

# ***Drosophila* CK2 phosphorylates Deadpan, a member of the HES family of basic-helix-loop-helix (bHLH) repressors**

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## **Abstract**

In *Drosophila*, protein kinase CK2 regulates a diverse array of developmental processes. One of these is cell-fate specification (neurogenesis) wherein CK2 regulates basic-helix-loop-helix (bHLH) repressors encoded by the *Enhancer of Split Complex* (*E(spl)C*). Specifically, CK2 phosphorylates and activates repressor functions of E(spl)M8 during eye development. In this study we describe the interaction of CK2 with an E(spl)-related bHLH repressor, Deadpan (Dpn). Unlike E(spl)-repressors which are expressed in cells destined for a non-neural cell fate, Dpn is expressed in the neuronal cells and is thought to control the activity of proneural genes. Dpn also regulates sex-determination by repressing *sxl*, the primary gene involved in sex differentiation. We demonstrate that Dpn is weakly phosphorylated by monomeric CK2 $\alpha$ , whereas it is robustly phosphorylated by the embryo-holoenzyme, suggesting a positive role for CK2 $\beta$ . The weak phosphorylation by CK2 $\alpha$  is markedly stimulated by the activator polylysine to levels comparable to those with the holoenzyme. In addition, pull down assays indicate a direct interaction between Dpn and CK2. This is the first demonstration that Dpn is a partner and target of CK2, and raises the possibility that its repressor functions might also be regulated by phosphorylation. (*Mol Cell Biochem* 274: 133–139, 2005)

*Key words:* CK2, Deadpan, *Drosophila*, HES, phosphorylation

## **Introduction**

Global signaling pathways are employed in a recurring fashion throughout development to regulate cell-fate specification and differentiation of diverse cell types. The roles of the Notch pathway during neurogenesis, myogenesis, egg chamber formation, etc., is just one notable example (reviewed in [1–4]). In *Drosophila*, during the process of neurogenesis, which occurs in the neuroectoderm, Notch orchestrates expression of bHLH transcription factors that either promote (proneural) or restrict (neurogenic) neuronal cell fate [5–7]. The former group includes bHLH activators encoded by the *achaete-scute* complex (*ASC*) or *atonal* (*ato*), whereas the

latter includes bHLH repressors encoded by the *Enhancer of Split* complex (*E(spl)C*). In the developing eye (for reviews see, [8–12]), Notch initially drives expression of Atonal [13–15], and this expression sets up neural competency in groups of cells called proneural clusters. However, with the exception of one cell from this cluster which goes on to adopt a neural fate, others are redirected to an alternate cell fate by expression of E(spl)-repressors [5, 14]. This inhibitory function of Notch has been termed ‘lateral inhibition’, and is critical for singling out cells of each proneural cluster that go on to differentiate as neurons [16, 17]. Thus, interference with the inhibitory phase of Notch elicits supernumerary neurons. In the eye, E(spl)M8 antagonizes the transcriptional functions

of Atonal via direct protein-protein interactions upon phosphorylation of E(spl)M8 (by CK2) at a highly conserved site [18]. As in the eye, bristle morphogenesis also depends on proneural factors encoded by *ASC* (Achaete, Scute, Lethal of Scute), and their transcriptional functions are similarly antagonized by E(spl) repressors [19–21]. However, during bristle morphogenesis, phosphorylation of E(spl) proteins by CK2 appears to be dispensable [18]. These results raise the possibility that the role of CK2 in regulation of E(spl) functions might be context specific.

The E(spl) proteins along with Hairy and Dpn constitute a group of evolutionarily conserved proteins that are collectively referred to as the HES (Hairy and Enhancer of Split) family [22–24], and they share some common modes of action. Accordingly, these proteins exhibit a number of conserved domains: a basic domain for DNA-binding, a helix-loop-helix domain for dimerization, an Orange-domain that determines specificity of interactions with proneural proteins, and an invariant C-terminal tetrapeptide, WRPW that recruits the co-repressor Groucho [25]. Given the structural similarity of these proteins, we reasoned that CK2 might also regulate Dpn via phosphorylation. In this report, we demonstrate an interaction of CK2 with Dpn.

