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...
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_CX_{9-29}CX_{1-3}HX_{9-29}C/H)X_{2-48}CX_{3-4}C). 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. SCFSkp2, 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 SCFSkp2 (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 SCFbw7

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**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., 2008a; Skaar et al., 2008b), 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 aspect of F-box protein activity 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 E3 enzyme (Wu et al., 2002).

### TABLE 1

<table>
<thead>
<tr>
<th>Tissue/cell type</th>
<th>F-box component</th>
<th>Substrate</th>
<th>Function</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraembryonic</td>
<td>Fbw2</td>
<td>GCM1</td>
<td>GCM1 required for development of extraembryonic tissue in mammals</td>
<td>Schreiber et al., 2000</td>
</tr>
<tr>
<td>Early embryo</td>
<td>β-TRCP</td>
<td>β-Catenin</td>
<td>Regulation of β-Catenin stability and transcriptional activity</td>
<td>Latres et al., 1999; Kitagawa et al., 1999; Hart et al., 1999</td>
</tr>
<tr>
<td>Cactus</td>
<td>β-TRCP</td>
<td>Ci</td>
<td>Regulation of Ci transcriptional activity</td>
<td>Jia et al., 2005; Wang and Li, 2006</td>
</tr>
<tr>
<td>Fbw7</td>
<td>Notch</td>
<td>β-Catenin</td>
<td>Phosphorylation-dependent degradation of Notch-ICD and regulation of transcription</td>
<td>Gupta-Rossi et al., 2001; Wu et al., 2001</td>
</tr>
<tr>
<td>Epithelia</td>
<td>β-TRCP</td>
<td>hDLG</td>
<td>Dlg inhibits epithelial differentiation in Drosophila; interaction with β-TRCP seen only with hDLG so far</td>
<td>Mantovani and Banks, 2003; Woods et al., 1996</td>
</tr>
<tr>
<td>Haematopoietic</td>
<td>Fbw7</td>
<td>TAp63γ</td>
<td>Possible role in epidermal differentiation via regulation of transcriptional activity</td>
<td>Gallegos et al., 2008</td>
</tr>
<tr>
<td>Muscle</td>
<td>MAFbx</td>
<td>MyoD</td>
<td>Promotes MyoD polyubiquitylation and degradation in vitro and in vivo</td>
<td>Lagrand-Cantaloube et al., 2009; Tintignac et al., 2005</td>
</tr>
<tr>
<td>Neural</td>
<td>β-TRCP</td>
<td>REST</td>
<td>Degradation of transcriptional repressor, promoting neuronal differentiation</td>
<td>Jogo et al., 2009</td>
</tr>
<tr>
<td>Neural crest</td>
<td>Fbw2</td>
<td>gcm</td>
<td>Degradation of gcm allows cell cycle exit and differentiation of glial progenitors in Drosophila</td>
<td>Ho et al., 2009; Hosoya et al., 1995</td>
</tr>
<tr>
<td>Neural crest</td>
<td>Ppa</td>
<td>Slug</td>
<td>Regulation of Slug stability during neural crest development</td>
<td>Box-Peralles et al., 2007</td>
</tr>
<tr>
<td>Fbw7</td>
<td>Skp2</td>
<td>Xic1</td>
<td>Regulation of primary neuronal differentiation in Xenopus</td>
<td>Vernon et al., 2006</td>
</tr>
<tr>
<td>Neural crest</td>
<td>Fbw7</td>
<td>Slug</td>
<td>Regulation of Slug stability during neural crest development</td>
<td>Almeida et al., 2010</td>
</tr>
</tbody>
</table>

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, SCFβ-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).

