Title
Discrete somatic niches coordinate proliferation and migration of primordial germ cells via Wnt signaling.

Permalink
https://escholarship.org/uc/item/43k7n01g

Journal
The Journal of cell biology, 214(2)

ISSN
0021-9525

Authors
Cantú, Andrea V
Altshuler-Keylin, Svetlana
Laird, Diana J

Publication Date
2016-07-11

DOI
10.1083/jcb.201511061

Peer reviewed
Discrete somatic niches coordinate proliferation and migration of primordial germ cells via Wnt signaling

Andrea V. Cantú, Svetlana Altshuler-Keylin, and Diana J. Laird

Department of Obstetrics, Gynecology and Reproductive Sciences, Center for Reproductive Sciences, Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, University of California, San Francisco, San Francisco, CA 94043

Inheritance depends on the expansion of a small number of primordial germ cells (PGCs) in the early embryo. Proliferation of mammalian PGCs is concurrent with their movement through changing microenvironments; however, mechanisms coordinating these conflicting processes remain unclear. Here, we find that PGC proliferation varies by location rather than embryonic age. Ror2 and Wnt5a mutants with mislocalized PGCs corroborate the microenvironmental regulation of the cell cycle, except in the hindgut, where Wnt5a is highly expressed. Molecular and genetic evidence suggests that Wnt5a acts via Ror2 to suppress β-catenin–dependent Wnt signaling in PGCs and limit their proliferation in specific locations, which we validate by overactivating β-catenin in PGCs. Our results suggest that the balance between expansion and movement of migratory PGCs is fine-tuned in different niches by the opposing β-catenin–dependent and Ror2-mediated pathways through Wnt5a. This could serve as a selective mechanism to favor early and efficient migrators with clonal dominance in the ensuing germ cell pool while penalizing stragglers.

Introduction

A central question in development is the extent to which cellular decisions are controlled by intrinsic or extrinsic cues. Primordial germ cells (PGCs), the precursors of eggs and sperm, are among the first embryonic lineages established (Laird et al., 2008). The subsequent migration of this small population through the early embryo to the nascent gonads is highly conserved among organisms (Nieuwkoop and Satasurya, 1979, 1981). Drosophila melanogaster and zebrafish have provided powerful models for identifying genes and pathways involved in PGC migration (Kunwar et al., 2006), although much less is known in mammals. After specification, mouse PGCs at embryonic day 7.5 (E7.5) migrate from the base of the allantois into the hindgut endoderm. They travel within the expanding hindgut epithelium and into the dorsal mesentery before colonizing the gonadal ridges by E11.5, after which sex-specific differentiation proceeds (Chiquoine, 1954; McLaren, 2003). This migration is asynchronous; those first to exit the hindgut are speculated to be “pioneer” germ cells that arrive first in the gonads and guide additional PGCs to their location (Gomperts et al., 1994).

Distinct from other models, mammalian PGC migration is concurrent with proliferation (Richardson and Lehmann, 2010), raising the question of how cells divide while moving. In mice, the number of PGCs expands from ~45 at E7.5 to ~200 at E9.5 (McLaren, 2003; Seki et al., 2007) and ~2,500 at E11.5 (Laird et al., 2011) and peaks to ~25,000 at E13.5 (Tam and Snow, 1981). Precise control of the cell cycle is suggested by differential rates of PGC proliferation during their migration (Seki et al., 2007); however, the underlying mechanisms remain unclear. Understanding this dynamic management of proliferation in PGCs could yield insights into the origin of germ cell tumors as well as evolutionary mechanisms that shape the gamete pool.

Several Wnt ligands have been implicated in PGC development: Wnt3 and Wnt3a in specification (Ohinata et al., 2009; Bialecka et al., 2012; Aramaki et al., 2013; Tanaka et al., 2013); Wnt5a and its receptor, Ror2, in migration (Laird et al., 2011; Chawengsaksophak et al., 2012); and Wnt4 in female sex differentiation (Vainio et al., 1999; Chassot et al., 2012). Both establishment and sex differentiation of PGCs use the β-catenin–dependent, canonical arm of the Wnt pathway (Chassot et al., 2008, 2011; Aramaki et al., 2013), but its function in migratory PGCs is unknown. This well-studied pathway regulates cell fate decisions and proliferation, and recent work suggests that the context of receptors and ligands determines the predominant downstream signaling pathway in an individual cell (van Amerongen and Nusse, 2009; van Amerongen et al., 2012). In this way, Wnts can be broadly used throughout development to simultaneously regulate many cell types and processes.

Here, we find that proliferation of PGCs during development varies according to successive embryonic location rather than by cell-intrinsic mechanisms. We show that distinct niches...
along the migratory route modulate the cell cycle in PGCs and perturbation of the noncanonical Wnt5a–Ror2 pathway disrupts germ cell proliferation specifically in the hindgut. We identify Wnt5a as a key regulator of PGC proliferation through its ability to dampen canonical, β-catenin–mediated Wnt activity and observe that the in vivo Wnt response in PGCs correlates with their rate of division. Thus, we find a novel mechanism of niche-specific regulation of the cell cycle via the balance of antagonistic Wnt signaling pathways during germ cell development.

### Results

**PGC proliferation during migration is associated with location**

Examination of PGCs through development previously identified a progressive increase in total number spanning the period from specification through sex differentiation (Tam and Snow, 1981; Seki et al., 2007). Subsequent analysis found that the rate of proliferation varied by embryonic age, with a slowing of the cell cycle around E9.0 (Seki et al., 2007). Using EdU incorporation to quantify cell proliferation (Fig. S1 A), we observed a similar relationship between the PGC cell cycle and embryonic age on a mixed genetic background (Fig. 1 A). However, because germ cell migration is largely individual and asynchronous, we noted that PGC locations were heterogeneous within a given embryonic age (Fig. 1 B). When indexed by location rather than age, the frequency of EdU+ PGCs increased in each successive migratory compartment, from hindgut epithelium to gonadal ridge mesenchyme ($P < 0.001$ by $\chi^2$; correlation coefficient = 0.991; Fig. 1 C). Importantly, this escalating rate of proliferation was observed within individual embryonic ages, particularly E9.5 and E10.5, where germ cell location is most diverse (Fig. S1, B–D). Additionally, the rate of proliferation remained largely constant in each location, irrespective of age. This trend held in C57BL6/CD1 embryos despite differing absolute levels of PGC proliferation (Fig. S1 E).