Dpn is a pan-neural bHLH protein with structural similarities to E(spl)-repressors [22, 26]. However, in contrast to E(spl)-repressors (which are expressed in cells destined for a non-neural fate), Dpn is expressed in neuronal precursors as soon as they are formed and plays important roles during neurogenesis. Consistent with this, loss of *dpn* has been reported to affect the function but not the gross morphology of the nervous system. Consequently, an absence of Dpn elicits weak motor activity and is lethal [26]. In addition, Dpn also plays an important role in sex determination [27, 28]. Sex determination, based on the ratio of X-chromosomes to the set of autosomes, initiates in the embryo and involves the functions of three types of genes; X-linked numerator elements such as *sis-a*, *sis-b* (*scute*), and *runt*, autosomally linked denominator elements (*dpn*), and maternal factors such as *daughterless* (*da*) and *extramacrochaetae* (*emc*) [28, 29]. The X/A ratio regulates the activity of a binary switch gene, *sex lethal* (*sxl*); when this ratio is 1:1 (females) *sxl* is active and directs feminization, whereas when it is 1:2 (males) *sxl* remains inactive. In this context, Dpn acts as a denominator element and one of its functions is to antagonize numerator derived elements, i.e., products of *sis-a* and *sis-b* (Scute) via protein-protein interactions [30]. As mentioned above, Scute also functions during neurogenesis. Thus, neurogenesis and sex determination share some common positive and negative elements for achieving cell fate specification. This supports the prescient suggestion that animal development employs conserved ‘functional gene cassettes’ reiteratively [31].

In this report, we demonstrate that another member of the HES family, Dpn, is also a physical partner and target of pro-

tein kinase CK2. Dpn contains two sites for phosphorylation by CK2. One of these is positionally conserved in a subdomain of some members of the HES family that regulates their interaction with proneural factors in a CK2-dependent manner. Taking into account the observation that CK2 profoundly influences repressor activity of E(spl)M8 [18], it would appear reasonable to suggest that this protein kinase might also regulate Dpn functions *in vivo*. The implications of these findings are discussed.

## Materials and methods

### *Yeast two-hybrid assay*

All manipulations involved in construction of the two-hybrid plasmids were carried out using standard methods, and employed vectors for expression of proteins as C-terminal fusions to the DNA-binding domain of LexA or the activation domain of VP16 [32]. The construction of plasmids expressing CK2/ $\beta$  as LexA- or AD-fusions has been previously described [33]. LexA-Deadpan was a generous gift from Zeev Paroush (Hebrew University, Jerusalem) and has been previously described [34]. Two-hybrid interactions were studied in yeast EGY048 containing plasmid pSH18-34. Various combinations of LexA- and AD-fusion plasmids were transformed into EGY048 using lithium acetate, and cultures were analyzed in triplicate for reporter gene (*LacZ*) expression using a solution-based assay as described [33].

### *Purification of GST-Dpn and phosphorylation by CK2*

A full length Dpn cDNA was subcloned into the vector pZEX wherein the cDNA is expressed as a C-terminal fusion with GST, and transformed in *E. coli* BL21. GST and GST-Dpn were expressed and purified essentially as described [33]. Following purification, the fusion protein was exchanged into storage buffer (50 mM Tris, pH 8.0, 0.5 mM EDTA, 10% glycerol, 200 mM NaCl, 1 mM PMSF) and concentrated using a Biomax-10K centrifugal filter device (Millipore). The concentration and purity were estimated by densitometry of Coomassie stained bands following SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and calibrated using known protein standards.

2  $\mu$ g of purified GST or GST-Dpn protein were subjected to phosphorylation in 50 mM Tris, pH 8.5, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 10  $\mu$ M ATP, 5  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP and CK2 (1  $\mu$ g/ml) in a total volume of 40  $\mu$ l. The reactions were terminated by boiling for 5 min following the addition of 10  $\mu$ l of 5x sample buffer [35]. Samples were separated by electrophoresis in 12% acrylamide gels containing sodium

dodecyl sulfate, stained with Coomassie, and the destained gels were exposed to Kodak XAR-5 film at room temperature. In order to study the effects of polybasic activators, reactions were supplemented with 0.68 mg/ml spermine, 100  $\mu$ g/ml poly(DL)lysine, or 125  $\mu$ g/ml protamine.

#### Direct protein-protein interactions

Interactions between Dpn and CK2 were assessed by pull down assays employing GST-fusion proteins and either CK2-holoenzyme or monomeric CK2 $\alpha$ . CK2 holoenzyme was purified from embryos according to Glover *et al.* [36], while CK2 $\alpha$  was over-expressed via functional complementation of the lethality of *ckal cka2 S. cerevisiae*, and purified to homogeneity as described [37]. The  $V_{max}$  of CK2 $\alpha$  is 0.4  $\mu$ mol/min/mg and that of the  $\alpha_2\beta_2$  holoenzyme is 1.6  $\mu$ mol/min/mg using partially hydrolyzed and dephosphorylated casein as a substrate. These values are similar to those reported earlier [36, 37].

