The tumour suppressor Lethal (2) giant discs is required for the function of the ESCRT-III component Shrub/CHMP4

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Summary

Recent work indicates that defects in late phases of the endosomal pathway caused by loss of function of the tumour suppressor gene *lethal (2) giant discs* (*lgd*) or the function of the ESCRT complexes I–III result in the ligand-independent activation of the Notch pathway in all imaginal disc cells in *Drosophila melanogaster*. *lgd* encodes a member of an uncharacterised protein family, whose members contain one C2 domain and four repeats of the DM14 domain. The function of the DM14 domain is unknown. We here report a detailed structure–function analysis of Lgd protein, which reveals that the DM14 domains are essential for the function of Lgd and act in a redundant manner. Moreover, our analysis indicates that the DM14 domain provides the specific function, whereas the C2 domain is required for the subcellular location of Lgd. We found that Lgd interacts directly with the ESCRT-III subunit Shrub through the DM14 domains. The interaction is required for the function of Shrub, indicating that Lgd contributes to the function of the ESCRT-III complex. Furthermore, our genetic studies indicate that the activation of Notch in ESCRT and *lgd* mutant cells occurs in a different manner and that the activity of Shrub and other ESCRT components are required for the activation of Notch in *lgd* mutant cells.

Key words: ESCRT complexes, Notch signalling, Shrub, Endosomal pathway, Lethal (2) giant discs, Protein trafficking, CHMP4, Snf7

Introduction

Short-range signalling through the Notch signalling pathway is repeatedly used throughout embryonic development of metazoans. In addition, it is required for homeostatic processes in mammals and its de-regulation often results in the development of diseases, such as cancer, in humans (Koch and Radtke, 2010). Thus, uncovering the regulation and signal transduction mechanism of the pathway is of pivotal interest in order to understand human development and formation of disease.

In *Drosophila melanogaster*, the Notch receptor undergoes two proteolytic events in response to binding of its ligands, Delta or Serrate, resulting in the release of the soluble intracellular domain (Nintra) into the cytosol (Bray, 2006). The first of these events is mediated by the ADAM metalloprotease Kuzbanian (Kuz) and removes the extracellular domain. This ectodomain shedding creates an intermediate called NEXT (Notch Extracellular Truncation), which is immediately cleaved by the γ-secretase complex to release Nintra. This translocates into the nucleus and associates with the CBF factor Suppressor of Hairless [Su(H)] to assemble a transcriptional activator complex that initiates expression of the target genes. Although described here for *Drosophila*, the principle mechanism of signalling through the Notch pathway is conserved in all metazoans.

Recent work has revealed that Notch can also be activated in a ligand-independent manner under certain circumstances, such as loss of function of the genes encoding members of the ESCRT complexes I–III or *lethal (2) giant discs* [*lgd*, also known as *l(2)gd1*] (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006; Lu and Bilder, 2005; Moberg et al., 2005; Thompson et al., 2005). *lgd* is a tumour suppressor gene, whose loss of function results in the overproliferation of imaginal disc cells in *Drosophila* (Bryant et al., 1993). We previously showed that the mutant phenotype is a consequence of the ectopic and ligand-independent activation of the Notch pathway in all imaginal disc cells in *Drosophila* (Childress et al., 2006; Jaekel and Klein, 2006).

lgd encodes a member of a protein family whose members are characterised by two types of domains, C2 and DM14 (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006) (Fig. 1). In many proteins, the C2 domain functions to tether proteins to membranes through binding to phospholipids (Gallagher and Knoblich, 2006). However, it can also mediate protein–protein interactions, e.g. through binding to phosphorylated tyrosine (Benes et al., 2005). The function of the DM14 domain is not known. It has been discovered in an in silico search of the *Drosophila* genome for novel protein domains (Ponting et al., 2001). Lethal (2) giant discs protein (Lgd) and most of its orthologues have four DM14 domains arranged in tandem in the N-terminus.

Mammals have two orthologues of Lgd in their genome, which we named Lgd1 and Lgd2 (Jaekel and Klein, 2006). The functions ascribed to these orthologues in cell culture studies are quite diverse. Lgd2 (also called CC2D1A, Aki or Freud-1) has been reported to act as a scaffold protein during EGF-R-signalling (Nakamura et al., 2008), as a potent activator of NF-κB signalling (Zhao et al., 2010) and appears to regulate cleavage of

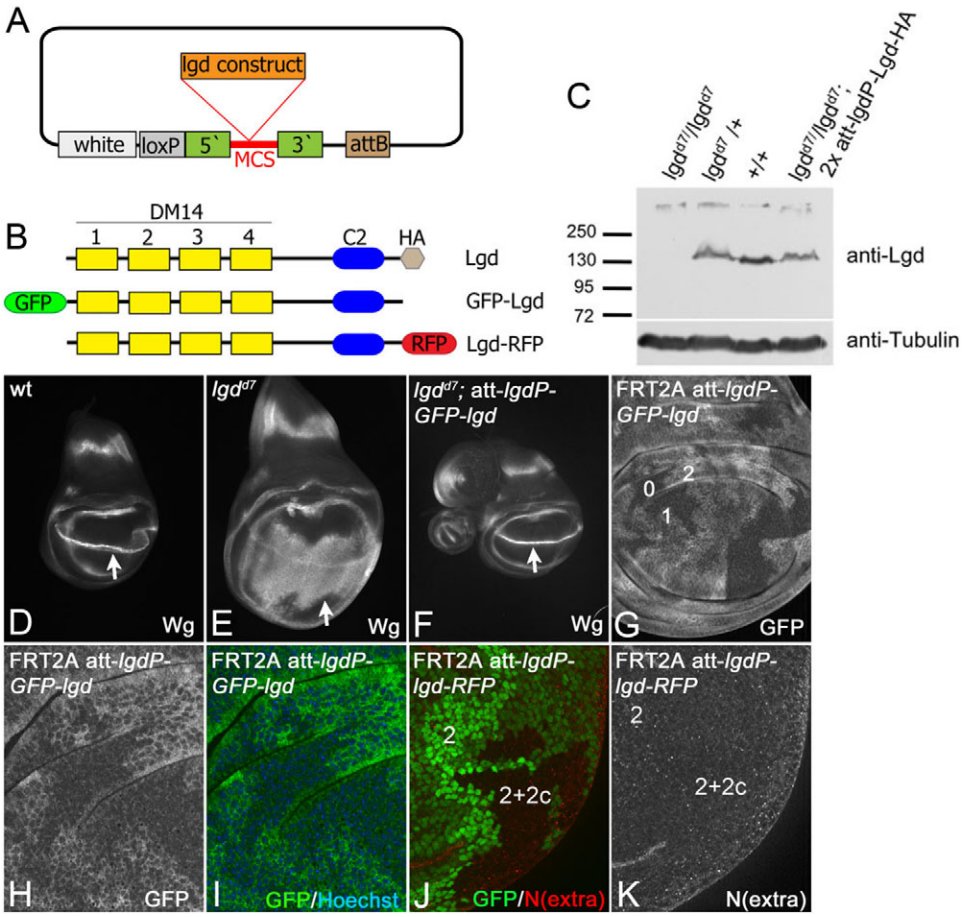

Fig. 1. Rescue assay for the functional analysis of Lgd. (**A**) Deletion constructs were inserted in the multiple cloning site (MCS), flanked by genomic sequences up- and downstream of the *lgd* transcription unit. (**B**) Full-length rescue constructs tested. (**C**) Expression of Lgd–HA compared to endogenous Lgd detected by anti-Lgd antibody staining. −/−, homozygous amorphic lgd^{d7} mutant flies. −/+, lgd^{d7} heterozygous flies. +/+, wild-type flies. −/−; c/c lgd^{d7} homozygous flies with two copies of *att-lgdP-lgd-HA* present in the genome. The comparison reveals that the amount of Lgd–HA produced by two copies of *att-lgdP-lgd-HA* is comparable to the lgd^{d7} (+/−) heterozygous flies. (**D–F**) Rescue experiment with *att-lgdP-GFP-lgd*. Expression of the Notch target gene *wg* is revealed by anti-Wg antibody staining. (D) Wild-type (wt) wing imaginal disc. Notch-dependent expression of Wg is restricted to the D/V boundary (arrow). (E) Its expression is dramatically expanded in lgd^{d7} null mutant. (F) Introduction of one copy of *att-lgdP-GFP-lgd* normalises the expression of Wg. (**G–I**) Subcellular localisation of GFP–Lgd. (G) Disc-bearing clones with no (0), one (1) or two (2) copies of *att-lgdP-GFP-lgd*. (H,I) Magnification of the area highlighted by the numbers in G. GFP–Lgd is evenly distributed throughout the cytosol and excluded from the nucleus. (**J**,**K**) Distribution of Notch in a disc carrying clones with two copies of *att-lgdP-lgd-RFP*, revealed with an antibody raised against the extracellular domain of Notch. No difference in the distribution of Notch is seen in cells with no (2) and two copies of *att-lgdP-lgd-RFP* (2+2c) in addition to the two endogenous copies of *lgd*.

the centrosome during mitosis (Nakamura et al., 2009). It has been further reported that both orthologues act as transcriptional repressors of the 5-HT1A receptor gene (Hadjighassem et al., 2009; Ou et al., 2003).

In *Drosophila*, Lgd is involved in constitutive trafficking of Notch through the endosomal pathway (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). Trafficking is initiated by endocytosis of Notch into early endosomal vesicles, which undergo homotypic fusion to form the early endosome. During maturation of the endosome, Notch is transferred into intraluminal vesicles (ILV). This step is necessary to transfer the intracellular domain of Notch into the lumen of the endosome and, thus, for complete degradation of the receptor upon fusion with the lysosome. The ILV-containing endosomes are called multivesicular bodies (MVB) and their formation is controlled by the activity of four ESCRT protein complexes (ESCRT-0 to ESCRT-III) that act in sequence (Williams and Urbe, 2007). The mature late endosome eventually fuses with lysosomes where its luminal content is degraded. Besides its role during endosomal trafficking, ESCRT complexes are required for autophagy, cytokinesis, budding of retroviruses (such as HIV and Ebola) from the plasma membrane, as well as in diseases such as cancer, cataract and neurodegeneration (McDonald and Martin-Serrano, 2009; Saksena and Emr, 2009).

