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Regulation of cell fate determination by Skp1-Cullin1-F-box (SCF) E3 ubiquitin ligases

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ABSTRACT The developing embryo is patterned by a complex set of signals and interactions resulting in changes in cell division, cell fate determination and differentiation. An increasing body of evidence points to the role of the ubiquitin proteasome system (UPS) and ubiquitin-mediated protein degradation as a major mechanism of protein regulation, crucial for control of developmental processes. The specific and irreversible signal generated by protein degradation can function as an integrator of cell signaling events, coupled with other post-translational protein modifications, but also as a master switch for differentiation in its own right. The UPS also displays more subtle mechanisms of regulating signaling than decreasing protein levels, such as proteolytic processing and altering subcellular localization. In particular, the SCF E3 ligase family plays pivotal roles in regulating diverse developmental events in varied species. This review will focus on the role played by SCF E3 ligases in cell fate determination and differentiation.

KEY WORDS: *differentiation, SCF, signaling, ubiquitylation, UPS*

Introduction

During embryogenesis, individual cells must respond to signaling within the developing embryo and elicit the appropriate response. Such responses involve changes in the level and/or activity of proteins and must be dynamic. Within the field of developmental biology, most emphasis has traditionally been placed on regulation of protein levels by control of transcription. However, it is becoming clear that many proteins are subject to regulated degradation and that this plays a critical regulatory role during embryogenesis. Regulated protein degradation has three key features: irreversibility, responsiveness and selectivity.

Regulated proteolysis of up to 90% of short-lived proteins is achieved by the ubiquitin proteasome system (UPS) (Ciechanover *et al*., 1984). Ubiquitin-mediated degradation is initiated by the covalent attachment of ubiquitin (Ub), a 76-amino acid protein, onto a substrate (Ciechanover *et al*., 1980a; Ciechanover *et al*., 1980b; Hershko *et al*., 1980; Wilkinson *et al*., 1980). Subsequent rounds of ubiquitylation attach additional Ubs to the first to build up a chain; chains of at least 4 Ubs then facilitate the recognition and destruction of the substrate by the 26S proteasome (reviewed in Pickart and Cohen, 2004; Wolf and Hilt, 2004).

The addition of Ub onto substrate proteins is catalysed by a multi-enzyme cascade (Fig. 1). Firstly, Ub is activated using energy from ATP hydrolysis, resulting in the fusion of AMP to the C-terminal carboxyl group. The active site cysteine of an E1 (Ub activating) enzyme can then form a thioester bond with activated Ub. Ub is then passed to the active site cysteine of an E2 (Ub conjugating) enzyme. The last enzyme in the cascade, an E3 (Ub ligase), facilitates the attachment of Ub onto the substrate protein from the E2 enzyme (Hershko *et al*., 1983; Pickart and Rose, 1985). Successive Ub moieties can be added to the first by a

Abbreviations used in this paper: APC/C, anaphase promoting complex/cyclosome; Arm, armadillo; bHLH, basic helix-loop-helix; β-TRCP, β-transducin repeat containing protein; Ci, Cubitus interruptus; CKI, cyclin dependent kinase inhibitor; Dlg, discs large; dpp, decapentaplegic; Fbw, F-box protein containing WD40 repeats; Fbx, F-box protein; GCM, glial cells missing; GSK3, glycogen synthase kinase 3; HECT, homologous to E6-associated protein C-terminus; Hh, hedgehog; IKK, IκB kinase; MAFbx, muscle atrophy F-box; NβT, neural β-tubulin; NC, neural crest; NF-κB, nuclear factor-κB; PKA, protein kinase A; Ppa, partner of paired; Ptc, patched; REST, RE1-silencing transcription factor; RING, really interesting new gene; SCF, Skp1-cullin1-F-box complex; Smo, smoothened; Ub, ubiquitin; UPS, ubiquitin proteasome system.

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sequential enzyme cascade. Alternatively, entire polyUb chains may be attached to the substrate protein by the action of an E4 enzyme, such as p300, which has been demonstrated to add chains of polyUb to p53 at sites previously monoubiquitylated by Mdm2 (Grossman *et al.*, 2003; reviewed in Hoppe, 2005). In humans, only 2 E1 enzymes and approximately 100 E2 enzymes have been characterised. By contrast, it is estimated that there may be as many as 1000 E3 ligases (reviewed in Hicke *et al.*, 2005) further divided into 3 classes: Homologous to E6-Associated Protein C-Terminus (HECT), Really Interesting New Gene (RING) and U-box.

Probably the most diverse family is that of the RING E3 ligases; there are almost 400 proteins with RING domains in the human genome, compared to around 38 with HECT domains (Semple, 2003). RING E3 ligases are characterised by the presence of a RING motif (consensus $CX_2CX_{9-39}CX_{1-3}HX_{2-3}[C/H]X_2CX_{4-48}CX_2C$). Based on bioinformatic data, RING E3 ligases can be further divided into single subunit and modular classes. Single subunit RING E3 ligases bind to both E2 enzymes and substrates. In the case of modular RING E3 ligases, the RING protein functions as part of a multi protein complex and substrates are recruited by a separate subunit (reviewed in Deshaies and Joazeiro, 2009). Examples include the Anaphase Promoting Complex/Cyclosome (APC/C) and Cullin-based RING E3 ligases. Cullin-based E3 ligases (reviewed in Petroski and Deshaies, 2005) use the RING protein Roc1 (also known as Rbx1 and Hrt1) to recruit E2 enzymes (Chen *et al.*, 2000; Furukawa *et al.*, 2002; Ohta *et al.*, 1999).

This review will concentrate on the role in development of the most well characterised sub-group of Cullin-based RING E3 ligases, the Skp1-Cullin1-F-box (SCF) E3 ligase complexes. Within the SCF complex, Cullin1 binds to Roc1, Skp1 and a variable F-box protein (Lyapina *et al.*, 1998; Wu *et al.*, 2000), and it is this latter component that confers the SCF complex designation, e.g. SCF^{Skp2}, where Skp2 is the F-box component. The SMART database (http://smart.embl-heidelberg.de/) gives an estimated 56 F-box proteins in humans, 77 in mice and 30-50 in *Xenopus laevis*, compared to the 600-700 found in the large gene networks of *Arabidopsis* and rice (Gagne *et al.*, 2002; Jain *et al.*, 2007; Kuroda *et al.*, 2002). In the crystal structure of SCF^{Skp2} (Schulman *et al.*, 2000; Zheng *et al.*, 2002), which is, to date, the only structure of a complete SCF complex described, Cullin1 forms a rigid, bi-lobed structure which acts as a 'molecular scaffold' on which to assemble the SCF complex. The C-terminal globular domain recruits Roc1, which in turn recruits the E2 enzyme. The Cullin1 N-terminal domain recruits Skp1, which then binds to the F-box protein substrate recognition subunit via interactions between the C-terminus of Skp1 and the F-box domain.

