Protein Kinase CK2 Phosphorylates a Conserved Motif in the Notch Effector E(spl)-My

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Short Report

Keywords: CK2, phosphorylation, Notch, bHLH, E(spl), HES

Posted Date: August 23rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-809298/v1>

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Abstract

Across metazoans, the effects of Notch signaling are mediated via the Enhancer of Split (E(spl)/HES) basic Helix-Loop-Helix-Orange (bHLH-O) repressors. Although conserved, sequence diversity is, in large part, restricted to the C-terminal domain (CtD), which separates the O-domain from the penultimate WRPW motif that binds the 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)-My 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 My homologues over 50×10^6 years of *Drosophila* evolution. Thus, a preponderance of E(spl) homologues 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.

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 (called Orange), followed by a C-terminal domain (CtD) of variable length, and terminate with an invariant WRPW motif through which they recruit the co-repressor Groucho (Gro, [3]). Since identification of the founding members, the *Drosophila* E(spl)-repressors, bHLH-O proteins have been found in all metazoans [4]. In addition to neurogenesis, these proteins are vital throughout development and regulate processes such as embryogenesis, myogenesis, somitogenesis, etc. [5, 6]. Despite their centrality to Notch biology, our understanding of the regulation of E(spl)/HES proteins by post translational modifications (PTM) 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 [7]. 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.

The E(spl) proteins in *Drosophila* are encoded by the E(spl)-Complex [2], which harbors seven repressors (M δ , My, M β , 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 50×10^6 years (MYR) of evolution. E(spl)-repressors are expressed in complex partly overlapping patterns that are tissue/compartment-specific [8], 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 [9], tissue-specific expression patterns, homo/hetero-dimerization specificities, and target gene selectivity [10]. Adding to this complexity is accumulating evidence that these proteins are regulated by phosphorylation [11].

We had previously reported that E(spl)-M8/M5/M7 are phosphorylated by protein kinase CK2 at a highly-conserved site in their CtD [12]. Modification of M8 was essential for early steps of eye development, but had a modest impact during genesis of the sensory bristles [13], two widely used readouts of Notch signaling. This regulation reflects conversion of M8 from a CtD-dependent 'cis'-inhibited state to an active repressor [14]. Activation by phosphorylation also requires MAPK [15], whose site resides in a Ser-rich region of the CtD, the P-domain. A similar P-domain in human HES6 is modified by CK2 + MAPK [16, 17], suggesting that PTM is an important, yet underappreciated, aspect of Notch signaling. Understanding the generality of PTM of E(spl)/HES repressors would, therefore, enable a better understanding of Notch biology.

The studies we report here uncover novel and direct interactions between CK2 and E(spl)-My. Our studies reveal that My is a bona fide target of this kinase, identify that the primary site for phosphorylation is uniquely positioned adjacent to the WRPW motif, and that this site is invariant in the My protein from 12 *Drosophila* species. Our studies reveal that a preponderance of E(spl) proteins are regulated by PTM, and provide new insights into the conservation of the kinase sites. Analysis of kinase-site specificity suggests that members of this class of bHLH-O proteins are functionally distinct, and differential regulation by PTM may fine tune Notch signaling.

Materials And Methods

Identification of CK2 Sites in E(spl)-My.

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)-My and generation of mutant versions of E(spl)-My.

The E(spl)-My open reading frame was PCR amplified from wild type *D. melanogaster* (*w¹¹¹⁸* flies) using primers complementary to the 5' and 3' ends of the intron-less My gene. The primers included EcoR1 and Xho1 restriction sites at the 5' and 3' ends, respectively. Site-directed mutagenesis was used to introduce Ala/Asp substitutions at predicted CK2 sites. All PCR-based constructs were verified by Sanger-sequencing.

Purification of GST-Fusion Proteins.

