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Slug stability is dynamically regulated during neural crest development by the F-box protein Ppa

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The neural crest is a population of stem-cell-like precursors found only in vertebrates. Slug, a member of the Snail family of zinc-finger transcriptional repressors, is a critical regulator of neural crest development and has also been implicated in the acquisition of invasive behavior during tumor progression. Despite its central role in these two important processes, little is known about the mechanisms that control the expression and/or activity of Slug. We demonstrate that Slug is a labile protein whose stability is positively reinforced through activation of the neural crest regulatory program. We identify Partner of paired (Ppa) as the F-box component of a modular E3 ligase, and show that it is expressed in neural crest-forming regions, and that it binds to and promotes ubiquitin-mediated proteasomal degradation of Slug. Misexpression of Ppa inhibits the formation of neural crest precursors, and Slug mutants in which Ppa binding has been abrogated rescue this inhibition. These results provide novel insight into the regulation of Slug, a protein that plays a central role in neural crest precursor formation, as well as in developmental and pathological epithelial to mesenchymal transitions.

KEY WORDS: *Xenopus*, Neural crest, Slug, Snail, Ppa, Ubiquitin

INTRODUCTION

The neural crest is a population of proliferative, migratory, multipotent progenitor cells found only in vertebrate embryos (Le Douarin and Kalcheim, 1999). The precursors of these cells arise from the ectoderm at the lateral edges of the neural plate during mid-gastrulation. Following neural tube closure, these cells undergo an epithelial to mesenchymal transition (EMT), delaminate from the dorsal aspect of the neural tube, and migrate extensively to populate distant sites throughout the embryo. Ultimately, neural crest cells produce most of the neurons and glia of the peripheral nervous system, melanocytes and nearly all of the elements of the craniofacial skeleton (Le Douarin and Kalcheim, 1999). Perhaps because of their contribution to such a large number of derivatives, defects in the neural crest are associated with an estimated half of all known birth defects (Hall, 1999). Moreover, a number of cancers of great clinical significance, including melanomas and gliomas, are neural crest-derived. Neuroblastomas, one of the most common pediatric solid tumors, originate from neural crest precursor cells themselves (Nakagawara and Ohira, 2004). Because a number of genes that are essential to neural crest development are misregulated in these tumors (Rosivatz et al., 2002; Rothhammer et al., 2004), examining the regulatory programs that control the normal development of neural crest cells can provide essential insights into how such programs are inappropriately reactivated during tumorigenesis.

As a population of proliferative, migratory, tissue-invasive stem cells, the neural crest shares a number of characteristics with metastatic tumor cells. For instance, the Snail family of transcriptional repressors are key molecular regulators of both neural crest development and tumor progression. Members of the Snail family, including the C_2H_2 zinc-finger transcription factors *Slug* and *Snail*, are among the earliest factors expressed in neural crest-forming regions (Nieto, 2002). Although Snail was first characterized in *Drosophila*, homologs have subsequently been isolated from many organisms including humans, non-vertebrate chordates, nematodes, annelids and mollusks (Manzanares et al., 2001). In *Xenopus*, *Slug* and *Snail* expression can be detected in neural crest-forming regions by late gastrula stages, and both factors are expressed at the lateral edges of the open neural plate in midbrain, hindbrain and spinal cord regions. *Snail* is also transiently expressed in the transverse neural fold, from which neural crest does not arise (Essex et al., 1993; Mayor et al., 1995).

The temporal and spatial expression of Snail-related factors prompted studies to probe their functional roles during neural crest precursor formation. In *Xenopus*, overexpression of dominant inhibitory forms of Slug leads to a loss of neural crest precursor formation, whereas ectopic wild-type Slug expands early neural crest marker expression (LaBonne and Bronner-Fraser, 1998; LaBonne and Bronner-Fraser, 2000). Similar effects have recently been reported in avian embryos (del Barrio and Nieto, 2002). In both avian and *Xenopus* embryos, treatment with Slug antisense oligos inhibits delamination of neural crest cells from the neural tube, resulting in defective migration (Carl et al., 1999; Nieto et al., 1994). Furthermore, the use of a hormone-inducible dominant negative form of Slug demonstrated that Snail-related factors play two temporally distinct roles in neural crest formation (LaBonne and Bronner-Fraser, 2000). Together, these results indicate that the neural crest can be categorized into at least two developmental stages (pre-migratory precursors and maturing, migratory cells), and that Snail-related factors have essential roles in both of these steps.

One hallmark of the Snail-related factors is their ability to promote EMTs during embryonic development (Bolos et al., 2003; Carver et al., 2001; Savagner, 2001). In addition to their role in triggering neural crest migration, Slug/Snail expression is also associated with a number of other developmental EMTs including mesoderm ingression (Nieto et al., 1994), forming heart cushions (Romano and Runyan, 2000) and palatal closure (Martinez-Alvarez et al., 2004). Significantly, many of the molecular and phenotypic changes associated with cells undergoing developmental EMTs are

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also characteristic of metastatic carcinoma cells (Vernon and LaBonne, 2004). Overexpression of Snail in epithelial cell lines is sufficient to induce these cells to undergo an EMT and acquire invasive properties (Cano et al., 2000; Thiery, 2002). Furthermore, whereas little or no *Snail* expression is detected in well-differentiated, non-invasive carcinomas, this factor is strongly expressed in a range of metastatic carcinoma cell lines as well as in many invasive human carcinomas (Batlle et al., 2000; Blanco et al., 2002; Cano et al., 2000).

Although Snail family members are critical for proper neural crest development and are implicated in the metastatic step of tumorigenesis, the mechanisms that control the expression or activity of these factors are poorly understood. For example, Snail and Slug are capable of inducing an EMT when ectopically expressed in epithelial cell lines, yet in embryos both of these factors are highly expressed in non-migratory neural crest precursor cells, and Slug is required for the formation of these precursor cells in *Xenopus*. Furthermore, in the non-vertebrate chordates *Ciona* and *Amphioxus*, *Snail* is expressed in a position analogous to where neural crest precursors form in vertebrates, yet these cells do not undergo an EMT or migrate (Corbo et al., 1997; Langeland et al., 1998). Taken together, these data point to post-transcriptional and/or post-translational regulation of Slug and Snail that is essential for precise control of the activity of these proteins, but is as of yet poorly understood.

We demonstrate here that the transcriptional repressor Slug is a highly unstable protein. Ubiquitin-mediated proteasomal degradation of this factor is opposed by activation of the neural crest regulatory program, components of which promote Slug stability. We map the portion of the Slug protein that confers instability, and show that this region is sufficient to transfer instability to an unrelated protein, Sox10. Furthermore, we show that Partner of paired (Ppa), an F-box-containing component of a modular E3 ubiquitin ligase, binds to the Slug protein and promotes its degradation. Morpholino-depletion of Ppa stabilizes Slug protein, whereas misexpression of Ppa promotes Slug turnover and inhibits the formation of neural crest precursors. These results shed important new light on the regulation of a protein that plays a central role in neural crest precursor formation as well as developmental and pathological EMTs.

MATERIALS AND METHODS

DNA constructs

Mouse *Snail* and *Xenopus Ppa* were obtained from ATCC (clone ID# 5121591; #3402730), *Drosophila Snail* was obtained from the DGRC (clone ID# 35237), *Ciona Snail* was a gift from M. Levine, *FoxD3* was a gift from N. Sasai, Ub^{K48R} was a gift from L. Hicke, and *Smad1* was a gift from A. Hemmati-Brivanlou. Epitope-tagged versions of all cDNAs were generated by amplifying the coding regions of these genes using low cycle-number PCR and a high fidelity polymerase (Tgo, Roche) and inserting them into the pCS2-MycC (provided by Robert Davis), pCS23xFlagC or pCS23xFlagN vector as noted. The pCS23xFlagC and N vectors were generated by oligo cloning multiple Flag sequences into the *Bam*HI/*Eco*RI or *Xba*I/*Sna*BI sites of pCS2+, respectively. The Slug alanine point mutants were generated using site-directed mutagenesis with complementary specific oligonucleotides (LY33,34AA sense: 5′-ATCATCTCCCCATTCGCAGCTGAGAGGTATCCTGTG-3′, antisense: 5′-CACAGGATACCTCTCAGCTGCGAATGGGGAGATGAT-3′) (VW58,59AA sense: 5′-TACAGCCCAATCACTGCGGCGACAGGACTGCTTCAC-3′, antisense: 5′- GTGAAGCAGTCCTGTCGCCGCAGTGATTGGGCTGTA-3′) in accordance with the protocol for the Quick Change kit from Stratagene. Deletion mutants were generated using the following primers: Slug N sense: 5′-ATGCCACGATCTTTTC-3′, antisense: 5′-GGGGTCGGAAAGTTTG-3′; Slug C sense: 5′-ATGGACTCGCATGCG-3′, antisense: 5′-ATGTGCTACACA-3′; ΔP38 sense: 5′-ATGCCTGTGTCTGTG-3′, ΔH64 sense: 5′-ATGCACCCACCGCTG-3′; Slug ΔP31-H64 sense1: 5′-ATGCCACGATCTTTT-3′, antisense1: 5′-TGGGGAGATGATCAC-3, sense2: 5′-CACCCACCGCTGCCC-3′, antisense2: 5′-ATGTGCTACACAGCA-3′. The SlugN-Sox10 fusion was made by amplifying amino acids 1-63 of Slug (sense: 5′-ATGCCACGATCTTTT-3′; antisense: 5′-AAGCAGTCCTGTCCA-3′) and fusing this fragment in the *Cla*I site upstream of *Sox*10 in the *Stu*I site of pCS2-MycC. All constructs were confirmed by sequencing.