To assess the cell cycle of PGCs under controlled conditions, we turned to our previously established ex vivo culture, in which PGCs can be maintained free of feeder cells or serum for $\sim 24$ h using defined medium and synthetic substrates (Laird et al., 2011). PGCs isolated at E9.5, E10.5, and E11.5 using the Oct4-ΔPE-GFP reporter (Anderson et al., 1999) were cultured in identical conditions. EdU analysis of these ex vivo cultures confirmed that PGC proliferation did not depend on age (Fig. 1 D). PGCs at E9.5 and E10.5 had identical rates of EdU incorporation, whereas the slight decrease in E11.5 PGCs is likely caused by the reduced cell survival in our culture conditions (Fig. 1 E). Thus, a correlation between the cell cycle rate and embryonic compartment in vivo suggests that location rather than intrinsic timing determines PGC proliferation.
Disruption of noncanonical Wnt signaling alters PGC proliferation in the hindgut

Our hypothesis that proliferation of PGCs is environmentally regulated predicts that disrupting their localization should affect proliferation by age, but not location. Genetic mutants of the noncanonical Wnt receptor Ror2 exhibit decreased efficiency of PGC colonization of the gonads (Laird et al., 2011). Although stragglers die in Ror2 Y324C embryos, the restoration of normal germ cell number on a Bax−/− background indicates that migration is the primary defect (Laird et al., 2011). Ror2 is broadly expressed in tissues critical for germ cell development, including somatic cells of the hindgut epithelium and surrounding dorsal mesentery (Yamada et al., 2010), as well as on migratory and postmigratory PGCs (Laird et al., 2011; Arora et al., 2014). As predicted, despite mislocalization of many Ror2 Y324C germ cells from E9.5−11.5 (Fig. S2 A), the frequency of EdU incorporation was similar between wild-type (WT) and Ror2 Y324C PGCs in the mesenchyme near the hindgut and subsequent mesentery and gonadal locations (Fig. 2 A). However, in the Ror2 Y324C hindgut, PGCs did not cycle according to their location but instead showed an aberrant increase in EdU incorporation; concurrently, elevated numbers of PGCs per histological section at E9.5 (Fig. 2 B) were indicative of an expansion in the overall number of PGCs at this age. EdU incorporation showed an increase in the hindgut of Ror2 Y324C mutants compared with WT at E9.5 (Fig. 2 C), but not at other locations or ages (Fig. S2, B and C), and the distribution of EdU-labeled PGCs at E9.5 was skewed toward the hindgut in Ror2 Y324C rather than the mesentery as in WT (Fig. 2 D). These data suggest that Ror2 signaling restricts PGC proliferation exclusively within the hindgut.

Wnt5a is the primary ligand for Ror2 (Hikasa et al., 2002; Oishi et al., 2003), and its expression along the PGC migratory route in the hindgut (Fig. S2 E), mesentery, and gonadal ridges (Laird et al., 2011; Fig. S3 E) coincides with expression of the Ror2 receptor by PGCs (Laird et al., 2011). Wnt5a+−/− embryos exhibit defects in the organization of the hindgut epithelium and reduced axial elongation (Yamaguchi et al., 1999; Cervantes et al., 2009). PGCs in these mutants migrate inefficiently (Fig. S2 D) and are diminished after E10.5 through an uptick in apoptosis (Laird et al., 2011; Chawengsaksophak et al., 2012). Our analysis of Wnt5a+−/− embryos found a similar, but less severe, phenotype than in Ror2 Y324C; altered distribution of PGCs was associated with increased proliferation in the hindgut at E9.5 (Fig. 2 E, G), whereas no differences at other locations or
embryonic ages were found (Fig. S2, F and G). This observed uptick in proliferation in the E9.5 hindgut most likely leads to an increased number of PGCs per histological section in Wnt5a−/− mutants at E9.5–10.5 (Fig. 2 F), corresponding to an overall increase in the PGC population at these ages. Strikingly, by E11.5, the number of PGCs per section was significantly decreased in both Wnt5a and Ror2 mutants, consistent with the previously described loss through mismigration and subsequent apoptosis. Although our observations of Wnt5a−/− confirmed the migratory phenotype of PGCs and ultimate reduction in their overall numbers (Laird et al., 2011; Chawengsaksophak et al., 2012), the altered distribution of proliferating Wnt5a−/− PGCs in the hindgut and ectopic locations (Fig. 2 H) suggests that WNT5a levels calibrate early PGC mitosis. Thus, we conclude that Wnt5a in the hindgut signals through Ror2 to limit PGC proliferation during migration.

**Wnt5a–Ror2 regulates the PGC cell cycle via suppression of the canonical Wnt pathway**

To identify the molecular mechanism connecting Wnt5a–Ror2 signaling to the cell cycle in this lineage, we interrogated transcriptional changes in Ror2Y324C PGCs from embryos at E9.5, when half or more are retained in the hindgut or nearby mesentery (Fig. 3 A). Microarray analysis of PGCs from single embryos showed significant misregulation of genes associated with the cell cycle and proliferation categories (Fig. 3 B). GSEA comparison of the Ror2Y324C microarray data with a curated list of cell cycle genes from the QIAGEN Cell Cycle PCR Array suggests an enrichment for Ror2Y324C-associated genes. P-value, enrichment score (ES), and normalized enrichment score (NES) are specified. Heatmap shows normalized microarray intensity values for the 15 most and 15 least enriched cell cycle–associated genes in Ror2Y324C PGCs as identified by the GSEA. For each gene, dark blue color indicates the lowest probe intensity value (zeroth percentile), dark red indicates the highest probe intensity value (100th percentile), and white indicates the middle 50th percentile of intensity values.
Examining specific changes in gene expression revealed no difference in levels of Ror2 between the WT and mutant groups, corroborating previous findings that the Ror2Y324C mutation is expressed but nonfunctional (Laird et al., 2011). Importantly, Ror family member Ror1 was found to have very low (1/10th levels of Ror2) and unchanging expression in PGCs across groups, eliminating the possibility of compensation for loss of Ror2 reported in other systems (Ho et al., 2012). Among >1.2-fold up-regulated transcripts in Ror2Y324C PGCs was Axin2, an established target of the canonical Wnt pathway (Niehrs, 2012). Ror2 has been linked to the suppression of canonical Wnt signaling using reporters of T-cell factor/lymphoid enhancer factor transcriptional activity (Mikels and Nusse, 2006; Mikels et al., 2009). To test this relationship, we generated compound mutants between Ror2Y324C and a null allele of β-catenin (Brault et al., 2001). Although homozygous β-catenin deletion causes lethality at gastrulation (Haegel et al., 1995; Huelsen et al., 2000), PGCs from Ror2Y324C/−β-catenin−/− embryos exhibited normal BrdU incorporation in ex vivo culture; this suggests that reduced dosage of β-catenin rescues the proliferation increase in Ror2Y324C PGCs (Fig. S3 A). Together, these expression and genetic studies suggest that that Ror2 opposes the β-catenin–dependent pathway in the regulation of PGC proliferation.

To explore the hypothesis that β-catenin acts downstream of Ror2 signaling to regulate the cell cycle in migratory PGCs, we compared our microarray data from Ror2Y324C PGCs to published data from β-catenin and TCF1 chromatin immunoprecipitation (ChIP) sequencing. Because of a dearth of ChIP-sequencing data in PGCs, the most relevant and comprehensive dataset available was from the mouse intestinal crypt (Schuijers et al., 2015). Despite known discrepancies between cell lineages, we found that 13% of 1.2-fold changed genes from our microarray analysis (dChip; Li and Wong, 2001; or Ingenuity Pathway Analysis; QIAGEN) were also bound by β-catenin (Fig. S3 B). GSEA comparison of β-catenin ChIP peaks from mouse intestine showed a significant enrichment in genes associated with the Ror2Y324C disruption in PGCs, including the targets Axin2 and Bmp7, among others (Fig. S3 C). These data suggest links among Wnt5α−>Ror2, the canonical Wnt pathway, and the PGC cell cycle.

