Expression and function of \textit{clift} in the development of somatic gonadal precursors within the \textit{Drosophila} mesoderm

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SUMMARY

The gonad forms from cells of two lineages: the germline and soma. The somatic gonadal cells generate the various cell types within the testis or ovary that support gametogenesis. These cells derive from embryonic mesoderm, but how they are specified is unknown. Here, we describe a novel regulator of \textit{Drosophila} gonadogenesis, \textit{clift}, mutations in which abolish gonad formation. \textit{clift} is expressed within somatic gonadal precursors as these cells first form, demonstrating that 9-12 cells are selected as somatic gonadal precursors within each of three posterior parasegments at early stages in gonadogenesis. Despite this early expression, somatic gonadal precursors are specified in the absence of \textit{clift} function. However, they fail to maintain their fate and, as a consequence, germ cells do not coalesce into a gonad. In addition, using \textit{clift} as a marker, we show that the anteroposterior and dorsoventral position of the somatic gonadal precursor cells within a parasegment are established by the secreted growth factor Wg, coupled with a gene regulatory hierarchy within the mesoderm. While loss of \textit{wg} abolishes gonadal precursors, ectopic expression expands the population such that most cells within lateral mesoderm adopt gonadal precursor fates. Initial dorsoventral positioning of somatic gonadal precursors relies on a regulatory cascade that establishes dorsal fates within the mesoderm and is subsequently refined through negative regulation by \textit{bagpipe}, a gene that specifies nearby visceral mesoderm. Thus, these studies identify essential regulators of gonadal precursor specification and differentiation and reveal novel aspects of the general mechanism whereby distinct fates are allocated within the mesoderm.

Key words: \textit{Drosophila}, gonadogenesis, \textit{clift}, \textit{eyes absent}, \textit{wingless}, \textit{bagpipe}, mesoderm

INTRODUCTION

The gonad forms during embryogenesis from germline and somatic cells. It is the interaction of these cell types within this organ that leads to the production of gametes in the adult. In many organisms, these two components are initially positioned apart from one another due to the early segregation of germ cells away from the rest of the developing embryo. It is not until a later stage that germ cells migrate back inside the embryo to contact somatic gonadal cells, with which they will condense into a gonad. Therefore, gonadogenesis provides an opportunity to examine the mechanisms whereby cells of separate lineages are specified, recognize and adhere to one another, and assemble into a functional organ.

In \textit{Drosophila}, the association of germ cells and somatic gonadal precursor cells (SGP cells) has been described (Brookman et al., 1992; Warrior, 1994; Boyle and DiNardo, 1995). The germ cells, after an initial displacement at the posterior pole of the embryo, reenter the embryo by squeezing through gut endoderm (Sonnenblick, 1941; Jaglarz and Howard, 1994, 1995; Warrior, 1994). Shortly afterwards, germ cells contact mesoderm and adhere to SGP cells that lie within the mesoderm of several posterior abdominal segments. Gonads form, however, at the most anterior of these segments. Therefore, germ cells and SGP cells must migrate anteriorly. They do so in close association with one another, eventually settling in the fifth abdominal segment and condensing into a gonad.

Although germ cells and SGP cells are associated throughout later stages of their migration to the gonad, germ cells are dispensable to gonadogenesis. In the absence of germ cells, SGP cells cluster into a gonad normally (Aboïm, 1945; Brookman et al., 1992; Warrior, 1994; Boyle and DiNardo, 1995). In contrast, SGP cells are essential for ensuring the incorporation of germ cells into the gonad. Mutations that affect SGP cell development disrupt germ cell migration to the gonad (Cumberledge et al., 1992; Brookman et al., 1992; Warrior, 1994; Boyle and DiNardo, 1995). Given this crucial role in directing gonadogenesis, we have focused on the development of SGP cells. Uncovering the regulatory pathways resulting in their specification and differentiation is a first step toward understanding the mechanisms of cellular recognition, guided migration and organogenesis used by these cells.

Despite the identification of the SGP cells and a description of their association with germ cells into a gonad, little is known about how these cells are generated within the mesoderm. SGP cells derive from segments of the embryo whose identity is
defined by the homeotic gene abdominal A and indeed, abd A function is required for their development (Cumberledge et al., 1992; Brookman et al., 1992; Warrior, 1994; Boyle and DiNardo, 1995). However, the mechanism whereby abd A provides this function or what additional factors are required for their specification is not known. Recently, several studies have identified both intrinsic and extrinsic factors that act to determine particular cell types within the mesoderm (Azpiazu and Frasch, 1993; Staeling-Hampton et al., 1994; Frasch, 1995; Carmena et al., 1995; Wu et al., 1995; Lawrence et al., 1995; Baylies et al., 1995; Baylies and Bate, 1996). To date, however, there has been little consideration of the gonadal soma. This is due in part to the lack of markers that identify SGP cells or mutations that specifically affect their development. Although such mutations could in theory be identified in screens for female and male sterility, no mutations that specifically affect the development and survival of the gonadal mesoderm have been isolated despite extensive screening (Schupbach and Wieschaus, 1991; Castrillon et al., 1993). Therefore, genes required for SGP development are likely to have other essential functions.

Based on these observations, we screened lethal collections to identify genes required for gonad formation. Here, we describe one such mutant, clift (cli, also known as eyes absent), mutations in which disrupt gonad formation. cli was originally identified as an embryonic lethal mutation with defects in head development (Nusslein-Volharr et al., 1984). It was later found to be alelic to eyes absent, a viable cli mutation with defects in photoreceptor cell differentiation and survival, and encodes a novel nuclear protein (Bonini et al., 1995). We find that cli also has an essential role in SGP cell development. In its absence, SGP cells do not differentiate and, as a consequence, germ cell migration is affected. Consistent with this requirement, we show that cli expression identifies SGP cells as these cells first form. Using cli expression as a marker, we identify essential regulators that act to ensure the precise allocation of SGP cells within the mesoderm.

