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Sex-Specific Deployment of FGF Signaling in *Drosophila* Recruits Mesodermal Cells into the Male Genital Imaginal Disc

Shaad M. Ahmad[1] and Bruce S. Baker[2,3]
[1]Department of Developmental Biology
Stanford University School of Medicine
Stanford, California 94305
[2]Department of Biological Sciences
Stanford University
Stanford, California 94305

Summary

A central issue in developmental biology is how the deployment of generic signaling proteins produces diverse specific outcomes. We show that *Drosophila* FGF is used, only in males, to recruit mesodermal cells expressing its receptor to become part of the genital imaginal disc. Male-specific deployment of FGF signaling is controlled by the sex determination regulatory gene *doublesex*. The recruited mesodermal cells become epithelial and differentiate into parts of the internal genitalia. Our results provide exceptions to two basic tenets of imaginal disc biology—that imaginal disc cells are derived from the embryonic ectoderm and belong to either an anterior or posterior compartment. The recruited mesodermal cells migrate into the disc late in development and are neither anterior nor posterior.

Introduction

Studies in model organisms have revealed two major themes common to the control of a diverse array of developmental processes. First, specific dedicated genetic regulatory hierarchies govern nearly every aspect of development and differentiation. Second, these regulatory hierarchies often function through regulatory molecules that individually are used to control diverse arrays of developmental processes. Examples include the Ras/mitogen-activated protein kinase pathway (Tan and Kim, 1999), cell-cell signaling molecules such as Notch and its ligands (Artavanis-Tsakonas et al., 1999), and morphogens such as Wingless/WNT (Cadigan and Nusse, 1997) and Dpp/TGFβ (Massague and Wotton, 2000), all generic regulatory molecules that are deployed to bring about the highly context-specific differentiation of multiple cell and tissue types. This presents one of the more intriguing questions in developmental biology: how are the dedicated regulatory hierarchies controlling individual developmental processes able to use generic regulatory molecules to achieve highly specific goals?

One set of signaling molecules used in developmental processes as varied as cell survival, apoptosis, proliferation, differentiation, matrix composition, chemotaxis, cell adhesion, migration, and growth of cell processes and found in species as diverse as vertebrates, *D. melanogaster*, and *C. elegans* are the fibroblast growth factors (FGFs) and their receptors (FGFRs; Szebenyi and Fallon, 1999). In *Drosophila*, one FGF, encoded by the *branchless* (*bnl*) gene, and two FGFRs, encoded by the *breathless* (*btl*) and *heartless* (*htl*) genes, are known. Bnl is necessary for the proper migration and branching of the tracheal cells (Sutherland et al., 1996). The Btl receptor is required initially for the proper development of the *Drosophila* tracheal system and later plays a role in the migration of specific CNS glial cells (Glazer and Shilo, 1991; Klämbt et al., 1992). The other FGFR, Htl, is essential for the early migration and patterning of the embryonic mesoderm (Beiman et al., 1996; Gisselbrecht et al., 1996). Here, we show that *bnl* and *btl*, under the control of the *Drosophila* sex determination hierarchy, function to recruit larval mesodermal cells to become part of the male genital imaginal disc.

Sexual differentiation in *D. melanogaster* involves integrating a binary fate choice (male or female) with the developmental decisions that specify the diverse tissues and organs of the fly. The genetic regulatory hierarchy governing sex specifies not only the fates of individual cells, but also controls the growth and morphogenesis of groups of cells, up to entire imaginal discs, and so provides an attractive system for examining how a dedicated regulatory hierarchy can deploy signaling molecules to coordinate the developmental fates of groups of cells. The sex determination hierarchy in *Drosophila* functions to deploy the sex-specific transcription factors encoded by the *doublesex* (*dsx*) and *fruitless* genes (Cline and Meyer, 1996; Baker et al., 2001). Here, we are concerned with the *dsx* branch of the hierarchy (summarized in Figure 1A), which is responsible for somatic sexual differentiation outside of the CNS. While relatively little is known about the direct target genes of the hierarchy (Burtis and Wolfner, 1992), recent reports have begun to illuminate how information about sex is integrated with that from other developmental hierarchies and how sex controls growth in imaginal discs. *dsx*, together with the homeotic genes *Abdominal-B* and *abdominal-A*, specifies the sexually dimorphic pigmentation patterns of the abdomen through their regulation of *bric-a-brac* (Kopp et al., 2000). The sex-specific patterns of growth of the genital imaginal disc are achieved through the action of *dsx* in the A/P organizers (Keisman et al., 2001), regions of the genital disc where it is thought that *dsx* in conjunction with homeotic genes modulates signaling by *wingless* (*wg*) and *decapentaplegic* (*dpp*; Keisman et al., 2001; Sánchez and Guerrero, 2001).

In *Drosophila*, imaginal discs give rise to the adult cuticular structures (Cohen, 1993). The genital imaginal disc is noteworthy because its derivatives, the genitalia and analia, show the greatest degree of sexual dimorphism in the adult fly (Figure 1B). Unlike the single segment-derived and paired thoracic discs, such as the wing and leg imaginal discs, there is a single genital disc comprised of three separate primordia, derived from embryonic segments A8, A9, and A10 (Nöthiger et al., 1977; Sánchez and Guerrero, 2001). Each of these primordia responds in a unique manner to the sex determination hierarchy (Figure 1B). In females, the A8-derived primordium grows to dominate the disc and gives rise to

