

Identification and characterization of proteins that interact with *Drosophila melanogaster* protein kinase CK2

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Abstract

D. melanogaster CK2 (DmCK2) is a highly conserved protein kinase that is composed of catalytic, α , and regulatory, β , subunits associated as an $\alpha_2\beta_2$ heterotetramer. In order to analyze the functions of CK2 in this metazoan model, we have used the two hybrid approach to identify interacting proteins. One of these cDNAs, DmA24, encodes a novel polypeptide with no homologs in GenBank, and is notable in that it contains a bipartite nuclear localization signal and two sites for phosphorylation by CK2. *In situ* hybridization to polytene chromosomes indicates that the DmA24 gene is located at the 61D interval of chromosome II a region that also harbors 3 additional genes with similar structure. DmA24p interacts with DmCK2 α , but not with DmCK2 β , demonstrating that this interaction is specific for the catalytic subunit of CK2. In addition, the protein is phosphorylated by the holoenzyme purified from *Drosophila* embryos. These studies identify DmA24p as a potentially new physiological partner of DmCK2. In addition, we also report the results of a large-scale screen that has identified a new set of DmCK2-interacting proteins. Most notable among these are Surf6, a nucleolar protein involved in RNA processing, and Spalt, a homeotic protein. (Mol Cell Biochem 227: 91–98, 2001)

Key words: protein kinase, CK2, *Drosophila*, two hybrid

Introduction

CK2 is an ubiquitous protein kinase that is highly conserved among eukaryotes [1, 2], and is capable of functioning as an oncogene [3, 4]. In general, CK2 is composed of catalytic (α) and regulatory (β) subunits that combine to form an $\alpha_2\beta_2$ holoenzyme, and distinct isoforms of both subunits have been described in a wide variety of organisms. CK2 is unique in its site selectivity in that it preferentially phosphorylates Ser/Thr residues that are followed by a stretch of acidic residues [2]. However, in at least one case, i.e. yeast Fpr3, the enzyme has been reported to phosphorylate Tyr residues [5]. Analysis of the phosphorylation of peptides suggests that the consensus site for CK2 can best be described as (S/T)xxD/E, and consistent with this, a number of proteins critical for tran-

scription, cell-cycle regulation, and signal transduction contain such site(s) and are known to be phosphorylated *in vitro* and *in vivo* [6]. *In vitro*, CK2 activity is inhibited by polyacidic compounds [7], and stimulated by polybasic compounds [8], but the *in vivo* relevance of these observations remains unclear. Comparisons between recombinant monomeric α subunit and native or reconstituted $\alpha_2\beta_2$ holoenzyme have revealed that the β subunit plays a complex, yet at times paradoxical, role in regulating the basal activity of the α subunit [9–12]. On the one hand, the β subunit stabilizes the α subunit against proteolysis and thermal denaturation and stimulates its activity approximately 5-fold against most substrates [13]; on the other hand, it negatively regulates phosphorylation of selected substrates, notably calmodulin [14, 15]. The β subunit also mediates stimulation of CK2 by poly-

basic compounds such as polylysine and protamine [15], although studies suggest that these compounds can exert their activating effects via the α subunit as well [14].

Genetic analyses in budding and fission yeast have demonstrated that the enzyme is essential for viability [16, 17] and is required for cell cycle progression in G1 and G2/M [18], for maintenance of cytoskeletal architecture [19], and for cytokinesis [17]. In contrast, analysis of CK2 in *Drosophila* has lagged despite the fact that the cDNAs encoding DmCK2 were the first to be isolated [20]. The absence of mutations in the genes encoding DmCK2 subunits has precipitated the use of alternative approaches. One such approach is the two hybrid strategy [21] to identify interacting proteins (many of which appear to be substrates of the enzyme), followed by genetic analysis using transgenes with alterations in the CK2-interaction domain and/or site(s) of phosphorylation. Such studies have demonstrated that CK2 negatively regulates the homeobox protein ANTP [22], and that this enzyme targets Dishevelled [23], a component of the Wntless signaling pathway, as well as the neurogenic transcriptional repressors such as m5, m7, and m8 [24]. These results suggest that CK2 plays a critical role in embryogenesis and cell-fate determination.