Embryological methods

All results shown are representative of at least three independent experiments. RNA for injection was produced in vitro from linearized plasmid templates using the Message Machine kit (Ambion). Collection, injection and in situ hybridization of *Xenopus* embryos was as described previously (LaBonne and Bronner-Fraser, 1998; Bellmeyer et al., 2003). The in situ probe for Ppa was generated by PCR using the primers: sense 5′-TCCGCATCACCGACA-3′ and antisense 5′-CAATACACCACATCC-3′ and then subcloned into the pGEMT vector (Promega). For 70 μm sections, specimens were post-fixed in 4% paraformaldehyde (PFA)/0.2% glutaraldehyde, embedded in 3% low melting point agarose and sectioned using a Leica VT1000M vibratome. The Ppa MO sequence is: 5′-AGACACGAGATGTGGGTCTCCATAG-3′ (targeted ATG is underlined). Where noted, embryos were treated with 10 μg/ml cycloheximide (Sigma) in 0.1× Marc's modified Ringers (MMR).

Immunoprecipitation and western blot analysis

For immunoprecipitations, embryos were collected at stage 10.5 and lysed in PBS+1% NP40 containing a protease inhibitor cocktail (Roche). Following centrifugation, cleared lysates were diluted with RIPA buffer and incubated with the indicated antibody [0.2 μg α-Myc (9E10, Santa Cruz) or α-FlagM2 (Sigma)] for 2 hours on ice, followed by a 2-hour incubation with protein A Sepharose beads. After extensive washing with RIPA buffer, SDS sample buffer was added to the beads and proteins were resolved by SDS-PAGE. Immunoblotting was performed using α-Flag (1:3000) or α-Myc (1:2000) antibodies as indicated. Labeled proteins were detected using HRP-conjugated secondary antibodies and enhanced chemiluminescence (Amersham).

Ubiquitination assays

The ubiquitination assay was modified from (McGarry, 2005). Fifty injected embryos were collected in an Eppendorf tube and washed four times with 1 ml extraction buffer containing protease inhibitors. Washed embryos were spun at 1000 ***g*** and all remaining liquid removed. Embryos were lysed by pipetting, using 500 μl of extraction buffer plus 20 μg/ml cytochalasin B, and then spun at 10,000 ***g*** at 4°C for 10 minutes. After removing the cytoplasmic layer to a new tube, energy mix (1/20 of volume) and cycloheximide (100 μg/ml) were added. The extract was then divided in half and treated with either 800 μM MG132 or dimethysulfoxide vehicle. Samples were taken at regular intervals for western blot analysis.

RESULTS

Slug is an unstable protein

Despite recent studies demonstrating that Snail family members are critical molecular regulators of neural crest development, and data implicating them in the acquisition of invasive behavior during tumor progression, little is known about the mechanisms that control the expression or activity of these factors. While investigating such mechanisms, we noted that both Slug and Snail are highly unstable proteins. Previous work in our laboratory had focused on the role of another labile protein, Id3, in the maintenance of the multipotent neural crest progenitor cell population (Light et al., 2005). Because the regulation of Id turnover by the ubiquitin-proteosome pathway is well documented, and because the half-lives of Id proteins have been previously calculated (Bounpheng et al., 1999; Fajerman et al., 2004), we compared the stability of Slug and Id3 in *Xenopus*. Embryos injected at the two-cell stage with mRNA encoding

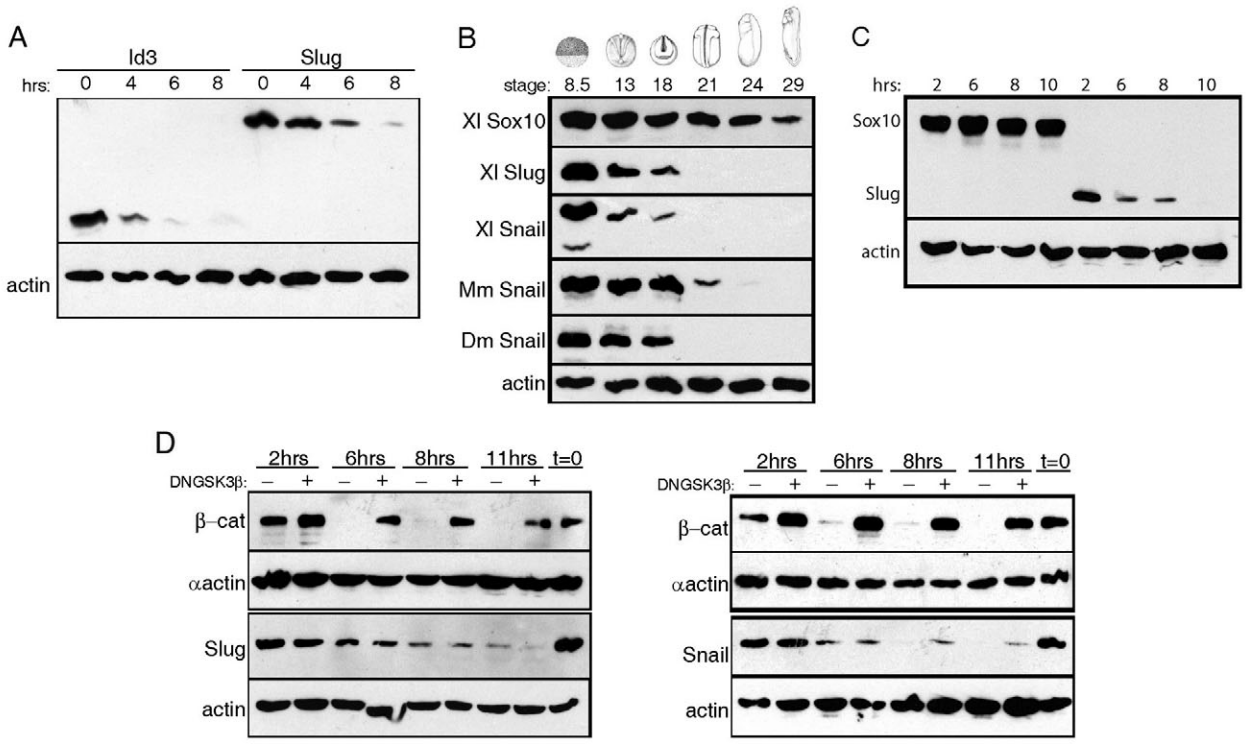

Fig. 1. Slug is an unstable protein. (**A**) Embryos were injected with mRNA encoding either Slug or Id3, allowed to develop to stage 8 and treated with cycloheximide (CHX) to prevent further protein synthesis. Western blot analysis demonstrates that Slug protein is highly unstable and is regulated in a time frame similar to that of Id3. (**B**) The instability of Snail family members is evolutionarily conserved. Western blot showing that in contrast to Xl Sox10, expression of Xl Slug, Xl Snail, Mm Snail and Dm Snail is greatly diminished or undetectable by migrating neural crest stages. All blots were stripped and re-probed with actin as a loading control. Representative actin shown is for Slug blot. (**C**) Embryos injected with mRNA encoding either Sox10 or Slug were treated with CHX at stage 8. Western blot analysis demonstrates that Sox10 has a significantly longer half-life than Slug. (**D**) Embryos co-injected with DNGSK3β and either β-catenin, Slug or Snail were treated with CHX at stage 8 and collected at the time points indicated. Whereas β-catenin was robustly stabilized by the presence of DNGSK3β, Snail was only modestly stabilized and Slug stability was not affected. Actin was used as a control.

epitope-tagged forms of Slug or Id3 were allowed to develop to stage 8 and were then treated with cycloheximide (CHX) to inhibit further protein synthesis. The embryos were collected at regular time intervals subsequent to CHX treatment and subjected to western blot analysis. These experiments confirmed that the Slug protein is highly unstable, and demonstrated that its half-life is comparable to that of Id3 in early embryos (Fig. 1A).