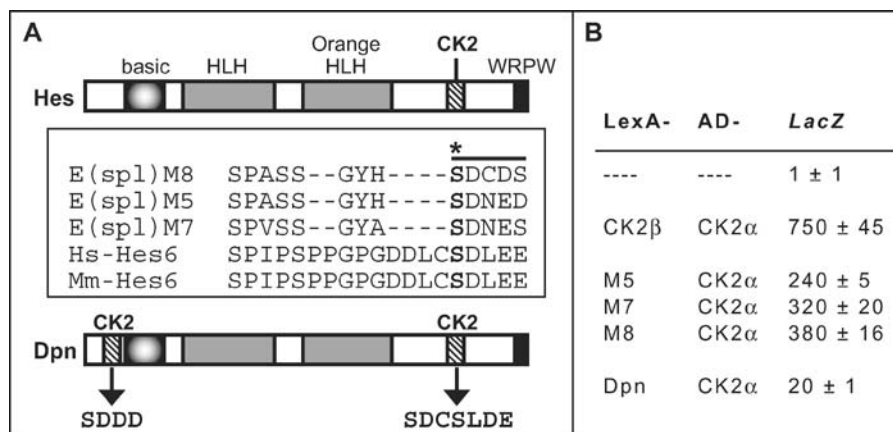
Two  $\mu$ g of purified GST or GST-Dpn were mixed with 25  $\mu$ l of glutathione-Sepharose 4B and incubated for 2 h at 4 °C. The Sepharose was separated by centrifugation for 1 min at 2000  $\times$  g, and the beads were washed twice with 500  $\mu$ l of wash buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 5% glycerol, 1 mM PMSF, and 0.1% Triton X-100) to remove unbound GST fusion proteins. The washed Sepharose, containing the immobilized GST fusion proteins, was then incubated with 100 ng of purified *Drosophila* CK2 and incubated for 3 h at 4 °C. The Sepharose was separated by centrifugation for 1 min at 2000  $\times$  g, and the su-

pernatant was recovered as unbound material. The pellets were washed two times for 5 min each, with 500  $\mu$ l of wash buffer. Sepharose-bound (pellet) and unbound (supernatant) fractions were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. CK2 was detected by Western blot analysis using primary antibody against CK2 at a dilution of 1:1000 and secondary antibody (goat-anti-rabbit IgG coupled to alkaline phosphatase, Bio-Rad) at a dilution of 1:3000. Immunoblots were visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate.

## Results and discussion

#### A positionally conserved CK2 site in HES-repressors

We had previously observed, using the yeast two hybrid assay, that a subset of E(spl)-repressors, i.e., M5, M7, and M8, robustly interact with CK2 $\alpha$  [33]. In addition, these three proteins are equivalently phosphorylated by monomeric CK2 $\alpha$  or the holoenzyme at a conserved CK2 site that is located in close proximity to the C-terminal Groucho binding WRPW motif (Fig. 1A). Furthermore, deletion of the CK2 site (SDCD) or replacement of the CK2 phosphoacceptor in M8 with Asp abolished interaction, suggesting that the CK2-site might, by itself, confer interaction. Given the overall structural conservation of the HES family, i.e., E(spl), Dpn, and Hairy, we, therefore, analyzed the sequence of Dpn to determine the presence of CK2 sites and their positional conservation, if any. This analysis revealed the presence



**Fig. 1.** Functional motifs in HES repressors. (A) Schematic representation of the functional motifs common to HES bHLH proteins. The structural/functional motifs are: basic domain (black box with halo), HLH and Orange domains (gray boxes), the C-terminal tetrapeptide, WRPW, that binds Groucho (black box), and the CK2 consensus site (checkerboard box). Size heterogeneities between HES members is not indicated. Inset shows the alignment of the sequences encompassing the CK2 site of a subset of HES members, and asterisk denotes the CK2-phosphoacceptor. (B) Comparative two hybrid interactions of E(spl)m5/7/8 and Dpn with CK2 $\alpha$ . Yeast EGY048 harboring the *LacZ*-expression plasmid, pSH18-34, was transformed with plasmids expressing the indicated protein pairs. Transformants were grown in galactose medium, and the levels of *LacZ* were determined as described [33]. *LacZ* activity is expressed in Miller Units, and the data shown is the average of 3 independent experiments.

of two potential sites, i.e., S<sup>9</sup>DDD and S<sup>408</sup>DCS<sup>411</sup>LDE (Fig. 1A). While the N-terminal site satisfies the requirement for an Asp/Glu at the  $n + 1$  and  $n + 3$  positions, the C-terminal site is lacking Asp/Glu at the  $n + 1$ . However, we note that a number of substrates where the  $n + 1$  position is not an Asp/Glu have been identified (reviewed in [38]). In Dpn, the first site is adjacent to the basic domain and harbors a single potential phosphoacceptor (Ser9). In contrast, the second site is located in the vicinity of the Groucho binding WRPW motif and contains two potential phosphoacceptors (Ser<sup>408</sup> and Ser<sup>411</sup>) that might be subject to hierarchical phosphorylation by CK2. Interestingly, the second site localizes to a region of Dpn which, although hypervariable amongst HES members, is positionally conserved in a number of repressors (M5/7/8, Hes6, etc.) [18]. In case of M8 and its murine homolog Hes6, this site is targeted by CK2 *in vitro*, and its perturbation dramatically affects their repressor activity *in vivo* [18, 33, 39].