In cells with mutant *lgd* (referred to here as '*lgd* cells'), Notch-containing late endosomes appear to be enlarged. However, the process in which Lgd is involved is not known. Genetic experiments indicate that the loss of function of *hrs* in *lgd* cells prevents the activation of the Notch pathway, suggesting that the function of Lgd is required downstream of Hrs (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). Hrs is a subunit of the ESCRT-0 complex, which initiates the sequential action of four ESCRT complexes during MVB formation. ESCRT-0 also contains STAM (signal-transducing

adaptor molecule) as a second subunit (Saksena and Emr, 2009). The genetic interaction with *hrs* raises the possibility that *lgd* might be involved in the ESCRT-mediated process. Indeed, genes that encode components of the ESCRT complexes I–III are also tumour suppressors whose loss of function activates the Notch pathway and results in overproliferation of imaginal disc cells (Moberg et al., 2005; Slagsvold et al., 2006; Thompson et al., 2005; Vaccari and Bilder, 2005). However, there are important differences in the phenotypes of ESCRT and *lgd* mutants. Loss of ESCRT function results in the loss of epithelial polarity, and mutant cells die if they are in competition with wild-type cells. The overproliferation of the disc is largely induced non-autonomously in surrounding wild-type cells (Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). By contrast, *lgd* cells are healthy, appear to out-compete their wild-type neighbours and overproliferate. Thus, it is also possible that the suppression of activation of Notch in *lgd* cells can be the result of the loss of an ESCRT-independent function of *hrs*. In agreement with this possibility is that, in contrast to loss of ESCRT-I–ESCRT-III function, loss of *hrs* function does not result in activation of Notch (Jaekel and Klein, 2006). The consequences of loss of *STAM* function for Notch signalling have not been reported.

Here, we report the further characterisation of the function of Lgd in *Drosophila*. We present a detailed structure–function analysis that uncovers a function for the as-yet uncharacterised DM14 domain as a protein interaction module. Our analysis further reveals an unexpected function for the C2 domain as a device to prevent nuclear mis-location of Lgd. Furthermore, we found that Lgd physically interacts with the ESCRT-III subunit Shrub/CHMP4/Snf7. Genetic interaction studies suggest an intimate functional relationship between *lgd* and *shrub* in vivo and indicate that Lgd contributes to ESCRT-III function.

Results

Structure–function analysis of Lgd

In order to obtain further insight in the function of Lgd, we generated various deletion constructs of *lgd* (see Figs 1, 2) and tested them in rescue assays. We initially designed a rescue assay based on the Gal4 system. However, we obtained confusing results that contradicted available information from analysis of *lgd* alleles. After many experiments, we concluded that the system is not well suited for our analysis because of the strong overexpression induced by it. To circumvent our problems, we developed a new assay (Fig. 1). We flanked our constructs with genomic sequences of 500 bp upstream and downstream of the *lgd* transcription unit (Fig. 1A). These sequences are part of a genomic rescue fragment, which is sufficient to completely rescue the *lgd* mutant phenotype (Childress et al., 2006). We surmised that they include the complete promoter of *lgd* (*lgdP*) and initiate expression of the constructs at the level of endogenous *lgd*. In addition, we used the phage-derived ϕC31-integrase system (Bischof et al., 2007) that allows the site-specific unidirectional insertion of constructs into the same genomic landing (attP) site and thus neutralises position effects. This new *att-lgdP* rescue assay allows the direct comparison of all constructs expressed close to the expression level of endogenous *lgd*. Western blot analysis revealed that the expression of a full-length construct (*att-lgdP-lgd-HA*) is about half that of the endogenous Lgd (Fig. 1B,C). We tested the rescue abilities of the *att-lgdP-lgd* full-length constructs in lgd^{d7} and lgd^{rsk73} null mutant animals. *lgd* mutant wing imaginal discs show expansion of Notch target gene expression, from their normal domain along the dorso-ventral (D/V) boundary of the wing primordium (Fig. 1D,E). We found that one copy of a construct tagged with GFP (*att-lgdP-GFP-lgd*) rescued the null mutant flies completely (Fig. 1D–F). The rescued flies developed to fertile adults and their wing imaginal discs showed normal expression of the Notch activity reporter Gbe+Su(H) and the endogenous target gene *wingless* (*wg*) (Fig. 1D–F and not shown). We observed a similar rescue with HA- and RFP-tagged Lgd variants (Fig. 1B, Fig. 2C and not shown). The complete rescue achieved with the full-length *lgd* constructs indicates that the genomic sequences used can provide sufficient expression of *lgd* in all tissues. In previous attempts with the Gal4 system, we were unable to achieve a complete rescue.

In order to monitor the subcellular distribution of GFP-tagged Lgd, we induced clones containing two copies of this construct by Flp-mediated recombination (FRT 2A att-*lgdP-GFP-lgd*). This recombination was necessary because the construct is expressed at very low levels making it difficult to detect a specific signal if one copy is present in the genome. By inducing clones, we generated cells that contained one, two or no copies of att-*lgdP-GFP-lgd* (Fig. 1G–I). Comparison of these regions revealed that GFP–Lgd was evenly distributed within the cytosol (Fig. 1H,I), as previously reported using antibody staining or tagged UAS constructs (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). No association with membrane structures was observed. Cells of clones that contained the two copies of the att-*lgdP-lgd-RFP* in addition to endogenous *lgd*, and therefore overexpressed Lgd, did not show enlargement of Notch-containing late endosomes, as observed upon expression of UAS *lgd* under control of the Gal4 system (Fig. 1J,K). This observation underscores the notion that the massive overexpression induced with the Gal4 system causes an artificial phenotype and that the results obtained with it are not useful in the analysis of a dynamic process such as endosomal trafficking.

Having established a suitable rescue assay, we investigated the importance and function of the recognisable domains of Lgd. If not stated otherwise, the rescue experiments were performed with one copy of a given att-*lgdP*-construct in the genome of lgd^{d7} null mutant flies (Figs 2, 3). Western blot analysis revealed that most deletion constructs were expressed at comparable levels, with the exception of those where the C2 domain was deleted (Fig. 2B). These constructs gave rise to significantly lower levels of protein (Fig. 2B). Because they are controlled by the same promoter and are inserted at the same genomic site, they are probably expressed at the same level as the other constructs. Therefore, it appears that the C2 domain is required for the stability of Lgd.

DM14 domains are essential and function in a redundant manner

In order to determine the importance of the DM14 domains for the function of Lgd, we tested several constructs that lacked various numbers of DM14 domains (Fig. 2A).

We found that a variant lacking all four domains (*lgdΔDM14*) was not able to rescue the *lgd* mutant phenotype (Fig. 2C,D), indicating that the DM14 domains are required for function. In addition, LgdΔDM14 caused an even greater enlargement of (Rab7-positive) late endosomes in lgd^{d7} null mutant cells. This is best seen in lgd^{d7} clones induced in the presence of *lgdΔDM14* in

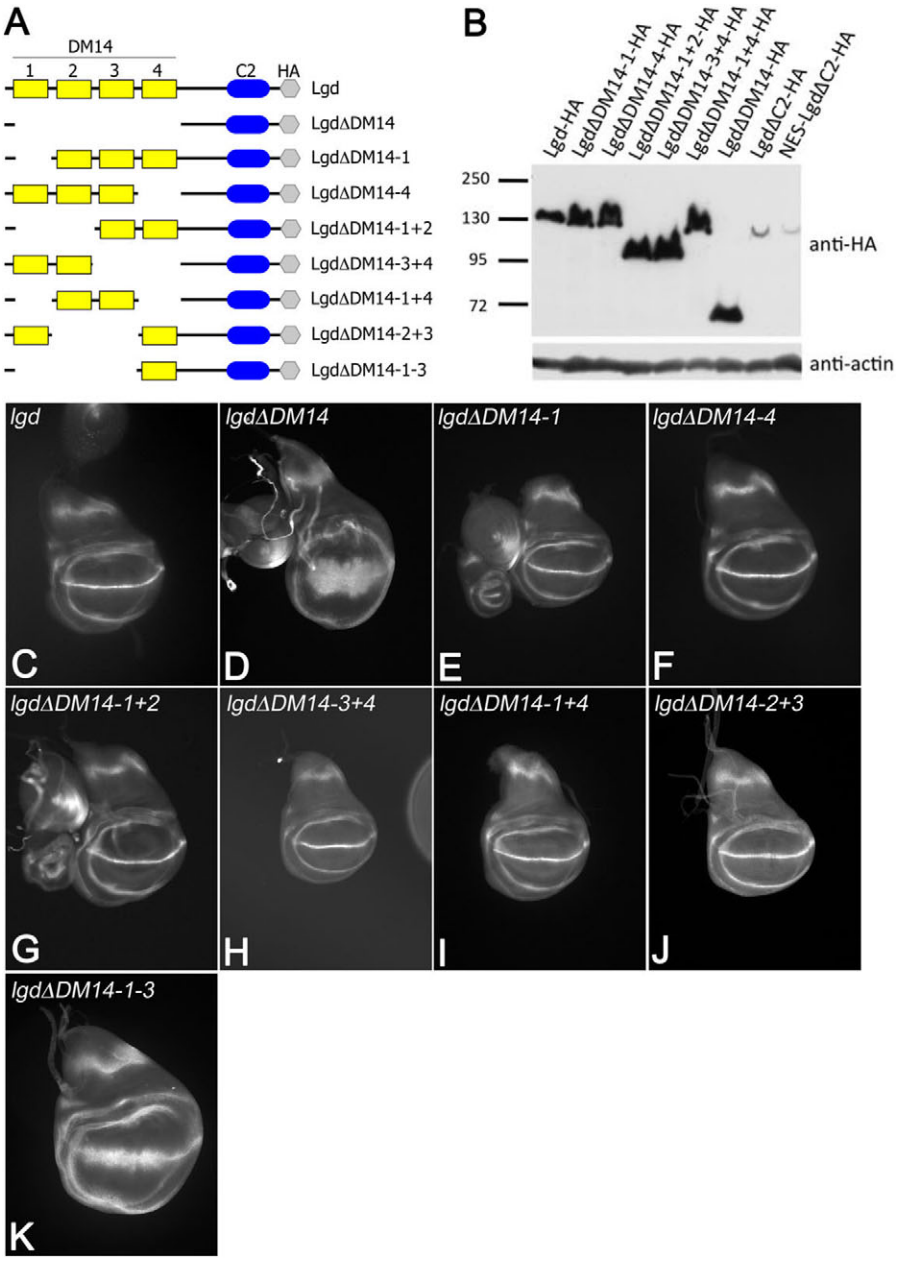

Fig. 2. Importance of the DM14 domains. (**A**) DM14 deletion constructs tested. (**B**) Western blot shows the protein expression of the constructs. All constructs are expressed at a similar level with the exception of those where the C2 domain is deleted. (**C–K**) Rescue activities of one copy of various constructs. Notch activity is revealed by anti-Wg antibody staining.

the genome (supplementary material Fig. S1A–F). The finding indicates that LgdΔDM14 has a negative effect on endosome morphology. Note that this effect is only observed in the absence of endogenous Lgd, indicating that LgdΔDM14 cannot compete with the full-length protein.