WNT5a from somatic cells suppresses canonical Wnt/β-catenin activity in PGCs

The observed changes in proliferation highlight the influence of the somatic cell microenvironment on PGCs, whereas the Ror2Y324C mutants implicate the canonical Wnt pathway in regulating their compartment-specific behaviors. To measure canonical Wnt responses in PGCs under controlled conditions, we returned to our ex vivo culture system using PGCs isolated from E9.5 tails (Bendel-Stenzel et al., 2000) as well as Oct4-ΔPE-GFP+ somatic cells from isolated gonadal ridges (Fig. S3 D). Somatic cells in the hindgut (E9.5 Ecad−) expressed Wnt antagonists Wnt5α and Dkk1, whereas the gonadal ridge somatic expressed Wnt5α and high levels of Sfrp1 (Fig. S3 E). Abundance of Dkk1 and Sfrp1 in the E9.5 Ecad− population was intermediate, suggesting that the mesenchyme surrounding the hindgut is a transitional zone during PGC migration.

Based on the genetic evidence that Wnt5α suppresses proliferation within the hindgut and molecular association between Ror2 and β-catenin targets, we pursued Wnt5α as a candidate secreted regulator of canonical Wnt signaling in PGCs. Exogenous WNT5α reduced levels of n-βcat in WT, E9.5 PGCs similarly to our tail somatic cells (Fig. 5 A), but the relatively uniform response suggested increased consistency throughout the culture environment. In contrast to WT, Ror2Y324C PGCs cultured with exogenous WNT5α did not dampen the Wnt response and instead activated the canonical pathway (Fig. S3 F). This suggests that Wnt5α acts primarily through the Ror2 receptor to inhibit β-catenin–dependent signaling, but when Ror2 is nonfunctional, Wnt5α signals through other receptors on PGCs.

To verify that altered levels of n-βcat correspond to transcriptional changes in our system, we interrogated expression of putative canonical Wnt target genes in ex vivo–cultured PGCs. After PGC culture in the presence of WNT5α, qRT-PCR revealed a decrease of several Wnt target transcripts established in other cell types including Axin2, Cdh1, Fn1, and Lef1 (Fig. 5 B); importantly, levels of the germ cell gene Dppa3(Stella) remained unaffected. Accordingly, we found that E-cadherin (Cdh1) protein was reduced in PGCs treated with Wnt5α (Fig. 5 C). These results confirm that changes in Wnt target gene transcription accompany changes in n-βcat observed in PGCs.

Although the aforementioned experiments show that WNT5α suppresses canonical Wnt activity in PGCs, they do not validate its relevance as a mediator of this suppression by somatic cell populations. Because of the expression of Wnt inhibitor Dkk1 in the hindgut and tail mesentery, we performed loss-of-function studies with mouse embryonic fibroblasts (MEFs) that express Wnt5α (Fig. 5 E) and produce a similar reduction in n-βcat of PGCs (Fig. 5 D) but have undetectable levels of Dkk1. Coculture of E9.5 PGCs with Wnt5α−/− MEFs alleviated the suppression of n-βcat observed with WT MEFs and led to increased Wnt response over that of isolated PGCs.
Moreover, the addition of exogenous WNT5a to the Wnt5a−/− MEF cocultures restored this suppression and reduced n-β-catenin in the PGCs. Thus, we conclude that Wnt5a secreted from somatic cells in the hindgut and surrounding mesenchyme serves to suppress canonical Wnt activity in migratory PGCs.

Specific somatic compartments modulate the canonical Wnt response in migratory PGCs

To assess canonical Wnt response at a single-cell level in vivo, we developed a protocol to quantitatively image and measure n-β-catenin in histological sections. After enzymatic treatment to render the nucleus accessible (Fig. S4 A), we measured the mean n-β-catenin immunofluorescence signal intensity within PGCs as compared with the mean intensity across all nuclei in an image (Fig. 6 A). This normalization enabled comparison of n-β-catenin intensity between histological sections and between slides.

On applying this method to sections from Ror2 Y324C embryos at E9.5, we found that overall levels of n-β-catenin in PGCs were higher than in WT (Fig. 6 B). This was also true when the data were separated by location (Fig. 6 C), consistent with elevated Wnt target genes in Ror2 Y324C microarray data. By E10.5, this increase in overall accumulation of n-β-catenin in Ror2 Y324C PGCs returned to WT levels (unpublished data), suggesting that over- active Wnt signaling is not sustained throughout the migratory period and supporting the decrease in overall proliferation at this age (Fig. S2 B).

To assess Wnt signaling in PGCs across embryonic ages, we studied WT embryos, in which we observed a wide range in levels of n-β-catenin, the highest being at E11.5 (Fig. S4 B). Mean n-β-catenin in PGCs in the E12.5 testis was comparatively low, reflecting the established repression during male sex differentiation (Chassot et al., 2011). Despite fluctuation of n-β-catenin in PGCs by age, the higher population mean in post-migratory PGCs at E11.5 compared with E9.5 (Fig. S4 B) again prompted our examination by location. From E9.5 to E11.5, we observed a progressive increase in n-β-catenin at each successive migratory location from the hindgut to the gonadal ridge (R² = 0.97 and P = 0.002 by regression analysis; Fig. 6 D). This pattern was retained at single ages, with lower levels of n-β-catenin in PGCs in the hindgut and adjacent mesenchyme compared with PGCs in mesentery or gonadal tissues (Fig. S4, C and D). PGC n-β-catenin did not vary with rostral-caudal position in the embryo (unpublished data), suggesting that a consistent environment within each compartment regulates the Wnt response in PGCs.
Genetic manipulation of β-catenin levels in PGCs alters proliferation

The parallel trajectories of proliferation and n-βcat together with the Wnt5a and Ror2 mutant defects suggest that suppression of canonical Wnt signaling slows proliferation during early PGC migration. To test this hypothesis, we bypassed the antagonistic effects of the hindgut and mesentery by over-activating β-catenin in migratory PGCs. We crossed mice carrying a gain-of-function (GOF) allele (Harada et al., 1999), β-cateninfloxE3/+ to mice with a drug-inducible Cre recombinase inserted into the Oct4 locus (Greder et al., 2012), Pou5f1Cre-ER/+. Tamoxifen administration at E7.75 or E8.25 of pregnancy induced excision of β-catenin exon 3, preventing degradation of the protein in PGCs (Fig. 7 A). Because of widespread expression of Oct4 through E7.5 (Downs, 2008), we tested this tamoxifen schedule in RosamTmG reporter mice (Muzumdar et al., 2007) crossed to Pou5f1Cre-ER/+ and observed overall specificity for excision in PGCs compared with their surrounding somatic tissues (Fig. S5 A). After activation of the Pou5f1Cre-ER allele, the resulting β-cateninGOF embryos exhibited an increase in the number of PGCs per section at E9.5 when compared with Cre-negative littermates (Fig. 7, B and C). No difference was observed in the frequency of apoptotic PGCs in mutants (unpublished data).