**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 localization 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 SCFβ-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, SCFβ-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 SCFβ-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 SCFβ-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 derepressed. 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*β-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*-mediated processing of *Ci* results in a repressive form (*CiRep*) rather than full destruction (Fig. 3). SCF*SLimB*-mediated processing of *Ci* is unusual, as ubiquitylation triggers partial proteolysis rather than full destruction (Fig. 3). SCF*SLimB*-mediated processing of *Ci* results in a repressive form (*CiRep*) rather than full destruction (Fig. 3). SCF*SLimB*-mediated processing of *Ci* results in a repressive form (*CiRep*) rather than full destruction (Fig. 3). SCF*SLimB*-mediated processing of *Ci* results in a repressive form (*CiRep*) rather than full destruction (Fig. 3). SCF*SLimB*-mediated processing of *Ci* results in a repressive form (*CiRep*) rather than full destruction (Fig. 3). SCF*SLimB*-mediated processing of *Ci* results in a repressive form (*CiRep*) rather than full destruction (Fig. 3). SCF*SLimB*-mediated processing of *Ci* results in a repressive form (*CiRep*) rather than full destruction (Fig. 3). SCF*SLimB*-mediated processing of *Ci* results in a repressive form (*CiRep*) rather than full destruction (Fig. 3). SCF*SLimB*-mediated processing of *Ci* results in a repressive form (*CiRep*) rather than full destruction (Fig. 3). SCF*SLimB*-mediated processing of *Ci* results in a repressive form (*CiRep*) rather than full destruction (Fig. 3). SCF*SLimB*-mediated processing of *Ci* results in a repressive form (*CiRep*) rather than full destruction (Fig. 3). SCF*SLimB*-mediated processing of *Ci* results in a repressive form (*CiRep*) rather than full destruction (Fig. 3). SCF*SLimB*-mediated processing of *Ci* results in a repressive form (*CiRep*) rather than full destruction (Fig. 3).

**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*β-TRCP*, which ubiquitylates *IκB* at lysines 21 and 22 (Maniatis, 1999) after modification of *IκB* by phosphorylation.

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**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*β-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*β-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 SCFβ-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 SCFβ-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 SClimb 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 SCFβ-TRCP is mediated by phosphorylation of a phosphoryodegron 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 SCFβ-TRCP and TAp63γ in a human keratinocyte cell line, HaCaT (Gallegos et al., 2008). Unexpectedly, the interaction with SCFβ-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 SCFβ-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, SCFβ-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 SCFβ-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, IkB, is targeted for degradation following phosphorylation by IKKB (reviewed in Karin and Ben-Neriah, 2000). The IKKB-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 IKKB 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 Dorsalis twist, ablation of which is associated with craniofacial abnormalities (Howard et al., 1997). Further, IKKB is a direct and indirect target of TAp63, both by direct binding to a p53-like consensus sequence on the IKKB promoter and by upregulation of the transactivators E1s-1 and GATA-3 (Candi et al., 2006; Gu et al., 2004; Sil et al., 2004). IKKB 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 SCFβ-TRCP E3 ligase. However, the exact level of crosstalk between these two transcription factors remains to be firmly established.

Other developmental signaling pathways

SCFβ-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
Intriguingly, adult and embryonic haematopoietic stem cells disassociate the gene expression signature of haematopoietic stem cells. Regulation of the level of c-Myc was found to be sufficient to direct the differentiation of primary neurons in Xenopus. This process is driven by a cascade of proneural basic Helix-Loop-Helix (bHLH) transcription factors, and the switch gene which, when overexpressed, caused an increase in the number of glial cells but not total number of cells in the nervous system (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., 1995), 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-

**Skp2, β-TRCP, FBW2 and neural differentiation**

**SCF** is an SCF complex containing the leucine rich repeat F-box protein Skp2 (also known as FBXL1). SCF 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) p27Kip1 (Carrano et al., 1999), p57Kip2 (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, SCFβ 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 ubiquitination of substrates by the SCFβ 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 SCFβ regulates this process. It is interesting to note in this regard that the CKI p57Kip2 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 p27Kip1 by SCFβ in mammals, Xic1 degradation in Xenopus by SCFβ 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 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 (Li et al., 2007).

Although a key determinant in neural differentiation, SCFβ 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 SCFβ-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 SCFβ during early neural differentiation (Westbrook et al., 2008).

**FBW2**, another F-box protein, currently has only one known substrate, **gcm**. In Drosophila, **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., 1995; Kim et al., 1998), there appears to be no functional conservation between Drosophila and mammals (Basyuk et al., 1999; Kanemura et al., 1999).
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 knockdown. 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, LaBonне 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 LaBonне, 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_{Fbw7} 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 Fbw7 ΔF-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 degradation (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 ubiquitination 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_{β-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_{β-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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