1 **Title:** Anchor negatively regulates BMP signaling to control *Drosophila* wing

2 development

3 **Running title:** A novel gene *anchor*

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8 **Key words:** Anchor, GPCR, BMP, *dpp*, wing veins, *Drosophlia*

9 **Summary statement:** The novel gene *anchor* is the ortholog of vertebrate GPR155,

10 which contributes to preventing wing disc tissue overgrowth and limiting the

11 phosphorylation of Mad in presumptive veins during the pupal stage.

12   **ABSTRACT**

13   G protein-coupled receptors play a particularly important function in many organisms.

14   The novel *Drosophila* gene *anchor* is the ortholog of vertebrate GPR155, and its

15   molecular function and biological process are not yet known, especially in wing

16   development. Knocking down *anchor* resulted in increased wing size and extra and

17   thickened veins. These abnormal wing phenotypes are similar to those observed in

18   gain-of-function of BMP signaling experiments. We observed that the BMP signaling

19   indicator p-Mad was significantly increased in *anchor* RNAi-induced wing discs in

20   larvae and that it also abnormally accumulated in intervein regions in pupae.

21   Furthermore, the expression of BMP signaling pathway target genes were examined

22   using a *lacZ* reporter, and the results indicated that *omb* and *sal* were substantially

23   increased in *anchor* knockdown wing discs. In a study of genetic interactions between

24   Anchor and BMP signaling pathway, the broadened and ectopic vein tissues were

25   rescued by knocking down BMP levels. The results suggested that the function of

26   Anchor is to negatively regulate BMP signaling during wing development and vein

27   formation, and that Anchor targets or works upstream of Dpp.

28 **INTRODUCTION**

29 *Drosophila,* as a practical model, is used to study the wing pattern formation.

30 Cells within wing discs must receive many different signals to form an appropriate

31 pattern. This variety of signals controls an enormous array of cellular processes, such

32 as proliferation, differentiation, apoptosis, and cell migration (Neufeld, et al., 1998;

33 Egoz-Matia, et al., 2011; Shbailat and Abouheif, 2013). The processes that control

34 vein formation during wing development are, in particular, mediated by many

35 different signaling pathways, including the Bone Morphogenetic Protein (BMP),

36 Epidermal Growth Factor (EGF), Hedgehog, Notch and Wnt pathways (Blair, 2007).

37 A BMP gradient is an important signaling mechanisms that is required for wing

38 patterning in *Drosophila*. BMPs are members of the transforming growth factor-β

39 (TGF-β) subfamily, the involvement of which is conserved in many patterning

40 processes across multicellular organisms (Clarke and Liu, 2008). In *Drosophila*, the

41 BMP family includes two classical ligands and three receptors. The ligands are the

42 BMP2/4 homolog Decapentaplegic (Dpp) and the BMP5/6/7/8 homolog Glass bottom

43 boat (Gbb) (Garcia-Bellido and Merriam, 1971; Wharton et al., 1991). The receptors

44 include two type I receptors, Thick-veins (Tkv) and Sax (Saxophone), and a type II

45 receptor, Punt (Put) (Affolter et al., 1994; Xie et al., 1994; Letsou et al., 1995). Dpp

46 provides a long range signal along the anterior-posterior (AP) boundary of the wing

47 disc, while Gbb provides a longer range signal than Dpp in the wing disc (Ray and

48 Wharton, 2001). Dpp and Gbb form homo- or heterogeneous dimers that elicit effects

49 during wing development (Israel et al., 1996). When a ligand binds to a receptor on

50   the surface of cell, the BMP family R-Smad member Smad 1/5/8 and the intracellular

51   Smads (R-Smads) are phosphorylated, allowing them to recruit Co-Smad (Smad 4,

52   Medea) and then translocate into the nucleus, where they regulate the transcription of

53   their target genes (Chacko et al., 2001; Shi and Massagué, 2003). BMP's target genes

54   include *optomotor blind* (*omb*) (Sivasankaran et al., 2000), *spalt* (*sal*) (Barrio and de

55   Celis, 2004), *daughter against dpp* (*dad*) (Tsuneizumi et al.1997), and *brinker* (*brk*).

56   In particularly, *brk* exprssion is repressed by Dpp (Campbell and Tomlinson, 1999).

57   These genes can affect cells fate decisions and tissue patterns to varying degrees. Dpp

58   is a typical factor that is involved in all currently characterized BMP signaling events

59   in *Drosophila*, and it performs developmental and genetic functions in the wing disc.

60   During larval development, Dpp is expressed in a strip along the AP boundary to

61   promote wing disc proliferation and specify presumptive vein formation (Capdevila

62   and Guerrero, 1994; Biehs et al., 1998). Knocking down Dpp signaling lead to the

63   development of small discs, reduced adult wing size and resulted in the loss of veins

64   (Spencer et al., 1982; Künnapuu et al., 2009). During the pupal stage, Dpp is localized

65   at the AP boundary and along presumptive veins. *dpp* mutants have the shortveins

66   phenotype, indicating that Dpp promotes the formation of distinct intervein patterns

67   (Segal and Gelbart, 1985). In addition, the ectopic activation of Dpp signaling induces

68   the overgrowth of wing tissues (Martín-Castellanos and Edgar, 2002; O'Keefe et al.,

69   2014). *gbb* is broadly expressed in the wing disc except at the AP boundary strip, and

70   *gbb* mutants exhibit phenotypes similar to those of *dpp* mutants, have small wing disc

71   and are missing veins (Khalsa et al., 1998; Ray and Wharton, 2001).

72   Unlike typical GPCRs, the mouse GPR155 sequence predicts that it has 17

73   transmembrane regions. Five variants of GPR155 mRNA have been identified,

74   including the Variant 1 and Variant 5 proteins, which have an intracellular DEP

75   domain near the C-terminal (Trifonov et al, 2010). GPR155 has only been reported in

76   mouse, and evidence suggests that GPR155 plays specific roles in Huntington disease

77   and autism spectrum disorders (Brochier et al., 2008). However, the molecular

78   function and biological processes that are affected by *Drosophila* GPR155 have not

79   been reported, including in wing development. *Drosophila* CG7510, which we have

80   called *anchor*, is an integral membrane protein that is the ortholog if vertebrate

81   GPR155, and it shares more than 37% homology with the mammalian GPR155. In

82   this study, we knocked down *anchor* using wing-specific Gal4 lines, and we observed

83   enlarged wing size, and a thickened and ectopic veins phenotype. During larval and

84   pupal stages, we detected ectopic levels of p-Mad, which is downstream of BMP

85   signaling, when *anchor* RNAi was applied. This also increased the levels of target

86   genes such as *sal* and *omb*. Taken together, those results suggest that the novel

87   functions of Anchor are mediated through the negative regulation of BMP signaling to

88   control wing development in *Drosophila*.

RESULTS

Locus of the *anchor* gene and homolog analysis

The transcription site of *anchor* is located at 74E2-74E3 on chromosome 3 in *Drosophila*, and its open reading frame (ORF) encodes a predicted protein that is 949 amino acid residues long (Fig. 1A). A membrane protein called Anchor was identified in a systematic search for genes that are homologous to known G-protein coupled receptors (GPCRs). The amino acid sequence of the Anchor gene contains 17 transmembrane domains and a C-terminal DEP domain (Fig. 1B). *anchor* encodes a protein with more than 37% homology to GPR155 in humans, chimpanzees, mice and rats (Fig. 1C). The conserved sequence in the DEP domain was first observed in three proteins: Dishevelled (*D. melanogaster*), which is an adaptor of the Wingless (Wnt) signaling pathway (Klingensmith et al., 1994); EGL-10 (*C. elegans*), which is a negative regulator of GPCR signaling (Koelle and Horvitz, 1996); and mammalian Pleckstrin, which mediates signaling in platelets and neutrophils (Kharrat et al., 1998).