**MATERIALS AND METHODS**

**Fly stocks**

Line 68-77 (Fig. 1A), is a P element transgenic line, which expresses LacZ in all somatic cells of the embryonic gonad (Simon et al., 1990; Boyle and DiNardo, 1995).

The cliDD and cliPH alleles were obtained from Bloomington and Tubingen stock centers and are described in Nusslein-Volharr et al. (1984). The eyal allele, a presumed null allele, is described in Bonini et al. (1993). All stocks are maintained over a balancer that carries a en promoter-LacZ transgene inserted at wingless (Pw+ wg1-en-11); Kassis, 1990). Phenotypes were examined in heteroallelic combinations, or over the deficiency Df(2L)P41, generated by the Bonini lab. hsp70-Eyall line K58.1 was constructed by the Bonini lab. The 1-7'-980 dpp LacZ transgenic line was provided by the Courey lab and is described in Huang et al. (1993).

The tin54O stock was provided to us by Manfred Frasch and is described in Azpiazu and Frasch (1993). tin mutant embryos were identified by the absence of even skipped staining in heart precursors (Azpiazu and Frasch, 1993). bagpipe null embryos were generated by crossing Df[3R]eD7, Pt(tinre58)TM3 ftzlacZ flies, carrying a tin rescue transgene, to Df[3R]eF1/TM3 ftzlacZ flies, also obtained from the Frasch lab.

**RESULTS**

**clift is required for the development of somatic gonadal precursors**

The embryonic gonad is composed of 8 to 15 germ cells (Fig. 1A, arrowhead) intermingled with approximately 35 somatic mesodermal cells (Fig. 1A, arrow; Sonnenblick, 1941). The gonad forms when germ cells, visualized with the vasa antibody (brown, Fig. 1B; Hay et al., 1988; Lasko and Ashburner, 1990) and SGP cells, visualized by the expression of the 412 retrotransposon (blue, Fig. 1B; Brookman et al., 1992) cluster together within parasegment (PS) 10 at 10 hours after egg laying (AEL). In a screen to identify genes that regulate the development of SGP cells (see MATERIALS AND METHODS), we identified clift (cli), mutations in which lead to the scattering of germ cells throughout posterior regions of the embryo at late stages of embryogenesis and the absence of most 412-expressing cells (Fig. 1C). The few 412-expressing cells observed in cli mutants are often associated with germ cells, indicating that some SGP cells do develop (Fig. 1C, arrowhead). However, 412-expressing cells and associated germ cells do not coalesce into a gonad, often remaining
clumped in posterior regions of the embryo. A second SGP marker, Dwnt-2 (Russell et al., 1992), is also not expressed in SGP cells in cli mutants (data not shown). These results demonstrate that cli is essential for gonadogenesis.

To determine the point at which gonad formation is affected in cli mutants, we followed the development of germ cells and SGP cells throughout earlier stages in gonadogenesis. The origin of the SGP cells, their association with germ cells and coalescence into a gonad can be followed by 412 expression. At 6 hours AEL, as germ cells complete migration through the midgut, 412 is expressed in broad bands in the mesoderm of PS2-PS14 (Fig. 2A). Initially, expression extends from the ventral midline laterally to approximately the level of the tracheal pit within overlying ectoderm (Fig. 2B). However, as germ cells contact mesoderm and segregate into bilateral groups, ventral-most cells within each PS lose 412 expression, such that by 7 hours AEL, only cells within dorsolateral regions of each PS continue expressing. SGP cells derive from the patches of 412-expressing cells within PS10, PS11 and PS12 (Fig. 2C; arrowhead). Those within PS11 and PS12 are already seen associating with germ cells (Fig. 2C; arrow). As development proceeds, SGP cells within PS11 and PS12, along with associated germ cells, migrate anteriorly towards PS10 and begin to condense. Throughout this process, 412 expression in more anterior PSs diminishes, such that by 9 hours AEL, expression is strongest in SGP cells lying along PS10/PS11 mesoderm (Fig. 2D, bracket). Finally, by 10 hours AEL, germ cells and 412-expressing SGP cells coalesce into a gonad at PS10 (above). Therefore, the 412 SGP marker is initially broadly expressed, then focusses down to SGP cells derived from PS10 through PS12.

In cli mutants, little or no 412 is expressed when SGP cells are normally segregating within the mesoderm (Fig. 2F). This suggests that cli function may be required for an early step in SGP cell development. Strikingly, cli function is also required for the early broad expression of 412 (Fig. 2E), normally observed long before SGP cells form.

Despite these dramatic affects on 412 expression, initial germ cell behavior is normal in cli mutants. Germ cells contact mesoderm and gather into bilateral groups in cli mutants as in wild type (Fig. 2F). They remain associated with lateral mesoderm throughout most of germ band retraction. Defects in germ cell migration in cli mutants are apparent, however, during migration in early germ-band-retracted embryos. Whereas germ cells normally appear lined up, closely adhering to one another in wild type, germ cells in cli mutants appear more dispersed, with some germ cells scattering throughout posterior regions (Fig. 2G, arrowheads). The stage at which this occurs is later than that observed in abd A mutants, which completely abolish SGP cells and result in scattering of germ cells during retraction (Warrior, 1994). This suggests that SGP cells may form in cli mutants despite the absence of 412 expression, thereby accounting for the initially normal germ cell behaviour. However, SGP cells may fail to maintain their fate and as a result, germ cells eventually disperse.

