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# An NRSF/REST-like repressor downstream of Ebi/SMRTER/Su(H) regulates eye development in *Drosophila*

**Leo Tsuda[1,4,*], Masako Kaido[1], Young-Mi Lim[1,4], Kagayaki Kato[1], Toshiro Aigaki[2] and Shigeo Hayashi[1,3,*]**

[1]Morphogenetic Signaling Group, Riken Center for Developmental Biology, Minatojima-Minamimachi, Chuo-ku, Kobe, Hyogo, Japan, [2]Department of Biological Sciences, Tokyo Metropolitan University, Hachioji-shi, Tokyo, Japan and [3]Department of Life Science, Kobe University Graduate School of Science and Technology, Kobe, Japan

**The corepressor complex that includes Ebi and SMRTER is a target of epidermal growth factor (EGF) and Notch signaling pathways and regulates Delta (Dl)-mediated induction of support cells adjacent to photoreceptor neurons of the *Drosophila* eye. We describe a mechanism by which the Ebi/SMRTER corepressor complex maintains *Dl* expression. We identified a gene, *charlatan* (*chn*), which encodes a C2H2-type zinc-finger protein resembling human neuronal restricted silencing factor/repressor element RE-1 silencing transcription factor (NRSF/REST). The Ebi/SMRTER corepressor complex represses *chn* transcription by competing with the activation complex that includes the Notch intracellular domain (NICD). Chn represses *Dl* expression and is critical for the initiation of eye development. Thus, under EGF signaling, double negative regulation mediated by the Ebi/SMRTER corepressor complex and an NRSF/REST-like factor, Chn, maintains inductive activity in developing photoreceptor cells by promoting *Dl* expression.**

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## Introduction

Highly ordered arrays of neuronal cells in sensory organs and in the central nervous system are produced by sequential inductive events involving intercellular signaling and cell fate determination (Stern, 2001). Intercellular signaling coordinates temporal and spatial expression of genes required for neuronal differentiation (Lee and Pfaff, 2001). The Notch signaling pathway plays important roles in the regulation of cell differentiation, proliferation and apoptosis in many developmental systems (reviewed in Lai, 2004). Binding of a ligand to the Notch receptor induces proteolytic cleavage of the transmembrane domain, with the subsequent release of the Notch intracellular domain (NICD) (Struhl and Adachi, 1998). NICD translocates to the nucleus where it forms a complex with the transcription factor, Suppressor of Hairless (Su(H)), which then activates gene transcription (Struhl and Adachi, 1998). In the absence of activated Notch, Su(H) acts as a transcriptional repressor of various genes involved in the development of mechanosensory cells and photoreceptor neurons in *Drosophila* (Barolo *et al*, 2000; Li and Baker, 2001). This Su(H)-mediated repression system is evolutionarily conserved. A human homolog of Su(H), RBPJκ, acts as either a transcriptional activator or repressor for many types of genes, including *IL-6*, *Hes1*, *p52/NFκB2* and *glial fibrillary acidic protein* (*GFAP*) (Kannabiran *et al*, 1997; Kao *et al*, 1998; Oswald *et al*, 1998; Hermanson *et al*, 2002).

RBPJκ-mediated repression requires the nuclear receptor corepressor (N-CoR) (Horlein *et al*, 1995) and/or the closely related silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) (Chen and Evans, 1995) to recruit histone deacetylases to target promoters (Kao *et al*, 1998; Hermanson *et al*, 2002; reviewed in Jepsen and Rosenfeld, 2002). N-CoR/SMRT corepressor function requires its association with transducin β-like 1 (TBL1; Guenther *et al*, 2000; Yoon *et al*, 2003; Perissi *et al*, 2004). TBL1 is an evolutionarily conserved F-box/WD40-repeat-containing protein originally identified as a candidate gene responsible for X-linked ocular albinism with late-onset sensorineural deafness (Bassi *et al*, 1999; Dong *et al*, 1999). It was recently shown that TBL1 function is not limited to transcriptional repression; it also converts transcriptional cofactors between repressive and stimulative states (Perissi *et al*, 2004).

The *Drosophila* homolog of TBL1, Ebi, was first identified as a downstream component of epidermal growth factor receptor (EGFR) signaling during eye development (Dong *et al*, 1999). Ebi activity in photoreceptor cells promotes expression of a Notch ligand, Delta (Dl), which is subsequently used to convert adjacent non-neural cells to cone cell fate (Flores *et al*, 2000; Tsuda *et al*, 2002). EGFR positively regulates *Dl* in photoreceptor cells, whereas Notch downregulates *Dl* (Tsuda *et al*, 2002). Previous studies have shown that a protein complex that includes Ebi, SMRTER (N-CoR/SMRT-related molecule in *Drosophila*; Tsai *et al*, 1999), Su(H) and strawberry notch (Sno; Majumdar *et al*, 1997) mediates EGFR signaling and positively regulates *Dl* expression (Tsuda *et al*, 2002). It is not clear how the protein complex containing Ebi and SMRTER, which primarily acts as a repressor, stimulates *Dl* because direct targets of this complex have not yet been identified.

*Corresponding author. L Tsuda, Department of Mechanism of Aging, National Institute for Longevity Sciences, 36-3 Gengo, Morioka-cho, Obu-city, Aichi 474-8522, Japan. Tel.: +81 562 46 2311 ext. 5054; Fax: +81 562 46 8461; E-mail: ltsuda@nils.go.jp or S Hayashi, Morphogenetic Signaling Group, Riken Center for Developmental Biology, 2-2-3, Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan. Tel.: +81 78 306 3185; Fax: +81 78 306 3183; E-mail: shayashi@cdb.riken.jp
[4]Present address: Department of Mechanism of Aging, National Institute for Longevity Sciences, Obu, Aichi 474-8522, Japan

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In this study, we report the identification of a gene, *charlatan* (*chn*), that is a direct target of the Ebi/SMRTER corepressor complex. *Chn* encodes a C2H2-type zinc-finger protein and was originally identified as a gene required for normal development of the peripheral nervous system (PNS; Kania *et al*, 1995). We found that Chn shares several characteristics with human neuronal restricted silencing factor/repressor element RE-1 silencing transcription factor (NRSF/REST), a repressor of various neuron-restricted genes (reviewed in Jones and Meech, 1999). *Dl* was identified as a direct target of Chn repressor. Taken together, these results show that Ebi/SMRTER/Su(H) corepressor complex mediates the crosstalk between EGFR and Notch by downregulating the NRSF/REST-like repressor, Chn, during photoreceptor cell development.

## Results

### *Ebi modulates the dual role of Su(H) as a repressor and an activator of chn expression during eye development*

The corepressor complex that includes Ebi, SMRTER and Su(H) is required for expression of *Dl* in *Drosophila* photoreceptor cells (Tsuda *et al*, 2002). To identify genetic loci that are transcriptionally repressed by the Ebi corepressor, we set up a screen using an ectopic gene expression system (Gene Search System; Toba *et al*, 1999). Insertion of a *Gene Search* (*GS*) vector, a modified P-element carrying the Gal4 upstream activating sequence ($UAS_G$) near its 3′ end, causes overexpression of a nearby gene under the control of the Gal4-$UAS_G$ system (Toba *et al*, 1999). We identified *GS* insertions into the *chn* locus (Kania *et al*, 1995), whose overexpression phenotype in the eye using an eye-specific Gal4 driver (*GMR-Gal4*) was modified by reducing *ebi* activity (Figure 1D and E). We thus studied the regulation of *chn* by Ebi-dependent transcriptional repression.

In third instar larval-stage eye discs, the *chn* transcript was highly expressed in the morphogenetic furrow (MF), where photoreceptor differentiation initiates, but was downregulated in cells in the later stage photoreceptor development (Figures 2A, A′ and 7D). In *ebi* mutant eye discs, however, *chn* expression became detectable in differentiating photoreceptor cells, and its expression in the MF was increased (Figure 2B and B′), suggesting that Su(H) in association with Ebi and SMRTER represses *chn* transcription in the eye disc.

