Yan, an ETS-domain transcription factor, negatively modulates the Wingless pathway in the *Drosophila* eye

Emily R. Olson, Raluca Pancratov*, Sujash S. Chatterjee*, Binita Changkakoty, Zeeshan Pervaiz
& Ramanuj DasGupta⁺

Cancer Institute and Department of Pharmacology, New York University School of Medicine, New York, USA

We report the identification of *yan*, an ETS-domain transcription factor belonging to the *Drosophila* epidermal growth factor receptor (DER) pathway, as an antagonist of the Wingless signalling pathway. We demonstrate that cells lacking *yan* function in the *Drosophila* eye show increased Wingless pathway activity, and inhibition of Wingless signalling in *yan*^{-/-} cells rescues the *yan* mutant phenotype. Biochemical analysis shows that Yan physically associates with Armadillo, a crucial effector of the Wingless pathway, thereby suggesting a direct regulatory mechanism. We conclude that *yan* represents a new and unsuspected molecular link between the Wingless and DER pathways.

Keywords: crosstalk; RNAi; Wingless; Wnt; Yan

EMBO reports (2011) 12, 1047–1054. doi:10.1038/embor.2011.159

INTRODUCTION

Only a few evolutionarily conserved signal-transduction pathways are used during metazoan development, and it is thought that signals are integrated at specific nodes of ‘crosstalk’. Proper orchestration of signalling pathways is essential for normal development, as dysregulation can result in developmental disorders and disease. Thus, the identification of such shared signalling nodes is of great interest.

The Wnt pathway is a highly conserved signalling cascade that is involved in several transcriptional and cellular responses (Michaelidis & Lie, 2008). In a resting cell, cytoplasmic Armadillo, the *Drosophila* orthologue of β -catenin, is phosphorylated by glycogen synthase kinase 3 β within the degradation complex that also includes Axin and antigen-presenting cells (Logan & Nusse, 2004). The phosphorylated Armadillo is targeted for ubiquitination and proteasome-mediated degradation. Stimulation by Wingless, the *Drosophila* Wnt1 orthologue, inhibits the degradation complex; stabilized Armadillo translocates into the nucleus, where, together with the lymphoid enhancer factor/T-cell factor (TCF)

family of transcription factors, it activates the expression of target genes (Miller *et al*, 1999; Staal & Clevers, 2000). In addition to the core components of the pathway, there are several examples of cross-regulatory influences on Wnt pathway activity by members of other signalling pathways, including the Hedgehog, Notch and platelet-derived growth factor pathways (Dasgupta, 2009).

In a whole-genome RNA interference (RNAi) screen to identify new modulators of the Wingless signalling pathway, we identified *yan* as a candidate negative regulator of the Wingless-responsive luciferase reporter, dTF12 (DasGupta *et al*, 2005). Yan encodes an ETS-domain transcription factor and is a core member of the *Drosophila* epidermal growth factor receptor (DER) signalling pathway (Rebay & Rubin, 1995). DER belongs to the receptor tyrosine kinase (RTK) family of receptors that use the mitogen-activated protein kinase pathway for signal transduction (Domínguez *et al*, 1998; Spencer *et al*, 1998). Activation of DER results in the phosphorylation of extracellular signal-regulated protein kinase (mitogen-activated protein kinase), which translocates into the nucleus and modulates the activity of its targets *yan* and *pnt* (O’Neill *et al*, 1994). Whereas *pnt* is an activator of DER target genes, *yan* is an inhibitor. Phosphorylation of Yan by phospho-extracellular signal-regulated protein kinase results in its nuclear export and rapid degradation; this allows phosphorylated Pnt to activate target-gene transcription.

In this report, we demonstrate that in addition to its role in repressing DER targets, *yan* negatively regulates Wingless signalling activity in the *Drosophila* eye. We also investigate molecular mechanisms that might underlie this new cross-regulatory interaction.

