Robinow Syndrome *DVL1* mutations disrupt morphogenesis and appendage formation in a Drosophila disease model

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Key words: Robinow Syndrome, disease model, DVL1, Wnt, limb formation, Drosophila

Abstract:

Robinow Syndrome is a rare developmental syndrome caused by mutations in numerous genes involved in Wnt signaling pathways. We previously showed that expression of patient variants in Drosophila and a chicken model disrupts the balance of canonical and non-canonical/PCP Wnt signaling. We also noted neomorphic effects that warranted further investigation. In this study, we examine morphological changes that occur as a result of one variant, DVL1$^{1519\Delta T}$, that serves as a prototype for the other mutations. We show that epithelial imaginal disc development is disrupted in legs and wings. Shortened leg segments are reminiscent of shortened limb bones seen in RS patients. We find that imaginal disc development is disrupted and accompanied by increased cell death, without changes in cell proliferation. Furthermore, we find altered dynamics of basement membrane components and modulators. Notably we find increased MMP1 expression and tissue distortion, which is dependent on Jnk signaling. We also find enhanced collagen IV (Viking) secreted from cells expressing DVL1$^{1519\Delta T}$. Through these studies we have gained more insight into developmental consequences of DVL1 mutations implicated in autosomal dominant Robinow Syndrome.

Introduction

Robinow Syndrome (RS) is a rare genetically heterogeneous disorder that is inherited as either an autosomal dominant or recessive disease. The two types can be distinguished by inheritance patterns and severity of phenotypes, with recessive typically leading to more severe clinical outcomes (Mazzeu *et al.*, 2007). In dominant RS, mutations have been identified in 7 genes and all give rise to a set of common phenotypes including “fetal facies” (hypertelorism or wide set eyes, wide nasal bridge, midfacial hypoplasia, micrognathia or smaller mandible and dental irregularities)(Zhang *et al.*, 2022). The limbs (especially the forelimbs) are characteristically shorter (mesomelia and brachydactyly) among other skeletal irregularities (Afzal *et al.*, 2000; Person *et al.*, 2010; Roifman *et al.*, 2015; White *et al.*, 2015, 2016, 2018; Abu-Ghname *et al.*, 2021). The mutations primarily affect components of Wnt signaling pathways, including *DVL1*, *DVL2*, *DVL3*, *WNT5A* and *FZD2*. Mutations in human Dishevelled 1 (*DVL1*) are the most commonly found variants implicated in autosomal dominant RS.

DVL1 mutations are characterized by a single nucleotide deletion in exon 14 which causes a frameshift that remarkably leads to an extended novel amino acid sequence shared amongst the variants, which replace at least 132 C-terminal amino acids found in wildtype DVL1 (White *et al.*, 2015; Hu *et al.*, 2022; Lima *et al.*, 2022; Zhang *et al.*, 2022). We have previously characterized the effects of three such variants ($DVL1^{1519\Delta T}$, $DVL1^{1529\Delta G}$ and $DVL1^{1615\Delta A}$; Fig. 1A) on Wnt signaling pathways using Drosophila and chicken models (Gignac *et al.*, 2023; Tophkhane *et al.*, 2024). By leveraging two model systems we were able to investigate the functional consequences of expression of *DVL1* variants relative to a reference wildtype human *DVL1* gene. We found that the variants disrupted the normal balance found in developing tissues between canonical β-catenin dependent Wnt signaling and non-canonical or planar cell polarity (PCP) Wnt signaling (Gignac *et al.*, 2023).

In our studies in developing Drosophila tissues, we expressed the human genes using the Gal4-UAS system which allowed us to precisely control the expression within domains of developing imaginal discs (Brand and Perrimon, 1993). We primarily characterized the effects in a small stripe of cells along the anterior-posterior boundary of the wing imaginal discs using the *dpp-Gal4* driver (Gignac *et al.*, 2023). In those studies, we were able to detect decreases in canonical Wnt pathway readouts as well as increased JNK/PCP signaling as assayed by JNK target gene expression as well as detecting the polarity of wing hairs. However, due to the small expression domain, the overall effects on development and viability were mild. We found that ubiquitous expression of DVL1 transgenes causes larval lethality, precluding an analysis of the effects. Thus, there is a need to evaluate the effects on tissue morphogenesis and appendage formation using other tools that allow us to study tissue development without causing early lethality.

In this study we demonstrate that expression of DVL1 RS mutations in broader domains compared to using *dpp-Gal4* disrupts morphogenesis of appendages and leads to severe disruptions in Drosophila development. Our studies revealed that the $DVL1^{1519\Delta T}$ variant can serve as a proxy for the other variants we previously studied and can reveal insights into changes in morphogenesis. Notably, we find severe disruptions in wing and leg formation that reveal that elevated cell death contributes to altered morphology while we found no elevated

proliferation. The effect on tissue morphology and expression of MMP1 are dependent on intact JNK signaling. Further, expression of the variant disrupts basement membrane integrity and cell apposition during metamorphosis. These effects provide insight into disruptions that may lead to altered limb formation in RS patients.

Results

DVL1$^{1519\Delta T}$ serves as a proxy for Robinow Syndrome *DVL1* variants

Our previous work has shown that when DVL1 variant expression was driven by *dpp-Gal4*, the adult wings displayed planar polarity defects as well as additional mild neomorphic phenotypes such as vein thickening, abnormalities in anterior cross vein, ectopic bristles on the wing margin of L3 longitudinal vein and a crease between L3-L4 veins. These phenotypes were exclusive to the variants only (Gignac *et al.*, 2023). Since the *dpp-Gal4* expression domain corresponding to the adult tissue is quite narrow, we wanted to express the variants by using drivers with larger expression domains. We also modulated the temperature at which the flies were raised, since Gal4 has enhanced activity at higher temperatures (Duffy, 2002). We first used *hedgehog-Gal4* (*hh-Gal4*) which is expressed in the posterior compartment of the wing and leg imaginal discs to assess phenotypes caused by human DVL1 proteins at 25°C (Fig. 1B). As shown in our previous work, wt*DVL1* had no effect on wing development (Fig. 1D). In contrast, *DVL1$^{1519\Delta T}$, DVL1$^{1529\Delta G}$* and *DVL1$^{1615\Delta A}$* all caused disruptions of the posterior domain in adult wings, which included thickening of longitudinal veins, severe reduction in wing tissue and some distortion of wing blade flatness (Fig. 1E-G). Based on these findings, and our previous work, we chose to focus subsequent studies on characterizing the effects of *DVL1$^{1519\Delta T}$* as a proxy for the three variants since it consistently phenocopied effects from *DVL1$^{1529\Delta G}$*. We showed that *DVL1$^{1615\Delta A}$* protein was less stable and when we drive its expression at higher levels, we observed similar effects as those found with the other variants (Gignac *et al.*, 2023). Thus, we are focusing here only on *DVL1$^{1519\Delta T}$* as a proxy for the other variants to gain insight into the functional consequences of an RS mutation on development.

DVL1$^{1519\Delta T}$ induces abnormal morphology in the notum and appendages

When expressed with the *hh-Gal4* driver at 25°C, the *DVL1$^{1519\Delta T}$* variant caused the development of smaller, blistered wings that had abnormally enlarged veins (Fig. 1E). In addition to these wing phenotypes, the flies had difficulty eclosing from their pupal cases and often could not walk, leading them to die after falling on the food due to an abnormal leg morphology. All 3 pairs of legs (termed L1-3) were malformed. The most distal part of the Drosophila leg called the tarsus contains 5 tarsal segments. In flies that expresses the *DVL1$^{1519\Delta T}$* variant in the *hh-Gal4* domain, the tarsi of of L3 often formed a curved structure that appears like a hook (Fig. 2x) which is never seen in wildtype flies. Furthermore, the femur and tibia segments were also shorter and appeared to have disrupted planar polarity.

When we expressed *DVL1$^{1519\Delta T}$* with *hh-Gal4* at higher levels by growing the flies at 29°C, we observed more severe phenotypes compared to the flies grown at 25°C (Fig. 2x). Variant expressing flies died inside their pupal cases at the pharate adult stage. Since the

expansion of wings occurs after eclosion, we were not able to image the adult wings. Variant expressing pupae were dissected out of their cases to document the disrupted morphogenesis (Fig. x). None of the wt*DVL1* expressing flies had difficulties eclosing at 29°C nor showed any obvious phenotypes (Fig. x).

We next expressed the $DVL1^{1519\Delta T}$ variant with the *ap-Gal4* driver which is expressed in the ventral compartment of the wing imaginal disc which gives rise to one side of the wing blade and the adult notum (or thorax) (Fig. 2x). During morphogenesis the two wing discs normally fuse to generate a seamless notum with a small posterior scutellum structure. Expression of the $DVL1^{1519\Delta T}$ variant disrupted wing development and wing expansion did not occur properly after eclosion, leading to shrivelled and blistered wings (Fig. 2x). The thorax of the flies was also abnormally developed. The flies had missing or malformed scutella and some of the thorax bristles were missing, and the remaining bristles showed PCP defects. No leg malformations were observed since *ap-Gal4* is not expressed in leg discs, enabling flies to eclose.