**Most other mesodermal cell types develop normally in cli mutants**

Our analysis of gonadogenesis in cli mutants suggests that cli may encode a specific regulator of SGP development. Alternatively, defects in SGP development could result from global defects within the mesoderm, suggesting cli functions in general mesodermal patterning. To distinguish these possibilities, we examined the specification and differentiation of other mesodermal cell types.

As SGP cells segregate within the mesoderm, progenitors for the heart, somatic and visceral musculature become apparent. For example, in wild type at 7 hours AEL, heart progenitors can be identified by the expression of tinman (tin) in cells along the dorsal-most edge of the mesoderm (Fig. 3A, arrow; Azpiazu and Frasch, 1993; Bodmer, 1993). In cli mutants, tin expression is indistinguishable from wild type (Fig. 3B, arrow), showing that heart progenitors are specified in the absence of cli function.

Similarly, cli function is not required for the specification of the visceral musculature. Precursors of the visceral musculature originate in patches of approximately 20 cells within each abdominal PS and are identified by the expression of the gene bagpipe, (bap, Azpiazu and Frasch, 1993). Once specified, these cells drop internally and fuse to generate a sheet of cells along the length of the embryo. This latter step can be visualized by the expression of Fasciclin III (Fas III; Fig. 3C, arrow; Snow et al., 1989; Azpiazu and Frasch, 1993). In cli mutants,
normal numbers of *bap*-expressing cells are specified (data not shown), drop interiorly and fuse (Fig. 3D, arrow).

We assayed for *cli* requirements within the somatic musculature by monitoring *nautilus* (*nau*) expression which identifies a subset of muscle precursors at 7 hours AEL (Michelson et al., 1990; Paterson et al., 1991). In *cli* mutants, *nau* expression is similar to wild type (compare Fig. 3E and F, arrowheads), suggesting that the early segregation of most muscle precursors is unaffected in *cli* mutants.

Thus, most mesodermal derivatives do not require *cli* function for their specification. As development proceeds in *cli* mutants, these lineages continue differentiating normally. For example, the development of the dorsal fat body, as visualized by the expression of *engrailed* (*en*), is normal (data not shown; Kassis, 1990). However, later development of body wall musculature is affected, as revealed by the expression of muscle Myosin (Kiehart and Feghali, 1986). Whereas wild-type embryos show a segmentally repeated array of elongated muscle fibers (Fig. 3G), muscle patterning in *cli* mutants appears moderately disorganized, with some fibers, such as the lateral longitudinal muscle either absent or reduced (Fig. 3H, arrow). Defects are most apparent in lateral muscle fibers, while more ventral (arrowhead, Fig. 3H) and dorsal muscles (not shown) appear relatively normal. These defects suggest a role for *cli* in the specification or differentiation of a subset of muscle fibers.

Overall, however, most mesodermal lineages are specified and develop normally in *cli* mutants. Therefore, the disruption of SGP cell development in *cli* mutants is unlikely to result from a general disorganization within the mesoderm. Given this relatively specific requirement for *cli*, we examined *cli* expression within the mesoderm to further characterize its role in SGP cell development.

**cli**t expression identifies somatic gonadal precursor cells

*cli* is expressed within the mesoderm from 4 to 6 hours AEL (Fig. 4A), as the ventrally derived invaginated mesoderm spreads dorsally along the ectoderm. Therefore, *cli* is present at a time consistent with its requirement for the early broad expression of 412. By 6½ hours AEL, *cli* expression is lost in most mesodermal cells. However, by 7 hours AEL, two distinct patterns emerge: a segmental repeat of 1-2 lateral cells (arrow, a muscle progenitor) and, in addition, three clusters of 9 to 12 cells within PS10, PS11 and PS12 (Fig. 4B, arrowhead). Here, we focus on the latter expression pattern.

Double labeling with the vasa antibody shows that the cells within PS11 and PS12 contact germ cells (Fig. 4C, brown), suggesting that these cells are SGP cells.

*cli* expression is maintained within SGP cells as these and associated germ cells begin migrating anteriorly towards PS10 (Fig. 4D, arrowhead). As the germ band retracts, however, *cli* expression restricts to the posterior half of the SGP cells (Fig. 4E, arrowhead). By 11 hours AEL, *cli* expression is further restricted to the posterior-most 10 to 12 SGP cells in the female gonad (Fig. 4F, arrowhead), while it is lost entirely from the male. The expression of Cli protein parallels that of *cli* transcripts throughout embryogenesis, although Cli protein persists longer within nuclei of anterior SGP cells (see Figs 6G and 8E).

This analysis suggests that *cli* expression identifies SGP cells that arise within PS10 through PS12 mesoderm. To confirm this, we examined whether *cli* expression required the function of a previously identified regulator of SGP cells, *abd*.

