Genetic characterization of *ebi* reveals its critical role in *Drosophila* wing growth

Steven J. Marygold,^{†,*} Cherryl D. Walker, Mariam H. Orme[‡] and Sally J. Leever

Growth Regulation Laboratory; Cancer Research UK London Research Institute; London, UK

[†]Current affiliation: Department of Genetics; University of Cambridge; Cambridge, UK; [‡]The Breakthrough Toby Robins Breast Cancer Research Centre; Institute of Cancer Research; London, UK

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Abbreviations: TBL1, transducin β -like 1; TBLR1, transducin β -like-related 1; SU(H), Suppressor of Hairless; EGFR, Epidermal growth factor receptor; WG, Wingless; TTK88, Tramtrack (88 kDa isoform); SMR, Smrter; HDAC3, Histone deacetylase 3; NCoR, nuclear receptor corepressor; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; InR/PI3K, Insulin receptor/Phosphoinositide-3-kinase; *vg*, *vestigial*; *sd*, *scalloped*; d-v, dorsal-ventral; AED, after egg deposition; *en*, *engrailed*; *da*, *daughterless*; Notch^{ICD}, Notch intra-cellular domain; SNPs, single nucleotide polymorphisms

The *ebi* gene of *Drosophila melanogaster* has been implicated in diverse signaling pathways, cellular functions and developmental processes. However, a thorough genetic analysis of this gene has been lacking and the true extent of its biological roles is unclear. Here, we characterize eleven *ebi* mutations and find that *ebi* has a novel role in promoting growth of the wing imaginal disc: viable combinations of mutant alleles give rise to adults with small wings. Wing discs with reduced EBI levels are correspondingly small and exhibit downregulation of Notch target genes. Furthermore, we show that EBI colocalizes on polytene chromosomes with Smrter (SMR), a transcriptional corepressor, and Suppressor of Hairless (SU(H)), the primary transcription factor involved in Notch signaling. Interestingly, the mammalian orthologs of *ebi*, transducin β -like 1 (TBL1) and TBL-related 1 (TBLR1), function as corepressor/coactivator exchange factors and are required for transcriptional activation of Notch target genes. We hypothesize that EBI acts to activate (de-repress) transcription of Notch target genes important for *Drosophila* wing growth by functioning as a corepressor/coactivator exchange factor for SU(H).

Introduction

Drosophila melanogaster EBI functions in diverse signaling pathways and cellular processes. It was originally characterized as a positive regulator of Epidermal growth factor receptor (EGFR) signaling during embryogenesis and adult eye development.¹ Subsequent studies have demonstrated a role for EBI in cell cycle regulation,² Notch pathway activation,^{3,4} and Wingless (WG) signal transduction,^{5,6} while genetic interactions implicate EBI in basolateral junction signalling,⁷ Ret signal transduction,⁸ and embryonic crystal cell development.⁹ Nonetheless, a thorough genetic characterization of *ebi* has not hitherto been performed and the full extent of its biological influence remains unclear.

EBI is a nuclear-localized protein of 700 amino acids comprising a LisH domain¹⁰ and an F box-like motif^{1,2} at the amino terminus, and a series of WD repeats¹¹ in the carboxy-terminal half (Fig. 1A and B). Several reports describe a transcriptional role for EBI, although the nature of this role is unresolved. Some studies cast EBI as a positive regulator of gene transcription.

For example, EBI associates physically with the 88 kDa isoform of the Tramtrack (TTK88) transcriptional repressor and promotes its proteasome-dependent degradation in response to an EGFR signal.^{1,2} EBI also forms a complex with the Suppressor of Hairless (SU(H)) transcription factor and the Smrter (SMR) corepressor.³ Here, similar to above, EGFR signaling acts via EBI in a process requiring the proteasome to alleviate SMR-SU(H)-mediated repression of target genes.^{3,12} In a third example, EBI is required for transcriptional activation mediated by Armadillo in response to WG stimulation.^{5,6}

In contrast, other reports implicate EBI in transcriptional repression. Two studies have found that EBI is in a complex with Histone deacetylase 3 (HDAC3) and either the Snail or SMYD4 transcriptional repressor.^{13,14} In both cases, EBI is thought to function with HDAC3 to repress target genes. In a third example, and in contrast to the earlier report in reference 3, EBI is proposed to mediate EGFR-dependent transcriptional repression as an integral part of an EBI-SMR-SU(H) corepressor complex.⁴

*Correspondence to: Steven J. Marygold; Email: sjm41@gen.cam.ac.uk
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Figure 1. Molecular characterization of *ebi* alleles. (A) Schematic of the EBI protein showing the location of mutations affecting the coding sequence; nonsense and frame-shift mutations are indicated above the schematic, missense mutations are shown below. L, LisH domain; F, F-box-like motif; W (on dark background), WD repeat predicted with high confidence; W (on light background), WD repeat predicted with low confidence. The LisH and WD domains shown are predicted by SMART.⁴⁴ (B) WD repeat consensus sequence (bold) and alignment of the eight predicted WD repeats in the EBI protein as predicted by SMART.⁴⁴ E values are given to the right of each domain sequence: repeats 4 and 8 are predicted with low confidence. Mutations shown in (A) are highlighted. (C) A prediction of the C-terminal β -propeller structure of EBI based on the solved structure of the β -subunit of transducin. The locations of the four amino acid substitutions associated with *ebi*^{E90} (C510Y), *ebi*^{2g2} (H533L), *ebi*^{2g1} (S553F) and *ebi*^{S602F} (S602F) mutations are shown. Note that the eighth, divergent WD repeat is not predicted to form part of the propeller, resulting in a seven-bladed structure.