$DVL1^{1519\Delta T}$ disrupts morphology in larval wing imaginal discs

The phenotypes observed in adult flies with the expression of the $DVL1^{1519\Delta T}$ variant led us to investigate the morphology of wing precursor tissues in larvae. We investigated the wing imaginal discs that expresses the variant using *ap-Gal4* and *hh-Gal4* drivers. We used *UAS-GFP* in these crosses to mark the domains in which transgenes were expressed. Imaginal discs comprise a columnar epithelium (disc proper) and a thin squamous peripodial cell layer. In our studies we focussed solely on the disc proper.

hh-Gal4 was used to drive expression of a control *UAS-RFP* line, wt*DVL1* and $DVL1^{1519\Delta T}$ at 29°C and imaginal discs were dissected from male and female third instar larvae and stained with DAPI and for F-actin to reveal tissue morphology (Fig. 3A-C). Orthogonal views of discs reveal the apical-basal orientation of the disc proper (small insets in 3A'-C'). Expression of wt*DVL1* did not disrupt wing disc morphology (Fig. 3B-B') and orthogonal views showed a normal disc structure. The activity of $DVL1^{1519\Delta T}$ in the posterior side of the wing imaginal discs induced extra folds in every direction, making the wing disc appear highly distorted. All parts of the wing disc, notum, hinge and wing pouch showed abnormal morphology. The orthogonal view from showed disruption of the arc-like form of the wing discs. With all the extra folds, the wing discs appeared highly overgrown and multilayered. The tissue in the presumptive notum and hinge above the wing pouch (labeled in Fig. 1B) appeared highly misfolded on the basal surface of the wing imaginal discs (Fig. 3C').

When the $DVL1^{1519\Delta T}$ variant was expressed in the dorsal compartment with *ap-Gal4* at 25°C, we observed that the expression domain in the wing disc (marked by FLAG) was distorted, and the dorso-ventral border was expanded towards the ventral compartment (Fig. 3E-E') compared to control discs (Fig. 3D-D'). By showing single sections in a progression from apical to basal we can reveal the distortion in the wing tissue (Fig. 3D', E'). We also observed that the highly regulated folding structure of the wing imaginal disc was altered, and more layers appeared to be formed above the wing pouch especially on the posterior side of the wing imaginal discs. The abnormal folding was also present on the dorsal domain, in the notum.

DVL1$^{1519\Delta T}$ induces apically and basally shifted cells

In Fig 3 we showed that the tissue was distorted along the apico-basal axis. To investigate this in more detail we generated flp-out clones expressing either control (*white-RNAi)*, wt*DVL1* or *DVL1*$^{1519\Delta T}$ using *actin-Gal4* in wing discs. Control and wt*DVL1* clones both showed normal tissue architecture, apico-basal polarity and E-cadherin protein levels and localization. In contrast, *DVL1*$^{1519\Delta T}$-expressing clones were apically and basally shifted. In some cases cells appear to be extruding from the basal side of the disc. In addition to the decreased levels of β-catenin/Armadillo which we previously found after expressing *DVL1*$^{1519\Delta T}$ (Gignac *et al.*, 2023), we now show that *DVL1*$^{1519\Delta T}$ expression was accompanied by a mild decrease in E-cadherin (E-cad) staining (Fig. 4F') and reduced E-cad at apical junctions (Fig. 4F").

DVL1$^{1519\Delta T}$ does not induce cell proliferation in wing imaginal discs

After observing the highly altered morphology in wing imaginal discs that express the *DVL1*$^{1519\Delta T}$ variant in *apterous* and *hedgehog* domains, we wanted to investigate whether cell proliferation was being induced to cause the extra fold formation. To assess the relative amount of cell proliferation occurring between the wt*DVL1* and variant expressing wing imaginal discs, we used an antibody against a commonly used specific mitotic marker, Phospho-histone H3 (PH3).

We investigated the levels of cell proliferation in wing imaginal discs that expresses either wt*DVL1* or *DVL1*$^{1519\Delta T}$ variant in the *hedgehog* domain in comparison to the control discs at 25°C (Fig. 5A-C"). The number of PH3 puncta in all three genotypes were very similar and the variance was very small (Fig. 5D). Statistical analyses showed no significant difference between the discs that express the *DVL1*$^{1519\Delta T}$ or wt*DVL1* and the control. From these results, we concluded that the abnormal morphology of the wing imaginal discs induced by the expression of *DVL1*$^{1519\Delta T}$ variant is not due to cell proliferation or tissue overgrowth.

DVL1$^{1519\Delta T}$ induces cell death in wing imaginal discs

To further investigate the reasons underlying the altered morphology in the wing imaginal discs and the adult tissues induced by the expression DVL1 variant, we investigated the relative amount of cell death occurring between wild type and variant forms of *DVL1*. Our earlier work has found that the DVL1 variant expression causes an imbalance in Wnt signalling and increases the activity of noncanonical Wnt signalling, which mediates cytoskeletal organization and apoptosis (Gignac *et al.*, 2023). We also showed that when DVL1 variants were expressed, JNK signalling was ectopically induced, which also regulates apoptosis (Gignac *et al.*, 2023). Considering these alterations in signalling and the smaller thorax and adult wing phenotypes induced by the expression of DVL1 variant in different domains, we scored cell death in larval wing imaginal discs using an antibody against the cleaved Death caspase-1 (Dcp1) protein (Song, McCall and Steller, 1997), which is an effector caspase, activated after the cell goes into apoptosis.

We induced the expression of either RFP, wt*DVL1* or *DVL1*$^{1519\Delta T}$ by using the *hh-Gal4* driver at 25°C (Fig. 5E-G"). We counted the number of Dcp1 puncta in the posterior domain of the wing imaginal discs (Fig. 5H). We found that both wt*DVL1* and *DVL1*$^{1519\Delta T}$ expression caused a significant increase in the Dcp1 puncta numbers compared to the control discs (Fig. 5H). *DVL1*$^{1519\Delta T}$ variant expressing discs had significantly higher numbers of Dcp1 puncta than the wt*DVL1* expressing discs (Fig. 5H).

With these results, we conclude that the expression of the *DVL1* RS variant leads to increased levels of apoptosis in wing imaginal disc epithelia. These results support our finding that the abnormal morphology is not caused due to an increase in cell proliferation. *DVL1*$^{1519\Delta T}$ expression also disrupts cytoskeletal organization as we showed for E-cadherin (Fig. 4).

DVL1$^{1519\Delta T}$ effects depend on JNK signaling

To probe if *DVL1*$^{1519\Delta T}$-induced phenotypes are caused by JNK activation downstream of PCP signaling, we expressed the dominant negative form of Basket (*Drosophila* JNK; bsk^{DN}) (Adachi-Yamada *et al.*, 1999) to inhibit JNK signaling while expressing the RS variant. We then monitored two well established targets of JNK signaling, the transcriptional reporter *puckered-lacZ* (*puc-lacZ)* (Martin-Blanco *et al.*, 1998) and Mmp1 protein levels (Uhlirova and Bohmann, 2006) (Fig. 6). We first used *ap-Gal4* to drive expression of *DVL1*$^{1519\Delta T}$ and then co-expressed either a benign *UAS-GFP* (Fig. 6A-B) or the *UAS-bsk*^{DN} transgene (Fig. 6C-D). Wing discs were stained with DAPI to reveal overall tissue morphology, and the expression of *puc-LacZ* and the *DVL1* transgene were detected with antibodies against β-galactosidase (Fig. 6A'-D') and the FLAG tag (Fig. 6A"-D"), respectively. Expression of *DVL1*$^{1519\Delta T}$ and GFP revealed the ectopic *puc-lacZ* staining and distorted *ap-Gal4* domain that we previously reported (arrow in Fig. 6B, Fig. 6B')(Gignac *et al.*, 2023). In contrast, co-expressing *bsk*^{DN} with the DVL1 variant suppressed the ectopic *puc-lacZ* expression and suppressed tissue distortion (Fig. 6C',arrow in D, D').

We carried out a similar experiment using *dpp-Gal4* to drive *DVL1*$^{1519\Delta T}$ in a narrow stripe along the anterior posterior boundary of the wing disc (Fig. 6E-H). We previously showed that expressing any of the three DVL1 variants with *dpp-Gal4* leads to ectopic Mmp1 expression (Gignac *et al.*, 2023), which we show here with *DVL1*$^{1519\Delta T}$ (Fig. 6E', F'). This effect was reversed by co-expression of *bsk*^{DN} (Fig. 6G', H'). The results confirm that the RS variant effects on tissue morphology and ectopic induction of *puc-lacZ* and MMP1 are dependent on JNK signaling.