To reveal the role of Su(H) as an activator, we examined *chn* expression when the level of *Su(H)* expression was reduced. It was shown previously that removing one copy of *Su(H)* suppresses the loss-of-*Dl* expression phenotype in *ebi* mutants (Figure 2F–H; Tsuda *et al*, 2002). We found that reducing one copy of *Su(H)* suppresses ectopic *chn* expression in *ebi* mutants (Figure 2C and C′), suggesting that ectopic expression of *chn* in *ebi* mutants (Figure 2B) is Su(H)-dependent. RT–PCR analysis of *chn* expression in *ebi*$^{-}$ eye discs differing in the dosage of *Su(H)* gene also supported these results (Supplementary Figure 1). Strong reduction of *Su(H)* expression alone reduced expression of *chn* in the MF, which became weaker and was slightly broader (Figure 2D). The phenotype of *ebi*, *Su(H)* double mutants was almost the same as *Su(H)* single mutants (Figure 2E, compare with B and D), suggesting that Su(H) acts as an activator in the

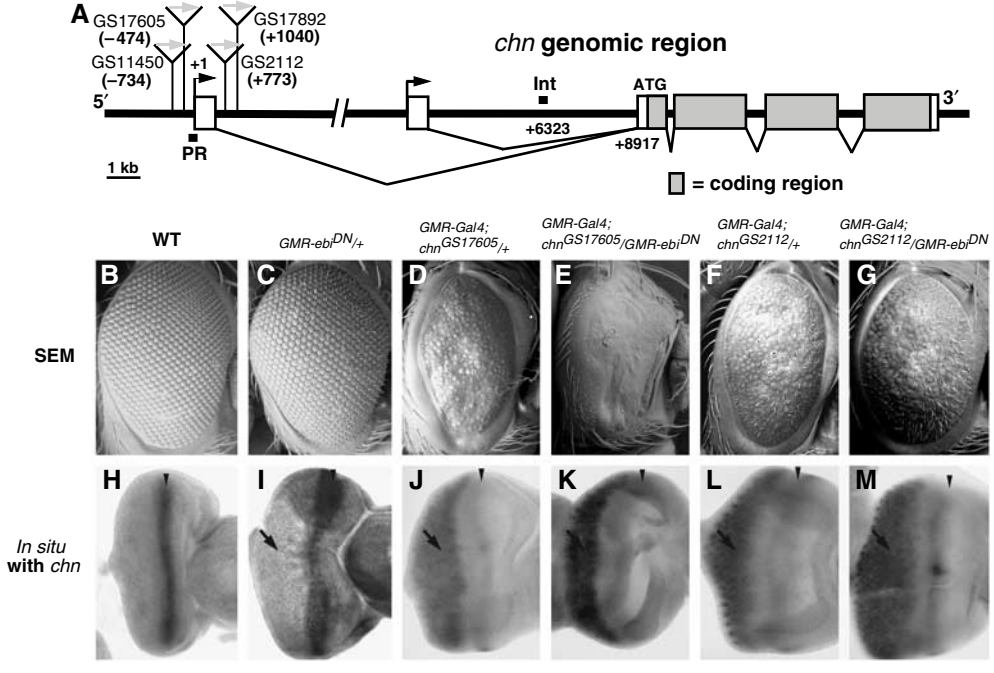


**Figure 1** Mapping of the target site of Ebi-dependent repression in the *chn* promoter. (**A**) Genomic region of *chn*. Boxes indicate exons, and filled portions represent the coding region. The locations of the four *GS* insertion sites are indicated with gray arrows. PR and Int represent the regions used for ChIP analysis. (**B–G**) Scanning electron micrographs of adult eyes. (**H–M**) *chn* mRNA expression patterns in eye imaginal discs. Samples shown in panels H and I were processed with a four-fold longer reaction time for RNA detection. One copy of *GMR-ebi*$^{DN}$ caused a slight increase in *chn* expression (I) and a mild rough eye phenotype (C). Both *chn*$^{GS17605}$ and *chn*$^{GS2112}$, driven by *GMR-Gal4*, caused abnormal eye morphologies (D, F) as well as ectopic expression of *chn* posterior to the MF (J, L, arrows). *GMR-ebi*$^{DN}$ markedly enhanced both the abnormal eye phenotype (E) and expression of *chn*$^{GS17605}$ (K). The phenotype caused by *chn*$^{GS2112}$ was not significantly altered by *GMR-ebi*$^{DN}$ (G, M). Anterior is at right. Arrowheads in panels H–M indicate the MF, and arrows indicate the region of ectopic *chn* expression.

 

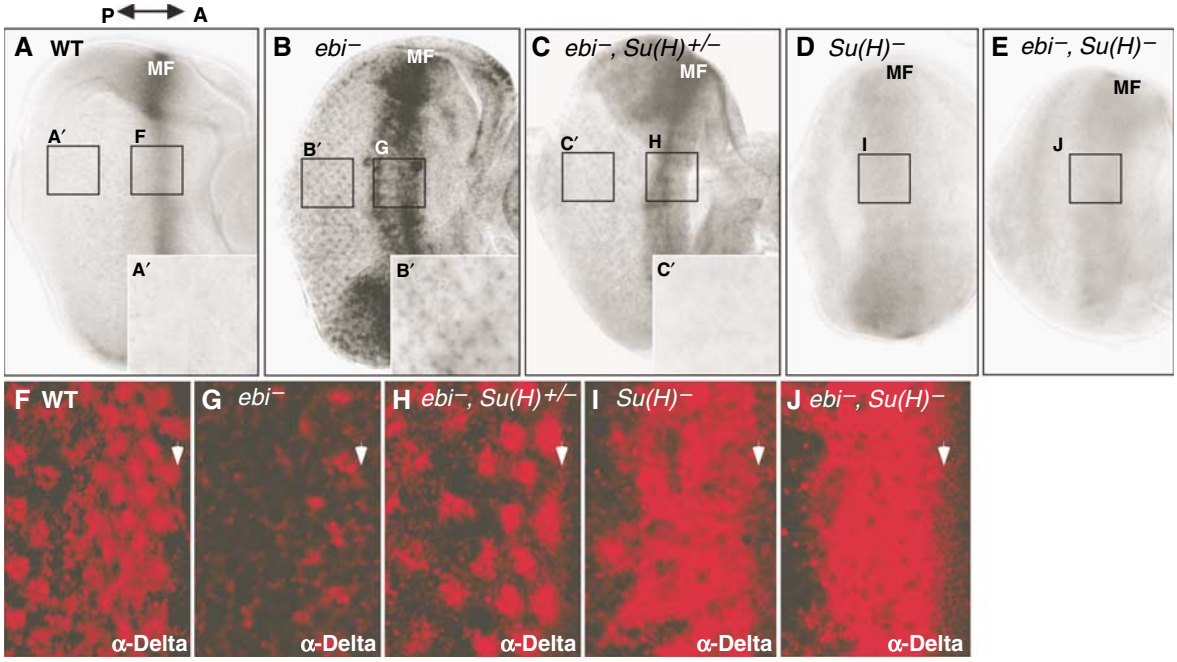


**Figure 2** Regulation of *chn* and *Dl* expression by *ebi* and *Su(H)*. (A–E) *In situ* hybridization patterns of *chn* mRNA expression in late third instar eye imaginal discs. The double arrow indicates anterior (A) and posterior (P). (**A**) In wild-type discs, *chn* is highly expressed around the MF. *chn* expression was reduced in differentiating ommatidia (**A′**). (**B**) $ebi^{P7}/ebi^{E4}$. In *ebi* mutants, *chn* expression was increased around the MF, and ectopic *chn* expression became detectable in differentiating photoreceptor cells (**B′**). (**C**) $ebi^{P7}Su(H)^{2}/ebi^{E4}, +$. (**D**) $Su(H)^{1}/Su(H)^{2}$. (**E**) $ebi^{P7}Su(H)^{2}/ebi^{E4}Su(H)^{1}$. (F–I) *Dl* expression was monitored by staining with anti-Dl. (**F**) Dl was detected in the MF (arrowhead) and in differentiating photoreceptor cells in wild-type eye discs. (**G**) In the *ebi* mutant, Dl levels were reduced in the MF and in photoreceptor cells. (**H**) $ebi^{P7}Su(H)^{2}/ebi^{E4} +$. (**I**) $Su(H)^{1}/Su(H)^{2}$. (**J**) $ebi^{P7}Su(H)^{2}/ebi^{E4}Su(H)^{1}$.

absence of Ebi. This might be due to dual functions of Su(H) as an activator or repressor (Barolo *et al*, 2000; Li and Baker, 2001). Hence, reducing the amount of Ebi in the corepressor complex involving Su(H) might convert Su(H) to an activator by permitting the replacement of the corepressor complex with NICD. The following molecular data support this hypothesis.

