Fly LMBR1/LIMR-type protein Lilipod promotes germ-line stem cell self-renewal by enhancing BMP signaling

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Limb development membrane protein-1 (LMBR1)/lipocalin-interacting membrane receptor (LIMR)-type proteins are putative nine-transmembrane receptors that are evolutionarily conserved across metazoans. However, their biological function is unknown. Here, we show that the fly family member Lilipod (Lili) is required for germline stem cell (GSC) self-renewal in the *Drosophila* ovary where it enhances bone morphogenetic protein (BMP) signaling. *lili* mutant GSCs are lost through differentiation, and display reduced levels of the Dpp transducer pMad and precocious activation of the master differentiation factor *bam*. Conversely, overexpressed Lili induces supernumerary pMad-positive *bamP-GFP*–negative GSCs. Interestingly, differentiation of *lili* mutant GSCs is *bam*-dependent; however, its effect on pMad is not. Thus, although it promotes stem cell self-renewal by repressing a *bam*-dependent process, Lilipod enhances transduction of the Dpp signal independently of its suppression of differentiation. In addition, because Lili is still required by a ligand-independent BMP receptor, its function likely occurs between receptor activation and pMad phosphorylation within the signaling cascade. This first, to our knowledge, in vivo characterization of a LMBR1/LIMR-type protein in a genetic model reveals an important role in modulating BMP signaling during the asymmetric division of an adult stem cell population and in other BMP signaling contexts.

stem cell | germline | Dpp | decapentaplegic | lipocalin receptor

The human lipocalin-interacting membrane receptor LIMR (also known as LMBR1L) and the closely related limb development membrane protein-1 (LMBR1) share a predicted multitransmembrane (TM) structure that is strikingly different from other well-characterized integral membrane receptors. LIMR was originally isolated by phage display through its interaction with lipocalin-1 (Lcn-1), a secreted lipid-binding carrier protein (1). Cell culture studies showed that LIMR could mediate the internalization of Lcn-1, suggesting a role in cell signaling (2). However, because the physiological roles of both Lcn-1 and LIMR are unknown, the significance of this observation is unclear. Less is known about the LMBR1 protein. Initially, the human locus was genetically linked to multiple congenital limb malformations. However, further studies of the human and mouse loci showed that the original association with limb defects was incidental because of the disruption of a long-range SHH enhancer located within an intron of *LMBR1* (3). To date, no loss-of-function or gain-of-function analyses of LIMR or LMBR1 have been reported in any model system, and studies in vertebrates may be complicated by functional redundancy between the two family members.

Drosophila contains a single uncharacterized LIMR-like protein, CG5807, which is the shared ortholog of both LIMR and LMBR1. We have investigated the fly gene in vivo and show here that it functions in the germ-line stem cells (GSC) of the *Drosophila* ovary. In the fruit fly, oocytes are continually produced by GSCs that are housed within a structure called germarium (Fig. 1*A*); here, two to three GSCs adhere to cells of the somatic niche, the cap cells (CC) (4, 5). As the GSCs divide, one daughter cell remains in contact with the CCs and maintains stem cell identity, whereas the other forms away from the niche and turns into a differentiating cystoblast (CB), the progenitor of egg chambers and ultimately oocytes.

The maintenance of ovarian stem cells is tightly regulated by multiple extrinsic and intrinsic factors. Some of these factors repress the differentiation program in the renewed GSC, whereas others relieve this repression in the CB. The major signaling system in this process is the BMP pathway (6). Specifically, the BMP2/4 ligand decapentaplegic (Dpp) is secreted by CCs and signals onto adjacent GSCs to prevent their differentiation. In the GSC, the signal is transmitted through the type I BMP receptor thickveins (Tkv) and the receptor-regulated R-SMAD Mothers against dpp (Mad) to the nucleus, thereby suppressing transcription of the differentiation-promoting factor Bag of marbles (Bam) (7, 8). Reduced BMP signaling favors differentiation resulting in stem cell loss; conversely, increased BMP signaling favors self-renewal resulting in GSC hyperplasia. In this context, modulators of signaling play a critical role in maintaining the optimal balance between self-renewal and differentiation. By enhancing BMP pathway activity in one daughter cell and antagonizing it in the other (9), they effect a dramatic switch from pathway on (in the renewed GSC) to pathway off (in the differentiating CB) during a single cell division.

We present here the first evidence to our knowledge of a physiological role for a LIMR/LMBR1-type protein in a model organism, showing that it contributes to the regulation of the BMP pathway in the female germ line. CG5807 is required in

Significance

Adult stem cells are maintained in an undifferentiated state in response to factors provided by their surrounding niche microenvironment. The *Drosophila* limb development membrane protein-1 (LMBR1)/lipocalin-interacting membrane receptor (LIMR) type transmembrane protein Lilipod (Lili) is required in ovarian germ-line stem cells (GSC) for their self-renewal. In the fly ovary, niche-secreted bone morphogenetic protein (BMP) ligands activate signaling in the GSC to suppress transcription of the differentiation factor Bam. Lili is required for this suppression and functions as a modulator of BMP signal transduction in this and other contexts. LMBR1/LIMR type transmembrane proteins are conserved throughout the Metazoa, and this study provides the first example to our knowledge of physiological function in a model organism.