RESULTS AND DISCUSSION

Yan misexpression inhibits Wingless pathway activity

We first examined the effects of *yan* on the fly-optimized Wingless-responsive TOPFlash reporter, dTF12. Knockdown of *yan* in *Drosophila* S2R+ cells increased the activity of dTF12 by approximately 2.5-fold only in Wingless-induced cells (Fig 1A), which suggested that *yan* does not influence the steady-state expression levels of endogenous Armadillo. Conversely, co-transfection of *wingless* complementary DNA (cDNA) with increasing amounts of a construct encoding a non-degradable form of *yan*, Yan^{ACT}, resulted in dose-dependent repression of

Cancer Institute and Department of Pharmacology, New York University School of Medicine, 522 First Avenue, Smilow Research Building, Room 1211, New York, New York 10016, USA

*These authors contributed equally to this work

⁺Corresponding author. Tel: +1 212 263 9247; Fax: +1 212 263 9210;

E-mail: ramanuj.dasgupta@nyumc.org

Received 13 January 2011; revised 7 July 2011; accepted 8 July 2011;
published online 26 August 2011

Fig 1 | Yan misexpression reduces Wingless pathway activity. (A) Double-stranded-RNA-mediated knockdown of *yan* in S2R+ cells activates the Wingless-responsive dTF12 luciferase reporter in the presence, but not in the absence, of Wingless induction, as compared with *gfp* knockdown (control). (B) Increasing amounts of *yan^{ACT}* cDNA results in a dose-dependent reduction of dTF12 on co-transfection with *wingless* cDNA. Error bars in panels A and B represent average variation in normalized luciferase reporter activity within four replica data points for each described condition. (C–E) The C96–GAL4 driver is specific to the wing margin (demonstrated by UAS–GFP) and alone does not affect Senseless expression (C–C'). Misexpression of UAS–Yan^{ACT} by C96–GAL4 (C96>Yan^{ACT}; E) results in reduction or complete loss of Senseless expression, similar to what is observed on C96–GAL4 misexpression of UAS–Axin (C96>Axin; D), a known negative regulator of the Wingless pathway. (F–J) In the embryo at stages 10 and 11, paired (*prd*)–GAL4 leads to expression of UAS–Axin–GFP in alternating segments (GFP expression; G–G'), resulting in a downregulation of Wingless-dependent Engrailed expression (G–G', white arrowheads) as compared with wild-type (F). Prd>Yan^{ACT} results in downregulation of Engrailed (H, white arrowheads), and coexpression of UAS–Arm* with UAS–Yan^{ACT} (prd>Arm*; Yan^{ACT}; I) restored Engrailed expression. Prd–GAL4 expression of dominant-negative DER (DER-DN; J) has no effect on En expression. (C–J) scale bar, 50 μ M. Arm, Armadillo; cDNA, complementary DNA; DER, *Drosophila* epidermal growth factor receptor; En, Engrailed; GFP, green fluorescent protein; RLU, relative luciferase units; Sens, senseless; UAS, upstream activating sequence; Wg, Wingless; WT, wild type.

dTF12 (Fig 1B). We used Yan^{ACT} because wild-type Yan often shows no effect when it is overexpressed (Rebay & Rubin, 1995). GAL4–upstream activating sequence (UAS) misexpression (Brand & Perrimon, 1993) of Yan^{ACT} had similar inhibitory effects on *in vivo* readouts of Wingless pathway activity. The Wingless target gene *senseless* was reduced in response to misexpression of Yan^{ACT} at the presumptive wing margin, and adult wings showed loss of sensory bristles and wing notching, similarly to in response to misexpression of UAS–Axin (Fig 1C–E, data not shown). In the

embryo, Wingless-dependent expression of *engrailed* (Vincent & Lawrence, 1994) was also reduced on misexpression of Yan^{ACT}, similarly to in response to misexpression of UAS–Axin (Fig 1F–H). Notably, Engrailed expression was restored on coexpression of UAS–Armadillo* with Yan^{ACT} (Fig 1I; supplementary Fig S1E–G online). Conversely, RNAi-mediated knockdown of *yan* resulted in expansion of Engrailed expression (supplementary Fig S1H, I' online), similarly to the activation of Wingless signalling on expression of Armadillo* alone (supplementary Fig S1J, K' online).

Changes in Wingless signalling activity, as measured by changes in Engrailed expression, were consistent with the secreted cuticle patterns, as overexpression of Yan led to phenotypes consistent with loss of Wingless signalling activity and vice versa (supplementary Fig S2 online). Notably, misexpression of a dominant-negative DER allele, DER-DN, had no effect on Engrailed expression or cuticle patterning (Fig 1J, data not shown), suggesting that the genetic interaction observed between Yan and the Wingless pathway is specific and independent of its function in the DER pathway. Taken together, these data indicated that Yan is able to antagonize Wingless pathway activity *in vivo*.