The instability of Slug and Id3 is distinct from what we observe with many other neural crest regulatory factors. For example, when similarly expressed in early embryos, the HMG-box transcription factor Sox10 can be detected on a western blot at least until swimming tadpole stages (Fig. 1B). By contrast, Slug protein levels in parallel embryos decline precipitously during migratory neural crest stages (Fig. 1B), demonstrating that Slug protein stability is dynamically regulated during the course of neural crest development. Importantly, the marked difference in stability noted between the Slug and Sox10 proteins is not due to differences in the stability of the injected RNAs as determined by RT-PCR (data not shown). Furthermore, in contrast to what we found with Slug and Id3 in the above CHX pulse-chase analyses, Sox10 was stable even in the absence of further protein synthesis (Fig. 1C). Together, these data indicate that the observed differences in protein levels reflect differences in protein half-life.

Given Slug's central role in neural crest development, it is likely that precise control over when and where the protein is present during this process is essential. To gain insight into the importance of protein turnover for the regulation of Slug function, we asked whether instability is a conserved feature of Snail family proteins. Embryos were injected with mRNA encoding *Xenopus* (Xl) Slug, *Xenopus* Snail, mouse (Mm) Snail, *Drosophila* (Dm) Snail, or with *Xenopus* Sox10 as a control, and samples were collected at blastula, gastrula, neurula and tailbud stages to compare the stability of the various proteins over time. Whereas Sox10 was extremely stable and expressed throughout the developmental time course examined, Xl Slug, Xl Snail, Mm Snail and Dm Snail proteins were all greatly diminished or undetectable by migratory neural crest stages (Fig. 1B), indicating that instability is an evolutionarily conserved feature of these proteins from invertebrates to mammals.

During the course of our studies, two groups reported that human (Hs) Snail is unstable in tissue culture cells, confirming that at least one member of this family is regulated by protein turnover in a context other than early embryonic cells. Moreover, although these two studies contained a number of conflicting findings, both reported that Hs Snail stability is regulated by glycogen synthase kinase β (GSK3β) phosphorylation and βtrcp-mediated ubiquitination (Yook et al., 2005; Zhou et al., 2004). Surprisingly, however, examination of the Slug coding sequence revealed that the well-defined βtrcp destruction motif ($DSGX_{2+n}S$) which is also found in β–catenin, IκB and Emi (Fuchs et al., 2004) is absent in Slug, making it unlikely that Slug is regulated via this mechanism. Nevertheless, we investigated whether Slug stability is regulated in the embryo by the GSK3β/βtrcp pathway in a manner similar to that suggested for Hs

Snail. Using β-catenin as a positive control, we investigated whether inhibiting GSK3β activity using a well-characterized dominant negative mutant (DNGSK3β) would alter Snail or Slug stability in the early embryo. We co-injected Snail, Slug or β-catenin along with DNGSK3β mRNA into two-cell stage embryos, treated the injected embryos with CHX at stage 8 and collected samples at the indicated time points. Whereas β-catenin was robustly stabilized by the presence of DNGSK3β, Slug protein stability was completely unaffected (Fig. 1D). A weak stabilization of Snail was noted in some experiments (Fig. 1D), suggesting that the GSK3β pathway may make a minor contribution to Snail regulation. However, our data indicate that Slug is not regulated by this mechanism.

Slug protein stability is regulated by neural crest transcription factors

Although numerous genes have been implicated in the establishment and maintenance of neural crest identity, the precise roles of individual factors and the interplay between these factors remains poorly understood (Heeg-Truesdell and LaBonne, 2004; Meulemans and Bronner-Fraser, 2004). As it was clear that Slug is regulated at the level of protein turnover, we next wished to determine whether the stability of Slug could be modulated by known neural crest regulatory factors. This was of particular importance given our experiments demonstrating that GSK3β does not regulate Slug stability.

A recent study reported that Slug overexpression was insufficient to promote neural crest formation in the trunk region of the avian spinal cord, but that the combined expression of Sox9, FoxD3 and Slug could induce formation of cells with the phenotypic and morphological characteristics of definitive neural crest. The mechanisms responsible for this combinatorial regulation were not investigated, however (Cheung et al., 2005). To determine whether FoxD3 and/or Sox9 modulate Slug stability, embryos were injected with Slug alone or together with FoxD3, Sox9 or both, treated with CHX at stage 8, and collected at regular time intervals for western blot analysis. We found that co-expression of either FoxD3 or Sox9 significantly enhanced Slug stability (Fig. 2A,B), but that co-expression of FoxD3 and Sox9 together did not result in any further stabilization (data not shown). These data indicate that, in addition to their individual and independent contributions to neural crest development, FoxD3 and Sox9 may promote this process through their ability to stabilize Slug.

The bHLH transcription factor Twist is also closely associated with the function of Snail family proteins. In *Drosophila*, Twist is required for the proper activation and maintenance of *snail* expression, and the Twist mutant phenotype can be significantly rescued by expression of Snail (Ip et al., 1994; Leptin, 1991). Furthermore, Twist activity has recently been linked to EMTs during tumor metastasis (Yang et al., 2004). Importantly, we found that, like Sox9 and FoxD3, co-expression of Twist was capable of stabilizing Slug (Fig. 2C), suggesting an additional neural crest regulatory function for this factor. To determine whether the ability to stabilize Slug was a general feature of neural crest regulatory factors, we asked whether Opl, a regulator of neural crest border fates, or Smad1, a downstream mediator of BMP signaling, were capable of stabilizing Slug. Interestingly, neither Opl nor Smad1 had any effect on Slug stability in these assays (Fig. 2D,E), indicating that the Slug stabilizing activities of Sox9, FoxD3 and Twist are specific. Finally, because many neural crest regulatory factors are involved in highly complex feedback loops, we investigated whether Snail was capable of stabilizing Slug. However, as with Opl and Smad1, we found that Snail overexpression had no effect on Slug stability (Fig. 2F).

The N-terminal half of the Slug protein confers its instability

Together, the experiments above demonstrate that Slug stability is dynamically regulated in early *Xenopus* embryos by a GSK3β/βtrcp independent process. An important first step in elucidating the mechanisms that do regulate Slug stability is to identify the region of the protein that confers instability. We therefore expressed mRNA encoding either the N- or C-terminal halves of Slug and used

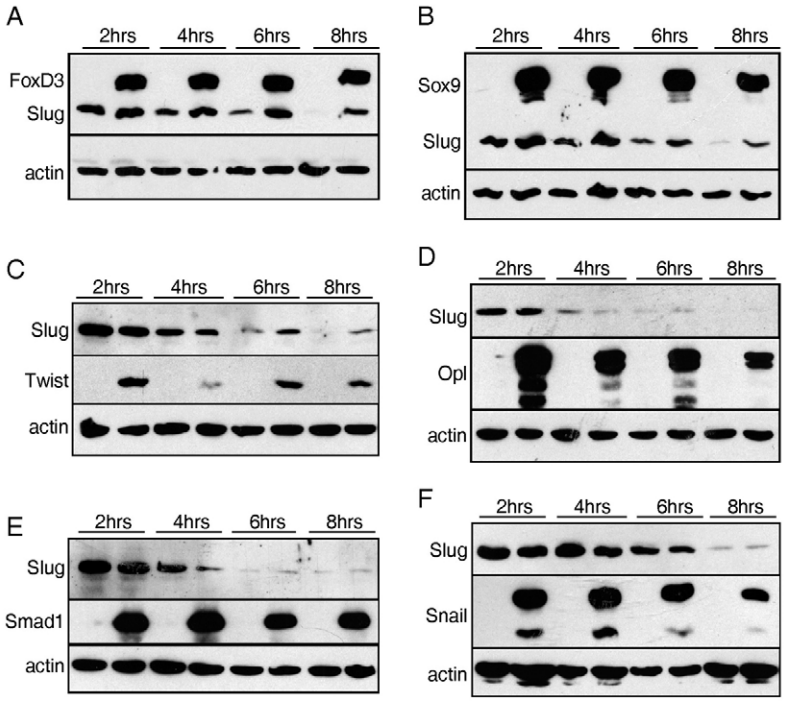

Fig. 2. Slug stability is positively regulated by activation of the neural crest regulatory program. (**A-F**) Embryos were co-injected with Slug and either FoxD3 (A), Sox9 (B), Twist (C), Opl (D), Smad1 (E) or Snail (F) and treated with CHX at stage 8 to prevent further protein synthesis. Samples collected at the time points indicated were subjected to western blot analysis. FoxD3, Sox9, and Twist all increased Slug stability, whereas Smad1, Snail and Opl were unable to do so. Actin was used as a control.

