Pygopus Residues Required for its Binding to Legless Are Critical for Transcription and Development*

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Fiona M. Townsley, Barry Thompson, and Mariann Bienz‡
From the Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, United Kingdom

Pygopus and Legless/Bel-9 are recently discovered core components of the Wnt signaling pathway that are required for the transcriptional activity of Armadillo/β-catenin and T cell factors. It has been proposed that they are part of a tri-partite adaptor chain (Armadillo>Legless>Pygopus) that recruits transcriptional co-activator complexes to DNA-bound T cell factor. Here, we identify four conserved residues at the putative PHD domain surface of Drosophila and mouse Pygopus that are required for their binding to Legless in vitro and in vivo. The same residues are also critical for the transactivation potential of DNA-tethered Pygopus in transfected mammalian cells and for rescue activity of pygopus mutant embryos. These residues at the Legless>Pygopus interface thus define a specific molecular target for blocking Wnt signaling during development and cancer.

Wnt signaling controls the transcription of genes that have important functions during normal and malignant development (1–3). A key effector of the canonical Wnt pathway is β-catenin, or Drosophila Armadillo. This protein is normally degraded rapidly, but is stabilized in response to Wnt signaling and translocates to the nucleus where it binds T cell factors (TCF)1 to stimulate the transcription of Wnt target genes. Thus, β-catenin/Armadillo functions as a transcriptional co-activator (4, 5). This activity is thought to involve the β-catenin-mediated recruitment of chromatin modifiers and remodelling factors such as CREB-binding protein (CBP) and Brg-1 (6–8).

Recently, two new core components of the Wingless signaling pathway have been discovered by Drosophila genetics, called Pygopus (Pygo) and Legless (Lgs) (9–12). These components are found in the nucleus, and have closely related counterparts in mammals (Pygo1, Pygo2, and Bel-9). The transcriptional activity of Armadillo/β-catenin depends on these factors. Pygo contains a PHD domain that binds to Lgs/Bel-9 in vitro, whereas Lgs/Bel-9 binds to the Armadillo repeat domain (ARD) of Armadillo/β-catenin. Thus, Lgs/Bel-9 serves as an adaptor between Armadillo/β-catenin and Pygo; the latter two proteins can be co-immunoprecipitated from Drosophila and mammalian cells. It has been suggested that Pygo and Lgs mediate transcriptional co-activation or access to chromatin, but their precise molecular functions are unknown.

One of the main interests in Pygo and Lgs/Bel-9 regarding cancer is that they provide potentially new targets for Wnt signaling inhibitors. Indeed, interference with hPygo1 or hPygo2 function by double-stranded RNA reduces the high levels of TCF/β-catenin-mediated transcription in colorectal cancer cells (10). Thus, we set out to characterize the interaction between Pygo and Lgs at the molecular and cellular biological levels. Here, we identify conserved residues within Pygo and mPygo1 that are required for their binding to Lgs in vitro and for their activity in mammalian transcription assays. Rescue assays in Drosophila demonstrate that these residues are essential for Pygo to function during development. We also identify additional conserved residues within the PHD domain of Pygo that contribute to its transcriptional activity in mammalian cells.

EXPERIMENTAL PROCEDURES

Plasmids and in Vitro Binding Assays—The PHD domains of Pygo (residues 735–815), mPygo1 (228–319), and full-length mPygo1 were subcloned into pGex-2TK (Amersham Biosciences) using standard PCR cloning procedures and were expressed in Escherichia coli BL21. LgsHD1 + 2 (232–555) was subcloned into pG778, 5S-labeled protein was generated by in vitro transcription/translation, and in vitro pull-down assays were performed as described (10). For expression in mammalian cells, full-length Lgs and LgsHD1 + 2 (9) were tagged N-terminally with the V5 epitope tag, full-length mPygo1, with a triple hemagglutinin (HA) tag, or with green fluorescent protein (GFP), and subsequently subcloned into pCDNA3.1 (Invitrogen). The following plasmids were also used for mammalian cell expression: HA-Pygo and HA-hPygo2 in pCDNA3.1, hPygo2 in pCS2 (10); hPygo1 (9); pCDNA-dTCF, pCS2-Armadillo (4); activated β-catenin (Δ45-β-catenin) (13); and hBel-9 in pCDNA3.1 (kindly provided by W. de Lau and H. Clevers). For GDB fusions, full-length Pygo and PygoΔPHD (1–749) were subcloned into pBIND (Promega); this vector also encodes renilla luciferase (as an internal control). pGLuc (Promega) was used to measure the transcriptional activity of GDB-Pygo fusions. Point mutations (see Fig. 1A) were introduced with the QuikChange mutagenesis kit (Stratagene), and each construct was confirmed by sequencing. mLoop1 is a quadruple (E756A, N758A, D759A, D761A), and mLoop2 is a quintuple mutant (L781A, T782A, E783A, A785V, L788A).

