UUCAA-3′ and *Mig-6(05)* siRNA was 5′-GCAGGGUAUCCAUUC UUUA-3′ with a 3′dTdT overhang. The nucleotide sequence of the human *ATR* siRNA was 5′-CCTCCGTGATGTTGCTTGA-3′ and *S6K1* siRNA was 5′-GGACATGGCAGGAGTGTTT-3′ with a 3′dTdT overhang. *ATM* siRNA used in this study was purchased from Ambion.

Viral infection

Retroviruses were generated by transfection of pMXs-Puro-Mig-6 into Platinum-A retroviral packaging cell line using Fugene 6 Reagent (Roche). Viral particles were collected 48 h after transfection. MDA-MB-231 cells were plated on 10-mm dishes at a density of 4×10^5 cells per dish. On the next day, the medium was replaced with 6 ml of viral supernatant containing 8 μg/ml Polybrene. At 48 h after retroviral infection, cells were selected with 1 μg/ml puromycin for 72 h, then pooled for further use.

Proliferation assay

HEK293 cells were seeded in 10-cm dishes with 60 000 cells per dish. After 24 h, cells were transfected with WT or mutant pcDNA3-FLAG-Mig-6 together with the pEGFP vector. Images were obtained of four fields per dish at 24, 36, 48, and 60 h after transfection. Green cells were counted as transfected cells.

MDA-MB-231 cells were seeded in 6-cm dishes, and transfected simultaneously with an siRNA against human *Chk1* or *Mig-6*, or a control siRNA. After 24 h, cells were reseeded into 96-well plates with 3500 cells per well, and proliferation assays were performed at 12, 24, 36, 48, and 60 h after replating, according to the manufacturer's instructions (CyQUANT® Cell Proliferation Assay Kit; Invitrogen).

MS analysis

Proteins were subjected to SDS–PAGE followed by in-gel digestion with trypsin. The obtained peptides were dried and then dissolved in 0.1% TFA, 2% ACN prior to LC-MS/MS analysis. Peptides were analysed using a nanoLC-MS/MS system, composed of an LTQ Orbitrap Velos (Thermo Fisher Scientific) coupled with a nanoLC (Advance, Michrom BioResources) and an HTC-PAL autosampler (CTC Analytics). Peptide separation was carried out using an in-house-pulled fused silica capillary (0.1 mm inside diameter, 15 cm length, 0.05 mm inside diameter at the tip), packed with a 3-μm C18 L-column. The mobile phases consisted of 0.1% formic acid (A) and 100% acetonitrile (B). Peptides were eluted by a gradient of 5–35% B for 40 min at a flow rate of 200 nl/min. CID spectra were acquired automatically in the data-dependent scan mode with the dynamic exclusion option. Full MS was obtained by Orbitrap in the range of m/z 300–2000 with resolution 30 000. The nine most intense precursor ions in the full MS spectra were selected for subsequent MS/MS analysis in an ion trap with the automated gain control mode. The lock mass function was activated to minimize mass error during analysis. The peak lists were generated by MSn.exe (Thermo Fisher Scientific) with a minimum scan/group value of 1, and were compared with the Human International Protein Index version 3.1.6 (57366 protein sequences; European Bioinformatics Institute) with the use of the MASCOT algorithm (ver. 2.1.4). Trypsin was selected to be the enzyme used, the permitted number of missed cleavages was set as two, and carbamidomethylation on cysteine was selected as the fixed modification. Oxidized methionine and phosphorylation on serine, threonine, and tyrosine were searched for as variable modifications. The precursor mass tolerances were 10 ppm and the tolerance of the MS/MS ions was 0.8 Da.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: NL and MK wrote the manuscript. NL and MM performed experiments. NL, MM SayuriS and MK analysed the data. NL, KK, YK, SenjiS, KIN, MN, HN, and MK designed the experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Amatschek S, Koenig U, Auer H, Steinlein P, Pacher M, Gruenfelder A, Dekan G, Vogl S, Kubista E, Heider KH, Stratowa C, Schreiber M, Sommergruber W (2004) Tissue-wide expression profiling using cDNA subtraction and microarrays to identify tumor-specific genes. *Cancer Res* **64**: 844–856

Anastasi S, Baietti MF, Frosi Y, Alemà S, Segatto O (2007) The evolutionarily conserved EBR module of RALT/MIG6 mediates suppression of the EGFR catalytic activity. *Oncogene* **26**: 7833–7846

Anastasi S, Fiorentino L, Fiorini M, Fraioli R, Sala G, Castellani L, Alemà S, Alimandi M, Segatto O (2003) Feedback inhibition by RALT controls signal output by the ErbB network. *Oncogene* **22**: 4221–4234

Anastasi S, Sala G, Huiping C, Caprini E, Russo G, Iacovelli S, Lucini F, Ingvarsson S, Segatto O (2005) Loss of RALT/MIG-6 expression in ERBB2-amplified breast carcinomas enhances ErbB-2 oncogenic potency and favors resistance to Herceptin. *Oncogene* **24**: 4540–4548

Chen MS, Ryan CE, Piwnica-Worms H (2003) Chk1 kinase negatively regulates mitotic function of Cdc25A phosphatase through 14-3-3 binding. *Mol Cell Biol* **23**: 7488–7497

Ferby I, Reschke M, Kudlacek O, Knyazev P, Pantè G, Amann K, Sommergruber W, Kraut N, Ullrich A, Fässler R, Klein R (2006) Mig6 is a negative regulator of EGF receptor-mediated skin morphogenesis and tumor formation. *Nat Med* **12**: 568–573

Fiorentino L, Pertica C, Fiorini M, Talora C, Crescenzi M, Castellani L, Alemà S, Benedetti P, Segatto O (2000) Inhibition of ErbB-2 mitogenic and transforming activity by RALT, a mitogen-induced signal transducer which binds to the ErbB-2 kinase domain. *Mol Cell Biol* **20**: 7735–7750

Fiorini M, Ballaro C, Sala G, Falcone G, Alemà S, Segatto O (2002) Expression of RALT, a feedback inhibitor of ErbB receptors, is subjected to an integrated transcriptional and post-translational control. *Oncogene* **21**: 6530–6539

Frangioni JV, Neel BG (1993) Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. *Anal Biochem* **210**: 179–187

Gonzalez FA, Raden DL, Davis RJ (1991) Identification of substrate recognition determinants for human ERK1 and ERK2 protein kinases. *J Biol Chem* **266**: 22159–22163

Guha U, Chaerkady R, Marimuthu A, Patterson AS, Kashyap MK, Harsha HC, Sato M, Bader JS, Lash AE, Minna JD, Pandey A, Varmus HE (2008) Comparisons of tyrosine phosphorylated proteins in cells expressing lung cancer-specific alleles of EGFR and KRAS. *Proc Natl Acad Sci USA* **105**: 14112–14117

Hackel PO, Gishizky M, Ullrich A (2001) Mig-6 is a negative regulator of the epidermal growth factor receptor signal. *Biol Chem* **382**: 1649–1662

Jean C, Hernandez-Pigeon H, Blanc A, Charveron M, Laurent G (2007) Epidermal growth factor receptor pathway mitigates UVA-induced G2/M arrest in keratinocyte cells. *J Invest Dermatol* **127**: 2418–2424

Kitagawa M, Higashi H, Jung HK, Suzuki-Takahashi I, Ikeda M, Tamai K, Kato J, Segawa K, Yoshida E, Nishimura S, Taya Y (1996) The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. *EMBO J* **15**: 7060–7069

Klein S, Levitzki A (2009) Targeting the EGFR and the PKB pathway in cancer. *Curr Opin Cell Biol* **21:** 185–193

Lam MH, Rosen JM (2004) Chk1 versus Cdc25: checking one's levels of cellular proliferation. *Cell Cycle* **3:** 1355–1357

Leitner A, Lindner W (2009) Chemical tagging strategies for mass spectrometry-based phospho-proteomics. *Methods Mol Biol* **527:** 229–243

Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, Donehower LA, Elledge SJ (2000) Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes Dev* **14:** 1448–1459

Mabuchi R, Sasazuki T, Shirasawa S (2005) Mapping of the critical region of mitogen-inducible gene-6 for NF-κB activation. *Oncol Rep* **13:** 473–476

Makkinje A, Quinn DA, Chen A, Cadilla CL, Force T, Bonventre JV, Kyriakis JM (2000) Gene 33/Mig-6, a transcriptionally inducible adapter protein that binds GTP-Cdc42 and activates SAPK/JNK. A potential marker transcript for chronic pathologic conditions, such as diabetic nephropathy. Possible role in the response to persistent stress. *J Biol Chem* **275:** 17838–17847

Mailand N, Falck J, Lukas C, Syljuåsen RG, Welcker M, Bartek J, Lukas J (2000) Rapid destruction of human Cdc25A in response to DNA damage. *Science* **288:** 1425–1429

Muslin AJ, Tanner JW, Allen PM, Shaw AS (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell* **84:** 889–897

Niida H, Nakanishi M (2006) DNA damage checkpoints in mammals. *Mutagenesis* **21:** 3–9

Niida H, Tsuge S, Katsuno Y, Konishi A, Takeda N, Nakanishi M (2005) Depletion of Chk1 leads to premature activation of Cdc2-cyclin B and mitotic catastrophe. *J Biol Chem* **280:** 39246–39252

O'Neill T, Giarratani L, Chen P, Iyer L, Lee CH, Bobiak M, Kanai F, Zhou BB, Chung JH, Rathbun GA (2002) Determination of substrate motifs for human Chk1 and hCds1/Chk2 by the oriented peptide library approach. *J Biol Chem* **277:** 16102–16115

Pante G, Thompson J, Lamballe F, Iwata T, Ferby I, Barr FA, Davies AM, Maina F, Klein R (2005) Mitogen-inducible gene 6 is an endogenous inhibitor of HGF/Met-induced cell migration and neurite growth. *J Cell Biol* **171:** 337–348

Puc J, Keniry M, Li HS, Pandita TK, Choudhury AD, Memeo L, Mansukhani M, Murty VV, Gaciong Z, Meek SE, Piwnica-Worms H, Hibshoosh H, Parsons R (2005) Lack of PTEN sequesters CHK1 and initiates genetic instability. *Cancer Cell* **7:** 193–204

Reschke M, Ferby I, Stepniak E, Seitzer N, Horst D, Wagner EF, Ullrich A (2010) Mitogen-inducible gene-6 is a negative regulator of epidermal growth factor receptor signaling in hepatocytes and human hepatocellular carcinoma. *Hepatology* **51:** 1383–1390

Ruan DT, Warren RS, Moalem J, Chung KW, Griffin AC, Shen W, Duh QY, Nakakura E, Donner DB, Khanafshar E, Weng J, Clark OH, Kebebew E (2008) Mitogen-inducible gene-6 expression correlates with survival and is an independent predictor of recurrence in BRAF (V600E) positive papillary thyroid cancers. *Surgery* **144:** 908–913 discussion 913-914

Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM, Abraham RT (1999) Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. *Cancer Res* **59:** 4375–4382

Schmitt E, Boutros R, Froment C, Monsarrat B, Ducommun B, Dozier C (2006) CHK1 phosphorylates CDC25B during the cell cycle in the absence of DNA damage. *J Cell Sci* **119:** 4269–4275

Shimada M, Niida H, Zineldeen DH, Tagami H, Tanaka M, Saito H, Nakanishi M (2008) Chk1 is a histone H3 threonine 11 kinase that regulates DNA damage-induced transcriptional repression. *Cell* **132:** 221–232

Tong J, Taylor P, Jovceva E, St-Germain JR, Jin LL, Nikolic A, Gu X, Li ZH, Trudel S, Moran MF (2008) Tandem immunoprecipitation of phosphotyrosine-mass spectrometry (TIPY-MS) indicates C19ORF19 becomes tyrosine-phosphorylated and associated with activated epidermal growth factor receptor. *J Proteome Res* **7:** 1067–1077

Tsunoda T, Inokuchi J, Baba I, Okumura K, Naito S, Sasazuki T, Shirasawa S (2002) A novel mechanism of nuclear factor κB activation through the binding between inhibitor of nuclear factor-κBα and the processed NH_2-terminal region of Mig-6. *Cancer Res* **62:** 5668–5671

Uto K, Inoue D, Shimuta K, Nakajo N, Sagata N (2004) Chk1, but not Chk2, inhibits Cdc25 phosphatases by a novel common mechanism. *EMBO J* **23:** 3386–3396

Woodgett JR, Gould KL, Hunter T (1986) Substrate specificity of protein kinase C. Use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements. *Eur J Biochem* **161:** 177–184

Xiao Z, Xue J, Semizarov D, Sowin TJ, Rosenberg SH, Zhang H (2005) Novel indication for cancer therapy: Chk1 inhibition sensitizes tumor cells to antimitotics. *Int J Cancer* **115:** 528–538

Xu DZ, Makkinje A, Kyriakis JM (2005) Gene 33 is an endogenous inhibitor of epidermal growth factor (EGF) receptor signaling and mediates dexamethasone-induced suppression of EGF function. *J Biol Chem* **280:** 2924–2933

Zhang XW, Pickin KA, Bose R, Jura N, Cole PA, Kuriyan J (2007) Inhibition of the EGF receptor by binding of MIG6 to an activating kinase domain interface. *Nature* **450:** 741–745

Zhang YW, Staal B, Su Y, Swiatek P, Zhao P, Cao B, Resau J, Sigler R, Bronson R, Vande Woude GF (2007) Evidence that MIG-6 is a tumour-suppressor gene. *Oncogene* **26:** 269–276

Zhang YW, Vande Woude GF (2007) Mig-6, signal transduction, stress response and cancer. *Cell Cycle* **6:** 507–513

Zhou BB, Elledge SJ (2000) The DNA damage response: putting checkpoints in perspective. *Nature* **408:** 433–439

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The SCF Ubiquitin Ligases Involved in Hematopoietic Lineage

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Abstract: The ubiquitin-proteasome system is involved in various cellular functions by regulating protein degradation. It has been shown previously that the SCF-type ubiquitin (E3) ligases are involved in cell cycle control. Here we review E3 ligases playing the crucial roles in the determination of cell fate during hematopoiesis. SCF^{Skp2} controls the degradation of CDK inhibitors, such as p21, p27 and p57, to regulate hematopoietic stem cell lineage. SCF^{Fbw7} targets several important proteins involved in hematopoiesis such as c-Myc, Notch and c-Myb. By controlling the precise levels of these proteins, E3 ligases are required for accurately determining hematopoietic lineage.