Loss of *yan* resembles gain of Wingless activity

We then performed loss-of-function analyses to see whether the inhibitory activity of *yan* towards Wingless signalling was physiologically relevant. Although we observed loss of Wingless pathway activity in the wing on Yan misexpression, Yan protein is not normally expressed in the wing (supplementary Fig S1D–D' online). Therefore, we turned to the developing eye, in which the functions of the DER and Wingless pathways have been well characterized (Freeman, 1997; Heberlein & Treisman, 2000; Voas & Rebay, 2004). The Wingless signalling pathway specifies the head-cuticle fates and inhibits expression of eye-specific genes (Baonza & Freeman, 2002), whereas the DER pathway is essential for the recruitment and maintenance of photoreceptors and for exiting the cell cycle. In the eye imaginal disc, Yan protein is restricted to the leading edge of the morphogenetic furrow and colocalizes with the morphogenetic furrow marker, *dpp-lacZ* (supplementary Fig S1B–B' online), and only is weakly expressed posterior to the morphogenetic furrow, whereas *wingless* is expressed anterior to and abutting Yan expression at the lateral margins of the eye disc (supplementary Fig S1A–A' online).

We used the Flp–FRT system (Xu & Rubin, 1993) to generate *yan*^{−/−} clones in the eye. *Yan*^{−/−} clones resulted in inhibition of photoreceptor development and, in clones near the periphery of the eye, an expansion of naked cuticle lacking photoreceptors (Fig 2C). In 5–10% of the adult eyes, we also observed aberrant structures from *yan*^{−/−} clones, including tube-like overgrowths (Fig 2D–D'). Loss of photoreceptor differentiation in *yan*^{−/−} clones was previously reported, which indicated an early, cell-autonomous requirement of *yan* for neuronal fate (Rogge *et al*, 1995). Moreover, we were intrigued by the similarity between the adult phenotypes shown by the *yan*^{−/−} clones with those of *axin*^{−/−} clones or Flip-Out clones expressing Armadillo* (Baonza & Freeman, 2002; Fig 2B,C).

We also observed patches of necrotic tissue in adult *yan*^{−/−} clones (Fig 2C, white arrowhead). We speculated that the cell death might result from Wingless-induced apoptosis during pupal stages, which normally occurs to clear incomplete ommatidial clusters (Lin *et al*, 2004). However, expression of UAS–p35 in *yan*^{−/−} clones did not rescue the adult phenotype, nor did we observe an increase in caspase 3 staining in *yan*^{−/−} clones (data not shown), suggesting that the cell-death phenotype is probably independent of apoptosis.

Molecular characterization in the eye imaginal disc also showed that Dacshund, an early eye-determining gene, is significantly reduced in *yan*^{−/−} clones at and posterior to the morphogenetic furrow where *yan* is expressed (Fig 2F,G'). This is similar to the

Dacshund repression observed in *axin*^{−/−} clones (Baonza & Freeman, 2002; data not shown). We also observed a loss of the R8-specific photoreceptor marker, Senseless (Fig 2H–H'''), as well as partial loss of the neuronal marker Elav, similar to what is observed on ectopic Wingless pathway activation in *axin*^{−/−} clones (supplementary Fig S4E–F' online). We generated an *in vivo* Wingless-responsive reporter, namely 'Fz3–RFP', by cloning red fluorescent protein (RFP) downstream of a 2.3-kb promoter region of the endogenous Wingless target gene, *fz3* (Sato *et al*, 1999). Fz3–RFP recapitulates endogenous Wingless signalling activity (Treisman & Rubin, 1995) in the eye imaginal disc (Fig 2I–I'), as well as other larval and embryonic tissues, and is responsive to the gain and loss of Wingless pathway activity (supplementary Fig S3 online). Examination of *yan*^{−/−} clones showed ectopic expression of Fz3–RFP in clones generated at or near the lateral margin of the eye disc (Fig 2J–J'), which corresponds to the endogenous source of Wingless. Notably, we did not observe Fz3–RFP activity in *yan*^{−/−} clones in the medial domain of the eye disc, and this is consistent with our results using the *in vitro* dTF12 reporter, which is only activated on *yan* knockdown in the presence of Wingless induction (Fig 1). However, we cannot rule out the possibility that the responsiveness of the artificial reporter, as opposed to the endogenous gene target *dac*, varies depending on the location of the clones within imaginal discs. Note the region-specific variability of reporter expression in *axin*^{−/−} clones (supplementary Fig S3D–D' online). Nevertheless, our results from the phenotypic characterization and modulation of Wingless-target and reporter genes are consistent with ectopic activation of the Wingless pathway within *yan*^{−/−} clones.