Cell Transfections and Transcription Activation Assays—293T cells were grown and transfected as described (10); unless specified otherwise, 1–2 μg of DNA were used per well of a six-well plate. Cells were harvested for transcription assays or fixed and stained with antibody 24 h after transfection as described (14). Rat anti-HA antibody (3F10; Roche Applied Science) and mouse anti-V5 antibody (Invitrogen) were used. TOPFLASH assays (see Fig. 3; Ref. 15) were performed as described (internal control, CMV-renilla; Ref. 14), using the Dual Luciferase Reporter Assay System (Promega). Relative luciferase values (see Fig. 4) were obtained from duplicate samples by dividing the firefly luciferase values obtained from pGLuc by the renilla luciferase values obtained from SV-renilla included in the GDB-Pygo fusion plasmids; averages from at least three different experiments were determined. GDB-Pygo fusions were not detectable by Western blotting, presumably because of lack of sensitivity of the anti-GAL4 antibody (Clontech), but each construct was detectable as HA-tagged protein and expressed at equivalent levels in transfected 293T cells (see Fig. 2, not shown).

Drosophila Strains and Rescue Analysis—Fly transformants of full-length HA-Pygo and various Pygo mutants (subcloned into pUAST; Ref.

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† To whom correspondence should be addressed. Tel.: 1223-402-055; Fax: 1223-412-142; E-mail: mb2@rnc-lmb.cam.ac.uk.
‡ The abbreviations used are: TCF, T cell factor; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; Pygo, Pygopus; Lgs, Legless; ARD, Armadillo repeat domain; HA, hemagglutinin; GFP, green fluorescent protein; NLS, nuclear localization signal.

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16) were generated by standard procedures. A transformant-bearing untagged Pygo (11) was also used. Overexpression in the wing and co-expression in the eye with activated Armadillo (8) were used for comparative assessment of the activities of wild-type and mutant Pygo among the different transformant lines. These revealed only small variations between different lines, most of which express comparable levels of protein as judged by Western blot analysis (with anti-HA antibody; see above).

For the rescue assays, wild-type and mutant HA-tagged Pygo were expressed with the ubiquitous driver daughterless GAL4 in germ-line clones of the null allele pygoS123 and of the hypomorphic allele pygoS28 (10). pygo mutants lacking maternal and zygotic Pygo were identified on the basis of GFP-marked balancers; GFP-negative embryos lack maternal and zygotic pygo function; one-half of these carry the GAL4 driver and thus express the Pygo rescue construct. Each construct was tested in two to three independent experiments. In the case of wild-type Pygo or mLoop1, the GFP-negative embryos fell into two clear classes of homogeneous phenotypes: (i) denticle lawns as observed after loss of maternal and zygotic pygo function (see Fig. 5B), or (ii) rescued cuticles (see Fig. 5D) as those observed after zygotic (paternal) rescue (see Fig. 5C). In the case of pygoS28, the "rescued cuticle" class looked essentially wild-type, with only minor segmentation defects (like the paternally rescued pygoS28 cuticles; not shown). The following percentages of rescued cuticles were observed: pygoS123, 64%, 65%, and 47% (wild-type Pygo; total of 79 embryos scored); 53%, 59%, and 50% (mLoop1; total of 74 embryos scored); pygoS28, 50%, 50%, and 51% (wild-type Pygo; total of 81 embryos scored); 53%, 52%, and 43% (mLoop1; total of 99 embryos scored). In the case of mLoop2, 100% denticle lawn cuticles were observed (for pygoS123, a total of 79 GFP-negative embryos were scored, see Fig. 5D; for pygoS28, a total of 33 GFP-negative embryos were scored, not shown), which is indistinguishable from those due to maternal and zygotic pygo loss (see Fig. 5B; not shown).

