Fbl12 did not affect the upper limit of overexpressed p21 (Fig.3E), implying that an upper limit of p21 expression level is already dictated innately. Probably, overexpression of p21 reaches the saturation condition in cells. Next, we investigated the mechanisms of how Fbl12 regulates the amount of p21. Previously, it has been reported that the p21 binding proteins block the default degradation after newly synthesis of p21 (14-16). Additionally, Fbl12 negatively regulates cellular proliferation. Therefore, we hypothesized that Fbl12 modulates the binding affinity of p21 with target protein involved in cell cycle, leading to a delay of degradation. To examine this, we tested whether Fbl12 regulates the interaction between p21 and CDK2, which is one of the targets of p21 (36). Interestingly, expression of Fbl12 clearly enhanced their binding ability (Fig. 3F), demonstrating that the mixed-type ubiquitination synthesized by Fbl12 promotes their interaction. This finding may support the idea that binding proteins, such as CDK2, protect p21 from proteasome-dependent degradation.

285

286 **PA28γ associates with SCF^{Fbl12}**

287 To understand the precise mechanisms by which SCF^{Fbl12} governs the amount of p21 in cells, we
288 performed proteomics analysis by using LC-MS/MS system to identify the other Fbl12 binding
289 proteins. Consequently, we identified 27 proteins as Fbl12-binding targets (Table 1). One of the
290 most interesting proteins was PA28γ. PA28γ forms a homohexameric ring and acts as a
291 proteasome activator (37). Recent studies have reported that PA28γ directly associates with p21,
292 leading to p21 degradation independently of ubiquitination (25, 26). Thus, it is likely that PA28γ
293 is involved in the control of p21 expression levels in concert with Fbl12. Before pursuing this
294 hypothesis, we verified the interaction between Fbl12 and PA28γ. Myc-PA28γ was significantly
295 associated with Flag-Fbl12 (Fig. 4A). In the reverse experiment, immunoprecipitation of
296 Flag-Fbl12, but not Flag-Skp2 (a structural homologue of Fbl12), caused
297 co-immunoprecipitation of Myc-PA28γ (Fig. 4B). We next determined the binding region using
298 deletion mutants. In consequence, we found that the F-box domain of Fbl12 is required for this
299 interaction (Fig. 4C). Furthermore, recombinant GST-PA28γ was associated with
300 His-Fbl12/Skp1 *in vitro*, suggesting that PA28γ is able to bind Fbl12 directly (Fig. 4D). To ask

301 whether these proteins colocalize in culture cells, we performed immunocytochemistry after
302 ectopic expression of EGFP-Fbl12. Both EGFP-Fbl12 and endogenous PA28γ colocalized in the
303 nucleus, supporting the finding that Fbl12 associates with PA28γ in the same subcellular region
304 (Fig. 4E). We then examined whether SCF^{Fbl12} ubiquitinates PA28γ as well as p21. The
305 ubiquitination level of Myc-PA28γ was comparable with the control sample, indicating that
306 SCF^{Fbl12} does not enhance ubiquitination of PA28γ (Fig. 4F). As the F-box domain, which is
307 essential to associate with Skp1 and form the SCF ubiquitin ligase complex, was important to
308 interact with PA28γ (Fig. 4C), we next asked whether Skp1 and the PA28γ-20S proteasome are
309 part of the same protein complex. When HA-Fbl12 was expressed in cells, Myc-PA28γ was
310 co-precipitated with Flag-Skp1. However, β5, which is a core subunit of the 20S proteasome,
311 was not included in this complex (Fig. 4G). These results suggest that SCF^{Fbl12} is associated with
312 PA28γ, which is free from the 20S proteasome. To further confirm this observation, we
313 performed a glycerol density gradient centrifugation. Most PA28γ was found in the lighter
314 fraction, and a small concentration of PA28γ was found in the heavier fraction, including the β5
315 subunit. Intriguingly, the fractions containing Fbl12 were different from β5 (Fig. 4H), supporting

316 the idea that SCF^{Fbl12} associates with a certain amount of PA28γ, which is free from the 20S
317 proteasome.
318
319 **PA28γ attenuates SCF^{Fbl12}-dependent p21 ubiquitination**
320 Previous studies have reported that PA28γ-20S proteasome promotes p21 degradation
321 independently of ubiquitination. Also, it has been reported that the defects in PA28γ delay the
322 degradation rate under normal condition (25, 26). To verify these results, we assessed if PA28γ
323 mediates both p21 degradation and amplification at least in our system. Consequently,
324 overexpression of PA28γ decreased p21 expression levels in several cell lines (Fig. 5A) and
325 attenuated cellular proliferation (Fig. 5B), demonstrating that PA28γ is involved in regulating
326 cellular proliferation via p21 expression level. Since p21 ubiquitination regulated by Fbl12 is
327 implicated in expression level and proliferation, we investigated if PA28γ has an effect on
328 SCF^{Fbl12}-induced p21 ubiquitination. As we described before, overexpression of Fbl12 increased
329 p21 ubiquitination. However, this effect was suppressed by PA28γ (Fig. 5C), suggesting that
330 PA28γ attenuates SCF^{Fbl12}-induced p21 ubiquitination. These observations prompt us to
331 investigate whether PA28γ controls p21 expression via incorporation into SCF^{Fbl12} complex. To

assess this, we performed immunoprecipitation assay. In contrast to the previous research, PA28γ did not show a clear binding to p21. However, this binding was dramatically increased when Fbl12 was co-expressed in cells (Fig. 5D), implying that PA28γ decreases p21 expression associated with the regulation of SCF^{Fbl12}.

UV stimulation induces p21 degradation through disassembly of protein complex.

Previous study has shown that UV irradiation induces p21 expression through p53 activation (3). On the other hand, several studies have shown that UV irradiation triggers rapid degradation of p21 (38-41). Thus, we examined whether Fbl12 suppresses UV-induced p21 turnover. Stimulation with UV for one hour markedly decreased the amount of endogenous p21 even in the presence of Fbl12 expression in HeLa cells (Fig. 6A). In addition, Fbl12 had little effect on the degradation rate of p21 in response to UV irradiation (Fig. 6B). Meanwhile, we did not observe a decrease in the amount of overexpressed-p21 level by the UV irradiation in our system (see Fig. 6C to 6F). Probably, p21 synthesis rate through an expression vector is higher than an endogenous p21 promoter. Taken together, our data suggest that expression of Fbl12 does not attenuate UV-induced p21 degradation. The question arises that why UV irradiation promotes

348 p21 degradation despite the existence of Fbl12. To answer this question, we investigated whether
349 UV stimulation alters the protein complex status. Consistent with our data in Figure 5D, Fbl12
350 promotes an association of p21 with PA28γ. On the other hand, this effect was attenuated by UV
351 irradiation (Fig. 6C). As PA28γ remained bound to the SCF^{Fbl12} complex after UV stimulation,
352 we hypothesized that the binding ability between p21 and Fbl12 was decreased in response to
353 UV stimulation. To confirm this idea, we conducted immunoprecipitation assay with or without
354 UV irradiation. In consequence, p21 was released from Fbl12 after UV stimulation (Fig. 6D),
355 suggesting that UV stimulation promotes disassembly of PA28γ-SCF^{Fbl12}-p21 complex, resulting
356 in promoting p21 degradation. Finally, we examined whether UV stimulation regulates the
357 ubiquitination status of p21 in our system. Stimulation with UV for one hour had a little effect on
358 the change of p21 ubiquitination in the absence of Fbl12 expression (Fig. 6E). Interestingly, this
359 stimulation slightly reduced the K63-linked ubiquitination even in the Fbl12-expressing cells
360 (Fig. 6F), demonstrating that UV stimulation may attenuate Fbl12-induced K63-linked
361 ubiquitination. Taken together, these data suggest that UV irradiation induces disassembly of
362 protein complex and attenuates K63-linked ubiquitination, leading to rapid degradation of p21.

