Traffic jam functions in a branched pathway from Notch activation to niche cell fate

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ABSTRACT

The niche directs key behaviors of its resident stem cells, and is thus crucial for tissue maintenance, repair and longevity. However, little is known about the genetic pathways that guide niche specification and development. The male germline stem cell niche in *Drosophila* houses two stem cell populations and is specified within the embryonic gonad, thus making it an excellent model for studying niche development. The hub cells that form the niche are specified early by Notch activation. Over the next few hours, these individual cells then cluster together and take up a defined position before expressing markers of hub cell differentiation. This timing suggests that there are other factors for niche development yet to be defined. Here, we have identified a role for the large Maf transcription factor Traffic jam (Tj) in hub cell specification downstream of Notch. Tj downregulation is the first detectable effect of Notch activation in hub cells. Furthermore, Tj depletion is sufficient to generate ectopic hub cells that can recruit stem cells. Surprisingly, ectopic niche cells in *tj* mutants remain dispersed in the absence of Notch activation. This led us to uncover a branched pathway downstream of Notch in which *Tj* functions to direct hub cell assembly in parallel to Tj dependent processes.

KEY WORDS: Traffic jam, Notch, Stem cell niche, Gonadogenesis, *Drosophila*

INTRODUCTION

Adult stem cells are a crucial component of most organs. Having the unique ability to either self-renew or replace differentiated cells, they function in steady-state homeostasis and can also be activated in response to injury (Barker et al., 2010). The instructive cues that guide stem cells to self-renew or differentiate often come extrinsically from the local microenvironment called the stem cell niche (Morrison and Spradling, 2008). Whereas previous work focused primarily on the identification and steady-state function of the stem cell niche, the mechanisms by which a niche is established during development are only more recently being addressed (Takeda et al., 2011; Yan et al., 2011; Sato et al., 2011). Several pathways required for niche cell specification have now been identified, including Sonic Hedgehog in the neurogenic niche, hormonal and Notch signaling in the *Drosophila* ovarian niche and Wnt signaling in the *C. elegans* germline niche (Wang et al., 2014b; Gancz et al., 2011; Song et al., 2007; Lam et al., 2006). Comprehensive analysis of the pathway targets and how they individually direct niche cell fate is limited in complex systems. Fortunately, the *Drosophila* testis germline stem cell (GSC) niche is well characterized and is established early within the male embryonic gonad. The niche cells can be unambiguously identified with multiple markers as early as the embryo-to-larval transition, and stem cells are recruited shortly thereafter (Le Bras and Van Doren, 2006; Sheng et al., 2009; Sinden et al., 2012). For this reason, the male gonad provides an ideal system to identify and dissect the pathways required for niche specification.

The male GSC niche is located at the apical tip of the testis and is composed of a small aggregate of post-mitotic somatic cells called hub cells (Brower et al., 1981; Le Bras and Van Doren, 2006). The hub cells secrete bone morphogenetic proteins (BMPs) and the cytokine Unpaired to activate self-renewal and adhesion in the GSCs (Kawase et al., 2004; Kiger et al., 2001; Leatherman and DiNardo, 2010; Shivdasani and Ingham, 2003; Tulina and Matunis, 2001). An additional component of the niche, the cyst stem cell (CySC) lineage, is maintained by Unpaired and Hedgehog secreted from the hub (Leatherman and DiNardo, 2008, 2010; Amoyel et al., 2013; Michel et al., 2012). Steady-state function of the niche is established within the larval gonad following hub cell specification and stem cell recruitment (Le Bras and Van Doren, 2006; Sheng et al., 2009; Sinden et al., 2012).

The specification of hub cells from a pool of somatic gonadal precursors (SGPs) occurs via Notch activation (Kitadate and Kobayashi, 2010; Okegbe and DiNardo, 2011). Around mid-embryogenesis, the germ cells travel through the gut, where they coalesce with SGPs. At this time, the SGPs are briefly exposed to an endodermally derived Delta ligand which activates Notch in some SGPs (Okegbe and DiNardo, 2011). Whereas Notch signaling is required early, cell surface and gene expression markers of hub cell fate are not detected until many hours later, at the end of embryogenesis. Thus, the hub only becomes identifiable in late-stage embryonic gonads after hub cells have sorted to an anterior position, accumulated significant levels of epithelial adhesion proteins and adopted cobblestone morphology. At this time, gene regulatory changes result in hub-specific expression of unpaired (Wawersik et al., 2005; Le Bras and Van Doren, 2006).

