Protein kinase CK2 phosphorylates a conserved motif in the Notch effector E(spl)-Mγ

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Abstract
Across metazoan animals, the effects of Notch signaling are mediated via the Enhancer of Split (E(spl)/HES) basic Helix–Loop–Helix–Orange (bHLH-O) repressors. Although these repressors are generally conserved, their sequence diversity is, in large part, restricted to the C-terminal domain (CtD), which separates the Orange (O) domain from the penultimate WRPW tetrapeptide motif that binds the obligate co-repressor Groucho. While the kinases CK2 and MAPK target the CtD and regulate Drosophila E(spl)-M8 and mammalian HES6, the generality of this regulation to other E(spl)/HES repressors has remained unknown. To determine the broader impact of phosphorylation on this large family of repressors, we conducted bioinformatics, evolutionary, and biochemical analyses. Our studies identify E(spl)-Mγ as a new target of native CK2 purified from Drosophila embryos, reveal that phosphorylation is specific to CK2 and independent of the regulatory CK2-β subunit, and identify that the site of phosphorylation is juxtaposed to the WRPW motif, a feature unique to and conserved in the Mγ homologues over 50 × 10⁶ years of Drosophila evolution. Thus, a preponderance of E(spl) homologues (four out of seven total) in Drosophila are targets for CK2, and the distinct positioning of the CK2 and MAPK sites raises the prospect that phosphorylation underlies functional diversity of bHLH-O proteins.

Keywords CK2 · Phosphorylation · Notch · bHLH · E(spl) · HES

Introduction
The bHLH proteins constitute a large group of transcription factors that regulate diverse aspects of cellular and developmental biology [1]. Among these are the diverse bHLH-O repressors [2] that mediate Notch signaling. In addition to the canonical bHLH domains, these proteins contain a second HLH domain (called Orange), which is followed by a C-terminal domain (CtD) of variable length, and terminate with an invariant WRPW motif through which they recruit the essential co-repressor Groucho (Gro, [3]). Since the identification of the founding members, the Drosophila E(spl)-repressors, bHLH-O proteins have been found in all metazoans [4], in which they serve as terminal effectors of Notch signaling. In addition to neurogenesis, these proteins are vital throughout animal development and regulate vital processes such as embryogenesis, myogenesis, somitogenesis, etc. [5, 6]. Despite their centrality to Notch biology and an extensive body of knowledge on the importance of post translational modifications (PTM) that impact Notch signaling (for review see [7]), our understanding of the regulation of E(spl)/HES proteins by PTMs such as phosphorylation remains incomplete.

Notch signaling is acknowledged to be a universal arbiter of cell fate choice [5], and is often affected in diseases such as cancers. Although Notch plays distinct tissue-specific roles, the underlying mechanism is largely similar. Signaling is triggered by the Delta/Serrate/Lag (DSL) family of ligands, involves cleavage and nuclear translocation of the Notch intracellular domain (NICD), and culminates with the expression of the E(spl)/HES repressors [8]. The accumulation of E(spl)/HES proteins in the signal receiving cell represses target genes, thereby altering cell fate. How such a deceptively simple signaling pathway acts across development remains to be fully understood. Complexity of this pathway, in part, reflects diversity of E(spl)/HES repressors, and studies in Drosophila have been at the forefront in...
understanding Notch biology and have informed studies in mammalian model organisms.

The E(spl) proteins in Drosophila are encoded by the E(spl)-Complex [2], which harbors seven repressors (M6, Mγ, Mβ, M3, M5, M7, and M8), their co-repressor Gro, and four Bearded-family bHLH genes that inhibit Notch signaling. This genetically dense locus (~ 60 Kb) is unique in that the organization and orientation of all genes is conserved in Drosophila species spanning 50 x 10^6 years (MYR) of evolution. E(spl)-repressors are expressed in complex partly overlapping patterns that are tissue/compartment-specific [9], and consequently, the functions of individual members have been gleaned from overexpression-based phenotypes [2]. These studies led to the view that bHLH-O members serve redundant functions [6]. Arguments against this view are the evolutionary conservation and molecular synteny of the E(spl)C [10], tissue-specific expression patterns, homo/hetero-dimerization specificities, and target gene selectivity [11]. Adding to this complexity is accumulating evidence that these proteins are regulated by phosphorylation [12].

