

# A dominant-negative form of the E3 ubiquitin ligase *Cullin-1* disrupts the correct allocation of cell fate in the neural crest lineage

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Selective protein degradation is an efficient and rapid way of terminating protein activity. Defects in protein degradation are associated with a number of human diseases, including potentially DiGeorge syndrome, which is characterised by abnormal development of the neural crest lineage during embryogenesis. We describe the identification of *Xenopus Cullin-1*, an E3 ubiquitin ligase, and show that blocking the function of endogenous *Cullin-1* leads to pleiotropic defects in development. Notably, there is an increased allocation of cells to a neural crest fate and within this lineage, an increase in melanocytes at the expense of cranial ganglia neurons. Most of the observed effects can be attributed to stabilisation of  $\beta$ -catenin, a known target of *Cullin-1*-mediated degradation from other systems. Indeed, we show that blocking the function of *Cullin-1* leads to a decrease in ubiquitinated  $\beta$ -catenin and an increase in total  $\beta$ -catenin. Our results show that *Cullin-1*-mediated protein degradation plays an essential role in the correct allocation of neural crest fates during embryogenesis.

**KEY WORDS:** *Cullin-1*, SCF, Neural crest,  $\beta$ -catenin, Ubiquitin, Protein degradation, *Xenopus*

## INTRODUCTION

During embryonic development the molecular profile of a cell has to be regulated in order to achieve cell specialisation. A large proportion of this regulation is carried out at the transcriptional level. However, it is becoming increasingly clear that proteins have to be actively removed from a cell if their function is no longer required. This removal is achieved by protein degradation via the 26S proteasome, which can degrade proteins down to peptides.

As not all proteins have to be degraded, an active selection mechanism is in place that marks the degradation targets by adding multiple ubiquitin molecules. This ubiquitination is catalysed by an enzyme cascade involving the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), the ubiquitin ligase (E3) (reviewed by Deshaies, 1999) and the still controversial E4, which is thought to facilitate the transition from monoubiquitination to polyubiquitination (Koegl et al., 1999).

The E3 ligases, which bind to a substrate and attach the ubiquitin molecule, are believed to provide substrate specificity. Structurally, E3 ligases can be subdivided into Ring-domain or HECT-domain based ligases. Both are protein motifs that enable the binding of the E2-conjugating enzyme, the protein that provides the ubiquitin molecules. Some E3 ligases, e.g. Smurf, Neuralized, Mind Bomb or Greul are single proteins. They usually contain the substrate-binding domain in the N terminus and the Ring or HECT domain in the C terminus. However, the majority of the E3 ligases consist of a protein complex where the tasks of substrate- and E2 binding are subdivided between separate proteins (Deshaies, 1999).

A well-known group of multi-subunit E3 ligases are the Cullin-Ring ubiquitin ligases (CLRs), which are based around a Cullin core. The function of Cullin proteins is to bind other proteins with its N

and C termini; hence, acting as a scaffold for the CLR complex. The archetypal CLR is the *Cullin-1*-based SCF (Skp1, Cullin-1, a Ring protein and an F-box protein) complex. The N terminus of Cullin-1 binds to a linking protein such as Skp1 and via this to an F-box protein that binds the substrate. It is believed that the substrate specificity of SCF stems from the F-box protein; examples of F-box proteins are cyclin F, cdc4,  $\beta$ -TrCP and Skp2 (Bai et al., 1996; Latres et al., 1999; Lisztwan et al., 1998; Skowyra et al., 1997; Yu et al., 1998). These are involved in the ubiquitination of cell cycle regulators such as p27<sup>Kip1</sup>, p21 and cyclin E (Nakayama et al., 2000; Wang et al., 1999; Yu et al., 1998), and transcription factors such as  $\beta$ -catenin, Gli, I $\kappa$ B and E2F (del Pozo et al., 2002; Ou et al., 2002; Jiang and Struhl, 1998; Yaron et al., 1998). The C terminus of Cullin protein binds to a Ring protein, e.g. Rbx-1, which in turn binds the E2 enzyme. There are at least seven known Cullin proteins in humans, which can assemble into different CLRs.

Many E3 ligases have been found to be involved in developmental processes. Among the single protein E3 ligases, GREUL1 (Borchers et al., 2002) anteriorises ectoderm when overexpressed in *Xenopus*, while Smurf degrades Smad1 in *Xenopus* (Zhu et al., 1999) and *Drosophila* (Podos et al., 2001), thereby limiting BMP signalling. Recently, ectodermin, a Smad4 ubiquitinating ligase has been shown to limit TGF- $\beta$  signalling in early *Xenopus* embryos and in human adult cells (Dupont et al., 2005). Interestingly, not all ubiquitination events necessarily result in degradation. For example Neuralized and Mind Bomb are E3 ligases that ubiquitinate the Notch ligand Delta, which subsequently leads to its internalisation and enhanced Notch signalling (Chen and Casey Corliss, 2004; Deblandre et al., 2001; Itoh et al., 2003; Le Borgne and Schweisguth, 2003). Among the multi-protein E3 ligases, Ozz promotes  $\beta$ -catenin degradation in myofibres (Nastasi et al., 2004), while APC/C (an E3 ligase complex whose subunit APC2 has Cullin protein homology), controls synapse size at the neuromuscular junction in *Drosophila* (van Roessel et al., 2004).

Although the structure of the SCF (the archetypal multiprotein CLR) is well understood (Zheng et al., 2002) and many of its targets have been identified, large gaps remain in our understanding of its

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function (Deshaies, 1999), particularly with regards to its role in a developmental context. Knockout studies of its core component *Cullin-1*, which should lead to the abolishment of all canonical SCF activity, have been carried out in *C. elegans*, mouse and *Arabidopsis* (Dealy et al., 1999; Kipreos et al., 1996; Shen et al., 2002; Wang et al., 1999). The resulting phenotypes were hyperplasia with a shortened G1 phase in *C. elegans*, and arrested embryogenesis in all cases. Similarly, loss of function of the Ring protein Roc1a, which provides the link between *Cullin-1* and the E2-conjugating enzyme, leads to embryonic lethality in *Drosophila* (Noureddine et al., 2002). In parallel, a role of the SCF complex in the *Drosophila* eye development is well documented (reviewed by Ou et al., 2003). Finally, RNAi against *Skp1* (the linking protein between Cullin proteins and the F-box protein) in *C. elegans* causes tissue hyperplasia and embryonic arrest (Nayak et al., 2002). Loss-of-function studies have also been carried out on the F-box components of the SCF. Mice with a mutation for *cyclin F* die at midgestation (Tetzlaff et al., 2004). In *Skp2*-deficient mice there was an increased accumulation of Cyclin E and p27 in the mutant cells but the mice were viable although slightly smaller (Nakayama et al., 2000). Finally, hypomorphic loss of function of the F-box protein *Slimb* (*Drosophila*  $\beta$ -TrCP) in a tissue-restricted manner leads to an accumulation of Ci (Gli) and Arm ( $\beta$ -catenin), which in turn results in supernumerary limbs (Jiang and Struhl, 1998).