DVL1$^{1519\Delta T}$ disrupts cell adhesion in pupal wings

We found that *DVL1*$^{1519\Delta T}$ caused dramatic phenotypes in adult wings by altering wing development, creating enlarged veins and decreasing the overall size of the wing blade. We also observed numerous defects in tissue morphology in larval imaginal discs. Based on these results, we wanted to examine pupal wings to gain insight into alterations happening during metamorphosis. Pupal wing developmental stages are well described for flies grown at 25°C (Waddington, 1940; Diaz de la Loza and Thompson, 2017; Bier, 2000). We looked at the wings

of pupae 28-30 hours after puparium formation (APF) since the wings at this stage have actin hairs and veins and reflect the "definitive shape" of the adult wings.

We used *dpp-Gal4* to express the control, wt*DVL1* and the RS variant along with UAS-GFP in a narrow domain which corresponds to the region between the presumptive 3rd and 4th longitudinal veins (L3 and L4)(Fig. 7A). Expression of wtDVL1 had no effect on pupal wing morphology as revealed by staining for F-actin (Fig. 7B-B'). When $DVL1^{1519\Delta T}$ was expressed in this domain, it caused an apparent blistering phenotype in the 28-30 hr APF pupal wings (Fig. 7C-C', arrow). At this stage of the pupal wing development, the vein pattern is established. This blister phenotype appeared to be due to a failure of the dorsal and ventral layers of the wing to adhere together to form the proper vein and wing blade structure. To examine this idea, we looked at a cross section of the wing blade in two regions – one where no DVL1 transgene was expressed (Fig. 7D, box E) and one within the region that appeared to be blistered (Fig. 7D, box E'). In normal wing tissue at 28-30 hrs APF the two sheets of epithelial cells can be seen to be tightly apposed at their basal surfaces, as their cell shapes are outlined by F-actin and DAPI reveals nuclear position (Fig. 7E). In contrast, in the region of the pupal wing that showed a blister, there is a large gap between the two sheets of cells and loose cells can be seen floating in the lumen (Fig. 7E').

An earlier study has shown that the overexpression of Matrix-metalloproteases (MMPs) can induce blister formation in pupal wings (Sun *et al.*, 2021). We previously showed that the RS variants induce ectopic MMP1 expression in wing imaginal discs (Gignac *et al.*, 2023). Thus, we investigated if the ectopic expression of MMP1 is also present in the pupal wing blisters. Staining of either control or *dpp*>wt*DVL1* pupal wings using an antibody against MMP1 showed very low-level expression (Fig. 7F'-G'). In contrast, we found that $DVL1^{1519\Delta T}$-induced blister shows elevated Mmp1 protein in 28-30hr APF pupal wings (Fig. 7H-H').

We next looked at the blisters more closely. The proper adhesion of the dorsal and ventral layers of the wings occur with the slow degradation of the basement membrane (Sun *et al.*, 2021). Since we hypothesized that there was an alteration in the adhesion process, we wanted to look at the basement membrane. We chose to target Drosophila Collagen IV called *Viking* (*Vkg*) as a proxy for the basement membrane since it is the most abundant component. We used Vkg-GFP expressing flies, which express an endogenously tagged Vkg-GFP fusion protein to visualize the basement membrane in pupal wings.

The variant and wt*DVL1* expression were driven by *dpp-Gal4* driver (Fig. 8B-C). We found that the Vkg-GFP is not abundant in the control pupal wing, we only observed a faint GFP signal withing the veins (Fig. Fig. 8A'-A"). We observed a sharper Vkg-GFP signal in the veins of the wt*DVL1* expressing pupal wings (Fig. 8B'-B"). The vein pattern was mostly comparable to control wings, however, L4 longitudinal vein of wt*DVL1* expressing pupal wings appeared malformed and sometimes bent. The pupal wings that express the $DVL1^{1519\Delta T}$ variant had the most intense Vkg-GFP signal (Fig. 8C'-C"). The veins and the area around the veins seemed to be filled with Vkg-GFP. The L3 longitudinal vein is enlarged, forming a blister-like structure. The region between the L3 and L4 longitudinal veins appeared much narrow supporting the idea that

Discussion

Robinow Syndrome mutations in the DVL1 gene cause a range of clinical phenotypes including bone malformations (Afzal *et al.*, 2000; Person *et al.*, 2010; Roifman *et al.*, 2015; White *et al.*, 2015, 2016, 2018; Abu-Ghname *et al.*, 2021). In our previous work we described that DVL1 frameshift mutations induce an imbalance in Wnt signaling pathways in chick and Drosophila models of RS (Gignac *et al.*, 2023). In that study we also described several neomorphic phenotypes in adult wings that suggested the variants could disrupt other developmental processes. Among these was an unusual crease in the region between longitudinal veins 3 and 4, as well as disruption in the crossvein. In this current study we have used Drosophila limbs and wings to investigate cellular changes that occur following expression of the prototypical RS variant $DVL1^{1519\Delta T}$.

DVL1 variants induce neomorphic phenotypes

Expression of $DVL1^{1519\Delta T}$ in broad domains within developing imaginal discs disrupted development of the organization of the columnar epithelial cells. These effects lead to f=dramatic effects in leg discs, causing shortening of limb segments analogous to bone shortening seen in RS patients. Furthermore, we found defects in the notum that suggested failure to form a seamless scar after the wing imaginal discs fuse during morphogenesis. Wing formation is severely disrupted and drove us to investigate wing disc phenotypes more thoroughly.

DVL1 variants disrupt morphology in imaginal wing discs

Based on the phenotypes we saw in the adult tissue that was expressing the DVL1 variant, we were curious to look at tissue morphology at earlier stages. We saw evidence of tissue malformation in imaginal wing discs with three different Gal4 drivers. In addition, the nuclei on the basal side appeared more punctate and spread apart, which could be indicative of cell death or migration (Dove, 2003; Bell and Lammerding, 2016). There was also a shift in the nuclear staining trending apically. The basally directed folding of the posterior side of wing discs was very striking and consistent across wing discs. One possibility for this folding could be tension induced from half the wing discs being overgrown while the other half was normal.

Robinow Syndrome variants induce cell death

We observed changes in tissue morphology and investigated if altered proliferation or cell death contribute to the effects. To our surprise, there was no difference in cell proliferation as measured by pH3 staining across all genotypes tested. In contrast, we found induction of cell death after expression of either wt*DVL1* or $DVL1^{1519\Delta T}$. Quantification showed that the variant significantly increased cell death relative to control and wt*DVL1*. These results suggest that altered cell death could contribute to the apparent misfolding of the disc by changing tension within the disc as cells die. Furthermore, dying cells often extrude basally from tissues (Fadul and Rosenblatt, 2018), which we see in the cross sections of flip out expression clones. These cells may contribute to the floating cells seen within the pupal blisters.

DVL1 variants disrupt dorso-ventral cell adhesion in pupal wings

The blistering in the pupal wings where DVL1 variants were expressed was intriguing. Previous studies have shown that the overexpression of Matrix metalloproteinase-2 (Mmp2) in

the wing tissue can induce blisters (Sun *et al.*, 2021). In that study, researchers hypothesize that in order for the dorsal and ventral layers of the wing to adhere, the basement membranes would need to be slowly degraded. If they were degraded too fast or not sufficiently, wing blisters would be induced. We had previously shown that MMP1 was induced in imaginal wing discs (Gignac *et al.*, 2023) and so we tested if this overexpression was sustained in the pupal wing and found that indeed the blisters were filled with secreted MMP1. While MMP1 preferentially cleaves the extracellular connections between adjacent cells and Mmp2 preferentially cleaves the connections between cells and the extracellular matrix, Drosophila studies have shown there is functional redundancy between them (Jia *et al.*, 2014). Future investigations will look into the integrity of the basement membrane in pupal wings where wt*DVL1* or *DVL1* variants are expressed to see if the overexpression of Mmp1, induced by the DVL1 variants, leads to the premature degradation of the basement membrane and thus interferes with dorso-ventral cell adhesion in early pupal stages. Of note, in our previous work we showed abnormal creases seen in adult wings that may be residual effects of the pupal blisters after they resolve.

In summary, our work has shown that DVL1 mutations implicated in RS can be modeled in Drosophila appendages where we can gain deeper understanding of the cellular consequences of dominant variant expression. The defects in leg segments is particularly intriguing given that RS is characterized by shortened limb bones. Future work will delve into signaling pathways that control leg specification, growth and morphology.

Materials and methods:

Drosophila Husbandry

Flies were raised on standard media. Stocks were kept at room temperature (~22°C) and crosses were performed at 25°C or 29°C as indicated. Three Gal4 driver fly lines were used to induce transgene expression: *dpp-Gal4, UAS-GFP* /TM6B (Swarup, Pradhan-Sundd and Verheyen, 2015), *Dll-lacZ/Cyo; Hh-Gal4/TM6B* (Hall *et al.*, 2017)*, apterous (ap)-Gal4* (Bloomington #3041). Additional stocks used were: *;Vkg-GFP;* (Flytrap) (Blaquiere *et al.*, 2018), *puc-LacZ* (Martin-Blanco *et al.*, 1998)*, w^{1118}*, *UAS-GFP*, *UAS-myr-RFP* (Bloomington #7118)*, UAS-bskDN* (Bloomington #6409) (Adachi-Yamada *et al.*, 1999).

