Senseless physically interacts with proneural proteins and functions as a transcriptional co-activator

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The zinc-finger transcription factor Senseless is co-expressed with basic helix-loop-helix (bHLH) proneural proteins in *Drosophila* sensory organ precursors and is required for their normal development. High levels of Senseless synergize with bHLH proteins and upregulate target gene expression, whereas low levels of Senseless act as a repressor in vivo. However, the molecular mechanism for this dual role is unknown. Here, we show that Senseless binds bHLH proneural proteins via its core zinc fingers and is recruited by proneural proteins to their target enhancers to function as a co-activator. Some point mutations in the Senseless zinc-finger region abolish its DNA-binding ability but partially spare the ability of Senseless to synergize with proneural proteins and to induce sensory organ formation in vivo. Therefore, we propose that the structural basis for the switch between the repressor and co-activator functions of Senseless is the ability of its core zinc fingers to interact physically with both DNA and bHLH proneural proteins. As Senseless zinc fingers are ~90% identical to the corresponding zinc fingers of its vertebrate homologue Gfi1, which is thought to cooperate with bHLH proteins in several contexts, the Senseless/bHLH interaction might be evolutionarily conserved.

KEY WORDS: *Drosophila*, Senseless

INTRODUCTION

The *Drosophila* zinc-finger (Zn-finger) transcription factor Senseless (Sens) and its homologues have been shown to be involved in a variety of cell biological and developmental processes, including cell fate specification, differentiation, neurodegeneration, proliferation, apoptosis and stem cell self-renewal (Cameron et al., 2002; Chandrasekaran and Beckendorf, 2003; Frankfort et al., 2001; Gilks et al., 1993; Grimes et al., 1996; Hock et al., 2004; Hock et al., 2003; Jia et al., 1996; Karsunky et al., 2002; Nolo et al., 2000; Saleque et al., 2002; Shroyer et al., 2005; Tong et al., 1998; Tsuda et al., 2005; Zeng et al., 2004). Although the functions of *sens* and its *C. elegans* homologue *pattern of gene expression-3* (*pag-3*) have been mainly studied in the context of the nervous system, most of the studies of the vertebrate homologues growth factor independence 1 (*Gfi1*) and *Gfi1b* have focused on hematopoietic development. However, recent work has provided strong evidence that *Gfi1* is required in the nervous system (Dufourcq et al., 2004; Tsuda et al., 2005; Wallis et al., 2003).

Indeed, the *Gfi1* loss-of-function phenotype in mouse inner ear hair cells is quite similar to the loss-of-function phenotype of *sens* in embryonic sensory organs (Nolo et al., 2000; Wallis et al., 2003). In addition, expression of *sens* and *Gfi1* in fly and mouse sensory organ precursors depends on the activity of fly basic helix-loop-helix (bHLH)-type proneural proteins and their mouse orthologues, respectively (Bertrand et al., 2002; Brown et al., 2001; Hassan and Bellen, 2000; Jafar-Nejad et al., 2003; Nolo et al., 2000; Wallis et al., 2003). Moreover, *sens* and proneural genes function in the same pathway during sensory organ development, and there is evidence for Gfi1/Gfi1b cooperation in vertebrate tissues (Brown et al., 2001; Frankfort et al., 2001; Frankfort et al., 2004; Jafar-Nejad et al., 2003; Kazanjian et al., 2004; Nolo et al., 2000; Shroyer et al., 2005; Wallis et al., 2003). Altogether, these observations strongly suggest that some mechanisms involved in the expression and function of Gfi1/Gfi1b are evolutionarily conserved.

Gfi1 proteins are C2H2-type Zn-finger nuclear proteins. There are six Zn fingers in Gfi1 and Gfi1b, five in PAG-3 and four in Sens. There is ~88-89% sequence identity between Sens Zn fingers and the four C-terminal-most Zn fingers of Gfi1b and PAG-3 (Jafar-Nejad and Bellen, 2004). Both Gfi1 and Gfi1b have been shown to bind to the same consensus DNA element [taATCact(a/t)gca; with the core sequence in uppercase] using their Zn-finger domains. There is ample evidence that both proteins function as DNA-binding transcriptional repressors (Doan et al., 2004; Grimes et al., 1996; Jegalian and Wu, 2002; Tong et al., 1998; Vassen et al., 2005; Zweidler-Mckay et al., 1996), although it has been suggested that they can also act as transcriptional activators (Osawa et al., 2002; Shariina et al., 2003). As predicted from the evolutionary conservation of their Zn fingers, PAG-3 and Sens strongly bind to the Gfi1b consensus binding site (Aamodt et al., 2000; Jafar-Nejad et al., 2003). Indeed, the sequence identity between the Zn fingers of the Gfi1 proteins goes beyond what is necessary to bind the same DNA sequence, suggesting that Gfi1 proteins might play conserved functional roles other than DNA binding (Jia et al., 1997).