RESULTS

In Vitro Binding between Pygo and Lgs—To determine the residues within the PHD domain of Pygo that are required for its binding to Lgs, we individually mutated each conserved residue predicted to be at the surface (17, 18) and also the second of the putative zinc-coordinating cysteine residues (Cys-2; mutation C753A) (Fig. 1, A and B). We expressed the wild-type and mutant domains fused to glutathione S-transferase-PHD domains of Pygo (C), or mPygo1 (D), as indicated above lanes. Input was 10% of the total binding reaction.
Armadillo (Fig. 1C, lane 14). Furthermore, the C753A mutation drastically reduces the binding of the PHD domain to Lgs (Fig. 1B, lane 5), confirming the predicted structural importance of Cys-2. Of the other 15 point mutations, only 3 have a comparable effect on binding (namely L781A, T782A, and L789A) and one further mutation (A785V) reduces the binding significantly (Fig. 1C, lanes 8–10, 20). The other mutations have little or no effect on the binding of Pygo to Lgs (Fig. 1C). These results identify Leu-781, Thr-782, Ala-785, and Leu-789 as four Pygo residues that are critical for the binding of Pygo to Lgs.

Interestingly, these residues are all located in the same loop (Loop 2) between the zinc-coordinating cysteines Cys-5 and Cys-6 (Fig. 1B). Furthermore, if the PHD domain is projected onto the structurally related RING finger domain (from c-Cbl, a Glu-3 ubiquitin ligase), the four Loop2 residues from Pygo span a region that corresponds to an α-helical surface portion of c-Cbl that contacts its binding partner Rad18 (a Glu-3 ubiquitin ligase) (19). Conversely, Loop1 residues (between Cys-2 and Cys-3; Fig. 1B) are predicted to be on the opposite surface of the PHD domain; Loop1 mutations do not substantially affect the binding between Pygo and Lgs (Fig. 1B, lanes 6, 7, 15–17). Thus, the Loop1 surface does not seem to be involved in binding to Lgs, whereas the above-mentioned four Loop2 residues of Pygo may all directly contact Lgs.

These Loop2 residues are either identical (Thr-782, Ala-785, Leu-789) or substituted by a similar amino acid (methionine instead of Leu-781) in mouse and human Pygo proteins (Fig. 1A). To test the function of the corresponding mammalian residues, we mutated two on each surface and also the structural Cys-2 residue in mPygo1 (Fig. 1A) and compared their in vitro binding to Lgs HD1 + 2 to that of wild-type mPygo1. This confirmed that Cys-2 and the two mutated Loop2 residues are critical for binding between mPygo1 and Lgs (Fig. 1D, lanes 5, 8, 9), whereas the Loop1 mutations do not substantially affect the binding (Fig. 1D, lanes 6, 7). Thus, the binding between Lgs and Pygo proteins is highly comparable between Drosophila and mammals.

