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A Gain-of-Function Suppressor Screen for Genes Involved in Dorsal–Ventral Boundary Formation in the Drosophila Wing

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ABSTRACT

The Drosophila wing primordium is subdivided into a dorsal (D) and a ventral (V) compartment by the activity of the LIM-homeodomain protein Apterous in D cells. Cell interactions between D and V cells induce the activation of Notch at the DV boundary. Notch is required for the maintenance of the compartment boundary and the growth of the wing primordium. *Beadex,* a gain-of-function allele of *dLMO,* results in increased levels of dLMO protein, which interferes with the activity of Apterous and results in defects in DV axis formation. We performed a gain-of-function enhancer-promoter (EP) screen to search for suppressors of *Beadex* when overexpressed in D cells. We identified 53 lines corresponding to 35 genes. Loci encoding for micro-RNAs and proteins involved in chromatin organization, transcriptional control, and vesicle trafficking were characterized in the context of *dLMO* activity and DV boundary formation. Our results indicate that a gain-of-function genetic screen in a sensitized background, as opposed to classical loss-of-function-based screenings, is a very efficient way to identify redundant genes involved in a developmental process.

In multicellular organisms, initially homogenous sheets of cells are often subdivided into adjacent cell populations by the activity of certain transcription factors (reviewed in Irvine and Rauskolb 2001). In many cases, cell interactions between these populations lead to the restricted expression of signaling molecules at their boundaries, which organize growth and/or the pattern of nearby cells. The stability of these boundaries frequently relies on the acquisition of differential cell affinities between adjacent populations. When these boundaries behave as lineage restriction boundaries, these populations are called compartments (García-Bellido *et al.* 1973). The Drosophila wing primordium, a monolayered epithelium that gives rise to the adult wing and part of the thorax, is subdivided into an anterior and a posterior compartment by the activity of the homeodomain transcription factors Engrailed and Invected in posterior cells (García-Bellido and Santamaria 1972; Lawrence and Morata 1976; Tabata *et al.* 1995; Zecca *et al.* 1995). During larval development, the wing primordium suffers a secondary compartment subdivision. The activity of the LIM-homeodomain transcription factor Apterous (Ap) is responsible for this later subdivision into a dorsal (D) and a ventral (V) compartment (Diaz-Benjumea and Cohen 1993).

Ap has three functions in wing development. It is responsible for the establishment of the Notch-dependent signaling center, the generation of a lineage restriction at the DV boundary, and the acquisition of a dorsal identity during cell differentiation. Ap exerts these functions through three classes of target genes. The complementary expression of Serrate and Delta, two ligands of the receptor Notch, to D and V cells, respectively, initiates a cascade of short-range cell interactions that lead to the activation of Notch at the DV boundary (Figure 1B). Dorsally expressed Serrate and ventrally expressed Delta activate Notch symmetrically in cells on both sides of the DV compartment boundary (Diaz-Benjumea and Cohen 1993; de Celis *et al.* 1996b; Doherty *et al.* 1996). Expression of the glycosyltransferase Fringe in D cells makes them more sensitive to Delta and less sensitive to Serrate (Bruckner *et al.* 2000; Moloney 2000; Munro and Freeman 2000), thus polarizing Notch activation toward the DV boundary. Notch activation induces Wingless (Wg) expression in cells along this boundary. The combined activity of Notch and Wg organizes the growth and patterning of the whole wing primordium (Giraldez and Cohen 2003). The transmembrane proteins Capricious (Caps) and Tartan (Trn) belong to the second class of Ap target genes that contribute to the generation of an affinity difference between D and V cells (Milán *et al.* 2001a). Finally, the activity of the

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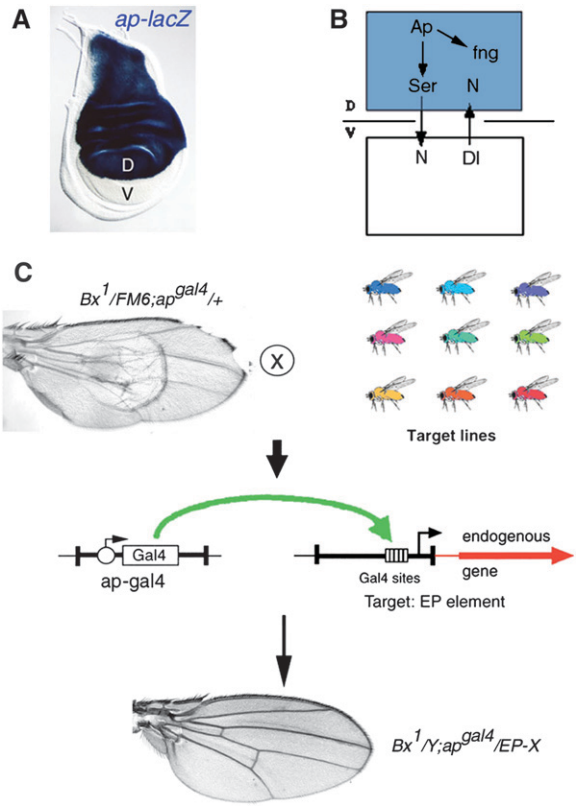

FIGURE 1.—Design of the genetic screen for suppressors of the *Beadex* wing phenotype. The wing primordium is subdivided into a dorsal (D) and a ventral (V) compartment by the restricted expression and activity of Apterous (Ap) in D cells. (A) *ap-lacZ* expression in a third instar wing disc visualized by histochemical staining for β-gal activity. (B) Early in development, Serrate (Ser) signals to V cells to activate Notch (N). Likewise, Delta (Dl) signals to D cells to activate Notch modified by Fringe (Fng) along the DV boundary. (C) *Beadex*^{1}*/FM6; apterous*^{Gal4}*/CyO* flies, which have a strong loss of wing-margin phenotype, were crossed with a large number of independent EP-containing lines. Gal4 expressed in D cells should bind to Gal4 binding sites within the target element enhancer and activate an adjacent endogenous gene X. Those lines that rescued the wing-margin phenotype were selected.

homeodomain protein Msh, another Ap target, confers D identity (MILÁN *et al.* 2001b).

The activity of Ap must be tightly regulated during development to allow the dynamic change in the expression pattern of Serrate and Delta. Later in development, these proteins are restricted to the presumptive vein tissue to help define the width of the adult longitudinal wing veins (DE CELIS *et al.* 1997). Ap activity depends on the formation of a higher order complex, in which two molecules of Ap are bridged by a dimer of its cofactor, the LIM-domain binding protein dLDB/Chip (FERNANDEZ-FUNEZ *et al.* 1998; MILÁN and COHEN 1999; VAN MEYEL *et al.* 1999). The level of Ap activity is regulated during wing development by expression of another LIM-domain protein, dLMO (MILÁN *et al.* 1998; SHORESH *et al.* 1998; ZENG *et al.* 1998). dLMO competes with Ap for binding to its cofactor Chip/dLDB, contributes to reducing the activity of Ap, and facilitates the transition in the expression pattern of Serrate and Delta, which, late in development, become symmetrically expressed along the wing veins in both D and V compartments (MILÁN and COHEN 2000).

To further our understanding of the process of boundary formation in the Drosophila wing, we performed a gain-of-function suppressor screen. This screen is based on the capacity of genes to bypass the requirement of Ap protein activity in DV boundary formation when they are overexpressed in the domain of *ap*. Here we identify, characterize, and discuss four classes of genes in the context of DV boundary formation or dLMO activity: chromatin organization genes, transcription factors, micro-RNAs, and proteins involved in vesicle trafficking and membrane fusion.

MATERIALS AND METHODS

Drosophila strains: *Bx*^{1}, *ap*^{gal4}, and *ap-lacZ* are described in MILÁN *et al.* (1998). *UAS-Caps*, *UAS-Trn*, *caps*$^{65.2}$, and *trn*$^{25/4}$ are described in MILÁN *et al.* (2001a). *Ax*^{M1} is described in PEREZ *et al.* (2005). *lilli*^{4u5} and *lilli*^{632} are described in WITTWER *et al.* (2001). *UAS-p35* is described in HAY *et al.* (1995). *UAS-mtv* and *mtv*^{6} are described in FUNAKOSHI *et al.* (2001). Other stocks are described in FlyBase. The following Drosophila genotypes were used to generate loss-of-function clones:

hs-FLP; lilli^{4u5} *FRT40A/Ubi-GFP FRT40A*
hs-FLP; cbt^{E1} *FRT40A/Ubi-GFP FRT40A*
hs-FLP; cbt^{E28} *FRT40A/Ubi-GFP FRT40A*
hs-FLP; nmd^{K10909} *FRT40A/Ubi-GFP FRT40A*
hs-FLP; FRT42D mtv^{6}*/FRT42D Ubi-GFP*
hs-FLP; FRT42D l(3)04708/FRT42D Ubi-GFP
hs-FLP; nuf^{KG02305} *FRT80/Ubi-GFP FRT80*
hs-FLP; draper^{d5} *FRT80/Ubi-GFP FRT80*
hs-FLP; draper^{d19} *FRT80/Ubi-GFP FRT80*
hs-FLP; FRT82 tara^{1}*/FRT82 Ubi-GFP.*

Larvae were heat-shocked for 1 hr at 37° and dissected 60 hr later.

Crossing scheme: In a cross, four virgins *of a Bx*^{1}*/FM6; ap*^{gal4}*/CyO* stock were mated with 2–3 males of 4200 independent *w; EP (white+)* insertions (RORTH *et al.* 1998), generated by the groups of S. Cohen, A. Ephrussi, M. Mlodzik, and P. Rorth (EMBL, Heidelberg, Germany) and kindly maintained by Günter Brönner in Göttingen (Germany). At least 10 *Bx*^{1}*/Y; ap*^{gal4}*/EP* males per cross were scored for their wing phenotype. Candidate enhancer-promoter (EP) lines were tested with the *Bx*^{1} stock for their capacity to rescue the wing phenotype in a Gal4-independent manner. Candidate EP lines were also tested for their capacity to cause a gain-of-function phenotype when overexpressed with the *ptc-gal4*, *en-gal4*, and *ap-gal4* wing drivers. Wings were mounted in Faure's medium.