Some evidence for a role of the SCF in early vertebrate development comes from studies on the F-box protein  $\beta$ -TrCP (Latres et al., 1999). Overexpression of a dominant-negative  $\beta$ -TrCP in early *Xenopus* embryos caused secondary axes (Lagna et al., 1999; Marikawa and Elinson, 1998). This is consistent with the proposed role of  $\beta$ -TrCP in  $\beta$ -catenin degradation, which is a well known mode of regulating  $\beta$ -catenin during the establishment of the axis in *Xenopus* and mouse (reviewed by Huelsken and Birchmeier, 2001; Weaver and Kimelman, 2004). Although these studies indicate the importance of the SCF during very early vertebrate embryonic development, its role in later developmental events remains to be characterised.

This characterisation is especially important as it has been recognised that protein degradation deficiencies underlie many human diseases (reviewed by Sakamoto, 2002). A number of cancers appear to be caused by the deregulation of protein degradation. For example, cervical cancer is thought to be due to the excessive destruction of the tumour suppressor p53 (reviewed by Sakamoto, 2002) and in breast cancer cells high levels of cyclin E coincided with a mutation in the F box protein *Cdc4* (Rajagopalan et al., 2004; Strohmaier et al., 2001). In addition, neurodegenerative diseases such as Parkinson's disease (Staropoli et al., 2003) and Angelman syndrome (Kishino et al., 1997; Matsuura et al., 1997) also appear to be the consequence of mutations in E3 ligases. DiGeorge syndrome, a birth defect characterised by misregulation of neural crest cells during early development, has been associated with a deletion of *UFD1* (Yamagishi et al., 1999) (reviewed by Baldini, 1999). This protein appears to be involved in the linking step between polyubiquitination and proteasome-mediated degradation (Bays and Hampton, 2002). A definitive causal relationship between *UFD1* and DiGeorge syndrome has not been proven yet, and there are other good candidates deleted in this syndrome, including TBX1 (reviewed by Baldini, 2005). In addition, several of the syndrome's neural crest defects can be recapitulated by inactivation of TGF- $\beta$  signalling (Wurdak et al., 2005). Nevertheless, the large variety of defects caused by aberrant protein degradation (reviewed by Sakamoto, 2002) indicates that this process is vital in many tissues and stages during development. The

investigation of protein degradation during development should contribute towards our understanding of the basis of such diseases known as 'ubiquitinopathies'.

Here, we present the functional analysis of *Xenopus Cullin-1* in a developmental context. We show that *Cullin-1* is expressed throughout early *Xenopus* development and is enriched in neural tissue. Overexpression of a truncated dominant-negative form leads to an accumulation of  $\beta$ -catenin, one of its degradation targets, and a reduction of ubiquitinated  $\beta$ -catenin, suggesting that it prevents  $\beta$ -catenin degradation. Embryos where the dominant-negative *Cullin-1* is overexpressed, display a range of embryological defects most noticeably an upregulation of melanocytes, a neural crest derived tissue. Analysis with molecular markers at different stages in development suggests that this increase is due both to an increased allocation of ectodermal tissue to the neural crest and to an increased allocation of neural crest progenitors to the melanocyte lineage. Our findings demonstrate for the first time a link between *Cullin-1* function and neural crest development. Moreover, these findings demonstrate that there is a requirement for protein degradation that goes beyond the very early embryonic stages and is essential for the correct cell fate allocation throughout development.

## MATERIALS AND METHODS

### Embryo culture and injections

*X. laevis* embryos were obtained by hormone induced egg laying and in vitro fertilisation by standard methods. Injections were carried out with in vitro transcribed capped RNA anally into one blastomere at the two-cell stage. RNA was injected in a volume of 10 nl, at doses ranging from 10 pg to 3 ng, and 200–300 pg of *lacZ* RNA was co-injected as a lineage tracer. As a control, *lacZ* RNA was injected alone at the highest dose employed for the experimental RNA. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967) and grown to the desired stage. They were then fixed in MEMFA and stained with X-gal as described (Bourguignon et al., 1998).

### In situ hybridisation

In situ hybridisation was performed as previously described (Harland, 1991). Probes were made from digoxigenin-labelled antisense RNA. At neurula stages, embryos were analysed with the *X. laevis* molecular marker *Sox3* (Penzel et al., 1997). Moreover, the *X. tropicalis Sox9* (Spokony et al., 2002), *Slug* (Mayor et al., 1995), *Sox10* (Aoki et al., 2003; Honore et al., 2003) and *Zic3* (Nakata et al., 1997) were also used. At tadpole stages, embryos were analysed for *N-tubulin*, a marker for differentiated neurons (Chitnis and Kintner, 1995; Oschwald et al., 1991). The chromogenic reaction was carried out with either nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) or with 5-bromo-6-chloro-3-indolyl-phosphate (magenta phos). In order to obtain sections, embryos were embedded in gelatine albumin and 30  $\mu$ m sections were cut using a vibratome.

### Western blot analysis

Embryos were snap-frozen at the desired stage. Proteins from 20 embryos were extracted in 150  $\mu$ l 15 mM Tris-HCl pH 7.5 containing the Complete Protease Inhibitor (Roche). Excessive yolk was removed by the addition of an equal volume of Freon (1,1,2-Trichlorotrifluoroethane, Sigma F5217). Proteins were separated in 8% SDS acrylamide gels and transferred onto nitrocellulose membranes. Blots were hybridised with a rabbit polyclonal anti  $\beta$ -catenin (1/500, Santa Cruz-H102), a mouse monoclonal *Cullin-1* (1/200, Santa Cruz- Cul1-D5) or a mouse monoclonal anti alpha tubulin (1/5000, clone DM1A, Sigma) antibody. Between different antibody stainings the blots were stripped for 1 hour at 55°C in 100 mM  $\beta$ -mercaptoethanol containing stripping solution (62.5 mM Tris-HCl pH 6.7, 2% SDS). Goat-anti-mouse-HRP or goat-anti-rabbit-HRP secondary antibodies were used (1/2500, Jackson Immunoresearch Laboratories). The blots were processed with ECL or ECL plus (Amersham), and the chemiluminescent signal was detected on medical film.