In Vivo Binding between Pygo and Lgs—Next, we introduced a subset of the same mutations into full-length Pygo and expressed wild-type and mutant Pygo together with Lgs HD1 + 2 in mammalian 293T cells. In the absence of Pygo, the Lgs fragment is largely cytoplasmic in these cells, although some nuclear staining is also observed (Fig. 2A). However, after co-transfection with Pygo, which itself is exclusively nuclear in these cells (10), HD1 + 2 is shifted efficiently into the nucleus (Fig. 2B), most likely because of its binding to Pygo. In support of this, HD1 + 2 is also shifted into the nucleus by the C-terminal fragment of Pygo that spans the PHD domain (Fig. 2C), whereas its nuclear-cytoplasmic distribution does not change if it is co-transfected with a Pygo deletion that lacks the PHD domain (Fig. 2D). The same is true for the Cys-2 mutation (Fig. 2E), although Cys-2 appears to aggregate somewhat in the nucleus (presumably because of misfolding), trapping the nuclear HD1 + 2 into these nuclear dots. Importantly, neither...
FIG. 3. The transactivation potential of Pygo proteins in mammalian cells. 293T cells were co-transfected with pTOPFLASH and plasmids encoding various Pygo proteins (A, 350 ng; B and C, 250 ng; D, 300 ng), activated β-catenin (Δ45-β-cat; 300 ng, or as indicated) or Armadillo (Arm) (C, 250 ng; D, 100 ng), dTCF (250 ng), and Lgs or LgsHD1 + 2 (300 ng or 600 ng, as indicated); relative luciferase values were determined (internal control, CMV-renilla; see “Experimental Procedures”). hPygo1 (B) was obtained from Ref. 9. Note that these transcription assays are exquisitely sensitive to the amounts of co-transfected activated β-catenin or Armadillo; the highest amounts used (300 ng) are still rate-limiting. Results similar to those shown in (D) were obtained if 50 ng (instead of 100 ng) of co-transfected Armadillo were used (not shown). The mild stimulation of TOPFLASH activity that can be observed with Pygo proteins in the presence of low amounts of Δ45-β-catenin (A) or Armadillo (D) appears to be insignificant because the same stimulation is seen also with mPygo1 lacking its C terminus (HA-mPygoΔPHD; see A).
of the two Loop2 mutations we tested (L789A and L781A) affects the nuclear-cytoplasmic distribution of HD1 + 2 (Fig. 2F; not shown), whereas the two Loop1 mutants (N758A and D761A) are fully capable of shifting HD1 + 2 into the nucleus (Fig. 2G; not shown). Thus, the Loop2 residues are critical for the in vivo interaction between Pygo and Lgs, whereas the Loop1 residues are irrelevant for this interaction.

The same is essentially true for mPygo1. The full-length protein shifts HD1 + 2 into the nucleus as efficiently as Pygo (Fig. 2F), as do the two Loop1 mutants in mPygo1 (Q254A and N251A; Fig. 2K; not shown). In contrast, no change is seen with the Cys-2 mutant (Fig. 2L), nor with the Loop2 mutant L282A (Fig. 2M), confirming the importance of this loop. The other Loop2 mutant (T275A) still facilitates nuclear uptake of HD1 + 2 (Fig. 2N) and is thus not severe enough to abolish the in vivo interaction between mPygo1 and Lgs, despite reducing their in vitro binding (Fig. 1D, lane 8).

We also tested the conserved N-terminal residues of Pygo for their role in its in vivo interaction with Lgs. These include a nuclear localization signal (NLS) and the N-box (10) that spans a series of proline residues and an NPFXD motif (Fig. 1A). The latter can be an internalization signal (20) and binds to a variety of endocytic proteins (21). We generated three sets of point mutations in the N terminus (Fig. 1A) and tested the mutants in transfected 293T cells with and without HD1 + 2. Each of these mutants is fully competent in shifting Lgs to the nucleus (Fig. 2, H and I; not shown), confirming that they are not required for the Lgs>Pygo interaction in the context of full-length Pygo. Notably, the Nlns mutation only weakly reduces the nuclear accumulation of Pygo (Fig. 2H), which is consistent with our observation that the C terminus of Pygo is nuclear despite not containing an NLS (Fig. 2C). Thus, the NLS and the C terminus of Pygo function redundantly to mediate its nuclear accumulation. In the case of the C terminus, this may be caused by a nuclear protein that binds to the PHD domain and anchors it in the nucleus.

Transcriptional Activation by DNA-tethered Pygo—It has been reported (15) that overexpressed hPygo1 stimulates the transcription of a luciferase reporter linked to TCF-binding sites (pTOPFLASH) in transfected 293 cells ~30-fold if co-expressed with activated β-catenin, suggesting that Pygo may act as a transcriptional co-activator (9). However, in our own experiments in transfected 293T cells, we observed, at best, a very mild transcriptional stimulation (~2×) with any of the mammalian Pygo proteins in the presence of varying amounts of activated β-catenin (Fig. 3, A and B). However, this stimulation seems to be insignificant because we see the same stimulation with a Pygo protein whose PHD domain has been deleted (Fig. 3A). The same is true for Drosophila Pygo, which did not stimulate TCF-mediated transcription by itself, in the presence of Armadillo, and/or in the presence of tTCF (Fig. 3C). Notably, co-expression of increasing amounts of Lgs, Lgs HD1 + 2, or hBcl-9 does not improve the transcriptional stimulation (Fig. 3D; not shown). Therefore, we conclude that overexpressed Pygo proteins cannot stimulate the transcription of TCF target genes in these cells, neither alone nor in the presence of co-expressed Lgs/Bcl-9 or TCF.