We had previously reported that E(spl)-M8/M5/M7 are phosphorylated by protein kinase CK2 at a highly conserved site in their CtD [13]. This modification of M8 was essential for early steps of eye development, but had a more modest impact during genesis of the sensory bristles [14], two widely used readouts of Notch signaling during Drosophila neurogenesis. This regulation reflects conversion of M8 from a CtD-dependent ‘cis’-inhibited state to an active repressor [15]. Activation by phosphorylation also requires MAPK [16], whose recognition site resides in a Ser-rich region of the CtD, which we have termed the P-domain [14, 16–19]. In addition, the phosphatase PP2A targets the P-domain of E(spl) proteins and opposes the activation of M8, suggesting that dynamic phosphorylation and dephosphorylation may fine tune the activities of E(spl) repressors during Notch-mediated tissue patterning [18]. It is noteworthy that a similar P-domain in human HES6 is modified by CK2 at a CtD-dependent ‘cis’-inhibited state to an active repressor [15]. Activation by phosphorylation also requires MAPK [16], whose recognition site resides in a Ser-rich region of the CtD, which we have termed the P-domain [14, 16–19]. In addition, the phosphatase PP2A targets the P-domain of E(spl) proteins and opposes the activation of M8, suggesting that dynamic phosphorylation and dephosphorylation may fine tune the activities of E(spl) repressors during Notch-mediated tissue patterning [18]. It is noteworthy that a similar P-domain in human HES6 is modified by CK2 at a CtD-dependent ‘cis’-inhibited state to an active repressor [15]. Activation by phosphorylation also requires MAPK [16], whose recognition site resides in a Ser-rich region of the CtD, which we have termed the P-domain [14, 16–19]. In addition, the phosphatase PP2A targets the P-domain of E(spl) proteins and opposes the activation of M8, suggesting that dynamic phosphorylation and dephosphorylation may fine tune the activities of E(spl) repressors during Notch-mediated tissue patterning [18]. It is noteworthy that a similar P-domain in human HES6 is modified by CK2 at a CtD-dependent ‘cis’-inhibited state to an active repressor [15]. Activation by phosphorylation also requires MAPK [16], whose recognition site resides in a Ser-rich region of the CtD, which we have termed the P-domain [14, 16–19]. In addition, the phosphatase PP2A targets the P-domain of E(spl) proteins and opposes the activation of M8, suggesting that dynamic phosphorylation and dephosphorylation may fine tune the activities of E(spl) repressors during Notch-mediated tissue patterning [18]. It is noteworthy that a similar P-domain in human HES6 is modified by CK2 at a CtD-dependent ‘cis’-inhibited state to an active repressor [15]. Activation by phosphorylation also requires MAPK [16], whose recognition site resides in a Ser-rich region of the CtD, which we have termed the P-domain [14, 16–19]. In addition, the phosphatase PP2A targets the P-domain of E(spl) proteins and opposes the activation of M8, suggesting that dynamic phosphorylation and dephosphorylation may fine tune the activities of E(spl) repressors during Notch-mediated tissue patterning [18].

Materials and methods
Identification of CK2 sites in E(spl)-Mγ

The ‘sequence motif’ search tool at prosite.expasy.org was used to analyze all seven E(spl) proteins for consensus (S-D/E-X-D/E) sites for CK2. The primary sequences of the Drosophila orthologues were downloaded from ‘flybase.org’ and aligned using the online software ‘MUSCLE’ using default parameter settings.

Isolation of E(spl)-Mγ and generation of mutant versions of E(spl)-Mγ

The E(spl)-Mγ open reading frame was PCR amplified from wild-type D. melanogaster (w^1118) flies using primers complementary to the 5’ and 3’ ends of the intron-less Mγ gene. The primers included EcoR1 and Xho1 restriction sites at the 5’ and 3’ ends, respectively. The PCR product was cloned into the vector pBluescript-II (Stratagene/Agilent) and sequenced to verify identity to the reported gene/genome sequence (Flybase ID, FBgn0002735). This entry clone was then used to PCR amplify the N-terminal and C-terminal domains of E(spl)-Mγ, i.e., Mγ-NTD and Mγ-CTD, respectively. Subsequently, site-directed mutagenesis was used to introduce Ala substitutions at predicted CK2 sites. All PCR-based constructs were verified by Sanger-sequencing.

Purification of GST-fusion proteins

Plasmid pZEX [13] was used to express recombinant proteins as GST fusions in E. coli BL21 harboring plasmid pT-TRX [22]. Cultures were grown to an OD_600 of 0.7 in 2xYTA containing 150 μg/mL ampicillin and 15 μg/mL chloramphenicol, as previously described by us [13, 23, 24]. Protein expression was induced with 1 mM IPTG at 30°C for ~ 3 h. Cells were resuspended in 8 mL of phosphate-buffered saline (PBS) containing 5 mM EDTA, 0.1 mM PMSF, 2 μg/mL Leupeptin (Sigma-Aldrich), and 0.2% β-mercaptoethanol, treated with lysozyme (1 mg/mL) on ice for 30 min, and disrupted by sonication for ~ 30 s. Lysed samples were treated with 1% Triton-X100 at 4°C for 1 h. Insoluble material was removed by centrifugation, and the supernatant incubated with glutathione-beads for 2 h at 4°C. Beads were washed with 10 bed volumes of PBS containing 5 mM EDTA and 0.1 mM PMSF. Bound protein was eluted with 15 mM reduced glutathione, 50 mM Tris pH-8.0, and exchanged into storage buffer (10 mM Tris pH-8.0, 0.5 mM EDTA, 5% glycerol, 150 mM NaCl, 0.1 mM PMSF, 0.02% Sodium...
Azide) using an Amicon Ultra-15 device (10,000 MW cut-off). The concentration and purity were determined by SDS-PAGE compared to protein standards (Fermentas).

Protein kinase CK2 phosphorylation assays

CK2 assays were performed at 25°C in 50 mM Tris, pH 8.5, 100 mM NaCl, 10 mM MgCl₂, 10 μM ATP, 2.5 μCi of [γ-32P]-ATP and ~2 μg of GST fusions in a volume of 40 μL, as described [25]. Reactions were initiated with 20 ng native CK2-holoenzyme purified from Drosophila embryos or recombinant monomeric CK2α from a yeast (complementation-based) expression system [26]. CK2 modulators were added at the following concentrations: GTP (60 μM), Heparitin (1 μg/mL), Protamine (125 μg/mL), Spermine (500 μg/mL), and Poly-(D/L)-lysine (100 μg/mL), as has previously been described [27, 28]. Reactions were terminated with 10 μL 5 × sample buffer (312 mM Tris–Cl, pH 6.8, 10% SDS, 25% β-mercaptoethanol and 40% glycerol). Samples were resolved by SDS-PAGE, stained with Coomassie blue, and subject to autoradiography.