Knocking down *anchor* induced extra vein formation in adult flies

To study the *in vivo* role of Anchor in *Drosophila* development, we used an *anchor* RNAi attached to a ubiquitously expressed promoter (e.g., *Actin-Gal4* and *Tubulin-Gal4*). Unfortunately, the RNAi induced a severe embryonic lethal phenotype. Next, we combined the *anchor* RNAi with *Hs-Gal4* at 25°C and then shifted the temperature to 37°C for 90 min each day beginning in the third instar larval stage and

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110   continuing until the flies were adults. Surprisingly, we found that the adult wings of

111   these flies exhibited abnormal wing veins (data not shown). These results led us to

112   speculate that the novel gene *anchor* plays an important role in *Drosophila* wing

113   development. To determine whether the abnormal wing phenotypes were caused by

114   the autonomous loss of the Anchor protein, we used *A9-Gal4* and *MS1096-Gal4* to

115   ubiquitously knockdown *anchor* in the wing blade. Unexpectedly, these flies

116   displayed thicker and more veins than the wings in the control flies (Fig. 2A-B, H-I);

117   When we used the *dpp-Gal4*, *ptc-Gal4* and *en-Gal4* drivers, we also observed ectopic

118   vein tissues in the *anchor* RNAi-induced adult wings (Fig. 2C-E, J-L), and we

119   observed that the extra veins in these flies were restricted to the AP boundary and the

120   anterior or posterior compartment of the wing. *ap-Gal4* is specifically expressed in

121   the dorsal cells of the L3 wing disc, while *brk-Gal4* is expressed in the tissues

122   surrounding the wing pouch cells in the L3 wing disc and the intervein cells in pupal

123   wings (Campbell and Tomlinson, 1999; Sotillos and De Celis, 2005). As expected, we

124   observed wing expansion defects when we used the *ap-Gal4* and *brk-Gal4* drivers. In

125   addition, a blistering phenotype was observed in *ap>anchor* RNAi wings (Fig. 2 F-G,

126   M-N).

127   We also observed a moderate but consistent increase in wing size in the

128   *A9>anchor* RNAi flies, in which the wings were more than 17% larger than those in

129   the controls (Fig. S1A, C). The distance between L3 and L4 was increased by

130   approximately 29% in the *ptc>anchor* RNAi wings (Fig. S1B, D). In addition, we

131   used another *anchor* RNAi construct (v105969) in combination with the

132   *MS10986-Gal4* and *A9-Gal4* drivers, and we observed that extra veins developed and

133   that wing size was increased (Fig. S2). To provide further confirmation that Anchor is

134   involved in wing development, we constructed transgenic flies in which the entire

135   *anchor* coding region was ectopically expressed. We then combined these flies with

136   *A9>anchor* RNAi flies. As expected, the extra vein phenotype was clearly rescued in

137   flies carrying a functional full-length *anchor* (Fig. S3A, B). *anchor*[P] is a *P*-element

138   insertion in the 5' UTR region of *anchor* that encodes a Gal4-responsive enhancer.

139   Similar to previous results, we observed that overexpressing *anchor*[P] using the

140   *A9-Gal4* driver as the background for *anchor* RNAi resulted in the rescue of the

141   thickened wing vein phenotype (Fig. S3C, D). These observations suggest that Anchor

142   plays an important role in wing development and especially in wing vein formation.

143   Furthermore, the phenotype of increased vein tissues in the *anchor* RNAi wings is

144   similar   to   that   observed   in   gain-of-function   BMP   signaling   experiments

145   (Martín-Castellanos and Edgar, 2002; O'Keefe et al., 2014). We therefore proposed

146   that Anchor may be involved in BMP signaling during *Drosophila* wing development.

147   **The control of Anchor wing size was mediated by an increase in cell proliferation**

148   **and size in imaginal discs**

149   To determine the biological function of Anchor during wing development, we

150   first analyzed the expression pattern of *anchor* using *in situ* hybridization. We

151   observed that *anchor* is expressed in a generalized manner in imaginal discs (Fig. S4A,

152   C). However, we observed significantly reduced *anchor* levels in RNAi flies that also

153   expressed *MS1096-Gal4* and *A9-Gal4* (Fig. S4B, D). This result further confirmed

154   that the increased wing vein phenotype resulted from low expression levels of the

155   *anchor* gene. We also observed that imaginal discs were enlarged in *anchor* RNAi

156   flies, which led us to speculate that Anchor is required to inhibit excessive cell

157   proliferation in imaginal discs. Wings cell proliferation begins during the larvae stage,

158   and the size of the adult wing is predetermined by the final size of the wing imaginal

159   disc (Day and Lawrence, 2000). The enlarged wing size observed in the *anchor* RNAi

160   flies may have resulted from an increase in cell number or cell size. To explore this,

161   we first analyzed cell proliferation using phospho histone H3 antibodies (PH3), which

162   stain dividing cells that are in M phase. The numbers of PH3$^+$ cells were significant

163   higher, by 42%, in the *A9>anchor* RNAi wing discs than in the controls (Fig. 3A, B,

164   G). We further identified the L3 wing blade using Wg antibodies and found that the

165   size of the wing blade in *A9>anchor* RNAi flies was significantly increased by 40%

166   (Fig. 3A', B', H). Unexpectedly, we also found that Wg expression levels were

167   significantly increased (Fig. 3A', B', I). Next, we compared cell size using phalloidin

168   staining and found that the average size of cells in the *A9>anchor* RNAi flies was

169   higher, by 32%, than the cell size in the controls (Fig. 3D, E, J). To validate our

170   proposal that Anchor is involved in controlling wing and cell size, we overexpressed

171   the full-length *anchor* gene in combination with *A9>anchor* RNAi in flies. As

172   expected, this combination completely rescued the phenotype, including the number

173   of PH$^+$ cells, cell size, wing blade and Wg expression levels. Wing size was, in

174   particular, completely rescued (Fig. 3C, C', F and G-J).

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175 Next, we analyzed apoptopic cell death using a direct TUNEL labeling essay.

176 However, we did not observe any difference between the *anchor* knockdown and

177 control wing discs (data not shown), indicating that the hyperproliferative wing discs

178 of the *anchor* RNAi flies as not caused by apoptosis-induced compensatory

179 proliferation. Hence, although *anchor* RNAi caused the transformation of interveins

180 into more densely packed vein tissues, we also observed an increase in wing size, and

181 these results demonstrate that the mechanism by which Anchor controls wing size is

182 mainly mediated by the regulation of cell proliferation and cell size in imaginal discs.

