Knockout Mouse Models Provide Insight into the Biological Functions of CRL1 Components

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Abstract

The CRL1 complex, also known as the SCF complex, is a ubiquitin ligase that in mammals consists of an adaptor protein (SKP1), a scaffold protein (CUL1), a RING finger protein (RBX1, also known as ROC1), and one of about 70 F-box proteins. Given that the F-box proteins determine the substrate specificity of the CRL1 complex, the variety of these proteins allows the generation of a large number of ubiquitin ligases that promote the degradation or regulate the function of many substrate proteins and thereby control numerous key cellular processes. The physiological and pathological functions of these many CRL1 ubiquitin ligases have been studied by the generation and characterization of knockout mouse models that lack specific CRL1 components. In this chapter, we provide a comprehensive overview of these mouse models and discuss the role of each CRL1 component in mouse physiology and pathology.

Keywords

Knockout mouse models · CUL1 · SKP1 · F-box proteins

Abbreviations

APC/C	Anaphase-promoting complex/cyclosome
ASK1	Apoptotic signal-regulating kinase 1
BACE1	β-site amyloid precursor protein-cleaving enzyme 1
BMP	Bone morphogenetic protein
CDK1	Cyclin-dependent kinase 1
CML	Chronic myeloid leukemia
CP110	Centrosomal protein 110
CRL	Cullin-RING ubiquitin ligase
CRY	Cryptochrome
CUL1	Cullin-1
DiPIUS	Differential proteomics-based identification of ubiquitylation substrates
DKO	Double knockout
DP	Double positive
EMI1	Early mitotic inhibitor 1
EMI2	Early mitotic inhibitor 2
F-box	Cyclin F-box
FBXL	LRR-containing F-box proteins
FBXO	Other domain-containing F-box proteins
FBXW	WD40 domain-containing F-box proteins
GC	Germinal center

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GSTP1	Glutathione S-transferase π1
HECT	Homologous to E6-associated protein C-terminus
HSC	Hematopoietic stem cell
IRE	Iron-responsive element
IRP	Iron-regulatory protein
JNK	c-Jun NH2-terminal kinase
KO	Knockout
LAP	Leukemia-associated protein
LIC	Leukemia-initiating cell
LRR	Leucine-rich repeat
MAFbx	Muscle atrophy F-box protein
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
NASH	Nonalcoholic steatohepatitis
NSPC	Neural stem/progenitor cell
PD-1	Programmed cell death-1
PD-L1	Programmed cell death-ligand 1
PER	Period
PHD	Plant homeodomain
PI3K	Phosphoinositide 3-kinase
PRC	Polycomb repressive complex
PTEN	Phosphatase and tensin homolog
RBR	RING between RING
RBX1	RING box 1, also known as regulator of Cullins 1 or ROC1
RIM1	Rab3-interacting molecule 1
RING	Really interesting new gene
ROS	Reactive oxygen species
RRM2	Ribonucleotide reductase 2
SASP	Senescence-associated secretory phenotype
SCF	SKP1–CUL1–F-box protein
Scg2	Secretogranin 2
SKP1	S-phase kinase-associated protein 1
SKP2	S-phase kinase-associated protein 2
SREBP	Sterol regulatory element-binding protein
SSC	Spermatogonial stem cell
T-ALL	T-cell acute lymphoblastic leukemia
U-box	UFD2-box
β-TrCP	β-transducin repeat-containing protein

10.1 Introduction

Ubiquitylation is a posttranslational protein modification that has many regulatory roles in eukaryotic cells, with one prominent role being the marking of target proteins for degradation by the 26S proteasome and the consequent activation or inhibition of a particular cellular process (Nakagawa and Nakayama 2015; Swatek and Komander 2016). Protein ubiquitylation is mediated by three enzymatic reactions: ubiquitin activation by an E1 ubiquitin-activating enzyme, ubiquitin conjugation to an E2 ubiquitin-conjugating enzyme, and transfer of ubiquitin from E2 to the substrate protein by an E3 ubiquitin ligase (Kleiger and Mayor 2014). The specificity of ubiquitylation is conferred by the E3 ubiquitin ligases, which physically interact with their cognate substrates.

E3 enzymes are categorized into three major classes on the basis of their domain structure and catalytic mechanism (Metzger et al. 2012): (1) RING (really interesting new gene) finger or RING- related PHD (plant homeodomain), LAP (leukemia-associated protein), or U (UFD2)-box domain families (Deshaies and Joazeiro 2009), (2) the HECT (homologous to E6-associated protein C-terminus) domain family (Rotin and Kumar 2009), and (3) the RBR (RING between RING) finger domain family (Smit and Sixma 2014). Whereas some E3 ligases function as a monomer, others exist as a complex that includes both an E2 binding component and a substrate binding component.

The CRL1 (Cullin-RING ubiquitin ligase 1) complex, also known as the SCF (SKP1–CUL1–F-box protein) complex, is a multisubunit RING finger-type ubiquitin ligase and a founding member of the CRL family, which also includes CRL2, CRL3, CRL4A, CRL4B, CRL5, CRL7, CRL9, and APC/C (anaphase-promoting complex/cyclosome) (Petroski and Deshaies 2005). The core of the CRL1 complex consists of three proteins: the scaffold protein CUL1 (Cullin-1), the RING finger protein RBX1 (RING box protein 1; also known as regulator of Cullins 1, or ROC1), and the adaptor protein SKP1 (S-phase kinase-associated protein 1). SKP1 links CUL1 to one of the F-box proteins that direct the CRL1 complex to its targets for ubiquitylation. Each F-box protein contains an F-box (cyclin F-box) domain that is necessary for the association with SKP1, as well as a substrate binding domain such

as a WD40 domain or LRR (leucine-rich repeat) domain. The human and mouse genomes encode ~70 F-box proteins, which have been classified into three categories: (1) FBXWs (WD40 domain-containing F-box proteins), (2) FBXLs (LRR-containing F-box proteins), and (3) FBXOs (other domain-containing F-box proteins) (Jin et al. 2004). Several knockout (KO) mouse models for components of the CRL1 complex have been established in order to facilitate investigation of their in vivo roles and suitability as drug targets. We here provide a comprehensive overview of such mice model and discuss how they have shed light on the physiological and pathological functions of the CRL1 complex.

10.2 CUL1

CUL1 serves as a scaffold protein of CRL1 by connecting the RBX1–E2 module and the SKP1–F-box protein module for substrate binding, and it is thus an essential component. Studies of *Cul1*-deleted mice were first reported in 1999 by two research groups (Dealy et al. 1999; Wang et al. 1999). Heterozygous mice manifested no visible pathology, but no $Cul1^{-/-}$ mice were born, indicating that CUL1 is required for embryonic development. Analysis of pregnant females revealed that $Cul1^{-/-}$ embryos underwent implantation in the uterine wall but that they failed to develop further and died between embryonic day (E) 6.5 and E7.5 before gastrulation. Accumulation of cyclin E protein, without a concomitant increase in the amount of cyclin E mRNA, was apparent in the $Cul1^{-/-}$ embryos and blastocysts, indicating that cyclin E is a physiological substrate of CRL1 in early embryos. Whether or how the accumulated cyclin E is responsible for the embryonic lethality by CUL1 KO remains to be tested.

10.3 SKP1

SKP1 is an adaptor protein that mediates the association of CUL1 with an F-box protein. As far as we are aware, a SKP1 KO mouse has not yet been described, but our unpublished results indicated that SKP1 KO mice died in utero early in the embryonic development. A transgenic mouse that expresses an NH_2-terminal SKP1-binding fragment of CUL1 (CUL1-N252) under the control of the *Cd4* gene enhancer and promoter in T lymphocytes has been analyzed to provide insight into the in vivo function of SKP1 (Piva et al. 2002). CUL1-N252 binds to SKP1 but lacks the RBX1 binding region, and it thus inhibits the interaction of SKP1 with endogenous CUL1-RBX1. The transgenic animals at 6–8 weeks of age did not show a defect in T-cell differentiation but manifested a decrease in the number as well as hypoplasia of T cells in lymphoid organs as a result of their impaired proliferation. The accumulation of cyclin E, β-catenin, and the cyclin-dependent kinase inhibitor p27 was detected in the thymus of these mice, again implicating these proteins as physiological targets of CRL1. Curiously, despite the low index of T-cell proliferation in young adults, >80% of CUL1-N252 transgenic mice developed T-cell lymphoma and died between 4 and 16 months of age. These lymphomas showed marked karyotype heterogeneity associated with amplification and overexpression of the *Myc* gene, suggesting that SKP1 plays a key role in the maintenance of genome stability and prevention of neoplastic transformation. The mechanistic link between the accumulation of CRL1 substrates and T-cell depletion or *Myc* amplification remains uncharacterized.

