The F-box protein Ppa is a common regulator of core EMT factors Twist, Snail, Slug, and Sip1

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The neural crest is a proliferative, multipotent stem cell population that arises during early vertebrate development (LaBonne and Bronner-Fraser, 1999; Le Douarin and Kalcheim, 1999). Neural crest cells form at the neural plate border and give rise to a diverse set of derivatives that includes neurons and glia of the peripheral nervous system, facial cartilage and bone, and melanocytes (Le Douarin and Kalcheim, 1999). Neural crest cells undergo an epithelial–mesenchymal transition (EMT) and acquire migratory and invasive behavior (Gammill and Bronner-Fraser, 2003; Tucker, 2004; Duband, 2006; Thiery and Sleeman, 2006; Yang and Weinberg, 2008) to disperse to their diverse target sites.

Many cells in the developing embryo undergo at least one round of EMT before terminally differentiating. In addition to the neural crest, this process has been studied in other developmental contexts, including the ingression of the mesoderm, formation of the cardiac valves, and development of the secondary palate (Markwald et al., 1977; Bolender and Markwald, 1979; Griffith and Hay, 1992; Leptin, 1999; Locascio and Nieto, 2001; Shook and Keller, 2003). Premigratory neural crest cells express a network of transcription factors that includes Snail, Slug, Twist, Foxd3, and one or more SoxE family factors (Taylor and LaBonne, 2007; Sauka-Spengler and Bronner, 2010), many of which are essential for both the formation of the precursor population and for their subsequent EMT and migration. These same factors have been found to contribute to developmental EMTs in other embryonic tissues (Thiise et al., 1987; Leptin and Grunewald, 1990; Leptin, 1999; Carver et al., 2001; Vernon and LaBonne, 2004, 2006; Shelton and Yutzey, 2008; Yang and Weinberg, 2008; Thiery et al., 2009).

It is increasingly recognized that epithelial tumor cells must also undergo an EMT to disseminate and form secondary metastases (Roussos et al., 2010). Importantly, developmental EMT regulatory factors are inappropriately expressed or misregulated in a wide array of human cancers, and this correlates with tumor aggressiveness and poor patient outcomes (Huber et al., 2005). Multiple signaling pathways, including TGF-β, Wnt, Notch, and receptor tyrosine kinase–mediated signals, have all been implicated as upstream initiators of the EMT process in tumor cells (Moustakas and Heldin, 2007; Thiery et al., 2009). However, these diverse upstream signals all appear to converge on a common set of core EMT regulatory factors that includes Snail, Slug, Twist, and Sip1 (also known as ZEB2; Yang and Weinberg, 2008; Thiery et al., 2009). Because developmental and pathological EMTs are controlled by the same core
regulatory factors, acquiring a better understanding of the mechanisms by which these factors control epithelial plasticity during neural crest development may also provide essential insights into how their misregulation contributes to metastasis.

The best studied of the core EMT regulatory factors are the Snail family repressors Snail and Slug (Snail2; Nieto, 2002; Wu and Zhou, 2010), which have been shown to regulate several genes involved in cell adhesion and cell junctions (Batlle et al., 2000; Cano et al., 2000; Hajra et al., 2002). During *Xenopus laevis* neural crest development, Snail family factors play temporally distinct roles in both the formation of neural crest precursors and the subsequent EMT/migration of these cells (LaBonne and Bronner-Fraser, 2000). Thus, the activity of these factors must be regulated to ensure that they only mediate EMTs in the appropriate cellular context. We have previously demonstrated that the cellular levels of the Slug protein are key to determining the effects of its expression in neural crest cells (Vernon and LaBonne, 2006). Slug/ Snail protein levels are regulated by the ubiquitin–proteasome system (UPS), and, in embryos, they are targeted for degradation by a leucine-rich repeat containing F-box protein Partner of paired (Ppa). Ppa serves as the substrate recognition component of an Skp–Cullin–F-box E3 ubiquitin ligase and is dynamically expressed in neural crest–forming regions. More recently, the human homologue of Ppa, FBXL14, was shown to target Snail for UPS-mediated degradation in tumor cells (Viñas-Castells et al., 2010), indicating that this level of control is conserved across developmental and pathological EMTs.

