The Protein Phosphatase 4 complex promotes the Notch pathway and *wingless* transcription

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Summary statement: The protein phosphatase 4 complex promotes Notch signaling and target gene expression during Drosophila wing development.

Abstract:

The Wnt/Wingless (Wg) pathway controls cell fate specification, tissue differentiation and organ development across organisms. Using an *in vivo* RNAi screen to identify novel kinase and phosphatase regulators of the Wg pathway, we identified subunits of the serine threonine phosphatase Protein phosphatase 4 (PP4). Knockdown of the catalytic and the regulatory subunits of PP4 cause reductions in the Wg pathway targets Senseless and Distal-less. We find that PP4 regulates the Wg pathway by controlling Notch-driven *wg* transcription. Genetic interaction experiments identified that PP4 likely promotes Notch signaling within the nucleus of the Notch-receiving cell. Although the PP4 complex is implicated in various cellular processes, its role in the regulation of Wg and Notch pathways was previously uncharacterized. Our study identifies a novel role of PP4 in regulating Notch pathway, resulting in aberrations in Notch-mediated transcriptional regulation of the Wingless ligand. Furthermore, we show that PP4 regulates proliferation independent of its interaction with Notch.

Introduction

The progression from a fertilized egg into a multicellular organism is a complex process, requiring proliferation and intricate cell-cell communication between individual cells for the eventual formation of tissues and organs. Only a handful of evolutionarily conserved signal transduction pathways are used reiteratively, both spatially and temporally to control development. In metazoans, the Wnt signaling [Wingless (Wg) in Drosophila] pathway regulates growth and proliferation, cell-fate differentiation, stem-cell renewal and homeostasis (Clevers and Nusse, 2012; Swarup and Verheyen, 2012). Wnt signaling alone does not control all these processes; its activity is extensively regulated by other signaling pathways and cellular mechanisms (Collu et al., 2014; Itasaki and Hoppler, 2010; Kim and Jho, 2014; Zeller et al., 2013). Determining how these interactions occur is critical for understanding basic cellular function and disease progression, as the disruption of the Wnt pathway has been implicated in a variety of developmental disorders and cancer (Clevers and Nusse, 2012).

The Drosophila wing imaginal disc is a powerful tool for studying Wg signaling (Swarup and Verheyen, 2012). In the developing wing disc the Wg ligand is expressed throughout different stages of disc development. At the end of the larval third instar stage, Wg expression is

56 confined to the presumptive wing margin along the dorsal/ventral (D/V) boundary, which
57 controls patterning and fate specification (Couso et al., 1994; Williams et al., 1993). Wg
58 produced in this narrow band of cells is secreted and acts as a morphogen to induce the nested
59 expression of target genes including Senseless (Sens) and Distal-less (Dll), in the flanking non-
60 boundary cells (Neumann and Cohen, 1997; Zecca et al., 1996).

61 The directed expression of Wg at the D/V boundary requires the transmembrane receptor
62 Notch in these boundary cells. The Notch ligands Delta (Dl) and Serrate (Ser) signal from the
63 flanking non-boundary cells, inducing proteolytic cleavages of Notch to generate a free Notch
64 intracellular domain (N^{ICD}) (Bray, 2016; Fortini, 2009). N^{ICD} translocates to the nucleus where it
65 binds transcriptional co-activators and DNA binding proteins to initiate target gene transcription,
66 including *wg* and *cut* (de Celis et al., 1996; Rulifson and Blair, 1995). The absence of Notch
67 results in reduced *wg* transcription and therefore reduced Wnt pathway activation (Rulifson and
68 Blair, 1995).

69 Both Notch and Wg signaling act to regulate common developmental processes such as
70 tissue patterning, fate specification and growth of different Drosophila appendages (Hing et al.,
71 1994). These two pathways share a number of common regulators which affect the activity of
72 their signaling outcome. In an *in vivo* RNAi screen to identify novel kinase and phosphatase
73 modulators of the Wg pathway, we found that the components of the Protein Phosphatase 4
74 (PP4) complex appeared to promote Wg signaling (Swarup et al., 2015). The serine threonine
75 phosphatase PP4 belongs to the Protein Phosphatase 2A (PP2A) group of phosphatases (Cohen
76 et al., 2005). Similar to what is found with PP2A, PP4 forms a heterotrimeric complex, which in
77 Drosophila consists of a catalytic subunit, Protein Phosphatase 4-19C (PP4-19C), and two
78 regulatory subunits called Protein Phosphatase 4 Regulatory subunit 2-related protein (PPP4R2r)
79 and PP4R3/Falafel (Flfl) (Cohen et al., 2005; Gingras et al., 2005).

80 PP4 is a highly conserved phosphatase seen across metazoans, and has been implicated in
81 a wide range of cellular processes, including chemotaxis in slime molds (Mendoza et al., 2007),
82 developmental signaling pathways such as Hedgehog (Jia et al., 2009), JNK (Huang and Xue,
83 2015; Zhou et al., 2002), Insulin-like growth factor (Mihindukulasuriya et al., 2004), as well as
84 TOR (Raught et al., 2001). The major functional role of PP4 is as a key regulator in cell cycle
85 progression and regulation of cell division (Helps et al., 1998; Huang et al., 2016). No previous
86 studies have implicated PP4 in Notch or Wnt/Wg signaling.

In this study we demonstrate that our previous observations of reduced Wg signaling due to knockdown of PP4 components are due to effects on *wg* transcriptional regulation by Notch. Using genetic interaction studies and expression of mutant Flfl, we determine that PP4 promotes the activity of nuclear Notch. We further elucidated that the function of PP4 in promoting Notch signaling was independent of its previously described role in cell cycle progression and proliferation. Taken together we have identified a novel role for PP4 in promoting Notch signaling and expression of *wg* during Drosophila development.

Results

PP4 promotes Wg signaling in the Drosophila wing imaginal disc

In a screen for modifiers of Wg signaling in the Drosophila wing imaginal disc, three components of PP4 were found to reduce Wg target genes following their knockdown through RNAi (Swarup et al., 2015). An involvement of PP4 in Wg signaling has not been previously identified, so we were curious to determine mechanistically how PP4 may be involved in regulating the output of the Wg pathway. In the developing wing imaginal disc the Wg target genes *Sens* and *Dll* are expressed in a distinct nested pattern along the dorsoventral (D/V) boundary (Fig. 1A, A'). We utilized *dpp-GAL4* expressed in a narrow band of cells along the anterior/posterior (A/P) boundary (marked by GFP; Fig. 1B', B") to express RNAi constructs to knockdown expression of the individual PP4 components. The knockdown of the catalytic subunit *PP4-19C* caused a strong reduction in Sens and Dll (Fig. 1C, C'). Reduction of the PP4 targeting subunits *ppp4R2* or *flfl* via RNAi caused reduced Dll protein levels (Fig. 1E', G'), but did not noticeably affect Sens (Fig. 1E, G).

To confirm that the reduction of PP4 components did not critically affect cell viability as loss of PP4 has been shown to promote JNK-dependant cell death (Huang and Xue, 2015), discs were stained for the apoptotic marker, cleaved caspase-3 (C. Casp-3). Compared to control cells expressing GFP (Fig. 1B, B"), reduction of any individual PP4 component did not increase levels of apoptosis within the *dpp* domain of the imaginal disc (Fig. 1D, F, H). Following Wg pathway activation, the key effector protein Arm is stabilized at the highest concentration in two bands of cells flanking the Wg-producing cells of the D/V boundary (Fig. 1I, I') (Peifer et al., 1991).