10.4 RBX1/ROC1

RBX1 (also known as ROC1) serves as an E2 module for multiple CRL complexes. Targeted inactivation of RBX1 in mice resulted in embryonic death at ~E7.5 due to failure of cell proliferation in association with the accumulation of p27, a known substrate of CUL1 and CUL4 (Tan et al. 2009; Zhou et al. 2013). Simultaneous ablation of p27 extended the embryonic life span to E9.5, indicating that p27-mediated proliferation arrest contributes to the early death of RBX1 KO

embryos. The fact that these embryos did not survive to term, however, also indicates that the role of p27 is no longer critical beyond E9.5 and that the accumulation of other substrates of CRLs that rely on RBX1 plays a role in late embryonic mortality.

10.5 FBXW1 (β-TrCP1) and FBXW11 (β-TrCP2)

β-TrCP (β-transducin repeat-containing protein) is encoded by a single gene in invertebrates, but mammalian genomes harbor two different *β-TrCP* genes that encode proteins—β-TrCP1 (FBXW1) and β-TrCP2 (FBXW11)—that share 88% amino acid sequence identity. Molecular characterization of β-TrCP was accelerated by the identification in substrate proteins of a short peptide motif—the β-TrCP degron (DSGXXS)—that is recognized by the WD40 domain of β-TrCP when its two serine residues are phosphorylated. To date, >50 proteins have been identified as targets of β-TrCP-mediated ubiquitylation, with such ubiquitylation playing a role in key signaling pathways including the Wnt (through β-catenin degradation), Sonic hedgehog (through Gli processing and degradation), and NF-κB (through IκB degradation and NF-κB/Rel processing) signaling pathways (Frescas and Pagano 2008; Nakayama and Nakayama 2006), suggesting that the loss of β-TrCP would be detrimental to normal physiology in mice. However, β-TrCP1 KO mice showed no gross tissue abnormalities up to 16 months of age, with only moderate disruption of spermatogenesis and fertility being apparent in male mice (Guardavaccaro et al. 2003; Nakayama et al. 2003) as well as loss of a specific cell type in the retina in both sexes (Baguma-Nibasheka and Kablar 2009). Given that the biochemical functions of β-TrCP1 and β-TrCP2 were thought to be indistinguishable, one interpretation of these findings could be that β-TrCP2 continued to target β-TrCP substrates for ubiquitylation in the absence of β-TrCP1. To test this hypothesis, we then generated β-TrCP2 KO mice and surprisingly found that these mice were not recovered at birth but were absorbed before E9.5 (Nakagawa et al. 2015), indicating that β-TrCP1 could not compensate for the loss of β-TrCP2 during embryonic development. As is often observed for embryos that die around this time, we detected an abnormal vasculature in the yolk sac and massive apoptosis throughout the embryo itself, probably due to malnutrition. However, the molecular targets of β-TrCP2 that are responsible for this phenotype remain to be identified.

Simultaneous β-TrCP1 KO and doxycycline-inducible β-TrCP2 knockdown throughout the body of adult mice was found to result in a pronounced testicular phenotype characterized by impairment of spermatogenesis without any notable abnormality in other tissues, with this phenotype being attributed to accumulation of the β-TrCP substrate SNAIL in the testis (Kanarek et al. 2010). However, the widespread expression of β-TrCP1/2 in the testis, including in male germ cells and Sertoli cells, made it difficult to elucidate the molecular mechanism underlying the contribution of β-TrCP to spermatogenesis. We therefore generated male germ cell-specific and Sertoli cell-specific β-TrCP1/2 DKO (double knockout) mice by combining whole-body β-TrCP1 KO and Cre-mediated, cell type-specific β-TrCP2 KO with the use of a *Stra8–Cre* transgene for germ cells or an *Amh–Cre* transgene for Sertoli cells. Loss of β-TrCP1/2 in male germ cells did not result in the accumulation of the transcription factor SNAIL in these cells, even though the mice were completely sterile (Nakagawa et al. 2017). To identify the β-TrCP substrate (or substrates) responsible for this failure of spermatogenesis, we searched for proteins annotated as spermatogenic regulators that contain the β-TrCP degron. This search identified DMRT1 (doublesex- and Mab3-related transcription factor 1) as a novel substrate of β-TrCP at the mitosis-to-meiosis transition, and we showed that the impaired degradation of DMRT1 inhibits entry into meiosis and was thus responsible, at least in part, for the spermatogenic failure in the β-TrCP1/2 DKO mice. The lack of a change in SNAIL protein level in our germ cell-specific β-TrCP1/2 KO mice led us to hypothesize that SNAIL might be regulated by β-TrCP1/2 in

Sertoli cells and that β-TrCP1/2 in such cells also might play a key role in male germ cell development. Indeed, Sertoli cell-specific β-TrCP1/2 DKO male mice were sterile and showed accumulation of SNAIL in Sertoli cells as well as downregulation of the expression of E-cadherin—which is encoded by a SNAIL target gene—at the periphery of seminiferous tubules (Morohoshi et al. 2019). These results indicate that β-TrCP1/2 regulate the interaction between Sertoli cells and germ cells through degradation of SNAIL in Sertoli cells, with such regulation being critical for male germ cell development.

Intestinal epithelium-specific and neuron-specific β-TrCP1/2 DKO mice have also been investigated. Given that the accumulation of β-catenin as a result of mutation of its β-TrCP degron has been shown to drive colorectal carcinogenesis (Kuipers et al. 2015; Sparks et al. 1998), loss of β-TrCP in the intestinal epithelium was also expected to give rise to colorectal neoplasia. However, tamoxifen-induced loss of β-TrCP1/2 in the intestinal epithelium of adult mice (achieved with the use of a *villin–Cre–ER* transgene) resulted in death within 1 week associated with disruption of the colonic epithelial barrier and severe inflammation dependent on the induction of interleukin-1β (Kanarek et al. 2014). The mutant mice showed an abnormal mitotic index as well as DNA damage in the intestine, indicating that chromosomal instability was responsible for the excessive induction of interleukin-1β. How the loss of β-TrCP1/2 results in chromosomal instability remains to be elucidated, however.

Analysis of neuron-specific DKO mice has also revealed that β-TrCP1/2 play a critical role in the generation of circadian rhythm in the brain. The circadian clock in mammals is dependent on a cell-autonomous, negative-feedback loop in which the clock proteins CLOCK and BMAL1 activate the expression of PER1 (Period 1) and PER2 as well as CRY1 (cryptochrome 1) and CRY2. PER1/2 and CRY1/2 then repress the activity of CLOCK/BMAL1, closing the autoregulatory loop (Takahashi 2017). In addition to transcriptional regulation, PER1/2 and CRY1/2 are subjected to proteasomal degradation directed by F-box proteins so as to ensure the accuracy of the 24-hour rhythm. PER1/2 are targeted for degradation by β-TrCP, whereas CRY1/2 are targeted by FBXL3. Consistent with this scenario, mice with only one (β-*TrCP1*$^{+/-}$$\beta$-*TrCP2*$^{-/-}$) or no ($\beta$-*TrCP1*$^{-/-}$-$\beta$-*TrCP2*$^{-/-}$) *β-TrCP* allele as a result of tamoxifen-induced systemic deletion of the *β-TrCP2* gene on the β-*TrCP1*$^{+/-}$ or β-*TrCP1*$^{-/-}$ background, respectively, showed longer circadian periods and arrhythmicity in association with the stabilization and accumulation of PER proteins (D'Alessandro et al. 2017). The same phenotypes were also observed in neuron-specific β-TrCP1/β-TrCP2 DKO mice generated with the use of an *Scg2 (secretogranin 2)–TA* and *TetO–Cre* system driven by the neuron-specific promoter of the *Scg2* gene, indicating that β-TrCP regulation of PER in neurons is key to the robustness of the circadian rhythm in mice. Whether β-TrCP substrates other than PER proteins also contribute to circadian rhythmicity remains to be determined.

10.6 FBXW5

NASH (nonalcoholic steatohepatitis) is the most prevalent liver disease and a major cause of cirrhosis and hepatocellular carcinoma (Younossi et al. 2018). ASK1 (apoptotic signal-regulating kinase 1, also known as MAP 3K5) has been identified as a therapeutic target for NASH, with an ASK1 inhibitor currently undergoing phase III trials (Sumida and Yoneda 2018). FBXW5 was recently found to function as an activator of ASK1 by mediating its Lys^{63}-linked ubiquitylation. Its contribution to the pathogenesis of NASH was also demonstrated by the observation that hepatocyte-specific FBXW5 KO mice (generated with an *albumin–Cre* transgene) are resistant to the development of hepatic steatosis, fibrosis, and inflammation induced by a high-fat, high-cholesterol diet (Bai et al. 2019). Molecular analysis revealed that the activation of ASK1 and its downstream kinases JNK (c-Jun NH_2-terminal kinase) and p38 MAPK (mitogen-activated protein kinase) induced by this diet in the liver of

wild-type mice was attenuated in the KO mice, indicating that FBXW5 is required for aberrant activation of ASK1 in response to metabolic stress in the liver. Ablation of ASK1 in a hepatocyte cell line markedly inhibited the enhancing effects of FBXW5 overexpression on the activation of JNK and p38 MAPK as well as on lipid accumulation induced by exposure to palmitic acid and oleic acid. These observations support the notion that suppression of FBXW5 is also a promising strategy for the treatment of NASH. A detailed phenotypic analysis of whole-body FBXW5 KO mice is awaited for the assessment of the side effects of FBXW5 inhibition.