*sens* was isolated in a genetic screen designed to identify novel genes involved in the development of the embryonic peripheral nervous system (PNS) in *Drosophila* (Salzberg et al., 1994). *sens* is expressed in sensory organ precursors and their progeny in both embryonic and adult PNS. In *sens* mutant embryos, sensory organ precursors (SOPs) form and divide but fail to differentiate properly, and instead undergo apoptosis (Nolo et al., 2000; Salzberg et al., 1994). Adult PNS development is also impaired in *sens* mutant clones, indicating that *sens* is required for the development of most or all sensory organs in flies. Moreover, ectopic expression of *sens*...
induces ectopic sensory organ formation, suggesting that Sens can play an instructive role in this process (Jafar-Nejad et al., 2003; Nolo et al., 2000). In Drosophila, the bHLH proneural proteins are involved in the selection of SOPs and implementation of neuronal fate in sensory lineages (Bertrand et al., 2002; Culi and Modolell, 1998; Goulding et al., 2000; Huang et al., 2000; Jarman et al., 1993; Modolell, 1997; Villares and Cabrera, 1987). As DNA-binding transcriptional activators, proneural proteins bind E-box elements and drive gene expression (Cabrera and Alonso, 1991; Murre et al., 1989; Van Doren et al., 1991). Given the evolutionary conservation of the role of bHLH proteins in neurogenesis, understanding how bHLH proteins regulate the expression of their target genes is of interest (Bertrand et al., 2002; Hassan and Bellen, 2000). We have previously presented in vivo and in vitro evidence that on a proneural target gene enhancer with multiple E-boxes and a Sens-binding site (S-box), Sens can function as a repressor or activator, depending on the level of Sens relative to proneural proteins (Jafar-Nejad et al., 2003). Although our findings suggested that binding of Sens to DNA might oppose its transcriptional synergism with proneural proteins, the structural basis for this synergism and the mechanism for the switch between the repressor and activator functions of Sens remain unknown.

Here, we dissect the mechanism of the transcriptional activation and repression mediated by the Sens protein on bHLH target enhancers. We present evidence that besides binding DNA, the core Zn fingers of Sens physically interact with proneural proteins, and therefore are responsible for both repression and co-activation. The DNA-binding-dependent repression and the bHLH-binding-dependent co-activator functions of Senseless are separable both in transcription assays and in vivo. We propose that differential affinity of the Sens Zn fingers for their DNA recognition site versus proneural proteins allows dual function in the transcriptional regulation of target genes.

MATERIALS AND METHODS
Fly strains, genetics and immunohistochemistry
The following fly lines were used in this study; y w; Canton S, UAS-sens (C6) (Nolo et al., 2000); C684-Gal4 (Manseau et al., 1997); y w; UAS-FLP; C684-Gal4 y + FRT80B, UAS-sens-1CC, UAS-sens-2CC, UAS-sens-3CC, UAS-sens-4CC, y w; sens-g; sens(2) red E/TM3, Kr-GFP, y w; sens-1CCg; sens(2) red E/TM3, Kr-GFP, y w; sens-3CCg; sens(2) red E/TM3, Kr-GFP (this study); and Eg-Gal4/TM6B (Pi et al., 2001). To rescue the adult mitotic clones of sens+ with sens genomic transgenes (sens-g, sens-1CCg or sens-3CCg), L+ Tb1 progeny of the following cross were inspected for regions of bristle loss or yellow bristles; y w; UAS-FLP; C684-Gal4 y + FRT80B × y w; sens-Xg/L; sens(2) FRT80B/TM6, Tb. Quantification of the post-orbital bristles was performed in y w eyeless-FLP; sens(2) FRT80B/w+ M(3)67C FRT80B (sens clones with no rescue) and y w eyeless-FLP; sens-Xg/s; sens(2) FRT80B/w+ M(3)67C FRT80B flies, in which sens-Xg stands for sens-g, sens-1CCg, sens-2CCg, sens-3CCg or sens-4CCg. Embryo collection, fixation and staining were performed using standard procedures (Bellen et al., 1992). Primary antibodies used in this study are rat α-Elav 1:500 (7E8A10; DSHB) (O’Neill et al., 1994), guinea pig anti-Sens 1:1000 (Nolo et al., 2000) and mouse mAb 2B11 1:100 (DSHB) (Zipursky et al., 1984). Secondary antibodies were from Molecular Probes (Alexa488-conjugated) and from Jackson ImmunoResearch Laboratories (Cy3 and Cy5 conjugated), and were used at 1:500.

Preparation of the mutant sens genomic rescue and UAS constructs
sens open reading frame cloned in pBluescript was mutated using the Quikchange site-directed mutagenesis kit (Stratagene) to generate Zn-finger mutant versions of sens, which were then transferred to the pUAST vector. A 1769 bp fragment that includes the coding region for the Zn-finger domains of sens in the genomic construct of sens was obtained by digesting the sens genomic rescue fragment with RsuII and AarII restriction enzymes. This 1769 bp RsuII-AarII fragment was cloned into a modified version of the pBluescript vector using AarII and RsuII sites. The Quikchange site directed mutagenesis kit was used to change the cysteines in Zn-finger domains into alanines. The mutant 1769 bp AarII-RsuII fragment was then excised from the pBluescript vector and cloned into the 21 kb pCaSpeR4-sens genomic rescue construct, which was fully digested with RsuII and partially digested with AarII. Colonies were screened by PCR and positive colonies were sequenced to determine the correct insertions.