### Immunoprecipitations

H293T cells were cultured in DMEM + 10% foetal calf serum. Confluent cultures were transfected with DNA using Lipofectamine 2000 (Invitrogen). Empty pCS2 vector DNA was used when not all of the constructs were employed. The following CMV expression constructs were used for the transfections. *X. laevis*  $\beta$ -catenin was N-terminally HA-tagged and cloned into the expression vector pCS107. pCW7 Myc-His<sub>6</sub>-Ubiquitin (a gift from M. Goldberg) and either the truncated pCS107 *Cullin-1* (*Cull1-C75* and *Cull1-C477*) or the full-length pCS107 *Cullin-1* (TEgg108117) or *cullin-3* (TNeu078f05) were used. Thirty-six hours after transfection, 10  $\mu$ M proteasome inhibitor MG132 was added to allow accumulation of ubiquitinated proteins. After 48 hours, cells were harvested (10 cm dish/transfection) and proteins were extracted. To reduce non-specific binding, lysates were pre-cleared with mouse IgG and Protein G beads for 1 hour. They were then incubated with anti-Myc-antibody-coupled agarose (Santa Cruz, 9E10, sc40-AC) overnight. The next day, the beads were washed thoroughly and the bound proteins were eluted in sample buffer. The eluate was separated on 8% SDS acrylamide gels and westerns were performed with anti-HA-HRP (1/5000 Roche). Approximately 10% of the lysate was retained as a control. Those samples were separated on 8% SDS acrylamide gels and westerns were performed as described above.

## RESULTS

### Cullin-1 is a very conserved protein among vertebrate species

*Cullin-1* was identified during a gain-of-function screen in *X. tropicalis* for genes affecting neural development (Voigt et al., 2005). The active clone in the screen was a C-terminally truncated form, missing the last 75 amino acids (clone TGas038c21, thereafter referred to as *Cul1-C75*; Fig. 1A,B). A full-length *Xenopus tropicalis* *Cullin-1* clone was subsequently identified in an EST database that allows full coding sequence predictions (TEgg108117) (Gilchrist et al., 2004) and was fully sequenced. *Xenopus tropicalis* *Cullin-1* is 99.6% identical to mouse and human *Cullin-1* at the protein level (Fig. 1A). Sequence comparison showed that the truncated clone lacks a large region of the Rbx-1 binding domain, the SCF subunit that binds to the E2 conjugating enzyme and provides the SCF with ubiquitin molecules (Zheng et al., 2002) (Fig. 1A-C). This truncation is very likely to inhibit the interaction between *Cullin-1* and *Rbx-1* (Fig. 1C). Moreover, the neddylation site, which is thought to activate the SCF functionally by attaching a Nedd8 molecule to *Cullin-1* (Lammer et al., 1998; Morimoto et al., 2000; Osaka et al., 1998) (star in Fig. 1A), is located in the missing area.

### Cullin-1 is expressed during embryonic development and is enriched in neural tissue

*Cullin-1* RNA is ubiquitously found throughout the blastula and gastrula embryo. At neural plate stages, it is enriched in the area of the neural ectoderm, particularly in the anterior region. In the tadpole, expression is enriched in the neural tube, eye and branchial arches (Fig. 2A) (see also Voigt et al., 2005).

Western blot analysis showed that there is a large supply of maternal *Cullin-1* protein in the egg and that the protein level remains near constant throughout early embryonic development. A slight increase is observed during late neurula/early tailbud stages (stage 19-28) (Fig. 2B).

### Ectopic truncated and full-length *Cullin-1* leads to an increase of $\beta$ -catenin levels in *Xenopus* embryos

A number of embryos that were injected with *Cull1-C75* displayed secondary axes, a telltale sign of ectopic  $\beta$ -catenin stabilisation. As *Cul1-C75* lacks a large part of the interaction domain with Rbx-1,

we anticipated that it would act as dominant negative of endogenous *Cullin-1*, i.e. inhibit the formation of active complexes by titrating out the remaining proteins in the complex. This in turn would lead to a stabilisation of  $\beta$ -catenin. Indeed, an increase in  $\beta$ -catenin was observed in *Cull1-C75* embryos at stage 8 and stage 19 (Fig. 3A,C). One might expect that overexpression of the full-length clone would have the opposite effect, i.e. it would enhance the degradation of  $\beta$ -catenin. Contrary to our expectations, overexpression of the full-length clone had the same effect as the truncated form, leading to an increase of  $\beta$ -catenin levels at stage 10 and stage 19 (Fig. 3B,C). Both constructs produced proteins of the expected molecular weight when overexpressed in the embryo (Fig. 3C). To find out whether a very low dose of overexpressed *Cullin-1* might lead to a downregulation of  $\beta$ -catenin, we injected doses of *Cullin-1* ranging from 10 pg to 3 ng into embryos. A decrease in endogenous  $\beta$ -catenin was not observed in any of the doses, whereas an increase was observed with doses as low as 50 pg (Fig. 3D).

### Overexpression of truncated and full-length *Cullin-1* in H293T cells leads to a decrease of ubiquitinated $\beta$ -catenin

If the endogenous SCF complex was inhibited, we would expect that the increase in  $\beta$ -catenin would be due to a reduction of its ubiquitination. Therefore, we examined the levels of ubiquitinated  $\beta$ -catenin when *Cullin-1* or *Cull1-C75* were overexpressed. We transfected H293T cells with *HA- $\beta$ -catenin* and *Myc-His-Ubiquitin* in the presence of 10  $\mu$ M MG132, a proteasome inhibitor (for the last 12 of 48 hours). The addition of the inhibitor allowed us to detect ubiquitinated forms of  $\beta$ -catenin that are normally rapidly degraded (Fig. 4). We then immunoprecipitated ubiquitinated proteins with an anti-Myc antibody, separated the bound proteins on an SDS/Page gel and used anti HA antibody to detect ubiquitinated  $\beta$ -catenin levels. We found that co-expression of *Cullin-1* or *Cull1-C75* led to a decrease of ubiquitinated  $\beta$ -catenin (Fig. 4).

