Published in final edited form as:

Genesis. 2009 July ; 47(7): 456–468. doi:10.1002/dvg.20521.

On the mechanism underlying the divergent retinal and bristle defects of M8* (*E(spl)D*) in *Drosophila*

Bhaskar Kahali, Anasua Bose, Umesh Karandikar, Clifton P. Bishop, and Ashok P. Bidwai*

Department of Biology, Life Sciences Building, 53 Campus Drive, West Virginia University, Morgantown, WV 26506-6057

Abstract

Our results, using endogenous mutants and Gal4-*UAS* driven transgenes, implicate multisite phosphorylation in repression by *E(spl)M8*. We propose that these phosphorylations occur in the morphogenetic furrow (MF) to reverse an auto-inhibited state of M8, enabling repression of Atonal during R8 specification. Our studies address the paradoxical behavior of M8*, the truncated protein encoded by *E(spl)D*. We suggest that differences in N signaling in the bristle versus the eye underlie the antimorphic activity of M8* in *N*⁺ (ectopic bristles) and hypermorphic activity in *N*^{sp} (reduced eye). Ectopic M8* impairs eye development (in *N*^{sp}) only during establishment of the *atonal* feedback loop (anterior to the MF), but is ineffective after this time point. In contrast, a CK2 phosphomimetic M8 lacking Groucho (Gro) binding, M8SDΔGro, acts antimorphic in *N*⁺ and suppresses the eye/R8 and bristle defects of *N*^{sp}, as does reduced dosage of *E(spl)* or CK2. Multisite phosphorylation could serve as a checkpoint to enable a precise onset of repression, and this is bypassed in M8*. Additional implications are discussed.

Introduction

The *Drosophila* sensory organs (eye and bristles) have been widely studied as models for neuronal specification and have provided important insights into the mechanisms underlying neurogenesis. The onset of neural cell fate specification in these sense organs involves expression of the basic-helix-loop-helix (bHLH) transcriptional activators encoded by the *achaete-scute Complex* (*ASC*) and *atonal* (*ato*), which are essential for formation of groups of equipotent cells, the proneural clusters (PNC's). Specification of the bristle-PNC's requires the *ASC* activators, whereas those that give rise to the 'founding' R8 photoreceptors require *Ato* (Ghyssen and Richelle, 1979; Jarman *et al.*, 1994; Modolell and Campuzano, 1998; Skeath and Carroll, 1991). In either case, however, only a single cell from each PNC goes on to form the bristle sensory organ precursor (SOP) or the R8 cell, a process that is vital for stereotyped patterning of these sensory organs (reviewed in (Baker, 2002; Bertrand *et al.*, 2002; Frankfort and Mardon, 2002; Gomez-Skarmeta *et al.*, 2003).

R8/SOP selection involves inhibitory Notch (N) signaling between the future R8/SOP and its immediate neighbors in a PNC (reviewed in (Artavanis-Tsakonas *et al.*, 1999; Bray, 2006; Mumm and Kopan, 2000)). This signaling involves interaction of the ligand Delta (DI) that is expressed at the highest levels in the future R8/SOP, and the N receptor in adjacent cells (Parks *et al.*, 1995; Simpson *et al.*, 1992). As a result, N is cleaved to release its intracellular domain (N^{icd}). This fragment translocates to the nucleus and along with Suppressor of Hairless (Su (H)) elicits transcription of the bHLH repressors encoded by the *Enhancer of split Complex*, *E(spl)C* (Bailey and Posakony, 1995; de Celis *et al.*, 1996; de la Concha *et al.*, 1988; Jennings

*To whom correspondence should be addressed; Tel: 304-293-5201; FAX: 304-293-6363; abidwai@wvu.edu.

et al., 1994; Lecourtois and Schweisguth, 1995). The E(spl) repressors in a complex with Gro (Paroush *et al.*, 1994) then antagonize Ato/ASC to redirect cell fate away from the (default) R8/SOP fate. This process, termed ‘lateral inhibition’ (Baonza and Freeman, 2001; Lehmann *et al.*, 1983; Simpson, 1990), thus ensures the emergence of a single R8/SOP from each PNC.

Given the importance of E(spl) for lateral inhibition, numerous studies have identified functional domains and their roles in repression. These are: basic (DNA binding), HLH (E(spl) dimerization), Orange (interactions with ASC/Ato) and a C-terminal WRPW tetrapeptide (Gro-binding). Structure function analysis suggests that loss of HLH, Orange or WRPW domains, individually, render E(spl) proteins non-functional (Giebel and Campos-Ortega, 1997). The role of WRPW is, perhaps, the best understood, since Gro is an essential co-repressor for all E(spl) members (Fisher *et al.*, 1996; Paroush *et al.*, 1994). One exception, however, is *E(spl) D*, a unique dominant mutant allele of the *E(spl)* member *m8* that abrogates eye development in the presence of the *split* allele of *N*, i.e., *N^{split}*.

E(spl)D harbors two mutations; one stabilizes the *m8* transcript, and the other truncates the protein after the Orange domain. This truncated protein called M8* lacks 56 C-terminal residues including WRPW, and thus cannot bind Gro (Nagel and Preiss, 1999; Tietze *et al.*, 1992). Importantly, over-expression of a *UAS-m8** construct during eye development mimics the effects of *E(spl)D*, indicating that the truncation in M8* underlies its enhancement of *N^{split}*. It has, therefore, been suggested that *E(spl)D* is a *gro*-independent hypermorph (Nagel *et al.*, 1999). However, in *N⁺* flies, M8* acts as an antimorph and elicits ectopic bristles, an unexpected finding since removal of the C-terminal tetrapeptide WRPW *per se* from full-length M8 renders the protein non-functional (Giebel and Campos-Ortega, 1997). The mechanism underlying these divergent activities of M8* in *N⁺*-vs-*N^{split}* remained unresolved.

Studies conducted by our laboratory have demonstrated that protein kinase CK2 phosphorylates M8 at Ser¹⁵⁹ in the C-terminal domain (CtD (Trott *et al.*, 2001)) that is missing in M8*. The role of this modification was suggested by the finding that the CK2 ‘phosphomimetic’ variant (M8S¹⁵⁹D) blocked R8 specification and eye development, and mimicked the effects of M8*, albeit in *N⁺* flies (Karandikar *et al.*, 2004). No such effects were seen with wild type M8. The observation that M8* and M8SD, but not M8, interact strongly with Ato (in yeast), raised the possibility that the non-phosphorylated CtD ‘autoinhibits’ Orange to restrain repression by M8 ((Karandikar *et al.*, 2004), see Fig. 1b). In the case of M8, phosphorylation would displace the CtD to expose Orange and enable repression, and the absence of this autoinhibitory region in M8* might thus enable it to precociously bind and antagonize Ato.

Given the similar eye phenotypes elicited by M8SD and M8*, we hypothesized that M8SD might provide a means to uncover the mechanism underlying the divergent activities of M8* in *N⁺*-vs-*N^{split}*, and further define the regulatory influence of phosphorylation on repression by E(spl) *in vivo*. Since M8* is unable to bind Gro, we sought to assess the role of WRPW in the retinal defects of M8SD, with the expectation that eliminating the ability of M8SD to bind Gro might render it *N^{split}*-dependent, akin to M8*. We describe here the unexpected antimorphic activity of this variant, which we call M8SDΔGro. In *N⁺* flies, M8SDΔGro elicits supernumerary (ectopic) bristles and potently suppresses the bristle loss phenotype of ectopic M8, but does not interfere with R8 specification or eye development, effects similar to those reported for M8* (Giebel and Campos-Ortega, 1997; Nagel *et al.*, 1999). However, M8SDΔGro and M8* behave very differently in the *N^{split}* background. While M8* exacerbates the reduced eye of *N^{split}*, M8SDΔGro suppresses the eye defects, especially the specification of the R8 cells. This suppression is specific to the time and place where R8 photoreceptors are specified, i.e., the morphogenetic furrow (MF). The possibility that the antimorphic activity of M8SDΔGro mediates its effects in *N⁺* or in *N^{split}* is supported by our findings that *Df*

(3R)^{BX22}, a deficiency that uncovers *m5*, *m7*, *m8* and *gro*, also suppresses *N^{spl}*, as does knockdown of CK2. No antimorphic behavior in *N⁺*, or suppression of *N^{spl}* is seen with the Gro-neutralized variant of M8 or with its non-phosphorylatable form M8S^{159A}. Our studies suggest that differences in N signaling between the bristle and the eye, rather than different mechanisms, underlie the antimorphic and hypermorphic activities of M8* in *N⁺* and *N^{spl}*, respectively.