EMSA, S2 cell transfection and luciferase assays
EMSA was performed as described previously (Ou et al., 2000). The proteins were in vitro translated using TNT Quick Coupled Transcription/Translation System kit (Promega). The R21 optimal Gfi1/Sens-binding sequence (Jafar-Nejad et al., 2003; Zweidler-Mckay et al., 1996) and the Sens-binding site on the ac promoter were used as the probes. Fifty times unlabeled probe was used as cold competitor. Transfection and luciferase assays were performed as described by Jafar-Nejad et al. (Jafar-Nejad et al., 2003). E-box and S-box mutagenesis were performed using the Quikchange site-directed mutagenesis kit. E-boxes were mutated from CANTTG to AANNTT, and the S-box core was mutated from AACGTG to GGCTC. All constructs were verified by sequencing. The primer sequences are available upon request.

GST pull-down experiments
GST fusion proteins were expressed using pGEX-4T1 vector (Amersham Biosciences) in BL-21 pLys(S) cells (Novagen). sens and different sens fragments were cloned into pGBK7 vector (Clontech) in-frame with the N terminal c-myc tag and in vitro translated using TNT Quick Coupled Transcription/Translation System kit (Promega). The same protocol as described by Giagtzoglu et al. (Giagtzoglu et al., 2003) was used to perform GST pull-down experiments. Detection was performed by western blot using anti-c-myc antibody (9E10, DSHB).

Co-immunoprecipitations
sens open reading frame was cloned into pCMV-HA vector (Clontech) in-frame with the N-terminal HA tag.achaete open reading frame was cloned into p3XFLAG-CMV-10 expression vector (Sigma) in-frame with N-terminal 3×FLAG tag. Lipofectamine 2000 (Invitrogen) was used to transfect COS-7 cells according to the manufacturer’s protocol. A total of 24 μg of DNA was transfected per 100 mm culture dish. Thirty-six hours after transfection cells in each culture dish were washed twice with cold PBS and were then harvested in 700 μl IP buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP40, 5 mM EDTA, 0.1 mM PMFS and Complete protease inhibitor (Roche)] for 45 minutes to obtain whole cell lysate. Upon centrifugation at 16,000 g, the supernatant was used for the IP reaction. Anti-HA monoclonal agarose conjugate (Sigma) was used for Co-IP reactions based on the product user manual. For washing steps the above-mentioned IP buffer was used.

Statistical analysis of the post-orbital bristle numbers
One-way ANOVA with Schefte error protection was used to determine the statistical significance of the differences between the number of post-orbital bristles in the genotypes under study. The P values are determined using ‘t’ test for independent samples’.

RESULTS
Zn finger 1, 2 and 3 are required for Sens DNA binding
We have previously shown in an S2 cell transcription assay that the Sens protein can act as a transcriptional repressor and activator, depending on its relative abundance to the proneural proteins (Jafar-Nejad et al., 2003). The reporter construct used in that study consists of the ac proximal enhancer/promoter region upstream of the firefly luciferase coding sequence (ac-luc; Fig. 1A). This ac enhancer contains a Sens-binding site (S-box) and three E-boxes, known binding sites for proneural proteins. Proneural proteins heterodimerize with Daughterless (Da) via their bHLH domains and
bind to the E-boxes on ac-luc to upregulate transcription (Cabrera and Alonso, 1991; Caudy et al., 1988; Van Doren et al., 1991; Van Doren et al., 1992). Depending on the amount of ac and da expression constructs transfected, the luciferase expression from this reporter can be increased 10 to 1000 times the basal level. To obtain the optimal sensitivity in the transcription assays, we use low levels of proneural expression constructs (1-2 ng) to assess the transcriptional activation potential of Sens (activation assay), and higher levels of proneurals (10 ng) to assess the transcriptional repression potential of Sens (repression assay). In the absence of Ac and Da, Sens does not activate or repress ac transcription.

Based on evolutionary conservation with its vertebrate homologues, Sens can be divided into two domains: an N-terminal domain of 414 amino acids, which shows little homology with other GPS proteins, and a C terminal domain of 127 amino acids, which exhibits strong homology with other GPS proteins and contains four highly conserved C2H2-type Zn fingers. We aligned Sens to its closest homologue from the mosquito *Anopheles gambiae*, which is thought to have diverged from *Drosophila* about 180 million years ago (Devenport et al., 2000), and found nine conserved stretches of 6-10 amino acids in the Sens N-terminal domain. Mutational analysis of the conserved stretches followed by transcription assays indicate that the individual conserved motifs in the N-terminal domain are not important for the activation and repression mediated on ac by Sens (data not shown).

The four C2H2-type Zn-finger domains of the GPS proteins, which mediate DNA binding, are shown in Fig. 1B (Nolo et al., 2000; Zweidler-Mckay et al., 1996). Deletion analysis of Gfi1 Zn
fingers has shown that Zn fingers 3-5 of Gfi1, which correspond to Zn fingers 1-3 of Sens, are required for DNA binding (Zweidler-Mckay et al., 1996). To begin to assess the precise role of individual Zn fingers in the repressor and activator functions of Sens, we mutated each Zn finger in Sens and examined the ability of the mutant Sens proteins to bind DNA in electromobility shift assays (EMSA). We generated two types of mutants for each Zn finger. In the first group (Sens-1CC, Sens-2CC, Sens-3CC and Sens-4CC), we mutated the two cysteines in the C2H2 structure to alanines (Fig. 1B, stars). These mutations probably disrupt the structure of the individual Zn fingers. In the second group (Sens-1RTT, Sens-2QDK, Sens-3QNT and Sens-4RDR), we altered the amino acids that have been predicted to directly contact DNA to alanines (Fig. 1B, boxes) (Blancafort et al., 2004), these mutations should abolish direct contact with specific DNA targets but at least partially preserve the overall Zn-finger structure.