Further insights into the mechanism of action of EBI come from studies of the mammalian orthologs, TBL1 and TBLR1. The two mammalian proteins share >90% amino acid identity with each other and >75% identity with *Drosophila* EBI within the functional domains. TBL1 and TBLR1 are core components of the HDAC3-containing nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) transcriptional corepressor complexes.¹⁵ These associations mirror those found in flies as NCoR and SMRT are the mammalian counterparts of *Drosophila* SMR, and both SMR and HDAC3 associate with EBI.^{3,13,14} Interestingly, while TBL1 and TBLR1 are required to mediate transcriptional repression by the NCoR/SMRT complex, they are also necessary for ubiquitylation-dependent dissociation of the corepressors upon receipt of an activating signal.¹⁶ In this way, TBL1 and TBLR1 serve as transcriptional corepressor/coactivator exchange factors. Several transcription factors have been shown

to require the exchange function of TBL1/TBLR1 in order to activate transcription, including NF κ B, CBF1 (the mammalian ortholog of *Drosophila* SU(H)) and most nuclear receptors.¹⁷ The possibility that *Drosophila* EBI has bifunctionality similar to mammalian TBL1/TBLR1 is attractive but has not been explored to date.

We originally identified two *ebi* mutations as enhancers of a small-wing phenotype resulting from inhibition of Insulin Receptor/Phosphoinositide-3-Kinase (InR/PI3K) signalling.¹⁸ Here, we confirm that EBI has a critical role in promoting growth of the wing. We also find that Notch target genes are downregulated in *ebi* mutant wing discs and that an EBI-SMR-SU(H) complex exists at discrete sites on polytene chromosomes. We hypothesize that EBI facilitates the conversion of SU(H) from a transcriptional repressor to an activator, thereby promoting expression of Notch target genes important for wing growth.

Table 1. Molecular nature of *ebi* alleles

Allele	Nucleotide change	Effect on coding region	Reference
Missense mutations			
<i>E90</i>	TGC>TAC	C510Y in 4 th WD repeat	1
<i>2g2</i>	CAC>CTC	H533L in 5 th WD repeat	This study
<i>2g1</i>	TCT>TTT	S553F in 5 th WD repeat	This study
<i>S602F</i>	TCC>TTC	S602F in 6 th WD repeat	2
<i>WKS-24</i>	AGT>AAT	S686N in 8 th WD repeat	This study
Frame-shift mutations			
<i>E4</i>	ATG>ATC	Abrogates initiating ATG	1
<i>delta1</i>	8 bp deletion	Frame-shift from codon 294; new STOP at codon 334	This study
Nonsense mutations			
<i>W418X</i>	TGG>TGA	W418X in 2 nd WD repeat	This study
<i>CCS-8</i>	TGG>TAG	W501X in 4 th WD repeat	This study
Non-coding sequence mutations			
<i>k16213</i>	~10.7 kb <i>P[lacW]</i> insertion in 5' UTR	NA	36
<i>P7</i>	~2.5 kb remnant of <i>P[lacW]ebi^{k16213}</i>	NA	This study

Results

***ebi* mutations dominantly enhance small-wing phenotypes.** The *E-2g¹* and *E-2g²* mutations were isolated as dominant enhancers of a small-wing phenotype induced by inhibition of the InR/PI3K pathway.¹⁸ Both mutations also dominantly enhance the small-wing and wing venation phenotypes caused by antagonizing EGFR signaling in the wing.¹⁸ Subsequent mapping and sequencing (see Materials and Methods) revealed that *E-2g¹* and *E-2g²* disrupt the *ebi* gene and we therefore refer to these mutations as *ebi^{2g1}* and *ebi^{2g2}*. Both mutations result in amino acid substitutions at conserved positions within the WD repeat consensus (Table 1 and Fig. 1). Interestingly, the residues affected by *ebi^{2g1}* and *ebi^{2g2}* are predicted to be hydrogen-bonded together within the fifth β -propeller blade of the EBI protein (Fig. 1C). As such, these mutations are expected to have similar deleterious effects on the protein, which is borne out in the similar phenotypes displayed by the mutant animals (see below).

We obtained and characterized several additional *ebi* mutations (Table 1 and Fig. 1; see also Materials and Methods) and tested them in our wing assays. All mutations dominantly enhance the InR/PI3K-sensitized phenotype and most dominantly enhance the EGFR-sensitized phenotypes (Table 2). Importantly, all nine additional *ebi* alleles fail to complement *ebi^{2g1}* and *ebi^{2g2}*. These data confirm that mutations at the *ebi* locus are responsible for the observed genetic interactions and suggest that EBI normally acts to promote wing growth.

***ebi* interacts genetically with *vestigial* and *scalloped*.** Both the InR/PI3K- and EGFR-sensitized wing phenotypes alluded to above are the result of GAL4-mediated expression of a UAS-containing transgene.¹⁹ Thus, a trivial explanation of the genetic interactions between *ebi* and the InR/PI3K and EGFR pathways might be that reduced *ebi* dosage results in increased expression or stability of the GAL4 protein, leading to hyper-activation of the UAS transgenes and enhancement of the phenotypes. To

test this possibility and to further explore the role of *ebi* in wing development, we tested for genetic interactions between *ebi* and *vestigial* (*vg*) or *scalloped* (*sd*), two genes which are critical for wing growth and patterning.²⁰

Flies heterozygous for both the *vg¹* mutation and an *ebi* mutation (*ebi^{2g1}*, *ebi^{2g2}* or *ebi^{E4}*) display wing notching and wing venation phenotypes that are not observed in any single heterozygote (data not shown). More dramatic effects are observed in *sd^{ETX4}* hemizygous male flies that are also heterozygous for an *ebi* mutation (Fig. 2A–F and Table 2). In the most severe cases, the wing is reduced to little more than a stump (Fig. 2F). These data provide additional evidence that *ebi* has an important function during wing growth/development and indicate that *ebi* was identified in our original screen for this reason rather than indirect effects on GAL4.