10.7 FBXW7 (SEL-10, hCDC4, or hAGO)

FBXW7 (also known as FBW7, SEL-10, hCDC4, or hAGO) is best characterized as a tumor suppressor protein. Consistently, it has been demonstrated that FBXW7 ubiquitylates and degrades several oncogenic proteins, and loss-of-function mutations in *Fbxw7* gene are frequently observed in a variety of human cancers (Davis et al. 2014; Shimizu et al. 2018). KO mouse studies not only confirmed anti-oncogenic properties and its mechanisms of FBXW7 but also revealed its contribution to cellular differentiation in relation to tissue and cancer stem cell maintenance.

Fbxw7$^{-/-}$ embryos died in utero at E10.5 with impaired vascular development in the brain and the yolk sac (Tetzlaff et al. 2004b; Tsunematsu et al. 2004). This impairment in vascular development was attributed to a defect in endothelial cells, given that Fbxw7 mRNA was detected predominantly in endothelial lineages at E9.5 and also mesoderm-derived para-aortic splanchnopleural explants isolated from *Fbxw7*$^{-/-}$ embryos did not form vascular network on stromal cells in vitro (Tsunematsu et al. 2004). Since FBXW7 substrate NOTCH4 was accumulated in extracts prepared from *Fbxw7*$^{-/-}$ embryos and its downstream target Hey1 mRNA was markedly increased in the vessels of these embryos, endothelial abnormalities are likely resulted from the dysregulation of Notch signaling. However, genetic rescue experiments were not performed to confirm that NOTCH4 accumulation is the primary cause of embryonic lethality. *Fbxw7*$^{+/-}$ mice appeared healthy and fertile but showed increased susceptibility to radiation-induced tumorigenesis, especially in the background of *p53*$^{+/-}$ or *Pten*$^{+/-}$ (Kwon et al. 2012; Mao et al. 2004), supporting the notion that FBXW7 is a tumor suppressor.

A high frequency of *Fbxw7* gene mutations is observed in T-ALL (T-cell acute lymphoblastic leukemia) (Akhoondi et al. 2007; Malyukova et al. 2007; Maser et al. 2007; O'Neil et al. 2007; Thompson et al. 2007). In agreement with this observation, mice in which *Fbxw7* gene was conditionally deleted in T lymphocytes with the use of a *Lck–Cre* or a *Cd4–Cre* transgene exhibited a thymic hyperplasia and subsequent development of T-cell lymphoma (Onoyama et al. 2007; Thompson et al. 2008). NOTCH1, NOTCH3, and MYC, positive regulators of cell cycle progression, were accumulated in FBXW7-deficient immature T cells, and these cells could not stop proliferation at CD4 and CD8 DP (double positive) stage when wild-type cells normally exit cell cycle. Importantly, this defect of cell cycle arrest was rescued by deletion of *Myc* gene, demonstrating that the accumulation of MYC in FBXW7-deficient DP thymocytes is responsible for the failure of cell cycle exit and the consequent hyperproliferation phenotype. In contrast to immature T cells, mature FBXW7-deficient T cells underwent apoptosis in response to mitogenic stimulation with the accumulation of MYC and p53. This abnormal apoptosis could be corrected by the additional deletion of *p53* gene. Since p53 did not seem to be a substrate of FBXW7, it is likely that accumulation of p53 was caused by increased MYC protein level (Zindy et al. 1998). These data demonstrated that FBXW7 regulates proliferation and apoptosis in a manner dependent on the differentiation stage in T-cell lineage.

Conditional deletion of *Fbxw7* in murine HSCs (hematopoietic stem cells) with the use of *Mx1–Cre* transgene and poly(I:C) injection led to severe leukopenia resulting from loss of self-

renewal activity and exhaustion of quiescent HSCs with accumulation of NOTCH1 and MYC (Matsuoka et al. 2008; Thompson et al. 2008). Since enforced MYC expression in HSCs also led to loss of self-renewal activity (Wilson et al. 2004), MYC appears to be the main substrate of FBXW7 in HSC maintenance. Interestingly, most of the HSC-specific FBXW7-deficient mice that did not exhibit leukopenia showed extrathymic development of DP T cells and T-ALL in which p53 protein level is reduced (Matsuoka et al. 2008). In addition, loss of p53 significantly promoted T-cell leukemogenesis in these mice. These findings suggest that deletion of FBXW7 provides a selective advantage to hematopoietic cells that harbor suppressed p53 function. How FBXW7-deficient cells evaded cell death in the setting of elevated MYC protein level has remained elusive, but anti-apoptotic MCL-1 might be involved because it was shown to be a substrate of FBXW7 and accumulated in thymic lymphoma and acute lymphoblastic leukemia cells from mice with the T-cell lineage-specific deletion of FBXW7 (Inuzuka et al. 2011).

The role of FBXW7 in tumor metastasis through regulating microenvironment was also revealed by the analysis of mice in which FBXW7 is depleted in HSCs with the use of a *Mx1–Cre* transgene and poly(I:C) injection (Yumimoto et al. 2015). These mice were susceptible to metastasis of injected melanoma and lung cancer cells and breast cancer cells to the lung. Mechanistically, it was demonstrated that FBXW7 KO in bone marrow-derived stromal cells resulted in the accumulation of NOTCH1, leading to the upregulation of CCL2 chemokine which likely promoted the formation of metastatic niches through recruitment of monocytic myeloid-derived suppressor cells and macrophages. These results suggest that FBXW7 antagonizes cancer development in both cell-autonomous and non-cell-autonomous manners.

Mice with HSC population expressing BCR–ABL, a fusion protein found in the CML (chronic myeloid leukemia) patients, develop CML, and thus these mice serve as a mouse model of CML pathogenesis (Pear et al. 1998). Intriguingly, loss of FBXW7 in BCR–ABL-expressing cells (generated by retroviral introduction of BCR–ABL to isolated HSCs from *Mx1–Cre; Fbxw7*$^{flox/flox}$ mice combined with tamoxifen injection) was shown to suppress progression of CML through enhanced apoptosis resulting from accumulation of MYC and p53 (Reavie et al. 2013; Takeishi et al. 2013). Analysis of FBXW7-ablated CML leukemic cells further elucidated the reduction of quiescent LICs (leukemia-initiating cells), indicating that FBXW7 is essential for the maintenance of LIC dormancy. Consistent with the hypothesis that LIC dormancy is the mechanism by which CML shows resistance to anticancer drugs, combination of FBXW7 KO and an anticancer drug (either imatinib or cytosine arabinoside) was found to markedly enhance the anticancer effect, supporting the notion that the "wake-up" therapeutic strategy combining anticancer agents with FBXW7 inhibition which sensitizes LICs to these drugs by putting LICs out of dormant state is potentially effective for eradicating LICs (Takeishi and Nakayama 2016).

In contrast to T-ALL, HSC-specific FBXW7-deficient mice did not develop B-cell lymphoma. Consistently, the mutation rate of *Fbxw7* gene is extremely low in human B-cell lymphoma (Akhoondi et al. 2007; Song et al. 2008). To examine the difference in sensitivity to *Fbxw7* mutation in T lymphocytes and B lymphocyte, we recently generated B-lymphocyte-specific FBXW7 KO mice with the use of a *Cd19–Cre* transgene and found that FBXW7 is essential for B-lymphocyte survival (unpublished data), at least in part by destabilizing nuclear NF-κB2/p100 which was previously reported to be a substrate of FBXW7 (Arabi et al. 2012; Busino et al. 2012; Fukushima et al. 2012).

