Lgd regulates the activity of the BMP/Dpp signalling pathway during Drosophila oogenesis

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ABSTRACT

The tumour suppressor gene lethal (2) giant discs (lgd) is involved in endosomal trafficking of transmembrane proteins in Drosophila. Loss of function results in the ligand-independent activation of the Notch pathway in all imaginal disc cells and follicle cells. Analysis of lgd loss of function has largely been restricted to imaginal discs and suggests that no other signalling pathway is affected. The devolution of Lgd to the Notch pathway was puzzling given that lgd loss of function also affects trafficking of components of other signalling pathways, such as the Dpp (a Drosophila BMP) pathway. Moreover, Lgd physically interacts with Shrub, a fundamental component of the ESCRT trafficking machinery, whose loss of function results in the activation of several signalling pathways. Here, we show that during oogenesis lgd loss of function causes ectopic activation of the Drosophila BMP signalling pathway. This activation occurs in somatic follicle cells as well as in germline cells. The activation in germline cells causes an extra round of division, producing egg chambers with 32 instead of 16 cells. Moreover, more germline stem cells were formed. The lgd mutant cells are defective in endosomal trafficking, causing an accumulation of the type I Dpp receptor Thickveins in maturing endosomes, which probably causes activation of the pathway. Taken together, these results show that lgd loss of function causes various effects among tissues and can lead to the activation of signalling pathways other than Notch. They further show that there is a role for the endosomal pathway during oogenesis.

KEY WORDS: Cc2d1a, Aki, Tape, Dpp, Lgd [L(2)gd1], Notch signalling, Endocytosis

INTRODUCTION

In recent years, it has been established that the tumour suppressor gene lethal (2) giant discs (lgd) [l(2)gd1 – FlyBase] has a function in the endosomal pathway and in the regulation of the activity of the Notch pathway in Drosophila melanogaster (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006; Schneider et al., 2013; Troost et al., 2012). Loss of its function leads to a defect in endosomal trafficking of transmembrane proteins, which accumulate in maturing endosomes (MEs). This defect causes the constitutive activation of the Notch pathway in a ligand-independent manner. lgd encodes a member of an evolutionary conserved family whose members contain four repeats of the novel DM14 domain followed by one C2 domain. A mutation in one of the two human orthologs, LGD2 (also named Aki, Freud-1, TAPE and Cc2d1a), causes mental retardation (Basel-Vanagaite et al., 2006), and this protein might be a tumour suppressor in liver cells (Zender et al., 2008).

In Drosophila the Notch receptor is activated by two ligands, Delta (DI) and Serrate (Ser) (reviewed by Bray, 2006). Their binding initiates the S2-cleavage of the extracellular domain of Notch (NECD). The cleaved NECD is then trans-endocytosed together with the ligand into the signal-sending cell. The cleavage is performed by a metalloproteinase encoded by kuzbanian (kuz) in Drosophila. The resulting intermediate Notch extracellular truncation (NEXT) fragment is cleaved by the γ-secretase complex, which contains Aph-1 and Presenilin (Pan). This S3-cleavage releases the Notch intracellular domain (NICD) into the cytosol from where it migrates into the nucleus to activate the target genes together with Suppressor of Hairless [Su(H)]. Previous work has shown that the constitutive activation of Notch observed in lgd mutant cells requires the activity of the γ-secretase complex (Jaekel and Klein, 2006).

Notch traffics constitutively through the endosomal pathway to be degraded in the lysosome (Jékely and Rørth, 2003). Trafficking is initiated by endocytosis, which results in the formation of early endosomal vesicles (reviewed by Huotari and Helenius, 2011). These vesicles fuse to form the early endosome (EE). Receptors destined for degradation remain in the ME, which eventually fuses with the lysosome where the cargo is degraded. During maturation of the endosome, receptors are concentrated in domains of its limiting membrane (LM) and are translocated into the lumen of the ME through pinching off this part as intraluminal vesicles (ILVs). The formation of ILVs achieves the separation of the NICDs of receptors from the cytosol. This step is important for the complete degradation of receptors as well as the termination of signalling through activated receptors.

The events in the endosomal pathway are controlled by small GTPases, chiefly Rab5 and Rab7 (Huotari and Helenius, 2011). Rab5 orchestrates events in the EEs, such as the initiation of ILV formation through recruitment of endosomal sorting complexes required for transport (ESCRT)-0, the first of five sequentially acting complexes, termed ESCRT-0–ESCRT-III and Vps4 (reviewed by Hurley, 2010). ESCRT-0 initiates the sequential recruitment of the complexes and concentrates ubiquitylated receptors in regions of ILV formation. ESCRT-0 consists of Hrs and Stam. The central component of the last acting ESCRT-III complex in Drosophila is encoded by shrub. It encodes the Drosophila ortholog of mammalian CHMP4 family proteins and yeast Snf7. Shrub is recruited to the LM of the MEs where it polymerises into filaments to perform the abscission of ILVs in concert with the Vps4 complex. As a result of the action of the ESCRT complexes, the ME contains an increasing number of ILVs and is called a multi-vesicular body (MVB). A recent paper provides evidence that suggests that LGD2 also interacts with CHMP4 during budding of HIV (Martinelli et al., 2012; Usami et al., 2012). Although the loss of the function of the ESCRT-I, -II and -III results...
in activation of the Notch and other signalling pathways in *Drosophila*, that of ESCRT-0 does not (Jaekel and Klein, 2006). An explanation for this puzzling fact is that ESCRT-0 performs an additional function that is required for activation in ESCRT-I, -II and -III mutants. This might consist of clustering of cargo at sites of ILV formation (Tognon et al., 2014). The additional function of ESCRT-0 is also required for the activation of the Notch pathway in *lgd* mutant cells because concomitant loss of ESCRT-0 and Lgd activity suppresses Notch activation (Gallagher and Knoblich, 2006; Jaekel and Klein, 2006).