[3]Correspondence: bbaker@cmgm.stanford.edu

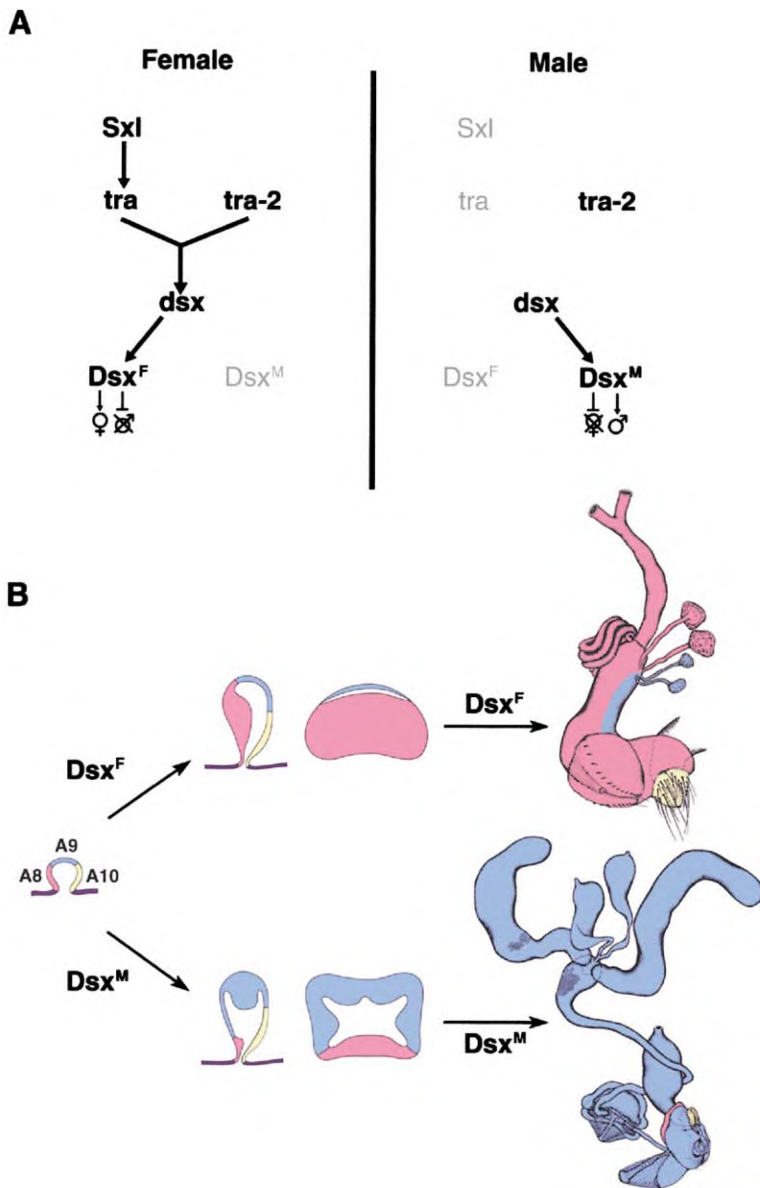

Figure 1. The Sex Determination Hierarchy and Its Effects on the Genital Disc

(A) Abbreviated version of the sex determination hierarchy in *D. melanogaster*. The female-specific *Sex-lethal* (*Sxl*) gene directs the female-specific splicing of the *transformer* (*tra*) pre-mRNA; only the female-specific *tra* mRNA encodes Tra protein. Tra and the *transformer-2* (*tra-2*) gene product direct the female-specific splicing of the *doublesex* (*dsx*) pre-mRNA to produce an mRNA encoding Dsx^F. The Dsx^F protein represses male differentiation genes and activates female differentiation genes. Males lack Sxl protein; consequently, Tra protein is not produced, and in its absence the *dsx* pre-mRNA is spliced by default into a male-specific mRNA encoding Dsx^M, a protein that represses female differentiation genes and activates male differentiation genes.

(B) Growth and differentiation of the genital disc in response to the sex determination hierarchy. Note that the function of the appropriate Dsx protein is needed both to bring about the growth of the relevant primordium and to bring about the subsequent differentiation of the developed primordia.

most of the female genitalia; the A9-derived primordium develops into the parovaria, part of the internal female genitalia; and the A10-derived primordium develops into the female analia (Nöthiger et al., 1977; Keisman et al., 2001). In males, the converse happens: the A9-derived primordium grows to dominate the disc and gives rise to all of the male genitalia; the A8-derived primordium gives rise to a miniature male eighth tergite; and the A10-derived primordium develops into the male analia (Nöthiger et al., 1977; Keisman et al., 2001).

We found that the genes *bnl*, which encodes a fibroblast growth factor (FGF), and *btl*, which encodes its receptor (FGFR; Sutherland et al., 1996; Glazer and Shilo, 1991; Klämbt et al., 1992), are both expressed in the mature male genital disc but not in the mature female genital disc. The *btl*-expressing cells are not originally a part of the male disc; rather, they are actively recruited into it during late larval development. Unlike the rest of the cells in the disc, which are derived from the embryonic ectoderm, these recruited *btl*-expressing cells originate from the mesoderm and initially express the mesodermal marker *twist*. After being recruited into the disc, they define a novel third compartment that is clonally distinct from the anterior and posterior compartments. Subsequently, the *btl*-expressing cells become epithelial (losing *twist* expression and gaining expression of the marker Coracle, a component of septate junctions in epithelia), and give rise to the paragonia and vas deferens, components of the internal male genitalia. The presence of the FGF encoded by *bnl* in the ectoderm-derived cells of the male genital discs induces the FGFR-expressing mesodermal cells to migrate into the male discs. The male-specific deployment of FGF signaling is a consequence of *bnl* expression being repressed in female discs by Dsx^F, the female-specific Doublesex protein. Our results thus constitute a demonstration of how the sex determination hierarchy acts through other genes to direct the construction of sex-specific tissues.

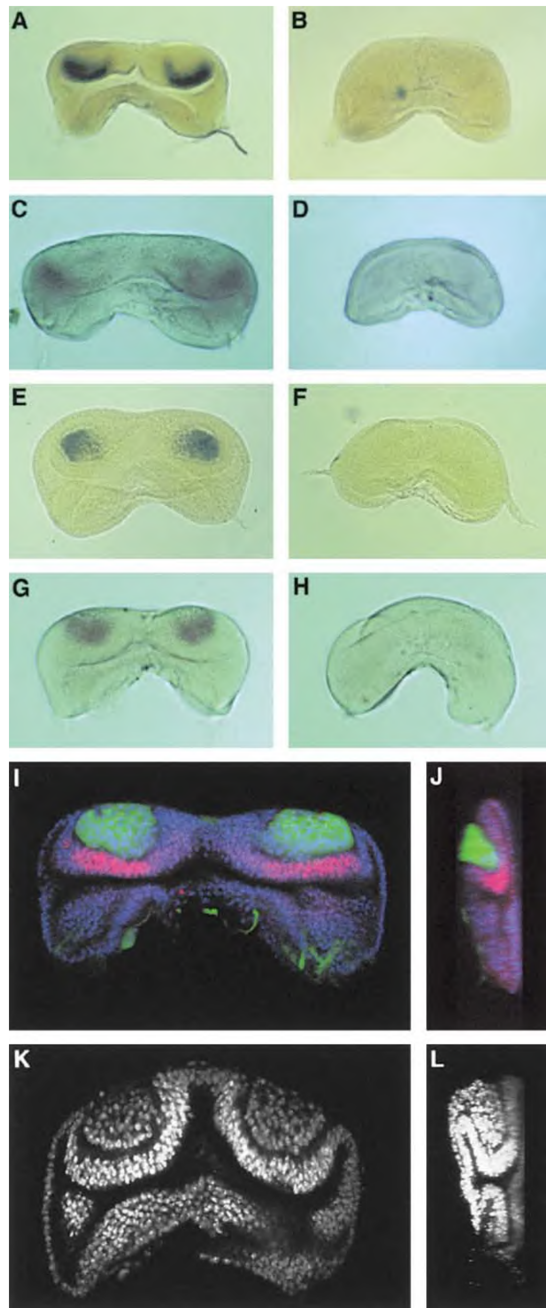

Figure 2. Male-Specific Expression of *bnl* and *btl*

(A–H) Expression patterns of the *bnl* and *btl* enhancer traps and the expression patterns of the endogenous *bnl* and *btl* transcripts. (A), (C), (E), and (G) are male genital discs, while (B), (D), (F), and (H) are female discs. Expression patterns for the enhancer trap for *bnl* (A and B), for the *bnl* transcript (C and D), for the enhancer trap for *btl* (E and F), and for the *btl* transcript (G and H). Enhancer traps were detected by X-gal staining and the endogenous transcripts by in situ hybridization.

(I) Confocal section through a male genital disc (nuclei in blue), showing *bnl* (magenta) and *btl* (green) expression in adjacent domains. The disc was from a larva of the genotype *UAS-GFP/+; bnl-lacZ/btl-GAL4*. Antibodies against β-galactosidase were used to visualize the *bnl* expression domains, while *GFP*-expressing cells marked the *btl* expression domains.

