A Context-Dependent Role for the RNF146 Ubiquitin Ligase in Wingless/Wnt Signaling in *Drosophila*

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ABSTRACT Aberrant activation of the Wnt signal transduction pathway triggers the development of colorectal cancer. The ADP-ribose polymerase Tankyrase (TNKS) mediates proteolysis of Axin—a negative regulator of Wnt signaling—and provides a promising therapeutic target for Wnt-driven diseases. Proteolysis of TNKS substrates is mediated through their ubiquitination by the poly-ADP-ribose (pADPr)-dependent RING-domain E3 ubiquitin ligase RNF146/Iduna. Like TNKS, RNF146 promotes Axin proteolysis and Wnt pathway activation in some cultured cell lines, but in contrast with TNKS, RNF146 is dispensable for Axin degradation in colorectal carcinoma cells. Thus, the contexts in which RNF146 is essential for TNKS-mediated Axin destabilization and Wnt signaling remain uncertain. Herein, we tested the requirement for RNF146 in TNKS-mediated Axin proteolysis and Wnt pathway activation in a range of *in vivo* settings. Using null mutants in *Drosophila*, we provide genetic and biochemical evidence that Rnf146 and Tnks function in the same proteolysis pathway *in vivo*. Furthermore, like Tnks, *Drosophila* Rnf146 promotes Wingless signaling in multiple developmental contexts by buffering Axin levels to ensure they remain below the threshold at which Wingless signaling is inhibited. However, in contrast with Tnks, Rnf146 is dispensable for Wingless target gene activation and the Wingless-dependent control of intestinal stem cell proliferation in the adult midgut during homeostasis. Together, these findings demonstrate that the requirement for Rnf146 in Tnks-mediated Axin proteolysis and Wingless pathway activation is dependent on physiological context, and suggest that, in some cell types, functionally redundant pADPr-dependent E3 ligases or other compensatory mechanisms promote the Tnks-dependent proteolysis of Axin in both mammalian and *Drosophila* cells.

KEYWORDS ADP-ribosylation; Axin; beta-catenin; Iduna; intestinal stem cell; midgut; RNF146; Tankyrase; Wingless; Wnt

THE Wnt/Wingless signal transduction pathway directs fundamental processes during metazoan development (Nusse and Clevers 2017) . Inhibition of Wnt signaling underlies numerous birth defects, and inappropriate activation of Wnt signaling is associated with several cancers, including the vast majority of colorectal cancers. Thus, understanding the basic mechanisms that underlie activation of the Wnt pathway will facilitate the design of innovative strategies for the treatment of several human diseases. Regulation of the stability of β-catenin/Armadillo, the central transcriptional activator in the pathway, dictates the level of Wnt pathway activity. A "destruction complex," which includes the concentration-limiting scaffold protein Axin, the tumor suppressor Adenomatous polyposis coli (APC), and glycogen synthase kinase 3, targets β-catenin for proteasomal degradation. Wnt stimulation inhibits the destruction complex and induces the recruitment of Axin to an activation complex termed the "signalosome," thus stabilizing β-catenin and promoting activation of the pathway. The regulation of Axin holds significant clinical relevance, as the level of Wnt signaling is modulated by Axin activity (Liu and He 2010).

Therapeutic agents that selectively target the Wnt pathway are a priority for the field, but have yet to enter the clinic (Lum and Clevers 2012). A promising lead was suggested by previous small molecule screens that identified Tankyrase (TNKS)—the enzyme that mediates the poly-ADP-ribosylation of Axin—as a novel therapeutic target for Wnt-driven diseases (Huang *et al.* 2009). Poly-ADP-ribosylation mediated by TNKS promotes Wnt signaling by targeting Axin for proteasomal degradation, and thereby stabilizing β-catenin. TNKS inhibitors impede the Wnt-dependent proliferation of

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cultured cells, and, in mice with conditional targeted deletion of *Apc*, TNKS inhibitors disrupt the growth of colonic adenomas, raising the possibility that these agents hold clinical promise (Waaler *et al.* 2012; Lau *et al.* 2013).

In mammalian cultured cells, the targeting of Axin, and of TNKS itself, for proteasomal degradation through TNKS-dependent poly-ADP-ribosylation requires their subsequent ubiquitination by the poly-ADP-ribose (pADPr)-dependent RING-domain E3 ubiquitin ligase RNF146/Iduna (Callow *et al.* 2011; Kang *et al.* 2011; Zhang *et al.* 2011; DaRosa *et al.* 2015). Furthermore, RNF146 also promotes Axin degradation in *Xenopus* embryos (Zhu *et al.* 2018). Thus, in principle, RNF146 could potentially provide another therapeutic target for Wnt-driven cancer. However, in contrast with the effects of TNKS inhibition, depletion of RNF146 neither stabilized Axin nor inhibited the transcriptional activation of Wnt target genes in colorectal carcinoma cell lines harboring truncations in APC (Callow *et al.* 2011). These findings raised the question of whether RNF146 is indeed essential for all TNKS-mediated Axin degradation *in vivo*, and whether RNF146 is a viable therapeutic target for Wnt-driven disease. An *in vivo* mouse model for RNF146 inactivation to address this question has not yet been reported.

Herein, we sought to test the extent to which RNF146 is essential for TNKS-mediated Axin proteolysis and Wnt signaling in a range of *in vivo* contexts. We built upon a previously established genetic model that demonstrated evolutionary conservation in Tnks function in *Drosophila*. These studies revealed that Tnks-mediated ADP-ribosylation targets Axin for proteolysis under basal conditions throughout development, and that the *in vivo* requirement for Tnks is context-dependent (Wang *et al.* 2016b,c). Specifically, in the adult *Drosophila* intestine, where gradients of Wingless signaling exist at high levels at each compartment boundary, and decrease as a function of distance from these boundaries (Buchon *et al.* 2013; Tian *et al.* 2016), Tnks is essential for transcriptional activation of target genes in regions where Wingless is present at low concentration and controls the Wingless-dependent regulation of intestinal stem cell (ISC) proliferation (Tian *et al.* 2016; Wang *et al.* 2016c). Furthermore, Tnks also serves to buffer Axin activity in other *in vivo* contexts, by ensuring that Axin levels remain below the threshold at which Wingless pathway activation is inhibited (Wang *et al.* 2016b; Yang *et al.* 2016). For example, Tnks is required for the Wingless-dependent specification of cell fate in the embryonic epidermis when endogenous Axin levels are increased by only twofold (Yang *et al.* 2016), and also serves this function in Wingless-dependent cell fate specification in the larval wing imaginal disc and in the pupal abdomen.