Constructs lacking either one or a combination of two DM14 domains (*lgdΔDM14-1*, *lgdΔDM14-4*, *lgdΔDM14-1+2*, *lgdΔDM14-3+4*, *lgdΔDM14-1+4*, *lgdΔDM14-2+3*) were functional and rescued the amorphic lgd^{d7} phenotype, giving rise to adult fertile flies (Fig. 2E–J). By contrast, a construct with only the fourth domain (*lgdΔDM14-1-3*) had only weak rescue capabilities (Fig. 2K). This construct also produces a similar endosomal phenotype to that observed with *lgdΔDM14* (not shown). Thus, it appears that the DM14 domains function in a redundant manner and any combination of two out of the four is sufficient for function.

The C2 domain is required for the localisation of Lgd within the cytosol

Previous reports showed that Lgd is located within the cytosol with no obvious association with endosomes or other membrane structures (Childress et al., 2006) (see above). Nevertheless, the C2 domain frequently functions to tether correspondent proteins to membranes through phospholipid binding. In agreement with this, it has been shown that a variant encoding little more than the C2 domain of Lgd is able to bind to phospholipids in vitro (Gallagher and Knoblich, 2006). Therefore, we tested whether this variant (UAS *C2-RFP*, Fig. 3A) binds to endosomes if expressed with the Gal4 system in imaginal disc cells (Fig. 3B,C). We found that C2-RFP, like full-length Lgd-RFP, was distributed throughout the cytosol with no obvious association with membranes (Fig. 3B,C). This suggests that the C2 domain of Lgd does not mediate membrane binding in vivo.

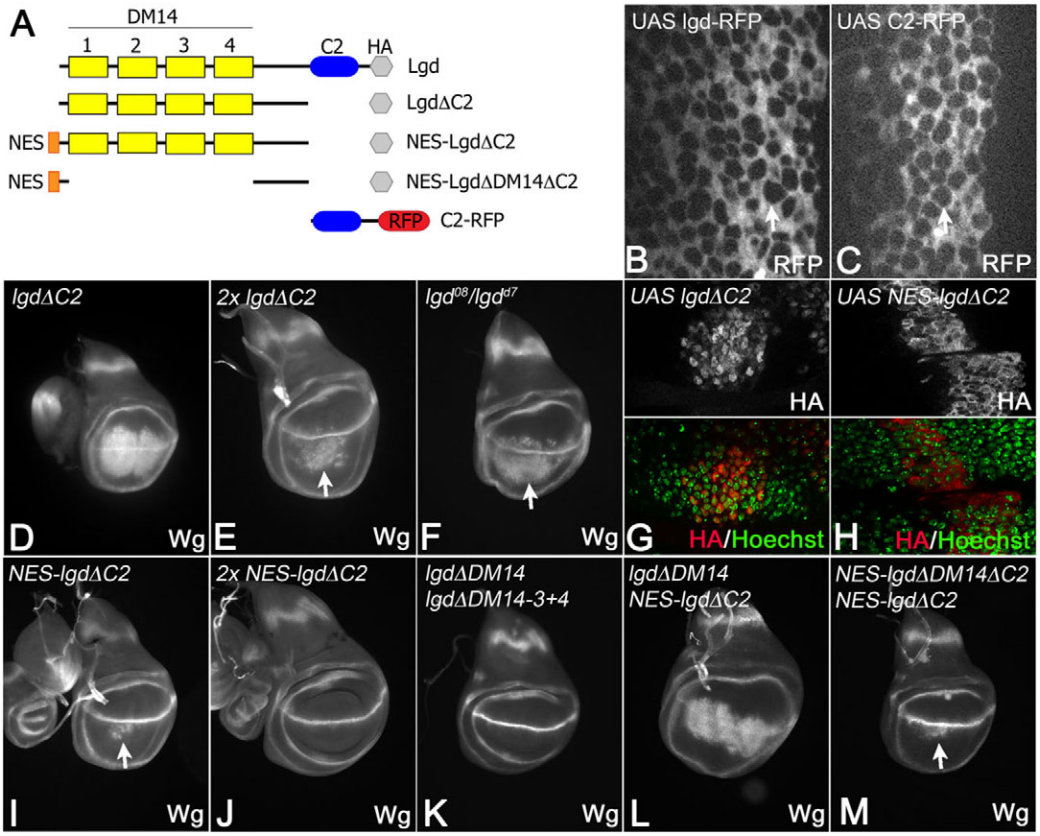

Fig. 3. Function of the C2 domain. (**A**) Constructs tested. (**B**,**C**) Expression of UAS *lgd-RFP* (B) and UAS C2-RFP (C) with *ptc*Gal4. The protein is distributed throughout the cytosol in both cases and excluded from the nucleus (arrows). (**D**–**J**) Rescue activities of various C2 domain deletion constructs. (D) One copy of *att-lgdP-lgdΔC2* does not lead to a significant rescue of the lgd^{d7} mutant phenotype (compare with Fig. 1E). (E) Two copies of *att-lgdP-lgdΔC2* result in a weak rescue with residual ectopic expression of Wg (arrow). (F) The phenotype of lgd^{08}/lgd^{d7} wing imaginal discs. The arrow highlight the weak ectopic expression of Wg. (G) Comparison of the HA and the nuclear Hoechst signal reveals that most of the LgdΔC2-HA is localised in the nucleus. (H) Most of the NES-LgdΔC2 is present the cytosol. (I) *att-lgdP-NES-lgdΔC2* results in a much better rescue than *att-lgdP-lgdΔC2* (compare with D). Arrow highlights the weak ectopic expression of Wg. (J) Two copies of *att-lgdP-lgdΔC2* result in a even better rescue (compare with I). (**K**–**M**) Competition between *att-lgdP-lgdΔDM14* and other constructs. *att-lgdP-lgdΔDM14* fails to rescue the lgd^{d7} null mutant phenotype (see Fig. 2D). (K) The additional presence of any construct with at least two DM14 domains can do so. *att-lgdP-lgdΔDM14-3+4* is shown as an example. (L) By contrast, the rescue by *att-lgdP-NES-lgdΔC2* is strongly suppressed in the presence of *att-lgdP-lgdΔDM14* (compare with G). (M) *att-lgdP-NES-lgdΔC2* is able to rescue the *lgd* phenotype in the presence of *att-lgdP-lgdΔDM14DC2* (compare with G). Arrow indicates the ectopic expression of Wg.

Next, we tested the activity of a construct that lacked the C2 domain (att-*lgdP-lgdΔC2*, Fig. 3A,D). This construct was unable to rescue the *lgd* mutant phenotype in our rescue assay, indicating that the C2 domain is essential for the function of Lgd (Fig. 3D, compare with Fig. 1E). However, if present in two copies, the construct showed weak rescue abilities (Fig. 3E), indicating that it has some residual function. The result is in good agreement with the behaviour of a previously isolated allele, lgd^{08}, which encodes a similar protein lacking the C2 domain (Gallagher and Knoblich, 2006). lgd^{08} caused a hypomorphic phenotype in heterozygousity with the null allele lgd^{d7} (Fig. 3F).

In our initial experiments with the Gal4 system, we noticed that LgdΔC2 became enriched in the nucleus (Fig. 3G). Thus, it might be deficient in function because of its mis-localisation into the wrong cellular compartment. To test this possibility, we added a nuclear export sequence (NES) to the N-terminus of LgdΔC2 (att-*lgdP*-NES-*lgdΔC2*), which re-localised it within the cytosol (Fig. 3H). Indeed, we found that *att-lgdP*-NES-*lgdΔC2* was able to partially rescue the *lgd* mutant phenotype in wing imaginal discs if present in one copy (Fig. 3I). If two copies were present, the rescued wing imaginal discs looked normal (Fig. 3J, compare with Fig. 1D). Furthermore, the rescued animals developed to the pharate adult stage, which is never observed in the absence of the construct. The pharate adults displayed mild defects, such as double sockets and loss of bristles, which are also seen in hypomorphic lgd^{d7}/lgd^{SH495} flies (not shown). The partial functionality of NES-*lgdΔC2* was further demonstrated by the observation that lgd^{d7}/lgd^{SH495} hypomorphic flies, which normally die as pharate adults (Jaekel and Klein, 2006), hatched and looked normal if just one copy of NES-*lgdΔC2* was present. However, we did not achieve a complete rescue of amorphic lgd^{d7} flies, even in the presence of two copies of NES-*lgdΔC2*. This was indicated by the failure to obtain living adults of this genotype.