Results

The eye/R8 defects of M8SD are rescued by increased dosage of Ato

The retinal defects of M8SD mimicked those of M8*, albeit in *N⁺* flies. Since the effects of *E (spl)D* (M8*) are sensitive to Ato dosage (Nagel and Preiss, 1999), we first tested if this is the case for M8SD. Expression of M8SD with *scaGal4* elicits a severely reduced eye (~15 ommatidia, (Karandikar *et al.*, 2004)), and the possibility remained that this is a limit phenotype, i.e., beyond a threshold and not amenable to modulation. We therefore used the weaker driver *109-68Gal4* (active in PNC's and in R8's (Powell *et al.*, 2004)). Expression of M8SD with *109-68Gal4* elicits a moderately reduced eye at 24°C (Fig. 1c), and eye discs display sporadic and inconsistent levels of Senseless (Sens) along the DV or AP axis (Fig. 1c'). Furthermore, ommatidial size varied upon expression of M8SD (Fig. 1c) and staining of discs with Sens+ELAV showed photoreceptor clusters with reduced numbers of secondary R-cells (data not shown). Since Sens is essential for R8 differentiation (Frankfort *et al.*, 2001; Nolo *et al.*, 2000), the possibility arises that the remaining R8's are likely to be functionally compromised, and might not recruit secondary photoreceptors normally. Both, the eye and R8 defects of M8SD were suppressed by co-expression of a *UAS-ato* construct (Fig. 1e, e'), suggesting that Ato is an *in vivo* target of M8SD (as is the case for M8*). Expression of *UAS-ato*, by itself, does not elicit overt eye/R8 defects at 24°C (Fig. 1d, d'), reflecting the observation that *109-68Gal4/+; UAS-ato/+* flies display rough eyes and supernumerary R8's in a more pronounced manner only at 29°C (White and Jarman, 2000).

Loss of Gro-binding abolishes the reduced eye of M8SD, but not its bristle defects

To remain consistent with previous studies, we abolished Gro-binding by removal of the WRPW motif (Paroush *et al.*, 1994). Henceforth, we refer to these variants as M8ΔGro, M8SAΔGro and M8SDΔGro (Fig. 2a). In contrast to the full-length proteins, these variants do not interact with Gro, as is also the case with M8* (Fig. 2b). For *in vivo* analysis, we generated multiple independent insertions of UAS-constructs encoding all three constructs (Fig. 2a); the single insertion of *UAS-m8** has been previously used by others and us (Karandikar *et al.*, 2004; Nagel *et al.*, 1999).

As previously described (Karandikar *et al.*, 2004), expression of M8SD with *scaGal4* elicited a severely reduced eye and loss of the scutellar macrochaetes (MC's) and interommatidial bristles (IOB's), whereas wild type M8 only elicits loss of the MC's and IOB's (Fig. 2c, g, g'). Expression of M8* did not elicit a reduced eye, as these studies were conducted in *N⁺* flies. To a large extent, these *in vivo* effects of M8SD and M8 were neutralized by removal of WRPW (Fig. 2c). However, M8SDΔGro, in particular, was not rendered non-functional. Its expression with *scaGal4* elicited ectopic MC's in 73% flies, a penetrance that was significantly higher than the baseline bristle defects of *scaGal4/+* flies (19%, Fig. 2c). Importantly, this phenotype was not intrinsic to any of the 14 independent *UAS-m8SDΔGro* insertions, but was observed only upon their expression with *scaGal4* (data not shown). Expression of M8* also elicited ectopic MC's with a similarly enhanced penetrance (~81%, Fig. 2c). A virtually identical phenotype has been reported by Giebel and Campos-Ortega (1997) using *scaGal4*, and interpreted as dominant-negative (antimorphic) effects. Expression of M8ΔGro or M8SAΔGro was without effect in multiple (14 and 6, respectively) independent lines; in either case ~15–

20% flies displayed ectopic MC's, numbers that were indistinguishable from the baseline defects in *scaGal4/+* flies alone (Fig. 2c, and data not shown). Thus, the elicitation of ectopic MC's requires removal of the entire CtD (M8*), or removal of WRPW in conjunction with a phosphomimetic Asp in place of Ser¹⁵⁹ (M8SDΔGro).

M8SDΔGro mimics the ectopic MC phenotype of M8*

To independently evaluate the ectopic MC's of M8SDΔGro, we employed the Gal4-driver *G455.2*, since its intrinsic MC defects are lower than those of *scaGal4* and because its expression is restricted to the scutellum (Giebel and Campos-Ortega, 1997). In the case of *G455.2/+* flies, ~11% display ectopic MC's, and this number was used as the baseline for comparisons (Fig. 2d). As with *scaGal4* (see above), *G455.2* mediated expression of M8SDΔGro led to ectopic MC's in 67% of the flies, and a similar number (74%) was seen upon expression of M8* (Fig. 2d). Once again, expression of M8ΔGro or M8SAΔGro was without effect, as their ectopic MC phenotypes were indistinguishable from the baseline MC defects in *G455.2/+* flies (Fig. 2d). The non-functional behavior of M8ΔGro has been previously reported (Giebel and Campos-Ortega, 1997). No MC defects (ectopic or missing) were intrinsic to multiple independent insertions of *UAS-m8SDΔGro* (14 lines), *UAS-m8ΔGro* (14 lines), *UAS-m8SAΔGro* (6 lines), or *UAS-m8** (1 line), by themselves (data not shown). Thus, expression of M8SDΔGro or M8* was required for eliciting the ectopic MC defects. Moreover, ectopic MC's were elicited with similar penetrance upon expression of all 14 *UAS-m8SDΔGro*, but none of the *UAS-m8ΔGro* or *UAS-m8SAΔGro* insertions (data not shown). As controls, we tested full-length M8 or M8SD (6 *UAS*-lines each), and found that both elicited potent loss of MC's (Fig. 2e).

M8SDΔGro exhibits antimorphic properties

If M8SDΔGro is antimorphic, the penetrance of its ectopic MC phenotype should be enhanced by a reduction in *E(spl)* dosage. For these studies, we used *Df(3R)^{BX22}* (uncovers *m5*, *m7*, *m8* and *gro* (Delidakis *et al.*, 1991)), which was brought through males to avoid maternal effects. The baseline bristle defects of *G455.2/+* flies are not significantly modified (enhanced/suppressed) by *Df(3R)^{BX22}* (Fig. 2e). In contrast, expression of M8SDΔGro in *Df(3R)^{BX22}/+* flies elicited ectopic MC's with a modestly higher penetrance than in a background wild type for the *E(spl)C* (82-vs-67%, Fig. 2d, e). Expression of M8ΔGro or M8SAΔGro in *Df(3R)^{BX22}* elicited ectopic MC's whose penetrance was indistinguishable from the baseline numbers in the relevant controls (*G455/+* or *G455/+; Df(3R)^{BX22}/+*), suggesting that both are non-functional (Fig. 2e, and data not shown). The potent loss of MC's with M8 or M8SD was not affected by *Df(3R)^{BX22}* (Fig. 2e), reflecting the suggestion that halved *gro* dosage is not rate limiting for repression by *E(spl)* (Nagel and Preiss, 1999).