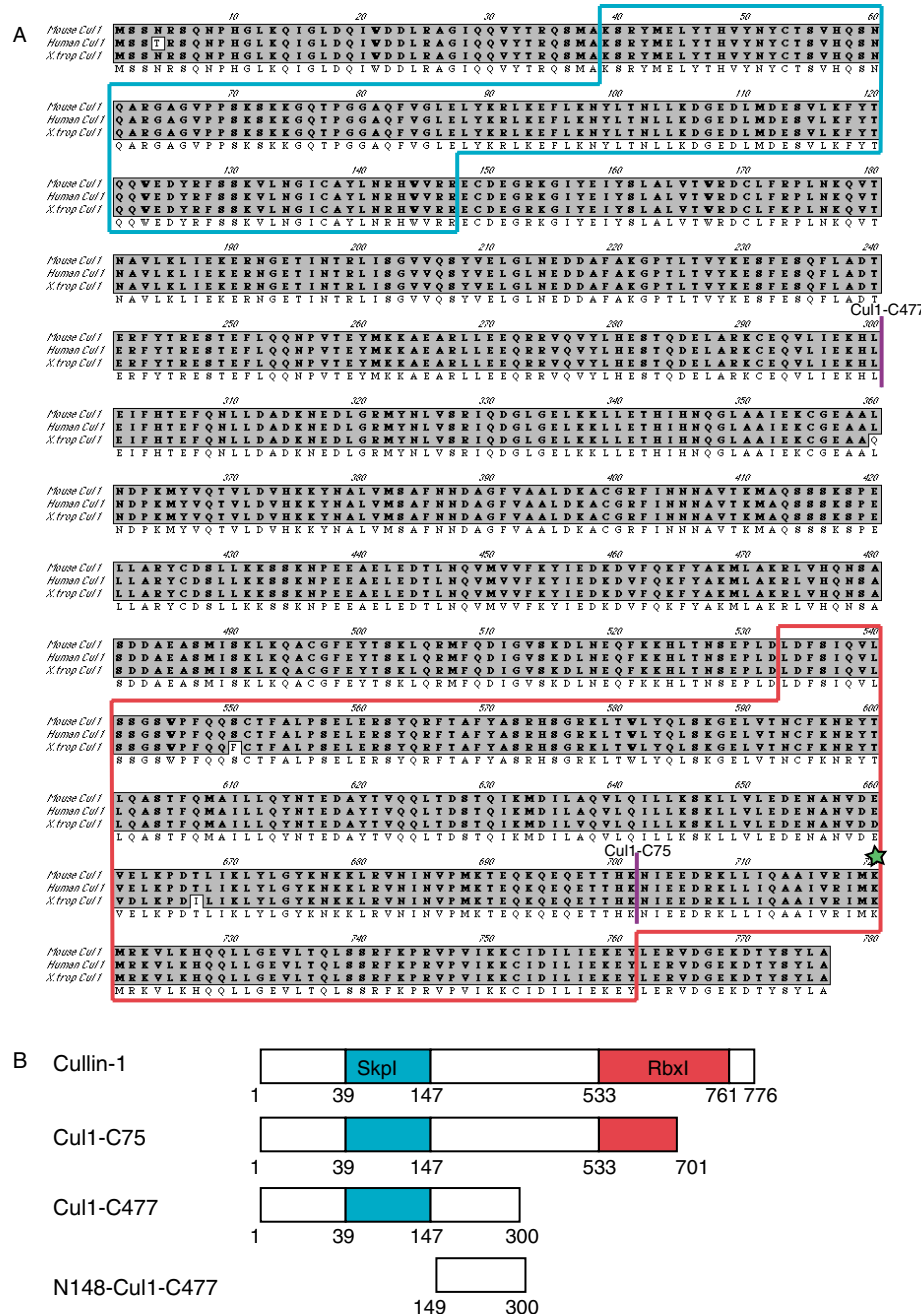
The increase of total  $\beta$ -catenin, together with the decrease of ubiquitinated  $\beta$ -catenin, suggests that the  $\beta$ -catenin ubiquitination machinery is inhibited, indicating that ectopic full-length or truncated *Cullin* proteins inhibit the SCF complex, most probably acting as dominant negatives of endogenous *Cullin* proteins. However, the *Cul1-C75* form of *Cullin-1* lacks only part of the Rbx1 interaction domain; therefore, it is formally possible that it retains wild-type activity. In this scenario, neither *Cul1-C75* nor *Cullin-1* act as dominant negatives when overexpressed, but they both act as wild-type instead. To address this possibility, we have constructed a severely truncated form of *Cullin-1* in the C-terminus that lacks the entire Rbx1 interacting domain, *Cul1-C477* (Fig. 1B). When overexpressed in H293T cells, *Cul1-C477* reduced the level of ubiquitinated  $\beta$ -catenin and indeed did so to a greater extent than either the full-length *Cullin-1* or the *Cul1-C75* construct (Fig. 4). Taken together, these results suggest that both the truncated and full length *Cullin-1* act as dominant negatives when overexpressed, most likely by inhibiting the activity of endogenous *Cullin-1* by titrating out the N-terminal components of the SCF complex into inactive or partial complexes.

Thus, these constructs provided us with an opportunity to study the effect of inhibiting endogenous *Cullin* proteins in development. Alternative means of inhibiting endogenous *Cullin* proteins such as ATG or splice site directed morpholinos in *X. tropicalis* were not effective, perhaps owing to the abundance of maternally supplied protein (data not shown).

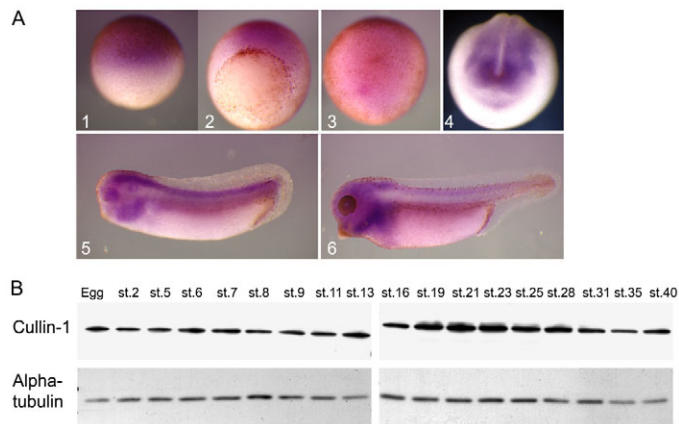
### Inhibition of endogenous *Cullin-1* in *Xenopus* embryos causes a complex phenotype

Having established that ectopic *Cullin-1* acts as a dominant negative, we carried out all of the following experiments with the full-length and the truncated Cullin-1 protein (Cul1-C75). The same phenotypes were observed with both forms. The phenotypes were complex but several distinct features could be observed. Duplicated

axes, a known consequence of ectopic  $\beta$ -catenin/*Wnt* signalling, were seen with low frequency (around 10%), presumably owing to targeting the injections to the animal rather than to the marginal area of the embryo (Fig. 5, Fig. 6D). The next most frequent phenotype were eye defects that ranged from malformations to complete absence of eye development (missing eyes scored in Fig. 5, shown in Fig. 6C). Ectopic tissue, particularly in the form of epidermal



**Fig. 1. Sequence and constructs for *Xenopus Cullin-1*.** (A) Protein alignment of the mouse, human and *X. tropicalis* Cullin-1. The full-length protein is 99.6% identical at the amino acid level. The Rbx-1- and Skp1-binding domains are boxed in red and blue, respectively. (B) Diagrammatic representation of *Cullin-1* constructs. Two C-terminally truncated versions of Cullin-1 were used. Cul1-C75 and Cul1-C477 are missing 75 and 477 amino acids from the C terminus respectively (C-terminal truncations indicated by purple bars in A). As a consequence, *Cul1-C477* contains none and *Cul1-C75* only contains a subset of the Rbx-1-binding sites. Moreover, lysine 720, the neddylation site (green star in A) is missing in both constructs. These constructs should lose or have reduced binding ability to Rbx-1, one of the components of the SCF complex, and are likely to interfere with the correct formation of the endogenous SCF. A fourth construct (N148-Cul1-C477), missing the 148 N-terminal and the 477 C-terminal amino acids, was also created. This deletes both the *Skp-1*- (yellow) and the *Rbx-1*- (red) binding domains and should not interfere with the endogenous SCF complex. (C) A wild-type SCF complex (with its substrate  $\beta$ -catenin) versus a complex based on the truncated Cullin proteins (Cul1-C75). In the wild-type scenario, Cullin proteins provide the bridge between the F-box protein that binds  $\beta$ -catenin and the E2 conjugating enzyme that supplies ubiquitin molecules. The truncation is likely to inhibit the interaction between the Cul1-C75 and Rbx-1/ E2, hence preventing SCF function. Polyubiquitination of  $\beta$ -catenin is shown by a series of grey circles, which are missing in the Cul1-C75-based SCF complex.