The delay in differentiation suggests that there are unknown intermediate effectors in hub cell specification downstream of Notch. The transcription factor Bowl and receptor tyrosine kinase (RTK) signaling have also been implicated in hub cell fate determination; however, their relationship to Notch activation has not been elucidated (DiNardo et al., 2011; Kitadate et al., 2007; Kitadate and Kobayashi, 2010). This work focuses on the role of the large Maf transcription factor Traffic jam (Tj) during hub cell specification. Previous work has shown that *tj* is expressed in SGPs and is required in gonad morphogenesis (Li et al., 2003). Additionally, Tj can suppress accumulation of the septate junction protein Fasciclin III (FasIII) and its RNA in the somatic cells of the adult testis and ovary (Li et al., 2003). Here, we show that (1) Tj represses markers of hub cell fate and niche signaling, (2) Tj is downregulated in a subset of anterior gonadal cells and (3) Notch is
required for this suppression. Finally, we show that Tj acts along one arm of a pathway downstream of Notch activation for hub cell specification, whereas Bowl acts in a parallel arm to direct hub cell assembly.

RESULTS

Tj is downregulated in hub cells

Traffic jam (tj) is initially expressed in SGPs upon contact with germ cells around stage 12 of embryogenesis (Li et al., 2003). Following compaction into a spherical gonad, Tj protein accumulated at similar levels in all SGPs, whereas the hub cell marker FasIII was not detected (Fig. 1A). Following niche formation, gonads contained an anteriorly localized aggregate of hub cells identified by FasIII enrichment (Fig. 1C). Given that Tj is downregulated in hub cells, we tested whether presumptive hub cells could be identified prior to niche formation by reduced Tj accumulation.

We observed a reduction of Tj protein accumulation among a subset of anterior SGPs prior to niche formation (Fig. 1B, arrows). Anecdotally, prior lineage tracing had revealed that hub cells are derived from among the anterior half of SGPs (Le Bras and Van Doren, 2006; DiNardo et al., 2011, and L.W., unpublished data). We quantified Tj accumulation by averaging signals from the anterior half of SGPs and by comparing that with the posterior half. During stage 15, the anterior-to-posterior ratio was 1.0±0.15 (s.d.) (n=5 gonads) but decreased to 0.7±0.08 (n=6) during stage 16, confirming that SGPs in the anterior had reduced Tj signal compared with those in the posterior (P=0.0016; Student’s t-test). Some anterior SGPs maintained high Tj levels (Fig. 1B). This is consistent with the fact that not all anterior SGPs contribute to hub, and suggests that our quantification of anterior versus posterior enrichment is a conservative estimate of the downregulation of Tj in hub-specified cells. Furthermore, prior to assembly of the hub at the anterior, we detected faint puncta of FasIII around Tjlow SGPs (Fig. 1B, arrows) but not around Tjhigh SGPs. By L1, the ratio of average pixel intensity in hub cells compared with the first two tiers of somatic cells around the hub was 0.3±0.05 (n=7). These data strongly suggest that Tj downregulation is evident prior to other markers of hub cell fate, including FasIII enrichment. We hypothesized that Tj repression might be a crucial step that instructs hub cell differentiation.

tj represses hub cell differentiation

If Tj restricts hub cell fate in SGPs, we would expect global loss of Tj to result in more SGPs adopting hub cell fate. To test this, we examined tj mutant gonads for epithelial and niche-specific gene expression markers. Control gonads accumulated FasIII at interfaces between hub cells (Fig. 2A, asterisk). In tj mutant gonads, we always observed an endogenous hub consisting of a cluster of FasIII-enriched cells assembled at the anterior with cobblestone morphology (Fig. 2B, asterisk). However, in addition, there were FasIII-enriched cells that did not assemble at the anterior, but instead remained dispersed and intermingled with germ cells (Fig. 2B, arrows). The average number of cells in tj mutants that accumulated FasIII on one gonad was 42±10.7 (n=18) compared with 10.8±3.7 in controls (n=18), whereas the total number of SGPs was similar (53.9±4.6, n=15; 50.4±5; n=14). We found that E-cadherin, which is enriched in endogenous hub cells, also accumulated ectopically (supplementary material Fig. S1).