The modest (but reproducible) enhancement by *Df(3R)^{BX22}* of the MC defects of M8SDΔGro, raised the possibility that this reflects the timing of expression with *G455.2*. While *G455.2* is PNC-specific (Giebel and Campos-Ortega, 1997), it is unknown if expression with this driver closely correlates with the onset of lateral inhibition or is delayed. Although this potential delay does not hamper MC loss by full-length (functional) M8 or M8SD (Fig. 2e), the timing of expression would be important for an antimorph of *E(spl)* proteins, which are thought to function as dimers (Alifragis *et al.*, 1997). If expression with *G455.2* were delayed (even slightly), ectopic M8SDΔGro would be less efficiently incorporated into endogenous *E(spl)* dimers, thus dampening its activity, leading to an underestimate of its 'strength'.

We thus tested if simultaneous co-expression of M8SDΔGro would attenuate the potent MC loss of *G455.2/+; UAS-m8/+* flies (Fig. 2f). This is, indeed, the case. Importantly, 87% flies co-expressing M8+M8SDΔGro displayed 4 MC's/scutellum, a number similar to that in *G455.2/+* flies (89%, see Fig. 2f). No such suppression was observed upon co-expression of

M8ΔGro, a variant that does not exhibit any antimorphic properties (Fig. 2f). Co-expression of a *UAS-LacZ* construct did not attenuate the MC loss of M8 (Fig. 2f), or the ectopic MC phenotype of M8SDΔGro (data not shown), indicating that suppression by M8SDΔGro does not involve competition by two *UAS*-constructs for a rate-limiting amount of Gal4. Thus, the well established antimorphic behavior (ectopic MC's) of M8* (Giebel and Campos-Ortega, 1997) is only seen with the phosphomimetic variant M8SDΔGro.

Compromising CK2 inhibits repression by ectopic M8

We have previously tested and found that M8 and M8SD elicit bristle loss with similar potencies (Karandikar *et al.*, 2004). However, the possibility remained that these are limit phenotypes. Given the antimorphic activity of M8SDΔGro, we tested whether compromising CK2 would impair the ability of ectopic M8 to elicit loss of MC's, microchaetes (mc's), and IOB's (Fig. 2g, g'). Indeed, these effects were suppressed by the simultaneous expression of a *UAS-CK2α-RNAi* construct (Fig. 2h, h'), which specifically targets the catalytic (α) subunit of *Drosophila* CK2 (Bose *et al.*, 2006). Similar results were obtained upon co-expression of *UAS-Tik* (Bose *et al.*, 2006), a dominant-negative construct that encodes a catalytically dead CK2αsubunit (data not shown). Thus, the autoinhibition attributed to the CtD and its regulation by (CK2) phosphorylation also appear to influence the interaction of M8 with ASC members.

M8SDΔGro suppresses the retinal defects of *N^{spl}*

Due to the similar behavior of M8* and M8SDΔGro during bristle and retinal development in *N⁺* flies, we next employed *N^{spl}*, a gain of function allele that renders R8 precursors' sensitive to inhibitory N signaling (Li *et al.*, 2003). This inappropriate N activity negatively impacts R8 specification and differentiation, and consequently *N^{spl}/Y* flies display a uniformly rough and a reduced eye (Fig. 3a). As expected, and previously described (Nagel *et al.*, 1999), expression of M8* elicited a severely reduced eye in *N^{spl}/Y* animals (Fig. 3g). Unexpectedly, however, expression of M8SDΔGro with *h^{H10}Gal4* suppressed the rough and reduced eye of *N^{spl}* and appeared to restore ommatidial phasing (see ventral half of eye, Fig. 3b); similar results were obtained upon expression of all 14 independent *UAS-m8SDΔGro* insertions (data not shown). Expression of M8ΔGro or M8SAΔGro (with *h^{H10}Gal4*) did not enhance or suppress the rough and reduced eye of *N^{spl}* males in multiple independent lines (Fig. 3h and data not shown).

To further assess these findings, we next tested for the spatial requirements for suppression of *N^{spl}* by M8SDΔGro, by employing drivers whose expression domains relative to the MF are well known. These are *109-68Gal4* (within the MF) and *gmrGal4* (posterior to the MF and after R8 patterning). We find that expression of M8SDΔGro with *109-68Gal4* also suppressed the rough and reduced eye of *N^{spl}* (Fig. 3c), whereas its expression with *gmrGal4* was without effect (Fig. 3d). A similar study with *scaGal4* (whose expression also occurs within the MF) was not conducted because *sca* is a modifier of *N^{spl}* (Brand and Campos-Ortega, 1990). As with *h^{H10}Gal4*, expression of M8ΔGro or M8SAΔGro with *109-68Gal4* was without effect in multiple independent insertions (data not shown, and see below). As added controls, we have confirmed that 1) *N^{spl}/Y; UAS-m8SDΔGro/+*, 2) *N^{spl}/Y; h^{H10}Gal4/+* or 3) *N^{spl}/Y; 109-68Gal4/+* flies all display rough and reduced eyes characteristic of *N^{spl}* males (data not shown, and see below); thus expression of M8SDΔGro was required for suppression.

To quantify the efficiency of rescue, we determined the ommatidial (facet) numbers in ≥ 15 flies of each of the relevant genotypes as described (Jones *et al.*, 2006). In this analysis, *N^{spl}* males, which display 323 ± 10 ommatidia, represent the baseline (Fig. 3i). Unlike the severely reduced eye of M8* (≤ 15 facets), expression of M8SDΔGro significantly increased facet numbers (Fig. 3i). This effect was stronger when this variant was expressed with *109-68Gal4* (566 ± 13 facets) as compared to *h^{H10}Gal4* (521 ± 15 facets). Consistent with the adult eye phenotype, expression of M8SDΔGro with *gmrGal4* resulted in 334 ± 16 facets, a

number virtually identical to that in N^{spl} (Fig. 3i). Thus, M8SDΔGro is ineffective in modulating N^{spl} when expressed posterior to the MF and after R8 specification. Facet numbers did not increase upon expression of M8ΔGro or M8SAΔGro (Fig. 3i), suggesting that these variants are likely nonfunctional (Fig. 3i). As controls, we tested and found that N^{spl}/Y ; $h^{H10}Gal4/+$ or N^{spl}/Y ; $109-68Gal4/+$ flies displayed facet numbers similar to that in N^{spl}/Y (the baseline), indicating that these Gal4 drivers, by themselves, did not modify N^{spl} (Fig. 3i).

To assess whether M8SDΔGro restored R8 patterning and differentiation, we immunostained eye discs for Ato and Sens, respectively. Consistent with the findings of others (Li *et al.*, 2003; Nagel and Preiss, 1999), Ato expression is inconsistent in the MF of N^{spl} eye discs, and R8 differentiation is aberrant (note spacing defects in Ato- and Sens-positive cells in Fig. 3a'). In contrast, M8SDΔGro appears to restore Ato expression in the MF, and the spacing of the (Sens-positive) R8 cells (Fig. 3c') appears close to that in the wild type (see Fig. 1).

The retinal defects of N^{spl} are suppressed by reduced $E(spl)$ - or CK2-dosage

Given that N^{spl} is a gain of function (see above), its retinal defects should be sensitive to $E(spl)$ -dosage. Consistent with this prediction, $Df(3R)^{BX22}$ also suppressed the rough and reduced eye of N^{spl} (Fig. 3e), and increased facet numbers (478 ± 19 , Fig. 3i) to levels (~ 470 facets) reported previously (Parks *et al.*, 1995).

We next tested whether the retinal defects of N^{spl} are modulated by reduced CK2 dosage. Based on our finding that knockdown of CK2 antagonizes repression by ectopic M8 (Fig. 2g, h), we reasoned that reduced CK2 levels/activity should attenuate phosphorylation of (endogenous) M8 and thus suppress N^{spl} . Indeed, we find that $h^{H10}Gal4$ expression of a $UAS-CK2\alpha-RNAi$ construct suppressed the rough and reduced eye of N^{spl} (Fig. 3f), and increased facet numbers to levels (473 ± 23) that approached those with $Df(3R)^{BX22}$ (Fig. 3i). Similar results were obtained upon co-expression of $UAS-Tik$ (Bose *et al.*, 2006), a CK2 dominant-negative construct (data not shown). Similar to our results with M8SDΔGro, expression of the $UAS-CK2\alpha-RNAi$ construct restored Ato expression levels at the anterior margin of the MF and patterning of Sens-positive cells posterior to the MF (data not shown).