The described experiments do not allow ascertaining whether the failure of NES-*lgdΔC2* to completely rescue the amorphic *lgd* phenotype was due to its significantly lower expression level (see Fig. 2B) or to loss of a specific function mediated by the C2 domain. Nevertheless, they indicate that the C2 domain has a

second function besides protein stabilization, which is the localisation of Lgd within the cytosol.

The C2 domain appears to be required for proper function of Lgd in the cytosol

Our analysis revealed that *lgdΔDM14* affects the morphology of late endosomes in the absence of endogenous *lgd*. We wondered which part of the remaining sequence of *lgdΔDM14* is responsible for this effect. As an obvious candidate, we deleted the C2 domain in *lgdΔDM14*. To assure cytosolic localisation of this construct, we added an NES signal (att-*lgdP-NES-lgdΔDM14ΔC2*). We found that NES-*lgdΔDM14ΔC2* failed to cause an enlargement of endosomes (supplementary material Fig. S2A), indicating that the C2 domain is responsible for the effect of LgdΔDM14 on endosomes. As expected, the construct also failed to rescue the *lgd* mutant phenotype even if present in two copies (supplementary material Fig. S2B). This finding suggests that the non-functional LgdΔDM14 interacts with an unknown element of the endosomal machinery via its C2 domain and causes the observed endosomal effect. Thus, the C2 domain probably mediates an interaction in the cytosol with an unknown factor, in addition to its function in cytosolic localisation.

To gain further evidence for this notion, we set up a competition assay based on the observation that LgdΔDM14 can affect endosome morphology only in the complete absence of endogenous Lgd (Fig. 3K–M). We asked which of our deletion constructs could suppress the effects caused by *lgdΔDM14* in *lgd* mutant cells if additionally present. We found that constructs that contained a C2 domain and two or more DM14 domains rescued the *lgd* mutant phenotype (Fig. 3K). By contrast, NES-*lgdΔC2* was unable to rescue *lgd* mutant flies, although it partially did so in the absence of *lgdΔDM14* (Fig. 3L). In addition, it failed to suppress the endosomal phenotype induced by *lgdΔDM14* (not shown). Moreover, NES-*lgdΔC2* rescued the *lgd* mutant phenotype in the presence of *NES-lgdΔDM14ΔC2* (Fig. 3M), indicating that the C2 domain of *lgdΔDM14* prevents the partial rescue by NES-*lgdΔC2*.

Together, these results raise the possibility that the C2 domain also mediates an interaction within the cytosol that is necessary for Lgd function. The fact that *lgdΔDM14* does not possess any rescue activity, but NES-*lgdΔC2* does, suggests that the DM14 domains provide the Lgd-specific function, whereas the C2 domain provides a general function, e.g. concentrating Lgd at the site of action.

Identification of Shrub as an interaction partner of Lgd

In order to identify the molecular process in which Lgd is involved, we performed a genetic modifier screen to uncover genes that interact with *lgd*. We searched for deficiencies that modify the phenotype caused by overexpression of UAS *lgd* in the wing blade with C5-Gal4 (Fig. 4A–E). Two overlapping deficiencies, *Df(2R)Np5* and *Df(2R)w45-30n*, strongly modified the overexpression phenotype (Fig. 4B–D), indicating that they uncover genes that might stand in a functional relationship with *lgd*. Both deficiencies uncover *shrub*, which encodes the orthologue of the ESCRT-III subunit Snf7/CHMP4/Vps32 (Sweeney et al., 2006). Indeed, we found that heterozygousity of the null allele $shrub^{4-1}$ caused a similar modification of the *lgd* overexpression phenotype (Fig. 4E), indicating that the loss of one copy of *shrub* is probably responsible for the modification caused by the two deficiencies.

Lgd physically interacts with Shrub through its DM14 domains

To investigate the relationship between Lgd and Shrub, we performed coimmunoprecipitation experiments (Fig. 4F–I). In a first set of experiments we used a GFP-tagged version of Shrub (Shrub–GFP) (Sweeney et al., 2006) together with a HA-tagged Lgd construct and expressed them in larval tissue. In order to avoid strong overexpression, we performed the experiments using *heatshock* (*hs*)Gal4 without heat-shock. We found that Shrub–GFP coimmunoprecipitated with Lgd–HA and vice versa (Fig. 4F). Furthermore, we were able to coimmunoprecipitate endogenous Shrub with Lgd–HA (Fig. 4G). The results indicate that Lgd can physically interact with Shrub.

The interaction between Lgd and Shrub could be direct or mediated by another protein acting as an adaptor. We tested these possibilities with an in vitro binding assay using bacterially expressed Lgd and Shrub. We found that Lgd can directly bind to Shrub in this GST-pulldown assay (Fig. 4H), indicating that the interaction between the two proteins appears to be direct.

To determine which part of Lgd is required for the interaction with Shrub, we used our HA-tagged UAS deletion constructs in coimmunoprecipitation experiments (expressed with *hs*Gal4; Fig. 4I). We found that a construct lacking all four DM14 domains could not be coimmunoprecipitated with Shrub, indicating that they are necessary for the interactions with Shrub. By contrast, variants of Lgd lacking the C2 domain could still be coimmunoprecipitated. Thus, the DM14 domains are required for the physical interaction with Shrub.

Lgd and Shrub interact in vivo

We uncovered the relationship between Lgd and Shrub in a genetic screen using the overexpression phenotype of Lgd. Because of the described artefacts produced by overexpression of Lgd, the uncovered genetic interaction might have no biological relevance. Therefore, we wanted to test the biological relevance of the uncovered interaction between Lgd and Shrub in an additional manner and looked for genetic interactions between mutant alleles of the two genes. The hypomorphic allelic combination lgd^{d7}/lgd^{SH495} allows flies to develop to pharate adults, which display defects characteristic for a slight ectopic activation of the Notch pathway (Jaekel and Klein, 2006). The null allele $shrub^{4-1}$ does not cause any observable phenotype in heterozygousity, but is embryonic lethal in homozygousity (Sweeney et al., 2006). However, we found that flies carrying the hypomorphic allelic combination lgd^{d7}/lgd^{SH495} and are heterozygous for $shrub^{4-1}$ (lgd^{d7} +/lgd^{SH495} $shrub^{4-1}$) die at the early third larval instar stage. The time of death is earlier than for *lgd* null mutants, which die during early pupal stages (Buratovich and Bryant, 1995). This finding reveals an intimate relationship between the two genes and suggests that the loss of *lgd* function affects the activity of *shrub*.

The early time point of death of lgd^{d7} +/lgd^{SH495} $shrub^{4-1}$ flies made the analysis of their imaginal discs difficult because they are very small at that time of development (supplementary material Fig. S2C,D). The cells of these discs contain dramatically enlarged Wg-positive vesicles (supplementary material Fig. S2D). To be able to better analyse lgd^{d7} +/lgd^{SH495} $shrub^{4-1}$ cells, we rescued the *lgd* mutant situation by introducing our *att-lgdP-lgd-RFP* rescue construct located on a FRT 2A chromosome (Fig. 5A). The presence of the FRT

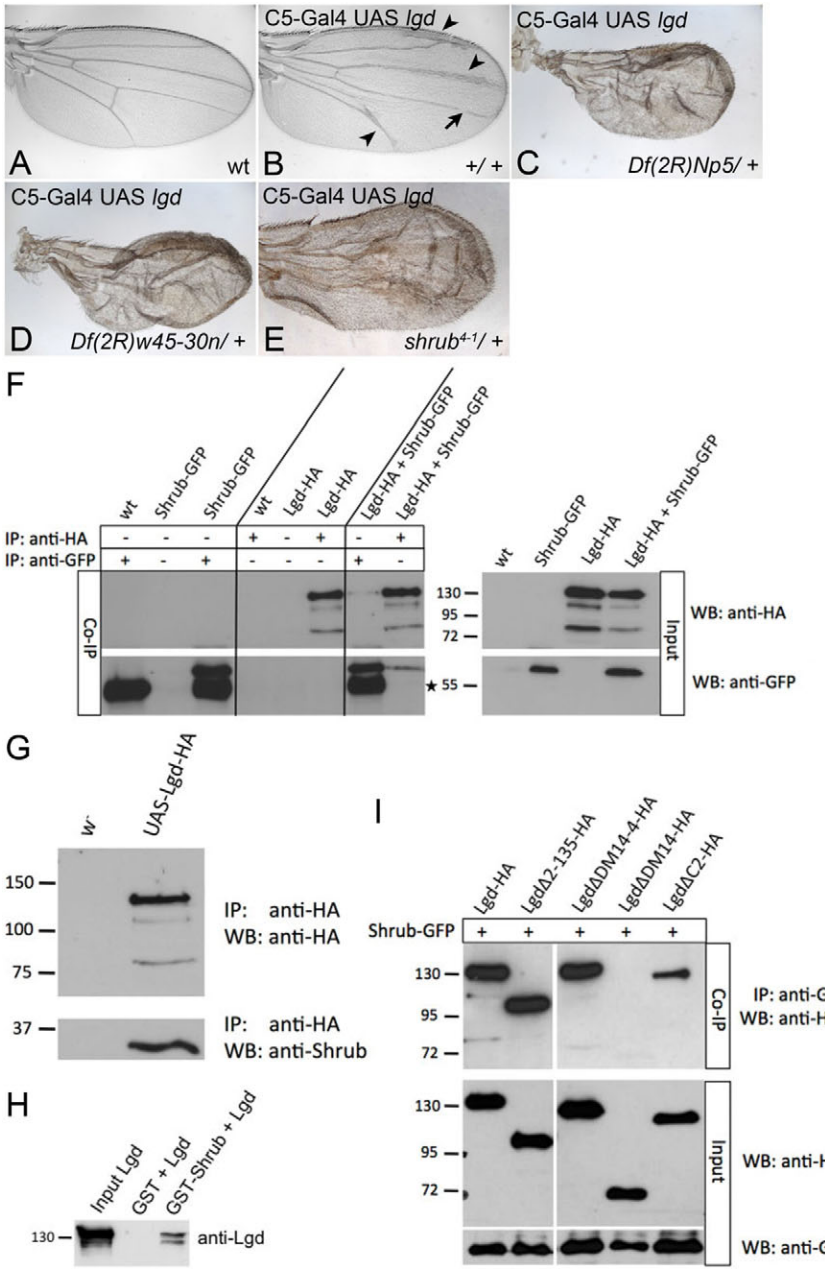

Fig. 4. Lgd physically interacts with Shrub. (**A**–**E**) Genetic modifier screen that identified Shrub as interaction partner. (A) Wild-type wing. (B) Wing phenotype caused by overexpression of UAS *lgd* with C5-Gal4. The overexpression causes partial thickening and short interruptions of wing veins (arrowheads and arrow, respectively). (C,D) The overexpression phenotype is modified by the presence of *Df(2R)Np* (C) and *Df(2R)w45-30n* (D). (E) A similar modification is observed by the presence of $shrub^{4-1}$. (**F**) Reciprocal coimmunoprecipitation of Shrub–GFP with Lgd–HA expressed with *hs*Gal4 without heat-shock. Asterisk highlights the IgG band. (**G**) Coimmunoprecipitation of endogenous Shrub with Lgd–HA expressed with *hs*Gal4 without heat-shock. (**H**) GST pull-down experiment using bacterially expressed Lgd and Shrub. (**I**) Immunoprecipitation of various deletion constructs with Shrub–GFP. All constructs are expressed with *hs*Gal4 without heat-shock. Only the variant lacking the DM14 domains is not precipitated.