183 **Knocking down *anchor* induced BMP downstream target genes in imaginal discs**

184 The ectopic veins phenotype in the *anchor* RNAi wing disc was similar to that

185 observed in gain-of-function of BMP signaling experiments, which were

186 demonstrated by inducing the excessive phosphorylation of Mad (Mothers against dpp)

187 (Haerry, 2010). Therefore, we next analyzed BMP signaling activity in knockdown

188 *anchor* flies using p-Mad antibodies. We observed that p-Mad had a normal

189 expression pattern along the AP boundary in *A9>anchor* RNAi discs but that its total

190 intensity was clearly enhanced (Fig. 4A, B, K). Ectopically expressing either Dpp or

191 excessively activating the Dpp receptor caused the overgrowth of the wing disc and

192 increased the expression of its downstream targets (Nellen et al., 1996. Haerry et al.,

193 1998). The markers *omb*, *sal*, *dad* and *brk* contain p-Mad dependent expression

194 domains and are expressed along the AP boundary (Minami et al., 1999). Therefore,

195 we analyzed the downstream transcriptional targets of p-Mad to identify the point of

196 coupling between p-Mad and its targets in *anchor* knockdown flies. We showed that

197 the brightness and widths of the *omb* and *sal* expression domains were strongly

198 increased in *anchor* knockdown discs; however, *dad* and *brk* levels were similar to the

199 levels observed in the control, and their total intensity was not changed (Fig. 4C-J,

200 L-O). In pupal wings, *dad* levels were significantly increased; however, *brk* levels

201 were reduced (Fig. S5). This result suggested that there is antagonism between

202 Anchor and BMP signaling during wing development. However, the increased levels

203 of p-Mad were not enough to induce changes in *dad* and *brk* levels in the imaginal

204 disc.

205 **Anchor maintains the normal presumptive veins formation through limit BMP**

206 **signaling in pupa stage**

207 During metamorphosis, Dpp signaling is dramatically altered in the *Drosophila*

208 wing. In the imaginal wing disc, the level of p-Mad reflects the gradient of Dpp, and

209 this pattern is maintained during the early stages of wing metamorphosis. The p-Mad

210 gradient is then lost and re-localized to presumptive veins (de Celis, 1997; O'Keefe et

211 al., 2014) (Fig. 5A-A', C-C'). Because of these shifts in the Dpp signaling zone, we

212 hypothesized that *anchor* may regulate presumptive vein cell differentiation. We

213 stained the intervein regions with anti-*Drosophila* Serum Response Factor (DSRF).

214 We showed that p-Mad is roughly overexpressed, that its pattern is disordered in

215 *anchor* knockdown pupal wings, and that edge veins excessively accumulated (Fig.

216 5B-B', D-D'). This result indicates that p-Mad is also dramatic increased during the

217 early pupal stage in *anchor* knockdown flies, which led to an ectopic vein phenotype

218 in adult wings. Therefore, we concluded that *anchor* is essential for controlling BMP

219 signaling to safeguard the proper formation of normal wing veins during early pupal

220 stages.

221 **Anchor targets *dpp* or works upstream of *dpp* to antagonize BMP signaling**

222 During the pupal stage, Dpp signaling is critically required for wing vein cell

223 differentiation and for these cells to acquire the appropriate location (de Celis, 1997).

224 Ectopically expressing either *dpp* or activating Dpp signaling resulted in thickened or

225 extra veins (O'Keefe et al., 2014). To further investigate the relationships between

226 Anchor and Dpp signaling, we used a genetic approach to perform a rescue

227 experiment. Downregulating the Dpp signaling mediators *med* and *mad* using RNAi

228 in flies induced the removal of parts of L3, L4 and L5 (Fig. 6G-H). However,

229 combining the depletion of *med* or *mad* with *A9>anchor* RNAi flies limited the width

230 of veins (Fig. 6A, M-N). Similarly, knocking down *anchor* using the background null

231 allele *mad^{12}* also rescued the broadening of vein tissues (Fig. 6B-C). This result

232 indicated that *anchor* is required to limit vein tissue formation, while *med* or *mad* are

233 downstream effectors of *anchor*. We also investigated the genetic relationship

234 between *anchor* and Tkv, Sax or Put receptors, which are involved in BMP signaling.

235 The hypomorphic alleles *tkv^7*, *tkv^{427}* and *sax^4* or the RNAi flies *A9>sax* RNAi and

236 *A9>put* RNAi resulted in significantly suppressed thickened vein tissue that was

237 caused by a reduction in *anchor* levels (Fig. 6A-B, D-F, I-J, O-P). The *mad^{12}/+*,

238    $sax^4/+$, $tkv^7/+$, and $tkv^{427}/+$ flies exhibited normal adult wing phenotypes (data not

239   shown).

240   In addition, knocking down *dpp* or *gbb* severely depleted vein tissue and reduced

241   wing size (Fig. 6K-L). However, knocking down *dpp* in *A9>anchor* RNAi flies did

242   not rescue the loss of wing vein phenotype, and the adult wing veins displayed a

243   phenotype that was similar to the phenotype caused by the *A9>dpp* RNAi (Fig. 6

244   K-Q). In addition, the reduced wing size and loss of veins that were caused by *gbb*

245   RNAi were rescued when the RNAi was combined with *A9>anchor* RNAi flies,

246   although the distal veins remained slightly thickened (Fig. 6B, L-R). This result

247   indicates that Dpp is required for the ectopic vein formation that is caused by

248   knocking down *anchor*. Thus, the *dpp* depletion phenotype was epistatic to the

249   reduction in *anchor*, which shows that Dpp is required for the reduction in wing veins

250   that was observed in the *anchor* phenotype. The genetic epistasis test suggested that

251   *anchor* is likely to function upstream of Dpp signaling or by targeting Dpp through a

252   parallel pathway.

13

DISCUSSION

In this study, we have analyzed the function of novel gene *anchor* in wing development. Inducing the wing-specific knockdown of *anchor* resulted in the formation of thickened and ectopic veins. Moreover, this phenotype was similar to that observed in gain-of-function BMP signaling experiments. In addition, we also observed enlarged wing blades and an increase in wing size in the *anchor* RNAi flies because of the high number of PH3-positive cells and enlarged wing blade cells. In addition, the intensity profile for p-Mad was enhanced in *anchor* RNAi-induced imaginal discs. Hence, Anchor substantially contributed to limiting the phosphorylation of Mad in presumptive veins during the pupal stage, and accordingly, the BMP signaling targets genes were significantly up-regulated. In genetic interaction experiments, we found that *anchor* was most likely functioning upstream of *dpp* or that it targeted *dpp*. We concluded that Anchor functions during wing vein development to inhibit BMP signaling.

Anchor regulates cell proliferation in wing discs

When the *anchor* RNAi was expressed throughout the wing disc in *Gal4* fly lines, it caused an increase in wing size in larvae and adults. Many different types of signals participate in cell proliferation within developing tissues. For example, Wg and Dpp signals mediate wing disc growth in different ways. The morphogen Wg is expressed at the wing pouch boundary to delineate the domain of the wing blade within the wing disc. The involvement of Wg signaling in compensatory proliferation has been

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274   explored by examining its expression in apoptotic cells. In apoptotic cells, ectopic Wg

275   signaling induced compensatory proliferation during mitosis (Martín et al., 2009).

276   However, when we used TUNEL assays, the number of apoptotic cells was not

277   changed when Wg intensity was increased in *anchor* RNAi-induced wing discs.