Fbxw7 mutations are also found in other human cancers, including early stage human colon adenomas (Rajagopalan et al. 2004). Deletion of FBXW7 in mouse intestinal epithelium with the use of a *villin–Cre* transgene was reported to cause an increase in transit-amplifying progenitor cells accompanied by a reduction of goblet and Paneth cells (Babaei-Jadidi et al. 2011; Sancho et al. 2010). These mice developed polyp-

like structures and adenomas, but not carcinomas, indicating that loss of FBXW7 alone is not sufficient to cause intestinal carcinomas. When *Apc*^{min} mono-allele bearing a nonsense mutation at codon 850 of the *Apc* gene was introduced (Fodde 2002), adenoma was developed with much shortened latency. Even in this condition, neoplasms did not progress beyond the adenoma stage and were neither invasive nor metastatic. Intestinal extracts prepared from FBXW7-deficient mice exhibited increased abundance of NOTCH1, NOTCH4, and c-JUN. Importantly, *c-Jun* gene deletion in *Apc*$^{min/+}$, *villin–Cre*, and *Fbxw7*$^{flox/flox}$ mice reduced tumor area to the size comparable with *Apc*$^{min/+}$ tumors, indicating that accumulated c-JUN is responsible for the increase in tumor size resulting from loss of FBXW7 (Sancho et al. 2010). In contrast to heterozygous deletion of *Apc*, *p53* codeletion with *Fbxw7* in the intestinal epithelium caused penetrant, aggressive, and metastatic adenocarcinomas (Grim et al. 2012). These tumors exhibited a sign of chromosomal instability, a commonly observed phenotype in human colorectal cancers. Although the molecular mechanisms were not evident, it was also noted that loss of p53 reverted the number of Paneth cells, but not goblet cells (Grim et al. 2012). These results suggested that FBXW7 contributes to not only proliferation arrest but also differentiation of intestinal cells, some of which depend on p53 activity.

The significant role of FBXW7 in cellular differentiation was also illustrated by the analysis of hepatocyte-, neural cell-, or spermatogonium-specific FBXW7 KO mice. Hepatocyte-specific FBXW7 KO mice with the use of an *albumin–Cre* or a *Mx1–Cre* transgene combined with poly (I:C) injection exhibited hepatomegaly and steatohepatitis probably as a result of stabilization of SREBP (sterol regulatory element-binding protein) and alteration of its downstream target gene expression (Onoyama et al. 2011). Loss of FBXW7 also caused skewed hepatic differentiation toward the cholangiocyte lineage rather than the hepatocyte lineage with accumulation of NOTCH1. Concomitant deletion of NOTCH cofactor RBPJ could rescue this defect, indicating that the skewed developmental orientation of hepatic stem cells to the cholangiocyte lineage is dependent on NOTCH1 accumulation induced by the loss of FBXW7.

Mice with NSPC (neural stem/progenitor cell)-specific deletion of FBXW7 with the use of a *nestin–Cre* transgene exhibited impaired stem cell differentiation to the neurons and increased progenitor cell death. These mice died perinatally due to the absence of suckling behavior (Hoeck et al. 2010; Matsumoto et al. 2011). Genetic and pharmacologic rescue experiments demonstrated that accumulated NOTCH1 was responsible for differentiation defects, while increase in c-JUN contributed to neural cell viability.

Deletion of FBXW7 in SSCs (spermatogonial stem cells) with the use of a *Stra8–Cre* transgene led to accumulation of undifferentiated spermatogonia with concomitant reduction of differentiating and mature germ cells (Kanatsu-Shinohara et al. 2014). Among the FBXW7 substrates tested, accumulation of MYC and cyclin E1 was noted in germ cells from FBXW7-deficient pup testes, and overexpression of MYC, but not cyclin E1, phenocopied effects of FBXW7 loss to enhance colonization of germ line stem cells. These data demonstrated that FBXW7 counteracts with positive regulators of self-renewal by degrading MYC to induce differentiation of SSCs.

Taken together, these various observations suggest that FBXW7 plays a critical role in proliferation and tumorigenesis as well as stem cell differentiation in different tissues by targeting tissue-specific substrates for degradation.

10.8 FBXW8

FBXW8 is a placenta- and embryo-specific F-box protein that binds to CUL1 through SKP1 but also interacts directly with CUL7. Although the significance of this association with two cullins is unknown, the fact that *FBXW8* mutations have not been identified in individuals with 3M syndrome, a rare growth disorder that is thought to be caused by the loss of CUL7 function, has suggested that FBXW8 is not absolutely required for CUL7 function (Li et al. 2014; Yan et al.

2014). This conclusion has also been supported by studies of KO mice.

About two-thirds of FBXW8 KO mouse embryos were found to die in utero beginning at E12.5 as a result of abnormal placental development and growth retardation, whereas the remaining one-third of embryos grew to adulthood but were smaller than their littermates throughout life (Tsunematsu et al. 2006; Tsutsumi et al. 2008). Histological analysis revealed aberrant development of spongiotrophoblasts in the placenta of the KO mice. Although CUL7 KO mice also exhibited an abnormally thin spongiotrophoblast layer, they did not show marked embryonic mortality. However, CUL7 KO neonates died soon after birth as a result of respiratory failure (Arai et al. 2003), which was not apparent in FBXW8 KO mice. Together with the observation that the stability of the FBXW8 protein was decreased in the absence of CUL7 (Arai et al. 2003; Tsunematsu et al. 2006), these results suggest that the role of CUL7 in placental development relies, at least in part, on FBXW8, whereas that in neonatal, lung function is independent of FBXW8. The FBXW8 substrates responsible for defective placental development in FBXW8 KO mice remain to be identified.

Phenotypes of FBXW protein KO mice described herein with identified primary substrates are summarized in Table 10.1.

10.9 FBXL1 (SKP2)

SKP2 (S-phase kinase-associated protein 2), also known as FBXL1, is the prototypical and best-characterized mammalian FBXL protein. SKP2 promotes cell proliferation by targeting negative regulators of the cell cycle for degradation, and its role as an oncoprotein has been supported by the fact that it is overexpressed in many cancers (Frescas and Pagano 2008; Nakayama and Nakayama 2006). SKP2 KO mice were found to be born approximately in the expected Mendelian ratio, but they were smaller than their wild-type littermates (Nakayama et al. 2000). The cellular phenotypes of these mice included nuclear enlargement and polyploidy in the liver, lung, kidney, and testis as well as an increased number of centrosomes in mouse embryonic fibroblasts. Providing support for a critical role of SKP2 in tumor progression, SKP2 KO mice were shown to be resistant to tumor development induced by loss of either the tumor suppressor proteins $p19^{ARF}$ or PTEN (phosphatase and tensin homolog) (Lin et al. 2010).

Although many substrates of SKP2 have been identified in cultured cells (Frescas and Pagano 2008; Nakayama and Nakayama 2006), p27 seems to be a key substrate, given that SKP2 KO mice showed marked accumulation of p27 (Nakayama et al. 2000) and that prominent cellular phenotypes of these mice were no longer apparent in SKP2 and p27 DKO mice (Kossatz et al. 2004; Nakayama et al. 2004).

10.10 FBXL3 and FBXL21

Screening of randomly mutagenized mice to detect genes related to circadian rhythm led to the identification of the *after-hours* and *overtime* mutants, both of which manifested a prolonged circadian cycle. Genetic analysis of both these mice revealed mutations in *Fbxl3*, the loss of function of which was found to result in the accumulation of CRY1/2 proteins (Busino et al. 2007; Godinho et al. 2007; Siepka et al. 2007).

Interestingly, FBXL21, a paralog of FBXL3, interacts more strongly with CRY1/2 than does FBXL3 and protects CRY1/2 from $CRL1^{FBXL3}$ activity, resulting in CRY1/2 stabilization (Hirano et al. 2013; Yoo et al. 2013). Importantly, the lengthening of the circadian cycle apparent in FBXL3 KO mice was partially reversed by simultaneous deletion of *Fbxl21*, providing support for the notion that FBXL21 antagonizes the function of FBXL3 in regulation of CRY1/2 stability and circadian rhythm in mice (Hirano et al. 2013). In contrast to FBXL3 KO mice, FBXL21 single KO mice did not exhibit abnormal rhythmic behaviors with a period indistinguishable from that of wild-type mice. However, these mice exhibited a decrease in wheel-running activities near the

Table 10.1 Phenotypes of FBXW protein KO mice including identified primary substrates