To determine protein-DNA interactions and relative binding affinities of the mutant Sens proteins for DNA, we used two different probes in EMSA assays. To detect weak protein-DNA interactions, we used as a probe a previously characterized Gfi1-binding site called R21, to which the wild-type Sens is able to bind strongly (Fig. 1C, lane 2). As shown in Fig. 1C, Sens-1CC, Sens-2CC and Sens-3CC proteins lose their ability to bind the R21 probe, suggesting that Zn fingers 1, 2 and 3 are required for DNA binding (Fig. 1C, lanes 4, 6, 8). However, in agreement with Gfi1 data (Zweidler-Mckay et al., 1996), Sens-4CC can bind DNA, suggesting that Zn finger 4 is not essential for DNA binding (Fig. 1C, lane 10). As shown in Fig. 1C, the second group of Sens Zn-finger mutants behave somewhat differently in the EMSA. Sens-2QDK, Sens-3QNT and Sens-4RDR behave similarly to their CC counterparts, indicating that the amino acids predicted to directly contact the R21 probe in Zn fingers 2 and 3 are crucially important for DNA binding. However, unlike Sens-1CC, Sens-1RTT is still able to bind the R21 probe, albeit weaker than wild-type Sens and Zn finger 4 mutants (Fig. 1C, lane 12). This difference suggests that although Zn finger 1 is required for DNA binding, its role in DNA binding is more complex than a direct contact between the R21 amino acids and DNA.

We also used the S-box in the ac promoter as a probe in the EMSA assay to determine the binding affinities of mutant Sens proteins for the endogenous Sens-binding site. Wild-type Sens and Sens-4CC but not Sens-1CC, Sens-2CC nor Sens-3CC are able to bind to the S-box probe (data not shown). Moreover, in line with the R21 data, the Sens-1RTT binds much weaker than wild-type Sens and Sens-4CC. Note that the Sens-4CC binding affinity for the S-box is weaker than wild-type Sens, suggesting that although Zn finger 4 is not essential for DNA binding, it may increase the strength of Sens-DNA interaction.

### Zn fingers play different roles in the activation and repression conferred by Sens

To determine the importance of each Zn-finger domain for the activation and repression mediated by Sens, we tested the mutants in the S2 cell transcription assay. In our activation assay (ac-da, 2ng), wild-type Sens can synergize with Ac-Da and increase the transcription induced by Ac-Da about 18 times (Fig. 1D). Sens-2CC and Sens-3CC failed to synergize with Ac-Da (Fig. 1D). Sens-4CC and especially Sens-1CC exhibited significantly less synergy than wild-type Sens. Western blot analysis shows that wild-type Sens and all mutant Sens constructs are expressed at similar levels, indicating that the difference between the activator potential of mutant versions of Sens is not simply due to their level of expression or their stability (data not shown). Similar results were obtained for Sens-1RTT, Sens-2QDK, Sens-3QNT and Sens-4RDR (data not shown). These data indicate that all Zn fingers cooperate in the Sens/bHLH synergism. However, Zn fingers 2 and 3 are indispensable for this process.

To test the ability of the Zn-finger mutants to repress ac transcription, we used our ‘repression assay’. Low levels of wild-type Sens repress transcription in this assay and as the Sens to proneural ratio increases, the Sens activity switches from a repressor to an activator (Fig. 1E,F) (Jafar-Nejad et al., 2003). Sens-4CC and Sens-4RDR behave essentially as wild-type proteins in this assay (Fig. 1E,F). By contrast, mutations in Zn fingers 1, 2 or 3 abolish the repression function of Sens, corroborating the correlation between Sens DNA binding and repression. Interestingly, Sens-1CC and Sens-1RTT display transcriptional activation at a lower Sens to proneural ratio compared with the wild-type Sens (Fig. 1E,F), providing further evidence for the negative contribution of Sens DNA-binding to its ability to synergize with proneural proteins. Similar to the data obtained from the ‘activation assay’ (Fig. 1D), Sens proteins with mutations in Zn finger 2 or 3 do not show any premature synergism with Ac-Da (Fig. 1E,F), highlighting the role of these core Zn fingers in both synergism and repression. Together, these data indicate specific roles for the Zn fingers in repression and activation (Table 1).