(J) Digital reconstruction of a parasagittal section through the same disc, based on a confocal Z series, with the anterior at the top and the ventral surface to the left.

(K–L) A confocal section through another male genital disc (nuclei in white) (K) and a reconstruction of a parasagittal section through that disc (L) for comparison.

In addition, our findings demonstrate some striking parallels between the roles of FGF signaling in sexual differentiation in *Drosophila* and mice.

These findings also significantly modify several commonly held tenets with respect to *Drosophila* imaginal disc development. It is generally accepted that *Drosophila* imaginal discs are derived solely from precursor cells allocated from the embryonic ectoderm and that discs subsequently grow by cell division rather than by cell recruitment (Cohen, 1993). In addition, it is generally accepted that imaginal discs are subdivided into anterior and posterior compartments, with all cells in a disc having either an anterior or posterior identity (García-Bellido et al., 1973; Eaton and Kornberg, 1990; Kornberg et al., 1985). Our results show that in the *Drosophila* male genital disc, cells derived from the mesoderm are recruited into the disc during late larval development and that these recruited mesodermal cells belong to neither the anterior nor the posterior compartment.

Results

Male-Specific Expression of *bnl* and *btl*

The extensive sexual dimorphisms of the genitalia and analia (Figure 1B) suggested that the genital disc would be relatively enriched in genes expressed downstream of *dsx*. To identify such genes, a random collection of enhancer traps was screened for sex-specific expression patterns in late third instar genital discs. Enhancer trap insertions in the *bnl* and *btl* genes were both isolated as enhancer traps expressed in male but not female genital discs (Figures 2A, 2B, 2E, and 2F). The sex specificity and the spatial patterns of expression of these enhancer traps accurately reflect the expression of the *bnl* and *btl* genes in the genital disc (Figures 2C, 2D, 2G, and 2H). Of the three primordia that comprise the genital disc, *bnl* and *btl* are both expressed in only one: the A9-derived developing “male” primordium. *bnl* and *btl* are also expressed in adjacent domains: *bnl* is expressed at the base of two bilateral bowl-like infoldings of the disc epithelium, while *btl* is expressed in a group of loosely packed cells that fills these bowls and extends over the anterior and ventral surfaces of the disc (Figures 2I–2L).

Recruitment of *btl*-Expressing Cells

The juxtaposition of *btl*- and *bnl*-expressing cells suggested that their proximity to one another might be the result of FGF-mediated cell-cell signaling. We therefore determined the locations of *btl*-expressing cells in male genital discs at different stages of larval development (Figure 3). At early third instar (70–75 hr after egg laying), while a few *btl*-expressing cells were associated with the external surface of the disc, none were detected inside the disc. In mid-third instar (89–99 hr AEL), the *btl*-expressing cells were lying on the external surface

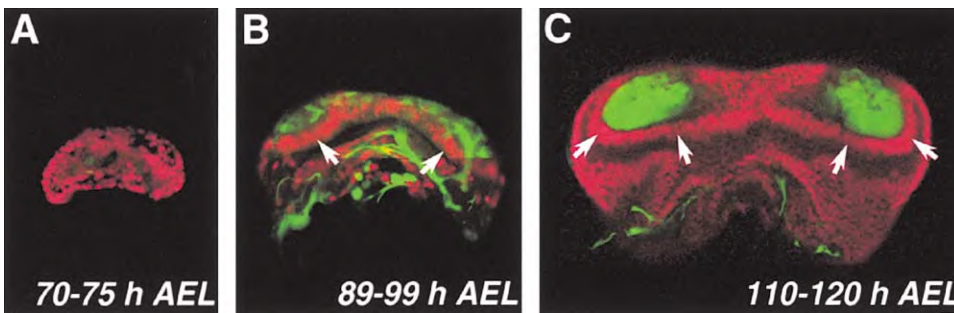

Figure 3. The *btl*-Expressing Cells Are Recruited into the Male Genital Disc

btl-expressing cells (green) and the ectoderm-derived *esg*-expressing disc cells (red) in confocal sections through an early third instar male disc (A), a mid-third instar male disc (B), and a late third instar male disc (C). Note the gradual migration of *btl*-expressing cells into the disc, from no *btl*-expressing cells inside the disc in (A) to completely filling deep invaginations in the disc epithelium in (C). Note also that the *btl*-expressing cells and the *esg*-expressing cells do not overlap. The discs were from larvae of the genotype *esg-lacZ/UAS-GFP; btl-GAL4/+*. Antibodies against β-galactosidase were used to visualize the *esg*-expressing cells, while *GFP* expression marked the *btl*-expressing cells.

of the disc, as well as adjacent to, and filling shallow invaginations in the disc epithelium. And by late third instar (110–120 hr AEL), these invaginations had become much deeper and were completely filled by *btl*-expressing cells. Thus, these *btl*-expressing cells are not originally a part of the disc epithelium but are recruited into invaginations in the epithelium during the third instar. Note also that unlike the disc epithelium, the *btl*-expressing cells in the third instar disc do not express *escargot* (*esg*; Figure 3), a classical marker for ectoderm-derived imaginal cells (Whiteley et al., 1992; Hayashi et al., 1993), indicating that the *btl*-expressing cells have a different origin than do the other cells of the disc.

Mesodermal Origin of the *btl*-Expressing Cells

A possible origin of these *btl*-expressing cells is suggested by the fact that the basal membranes of the wing and leg imaginal discs are associated with embryonic mesoderm-derived adepithelial cells, which express the mesodermal marker *twist (twi)* and are the precursors of the adult muscles attached to the disc derivatives (Bate, 1993). The *btl*-expressing cells in the male genital disc start out as *twi*-expressing cells but turn off *twi* expression as they migrate deeper into the disc (Figure 4). By using appropriate markers (e.g., antibodies against Coracle, a protein localized to septate junctions lying just below the apical membrane) to determine the polarity of the genital disc epithelia, we found that the *btl*-expressing cells were associated only with the basal membrane of the disc epithelia (data not shown). This led us to conclude that the *btl*-expressing cells are mesodermal in origin and derived from the adepithelial cells associated with the genital disc. Note that these conclusions run contrary to the generally accepted view that the disc is derived solely from ectoderm-derived founding cells set aside during embryonic development.

Bnl Recruits the *btl*-Expressing Cells into the Disc

How is the migration of these mesoderm-derived *btl*-expressing cells into the male genital disc brought about? That *bnl* is expressed in the male genital disc epithelium and *btl* in the cells that migrate into the disc and come to lie adjacent to the FGF-expressing cells, suggested that the FGF could be acting as an instructive cue to recruit the *btl*-expressing cells into the disc, similar to the role it plays in tracheal development (Sutherland et al., 1996). To test this hypothesis, we misexpressed *bnl* in other regions of the disc and examined the effect of such ectopic *bnl* expression on the migration of *btl*-expressing cells. We observed that ectopic expression of *bnl* was sufficient to recruit *btl*-expressing cells into locations where they are not observed in wild-type (Figure 5), indicating that the FGF was acting as an instructive cue to direct migrations of cells expressing Btl, its receptor.