The suppression of N^{spl} by reduced $E(spl)$ or CK2 dosage suggest that phosphorylation does augment repression by M8 (Fig. 2). Given the MF-specificity of M8SDΔGro and its effects on Ato and Sens expression, it is likely that its antimorphic activity underlies suppression of N^{spl} .

M8SDΔGro suppresses the bristle defects of N^{spl}

We next assessed for modulation of the MC defects of N^{spl} . In general, 85–90% of N^{spl} males exhibit split and missing MC's, whereas they display ectopic MC's with attenuated ($\sim 45\%$) severity (Fig. 4a, d). Expression of M8SDΔGro potently suppressed the bristle defects of N^{spl} , such that only ~ 1 –2% of N^{spl} males displayed split, missing or ectopic MC's (Fig. 4d). Moreover, a majority of these flies displayed 4 MC's/scutellum (Fig. 4c), similar to wild type flies. Similar results were obtained upon expression of multiple (14) independent $UAS-m8SD\Delta Gro$ insertions (inset in Fig. 4). Consistent with our findings in the eye (see above), a reduction in $E(spl)$ dosage ($Df(3R)^{BX22}$), or a reduction of CK2 dosage ($CK2\alpha-RNAi$) also suppressed the split and missing MC's of N^{spl} , but did not modulate the ectopic MC phenotype (Fig. 4d). Under similar conditions, expression of M8*, M8ΔGro or M8SAΔGro did not suppress (or enhance in the case of M8*) the bristle defects of N^{spl} (Fig. 4b, d). As controls, we tested and found that N^{spl}/Y ; $h^{H10}Gal4/+$, N^{spl}/Y ; $UAS-m8SD\Delta Gro/+$, or N^{spl}/Y ; $UAS-CK2\alpha-RNAi/+$ flies all displayed bristle defects whose severity was indistinguishable from that of N^{spl} (data not shown). Thus M8SDΔGro is the only $E(spl)$ variant that suppresses the neural defects of N^{spl} , and its effects are mimicked by reduced CK2- or $E(spl)$ -dosage.

Discussion

The mechanisms by which E(spl) proteins mediate repression have emerged from investigations into loss/gain-of-function mutations, and in vivo assays using site-specific variants. These studies indicate that homo/heterodimers of E(spl) proteins in a complex with Gro antagonize ASC/Ato through DNA-binding and protein-protein interactions. It was initially thought that transcription of *E(spl)* in response to inhibitory N signaling and the accumulation of these proteins was, by itself, sufficient for repression of the neural fate in the non-SOP/R8 cells. Studies on the retinal defects of *E(spl)D* suggest that this is, perhaps, an oversimplification. This mutation encodes a truncated protein (M8*) and increases transcript stability in the MF of the eye disc, the site of expression of endogenous *m8* (Nagel and Preiss, 1999). Despite its inability to bind Gro, M8* severely impairs eye development in the presence of *N^{spl}*, leading to the proposal that *E(spl)D* is a *gro*-independent hypermorph (Nagel *et al.*, 1999). This possibility is enigmatic since Gro is essential for repression. Moreover, overexpression of wild-type M8 in *N^{spl}* males does not elicit a reduced eye, suggesting that repression by E(spl) is regulated through the CtD that is lacking in M8*. The severe reduced eye of M8SD, and that CK2-RNAi compromises lateral inhibition implicated this kinase in repression by M8 (Bose *et al.*, 2006; Karandikar *et al.*, 2004). Our studies on the antimorphic activity of the phosphomimetic variant M8SDΔGro, its ability to suppress *N^{spl}*, and the spatial requirements for the retinal defects of M8SD-vs-M8*, collectively, suggest that spatially controlled, multi-site phosphorylation lies at the heart of repression by M8. These studies provide new insights into repression by M8, and suggest an alternative mechanism for the retinal defects of *E(spl)D*.

Our studies provide strong evidence that M8SDΔGro exhibits key characteristics of an antimorph. It elicits ectopic bristles akin to the loss of *E(spl)C*, the severity of this phenotype is enhanced (albeit modestly) by a deficiency in the *E(spl)C*, and it potentially neutralizes the bristle loss phenotype of ectopic M8. Importantly, this antimorphic activity is observed upon expression of all 14 independent M8SDΔGro insertions using two distinct drivers (*scaGal4* and *G455.2*). This, however, is not the case for M8ΔGro or M8SAΔGro, neither of which elicits any overt (bristle) phenotypes and might thus be non-functional. The non-functional (or loss of function) behavior of M8ΔGro has been reported by others as well (Giebel and Campos-Ortega, 1997). While we have tested multiple independent M8ΔGro or M8SAΔGro insertions, it is formally possible that this reflects attenuated expression, or instability in vivo. This caveat does not apply to M8* or M8SDΔGro, since both variants elicit ectopic bristles as previously described for loss of *N* or *E(spl)* functions. Thus the antimorphic activity of M8* in *N⁺* flies is mimicked only by M8SDΔGro, among the variants tested. Giebel and Campos-Ortega (1997) presciently suggested that the antimorphic activity of M8* might reflect regulatory influence of the region between Orange and WRPW (lacking in M8*) on E(spl) activity, even though the role of phosphorylation by CK2 was unknown at that time.

It is in *N^{spl}* that dramatic differences are observed between M8* and M8SDΔGro. Our analysis is of interest because, to our knowledge, M8SDΔGro is the only variant that suppresses the retinal and bristle defects of *N^{spl}* (Fig. 5a). Several observations suggest that the antimorphic activity of M8SDΔGro underlies suppression of *N^{spl}*. First, this variant elicits rescue only when expressed anterior to, or within, the MF, and restores Ato- and Sens-expression (Fig. 3). Thus suppression by M8SDΔGro closely correlates, in time and space, to a region of the eye disc wherein the founding R8 photoreceptors are specified, a process that is impaired by *N^{spl}* (see below). Second, suppression is also seen with reduced *E(spl)* dosage (*Df(3R)^{BX22}*, Fig. 3). Of the *E(spl)* members uncovered by this deficiency (*m5*, *m7*, *m8*), only *m8* is expressed in the MF of the eye disc (Cooper *et al.*, 2000; Ligoxygakis *et al.*, 1998). The possibility thus arises that the effects of M8SDΔGro are directed at attenuating this endogenous activity. Third, suppression of *N^{spl}* is also seen upon reduced CK2 levels (Fig. 3), a condition that strongly

attenuates repression by ectopic M8 (Fig. 2). This behavior of M8SDΔGro is in contrast to the severe enhancement by M8*. Based on the autoinhibition model (Fig. 1b), one would have predicted that M8SDΔGro should have enhanced, rather than suppressed, *N^{spl}*. A refinement of the autoinhibition model (see below) and a fundamental difference in N signaling between the bristle and the eye may provide an explanation for these unexpected results.

In the case of the MC's, expression of ASC is under the control of pre-pattern factors, but not N signaling (reviewed in (Gomez-Skarmeta *et al.*, 2003)). Consequently, loss of N signaling compromises lateral inhibition, and elicits ectopic bristles, as is seen with the loss of *E(spl)* C. While all *E(spl)* members repress as dimers (Alifragis *et al.*, 1997), the underlying reason(s) have remained unclear. It has been suggested that dimers of bHLH proteins, such as Drosophila Hair or human Hes6, might be necessary for proper Gro-recruitment, possibly by the presentation of dual WRPW-motifs (Belanger-Jasmin *et al.*, 2007; Jennings *et al.*, 2008). If so, dimerization of M8* or M8SDΔGro with endogenous *E(spl)* proteins would impair Gro-recruitment and repression. This possibility is supported by our findings that M8SDΔGro potentially neutralizes repression by ectopic M8 (Fig. 2), and that these proteins form direct complexes (in yeast two-hybrid assays, data not shown). Thus the antimorphic behavior of M8* and M8SDΔGro in *N⁺* might reflect neutralization of endogenous *E(spl)*.

