Genetic Annotation of Gain-Of-Function Screens Using RNA Interference and *in Situ* Hybridization of Candidate Genes in the *Drosophila* Wing

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ABSTRACT Gain-of-function screens in *Drosophila* are an effective method with which to identify genes that affect the development of particular structures or cell types. It has been found that a fraction of 2–10% of the genes tested, depending on the particularities of the screen, results in a discernible phenotype when overexpressed. However, it is not clear to what extent a gain-of-function phenotype generated by overexpression is informative about the normal function of the gene. Thus, very few reports attempt to correlate the loss- and overexpression phenotype for collections of genes identified in gain-of-function screens. In this work we use RNA interference and *in situ* hybridization to annotate a collection of 123 P-GS insertions that in combination with different Gal4 drivers affect the size and/or patterning of the wing. We identify the gene causing the overexpression phenotype by expressing, in a background of overexpression, RNA interference for the genes affected by each P-GS insertion. Then, we compare the loss and gain-of-function phenotypes obtained for each gene and relate them to its expression pattern in the wing disc. We find that 52% of genes identified by their overexpression phenotype are required during normal development. However, only in 9% of the cases analyzed was there some complementarity between the gain- and loss-of-function phenotype, suggesting that, in general, the overexpression phenotypes would not be indicative of the normal requirements of the gene.

GAIN-OF-FUNCTION screens have been systematically used in *Drosophila* to identify sets of genes that when overexpressed result in a particular phenotypic outcome (St Johnston 2002; Molnar *et al.* 2006a). This modality of genetic screen has several advantages, for example, the possibility of targeting the misexpression to a particular tissue at a predetermined time point, the existence of large collections of well-characterized transposable elements insertions harboring Upstrean Activating Sequence (UAS) from yeast, the facility with which new insertions can be generated and mapped to the genome, and the assignation of the misexpression effects to particular genes. Gain-of-function screens also allow selecting those genes with elusive or no loss-of-function phenotype, due, for example, to gene redundancy. For these reasons, gain-of-function screens constitute an effective entry point from which to characterize the requirements and molecular function of a gene in a particular developmental process. In addition, and for those genes affecting cell-fate choices such as several transcription factors and signaling molecules, there is a reasonably good complementarity between the loss-of-function and the gain-of-function phenotypes (Molnar *et al.* 2011). This complementarity allows refininement of the function assigned to the gene, as well as identification of additional components of signaling pathways by the consequences of their overexpression.

As with any screen, the gain-of-function approach also has several drawbacks. First is the possibility of unspecific effects of the misexpression, which could result in interesting phenotypes unrelated to the normal function of the gene (de Celis and Molnar 2010). Although this is likely the case for an unknown fraction of overexpressed genes, it is interesting to point out that the overlap of genes selected comparing different screens is low, suggesting some degree of specificity relative to the cell type or process under examination. Also, in some cases it is difficult to establish which gene in the proximity of the P-UAS insertion is responsible

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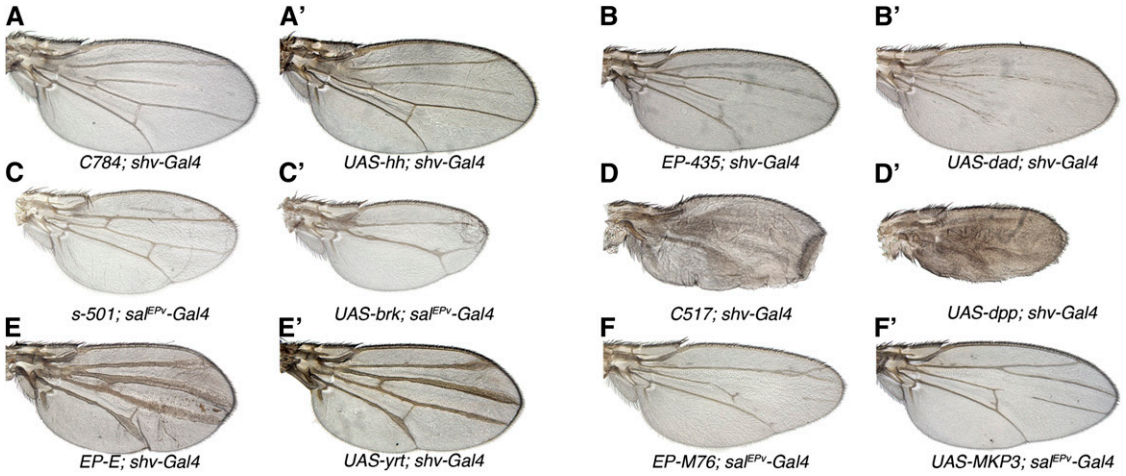


Figure 1 Similarity between *P-GS/Gal4* and *UAS-X/Gal4* phenotypes for representative examples of previously characterized genes that were isolated in the P-GS screens. (A and A′) *C784/shv–Gal4* (A) and *UAS–hh/shv–Gal4* (A′) showing loss of the L3 vein. (B and B′) *EP-435/shv-Gal4* (B) and *UAS-dad/shv–Gal4* (B′) showing loss of longitudinal veins. (C and C′) *s-501/sal^{EPv}–Gal4* (C) and *UAS-brk/sal^{EPv}–Gal4* showing a reduced wing size and the loss of longitudinal veins. (D and D′) *C517/shv–Gal4* (D) and *UAS–dpp/shv–Gal4* (D′) showing the formation of ectopic vein tissue. (E and E′) *EP-E/shv–Gal4* (E) and *UAS–yrt/shv–Gal4* (E′), showing a similar phenotype of thick veins. (F and F′) *sal^{EPv}–Gal4/EP-M76* (F) and *sal^{EPv}–Gal4/UAS–MKP3* (F′) showing a similar loss of vein phenotype. The *shv–Gal4* and *sal^{EPv}–Gal4* drivers are expressed in the pupal veins and in the central region of the wing blade, respectively.

for the overexpression phenotype, and this is particularly critical when the transposable element contains two sets of UAS sequences and consequently affects the expression of several genes simultaneously (Toba *et al.* 1999). Finally, it is not yet clear for most genes to what extent the loss- and gain-of function phenotypes are related to each other, as in most gain-of-function screens there had not been a systematic effort to compare both phenotypes. In fact, to our knowledge, in only one case, screening for defects in neuromuscular junctions, has this been attempted, and the authors reported that most loss- and gain-of-function phenotypes are unrelated to each other (Kraut *et al.* 2001). It is not yet known, however, whether it is a general rule that a loss-of-function phenotype cannot be predicted from the analysis of the corresponding gain-of-function phenotype. Similarly, it is not clear whether the genes causing the gain-of-function phenotype do so mostly due to overexpression or by ectopic expression.

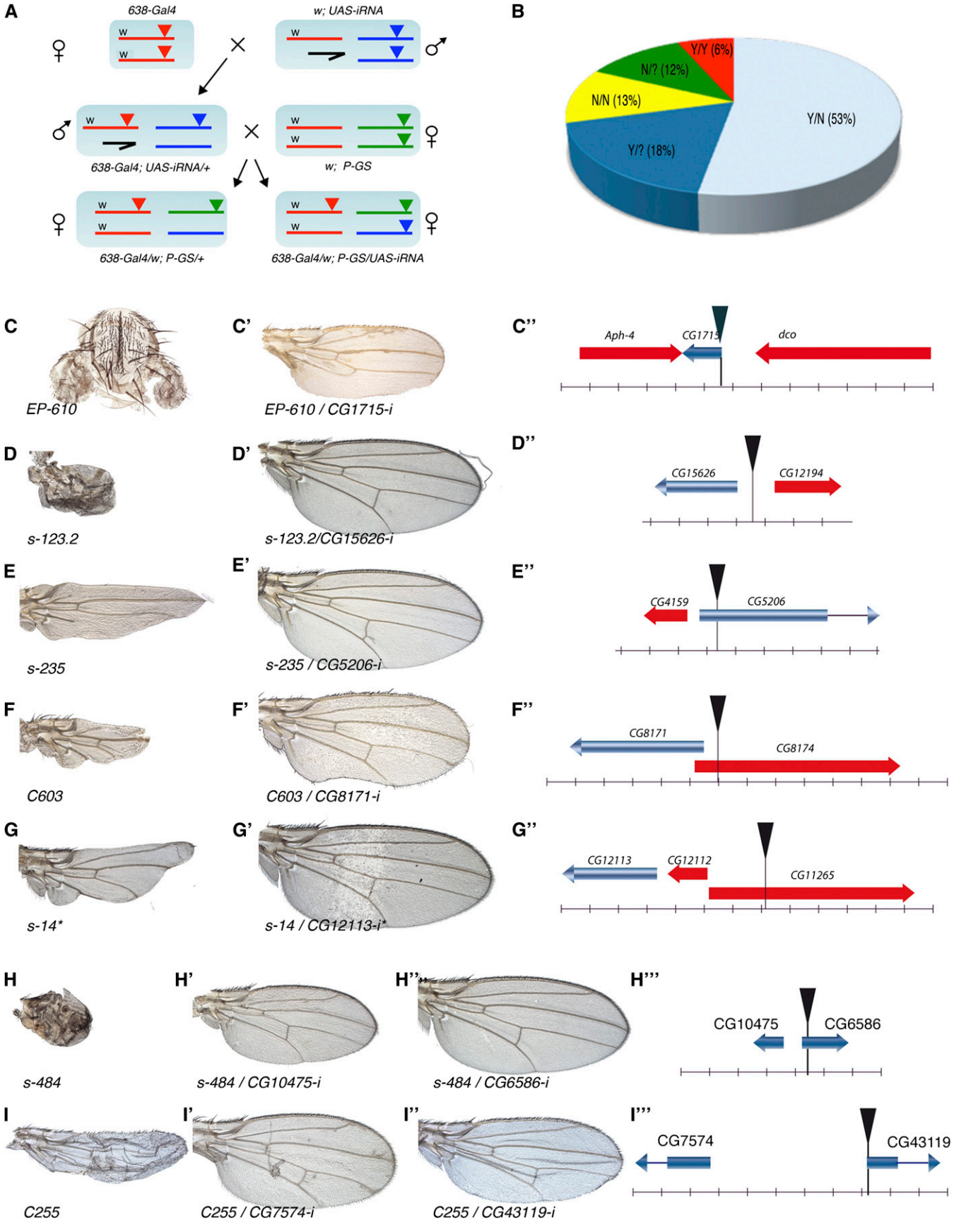
We recently described the results of two large-scale screens using the P-GS element (Toba *et al.* 1999), aimed to identify genes affecting, by over- or ectopic expression, the development of the wing and the differentiation of the veins (Molnar *et al.* 2006b; Cruz *et al.* 2009). The P-GS element has been used in several gain-of-function screens, and a large number of insertions are available through the *Drosophila* Genetic Resource Center at Kyoto. This *P*-element contains two sets of UAS sequences, one in each of its ends, and consequently, it can activate the expression of genes situated at both sides of the insertion site in response to Gal4 (Toba *et al.* 1999). In fact, this has been experimentally confirmed in a number of cases by *in situ* hybridization, where not only the adjacent genes to the P-GS element but also other genes located more distant to the *P*-element can be expressed in response to Gal4 (Molnar *et al.* 2006b). This characteristic of the P-GS favors the efficient recover of novel insertions based on their overexpression phenotypes, but at the same time complicates the assignation of these phenotypes to individual genes. In fact, our screens were successful in isolating with high efficiency novel insertions due to the phenotype of *P-GS/Gal4* combinations, but the gene or genes actually causing these phenotypes were not identified, due to the characteristics of the P-GS element.

In this work, we develop a strategy by which to annotate these screens that can be extended to any screen using P-UAS elements. Our main objective is to unambiguously determine which of the genes overexpressed in a *P-GS/Gal4* combination is responsible for the resulting phenotype. To do this, we introduce UAS-driven RNA interference (RNAi), targeting each of the candidate genes in the *P-GS/Gal4* background, and looked for an effective rescue of the overexpression phenotype due to the presence of the RNAi. We applied this strategy to a subset of 123 P-GS insertion sites and show that it is effective in the majority of cases to assign for each P-GS insertion a gene causing the overexpression phenotype. In addition, we also describe the expression patterns of the candidate genes in the wing disc and find that most of them are expressed in wild-type discs, suggesting that they have some role during normal wing disc development. Finally, we analyzed the loss-of-function phenotypes for the candidate genes in *UAS-RNAi/Gal4* combinations. This allows us to compare the loss- and gain-of-expression phenotypes for genes that have not yet been characterized in depth.

Material and Methods

Drosophila strains

We used the following stocks: the Gal4 lines *638-Gal4* (Molnar *et al.* 2006b), *sal^{EPv}–Gal4* (Cruz *et al.* 2009), and *shv^{3Kpn}–Gal4*


A
638-Gal4
w; UAS-iRNA
638-Gal4; UAS-iRNA/+
w; P-GS
638-Gal4/w; P-GS/+
638-Gal4/w; P-GS/UAS-iRNA
B
Y/Y (6%)
N/? (12%)
N/N (13%)
Y/N (53%)
Y/? (18%)
C
EP-610
C'
EP-610 / CG1715-i
C''
Aph-4
CG1715
dco
D
s-123.2
D'
s-123.2/CG15626-i
D''
CG15626
CG12194
E
s-235
E'
s-235 / CG5206-i
E''
CG4159
CG5206
F
C603
F'
C603 / CG8171-i
F''
CG8171
CG8174
G
s-14*
G'
s-14 / CG12113-i*
G''
CG12113
CG12112
CG11265
H
s-484
H'
s-484 / CG10475-i
H''
s-484 / CG6586-i
H'''
CG10475
CG6586
I
C255
I'
C255 / CG7574-i
I''
C255 / CG43119-i
I'''
CG7574
CG43119

(Sotillos and de Celis 2006), the expression of which is restricted to the wing blade (*638–Gal4*), the central region of the wing blade (sal^{EPv}–*Gal4*), and the pupal veins (shv^{3Kpn}–*Gal4*). We also used the following UAS lines: *UAS–GFP* (Ito *et al.* 1997), *UAS–dicer* (Dietzl *et al.* 2007), *UAS–hh* (Ingham and Fietz 1995), *UAS–dad* (Tsuneizumi *et al.* 1997), *UAS–dpp* (Staehling-Hampton and Hoffmann 1994), *UAS–brk (REF)*, *UAS–yrt* (Molnar *et al.* 2006b), and *UAS–MKP3* (C. Molnar and J. F. de Celis, unpublished results). The P-GS lines used are described in (Molnar *et al.* 2006b) and (Cruz *et al.* 2009) and the UAS–RNAi lines were obtained from VDRC (Dietzl *et al.* 2007) and NIG-FLY (http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp) stock centers (Supporting Information, Table S1). Unless otherwise stated, crosses were performed at 25°. The phenotypic analysis of *UAS–RNAi* lines was carried out in a *UAS–dicer* background, whereas the rescue experiments were performed in the absence of *dicer* overexpression. Wings were mounted in lactic acid:ethanol (1:1) and photographed with a Spot digital camera and a Zeiss Axioplan microscope. Lines not described in the text can be found in FlyBase.