Keywords: Ubiquitin, proteasome, E3 ligase, SCF complex, hematopoiesis, Fbw7, Skp2, Fbw1, phosphorylation, Hematopoietic stem cells.

OVERVIEW OF THE SCF-TYPE UBIQUITIN LIGASES

The cellular levels of many proteins are carefully regulated by the ubiquitin-proteasome system (UPS), one of the degradation mechanisms in the cell. A ubiquitin ligase (E3) polyubiquitylates its specific substrates by collaborating with a ubiquitin-activating enzyme (E1) and a ubiquitin-conjugation enzyme (E2). The polyubiquitin-modified substrates are then degraded by the 26S-proteasome complex [1, 2]. The specificity of the ubiquitylation process is achieved by over 1000 different E3 ubiquitin ligases. E3 ligases are classified in four types of single ring-finger, complexed ring-finger, HECT and U-box. One of major complexed ring-finger E3 ligases is the SCF (Skp1-Cul1-F-box protein)-type E3. The SCF complex consists of four subunits: the RING-finger protein Rbx1/Roc1, the scaffold protein Cul1, the adaptor protein Skp1 and an F-box protein that has a Skp1-binding domain and a substrate-recognition domain [3, 4] Fig. (**1**). Sixty-nine F-box proteins are found in the human genome, whereas Rbx1/Roc1, Cul1, and Skp1 are all unique and common elements in the SCF complex [5-7]. Because it is speculated that one F-box protein targets several proteins for degradation, SCF-type E3s control the degradation of hundreds of cellular proteins. The recognition of the F-box protein with its substrate is often dependent on post-translational modification of the substrate, such as phosphorylation [4, 8] Fig. (**1**). Once a substrate is modified, it is recognized by a specific F-box protein, ubiquitylated and subsequently degraded by the proteasome.

PROTEINS INVOLVED IN THE HEMATOPOIETIC LINEAGE

Hematopoietic stem cells (HSCs) reside in the bone marrow (BM) and give rise to both lymphoid (B-cells, T-cells and natural killer cells) and myeloid (red blood cells, neutrophils, basophils, eosinophils, monocytes, macrophages, dendritic cells and platelets) lineages [9] Fig. (**2**). During homeostasis, HSCs remain dormant and harbor multilineage self-renewal activity. HSCs exit quiescence and enter into cell cycle for self-renewal and production of new blood cells in response to hematopoietic stresses such as BM injury, G-CSF stimulation or bleeding [9, 10]. After the re-establishment of homeostasis, activated HSCs return to dormancy [10]. Several signaling pathways and molecules have been found to control the fate of HSCs, including Notch [11, 12], Wnt [13], c-Myc [14, 15] and cell cycle regulators.

Some of these regulatory molecules, such as c-Myc, c-Myb, Notch, and cell cycle regulators, are targeted for degradation by SCF-type ubiquitin ligases to regulate the hematopoietic lineage. The targets and function of some of these SCF complexes, including Skp2, Fbw1, and Fbw7, are illustrated in Fig. (**2**) and described below.

TARGETS AND FUNCTION OF SCF^{SKP2} IN HEMATOPOIESIS

HSCs can replicate indefinitely, whereas the number of divisions of progenitor cells is limited according to their differentiation. This is regulated by cell cycle modulators such as CDK/cyclin dimers whose activity is repressed by CDK inhibitors (CKIs), particularly $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ (hereafter, p21, p27 and p57, respectively).

Disruption of the *p21* gene in mice induced stem cell expansion under stress conditions that normally require cell-cycle arrest to prevent premature stem cell depletion and hematopoietic death [16]. However, p21 does not contribute to the proportion of the cells in G_0 phase during steady-state hematopoiesis [17]. Therefore, p21 may be more important in the maintenance of HSCs under stress conditions.

Among the CKIs, p57 specifically shows higher expression in HSCs compared to non-HSCs [18]. Recently, two groups have shown that p57 is a crucial brake for cycling

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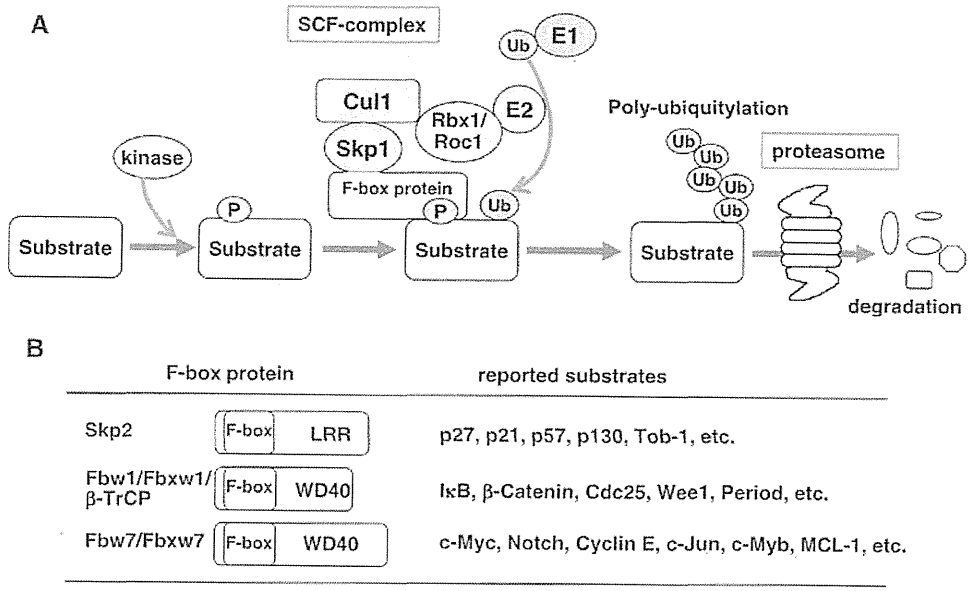

Fig. (1). SCF-type E3 ubiquitin ligases SCF^{Skp2}, SCF^{Fbw1} and SCF^{Fbw7} A. SCF-type E3 ligases consist of four subunits: Rbx1/Roc1, Cul1, Skp1 and an F-box protein. The target proteins of SCF^{Skp2}, SCF^{Fbw1} and SCF^{Fbw7} as indicated in **B** below are often phosphorylated prior to its recognition by the specific F-box protein. The SCF complex ubiquitylates the substrate in collaboration with E1 and E2 enzymes, followed by ubiquitin-dependent degradation in the 26S proteasome.

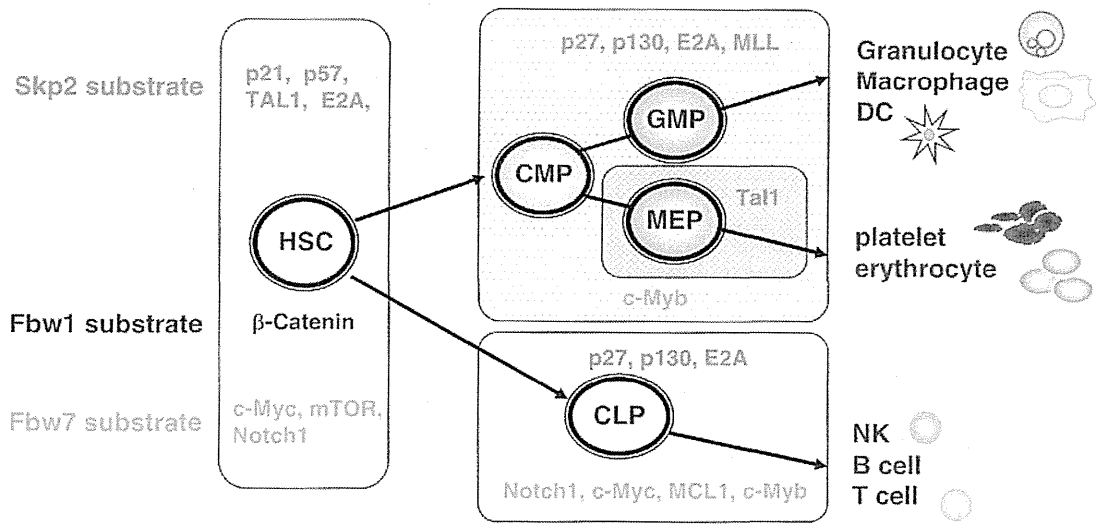

Fig. (2). Degradation targets of SCF type E3s in hematopoietic lineage. Skp2 has roles in the maintenance of HSC and progenitors. It may be also involved in the onset of leukemia. Fbw1 promotes the self-renewing of HSCs. Fbw7 regulates proliferation of HSCs and progenitors as a tumor suppressor, and the overexpression of its targets is frequently detected in T-ALL. The substrates of Skp2, Fbw1 and Fbw7 are shown by red, black and blue characters, respectively. CMP indicates common myeloid progenitor; GMP, granulocyte-macrophage progenitor; MEP, megakaryocyte-erythrocyte progenitor; CLP, common lymphoid progenitor.

HSCs and links self-renewal activity to cell cycle quiescence. Matsumoto *et al.* noticed the defect in the self-renewal capacity of p57-deficient HSCs and a reduction of the proportion of the cells in G_0 phase, then they described that p57 was required for quiescence and maintenance of adult HSCs [19]. Furthermore, an additional ablation of *p21* in a *p57*-null background resulted in a further decrease in the *in vitro* colony-forming activity of HSCs [20]. Although Zou *et al.* demonstrated that p57 and p27 cooperated to maintain HSCs quiescence, the importance of p27 alone in HSCs is unclear [21]. Depletion of p27 does not affect stem cell number, cell cycling, or self-renewal, despite an increase in progenitor proliferation and pool size [20]. However Matsumoto *et al.* showed that the HSCs abnormalities of p57-deficient mice were rescued by knocking in the *p27* gene at the *p57* locus [19].

In splenocytes, the absence of p27 can be compensated by an RB family member, p130RB2. In the spleens of *p27-/-*;

p130RB2-/- mice, proliferation of B- and T-cells, erythroid progenitors, monocytes and macrophages were all significantly augmented compared with their *p27-/-* counterparts [22]. The peculiar oscillation of each Skp2 substrate in HSCs may be responsible for the role of Skp2 in the maintenance of HSCs status. However, given the shared functions of the CKIs regulated by Skp2, the loss of one CKI may be compensated by the others in the maintenance and development of HSCs and progenitors.

The protein levels of p21, p27, p57 and p130RB2 are controlled posttranslationally by Skp2-dependent ubiquitylation and proteasome degradation. Skp2 expression is induced in HSCs and progenitors in response to hematopoietic stress. In *Skp2*-desrupted mice, the inability to rapidly and efficiently enter the cell cycle after 5-FU treatment and transplantation, leads to enhanced HSCs quiescence and impaired regeneration during hematopoiesis [23]. Therefore it is thought that the reduction in cell cycle in bone marrow cells in the absence of Skp2 is caused by an accumulation of CKIs [23]. The opposite observation has been also reported in that Skp2-deficiency leads to reduced quiescence and increased HSCs cycling and neither p21 nor p27 were stabilized in HSCs [24] Fig. (**2**). This discrepancy might be caused by differences in the genetic background of the knockout mice and/or experimental strategy. It is necessary to elucidate how Skp2 distinguishes between similar proteins to maintain the appropriate cell fate in different environments and stages.

Cyclin D1 is the catalytic subunit of CDK4/6 and thereby induces cell cycle progression in quiescent HSCs *via* phosphorylation of retinoblastoma protein (pRB). Deletion of Skp2 in HSCs induces expression of Cyclin D1 [24]. Moreover, the transport of Cyclin D1 into the nucleus by heat shock cognate protein 70 (HSC70), prevents the binding between Cyclin D1 and cytosolic p27 or p57 [21]. These results suggest that Skp2 may play multiple roles in Cyclin D1 regulation including transcriptional repression and the functional activation through the depletion of p27 and p57.

T-cell acute lymphocytic leukemia (T-ALL) protein 1 (Tal1) gene gets the gain-of-functional mutation frequently in pediatric T-ALL. *Tal1*-disrupted mice are embryonic lethal caused by a failure in blood production [25]. Tal1 expression is transient and confined to HSCs and megakaryocytic/erythrocytic progenitor cells (MEP). Tal1 expression is rapidly extinguished once cells begin to differentiate and lose their proliferative potential, with the exception of erythropoiesis and megakaryopoiesis [26]. This is consistent with its fundamental role in the regulation of erythroid cell- and megakaryocyte-specific gene expression programs. Tal1 contains a basic helix-loop-helix (bHLH) domain and forms heterodimers with other bHLH proteins including E2A. In hematopoietic cells, these heterodimers are a part of a transcriptional complex regulating target genes associated with hematopoietic development and leukemia [26, 27]. Ubiquitylation and degradation of both E2A and Tal1 are Skp2-dependent [28, 29]. In lymphoid cell lines, the interaction between E2A and Skp2 is governed by MAP kinase activity, which is induced by Notch signaling [29]. Expression of Tal1 is not detected in normal lymphoid cell lines, suggesting that its excess in T-cells might contribute to leukemogenesis. It is possible that Tal1 in T-cells might be regulated by a signaling system same as that of E2A.

The mixed lineage leukemia (MLL) gene encodes a DNA-binding protein that methylates lysine 4 on histone H3 and positively regulates gene expression including multiple Hox genes [30]. MLL works as a major regulator of hematopoiesis [31], and hence its chromosomal aberration or gene amplification is a cause of acute myeloid and lymphoid leukemias [32]. In mice overexpressing the fusion MLL-ENL (11-19 leukemia protein), which is frequently found in clinical samples, leads to myelomonocytic leukemias derived from HSCs or progenitor cells [33]. Possibly through the activation of leukemogenic gene-expression, MLL fusion proteins efficiently transform hematopoietic cells into leukemia stem cells [30]. MLL is regulated by two different UPS complexes, SCF^{Skp2} and APC/C^{Cdc20}, throughout the cell cycle [31]. However, these UPS activities are ineffective against MLL-ENL fusion proteins [31]. Skp2-mediated degradation of MLL is inhibited by ATR mediated phosphorylation in response to genotoxic stress in S phase [34]. Although it has not been clarified whether this system functions in hematopoietic lineages, the ATR-induced increase of MLL levels may contribute to cancer onset.