Discussion

We have shown here that Fbl12 increases p21 expression levels through their interactions. SCF^{Fbl12} enhances p21 mixed-type ubiquitination and this effect was suppressed by PA28γ. Consequently, expression of Fbl12 extended the half-life of p21. Furthermore, UV stimulation promotes disassembly of PA28γ-SCF^{Fbl12}-p21 complex, reduces mixed-type ubiquitination of p21, resulting in its degradation. These data demonstrate that Fbl12 controls the intracellular concentration of p21 and Fbl12-induced mixed-type ubiquitination is a key event that prevents p21 from default degradation via binding proteins (Fig.7).

Recent studies have shown that p21 associates with binding proteins immediately after the synthesis, resulting in its stabilization (14-16). Probably, this binding might hamper K48-linked ubiquitination or recognition by proteasome. In our study, we found that SCF^{Fbl12} enhances mixed-type ubiquitination, including K63-linkage ubiquitin chain, leading to an increase of p21 expression. Although the mechanisms inferred from our findings have not been clarified, several studies have posited a relationship between the SCF complex and the K63-linked ubiquitin chain. For example, one of the F-box proteins, Fbxl21 ubiquitinates CRY

379 to control the circadian rhythms (42, 43). It was also reported that SCF^{Fbxl21} mediates the
380 formation of K63- and K11-linked polyubiquitin chains, implying that the linkage modes of
381 ubiquitin chain might be a determinant of CRY stability (42). Additionally, $SCF^{\beta\text{-}TrCP}$ competes
382 with SCF^{Fbxw7}, and leads to K63-linked ubiquitin chain conjugation on c-Myc that eventually
383 leads to the blockage of c-Myc from proteasomal degradation (44). Given that the conjugation of
384 ubiquitin chains of more than four molecules is thought to be necessary for the recognition of
385 26S proteasome (45), insertion of K63-linked ubiquitination into K48-linked ubiquitin chain may
386 hamper the preferred conformation of the polyubiquitin chain, resulting in the decrease of
387 affinity for 26S proteasome. Since we observed p21 degradation attenuated by Fbl12
388 overexpression along with the rapid p21 synthesis (Fig. 3B), p21 degradation rate might be
389 surpassed by its synthesis rate. Consequently, p21 expression level appears to increase
390 dependently on Fbl12. Alternatively, it is possible that the K63-linked ubiquitination competes
391 with K48-linked ubiquitination on the same lysine site(s) of target proteins in a similar manner to
392 that of c-Myc.

393 It has been shown that the E2s, which are ubiquitin-conjugating enzymes, are
394 important for the determination of ubiquitin-linkage mode (46). Usually, SCF complex seems to

395 conjugate K48-linked ubiquitination with target protein via the recruitment of specific E2
396 enzymes, such as UBE2R1 and UBE2D2. On the other hand, recent studies have reported that
397 the heterodimer of UBE2N with UBE2V1 plays important roles in the formation of K63-linked
398 ubiquitin chain (46). The mechanisms of how SCF complex mediates mixed-type ubiquitination
399 remain unclear. It has been known that Ubc4, which is a yeast homolog of UBE2D2, is involved
400 in the formation of not only K48-linked ubiquitination but also K63-linked ubiquitination under
401 stress condition (47). In addition, UBE2D2 is responsible for SCF^{Fbl12}-induced p57
402 ubiquitination (28). Thus, UBE2D2 is thought to be a primary candidate that controls
403 SCF^{Fbl12}-dependent mixed-type ubiquitination. However, our preliminary experiments have
404 shown that the combination between SCF^{Fbl12} and UBE2D2 had a little effect on the
405 enhancement of p21 ubiquitination in our *in vitro* ubiquitination assay (data not shown). This
406 suggests that other specific E2 enzyme is responsible for this cascade reaction in cells.
407 Alternatively, it is plausible that other unknown factors play important roles in the regulation of
408 linkage specificity and activity of SCF^{Fbl12}. Indeed, expression of PA28γ decreases the
409 ubiquitination level of not only p21 but also possibly by autoubiquitinated Fbl12 (Fig. 5C).
410 Probably, PA28γ might alter the structural distance from E2 enzyme to the substrate on the

411 SCF^{Fbl12} complex, resulting in a decrease of ubiquitin ligase activity. The biological relevance
412 that underlie PA28γ-regulated p21 turnover are yet to be elucidated. Previously, several groups
413 have shown that PA28γ promotes ubiquitin-independent degradation of p21 through their
414 binding. (25, 26). We report here that an increment of PA28γ negatively regulates the amount of
415 p21. As PA28γ level has been reported to be upregulated by NF-κB activation (48), the
416 regulation of the amount of PA28γ could mediate a pivotal process that controls p21 expression
417 level regulated by stress responses.

418 Recently, we have reported that UV irradiation induces Fbl12ΔF transcription via the
419 alternative promoter, leading to an involvement of Fbl12 regulation (49). Since overexpression
420 of Fbl12ΔF does not affect the cellular proliferation, it is possible that Fbl12-related pathways
421 are linked to UV-induced DNA damage response. Interestingly, we occasionally observed
422 punctate structure co-localized with Fbl12 in nucleus after UV irradiation (data not shown). It
423 has also been reported that PA28γ translocates to Cajal bodies in response to UV, leading to an
424 enhancement of their degradation (50). These results have suggested that the SCF^{Fbl12}-PA28γ
425 protein complex is a potent mediator that regulates nuclear integrity following DNA damage
426 response. Since defect in *Fbxl12* gene generated by CRISPR/Cas9 had little effect on the

proliferation under basal condition (data not shown), our finding could be involved in not only cellular proliferation but also in DNA damage responses, as well as Fbl12ΔF. One potential mechanism is that Fbl12 activity is augmented by stress stimulation such as UV. Intriguingly, Fbl12 is thought to be phosphorylated at Ser-124 (http://www.phosphosite.org/). This observation suggests that Fbl12 activity is modulated by phosphorylation in response to the stress stimulation. However, the precise mechanisms that underlie the control of this mechanisms mediated by SCF^{Fbl12}-PA28γ proteins remain unclear. This will need to be investigated more thoroughly in the future.

Taken together, we found that Fbl12 binds and ubiquitinates p21. This ubiquitination is associated with the stability of p21. In addition, we found that Fbl12 regulates default degradation under basal condition but not under UV-stimulated condition. Therefore, our findings provide novel mechanisms that underlie the regulation of p21 expression level in cells.

References

1. **Abbas T, Dutta A.** 2009. p21 in cancer: intricate networks and multiple activities. Nat Rev Cancer **9:**400-414.

2. **Gsponer J, Futschik ME, Teichmann SA, Babu MM.** 2008. Tight regulation of unstructured proteins: from transcript synthesis to protein degradation. Science **322:**1365-1368.

3. **el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B.** 1993. WAF1, a potential mediator of p53 tumor suppression. Cell **75:**817-825.

4. **Gartel AL, Najmabadi F, Goufman E, Tyner AL.** 2000. A role for E2F1 in Ras activation of p21(WAF1/CIP1) transcription. Oncogene **19:**961-964.

5. **Narla G, Heath KE, Reeves HL, Li D, Giono LE, Kimmelman AC, Glucksman MJ, Narla J, Eng FJ, Chan AM, Ferrari AC, Martignetti JA, Friedman SL.** 2001. KLF6, a candidate tumor suppressor gene mutated in prostate cancer. Science **294:**2563-2566.

6. **Mukherjee S, Conrad SE.** 2005. c-Myc suppresses p21WAF1/CIP1 expression during estrogen signaling and antiestrogen resistance in human breast cancer cells. J Biol Chem **280:**17617-17625.

7. **Jung P, Menssen A, Mayr D, Hermeking H.** 2008. AP4 encodes a c-MYC-inducible repressor of p21. Proc Natl Acad Sci U S A **105:**15046-15051.

8. **Li Y, Dowbenko D, Lasky LA.** 2002. AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival. J Biol Chem **277:**11352-11361.

9. **Zhou BP, Liao Y, Xia W, Spohn B, Lee MH, Hung MC.** 2001. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nat Cell Biol **3:**245-252.