Cells are apically constricted in *yan*^{−/−} clones

We also observed an apparent increase in Armadillo immunofluorescence within *yan*^{−/−} clones at and posterior to the morphogenetic furrow (Fig 2H–H'''), although we did not observe any changes in levels of *armadillo* messenger RNA (mRNA), Wingless or *wingless* mRNA within *yan*^{−/−} clones (data not shown). As Armadillo is a component of the adherens junction, we examined cell morphology in *yan*^{−/−} clones. In wild-type eye imaginal discs, cells within the morphogenetic furrow are in a lower plane and are apically constricted. Posterior to the morphogenetic furrow, cells relax and Armadillo localizes to evenly spaced 'rosettes' where it is incorporated into the adherens junction between the differentiating photoreceptors (Fig 3A–A'). In *yan*^{−/−} clones, cells remain apically constricted posterior to the morphogenetic furrow (Fig 3B–B'). Although *yan*^{−/−} clones are not rounded, they segregate away from neighbouring wild-type cells as they remain in the lower plane of the morphogenetic furrow (data not shown). We observe similar apical constriction in cells within *axin*^{−/−} clones in the eye (Fig 3C–C') and the wing (supplementary Fig S4A–A' online). Comparable observations have been reported for cells lacking antigen-presenting cell function in the larval optic lobe (Hayden *et al*, 2007), and we found that cells responding to endogenous Wingless signalling at the presumptive wing margin are also apically constricted (supplementary Fig S4C,D online). To ensure that apical constriction was not due to premature activation of DER signalling in *yan*^{−/−} clones, we also generated Flip-Out clones expressing an activated allele of DER, DER^{Δtop}. Although we observed the expected ectopic differentiation of photoreceptor clusters, apical

Fig 2 | Wingless signalling is activated in *yan*^{-/-} clones. (A–C) Generation of *yan*^{-/-}-null clones results in patches of necrotic tissue, naked-cuticle-lacking photoreceptors (white and black arrowheads, C) and tubular outgrowths (yellow arrowheads, D–D') in adult *Drosophila* eyes, as compared with wild type (A). Similar adult phenotypes were observed in *axin*^{-/-} clones (B). (E–H'') Within *yan*^{-/-} clones, the early eye-determining gene Dacshund is reduced (F–G'), the R8 photoreceptor marker Sens is lost and Armadillo immunofluorescence is increased (H–H'') posterior to the morphogenetic furrow (white arrowheads throughout). (I–J'') Fz3–RFP is an *in vivo* reporter and overlaps with endogenous Wingless expression (as monitored using *wg-lacZ*, I) at the lateral margins of the eye disc. Fz3–RFP is ectopically expanded in *yan*^{-/-} clones near the lateral margins (yellow arrowhead, J'–J''). Anterior is to the left. Arm, Armadillo; Dac, Dacshund; GFP, green fluorescent protein; PR, photoreceptor; RFP, red fluorescent protein; Sens, Senseless; Wg, Wingless; WT, wild type.

constriction was not evident in cells within the clones (Fig 3D–D'). We also generated *yan*^{-/-} mosaic analysis with a repressible cell marker (MARCM) clones (Lee & Luo, 2001) expressing *pnt* small-hairpin RNA and observed apical constriction and adult phenotypes similar to *yan*^{-/-} clones (supplementary Fig S4J–M online). Therefore, we concluded that apical constriction within *yan*^{-/-} clones was not a consequence of ectopic DER signalling, and is independent of *pnt* activity.