Molecular characterization of EP lines: To identify the genes isolated by their gain-of-function capacity to suppress the *Beadex*^{1} phenotype, flanking DNA was isolated by plasmid rescue and the genomic region immediately downstream of the EP element promoter (at the 3′ end of the *P* element) was sequenced. Flanking DNA was also isolated by inverse PCR to verify the presence of only one EP line per stock, and the genomic region immediately downstream of the EP element promoter was sequenced to confirm the plasmid rescue results. Details on the protocols followed can be found at the

Berkeley Drosophila Genome Project Web site (http://www.fruitfly.org/).

Antibodies and constructs: Monoclonal antibodies against Wingless (Wg) and Cut are described in the Developmental Hybridoma Bank. Antibody against dLMO is described in Milán *et al.* (1998) and was kindly provided by S. M. Cohen. Other antibodies are commercially available. *In situ* hybridization was carried out as in Milán *et al.* (1996). The dLMO 3′-UTR was amplified by PCR from genomic DNA and cloned into tubulin-enhanced green fluorescence protein (EGFP) as described in Brennecke *et al.* (2003). The *miR-14* hairpin was cloned downstream of dsRed2 in pUAST as described in Brennecke *et al.* (2003).

RESULTS AND DISCUSSION

Several ways to rescue the *Beadex*^{1} wing phenotype: *Beadex*^{1} (*Bx*^{1}) is a gain-of-function allele of *dLMO* that results in higher levels of *dLMO* mRNA in the developing wing imaginal disc (Milán *et al.* 1998; Shoresh *et al.* 1998; Zeng *et al.* 1998). dLMO protein competes with Ap for binding to its cofactor Chip. Consequently, the activity of the Ap protein is reduced in a *Bx*^{1} background, its target genes are not expressed at appropriate levels, and the activation of Notch at the DV boundary and the formation of the adult wing margin are compromised (Figure 2B). Activation of the Notch signaling pathway by means of a gain-of-function allele of *Notch* (*Abruptex*, de Celis and García-Bellido 1994) or reduced levels of *Hairless*, an antagonist of Notch signaling activity (Bang *et al.* 1995), rescued the *Bx*^{1} phenotype (Figure 2, I and J), indicating that the wing-margin defects of *Bx*^{1} wings are a direct consequence of reduced levels of Notch.

Overexpression of *ap* in its own expression domain (using the *ap*^{gal4} driver) rescues the *Bx*^{1} phenotype (in *Bx*^{1}*/Y; ap*^{gal4}*/UAS-ap* flies), and reduced levels of *ap* or *Chip* enhance the *Bx*^{1} phenotype (Milán *et al.* 1998, 2004). Two classes of Ap target genes contribute to the formation of the DV boundary. *fringe* and *Serrate* are directly involved in the activation of Notch at the DV boundary (Irvine and Wieschaus 1994; Diaz-Benjumea and Cohen 1995). As expected, overexpression of either of these genes in the *ap* expression domain rescues the *Bx*^{1} phenotype, and reduced levels of these two genes enhance it (Milán *et al.* 1998, 2004). The leucine-rich repeat (LRR) transmembrane proteins Caps and Trn are involved in the generation of an affinity difference between D and V cells. Reduced levels of *caps* or *trn* have been shown to enhance the *Bx*^{1} phenotype (Milán *et al.* 2001a; compare also Figure 2D and 2F with 2B), indicating that these two genes contribute to the formation of the DV boundary. We then monitored the capacity of overexpressed Caps or Trn to rescue the wing-margin defects of *Bx*^{1} adult wings. Overexpression of either of these two proteins suppressed the wing-margin defects (Figure 2, C and E). Interestingly, they also rescued the Notch activation levels at the DV boundary, as shown by the levels of Wg protein expression (compare Figure 2G and 2H and data not shown). Taken together, these results indicate that Caps and Trn contribute to the activation of Notch at the DV boundary, probably through the generation of a stable DV affinity boundary. The *Bx*^{1} wing-margin phenotype therefore appears to be a highly sensitive background in which to search for genes involved in Ap activity and Notch activation, and as such we used it in a gain-of-function-based screen, described in the next section.

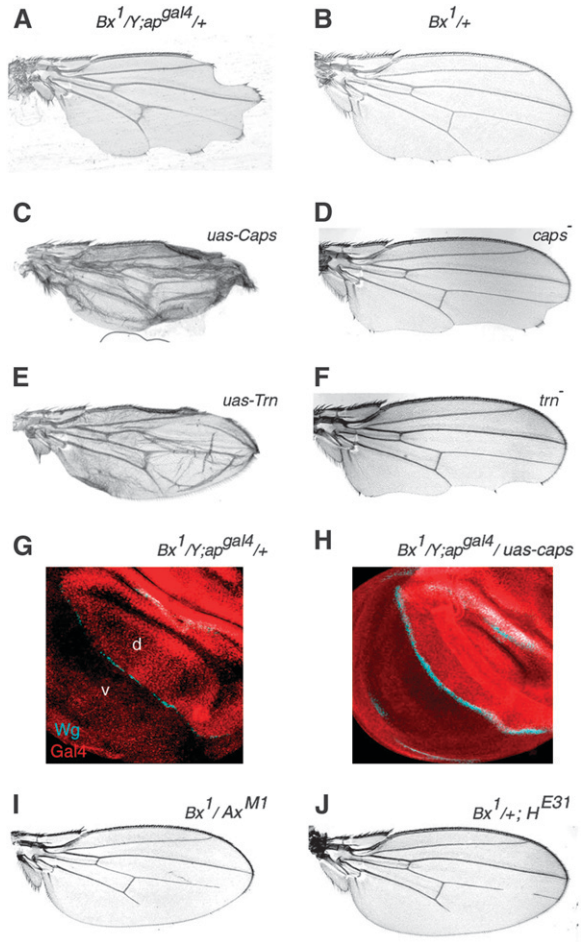

Figure 2.—Increased Notch activity or Caps/Tartan expression rescues the *Beadex* wing phenotype. (A, C, and E) Cuticle preparations of *Bx*^{1}*/Y; ap*^{gal4}*/+* (A), *Bx*^{1}*/Y; ap*^{gal4}*/uas-caps* (C) and *Bx*^{1}*/Y; ap*^{gal4}*/uas-trn* (E) adult wings. Note rescue of the wing-margin defects when Caps or Trn are expressed. Note also the blistered wing phenotype in C and E, probably due to defects in cell apposition between D and V wing surfaces in the presence of high levels of Caps or Trn expression. (B, D, and F) Cuticle preparations of *Bx*^{1}*/+* (B), *Bx*^{1}*/+, caps*$^{65.2}$*/+* (D), and *Bx*^{1}*/+, trn*$^{25/4}$*/+* (F) adult wings. Note enhancement of the wing-margin defects when either *caps* or *trn* are removed. (G and H) *Bx*^{1}*/Y; ap*^{gal4}*/+* (G) and *Bx*^{1}*/Y; ap*^{gal4}*/ uas-caps* (H) wing discs labeled to visualize Gal4 (red) and Wingless (Wg, blue) protein expression. Dorsal (d) and ventral (v) compartments are marked. (I and J) Cuticle preparations of *Bx*^{1}*/Ax*^{M1} (I) and *Bx*^{1}*/+; H*$^{E-31}$*/+* (J) adult wings. Note rescue of the wing-margin defects when compared to the *Bx*^{1}*/+* wing shown in B.

The suppression screen: A loss-of-function approach has been widely used in traditional genetic screenings but has several limitations. Redundancy between genes that have overlapping functions might partially or completely mask gene function, and an early phenotype caused by a mutation might prevent the detection of later phenotypes. The gain-of-function approach bypasses these two limitations and allows the examination of the misexpression of phenotypes in the biological context of choice. This approach has been shown to be a powerful tool in identifying genes involved in development (Rorth *et al.* 1998). Here we performed an EP-mediated overexpression screening, in which 4200 randomly inserted and independently generated EP insertions, which allow the misexpression of genes that lie immediately downstream of the point of insertion, were driven in the developing wing by a dorsal-specific Gal4 driver (ap^{gal4}) in a Bx^1 sensitized background (Figure 1C). Bx^1/Y; $ap^{gal4}/+$ male flies showed a strong scalloping phenotype (loss of wing-margin structures) as a result of reduced levels of Ap activity (note that ap^{gal4} is a loss-of-function allele of *ap*). Those EP insertions, which have the capacity to rescue the wing-margin phenotypes of these flies, drive candidate genes involved in DV boundary formation, either as positive regulators of Ap activity or as downstream genes involved indirectly or directly in the activation of Notch at the boundary. Fifty-three EP lines partially or totally rescued the scalloping phenotype. Most of these lines (47/53) showed a complete rescue. Table 1 describes the relationship of the EP insertion with known and predicted genes. These EP insertions correspond to 35 genes. The finding of EP lines that drive the expression of *fringe* (EPs *3-511*, *3-581*, *3-612*, and *3-934*), *ap* (EP *3-1583*) or *osa* (EPs *3-473*, *3-619*, *3-900*, *3-941*, *3-1074*, *3-1386*, and *3-1591*), a member of the Brahma chromatin-remodeling complex that binds Chip and modulates the expression of Ap target genes (Collins *et al.* 1999; Heitzler *et al.* 2003; Milán *et al.* 2004), confirms the success of our screen.

Most EPs were located in the correct location (5′ end) of the nearby genes that would generate Gal4-driven sense-strand full-length mRNAs. This result was also observed in other published EP screens (Rorth *et al.* 1998; Toba *et al.* 1999). A low percentage of EP lines were located in intronic or exonic sequences. Partial Gal4-driven sense-strand functional mRNAs might be generated in the former case. Note three EP lines were inserted in the first intron of *osa* (Table 1) and the rescue capacity of this gene has already been demonstrated by means of an *UAS-osa* transgenic construct (Milán *et al.* 2004). When inserted in exonic sequences, mutant alleles of the candidate genes might be produced. Note those EP lines inserted in *skuld*, *Draper*, and *nonmitochondrial derivative* (*nmd*) were located in exonic sequences (Table 1). The loss of function of these genes, and not its Gal4-mediated overexpression, was responsible for the Bx^1 rescue, as demonstrated by the dominant genetic interaction between Bx^1 and loss-of-function alleles of these genes (see below). Surprisingly, some EPs (*e.g.*, *3-1583* driving the expression of *ap*; *3-612* and *3-934* driving the expression of *fringe*) were located in the opposite direction, suggesting that the Gal4 binding sites were duplicated during transposition, or alternatively, that the EP drives the expression of 3′-located genes. Interestingly, a similar case was found in UAS transgenic constructs, in which a genomic fragment containing a micro-RNA in antisense orientation relative to the pUAST vector is transcribed in a Gal4-driven sense strand, probably as a result of the capacity of the Gal4-dependent transcription to increase the activity of the endogenous promoter (Brennecke *et al.* 2003).