10. **Rossig L, Jadidi AS, Urbich C, Badorff C, Zeiher AM, Dimmeler S.** 2001. Akt-dependent phosphorylation of p21(Cip1) regulates PCNA binding and proliferation of endothelial cells. Mol Cell Biol **21:**5644-5657.

11. **Barnouin K, Dubuisson ML, Child ES, Fernandez de Mattos S, Glassford J, Medema RH, Mann DJ, Lam EW.** 2002. H2O2 induces a transient multi-phase cell

cycle arrest in mouse fibroblasts through modulating cyclin D and p21Cip1 expression. J Biol Chem **277:**13761-13770.

12. **Kim GY, Mercer SE, Ewton DZ, Yan Z, Jin K, Friedman E.** 2002. The stress-activated protein kinases p38 alpha and JNK1 stabilize p21(Cip1) by phosphorylation. J Biol Chem **277:**29792-29802.

13. **Asher G, Reuven N, Shaul Y.** 2006. 20S proteasomes and protein degradation "by default". Bioessays **28:**844-849.

14. **Bornstein G, Bloom J, Sitry-Shevah D, Nakayama K, Pagano M, Hershko A.** 2003. Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. J Biol Chem **278:**25752-25757.

15. **Yamada K, Ono M, Perkins ND, Rocha S, Lamond AI.** 2013. Identification and functional characterization of FMN2, a regulator of the cyclin-dependent kinase inhibitor p21. Mol Cell **49:**922-933.

16. **Jascur T, Brickner H, Salles-Passador I, Barbier V, El Khissiin A, Smith B, Fotedar R, Fotedar A.** 2005. Regulation of p21(WAF1/CIP1) stability by WISp39, a Hsp90 binding TPR protein. Mol Cell **17:**237-249.

17. **Ferrandina G, Stoler A, Fagotti A, Fanfani F, Sacco R, De Pasqua A, Mancuso S, Scambia G.** 2000. p21WAF1/CIP1 protein expression in primary ovarian cancer. Int J Oncol **17:**1231-1235.

18. **Sarbia M, Stahl M, zur Hausen A, Zimmermann K, Wang L, Fink U, Heep H, Dutkowski P, Willers R, Muller W, Seeber S, Gabbert HE.** 1998. Expression of p21WAF1 predicts outcome of esophageal cancer patients treated by surgery alone or by combined therapy modalities. Clin Cancer Res **4:**2615-2623.

19. **Yu ZK, Gervais JL, Zhang H.** 1998. Human CUL-1 associates with the SKP1/SKP2 complex and regulates p21(CIP1/WAF1) and cyclin D proteins. Proc Natl Acad Sci U S A **95:**11324-11329.

20. **Starostina NG, Simpliciano JM, McGuirk MA, Kipreos ET.** 2010. CRL2(LRR-1) targets a CDK inhibitor for cell cycle control in C. elegans and actin-based motility regulation in human cells. Dev Cell **19:**753-764.

21. **Abbas T, Sivaprasad U, Terai K, Amador V, Pagano M, Dutta A.** 2008. PCNA-dependent regulation of p21 ubiquitylation and degradation via the CRL4Cdt2 ubiquitin ligase complex. Genes Dev **22:**2496-2506.

22. **Amador V, Ge S, Santamaria PG, Guardavaccaro D, Pagano M.** 2007. APC/C(Cdc20) controls the ubiquitin-mediated degradation of p21 in prometaphase. Mol Cell **27:**462-473.

23. **Lee EW, Lee MS, Camus S, Ghim J, Yang MR, Oh W, Ha NC, Lane DP, Song J.** 2009. Differential regulation of p53 and p21 by MKRN1 E3 ligase controls cell cycle arrest and apoptosis. EMBO J **28:**2100-2113.

24. **Bloom J, Amador V, Bartolini F, DeMartino G, Pagano M.** 2003. Proteasome-mediated degradation of p21 via N-terminal ubiquitinylation. Cell **115:**71-82.

25. **Li X, Amazit L, Long W, Lonard DM, Monaco JJ, O'Malley BW.** 2007. Ubiquitin- and ATP-independent proteolytic turnover of p21 by the REGgamma-proteasome pathway. Mol Cell **26:**831-842.

26. **Chen X, Barton LF, Chi Y, Clurman BE, Roberts JM.** 2007. Ubiquitin-independent degradation of cell-cycle inhibitors by the REGgamma proteasome. Mol Cell **26:**843-852.

27. **Touitou R, Richardson J, Bose S, Nakanishi M, Rivett J, Allday MJ.** 2001. A degradation signal located in the C-terminus of p21WAF1/CIP1 is a binding site for the C8 alpha-subunit of the 20S proteasome. EMBO J **20:**2367-2375.

28. **Kim M, Nakamoto T, Nishimori S, Tanaka K, Chiba T.** 2008. A new ubiquitin ligase involved in p57KIP2 proteolysis regulates osteoblast cell differentiation. EMBO Rep **9:**878-884.

29. **Natsume T, Yamauchi Y, Nakayama H, Shinkawa T, Yanagida M, Takahashi N, Isobe T.** 2002. A direct nanoflow liquid chromatography-tandem mass spectrometry system for interaction proteomics. Anal Chem **74:**4725-4733.

30. **Postow L, Funabiki H.** 2013. An SCF complex containing Fbxl12 mediates DNA damage-induced Ku80 ubiquitylation. Cell Cycle **12:**587-595.

31. **Mallampalli RK, Kaercher L, Snavely C, Pulijala R, Chen BB, Coon T, Zhao J, Agassandian M.** 2013. Fbxl12 triggers G1 arrest by mediating degradation of calmodulin kinase I. Cell Signal **25:**2047-2059.

32. **Nishiyama M, Nita A, Yumimoto K, Nakayama KI.** 2015. FBXL12-Mediated Degradation of ALDH3 is Essential for Trophoblast Differentiation During Placental Development. Stem Cells **33:**3327-3340.

33. **Varela I, Tarpey P, Raine K, Huang D, Ong CK, Stephens P, Davies H, Jones D, Lin ML, Teague J, Bignell G, Butler A, Cho J, Dalgliesh GL, Galappaththige D, Greenman C, Hardy C, Jia M, Latimer C, Lau KW, Marshall J, McLaren S, Menzies A, Mudie L, Stebbings L, Largaespada DA, Wessels LF, Richard S, Kahnoski RJ, Anema J, Tuveson DA, Perez-Mancera PA, Mustonen V, Fischer A, Adams DJ, Rust A, Chan-on W, Subimerb C, Dykema K, Furge K, Campbell PJ, Teh BT, Stratton MR, Futreal PA.** 2011. Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. Nature **469:**539-542.

34. **Yoshida Y, Murakami A, Tanaka K.** 2011. Skp1 stabilizes the conformation of F-box proteins. Biochem Biophys Res Commun **410:**24-28.

35. **Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F.** 2013. Genome engineering using the CRISPR-Cas9 system. Nat Protoc **8:**2281-2308.

36. **Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ.** 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell **75:**805-816.

37. **Mao I, Liu J, Li X, Luo H.** 2008. REGgamma, a proteasome activator and beyond? Cell Mol Life Sci **65:**3971-3980.

38. **Fotedar R, Bendjennat M, Fotedar A.** 2004. Role of p21WAF1 in the cellular response to UV. Cell Cycle **3:**134-137.

39. **Bendjennat M, Boulaire J, Jascur T, Brickner H, Barbier V, Sarasin A, Fotedar A, Fotedar R.** 2003. UV irradiation triggers ubiquitin-dependent degradation of p21(WAF1) to promote DNA repair. Cell **114:**599-610.

40. **Lee H, Zeng SX, Lu H.** 2006. UV Induces p21 rapid turnover independently of ubiquitin and Skp2. J Biol Chem **281:**26876-26883.

41. **Soria G, Podhajcer O, Prives C, Gottifredi V.** 2006. P21Cip1/WAF1 downregulation is required for efficient PCNA ubiquitination after UV irradiation. Oncogene **25:**2829-2838.