In this report, we demonstrate that *Drosophila* Rnf146/Iduna mediates the pADPr-dependent degradation of Tnks substrates, including Axin and Tnks itself, under basal conditions throughout development. We provide genetic and biochemical evidence that Tnks and Rnf146 function in the same pADPr-dependent proteolytic pathway, indicating that RNF146 function is evolutionarily conserved. Furthermore, like Tnks, Rnf146 promotes Wingless signaling in multiple *in vivo* contexts by buffering Axin levels such that they remain below the threshold that inhibits Wingless signaling. Surprisingly, however, and in contrast to Tnks, Rnf146 is dispensable in the adult midgut for both promoting Wingless target gene activation and for regulating the Wingless-dependent control of ISC proliferation. Together, these findings reveal a context-dependent role for RNF146 in Tnks-mediated Axin proteolysis and Wingless signaling *in vivo*, and support the hypothesis that, in specific contexts, functionally redundant pADPr-dependent E3 ligase(s) may mediate the proteolysis of Axin not only in mammalian cells, but also in *Drosophila* cells.

Materials and Methods

Drosophila stocks and transgenes

To generate deletions in *Rnf146*, *P{Δ2-3}99B* was used to mobilize the *P* element *EY09040* (Bloomington Drosophila Stock Center, BDSC) (Bellen *et al.* 2004), which is inserted in the first intron of *Rnf146*. By PCR screening, seven lines with deletions in *Rnf146* were identified, including $Rnf146^{36}$ and $Rnf146^{157}$. $Rnf146^{36}$ has a deletion of 5970 nucleotides (+774 to +6743 with reference to the *Rnf146* transcriptional start site); 14 nucleotides from *EY09040* remain at this site. $Rnf146^{157}$ has a deletion of 3540 nucleotides (+774 to +4313 with reference to the *Rnf146* transcriptional start site). Approximately 1.2K nucleotides from *EY09040* remain at this site. The *Rnf146* mutant alleles were recombined with *FRT2A* for clonal analysis.

Other stocks: $Tnks^{19}$ (Wang *et al.* 2016c), $Tnks^{503}$ (Wang *et al.* 2016c), *C765-Gal4* (BDSC) (Brand and Perrimon 1993), *71B-Gal4* (BDSC) (Brand and Perrimon 1993), *UAS Axin-V5* (Yang *et al.* 2016), *BAC Axin-V5* integrated at the *VK30* site (PBac{y[+]-attP-9A}VK00030) (Gerlach *et al.* 2014), *UAS-AxinΔTBD-V5*, *UAS-AxinΔRGS-V5*, *UAS-AxinΔArm-V5*, *UAS-AxinΔPP2A-V5*, *UAS-AxinΔDIX-V5* were integrated at the *attP33* site (Tacchelly-Benites *et al.* 2018), *esg-Gal4 UAS-GFP* (Micchelli and Perrimon 2006), *fz3-RFP* (Olson *et al.* 2011), *FRT82B* $pygo^{S123}$ (Thompson *et al.* 2002); *hsFLP1* (Golic and Lindquist 1989), *FRT82B arm-lacZ* (Vincent *et al.* 1994) (provided by J. Treisman, Skirball Institute, New York, NY), and *FRT2A ubi-GFPnls (BDSC#5825)*.

MARCM lines: MARCM 82B: *hsflp, UAS-mCD8:GFP; tub-Gal4, FRT82B, tub-Gal80* (Li *et al.* 2013).

MARCM 2A: *y w hs-flp; tub-Gal4 UAS-mCD8::*GFP^{LL5}*/CyO act-*GFP^{JMR1}*; FRT2A tub-*$Gal80^{LL9I}$ (Jiang and Reichert 2012).

Canton-S flies were used as wild-type controls. All crosses were performed at 25° unless otherwise indicated.

Clonal analysis

Mitotic mutant clones were generated by *FLP*-mediated recombination (Xu and Rubin 1993) in the larval wing imaginal

discs using *hsFLP1*. Clones were induced by subjecting first and second instar larvae to a 37° heat shock for 2 hr, and were detected by the loss of expression of an *arm-lacZ* or ubi-GFP transgene in third-instar larval wing imaginal discs.

Genotypes for generating mitotic clones in the wing were as follows:

Tnks mutant wing disc clones expressing *Axin-V5* or *Axin-V5* deletion mutants with the *71B* driver: *hsFLP1/+; UASAxin-V5/+; FRT82B Tnks^{19}/71B-Gal4 FRT82B arm-lacZ*.

Rnf146 mutant wing disc clones expressing *Axin-V5* or *Axin-V5* deletion mutants with the *C765* driver: *hsFLP1/+*; *UAS-Axin-V5/+*; *Rnf146 FRT2A*, *C765-Gal4/ubi-GFP FRT2A*.

Mitotic clones were generated in the intestine using the MARCM system (Lee and Luo 2001). For analysis of *fz3-RFP* expression, clones were induced in third-instar larvae by a single 2 hr heat shock and examined at 1–2 days after eclosion, as *fz3-RFP* expression becomes less homogenous with age.

Antibodies

The primary antibodies used for immunostaining were mouse anti-V5 (1:5000; Invitrogen), mouse anti-Wingless (1:200, 4D4 concentrated antibody; Developmental Studies Hybridoma Bank, DSHB), guinea pig anti-Senseless (1:1000) (Nolo *et al.* 2000), rabbit anti-β-gal (1:1000; MP Biomedicals), mouse anti-pADPr (1:1000, clone 10H; Tulip Biolabs), rabbit anti-GFP (1:200; Invitrogen). The primary antibodies used for immunoblotting were guinea pig anti-Axin (1:1000) (Wang *et al.* 2016c), guinea pig anti-Tnks (1:1000) (Wang *et al.* 2016c), rabbit anti-Kinesin Heavy Chain (1:10,000; Cytoskeleton), rabbit anti-Gluthathione-S-Transferase (1:10,000; Invitrogen) and rabbit anti-pADPr 96-10 (1:10,000) (Affar *et al.* 1999).

The secondary antibodies used for immunostaining were goat or donkey Alexa Fluor 488 or 555 conjugates (1:400; Invitrogen). The secondary antibodies used for immunoblotting were: goat anti-rabbit HRP conjugate (1:10,000; Bio-Rad), goat anti-mouse HRP conjugate (1:10,000; Bio-Rad), and goat anti-guinea pig HRP conjugate (1:10,000; Jackson ImmunoResearch).

Immunostaining and immunoblotting

For immunostaining, third-instar larval wing imaginal discs were dissected in PBS, fixed in 4% paraformaldehyde in PBS for 20 min, and washed with PBS with 0.1% Triton X-100, followed by incubation in PBS with 0.5% Triton X-100 and 10% BSA for 1 hr at room temperature. Incubation with primary antibodies was performed at 4° overnight in PBS with 0.5% Triton X-100. Incubation with secondary antibodies was for 2 hr at room temperature. Fluorescent images were obtained on a Nikon A1RSi confocal microscope and processed using Adobe Photoshop software.

