**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; Molemaar 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) (Hecth 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; FRT2A FRT82B /TM3 were mutagenized with EMS and crossed to females of the genotype w; Q1206-Gal4 UAS-flp; FRT2A /TM3. F1 flies of the genotype w; Q1206-Gal4 UAS-flp+/; FRT2A FRT82B /FRT2A FRT82B 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 FRT2A FRT82B chromosome. The wings of the resulting F1 flies were screened for wing notches and ectopic margin bristles. The F1 male flies with interesting wing phenotypes were further crossed with females of the genotype w, Dv/TM3. The resulting F2 flies with genotype of FRT2A FRT82B /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; FRT2A /TM3 or with w; vg Q1206-Gal4 UAS-flp; FRT82B /TM3. 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; FRT82B ubiquitin-GFP /TM6C females were crossed with FRT82B pygo (or axin or pygo-axin) /TM6B. Larvae of the genotype y w hsFLP; FRT82B pygo (or axin or pygo-axin) / FRT82B 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-DI at 1:500 (a gift from I. Duncan), monoclonal anti-Engrailed 4D9 at 1:500 (a gift from I. Duncan), monoclonal anti-Even-skipped 2B8 at 1:10 (IDSIB) and monoclonal anti-Arm N27A1 at 1:5 (IDSIB). 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 NcoI-EcoRI 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 EcoRI-XbaI 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).
Cell culture, transient transfections and reporter gene assays

Human 293T cells (5×10⁵) 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 (2×10⁶) 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 MgCl₂, 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 1 mM PMSF and 10 μl/ proteinase inhibitor lyser (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*GCGCCGTTTTCTT*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 0.1xMMR.

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.

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

<table>
<thead>
<tr>
<th>PCR primer</th>
<th>Reference</th>
<th>Sequence</th>
<th>Denat. temp°C</th>
<th>Anneal temp°C/ time (sec)</th>
<th>Extension temp°C/ time (sec)</th>
<th>Acquisition temp°C/ time (sec)</th>
</tr>
</thead>
</table>
| 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'-GCG AGG ATT TAG AGT TG-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 GGC 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 GGT GA-3 | 95 | 55/5 | 72/16 | 81/3 |
| Xbra       | Sun et al., 1999 | U: 5'-TTC 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 |
| Xnr3       | 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 CTC CAC ACA AGA TG-3' | 95 | 55/5 | 72/12 | 90/3 |
| Xsox17α    | 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 GGT TGC ATT GCT CGC-3 | 95 | 58/6 | 72/14 | 85/3 |

D, downstream primer; U, upstream primer.
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 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 pygoF66 (B) cause wing notching and the formation of ectopic bristles in nearby tissues. The wing phenotypes associated with pygoF66 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 pygo mutant embryos derived from germline clones are shown for pygoF15 (E), pygoF66 (F) and pygoF107(H). A pygo mutant embryo shown in G was derived from a homozygous pygoF15 mutant germline clone and paternally mutant for pygoF66.

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 pygoF66 and pygoF15, we generated embryos maternally mutant for pygoF107 and paternally mutant for pygoF66. These pygo mutant embryos, referred to as pygoF107 glc/ pygoF66, 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 pygoF107 glc/ pygoF66 embryos.

First, we examined the expression of en and wg in pygoF107 glc/ pygoF66 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
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. 2F), resembling the phenotype 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.

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 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.

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 pygoF107glc/pygoF66 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 pygoF107glc/pygoF66 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 pygoF15, 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 pygoF15. These results argue that Pygo is required for both Wg short- and long-range activities in wing patterning.
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 arr (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 pygoF15 (data not shown). As pygoF15 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 pygoF15. 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 pygoF15 (Fig. 3H,H'). We also observed no alteration of Dpp signaling in mosaic clones mutant for pygoF15, as analyzed by anti-phosphorylated MAD antibody staining (Tanimoto et al., 2000) (data not shown).
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’). In a axin-pygoF15 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’). This upregulated Arm protein is not diminished in clones mutant for axin-pygo (I,J,J’). There is no difference in the subcellular localization of Arm protein in clones of axin and axin-pygo (compare I,I’ with J,J’).

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-pygoF15 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-pygoF15 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 pygoF15 clones (see Fig. 1B,C). A axin somatic clone in the wing disc produces autonomously the expression of Ac (E,E’). In a axin-pygoF15 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’). This upregulated Arm protein is not diminished in clones mutant for axin-pygo (I,J,J’). There is no difference in the subcellular localization of Arm protein in clones of axin and axin-pygo (compare I,I’ with J,J’).
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\(^\prime\), I\(^\prime\prime\)). This upregulated Arm protein is not diminished in clones mutant for \( axin-pygo \) (Fig. 4J, J\(^\prime\), J\(^\prime\prime\)). 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...
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 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...
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 (Ausland 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) (Ausland 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) (Ausland 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 pygoF107 mutant. pygoF107 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 pygoF15 and pygoF66, 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)], pygoF107 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 pygo130 allele used in their work is a specific deletion in PHD domain. We have observed additional embryonic defects associated with both pygoF15 and pygoF66 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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