In an attempt to better define the role of DmCK2, we have used the two-hybrid approach to identify and characterize its physiological partners. We describe the isolation and characterization of a cDNA, DmA24, whose product interacts with DmCK2 α . The corresponding gene is localized to the 61D interval, a region that harbors three other genes which exhibit evidence of exon shuffling. Although the DmA24 protein is not homologous to other proteins with known functions, it contains a nuclear localization signal, and two sites for phosphorylation by CK2. Consistent with this, we demonstrate that DmA24p is phosphorylated by the embryo holoenzyme. The available data suggest that DmA24 is a newly identified physiological partner and substrate of DmCK2. In addition, we summarize the results of a second two hybrid screen which has identified proteins that are likely to play a role in transcription, RNA processing, and development.

Materials and methods

Yeast two-hybrid screening and β -galactosidase assay

All manipulations involved in construction of the two-hybrid plasmids were carried out as described [25]. The construction of plasmids expressing Dm α / β as fusions with the Gal4 DNA-binding domain (Gal4DB) and the Gal4 Activation-domain (Gal4AD) has been previously described [26]. Yeast

strain HF7C [27] expressing GAL4DB-Dm α was used to screen a 3- to 18-h *Drosophila* embryo two hybrid cDNA library [28]. Transformants exhibiting robust His-independent growth were counterscreened for expression of *LacZ* [29]. The library plasmids were recovered, and retested against GAL4DB-Dm α or GAL4DB-Dm β . Those cDNAs which induced expression of the reporter genes only in response to Dm α (a bait-specific manner) were sequenced on an Applied Biosystems model 373A DNA sequencer using the Prism Dye Terminator Cycle sequencing kit (Applied Biosystems).

Isolation of full-length clones encoding DmA24

One of the cDNAs, DmA24, isolated in this screen was missing its mature 5' and 3' ends. We, therefore, screened a commercially available 0–18 h *Drosophila* embryo cDNA library (Stratagene). The DmA24 cDNA was labeled by random hexamer primers (Boehringer Mannheim), and used to screen 200,000 plaques. Four positives were identified, converted into plasmids, and restriction mapped. The largest clone (DmA24-13) was sequenced in order to compile the full length sequence, and for subsequent analysis.

Interaction of full length DmA24 with DmCK2 α

Explicit interactions between full length DmA24 and Dm α were tested using the LexA system [30]. DNA corresponding to amino acids 1–387 of DmA24 was amplified from plasmid DmA24-13 by PCR, and subcloned into the EcoRI-BamHI site of plasmid LexA202 [30]. The construction of a plasmid expressing Dm α as an AD-fusion has been previously described [24]. EGY048 containing plasmid pSH18-34 was transformed with a plasmid expressing the AD-alone or AD-Dm α fusion protein using lithium-acetate [25]. A single transformant was subsequently retransformed with a plasmid expressing LexA-DmA24. Three independent transformants were grown in galactose medium, and assayed in triplicate for *LacZ* activity as described [29].

In situ hybridization to polytene chromosomes

Salivary glands from third instar larvae were isolated, dissected, and prepared for hybridization essentially as described [31]. The cDNA from clone DmA24 was labeled by nick translation using biotinylated dUTP (Boehringer-Mannheim). Probe binding was visualized using streptavidin coupled to alkaline phosphatase, and slides were photographed at 400 \times magnification.

Phosphorylation of DmA24 by CK2

Expression of GST-DmA24, purification of the fusion protein and phosphorylations were conducted essentially as described [24]. Phosphorylation reactions were carried out in 50 mM Tris, pH 8.5, 100 mM NaCl, 10 mM MgCl₂, 10 μM ATP, 5 μCi [γ -³²P]ATP and CK2 (1 μg/ml) in a total volume of 40 μl, at 25°C. The reactions were terminated by boiling for 5 min following the addition of 10 μl of 5 × sample buffer [32]. 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.