The ectopic cells also induced the niche gene expression marker unpaired (updRFP), similar to the endogenous hub (Fig. 2A,B). UpdRFP overlapped with FasIII accumulation in almost all cells, resulting in tj mutant gonads containing an average of 39.3±10.4 updRFP-expressing cells compared with 12.7±3.6 in controls. Furthermore, these cells were active in inducing Jak/STAT signaling in response to Unpaired. In control gonads, a somatic cell reporter for STAT induction (STAT-GFP) accumulated in the cytoplasm of the hub cells (Fig. 2C, asterisk) and in select somatic cells near the hub, presumably recruited as CySCs (Fig. 2C, arrow). In tj mutant gonads, GFP accumulated in most of the somatic cells, including those far from the endogenous hub (Fig. 2D, arrows), indicating that the ectopic hub cells activate STAT. Thus, tj mutants contained more hub cells than controls, indicating that Tj functions to repress hub cell differentiation in SGPs.

Ectopic stem cells are recruited in tj mutant gonads

If the ectopic hub cells in tj mutants act as niche, they should recruit and maintain stem cells. GSCs recruited to the hub stabilize STAT within the nucleus, which is required for E-cadherin-mediated attachment (Sheng et al., 2009; Yamashita et al., 2003; Leatherman and DiNardo, 2010). Just as in controls (Fig. 3A,C), tj mutants exhibited STAT accumulation within the GSCs contacting the endogenous hub (Fig. 3B, asterisk) and E-cadherin enrichment at their shared interface (Fig. 3D, asterisk). Germ cells contacting ectopic hub cells also accumulated STAT (Fig. 3B, arrows) and exhibited E-cadherin enrichment (Fig. 3D,D′, inset, arrowheads; N=10/11 gonads). Occasionally, we detected interfaces between ectopic hub cells and germ cells that were less enriched for E-cadherin (Fig. 3D,D′, N=1/11). We also staged germ cells by fusome morphology. In control gonads, GSCs surrounding the hub contained dot fusomes (Fig. 4A,C, arrowheads), whereas differentiating germ cells located farther from the hub accumulated...
branched structures (Fig. 4A,C, arrows). tj mutant gonads contained only dot or dumbbell-shaped fusomes at late L1 (Fig. 4B, arrowheads), signifying that cells had not progressed past the two-cell stage. In later developmental stages (L3), some branched fusomes were detected, but only among germ cells at some distance from ectopic hub cells (Fig. 4D, arrow). Strikingly, germ cells adjacent to ectopic hub cells often contained dot fusomes (Fig. 4D, arrowheads). As the ectopic cells were several tiers removed from the endogenous hub, these data strongly suggest that ectopic hub cells in tj mutants exhibit niche qualities.

We also examined tj mutant gonads for the presence of CySCs. During normal development, both Zfh1 and Eyes absent (eya) are expressed in SGP s prior to niche formation (Broihier et al., 1998; Boyle and DiNardo 1995). Following niche formation and CySC specification, Zfh1 is selectively enriched in somatic cells near the hub, while Eya accumulates in a complementary pattern in more distal, differentiating cells (Fig. 4E,G) (Sinden et al., 2012; Fabrizio et al., 2003). In late L1 tj mutant gonads, Zfh1-enriched, Eya-depleted somatic cells were detected more than two tiers from the endogenous hub, suggesting the presence of ectopic stem cells (Fig. 4F, arrowheads). However, we also detected Eya+ cells adjacent to ectopic hub cells (Fig. 4F, arrow). Given that eya was previously expressed in these cells, it is possible that its repression is delayed in tj mutants. Therefore, we examined L3 tj mutant gonads, days after CySC specification would have occurred. Mature tj mutant gonads contained Zfh1+ cells both adjacent to the endogenous hub and several tiers away, near the ectopic hub cells, while Eya+ cells were only found distal to any hub cells (Fig. 4H,H′, arrowheads, endogenous hub marked by asterisk). We conclude that tj mutants contain two pools of niche cells: an endogenous compact hub and dispersed hub cells, both of which can support GSCs and CySCs.

**Ectopic niche cells in tj mutants exhibit incomplete conversion to hub cell fate**

Whereas some of the ectopic cells were cuboidal, resembling endogenous hub cells (Fig. 2B, arrows), others were elongate with extensions that partially encysted germ cells, a quality also exhibited by CySCs (Fig. 3D′, arrow). This suggested that some of the ectopic cells in tj mutants were of mixed hub-CySC identity. To test this, we compared the levels of the CySC marker Zfh1 and cell cycle state, as only CySCs, and not hub cells, actively cycle. In late L1, Zfh1 was low in Fas3+ hub cells but was easily detectible in the first two tiers of somatic cells around the hub (Fig. 5A). Zfh1+ CySCs also incorporated 5-ethynyl-2′-deoxyuridine (EdU) (Fig. 5A, arrow), whereas Fas3+ hub cells did not (n=18 gonads). In tj mutants, we observed Fas3+ cells that accumulated Zfh1 at similar levels to CySCs (Fig. 5B, arrowheads). A subset of the Fas3+Zfh1hi cells incorporated EdU (Fig. 5C-C″, arrows; n=23 gonads). Spatially, these aberrantly cycling cells were located either among the outer cells of the endogenous hub or among the ectopic hub cells, but were never internal among the endogenous hub. This demonstrates that the ectopic hub cells in tj mutants exhibited some CySC behavior.