#### Two hybrid interaction of CK2 and Dpn

Given the strong two hybrid interaction of E(spl)M5/7/8 with CK2, and that interaction required integrity of the CK2 site, we sought to ask whether Dpn was also a partner of CK2. However, in an explicit test we observed that strength of the (two hybrid) interaction between LexA-Dpn and AD-CK2 $\alpha$  appeared marginal when compared to that between LexA-M8 and AD-CK2 $\alpha$  (Fig. 1B). This result was surprising because Dpn contains two CK2 sites, both of which are significantly more acidic than the single site in M5/7/8 (see Fig. 1A). We reasoned that the significantly attenuated Dpn-CK2 $\alpha$  interaction might reflect attenuated expression and/or instability of Dpn in yeast, or, perhaps, its ability to act as a repressor in yeast. An alternative possibility is that this interaction also requires CK2 $\beta$  (see below). If so, a direct biochemical route might be more informative to assess targeting of Dpn by CK2.

#### Deadpan is phosphorylated by CK2

To test if Dpn is a CK2 target we performed an *in vitro* phosphorylation assay. GST and GST-Deadpan were subjected to phosphorylation using purified monomeric CK2 $\alpha$  or CK2 holoenzyme. The former isoform is relevant to our two hybrid analysis, whereas the latter isoform mimics the environment most likely to be encountered *in vivo* and thus might be considered to be physiologically more relevant. Our results indicate that GST-Dpn is phosphorylated weakly by monomeric CK2 $\alpha$ , whereas it was robustly phosphorylated by the embryo-holoenzyme (Fig. 2). No phosphorylation of the GST affinity tag was observed for either isoform of CK2. These results suggest that phosphorylation of Dpn by CK2 is

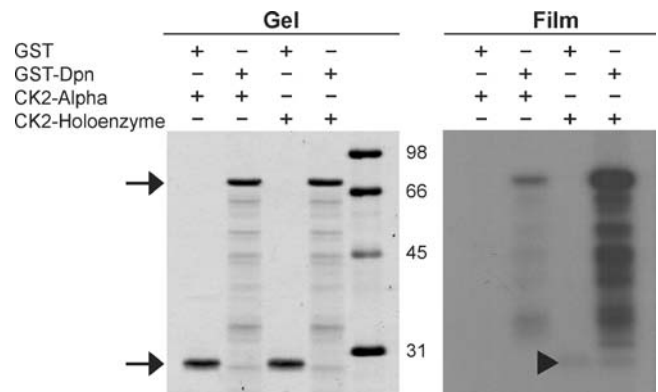


Fig. 2. Phosphorylation of Dpn by CK2. The indicated GST-fusion proteins were purified, and subjected to phosphorylation using the monomeric  $\alpha$  subunit, CK2 $\alpha$  or the  $\alpha_2\beta_2$  holoenzyme from *Drosophila* embryos. Samples were electrophoresed in 12% SDS-polyacrylamide gels, stained with Coomassie Blue (Gel) and autoradiographed (Film). The positions of GST and GST-Dpn are denoted by arrows, and the arrowhead indicates the autophosphorylated CK2 $\beta$  subunit.

positively influenced by the  $\beta$  subunit, and might explain its 'weak' interaction with CK2 $\alpha$  in yeast. We do not consider it likely that Dpn interacts exclusively via CK2 $\beta$ , because CK2 $\alpha$  exhibits phosphorylation of this bHLH protein, albeit weakly. The more likely scenario is that the Dpn interacts with CK2 via a binding site encompassing both subunits, i.e., the holoenzyme. Because this is the *in vivo* conformation of CK2 strengthens the notion that Dpn is a CK2 target. Comparative kinetic analysis with the two isoforms will be needed to address how CK2 $\beta$  enhances interaction and phosphorylation of Dpn.