Results and discussion

Two hybrid screen for CK2 interacting proteins

The yeast strain HF7C expressing GAL4DB-Dm α as a bait was used to screen a *D. melanogaster* embryo two hybrid cDNA library and resulted in the isolation of 15 *HIS*⁺/*LacZ*⁺ clones. As expected, these include DmCK2 β [33], DmCK2 β' [26], a novel isoform of the β subunit, and DmCK2 α [26]. We do not consider our isolation of Dm α (using Dm α as a bait) to imply that this protein can dimerize, but rather that the β/β' subunits of yeast CK2 mediate this interaction. This interpretation is consistent with the observation that this protein exhibits a strong two-hybrid interaction with the β' subunit of yeast CK2 (Trott and Bidwai, unpublished). The isolation of DmCK2 subunits thus serves as a valuable internal control. In addition, this screen resulted in the isolation of E(spl)m7, the ribosomal protein DmL22, and two novel proteins, DmA24, and DmA35. E(spl)m7 encodes a neurogenic transcriptional repressor [34], and we have recently demonstrated that m7, and two related proteins m5 and m8, interact with DmCK2, and all three proteins are phosphorylated by the embryo holoenzyme within a highly conserved PEST domain that negatively regulates function of these proteins *in vivo* [24]. Interestingly, DmL22 contains an hyperacidic C-terminal tail that also harbors a site for phosphorylation by CK2, SSNDDEDDDAE (see Table 1). In this regard, yeast and human L22 also exhibit an hyperacidic C-terminal tail, but lack the CK2 site. Given that this enzyme interacts strongly with polyAsp/Glu peptides [7], it is conceivable that this interaction may also be observed in humans and yeast, but the lack of conservation of the CK2 site makes it unclear whether phosphorylation will be meaningful in an evolutionary context. The functions of DmA24 and DmA35 are currently unknown, although both proteins contain consensus sites for phosphorylation by CK2, and are predicted to be nuclear localized with proposed roles in transcription. We

describe here the molecular and biochemical characterization of the protein encoded by the two hybrid clone, DmA24.

Nucleotide sequence of the DmA24 cDNA

The cDNA contained in the plasmid DmA24 was completely sequenced as described in Materials and methods. Sequencing revealed that this clone was lacking its 5' untranslated region (including the initiation codon), as well as its 3' untranslated region (by virtue of the absence of a polyA-tail). Most relevant to our two hybrid analysis, the encoded protein appeared to be lacking its mature N- and C-terminal ends and, in addition, contained a small peptide introduced at its C-terminus via translational read-through of the polylinker of the AD-vector [28]. In order to establish that the full length protein, rather than the aberrant C-terminal peptide, mediates interaction with Dm α , we have isolated full length clones encoding DmA24, and one of these, DmA24-13, was completely sequenced (Fig. 1). This cDNA (deposited as GenBank accession number AF090440) is ~1354 bp long and contains a major uninterrupted open reading frame of 1164 bp that encodes a 387 amino acid long polypeptide with a calculated molecular mass of 44 kDa, and a predicted pH of 9. In addition, clone DmA24-13 contains 47 nucleotides in the 5' untranslated region, and 144 nucleotides in the 3' untranslated region. Although the putative initiation methionine codon is not preceded by a Kozak-type consensus sequence, one in frame stop codon is located 15 nucleotides upstream of the AUG. The presence of an in-frame stop codon 5' to the initiation codon, perhaps, explains our inability to isolate full-length clones encoding DmA24, as the two hybrid interaction requires a productive fusion between Gal4AD and the cDNA. The 3' untranslated region terminates in an oligo(A) stretch, suggesting that the cDNA is full length with regard to its 3' untranslated region. However, because the potential polyA-addition signal, AATAAA, is not spaced at the expected distance with respect to the poly A-tail (see Fig. 1), it is unclear whether this is the preferred signal *in vivo*.

Two hybrid interaction of DmA24 with DmCK2 α

The two hybrid cDNA contained in clone DmA24 was tested for bait specificity via transformation of HF7C expressing Gal4DB-DmCK2 α or Gal4BD-DmCK2 β , and testing for induction of *HIS3* expression. As shown in Fig. 2A, DmA24 interacts with Dm α , but not with Dm β . The inability of the β subunit to interact with DmA24 is not attributable to a lack of expression, instability, or toxicity of Gal4DB-Dm β fusion protein in yeast as this very construct exhibits a robust interaction with Dm α [26]. Although not explicitly tested for, the inability of DmA24 to induce *HIS3* expression in combina-