Inhibition of Armadillo activity can rescue *yan*^{-/-} clones

On the basis of the molecular characterization of the *yan*^{-/-} clones, we proposed that the *yan* mutant phenotype could be at

least partly due to increased Wingless signalling activity. Therefore, we sought to rescue the *yan*^{-/-} phenotype by inhibiting Armadillo protein and Wingless activity within *yan*^{-/-} clones. We generated *yan*^{-/-} MARCM clones that expressed either Axin or a dominant-negative form of TCF, dnTCF (van de Wetering *et al*, 1997), both of which resulted in significant rescue of apical constriction, accompanied by restoration of Armadillo protein staining in stereotypical rosettes (Fig 4A–C'). We also observed the reappearance of Elav expression, although in large clones the rescue observed with dnTCF was not as robust as that observed with Axin (supplementary Fig S4G–I' online). These results indicate that there is both an Armadillo-associated morphological

Fig 3 | Cells are apically constricted in *yan*^{-/-} clones and *axin*^{-/-} clones in the eye. (A–A'') Wild-type control eye imaginal disc shows increased Armadillo immunofluorescence within the morphogenetic furrow (white arrowheads); higher magnification (A'–A'') shows that cells in the morphogenetic furrow are apically constricted and Armadillo localizes to the cell membrane. Posterior to the morphogenetic furrow, cells relax and Armadillo localizes to 'rosettes' incorporated into the adherents junctions between differentiating photoreceptors (yellow arrowheads, A''). (B–B'') Cells within *yan*^{-/-} clones remain apically constricted several rows posterior to the morphogenetic furrow (white arrowheads throughout), similar to what we observe in *axin*^{-/-} clones (C–C''). Activation of the DER pathway on generation of FO clones expressing an activated form of DER (FO > DER^{Δtop}, D–D'') results in ectopic Armadillo-positive rosettes, but does not result in apical constriction. Anterior is to the left. Scale bar, 25 μm. Arm, Armadillo; DER, *Drosophila* epidermal growth factor receptor; FO, Flip-Out; GFP, green fluorescent protein; WT, wild type.

component and Armadillo signalling component to the *yan*^{-/-} phenotype. Nevertheless, both Axin and dnTCF were able to rescue the adult *yan*^{-/-} phenotype to a similar extent, as both resulted in a significant rescue of photoreceptor loss and cell death, and only approximately 6–7% of adults showed subtle ommatidial morphological defects (mild defects, Fig 4F–H). This was in comparison to control *yan*^{-/-} MARCM clones expressing UAS–green fluorescent protein (GFP) or sibling control flies in which 17–20% of adults showed the *yan* mutant phenotype characterized in Fig 1 (severe defects and death, Fig 4D,E,H). Taken together, the ability of Axin or dnTCF expression, and not *pnt* knockdown (supplementary Fig S4J–M online), to rescue

yan^{-/-} phenotype indicates that increased Wingless/Armadillo signalling activity within *yan*^{-/-} clones contributes to the *yan*^{-/-} phenotype.

Yan and Armadillo proteins physically interact

We speculated that our results from the *in vitro* and *in vivo* genetic interaction studies (Fig 1; supplementary Figs S1,S2 online) might indicate a physical interaction between Yan and Armadillo proteins as a direct mechanism for the antagonistic influence of Yan on Armadillo activity. Indeed, coimmunoprecipitation assays showed that both endogenous and transfected Yan protein coimmunoprecipitated with Armadillo protein in nuclear extracts

Fig 4 | Expression of negative regulators of the Wingless pathway suppresses the *yan* mutant phenotype in the *Drosophila* eye. (A–A') Cells with control *yan*^{−/−} MARCM clones expressing UAS–GFP are apically constricted posterior to the morphogenetic furrow, whereas *yan*^{−/−} MARCM clones expressing UAS–Axin (B–B') or UAS–dnTCF (C–C') show suppression of the apical constriction phenotype. A–C' represent Z-projection images of the eye imaginal disc; anterior is to the left; white arrowheads in A–C' denote morphogenetic furrow; yellow asterisks in A–A' and C–C' denote areas with GFP marked tones in the peripodial membrane of the eye disc, hence absence of apical constrictions; scale bars, 25 μm. (D–H) Approximately 17–20% of control MARCM adults with genotype *yan*^{−/−}; TM6B (D) or *yan*^{−/−}; and UAS–GFP (E) show the *yan*^{−/−} phenotype, which includes necrotic patches and naked cuticle lacking photoreceptors (white and black arrowheads E–D; severe defects and death in H). *yan*^{−/−} MARCM clones expressing UAS–Axin (F) or UAS–dnTCF (G) show significant suppression of the *yan*^{−/−} phenotype, as only approximately 6–7% of adult flies showed mild defects in ommatidial morphology, as compared with sibling controls (H). Arm, Armadillo; dnTCF, dominant-negative TCF allele; GFP, green fluorescent protein; MARCM, mosaic analysis with a repressible cell marker; TCF, T-cell factor; UAS, upstream activating sequence; Wg, Wingless; WT, wild type.