Characterization of *ebi* mutations using *ebi^{P7}*. We used the weak *ebi^{P7}* allele^{1,3} to better characterize the developmental role of *ebi* and to assess the relative strength of the eleven *ebi* mutant alleles we had collected (Table 2). No or very few trans-heterozygous progeny are observed when the following mutations were combined with *ebi^{P7}*: *ebi^{2g2}*, *ebi^{E90}*, *ebi^{S602F}*, *ebi^{2g1}*, *ebi^{CCS-8}*. These are therefore the strongest alleles. Four other alleles—*ebi^{E4}*, *ebi^{delta1}*, *ebi^{W418X}* and *ebi^{k16213}*—are semi-viable in trans with *ebi^{P7}* and are therefore classed as intermediate strength alleles. Finally, *ebi^{WKS-24}* and *ebi^{P7}* are classed as the weakest alleles as *ebi^{WKS-24}/*ebi^{P7}** flies and *ebi^{P7}* homozygotes are fully viable. This ranking of *ebi* alleles based on viability in trans with *ebi^{P7}* closely mirrors the strengths of their genetic interactions with *sd^{ETX4}* (Table 2). Taken together, these phenotypes and genetic interactions order the eleven *ebi* mutations into a robust allelic series.

The phenotypic severity of the mutations also correlates with their molecular nature (compare Tables 1 and 2). For example, *ebi^{E90}*, *ebi^{2g2}*, *ebi^{2g1}* and *ebi^{S602F}* result in amino acid substitutions at the interfaces between propeller blades that likely disrupt propeller structure and/or stability (Fig. 1). Given that EBI is

Table 2. Summary of phenotypic data

<i>ebi</i> allele ^a	Genetic interactions ^b				Phenotypes in trans with <i>ebi</i> ^{P7}		
	<i>MS1096>Dp110^{KD}</i>	<i>MS1096>aos</i>	<i>sd^{ETX4}</i>		Viability ^c	Wing area ^d	Wing cell density ^e
	Small wing	Small wing	Wing vein loss	Wing notching			
<i>2g2</i>	++	++	++	+++++	0%	nd	nd
<i>E90</i>	+++	++	++	+++++	3%	nd	nd
<i>S602F</i>	++	++	++	+++++	3%	nd	nd
<i>2g1</i>	+++	++	++	+++++	5%	72%	116%**
<i>CCS-8</i>	+++	+++	+	+	7%	nd	nd
<i>E4</i>	++	++	+	++++	15%	86%	106%*
<i>delta1</i>	++	++	++	+++	17%	84%	98%
<i>W418X</i>	++	++	+	+++	17%	83%	102%
<i>k16213</i>	+	0	0	++	20%	82%	nd
<i>WKS-24</i>	++	0	0	+	29%	nd	nd
<i>P7</i>	++	0	0	0	34%	nd	nd

^a*ebi* mutations are listed here in decreasing order of their severity, based primarily on their viability *in trans* with *ebi*^{P7}. ^bThe number of '+' signs indicates the degree of enhancement compared to the control phenotype. '0' indicates there is no significant effect. For scoring wing size, + indicates that mean wing area is >95% that of control wings; ++, 91–95%; +++, <91%. (p values, calculated using Student's *t*-test, were <0.01 in each case.) For scoring the *MS1096>aos* vein phenotype, the integrity of wing veins L3 and L4 was quantitatively assessed. For scoring the *sd^{ETX4}* wing phenotype, wing notching and wing size were qualitatively assessed. ^cPercentage of *ebi* trans-heterozygous (non-balancer) progeny from a cross between *ebi*^{P7}/*In(2LR)Gla* flies and flies carrying the listed *ebi* mutation also balanced with *In(2LR)Gla*. 33% is expected if the *trans*-heterozygotes are fully viable. ^dMean wing area of trans-heterozygous flies, expressed as a percentage of the mean wing area of *ebi*^{P7}/+ control flies. 'nd', not done. ^eMean cell density (assessed by counting wing hairs within a unit area) of trans-heterozygous flies, expressed as a percentage of the mean cell density of *ebi*^{P7}/+ control flies. p-values were calculated using a two-tailed Student's *t*-test assuming equal variances: **p* < 0.01, ***p* < 0.001. 'nd', not done.

likely to function as a homo-tetramer,²¹ the proteins encoded by these four mutant alleles probably act as dominant negatives when present in the heterozygous condition. All of these alleles are classed as 'strong' in the phenotypic analyses. The medium strength alleles include *ebi*^{E4}, which was characterized as a null mutation,¹ and two mutations (*ebi*^{delta1} and *ebi*^{W418X}) that truncate the protein before or early within the WD repeat domain and are therefore also likely to be null alleles. Finally, the weakest alleles are *ebi*^{P7}, which does not affect the coding region, and *ebi*^{WKS-24}, which causes an amino acid substitution in the eighth WD repeat that is not predicted to form part of the tertiary structure (Fig. 1C).