**Fig. 2. Expression analysis of *Cullin-1*.** (A) RNA expression pattern of *Cullin-1* during early *X. tropicalis* development. *Cullin-1* is ubiquitous at blastula (1, lateral view) and gastrula (2, vegetal, 3, animal view) stages. At neurula stages (4, anterior view) it becomes enriched in neural tissue. At tadpole stages, it becomes enriched in the neural tube, eye and branchial arches (5+6, anterior towards the left, dorsal upwards). (B) *Cullin-1* protein presence during development. Westerns with an anti-*Cullin-1* antibody were carried out on protein extracts of whole *X. laevis* embryos at different developmental stages. *Cullin-1* protein is found throughout development. *Cullin-1* protein levels are slightly increased during late neurula/early tadpole stages.

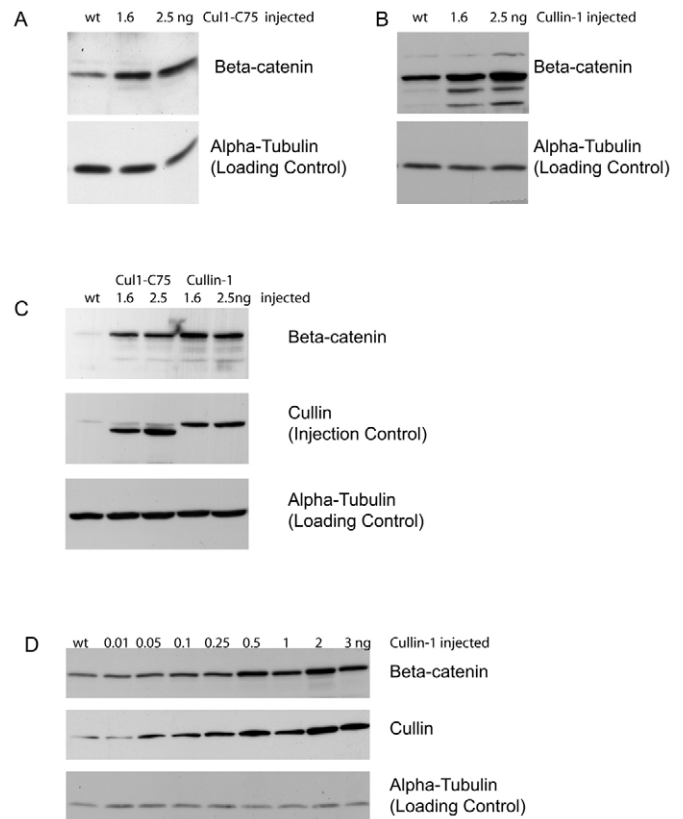
folds, was also observed with high frequency (Fig. 5, Fig. 6G). Finally, the most frequent phenotype was an increase in melanocytes, seen even at the lowest doses injected (250 pg) (Fig. 5, Fig. 6B,E,G).

Both constructs, *Cullin-1* or *Cull1-C75*, showed a dose response in relation to phenotypes found (Fig. 5). Interestingly, the incidence of ‘ectopic tissue’ and ‘missing eyes’ phenotypes was higher in the lower doses of the truncated than the full-length constructs (Fig. 5). This was consistent with our expectation that the truncated construct would be a more effective dominant negative than the full-length one. Injection of the severely truncated *Cul1-C477* that lacks the entire Rbx1 binding domain also gave rise to the same phenotype of duplicated axes and increased number of melanocytes (Fig. 7). Introducing an additional truncation in the N terminus, which is thought to interact with Skp1 (Fig. 1B, N148-*Cul1-C477*), renders the construct inactive when overexpressed in *Xenopus* embryos, as they show no phenotype (93%,  $n=66$ ).

As a test for the specificity of the *Cullin-1* phenotype, we overexpressed the *Xenopus tropicalis Cullin-3*, which is thought to be part of an E3 ligase complex that is similar to the SCF, but with different degradation targets. Although *Cullin-3* is also expressed ubiquitously during embryonic development, we did not detect a phenotype when it was overexpressed (data not shown).

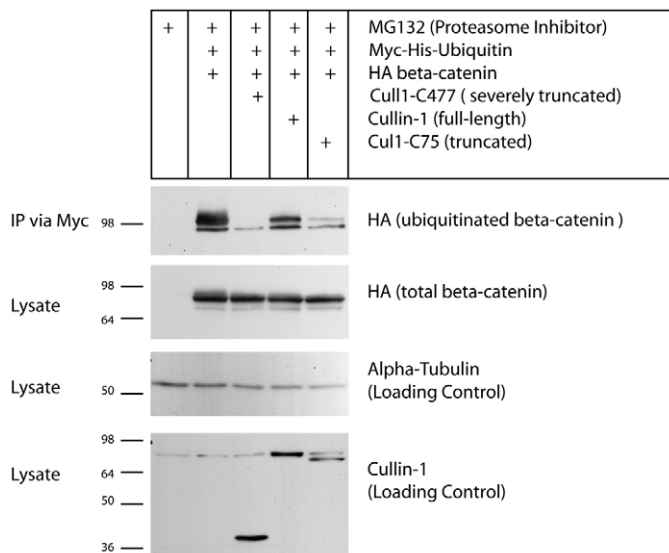
### Inhibition of *Cullin-1* leads to an increase of melanocytes at the expense of cranial ganglia

As mentioned above, the most frequent phenotype at tadpole stages were ectopic melanocytes (91%,  $n=228$ ). We quantitated the increase of melanocytes in transverse sections and found that there is an average 4.4-fold increase. As melanocytes are derived from neural crest tissue, which also gives rise to the cranial ganglia, we



**Fig. 3. *Cul1-C75* and *Cullin-1* RNA injections increase the level of  $\beta$ -catenin in the *Xenopus* embryo.** (A) 1.6 or 2.5 ng of *Cul1-C75* (truncated) capped RNA were injected animally into each blastomere of two-cell stage *X. laevis* embryos. Proteins were extracted at stage 10 and analysed for  $\beta$ -catenin levels. Injection led to an upregulation of  $\beta$ -catenin levels. Blots were stripped and analysed against  $\alpha$ -tubulin as a loading control. (B) The same was carried out for full-length *Cullin-1* RNA. Embryos were analysed at stage 8 and surprisingly an upregulation of  $\beta$ -catenin levels was also observed. (C)  $\beta$ -Catenin levels were also analysed at stage 19 (neurula stage), a time during which neural crest tissue becomes specified. As at stage 8 and 10, injections of 1.6 and 2.5 ng of either the *Cul1-C75* and the full-length *Cullin-1* lead to an increase in  $\beta$ -catenin levels. This was also found for stage 24 (data not shown). To determine whether both constructs are translated correctly, western blots were stripped and subsequently analysed for *Cullin-1* protein levels (with an antibody raised towards *Cullin-1*'s N-terminus). The full-length *Cullin-1* RNA (lane 4 and 5) results in higher levels of proteins of the correct size when compared with uninjected embryos (lane 1). A clear size difference between *Cul1-C75* and *Cullin-1* is also visible. (D) A wide range of *Cullin-1* RNA doses were injected (10 pg–3 ng into each blastomere at the two-cell stage) and embryos were analysed at stage 10. A decrease in  $\beta$ -catenin was never observed; indeed the increase of  $\beta$ -catenin correlates with the increased dose of *Cullin-1* injection.