We have recently shown that Lgd physically interacts with the ESCRT-III core component Shrub (Troost et al., 2012). This interaction is important for the full activity of Shrub in *in vivo*. Genetic experiments have revealed that the activation of Notch in *lgd* mutant cells requires the Rab7-mediated fusion of the ME with the lysosome (Schneider et al., 2013). Furthermore, every other manipulation that prevents fusion of the ME with the lysosome, such as loss of Rab7 function, suppresses activation of Notch in *lgd* mutant cells (Schneider et al., 2013). This requirement for fusion explains a paradox in the relationship between Lgd and Shrub: the activation of Notch in *lgd* mutant discs is suppressed if the activity of *shrub* is reduced to 50%, although their individual loss results in Notch activation (Troost et al., 2012). The paradox could be explained by the observation that the MEs of these cells lose association with Rab7 and fail to fuse with the lysosome (Schneider et al., 2013). Thus, Notch pathway activation fails because the endosomes are more dysfunctional than in *lgd* mutant cells.

In *Drosophila*, loss of function of ESCRT-I, -II and -III in imaginal discs results in the ectopic activation of the Notch pathway. In addition, the activity of the *Drosophila* BMP pathway, called the Decapentaplegic (Dpp) pathway, is enhanced (Thompson et al., 2005; Vaccari and Bilder, 2005). Moreover, loss of ESCRT function causes loss of epithelial integrity (Thompson et al., 2005; Vaccari and Bilder, 2005). However, our previous analysis in imaginal discs indicates that neither the activity of other major signalling pathways, such as the Dpp pathway, nor the epithelial integrity is affected by the loss of *lgd* function (Schneider et al., 2013). Hence, the phenotype of ESCRT mutants is more severe than that of loss-of-function *lgd* mutants despite their intimate relationship.

The analysis of signalling pathways in *lgd* mutants described above was restricted to imaginal discs and indicated that only the Notch pathway is affected. It is not known whether these pathways are affected in other mutant tissues. The development of the oocyte of *Drosophila* is an excellent system to study cellular processes, such as cell signalling and, thus, to answer this question (reviewed by Bastock and St Johnston, 2008). During oogenesis the progenies of a germline stem cell (GSC) develops through distinct and recognisable stages into a mature egg in the ovariole. The ovariole is subdivided into an anterior gerarium, which contains two kinds of stem cells and a posterior part that consists of a string of increasingly older egg chambers (ECs) with 16 germline cells (GCs) surrounded by a somatic follicle epithelium. The ECs bud off the gerarium and mature upon their posterior migration. The GSCs are located at the anterior tip of the gerarium and are surrounded by somatic cap cells, which form the niche required for their survival. The niche can accommodate 2-3 GSCs. These divide asymmetrically to give rise to another GSC and a cytoblast that begins to differentiate. The cytoblast undergoes four rounds of mitotic divisions to give rise to a cyst with 16 GCs. The cyst is encapsulated by somatic follicle cells (FCs) to generate the EC. The FCs are generated by two somatic stem cells located at the exit of the gerarium. One GC in each EC will differentiate into the oocyte, whereas the other 15 differentiate into polyploid nurse cells.

Several signals are required to maintain the GSC population. These are emitted at the tip of gerarium by cap cells (Harris and Ashe, 2011). The most important one is Dpp. Although anterior cap cells are probably responsible for the majority of the Dpp signal, the extent of its expression domain remains unclear (Harris and Ashe, 2011). Dpp activates the co-receptor Thickveins (Tkv; type 1) and type 2 receptors on GSC (Xie and Spradling, 2000). The result of Dpp signalling is the suppression of the expression of *bag of marbles* (*bam*) in the GSC. When a GSC divides, the daughter attached to the cap cell receives the Dpp signal, which suppresses *bam* expression and maintains the GSC fate. The non-contacting daughter cell does not receive sufficient signal and produces Bam, which causes its differentiation as a cystoblast. Thus, Bam silencing is the hallmark of asymmetry in the female germline of *Drosophila*.

The short range of the Dpp signal is achieved through degradation of the activated Tkv receptor in the cytoblast by a specific mechanism that involves the kinase Fused (Fu) and the E3 ligase Smurf (Xia et al., 2010). If the mechanism is disturbed, for example, by inactivation of *fu*, the number of GSCs increases and an extra round of division of the cyst cells occurs, frequently giving rise to ECs with 32 instead of 16 GCs (Narbonne-Reveau et al., 2006).