EST clones

We obtained EST cDNA clones from the *Drosophila* Genomics Resource Center (Table S2).

In situ hybridization

We used digoxigenin-labeled RNA probes synthesized from the corresponding linearized EST clones or from PCR products (Table S2). Third-instar larvae were dissected in PBS and fixed 30 min in 4% formaldehyde, washed three times for 5 min in PBT (PBS–0.1%Tween20), and fixed a second time for 20 min in 4% formaldehyde–PBT. After several washes in PBT, the dissected larvae were kept at −20° in hybridization solution, HS (50% formamide, 5× SSC, 100 μg/ml DNA salmon sperm, 50 μg/ml heparin, 0.1% Tween20). The hybridization was carried out overnight at 55° with the probe at 1/50 dilution previously denaturalized by 10 min incubation at 80°. Excess of probe was washed at 55° in HS, and discs were washed several times in PBT and incubated for 2 hr with anti-digoxigenin antibody (Roche, Indianapolis) in a 1:4000 dilution in PBT. The color reaction was carried out in 100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris–HCl pH 9.5, 0.1% Tween20, nitroblue tetrazolium chloride, and bromo-chloro-indolylphosphate (Roche). After the color developed, the discs were rinsed several times in PBT, dissected in 30% glycerol, and mounted in 70% glycerol. The discs were photographed with a Spot digital camera and a Zeiss Axioplan microscope.

Results

Identification of candidate genes

We know that several genes in the proximity of a P-GS insertion site are expressed, albeit at different levels, in response to Gal4 (Toba *et al.* 1999; Molnar *et al.* 2006b). To estimate how many of the overexpressed genes are related to the phenotype of each *P-GS/Gal4* combination, we first looked for all the P-GS insertions isolated in our screens that mapped close to a gene for which there is information about its role during wing development and its gain-of-function phenotype. In these cases, we compared the *P-GS/Gal4* and *UAS/Gal4* phenotypes and found that in most cases the gene closer to the insertion site causes the overexpression phenotype (93%, $n = 45$). This is certain for all P-GS insertions that possibly affect only one gene (100%, $n = 16$), but also for P-GS insertions likely affecting more than one gene (89%; $n = 29$) (Figure 1 and Table S3). In general, the phenotypes of the *P-GS/Gal4* and the *UAS/Gal4* were very similar for all cases analyzed (Figure 1 and data not shown). We assumed that this might be a general characteristic of P-GS elements inserted in any genomic location and consequently we define as candidates only those genes that are more proximal to either end of the P-GS element. In the cases analyzed so far, these are the genes whose expression is more robust in response to Gal4 (Molnar *et al.* 2006b).

Figure 2 Genetic identification of genes targeted by P-GS elements that cause the overexpression phenotype. (A) Scheme of the crosses made to combine the *638-Gal4* (red triangle), *UAS–RNAi* (blue triangle), and *P-GS* (green triangle) insertions. The progeny of the cross between *638-Gal4*; *UAS–RNAi/+* males and *P-GS* females results in two classes of females, those carrying the *UAS–RNAi* (third row, right) and those without this chromosome (third row, left). (B) Overall results of the rescue experiments expressed in percentages. The percentage of *P-GS* lines in which one UAS–RNAi rescues (Y) and one does not rescue (N) the *638-Gal4/P-GS* phenotype is indicated in light blue (53%, Y/N). The percentage of cases in which only one candidate was tested and rescued the *638-Gal4/P-GS* phenotype is indicated in dark blue (18%, Y/?). The percentages of P-GS lines in which no *UAS–RNAi* rescues the *638-Gal4/P-GS* phenotype are indicated in yellow (13%, N/N) and green (12%, N/?), for cases in which all candidate were tested (yellow) and for those in which only one candidate was tested (green). The percentage of *P-GS* lines in which the *UAS–RNAi* for both candidates were tested and rescued the *638-Gal4/P-GS* phenotype is indicated in red (6%, Y/Y). (C–G′′) Examples of *P-GS/Gal4* combinations (C–G) rescued by the expression of one UAS-RNAi (C′–G′), and genomic organization close to the P-GS insertion site (C′′-G′′). The *638-Gal4/P-GS* wings (left) and the *638-Gal4/P-GS*; *UAS-RNAi/+* wings (right wing) are shown in C–G and C′–G′, respectively. (C′′–G′′) Scheme of the genomic region (horizontal line with ticks marking kilobases) in which the P-GS element (black triangles) is inserted. The gene responsible of the overexpression phenotype is shown in blue, and other genes not related to the overexpression phenotype in red. (C and C′′) P-GS insertion *EP-610*. (D and D′′) P-GS insertion *s-123.2*. (E and E′′) P-GS insertion *s-235* (F and F′′) P-GS insertion *C603*. (G and G′′) P-GS insertion *s-14*, both phenotypes were obtained at 17°. (H–I′′′) Representative examples of cases in which two RNAi modify the phenotype of P-GS/Gal4 combinations. (H and H′′′) P-GS insertion *s-484* in the combinations *638-Gal4/+*; *s-484/+* (H), *638-Gal4/+*; *s-484/UAS-iCG10475* (H′), *638-Gal4/+*; *s-484/UAS-iCG6586* (H′′), and genomic map of the insertion site (H′′′). (I and I′′′) P-GS insertion *C255* in the combinations *638-Gal4/+*; *C255/+* (B), *638-Gal4/+*; *C255/UAS-iCG7574* (I′), *638-Gal4/+*; *C388 /UAS-iCG43119* (I′′), and genomic map of the insertion site (I′′′).

Table 1 P-GS insertion (Line), cytological localization (Cyto), number of insertions identified affecting the same genes (*N*), wing phenotype in combinations of the P-GS insertion with *shv–Gal4* (shv), *638-Gal4* (638), *253-Gal4* (253), and *sal^{EPv}–Gal4* (sal)

Line	Cyto	*N*	shv	638	253	sal	D5′ (kb)	Gene 5′	RNAi	*In situ*	Rescue	M	C	D3′ (kb)	Gene 3′	RNAi	*In situ*	Rescue	M
C644	87D9	3	CD	L	wt	S-P	0	*CG8031*	wt	wG	N	CGh	2	0	*CG7583 (CtBP)*	S-P/L	wG	Y	TF
C275	64E5	3	CD	L/S-P	wt	wS-P	1	*CG10578 (Dnaj-1)*	wt	PG	N	P	2	0	*CG5486 (Ubp64E)*	PL/sS	wG	Y	P
C278	88E4	10	V+	sN	+Mq	wN	0	*CG6499*	wt	nE	Y	M	2	0	*CG42404*	wt	nE	N	CG
C442	98A2	16	V−	S-P/nW	+/−Mq	S-P	0	*CG5643 (wdb)*	S	G	Y	PPh	2	1	*CG5692 (raps)*	wt	wG	N	CS
C495	99D1	1	V−	V−	wt	wt	0	*CG15525*	Bs	G	N	CGh	2	0	*CG11504*	wt	G	Y	CGh
C518	56E3	5	CD/−V/N	nW/L	−Mq	S-P	0	*CG9854 (hrg)*	wt	G	N	RNA	2	2	*CG11025 (Isopeptidase-T-3)*	wt	nE	Y	P
C526	29C4	2	V−/nW	nW	wt/+Mq	V−	5	*CG13398*	wt	G	N	CGh	2	0	*CG13388 (Akap200)*	Bs		Y	CS
C603	51F11	1	CD	nW	-Mq	S-P	0	*CG8171 (dup)*	sN/PL	wG	Y*	DNA	2	0	*CG8174 (SRPK)*	wt		N	CS
C7	49A4	1	V+/CD	L	wt	wt	3	*CG8834*	wt		N	M	2	0	*CG8525*	wt	P	Y	M
s-407.2	9F5	2	CD	nW	wt	S−P/CD	0	*CG1655 (sofe)*	wt	PG	N	CS	2	0	*CG2186*	sS	G	Y	CG
C76	9D2	1	V+	sN	+Mq	wt	0	*CG15302 (or9a)*	wt	G	Y	cs	2	0	*CG15304 (Neb-cGP)*	wt	PG	N	CG
C79	53F1	4	CD	L	wt	S-P	8	*CG8963*	Bs		N	RM	2	0	*CG9635 (RhoGEF2)*	V-	P	Y	CS
C861	92F1	1	V+	sN/LP	-Mq	S-P	0	*CG4241 (att-ORFA)*	wt	wG	N	M	2	1	*CG5180*	wt	PG	Y	CG
EP-234	21B4	1	V−	sN	-Mq	S-P	0	*CG11617*	wt	G	Y	TF	2	0	*CG11490*	wt	G	N	CS
EP-620	4E2	6	CD	S-P/N	L	S-P	2	*CG32767*	nW	PG	N	TF	2	0	*CG6775 (rugose)*	wt	nE	Y	CGh
EP-733	52F2	2		V−		V−	3	*CG10734*	wt		N	CG	2	0	*CG8434 (lbk)*	wt	P	Y	CA
EP-767.1	89 A8	1	V+	V−/N	wt	wt	0	*CG18740 (mor)*	L/V+	P	Y	TF	2	0	*CG4261 (Hel89B)*	wt	G	N	TF
EP-880	61C3	2	V+	V+/S	wt	wt	0	*CG13892 (Cypl)*	wt	nE	N	PP	2	0	*CG17090 (Hipk)*	sS/N/Bs	PG	Y	CS
EP-M89	45C3	1	V−	sN/nW	-Mq	CD/sS	1	*CG2072 (Mad1)*	wV−/N/S	G	Y	CGh	2	0	*CG1975 (Rep2)*	PL	G	N	CGh
s-12	42C6-7	2	wt	L	L/-Q	nW	0	*CG9432 (1(2)01289)*	wt		N	M	2	1	*CG3268 (phtf)*	wt	nE	Y	TF
s-123.2	25A8	2	CD	S-P/L	wt	S-P	0	*CG15626*	wt	G	Y	CGh	2	0	*CG12194*	wt/S	nE	N	Tra
s-156	66A19	1	Bs	L	+/−Mq	S-P	0	*CG8114 (pbl)*	sN/L	G	Y	CS	2	0	*CG8281*	wt		N	CG
C607	89D5-6	2	F	CD/S		s	0	*CG14905*	wt		N	CG	2	0	*CG6588 (Fas 1)*	Bs	G	Y	CA
s-186	63D1	1	wt	S-P/CD	-Mq	S-P	0	*CG32268 (dro6)*	wt	nE	N	CGh	2	0	*CG12008 (kst)*	wt	P	Y	CA
C327.2	57E6	2	wt	sN/V−	wt	s	0	*CG9847 (Fkbp13)*	wt	nE	N	PM	2	4	*CG15668 (Mesk2)*	wt	nE	Y	CS
s-235	92F2	3	CD	sN/−V	wt	N/S-P	1	*CG4159*	wt		N	M	2	0	*CG5206 (bon)*	PL/S/V+	wG	Y*	TF
s-244	30C7	1	L	L/nW		S-P	10	*CG4105 (Cyp4e3)*	wt		N	M	2	0	*CG3998 (zf30C)*	V−	G	Y	TF
C588	94E9	3	CD	L	-Mq	S-P	0	*CG10868 (orb)*	S	PG	Y	RM	2	0	*CG6759 (cdc16)*	sN/L	G	N	CC
s-286	67B1	2	V−	N/V−		S-P	3	*CG10923 (Klp67A)*	wt	G	N	Cy	2	0	*CG4205 (Fdxh)*	nW	G	Y	M
s-303	57F10	4	L	L	L/-Mq	S-P	2	*CG30403*	wt	nE	Y	TF	2	0	*CG17950 (HmgD)*	wt	G	N	TF
s-349	99A1	2	Bs	L	L	s	10	*CG14508*	wV+	wG	Y	M	2	0	*CG31044*	S/nW		N	CG
s-393	91D4	1	F	nW	-Mq	s/v−	10	*CG14291*	wt	G	Y	M	2	0	*CG17836 (Xrp1)*	wt		N	CGh
s-397	14C4	1	S,+q	S-P/nW			0	*CG9968 (Anxb11)*	Bs		N	CA-Cy	2	0	*CG32575 (hang)*	wt	G	Y	CGh
s-409	19B3	1	L	L	-Mq	S-P	3	*CG9576*	wt	PG	Y*	CGh	2	0	*CG9577*	wt	G	N	M
s-414	85F12	1	L	L	wt	s	0	*CG6203 (Fmrl)*	wt		N	RM	2	2	*CG3940*	wt	G	Y	M
s-439	95D10	6	Bs	sN/nW	-Mq	S-P	0	*CG5422 (Rox8)*	wt	G	Y	R	2	0	*CG5986*	wt	G	N	CG
s-44.2	29E4	2	CD	nW/L	+Mq	wN/S	0	*CG9310 (Hnf4)*	wt/Bs/dp	G	N	TF	2	5	*CG9314*	wt	P	Y*	M
s-460	30B12	1	L	S/q+	+Mq	S/q+	0	*CG12245 (gcm)*	wt	wG	Y	TF	2	8	*CG3841*	wt		N	M
s-523.2	46F1	2	L	V+	-Mq	S-P	2	*CG17753 (CCS)*	wt		N	M	2	0	*CG30011 (gem)*	wt	G	Y	TF
s-69	60 B4	1	CD,−V	sN/nW	+/−Mq	S-P	0	*CG3924 (Chi)*	nW	G	Y	TF	2	0	*CG3167 (man1)*	wBs	wG	N	CGh
s-395	4C13	1		L		S/CD	1	*CG2984 (Pp2C1)*	wt		N	CS	2	0	*CG6998 (ctp)*	sS	PG	Y	Cy
s-271	60B10	1	L	N/V−	-Mq/S	V−	0	*CG4012 (gek)*	S	G	N	CS	2	2	*CG11290 (enok)*	S/Bs	G	Y	TF
C618	89B12	10	N	wN/V+	-Mq		35	*CG4337 (mtssB)*	wS	PG	Y	DNA	2	0	*CG6889 (taranis)*	wt	G	N	CGh
s-253	18C8	1	L	L	-Mq	S-P	0	*CG12204*	S		N	CG	3	1/1	*CG3400 (Pfrk)/CG14200*	S-P	PG	Y	M
s-98	54C12	2	CD	N/CD	-Mq	S-P/E	0	*CG6477 (RboGAP54D)*	wt	G	Y	CS	2	0	*CG4924 (icln)*	wt	P	N	CGh/CS
s-14	7F4	1	L	L		wS-P	4/2	*CG12113 (IntS4)/CG12112*	N/wt	PG/¿?	Y*/N	CGh/CG	3	0	*CG11265 (Trf4-1)*	wt	nE	N	RNA
s-442	36D2	1	L	nW		S-P	0	*CG15150 (elfless)*	wt/V−	P	Y	TF	2	0	*CG15151 (PFE)*	F		N	CG
s-160	54C12	1	+V	sN/nW/L	-Mq	S-P	0	*CG30105*	wt		N	CG	2	0	*CG4943 (lack)*	wt	wG	Y	PP
s-143	88B1	2	wt	nW	wt	S-P	0	*CG8651 (trx)*	v+/−	PG	Y	TF	1		*CG12207*	wt		N	CGh
EP-610	100B1	2	F	nW	-Mq	S-P	0	*CG1715 (1(3)03670)*	S-P/nW	PG	Y	CGh	1						
EP-112	31A1	2	V−	V−/N	-Mq	wV−							1	0	*CG4799 (Pen)*	wt	PG	Y	CA
EP-37	25B10	3	V−	nW	-Mq	wS	0	*CG33113 (Rtnl1)*	wt	PG	Y	CS	1						
EP-666	73D1	2	Arr	S-P/nW	-Mq	wV−							1	0	*CG9712 (tsg101)*	wF/Bs/S/V+	PG	Y	PM
s-108	61C8	1	wt	wN	-Mq	Nw	0	*CG13894*	wt	PG	Y	TF	1						
S-120	67C10	1	F	nW	-Mq	S-P	0	*CG10574 (1-2)*	wt	G	Y	PPI	1						