***ebi* mutants have disproportionately small wings.** The most obvious phenotype shown by *ebi*^{P7} trans-heterozygous escapers is a reduction in wing size (Fig. 3A–C and Table 2). For example, the mean wing area of flies bearing *ebi*^{P7} in combination with an intermediate strength *ebi* allele is 82–86% that of *ebi*^{P7}/+ control wings. Cell density measurements reveal that the size of individual wing cells is not significantly reduced in most of these cases, meaning that such modest reductions in wing area are primarily the result of reduced cell number (Table 2). A more severe allelic combination reduces wing size further: the mean area of *ebi*^{P7}/*ebi*^{2g1} wings is 72% that of control wings and, in this case, a reduction in cell size makes a significant contribution to the small wing phenotype (Table 2). Gross defects in wing patterning are not apparent in any *ebi* trans-heterozygous combination.

Interestingly, the significant reduction in wing size in trans-heterozygous combinations of weak or intermediate *ebi* alleles is never accompanied by a significant decrease in the size of other organs or the whole organism. Male trans-heterozygous escapers

harboring the strongest *ebi* alleles occasionally show an overall reduction in body size (Fig. 3A shows a rare example), but their wings are still disproportionately small.

Other, less penetrant and more variable morphological defects observed in *ebi* trans-heterozygotes include stunted and ectopic macrochaetae in the notum, notching of the wing margin, slight roughening of the eye, malformed legs, and abnormal growths in the antennal region (Fig. 3D and E and data not shown).

The results above establish, for the first time, an important and peculiarly specific role for EBI in the growth of the wing. We therefore decided to characterize this requirement earlier in development by examining *ebi* mutant imaginal wing discs. (Other *ebi* phenotypes were not examined further).

***ebi* mutant wing discs are small and show reduced expression of Notch/SU(H) target genes.** First, we examined *ebi* trans-heterozygous wing discs from wandering stage third instar larvae immunostained for WG (Fig. 4). WG is expressed in two domains in the developing wing pouch (Fig. 4A): the ring of expression around the perimeter provides a useful read-out of wing pouch size, while the prominent stripe of WG expression across the middle of the pouch marks the dorsal-ventral (d-v) boundary and is dependent on Notch signalling.^{22,23} Larvae trans-heterozygous for *ebi*^{P7} and an intermediate strength *ebi* allele (*ebi*^{W418X} or *ebi*^{E4}) have visibly smaller wing pouches, though the WG pattern appears normal in most examples (Fig. 4B and C). Around 50% of flies with these genotypes survive to adulthood and display the small wing phenotype (Table 2). As expected, larvae that are trans-heterozygous for *ebi*^{P7} and a strong *ebi* allele (*ebi*^{2g2}, *ebi*^{S602F} or *ebi*^{2g1}) show a more severe phenotype: wing pouches are reduced to about a quarter of their normal

Figure 2. *ebi* interacts genetically with *sd* and *Notch*. (A) Control wing from *sd*^{ETX4} hemizygous male. (B–F) *sd*^{ETX4} hemizygous male wings that are also heterozygous for the *ebi* mutation shown. (G) Control wing from *N*^{nd-1} hemizygous male showing abnormal notching of the wing margin and thickening of wing veins. (H) Heterozygosity for *ebi*^{2g1} enhances both aspects of the *N*^{nd-1} wing phenotype.

size and the WG staining is generally weaker with breaks in the d-v stripe of expression (Fig. 4D–F). Flies with these genotypes rarely survive to adulthood (Table 2).

We initially sought to confirm and extend these results by inducing homozygous clones of strong/intermediate strength *ebi* alleles in wing imaginal discs. However, no or very few *ebi*^{E4}, *ebi*^{E90}, *ebi*^{2g2} or *ebi*^{W418X} clones survive to late third instar stage when induced at 48 hours after egg deposition (AED), and recovery could not be improved by inducing clones in a *Minute*¹ background. These data indicate that *ebi* is required for cell viability in the wing disc at this stage. Clones induced at 72 hours AED do survive to late third instar stage but were too small to be useful in our experiments.

As an alternative approach, we expressed dsRNA targeted against *ebi* mRNA to knock-down EBI expression in specific areas of the wing. Several independent *UAS-ebi*^{IR} transgenic lines were generated and all constructs gave similar results. Whereas expression of *UAS-ebi*^{IR} using the ubiquitous *da-GAL4* driver

results in pupal lethality (data not shown), expression with either *engrailed* (*en*)-*GAL4* or *MS1096-GAL4* results in viable flies that exhibit a reduction of overall wing size (Fig. 5A–D). *en-GAL4 UAS-ebi*^{IR} wings are reduced to 76–81% of the size of controls, with a greater reduction in the posterior compartment where *en-GAL4* is expressed (Fig. 5A and C). Similarly, *MS1096-GAL4 UAS-ebi*^{IR} wings are 69–78% the size of control wings, though these wings curve upwards owing to the predominant expression of *MS1096-GAL4* in the dorsal compartment of the wing disc (Fig. 5B and D). These significant reductions in wing size occur in the absence of gross patterning defects, consistent with the results obtained with *ebi*^{P7} trans-heterozygotes.

We then examined third instar wing discs from these animals to assess: (i) EBI expression levels, (ii) the size of the prospective wing pouch and (iii) activation of the Notch pathway (Fig. 5E–G). As expected, EBI levels are specifically reduced in the posterior of *en-GAL4 UAS-ebi*^{IR} discs (Fig. 5F) and are reduced to lower levels in the dorsal half of the wing pouch

Figure 3. *ebi* mutant adults have disproportionately small wings and bristle defects. (A) *ebi* trans-heterozygous escapers (right) have wings that are much smaller than the control (left) without proportionate decreases in the size of other body parts. (B and C) *ebi* trans-heterozygous mutant wings (C) are significantly smaller than control wings (B). (D and E) Macrochaetes on *ebi* trans-heterozygous mutant nota (E) appear stunted and form in ectopic positions (arrows) compared to controls (D).