F-box protein	Systemic or conditional KO	Phenotype of KO mice	Primary substrate	References
β-TrCP1 (FBXW1)	Whole body	Male subfertility, abnormal retinal development	ND	Baguma-Nibasheka and Kablar (2009), Guardavaccaro et al. (2003) and Nakayama et al. (2003)
β-TrCP2 (FBXW11)	Whole body	Embryonic lethality	ND	Nakagawa et al. (2015)
β-TrCP1+2	Spermatogonia	Male sterility	DMRT1	Nakagawa et al. (2017)
β-TrCP1+2	Sertoli cells	Male sterility	SNAIL	Morohoshi et al. (2019)
β-TrCP1+2	Intestinal epithelial cells	Disruption of colonic epithelial barrier	ND	Kanarek et al. (2014)
β-TrCP1+2	Neuronal cells	Abnormal circadian rhythm	PER1/2	D'Alessandro et al. (2017)
FBXW5	Hepatocytes	Resistance to nonalcoholic steatohepatitis	ASK1	Bai et al. (2019)
FBXW7	Whole body	Embryonic lethality	ND	Tetzlaff et al. (2004b) and Tsunematsu et al. (2006)
FBXW7	T cells	Development of T-cell lymphoma	MYC	Onoyama et al. (2007) and Thompson et al. (2008)
FBXW7	Hematopoietic stem cells	Loss of self-renewal activity	ND	Matsuoka et al. (2008) and Thompson et al. (2008)
FBXW7	Bone marrow stromal cells	Increased susceptibility to cancer metastasis	NOTCH1	Yumimoto et al. (2015)
FBXW7	Chronic myeloid leukemia cells	Reduction of quiescent leukemia-initiating cells	MYC	Reavie et al. (2013) and Takeishi et al. (2013)
FBXW7	Intestinal epithelial cells	Development of adenoma	c-JUN	Babaei-Jadidi et al. (2011) and Sancho et al. (2010)
FBXW7	Hepatocytes	Steatohepatitis	SREBP	Onoyama et al. (2011)
FBXW7	Hepatocytes	Skewed differentiation to the cholangiocytes	NOTCH1	Onoyama et al. (2011)
FBXW7	Neural stem/ progenitor cells	Impaired differentiation to the neurons	NOTCH1	Hoeck et al. (2010) and Matsumoto et al. (2011)
FBXW7	Neural stem/ progenitor cells	Reduced viability	c-JUN	Hoeck et al. (2010) and Matsumoto et al. (2011)
FBXW7	Spermatogonial stem cells	Impaired differentiation	MYC	Kanatsu-Shinohara et al. (2014)
FBXW8	Whole body	Partial embryonic lethality, abnormal placental development, small size	ND	Tsunematsu et al. (2006) and Tsutsumi et al. (2008)

ND not determined, *NA* not applicable

subjective dawn, though the underlying mechanism has yet to be elucidated (Hirano et al. 2013).

10.11 FBXL5

FBXL5 plays a central role in cellular iron metabolism. Cellular and organismal iron levels are strictly controlled by hormonal as well as cell-intrinsic mechanisms because iron is required for various biological processes but in excess contributes to the generation of toxic ROS (reactive oxygen species) (Hentze et al. 2010; Muckenthaler et al. 2017). In particular, the IRP (iron-regulatory protein) and IRE (iron-responsive element) system functions prominently to maintain the cellular iron level in balance (Wilkinson and Pantopoulos 2014). In response to iron deprivation, IRP1 loses its [4Fe–4S] cluster, and the apo-IRP1 protein then

binds to IREs present in the mRNAs for various proteins related to iron trafficking, storage, or utilization. This binding modulates production of the encoded proteins and thereby increases the availability of intracellular iron. In contrast to IRP1, IRP2 does not contain an Fe–S cluster that can function in iron sensing, but its abundance is regulated by FBXL5 in an iron-dependent manner. Under iron-replete conditions, FBXL5 constitutively ubiquitylates IRP2 and thereby targets it for proteasomal degradation. A decline in iron availability results in destabilization of FBXL5 and the consequent stabilization of IRP2. This scenario suggests that loss of FBXL5 would result in the accumulation of IRP2 even in the presence of sufficient iron, which in turn would lead to a buildup of iron, the excessive generation of ROS, and the induction of cellular damage. Disruption of *Fbxl5* in mice indeed resulted in constitutive accumulation of IRP2 and misexpression of its target genes (Moroishi et al. 2011). The FBXL5 KO mice died during embryogenesis at ~E8.5 as a result of overwhelming oxidative stress, most noticeably in extraembryonic tissues in which iron is supplied from the mother. Importantly, concomitant deletion of *Irp2* completely rescued the embryonic lethality induced by FBXL5 loss, showing that IRP2 is a primary substrate of FBXL5.

Specific deletion of FBXL5 in the liver (with the use of an *albumin–Cre* transgene), which controls systemic iron levels, also resulted in IRP2 accumulation in this organ, leading to iron buildup, liver damage, and the development of steatohepatitis (Moroishi et al. 2011). The serum iron concentration was also increased in these mice, whereas the secretion of hepcidin, which negatively regulates iron availability, was unexpectedly decreased, with this effect being attributable to a decrease in BMP (bone morphogenetic protein) signaling of unknown cause and contributing to systemic iron overload and increased sensitivity to a high-iron diet.

Given that iron overload is linked to several neurodegenerative diseases (Rouault 2013), NSPC-specific FBXL5 KO mice were generated with the use of a *nestin–Cre* transgene in order to investigate this relation (Yamauchi et al. 2017). These mice died within a day of delivery as a result of respiratory failure, indicative of disruption of the control of respiration by the nervous system. The brain of these FBXL5 KO mouse embryos revealed increased proliferation and attenuated neuronal differentiation of NSPCs. It also manifested increased levels of IRP2 and iron, resulting in the generation of ROS and activation of the PI3K (phosphoinositide 3-kinase)–AKT–mTOR (mammalian target of rapamycin) signaling pathway. Importantly, treatment with either a PI3K inhibitor or an mTOR inhibitor rescued the proliferation defect of FBXL5-deficient NSPCs in vitro, indicating that activation of ROS–PI3K–AKT–mTOR signaling by IRP2-mediated iron overload is responsible for the dysfunction of these cells.

Hematopoiesis is particularly sensitive to iron levels (Camaschella 2015; Fleming and Ponka 2012). Ablation of FBXL5 specifically in HSCs of mice with the use of an *Mx1–Cre* transgene and poly(I:C) injection resulted in impairment of the repopulation ability of these cells (Muto et al. 2017). This impairment was due to the accumulation of IRP2, iron overload, and oxidative stress that promoted exit of the cells from quiescence and their premature exhaustion. Importantly, ablation of IRP2 restored the repopulation ability of the FBXL5-deficient HSCs, indicating that FBXL5 supports HSC function primarily through suppression of IRP2 activity.

10.12 FBXL10 (KDM2B) and FBXL11 (KDM2A)

FBXL10 (also known as KDM2B) binds to SKP1 through its F-box domain, but it does not appear to associate with CUL1 (Koyama-Nasu et al. 2007). Instead, FBXL10 seems to function primarily in epigenetic chromatin regulation. FBXL10 binds to CpG islands in the genome through a CXXC domain and recruits PRC1 (polycomb repressive complex 1), which catalyzes ubiquitylation of histone H2A. In addition, FBXL10 removes methyl groups attached to Lys^{36} of histone H3 (H3K36) in a reaction mediated by its JmjC domain. FBXL10 exists as

two isoforms as a result of alternative use of exon 1, with the short isoform (isoform 2) lacking the JmjC domain. This situation affords the opportunity to investigate the function of the H3K36 demethylase activity of FBXL10 by targeted exon disruption. Mice lacking both isoforms of FBXL10 died in utero at ~E11.5 to E13.5 manifesting severe developmental abnormalities including small size, failure of neural tube closure, and limb and craniofacial malformation (Andricovich et al. 2016). The embryonic death was attributed primarily to defective hematopoiesis, given that the vasculature of the yolk sac was absent and that loss of FBXL10 only in the hematopoietic lineage (achieved with the use of a *Tie2–Cre* transgene) also induced death at the same embryonic stage as did systemic ablation. FBXL10 KO in adult hematopoietic stem cells with the use of a *Vav1–Cre* transgene or the *Mx1–Cre* transgene and poly(I:C) injection also resulted in a reduction in the number of long-term HSCs and defective lymphopoiesis, suggesting that FBXL10 is required for normal development of the hematopoietic lineage in both embryonic and adult mice. Transcriptomic analysis of hematopoietic progenitors of these HSC-specific FBXL10 KO mice revealed upregulation of the expression of PRC target genes as well as downregulation of that of transcription factor genes related to hematopoietic development. How many of these genes are direct targets of FBXL10 remains unknown, but chromatin immunoprecipitation sequencing analysis of human leukemic cell lines detected FBXL10 at the promoters of some genes whose expression was altered in the FBXL10 KO cells.

About one-half of mice with targeted disruption of the long isoform of FBXL10 (isoform 1) manifested failure of neural tube closure and exencephaly and died shortly after birth (Fukuda et al. 2011), indicating that isoform 2 is able to compensate for the loss of isoform 1 in embryonic hematopoiesis, which therefore must be independent of JmjC domain-catalyzed H3K36 demethylation. The molecular mechanism underlying these brain phenotypes is currently unknown. Disruption of only isoform 2, with isoform 1 remaining intact, also resulted in partial perinatal lethality associated with craniofacial abnormalities, cleft palate, and the eyes-open-at-birth phenotype (Boulard et al. 2016). Transcriptome-wide analysis of female embryos, which showed more severe phenotypes than did male embryos, revealed dysregulation of X-linked genes, possibly as a result of abnormal overexpression of *Xist*, which plays a key role in inactivation of one of the two X chromosomes in females. These observations implicated FBXL10 in X chromosome regulation, but again the molecular mechanism by which FBXL10 regulates *Xist* expression is not clear.