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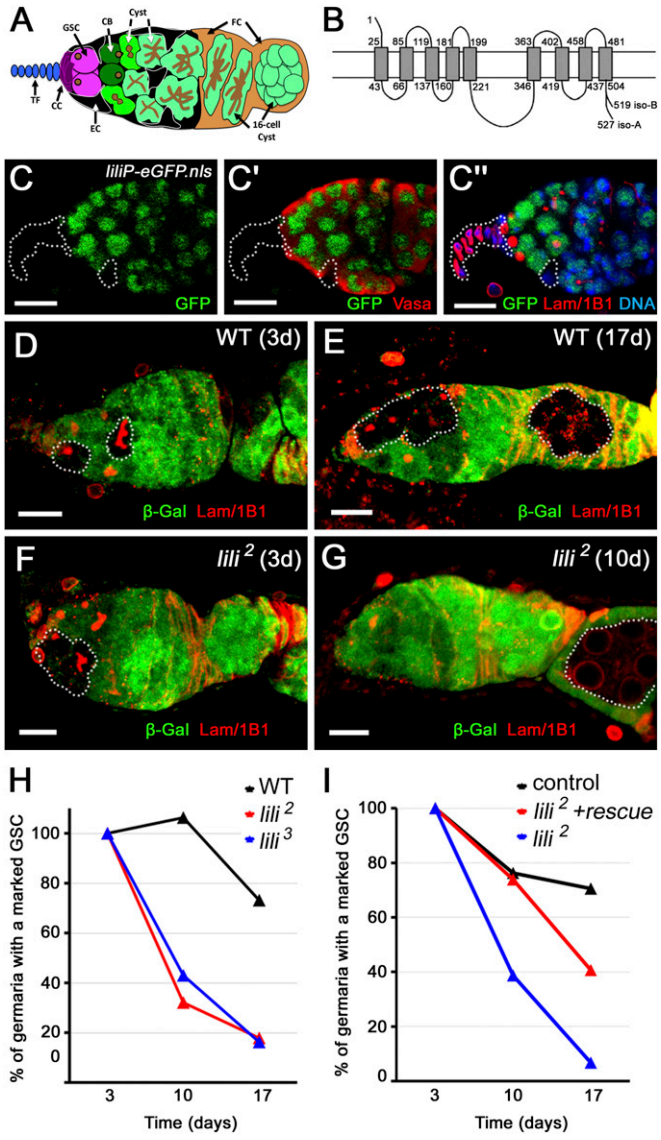

Fig. 1. *lili* is intrinsically required for GSC maintenance. (*A*) Illustration of *Drosophila* germarium highlighting germ line: GSC (magenta), CB (dark green), cysts (green and light green); and somatic cells of the niche: cap cells (CC; purple), terminal filament cells (TF; blue), and escort cells (EC; black). Follicle cells surrounding forming egg chambers are shown in light brown (FC). TFs and CCs show strong nuclear membrane staining with anti-Lamin C Ab and can be distinguished by their position and morphology at the anterior tip of the germarium. All germ-line cells express the cytoplasmic protein Vasa and can be distinguished by the presence of an intracellular structure (dark brown) detected by anti-Adducin Ab (1B1). This structure is called spectrosome and appears spherical in GSCs and CBs, whereas it is called fusome and appears branched across the interconnected cells of developing cysts. GSCs and CBs are distinguished by location: GSCs contact CCs and ECs, whereas CBs contact GSCs and ECs, but not CCs. (*B*) An illustration of predicted Lilipod topology. Isoforms A and B bear unique C termini (15 aa in A and 6 in B). (*C*–*C*′′) Anterior half of a *liliP-eGFP.nls* germarium stained for GFP (*lili*-reporter), Vasa (germ line), Lamin C (CC nuclear membrane), anti-1B1 (spectrosome/fusome), and DAPI (nuclei) as marked in images. Dashed outline mark TF and CCs, and one intercalating EC; none express *lili-eGFP.nls*. The continued presence of GFP in the differentiating germ line may reflect persistence of GFP protein. *liliP-eGFP.nls* must, however, be transcribed in GSCs. (*D*–*G*) Mosaic germaria containing clonal GSCs (β-gal negative) and progeny cysts (dashed outlines) at 3 (*D*) and 17 (*E*) d ACI stained with both anti-Lamin C and anti-1B1 (red) as well as anti–β-gal (green). (*D* and *E*) Mosaic germaria with control WT clones. (*F* and *G*) Mosaic germaria with *lili*^{2} homozygous mutant clonal GSCs and progeny at 3 d ACI (*F*), but only a clonal cyst and no *lili*^{2} mutant GSCs at 10 d ACI (*G*). (*H*) Progressive loss of *lili* mutant GSCs over time. Percentage of total germaria that contain at least one WT or mutant clonal GSC. (*I*) Expression of Lili (*nos-Gal4*^{vp16}*UASp-lili*^{A}) slows down *lili* mutant GSC loss. (Scale bars: 10 μm.)

GSCs to promote BMP signaling and ensure the suppression of *bam*. Its loss leads to stem cell differentiation and sterility over time. Conversely, its overexpression increases the number of BMP-responsive cells, thereby expanding the GSC compartment. We also show that CG5807 affects the signaling cascade between the Tkv receptor and the Mad transducer, and that it operates in other BMP-signaling contexts in the soma. Based on these findings, we have named the CG5807 gene *lilipod* (*lili*) to reflect its sequence conservation and its function as a "*LIMR-like promoter of ovarian dpp*."

Results

Lili Is Evolutionarily Conserved and Required in GSCs for Germ-Line Maintenance. The Lilipod protein shares 44% (71%) amino acid identity (similarity) with human LIMR and 43% (72%) identity (similarity) with human LMBR1 (Fig. S1). TMpred and TMHMM (v2.0) predict a structure with nine TM spanning regions, extracellular N- and intracellular C-termini, and a large 125-aa central loop (Fig. 1*B*), similar to that originally proposed for human LIMR (2). Homozygous mutant animals (*lili*^{1}, *lili*^{2}, or *lili*^{3} allele) died as young larvae (during L1 and early in L2), but could be rescued to viability by a genomic *p(lili*^{WT}) construct or by somatic expression of Lili isoform A (*hs-Gal4* or *actin5C-Gal4* with *UASt-lili*^{A}).

lili expression is reported in several adult tissues with highest enrichment in the ovary and testis (flybase.org). To confirm expression in the ovary, we used a *liliP-eGFP* reporter containing 626 bp of 5′-flanking genomic DNA (Fig. S2) and detected GFP protein in the GSCs and their progeny but not in the somatic niche (cap, escort, or terminal filament cells) (Fig. 1 *C*–*C*′′); at a later stage, expression is also found in the somatic follicular epithelium around the forming egg chambers (Fig. S3). Thus, *lili* is transcribed at least in the GSCs and the follicle cells.