Two different methods were used to validate the candidate genes whose overexpression was able to rescue the *Beadex* wing-margin phenotype. First, available UAS transgenes, or alternatively, available EP insertions located at the 5′ end of the candidate genes were tested for the ability to rescue, in a Gal4-dependent manner, the *Beadex* wing-margin phenotype. We found that in the cases analyzed (32/53 EPs) this was the case (Table 1). In some cases, UAS transgenes or other EP insertions were not available. We then carried out *in situ* hybridization with RNA-labeled probes of the genes downstream of the EP insertions. We found that in the cases analyzed (4) the genes are overexpressed in a Gal4-dependent manner (Figure 3). Our experience with EP insertions somehow indicates, however, that this might not be the best way for the validation, since many genes in the neighborhood can be upregulated upon Gal4 transcriptional activation. This is the reason we have not performed *in situ* hybridization for more EP insertions.

Since we performed a misexpression screening, several of the candidate genes able to rescue the Bx^1 phenotype may not be required for wing development and DV boundary formation in wild-type flies. To test this, we searched FlyBase for mutants in the candidate genes or deficiencies covering them, and checked whether there were any dominant interactions with Bx^1. $Bx^1/+$ females have a mild scalloping in the posterior compartment of the wing (Figure 2B). Males carrying a mutation in the candidate gene or a deficiency covering it were crossed with Bx^1 females, and the wing phenotype of the female progeny was tested for enhancement. The vast majority of the candidate genes showed a dominant interaction with $Beadex^1$ (Table 1; 28/34 genes tested; Figure 4). Some of the genes, when removed, rescued the Bx^1 phenotype (*nmd*, *skuld/pap*, and *Draper*, Figure 4R and data not shown), indicating that the suppression is due to the loss of function of the candidate gene.

Finally, we analyzed the capacity of the genes identified to cause a gain-of-function phenotype in the wing, in an otherwise wild-type background. For this purpose,

TABLE 1

List of suppressor lines

EPG	Closest gene	Percentage and type of rescue	Verification	Genetic interaction	Other phenotypes	3′ genomic sequence
2-18	*Sly*	Complete/GOF		*Df(2L)C144*	Loss of macrochaete	CTAACTCGTCGCCGTCGGCGACT[a]
2-27	*lilliputian*	Complete/GOF	***UAS-lilli***	***lilli*$^{l(2)00632}$**	Loss of macro-/microchaete	TGTTATTTGAAAACGTAAGTTCA
2-235	*miR-14*	Complete/GOF	***UAS-miR-14***	No interaction with *miR14*$^{\Delta 1}$		tggttcgcgtttttccgttctgc
2-356	*miR-14*	Complete/GOF	***UAS-miR-14***	No interaction with *miR14*$^{\Delta 1}$		gataaaacataaaacgtgtaataa
2-402	*miR-14*	Complete/GOF	***UAS-miR-14***	No interaction with *miR14*$^{\Delta 1}$		gataaaacataaaacgtgtaataa
2-408	*cabut*	Complete/GOF	*EP2237*	No interaction with *cbt*^{E28}		gttgagctcccatactctcctgctt
2-430	*CG15095*	Complete/GOF				ccgcacacgacgaaggcacccgat
2-446	*nmd /Msp*	Complete/LOF		***nmd*^{cy4}**		GCCAACGTATTTGCTCTAAATAGA[b]
2-473	*mtv/sbb/bks*	Complete/GOF	***UAS-mtv***	***mtv*^{6}**	Loss of macrochaete	attgcgctctccctctctgtctc
2-760	*CG5890*	Complete/GOF		*Df(3R)Esp*^{l3}	Loss of macrochaete	TGGGGTTTGAATTTAAGTTAT
2-814	*miR-14*	Complete/GOF	***UAS-miR-14***	No interaction with *miR14*$^{\Delta 1}$	Loss of macrochaete	gttctgcgtttcattcgtcgtcgaa
2-865	*CG8405*	Partial/GOF		*Df(2R)Jp1*	Loss of macrochaete	cgctaaacgaaacaaagctcaattc
2-867	*Draper*	Complete/LOF		***EP522*, *drpr*$^{\Delta 5}$, *drpr*$^{\Delta 119}$**	Loss of macrochaete/Ectopic vein 2	GGTTTGAATGGCCAAAGTTTCNT[b]
2-1069	*chameau*	Complete/GOF		*Df(2L)spd*	Loss of macrochaete	CACCAACCTTCCACGCCCCTGGCAT[a,c]
2-1080	*CG4477*	Complete/GOF		*Df(3L)29A6*	Loss of micro-/macrochaete, Small D compartment	GGCCACGAAAGTTAGCAAGACTTAA
2-1142	*miR-14*	Complete/GOF	***UAS-miR-14***	No interaction with *miR14*$^{\Delta 1}$	Loss of macrochaete	ggcgcacatataaaacgtttatata
2-1279	*schnurri*	Complete/GOF	*EP2359*	***shn*^{1}, *shn*^{04738}**	Loss of macrochaete, Ectopic vein 2	CCACAGACGCAACAGTCTCGCTCAC
2-1583	*apterous*	Complete/GOF	***UAS-ap***	***ap*^{ugo35}**		Ccgggcaggcgattac[a]
2-1743	*schnurri*	Complete/GOF	*EP2359*	***shn*^{1}, *shn*^{04738}**	Loss of macrochaete, Small D compartment	TCTGAATGAATTTCGCTGGTCGCCT
3-26	*miR-282-RA*	Partial/GOF	*EP3041*	***Df(3L)HR370*, *Df(3L)HR232*, *NP0245***	Ectopic vein 2	agtgctgccccaaaagaacgatgct
3-28	*CG11399*	Complete/GOF	***GS11380/in situ* hybridization**	***EY11352***	dupl macrochaete, Large D compartment	GGCTCCGAGTTTCGTCG
3-364	*miR-279a*	Complete/GOF	*EP3069/ EP3626*	***Df(3R)3450*, *l(3)04708***	Small D compartment	tcaattaccaggcttaccagggt
3-378	*HLH-gamma*	Partial/GOF		*Df(3R)Esp*^{l3}	Loss of micro-/macrochaete	gatgttgcacacgtgcaacgccgagggaat
3-473	*osa*	Complete/GOF	***UAS-osa***	***osa*^{eld380}**		CTCTCAAGACAACGGCAGCGGCCAAAAG[c]
3-488	*Mcpc*	Complete/GOF		***Mcpc*^{564}**	Small D compartment, Loss of micro-/macrochaete	ctcacggaggtgcagcgatttctca

(*continued*)

TABLE 1

(Continued)

EPG	Closest gene	Percentage and type of rescue	Verification	Genetic interaction	Other phenotypes	3′ genomic sequence
3-511	*fng*	Complete/GOF	***UAS-fng***	***fng^{80}***		Ttcggcagctactgtggcacaagct[a]
3-532	*pap/skuld*	Complete/LOF		***EP3375, skd^{l0198}, skd^{l0197}***		GACGGGCAGCAGCATTCCTCTCA[c]
3-562	*Annexin IX*	Complete/GOF	*EY08209*	***l(3)neo54***	Loss of macro	Gagacgcacagccgcttgaagt[c]
3-581	*fng*	Complete/GOF	***UAS-fng***	***fng^{80}***		tggaccagctcaaagggaatt
3-612	*fng*	Complete/GOF	***UAS-fng***	***fng^{80}***		Gcttgtgccacagtagctgcc[a]
3-619	*osa*	Complete/GOF	***UAS-osa***	***osa^{eld380}***		GTCTGTGGACGGTCGTCAATGCGTGA[b]
3-637	*pointed*	Complete/GOF	***uas-pointedP2***	No interaction with *pnt$^{\Delta 88}$*	Blisters	cgtcggccttttttcggtttgtttctttt
3-732	*PAR-5/14-3-3ε*	Complete/GOF	***EP3423/in situ* hybridization**	*Df(3R)Cha7*		acagccactgtgcagcgtcgccga
3-797	*CG1943*	Partial/GOF		*Df(3R)Antp17, Df(3R)Tpl10*		*gccagacttcttcgagtagagccg*
3-826	*CG8369*	Complete/GOF	***In situ* hybridization**	*Df(3R)p40, Df(3R)p712*	Small D compartment	Atcgtcggcatgctggcaccttnta[a]
3-853	*pointed*	Complete/GOF	***uas-pointedP2***	No interaction with *pnt$^{\Delta 88}$*		cgtcggccttttttcggtttgtttctt
3-900	*osa*	Complete/GOF	***UAS-osa***	***osa^{eld380}***		ggctcgcttcttcgtcatgc
3-934	*fng*	Complete/GOF	***UAS-fng***	***fng^{80}***		Tgtgccacagtagctgc[a]
3-941	*osa*	Partial/GOF	***UAS-osa***	***osa^{eld380}***		CTCTCAAGACAACGGCAGCGGCC[c]
3-980	*CG8149*	Partial/GOF		*Df(3R)by10*		aactgaacctttggcgattccgattcgt
3-1005	*capicua*	Complete/GOF		***cic^{D49d}***		ttggagccgtgaaacgaggagag
3-1074	*osa*	Partial/GOF	***UAS-osa***	***osa^{eld380}***		GTGCTGCCCGTTTCATTCGTTTC[c]
3-1199	*CG 14073*	Complete/GOF	***In situ* hybridization**	*Df(3L)Cat*		acccaggttatttttgtcattcat
3-1386	*osa*	Complete/GOF	***UAS-osa***	***osa^{eld380}***		GTGCGTGTATAGCCGCCAAATGTGAAA[c]
3-1575	*taranis*	Complete/GOF	*EP3463*	***tara^{1}, tara^{3881}***	Loss of macro	ttaaagtgaactgcatta
3-1591	*osa*	Complete/GOF	***UAS-osa***	***osa^{eld380}***		gtgcgtgctacccgctctaatcttgt
3-1638	*pointed*	Complete/GOF	***uas-pointedP2***	No interaction with *pnt$^{\Delta 88}$*	Small D compartment, Loss of macrochaete	cgtcggccttttttcggtttgtttc
3-1715	*nuclear fallout*	Complete/GOF		***nuf^{KG02305}***	Small D comparment	agccaatgcaaaccggaaacaac
3-1729	*miR-279a*	Complete/GOF	*EP3069/ EP3626*	**Df(3R)3450/l(3)04708**	Small D compartment	gtgtgtgcaaatgatacaaagcaga
3-1789	γ-*SNAP*	Complete/GOF		*Df(3L)XS572*	Small D compartment	ATTCGGTGCCACATTGCCAAATCCC
3-1809	*CG14709*	Complete/GOF		*Df(3R)M-Kx1*		gtgccaggttaagatcagcgctctttgtgg
3-1859	*pointed*	Complete/GOF	***uas-pointedP2***	No interaction with *pnt$^{\Delta 88}$*	Loss of macrochaete	agccgagctaaaccgatccccgattt
3-2015	*Su(Tpl)/dEll*	Complete/GOF		***Su(Tpl)^{17}, Su(Tpl)$^{S-192}$***		GTTATCACCACCTCCCTCGTTT[a,c]