The divergent behaviors of M8SDΔGro and M8* in *N^{spl}* can be explained given the biphasic nature of N signaling during R8 specification, the first step in retinal histogenesis (reviewed in (Frankfort and Mardon, 2002; Hsiung and Moses, 2002)). Anterior to the MF, N elicits *ato* expression ('proneural enhancement'), whereas in the MF it elicits *E(spl)* expression and lateral inhibition (Ligoxygakis *et al.*, 1998). These functions are differentially affected in *N^{spl}*. It was initially thought that *N^{spl}*, which does not alter *E(spl)* expression, is compromised for *ato* expression (Nagel and Preiss, 1999). However, analysis of clones of *N^{spl}* (in *N^{spl}/+* females) indicates that *N^{spl}* renders R8 precursors sensitive to inhibitory N activity (Li *et al.*, 2003). This N activity negatively impacts R8 specification and differentiation. As a result, inductive signals such as Hedgehog and Decapentaplegic, which are secreted by differentiating photoreceptors posterior to the MF, are defective in *N^{spl}* (Li *et al.*, 2003). These signals are necessary for *ato* expression anterior to the MF. Thus, the reduced *ato* expression in *N^{spl}* reflects non-autonomous effects.

The identification of enhancers/suppressors of *N^{spl}* also supports the notion that this allele renders R8's sensitive to inhibitory N signaling. For example, the retinal defects of *N^{spl}* are suppressed by halved dosage of *E(spl)C* or *Dl* (Parks *et al.*, 1995), suggesting that under these conditions lateral inhibition is attenuated in the sensitized R8's. Similar effects are seen with halved dosage of *sca* (Baker *et al.*, 1990; Brand and Campos-Ortega, 1990), reflecting its role in repression of *ato* during formation of intermediate groups (Baker *et al.*, 1996). In contrast, reduced dosage of *da*, a co-activator of Ato/ASC, enhances *N^{spl}* (Brand and Campos-Ortega, 1990). *N^{spl}* is likely to also render the SOP's sensitive to inhibitory N signaling, given its diverse bristle defects (Brennan *et al.*, 1997). Thus, *N^{spl}* provides a background that is highly sensitive to levels/activities of the Ato/ASC activators as well as the *E(spl)* repressors.

It is in this (*N^{spl}*) background of reduced Ato that M8* displays hypermorphic effects. It is thought that the Ato-positive feedback loop, which mediates proneural gene 'self stimulation' in the future R8 cells (Sun *et al.*, 1998), involves cooperative interactions between adjacently positioned Ato+Da dimers (Powell *et al.*, 2004). Given the preferentially strong interaction of M8* with Ato and Da (Nagel *et al.*, 1999; Nagel and Preiss, 1999), it seems reasonable to suggest that M8* might impair the Ato feedback loop. Consistent with this possibility, Ato levels precipitously decrease anterior to the MF in *N^{spl}/Y*; *E(spl)D/+* or in *N^{spl}/Y*; *h^{H10}Gal4/UAS-m8** discs (Karandikar *et al.*, 2004; Nagel and Preiss, 1999). Given the already low Ato levels in *N^{spl}* (Fig. 3), this impairment will further attenuate Ato activity, which would then be

insufficient to support R8 specification and elicit loss of the eye field. Thus the behavior of M8* in *N^{spl}* is unlikely to reflect a hyperactive repressor (that functions without Gro), but instead reflects its ability to prevent upregulation of *ato* expression in the sensitized R8 precursors. Consistent with this, the effects of *E(spl)D* in *N^{spl}* females are enhanced by mutations in *ato* or *da* (Nagel and Preiss, 1999).

This interpretation accounts for the enhancement of *N^{spl}* by M8*, but not for its suppression by M8SDΔGro. Based on the autoinhibition model (Fig. 1), these two variants were expected to elicit similar outcomes. To resolve this paradox, we assessed the spatial requirements for the reduced eye of M8* in *N^{spl}* versus M8SD in *N⁺* (Fig. 5b). Surprisingly, expression of M8* in the MF of *N^{spl}* males (with *109-68Gal4*) has minimal effects, i.e., these flies display *N^{spl}* eyes (Fig. 5c). Thus, once the ‘Ato-self stimulatory’ loop is established and Ato levels rise M8* loses potency. This finding is in line with our suggestion that the dominant effects of M8* reflect impaired ‘Ato-self-stimulation’. The reduced eye of M8SD in *N⁺* is also specific to its expression domain (Fig. 5b). Unlike the strong loss of the eye field when expressed in the MF (*109-68Gal4*, Fig. 1) or *scaGal4* (Karandikar *et al.*, 2004), M8SD is ineffective when expressed anterior to the MF (*h^{H10}Gal4*, Fig. 5b, c). We interpret this MF specificity in the context of secondary phosphorylation.

The CK2 site in M8 resides in a region that harbors additional Ser residues (Fig. 5d). Importantly, these residues are conserved in *Drosophila* M8/5/7 and in human/murine Hes6. In the case of Hes6, secondary phosphorylation appears necessary for function in vivo (Belanger-Jasmin *et al.*, 2007). If this were to apply to *Drosophila* M8 as well, multi-site phosphorylation would be a prerequisite for repression. If CK2 phosphorylation served as a ‘priming’ step, ectopic M8SD would be precociously subject to multi-site phosphorylation, precipitating its strong repressor activity. This effect would be restricted, in that only when expressed in the MF (stage 2/3, Fig. 5b) would it demonstrate a phenotype. Its inability to elicit a reduced eye anterior to the MF (*h^{H10}Gal4*, Fig. 5c) might reflect a spatial context where the additional participating kinases have not yet been activated. Absent the secondary phosphorylation, M8SDΔGro might remain autoinhibited, i.e., refractory for Ato binding. When assayed in yeast (two-hybrid), M8SD interacts more strongly with Ato, as compared to M8 (Karandikar *et al.*, 2004), and the possibility remains that this variant is predisposed to secondary phosphorylation by endogenous yeast kinases, or that the observed interaction does not reflect the in vivo situation. In any case, M8SDΔGro retains the capacity to dimerize with, and neutralize, endogenous E(spl), and this antimorphic activity would enable Ato levels to rise to a threshold sufficient for specifying the R8 fate. This interpretation is further supported by our findings that Ato levels in the MF of *N^{spl}* discs are significantly enhanced by M8SDΔGro (Fig. 3c'), and that decreased *E(spl)*- or CK2-dosage also suppress the retinal/bristle defects of *N^{spl}* (Figs. 3, 4).

Examination of M8 indicates that Ser¹⁵¹ meets the consensus (PxSP, Fig. 5d) for the mitogen-activated protein kinases (MAPK). Indeed, diphospho-ERK (activated MAPK) is detected in intermediate group cells in the MF, leading to the suggestion that activity of the EGF receptor is required for lateral inhibition of R8 cells to occur promptly (Lesokhin *et al.*, 1999). It is, however, unknown if this regulation reflects modification of endogenous M8. The remaining sites in M8 might be modified by CK1 (Ser¹⁵⁴) and GSK3 (Ser¹⁵⁵), whose site recognition is influenced by the phosphorylation status of adjacent residues (reviewed in (Roach, 1991)). So far, R8 defects have not been described for mutations in either gene. It is noteworthy that the activities of CK2, CK1, GSK3 and the phosphatase PP2A regulate the clock protein Period (Kim and Edery, 2006; Lin *et al.*, 2002; Sathyanarayanan *et al.*, 2004). In this regard, PP2A has been implicated in N signaling (Abdelilah-Seyfried *et al.*, 2000). Irrespective if it is hierarchical or independent, multi-site phosphorylation of M8 might represent a developmental

‘checkpoint’ enabling repression to only occur in a precise spatial context and without a temporal lag.