The *btl*-Expressing Cells Define a Novel Compartment

All imaginal discs are thought to be divided into an anterior compartment, defined by the expression of genes like *cubitus interruptus (ci)*, and a posterior compartment, defined by the expression of genes such as *engrailed* and *invected* (García-Bellido et al., 1973; Eaton and Kornberg, 1990; Kornberg et al., 1985). However, by using markers for the anterior and posterior compartments, we found that the *btl*-expressing cells defined a third domain that was adjacent to, but outside of, both the anterior and posterior compartments (Figure 6A).

To address whether these *btl*-expressing cells defined a novel compartment, we asked whether random *GFP*-expressing clones induced at the first instar larval stage could cross between the *btl*-expressing domain and the rest of the disc. In 217 third instar male genital discs with *GFP*-expressing clones, no clone in the *btl*-expressing domain extended outside this domain (Figure 6B). Conversely, no clone in the rest of the genital disc ever included any *btl*-expressing cells, although a number of such clones lay adjacent to and often almost completely surrounded the *btl*-expressing domain (Figure 6C). Thus, the *btl*-expressing cells define a third compartment of the genital disc.

Epithelial Fate of the *btl*-Expressing Cells

While the *btl*-expressing cells appear loosely packed and distinct from the epithelium in which *bnl* is expressed in a late third instar male disc, by 8 hr after puparium formation (APF), the *btl*-expressing cells take on a single-layered, tightly packed morphology reminis-

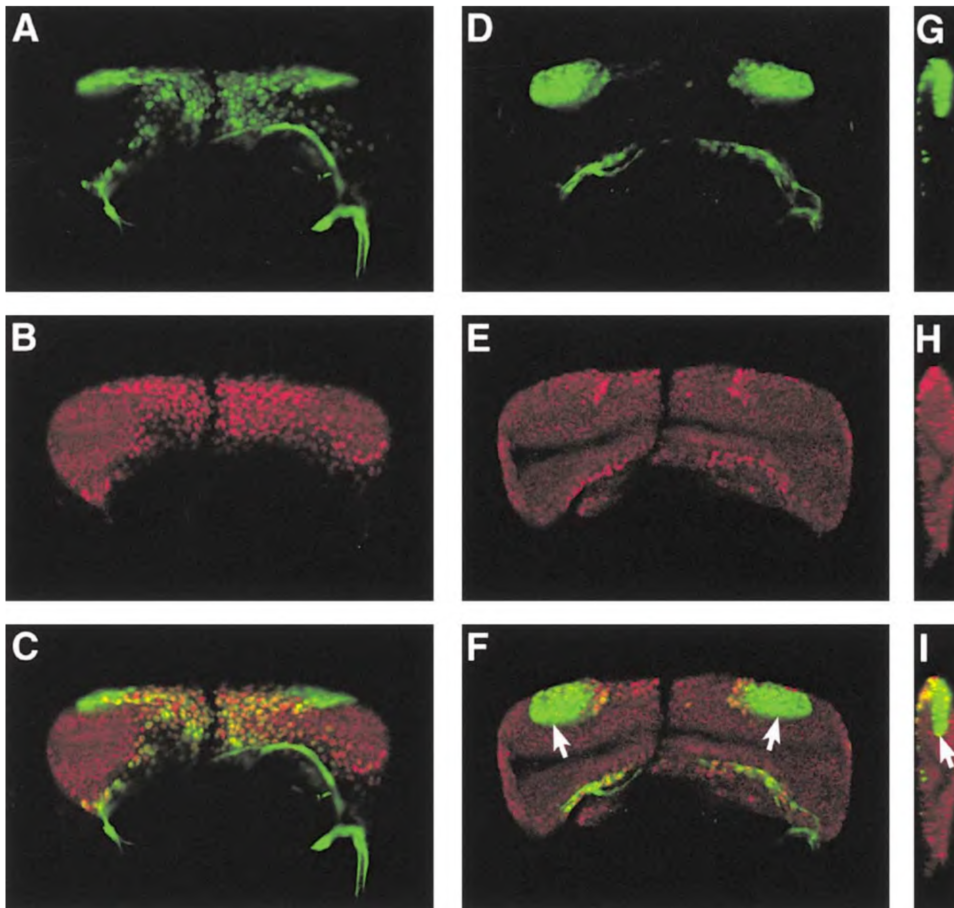

Figure 4. The *btl*-Expressing Cells Have a Mesodermal Origin

(A) Confocal section along the outer ventral surface of a male genital disc, showing the *btl*-expressing cells (green).

(B) The same section, now showing the cells expressing the mesodermal marker *twist* (red).

(C) A merge of (A) and (B), showing that *btl*-expressing cells on the outside of the ventral surface of the disc express *twist*.

(D–F) Another confocal section through the same disc, this time halfway between the dorsal and ventral surfaces, showing that as the *btl*-expressing cells migrate deeper into the disc, they lose *twist* expression.

(G–I) Reconstruction of a parasagittal section through the disc in (A–F). The disc was from a larva of the genotype *UAS-GFP.nls/+; btl-GAL4/+*. *twist* expression was detected with antibodies against the protein, while *GFP* expression marked the *btl*-expressing cells.

cent of epithelia and develop lumens that are not continuous with the original lumen in the genital disc (Figures 7A and 7B). Epithelia in invertebrates are characterized by the presence of septate junctions (the counterparts of the tight junctions in vertebrates) that lie just below the apical membranes (Bryant, 1997). By using antibodies against Coracle, a constituent protein of septate junctions (Fehon et al., 1994), we found that while the original ectoderm-derived cells of the genital disc all possessed septate junctions, *btl*-expressing cells all the way up to 6 hr APF lacked septate junctions, even though some of these *btl*-expressing cells appear epithelial morphologically (Figure 7G). By 20 hr APF, however, tight punctate localizations of Coracle were easily detectable in *btl*-expressing cells; this staining was indistinguishable from that at septate junctions in other parts of the genital disc (Figures 7H and 7I). Thus, the mesoderm-derived *btl*-expressing cells were converted into epithelia during pupal development. These mesoderm-derived epithelia and their enclosed lumens eventually develop into the vas deferens and paragonia, major components of internal male genitalia (Figures 7A–7F).

Sex-Specific Regulation of *bnl* and *btl*

A priori, there are two possible explanations for the male-specific expression of FGF. One possibility is that *bnl* is an A9-specific gene, being expressed only in males where the A9-derived primordium grows significantly. The other possibility is that *bnl* is a target of the sex determination hierarchy, being either repressed by the female-specific Dsx protein (Dsx^F) in females and/or activated by the male-specific Dsx protein (Dsx^M) in males. To distinguish between these possibilities, we generated feminized (Tra protein-expressing) clones of cells in the A9-derived primordium of wild-type male genital discs and examined the effects of these clones on *bnl* expression. Whenever feminized clones overlapped domains of *bnl* expression, the expression of *bnl* was repressed, indicating that it was cell-autonomous regulation by the sex determination hierarchy that was