Like Slug/Snail, Twist is a core EMT regulatory factor that can modulate the behavior and fate of cells in both development and cancer (Leptin, 1991; Chen and Behringer, 1995; Castanon and Baylies, 2002; O’Rourke and Tam, 2002; Soo et al., 2002; Yang et al., 2004; Thiery et al., 2009). Twist is a basic helix-loop-helix (bHLH) protein and is thus structurally unrelated to Slug and Snail. Like those proteins, however, Twist plays developmental roles in the mesoderm and neural crest and promotes EMTs and invasive behavior in tumor cells (Leptin, 1991; Chen and Behringer, 1995; Castanon and Baylies, 2002; O’Rourke and Tam, 2002; Soo et al., 2002; Yang et al., 2004; Thiery et al., 2009; Cakouros et al., 2010). Little is known about how Twist regulates EMTs during either embryonic development or tumor cell metastasis or how it is regulated such that it only mediates these changes in the correct cellular context.

Here, we demonstrate that Twist is a labile protein regulated by the UPS. We find that Twist, as well as another core EMT regulatory factor, Sip1 (ZEB2), binds to and is targeted for degradation by Ppa, the F-box protein previously shown to regulate Slug and Snail stability (Vernon and LaBonne, 2006). Together, these results point to the evolution of a common mechanism for controlling the function of three structurally unrelated families of factors that share conserved regulatory function. These findings have important implications for understanding the evolution of the neural crest as a migratory stem cell population and potentially for the control of EMT as a transient and reversible process.

![Figure 1](image.png)

**Results and discussion**

**Twist is an unstable protein**

The structurally diverse proteins Snail, Slug, Twist, and Sip1 comprise the core group of EMT regulatory factors. It is essential to understand how the activities of these factors are regulated such that they only mediate changes in cell behavior in appropriate contexts. It has recently been demonstrated that Snail family factors are regulated at the level of protein stability by targeting to the UPS (Zhou et al., 2004; Yook et al., 2005; Vernon and LaBonne, 2006; Viñas-Castells et al., 2010). This mechanism is exceptionally well suited to providing dynamic context-dependent control of proteins that must regulate essential targets without always inducing an EMT. In the neural crest, the tuning of Slug/Snail protein levels is mediated by the F-box protein Ppa, the substrate recognition component of a modular E3 ubiquitin ligase (Vernon and LaBonne, 2006).

To determine whether Twist function might also be regulated at the level of protein stability, we first examined whether Twist, like Slug and Snail, is a labile protein. Embryos were injected at the two-cell stage with mRNA encoding C-terminally epitope-tagged Twist and cultured until early blastula stages. Western analysis demonstrates Twist protein levels were observed to decrease over the course of 6 hours in cycloheximide (CHX)-treated embryos (Fig. 1 A and not depicted). To further determine whether Twist was unstable over developmental time, embryos expressing epitope-tagged Twist were cultured and collected over the course of progressively later embryonic stages, including gastrula, neurula, and tailbud stages. Twist protein levels were observed to decrease over the course of...
undergo caspase cleavage–dependent ubiquitination/degradation during apoptosis (Demontis et al., 2006). To determine whether Twist is also ubiquitinated in early embryos, we performed co-immunoprecipitation assays from embryos coinjected with Twist and ubiquitin. An abundant ladder of polyubiquitinated Twist protein was observed under these conditions, indicating that Twist ubiquitination does occur in early embryos (Fig. 2 A). To determine the region of Twist required for ubiquitination, a deletion series was constructed consisting of either the Twist N terminus (M1-R96), the Twist C terminus (A57-H166), or a form of Twist missing the C-terminal WR domain (Twist \( \Delta WR \), M1-V142; Fig. 2 B). When the ubiquitination of these Twist deletion
injected with mRNA encoding either Twist ΔWR or wild-type Twist, such that these proteins were expressed at initially equivalent levels. Injected embryos were cultured to blastula stages, CHX treated to prevent further protein synthesis, and collected at set time intervals for Western blot analysis. The deletion of the WR domain was found to substantially stabilize the Twist protein (Fig. 2 D). Similarly, the deletion of the WR domain was found to stabilize Twist over developmental time (Fig. 2 E).

Twist interacts with the E3 ubiquitin ligase Ppa

Because the WR domain is required for Twist ubiquitination, we speculated that the deletion of the WR domain might stabilize the Twist protein. To test this hypothesis, the stability of Twist ΔWR was compared with that of wild-type Twist. Embryos were injected with mRNA encoding either Twist ΔWR or wild-type Twist, such that these proteins were expressed at initially equivalent levels. Injected embryos were cultured to blastula stages, CHX treated to prevent further protein synthesis, and collected at set time intervals for Western blot analysis. The deletion of the WR domain was found to substantially stabilize the Twist protein (Fig. 2 D). Similarly, the deletion of the WR domain was found to stabilize Twist over developmental time (Fig. 2 E).

