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A third functional isoform enriched in mushroom body neurons is encoded by the Drosophila *14-3-3ζ* gene

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ABSTRACT

14-3-3 Proteins are highly conserved across eukaryotes, typically encoded by multiple genes in most species. Drosophila has only two such genes, *14-3-3ζ* (*leo*), encoding two isoforms LEOI and LEOII, and 14-3-3ε. We report a *bona fide* third functional isoform encoded by *leo* divergent from the other two in structurally and functionally significant areas, thus increasing 14-3-3 diversity in Drosophila. Furthermore, we used a novel approach of spatially restricted *leo* abrogation by RNA-interference and revealed differential LEO distribution in adult heads, with LEOIII enrichment in neurons essential for learning and memory in Drosophila.

1. Introduction

The 14-3-3 proteins comprise a family of small acidic molecules with apparently diverse cellular functions [1,2]. Each 14-3-3 protein consists of nine anti-parallel α-helices arranged in a characteristic U shape [1,3]. The four amino-terminal helixes are essential for dimerization [4] and 14-3-3s form homo and heterodimers [5]. Multiple family members are present in all eukaryotes examined [6,7], but the number of 14-3-3 isotypes differs per species. Animal family members are divided into two conservation groups based on protein sequence similarity. The typical group contains the β,γ,η, ζ and α, δ which are the phosphorylated forms of β and ζ respectively. Members of the typical group share 75–92% sequence identity, while ε, σ and τ of the atypical group are more dissimilar with the typical isotypes, yet 45–63% identical among them [8]. With the exception of σ which is epithelial cell-specific, all vertebrate isotypes are primarily expressed in neurons [8,9].

Unlike the seven distinct 14-3-3 genes in mammals, Drosophila contains two 14-3-3 genes, one from each conservation group [8,10]. The *D14-3-3ε* gene encodes a single protein, whereas, the *14-3-3ζ*, or *leonardo* was reported to encode two isoforms LEOI and LEOII arising by incorporating one of the mutually exclusive exons 6 and 6′ in the mRNA [10–12]. However, the recently annotated Drosophila genome predicted the existence of a third possible exon 6 (http://flybase.org/reports/FBgn0004907.html), potentially encoding a third, divergent LEO isoform. Because tissue-specific functional divergence has been reported for LEOI and LEOII [11], we tested the prediction of a third functional LEO isoform by expression analysis, genetic complementation tests and tissue specificity in its distribution.

2. Materials and methods

2.1. Drosophila culture, strains and genetics

Drosophila were cultured as before [11]. The UAS-leoI, and UAS-leoII transgenes have been described previously [11]. cDNAs for *UAS-leoIIIFlag* were obtained from Levitan et al. [13], subcloned into pUAST, transformants were generated and normalized to the Cantonised w^{1188} genetic background [11]. Transgenes on the third chromosome were used for ease of manipulations and introduced in the lethal leo^{p1188}/CyO or leo^{P2335}/CyO mutant backgrounds [10]. Two novel insertions in *leo* carrying the Gal4-encoding P{GawB} were characterized and obtained from the Japanese National Institute of Genetics. The homozygous viable leo^{NP0863} strain (*leoGal4*) and leo^{NP7346} which is homozygous lethal, neighboring previously described [14] lethal insertions (Fig. 1A). The *leoRNAi* transgenic line was obtained from the Vienna Drosophila Resource Centre and was normalized to Cantonised w^{1188}. *ElavGal4* has been described previously [15], whereas *ElavGal4;* leo^{P1188}/CyO was

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generated by standard crosses. To access rescue of the leo^{P1188} and leo^{P2335} homozygous lethality, leo^{P1188}/CyO and leo^{P2335}/CyO transgene-bearing males (i.e. leo^{P1188}/CyO; *LEOI*, etc.), were crossed *en masse* with *ElavGal4;* leo^{P1188}/CyO virgin females. Control crosses were w^{1118} virgin females crossed to the transgene-carrying strain (leo^{P1188}/CyO; *leoI*). The proportion of straight-winged progeny over the total was estimated for each cross.