prepared from S2R+ cells transfected with *armadillo* or *wingless* cDNAs (Fig 5B; supplementary Fig S5C,D online). We also observed coimmunoprecipitation between endogenous Yan and Armadillo *in vivo* with crude nuclear extracts prepared from *Drosophila* embryos expressing UAS–Armadillo* under the control of *daughterless*–GAL4 (Fig 5C).

Intriguingly, western blot analysis of nuclear extracts from S2R+ cells also showed a mobility shift of endogenous Yan protein in the presence of high levels of Armadillo due to transient transfection of either *armadillo* cDNA or increasing amounts of *wingless* cDNA (Fig 5A). This might be indicative of a Wingless/Armadillo-dependent post-translational modification of Yan,

Fig 5 | Yan and Armadillo proteins physically interact. (A) Western blot analysis showing mobility shift in Yan protein (red arrows) in S2R+ cells in the presence of increased levels of Armadillo on transient transfection of *armadillo* cDNA or increasing amounts of *wingless* cDNA. (B,C) Endogenous Yan protein coimmunoprecipitates with Armadillo in both S2R+ cells transiently transfected with *arm* cDNA (B) or in embryos with Da-GAL4 expression of UAS-Arm* (C). Notably, we observe that both Yan moieties coimmunoprecipitate with Armadillo (red and black asterisks, see text). D, a suggested model for the dual role of Yan during retinal development in *Drosophila*. Arm, Armadillo; cDNA, complementary DNA; *da*, *daughterless*; IB, immunoblot; insol., insoluble fraction; IP, immunoprecipitation; UAS, upstream activating sequence.

although our coimmunoprecipitation results indicated that this modification is not necessary for Yans associated with Armadillo. Although we observed that the slower-migrating moiety of Yan (red asterisks in Fig 5B,C) more-efficiently coimmunoprecipitated with Armadillo, in cultured S2R+ cells, this was not exclusive, and our *in vivo* coimmunoprecipitation results showed immunoprecipitation of both moieties (supplementary Fig S5A online). This suggested that depending on the cellular context, Armadillo can preferentially, although not exclusively, associate with the faster-migrating moiety of Yan (Fig 5C). We are intrigued by the modification of Yan as a consequence of its interaction with Armadillo, and studies are underway to investigate the precise mechanism and functional consequences for the regulation of the Wingless pathway activity.

On the basis of our *in vivo* and *in vitro* characterization of the antagonistic interaction between Yan and the Wingless pathway, we propose that *yan* has a dual role at the moving anterior/posterior boundary defined by the morphogenetic furrow (Fig 5D). In addition to the previously defined function of Yan in inhibiting premature photoreceptor recruitment (O'Neill et al, 1994; Rogge

et al, 1995), we suggest that Yan blocks Wingless signalling at the morphogenetic furrow, probably by regulating the activity of Armadillo protein (Fig 5D). By maintaining low Wingless signalling activity, we propose that Yan maintains the competence of these cells to adopt a retinal fate. In the absence of Yan, Armadillo is free to either participate in adherens junctions, thereby causing the apical constriction phenotype, or interact with TCF in the nucleus to activate target genes. We speculate that in *yan*^{-/-} clones generated far from a source of Wingless, most of the liberated Armadillo that does not localize to adherens junctions is degraded and is unable to robustly signal in the nucleus. This could explain why we observe apical constriction in all *yan*^{-/-} clones, but ectopic Fz3-RFP activation only in *yan*^{-/-} clones near the lateral margins of the eye disc. Similarly, expression of Axin—which can strongly downregulate both the membrane-directed and signalling-competent pool of liberated Armadillo—within *yan*^{-/-} clones results in a stronger rescue of apical constriction than the expression of dnTCF, which only interferes with the signalling-competent pool of Armadillo. These data indicate that both the junctional and nuclear signalling functions of Armadillo probably contribute to the *yan*^{-/-} phenotype.