GOF, gain of function; LOF, loss of function. Candidate genes validated with an UAS transgene, RNA probe (*in situ* hybridization) or genetic interaction with a mutant allele are in boldface type. Forty-one EP lines have been validated this way. Candidate genes validated with an EP insertion or genetic interaction with a deficiency are underlined. Ten EP lines have been validated this way.

[a] The EP insertion is pointing in reverse.

[b] The EP is sitting in an exon.

[c] The EP is sitting in an intron.

[d] http://flybase.org/.bin/fbsymq.html.

we used the following Gal4 drivers: *ap-gal4* (expressed in dorsal cells), *patched-gal4* (expressed along the anterior–posterior compartment boundary), and *engrailed-gal4* (expressed in the posterior compartment). Ectopic expression of *fringe* or *ap* in the V compartment, using the *patched-gal4* or *engrailed-gal4* drivers, induces ectopic wing-margin structures (Milán and Cohen 2003). When using the same Gal4 drivers, none of the lines identified, with the exception of those driving the expression of *fringe* or *ap*, caused this phenotype (data not shown). This observation indicates that the number of genes with an instructive role like *fringe*, *Serrate*, or *ap* is very low, and that the newly identified genes encode for proteins that modulate the activity of the elements or pathways involved in DV boundary formation. Many of these genes caused the loss of macro- and micro-chaetae in the adult notum (when expressed with the *ap-Gal4* driver), thereby resembling a Notch gain-of-function phenotype (Table 1, Heitzler and Simpson 1991). This suggests that the nature of the Bx^1 rescue by overexpression of these genes is through an increase in Notch activity levels, as occurred in an *Abruptex* or *Hairless* mutant background (Figure 2, I and J). Taken together, these results indicate that the designed suppression screen is efficient in identifying modulators of the pathways and elements involved in DV boundary formation.

Classes of genes: Of the 35 genes identified in the screen, 20 corresponded to genes previously characterized. Five genes [*ap*, *osa*, *fng*, *skuld/pap*, and *E(spl-γ)*] participate in Notch signaling and/or DV boundary formation in the Drosophila wing (Diaz-Benjumea and Cohen 1993; Irvine and Wieschaus 1994; de Celis *et al.* 1996a; Janody *et al.* 2003; Milán *et al.* 2004). Other genes are involved in other aspects of wing development (*e.g.*, *capicua* and *pointed* in EGF receptor signaling, *mtv* and *schnurri* in Dpp signaling) (Greider *et al.* 1995; Funakoshi *et al.* 2001; Roch *et al.* 2002) and Hedgehog signaling (Bejarano *et al.* 2007), suggesting that either different signaling pathways are closely coordinated during DV boundary formation or distinct pathways share common elements. A large group of genes with essential roles in other developmental processes were identified (*e.g.*, *PAR-5/14-3-3ε* in anterior–posterior axis formation in the oocyte, *cabut* in JNK signaling, and embryonic dorsal closure) (Benton *et al.* 2002; Muñoz-Descalzo *et al.* 2005). Many of these have not been tested for their role in DV boundary formation; however, they might also be involved in this developmental process. These genes are described in Table 2. Here we will further discuss the function of several of these genes.

Chromatin organization genes: Eukaryotic nucleosome assembly and higher-order packaging produce a general repression of gene expression. Remodeling of chromatin structure is required for gene activation. ATP-dependent protein complexes with chromatin-remodeling activity can change nucleosomal pattern and DNA packaging. In Drosophila, the *Polycomb* group of genes maintains repression of homeotic genes by inducing a repressive chromatin structure while some members of the *trithorax* group of genes suppress dominant *Polycomb* phenotypes (Kennison and Tamkun 1988). In our screen, we found two members of the *trithorax* group of genes: *taranis* (*3-1575*) and *osa* (*3-473, 3-619, 3-900, 3-941, 3-1074, 3-1386, 3-1591*), whose misexpression rescues the Bx^1 mutant phenotype (Figure 4N and Milán *et al.* 2004), and one member of the *Polycomb* group of genes: *chameau* (*2-1069*), whose, most probably, loss of function dominantly rescues the Bx^1 mutant phenotype (Figure 4E). *osa*, a *trithorax* gene, associates with the Brahma chromatin remodeling complex (Collins *et al.* 1999), binds and genetically interacts with Chip, the Ap cofactor (Heitzler *et al.* 2003), and modulates the expression of Ap target genes (Milán *et al.* 2004). *taranis*, another member of the *trithorax* group of genes, appears to be involved in integrating chromatin structure with cell-cycle regulation (Calgaro *et al.* 2002). Although *taranis* genetically interacts with *osa* and $Beadex^1$ (Figure 4T and Calgaro *et al.* 2002; Milán *et al.* 2004), clones of cells mutant for *taranis* did not affect DV boundary formation (supplemental Figure S1 at http://www.genetics.org/supplemental/), thus suggesting that the role of *taranis* in this process is redundant with another gene. *chameau*, a member of the *Polycomb* group of genes, is involved in gene silencing. The EP line *2-1069* is inserted pointing reverse in the fifth exon of *chameau* and it is thus supposed that it drives transcription of antisense strand *chameau* mRNA. Consequently, the rescued phenotype observed might be the result of decreased expression of *chameau*.

Genes involved in the control of transcription: The percentage of genes involved in transcriptional regulation, as detected in the screen, was very high, corresponding to almost half of the lines. Three genes have already been well-characterized for their role in DV boundary formation and/or Notch signaling in the Drosophila wing, including *ap* (*2-1583*, Figure 4H). *E(spl)-γ* (*3-378*, Figure 4K), a member of the *Enhancer of split* gene complex, is a downstream effector of Notch, acts as a transcriptional repressor in controlling neuronal cell fate decisions (Robey 1997), and belongs to the Hairy-related proteins with a proline basic HLH domain. *skuld/pap/TRAP240* (*3-532,* Figure 4L), a homolog *of TRAP240*, together with *kohtalo*, the *TRAP230* homolog, are the largest subunits of the Drosophila mediator complex. Proteins of this complex act as transcriptional coactivators that link specific transcription factors to RNA polymerase II and basal transcriptional machinery. *skuld* and *kohtalo* are required to maintain the difference in cell affinities between D and V cells (Janody *et al.* 2003). One might then expect that mutations in these genes would enhance, like *caps* and *tartan* mutant alleles do, the Bx^1 wing-margin phenotype (Milán *et al.* 2001a). However, this was not the case. The EP line *3-532*, which is inserted in the first

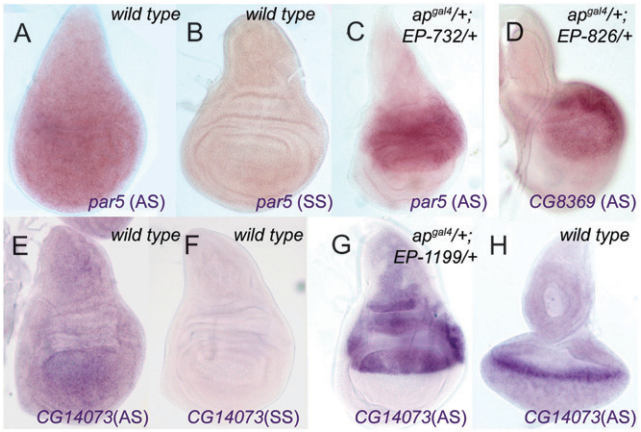

Figure 3.—*In situ* hybridization to *wild type* (A, B, E, F, and H), ap^{gal4}/+; *EP-732*/+ (C), ap^{gal4}/+; *EP-826*/+ (D), and ap^{gal4}/+; *EP-1199*/+ (G) wing (A–G) or eye-antenna (H) imaginal discs with anti-sense (AS) or sense (SS) *par 5* (A–C), *CG8369* (D), and *CG14073* (E–H) RNA probes. Note high levels of *CG14073* expression at the morphogenetic furrow of the eye-antenna imaginal disc.

intron, and loss-of-function mutations in *skuld* dominantly rescued the Bx^1 phenotype in a Gal4-independent manner (Figure 4R and data not shown), indicating that *skuld* might be involved in other aspects of wing development and not simply in controlling cell affinities.