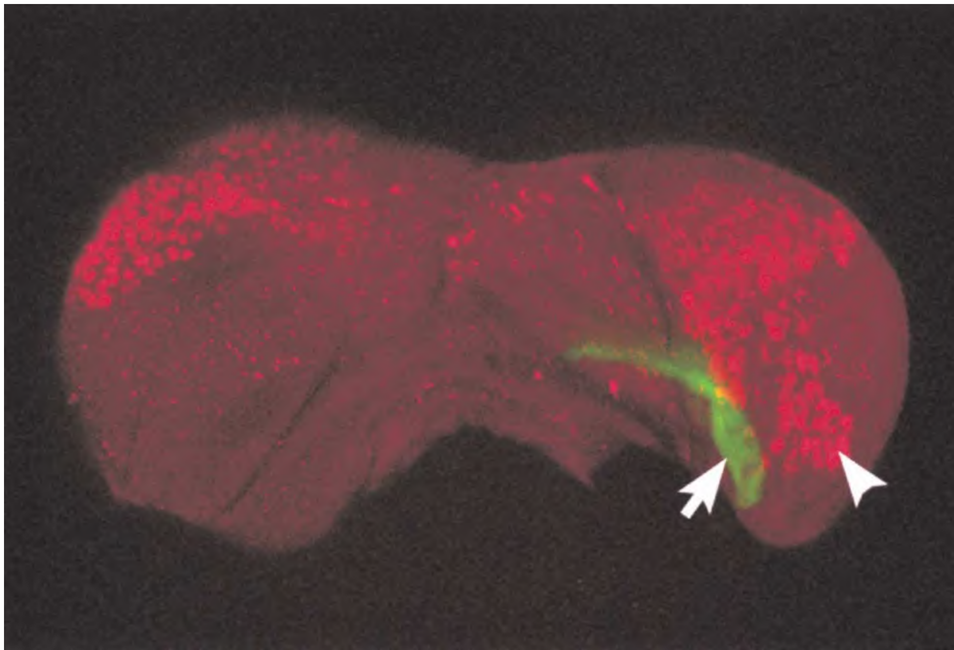

Figure 5. Bnl Recruits the *btl*-Expressing Cells into the Disc

Ectopic expression of *bnl* (green, arrow) in this male genital disc resulted in the migration of the *btl*-expressing cells (red) into a nearby ectopic domain (arrowhead) where they are not observed in wild-type (compare with the left side of this disc). The disc was from a heat-shocked larva of the genotype *act5C>CD2>GAL4/Y; UAS-bnl/UAS-GFP; btl-lacZ/hs-FLP*. *GFP* expression marked the clone of cells ectopically expressing *bnl*; antibodies against β-galactosidase were used to visualize the *btl*-expressing cells.

responsible for the male-specific expression of *bnl* in the genital disc (Figures 8A–8F).

When a feminized clone completely eliminated *bnl* expression from one side of a male disc, the lobe lacking *bnl* expression looked flattened (Figures 8D–8F). This was a consequence of *btl*-expressing cells not migrating into this lobe in the absence of Bnl protein, showing that *bnl* expression is not simply sufficient, but also necessary for the recruitment of *btl*-expressing cells. This observation suggests that *btl*, unlike *bnl*, is not a target of the sex determination hierarchy, and that the male-specific expression of *btl* in the genital disc is solely a consequence of Bnl recruiting the *btl*-expressing cells. To test this model, we generated feminized (Tra protein-expressing) clones among the *btl*-expressing cells and observed their effects on migration into the male genital disc. Feminized *btl*-expressing cells were recruited efficiently into the male discs, confirming that the sex hierarchy acts through *bnl* to induce the migration of *btl*-expressing cells (Figures 8G–8I). This model also implies that *btl*-expressing adepithelial cells should be associated with the ventral surface of the female genital disc, which they are (data not shown).

To examine how *dsx* regulated *bnl* expression, we compared *bnl* expression in wild-type genital discs and discs lacking *dsx* function. *bnl* is expressed in the A9-derived primordium of a wild-type male disc, where Dsx^M is present (Figure 8J), but is not expressed in the A8-derived primordium of a wild-type female disc, where Dsx^F is expressed (Figure 8K). However, in a disc in

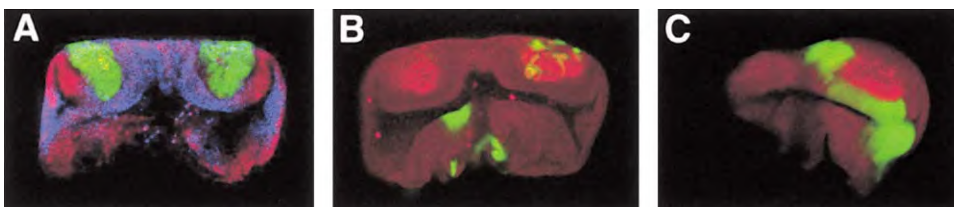

Figure 6. The *btl*-Expressing Cells Define a Novel Third Compartment that Is Neither Anterior Nor Posterior

(A) *btl*-expressing cells (green) lie adjacent to but outside both the *engrailed*-expressing posterior compartment (red) and the *ci*-expressing anterior compartment (blue). The disc was from a larva of the genotype *UAS-GFP/+; btl-GAL4/ci-lacZ*. *GFP* expression marked the *btl*-expressing cells; antibodies against β-galactosidase were used to visualize the anterior compartment; and antibodies against Engrailed and Invected were used to visualize the posterior compartment.

(B and C) Randomly generated *GFP*-expressing clones of cells (green) in the *btl*-expressing domain (red) do not cross over to the rest of the disc (B), and *GFP*-expressing clones (green) in the rest of the disc do not cross over into the *btl*-expressing domain (red) (C), indicating that the *btl*-expressing domain is a clonally distinct compartment. The discs used in (B) and (C) were from heat-shocked larvae of the genotype *act5C>CD2>GAL4/Y; UAS-GFP/+; btl-lacZ/hs-FLP*. Antibodies against β-galactosidase were used to visualize the *btl*-expressing cells.