One well-characterised event during oogenesis that is mediated through the Notch signalling pathway is the switch from the mitotic cycle to the endocycle in FCs of ECs, which occurs at between stage 6 and 7 (Bastock and St Johnston, 2008). This switch is triggered by a DI signal from the GCs and initiates the differentiation of FCs through activation of Hindsight (Hnt) expression. We have shown that the loss of function of *lgd* in ECs results in precocious activation of the Notch pathway and expression of Hnt (Schneider et al., 2013). As in wing discs, the activation was independent of Kuz, and depended on Hrs and fusion of the ME with the lysosome (Schneider et al., 2013). Hence, the activation is caused by the same mechanism as in the wing disc.

The consequence of loss of *lgd* function in the germline was investigated previously using the allele *lgd*Δ (Szabad et al., 1991). The authors observed ECs with more than 40 GCs, a feature characteristic of tumour suppressor mutants. However, the analysis was hampered by the uncertain nature of *lgd*Δ and the lack of appropriate markers.

Here, we further investigated the function of *lgd* during oogenesis. We found that its loss causes activation of the Dpp pathway in addition to the Notch pathway in FCs. In the germline, loss of function of *lgd* causes an extra mitotic division that produces ECs with 32 GCs. In addition, it causes formation of more GSCs. Both phenotypes rely on ectopic activation of the Dpp. Our results indicate that these phenotypes are caused by a failure to degrade Tkv. Moreover, we found that the loss of function of *shrub* results in a similar phenotype.

**RESULTS**

In order to test whether other signalling pathways besides the Notch pathway are activated as a consequence of loss of *lgd* function during oogenesis, we monitored the activity of the Dpp pathway and compared it with that of the Notch pathway. In the following, we first describe the effects of loss of *lgd* function in FCs and then turn to the germline.

**The Dpp pathway is activated in *lgd* mutant follicle cells**

*dad-lacZ* has been extensively used to detect the activity of the Dpp pathway. During normal development expression of *dad-lacZ* is...
restricted to the GSCs and initiated in FCs only from stage 9 onwards (Kai and Spradling, 2003) (Fig. 1C,D, arrow). In order to monitor the activity of the pathway in lgd mutant FCs, we induced lgd mutant clones and analysed the expression of dad-lacZ. We found that dad-lacZ is ectopically activated in all lgd FCs (Fig. 1E-H,M-P). Activation had already occurred by the time FCs of ECs had budded off the germarium (arrowhead in Fig. 1E-H). The activation is strictly cell-autonomous. We confirmed this finding using a dad-promoter-GFP construct (data not shown).

Given that loss of lgd results in activation of the Notch pathway, we tested whether dad-lacZ is a tissue-specific target of it. We therefore inactivated the pathway in lgd cells through inactivation of the γ-secretase component Aph-1 (Fig. 2A-H). Loss of aph-1 function suppresses ectopic Notch signalling in lgd FCs (Schneider et al., 2013). By contrast, we found that dad-lacZ was still expressed in lgd aph-1 clones, indicating that its expression in lgd FCs is independent of the activity of the Notch pathway (Fig. 2E-H, arrow).

We next inactivated the Dpp pathway in lgd FCs. As expected, no dad-lacZ expression was observed upon loss of tkv function alone (Fig. 2I-L, arrow). However, the ectopic expression of dad-lacZ was abolished in lgd tkv clones (Fig. 2M-P, arrow). Thus, ectopic activation of the Dpp pathway causes the observed ectopic expression of dad-lacZ in lgd cells and requires its receptor to do so. In contrast to dad-lacZ, Hnt was still precociously expressed in the lgd tkv FCs (Fig. 2Q-S, arrow). Given that Hnt is sufficient to drive the FCs into the endocycle, it is likely that the precocious switch to the endocycle observed in lgd mutants is unaffected by loss of Dpp signalling (Sun and Deng, 2007).

We have previously reported that the Dpp pathway is not ectopically activated in lgd mutant wing imaginal discs (Schneider et al., 2013). This conclusion was based on the analysis of other target genes. Here, we found that the expression of dad-lacZ was also unaffected by loss of lgd function in the wing disc (supplementary material Fig. S1A-D). Hence, activation of Dpp signalling is a tissue-specific response to loss of lgd function.

**Activation of the Dpp pathway occurs in a different manner to that of the Notch pathway in lgd FCs**

We were curious to test whether the activation of the Dpp pathway in lgd FCs depends on the function of hrs and occurs in the lysosome, as it is the case for the activation of the Notch pathway. However, expression of dad-lacZ was ectopically activated in lgd hrs FCs, indicating that the activation of the Dpp pathway is independent of the function of hrs (Fig. 3A-H, arrows). Dmon1 is required to recruit Rab7 to the endosome (Yousefian et al., 2013). The recruitment is necessary for the fusion of the ME with the lysosome. Hence, the MEs fail to fuse with the lysosome in Dmon1 cells. In agreement with our previous findings, the ectopic activation of Notch signalling, measured with activity reporter Gbe+Su(H), was abolished in lgd Dmon1 FCs and wing imaginal disc cells (Fig. 3L-P; supplementary material Fig. S1E,F). In contrast, dad-lacZ was expressed in lgd Dmon1 FCs, indicating that the ectopic activation of the Dpp pathway does not require ME-lysosome fusion (Fig. 3I-O). Taken together, these results suggest that the activation of the Dpp pathway in FCs occurs in a different manner to that of the Notch pathway.