Five genes have already been well-characterized for their role in other aspects of wing development. *capicua* (*3-1005*), a transcriptional repressor involved in wing-vein patterning (Roch *et al.* 2002), and *pointed* (*3-637*, *3-853*, *3-1638*, and *3-1859*), a gene encoding two ETS-related proteins, are two transcriptional mediators of the EGF receptor pathway in Drosophila. The restricted expression of the EGF receptor ligand vein in the most dorsal part of the early wing primordium induces the activation of the EGF receptor pathway and the expression of *ap*, thus defining the dorsal compartment (Wang *et al.* 2000; Zecca and Struhl 2002). Given that transheterozygous combinations for *pointed* loss-of-function alleles resemble the phenotypes caused by early depletion of the EGF receptor pathway (Scholz *et al.* 1993), the activity of the EGF receptor pathway might be mediated by *pointed*. The role of *capicua* in this process remains to be analyzed.

schnurri (*2-1279* and *2-1743*), a zinc finger-containing transcription factor, *master of thickveins* (*mtv*, *2-473*), a zinc finger-containing nuclear protein, and *lilliputian* (*lilli*, *2-27*), the only member of the fragile X/Burkitt's lymphoma family of transcription factors in Drosophila, are involved in various aspects of *dpp*-dependent patterning in embryonic and/or wing development (Aurora *et al.* 1995; Greider *et al.* 1995; Funakoshi *et al.* 2001; Su *et al.* 2001). Two distinct insertions driving the expression of *schnurri* were identified as suppressors of Bx^1 (Figure 4G) and an independently generated EP insertion driving *schnurri* expression (*EP2359*; Rorth *et al.* 1998) also suppressed the Bx^1 wing-margin phenotype. Overexpression of *lilli* or *mtv* in the D compartment of Bx^1 males completely rescued the wing-margin defects of the adult flies and Notch activity levels at the DV boundary, as shown by the expression of Wg protein (Figures 5, B and D, and 6A and data not shown). In these two cases, the rescue was verified with an UAS transgenic construct (Figure 5C and data not shown). Note the overexpression of *schnurri*, *lilli*, or *mtv* caused growth and wing-folding defects, indicating that these genes are involved in other developmental processes (Greider *et al.* 1995; Funakoshi *et al.* 2001; Su *et al.* 2001; Wittwer *et al.* 2001; Bejarano *et al.* 2007). In all cases, loss of one copy of the gene strongly enhanced the Bx^1 heterozygous adult wing phenotype (compare Figures 4O, 5A, and 6B with 2B). *schnurri* mutant cells have been previously shown to cause loss of wing-margin structures in the adult wing (Greider *et al.* 1995). We have analyzed the capacity of *lilli* or *mtv* mutant cells to activate the Notch pathway at the DV boundary. For this purpose, we monitored the expression of *wg* and *cut*, two target genes of Notch at the wing margin. Notch activity levels were strongly reduced in *mtv* mutant cells (Figure 5E). Consistent with this, *mtv* mutant clones induced loss of wing-margin structures in the adult (Figure 5, F and G). Loss of *lilli* caused a slight reduction in Wg expression levels (Figure 6, D–F) and did not produce any overt adult wing phenotype. Altogether, these results indicate that these three genes are directly or indirectly required for proper Notch activation at the DV boundary. Mtv protein is known to work in the same protein complex as Groucho (Bejarano *et al.* 2007), the founding member of a superfamily of transcriptional corepressors that operate in many signaling pathways, including Notch in the Drosophila wing. We would then like to speculate that Mtv exerts its function together with Groucho in the Notch pathway.

Finally, overexpression of *cabut* (*2-408*), a gene encoding a zinc finger transcription factor and involved in embryonic dorsal closure (Muñoz-Descalzo *et al.* 2005), rescued the Bx^1 phenotype (Figure 4B). It is interesting to note that loss of function mutations of *cabut* dominantly enhanced the Bx^1 phenotype and clones of cells mutant for *cabut* did not show any apparent wing phenotype (supplemental Figure S1 at http://www.genetics.org/supplemental/). These observations suggest that the role of *cabut* is redundant with another gene during wing development. *Su(Tpl)dEll*, a gene encoding an RNA polymerase II transcription elongation factor, is essential for development and strongly interacts with mutants in *Notch* and *cut* in the Drosophila wing (Eissenberg *et al.* 2002). We also verified that loss-of-function mutations of *Su(Tpl)dEll* dominantly enhanced the Bx^1 phenotype (Table 1).

***CG11399*, a suppressor of *Beadex*, encodes the Drosophila phosphorylated carboxy-terminal domain interacting protein 1 ortholog:** The EP insertion line

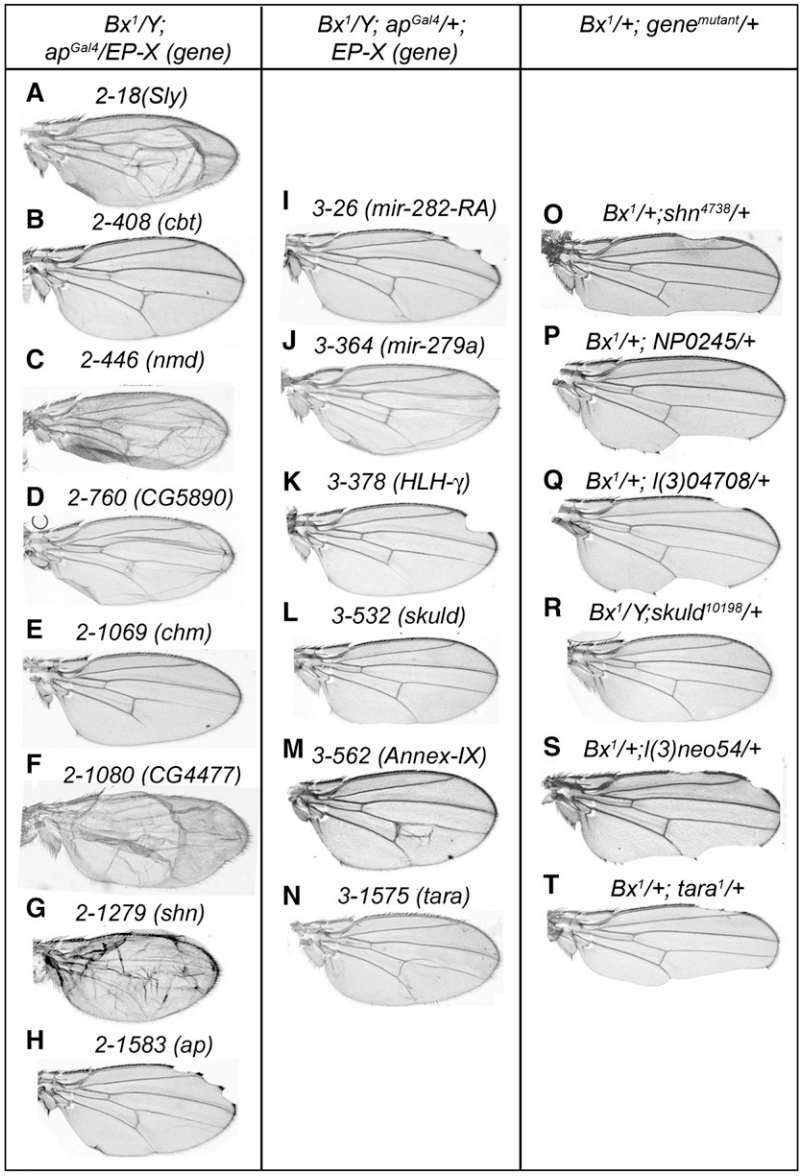

FIGURE 4.—Wing phenotypes of the suppressor lines. Cuticle preparations of adult wings are from the following genotypes: (A) *Bx*^{1}*/Y; ap*^{gal4}*/EP2-18* (*Sly*); (B) *Bx*^{1}*/Y; ap*^{gal4}*/EP2-408* (*cbt*); (C) *Bx*^{1}*/Y; ap*^{gal4}*/EP2-446* (*nmd*); (D) *Bx*^{1}*/Y; ap*^{gal4}*/EP2-760* (*CG5890*); (E) *Bx*^{1}*/Y; ap*^{gal4}*/EP2-1069* (*chm*); (F) *Bx*^{1}*/Y; ap*^{gal4}*/EP2-1080* (*CG4477*); (G) *Bx*^{1}*/Y; ap*^{gal4}*/EP2-1279* (*shn*); (H) *Bx*^{1}*/Y; ap*^{gal4}*/EP2-1583* (*ap*); (I) *Bx*^{1}*/Y; ap*^{gal4}*/EP3-26* (*miR-282-RA*); (J) *Bx*^{1}*/Y; ap*^{gal4}*/EP3-364* (*miR-279a*); (K) *Bx*^{1}*/Y; ap*^{gal4}*/EP3-378* (*HLH-γ*); (L) *Bx*^{1}*/Y; ap*^{gal4}*/EP3-532* (*skuld*); (M) *Bx*^{1}*/Y; ap*^{gal4}*/EP3-562* (*Annex-IX*); (N) *Bx*^{1}*/Y; ap*^{gal4}*/EP3-1575* (*tara*); (O) *Bx*^{1}*/+; shn*^{4738}*/+*; (P) *Bx*^{1}*/+; NP0245/+*; (Q) *Bx*^{1}*/+; l(3)04708/+*; (R) *Bx*^{1}*/Y; skuld*^{10198}*/+*; (S) *Bx*^{1}*/+; l(3)neo54/+*; and (T) *Bx*^{1}*/+; tara*^{1}*/+*.