However, if tethered to DNA with the DNA-binding domain of GAL4 (GBD-Pygo), full-length Pygo efficiently stimulates transcription of a luciferase reporter linked to GAL4 upstream activating sequences (Fig. 4). A similar stimulation has also been observed with a GDB fusion of Pygo that lacks its N terminus (12), suggesting that the latter is dispensable for transactivation. Consistent with this, the activity of GDB-Pygo is >10-fold reduced if its PHD domain is deleted (Fig. 4; note that the expression levels of the Pygo mutants are all comparable with those of full-length wild-type Pygo; Fig. 2). Likewise, the Cys-2 mutant is equally inactive in stimulating transcription (Fig. 4). Thus, Pygo can stimulate transcription efficiently if tethered to DNA; this activity depends on its PHD domain.

Next, we tested our mutants as GDB-Pygo fusions. Individual point mutations only mildly reduce transactivation, maximally ~2.8× (C753A; Fig. 4). Of the Loop2 mutations, the most severe one is L781A (~<2× reduction; Fig. 4). Interestingly, the most severe reduction (apart from C753A) is observed with the Loop1 mutation D761A (Fig. 4). Other Loop1 and Loop2 mutations also show mild effects, and multiple mutations in the same loop (mLoop1, mLoop2) have more severe effects, whereas maximal effects are seen with simultaneous mutations in both loops (Fig. 4). We conclude that residues in both surface loops of the PHD domain contribute to the transcriptional activity of DNA-tethered Pygo. This conclusion implies that this activity not only depends on its binding to Lgs, but also on its ability to bind to another protein. The latter is unlikely to be Pygo itself, because we have not been able to detect dimerization of Pygo nor of its C-terminal fragment in our in vitro or in vivo binding assays (not shown). Therefore, an unknown Loop1-binding protein seems to synergize with Lgs in transcriptional activation.

Loop2 Residues Are Essential for Rescue of pygo Mutants—
The transcriptional activity of DNA-tethered Pygo measured in our transfection assays is likely to depend on a variety of endogenous proteins, e.g. hPygo1 and hPygo2, which may mask the functional importance of specific residues under test. Thus, we used the GAL4 system to express three of the Pygo mutants in Drosophila embryos to see whether they could rescue pygo mutants that lack endogenous Pygo.

Wild-type Pygo expressed ubiquitously in the embryo rescues the "denticile lawn" phenotype of pygo mutants to a considerable extent (Fig. 5D, compare with A and B; see also "Experimental Procedures"). Indeed, the rescued cuticles look essentially the same as those from paternally rescued pygo A, compare with pygo A). However, this is not the case with maternally rescued pygo, where the rescued cuticles appear wild-type (Fig. 5D, compare with C and D). If we compare the rescued Loop1 residues are either not required in untethered Pygo, or they function redundantly with other Pygo sequences in flies. Alternatively, the loop1 mutant may not be completely defective in binding to its cognate protein. In contrast, the mLoop2 mutant does not rescue pygo mutants at all (Fig. 5F, compare with B;
notably, this mutant is expressed at normal levels in embryos and produces similar phenotypes in the wing and eye as does wild-type Pygo; see “Experimental Procedures”). The same results were obtained in rescue assays of a hypomorphic pygo allele (pygo<sup>z20</sup>); in this case, wild-type Pygo, Nnpf, and mLoop1 fully restore the cuticles to wild-type (not shown; see “Experimental Procedures”). Thus, the four conserved Pygo Loop2 residues implicated in Lgs binding are critical for the function of Pygo during embryonic development.

**DISCUSSION**

We have identified four conserved residues in the PHD domain of Pygo required for its binding to Lgs that are critical for its function in transcription and embryonic development. These residues are predicted to be in a contiguous surface patch of the PHD domain (17, 18) and are thus likely to contact Lgs directly. Given the functional importance of these Loop2 residues, it is likely that blocking their binding to Lgs/Bcl-9 will reduce Wnt signaling activity in normal and malignant cells.