In conclusion, our study shows a new and unsuspected function for *yan* in the negative regulation of Wingless signalling. These results might be relevant to the understanding of the molecular regulation of Wnt-RTK signalling crosstalk in human disease. The closest human homologue of *yan*, encoded by *TEL/ETV6* is known to be associated with leukaemia, which can also result from uncontrolled activation of Wnt signalling (Peeters et al, 1997; Roman-Gomez et al, 2007). These observations underscore the importance of defining the molecular nature of the crosstalk between Wnts and RTKs in both normal development and disease.

METHODS

Luciferase reporter assays. Transfections in S2R+ cells were carried out in 96-well plates, as described previously (DasGupta et al, 2005), using 100 ng total DNA including dTF12 (TOPFlash), PolIII-Renilla Luciferase and pAct or pAct-Wingless and 250 ng Yan double stranded RNA (Drosophila RNAi Screening Center (DRSC) amplicons tested include DRSC00801, DRSC32788 and DRSC32787). Quantities of 25, 50, 75 and 100 ng pMT-Yan^{ACT} (Ilaria Rebay) were transfected with 50 ng pAct-Wingless and 25 ng each of dTF12, PolIII-Renilla Luciferase.

Drosophila stocks and genetics. The following cell lines were obtained from stock collection centre in Bloomington, IN: UAS: Axin.GFP (*Axin*), dTCF-ΔN-1 (*dnTCF*), Arm*^{S10}, FRT40A ubiGFP. UAS-Yan^{ACT}, *yan*⁴⁴³ FRT40A and *yan*⁸⁸⁴ FRT40A were gifts from Ilaria Rebay. Hsflp ubiGFP, FRT40A, Tub-GAL80, Tub-GAL4/TM6B and *wingless*-LacZ were obtained from Jessica Treisman; Fz3-RFP transgenic lines were generated by BestGene Inc.; and *axin*^{-/-} FRT82B from Nicholas Tolwinski. Flip-Out clones UAS-Armadillo and UAS-Axin females were crossed to actin>STOP>GAL4 UAS-GFP, hslpMKRS/TM6c males. Flipase was induced by heat shocking larvae for 48–72 h after egg laying, at 38 °C for 70 min.

Plasmids and constructs. Fz3-RFP construct: 2333 base-pair fragment was PCR-amplified (F primer: 5'-GGGGTACCGAACC GAAGAGTT-GGCAGAGAG-3', R primer: 5'-CTAGCTAGCTTAG

TGGGTTTCAGGAGG-3') from the *Drosophila melanogaster* genomic region upstream of *frizzled3* translation start, flanked by 5' *KpnI* and 3' *NheI* sites, followed by ligation into pH-Stinger Red (Barolo et al, 2004); fly transgenic lines were generated by germline injection. PAct control plasmid and pAct-Arm S10-myc (Pai et al, 1997) were used in transient transfections.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

We thank members of the DasGupta and Dr E. Bach labs for discussions and N. Tolwinski, J. Treisman, H. Bellen and I. Rebay for fly stocks and reagents. We thank the *Drosophila* RNAi Screening Center (DRSC; <http://www.flyrnai.org/>) and the New York University-RNAi Screening facility (<http://rnai.med.nyu.edu/>) where primary and validation screens were performed. This work was supported by March of Dimes research grant (#10-363) to R.D. and the National Institutes of Health Developmental Genetics training grant to E.R.O.

Author contributions: E.R.O. and R.D. designed and carried out cell culture and fly experiments with assistance from B.C., S.S.C., Z.P.; R.P. and R.D. designed and generated the Fz3-RFP reporter; R.P. and B.C. conducted experiments to functionally test Fz3-RFP *in vivo*; E.R.O. and R.D. wrote the manuscript and compiled figures; E.R.O., R.P., S.S.C. and R.D. edited and approved the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