Results and discussion

E(spl)-Mγ harbors multiple consensus sites for CK2

We first aligned E(spl)-Mγ homologues from 12 species that represent ~50 × 10⁶ years (MYR) of Drosophila evolution [29]. All E(spl)-Mγ homologues harbor three conserved consensus sites for CK2 (Fig. 1A), fully meeting the strict recognition requirements for this kinase [30]. Unique among these are Thr⁴⁵ and Ser⁴⁸, which reside within the loop of the HLH domain (Fig. 1A). Modification of Ser⁴⁸ is predicted to elicit hierarchical phosphorylation of Thr⁴⁵, because Asp/Glu or pSer/pThr promotes recognition by CK2 with equal potency [31]. Interestingly, of the seven E(spl) bHLH-O proteins in Drosophila, E(spl)-Mγ is the sole member harboring potential site(s) for CK2 within the HLH domain. The third CK2 site (Ser¹⁹⁵) is juxtaposed to the WRPW motif (Fig. 1A), a feature also unique to E(spl)-Mγ. Notably, numerous Asp/Glu, a hallmark of 'high likelihood' targets of CK2, flank Ser¹⁹⁵. Although E(spl)-Mγ homologues from several Drosophila species harbor the insertion of one/two amino acids immediately N-terminal to Ser¹⁹⁵, in no case...
is the DS195EDEE motif perturbed (Fig. 1A, numbering reflects the D. melanogaster sequence).

We next determined if the insertion/deletion of two amino acids correlates to specific branches of the Drosophila family. Given the known evolutionary relationships between 12 species representing six major groups of fruit flies [29], it appears likely that E(spl)-Mγ from the Hawaiian, Virilis, Repleta, and Obscura groups (harboring an insertion of two residues) are the ancestral form (Fig. 1B). A one residue deletion occurred ~ 37 × 10^6 MYR ago with the emergence of the Willistoni group, and both residues were lost ~ 13 × 10^6 MYR ago with the emergence of the Melanogaster group. The strong conservation of the C-terminal CK2 site despite the insertion/deletion suggests that the one/two residue insertion (in the ancestral species) is unlikely to alter protein structure and/or function.

**Phosphorylation of E(spl)-Mγ by CK2**

We next determined if E(spl)-Mγ is phosphorylated by CK2. This kinase exits as an α2β2 holoenzyme in all eukaryotes [32], and phosphorylates Ser/Thr (and in restricted cases Tyr) in a messenger-independent manner. Although phosphorylation is catalyzed by the CK2α subunit, the regulatory CK2β subunit promotes or inhibits recognition in a target-specific manner [25, 33]. We thus used native α2β2 holoenzyme (CK2-HoloE) purified from Drosophila embryos and monomeric (recombinant) CK2α purified from yeast rescued by a cDNA encoding Drosophila CK2α [26]. Both enzyme forms are pure based on Coomassie staining (Fig. 2, Gel). In the case of CK2-HoloE, the weak staining of CK2β relative to CK2α (Fig. 2) does not reflect sub-stoichiometric levels, as this enzyme preparation has a sedimentation coefficient of 6.4S (see Inset in Fig. 2), consistent with the α2β2 conformation, and reflects weak staining of CK2β from flies and mammals [27, 34]. Both forms display kinetic properties and ATP/GTP-dependency identical to those reported by others and us [26, 35]. Importantly, both enzyme preparations require ~ 100 mM NaCl for optimal activity and display substrate-specificity identical to that revealed by the Pinna and Krebs laboratories [30, 36, 37]. Together, these parameters and properties are highly CK2-specific.

The two isoforms of CK2 were used to phosphorylate E(spl)-Mγ. In these assays, 20 ng of CK2α or CK2-HoloE was used and thus proteins with the mobility of CK2-α/β are not seen in SDS-PAGE gels stained with Coomassie (Fig. 2). E(spl)-Mγ was tested as a GST fusion, while GST-alone and GST-E(spl)-M8 served as negative and positive controls, respectively. As shown in Fig. 2, E(spl)-Mγ was robustly phosphorylated by both CK2α and CK2-HoloE. Phosphorylation is specific to E(spl)-Mγ, as GST was not phosphorylated. The band intensities suggest that CK2-HoloE more efficiently phosphorylates E(spl)-Mγ than does CK2α. This difference likely reflects (~ fourfold) greater activity of the holoenzyme [26], rather than a requirement for CK2β. Phosphorylation of E(spl)-M8 (positive control) was observed with both isoforms, although CK2β appeared to modestly diminish phosphorylation, even though this modification has been evinced in vivo readout assays for Notch signaling. Thus, E(spl)-Mγ is targeted by native Drosophila CK2 independent of CK2β.

![Fig. 2](https://example.com/figure2.png)

**CK2 phosphorlataes E(spl)-Mγ.** The indicated proteins were phosphorylated using monomeric CK2α or the α2β2 holoenzyme (CK2-HoloE). Proteins were separated by SDS-PAGE, stained with coomassie (Gel), and autoradiographed (Film). Boxed areas denote GST-alone and partial degradation products of GST-M8, neither of which are targeted by CK2α or CK2-HoloE. Asterisk denotes close migration of weakly autophosphorylated CK2β and non-phosphorylated GST. Gray inset box indicates physical and kinetic parameters of the purified CK2-HoloE and monomeric CK2α used in the studies.

| Protein      | CK2-α | CK2-HoloE |
|--------------|-------|-----------|
| Sed. Coeff.  | 3.1 S | 6.4 S     |
| Km ATP       | 15 µM | 15 µM     |
| Km GTP       | 65 µM | 65 µM     |

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Modulation of CK2-dependent targeting of E(spl)-My