TARGETS AND FUNCTION OF SCF^{FBW1} IN HEMATOPOIESIS

β-catenin acts as a coactivator for transcription factors such as TCF and LEF, both of which function in lymphoid cells. Cytoplasmic β-catenin forms a complex with APC, axin and GSK-3β [35]. β-catenin is phosphorylated by GSK-3β in the complex and is subsequently recognized and degraded by the Fbw1 (Fbxw1/ β-TRCP/ FWD1)-mediated UPS [36, 37]. Fbw1 recognizes a consensus motif D-pS-G-X-X-pS within the substrate in a phosphorylation-dependent manner [37]. The canonical Wnt signal pathway inhibits GSK3 activity, thereby stabilizing β-catenin and promoting its translocation into the nucleus. Fine tuning Wnt stimulation is essential for hematopoiesis. An increase in Wnt signaling, which stabilizes β-catenin, maintains the self-renewal capacity of HSCs by upregulating HoxB4 and Notch1 [38-41]. The effects of a reduction of β-catenin, however, are controversial. Zhao *et al.* reported that loss of β-catenin impaired the progression of CML, however, Jeannet *et al.* and Koch *et al.* observed that the absence of β- and γ-catenin did not perturb hematopoiesis or lymphopoiesis [42-44]. In adult mammals, hematopoiesis primarily takes place within bone marrow. β-catenin-deficient bone marrow is able to maintain HSCs but exhibits a decreased capacity to support primitive progenitors and decreased in the numbers of osteoblasts which have a functional role in regulating hematopoiesis through production of soluble molecules, such as stem cell factor [45]. These findings show that the quantitative regulation of β-catenin levels is necessary for normal hematopoietic lineage Fig. (**2**). This raises the possibility that not only Wnt signaling levels but also Fbw1 activity participates in regulating the hematopoietic lineage.

NFκB is an antiapoptotic and oncogenic transcription factor whose constitutively increased activation is detected in hematologic malignancies [46]. Normal HSCs lack constitutively active NFκB, despite their requirement of cytokine-

and growth factor-induced NFκB activation for survival and differentiation [47]. The activity of NFκB is inhibited by its dimerization with IκB, whose level is regulated by Fbw1-mediated UPS [48]. NFκB is also negatively regulated by PDLIM2 (also known as SLIM) in splenic $CD11c^{+}$ cells, which acts as a nuclear ubiquitin E3 ligase for the p65 subunit of NFκB [49]. The repression of constitutively active NFκB is expected to be a molecular targeting strategy effective in treating multiple myeloma (MM). Bortezomib, which is a reversible inhibitor of the 26S proteasome, induces apoptosis through the inhibition of IκB degradation and the prevention of nuclear translocation of NFκB, in MM cells [50]. Because 26S proteasome is a basis component of UPS-mediated degradation system, other substrates, which are also stabilized by Bortezomib, are likely to play additional roles in MM.

Fbw1 has an important role in the immune response. Human immunodeficiency virus type-1 (HIV-1) infection induces the expression of T-cell surface receptor CD4. This receptor, which prevents efficient viral replication and spread of HIV-1, is degraded by Fbw1-mediated UPS [51]. Moreover, Tetherin (BST-2/CD317), a host restriction factor that strongly inhibits the release of virions from the host cell surface, is another substrate of Fbw1 [52]. Although CD4 and Tetherin are unlikely natural substrates for Fbw1, HIV-1 viral membrane-associated protein U (Vpu) functions as an adaptor to link Fbw1 to both targets [51-53]. These interactions depend on two phosphorylation sites, which are located within a conserved Fbw1 recognition motif in Vpu [51]. It is likely that the degradation of CD4 and Tetherin by Fbw1 accelerates viral replication. Therefore, inhibition of Vpu phosphorylation might be an effective molecular targeted therapy for abrogating the infectious spread of HIV-1.

TARGETS AND FUNCTION OF SCF^{FBW7} IN HEMATOPOIESIS

The expression level of c-Myc in HSCs is dependent on the differentiation state of HSCs. The reduction of c-Myc levels is regulated by posttranscriptional mechanisms, not transcriptional control [54]. c-Myc, which is a target for Fbw7-mediated UPS, was upregulated in HSCs of *Fbw7*-deficient mice and was correlated with a corresponding loss of quiescent HSCs in these animals, but other cell cycle targets for Fbw7, such as Cyclin E and c-Jun, were not affected by the loss of Fbw7 [55, 56]. This suggests that the accumulation of c-Myc in HSCs induced their entry into the cell cycle Fig. (**2**). Reavie *et al.* demonstrated that decreasing c-Myc levels by generating $Fbw7^{F/F}/Myc^{F/+}$ mice rescued the *Fbw7* deficiency-induced HSCs phenotype [54]. By fine tuning the expression levels of c-Myc by means of Fbw7-mediated degradation, the degree of cell cycle entry of HSCs can be carefully regulated.

Mammalian target of rapamycin (mTOR) is another target for ubiquitylation and subsequent degradation by Fbw7 [57]. Therefore, the loss of *Fbw7* also increases mTOR signaling. It has been reported that aging increases mTOR activity in HSCs, while its inhibition by rapamycin can restore the self-renewal and hematopoiesis of HSCs [58]. The loss of a number of negative regulators of mTOR, such as Fbw7, PTEN, PML and TSC1, leads to the hyperproliferation and subsequent exhaustion of HSCs [59]. This suggests that mTOR may participate in Fbw7-dependent functions in HSCs Fig. (**2**).

The Notch family plays a crucial role in the determination of cell fate in development [60]. Notch is a transmembrane receptor consisting of a large extracellular unit which can associate with ligands in a calcium-dependent manner. The binding of these ligands, such as Jagged1, Jagged2, and Delta1, leads to the proteolytic cleavage of Notch1. The cleaved intracellular tail of Notch1 is then transported into the nucleus, where it acts as a transcription factor. This activation of Notch1 induces changes in self-renewal, proliferation and differentiation of HSCs and progenitors [61].

The contribution of Notch1 in the hematopoietic abnormalities in *Fbw7*-deficient mice is still unclear. The expression of some key hematopoietic regulating factors, such as GATA-2 and HES, are regulated by the Notch signal pathway [62, 63]. Furthermore, genetic mutations in *NOTCH1* are frequently detected in T-ALL patients. Most of these mutations lead to a truncation of the C-terminal domain, which is the recognition site for Fbw7. These mutations would then result in a loss of UPS-mediated degradation and an increase in Notch1 activity [64, 65]. Furthermore, inactivation of Fbw7 by genetic mutation, which is also highly detected in human T-ALL, interferes the normal Notch1 regulation [64]. This gain-of-function of Notch1 plays an important role in initiating cell transformation and the onset of T-ALL, because Notch1 is essential during normal T-lymphocyte development [65, 66].

The loss of Fbw7 will have not only affects Notch1 signaling in T-cells, but on other Fbw7-dependent targets as well Fig. (**2**). Mice with conditional inactivation of Fbw7 in the T-cell lineage develop thymic hyperplasias and thymic lymphomas. The protein levels of not only Notch1 but also c-Myc are increased in CD4+/CD8+ double positive (DP) thymocytes of these mice [67]. An increase in c-Myc would then contribute to cell cycle entry of these cells. FACS analysis of BM and thymocytes from Fbw7-deficient leukemic mice depicts an accumulation of DP and a loss of CD4-/CD8- double negative (DN) and single positive (CD4+ or CD8+) populations. This is consistent with the significant levels of Notch1 and c-Myc that are found in thymocytes [55]. Additionally, more than 50 % of Fbw7-deficient mice also developed T-ALL [55]. Further analysis of double conditional knockout (cKO) of *Fbw7* and *c-Myc* or *RBP-J*, an essential mediator of Notch signaling, have helped to clarify the roles each plays in the immature T-cells. The accumulation of Notch1 depends on the stage of the T-cell but c-Myc levels are stage-independent [67]. The aberrant accumulation of c-Myc and Notch1 by depletion of Fbw7 both might disturb the early stage of T-cell development leading to the onset of T-ALL.

In the T-cell lineages, the hematopoietic transcription factor c-Myb is involved in the maturation process at several key steps [68, 69]. c-Myb is short-lived protein whose UPS-mediated degradation is catalyzed by Fbw7 [70, 71]. Genetic mutations of c-Myb which lead to its overexpression have been found in T-ALL [72]. In addition to mutations in *c-Myb*, the accumulation of c-Myb protein can also be caused by defects in Fbw7-mediated degradation, which might also

Table I. The Function of Substrates of SCF-Type E3 Ligase in Blood Cells

Cell Status	E3 Ligase	Substrate	Function	Ref.
Stem cell	Skp2	p21	cell cycle arrest under the stress conditions	[20]
		p57	brake for cycling HSCs	[19]
		p21 & p57	maintainance of quiescence	[21]
		Tal1	maintain of HSCs pool	[26]
		E2A	maintain of HSCs pool	[26]
	Fbw7	c-Myc	brake for cycling HSCs, regulation of polarity	[54, 55]
		mTOR	maintainance of self-renewal and hematopoiesis	[58]
		Notch1	maintainance of self-renewal and balance between HSCs and progenitors	[61]
	Fbw1	β–Catenin	maintainance of self-renewal and multilineage differentialtion	[39-41]
Progenitor	Skp2	p27	repression of proliferation	[20]
		p130 & p27	cooperate to regulate hematopoitic cells proliferation, repression of proliferation of splenic progenitor	[22]
		Tal1	regulation of erythroid cells and megakaryocytes specific expression programs	[26]
		E2A	promoting the differentiation of lymphoid and erythroid progenitor cells	[26]
		MLL	regulation of hematopoiesis	[76]
	Fbw7	Notch1	T-cell maturation	[65], [66]
		c-Myc	development and cell proliferation of T-cell lineage	[67]
		c-Myb	promoting the differentiation of erythroid and lymphoid cells	[68], [69]
		MCL1	maintainance of proliferation of T-cell progenitors	[77]

contribute to the onset of T-ALL. c-Myb is expressed in immature progenitors of all hematopoietic lineages and is associated with the regulation of proliferation, differentiation and survival [73]. Constitutive expression of c-Myb prevents the terminal differentiation of erythrocytes and megakaryocytes and abolishes B-lymphocyte development [74]. c-Myb-depleted mice show aberrant hematopoietic lineages, favoring differentiation toward macrophages and megakaryocytes [68, 75]. Interestingly, Fbw7-deficient mice have aberrant levels of peripheral blood cells and low levels of platelets [55]. Because the loss of Fbw7 has the opposite effect of c-Myb depletion on megakaryocytes, the Fbw7 target influencing platelet numbers seen in *Fbw7* cKO mice is likely to be c-Myb.

MCL1, a pro-survival BCL2 family member, is frequently overexpressed in leukemia, although the mechanisms are not fully understood [76]. MCL1 transgenic female mice expand the DN1 subset containing the most primitive cells, including the rare early T-cell progenitors [77]. MCL1 has a rapid turn-over rate, and is quickly degraded by apoptotic inducing signals. Three types of E3 ligases have been identified for MCL1, Mule (a HECT-type E3 ligase), Fbw1 and Fbw7 [78-80]. It has been shown that a loss of Fbw7 that leads to an accumulation of MCL1 induces leukemia [80]. DNA damage activates GSK3β and results in phosphorylation of MCL1, and both Fbw1 and Fbw7 regulate GSK3β mediated phosphorylation-dependent degradation of MCL1 [79, 80]. Both Mule and Fbw1 contribute to the UV- or staurosporine-induced MCL1 degradation [78, 79].

GSK3 often works as a coregulator for the turnover of Fbw7 substrates. GSK3 phosphorylates threonine or serine residues of the CPD (Cdc4 phosphodegron) in each substrate, which then recruits Fbw7 to the substrates. Three arginine residues in the WD40 repeats region of Fbw7 comprise the binding pocket for the recognition of the phosphorylated substrates, such as c-Myc, Notch1, c-Myb and MCL1. These are all involved in hematopoietic lineage and are lost after a specific period during development Fig. (**2**). Therefore, mutation of the CPD in the substrate or of the arginines in the WD40 domain of Fbw7 results in loss of Fbw7-mediated target degradation [4, 56]. Interestingly, while the CPD sequences of the substrates are highly conserved across species, the CPD in mouse c-Myb is not conserved in human c-Myb, which is degraded independently of GSK3 [81]. This suggests that not only GSK3 activity but also other regulatory signals participate in regulating c-Myb

in humans. Further studies are required to clarify the roles of different degradation pathways regulating hematopoietic lineages.

PERSPECTIVES

As illustrated in this review, Fbw7, Skp2 and Fbw1 regulate the cellular levels of hematopoietic transcription factors and play crucial roles for the development of hematopoietic lineages. Therefore, mutations that affect the activity of these SCF-type E3 ligases also affect hematopoiesis and makes the cells more susceptible to malignancies. There are many reports of Skp2-overexpression in malignant cells; therefore, it is possible that a Skp2 inhibitor may be a novel anticancer drug for hematopoietic cancers. By contrast, Fbw7 targets oncogenic proteins for degradation and is considered a tumor suppressor protein. Considering the frequent deletion or mutations of the *Fbw7* gene in T-ALL, the loss of Fbw7 activity might contribute to the onset of this cancer. Therefore, Fbw7 may be useful for a novel diagnostic marker for T-ALL.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

UPS	=	Ubiquitin-Proteasome System
SCF	=	Skp1-Cul1-F-box Protein
HSCs	=	Hematopoietic Stem Cells
BM	=	Bone Marrow
CDK	=	Cyclin-Dependent Kinase
CKI	=	CDK Inhibitor
T-ALL	=	T-Cell Acute Lymphocytic Leukemia
HIV-1	=	Human Immunodeficiency Virus Type-1
mTOR	=	Mammalian Target of Rapamycin
DP	=	CD4+/CD8+ Double Positive
DN	=	CD4-/CD8- Double Negative
cKO	=	Conditional Knockout
GSK	=	Glycogen Synthase Kinase
CPD	=	Cdc4 Phosphodegron

REFERENCES

[1] Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem 1998; 67: 425-79.