The most parsimonious interpretation of our studies is that it is the bypass of the multi-site phosphorylation, rather than Gro-binding, that underlies the dominant behavior of M8*. Our studies provide a putative mechanism to address the paradoxical behavior of M8*, and further illustrate the complex regulatory role of phosphorylation of M8. Future efforts to parse which of these Ser residues are modified, in addition to Ser¹⁵⁹ (the CK2 site), the identity of the kinase (s), and the signaling processes will be required to more fully define the mechanism by which spatially controlled multi-site phosphorylation regulates repression by M8 during lateral inhibition and neural selection.

Methods

Plasmid construction and germline transformations

The construction of variants of M8 harboring Ala/Asp in place of Ser¹⁵⁹ (M8SA and M8SD) has been described previously (Trott *et al.*, 2001). The Gro-compromised variants were generated by the substitution of a stop codon in place of the W¹⁷⁶ of the WRPW motif. All constructs were sequenced to confirm the presence of only the intended mutations. For *in vivo* expression, cDNA's were cloned into the EcoRI and BamHI sites of the plasmid pUAST (Brand and Perrimon, 1993), and germ line transformants were generated as described (Rubin, 1983). *w*⁺ progeny were identified and the location of insertions was determined via crosses to lines harboring chromosomes carrying dominant visible markers. At least 6–14 independent insertions of each construct have been used in these studies. *UAS-m8**, *UAS-CK2α-RNAi* and *UAS-Tik* lines have been previously described (Bose *et al.*, 2006; Nagel *et al.*, 1999).

Protein-protein interactions

Protein-protein interactions were analyzed by the LexA-based version of the yeast interaction trap (Gyuris *et al.*, 1993). Bait and prey constructs were expressed as C-terminal fusions with the DNA-binding domain of LexA or the activation domain (AD) of B42, and interactions were assessed in yeast EGY048. *LacZ* activity was determined for at least three independent transformants, each in triplicate, employing XGal or ONPG as substrates, as described (Trott *et al.*, 2001). *LacZ* activity was determined using the formula $1000 \times \text{OD}_{420} / (\text{Tx} \times \text{V} \times \text{OD}_{600})$, where T is minutes and V is the concentration factor of the assay.

Fly stocks, crosses and phenotypes

Flies were raised at 24°C on standard Yeast-Glucose medium. The *Gal4* drivers used in these studies were generously provided by other researchers or obtained from the Bloomington Stock Center (denoted by the prefix B). These drivers are *G455.2* (Giebel and Campos-Ortega, 1997), *scaGal4* (Nakao and Campos-Ortega, 1996), *109-68Gal4* (Jarman and Ahmed, 1998), and *h^{H10}Gal4* (Huang and Fischer-Vize, 1996). *UAS-m8**, and *UAS-ato* flies were gifts of Drs. Anette Preiss and Yuh Nun Jan, respectively.

All crosses were performed at 24°C. Fly heads were dehydrated by sequential passes through a graded alcohol series (25-50-75-absolute) and finally through Hexamethyldisilizane. Heads were mounted on EM stubs, dried for 24 hours, sputter coated with gold, and examined with a JEOL-6400 scanning electron microscope at an accelerating voltage of 20 kV. For bristle phenotypes, newly eclosed adults were photographed. For quantitative analysis of the bristle phenotypes, multiple crosses were established ($\geq$ triplicates), and adults were scored for bristle artifacts. In every case multiple independent insertions of *UAS*-constructs were used.

Immunostaining and confocal microscopy

Imaginal discs were isolated from late third instar larvae and processed as described (Kavler *et al.*, 1999) with modifications. Discs were fixed in 4% paraformaldehyde in 1x phosphate buffered saline (PBS) for 15 minutes, and washed three times with PBS containing 0.1% Triton X-100 (PBS-TX). The discs were incubated for 12 hours at 4°C in PBS-TX containing 5% normal goat serum and then immunostained. The following antibodies were used in this study: rabbit anti-Ato (1:1000, gift of Yuh Nun Jan), guinea pig anti-Sens (1:800, gift of Hugo Bellen) and mAb-ELAV (1:1000, DHSB, Iowa City). Secondary antibodies (Molecular Probes) were goat-anti rabbit-IgG coupled to Alexa Fluor 594 (1:1000), donkey anti-guinea pig-IgG coupled to Alexa Fluor 488 (1:1000), and goat anti-mouse-IgG coupled to Alexa Fluor 594 (1:1000). Discs were mounted in Vectashield. An Olympus FluoView (FV1000) was used for confocal imaging. Images in Figs. 1 and 3 represent scans acquired every 1 µm along the apicobasal axis of the discs and then compressed as a Z-stack.

Acknowledgments

We thank Philip Keating for comments on the manuscript, and Sophia Zhang for technical assistance. We are indebted to Anette Preiss for *UAS-m8** flies, Yuh Nung Jan for *UAS-ato* and *109-68Gal4* flies and anti-Ato antibodies, Hugo Bellen for anti-Sens antibodies, J. Fischer-Vize for *h^{H10}Gal4* flies and Christos Delidakis for the *Dff(3R)^{boss14}* and *Dff(3R)^{BX22}* flies. We thank Diane Schwegler-Berry (NIOSH, CDC) for assistance with electron microscopy. This work was supported by a National Institutes of Health Grant EY015718 to A.P.B.

References

- Abdelilah-Seyfried S, Chan YM, Zeng C, Justice NJ, Younger-Shepherd S, Sharp LE, Barbel S, Meadows SA, Jan LY, Jan YN. A gain-of-function screen for genes that affect the development of the Drosophila adult external sensory organ. *Genetics* 2000;155:733–752. [PubMed: 10835395]
- Alifragis P, Poortinga G, Parkhurst SM, Delidakis C. A network of interacting transcriptional regulators involved in Drosophila neural fate specification revealed by the yeast two-hybrid system. *Proc Natl Acad Sci U S A* 1997;94:13099–13104. [PubMed: 9371806]
- Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science* 1999;284:770–776. [PubMed: 10221902]
- Bailey AM, Posakony JW. Suppressor of *hairless* directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. *Genes & Dev* 1995;9:2609–2622. [PubMed: 7590239]
- Baker, NE. Notch and the patterning of ommatidial founder cells in the developing Drosophila eye. In: Moses, K., editor. *Drosophila eye development*. Heidelberg: Springer-Verlag; 2002. p. 35-58.
- Baker NE, Mlodzik M, Rubin GM. Spacing differentiation in the developing Drosophila eye: a fibrinogen-related lateral inhibitor encoded by *scabrous*. *Science* 1990;250:1370–1377. [PubMed: 2175046]
- Baker NE, Yu S, Han D. Evolution of proneural atonal expression during distinct regulatory phases in the developing Drosophila eye. *Curr Biol* 1996;6:1290–1301. [PubMed: 8939576]
- Baonza A, Freeman M. Notch signalling and the initiation of neural development in the Drosophila eye. *Develop* 2001;128:3889–3898.
- Belanger-Jasmin S, Llamas E, Tang Y, Joachim K, Osiceanu AM, Jhas S, Stifani S. Inhibition of cortical astrocyte differentiation by *Hes6* requires amino- and carboxy-terminal motifs important for dimerization and phosphorylation. *J Neurochem* 2007;103:2022–2034. [PubMed: 17868320]
- Bertrand N, Castro DS, Guillemot F. Proneural genes and the specification of neural cell types. *Nat Rev Neurosci* 2002;3:517–530. [PubMed: 12094208]
- Bose A, Kahali B, Zhang S, Lin J-M, Allada R, Karandikar U, Bidwai A. Drosophila CK2 regulates lateral-inhibition during eye and bristle development. *Mech Dev* 2006;123:649–664. [PubMed: 16930955]
- Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Develop* 1993;118:401–415.