Figure 4. *ebi* mutant wing discs are small and show aberrant WG expression. Third instar wing imaginal discs showing immunostaining of WG. Anterior is to the left, and dorsal is uppermost. (A) Control disc. (B–F) *ebi* trans-heterozygous mutant discs showing phenotypes of increasing severity. The wing pouch is smaller than the control in all genotypes and WG expression is disrupted in the more severe cases.

compared to the ventral half in *MS1096-GAL4 UAS-ebi^{IR}* discs (Fig. 5G). Thus, in both cases, we could assess the effect of knock-down of EBI in one half of the wing pouch with the other half acting as a convenient internal control.

We used the *vg* quadrant enhancer reporter (*vg^{QE}-lacZ*) in these experiments as a measure of wing pouch size.²⁴ Expression of *UAS-ebi^{IR}* using either GAL4 driver reduces the overall size of the wing pouch compared to controls, with the half of the pouch targeted for RNAi being more severely affected than the internal control (Fig. 5E'–G'). This is consistent with the adult phenotypes (Fig. 5A–D) and the trans-heterozygous mutant discs (Fig. 4).

The interruptions observed in the d-v stripe of WG expression in *ebi* trans-heterozygotes (Fig. 4) suggested that a reduction in functional EBI compromises Notch signaling in the wing disc.^{22,23} Indeed, this idea is supported by the strong genetic interactions observed between *ebi* and genes encoding Notch pathway components in wing assays (Fig. 2G and H and data not shown). We wished to examine this hypothesis further using an alternative approach and used the *mβ1.5-lacZ* reporter²⁵ to examine the status of Notch/SU(H)-mediated transcription in *UAS-ebi^{IR}* discs. *mβ1.5-lacZ* is normally expressed in a stripe at the d-v margin of the wing disc and in broad regions on either side that correspond to the intervein regions of the adult wing²⁵ (Fig. 5E''). Significantly, *mβ1.5-lacZ* is reduced when EBI levels are knocked down by RNAi (Fig. 5F'' and G''). The effect is relatively weak using *en-GAL4*, where the reduction in *mβ1.5-lacZ* expression is

restricted to the prospective wing blade regions in the posterior half of the disc (Fig. 5F''), while a clear reduction in reporter activity is observed throughout the dorsal half of the wing pouch when *UAS-ebi^{IR}* is expressed using the stronger *MS1096-GAL4* driver (Fig. 5G'').

Our observations in *ebi* mutant larvae demonstrate that EBI is required to promote growth and Notch/SU(H) signaling in the developing wing pouch, and lead us to infer that EBI may play a role in upregulating Notch/SU(H) target genes required for wing growth. This conclusion is consistent with two previous observations. First, *ebi* mutant wing discs were found to have reduced expression of the Notch ligand Delta at the d-v boundary,³ which supports the idea of EBI having a positive influence on Notch pathway activation. Second, the wing pouch of discs mutant for *Notch* or *Su(H)* was found to be abnormally small,^{22,26} which demonstrates that reduced Notch signaling (in our case via mutation of *ebi*) does indeed retard wing growth.

EBI colocalizes with SU(H) and SMR on polytene chromosomes. Previous work has shown that EBI and SU(H), together with the transcriptional corepressor SMR, can be co-immunoprecipitated when transiently expressed in S2 cells,³ suggesting that the three proteins exist in a complex. However, these associations have not previously been examined in conditions where the proteins are expressed at their normal endogenous levels in vivo. We sought to gain further insight into the relationship between EBI and SU(H) by immunostaining polytene chromosomes from larval salivary glands.

Figure 5. RNAi-knockdown of *ebi* reduces wing size and Notch target gene expression. (A–D) Adult wings with genotypes as shown. Wings expressing an *ebi* RNAi transgene (C and D) are smaller than their respective controls (A and B). (E–G) Third instar wing imaginal discs showing immunostaining of EBI (first column), *vg^{QE}-lacZ* (second column) or *mβ1.5-lacZ* (third column). Anterior is to the left, and dorsal is uppermost. The area of *vg^{QE}-lacZ* expression and the absolute levels of EBI and *mβ1.5-lacZ* expression are reduced when *ebi* RNAi is targeted to the posterior (F–F'') or dorsal (G–G'') compartments of the wing disc compared to controls (E–E''). The images shown in the first two columns are of the same disc; the image in the third column is of a different disc. Genotypes: (E and E') *vg^{QE}-lacZ/+*, (E'') *mβ1.5-lacZ/+*, (F and F') *en-GAL4 UAS-ebi^{IR.f6}/vg^{QE}-lacZ*, (F'') *en-GAL4 UAS-ebi^{IR.f6}/mβ1.5-lacZ*, (G and G') *MS1096-GAL4 UAS-ebi^{IR.f5}/+*; *vg^{QE}-lacZ/+*, (G'') *MS1096-GAL4 UAS-ebi^{IR.f5}/+*; *mβ1.5-lacZ/+*.