278   Therefore, increased Wg intensity was another factor that enlarged wing blades in the

279   *anchor* RNAi flies. The morphogen Dpp is expressed along the AP boundary, where it

280   forms a gradient during wing disc development. Dpp signaling is thought to promote

281   growth and proliferation. When *dpp* was overexpresses or enhanced in the wing disc,

282   it led to overgrowth or enlarged wing size in adult flies (Martín-Castellanos and Edgar,

283   2002; O'Keefe et al., 2014). We observed a dramatic increase in the levels of p-Mad,

284   *sal* and *omb* in the *anchor* knockdown flies, which indicated that Anchor opposed

285   Dpp signaling to inhibit cell proliferation in wing discs.

286   **Dpp signaling rebuilds after pupariation**

287   In wing imaginal discs, Dpp is expressed in a strip of cells close to the anterior

288   edge of the AP boundary. It transmits its signal via receptor complexes and transducer

289   dimers (Israel et al., 1996). After pupariation, Dpp signaling continues to be expressed

290   at the AP boundary and also appears along presumptive veins. This shift in expression

291   results in subsequent changes in p-Mad and downstream targets. At 24 hr APF, p-Mad

292   expression is restricted to presumptive and marginal vein cells. *omb* is expressed in

293   the distal edge of the pupal wing, and *dad* expression is broadly activated in cells

294   corresponding to presumptive veins. At the same time, the expression of *brk* is

295 eliminated from presumptive veins and becomes restricted to intervein cells (Sotillos

296 and De Celis, 2005). p-Mad levels are substantially increased in pupal wings that

297 express low levels of *anchor* at 24 hr APF. Its transcriptional targets, including *omb*

298 and *dad*, are also substantially increased (Fig. S3). Consistent with p-Mad expansion

299 in intervein region, brk expression is abolished from essentially intervein cells in

300 *anchor* RNAi pupal wing. These results are evidence that *anchor* is involved in Dpp

301 signaling and that it maintains the position of wing veins and distinguishes the

302 boundaries between veins and interveins.

303 **anchor interactions with ligands antagonize BMP**

304 Our experiments reveal the following important factors that contribute to the

305 connection between Anchor and BMP signaling: (1) decreasing *anchor* levels in the

306 wing discs induces the overexpression of BMP signaling factors, which increases

307 wing blade size and adult wing size; (2) in these flies, the levels of BMP signaling

308 effectors, including p-Mad, *sal*, and *omb*, are generally increased in the wing discs,

309 with p-Mad levels being especially increased during the pupal stage; and (3)

310 mutations in BMP signaling factors rescued the ectopic phenotypes observed in

311 *A9>anchor* RNAi-induced flies to varying degrees. These data indicate that in the

312 absence of *anchor*, BMP signaling is enhanced.

313 In normal wing disc cells, the Dpp ligand prefers to bind to Tkv, while the Gbb

314 ligand has a higher affinity for Sax. The type I receptor Tkv is required for the

315 transcription of all BMP signaling target genes during wing development, and Sax

316 promotes BMP signaling by forming a receptor complex with Tkv. In wing discs, Tkv

317 and Sax can form three types of receptor dimer complexes: Tkv-Tkv, Tkv-Sax and

318 Sax-Sax. The Tkv-Sax receptor complexes are probably the mechanism by which Sax

319 contributes to promoting BMP signaling, which initiates a more robust intracellular

320 phosphorylation cascade than the Tkv-Tkv dimers (Bangi and Wharton, 2006).

321 However, Sax-Sax dimers initiate almost no signaling (Weis-Garcia and Massagué,

322 1996; Haerry, 2010). The Tkv-Sax receptor complexes result in more substantial

323 signaling than the other dimer complexes. Anchor appears to preferentially bind to the

324 ligand Dpp (Fig. 7E). Knocking down *anchor* induced p-Mad levels to increase,

325 which caused adult wings to exhibit thickened and ectopic veins. Anchor was not

326 available to control the ligand Dpp, which could then induce the increased

327 phosphorylation of Tkv homodimers (Fig. 7A and F). We found that overexpressing

328 *tkv* or *dpp* enhanced the extent of ectopic vein tissue to background *anchor*

329 knockdown levels (Fig. 7B and C). Compared to the effects of the *anchor* RNAi,

330 overexpressing *tkv* or *dpp* increased the number of Tkv receptor homodimers, which

331 led to a moderate increase in phosphorylation (Fig. 7G). Substantially overexpressing

332 *gbb* allowed Dpp and Gbb to activate Tkv-Sax heterodimers to undergo high levels of

333 phosphorylation in *anchor* deletion mutants (Fig. 7D and H). Therefore, we

334 hypothesized that Anchor restricts the ligand Dpp from inducing the excessive

335 formation of heterodimers or homodimers, which led to a large increase in p-Mad

336 levels.

337   When we simultaneously reduced *anchor* and *gbb* expression, we observed adult

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338   wing veins that displayed normal longitude veins but distal veins that were slightly

339   broadened (Fig.6B, L, R). This phenomenon indicated that *anchor* affected Gbb

340   signaling but did not directly interact with the ligand Gbb. When we simultaneously

341   reduced *anchor* and *dpp* levels, we observed adult wing veins that displayed a

342   phenotype like that of the *dpp* RNAi-induced flies, which lost almost all of their veins

343   (Fig. 6B, K and Q). These data further confirm that *anchor* is likely to function either

344   upstream of Dpp signaling or through a parallel pathway. We propose the hypothesis

345   that Anchor traps the Dpp ligand to negatively regulate the BMP signaling pathway

346   during wing development.

18

347   **METERIALS AND METHODS**

348   **Fly stocks**

349   The two *anchor* RNAi forms (v8532, v105969) were obtained from the Vienna

350   *Drosophila* RNAi Stock Center (VDRC). The *med* RNAi, *mad* RNAi, *dpp* RNAi, *gbb*

351   RNAi, *sax* RNAi, and *put* RNAi were obtained from Tsinghua *Drosophila* model

352   animal center. The *dpp-Gal4*, *ap-Gal4*, *patched-Gal4*, *brk-Gal4*, *en-Gal4*, *omb-lacZ*,

353   *dad-lacZ*, *brk-lacZ*, null allele *Mad^{12}*, *tkv^{427}*, *tkv^7* and *sax^4* flies were described

354   previously (Liu et al., 2011). The *sal-lacZ* was generously provided by J Shen

355   (University of China Agriculture). The *UAS-anchor* was obtained by germline

356   transformation. A strain in which *anchor^{G9098}* was inserted using *P* elements into the

357   5' region of the *anchor* gene was purchased from GenExel (Daejeon, Korea). Other

358   strains used in this study included *UAS-dpp^{19B5}*, *UAS-gbb^{99A2}*, *UAS-tkv^{1A3}*, *A9-Gal4*,

359   *MS1096-Gal4*, *ptc-Gal4*, and *w^{1118}*. All genotypes were bred into the *w^{1118}* background.

360   In this study, we used another *anchor* RNAi construct (v105969) to knockdown

361   *anchor* levels, as shown in Fig. S2.

362   ***In Situ* Hybridization**

363   The partial sequence of *anchor* (1-600 bp) was amplified from cDNA using PCR and

364   subcloned into the pSPT18/19 vector. Digoxigenin-labeled riboprobes were prepared

365   using T7 RNA polymerase and a DIG RNA labeling kit (Roche). RNA *in situ*

366   hybridization on third instar larval (L3) wing discs was carried out according to a

367   standard protocol. The tissue was fixed in RNAse-free 4% paraformaldehyde in PBS

368   for 1 hr. The wing discs were prehybridized with 100 μg of salmon sperm DNA/ml in

369   hybridization buffer at 55°C for 1 hr. Hybridizations were performed overnight at

370   55°C in hybridization solution containing an *anchor* probe. The wing discs were

371   incubated with alkaline phosphatase-conjugated anti-DIG antibodies (Roche) for 2 hr

372   at room temperature, and hybridization signals were visualized using BCIP/NBT.