![Fig. 2. *cli* is required for expression of the SGP marker, 412. Embryos labeled with 412 (blue) and Vasa (brown). (A) Wild type, 6 hours AEL; 412 expression is initiated in broad bands within the mesoderm of PS 2-14, as germ cells (brown) complete migration through the midgut. (B) Top view of embryo shown in A, looking down on PS10 through PS12 of the extended germ band (arrowheads). 412 expression extends from the ventral midline (white arrowhead) to the position of the tracheal pits (just below the plane of focus, arrowheads). White arrows indicate position of wing-expressing ectodermal cells within the posterior PS. (C) Restricted 412 expression at 7 hour AEL. Germ cells (arrow) have segregated into bilateral groups and begun contacting 412-expressing SGP cells specified within PS10-PS12 (arrowheads). (D) 9 hours AEL; 412 expression is strongest in SGP cells associated with germ cells aligned in posterior regions of the embryo (bracket). Note that there is still substantial 412 expression in anterior regions. (E) *cli* mutant, 6 hours AEL; no 412 is expressed. (F) *cli* mutant, 8 hours AEL; germ cells contact mesoderm and segregate into bilateral groups normally. (G) *cli* mutant, 9 AEL; defects in germ cell migration are observed. Germ cells appear more dispersed compared to wild type (D), with some scattering (arrowheads). Bar scale represents 90 μm in A, E, D and G, 45 μm in B, C and F.
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A (Lewis, 1978; Szabad and Nöthiger, 1992; Cumberledge et al., 1992; Brookman et al., 1992; Boyle and DiNardo, 1995; Greig and Akam, 1995). In \textit{abd A} mutants, no \textit{nau} expression within PS10-12 is detected at 7 hours AEL (Fig. 4G), although early, broad expression of \textit{nau} within the mesoderm is unaffected (data not shown). In addition, when \textit{abd A} is ectopically expressed at 4 hours AEL, \textit{cli} expression is induced in patches of mesodermal cells in anterior PSs (Fig. 4H). These ectopic \textit{cli}-expressing cells differentiate as SGP cells, since they express the differentiation marker \textit{nau} at later stages (data not shown; Boyle and DiNardo, 1995). Therefore, \textit{cli} is an early and specific marker of SGP cells.

\textbf{\textit{cli}} is required to maintain somatic gonadal precursor fates within the mesoderm

Since \textit{cli} is initially expressed throughout the mesoderm, then restricts to those cells eventually selected as SGP cells and is required for their development, we wondered whether \textit{cli} function is required for the initial segregation of SGP cells. To assay whether SGP cells fail to segregate in \textit{cli} mutants, or segregate but fail to maintain their fate, we examined \textit{cli} expression in embryos mutant for the \textit{cli}^{HE} allele. This allele, although a strong loss of function allele, produces a transcript, thereby allowing us to examine early stages of SGP cell development. In the absence of \textit{cli} function, we see \textit{cli} expression in PS10-PS12 mesoderm at 7 hours AEL (Fig. 5B, arrowheads). In addition, these cells are often observed contacting germ cells (data not shown). Thus, \textit{cli} function is not essential for the initial selection of SGP cells out of the mesoderm. Consistent with this, ectopic expression of \textit{cli} by a heat-shock inducible promoter at various stages of development in wild-type embryos does not alter the number of SGP cells specified (data not shown). Therefore, \textit{cli} function is not sufficient to promote SGP cell fates, nor is it an essential component of the initial selection process. \textit{cli} function is required, however, to maintain SGP fates within the mesoderm. Examination of later stages in development shows that \textit{cli} expression drops off dramatically within the mesoderm such that fewer cells can be detected by 8 hours AEL compared to wild type (Fig. 5C,D). As seen with 412 expression, these few \textit{cli}-positive cells often adhere to germ cells (not shown). However, germ cells and SGP cells fail to complete their migration and coalescence into a gonad. Therefore, \textit{cli} function is required to maintain SGP fates within the mesoderm.
Having identified an early and specific marker for SGP cells, we have mapped their origin more precisely in order to identify regulators that act to determine SGP fates within the mesoderm.

**Wingless signalling defines the anteroposterior position of somatic gonadal precursors in a PS**

SGP cells develop in distinct patches within each of three PSs. The anteroposterior position of each cluster within a PS can be determined relative to mesodermal Eve expression. Eve is expressed in a subset of pericardial cells and a dorsal muscle progenitor that lie at the posterior margin of each PS, just below Wg-expressing ectodermal cells (Fig. 6A-D; black arrowheads in A,B and D; Lawrence et al., 1995). We find that early, broad 412 expression maps approximately one to two cells posterior to Eve-expressing cells (Fig. 6A, black arrowhead) showing that early 412 expression lies within the anterior half of each PS. However, as 412 expression restricts to SGP cells (Fig. 6B, brackets), staining cells appear shifted slightly, due to either de novo expression of our marker or cell movement, such that 412-expressing cells now lie in line with and just posterior to Eve cells (Fig. 6B, arrowhead). Similar staining is seen with the Cli marker at this stage (Fig. 6C,D, brackets in D). Therefore, SGP cells derive from a restricted region of each PS, their final position being in line with and just posterior to Eve-expressing cells (arrowhead). In addition, double labeling with a reporter lacZ construct that mimics ectodermal Wg expression shows that SGP cells are near Wg-expressing ectodermal cells (data not shown). Lastly, like Eve-expressing pericardial cells, SGP cell specification requires wg function (Wu et al., 1995; Lawrence et al., 1995; Warrior, 1994; M. B. and S. D., unpublished observations). These observations suggest that local and restricted Wg signaling to mesodermal cells may determine the position and number of SGP cells specified within a PS. To test this, we examined Cli expression in embryos ectopically expressing Wg under the control of a heat-shock inducible promoter (Noordermeer et al., 1992; see MATERIALS AND METHODS). Whereas untreated embryos show the normal patches of SGP cells within PS10-PS12 mesoderm at 7 hours AEL (Fig. 6D), embryos given a 30 minute heat-shock treatment at 4-5 hours AEL for ectopic Wg expression show an approximately three-fold increase in SGP cells, such that almost all cells within the lateral mesoderm of PS10-PS12 express Cli (Fig. 6E).