sequences allows the removal of *lgd-RFP* in cells by Flp-mediated recombination and, thus, the induction of lgd^{d7} +/lgd^{SH495} $shrub^{4-1}$ cell clones (Fig. 5A). The lgd^{d7} +/lgd^{SH495} $shrub^{4-1}$ cells contained large Notch-positive endosomes, which were not observed in adjacent $shrub^{4-1}$/+ cells or in cells of lgd^{d7}/lgd^{SH495} control clones generated in the same manner (Fig. 5B,C, data not shown, see also supplementary material Fig. S3). The large endosomes were decorated with Spinster and Hrs (Fig. 5C–G). The apico–basal polarity of the lgd^{d7} +/lgd^{SH495} $shrub^{4-1}$ cells was not disturbed, as indicated by the normal apical localisation of Notch (not shown). In contrast to our expectations, we did not detect ectopic activation of the Notch pathway (Fig. 5H,I). We were able to recapitulate the genetic interactions by expressing an *lgd*–RNAi construct in *shrub* heterozygous flies (T.T. and T.K., unpublished data). In a similar experiment, we failed to observe similar genetic interactions of *lgd* and *vps20*, which encodes another component of the ESCRT-III complex (Vaccari et al., 2009) (data not shown). This suggests that *lgd* and *shrub* have a specific relationship.

Together, these results indicate that the physical interaction between Lgd and Shrub we have uncovered in vitro is important for their in vivo function.

Four DM14 domains provide robustness to the interaction between Lgd and Shrub

Our analysis indicated that any combination of two of the four DM14 domains is sufficient for function of Lgd. In order to further investigate the functional redundancy among the DM14 domains in Lgd, we tested the rescue abilities of our deletion constructs in the sensitised lgd^{d7} +/lgd^{SH495} $shrub^{4-1}$ situation

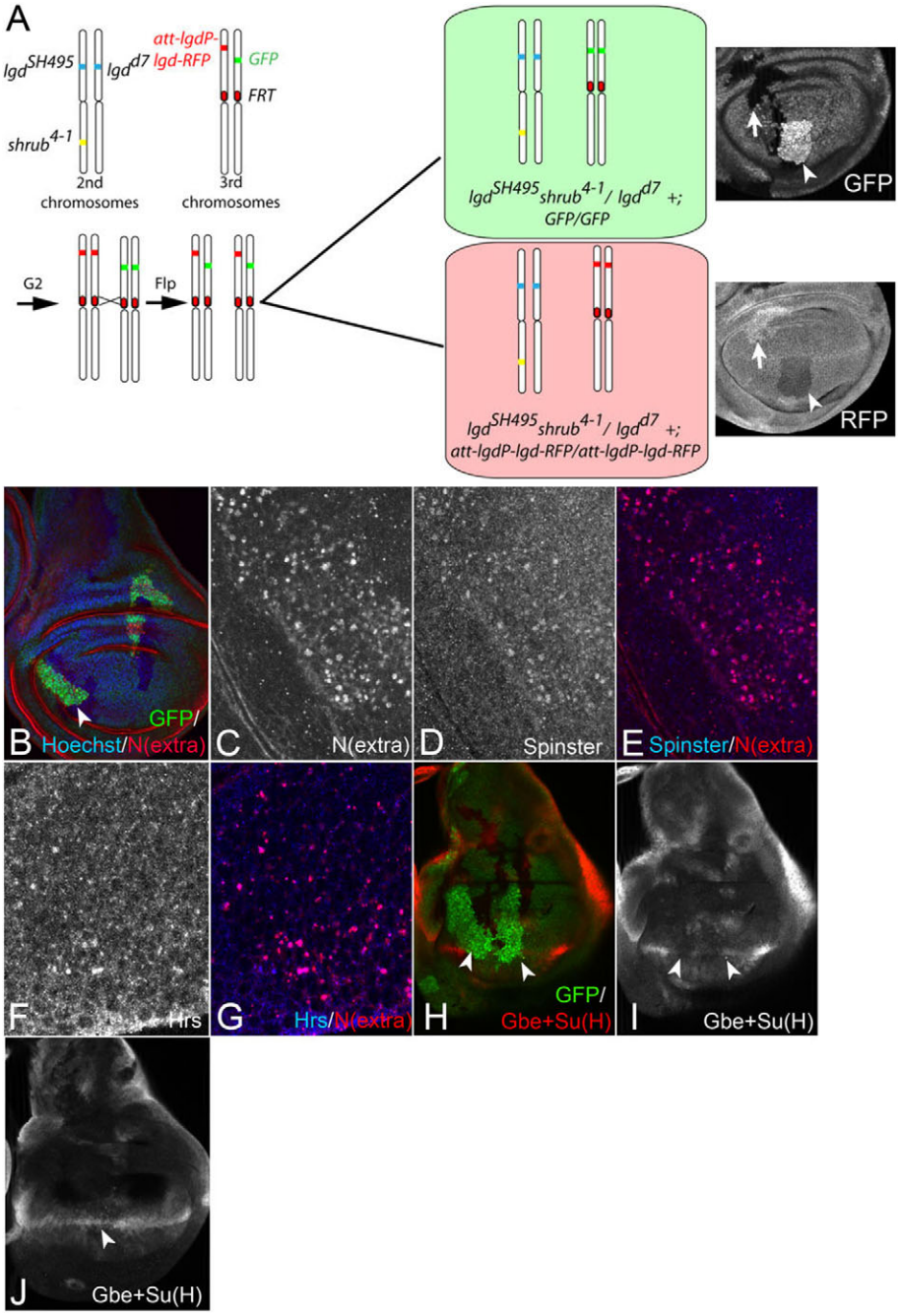

Fig. 5. Analysis of lgd^{SH495} $shrub^{4-1}/lgd^{d7}$ + cells. (**A**) Generation of lgd^{SH495} $shrub^{4-1}/lgd^{d7}$ + cell clones using a third chromosomal rescue construct (*att-lgdP-lgd-RFP*) on a FRT2A chromosome. The rescued cells do not show any recognisable endosomal abnormalities. The FRT2A site allows the Flp-mediated removal of the rescue construct from cells and thus the induction of lgd^{SH495} $shrub^{4-1}/lgd^{d7}$ + cells. These cells are labelled by the presence of two copies of the GFP construct and loss of RFP (arrowheads). (**B–G**) lgd^{SH495} $shrub^{4-1}/lgd^{d7}$ + cells contained dramatically enlarged endosomes that are positive for Spinster (B–E) and Hrs (F–G). (**H–J**) The Notch pathway is not activated in lgd^{SH495} $shrub^{4-1}/lgd^{d7}$ + cells, indicated by the absence of ectopic expression of the Notch sensor Gbe+Su(H)-*lacZ*. Arrowheads highlight the clones. (J) The same disc as in I in another focal plane to show that the expression of Gbe+Su(H) along the D/V boundary (arrowhead) is not interrupted.

(Fig. 6A–G). We found that the introduction of full-length *lgd* constructs rescued all aspects of lgd^{d7} +/lgd^{SH495} $shrub^{4-1}$ mutants, giving rise to fertile flies. The expression of Wg in the wing imaginal disc of these flies was normal (Fig. 6A–C). As expected, the constructs lacking all four (*lgdΔDM14*) or the first three DM14 domains (*lgdΔDM14-1-3*) were unable to rescue the mutant phenotype (not shown). By contrast, constructs lacking two domains partially rescued the lgd^{d7} +/lgd^{SH495} $shrub^{4-1}$ mutant flies (Fig. 6A,C–G). Flies of this genotype develop further than lgd^{d7} +/lgd^{SH495} $shrub^{4-1}$ flies, which enabled us to monitor their wing imaginal discs. In these discs, Notch was slightly activated ectopically, as revealed by the weak ectopic expression of Wg. The degree of ectopic expression varied among the constructs tested, indicating differences in their rescue abilities (Fig. 6D–G, arrows). The weakest rescue was found with constructs that lacked the second DM14 domain (Fig. 6D,G). This suggests that the second DM14 domain has the greatest importance for the function of Lgd. Note that all constructs tested were able to completely rescue lgd^{d7} amorphic flies (see Fig. 2).

The results show that reduction of the activity of *shrub* by half reveals a functional impairment of constructs that lack two DM14 domains. It therefore appears that the presence of four DM14 domains provides robustness to the function of Lgd. In addition, the results provide evidence that the DM14 domains mediate the interaction between Lgd and Shrub in vivo.