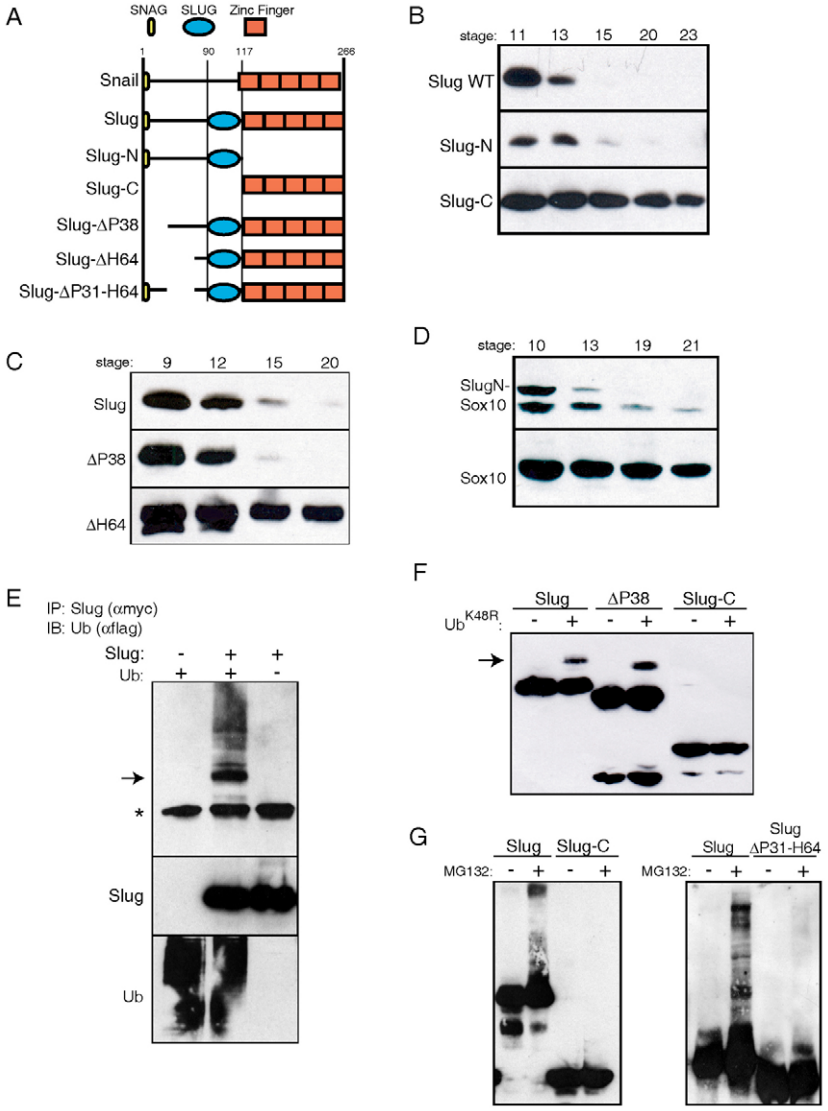

Fig. 3. Sequences within the N terminus of Slug are responsible for its ubiquitin-mediated proteasomal degradation. (**A**) Schematic illustrating the mutant constructs used in these experiments. (**B**) Western blot demonstrating that the C-terminal zinc fingers of Slug are highly stable, but the stability of the N terminus is comparable to that of the intact protein. (**C**) Western blot analysis was used to monitor the relative stability of Slug with a series of Slug deletion mutants. When compared to the full-length protein, the stability of ΔP38 was unchanged but the ΔH64 mutant was greatly stabilized. (**D**) Western blot comparing the stability of Sox10 and a Slug N-Sox10 fusion protein. Addition of amino acids M1-L63 of Slug to Sox10 was sufficient to render it unstable (double bands represent a partial degradation product). (**E**) Poly-ubiquitinated forms of Slug were immunoprecipitated from lysates of embryos co-injected with tagged forms of Slug and ubiquitin. Slug is indicated by an arrow. In all blots an asterisk indicates IgG background band. (**F**) Embryos were co-injected with a mutant form of Ub (Ub^{K48R}) and either Slug, ΔP38, or Slug-C and collected for western blot analysis. Whereas Slug and the unstable deletion mutant ΔP38 both incorporate Ub^{K48R} (arrow), the stable C-terminal zinc fingers do not. (**G**) Western blot demonstrating the accumulation of higher molecular mass forms of wild-type Slug, but not the Slug-C or Slug ΔP31-H64 deletion mutants, in response to treatment with the proteasomal inhibitor, MG132.

western blot analysis to compare the stability of these truncation mutants. These experiments indicated that the zinc-finger-containing C terminus of Slug was highly stable, whereas the stability of the N terminus was comparable to that of the full length protein (Fig. 3B). As the instability-conferring region of Slug clearly mapped to the N-terminal half of the protein, we next constructed a series of mutants that deleted progressively larger regions of the N terminus and assayed their stability. We found that whereas deletion of the first 38 amino acids of Slug had no effect on stability, deletion of the first 64 amino acids greatly stabilized the protein (Fig. 3C). Because the sequence between amino acids P38 and H64 appeared to be necessary for Slug instability, we next investigated whether appending this region to an unrelated protein would be sufficient to render that protein unstable. The N-terminal amino acids (M1-L63) of Slug were fused in frame to the N terminus of Sox10, a protein that is normally highly stable (Fig. 1B). Wild-type Sox10 or the SlugN-Sox10 fusion was then expressed in early embryos and their stability compared by western blot analysis. We found that the SlugN-Sox10 fusion protein was significantly less stable than wild-type Sox10, demonstrating that the region spanning amino acids M1-L63 of Slug was sufficient to transfer instability to Sox10 (Fig. 3D).

Many labile transcription factors are regulated via ubiquitin-mediated proteasomal degradation. To determine whether Slug is regulated in this manner, we performed co-immunoprecipitation assays from embryos co-injected with Slug and ubiquitin and observed strong poly-ubiquitination of the protein (Fig. 3E). Next we wished to identify the portion of the Slug protein that undergoes ubiquitination. To maximize the sensitivity of these experiments, we utilized a ubiquitin mutant (Ub^{K48R}) that can be conjugated to substrates, but that cannot form polyubiquitin chains or promote degradation (Chau et al., 1989; Finley et al., 1994; Hochstrasser et al., 1991). We co-injected Slug, the ΔP38 mutant or Slug-C with Ub^{K48R} and performed western blots to detect ubiquitin incorporation. Whereas full-length Slug and the unstable ΔP38 mutant were both clearly ubiquitinated in this assay, the stable C-terminal zinc finger mutant was not (Fig. 3F). To confirm and extend these findings, we examined whether Slug was polyubiquitinated and degraded in a proteasome-dependent fashion. Lysates from embryos injected with mRNA encoding Slug or the Slug-C mutant were treated with either DMSO or the proteasome inhibitor MG132. Accumulation of polyubiquitinated species was dramatically increased in the Slug sample treated with MG132 as compared to both the control DMSO-treated samples and the MG132 treated

Slug-C sample (Fig. 3G). These data confirm that Slug is targeted for proteasomal degradation via poly-ubiquitination of the N-terminal portion of the protein.

The F-box protein Ppa is expressed in the neural crest and binds to Slug

We next sought to identify E3 ubiquitin ligases that might regulate Slug turnover. Interestingly, although most E3 ligases have been reported to be broadly expressed, the F-box protein Ppa shows developmentally restricted expression in both *Drosophila* and zebrafish embryos (Das et al., 2002; Raj et al., 2000). The *Xenopus* homolog of Ppa had been previously isolated in our laboratory to examine a putative role in regulating neural plate border cell types. Similar to *Drosophila* and zebrafish Ppa, the *Xenopus* Ppa protein contains an F-box together with 11 leucine rich repeats (LRRs) and is 95% identical to the previously identified zebrafish ortholog (see Fig. S1 in the supplementary material). F-box-containing proteins function as substrate recognition subunits for SCF (Skp1-Cullin-F-box) ubiquitin ligases, and consistent with this we found that *Xenopus* Ppa binds to Skp1 through its F-box (Fig. S1 in the supplementary material). Whereas the N-terminal region of *Drosophila* Ppa contains a putative alanine/histidine/proline-rich repressor domain and a PEST domain (Raj et al., 2000) these motifs are not conserved in any other known Ppa homologs, including *Xenopus* Ppa.

