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pygopus encodes a nuclear protein essential for Wingless/Wnt signaling

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SUMMARY

The Wingless (Wg)/Wnt signal transduction pathway regulates many developmental processes through a complex of Armadillo(Arm)/β-catenin and the HMG-box transcription factors of the Tcf family. We report the identification of a new component, Pygopus (Pygo), that plays an essential role in the Wg/Wnt signal transduction pathway. We show that Wg signaling is diminished during embryogenesis and imaginal disc development in the absence of *pygo* activity. Pygo acts downstream or in parallel with Arm to regulate the nuclear function of Arm protein. *pygo* encodes a novel and evolutionarily conserved nuclear protein bearing a PHD finger that is essential for its activity. We further show that Pygo can form a complex with Arm in vivo and possesses a transcription activation domain(s). Finally, we have isolated a *Xenopus* homolog of *pygo* (*Xpygo*). Depletion of maternal *Xpygo* by antisense deoxyoligonucleotides leads to ventralized embryonic defects and a reduction of the expression of Wnt target genes. Together, these findings demonstrate that Pygo is an essential component in the Wg/Wnt signal transduction pathway and is likely to act as a transcription co-activator required for the nuclear function of Arm/β-catenin.

Key words: *Drosophila*, *Xenopus*, *pygopus*, Wingless, Wnt, Signaling

INTRODUCTION

Wingless (Wg)/Wnt proteins are secreted glycoproteins that have diverse and profound roles in animal development. They exert their biological roles by activating a conserved signal transduction pathway to regulate the expression of downstream target genes (reviewed by Wodarz and Nusse, 1998). Genetic and biochemical studies in both *Drosophila* and vertebrates have identified many conserved components of this pathway. A key effector in the pathway is *Drosophila* Armadillo (Arm) and its vertebrate homolog β-catenin. In the absence of Wg/Wnt signaling, the cytosolic Arm/β-catenin is maintained at low levels through a degradation complex consisting of the serine/threonine kinase Shaggy/Zeste-white 3 (Siegfried et al., 1992) or its vertebrate homolog GSK-3β (Yost et al., 1996), the scaffold protein Axin (Hamada et al., 1999; Zeng et al., 1997), the tumor suppressor gene product adenomatous polyposis coli [*APC* (McCartney et al., 1999; Rubinfeld et al., 1996)] and CK1α (Liu et al., 2002). Binding of Wg/Wnt to cell surface receptors of the Frizzled (Bhanot et al., 1996; Yang-Snyder et al., 1996) and LRP families (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000), as well as to cell-surface proteoglycans (Lin and Perrimon, 2000), leads to an accumulation of Arm/β-catenin (Larabell et al., 1997; Peifer et al., 1994b; Riggleman et al., 1990). The accumulated Arm/β-catenin translocates into the nucleus where it binds to the HMG-box transcription factor protein Pangolin(Pan)/dTcf (Brunner et al., 1997; Riese et al., 1997; van de Wetering et al., 1997) in *Drosophila* or Lef-1/TCF (Behrens et al., 1996; Molenaar et al., 1996), activating expression of Wg/Wnt target genes.

Although the importance of the Pan/Arm and Tcf/β-catenin complexes in the activation of Wg/Wnt target genes is well established, their mechanism(s) of action is poorly understood. Arm/β-catenin is composed of 12 imperfect repeats (Arm repeats) flanked by unique N- and C-terminal domains (Peifer et al., 1994a; Peifer et al., 1992). Arm/β-catenin interacts with various molecules that regulate its nuclear activity. The central Arm repeats in Arm/β-catenin can interact with Pan/Tcf. In the absence of Wg/Wnt signaling, Pan/Tcf functions as a repressor to suppress the expression of many Wg/Wnt target genes. Studies in *Drosophila* and *Xenopus* indicate that repression is mediated by Groucho (Gro) co-repressors (Cavallo et al., 1998; Roose et al., 1998) and Osa-containing Brahma chromatin remodeling complexes (Collins and Treisman, 2000). In this regard, Wg/Wnt signaling permits accumulated Arm/β-catenin to translocate to the nucleus to relieve the repression mediated by Gro and Osa/Brm complexes. Arm/β-catenin can also interact with various factors that positively regulate its nuclear activity. It has been proposed that the C-terminal region of Arm/β-catenin serves as a transcription activation domain. This domain can function as a transcriptional activator when fused to a DNA-binding domain (van de Wetering et al., 1997), and a mutant Arm lacking this domain is defective in Wg signaling (Cox et al., 1999; van de Wetering et al., 1997). In *Drosophila*, the zinc-finger protein Teashirt can bind to the C-terminal domain of Arm and acts as a regionalized factor in the trunk of

the embryo, which is required for the specification of the late Wg-signaling events (Gallet et al., 1999). However, other experiments suggest that Arm/β-catenin can recruit the transcriptional machinery via multiple contact sites. For example, it has been reported that β-catenin can interact directly in vitro with the TATA-binding protein (TBP) (Hecht et al., 1999) through both its N-terminal and Arm repeat regions. It is also reported that Pontin52, a protein that interacts with TBP, can bind to N-terminal Arm repeats (Bauer et al., 1998). Taken together, these results suggest that regulation of gene expression by Arm/β-catenin is likely to be mediated by multiple protein complexes. Identification of molecule(s) required for the in vivo function of Arm/β-catenin will be essential to elucidate the molecular mechanism(s) by which the Arm/β-catenin-Tcf complex activates Wg/Wnt target gene expression.

In a genetic screen to identify genes involved in Wg signaling, we have isolated a novel gene that was named *pygopus* (*pygo*). We show that loss of *pygo* function leads to defects associated with Wg signaling in both embryogenesis and imaginal disc development. Pygo acts downstream or in parallel with Arm to regulate the nuclear function of Arm protein. Consistent with our genetic analysis, we have shown that *pygo* encodes a novel nuclear protein containing a PHD (plant homology domain) finger, a domain shared by many proteins that play roles in chromatin remodeling and transcription co-activation. The PHD finger of Pygo is crucial for its function, as a point mutation in the PHD finger disrupts Wg signaling. We provide further biochemical evidence to demonstrate that Pygo can form a complex with Arm in vivo and contains a transcription activation domain(s). Finally, we have isolated a *Xenopus* homolog of *pygo* (*Xpygo*). Depletion of maternal *Xpygo* by antisense deoxyoligonucleotides leads to ventralized embryonic defects and a reduction in Wnt target gene expression. Together, our results provide both genetic and molecular evidence for a role of Pygo in Wnt/Wg signaling. Our independent findings in this work are consistent with and further advance the recent studies by other groups of Pygo (Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002).

MATERIALS AND METHODS

Genetic screen, mapping and identification of *pygo* mutations

We devised a genetic screen that used the 'direct mosaic' system (Duffy et al., 1998) by using the Gal4/UAS system (Brand and Perrimon, 1993) to control the expression of the yeast recombinase, Flipase (Flp). A wing-specific Gal4 line, *vg Q1206-Gal4* (Simmonds et al., 1995) was used to drive high level expression of Flp in the dorsoventral compartment boundary of the wing imaginal disc. Males of the genotype *w; FRT^{2A} FRT^{82B} e /TM3* were mutagenized with EMS and crossed to females of the genotype *w; vg Q1206-Gal4 UAS-flp; FRT^{2A} FRT^{82B}/TM*3. F_1 flies of the genotype *w; vg Q1206-Gal4 UAS-flp/+; FRT^{2A} FRT^{82B} e / FRT^{2A} FRT^{82B}* express Flp primarily in wing imaginal cells under *vg Q1206-Gal4* control. This Flp activity mediates a high frequency of mitotic recombination, generating clones of cells homozygous for the mutagenized *FRT^{2A} FRT^{82B} e* chromosome. The wings of the resulting F1 flies were screened for wing notches and ectopic margin bristles. The F_1 male flies with interesting wing phenotypes were further crossed with females of the genotype *w, Dr/TM3*. The resulting F_2 flies with genotype of *FRT^{2A} FRT^{82B} e/TM3* were isolated based on the ebony marker. We further determined the chromosome arm of interesting mutations by crossing them to the stocks of genotype *w; vg Q1206-Gal4, UAS-flp; FRT^{2A} /TM*3 or with *w; vg Q1206-Gal4 UAS-flp; FRT^{82B} /TM*3. Approximately 100,000 F1 flies were screened, leading to the identification of five *pygo* alleles.

The locations of *pygo* mutations were determined on the right arm of third chromosome as described above. These alleles were then grouped by a complementation test. In an epistasis experiment as described in Fig. 3, we determined that *pygo* was located near *axin*. We further mapped the *pygo* locus within 0.6 cytological units to the left or one unit to the right of *axin* by a standard genetic method. In this region, we identified a total of 30 genes that are putative nuclear proteins. Two of them have P-element insertions. In a complementation test, EP(3)1076 was identified as a candidate for a gene encoding Pygo.