Structural studies are also providing insight into further mechanisms of SCF activity. Many F-box proteins interact with their cognate substrates only after the substrates have been post-translationally modified, adding an extra level of regulation. Although the most widely reported prior modification is phosphorylation, substrates have also been reported to require acetylation, glycosylation or nitration (Guinez *et al.*, 2008; Hwang *et al.*, 2010). For instance, it is known that degradation of cyclin E by SCF^{Fbw7}

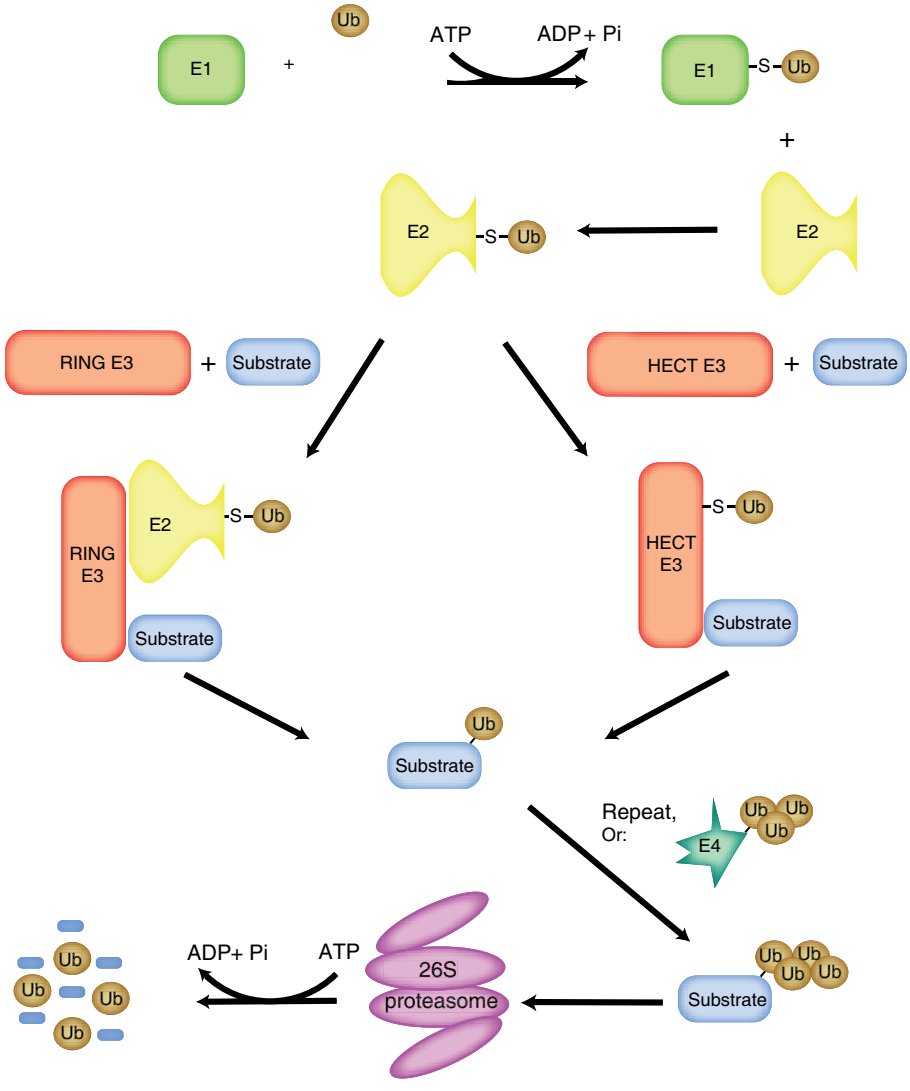

Fig. 1. Schematic of Ub mediated protein degradation. *Ub is first covalently linked to an E1 (Ub-activating) enzyme using energy from ATP hydrolysis before being shuttled to an E2 (Ub-conjugating) enzyme. Ub is then either conjugated directly to a HECT E3 ligase before transfer to the substrate or the E2-Ub is recruited via a RING E3 ligase into a complex containing the substrate. Note that all Ub conjugation from E1 to E3 is via thioester linkage to a cysteine sidechain sulfur. Further attachment of Ub to internal lysines on the original substrate Ub is achieved either by repetition of the above scheme or the action of an E4 enzyme, which transfers polyUb chains to monoubiquitylated substrate ubiquitin. A chain of four or more K48-linked polyUb targets the substrate to the 26S proteasome where it is unfolded and degraded in an ATP-dependent manner into small peptides with concurrent deubiquitylation to recycle Ub.*

is triggered only following phosphorylation at multiple sites (Ye *et al.*, 2004). Binding partners increase or inhibit the activity of SCF complexes and in particular binding or covalent modification of the C-terminal winged helix bundle domain of the Cullin subunit plays an important role in regulating SCF activity (Duda *et al.*, 2008; Liu *et al.*, 2002). Versatility in substrate specificity for the SCF E3 ligases is provided by the recognition subunit F-box proteins, which bind distinct substrates. Structural analysis of SCF complexes and their cognate substrates is beginning to reveal a wide range of mechanisms for substrate recognition. For example, the atypical F-box protein Fbx4 contains a GTPase domain which is crucial for the binding of a globular domain of its TRF1 substrate (Zeng *et al.*, 2010). By contrast, the *Arabidopsis* F-box protein TIR1 requires only the presence of the plant hormone auxin in order to bind to its cognate substrates, the Aux/IAA proteins (Kepinski and Leyser, 2004). Structural studies have revealed that TIR1 is itself the sensor of auxin and that the binding of auxin to TIR1 is necessary to complete the docking site for Aux/IAA proteins (Tan *et al.*, 2007). Thus, although the F-box motif provides a consistent recognition motif for binding to the SCF scaffold, the mechanism of substrate recognition by the F-box protein varies.

A number of F-box proteins have exhibited roles in development through regulation of substrate levels (see Table 1). This review will focus on the role that F-box proteins play in cell fate determination and signaling during embryogenesis and organogenesis. Although many F-box proteins are also involved in the degradation of cell cycle components (Skaar *et al.*, 2009a; Skaar *et al.*, 2009b), this aspect of F-box protein activity has been previously described in detail (reviewed in Ang and Harper, 2004; Skaar and Pagano, 2009) and this role in development will not be considered here.