To investigate *lili*'s function in the germ line, we analyzed homozygous mutant GSC clones induced in heterozygous females by the FLP/FRT method. Clonal GSCs and their progeny were identified by loss of the constitutive β-galactosidase marker *arm-lacZ* and GSC maintenance was assessed at days 3, 10, and 17 after clonal induction (ACI). Control GSCs (wt) were maintained over time, with 75–85% of germaria still containing a clonal GSC at 17 d ACI (level at 3 d ACI set as 100%) (Fig. 1 *D*, *E*, and *H* and Dataset S1). By contrast, at 10 and 17 d ACI, the frequency of *lili* mutant GSCs had dropped drastically (Fig. 1 *F*–*H* and Dataset S1). As expected, mutant stem cell loss was generally accompanied by replacement with a wt stem cell (Fig. 1*G*). To confirm that this phenotype was due to loss of Lili, we rescued the stem cell loss by driving expression of the wt protein in *lili*^{2} mutant GSCs (i.e., mutant clones in a *nos-Gal4*^{vp16} *UASp-lili*^{A} background) (Fig. 1*I* and Dataset S1).

Altogether, these data establish that *lili* is expressed and required in the female germ line, where it promotes GSC maintenance.

lili Mutant GSCs Are Lost Through Differentiation. Failure to self-renew is only one possible cause of stem cell loss, which can also result from cell death or, in mosaic germaria, from diminished fitness of mutant GSCs vis-à-vis WT ones (competition). Hence, we specifically investigated these possibilities in the case of *lili*.

We assessed apoptotic death by staining mosaic ovaries for activated Caspase-3 at 8 d ACI. As for wild-type clones (Fig. 2*A*), we did not detect any activated Caspase-3 staining in *lili*^{2} or *lili*^{3} mutant GSCs (n = 40 and n = 47, respectively) (Fig. 2*B*). Cell death was also not observed in WT or *lili* mutant egg chambers, but was clearly detected in stage 7–8 egg chambers when induced by poor nutrition (Fig. S4). In agreement with this finding, morphologically normal *lili* mutant cysts, egg chambers and eggs were regularly observed after clonal induction (Fig. 1 *F* and *G*). Altogether, these data indicate that *lili* is not required for GSC viability.

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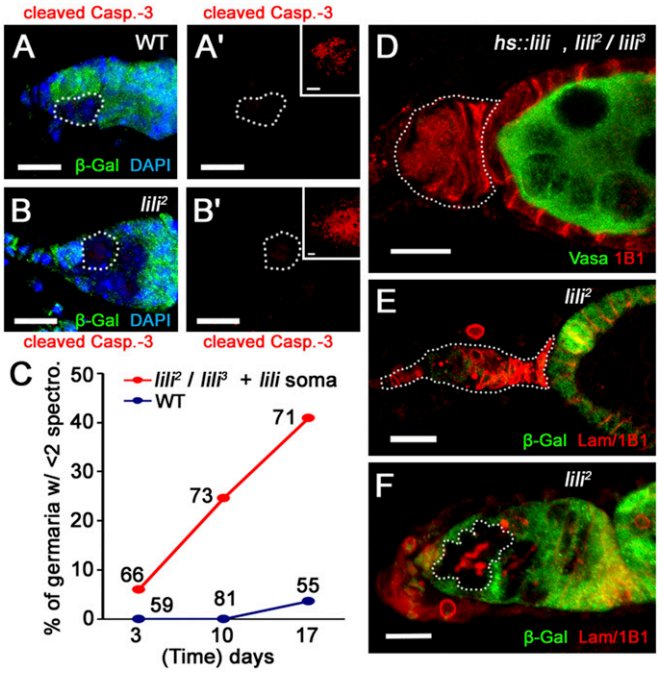

Fig. 2. Loss of *lili* mutant GSCs is not caused by cell death or cell competition. (*A* and *B*) Germaria containing either WT control (*A*) or *lili* mutant (*B*) clonal GSCs (β-gal negative; dashed lines) do not show any activated Caspase-3 staining (red) (*A′* and *B′*). Positive cell death control (anti-activated Caspase-3 Ab; red) stage 7–8 egg chambers are shown in *Insets* (Fig. S4). Clone tissue is marked by absence of β-gal (green). (*C*) GSCs are lost over time in *hs-Gal4/UASt-lili*^{A}*; lili*^{2}*/lili*^{3} females. (*D*) Example of a "GSC-depleted" germarium (dashed outline) stained for the germ line (Vasa; green) and membranes/spectrosomes/fusomes (1B1; red) from a 10-d-old *hs-Gal4/UASt-lili*^{A}*; lili*^{2}*/lili*^{3} female. (*E*) A similarly depleted germarium (dashed outline) attached to a *lili*^{2} mutant clonal egg chamber (β-gal present in the soma but not the germ line) stained for the spectrosome, fusome, and cell membranes marker combination Lamin C/1B1 (red). (*F*) Example of a GSC-depleted germarium showing developing *lili*^{2} clonal cysts (dashed outline; β-gal absent) at the site normally occupied by GSCs, staining is with anti-LamC/anti-1B1 combination (red; branched fusome is clearly visible in clonal cyst). (Scale bars: 10 μm.)