To identify molecular lesions associated with *pygo* alleles, genomic DNA was prepared from larvae homozygous for *pygo* alleles and amplified by PCR using synthetic oligonucleotide primers against the *pygo* gene. Mutations were identified by sequencing the PCR products in both directions.

Generation of clones for phenotypic analysis

Females with germline clones were generated using the autosomal 'FLP-DFS' technique (Chou and Perrimon, 1996) as described (Häcker et al., 1997). Imaginal disc clones of *pygo, axin or pygo-axin* mutant cells marked by the absence of the *ubiquitin-GFP* marker gene were generated using the FLP/FRT method (Xu and Rubin, 1993) as follows: *y w hsflp; FRT^{82B} ubiquitin-GFP /TM6C* females were crossed with *FRT^{82B} pygo* (or *axin* or *pygo-axin*) */TM6*B. Larvae of the genotype *y w hsflp; FRT^{82B} pygo*(or *axin* or *pygo-axin*) / *FRT^{82B} ubiquitin-GFP* were heat shocked for 3 hours at 38°C during the first instar or second instar larval stage. Imaginal discs from non-Tubby third instar larvae were dissected and stained.

Antibody staining and in situ hybridization

Fixation of embryos and imaginal discs and antibody staining procedures were performed as described (Häcker et al., 1997). Antibody dilutions were as follows: rabbit anti-Wg at 1:500 (a gift from S. Cumberledge), rabbit anti-Labial at 1:200 (a gift from T. Kaufman), rabbit anti-GFP (Clontech) at 1:500, monoclonal anti-Dll at 1:500 (a gift from I. Duncan), monoclonal anti-Engrailed 4D9 at 1:500 (Iowa Developmental Studies Hybridoma Bank; IDSHB), monoclonal anti-Ac at 1:1 (IDSHB), monoclonal anti-Even-skipped 2B8 at 1:10 (IDSHB) and monoclonal anti-Arm N27A1 at 1:5 (IDSHB). Fluorescent-conjugated secondary antibodies are from Jackson ImmunoResearch Laboratories.

In situ hybridization of whole-mount embryos was carried out with a digoxigenin-labeled RNA probe as described previously (Häcker et al., 1997).

Molecular cloning

A *Drosophila pygo* cDNA containing the entire coding region was obtained from a 0- to 4-hour-old *Drosophila* embryonic cDNA library (Brown and Kafatos, 1988) by PCR using synthetic oligonucleotide primers. To isolate *Xenopus pygo* cDNA, we designed RACE PCR primers based on the sequence information in the EST database (GenBank at NCBI, Accession Number, BF427056), encoding the putative *Xenopus* Pygo protein. These primers were used in conjunction with the Clontech SMART RACE kit, which generated a 5′ and a 3′ RACE fragment, which were shown to overlap and to contain a complete open reading frame by sequence analysis (GenBank Accession Number, AF521655).

A Myc-tagged Pygo construct was made by cloning the coding region into *Nco*I-*Eco*RI site in frame with five Myc-epitope tags in pCS2+MT vector (Rupp et al., 1994). A GAL4 DB-Pygo construct was made by cloning the coding region of Pygo from amino acids 105 to 815 into *Eco*RI-*Xba*I site in PM1 vector (Sadowski et al., 1992). An Arm-HA construct contains HA-tagged Arm (amino acid 128 to 844) cloned in pCS2 vector (Rupp et al., 1994).

Table 1. PCR primer pairs and PCR cycling conditions used with the LightCycler™

PCR primer pair	Reference	Sequence	Denat. temp°C	Anneal temp°C/ time (sec)	Extension temp°C/ time (sec)	Acquisition temp°C/ time (sec)
Bmp4	New	U: 5′-ACC CAT AGC TGC AAA TGG AC-3′ D: 5′-CAT GCT TCC CCT GAT GAG TT-3′	95	55/5	72/12	81/3
Chordin	XMMR	U: 5-AAC TGC CAG GAC TGG ATG GT-3 D: 5-GGC AGG ATT TAG AGT TGC TTC-3	95	55/5	72/12	81/3
Goosecoid	New	U: 5′- TTC ACC GAT GAA CAA CTG GA-3′ D: 5′- TTC CAC TTT TGG GCA TTT TC-3′	95	55/5	72/11	82/3
ODC	Heasman et al., 2000	U: 5-GCC ATT GTG AAG ACT CTC TCC ATT C-3 D: 5-TTC GGG TGA TTC CTT GCC AC-3	95	55/5	72/12	83/3
Siamois	Heasman et al., 2000	U: 5-CTG TCC TAC AAG AGA CTC TG-3 D: 5-TGT TGA CTG CAG ACT GTT GA-3	95	55/5	72/16	81/3
Xbra	Sun et al., 1999	U: 5-TTC TGA AGG TGA GCA TGT CG-3 D: 5-GTT TGA CTT TGC TAA AAG AGA CAG G-3	95	55/5	72/8	75/3
*Xnr*3	Kofron et al., 1999	U: 5-CTT CTG CAC TAG ATT CTG-3 D: 5-CAG CTT CTG GCC AAG ACT-3	95	57/5	72/10	79/3
Xpygo	New	U: 5-CAA TGG GAA CCA ACC AAA CT-3′ D: 5-ACG ATG CCT CAC ACA AGA TG-3′	95	55/5	72/12	90/3
Xsox17 α	Xanthos et al., 2001	U: 5-GCA AGA TGC TTG GCA AGT CG-3 D: 5-GCT GAA GTT CTC TAG ACA CA-3	95	58/5	72/8	85/3
Xwnt8	Ding et al., 1998	U: 5-CTG ATG CCT TCA GTT CTG TGG-3 D: 5-CTA CCT GTT TGC ATT GCT CGC-3	95	58/6	72/14	85/3

D, downstream primer; U, upstream primer.

Cell culture, transient transfections and reporter gene assays

Human 293T cells (5×10^5) were seeded into 35 mm tissue culture dishes and transiently transfected with 2 μg pG5E1b-luciferase reporter and 2 μg plasmids expressing Gal4 DB domain or its fusion proteins using polyfect transfection reagent (Qiagen). A 0.5 μg CMV-β-Gal plasmid was co-transfected as an internal control. Cells were harvested after 36 hours and lysed in 1% Triton X-100, 100 mM Tris (pH 7.8), 2 mM EDTA, 2 mM DTT, 2 μg/ml Aprotinin, 2 μg /ml Leupeptin, 2 μg /ml Pepstatin and 100 μg/ml PMSF. Luciferase activity was determined on Monolight™ 3010 (PharMingen).

Immunoprecipitation and western blotting

293 T cells (2×10^6) were transfected with 4 μg of expression vector for Myc-tagged Pygo and 4 μg of expression plasmid for HA-tagged Arm. Cells were harvested 36 hours later and lysed in 1 ml of 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 1 mM PMSF and 10 ml/ proteinase inhibitor tablet (Roche Molecular Biochemicals) on ice for 30 minutes. After clearance, one half of each lysate was used for immunoprecipitation with 1.0 μg of anti-Myc antibody (9E10, Roche Molecular Biochemicals) for 3 hours at 4°C in the presence of a 12.5 μl bed volume of protein-Sepharose (Amersham Pharmacia) preincubated in cell lysates from non-transfected cells supplemented with 1% bovine serum albumin. Immunoprecipitates were washed once with 1 ml of lysis buffer and three times with 1 ml of 20 mM Tris-HCl pH 7.6, 100 mM NaCl, 1 mM EDTA and 0.1% Nonidet P-40 for 10 minutes at 4°C. Material bound to the beads was eluted in SDS-loading buffer, resolved by SDS-PAGE on 10% gels, transferred onto PVDF membrane (BioRad) and analyzed by western blotting using the ECL detection system (Amersham Pharmacia).

Xenopus oocyte and embryo manipulation

Oocytes were manually defolliculated and cultured as described (Kofron et al., 1999). They were injected with the antisense oligodeoxynucleotide (oligo), cultured at 18°C in oocyte culture medium (OCM), colored with vital dyes and fertilized using the host transfer technique as described previously (Zuck et al., 1998). The antisense *Xpygo* oligo was an HPLC-purified chimeric phosphorothioate/phosphodiester oligo with the base composition 5′ T*T*T*GCGCCGTTTCTT*C*T*C 3′, where * indicates a phosphorothioate bond (Gibco). The oligo was resuspended in sterile filtered water and injected vegetally in doses of 2-2.5 ng. Eggs were stripped and fertilized using a sperm suspension and the embryos were cultured in 0.1×MMR.

Analysis of gene expression using real-time RT-PCR

Total RNA was prepared from oocytes and embryos and cDNA synthesized as described elsewhere (Kofron et al., 2001). Real-time RT-PCR and quantitation using the LightCycler™ System (Roche) was carried out as described (Kofron et al., 2001). The PCR primer pairs and cycling conditions are listed in Table 1. Relative expression values were calculated by comparison to a standard curve generated by serial dilution of uninjected control cDNA. Samples were normalized to levels of *ornithine decarboxylase* (*ODC*), which was used as a loading control. Samples of water alone or controls lacking reverse transcriptase in the cDNA synthesis failed to give specific products in all cases.