F-box proteins and signaling: patterning in the early embryo

Several F-box proteins have key roles in major signaling pathways involved in patterning of the embryo, for instance *β-Transducin Repeat Containing Protein* (*β-TRCP*), which plays pleiotropic roles in regulation of cell signaling. Indeed, *β-TRCP* is one of the best studied of this class of E3 ubiquitin ligases because of its multiple important substrates. *Xenopus* has 2 F-box *β-TRCP* genes, *β-TRCP1* and *β-TRCP2* (also known as *FBXW1 and FBXW11* respectively). 4 transcripts of *β-TRCP* are expressed in *Xenopus*, which differ in the presence or absence of amino acid sequences at the N- or C-termini (Ballarino *et al.*, 2004; Ballarino *et al.*, 2002). Similarly, 2 distinct genes exist in humans with multiple isoforms expressed (Fuchs *et al.*, 1999; Suzuki *et al.*, 1999). Recent evidence suggests that different isoforms of *β-TRCP* play different roles in development, with differing tissue-specific expression in mouse, while assays in *Xenopus* demonstrate differential isoform activity (Seo *et al.*, 2009).

β-TRCP recognises substrates via binding to seven WD40 repeats present in its C-terminus and phosphorylation of substrates is a prerequisite for binding (for example, Winston *et al.*, 1999). Most β-TRCP substrates identified to date have a specific phosphodegron (DpSGϕXpS, where ϕ is a hydrophobic amino acid, and p denotes phosphorylation), a motif which, when phosphorylated, allows targeting of the substrate for degradation (reviewed in Ang and Wade Harper, 2005; Jin *et al.*, 2003; Winston *et al.*, 1999). In addition, lysines that are 9-13 amino acids N-terminal to this phosphodegron are preferentially ubiquitylated. This is due to structural constraints associated with optimal presentation of the substrate to the E2 enzyme (Wu *et al.*,

TABLE 1

SUMMARY OF F BOX PROTEINS INVOLVED IN CELL FATE DETERMINATION

Tissue/cell type	F-box component	Substrate	Function	Ref
Extraembryonic	Fbw2	GCM1	GCM1 required for development of extraembryonic tissue in mammals	Schreiber *et al.*, 2000
Early embryo	β-TRCP	β-Catenin	Regulation of β-Catenin stability and transcriptional activity	Latres *et al.*, 1999; Kitagawa *et al.*, 1999; Hart *et al.*, 1999
		Cactus	Regulation of Dorsal transcriptional activity and dorsal-ventral patterning in *Drosophila*	Belvin *et al.*, 1995; Maniatis, 1999
		Ci	Regulation of Ci transcriptional activity	Jia *et al.*, 2005; Wang and Li, 2006
	Fbw7	Notch	Phosphorylation-dependent degradation of Notch-ICD and regulation of transcription	Gupta-Rossi *et al.*, 2001; Wu *et al.*, 2001
Epithelia	β-TRCP	hDLG	Dlg inhibits epithelial differentiation in *Drosophila*, interaction with β-TRCP seen only with hDLG so far	Mantovani and Banks, 2003; Woods *et al.*, 1996
		IκB	NF-κB signalling implicated in proliferation and differentiation of basal layer of epidermis	Hu *et al.*, 1999, Takeda *et al.*, 1999
		TAp63γ	Possible role in epidermal differentiation via regulation of transcriptional activity	Gallegos *et al.*, 2008
Haematopoietic	Fbw7	c-Myc	Abnormal thymocyte development due to aberrant c-myc regulation; regulates haematopoietic stem cell gene expression signature	Onoyama *et al.*, 2007, Reavie *et al.*, 2010
		Notch	Negative regulation of Notch signalling in haematopoietic, vascular and cardiac development in mice	Tetzlaff *et al.*, 2004
Muscle	MAFbx	MyoD	Promotes MyoD polyubiquitylation and degradation *in vitro* and *in vivo*	Lagirand-Cantaloube *et al.*, 2009, Tintignac *et al.*, 2005
		myogenin	Promotes myogenin polyubiquitylation and degradation	Jogo *et al.*, 2009
Neural	β-TRCP	REST	Degradation of transcriptional repressor, promoting neuronal differentiation	Chong *et al.*, 1995, Westbrook *et al.*, 2008
	Fbw2	gcm	Degradation of gcm allows cell cycle exit and differentiation of glial progenitors in *Drosophila*	Ho *et al.*, 2009, Hosoya *et al.*, 1995
	Skp2	Xic1	Regulation of primary neuronal differentiation in *Xenopus*	Boix-Perales *et al.*, 2007
Neural crest	Ppa	Slug	Regulation of Slug stability during neural crest development	Vernon *et al.*, 2006
	Fbw7	Unknown	Fbw7 necessary for development of neural crest	Almeida *et al.*, 2010

Known SCF substrates per tissue and cell type for each F-box protein are summarized along with their roles.

2003). The phosphodegron is a highly efficient binding motif that can act as a transferable destruction signal (Wulczyn *et al.*, 1998).

A role for *β-TRCP* was first identified from studies with the *Drosophila* orthologue, *Slimb* (Jiang and Struhl, 1998). Loss of function of *Slimb* resulted in the accumulation of the transcription factors *Armadillo/β-catenin* (*Arm*) and *Cubitus interruptus* (*Ci*), components of the *Wnt* and *Hedgehog* (*Hh*) signaling pathways, respectively. It was proposed that *Slimb* negatively regulates these pathways through proteolysis of Arm and Ci. Since then, SCF$^{\beta\text{-TRCP}}$ complexes have been demonstrated to degrade a large number of substrates, many of which have roles during development (reviewed in Fuchs *et al.*, 2004).