### *Mapping of the target site of Ebi-mediated repression in the chn promoter region*

To reveal the molecular nature of transcriptional regulation of *chn* by Su(H), we searched for Su(H) target sites in the genomic region of *chn* (Figure 1A). As Su(H) binds slightly degenerate sequences (Flores *et al*, 2000), it was not easy to identify the functional Su(H) binding region from a simple genomic search. We thus took an alternative approach to map the *chn* genomic region, which is regulated by Su(H) in the normal chromosomal context. Ebi-mediated repression involves SMRTER, a corepressor that recruits histone deacetylases and induces the formation of inactive chromatin (Tsai *et al*, 1999), which spreads from the site where Su(H) recruits the corepressor complex. Promoters near the Su(H)-binding site are thus expected to be downregulated in an Ebi-dependent manner. We identified four insertion lines of the *GS* vector in the *chn* promoter region (Figure 1A). All these *GS* lines caused ectopic expression of *chn* with consequent abnormal eye morphology when they were crossed with *GMR-Gal4* (Figure 1D, F, J and L; data not shown). If the effect of the Ebi/SMRTER corepressor complex reaches the $UAS_G$ in those insertions, reduction of Ebi activity will derepress $UAS_G$ and further enhance activation by *GMR-Gal4*. One copy of a dominant-negative construct of *ebi* (*GMR-*$ebi^{DN}$; Dong *et al*, 1999) caused only a mild defect in eye morphology and weak, if any, ectopic expression of *chn* (Figure 1C and I). *GMR-*$ebi^{DN}$ strongly enhanced the overexpression phenotype of $chn^{GS17605}$ and $chn^{GS11450}$, which contained *GS* vector insertions (−474 and −734, respectively) upstream of the transcriptional start site (Figure 1A, and compare E and K to D and J; data not shown). However, *GMR-*$ebi^{DN}$ failed to enhance the overexpression phenotype of other *GS* lines ($chn^{GS2112}$ and $chn^{GS17892}$) that were inserted downstream (+773 and +1040, respectively) of the first exon (compare Figure 1G and M to F and L; data not shown). As a control, we determined whether *GMR-*$ebi^{DN}$ affects the overexpression phenotype of $UAS_G$-*chn* cDNA inserted outside of the *chn* locus. No strong genetic interaction was observed (data not shown). From these results, we concluded that Ebi-dependent transcriptional repression is targeted to the proximity of the transcriptional initiation site of the *chn* promoter.

### *Ebi and SMRTER associate with the chn promoter in vivo*

We searched for potential Su(H)-binding sites around the transcriptional initiation site of *chn* and found two sequences, S1 and S2, with similarity to the consensus sequences for Su(H) binding (5′-RTGRGAR-3′; Nellesen *et al*, 1999) within the fragment PR(*chn*) (Figures 1A, 3A and B). The S1 and S2 sites were indeed bound by His-Su(H) in an electrophoretic mobility shift assay (EMSA; Figure 3C). The binding was competed out by unlabeled oligonucleotides containing S1, S2 or a consensus Su(H) sequence (E(spl)mγ), but not by

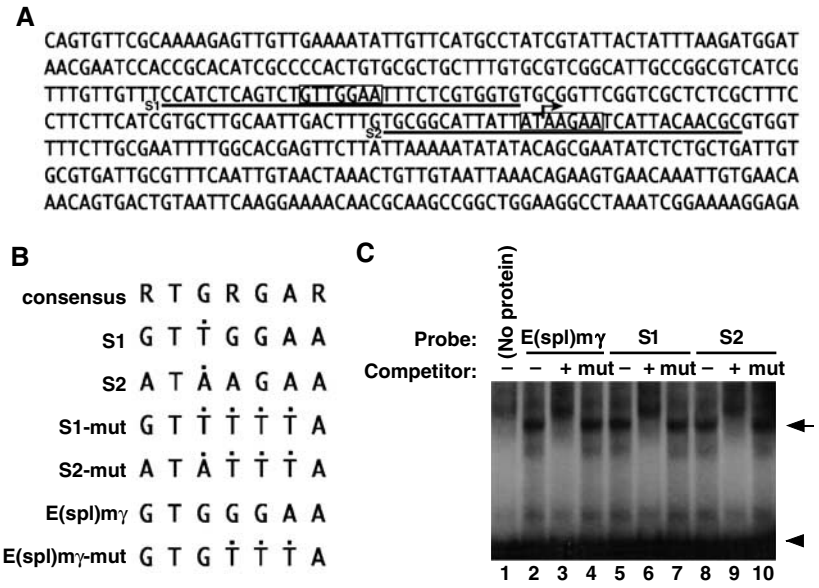


**Figure 3** Su(H) binds to the promoter region of *chn*. (**A**) The sequence of PR(*chn*) in the promoter region of *chn* (Figure 2A). Degenerate Su(H)-binding consensus sites are boxed (S1 and S2, sequences used for EMSA, are underlined). The arrow represents a putative transcriptional initiation site. (**B**) Core sequences of oligonucleotides used for EMSA. Black dots indicate positions that differ from the Su(H)-binding consensus (5′-RTGRGAR-3′). (**C**) EMSA using a His fusion of recombinant Su(H). E(spl)mγ is a positive control previously known to bind to Su(H) (lane 1, arrow). Ten-fold excess of unlabeled probe inhibited complex formation (lane 2), but the mutant form of the probe failed to compete (lane 3). S1 and S2 sequences also formed a complex with Su(H) (lanes 4 and 7). Ten-fold excess of unlabeled probes inhibited complex formation (lanes 5 and 8), but competitor containing consensus sequence mutations abrogated the competition (lanes 6 and 9). Arrowhead represents a position of free probe.

mutant forms of S1, S2 or E(spl)mγ sequences (Figure 3B and C), suggesting that Su(H) binds specifically to S1 and S2 sites.

We used chromatin immunoprecipitation (ChIP) to test whether PR(*chn*) is a target of Ebi-mediated repression *in vivo*. Eye disc chromatin from third instar larvae was fixed and immunoprecipitated by anti-Ebi. This procedure efficiently recovered fragment PR(*chn*) but not the intron fragment of *chn* genomic region Int(*chn*), which was used as a negative control (Figure 4A). Using the same procedure, we detected the association of SMRTER with PR(*chn*), as expected because Ebi and SMRTER associate with Su(H) (Tsuda *et al*, 2002). Association of Ebi and SMRTER was also detected using the promoter region of *hsp27*(*hsp27*), to which SMRTER is recruited by the ecdysone receptor (Figure 4A; Tsai *et al*, 1999; Sawatsubashi *et al*, 2004).

### *Mutually exclusive binding of NICD and Ebi/SMRTER to the Su(H)-binding site of the chn promoter*

We showed that *chn* is positively regulated by Su(H) at the MF (Figure 2). We therefore analyzed whether NICD, a positive regulator of Su(H) under control of Notch signaling, is also recruited to PR(*chn*) in the eye disc. Using the antibody against NICD, we showed that NICD is indeed recruited to the promoter region of *E(spl)mδ*,which is shown to be a target site of NICD/Su(H) in the eye imaginal disc (Cooper and Bray, 1999; Figure 4A). We also detected that NICD is recruited to PR(*chn*) and but not to Int(*chn*) or *hsp27* (Figure 4A). To determine the relationship between NICD and the Ebi/SMRTER complex bound to PR(*chn*), we conducted a sequential ChIP analysis (Figure 4B). PR(*chn*) was specifically recovered after two rounds of immunoprecipitation—with anti-Ebi followed by anti-SMRTER—although the microsatellite sequence, which was used for negative control, was not, suggesting that Ebi and SMRTER form a complex bound to PR(*chn*) in the eye disc. On the other hand, sequential immunoprecipitation with anti-Ebi and anti-NICD failed to recover PR(*chn*) (Figure 4B). These results suggest that an activator, NICD, and a corepressor, Ebi/SMRTER, are targeted to a common Su(H)-binding site, PR(*chn*), in the *chn* promoter in a mutually exclusive manner.