RESULTS

Identification of *pygo* as a new segment polarity gene required for Wg signaling

To identify genes involved in Wg signaling, we devised a genetic screen that used the ‘direct mosaic’ system (Duffy et al., 1998). We used a wing specific Gal4 line, *vg Q1206-Gal4*, to drive a high level expression of UAS-flipase that induces mitotic recombination with very high frequency in a developing wing. Wg is expressed in a narrow strip of cells at the dorsoventral (DV) compartment boundary in the wing

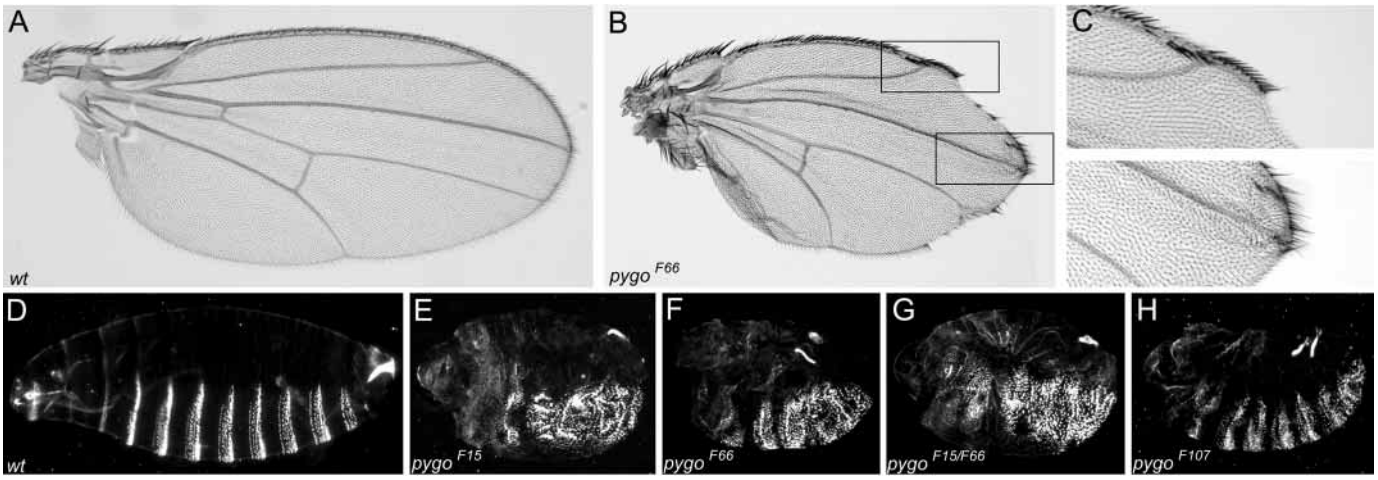

Fig. 1. Identification of *pygo* as a new segment polarity gene required for Wg signaling. Wings are oriented proximal towards the left and anterior upwards (A-C). A wild-type wing is shown in A. A wing with somatic clones of $pygo^{F66}$ is shown in B. The enlarged parts of the wing in B are shown in C. Clones of $pygo^{F66}$ (B) cause wing notching and the formation of ectopic bristles in nearby tissues. The wing phenotypes associated with $pygo^{F66}$ are fully penetrant. Virtually identical results were obtained from other *pygo* alleles. The cuticle phenotypes of wild-type (D) and *pygo* mutant embryos (E-H) are shown. All embryos are oriented anterior towards the left and dorsal upwards. The *pygo* mutant embryos in E-H were derived from homozygous *pygo* mutant germline clones (see Materials and Methods). The wild-type embryo (D) forms a segmented larval cuticle decorated with denticles spaced by naked cuticle. *pygo* mutant embryos (E-H) form unsegmented cuticles that produce 'lawn' of denticle hairs. Homozygous mutant embryos derived from germline clones are shown for $pygo^{F15}$ (E), $pygo^{F66}$ (F) and $pygo^{F107}$(H). A *pygo* mutant embryo shown in G was derived from a homozygous $pygo^{F15}$ mutant germline clone and paternally mutant for $pygo^{F66}$.

imaginal disc. Wg signaling directs the formation of wing margin bristles and controls the growth and patterning in surrounding cells of the presumptive wing blade (Neumann and Cohen, 1997; Zecca et al., 1996). Wg also plays a role in downregulating its own expression in cells immediately adjacent to the DV boundary (Rulifson et al., 1996). Hence, mutations that block Wg signaling cause a loss of wing margin bristles and deletions of nearby portions of the wing. In addition, a reduction of Wg signaling in cells adjacent to the DV boundary induces the expression of Wg that leads to the formation of ectopic bristles in nearby wild-type tissue. From this genetic screen, we recovered five recessive alleles of a locus on the third chromosome. We named this novel gene *pygopus* (*pygo*). *pygo* mutant clones in wing exhibit wing nicks with ectopic margin bristles in nearby wild-type tissue (Fig. 1A-C). The wing phenotypes associated with *pygo* mutations resemble those associated with mutations in *dishevelled* (*dsh)* (Rulifson et al., 1996) and the *frizzled-frizzled 2* (*fz-fz2)* double mutant (Chen and Struhl, 1999), suggesting that Pygo plays a role in Wg signaling.

To confirm that Pygo is required for Wg signaling, we examined the embryonic cuticle patterning associated with *pygo* mutations. Animals zygotically mutant for *pygo* appear to have normal cuticle patterning (data not shown) and survive until the third instar larval or early pupal stages. However, homozygous mutant embryos derived from females lacking maternal *pygo* activity die with a strong segment-polarity phenotype (Fig. 1E-H). Instead of the wild-type segmentally repeated pattern of denticle belts interspersed by naked cuticle, such embryos display a lawn of denticle belts and fail to secrete naked ventral cuticle. The cuticle phenotype of *pygo* is reminiscent of those associated with mutations in essential Wg signaling components (Nusslein-Volhard and Wieschaus, 1980), providing genetic evidence that *pygo* is a novel segment-polarity gene involved in Wg signaling.

Among three independent alleles that we examined, both $pygo^{F66}$ and $pygo^{F15}$ are relatively strong and are likely to represent null alleles (Fig. 1E-G). The cuticle defects associated with $pygo^{F107}$ are somewhat weaker (Fig. 1H). The maternal effects associated with $pygo^{F107}$ are completely paternally rescuable. However, zygotically wild-type embryos derived from females lacking maternal materials for $pygo^{F66}$ or $pygo^{F15}$ develop pair-rule like phenotypes that exhibit denticle deletions (data not shown). These results suggest that $pygo^{F107}$ is a unique allele specific to Wg signaling and that maternal *pygo* is also likely to be involved in developmental processes other than Wg signaling.

Pygo is required for Wg signaling in various embryonic developmental processes

Wg signaling is required for various developmental processes during embryogenesis. To further determine the possible roles of Pygo in Wg signaling during embryogenesis, we examined several well-defined Wg signaling events in embryos mutant for *pygo*. Owing to the complication of additional defects associated with the *pygo* null alleles $pygo^{F66}$ and $pygo^{F15}$, we generated embryos maternally mutant for $pygo^{F107}$ and paternally mutant for $pygo^{F66}$. These *pygo* mutant embryos, referred to as $pygo^{F107}$glc/ $pygo^{F66}$, were used for all the analyses of Wg signaling events during embryogenesis described below. We found that *pygo* mutant embryos fail to transduce Wg signaling. However, no other defects have been identified in $pygo^{F107}$glc/ $pygo^{F66}$ embryos.

First, we examined the expression of *en* and *wg* in $pygo^{F107}$glc/ $pygo^{F66}$ embryos. In the ventral embryonic epidermis, the expression of *wg* and *en* is initiated by pair-rule and gap genes. At stage 10, Wg signaling is required for