118 Expression of *flfl-RNAi* in the posterior domain of the wing imaginal disc, using *engrailed (en)-*
119 *Gal4* (Fig. 1I, J), caused a reduction of stabilized Arm (Fig. 1J, arrow in J'). In subsequent
120 experiments, we utilized *flfl-RNAi* to reduce PP4 activity, as it has been previously confirmed as
121 a functional indicator of the entire complex (Sousa-Nunes et al., 2009). Together, these data
122 suggest PP4 is required for promoting Wg pathway activation.
123
124
125 *PP4 promotes Wg signaling through Notch pathway activation*
126
127 As the reduction of Wg target genes and Arm was apparent upon knockdown of PP4
128 components, we next wanted to look at the Wg ligand and its transcription. In third instar wing
129 imaginal discs, *wg* is transcribed, translated, and undergoes post-translational modification,
130 which can affect its stability, before being secreted to activate the Wg pathway in neighbouring
131 cells (Franch-Marro et al., 2008; Tanaka et al., 2002). Utilizing Wg antibodies, and the *wg*
132 transcriptional reporter, *wg-lacZ*, we could identify any defects in the ligand's transcription,
133 processing, or stability. In a wild type wing imaginal disc, Wg and *wg-lacZ* expression are
134 refined along the D/V boundary in a narrow band 2-3 cells wide (Fig. 2A, B). Expression of *flfl-*
135 *RNAi* in the posterior domain of the wing disc, using *hedgehog* (*hh*)-*Gal4*, resulted in a reduction
136 of both total Wg protein levels and transcription (Fig. 2E, F) suggesting PP4 is involved in
137 regulation of *wg* transcription.
138 *wg* transcription is controlled along the D/V boundary of the wing disc by Notch
139 signaling (Rulifson and Blair, 1995). We next wished to determine if PP4 regulation of *wg*
140 transcription is mediated through the involvement of the Notch signaling pathway. *cut*, another
141 Notch target gene (de Celis et al., 1996), is expressed in a similar pattern as Wg along the D/V
142 boundary (Fig. 2C). The reduction of *flfl* in the posterior domain of the wing disc via RNAi
143 resulted in a strong loss of Cut expression, indicating an overall reduction in Notch signaling
144 (Fig. 2G). Looking at the Notch ligand Delta (Dl) which is enriched in the cells adjacent to the
145 D/V boundary (Fig. 2D), it was apparent that *flfl-RNAi* expressed in the posterior domain of the
146 disc, resulted in reduced Dl and a failure of its refinement (Fig. 2H). We could not discern if this
147 effect on Dl is from upstream regulation of *Dl* expression, or on Notch activation itself, as the
148 refinement of Dl involves a cis/trans feedback mechanism with N for pattern refinement, through

149 lateral inhibition of each gene (Axelrod, 2010). Together, these results demonstrate that PP4
150 normally appears to influence Notch signaling to promote multiple pathway targets including *wg*.
151
152 *PP4 promotes Notch signaling in the Notch signal receiving cells.*
153
154 Having identified that Flfl, and by extension PP4, is involved in promoting Notch
155 signaling we sought to further elucidate how. During wing imaginal disc development Notch and
156 its ligands Dl and Serrate (Ser) undergo refinement through lateral inhibition resulting in high
157 levels of active Notch (N^{ICD}) being expressed along the D/V boundary and suppressed in the
158 flanking cells (Fig. 3D arrow head), which conversely have high levels of Dl (Fig. 2D) and Ser
159 (de Celis et al., 1996). This feed forward loop of lateral inhibition creates Notch signal-sending
160 cells (cells flanking the D/V boundary), and signal-receiving cells (D/V boundary cells) with
161 active Notch signaling (Axelrod, 2010).
162 To further analyze the role of the PP4 complex in the complementary ligand-expressing
163 and Notch-expressing cells, we used *C5-Gal4* and *wg-Gal4* respectively, to express *flfl-RNAi* in
164 the wing imaginal disc. *C5-Gal4* is expressed in N ligand expressing cells flanking the D/V
165 boundary (Fig. 3E), while *wg-Gal4* is expressed along the D/V boundary, in the active N signal
166 receiving cells (Fig. 3J). By looking at Wg and Notch target genes, we could determine in which
167 cells Flfl and by extension PP4 is working to affect the Notch pathway. *C5>flfl-RNAi* appeared
168 to have no affect on *Dll* expression (Fig. 3A, F), Wg (Fig. 3B, G), or Cut (Fig. 3C, H). The
169 enrichment of the N^{ICD} along the D/V boundary also appeared to be unaffected (Fig. 3D, I
170 arrowheads). *wg>flfl-RNAi* gave very contrasting results. Knockdown of *flfl* in the Notch
171 receptor expressing cells resulted in a complete loss of *Dll-lacZ (*Fig. 3K), strong reduction of
172 Wg (Fig. 3L), loss of Cut (Fig. 3M), and a failure of enrichment of N^{ICD} (Fig. 3N). Knockdown
173 of the other components of PP4 using *wg-Gal4*, but not *C5-gal4*, also caused loss of Wg
174 expression (Fig. S1A-D). Taken together, these results suggest that PP4 functions within the
175 Notch-expressing cell to promote full pathway activation and target gene expression.
176
177 *PP4 functions within the nucleus to promote Notch signaling.*
178

179 To further refine where PP4 functions within the Notch signal receiving cell, we utilized
180 mutant transgenes of Flfl and N in the adult Drosophila wing. During pupal wing
181 metamorphosis, the activation and refinement of Dl and N are required to refine adult vein
182 formation (Huppert et al., 1997). Notch signaling is also critical for the development of sensory
183 bristles along the adult wing margin (reviewed in Posakony, 1994). Any developmental defects
184 from expression of the various Flfl transgenes in adult wings could provide insight into PP4's
185 role in the Notch pathway. Using *MS1096-Gal4*, which is expressed across the entire developing
186 wing pouch, to ectopically express wild type Flfl or a cytoplasmic form, $Flfl^{\Delta 3NLS+2NES}$ (Flfl-
187 cyto), had no effect on the adult wing compared to wild type (Fig. 4A-C). Endogenous Flfl is a
188 predominantly nuclear protein and the wild type Flfl transgene has been shown to function
189 similarly (Sousa-Nunes et al., 2009). Knockdown of *flfl* via RNAi induced ectopic and thicker
190 veins in the adult wing (Fig. 4D), a hallmark of reduced Notch activity (Huppert et al., 1997).
191 This phenotype could be suppressed by reintroduction of the wild type *flfl* transgene (Fig. 4E),
192 but not with expression of *flfl-cyto* (Fig. 4F). This suggests that Flfl functions within the nucleus,
193 rather than the cytoplasm, to promote Notch.