Note, that weak ectopic activation of dad-lacZ can also be observed in the Dmon1+/ lgd/ double heterozygous cells (Fig. 3L-K, red arrow). Moreover, the activation of the reporter is stronger in lgd Dmon1 cells than in lgd cells (Fig. 3I-O, compare with Fig. 1E-H). Given that the loss of Dmon1 function does not result in activation of dad-lacZ expression, this finding indicates that the loss of Dmon1 function enhances the ectopic activation of the Dpp pathway caused by loss of lgd function (Fig. 3I-P).

**Loss of lgd function in GCs causes a defect in protein degradation**

Next, we investigated the consequences of loss of function of lgd in the female germline using clonal analysis. We found that lgd GCs contained huge endosomes, which were positive for the endosomal markers Rab5 and Rab7, identifying them as MEs.

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**Fig. 1. Loss of lgd function results in ectopic activation of expression of dad-lacZ in FCs.** (A-D) Overview of expression of dad-lacZ and Bam-GFP in a wild-type (wt) ovariole. Expression of dad-lacZ occurs in the two GSCs in the germarium (arrow). Bam-GFP is expressed in cystoblasts and upregulated during the mitotic cyst stages in the gerarium (arrowhead). (E-H) An ovariole where most of the FCs are mutant for lgd, indicated by the loss of RFP. These FCs activate expression of dad-lacZ (arrows). The red arrow highlights an older stage EC (stage 8/9) with lgd mutant FC; the expression of dad-lacZ is still maintained. The arrowhead points to mutant dad-lacZ-positive FCs at the exit of the gerarium. (I-L) Magnification of egg chambers of the wild-type ovariole shown in A-D. The focus is on the FCs, which do not express dad-lacZ. (M-P) Magnification of egg chambers of the ovariole with lgd mutant clones shown in E-H. The mutant FCs (labelled by loss of RFP) express dad-lacZ (arrows).
The enlargement of MEs was also previously observed in lgd mutant FCs and indicates a defect in endosome maturation (Schneider et al., 2013). Using the Gbe+ Su(H) reporter, we fail to observe activation of the Notch pathway in lgd GCs (data not shown). Thus, we were surprised to observe the NECD in mutant MEs (Fig. 4A-J). The NECD could stem from the GCs, which weakly express Notch. In this case, one would also expect to detect the NICD. Alternatively, the NECD in MEs could be the consequence of transendocytosis, which occurs during Dl signalling from GCs to FCs (Parks et al., 2000). In this case, NICD should be absent from GCs. To investigate the origin of NECD, we used a gene trap in Notch, which encodes a functional variant with YFP inserted in the NECD (YFP-Notch) (Rees et al., 2011). We used YFP-Notch in combination with an antibody directed against NICD to detect both parts of the receptor. In lgd GCs we detected the YFP-NECD, but not the NICD (Fig. 4N-Q). Thus, the GCs appear to take up NECD from the surrounding Notch-expressing FCs. In agreement with this conclusion, we found that Dl colocalises with NECD in the MEs of lgd GCs (supplementary material Fig. S2A-J). Moreover, we found that Dl lgd GCs failed to incorporate NECD, whereas lgd GCs in other ECs of the same ovariole did so (supplementary material Fig. S2K-N). This result confirms that the incorporation of NECD is a consequence of signalling from GCs to FCs. Given that accumulation of NECD is hardly seen in wild-type GCs, it is likely that the lgd GCs are defective in degradation of Dl-NECD and other transmembrane proteins. The defect reveals the trans-endocytosis event during Notch signalling in an unprecedented clarity.

(Fig. 4A-J, arrowheads). The enlargement of MEs was also previously observed in lgd mutant FCs and indicates a defect in endosome maturation (Schneider et al., 2013). Using the Gbe+ Su(H) reporter, we fail to observe activation of the Notch pathway in lgd GCs (data not shown). Thus, we were surprised to observe the NECD in mutant MEs (Fig. 4A-J). The NECD could stem from the GCs, which weakly express Notch. In this case, one would also expect to detect the NICD. Alternatively, the NECD in MEs could be the consequence of transendocytosis, which occurs during Dl signalling from GCs to FCs (Parks et al., 2000). In this case, NICD should be absent from GCs. To investigate the origin of NECD, we used a gene trap in Notch, which encodes a functional variant with YFP inserted in the NECD (YFP-Notch) (Rees et al., 2011). We used YFP-Notch in combination with an antibody directed against NICD to detect both parts of the receptor. In lgd GCs we detected the YFP-NECD, but not the NICD (Fig. 4N-Q). Thus, the GCs appear to take up NECD from the surrounding Notch-expressing FCs. In agreement with this conclusion, we found that Dl colocalises with NECD in the MEs of lgd GCs (supplementary material Fig. S2A-J). Moreover, we found that Dl lgd GCs failed to incorporate NECD, whereas lgd GCs in other ECs of the same ovariole did so (supplementary material Fig. S2K-N). This result confirms that the incorporation of NECD is a consequence of signalling from GCs to FCs. Given that accumulation of NECD is hardly seen in wild-type GCs, it is likely that the lgd GCs are defective in degradation of Dl-NECD and other transmembrane proteins. The defect reveals the trans-endocytosis event during Notch signalling in an unprecedented clarity.