2.2. Sequence alignments and analysis

leo genomic sequence was obtained from Flybase (http://www.flybase.org) and GeneDoc (http://www.nrbsc.org/gfx/genedoc/index.html) was used for protein sequence alignment. The following FLYBASE identification numbers are assigned to the distinct transcripts:

Transcript type	FLYBASE transcript	FLYBASE ID#	NCBI RefSeq
leo I	14-3-3zeta-RD	FBtr0088414	NM_057537
	14-3-3zeta-RE	FBtr0088415	NM_ 165740
	14-3-3zeta-RJ	FBtr0100183	NM_001014515
leo II	14-3-3zeta-RA	FBtr0088413	NM_165741
	14-3-3zeta-RB	FBtr0088412	NM_165742
	14-3-3zeta-RG	FBtr0088417	NM_165745
	14-3-3zeta-RH	FBtr0088418	NM_206070
	14-3-3zeta-RI	FBtr0100182	NM_0010114516
leo III	14-3-3zeta-RC	FBtr0088419	NM_165743
	14-3-3zeta-RF	FBtr0088416	NM_165744

2.3. Western blotting

Four heads were homogenized in 40 μl of lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, containing protease and phosphatase inhibitors-Sigma). Sample equivalence in total protein was confirmed using the Quant-iT protein assay (Molecular Probes). Laemmli buffer was added, samples were boiled for 5 min at 92 °C, centrifuged for 5 min at 14,000×*g*, proteins were separated by SDS gel electrophoresis, transferred on PVDF membrane and probed with rabbit anti-LEO pAb [14] at 1:20,000 and anti-syntaxin mAb (8C3, DSHB) at 1:5000, followed by appropriate secondary HRP-conjugate Ab (1:5000; Jackson ImmunoResearch) and visualized by chemiluminescence.

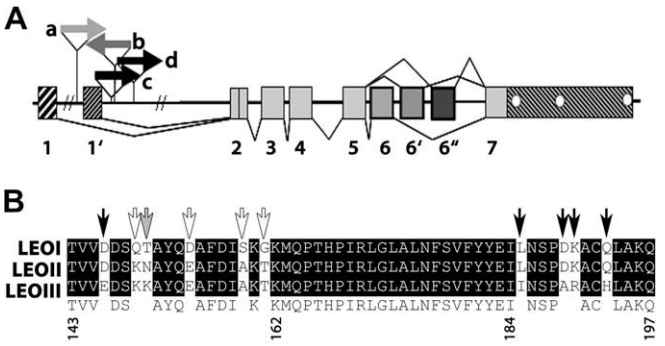
2.4. Reverse transcription, qualitative and real-time PCR

RNA was prepared and qualitative PCR performed as before [10], with typical annealing temperature 57 °C. Quantitative PCR Reactions were performed using the MiniOpticon System (Biorad), with Platinum SYBR Green qPCR Supermix UDG (Invitrogen). Transcripts of each *leo* isoform were amplified with the following primer sets:

	Forward	Reverse
leo I	LeoCOM-177F	LeoI- 465R
leo II	LeoII- 461F	LeoII- 565R
leo III	LeoCOM-177F	LeoIII-553R

LeoCOM-177F: GTCATCGTGGCGTGTCATC, LeoI-465R: CCCTTGCTAATGTCAAAT, LeoII-461F: AGGCGTTCGATATTGCAAAAACC, LeoII-565R: AGCCAATTGGCAAGCTTTGTC and LeoIII-553R: CCTCGCTGGTGAATTGAT.

act5C mRNA was amplified as internal standard. For each sample, *leo* and *act5C* were assayed in triplicate in separate wells and monitored with the MJ Opticon Monitor Analysis software (v3.1) and data quantified with the method described by Pfaffl and Hageleit [16]. Results presented are the average of three runs from two independent reverse transcription reactions. Student's *t*-tests were used to probe the significance of the differences between RNAi-expressing strains and their respective controls. Multiple comparisons using Tukey–Kramer HSD tests were utilized when appropriate as indicated.