3-28 directed the expression of *CG11399* (Figure 7, A–D) and, when overexpressed in the D compartment, completely rescued the *Bx*^{1} phenotype (Figure 7E). Another independently generated EP insertion (GS11380) drove the expression of *CG11399* and also rescued the wing-margin defects (data not shown). Interestingly, when *CG11399* was overexpressed in the notum (in *ap*^{gal4}*/+; EP3-28/+* or *ap*^{gal4}*/+; GS11380/+* flies), macro- and micro-chaetae were absent, thereby resembling a Notch gain-of-function phenotype (Figure 7, N and O). Consistent with this, an EP insertion located in the fifth protein encoding exon of *CG11399* and most probably behaving as a loss-of-function allele of *CG11399* (note it has a mild wing-vein phenotype that is also reproduced in a hemizygous condition over a deficiency, Figure 7, G and H) enhanced the wing-margin and -vein defects of *N*^{ND1}, a hypomorphic allele of *Notch* (compare Figure 7, I and J), and *dx*^{enu}, a hypomorphic allele of *deltex*, a positive modulator of a Notch receptor (MATSUNO *et al.* 1995; compare Figure 7, K and L). *CG11399* encodes the Drosophila ortholog of human phosphorylated carboxy-terminal domain (CTD) interacting factor 1 (PCIF1), a nuclear WW domain-containing protein (FAN *et al.* 2003). Phosphorylation of the CTD of the largest subunit of RNA polymerase II is crucial in transcription elongation and in coupling transcription to pre-mRNA processing. The WW domain of PCIF1 directly and preferentially binds to the phosphorylated CTD compared to the unphosphorylated CTD (FAN *et al.* 2003). PCIF1 may play a role in mRNA synthesis by modulating RNA polymerase II activity. Our results suggest that the Drosophila PCIF1 ortholog, encoded by *CG11399*, participates in modulating the transcription of certain mRNAs involved in Notch signaling. It is interesting to note that *CG11399* specifically modulates the Notch pathway and does not

TABLE 2

Classes of suppressor lines

Type of genes	Genes	EP line	Description of gene	References
Epigenetic mechanisms	*osa*	*3-473*, *3-619*, *3-900*, *3-941*, *3-1074*, *3-1386*, *3-1591*	A trithorax group gene that associates with the Brahma chromatin remodeling complex. Osa binds Chip and modulates the expression of Ap target genes.	Heitzler *et al.* (2003) Milán *et al.* (2004)
	taranis	*3-1575*	A novel trithorax-group member, involved in maintaining an active chromatin structure; it genetically interacts with *osa*	Calgaro *et al.* (2002)
	chameau	*2-1069*	Histone acetyltransferase activity, involved in chromatin-mediated gene silencing	Grienenberger *et al.* (2002)
Transcription	*apterous*	*3-1583*	LIM-homeodomain transcription factor, involved in DV boundary formation in the wing	Diaz-Benjumea and Cohen (1993)
	E(spl)-γ	*3-378*	HLH-containing transcription factor that serves as an important effector of Notch to control neuronal cell fate	Bray (1997)
	skuld/pap	*3-532*	RNA polymerase II transcription mediator activity, involved in maintaining the compartment affinity boundaries	Janody *et al.* (2003)
	pointed	*3-637*, *3-853*, *3-1638*, *3-1859*	Type II transforming growth factor-β receptor in the nucleus that regulates transcription of aos	Hsu and Schulz (2000)
	capicua	*3-1005*	Transcription repressor acting in EGFR signaling to induce vein/intervein cell fate decision	Roch *et al.* (2002)
	schnurri	*2-1279*, *2-1743*	Zinc finger transcription factor involved in both activation and repression of Dpp target genes	Greider *et al.* (1995)
	mtv/sbb/bks	*2-473*	Zinc finger protein, interacts with Groucho to repress hh expression in the anterior compartment of the wing disc	Bejarano *et al.* (2007)
	lilliputian	*2-27*	Nuclear protein involved in the control of cell size	Wittwer *et al.* (2001)
	cabut	*2-408*	Nuclear protein involved in embryonic dorsal closure	Muñoz-Descalzo *et al.* (2005)
	Su(Tpl)/dEll	*3-2015*	RNA polymerase II transcription elongation factor activity[a] Strong interaction with Cut and Notch	Eissenberg *et al.* (2002)
	CG8149	*3-980*	SAP domain/DNA binding domain containing protein	
	CG11399	*3-28*	WW domain protein involved in coactivation of transcription and modulation of RNA polymerase II activity	
Micro-RNAs	*mir-14*	*2-235*, *2-356*, *2-402*, *2-814*, *2-1142*,	Micro-RNA that suppresses cell death	Xu *et al.* (2003)
	mir-282-RA	*3-26*		
	mir-279a	*3-364*, *3-1729*		

(*continued*)

TABLE 2

(Continued)

Type of genes	Genes	EP line	Description of gene	References
Membrane trafficking	γ-*Snap*	*3-1789*	γ-soluble NSF attachment protein, involved in synaptic vesicle priming and protein transport, involved in the Notch signaling pathway	Stewart *et al.* (2001)
	Sly	*2-18*	SNARE-binding activity, involved in protein targeting/intracellular protein transport and a component of the membrane	
	nuf	*3-1715*	Nuf is required for localizing Rab11 to the recycling endosome and for recruiting proteins to the plasma membrane during cellularization.	Emery *et al.* (2005)
	Annexin IX	*3-562*	Calcium-dependent phospholipid binding activity, involved in cytoskeletal interaction and intracellular signaling	
Others	*fringe*	*3-511*, *3-581*, *3-612*, *3-934*	Target of Apterous encoding a glycosyltransferase activity involved in Notch signaling	Irvine and Wieschaus (1994)
	*PAR-5/14-3-3*ε	*3-732*	Protein kinase activity/Ras signal transduction[b]/ anterior-posterior polarization in Drosophila	Benton *et al.* (2002)
	Draper	*2-867*	Extracellular matrix structural constituent[c] involved in phagocytosis of apoptotic cells by hemocytes	
	CG8369	*3-826*	Kazal-type serin protease inhibitor	
	CG15095	*2-430*	General substrate transporter of the Major facilitator superfamily	
	CG14709	*3-1809*	Fatty acid binding activity; transporter activity[d]	
	nmd/Msp	*2-446*	ATPase in ER probably involved in intracellular protein transport	
	Mcpc	*3-488*	Mitochondrial phosphate carrier protein	
	CG5890	*2-760*	Calcium[e] binding protein	
	CG8405	*2-865*	K+ channel	
	CG4477	*2-1080*	Peptidase	
	CG1943	*3-797*	Unknown	
	CG14073	*3-1199*	Four Ankyrin Repeats. It binds through *CG4195* to two additional proteins involved in chromatin silencing: *polyhomeotic-p* (*CG18412*) and *polyhomeotic-d* (*CG3895*).	Giot *et al.* (2003)

[a] http://flybase.bio.indiana.edu/cgi-bin/goreport?GO:0016944
[b] http://flybase.org/.bin/goreport?GO:0007265
[c] http://flserver.gen.cam.ac.uk:7081/.bin/goreport?GO:0005201
[d] http://fbserver.gen.cam.ac.uk:7081/.bin/goreport?GO:0005215
[e] http://fbserver.gen.cam.ac.uk:7081/.bin/goreport?GO:0005102

appear to modulate other active pathways in the Drosophila wing, since overexpression or loss of *CG11399* activity did not cause any other wing phenotype.

micro-RNAs: Three micro-RNAs, small regulatory RNAs that are between 21 and 25 nucleotides in length and repress gene function through interactions with target mRNAs, were identified in the screen: *miR-14* (*2-235*, *2-356*, *2-402*, *2-814*, and *2-1142*), *miR-282-RA* (*3-26*, Figure 4I) and *miR-279a* (*3-364* and *3-1729*, Figure 4J and data not shown, see Tables 1 and 2). Among these, *miR-14* was identified in a previous screen as a cell death suppressor (Xu *et al.* 2003). Five distinct insertions driving the expression of *miR-14* were identified as suppressors of Bx^1 and an *UAS-miR-14* transgenic construct also

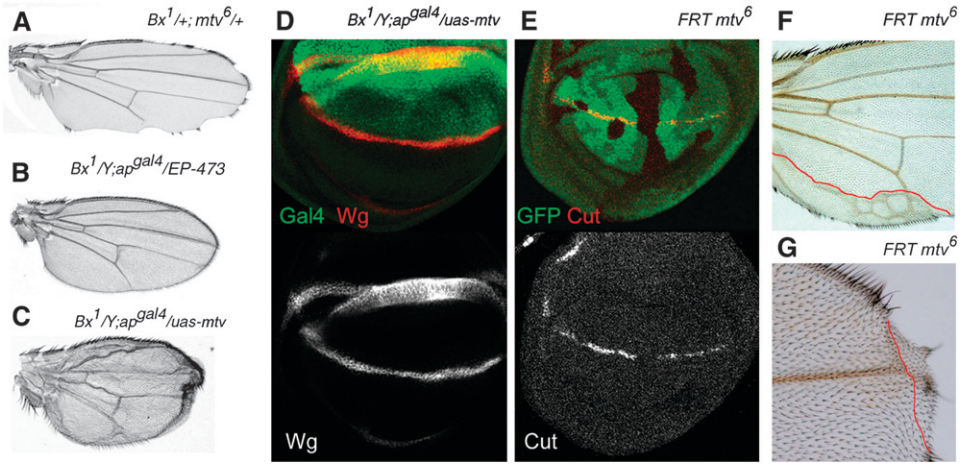

FIGURE 5.—*master of thickveins* as a suppressor of the the *Beadex*^{1} phenotype. (A–C) Cuticle preparations of *Bx*^{1}*/+; mtv*^{6}*/+* (A), *Bx*^{1}*/Y; ap*^{gal4}*/EP-473* (B), and *Bx*^{1}*/Y; ap*^{gal4}*/uas-mtv* (C) adult wings. (D) *Bx*^{1}*/Y; ap*^{gal4}*/uas-mtv* wing imaginal discs labeled to visualize Gal4 (green) and Wingless (Wg, red in the top and white in the bottom) protein expression. (E) Clones of cells mutant for *master of thickveins* (*mtv*^{6}) and labeled by the absence of the GFP marker (green). Cut protein expression is shown in red (top) or white (bottom). Note loss of Cut expression in clones abutting the DV boundary. (F and G) Clones of cells mutant for *master of thickveins* (*mtv*^{6}), labeled by the *forked* (*f*^{36a}) cuticle marker and marked by a red line. Note loss of wing-margin structures and ectopic vein tissue.

suppressed the *Bx*^{1} wing phenotype (Figure 8, A, B, and D). Expression of *miR-14* increased Notch activity levels at the boundary of *Bx*^{1} wing discs, as shown by the expression of Wg (Figure 8, F and G). Overexpression of *miR-14* has been reported to suppress cell death induced by multiple stimuli (XU *et al.* 2003). In this context, it is interesting to note that loss of Notch activity causes cell death in the wing disc (YE and FORTINI 1999) and it has been postulated that this cell death causes defects in the adult wing margin (ADACHI-YAMADA *et al.* 1999). We then analyzed the capacity of suppressed cell death to bypass the requirement for Notch in cell survival and, consequently, to rescue the wing-margin defects of *Bx*^{1} wings. For this purpose, we used the P35 baculovirus protein, which strongly inhibits caspase enzymatic activity in Drosophila tissues (HAY *et al.* 1995) and the Drosophila inhibitor of caspases *DIAP1* (WANG *et al.* 1999). Expression of *p35* or *DIAP1* in the D compartment of *Bx*^{1} flies did not rescue the wing-margin defects (Figure 8, C and E). Wg expression levels in boundary cells also were not increased (data not shown). Taken together, these results indicate that the capacity of *miR-14* to rescue the *Bx*^{1} wing-margin phenotype is not a consequence of suppressed cell death, and the loss of wing-margin structures in this mutant background is not a direct result of cell death. It appears then as if the wing-margin defects in the absence of Notch signaling might be a consequence of impaired patterning of the wing margin as well as a failure of growth and not a direct consequence of cell death.