- Baonza A, Freeman M (2002) Control of *Drosophila* eye specification by Wingless signalling. *Development* **129**: 5313–5322
- Barolo S, Castro B, Posakony JW (2004) New *Drosophila* transgenic reporters: insulated P-element vectors expressing fast-maturing RFP. *Biotechniques* **36**: 436–440, 442
- Brand A, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**: 401–405
- Dasgupta R (2009) Functional genomic approaches targeting the wnt signaling network. *Curr Drug Targets* **10**: 620–631
- DasGupta R, Kaykas A, Moon RT, Perrimon N (2005) Functional genomic analysis of the Wnt–wingless signaling pathway. *Science* **308**: 826–833
- Dominguez M, Wasserman JD, Freeman M (1998) Multiple functions of the EGF receptor in *Drosophila* eye development. *Curr Biol* **8**: 1039–1048
- Freeman M (1997) Cell determination strategies in the *Drosophila* eye. *Development* **124**: 261–270
- Hayden MA, Akong K, Peifer M (2007) Novel roles for APC family members and Wingless/Wnt signaling during *Drosophila* brain development. *Dev Biol* **305**: 358–376
- Heberlein U, Treisman JE (2000) Early retinal development in *Drosophila*. *Results Probl Cell Differ* **31**: 37–50
- Lee T, Luo L (2001) Mosaic analysis with a repressible cell marker (MARCM) for *Drosophila* neural development. *Trends Neurosci* **24**: 251–254
- Lin HV, Rogulja A, Cadigan KM (2004) Wingless eliminates ommatidia from the edge of the developing eye through activation of apoptosis. *Development* **131**: 2409–2418
- Logan CY, Nusse R (2004) The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* **20**: 781–810
- Michaelidis TM, Lie DC (2008) Wnt signaling and neural stem cells: caught in the Wnt web. *Cell Tissue Res* **331**: 193–210
- Miller JR, Hocking AM, Brown JD, Moon RT (1999) Mechanism and function of signal transduction by the Wnt/β-catenin and Wnt/Ca2+ pathways. *Oncogene* **18**: 7860–7872
- O'Neill EM, Rebay I, Tjian R, Rubin GM (1994) The activities of two Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway. *Cell* **78**: 137–147
- Pai LM, Orsulic S, Bejsovec A, Peifer M (1997) Negative regulation of Armadillo, a Wingless effector in *Drosophila*. *Development* **124**: 2255–2266
- Peeters P et al (1997) Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor-associated kinase JAK2 as a result of t(9;12) in a lymphoid and t(9;15;12) in a myeloid leukemia. *Blood* **90**: 2535–2540
- Rebay I, Rubin GM (1995) Yan functions as a general inhibitor of differentiation and is negatively regulated by activation of the Ras1/MAPK pathway. *Cell* **81**: 857–866
- Rogge R, Green PJ, Urano J, Horn-Saban S, Mlodzik M, Shilo BZ, Hartenstein V, Banerjee U (1995) The role of yan in mediating the choice between cell division and differentiation. *Development* **121**: 3947–3958
- Roman-Gomez J, Cordeu L, Agirre X, Jimenez-Velasco A, San Jose-Eneriz E, Garate L, Calasanz MJ, Heiniger A, Torres A, Prosper F (2007) Epigenetic regulation of Wnt-signaling pathway in acute lymphoblastic leukemia. *Blood* **109**: 3462–3469
- Sato A, Kojima T, Ui-Tei K, Miyata Y, Saigo K (1999) Dfrizzled-3, a new *Drosophila* Wnt receptor, acting as an attenuator of Wingless signaling in wingless hypomorphic mutants. *Development* **126**: 4421–4430
- Spencer SA, Powell PA, Miller DT, Cagan RL (1998) Regulation of EGF receptor signaling establishes pattern across the developing *Drosophila* retina. *Development* **125**: 4777–4790
- Staal FJ, Clevers H (2000) Tcf/Lef transcription factors during T-cell development: unique and overlapping functions. *Hematol J* **1**: 3–6
- Treisman JE, Rubin GM (1995) wingless inhibits morphogenetic furrow movement in the *Drosophila* eye disc. *Development* **121**: 3519–3527
- van de Wetering M et al (1997) Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene dTCF. *Cell* **88**: 789–799
- Vincent JP, Lawrence PA (1994) *Drosophila* wingless sustains engrailed expression only in adjoining cells: evidence from mosaic embryos. *Cell* **77**: 909–915
- Voas MG, Rebay I (2004) Signal integration during development: insights from the *Drosophila* eye. *Dev Dyn* **229**: 162–175
- Xu T, Rubin GM (1993) Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**: 1223–1237