Ppa is not expressed in neural crest-forming regions at late gastrula and early neural plate stages, a time when *Slug* function is known to be essential for the establishment of the progenitor cell population (Fig. 4Aa). However, *Ppa* becomes highly expressed in neural crest cells as the neural folds are closing (Fig. 4Ab-d), and is expressed in at least some migrating neural crest cells (Fig. 4Ae,f). Because expression of *Ppa* appeared to be dynamic, and it was not expressed in neural crest precursors at all stages, we next directly compared its expression to that of *Slug* using double whole mount in situ hybridization. Again, these experiments clearly indicated that *Ppa* expression entirely co-localizes with that of *Slug* as the neural folds are closing (Fig. 4Ba,b). Interestingly, however, by stage 20, just prior to the onset of neural crest migration in cranial regions, *Ppa* expression is downregulated/lost in a subset of neural crest precursors (Fig. 4Bc). Similarly, at early migratory stages it is clear that many neural crest cells proximal to the neural tube (e.g. cells that have just begun migration) do not express *Ppa* (Fig. 4Bd). To obtain better resolution, we examined the co-localization of *Ppa* and *Slug* expression in sections. Interestingly, we noted that in rostral regions of stage 15 embryos, where neural crest precursors are already specified and where cranial neural folds are closing, expression of *Slug* is contained completely within the *Ppa* expression domain (Fig. 4Ca). However, neural crest precursors are specified in a rostral-to-caudal progression, and when we examined more caudal sections from the same embryo, at axial levels where neural crest precursors are likely to be newly induced, we noted that the domains of *Slug* staining lay largely outside the *Ppa* expressing region (Fig. 4Cb). Sections through embryos in which neural crest cells are initiating migration in cranial (Fig. 4Cc) or spinal cord (Fig. 4Cd) regions also show loss of *Ppa* expression in some *Slug*-expressing cells. Together, these findings are consistent with a role for *Ppa* in controlling Slug protein levels such that they are maximized at times when Slug is known to play critical and essential roles in neural crest development. Interestingly, we also found that in embryos injected with either FoxD3 or Sox9, expression of *Ppa* was significantly reduced (Fig. S1 in the supplementary material). By contrast, Ppa protein levels are unaffected by exogenously coexpressed FoxD3 or Sox9 (data not shown). Regulation of *Ppa* at the level of transcription provides one potential mechanism via which these neural crest regulatory factors may act to stabilize Slug protein.

Since the expression of *Ppa* is spatially and temporally consistent with a role in regulating Slug stability at key stages of neural crest development, we asked whether Slug and Ppa could interact in vivo.

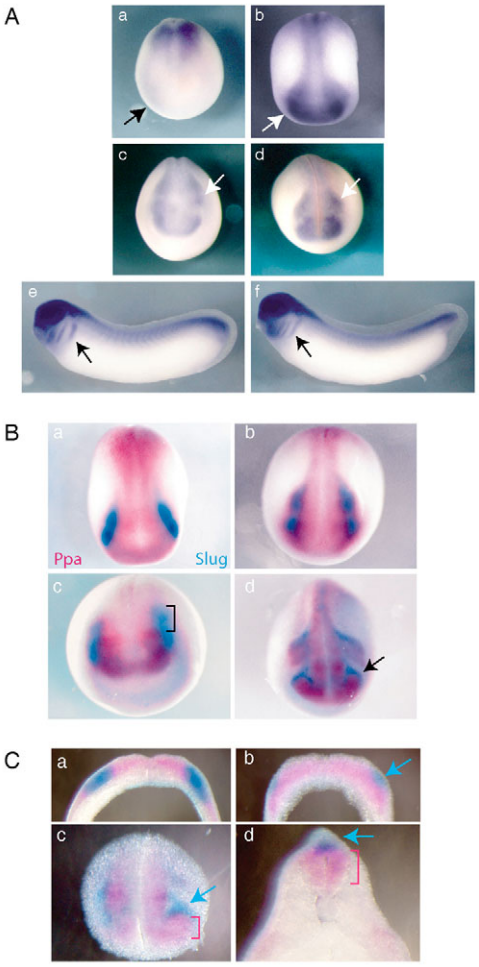

Fig. 4. *Ppa* is dynamically expressed in neural crest-forming regions. (**A**) In situ hybridization showing the developmental expression of *Ppa* at stage 13 (a) stage 16 (b) stage 19 (c), stage 22 (d) stage 24 (e) and stage 28 (f). Strong expression is seen in the neural crest region of late neural plate stage embryos (b,c, arrows) and in the migrating neural crest at tailbud stages (d-f, arrows). By contrast, no *Ppa* expression is seen in neural crest precursor-forming regions at stage 13 (a, arrow). (**B**) Double in situ hybridizations comparing the expression patterns of *Ppa* (pink) and *Slug* (light blue). At stages 15 (a) and 18 (b), *Ppa* and *Slug* expression is entirely overlapping. However, at stages 20 (c) and 22 (d), *Ppa* and *Slug* expression patterns are only partially coincident. Bracket in c indicates region of *Slug*-expressing cells that do not expess *Ppa*. Arrow in d indicates migratory neural crest cells that express *Slug* but not *Ppa*. (**C**) Vibratome sections of double in situ hybridizations demonstrate the complete colocalization of *Ppa* and *Slug* expression in the rostral region of a stage 15 embryo (a). A more caudal section from the same embryo as in a, showing that *Ppa* is not expressed in the newly induced, *Slug*-expressing neural crest precursors (b, arrow). Sections through cranial (c) and spinal cord (d) regions of a stage 24 embryo. *Slug* is expressed in the earliest migrating neural crest (c,d, arrow) but *Ppa* is excluded from these cells (c,d, bracket).