**Loss of lgd function in the germline results in formation of additional GCs**

In order to confirm that loss of function of lgd results in the formation of supernumerary GCs, we analysed lgd-null mutant germline clones. lgd is a null allele that fails to produce detectable levels of proteins (Childress et al., 2006; Jaekel and Klein, 2006). In agreement with the report of Szabad et al. (1991), we found that a fraction of ECs with an lgd mutant germline contained supernumerary GCs (Fig. 5A-L). Quantification of this phenotype revealed that it occurs in 15% of the analysed ECs (Fig. 6; n=500). These ECs contained exactly twice as much GCs as normal ones (32 cells; see supplementary material Movie 1). The nuclei of these GCs are smaller than that of normal GCs, indicating a lower degree of endoreplication (compare Fig. 5H with K, arrowhead). We observed the same phenotype using the independently induced null allele lgd (Fig. 6). Moreover, the phenotype was rescued by the presence of two copies of an lgd-RFP rescue construct (Troost et al., 2012) (Fig. 6). Thus, the 32 GCs phenotype is caused by the loss of lgd function.

ECs with 32 GCs could result from fusion of two normal ECs or from an additional round of division of the GCs. The two possibilities can be discriminated by determining the number of oocytes: if fusion occurs, two oocytes would be present in the EC, whereas one would be present if an extra round of division occurs. Orb is a specific oocyte marker. Only one Orb-positive GC was present in ECs with 32 GCs (Fig. 5M-O). Moreover, YFP-Tkv accumulates in the oocyte and can be therefore used as a marker (see below). In ECs with 32 cells, only one cell accumulated YFP-Tkv.
The oocyte is the only cell in the egg chamber that maintains its diploid state. Its nucleus is therefore smaller than that of the polyploid nurse cells. Only one small nucleus was present at the posterior end of lgd ECs with 32 FCs (Fig. 5K, arrow). Thus, it appears that the 32 GC phenotype is caused by an additional round of division.

We tested whether we could detect an additional round of division in lgd mutant germaria using the mitotic marker Cyclin A (CycA, Fig. 5S-X). CycA is expressed in early, but not older posterior cysts (Fig. 5V-X, arrowhead). We observed a fraction of lgd germaria (14%, n=21) where all cysts express CycA (Fig. 5V-X, arrowhead). The frequency is similar to that of EC with 32 cells (Fig. 6). This finding further supports our notion that a fraction of lgd cysts undergo an extra round of mitotic division.

**Ectopic activation of the Dpp pathway causes the formation of ECs containing 32 GCs**

Next, we tested whether the ectopic activation of the Dpp pathway is required for the formation of supernumerary GCs upon loss of lgd function. Therefore, we monitored lgd tkv germline clones and failed to detect ECs with 32 GCs in this situation (Fig. 6). Thus, ectopic Dpp signalling is required for the extra round of division of lgd GCs. Ectopic activation of dad-lacZ in lgd GCs was observed irrespective of whether the ECs contained 16 or 32 cells (Fig. 7B, red and yellow arrows). Hence, activation of the Dpp pathway is required, but is not sufficient, for the extra round of cell division of mutant germlines. Alternatively, the levels of Dpp signalling might fluctuate around threshold levels required for extra division.

The main function of Dpp signalling in the germarium is to suppress expression of Bam in GSCs (Bastock and St Johnston, 2008). Consequently, expression of Bam is initiated in cystoblasts and strongly upregulated during mitotic cyst stages in the germarium (Fig. 7F-H, wt). We found that expression of Bam-GFP was altered in many lgd mutant germaria. In some cases the Bam-GFP expression was suppressed, whereas in other cases no obvious changes were observed (Fig. 7F-L). Nevertheless, expression of high Bam and dad-lacZ were mutually exclusive. Moreover, in germaria with high Bam-GFP expression, dad-lacZ reappeared in older lgd cysts with no expression of Bam (yellow arrow in Fig. 7I-L). These findings reconfirm that Dpp is activated and suggest that its activity fluctuates around the threshold level required to suppress the expression of Bam.

During this analysis, we observed more dad-lacZ-positive cells at the tip of the germarium where the GSCs are located (Fig. 7J,L,
These cells were Bam negative, indicating that they are GSCs. Counting revealed that lgd germaria contained one additional GSC (Fig. 8), suggesting an increase of the range of the Dpp signal.

The Tkv receptor accumulates in MEs of lgd cells

The above data indicate that ectopic activation of the Dpp pathway in lgd mutant GCs is dependent on Tkv and show that the cells are defective in degradation of transmembrane proteins. Thus, we asked whether the degradation of Tkv is defective in lgd mutant GCs. In order to monitor the expression of Tkv, we used a gene trap where YFP is inserted in the N-terminus of Tkv (YFP-Tkv) (Kyoto Stock Center). This variant is fully functional. Given that expression of Tkv during oogenesis has not been described, we provide a detailed description in supplementary material Fig. S3. In short, Tkv is expressed in all cells of the germarium at different levels during normal oogenesis. The loss of lgd function results in a strong accumulation of YFP-Tkv in the NECD-positive enlarged vesicles of GCs (Fig. 9A-H, arrowheads in F-H). The majority of these vesicles were Rab7 positive, identifying them as MEs (Fig. 9I-L). We found a similar accumulation in Notch-positive MEs of lgd mutant FCs (Fig. 9M-P, arrow). Taken together, the results suggest that the degradation defect also causes the accumulation of Tkv in mutant MEs.