Since overexpression of Wg also has dramatic effects on the generation of cell types within the ectoderm (Noordermeer et al., 1992), it is possible the effects that we observe on SGP cell development are a secondary consequence of patterning defects generated within the ectoderm. To address whether the expansion of the SGP population is a direct response of mesodermal cells to Wg signaling, we activated the Wg signal transduction pathway specifically in mesodermal cells. To accomplish this, we used a constitutively activated form of Armadillo (ArmS10, see MATERIALS AND METHODS),

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**Fig. 4. cli expression identifies SGP cells at early stages.** cli RNA expression (blue) and Vasa staining (brown). (A) 6 hours AEL; cli expression within mesoderm. (B) Abdominal region of embryo, 7 hour AEL. cli expression is lost within mesoderm except for SGP cells specified within PS10, PS11 and PS12 (arrowheads). Expression is also seen in a lateral muscle precursor (arrow) as well as in the ectoderm. (C) Magnified view of embryo in B. SGP cells within PS12 contact germ cells (arrowhead). (D) 8 hours AEL; cli-expressing SGP cells associated with germ cells throughout germ band retraction, as germ cells and SGP cells (arrowhead) begin migrating anteriorly. (E) 9 hours AEL; focusing on the PS10-PS11 region. cli expression within SGP cells restricts posteriorly (arrowhead), as SGP cells and associated germ cells begin to condense. (F) 10 hour AEL; coalesced gonad. cli expression is restricted to posterior SGP cells in females (arrowhead). (G) abd A mutant, 7 hours AEL; no cli expression is observed in SGP cells. (H) Hs-abd A, 7 hours AEL; ectopic expression of abd A at 4 hours AEL results in induction of patches of cli expression in anterior PSs (arrowheads). Scale bar represents 90 μm in A, 45 μm in B,D,G and I, and 21 μm in C,E and F.
Somatic gonadal precursor cells develop adjacent to visceral mesoderm

We next investigated the dorsoventral positioning of SGP cells. An early event in the establishment of distinct fates along this axis occurs with the split into dorsal and ventral domains. This regionalization relies upon induction by the transforming growth factor β homologue dpp (Padgett et al., 1987; Staehling-Hampton et al., 1994; Frasch, 1995). dpp, expressed in dorsal ectoderm, maintains the expression of tin in underlying mesoderm, thereby allowing the specification of dorsal cell types such as heart and visceral mesoderm (diagramed in Fig. 7B; Azpiazu and Frasch, 1993; Bodmer, 1993). To identify regulators that control the dorsoventral aspect of SGP specification, we sectioned stained embryos to determine the position at which SGP cells develop relative to other mesodermal derivatives.

cli is initially expressed throughout the mesoderm as the invaginated mesoderm flattens into a monolayer and begins spreading dorsally along the ectoderm (data not shown). As the migrating mesodermal cells extend to their dorsal limit (Fig. 7C, arrowhead), cli expression is lost from dorsal-most cells such that, by 5 hours AEL, expression is limited to ventral and lateral mesodermal cells.

This restriction in cli expression coincides with the subdivision of the mesoderm into dorsal and ventral domains through induction by dpp. To map the dorsal-most limit of cli expression at this stage, we double-labeled embryos carrying a dpp-lac Z reporter gene (Huang et al., 1993). cli expression within mesoderm extends to and overlaps slightly with dpp-expressing ectodermal cells (Fig. 7C, arrow). Subsequent to this, cli expression within the mesoderm declines dramatically, except for expression in SGP cells, here shown contacting germ cells (Fig. 7D, arrowhead).

These SGP cells are likely to derive from the dorsal-most cli-expressing cells near overlying dpp-expressing ectodermal cells (Fig. 7B). This would place the SGP cells adjacent to prospective visceral mesoderm, a dorsal mesodermal cell type. To confirm this, we examined 412 expression, which initiates expression slightly later than cli but persists longer and, thus, can be mapped relative to the visceral mesoderm marker, Fas III. Indeed most Fas III-expressing cells are dorsal to the 412 expression domain (Fig. 7E). Note, however, that at this stage, the patches of visceral mesodermal cells are in the process of dropping interiorly, so that some Fas III-expressing cells lie adjacent and interior to 412-expressing cells. At the same time, 412-expressing cells appear to extend further dorsally. In subsequent stages, high level 412 expression is maintained in SGP cells lying adjacent to Fas III-staining cells (Fig. 7F, arrowhead), now positioned interior and adjacent to the midgut. Although we cannot exclude the possibility of de novo 412 expression, the simplest hypothesis is that the SGP cells derive from the leading edge of 412-expressing cells. Thus, we believe that SGP cells originate in regions just ventral to the visceral mesoderm precursors, near the boundary of dpp expression in overlying ectodermal cells.

Somatic gonadal precursor development depends on tinman

Since tin appears to mediate the role of dpp in specifying dorsal mesoderm, we tested whether tin function is also required for SGP cell development. In tin mutants, few or no SGP cells are detected at 7 hours AEL by cli expression (Fig. 8B), although early, broad cli expression within the mesoderm is not affected
In addition, germ cells are scattered at late stages and no gonad forms (data not shown). Thus, SGP cells, like other dorsal mesodermal cell types, require tin function.