To further confirm that CK2-holoE was responsible for phosphorylation of My, we tested for modulation by competitor (GTP), inhibitor (Heparin), or polybasic activators, as previously reported [25]. We exploited the unusual property that CK2 can use ATP/GTP as phosphate donors [27]. Assays contained 15 μM [γ 32P]-ATP (1xKm) and supplemented with 60 μM cold-GTP (at its Km). In this assay, competition by GTP should diminish phosphorylation, if the enzyme is CK2. Indeed, GTP strongly decreased 32P-incorporation into GST-Mγ (Fig. 3A). Likewise, Heparin (1 μg/mL), a CK2 inhibitor [38], strongly diminished phosphorylation of GST-E(spl)-Mγ. We next tested polybasic compounds that stimulate CK2-HoloE through interaction with CK2β [25]. All three (protamine, spermine, and poly-lysine) modestly increased phosphorylation of GST-Mγ. As GST-alone is not phosphorylated by CK2-HoloE in the absence of these effectors (Fig. 3), or in their presence (data not shown and see [25]), the phosphorylation observed in Figs. 2 and 3 reveals a specific interaction of CK2 with My. These studies strongly suggest that E(spl)-My is a bona fide substrate of CK2.

Identify the CK2 site(s) in E(spl)-My

We next sought to identify CK2 target sites. Because My harbors three predicted CK2 sites, it was divided into two parts; My-NTD contains the bHLH-O domains, whereas My-CtD contains residues of the CtD including WRPW (Fig. 4A). These constructs separate the CK2 sites within the HLH domain (T45LES48EGE) from that (S195EDE) proximal to WRPW. Wild-type and Ala mutants of My-NTD and My-CtD were purified as GST fusions. For reasons that are unclear, My-NTD was poorly expressed, unstable, and sensitive to degradation/ proteolysis despite the use of protease deficient E. coli cells and the inclusion of protease inhibitor cocktails. As an alternative, full-length My and its Ala derivative mutants were used for mapping sites of phosphorylation.

Wild-type My and its Ala mutants were tested for phosphorylation by CK2-HoloE. Analysis with monomeric CK2α was deemed unnecessary given CK2β-independent targeting of My (Fig. 2). In brief, 20 ng of CK2-HoloE was used to phosphorylate 0.5–1.0 μg of My variants under conditions (10 min) where enzyme activity is linear. As shown in Fig. 4B, My-CtD was efficiently phosphorylated by CK2, and this was abrogated by replacing Ser195 with Ala (Fig. 4B). Under similar conditions CK2 did not phosphorylate GST-alone. We next tested My-NTD and its Ala mutants. While our studies with My-NTD did not reveal any discernible phosphorylation (data not shown), problems with yields and quality remained. We therefore generated My-FL (full-length) with an Ala substitution at Ser195. We reasoned that if this variant was refractory to phosphorylation, it would demonstrate that the two closely juxtaposed and proximal sites Thr45 and Ser48 in the T45LES48EGE motif (see schematic in Fig. 4A) are not targeted by CK2. Indeed, we find that unlike wild-type My-FL, its Ser195A mutant was completely refractory to phosphorylation (Fig. 4B), establishing that the CK2 modifies My solely at Ser195 in its CtD. While the reasons why My-NTD is refractory to CK2 are unclear, but we speculate that localized folding may render Thr45/ Ser48 (in the HLH domain) inaccessible to CK2 and hence refractory to phosphorylation.

The importance of PTM of E(spl)/HES proteins via phosphorylation

The phosphorylation of E(spl)-My reveals that a preponderance (four out of seven) of E(spl)C repressors are targeted by CK2. The physiological consequences of phosphorylation are best understood for E(spl)-M8, whose regulation by phosphorylation has been studied during eye and bristle development [14, 16]. In either context, the CK2 and MAPK sites (the P-domain) auto-inhibit the O-domain via protein–protein interactions [15]. Phosphorylation overrides auto-inhibition to uncover the O-domain, thereby permitting E(spl)-M8 repressor activity to manifest. Accordingly, removal of the CtD or just the P-domain elicits constitutive (phosphorylation-independent) repression by E(spl)-M8.
More recent studies ([12], and Majot and Bidwai, unpublished) are revealing that M8 is likely to be regulated through additional PTMs and phosphorylation-dependent degradation (see below). The P-domain of M8 harbors two additional Ser residues (Ser154 and Ser155), which meet the consensus for CK1, a kinase that phosphorylates Ser with pSer/Asp/Glu at the n+4-to-n+9 positions [39, 40]. CK2 targeting of M8 (at Ser159) may thereby favor (hierarchical) CK1-dependent modification of Ser154 and Ser155. This, in turn, generates the pSpSGYHpSDCD 'phospho-degron' (Fig. 4C), which meets the strict consensus for degradation via Slimb/βTrCP [12]. The M8-Slimb/βTrCP interaction has been reported in an in vitro (Drosophila S2) cell-based assay [41], and our studies (unpublished) reveal that mutations in CK1 or Slimb/βTrCP genes strongly diminish in vivo repressor activity of M8. Together, these results suggest that controlled activation of E(spl) proteins (by CK2 and MAPK) and degradation via CK1 and Slimb/βTrCP impact these terminal effectors of Notch signaling during neurodevelopment. A similar sequence is conserved in M5 and M7.
(Fig. 4C). The spacing of the kinase motifs in the P-domain is likely to be germane to understanding the function and regulation of these bHLH repressors. HES6, the mammalian homologue of E(spl)-M8 also conserves similarly positioned CK2, MAPK, and CK1 sites in its P-domain (not shown). We interpret our findings on Mγ in light of those with M8.