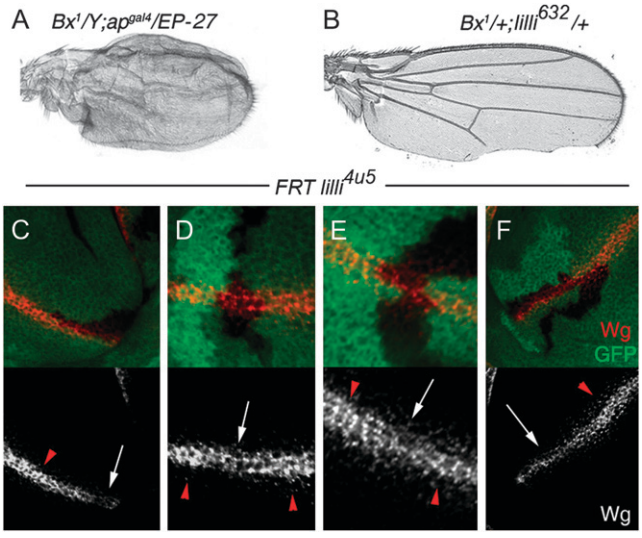

FIGURE 6.—*lilliputian* as a suppressor of the the *Beadex*^{1} phenotype. (A and B) Cuticle preparations of *Bx*^{1}*/Y; ap*^{gal4}*/EP-27* (A) and *Bx*^{1}*/+; lilli*^{632}*/+* (B) adult wings. (C–F) Clones of cells mutant for *lilliputian* (*lilli*^{4u5}) and labeled by the absence of the GFP marker (green). Wingless (Wg) protein expression is shown in red (top) or white (bottom). Note reduced levels of Wg protein expression in clones abutting the DV boundary (white arrows) when compared to the endogenous level (red arrowhead) of Wg expression.

In the last few years, several groups have carried out computational identifications of *micro-RNA* target genes by looking for target sites located in the 3′-UTRs of the mRNAs. Interestingly, *Bx/dLMO* was identified as a potential *miR-14* target gene (STARK *et al.* 2003). We then monitored the capacity of *miR-14* to reduce the levels of dLMO protein in the wing disc as well as to phenocopy a *dLMO* (held-up wing) loss-of-function phenotype. The phentoype of *ap*^{gal4}*;UAS-miR-14* flies resembled the held-up wing phenotype of *dLMO* mutant flies (Figure 8, H and I), and the expression levels of dLMO protein were reduced by overexpression of *miR-14* in the wing disc (Figure 8, J–L). We then monitored the capacity of *miR-14* to regulate the expression level of a *dLMO* 3′-UTR sensor transgene consisting of the *dLMO* 3′-UTR cloned into the tubulin-promoter-EGFP reporter plasmid. The *dLMO* 3′-UTR sensor transgene was expressed uniformly in the wing imaginal disc (Figure 8N). Gal4-dependent expression of *miR-14* reduced expression of the *dLMO* 3′-UTR sensor transgene (Figure 8M). Although *miR-14* is expressed during larval stages (GRUN *et al.* 2005), loss of *miR-14* did not show any

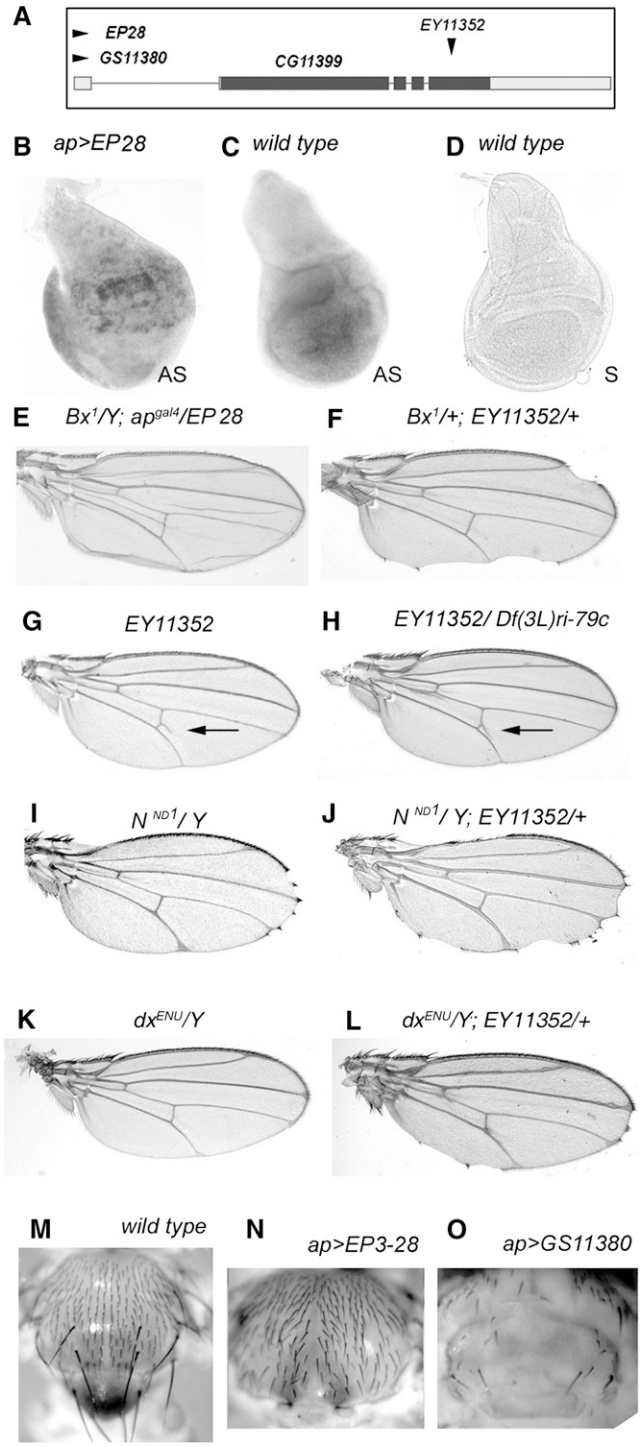

FIGURE 7.—*CG11399*, the Drosophila phosphorylated C-terminal domain interacting factor, suppresses the *Beadex*^{1} phenotype. (A) Genomic map of the *CG11399* region. Exons are shown as boxes, and the ORF is marked in black. *CG11399* was identified as a suppressor of the *Beadex*^{1} phenotype by the EP-3-28 insertion (black arrowhead). Two other EP lines (GS11380 and EY11352, black arrowheads) are shown. (B–D) *In situ* hybridization to *ap*^{gal4}*/+; EP-28/+* (B) and *wild-type* (C and D) wing imaginal discs with an anti-sense (B and C) and sense (D) *CG11399* RNA probe. (E–L) Cuticle preparations of *Bx*^{1}*/Y; ap*^{gal4}*/EP-28* (E), *Bx*^{1}*/+; EY11352/+* (F), *EY11352* (G), *EY11352/Df(3L)ri-79c* (H), *N*^{ND1}*/Y* (I), *N*^{ND1}*/Y; EY11352/+* (J), *dx*^{ENU}*/Y* (K), and *dx*^{ENU} */Y; EY11352/+* (L) adult wings. Note ectopic vein tissue marked by a black arrow in G and H. (M–O) Cuticle preparations of *wild-type* (M), *ap*^{gal4}*/+; EP-28/+* (N), and *ap*^{gal4}*/+; GS11380/+* (O) adult nota. Note loss of macrochaetae in N and loss of both macro- and microchaetae in O.

overt wing phenotype nor did it enhance the wing-margin defects of *Bx*^{1}*/+* wings. Thus, the direct regulation of dLMO protein levels by *miR-14* might be required in other developmental contexts in which dLMO activity is involved (TSAI *et al.* 2004).

Membrane fusion and vesicle trafficking: In many signal transduction pathways, vesicle trafficking of ligands or receptors is a key regulatory event (reviewed in GONZALEZ-GAITAN 2003). SNARE proteins play a central role in intracellular membrane fusion and vesicle trafficking (reviewed in JAHN and SCHELLER 2006). The interaction of SNAREs present on two opposing membranes is generally believed to provide the driving force to initiate membrane fusion. We identified two genes involved in SNARE-dependent membrane fusion in our screen: γ-soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein (SNAP) (*3-1789*) and Slh (2-18, Figure 4A), the Drosophila ortholog of the Sec1p/Munc18 protein. SNAPs are highly conserved proteins that participate in intracellular membrane fusion and vesicular trafficking. They recruit NSF to the membrane after being bound to specific membrane receptors termed SNAREs (SNAP receptor). The complex, which is then disrupted upon ATP hydrolysis by NSF, is a prerequisite of membrane fusion. Sec1/Munc18 proteins are required for the controlled assembly of SNARE complexes and are essential for membrane fusion at the plasma membrane. The nature of the suppression of the *Bx*^{1} phenotype by γ-SNAP or Slh overexpression may rely on elevated levels of transmembrane or secreted proteins involved in Notch signaling and/or DV boundary formation in the plasmatic membrane.