### *chn encodes a C2H2-type zinc-finger protein with similarity to human NRSF/REST*

Chn is a 1108-amino-acid protein with multiple C2H2-type zinc-finger motifs (Figure 5A; Escudero *et al*, 2005; Reeves and Posakony, 2005). Although we could not detect any highly homologous gene within the mammalian genome using BLAST, we found a small sequence of similarity between the N-terminal zinc-finger motif of Chn and the fifth zinc-finger of human NRSF/REST (Figure 5B). We found that Chn has several structural and functional similarities to human NRSF/REST, as follows. First, Chn and NRSF/REST each contain an N-terminal region with multiple zinc-finger motifs (five motifs in 264 residues in Chn and eight motifs in 251 residues in NRSF/REST), followed by a cluster of S/T-P motifs (serine or threonine followed by a proline) and a single zinc-finger motif at the C terminus (Figure 5A). Second, the C-terminal region of NRSF/REST binds a corepressor, CoREST, which serves as an adaptor molecule to recruit a complex that imposes silencing activities (Andres *et al*, 1999; Lunyak *et al*, 2002). We found that the *Drosophila* homolog of CoREST (dCoREST) (Andres *et al*, 1999; Dallman *et al*, 2004) can associate with the C-terminal half of Chn in cultured S2 cells (Figure 5C). Finally, NRSF/REST binds to *NRSE/RE1*, a 21-bp sequence located in the promoter region of many types of neuron-restricted genes, via the N-terminal zinc-

 

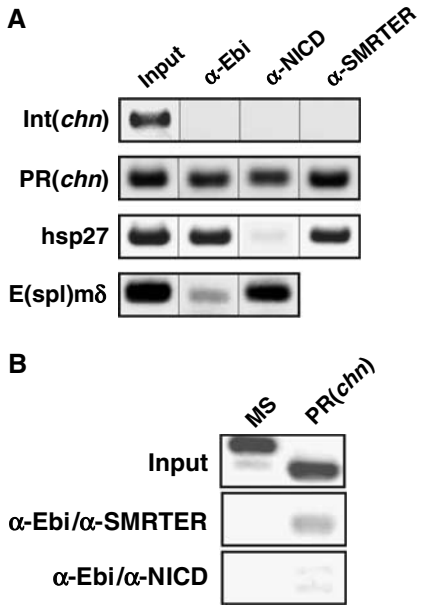


**Figure 4** Occupancy of the *chn* promoter by Ebi, SMRTER and NICD *in vivo*. (**A**) ChIP assay of third instar eye imaginal discs using anti-Ebi, anti-SMRTER or anti-NICD. PR(*chn*) and Int(*chn*) are derived from the *chn* genomic region (Figures 1A and 3A), and hsp27 is a fragment of the *hsp27* promoter region (Tsai *et al*, 1999; Sawatsubashi *et al*, 2004). E(spl)mδ is a fragment of the *E(spl)mδ* promoter region (Cooper and Bray, 1999). (**B**) Sequential ChIP assay. Crosslinked chromatin was immunoprecipitated with anti-Ebi followed by a second immunoprecipitation with anti-SMRTER or anti-NICD. MS is the sequence from microsatellite DNA (Sawatsubashi *et al*, 2004). Although anti-SMRTER precipitated PR(*chn*) from Ebi-containing chromatin, anti-NICD did not.

finger motifs (Chong *et al*, 1995; Schoenherr and Anderson, 1995). We found that a recombinant protein containing the N-terminal zinc-fingers of Chn bound specifically to the *NRSE/RE1* sequence *in vitro* (Figure 5D, left panel). Thus, the structural similarity to NRSF/REST, binding to dCoREST and the DNA-binding specificity of Chn suggest that it is a candidate for a functional *Drosophila* homolog of NRSF/REST.

### *Identification of putative Chn target genes*

If Chn acts as a regulator of neural-related functions, as suggested for NRSF/REST, then Chn would be expected to bind to a regulatory region common to many types of neural-related genes in *Drosophila*. We identified numerous sequences similar to *NRSE/RE1* in the *Drosophila* genome, and their binding to Chn was assessed by EMSA (data not shown). Using these sequences, we derived a consensus binding sequence for Chn (Chn-binding element (CBE), 5′-BBHASMVMMVCNGACVKNNCC-3′). We searched for and identified 26 CBEs within 10 kb of annotated genes from the *Drosophila* genome (version 3.2) (Table I). Binding to Chn was confirmed for 18 CBEs using EMSA competition assay (Table I). Genes containing the CBE include dopamine receptor 2 (*DopR2*) and the potassium channel, *ether-a-go-go*, for which the mammalian homologs are target genes of NRSF/REST (Schoenherr *et al*, 1996; Chen *et al*, 1998; Lunyak *et al*, 2002; Bruce *et al*, 2004). These results suggest that the CBE is a good indicator of Chn binding sites and that Chn regulates many types of neural-related genes, as is implicated for NRSF/REST (Jones and Meech, 1999; Bruce *et al*, 2004). However, we found that divergent forms of CBE adjacent to *hairy* and *extra-macrochaetae* were bound specifically by Chn (Table I, asterisks). Likewise, some of the CBE sites failed to bind to Chn. Thus, a further refinement will be necessary to predict a definitive set of Chn binding sites in the *Drosophila* genome.

### *Chn represses Dl in photoreceptor neurons*

We found that the genomic region of *Dl* has two divergent CBEs (Table I), suggesting that *Dl* is a target of Chn (Figure 5D, right panel; data not shown). *Dl* was normally expressed in photoreceptor cells, where *chn* is repressed (Figure 2A and F). Upon loss of *ebi* function, *chn* was ectopically expressed and Dl levels were reduced (Figure 2B and G, and Supplementary Figure 1). *Dl* expression was elevated in a double mutant of *ebi* with *Su(H)* or with mutant *Su(H)*, in which *chn* expression in the MF was reduced (Figure 2D, E, I and J). The apparent inverse relationship between *chn* and *Dl* expression suggests that Chn might repress *Dl*. To confirm this hypothesis, we ectopically expressed *chn* in photoreceptor cells after commitment to neuronal differentiation and monitored the resultant Dl levels. Ectopic expression of *chn* reduced Dl levels (Figure 6D, compare to A). Furthermore, Cut expression in cone cells, which requires Dl as an inductive signal from photoreceptor cells (Flores *et al*, 2000; Tsuda *et al*, 2002), was strongly reduced (Figure 6F, compare to C), and cone cells failed to develop as evidenced by defective lens secretion owing to overexpression of *chn* (Figure 1D and F). In contrast, Cut expression in glia, which are located beneath the photoreceptor cells and recruited from the brain region (Hummel *et al*, 2002), was not affected (Figure 6I and J). Despite the strong reduction in the Dl level, *chn* overexpression did not affect the expression of the neuronal marker Elav (Figure 6E). These results indicate that *chn* specifically blocks cone cell induction by photoreceptor cells by repressing *Dl* while leaving neuronal differentiation intact. Using ChIP assays, we detected preferential association of Chn with the divergent CBEs in the *Dl* genomic region (Dl CBE1 and CBE2; Table I and Figure 5E), whereas no significant association with Ebi was detected, which was used as a negative control (Figure 5E). These results suggest that Chn can interact with CBEs on the genomic region of *Dl* and regulate the expression level of *Dl*.

The C-terminal zinc-finger motif of NRSF/REST is essential for its repression activities (Tapia-Ramirez *et al*, 1997). We found that a point mutation introduced into the C-terminal zinc-finger motif of Chn (Figure 5A, $chn^{C1049A}$) abrogated its ability to repress *Dl* expression (Figure 6G, compare to D), demonstrating another correlation between Chn and NRSF/REST.