examined the formation of cranial nerves, using *N-tubulin* as a marker (Chitnis and Kintner, 1995; Oswald et al., 1991). We found that *N-tubulin* was not disrupted in the brain or in the spinal cord, except for defects as a consequence of secondary axes. However, *N-tubulin*-positive cranial ganglia were severely disrupted or absent in 74% ( $n=35$ ) of the embryos (Fig. 6B,C,E,F, arrows). This finding suggests that increased melanocytes are produced at the expense of cranial neurons.

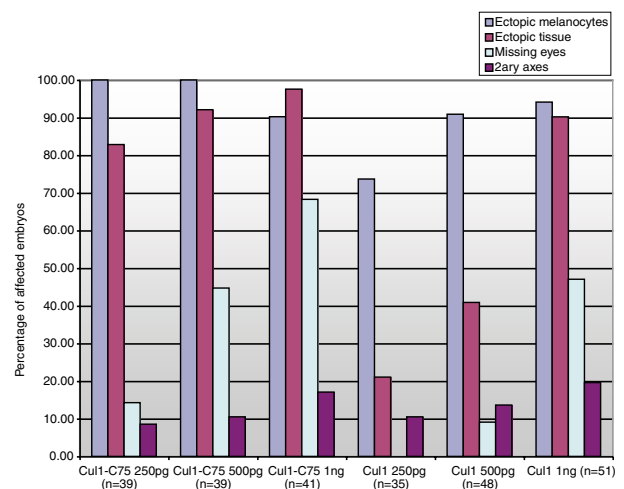


**Fig. 4. *Cullin-1*, *Cul1-C75* and *Cul1-C477* decrease  $\beta$ -catenin ubiquitination in cell lines.** H293T cells were transfected with *Myc-His-Ubiquitin*, *HA- $\beta$ -catenin* and either *Cul1-C477*, *Cullin-1* or *Cul1-C75*. After 36 hours, 10  $\mu$ M MG132 (a proteasome inhibitor) was added for 12 hours to accumulate ubiquitinated protein. All ubiquitinated protein was immunoprecipitated (IP) via Myc. Ubiquitinated  $\beta$ -catenin was visualised with an anti-HA antibody. Co-expression of any of the *Cullin-1* gene constructs led to a reduction of the levels of ubiquitinated  $\beta$ -catenin. The severity of reduction corresponded to the amount of truncation, with the most truncated construct *Cul1-C477* causing the most severe reduction (lane 3 IP), the *Cul1-C75* (lane 5) causing a strong reduction and full-length *Cullin-1* (lane 4) causing some reduction. This suggests that *Cullin-1*, *Cul1-C75* and *Cul1-C477* inhibit  $\beta$ -catenin ubiquitination, probably by inhibiting the endogenous SCF complex. As a control, ~10% of the lysate was removed previous to the IP and analysed for total levels of  $\beta$ -catenin by anti-HA staining. Co-transfection of *Cullin-1* led to increased  $\beta$ -catenin levels in the lysate control in 4/6 experiments (not shown here). An increase in total  $\beta$ -catenin is not always detected in these experiments because of the high levels of exogenous  $\beta$ -catenin and because the ubiquitinated form is only a fraction of the total  $\beta$ -catenin.

### Early neural crest markers are expanded at the expense of neural tissue when *Cullin-1* function is inhibited

An increase in melanocytes could also be the consequence of an increased allocation of neural tissue into the neural crest lineage early on. Therefore, we investigated whether early neural crest formation was affected by looking at the expression of the early cranial neural crest markers *Slug*, *Zic3*, *Sox9* and *Sox10* in neural plate stage embryos. Although some of these genes also mark the trunk neural crest (e.g. *Sox10*), at the neural plate stage they are most helpful in visualising the cranial neural crest, which is particularly prominent at this stage. All markers were increased when *Cul1-C75* or *Cullin-1* were overexpressed, but most predominantly *Zic3* (Fig. 8A). Surprisingly, in a small number of cases *Sox9* was reduced (8% reduced, 51% expanded,  $n=86$ ). *Sox10* was reduced and expanded with equal frequency (18% in both cases,  $n=66$ , not shown).

Interestingly, these markers showed that the expansion of neural crest often occurred in the anterior most neural plate of experimental embryos, an area that is normally crest free. When the injected RNA was localised to the anterior neural plate (the area adjacent to where neural crest tissue is found) *Sox3*, a neural plate marker, was often



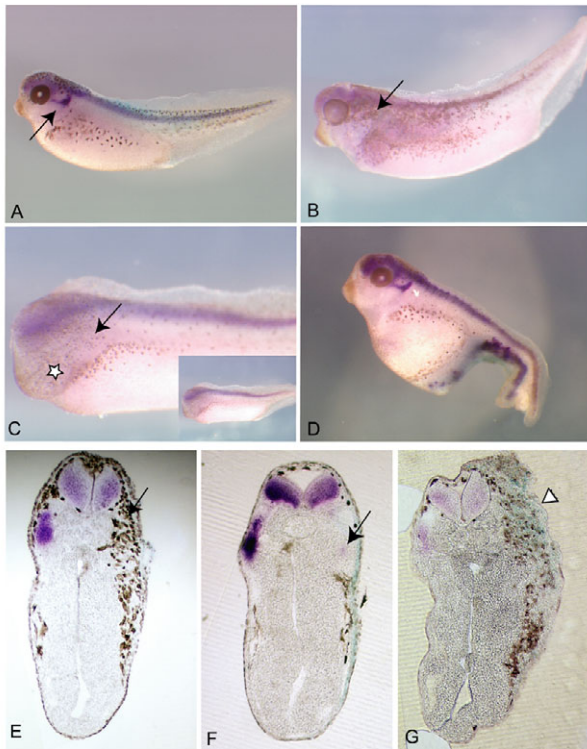
**Fig. 5. Comparative dose response of *Cul1-C75* and *Cullin-1* in inducing developmental abnormalities.** Embryos were injected with different doses of *Cul1-C75* or *Cullin-1* RNA anally into one blastomere at two-cell stage and grown to tadpole stages (stage 39/40). Morphological analysis was carried out by comparing the uninjected with the injected side and embryos were scored for defects. The phenotype predominantly consisted of ectopic melanocytes, ectopic tissue (such as epidermal folds), eye defects and secondary axes. The most robust phenotype was ectopic melanocytes, which could be seen in almost all of the injected embryos. The dose of RNA injected correlates with the penetrance of the phenotype. The truncated *Cullin-1* construct (*Cul1-C75*) was more potent at causing a phenotype than the full-length construct.

inhibited in that area (Fig. 8B). These findings suggest that inhibition of endogenous *Cullin-1* function leads to ectopic neural crest at the expense of neural plate tissue in the anterior neural plate.