FBXL11 (also designated as KDM2A) has a structure similar to that of its paralog FBXL10, but it has not been shown to associate with PRC1 (Wu et al. 2013). FBXL11 deficiency in mice led to severe growth retardation with embryonic death at E10.5 to E12.5. The brain of the mutant mice manifested reduced cell proliferation, increased apoptosis, and arrested neuronal differentiation (Kawakami et al. 2015). Although expression of FBXL11 was found to be ubiquitous in the embryo proper, it was not examined in extraembryonic tissues. The mechanism by which loss of FBXL11 leads to embryonic death therefore remains to be investigated.

10.13 FBXL12

Four-fifths of FBXL12 KO mice were found to die soon after birth as a result of intrauterine growth retardation and a consequent inability to compete with their wild-type or heterozygous littermates for suckling (Nishiyama et al. 2015). The remaining one-fifth of the homozygous mutant embryos grew to adulthood but were smaller compared with $Fbxl12^{+/-}$ or $Fbxl12^{+/+}$ mice. FBXL12 is expressed specifically in the junctional zone of the mouse placenta, and loss of FBXL12 led to a reduction in the numbers of both spongiotrophoblasts and glycogen cells in this zone. A search for substrates of FBXL12 in a human trophoblast cell line with the use of the DiPIUS (differential proteomics-based identification of ubiquitylation substrates) system (Yumimoto et al. 2012) identified ALDH3A1

and ALDH3A2, enzymes that oxidize aldehydes to generate carboxylic acids. Indeed, these proteins were found to accumulate in the FBXL12 KO placenta, and inhibition of ALDH3 activity rescued the defective differentiation of trophoblast stem cells, indicating that accumulation of ALDH3 family proteins and a consequent increase in the concentration of toxic aldehydes were at least partially responsible for the placental abnormality of FBXL12 KO mice.

In adult mice, FBXL12 is highly expressed in the thymus, and examination of FBXL12 KO mice that survived the neonatal stage revealed impaired differentiation of CD4/CD8 DP T cells into CD4 or CD8 single-positive cells in association with the accumulation of ALDH3 (Nita et al. 2016), indicating that FBXL12 contributes to T-cell differentiation in adult mice by regulating the abundance of ALDH3.

10.14 FBXL20 (SCRAPPER)

FBXL20, also known as SCRAPPER, is highly expressed in the brain, where its localization at synapses is mediated by a fatty acid attached to its CAAX domain (Yao et al. 2007). FBXL20 directly binds to and ubiquitylates the presynaptic protein RIM1 (Rab3-interacting molecule 1) both in vitro and in vivo, and FBXL20 KO mice were found to show increased neurotransmitter release, probably as a result of the accumulation of RIM1. The studied mice had a hybrid 129Sv × C57BL/6 background and showed stochastic death shortly after birth, a reduced life span for those that survived the neonatal period, and a smaller body size compared with wild-type littermates. A subsequent study found that the lethality of *Fbxl20* deletion was more pronounced for mice backcrossed to the C57BL/6J background (Yao et al. 2011), indicating that genetic background markedly affects this phenotype of FBXL20 KO mice, although the factors contributing to this effect remain unknown.

Behavioral analysis revealed a lower level of freezing induced by foot shock or by subsequent placement in the apparatus where the foot shock had been delivered for $Fbxl20^{+/-}$ mice compared with wild-type littermates. Combined with the observed high level of expression of FBXL20 in the hippocampus (Yao et al. 2007), these results suggested that FBXL20 plays a role in the formation of contextual fear memories in this region of the brain. Whether or how RIM1 accumulation contributes to this phenotype awaits clarification.

Phenotypes of FBXL protein KO mice with identified primary substrates are summarized in Table 10.2.

10.15 FBXO1 (Cyclin F)

The F-box domain was first identified in cyclin F, also known as FBXO1. Similar to cyclin A, the expression of cyclin F begins in S phase of the cell cycle, peaks in G_2, and declines as cells enter mitosis (Bai et al. 1994). These changes in expression level during the cell cycle and the identification of cell cycle-related substrates for cyclin F such as RRM2 (ribonucleotide reductase 2) and CP110 (centrosomal protein 110) indicated that cyclin F contributes to regulation of cell proliferation (D'Angiolella et al. 2013). Loss of cyclin F in mice induced embryonic death between E9.5 and E10.5 as a result of extraembryonic defects (Tetzlaff et al. 2004a). Cyclin F was found to be expressed at a high level in the chorionic trophoblast layer, and the absence of cyclin F led to a reduced level of cell proliferation in this region and impairment of chorioallantoic fusion, which connects the mother to the embryo to allow the exchange of nutrients and metabolic waste products. Whether the attenuated trophoblast proliferation was causal for or merely correlative with the placental failure was not determined, and the cyclin F substrates responsible for this phenotype were not identified. Although cyclin F is expressed in all dividing tissues in both embryos and adult mice, conditional ablation of cyclin F in the eye (with the use of a *Pax6–Cre* transgene), bone (with an *α1-collagen–Cre* transgene), or gut and bladder (with an *FABP–Cre* transgene) did not give rise to notable defects, indicating that cyclin F is not essential for the development and physiology of at least these tissues (Tetzlaff et al. 2004a).

Table 10.2 Phenotypes of FBXL protein KO mice including identified primary substrates

SKP2 (FBXL1)	Whole body	Small size, polyploidy	p27	Kossatz et al. (2004) and Nakayama et al. (2000, 2004)
FBXL3	Whole body	Abnormal circadian rhythm	CRY1/2	Godinho et al. (2007) and Siepka et al. (2007)
FBXL5	Whole body	Embryonic lethality, abnormal placental development	IRP2	Moroishi et al. (2011)
FBXL5	Hepatocytes	Steatohepatitis	IRP2	Moroishi et al. (2011)
FBXL5	Neural stem/progenitor cells	Neonatal lethality, abnormal neuronal development	IRP2	Yamauchi et al. (2017)
FBXL5	Hematopoietic stem cells	Reduced number of hematopoietic stem cells	IRP2	Muto et al. (2017)
FBXL10 (KDM2B)	Whole body	Embryonic lethality	ND	Andricovich et al. (2016), Boulard et al. (2016) and Fukuda et al. (2011)
FBXL10	Hematopoietic stem cells	Reduced number of hematopoietic stem cells	ND	Andricovich et al. (2016)
FBXL11 (KDM2A)	Whole body	Embryonic lethality	ND	Kawakami et al. (2015)
FBXL12	Whole body	Partial neonatal lethality, abnormal placental development, small size	ALDH3	Nishiyama et al. (2015)
FBXL12	T cells	Abnormal T-cell development	ALDH3	Nita et al. (2016)
FBXL20	Whole body	Partial neonatal lethality, abnormal memory formation	ND	Yao et al. (2007, 2011)
FBXL21	Whole body	Decreased activities near the subjective dawn	CRY1/2	Hirano et al. (2013)

ND not determined, *NA* not applicable

Interest in cyclin F was recently ignited by the identification of mutations in its gene associated with familial and sporadic amyotrophic lateral sclerosis and frontotemporal dementia (Williams et al. 2016). Mouse models expressing these mutant forms of cyclin F have not yet been described, but their establishment is likely to provide important insight into pathological mechanisms.

10.16 FBXO2

FBXO2 is expressed in the brain, ears, and testis (Erhardt et al. 1998; Thalmann et al. 1997; Yoshida et al. 2003), specifically recognizes N-linked high-mannose oligosaccharides attached to proteins, and targets for ubiquitylation and degradation N-glycosylated proteins such as β1-integrin (Yoshida et al. 2002), the NR1 subunit of the NMDA subtype of glutamate receptors (Kato et al. 2005), and BACE1 (β-site amyloid precursor protein-cleaving enzyme 1), the latter of which contributes to amyloidosis in Alzheimer's disease (Gong et al. 2010). Mice with targeted deletion of FBXO2 developed accelerated age-related hearing loss starting at ~2 months of age without noticeable pathology in the brain (Nelson et al. 2007). Cellular degeneration was found to begin in the epithelial support cells of the organ of Corti and progressed to hair cells and the spiral ganglion. These observations thus indicated that FBXO2 is essential for homeostasis of the inner ear and that it acts to prevent age-related hearing loss. Of note, the abundance of SKP1 in the cochlea was reduced in parallel with the loss of FBXO2, suggesting that FBXO2 somehow stabilizes SKP1 in the ear. The relation between hearing loss and the potential accumulation of N-glycosylated proteins in the FBXO2 KO mice, however, remains unclear.