- Brand M, Campos-Ortega JA. Second-site modifiers of the split mutation of Notch defines genes involved in neurogenesis in *Drosophila melanogaster*. *Dev Genes Evol* 1990;198:275–285.
- Bray SJ. Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* 2006;7:678–689. [PubMed: 16921404]
- Brennan K, Tateson R, Lewis K, Arias AM. A Functional Analysis of Notch Mutations in *Drosophila*. *Genetics* 1997;147:177–188. [PubMed: 9286678]
- Cooper MTD, Tyler DM, Furriols M, Chalkiadaki A, Delidakis C, Bray SJ. Spatially Restricted Factors Cooperate with Notch in the Regulation of Enhancer of split Genes. *Dev Biol* 2000;221:390–403. [PubMed: 10790334]
- de Celis JF, de Celis J, Ligoxygakis P, Preiss A, Delidakis C, Bray SJ. Functional relationship between Notch, Su(H) and the bHLH genes of the E(spl) complex: the E(spl) genes mediate only a subset of Notch activities during imaginal development. *Develop* 1996;122:2719–2928.
- de la Concha A, Dietrich U, Weigel D, Campos-Ortega JA. Functional interactions of the neurogenic genes of *Drosophila*. *Genetics* 1988;118:499–508. [PubMed: 17246418]
- Delidakis C, Preiss A, Hartley DA, Artavanis-Tsakonas S. Two genetically and molecularly distinct functions involved in early neurogenesis reside within the enhancer of split locus of *Drosophila melanogaster*. *Genetics* 1991;129:803–823. [PubMed: 1752423]
- Fisher AL, Ohsako S, Caudy M. The WRPW motif of the hairy-related bHLH repressor proteins acts as a 4-amino acid transcriptional repression and protein-protein interaction domain. *Mol Cell Biol* 1996;16:2670–2677. [PubMed: 8649374]
- Frankfort BJ, Mardon G. R8 development in the *Drosophila* eye: a paradigm for neural selection and differentiation. *Develop* 2002;129:1295–1306.
- Frankfort BJ, Nolo R, Zhang Z, Bellen H, Mardon G. senseless repression of rough is required for R8 photoreceptor differentiation in the developing *Drosophila* eye. *Neuron* 2001;32:403–414. [PubMed: 11709152]
- Ghysen A, Richelle J. Determination of sensory bristles and pattern formation in *Drosophila*. II. The achaete-scute locus. *Dev Biol* 1979;70:438–452. [PubMed: 478169]
- Giebel B, Campos-Ortega JA. Functional dissection of the *Drosophila* enhancer of split protein, a suppressor of neurogenesis. *Proc Natl Acad Sci U S A* 1997;94:6250–6254. [PubMed: 9177203]
- Gomez-Skarmeta JL, Campuzano S, Modolell J. Half a century of neural pre patterning: the story of a few bristles and many genes. *Nat Rev Neurosci* 2003;4:587–598. [PubMed: 12838333]
- Gyuris J, Golemis E, Chertkov H, Brent R. Cdi1, a human G1 and S phase protein phosphatase that associates with cdk2. *Cell* 1993;75:791–803. [PubMed: 8242750]
- Hsiung F, Moses K. Retinal development in *Drosophila*: specifying the first neuron. *Hum Mol Genet* 2002;11:1207–1214. [PubMed: 12015280]
- Huang Y, Fischer-Vize J. Undifferentiated cells in the developing *Drosophila* eye influence facet assembly and require the Fat facets ubiquitin-specific protease. *Develop* 1996;122:3207–3216.
- Jarman AP, Ahmed I. The specificity of proneural genes in determining *Drosophila* sense organ identity. *Mech Dev* 1998;76:117–125. [PubMed: 9767145]
- Jarman AP, Grell EH, Ackerman L, Jan LY, Jan YN. *atonal* is the proneural gene for *Drosophila* photoreceptors. *Nature* 1994;369:398–400. [PubMed: 8196767]
- Jennings B, Preiss A, Delidakis C, Bray SJ. The Notch signaling pathway is required for *Enhancer of split* bHLH protein expression during neurogenesis in *Drosophila*. *Develop* 1994;120:3537–3548.
- Jennings BH, Wainwright SM, Ish-Horowicz D. Differential in vivo requirements for oligomerization during Groucho-mediated repression. *EMBO Rep* 2008;9:76–83. [PubMed: 18034187]
- Jones C, Reifegerste R, Moses K. Characterization of *Drosophila* mini-me, a Gene Required for Cell Proliferation and Survival. *Genetics* 2006;173:793–808. [PubMed: 16547096]
- Karandikar U, Trott RL, Yin J, Bishop CP, Bidwai AP. *Drosophila* CK2 regulates eye morphogenesis via phosphorylation of E(spl)M8. *Mech Dev* 2004;121:273–286. [PubMed: 15003630]
- Kavler J, Fu W, Duan H, Noll M, Posakony JW. An essential role for the *Drosophila* Pax2 homolog in the differentiation of adult sensory organs. *Develop* 1999;126:2261–2272.

- Kim EY, Edery I. Balance between DBT/CKIepsilon kinase and protein phosphatase activities regulate phosphorylation and stability of *Drosophila* CLOCK protein. *Proc Natl Acad Sci U S A* 2006;103:6178–6183. [PubMed: 16603629]
- Lecourtois M, Schweisguth F. The neurogenic suppressor of hairless DNA-binding protein mediates the transcriptional activation of the enhancer of split complex genes triggered by Notch signaling. *Genes & Dev* 1995;9:2598–2608. [PubMed: 7590238]
- Lehmann R, Jimenez F, Dietrich U, Campos-Ortega JA. On the phenotype and development of mutants of early development in *Drosophila melanogaster*. *Roux's Arch Dev Biol* 1983;192:62–74.
- Lesokhin AM, Yu S-Y, Katz J, Baker NE. Several levels of EGF receptor signaling during photoreceptor specification in wild-type, ellipse, and null mutant *Drosophila*. *Dev Biol* 1999;205:129–144. [PubMed: 9882502]
- Li Y, Lei L, Irvine KD, Baker NE. Notch activity in neural cells triggered by a mutant allele with altered glycosylation. *Develop* 2003;130:2829–2840.
- Ligoxygakis P, Yu SY, Delidakis C, Baker NE. A subset of Notch functions during *Drosophila* eye development require Su(H) and E(spl) gene complex. *Develop* 1998;125:2893–2900.
- Lin JM, Kilman VL, Keegan K, Paddock B, Emery-Le M, Rosbash M, Allada R. A role for casein kinase 2alpha in the *Drosophila* circadian clock. *Nature* 2002;420:816–820. [PubMed: 12447397]
- Modolell J, Campuzano S. The achaete-scute complex as an integrating device. *Int J Dev Biol* 1998;42:275–282. [PubMed: 9654009]
- Mumm JS, Kopan R. Notch signaling: From the outside in. *Dev Biol* 2000;228:151–165. [PubMed: 11112321]
- Nagel A, Yu Y, Preiss A. Enhancer of Split [E(spl)D] is a Gro-independent, hypermorphic mutation in *Drosophila*. *Dev Genet* 1999;25:168–179. [PubMed: 10440851]
- Nagel AC, Preiss A. Notch spl is deficient for inductive processes in the eye, and E(spl)D enhances split by interfering with proneural activity. *Dev Biol* 1999;208:406–415. [PubMed: 10191054]
- Nakao K, Campos-Ortega JA. Persistent expression of genes of the Enhancer of Split Complex suppress neural development in *Drosophila*. *Neuron* 1996;16:275–286. [PubMed: 8789943]
- Nolo R, Abbott LA, Bellen HJ. Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in *Drosophila*. *Cell* 2000;102:349–362. [PubMed: 10975525]
- Parks AL, Turner R, Muskavitch MAT. Relationship between complex Delta expression and the specification of retinal cell fates during *Drosophila* eye development. *Mech Dev* 1995;50:201–216. [PubMed: 7619731]
- Paroush Z, Finley RL, Kidd T, Wainwright SM, Ingham PW, Brent R, Ish-Horowicz D. Groucho is required for *Drosophila* neurogenesis, segmentation, and sex determination and interacts directly with hairy related bHLH proteins. *Cell* 1994;79:805–815. [PubMed: 8001118]
- Powell LM, Zur Lage PI, Prentice DR, Senthinathan B, Jarman AP. The proneural proteins Atonal and Scute regulate neural target genes through different E-box binding sites. *Mol Cell Biol* 2004;24:9517–9526. [PubMed: 15485919]
- Roach P. Multisite and hierarchal protein phosphorylation. *J Biol Chem* 1991;266:14139–14142. [PubMed: 1650349]
- Rubin G. Vectors for P element-mediated gene transfer in *Drosophila*. *Nuc Acids Res* 1983;11:6341.
- Sathyanarayanan S, Zheng X, Xiao R, Sehgal A. Posttranslational regulation of *Drosophila* PERIOD protein by protein phosphatase 2A. *Cell* 2004;116:603–615. [PubMed: 14980226]
- Simpson P. Lateral inhibition and the development of the sensory bristles of the adult peripheral nervous system of *Drosophila*. *Develop* 1990;109:509–519.
- Simpson P, Bourouis M, Heitzler P, Ruel L, Haenlin M, Romain P. Delta, notch, and shaggy: elements of a lateral signaling pathway in *Drosophila*. *Cold Spring Harb Symp Quant Biol* 1992;57:391–400. [PubMed: 1339674]
- Skeath JB, Carroll SB. Regulation of achaete-scute gene expression and sensory organ pattern formation in the *Drosophila* wing. *Genes & Dev* 1991;5:984–995. [PubMed: 2044964]
- Sun Y, Jan L, Jan Y. Transcriptional regulation of atonal during development of the *Drosophila* peripheral nervous system. *Develop* 1998;125:3731–3740.