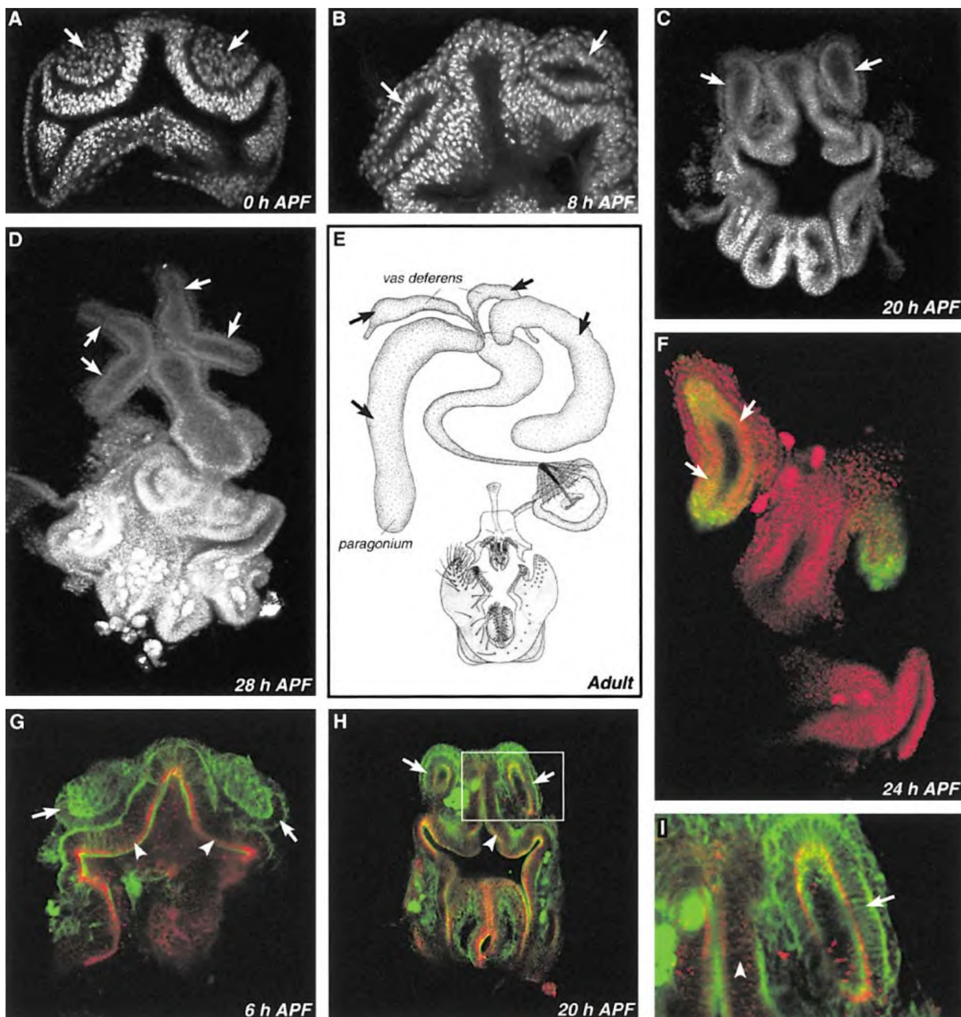

Figure 7. Fate of the *btl*-Expressing Cells in the Male Genital Disc

(A–E) The *btl*-expressing cells (arrows) acquire an epithelial morphology, develop distinct lumens of their own, and eventually become the paragonia and vas deferens. (A)–(D) are confocal images at different periods after puparium formation (APF) showing nuclei, and (E) is an illustration based on Epper (1980).

(F) Confocal image of a 24 hr APF male genital disc (nuclei in red) confirming that it is indeed the *btl*-expressing cells (green, arrows) that are becoming epithelial and developing a lumen. The developing genital disc was from a fly of the genotype *UAS-GFP/+; btl-GAL4/+*; thus, *GFP* expression marked the *btl*-expressing cells.

(G) Confocal image of a 6 hr APF male genital disc (cytoskeleton in green) showing that while the original ectoderm-derived disc epithelia (arrowheads) contain septate junctions marked by Coracle localization (red), the *btl*-expressing cells at this point do not, even though some of these cells (arrows) have acquired a distinct epithelial morphology.

(H) Confocal image of 20 hr APF male genital disc (cytoskeleton in green), showing that both the original ectoderm-derived disc epithelia (arrowhead) and the epithelia derived from the *btl*-expressing cells (arrows) now contain septate junctions marked by the presence of Coracle (red).

(I) Magnification of box in (H), showing the epithelia derived from the *btl*-expressing cells (arrow) in greater detail.

which neither Dsx protein is expressed, both the A8 and A9 primordia proliferate and *bnl* expression is seen in both primordia (Figure 8L). That the A8 primordium grows in both wild-type and *dsx* mutant females but *bnl* is expressed in the A8 primordium only when the Dsx^F protein is absent, implies that *bnl* expression is repressed in the female genital disc by the presence of Dsx^F protein.

Answer to a *dsx* Puzzle

The ectopic expression of *bnl* in the A8-derived "female" primordia of discs lacking *dsx* function offers an explanation for a puzzling observation first made by Hildreth (1965): while wild-type males have only two paragonia, *dsx* mutant flies often have as many as four paragonia-like structures. Our finding that the ectopic expression of *bnl* in flies mutant for *dsx* results in *btl*-expressing cells from the ventral surface of the disc being recruited into two ectopic invaginating pockets in the A8-derived female primordium of the disc, in addition to the original bowls in the A9-derived primordium (Figures 8M–8O), taken together with the previous observation that the extra paragonia in *dsx* mutants arise from the female primordium (Epper, 1981), suggests that these ectopic

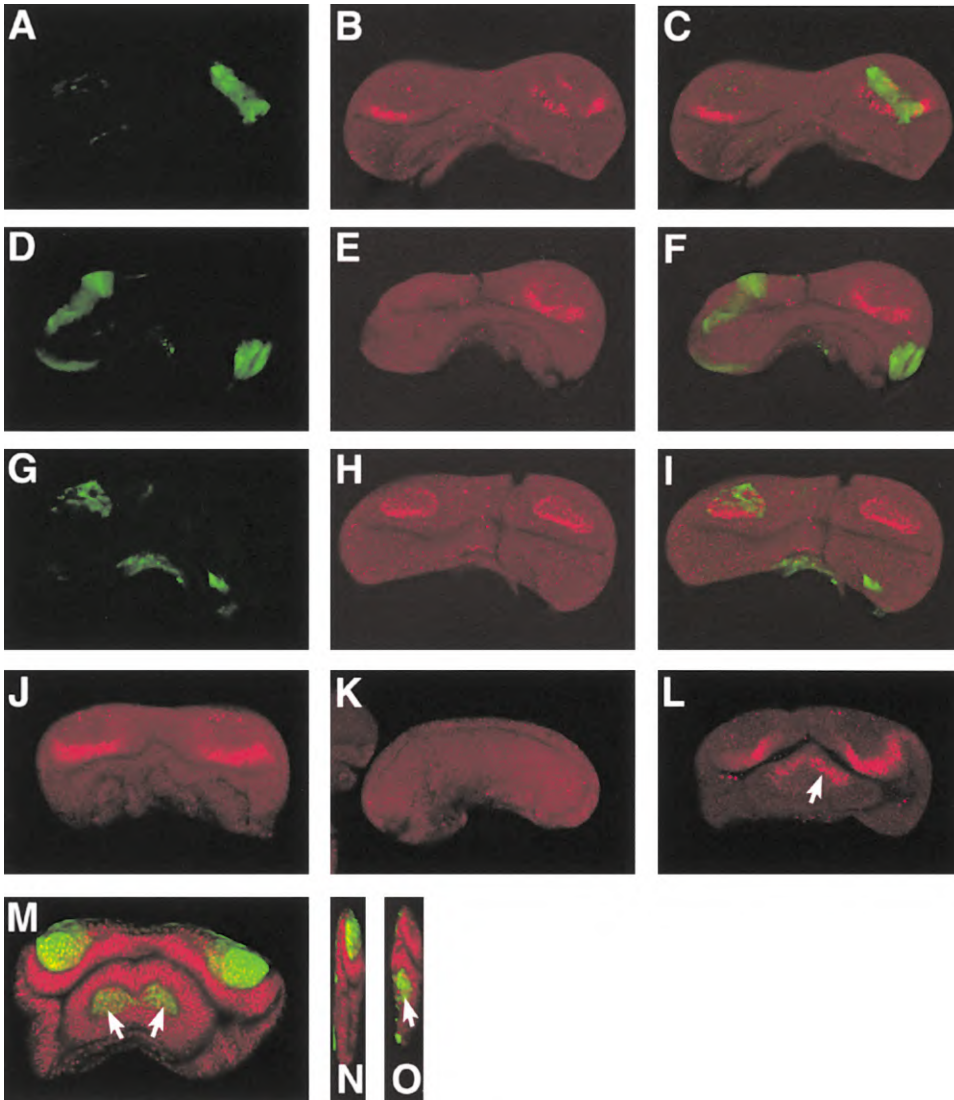

Figure 8. Sex-Specific Regulation of *bnl* and *btl*

(A–F) *bnl* is a target of the sex determination hierarchy, downstream of *tra*.