For larval lysates, third-instar larvae were dissected to remove salivary glands, fat body, and gut tissues in cold PBS. After removal of PBS, 4× Laemmli loading buffer supplemented with 1 M DTT was added and the lysates were vortexed briefly. For lysates from embryos, embryos were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1% NP-40, 10% glycerol, 1.5 mM EDTA (pH 8.0)], supplemented with phosphatase and protease inhibitor cocktail (1:100; Thermo Scientific) and 1 μM of the poly (ADP-ribose) glycohydrolase inhibitor ADP-HPD (Enzo Life Sciences). All lysates were incubated for 5 min at 100° before SDS-PAGE analysis. For detection of pADPr in immunoblots: third-instar larvae of the indicated phenotypes were dissected in cold 1× PBS supplemented with protease inhibitor cocktail ProteaseArrest (1:100; GBiosciences) and 1 μM of the poly(ADP-ribose) glycohydrolase inhibitor ADP-HPD (Enzo Life Sciences). Tissues were lysed as described above and samples were resolved by SDS-PAGE using 4–15% gradient gels (Bio-Rad). Quantification of immunoblots was performed with ImageJ (Wayne Rasband, National Institutes of Health).

Quantification and statistics

Progenitor cells were identified by *esg* > *GFP*. For quantification, the number of total progenitor cells was counted in a field of 0.032 mm^2 within the R5a midgut region (Buchon *et al.* 2013). For quantification of pH 3-positive cells, the total number of pH 3$^+$ cells in the posterior midgut of the indicated genotypes was counted. All *t*-tests were performed using Prism (GraphPad Software).

Data availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article and figures. Supplemental material available at Figshare: https://doi.org/10.25386/genetics.7523393.

Results

Drosophila Rnf146/Iduna targets Tnks substrates for degradation in vivo

In vitro studies have revealed that human RNF146/Iduna, a pADPr-dependent RING-domain E3 ubiquitin ligase, promotes Wnt signaling in some cultured cell lines by targeting Axin for degradation, whereas in colonic carcinoma cells, RNF146 is dispensable for this process (Callow *et al.* 2011; Zhang *et al.* 2011). We sought to determine whether RNF146 is essential for Wnt signaling in distinct *in vivo* contexts. We hypothesized that, if the ability of Tnks to promote Wingless signaling is fully dependent on RNF146, the *in vivo* requirements for Rnf146 would be similar to those of Tnks. The single *Drosophila* Rnf146 homolog shares sequence homology with mammalian RNF146 in their two major domains: the RING domain, which is predicted to interact with an E2 ubiquitin conjugating enzyme, and the poly-ADP-ribose (pADPr)-binding WWE domain (Figure 1A). Indeed, all amino acids within the human RNF146 WWE domain that

bind pADPr (Wang *et al.* 2012) are identical in *Drosophila* Rnf146 (Figure 1A).

To study Rnf146 function *in vivo*, we generated *Drosophila Rnf146* mutants by inducing imprecise excision of a transposon inserted within the first intron of the *Rnf146* gene. We isolated several deletions that eliminate most of the open reading frame, including $Rnf146^{36}$ and $Rnf146^{157}$ (Figure 1B). Like *Tnks* null mutants, *Rnf146* null mutants (lacking both maternal and zygotic *Rnf146*) are viable and display no overt external patterning defects.

We tested the hypothesis that proteins normally targeted for pADPr-directed degradation would accumulate after their ADP-ribosylation in *Rnf146* mutants using an antibody directed against pADPr in immunoblots. We found a marked increase in levels of proteins modified by pADPr in *Rnf146* mutant larval lysates, but that these levels were restored to wild-type levels by concomitant inactivation of *Rnf146* and *Tnks* in *Rnf146 Tnks* double null mutants (Figure 1C). These results support the conclusion that Tnks and Rnf146 function in the same pADPr-dependent proteolysis pathway in *Drosophila*. To determine whether the pADPr-dependent protein turnover mediated by Rnf146 is a cell autonomous function, we examined clones of *Rnf146* null mutant cells in third-instar larval imaginal discs by immunostaining with a pADPr antibody. We observed an increase in pADPr staining intensity that is confined to all *Rnf146* mutant cells in all discs examined and eliminated by concomitant inactivation of *Tnks* (Figure 1, D–I and Figure S1). These findings suggested that *Drosophila* Rnf146, like human RNF146, is a pADPr-directed ubiquitin ligase that promotes turnover of proteins ADP-ribosylated by Tnks, and that Rnf146 activity is cell autonomous and ubiquitous.

Rnf146 mediates the pADPr-directed proteolysis of Axin and Tnks in vivo

Given that human RNF146 regulates Wnt signaling in cultured human cells through Tnks-dependent Axin degradation, and that human RNF146 promotes the turnover of both Tnks and Axin, we sought to determine whether this activity is functionally conserved in *Drosophila* Rnf146. In comparison with wild type, Tnks levels are markedly increased in lysates from *Rnf146* mutant larvae and embryos (Figure 2A and Figure S2A). Furthermore, immunostaining of third-instar larval imaginal discs with a Tnks antibody revealed a cell autonomous increase in Tnks staining in clones of *Rnf146* null mutant cells (Figure2, C–E and Figure S2, B–D). These findings provide *in vivo* evidence that the essential role of RNF146 in Tnks degradation is evolutionarily conserved, demonstrate that this requirement for Rnf146 is cell-intrinsic, and indicate that Rnf146 destabilizes Tnks at multiple developmental stages.

To determine whether *Drosophila* Rnf146 also promotes the turnover of Axin *in vivo*, we examined lysates from *Rnf146* mutant larvae in immunoblots with an Axin antibody. By comparison with wild type, Axin levels are increased in *Rnf146* mutant larvae and embryos (Figure 2B and Figure S2A). The magnitude of this increase in Axin levels is comparable upon Tnks loss, Rnf146 loss, or concomitant loss of Tnks and Rnf146, as revealed in lysates from *Rnf146 Tnks* double mutant larvae (Figure 2B). These *in vivo* findings also support the conclusion that Tnks and Rnf146 function in the same pADPr-dependent degradation pathway. Importantly, Rnf146 targets Axin for degradation even prior to 3 hr of embryonic development (Figure S2A), when *wingless* expression is first detectable. Therefore, like Tnks, Rnf146 also targets Axin for proteolysis independently of Wingless pathway activation. To determine whether Rnf146 promotes Axin degradation in a cell autonomous manner, we generated *Rnf146* mutant clones in transgenic flies that express a V5-tagged Axin under the control of its endogenous promoter/enhancer (BAC Axin-V5) (Gerlach *et al.* 2014). As anticipated, Axin-V5 levels were increased in a cell-autonomous manner within *Rnf146* mutant clones (Figure 2, F–H). Together, these findings indicate that *Drosophila* Rnf146 is required for the turnover of both Tnks and Axin at multiple developmental stages, and that human and *Drosophila* Rnf146 share evolutionarily conserved roles in Tnks-dependent Axin destabilization.