Plasmid pZEX was used to express recombinant proteins as GST fusions in *E. coli* BL21 harboring plasmid pT-TRX [18]. Cultures were grown to an OD₆₀₀ of 0.7 in 2xYTA containing 150 µg/mL ampicillin and 15 µg/mL chloramphenicol. Protein expression was induced with 1 mM IPTG at 30C for ~ 3 hours. Cells were resuspended in 8 mL of phosphate buffered saline (PBS) containing 5 mM EDTA, 0.1 mM PMSF and 0.2% β-mercaptoethanol, treated with lysozyme (1 mg/mL) on ice for 30 minutes, and disrupted by sonication for ~ 30 seconds. Lysed samples were treated with 1% Triton-X100 at 4C for 1 hour. Insoluble material was removed by centrifugation, and the supernatant incubated with glutathione-beads for 2 hours at 4C. 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 cutoff). The concentration and purity were determined by SDS-PAGE compared to protein standards (Fermentas).

Protein Kinase CK2 Phosphorylation Assays.

CK2 assays were performed at 25C 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 [19]. Reactions were initiated with 20 ng native CK2-holoenzyme purified from *Drosophila* embryos or recombinant monomeric CK2α from a yeast expression system [20]. CK2 modulators were added at the following concentrations; GTP (60 µM), Heparin (1 µg/mL), Protamine (125 µg/mL), Spermine (500 µg/mL) and Poly-(D/L)-lysine (100 µg/mL). Reactions were terminated with 10 µL 5x 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)-My harbors multiple consensus sites for CK2.

We first aligned E(spl)-My homologues from 12 species that represent ~ 50x10⁶ years (MYR) of *Drosophila* evolution [21]. All E(spl)-My homologues harbor three conserved consensus sites for CK2 (Fig. 1A), fully meeting the strict recognition requirements for this kinase [22]. 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 promote recognition by CK2 with equal potency [23]. Interestingly, of the seven E(spl) bHLH-O proteins in *Drosophila*, E(spl)-My 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)-My. Notably, numerous Asp/Glu, a hallmark of 'high likelihood' targets of CK2, flank Ser¹⁹⁵. Although E(spl)-My 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 [21], it appears likely that E(spl)-My from Hawaiian, Virilis, Repleta and Obscura groups (harboring an insertion of two residues) are the ancestral form (Fig. 1B). A one residue deletion occurred ~ 37x10⁶ MYR ago with the emergence of the Willistoni group, and both residues were lost ~ 13x10⁶ 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)-My by CK2.

We next determined if E(spl)-My is phosphorylated by CK2. This kinase exists as an $\alpha 2\beta 2$ holoenzyme in all eukaryotes [24], and phosphorylates Ser/Thr/Tyr in a messenger-independent manner. Although phosphorylation is catalyzed by CK2 α , the regulatory CK2 β subunit promotes or inhibits recognition in a target-specific manner [19, 25]. We thus used native $\alpha 2\beta 2$ holoenzyme (CK2-HoloE) purified from *Drosophila* embryos and monomeric (recombinant) CK2 α purified from yeast rescued by a cDNA encoding *Drosophila* CK2 α [20]. Both enzyme forms are pure based on Coomassie staining (Fig. 2, Gel). In the case of CK2-HoloE, the weak staining of CK2 β relative to CK2 α (Fig. 2) does not reflect substoichiometric levels, as this enzyme preparation has a sedimentation coefficient of 6.4S (data not shown), consistent with the $\alpha 2\beta 2$ conformation, and reflects weak staining of CK2 β from flies and mammals [26, 27]. Both forms display kinetic properties and ATP/GTP-dependency identical to those reported by others and us [20, 28].

The two isoforms of CK2 were used to phosphorylate E(spl)-My. In these assays, 20 ng of CK2 α or CK2-HoloE were 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)-My 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)-My was robustly phosphorylated by both CK2 α and CK2-HoloE. Phosphorylation is specific to E(spl)-My, as GST was not phosphorylated. The band intensities suggest that CK2-HoloE more efficiently phosphorylates E(spl)-My than does CK2 α . This difference likely reflects (~ 4-fold) greater activity of the holoenzyme [20], rather than a requirement for CK2 β . Phosphorylation of E(spl)-M8 (positive control) was observed with both

isoforms, although CK2 β appeared to modestly diminish phosphorylation. Thus, E(spl)-My is targeted by native *Drosophila* CK2 independent of CK2 β .