#### Direct interaction of Dpn and CK2

Although the phosphorylation analysis suggest that Dpn interacts preferentially with the holoenzyme, two hybrid analysis with this isoform *per se* has been precluded because yeast strains that express equivalent amounts of CK2 $\alpha$  and CK2 $\beta$  are currently unavailable. We have, therefore, assessed the ability of Dpn to form a direct complex with embryo-CK2 or CK2 $\alpha$ . GST-alone and GST-Dpn were purified, immobilized on glutathione-sepharose, and tested for complex formation with the two isoforms of CK2. The presence of CK2 in the bound (pellet) and unbound (supernatant) fractions was assessed by Western blotting using an antisera which recognizes both ( $\alpha$  and  $\beta$ ) subunits of CK2 [40]. As expected, incubation of CK2 with sepharose beads did not result in immunoreactive material in the pellet fraction (data not shown). In addition, incubation of GST-beads (Fig. 3, lanes 1 and 3) also did not result in any immunoreactive material in the pellet, indicating that neither isoform interacts with GST consistent with their

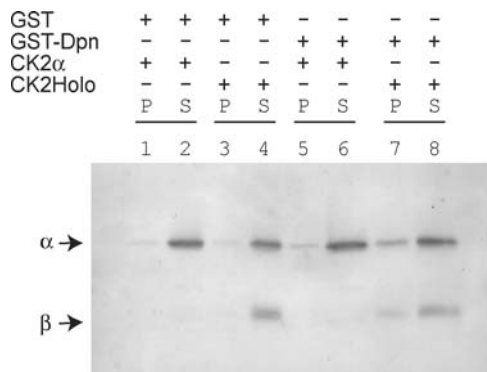


Fig. 3. Interaction of Dpn with CK2 $\alpha$  or CK2-holoenzyme. Bacterially expressed GST alone or a GST-Dpn fusion protein were immobilized on glutathione-sepharose beads, and incubated with either monomeric  $\alpha$  subunit (CK2 $\alpha$ ), or the  $\alpha_2\beta_2$  holoenzyme from *Drosophila* embryos (CK2Holo). The beads were separated from the unbound material, and the bead bound (P, pellet) and the unbound (S, supernatant) samples were examined for the presence of CK2 by Western blotting. The arrows indicate immunoreactive bands corresponding to CK2 $\alpha$  and CK2 $\beta$ .

inability to phosphorylate this affinity tag (see Fig. 2). Incubation of GST-Dpn beads with CK2 $\alpha$  resulted in a minor amount of immunoreactive material in the pellet (Fig. 3, lane 5). In contrast, incubation of GST-Dpn beads with embryo-CK2 (Fig. 3, lane 7) resulted in significantly greater amounts of immunoreactive material in the pellet, demonstrating that Dpn and CK2-holoenzyme interact directly. These binding data appear to qualitatively mirror the phosphorylation data (see Fig. 2), and we estimate that  $\sim 20\%$  of the holoenzyme interacted with Dpn. Given the experimental conditions of these assays, CK2-holoenzyme contributed half the amount of catalytic subunit compared to CK2 $\alpha$  alone, suggesting that complex formation appears to be relatively efficient for the holoenzyme. These results demonstrate that the Dpn-CK2 in-

teraction is direct. In addition, complex formation occurs in the absence of MgATP, in line with previous analysis of the interaction of this enzyme with M5/7/8, ZFP35, etc. [41, 42].

#### Effect of polybasic compounds on the phosphorylation of Dpn

Our observations of a direct CK2-Dpn complex and its preferential phosphorylation by the holoenzyme, suggested for a positive role for CK2 $\beta$ . The marginal ability of CK2 $\alpha$  to phosphorylate Dpn (see above), and that CK2 $\beta$  mediates activation by polybasic effectors [43, 44], led us to assess whether phosphorylation was responsive to polybasic activation. The marginal phosphorylation of Dpn by CK2 $\alpha$  was unaffected by either spermine or protamine, but was dramatically stimulated by poly(DL)lysine (Fig. 4, compare lanes 5–8). The stimulatory effects of poly(DL)lysine are not due to non-specific phosphorylation, because GST is not phosphorylated in its presence (Fig. 4, compare lanes 1 and 3). In contrast, phosphorylation of Dpn by embryo-CK2 was unresponsive to further activation by these effectors (Fig. 4, lanes 13–16). These results suggests that phosphorylation of Dpn by embryo-CK2 is unresponsive to further activation, and supports the notion that substrates that are efficiently phosphorylated, e.g., the RII subunit of PKA, topoisomerase II, etc., are generally refractory to these activators [43].

#### Implications of Phosphorylation of Deadpan

While the mechanism by which Dpn functions during neurogenesis remains to be resolved, its role(s) during sex determination are much better understood. In either case, however, one common feature of its functions is antagonism of