373   Finally, the slides were mounted using Vectashield mounting medium (Vector

374   Laboratories) and analyzed using an Axioskop 2 plus microscope (Zeiss). All

375   experiments were independently repeated at least three times

376   **Immunohistochemistry**

377   Wing imaginal discs obtained from third instar larvae were fixed in 4%

378   paraformaldehyde for 1 hr at room temperature. The wing discs were then placed in

379   blocking buffer (PBS plus 0.1% Tween 20 and 5% normal goat serum) for 1 hr at

380   room temperature. To prepare pupal wings, late third instar larvae were selected and

381   allowed to develop for 24 hr after puparium formation (APF) at 29°C. Whole pupae

382   (24 hr APF) were removed from their pupal cases and fixed in 3.7% formaldehyde for

383   2 hr at room temperature. The pupal wings were dissected in 0.3% PBST (0.3%

384   TritonX-100 in PBS), incubated in blocking buffer (PBS containing 0.3% Triton

385   X-100, 2% BSA, and 2% normal goat serum) for 1 hr (Liu et al., 2011). The wings

386   were incubated in primary antibodies overnight at 4°C and then incubated with

387   secondary antibodies according to standard methods. Finally, the wings were mounted

388   in Vectashield fluorescent mounting medium (Vector Laboratories) or Prolong

389   diamond antifade mountant (Molecular probes). The tissues were analyzed using a

390   LSM 510 META confocal microscope (Zeiss) or an Axioskop 2 plus microscope

391   (Zeiss). The following primary antibodies were used: mouse anti-β-gal (1:200,

392   Promega), rabbit anti-pSmad1 (1:50, a gift from Ed Laufer, Columbia University,

393   New York) (Vargesson and Laufer, 2009), rabbit phospho-H3 (1:800, Upstate), and

394   mouse anti-DSRF (1:100, Active Motif). Secondary antibodies were conjugated with

395   Alexa Fluor 488 and Alexa Fluor 568 (Molecular probes) and used at a 1:200 dilution.

396   All experiments were independently repeated at least three times.

397   **Transgenic constructs**

398   To generate a *UAS-anchor* strain, the full-length *anchor* coding sequence was PCR

399   amplified and cloned into pUAST between the underlined restriction sites. The

400   following   primers   were   used:

401   5′-AAAGAATTCATGGACAGCTCCATGTACTACG-3′   and   5′-

402   AAACTCGAGCTATATGCGACTGCAGAAAT-3′.   Transgenic   flies   were   then

403   generated using standard methods.

404   **Statistical Analysis**

405   Images were acquired using a Zeiss fluorescence microscope. All numerical data,

406   including wing size, imaginal disc size, cell size, cell number and intensity values,

407   were analyzed using image J. The statistical analyses were performed using a

408   two-tailed unpaired Student′s *t*-test with Prism software (GraphPad 6.0). ****$P<0.005$

21

409   was considered significant, and ***$P<0.001$ was considered more significant. "ns"

410   indicates no significant difference. Error bars in the graph indicate SEM.

Competing interests

418   The authors declare no competing or financial interests.

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545   1756-1759.

546 **Figure legends:**

547 **Fig. 1. Molecular characteristics of *Drosophila* Anchor.**

548 (A) The transcriptional unit containing *anchor* is located at 74E2-74E3 on

549 chromosome 3. (B) Diagram of the predicted secondary structure of Anchor. Potential

550 17-transmembrane domains and a DEP domain were identified using TMHMM

551 software (http://www.cbs.dtu.dk/services/TMHMM/). The transmembrane domain

552 amino acids are indicated by red lines. (C) Protein alignment was performed using

553 Clustal X with the following sequences: fruit fly (*Drosophila melanogaster*,

554 NP_648998.2), human (*Homo sapiens*, NP_001253979.1), chimpanzee (*Pan*

555 *troglodytes*, XP_003309468.1), mouse (*Mus musculus*, NP_001177226.1), and rat

556 (*Rattus norvegicus*, NP_001101281.1). The fruit fly *anchor* shared 37% homology

557 with the human sequence, 37% with the chimpanzee sequence, 38% with the mouse

558 sequence, and 38% with the rat sequence.

559 **Fig. 2. Wing-specific knockdown of *anchor* resulted in thickened and extra veins**

560 **in adult flies**

561 (A-G) Control wing of a female *Gal4*/*w*^*1118*^ (*Gal4>+*) fly. The wing-specific *Gal4*

562 driver included *A9-Gal4, MS1096-Gal4, dpp-Gal4, ap-Gal4, ptc-Gal4, brinker-Gal4,*

563 and *en-Gal4.* Longitudinal veins (L2-L5) and crossing veins (ACV, PCV) are

564 indicated in A. (H-N) The *anchor* RNAi flies were crossed with flies carrying a

565 wing-specific *Gal4* driver. These flies displayed wing blades with reduced sizes in

566   differing compartments. (H) The *A9>anchor* RNAi, (I) *MS1096>anchor* RNAi, (J)

567   *dpp>anchor* RNAi, (K) *ptc>anchor* RNAi, and (L) *en>anchor* RNAi resulted in the

568   formation of ectopic and thickened veins. (M) The *ap>anchor* RNAi resulted in wing

569   blistering (arrow). (N) The *brk>anchor* RNAi resulted in reduced wing size and

570   patterning defects. All images were acquired at the same magnification, and all

571   crosses were performed at 29°C (A-C, H-J) or 25°C (D-G, K-N). Scale bar: 200 μm.

572   **Fig. 3. Analysis of cell proliferation and cell size in *anchor* RNAi imaginal discs**

573   (A-C') Analysis of mitosis and wing blade development in *A9>+*, *A9>anchor* RNAi

574   and *A9>anchor RNAi/UAS-anchor*-induced flies. Cell proliferation was monitored

575   using PH3 (green), wing blade morphology was detected using Wingless (Wg, red),

576   and the wing blade is outlined by a white dotted line. The PH3$^+$ cells in the area

577   containing the wing blade and wing blade sizes were significantly increased in

578   *A9>anchor* RNAi-induced flies (B-B'). This phenotype was completely rescued by

579   *UAS-anchor* (C-C'). (D-F) Cell outlines were visualized using phalloidin (green), the

580   white squares in the discs are shown at a higher magnification (Scale bar =10μm), and

581   cell size was increased in the *anchor* RNAi flies. (G-J) Quantification of PH3$^+$ cell

582   numbers, wing blade size, Wg labeling intensity and the average size of a single cell

583   in the dorsal area of the wing pouches shown in A-F. All combinations were grown at

584   29°C and a select group of female larvae were dissected. Scale bar: 50μm

585 **Fig. 4. Analysis of BMP signaling pathway activity in *anchor* RNAi flies**

586 (A-B, K) Immunohistochemical staining for p-Mad (green) in *anchor* RNAi flies

587 showed that the p-Mad expression domain was larger in the mutant wing discs than in

588 the control discs (A-B). The canonical late L3 p-Mad profile is outlined with blue and

589 orange lines in *A9>anchor* RNAi and *A9>+* flies in the dorsal wing pouch, as shown

590 in A and B (K). (C-J) The expression levels of BMP target genes, including *omb*, *sal*,

591 *dad* and *brk*, were determined using *lacZ* reporter genes. The levels of *omb* and *sal*

592 were significantly increased (C-F); however, *dad* and *brk* levels were not significant

593 altered and were similar to the levels observed in the controls (G-J). (L-O)

594 Quantification of the total intensity values of the four BMP target genes shown in C-J.

595 All combinations were grown at 29°C, and female larvae were dissected. Scale bar:

596 50 µm.