194 To confirm the hypothesis that Flfl likely acts in nuclear Notch signaling, we expressed a
195 construct encoding the intracellular domain of Notch that localizes to the nucleus (N^{nucl})(Rebay
196 et al., 1993). This activated nuclear Notch suppressed wing vein formation and the formation of
197 sensory bristles (Fig. 4G, G') compared to wild type (Fig. 4A). Notch-dependent activation of *wg*
198 expression is essential for Wg signaling to induce expression of proneural genes like *Sens* for the
199 specification of sensory organ precursor (SOP) cells (Nolo et al., 2000). SOPs then divide and
200 differentiate giving rise to the sensory bristles in the adult fly via Notch signaling (Guo et al.,
201 1996; Hartenstein and Posakony, 1990). Although ectopic Notch signaling increases the number
202 of SOPs in the wing disc via Wg, they do not differentiate correctly resulting in double socket
203 cells, and loss of bristle cells (Guo et al., 1996). Knockdown of *flfl* in the N^{nucl} expressing cells
204 was able to partially recover vein loss, but did not significantly rescue the bristle defect (Fig. 4H
205 arrow, H'). Conversely, the expression of Flfl or Flfl-cyto had no affect on the N^{nucl} phenotype
206 (Fig. 4I-J').We interpret the inability to specifically rescue the bristle defect as being due to the
207 combination of the strength of N^{nucl} as well as an incomplete knockdown of *flfl* via RNAi. To
208 confirm that this phenotype could be rescued in our assay, we tested whether loss of *wg* could
209 rescue the effect since up-regulated target genes expression causes the N^{nucl} phenotype.

210 Expression of a weak *wg-RNAi* transgene was able to induce sporadic sensory bristle loss (Fig.
211 4K, K' arrow), due to reduced SOPs (Nolo et al., 2000; Parker et al., 2002). When combined
212 with N^{nucl}, *wg-RNAi* can partially suppress the overactive Notch phenotype of inhibited bristle
213 formation (Fig. 4L, L' arrowhead).

214

215 *Flfl is required for proliferation and maintenance of overall tissue size independent of Notch*
216 *signaling.*

217

218 Adult flies with reduced *flfl* expression displayed smaller wing blades compared to
219 control flies (Fig. 4A, D). This was expected given PP4's known role in cycle progression and
220 growth (Helps et al., 1998; Huang et al., 2016; Martin-Granados et al., 2008; Zhuang et al.,
221 2014). Notch has been implicated in cell proliferation in the wing imaginal disc, but a direct
222 mechanism for its involvement is not fully understood (Baonza and Garcia-Bellido, 2000;
223 Giraldez and Cohen, 2003; Go et al., 1998). We quantified the area of the adult wings of the
224 different genotypes, in order to determine if PP4/Flfl's role in growth is mediated through Notch
225 signaling. Overexpression of Flfl and Flfl-cyto had no significant effect on wing size compared
226 to wild type (Fig. 5A, box plot a,b,c). Knockdown of *flfl* resulted in a ~28% reduction in wing
227 size, and could be fully rescued by the wild type *flfl* transgene (Fig. 5A, box plot d,e). Flfl-cyto
228 was able to slightly rescue the growth defect from *flfl-RNAi*, but not to a significant level (Fig.
229 5A, box plot f). The ability to rescue partially may be due to PP4's role in mitotic progression
230 after nuclear envelope break down at prometaphase, allowing for Flfl-cyto to perform its
231 function at this step in mitosis (Huang et al., 2016). Wings expressing N^{nucl} did not have a
232 significantly smaller area than wild type wings, and were unable to rescue the growth defect
233 from *flfl-RNAi* (Fig. 5A, box plot g,h). As N^{nucl} is unable to rescue the growth defect from
234 reduced Flfl levels, it indicates that PP4/Flfl's role in regulating growth is likely independent to
235 its function in propagating Notch signaling. An alternative interpretation could be that Flfl acts
236 downstream of N^{nucl} or that Flfl is required for proper Notch function in the proliferation.

237 To determine if the growth defects from loss of PP4 were due to decreased cell
238 proliferation or overall cell size, we looked at the developing wing imaginal disc. We utilized *en-*
239 *Gal4* driving GFP to mark the posterior portion of the wing disc, representing roughly 50% of
240 the overall tissue (Fig. 5B). We compared the size of the GFP-positive region to the control

241 anterior side of the disc in different genotypes to determine the effects that changes in the levels
242 of Flfl have on tissue growth. In control discs expressing *UAS-lacZ*, the ratio between the
243 internal control area of the anterior, to the GFP positive posterior was equal (Fig. 5B, E).
244 Overexpression of Flfl in the posterior domain did not affect the posterior/anterior area ratio
245 (Fig. 5C, E). However, reduction of *flfl* via RNAi resulted in a significant decrease in size of the
246 posterior domain (Fig. 5D, E).

247 We simultaneously looked at the number of mitotic cells in these genotypes using a
248 phospho-Histone H3 (Ser10) (PH3) antibody. There was no significant difference between our
249 control discs and those overexpressing of Flfl (Fig. 5B', C', F). Surprisingly, we found that *flfl-*
250 *RNAi* tissue had significantly elevated levels of PH3 positive cells (Fig. 5D', F). This result was
251 perplexing considering the decreased tissue size, yet apparent increase in proliferation rate. As
252 PH3 marks condensed chromatin prior to chromosomal segregation in cells along the G2/M
253 transition (Hendzel et al., 1997), it is possible that *flfl-RNAi* cells were arrested during early
254 mitosis, and had not undergone mitotic exit. Previously, Sousa-Nunes et al. (2009) identified a
255 similar effect. Drosophila *flfl/+* brains exhibited much lower rates of Bromodeoxyuridine (BrdU)
256 incorporation and reduced proliferation, yet exhibited elevated levels of PH3 positive cells,
257 demonstrating *flfl* is important for mitotic progression. Similar results have been found in
258 multiple cases (Huang et al., 2016; Martin-Granados et al., 2008). Our results suggest a similar
259 function for PP4/Flfl in the wing disc, where PP4 is critical for cell cycle progression and mitosis
260 affecting proliferation rates and overall tissue size, yet this function is independent of its role in
261 promoting Notch signaling.
262
263 *aPKC is not involved with PP4 and Notch signaling in the Drosophila wing imaginal disc*
264
265 We examined whether PP4 mechanistically interacted with other known modulators of
266 the Notch pathway. One well-characterized regulator is atypical protein kinase C (aPKC). aPKC
267 is a protein kinase widely studied for its role in developmental processes, including asymmetric
268 cell division (ACD). The regulators of ACD (including aPKC, Bazooka, and Crumbs) act
269 upstream of Notch signaling and determine the identity of the Notch signal sending and signal
270 receiving cells (reviewed in Knoblich, 2008). aPKC also promotes the Notch pathway by
271 inhibiting Numb-mediated endocytosis of the Notch pathway components (Frise et al., 1996;

272 Smith et al., 2007; Wang et al., 2006). Previous studies have shown a role for PP4/Flfl in the
273 localization of the Miranda complex to promote neuroblast ACD in Drosophila by acting
274 downstream or parallel to aPKC (Sousa-Nunes et al., 2009). Since such a role has not been
275 identified in a proliferating epithelium like the wing disc we sought to investigate if this
276 mechanism was conserved during the development of the wing disc.