Loss of SCs in mosaic germaria can also be due to competition. This phenomenon occurs when metabolically less fit mutant cells compete for growth-promoting signals with neighboring WT cells; as a result, the mutant cells are eliminated and replaced by their WT competitors. In the germ line, this elimination occurs when less-fit mutant SCs are displaced from the niche by the healthier wt SCs, and are thereafter induced to differentiate (9, 10). A hallmark of this phenomenon is that in-niche retention of mutant SCs can be restored by absence of wt competitors (as shown for GSCs mutant for *dm*, the fly MYC homolog; ref. 10). To investigate this possibility, we turned to *lili*^{2}*/lili*^{3} transheterozygote females rescued by *hs-Gal4*–driven expression of *UASt-lili*^{A}, relying on the preferentially somatic expression from the UASt vector (11). In this background (somatically-rescued *lili*^{2}*/lili*^{3} females), the all-mutant germ line still showed progressive GSC loss despite the lack of WT SC (no competition) (Fig. 2 *C* and *D*). Results consistent with this interpretation were also obtained from our clonal analyses (scoring the rare all-mutant samples presumably due to multiple FLP/FRT events in one germarium). In wt controls, germaria with all-clonal germ line were still present and normal at day 17 (Dataset S2). In contrast, when inducing *lili* mutant GSCs, no normal germaria with all-mutant germ line were observed at the later time point (although present at day 3 ACI). We detected instead "depleted" germaria, containing either no germ line at all or only mutant differentiating cysts (Fig. 2 *E* and *F*), and "arrested" germaria, with mutant spectrosome-containing cells but no cysts, right next to developing egg chambers (Dataset S2). Thus, loss of *lili* mutant GSCs in mosaic germaria does not result simply from competition between mutant and WT stem cells for critical resources.

Collectively, these findings suggest that Lili is required in GSCs for the promotion of self-renewal over differentiation, rather than GSC survival or cell fitness.

BMP Pathway Gain of Function Requires lilipod. As mentioned in Introduction, BMP signaling plays a central role in balancing self-renewal versus differentiation, and the Dpp ligand signals to the GSC through the Tkv receptor to suppress expression of the potent differentiation factor Bam. In fact, either loss of BMP signaling or expression of exogenous Bam are sufficient to trigger GSC differentiation in the niche and, thus, SC loss (6, 12). Conversely, excessive BMP pathway activity or loss of *bam* delays differentiation and results in accumulation of stem cells outside the niche (GSCs hyperplasia) (6, 13). To investigate whether loss of *lili* mutant GSC might be due to a perturbation in BMP signaling, we investigated the relationship between *lili* and the pathway components *bam* and *tkv*.

To assess whether pathway activity was sensitive to *lili* gene dosage, we relied on a genetic background that is hyperactive for BMP signaling but sensitized to the dosage of pathway components. Germ-line expression of a constitutively active ligand-independent form of the Tkv receptor (*nos-Gal4*^{vp16} *UASp-tkv*^{CA}) induces a tumorous phenotype (all GSC-like cells no cysts) that can be partially suppressed by loss of one WT allele ($^{-/+}$) of pathway components (14, 15). Interestingly, in this test, *lili* mutant alleles acted as dominant suppressors of the tumorous phenotype by restoring the presence of dividing cysts (Fig. 3 *A* and *B*). In this *nos-Gal4*^{vp16} *UASp-tkv*^{CA} *lili*^{2}/+ background, expression of the differentiation factor Bam was observed in 56% of the ovarioles (27 of 48 germaria), compared with never in *nos-Gal4*^{vp16} *UASp-tkv*^{CA} ovarioles (0 of 48 germaria) (Fig. 3 *C* and *D*). Importantly, heterozygosity for *lili* does not by itself promote differentiation, i.e., it does not cause GSC loss (*lili*^{3}/+ contained 5.40 ± 0.20 spectrosomes, $n = 60$ germaria; wild-type contained 5.14 ± 0.14 spectrosomes, $n = 100$). Hence, the sensitivity of Tkv^{CA} to *lili* gene dosage most likely reflects a limiting effect of *lili* on signaling by the BMP receptor.

Next, we reasoned that if Lili promotes GSC maintenance through *dpp* signaling, then it should contribute to the repression of *bam*, and the differentiation of *lili* mutant stem cells should be entirely *bam*-dependent (16–18). To test this prediction, we analyzed double-mutant *lili bam* GSCs. Maintenance of single mutant (*lili*^{2} or *bam*$^{\Delta 86}$) and double mutant (*lili*^{2} *bam*$^{\Delta 86}$ or *lili*^{3} *bam*$^{\Delta 86}$) GSC clones was assessed on day 3, 10, and 17 ACI. As expected, *lili*^{2} mutant SCs were lost over time, whereas *bam*$^{\Delta 86}$ mutant stem cells failed to properly differentiate, causing the accumulation of spectrosome-containing cells throughout the germarium (Fig. 3 *E* and *F*). In the double mutants, loss of *bam* blocked the differentiation of *lili* mutant GSCs, resulting in the accumulation of undifferentiated, spectrosome-containing *lili bam*$^{\Delta 86}$ cells (Fig. 3 *G*–*I* and Dataset S3). No *lili bam*$^{\Delta 86}$ double mutant germ-line cysts were observed at any time point, demonstrating that *lili* mutant GSCs completely depend on *bam* for differentiation. Interestingly, *lili bam* GSCs persisted in the niche longer than WT SCs, similarly to single mutant *bam* cells (19), pointing to a possibly enhanced association with the soma.

These findings suggest that *lili* functions to promote self-renewal over differentiation through the BMP pathway.

pMad Is Decreased in lilipod Mutant GSCs. The genetic data described above were consistent with a model whereby Lili promoted signaling by Dpp. Hence, we decided to directly assess the activity of the *dpp* pathway in *lili* mutant GSCs.