C

PROTEIN SEQUENCE IDENTITY (%)				
	H14-3-3			
D14-3-3	14-3-3ζ	14-3-3β	14-3-3γ	14-3-3ε
LeoI	**80**	**80**	69	65
LeoII	**78**	**78**	69	69
LeoIII	**72**	**74**	**74**	69
D14-3-3ε	61	63	58	**92**

Fig. 1. Alternative isoforms are encoded by the *leo* gene. (A) *leo* exons are represented with boxes and introns with lines. The splicing pattern is indicated by lines connecting the exons and the mutually exclusive exons 6, 6 and 6 are shaded grey. The alternative 5 untranslated exons are indicated by hatched patterns, as is the untranslated portion of exon 7 bearing the three polyadenylation sites (open circles). The relative locations of transposons flanking exon 1 is shown by the arrows, which indicate transcription of the bacterial *lacZ* reporter gene in (c) P2335 and (d) P1188 (black arrows) and the yeast *gal4* transcription factor in (a) NP0863 (light grey) and (b) NP7346 (darker grey). (B) An alignment of the 54 predicted amino acids encoded by the alternative exons differentiating LEOI, LEOII and LEOIII. Position relative to the entire protein is denoted below the consensus. Conserved residues are shaded in black and include the invariant sequence of amino acids 162-184. Black arrows denote LEOIII-specific differences, open arrows LEOI-specific differences, while the gray arrow denotes a highly variable position. (C) The identity of the four Drosophila 14-3-3 isoforms within exon 6 with their closest human homologs.

2.5. Immunocytochemistry in whole mount brains

Brains were dissected and fixed for 20 min in 4% PFA in PBS. Blocking was at room temperature for 1 h in 10% Normal Goat Serum in PBHT (0.02 M PO_4, 0.5 M NaCl, 0.2% Triton X-100, pH 7.4). Anti-LEO pAb [14] was used (1:2000) over night at 4 °C. After washing, anti-rabbit IgG Alexa Fluor-conjugated (Molecular Probes) secondary antibody was applied at 1:500 for minimally 4 h at room temperature, several washes were performed and samples were mounted in fluorescent mounting medium (Dako). Confocal images were obtained with a Biorad Radiance 2100 system.

3. Results and discussion

The structure of *leo* transcripts and putative splicing pattern according to Flybase's annotated genomic sequence is shown in

Fig. 1A. This predicts a novel LEO isoform incorporating the novel exon 6. The sixth exon encodes helices F and G in their entirety and about half of helix H. The amino acid differences within the alternative exons are summarized in Fig. 1B. There are two clusters of distinct amino acids flanking the invariant sequence (Supplemental Fig. 1), characteristic of helix G [4]. Differences at the amino-terminal side are confined within helix F and mostly distinguish LEOI from LEOII [10]. The LEOII-specific amino-acids are also shared by LEOIII, except for the conservative change Asp^{146} to Glu, specific for LEOIII. A difference between LEOII and LEOIII is at position 150, which is distinct in all three isoforms and in LEOIII adds a second basic Lys in a row. However, LEOIII is most distinct from the other two isoforms at the carboxy-terminal side of the conserved block, where LEOI and LEOII do not differ. This region forms the loop between helices G and H and thus is likely to affect their spatial arrangement. Following the conservative Ile in place of the typical Leu^{183}, there is a drastic change from acidic Asp^{187} to Ala, a conservative Lys^{188} to Arg and a $Glut^{191}$ to His in LEOIII (Fig. 1B). Thus, LEOIII differs by 10 amino acids from LEOI and 6 from LEOII. Because they are likely involved in ligand binding [5], these differences are potentially functionally significant. In fact, sequence alignment of the alternative exons of *leo* with the human 14-3-3s along their respective exon indicates LEOIII slightly (2%) more similar with the β and γ proteins rather than ζ (Fig. 1C).

To verify that exon 6″ is indeed incorporated in *leo* transcripts, we used an exon-specific reverse primer and a forward primer within common exon 4, for oligo dT-initiated Reverse Transcription coupled to the Polymerase Chain Reaction (RT–PCR) as described previously [10]. In addition, to determine whether the putative *leolll* RNA is expressed tissue-specifically, we used RNA from different developmental stages and distinct adult tissues. *Bona fide* transcripts incorporating exon 6″ were found in all Drosophila life stages and tissues examined, as for *leol* and *leoll* (Fig. 2A). In agreement with this conclusion, we identified a *leolll* cDNA clone (out of 14 fully sequenced) in an adult head library (data not shown). Furthermore, we obtained an independently isolated *leo* cDNA clone [13] and upon sequencing we discovered it represented the *leolll* transcript. Collectively, these data strongly indicate that the Drosophila *leo* gene encodes three distinct protein isoforms.