### *Double-negative regulation of Dl by Ebi and Chn*

To obtain *in vivo* evidence for the role of Ebi-mediated *chn* repression in eye development, we isolated new *chn* mutations (see Materials and methods). $chn^{1}$ contained a small deletion that caused a frame shift after amino acid 94, likely yielding a null allele (Figure 5A). Interference of Ebi function in photoreceptor cells by the strong hypomorphic allele of *ebi* abrogated *Dl* expression (Figure 2F and G); however, redu-

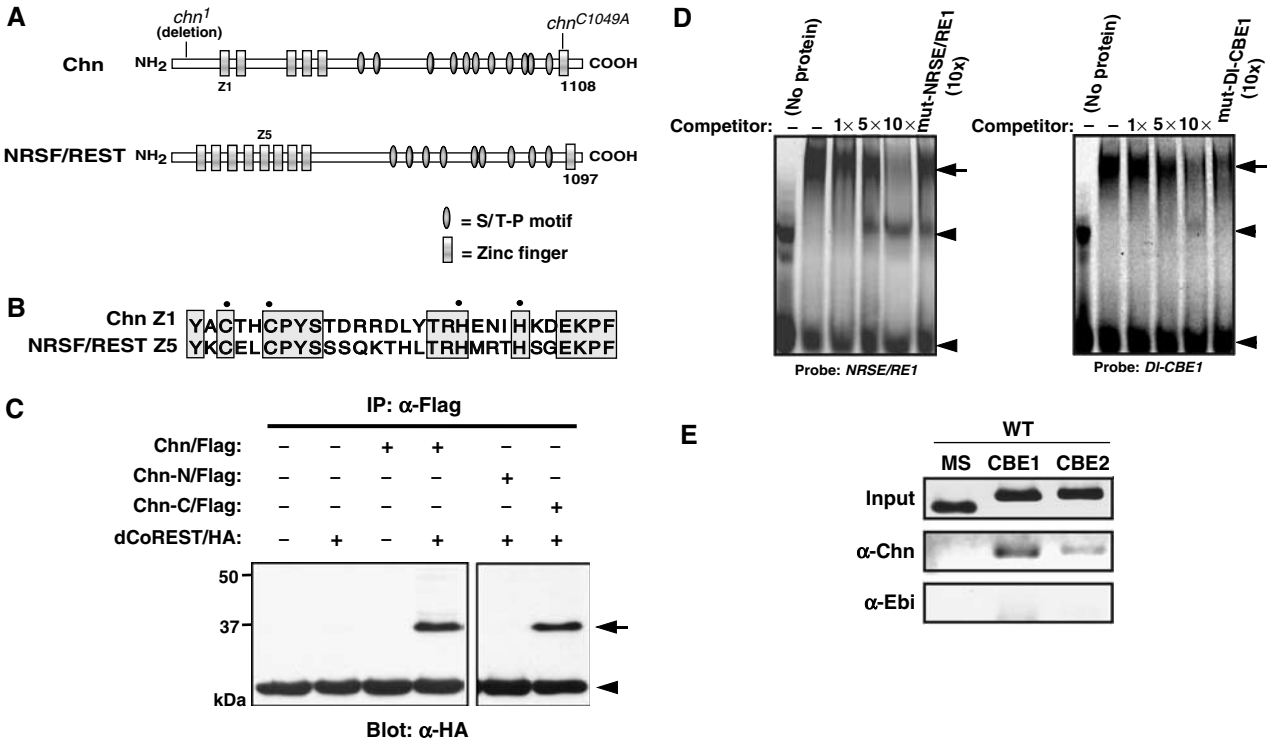


**Figure 5** Common properties of Chn and NRSF/REST. (**A**) Schematic diagram of Chn and human NRSF/REST. Mutation sites of EMS-induced (*chn*$^{1}$) and mutagenized (*chn*$^{C1049A}$) *chn* are shown above the Chn diagram. (**B**) Sequence comparison between the first zinc-finger motif in Chn (Chn Z1) and the fifth zinc-finger motif in human NRSF/REST (NRSF/REST Z5). (**C**) Immunoprecipitation assay. Chn and dCoREST associate in cultured S2 cells. Chn-N/Flag and Chn-C/Flag contain residues 1–654 and 655–1108, respectively, and are tagged with a single Flag epitope at the C-terminus. dCoREST/HA contains the smallest splice variant of dCoREST, which has EML2 and SANT domains (Dallman *et al*, 2004) and a single HA epitope at the N-terminus. The arrow indicates the position of dCoREST/HA. The arrowhead indicates the position of IgG. (**D**) Chn binds to the *NRSE/RE1* and *Dl-CBE1* sequences. EMSA was performed using *NRSE/RE1*, a 21-bp recognition sequence of NRSF/REST (Table I), or *Dl-CBE1* (Table I) as a probe. A GST fusion of N-terminal zinc-finger domains of Chn formed a specific complex with the probe (arrow). Arrowheads denote a nonspecific shifted band (upper) and free probe (lower). Up to 10-fold molar excess of unlabeled probe was used as competitor. The mutant forms of *NRSE/RE1* or *Dl-CBE1* failed to compete. (**E**) ChIP assay of third instar eye imaginal discs. Anti-Chn preferentially precipitated the genomic regions containing CBE1 (Dl CBE1) or CBE2 (Dl CBE2) (Table I), but anti-Ebi did not (WT).

cing one copy of *chn* completely suppressed this phenotype (Figure 6K and L). This strong genetic interaction between *ebi* and *chn* suggests that the downregulation of *chn* activity in photoreceptor cells seems to be responsible for the proper development of photoreceptor neurons. Combined with the strong repression of *Dl* by Chn, these results suggest that an important function of Ebi in photoreceptor cells is to repress *chn* to permit expression of *Dl*; this, in turn, induces cone cell differentiation.

To determine if Chn is required at the stage when photoreceptor cells express *Dl* to induce cone cell development, we studied phenotypes of small clones of *chn*$^{1}$ cells. The *chn*$^{1}$ clones present within or behind the MF were normal, as assessed by Elav expression, ommatidium assembly and progression of the MF (data not shown), suggesting that eye development can proceed without *chn* function once the MF has initiated. Although *chn* is highly expressed in the MF (Figure 2A), the apparent lack of a requirement for *chn* in MF progression might be owing to genes with overlapping functions or to a possible long half-life of Chn.

### *Chn-mediated repression of Dl is essential for the initiation of eye development*

In the eye disc at the early third instar, Chn was expressed at the edge of the eye disc, the site of MF initiation (Figure 7A). To address the role of *chn* in the early stage of the eye development, large clones of cells homozygous for *chn*$^{1}$ were induced in the eye by the FLP/FRT technique. Mutant animals had substantially smaller eyes (Figure 7B), similar to the mutant phenotype of *thick vein* (*tkv*; Figure 7C). Tkv is a receptor for Decapentaplegic and is required for the initiation of the MF (Borod and Heberlein, 1998), suggesting that *chn* is also required at the early stage of photoreceptor cell development. In eye discs bearing *chn*$^{1}$ mutant clones, the number of photoreceptor cells was greatly reduced and the remaining photoreceptor cells were always associated with Chn-expressing cells that escaped FLP recombination (Figure 7E, arrowheads and arrows). Although *chn*$^{1}$ is embryonic lethal, *chn*$^{1}$/*chn*$^{9}$ heterozygote yielded a few escapers that survived until the late third instar larval stage. Eye discs of those larvae were small, and the MF was absent (Figure 7G, compare to F). Only a few patches of Elav-positive neurons were observed, which apparently formed independently of the MF (Figure 7G, arrow).

These phenotypes suggest that eye development cannot initiate without *chn*. Furthermore, we found that Dl was substantially increased at the posterior edge of eye discs having large *chn*$^{1}$ mutant clones (Figure 7H, arrow). This ectopic expression of *Dl* likely accounts for the failure to initiate eye development, as a high level of *Dl* in cells autonomously

 

**Table I** Putative Chn target genes containing *CBE* and/or its variant sequences adjacent to the coding sequence, based on informatics and binding activity