Pathway activity can be monitored at the level of activated Mad by using the anti-pMad Ab, and at the level of *bam*-silencing using the *bamP-GFP* reporter. We therefore compared pMad and *bamP-GFP* expression in germaria containing, still in the niche, an unmarked clonal GSC (either WT control or *lili*^{2} mutant clone) next to a β-galactosidase–marked WT GSC (at 8 d ACI). As expected, pMad expression was robust and *bamP-GFP* was undetectable in all WT GSCs, clonal and nonclonal. By contrast, a large fraction of

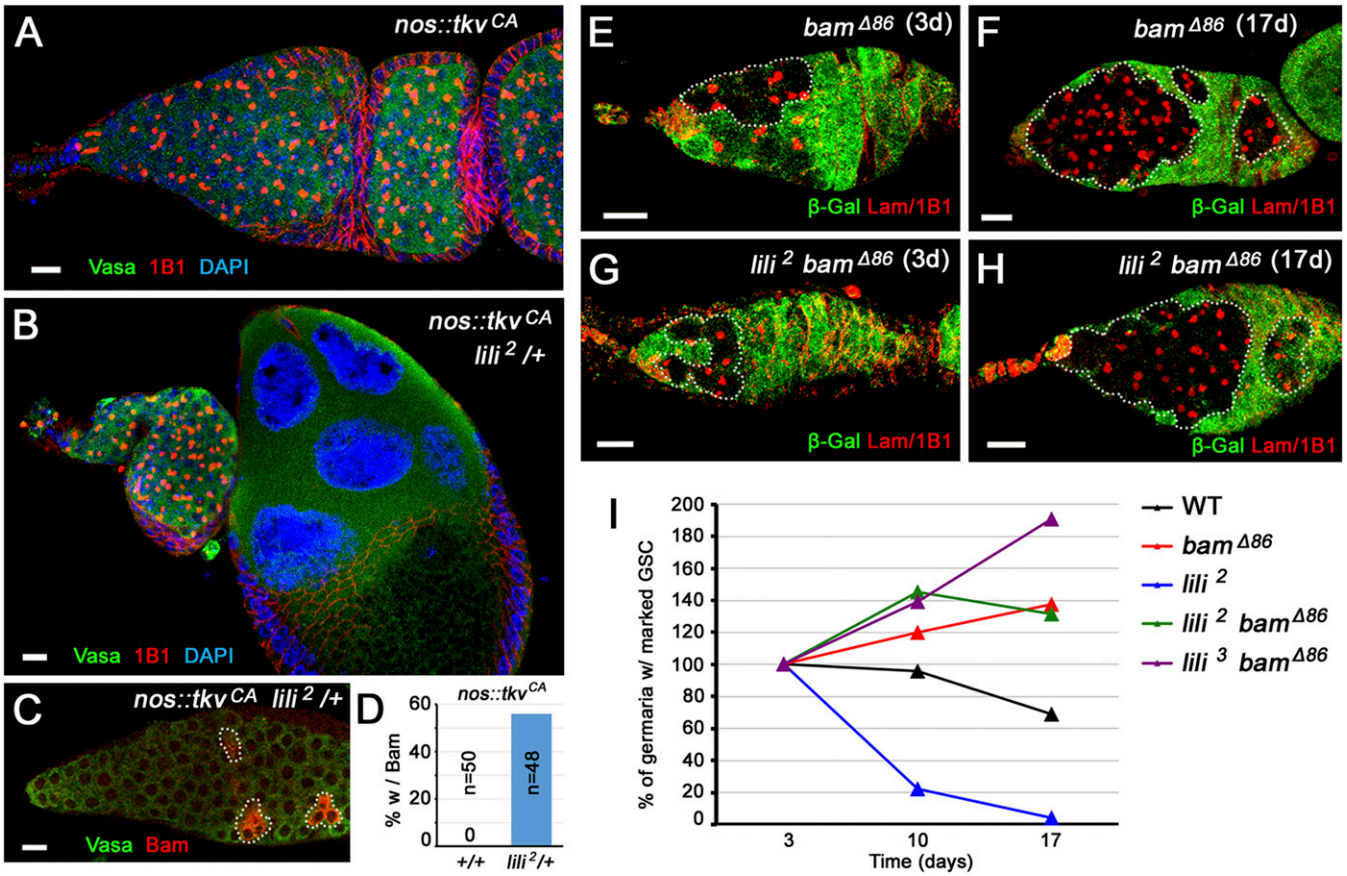

Fig. 3. *lili* dominantly suppresses hyperactivated BMP pathway. (*A* and *B*) Germaria from either a *nos-Gal4^{vp16} UASp-tkv ^{CA}* female (*A*) or a *nos-Gal4^{vp16} UASp-tkv ^{CA}* female heterozygous for lili (*lili ^{2}/+*) (*B*) stained with anti-Vasa and anti-1B1 (*A* and *B*). (*C* and *D*) In *nos-Gal4^{vp16} UASp-tkv ^{CA}* females heterozygous for lili (*lili^{2}/+*), induction of Bam expression (anti-Bam Ab; red) is detected in the tumorous germ line (anti-Vasa Ab; green): detection in germarium (*C*) and quantification (*D*). Dashed outlines mark germ-line cells expressing Bam. (*E–H*) Germaria containing homozygous *bam^{Δ86}* (*E* and *F*) or *lili^{2} bam^{Δ86}* (*G* and *H*) clonal GSCs at 3 and 17 d ACI stained with anti–β-gal (green) and anti-Lamin C/anti-1B1 (red). Dashed outlines mark clonal germ-line cells. (*I*) Percentage of germaria containing a homozygous clonal *lili bam^{Δ86}* double mutant GSC, *lili^{2}*, *or bam^{Δ86}* single mutant GSC, or control clonal GSC at 3, 10, and 17 d ACI. Increased accumulation of *bam^{Δ86}* GSC clones was reported by Jin et al. (19), due to increased competitiveness for the niche compared with wild-type GSCs; *lili bam^{Δ86}* double mutant GSCs show a similar effect. (Scale bars: 10 μm.)

lili^{2} mutant GSCs showed greatly reduced pMad levels compared with their neighboring WT stem cell (39%; 14 of 36 mutant-WT GSC pairs showed clearly lower pMad in the mutant SC; $P < 0.005$) (Fig. 4 *A* and *A′*). Conversely, a precocious, although still weak, up-regulation of *bamP-GFP* expression could be seen in many *lili* mutant GSCs (21%; 4 of 19 mutant-WT GSC pairs showed clearly higher GFP levels in the mutant SC; $P < 0.005$) (Fig. 4 *B* and *B′*). Considering the short experimental window between sufficient degradation of Lili protein after ACI and consequent stem cell loss to differentiation, these levels of pMad reduction and *bam* induction are significant.