Examination of proliferation by EdU incorporation in vivo did not reveal differences in labeled PGCs in β-cateninGOF mutants (Fig. S5 C). Curiously, EdU labeling was aberrantly high in the control PGCs relative to PGCs in mice of mixed genetic background (Fig. 1 C), suggesting that cell cycle is altered by exposure to tamoxifen or differs in the C57BL6 background as compared with mixed background controls. To circumvent this issue and improve temporal control over the timing of excision, we cultured E9.5 PGCs from single Pou5f1Cre-ER/+; Oct4-ΔPE-GFPtg/+; β-cateninfloxE3/+ embryos with their corresponding WT tail soma to simulate the Wnt-suppressive in vivo environment. After adding 4OH-tamoxifen (Fig. 7 A), we observed a similar frequency of EdU incorporation after 10 h in culture. However, by 17 h, PGCs from Cre+ (GOF) embryos exhibited an increased proliferation rate over PGCs from controls (Fig. 7 D), suggesting that intrinsic overactivation of the canonical Wnt pathway allows PGCs to bypass the suppression...
by neighboring somatic cells. This result corroborates the up-tick in overall numbers of PGCs counted at E9.5–10.5 in vivo (Fig. 7 B). Thus, ectopic activation of the canonical Wnt pathway in migratory PGCs is sufficient to alter their proliferation in environments associated with early stages of migration. Interestingly, our cumulative data show a mislocalization of PGCs along the migratory route of GOF mutants, indicative of a migration defect (Fig. S5 B); this concomitant perturbation of migration and proliferation is consistent with PGC phenotypes in Wnt5a and Ror2 mutants and the antagonism between β-catenin and Wnt5a–Ror2 pathways.

To further interrogate this relationship, we conducted genome-wide transcriptional analysis on E9.5 β-catenin

Discussion

Here, we examined the proliferation of PGCs during migration and showed that their expansion is regulated by the somatic compartments through which they move. We identified canonical Wnt signaling as a mechanism that controls this proliferation and the Wnt5a–Ror2 axis as a key suppressor of Wnt activity during transit through the hindgut and surrounding mesenchyme (Fig. 8). We observed a steady rise in the PGC Wnt response along the migratory route, concomitant with increased cycling, and demonstrated that excessive proliferation...
Figure 7. Overactivation of β-catenin in PGCs leads to an increase in proliferation and misregulation of genes in common with Ror2 Y324C. (A) Schematic of breeding and tamoxifen exposure to generate control (Cnt, β-catenin GOF/+; Pou5f1+/+) and β-cateninGOF (β-cateninGOF/+; Pou5f1Cre-ER/+) embryos in vivo and cells ex vivo. (B) Mean number of PGCs counted in histological sections of Cnt and β-cateninGOF embryos at different ages. n = 13 Cnt embryos and 15 β-cateninGOF embryos. P-values by Student’s t-test for section counts. Estimates for total numbers of Cnt and β-cateninGOF PGCs per embryo were calculated using the multiplier of 100 × for E9.5, 125 × for E10.5, and 150 × for E11.5 based on cell counts reported in the literature. (C) Oct4-ΔPE-GFP+ PGCs (gray) in E9.5 littermates. Bar, 100 µm. (D) Rate of in vitro EdU incorporation in Cnt and β-cateninGOF PGCs cultured for 10–22 h with 4-OHT. n = 4 litters; 794–818 cells; *, P < 0.05 by Student’s t-test; error bars indicate standard deviation. (E) Microarray data from β-cateninGOF PGCs compared with WT shown as log fold change versus log p-values for all annotated microarray probes (Gladstone Bioinformatics Core [GBC]). Each dot represents a single probe. Blue dots show genes with P < 0.05 and a fold-change >1.2 (not log scale). Orange dots show significant genes that overlap with Ror2 Y324C microarray hits (dChip) and β-cateninGOF microarray hits (GBC). Each dot represents a single probe. Blue dots show genes with P < 0.05 and a fold-change >1.2 (not log scale). Orange dots show significant genes that overlap with Ror2 Y324C microarray hits (dChip) and β-cateninGOF microarray hits (GBC). (F) Functional analysis of >1.2-fold microarray hits by Ingenuity Pathway Analysis find cell cycle–associated genes to be significantly misregulated. Number of genes associated with each Gene Ontology category is shown on the right. Example genes of interest for cell cycle and cell growth and proliferation categories are listed. (G) Number of genes that overlap between Ror2 Y324C microarray hits (dChip) and β-cateninGOF microarray hits (GBC). (H) GSEA comparison of the Ror2 Y324C microarray dataset with β-cateninGOF misregulated genes suggests an enrichment for genes associated with both mutations. P-value, enrichment score (ES), and normalized enrichment score (NES) are specified. Heatmap shows normalized microarray intensity values for the 20 most and 20 least enriched genes in Ror2 Y324C PGCs that are misregulated in the β-cateninGOF microarray dataset. For each gene, dark blue color indicates the lowest probe intensity value (zero percentile), dark red indicates the highest probe intensity value (100th percentile), and white indicates the middle 50th percentile of intensity values.
during early PGC migration can be induced by autonomously over-activating \( \beta\)-catenin. Together, these results identify a yin and yang of Wnts in proliferation and migration: canonical Wnt signaling drives the expansion of migratory PGCs, counterbalanced in specific niches by Wnt5a–Ror2, which promotes their movement.

Previous examination of the Wnt5a\(^{−/−}\) and Ror2\(^{−/−}\) mutants identified defects in the migration and survival of PGCs (Laird et al., 2011; Chawengsaksophak et al., 2012). Our current studies have uncovered an additional role for these genes in regulating PGC proliferation during the early and most active stages of their migration. Historically, Wnt5a is considered a noncanonical Wnt ligand (Moon et al., 1993; Wong et al., 1994); however, recent work in vivo has demonstrated its capacity to activate or antagonize the canonical Wnt pathway in a tissue-specific context (van Amerongen et al., 2012). Proposed mechanisms for Wnt5a-induced attenuation of the canonical Wnt pathway include competition for binding Frizzled receptors, simultaneous engagement of different receptors, and blocking transcription downstream of \( \beta\)-catenin (Mikels and Nusse, 2006; Yamamoto et al., 2008; Sato et al., 2010). This final mechanism is ruled out in PGCs by our observation that decreased n-\( \beta\)cat levels accompany diminished Wnt target transcripts after WNT5a treatment.

Our studies confirm that Ror2 is the relevant receptor for mediating Wnt5a-induced attenuation of \( \beta\)-catenin–dependent signaling in migratory PGCs, consistent with previous biochemical studies (Mikels and Nusse, 2006). The permissive role of Wnt5a in PGC migration (Laird et al., 2011), together with its repression of proliferation, leads to the model that Wnt5a acts through Ror2 to balance the promotion of PGC movement while limiting mitosis. This balance may be particularly important for known periods of active migration, such as egress from the hindgut, which is when we observe the strongest effect. Considering the potential conflicts posed to migration by cell division (loss of adhesion and orientation, as well as spatial constraints in tightly packed tissue), negotiation of both by Wnt5a–Ror2 offers a single solution. The identification of Wnt5a and Ror2 as prognostic biomarkers for tumor invasiveness (Weeraratna et al., 2002; Edris et al., 2012; Lu et al., 2012) raises the possibility that cancer cells exploit the same mechanism to regulate metastasis and growth.

Our study identifies the Wnt pathway as a mechanism for extrinsic regulation of proliferation in migratory PGCs. Previous work demonstrated a mitogenic role for KitL–cKit in PGCs, and dynamic expression of KitL in somatic compartments of the migratory route provides temporal specificity of signaling in each niche (Runyan et al., 2006; Gu et al., 2009). Prior studies also identified the chemokine SDF1 as a potential mitogen for PGCs primarily in the gonad (Ara et al., 2003; Molyneaux et al., 2003). Both pathways may function redundantly with Wnt signaling to regulate PGC proliferation during migration. Redundancy in the control of PGC division may have arisen as an advantage in the face of consequences for reproduction: sterility from insufficient PGCs or germ cell tumors from their unchecked proliferation. “Agreement” between multiple mechanisms of cell cycle control in PGCs may have evolved to prevent tumor formation and safeguard against overrepresentation of germ line variants with elevated proliferation (Burt and Trivers, 2006). However, our data suggest that in a WT context, the balance between migration and proliferation could serve as a selective mechanism to reward early and efficient migrators—“pioneer” germ cells (Gomperts et al., 1994)—with clonal dominance in the ensuing germ cell pool, while penalizing stragglers with reduced time for expansion.