277 Using *MS1096-Gal4* to express *aPKC-RNAi* in the wing disc resulted in small, crumpled
278 adult wings, with only sporadic sensory bristles (Fig. S2B), indicating disrupted patterning and
279 growth. Overexpression of an aPKC transgene did not induce any visible phenotype (Fig. S2C),
280 and was able to rescue the *aPKC-RNAi* phenotype, confirming the phenotype seen from the
281 RNAi was directly a result of loss of *aPKC* (Fig. S2D). The knockdown of *flfl* via RNAi or over
282 expression of the wild type *flfl* transgene had no affect on the *aPKC-RNAi* phenotype (Fig. S2E,
283 F). Importantly, expression of N^{nucl} was unable to rescue the crumpled wing from *aPKC*
284 knockdown, indicating aPKC is most likely not a direct upstream regulator of Notch in the wing
285 imaginal disc (Fig. S2G). In addition to this, overexpression of *aPKC* had no effect on the *flfl-*
286 *RNAi* vein thickening compared to *flfl-RNAi* alone (Fig. 4D, S2H). Although aPKC is involved
287 with PP4 and Notch signaling in ACD (Sousa-Nunes et al., 2009; Zhang et al., 2016), it does not
288 appear to be directly involved in Notch signaling and pattering in the developing wing imaginal
289 disc.
290
291
292 **Discussion**
293
294 An *in vivo* RNAi screen initially identified three components of the PP4 enzyme complex
295 as modulators of endogenous Wg signaling during wing development (Swarup et al., 2015). The
296 PP4 complex in *Drosophila melanogaster* has been implicated in many signaling pathways,
297 cellular functions and developmental processes, yet its role in regulating Wg signaling was
298 previously uncharacterized. In this study, we revealed that the effect on Wg pathway was at the
299 level of expression of the Wg ligand, by promotion of Notch signaling. Notch signaling regulates
300 the precise expression of Wg in the cells of the D/V boundary of the wing imaginal disc. This
301 expression is required for specification of the wing margin and bristle structures (Couso et al.,
302 1994; de Celis et al., 1996; Neumann and Cohen, 1996).

303 A partial knockdown of Flfl, PP4-19C and PPP4R2 by RNAi in cells along the A/P
304 boundary of the wing imaginal discs was able to effectively reduce Wg target genes, yet did not
305 induce elevated levels of JNK-mediated cell death as previously reported (Huang and Xue,
306 2015). Cell death was inducible upon stronger expression of the RNAi using *act-Gal4* with heat-
307 shock inducible flip-out clones (data not shown). The knockdown of Flfl was further found to
308 reduce expression of the Wg ligand as well as other Notch pathway target genes, implicating PP4
309 in the Notch pathway. Reduction of PP4 proteins in the D/V boundary all resulted in reduced Wg
310 expression, while their knockdown in neighbouring Dl- and Ser-expressing cells had no effect.
311 This result highlights that the PP4 complex acts in boundary cells to regulate Notch-dependent
312 gene expression.

313 Previously PP4 has been indirectly associated with Notch signaling in Drosophila for its
314 involvement in asymmetrical cell division (ACD) of the developing neuroblasts (Sousa-Nunes et
315 al., 2009; Zhang et al., 2016). While PP4 acts in concert with aPKC and Notch signaling to drive
316 proper ACD, we were unable to identify a role for aPKC in Notch signaling in the epithelial cells
317 of the wing imaginal disc. However, further genetic interaction experiments also identified that
318 PP4's involvement in Notch signaling in the wing imaginal disc appears to be independent of
319 PP4's role in cell cycle progression and tissue growth. These results demonstrated that although
320 both PP4 and Notch are required for cell proliferation (Giraldez and Cohen, 2003; Go et al.,
321 1998; Huang et al., 2016), in the wing imaginal disc it is likely not through the same mechanism.

322 Subsequent genetic interaction studies revealed that most likely Flfl acts to promote
323 Notch through its role in the nucleus. A cytoplasmic form of Flfl could not recue the phenotypes
324 generated by *flfl-RNAi* in the adult wing, while expression of a wildtype transgene could. The
325 required function of Flfl in the nucleus was further bolstered by the fact that the wing phenotype
326 induced by activated nuclear N was partially suppressed by *flfl-RNAi*. As the N^{ICD} enters the
327 nucleus and binds to Suppressor of Hairless and Mastermind to initiate target gene transcription,
328 a multitude of cofactors must be recruited, while others must be removed, from the
329 transcriptional initiation site (reviewed in Bray, 2016). This includes the inhibition of histone
330 deacetylase (HDAC) co-repressor complexes (Kao et al., 1998). PP4 has been previously
331 identified to dephosphorylate and inhibit HDAC activity, while its depletion stimulates HDACs
332 (Zhang et al., 2005). Taken together a possible mechanism for PP4 to promote Notch signaling is
333 through the dephosphorylation of HDACs. This could allow for increased chromatin

334 remodelling, which is needed for the binding of other transcriptional co-factors to ensure full
335 transcriptional initiation of target genes (reviewed Bray, 2016). This is just one possibility, as
336 PP4 may be responsible for the dephosphorylation and modulation of any number of components
337 that cooperate with transcription factors, or regulate the activity of N^{ICD} leading to appropriate
338 target gene expression. Future studies will hopefully address the exact mechanism PP4 plays in
339 promoting nuclear Notch signaling for full expression of target genes like *wg*.
340

341 **Methods and Materials**

342

343 **Fly Strains and Crosses**

344

345 Fly strains and crosses were raised on standard medium at 25°C unless stated otherwise. w^{1118}
346 was utilized as wild type. In assays examining genetic interactions between two UAS-driven
347 transgenes, control crosses were performed with *UAS-lacZ* and *UAS-gfp* to eliminate effects
348 caused by the titration of Gal4. The following fly strains were used in this study: *UAS-GFP,*
349 *UAS-lacZ/TM6B*, *UAS-dicer, dpp-Gal4*, *Dll-lacZ*, *MS1096-Gal4, UAS-aPKC-GFP* (obtained
350 from the Bloomington Drosophila Stock Center), *UAS-GFP*, *C5-Gal4 (*Hugo Bellen*), UAS-*
351 *flflΔ3NLS+2NES (flfl-cyto)* (Gregory Somers), *UAS-flfl (*Zoltan Lipinszki*), wg-lacZ/CyO and*
352 *UAS-N[nucl]* (Spyros Artavanis-Tsakonas), *UAS-flfl-RNAi* (VDRC 24143,103793), *UAS-PP4-*
353 *19C-RNAi* (VDRC 25317, 103317, 43250), *UAS-PPP4R2R-RNAi* (VDRC 25445, 105399), *UAS-*
354 *aPKC-RNAi (*VDRC 2907,105624*)* [obtained from the Vienna Drosophila Resource Center
355 (Dietzl et al., 2007)], *hh-Gal4/TM6B*, *UAS-wg-RNAi, wg-Gal4 (*ND382*)* and *en-Gal4,UAS-GFP*
356 (gifts from Konrad Basler, Institute of Molecular Life Sciences University of Zurich,
357 Switzerland).
358

359 Crosses involving *C5-Gal4* were performed at 29°C to induce maximal Gal4 expression in the
360 developing wing disc.