Figure 6 (See opposite page). EBI colocalizes with SMR and SU(H) on polytene chromosomes. (A–F) The same polytene chromosome spread from wild-type salivary glands immunostained for the antigens indicated in each part. (A–C) Single channel images. (D–F) Two channel images showing overlap of signals; overlap is magenta in (D), cyan in (E) and yellow in (F). Examples of distinct bands that appear to be SU(H)-positive only (arrows), EBI/SMR-positive only (solid arrowheads), or positive for all three antigens (open arrowheads) are indicated.

We find that EBI is localized to many discrete sites on polytene chromosomes (Fig. 6A), demonstrating that EBI is an abundant chromosome-associated protein in vivo. SMR is also detected at specific sites (Fig. 6B), as demonstrated previously.²⁷ Strikingly, EBI and SMR appear to completely colocalize (Fig. 6D). SU(H) could also be detected at several distinct chromosomal sites (Fig. 6C), many of which are also positive for EBI/SMR (Fig. 6E and F, open arrowheads). Nevertheless, SU(H) is also clearly detected at sites that lack EBI/SMR (arrows in Fig. 6), and conversely, EBI/SMR is detected at sites that apparently lack SU(H) (solid arrowheads in Fig. 6).

The complete colocalization of EBI and SMR suggests they are obligate partners on DNA, which would mean that EBI is a constitutive component of the SMR corepressor complex. The many cases where the EBI-SMR complex also colocalizes with SU(H) probably represent the complex repressing transcription of SU(H) target genes. The existence of SU(H)-negative, EBI-SMR-positive sites indicates that EBI-SMR can be recruited to DNA via factors other than SU(H), which is consistent with previous reports in references 27 and 28 (also see Introduction). Sites that are SU(H)-positive but EBI-SMR-negative could be examples of SU(H)-mediated transcriptional activation where EBI-SMR has been lost from the complex. Alternatively, they may be cases of SU(H) repressing transcription independently of EBI-SMR; for example, sites where SU(H) is bound to other corepressors such as Hairless, CtBP or Groucho.²⁹

Discussion

EBI promotes wing growth. The *ebi* gene has been implicated in several different developmental processes but a function in promoting wing growth has not been described. We originally identified *ebi* mutations as dominant enhancers of a small-wing phenotype,¹⁸ suggesting a growth-promoting role for the wild type gene product. In this report, we have confirmed through genetic analyses that *ebi* is indeed required by the developing wing to attain its normal size. We found that *ebi* mutant wings comprise fewer cells than controls, though whether this is the result of decreased cell proliferation and/or increased apoptosis during wing development remains to be determined. The size of *ebi* mutant wing cells is also reduced in the most severe cases, but again, the root cause of this effect is unclear at this time.

The most striking phenotype of viable *ebi* mutants is the *disproportionate* reduction in wing size—the size of other organs is not similarly decreased if affected at all. It is possible that the two-dimensional nature of the adult wing exaggerates the size reduction, while reductions in the size of other structures such as the eye, head, legs or body are less apparent. However, the strong genetic interactions between *ebi* and the *vg* and *sd* genes, which are specifically required for wing and haltere growth and development,²⁰ support a prominent role for EBI in wing growth. This specificity is not the result of wing-specific expression as EBI is widely expressed in larvae,¹ but it could be explained if EBI is a target of signals predominantly active in the wing and/or if EBI promotes expression of genes that are particularly important for wing development. Alternatively, the fact that *Su(H)* expression is

relatively high in the wing pouch region of wing imaginal discs²⁶ could explain the sensitivity of this organ to *ebi* dosage, given the involvement of EBI in Notch-SU(H) signaling (discussed below).

While our data reveal a growth-promoting role of EBI, Boulton et al.² reported that EBI inhibits cell cycle progression. These contradictory observations may be explained by tissue/stage-specific roles of EBI: its role in preventing S phase entry was characterized in the embryonic nervous system and the developing eye, whereas our results describe a positive role for EBI in the growth of the wing.

EBI potentiates Notch signaling in vivo. Several lines of evidence now indicate that EBI influences Notch signal transduction. First, *ebi* interacts genetically with genes encoding core components of the Notch pathway³ (this work). Second, *ebi* mutants exhibit supernumerary macrochaetae in the notum, occasional notching of the wing margin and a reduction in the size of the pouch region of the wing imaginal disc (this work), phenotypes that are reminiscent of Notch pathway mutants.^{22,26,30,31} Third, mutation of *ebi* results in downregulation of the Notch target genes *wg* and *E(spl)mβ* in wing imaginal discs (this work) and *cut* in eye imaginal discs.³ Fourth, EBI interacts physically with SU(H),³ which is the key transcription factor downstream of Notch, and colocalizes with it on polytene chromosomes (this work). Fifth, EBI orthologs are required for Notch-dependent gene activation in mammalian cells.¹⁷ These observations indicate that EBI potentiates Notch signaling in vivo.

Admittedly, *ebi* mutants do not display canonical Notch loss-of-function phenotypes, such as consistent notching of the wing margin, additional wing vein material, an increased density of notal microchaetae, or an embryonic neurogenic phenotype. Neither has *ebi* been identified in whole genome screens for Notch pathway regulators.³²⁻³⁴ This suggests that EBI may only be relevant to Notch signal transduction in particular contexts. For example, SU(H) may form distinct corepressor complexes of which only a subset involve the EBI-SMR complex.²⁹ In this case, genetic manipulation of *ebi* will affect only Notch target genes regulated by SMR, and will therefore result in a correspondingly narrow subset of phenotypes.