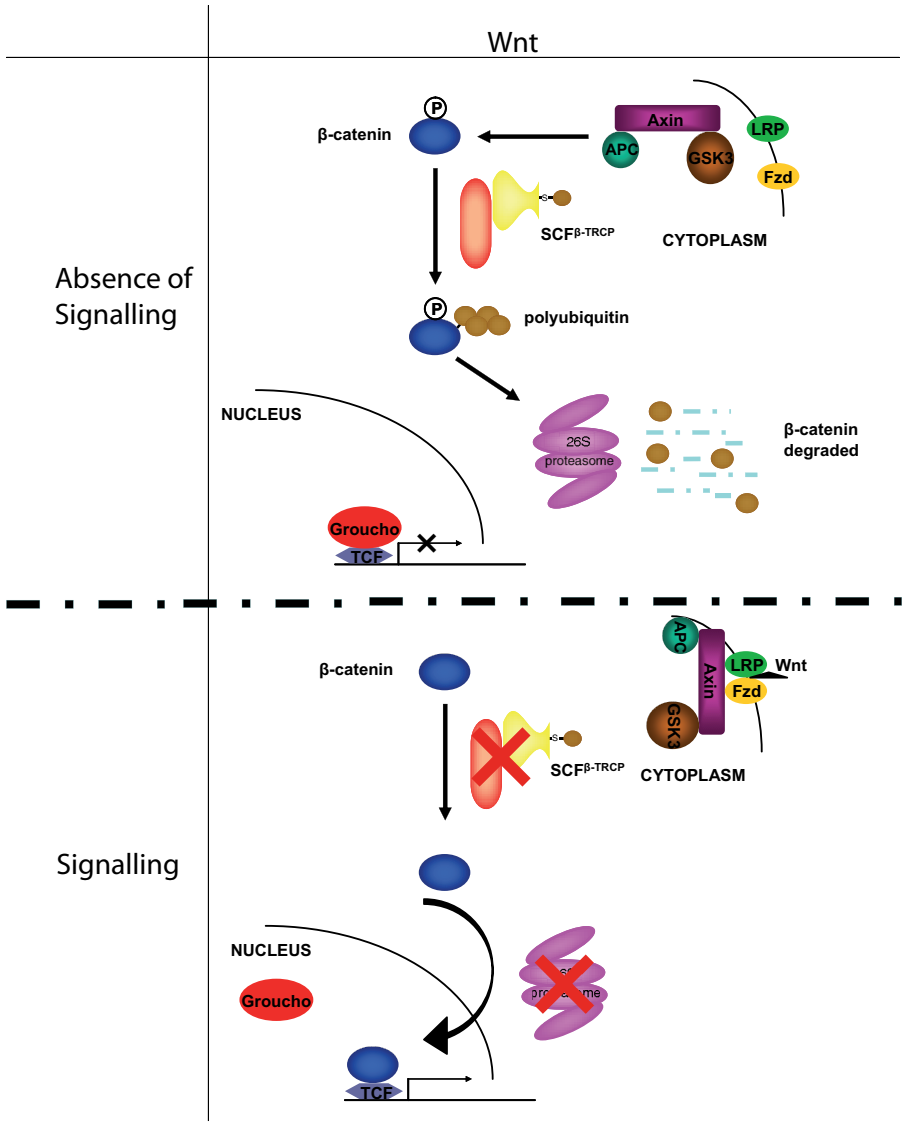

Fig. 2. Regulation of Wnt signaling by Ub mediated protein degradation. *The Wnt pathway is shown in the presence and absence of Wnt, leading to activation and inhibition, respectively, of β-catenin transcriptional activity. In the absence of Wnt, β-catenin is phosphorylated by GSK3 and targeted for degradation by SCF$^{\beta\text{-TRCP}}$. When Wnt is present, binding by the Fzd receptor leads to the complex containing GSK3 being bound at the membrane and unavailable to phosphorylate β-catenin. β-catenin is therefore not degraded and enters the nucleus to form a transcriptionally active complex with TCF, displacing the repressor, Groucho. APC, adenomatous polyposis coli; Fzd, frizzled; GSK3, glycogen synthase kinase 3; LRP, low density lipoprotein receptor related protein; TCF, T cell factor. P is used to denote phosphorylation.*

Wnt signaling

The transcription factor *β-catenin* mediates signaling via the canonical *Wnt* pathway, which regulates multiple developmental processes, for instance dorsal-ventral axis formation in *Xenopus*. β-catenin has an asymmetric localisation in the early *Xenopus* embryo, concentrated on the future dorsal side of the embryo, allowing the expression of dorsal-specific genes. Elevations in dorsal β-catenin levels are attributed to activation of *Wnt* signaling (Larabell *et al.*, 1997); β-catenin is degraded by Ub-mediated proteolysis, and removal of glycogen synthase kinase 3β (GSK3β) phosphorylation sites or activation of *Wnt* signaling stabilises the protein (Aberle *et al.*, 1997). This has led to a model whereby, in the absence of *Wnt* signaling, β-catenin is degraded in a manner dependent upon phosphorylation at GSK3β sites (Fig. 2), but in response to *Wnt* signaling, β-catenin is stabilised and can promote gene expression. *β-TRCP* has been characterised as a negative regulator of *Wnt* signaling; overexpression of *β-TRCP* reduces formation of *Wnt8*-induced secondary axes in *Xenopus*, and inhibition of SCF$^{\beta\text{-TRCP}}$ using a dominant negative F-box deleted (ΔF-box) mutant results in formation of secondary axes (Lagna *et al.*, 1999; Marikawa and Elinson, 1998). The latter effect is inhibited by co-overexpression of mediators of the *Wnt* pathway (Marikawa and Elinson, 1998). Subsequently, SCF$^{\beta\text{-TRCP}}$ was demonstrated to be the E3 ligase for β-catenin (Fuchs *et al.*, 1999; Hart *et al.*, 1999; Latres *et al.*, 1999).

A distinct role has been identified for SCF $^{\beta\text{-TRCP}}$ in neural crest formation. Neural crest (NC) development depends on the activity of the *Snail* family of transcription factors, which trigger the epithelial to mesenchymal transition, via repression of *E-cadherin* that results in the migration of NC cells from the neural tube throughout the embryo. Work in several cell lines has demonstrated that *Wnt* signaling leads to stabilisation of Snail through inhibition of SCF$^{\beta\text{-TRCP}}$ mediated degradation. GSK3β targets human Snail for phosphorylation at serines between amino acids 92-120 and this is required for nuclear export, β-TRCP binding and proteasomal degradation (Yook *et al.*, 2005; Zhou *et al.*, 2004).

Hh signaling

The *Hh* signaling pathway is involved in a range of patterning processes during development, many of which have been identified using *Drosophila* as a model system (reviewed in Ingham and McMahon, 2001; Ingham and Placzek, 2006). In the absence of signaling, a G-protein coupled receptor, *Smoothened* (*Smo*), is