To find further evidence that the degradation defect causes ectopic activation of the Dpp pathway, we prevented degradation in the lysosome completely. Loss of Dmon1 function prevents fusion of the ME with the lysosome (Yousefian et al., 2013). Hence, the lifetime of MEs is dramatically increased and degradation of cargo prevented. We found that loss of Dmon1 function in the germline fails to activate the Dpp pathway (supplementary material Fig. S4A-D). However, concomitant loss of Dmon1 and lgd function resulted in enhanced activation of the Dpp pathway, which manifests itself through expression of dad-lacZ in most GCs of the germarium (Fig. 9Q-T). Moreover, we observed that all GCs in the germaria contained a spectrum, which is a hallmark of GSCs (Fig. 9Q-T, arrowheads in S,T). This tumorous germarium phenotype is also observed upon ectopic activation of the Dpp pathway (Xie and Spradling, 2000). We also observed weak dad-lacZ expression in double heterozygous Dmon1 and lgd mutant FCs, indicating that further delaying the degradation of Tkv in lgd GCs increases the activation of the Dpp pathway (arrow in Fig. 9Q-T). Given that loss of Dmon1 function alone does not activate the pathway, the lgd mutant cells must have an additional defect in trafficking of Tkv that is required for the activation of the pathway besides delaying degradation.

Loss of function shrub causes activation of the Dpp pathway

Lgd is required for the full function of Shrub (Troost et al., 2012). Moreover, loss of shrub function causes also ectopic activation of the Notch pathway in FCs (Vaccari et al., 2009). Therefore, we were curious whether the loss of shrub function causes the activation of the Dpp pathway during oogenesis. We found that dad-lacZ was also activated ectopically and cell-autonomously in shrub FC clones, suggesting that the Dpp pathway is activated (Fig. 10A,B). shrub germline clones survived poorly and could not be analysed properly. However, we found that the reduction of shrub function by 50%, achieved through heterozygosity of a null allele (shrub<sup>y<sup>+/+</sup></sup>), caused ectopic activation of dad-lacZ (Fig. 10C,D). Furthermore, we observed that 30% of the ECs contained 32 GCs (Fig. 6). Hence,
the reduction of shrub activity in GCs causes defects similar to that of loss of lgd function.

We further found that the shrub/+ germaria contained more dad-lacZ GSCs than the wild-type, indicating that the range of the Dpp signal had increased (4.9 cells versus 2.6 cells; Fig. 8 and Fig. 10C-F, arrowhead). Likewise, expression of Bam-GFP varied in shrub/+ germaria. We found clearly abnormal expression of Bam-GFP in some germaria, where some of the cells in the Bam-GFP domain still expressed dad-lacZ but not Bam-GFP (Fig. 10E,F, arrows). In contrast the expression was close to normal in other germaria (Fig. 10C,D).

Despite the similarity in phenotype of lgd and shrub mutants, the fraction of egg chambers with 32 GCs decreased from 30% to 7% in shrub lgd double heterozygous flies (shrub lgd/+; Fig. 6). This suggests that the two genes have an antagonistic relationship. However, the double heterozygous germaria exhibited abnormal Bam-GFP expression and contained as many additional dad-lacZ-positive GSCs as described for shrub heterozygous flies (Fig. 10G,H, arrows and arrowheads, Fig. 8).

### Functional interactions with Fu during germline development

The phenotype of lgd in the germline resembles that of fu mutants (Narbonne-Reveau et al., 2006). Therefore, we tested whether lgd and shrub have a functional relationship with fu. Indeed, we found that this is the case: loss of one copy of fu increased the number of GSCs by two (4.5), whereas loss of lgd function results in an increase by one (3.6; Fig. 8). However, the loss of one copy of fu in an lgd mutant germline increased the number of GSCs to 7 (Fig. 8). This suggests a further increase in the effective range of the Dpp pathway. Surprisingly, we detected a decrease in the number of ECs with 32 GCs in this genotype instead of the expected increase (Fig. 6). Nevertheless, both results indicate an existing functional relationship between both loci that might be more complicated than anticipated.

Similarly, loss of one copy of fu also enhanced the shrub heterozygous phenotype in the germline: the number of GSCs increased from 4.9 (shrub/+ to 6.7 (fu/+; shrub/+; Fig. 8). Moreover, the percentage of ECs containing 32 GCs increased from 29% (shrub/+) to 52% in fu/+; shrub/+ animals (Fig. 8). Note, that the loss of one copy of fu alone failed to produce egg chambers with 32 GCs. These findings reveal a strong synergistic functional relationship between shrub and fu.