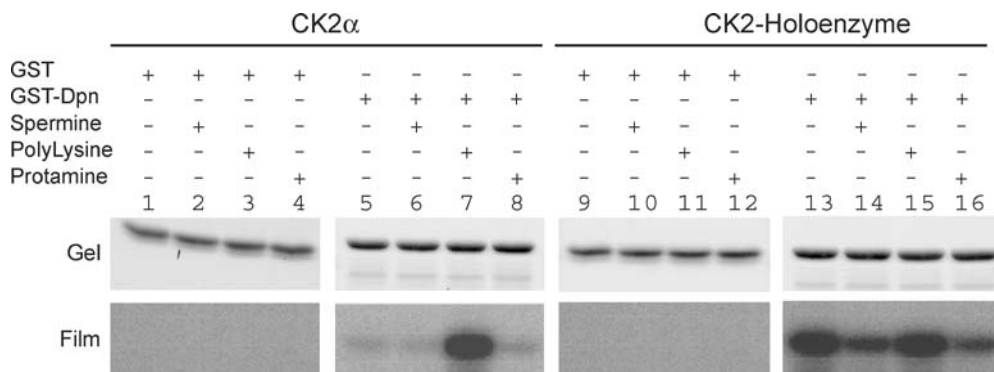


Fig. 4. Effect of polybasic activators on phosphorylation of Dpn. GST-alone or GST-Dpn were purified, and subjected to phosphorylation using the monomeric  $\alpha$  subunit (CK2 $\alpha$ ) or the  $\alpha_2\beta_2$  holoenzyme from *Drosophila* embryos. Samples were phosphorylated with either CK2 $\alpha$  (lanes 1–8) or the holoenzyme (lanes 9–16). Phosphorylations were conducted in the absence of any effector (lanes 1, 5, 9, and 13), or in the presence of 0.68 mg/ml spermine (lanes 2, 6, 10, and 14), 100  $\mu\text{g/ml}$  poly(DL)lysine (lanes 3, 7, 11, and 15), and 125  $\mu\text{g/ml}$  protamine (lanes 4, 8, 12, and 16). Samples were electrophoresed in 12% SDS-polyacrylamide gels, stained with Coomassie Blue (Gel, upper panels), and autoradiographed (Film, lower panels).

ASC, whereby Dpn represses transcription of ASC via DNA-binding [45]. In line with this, ectopic expression of *dpn* reduces ASC activity, suggesting a negative interaction between these two loci. It is noteworthy that a similar function is ascribed to HES repressors as well, although in their case DNA-binding as well as direct interactions with proneural factors (ASC and Atonal) are known to be required for antagonism [17, 23, 46].

How might phosphorylation of *dpn* regulate its *in vivo* functions? It is difficult to propose this with certainty based solely on *in vitro* analysis. However, based on the extensive body of genetic and molecular analysis on Dpn to date, and the emerging notion that CK2 profoundly influences the activity of the related repressor, E(spl)M8, during eye development [18], some possibilities can be predicted. As stated above, CK2 phosphorylation regulates repressor activity of M8 and replacement of the phosphoacceptor with Asp generates a dominant allele that is severely exacerbated for its antineurogenic functions. A similar CK2 dependent mechanism might also underlie the interaction of M8 with the ASC-bHLH activator, Lethal of Scute (Karandikar and Bidwai, unpublished). In a similar vein, it is conceivable that phosphorylation of Dpn might augment its ability to antagonize ASC-derived bHLH activators by either modulating DNA binding or direct protein-protein interactions. CK2 is known to regulate DNA-binding as well as protein-protein interactions [18, 39, 47, 48].

#### *bHLH repressors and CK2, a recurring theme during neurogenesis*

The development of nervous system is regulated by the interplay between proneural proteins and their repressors. A general strategy during neurogenesis appears to be the conferring of neural potential on a field of cells, from which arises a precise pattern of neural and accessory cell fates through this interplay and, as such, this mechanism also appears to be involved in other cell fate decisions. It is increasingly becoming apparent that cell fate choice is unlikely to be based simply on the levels of an activator versus its cognate repressor. Rather, this interplay must also be modulated in a spatial and temporal context. In such a scenario, regulation of protein turnover, presence or absence of cofactors, and regulatory modifications, etc., might provide a means to achieve 'fine tuning' of this interplay. In this context, protein kinases and/or phosphatases might provide a simple bistable mechanism to 'fine tune' the developmental outcome [20]. Such a mechanism is beginning to emerge for regulation of repression by E(spl)M8 and its mammalian counterpart, Hes6 [18, 39]. In both, phosphorylation by CK2 regulates their ability to interact with and antagonize proneural factors. Given the expanding repertoire of HES proteins that are targeted by

CK2, it would not come as a surprise that a similar mechanism might also be employed for regulation of another HES member, Dpn.

Among the HES members that are CK2 targets, Dpn differs from E(spl) in a number of ways. While E(spl) transcription (by Su(H)) occurs in response to an activated Notch receptor, Dpn has been thought to be Notch-independent, although it contains binding sites for Su(H) in a region that recapitulates PNS/CNS specific expression [49, 50]. Furthermore, E(spl) repressors block proneural proteins in cells undergoing lateral inhibition, whereas Dpn achieves a similar outcome but in neural cells [20, 21, 51, 52]. The remarkable conservation of CK2 by itself (reviewed in [53, 54]), and its ability to modulate the activity of repressors in different developmental contexts might be indicative of its selection as a general modulator of cell fate determination.