PGCs interact with a diversity of tissues as they move from the developing endoderm to the gonadal ridges yet are able to retain their germ cell characteristics. Migration through these microenvironments supports their survival, regulates their proliferation, and maintains their unique pluripotency program. Wylie et al. proposed that PGCs thus migrate along a “traveling niche,” which provides a spatiotemporal environment analogous to a stem cell niche that maintains germ cell properties despite spanning diverse regions of the embryo (Gu et al., 2009). The departure from this niche by mismigration leads to either cellular demise or loss of PGC identity through transformation to pluripotent embryonic carcinoma cells (Matsui et al., 1992; Resnick et al., 1992). Our data suggest that WNT5a is a component of this dynamic niche, maintaining the migratory capability of PGCs while balancing their proliferation; however, it is likely that additional factors modulate the \( \beta\)-catenin–dependent response in PGCs. The expression of multiple and varied Wnt regulators by the somatic environment is suggested by the elevated Wnt response in WT gonadal PGCs (Fig. 6 D), increased n-\( \beta\)cat in PGCs cocultured with Wnt5a\(^{−/−}\) MEFs (Fig. 5 D), and differences in cell cycle response by location despite similar overactivation of Wnt signaling in E9.5 Ror2\(^{−/−}\) PGCs (Fig. 6 C). Other Wnt ligands with known expression in the embryonic posterior or in tissues of the PGC migratory route include Wnt3a (Takada et al., 1994), Wnt4 (Vainio et al., 1999), and Wnt11 (Kispert et al., 1996). Interactions between alternative Wnt modulatory factors may also be important. For example, WNT5a in the gonad could be neutralized by high levels of SFRP1 (Fig. S3 E) to enable robust canonical Wnt activity.
in PGCs. We speculate that the expansion of migratory PGCs is achieved by calibration of their canonical Wnt activity in each somatic compartment by a distinct composition of activators and inhibitors. Our investigation into the dynamic microenvironment of migratory PGCs using ex vivo, coculture approaches paves the way for further characterization of each embryonic compartment and the cellular interactions between PGCs different somatic cell populations.

The balancing regulation of motility and proliferation by the microenvironment through noncanonical and canonical Wnt pathways may function similarly in other itinerant cell lineages in development, such as hematopoietic stem cells and neural crest. Further exploration of these microenvironments at both the cellular and mechanistic levels will enhance understanding of the interplay between migration of precursor populations and other simultaneous cell processes critical to their development.

Materials and methods

Animals

WT embryos were generated by mating CD1 females with homozygous Oct4-ΔPE-GFP males (MGI:5057158, multiple-copy transgene insertion; Anderson et al., 1999). Other mouse strains were maintained on a mixed or C57BL6 background, including; Ror2+/- (MGI:5305088, ENU-induced point mutation; Laird et al., 2011), Wnt5a (MGI:1857617, targeted deletion; Yamaguchi et al., 2001) to Zp3cre/+ (MGI:5049897, targeted knockin; Greder et al., 2012), Rosa26loxP (MGI:3716464, targeted knockin; Muzumdar et al., 2007), and β-catenin+/- (MGI:1858008, targeted floxed allele; Harada et al., 1999). β-cateninloxPloxP mice were created by crossing β-cateninloxPloxP mice (MGI:2148567, targeted floxed allele; a gift from K. Hadjantonakis (Memorial Sloan Kettering Cancer Center, New York, NY); Brault et al., 2001) to Zp3flox/flox (MGI:2176187, transgene insertion; de Vries et al., 2000) for germline deletion.

Embryos were dissected from timed matings and staged by the following anatomical landmarks: 20−25 somite pairs was designated E9.5, 26−28 as E9.75, 30−33 as E10.25, 34−37 as E10.5, 38−41 as E10.75, and 45−48 as E11.25 (Kaufman, 1992). Genotypes were determined by PCR. All mouse work was performed under the University of California, San Francisco, Institutional Animal Care and Use Committee guidelines in an approved facility of the Association for Assessment and Accreditation of Laboratory Animal Care International.

Tamoxifen and EdU injections

To generate β-catenin GOF PGCs, β-cateninloxPloxPflox/Elac2 females were mated with Pou5f1Cre+/-; Oct4-ΔPE-GFPm/+ males. Tamoxifen (#T5648; Sigma-Aldrich) was suspended at 20 mg/ml in sunflower seed oil (#S5007; Sigma-Aldrich) and administered via intraperitoneal injection on day E7.75 at 5 mg/30g mouse weight. 4-Hydroxytamoxifen (#H7904; Sigma-Aldrich) was added at 1 µM to cells in culture at 0−3 h after seeding.

EdU (#C10338, Click-It Imaging kit; Thermo Fisher Scientific) was suspended at 2.5 mg/ml in PBS and administered at 25 µg/g mouse weight via i.p. injection 3−4 h before embryo dissection. In ex vivo culture, EdU was added to cells at 10 µM 0−3 h after seeding.

Flow cytometry and qRT-PCR

Embryos were dissected at E9.5−11.5 in cold PBS and the posterior fragment or gonads dissociated in 0.25% trypsin/EDTA for 3 min at 37°C followed by 1 mg/ml DNase I to dissolve any lingering clumps of tissue. Dead cells were excluded on the basis of Sytox Blue (Invitrogen) signal. PGCs were delineated as Oct4-ΔPE-GFP+. Hindgut cells were live-stained for E-cadherin (1:200; #13−1900; Invitrogen) and sorted away from E-cad−/GFP+ PGCs. Gonadal somas was microdissected away from the mesonephros and depleted of GFP+ PGCs. Each cell type was sorted directly into lysis buffer and extracted with the appropriate RNAs kit (QIAGEN), DNase I treated, and reverse-transcribed with qScript (Quanta Biosciences) or SuperScript III (Invitrogen). PCR primers were designed with Primer Express software (Applied Biosystems) or Primer-BLAST (NCBI). Amplification was performed with 50 or 100 cell equivalents of cDNA on a Mastercycler EP (Eppendorf) using the following primer sets for selected Wnt target genes from the Wnt Homepage (http://web.stanford.edu/group/nusselab/cgi-bin/wnt): Wnt5a: 5′−CCAGTTCCGGCATCAGGAGATGGA-3′, 5′−CACCCGGCTACGTAGAAGCCCG-3′; Wnt3a: 5′−GCCACCGTGCACACACGC-3′, 5′−AGCGAGGGCATGGCACAG-3′; Dkk1: 5′−GCTTCGCGAAAGGGAAGCGGCA-3′, 5′−GGTGGTACGCCGCAACAACT-3′; Fzd6: 5′−CTGGCCGACCCGGATGTGA-3′, 5′−TGCGCTCTGGCCAAAGACTACAGC-3′; Cnrd1: 5′−CTGAAACCTGCGGAGCCCAAACA-3′, 5′−TTTCGTCACGACCGAGAGCGA-3′; Cdh1: 5′−CCCTCTATGGCGTCCCCAGT-3′, 5′−GGTGGTACGCCGCAACACAACT-3′; 5′−GCCACAGACCGGGACGG-3′; Lef1: 5′−TGCGTCGTCGCTGGCTGCTT-3′, 5′−TTCAACCAGGGCGCCAGAAAC-3′; Dpaa5: 5′−AAAAAGCTGCGGAGAAGTTAGA3′, 5′−AAATCTTCGTCGATTTCGCACACAC-3′, 5′−GACTTCACAGCAATCCTCCAC-3′, 5′−AGGAAAGCTGGAAAGGACGGC-3′, 5′−AGGGTGTCCTTGCTACAGCAG-3′, 5′−AACTTTGAGGGCCACAGGAA-3′.