inhibited by a multipass transmembrane receptor for Hh, *Patched* (*Ptc*). Binding of secreted Hh proteins to Ptc alleviates inhibition of Smo and results in the activation of signaling within the cell. The transcription factor *Ci* (the *Gli* family in mammals) is the major mediator of *Hh* signaling in cells. In the absence of *Hh* signaling, *Ci* is a transcriptional repressor for genes such as *Decapentaplegic* (*dpp*). However, when *Hh* signaling is activated, *dpp* expression is de-repressed. The duality of *Ci* activity is achieved by proteolytic processing; full length Ci (Ci155) is a transcriptional activator and a C-terminally truncated form (Ci75) is a repressor. It appears that processing of Ci to repressor forms is mediated by the SCF^{Slimb} complex, the *Drosophila* homologue of $SCF^{\beta\text{-}TRCP}$. Recruitment of SCF^{Slimb} to Ci requires phosphorylation of Ci protein at multiple residues in the C-terminus by Protein Kinase A (PKA) and GSK3. This facilitates further phosphorylation by Casein Kinase I A and E, followed by SCF^{Slimb} recruitment (Jia *et al.*, 2005; Smelkinson *et al.*, 2007). SCF^{Slimb}-mediated processing of Ci is unusual, as ubiquitylation triggers partial proteolysis rather than full destruction (Fig. 3). SCF^{Slimb} activity must be inhibited following *Hh* pathway activation; this allows accumulation of the Ci activator form (Ci155) rather than its repressor form (Ci75). The situation in mammals is more complex. There are 3 Gli proteins, homologues of Ci, Gli1, Gli2 and Gli3. Mouse Gli3 is efficiently processed to a repressive form (Pan *et al.*, 2006), most likely by $SCF^{\beta\text{-}TRCP}$ (Wang and Li, 2006), whilst *Gli2* is important for transcriptional activation. In mouse, Gli2 is inefficiently processed to the repressive form and instead can be degraded fully by the $SCF^{\beta\text{-}TRCP}$ complex (Bhatia *et al.*, 2006; Pan *et al.*, 2006).

NF-κB signaling

Nuclear factor-κB (*NF-κB*) was first identified as a transcription factor involved in expression of the *immunoglobulin κ light chain* gene in B cells (Sen and Baltimore, 1986). It is a member of the *Rel* family of transcription factors, of which there are three genes in *Drosophila*: *Dorsal*, *Dif* and *Relish* (Dushay *et al.*, 1996; Ip *et al.*, 1993; Steward, 1987). Also important in mounting an effective immune response, the developmental role played by *NF-κB* was elucidated through genetic analysis of signaling by its *Drosophila* homologue, *Dorsal* (reviewed in Karin and Ben-Neriah, 2000). Ablation of *Dorsal* activity resulted in embryos lacking ventral structures, which require nuclear localisation of *Dorsal* at the ventral side of the embryo. *Dorsal* is usually bound in the cytoplasm by its inhibitor, *Cactus*, a homologue of mammalian *IκB* (Geisler *et al.*, 1992), such that its nuclear localisation signal is obscured and entry to the nucleus does not occur (Henkel *et al.*, 1992; Wu and Anderson, 1998). Ventral activation of the *IL-1 receptor* homologue, *Toll* (Hashimoto *et al.*, 1988), leads to phosphorylation and degradation of *Cactus*, allowing *Dorsal* to dimerise and enter the nucleus as an active complex (Belvin *et al.*, 1995).

The SCF complex responsible for targeting *IκB* for degradation is, once again, $SCF^{\beta\text{-}TRCP}$, which ubiquitylates *IκB* at lysines 21 and 22 (Maniatis, 1999) after modification of *IκB* by phosphoryla-

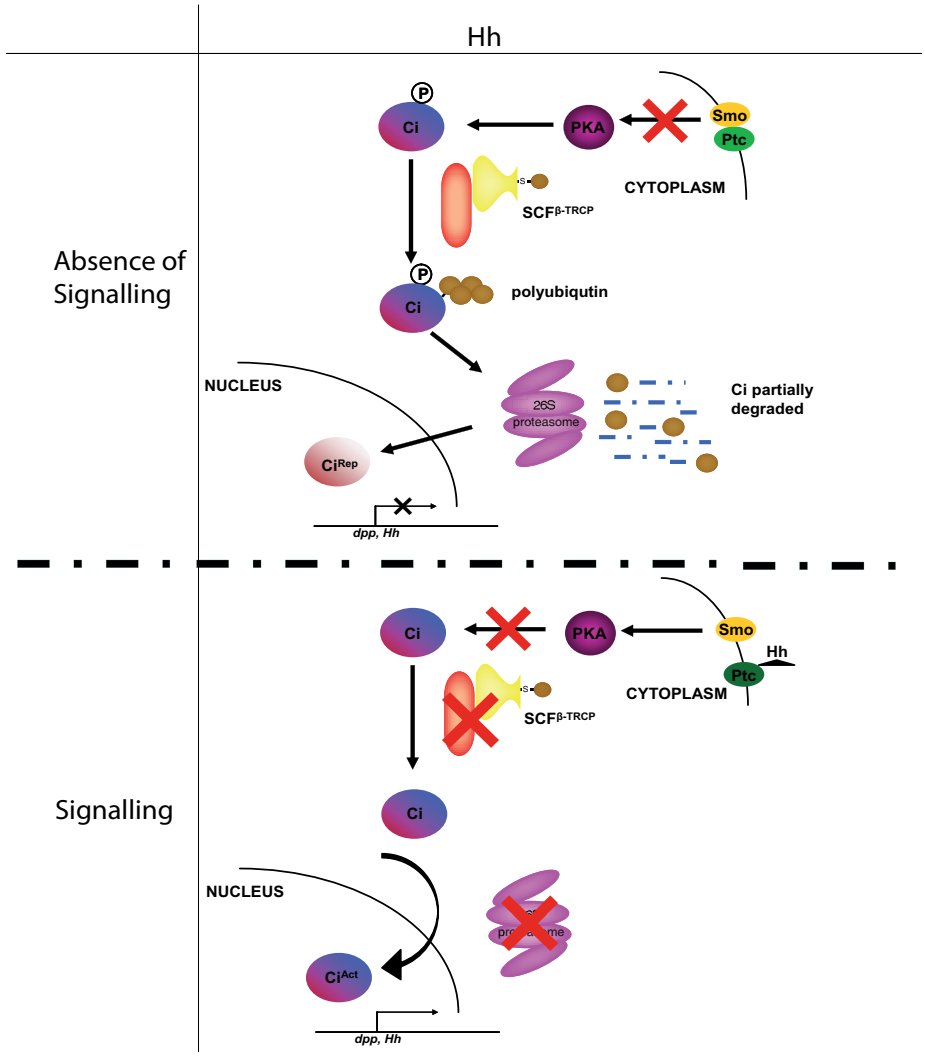

Fig. 3. Regulation of Hh signaling by Ub mediated protein processing. *The Hh pathway is shown in the presence and absence of Hh, leading to activation and inhibition, respectively, of Ci transcriptional activity. In the absence of Hh, Ci is phosphorylated by PKA, active in the presence of inhibited Smo, and polyubiquitylated by $SCF^{\beta\text{-}TRCP}$. This targets Ci for partial proteolysis by the 26S proteasome, leading to the formation of a transcriptional repressor form. When Hh is present, the binding of Hh to Ptc alleviates repression of Smo and inhibits activation of PKA and phosphorylation of Ci. Ci therefore enters the nucleus in a transcriptionally active form that has not been proteolysed. Note the similarities between the Wnt and Hh pathways: signaling at the external surface of the membrane inhibits intracellular signaling and phosphorylation of a transcription factor, which in turn inhibits targeting by $SCF^{\beta\text{-}TRCP}$ to the 26S proteasome. Ci, Cubitus interruptus (Act and Rep are used to denote activator and repressor forms, respectively); dpp, decapentaplegic; Hh, Hedgehog; PKA, protein kinase A; Ptc, Patched; Smo, Smoothened. P is used to denote phosphorylation.*