To address the question of whether LEOIII is a functional protein, we investigated the ability of *UAS-leolll* transgenes to reverse the homozygous lethality associated with previously reported [10,14], transposon mutations in the gene. We introduced the *UAS-leolll*, UAS-leol and *UAS-leoll* transgenes in leo^{P1188}/+ and leo^{P2335}/+ heterozygotes and determined the level of mutant homozygotes recovered upon their pan-neuronal expression as described previously [11]. All crosses were performed in triplicate, the data were pooled and displayed in Fig. 2B. These results demonstrate that pan-neuronal expression of *UAS-leoIII* resulted in recovery of significantly more leo^{P1188} homozygotes and leo^{P1188}/leo^{P2335} heteroallelics than obtained from control crosses and UAS-leoI *I* was also effective in rescuing leo^{P1188} homozygotes (Fig. 2B). Similar results were obtained with independent transgenes (not shown). To determine whether this difference resulted from position-effect mediated variation in transgene expression, we determined the relative transgene-specific RNA levels using Quantitative RT–PCR (Q-RT–PCR). The results in Fig. 2C demonstrate that although the relative levels of UAS-*leoI* and UAS-*leoII* transcripts were similar, UAS-*leoIII* transcripts were actually less abundant. Therefore, enhanced *UAS-leoIII* transgene expression was probably not the reason for increased rescue from lethality and unless LEOIII is more stable than the other isoforms, the difference likely underlies functional differences. Nonetheless, these results indicate that *leoIII* transcripts encode a functional LEO III isoform.

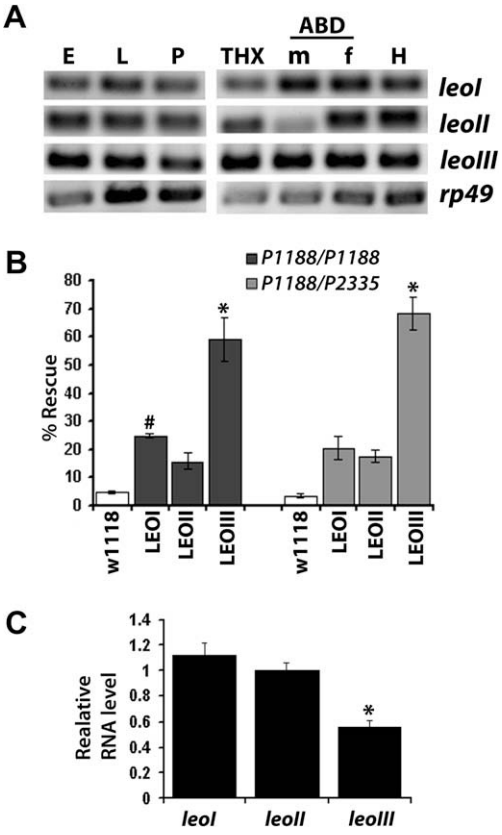


Fig. 2. The ubiquitous LEOIII RNA encodes a functional isoform. (A) The distribution of three predicted *leo* mRNAs was investigated with qualitative RT–PCR. The tissues sampled were: mid-development embryos (E), second instar larvae (L), mid-pupation pupae (P) and from adult tissues, pooled thoraces (THX) of both sexes, male (m) and female (f) abdomens (ABD) and pooled heads from both sexes (H). The *rp49* transcript was co-amplified with the *leo* mRNAs as a measure of reaction success. (B) Rescue of the lethality of leo^{P1188} homozygotes (dark grey bars) and heteroallelics with leo^{P2335} (light gray bars), by single isoforms-expressing transgenes expressed pan-neuronally. Rescue reflects the number of non-CyO progeny, denoting leo^{P1188} homozygotes or leo^{P1188}/leo^{P2335} heteroallelics recovered as a percent of expected if the homozygotes (or heteroallelics) were fully viable. Controls were derived by crossing with w^{1118}. Asterisks indicate highly significant differences ($P < 0.001$ LSM contrast analysis) from controls (open bars), whereas # indicate differences at $P < 0.005$. (C) Quantification of the expression levels of the isoform-specific transgenes in leo^{P1188} homozygotes relative to that of the endogenous actin5C. Identical primers for RT and PCR were used such that transcript levels may be comparable. The mean ± its standard error (S.E.M.) is shown for three independent experiments consisting of a triplicate determination for each ratio. The level of *LEOIII*-specific transcript was significantly different (Tukey–Kramer $\alpha = 0.01$, denoted by the asterisk) from the others.