| Genes (target sites) | Sequence comparison | Binding | Location/direction |
|---|---|---|---|
| (NRSE/RE1) | TTCAGCACCACGGACAGCGCC | + | |
| (CBE) | BBHASMVMMVCNGACVKNNCC | | |
| *Neural functions/expressions* | | | |
| Mnt | GTTACCGACGCCGACGGTACC | + | 1st intron/R |
| HMG protein Z | CCCACCCCCCCTGACCGACCC | + | 1st intron/R |
| allatostatin C receptor2 | TTTAGCCAAGCCGACAGATCC | – | 1st intron/R |
| Arrestin 2 | TGAAGACCCACGGACAGGGCC | + | 0 kb upstream/F |
| Olfactory-specific E | TCCACCCCCACCGACGTTCCC | + | 3 kb upstream/R |
| Dystroglycan | CTTAGAACAGCCGACGTCCCC | + | 1st intron/R |
| nAcRβ-64B | CGCACCCAAACTGACGGCTCC | – | 5′ UTR/F |
| Lar | CGAAGAACAACTGACGTAGCC | + | 4 kb upstream/F |
| ether-a-go-go | GTCACAGACACAGACCTAGCC | + | 1st intron/R |
| Dopamine receptor2 | CCAAGACAAACAGACGGCCCC | + | 3 kb downstream/F |
| kekkon-2 | GCCACCCACACCGACATTTCC | + | 5′ coding/F |
| shaking B | TTAACAGCACCTGACCGACCC | + | 2nd intron/F |
| unc-115 | TCTACACACCCCGACATGGCC | + | 0 kb upstream/R |
| brinker | GCAACAGCAGCAGACATTTCC | – | 3′ coding/F |
| bifid | GGCAGCGACGCTGACGTCGCC | + | 2nd intron/R |
| Deformed | TGCACCACCGCCGACGGCACC | + | 5′ coding/R |
| Socs 16D | TCCAGCGAACCGGACGGCTCC | – | 5th exon/F |
| Saliva | TTTAGCAAACCTGACATTGCC | – | 3rd intron/R |
| hairy (CBE1) | CGCAGCAACACAGACCGCCCC | + | 9 kb upstream/F |
| *hairy (CBE2) | GCCACCAAAAAAGACGTAAGT | + | 2nd exon/F |
| emc (CBE1) | TCCACCAAAGCAGACCTCTCC | + | 5′ UTR/F |
| *emc (CBE2) | CGCACCACCCATGACAGAGCC | + | 3 kb downstream/R |
| *Dl (CBE1) | TTCAGCACCACCGCCATTGGT | + | 2nd intron/F |
| *Dl (CBE2) | CCCAGCCCCACGGCCATATCC | + | 10 kb downstream/F |
| *Oogenesis/cytoskeleton* | | | |
| spire | TGAAGAAAAACCGACATAACC | – | 1st intron/F |
| orb | CCCACAGCAGCCGACCGACCC | + | 1st intron/R |
| lkb1 | TCAACAGAAACGGACAGATCC | – | 0.1 kb upstream/R |
| Death caspase-1 | CGCACCCACACGGACGTACCC | + | 1 kb upstream/R |
| D/V axis/defence | | | |
| Tehao | GCAACAAAAACTGACGTCTCC | – | 10 kb upstream/F |
| *Cardiac cell development* | | | |
| tincar | CCAACCCAACCTGACCTGACC | + | 7 kb upstream/R |

(+) Positive binding to Chn zinc-finger domain, based on EMSA. (–) Very weak binding, if detectable, to Chn zinc-finger domain. Asterisks represent variant CBE sequences. Black dots indicate positions that differ from the CBE consensus. F and R represent the forward and reverse directions, respectively, against the coding sequences of the candidate target genes. nAcRβ-64B, nicotinic acetylcholine receptor beta 64B; Socs16D, suppressor of cytokine signaling at 16D; emc, extra macrochaetae; orb, oo18 RNA-binding protein. Single letter abbreviations: B = C, G or T; H = A, C or T; S = G or C; M = A or C; V = A, C or G; N = A, G, C or T; K = G or T.

inhibits *Notch* (Jacobsen *et al*, 1998), which is required to initiate the MF (Kumar and Moses, 2001). On the other hand, *Dl* remained repressed in discs containing the *tkv* mutant clone (Figure 7I, arrow).

## Discussion

### *Functional similarity of Chn to human NRSF/REST*

Although it has been established that mammalian NRSF/REST is a key regulator of neuron-specific genes (Jones and Meech, 1999), attempts to isolate invertebrate homolog of NRSF/REST have so far failed to identify a true homologous factor in invertebrates (Dallman *et al*, 2004). The properties of Chn, including the similarity in DNA-binding specificity, association with CoREST and transcriptional repressor activity, suggest that Chn is a strong candidate for a functional *Drosophila* homolog of NRSF/REST. *chn* was originally identified by its requirement in the development of the PNS (Kania *et al*, 1995; Escudero *et al*, 2005; Reeves and Posakony, 2005). We identified a number of candidate target genes of Chn, a large fraction of which is implicated in neural function and/or gene expression (Table I). We expect that further analysis of these candidates will provide valuable information about *chn* function *in vivo*, which may be extended to the understanding of NRSF/REST.

### *Regulation of Dl and the initiation of eye development by chn*

The Chn mutation blocked eye development by preventing the initiation of MF, a process requiring Notch signaling (Kumar and Moses, 2001; Figure 7). This phenotype is likely owing to a loss of Notch function, because elevated *Dl* expression is known to block Notch signaling. The function of Chn during the early stage of eye development might be to regulate *Notch* signaling at an appropriate level by downregulating *Dl* (Figure 8B). It is possible that Chn-mediated modulation places a variety of Notch functions in eye (Baonza and Freeman, 2001; Kumar and Moses, 2001; Li and Baker, 2001; Tsuda *et al*, 2002; Kenyon *et al*, 2003; Baonza and Freeman, 2005) under the influence of EGFR signaling and provides flexibility in its regulation.

Although *chn* is expressed in the MF (Figure 2), our genetic analyses show that small clones of *chn* mutant cells permit

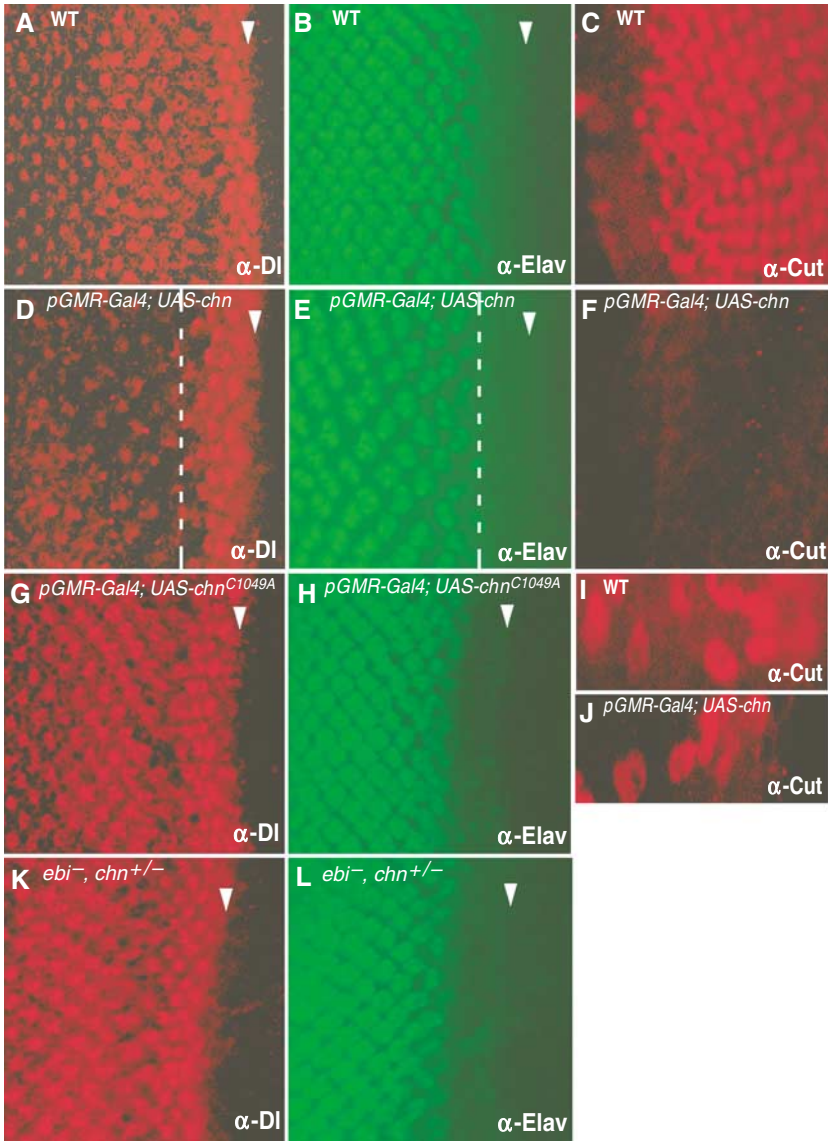


**Figure 6** *chn* represses *Dl* expression in photoreceptor cells. Eye imaginal discs in third instar larvae stained for Dl (**A**, **D**, **G**, **K**), Elav (a neuronal marker; **B**, **E**, **H**, **L**) or Cut (**C**, **F**, **I**, **J**). Panels A–C and I, wild type; panels D–F and J, *GMR-Gal4; UAS-chn/+*; panels G and H, *GMR-Gal4; UAS- chn$^{C1049A}$/+*. The Dl level was severely reduced by *chn* overexpression (D), whereas the Elav level was unchanged (E). The level of Cut in cone cells was reduced by *chn* overexpression (F) but was not affected in glia cells (I, J). A mutated form of *chn* (*chn$^{C1049A}$*) could not repress *Dl* expression (G), whereas the Elav level was unchanged (H). (K, L) *ebi$^{E4}$*, *chn$^{1}$/ebi$^{111}$*, +. Reducing a copy of *chn* suppressed the defect of *Dl* expression in the *ebi* mutant. Arrowheads indicate the MF. The broken white line represents the point at which *GMR-Gal4* expression began. Anterior is at right.

progression of the MF and photoreceptor differentiation (data not shown). We speculate that the repressive effect of Chn is overcome by other signals in the MF, such as hedgehog signaling, which strongly induces *Dl* (Greenwood and Struhl, 1999).