While the E(spl)/HES proteins are conserved across the bHLH-O region, sequence divergence is largely restricted to the CtD, which is of variable length and composition. Might CtD divergence underlie functional diversity among this family of repressors? Several lines of evidence support this possibility. (1) The CtDs of E(spl)-M8/M5/M7/Mγ are notable in that they are rich in Ser/Thr residues. Of these the CK2 site is present in all four members, whereas that for MAPK has been lost from only M7 (Fig. 4C). This pattern of phosphorylation-site maintenance/loss is invariant in homologues across 12 Drosophila species (gray box in Fig. 4C), a strong evolutionary argument that this correlation is not merely incidental. (2) It is increasingly being realized that phosphorylation often targets ‘intrinsically disordered’ (ID) regions, which are rich in Ser/Thr residues [42, 43]. Accordingly, the CtDs of M8/M5/M7/Mγ (and HES6) are predicted to be disordered [12], but not for those members lacking phosphorylation sites such as E(spl)-Mδ [15]. It is of interest to note that targeted knockdown of CK2 (by RNAi) or overexpression of the phosphatase PP2A strongly antagonizes repression by M8 [18], but neither has an effect on Mδ, which is not phosphorylated by CK2 [17]. While the CK2 and/or MAPK sites of M8/M5/M7 are contained within the ID-region, Mγ is unique in that the CK2 and MAPK sites flank the ID-region [12]. The differential placement of phosphorylation sites relative to regions of ID raises the prospect that phosphorylation effects on Mγ are unlikely to mimic those with M5, M7, M8, and HES6. Indeed, in vivo studies during eye/bristle development reveal that Asp mutations at the CK2 and MAPK sites on M8 potently enhance repressor activity, whereas they have no discernible effects on M5 [16], Kim and Bidwai, unpublished. (3) Unlike the P-domain of M8, which directly mediates auto-inhibition, the CK2 and MAPK sites of Mγ are separated by 31 residues, making it likely that CtD-Orange interactions would be different from those in M8. Evidence in favor of this will require knowledge of residues of the O-domain that directly contact the CtD and participate in auto-inhibition. (4) As noted above, M8/M5/M7 conserve a CK2+CK1-dependent phospho-degron, pSpSGYHpS (Fig. 4C). It is difficult to reconcile a similar mechanism for Mγ-turnover, because its SSYAGS sequence does not meet the consensus for Slimb-binding (Fig. 4C), and the absence of Asp/Glu residues C-terminal to this sequence and displacement of the CK2 site preclude the formation of a ‘phospho-degron’ through hierarchical phosphorylation. Degradation may therefore be of lesser importance for Mγ. We do, however, note numerous conserved Ser/Thr in the linker separating the CK1 and CK2 sites (see Fig. 4C), raising the prospect that distinct kinases may impact Mγ. (5) The juxtaposition of the CK2 site to WRPW is unique to Mγ (Fig. 4C), and it is unknown if CK2 influences Groucho binding. If this were the case, CK2 would modulate formation of an Mγ-Groucho complex, thereby imposing regulation distinct from that for M8, whose interaction with Groucho is phosphorylation-independent [14]. (6) The ability of E(spl) proteins to repress other bHLH factors such as Atonal hinges upon many factors, one of which is the formation of homo/heterodimers [11]. The possibility that phosphorylation of Mγ may bias partner preference for dimerization remains unexplored. Systematic biochemical and genetic analyses are required to identify which E(spl) proteins hetero-dimerize with Mγ, and if phosphorylation influences their binding with bHLH factors other than Atonal.

**An expanding role for PTM regulation of Notch signaling**

Among the several highly conserved signaling pathways that regulate animal development, the Notch pathway is deceptively simple. Specifically, activation involves cleavage of the ligand-bound Notch, thereby releasing the NICD, which translocates to the nucleus and regulates gene expression in concert with effectors such as Su(H). It has been somewhat paradox how such a simple pathway can orchestrate diverse developmental programs. Since the discovery of the first Notch mutations, named to reflect a ‘notched wing’ phenotype in Drosophila, it has become clear that this pathway is vital for embryogenesis, neurogenesis, organogenesis, and the morphogenesis of adult structures. Furthermore, studies in mammalian model organisms have revealed additional roles in myogenesis, sprouting angiogenesis, kidney, liver, and heart development, stem cell maintenance, and deregulated signaling has been implicated in diverse cancers such as lung, breast, colorectal, prostate, and pancreatic cancers, as well as osteogenic sarcomas [44–46]. These clinical findings led to significant efforts to develop inhibitors of Notch signaling as a therapeutic [45, 47], but many of these have not borne fruit, perhaps reflecting the broad pleiotropic roles of this signaling pathway and its regulation by an array of PTMs (reviewed in [7]), and its intersection (crosstalk) with other signaling pathways.

Emerging evidence has revealed that Notch signaling involves significant crosstalk with other developmentally important pathways such as EGFR and Wingless/WNT, which are themselves involved with diverse cancers. In addition, an array of PTMs lie at the heart of Notch signaling—likely enabling diversity of signaling in both temporal and spatial contexts (reviewed in [7, 48]). This diversity of PTMs targets not only the Notch receptor, but many of the
pathway components. These include PTMs affecting the receptor such as glycosylation, O-glucosylation, O-fucosylation, hydroxylation, acetylation, methylation, and phosphorylation and dephosphorylation, to name a few. Some of these PTMs regulate receptor trafficking, localization, maturation, turnover, and signal strength and/or duration. Another aspect is signal specificity, first identified during early Drosophila eye development, when Notch first signals in a Su(H)- and E(spl)/HES-independent manner, and shortly thereafter (≤ 30 min later) switches into a Su(H)- and E(spl)/HES-dependent mode [49–51]. It remains unknown how Notch switches between these two modalities, and the possibility remains that this involves spatial and/or temporal differences in PTMs outlined by Antfolk and coworkers [7]. Similarly, PTMs also target the Notch ligands, and transcriptional complexes (Su(H)/CSh and MAML). It has accordingly been suggested that regulation of protein stability, protein–protein interactions, localization, and activity may confer fine-tuning of Notch, without which it may be unable to fulfill its diverse roles [7, 46, 48, 52]. Given our findings on CK2 regulation of E(spl)/HES proteins, it will be of interest to determine if mutations in CK2 genes may be linked to aberrant Notch pathologies. For example, several mutations have now been identified in both human CSNK2A1 (encoding CK2α) and CSNK2B (encoding CK2β) genes, and these are involved with a constellation of neurodevelopmental disorders including delayed development, intellectual disability, etc., that are collectively called ‘Okur-Chung’s Neuropathy’ [53–57]. It remains unknown if these pathologies involve aberrant Notch signaling dynamics or specificity.

