Deltex modulates Dpp morphogen gradient formation and affects the Dpp signaling in *Drosophila*

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Abstract

Deltex (Dx) is a context-dependent regulator of Notch signaling and regulates Notch signaling in a non-canonical fashion by facilitating the endocytosis of its receptor. In an RNAi-based modifier screen of kinases and phosphatases Thickveins (Tkv), the receptor of Decapentaplegic (Dpp), was identified as one of the interactors of Dx. Dpp, a *Drosophila* TGF-β/Bone Morphogenetic Protein homolog acts as a morphogen to specify cell fate along the anterior-posterior axis of the wing. Tight regulation of Dpp signaling is thus indispensable for its proper functioning. Here we present Dx as a novel modulator of Dpp signaling. We show evidence for the very first time that *dx* genetically interacts with *dpp* and its pathway components. Immunocytochemical analysis shows that Dx co-localizes with Dpp and its receptor Tkv in the *Drosophila* third instar larval tissues. Further, Dx is also seen to modulate the expression of *dpp* and its target genes. Here, we attribute this modulation to the endocytosis and trafficking of Dpp through Dx. This study thus presents a whole new avenue of Dpp signaling regulation via the cytoplasmic protein Dx.

Introduction

Dpp is a member of the TGFβ superfamily of signaling molecules, similar to vertebrate BMP2 and BMP4 (Bone Morphogenetic Protein 2 and 4), and is involved in many developmental processes ranging from cell division to cell-fate determination (Affolter and Basler, 2007; Kicheva and González-Gaitán, 2008; Perrimonet al., 2012). Any alteration in the pathways often results in human disease, including developmental disorders, vascular diseases, and cancer (Blobe et al., 2000; Massagué et al., 2000). Proper regulation of Dpp signaling is thus indispensable for its functioning, hence multiple genes potentially regulate the Dpp signaling cascade.

Dpp is expressed in a narrow stripe of anterior cells at the anterior-posterior (A/P) boundary of the wing disc (Affolter and Basler, 2007; Basler and Struhl, 1994). The secreted Dpp dimer binds and activates the receptor complex, Thickvein (Tkv) and Punt (Put), which in turn phosphorylates Mad (Mothers against Dpp), the downstream target of Tkv/Punt kinase along the Dpp domain (Affolter and Basler, 2007). Once phosphorylated, pMad forms a complex with a co-mediator Medea and translocate to the nucleus, thereby activating the transcription of the downstream target genes, such as *spalt (sal)* and *optomotor-blind (omb)*, at different extracellular concentrations of Dpp (Lecuit et al., 1996; Shi and Massagué, 2003).

Since the different concentration of Dpp activates different target gene expression, it is a prerequisite to understand the mechanism of Dpp gradient formation. A promising model of Dpp gradient establishment was proposed by Entchev et al, where it was reported that Dpp gradient is formed via intracellular trafficking initiated by receptor-mediated endocytosis of the ligand in the receiving cells, and the gradient is maintained by endocytic sorting of Dpp towards recycling versus degradation (Entchev et al., 2000). A similar mechanism of Notch signaling regulation has also been reported where Dx is required for the transportation of Notch from the cell membrane to the endosomal vesicle, where the

Notch protein is either recycled or degraded depending upon their ubiquitination state (Hori et al., 2004; Yamada et al., 2011).

Dx is a cytoplasmic interactor of Notch and regulates Notch signaling in a context-dependent manner (Diederich et al., 1994; Dutta et al., 2017; Matsuno et al., 1995; Matsuno et al., 1998; Mishra et al., 2014). The E3 ubiquitin ligase Dx plays an important role in determining Notch stability by mono- or polyubiquitinating the protein depending on the cellular context (Baron, 2012; Hori et al., 2011; Mukherjee et al., 2005; Wilkin et al., 2008). In a more recent study, Dx has been reported as an interactor of Eiger, the TNF (Tumor Necrosis Factor) homolog, and is involved Eiger-mediated JNK activation (Dutta et al., 2018). Moreover, we have also reported that Dx synergizes with TRAF6, the adaptor molecule in the JNK cascade, and activates JNK signaling at a level downstream of the ligand-receptor interaction (Sharma et al., 2020). A novel function of Dx in Toll pathway activation has also been reported lately, where Dx is shown to activate the Toll pathway in a JNK-independent manner (Sharma et al., 2021).

In the present study, we report that *dx* genetically interacts with different alleles of Dpp pathway components during wing development. Moreover, Dpp-induced wing phenotype is also seen to be modulated in Dx over-expression background. Immuno-cytochemical study further revealed that Dpp and its receptor Tkv localize with Dx in the same cytoplasmic vesicle. The expression of Dpp and its downstream targets, Mad and Spalt, also gets modulated upon Dx over-expression and this modulation is attributed to Dx-mediated Dpp trafficking.

Results and Discussion:

deltex genetically interacts with Dpp pathway components

Kinases and phosphatases have the potential of switching ON and switching OFF a gene and in turn, the cascade associated with it. To check whether kinases have some role in Dx-associated phenotypes, we performed an RNAi-based modifier screen with kinases and phosphatases that regulate a broad array of biological and cellular processes. We identified *Thickveins (tkv)*, as a potent interactor of Dx (Fig. S1). When *tkv* was downregulated in the dorsal-ventral boundary a crumpled wing phenotype was observed. This got rescued appreciably when *dx* was over-expressed in the same background (Fig. S1 C2-C3).

Tkv is the receptor of the ligand Dpp and hence it was intriguing to study the role of Dx in Dpp pathway. Dpp signaling plays a prominent role in wing development, and a reduction in Dpp level affects the differentiation of veins and cross-veins (Segal and Gelbart, 1985; Spencer et al., 1982). Loss-of-function mutation in either at the ligand, receptor, or transcription factor level of the Dpp pathway component disturbs wing vein formation and wing growth (Affolter and Basler, 2007). On the contrary, ectopic activation of Dpp signaling results in the formation of extra veins (Affolter and Basler, 2007; Eivers et al., 2009; Sotillos and De Celis, 2005). *dx* on the other hand show haplosufficiency, where heterozygous females do not exhibit any visible phenotype. However, hemizygous flies show wing vein thickening (Fig.1 A2 and A3).