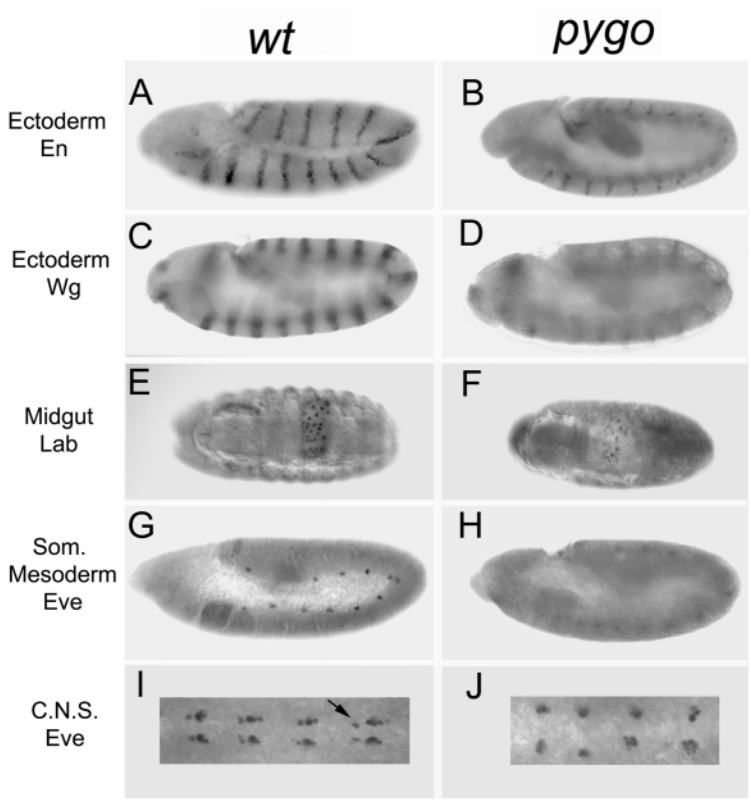

Fig. 2. Pygo is required for Wg signaling in various embryonic developmental processes. Wild-type (wt) and *pygo* mutant embryos are shown for expression of En (A,B), Wg (C,D), Labial (E,F) and Eve (G-J). All the *pygo* mutant embryos were derived from homozygous $pygo^{F107}$ mutant germline clones and paternally mutant for $pygo^{F66}$. En is normally expressed in stripes in the ectoderm of the thorax and abdomen during stage 10 (A). Wg is also expressed in stripes in the ectoderm of the thorax and abdomen (C) during stage 10. Both Wg and En stripes are strikingly reduced in *pygo* mutant embryos at stage 10 (B,D). The endoderm is normally subdivided into discrete domains by constrictions imposed by the visceral mesoderm and Labial is expressed in one of these domains (E). Labial expression is diminished in a *pygo* mutant embryo (F). Eve is normally expressed in specific subsets of cells derived from the somatic mesoderm that will form the heart (G) and also expressed in specific neurons in the central nervous system (CNS), including the RP2 neurons (arrow) (I). (H,J) In *pygo* mutant embryos, the expression of Eve-positive cells is absent in the somatic mesoderm cells (H) and in the RP2 neurons (J), respectively.

maintenance of *en* transcription. Subsequently, En, through a signaling pathway mediated by Hh, is also required for the maintenance of *wg* transcription. In a *wg* mutant, the expression of *wg* and *en* initiates correctly, but fades at stage 10 (DiNardo et al., 1988; Martinez Arias et al., 1988). In *pygo* mutant embryos, the En expression began to fade by stage 10 (Fig. 2B). Similarly, the expression of Wg protein is greatly reduced (Fig. 2D). These results are consistent with a role for Pygo in Wg signaling.

Second, the development of the midgut requires Wg signaling. In wild-type embryos, Wg signaling from the visceral mesoderm up-regulates expression of the homeotic gene labial (lab) in the endoderm. The expression of Lab is not induced to the wild-type level in embryos mutant for wg, dsh, arm and fz-fz2 (Bhanot et al., 1999; Chen and Struhl, 1999; Hoppler and Bienz, 1995; Immergluck et al., 1990; Yu et al., 1996). In $pygo^{F107}$glc/ $pygo^{F66}$ embryos, the expression of Lab is also strikingly reduced (Fig. 2F), resembling the phenotype produced by loss of Wg signaling.

Third, Wg signaling is required to specify cardiac precursor cells by maintaining the expression of the homeobox gene *tinman* (*tin*) in the cardiac mesoderm (Park et al., 1996). Even-skipped (Eve) protein is expressed in a subset of myoblasts that will give rise to the heart at stage 10/11 (Fig. 2G). The presence of these Eve-expressing cells is strictly dependent on Wg signaling (Lawrence et al., 1995; Wu et al., 1995). In $pygo^{F107}glc/\ pygo^{F66}$ mutant embryos, these eve-expressing cells are also absent (Fig. 2H), suggesting that *pygo* is required for Wg signaling in specifying cardiac precursor cells.

Finally, in the embryonic central nervous system, Wg signaling is required to specify the neuroblasts that produce the RP2 motoneurons in each segment (Bhanot et al., 1999; Bhat, 1996; Chen and Struhl, 1999; Chu-LaGraff and Doe, 1993). These neurons can be easily visualized because they express Eve protein (Fig. 2I). These Eve-positive neurons are not present in wg or fz-fz2 mutant embryos (Bhanot et al., 1999; Chen and Struhl, 1999). In $pygo^{F107}$glc/ $pygo^{F66}$ embryos, these Eve-expressing neurons are also missing (Fig. 2J).

In summary, the observation of *wg*-like phenotypes in various developmental processes in *pygo* mutant embryos provide strong evidence that *pygo* is involved in Wg signaling during embryogenesis.

Pygo is required for Wg signaling in imaginal disc development

We further examined the role of Pygo in Wg signaling during imaginal disc development. In the wing disc, Wg acts both as a short-range inducer and as a long-range morphogen (Neumann and Cohen, 1997; Zecca et al., 1996). In the DV boundary, Wg acts at short range to specify the expression of the *achaete scute complex* (*asc*), which can be detected by antibody against Achaete (Ac) protein (Couso et al., 1994). To pattern the entire wing, Wg functions as a long-range morphogen (up to 20-30 cell diameters away from its site of synthesis) to trigger a graded transcriptional response of target genes, including *distalless* (*dll*) and *vestigial* (*vg*) (Neumann and Cohen, 1997; Zecca et al., 1996). We examined the expression of Ac, Dll and Vg in wing discs. As shown in Fig. 3, in mosaic clones mutant for $pygo^{F15}$, the expression of Ac is completely abolished (Fig. 3B,B′), and Dll expression is also markedly diminished (Fig. 3D,D′). Pygo is likely to function cell-autonomously in Wg signaling as the expression of both Ac and Dll are diminished in all of the cells mutant for $pygo^{F15}$. These results argue that Pygo is required for both Wg short- and long-range activities in wing patterning.