### *chn and the crosstalk between EGFR and Notch signaling*

Developing photoreceptor cells are exposed to the EGFR ligand, Spitz, and the Notch ligand, Dl, and each cell must assess the level of the two signals and respond appropriately to perform each task of photoreceptor cell specification and induction of non-neural cone cells. We investigated this question by studying the expression of Dl in photoreceptor cells. We identified *chn* as a direct target of Ebi/SMRTER-dependent transcriptional repression and as a repressor of *Dl* expression. The abrogated expression of *Dl* in *ebi* mutants was recovered by reducing one copy of *chn* (Figure 6K), suggesting that the negative regulation of *chn* by *ebi* is indeed prerequisite for photoreceptor cell development.

Our genetic data suggest that Su(H) may activate or repress *chn* expression (Figure 2). This idea is supported by data showing that Ebi/SMRTER and NICD are recruited to the promoter region of *chn* (Figure 4A). The Ebi/SMRTER complex formed in this region did not contain any detectable level of NICD, suggesting that the binding of Ebi/SMRTER and NICD to this region may be mutually exclusive (Figure 4B), and therefore we expect that a regulatory system controls the balance between the active and repressive

 

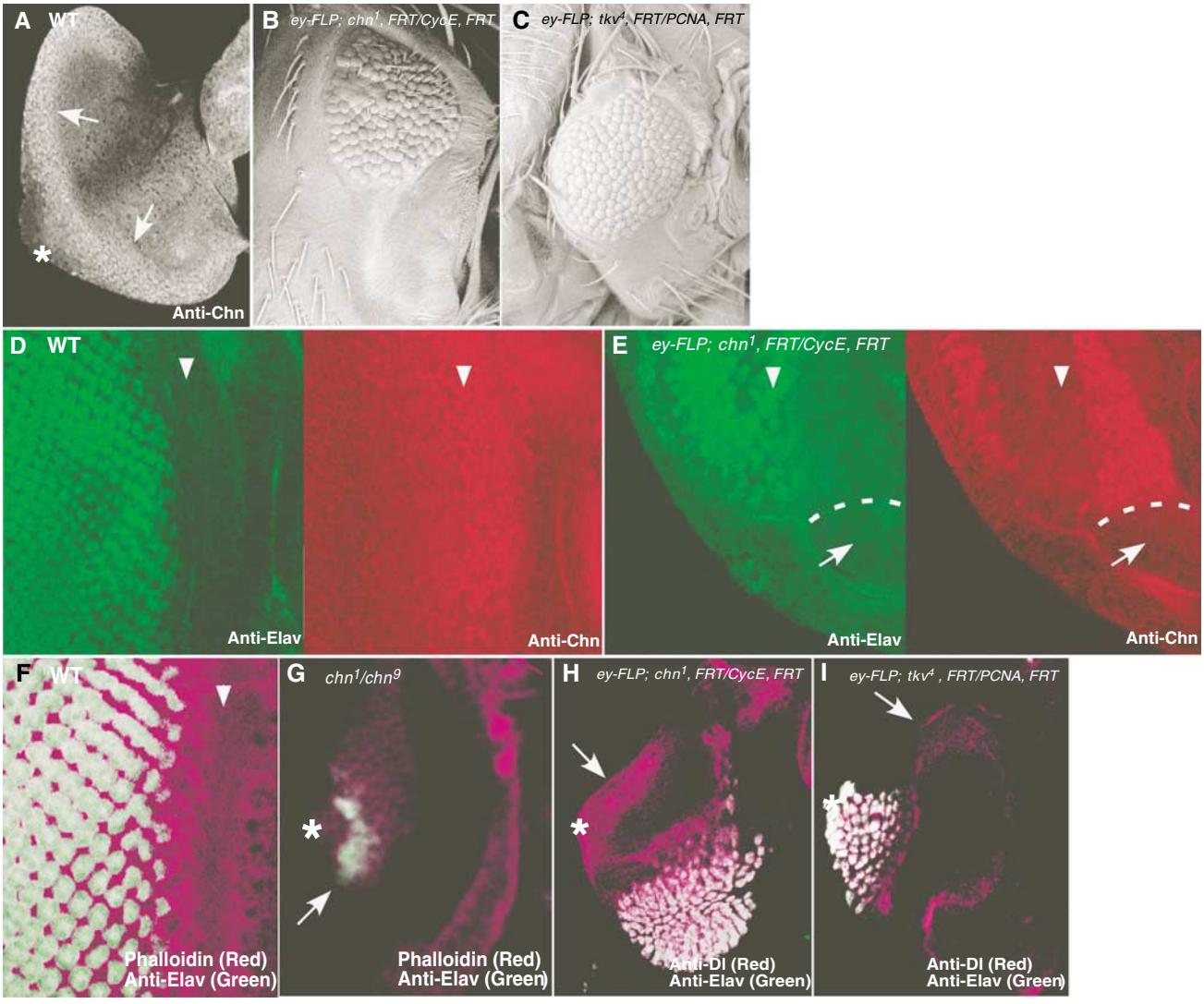


**Figure 7** *chn* is required for eye morphogenesis. (**A**) In wild-type eye discs from early third instar larvae, Chn expression was detected at the posterior edge of the disc using anti-Chn antibody (arrows). (**B, C**) Adult eye morphology. (B) *ey-FLP; chn*$^{1}$*, FRT42D/PCNA*$^{775}$*, FRT42D*. Large clones of *chn*$^{1}$ were formed and caused the small eye phenotype. (C) *ey-FLP; tkv*$^{4}$*, FRT40A/CycE*$^{AR95}$*, FRT40A*. A large clone of *tkv*$^{4}$ showed a similar eye defect. (**D**) In wild-type eye disc, Elav (green) was expressed behind the MF (left panel), marked with Chn (red; right panel). (**E**) In eye discs with large clones of *chn*$^{1}$, Elav-positive cells were greatly reduced in number (green; left panel, arrowheads) and were always associated with residual *chn* expression (red; right panel). White dots represent the border of *chn*$^{1}$ clone (arrows). (**F**) Wild-type eye disc stained with phalloidin (purple) and anti-Elav (green). (**G**) *chn*$^{1}$*/chn*$^{9}$. Strong *chn* mutations eliminated the MF, whereas a few Elav-positive cells can be observed (arrow). (**H**) In eye discs with large clones of *chn*$^{1}$, ectopic expression of *Dl* was detected at the posterior edge of the disc (arrow). (**I**) Large clone of *tkv*$^{4}$ did not induce ectopic *Dl* expression (arrow). Asterisks represent the position of the optic stalk where the MF propagation initiates, and arrowheads represent the position of the MF.

states of Su(H) (Figure 8A). Taken together, these results suggest that *chn* is a key factor in the crosstalk between two major signal transduction pathways: the EGFR-dependent pathway and the Notch/Delta-dependent pathway (Figure 8A).

In the mammalian system, competition between SMRT and NICD for interaction with RBPJκ determines the state of RBPJκ-dependent transcriptional activity (Kao *et al*, 1998). Extracellular signaling may modulate this competition, as diverse signaling pathways modulate the functions of N-CoR/SMRT (Lavinsky *et al*, 1998). Our findings would prompt investigations of potential interaction of two repression systems of NRSF/REST and N-CoR/SMRT, and their regulation by Notch and EGF signaling in mammalian neuronal differentiation.

## Materials and methods

### *Drosophila* stocks

The following stocks were used in this study: *Oregon-R*, *GMR-ebi*$^{DN}$, *ebi*$^{E4}$, *ebi*$^{D7}$ and *ebi*$^{I11}$ (Dong *et al*, 1999; Tsuda *et al*, 2002); *Su(H)*$^{2}$ and (*Su(H)*$^{1}$ (Schweisguth and Posakony, 1992); *tkv*$^{4}$ (Burke and Basler, 1996); *ey-FLP* (Newsome *et al*, 2000); *da-Gal4* (Nakao and Campos-Ortega, 1996). *GMR-Gal4* (Hay *et al*, 1994) was kindly provided by Dr M Yamaguchi (Kyoto Institute of Technology). *cycE*$^{AR95}$*FRT40A/CyO* and *PCNA*$^{775}$*FRT42D/CyO* were kindly provided by Dr CH Lee (NIH).