To address the functional relationship between *dx* and *dpp*, we further investigated whether mutations in *dx* and *dpp* display genetic interactions. It is interesting to note that both the *dpp* alleles (dpp^5 and dpp^6) show lethality in homozygous condition; however, in heterozygous condition, they do not exhibit any phenotype. When loss of function allele of *dpp* was brought in trans-heterozygous combination (*dx*/+; *dpp*/+), showed wings that were

indistinguishable from the wild type (data not shown). However,reducing the dose of *dpp*, in *dx* null background showed enhanced notching and wing vein thickening when compared with *dx* hemizygotes in an otherwise wild type background (Fig.1B1-C3). Given that genetic interaction between *dx* and *dpp* was observed in the absence of all *dx* functions, it is explicit that a complete absence of *dx* creates a sensitized genetic background that makes the development of the wing margin sensitive to a decrease in the dosage of *dpp*.

We also checked if *dx* interacts with other Dpp pathway components, viz. the receptor *tkv*, and the downstream target *Mad*. The loss of function alleles of *tkv* (*tkv*^{8} and *tkv*^{12}) and *Mad* (*Mad*^{k00237}) display wing nicking and vein thickening phenotype with *dx* hemizygotes in trans-heterozygous condition (Fig.1D1-F3). Together, these results suggest that *dx* and *dpp* genetically interact, and may have a functional implication on wing development.

Deltex physically interacts with Dpp and co-localizes in the same sub-cellular compartment

To investigate further we checked the sub-cellular localization of Dpp and Dx. Dpp being a morphogen show a diffused Dpp expression in endogenous condition (Teleman and Cohen, 2000), however, when co-expressed with Dx, punctate expression of Dpp was observed that strongly co-localizes with Dx vesicle in the same sub-cellular compartment (Fig. 2A1-A4). Moreover, we also observed a sparse localization of smaller endogenous Dpp puncta with FLAG-tagged *UAS-Dx* (Fig. S2 A1-A4). Interestingly in the absence of Dx, a less punctate Dpp expression was observed (Fig. S2 B1-B3). We also tried to check the localization status of Tkv along with Dx, and we found a strong co-localization, when Dx and Tkv were over-expressed with *dpp-GAL4* in the same sub-cellular compartment (Fig. 2B1-B4).

Previous reports have shown the potent role of Dx in vesicular trafficking of Notch from the cell membrane to the cytoplasmic vesicles (Hori et al., 2004; Wilkin et al., 2008) therefore we hypothesize if Dx binds with Dpp and facilitates trafficking of Dpp, like that of Notch. To address this hypothesis, it was crucial to check if Dpp physically interacts with Dx. Co-immunoprecipitation experiment with FLAG-tagged Dx revealed that FLAG-tagged Dx pulled down Dpp-GFP when co-expressed in eye tissue (Fig. 2C). Likewise, FLAG-tagged Dx was immunoprecipitated with GFP antibody when the two proteins were expressed together (Fig. 2D). Additionally, we also checked if Dx physically interacts with the receptor Tkv, and found that FLAG-tagged Dx pulled down Tkv-GFP when co-expressed in the eye tissue (Fig. 2E).

Taken together, our data suggest that there is a direct interaction of Dx with Dpp and its receptor Tkv and we hypothesize that Tkv possibly facilitates the binding of cytoplasmic Dx to Dpp. The functional implication of this interaction though is a crucial aspect of investigation.

Dx modulates Dpp signaling activity

Dpp over-expression with *C96-GAL4* inflicts an enhanced wing vein thickening and ectopic veins (Fig. 3A3). Interestingly, induction of Dx in Dpp over-expressed flies results in a reduction of vein thickening and ectopic vein formation (Fig. 3A4). Besides, lowering the dose of *Tkv* in the wing margin, induces wing serration which was significantly rescued when Dx was over-expressed in the same background (Fig. 3B1-B4: n=100). On the contrary, reducing the dose of Dx in Dpp over-expressed and Tkv under-expressed flies render no phenotype (Data not shown). We hereby hypothesize that over-expression of Dx might facilitate the proper distribution of Dpp. The results here indicate the potential role of Dx in modulating the phenotype associated with Dpp signaling.

To further investigate the involvement of Dx in Dpp signaling regulation, we tried to observe if the expression of Dpp and its target is affected by *dx* activity. Dpp organizes the *Drosophila* wing patterning by acting as a graded morphogen. The gene is transcribed in a narrow stripe along the A/P compartment boundary of the wing imaginal disc. However, when *FLAG-Dx* was over-expressed inthe dorsal compartment of the wing imaginal disc, a broader Dpp stripe was seen, suggesting a wider gradient of Dpp, compared to that of the wild type (Fig. 3C3). A similar broadening of Dpp expression domain was observed when Dx was over-expressed with A/P domain-specific *dpp-GAL4* (Fig. S3 A3).

In addition, upon expression of Dx at the A/P border cells with *dpp-GAL4*, pMad expression was abnormally altered (Fig. 3D3), the latter being the downstream signal transducer to Dpp. pMad levels in general, are high in the central region of the wing disc and decline gradually towards the anterior and posterior distal cells. As there are no other transducers of Dpp signaling known, pMAD levels are taken to reflect the intensity of Dpp signal transduction activity. pMad regulates the expression of the Dpp target genes (*spalt, optomotor blind,* and *vestigial),* in a Dpp-dependent manner in domains that straddle the Dpp stripe, with Spalt having the narrowest domain and Vestigial the widest (Hamaratoglu et al., 2014). Of the above-mentioned markers, we checked the expression of Spalt (Sal) in Dx over-expressed discs. Spalt expresses in the wing pouch in a broad stripe of cells centered along the anterior-posterior compartment boundary (de Celis et al., 1996; Nellen et al., 1996). An ectopic Spalt expression was observed in the posterior compartment of the disc where Dx was over-expressed with *en-GAL4* (Fig. 3E3). In addition to it, the localization of Spalt was also altered upon Dx over-expression and a more elongated Spalt expression with a tapering end towards the ventral region of the disc was observed.

These results indicate that Dx may facilitate Dpp signaling via enhancing the traffickingof the morphogen Dpp, thereby altering the effector target gene expression.