We next tested whether other aspects of hub cell fate differed between the endogenous and ectopic hub cells. Hedgehog secreted from the hub activates signaling within the CySC lineage. This is visualized by punctate accumulation of the receptor Patched (Ptc), representing trafficking intermediates (Amoyel et al., 2013; Michel et al., 2012). In late L1 controls, Ptc is enriched around the hub (Fig. 6A, asterisk) and the first two tiers of Zfh1+ CySCs (Fig. 6A, arrows). In tj mutant gonads, Ptc was detected within the endogenous hub (Fig. 6B, asterisk) and within CySCs adjacent to the endogenous hub. However, Ptc staining was also observed around Zfh1+ cells (Fig. 6B, arrow) several tiers from the endogenous hub, yet adjacent to ectopic hub cells (Fig. 6B, arrowhead). We also examined ligand expression using a lacZ enhancer trap for hedgehog (hhlacZ). As in controls (Fig. 6C), the endogenous hub cells in tj mutants co-expressed hedgehog with unpaired (Fig. 6D, asterisk); however, the ectopic unpaired-expressing cells did not express hedgehog (Fig. 6D, arrowheads) even though there was ectopic activation of the pathway. Furthermore, L3 tj mutant gonads accumulated hhlacZ only within the endogenous hub and not ectopic hub cells (data not shown). Perhaps the ectopic hub cells expressed levels of hhlacZ below our limits of detection. Alternatively, as signaling normally occurs in CySCs, and not in their differentiated daughter cells, it is possible that the ectopic CySCs recruited in tj mutant gonads are all competent to respond to Hedgehog secreted from the endogenous hub.
**Fig. 3. Germ cells adjacent to ectopic hub cells accumulate STAT and E-cadherin.** (A,B) Control (A) and \( tj \) (B) mid-L1 gonads stained with anti-Vasa (red), anti-GFP (\( updGFP \)) and anti-STAT (white). (A,A′) In a control gonad, STAT accumulates within GSCs (arrowheads) attached to the hub (asterisks) but not gonialblasts (arrows). (B-B″) In a \( tj \) mutant gonad, STAT accumulates in germ cells (arrows) adjacent to ectopic hub cells (arrowheads) that are not contacting the endogenous hub (asterisk). (C,D) Control (C) and \( tj \) (D) late-L1 gonads stained with anti-Vasa (red), anti-GFP (\( updGFP \)) and anti-E-cadherin (white). (C,C′) Control gonad showing E-cadherin enrichment between GSCs and hub cells (arrowheads, inset), and lesser enrichment around a differentiating germline cyst distal to the hub (arrows). (D,D′) A \( tj \) mutant gonad, with E-cadherin enriched between GSCs and endogenous hub cells (asterisks) and ectopic hub cells (arrows, inset). (D″,D‴) In a different \( tj \) mutant gonad, a germ cell exhibits little E-cadherin at the interface with an ectopic hub cell (arrows).
cell specification might also require activation of Bowl in parallel to Tj downregulation. If this were true, activating Bowl in the ectopic hub cells in \( tj \) mutants should allow them to convert more fully to hub cell fate. To test this, we activated Bowl by using \( upd\text{gal4} \) to express Drumstick (Drm), a protein that binds and sequesters Lines, preventing its association with Bowl, thereby allowing Bowl to regulate transcription (Hatini et al., 2005). As expected, the hub was located at the anterior in control gonads, as judged by FasIII and N-cadherin (an additional adhesion protein enriched between hub cells) (Fig. 8A, asterisk). In \( tj \) mutant gonads, N-cadherin and FasIII were co-enriched on both endogenous (Fig. 8B, asterisk) and ectopic hub cells (\( upd\text{GFP} \)). Branched fusomes are observed distal to endogenous and ectopic hub cells (arrow). (E-H) Control (E,G) and \( tj \) (F,H) gonads stained with anti-GFP (\( upd\text{GFP} \)), anti-Zfh1 (white) and anti-Eya (red). In L1 (E) and L3 (G) control gonads, Zfh1+Eya\(^-\) nuclei are found closest to the hub (asterisks), whereas Eya accumulates distally. Peripheral Zfh1\(^+\) nuclei mark the gonadal sheath. Fully differentiated cyst cells with large Eya\(^-\)Zfh1\(^+\) nuclei are detected farthest from the hub in L3 (G, arrows). In L1 (F) and L3 (H) \( tj \) mutant gonads, Zfh1+Eya\(^-\) nuclei (F, arrowheads) are found several cell diameters from the endogenous hub (hub is not in focal plane in F, hub is marked by asterisk in H). In L1, Eya\(^-\) nuclei can be found adjacent to ectopic hub cells (F, arrow). At L3, Eya\(^-\) nuclei are only found distal to both endogenous and ectopic hub cells (H, arrowheads). Fully differentiated cyst cells that accumulate Eya only are not detected in L3 \( tj \) mutants.