(continued)

Table 1, *continued*

Line	Cyto	N	shv	638	253	sal	D5′ (kb)	Gene 5′	RNAi	*In situ*	Rescue	M	C	D3′ (kb)	Gene 3′	RNAi	*In situ*	Rescue	M
s-121.2	19F6	2	CD	S/L	S	S-P							1	0	*CG1417 (slgA)*	wt	P	Y	M
s-153.1	56D3	2	wt	L	wt	S-P	0	*CG17246 (SdhA)*	PL/Bs/F	PG	Y	M	1						
s-415	85D1	3	F	nW	-Mq	S-P	0	*CG9755 (pum)*	wV+	G	Y	RB	1						
s-425.1	88A3	5	L	L/S/F/N	wt	s	0	*CG9924 (rdx)*	PL/S/F/Bs	G	Y	CGh	1						
s-436	8E6-7	1	CD	sN	wt	s	0	*CG42611 (Megalin)*	V−/wN	wG	Y*	CG	1						
s-518a	90F11	1	CD	sN/L	-Mq	s							1	2	*CG18599*	sN	PG	Y*	CGh
s-60	33A1	7	F/CD	Ns	wt	wS-P	0	*CG14938 (crol)*	wF/N	G	Y	TF	1						
s-72	51A4	1	CD	L	L/-Mq	S-P							1	0	*CG17390 (oaz)*	wt	P	Y*	TF
s-132	35D1	1	CD	sN/nW/L	wt	wS-P	0	*CG42616 (Cul-3)*	F/ML	wG	Y*	PP	1						
s-171.1	85F9	1	−V	L	S	S	14	*CG5361*	wt	wG	Y	PP	2	2	*mir317*				
s-206	86F5	1	Bs	S/V−	wt	S-P	0	*CG6923*	sS/ML	PG	Y	PM	2	0	*CG43062*				CG
s-422	97E10	1	CD	L	-Mq	S-P	0	*CG18766*	wS-P	G	Y	CGh	2	6	*CG5930 (TfIIA-L)*				RNA
s-66	68D2	1	wt	sN	wt	N	4	*CG32085*				CGh	2	0	*CG7334 (Sug)*	wt	PG	Y	M
s-480	54C3-7	3	Arr	sN/−V	wt	N/S-P	0	*CG6510 (Rpl18)*				RB	2	0	*CG4903 (MESR4)*	sS/V+	G	Y	TF
C277	39B4	8	N+CD	Bs	wt	wt	0	*CG31626*		wG/nE		CGh	2	0	*CG8676 (Hr39)*	wt	wG	Y	TF
C432	67C4	1	CD	L	wt	S-P	0	*CG6757 (SH3PX1)*	wt	G	Y	L	2	0	*CG16707 (vsg)*		G		CGh
s-254	32E2	2	V−	S-P	-Mq	s	0	*CG6392 (cmet)*	S	PG	Y	CY	2	0	*CG32955 (Cana)*				CY
EP-339	35E2	6	V−	V−	-Mq	q+	0	*CG4993 (PrL-1)*		G		CS	2	6	*CG4930*	wt	PG	Y	CG
EP-439	57F10	1	V−	S-P	-Mq	S-P	0	*CG30404 (tango11)*	wV+			CGh	2	0	*CG17952*	wt	P	Y	CA
EP-J59	55B8	1	Dif	nW	-Mq	S-P	7	*CG5748 (Hsf)*				TF	2	0	*CG5119 (pAbp)*	EPL	G	Y*	RM
EP-708	60D9	1	V−	V−	-Mq	wt	0	*CG13594*	PL/sN	G	Y	CG	2	0	*CG3616 (Cyp9c1)*				M
EP-471	21B2	2	V+	sN	+Mq	N	2	*CG33635*				M	2	0	*CG18497 (spen)*	V−L	G	Y	TF
EP-450	62A6-7	1	Arr	S	S	S/F	0	*CG12086 (cue)*	wt/ML	PG		CGh	2	0	*CG1009 (Psa)*	wt	PG	Y	P
s-456	2C2	S	F	L	S	S	3	*CG4406*		G		PP	2	0	*CG4399 (east)*	F		Y*	P
C684	7C4	1	L	sN	wt	S/wN	0	*CG10777*	wt	G	Y	TF	2	3	*CG42593*				CGh
C474.1	77B4	2	N	sN	+		0	*CG5585*	S/cvp	G	Y	CGh	2	0	*CG5605 (eRF1)*	ELL/N/S	G		P
EP-773	55F6	1	N+CD	N	+		0	*CG15098*				CG	2	0	*CG15083*	wV+	G	Y	CG
C840	88C6	1	N	+V	-Mq		0	*CG7832 (l(3)L1231)*	S/V+	G	Y	CG	2	8	*CG3505*				P
s-40	9F2	1	CD	nW	wt	S-P	0	*CG1691 (Imp)*	wt	G	N	RB	1						
C67	68A1	2	Arr	L		v+	0	*CG12296 (klu)*	wt	PG	N	TF	1	37	*CG7923 (Fad2)*	wt		N	M
C367	55B7	3	CD	L	-Mq	S-P	3	*CG5753 (stau)*	wt	G	N	RNA	2	0	*CG12767 (Dip3)*	wt	G	N	TF
C409	13D4	1	+	L		wt	0	*CG9215*	wt	PG	N	TF	2	0	*CG8097*	wt	G	N	RNA
EP-572	93B3	1	V−	S-P/L	-Mq	V−,N	0	*CG3593 (r-l)*	wt	G	N	M	2	0	*CG5737 (dmrt93B)*	wt		N	TF
EP-M	36C5	2	N/Dif	sN/L	L	V−	1	*CG6667 (dorsal)*	wt	G	N	TF	2	2	*CG5050*	wt		N	CGh
s-529	26A1	1	F	S-P	-Mq	S-P	3	*CG9021*	wt		N	CG	2	0	*CG14001 (bchs)*	wt	wG	N	P
s-532	46C2	1	wt	nW	wt	S-P/CD	0	*CG1513*	wt	PG	N	M	2	0	*CG30007*	wt	nE	N	CG
s-116	57A6	1	wt	N	wt	Nw	0	*CG13432 (qsm)*	N/dp	PG	N	CGd	2	5	*CG13425 (bancal)*	wt	PG	N	RNA
s-477	52A5	1	wt	N/V−	wt	N	0	*CG12964*	wt	P	N	CGh	2	2	*CG12960*	wt		N	CG
s-382	70D7	1	L	L	-Mq	S-P/nW	0	*CG3836 (stwl)*	wt		N	TF	2	0	*CG3919*	wt		N	TF
s-256	60E8	1	F	N	-Mq/S	wt	0/4	*CG2790*	S	nE	N	PM	2	0	*CG12851*	wS	PG	N	CG
EP-687	54F1	1	CD	N	-Mq	wt						PP	2	0/2	*CG11430 (olft86-F)/CG30323*	ELL/S	G	¿?/N	PP
EP-596	58F4	1	F	nW/L	L	wt	5/5	*CG43325/CG43326*	wt		N	CG	4	0	*CG42566/CG4250*		¿?/NE		CGd
EP-536	88D2	1	V+	N	wt	N	0	*CG7425 (eff)*	V−/PL	G		PP	2	0	*CG43162*	wt	G	N	CG
EP-319	42A4	2	CD	N	-Mq	CD	0	*CG12051 (Act42A)*	L/S/V−	G		CY	2	0	*CG7865 (PNGase)*	wt	nE	N	P
EP-24	29A1	4	V+	nW/S-P	+/−Mq	V−	0	*CG8049 (Btk29A)*	wt	PG		CS	2	3	*CG7851 (Scgalpha)*				CA
s-178	82D6	3	L	L	S/L	Ns/PL	0	*CG42574*	wt		N	CG	2	7	*CG1129*				M
s-338	49E7	1	wt	S-P	-Mq	S-P	16	*CG3886 (Psc)*	L		N	TF	2	0	*CG3905 (Su(z)2)*	Bs/F/V−	wG		TF
s-294	18E1	1	F/CD	L	-Mq	S-P	2	*CG14229*	nW−sN	wG		CG	2	0	*CG12530 (Cdc42)*	wt	G	N	CC
EP-501	72D6	1	V−	V−	wt	wt	2	*CG5215 (Zn72D)*	wt	G	N	RB	2	0	*CG5444 (Taf4)*	EPL	G		TF
EP-108	30F4	1	V+	sN	-Mq	V+	0	*CG5838 (Dref)*	sN	P	N	TF	2	0	*CG4651 (RpL13)*	nW/PL			RNA
EP-165	9E1	6	V+	V+/Bs	wt	wt	0	*CG32676*	sN/nW	G		PP	2	0	*CG1799 (ras)*	dp/ML	wG	N	M
C407	93B2	3	V+	sN	wt	wt	13	*CG31191*	wt	PG	N	CGh	2	0	*CG5670 (Atpalpha)*	L/sF	W		M
C717	30B5	3	V+/CD	N	-Mq	−V/sN	4	*CG4405 (jp)*	nW/L	PG		CY	2	0	*CG3838*	wt	G	N	DNA
s-125.1	87F9-11	2	wt	nW	wt	H	0	*CG9591 (omd)*	nW	PG	Y	R	2	0	*CG9351 (flfl)*	V+/S	G	Y	M
s-484	65A4	1	Ns	sN/nW	-Mq	S-P	0	*CG10475 (Jon65Ai)*	wt	G	Y	PM	2	0	*CG6586 (tan)*	wt	PG	Y	CG
C388	30B8	4	CD	S/F	-Mq	wE	0	*CG4422 (Gdi)*	L/sS-P	PG	Y*	CS	2	0	*CG33298*	wt	wG	Y	M

(continued)

Table 1, *continued*

Line	Cyto	*N*	shv	638	253	sal	D5′ (kb)	Gene 5′	RNAi	*In situ*	Rescue	M	C	D3′ (kb)	Gene 3′	RNAi	*In situ*	Rescue	M
EP-284	60E1	1	V+	S-P	wt	wN	0	*CG16932 (Eps15)*	wt	PG	Y	M	2	5	*CG3594 (Eap)*	wt	wP	Y	RNA
EP-323	37B11	2	V−	S-P	-Mq	wS-P	0	*CG15173*	wt	G	Y	CGh	2	0	*CG10473 (Acn)*	wS	PG	Y	P
C98	35F1	6	V−/N	V−/N	-Mq	wt	0	*CG7664 (crp)*	S/F/PL	PG	Y*	TF	2	1	*CG4132 (pkaap)*	S	wG	Y	CS
s-535	5B5	5	V−	N/V−	+Mq	wt	0	*CG3171 (Tref)*	wS	G	Y	CS	2	0	*CG15779 (Gr5a)*	wt	G	Y	CS
C255	66B3	1	CD	L	-Mq	wt	5	*CG7574 (bipl)*	V+	G	Y*	CG	2/CR	0	*CG43119 (Ect4)*	wt	nE	Y	CG

The genes located left or right to the insertion site are indicated as gene 5′ and gene 3′, respectively, and to the right of each gene is the distance of their 5′ coding region to the insertion site in kilobases (D5′ and D3′), their UAS–RNAi phenotype (RNAi), expression pattern (*in situ*), and molecular nature (M). The number of gene cadidates to cause the overexpression phenotypes is shown in the C column. We used the following abbreviations: for the phenotype columns, wt (no phenotype), CD (cell differentiation phenotype), V+ and V− (extra and loss of veins, respectively), N (wing margin phenotype), nW (lack of wing), Bs (wings with blisters), F (folded wings), L (lethal combination), S (wing size reduction), S-P (defects in wing size and pattern), +Mq (extra-macrochaetae), −Mq (loss of macrochaetae), q+ (extra chaetae in the wing blade); for the *in situ* columns, wG (low level of generalized expression), G (generalized expression), PG (generalized expression with higher levels in the wing veins and/or wing margin, P (distinct pattern of expression), nE (no expression detected); for the Molecular nature columns, CG (protein found only in Drosophilids), CGh (protein with conserved structural motives), M (protein involved in general metabolism), PM (protein metabolism), RNA (RNA metabolism), DNA (DNA metabolism), CS (cell signaling), Cy (cytoskeleton), TF (transcription factor). The rescue columns indicate whether the expression of the gene RNAi rescues (Y) or fails to rescue (N) the *638-Gal4/P-GS* phenotype.