Dx facilitates Dpp trafficking

Although Dpp is the most extensively studied morphogen, the molecular mechanism by which the Dpp gradient is formed and sustained is poorly understood. Endocytosis of Dpp however, is proposed to play a potent role in the formation of the Dpp gradient (Entchev et al., 2000). Dx plays a key role in receptor-mediated endocytosis of Notch (Hori et al., 2004; Hori et al., 2011). Dx depletes Notch from the apical cell surface and leads to its accumulation in the late endosomal vesicles. Besides, if the transport of Notch into the late endosome was impaired, it hampered Dx-mediated Notch signal activation (Hori et al., 2004). These reports prompted us to study the role of Dx in Dpp trafficking.

We first tried to check, if Dx co-localizes with key Rab components along with Dpp, to ensure that all the three molecules are in the same vesicle. We over-expressed *dpp-GFP*, and *FLAG-Dx* in the third instar wing imaginal disc with *dpp-GAL4* and checked if the two proteins co-localizes with early endosome marker Rab5. Since we have already observed that Dx and Dpp do physically interact (Fig. 2), we focused if Dx and Dpp positive vesicles are Rab5 positive too. Our results show that Dx co-localizes with Dpp and Rab5 (Fig. S4 A1-B6), and there is a possibility of these three proteins being in a trimeric complex. We next questioned if this complex has some role in Dpp trafficking. We centered our study on Rab7, since Rab7 targets endocytic cargo from early to late endosome and lysosome for degradation (Méresse et al., 1995; Vitelli et al., 1997), and Dx promotes accumulation of Notch in the late endosomal compartment where Dx co-localizes with Notch and Rab7 (Hori et al., 2004).

We tried to monitor the Dpp signaling range in Dx context. For this, we checked the status of Dpp in Rab7 over-expression background. A reduction in Dpp gradient was observed on ectopically expressing Rab7, however, this reduction got rescued when Dx was brought in the same background (Fig 4. A-C). Moreover, we also visualized the expression of Dpp

target Spalt. When *UAS-Rab7* was ectopically expressed in the posterior compartment of the wing disc, a reduction in Spalt expression was observed (Fig. 4E1-E2) (Entchev et al., 2000), indicating that sorting of endocytic cargo towards degradation limits the range of Dpp signaling. This reduction in Spalt expression upon Rab7 over-expression was however rescued, when Dx was co-expressed in the same sub-cellular compartment (Fig. 4F1-F2).

In conclusion, we have uncovered an important function of the cytoplasmic protein Dx in the gradient formation of the morphogen Dpp. Our studies show that Dx genetically and physically interacts with Dpp and Tkv. Our results further reveal that rab7 along with Dx expands the gradient of Dpp and its target Spalt. We attribute this expansion to Dx-mediated Dpp trafficking; however, further analysis is required to unravel the detailed mechanistic aspect of Dpp endocytosis and trafficking via Dx.

Material and Methods:

Drosophila Genetics:

All fly stocks were maintained on standard cornmeal/yeast/molasses/agar medium at 25°C as per standard procedures. *UAS-FLAG-Dx, dx,* and dx^{152} were kindly provided by Prof. Spyros Artavanis Tsakonas. tkv^{8} and tkv^{12} were kind gift from Prof. Yu Cai. *UAS- tkv-GFP* line was obtained from Prof. Thomas Kornberg. dpp^{5} (BL-20620), dpp^{6} (BL-2071), Mad^{k00237} (BL-10474), *UAS-dpp-GFP* (BL-53716), *UAS-Rab7-GFP* (BL-42706), *en-GAL4, dpp-GAL4, ap-GAL4, GMR-GAL4,* and *C96-GAL4* stocks were obtained from Bloomington Stock Center. *UAS-tkv-IR* (VDRC-105834) was obtained from Vienna Drosophila Resource Center.

All crosses were performed at 25°C unless otherwise mentioned. To induce the expression of the gene in the specific domain GAL4-UAS binary system was used (Brand and Perrimon, 1993). Combination stocks were made with help of appropriate genetic crosses.

Immunoprecipitation and Immunoblotting

For immunoprecipitation of Dx and Dpp, protein lysates were prepared in 1X RIPA buffer from *Drosophila* head tissue expressing *UAS-FLAG-Dx*; *UAS-Dpp-GFP*, *UAS- FLAG-Dx*, and only *UAS-Dpp-GFP* using *GMR-GAL4* driver. Crude lysate containing 2mg of total protein was mixed with 5µl of anti-FLAG or anti-GFP antibody and 30µ1of protein A/G beads and kept overnight for an end-over-end rotation at 4°C. Only *UAS-dpp-GFP* or *UASFLAG-Dx* lysate was used for control samples. Beads were collected after washing thrice with 1X RIPA buffer and separated on 12% denaturing SDS polyacrylamide gel followed by transfer onto Immuno-blot PVDF membranes (Bio-Rad). After washing for 10 min in TBST [Tris base (50mM), NaCl (150mM), Tween-20 (0.1%)], and blocking (4% skimmed milk in TBST) for 30min, blots were probed with rabbit anti-GFP antibody in 1:1500 dilution (Invitrogen #PA1-980A) or rabbit anti- FLAG antibody in 1:1000 dilution (Sigma #F7425). Again, after washing thrice in TBST and another blocking for 1 hr, goat anti-rabbit IgG-AP conjugate in 1:2000 dilution (Molecular Probes) in blocking solution was added for 90 min followed by three washings in TBST. Color was detected by Sigma FAST BCIP/NBT (Sigma). Similar approach was followed to perform immunoprecipitation of Dx and Tkv.

Immunocytochemistry and Microscopy

Drosophila third instar larval wing discs were dissected out in ice-cold 1X-PBS, and tissues were fixed in a 1:1 mixture of 3% paraformaldehyde in PBS and at room temperature for 1 min, followed by a second fixation in 3% paraformaldehyde and 5% DMSO for 20 min. Immunostaining was done as described previously (Sharma et al., 2020). Following primary antibodies were used in this study: Rabbit anti-Flag (1:00, Sigma #F7425), (Rabbit Phospho-Smad1/5 (1:50, Cell Signaling Technology #41D10), Rabbit anti-Dpp (1:100, kindly gifted by Prof. Matthew Gibson), Guinea pig anti-Spalt (1:20,000, kindly gifted by Prof. Antonia Monteiro), Guinea pig anti-Rab5 (1:1000, a generous gift from Prof. Akira Nakamura), Alexa488-, or Alexa555-, or Alexa405-conjugated secondary antibodies (1:200, Molecular Probes) were used to detect the primary antibodies. Imaging was done in Carl Zeiss LSM 780 laser scanning confocal microscope and images were processed with Adobe Photoshop7.