Generation of somatic flip-out clones

To generate heat-shock inducible actin flip-out clones, *yw,hsFlp[122];;act>CD2>Gal4, UAS-RFP/TM6B* was used. A 10 min heat shock (37°C) was applied to larvae grown for two days after egg laying (25°C). After heat shock, the larvae were grown at 29°C and dissected when they reached third instar larval stage.

Dissections

Wing imaginal discs: Third instar larval wing imaginal discs were dissected out of larvae in cold PBS. The top half of the larvae were separated and flipped inside out to expose the imaginal

discs. Fat tissue and the guts were removed while leaving the imaginal tissue attached to the cuticle.

Pupal wings: Pupal wings were dissected as described by Bolatto and coworkers (Bolatto et al., 2017) with some modifications. 0hr APF pupae were collected and placed ventral side down onto a microscope slide covered with double-sided tape. The microscope slides were placed at 25°C until the desired stage of development. Pupal cases were opened with forceps and naked pupae were transferred onto a new microscope slide with double-sided tape and covered with 4% paraformaldehyde (PFA) in PBS. After a 30-minute incubation, pupal wings were dissected out and placed into a 4% PFA filled well of an HLA plate for an additional 15-minute incubation. All immunofluorescence experiments with pupal wings were performed in Greiner HLA Terasaki multiwell (54-well) plates rather than Eppendorf tubes.

Pharate Pupae: Pupae were gently pulled off the sides if vials and placed on double sided tape on a microscope slides. Forceps were used to pry open the operculum and tear away the case. Pharates were then photographed using a Leica dissection microscope and an iPhone.

Adult wings: Adult flies of the target genotype were collected in small glass vials filled with 70% ethanol. The wings were dissected using forceps in 95% ethanol in dissection dishes and mounted immediately. Aquatex (EMD Chemicals) was used as mounting media. The slides were baked overnight at 65°C incubator with small weights on them.

Immunofluorescence staining, microscopy, and image processing

Tissue was dissected in phosphate-buffered saline (PBS) and fixed in 4% PFA at room temperature for 15 minutes. Samples were washed twice for 10 minutes with PBS with 0.1% Triton X-100 (PBS-T). Following a 1-hour block with 5% bovine serum albumin diluted in PBST at room temperature, samples were incubated overnight with primary antibodies at 4°C. The following primary antibodies were used:

List of primary antibodies used in this study

Antibody	Dilution	Clone	Distributor
Mouse anti-FLAG	1:500	M2	Sigma
Rabbit anti-FLAG	1:500	SIG1-25	Sigma
Rabbit anti-Dcp1	1:500	Asp215	Cell Signaling
Rabbit anti-PH3 Ser10	1:500	D2C8	Cell Signaling
Mouse anti-Armadillo	1:50	N2 7A1	DSHB
Mouse anti-β-Galactosidase	1:50	40-1a	DSHB
Rabbit-anti-GFP	1:500	A-11122	Thermo Fisher
Mouse anti-Mmp1	1:100 (each)	3A6B4, 3B8D12, 5H7B11	DSHB

rat anti-DE-Cadherin	1:50	DCAD2	DSHB

Samples were washed twice for 10 minutes with PBS-T (0.5% Triton-X100 in PBS) and incubated with Cy3 and/or Alexa Fluor 647-conjugated secondary antibody (1:500, Jackson ImmunoResearch Laboratories), DAPI (4',6-Diamidino-2-Phenylindole) and/or Rhodamine Phalloidin (Invitrogen™ R415). for 2 hr at room temperature. After two 10- minute washes, samples were mounted in 70% Glycerol in PBS or VECTASHIELD® Antifade Mounting Medium (Vector Labs) and imaged using a Nikon Air laser-scanning confocal microscope or a Zeiss LSM880 with Airyscan confocal microscope. Images were processed with ImageJ, FIJI software (Schindelin *et al.*, 2012; Schneider, Rasband and Eliceiri, 2012) and are presented as Z-stack maximum intensity projections unless otherwise stated.

Quantification of cell death and cell proliferation using Dcp1 and PH3 staining

After completing the staining and mounting steps, wing imaginal discs were imaged as described in the previous section. Using FIJI (Schindelin *et al.*, 2012), the *ap-GFP* or *hh-Gal4* domains on wing imaginal discs were marked. PH3 or Dcp1 positive cells were counted within each region automatically using the Analyze → Analyze Particles tool after thresholding. The difference between the number of PH3 or Dcp1 puncta within the *apterous* or *hedgehog* domains were then plotted.

Statistical analyses

Statistical analyses of the plotted data of mRNA and protein expression were done using one-way analysis of variance (ANOVA). For immunofluorescence experiments, paired (Arm stability within 1 wing disc) or unpaired (all other experiments) t-tests were performed to determine p-values. All statistical analyses were performed using GraphPad Prism 9.3.1 (GraphPad Software, San Diego, USA). Analyzed data with $p<0.05$ was considered statistically significant. Significance depicted as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns = not significant.

Acknowledgements:

We are grateful to the Developmental Studies Hybridoma Bank (IA, USA) and the Bloomington Drosophila Stock Centers for providing antibodies and fly strains. The images in Fig. 1A-B were created with BioRender.

Funding:

This work was funded by the Canadian Institutes of Health Research (grant PJT- 166182 to J.M.R. and E.M.V.).

Figure legends:

Fig. 1 *DVL1$^{1519\Delta T}$* serves as a proxy for Robinow Syndrome variants

(A) Graphical representation of human DVL1 protein and three variants associated with Robinow Syndrome. In each variant, a single nucleotide deletion results in a frameshift which causes the addition of a lengthy novel peptide (indicated in yellow) which is shared in large part between all three variants. (B) The *hedgehog-Gal4* (*hh-Gal4*) driver is expressed in the posterior domain of wing imaginal discs which corresponds to the posterior half of adult wings (indicated by green shading). The presumptive notum, hinge and wing pouch domains are indicated in the disc. (C-G) Representative adult female wings from crosses grown at 25°C of the following genotypes: (C) control *hh>RFP* (D) *hh>wtDVL1*, (E) *hh>DVL1$^{1519\Delta T}$,* (F) *DVL1$^{1529\Delta G}$ and* (G) *DVL1$^{1615\Delta A}$.*

Fig. 2 *DVL1$^{1519\Delta T}$* induces abnormal morphology in adult fly appendages and thorax

(A-C) Male adults from crosses grown at 25°C of the indicated genotypes. Leg and wing defects are visible in the variant (C). Single third legs (L3) showing of the indicated genotypes. (A”-C”) Males from crosses grown at 29°C to drive higher transgene expression. Pharate pupa in C” was dissected from pupal case. (D-F) Females from crosses of the indicated genotypes grown at 25°C. (D-F) The adult notum and scutellum. (D’-F’) Adult wings of indicated genotypes.

Fig. 3 *DVL1$^{1519\Delta T}$* variant disrupt morphology in larval wing imaginal discs

(A-C) Wing imaginal discs from female larvae grown at 29°C and using *hh-Gal4* to express transgenes. Images showing DNA (DAPI, blue), F-actin (red) for (A, A’) control *UAS-RFP* (B-B’) wt*DVL1* and (C-C’) *DVL1$^{1519\Delta T}$* wing discs mounted on bridged slides to retain their three-dimensional shape. (A-C) Maximum projections, (A’-C’) single plane images with orthogonal views of the tissue. Scale bar = 100 µm. (D-E) Wing imaginal discs from female larvae grown at 25°C and using *ap-Gal4* to express control and *DVL1$^{1519\Delta T}$* transgenes, as indicated. DAPI (blue) and anti-FLAG or GFP (magenta) were used to show the cells expressing transgenes in merged images (D, E) while single channel DAPI (white) staining shown is in D’, E’ to highlight tissue morphology. Single plane images are organized apical to basal as labelled.

Fig. 4 Epithelial organization is disrupted by *DVL1$^{1519\Delta T}$*

(A-B”) Control heat shock inducible flip out clones in wing discs are positively marked with RFP. DNA is labelled with DAPI (blue). (B, D, F) Zoomed in regions highlighted by yellow boxes in A, C, E. (B-B”) E-cadherin protein localization in at the apical surface (B’) and orthogonal view (B”) shows apical enrichment in columnar epithelial cells of disc. Cells in flip out clones are marked with RFP and extend along the apical-basal axis of the disc. (C-D”) Clones of cells expressing wt*DVL1*. (F-F”) Clones of cells expressing *DVL1$^{1519\Delta T}$*. In F”, cells are seen at the apical and basal surfaces of disc, and E-cadherin staining appears reduced (arrow).