597 **Fig. 5. Increased and ectopic accumulation of p-Mad in presumptive veins and at**

598 **the margin in *anchor* RNAi flies during pupal development**

599 Immunochemical staining showing the wing blade and interveins was performed

600 using p-Mad (green) and DRSF (red) antibodies at 24 hr APF. p-Mad was localized to

601 presumptive veins and the anterior margin in control pupal wings (A-A' C-C'). After

602 *anchor* knockdown, pupal wings displayed a massive amount of ectopic p-Mad, and it

603 accumulated in cells near the distal wing margin (B-B', D-D'). All combinations were

604 grown at 29°C. Scale bar: 100 µm.

31

605 **Fig. 6. Anchor antagonizes the BMP signaling pathway.**

606 (A, B) *A9>anchor* RNAi resulted in broadened L3, L5 and distal marginal veins and

607 ectopic L5 and PCV veins, as indicated by arrows. (C) *A9>anchor* RNAi/*mad*[12]

608 suppressed the development of distal marginal veins and broadened L5 (C versus B,

609 arrows). (D) *A9>anchor* RNAi/*sax*[4] suppressed the broadening of distal marginal

610 veins (D versus B, arrows). (E) *A9>anchor* RNAi/*tkv*[7] suppressed the broadening of

611 L5 (E versus B, arrow). (F) *A9>anchor* RNAi/*tkv*[427] suppressed the broadening of

612 distal marginal veins and L5 (F versus B, arrow). (M) *A9>anchor* RNAi/*med* RNAi

613 suppressed the broadening of distal marginal veins (M versus A, arrow), and the low

614 expression of *med* resulted in shortened L3, L4 and L5 (M versus G). (N) *A9>anchor*

615 RNAi/*-mad* RNAi suppressed the broadening of L5 (N versus A, arrow), and the low

616 expression of *mad* resulted in shortened L3, L4 and L5 (N versus H). (O) *A9>anchor*

617 RNAi/*sax* RNAi suppressed the broadening of L2 (O versus A, arrow). (P)

618 *A9>anchor* RNAi/*put* RNAi resulted in a phenotype similar to that of the *put* RNAi

619 (P versus J), and the missing PCV and missing portion of L3-5 resulted from the low

620 expression of *put*. (Q) *A9>anchor* RNAi/ *dpp* RNAi resulted in a phenotype similar

621 phenotype to that of the *dpp* RNAi (Q versus K), and the missing veins resulted from

622 the low expression of *dpp*. (R) *A9>anchor* RNAi/ *gbb* RNAi suppressed the

623 broadening of L3, L5 and distal marginal veins (R versus A), and the missing PCV

624 and the missing portion of L5 was caused by the low expression of *gbb* (R versus L).

625 Other details of these experiments are analyzed in Table S1. Scale bar: 200 μm.

626   **Fig. 7. Hypothetical Models explaining the opposing effects of Anchor on the**

627   **BMP signaling pathway.**

628   (A-D) Inducing the overexpression of *tkv* or *dpp* (BMP effectors) in *anchor* RNAi

629   flies enhanced the thickened and ectopic vein phenotype (B and C, arrows). Similar to

630   the effect of overexpressing *gbb*, these flies formed massively ectopic veins (D). All

631   of the flies were grown at 25°C, scale bar: 200 μm. (E) A model showing both the

632   antagonistic functions and the signaling functions of *anchor* in wing disc cells. The

633   ligands displayed preferential affinity to different receptors, with Dpp preferentially

634   binding to Tkv receptors and Gbb preferentially binding to Sax receptors. The

635   different receptor complexes induced varying degrees of phosphorylation degrees.

636   Tkv-Sax receptor complexes mediated more significant levels of signaling (thickened

637   arrow) than were initiated by Tkv-Tkv complexes (thin arrow). However, Sax-Sax

638   complexes did not phosphorylate Mad. Anchor restricts the free ligand Dpp from

639   being released from the intracellular compartment, to some extent. (F) Cells

640   expressing low levels of *anchor* failed to limit the ligand Dpp, resulting in more Dpp

641   binding to each to increase the levels of p-Mad. (G) Overexpressing *dpp* or tkv in

642   *anchor* RNAi cells contributed to a higher level of p-Mad than was observed in

643   *anchor* RNAi cells (compare C to A). (H) In the absence of *anchor*, overexpressing

644   *gbb* led to the ligands inducing the formation of heterodimers. These dimers bind to

645   Tkv-Sax receptor complexes to induce the levels of p-Mad to increase (compare D to

646   A).

33

647   **Fig. S1**. **Wing-specific knockdown of *anchor* caused enlarged wing size in adult**

648   **flies**

649   (A) Overlapped adult wings obtained from *A9>+* and *A9>anchor* RNAi flies show

650   that wing size was larger in the anchor RNAi flies. (B) Overlapped adult wings

651   obtained from *ptc>+ and ptc>anchor* RNAi flies show that the distance between L3

652   and L4 was longer in the *anchor* RNAi wings. (C, D) The quantification of wing size

653   and the distance between L3 and L4 are compared in A and B. All flies were grown at

654   25°C, scale bar: 200 μm.

655   **Fig. S2**. **Knockdown of *anchor* using *anchor* RNAi (v105969) resulted in a**

656   **phenotype similar to that of *anchor* RNAi (v8532) flies, which displayed extra**

657   **veins and larger wings**

658   (A-F) Knocking down *anchor* using *MS1096>anchor* RNAi (v105969) and

659   *A9>anchor* RNAi (v105969) resulted in extra veins and enlarged wings, similar to the

660   anchor RNAi (v8532)   phenotype. (G, H) The quantification of wing size, as

661   compared in C and F. All flies were grown at 29°C, scale bar: 200 μm.

662   **Fig. S3. Overexpressing *anchor* rescued the ectopic phenotypes in *anchor* RNAi**

663   **flies**

664   Overexpressing full length *anchor* using *UAS-anchor* or *anchor^P* in RNAi flies

665   significantly suppressed extra vein formation in adult flies (B compared to A). The

666   *anchor^P* flies contained a *P*-element insertion in the 5' UTR region of *anchor* that