Is EBI a corepressor/coactivator exchange factor for SU(H)? Work by Tsuda and colleagues has shown that EBI can form a tri-partite complex with SU(H) and the transcriptional corepressor SMR.³ They suggest that, in the presence of an EGFR signal, an EBI-SMR corepressor complex out-competes the coactivation complex containing the Notch intra-cellular domain (Notch^{ICD}), and thereby represses Notch/SU(H) target genes such as *charlatan*.⁴ Our polytene data are consistent with this model: EBI appears to be a constitutive component of the DNA-bound SMR corepressor complex, and EBI-SMR frequently colocalizes with SU(H) where the three proteins presumably act together to repress transcription of target genes.

However, a model in which EBI is an obligate transcriptional corepressor of SU(H) does not fit easily with the evidence that EBI has a positive role within the Notch pathway, or the observations that EBI promotes gene expression downstream of other signaling pathways.^{1,2,6} Moreover, such a model does not take account of the evidence from the mammalian system.

CBF1 is the mammalian ortholog of SU(H), and NCoR and SMRT are the mammalian counterparts of Drosophila SMR. As in Drosophila, the NCoR and SMRT corepressors associate with CBF1 to mediate repression of Notch target genes.³⁵ CBF1/SU(H) is a bifunctional transcription factor:²⁹ it represses genes in the absence of Notch^{ICD}, and activates them in the presence of Notch^{ICD}. Significantly, TBL1 and TBLR1, the mammalian orthologs of EBI, are core components of the NCoR and SMRT corepressor complexes, but are also necessary to convert CBF1 from a repressor to an activator upon stimulation by Notch.¹⁷ TBL1 and TBLR1 recruit the proteasome to the promoter where they mediate ubiquitylation-dependent dismissal of the NCoR or SMRT corepressor complex.

Taking account of all available data, we propose the following model. In the absence of an activating signal, EBI is an integral part of a complex containing the SMR corepressor and SU(H), and this complex represses target genes. Upon activation, EBI participates in the dissociation or degradation of the corepressor(s) via the proteasome, and facilitates the recruitment of coactivators, including Notch^{ICD}, resulting in transcription of Notch/SU(H) target genes—in our case, genes required to promote growth of the Drosophila wing. The identity of the signal(s) that activate the exchange function of EBI in the context of the developing wing is not known, but based on our work and that of others, EGFR or InR/PI3K or Notch itself are all excellent candidates.

In summary, we hypothesize that EBI has both a negative and positive function in transcriptional regulation. Such bifunctionality is unlikely to be specific to regulation of SU(H), and we suspect it is also applicable to the other transcription factors with which EBI has been shown to associate, such as TTK88, Armadillo, Snail and SMYD4.^{1,2,13,14} This view could explain the apparently contradictory functions ascribed to EBI in the literature as, depending on the biological context, either the positive or negative transcriptional role of EBI will be evident.

Materials and Methods

ebi mutations. *E-2g¹* (FBal0190862) and *E-2g²* (FBal0191945) were initially identified as dominant enhancers of a small-wing phenotype and defined a lethal complementation group on chromosome 2.¹⁸ The *E-2g¹*-bearing chromosome was used to map the locus as described in reference 18. Meiotic recombination mapping using visible markers and single nucleotide polymorphisms (SNPs) delimited the *E-2g*-containing region to approximately 2 Mb at the tip of 2L (computed cytology 21A1–22B8). We then used deficiency chromosomes affecting this region to map *E-2g¹* and *E-2g²* by lethal complementation analysis. (The enhancer mutation and the lethal locus cosegregated in all recombinants examined, so we were reasonably confident that these traits mapped to the same gene). *Df(2L)al* (FBab0001692), a well characterized deficiency with breakpoints 21C1;21C7 and which uncovers 25 genes, failed to complement the lethality of both mutations. Available stocks containing mutations in candidate genes were obtained and *ebi^{k16213}* (FBal0064059) was found to be lethal in trans to both *E-2g¹* and *E-2g²*. Both *E-2g* mutations failed to complement all other *ebi* mutations obtained,

confirming allelism. Finally, we sequenced the *ebi* gene (located at 21C2) in both mutants and found point mutations resulting in amino acid substitutions in each case (Table 1 and Fig. 1). Thus *E-2g¹* and *E-2g²* were renamed *ebi^{2g1}* and *ebi^{2g2}*, respectively.

The homozygous lethal *P{lacW}ebi^{k16213}* line³⁶ was obtained from the Bloomington Drosophila Stock Center (FBst0011166). Crosses with the weak *ebi^{p7}* mutation (FBal0249412) revealed that the *P{lacW}ebi^{k16213}* chromosome contained a second-site lethal mutation. This was removed by recombination, although the ‘cleaned’ *P{lacW}ebi^{k16213}* chromosome remained homozygous lethal.

ebi^{E4} (FBal0096973), *ebi^{E90}* (FBal0096972) and *ebi^{p7}* have been described in reference 1; *ebi^{E4}* and *ebi^{E90}* were obtained from Larry Zipursky, and *ebi^{p7}* from Leo Tsuda. *ebi^{p7}* is an imprecise excision of the *P{lacW}ebi^{k16213}* insertion,¹ although its molecular characterization has not previously been reported. Through PCR and DNA sequencing, we found that the *ebi^{p7}* chromosome retains an approximately 2.5 kb remnant of the original ~10.7 kb *P{lacW}* element with both the 5' and 3' *P{lacW}* sequences intact, suggesting that the *ebi^{p7}* excision is an internal deletion of *P{lacW}ebi^{k16213}* of approximately 8.2 kb. The original *ebi^{p7}* chromosome we obtained contained a second-site lethal (presumably the same one present in the *P{lacW}ebi^{k16213}* parental chromosome) and was homozygous lethal. We removed this second-site lethal by recombination, after which the *ebi^{p7}* chromosome was homozygous viable.