**bagpipe restricts somatic gonadal precursor development to lateral regions of the mesoderm**

How the dorsal mesoderm is further subdivided into cardiac, dorsal muscle, visceral and SGP cells is unclear. However, previous analysis indicated that, once this further subdivision is made, there may be regulatory interactions between the developing primordia to help refine the position of each. In embryos with a weak allele of the visceral mesoderm regulator *bap*, cells that normally give rise to visceral mesoderm are transformed into other mesodermal fates. These include most often lateral somatic muscles, but occasional contribution to the gonad was also observed, suggesting that *bap* normally repressed the formation of these cell types (Azpiazu and Frasch, 1993). To test whether the specification of SGP cells is negatively regulated by *bap*, we first examined double-labeled embryos to determine if any cells co-express *Cli* and *bap* in the region from which SGP cells are selected.

*bap* expression identifies visceral mesoderm primordia as patches of approximately 20 dorsolaterally located cells in each abdominal PS that subsequently drop internally to form the visceral mesoderm (Azpiazu and Frasch, 1993). At this time, *Cli* is broadly expressed at high levels in ventral and lateral mesoderm, extending just into the *bap* domain, where a small number of cells that co-express *bap* and *Cli* are found (Fig. 8C, arrowheads). These co-expressing cells lie near the region from which the SGP population is derived. Thus, *bap* may normally negatively regulate SGP fate by repressing *Cli* expression within these cells.

We next re-investigated whether *bap* regulates SGP cell fate specification, by assaying *bap* null embryos, since the previous study used a *bap* hypomorphic mutation. We find that the patch of *Cli*-expressing SGP cells at 7 hours AEL has expanded. Whereas the total number of SGP cells averages 25-37 in wild type (Fig. 8A; Fig. 8F, 7 hours), we find 40-55 cells in *bap* mutants (Fig. 8D; Fig. 8F, 7 hours). Although it is difficult to determine precisely the position of the extra SGP cells, they often appear in slightly more dorsal regions in the mesoderm compared to wild type. This would be consistent with extra SGP cells being recruited from cells that would normally become visceral mesoderm, which lies just dorsal to the developing SGP cells.

The extra *Cli*-expressing cells are SGP cells since extra cells are also found within the formed gonad (Fig. 8E). SGP cells within *bap* mutant gonads ranges from 50 to 60 total cells, compared to the 35-45 cells found in wild-type gonads (Fig. 8F, 13 hrs). These results show that wild-type *bap* function...
represents SGP cells (arrowhead) in lateral mesoderm. These cells lie adjacent to visceral mesoderm which has dropped interiorly and now contacts the gut. Bar represents 90 μm.

Fig. 7. SGP cells arise just ventral to visceral mesoderm. (A) 5 hours AEL; cli RNA expression; line indicates position of transverse sections shown in C-F. (B) Diagram of transverse section of embryo, 7 hours AEL, illustrating early subdivisions within the mesoderm. Due to the extended germ band, mesodermal cells lie within both the top and bottom half of each section. (g) Midgut. The dorsal-most mesodermal cells underlie dpp-expressing ectoderm, indicated by the thick black lines. dpp maintains tin expression within underlying mesoderm, which subsequently generates heart precursors (red), visceral mesoderm (yellow) and dorsal muscles (not shown). SGP cells (blue) lie adjacent to visceral mesoderm. (C-F) 10 μm thick transverse sections. (C) 5 hours AEL; cli RNA (blue) and dpp-lacZ (brown), which marks the extent of dpp expression within the ectoderm (arrow). Arrowhead marks the dorsal limit of the extended mesoderm; asterix marks the ventral midline. cli expression extends to, and overlaps slightly with adjacent dpp-expressing ectoderm. (D) 7 hours AEL; cli expression (blue) within the mesoderm diminishes dramatically except for a small number of SGP cells within lateral regions (arrowhead) that begin contacting germ cells (brown). Arrows point to cli expression in CNS and muscle precursors. (E,F) 412 (blue) and the visceral mesoderm marker Fas III (brown). (E) 6 hours AEL; 412-expressing cells are ventral and adjacent to visceral mesoderm (arrow) which is in the process of dropping interiorly. (F) 7 hours AEL; 412 expression has restricted to SGP cells (arrowhead) in lateral mesoderm. These cells lie adjacent to visceral mesoderm which has dropped interiorly completely and now contacts the gut. Bar represents 90 μm for A and 47 μm for C-F.

DISCUSSION

Gametogenesis entails a complex developmental program that ensures the continuous generation of mature gametes from germline stem cells. This differentiation program is supported by a variety of somatic cell types, each of which derives from the population of SGP cells found clustered in the embryonic gonad. To begin to investigate how these cells differentiate and sustain the germline, we have been focusing on the development of SGP cells during embryogenesis. Our studies of the generation of SGP cells within the mesoderm not only uncover essential regulators of their specification and differentiation but also reveal novel aspects of the general mechanism whereby cell fates are allocated within the mesoderm.

Segregation of somatic gonadal precursor cells within the mesoderm

Until recently, little has been known about the mechanism whereby SGP fates are determined within the mesoderm. Previous analysis illustrated that they arise as patches of cells from three posterior abdominal PS at mid embryogenesis (Brookman et al., 1992; Warrior, 1994; Boyle and DiNardo, 1995; Greig and Akam, 1995). In addition, using 412 expression as a marker, these studies established that the restriction of SGP cell specification to these PSs depends on the function of the homeotic gene abd A. Using the more specific marker cli, we have confirmed these results and demonstrated a role for abd A in SGP cells as these cells first form. Thus, ectopic expression of abd A results in SGP cells being specified within more anterior PS, while loss of abd A expression abolishes SGP cells. Thus, abd A function determines the competence of a PS for SGP cell induction. abd A does not, however, instruct SGP cell number or position within a PS, since SGP cells induced in anterior segments still show reiterated patches of expression. Therefore, abd A function is permissive. Here, we show that the anteroposterior and dorsoventral position of SGP cells within a PS is established by ectodermal signals generated by the secreted growth factor Wg, coupled with a gene regulatory hierarchy within the mesoderm.