The WR domain renders Twist unstable

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Twist interacts with the E3 ubiquitin ligase Ppa

Polyubiquitin-mediated proteasomal targeting is controlled by a series of enzymes that includes an activating enzyme (E1), a
conjugating enzyme (E2), and an E3 ubiquitin ligase that also confers substrate recognition (Ho et al., 2006). Ppa, an F-box protein that targets Slug/Snail for degradation, is dynamically expressed during neural crest development (Vernon and LaBonne, 2006). Because Twist, like Slug/Snail, is a core EMT regulatory factor that is regulated by ubiquitination, we asked whether Ppa might also play a role in controlling Twist stability. First, we sought to determine whether Ppa could physically interact with Twist. In a coimmunoprecipitation assay from injected embryo lysates, Ppa was shown to strongly interact with both Snail and Twist but not with Sox10, an essential neural crest regulatory factor that has not been linked to either developmental or pathological EMTs (Fig. 3 A).

Although the Twist WR domain is required for Twist ubiquitination, the domain itself contains no lysine residues that could function as ubiquitin acceptor sites. We therefore asked whether the WR domain mediates interaction with Ppa. Full-length Twist, or Twist ΔWR, was coexpressed with Ppa in Xenopus embryos, and binding was assayed by coimmunoprecipitation. The deletion of the WR domain led to loss of interaction with Ppa (Fig. 3 C), suggesting that the role of the WR domain in regulating Twist stability may be the recruitment of the ubiquitination machinery via Ppa. To test whether the WR domain contained all sequences necessary for Ppa recruitment, we asked whether this domain would be sufficient to confer the ability to interact with Ppa onto another protein. We chose the ubiquitously expressed bHLH protein E12, which does not interact with Ppa (Fig. 3 C). We found that a fusion protein in which the Twist WR domain was linked in frame to the E12 C terminus (E12-WR; Fig. 3 B) strongly interacted with Ppa in coimmunoprecipitation assays, indicating that the WR domain alone was sufficient to mediate this interaction (Fig. 3 C). Slug and Snail were previously shown to interact with Ppa via an extended hydrophobic region in their N termini that has little sequence similarity with the WR domain of Twist (Fig. 3 D; Vernon and LaBonne, 2006).

**Ppa is an endogenous regulator of Twist stability**

If Ppa functions as the endogenous E3 recognition subunit for Twist ubiquitination in early Xenopus embryos, overexpression of Ppa should increase the rate of Twist turnover, whereas down-regulation of endogenous Ppa should increase Twist stability. To test the first hypothesis, the relative stability of Twist was compared in the presence or absence of overexpressed Ppa. We found that coexpression of Twist and Ppa significantly accelerated Twist protein turnover (Fig. 4 A), consistent with a role for Ppa in targeting Twist for proteasomal degradation. To test whether endogenous Ppa controls Twist stability, Ppa was down-regulated using previously characterized translation blocking morpholinos (MOs; Vernon and LaBonne, 2006). The stability of Twist protein was compared in Ppa-depleted embryos versus embryos coinjected with Twist and control MO. Depletion of endogenous Ppa led to significant stabilization of Twist (Fig. 4 B), confirming that Ppa is an endogenous regulator of Twist stability.