Fig. 5 DVL1$^{1519\Delta T}$ does not promote proliferation but increases cell death

(A-C) Merged confocal microscopy images showing the *hh-Gal4* UAS transgene expression domain (anti-FLAG or RFP, red), DNA (DAPI, blue) and PH3 (white) staining in (A) control, (B) wt*DVL1* and (C) *DVL1$^{1519\Delta T}$* wing discs grown at 25°C. (A’, B’, C’) The *hh-Gal4* domain of

representative wing discs is shown with the RFP or FLAG staining images. (A'', B", C'') PH3 staining for all genotypes are shown. (D) The number of PH3 puncta counted within the *hh-Gal4* domain are plotted. (E-G) Merged confocal microscopy images showing the *hh-Gal4* UAS transgene expression domain (anti-FLAG or RFP, red), DNA (DAPI, blue) and anti-Dcp1 (white) staining in (E) control, (F) wt*DVL1* and (G) $DVL1^{1519\Delta T}$ wing discs grown at 25°C. (H) The number of Dcp1 puncta counted within the *hh-Gal4* domain are plotted. Sample size is shown under each genotype. Statistics were performed with ANOVA. Scale bar = 100 µm.

Fig. 6 $DVL1^{1519\Delta T}$ effects are mediated through JNK signaling

(A-D') *ap-Gal4* was used to express $DVL1^{1519\Delta T}$along with either control *UAS-GFP* (A-B") or $UAS\text{-}bsk^{DN}$ (C-D"). (B, D) Magnified views of regions shown in white boxes in A and C. Wing discs were stained with DAPI to reveal tissue organization, and the transcriptional reporter *puckered-lacZ* (*puc-lacZ*) as a readout of Jnk signalling. Anti-FLAG antibodies were used to reveal cells expressing transgenes. (E-H") dpp-Gal4 was used to express $DVL1^{1519\Delta T}$along with either control *UAS-GFP* (E-F") or $UAS\text{-}bsk^{DN}$ (G-H"). (F, H) Magnified views of regions shown in white boxes in E and G. Antibodies were used to detect MMP1 protein levels and FLAG.

Fig. 7 Pupal wing development is disrupted by expression of a DVL1 variant

The *dpp-Gal4>UAS-GFP* expression domain is shown in green and actin (rhodamine-phalloidin, red) staining in (A) control, (B) wt*DVL1* and (C) $DVL1^{1519\Delta T}$ pupal wings dissected 28hrs after puparium formation (APF). Single channel actin (white) stain shown in (A', B', C'). Arrow indicates pupal wing blister. (D) $DVL1^{1519\Delta T}$ pupal wing in which areas shown in E and E' are indicated. Cross sections outside (E) and inside (E') of the *dpp-Gal4* expression domain are shown. (E-E') Single plane images of orthogonal views showing F-Actin (green) and DNA (blue) staining in 28hr APF $dpp{>}DVL1^{1519\Delta T}$ pupal wings above (A) and inside (B) of a wing blister. Crosses were performed at 25°C and female and wings are shown.

Fig. 8 $DVL1^{1519\Delta T}$ induces Viking accumulation

28hr APF pupal wings showing F-Actin (red) and Vkg-GFP (green) staining in control (A), wt*DVL1*- expressing (B) or $DVL1^{1519\Delta T}$-expressing (C) tissue at 10x zoom. 63x zoomed in images of the region marked by the yellow squares in A-C are shown in A'-C' respectively also with DAPI staining showing the cell nuclei in blue. Crosses were performed at 25°C. Scale bar = 100 µm.

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Fig. 1 DVL1$^{1519\Delta T}$ serves as a proxy for variants

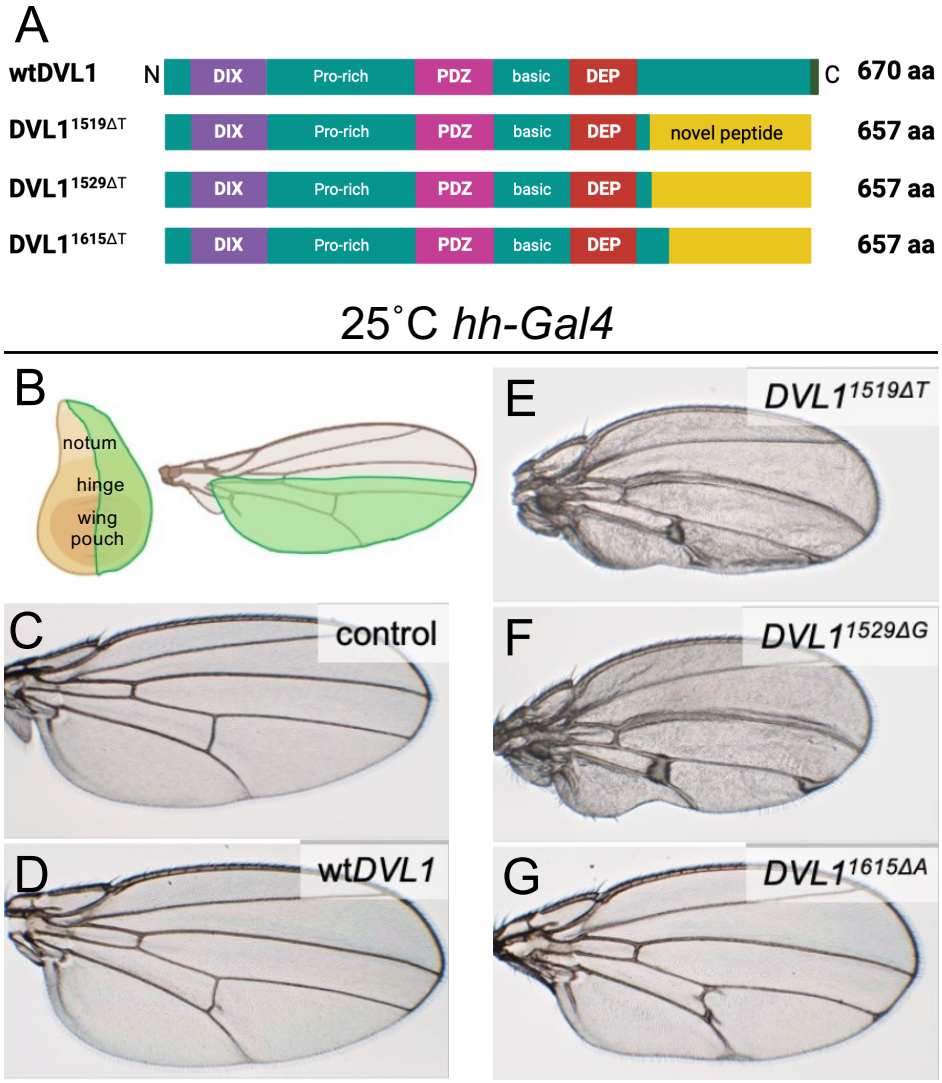
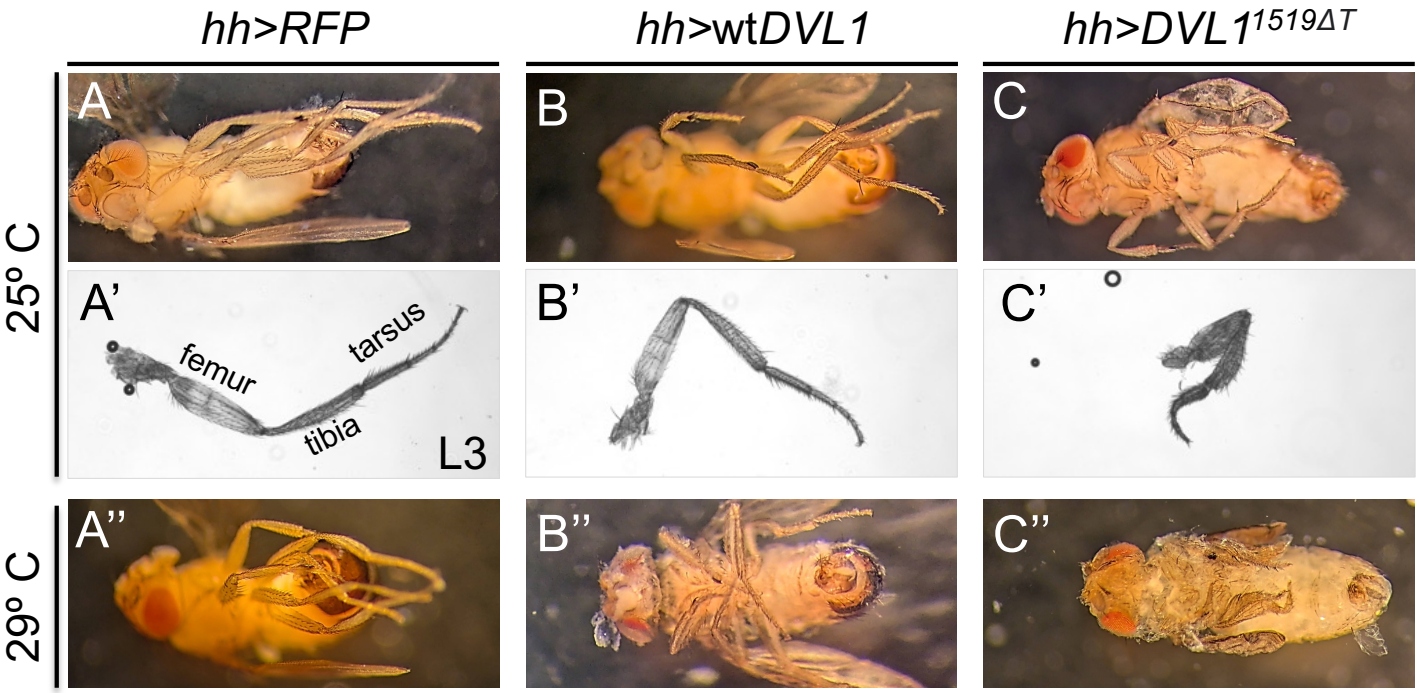