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Fig. 4. Ectopic hub cells maintain germline and cyst stem cells. (A-D) Control (A,C) and \( tj \) (B,D) gonads stained with anti-Vasa (red), anti-GFP (\( upd\text{GFP} \)) and anti-\( \alpha \)-spectrin (white) to mark fusomes. L1 (A) and L3 (C) control gonads exhibit dot fusome-containing germ cells (arrowheads) only around the hub (asterisks), whereas branched fusomes are observed several tiers away (arrows). (B) L1 \( tj \) mutant gonad exhibits only dot or dumbbell-shaped fusomes (arrowheads). (D) In an L3 \( tj \) mutant gonad, dot fusome-containing germ cells (arrowheads) are found several tiers from the endogenous hub (asterisk), yet adjacent to ectopic hub cells (\( upd\text{GFP} \)). Branched fusomes are observed distal to endogenous and ectopic hub cells (arrow). (E-H) Control (E,G) and \( tj \) (F,H) gonads stained with anti-GFP (\( upd\text{GFP} \)), anti-Zfh1 (white) and anti-Eya (red). In L1 (E) and L3 (G) control gonads, Zfh1+Eya\(^-\) nuclei are found closest to the hub (asterisks), whereas Eya accumulates distally. Peripheral Zfh1\(^+\) nuclei mark the gonadal sheath. Fully differentiated cyst cells with large Eya\(^-\)Zfh1\(^+\) nuclei are detected farthest from the hub in L3 (G, arrows). In L1 (F) and L3 (H) \( tj \) mutant gonads, Zfh1+Eya\(^-\) nuclei (F, arrowheads) are found several cell diameters from the endogenous hub (hub is not in focal plane in F, hub is marked by asterisk in H). In L1, Eya\(^-\) nuclei can be found adjacent to ectopic hub cells (F, arrow). At L3, Eya\(^-\) nuclei are only found distal to both endogenous and ectopic hub cells (H, arrowheads). Fully differentiated cyst cells that accumulate Eya only are not detected in L3 \( tj \) mutants.

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Fig. 5. Ectopic hub cells in \( tj \) mutants accumulate Zfh1 and cycle. Late L1 control (A) and \( tj \) mutant (B,C) gonads stained with anti-FasIII (green), anti-Zfh1 (red) and EdU (white). (A,A′) In a control gonad, Zfh1 is low in FasIII\(^+\) hub cells but accumulates in nearby CySCs, one of which incorporates EdU (arrows). (B,B′) A \( tj \) mutant gonad contains Zfh1\(^+\)FasIII\(^+\) hub cells (arrowheads). (C-C′) In a different focal plane within the same \( tj \) mutant gonad, a Zfh1\(^+\)FasIII\(^+\) ectopic hub cell incorporates EdU (arrows).
endogenous hub cells, appearing more compact and less elongate than the ectopic hub cells of tj mutants, and adopting a more cuboidal morphology. We quantified the rescue by counting anteriorly associated, N-cadherin-enriched hub cells. Control gonads exhibited 13.7±1.3 or 14.3±3.5 anterior hub cells (upd>uas gfp; upd>uas drm, respectively) and tj mutants averaged 15.5±4.1 cells. However, tj mutants with activated Bowl (tj; upd>drm) averaged significantly more anteriorly assembled hub cells (19.5±4.4; *P*<0.004). Additionally, eight out of 21 tj mutants with activated Bowl had greater than 22 anterior hub cells compared with only two out of 21 tj single mutants (Fig. 8D). We conclude that Bowl activation is sufficient for proper hub cell aggregation and assembly at the anterior and is probably a Notch target that functions in parallel to Tj downregulation.