To establish whether the decrease in pMad was a consequence of the precocious activation of *bam*, we then assessed pMad signaling in *lili^{2} bam^{Δ86}* double mutant GSCs. As reported (16, 20), pMad expression remained high in *bam^{Δ86}* mutant SCs in contact with cap cells (Fig. 4 *C* and *C′*). By contrast, pMad was reduced in nearly 60% of the *lilipod^{2} bam^{Δ86}* GSCs within the niche relative to their neighboring nonclonal GSC (reduced in 13 of 23 mutant-WT pairs; Fig. 4 *D* and *D′*). Hence, the disruption of BMP signaling in *lili* mutant GSCs is independent of *bam* activation, differentiation, or exit from the niche.

In conclusion, Lili is required to maintain normal levels of BMP signal transduction in the GSC and it does so independently of its ability to suppress differentiation.

Lilipod Overexpression Induces Supernumerary pMad^{+} bamP-GFP$^{−}$ GSCs. Lastly, to test whether Lili overexpression is not only required but also sufficient to promote self-renewal over differentiation, we expressed the protein ubiquitously (*hs-Gal4*) or in the germ line (*nos-Gal4^{VP16}*) and then assessed the number of spectrosome-containing cells (GSCs + CBs) per germarium. A significant increase was observed in both genetic backgrounds (Fig. 5 *A–B′*). A single heat-shock pulse of homozygous *hs-Gal4 UASp-lili^{A}* females was sufficient to induce a doubling of the average number of spectrosome-containing cells from 5.27 ± 0.03 ($n = 48$) in Gal4-only control to 10.38 ± 0.40 ($n = 58$) when *UASp-lili^{A}* was also present (Fig. 5 *A* and *A′*). In the case of *nos-Gal4^{vp16} UASp-lili^{A}* flies (single transgenes), the increase was more modest but still significant, from 5.06 ± 0.12 ($n = 121$) spectrosomes per germarium in *nos-Gal4^{vp16} UASp-lacZ* controls to 6.21 ± 0.15 ($n = 107$) in *nos-Gal4^{vp16} UASp-lili^{A}* ($P < 0.005$) (Fig. 5 *B* and *B′*). Importantly, this was accompanied by corresponding increases in pMad-positive (Fig. 5 *C* and *C′*) and *bam-P-GFP*–negative spectrosome-containing cells (Fig. 5 *C–D′*). This effect is similar to the GSC expansion induced by *nos-Gal4*–driven expression of the WT Tkv receptor (21).

Consistent with a model of GSC expansion through enhanced Dpp signaling, impairment of the Dpp pathway within Lili-overexpressing GSCs completely suppressed the supernumerary-GSCs phenotype. In fact, the coexpression of the inhibitory SMAD Dad with Lili (*nos-Gal4^{vp16} UASp-lili^{A} UASp-Dad*) or the induction, by single heat shock, of Bam in Lili-overexpressing cells (*hs-Bam* with *nos-Gal4^{vp16} UASp-lili^{A}*) resulted in GSCs loss through precocious differentiation (Fig. 5 *E–H*). Thus, the Lili-induced expansion of the GSC compartment appears to be Dpp-dependent.

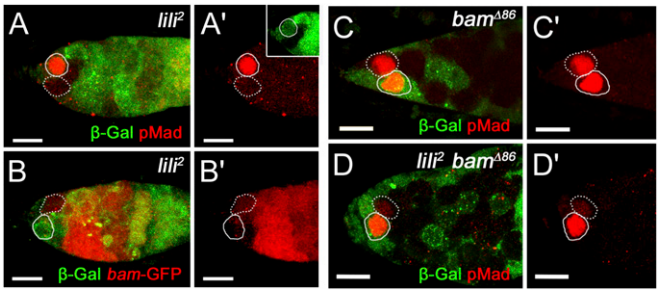

Fig. 4. *lili* is required for BMP signaling in the GSC. In all images, homozygous clonal GSCs are marked by a dashed outline, whereas neighboring nonclonal GSCs are marked by a solid outline. (*A*–*B*′) Germaria containing a *lili*^{2} mutant GSC (β-gal negative; green) next to a WT GSC (β-gal positive; green) stained for pMad (*A* and *A*′) or *bamP-GFP* (*B* and *B*′) expression (both shown in red). The *lili* mutant GSC have lower pMad and higher *bamP-GFP* compared with neighboring WT GSCs. (*C* and *C*′) Germarium containing a *bam*$^{\Delta 86}$ mutant GSC (β-gal negative; green) next to a WT GSC (β-gal positive; green) stained for anti-pMad (red). Robust pMad was present in all clonal GSCs observed ($n = 38$). (*D*) Germarium containing a clonal *lili*^{2} *bam*$^{\Delta 86}$ double mutant GSC (β-gal negative; not green) next to a WT GSC (β-gal positive; green) stained for pMad (red). Loss of pMad is observed even in the absence of differentiation. (Scale bars: 10 μm.)