In summary, our studies reveal that a preponderance of E(spl) proteins are targets of CK2 and contain highly conserved sites for several additional kinases such as MAPK and CK1, thereby implicating multi-site phosphorylation in regulating Notch signaling pathway output. These sites of phosphorylation co-localize with regions predicted to be intrinsically disordered, but are positioned differently in each isoform, raising the prospect that these bHLH repressors and their PTMs are unlikely to serve redundant functions. Although the roles of multi-site phosphorylation of M7, M5, My, and HES6 remain unresolved, our studies on My suggest that PTM by phosphorylation is more central to these Notch effector proteins than has been recognized. Future efforts are needed to clarify the roles of phosphorylation in dimerization of E(spl) proteins, their ability to bind and repress proneural bHLH factors, and determine if these modifications underlie tissue-specific roles of Notch signaling during development of the nervous system and elsewhere. Given that these bHLH-O effectors are expressed in a tissue-specific manner, a better and more detailed understanding of their regulation by PTMs may provide a unique opportunity to interrogate and interfere with the functions of specific members through kinase-specific inhibitors [58], many of which have had success in the clinic.

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References

1. Dambly-Chaudiere C, Vervoort M (1998) The bHLH genes in neural development. Int J Dev Biol 42:269–273
2. Delidakis C, Monastirioti M, Magadi SS (2014) E(spl): genetic, developmental, and evolutionary aspects of a group of invertebrate Hes proteins with close ties to Notch signaling. Curr Top Dev Biol 110:217–262. https://doi.org/10.1016/B978-0-12-405943-6.00006-3
3. Paroush Z, Finley RL, Kidd T, Wainwright SM, Ingham PW, Brent R, Ish-Horowicz D (1994) Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with hairy related bHLH proteins. Cell 79:805–815
4. Sun H, Ghaffari S, Taneja R (2007) bHLH-Orange transcription factors in development and cancer. Transl Oncogenom 2:105–118
5. Ehebauer M, Hayward P, Arias AM (2006) Notch, a universal code availability Not Applicable.

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References

1. Dambly-Chaudiere C, Vervoort M (1998) The bHLH genes in neural development. Int J Dev Biol 42:269–273
2. Delidakis C, Monastirioti M, Magadi SS (2014) E(spl): genetic, developmental, and evolutionary aspects of a group of invertebrate Hes proteins with close ties to Notch signaling. Curr Top Dev Biol 110:217–262. https://doi.org/10.1016/B978-0-12-405943-6.00006-3
3. Paroush Z, Finley RL, Kidd T, Wainwright SM, Ingham PW, Brent R, Ish-Horowicz D (1994) Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with hairy related bHLH proteins. Cell 79:805–815
4. Sun H, Ghaffari S, Taneja R (2007) bHLH-Orange transcription factors in development and cancer. Transl Oncogenom 2:105–118
5. Ehebauer M, Hayward P, Arias AM (2006) Notch, a universal code availability Not Applicable.

Declarations

Conflicts of interest The authors have not disclosed any competing interests.

Ethics approval Not applicable.

Consent to participate Not applicable.