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## References

1. Artavanis-Tsakonas S, Matsuno K, Fortini ME: Notch signalling. *Science* 268: 225–232, 1995
2. Blaumuller CM, Artavanis-Tsakonas S: Comparative aspects of Notch signaling in lower and higher eukaryotes. *Perspect Dev Neurobiol* 4: 325–343, 1997
3. Mumm JS, Kopan R: Notch signaling: from the outside in. *Dev Biol* 228: 151–165, 2000
4. Lai EC: Notch signaling: control of cell communication and cell fate. *Development* 131: 965–973, 2004
5. 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*. *Development* 120: 3537–3548, 1994
6. Culi J, Modolell J: Proneural gene self-stimulation in neural precursors: an essential mechanism for sense organ development that is regulated by Notch signaling. *Genes Develop* 12: 2036–2047, 1998
7. Dambly-Chaudiere C, Vervoort M: The bHLH genes in neural development. *Int J Dev Biol* 42: 269–273, 1998
8. Freeman M: Cell determination strategies in the *Drosophila* eye. *Development* 124: 261–270, 1997
9. Jarman AP: Developmental genetics: vertebrates and insects see eye to eye. *Curr Biol* 10: 2000
10. Kumar J, Moses K: Transcription factors in eye development: a gorgeous mosaic. *Genes Develop* 11: 2023–2028, 1997
11. Pichaud F, Treisman J, Desplan C: Reinventing a common strategy for patterning the eye. *Cell* 105: 9–12, 2001
12. Voas MG, Rebay I: Signal integration during development: insights from the *Drosophila* eye. *Dev Dyn* 229: 162–175, 2004
13. Jarman AP, Grell EH, Ackerman L, Jan LY, Jan YN: *atonal* is the proneural gene for *Drosophila* photoreceptors. *Nature* 369: 398–400, 1994
14. 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. *Development* 125: 2893–2900, 1998