361 **Immunofluorescence of wing imaginal discs**

362

363 Third-instar larvae were dissected in phosphate-buffered saline (PBS). Wing imaginal discs were
364 fixed in 4% paraformaldehyde at room temperature for 20 minutes and then washed 3 times for 5

365 minutes in PBS. Discs were blocked [2% BSA diluted in PBS 0.1% Triton X-100 (PBST)] for 45
366 minutes at room temperature, followed by incubation with primary antibodies (diluted in block)
367 overnight at 4°C. Tissue was then washed 3 times in PBST and incubated with secondary
368 antibodies (diluted in block) at room temperature for 1.5 hours. Tissue was then washed a final
369 three times in PBST and mounted in a 70% glycerol solution. The following primary antibodies
370 and dilutions were used: mouse anti-β-galactosidase (1:2000 Promega), mouse anti-Wg (1:100
371 DSHB), mouse anti-Cut (1:50 DSHB), mouse anti-N^{ICD} (1:50 DSHB), mouse anti-Delta (1:50
372 DSHB), mouse anti-Arm (1:50 DSHB), rabbit anti-cleaved Caspase 3 (1:100 Cell Signaling),
373 rabbit anti-PH3 (1:200 Cell Signaling), guinea pig anti-Sens (1:500, a gift from Hugo Bellen,
374 Dept. of Molecular and Human Genetics, Baylor College of Medicine, USA), mouse anti-Dll
375 (1:300, a gift from Ian Duncan, Dept. of Biology, Washington University in St. Louis, USA).
376 Secondary antibodies (Jackson ImmunoResearch) were used at a 1:200 dilution.
377
378 **Adult wing mounting**
379
380 Adult wings were dissected in 95% ethanol followed by mounting in Aquatex (EMD Chemicals
381 Inc.) A minimum of 8 wings were mounted per genotype for analysis.
382
383 **Imaging, Analysis and Quantification**
384
385 Fluorescent images were taken with a Nikon A1R laser scanning confocal microscope and
386 processed using Adobe Photoshop CS6. Adult wings were imaged with an Axioplan 2
387 microscope. Adult wing and wing disc areas were quantified using ImageJ software. PH3 cell
388 counts were performed using the ImageJ plugin, "Cell Counter". To compare PH3 positive cell
389 counts per genotype, counts were converted as a ratio of PH3 cells/area, then analysed as
390 experimental condition over control tissue. Significance between groups was assessed by one-
391 way analysis of variance (ANOVA), and $p<0.01$ was considered significant unless stated
392 otherwise.
393

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Competing Interests

The authors declare that they have no competing interests.

Author contributions

E.T.H., T.P.-S. and E.M.V. designed the experiments, E.T.H., T.P.-S., F.S. and E.M.V. performed the experiments, and E.T.H., T.P.-S. and E.M.V analyzed the data and wrote the manuscript.

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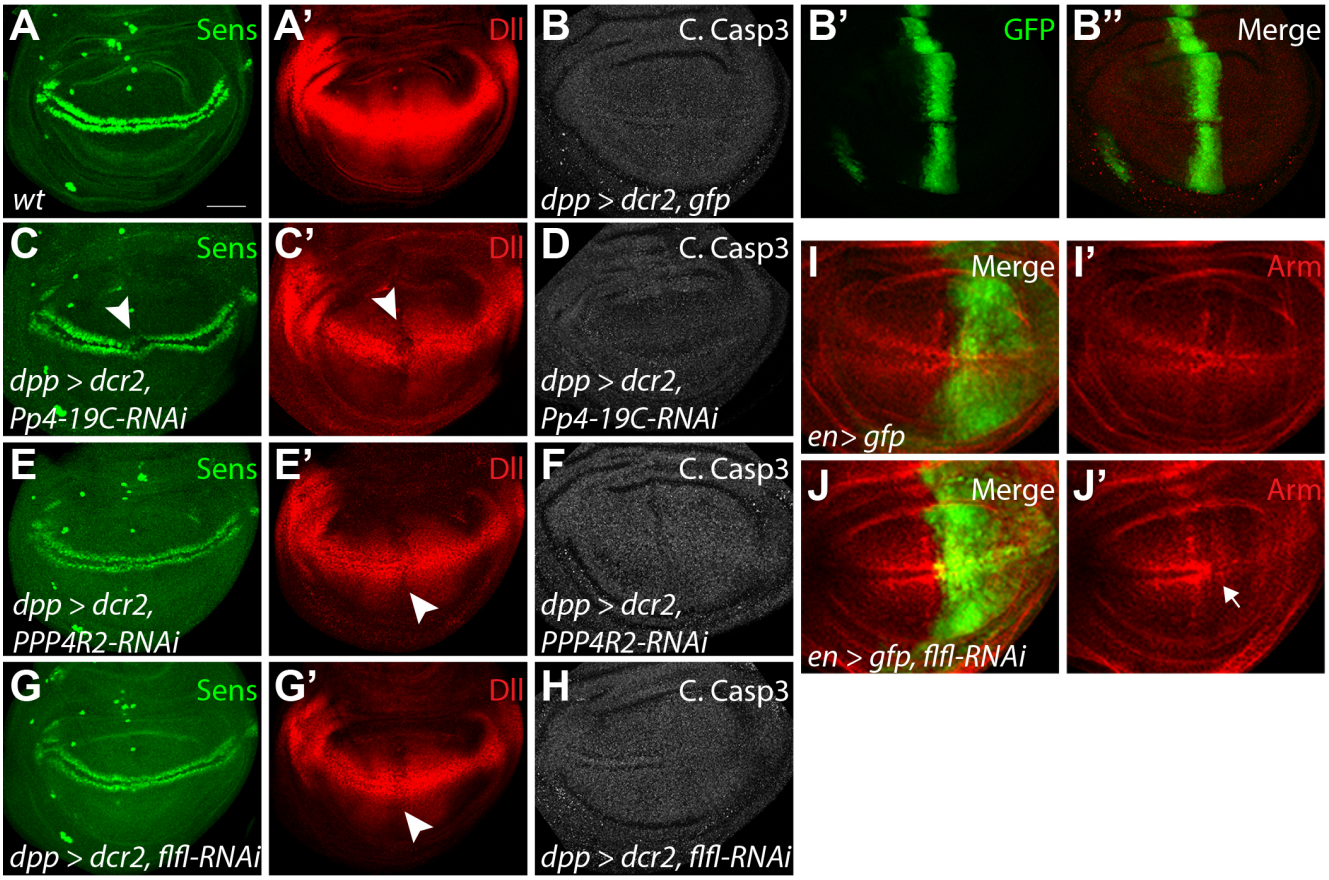
548

549 **Figure Legends:**

550 **Figure 1. Reduction of PP4 subunits inhibits Wg pathway activation without inducing cell**
551 **death.** (A-B'') Wild-type expression pattern of Wg target genes Sens (A), and Dll (A'), as well
552 as cleaved caspase 3 (B) with normal expression pattern of *dpp-Gal4* (B', B") along the
553 anterior/posterior boundary in the developing wing disc. (C-D) The knockdown of PP4-19C with
554 RNAi along the anterior/posterior boundary causes a loss of Sens (C, arrow head) and Dll (C',
555 arrow head), but does not elevate C. Caspase 3 activity (D). (E-F) Knockdown of PPP4R2 was
556 able to effectively reduce Dll (E', arrow head), but did not affect Sens (E) or C. Casp3 (F). (G-H)
557 Flfl knockdown did not affect Sens (G) or C. Casp3 levels (H), but did reduces Dll (G', arrow
558 head). Normal expression pattern of *en-Gal4* (I) in the posterior domain of the wing disc, shown
559 with Arm (I, I'). (J-J') The knockdown of Flfl in the posterior domain (shown with GFP) induced
560 a marked reduction in stabilized Arm (J'). Scale bar: 50 µm.