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References

1. Dambly-Chaudiere C, Vervoort M (1998) The bHLH genes in neural development. Int J Dev Biol 42:269–273
2. Delidakis C, Monastirioti M, Magadi SS (2014) E(spl): genetic, developmental, and evolutionary aspects of a group of invertebrate Hes proteins with close ties to Notch signaling. Curr Top Dev Biol 110:217–262. https://doi.org/10.1016/B978-0-12-405943-6.00006-3
3. Paroush Z, Finley RL, Kidd T, Wainwright SM, Ingham PW, Brent R, Ish-Horowicz D (1994) Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with hairy related bHLH proteins. Cell 79:805–815
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9. Cooper MTD, Tyler DM, Furtiols M, Chalkiadaki A, Delidakis C, Bray SJ (2000) Spatially restricted factors cooperate with notc in the regulation of enhancer of split genes. Dev Biol 221:390–403
10. Maier D, Marte BM, Schafner W, Yu Y, Preiss A (1993) Drosophila evolution challenges postulated redundancy in the E(spl) gene complex. Proc Natl Acad Sci U S A 90:5464–5468
11. Alifragis P, Poortinga G, Parkhurst SM, Delidakis C (1997) 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 94:13099–13104
12. Majot AT, Sizemore TS, Bandopadhayay M, Jozwick LM, Bidwai AP (2015) Protein kinase CK2: a window into the posttranslational regulation of the E(spl)/HES repressors from invertebrates and vertebrates. In: Ahmed K, Issinger O-G, Szszyka R (eds) Protein kinase CK2 cellular function in normal and disease states. Springer, New York, pp 81–108
13. Trott RL, Kalive M, Paroush Z, Bidwai AP (2001) 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
14. Karandikar U, Trott RL, Yin J, Bishop CP, Bidwai AP (2004) Drosophila melanogaster CK2 regulates eye morphogenesis via phosphorylation of E(spl)M8. Mech Dev 121:273–286
15. Kahali B, Kim J, Karandikar U, Bishop CP, Bidwai AP (2010) Evidence that the C-terminal domain (Cd) autoinhibits neural repression by Drosophila E(spl)M8. Genesis 48:44–55
16. Bandopadhayay M, Bishop CP, Bidwai AP (2016) The Conserved MAPK Site in E(spl)-M8, an Effector of Drosophila Notch Signaling. Controls Repressor Activity during Eye Development. PLoS ONE 11:e0159508. https://doi.org/10.1371/journal.pone.0159508
17. Majot AT, Bidwai AP (2017) Analysis of transient hypermorphic activity of E(spl)D during R8 specification. PLoS ONE 12:e0186439. https://doi.org/10.1371/journal.pone.0186439
18. Bose A, Majot AT, Bidwai AP (2014) The Ser/Thr phosphatase PP2A regulatory subunit Widerborst inhibits notch signaling. PLoS ONE 9:e101884. https://doi.org/10.1371/journal.pone.0101884
19. Kahali B, Bose A, Karandikar U, Bishop CP, Bidwai A (2009) On the mechanism underlying the divergent retinal and bristle defects of M8* (E(spl)D) in Drosophila. Genesis 47:456–468. https://doi.org/10.1002/dvg.20521
20. Belanger-Jasmin S, Llamosas E, Tang Y, Joachim K, Osiceanu AM, Jhas S, Stifani S (2007) Inhibition of cortical astrocyte differentiation by Hes6 requires amino- and carboxy-terminal motifs important for dimerization and phosphorylation. J Neurochem 103:2022–2034
21. Gratton M-O, Torban E, Jasmin SB, Theriault FM, German MS, Stifani S (2003) Hes6 promotes cortical neurogenesis and inhibits Hes1 transcription repression activity by multiple mechanisms. Mol Cell Biol 23:6922–6935
22. Yasukawa T, Kani-Ishii C, Maekawa T, Fujimoto J, Yamamoto T, Ishii S (1995) Increase of solubility of foreign proteins in Escherichia coli by coproduction of the bacterial thioredoxin. J Biol Chem 270:25328–25331
23. Kalive M, Trott RL, Bidwai AP (2001) 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
24. Trott RL, Kalive M, Karandikar U, Rummr R, Bishop CP, Bidwai AP (2001) Identification and characterization of proteins that interact with Drosophila melanogaster protein kinase CK2. Mol Cell Biochem 227:91–98
25. Bidwai AP, Reed JC, Glover CVC (1993) The phosphorylation of calmodulin by the catalytic subunit of casein kinase II is inhibited by the regulatory subunit. Arch Biochem Biophys 300:265–270
26. Bidwai AP, Hanna DE, Glover CVC (1992) The free catalytic subunit of casein kinase II is not toxic in vivo. J Biol Chem 267:18790–18796
27. Glover CVC, Shelton ER, Brutlag DL (1983) Purification and characterization of a type II casein kinase from Drosophila melanogaster. J Biol Chem 258:3258–3265
28. Meggio F, Boldyreff B, Marin O, Marchiotor F, Perich JW, Issinger OG, Pinna LA (1992) The effect of polylysine on casein-kinase-2 activity is influenced by both the structure of the protein/peptide substrates and the subunit composition of the enzyme. Eur J Biochem 205:939–945
29. Beverley SM, Wilson AC (1984) Molecular evolution in Drosophila and the higher Diptera II. A time scale for fly evolution. J Mol Evol 21:1–13
30. Kuenzel EA, Mulligan JA, Sommecorno J, Krebs EG (1987) Substrate specificity determinants for casein kinase II as deduced from studies with synthetic peptides. J Biol Chem 262:9136–9140
31. Hruby TW, Rouach P (1990) Phosphoserine in peptide substrates can specify casein kinase II action. Biochim Biophys Acta 103:1590–1593
32. Bandopadhayay M, Arbet S, Bishop CP, Bidwai AP (2017) Drosophila protein kinase CK2: genetics, regulatory complexity and emerging roles during development. Pharmaceuticals (Basel). https://doi.org/10.3390/pharmaceutics8020004
33. Kahali B, Trott R, Paroush Z, Allada R, Bishop CP, Bidwai AP (2008) Drosophila CK2 phosphorylates Hairy and regulates its activity in vivo. Biochim Biophys Acta 1773:637–642
34. Dahmus GK, Glover CVC, Brutlag D, Dahmus ME (1984) Similarities in structure and function of calf thymus and Drosophila casein kinase II. J Biol Chem 259:9001–9006
35. Lin WJ, Tuazon PT, Traugh JA (1991) Characterization of the catalytic subunit of casein kinase II expressed in Escherichia coli and regulation of activity. J Biol Chem 266:5664–5669
36. Meggio F, Marin O, Pinna LA (1994) Substrate specificity of protein kinase CK2. Cell Mol Biol Res 40:401–409
37. Kuenzel EA, Krebs EG (1985) A synthetic substrate specific for casein kinase II. Proc Natl Acad Sci U S A 82:737–741
38. Hathaway GM, Lubben TH, Traugh JA (1980) Inhibition of casein kinase II by heparin. J Biol Chem 255:8038–8041
39. Marin O, Bustos VH, Cesarlo L, Meggio F, Pagano MA, Antonelli M, Allende CC, Pinna LA, Allende JE (2003) A noncanonical sequence phosphorylated by casein kinase 1 beta-catenin may play a role in casein kinase 1 targeting of important signaling proteins. Proc Natl Acad Sci U S A 100:10193–10200. https://doi.org/10.1073/pnas.1733909100
40. Price MA (2006) CKL, there’s more than one: casein kinase I evolution challenges postulated redundancy in the E(spl) gene network of interacting transcriptional regulators involved in Drosophila. Mol Biol Cell 17:933–943
41. Guruharsha KG, Rual JF, Zhai B, Mintseris J, Vaidya P, Vaidya N, Beeken C, Wong C, Rhee DY, Cenaj O, McKillop E, Shah S, Stapleton M, Wan KH, Yu C, Parsa B, Carlson JW, Chen X, Kapadia B, Vijayaraghavan K, Gygi SP, Celniker SE, Obar RA, Artavasds-Tsakonas S (2011) A protein complex network of Drosophila melanogaster. Cell 147:690–703. https://doi.org/10.1016/j.cell.2011.08.047
42. Tompa P (2012) Intrinsically disordered proteins: a 10-year recap. Trends Biochem Sci 37:509–516. https://doi.org/10.1016/j.tibs.2012.08.004
43. Chakrabortee S, Byers JS, Jones S, Garcia DM, Bhullar B, Chang A, She R, Lee L, Fremin B, Lindquist S, Jarosz DF (2016) Intrinsically disordered proteins drive emergence and inheritance of biological traits. Cell 167(369–381):e12. https://doi.org/10.1016/j.cell.2016.09.017
44. Tao J, Jiang MM, Jiang L, Salvo JS, Zeng HC, Dawson B, Bertin TK, Rao PH, Chen R, Donehower LA, Gannon F, Lee BH (2014)
Notch activation as a driver of osteogenic sarcoma. Cancer Cell 26:390–401. https://doi.org/10.1016/j.ccr.2014.07.023