We hypothesized that the Tnks and Axin that accumulates in *Rnf146* mutants would be ADP-ribosylated. To test this hypothesis, we performed pull down assays with the Trp-Trp-Glu (WWE) domain of Rnf146, which binds robustly to pADPr in Tnks substrates (Zhang *et al.* 2011; Wang *et al.* 2012). We previously performed three distinct controls that verified the specificity of the GST-WWE pull downs for detection of Tnks-dependent Axin ADP-ribosylation *in vivo*: Axin pull down was abolished in *Tnks* null mutants (Wang *et al.* 2016a; Yang *et al.* 2016), or when the Tnks binding domain within Axin was deleted (Yang *et al.* 2016), or by the GST-WWE^{R164A} negative control (Yang *et al.* 2016), in which an arginine-to-alanine substitution in the RNF146 WWE domain abolishes interaction with poly-ADP-ribose (Zhang *et al.* 2011). In lysates from *Rnf146* mutant larvae, ADP-ribosylated Tnks and Axin were pulled down by WWE, but not the WWE^{R163A} mutant control (Figure 2I). Mobility shifts in ADP-ribosylated Axin were small, consistent with previous findings that ADP-ribosylation does not alter the electrophoretic mobility of Axin (Huang *et al.* 2009; Zhang *et al.* 2011) (Figure 2I). To determine the extent to which ADP-ribosylated Axin accumulates in *Rnf146* mutants, we performed WWE pulldowns with lysates from wild-type *vs.* *Rnf146* mutant larvae. ADP-ribosylated Axin was not detected in wild-type larvae, indicating that ADP-ribosylated Axin is turned over rapidly *in vivo*. By comparison, ADP-ribosylated Axin levels were markedly increased in *Rnf146* mutants, despite the fact that elimination of Rnf146 resulted in only a moderate increase in total levels of Axin protein (Figure 2J). Taken together, these data provide evidence that like human RNF146, *Drosophila* Rnf146 targets both ADP-ribosylated Tnks and ADP-ribosylated Axin for degradation *in vivo*.

Rnf146 promotes Wingless signaling by buffering Axin activity

Tnks inactivation increases Axin levels by only twofold to threefold, which is below the threshold at which Axin levels

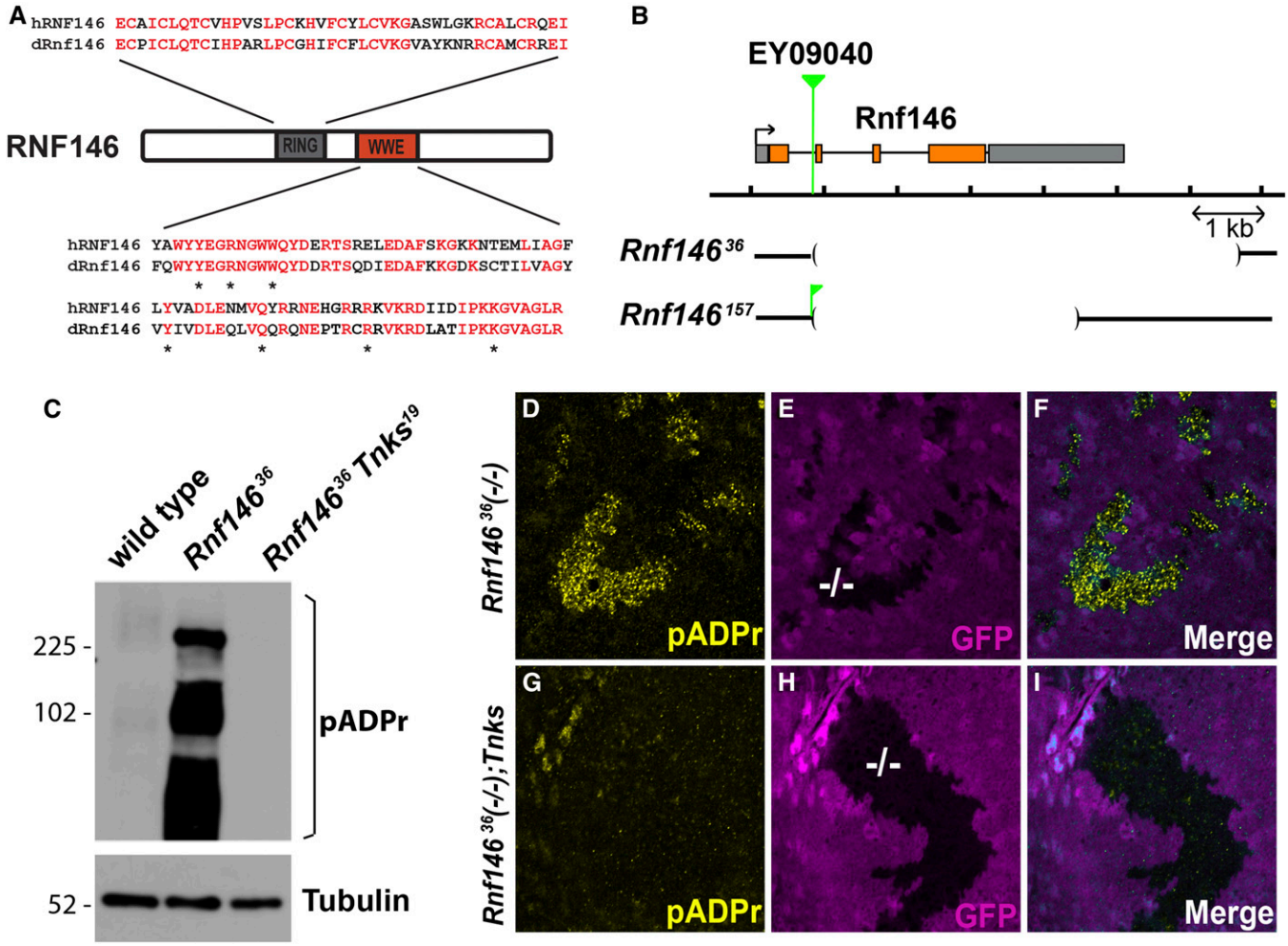

Figure 1 *Drosophila* Rnf146/Iduna targets Tnks substrates for degradation *in vivo*. (A) Schematic representation of the domain structure of RNF146. Residues in human RNF146 and the *Drosophila* homolog that are identical are shown in red. All residues in the human RNF146 WWE domain that bind poly-ADP-ribose are identical in *Drosophila* Rnf146 and indicated with asterisks. (B) Schematic representation of *Drosophila* Rnf146 genomic region and deletions in two *Rnf146* mutants. Insertion site of the P element *EY09040* is indicated (green). A fragment of the P element *EY09040* remains in the $Rnf146^{157}$ mutant. (C) Lysates from wild-type and *Rnf146* mutant larvae were analyzed by immunoblot using pADPr antibody. Tubulin was used as a loading control. The levels of poly-ADP-ribosylated proteins are increased in *Rnf146* null mutant third-instar larvae, but revert to baseline in *Rnf146 Tnks* double null mutants. (D–I) Third-instar larval wing imaginal discs with $Rnf146^{36}$ null mutant clones marked by the absence of GFP (−/−; magenta) were stained with indicated antibodies. Proteins modified by poly-ADP-ribose (D) accumulate cell autonomously in $Rnf146^{36}$ mutant clones but revert to baseline upon concomitant inactivation of Tnks ($Tnks^{19}$) (G–I). Blue is the merge of magenta and yellow.