Next, we selected 123 of the total 409 insertion sites identified in the wing screens that used the P-GS elements. The criterion to choose these lines was mainly the interest of the overexpression phenotype. In this manner, we chose those lines that when combined with wing-specific Gal4 drivers had strong effects on the size of the wing, the pattern of longitudinal veins, or the integrity of the wing margin (see Figure S1, Figure S2, and Figure S3). In addition, the 123 selected lines correspond to candidate genes whose possible functions during wing development are not known. For most of the P-GS lines selected we obtained the UAS–RNAi constructs targeting the genes more proximal to the insertion site (see Table S1) and combined them into a *638-Gal4/+*; *P-GS/+* genetic background (Figure 2A). Our rationale was that the expression of the RNAi targeting the gene causing the phenotype of the *638-Gal4/P-GS* combination would suppress or at least reduce this phenotype. In these cases, the female progeny of the cross between *638-Gal4*; *UAS-RNAi/+* males and P-GS females can be classified into two classes, which we assume correspond to females of *638-Gal4/+*; *P-GS/+* genotype showing the overexpression phenotype, and to *638-Gal4/+*; *P-GS/UAS–RNAi* females, which gave a wild type or a weaker version of the overexpression phenotype (Figure 2A). The complete results of these combinations are presented in Table 1, Figure S1, Figure S2, and Figure S3, and some illustrative examples are shown in Figure 2. We found that in 65 cases in which all candidate genes were tested only one RNAi construct efficiently rescues the *638-Gal4/P-GS* phenotype (Figure 2B, Table 1, Figure S1, and Figure S2). In 49 of these 65 P-GS insertions there were two candidate genes (see, for examples, Figure 2, D–G′′), whereas in 16 only one gene was close to the insertion site and oriented appropriately with respect to the EP insertion (see, for example, Figure 2, C–C′′). We also found 14 cases in which none of the RNAi introduced into the *638-Gal4/P-GS* background modified the overexpression phenotype (Table 1). In 4 of these cases there was only one candidate gene, and the failure of the corresponding RNAi to affect the overexpression phenotype under the conditions in which we used them suggests that these RNAi were not effective enough to reduce the levels of the overexpressed transcript. The fraction of false negatives is similar to that found in large-scale screens using the UAS–RNAi collection (Mummery-Widmer *et al.* 2009). In addition, we also found 8 cases in which two different RNAi suppress the overexpression phenotype of a particular P-GS insertion (Table 1, see examples in Figure 2, H-I′′′). In three of these cases the efficiency of rescue was different for each of the two RNAi used (Figure 2, H–H′′, and data not shown), whereas in 5 cases both RNAi result in a similar rescue (Figure 2, I–I′′, and data not shown). These cases might correspond to those in which at least two genes contribute to the overexpression phenotype or to cases in which the presence of additional UAS sequences "titrate out" the Gal4 protein, resulting in an unspecific reduction of Gal4 driven expression. Finally, in 36 cases we checked only

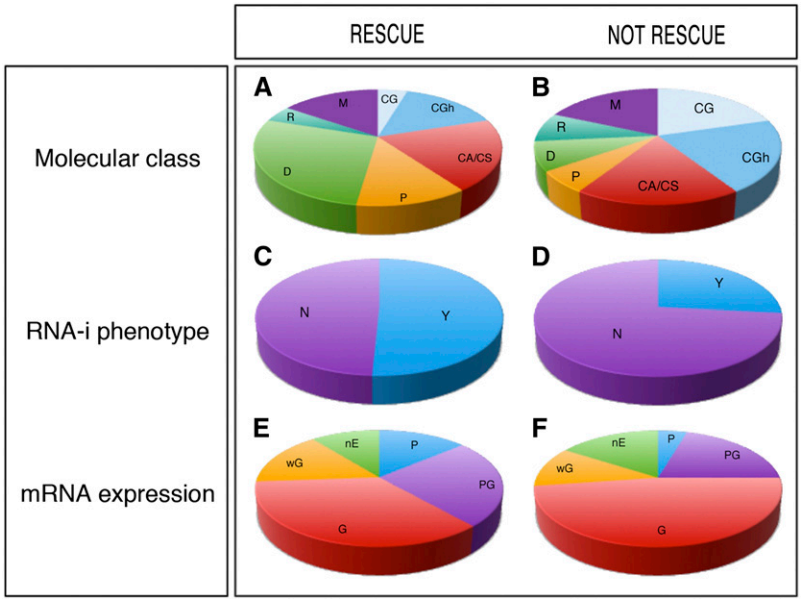


Figure 3 Numerical percentages of the molecular classes, RNAi phenotypes, and expression patterns for the annotated genes. (A and B) Molecular classes found for genes rescuing (A) and not rescuing (B) the *P-GS/Gal4* phenotype. CG (light blue): proteins without orthologous outside *Drosophila* and without annotated functional domains. CGh (dark blue): proteins without orthologous outside *Drosophila* but with conserved functional domains. CA/CS (red): proteins involved in cell adhesion or cell signaling. P (orange): proteins involved in protein metabolism. D (light green): Proteins involved in DNA metabolism. R (dark green): proteins involved in RNA metabolism. M (purple): proteins involved in general metabolic functions. (C and D) Percentage of *Gal4/UAS-RNAi* combinations showing a mutant phenotype in the wing for genes that rescue the *P-GS/Gal4* phenotype (C) and for genes that do not rescue this phenotype (D). Y: *Gal4/UAS-RNAi* displaying a wing phenotype (blue). N: *Gal4/UAS-RNAi* not displaying a wing phenotype (purple). (E and F) Percentage of expression patterns in the wing disc found for genes that rescue (E) and for genes that do not rescue (F) the *Gal4/P-GS* phenotype. P (blue): genes expressed in a distinct pattern. PG (purple): genes expressed in a generalized manner but with clear differences in levels of expression related to the presumptive wing margin or wing veins. G (red): genes expressed in a generalized and homogeneous manner. wG (orange): genes expressed at low levels and in a generalized and homogeneous manner. nE (green): genes for which we did not detect expression in the wing disc.

one of the two candidate genes, obtaining a positive result (rescue of the *638-Gal4/P-GS* phenotype) in 22 and a negative result in 14 cases (Table 1 and Figure S3).

Molecular nature and expression pattern of the candidate genes

The identification of the genes responsible for the *638-Gal4/P-GS* phenotype allowed us to make a more precise molecular annotation of the genes altering wing development when overexpressed. We found a clear enrichment of proteins related to transcription and chromatin structure for those genes causing overexpression phenotypes compared to those not related to the phenotype (26% compared to 7%, Figure 3, A and B). On the contrary, the class of genes without conserved representatives outside insects (CG and CGh classes; see Table 1) is more represented in the group of genes not causing an overexpression phenotype (47% compared to 22%, Figure 3, A and B). Other molecular classes are similarly represented in both groups of genes (Figure 3, A and B).

We also made *in situ* hybridization for most of the genes annotated in this work (Figure 3, E and F, Figure 4, Figure S4, Figure S5, and Figure S6). The more frequent class of expression patterns corresponds to genes expressed in a generalized manner in the wing disc (50 and 59% of cases in the rescuing and nonrescuing groups, respectively, see Figure 3, E and F, and Figure 4, C–E). For a considerable fraction of genes, we found expression patterns in which, above a background of general expression, we could detect different levels of mRNA enrichment associated with the developing veins or the presumptive wing margin (25 and 20% of cases in the rescuing and nonrescuing groups, respectively; see Figures 3, E and F, Figure 4, F–H, and Figure S4, Figure S5, and Figure S6). The main difference between the rescuing and nonrescuing classes was found in cases in which the expression of the gene is detected in a restricted pattern (14 and 5% of cases in the rescuing and nonrescuing groups, respectively (Figures 3, E and F, Figure 4, I–J, and Figure S4, Figure S5, and Figure S6). Genes not normally expressed constitute the 11 and 16% of cases in the rescuing and nonrescuing groups, respectively (Figure 3, E and F, Figure 4, A and B, and Figure S4, Figure S5, and Figure S6). In this manner, we can conclude that an estimated fraction of 11% of the genes selected cause a phenotype in the wing due to ectopic expression, whereas in the remnant 89% of cases the phenotype is due to the expression of the gene at higher than normal levels, but in places where the gene is normally expressed.

Loss-of-function phenotype of candidate genes

The availability of the UAS-RNAi constructs also made it possible to check for the loss-of-function phenotypes of those genes that were selected on the basis of their gain-of expression phenotype. We found that only for a fraction of the selected genes (51%) the expression of their RNAi results in a phenotype in the wing (Figure 3, C and D). These combinations were done in a *UAS–dicer* background, and the phenotypes consisted in changes in the size of the wing (13%; Figure 5, A, C, and G, Figure S7, and Figure S9), the size and the pattern of veins (10%; Figure 5, A, D, and E, Figure S7, and Figure S9), the differentiation of veins (10%; Figure 5, A, D, and E, Figure S7, and Figure S9), the integrity of the wing margin (6%; Figure 5, E, F, and I, Figure S7, and Figure S9), or the adhesion between the dorsal and ventral wing surfaces (10%, including those causing folded wings; Figure 5H, Figure S7, and Figure S9). For the remnant

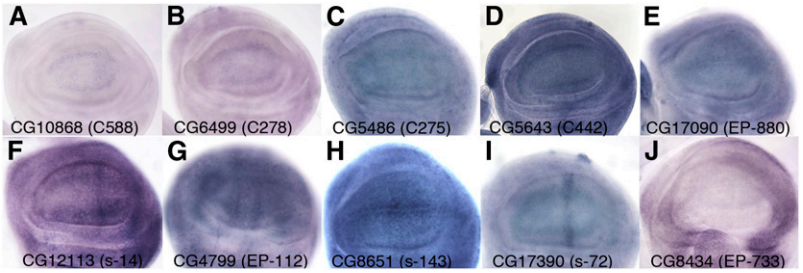


Figure 4 Representative examples of expression patterns for genes that rescue the phenotype of *P-GS/638-Gal4* combinations. (A) No expression in the wing disc (*CG10868*). (B) Weak and generalized expression (*CG6499*). (C–E) Generalized expression of *CG5486* (C), *CG5643* (D), and *CG17090* (E). (F–H) Generalized expression with regions of preferential accumulation. *CG12113* (F), *CG4799* (G), and *CG8651* (H). (I and J) Expression in a distinct spatial pattern of *CG17390* (I) and *CG8434* (J). In all cases the pictures show the wing region of late third-instar wing disc hybridized with the corresponding RNA probes.

49% of cases we could not detect a loss-of-function phenotype (Figure 3, C and D, and Figure S8), either because the gene is not normally required during wing development or because the reduction resulting from RNAi expression is not strong enough to identify a requirement. The expression of RNAi directed against the genes that were identified as not causing a gain-of-expression phenotype resulted in wild-ype wings in the 73% of cases analyzed (Figure 3, C and D). In general, genes whose expression was not detected by *in situ* hybridization did not result in a loss-of-function phenotype (15 *vs.* 1 cases in total)

The loss-of-function phenotype gives a clear indication of the normal requirements of the gene during development. For some genes, such as those involved in signaling, or for transcription factors affecting cell-fate choices, there is a good complementarity between the loss- and gain-of expression phenotype (Molnar *et al.* 2011). In this manner, mutations reducing EGFR activity, for example, cause loss of veins, whereas the increase in EGFR causes the formation of ectopic veins (Sturtevant and Bier 1995; Guichard *et al.* 1999). Similarly, loss of the proneural genes causes a loss of bristles phenotype, whereas ectopic expression of these genes results in the differentiation of supernumerary bristles (Campuzano *et al.* 1986; Balcells *et al.* 1988). We compared the loss- and gain-of-expression phenotypes for the collection of genes analyzed, which belong to a variety of molecular classes, and could not find such a complementarity. Thus, in 59% of the cases analyzed, the loss- and gain-of-expression phenotype seem unrelated to each other (Table 1, Figure 6E, Figure S7, and Figure S9), and in only 9% of the cases was there some complementarity between the gain- and loss-of-function phenotype (Figures 6, C and D, Figure S7, and Figure S9). More intriguingly, in 32% of cases, the loss- and gain-of-expression phenotype looked similar, suggesting a dominant–negative effect of the overexpression (Figure 6, A and B, Figure S7, and Figure S9).