Discussion

In this study, we show that the LIMR/LMBR1 type protein Lilipod functions to promote germ-line stem cell self-renewal in the *Drosophila* ovary. Lili is intrinsically required in GSCs to promote self-renewal over differentiation with loss or gain of Lili resulting in precocious or delayed differentiation, respectively. We show that Lili loss or gain affects the level of BMP pathway activity in the early germ-line lineage. Whereas precocious differentiation of *lili* mutant GSCs is entirely Bam-dependent, Lili's modulation of Dpp signaling in the GSC is independent of the differentiation program, acting on a step in the cascade between the activated BMP receptor and the pMad transducer.

The activity of the BMP pathway is tightly regulated in the ovarian stem cell niche to maintain homeostatic balance between self-renewal and differentiation. This regulation of Dpp signaling involves multiple positive and negative modulators (21–23) in both GSC and CB daughter cells. In the GSC, germ-line factors positively (Lis-1) and negatively (the I-SMAD Dad) regulate the transduction of the signal intracellularly for an optimal balance of differentiation and self-renewal. They achieve this goal by modulating the activation and/or stability of the transducer Mad/pMad and the receptor Tkv (21, 22). In the forming CB, reduced signaling, as the cell moves away from the source of Dpp (the niche), leads to the stabilization of factors (Fu kinase and Smurf E3 ligase) that promote the degradation of Tkv and Mad/pMad (22). This step is then followed by a Bam-dependent shutdown of Mad synthesis through the translational repressor Brat (23). Ultimately, these mechanisms work to prevent the precocious differentiation of the SCs or their tumorous expansion.

The marked reduction in pMad levels within *lili* mutant GSCs suggests that Lili either enhances the activity or protects the stability of signaling components. The finding that removal of Bam from *lili* mutant GSCs suppresses their precocious differentiation without restoring normal levels of pMad indicates that Lili's effect on the cascade occurs upstream of *bam*. Moreover, because the Tkv^{CA} receptor induces tumor formation even in the absence of functional Dpp ligand (24), the sensitivity of the Tkv^{CA} tumorous phenotype to *lili* gene dosage (Fig. 3*B*) places the activity of Lili downstream of the ligand–receptor interaction. We conclude, therefore, that *lilipod* modulates the intracellular transduction of the Dpp signal somewhere between the Tkv–Punt interaction (required even for Tkv^{CA}) and phosphorylated pMad levels. It remains to be seen whether Lilipod functions directly with receptor components and/or the transducer Mad (to enhance interactions, activity, or stability) or indirectly (through novel or already known regulators, such as Lis-1, Dad, or Fu/Smurf). However, loss- and gain-of-functions phenotypes for these factors are not readily compatible with the ones described here for *lili*; for instance, neither Lis-1 overexpression nor *dad* loss-of-function have been

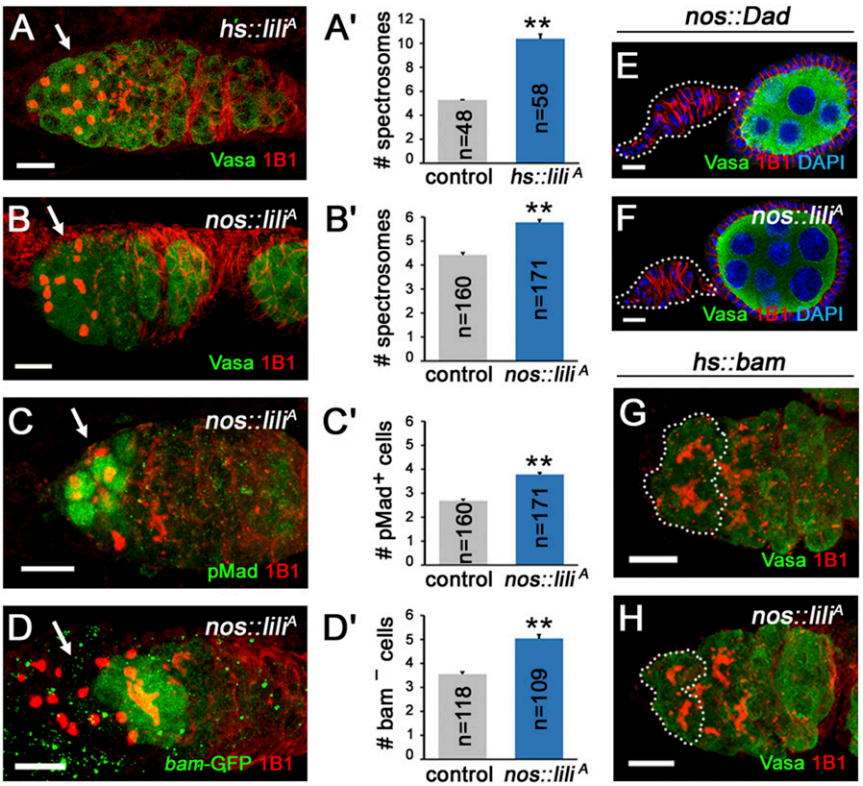

Fig. 5. Overexpression of Lilipod results in ectopic GSC-like cells and increased BMP signaling activity. (*A* and *B*) Induction of Lili expression in *hs-Gal4*^{vp16} *UASp-lili*^{A} females leads to an increase in GSC numbers. See example of germarium stained with 1B1 (red) and Vasa (green) to identify spectrosome-containing germ-line cells in *A*, quantification compared with *hs-Gal4*^{vp16} only control in *A*′. (*B* and *C*) Germaria from *nos-Gal4*^{vp16} *UASp-lili*^{A} females also show a reproducible but more modest expansion of the GSCs+CBs compartment shown as spectrosome-containing cells in *A* (1B1 in red and Vasa in green). That this expansion reflects an increase in pMad-positive, *bamP-GFP*–negative GSCs is shown in *C* (1B1 in red and pMad in green) and *D* (1B1 in red and *bamP-GFP* in green). All quantifications compared with *nos-Gal4*^{vp16} controls are shown to the right (*B*′–*D*′). (*E* and *F*) Germaria from *nos-Gal4*^{vp16} *UAS-Dad* (*E*) and *nos-Gal4*^{vp16} *UAS-Dad UASp-lili*^{A} (*F*) females both show complete loss of GSCs, dashed outlines mark depleted germaria, and notice attached egg chamber at right. (*G* and *H*) A HS-induced pulse of Bam expression either in *nos-Gal4*^{vp16} control (*C*) or *nos-Gal4*^{vp16} *2XUASp-lili*^{A} (*D*) germaria results in forced differentiation of the germ line as shown by 1B1-stained fusomes in Vasa-positive cells in the GSCs position. Phenotypes shown in *F* and *H* were as penetrant as in controls (*E* and *G*). (Scale bars: 10 μm.)