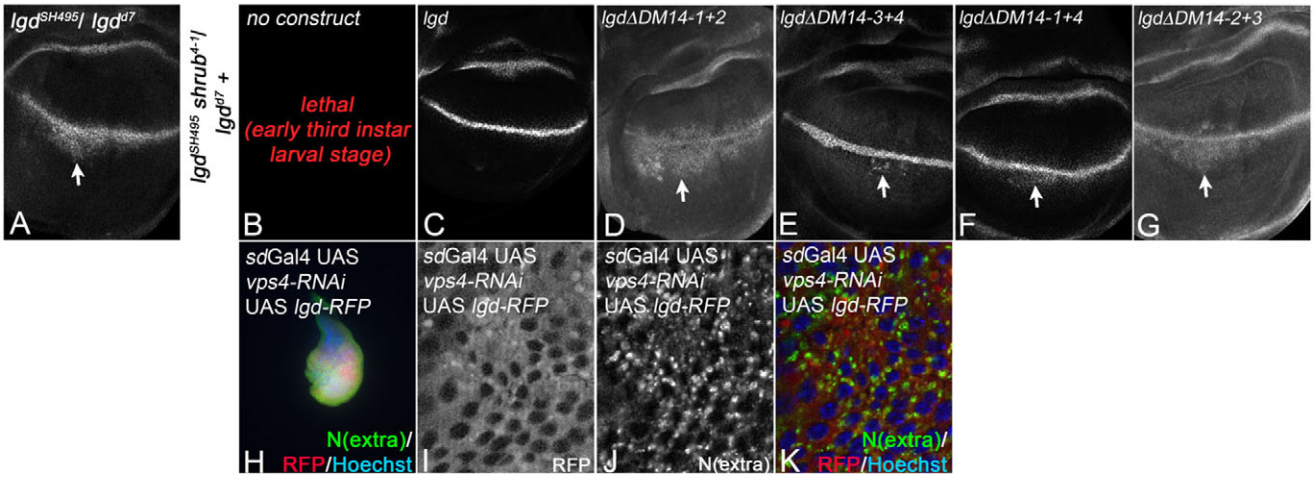

Fig. 6. Rescue of lgd^{SH495} $shrub^{4-1}/lgd^{d7}$ + wing imaginal discs with various deletion constructs. (**A**) The phenotype of the lgd^{SH495}/lgd^{d7} hypomorphic combination in the wing disc. The arrow points to a region with ectopic expression of Wg. (**B–G**) Rescue of lgd^{SH495} $shrub^{4-1}/lgd^{d7}$ + flies. (B) The flies die at the early stages of third larval instar in the absence of constructs. (C–G) Although Lgd-HA leads to a complete rescue (C), ectopic expression of Wg is still recognisable in the rescue experiment with construct encoding variants with two DM14 domains (arrows in D–G). Note that the constructs without the second DM14 domain (D,G) have less rescue abilities than the ones with it (E,F). (**H–K**) Coexpression of UAS *vps4-RNAi* and UAS *lgd-RFP*. Depletion leads to Notch-positive enlarged endosomes (J). Nevertheless, Lgd–RFP (I) is not associated with these endosomes (J,K).

Interaction between Lgd and Shrub appears to occur within the cytosol

The members of the ESCRT-III complex cycle between the cytosol where they exist in the monomeric closed form and the endosomal membrane where they assemble into the complex (Babst, 2005). The displacement of the subunits from the endosomal membrane requires the activity of the ATPase Vps4 (Babst, 2005). Loss of *vps4* function results in the arrest of ESCRT complexes on the endosomal membrane and ESCRT dysfunction (Babst, 2005). Indeed, location of ESCRT subunits on the endosomal membrane can only be detected if the cells are depleted of Vps4 (Babst et al., 2002). In normal cells, the subunits appear to be located in the cytosol. Because Lgd interacts with the ESCRT-III subunit Shrub, it is possible that Lgd cycles together with Shrub. Thus, a possible endosomal location might be only observed if the function of Vps4 is depleted. We depleted the function of Vps4 by expressing an UAS *vps4*-RNAi construct along with UAS *lgd-RFP* and monitored the cellular location of Lgd–RFP in the Vps4-depleted cells. Even under these circumstances, Lgd was not associated with endosomes or other membranes (Fig. 6H–K), suggesting that Lgd and Shrub interact in the cytosol.

Loss of ESCRT function suppresses the activation of Notch in *lgd* cells

An antagonistic relationship between *lgd* and *hrs*, which encodes a component of the ESCRT-0 complex, has been reported recently (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). We here found that loss of function of the other subunit of ESCRT-0, *stam*, also suppressed the activation of Notch in *lgd* cells [UAS *stam*-RNAi expressed with *scalloped* (*sd*)Gal4 or *hedgehog* (*hh*)Gal4 in *lgd* mutant discs] (Fig. 7A,E). This finding suggests that it is the loss of the ESCRT-0 function that prevents activation of Notch in *lgd* cells and not a possible ESCRT-0-independent function of Hrs.

It is possible that ESCRT-0 has another function, which is independent of MVB formation. The loss of this function might suppress the activation of Notch in *lgd* cells. Thus, we monitored the activity of Notch in *lgd* cells where ESCRT-III is impaired. Although the loss of *shrub* as well as the loss of *lgd* function results in ectopic activation of Notch (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006; Sweeney et al., 2006), we found no activation in lgd^{d7} +/lgd^{SH495} $shrub^{4-1}$ cells (Fig. 5H,I). Given the dramatic effect on endosome morphology observed in these cells, one could also expect an enhancement of the weak Notch activation normally observed in the hypomorphic *lgd* genotype. However, we were unable to detect activation of Notch in lgd^{d7}/lgd^{SH495} cell clones (not shown) and it is possible that the enhancement by loss of one copy of *shrub* is insufficient to enhance activation of Notch under these circumstances. Alternatively, *shrub* and *lgd* might function antagonistically to each other with respect to Notch activation. In order to resolve this question, we monitored the activity of Notch in cells that are homozygous for the null allele lgd^{d7} and heterozygous for $shrub^{4-1}$. Notch activation could be reliably detected in lgd^{d7} clones with Gbe+Su(H)-*lac*Z (Fig. 7B–D). This ectopic activation was completely suppressed in lgd^{d7} $shrub^{4-1}/lgd^{d7}$ + cell clones (Fig. 7F–H). In addition, we observed an endosomal phenotype in lgd^{d7} $shrub^{4-1}/lgd^{d7}$ + cells that was similar but stronger than in the hypomorphic lgd^{SH495} $shrub^{4-1}/lgd^{d7}$ + situation described above (supplementary material Fig. S3A–G). This indicates that the reduction in *shrub* activity suppresses the activation of Notch in *lgd* cells. In a further experiment, we expressed a UAS *myc-shrub* construct in lgd^{d7} mutant wing imaginal discs. Expression of this construct resulted in an enlargement of Notch-containing endosomes (Fig. 7I), indicating that its overexpression resulted in a negative effect on endosome function. When this construct was expressed in the posterior half of lgd^{d7} discs by *hh*Gal4, the activity of Notch was normalised (Fig. 7J), indicating that depletion of *shrub* function in *lgd* cells suppresses the activation of Notch.

Subsequently, we depleted *lgd* cells of the function of *vps20*, which encodes another ESCRT-III component. We found that

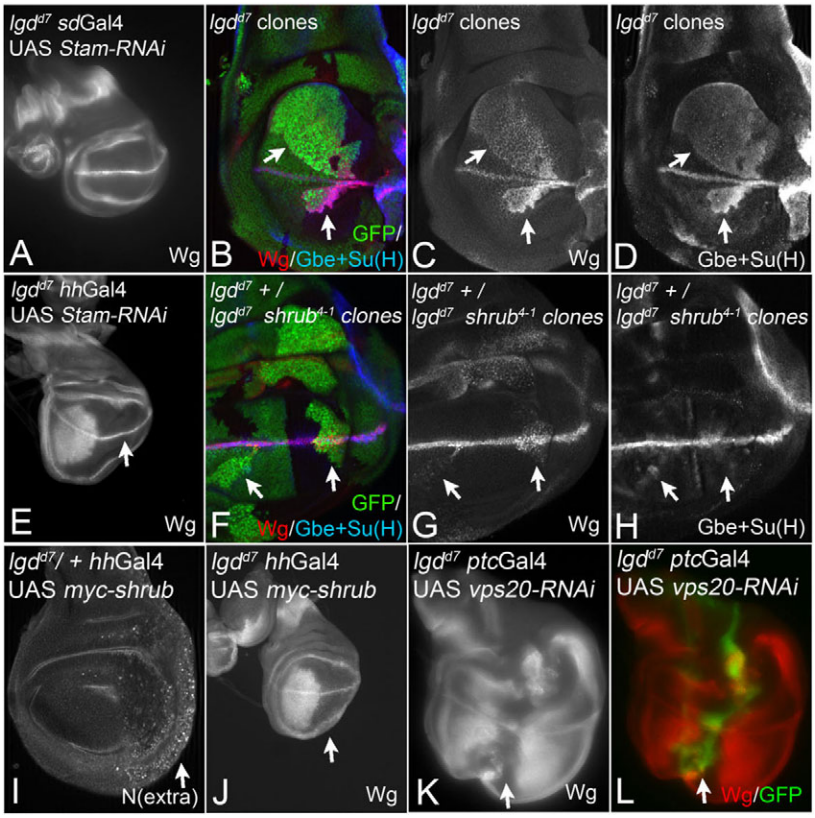

Fig. 7. Relationship between *lgd* and ESCRT genes. (**A**,**E**) Expression of UAS *Stam-RNAi* with *sd*Gal4 or *hh*Gal4 in lgd^{d7} wing imaginal discs results in normalisation of the expression of Wg. The arrow in E highlights the normalised expression. (**B**–**D**) Notch is ectopically activated in lgd^{d7} clones (clones are highlighted by the arrows), revealed by the ectopic expression of Wg and Gbe+Su(H)-*lac*Z. (**F**–**H**) Loss of one copy of *shrub* results in the suppression of Notch activation in *lgd* cells. Arrows highlight the lgd^{d7} $shrub^{4-1}$/lgd^{d7} + clones. No ectopic expression of Wg (G) and Gbe+Su(H)-*lac*Z (H) is observed. (**I**,**J**) Expression of UAS *myc-shrub* with *hh*Gal4 in lgd^{d7} mutant wing discs results in the formation of large Notch-containing vesicles (I) and the suppression of the ectopic activation of Wg (arrow in J). (**K**,**L**) Depletion of Vps20 through expression of a *vps20*-RNAi construct with *ptc*Gal4 results in the suppression of ectopic expression of Wg (arrow).

expression of UAS *vps20*-RNAi also resulted in suppression of Notch signalling in *lgd* null mutant discs (Fig. 7K,L). Thus, loss of ESCRT-III function suppresses the activation of Notch in *lgd* cells.