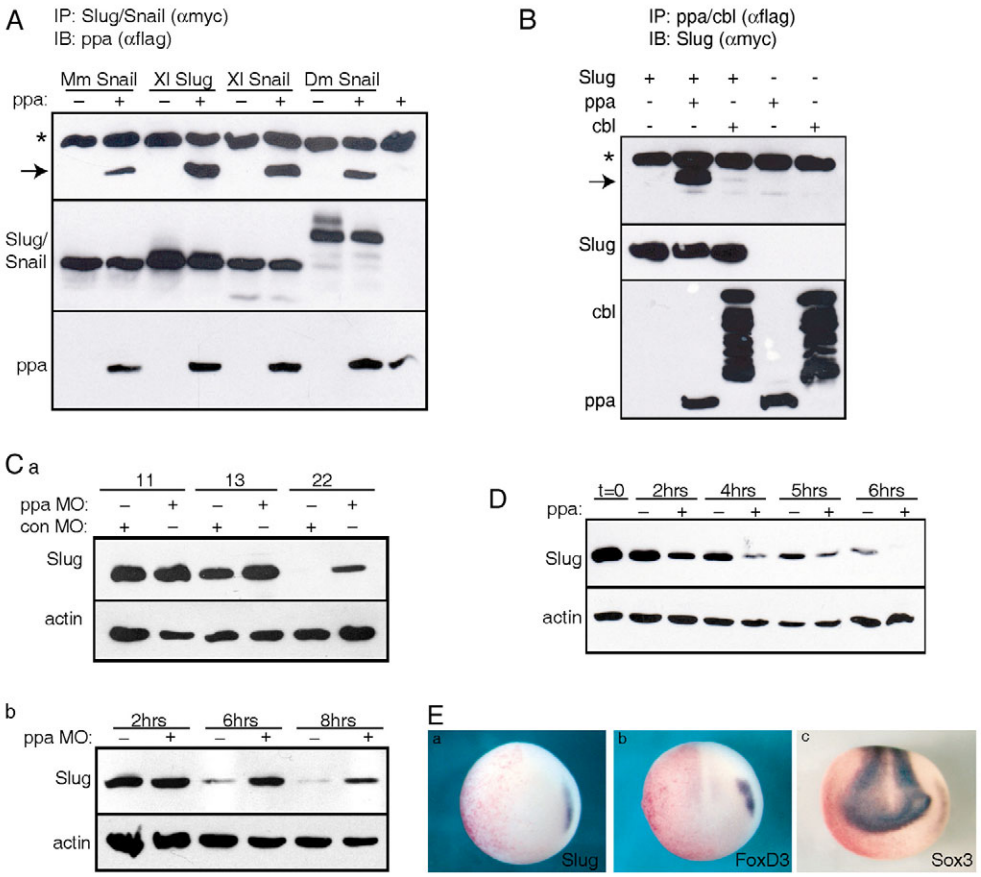

Fig. 5. Slug binds to and is regulated by Ppa. (**A**) Slug was immunoprecipitated from lysates of embryos co-injected with tagged forms of Slug and Ppa using an α-Myc antibody. Immunoprecipitates were resolved by SDS-PAGE and bound Ppa was detected by α-Flag western (arrow). Interaction with Ppa is a conserved feature of Snail family proteins including those of *Xenopus*, mouse, and *Drosophila* Snail. (**B**) Slug was assayed for its ability to interact with the E3 ligase hcbl in co-IP experiments. Whereas Ppa efficiently binds Slug (arrow), hcbl does not. (**C**) Embryos were co-injected with Slug and control morpholino (con MO) or Ppa MO and allowed to develop normally (a) or were treated with CHX (b). The loss of Ppa mediated by the Ppa MO greatly stabilizes Slug protein. (**D**) Embryos injected with Slug alone or together with Ppa were treated with CHX at stage 8 and collected at the time points indicated. Slug protein is significantly destabilized by co-expression of Ppa. Actin is used as a control. (**E**) Embryos were injected in one of two cells at the two-cell stage with mRNA encoding Ppa and the lineage tracer, nβ-gal (red, injected side to the left). Expression of *Slug* (a), *FoxD3* (b) and *Sox3* (c) were examined at stage 15 by in situ hybridization. Misexpression of Ppa results in failure of neural crest precursor formation and expansion of neural progenitors.

Co-immunoprecipitation experiments demonstrated a strong interaction between these two proteins (Fig. 5A). By contrast, Slug does not bind to an unrelated E3, cbl (Fig. 5B). Moreover, we found that Mm Snail, Xl Snail and Dm Snail also bind to Ppa in co-immunoprecipitation assays, demonstrating that this interaction is conserved amongst Slug/Snail family members and their orthologs (Fig. 5A).

If Ppa functions as an E3 recognition subunit for Slug, then downregulating Ppa would be expected to have a stabilizing effect on Slug protein and, conversely, overexpressing Ppa should increase the rate of Slug turnover. To test the first hypothesis, we utilized a Ppa morpholino (Ppa MO) to specifically deplete Ppa protein from early *Xenopus* embryos (data not shown). The relative stability of Slug protein was compared in embryos co-injected with Slug and a control morpholino (con MO) against embryos co-injected with Slug and the Ppa MO. Importantly, we found that Slug protein was significantly stabilized in Ppa-depleted embryos (Fig. 5Ca,b). In contrast, parallel experiments demonstrated that co-expression of Ppa with Slug accelerated Slug protein turnover, consistent with Ppa functioning as an E3 for Slug (Fig. 5D).

Given that Ppa regulates the turnover of a key neural crest regulatory factor, overexpression of Ppa might be expected to have deleterious consequences for neural crest precursor formation. To test this possibility, embryos were injected in one of two cells with *Ppa* mRNA and the lineage tracer, nβ-gal, and the expression of neural crest markers was examined at neural plate stages by in situ hybridization. *Ppa* injection caused the downregulation or loss of a wide variety of early neural crest markers including *Slug* (100%, *n*=18), *FoxD3* (100%, *n*=23), *Twist* (100%, *n*=19) and *Sox10* (100%, *n*=20) and this disruption persisted to migratory stages (Fig. 5Ea,b and data not shown). We found that the ability of Ppa to reduce the expression of neural crest markers was dependent on the presence of an intact F-box (data not shown). Importantly, Ppa overexpression did not perturb the expression of *muscle actin*, demonstrating that the observed loss of neural crest precursors is not due to an underlying defect in the mesodermal cell population (data not shown). Furthermore, Ppa injection led to expanded expression of the neural plate marker *Sox3* (90%, *n*=20), suggesting that cells overexpressing Ppa adopt a neural fate instead of becoming neural crest precursors (Fig. 5Ec).

We next sought to determine the extent to which the loss of neural crest cell formation in Ppa-injected embryos was due to effects on Slug. Since individual E3s are known to have multiple targets (Pickart, 2001), the most effective means of specifically disrupting the Ppa-Slug interaction required identifying and eliminating the sequence in Slug responsible for Ppa binding. Consistent with their relative stability, we found that the N terminus of Slug bound to Ppa in co-immunoprecipitation (IP) experiments, but the C terminus did not (Fig. 6A). Because transfer of the N-terminal 63 amino acids of Slug to Sox10 rendered Sox10 unstable (Fig. 3D), we tested whether the ability to bind Ppa had also been conferred on the SlugN-Sox10 fusion. Embryos were co-injected with wild-type Slug, Sox10 or SlugN-Sox10 mRNA along with Ppa, and were harvested at gastrula stages for IP/western blot analysis. These experiments demonstrated that Slug-N was sufficient to confer Ppa binding ability on Sox10 (Fig. 6B), providing a potential mechanism to explain the instability of the SlugN-Sox10 fusion protein.

To more precisely map the Ppa interacting domain of Slug, we utilized the N-terminal deletion mutant series previously generated to examine Slug instability. We found that Ppa bound well to the ΔP31 mutant, but that deletion of an additional seven amino acids (ΔP38) caused this interaction to weaken significantly (Fig. 6C). Together with our previous experiments showing that the addition of amino acids M1-H64 was sufficient to transfer both Ppa binding and instability to Sox10, these data indicated that Ppa binding was mediated by a region spanning amino acids P31 to H64. To confirm these results, we constructed an internal deletion mutant that removed these amino acids. Co-immunoprecipitation experiments demonstrated that the deletion mutant ΔP31-H64 was no longer capable of binding to Ppa, indicating that this sequence was necessary for the E3 to interact with Slug (Fig. 6D). Consistent with these findings, deletion of this region also abolished the accumulation of poly-ubiquitinated Slug proteins following MG132 treatment (Fig. 3G), further confirming the role of Ppa as a bona fide E3 subunit for Slug.

Disruption of the Ppa binding domain stabilizes Slug

Examination of the region between P31 and H64 of Slug did not reveal any defined protein interaction motifs. This was not particularly surprising since few sequence elements or structural features constituting such a destabilizing signal or degron have been well characterized to date. Nevertheless, whereas some proteins, such as β-catenin, are targeted for degradation by short, highly conserved peptide sequences, other proteins seem to depend on an extended protein surface or prefolded structure to trigger their proteolysis (Laney and Hochstrasser, 1999). Ppa contains 11 leucine-rich repeats, which generally mediate protein-protein interactions and can interact with extended hydrophobic regions (Kobe and Kajava, 2001). Interestingly, we noted that the region between P31-H64 of Slug was rich in hydrophobic amino acids (Fig. 7A).

To test the hypothesis that this hydrophobic region might mediate Slug's interaction with Ppa, we introduced point mutations designed to disrupt such an interaction. In Slug LY33,34AA (Slug$^{\psi 1}$) and Slug VW58,59AA (Slug$^{\psi 2}$), target hydrophobic amino acids were mutated to alanines (Fig. 7A). The ability of these Slug mutants to bind Ppa was then examined in co-immunoprecipitation assays. Although neither of these mutations alone was sufficient to disrupt the Slug-Ppa interaction, in combination they abolished Ppa binding (Fig. 7B). These data support a model in which Ppa interacts with Slug using extended contacts over a stretch of hydrophobic residues. Snail family orthologs all contain a comparable hydrophobic amino acid-rich region, further suggesting that these proteins interact with Ppa using a similar motif.