15. White N, Jarman A: *Drosophila atonal* controls photoreceptor R8-specific properties and modulates both receptor tyrosine kinase and Hedgehog signalling. *Development* 127: 1681–1689, 2000
16. Nagel A, Yu Y, Preiss A: *Enhancer of Split [E(spl)D]* is a Gro-independent, hypermorphic mutation in *Drosophila*. *Develop Genet* 25: 168–179, 1999
17. 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* 208: 406–415, 1999
18. Karandikar U, Trott RL, Yin J, Bishop CP, Bidwai AP: *Drosophila* CK2 regulates eye morphogenesis via phosphorylation of E(spl)M8. *Mech Develop* 121: 273–286, 2004
19. Heitzler P, Bourouis M, Ruel L, Carteret C, Simpson P: Genes of the *Enhancer of split* and *achaete-scute* complexes are required for a regulatory loop between Notch and Delta during lateral signalling in *Drosophila*. *Development* 122: 161–171, 1996
20. Modolell J, Campuzano S: The *achaete-scute* complex as an integrating device. *Int J Dev Biol* 42: 275–282, 1998
21. Campos-Ortega JA: The genetics of the *Drosophila achaete-scute* gene complex: a historical perspective. *Int J Dev Biol* 42: 291–297, 1998
22. Delidakis C, Artavanis-Tsakonas S: The *enhancer of split [E(spl)]* locus of *Drosophila* encodes seven independent helix-loop-helix proteins. *Proc Natl Acad Sci USA* 89: 8731–8735, 1991
23. 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 USA* 94: 13099–13104, 1997
24. Maier D, Marte BM, Schafer W, Yu Y, Preiss A: *Drosophila* evolution challenges postulated redundancy in the *E(spl)* gene complex. *Proc Natl Acad Sci USA* 90: 5464–5468, 1993
25. Fisher AL, Caudy M: Groucho proteins: transcriptional corepressors for specific subsets of DNA binding transcription factors in vertebrates and invertebrates. *Genes Develop* 12: 1931–1940, 1998
26. Bier E, Vassin H, Young-Shepherd S, Jan LY, Jan Y: *deadpan*, an essential pan-neural gene in *Drosophila*, encodes a helix-loop-helix protein similar to the hairy gene product. *Genes Develop* 6: 2137–2151, 1992
27. Erickson JW, Cline TW: Key aspects of the primary sex-determination mechanism are conserved across the genus *Drosophila*. *Development* 125: 3259–3268, 1998
28. Cline TW: The *Drosophila* sex determination signal: how do flies count to two? *Trends Genet* 9: 385–390, 1993
29. Parkhurst SM, Meneely PM: Sex determination and dosage compensation: lessons from flies and worms. *Science* 264: 924–932, 1994
30. Liu Y, Belote JM: Protein-protein interactions among components of the *Drosophila* primary sex determination signal. *Mol Gen Genet* 248: 182–189, 1995
31. Jan YN, Jan LY: Functional gene cassettes in development. *Proc Natl Acad Sci USA* 90: 8305–8307, 1993
32. Gyuris J, Golemis E, Chertkov H, Brent R: Cdi1, a human G1 and S phase protein phosphatase that associates with cdk2. *Cell* 75: 791–803, 1993
33. 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* 276: 2159–2167, 2001
34. 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* 79: 805–815, 1994
35. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227: 680–685, 1970
36. Glover CVC, Shelton ER, Brutlag DL: Purification and characterization of a type II casein kinase from *Drosophila melanogaster*. *J Biol Chem* 258: 3258–3256, 1983
37. Bidwai AP, Hanna DE, Glover CVC: Purification and characterization of Casein Kinase II (CKII) from  $\Delta$ CKA1  $\Delta$ CKA2 *S. cerevisiae* rescued by *Drosophila* CKII subunits. *J Biol Chem* 267: 18790–18796, 1992
38. Meggio F, Pinna LA: One-thousand-and-one substrates of protein kinase CK2. *FASEB J* 17: 349–368, 2003
39. Gratton M-O, Torban E, Jasmin SB, Theriault FM, German MS, Stifani S: Hes6 promotes cortical neurogenesis and inhibits hes1 transcription repression activity by multiple mechanisms. *Mol Cell Biol* 23: 6922–6935, 2003
40. Dahmus GK, Glover CVC, Brutlag D, Dahmus ME: Similarities in structure and function of calf thymus and *Drosophila* casein kinase II. *J Biol Chem* 259: 9001–9006, 1984
41. Kalive M, Trott RL, Bidwai AP: A gene located at 72A in *Drosophila melanogaster* encodes a novel zinc-finger protein that interacts with protein kinase CK2. *Mol Cell Biochem* 227: 99–105, 2001
42. Trott RL, Kalive M, Karandikar U, Rummer R, Bishop CP, Bidwai AP: Identification and characterization of proteins that interact with *Drosophila melanogaster* protein kinase CK2. *Mol Cell Biochem* 227: 91–98, 2001
43. Bidwai AP, Reed JC, Glover CVC: The phosphorylation of Calmodulin by the catalytic subunit of casein kinase II is inhibited by the regulatory subunit. *Arch Biochem Biophys* 300: 265–270, 1993
44. Meggio F, Boldyreff B, Issinger O-G, Pinna LA: Casein kinase 2 down regulation and activation by polybasic peptides are mediated by acidic residues in the 55–64 region of the b-subunit: a study with calmodulin as phosphorylatable substrate. *Biochem J* 33: 4336–4342, 1994
45. Winston RL, Millar DP, Gottesfeld JM, Kent SB: Characterization of the DNA binding properties of the bHLH domain of Deadpan to single and tandem sites. *Biochemistry* 38: 5138–5146, 1999
46. Giagtzoglou N, Alifragis P, Koumbanakis KA, Delidakis C: Two modes of recruitment of E(spl) repressors onto target genes. *Development* 130: 259–270, 2003
47. Luscher B, Christenson E, Litchfield DW, Krebs EG, Eisenman RN: Myb DNA binding inhibited by phosphorylation at a site deleted during oncogenic activation. *Nature* 344: 517–522, 1990
48. Luscher B, Kuenzel EA, Krebs EG, Eisenman RN: Myc oncoproteins are phosphorylated by casein kinase II. *EMBO J* 8: 1111–1120, 1989
49. Emery JF, Bier E: Specificity of CNS, PNS regulatory subelements comprising pan-neural enhancers of the *deadpan* and *scratch* genes is achieved by repression. *Development* 121: 3549–3560, 1995
50. Rebeiz M, Reeves NL, Posakony JW: SCORE: a computational approach to the identification of cis-regulatory modules and target genes in whole-genome sequence data. Site clustering over random expectation. *Proc Natl Acad Sci USA* 99: 9888–9893, 2002
51. Wrischnik LA, Timmer JR, Megna LA, Cline TW: Recruitment of the proneural gene *scute* to the *Drosophila* sex-determination pathway. *Genetics* 165: 2007–2027, 2003
52. Deshpande G, Stuke J, Schedl P: *scute (sis-b)* function in *Drosophila* sex determination. *Mol Cell Biol* 15: 4430–4440, 1995
53. Bidwai AP: Structure and function of casein kinase II. *Recent Res Devel Mol Cell Biol* 1: 51–82, 2000
54. Glover CVC: On the physiological role of casein kinase II in *Saccharomyces cerevisiae*. *Prog Nuc Acid Res Mol Biol* 59: 95–133, 1998