### Genetics

Genetic modifiers of *ebi*$^{DN}$-induced rough eye phenotype were identified by mating males of *GS* insertion lines with *GMR-Gal4; GMR-ebi*$^{DN}$*/CyO* females. From the progeny of ~6500 crosses, we identified 17 *GS* lines in which overexpression-based rough eye phenotype was altered by *ebi*$^{DN}$.

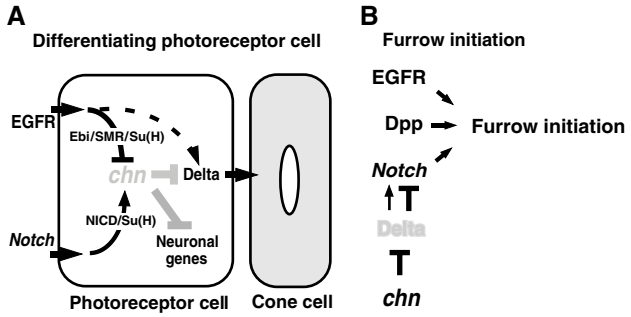


**Figure 8** Models for *chn* function during photoreceptor cell development. (**A**) *chn* is not expressed in photoreceptor cells because of repression by Ebi/SMRTER(SMR)/Su(H), which antagonizes activation by NICD/Su(H). In the absence of Chn, *Dl* is derepressed and induces cone cell differentiation. Elevation of EGFR signaling causes ectopic expression of *Dl* (Tsuda *et al*, 2002), suggesting that EGFR signaling activates *Dl* expression independently of Ebi/SMR/Su(H) activity (dotted line). (**B**) At the initiation of the MF, *Dl* expression seems to be kept at a low level by *chn* function. Elevation of *Dl* in the *chn* mutant might downregulate *Notch*, which is required for the initiation of the MF (Kumar and Moses, 2001).

Ethylmethanesulfonate (EMS)-induced *chn* mutations were isolated by reversion of lethality due to *chn* overexpression. $chn^{GS2112}$ males treated with EMS were crossed to *da-Gal4* females, and four revertant lines were established. The mutated site in $chn^{1}$ was determined by RT–PCR and sequencing, which identified a small deletion between 280 and 296 bp that caused a frame shift mutation. $chn^{9}$ is a genetically strong hypomorphic allele.

A large clone of *chn* or *tkv* was induced as previously described (Clandinin *et al*, 2001). Eye-specific mosaic flies were generated by FLP/FRT-induced sister chromatid recombination using *ey-FLP* and a recessive cell-lethal mutation ($cycE^{AR95}$ or $PCNA^{775}$) to eliminate the twin-spot (Stowers and Schwarz, 1999; Newsome *et al*, 2000).

### Molecular biology and biochemistry

A 3.3-kb *chn* coding region was isolated using PCR from a 0–4 h embryonic cDNA library (Brown and Kafatos, 1988) and cloned into *pUAST*. The Chn zinc-finger domain (residues 297–610) was also amplified by PCR and cloned into *pGEX4T* (Amersham Biosciences), and the GST fusion protein was isolated using the standard protocol. The Su(H) DNA-binding domain (residues 126–528) was also cloned into *pET28*(*a*+) (Novagen), and the His-Su(H) fusion protein was isolated and used for EMSA. Full length (chn; 1–3327 bp), N-terminal (chn-N; 1–1962 bp) and C-terminal (chn-C; 1963–3327 bp) regions of Chn with flag epitope were amplified by PCR and cloned into *pUAST* for transfection into S2 cells. Immunoprecipitation was performed essentially as described (Lim *et al*, 2000). S2 cell transfection experiments were performed as the manufacturer's protocol (Qiagen).

### Oligonucleotides

For the Chn binding assay, we chemically synthesized partially double-stranded 31-bp oligonucleotides containing a 21-bp NRSE (or NRSE-like) region and 5 bp each of terminal sequences. This double-stranded oligonucleotide was labeled with Cy5-dCTP and Klenow fragment of DNA polymerase I. For the Su(H) binding assay, we used $^{32}$P-labeled double-stranded 31-bp oligonucleotides containing 7-bp Su(H) recognition sequences (or alternative forms) and a 24-bp linker sequence. Oligonucleotide sequences are presented in Supplementary data.

### Antibodies

The following antibodies were used: rabbit anti-Ebi (Dong *et al*, 1999), rabbit anti-SMRTER (a gift from Dr R Evans, Salk Institute), mouse anti-NICD (Developmental Studies Hybridoma Bank (DSHB), University of Iowa), mouse anti-Dl (Parks *et al*, 1995), rat anti-Elav (O'Neill *et al*, 1994) and mouse anti-Cut (DSHB). Secondary antibodies were conjugated with Alexa488 or Cy3 (Jackson Labs). Polyclonal anti-Chn was raised (MBL Ltd) by injecting rabbits with purified Chn zinc-finger domain (residues 297–610) fused with GST.

### In situ hybridization and immunocytochemistry

*In situ* hybridization on whole-mount *Drosophila* eye discs was performed according to Tautz and Pfeifle (1989). Single-stranded antisense digoxigenin-containing RNA probes were prepared using the Genius kit (Boehringer Mannheim). Immunocytochemistry on imaginal discs was performed essentially as described (Majumdar *et al*, 1997).

### EMSA

*In vitro* DNA and protein interactions were detected by EMSA. Up to 1 μg purified GST-fusion protein was incubated on ice for 2 min in 20 μl of buffer (10 mM HEPES pH 7.6, 1 mM EDTA, 1 mM DDT, 32 mM KCl, 10% (v/v) glycerol containing 5% Ficoll 400 (Sigma), 500 ng/ml poly(dI-dC) and 1 μg of sonicated calf thymus DNA (average size, 0.2 kb). When necessary, unlabeled oligonucleotide competitors were added at this step. Then, double-stranded Cy5-labeled synthetic oligonucleotide (50 pg) was added, and the mixture was incubated for 10 min on ice. DNA–protein complexes were resolved on a 5% polyacrylamide gel in TAE at 4°C. Signals were detected by a fluorescent gel scanner (Typhoon, Amersham Biosciences). In the case of Su(H), we used $^{32}$P-labeled oligonucleotide (50 pg) probe and electrophoresis in a 1% agarose gel in TAE at 4°C. Signals were detected by autoradiography. CBEs listed in Table I were tested for binding to Chn by performing EMSA using Cy5-labeled probes under a condition without nonspecific competitors. Those probes that showed association to Chn were further verified by EMSA competition assays in the standard condition.

### ChIP analysis

Third instar larval eye discs (200) of wild type (Oregon R) or *pGMR-Gal4; UAS-chn* were dissected in PBS from the eye–antenna complex, fixed with 1% formaldehyde for 15 min at room temperature and treated as described (Hecht and Grunstein, 1999). Crosslinked adducts were resuspended and sonicated, resulting in DNA fragments of 500–1000 bp. Immunoprecipitation was performed using the antibodies described above. Protein-bound, immunoprecipitated DNA was dissolved in TE buffer and incubated at 65°C for 6 h. Digestion buffer (10 mM Tris–HCl, 100 mM NaCl, 25 mM EDTA, pH 8.0) was added to the sample and incubated for 1 h at 45°C with 0.1 mg/ml proteinase K (Sigma). DNA was purified using a Qiagen column (Qiagen Ltd) and used as template for 40 cycles of PCR.

Sequential immunoprecipitation was performed by eluting the antibody–chromatin–DNA complex from the protein-A column using DTT treatment before the addition of the second antibody.

### mRNA quantification

Poly A+ mRNA, isolated from 20 eye-antennal discs of third instar larvae using an mRNA purification kit (Amersham Biosciences), was reverse transcribed with SuperScript II (Invitrogen) reverse transcriptase using oligo-dT as a primer. mRNA was quantified using a Smert Cycler (TAKARA) with SYBR Green PCR Master mix (TOYOBO). Data obtained from duplicate mRNA preparations were standardized using *elav* mRNA as a control.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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