10.17 FBXO4

FBXO4 binds to αB-crystallin (also known as HSPB5), and the two proteins assemble with the other CRL1 components to form an active

ubiquitin ligase that has been thought to target cyclin D1 for degradation and thereby to suppress oncogenesis (Barbash et al. 2008; Lin et al. 2006). *Fbxo4*$^{+/-}$ and *Fbxo4*$^{-/-}$ mice were thus found to accumulate cyclin D1 and to develop multiple types of tumor, including lymphoma, histiocytic sarcoma, and, less frequently, mammary and hepatocellular carcinoma (Vaites et al. 2011). In contrast to these findings, FBXO4 KO mice generated by our group did not show accumulation of cyclin D1 or development of tumors for up to 1 year of age (Kanie et al. 2012). We also obtained evidence that CRL1 does not play a role in cyclin D1 degradation, despite several previous studies having indicated that FBXO4 (Barbash et al. 2008; Lin et al. 2006), FBXW8 (Okabe et al. 2006), SKP2 (Yu et al. 1998), and FBXO31 (Santra et al. 2009) ubiquitylate cyclin D1 for proteasomal degradation. The reason of these discrepancies remains unknown.

10.18 FBXO5 (EMI1) and FBXO43 (EMI2)

EMI1 (early mitotic inhibitor 1), also known as FBXO5, plays a key role in cell cycle progression. Although EMI1 forms a CRL1 ubiquitin ligase (Marzio et al. 2019; Reitsma et al. 2017), the primary function of EMI1 is thought to be inhibition of APC/C ubiquitin ligase activity in G_1 phase in order to promote DNA replication, in S phase to prevent DNA rereplication, and in G_2-M phase to ensure proper mitotic progression (Lara-Gonzalez et al. 2017). The loss of EMI1 would therefore be expected to impair cell cycle progression, an effect not compatible with embryonic development. Indeed, EMI1 KO mouse embryos were found to die at the preimplantation stage, with the EMI1-deficient cells manifesting abnormal mitosis (Lee et al. 2006). However, EMI1-deficient embryos showed no obvious defects in entry into or progression through S phase, indicating that EMI1 plays a nonredundant role in M-phase progression during early embryonic development but that its role in S-phase regulation is redundant and can be undertaken by other proteins.

The EMI1 paralog EMI2 (early mitotic inhibitor 2, also known as FBXO43) contributes specifically to germ cell development. Although the ability of EMI2 to inhibit APC/C activity appears to be redundant with that of EMI1, the expression of EMI2 is restricted to reproductive organs where germ cells are produced (Shoji et al. 2006). In frog and mouse oocytes, the role of EMI2 as a component of cytostatic factor, which is required for the establishment and maintenance of meiotic metaphase II arrest, has been well characterized (Madgwick and Jones 2007). Consistent with this role, female EMI2 KO mice were found to be viable but infertile, with their oocytes showing defects in meiosis II (Gopinathan et al. 2017). Male EMI2 KO mice were also sterile, indicating that EMI2 is essential for meiosis in both males and females. In spermatogenesis, EMI2 was found to be essential for meiosis I progression at the early diplotene stage. Increased APC/C activity in the absence of EMI2 would be expected to result in excessive degradation of its substrate cyclin B and consequent inactivation of CDK1 (cyclin-dependent kinase 1). Importantly, crossing of EMI2 KO mice with a knock-in mouse line with elevated CDK1 activity rescued the meiosis I arrest apparent in EMI2-deficient male germ cells, supporting the notion that APC/C inhibition is the primary function of EMI2 in early spermatogenesis.

10.19 FBXO7

A genome-wide association study identified autosomal recessive mutations in *FBXO7* (also known as *PARK15*) in individuals with a juvenile form of familial Parkinson's disease (Shojaee et al. 2008). Parkinson's disease is typically characterized by motor deficits due to loss of midbrain dopaminergic neurons with intracellular inclusions containing aggregates of α-synuclein (Kalia and Lang 2015; Poewe et al. 2017). Support for a causative role of FBXO7 loss in this disease was provided by analysis of FBXO7 KO mice (Vingill et al. 2016). Systemic deletion of FBXO7 in mice thus gave rise to defects in locomotor activity and death within 1 month after birth. Conditional

FBXO7 KO in the forebrain with the use of a *Nex–Cre* transgene also resulted in the development of motor defects, whereas loss of FBXO7 in catecholaminergic (including dopaminergic) neurons induced with a *tyrosine hydroxylase–Cre* transgene gave rise to much milder motor coordination problems that were apparent only in older mice challenged with forced movement. These results demonstrated a key role for FBXO7 expressed in forebrain non-dopaminergic neurons in coordination with accurate movement in mice. The same study identified the PSMA2 core subunit of the proteasome as a substrate of FBXO7. $CRL1^{FBXO7}$ was thus found to mediate the polyubiquitylation of PSMA2 via a ubiquitin-Lys^{63} linkage, but this modification did not result in the degradation of PSMA2. Loss of FBXO7 resulted in the attenuation of the assembly and activity of the proteasome, indicating that FBXO7 promotes proteasome function through ubiquitylation of PSMA2. Although proteasome dysfunction has been linked to Parkinson's disease, it remains unclear whether dysregulation of the proteasome is the primary cause of the motor deficits in FBXO7 KO mice.

10.20 FBXO8

Downregulation of FBXO8 expression is frequently observed in glioma as well as in hepatocellular, gastric, and colorectal cancer (Wang et al. 2013, 2017; Wu et al. 2015; Yu et al. 2014). FBXO8 KO mice were found to be more susceptible than their wild-type littermates to the development of colorectal cancer induced by azoxymethane and dextran sodium sulfate (FeiFei et al. 2019). Proteomics analysis of proteins associated with FBXO8 identified GSTP1 (glutathione S-transferase π1), which was also shown to be ubiquitylated by FBXO8 and thereby targeted for degradation. The abundance of GSTP1 was shown to be negatively correlated with that of FBXO8 in both human and mouse colorectal cancer. In addition, FBXO8 knockdown increased cell proliferation and attenuated apoptosis in human colorectal cancer cells, and both of these effects were prevented by simultaneous knockdown of GSTP1. These results thus implicated GSTP1 as a primary target of FBXO8 for suppression of colorectal carcinogenesis.

10.21 FBXO11 (UBR6)

A screen for deafness in a large-scale mouse mutagenesis program identified the *Jeff* line with a mutation in *Fbxo11* (also known as *Ubr6*) that resulted in a glutamine-to-leucine substitution at amino acid position 491 (Hardisty-Hughes et al. 2006; Nolan et al. 2000). Deafness in *Jeff* heterozygotes was attributed to chronic inflammation of the middle ear that developed at weaning and raised the threshold for a cochlear nerve response (Hardisty et al. 2003). All *Jeff* homozygotes died shortly after birth as a result of respiratory problems (Hardisty-Hughes et al. 2006).

Biochemical analysis to identify FBXO11 substrates pinpointed BCL6, which plays an important role in the initiation and maintenance of the GC (germinal center) reaction in the spleen (Duan et al. 2012). Targeted deletion of *Fbxo11* in GC-derived B cells with the use of a *Cγ1–Cre* transgene resulted in the accumulation of BCL6 and an increase in the number of GC B cells, leading to the development of lymphoproliferative disease (Schneider et al. 2016). Given that these phenotypes are also observed in mice that overexpress BCL6 as a result of a chromosomal translocation that juxtaposes the BCL6 and immunoglobulin heavy chain genes and which is analogous to that detected in humans with diffuse large B-cell lymphoma (Cattoretti et al. 2005), BCL6 can be regarded as a primary substrate of FBXO11 for maintenance of GC homeostasis. Whether BCL6 also contributes to hearing loss in *Jeff* mice remains to be determined.

De novo heterozygous loss-of-function mutations in *FBXO11* were recently identified in individuals with syndromic intellectual disability (Fritzen et al. 2018; Gregor et al. 2018; Jansen et al. 2019). Further studies of FBXO11 KO mice focusing on the nervous system may shed light on the pathogenesis of this disease.

10.22 FBXO15

The *FBXO15* gene was identified as a target of the transcription factors Oct3/4 and Sox2, which are essential for maintenance of undifferentiated embryonic stem cells (Tokuzawa et al. 2003). Although FBXO15 is specifically expressed in early embryos as well as in adult reproductive organs, FBXO15 KO mice showed no gross abnormalities, with embryonic development and fertility appearing to be unaffected (Tokuzawa et al. 2003), indicating that FBXO15 is dispensable for normal development and physiology in mice.