- Tietze K, Oellers N, Knust E. Enhancer of SplitD, a dominant mutation of *Drosophila*, and its use in the study of functional domains of a helix-loop-helix protein. *Proc Natl Acad Sci U S A* 1992;89:6152–6156. [PubMed: 1631102]
- Trott RL, Kalive M, Paroush Z, Bidwai AP. *Drosophila melanogaster* casein kinase II interacts with and phosphorylates the basic-helix-loop-helix (bHLH) proteins m5, m7, and m8 derived from the Enhancer of split complex. *J Biol Chem* 2001;276:2159–2167. [PubMed: 11208814]
- White N, Jarman A. *Drosophila atonal* controls photoreceptor R8-specific properties and modulates both receptor tyrosine kinase and Hedgehog signalling. *Develop* 2000;127:1681–1689.

Figure 1. Role of phosphorylation of M8 and suppression of eye defects of M8SD with increased dosage of Ato

(a) Conserved domains in M8, M5, and M7. The C-terminal domain (CtD) harbors a CK2 phosphorylation site (SDCD). (b) Regulation of the antagonism of Ato upon phosphorylation of M8 (color of boxes correspond to those in panel a). The ability of M8SD to antagonize Ato and block eye development occurs in N^{+} flies, whereas M8* requires N^{spl} . (c–e) The eye defects of M8SD are suppressed by increased Ato dosage. The genotypes are, (c) *109-68Gal4/+; UAS-m8SD/+*, (d) *109-68Gal4/+; UAS-ato/+*, and (e) *109-68Gal4/+; UAS-m8SD/UAS-ato*. Magnifications are 200x. (c', d', e') Eye discs were immunostained with α -Sens; WT denotes wild type. Arrows denote the direction of MF progression. The genotype of discs in c', d' and e' are as in panels c, d and e, respectively.

a: E(spl) constructs **# UAS-lines**

1 (Nagel et al, 1999)

14

6

14

b: Protein-interactions

	Gro	Ato
M8*	-	+
M8	+	-
M8ΔGro	-	-
M8SD	+	+
M8SDΔGro	-	+
M8SA	+	-
M8SAΔGro	-	-

c: Phenotypes (with *scaGal4*)

	Eye	IOB	MC
<i>scaGal4/+</i>	--	--	19% Ectopic
M8SD	Reduced	Loss	Loss
M8SDΔGro	NE	NE	73% Ectopic ←
M8	NE	Loss	Loss
M8ΔGro	NE	NE	15% Ectopic
M8*	NE	NE	81% Ectopic ←

Bristle phenotypes (n≥200) **Figure 2. M8SDΔGro exhibits antimorphic activity**

(a) Schematic of variants. The triangles denote removal of WRPW (ΔGro), and S/A/D represent variants harboring Ser/Ala/Asp at position 159, respectively. (b) Yeast two hybrid interactions. (c) Crosses between *scaGal4* and *UAS*-constructs were conducted at 24°C; NE denotes no effect. The inability of M8* to elicit any eye defects is because crosses were conducted in *N*⁺ flies. (d–f) Bristle phenotypes; the genotypes are as indicated. (g–h) The IOB, MC and mc loss of ectopic M8 are antagonized by co-expression of a *UAS-CK2α-RNAi* construct.

Figure 3. M8SDΔGro suppresses the retinal defects of N^{spl}
 (a–g) Eye phenotypes; the Gal4 drivers are, $h^{H10}G$ ($h^{H10}Gal4$), $109-68G$ ($109-68Gal4$), and $gmrG$ ($gmrGal4$). The genotypes are, (a) N^{spl}/Y , (b) $N^{spl}/Y; UAS-m8SD\Delta Gro/+; h^{H10}Gal4/+$, (c) $N^{spl}/Y; 109-68Gal4/UAS-m8SD\Delta Gro$, (d) $N^{spl}/Y; UAS-m8SD\Delta Gro/+; gmrGal4/+$, (e) $N^{spl}/Y; Df(3R)BX22/+$, (f) $N^{spl}/Y; UAS-CK2\alpha-RNAi/+; h^{H10}Gal4/+$, and (g) $N^{spl}/Y; UAS-m8^*/+; h^{H10}Gal4/+$. Magnifications are 200x. (h) Summary of effects on N^{spl} . The number in parenthesis denotes number of insertions tested. (i) Quantitative analysis of suppression of N^{spl} (facet counts). The numbers correspond to the genotypes in panels a–g, and asterisks denote P -values < 0.0001 . (a', c') Eye discs were immunostained with $\alpha-Ato$ and $\alpha-Sens$.

Genotypes of discs in a' and c' correspond to those in panels a and c, respectively, and arrows denote MF progression.

Figure 4. M8SDΔGro suppresses the bristle defects of N^{spl}

(a–c) Nota of flies. Genotypes are, (a) N^{spl}/Y , (b) $N^{spl}/Y; UAS-m8^*/+; h^{H10}Gal4/+$, and (c) $N^{spl}/Y; UAS-m8SD\Delta Gro/+; h^{H10}Gal4/+$. Asterisks denote missing bristles, and arrows denote split bristles, both characteristic of N^{spl} males. (d) Modulation of N^{spl} bristle defects by M8-variants, reduced $E(spl)$ - or CK2-dosage. UAS constructs were expressed with $h^{H10}Gal4$. Inset shows the number of insertions tested; in all cases ≥ 500 flies of the relevant genotype were assessed for MC defects. Arrows denote strong suppression and arrowheads denote moderate suppression.

Figure 5. Modulation of *N^{sp}* and MF-specificity

(a) M8SDΔGro, reduced *E(spl)*- or reduced CK2-dosage suppress the retinal defects of *N^{sp}*, whereas M8* enhances. (b) MF specificity of M8* and M8SD. Schematic of cells spanning stages 1–4 of the MF are shown relative to the Gal4 drivers. *ato* expression in response to N signaling ('proneural enhancement') is denoted as a shaded triangle. Retinal phenotypes of M8* (in *N^{sp}*) and M8SD (in *N⁺*). Black box denotes a region of expression leading to a reduced eye and strikethrough denotes no retinal phenotypes. (c) Adult eye phenotypes of the indicated genotypes. (d) Conserved secondary phosphorylation sites (black dots) in *Drosophila E(spl)* and human (h) and murine (m) Hes6. Numbers above alignment refer to the M8 protein. Ser¹⁵⁹ and Ser¹⁵¹ meet the consensus recognition motifs for CK2 and MAPK, respectively.