tion (Alkalay *et al.*, 1995). Inhibition of the 26S proteasome also inhibits *NF-κB* signaling, suggesting that the post-translational modifications that occur to *IκB* are insufficient to cause dissociation from *NF-κB* (Lin *et al.*, 1995). Intriguingly, a role for *NF-κB* signaling in dorsal-ventral patterning in vertebrates has not been established and *NF-κB1*-null mice do not display any gross developmental abnormalities (reviewed in Attar *et al.*, 1997). However, *NF-κB* signaling does seem to play a role in the formation of the epidermis, as knockout of a kinase responsible for phosphorylation of *IκB*, *IκB kinase α (IKKα)*, leads to severe deformity and death of neonates 4 hours post-partum due to thickening of the epidermis (see below).

The examples given above highlight several common features of signaling regulated by $SCF^{\beta\text{-}TRCP}$. Most notably, they illustrate how the UPS can produce a rapid response to signaling events. For instance, in the absence of *Wnt* signaling, β-catenin is degraded by $SCF^{\beta\text{-}TRCP}$, while activation of *Wnt* signaling rapidly stabilises the protein through inhibition of GSK3β. This allows a much faster response than if β-catenin needed to be synthesised *de novo*. Similarly, the response allowed by the switching of Ci from a repressor to an activator form, following inhibition of SCF^{Slimb} by *Hh* signaling, is more rapid than that allowed by changes in expression of repressor and activator genes. It is also noteworthy that, in all these cases, signaling begins with kinases and, for instance, targeting of the substrate to $SCF^{\beta\text{-}TRCP}$ is mediated by phosphorylation of a phosphodegron motif. Integration of the UPS and phosphorylation cascades allows a fine-tuning of the system by combining reversible and irreversible aspects of regulation.

β-TRCP and epidermal development

The epidermis consists of a stratified epithelium that is made up of keratinocytes. Mitotically active keratinocytes reside in the inner basal layer and continuously renew the surface of the epidermis by detaching from the basement membrane and migrating to the outer, terminally differentiated, layer. The transcription factor *p63*, a member of the *p53* family, is crucial for the differentiation of keratinocytes and *p63*-null mice lack epidermis, epidermal structures and squamous epithelia (Mills *et al.*, 1999; Yang *et al.*, 1999). The gross manifestation of the lack of epidermis is the truncation of the limbs and severe craniofacial abnormalities.

There are 6 isoforms of *p63* resulting from differential promoter usage, producing the full N-terminal TAp63 and the N-terminally truncated ΔNp63, and alternative splicing at the 3' end of the transcripts, to produce the α, β and γ isoforms of both TAp63 and ΔNp63. Both TAp63 and ΔNp63 are transcriptionally active, although only TAp63 contains the transactivation domain (reviewed in Candi *et al.*, 2008). In mature epidermis, ΔNp63α appears to be the major isoform expressed in proliferating keratinocytes in the basal layer, but not present in suprabasal layers, although several isoforms are claimed to be required for normal stratification in the embryo (Gu *et al.*, 2006; Koster and Roop, 2004). It has been reported that there is an interaction between endogenous $SCF^{\beta\text{-}TRCP}$ and TAp63γ in a human keratinocyte cell line, HaCaT (Gallegos *et al.*, 2008). Unexpectedly, the interaction with $SCF^{\beta\text{-}TRCP}$ increases the half life of p63 and ubiquitylation of TAp63γ appears to increase its transcriptional activity by around 50%, as assessed by RT-PCR. Although it is likely that $SCF^{\beta\text{-}TRCP}$ has a role in epidermal development *in vivo* there are, as yet, no data to confirm this.

In contrast to its role in promoting the stability and activity of *p63*, $SCF^{\beta\text{-}TRCP}$ also interacts with *hDLG*, the human homologue of *Drosophila discs large* (*Dlg*). Mutations in *Dlg* result in invasive growth of epithelial cells in *Drosophila* (Woods *et al.*, 1996) and *hDLG* is recruited to the plasma membrane by E-cadherin cell-cell adhesion, where it organises junction structures and the actin cytoskeleton (Ide *et al.*, 1999; Reuver and Garner, 1998). Interaction with $SCF^{\beta\text{-}TRCP}$ promotes the ubiquitylation and degradation of hDLG (Mantovani and Banks, 2003). The interaction appears to be promoted by phosphorylation of the SH3 domain of hDLG (Mantovani *et al.*, 2001), although the physiological relevance of this interaction remains unclear.

As well as the central role played by *p63* in epidermal formation and stratification, it appears that *NF-κB* signaling may also play a role in the differentiation of epidermal cells. The inhibitory binding partner of *NF-κB*, *IκB*, is targeted for degradation following phosphorylation by *IKK*, (reviewed in Karin and Ben-Neriah, 2000). The *IKKα*-null mouse appears to phenocopy the *p63*-null mouse, as at a superficial level the neonates lack limbs and show aberrant craniofacial development (Hu *et al.*, 1999; Takeda *et al.*, 1999). Closer inspection of the *IKKα* mutants shows that skeletal organisation is approximately wild type, but the epidermis is 5- to 10-fold thicker and so limbs cannot emerge out of the thickened skin. The epidermis is composed of a single layer and it would appear that the loss of *NF-κB* signaling leads to gross overproliferation of the basal layer.

Intriguingly, in *Drosophila* one of the target genes of *Dorsal* is *twist*, ablation of which is associated with craniofacial abnormalities (Howard *et al.*, 1997). Further, *IKKα* is a direct and indirect target of TAp63, both by direct binding to a *p53*-like consensus sequence on the *IKKα* promoter and by upregulation of the transactivators *Ets-1* and *GATA-3* (Candi *et al.*, 2006; Gu *et al.*, 2004; Sil *et al.*, 2004). IKK interacts with ΔNp63α and promotes its Ub mediated degradation (Chatterjee *et al.*, 2010), suggesting that *NF-κB* and *p63* share multiple components which regulate their activities, the most prominent being the $SCF^{\beta\text{-}TRCP}$ E3 ligase. However, the exact level of crosstalk between these two transcription factors remains to be firmly established.