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563 **Figure 2. PP4 promotes Wg signaling through Notch pathway activation.** (A-D) Wild-type
564 pattern of Wg protein (A), *wg* transcription (B), Cut protein (C), and Dl protein (D) in the
565 developing wing disc. (E-H) Using *hh-Gal4*, expressed in the posterior domain of the wing disc
566 (right of the dotted line), to express *flfl-RNAi*, caused a reduction in total Wg protein levels (E),
567 as well as *wg* transcription (F), and induced a loss of Cut (G), and Dl (H). Scale bar: 50 µm.

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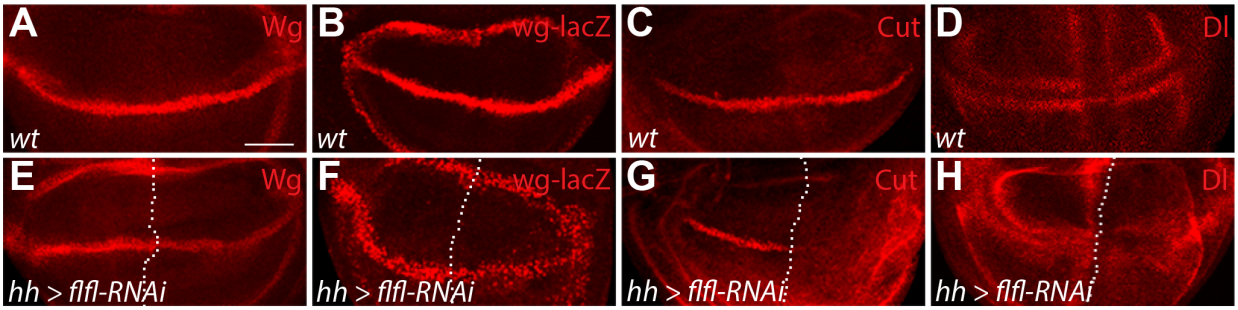

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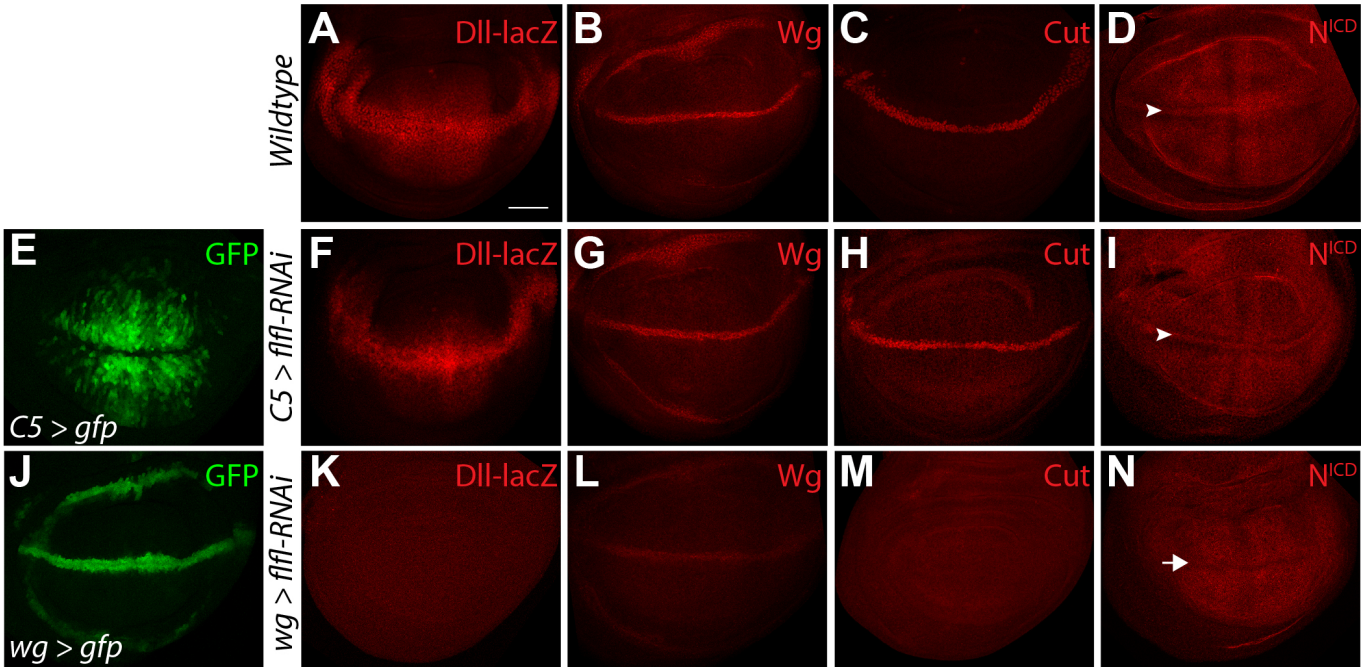
571 **Figure 3. PP4 promotes Notch signaling in the Notch-signal receiving cells.** (A-D) Wild-type
572 expression pattern of *Dll-lacZ* (A), Wg (B), Cut (C), and N^{ICD} (D) in the developing wing disc.
573 N^{ICD} is enriched along the dorsal/ventral (D/V) boundary (D, arrowhead) and suppressed in the
574 adjacent cells. (E) Expression pattern of *C5-Gal4* driving GFP in the D/V boundary flanking
575 cells of the wing pouch. (F-I) The knockdown of *flfl* with RNAi in the D/V boundary flanking
576 cells does not affect *Dll-lacZ* (F), Wg (G), Cut (H), or N^{ICD} (I). (J) Expression pattern of *wg-Gal4*
577 driving GFP along the D/V boundary. (K-N) Knockdown of *flfl* in the D/V boundary cells causes
578 a loss of *Dll-lacZ* (K), strong reduction of Wg (L), loss of Cut (M), and a failure of N^{ICD}
579 enrichment along the D/V boundary (N, arrow). Scale bar: 50 µm.



