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

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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
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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 3β within the degradation complex that also includes Axin and antigen-presenting cells (Logan & Nusse, 2004). The phosphorylated Armadillo is targeted for ubiquitination and proteosome-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 phosphorylation of 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, YanACT, resulted in dose-dependent repression of

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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 yanACT 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–C0). Misexpression of UAS–YanACT by C96–GAL4 (C96 > YanACT; E0) 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–G0), resulting in a downregulation of Wingless-dependent Engrailed expression (G–G0, white arrowheads) as compared with wild-type (F). Prd > YanACT results in downregulation of Engrailed (H, white arrowheads), and coexpression of UAS–Arm* with UAS–YanACT (prd > Arm*; YanACT; 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 μM. 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 YanACT 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 YanACT had similar inhibitory effects on in vivo readouts of Wingless pathway activity. The Wingless target gene senseless was reduced in response to misexpression of YanACT 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 YanACT, similarly to in response to misexpression of UAS–Axin (Fig 1F–H). Notably, Engrailed expression was restored on coexpression of UAS–Armadillo* with YanACT (Fig 1I; supplementary Fig S1E–G online). Conversely, RNAi-mediated knockdown of yan resulted in expansion of Engrailed expression (supplementary Fig S1I’ 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 affect 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 Dachshund, 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 Dachshund 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**(w)**), 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**(w)**), 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. As yan is expressed anterior to and abutting Yan expression at the lateral margins of the eye disc (supplementary Fig S1A–A* online), yan is expressed anterior to and abutting Yan expression at the lateral margins of the eye disc (supplementary Fig S1A–A* online), yan is expressed anterior to and abutting Yan expression at the lateral margins of the eye disc (supplementary Fig S1A–A* online).
constriction was not evident in cells within the clones (Fig 3D–D’). We also generated yan+/C0+/C0+ 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+/C0+/C0+ clones (supplementary Fig S4J–M online). Therefore, we concluded that apical constriction within yan+/C0+/C0+ 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
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.

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**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\(^{top}\), 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.
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.
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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 SC). 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 SD). 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 SD). 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 Fz–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 dTIF12 (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-YanACT (Ilaria Rebay) were transfected with 50 ng pAct-Wingless and 25 ng each of dTIF12, 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*510, FRT40A ubiGFP. UAS-YanACT, yan445, FRT40A and yan884, FRT40A were gifts from Ilaria Rebay. Hsfp ubiGFP, FRT40A, Tub–GAL80, Tub–GAL4/TM6B and wingless–LacZ were obtained from Jessica Treisman; Fz–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, hsfpMKRS/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.** Fz–RFP construct: 2333 base-pair fragment was PCR-amplified (F primer: 5’-GGGGTACCGGAAGGAAGGTTTTG-3’, R primer: 5’-CTAGCTAGCTAG-3’).
TGGGTTCAGGG-3’) from the Drosophila melanogaster genomic region upstream of frizzled3 translation start, flanked by 5’ Kpn1 and 3’ Nhel 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.embo-reports.org).

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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.

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