Rab11 is involved in controlling vesicular protein transport through recycling endosomes to the plasma membrane (PFEFFER and AIVAZIAN 2004). Dominant-negative forms of Rab11 inhibit the recycling of endocytosed transmembrane proteins to the plasma membrane, thereby suggesting that Rab11 regulates trafficking of vesicular cargo through the recycling endosomal compartment. Nuf (nuclear fallout) is a homolog of arfophilin-2, an ADP ribosylation factor effector that binds Rab11 and influences recycling endosome (RE) organization (HICKSON *et al.* 2003). Nuf and Rab11 are mutually required for their localization to the RE. Delta has been shown to pass through the recycling endosome which is marked by Rab 11 and Nuf, an essential step for its activity as a Notch ligand (EMERY *et al.* 2005). We identified *nuf* as a gain-of-function suppressor of the *Bx*^{1} phenotype (line 3-1715) and loss-of-function alleles of *nuf* enhanced the wing-margin defects of *Bx*^{1}*/+* heterozygous flies (Table 1).

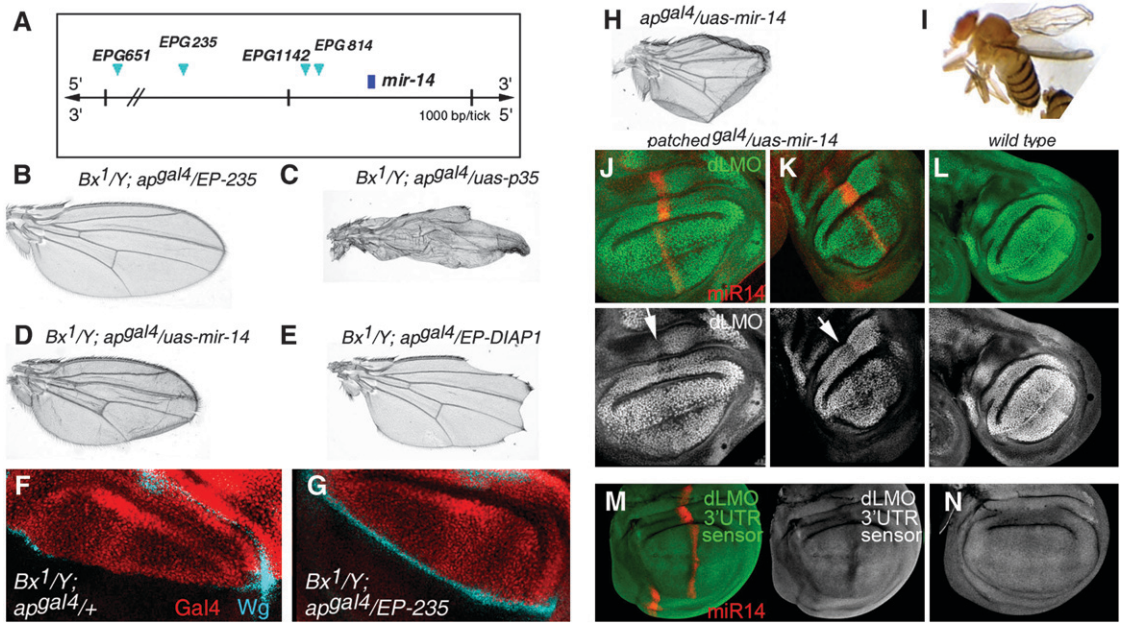

Figure 8.—*miR-14* as a suppressor of the *Beadex*^{1} phenotype: genomic map of the *miR-14* region (blue box). *miR-14* was identified as a suppressor of the *Beadex*^{1} phenotype by four EP insertions (blue arrowheads). (B–E) Cuticle preparations of *Bx*^{1}*/Y; ap*^{gal4}*/EP-235* (B), *Bx*^{1}*/Y; ap*^{gal4}*/uas-p35* (C), *Bx*^{1}*/Y; ap*^{gal4}*/uas-miR-14* (D), and *Bx*^{1}*/Y; ap*^{gal4}*/EP-DIAP1* (E) adult wings. (F and G) *Bx*^{1}*/Y; ap*^{gal4}*/+* (F) and *Bx*^{1}*/Y; ap*^{gal4}*/ EP-235* (G) wing discs labeled to visualize Gal4 (red) and Wingless (Wg, blue) protein expression. (H) Cuticle preparation of a *ap*^{gal4}*/uas-miR-14* adult wing. (I) *ap*^{gal4}*/uas-miR-14* adult fly. Note the *held-up* wing phenotype. (J–L) *ptc*^{gal4}*/uas-miR-14* (J and K) and *wild-type* (L) wing discs labeled to visualize GFP (red) and dLMO (green, top, or white, bottom) protein expression. Note reduced levels of dLMO protein in the GFP domain (white arrow). (M and N) Expression of the *dLMO* 3′-UTR sensor transgene (green or white) in *ptc*^{gal4}*/uas-miR-14* (M) or wild-type wing discs (N). The sensor transgene was downregulated by *miR-14* overexpression.

Clones of cells mutant for *nuf* did not affect Notch activity levels at the DV boundary, as shown by the expression levels of Wg (supplemental Figure S1 at http://www.genetics.org/supplemental/). Thus, enhanced recycling of endosomal Delta, and probably other proteins involved in DV boundary formation, might increase, directly or indirectly, Notch signal and rescue the wing-margin defects of *Bx*^{1} adult wings. This recycling might be required to modulate Notch activity levels at the DV boundary but does not appear to be a strict requirement for this process.

Finally, we identified Annexin IX (*3-562*, Figure 4M) as a gain-of-function suppressor of *Beadex*. Annexin IX is a member of the annexin family of intracellular Ca^{2+}-dependent lipid-binding proteins. Interestingly, some members of this family are found in apical transport vesicles in Madin–Darby canine kidney cells and may be involved in apical delivery of *trans*-Golgi network-derived vesicles (reviewed in Harder and Simons 1997). Taken together, the finding of genes as gain-of-function suppressors of the *Bx*^{1} wing-margin phenotype and encoding for proteins involved in membrane fusion, endosome recycling, and vesicle trafficking indicates that the relative levels at the membrane of transmembrane proteins or secreted molecules are crucial and probably modulated during wing development.

Conclusions: Here we show that a gain-of-function EP-based screen in a *Bx*^{1}-sensitized background to search for suppressors of the wing-margin phenotype is efficient in identifying known and new genes involved in DV boundary formation as well as in the regulation of *Beadex/dLMO* gene activity. Dominant genetic interactions of *Bx*^{1} with loss-of-function alleles of the suppressor genes identified have demonstrated that the vast majority are involved in wing development. This is in contrast with classic EP screens based on the gain-of-function phenotype of candidate genes, in which the number of genes not participating in the developmental context of interest is relatively higher. We have shown that many of the *Bx*^{1} suppressors involved in DV boundary formation are not essential during wing development (*i.e.*, *taranis*, *nmd*, *nuf*, *draper*, and *cabut*; supplemental Figure S1 at http://www.genetics.org/supplemental/). This observation suggests that these suppressors share redundant activities with other gene products. The EP gain-of-function approach has also been shown to be extremely efficient in unraveling new roles for the recently identified microRNAs (miRs, *e.g.*, Brennecke *et al.* 2003; Nairz *et al.* 2006). Loss-of-function-based forward genetic screenings have not been as productive in this respect, probably because of the reduced size of these miRs or their redundant activities. Taken together, a suppressor EP-

based gain-of-function screen in a sensitized background provides a suitable combination to identify new genes, including miRs and redundant genes, involved in a given process.

Redundancy and regulatory feedback loops contribute to the robustness of gene regulatory networks (Stelling *et al.* 2004). Classical loss-of-function-based forward genetic screenings have been highly productive in identifying genes that behave as hubs in these networks (Friedman and Perrimon 2007). Essential genes in yeast are among those most highly connected (Barabasi and Oltvai 2004). However, forward genetic screenings are not as effective in identifying redundant genes or regulators of these feedback loops, whose loss of function might not show any overt phenotype. More quantitative *in vivo* genetic screenings, such as the one done recently in Drosophila for bristle number (Norga *et al.* 2003), or, alternatively, cell culture-based RNAi quantitative screenings have been more efficient in this regard (Friedman and Perrimon 2007). Our results indicate that an EP-based gain-of-function *in vivo* genetic screen in a sensitized background is a strong alternative for the identification of redundant genes or regulators of feedback loops involved in developmental gene regulatory networks.

Here we have identified, characterized, and discussed four classes of genes in the context of DV boundary formation or dLMO activity: chromatin organization genes, transcription factors, miRs, and proteins involved in vesicle trafficking and membrane fusion. Several conclusions can be drawn. Among the genes involved in chromatin organization, *Osa* binds Chip and modulates the expression of Ap target genes (Milán *et al.* 2004). Several transcription factors involved in other signaling pathways during wing development have also been shown to act as Bx^1 suppressors, suggesting that Notch and these pathways share common elements or that these pathways collaborate with Notch in boundary formation. The finding of genes encoding for proteins that participate in distinct aspects of vesicle trafficking and membrane fusion indicates that the sorting of sufficient levels of certain molecules, including Notch and its ligand Delta, toward the plasma membrane is especially critical to reach appropriate levels of Notch activity at the DV boundary. Consistent with this, it is interesting to note that overexpression of these genes in an otherwise wild-type background does not show any overt wing phenotype, suggesting that the activity of the Notch pathway is finely regulated and buffered during boundary formation (Rulifson *et al.* 1996; Herranz *et al.* 2006).

The screen was designed and performed to find new genes involved in Ap and/or dLMO activity, as well as new Ap target genes involved in DV boundary formation. Although genes known to participate in DV boundary formation, like *fringe* or *osa*, were scored several times, we did not identify new transmembrane proteins or cell adhesion molecules involved in the generation of an affinity difference between D and V cells. *P* elements are known for their preferential insertion in certain regions of the genome called hot spots. The gene or genes involved in this process might be located in the so-called cold spots, thus suggesting that a distinct transposable element, like the lepidopteran *piggyBac*, with a different profile of hot spots and cold spots (Thibault *et al.* 2004), is a good candidate to search, on a similar suppressor gain-of-function basis, for thesekind of genes.

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