(A) Tra protein-expressing, and thus feminized, clones (green) in the A9-derived male primordium in a confocal section through a male genital disc.

(B) *bnl* expression (red) in the same confocal section.

(C) Merge of (A) and (B), showing that expression of *bnl* is repressed where it is overlapped by the feminized clone.

(D–F) Confocal section through another male genital disc where a Tra protein-expressing (feminized) clone (green) completely eliminates *bnl* expression (red) from the left side of the disc, and that side looks flattened due to a failure to recruit *btl*-expressing cells into the disc. The discs used in (A)–(F) were from heat-shocked larvae of the genotype *act5C*>*CD2*>*GAL4/Y; UAS-tra/UAS-GFP; bnl-lacZ/hs-FLP*. *GFP* expression marked the clone of cells ectopically expressing Tra protein; antibodies against β-galactosidase were used to visualize *bnl* expression.

(G–I) *btl* is not a direct target of the sex determination hierarchy.

(G) Confocal section through a male disc, showing Tra protein-expressing clones (green).

(H) The same confocal section, showing the *btl*-expressing cells (red).

(I) Merge of (G) and (H), demonstrating that *btl*-expressing cells that also express Tra protein are recruited efficiently into the disc. The disc used in (G)–(I) was from a heat-shocked larva of the genotype *act5C*>*CD2*>*GAL4/Y; UAS-tra/UAS-GFP; btl-lacZ/hs-FLP*. *GFP* expression marked the clone of cells ectopically expressing Tra protein; antibodies against β-galactosidase were used to visualize the *btl*-expressing cells.

(J–L) *bnl* is downstream of *dsx*, and its expression (red) is repressed by Dsx^F.

(J) Confocal section through a wild-type male (XY) genital disc; only Dsx^M is present and *bnl* is expressed (red) in the A9-derived primordium.

(K) Confocal section through a wild-type female (XX) disc; only Dsx^F is present and *bnl* is not expressed in the A8-derived primordium.

(L) Disc from a XX; dsx^1/dsx^{M+R15} larva (lacking both Dsx^M and Dsx^F proteins); *bnl* is expressed (red) both in the A9-derived primordium, and also, ectopically, in the A8-derived primordium (arrow). All three discs in (J)–(L) were from larvae that also carried the *bnl-lacZ* enhancer trap; thus, antibodies against β-galactosidase were used to visualize *bnl* expression.

(M–O) Ectopic expression of *bnl* in the A8-derived female primordium of a disc (nuclei in red) lacking *dsx* function results in ectopic invaginating pockets (arrows) of *btl*-expressing cells (green) being recruited into this primordium.

(M) Confocal section through the disc.

(N and O) Reconstructions of parasagittal sections showing *btl*-expressing cells (green) being recruited into the A9-derived male primordium and, ectopically, into the A8-derived female primordium, respectively. The disc used in (M)–(O) was from a larva of the genotype XX; *UAS-GFP/+; btl-GAL4* dsx^1/dsx^{M+R15}; thus, *GFP* expression was used to visualize the *btl*-expressing cells.

pockets of *btl*-expressing cells give rise to the supernumerary paragonia.

Discussion

We have shown that, contrary to accepted views of imaginal disc development, cells derived from the mesoderm are actively recruited into the male genital disc during larval development, where they comprise a novel third compartment. These mesoderm-derived cells undergo a mesodermal-to-epithelial transition and give rise to a major portion of the internal adult male genitalia. That similar FGF signaling also occurs in other imaginal discs is indicated by the finding that migration of *btl*-expressing cells in response to the localized expression of *bnl* also occurs in wing discs (M. Sato and T.B. Kornberg, personal communication).

Our findings, taken together with previous results, indicate that *dsx* modulates both cell-cell signaling and the response of cells to such signals in the genital disc. The sex of the A/P organizer, a *wg*- and *dpp*-expressing strip of cells at the anterior/posterior compartment border, controls the sex-specific patterns of growth throughout the genital disc (Keisman et al., 2001; Sánchez and Guerrero, 2001). In addition, *dsx* functions cell autonomously to sex specifically modulate the response of *dachshund* to *wg* and *dpp* signaling (Keisman and Baker, 2001). The results we present here show that the sex determination hierarchy also functions cell autonomously in the genital disc to direct the sex-specific deployment of another signaling system: *bnl* expression in the genital disc depends on the sex of the individual *bnl*-expressing cells. This multifaceted role of the sex determination hierarchy in the genital disc, acting through the Dsx proteins to regulate several different levels of disc development, shows a remarkable similarity to the role of the homeotic gene *Ultrabithorax* (*Ubx*), which controls the growth of the haltere disc by regulating the expression of *wg* and also acts cell autonomously to regulate the activation of *wg* and *dpp* target genes (Weatherbee et al., 1998). Both *Ubx* and *dsx* direct entire discs down specific developmental paths.

Given that *bnl* is regulated cell autonomously by Dsx^F, an obvious question is whether the Dsx^F protein directly represses *bnl*. In this regard, we note that 0.7 kb and 1.6 kb upstream of the putative *bnl* transcriptional start site (Adams et al., 2000; Rubin et al., 2000), there are clusters of 5 and 4 sites respectively with at most a 1 bp mismatch to the 13 bp consensus Dsx binding site sequence (Burtis et al., 1991; Erdman et al., 1996). This is reminiscent of the 3 Dsx binding sites in a 76 bp stretch of an enhancer for the *Yolk protein* (*Yp*) genes, the only known direct targets of *dsx* (Burtis et al., 1991; Coschigano and Wensink, 1993).

The *Drosophila* sex determination hierarchy acts at multiple levels to control sexual differentiation. Some terminal differentiation genes like the *Yp* genes are direct transcriptional targets of the Dsx proteins and are continuously subject to their regulation (Belote et al., 1985; Burtis et al., 1991). In other cases, the direct targets of *dsx* appear to be genes involved in initiating the differentiation of sex-specific tissues; genes expressed subsequently in these sex-specific tissues are governed by a tissue differentiation program, rather than being directly controlled by the sex hierarchy (Burtis and Wolfner, 1992). It seems likely that the targets through which *dsx* initiates formation of such sex-specific tissues will be the genes where information from several developmental hierarchies is integrated to direct the differentiation of tissues.