Fig. 2 *DVL1*$^{1519\Delta T}$ induces abnormal morphology in adult fly appendages and thorax



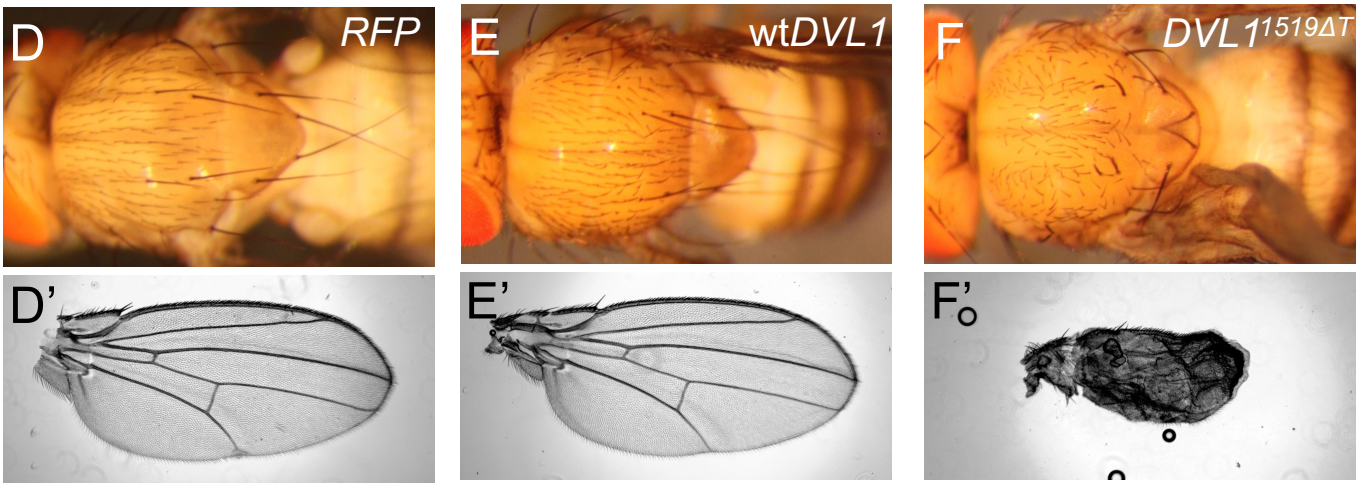


Fig. 3 *DVL1*$^{1519\Delta T}$ variant disrupt morphology in larval wing imaginal discs