ebi^{S602F}, *ebi^{W418X}* and *ebi^{delta1}* were obtained from Pascal Heitzler. *ebi^{S602F}* is identical to the *ebi^{CC3}* allele (FBal0119260,²). *ebi^{delta1}* is an 8 bp deletion starting in codon 294 which changes the reading frame and introduces a new stop codon at codon 334, which would truncate the protein before the WD repeat region (Table 1 and Fig. 1). (*ebi^{delta1}* is similar, but not identical to the *ebi^{CC4}* mutation²). *ebi^{W418X}* is a previously undescribed mutation that converts codon 418 to a stop codon, thereby truncating the protein within the 2nd WD repeat. (We were unable to obtain a stock harboring the *ebi^{CC1}* mutation).²

ebi^{CCS-6} (FBal0155358), *ebi^{CCS-8}* (FBal0155357) and *ebi^{WKS-24}* (FBal0155351) were obtained from the Bloomington Drosophila Stock Center and were originally generated by Exelixis Inc. Their molecular characterization has not previously been reported. The *ebi^{CCS-6}* stock contained viable homozygotes, and *ebi^{CCS-6}/ebi^{E90}* and *ebi^{CCS-6}/ebi^{2g1}* trans-heterozygotes are completely viable and aphenotypic. We concluded that the *ebi* mutation in this stock is either extremely weak or has been lost, and *ebi^{CCS-6}* was not studied further. In contrast, *ebi^{WKS-24}* and *ebi^{CCS-8}* are each lethal in combination with *ebi^{2g1}* or *ebi^{E90}*, and DNA sequencing found missense or nonsense mutations in the *ebi* coding region (Table 1 and Fig. 1).

All *ebi* mutations were recombined with FRT40A and balanced with *In(2LR)Gla* or *CyO*. The homozygous lethality of *ebi^{k16213}*, *ebi^{WKS-24}*, *ebi^{E4}*, *ebi^{E90}*, *ebi^{2g2}* and *ebi^{W418X}* FRT40A recombinants could be rescued with an *ebi* genomic rescue construct (FBtp0010759¹), thus demonstrating that these chromosomes lacked secondary lethal loci. These chromosomes were used to make *ebi* mutant clones in the wing imaginal disc.

Seven independent *UAS-ebi^{IR}* lines were established with insertions on chromosomes X, 2 and 3. Based on the relative strengths

of phenotypes observed with the *MS1096-GAL4* (FBtri0002374) driver line, insertions f6 and f28 were classified as strong expressers, insertions m17 and f5 as medium expressers and m7, m8 and m12 as weak expressers. Insertion f5 on chromosome X was recombined with *MS1096-GAL4*, and insertion f6 on chromosome 2 was recombined with *en-GAL4* (FBtp0055689) for the experiments shown in Figure 6.

Other fly stocks. The *MS1096-GAL4 UAS-Dp110^{KD}* (*MS1096>Dp110^{KD}*) and *MS1096-GAL4 UAS-aos* (*MS1096>aos*) stocks have been described in reference 18; *vg^{QE}-lacZ* (FBtp0001363²⁴) was obtained from Sean Carroll; *mB1.5-lacZ* (FBtp0012943²⁵) was obtained from Sarah Bray; *hsFLP; arm-lacZ M(2)z FRT40A* (FBst1001673) was obtained from Antonio Garcia-Bellido; *vg^l* (FBal0017868), *sd^{ETX4}* (FBal0032671), *N^{md-1}* (FBal0012890), *Df(2L)al*, *MS1096-GAL4*, *en-GAL4* and *da-GAL4* (FBtp0001168) were obtained from the Bloomington Drosophila Stock Center. Oregon-R or *u¹¹⁸* (FBst0006326) were used as wild type controls.

Phenotypic analyses. Flies were maintained and crossed as described in reference 18. Wing preparations, wing size measurements and wing cell density measurements were performed as described in reference 37.

Molecular biology. Isolation of genomic DNA from flies, primer design, PCRs, SNP identification and genotyping, DNA sequencing, and sequence analysis were performed as described in references 18 and 37. For the *UAS-ebi^{IR}* transgenics, a 553 bp region within the 2nd exon of *ebi* was amplified and inserted in the pWIZ vector³⁸ as a head-to-head inverted repeat in a two-step cloning procedure. The insert was verified by DNA sequencing and used to transform flies using standard methods. Primers and SNPs are available upon request.

Immunofluorescence. Imaginal disc and polytene chromosome dissection and immunolabeling were performed as

described in reference 39. Primary antibodies used were: mouse anti-WG 1:500 (Developmental Studies Hybridoma Bank), mouse anti-EBI 1:1,000,² rabbit anti-βgal 1:10,000 (Cappell), rabbit anti-SMR 1:100,²⁷ and rat anti-SU(H) 1:100.⁴⁰ Alexa Fluor secondary antibodies and TO-PRO-3 were obtained from Invitrogen. Imaging was performed using a Zeiss LSM 510 confocal microscope. Images were processed in ImageJ and assembled using Adobe Illustrator.

Bioinformatics. The EBI protein sequence was obtained from FlyBase⁴¹ and functional domains were predicted using Pfam,⁴² Prosite⁴³ and SMART.⁴⁴ Results from SMART are presented herein. Modelling of EBI C-terminal structure (Fig. 1C) was based on the structure of the beta-subunit of the heterotrimeric G-protein transducin (Protein Data Bank ID: 1GOT).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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