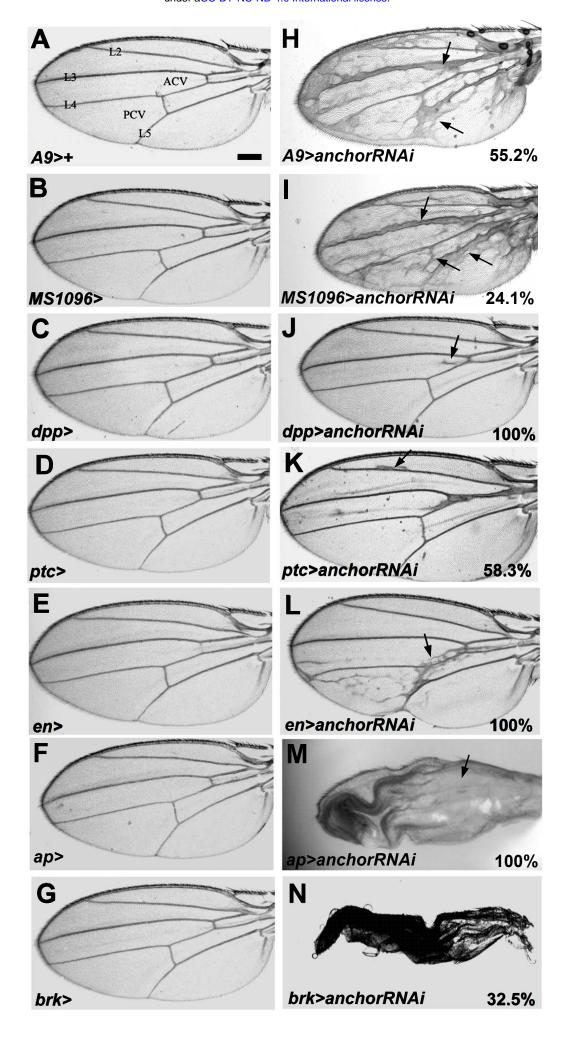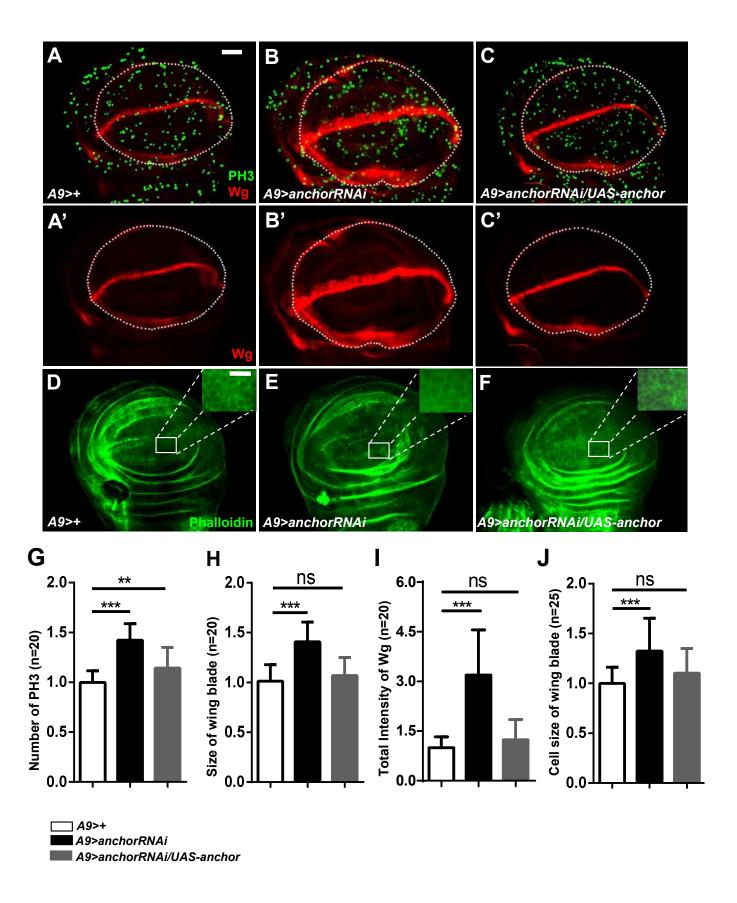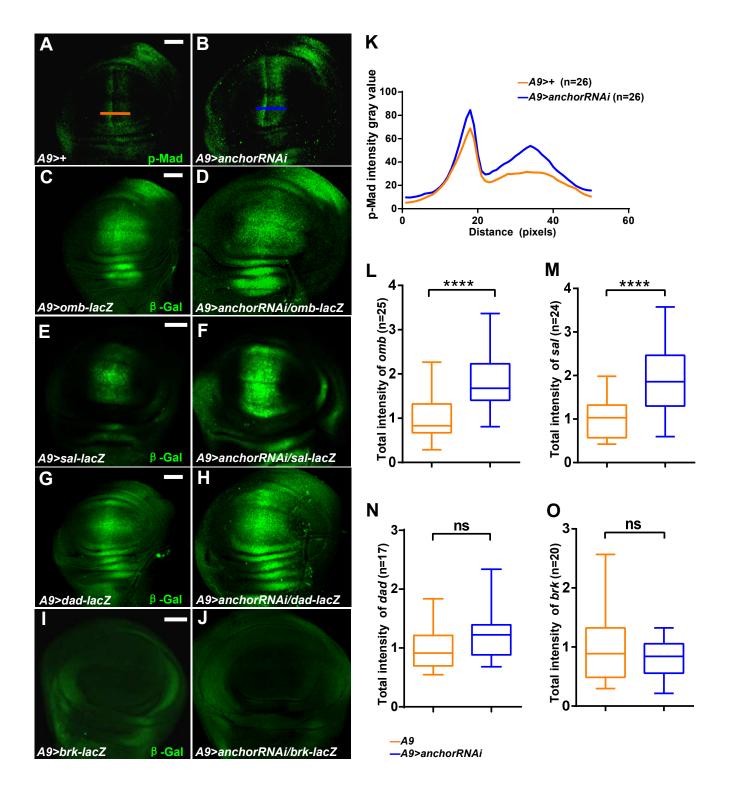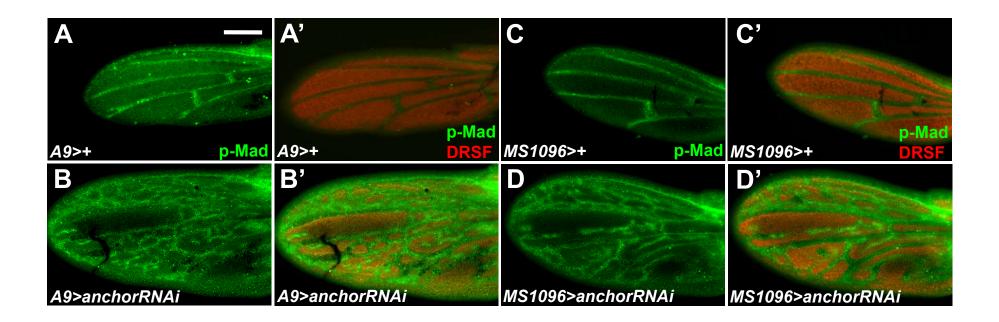667   encoded a Gal4-responsive enhancer (D compared to C). All crosses were performed

668   at 18°C (A-B) or 25°C (C-D). Scale bar: 200 μm.

669   **Fig. S4. Distribution of *anchor* gene expression in wing discs.**

670   *In situ* hybridization was performed using an *anchor*-specific probe in L3 wing discs.

671   (A, C) In control discs, the *anchor* gene is expressed in a generalized manner. (B, D)

672   When an *anchor* RNAi driven by *MS1096-Gal4* or *A9-Gal4* was used, we observed a

673   significant reduction in *anchor* gene levels. In addition, *anchor* RNAi caused the wing

674   blade region to overgrown within the imaginal disc. All combinations were grown at

675   29°C, and selected female larvae were dissected. Scale bar: 50 μm.

676   **Fig. S5. Knocking down *anchor* shifted *dad* and *brk* levels in pupal larvae.**

677   The expression levels of the BMP targets *dad* and *brk* were assayed using *lacZ*

678   reporter genes. The *dad* expression domain was significantly expanded in

679   presumptive veins, and *brk* expression was reduced. All combinations were grown at

680   29°C, scale bar: 100 μm.