42. **Hirano A, Yumimoto K, Tsunematsu R, Matsumoto M, Oyama M, Kozuka-Hata H, Nakagawa T, Lanjakornsiripan D, Nakayama KI, Fukada Y.** 2013. FBXL21 regulates oscillation of the circadian clock through ubiquitination and stabilization of cryptochromes. Cell **152:**1106-1118.

43. **Yoo SH, Mohawk JA, Siepka SM, Shan Y, Huh SK, Hong HK, Kornblum I, Kumar V, Koike N, Xu M, Nussbaum J, Liu X, Chen Z, Chen ZJ, Green CB, Takahashi JS.** 2013. Competing E3 ubiquitin ligases govern circadian periodicity by degradation of CRY in nucleus and cytoplasm. Cell **152:**1091-1105.

44. **Popov N, Schulein C, Jaenicke LA, Eilers M.** 2010. Ubiquitylation of the amino terminus of Myc by SCF(beta-TrCP) antagonizes SCF(Fbw7)-mediated turnover. Nat Cell Biol **12:**973-981.

45. **Saeki Y, Isono E, Oguchi T, Shimada M, Sone T, Kawahara H, Yokosawa H, Toh-e A.** 2004. Intracellularly inducible, ubiquitin hydrolase-insensitive tandem ubiquitins inhibit the 26S proteasome activity and cell division. Genes Genet Syst **79:**77-86.

46. **Ye Y, Rape M.** 2009. Building ubiquitin chains: E2 enzymes at work. Nat Rev Mol Cell Biol **10:**755-764.

47. **Arnason T, Ellison MJ.** 1994. Stress resistance in Saccharomyces cerevisiae is strongly correlated with assembly of a novel type of multiubiquitin chain. Mol Cell Biol **14:**7876-7883.

48. **Sun J, Luan Y, Xiang D, Tan X, Chen H, Deng Q, Zhang J, Chen M, Huang H, Wang W, Niu T, Li W, Peng H, Li S, Li L, Tang W, Li X, Wu D, Wang P.** 2016. The 11S Proteasome Subunit PSME3 Is a Positive Feedforward Regulator of NF-kappaB and Important for Host Defense against Bacterial Pathogens. Cell Rep **14:**737-749.

49. **Tsuruta F, Kim J, Fukuda T, Kigoshi Y, Chiba T.** 2015. The intronic region of Fbxl12 functions as an alternative promoter regulated by UV irradiation. Biochem and Biophys Rep **3:**100-107.

50. **Cioce M, Boulon S, Matera AG, Lamond AI.** 2006. UV-induced fragmentation of Cajal bodies. J Cell Biol **175:**401-413.

Acknowledgments

We thank Dr. Toru Natsume (AIST, Japan) for helping the proteomics experiments, Takuma Aihara for critical reading of the manuscript, and the members of the Chiba laboratory for helpful discussions and technical support. This work was supported by Grant-in-Aid from Ministry of Education, Science, Sports and Culture of Japan (JSPS KAKENHI 23770218 to FT).

Figure legends

Figure 1. Fbl12 promotes p21 ubiquitination

(A) Co-immunoprecipitation of Myc-Fbl12 and Flag-p21 in HEK 293T cells. **(B)** GST pull down of His-Fbl12/Skp1 and either GST or GST-p21. The precipitated proteins were analyzed by immunoblot analysis. **(C)** Schematic structure of Fbl12. F-Box: F-Box domain; LRR: leucine-rich repeat (upper panel). Co-immunoprecipitation of p21 and either EGFP-Fbl12, EGFP-Fbl12 F-box, or EGFP-Fbl12ΔF (lower panel). **(D)** Schematic structure of p21. CDI: CDK inhibitor domain; NLS: nuclear localization signal (upper panel). Co-immunoprecipitation of Flag-Fbl12 and either EGFP-p21, EGFP-p21ΔNLS, or EGFP-p21NLS (lower panel). (**E**) Ubiquitinated proteins were purified from denatured cell lysates using Talon metal affinity resin, and analyzed by immunoblot analysis.

F**igure 2. Fbl12 increases p21 expression levels associated with mixed-type ubiquitination**

(A) HEK 293, HeLa, and HCT116 cells were transfected with either Myc-Fbl12 or control vector. Cell lysates were subjected to immunoblot analysis. **(B)** *Fbxl12*-deficient cell lysates were

analyzed by immunoblot analysis. **(C)** HEK 293 cells were treated with siFbl12 (200 ng/ml or 400 ng/ml) and incubated for 1 or 2 days. Cell lysates were subject to immunoblot analysis (upper panel). The qPCR analysis of Fbl12 mRNA in esiRNA-treated HEK 293 cells. Results were normalized to actin expression (n=3, mean ± SEM, *P<0.05 by student's t-test) (lower panel). **(D)** qPCR analysis of p21 mRNA in either Flag-Fbl12-expressing cells (left) or siFbl12-treated cells (right). Results were normalized to actin expression (n=3, mean ± SEM, N.S., not significant by student's t-test). **(E)** HEK 293 cells were transfected with either Flag-Fbl12 or Flag-Fbl12ΔF. Cell lysates were subjected to immunoblot analysis. **(F)** His-p21 were purified from denatured cell lysates using Talon metal affinity resin and analyzed by immunoblot analysis. **(G and H)** Ubiquitinated proteins were purified from denatured cell lysates using Talon metal affinity resin and analyzed by immunoblot analysis. **(I)** Ubiquitinated proteins were purified from denatured *Fbxl12*-deficient cell lysates using Talon metal affinity resin and analyzed by immunoblot analysis.

Figure 3. Fbl12 suppresses default degradation of p21

(A) HEK293 cells were transfected with either control or Myc-Fbl12 plasmids. The cellular proliferation was analyzed using cell counting kit-8 (n=3, mean ± SEM, **P<0.01 by student's t-test). **(B)** HEK 293 cells were transfected with either Myc-Fbl12 or control vector. Cells were incubated with 20 μM MG132 for the indicated time, and were then subjected to immunoblot analyses (left panel). Data were quantified using Image J software. These data are representative of three independent experiments (right panel). **(C)** HEK 293 cells were transfected with either Myc-Fbl12 or control vector. Cells were incubated in the absence or presence of 50 μg/ml CHX for the indicated time, and were then subjected to immunoblot analyses (left panel). Data were quantified by using Image J software. These data are representative of three independent experiments (right panel). **(D)** HEK 293 cells were transfected with either Myc-Fbl12 or control vector. Cells were incubated in the absence or presence of 50 μg/ml CHX together with 20 μM MG132 for the indicated time, and were then subjected to immunoblot analyses (left panel). Data were quantified using Image J software. These data are representative of three independent experiments (right panel). **(E)** HEK 293 cells were transfected with indicated vectors. Cells were incubated in the absence or presence of 50 μg/ml CHX for the indicated time, and were then subjected to immunoblot analyses. **(F)** Co-immunoprecipitation of Flag-Fbl12, p21 and

Myc-CDK2 in HEK 293 cells. Ectopic expression of Fbl12 promoted the association of p21 with CDK2.

Figure 4. PA28γ associates with SCF^{Fbl12}

(A) Co-immunoprecipitation of Flag-Fbl12 and Myc-PA28γ. **(B)** Co-immunoprecipitation of Flag-tagged F-box proteins (Fbl12 or Skp2) and Myc-PA28γ. **(C)** Co-immunoprecipitation of Myc-PA28γ and either Flag-Fbl12 or Flag-Fbl12ΔF. (**D**) GST pull down of His-Fbl12/Skp1 and either GST or GST-PA28γ. The precipitated proteins were analyzed by immunoblot analysis. **(E)** Fluorescent images of HeLa cells expressing EGFP-Fbl12. Cells were stained with anti-GFP and anti-PA28γ antibodies. Bar: 20 μm. **(F)** Ubiquitinated proteins were purified from denatured cell lysates using Talon metal affinity resin and analyzed by immunoblot analysis. **(G)** Co-immunoprecipitation of Flag-Skp1, HA-Fbl12, and Myc-PA28γ in HEK 293T cells. **(H)** Cell lysates separated by a glycerol density gradient centrifugation were subjected to immunoblot analysis. Flag-Fbl12 and PA28γ form a complex smaller than the 20S.