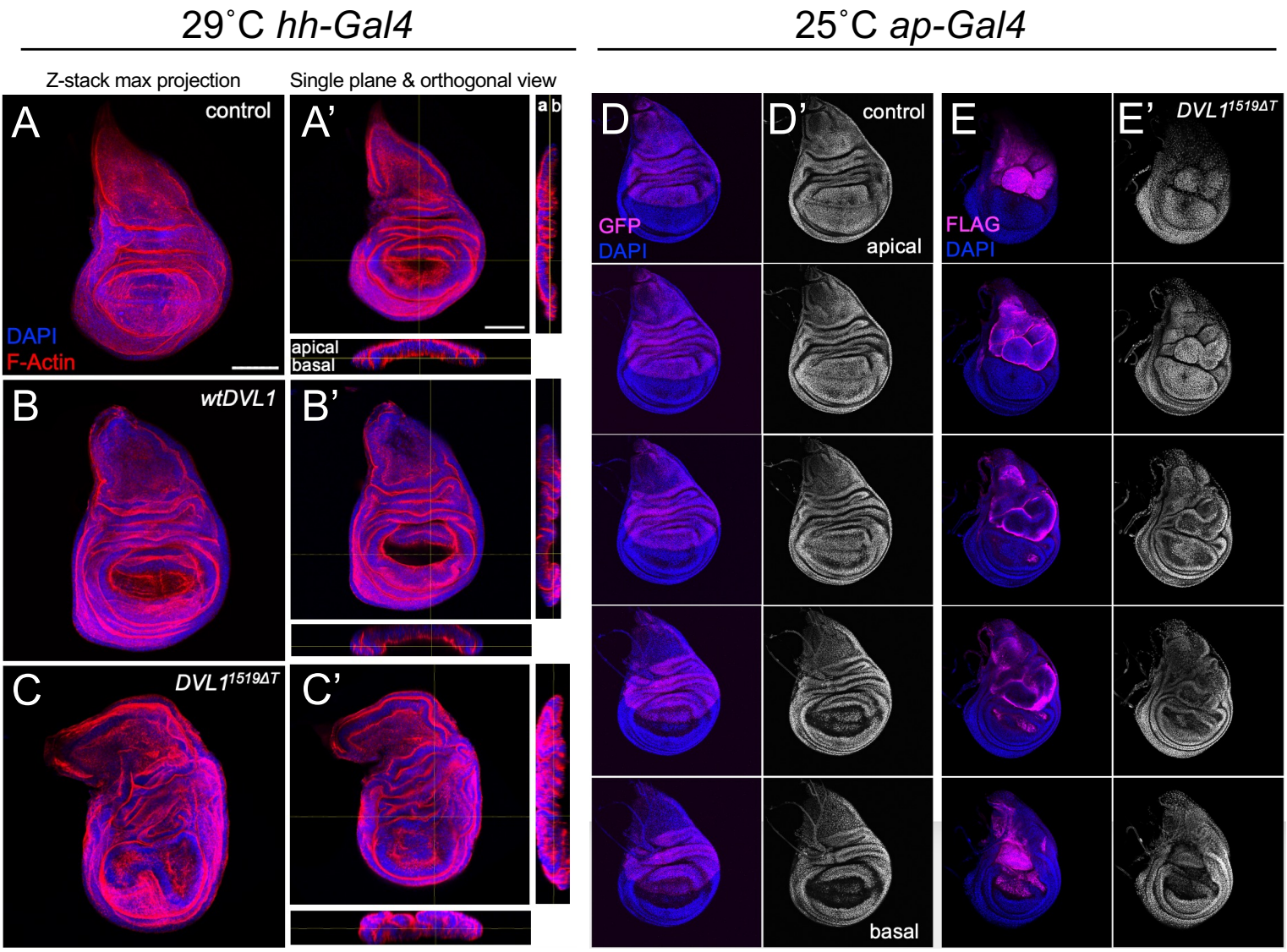


Fig. 4 DVL variant can alter epithelial organization

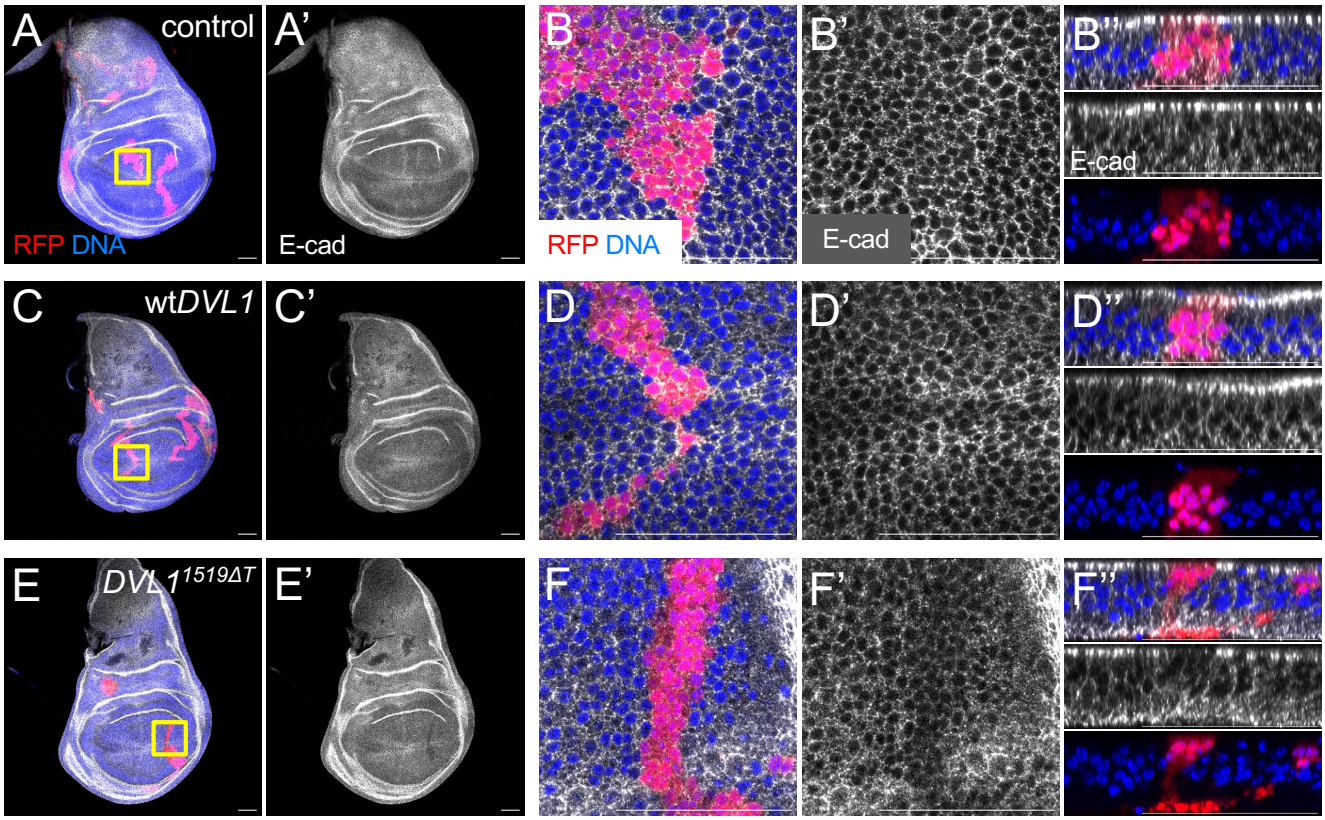


Fig. 5 *DVL1$^{1519\Delta T}$* does not promote proliferation but causes elevated cell death

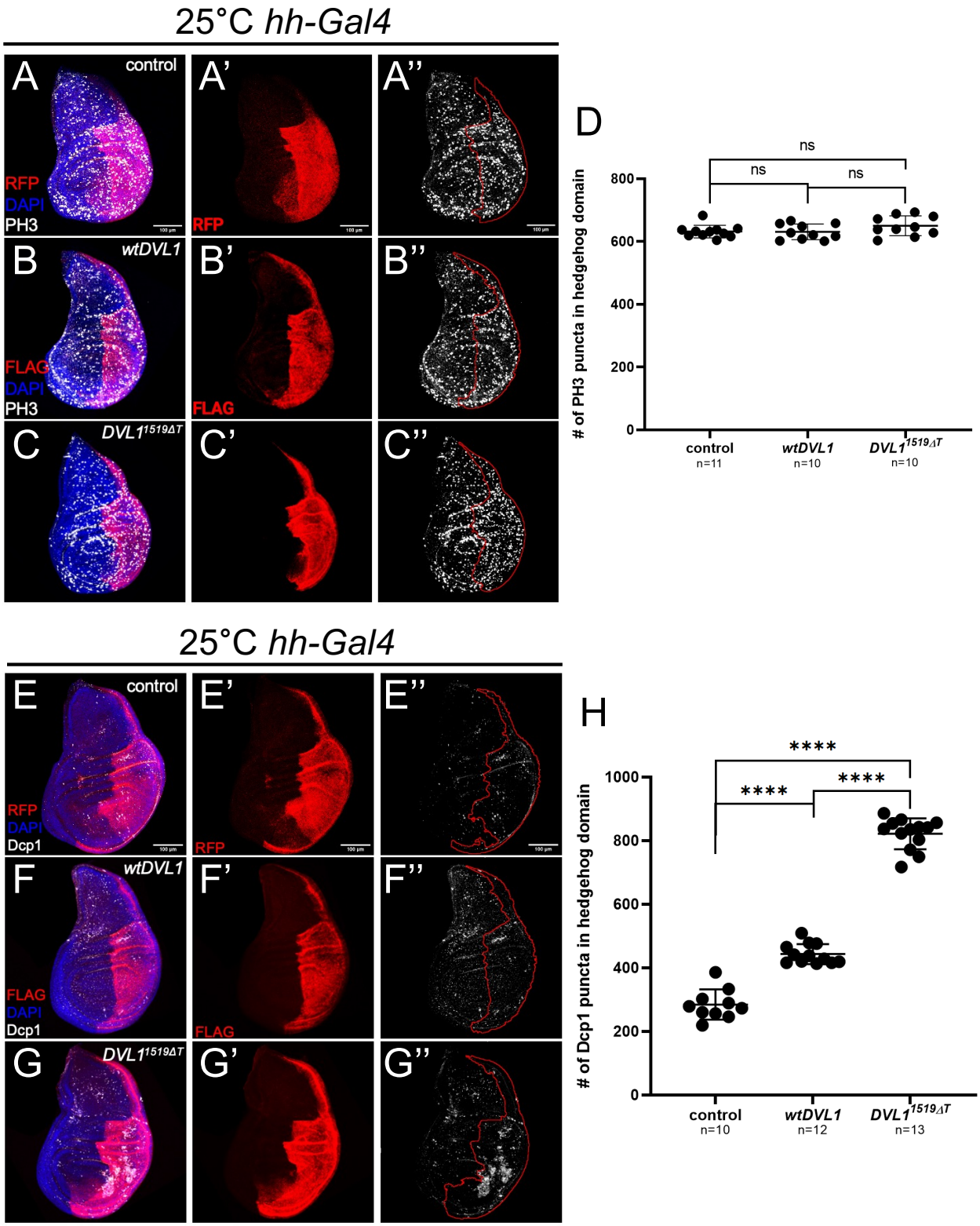


Fig. 6 *DVL1*$^{1519\Delta T}$ effects depend on JNK signaling

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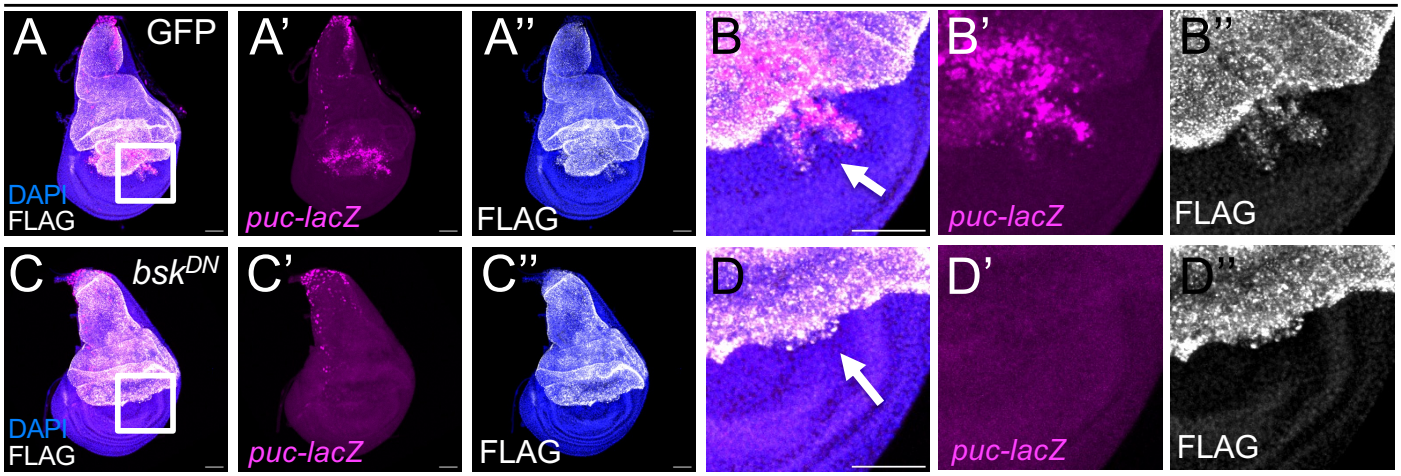