[2] Nakayama KI, Nakayama K. Ubiquitin ligases: cell-cycle control and cancer. Nat Rev Cancer 2006; 6: 369-81.

[3] Frescas D, Pagano M. Deregulated proteolysis by the F-box proteins SKP2 and β-TrCP: tipping the scales of cancer Nat Rev Cancer 2008; 8: 438-49.

[4] Welcker M, Clurman BE. FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. Nat Rev Cancer 2008; 8: 83-93.

[5] Jin J, Cardozo T, Lovering RC, Elledge SJ, Pagano M, Harper JW. Systematic analysis and nomenclature of mammalian F-box proteins. Gene Dev 2004; 18: 2573-80.

[6] Skaar JR, Pagano M. Control of cell growth by the SCF and APC/C ubiquitin ligases. Curr Opin Cell Biol 2009; 21: 816-24.

[7] Cardozo T, Pagano M. The SCF ubiquitin ligase: insights into a molecular machine. Nat Rev Mol Cell Biol 2004; 5: 739-51.

[8] Kitagawa K, Kotake Y, Kitagawa M. Ubiquitin-mediated control of oncogene and tumor suppressor gene products. Cancer Sci 2009; 100: 1374-81.

[9] Schroeder T. Hematopoietic stem cell heterogeneity: subtypes, not unpredictable behavior. Cell Stem Cell 2010; 6: 203-7.

[10] Wilson A, Laurenti E, Oser G, *et al.* Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell 2008; 135: 1118-29.

[11] Clements WK, Kim AD, Ong KG, Moore JC, Lawson ND, Traver D. A somitic Wnt16/Notch pathway specifies haematopoietic stem cells. Nature 2011; 474: 220-4.

[12] Loeffler D, Kokkaliaris KD, Schroeder T. Wnt to notch relay signaling induces definitive hematopoiesis. Cell Stem Cell 2011; 9: 2-4.

[13] Duncan AW, Rattis FM, DiMascio LN, *et al.* Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat Immunol 2005; 6: 314-22.

[14] Hoffman B, Amanullah A, Shafarenko M, Liebermann DA. The proto-oncogene c-myc in hematopoietic development and leukemogenesis. Oncogene 2002; 21: 3414-21.

[15] Laurenti E, Varnum-Finney B, Wilson A, *et al.* Hematopoietic stem cell function and survival depend on c-Myc and N-Myc activity. Cell Stem Cell 2008; 3: 611-24.

[16] Cheng T, Rodrigues N, Shen H, *et al.* Hematopoietic stem cell quiescence maintained by $p21^{cip1/waf1}$. Science 2000; 287: 1804-8.

[17] van Os R, Kamminga LM, Ausema A, *et al.* A Limited role for $p21^{cip1/waf1}$ in maintaining normal hematopoietic stem cell functioning. Stem Cells 2007; 25: 836-43.

[18] Umemoto T, Yamato M, Nishida K, Yang J, Tano Y, Okano T.$p57^{Kip2}$ is expressed in quiescent mouse bone marrow side population cells. BBRC 2005; 337: 14-21.

[19] Matsumoto A, Takeishi S, Kanie T, *et al.* p57 is required for quiescence and maintenance of adult hematopoietic stem cells. Cell Stem Cell 2011; 9: 262-71.

[20] Cheng T, Rodrigues N, Dombkowski D, Stier S, Scadden DT. Stem cell repopulation efficiency but not pool size is governed by $p27^{kip1}$ Nat Med 2000; 6: 1235-40.

[21] Zou P, Yoshihara H, Hosokawa K, *et al.* $p57^{Kip2}$ and $p27^{Kip1}$ cooperate to maintain hematopoietic stem cell quiescence through interactions with Hsc70. Cell Stem Cell 2011; 9: 247-61.

[22] Soeiro I, Mohamedali A, Romanska HM, *et al.* $p27^{Kip1}$ and p130 cooperate to regulate hematopoietic cell proliferation *in vivo*. Mol Cell Biol 2006; 26: 6170-84.

[23] Rodriguez S, Wang L, Mumaw C, *et al.* The SKP2 E3 ligase regulates basal homeostasis and stress-induced regeneration of HSCs. Blood 2011; 117: 6509-19.

[24] Wang J, Han F, Wu J, *et al.* The role of Skp2 in hematopoietic stem cell quiescence, pool size, and self-renewal. Blood 2011; 118: 5429-38.

[25] Porcher C, Swat W, Rockwell K, Fujiwara Y, Alt FW, Orkin SH. The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. Cell 1996; 86: 47-57.

[26] Anantharaman A, Lin, IJ, Barrow J, *et al.* Role of Helix-Loop-Helix proteins during differentiation of erythroid cells. Mol Cell Biol 2011; 31: 1332-43.

[27] O'Neil J, Shank J, Cusson N, Murre C, Kelliher M. TAL1/SCL induces leukemia by inhibiting the transcriptional activity of E47/HEB. Cancer Cell 2004; 5: 587-96.

[28] Nie L, Xu M, Vladimirova A, Sun XH. Notch-induced E2A ubiquitination and degradation are controlled by MAP kinase activities. EMBO J 2003; 22: 5780-92.

[29] Nie L, Wu H, Sun XH. Ubiquitination and degradation of Tal1/SCL are induced by notch signaling and depend on Skp2 and CHIP. J Biol Chem 2008; 283: 684-92.

[30] Krivtsov AV, Armstrong SA. MLL translocations, histone modifications and leukaemia stem-cell development. Nature Rev 2007; 7: 823-33.

[31] Liu H, Takeda S, Cheng EH, Hsieh JJ. Biphasic MLL takes helm at cell cycle control. Cell Cycle 2008; 7: 428-35.

[32] Ayton PM, Cleary ML. Molecular mechanisms of leukemogenesis mediated by MLL fusion proteins. Oncogene 2001; 20: 5695-707.

[33] Cozzio A, Passegué E, Ayton PM, Karsunky H, Cleary ML, Weissman IL. Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. Genes Dev 2003; 17: 3029-35.

[34] Liu H, Takeda S, Kumar R, *et al.* Phosphorylation of MLL by ATR is required for execution of mammalian S-phase checkpoint. Nature 2010; 467: 343-6.

[35] Akiyama T. Wnt/β-catenin signaling. Cytokine Growth Factor Rev 2000; 11: 273-82.

[36] Hart M, Concordet JP, Lassot I, *et al.* The F-box protein β-TrCP associates with phosphorylated β-catenin and regulates its activity in the cell. Curr Biol 1999; 9: 207-10.

[37] Kitagawa M, Hatakeyama S, Shirane M, *et al.* An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of β-catenin. EMBO J 1999; 18: 2401-10.

[38] Baba Y, Yokota T, Spits H, Garrett KP, Hayashi S, Kincade PW. Constitutively active β-Catenin promotes expansion of multipotent hematopoietic progenitors in culture. J Immunol 2006; 177: 2294-303.

[39] Kirstetter P, Anderson K, Porse BT, Jacobsen SE, Nerlov C. Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. Nat Immunol 2006; 7: 1048-56.

[40] Scheller M, Huelsken J, Rosenbauer F, *et al.* Hematopoietic stem cell and multilineage defects generated by constitutive β-catenin activation. Nat Immunol 2006; 7: 1037-47.

[41] Reya T, Duncan AW, Ailles L, *et al.* A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 2003; 423: 409-14.

[42] Zhao C, Blum J, Chen A, *et al.* Loss of β-catenin impairs the renewal of normal and CML stem cells *in vivo*. Cancer Cell 2007; 12: 528-41.

[43] Jeannet G, Scheller M, Scarpellino L, *et al.* Long-term, multilineage hematopoiesis occurs in the combined absence of β-catenin andf γ-catenin. Blood 2008; 111: 142-9.

[44] Koch U, Wilson A, Cobas M, Kemler R, Macdonald HR, Radtke F. Simultaneous loss of β- and γ-catenin does not perturb hematopoiesis or lymphopoiesis. Blood 2008; 111: 160-4.

[45] Nemeth MJ, Mak KK, Yang Y, Bodine DM. β-Catenin expression in the bone marrow microenvironment is required for long-term maintenance of primitive hematopoietic cells. Stem Cells 2009; 27: 1109-19.

[46] Braun T, Carvalho G, Fabre C, Grosjean J, Fenaux P, Kroemer G. Targeting NF-κB in hematologic malignancies. Cell Death Differ 2006; 13: 748-58.

[47] Bottero V, Withoff1 S, Verma IM. NF-κB and the regulation of hematopoiesis. Cell Death and Diff 2006; 13: 785-97.

[48] Hatakeyama S, Kitagawa M, Nakayama K, *et al.* Ubiquitin-dependent degradation of IκBα is mediated by a ubiquitin ligase Skp1/Cul 1/F-box protein FWD1. Proc Natl Acad Sci USA 1999; 96: 3859-63.

[49] Tanaka T, Grusby MJ, Kaisho T. PDLIM2-mediated termination of transcription factor NF-kB activation by intranuclear sequestration and degradation of the p65 subunit. Nat Immunol 2007; 8: 584-91.

[50] Frankland-Searby S, Bhaumik SR. The 26S proteasome complex: An attractive target for cancer therapy. BBA 2012; 1825: 64-76.

[51] Margottin F, Bour SP, Durand H, *et al.* A novel human WD protein, h-βTrCP, that interacts with HIV-1 Vpu connects CD4 to the ER degradation pathway through an F-box motif. Mol Cell 1998; 1: 565-74.

[52] Dougras JL, Viswanathan K, McCarroll MN, Gustin JK, Früh K, Moses AV. Vpu directs the degradation of the human immunodeficiency virus restriction factor BST-2/Tetherin *via* a βTrCP-dependent mechanism. J Virol 2009; 83: 7931-47.

[53] Dubé M, Bego MG, Paquay C, Cohen ÉA. Modulation of HIV-1-host interaction: role of the Vpu accessory protein. Retrovirology 2010; 7: 114.

[54] Reavie L, Della Gatta G, Crusio K, *et al.* Regulation of hematopoietic stem cell differentiation by a single ubiquitin ligase-substrate complex. Nat Immunol 2010; 11: 207-15.

[55] Matsuoka S, Oike Y, Onoyama I, I *et al.* Fbxw7 acts as a critical fail-safe against premature loss of hematopoietic stem cells and development of T-ALL. Genes Dev 2008; 22: 986-91.

[56] Thompson BJ, Jankovic V, Gao J, *et al.* Control of hematopoietic stem cell quiescence by the E3 ubiquitin ligase Fbw7. J Exp Med 2008; 205: 1395-408.

[57] Mao JH, Kim IJ, Wu D, *et al.* FBXW7 targets mTOR for degradation and cooperates with PTEN in tumor suppression. Science 2008; 321: 1499-502.

[58] Chen C, Liu Y, Liu Y, Zheng P. mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells. Sci Signal 2009; 2:75.

[59] Li J. Quiescence regulators for hematopoietic stem cell. Exp Hematol 2011; 39: 511-20.

[60] Lobry C, Oh P, Aifantis I. Oncogenic and tumor suppressor functions of Notch in cancer: it's NOTCH what you think. J Exp Med 2011; 208: 1931-5.

[61] Stier S, Cheng T, Dombkowski D, Carlesso N, Scadden DT. Notch1 activation increases hematopoietic stem cell self-renewal *in vivo* and favors lymphoid over myeloid lineage outcome. Blood 2002; 99: 2369-78.

[62] Guzman ML, Neering SJ, Upchurch D, *et al.* Nuclear factor-κB is constitutively activated in primitive human acute myelogenous leukemia cells. Blood 2001; 98: 2301-7.

[63] Kumano K, Chiba S, Shimizu K, *et al.* Notch1 inhibits differentiation of hematopoietic cells by sustaining GATA-2 expression. Blood 2001; 98: 3283-9.

[64] Malyukova A, Dohda T, von der Lehr N, *et al.* The tumor suppressor gene hCDC4 is frequently mutated in human T-cell acute lymphoblastic leukemia with functional consequences for Notch signaling. Cancer Res 2007; 67: 5611-6.

[65] Thompson BJ, Buonamici S, Sulis ML, *et al.* The SCF^{FBW7} ubiquitin ligase complex as a tumor suppressor in T cell leukemia. J Exp Med 2007; 204: 1825-35.

[66] Zúñiga-Pflücker JC. T-cell development made simple. Nat Rev Immunol 2004; 4: 67-72.

[67] Onoyama I, Tsunematsu R, Matsumoto A, *et al.* Conditional inactivation of Fbxw7 impairs cell-cycle exit during T cell differentiation and results in lymphomatogenesis. J Exp Med 2007; 204: 2875-88.

[68] Emambokus N, Vegiopoulos A, Harman B, Jenkinson E, Anderson G, Frampton J. Progression through key stages of haemopoiesis is dependent on distinct threshold levels of c-Myb. EMBO J 2003; 22: 4478-88.

[69] Anderson MK. At the crossroads: diverse roles of early thymocyte transcriptional regulators. Immunol Rev 2006; 209: 191-211.

[70] Kanei-Ishii C, Nomura T, Takagi T, Watanabe N, Nakayama KI, Ishii S. Fbxw7 acts as an E3 ubiquitin ligase that targets c-Myb for nemo-like kinase (NLK)-induced degradation. J Biol Chem 2008; 283: 30540-8.

[71] Kitagawa K, Hiramatsu Y, Uchida C, *et al.* Fbw7 promotes ubiquitin-dependent degradation of c-Myb: involvement of GSK3-mediated phosphorylation of Thr-572 in mouse c-Myb. Oncogene 2009; 28: 2393-405.

[72] Lahortiga I, De Keersmaecker K, Van Vlierberghe P, *et al.* Duplication of the MYB oncogene in T cell acute lymphoblastic leukemia. Nat Genet 2007; 39: 593–5.