45. Majumder S, Crabtree JS, Golde TE, Minter LM, Osborne BA, Miele L (2021) Targeting notch in oncology: the path forward. Nat Rev Drug Discov 20:125–144. https://doi.org/10.1038/s41573-020-00091-3

46. Aster JC, Pear WS, Blacklow SC (2017) The varied roles of notch in cancer. Annu Rev Pathol 12:245–275. https://doi.org/10.1146/annurev-pathol-052016-100127

47. Andersson ER, Lendahl U (2014) Therapeutic modulation of Notch signalling—are we there yet? Nat Rev Drug Discov 13:357–378. https://doi.org/10.1038/nrd4252

48. Bray SJ (2016) Notch signalling in context. Nat Rev Mol Cell Biol 17:722–735. https://doi.org/10.1038/nrm.2016.94

49. Baonza A, Freeman M (2001) Notch signalling and the initiation of neural development in the Drosophila eye. Develop 128:3889–3898

50. Ligoxygakis P, Yu SY, Delidakis C, Baker NE (1998) A subset of Notch functions during Drosophila eye development require Su(H) and E(spl) gene complex. Develop 125:2893–2900

51. Lubensky DK, Pennington MW, Shraiman BI, Baker NE (2011) A dynamical model of ommatidial crystal formation. Proc Natl Acad Sci U S A 108:11145–11150. https://doi.org/10.1073/pnas.1015302108

52. Borggrefe T, Giaimo B (2018) Molecular mechanisms of notch signaling. Advances in experimental medicine and biology. Springer, Berlin

53. Owen CI, Bowden R, Parker MJ, Patterson J, Patterson J, Price S, Sarkar A, Castle B, Deshpande C, Splitt M, Ghali N, Dean J, Green AJ, Crosby C, Tatton-Brown K (2018) Extending the phenotype associated with the CSNK2A1-related Okur-Chung syndrome-A clinical study of 11 individuals. Am J Med Genet A. https://doi.org/10.1002/ajmg.a.38610

54. Chiu ATG, Pei SLC, Mak CCY, Leung GKC, Yu MHC, Lee SL, Vreeburg M, Pfundt R, van der Burgt I, Kleefstra T, Frederic TM, Nambot S, Faivre L, Bruel AL, Rossi M, Isidor B, Kury S, Cogne B, Bensard T, Willems M, Reijnders MRF, Chung BHY (2018) Okur-Chung neurodevelopmental syndrome: Eight additional cases with implications on phenotype and genotype expansion. Clin Genet 93:880–890. https://doi.org/10.1111/cge.13196

55. Nakashima M, Tohyama J, Nakagawa E, Watanabe Y, Siew CG, Kwong CS, Yamoto K, Hirai T, Fukuda T, Kaname T, Nakabayashi K, Hata K, Ogata T, Saito H, Matsumoto N (2019) Identification of de novo CSNK2A1 and CSNK2B variants in cases of global developmental delay with seizures. J Hum Genet 64:313–322. https://doi.org/10.1038/s10038-018-0559-z

56. Poirier K, Hubert L, Viot G, Rio M, Billuart P, Besmond C, Bienvenu T (2017) CSNK2B splice site mutations in patients cause intellectual disability with or without myoclonic epilepsy. Hum Mutat 38:932–941. https://doi.org/10.1002/humu.23270

57. Colavito D, Del-Giudice E, Ceccato C, Dalle-Carbonare M, Leon A, Suppiej A (2018) Are CSNK2A1 gene mutations associated with retinal dystrophy? Report of a patient carrier of a novel de novo splice site mutation. J Hum Genet. https://doi.org/10.1038/s10038-018-0434-y

58. Ferguson FM, Gray NS (2018) Kinase inhibitors: the road ahead. Nat Rev Drug Discov 17:353–377. https://doi.org/10.1038/nrd.2018.21

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