Discussion

Gain-of-function screens constitute an effective method with which to identify genes that, for some reason, are not amenable to a conventional loss-of-function approach. The main assumption justifying the use of the overexpression to determine gene functions is that the phenotype caused by the presence of a protein at higher than normal levels is informative with respect to its normal function. However, this has been proved only for a limited set of genes, which, in general, were first thoroughly analyzed in loss-of-function conditions. Some paradigmatic examples are the cases of the homeotic and proneural genes, where the phenotype of the dominant alleles *Contrabithorax* and *Hairywing*, respectively, is complementary to the phenotype of the corresponding loss-of-function alleles (Balcells *et al.* 1988; Gonzalez-Gaitan *et al.* 1990). In this manner, the analysis of these alleles was instrumental in assigning instructive functions for the corresponding gene products. These cases, however, were shown to correspond to ectopic expression of the genes, rather than mere overexpression (Cabrera *et al.* 1985; Balcells *et al.* 1988; Gonzalez-Gaitan *et al.* 1990). Furthermore, the relationships between the normal function of Ultrabithorax or Scute and the phenotypes obtained when these proteins are overexpressed in their normal domains of expression are much more complex to dissect (Chang *et al.* 2008; Garaulet *et al.* 2008). Another group of genes for which it is assumed that the consequences of the overexpression and the loss-of-function are opposite are those encoding components of signaling pathways. In this case, however, the comparison with the loss-of-function is generally done with the expression of constitutively activated forms of the protein, such as the intracellular fragment of Notch (Notch^{intra}), phosphomimic forms of transmembrane receptors, or GTPase proteins that are artificially maintained in the GTP-bounded form. When wild-type proteins are overexpressed, the results tend to be much more complicated. For example, overexpression of the full-length receptor Notch generates a phenotype of thicker veins that is more similar to the loss-of-function of the gene than to the expression of the Notch^{intra}-activated form (de Celis and Bray 1997). Similarly, the overexpression of the receptor Thickveins results in phenotypes similar to a partial loss-of-function of its ligand, Decapentaplegic (Dpp) (Lecuit and Cohen 1998). It holds true, however, that the analysis of these effects revealed novel and interesting aspects of the cell biology of the corresponding pathways, such as the necessity of a correct balance between the amount of Notch and its ligands (de Celis and Bray 1997; Huppert *et al.* 1997) and a role of the receptor Thickveins in the sequestering and turnover of its ligands (Lecuit and Cohen 1998). In these examples, the overexpression or ectopic expression was useful in assigning normal functions to the wild-type protein, but to what extent this is the case for the generality of proteins is unknown.

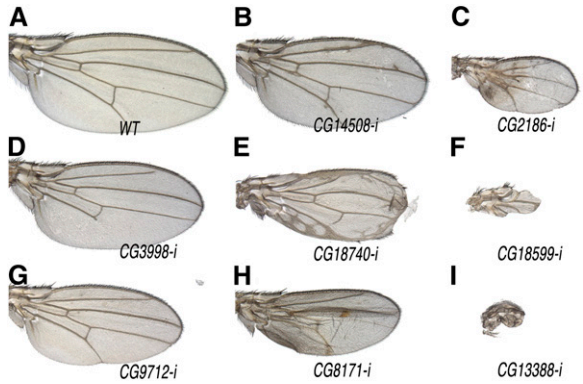


Figure 5 Representative examples of loss-of-function phenotypes for genes that rescue the *P-GS/638-Gal4* combinations. (A) Wild-type wing. (B–I) Wings of *UAS–dicer/+*; *nub–Gal4/UAS–RNAi* genotype illustrating the formation of extra-vein tissue (B; UAS–CG14508-i), reduced wing size and thicker veins (C; UAS–CG2186-i), loss of veins (D; UAS–CG3998-i), extra veins and loss of wing margin (E; UAS–CG18740-i), extreme loss of wing margin (F; CG18599-i), reduced wing size (G; CG9712-i), blistered wings (H; CG8171-i), and extreme loss of wing tissue (I; CG13388-i).

The use of overexpression to identify candidate genes affecting a particular process was busted when the UAS sequences were incorporated into the *P*-element, allowing the screen of large collections of P-UAS elements (Rorth *et al.* 1998). These screens have been applied to a variety of processes, such as imaginal development (Rorth 1996; Tseng and Hariharan 2002; Cruz *et al.* 2009), vein patterning (Molnar *et al.* 2006b), sensory organ formation (Abdelilah-Seyfried *et al.* 2001), motor axon guidance (Kraut *et al.* 2001), and many others (Huang and Rubin 2000; Pena-Rangel *et al.* 2002; McGovern *et al.* 2003; Nicolai *et al.* 2003; Hall *et al.* 2004; Schulz *et al.* 2004). In all these cases the screens were successful in identifying candidate genes affecting the process of interest when overexpressed, with a fraction of positive insertions of between 1 and 10% depending on the P-UAS element used and the particular experimental system. However, these screens did not unambiguously identify the genes causing the overexpression phenotype, and only in one case was there some attempt to correlate these phenotypes with the corresponding loss-of-function phenotypes (Kraut *et al.* 2001). In this last case, it was found that loss- and gain-of-function phenotypes were not generally opposite in sign and, therefore, that a gain-of-function phenotype could not predict the corresponding loss-of-function phenotype.

In this work, we aimed to annotate two gain-of-function screens that used the bidirectional P-GS element (Toba *et al.* 1999; Molnar *et al.* 2006b; Cruz *et al.* 2009), by identifying the genes causing the overexpression phenotype, their expression patterns, and the relationships between the gain- and loss-of-function phenotypes for this set of genes. To this end, we selected 123 P-GS insertions affecting candidate genes whose possible functions during wing development are not known. We expect that these insertions constitute a representative sample, as they affect candidate genes belonging to very different molecular categories, and are located in genomic regions of diverse complexity. To identify for each P-GS insertion site the gene causing the overexpression phenotype, we first defined the set of candidate genes as those more proximal to either end of the P-GS element and transcribed in the orientation that the UAS sequence is driving the overexpression. This is the case for genes with a previously known gain-of-function phenotype, and we expect that the expression of genes more proximal to the P-GS in response to Gal4 is more robust than that of genes located farther away from the insertion site (Molnar *et al.* 2006b). We introduced in the *Gal4/P-GS* background RNAi targeting the candidate genes for each insertion, expecting that the expression of at least one RNAi would reduce the Gal4/P-GS phenotype. This was the case for a considerable fraction of P-GS insertions for which we could test all annotated candidate genes (75%), indicating that this strategy can identify the gene/s causing the overexpression phenotype. In a fraction of cases (16%) we could not find any candidate gene modifying the Gal4/P-GS phenotype. These results imply that the rescues were not due to a titration of the Gal4 due to the presence of additional UAS sequences, a result we confirmed introducing UAS–GFP construct in six Gal4/P-GS backgrounds (not shown). More likely, the false-negative cases of nonrescue were due to inefficient gene silencing caused by either insufficient levels of RNAi expression driven by the UAS–RNAi constructs or by ineffective RNA interference. This result is not surprising, because an estimated fraction of 29% of UAS–RNAi lines is inefficient to cause gene silencing (Mummery-Widmer *et al.* 2009). In addition, we also found a fraction of cases (9%) in which two candidate genes rescued to some extent the Gal4/P-GS phenotype, which we assume corresponds to cases in which two neighbor genes need to be overexpressed simultaneously to result in an overexpression phenotype. We did not test all candidate genes for a 29% of P-GS insertions, either because the RNAi were not available or because some of the combinations required for testing the rescue were lethal.

We searched for some common aspect shared by genes causing wing overexpression phenotypes, in either molecular nature or expression patterns, and also examined the similarities between their gain- and loss-of-function phenotypes. With respect to the molecular nature, we found only significant differences in the DNA-related class, which is enriched in the group of proteins causing an overexpression phenotype (28% *vs.* 8%), and in the CG/CGh class, which is less represented in this group (19% *vs.* 40%). Other molecular classes, such as proteins involved in RNA, protein, or general metabolism, cell adhesion, and cell signaling are similarly represented in the groups causing and not causing the overexpression phenotype. We also annotated the number of protein–protein interactions (Murali *et al.* 2011) identified for the genes described in our screen, reasoning that proteins engaged in interactions with many partners might be enriched in the class of proteins causing overexpression phenotypes. However, we could not find any difference in

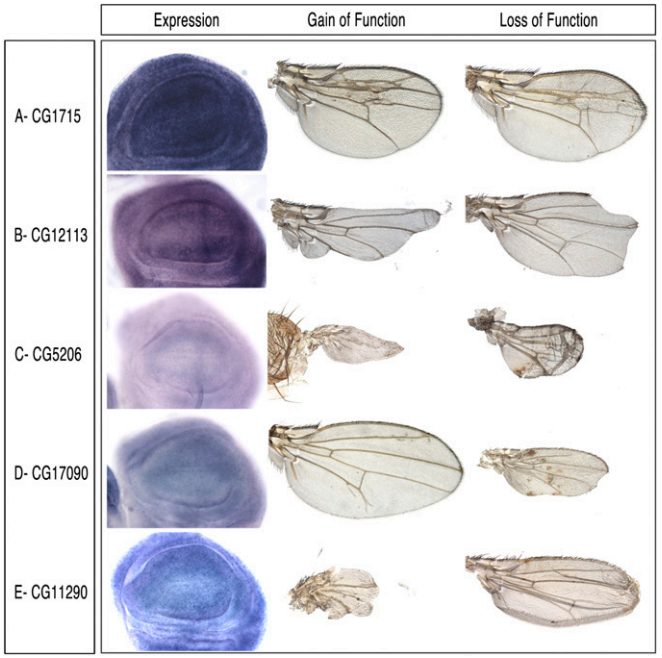


Figure 6 Representative examples of expression patterns and the gain- and loss-of-function phenotypes of the corresponding gene. (A) Expression of *CG1715* and wing phenotypes of its gain (*EP–610/sal^{EPv}–Gal4*) and loss-of-function (*sal^{EPv}–Gal4/UAS–iCG1715*) phenotypes. These phenotypes are similar and consist of the reduction of the size of the interveins. (B) Expression of *CG12113* and its gain (*638-Gal4/s-14*, grown at 17°) and loss-of-function (*UAS–dicer/+*; *nub–Gal4/UAS–iCG12113*) phenotypes. These phenotypes are similar and consist in the loss of wing margin tissue. (C) Expression of *CG5206* and its gain (*638-Gal4/s-235*) and loss-of-function (*UAS–dicer/+*; *nub–Gal4/UAS–iCG5206*) phenotypes. These phenotypes are complementary, consisting of loss of veins and wing margin (overexpression) and differentiation of thicker veins (loss-of-function). (D) Expression of *CG17090* and its wing phenotypes of gain (*638-Gal4/EP-880*) and loss-of-function (*UAS–dicer/+*; *nub–Gal4/UAS–iCG17090*). These phenotypes are complementary with respect to the effects on the size of the wing. (E) Expression of *CG11290* and its gain (*638-Gal4/s-271*) and loss-of-function (*UAS–dicer/+*; *nub–Gal4/UAS–iCG11290*) phenotypes. These phenotypes are unrelated to each other and consist of severe notching of the wing (overexpression) and blistered wing (loss-of-function).

this respect between the proteins causing or not causing an overexpression phenotype analyzed in this work (Figure S10). Finally, we compared the genes identified in eight published screens using the EP or P-GS transgenes with our data. These screens were designed to isolate genes affecting antennal formation (Zhang *et al.* 2006), germ cell development (Schulz *et al.* 2004), eye disc growth (Tseng and Hariharan 2002), CNS (McGovern *et al.* 2003), axon guidance (Kraut *et al.* 2001), sensory organ formation in the thorax and dorsal closure (Abdelilah-Seyfried *et al.* 2001; Pena-Rangel *et al.* 2002), and muscle development (Staudt and Al 2005). Of the total of 394 genes identified in these screens, 88% are unique for each screen, 9% of the genes were identified in two screens, and only 3% were found in three or four screens (data not shown). This result indicates that the experimental system in which the overexpression is tested determines to a large extent whether a protein interferes or not with the development of that system. This conclusion implies that the overexpression phenotype presents strong cell-type specificity and argues against unspecific effects of the overexpression affecting common cellular processes that result in tissue-specific phenotypes.

To discuss the possible roles during wing development of the genes identified in this annotation is beyond the scope of this work. However, we found that 52% of genes causing overexpression phenotypes also result in a loss-of-function phenotype. All these genes were normally expressed in the wing disc, suggesting that their gain-of-function phenotype corresponds to overexpression and not to ectopic expression. For the remnant 48% of cases (31 out of 65), we did not detect a loss-of-function phenotype. Only in 6 of these cases we failed to detect expression of the gene in wild-type discs, indicating that the overexpression phenotype corresponds to cases of ectopic expression. The remnant 25 cases correspond to genes that are normally expressed in the wing disc, but either their function is not required during wing development or, more likely, the reduction resulting from the expression of the RNAi is not strong enough to generate a loss-of-function phenotype. The comparison of the gain- and loss-of-function phenotypes for 34 cases is revealing, because for the majority of them (59%) there is no complementarity between these phenotypes. We could find only some complementarity between loss- and gain-of-function phenotypes for 9% of these 34 cases. Interestingly, the loss- and gain-of expression phenotype looked similar in the remaining 32% of cases, suggesting a dominant-negative effect of the overexpression. With these numbers, we think that the gain-of-expression phenotypes might be a convenient entry point for identifying genes affecting a particular developmental system, but that in very few cases these phenotypes would be indicative of the specific requirements of the gene. On the positive side, the analysis of the loss-of-function conditions indicates that at least half of the genes (52%) identified by their overexpression phenotype are required during normal development, validating the gain-of-function approach as a way to identify genes required for a particular developmental system.