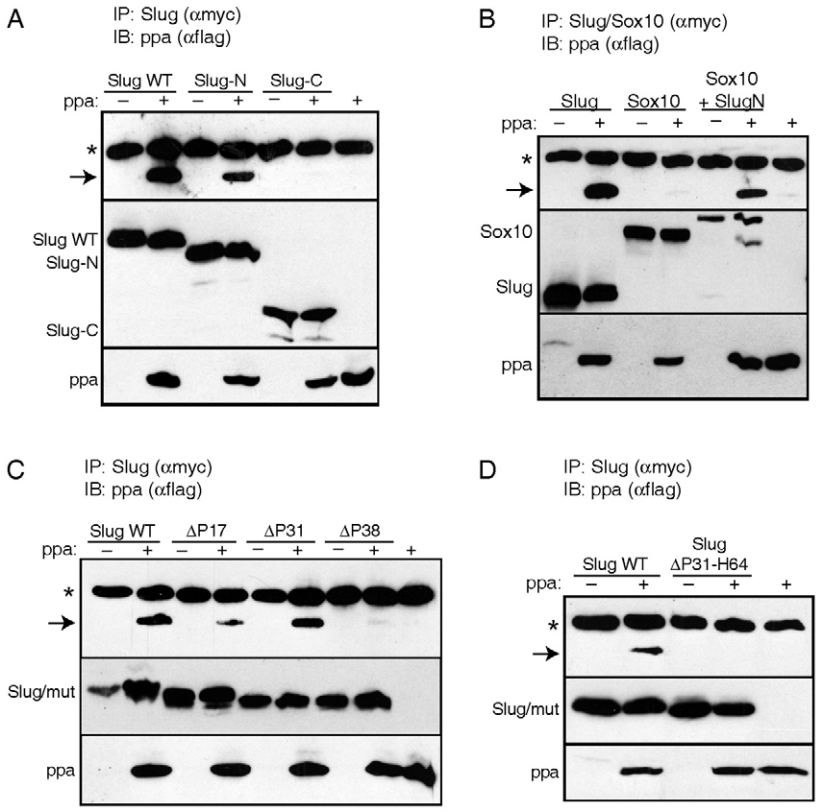

Fig. 6. The N terminus of Slug binds to Ppa and can transfer this interaction to Sox10. (**A**) Embryos were co-injected with Ppa and wild-type Slug, Slug-N or Slug-C. Following immunoprecipitations using an α-Myc antibody (Slug/mutants), bound Ppa (arrow) was detected by α-Flag immunoblot. Ppa binds the N terminus of Slug, but not the C terminus. (**B**) Embryos co-injected with Ppa and Slug, Sox10 or a Slug N-Sox10 fusion construct were collected for IP/western blotting. The addition of Slug M1-H64 is sufficient to transfer Ppa binding to Sox10 (arrow). (**C**) Serial Slug deletion mutants were co-injected with Ppa to map the Ppa binding region. (**D**) Co-immunoprecipitation experiments showing that a Slug mutant with deletion of amino acids P31 to H64 no longer interacts with Ppa.

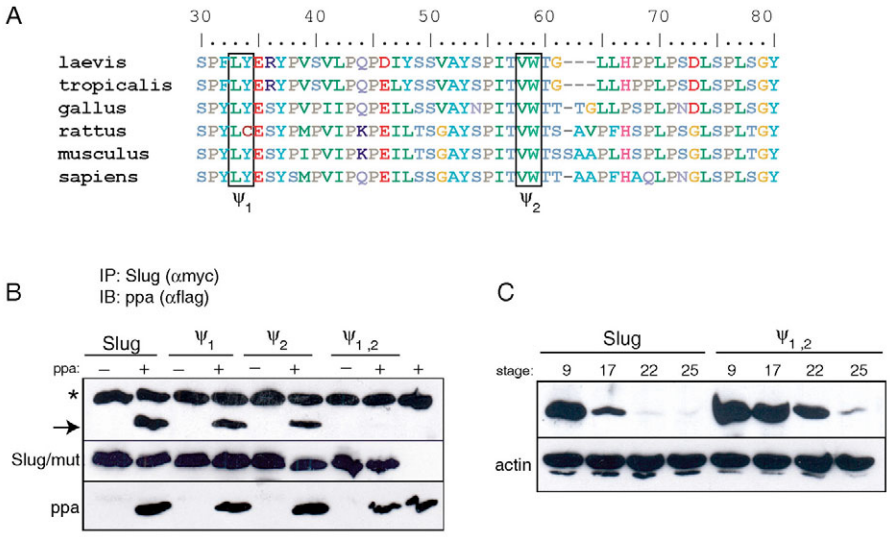

Fig. 7. Point mutations in a hydrophobic rich region of the Slug N terminus prevents interaction with Ppa and stabilizes the protein. (**A**) Alignment showing the conserved hydrophobic stretch of amino acids found in Slug proteins. laevis, *Xenopus laevis*; tropicalis; *Xenopus tropicalis*; gallus, *Gallus gallus*; rattus, *Rattus rattus*; musculus, *Mus musculus*; sapiens, *Homo sapiens*. (**B**) Wild-type Slug, or Slug in which target hydrophobic amino acids had been mutated (LY33, 34 VW58, 59 AA) were assayed in co-IP experiments for interaction with Ppa. Whereas Slug, Slug$^{\psi 1}$ and Slug$^{\psi 2}$ can efficiently bind Ppa (arrow), the combined mutant (Slug$^{\psi 1,2}$) no longer interacts (* indicates IgG background band). (**C**) Embryos injected with wild-type Slug or Slug$^{\psi 1,2}$ were collected at the stages indicated. Western blots from injected embryo lysates demonstrate that Slug$^{\psi 1,2}$ is significantly more stable than is wild-type Slug.

Having identified point mutations that abrogate the ability of Ppa to bind Slug, we investigated whether disrupting this interaction affected the stability of Slug protein. Embryos injected with mRNA encoding wild-type Slug or Slug$^{\psi 1,2}$ were collected at progressively later stages of development to compare the stability of these two proteins by western blot. We found that Slug$^{\psi 1,2}$ protein is more stable than wild-type Slug, indicating that elimination of Ppa binding protects Slug from degradation (Fig. 7C). To determine if stabilizing Slug had consequences for normal neural crest development, Slug or Slug$^{\psi 1,2}$ mRNA was injected together with the lineage tracer nβ-gal into one cell of two-cell stage embryos. In situ hybridization of embryos at neural plate stages revealed that Slug and Slug$^{\psi 1,2}$ both potently expanded the neural crest precursor population (Fig. 8Aa,e), indicating that the stabilizing mutations do not have antimorphic effects on Slug function. When sibling embryos were examined at stage 24, Slug-injected embryos showed an increased number of migratory neural crest cells, consistent with the expansion seen in the progenitor pool at earlier stages (compare Fig. 8Ab with 8Ac). Interestingly, the cranial region of many embryos injected with Slug$^{\psi 1,2}$ had areas that were cleared of migrating *Sox10*-expressing neural crest (compare Fig. 8Af with 8Ag), suggesting that prolonged expression of high levels of Slug protein after the onset of neural crest migration is incompatible with the normal development of these cells. Importantly, however, these

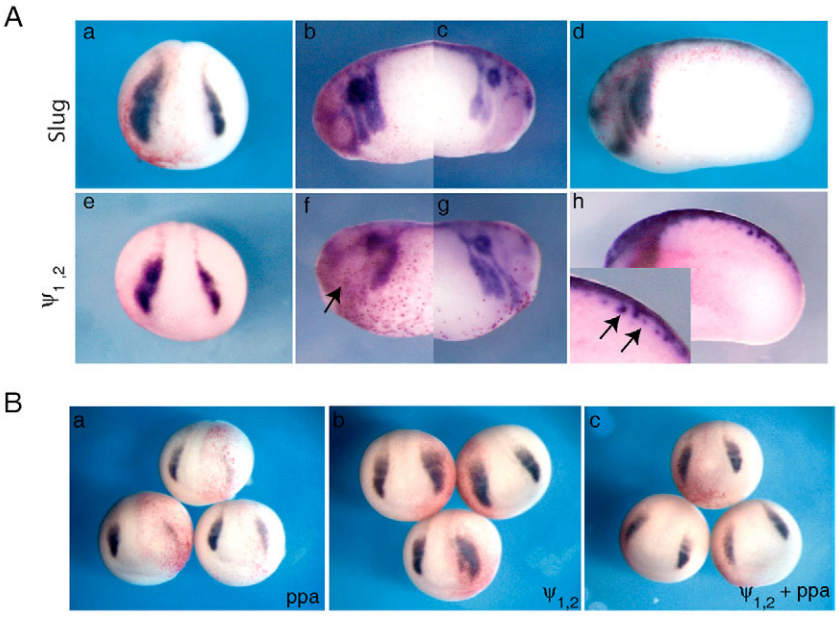

Fig. 8. A stabilized Slug mutant rescues neural crest formation in Ppa-injected embryos. (**A**) Embryos were injected with Slug (a-d) or Slug$^{\psi 1,2}$ (e-h), together with the lineage tracer nβ-gal (red) and in situ hybridization was used to analyze neural crest precursor formation and migration. Both Slug (a) and Slug$^{\psi 1,2}$ (e) expand *Slug* expression at stage 15. Slug overexpression increases the number of migratory neural crest cells at stage 24 (b, injected side; c, control side) whereas Slug$^{\psi 1,2}$ injection results in a clearing of *Sox10*-positive cells (f, injected side; g, control side). The presence of premature, ectopic migratory neural crest cells is observed in embryos overexpressing Slug$^{\psi 1,2}$ (h, inset) but not in embryos injected with Slug (d). (**B**) Embryos were injected in one of two cells with Ppa (a) Slug$^{\psi 1,2}$ (b) or Ppa and Slug$^{\psi 1,2}$ (c) along with the lineage tracer nβ-gal (red). Ppa prevents neural crest precursor formation (a), whereas Slug$^{\psi 1,2}$ expands this cell population (b), as assayed by *Slug* expression. Co-injection of Slug$^{\psi 1,2}$ with Ppa rescues neural crest precursor formation (c).

effects do not represent antimorphic activities of the mutant protein, as expression of high levels of wild-type Slug can also have similar consequences (data not shown). Another noteworthy phenotypic consequence of the stabilized Slug protein was the presence of premature, ectopic migratory neural crest cells in spinal cord regions (Fig. 8Ah), further supporting a role for Snail family proteins in promoting EMTs and migratory behavior.