Are there differences in the tissue distribution of the three LEO isoforms? The anti-LEO antibody recognizes all three isoforms as expected (Supplemental Fig. 2), given that it is polyclonal, raised against the entire protein [14] and the differences among the isoforms are small and non-contiguous. Therefore, we capitalized on the availability of Gal4 drivers and transgenes able to abrogate LEO levels by RNA-interference (RNAi). We drove such transgenes with different tissue-specific Gal4 drivers and assessed the consequences on the levels of each *leo* transcript using Q-RT–PCR. We validated the assay using adult flies null for *leo*. These animals can be obtained as heteroallelics of leo^{P1188} with a novel lethal insertion, leo^{NP7346} (Fig. 1A). Although leo^{NP7346}, as leo^{P1188} homozygotes are fully lethal, such heteroallelic animals can be obtained, albeit relatively rarely, comprising 12–18% of the progeny of such a cross. In agreement with prior results [10], these animals do not exhibit external defects or grossly aberrant behaviors and appear devoid of LEO (Fig. 3A). As expected, Q-RT–PCR of RNA from

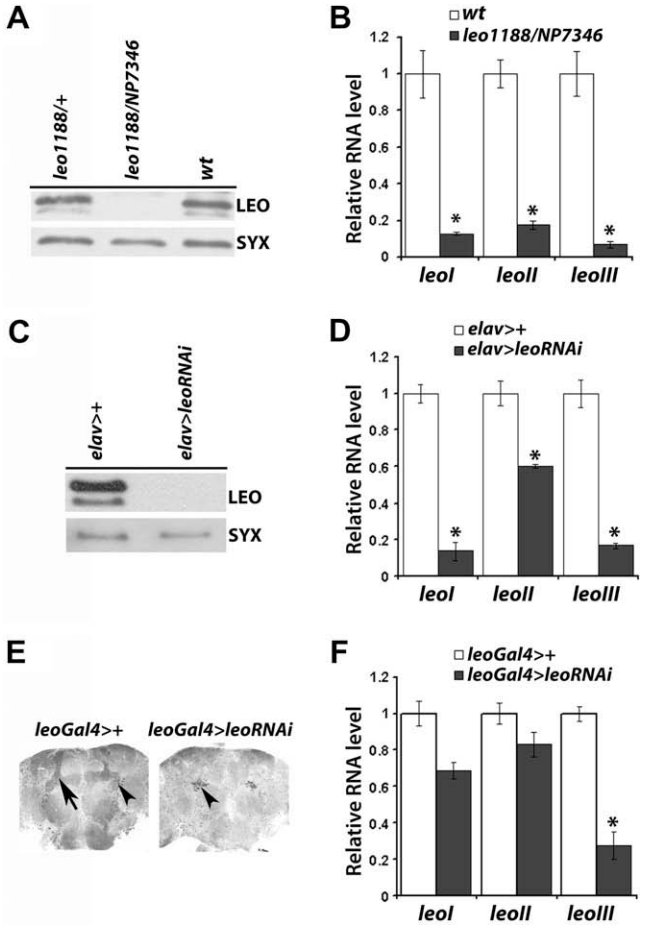


Fig. 3. Spatial distribution LEO isoforms. (A) A western blot of head lysates demonstrating that leo^{P1188}/leo^{NP7346} lack detectable LEO protein levels. Syntaxin (SYX) was used as a loading control. (B) Quantitative RT–PCR of all three LEO isoforms in adult heads of the protein null leo^{P1188}/leo^{NP7346} animals with actin5C internal reference. The leo/actin5C ratios were determined in triplicate for each experiment and three such experiments using independently isolated RNA samples were averaged and shown ± S.E.M. Paired Student's *t*-tests indicated highly significant differences ($P < 0.001$) with the respective controls. (C) A western blot of brain lysates from animals expressing *leoRNAi* under *leoGal4* demonstrating the reduction to near loss of LEO protein in that tissue. (D) Quantitative RT–PCR of RNA isolated from whole heads of animals with LEO abrogated in the nervous system. All experimental *leo* RNAs were significantly different than those in controls (*t*-tests, $P < 0.001$). (E) Whole mount confocal image stacks of anti-LEO immunohistochemistry in dissected brains of the indicated genotypes. Arrow points to the mushroom body neurons, where LEO protein distributes preferentially in control animals and the staining is absent in animals expressing the *leoRNAi* specifically in these neurons. Arrowheads point to the ellipsoid body neuron somata where staining remains in the later animals. (F) Quantitative RT–PCR of RNA isolated from heads of animals with RNAi-abrogated LEO in the mushroom bodies. RNA levels of *LEOIII* was significantly different from respective controls (*t*-tests, $P < 0.001$), whereas those of *LEOI* and *LEOII* were not ($P = 0.09$ and $P = 0.27$, respectively).