### DISCUSSION

The previously conducted analysis of the function of lgd was largely restricted to imaginal discs. It suggested that loss of lgd function specifically activates the Notch pathway. Moreover, we showed that the loss of lgd function in FCs results in activation of the Notch pathway in the same manner as in imaginal disc cells. The deviation of Lgd to the Notch pathway was puzzling, given that it also controls trafficking of components of several signalling pathways, such as Tkv (Jaekel and Klein, 2006). Lgd functionally interacts with Shrub, whose loss of function affects several signalling pathways in imaginal disc cells. Thus, it would be conceivable that these pathways are also activated in lgd mutant cells. Here, we show that the Dpp pathway is ectopically activated upon loss of function of lgd in the ovaries. This activation occurs in FCs as well as GCS. Only the Dpp, and not the Notch, pathway is activated in lgd mutant GCs. Our observation is different from what has been reported for mutants of the ESCRT-II component Vps25, where Dpp signalling was enhanced, but the pathway not ectopically activated (Thompson et al., 2005).

We failed to detect any phenotype that could be attributed to the observed ectopic activation of the Dpp pathway in lgd mutant FCs, but found that its ectopic activation in the mutant germline causes an extra round of cell division. Moreover, more GSCs were observed and
expression of Bam-GFP was suppressed in a fraction of germaria. These findings suggest that the range of the Dpp signal is extended and, thus, maintains the GSC fate in more distantly located cells.

It has been recently shown that the Dpp-activated form of the Tkv receptor is specifically degraded in cyst cells to suppress the ectopic activation of the pathway (Xia et al., 2010). This degradation through the endosomal pathway is mediated by a complex consisting of the E3 ligase Smurf and Fu, and restricts the activity of the pathway to GSCs in the cap cell niche. The loss of fu function causes a loss of degradation of the activated receptor in progenies of the GSCs and, thus, increases the range of the pathway in GCs. Hence, ectopic activation occurs cell autonomously, but is induced by the Dpp ligand. The ectopic activity induced by loss of fu function causes a variety of phenotypes, including egg chambers that contain 32 GCs, as we have observed upon loss of lgd function here (Narbonne-Reveau et al., 2006). We have previously shown that loss of function of lgd results in a defect in the degradation of Tkv and other cargo proteins, such as Notch and DI, in FCs and GCs (Jaekel and Klein, 2006). We also found that the range of the Dpp pathway is increased in the absence of lgd function. It is therefore likely that the activation of the pathway is caused by a general failure of degradation that also affects the degradation of the activated form of Tkv. In further support of this notion, we discovered genetic interactions between fu, lgd and shrub.

Fig. 6. Frequency of egg chambers with 32 GCs in various genotypes.

Fig. 7. Loss of lgd function in GCs results in activation of Dpp signalling. (A,B) An ovariole with an lgd germline (RFP negative). Expression of dad-lacZ is activated in GCs up to stage 6 (arrowhead). The white arrow highlights an egg chamber that emerges from the germarium and expresses dad-lacZ. The red arrow highlights a stage 2 EC where the dad-lacZ expression peaks. This EC contains 32 GCs. The yellow arrow highlights an EC with 16 GCs. (C,E) A stage 2 EC with a lgd tkv germline (arrow). (F-H) A wild-type (wt) germarium next to one with an lgd germline (RFP negative). Expression of Bam-GFP is upregulated in the mitotic cyst stages in the wild type, whereas it is strongly reduced in the mutant germarium (arrowheads). (I-L) A germarium with an lgd germline where the expression of Bam-GFP is unaffected and the upregulation of expression in mitotic cyst stages is clearly recognisable (arrowhead, compare with the wild-type germarium in F-H). Note, that the mutant germarium contains four dad-lacZ-expressing Bam-GFP-negative GSCs (arrow in J,L). The yellow arrow points to a single GC of an abutting egg chamber that expresses dad-lacZ.
We have proposed that in lgd cells, transmembrane proteins destined to become degraded in the lysosome are not completely incorporated in ILVs of MEs (Schneider et al., 2013). This hypothesis has been strongly supported by recent work of the Schweisguth laboratory (Couturier et al., 2014). Consequently, a fraction of Notch and Tkv remains at the LM and their intracellular domains stay in contact with the cytosol. Activated Tkv in this fraction continues to signal as long as the ME exists. We therefore propose that it is the defect in incorporation of activated Tkv into ILVs that causes the ectopic activation of the Dpp pathway. In this scenario, defects that increase the lifetime of the lgd mutant ME, such as loss of Dmon1 function, enhances and prolongs the activity of the Dpp pathway. This is what we have observed. The incorporation of transmembrane proteins into ILVs requires their previous ubiquitylation by E3 ligases. Hence, the loss of the function of the E3 ligase Fu, which normally ubiquitylates activated Tkv, also prevents incorporation of activated Tkv in ILVs.

Fig. 8. Number of GSCs in wt, lgd mutant and shrub and fu heterozygous germlines. *P<0.05, compared with wt; **P<0.05, lgd compared with fu+/lgd; t-test, normal distribution.