[73] Oh IH, Reddy EP. The myb gene family in cell growth, differentiation and apoptosis. Oncogene 1999; 18: 3017–33.

[74] Sakamoto H, Dai G, Tsujino K, *et al.* Proper levels of c-Myb are discretely defined at distinct steps of hematopoietic cell development. Blood 2006; 108: 896-903.

[75] Metcalf D, Carpinelli MR, Hyland C, *et al.* Anomalous megakaryocytopoiesis in mice with mutations in the c-Myb gene. Blood 2005; 105: 3480-7.

[76] Craig, RW. MCL1 provides a window on the role of the BCL2 family in cell proliferation, differentiation and tumorigenesis. Leukemia 2002; 16: 444-54.

[77] Gui J, Morales AJ, Maxey SE, *et al.* MCL1 increases primitive thymocyte viability in female mice and promotes thymic expansion into adulthood. Int Immunol 2011; 23: 647-59.

[78] Zhong Q, Gao W, Du F, Wang X. Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. Cell 2005; 121: 1085-95.

[79] Ding Q, He X, Hsu JM, *et al.* Degradation of Mcl-1 by β-TrCP mediates glycogen synthase kinase 3-induced tumor suppression and chemosensitization. Mol Cell Biol 2007; 27: 4006-17.

[80] Inuzuka H, Shaik S, Onoyama I, *et al.* SCF^{FBW7} regulates cellular apoptosis by targeting MCL1 for ubiquitylation and destruction. Nature 2011; 471: 104-9.

[81] Kitagawa K, Kotake Y, Hiramatsu Y, *et al.* GSK3 regulates the expressions of human and mouse c-Myb *via* different mechanisms. Cell Div 2010; 5: 27.

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Regulation of DNA Replication Licensing

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Abstract: In eukaryotic cells, DNA replication is tightly regulated to occur only once per cell cycle. DNA licensing is a mechanism to guarantee this aim; that is, licensing of replication initiation is permitted during late M phase to G1 phase. The license is canceled by the start of DNA replication. Once DNA replication begins, the license is never given until the next late M phase. The licensing corresponds to the process of assembling components of the pre-replication complex (pre-RC) on the replication origin DNA. This pre-RC is the target of several different regulation systems to prevent re-replication of DNA during a single cell cycle. In this review, the regulation mechanisms mainly in mammals to control assembling components of the pre-RC will be discussed.

Keywords: CDC10-dependent transcript 1 (CDT1), cell division cycle 6 (CDC6), DNA replication, high mobility group AT-hook 1 (HMGA1), histone acetyltransferase binding to ORC (HBO1), homeobox protein Hox-D13 (HOXD13), licensing, mini chromosome maintenance (MCM), origin recognition complex (ORC), prereplication complex (pre-RC).

INTRODUCTION

Vast amounts of information in the genome are quickly duplicated in eukaryotic cells. To maintain the information, DNA replication must occur only once per cell cycle for all chromosome sequences. To duplicate DNA rapidly, eukaryotic cells have multiple replication origins. This means that even if one replication origin has fired, another origin may remain silent. The eukaryotic cell has to develop multiple systems to distinguish origins that have already fired from those that have not fired. It is well known that the pre-replication complex (pre-RC) is a target of the mechanisms to restrict DNA replication to a single round per cell cycle. Essential components of the pre-RC are the origin recognition complex (ORC), cell division cycle 6 (CDC6), CDC10-dependent transcript 1 (CDT1), and mini chromosome maintenance (MCM) 2–7 complex. Recently, a family member of the high-mobility group proteins, HMGA1a; a Hox protein belonging to the large family of homeodomain-containing DNA-binding proteins, HOXD13; a member of the MCM family, MCM9; and a histone acetyltransferase, HBO1, were reported to facilitate pre-RC formation.

Replication licensing begins when ORC binds a replication origin. In fission and budding yeast, ORC binds the replication origin throughout the cell cycle; in higher eukaryotes, ORC is transiently released from the origin in M-phase. HMGA1 is indicated to target ORC to AT-rich chromatin regions. Chromatin-bound ORC recruits CDC6 and CDT1 to a replication origin. HOXD13 interacts with the CDC6. These proteins act as a platform for the MCM2–7 complex to load the complex on the replication origin. MCM9 and HBO1 directly bind CDT1, and they exist at the replication origin. Both factors facilitate MCM2–7 complex loading on the origin. This step-by-step process is regulated during the cell cycle by various mechanisms, and it is very important for preventing the re-initiation of DNA replication from origins that have already fired during a single cell cycle.

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PRE-RC COMPONENTS

ORC

ORC was identified as an associated protein at autonomously replicating sequences (ARSs) in budding yeast [1]. ORC is composed of six subunits (ORC1–6) and acts as an AAA^+ ATPase. Although yeast ORC binds to specific DNA sites, metazoan ORC preferentially binds to AT-rich sequence with nanomolar affinity. In mammalian cells, the amounts of ORC subunits, except ORC1, are constant throughout cell cycle. ORC1 is regulated by phosphorylation and ubiquitylation. In hamster cells, the largest subunit ORC1 binds CDK2/Cyclin A and is phosphorylated at G2/M phase. This phosphorylation reduces the affinity of ORC1 to chromatin [2]. In human cells, ORC1 is selectively polyubiquitinated by SCF^{SKP2} ubiquitin ligase and degraded by 26S proteasome during S phase [3]. In HeLa and CHO cells, ORC1 modifications such as mono-ubiquitylation and hyperphosphorylation that occur normally during S and G2-M phases, respectively, can cause ORC1 to accumulate in the cytoplasm [4]. Thus, ORC activity is lost during S phase to late M phase by suppressing ORC1. At the onset of mitosis, non-modified ORC1 is supplied. Another ORC subunit, ORC2, is also modified. Polo-like kinase 1 (PLK1) phosphorylates ORC2, and ORC2 phosphorylation is enhanced by genotoxic stress. This phosphorylation associates with DNA replication origins to maintain the pre-RC during DNA replication stress [5].

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CDC6

CDC6 was originally identified from cell cycle mutants of budding yeast [6]. CDC6 is another AAA^+ ATPase and is an essential molecule for replication licensing. Chromatin-bound ORC recruits both CDC6 and CDT1 to the replication origin. CDC6, together with ORC and CDT1, makes a platform to load the MCM2–7 complex onto chromatin. Another MCM family member, MCM8, interacts with ORC2 and CDC6. MCM8 is involved in CDC6 recruitment to the origin [7]. In human cells, CDC6 is destabilized in G1 by the ubiquitin-proteasome. A ubiquitin ligase, APC/C^{CDH1}, acts as an E3 ligase [8, 9]. In S phase, a part of CDC6 is phosphorylated by CDK2/Cyclin A and is exported to the cytoplasm to prevent re-replication [10-12]. Prior to this phosphorylation, CDC6 is acetylated by a histone acetyltransferase (HAT), GCN5 [13]. GCN5 specifically acetylates CDC6 at three lysine residues flanking its cyclin-docking motif and is required following phosphorylation. These modifications are necessary for the translocation of CDC6.

CDT1

In eukaryotes, Cdc10-dependent transcript 1 (CDT1) was identified in fission yeast as a target of the CDC10 transcription factor [14]. CDT1 is a major target to prevent rereplication in metazoans. In mammals, two different proteolysis pathways degrade CDT1. One pathway, the SCF^{SKP2} E3 ubiquitin ligase-dependent pathway, requires phosphorylation by CDK2/Cyclin A [15-17]. However, the SCF^{SKP2}-dependent degradation may be a minor pathway because the total CDT1 protein amount was not affected in *Skp2-/-* mouse embryonic fibroblasts. This phenomenon can be explained by another PCNA-dependent proteolysis pathway. $CUL4\text{-}DDB1^{CDT2}$ is another ubiquitin ligase for CDT1. CDT1 protein levels peak in G1/S phase. CDT1 is degraded in S phase by predominantly $CUL4\text{-}DDB1^{CDT2}$. This catalysis involves CDT1 and $CUL4\text{-}DDB1^{CDT2}$ binding chromatin-bound PCNA. $CUL4\text{-}DDB1^{CDT2}$ also ubiquitylates CDT1 after DNA damage. It would be important to prevent rereplication at G2 phase because DNA damage induces checkpoint activation. The checkpoint signaling suppresses CDK activity. As a result, low CDK activity would trigger reassembly of the pre-RC components onto replication origins. When DNA damage occurs in G1 phase, CDT1 is rapidly degraded. A recent paper reported that pre-RC components including CDT1 dynamically interact with chromatin throughout G1 phase [18]. The reason for the rapid elimination of CDT1 from replication origins in G1 phase remains to be elucidated.

Geminin is an inhibitor of replication licensing. It binds CDT1 and is recruited to replication origins. The interaction between geminin and CDT1 inhibits the loading of the MCM complex onto chromatin. Geminin was originally identified in *Xenopus* as a substrate of APC/C^{CDC20} and APC/C^{CDH1} [19]. Geminin degradation allows pre-RC formation in G1 phase. CDT1 ubiquitylation is prevented by geminin interaction. This process is important to supply enough CDT1 in G1 phase.

MCM2–7

The MCM2–7 heterohexamer is predicted to be a DNA helicase. It was identified in the budding yeast as mutants defective in the maintenance of minichromosomes. CDC6 and CDT1 recruit MCM2–7 to the replication origin. ORC and CDC6 ATPases stimulate MCM2–7 loading on replication origins [20, 21].

Although DNA helicase activity was not detected *in vitro* for a long time, immuno-depletion of MCM2–7 from *Xenopus* egg extract inhibited DNA unwinding activity, suggesting that MCM2–7 was implicated in DNA helicase activity [22]. Quite recently, Bochman and Schwacha reported MCM2–7 helicase activity. Unlike MCM4,6,7, MCM2–7 helicase activity was strongly anion dependent [23]. Each MCM subunit includes a C-terminal AAA^+ domain and a distinct N-terminal domain. A recent study indicated that a double hexamer MCM complex that was connected head-to-head *via* N-terminal rings was loaded on the DNA, and it encircled double-stranded DNA [24].

Recently, MCM protein dynamics in living mammalian cells were investigated. These proteins firmly bound chromatin throughout G1 phase. Chromatin bound each of the MCM2–7 subunits, and this interaction gradually increased from late M phase until the initiation of S phase [25]. This report also indicated that the unloading of MCM proteins from chromatin required DNA replication.

Co-Factors Facilitating Pre-RC Formation

HMGA1a

HMGA1a was indicated to target ORC subunits to DNA [26]. In *S. cerevisiae*, a sequence of the replication origin is defined and named the autonomously replicating sequence (ARS). In contrast to budding yeast, a common sequence for the mammalian replication origin has not been identified. How ORC subunits recognize origins is largely unknown. In *Schizosaccharomyces pombe*, origin sequences contain AT-rich sequences. SpORC subunits are recruited to the origin *via* the SpORC4 subunit. SpORC4 contains nine AT-hook motifs. In mammalian cells, the HMGA family of proteins contains the AT-hook motif. One of the members, HMGA1a, specifically binds to the minor groove of AT tracks [27]. Targeting HMGA1a to the special site on the plasmid recruits ORC and generates an artificial origin of DNA replication in cells.

HOXD13

HOXD13 is a member of the HOX family of proteins that belongs to the large family of homeodomain-containing DNA-binding proteins. HOX proteins have been found to associate with the replication licensing regulator geminin [28]. Some members of the HOX proteins have been shown to bind replication origins [29-31]. HOXD13, a member of the HOX proteins, binds human replication origins during the G1 phase of the cell cycle. It interacts with CDC6 and promotes pre-RC formation [32]. Endogenous HOXD13 accelerates DNA synthesis initiation. Geminin, which interacts with CDT1 and inhibits pre-RC formation, also interacts with HOXD13 and inhibits HOXD13-mediated pre-RC formation. HOX family proteins might have an important role in the assembly of the pre-RC.

HBO1

HBO1 (histone acetyltransferase binding to ORC) is a MYST family histone acetyltransferase. It was first identi-

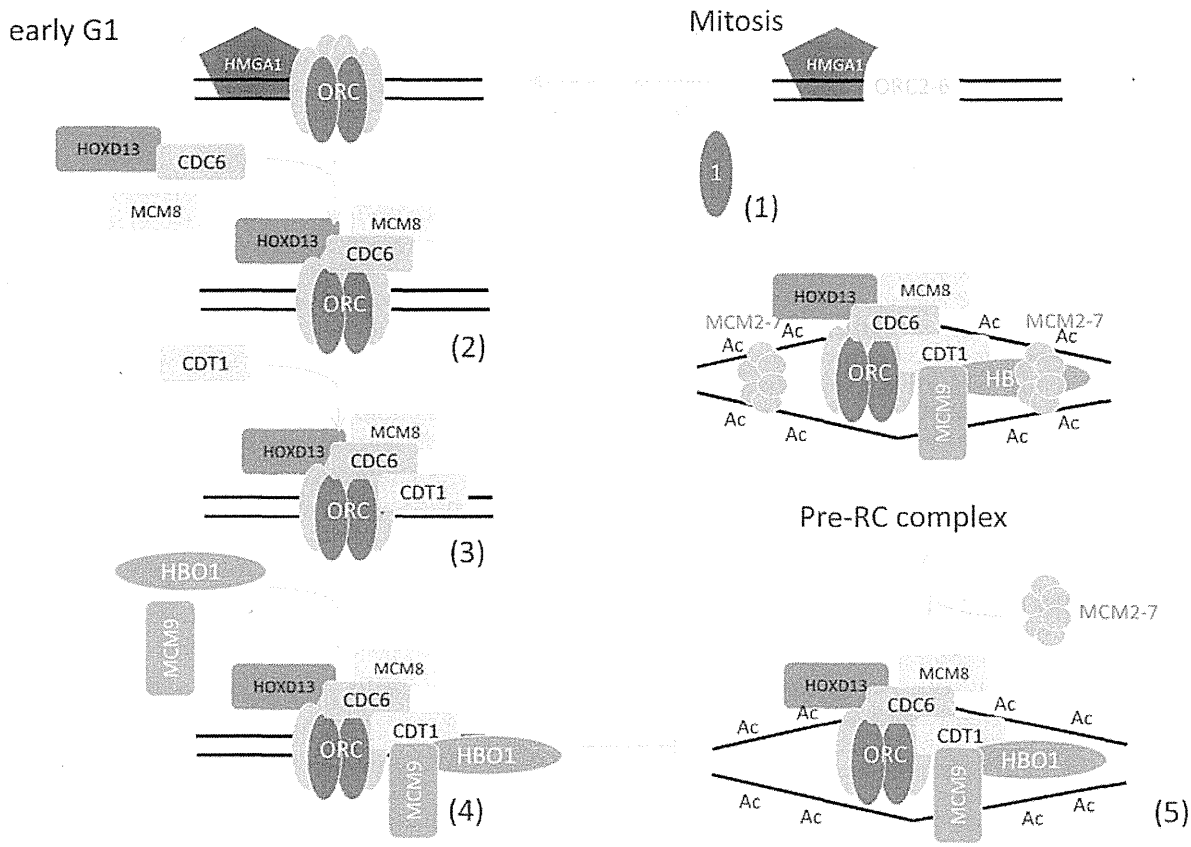

Fig. (1). Prereplication complex (pre-RC) formation during mitosis to G1 phase. (**1**) ORC1 is removed during S and G2-M phases; thus, ORC activity is reconstructed at mitosis. At the onset of mitosis, non-modified ORC1 is supplied. HMGA1 targets ORC to the replication origin; (**2**) Chromatin-bound ORC recruits CDC6 and MCM8 to the origin. HOXD13 interacts with CDC6 at replication origins; (**3**) CDT1 binds to ORC and CDC6 to localize to the origin; (**4**) CDT1 recruits co-factors, HBO1 and MCM9, to facilitate MCM2–7 loading at the origin; (**5**) Chromatin acetylation around the origin is required for proper MCM2–7 loading.