the heads of such animals indicated near absence of all three *leo* transcripts compared to the respective relative levels in controls (Fig. 3B).

14-3-3s are largely neuronal proteins, but also present in other tissues [8,17] and LEO is also distributed widely [10]. Therefore, to determine whether LEO isoforms may be distributed differentially in Drosophila, we used RNAi-mediated abrogation to eliminate them specifically within the adult nervous system using the pan-neuronal *ElavGal4* driver. Flies lacking LEO in the nervous system were viable and did not exhibit obvious external defects. Western blots from single dissected brains of such adults confirmed the lack of LEO in the nervous system (Fig. 3C). Samples for Q-RT–PCR were prepared from entire heads (brain, head fat and muscles, cuticle and external compound eye), to determine whether any of the LEO isoforms was present outside the nervous system. The signals for *leoI* and *leoIII* transcripts were highly diminished and near zero in heads from such adults. However, although reduced compared to controls, *leoII*-specific signal remained relatively elevated (Fig. 3D). This is in contrast to the near elimination of the *leoII*-specific signal in the genetic nulls (Fig. 3B) and suggests that of the three isoforms, LEOII is also present outside the nervous system in adult heads.

LEO is preferentially distributed in the mushroom bodies [14], neurons with critical roles in higher order behaviors including learning and memory [18]. Therefore, to determine whether a particular LEO isoform is preferentially present within the mushroom bodies, we expressed the *leoRNAi* transgene with *leoGal4* (see Section 2), a novel insertion in the gene (Fig. 1A), which expresses Gal4 nearly exclusively within these neurons (Supplemental Fig. 2). Fig. 3E demonstrates lack of LEO within the mushroom bodies upon *leoRNAi* expression with *leoGal4*. However, it remained unaltered in ellipsoid body neurons where LEO is also abundant [10,14] and in the remainder of the brain, attesting to the specificity of the driver. Q-RT–PCR of RNAs from the heads of such animals indicated a prominent reduction for *leoIII*, a smaller non-significant reduction for *leoI*, whereas *leoII* did not change appreciably (Fig. 3D). These results indicate that LEOIII is preferentially distributed in mushroom body neurons. LEOI and LEOII may also be marginally present within these neurons. These results contrast our previous report [10] indicating that LEOII was a mushroom body-specific isoform. Upon closer examination, the leo*II*-"specific" primer used in that study could also hybridize to *leoIII* transcripts, because of the very low sequence divergence at the 5 of exons 6 and 6 where the primer annealed. We posit then, that in these experiments the mushroom body-preferential *leoIII* was abrogated by ablating these neurons and it was reported erroneously as *leoII* because of the sequence overlap and lack of information about *leoIII*.

Given the functional specialization of the mushroom bodies, preferential distribution of LEOIII therein may also reflect functional differences among the isoforms in these neurons consistent with the sequence divergence within alternate sixth exons. The preferential distribution of LEO isoforms at least in the adult Drosophila head, indicates that alternative splicing of the distinct sixth exons is not stochastic, but rather regulated, an event that may even occur in other tissues and life stages.

Interestingly, vertebrate and especially mammalian species appear to employ multiple 14-3-3 proteins despite their unusually high sequence identity, perhaps because small changes in relevant amino acids result in significant functional consequences. In contrast, the more compact Drosophila genome seems to have coped with encoding this structurally small, but apparently functionally significant diversity by alternative splicing of a single transcript, rather than multiple distinct 14-3-3 encoding genes. This appears to have arisen by simple exon duplication and divergence, whereas in the case of mammals entire gene(s) were likely duplicated and diverged.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.08.003.

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