Figure 5. PA28γ attenuates SCF^{Fbl12}-induced p21 ubiquitination

(A) HEK 293, HeLa, and HCT116 cells were transfected with either Myc-PA28γ or control vector. Cell lysates were subjected to immunoblot analysis. **(B)** HEK 293 cells were transfected with either control or Myc-Fbl12 plasmids. The cellular proliferation was analyzed using cell counting kit-8 (n=3, mean ± SEM, *P<0.05 by student's t-test). (**C**) Ubiquitinated proteins were purified from denatured cell lysates using Talon metal affinity resin and analyzed by immunoblot analysis. Expression of PA28γ reduced SCF^{Fbl12}-induced p21 ubiquitination. **(D)** Co-immunoprecipitation of Flag-Fbl12, p21 and Myc-PA28γ in HEK 293T cells. Ectopic expression of Fbl12 promoted the association of p21 with PA28γ.

Figure 6. UV stimulation induces p21 degradation through disassembly of protein complex.

(A) Fluorescent images of HeLa cells expressing EGFP-Fbl12. Cells were stimulated by 12 $\mu W/cm^2$ UV, and were then subjected to immunocytochemistry using anti-GFP and anti-p21 antibodies (left). Arrowheads indicate Fbl12-expressing cells. Bar: 20 μm. Ratiometric measurement of p21 to Hoechst fluorescence observed in cells expressing EGFP-Fbl12 before and after stimulation with UV (right) (*n* >100; mean ± SD; ***, P < 0.001 by one-way ANOVA). **(B)** HEK 293 cells were transfected with either Myc-Fbl12 or control vector. Cells were

stimulated by 12 μW/cm^2 UV, and were then subjected to immunoblot analysis (left). Expression level was quantified using Image J softwares (right). **(C)** Co-immunoprecipitation of Flag-Fbl12, p21 and Myc-PA28γ in HEK 293 cells. UV irradiation promoted the dissociation of p21 from Fbl12-PA28γ complex. **(D)** Co-immunoprecipitation of Flag-Fbl12 and p21 in HEK 293 cells. UV irradiation promoted the dissociation of p21 from Fbl12. (**E and F**) HEK 293 cells were stimulated with UV in the presence or absence of Fbl12 expression. Ubiquitinated proteins were purified from denatured cell lysates using Talon metal affinity resin, and analyzed by immunoblot analysis.

Figure 7. Model for the regulation of p21 turnover

SCF^{Fbl12} promotes mixed-type ubiquitination of p21, and attenuates the default degradation under basal condition, and this effect is cancelled by PA28γ (upper). UV stimulation promotes disassembly of PA28γ-SCF^{Fbl12}-p21 complex and induces rapid degradation of p21 (lower). Other E3 ligase may ubiqutinate free p21 in cells.