PGC ex vivo culture

Oct4-ΔPE-GFP+ PGCs sorted from E9.5 embryos were seeded in chambered slides (Lab-Tek II) coated with 1 mg/ml Matrigel (#354234; BD) then incubated at 37°C in 5% CO2 with DMEM/15% Knockout Serum Replacement (Invitrogen), 1,000 U/ml LIF (EMD Millipore), 5 µM Forskolin (#F3917; Sigma-Aldrich), and 50 ng/ml SCF (#PMC2115; R&D Systems). PGC numbers were counted at 3 h in culture and before time of fixation (18−24 h). After culture, cells were fixed for 10−15 min in 4% PFA and immunostained.

Somatic cells from E9.5 WT tails were digested using 0.25% trypsin/EDTA and cultured directly with endogenous PGCs. MEFs were generated from the posterior region of E13.5 WT and Wnt5a−/− embryos. Embryonic tissue was trypsinized for 30−45 min at 37°C and triturated in DMEM/10% fetal calf serum. Cells were grown to confluency for 3−4 d, passed to expand the population, and frozen in liquid nitrogen until use (Hogan et al., 1994). Conditioned media from E9.5 tail somatic cells was collected 16−20 h after dissociated postembryos were seeded in culture.

For BrdU experiments, hindguts and dorsal mesentery from E9.5 to 10.5 embryos were dissociated in 0.25% trypsin with 1 mg/ml DNase I at 37°C for 5 min. Digestion was stopped with FCS, and suspensions were seeded onto mitomycin C−treated STO fibroblast monolayers in chambered slides (Lab-Tek II), then incubated for 24 h at 37°C in 5% CO2 with DMEM/10% FCS. After a 1-h treatment with 10 ng/ml BrdU, cells were fixed for 10 min in 4% PFA and immunostained.

Immunostaining

Embryos fixed in 4% PFA were embedded in OCT and cryosectioned at a thickness of 10 µm. Slides were blocked 1 h in 10% calf serum + 0.1% Triton X-100 in PBS and stained overnight at 4°C in the blocking
buffer followed by three 15-min washes in PBS. Primary antibodies used included β-catenin (rabbit, 1:25; #9828; Cell Signaling Technology), Axin2 (goat, Conductin M-20, 1:100; #sc-8570; Santa Cruz Biotechnolog, Inc.), GFP (chicken, Aves GFP-1020, 1:200); SSEA1 (mouse IgM, 1:200; MC-480; Developmental Studies Hybridxoma Bank), vasa (rabbit, 1:400; ab13840-100; Abcam), cleaved PARP (rabbit, 1:50; #9544s; Cell Signaling Technology), E-cadherin (rat, 1:200; #13-1900; Invitrogen), Wnt5a (goat, 1:20; #AF645; R&D Systems), and Stella (rabbit, 1:100; ab19878; Abcam). Histological staining for β-catenin was preceded by 10 min additional fixation in 4% PFA and 3 min treatment in undiluted Ficin (Invitrogen) at room temperature followed by a quick PBS wash. This treatment was not necessary for β-catenin staining on cultured cells. EdU was labeled per kit protocol (#C10338 or C10340; Thermo Fisher Scientific). Secondary antibodies were purchased from Invitrogen and incubated for 1 h in blocking buffer at room temperature at 1:200. Nuclei were labeled with DAPI or Hoechst (1:1,000; Roche or Sigma-Aldrich). Sections were mounted in Vectashield (Vector Laboratories).

Image collection and analysis
Bright-field imaging was performed on an Olympus MVX10 stereo-microscope and Olympus acquisition software. Confocal imaging was performed at room temperature with a 20× 0.7 dry objective or 40× 0.95 oil-immersion objective on an SP5 TCS microscope (Leica) equipped with 405-, 488-, 543-, 594-, and 633-nm lasers. Use of the 20× objective typically required the addition of a 2× digital zoom for optimal visualization of PGCs for quantification. Files of 1,024 × 1,024 pixel images with 1- to 5-µm z-stacks were captured by a scanner with maximal frame resolution and acquisition software (Leica).

In vivo, mean nuclear β-catenin intensity was measured by comparing the signal within individual PGC nuclei to the mean signal of all nuclei in the image (>700). We devised a protocol in Velocity imaging processing software (PerkinElmer) to accurately obtain β-catenin signal intensity in each cell. PGCs were identified in the GFP or Hoechst channel using the same task commands for in vivo measurements to collect signal intensity. Normalization in ex vivo cultured PGCs was calculated as follows: individual PGC n-β-caten/mean n-β-caten of control well (ISO).

Microarray analysis
Targeted gene expression of E9.5 PGCs was analyzed using a GeneChip Mouse Gene 1.0 ST Array (Affymetrix). Oct4-ΔPE-GFP+ PGCs from single embryos were sorted into lysis buffer, extracted with RNeasy Micro kit (74004; QIAGEN), and sent to the University of California, San Francisco, Gladstone Genomics Core Facility for small sample amplification, microarray processing, and probe intensity normalization into a logarithm base 2 value. Intensity values for select Wnt target genes were normalized to housekeeping probe Gapdh. The raw data files can be downloaded from the Gene Expression Omnibus (GSE60179).

Bioinformatics
Microarray data were analyzed by AVC with dChip (Li and Wong, 2001) and Ingenuity Pathway Analysis (QIAGEN) to identify misregulated genes and associated functional categories. Additional analysis was performed by the Bioinformatics Core at the Gladstone Institutes, University of California, San Francisco. Microarrays were normalized for array-specific effects using Affymetrix’s “Robust Multi-Array” normalization. This was performed using the Affymetrix tool “apt-probeset-summarize,” which is part of the “Affymetrix Power Tools” package, available at http://www.affymetrix.com/ESTORE/PARTNERS_PROGRMS/PROGRAMS/DEVELOPER/TOOLS/PWERTOOLS.AFFX. Normalized arrays values were reported on a log2 scale. (Mean normalized expression is typically ~7.0.) For statistical analyses, we removed all probe sets where no experimental groups had a mean log2 intensity >3.0. This is a standard cutoff, below which expression is indistinguishable from background noise. Linear models were fitted for each gene using the Bioconductor “limma” package in R (Gentleman et al., 2004; Smyth, 2004). Moderated t-statistics, fold change, and the associated p-values were calculated for each gene. To account for the fact that thousands of genes were tested, we reported false discovery rate (FDR)-adjusted values, calculated using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). FDR values indicate the expected fraction of falsely declared differentially expressed (DE) genes among the total set of declared DE genes (i.e., FDR = 0.15 would indicate that ~15% of the declared DE genes were expected to be caused by experimental noise instead of actual differential expression). Batch effects between the Ror2Y324C and β-cateninGOF microarray datasets were adjusted using “removeBatchEffect” in “limma” package in R. Comparisons were conducted on n = 4 WT and n = 4 Ror2Y324C embryos for the Ror2Y324C dataset and n = 5 WT and n = 2 β-cateninGOF embryos for the β-cateninGOF dataset.