### Zn fingers 2 and 3 are required for bristle specification

To explore the role of DNA-binding and repressor versus activator function of Sens in its ability to induce extra SOPs in vivo, we employed the Gal4/UAS system to express Zn-finger mutant versions of Sens in flies (Brand and Perrimon, 1993). Wild-type sens and the CC mutants were ectopically expressed using the thorax-specific driver Eq-Gal4 (Pi et al., 2001; Tang and Sun, 2002). Expression of wild-type Sens using this driver induces numerous extra bristles on the thorax (Fig. 2B) (Pi et al., 2004). Sens-1CC and Sens-4CC are both able to induce many extra bristles (Fig. 2C,F). However, although immunohistochemical staining with anti-Senseless antibody indicates comparable expression levels from all transgenes (data not shown), Sens-2CC and 3CC are unable to induce extra sensory organs (Fig. 2D,E). As Sens-1CC is unable to bind DNA, the data suggest that DNA binding is not essential for SOP induction upon Sens overexpression. Moreover, the inability of Sens-2CC and Sens-3CC to generate extra sensory organs correlates well with their inability to synergize with Ac-Da in transcription assays (Table 1) and suggests a link between Sens transcriptional synergism and SOP induction.

### Table 1. Comparison of the in vitro and in vivo roles of Sens Zn fingers

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Summary of the data obtained from EMSA, transcription assay, overexpression (OE) and rescue experiments performed using wild-type and Zn-finger mutant versions of Sens. ND, not determined.
Mechanisms of Senseless synergism

DNA binding is not essential for some of the Sens functions in vivo

So far, our data suggest that Sens DNA binding is dispensable for adult bristle formation by Sens. To better assess the in vivo role of DNA-binding in Sens function, we performed in vivo rescue experiments. We generated four sens genomic rescue constructs, each mutatet in one of the Zn fingers (sens-1CCg, sens-2CCg, sens-3CCg, sens-4CCg), and established several independent transgenic lines for each construct. We first determined the ability of sens-g, Sens-1CCg and Sens-3CCg mutant genomic transgenes to rescue the external sensory organs in adult sensE2 clones, which lose almost all mechanosensory bristles (Fig. 3A). As shown in Fig. 3B, sens-g is able to rescue all the bristles in the sensE2 clones. sens-1CCg transgene is able to rescue many bristles in a sensE2 clone, as evidenced by the presence of yellow bristles (Fig. 3C). However, sens-3CCg fails to rescue the bristle loss phenotype in mutant clones (Fig. 3D). To compare the in vivo function of the wild-type and mutant sens genomic transgenes in a quantitative manner, we generated large adult sens mutant clones in the head using the eyeless-Flp system (Newsome et al., 2000), and tested each sens genomic transgene for its ability to rescue the loss of post-orbital bristles in sens clones. In animals with sens mutant clones but without a rescue transgene, the number of post-orbital bristles is less than a third of the wild-type number (Fig. 3E and data not shown).

A wild-type genomic transgene as well as sens-4CCg fully rescues the bristle loss (Fig. 3E an data not shown), and sens-1CCg causes a partial restoration to about two-thirds of the wild type. By contrast, sens-2CC or sens-3CC do not rescue (Fig. 3E and data not shown). In addition, the rescue data for bristles perfectly correlate with the rescue data of eye size and morphology (data not shown), where Sens has been shown to be required for R8 photoreceptor specification (Frankfort et al., 2001). As 1CC, 2CC and 3CC all disrupt the DNA binding and 1CC partially preserves the synergism, these data corroborate our cell culture and in vivo overexpression observations and indicate that the sens requirement for adult bristle development corresponds to the activator function of Sens.

We also tested sens-1CCg and sens-3CCg transgenes for their ability to rescue the lethality and the PNS development in sens mutant embryos. Unlike the wild-type sens rescue transgene (sens-g), sens-1CCg and sens-3CCg are not able to rescue the lethality of
embryos that are homozygous for a null allele of sens (Table 1). Staining for neuronal markers reveals a significant rescue of PNS development with the sens-1CCg transgene (data not shown). However, the rescue is not complete, as some of the neurons are lost. In addition, the neurons exhibit differentiation, axon guidance and fasciculation defects (data not shown), indicating that Sens is required for neuronal differentiation in the embryonic PNS. By contrast, sens-3CCg does not rescue the PNS defects associated with sens(2) (data not shown, Table 1), although staining with anti-Sens antibody shows that both transgenes are able to produce similar levels of Sens protein (data not shown). As Sens-1CC and Sens-3CC have no DNA-binding activity in our assay conditions, the data indicate that some key aspects of Sens function in embryonic sensory organ development are not dependent on DNA binding.

To further support the notion that Sens DNA-binding is dispensable for adult bristle formation, we generated phosphomimetic mutations in each of the three linker motifs in Sens Zn-finger domain by changing the first serine or threonine residues (Fig. 1B, circles) to glutamic acid, and tested them in vitro and in vivo. It has been shown that phosphorylation of the first threonine or serine residue in the conserved linker motifs that connect adjacent C2H2-type Zn fingers results in a severe decrease in the ability of the corresponding Zn-finger protein to bind DNA, regardless of their specific recognition site (Dovat et al., 2002; Jantz and Berg, 2004). EMSA assays indicated that phosphomimetic mutation in linker 3 did not cause any detectable effect on Sens-DNA interactions and phosphomimetic mutation in linker 1 caused a slight decrease in the affinity of Sens for DNA (Fig. 4A). However, we did not detect DNA-binding for Sens with a phosphomimetic mutation in linker 2 (Sens-L2-T-E, Fig. 4A). We next performed ‘activation assays’ in S2 cells and found that, similar to Sens-1CC, Sens-L2-T-E can partially synergize with Ac-Da (data not shown). Finally, we generated transgenic flies that express Sens-L2-T-E and ectopically expressed mutant Sens using the Eq-Gal4 driver to test if it can induce extra SOP formation. We found that although weaker than wild-type Sens, misexpression of Sens-L2-T-E can induce numerous extra bristles (Fig. 4B,C). These data support the notion that Sens DNA-binding is not essential for the generation of extra bristles in vivo and further establish a correlation between the Sens-proneural synergism in cell culture and bristle induction by Sens in vivo.