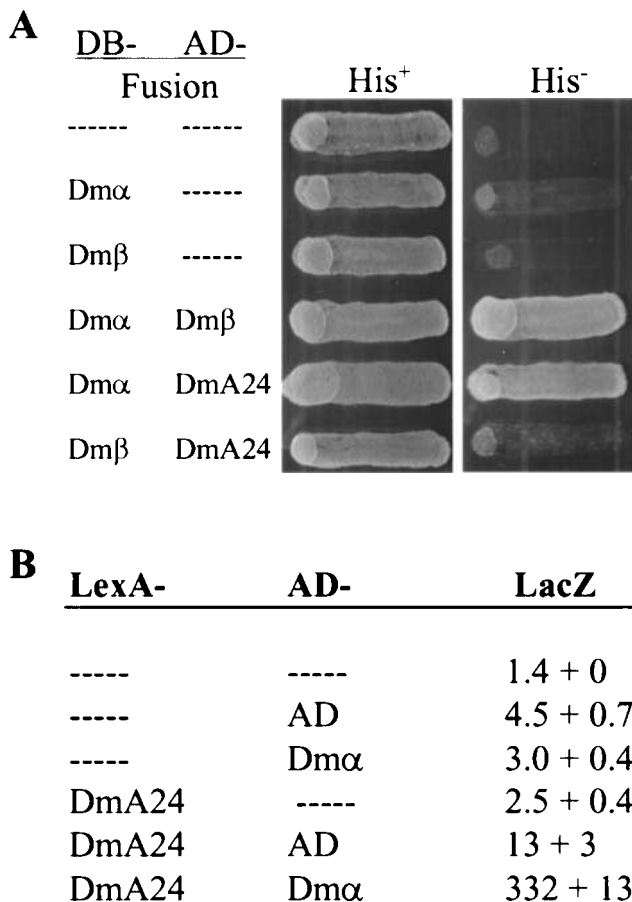


Fig. 2. Two hybrid interactions of DmA24 with DmCK2 subunits. (A) *S. cerevisiae* strain HF7C was transformed with plasmids expressing the indicated fusions with GAL4DB or GAL4AD. A dashed line indicates the absence of a plasmid. Following growth in dropout medium, cells were plated onto complete (His⁺) or minimal media lacking histidine (His⁻) and incubated at 29°C for 3–4 days. (B) Yeast EGY048 was transformed with plasmids expressing the LexA-DmA24 in combination with either the AD alone or the AD-DmCK2 α fusion protein. Transformants were grown in galactose media, and *LacZ* activity was determined in triplicate as described [29], and is expressed in Miller Units.

however, be noted that a low level of sequence homology was found with the non-histone chromosomal protein Prod, which is associated with centromeric regions of *Drosophila* chromosomes, although the level of sequence conservation is, at best, modest (data not shown). The predicted DmA24p sequence contains no signal sequences suggesting that it is neither secreted nor sequestered into cellular organelles. However, a search for motifs indicate the presence of a bipartite nuclear localization motif, and 2 consensus sites for phosphorylation by CK2, WS²³⁵DDE and FT²⁸³DEEE (Fig. 3A), both of which satisfy the requirement for an Asp/Glu at the n+1 and n+3 positions [35, 36].

We were also interested in determining whether the interaction of DmA24 with DmCK2 elicits phosphorylation of this

protein. Phosphorylations were conducted using two isoforms of CK2, i.e. the monomeric α subunit purified from a yeast expression system [9], and the holoenzyme purified from embryos [37]. The former isoform is relevant to our two hybrid analysis (see Fig. 2), whereas the latter isoform mimics the environment likely to be encountered *in vivo*. The results demonstrate that GST-DmA24 is phosphorylated by the embryo-holoenzyme, but very weakly, or not at all, by the monomeric α subunit (Fig. 3B, compare lanes 1 and 2). We have also tested for the effects of poly(DL)lysine, and find that this polybasic activator stimulates α subunit mediate phosphorylation of DmA24 modestly, whereas it dramatically stimulates phosphorylation by the embryo holoenzyme (data not shown). We have deferred from conducting parallel analysis on the phosphorylation of GST, because we have previously demonstrated that this affinity tag is not phosphorylated by either isoform of CK2 in the absence or the presence of polybasic activators [24]. These results suggest that insofar as phosphorylation of DmA24 is concerned, the β subunit plays a positive role. Comparative kinetic analysis with the two isoforms will be needed to address whether the β subunit enhances phosphorylation by modulating the k_m and/or the V_{max} . However, our observation that β alone does not exhibit a two hybrid interaction with DmA24, taken together with the phosphorylation analysis, suggest that the physical interaction of CK2 α with DmA24 may be greatly enhanced in the presence of CK2 β , i.e. in the context of the holoenzyme. However, such analysis is currently precluded by the unavailability of a yeast strain that produces DmCK2 holoenzyme suitable for two hybrid analysis. That DmA24p interacts with CK2 and is phosphorylated as well is in line with similar analysis from a number of laboratories demonstrating that this enzyme exists in a complex *in vivo* with many of its substrates such as, topoisomerase II [38–40], HSP90 [41], ANTP [22], m5/7/8 [24], etc.