GSEA of the Ror2Y324C microarray dataset was compared with lists of cell cycle genes (Cell Cycle PCR Array; QIAGEN), β-catenin ChIP peaks called from mouse intestinal crypts (Schuijers et al., 2015), and misregulated genes identified in the β-cateninGOF microarray dataset. Analyses were conducted using GSEA software downloaded from the Broad Institute at http://software.broadinstitute.org/gsea/index.jsp (Mootha et al., 2003; Subramanian et al., 2005). Files input comprised of Ror2Y324C expression data (expression dataset, .txt file), gene lists of interest (gene sets database, .gmt file), labels for the expression data (phenotype labels, .cls file), and annotation of Affymetrix MoGene 1.0 ST microarray chips provided by the Broad Institute (Chip platform, .chip file). Details for the creation of each file type can be found in the GSEA user guide. Each analysis was performed as a Ror2Y324C versus WT comparison and ran 1,000 permutations where the dataset was not collapsed to gene symbols (marked “false”), “phenotype” was selected as the permutation type, and minimum/maximum sizes were 20/5,000.

Statistics
Statistical tests including the Student’s t test, Fisher’s exact test, χ2 test, correlation coefficient, regression analysis, and analysis of variance were performed in Excel (Microsoft), Prism (GraphPad), and VassarStats.
Online supplemental material

Fig. S1 shows examples of EdU labeling in embryo sections and frequency of EdU incorporation by location at specific ages and in different genetic backgrounds. Fig. S2 shows PGC distribution in Ror2<sup>Y324C</sup> and Wnt5a<sup>−/−</sup> embryos by age, frequency of EdU incorporation by location at specific ages in each mutant, and localization of WNT5A in the hindgut. Fig. S3 shows rescue of BrdU incorporation in Ror2<sup>Y324C</sup> mutants by β-catenin haploinsufficiency, overlap and GSEA enrichment of Ror2<sup>Y324C</sup> and Wnt5a<sup>−/−</sup> embryos by age, frequency of EdU incorporation by location at specific ages in each mutant, and localization of PGCs treated with exogenous WNT5a. Fig. S4 shows the effect of Ficin treatment on revealing nuclear β-catenin signal in histological sections and quantification of nuclear β-catenin intensity in PGCs in all migratory ages and in specific locations by age. Fig. S5 shows Pou5f1cre-ER<sup>−/−</sup> activity with tamoxifen exposure at E8.25 and PGC distribution and EdU incorporation across embryonic locations in β-catenin<sup>GOF</sup> mutants. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201511061/DC1.

Acknowledgments

The authors thank A. Brack, R. Arora, D. Nguyen, B. Reznik, N. Meyer, B. DeVeale, and K. Ebata for feedback on versions of this manuscript; M. Kissner and A.D.J. Ross for technical support; J. Schuijers and H. Clevers for sharing ChIP annotations and data; A. Williams at the Gladstone Bioinformatics Core for statistical analysis of microarray data; and A. Laird for guidance on statistics.

This work was supported by a National Science Foundation postdoctoral fellowship to A.V. Cantú, the University of California, San Francisco Program for Breakthrough Biomedical Research, and National Institutes of Health grants 1R21ES023297-01 and DP2OD007420 to D.J. Laird.

The authors declare no competing financial interests.

Author contributions: A.V. Cantú, S. Altshuler-Keylin, and D.J. Laird designed the study; A.V. Cantú and D.J. Laird wrote the manuscript; and all authors carried out experiments and contributed to data analysis.

Submitted: 16 November 2015
Accepted: 15 June 2016

References

Anderson, R., R. Fässler, E. Georges-Labouesse, R.O. Hynes, B.L. Bader, J.A. Kreidberg, K. Schaible, J. Heasman, and C. Wylie. 1999. Mouse primordial germ cells lacking β1 integrins enter the germline but fail to migrate normally to the gonads. Development. 126:1655–1664.
Ara, T., Y. Nakamura, T. Egawa, T. Sugiyama, K. Abe, T. Kishimoto, Y. Matsui, and T. Nagasawa. 2003. Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1. Dev. Biol. 257:308–318.
Aramaki, S., K. Hayashi, K. Kurimoto, H. Ohta, Y. Yabuta, H. Iwanari, Y. Mochizuki, T. Hamakubo, Y. Kato, K. Shihara, and M. Saitou. 2013. Mesodermal factor T specifies mouse germ cell fate by directly regulating oogonia differentiation and entry into meiosis in the mouse fetal ovary. PLoS One. 8:e625641. http://dx.doi.org/10.1371/journal.pone.0062564
Chassot, A.A., E.P. Gregoire, R. Lavery, M.M. Taketo, D.G. de Rooy, I.R. Adams, and M.C. Chabossier. 2011. Rspo1/β-catenin signaling pathway regulates oogonia differentiation and entry into meiosis in the mouse fetal ovary. Stem Cells. 29:274–285. http://dx.doi.org/10.1002/stem.11059323
Chiquoine, A.D. 1954. The identification, origin, and migration of the primordial germ cells in the mouse. Anat. Rec. 118:135–146. http://dx.doi.org/10.1002/ar.1951180202
de Vries, W.N., L.T. Binns, K.S. Fancher, J. Dean, R. Moore, R. Kemler, and B.B. Knowles. 2000. Expression of Cre recombinase in mouse oocytes: a means to study maternal effect genes. Genesis. 26:110–112. http://dx.doi.org/10.1002/(SICI)1526-680X(200002)26:2<110::AID-GENE2>3.0.CO;2-8
Downs, K.M. 2008. Systematic localization of Oct-3/4 to the gastrulating mouse conceptus suggests manifold roles in mammalian development. Dev. Dyn. 237:464–475. http://dx.doi.org/10.1002/dvdy.21438
Ediris, B., J. Espinosa, T. Mühlenberg, A. Mikels, C.H. Lee, S.E. Steigen, S. Zhu, K.D. Montgomery, A.J. Lazar, D. Lev, et al. 2012. ROR2 is a novel prognostic biomarker and a potential therapeutic target in leiomyosarcoma and gastrointestinal stromal tumour. J. Pathol. 227:223–233. http://dx.doi.org/10.1002/path.3986
Gentleman, R.C., V.J. Carey, D.M. Bates, B. Bolstad, M. Dettling, S. Dudoit, J.C. Irizarry, V. de Kerkhof, D. Leiden, S. Li, M.J. Abedin, A. Sajini, Y. Segal, J.M.W. Slack, R. Moore, R. Kemler, and B.B. Knowles. 2000. Expression of Cre recombinase in mouse oocytes: a means to study maternal effect genes. Genesis. 26:110–112. http://dx.doi.org/10.1002/(SICI)1526-680X(200002)26:2<110::AID-GENE2>3.0.CO;2-8
Gu, Y., C. Runyan, A. Shoemaker, A. Surani, and C. Wylie. 2009. Steel factor controls primordial germ cell survival and motility from the time of their colonization of the gonads by mid-gestation. Dev. Biol. 328:1253–1264.
Hagele, H., L. Larue, M. Ohsugi, L. Fedorov, K. Herrenknecht, and R. Kemler. 1995. Lack of β-catenin affects mouse development at gastrulation. Development. 121:3529–3537.
Harada, N., Y. Tamai, T. Ishikawa, B. Sauer, K. Takaku, M. Oshima, and M.M. Taketo. 1999. Intestinal polyposis in mice with a dominant stable mutation of the β-catenin gene. EMBO J. 18:5931–5942. http://dx.doi.org/10.1093/emboj/18.21.5931

Hikasa, H., M. Shibata, I. Hiratani, and M. Taira. 2002. The Xenopus receptor tyrosine kinase Xor2 modulates morphogenetic movements of the axial mesoderm and neuroectoderm via Wnt signaling. Development. 129:227–233.