Fig. 9. Loss of lgd function causes accumulation of YFP-Tkv in FCs and GCs. Clones are labelled by the absence of RFP. (A,B) A wild-type (wt) ovariole next to an ovariole with lgd mutant GCs. The mutant GCs contain enlarged YFP-Tkv-positive vesicles (arrowhead). (C-E) Magnification of the mutant GCs highlighted in A,B with an arrowhead. (F-H) EC with an lgd mutant germline. The Tkv-containing vesicles also contain NECD (arrowheads). (I-L) The enlarged vesicles are positive for Rab7. (M-P) Accumulation of YFP-Tkv in endosomes of lgd FCs. (P) Surface view on the egg chamber shown in M-O. (Q-T) A germarium with an lgd Dmon1 germline (arrowhead). Different optical planes of the germarium shown in Q and R. The arrowheads point to the spectrosomes. The arrow in Q-T points to the double heterozygous FCs that weakly express dad-lacZ.
We found that loss of shrub function causes a similar defect to that of lgd. Shrub is the central element of the ESCRT-III complex, which is required for ILV scission and interacts physically with Lgd (Troost et al., 2012). Loss of shrub function is expected to result in all Tkv remaining on the LM. Thus, it was no surprise to see that loss of its function in the FCs also causes the activation of the Dpp pathway. More surprising was the finding that reduction of Shrub activity by only 50% is sufficient to initiate the activation of the Dpp pathway in the germline. Given that those phenotypes of shrub+/ cells are stronger than those of lgd mutant cells, it is likely that reduction of the activity of shrub by 50% causes a larger fraction of Tkv to remain on the LM than in lgd mutant cells. The phenotypes of shrub+/ cells are strongly enhanced by heterozygosity of fu, indicating that there is an intimate functional relationship and that, as in the case of lgd, a defect in Tkv degradation causes the activation of the Dpp pathway.

Because of the similarity in the phenotype and their intimate relationship, it is puzzling to find an antagonistic relationship between shrub and lgd. However, we observed this antagonism only in respect to the formation of supernumerary GCSs, but not in respect to formation of supernumerary dad-lacZ-positive GCSs. A similar complex situation was found for the relationship of shrub and lgd in imaginal discs, where the reduction of shrub activity suppressed the activation of the Notch pathway, but enhanced the morphological defect of endosomes in lgd cells (Troost et al., 2012). Finding explanation for this complex relationship will be key to understanding the function of Lgd. One possibility is that the function of the ME is disturbed more severely in the double mutants than in each single mutant. In agreement with this is our observation in the imaginal disc, where the MEs lost their association with Rab7 in shrub lgd/lgd+ cells (Schneider et al., 2013).

**MATERIALS AND METHODS**

**Drosophila genetics**

The fly stocks used were as follows: tub rhab5-CFP tub rhab7-YFP (Marois et al., 2006), GbeaSu(H)-nlsGFP (de Navascues et al., 2012), bam-GFP (kindly provided by Masahiko Takemura, Department of Biology, Graduate School of Science, Kobe, Japan), N-YFP (Rees et al., 2011), dad-lacZ (Casaneuve and Ferguson, 2004) and lgdP-lgd-RFP (Troost et al., 2012). Mutant stocks were as follows: shrub+/− FRTG13 (Sweeney et al., 2002), lgdP7 aph-1D35 FRT40A (Schneider et al., 2013), hrsD28 FRT40A (Lloyd et al., 2002), tkvA12 FRT40A (Terracol and Lengyel, 1994), lgdG7 tkvA12 FRT40A (this study), aph-1D35 FRT40A (Hu et al., 2002), Dpp10 FRT82B (Michelli et al., 1997), lgdG7 FRT40A, lggG873a FRT40A and lggG7 hrsD28 FRT40A (Jaekel and Klein, 2006), Dmon1mut4 FRT40A (Yousefian et al., 2013), lgdG7 Dmon1mut4 FRT40A (this study), fu1 (Nüsslein-Volhard and Wieschaus, 1980) and lggG7 shrubG7-1 FRT40A (Nadja Ohlenhard, diploma thesis, Heinrich-Heine-Universität, Düsseldorf). Other lines were: Tkv-YFP (Kyoto Stock Center), dad-GFP (Ninov et al., 2010) and PCNA-GFP (Thacker et al., 2003).

**Clonal analysis**

 Follicle cell clones were generated with the Flp/FRT system (Xu and Rubin, 1993) and induced in adult females by applying a 2-h heat shock (37°C) on two consecutive days. Flies were kept at 25°C and ovaries were dissected 2 days after heat shock. Germline clones were induced in the first-instar larvae (24-48 h after egg laying) by applying a 90-min heat shock (37°C). Larvae were kept at 25°C and ovaries were dissected 2 days after eclosion.

**Immunostaining and microscopy**

Antibody staining was performed according to standard protocols. Ovaries were additionally stained with the nuclear marker Hoechst 33258. Antibodies used were: the mouse anti-Notch antibody against the extracellular domain (C458.2H), mouse anti-Notch antibody against the intracellular domain, and anti-DI, anti-Orb, anti-CycA and anti-Hnt antibodies (169) (all obtained from DSHB Iowa). Further antibodies used in this work were: rabbit anti-IgG (Cappel), anti-Rab7 (abcam), anti-Spectrin (DSHB) antibodies. Fluorochrome-conjugated secondary antibodies were purchased from Invitrogen, Molecular Probes. Images were obtained with a Zeiss AxioImager Z1 Microscope equipped with a Zeiss Apotome.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

K.S.M and M.S. designed and performed experiments; T.K. designed and supervised experiments and wrote the manuscript.

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