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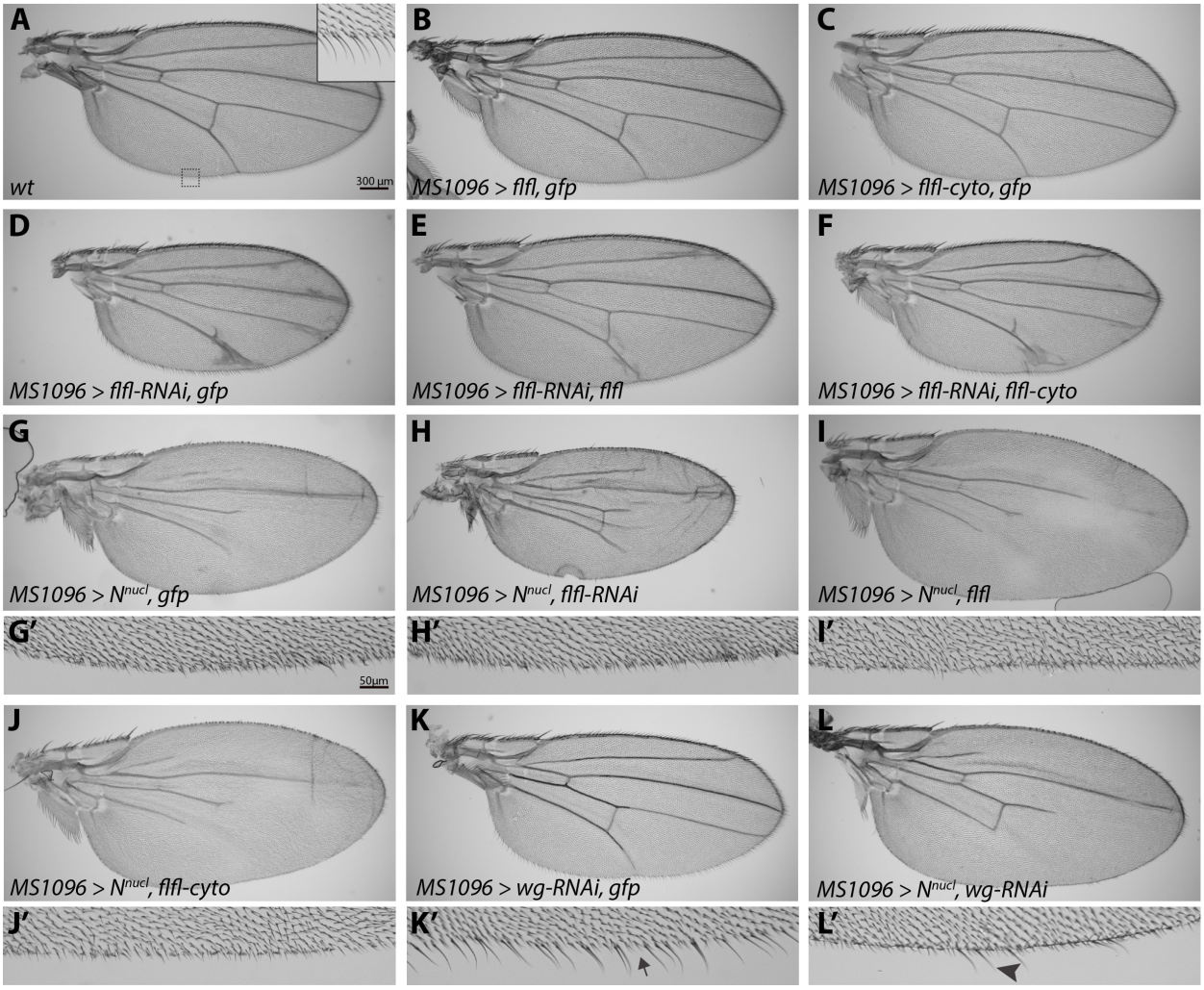
582 **Figure 4. PP4 likely functions within the nucleus to promote Notch.** (A-C) Adult wild-type
583 wing and margin (A, inset). Over expression of Flfl (B), or Flfl-cyto(C) throughout the entire
584 wing does not induce any noticeable phenotype. (D-E) Knockdown of Flfl induces ectopic veins
585 and thickening of veins, as well as a reduced wing size (D). This effect can be primarily rescued
586 by reintroduction of a full length Flfl transgene (E), but not by Flfl-cyto (F). (G-L') Over-
587 expression of N^{nucl} induces a loss of wing veins (G) and wing margin bristles (G'). Knockdown
588 of *flfl* induced a mild rescue the N^{nucl} loss of vein phenotype, and still reduces the overall wing
589 size (H, H'). The over expression of Flfl (I, I'), or Flfl-cyto (J, J') did not disrupt the N^{nucl}
590 phenotype. (K-L') Expression of *wg-RNAi* in the wing induced sporadic loss of margin bristles
591 (K, K' arrow). N^{nucl} with *wg-RNAi* is able to maintain several margin bristles (L, L' arrowhead).



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594 **Figure 5. Flfl is required for proliferation and overall tissue size independent of Notch**
595 **signaling.** (A) Box plots representing total wing area of genotypes shown in Fig. 4 (n = 8-13).
596 Over expression of Flfl (b) or Flfl-cyto (c) did not affect wing size compared to wild type (a).
597 *flfl-RNAi* caused a significant reduction in wing size (d). The *flfl-RNAi* size defect could be fully
598 rescued by reintroduction of full length Flfl (e), but no effect was seen with Flfl-cyto (f). Wings
599 expressing N^{nucl} (g) are slightly smaller than wild type wings. N^{nucl} and *flfl-RNAi* wings (h) have
600 a significant size reduction compared to wild type (a), equivalent to that of *flfl-RNAi* alone (d).
601 Data are presented as box plot 25-75 percentile, whiskers 10-90 percentile, (—) median, (+)
602 mean, (•) outliers, letters above representing significance from corresponding genotypes,
603 ($p<0.01$) generated from one-way ANOVA. (B-F) The normal expression pattern of *en-Gal4*
604 marked by GFP (B) in the posterior domain of the developing wing disc, shown with mitotic cell
605 marker PH3 (B') and represented as a ratio of posterior domain vs. the anterior control (n = 8)(E,
606 F). Over expression of Flfl in the posterior domain had no effect on area (C, E) or proliferation
607 rate (n = 7)(C' F). The knockdown of Flfl with RNAi in the posterior domain induced a
608 significant reduction in area (D, E), and exhibited a significantly higher number of PH3
609 positively marked cells (n=9)(D' F). Data are presented as means ± s.d., (*) $p<0.05$, (**) $p<0.01$
610 generated from one-way ANOVA. Scale bar: 50 µm.

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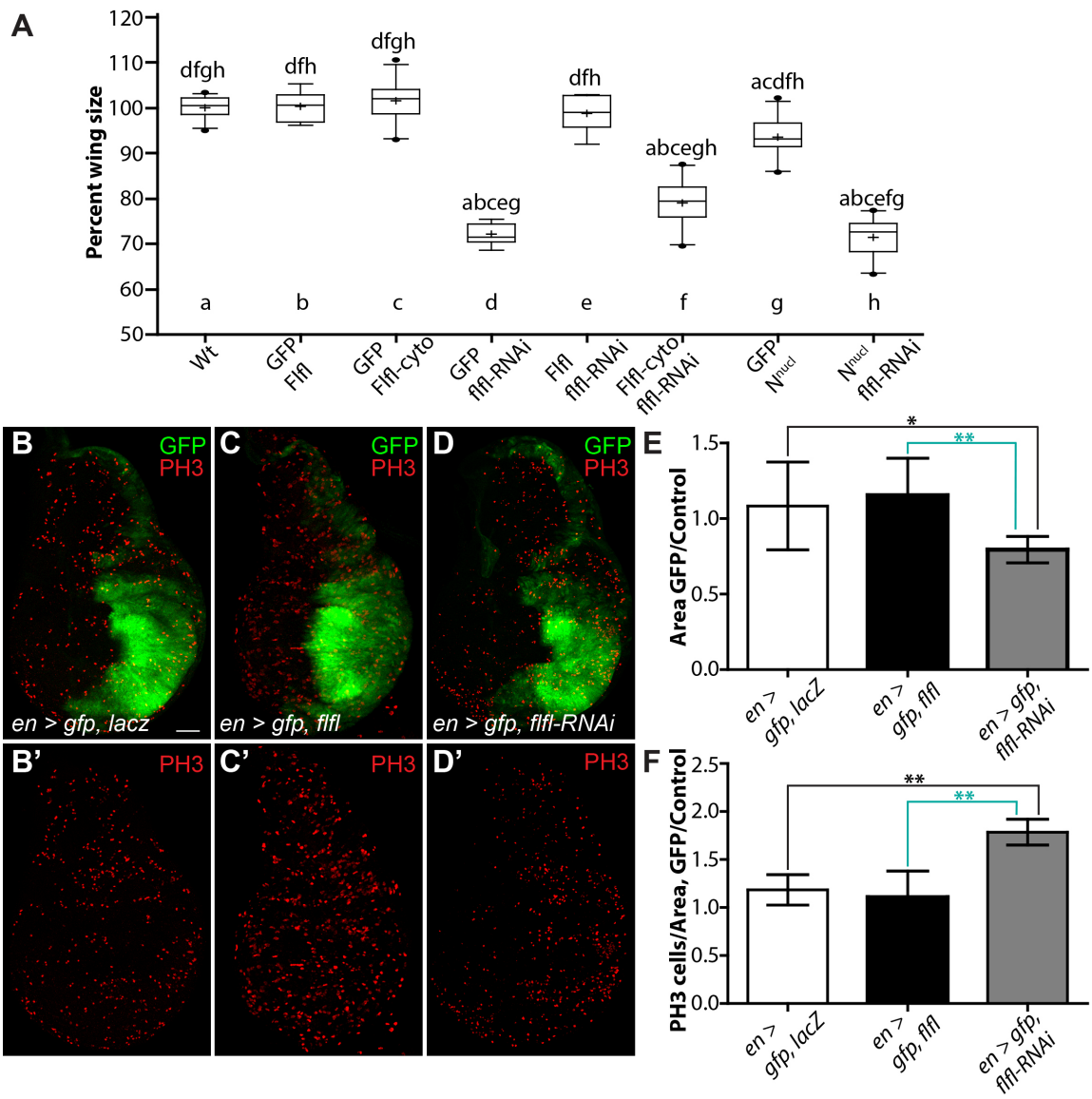
A
Percent wing size
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GFP Flfl
GFP Flfl-cyto
GFP flfl-RNAi
Flfl flfl-RNAi
Flfl-cyto flfl-RNAi
GFP Nnucl
Nnucl flfl-RNAi
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en > gfp, lacz
C
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PH3
en > gfp, flfl
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GFP
PH3
en > gfp, flfl-RNAi
B'
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Area GFP/Control
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en > gfp, lacZ
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en > gfp, lacZ
en > gfp, flfl
en > gfp, flfl-RNAi