fied as an ORC1-binding protein in human [33]. HBO1 was also identified as a MCM2-binding protein [34]. HBO1 is recruited to replication origins in a CDT1-dependent manner and enhances CDT1 licensing activity [35]. The acetyltransferase activity of HBO1 is essential to accelerate MCM2–7 complex loading onto chromatin. HBO1 activity is inhibited by geminin [36]. The HBO1 complex includes JADE-1 and bind to histone H3. The PHD (plant homology domain) finger domain in JADE-1 interacts with the N-terminus of histone H3 with different specificity with respect to its methylation states [37, 38]. Recently, Miotto and Struhl reported that HBO1 recruitment to origins by CDT1 was inhibited by JNK1 phosphorylation of CDT1 under non-genotoxic stress conditions [39]. These observations suggest that the HBO1 histone acetyltransferase activity changes the environment of a replication origin to relax its form. Such a conformational change of the chromatin would be important for loading the MCM complex onto the replication origin.

MCM9

MCM9 is a member of the MCM2–8 family. MCM9 exists only in vertebrates. The N-terminus of MCM9 conserves the MCM2-8 signature; however, the MCM9 C-terminus is significantly different. Lutzmann and Méchali recently reported that MCM9 was required to load MCM2–7 onto chromatin in the *Xenopus* system [40]. MCM9-depletion abolished replication of sperm chromatin. They also showed that the interaction between MCM9 and CDT1 was important for their stability, and this interaction prevented an excess of geminin on chromatin during the licensing reaction. In contrast to *Xenopus*, Hartford *et al.* reported that mammalian MCM9 was dispensable for pre-RC formation and DNA replication in mice. They concluded that MCM9 is important for germ-line stem-cell maintenance and proliferation [41].

Dynamic Establishment of Replication Licensing

Licensing is required for accurate DNA replication that is precisely restricted to once per cell cycle. The eukaryotic origin is marked by the binding of the ORC complex in late M phase. HMGA1a protein might assist ORC complex binding to the origins. Then, CDC6 and CDT1 recruit the MCM2–7 complex to the origin with co-factors, HOXD13, HBO1 and MCM9, throughout G1 phase Fig. (**1**). The dynamics of replication licensing in living *Caenorhabditis elegans* embryos was analyzed using video microscopy [42]. The authors proposed a model of a licensed origin. They observed that the dissociation of chromatin-bound ORC and

CDC6 was promoted by MCM2–7 complex loading. This negative feedback loop would prevent excessively large gaps between adjacent origins.

Recent studies concerning origin licensing in living cells indicate that members of the pre-RC, except the MCM2–7 complex, bound and dissociated from the chromatin with flexibility during late M phase to late G1 phase. Once origins are licensed (MCM2–7 complex loading is established), the origin would never be canceled until DNA replication has started. However, one question about the dynamics of pre-RC establishment has appeared. If DNA damage is suffered during the period of the establishment, would any checkpoint systems suppress pre-RC formation? Some of the well-known checkpoint targets are cyclin-dependent kinases (CDKs). However, CDKs are inactive during late M phase to late G1 phase in a normal cell cycle. To maintain genome integrity, pre-RC formation would be an effective target for DNA damage checkpoints.

Perspectives

These findings about DNA replication initiation will be useful for understanding the DNA damage response in anti-cancer drug treatments. However, they may not directly provide novel molecular targets for cancer therapy because differences in these mechanisms between normal cells and cancer cells should be elucidated. Further study is required to investigate whether a pre-RC–regulating molecule such as HBO1 may be a novel target for immunosuppressive drugs to inhibit lymphocyte proliferation.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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REFERENCES

[1] Diffley JF, Cocker JH. Protein-DNA interactions at a yeast replication origin. Nature 1992; 357: 169-72.

[2] Li CJ, Vassilev A, DePamphilis ML. Role for Cdk1 (Cdc2)/cyclin A in preventing the mammalian origin recognition complex's largest subunit (Orc1) from binding to chromatin during mitosis. Mol Cell Biol 2004; 24: 5875-86.

[3] Méndez J, Zou-Yang XH, Kim SY, Hidaka M, Tansey WP, Stillman B. Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. Mol Cell 2002; 9: 481-91.

[4] Saha T, Ghosh S, Vassilev A, DePamphilis ML. Ubiquitylation, phosphorylation and Orc2 modulate the subcellular location of Orc1 and prevent it from inducing apoptosis. J Cell Sci 2006; 119: 1371-82.

[5] Song B, Liu XS, Davis K, Liu X. Plk1 phosphorylation of Orc2 promotes DNA replication under conditions of stress. Mol Cell Biol 2011; 31: 4844-56.

[6] Liszlewicz J, Godany A, Agoston DV, Küntzel H. Cloning and characterization of the Saccharomyces cerevisiae CDC6 gene. Nucleic Acids Res 1988; 16: 11507-20.

[7] Volkening M, Hoffmann I. Involvement of human MCM8 in prereplication complex assembly by recruiting hcdc6 to chromatin. Mol Cell Biol 2005; 25: 1560-8.

[8] Méndez J, Stillman B. Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. Mol Cell Biol 2000; 20: 8602-12.

[9] Petersen BO, Wagener C, Marinoni F, *et al.* Cell cycle- and cell growth-regulated proteolysis of mammalian CDC6 is dependent on APC-CDH1. Genes Dev 2000; 14: 2330-43.

[10] Jiang W, Wells NJ, Hunter T. Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. Proc Natl Acad Sci USA 1999; 96: 6193-8.

[11] Petersen BO, Lukas J, Sørensen CS, Bartek J, Helin K. Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. EMBO J 1999; 18: 396-410.

[12] Delmolino LM, Saha P, Dutta A. Multiple mechanisms regulate subcellular localization of human CDC6. J Biol Chem 2001; 276: 26947-54.

[13] Paolinelli R, Mendoza-Maldonado R, Cereseto A, Giacca M. Acetylation by GCN5 regulates CDC6 phosphorylation in the S phase of the cell cycle. Nat Struct Mol Biol 2009; 16: 412-20.

[14] Hofmann JF, Beach D. cdt1 is an essential target of the Cdc10/Sct1 transcription factor: requirement for DNA replication and inhibition of mitosis. EMBO J 1994; 13: 425-34.

[15] Li X, Zhao Q, Liao R, Sun P, Wu X. The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. J Biol Chem 2003; 278: 30854-8.

[16] Liu E, Li X, Yan F, Zhao Q, Wu X. Cyclin-dependent kinases phosphorylate human Cdt1 and induce its degradation. J Biol Chem 2004; 279: 17283-8.

[17] Sugimoto N, Tatsumi Y, Tsurumi T, *et al.* Cdt1 phosphorylation by cyclin A-dependent kinases negatively regulates its function without affecting geminin binding. J Biol Chem 2004; 279: 19691-7.

[18] Xouri G, Squire A, Dimaki M, *et al.* Cdt1 associates dynamically with chromatin throughout G1 and recruits Geminin onto chromatin. EMBO J 2007; 26: 1303-14.

[19] McGarry TJ, Kirschner MW. Cyclin-dependent kinases phosphorylate human Cdt1 and induce its degradation. Cell 1998; 93: 1043-53.

[20] Bowers JL, Randell JC, Chen S, Bell SP. ATP hydrolysis by ORC catalyzes reiterative Mcm2-7 assembly at a defined origin of replication. Mol Cell 2004; 16: 967-78.

[21] Speck C, Chen Z, Li H, Stillman B. ATPase-dependent cooperative binding of ORC and Cdc6 to origin DNA. Nat Struct Mol Biol 2005; 12: 965-71.

[22] Shechter D, Ying CY, Gautier J. DNA unwinding is an Mcm complex-dependent and ATP hydrolysis-dependent process. J Biol Chem 2004; 279: 45586-93.

[23] Bochman ML, Schwacha A. The Mcm2-7 complex has *in vitro* helicase activity. Mol Cell 2008; 31: 287-93.

[24] Remus D, Beuron F, Tolun G, Griffith JD, Morris EP, Diffley JF. Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing. Cell 2009; 139: 719-30.

[25] Kuipers MA, Stasevich TJ, Sasaki T, *et al.* Highly stable loading of Mcm proteins onto chromatin in living cells requires replication to unload. J Cell Biol 2011; 192: 29-41.

[26] Reeves R, Nissen MS. The A.T-DNA-binding domain of mammalian high mobility group I chromosomal proteins. A novel peptide motif for recognizing DNA structure. J Biol Chem 1990; 265: 8573-82.

[27] Thomae AW, Pich D, Brocher J, *et al.* Interaction between HMGA1a and the origin recognition complex creates site-specific replication origins. Proc Natl Acad Sci USA 2008; 105: 1692-7.

[28] Luo L, Yang X, Takihara Y, Knoetgen H, Kessel M. The cell-cycle regulator geminin inhibits Hox function through direct and polycomb-mediated interactions. Nature 2004; 427: 749-53.

[29] Comelli L, Marchetti L, Arosio D, *et al.* The homeotic protein HOXC13 is a member of human DNA replication complexes. Cell Cycle 2009; 8: 454-9.

[30] de Stanchina E, Gabellini D, Norio P, *et al.* Selection of homeotic proteins for binding to a human DNA replication origin. J Mol Biol 2000; 299: 667-80.

[31] Gabellini D, Colaluca IN, Vodermaier HC, *et al.* Early mitotic degradation of the homeoprotein HOXC10 is potentially linked to cell cycle progression. EMBO J 2003; 22: 3715-24.

[32] Salsi V, Ferrari S, Ferraresi R, Cossarizza A, Grande A, Zappavigna V. HOXD13 binds DNA replication origins to promote origin licensing and is inhibited by geminin. Mol Cell Biol 2009; 29: 5775-88.

[33] Iizuka M, Stillman B. Histone acetyltransferase HBO1 interacts with the ORC1 subunit of the human initiator protein. J Biol Chem 1999; 274: 23027-34.
[34] Burke TW, Cook JG, Asano M, Nevins JR. Replication factors MCM2 and ORC1 interact with the histone acetyltransferase HBO1. J Biol Chem 2001; 276: 15397-408.
[35] Miotto B, Struhl K. HBO1 histone acetylase is a coactivator of the replication licensing factor Cdt1. Genes Dev 2008; 22: 2633-8.
[36] Miotto B, Struhl K. HBO1 histone acetylase activity is essential for DNA replication licensing and inhibited by Geminin. Mol Cell 2010; 37: 57-66.
[37] Foy RL, Song IY, Chitalia VC, *et al.* Role of Jade-1 in the histone acetyltransferase (HAT) HBO1 complex. J Biol Chem 2008; 283: 28817-26.
[38] Saksouk N, Avvakumov N, Champagne KS, *et al.* HBO1 HAT complexes target chromatin throughout gene coding regions *via* multiple PHD finger interactions with histone H3 tail. Mol Cell 2009; 33: 257-65.
[39] Miotto B, Struhl K. JNK1 phosphorylation of Cdt1 inhibits recruitment of HBO1 histone acetylase and blocks replication licensing in response to stress. Mol Cell 2011; 44: 62-71.
[40] Lutzmann M, Méchali M. MCM9 binds Cdt1 and is required for the assembly of prereplication complexes. Mol Cell 2008; 31: 190-200.
[41] Hartford SA, Luo Y, Southard TL, Min IM, Lis JT, Schimenti JC. Minichromosome maintenance helicase paralog MCM9 is dispensible for DNA replication but functions in germ-line stem cells and tumor suppression. Proc Natl Acad Sci USA 2011; 108: 17702-7.
[42] Sonneville R, Querenet M, Craig A, Gartner A, Blow JJ. The dynamics of replication licensing in live Caenorhabditis elegans embryos. J Cell Biol 2012; 196: 233-46.