dpp-Gal4> DVL1$^{1519\Delta T}$ +

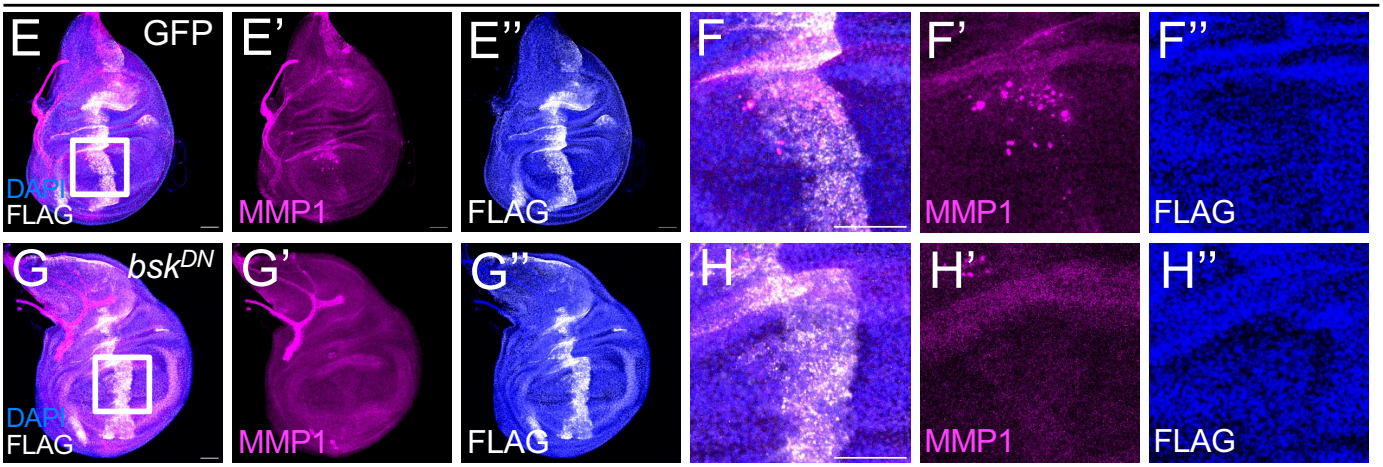

Fig. 7 *DVL1*$^{1519\Delta T}$ disrupts pupal wing development

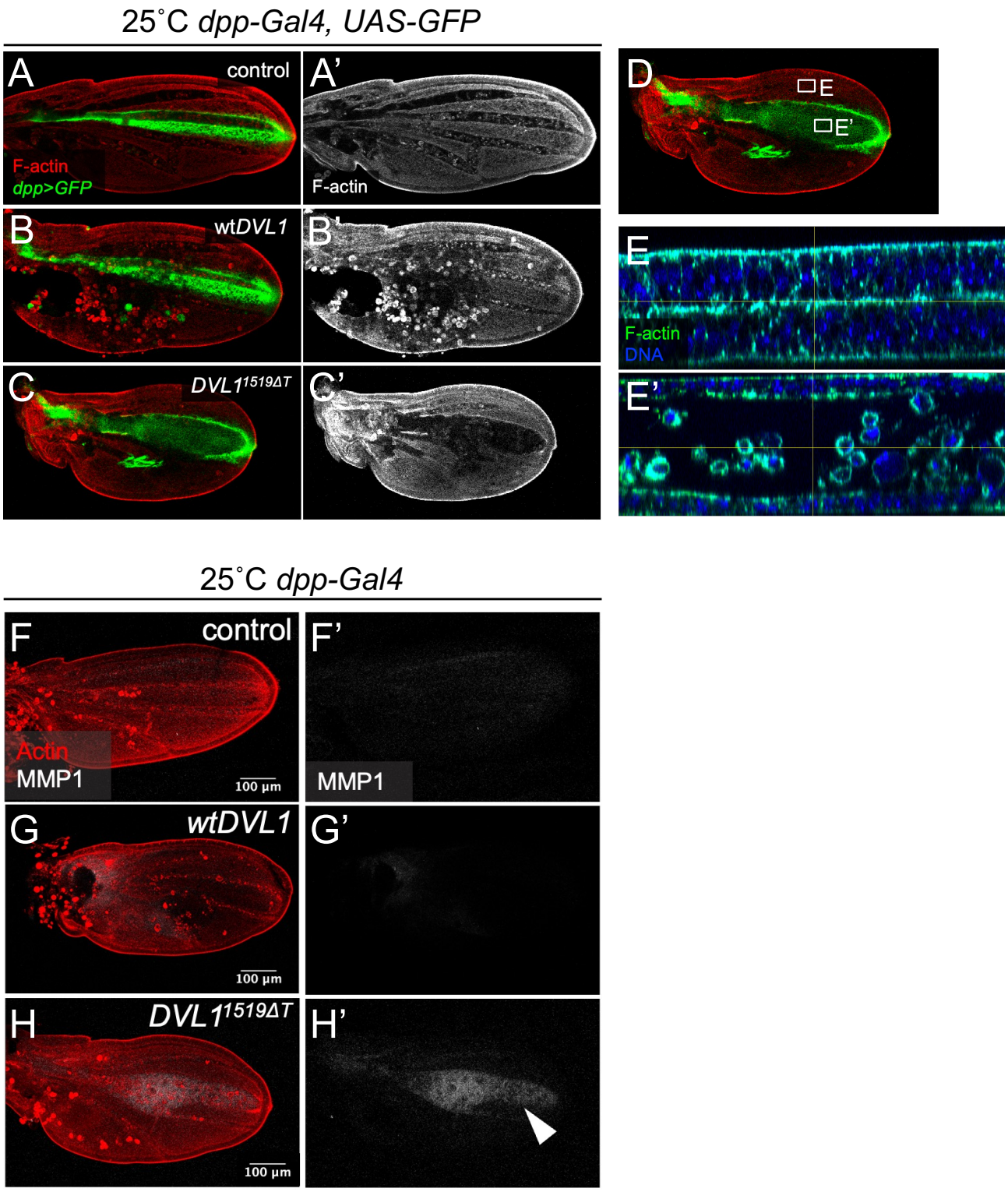
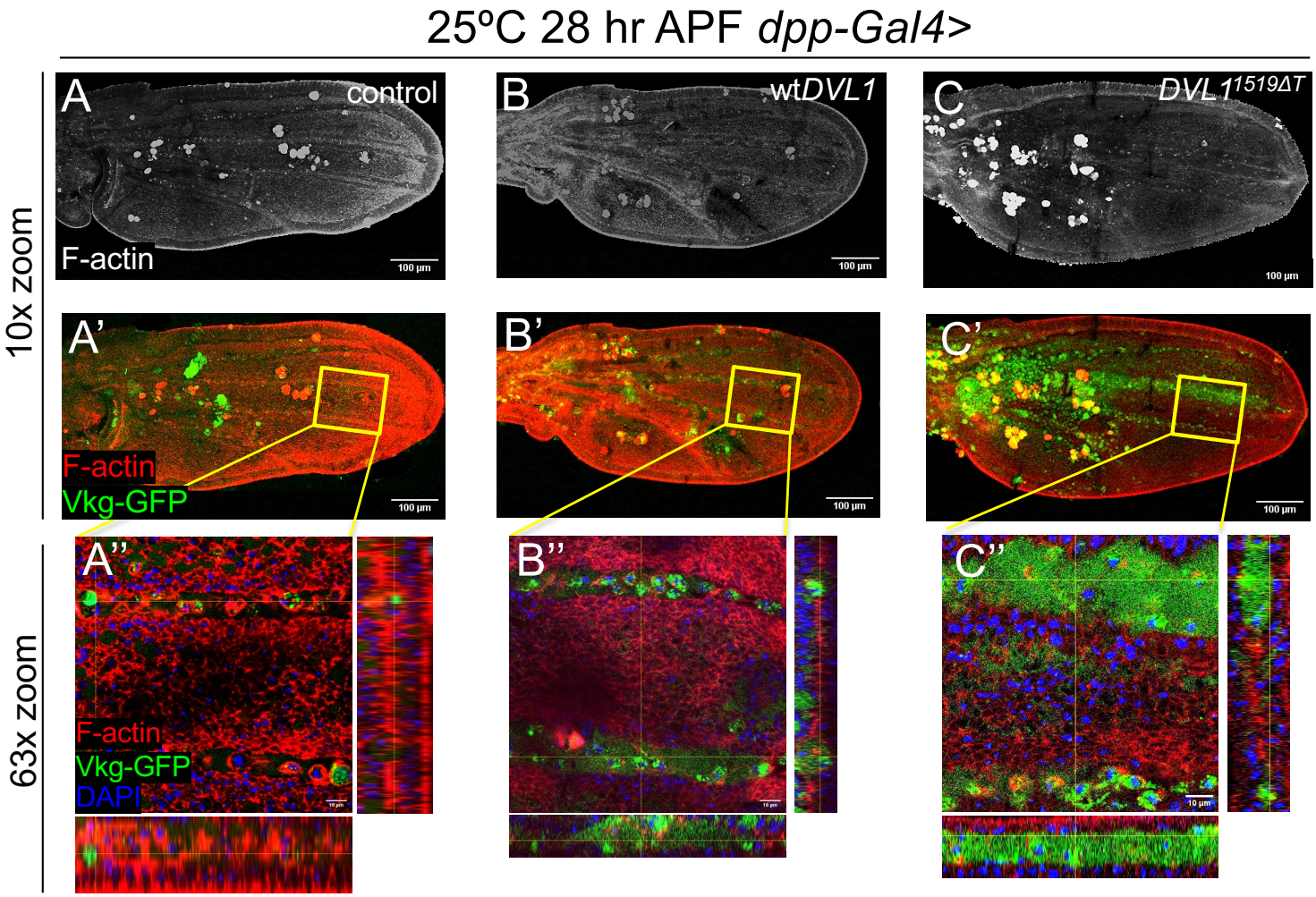
Fig. 8 *DVL1*$^{1519\Delta T}$ induces Viking accumulation