Acknowledgments

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GENETICS

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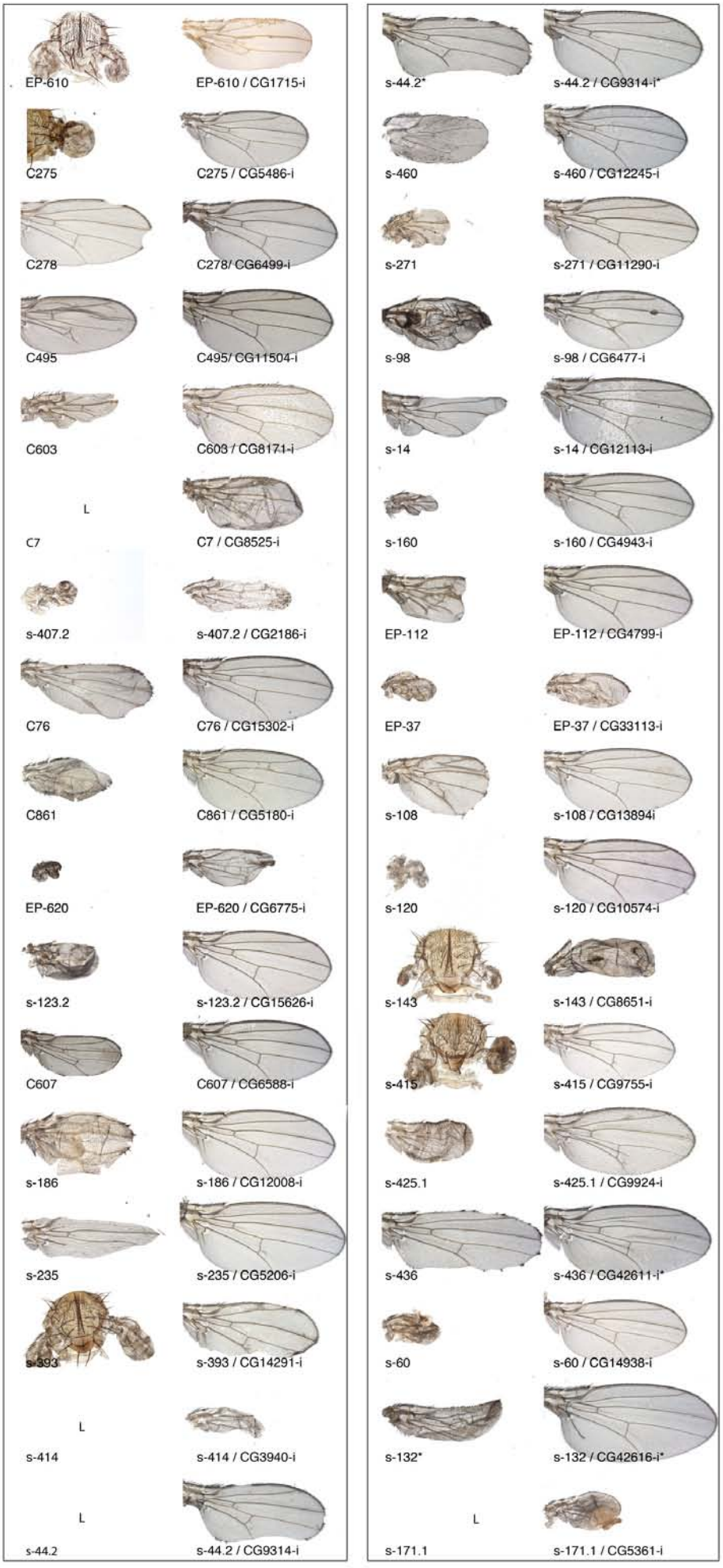
Genetic Annotation of Gain-Of-Function Screens Using RNA Interference and *in Situ* Hybridization of Candidate Genes in the *Drosophila* Wing

Cristina Molnar, Mar Casado, Ana López-Varea, Cristina Cruz, and Jose F. de Celis

DOI: 10.1534/genetics.112.143537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C861
C861 / CG5180-i
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s-123.2 / CG15626-i
C607
C607 / CG6588-i
s-186
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Figure S1 Rescue of *638-Gal4/P-GS* phenotype by RNA interference of only one gene adjacent to the insertion site. Each pair of pictures illustrate representative wings of *638-Gal4/P-GS* combinations (first and third columns) and *638-Gal4/P-GS; UAS-RNAi/+* (second and fourth columns) genotypes. In each picture is indicated the corresponding *P-GS* and *UAS-RNAi* lines. All cases correspond to *P-GS* insertions in which the combination was made for all candidate genes, and only one of the RNAis rescued the *638-Gal4/P-GS* phenotype. L indicates lethal combinations.

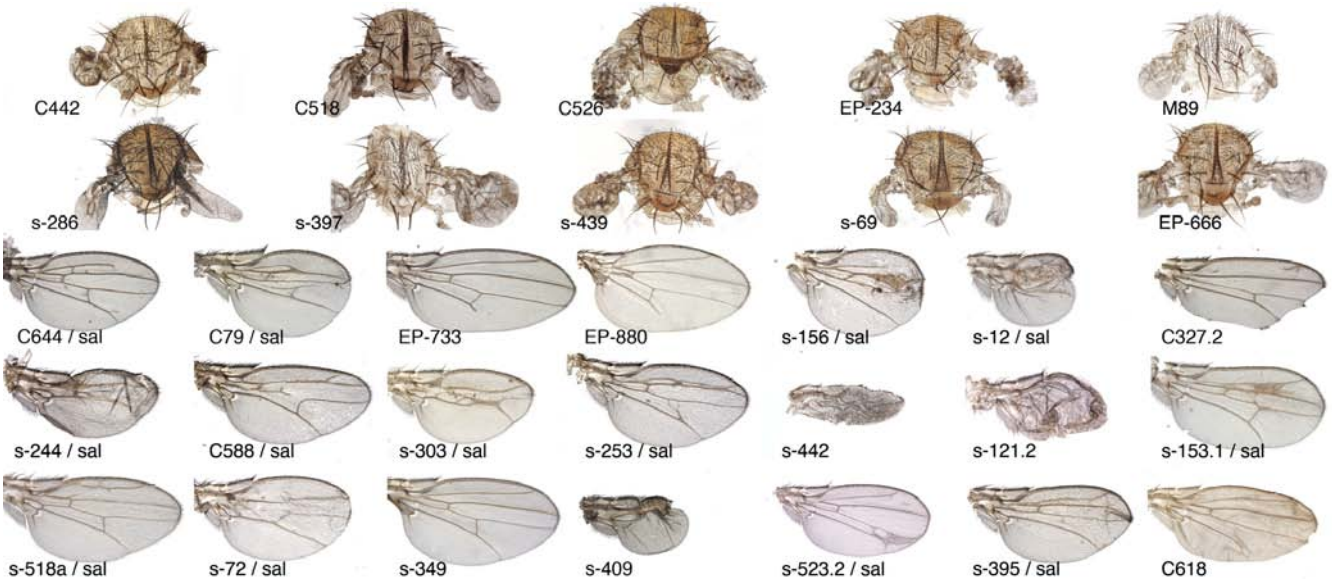

Figure S2 Phenotype of *638-Gal4/P-GS* combinations. Pictures of representative *638-Gal4/P-GS* or *sal^{EPv}-Gal4/P-GS* wings which phenotype is rescued to wild type by the expression of one UAS-RNAi corresponding to a candidate gene. The name of each P-GS line is indicated in the left-bottom corner of each picture. sal represents *sal^{EPv}-Gal4*, and all other cases correspond to *638-Gal4* combinations.

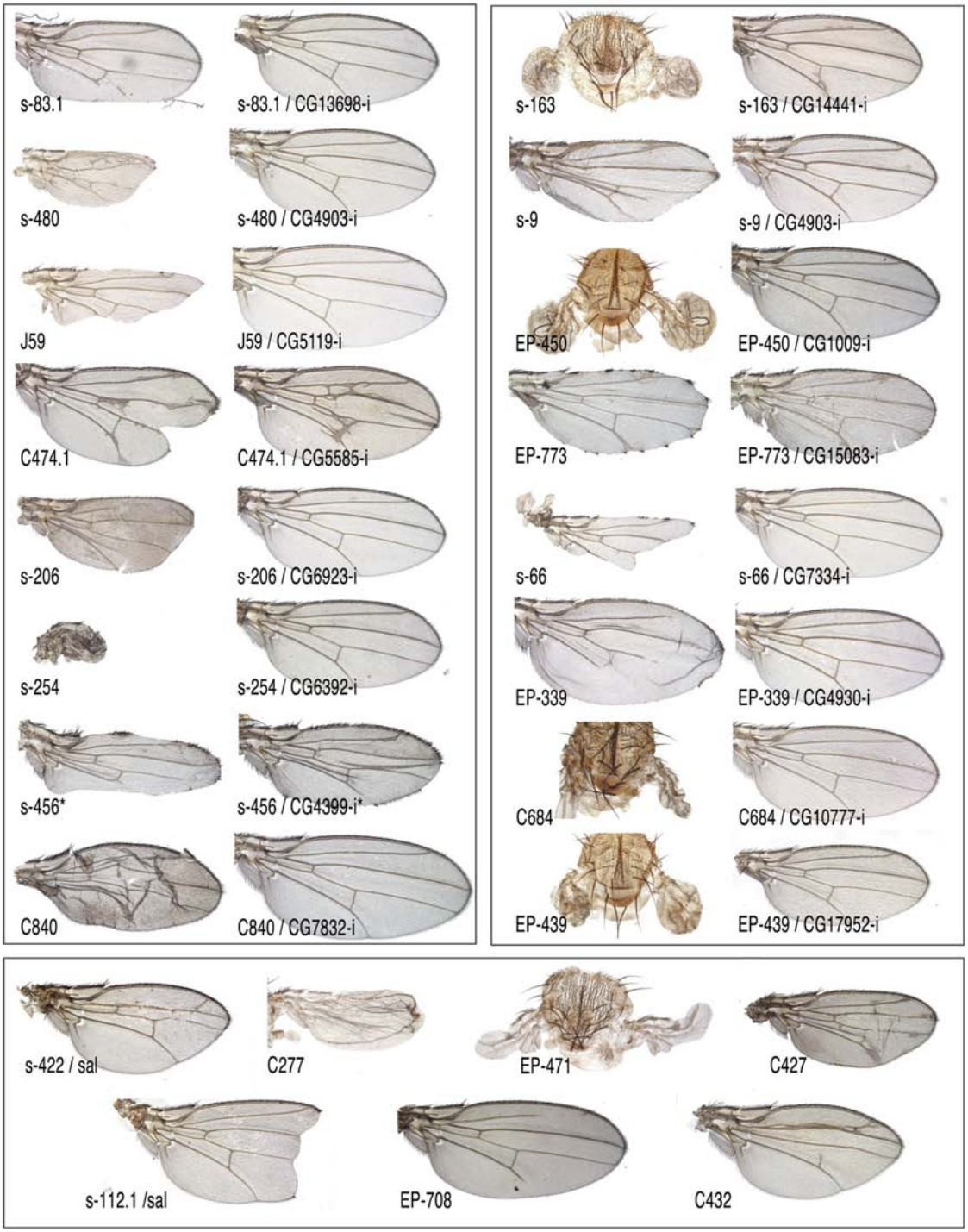

Figure S3 Rescue of *638-Gal4/P-GS* phenotype by at least the RNA interference of one gene adjacent to the insertion site. Each pair of pictures illustrate representative wings of *638-Gal4/P-GS* combinations (first and third columns) and *638-Gal4/P-GS; UAS-RNAi/+* (second and fourth columns) genotypes. The bottom box shows wings of *638-Gal4/P-GS* or sal^{EPv}*-Gal4/P-GS* (sal) genotype in which the RNAi of at least one candidate gene rescued the *638-Gal4/P-GS* phenotype. In each picture is indicated the corresponding P-GS and UAS-RNAi lines. All cases correspond to P-GS insertions in which the *638-Gal4/P-GS; UAS-RNAi/+* combination was made only for one candidate gene that rescued the *638-Gal4/P-GS* phenotype.