**Ppa also regulates the core EMT factor Sip1**

The remarkable finding that zinc finger EMT regulatory factors Slug/Snail and the structurally unrelated bHLH factor Twist are all targeted by Ppa for ubiquitin-mediated proteasomal degradation led us to ask whether this could be a common mechanism for controlling the function of core EMT regulatory factors. Another factor included in this group is Sip1 (Smad-interacting protein-1, also known as ZEB2), a zinc finger/homeodomain transcriptional repressor that belongs to the δEF1 family of proteins (Verschueren et al., 1999). Sip1 has been linked to EMT/metastasis in a broad array of cancers (Rosivatz et al., 2002; Maeda et al., 2005; Peinado et al., 2007; Polyak and Weinberg, 2009) and is expressed in cranial neural crest cells at migratory stages (van Grunsven et al., 2000). Similar to Slug, Snail, and Twist, Sip1 has been shown to down-regulate epithelial cadherin (Comijn et al., 2001; Maeda et al., 2005; Peinado et al., 2007). We therefore sought to determine whether Sip1, like Slug, Snail, and Twist, is regulated by the UPS. We found that Sip1 can be ubiquitinated and that it physically interacts with Ppa in coimmunoprecipitation assays (Fig. 5 A). The sequences required for Ppa recruitment lie predominantly in the N terminus of the protein, upstream of the Smad-binding domain (unpublished data). Consistent with its ability to recruit Ppa, we found that Sip1 is a labile protein and further found that coexpression with Ppa dramatically increases the rate of Sip1 turnover (Fig. 5 B). Together, these findings indicate that a common mechanism has evolved for coordinate regulation of structurally diverse group of proteins (Fig. 5 C) that are functionally linked through their shared regulation of the neural crest, EMT, and invasive cell behavior.

**A common, evolutionarily conserved regulatory mechanism**

The core EMT regulatory factors Snail, Slug, Twist, and Sip1 are often coexpressed and likely play coordinated roles in the cellular and morphological changes underlying this transition in both developmental and pathological contexts (Rosivatz et al., 2002; Takahashi et al., 2004; Martin et al., 2005;
Peinado et al., 2007; Foubert et al., 2010; Taube et al., 2010; Montserrat et al., 2011). In Xenopus, Slug, Snail, and Twist play required roles in establishing neural crest precursor cells long before these cells undergo an EMT and become migratory (Hopwood et al., 1989; LaBonne and Bronner-Fraser, 2000; Linker et al., 2000; van Grunsven et al., 2000; Aybar et al., 2003). Precisely how this group of core factors functions to coordinately regulate neural crest development remains an area of active investigation. Moreover, little is understood about how these structurally distinct factors are themselves regulated to ensure that EMTs only occur in the correct time and place.

Numerous studies analyzing the transition from the epithelial to mesenchymal state in cultured cells have focused on the ability of individual factors to promote this complex cellular program (Peinado et al., 2004; Yang et al., 2004, 2010; Moody et al., 2005; Yook et al., 2005; Bindels et al., 2006; Medici et al., 2008; Ansieau et al., 2010; Browne et al., 2010; Fu et al., 2011). However, studies of these proteins in embryonic contexts suggest that the core EMT regulatory factors are more likely to act...
in concert, with each controlling distinct subsets of the necessary structural and behavioral changes associated with EMT-like processes (Grunewald, 1990; Leptin and Grunewald, 1990; Leptin, 1991; Seher et al., 2007; Wu et al., 2007; Martin et al., 2009; Martin et al., 2010). For example, during ventral furrow formation in Drosophila melanogaster, Twist and Snail have been shown to differentially regulate the pulsed constrictions undergone by mesoderm-fated cells (Martin et al., 2009). In order for presumptive mesodermal cells to properly invaginate during gastrulation, Snail must first induce cellular contractions, followed by Twist stabilization of the constricted state. Thus, although both factors play required roles in this process, the functional output of the two proteins is quite different in this context. This seems likely to prove true in other cell and developmental contexts as well, highlighting the importance of understanding the mechanisms by which the activities of the core EMT regulatory factors are coordinately regulated. Consistent with the cellular level of these proteins being a critical aspect of the coordinated control of their function, it has proven difficult to achieve a full rescue of the effects of Ppa up-regulation by expressing even combinations of the target factors (Fig. S1).

In this paper, we demonstrate that Twist, like Snail family proteins, is a labile protein whose activity is regulated by the UPS. We further show that Twist is targeted for UPS-mediated degradation by Ppa, the same F-box protein that regulates Snail stability, and that this regulation is dependent on the WR domain. Remarkably, we find that Ppa also controls the levels of another core EMT regulatory factor, the δEF1 family protein Sip1, a zinc finger/homeodomain protein that is not a member of the Snail superfamily. Together, these findings indicate that a common regulatory mechanism has evolved to control the activity of a core group of functionally linked but structurally diverse factors (Fig. 5, C and D).