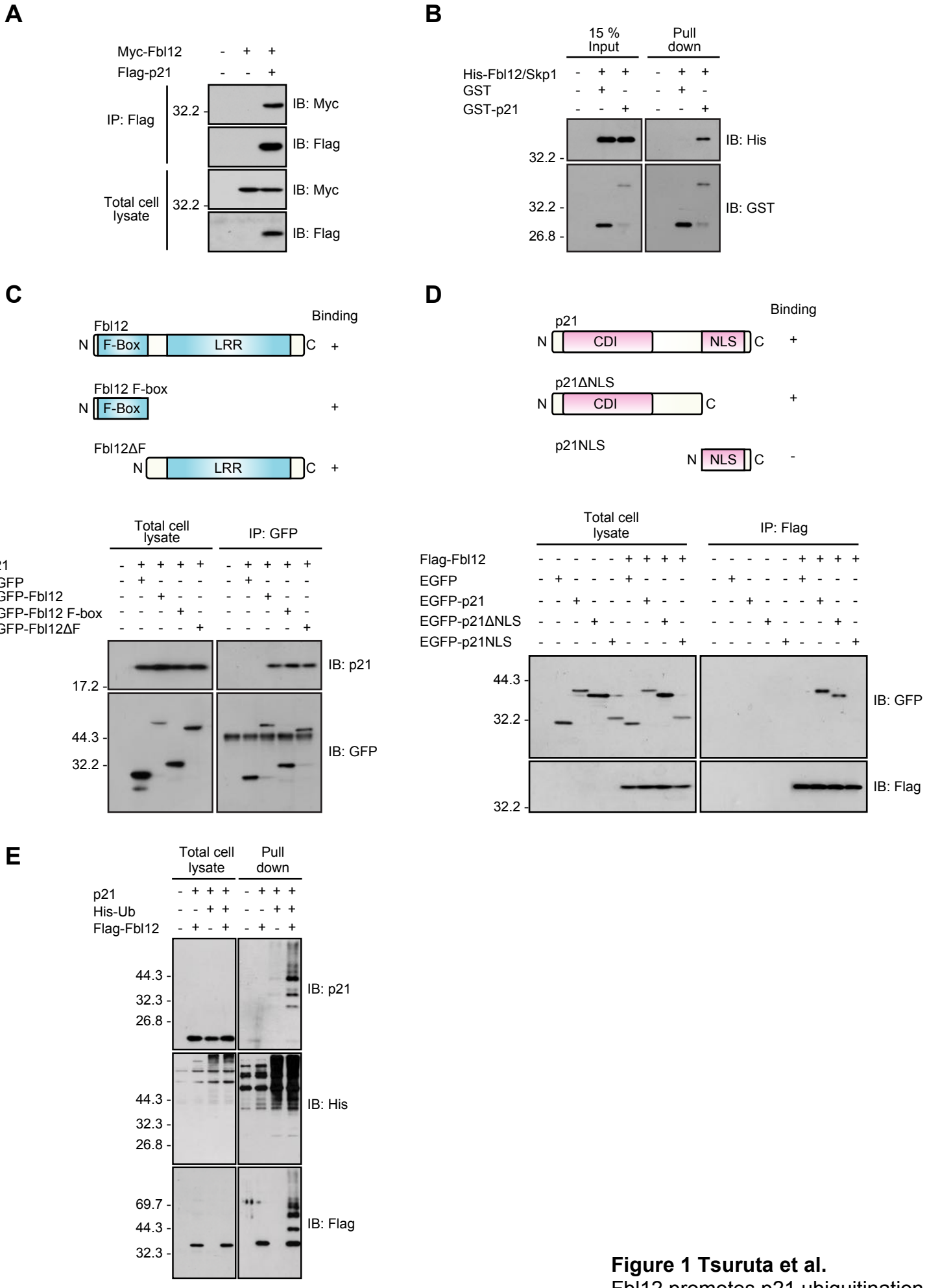

Figure 1 Tsuruta et al.
Fbl12 promotes p21 ubiquitination

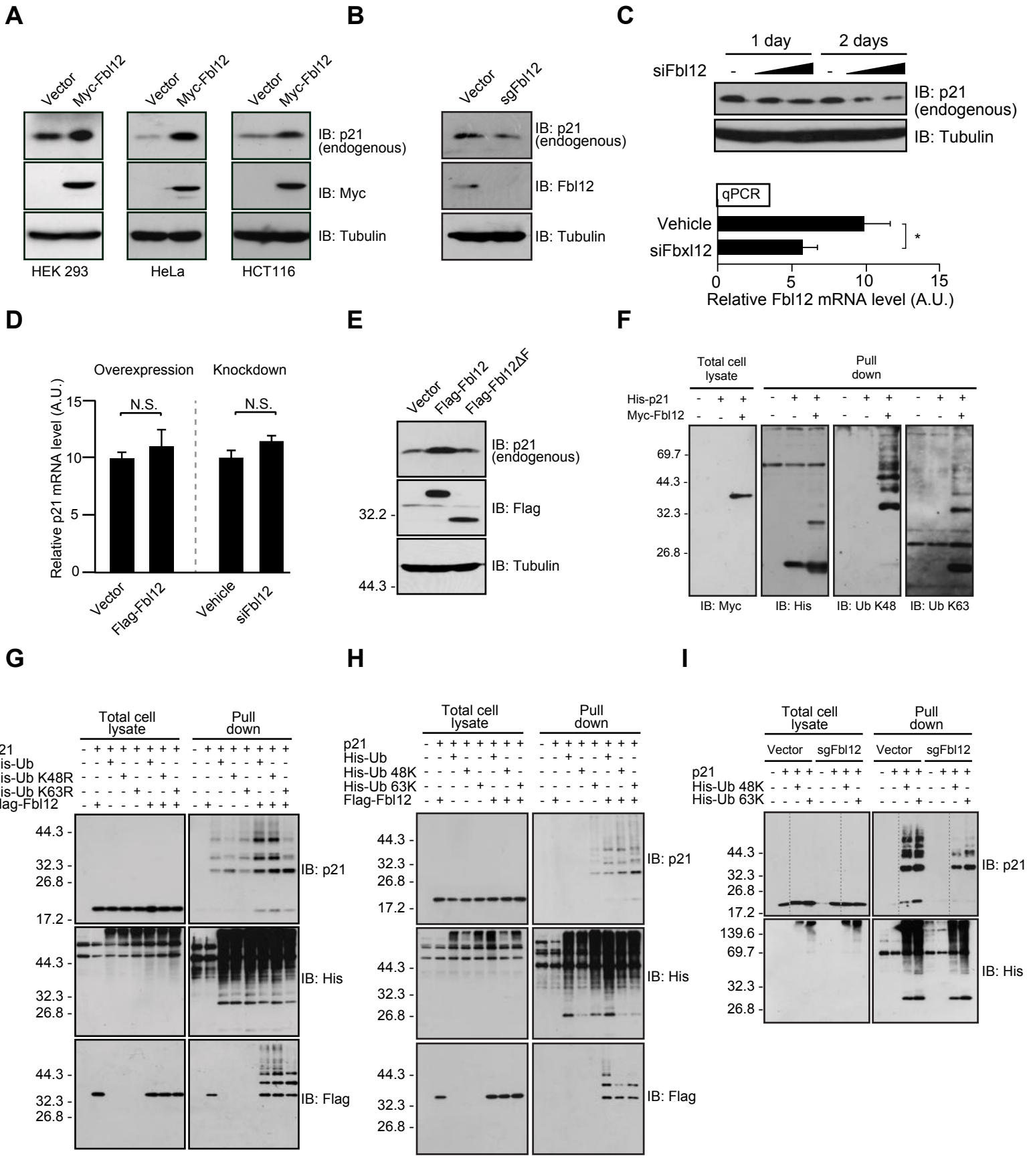

Figure 2 Tsuruta et al.
Fbl12 increases p21 expression levels associated with mixed-type ubiquitination