Since overexpression of Ppa mRNA leads to a nearly complete loss of neural crest precursor cells (Fig. 5E), we examined whether expression of the Slug$^{\psi 1,2}$ mutant, which is resistant to Ppa-mediated degradation, was sufficient to restore neural crest precursor formation in Ppa-injected embryos. We injected mRNA encoding Ppa, Slug$^{\psi 1,2}$, or both together with nβ-gal into one cell of two-cell stage embryos. Injected embryos were cultured to neural plate stages when the expression of neural crest precursor markers was examined by in situ hybridization. Expression of Ppa alone dramatically reduced the expression of *Slug* (100%, *n*=14) (Fig. 8Ba), whereas expression of Slug$^{\psi 1,2}$ expanded the neural crest precursor cell population (93%, *n*=29) (Fig. 8Bb). Importantly, we found that co-expression of Slug$^{\psi 1,2}$ could rescue the Ppa-mediated loss of neural crest precursors (82%, *n*=23; Fig. 8Bc), suggesting that Slug is the major target of Ppa regulation in these cells. Whereas wild-type Slug is also capable of rescuing the Ppa-overexpression neural crest defect when expressed at high levels, the Slug$^{\psi 1,2}$ mutant that does not bind to Ppa rescues this phenotype much more effectively, and this is particularly evident at lower doses (data not shown).

DISCUSSION

Multiple levels of regulation, including transcriptional, post-transcriptional and post-translational modification, are employed to ensure tight control of the activity of key regulatory proteins. An increasing number of transcription factors that play key roles in embryonic development, including Runx2 (Shen et al., 2005), Smads (Izzi and Attisano, 2004) and MyoD (Tintignac et al., 2005), are known to be regulated at the level of protein turnover. Slug and Snail are excellent examples of proteins for which such precise and accurate control is necessary, given their critical roles in both neural crest precursor cell formation and migration, and their ability to induce EMTs when overexpressed in epithelial cells. Despite their developmental and clinical importance, however, the mechanisms via which Snail family members control these processes are poorly understood, and even less is known about the ways in which these transcriptional repressors are themselves regulated. In this paper, we demonstrate that Slug is a labile protein, the expression of which is dynamically regulated by the opposing forces of neural crest regulatory factors and the ubiquitin-mediated proteasomal degradation pathway.

Several recent studies have investigated the functional roles of Snail-related factors during neural crest development and their involvement in the metastatic progression of tumors. However, although much emphasis has been placed on the ability of Snail family members to induce both physiological and pathological EMTs, the expression of these genes is not always sufficient to induce this dramatic morphological transformation. For example, *Xenopus* Slug is highly expressed in neural crest precursors, and its activity is required for their formation, but these cells are epithelial and do not undergo an EMT or migrate until much later in development. Furthermore, Snail mRNA expression is generally inversely correlated with E-cadherin, but some cell lines show high levels of both genes (Dominguez et al., 2003). Indeed, recent studies have suggested that only mutant forms of Snail are capable of downregulating E-cadherin activity or inducing an EMT when overexpressed in certain epithelial cell lines (Yook et al., 2005; Zhou et al., 2004). Together, these data point to a dynamic, context-dependent regulation of Slug and Snail that allows them to regulate essential targets without always inducing an EMT.

The non-vertebrate chordate *Amphioxus* provides a model for considering this aspect of Slug regulation. Despite the presence of seemingly appropriate signaling conditions, the induction of early patterning genes such as *Pax3/7* (Holland et al., 1999) and *Opl* (Gostling and Shimeld, 2003), and the expression of *Snail* (Langeland et al., 1998) at the neural plate border, *Amphioxus* lacks definitive neural crest (Holland and Holland, 2001). This deficiency has been hypothesized to be due to the absence of other neural crest specifiers such as Sox9, FoxD3 and Twist (Yu et al., 2002). Although these transcription factors have been shown to confer different aspects of the neural crest phenotype, their concerted activity is important for the proper development of these cells. Here we demonstrate that the Slug protein is stabilized by co-expression of Sox9, FoxD3 or Twist, while overexpression of other neural crest regulatory factors including Opl, Smad1 or Snail has no effect on Slug stability. We suggest that one way in which Sox9, FoxD3 and Twist, which are not expressed at the neural plate border in non-vertebrate chordates, may contribute to neural crest formation is by stabilizing Snail family proteins, thus altering their functional capacity to promote neural crest development. We are currently investigating the mechanism(s) underlying the ability of these neural crest transcription factors to influence Slug protein stability, and one means by which they appear to do so is by regulating the expression of Ppa. We found that expression of either FoxD3 or Sox9 reduced the expression of *Ppa* as detected by in situ hybridization (Fig. S1 in the supplementary material).

We show here that Slug is a target of the ubiquitin-mediated proteasomal degradation pathway. Despite providing most of the substrate binding specificity to this pathway (Hershko and Ciechanover, 1998), most E3 ligases examined thus far have broad, diffuse expression patterns in early embryos. A noteworthy exception to this is Ppa, an F-box protein first characterized in *Drosophila*, where it was one of only two E3s found to have specifically localized mRNA transcripts (Das et al., 2002; Raj et al., 2000). We report here that *Xenopus Ppa* is expressed at the neural plate border and in migrating neural crest cells, and that Ppa directly binds to Slug and promotes its degradation. Significantly, we find that ectopic Ppa expression leads to an almost complete loss of neural crest precursor cells. Morpholino-mediated depletion of Ppa leads to the stabilization of Slug protein (Fig. 5C) and also leads to aberrant neural crest development (A.E.V. and C.L., unpublished). The latter finding is of interest, but it must nevertheless be interpreted with caution since the range of substrates regulated by Ppa remains unknown. Instead, to specifically address the significance of Ppa regulation for Slug stability and function, we mapped the Ppa interaction site to a region in the Slug N terminus that is rich in hydrophobic amino acids and characterized minimal mutations that disrupt the interaction between these two factors. These mutations significantly stabilize the Slug protein, and can rescue neural crest precursor formation in Ppa-injected embryos, suggesting that Slug is the major target of Ppa regulation in neural crest cells. Finally, we demonstrate that the persistence of too much Slug protein in migratory neural crest cells severely disrupts their development, consistent with a need for tight control over the levels and activity of Slug protein in early embryos.

Importantly, although two recent studies using mammalian cell cultures implicated the GSK3β/βtrcp pathway in the regulation of Hs Snail stability, we find that this pathway does not regulate Slug

stability in *Xenopus* embryos. In marked contrast to the robust stabilization of β-catenin, we found that blocking GSK3β activity had no effect on Slug stability. This is consistent with the absence in Slug of the βtrcp binding site present in human and mouse Snail proteins. Interestingly, however, the GSK3β/βtrcp pathway also appears to play, at most, a minimal role in regulating Snail stability in *Xenopus* embryos. Because Snail family proteins fulfill many essential functions in organisms ranging from arthropods to vertebrates, the existence of multiple, context-dependent mechanisms for controlling the activity and stability of these proteins makes considerable sense. Ppa, the F-box protein characterized here, can promote the degradation of Snail family proteins from *Drosophila* through to mammals (Fig. 5A; A.E.V. and C.L., unpublished), suggesting that this is a highly conserved mechanism for regulating Slug/Snail activity, and therefore is likely to be of significance beyond its role in neural crest development. Although additional E3s that modulate the levels, and thus the activity, of these critical regulatory factors may yet be described, determining the contribution of Ppa to the control of Slug/Snail-mediated EMTs during tumor progression will be important.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/17/3359/DC1

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