Phosphorylation status of the Kep1 protein alters its affinity for its protein binding partner alternative splicing factor ASF/SF2

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Mutations in the *Drosophila* *kep1* gene, encoding a single maxi KH (K homology) domain-containing RNA-binding protein, result in a reduction of fertility in part due to the disruption of the apoptotic programme during oogenesis. This disruption is concomitant with the appearance of an alternatively spliced mRNA isoform encoding the inactive caspase *dredd*. We generated a Kep1 antibody and have found that the Kep1 protein is present in the nuclei of both the follicle and nurse cells during all stages of *Drosophila* oogenesis. We have shown that the Kep1 protein is phosphorylated in ovaries induced to undergo apoptosis following treatment with the topoisomerase I inhibitor camptothecin. We have also found that the Kep1 protein interacts specifically with the SR (serine/arginine-rich) protein family member ASF/SF2 (alternative splicing factor/splicing factor 2). This interaction is independent of the ability of Kep1 to bind RNA, but is dependent on the phosphorylation of the Kep1 protein, with the

interaction between Kep1 and ASF/SF2 increasing in the presence of activated Src. Using a CD44v5 alternative splicing reporter construct, we observed 99% inclusion of the alternatively spliced exon 5 following *kep1* transfection in a cell line that constitutively expresses activated Src. This modulation in splicing was not observed in the parental NIH 3T3 cell line in which we obtained 7.5% exon 5 inclusion following *kep1* transfection. Our data suggest a mechanism of action in which the *in vivo* phosphorylation status of the Kep1 protein affects its affinity towards its protein binding partners and in turn may allow for the modulation of alternative splice site selection in Kep1–ASF/SF2-dependent target genes.

Key words: alternative splicing, apoptosis, *Drosophila*, Kep1 protein, oogenesis, phosphorylation.

INTRODUCTION

The past few years have seen the completion of a number of different genomes sequenced. It has become apparent that although the sizes of the genomes vary, the number of genes present in different animal species remains relatively constant. Thus the diversity seen among different members of the animal kingdom results in part in the ability to generate protein isoforms from the same genomic loci using the process of alternative splicing. The *Drosophila* genome contains approx. 13 500 annotated genes, yet the number of polypeptide species present has been estimated to be 21 376.

Alternative splicing has been shown to play a role in apoptosis (reviewed in [1]). Examples of alternative splicing for the genes encoding the caspase Ich-1 [2], Bcl-X [3], CED-4 (cell-death determining-4) [4] and caspase 9 [5] have been documented. In all of these cases, the spliced isoforms show the opposite apoptotic functions of their parental products. Thus regulation of RNA processing may be a general mechanism of modulating the balance between active and repressor forms of proteins involved in the apoptotic pathway.

We have previously demonstrated that the Kep1 protein has a nuclear distribution [6] and that a mutation in the *kep1* gene results in the appearance of an alternatively spliced caspase-8-like *dredd* transcript in *Drosophila* ovaries [7]. This alternative isoform contains intron II resulting in the generation of a premature stop codon. Ovary extracts isolated from *kep1*^{-/-} mutants show a severe reduction in caspase-8-like activity [7].

kep1 encodes a gene product containing a single KH (K homology) domain belonging to the STAR (signal transduction and activator of RNA) family. The mammalian Sam68 protein is the prototypical member of this family. Based on the presence of both SH2 (Src homology 2) and SH3 domains in Sam68 and the loss of homopolymeric RNA-binding capacity following p59^{lyn} kinase phosphorylation [8], members of this family were postulated to directly link signal transduction and RNA metabolism [9].

In the present study we provide evidence that the Kep1 protein interacts specifically with ASF/SF2 (alternative splicing factor/splicing factor 2). This interaction is independent of the presence of RNA and the ability of Kep1 to bind RNA. However, this interaction is increased when activated Src protein kinase is present. These findings suggest a mechanistic function for the role of the Kep1 protein in splice site selection.

EXPERIMENTAL

DNA constructs

Activated Src and kinase dead Src constructs were obtained from Upstate Biotechnology (Charlottesville, VA, U.S.A.). The CD44v5 splicing reporter construct (pETv5) was a gift from H. König (Institut für Toxikologie und Genetik, Karlsruhe, Germany) [10]. PCR was used to construct the GST (glutathione S-transferase)–Kep1 expression vector. Oligonucleotides were designed to introduce the PCR product in frame with GST in the EcoRI/HindIII sites of the pGEX KG vector [11].