inhibit activation of the Wingless pathway. However, an increase in Axin to levels that do not inhibit Wingless signaling in wild type are sufficient to inhibit Wingless-dependent cell fate specification in *Tnks* mutants, suggesting that Tnks serves to buffer Axin activity (Wang *et al.* 2016b). As our findings indicated that Tnks and Rnf146 likely function in the same proteolysis pathway, we postulated that Rnf146 would also promote Wingless signaling by maintaining Axin levels below this *in vivo* threshold. To test this hypothesis, we first expressed an *Axin-V5* transgene near physiological levels using the *71B-Gal4* driver, which drives expression in the dorsal and ventral regions of the pouch of third-instar larval wing imaginal discs (Wang *et al.* 2016b). As demonstrated previously, under these conditions, the expression of both *wingless* and the Wingless target gene *senseless* at the dorsoventral boundary of the third-instar larval wing imaginal discs are indistinguishable from wild type (Figure 3, A and B), as is patterning of the adult wing margin (Figure 3, C and D). However, under the same conditions, inactivation of *Rnf146* resulted in the loss of *senseless* expression and aberrant expansion of *wingless* expression at the dorsoventral boundary of larval wing discs, loss of sensory bristles at the adult wing margin, notched wings, and ectopic bristles within the wing blade (Figure 3, E–H, 100% penetrance, $n = 27$); each of these defects is indicative of the inhibition of Wingless signaling. These findings indicate that increases in Axin levels that are within the physiological threshold in wild-type flies inhibited Wingless signaling if Rnf146 activity is lost.

To determine if Rnf146 regulates Wingless signaling in other physiological contexts, we examined a different

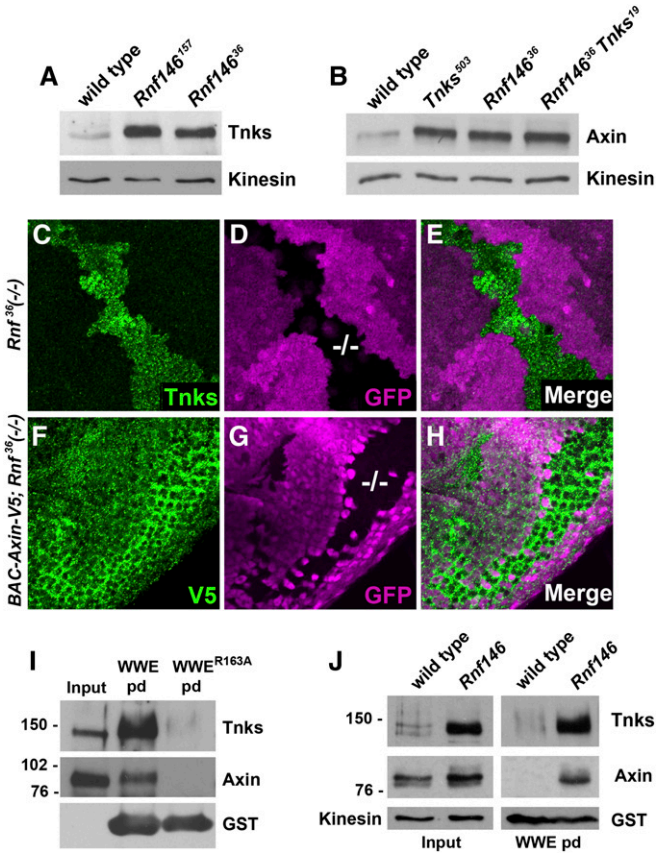

Figure 2 *Drosophila* Rnf146 mediates ADP-ribose-directed destabilization of Axin and Tnks *in vivo*. (A) Immunoblot of lysates from wild-type and *Rnf146* null mutant larvae probed with Tnks and Axin antibody. Tnks and Axin protein levels are increased in *Rnf146* mutant larvae. Kinesin was used as a loading control. (B) Immunoblot of lysates from wild type, *Tnks* null mutant, *Rnf146* null mutant, and *Rnf146 Tnks* double null mutant larvae probed with Axin antibody. Axin protein levels are increased in *Tnks* and *Rnf146* mutants, but not further increased in *Rnf146 Tnks* double mutants. Kinesin was used as a loading control. (C–E) Third-instar larval wing imaginal discs with $Rnf146^{36}$ null mutant clones marked by the absence of GFP (−/−; magenta) were stained with indicated antibodies. Tnks accumulates cell autonomously in $Rnf146^{36}$ mutant clones. (F–H) Eye imaginal discs from third-instar larvae expressing a *BAC Axin-V5* transgene with *Rnf146* null mutant clones were stained with indicated antibodies. *Rnf146* mutant clones were marked by the absence of GFP (−/−; magenta). (I) Detection of poly-ADP-ribosylated Tnks and ADP-ribosylated Axin using the GST-WWE pulldown assay. (J) poly-ADP-ribosylated Tnks and poly-ADP-ribosylated Axin accumulated in *Rnf146* null mutants.

developmental stage and tissue. As demonstrated previously, when an *Axin-V5* transgene is expressed in the pupal abdomen with the *71B-Gal4* driver, Wingless-dependent cell fate specification is indistinguishable from wild-type, as revealed by the size, morphology, spacing, and number of sternites in adults (Wang *et al.* 2016b) (Figure 3I). In contrast, under the same conditions, the abdomens of *Rnf146* mutant adults displayed a reduced number of sternites and sternal bristles, a decreased size in the sternites that remained, and expansion of the pleura (Figure 3J, 100% penetrance, $n = 27$). These phenotypes indicate the loss of Wingless signaling, as observed previously in *wingless* mutants, or upon inactivation of the Wingless pathway transcriptional activators dTCF/Pangolin and Legless/BCL9 (Baker 1988; Brunner *et al.* 1997; Kramps *et al.* 2002). Thus, as observed in the wing, increases in Axin transcription that are compatible with normal development in the wild-type abdomen inhibit Wingless-dependent developmental processes upon Rnf146 inactivation. These findings suggested that by targeting ADP-ribosylated Axin for degradation, Rnf146 serves to buffer Axin activity in multiple *in vivo* contexts.