Other developmental signaling pathways

$SCF^{\beta\text{-}TRCP}$ is not the only SCF complex to play a role in major signaling pathways. Mammalian *FBW7* was initially identified as an F-box protein in a yeast screen for effectors of the cell cycle and termed *cdc4* (Nurse *et al.*, 1976). The *Caenorhabditis elegans* homologue, *SEL-10*, was found through mutational analysis to be responsible for the degradation of the *Notch* intracellular domain, the effector of *Notch* signaling, and thus termination of the *Notch* signal (Gupta-Rossi *et al.*, 2001; Wu *et al.*, 2001). In *C. elegans*, mutation of *SEL-10* resulted in aberrant vulval development, a process dependent upon *Notch* signaling (Hubbard *et al.*, 1997). In mice, knockout of *FBW7* results in embryonic lethality at E10.5 through a combination of aberrant haematopoietic and vascular development and heart maturation defects (Tetzlaff *et al.*, 2004). Defects in neural tube closure and development of all brain regions were also observed at E9.5. Intriguingly $FBW7^{+/-}$ mice appear grossly phenotypically normal up to 1 year of age and, despite reports of mutation of *FBW7* in T-ALL cell lines and

patient samples (O'Neil *et al.*, 2007), did not display an increased incidence of tumorigenesis (Tetzlaff *et al.*, 2004).

However, a role for *FBW7* as a tumour suppressor has been observed in the absence of *p53* activity (Mao *et al.*, 2004). Most recently, conditional inactivation of *FBW7* in murine T cells was found to increase the number of double-positive thymocytes but not single-positive thymocytes, due to increased apoptosis of double-positive thymocytes (Onoyama *et al.*, 2007). This is suggestive of a developmental block in thymocyte maturation and is supported by an increased incidence of thymic lymphoma in Lck-Cre/*FBW7*$^{F/F}$ mice resulting from clonal expansion of progenitors bearing an immature, double-positive phenotype. These abnormalities in thymocyte development were also observed in CD4-Cre/*FBW7* $^{F/F}$/*RBP-J*$^{F/F}$ mice but not in CD4-Cre/*FBW7*$^{F/F}$/*c-Myc*$^{F/F}$ mice, leading the authors to conclude that abnormal thymocyte development arises due to disregulation of *c-Myc* and not *Notch* signaling (Onoyama *et al.*, 2007). More recently, *FBW7* has been found to play a more general role in haematopoiesis, as regulation of the level of c-Myc was found to be sufficient to direct the gene expression signature of haematopoietic stem cells. Intriguingly, adult and embryonic haematopoietic stem cells displayed different responses to c-Myc levels at the level of gene expression (Reavie *et al.*, 2010).

Skp2, β-TRCP, FBW2 and neural differentiation

SCF^{Skp2} is an SCF complex containing the leucine rich repeat F-box protein *Skp2* (also known as *FBXL1*). SCF^{Skp2} has been shown to ubiquitinate a number of cell cycle substrates, including c-Myc (Kim *et al.*, 2003; von der Lehr *et al.*, 2003), Cyclin E (Nakayama *et al.*, 2000), the cyclin dependent kinase inhibitors (CKI) p27^{Kip1} (Carrano *et al.*, 1999), p57^{Kip2} (Kamura *et al.*, 2003) and the *Xenopus* CKI Xic1 (Lin *et al.*, 2006), and has been implicated in development of many cancers (Bashir *et al.*, 2004; Kitagawa *et al.*, 2008; Signoretti *et al.*, 2002). However, in addition to a central role in proteolysis of cell cycle regulators, SCF^{Skp2} may have additional functions in differentiation and development.

Recent work has highlighted a role for *Skp2* during neural development in *Xenopus.* Primary neurogenesis in this species results in differentiation of neurons that mediate the early movements of the embryo. This process is driven by a cascade of proneural basic Helix-Loop-Helix (bHLH) transcription factors, resulting in the expression of markers of terminal neuronal differentiation, such as *neural β-tubulin* (*NβT*) (reviewed in Lee, 1997). Depletion of Skp2 protein using translation-blocking anti-sense morpholinos promotes primary neurogenesis, as assessed by expression of *NβT*, by a mechanism independent of changes in the cell cycle. Conversely, overexpression of *Skp2* inhibits formation of primary neurons and this inhibition occurs at an early point in the bHLH cascade (Boix-Perales *et al.*, 2007). *Skp2*-mediated inhibition of this process is likely to occur via ubiquitylation of substrates by the SCF^{Skp2} complex, as overexpression of a ΔF-box form of *Skp2*, which can no longer bind to Skp1 and therefore the rest of the SCF complex, has no effect on formation of primary neurons (Boix-Perales *et al.*, 2007). As the CKI *Xic1* is required for differentiation of primary neurons in *Xenopus* (Vernon *et al.*, 2003), degradation of Xic1 in neural precursors may be the major mechanism by which SCF^{Skp2} regulates this process. It is interesting to note in this regard that the CKI p57^{Kip2} has been reported to associate with several proteins involved in differentiation, such as MyoD (reviewed in Besson *et al.*, 2008; Reynaud *et al.*, 2000). However, unlike the degradation of the CKI p27^{Kip1} by SCF^{Skp2} in mammals, Xic1 degradation in *Xenopus* by SCF^{Skp2} does not require prior phosphorylation of its CKI target (Lin *et al.*, 2006).

The stability of Skp2 itself is regulated by the UPS in mammals, and the E3 ligase responsible is the APC/C coupled to the substrate recognition subunit, Cdh1 (Bashir *et al.*, 2004; Wei *et al.*, 2004). Degradation of Skp2 by APC/C^{Cdh1} is also important for myogenesis, as depletion of Cdh1 by siRNA in the mouse skeletal muscle cell line, C2C12, reduces cellular elongation and myogenic fusion (Li *et al.*, 2007). It was found that the attachment of Ub to and degradation of Skp2 was greatly reduced in Cdh1-depleted cells when compared to control C2C12 cells. The authors speculated that the increase in Skp2 levels in Cdh1-depleted cells would lead to reduced levels of p21 and p27, cell cycle regulators which are crucial for muscle differentiation (Vernon and Philpott, 2003; Zhang *et al.*, 1999). However, it was also suggested that the myogenic transcription factor myf5 is a target for APC/C^{Cdh1} (Li *et al.*, 2007).

Although a key determinant in neural differentiation, SCF^{Skp2} is not the only E3 ligase to have been implicated in this process. Recently it has been shown that the master repressor of neuronal gene expression, *RE1 silencing transcription factor* (*REST*), is a substrate for SCF$^{β\text{-}TRCP}$-mediated degradation (Chong *et al.*, 1995; Schoenherr and Anderson, 1995; Westbrook *et al.*, 2008). However, the functional relevance of this interaction to neural development is not clear, as the *in vivo* data presented are mostly obtained from epithelial cells or cell lines of non-neural origin. Nevertheless, data from neural stem and progenitor cells seem to suggest that endogenous REST stability is regulated by SCF$^{β\text{-}TRCP}$ during early neural differentiation (Westbrook *et al.*, 2008).