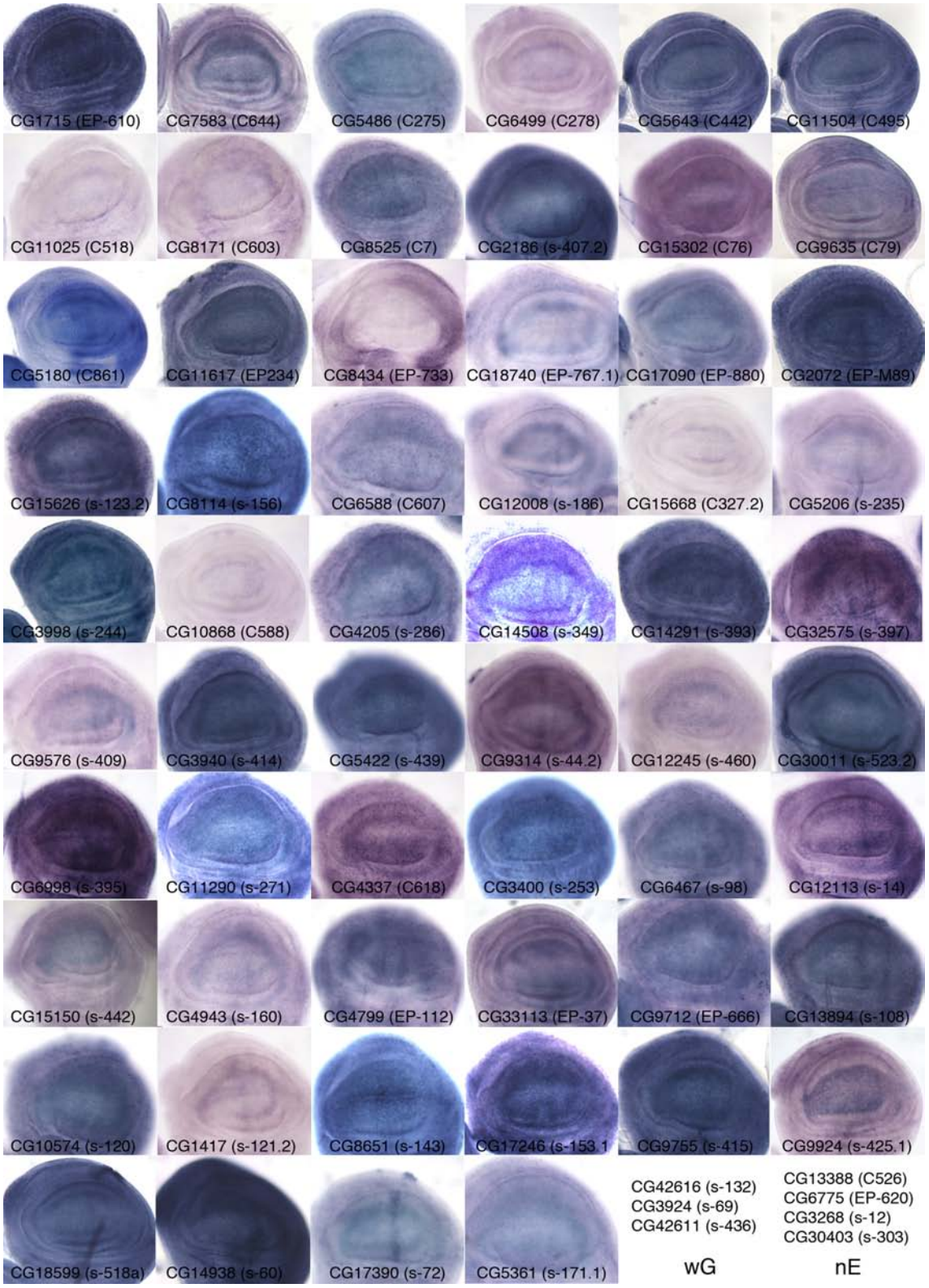

Figure S4 Expression pattern of the genes unambiguously identified as causing the over-expression phenotype of *638-Gal4/P-GS* combinations. Each picture shows the wing blade and wing hinge region of a late third instar wing disc hybridized with a RNA probe for the gene indicated at the bottom. The last two right-bottom panels indicate genes with weak and generalised expression (wG) or with no expression detected (nE).

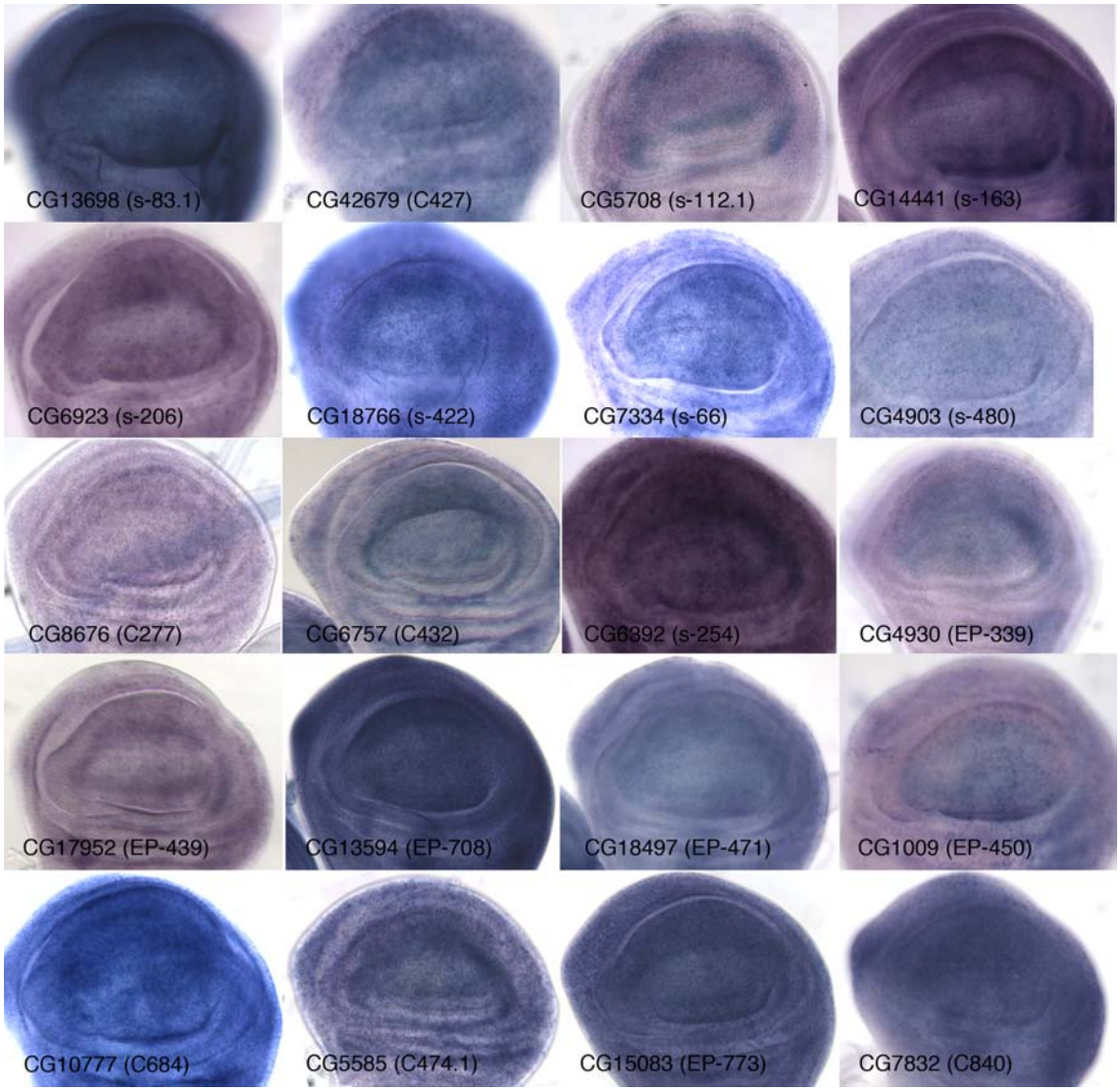

Figure S5 Expression pattern of the genes likely causing the over-expression phenotype of *638-Gal4/P-GS* combinations. Each picture shows the wing blade and wing hinge region of a late third instar wing disc hybridized with a RNA probe for the gene indicated at the bottom. In these cases not all candidate genes were tested.

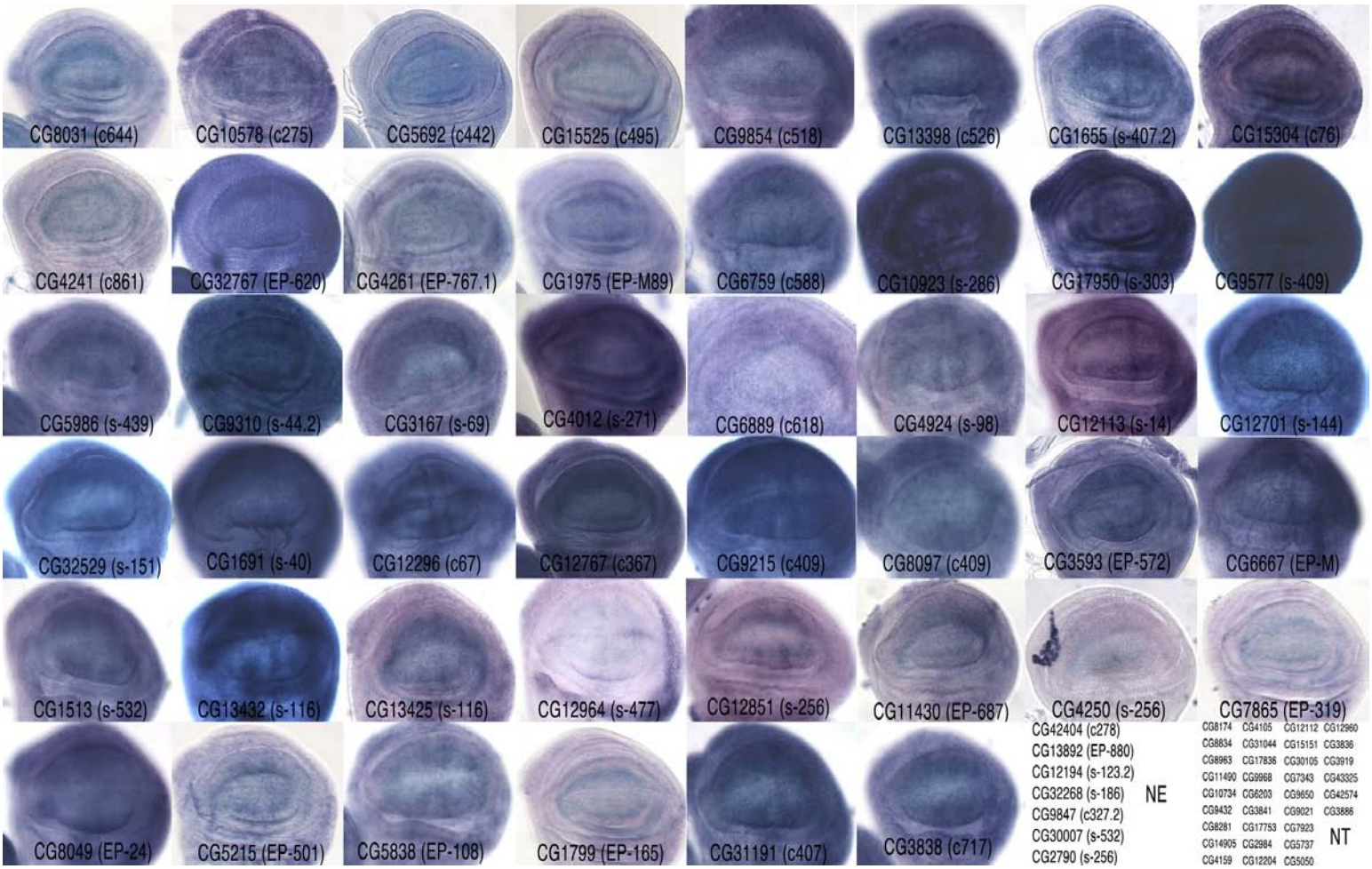

Figure S6 Expression pattern of the genes neighbor to P-GS insertions whose RNAi did not rescue the over-expression phenotype of *638-Gal4/P-GS* combinations. Each picture shows the wing blade and wing hinge region of a late third instar wing disc hybridized with a RNA probe for the gene indicated at the bottom. Last two right-bottom panels indicate genes not expressed (NE) and those not tested (NT).

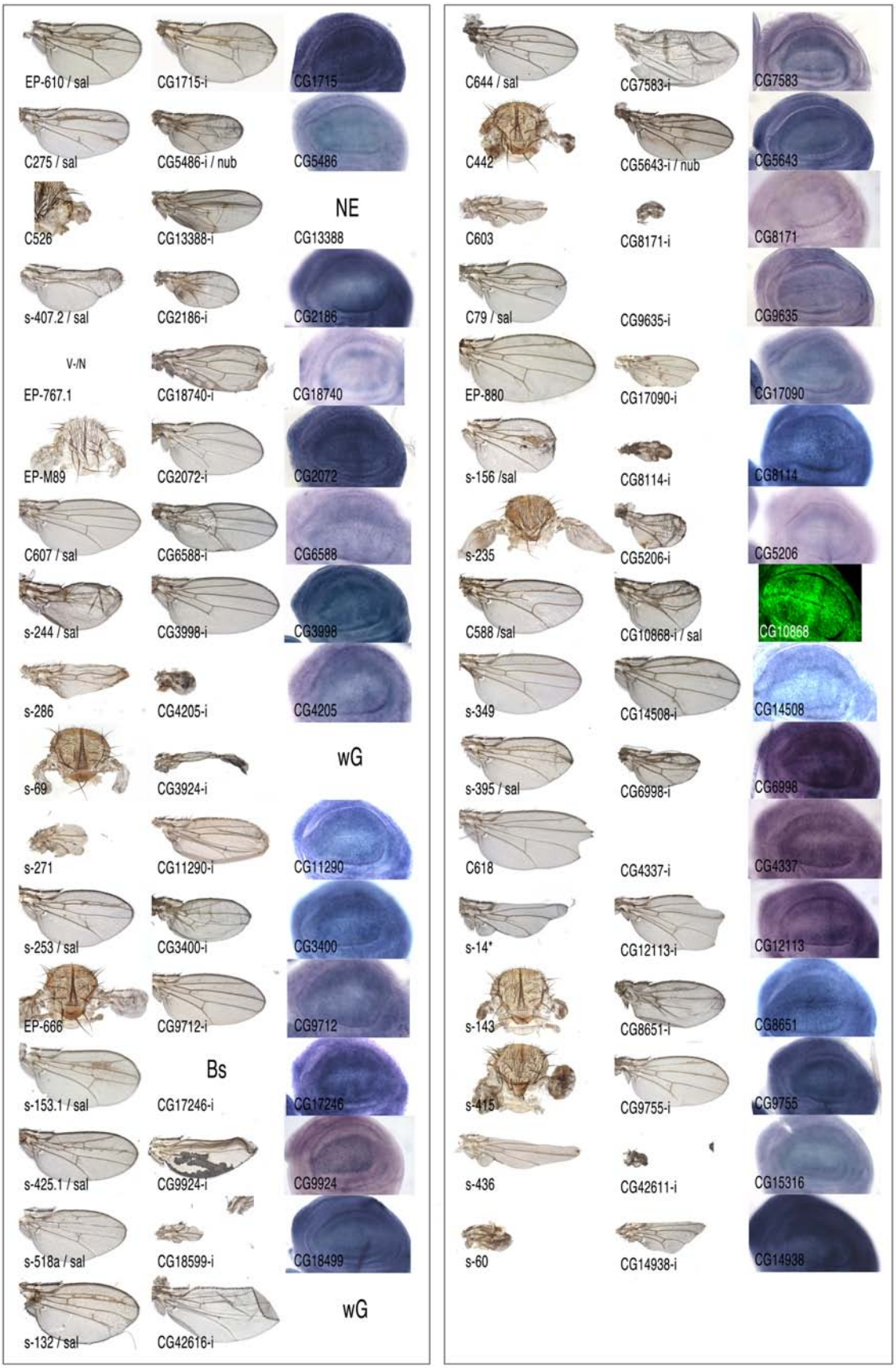

Figure S7 Gain- and loss-of-function phenotypes and expression pattern of genes causing the over-expression phenotype of *638-Gal4/P-GS* combinations. Each column of pictures shows representative wings of *638-Gal4/P-GS* or *sal^{EPv}-Gal4/P-GS* (sal) combinations (first column in each box) and *638-Gal4/UAS-RNAi* or *nub-Gal4/UAS-RNAi* combinations (nub; second column in each box). In each picture is indicated the corresponding P-GS insertion and UAS-RNAi lines. The third column in each box show the expression pattern in the wing blade and wing hinge of the corresponding genes. The expression of *CG10868* corresponds to a protein-trap insertion in the gene. The rest correspond to late third instar wing disc hybridized with a RNA probe for the gene indicated. NE: no expression detected, wG: weak and generalised expression, Bs: blister phenotype.