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The Amelioration of Renal Damage in Skp2-Deficient Mice Canceled by p27 ^{Kip1} Deficiency in Skp2$^{-/-}$ p27$^{-/-}$ Mice

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Abstract

SCF-Skp2 E3 ubiquitin ligase (Skp2 hereafter) targets several cell cycle regulatory proteins for degradation via the ubiquitin-dependent pathway. However, the target-specific physiological functions of Skp2 have not been fully elucidated in kidney diseases. We previously reported an increase in Skp2 in progressive nephropathy and amelioration of unilateral ureteral obstruction (UUO) renal injury associated with renal accumulation of p27 in Skp2$^{-/-}$ mice. However, it remains unclear whether the amelioration of renal injury in Skp2$^{-/-}$ mice is solely caused by p27 accumulation, since Skp2 targets several other proteins. Using Skp2$^{-/-}$p27$^{-/-}$ mice, we investigated whether Skp2 specifically targets p27 in the progressive nephropathy mediated by UUO. In contrast to the marked suppression of UUO renal injury in Skp2$^{-/-}$ mice, progression of tubular dilatation associated with tubular epithelial cell proliferation and tubulointerstitial fibrosis with increased expression of collagen and α-smooth muscle actin were observed in the obstructed kidneys in Skp2$^{-/-}$p27$^{-/-}$ mice. No significant increases in other Skp2 target proteins including p57, p130, TOB1, cyclin A and cyclin D1 were noted in the UUO kidney in Skp2$^{-/-}$ mice, while p21, c-Myc, b-Myb and cyclin E were slightly increased. Contrary to the ameliorated UUO renal injure by Skp2-deficiency, the amelioration was canceled by the additional p27-deficiency in Skp2$^{-/-}$p27$^{-/-}$ mice. These findings suggest a pathogenic role of the reduction in p27 targeted by Skp2 in the progression of nephropathy in UUO mice.

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Introduction

Cell proliferation is a basic biological mechanism that is controlled by a network of proteins including cyclins, cyclin-dependent kinases (CDKs) [1] and cyclin-dependent kinase inhibitors (CKIs) [2]. The CKI p27^{Kip1} (p27), a known negative regulator of the cell cycle, is abundantly expressed in most normal quiescent cells, and its level declines when cells are stimulated to proliferate in response to mitotic stimuli [3,4]. *In vitro* studies have shown that experimentally reducing the level of p27 protein augments the proliferative response to mitogens [5,6], while forced overexpression of p27 inhibits cell proliferation [4]. The level of p27 protein is controlled not only by transcriptional activation but also by proteolytic degradation of p27 protein via the ubiquitin-proteasome system as a post-translational regulation. Consequently, G1-cyclin-CDK complexes become activated to phosphorylate retinoblastoma protein and advance the cell cycle from G1 to S phase [7,8].

The ubiquitin-proteasome pathway of protein degradation plays an important role in controlling the abundance of cell cycle regulatory proteins [9,10]. The rapidity and substrate specificity of protein degradation through the ubiquitin-proteasome pathway are consistent with its role in controlling the fluctuations in the intracellular concentrations of cyclins and CKIs. Skp2 is known to be the F-box protein component of an SCF-type ubiquitin ligase that interacts with p27, and the SCF-Skp2 complex promotes p27 degradation by ubiquitination [11,12]. Skp2$^{-/-}$ mice have been reported to show cellular accumulation of p27 [13]. Moreover, cdc kinase subunit 1 (Cks1) is required for degradation of p27 mediated by Skp2 [14]. It has also been reported that Skp2 targets several cell cycle regulatory proteins including p27, p21, p57, cyclin E, cyclin A and cyclin D1 for degradation via the ubiquitin-dependent pathway [15]. However, it remains unclear which proteins are targeted by Skp2 for degradation in specific biological processes or diseases.

In the kidney, cell proliferation is supposed to be a pivotal response to damage, and culminates in the development of renal injury and fibrosis. Proliferation of tubular cells is a characteristic feature of obstructed kidneys in unilateral ureteral obstruction (UUO). UUO is a representative model of progressive tubulointerstitial injury that is suitable for investigating the cellular and molecular events that occur during the progression of renal fibrosis associated with cell proliferation and apoptosis [16,17]. An imbalance between cell proliferation and apoptosis has been shown to lead to unchecked apoptosis, resulting in progressive cell loss, renal tubular atrophy and interstitial fibrosis [18].

It has been reported that both the mRNA and protein levels of the CDKIs p27 and p21 are upregulated at an early stage in the obstructed kidneys of UUO mice [19–21]. Marked increases in renal tubular epithelial cell proliferation and apoptosis are also observed in the obstructed kidneys in p27$^{-/-}$ mice [22]. Since upregulation of p27 safeguards against excessive renal epithelial cell proliferation, p27 may be involved in protecting cells and tissues against inflammatory injury. On the other hand, no significant changes in tubular epithelial cell proliferation and apoptosis are found in the obstructed kidneys in p21$^{-/-}$ mice, despite the proliferation of interstitial cells, especially myofibroblasts [23]. Unlike p27, p21 limits the magnitude of early myofibroblast proliferation, but does not seem to be essential for the regulation of tubular epithelial cell proliferation and apoptosis following UUO [23]. These studies suggest differential regulatory roles for the CDKIs p27 and p21 in UUO kidneys.

Recently, we reported that Skp2 mRNA was increased in UUO kidneys and that the progression of fibrotic tubulointerstitial damage in UUO kidneys was attenuated in Skp2$^{-/-}$ mice [24]. Although the p27 protein level was increased in the obstructed kidneys in wild-type (WT) mice, it was significantly higher in Skp2$^{-/-}$ mice. p27 accumulation, which results from SCF-Skp2 ubiquitin ligase deficiency in Skp2$^{-/-}$ mice, inhibited the renal tubular epithelial cell proliferation, and was involved to the amelioration of the renal damage induced by obstructive nephropathy. Moreover, we found upregulation of not only Skp2, but also Cks1, an essential cofactor for the SCF-Skp2 ubiquitin ligase in targeting p27, which were induced by activation of the TNF-α/NF-κB pathway in two models of chronic progressive nephropathy, namely UUO mice and chronic anti-thymocyte serum nephropathy rats [25]. However, because Skp2 has multiple targets including p21, p57, c-Myc, p130 and TOB1 in addition to p27 [26–30], little is known about the specific target of Skp2 in renal lesions.

In the present study, we investigated whether degradation of p27 targeted by Skp2 is required for the development of tubulointerstitial injury in UUO kidneys by comparing WT, Skp2$^{-/-}$ and p27$^{-/-}$ mice with Skp2$^{-/-}$p27$^{-/-}$ mice. Contrary to the amelioration of UUO renal injury by Skp2-deficiency, the amelioration was abolished by the additional deficiency of p27 in Skp2$^{-/-}$p27$^{-/-}$ double knockout mice. These findings suggest that p27 is the key molecule targeted by Skp2 that is involved in the progression of renal injury in UUO mice.

Results

Levels of Skp2 target proteins in the UUO kidney

First, we investigated the levels of Skp2 target proteins in UUO renal injury in WT and Skp2$^{-/-}$ mice. Consistent with our previous report [24], accumulation of p27 and p21 was observed in the UUO kidneys in Skp2$^{-/-}$ mice (Figure 1). The levels of c-Myc, b-Myb and cyclin E were also slightly increased, whereas p57, p130, TOB1, cyclin A and cyclin D1 did not accumulate, in the UUO kidneys in Skp2$^{-/-}$ mice. These findings suggest that the increased Skp2 promoted the degradation of p27, p21, c-Myc, b-Myb and cyclin E in the UUO kidneys. However, it remains unclear which protein degradation targeted by Skp2 plays an important role in the progression of the obstructive nephropathy. To address this question, we first focused on p27 and compared its levels in UUO renal injury in Skp2$^{-/-}$p27$^{-/-}$ double-knockout mice with those in WT, Skp2$^{-/-}$ and p27$^{-/-}$ mice.

Genotypes of Skp2$^{-/-}$, Skp2$^{-/-}$p27$^{-/-}$ and p27$^{-/-}$ mice

The genotypes of the Skp2$^{-/-}$, Skp2$^{-/-}$p27$^{-/-}$ and p27$^{-/-}$ mice were confirmed by PCR (Figure S1A). p27 level was increased in Skp2$^{-/-}$ mice comparing with wild-type mice after UUO operation, whereas p27 protein was not detected in both p27$^{-/-}$ and Skp2$^{-/-}$p27$^{-/-}$ mice (Figure S1B).

Diminished ameliorative effect of Skp2-deficiency on the obstructive renal injury in Skp2$^{-/-}$p27$^{-/-}$ mice

In accordance with our previous report [24], remarkable amelioration of the tubulointerstitial fibrosis and significant decreases in the numbers of dilated tubules, tubular cells and interstitial cells were noted in the UUO kidneys in Skp2$^{-/-}$ mice compared with WT mice. In contrast, the amelioration of the UUO renal injury noted in Skp2$^{-/-}$ mice was almost completely abolished, and instead rather aggravated, by the additional p27-deficiency in Skp2$^{-/-}$p27$^{-/-}$ mice (Figure 2, A–E). Aggravation of the UUO renal injury was also observed in p27$^{-/-}$ mice. There were no significant renal histological changes in the non-obstructed CLK kidneys in all genotypes of mice.

Diminished suppressive effect of Skp2-deficiency on tubular epithelial cell proliferation and apoptosis in UUO kidneys in Skp2$^{-/-}$p27$^{-/-}$ mice

The number of Ki67-positive proliferative tubular epithelial cells per tubule was significantly increased in the UUO kidneys in WT mice, but was suppressed in Skp2$^{-/-}$ mice. However, the suppression of tubular epithelial cell proliferation noted in the UUO kidneys in Skp2$^{-/-}$ mice was diminished in Skp2$^{-/-}$ p27$^{-/-}$ mice (Figure 3, A–F). A marked increase in proliferative tubular cells was observed in p27$^{-/-}$ mice. The number of Ki67-positive interstitial cells was also increased in the UUO kidneys in WT mice compared with the CLK kidneys, and was slightly

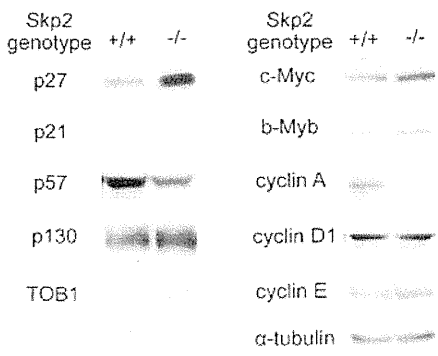

Figure 1. Immunoblot analyses of the previously reported Skp2 target proteins in UUO kidneys. The levels of the previously reported Skp2 target proteins were detected by western blot analysis in the obstructed kidneys in WT (Skp2$^{+/+}$) and Skp2$^{-/-}$ mice at 7 days after UUO. α-tubulin was evaluated as an internal control.
doi:10.1371/journal.pone.0036249.g001

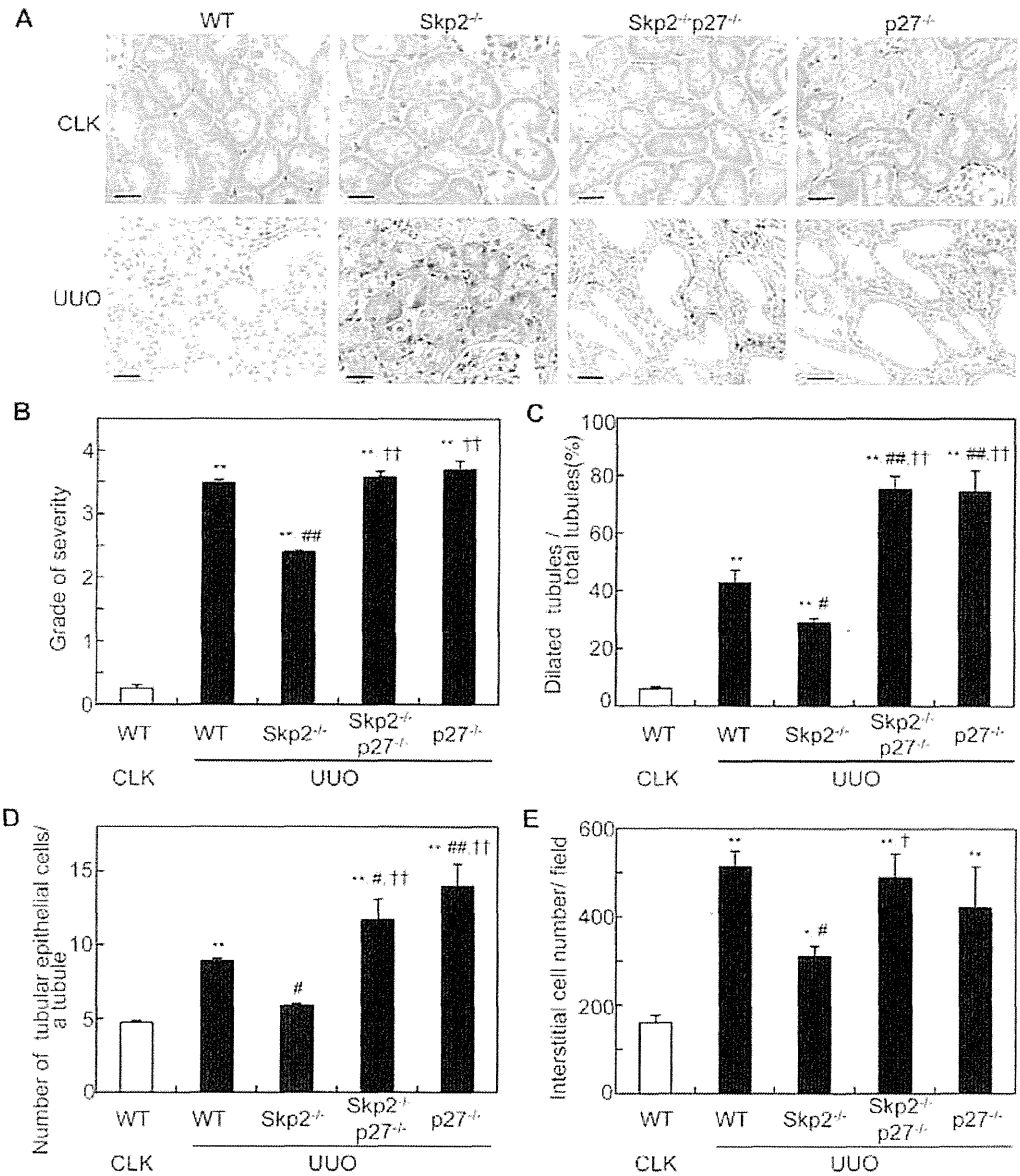

Figure 2. Levels of UUO renal injury. (**A**) Representative microscopic images of CLK and UUO kidneys in WT, Skp2$^{-/-}$, Skp2$^{-/-}$p27$^{-/-}$ and p27$^{-/-}$ mice (Masson's trichrome staining; scale bars: 50 μm). Increases in the interstitial area, tubular dilatation and atrophy, and interstitial cell infiltration are observed in the UUO kidneys in WT mice. However, the severities of these lesions are markedly less in the UUO kidneys in Skp2$^{-/-}$ mice. In contrast, aggravation of the UUO renal injury is noted in Skp2$^{-/-}$p27$^{-/-}$ and p27$^{-/-}$ mice. (**B**) The severity of fibrotic tubulointerstitial lesions was graded semiquantitatively as follows: 0, absent (0%); 1, weak (≤10%); 2, mild (>10 to ≤30%); 3, moderate (>30 to ≤50%); 4, strong (>50%) in WT, Skp2$^{-/-}$, Skp2$^{-/-}$p27$^{-/-}$ and p27$^{-/-}$ mice at 7 days after UUO. (**C–E**) The numbers of dilated tubules (**C**), renal tubular epithelial cells in a tubule (**D**), and tubular interstitial cells (**E**) were counted and evaluated statistically in the UUO kidneys in WT, Skp2$^{-/-}$, Skp2$^{-/-}$p27$^{-/-}$ and p27$^{-/-}$ mice at 7 days after UUO. The CLK kidneys in WT mice were evaluated as controls. *P<0.05, **P<0.01 versus WT CLK kidneys, $^{\#}P$<0.05, $^{\#\#}P$<0.01 versus WT UUO kidneys and $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01 versus Skp2$^{-/-}$ UUO kidneys.
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decreased in the UUO kidneys in Skp2$^{-/-}$ mice (Figure 3, A–E and G). On the other hand, marked increases in Ki67-positive proliferative interstitial cells were observed in the UUO kidneys in Skp2$^{-/-}$p27$^{-/-}$ and p27$^{-/-}$ mice.