10.23 FBXO22

Cellular senescence has emerged as a mechanism of proliferative arrest that limits tumorigenesis but also drives aging-related pathologies as a result, at in least in part, of cytokine secretion related to the SASP (senescence-associated secretory phenotype) (Hinds and Pietruska 2017). Transcriptome-wide analysis of senescent cells identified FBXO22 mRNA as a transcript that was upregulated at later time points of senescence induction (Johmura et al. 2016). The *FBXO22* gene was shown to be a transcriptional target of p53, a critical player in the induction of cellular senescence, and the FBXO22 protein was found to ubiquitylate p53 and thereby target it for degradation. This negative feedback loop serves to maintain an adequate level of p53 and to result in the upregulation of the cell cycle inhibitor $p16^{INK4A}$ and promotion of SASP. FBXO22 KO mice were found to be born but to be smaller than their wild-type littermates and to manifest accumulation of p53 and a concomitant reduction in the amount of p16 in all tissues tested. The SASP and aging-related phenotypes of these mice as well as their susceptibility to tumorigenesis remain to be investigated.

10.24 FBXO30

FBXO30 KO mice showed no gross abnormalities with the exception of a nursing problem evident in female mice (Liu et al. 2016). Histological analysis of pregnant FBXO30 KO mice revealed mammary gland atrophy with few glands containing milk droplets as a result of a defect in the production of mature luminal epithelial cells. Mass spectrometric analysis of FBXO30-associated proteins identified the motor protein Eg5 (also known as KIF11) as a substrate for FBXO30-mediated ubiquitylation and degradation, which occur specifically in S phase of the cell cycle, when FBXO30 is highly expressed. The accumulation of Eg5 apparent in FBXO30-deficient mammary gland epithelial cells in vitro was shown to result in centrosome amplification, the formation of multipolar or multiple bipolar spindles, abnormal separation of chromosomes, and cell cycle arrest. These phenotypes were rescued by depletion of Eg5 or treatment of these cells with an Eg5 inhibitor. Furthermore, Eg5 accumulated in the mammary glands of FBXO30 KO mice, and treatment of these animals with an Eg5 inhibitor rescued the defect in mammopoiesis. These observations thus supported the notion that Eg5 is a primary substrate of FBXO30 in mammary gland development.

10.25 FBXO32

FBXO32, also known as MAFbx (muscle atrophy F-box protein) or atrogin-1, is a striated muscle-specific F-box protein whose expression in mice is induced by stimuli that trigger muscle atrophy such as food deprivation, immobilization, denervation, hind limb suspension, glucocorticoid treatment, and cachexia (Bodine and Baehr 2014; Bodine et al. 2001; Gomes et al. 2001). FBXO32 KO mice were found to grow to adulthood without obvious abnormalities, but they died at ~16–18 months of age as a result of congestive heart failure (Zaglia et al. 2014). Cutting of the sciatic nerve as a model of denervation results in a loss of gastrocnemius and tibialis anterior muscle mass, and this effect was attenuated in FBXO32 KO mice, further implicating FBXO32 in muscle atrophy (Bodine et al. 2001). Identified substrates of FBXO32 include the transcription factor MyoD (Tintignac

et al. 2005) and eukaryotic translation initiation factor 3 subunit F (Lagirand-Cantaloube et al. 2008), but both of these proteins were identified in a cultured myoblast cell line, with further in vivo analyses being required to determine whether either plays a role in muscle sparing in FBXO32 KO mice.

10.26 FBXO38

Cancer cells acquire resistance to attack by cytotoxic T cells through expression of ligands that bind to receptors on the T cells and thereby suppress their function. One of the best characterized receptor–ligand pairs in this regard is PD-1 (programmed cell death-1) and PD-L1 (programmed cell death-ligand 1), and inhibitory antibodies to these proteins have recently been introduced for cancer immunotherapy (Sharma and Allison 2015). Proteome-wide analysis of PD-1 binding proteins identified FBXO38, which was shown to ubiquitylate PD-1 for degradation (Meng et al. 2018). Generation of T-cell-specific FBXO38 KO mice with the use of a *Cd4–Cre* transgene revealed that FBXO38 deficiency resulted in increased expression of PD-1 in tumor-infiltrating cytotoxic T cells and consequent acceleration of tumor growth. PD-1 blockade normalized the rate of tumor growth in these mice, supporting the notion that PD-1 is a primary substrate of FBXO38 in suppression of tumor progression.

10.27 FBXO45

FBXO45 contains an F-box domain that binds to SKP1, but, like FBXL10, it does not appear to associate with CUL1. Instead, it relies on another RING finger domain-containing protein—PAM in human, PHR in mouse—for the E2 recruitment required for ubiquitin ligase activity (Saiga et al. 2009). FBXO45 is specifically expressed in the nervous system, and FBXO45 KO mice die during embryogenesis as a result of abnormal innervation of the diaphragm, impaired synapse formation, and aberrant development of axon tracts in the brain, implicating FBXO45 in neural development (Saiga et al. 2009). FBXO45 has been shown to regulate neurotransmission by inducing the degradation of the synaptic protein Munc13-1 (Tada et al. 2010), suggesting that Munc13-1 accumulation may be responsible for the abnormal development of the nervous system in FBXO45 KO mice.

Phenotypes of FBXO protein KO mice with identified primary substrates are summarized in Table 10.3.

10.28 Summary

Although the in vivo functions of more than half of all F-box proteins remain to be investigated with KO mouse models, the available data for the other F-box proteins reviewed in this chapter indicate that the CRL1 ubiquitin ligase plays a key role in a wide variety of physiological and pathological processes in mice.

Consistent with the estimate that 25%–30% of all KO mouse models show embryonic or neonatal mortality (Adams et al. 2013), deletion of 11 of the 27 F-box protein genes examined to date has been found to result in death in utero as a result of extraembryonic (β-TrCP2, FBXW8, FBXL5, FBXL10, FBXL12, cyclin F), embryonic (EMI1, FBXO45), or both extraembryonic and embryonic (FBXW7) abnormalities. FBXL11 and FBXL20 KO mice remain to be further analyzed to determine whether extraembryonic or embryonic defects are responsible for the embryonic lethal phenotype.

Characterization of several KO mouse models (β-TrCP1+2, FBXL3, FBXL5, FBXL21, FBXO2, FBXO7, FBXO11, and FBXO45) has revealed a key role for CRL1 in nervous system development or function. The association of mutations in several human F-box protein genes with neurological diseases—such as mutations of the cyclin F gene with amyotrophic lateral sclerosis and frontotemporal dementia, of *FBXO7* with Parkinson's disease, and of *FBXO11* with syndromic intellectual disability—emphasizes the importance of CRL1 in the physiology and pathology of the brain. The roles of F-box proteins in cancer have also been well studied, with KO mice having been invaluable tools for

Table 10.3 Phenotypes of FBXO protein KO mice including identified primary substrates

Cyclin F (FBXO1)	Whole body	Embryonic lethality	ND	Tetzlaff et al. (2004a)
Cyclin F	Retinal cells	Not detected	NA	Tetzlaff et al. (2004a)
Cyclin F	Osteoblasts	Not detected	NA	Tetzlaff et al. (2004a)
Cyclin F	Intestinal and bladder epithelial cells	Not detected	NA	Tetzlaff et al. (2004a)
FBXO2	Whole body	Deafness	ND	Nelson et al. (2007)
FBXO4	Whole body	Not detected, or tumor development	Cyclin D1	Vaites et al. (2011) and Kanie et al. (2012)
FBXO5 (EMI1)	Whole body	Embryonic lethality	ND	Lee et al. (2006)
FBXO7	Whole body	Parkinsonism	ND	Vingill et al. (2016)
FBXO8	Whole body	Promotion of colorectal cancer	GSTP1	FeiFei et al. (2019)
FBXO11 (UBR6)	Whole body	Deafness (heterozygous), perinatal lethality (homozygous)	ND	Hardisty-Hughes et al. (2006) and Hardisty et al. (2003)
FBXO11	Germinal center B cells	Lymphoproliferative disease	BCL6	Schneider et al. (2016)
FBXO15	Whole body	Not detected	NA	Tokuzawa et al. (2003)
FBXO22	Whole body	Small size	p53	Johmura et al. (2016)
FBXO30	Whole body	Female nursing problem, abnormal mammary gland development	Eg5	Liu et al. (2016)
FBXO32	Whole body	Resistance to muscle atrophy, congestive heart failure	ND	Bodine et al. (2001) and Zaglia et al. (2014)
FBXO38	T cells	Enhanced tumor growth	PD-1	Meng et al. (2018)
FBXO43 (EMI2)	Whole body	Male and female sterility	ND	Gopinathan et al. (2017)
FBXO45	Whole body	Neonatal lethality, abnormal neural development	ND	Saiga et al. (2009)

ND not determined, *NA* not applicable

the evaluation of in which tissues and how these proteins contribute to tumor suppression or promotion (Wang et al. 2014).

Given that most F-box proteins remain functional orphans, the generation and characterization of KO mice for these proteins combined with biochemical analysis to identify relevant substrates will expand the range of physiological functions of CRL1. Further, such studies for all F-box proteins have the potential to provide a basis for the development of drugs that target F-box proteins themselves or their regulators or substrates. Such drugs may prove effective for the treatment of various diseases including neurodegenerative disorders and cancer.

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