Although we observed a rescue of hub assembly, we detected no change in the number of cells expressing hhlacZ (tj; upd>drm=16 cells; *n*=7) compared with controls (upd>drm=16.2; *n*=5, upd>gfp=18; *n*=6, tj=18; *n*=8). Lastly, EdU pulse-labeling showed that ectopic hub cells continued cycling with Bowl

Fig. 6. Hedgehog signaling range is broader in tj mutants. (A,B) Control (A) and tj mutant (B) gonads stained with anti-Zfh1 (red), anti-GFP (updGFP) and anti-Patched (Ptc) (white). (A-A″) In a control gonad, Ptc is only detected on hub cells (asterisks) and Zfh1+ cells near the hub (arrows). (B-B″) In a tj mutant gonad, Ptc accumulates within the endogenous hub (asterisks) and adjacent somatic cells but also on an ectopic hub cell (arrowheads) and an adjacent somatic cell (arrows) several tiers from the endogenous hub. (C,D) L1 control (C) and tj mutant (D) gonads stained with anti-Tj (white), anti-GFP (updGFP) and anti-lacZ (hhlacZ). (C-C″) Control gonad showing lacZ co-expressed with GFP in hub cells. (D-D″) In a tj mutant gonad, lacZ is co-expressed with GFP in endogenous hub (asterisks) but is absent in ectopic GFP+ cells (arrowheads). tj^e02 allele encodes for truncated Tj protein, resulting in cytoplasmic staining (Li et al., 2003).

Fig. 7. Tj functions downstream of Notch activation in a branched pathway to specify hub cells. (A-D) Stage 17/L1 control (A), Notch (B), tj (C) and N;tj (D) mutant gonads stained with anti-Vasa (red), anti-FasIII (green) and anti-Tj (white). (A,A′) Control gonad containing FasIII+ Tj^hox hub cells (asterisks). (B,B′) Notch mutant gonad accumulates Tj in all SGPs and lacks FasIII enrichment. (C,C′) tj mutant gonad contains an endogenous FasIII-enriched hub (asterisks) and ectopic FasIII-enriched cells (arrows). (D,D′) In an N;tj mutant gonad, SGPs accumulate FasIII but fail to organize as an anterior, compact aggregate. (E-H′) Stage 17/L1 control (E), Notch (F), tj (G) and N;tj (H) mutant gonads stained with anti-Zfh1 (red), anti-GFP (STAT-GFP) and anti-Tj (white). (E,E′) In a control gonad, GFP is detected within the hub (asterisk) and gonadal sheath. (F,F′) In a Notch mutant gonad, GFP is detected only within the sheath. (G,G′) In tj (G) and N;tj (H) mutant gonads, GFP is detected in most somatic cells (G′,H′).
activation (data not shown), suggesting the existence of a Notch effector in addition to the two branches described here, or an unknown, parallel input to hub cell fate.

DISCUSSION

Previous genetic and lineage tracing data demonstrated that a subset of SGPs is Notch-activated early during gonadogenesis (Okegbe and DiNardo, 2011). Several hours later, these cells assemble into a compact niche that expresses various factors required for stem cell recruitment, attachment and self-renewal (Le Bras and Van Doren, 2006) (Fig. 9). Here, we have dissected the pathway downstream of Notch activation in hub cell specification. Specifically, Notch activation downregulates Traffic jam, thus relieving repression of unpaired and allowing for the accumulation of multiple adhesion proteins in these cells. In a parallel arm, Bowl activation mediates proper assembly and aggregation of hub cells at the anterior, possibly through regulation of F-actin. Based on our data in the adult testis, Hedgehog is placed downstream of Bowl activation. This is consistent with downregulation of Tj in the few hub cells that are specified. Furthermore, forced activation of Notch only moderately increases the number of hub cells (Kitadate and Kobayashi, 2010), and these extra hub cells also exhibited reduced Tj accumulation (L.W., unpublished data). Although this might suggest direct control of Tj by Notch, the situation is more complex. The fact that Notch activation is insufficient for hub cell fate, combined with the fact that a significant period exists between the requirement for Notch and a detectable reduction of Tj protein (∼6 h), suggests that Notch repression of Tj probably occurs through intermediate effectors. It was recently shown that robust Tj accumulation in early-stage SGPs requires the gene midline, which encodes a T-box 20 transcription factor (Tripathy et al., 2014). In other tissues, Midline can antagonize Notch signaling and is repressed in Notch-activated cells (Das et al., 2013). Therefore, it would be interesting to determine whether midline is regulated by Notch in SGPs.