Intensity Profiling

Intensity profiles were generated by ImageJ software. The average intensity for each image was measured using the plot function. For measuring gradient profiles in ImageJ, we used average projections of five consecutive slices spanning the disc in triplicates. Gradient profiles were extracted using ImageJ software. For Fig. 3 and Supplementary Fig. 3, the entire pouch up to the edge of the wing disc was measured. For Fig. 4, the pouch region was measured. Measurement of Spalt (Sal) domain was done taking into consideration the A/P boundary.

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Competing interests

The authors declare no competing interest.

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Figures

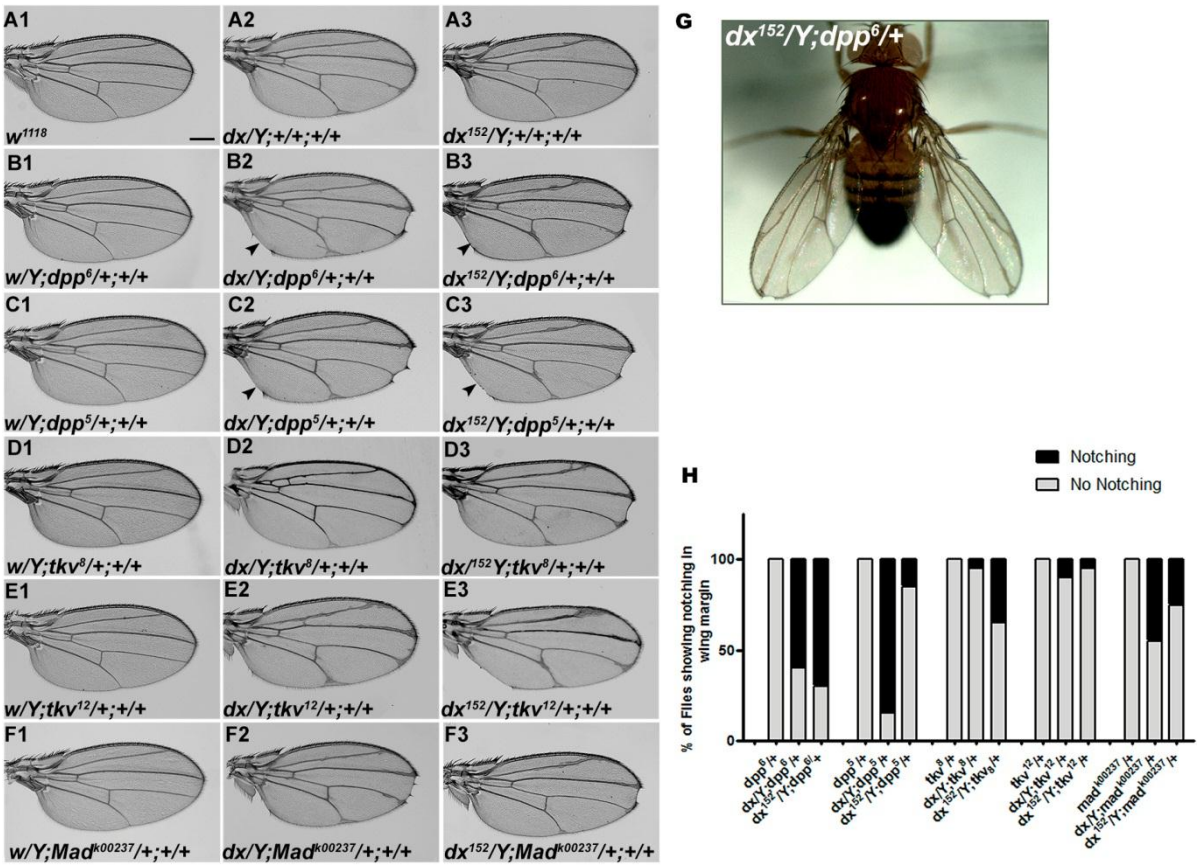

Fig. 1. *dx* genetically interacts with *dpp* and its pathway components. (A1-F3) Representative wings from males with indicated genotypes. *dx* (A2) and dx^{152} (A3) hemizygous flies show extra vein material at the distal end of the wing. (B2, B3, and C2, C3) Both the *dx* allele show an enhancement of wing vein thickening along with wing notching in hemizygous combination with different alleles of *dpp* (dpp^{6} and dpp^{5}) heterozygotes. (D2, D3, and E2, E3) Different alleles of *tkv* (tkv^{8} and tkv^{12}) in trans-heterozygous conditions show enhanced vein thickening and wing nicking phenotype with *dx* hemizygotes. (F2, F3) *dx* alleles show strong genetic interaction with the Dpp target gene *Mad*, where a loss of function allele of *Mad* (Mad^{k00237}) shows wing nicking phenotype with *dx* alleles in hemizygous condition. (G) Representative adult fly of the indicated genotype. Note the spread-out wing. (H) Graph showing the frequency of wing notching phenotypes observed in different genetic combinations (n=100). Scale bar: A1-F3: 200µm.