**Proneural proteins are able to recruit Sens to the ac regulatory region**

The proximal ac enhancer used in the ac-luc reporter has three E-boxes as well as one S-box (Fig. 1A). Removal of the three E-boxes shows that they are important for transcriptional activation by Ac-Da or Sc-Da in S2 cell co-transfection experiments and for proper expression of an ac-lacZ transgene in vivo (Martinez et al., 1993; Van Doren et al., 1992). As our data indicate that binding of Sens to the S-box in the ac enhancer is not necessary for Sens-proneural synergism, we assessed the role of each E-box in this process. To this end, we amplified a reporter with mutations in various combinations of E-boxes. wt, wild type; E1-3, mutant E-boxes 1-3 (see Fig. 1A). Blue bars represent the presence, and red bars represent the absence, of the S-box on the ac-luc reporter. The absence of the S-box does not affect the expression mediated by Ac and Da when no Sens protein is present. (B) Amplification of the expression induced by Ac and Da upon addition of Sens. The absence of the S-box significantly increases the ability of Sens to synergize with proneural proteins on the ac-luc reporter.
end, we generated ac-luc reporter constructs with all combinations of single, double and triple mutant E-boxes in the presence and absence of the S-box.

We first established the contribution of each E-box to the transcriptional activity conferred by the Ac and Da in the absence of Sens. As shown in Fig. 5A, luciferase expression is highest when all three E-boxes are present and lowest when all three E-boxes are mutated, in agreement with previous observations (Van Doren et al., 1992). Although each individual E-box seems to contribute to the wild-type level of transcription, E1 is more important than E2 and E3. Similar results were obtained when low levels of ac-da (2 ng each) were used in transfection assays (data not shown). These data also serve as a control to show that when the sens expression vector is not co-transfected with ac-da, presence or absence of the S-box does not affect the luciferase expression mediated by Ac-Da.

We next co-transfected sens together with proneurals and calculated the ratio between luciferase expression conferred by Ac-Da in the presence and absence of Sens as an amplification ratio (Fig. 5B). Absence of the S-box in an otherwise wild-type construct results in a significant enhancement of luciferase expression in the presence of Sens (Fig. 5B). The amplification ratio becomes even higher in E1, E2 and E3 single mutants. Moreover, when the S-box is mutated, even the presence of E1 or E2 site alone is enough to mediate Sens-proneural synergism. However, when Ac and Da are not able to bind DNA (triple E1, E2, E3 mutant), Sens is unable to synergize with proneural proteins. Together, these data strongly suggest that Sens can be recruited to the ac promoter by the Ac-Da protein complex to potently activate transcription.

Sens interacts with proneural proteins via its Zn-finger domains

As the activator function of Sens fully depends on the presence of proneural proteins and at least one intact E-box in the enhancer, we hypothesize that Sens is recruited to the ac enhancer not only via DNA interaction but also through interaction with proneural proteins. To test this hypothesis, we first performed a co-IP experiment by expressing HA-tagged Sens and Flag-tagged Ac in Cos-7 cells. Indeed, antibodies against HA-Sens permitted precipitation of the Flag-Ac protein, suggesting that Ac and Sens interact in vivo (Fig. 6A). To test if Sens and other proneural proteins also interact, we examined if GST-tagged Ac, Scute (Sc) and Atonal (Ato) can pull down tagged Sens. We find that Sens physically interacts with all of the bHLH-type proneural proteins tested here (Fig. 6B) and also with the ubiquitously expressed bHLH-type.
protein Daughterless (Jafar-Nejad et al., 2006), in agreement with the observation that Sens can synergize with various proneural proteins in vivo (Nolo et al., 2000; Quan et al., 2004).

To identify the Sens domain that binds to proneural proteins, we performed GST pull-down experiments using GST-Scute and several fragments of the Sens protein that are tagged with c-myc. As shown in Fig. 6C, the Zn-finger domains of Sens mediate the interaction between Sens and Scute. In addition, the data also show that the Zn-finger domains alone are sufficient to mediate the interaction.