In summary, the experiments indicate that the activity of the ESCRT complexes is required for activation of Notch in *lgd* cells. The common function mediated by these complexes is formation of ILVs. Hence, it appears that formation of ILVs is a prerequisite for activation of Notch in *lgd* cells.

Discussion

Here we report the results of a detailed structure–function analysis of Lgd, a member of a recently discovered protein family whose hallmark is the possession of four tandem repeats of the uncharacterised DM14 domain. Although a recent study has reported a similar analysis for human Lgd2 in cell culture (Zhao et al., 2010), this is the first comprehensive analysis of a member of this uncharacterised protein family in an animal model. For the analysis we had to develop a new assay system that assured expression of the constructs at the level of endogenous *lgd*. This was necessary because we found that the process of protein trafficking is very sensitive to overexpression of Lgd. Thus, data obtained by overexpression of Lgd proteins (e.g. in cell culture) must be interpreted with great caution. This notion can probably be extended to other elements of the endosomal pathway, because we and others previously noticed dramatic changes in endosome morphology if other endosomal proteins, such as FYVE–GFP, Rab5–GFP or Rab7–GFP, are expressed with the Gal4 system (Jaekel and Klein, 2006; Wucherpfennig et al., 2003). Moreover, we found that overexpression of these proteins suppresses the activation of Notch in *lgd* cells (Jaekel and Klein, 2006). These findings indicate that the overexpression of endosomal proteins induces significant changes in protein trafficking through the endosomal pathway.

Using our assay, we found that the DM14 domains are important for the function of Lgd and that they constitute novel modules for direct interaction with a core member of the ESCRT-III complex during protein trafficking. Moreover, our analysis reveals that the DM14 domains provide the specific function of Lgd and function in a redundant manner. Using cell culture, Nakamura and colleagues provided evidence that the fourth DM14 domain of Lgd2 is especially important for its function as a scaffold protein that is required for PDK1/Akt signalling activated by the EGF pathway (Nakamura et al., 2008). However, we could not detect any specific importance of the fourth DM14 domain in our experiments in *Drosophila*. In our assay, any combination of two of the four domains appears to be sufficient for Lgd function and can rescue the *lgd* mutant phenotype.

However, this notion holds true only if the concentration of Shrub is normal. In situations where the activity of Shrub is reduced ($shrub^{4-1}$/+), variants with four domains can provide more activity and assure sufficient interaction to maintain correct endosomal trafficking. This was already observed in animals that are hypomorphic for *lgd* (lgd^{d7}/lgd^{SH495} $shrub^{4-1}$). In other words, four DM14 copies enable the organism to tolerate the *lgd shrub* double heterozygous situation. Because almost all Lgd-like proteins discovered so far have four copies, it is likely that this ability endows members of the family with a functional robustness that is evolutionarily advantageous. The rescue experiments in the sensitized *lgd* +/*lgd* $shrub^{4-1}$ backgrounds also suggest that the second DM14 domain is of greatest importance for the function of Lgd in *Drosophila*. This is in

contrast to results of cell culture experiments for human Lgd2 (Nakamura et al., 2008). However, it is important to point out that most of the evidence for function in mammals is obtained with cell culture experiments, which often involve the overexpression of Lgd orthologues at levels way above endogenous levels. Given our great difficulties in gaining sensible results using the Gal4 system, we believe these data should be interpreted carefully.

It has been previously shown that the C2 domain of Lgd can bind to certain phospholipids, such as phosphatidylinositol 3-phosphate, phosphatidylinositol 4-phosphate and phosphatidylinositol 5-phosphate, in an in vitro assay (Gallagher and Knoblich, 2006). Furthermore, cell fractionation experiments using cytosolic extracts from wild-type and the lgd^{08} mutant animals that encode a variant lacking the C2 domain, suggest that a small fraction is associated with the membrane in a C2-dependent manner. These biochemical data are contrasted by microscopy studies, which reported a cytosolic distribution of Lgd without any obvious association with membrane structures (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). In agreement, we found that tagged Lgd variants expressed at the endogenous level are localised within the cytosol. Moreover, we found that a *lgd* construct, encoding little more than the C2 domain and virtually identical to the Lgd fragment used in the in vitro phospholipid binding assay (Gallagher and Knoblich, 2006), is located in the cytosol similarly to Lgd. The discrepancy between the biochemical and microscopy data might be explained by the possibility that only a small fraction of Lgd (which cannot be detected in antibody staining) is associated with membranes. However, knowing that Lgd interacts with Shrub, it is surprising that we did not find any obvious association of Lgd even upon depletion of Vps4, although the ESCRT-III complex is locked on the endosomal membrane in this situation. One would expect that the membrane-associated fraction of Lgd should be increased in this situation. Thus, we believe that Lgd is located within the cytosol. This notion is further supported by the fact that variants of Lgd that lack the C2 domain can rescue the *lgd* mutant phenotype to a high degree, although they are produced at a much lower level than the other constructs tested and than endogenous Lgd.

We could determine three distinct functions for the C2 domain. The first function is that it provides protein stability, because we found that the constructs encoding variants without the C2 domain give rise to significantly lower amounts of protein than variants with the domain. The second function is the localisation of Lgd within the cytosol. This function provides an explanation for the discrepancy between the in vivo and biochemical studies, because we found that variants without the C2 domains are located in the nucleus. The reason for the mis-localisation of Lgd variants that lack the C2 domain is unclear at the moment. We have not discovered a cryptic nuclear localisation sequence (NLS) within Lgd. Thus, it is possible that it is transported in the nucleus in complex with another protein that contains an NLS.

The presented results suggest a third function for the C2 domain, because we found that LgdΔDM14, which cannot provide any specific function in the rescue assay, can out-compete NESLgdΔC2 in a C2-dependent manner and thereby prevent the partial rescue of *lgd* mutants. A likely possibility is that the C2 domain mediates an interaction with other proteins that results in concentration of Lgd at the site of action within the cytosol. In agreement with this possibility, recent reports have shown that the C2 domains of Nedd4L, PKC and PKCε mediate protein–protein interactions (Benes et al., 2005). Furthermore, human Lgd2/CC2D1A appears to interact via its C2 domain with the E2 enzyme Ubc13 during NF-κB signalling (Zhao et al., 2010). Therefore, we favour the possibility that the C2 domain of Lgd mediates protein–protein interactions instead of localising Lgd to a distinct membrane. It is possible that the cytosolic interaction prevents Lgd from migrating into the nucleus.

Recent results obtained in mammalian cell culture experiments suggest that human Lgd1 and Lgd2 might also act as transcriptional repressors (Hadjighassem et al., 2009; Ou et al., 2003). We find that Lgd requires location within the cytosol for its function. Hence, our results are not easily compatible with a function as a transcription factor, as suggested for human Lgd1 and Lgd2, and we believe that a gene regulatory function for Lgd in *Drosophila* is unlikely.

Lgd, the ESCRT machinery and Notch activation

Previous work has established that loss of function of ESCRT-I–ESCRT-III complexes results in non-autonomous and autonomous cell proliferation and activation of the Notch pathway (Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). In addition, the mutant cells lose their epithelial organisation and eventually die (Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). Although loss of function of *lgd* results in activation of the Notch pathway and overproliferation, these effects are cell-autonomous, and the mutant cells do not lose their polarity and survive well. Thus, the phenotypes of the two groups overlap, but are not identical. Nevertheless, we have found an intimate relationship between the ESCRT-III component Shrub and Lgd. Both proteins physically interact and this direct interaction is important in vivo, as indicated by the strong genetic interactions uncovered between the two genes. Importantly, we observed that the time of death for a hypomorphic allelic combination of *lgd*, which normally results in pharate adults, is earlier than that of *lgd* null mutants if the activity of *shrub* is reduced by half. The earlier time of death suggests that the function of *shrub* is impaired upon loss of *lgd* function. Thus, it appears that the physical interaction with Lgd is required for the proper function of Shrub. Because the loss-of-function phenotype of *shrub* is more deleterious and includes more aspects than that of *lgd*, it is likely that *lgd* contributes to, but is not absolutely required for, the function of *shrub*. Either loss of *lgd* results in the loss of one distinct aspect of Shrub function or it reduces its activity beyond a threshold that is required for complete function. Our finding that overexpression of Shrub can rescue the *lgd* phenotype supports the second possibility. Recent work suggests that Shrub forms long homopolymers on the cytosolic surface of the endosomal membrane. This polymerisation is required for the abscission of vesicles into the lumen of the maturing endosome (Saksena et al., 2009). In order to polymerise, Shrub has to be converted from a closed cytosolic into the open form (Babst et al., 2002). After ILV formation, Shrub becomes converted into the closed form by Vps4, with consumption of ATP. Because our data suggest that Shrub and Lgd interact in the cytosol, it is possible that Lgd somehow helps to prepare Shrub for the next round of polymerisation on the endosomal membrane.

The presented genetic studies suggest an antagonistic relationship between Lgd and several components of the ESCRT complexes with respect to Notch activation. This implies that activation of Notch in *lgd* cells depends on the function of the ESCRT complexes and therefore indicates that it

must occur in a different manner in *lgd* cells to that in ESCRT-mutant cells. Our results suggest that loss of *lgd* function somehow affects the activity of Shrub, which in turn results in the activation of Notch. It is important to point out that the antagonism between *lgd* and ESCRT is observed only with respect to activation of Notch signalling. With respect to endosome morphology, they appear to act synergistically because a reduction of *shrub* function by half results in a dramatic enlargement of endosomes of *lgd* hypomorphic cells, which normally do not exhibit such a defect. Our results therefore reveal a complex relationship between Lgd and the ESCRT function and further work is required to resolve this relationship in detail.