## DISCUSSION

We have shown that the overexpression of a C-terminally truncated form of the E3 ubiquitin ligase *Cullin-1* leads to a complex phenotype. A number of distinct developmental defects were observed, namely double axis, abnormal ectodermal growths and reduced or missing eyes. The most striking defect observed was a large increase of melanocytes. This was brought about both by an increased, frequently ectopic, early allocation of cells to neural crest fate and by an increased allocation to a melanocyte fate within the neural crest lineage at the expense of cranial sensory neuron fate.

We attribute these effects to the inhibition of the activity of the endogenous SCF complex. The C-terminally truncated *Cullin-1* is missing the Rbx1-binding site and therefore would act as a dominant negative by titrating out endogenous components into inactive complexes (Fig. 1C). This was substantiated by an increase in one of the *Cullin-1* degradation targets,  $\beta$ -catenin, accompanied by a decrease of the ubiquitinated form. Interestingly, the same phenotypic and biochemical effects were observed after overexpressing the wild type *Cullin-1*. Obtaining the same effect with a truncated and a full-length clone is not without precedent: *Neuralized* is another case of a ubiquitin ligase where overexpression of a truncated, dominant-negative form and a full-length clone have the same effect (Deblandre et al., 2001). In another study, Mistry et al. (Mistry et al., 2004) found that overexpression and loss of *Cullin-3* leads to an accumulation of Ci155, the *Drosophila* homologue of the Gli genes.

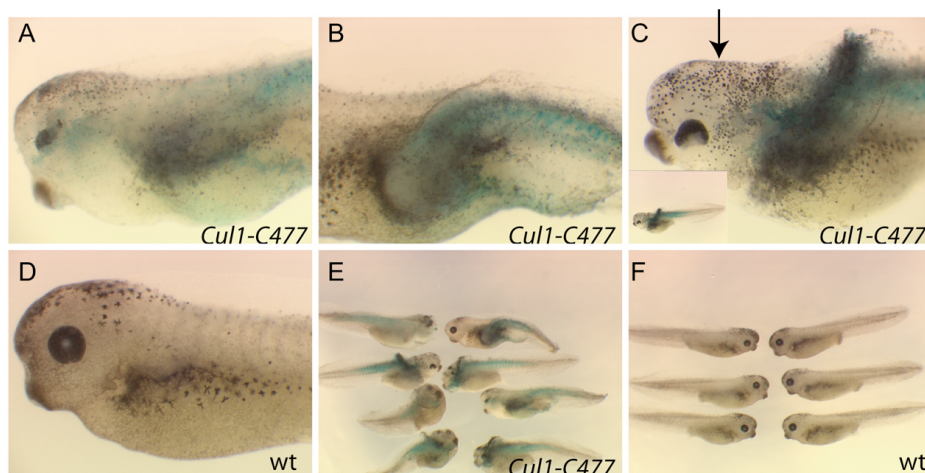


**Fig. 6. Phenotypes observed in *Cul1-C75* and *Cullin-1* injected embryos.** (A) Wild-type embryo for comparison. The most striking phenotype observed was an increase in melanocytes (B), but eye defects (C, star represents where the eye should be) and secondary axes (D) were also commonly observed (see Fig. 5 for quantification). Embryos were hybridised in situ with a probe for *N-tubulin*, a marker for differentiated neurons (purple). Ectopic *N-tubulin* can be observed in the secondary axis (D). The lineage tracer *lacZ* (light-blue staining) indicates the area of injection. Lateral view with anterior towards the left and dorsal towards the top. The injected side is shown. Transverse sections of the embryos show clearly the large upregulation of melanocytes (E-G) and the ectopic tissue (white arrowhead) in the injected side. Neural differentiation in the neural tube is not affected (as marked by *N-tubulin*); however, cranial ganglia neurons (*N-tubulin* positive, arrows) are absent (compare B,C with A and E,F with control side). Transverse view with dorsal towards the top and the injected side on the right.

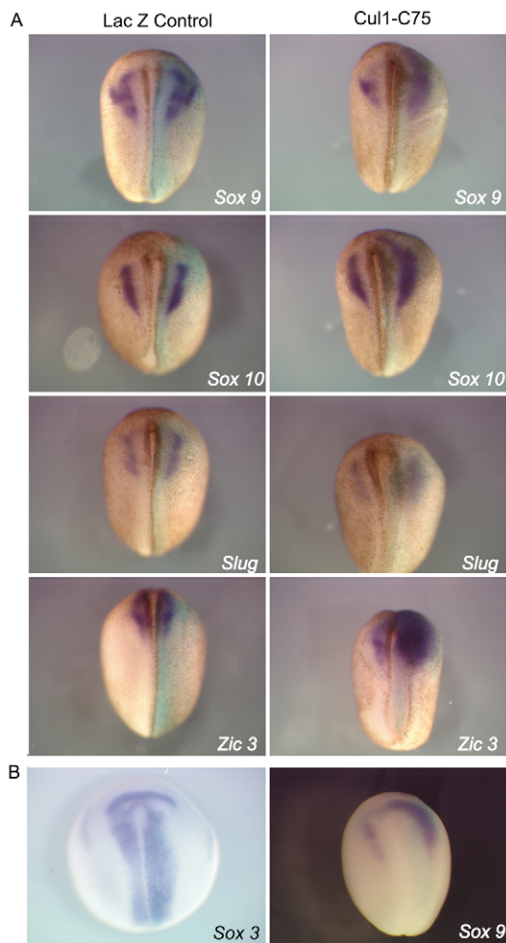
We suggest that the full-length construct also acts as a dominant negative when overexpressed, possibly by titrating out N-terminal and C-terminal binding proteins into partial complexes. As expected from this model, a construct that lacks both the N-terminal and C-terminal domain had no effect in development. We cannot exclude the possibility that the dominant-negative construct disrupts some other ubiquitin ligase that is involved in  $\beta$ -catenin degradation and shares binding partners with the SCF (reviewed by Wu et al., 2004). However, the presence of *Cullin-1* in neural tissue at the right time makes it very likely that Cullin-1 is the primary target of the

dominant-negative construct. Furthermore, a dominant-negative Cullin-1 partner  $\beta$ -TrCP shows similarities with our dominant-negative Cullin-1 construct (duplicated axes) (Marikawa and Elinson, 1998) further supporting the notion that it is an SCF complex that is disrupted in our study.