As mentioned above, the full length DmA24 sequence did not exhibit significant homology to other proteins. During this analysis, we discovered that the C-terminal 50 amino acids of the DmA24 polypeptide exhibit an inordinate level of conservation with the C-terminal regions of proteins encoded by the CG13896, CG13895, and CG13894 genes. We have, therefore, aligned this region in order to emphasize the overall level of sequence similarity (Fig. 3C). This region does not exhibit similarity to any other proteins, thus precluding any predictions of its potential functions. That DmA24 forms a direct complex with DmCK2 α , and is phosphorylated by the embryo holoenzyme strongly suggest that this protein represents a new physiological partner and substrate of CK2. Although the functions of the corresponding gene are unclear at present, the recently initiated effort to isolate systematic mutations in all of the genes will clarify the phenotypes associated with loss of DmA24, and should facilitate analysis of the role of its phosphorylation by DmCK2.

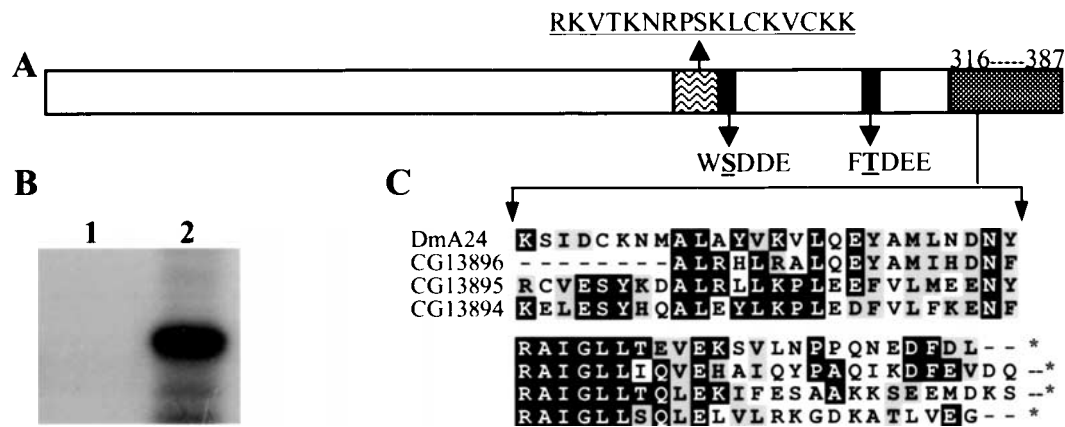


Fig. 3. Functional motifs in DmA24, and phosphorylation by CK2. (A) The motifs contained in DmA24 are a nuclear localization signal (cross-hatched box), two sites for phosphorylation by CK2 (black box), and the shaded box corresponds to sequences exhibiting a high level of conservation to the C-termini of proteins encoded by three other genes, CG13896, CG13895, and CG13894. (B) GST-DmA24 was subjected to phosphorylation using the monomeric α subunit (lane 1) or the embryo holoenzyme (lane 2). (C) Sequences encompassing the penultimate 50 amino acids of DmA24 were aligned to the corresponding regions of the indicated proteins. Identical residues = white letters on a black background; shaded letters = conservative substitutions; *C-terminal end.

Summary of second round of two hybrid screens

As mentioned above, our preliminary two hybrid screens identified a limited repertoire of proteins. We have, therefore, rescreened the embryonic two hybrid cDNA library at greater saturation. We have screened 40×10^6 independent transformants, and have isolated approximately 250 *His⁺/LacZ⁺* clones. From this pool, we have so far analyzed 75 clones that activate transcription of *HIS3* and *LacZ* at varying levels in a bait-specific manner. Because β/β' cDNAs are isolated at a relatively high frequency when Dm α is used as a bait [26,

33], we culled the available pool for clones encoding β/β' via PCR using cDNA-specific primers. The remaining clones were sequenced in order to identify the encoded protein (see Table 1).