CK2-specific targeting of E(spl)-My revealed through effectors.

It remained formally possible that a minor enzyme contaminant (other than CK2) was responsible for phosphorylation of My. To exclude this, My phosphorylation by CK2-HoloE was tested in the presence of competitor (GTP), inhibitor (Heparin), or polybasic activators, as previously reported [19]. We exploited the unusual property that CK2 can use ATP/GTP as phosphate donors [27]. Assays contained 15 μ M [γ ³²P]-ATP (1xKm) and supplemented with 60 μ M cold-GTP. In this assay, competition by GTP should diminish phosphorylation, if the enzyme is CK2. Indeed, GTP strongly decreased ³²P-incorporation into GST-My (Fig. 3A). Likewise, Heparin (1 μ g/mL), a CK2 inhibitor [29], strongly diminished phosphorylation of GST-E(spl)-My. We next tested polybasic compounds that stimulate CK2-HoloE through interaction with CK2 β [19]. All three (protamine, spermine and poly-lysine) modestly increased phosphorylation of GST-My. 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 [19]), the phosphorylation observed in Figs. 2 and 3 reveal a specific interaction of CK2 with My. These studies confirm E(spl)-My as 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 were tested for phosphorylation by CK2-HoloE. Analysis with monomeric CK2 α was deemed unnecessary given CK2 β -independent targeting of My (Fig. 2). In brief, 20 ng of CK2-HoloE were used to phosphorylate 0.5-1.0 μ g 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 My-NTD-S⁴⁸A was very weakly phosphorylated, neither My-NTD-T⁴⁵A nor My-NTD-T⁴⁵+S⁴⁸A were phosphorylated (Fig. 4A). We thus tested full-length My with the S¹⁹⁵A mutant, and find that, unlike wild-type E(spl)-My (Fig. 2), the single-site mutant FL-S¹⁹⁵A was not phosphorylated (Fig. 4B). Therefore, the faint band with My-NTD-S⁴⁸A (boxed area in Fig. 4B) more likely represent an *in vitro* artifact. These results demonstrate that CK2 modifies My only at Ser¹⁹⁵ in its CtD (Fig. 4A). Localized folding may render Ser⁴⁸/Thr⁴⁵ (in the HLH domain) refractory/inaccessible to CK2.

The importance of PTM of E(spl)/HES proteins.

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 [13, 15]. In either context, the CK2 and MAPK sites auto-inhibit the O-domain via protein-protein interactions. Phosphorylation overrides auto-inhibition to uncover the O-domain, thereby permitting E(spl)-M8 repressor activity. Accordingly, removal of the CtD or just the P-domain elicits constitutive (PTM-independent) repression by E(spl)-M8 [15]. More recent studies ([11], and Majot and Bidwai, unpublished) are revealing that M8 is likely to be regulated through PTM-dependent degradation. 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 [30, 31]. CK2 targeting of M8 (at Ser¹⁵⁹) may thereby favor 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/ β TrCP [11]. The M8-Slimb/ β TrCP interaction has been reported [32], but its phospho-dependency remains unresolved. 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 My 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 amongst this family of repressors? Several lines of evidence support this possibility. 1) The CtDs of E(spl)-M8/M5/M7/My 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 PTM-site maintenance/loss is invariant in homologues across 12 *Drosophila* species (grey box in Fig. 4C), a strong evolutionary argument that this correlation is not merely incidental. 2) It is increasingly being realized that PTM often targets 'intrinsically disordered' (ID) regions, which are rich in Ser/Thr/Asp/Glu residues [33, 34]. Accordingly, the CtDs of M8/M5/M7/My (and HES6) are predicted to be disordered [11], but not for those lacking PTM sites such as E(spl)-M δ [14]. While the CK2 and/or MAPK sites of M8/M5/M7 are contained within the ID-region, My is unique in that the CK2 and MAPK sites flank the ID-region [11]. The differential placement of PTM sites relative to regions of ID raises the prospect that phosphorylation effects on My are unlikely to mimic those with M5, M7, M8 and HES6. Indeed, *in vivo* studies in the eye/bristle reveal that Asp mutations at the CK2 and MAPK sites on M8 potently enhance repressor activity, whereas they have no discernible effects on M5 ([15], Kim and Bidwai, unpublished). 3) Unlike the P-domain of M8, which directly mediates auto-inhibition, the CK2 and MAPK sites of My 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 mentioned above, M8/M5/M7 conserve a