Compared with the CLK kidneys in WT mice, the numbers of TUNEL-positive apoptotic tubular and interstitial cells were increased in the UUO kidneys in WT mice, but were significantly fewer in Skp2$^{-/-}$ mice. In contrast, the suppression of TUNEL-positive apoptotic tubular and interstitial cells in the UUO kidneys noted in Skp2$^{-/-}$ mice was diminished by the additional p27-deficiency in Skp2$^{-/-}$p27$^{-/-}$ mice (Figure 4, A–G). The increases in the numbers of TUNEL-positive apoptotic tubular and interstitial cells were also observed in the UUO kidneys in p27$^{-/-}$ mice.

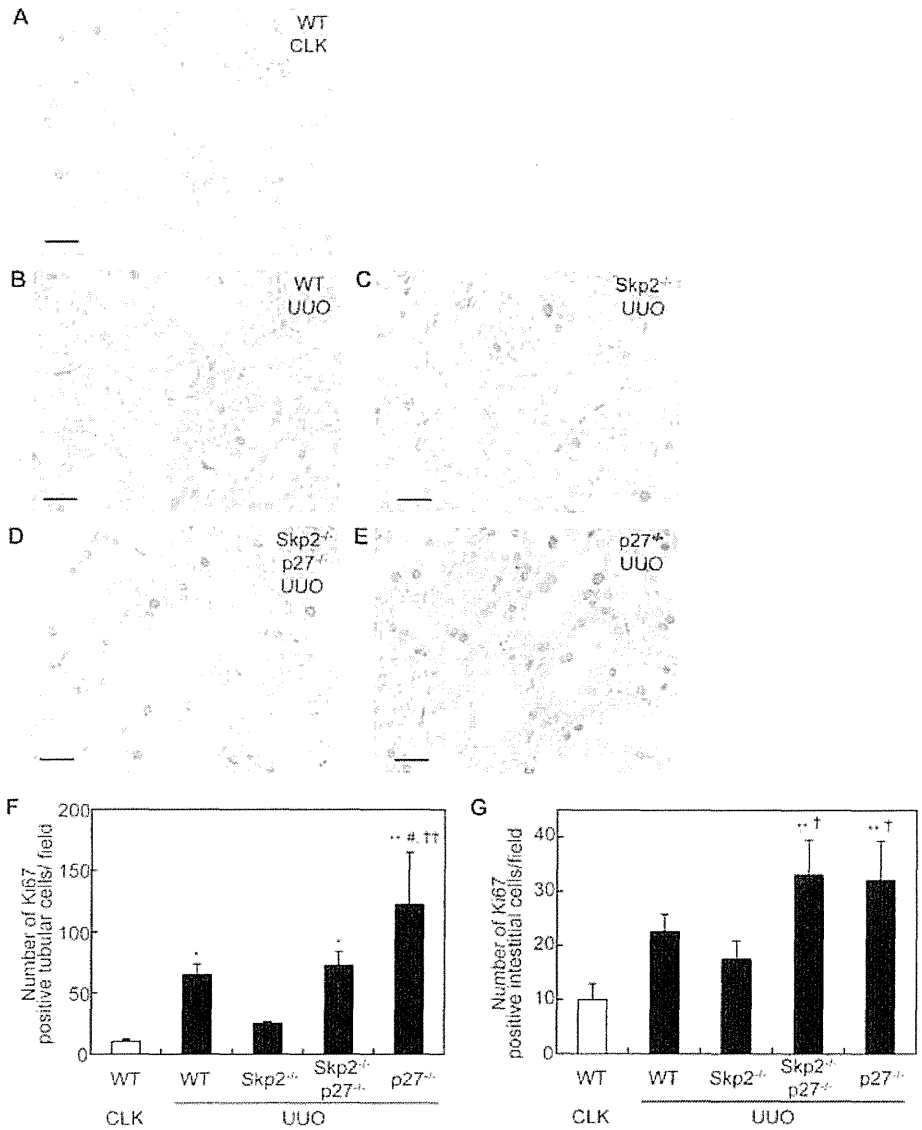

Figure 3. Numbers of Ki67-positive proliferative tubular epithelial and interstitial cells in UUO kidneys. (**A–E**) Sections of CLK kidneys in WT mice (**A**) and UUO kidneys in WT (**B**), Skp2$^{-/-}$ (**C**), Skp2$^{-/-}$p27$^{-/-}$ (**D**) and p27$^{-/-}$ (**E**) mice were subjected to immunostaining with an anti-Ki67 antibody, scale bars: 50 μm (**F, G**) The numbers of Ki67-positive tubular epithelial cells (**F**) and Ki67-positive interstitial cells (**G**) in the UUO kidneys were counted in the mice of each genotype. The CLK kidneys in WT mice were evaluated as controls. *$P<0.05$, **$P<0.01$ versus WT CLK kidneys, $^{\#}P<0.05$ versus WT UUO kidneys and $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ versus Skp2$^{-/-}$ UUO kidneys.
doi:10.1371/journal.pone.0036249.g003

Diminished suppressive effect of Skp2-deficiency on progression of tubulointerstitial fibrosis in UUO kidneys in Skp2$^{-/-}$p27$^{-/-}$ mice

Comparing with the CLK kidneys in WT mice, type I collagen-positive interstitial area was significantly increased in the UUO kidneys in WT mice, but the increment was significantly suppressed in Skp2$^{-/-}$ mice. On the other hand, the suppression was markedly diminished by the additional p27-deficiency in the UUO kidneys in Skp2$^{-/-}$p27$^{-/-}$ mice (Figure 5B). Similarly, the interstitial areas positive for α-SMA and F4/80 were markedly increased in the UUO kidneys in WT mice. Although these increments were significantly suppressed in Skp2$^{-/-}$ mice, the suppression was almost completely abolished by the additional p27-deficiency in Skp2$^{-/-}$p27$^{-/-}$ mice (Figure 5, A, C and D). Increases in type I collagen-positive fibrotic interstitial area, α-SMA and interstitial migration of F4/80-positive macrophages were also observed in the UUO kidneys in p27$^{-/-}$ mice. In addition, we performed QRT-PCR to measure the mRNA expression levels of COL I, α-SMA, F4/80 and fibronectin. As shown in Figure S2, comparing with the CLK kidneys in WT mice, mRNA expression levels of type I collagen, α-SMA, F4/80 and fibronectin were increased in the UUO kidneys in WT mice, but the increments were suppressed in Skp2$^{-/-}$ mice. On the other hand, the suppression was diminished by the additional p27-deficiency in the UUO kidneys in Skp2$^{-/-}$p27$^{-/-}$ mice (Figure S2). These tendencies were consistent with immunohistochemical data in Figure 5A. We also found that vimentin-positive interstitial

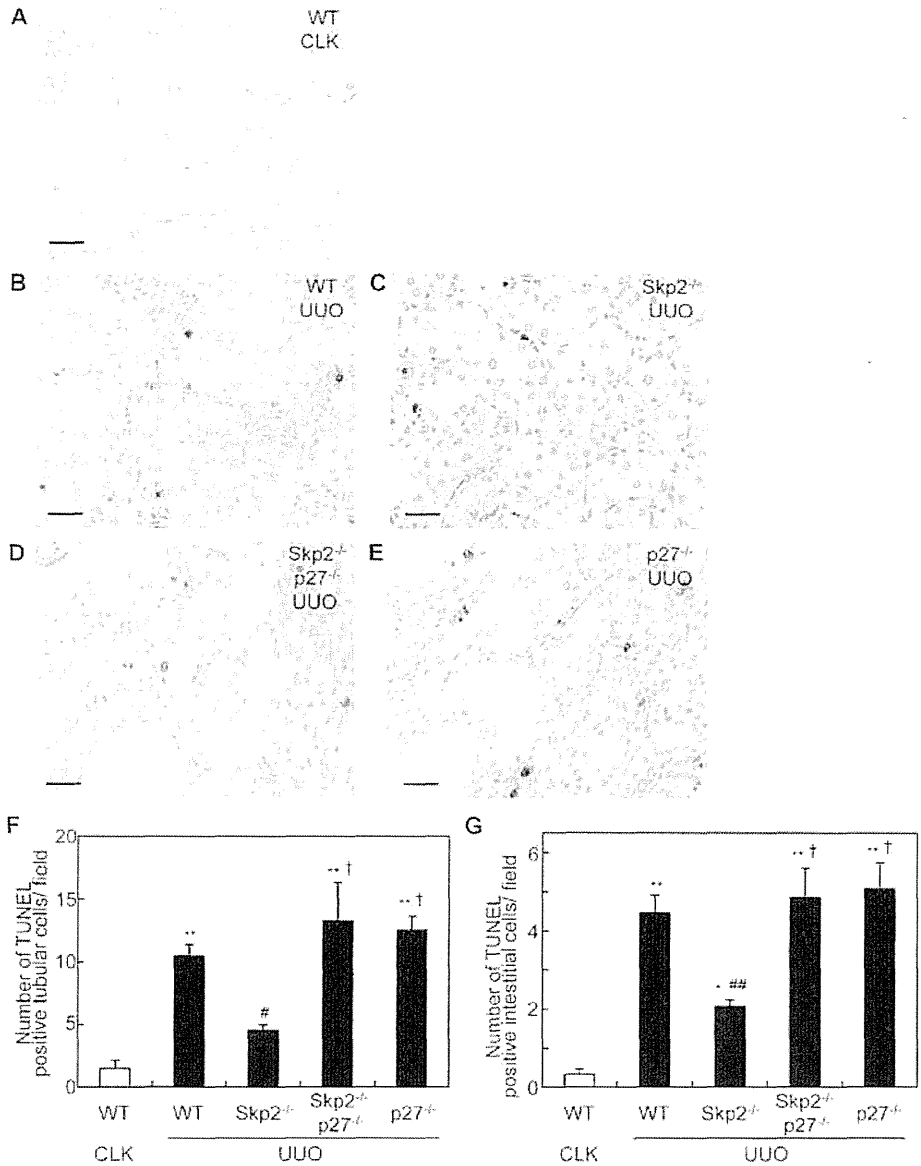

Figure 4. Numbers of TUNEL-positive apoptotic tubular epithelial and interstitial cells in UUO kidneys. (**A–E**) Sections from CLK kidneys in WT mice (**A**) and UUO kidneys in WT (**B**), Skp2$^{-/-}$ (**C**), Skp2$^{-/-}$p27$^{-/-}$ (**D**) and p27$^{-/-}$ (**E**) mice were subjected to TUNEL staining, scale bars: 50 μm. (**F, G**) The numbers of TUNEL-positive tubular epithelial cells (**F**) and TUNEL-positive interstitial cells (**G**) in the UUO kidneys were counted in the mice of each genotype. The CLK kidneys in WT mice were evaluated as controls. *$P<0.05$, **$P<0.01$ versus WT CLK kidneys, $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus WT UUO kidneys and $^{\dagger}P<0.01$ versus Skp2$^{-/-}$ UUO kidneys.
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area was significantly increased in the UUO kidneys in WT mice, but the increment was suppressed in Skp2$^{-/-}$ mice (Figure S3). On the other hand, the suppression was markedly diminished by the additional p27-deficiency in the UUO kidneys in Skp2$^{-/-}$p27$^{-/-}$ mice. These results strongly suggested that the Skp2/p27 pathway may contribute to EMT in progressive UUO renal injury.

Discussion

SCF-Skp2 is an important E3 ligase that targets several cell cycle regulatory proteins. However, it has not been fully elucidated which protein degradation targeted by Skp2 contributes to the physiological and pathological events in progressive renal injury. In Skp2$^{-/-}$ mice, we previously reported that accumulation of p27, which had escaped from Skp2-mediated ubiquitin-proteasomal degradation, resulted in the suppression of tubular epithelial cell proliferation, tubular dilatations and tubulointerstitial fibrosis in UUO renal injury [24]. In the present study, we compared the UUO renal injury among WT, Skp2$^{-/-}$, Skp2$^{-/-}$p27$^{-/-}$ and p27$^{-/-}$ mice, and found that 1) the UUO renal injury was less severe in Skp2$^{-/-}$ mice, in which renal accumulation of p27 was noted, 2) the amelioration of UUO renal injury noted in Skp2$^{-/-}$ mice, was almost completely abolished, and instead rather