An in vivo analysis of domains required for Tnks- and Rnf146-dependent degradation of Axin

To further test the hypothesis that Tnks and Rnf146 act in the same pathway to mediate Axin proteolysis, we performed a structure-function analysis to compare the domains required for Axin degradation by Tnks or Rnf146. We previously generated a series of *Axin* transgenes with deletions in domains required for Axin's interaction with other Wingless pathway components, including Tnks (*AxinΔTBD-V5*), the tumor suppressor Apc (*AxinΔRGS-V5*), the transcriptional activator Armadillo (*AxinΔArm-V5*), the phosphatase PP2A (*AxinΔPP2A-V5*), and the signalosome component Dishevelled (*AxinΔDIX-V5*) (Tacchelly-Benites *et al.* 2018). As demonstrated previously, immunostaining of wild-type Axin-V5 expressed in larval wing discs using the *71B-Gal4* driver, which drives expression in the dorsal and ventral wing pouch, revealed a marked increase in Axin-V5 levels in *Tnks* mutant clones (Figure 4, A–C) (Wang *et al.* 2016b), indicating that V5-tagged Axin is subject to Tnks-dependent degradation *in vivo*. A similarly marked increase in Axin-V5 levels was present in *Rnf146* null mutant clones (Figure 4, G–I). Consistent with previous work (Wang *et al.* 2016b), deletion of the TBD, but not the RGS domain, abolished the ability of Tnks to promote Axin degradation (Figure 4, D–F and Figure S3, A–C). Similarly, deletion of the TBD, but not the RGS domain, also abolished the ability of Rnf146 to promote Axin degradation (Figure 4, J–L and Figure S4, A–C). Together, these *in vivo* results support that hypothesis that Tnks and Rnf146 act in the same Axin proteolysis pathway.

Similar to Axin-V5, the levels of AxinΔArm-V5 were increased in both *Tnks* mutant and *Rnf146* mutant wing disc clones (Figures S3, D–F and S4, D–F), indicating that the Arm-binding domain is dispensable for Tnks-dependent Axin degradation. Analysis of AxinΔPP2A-V5 and AxinΔDIX-V5 in the same assay revealed some disruption of Axin regulation by Tnks or Rnf146 in the majority of wing disc clones. In many cells, AxinΔPP2A-V5 and AxinΔDIX-V5 were present at the same levels in *Tnks* null mutant clones or *Rnf146* null mutant clones *vs.* in the neighboring cells outside these clones, suggesting loss of Axin destabilization in most clones (Figures S3, G–I and M–O and S4, G–I and M–O); however, we observed that, occasionally, the levels of AxinΔPP2A-V5 and AxinΔDIX-V5 were increased within some cells in *Tnks* mutant or *Rnf146* mutant wing disc clones, suggesting that Tnks- and Rnf146-mediated degradation remained at least

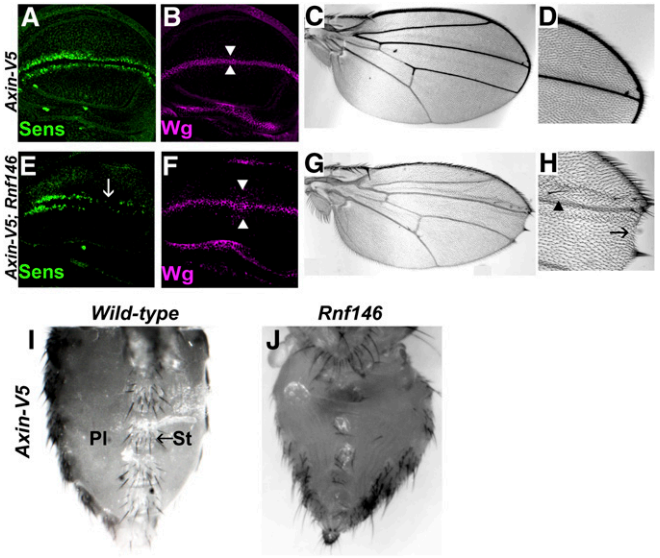

Figure 3 Rnf146 prevents the accumulation of excess Axin levels and promotes Wingless signaling. (A and B) Confocal images of third-instar larval wing imaginal discs expressing *Axin-V5* with the *71B-Gal4* driver, which drives expression in the dorsal and ventral regions of the pouch of third-instar larval wing imaginal discs. Expression of *Axin-V5* does not disrupt the Wingless target gene *senseless* (A), *wingless* expression (B) or cell fate in the adult wing (C and D). (E–H) Expression of *Axin-V5* in $Rnf146^{36}/Rnf146^{157}$ null mutants causes disruption of Senseless [(E), arrow] and a slight increase in the number of cells expressing Wingless [(F), arrowheads]. In the adult wing, expression of *Axin-V5* in *Rnf146* null mutants results in the loss of wing blade tissue (G), loss of sensory bristles at the wing margin [(H), arrow] and ectopic bristles in the wing blade [(H), arrowhead]; 100% of *Rnf146* mutants had wing margin defects, $n = 13$. (I) Ventral abdomen of wild-type adult female expressing *Axin-V5* with the *71B-Gal4* driver. Sternites and sternal bristles (St) marked by arrow; pleura (Pl). Some sternites and sternal bristles are lost in $Rnf146^{36}/Rnf146^{157}$ mutants (J), indicating that Rnf146 promotes Wingless-dependent cell fate specification.

partially intact (Figures S3, J–L and P–R and S4, J–L and P–R). Together, our results indicate that the same domains are required for Axin degradation by Tnks or Rnf146, with not only the Tnks-binding domain, but also the PP2A-binding and DIX domains important in this process. These results provide further *in vivo* evidence for the existence of a Tnks-Rnf46 degradation pathway.

Rnf146 is dispensable for Wingless-dependent regulation of ISC proliferation

Tnks is dispensable for the majority of Wingless-dependent developmental processes under standard laboratory conditions, but is essential for the Wingless-dependent control of ISC proliferation during homeostasis in the adult midgut (Wang *et al.* 2016c). In this physiological context, the role of Tnks in targeting Axin for degradation is essential to regulate Wingless signaling and thus ISC proliferation. To determine whether Rnf146 similarly regulates ISC proliferation during homeostasis, we compared the number of midgut progenitor cells, which we identified by their expression of the transcription factor *escargot* (*esg*) (Micchelli and Perrimon 2006), in controls *vs.* *Rnf146* mutants or *Tnks* mutants. In contrast with *Tnks* mutants, *Rnf146* mutants did not exhibit an increased number of progenitor cells during homeostasis at 14 days after eclosion (Figure 5A and Figure S5, A–C). To directly compare the proliferation rate of ISCs, we identified dividing ISCs with phospho-histone H3 (pH3)—a marker for mitosis. Quantification indicated that in contrast with *Tnks* mutants, no significant difference in the ISC proliferation rate existed between controls and homozygous *Rnf146* mutants (Figure 5B). To rule out genetic background or the accumulation of suppressors that could mask an increased ISC proliferation rate, we tested the *Rnf146* null mutants in *trans* to a deficiency that deletes the entire *Rnf146* locus. Analysis of these transheterozygotes also revealed no significant increase in the ISC proliferation rate (Figure 5B). Therefore, we conclude that, in contrast with Tnks, Rnf146 is not required to regulate ISC proliferation during homeostasis in the adult midgut, suggesting that Rnf146 is dispensable for the regulation of Axin and Wingless signaling in this context.