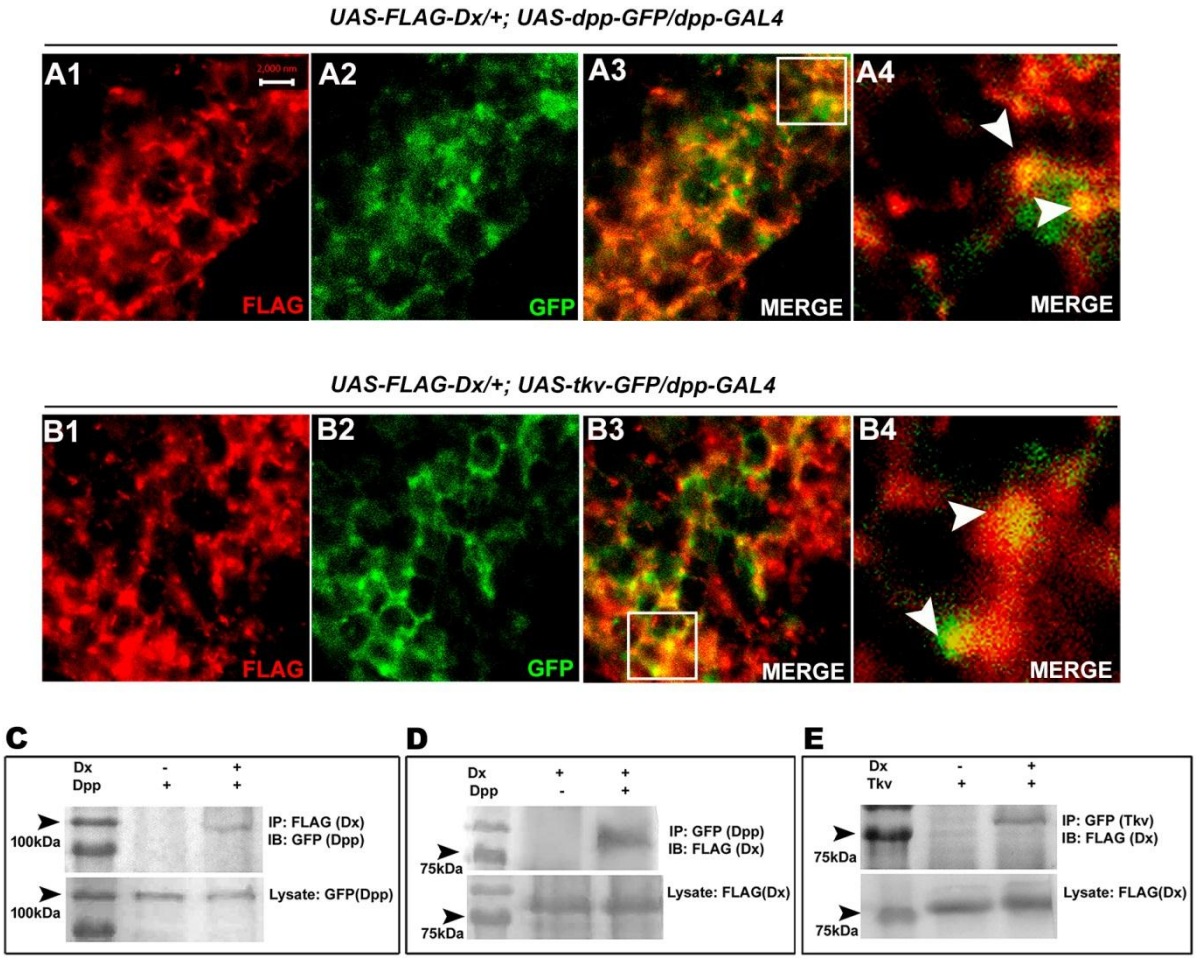

Fig. 2. Dx physically interacts with Dpp and Tkv and co-localizes in the same subcellular compartment. (A1-A4) *dpp-GFP* and *FLAG-Dx* were co-expressed under the control of *dpp- GAL4.* The merged panel (A3 and A4) shows that the two proteins co-localize in the cytoplasmic vesicles. (B1-B4)) *Tkv-GFP* co-localizes with Dx when the two genes were expressed together. B3 and B4 show the merge panel respectively. A4 and B4 are enlarged images of the squares in A3 and A4 respectively. (C and D) Co-immunoprecipitation of FLAG-Dx and Dpp-GFP. (C). FLAG immunoprecipitated Dpp-GFP was analyzed by Western blotting with anti-GFP antibody. The lower blot shows the level of protein in the lysates respectively. (D) GFP immunoprecipitated FLAG-Dx was detected by anti-FLAG antibody. (E) Co-immunoprecipitation of FLAG-Dx and Tkv-GFP. GFP Immunoprecipitated Tkv-GFP was detected by anti-FLAG antibody. Scale bar: A1-B4: 2µm.