35

A Distal ← 3L: 74E2-74E3 → Proximal

Vps60 — anchor — CR44566

B Cyomembrane / Outside / Transmembrane / Inside / DEP

C

A *A9>+*

B *MS1096>*

C *dpp>*

D *ptc>*

E *en>*

F *ap>*

G *brk>*

H *A9>anchorRNAi* 55.2%

I *MS1096>anchorRNAi* 24.1%

J *dpp>anchorRNAi* 100%

K *ptc>anchorRNAi* 58.3%

L *en>anchorRNAi* 100%

M *ap>anchorRNAi* 100%

N *brk>anchorRNAi* 32.5%

A A9>anchorRNAi 29℃
B A9>anchorRNAi 25℃
C A9>anchorRNAi/mad¹²/+ 25℃
D A9>anchorRNAi/sax⁴/+ 25℃
E A9>anchorRNAi/tkv⁷/+; 25℃
F A9/anchorRNAi>tkv⁴²⁷/+ 25℃
G A9>medRNAi 29℃
H A9>madRNAi 29℃
I A9>saxRNAi 29℃
J A9>putRNAi 25℃
K A9>dppRNAi 25℃
L A9>gbbRNAi 25℃
M A9>anchorRNAi/medRNAi 29℃
N A9>anchorRNAi/madRNAi 29℃
O A9>anchorRNAi/saxRNAi 29℃
P A9>anchorRNAi/putRNAi 25℃
Q A9>anchorRNAi/dppRNAi 25℃
R A9>anchorRNAi/gbbRNAi 25℃

A *A9>anchorRNAi*

B *A9>anchorRNAi/UAS-tkv*

C *A9>anchorRNAi/UAS-dpp*

D *A9>anchorRNAi/UAS-gbb*

E Anchor / DEP / Dpp / Tkv / Gbb / Sax / Mad / p-Mad

F DEP / Mad / p-Mad

G DEP / Mad / p-Mad

H DEP / Mad / p-Mad

A

a

b

A9>+//A9>anchorRNAi

B

a

b

ptc>+ //ptc>anchorRNAi

C

****

Size of adult wing (n=16)

1.6

1.4

1.2

1.0

0.8

A9>+   A9>anchorRNAi

D

****

Distance between L3 and L4 (n=16)

1.6

1.4

1.2

1.0

0.8

ptc>+   ptc>anchorRNAi

A *A9>anchorRNAi*

B *A9>anchorRNAi/UAS-anchor*

C *A9>anchorRNAi*

D *A9>anchorRNAi/anchor^P*

A. *MS1096>+*

B. *MS1096>anchorRNAi*

C. *A9>+*

D. *A9>anchorRNAi*

A A9 >dad-lacZ

B A9 > anchorRNAi/dad-lacZ

β -Gal
DAPI

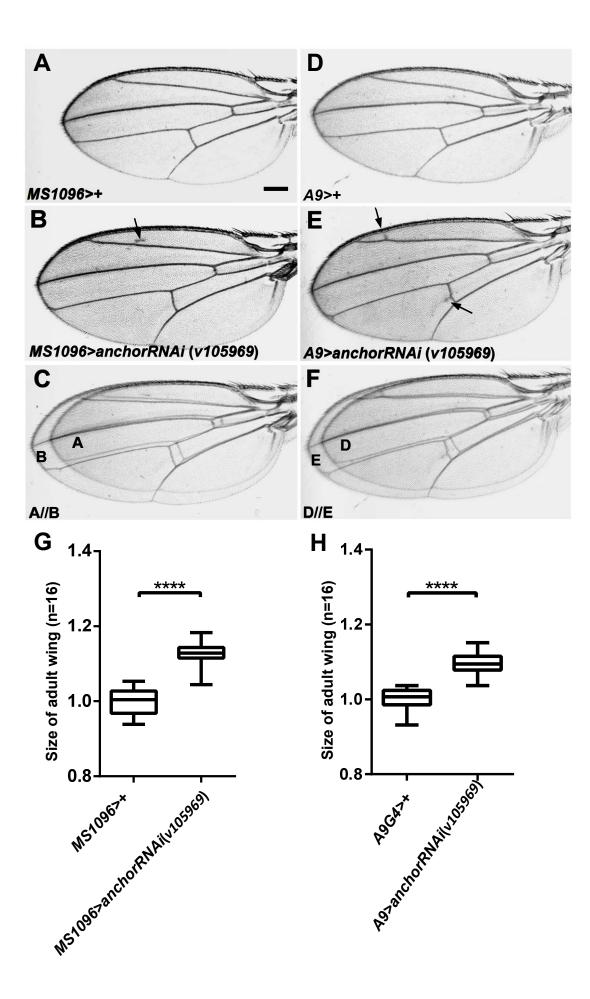**C** A9 >brk-lacZ

D A9 >anchorRNAi/brk-lacZ

β -Gal
DAPI

Table S1. Summary wing phenotypes of *anchor* RNAi combinations

Genotype	thickened veins					Extra veins					Number of wings
	L2	L3	L4	L5	Distal marginal vein	Before and L2	Between L2 and L3	Between L3 and L4	Between L4 and L5	Behind L5	
A9>anchorRNAi ▽	72.92%	100.00%	66.67%	79.17%	100.00%	100.00%	86.11%	100.00%	100.00%	100.00%	108
A9>medRNAi ▽	67.31%	-	-	7.69%	32.69%	22.12%	37.50%	-	36.54%	-	104
A9>madRNAi ▽	52.88%	15.63%	-	33.33%	35.00%	31.67%	82.50%	-	42.50%	10.83%	120
A9>saxRNAi ▽	-	-	-	-	-	-	-	-	-	-	24
A9>anchorRNAi/medRNAi ▽	53.66%	48.81%	32.14%	30.95%	17.86%	20.24%	29.76%	55.95%	32.14%	26.19%	84
A9>anchorRNAi/madRNAi ▽	40.83%	19.17%	32.50%	50.83%	38.33%	77.50%	93.33%	82.50%	83.33%	58.33%	120
A9>anchorRNAi/saxRNAi ▽	18.18%	67.05%	-	48.86%	80.68%	100.00%	59.03%	87.75%	93.75%	59.38%	88
*A9>anchorRNAi**	36.67%	63.33%	55.00%	40.00%	100.00%	100.00%	85.00%	100.00%	100.00%	100.00%	120
*A9>anchorRNAi/mad^{12} /+**	20.21%	74.47%	30.85%	56.38%	78.72%	90.42%	60.64%	74.47%	82.98%	67.02%	94
*A9>anchorRNAi/sax^4 /+ **	27.55%	36.73%	9.18%	44.89%	38.78%	72.45%	54.08%	100.00%	100.00%	89.79%	98
*A9>anchorRNAi/tkv^7 /+ **	18.89%	71.11%	23.33%	56.25%	65.55%	100.00%	58.89%	100.00%	78.89%	81.11%	90
*A9>anchorRNAi/tkv^{d27} /+**	11.76%	62.75%	32.35%	43.33%	54.90%	100.00%	41.18%	100.00%	100.00%	81.37%	102
*A9>putRNAi **	100.00%	-	-	-	-	12.50%	6.25%	-	9.38%	-	32
*A9>dppRNAi**	-	-	-	-	-	-	-	-	-	-	63
*A9>gbbRNAi**	-	-	-	-	-	-	-	-	-	-	94
*A9>anchorRNAi/putRNAi**	100.00%	2.78%	-	-	-	22.22%	19.44%	-	38.89%	13.89%	36
*A9>anchorRNAi/dppRNAi**	-	-	-	-	-	-	-	-	-	-	55
*A9>anchorRNAi/gbbRNAi**	-	-	-	-	73.81%	29.76%	75.00%	63.09%	-	48.81%	84
*A9>anchorRNAi/anchor ^{P} **	17.59%	54.63%	16.67%	36.11%	100.00%	100.00%	53.70%	100.00%	84.26%	85.19%	108
A9>anchorRNAi △	21.43%	22.62%	-	-	72.62%	26.19%	53.57%	94.05%	100.00%	23.81%	84
A9>anchorRNAi/UAS-anchor^{ii} △	6.09%	-	-	-	47.56%	4.88%	29.88%	76.22%	75.00%	21.34%	164
A9>anchorRNAi/UAS-anchor^{II} △	12.50%	-	-	-	75.00%	-	25.00%	87.50%	81.25%	-	20

▽ Grow up at 29℃
* Grow up at 25℃
△ Grow up at 18℃