CK2 + CK1-dependent phospho-degron, pSpSGYHpS (Fig. 4C). It is difficult to reconcile a similar mechanism for My-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 My. 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 My. 5) The juxtaposition of the CK2 site to WRPW is unique to My (Fig. 4C), and it is unknown if CK2 influences Groucho binding. If this were the case, CK2 would modulate formation of an My-Groucho complex, thereby imposing regulation distinct from that for M8, whose interaction with Groucho is phosphorylation-independent [13]. 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 [10]. The possibility that phosphorylation of My may bias partner preference for dimerization remains unexplored. Systematic biochemical and genetic analyses are required to identify which E(spl) proteins hetero-dimerize with My, and if phosphorylation influences their binding with bHLH factors other than Atonal.

In summary, our studies reveal that a preponderance of E(spl) proteins are targets of CK2 and conserve a site for MAPK, implicating these kinases 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 do not serve redundant (anti-neural) functions. Although the roles of multi-site phosphorylation of M7, M5, My and HES6 remain unresolved, our studies on My suggest that PTM is more central to these 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.

Declarations

Funding	Not Applicable
Conflicts of interest/ Competing interests	Not Applicable
Availability of data and material	All data are part of submitted manuscript
Code availability	Not Applicable
Authors' contributions	<p>LJ and AB contributed to design of the work, as well as acquisition, analysis, and interpretation of data.</p> <p>LJ and AB drafted the work.</p> <p>LJ and AB approved the version to be published.</p> <p>LJ and AB agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.</p>
Ethics approval	Not Applicable
Consent to participate	Not Applicable
Consent for publication	Not Applicable

ACKNOWLEDGEMENTS:

The authors thank Mohna Bandyopadhyay and Sophia Zhang for technical assistance.

DATA AVAILABILITY STATEMENT:

All data generated or analyzed during this study are included in this published article

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Figures

Figure 1

Conserved sites for CK2 in E(spl)-My. A) Functional domains in My; the Orange domain forms an HLH motif. Alignment of My from 12 *Drosophila* species illustrating the conserved CK2 sites; residues in green meet the consensus for CK2. The arrowhead denotes the insertion of one/two residues in *Drosophila* My homologues. Members of the Melanogaster group (Mel-Group) are in panel B. B) Phylogenetic

relationship between *Drosophila* species analyzed in panel-A. Note that loss of one/two residues closely parallels divergence of the major groups.

Figure-2

Figure 2

CK2 phosphorylates E(spl)-My. 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-Holo-E. Asterisk denotes close migration of weakly autophosphorylated CK2β and non-phosphorylated GST.

Figure-3

Figure 3

Specificity of CK2 phosphorylation of E(spl)-My. Full-length GST-M γ was phosphorylated using the $\alpha 2 \beta 2$ holoenzyme (CK2-HoloE). Assays were conducted in the absence or presence of the indicated

modulators. Proteins were separated by SDS-Page, stained with coomassie (Gel), and autoradiographed (Film).

Figure-4

Figure 4

Identify CK2 sites in E(spl)-My. A) Location of the CK2 sites relative to functional domains. Constructs include full-length My, the region from the N-terminus-through-Orange (My-NtD) and the C-terminal Domain (My-CtD). B) Proteins were phosphorylated using the $\alpha 2 \beta 2$ holoenzyme (CK2-HoloE).

Phosphorylated proteins were separated by SDS-Page, stained with coomassie (Gel), and autoradiographed (Film). Dashed-box denotes absence of phosphorylation of GST alone, and solid box denotes weak phosphorylation of Ala mutants of My-NtD. C) Alignment of D. melanogaster M8/M5/M7/My. Fractional conservation of the MAPK and/or CK2 sites in 12 Drosophila species is indicated in grey box.