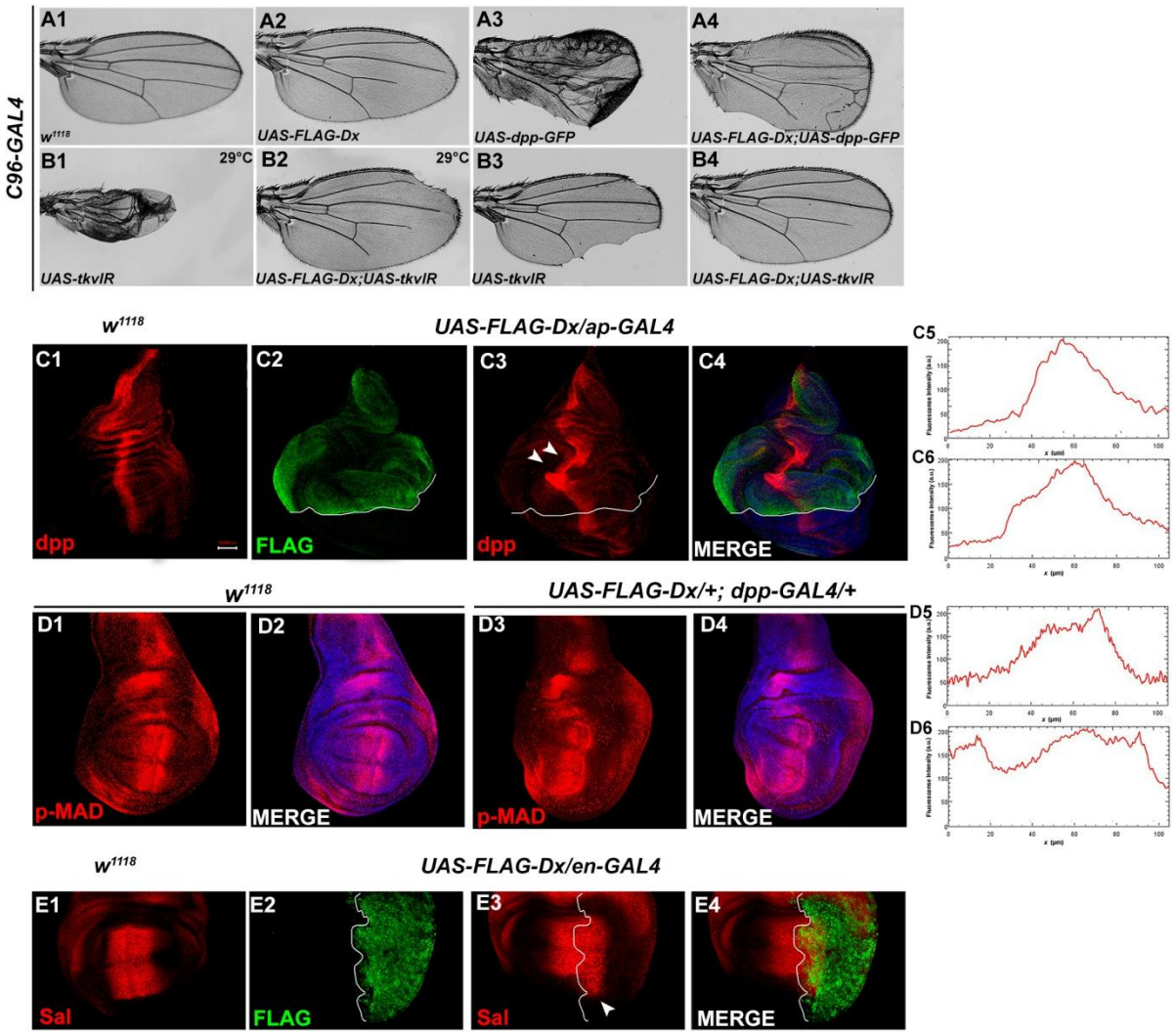

Fig. 3. Dx modulates the expression of Dpp and its targets, Spalt and Mad. (A1-B4) Representative image of wild-type wing (A1). Over-expression of Dpp in the *Drosophila* wing by *C96-GAL4* (A3) shows excessive extra vein material and wing margin serration. Expression of Dx in the same background rescued the Dpp gain of function effect (A4). (B1- B4) Dx also rescues the Tkv loss of function effect (n=100). (C1-C4) Over-expression of Dx with *ap-GAL4* results in the broadening of the Dpp diffusion domain. (D1-D4) Dx over-expression modulates the expression of pMad. D2 and D4 show merged images of pMad and DAPI. (E1-E4) Ectopic Spalt expression was observed in the posterior domain of the wing disc when Dx was driven with *en-GAL4* (marked by arrowhead). C5, C6, and D5, D6 are average fluorescence intensity profiles of Dpp and pMAD expression. Scale bar: A1-B4: 200µm. C1-E4: 50µm.

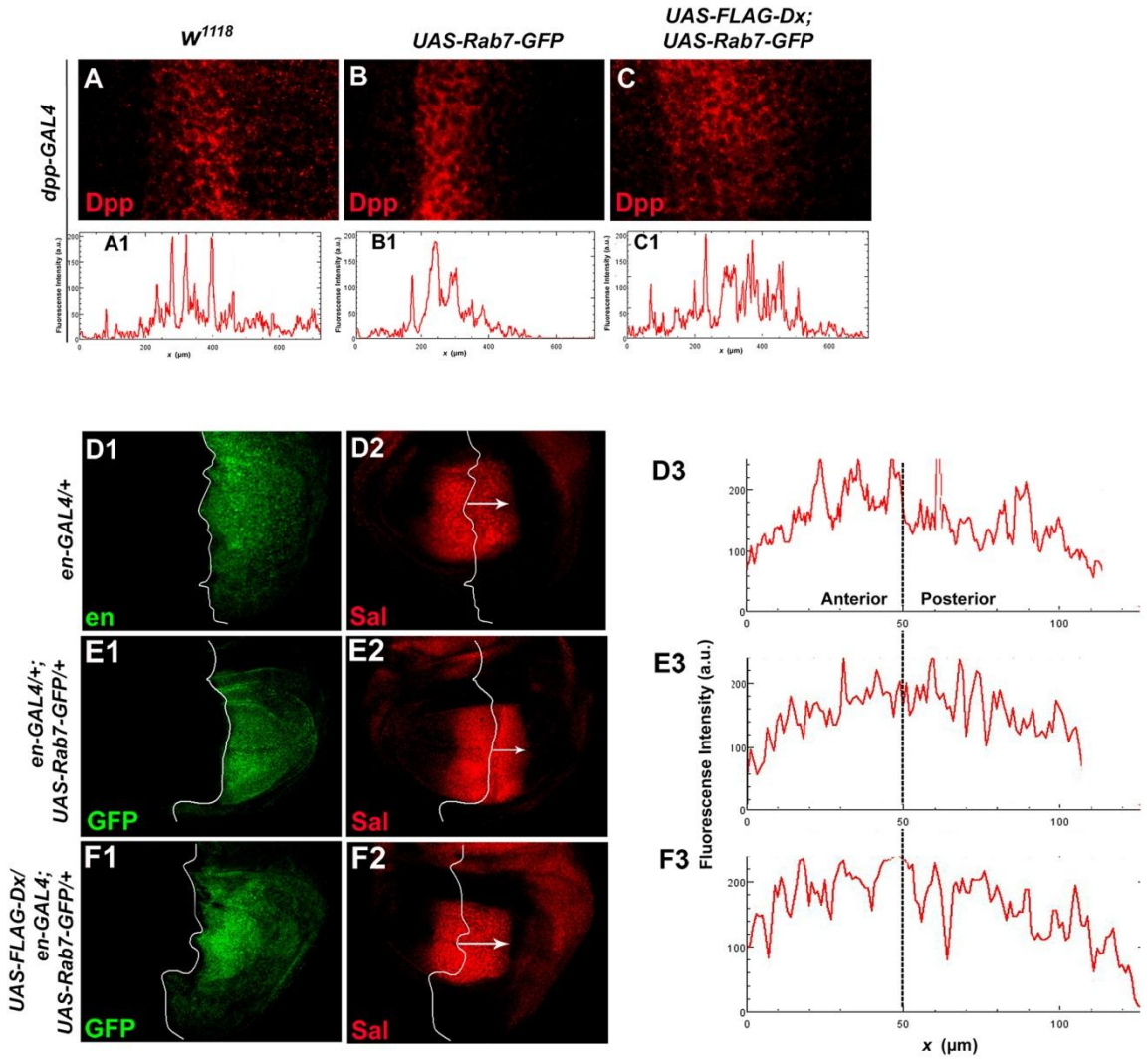

Fig. 4. Dx modulates the Dpp signaling range in Rab7 mutant. (A-C1) Dpp expression in the mentioned genotypes. Note the reduction of Dpp gradient in Rab7 over-expressed condition (B). Over-expression of Dx in the same background amplifies the Dpp gradient (C). (A1-C1) shows the average intensity profiles of (A-C). (D1-D2) Wild type Spalt expression. (E1-E2) Rab7 over-expression with *en-GAL4*. Note the reduction in the expression range of Spalt in the posterior domain of the wing imaginal disc. (F1-F2) Dx over-expression in the same background amplifies the Spalt expression gradient in the posterior domain of the disc. The white arrow marks the domain range respectively. (D3-F3) show the average fluorescence intensity profile of Spalt expression. Scale bar: A-C: 5μm. D1-D2, E1-E2, F1-F2: 50μm.