Notch also acts in specifying the female germline stem cell niche in Drosophila (Song et al., 2007). As these niche cells also express traffic jam, it is worth investigating whether Traffic jam mediates Notch signaling during germlium niche specification. Furthermore, the regulatory relationship we identified in the male gonad between Notch and the Maf factor Tj might also apply in mammals. Interestingly, both c-Maf and MafB are expressed in somatic cells intermingled with the germ line in the developing mammalian gonad (DeFalco et al., 2011). Additionally, Notch signaling restricts Leydig cell differentiation within the interstitial...
compartment at the same developmental timepoint (Tang et al., 2008). Our data suggest that it would be reasonable to test whether Notch and Maf factors function together in specifying mammalian gonadal cell types.

**Traffic jam represses cytokine signaling**

We found that Tj depletion relieves repression of unpaired in SGPs, allowing them to activate Jak/STAT; thus bypassing one role for Notch. Recent work showed that Tj functions in border cell migration, in which it also modulates the Jak/STAT pathway. In that case, Tj enhances expression of the Jak/STAT pathway antagonist Suppressor of cytokine signaling E (Socs36E – Flybase.org) (Gunawan et al., 2013). Whereas we found that Tj functions by repressing expression of the ligand, both studies support a role in which Tj attenuates STAT signaling. Notably, in T helper cells, a large Maf factor also regulates expression of cell type-specific cytokines that activate STAT signaling (Ho et al., 1996; Kim et al., 1999). In this system, e-Maf activates expression of the ligand rather than repressing it; however, large Mafs can activate or repress transcription depending on context (Kataoka, 2007). These examples highlight recurring evidence of cooperation between Maf factors and cytokine signaling.

**Bowl mediates hub cell assembly**

Proteins representing different adhesive complexes accumulate on hub cells, including FasIII, E-cadherin and N-cadherin (Brower et al., 1981; Le Bras and Van Doren, 2006). However, depleting one or several subsets of these factors has little effect on hub cell assembly or aggregation at the anterior (M. Van Doren, personal communication). This suggests either significant redundancy in hub cell adhesion or that a yet unidentified factor is responsible for mediating the proper aggregation and assembly of hub cells. In tj mutants, ectopic hub cells were enriched for multiple, different epithelial complexes (FasIII and E-cadherin or FasIII and N-cadherin), yet these cells did not exhibit compact, anterior assembly. This suggests a requirement for an unknown factor. As activating Bowl was sufficient to significantly rescue hub cell assembly, perhaps Bowl regulates this factor. Bowl and Drumstick, both members of the Odd-skipped family of zinc-finger proteins, regulate morphological changes in the developing Drosofila leg (Hao et al., 2003). Overexpression of another family member, Odd, results in cell-autonomous and non-autonomous morphological changes and increases in F-actin (Hao et al., 2003). Therefore, examining changes in F-actin enrichment and identifying actin regulators downstream of Bowl would reveal the mechanism for hub assembly.

We have not clarified the epistatic relationship between bowl and Notch, which can be complex. In leg development, bowl expression is induced by Notch signaling (Hao et al., 2003; de Celis Ibeas and Bray, 2003). Furthermore, Bowl can repress delta in the Notch-activated cell, thus stabilizing a Notch signaling interface at leg segment boundaries (Greenberg and Hatini, 2012). In the wing, however, Bowl modulates Notch signaling by reducing availability of the Notch co-repressor Groucho (Benitez et al., 2009). In the gonad, mutants for either Notch or bowl have fewer hub cells, although the Notch phenotype is more severe (Kitadate and Kobayashi, 2010; Okegbe and DiNardo, 2011; DiNardo et al., 2011). Due to this and to the early requirement for Notch in SGPs, we suspect that Notch functions upstream of Bowl, forming a pathway parallel to Notch and Tj (Fig. 9). Whether the Notch-Bowl arm results in hedgehog expression is unresolved. Similar to the complex relationship between Bowl and Notch, Hedgehog signaling can either promote Bowl accumulation (in the epidermis) or Bowl can induce hedgehog expression (in retinogenesis) (Hatini et al., 2005; Bras-Pereira et al., 2006). Whereas we did not detect hhlacZ induction in gonads activated for Bowl, inviability prevented us from examining later timepoints. Previously, we observed hhlacZ induction five days after clonal activation of Bowl in adult CySCs (DiNardo et al., 2011). Given that CySCs are derived from SGPs, we favor the idea that Bowl can induce hedgehog.