Ho, H.Y., M.W. Susman, J.B. Bikoff, Y.K. Ryu, A.M. Jonas, L. Hu, R. Kuruvilla, and M.E. Greenberg. 2012. Wnt5a-Ror-Dish Devilled signaling constitutes a core developmental pathway that controls tissue morphogenesis. Proc. Natl. Acad. Sci. USA. 109:4044–4051. http://dx.doi.org/10.1073/pnas.120421109

Hogan, B., R. Beddington, F. Constantini, and E. Lacy. 1994. Manipulating the Mouse Embryo. 3rd. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 800 pp.

Huelsken, J., R. Vogel, V. Brinkmann, B. Erdmann, C. Birchmeier, and W. Birchmeier. 2000. Requirement for β-catenin in anterior-posterior axis formation. J. Cell Biol. 148:567–576. http://dx.doi.org/10.1083/jcb.148.3.567

Jamora, C., R. DasGupta, P. Kocjniewski, and E. Fuchs. 2003. Links between signal transduction, transcription and adhesion in epithelial bud development. Nature. 422:317–322. http://dx.doi.org/10.1038/nature01458

Jho, E.H., T. Zhang, C. Domon, C.K. Jou, N.F. Freud, and F. Costantini. 2002. Wnt/beta-catenin/TCF signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol. Cell. Biol. 22:1172–1183. http://dx.doi.org/10.1128/MCB.22.4.1172-1183.2002

Kaufman, M.H. 1992. The Atlas of Mouse Development. Elsevier Academic Press, London. 512 pp.

Kispert, A., S. Vainio, L. Shen, D.H. Rowitch, and A.P. McMahon. 1996. Proteoglycans are required for maintenance of Wnt-11 expression in the uterine tips. Development. 122:3637–3637.

Kunwar, P.S., D.E. Siekhuis, and R. Lehmann. 2006. In vivo migration: a germ cell perspective. Annu. Rev. Cell Dev. Biol. 22:237–265. http://dx.doi.org/10.1146/annurev.cellbio.22.010305.103337

Laird, D.J., U.H. von Andrian, and A.J. Wagers. 2008. Stem cell trafficking in tissue development, growth, and disease. Cell. 132:612–630. http://dx.doi.org/10.1016/j.cell.2008.01.041

Laird, D.J., S. Altshuler-Keelyn, M.D. Kissner, X. Zhou, and K.V. Anderson. 2011. Ror2 enhances polarity and directional migration of primordial germ cells. PLoS Genet. 7:e1002428. http://dx.doi.org/10.1371/journal.pgen.1002428

Li, C., and W.H. Wong. 2001. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc. Natl. Acad. Sci. USA. 98:31–36. http://dx.doi.org/10.1073/pnas.98.1.31

Logan, C.Y., and R. Nusse. 2004. The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. 20:781–810. http://dx.doi.org/10.1146/annurev.cellbio.20.010403.113126

Lu, B.J., Y.Q. Wang, X.J. Wei, L.Q. Rong, D. Wei, C.M. Yan, D.J. Wang, and J.Y. Sun. 2012. Expression of Wnt-5a and ROR2 correlates with disease severity in osteosarcoma. Mol. Med. Rep. 5:1033–1036. http://dx.doi.org/10.3892/mmr.2012.772

Lustig, B., J. Jerchow, M. Sachs, S. Weiler, T. Pietsch, U. Karsten, M. van de Wetering, H. Clevers, P.M. Schlag, W. Birchmeier, and J. Behrens. 2002. Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol. Cell. Biol. 22:1184–1193. http://dx.doi.org/10.1128/MCB.22.4.1184-1193.2002

Matsui, Y., K. Zsebo, and B.L.M. Hogan. 1992. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell. 70:841–847. http://dx.doi.org/10.1016/0092-8674(92)90317-6

McLaren, A. 2003. Primordial germ cells in the mouse. Dev. Biol. 262:1–15. http://dx.doi.org/10.1016/S0012-1606(03)00214-8

Mikels, A.J., and R. Nusse. 2006. Purified Wnt5a protein activates or inhibits β-catenin-TCF signaling depending on receptor context. PLoS Biol. 4:e115. http://dx.doi.org/10.1371/journal.pbio.0040115

Mikels, A., Y. Minami, and R. Nusse. 2009. Ror2 receptor requires tyrosine kinase activity to mediate Wnt5a signaling. J. Biol. Chem. 284:30167–30176. http://dx.doi.org/10.1074/jbc.M109.041715

Molyneux, K.A., H. Zinszner, P.S. Kunwar, K. Schabl, J. Stebler, M.J. Sunshine, W. O'Brien, E. Ruz, D. Littman, C. Wylie, and R. Lehmann. 2003. The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. Development. 130:4279–4286. http://dx.doi.org/10.1242/dev.006640

Moon, R.T., R.M. Campbell, J.L. Christian, L.L. McGrew, J. Shih, and S. Fraser. 1993. Wnt-5A: a maternal Wnt that affects morphogenetic move-
Weeraratna, A.T., Y. Jiang, G. Hostetter, K. Rosenblatt, P. Duray, M. Bittner, and J.M. Trent. 2002. Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. *Cancer Cell.* 1:279–288. http://dx.doi.org/10.1016/S1535-6108(02)00045-4

Wong, G.T., B.J. Gavin, and A.P. McMahon. 1994. Differential transformation of mammary epithelial cells by Wnt genes. *Mol. Cell. Biol.* 14:6278–6286. http://dx.doi.org/10.1128/MCB.14.9.6278

Yamada, M., J. Udagawa, A. Matsumoto, R. Hashimoto, T. Hatta, M. Nishita, Y. Minami, and H. Otani. 2010. Ror2 is required for midgut elongation during mouse development. *Dev. Dyn.* 239:941–953. http://dx.doi.org/10.1002/dvdy.22212

Yamaguchi, T.P., A. Bradley, A.P. McMahon, and S. Jones. 1999. A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development.* 126:1211–1223.

Yamamoto, S., O. Nishimura, K. Misaki, M. Nishita, Y. Minami, S. Yonemura, H. Tarui, and H. Sasaki. 2008. Cthrc1 selectively activates the planar cell polarity pathway of Wnt signaling by stabilizing the Wnt-receptor complex. *Dev. Cell.* 15:23–36. http://dx.doi.org/10.1016/j.devcel.2008.05.007

Yan, D., M. Wiesmann, M. Rohan, V. Chan, A.B. Jefferson, L. Guo, D. Sakamoto, R.H. Caothien, J.H. Fuller, C. Reinhard, et al. 2001. Elevated expression of axin2 and hnk1 mRNA provides evidence that Wnt/L-catenin signaling is activated in human colon tumors. *Proc. Natl. Acad. Sci. USA.* 98:14973–14978. http://dx.doi.org/10.1073/pnas.261574498