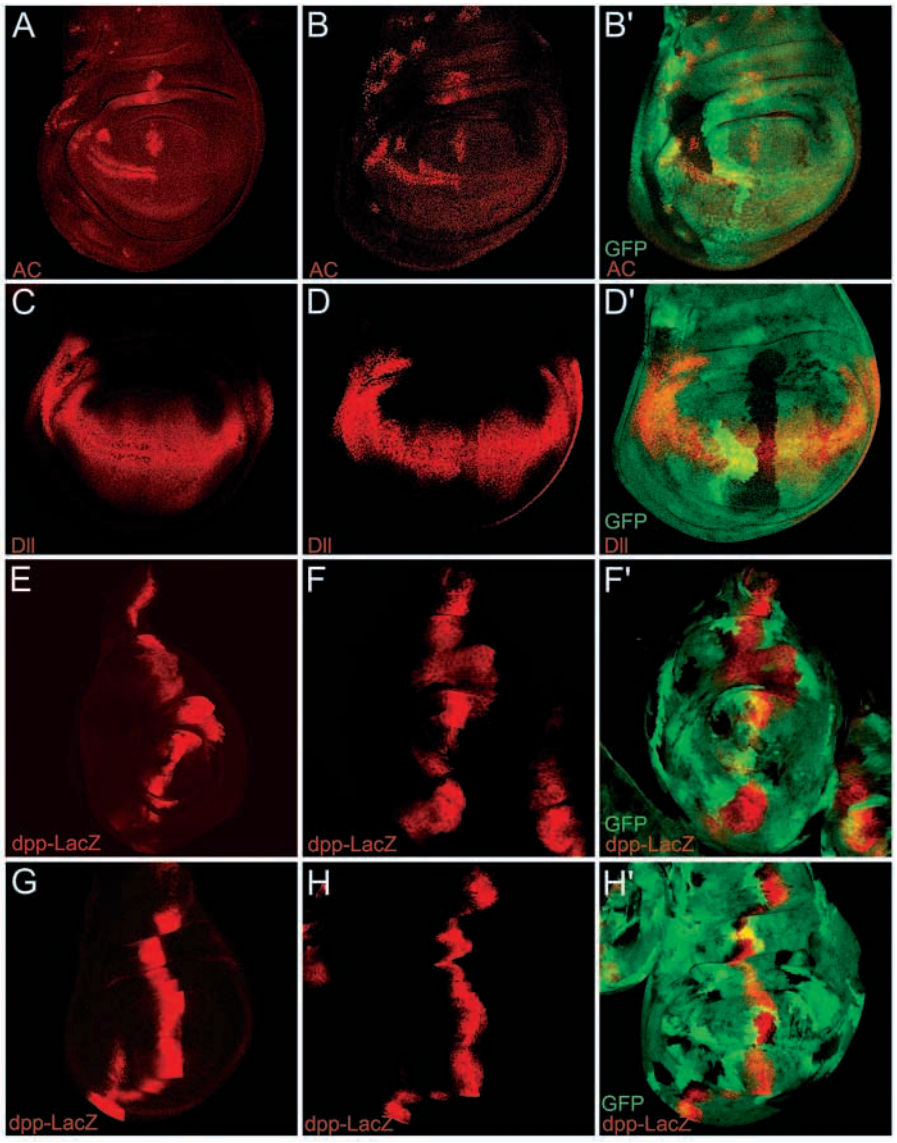

Fig. 3. Pygo is required for Wg signaling in imaginal disc development. In these images, anterior is towards the left, dorsal is upwards. All the discs were derived from third instar larvae. Somatic clones mutant for *pygo*^{F15} are marked by the absence of GFP shown in green. A complex pattern of Achaete (Ac) expression is shown in a wing disc from a wild-type third instar larva (A). Wg acts at short range to induce the expression of Ac at the DV boundary of anterior wing pouch (A). In a mosaic clone mutant for *pygo*^{F15}, which is marked by absence of GFP, the expression of Ac protein is abolished (B,B′). Note that a patch of Ac-positive cells within the clone is Wg independent. Therefore the expression of Ac in these cells is not diminished. In response to Wg signaling, Dll is expressed in a graded manner with highest expression in DV boundary in a wild-type wing disc (C). Dll expression is diminished autonomously in a *pygo*^{F15} mosaic clone (D,D′). In a wild-type leg disc, Dpp-*lacZ* expression is repressed by Wg signaling in the ventral anterior quadrant (E). In a *pygo*^{F15} mosaic clone of the leg disc, Dpp-*lacZ* expression is de-repressed (F,F′). In a wild-type wing disc, Dpp-lacZ expression is induced by Hh signaling at the AP boundary. The expression of Dpp-*lacZ* is not altered in mosaic clones mutant for *pygo*^{F15} (H,H′).

Although the expression of Ac is completely abolished (Fig. 3B,B′), the mutant clones still express some Dll and they seem to be surviving better than *arrow* (*arr*) and *Fz-fz2* (Chen and Struhl, 1999; Wehrli et al., 2000). Furthermore, we observed no obvious reduction in the expression of Vg in clones mutant for *pygo*^{F15} (data not shown). As *pygo*^{F15} is a null allele, this result suggests that Pygo is likely required for high threshold activity of Wg signaling. Alternatively, the residual activity of Wg signaling may be due to remaining Pygo protein in mutant clones.

We also examined the activity of Pygo in Wg signaling in the leg disc. The patterning of the leg is controlled by Wg and Decapentaplegic (Dpp). The expression of *dpp* and *wg* are maintained by mutual repression: Dpp signaling blocks *wg* transcription, whereas Wg signaling attenuates *dpp* transcription (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996). *wg* is expressed in a ventral quadrant where the expression of *dpp* is repressed (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996). If Wg signal transduction is blocked, as in clones of cells mutant for *dsh* (Heslip et al., 1997) and *arr* (Wehrli et al., 2000), *dpp* expression is de-repressed and patterning is disrupted. Similarly, *dpp* expression is de-repressed in clones of cells mutant for *pygo* in the ventral anterior quadrant (Fig. 3F,F′). Taken together with the results from wing imaginal disc, these findings indicate that Pygo is necessary in cells responding to Wg input, for both positive and negative gene regulation.

In contrast to the multiple functions of Pygo during embryogenesis, its activity is very specific to Wg signaling in wing patterning. Both Hedgehog (Hh) and Dpp signaling events are unaffected by null mutant *pygo*^{F15}. In the wing disc, Hh is expressed in the posterior (P) compartment from which it emanates to activate expression of *dpp* at the anterior/posterior boundary (Basler and Struhl, 1994; Tabata and Kornberg, 1994). The expression of *dpp* is not altered in mosaic clones mutant for *pygo*^{F15} (Fig. 3H,H′). We also observed no alteration of Dpp signaling in mosaic clones mutant for *pygo*^{F15}, as analyzed by anti-phosphorylated MAD antibody staining (Tanimoto et al., 2000) (data not shown).

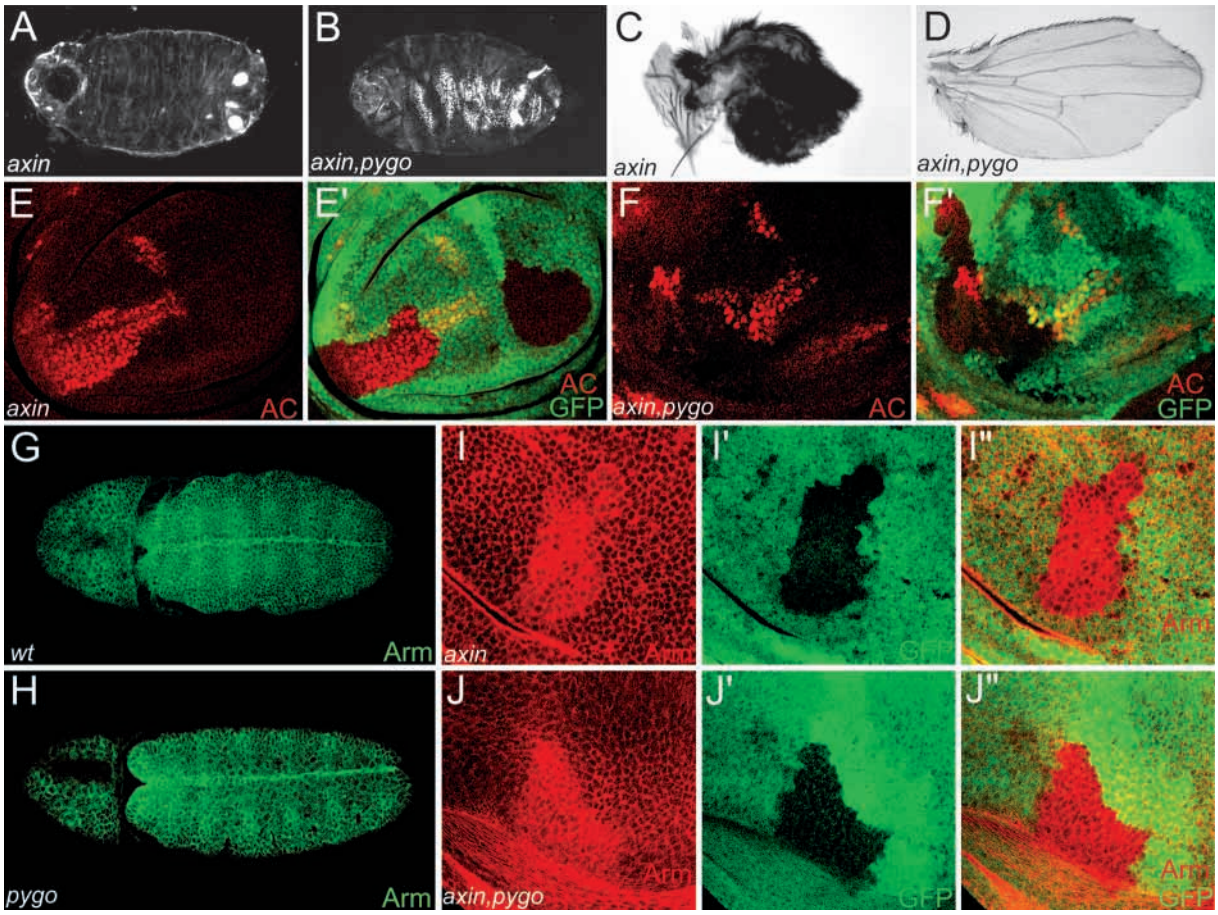

Fig. 4. Pygo acts downstream or in parallel with Arm to regulate nuclear Arm activity. An embryo lacking both maternal and zygotic *axin* exhibits the characteristic naked cuticle phenotype associated with constitutive Wg signaling (A). In a *axin-pygo*^{F15} double null embryo, the naked cuticle phenotype is reversed and exhibits a 'lawn' of denticles phenotype (B). A wing bearing somatic clones of *axin* produced by *vg Q1206-Gal4*/UAS flipase, is shown in C. Numerous bristles were produced by clones mutant for *axin* (C). A wing with *axin-pygo*^{F15} somatic clones (D) exhibits no bristle within the wing and produces notching and the formation of ectopic bristles in nearby tissues, which is similar to the wing bearing *pygo*^{F15} clones (see Fig. 1B,C). A *axin* somatic clone in the wing disc produces autonomously the expression of Ac (E,E′). In a *axin-pygo*^{F15} somatic clone, no induction of Ac expression is observed (F,F′). In a wild-type embryo, Arm protein levels are upregulated in a segmentally repeated fashion in the ventral ectoderm (G). The expression of Arm protein remains in a segmentally repeated fashion in embryos mutant for *pygo* (H). In a wing disc, Arm protein is strikingly upregulated in a clone mutant for *axin* (I,I′,I″). This upregulated Arm protein is not diminished in clones mutant for *axin-pygo* (J,J′,J″). There is no difference in the subcellular localization of Arm protein in clones of *axin* and *axin-pygo* (compare I,I′,I″ with J,J′,J″).