reported to induce GSC expansion and down-regulation of Fu or Smurf has a much stronger effect than Lili overexpression (6, 21, 22). Nonetheless, these possibilities remain to be explored through further studies of *lili* in vivo and in vitro.

Does *lili* function as a universal modulator of BMP signaling or as a factor specific to the germ line? Whereas a thorough investigation of this issue is beyond the scope of this work, *dpp–lili* interactions in the soma point to roles in other BMP-signaling events. In fact, *lili* alleles dominantly enhance hypomorphic *dpp* mutant phenotypes observed in wings (Fig. S5 *A–G*) and embryonic cuticles (Fig. S5*H*), such that the range of mutant defects observed is more severe in *dpp/dpp; lili/+* combinations than in *dpp/dpp* alone, and, conversely, introduction of one *dpp* mutant allele in a *lili* homozygous background has severe consequences for embryonic patterning. In addition, the lethality of *lili* alleles (delayed to the larval stage by a strong maternal contribution) can be rescued by ubiquitous low-level expression of exogenous Dpp (Fig. S5*I*). Although these findings do not exclude other non-Dpp-related functions for Lili, they confirm its significant role in regulating Dpp signaling in multiple developmental contexts.

Lastly, the high degree of sequence and structural similarity between LIMR and LMBR1 proteins from different species suggests conservation of function. The implication of *Drosophila* Lili in BMP signaling provides strong evidence for a signaling-related role for proteins of this kind. Given also the expression of a number of lipocalin-like factors in *Drosophila*, the fly offers an ideal system in which to dissect the function of Lili and investigate its possible regulation by a ligand.

Materials and Methods

Genetics. Flies were grown at 25 °C unless otherwise stated. $lili^{2}$; $lili^{3}$ mutant alleles were generated by imprecise excision from P-element shown in Fig. S2 and delete the ORF in exon 1. The $lili^{1}$ allele introduces a stop codon at position 69 and was isolated from chromosome l(3)96Bb^{2} (BL4526), which contains also a lethal hit in the *Vps22* locus. The following chromosomes were generated by recombination: FRT $lili^{1}$; FRT $lili^{2}$; FRT $lili^{3}$; FRT $lili^{2}$ $bam^{\Delta 86}$; *FRT* $lili^{3}$ $bam^{\Delta 86}$. Other stocks: *hs-Gal4* (BL1799), *Act5C-Gal4* (BL3954), *nos-Gal4*^{vp16} (BL4937), *UAS-dpp* (BL1486), $bam^{\Delta 86}$ (BL5427), *bamP-GFP* (25), *UASp-Dad* (15), *hs-bam* (BL24636), *UASp-tkv*^{CA} (16), *dpp*^{d5} (BL2071), *dpp*^{d6} (BL2062), and dpp^{hr56} (BL36528); a *dpp*^{hr56}; $lili^{3}$ recombinant stock was generated in the laboratory. GSC clones with marking were generated by FLP/FRT-mediated recombination by standard techniques (26) using *FRT-82B arm-lacZ* (BL7369). To induce *lili* mutant GSCs, 3- to 5-d-old adult females with a genotype of *hs-FLP/+; FRT*$_{82B}$ *armadillo-lacZ/FRT*$_{82B}$ *lili* ($lili^{1}$, $lili^{2}$, $lili^{3}$, or *FRT*$_{82B}$ WT control) were heat-shocked at 37 °C for 1 h, twice per day, for 2 d. Bam expression was induced by a single 1-h heat shock, and ovarioles were scored 24 h later; Dad expression was induced through *UAS-Dad* and scored at day 3 after eclosion. All scoring was blind. Transgenic lines in w^{1118} were generated in-house: *p(lili),* 10,023-bp genomic DNA in pCaSpeR4; *liliP-eGFP.nls,* reporter containing upstream DNA through start codon; for the *UASt-lili*^{A}*-Myc*, *UASp-lili*^{A}, and *UASp-eGFP-lili*^{A} expression constructs, cDNAs (isoform A) of identical sequence were obtained from BDGP (GH12663; GenBank AF132157) and by RT-PCR and cloned in UASt and UASp vectors.

Immunohistology. Primary antibodies: mouse anti-Hts (1B1, 1:50; DSHB), mouse anti-Lamin C (LC28.26, 1:25; DSHB), rat anti-DE-cadherin (DCAD2, 1:50; DSHB), mouse anti-β-galactosidase (1:1,250; Promega), rabbit anti-β-galactosidase (1:2,000; Cappel), rabbit anti-GFP (1:10,000; Invitrogen), rabbit anti-Vasa (1:10,000; gift from P. Lasko, McGill University, Montreal), mouse anti-Bam (1:400; DSHB concentrated), rabbit anti-Cleaved caspase-3 (1:100; Cell Signaling), and rabbit anti-human p423/425 Smad3 (1:150; abcam52903) (27, 28). Secondary antibodies: Anti-mouse, anti-rat, and anti-rabbit Cy2-, Cy3- or Cy5-conjugated (1:200; Jackson Immuno Research Laboratories). Images were obtained with a Leica DM5500Q confocal system and processed with Adobe Photoshop.

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