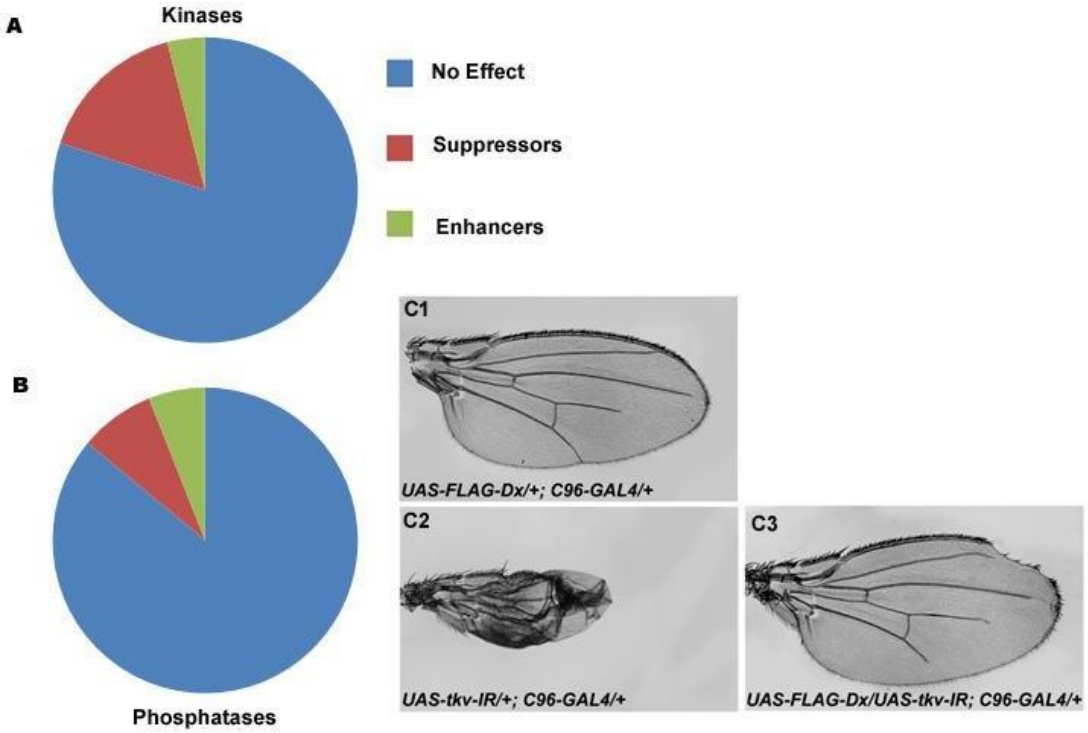

Fig. S1. RNAi-based genetic screen for modifiers of Dx induced phenotype in the *Drosophila* wing. (A and B) Breakdown of screen results by kinases and phosphatases respectively. (C3) RNAi-based modifier screen showed *tkv* genetically interacts with *dx*. (C1) Over-expression of Dx with *C96-GAL4* resulted in mild bristle loss. (C2) *Tkv-RNAi* showed flies with massive morphological defects which got rescued to a major extent when Dx was expressed in the same background (C3). Scale bar: C1-C3: 200µm.

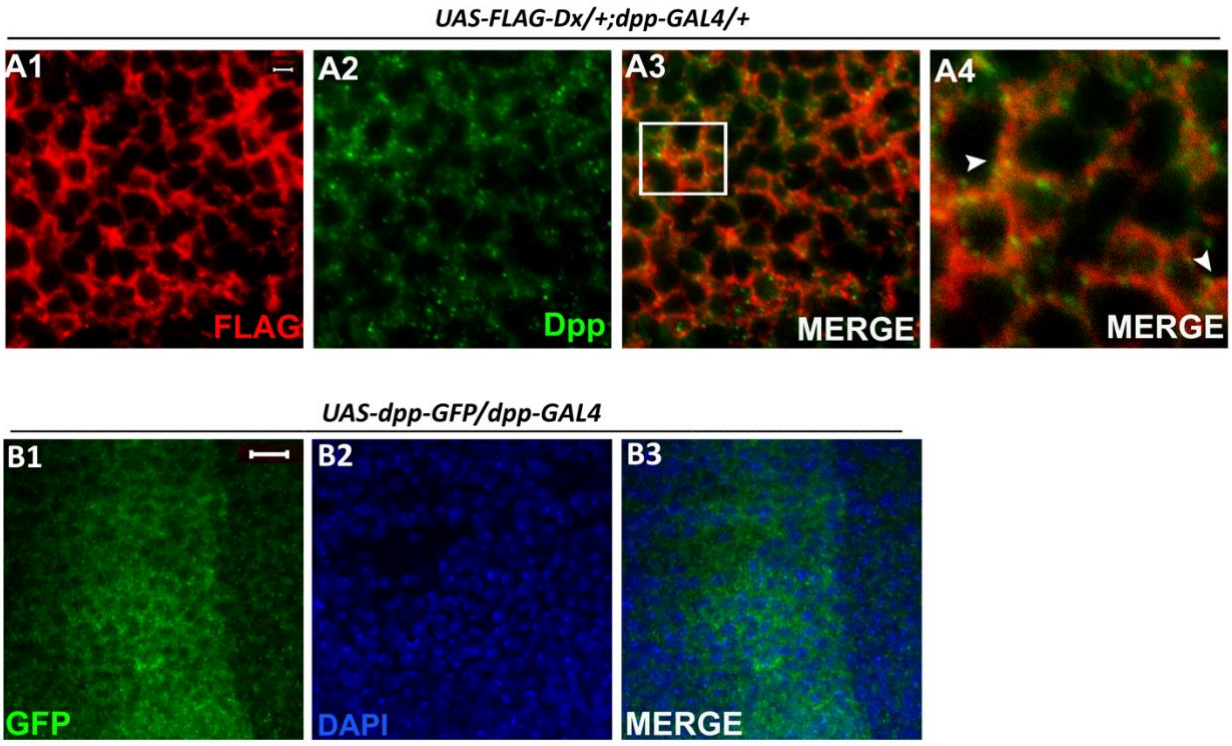

Fig. S2. Endogenous Dpp co-localizes with Dx. (A1-A4) UAS-FLAG-Dx co-localizes with endogenous Dpp. Note the smaller Dpp puncta of endogenous Dpp. A4 is the enlarged image of A3. Arrow marks the co-localized spots. (B1-B3) *UAS-Dpp GFP* expression under *dpp-GAL4* showed a diffused and less punctate Dpp expression. Scale bar: A1-A3: 2µm, B1-B3: 5µm.