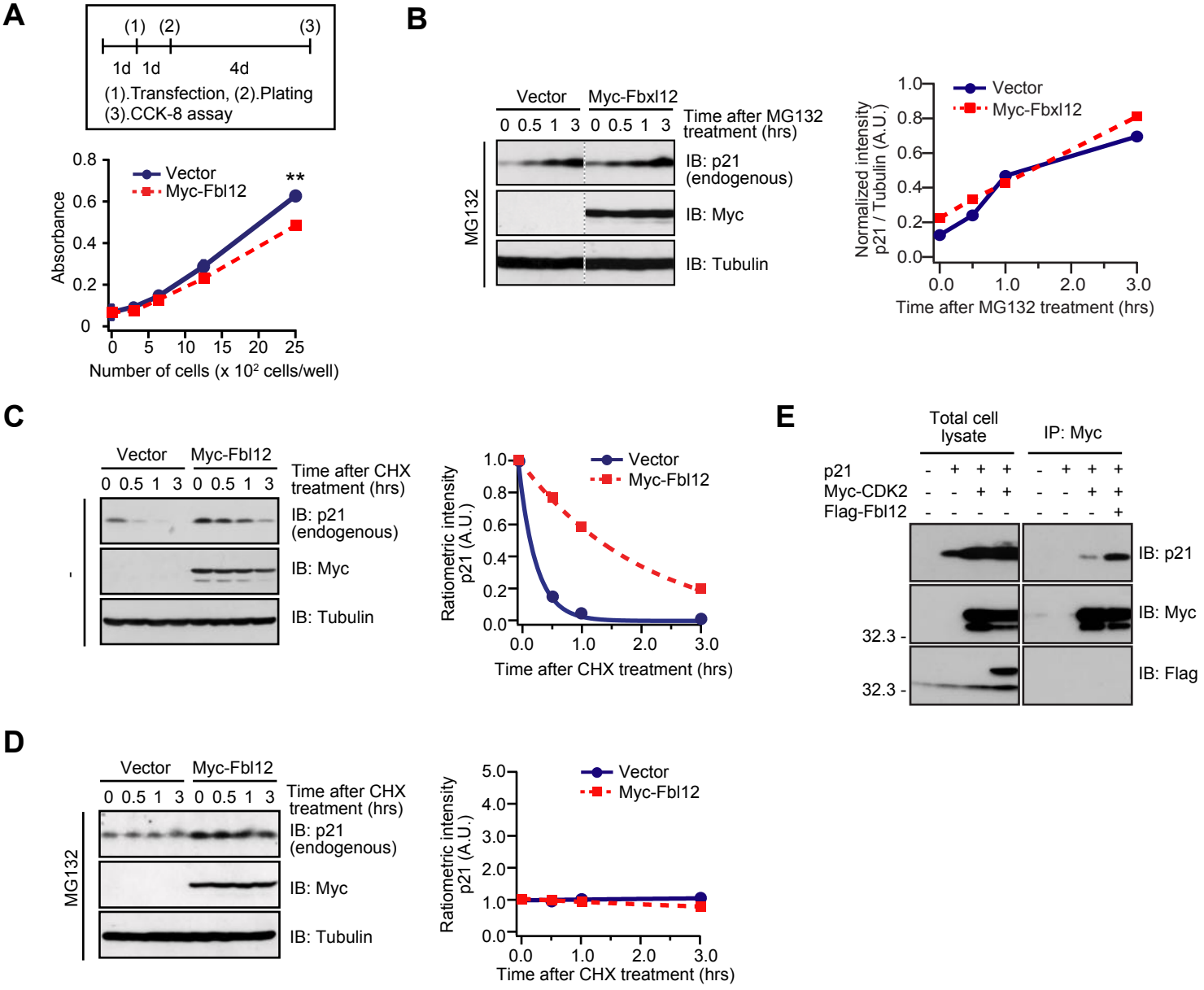

Figure 3 Tsuruta et al.
Fbl12 suppresses default degradation of p21

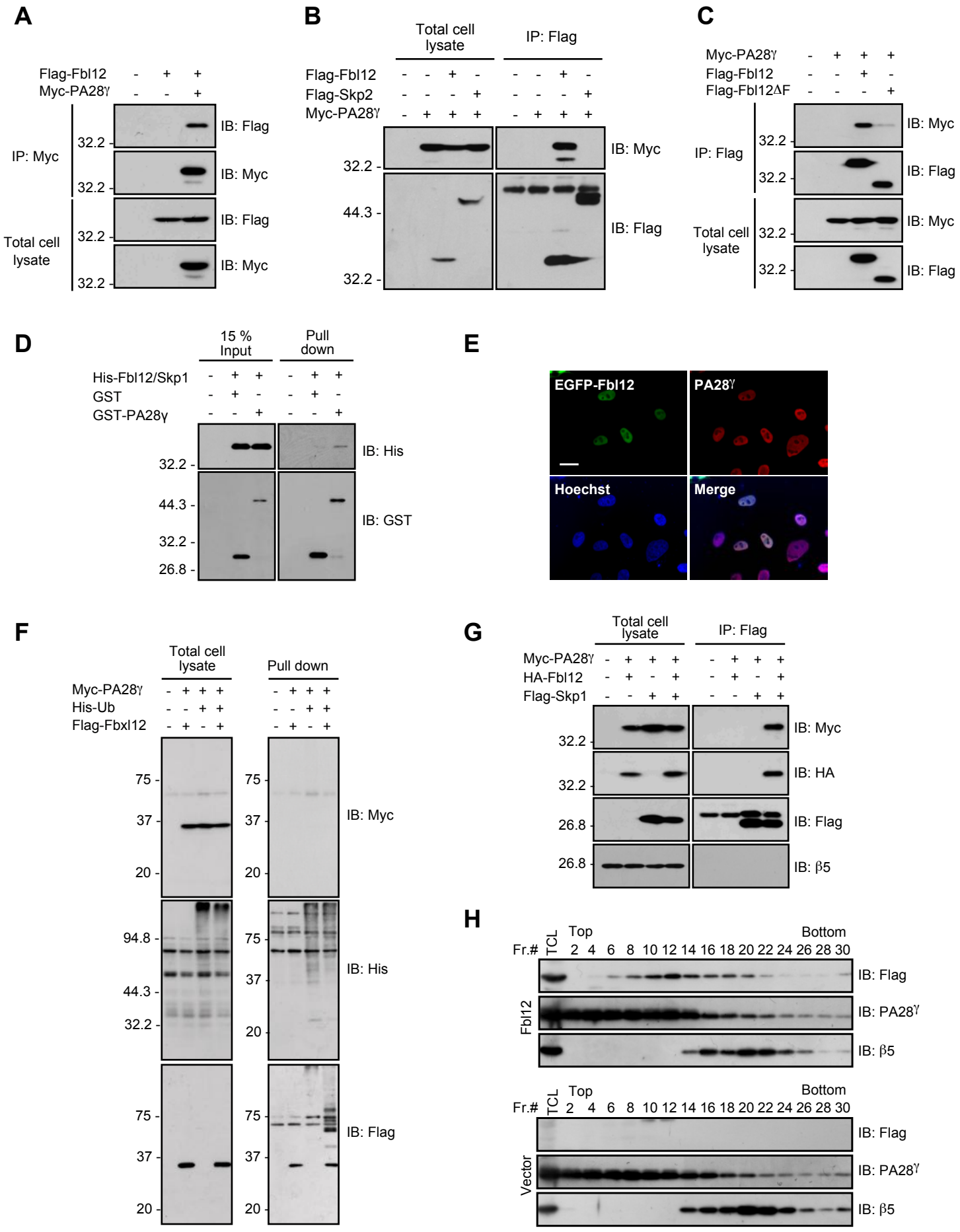

Figure 4 Tsuruta et al.
PA28γ associates with SCF^{Fbl12}

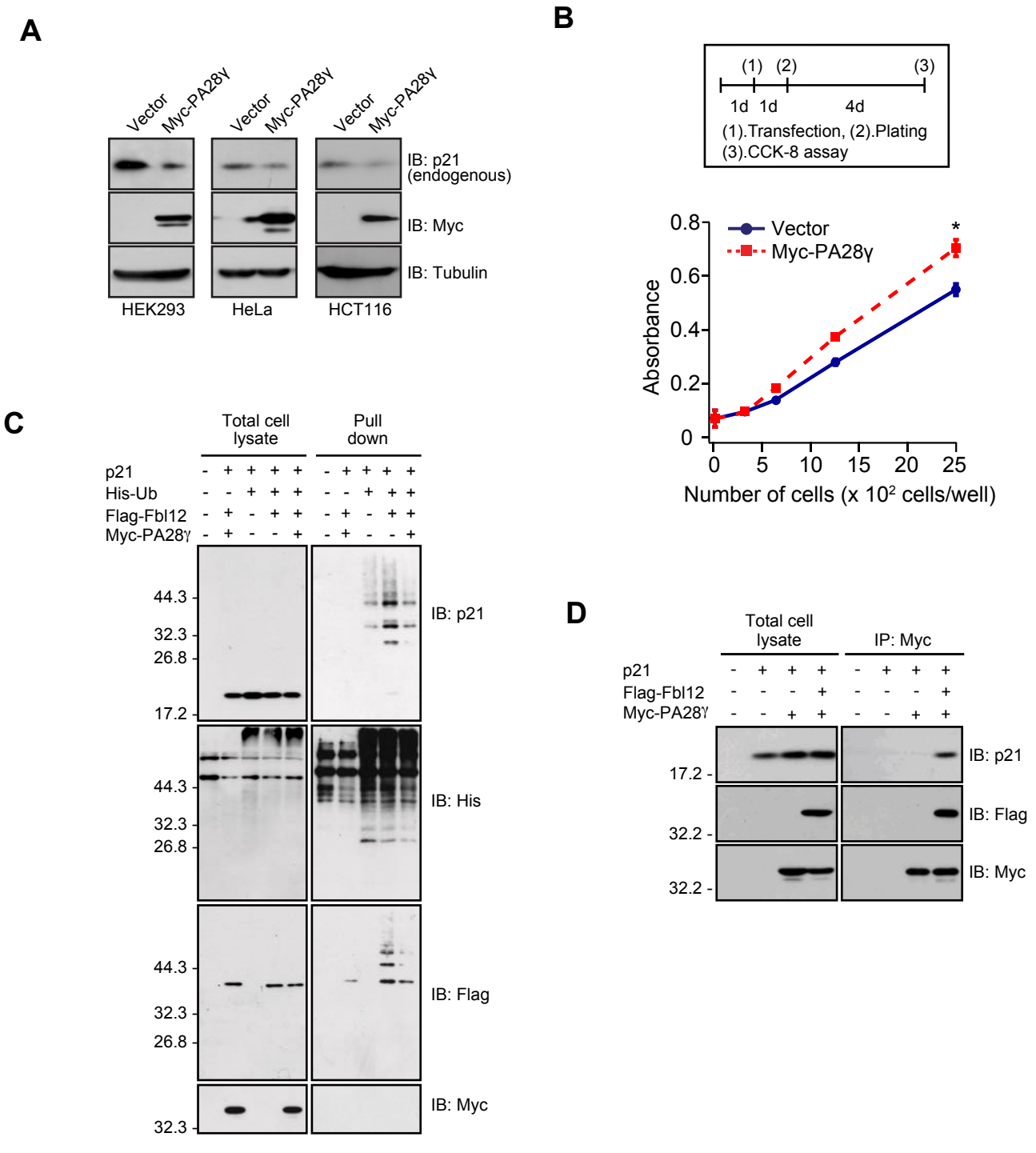

Figure 5 Tsuruta et al.
PA28γ attenulates SCF^{Fbl12}-dependent p21 ubiquitination