To further test this conclusion, we analyzed the effects of Rnf146 loss on the activation of Wingless target gene expression in the adult midgut. In the adult intestine, Wingless pathway activity peaks at major compartment boundaries, decreases as a function of distance from these boundaries, and is present at a low levels within compartments (Buchon *et al.* 2013; Tian *et al.* 2016). Previous work revealed that during adult midgut homeostasis, Tnks is critical for the activation of Wingless target gene expression in compartments, where Wingless pathway activity is relatively low, but dispensable for pathway activation at the compartment boundaries, where Wingless pathway activity is high (Wang *et al.* 2016c) (Figure 5, A–C). We sought to determine if Rnf146 similarly promotes Wingless target gene activation in the adult midgut by examining the expression of *fz3-RFP*, a Wingless target gene reporter (Sato *et al.* 1999; Sivasankaran *et al.* 2000). As reported previously, the expression of *fz3-RFP* in differentiated enterocytes (ECs) requires Wingless signaling (Tian *et al.* 2016; Wang *et al.* 2016c), and is completely lost upon inactivation of the essential Wingless signaling transcription cofactor Pygopus (Pygo) (Kramps *et al.* 2002; Parker *et al.* 2002; Thompson *et al.* 2002; Tian *et al.* 2016; Wang *et al.* 2016c) (Figure 5, F–H). However, *fz3-RFP* expression was unchanged in *Rnf146* mutant clones, even within the compartment where Wingless signaling activity is relatively low (Figure 5, I–K). Taken together, these findings indicate that, in contrast with Tnks, Rnf146 is dispensable for regulating Wingless signaling and ISC proliferation in the adult midgut during homeostasis.

Discussion

In principle, as the pADPr-directed E3 ligase RNF146/Iduna is known to target Axin for TNKS-mediated proteasomal degradation in mammalian cells, it could provide an effective therapeutic target to inhibit Wnt signaling (Huang *et al.* 2009; Callow *et al.* 2011; Zhang *et al.* 2011). However, previous

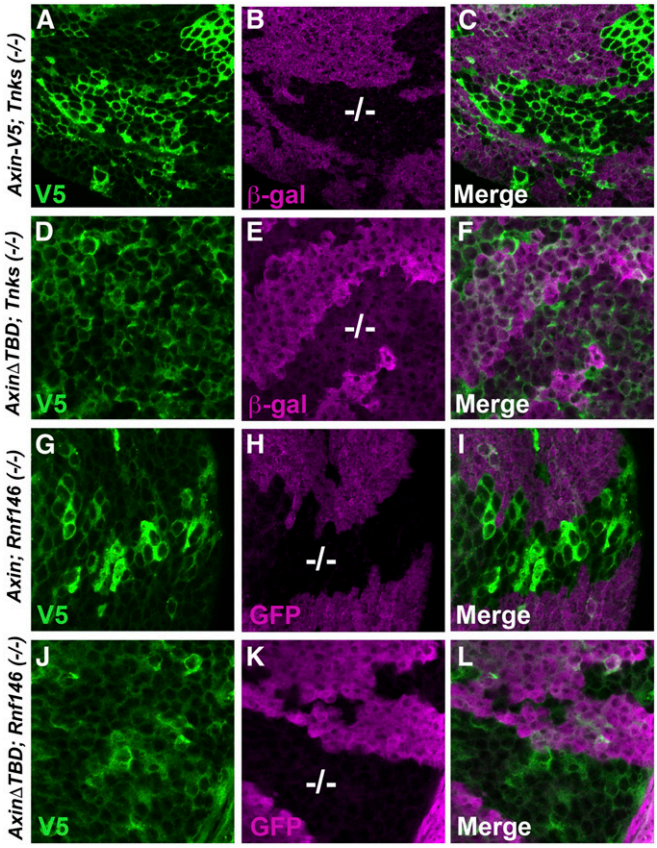

Figure 4 The Tankyrase binding domain in Axin is required for Tnks- and Rnf146-dependent Axin degradation. Confocal images of third-instar larval wing imaginal discs expressing indicated Axin transgenes with the *71B-Gal4* driver, which drives expression in the dorsal and ventral regions of the pouch of third-instar larval wing imaginal discs. Axin-V5 or an Axin deletion mutant that lacks the Tankyrase binding domain (AxinΔTBD-V5) were stained with V5 antibody (green). *Tnks* mutant clones were marked by the lack of β-gal staining (−/−; magenta), and *Rnf146* mutant clones were marked by the lack of GFP staining (−/−; magenta). The levels of wild-type Axin are increased in *Tnks* mutant clones (A–C) and in *Rnf146* mutant clones (G–I) whereas the levels of AxinΔTBD are indistinguishable inside and outside both *Tnks* mutant clones (D–F) and *Rnf146* mutant clones (J–L).

studies revealed that RNF146 is essential for this function in only some cultured cell lines, whereas in others, including colorectal cancer cells with truncations in APC, RNF146 is dispensable for Axin destabilization and Wnt signaling. Therefore, the extent to which RNF146 is required to regulate Wnt-dependent processes *in vivo* has remained unclear, as is the extent to which RNF146 could provide a viable therapeutic target for Wnt-driven diseases. Herein, we discovered that RNF146/Iduna function is conserved in *Drosophila*, and we evaluated the extent to which RNF146 is required for TNKS-dependent Wingless pathway regulation *in vivo*. Following the isolation of null mutants, we provide multiple lines of *in vivo* evidence that *Drosophila* Rnf146/Iduna mediates the pADPr-directed proteolysis of Tnks substrates. We demonstrate by both immunostaining and immunoblotting of null mutant larval imaginal discs that the level of pADPr-modified proteins is markedly increased *in vivo* following Rnf146 inactivation, but reverts to baseline upon concomitant inactivation of Tnks. These findings suggest that most, if not all Rnf146 substrates in larval imaginal discs require poly-ADP-ribosylation that is catalyzed specifically by Tnks, rather than by other ADP-ribose polymerases.

Our findings demonstrate that, as in cultured mammalian cells, *Drosophila* Axin and Tnks both serve as Rnf146 substrates *in vivo*. We show that following Rnf146 inactivation in larvae, both Axin and Tnks levels increase *in vivo*, with even larger relative increases in the levels of ADP-ribosylated Axin and ADP-ribosylated Tnks. Like Tnks, Rnf146 promotes Axin degradation ubiquitously and prior to the initiation of Wingless signaling, suggesting that Tnks and Rnf146 participate in a constitutive pathway for Axin proteolysis that acts under basal conditions but is necessary to promote signaling following Wingless stimulation. Our findings demonstrate that the same domains in Axin are required for Tnks- and Rnf146-dependent degradation, providing additional *in vivo* evidence supporting the conclusion that Tnks and Rnf146 function in the same pADPr-dependent pathway for Axin proteolysis. Although the ADP-ribosylation sites and the pADPr-dependent ubiquitination sites that target Axin for degradation are not yet known, our findings indicate that these sites are likely to be found in the domains that we identified herein as essential for Tnks- and Rnf146-mediated Axin degradation.