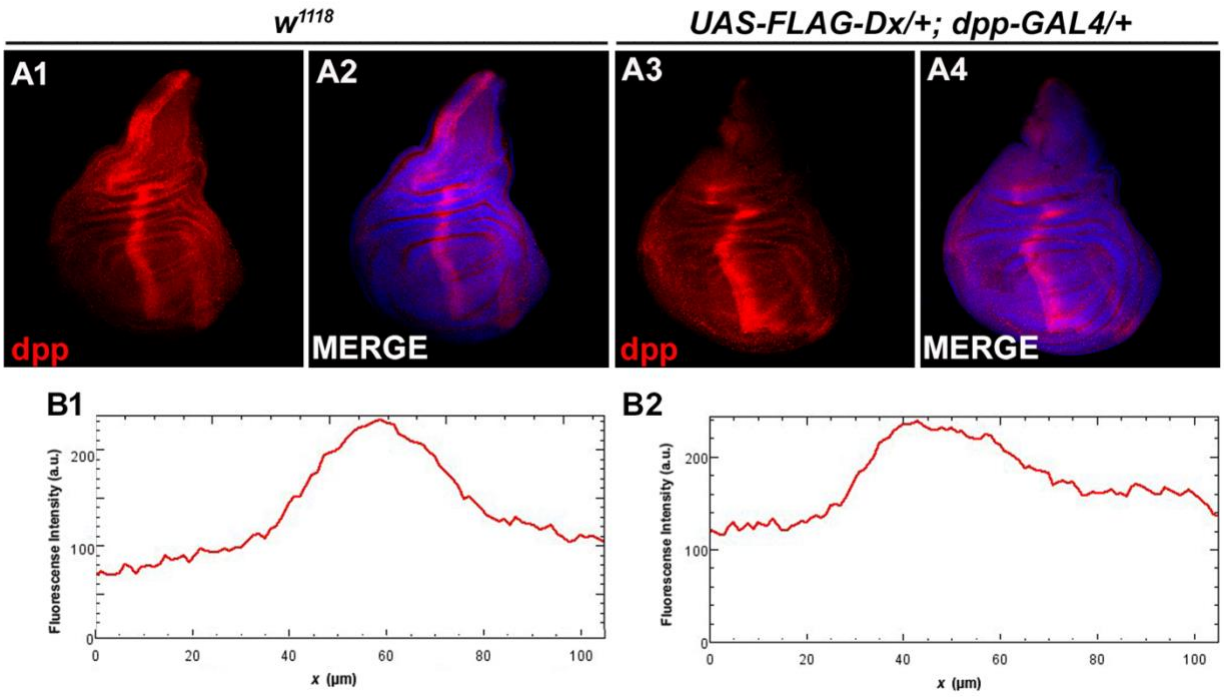

Fig. S3. Dx broadens the Dpp expression gradient. (A1-A4) A broadening of the Dpp expression gradient was observed when Dx was expressed with A/P domain-specific *dpp-Gal4.* B1 and B2 show the average intensity profile of Dpp expression. Scale bar: A1-A4: 50µm.

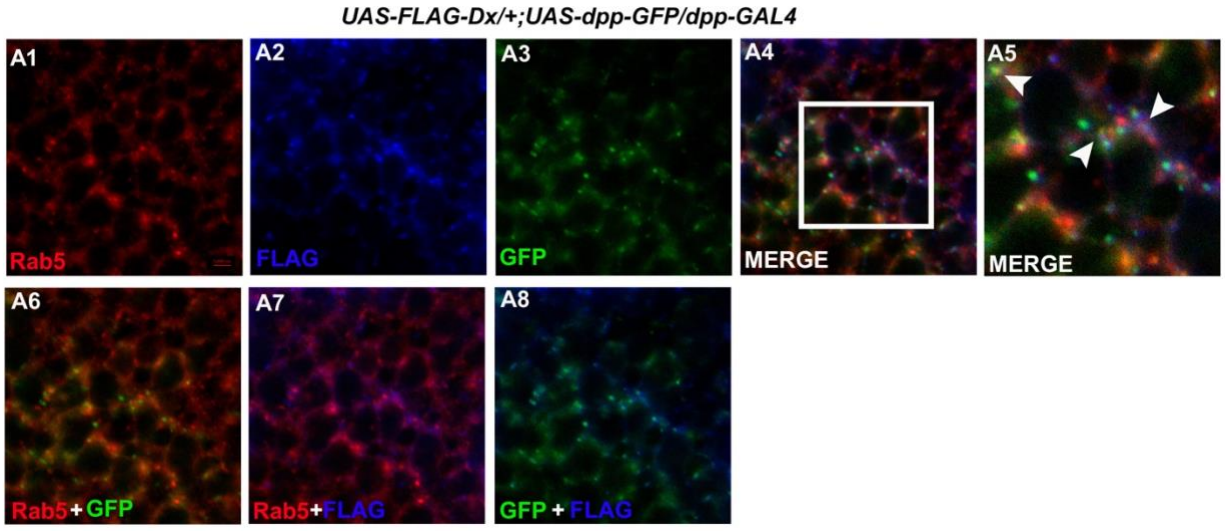

Fig. S4. Dx co-localizes with Dpp and Rab5 in the same subcellular compartment. (A1-A5) FLAG-tagged Dx and dpp-GFP were co-expressed under the control of *dpp-GAL4* driver. (A4 and A5) Dx and Dpp co-localize with Rab5 in the same subcellular compartment. A5 is the enlarged image of the square in A4. The arrow highlights the co-localized spots. (A6-A8) Merged images of respective combinations. Scale bar: A1-A4, A6-A8: 5µm.