It is intriguing to hypothesize that in circumstances in which the functional output of multiple components of a gene regulatory network must be tightly coordinated, it might be advantageous to control their function collectively via a common regulatory mechanism. Moreover, as it appears that the intricate cellular and behavior changes mediated by the core EMT regulatory factors must be tightly controlled in a space- and time-correlated manner (Martin et al., 2009), differential sensitivity to Ppa could contribute to the proper coordinated action of these factors. Finally, our finding that there is a common regulatory mechanism for the core EMT regulatory factors in neural crest cells suggests that this may also prove true during tumor progression, thus highlighting Ppa as a potential focal point for therapeutic intervention aimed at halting metastasis.

**Materials and methods**

**DNA constructs**

Xenopus Ppa was obtained from American Type Culture Collection (clone ID 403279). Epitope-tagged versions of all cDNAs were generated by amplifying the coding sequence and inserting them into pcS2-MycC or pcS2-FlagC vectors. Xenopus Twist deletion mutants were generated using the primers Twist N-terminal sense 5'-ATGATGCCAGAAGA-3' and antisense 5'-TCTCAGGGCAAGC-3', Twist C-terminal sense 5'-ATGCGGCCAGG-3', and Twist WR sense 5'-ATGATCCAGAAGA-3' and antisense 5'-CACATAACTGCAGCTGGC-3'. The E12-WR domain fusion construct was generated by inserting the WR domain sequence 5'-GGGCGATAGAAGGCTCAGATCGCTCCTTCCGTGTGGGAGTATGGAGGCTGCGTTGTCTGGTGAC-3' into the EcoRI site of Xenopus E12 in pcS2-MycC vector. Xenopus Sip1 in the vector pcS2* was obtained from A. Eisaki (University of Tokyo, Tokyo, Japan; Eisaki et al., 2000) and subcloned into the pcS2-MycC vector. 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 (Invitrogen). Embryos were injected at the two-cell stage unless otherwise noted and were collected at the indicated stage. In situ hybridization of Xenopus embryos was performed with digoxigenin-labeled RNA probes using the standard protocol (LaBonne and Brömer-Fraser, 1998; Bellmeyer et al., 2003) and developed using BM purple substrate (Roche). Embryo images were collected on a dissecting microscope (SZX12; Olympus) fitted with a 10x objective and a digital camera (Q-Color5; Olympus). Composite images were assembled using Photoshop [Adobe]. The Ppa MO sequence is 5'-AGAAGACGAGATGAGGTCTCCATACG-3' (the initiation codon is underlined) and was characterized in Vernon and LaBonne (2006). Where noted, embryos were treated with 10 µg/ml CHX (Sigma-Aldrich) in 0.1x Marc’s modified Ringer’s.

**Immunoprecipitation and Western blot analysis**

For immunoprecipitations, embryos were collected at stage 10, lysed in PBS + 1% NP-40 containing a protease inhibitor cocktail (Roche), and incubated with the indicated antibody (0.2 µg α-Myc [9E10; Santa Cruz Biotechnology, Inc.] or 0.2 µg α-FlagM2 affinity purified [Sigma-Aldrich]) for 2 h on ice followed by a 2-h incubation with protein A-Sepharose beads. Immunoprecipitations were washed with radiocimmunoprecipitation assay buffer and resolved by SDS-PAGE. Immunoblotting was performed using α-Myc (1:2,000), affinity-purified α-FlagM2 (1:3,000), or α-actin (1:1,000; Sigma-Aldrich) antibody as indicated. Labeled proteins were detected using HRP-conjugated secondary antibodies and enhanced chemiluminescence [GE Healthcare].

**Ubiquitination/protein stability assays**

To detect ubiquitination, embryos were co-injected with mRNA encoding Flag epitope-tagged ubiquitin and myc epitope-tagged target substrate (Twist or Sip1). Embryos were collected at stage 10, and coimmunoprecipitations of the proteins were followed as described in the previous section. To determine the protein stability of desired proteins, Xenopus embryos were collected at stage 8 for time point 0 (t = 0). Embryos were then treated with 10 µg/ml CHX in 0.1x Marc’s modified Ringer’s and collected at hourly time points.

**Online supplemental material**

Fig. S1 shows the rescue of neural crest in Ppa-injected embryos by the core EMT factors and shows in situ hybridization examining the extent to which individual and combined expression of core EMT factors, including Twist, rescues the loss of neural crest that accompanies Ppa misexpression. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201012085/DC1.

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**References**


Batlle, E., E. Sancho, C. Franci, D. Domínguez, M. Monfar, J. Baulida, and A. García De Herreros. 2000. The transcription factor snail is a repressor of