Our model suggests that Tj and Bowl mediate many aspects of hub cell fate downstream of Notch signaling. As noted above, a fraction of hub cells are still specified in bowl mutants, and these appear to assemble anteriorly (DiNardo et al., 2011). This could suggest that only a subset of hub cells require Bowl for proper assembly (perhaps centrally located SGPs that need to move anteriorly). Alternatively, there could be an additional, unknown Notch effector that can compensate for bowl. Indeed, there is room for some complexity as RTK signaling represses hub cell fate in posterior SGPs and its interplay with the Notch pathway is yet unclear. In this regard, it is intriguing that tj mutant SGPs with forced activation of Bowl continue to cycle, whereas endogenous hub cells are quiescent (Fig. 6D and data not shown). Interestingly, we detected EGFR pathway activity in some ectopic hub cells (supplementary material Fig. S3D). EGF signaling mediates the proliferation of Drosofila intestinal and gastric stem cells (Buchon et al., 2010; Jiang et al., 2011; Xu et al., 2011; Bitue and Jasper, 2011; Wang et al., 2014a). Perhaps the ectopic hub cells in tj mutants remain cycling due to an inability to repress EGF signaling. With the pathway for hub cell specification now more clearly delineated (Fig. 9), future work can address the intersecting cell biological and gene expression targets of multiple pathways required for hub cell fate.

**Materials and Methods**

**Fly stocks**

Fly lines used were y1; y+ tj103 (D. Godt, University of Toronto, Canada), unpaired-GAL4 UAS GFP (FBtp0016756), UAS-RedStinger (FBti0040830) (updRFP), 10X-STATGFP (E. Bach, New York University, USA), N264.39 (FBA0029934), hedgehog-lacZ (FBst0005530), GTRACE (FBti0028281), UAS drm (J. A. Lengyel, University of California, Los Angeles, USA). Heterozygous siblings or w1118 were used as controls.

**Immunostaining**

Embryos were collected and aged 22-24 h in a humidified chamber to first-instar larvae or 36-48 h for late L1. Hatched larvae were dissected in Ringer solution and the internal organs were gently massaged out. Unhatched larvae were dechorionated, hand-devitellinized and dissected as described above. Tissue was fixed in 4% formaldehyde, Ringer instar larvae or 36-48 h for late L1. Hatched larvae were dissected in Ringer’s solution and the internal organs were gently massaged out. Unhatched larvae were dechorionated, hand-devitellinized and dissected as described above. Tissue was fixed in 4% formaldehyde, Ringer’s solution and 0.1% Triton-X-100 for 20 min (to reveal dPERK, tissue was fixed in 8% formaldehyde for 25 min), washed in PBS plus Triton-X-100 and blocked for 1 h in 4% normal serum. Primary antibodies were used overnight at 4°C or for 4 h at room temperature. Alexa Fluor-conjugated secondary antibodies donkey anti-chicken 488, anti-mouse 647, anti-mouse 555, anti-mouse 488, anti-rabbit 555, anti-rat 647, anti-guinea pig 555 and anti-goat 647 were used at 1:400 (all Jackson ImmunoResearch) and dpERK (1:100; Cell Signaling, 4370); mouse antibodies against Fasciclin III (1:50; DSHB), Eya 10H6 (1:20; DSHB), alpha-spectrin (1:200; DSHB), Patched (1:50, DSHB) and B-gal (1:10,000; Promega, Z3781); rat antibodies against DE-cadherin (1:20, DSHB, DCAD2) and DN-Cadherin (1:20, DSHB); guinea pig anti-Traffic jam (1:10,000; a gift from D. Godt, University of Toronto, Canada);
goat anti-Vasa (1:400; Santa Cruz, sc-26875); and chicken anti-GFP (1:1000; Invitrogen, A10262).

**Fluorescence imaging quantification**

Z-stacks were obtained through the depth of the gonad, using a Zeiss Axioplan microscope with an apotome attachment. Immunofluorescence signals of Tj were quantified using Metamorph by measuring nuclear pixel intensity.

**S phase labeling**

Dissected tissues were incubated for 30 min in 10 μM EdU and then fixed. EdU incorporation was visualized using the Click-iT EdU Alexa Fluor 647 Kit (Invitrogen).

**Sex identification and genotyping**

Embryos were staged according to Campos-Ortega and Hartenstein (1985). Male embryos and larvae were identifiable due to the larger size of the gonad. For other cases, sex was determined by immunostaining male-specific SGPs. Fluorescent balancer chromosomes [P[w+ TM6 Hu ubi-GFP] (FBst0004533) or P[w- CyO act-GFP] (FBst0004887)] distinguished heterozygous from homozygous mutant larvae. Notch mutants were identified by their neurogenic phenotype.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

S.D. and L.W. conceived the project and designed experiments. L.W. performed experiments and analyzed results. L.W. wrote and S.D. edited the manuscript.

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**Supplemental material**

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