How Pygo and Lgs function molecularly in the transcription of Wnt target genes is still unclear. It has been suggested (9) that Pygo is recruited to DNA-bound TCF through Lgs/Bcl-9 and Armadillo/β-catenin and that the N terminus of Pygo recruits a co-activator complex or components of the general transcription machinery. Its PHD domain has been implicated in the former, and our work demonstrates the importance of specific Loop2 residues in this recruitment step.

As yet, there is no direct evidence for the functional importance of the N terminus of Pygo. This portion of the protein is not required for transactivation by DNA-tethered Pygo (12) (Fig. 4). We also failed to detect a function of the conserved NPFXXD motif within the N-box. However, it is possible that our mutations have not inactivated the N terminus sufficiently to reveal its function. Also, the N terminus may act redundantly with other Pygo sequences.

Perhaps our most puzzling result is the striking difference in the transactivation potential of untethered Pygo versus DNA-tethered Pygo: the latter is highly active in stimulating transcription (Fig. 4; Ref. 12), whereas the former is essentially inactive in transfected 293T cells (Fig. 3). Similar results were obtained by other investigators<sup>2</sup> who also found that overexpressed untethered Pygo proteins do not stimulate TOPFLASH transcription either in transfected 293 or 293T cells, even in the presence of co-expressed Bcl-9. Therefore, the suggestion that Pygo may act as a transcriptional co-activator (9) may need revisiting. But how can the difference in activity between untethered and DNA-tethered Pygo be explained? Because our transcription assays measured the effects of overexpressed protein in conjunction with endogenous factors, a stimulatory effect could only be observed if none of these are limiting. Given that the direct linkage of Pygo to a DNA-binding domain confers on it high transactivation potential, this implies that the rate-limiting step in these assays is the recruitment of Pygo to DNA. Recall that this apparently involves the three proteins TCF>β-catenin>Lgs, but none of these are likely to limit the function of exogenous (untethered) Pygo; its transactivation potential cannot be revealed even if these factors are co-overexpressed (Fig. 3). However, one possible explanation is that the direct tethering of Pygo to DNA by means of a DNA-binding domain is likely to be far more efficient than indirect recruitment by means of a three-protein adaptor chain. Bypassing this chain may allow us to detect the intrinsic transactivation potential of Pygo.

Our transcription assays have identified conserved Loop1 residues that contribute to the transactivation potential of DNA-tethered Pygo. These Loop1 residues are predicted to be at a PHD domain surface opposite to its putative Lgs-interacting surface (17, 18). They do not seem to be involved in intramolecular interactions nor in homo-dimerization, nor is this surface of the PHD domain predicted to be involved in the putative binding to phosphoinositides (22), so they are likely to mediate binding to an unknown ligand. Our rescue assays in embryos have not mirrored their functional importance in mammalian transcription assays for a number of possible reasons (see above). The ultimate proof of their functional significance will have to await the identification of their cognate Loop1-binding ligand.

**CONCLUSIONS**

An early if not initiating event in colorectal tumorigenesis is the loss of the Adenomatous polyposis tumor suppressor that results in activated TCF-mediated transcription (2, 3). Elevated TCF-mediated transcription can also be caused by activating mutations in β-catenin, which are found in colorectal and many other types of cancers (23). Blocking TCF-mediated transcription in colorectal cancer cells reduces their proliferative potential (24) and may thus attenuate the progression of colorectal tumors. Until recently, the only molecular candidate target for disruption has been the TCF/β-catenin interface. However, TCF factors bind to the same surface of β-catenin as some of its negative regulators and mimic their interaction with β-catenin (25), so this interface is not an ideal target. Pygo and Lgs/Bcl-9 provide new potential targets, with two unique binding surfaces that do not seem to be shared with other binding partners. The four conserved Loop2 residues of Pygo that we have shown to be critical for Lgs binding and in vivo function define the interaction between the two proteins at the molecular level and may help to develop their interface as a suitable molecular target for disruption in cancer.

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<sup>2</sup> W. de Lau and H. Clevers, personal communication.
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Note Added in Proof—Due to an error by the Endnote program, the name of the senior author of Ref. 12 was left off the reference. We apologized to the author for this error. The correct reference should read: Belenkaya, T. Y., Han, C., Standley, H. J., Lin, X., Houston, D. W., Heasman, J., and Lin, X. (2002) Development 129, 4089–4101.

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