FBW2, another F-box protein, currently has only one known substrate, *glial cells missing homologue 1* (*GCM1*), an interaction observed in a placental cell line (Chiang *et al.*, 2008). In *Drosophila*, *glial cells missing* (*gcm*) was first identified as a glial fate switch gene which, when overexpressed, caused an increase in the number of glial cells but not total number of cells in the nervous system (Hosoya *et al.*, 1995; Jones *et al.*, 1995). More recent work suggests that *gcm* degradation allows cell cycle exit and differentiation of glial progenitors in *Drosophila* (Ho *et al.*, 2009). Rapid degradation of gcm allows the daughter cells of the thoracic neuroglioblast, NB6-4T, which expresses *gcm* at low levels, to adopt differing cell fates following asymmetric segregation of *gcm* transcript. Recently, a role for *gcm* in the differentiation of the *Drosophila* haemocyte lineage has also been reported (Jacques *et al.*, 2009). Intriguingly, although the mammalian homologues *GCM1* and *GCM2* share high sequence homology in the DNA binding domain and conservation of domain structure (Akiyama *et al.*, 1996; Altshuller *et al.*, 1996; Kim *et al.*, 1998), there appears to be no functional conservation between *Drosophila* and mammals (Basyuk *et al.*, 1999; Kanemura *et al.*, 1999).

GCM1 is mainly expressed in the placenta, with lower levels of expression in the thymus, whilst *GCM2* is expressed in the developing mouse parathyroid gland (Basyuk *et al.*, 1999; Kim *et al.*, 1998). In the placenta, *GCM1* is absolutely required for expression of the fusogenic protein syncytin and knockout leads to embryonic lethality at E9.5-10 due to aberrant labyrinth forma-

tion (Anson-Cartwright *et al.*, 2000; Schreiber *et al.*, 2000). In contrast to the extraembryonic tissue, there appear to be no embryonic abnormalities associated with *GCM1* knockout. Although little is known about the role of degradation in the regulation of *GCM1*, it is noteworthy that both *Drosophila* and mammalian homologues have a conserved role for the UPS despite a complete lack of conservation of developmental function.

Ppa, Fbw7 and neural crest development

The transcription factor *Slug* is required for NC development in *Xenopus* (for example, LaBonne and Bronner-Fraser, 2000), and the degradation of Slug by the F-box protein Partner of Paired (Ppa, also known as FBXL14 in vertebrates) has been reported in *Xenopus* (Vernon and LaBonne, 2006). Using overexpression and knock-down techniques, it was demonstrated that SCF^{Ppa}-mediated degradation of Slug was important for patterning the neural plate border; overexpression of *Ppa* expanded the neural plate, at the expense of the NC, whereas by contrast, overexpression of a *Ppa*-refractory *Slug* mutant expanded the NC and led to premature migration of NC cells in the spinal cord. It is interesting to note that two key regulators of NC development, *Slug* and *Snail*, are degraded by SCF E3 ligases (SCF^{Ppa} and $SCF^{\beta\text{-}TRCP}$ respectively, see above).

A further role for F-box proteins in the development of the NC has been recently described in *Xenopus*, where the function of *Fbw7* was perturbed by expression of an *Fbw7ΔF-box* mutant (Almeida *et al.*, 2010). Loss of Fbw7 activity led to reduced expression of both *Slug* and *Snail*, as well as *c-Myc*, and the loss of NC-derived tissues, such as melanocytes. The activity of Fbw7 appeared to be required specifically for development of NC, as expression of early markers in other tissues was unperturbed when Fbw7 activity was inhibited. Thus it appears that several stages of NC development are regulated by SCF complexes.

MAFbx and myogenesis

Muscle Atrophy F-box (*MAFbx*), also known as *Atrogin-1* and *FBXO32*, was first identified as an E3 ligase that could target MyoD, the master regulator of myogenesis, for Ub-mediated proteolysis (Davis *et al.*, 1987; Lassar *et al.*, 1991) and is a muscle-specific gene upregulated during muscle atrophy (Bodine *et al.*, 2001; Gomes *et al.*, 2001). In C2C12 cells, *MAFbx* expression increased during *ex vivo* differentiation (Tintignac *et al.*, 2005).

MAFbx was first characterised following a yeast two-hybrid screen using Skp1 binding proteins as prey and MyoD as bait (Tintignac *et al.*, 2005), establishing that the two proteins interacted via an LXXLL motif on MyoD. This suggests that, unusually for an F-box protein, MAFbx recognises MyoD independently of MyoD phosphorylation state. Overexpression of *MAFbx* reduced the half-life of MyoD and also increased the ubiquitylation of MyoD. Conversely, inhibition of *MAFbx* using a dominant negative ΔF-box construct (*MAFbxΔF*) increased the half-life of MyoD. As well as the considerable evidence for MyoD degradation by MAFbx *in vitro*, a recent report has also demonstrated increased polyubiquitylation of MyoD following transfection of *MAFbx*, but not *MAFbxΔF*, into C2C12 cells (Lagirand-Cantaloube *et al.*, 2009). A direct interaction between SCF^{MAFbx} and myogenin, a bHLH transcription factor downstream of MyoD, has been demonstrated and SCF^{MAFbx} was seen to promote polyubiquitylation and degradation of myogenin *in vivo* (Jogo *et al.*, 2009).

Conclusions

The UPS is well known for its housekeeping role in protein turnover but it is becoming increasingly clear that it also plays a crucial role in dynamic processes involved in development, where ubiquitylation can result in either protein destruction, proteolytic processing or change in sub-cellular localization. Single SCF E3 ligase complexes may have multiple targets, exemplified by $SCF^{\beta\text{-}TRCP}$, which potentially results in co-ordination of developmental signaling pathways, while single targets can be ubiquitylated by more than one E3 ligase complex. The selectivity, irreversibility and responsiveness of SCF complexes make them excellent candidates for developmental regulation, while substrate ubiquitylation is also often regulated by further post-translational substrate modification such as phosphorylation, which can fine-tune cellular responses.

Such complexity, illustrated well by the multiple roles of $SCF^{\beta\text{-}TRCP}$, often makes it difficult to define the role of individual E3 ligases in distinct developmental events. However, the use of multiple model systems and analysis of individual substrates can both facilitate this reductionist approach to identifying the role of distinct SCF complexes, and allow us to explore the role of multiple ubiquitylation pathways in regulating complex developmental events. There is clearly a lot to learn.

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