Our cell culture, ectopic expression and rescue experiments indicate that Zn finger 2 and Zn finger 3 are indispensable for Sens function in cell culture and in vivo. To examine if Zn finger 2 and Zn finger 3 are involved in the interaction between Sens and proneural proteins, we deleted each Sens Zn finger individually and tested mutant Sens proteins in GST pull-down assays for their ability to interact with Scute. We find that Sens is still able to interact with Scute in the absence of any of the four Zn fingers (Fig. 6D, lanes b-e), indicating that the Sens-bHLH interaction does not depend on a single Zn finger. However, when both Zn finger 2 and Zn finger 3, and the linker between them are removed, Sens is no longer able to interact with Scute (Fig. 6D, lane f). These data indicate that the core region of the Sens Zn-finger domain, which contains Zn finger 2 and Zn finger 3, mediates the interaction between Sens and Scute. To further support the notion that Sens-proneural interaction is required for bristle formation, we tested the mutant Sens proteins in GST pull-down assays for their ability to interact with Scute. We find that Sens is still able to interact with Scute (Fig. 6E, lanes b-e), indicating that the Sens-bHLH interaction does not depend on a single Zn finger. However, when both Zn finger 2 and Zn finger 3, and the linker between them are removed, Sens is no longer able to interact with Scute (Fig. 6E, lane f). These data indicate that the core region of the Sens Zn-finger domain, which contains Zn finger 2 and Zn finger 3, mediates the interaction between Sens and Scute. To further support the notion that Sens-proneural interaction is required for bristle formation, we tested the mutant Sens proteins in GST pull-down assays for their ability to interact with Scute. We find that Sens is still able to interact with Scute (Fig. 6E, lanes b-e), indicating that the Sens-bHLH interaction does not depend on a single Zn finger. However, when both Zn finger 2 and Zn finger 3, and the linker between them are removed, Sens is no longer able to interact with Scute (Fig. 6E, lane f). These data indicate that the core region of the Sens Zn-finger domain, which contains Zn finger 2 and Zn finger 3, mediates the interaction between Sens and Scute. To further support the notion that Sens-proneural interaction is required for bristle formation, we tested the mutant Sens proteins in GST pull-down assays for their ability to interact with Scute. We find that Sens is still able to interact with Scute (Fig. 6E, lanes b-e), indicating that the Sens-bHLH interaction does not depend on a single Zn finger. However, when both Zn finger 2 and Zn finger 3, and the linker between them are removed, Sens is no longer able to interact with Scute (Fig. 6E, lane f). These data indicate that the core region of the Sens Zn-finger domain, which contains Zn finger 2 and Zn finger 3, mediates the interaction between Sens and Scute.

As the ac promoter may have other binding sites for unknown factors that are also involved in the synergism between Sens and proneural proteins, we performed another set of transcription assays on an artificial reporter consisting of five UAS sites and a thymidine kinase promoter, UAS-tk-luc (Fig. 7A). Giagtzoglou et al. recently showed that the Scute protein is able to induce luciferase expression from the UAS-tk-luc reporter when fused with the Gal4-DNA binding domain (Gal4DBD) (Giagtzoglou et al., 2005). If binding of proneural proteins is enough to recruit Sens to an enhancer, we anticipate synergism in this assay when Scute or Achaete is fused to the Gal4DBD. As shown in Fig. 7B, Sens is able to synergize with full-length Scute and Achaete, strongly suggesting that Sens is brought to the enhancer via proneural proteins.

E(spl) proteins are known to be negative regulators of proneural protein expression and function (Culi and Modolell, 1998; Delidakis and Artavanis-Tsakonas, 1992; Giagtzoglou et al., 2003; Jimenez and Ish-Horowicz, 1997; Knust et al., 1992). E(spl)m7 was shown to inhibit Scute-mediated transcriptional activation by binding to Sc directly (Giagtzoglou et al., 2005). As shown in Fig. 7C, Sens is not able to synergize with the Scute protein in the presence of the E(spl)m7, providing further support to the hypothesis that Sens can only synergize with proneural proteins when proneural proteins are able to induce transcription.

Our previous data indicate that repression mediated by low levels of Sens requires DNA binding, and that Sens DNA binding has a negative role on the synergism between Sens and proneurals. To test if proneural proteins can synergize with Sens when it is tethered to DNA, we generated Sens-Gal4DBD fusion protein. This fusion protein significantly represses the basal level of luciferase expression from an artificial reporter consisting of five UAS sites fused with the Gal4-DNA binding domain (Gal4DBD) (Giagtzoglou et al., 2005). If binding of proneural proteins is enough to recruit Sens to an enhancer, we anticipate synergism in this assay when Scute or Achaete is fused to the Gal4DBD. As shown in Fig. 7D, Sens is able to synergize with full-length Scute and Achaete, strongly suggesting that Sens is brought to the enhancer via proneural proteins. These observations provide further evidence that Sens, when bound to DNA, indeed acts as a repressor.

Fig. 7. Sens can synergize with proneural proteins on the UAS-tk-luc reporter in S2 cell transcription assays. (A) Schematics of the UAS-tk-luc reporter construct. (B) S2 cell transcription assays using UAS-tk-luc reporter. Sens expression alone does not have a significant effect on the basal expression of the UAS-tk-luc reporter. However, Sens is able to synergize with Sc and Ac. (C) E(spl)m7 strongly inhibits the expression induced by the Sc-Gal4DBD and the synergism between Sens and Sc-Gal4DBD. (D) When fused to Gal4DBD, Sens acts as a strong repressor on the UAS-tk-luc reporter. Ac and Sc fail to synergize with DNA-bound Sens.
DISCUSSION
Sensory organ precursor development in flies requires the function of proneural proteins and Sens (Frankfort et al., 2001; Frankfort et al., 2004; Jafar-Nejad et al., 2003; Koelzer and Klein, 2003; Nolo et al., 2000). Expression of sens is under the direct control of the proneural proteins (Jafar-Nejad et al., 2003) and SOPs co-express proneural proteins and Sens (Jafar-Nejad et al., 2003; Nolo et al., 2000, zur Lage et al., 2003). We have previously shown that high levels of Sens synergize with proneural proteins to upregulate the expression of the ac gene via the ac proximal enhancer, a known target of proneural proteins (Jafar-Nejad et al., 2003; Van Doren et al., 1992). However, low levels of Sens oppose the activator function of proneural proteins on this enhancer. Intriguingly, our data suggested that Sens DNA binding has a negative role in the synergism between proneural proteins and Sens, but the molecular mechanism underlying this dual role in transcriptional regulation was not elucidated.