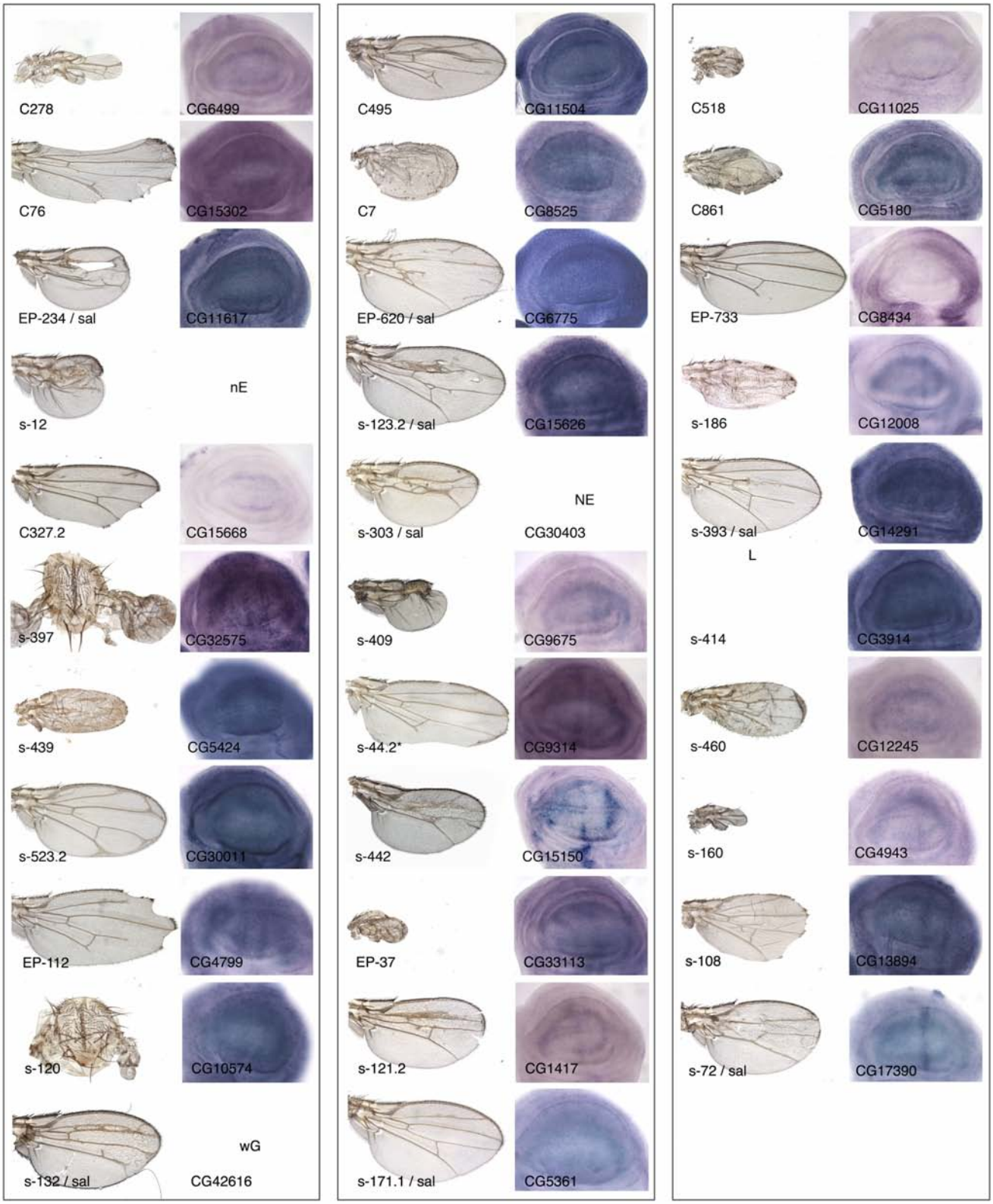

Figure S8 Gain-of-function phenotypes and expression pattern of genes causing the over-expression phenotype of *638-Gal4/P-GS* combinations. Each box shows representative wings of *638-Gal4/P-GS* or *sal*^{EPv}*-Gal4/P-GS* (sal) combinations (first column in each box) and the expression pattern in the wing blade and wing hinge of the corresponding gene (second column in each box). In these cases, the expression of the RNAi was able to rescue the over-expression phenotype, but did not caused a loss-of-function phenotype on their own. nE: no expression detected, wG: weak and generalised expression, L: lethal combination.

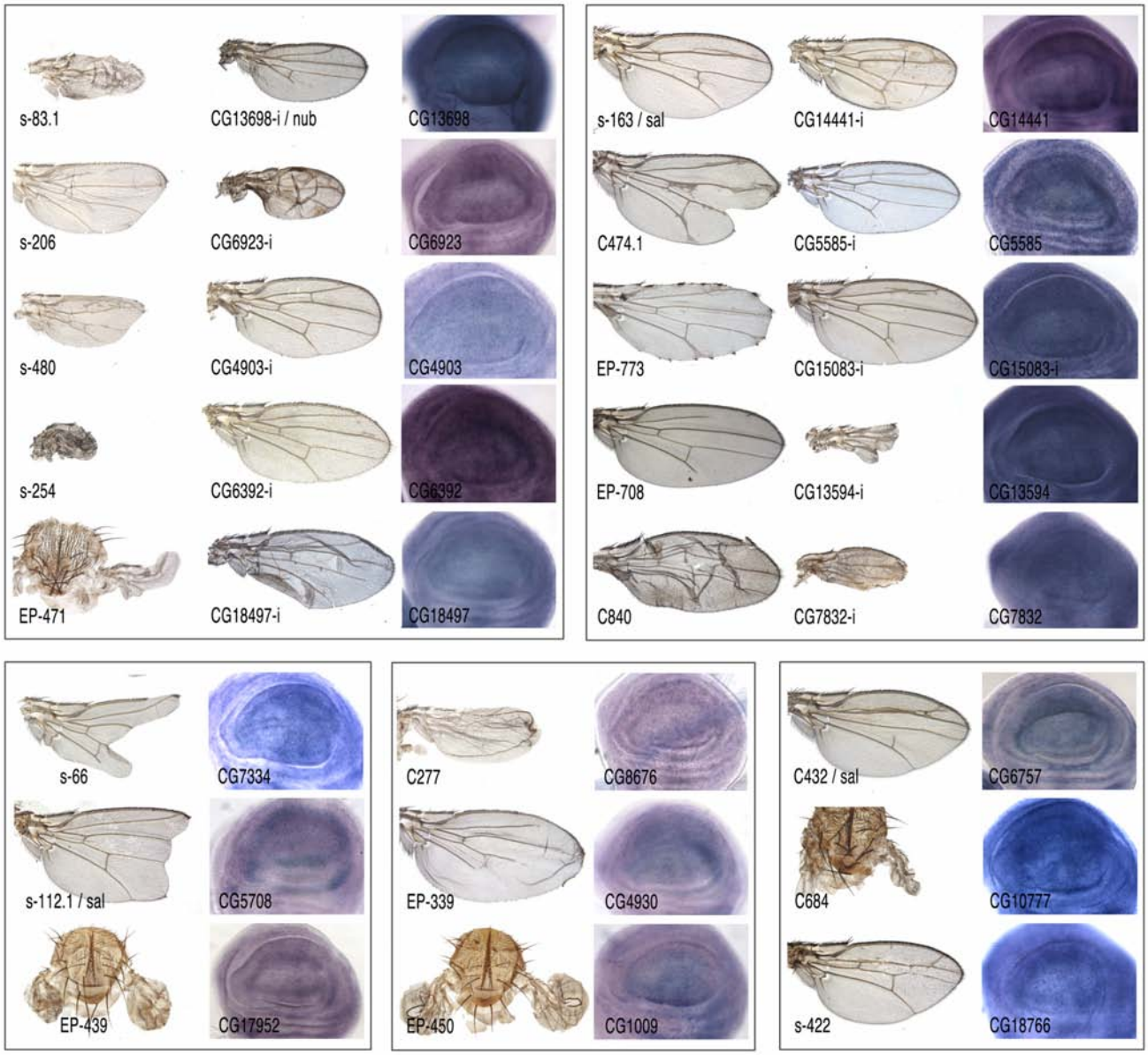

Figure S9 Gain- and loss-of-function phenotypes and expression pattern of genes likely causing the over-expression phenotype of *638-Gal4/P-GS* combinations. The top two boxes correspond to genes whose RNAi rescue the phenotype of *638-Gal4/P-GS* combination and also cause a loss-of-function phenotype in *638-Gal4/UAS-RNAi* combinations. The bottom three boxes correspond to genes whose RNAi rescue the phenotype of *638-Gal4/P-GS* combination, but did not cause a loss-of-function phenotype in *638-Gal4/UAS-RNAi* combinations. The expression pattern of each candidate gene are shown to the right in each box. In each picture is indicated the corresponding P-GS insertion and UAS-RNAi lines.

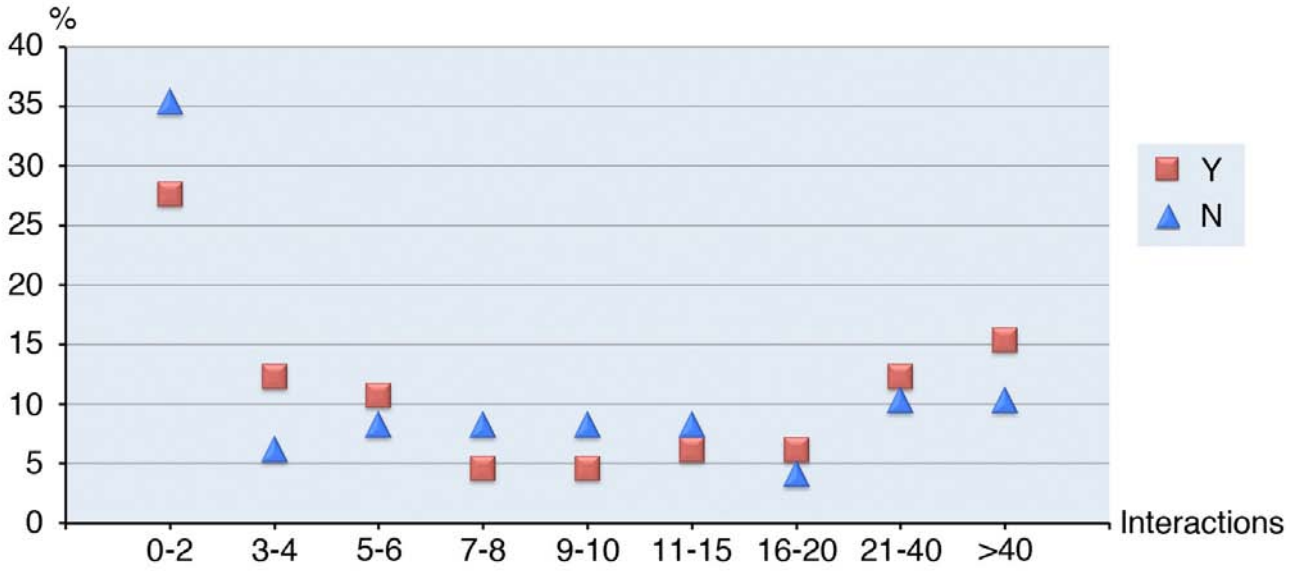

Figure S10 Number of molecular interactions described for genes that cause (Y, red squares) or fail to cause (N; blue triangles) an over-expression phenotype.

Tables S1-S3

Tables S1-S3 are available for download as Excel files at

http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.112.143537/-/DC1.

Table S1 UAS-RNAi used in this work indicating the strain (ID), name of the gene (Gene), corresponding P-GS insertion (P-GS) and chromosome in which the UAS-RNAi is inserted (Cro).

Table S2 Name of EST clones used to generate RNA probes for in situ hybridization. All cases in which the probe was synthetize from a PCR-amplified fragment are indicated as "PCR". The blue shadowing indicates cases for which we did not attempt the in situ hybridization.

Table S3 P-GS lines (Line) inserted in the proximity of known genes causing an over-expression phenotype (indicated in bold) similar to the corresponding UAS lines in combination with Gal4 drivers. Abbreviations: no phenotype (wt), Notched wings (N), extra-veins (V+), loss of veins (V-), effects on wing size and vein pattern (S-P), Blistered wings (Bs), Folded wings (F), wing absence (nW), cell differentiation phenotype (CD), lethal combination (L), wing to haltere transformation (H).