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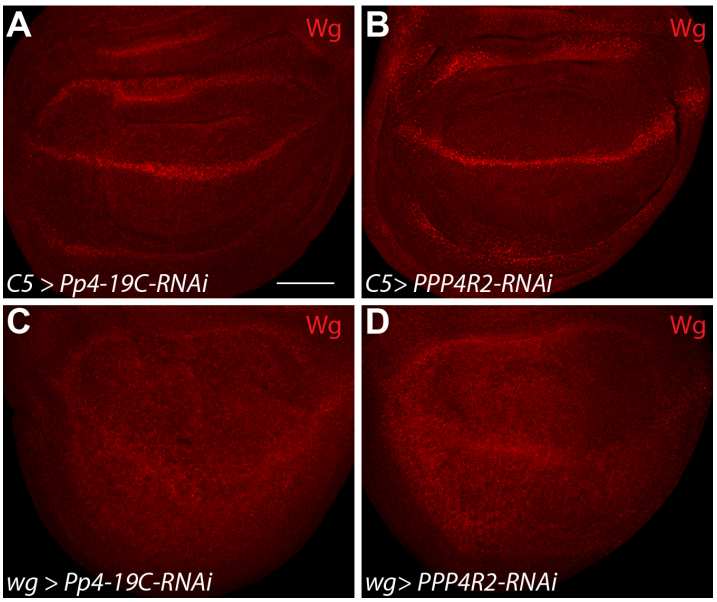
614 **Hall, Pradhan-Sundd *et al.* - Supplemental Information**

615

616 **Supplemental Figure 1. PP4 subunits promote Notch signaling in the Notch receiving cells.**
617 (A, B) *C5-Gal4* expressing *Pp4-19C-RNAi* or *PPP4R2-RNAi* along the D/V boundary flanking
618 cells of the wing pouch did not affect Wg protein levels or patterning. (C, D) *wg-Gal4* expression
619 of *Pp4-19C-RNAi* or *PPP4R2-RNAi* resulted in strong reduction of Wg in the wing disc. Scale
620 bar: 50 µm.



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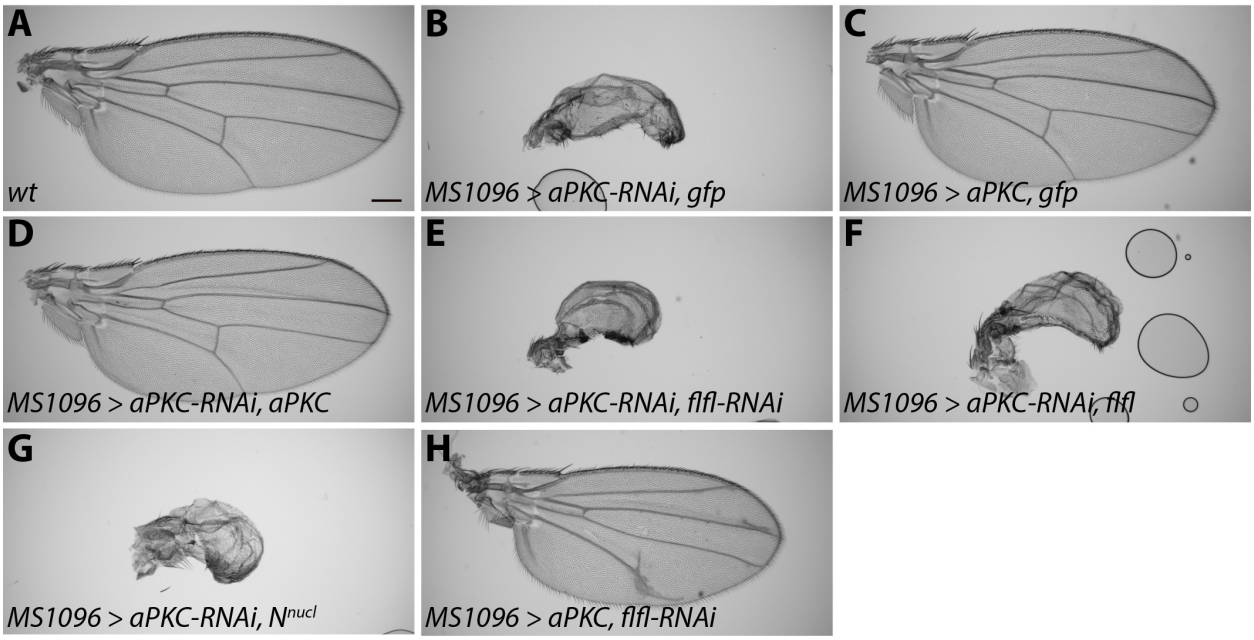
Supplemental Figure 2. aPKC does not promote Notch signaling in the Drosophila wing.(A) Adult wild-type wing. (B) Knockdown of aPKC with RNAi throughout the entire wing induces small crumpled, blistered, and malformed wings. (C-D) Over expression of an aPKC transgene induces no visible phenotype (C), but can fully rescue the effects of *aPKC-RNAi* (D). (E-G) The knockdown of Flfl (E), or its over expression (F) had no affect on the *aPKC-RNAi* phenotype. N^{nucl} was also unable to rescue the *aPKC-RNAi* phenotype (G). (H) Over expression aPKC does not affect the *flfl-RNAi* phenotype. Scale bar: 300 µm.