Pygo acts downstream or in parallel with Arm to regulate nuclear Arm activity

Having established the requirement of Pygo for Wg signaling in both embryogenesis and disc development, we next analyzed the position of Pygo action along the Wg signal transduction pathway. We first used the *Drosophila axin* mutant for an epistasis analysis. Axin is a scaffold protein required for efficient Arm degradation in the cytoplasm by facilitating the formation of the cytoplasmic destruction complex (Hamada et al., 1999; Salic et al., 2000; Willert et al., 1999a; Willert et al., 1999b). Loss of function of *axin* causes a constitutive activation of Wg signaling and thus results in phenotypes opposite to those of *wg* and *pygo*. If Pygo acts downstream of Axin, then a reduction in *pygo* should suppress a constitutively activated Wg signaling associated with the *axin* mutant. We used a *axin* null mutant to make a double *pygo-axin* mutant. As shown in Fig. 4, embryos mutant for null *axin* develop a 'naked cuticle' that lacks ventral denticles (Fig. 4A). By contrast, embryos that are double mutant for both *axin* and *pygo* exhibit 'a lawn of denticles' phenotype (Fig. 4B), resembling *pygo* embryos. We generated mosaic clones mutant for *axin* or *axin-pygo* using the 'direct mosaic system' by *vg Q1206-Gal4/* UAS-Flp. Mosaic clones of *axin* produce numerous ectopic bristles within the wing (Fig. 4C) and induce high level expression of Ac protein cell-autonomously (Fig. 4E,E′). By contrast, clones of *axin-pygo* cause wing notching phenotypes and the formation of ectopic margin bristles nearby wing notching (Fig. 4D), resembling the phenotypes associated with clones of *pygo* mutant cells (Fig. 1B). No induction of Ac expression was observed in *axin-pygo* mutant clones (Fig. 4F,F′). These results place Pygo downstream of Axin in the Wg signal transduction pathway.

We next examined the levels of Arm protein in the *pygo*

mutant. In response to Wg signaling, Arm protein levels are upregulated in a segmentally repeated fashion in the ventral ectoderm (Peifer et al., 1994b; Riggleman et al., 1990) (Fig. 4G). This expression pattern of Arm protein is disrupted in *wg* embryos (Peifer et al., 1994b; Riggleman et al., 1990). By contrast, Arm protein expression remains in a segmentally repeated fashion in embryos mutant for *pygo* (Fig. 4H). Furthermore, Arm protein is strikingly upregulated in clones mutant for *axin* (Fig. 4I,I′,I″). This upregulated Arm protein is not diminished in clones mutant for *axin-pygo* (Fig. 4J,J′,J″). We also observed no difference in subcellular localization of Arm protein in clones of *axin* and *axin-pygo (*Fig. 4I,I′,I″ and Fig. 4J,J′,J″). These results further suggest that Pygo is not involved in the post-translation control or subcellular localization of Arm protein.

In summary, from the experimental results described in Fig. 4, we conclude that Pygo acts downstream or in parallel with nuclear Arm protein.

pygo encodes a novel nuclear protein bearing a PHD finger

The mosaic clonal analyses and genetic epistasis studies place the Pygo activity downstream or in parallel with Arm protein, suggesting that *pygo* is likely to encode a nuclear protein. We mapped the *pygo* gene to cytological position 100 C6 near *axin* (see Material and Methods). Searches of annotated genome databases in this region identified several candidates bearing a nuclear localization signal (NLS). In a complementation test with available P-element insertions, we identified EP (3)1076 as a candidate for the gene encoding *pygo*. The following lines of evidence support our conclusion. First, the EP(3)1076 line fails to complement with any of our five *pygo* alleles. Second, The P-element in EP(3)1076 line is inserted 500 base pair (bp) upstream of a putative start codon of gene CG11518 that contains an open reading frame (ORF) encoding a putative protein of 815 amino acids (Fig. 5A). We identified point mutations in this ORF for all three *pygo* alleles characterized (Fig. 5B). $pygo^{F66}$ and $pygo^{F15}$ are nonsense mutations predicted to cause termination of translation at amino acid residues 69 and 104, respectively. Finally, a Pygo-GFP fusion protein is localized to the nucleus when expressed in human 293T cells (data not shown).

Pygo is a novel nuclear protein bearing a putative nuclear localization signal (NLS) in the N-terminal region (Fig. 5A). Searches of genome databases identified two homologs in both human and mouse (Fig. 5B). The most striking homology region is located in the C-terminal domain that contains 60 amino acids exhibiting high homologies to the PHD (plant homology domain) finger (Aasland et al., 1995). This domain contains the Cys4-His-Cys3 sequence of a PHD finger that has been found in an increasing number of proteins with roles in regulating transcription via modification of chromosome structure (Aasland et al., 1995). Interestingly, $pygo^{F107}$ is a point mutation that converts amino acid 802 cysteine into tyrosine, which is the conserved cysteine in the PHD finger, suggesting that the PHD finger in Pygo is essential for its function in Wg signaling.

Pygo protein forms a complex with Arm in vivo and contains a transactivation domain(s)

We have presented evidence that Pygo is a nuclear protein acting downstream or in parallel with Arm to regulate the nuclear function of Arm protein. One possible role of Pygo is to function as a co-activator that may further link Arm protein to the basal transcription machinery and/or to a chromatin remodeling complex(es). To test this possibility, we performed two experiments. First, we conducted a co-immunoprecipitation experiment to examine whether Arm and

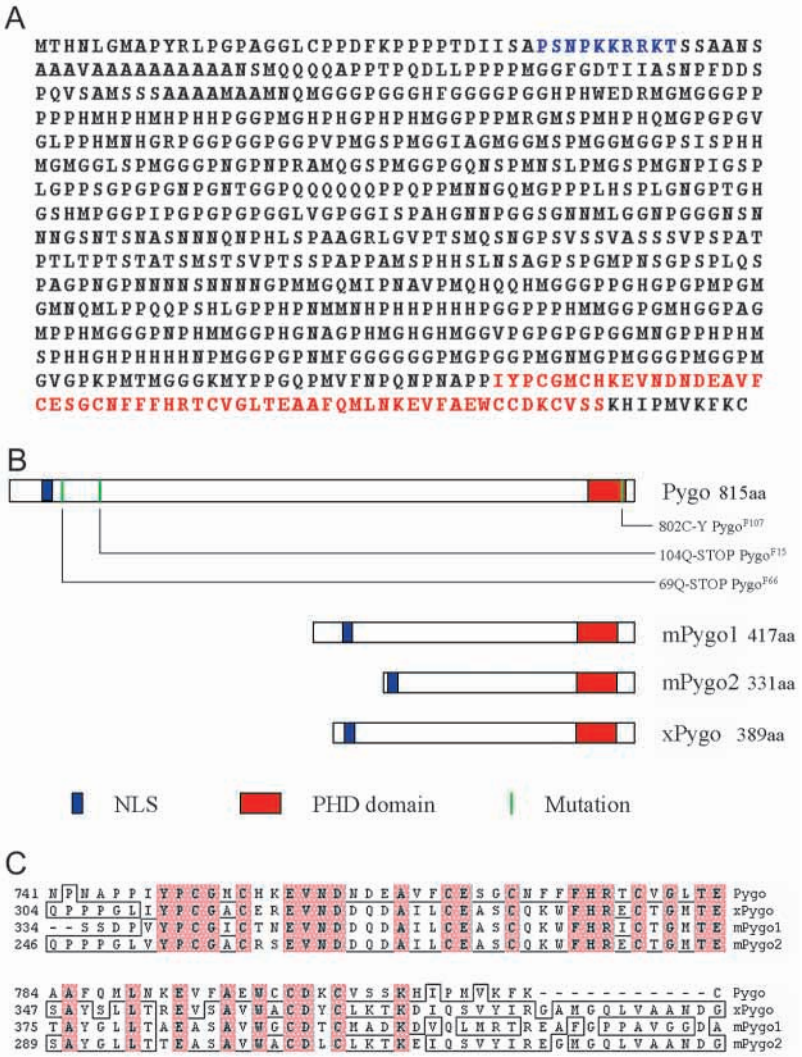

Fig. 5. *pygo* encodes a novel putative nuclear protein with a PHD finger domain. The amino acid sequence of *Drosophila* Pygo is shown in (A). A putative nuclear localization signal (NLS) is found in the N-terminal region (blue) and a PHD finger is found in C-terminal region (red). Three *pygo* mutants were sequenced and the mutations (green bars) are shown in B. Homologs of Pygo are also found in *Xenopus* and *Mus musculus* (B). They all have a NLS (blue box) at the N terminus and a PHD domain (red box) at the C terminus. The C-terminal regions of the four proteins are very conserved, and the alignment is show in C, which includes the PHD domains and the flanking sequences. The identical residues among four proteins are shown in pink and the similar residues are boxed (C).