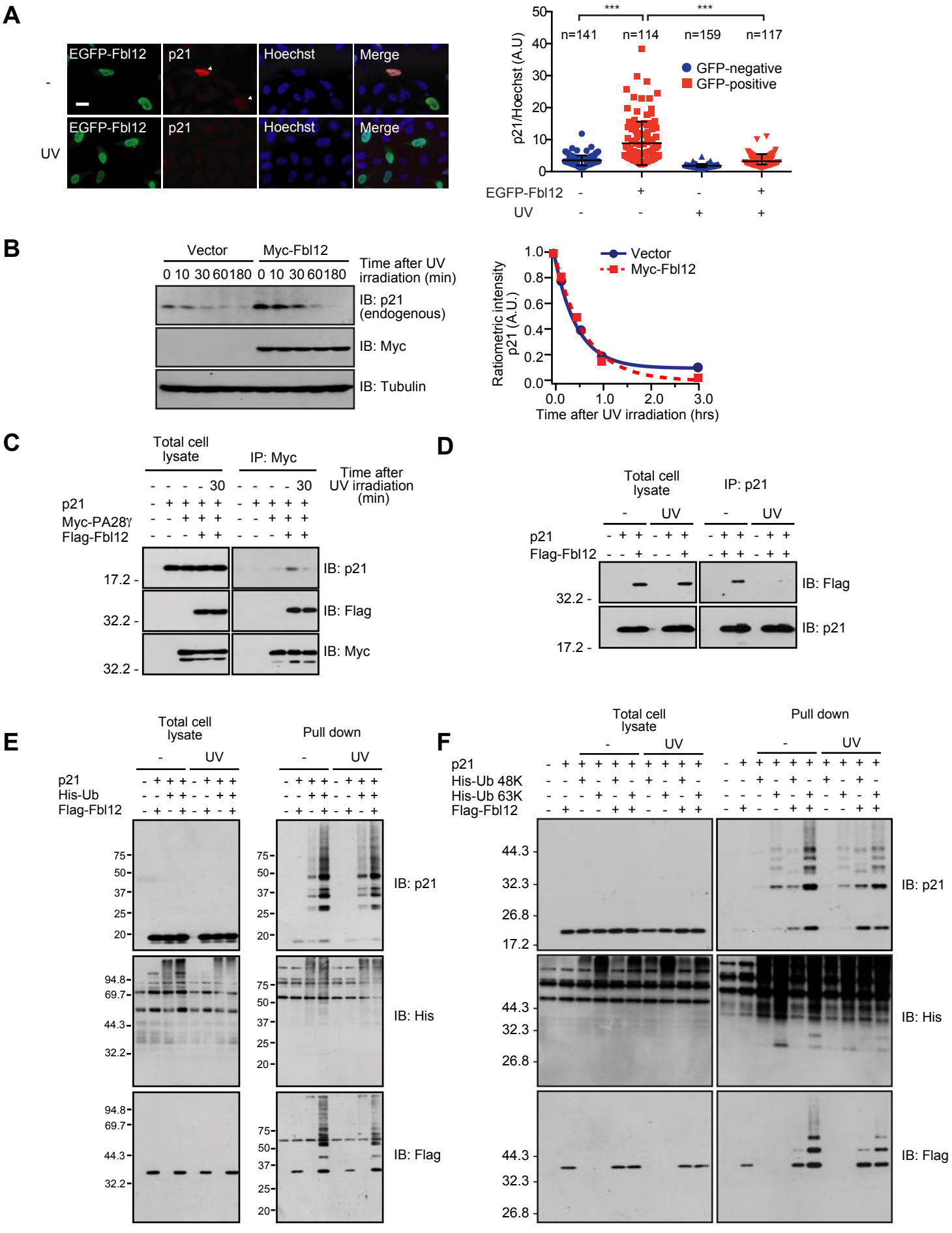

Figure 6 Tsuruta et al.
UV stimulation induces p21 degradation through disassembly of protein complex.

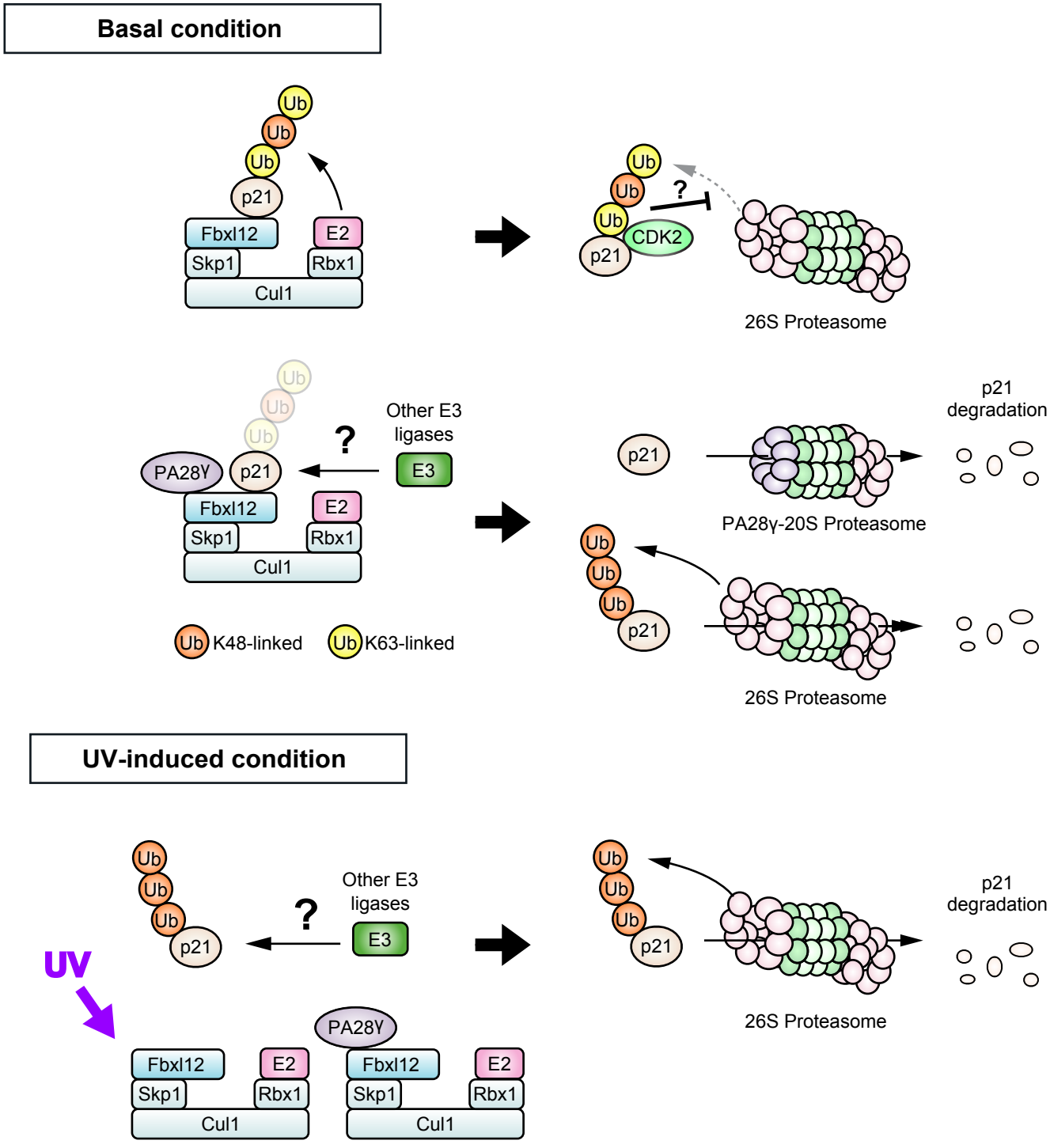

Figure 7 Tsuruta et al.
Model for the regulation of p21 turnover

Table 1.

Gene symbol	Gene description
PSME3 (PA28γ)	proteasome activator subunit 3; isoform 1
SKP1	S-phase kinase-associated protein 1; isoform b
CUL1	cullin 1
TCEB1	elongin C
UBR5	ubiquitin protein ligase E3 component n-recognin 5
AKAP8L	A kinase anchor protein 8-like
PPM1G	protein phosphatase 1G
PPP5C	protein phosphatase 5, catalytic subunit
PRKAR1A	cAMP-dependent protein kinase, regulatory subunit alpha 1
GPRASP2	G protein-coupled receptor associated sorting protein 2
IPO5	importin 5
NAP1L1|NAP1L4	nucleosome assembly protein 1-like; 1 or 4
NAP1L4	nucleosome assembly protein 1-like 4
DDB1	damage-specific DNA binding protein 1
TIMM13	translocase of inner mitochondrial membrane 13
NIP30	hypothetical protein LOC80011
RNF219	ring finger protein 219
SAPS3	SAPS domain family, member 3
LOC152667|NIP30	NIP30-like family
TTC9C	tetratricopeptide repeat domain 9C
FKBP5	FK506 binding protein 5
MAGED1	melanoma antigen family D, 1
CACYBP	calcyclin binding protein; isoform 2
CALM1|CALM2|CALM3	calmodulin; 1 or 2 or 3
CALM1|CALM2|CALM3|CALML3	calmodulin 1 or calmodulin 2 or calmodulin 3 or calmodulin-like 3
CALU	calumenin
RCN2	reticulocalbin 2, EF-hand calcium binding domain

Proteins identified by mass spectrometry of Fbl12 immunoprecipitates