Because activation of Notch is not possible without release of the Notch intracellular domain (NICD) into the cytosol, we assume that a fraction or all of Notch must somehow remain at the limiting membrane of the endosome and is not incorporated into ILVs in *lgd* cells. There are three possibilities for how this might be achieved: no ILVs form; Notch might not be efficiently incorporated into the ILVs; or ILVs might back-fuse with the limiting membrane of the maturing endosome. Back-fusion has been documented to occur in vertebrate cells (Falguieres et al., 2009). Our results suggest that loss of *lgd* function results in a reduction in the activity of Shrub. Therefore, we favour the possibility that the loss of *lgd* function results in a less efficient incorporation of Notch into the ILVs due to the reduced activity of Shrub.

How is Notch that remains in the limiting membrane activated (see supplementary material Fig. S4 for a cartoon)? Activation of Notch in *lgd* cells is independent of the ligands, but dependent on the γ-secretase complex (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). The S3 cleavage of Notch mediated by γ-secretase requires previous shedding of its ectodomain (De Strooper et al., 1999; Struhl and Greenwald, 2001). This is normally performed by the ligand-dependent S2 cleavage through Kuz. Thus, we think that ectodomain shedding must occur in an alternative, ligand-independent manner in *lgd* cells. A possibility is that the ectodomain that reaches into the endosomal lumen might simply change its conformation because of the increasing acidification in the lumen. This conformational change might allow Kuz to access its normally hidden cleavage site in Notch to cleave the ectodomain independently of the ligands. Alternatively, the ectodomain might be degraded by the peptidases that become activated in the acidic environment of the late endosome or lysosome. The resulting NEXT-like intermediate could be cleaved by γ-secretase, and the intracellular domain would be released into the cytosol. In the second scenario, activation of Notch could be independent of Kuz. Thus, it is important to determine whether Kuz is required for the ectopic activation of Notch in *lgd* cells. It is also important to determine whether Notch is activated in the maturing–late endosome or in the lysosome: One possibility to explain the puzzling antagonism observed between Lgd and several components of the ESCRT complexes with respect to Notch activation could be explained by the synergistic endosomal morphology phenotype: if activation of Notch occurs in the lysosome and the synergism between *lgd* and ESCRT mutants on endosome morphology prevents fusion of the late endosome with the lysosome (e.g. through loss of association with the HOPS tethering complex), Notch activation would be suppressed in double mutant cells.

In any case, the data so far available suggest that Lgd is a general component of the endosomal machinery and that the activation of Notch in *lgd* cells is probably not caused by a specific defect in Notch regulation. It occurs because of a general defect in endosomal trafficking and because of the extraordinary mechanism of Notch activation.

So far no function in endosomal trafficking has been described for the mammalian orthologues. However, our data provide overwhelming evidence that Lgd is involved in endosomal trafficking in *Drosophila*. In addition, work by Tsang and colleagues suggests that the functional relationship between Lgd and Shrub is conserved in humans (Tsang et al., 2006). These authors have performed a yeast-two-hybrid screen in which they searched for proteins that interact with the ESCRT components. Among the identified proteins was human Lgd2/CC2D1A, which interacted with all three Shrub orthologues, CHMP4A, CHMP4B and CHMP4C.

Materials and Methods

Drosophila genetics

UAS lines used were UAS *Stam-RNAi* (#VDRC 22497), UAS *vps20-RNAi* (#VDRC 47653), UAS *vps4-RNAi* (VDRC #35126), UAS-*shrub-GFP* (Sweeney et al., 2006), and UAS *myc-shrub* (this work).

Gal4 lines were *hh*Gal4 (Tanimoto et al., 2000), *patched* (*ptc*)Gal4 (Speicher et al., 1994), *hs*Gal4 (Bloomington Stock Center), C5-Gal4 (Bloomington Stock Center) and *sd*Gal4 (Klein et al., 1997).

Other lines used were Gbe+Su(H)-*lac*Z (Furriols and Bray, 2001), *wg-lac*Z (Kassis et al., 1992), lgd^{17} FRT40A, lgd^{SH495} (Oh et al., 2003), lgd^{08} (Gallagher and Knoblich, 2006), $shrub^{4-1}$ FRTG13 (Sweeney et al., 2006), *Df(2R) Np5* and *Df(2R) w45-30n* (Bloomington Stock Center), *tub. rab5-CFP* and *tub. rab7-YFP* (Marois et al., 2006).

Clonal analysis

Clones were generated with the Flp/FRT system (Xu and Rubin, 1993) and induced at the first larval instar (24-48 hours after egg laying) by applying a 1-hour heat shock (37℃).

Generation of constructs and vectors

UAS *lgd* constructs were generated by PCR (splicing over extension) (Horton et al., 1989) using pUAST-*lgdHA* (Jaekel and Klein, 2006) as template. Amplified sequences were cloned into pUAST or *plgdPattB* using *Not*I and *Kpn*I (Brand and Perrimon, 1993). All constructs were sequenced prior to injection. Primer sequences are available from the authors upon request.

To generate the *plgdP-attB* rescue vector, the genomic region upstream and downstream (548 bp upstream of the translational start and 553 bp downstream of the translational stop of lgd/CG4713 harbouring a minimal MCS containing *Not*I, *Xho*I and *KpnI*,) were synthesized (Genscript, Scotch Plains, NJ). The DNA fragment was subcloned (out of pUC47) into *pattB* (Bischof et al., 2007) with *Bam*HI and *Xba*I.

The UAS *myc-Shrub* construct was generated by amplification of the corresponding cDNA (DGRC, GH13992). The N-terminal *myc*-tag fragment was cloned into the pUAST vector using *Eco*RI and *Xba*I.

Microscopy

Antibody staining was performed according to standard protocols. Antibodies used were anti-Wg antibody, mouse Notch antibodies against the extracellular (C458.2H) and intracellular (C17.9C6) domains, and anti-Cut (2B10) all from the Developmental Studies Hybridoma Bank (DSHB); anti-Rab7 (Tanaka and Nakamura, 2008); anti-Spin (Sweeney and Davies, 2002); anti-Hrs (Lloyd et al., 2002); and anti-HA (Roche). Fluorochrome-conjugated secondary antibodies were purchased from Invitrogen/Molecular Probes. Images were obtained with a Zeiss AxioImager Z1 Microscope equipped with a Zeiss Apotome. Nuclei were stained with Hoechst 33258 dye.

Western blot and coimmunoprecipitation

For western blots and coimmunoprecipitations, wandering third instar larvae were dissected in ice-cold PBS and homogenized in lysis buffer [10% glycerol, 50 mM HEPES pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA and 4 µl/ml protease inhibitor cocktail (Sigma)]. After 15 minutes of incubation on ice the lysates were centrifuged for 15 minutes at 4℃.

For western blots, SDS sample buffer was added to the supernatants, which were run on 10% SDS-PAGE gels according to standard procedures. After blocking (5%

milk powder in PBS), the blots were probed using the following antibodies (diluted in 2% milk powder in PBS): anti-HA (1:3000; 3F10, Roche), anti-GFP (1:2,500; TP401, Torrey Pines Biolabs), anti-shrub (1:1000; gift from Fen-Biao Gao, University of Massachusetts Medical School, Worcester, MA), anti-actin (1:10,000; Sigma), anti-tubulin (1:50,000; Sigma), anti-Lgd (1:3000; this work). HRP-conjugated secondary antibodies (Dianova) were used at 1:5000.

For coimmunoprecipitation, 30 larvae were homogenized in 150 µl lysis buffer (as described) and the supernatants were incubated with 1 µl anti-HA or anti-GFP overnight at 4℃. After incubation with 18 µl protein G-Sepharose 4 Fast Flow beads (GE-Healthcare) for 2 hours, the immunocomplexes were washed twice with lysis buffer and dissolved in SDS sample buffer prior loading onto 10% SDS-PAGE gels.

Generation of antibody against Lgd

A DNA fragment encoding the amino acid residues 362–663 of Lgd was amplified by PCR and cloned into pGEX-6P-2 (GE Healthcare) to generate a GST-tagged fusion protein. After expression in *Escherichia coli* (C41, OverExpress), the protein fragment was purified using glutathione Sepharose 4B medium (GE Healthcare) according to the user manual. After elution, the GST fusion part was removed by PreScission protease cleavage (GE Healthcare) and the Lgd fragment injected into guinea pigs (Cocalico Biologicals, Reamstown, PA). Specificity of the Lgd antibody was assessed by immunoblotting and immunostaining (data not shown).

Pull-down assay

The *shrub* cDNA (DGRC, GH13992) was cloned into the pGEX-4T1 vector (GE-Healthcare) to generate a GST fusion protein, and the full-length *lgd* cDNA was cloned into the pQE-30 Xa vector (Qiagen) to generate a His-tagged fusion protein. Expression of the two proteins in DH5α cells (Invitrogen) was induced by application of 1 mM IPTG (at 30℃). After purification with glutathione Sepharose 4B (GE Healthcare) and Ni-NTA-agarose (Qiagen), respectively, the His–Lgd protein was eluted from the agarose while the GST–Shrub remained coupled to the Sepharose beads. For the pull-down assay, equal amounts of His–Lgd and Sepharose-bound GST–Shrub (or Sepharose-bound GST alone as control) were incubated in pull-down buffer (20 mM Tris pH 8.0, 0.2 M EDTA, 1 mM DTT, 0.1 M NaCl, 0.2% IGEPAL, 1 µM Pepstatin, 0.8 mM Pefabloc, 0.3 µM Aprotinin and 5 µM Leupeptin). After incubation overnight at 4℃, the Sepharose beads were pelleted and washed with pull-down buffer. The protein complexes were dissolved using SDS sample buffer and analyzed via western blot.

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