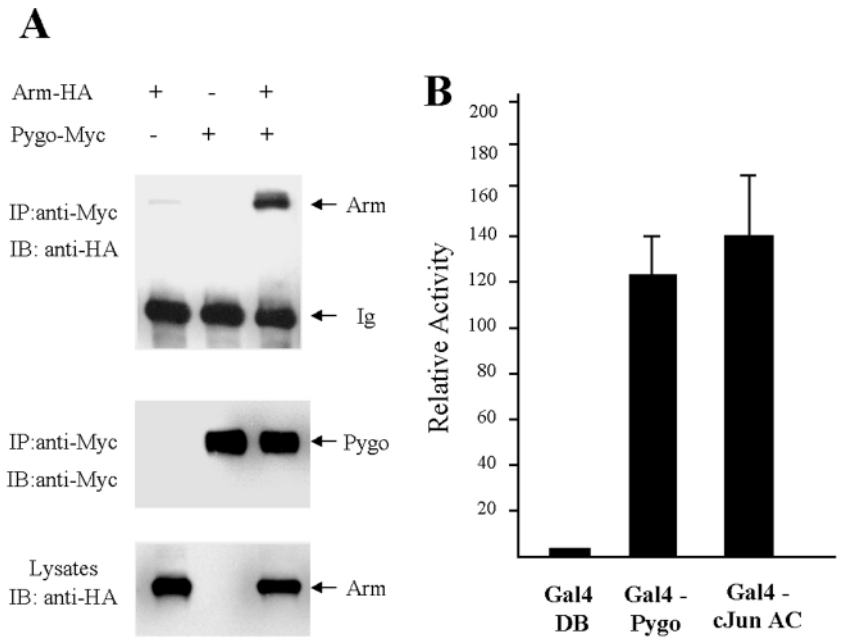

Fig. 6. Pygo protein forms a complex with Arm in vivo and contains a transactivation domain(s). (A) Co-immunoprecipitation of Pygo and Arm. 293T cells were transfected with plasmids expressing either Myc-tagged Pygo (amino acid 105 to 815) or HA-tagged Arm (amino acid 128 to 844) alone and together as indicated. Whole-cell lysates were prepared 36 hours after transfection. Lysates were immunoprecipitated with mouse monoclonal anti-Myc antibody. Immunoprecipitated materials and a fraction of each lysate were resolved by SDS-PAGE and analyzed by western blotting with antibodies as shown. IP, immunoprecipitation; IB, immunoblot. (B) Analysis of the transcription activation domain(s) in Pygo. Cells were transfected with the pG5E1b-luciferase reporter construct (Hsu et al., 1994) and with vectors expressing GAL4 DNA-binding domain alone (pM1) (Sadowski et al., 1992) or with GAL4-Pygo fusion protein. A GAL4-Jun AC-containing Jun activation domain (amino acids 5 to 89) fused with GAL4 was used as a positive control. Luciferase acitivities are expressed as relative activities compared with cells transfected with the plasmid containing the GAL4 DNA-binding domain alone.

Pygo proteins are present in a complex(es). HA-tagged Arm and Myc-tagged Pygo were either expressed individually or in combination in human 293T cells. Upon immunoprecipitation of Myc-tagged Pygo from cellular lysates of transfected cells, the Arm protein could be detected by western blotting in the immunoprecipitate (Fig. 6A), suggesting that Arm and Pygo proteins are present in a complex(es). Second, we wanted to determine whether Pygo contains any transactivation domain(s), by fusing a nearly full-length Pygo protein to a GAL4 DNA-binding domain. The Gal4-Pygo fusion protein strongly activated the transcription from a promoter containing Gal4 binding sites, suggesting that Pygo contains a transactivation domain(s) (Fig. 6B). Together, these findings demonstrate that Pygo protein forms a complex with Arm and possesses transactivation domain(s), two important properties characteristic of a co-activator (Maniatis et al., 1987; Tjian and Maniatis, 1994).

Kramps et al. have shown that Pygo interacts with Arm via Legless, an adaptor protein that links Pygo to Arm (Kramps et al., 2002). As the human homologs of Legless, BCL9 and its related protein are likely to be present in 293T cells, it is possible that the Arm-Pygo complex we observed may contain BCL9 and its related protein (Kramps et al., 2002).

Xenopus Pygo is required for Wnt signaling

The Wg/Wnt signal transduction pathway is conserved in both vertebrates and invertebrates (reviewed by Wodarz and Nusse, 1998). We thus examined whether Pygo is required for Wnt signaling in *Xenopus*. Using available databases, we identified a *Xenopus* oocyte EST with homology to human *pygo* over the PHD domain, likely to be the *Xenopus* homologue of *pygo*. We obtained a full-length cDNA of *Xenopus pygo* (*Xpygo*) using a RACE cDNA amplification strategy. Sequence comparison showed that XPygo has 40% and 70% similarities to the mouse Pygo1 and mouse Pygo2 respectively, particularly over the PHD domain terminus (Fig. 5C).

Wnt signaling is required for dorsal axis formation during *Xenopus* embryonic development through the activation of response genes (Harland and Gerhart, 1997). To examine the role of Xpygo in early *Xenopus* development, we designed antisense deoxyoligonucleotides for injection into *Xenopus* oocytes, to deplete the maternal store of *Xpygo* mRNA. The oligo that gave the best depletion, as assayed by real-time RT-PCR, was prepared in a modified chimeric phosphorothioate/phosphodiester version, and injected vegetally into oocytes. This oligo (2 ng) depleted maternal *Xpygo* mRNA to approximately 25% of control levels (Fig. 7A). Although higher doses gave better depletion, they were also more toxic once the oocytes were fertilized. Embryos obtained via the host transfer procedure from oligo-injected oocytes showed a ventralized phenotype at the tailbud stage, lacking head and tail structures, with both the anteroposterior and dorsoventral axes affected by loss of *Xpygo* (Fig. 7B). This ventralized phenotype was also seen by molecular analysis of sibling embryos at the late blastula and early gastrula stages, as expression of the dorsal markers *chordin*, *Xnr3*, *siamois* and *goosecoid* was reduced in injected embryos at stages 9.5 and 10.5, relative to control uninjected embryos (Fig. 7C). We note that *Xnr3* was more affected by loss of *Xpygo* than *siamois*, even though both genes are known to be direct targets of the Wnt dorsalization pathway (Brannon et al., 1997; McKendry et al., 1997). We did not observe a significant change in the expression of the pan-mesodermal marker *Xbra*, the ventral markers *Xwnt8* and *Bmp4*, or the endodermal marker Xsox17α at these stages (Fig. 7C).

DISCUSSION

Drosophila Arm and its vertebrate homolog β-catenin are key components in the Wg/Wnt signal transduction pathway that plays essential roles in numerous developmental processes. In response to Wg/Wnt signaling, up-regulated Arm/β-catenin enters the nucleus to form a bipartite transcription factor