Wingless defines the anteroposterior position of somatic gonadal precursor cells within a PS

Our results indicate that restricted Wg expression determines the position at which SGP cells develop within a PS. While loss of Wg function abolishes SGP cells (Warrior, 1994; M. B. and S. D., unpublished observations), global expression of Wg, or activation of its signal transduction pathway within the mesoderm, results in almost all dorsolateral mesodermal cells within the PS acquiring SGP fates and populating the gonad. Thus, Wg input instructs SGP fate within PS10-PS12 mesoderm as long as these cells have acquired appropriate dorsoventral cues.

Wg is required in the generation of other cell types within the mesoderm, although this requirement appears to be permissive rather than instructive (Lawrence et al., 1995; Baylies et al., 1995; Ranganayakulu et al., 1996; Park et al., 1996). For example, absence of Wg function results in the loss of nau
expression in a subset of muscle progenitors, while uniform Wg expression restores them to Wg mutant embryos. Ectopic Wg expression, however, has no affect on the number or position of nau-expressing muscle progenitors specified (Baylies et al., 1995). Wg also appears to function permissively in the development of Eve-expressing heart precursors and a dorsal muscle progenitor. Ectopic Wg expression expands the population of Eve-expressing cells (Lawrence et al., 1995), just as for SGP cells. However, unlike for SGP cells, ectopic Eve-expression is transient and remains restricted to the posterior-most cells within each PS. Thus, Wg input is not sufficient to position these cell fates within a PS. Indeed, more recent evidence suggests that Wg input determines only the competence of a region for the specification of Eve cells, allowing other signaling pathways to specify cell fate (A. Michelson, personal communication).

Since our results demonstrate that Wg signaling can be instructive, at least for SGP cell fates, taken together these observations extend our understanding of how different cell types are generated within the mesoderm. One signaling molecule, Wg, can act as a competence factor for one cell type while instructing the fate of another. Although it is not known what regulates the different responses to Wg within these mesodermal lineages, in the epidermis, Wg signaling at different times in development has distinct outputs. Early, Wg signaling maintains the expression of a target gene, engrailed, whereas later Wg input specifies engrailed cell fate (Dougan and DiNardo, 1992). Therefore, it is possible that the distinct responses to Wg signalling within the mesoderm are a result of Wg action at different times in development. However, Wg appears to be required during the same early time period to specify heart precursors as SGP cells (Wu et al., 1995; M. B. and S. D., unpublished observations).

Given that our mapping places some SGP cells several cell diameters posterior to Wg-expressing cells, perhaps Wg acts indirectly, generating a secondary signal to determine SGP fates. If so, such a signal must originate within the mesoderm, since activation of the Wg pathway specifically within mesoderm is sufficient to specify SGP fates. Alternatively, Wg may signal directly to determine SGP position within a PS, spreading across several cell diameters. It is possible, however, that spread of Wg protein is not required. SGP cells may be more closely positioned to Wg-expressing cells earlier, when Wg is required to generate SGP fate. Rearrangements within the mesoderm may have taken place to position SGP cells further from Wg-expressing cells by the time these cells can be identified by our markers.

The SGP population expands primarily along the anteroposterior axis in response to global Wg expression. Therefore, additional regulators act along the dorsoventral axis to specify SGP cells.

**Fig. 8.** SGP cell development requires tin function and is negatively regulated by bap. (A-E) anti-Cli staining (brown). (A) Wild type, 7 hours AEL; Cli-expressing SGP cells (arrowhead). (B) tin mutant, 7 hours AEL; few or no SGP cells are specified (arrow points to single Cli-expressing SGP cell within PS12). (C) Wild type, PS10-12 region, 6 hour AEL, double labeled with Cli (brown) and bap RNA (blue). Dorsal (d) is down, ventral (v), up. A small number of Cli-expressing cells that lie at the ventral border of the bap domain are doubly labeled (arrowheads). (D) bap null mutant, 7 hours AEL; increased numbers of SGP cells are specified (arrowheads). (E) bap null mutant, 13 hours AEL; dissected gonad showing extra SGP cells are also found within the formed gonad (arrow in E). (F) Histogram showing average number of SGP cells specified in 13 wild-type (s.d.=6.93) and 13 tin mutant embryos (s.d.=5.68) at 7 hours AEL. Increased numbers are also found incorporated into the gonad at 13 hours AEL, comparing 10 tin mutants (s.d.=4.98) with 10 wild-type embryos (s.d.=6.05). One-tailed t-test P<0.05. Scale bar represents 45 μm in A, B and D; 20 μm in C and E.

**Positioning somatic gonadal precursors along the dorsoventral axis requires tinman function and is negatively regulated by bagpipe**

Our results show that SGP cells, like other dorsal cell types such as visceral mesoderm and heart precursors, require tin function (Bodmer, 1993; Azpiazu and Frasch, 1993). These results suggest that the initial positioning of SGP cells along the dorsoventral axis relies on a regulatory cascade that establishes a domain of mesodermal cells competent to develop dorsal cell fates. This regionalization is determined by an inductive signal by overlying dpp-expressing ectodermal cells (Staehling-Hampton et al., 1994; Frasch, 1995; Maggert et al., 1995). dpp maintains tin expression in underlying mesoderm, thereby allowing the specification of dorsal cell types.