Based on our current data, we propose the following model for the role of Sens in transcriptional regulation of proneural target genes in sensory precursors. Early in the proneural cluster, proneural gene expression is under the control of proneural and E(spl) proteins. At this stage, proneural genes start to engage in a positive autoregulatory loop by binding to the E-boxes in their own enhancers (Culi and Modolell, 1998; Van Doren et al., 1992). Initially, low levels of Sens bind DNA rather than the proneural proteins via its Zn fingers because it has a higher affinity for DNA (Fig. 8A). When bound to DNA, Sens acts as a repressor. As Sens interacts with several E(spl) proteins (Jafar-Nejad et al., 2003), recruitment of E(spl) through Sens might contribute to the negative regulation of proneural target enhancers (Fig. 8A). As the level of proneural proteins increases, proneural proteins induce more Sens expression. This will lead to saturation of the S-boxes (Fig. 8B).

Additional Sens will bind proneural proteins via its core Zn-finger domains and act as a co-activator to increase the transcription induced by proneural proteins (Fig. 8C). We propose that the switch between the repressor and co-activator functions of Sens depends on the conformational state of its Zn fingers. In this model, binding to proneural proteins will allow the Sens Zn fingers to adopt an alternative conformation compared to the DNA-bound state. This will enable Sens to cooperate with co-activators already recruited by proneural proteins, or to recruit new co-activators to further increase the ability of proneural proteins to increase the expression of their target genes in some contexts. This conformation-based hypothesis is supported by our observation that even point mutations in Sens Zn fingers that are dispensable for proneural interaction still cause severe reduction in the synergism between Sens and proneural proteins.

Multiple lines of evidence suggest that Sens acts as a transcriptional co-activator for bHLH proneural proteins. First, Sens is required for the upregulation and maintenance of proneural gene expression in the wing margin chemosensory SOPs (Nolo et al., 2000). Second, Sens synergizes with proneural proteins to upregulate the expression of the ac proximal enhancer in S2 cell assays (Jafar-Nejad et al., 2003). Third, ectopic expression of Sens induces ectopic proneural gene expression (Lai, 2003; Nolo et al., 2000). Fourth, Sens physically binds bHLH proteins via the core region of its Zn-finger domain. Fifth, Sens can not induce transcription in the absence of proneural proteins. It should be mentioned that our in vitro and in vivo observations indicate that DNA binding is not essential for the ability of Sens to act as a co-activator and to induce SOP formation. Therefore, as SOPs accumulate high levels of both proneural proteins and Sens, it is likely that proneural target enhancers that do not contain a Sens-binding site might also be a target for proneural-Sens transcriptional synergism.

Similar to its vertebrate homologues (Grimes et al., 1996; Tong et al., 1998), Sens can function as a transcriptional repressor when bound to DNA (Fig. 7). Mutational analysis of Sens Zn fingers also indicates a link between DNA binding and the repressor function of Sens: those Sens mutants that do not bind DNA (1CC, 2CC and 3CC) fail to repress ac transcription, whereas mutating Zn finger 4, which does not play a major role in DNA binding, does not affect the repressor function of Sens. Although the repressor function seems to be less crucial than the co-activator function in vivo, our data suggest that the repressor function of Sens also contributes to its role in PNS development.

Sens physically interacts with proneural proteins via its Zn-finger domains, which are highly conserved between Sens and its vertebrate homologues. In addition, Sens can synergize with the mouse Ato homologue Math1 (Atoh1 – Mouse Genome Informatics), when the two proteins are co-expressed in flies (Quan et al., 2004). Together, these observations suggest that the Sens-bHLH interaction is evolutionarily conserved. In other words, vertebrate bHLH proteins such as Math1, Mash1 (ASecl – Mouse Genome Informatics) and Math5 (Atoh7 – Mouse Genome Informatics), which are co-expressed with Gfi1 in mouse tissues (Kazanjian et al., 2004; Wallis et al., 2003; Yang et al., 2003), might be able to recruit Gfi1 to their target enhancers.

![Fig. 8. A model for the dual role of Sens Zn fingers in the transcriptional regulation of proneural target genes.](image-url)

E represents E-box, S represents S-box. The four ovals in Sens depict Zn fingers. See Discussion for details.
In conclusion, our data suggest that Sens, a C2H2-type Zn-finger protein, binds to bHLH proneural proteins via its core Zn-finger domains and acts as a co-activator of the expression induced by proneural proteins. Sens can bind to various bHLH proteins and synergize with fly proteins, as well as some of their vertebrate homologues in vivo. These data, together with other examples of Zn-finger/bHLH synergism (Bellefroid et al., 1996), suggest that physical and genetic interactions of this type might be a common mechanism for Zn-finger/bHLH cooperation during development.

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