As expected, we have identified the α and β/β' subunits of DmCK2, as well as L22 and E(spl)m7. In addition, the second screen yielded the ribosomal protein L7, which does not contain consensus sites for phosphorylation by CK2. Three proteins with predicted signal transduction functions are CG15784, CG8159, and CG1603, and all three proteins contain CK2 sites. The most noteworthy of these are the

Table 1. Proteins that interact with the catalytic subunit of DmCK2

Protein ^a	Function	CK2 site ^b
DmCK2 β	CK2 subunit	M ¹ SSSEE
DmCK2 β'	CK2 subunit	M ¹ TDSDE
DmCK2 α	CK2 subunit	None
L22	Ribosomal protein	SSNDDEDDDAE*
L7	Ribosomal protein	None
E(spl)m7	Repressor of neurogenesis	S ¹⁵⁴ DCD
CG15784	Unknown	T133SSSSSSSSSSSSSSSSSSSSSEDEE S376SSSSSSSSSSSSSSSSSSSSSDGE
CG8159	Putative transcription factor	S ¹⁵⁴ SSTEDD
CG1603	Putative transcription factor	T ¹⁵⁰ DDDD
Surf6	RNA processing in nucleolus	EDEET ⁵⁹ YEE DS ⁸⁷ DDVEDE
Spalt	Homeotic	E ²⁷⁴ EDTEEDADQEQQEQFIDTYEEEE

^aProtein names beginning with the prefix CG refer to expressed genes whose functions are presently unknown. The names used have been assigned by Berkeley Drosophila Genome Project. ^bThe CK2 consensus motif is shown with a superscript indicating the position of the site relative to the full length protein. *C-terminus.

T¹³³S₂₂EDEE and S³⁷⁶S₂₁DGE sites in CG15784, a protein predicted to contain coiled-coils and three nuclear localization signals. Experiments to address phosphorylation of this protein are currently underway. It should be noted that if this is in fact the case, the former site could theoretically account for phosphorylation stoichiometry of ≥ 25 on a mole/mole basis. Two proteins identified in this screen that were not previously known to interact with CK2 are Surf6 and Spalt. Although the function of the former protein is not known in *Drosophila*, its human homolog is localized to the nucleolus, and is involved in RNA processing [42]. Spalt is an evolutionarily conserved protein which provides homeotic gene function in the head and tail region of the *Drosophila* embryo [43]. Like the case with ANTP, Spalt contains 3 consensus sites for CK2 in the vicinity of its homeobox domain, suggesting that the activity of this protein may also be restricted to regions of the embryo via phosphorylation. Future studies aimed at biochemical and molecular genetic analysis of phosphorylation of Surf6 may provide clues to the role of this enzyme in regulating nucleolar functions, whereas analysis with E(spl)m7 and Spalt should clarify the role of this enzyme in a developmental context.

Interestingly, most of the proteins identified contain CK2 sites and are predicted to be localized to the nucleus or the nucleolus. This not surprising given that CK2 functions extensively in both of these cellular locales [44]. Given our results with the E(spl) family [24], wherein ability to be phosphorylated is the basis for interaction with CK2, one would predict that the proteins we have isolated may in part be due to the presence of sites for modification by CK2. However, none of the potentially best substrates for DmCK2 that were predicted from a genome-wide analysis in *Drosophila* [45], have so far been identified in our two hybrid screens. A notable example is *Drosophila* D1 [46], which is an excellent substrate for DmCK2 *in vitro* and *in vivo*, but does not exhibit a two hybrid interaction with either subunit (Bidwai, unpublished). In addition, results with yeast Fpr3 [5, 47] suggest that secondary structure constraints do influence interaction and/or phosphorylation, and suggest that the mechanism of interaction of this enzyme with these potential substrates may be distinct in each case. Surprisingly, our two hybrid screens appear to be lacking for many highly conserved proteins that have been identified in similar screens with the human enzyme [48, 49]. One possible reason underlying the isolation of distinct proteins in our screens could be the utilization of embryonic libraries, which might be underrepresented for transcripts encoding proteins that are maternally deposited, or represent genes that do not become transcriptionally active until, perhaps, the larval, pupal, or adult stages. Future screens utilizing libraries spanning a wider developmental time-scale will, therefore, be needed to identify those genes that are stage specific, and whose products interact with CK2.

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