Once this initial subdivision has occurred, distinct progenitors for each of the cell types derived from this region are precisely
defined. How this occurs is unclear. Perhaps there is a continued requirement for dpp, acting in a second step to establish different fates along the dorsoventral axis in a concentration-dependent manner, such as has been proposed for the determination of cell fates by dpp in the ectoderm (Ferguson and Anderson, 1992; Wharton et al., 1993). At present, however, there is little evidence of such a mechanism acting within the mesoderm. The positioning of visceral precursors within dorsal mesoderm appears to be determined simply by the extent of dpp expression in overlying ectoderm. The ventral limit of the visceral mesoderm coincides with that of ectodermal dpp and broadening dpp expression within ectoderm results in the concomitant expansion of visceral mesoderm (Staehling-Hampton et al., 1994; Frasch, 1995). The enlarged dpp expression domain, however, has no effect on the number or position of cardiac or dorsal muscle precursors (Frasch, 1995). Therefore, positioning of these progenitors must rely on additional regulators to further subdivide the mesoderm. In fact, some determinants for cardiac mesoderm are being identified, indicating that a hierarchy of signals act in combination with dpp to determine these fates (A. Michelson, personal communication).

In our analysis, the SGP cells are unambiguously identified for the first time by the specific expression of Cli in small clusters within PS10, PS11 and PS12 at 7 hours AEL. Their position is within dorsal mesoderm, adjacent to the internally located visceral mesoderm. However, we place the origin of SGP cells just ventral to visceral mesoderm. First, SGP cells are found at the same anteroposterior register as visceral mesoderm. Second, initial mesodermal expression of both the 412 and cli markers extends from ventral mesoderm laterally to the domain of visceral mesoderm cells, as marked by bap and Fasciclin III expression. As visceral mesoderm drops internally, the leading edge of 412-expressing cells extends further dorsally, leaving the final position of the SGP cells at these slightly more dorsal positions. Although it remains possible that there is de novo 412 and Cli expression at these more dorsal positions, we view this as less likely. Recent data indicate that all other cells in the dorsal mesoderm at this anteroposterior register are allocated to visceral or heart progenitor fate, leaving no mesodermal cells to take on the SGP fate (Azpiazu et al., 1996).

At present, it is still unclear what might specify SGP cell fate at the ventral border of visceral mesoderm. Our results do, however, indicate that cross regulatory interactions are important in refining this patterning. A significant increase in the number of SGP cells specified is observed when the dorsally adjacent visceral mesoderm is eliminated. Such cross-regulatory interactions are central to patterning within many tissues (Kellerman et al., 1990; Brook and Cohen, 1996; Jiang and Struhl, 1996). Thus, we view it likely that the repression of SGP fate by bap reveals a common mechanism used between nearby developing mesodermal primordia to ensure the precise allocation of each within the mesoderm.

**clif** function within somatic gonadal precursors

Our analysis of cli expression shows that, after an initial burst throughout the mesoderm, cli expression restricts to SGP cells. Despite this broad cli expression and its requirement for the early expression of 412, our results indicate that cli is not essential for the initial selection of SGP cells within the mesoderm. In the absence of cli function, cli-expressing SGP cells do form and initially associate with germ cells, indicating that early steps in their development have occurred. Therefore, it is unclear what role, if any, is played by early mesodermal expression of cli.

Perhaps cli function at this stage is functionally redundant with an as yet unidentified gene that, like cli, acts to establish SGP cell fates, but does so independently of initiating 412 expression.

Our results do, however, reveal an essential requirement for cli in the maintenance of SGP cells. Shortly after SGP cells are specified in cli mutants, we observe a reduction in the number of cli-expressing SGP cells, such that by germ band retraction, few SGP cells remain. Those few SGP cells that are present, however, fail to complete their migration to the position of gonad formation. As a consequence, germ cells are not incorporated into a gonad, remaining scattered throughout posterior regions of the embryo. Although our experiments do not allow us to determine the ultimate fate of the mutant SGP cells, perhaps SGP cells fail to differentiate properly and as a consequence die.

The mechanism whereby cli maintains SGP cell fates is unclear. The molecular nature of cli provides little insight into its function, as it encodes a novel nuclear protein with no obvious motifs (Bonini et al., 1993). Previous studies have shown that cli functions elsewhere, for instance, in head morphogenesis (Nusslein-Volhard et al., 1984), and for the differentiation and/or survival of cells within the eye imaginal disk (Bonini et al., 1993). In viable cli mutants, the onset of photoreceptor cell differentiation fails to occur. As a consequence, cells die by programmed cell death. The targets of cli function, however, within these other tissues, as in SGP cells, are currently unknown.

At present, we do know a little about the regulatory pathway leading to the specification and differentiation of SGP cells. Previous studies have suggested that abd A function is required in a second step in gonadogenesis, distinct from its earlier role in competence. In embryos carrying mutations in the iab-4 cis-regulatory region of abd A, germ cells initially associate with SGP cells, but SGP cells fail to coalesce into a gonad at PS10 and germ cells scatter throughout the embryo at early stages of retraction (Warrior, 1994; Boyle and DiNardo, 1995). The extent of germ cell scattering in iab-4 mutants is more severe than that observed in cli mutants, suggesting that cli may act downstream of late abd A function in gonadogenesis. Given that ectopic expression of cli alone has no affect on gonad formation, it is unlikely to be the sole target of late abd A function, suggesting that other genes required for the differentiation and function of SGP cells remain to be identified.

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