Our results suggest that *bnl* is one of the genes used by the sex determination hierarchy to direct the construction of sex-specific tissues. Bnl recruits *btl*-expressing cells into the male genital disc, and the recruited cells eventually form the paragonia and vas deferens, tissues that are present only in males. Moreover, three genes expressed in the paragonia, the male-specific transcripts *316*, *355a*, and *355b,* have been shown to be regulated in a tissue-specific rather than sex-specific manner: while transcription of these three male-specific RNAs begins in the late pupal period, their expression is governed by the sex hierarchy acting earlier, during the third larval instar—the period when the expression of *bnl* recruits the paragonia-forming *btl*-expressing cells into the male genital disc (DiBenedetto et al., 1987; Chapman and Wolfner, 1988). Thus, the sex-specific expression of the *mst*s is achieved by *dsx* acting through *bnl* to generate the sex-specific tissue, the paragonia, in which the *mst*s are subsequently expressed.

bnl also appears to be a gene where information from other regulatory hierarchies and the sex determination hierarchy are integrated in the male genital disc. The genetic hierarchies that control pattern formation and confer positional identity in the thoracic imaginal discs have previously been shown to function analogously in the genital disc (Freeland and Kuhn, 1996; Chen and Baker, 1997; Casares et al., 1997; Sánchez et al., 1997; Emerald and Roy, 1998). The fact that the *bnl* expression domain is limited to two specific subsets of the ectoderm-derived disc epithelia in males implies that *bnl* is also regulated by these pattern formation hierarchies. One area of future exploration will be examining how this coordinated regulation of *bnl* by *dsx* and the genes involved in pattern formation is brought about.

An intriguing aspect of our findings was the gradual transition of the *btl*-expressing cells, upon recruitment into the male genital disc, from *twi*-expressing mesodermal cells to epithelial cells with septate junctions. It is not clear if this transformation is also a consequence of FGF signaling, or if it is brought about by a different process. However, three separate observations suggest a role for *bnl* and *btl* in this mesoderm-epithelial transition: (1) FGF signaling mediates this process in mice—during kidney development, FGF2 and leukemia inhibiting factor (LIF) secreted from the epithelial ureteric bud induce the conversion of the undifferentiated mesoderm-derived metanephric mesenchyme to the epithelial tubular structures of the nephron (Perantoni et al., 1995; Barasch et al., 1999), (2) the converse process can also be mediated by FGF signaling—*FGFR1* regulates the morphogenetic movement and cell fate specification events during gastrulation in mice; it orchestrates the epithelial to mesenchymal transition during morphogenesis at the primitive streak and specifies the mesodermal cell fate of these mesenchymal cells (Ciruna and Rossant, 2001), and (3) *stumps*, a gene acting downstream of the FGFR-encoding *btl* (Michelson et al.,

1998), has its expression elevated in the *btl*-expressing cells undergoing the transition into epithelial cells in the genital disc (S.M.A., unpublished data).

Finally, we note that there are striking parallels between the roles of the FGF in sexual differentiation in the fly and *FGF9* in sexual differentiation in mice. *FGF9* is required for testicular embryogenesis in mice, and in its absence XY mice undergo male-to-female sex reversal (Colvin et al., 2001). *FGF9* is expressed in the early embryonic gonads of male mice, not in the gonads of female mice, and not in the mesonephros of either sex, while *bnl* is expressed in the male genital disc, not in the female genital disc, and not in the *btl*-expressing mesodermal cells that are recruited into the male disc. The mesonephric cells migrate into only the male gonads, and the *btl*-expressing cells are recruited only into the male genital disc. Exogenous FGF9 induces mesonephric cell migration into female gonads, while ectopic expression of *bnl* is sufficient to recruit the *btl*-expressing cells into the female primordium of a *dsx* disc. The *btl*-expressing cells are mesodermal in origin, eventually undergo a transition into epithelial cells, and give rise to the vascular paragonia and vas deferens. The mesonephros, too, is derived from the mesoderm, and mesonephric cell migration into the testis contributes to the vascular endothelial, myoepithelial, and peritubular myoid cell populations. Given that there is considerable variation in the earlier aspects of sex determination across species (Marín and Baker, 1998), these findings suggest a possible conserved role for FGF signaling in later aspects of sexual differentiation.

Experimental Procedures

Drosophila Stocks

The following enhancer traps, mutant alleles, and transgenes were used in this study: *bnl-lacZ*, aka *l(3)06916* (a lethal insertion into the *bnl* locus of an enhancer trap carrying a *lacZ* reporter; Sutherland et al., 1996; Spradling et al., 1999); *btl-lacZ*, aka *l(3)00208* (a lethal insertion into the *btl* locus of an enhancer trap carrying a *lacZ* reporter; Spradling et al., 1999); *btl-GAL4* (Shiga et al., 1996); *ci-lacZ*, aka *7.1-ci* (Schwartz et al., 1995); *esg-lacZ*, aka *l(2)5729* (an enhancer trap for *esg* that faithfully recapitulates *esg* mRNA expression; Hayashi et al., 1993); *Actin>CD2>GAL4* (Pignoni and Zipursky, 1997); *UAS-bnl* (Sutherland et al., 1996); *UAS-tra* (Ferveur et al., 1995); *UAS-GFP*, aka *P{UAS-GFP.S65T}T10* (described in Flybase); *UAS-GFP.nls* (also described in Flybase); dsx^1 (a null allele); and dsx^{M+R13} (another null allele).

Detection of *bnl* and *btl* Expression

In situ hybridization with antisense *bnl* and *btl* riboprobes were carried out as described (Kozopas et al., 1998) to visualize the expression patterns of the *bnl* and *btl* transcripts. Since the *bnl* and *btl* enhancer traps and reporters (*bnl-lacZ*, *btl-lacZ*, and *btl-GAL4* driving either *UAS-GFP* or *UAS-GFP.nls*) faithfully recapitulated the expression patterns of the *bnl* and *btl* transcripts (Figure 2), *bnl* and *btl* expression were subsequently followed by examining the expression patterns of these enhancer traps and reporters. X-Gal staining was carried out as described (Sullivan et al., 2000).

Antibody Staining

Tissues were labeled as described (Xu and Rubin, 1993) with antibodies against β-galactosidase (Promega), Engrailed and Invected (Developmental Studies Hybridoma Bank), Coracle (a gift from R.G. Fehon), and Twist (a gift from S. Roth). Appropriate Cy-2-, Cy-3-, and Cy-5-conjugated antibodies (Jackson Immunoresearch) were used as secondary antibodies. Where noted, discs and their derivatives were counterstained either with propidium iodide (PI) to visualize nuclei, or with Alexa Fluor 660 phalloidin (Molecular Probes) to visualize the actin cytoskeleton.

Ectopic Expression Using the Flip-Out GAL4 System

Clones that ectopically expressed the genes indicated below were created using the flip-out GAL4 system (Ito et al., 1997; Pignoni and Zipursky, 1997). *GFP*-expressing clones which showed that the *btl*-expressing cells defined a novel compartment were generated by heat shocking larvae of genotype *act5C>CD2>GAL4/Y; UAS-GFP/+; btl-lacZ/hs-FLP*, *GFP*-expressing clones that ectopically expressed the Bnl protein were generated by heat shocking larvae of genotype *act5C>CD2>GAL4/Y; UAS-bnl/UAS-GFP; btl-lacZ/hs-FLP*, and *GFP*-expressing clones that expressed Tra in male discs were generated by heat shocking larvae of genotype *act5C>CD2>GAL4/Y; UAS-tra/UAS-GFP; btl-lacZ/hs-FLP* or *act5C>CD2>GAL4/Y; UAS-tra/UAS-GFP; bnl-lacZ/hs-FLP* with a single 38°C 30 min heat shock pulse at first instar.

Microscopy and Imaging

Confocal images were collected using a Bio-Rad MRC1024 confocal microscope. Combined confocal images and reconstructions were made using NIH Image and Lasersharp software (Bio-Rad).

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