Our discovery that the function of mammalian RNF146 in Axin degradation is evolutionary conserved in *Drosophila* allowed us to investigate whether Rnf146 promotes Wingless signaling *in vivo*, and whether Tnks and Rnf146 possess identical roles in Wingless-dependent processes. We achieved this by comparing the consequences of their inactivation *in vivo* in multiple physiological contexts, and by determining whether there exist differences that may underlie their disparate requirements in Axin degradation *vs.* Wnt signaling in cultured mammalian cells. The threshold at which Axin levels inhibit Wingless signaling is between threefold and ninefold above the endogenous Axin level in wing discs (Peterson-Nedry *et al.* 2008; Wang *et al.* 2016b) and approximately fourfold to eightfold in embryos (Yang *et al.* 2016; Schaefer *et al.* 2018). Like inactivation of Tnks (Feng *et al.* 2014; Wang *et al.* 2016b), inactivation of Rnf146 has no overt effects on Wingless-dependent patterning of imaginal discs or the expression of Wingless target genes in larval wing discs at endogenous Axin levels. Herein, we demonstrate that an increase in Axin to levels that are still compatible with normal development in wild-type flies inhibit Wingless signaling in *Rnf146* null mutants. These Wingless signaling defects in cell fate specification in larval wing imaginal discs and the pupal abdomen in *Rnf146* mutants phenocopy *Tnks* mutants. Thus, Rnf146 is essential for the role of Tnks in preventing Axin accumulation above a threshold level, and, thereby, serves to buffer the negative regulation of Wingless signaling by Axin in several physiological contexts.

However, in one physiological context, the consequences of Tnks inactivation differ markedly from that of Rnf146

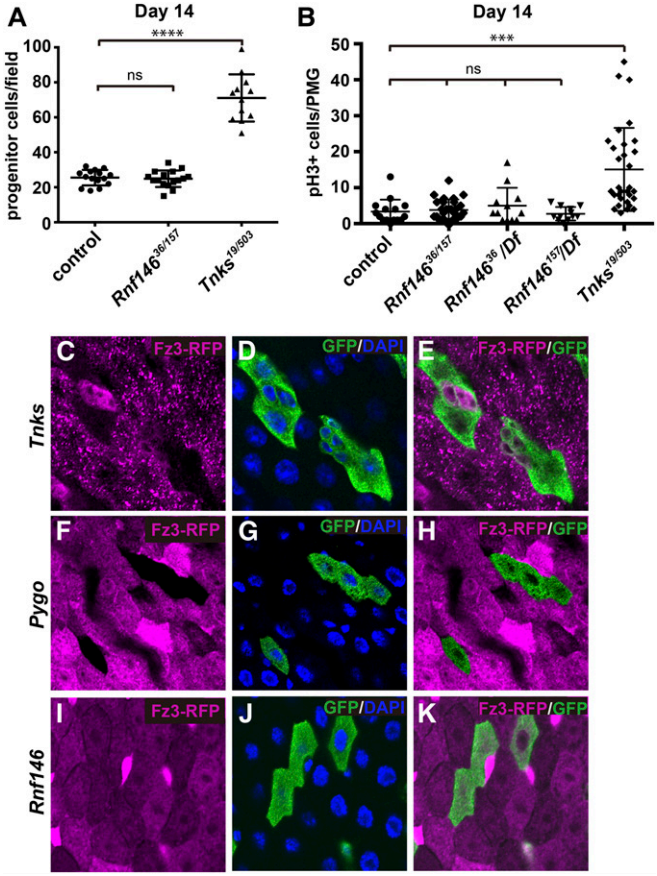

Figure 5 Rnf146 is dispensable for Wingless-dependent regulation of ISC proliferation and Wingless target gene activation in the adult midgut. (A) Quantification of the relative number of progenitor cells in wild-type (n = 15), *Rnf146* mutants (n = 16), or *Tnks* mutants (n = 12) at 14 days posteclosion. Values are presented as mean ± SD **** $P < 0.0001$ (t-test), ns: nonsignificant. (B) Quantification of pH 3-positive cells in posterior midguts (PMG) of wild-type, *Rnf146* null mutants, or *Tnks* null mutants at 14 days post-eclosion. Values are presented as mean ± SD *** $P < 0.001$, (t-test), ns: nonsignificant. Numbers of flies examined for each genotype (from left to right) are 15, 27, 12, 12 and 31 respectively. (C–E) *fz3-RFP* expression in ECs is dependent on Wingless signaling activation. Fz3-RFP (magenta) is absent in *Tnks*^{19} null mutant clones that are at a distance from the midgut–hindgut boundary (marked by GFP). (F–H) Fz3-RFP (magenta) is absent in all *pygo*^{S123} null mutant clones (marked by GFP). (I–K) Rnf146 is dispensable for *fz3-RFP* expression in all regions examined, regardless of distance from the compartment boundary. Mutant clones (marked by GFP) were induced in third-instar larvae and examined 1–2 days after eclosion.

inactivation. During homeostasis in the adult intestine, the regulation of Axin by Tnks is essential for the Wingless-dependent control of ISC proliferation in the epithelium (Wang *et al.* 2016c). In this context, Tnks is essential for the expression of Wingless target genes in the midgut compartments, where Wingless pathway activity is relatively low, but dispensable at the compartment boundaries, where Wingless pathway activity is high. Tnks inactivation results in phenotypes that are also observed upon inactivation of positive regulatory Wingless pathway components, and results in ISC overproliferation driven by the nonautonomous activation of JAK/STAT signaling in the epithelium. Importantly, reduction in the gene dosage of *Axin* by one-half suppresses this overproliferation phenotype, providing evidence that aberrantly increased Axin levels underlie the deregulation of ISC proliferation in *Tnks* mutants. In sharp contrast with Tnks loss, inactivation of Rnf146 neither inhibits the transcriptional activation of a Wingless target gene nor results in increased ISC proliferation. Together, these findings indicate that in many, but not all, physiological contexts, Rnf146 serves as an essential E3 ubiquitin ligase for Tnks-mediated Axin proteolysis and thus for promoting Wingless signaling.

These results, together with previous studies in cultured mammalian cell lines, suggest that Rnf146 is dispensable for Tnks-dependent regulation of Wnt signaling in both *Drosophila* and mammalian intestinal cells. Our findings provide additional evidence to support the hypothesis that in specific cell types, functionally redundant pADPr-dependent E3 ligase(s) promote Tnks-dependent Axin proteolysis not only in mammalian cells (Callow *et al.* 2011), but also in *Drosophila* cells. Alternatively, other mechanisms may promote Axin degradation to compensate for the loss of Rnf146. If similar modes of Axin regulation exist in human cells, our results suggest that inhibition of RNF146 may indeed suffice to attenuate the aberrant activation of Wnt signaling in certain Wnt-driven disease contexts but not others, and that differences will likely be determined by the existence of underlying compensatory mechanisms for Axin proteolysis in specific cell types.

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