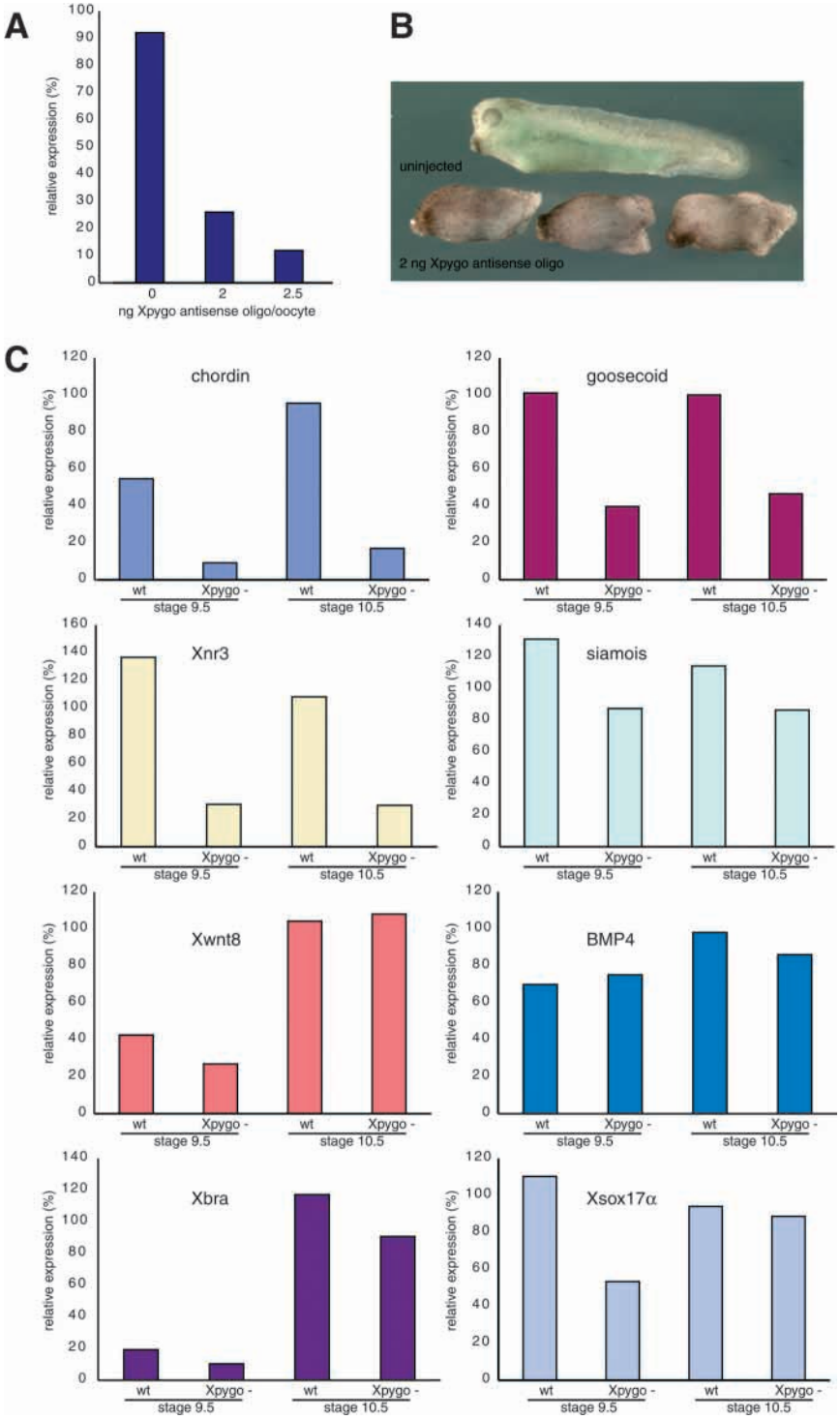

Fig. 7. Xpygo depletion results in a ventralized phenotype. (A) Maternal *Xenopus pygo* (*Xpygo*) mRNA transcripts are depleted in a dose-dependent manner upon injection of an antisense *Xpygo* oligo into oocytes. mRNA levels were assayed 24 hours after oligo injection. (B) Uninjected control embryo (above; blue) at stage 36, and embryos derived from sibling oocytes injected with antisense *Xpygo* (below; mauve). *Xpygo*$^{-}$ embryos are ventralized. (C) Relative expression of markers normalized to *ODC* in wild type and *Xpygo*$^{-}$ embryos at late blastula (9.5) and gastrula (10.5) stages. Expression of dorsal markers is reduced in *Xpygo*$^{-}$ embryos.

complex with TCF, which activates transcription of Wg/Wnt target genes. However, the mechanisms of how the Arm/β-catenin-TCF complex promotes target gene activation are poorly understood. In this report, we used a genetic screen in *Drosophila* to identify *pygo* encoding an essential component of the Wg signal transduction pathway. We further isolated *Xenopus pygo* and examined its role during *Xenopus* embryogenesis. Our findings provide strong genetic and molecular evidence that Pygo is an essential component in the Wg/Wnt signal transduction pathway.

As we were preparing this manuscript for publication, the molecular and phenotypic analysis of *legless* (Kramps et al., 2002) and *pygo* by others (Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002) were reported. In this report, we have provided more substantial analysis of *pygo* in Wg signaling in *Drosophila*. We have also provided the first evidence for a role of a *Xenopus pygo* in Wnt signaling during *Xenopus* embryogenesis. Our results are consistent with and complement others for a role of Pygo in Wg/Wnt signaling. Our results are also in agreement with a model that Pygo functions as a transcription co-activator required for the nuclear activity of Arm/β-catenin (Kramps et al., 2002).

Pygo is required for Wg/Wnt signaling in animal development

Our detailed functional analyses of the *pygo* mutant strongly argue that Pygo is an essential component in the Wg signal transduction pathway and is likely to be required universally for all the Wg signaling events in embryogenesis and imaginal disc development. Two lines of evidence support this conclusion. First, Wg signaling in *pygo* mutants is defective in all the embryonic developmental processes examined, including ventral cuticle patterning, midgut constriction, embryonic central nervous system and specification of cardiac precursor cells. Second, Pygo is required for cells to respond to Wg input, for both positive and negative gene regulation in imaginal disc development. This is in contrast to other genes such as *teashirt*, which is specifically required for a subset of late Wg-dependent functions in the embryonic trunk segments where the *teashirt* gene is expressed (Gallet et al., 1999). So far, we are not aware of any tissue in which Wg transduces its signaling in the absence of Pygo activity.

The Wg/Wnt signal transduction pathway is conserved in both vertebrate and invertebrate (reviewed by Wodarz and Nusse, 1998). Our loss-of-function studies in *Xenopus* provide

strong evidence that Pygo is also required for Wnt signaling in vertebrate development. The *Xenopus* homolog of Pygo, XPygo, shares a significant degree of homology with *Drosophila* Pygo, particularly in the PHD finger domain at the C-terminal region. Depletion of maternal *Xpygo* mRNA by antisense oligos led to ventralized embryonic defects and a reduction in the expression of various Wnt target genes. These results are consistent with the role of Pygo in *Drosophila*, suggesting that XPygo is crucial for Wnt signaling in embryonic development in *Xenopus*. Consistent with our results, Thompson et al. have shown that a disruption of human PYGO1 and PYGO2 by double-stranded (ds) RNA interference (RNAi) led to a reduction of the expression of β-catenin/TCF target gene expression in colorectal cancer cells (Thompson et al., 2002). Transfection of PYGO1 can enhance the TCF-mediated transcription in transient transfection assays (Kramps et al., 2002). Together, these results strongly suggest that Pygo is essential for Wnt signaling in vertebrates as well.

The PHD finger in Pygo is crucial for its function in Wg/Wnt signaling

pygo encodes a novel and evolutionarily conserved protein. The most strikingly homologous domain is located in the C-terminal region that contains a PHD finger domain. The PHD finger is a domain of 60 amino acids characteristically defined by seven cysteines and a histidine that are spatially arranged in a consensus of C4HC3 of varying lengths and composition (Aasland et al., 1995). This evolutionarily conserved domain is predicted to chelate two zinc ions and is similar to, but distinct from, other zinc-binding motifs such as the RING finger (Cys3-His-Cys4) and LIM domain (Cys2-His-Cys5) (Aasland et al., 1995; Borden, 1998; Capili et al., 2001; Wu et al., 1996). PHD finger domains have been found in many different proteins, including transcription factors and are the targets of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Aasland et al., 1995; Capili et al., 2001). In many cases, they serve as protein-protein interaction motifs involved in the formation of multi-protein complexes. Our genetic analyses provide strong evidence that the PHD finger in Pygo plays a crucial and specific role in Wg signaling. We have found that Wg signaling is defective in both embryogenesis and imaginal disc development in the $pygo^{F107}$ mutant. $pygo^{F107}$ contains a point mutation that converts amino acid 802 cysteine into tyrosine, which is the last conserved cysteine in the PHD finger. Both structural determination and mutational analysis suggest this is a critical residue for the function of the PHD finger domain (Capili et al., 2001). Interestingly, while both $pygo^{F15}$ and $pygo^{F66}$, two null alleles of *pygo,* have defects in addition to those in Wg signaling in embryogenesis [they exhibit pair-rule like phenotypes that have denticle deletions (data not shown)], $pygo^{F107}$ exhibits only defects specifically associated with Wg signaling. Thus, our results suggest that the PHD finger domain in Pygo may provide a specific motif that is dedicated to Wg signaling, possibly involving the formation of a Pygo-Arm multi-protein complex(es). Consistent with our studies, Kramps et al. have shown that Pygo interacts with Arm via Legless (Kramps et al., 2002). The PHD domain in Pygo is required for the interaction between Pygo and Legless. The $pygo^{130}$ allele used in their work is a specific deletion in PHD domain. We have observed additional embryonic defects associated with both $pygo^{F15}$ and $pygo^{F66}$ null alleles (data not shown). Similar results have also been observed by Parker et al. (Parker et al., 2002), suggesting that the remaining portion of Pygo has an additional role in embryonic development. We are currently investigating the role of Pygo in regulating pair-rule gene expression in embryonic development.

Mechanism(s) by which Pygo is involved in Wg signaling

To understand the molecular mechanism(s) by which Pygo participates in Wg signaling, we have carried out detailed genetic epistasis analysis and molecular studies. Our results support a model in which Pygo acts as a transcription co-activator required for activation of Wg/Wnt target genes. The following evidence supports this conclusion. First, our genetic epistasis analysis in both embryos and wing disc placed Pygo downstream of Axin. Further experiments have demonstrated that Pygo is not involved in the post-translation control and subcellular localization of Arm protein. These results thus provide strong evidence that Pygo acts downstream or in parallel with Arm to regulate the nuclear function of Arm activity. Second, consistent with genetic epistasis analysis, we found that Pygo contains a nuclear localization signal and is localized in the nuclei when Pygo-GFP fusion protein is expressed in 293T cells (data not shown). The co-immunoprecipitation experiment provided molecular evidence that Arm and Pygo proteins are present in vivo in a multi-protein complex. Finally, like many other co-activators that can activate transcription when fused to a DNA binding domain(s) (Maniatis et al., 1987; Tjian and Maniatis, 1994), we have also observed that Pygo has an intrinsic activation function when examined as a GAL4 fusion protein. Our results in this report are in agreement with the model proposed by Kramps et al., in which Pygo is linked to Arm protein via Legless and acts as a transcription co-activator required for the activity of Arm/β-catenin-Tcf complex. It remains to be determined whether Pygo recruits the Arm/β-catenin-Tcf complex to the basal transcriptional machinery or to chromatin remodeling complexes.

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