Although a number of target genes have been proposed as targets of the SCF complex, such as p27<sup>Kip1</sup>, p21 and cyclin E, many aspects of the phenotype that we have observed can be accounted for by the inappropriate accumulation of stabilised  $\beta$ -catenin. For example, it is well established that ectopic stabilisation of  $\beta$ -catenin leads to



**Fig. 7. Phenotype of a severely C-terminally truncated form of Cullin1, *Cul1-C477*.** In order to ensure that the C-terminally truncated *Cul1-C75* does not act as wild-type form of Cullin-1, we made a *Cullin-1* construct (*Cul1-C477*) that is lacking all of the C terminus (Fig. 1B). A 250 pg dose of this construct together with the lineage tracer *lacZ* (light blue) were injected anally at the two-cell stage into *X. laevis* embryos. Embryos were analysed at tadpole stages for morphological defects. Injected embryos (A-C,E) showed an accumulation of melanocytes (A-C, arrow in C), secondary axes (B) and ectopic tissue (C), all phenotypes previously observed with the other *Cullin-1* constructs. For comparison, un.injected control embryos are shown (D,F). This indicates that all of these constructs act in the same manner, which is most likely to be a dominant-negative mechanism. All embryos are shown laterally. In A-D, anterior is towards the left; in E,F, embryos are facing each other.



**Fig. 8. *Cul1-C75* RNA injections increase early neural crest markers.** (A) Embryos were injected with 500 pg of *Cul1-C75* or *Cullin-1* (same phenotype, but not shown) plus the lineage tracer *lacZ* (light blue) into one blastomere at the two-cell stage. As a control, 500 pg *lacZ* RNA was injected alone. Embryos were grown to neurula stage and analysed for the early neural crest markers *Sox9*, *Sox10*, *Slug* and *Zic3* (purple). Expression of all markers was expanded, predominantly into the anterior neural plate. In some cases, expression of *Sox9* and *Sox10* were reduced (not shown). Dorsal view with anterior towards the top. (B) When injections were targeted to the anterior neural plate, expression of *Sox3* (a marker for proliferating neural tissue) was reduced. This was in the same region where the expansion of neural crest markers (as shown for *Sox9*) was seen. Dorsal view with anterior towards the top.

duplicated axes (Funayama et al., 1995; Lagna et al., 1999; Marikawa and Elinson, 1998). Ectodermal growths could also be related to the proliferative effect of *Wnt/β-catenin* (Moon et al., 2004). As cell cycle regulators are among the known targets of *Cullin-1*, a direct effect of *Cullin-1* inhibition on cell cycle regulators is also a possible cause of these ectodermal growths (Nakayama et al., 2000; Wang et al., 1999; Yu et al., 1998).

The effect of blocking the SCF complex on neural crest can also be attributed to the increased level of  $\beta$ -catenin. It is believed that distinct waves of *Wnt* signalling first induce neural crest tissue and later on restrict neural crest lineages (Lewis et al., 2004). This is supported by many studies showing that *Wnt* signalling is necessary and sufficient for neural crest induction (reviewed by Huang and Saint-Jeannet, 2004; LaBonne and Bronner-Fraser,

1998; Wu et al., 2003; Yanfeng et al., 2003). Furthermore, *Wnt* signalling later pushes the neural crest lineage towards melanocyte development (Dorsky et al., 1998; Goodall et al., 2004; Hari et al., 2002; Jin et al., 2001; Saito et al., 2003; Takeda et al., 2000). Although the latter is widely accepted, it is not without controversy as constitutively active  $\beta$ -catenin expressed under the *Wnt1* promoter led to an upregulation of sensory neurons at the expense of melanocytes (Lee et al., 2004). The reason for this controversy is not understood and our and others results are not consistent with this view (see above).

We suggest that in truncated or full-length *Cullin-1* injected embryos increased  $\beta$ -catenin signalling is responsible, directly or indirectly, for the observed increased expression of the early neural crest markers *Slug*, *Sox9*, *Sox10* and *Zic3*. In turn, these would mediate the increased allocation to neural crest fate and in particular the melanocyte lineage at the expense of cranial neuron fate. Indeed, overexpression of  $\beta$ -catenin expands *Slug* expression at the expense of the pan-neural *Sox2* (LaBonne and Bronner-Fraser, 1998) and overexpression of *Sox10* leads to a massive increase of melanocytes, a phenotype that can also be induced by ectopic *Wnt1* (Honore et al., 2003). Overexpression of *Zic3* leads to neural and neural crest hyperplasia, concomitant with increased *Slug* expression, an increase in melanocytes, and loss of or deformed eyes (Nakata et al., 1997). This resembles the *Cullin-1* phenotype very closely. Within the neural crest lineage, *Wnt/β-catenin* signalling may promote directly the melanocyte fate by activating genes such as the transcription factor *MITF/nacre*, which is required for the development of melanocytes (reviewed by Yanfeng et al., 2003). In addition, *Sox10* transcriptionally regulates *mitfa* (Dutton et al., 2001; Elworthy et al., 2003).

Finally, the expansion of the neural crest domain in the anterior neural plate observed in truncated and full-length *Cullin-1* injected embryos is very similar to phenotypes observed in the intracellular *Wnt* signalling repressor *headless/tcf3* zebrafish mutant (Dorsky et al., 2003; Kim et al., 2000). Therefore, this effect can also be accounted for by increased/ectopic *Wnt/β-catenin* signalling in the anterior neural plate.

There are several *Wnt* genes expressed in the dorsal neural tube and neural crest that could mediate  $\beta$ -catenin activation (reviewed by Wu et al., 2003; Yanfeng et al., 2003) and several *Wnt* inhibitors could limit *Wnt* activity in this area. For example, extracellular *Wnt* inhibitors are expressed in the dorsal neural tube and these could limit *Wnt* signalling during neural and neural crest development (Baranski et al., 2000; Duprez et al., 1999; Jin et al., 2001; Ladher et al., 2000). Furthermore, intracellular *Wnt* inhibitors such as *tcf3* are thought to prevent *Wnt* signalling in the anterior neural plate (Dorsky et al., 2003; Kim et al., 2000). Here, we have shown that alongside these inhibitors, deactivation of *Wnt* signalling by ubiquitination and degradation of  $\beta$ -catenin is important for neural crest development.

Although protein degradation is a recognised means for limiting  $\beta$ -catenin signalling in early *Xenopus* development, where no zygotic transcription takes place, it was not known whether it is equally important in later vertebrate development. To date, most studies that implicate protein degradation in later developmental events come from *Drosophila* (Jiang and Struhl, 1998; Ou et al., 2003; van Roessel et al., 2004). Our results show that inhibition of *Wnt* and possibly other signalling pathways by *Cullin-1*-mediated proteolysis is a major contributor in the correct development of the neural crest lineage. If this process is misregulated, serious defects in the development of the neural crest can occur, such as, for example, in DiGeorge syndrome. Therefore, understanding how



protein degradation contributes to the specification of neural crest fates will lead to a better understanding of the aetiology of such disorders.

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