Abbreviations used: ASF, alternative splicing factor; ERK, extracellular-signal-regulated kinase; GFP, green fluorescent protein; GST, glutathione S-transferase; HEK, human embryonic kidney; KH, K homology; RBP-1, RNA-binding protein 1; RT, reverse transcriptase; SF2, splicing factor 2; SH, Src homology; SR, serine/arginine-rich; SRPK, SR protein kinase; STAR, signal transduction and activator of RNA.

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Oligonucleotides for *kep1* were 5'-CGCGAATTCTGATAAA-AATGGAAACCCCAAGC-3' and 5'-CGCAAGCTTCTATACG-GATTGGGCTTATAG-3'. PCR was for 30 cycles at 94 °C for 30 s, 60 °C for 30 s and 74 °C for 2 min using Vent polymerase and the Myc-tagged *kep1* construct in pBlueScript KS as a template [6]. The *kep1* I to N construct was generated by inverse PCR using the oligonucleotides 5'-AATGCCATTAAGGGTTCGAG-3' and 5'-CTTGCCTGGGTCTCCTCC-3' using the same PCR conditions as above except that the extension was for 4 min. The PCR product was ligated and the resulting plasmid containing the mutation was digested with EcoRI/XhoI to release the insert. The insert was subsequently cloned into the Myc-pcDNA plasmid [6] using the same restriction sites.

Antibodies

Monoclonal antibodies 16H3 [12] and 103 [13] directed against the SR (serine/arginine-rich) proteins were obtained from Zymed Laboratories (South San Francisco, CA, U.S.A.). Phosphotyrosine-specific PY99 and the GFP (green fluorescent protein) B2 antibodies were obtained from Santa Cruz Biotechnology. Kep1 antibody was generated by injecting rabbits with a GST–Kep1 fusion protein (Pocano Rabbit Farm and Laboratory, Canadensis, PA, U.S.A.). The Kep1 antibody was purified on a His–Kep1 column following standard methods.

Immunoprecipitations and Western blotting

COS-7, NIH 3T3 and/or HEK-293 (human embryonic kidney 293) cells were lysed 24 h following transfection in a buffer containing 300 mM NaCl, 1% Triton X-100, 20 mM Tris (pH 7.4) and protease inhibitors. Ovaries from well-fed 2–3-day-old flies were dissected in PBS and subsequently transferred to Schneider S2 medium containing 10% (v/v) fetal calf serum. Ovaries were incubated at 25 °C for 6 h either untreated or in the presence of 10 μ M camptothecin and subsequently lysed using the same buffer as above. Immunoprecipitations and Western blotting were carried out essentially as described in [6].

Protein–protein interactions and GST pull-downs

³⁵S-labelled proteins were generated using the TnT coupled transcription/translation system (Promega) according to the manufacturer's instructions using the following cDNAs: LD40489 for ASF/SF2, LD29830 for SRp54, LD32469 for SC35, AT29232 for B52, LD46359 for 9G8, GM02602 for RPB1 and RE47308 for RBP-1 (RNA-binding protein 1)-like protein. ³⁵S-Labelled proteins were mixed with 10 μ g of either GST or the GST–Kep1 fusion protein and tumbled for 4 h at 4 °C.

Real-time RT (reverse transcriptase)–PCR

NIH 3T3 cells and Src 3T3 cells were transfected using Lipofectase (Qiagen) and a 1:3 ratio of CD44v5/myc–kep1 constructs in 60 mm dishes. RNA and proteins were isolated using TRIzol[®] reagent (Invitrogen) 24 h following transfections. Isolated RNA (0.5 μ g/reaction) was subjected to RQ DNase treatment (Promega) for 30 min at 37 °C prior to being reverse transcribed using an oligo-dT primer and Superscript II RT (Invitrogen). Then, 5% of the reverse transcription reaction was amplified using the QuantiTect SYBR Green PCR kit (Qiagen) and the following oligonucleotides: 5'-ACCTTCAGACCTTGGCATG 3'- and 5'-AGGGTAGTGGTGGGCCTAGT-3', which are specific to exon 3 of rat insulin 2, which is common to both CD44 exon 5 included and excluded transcripts, and oligonucleotides 5'-CA-GCACCAGTGCTCATGGA-3' and 5'-GCTTGTCATGTGG-

Figure 1 Characterization of the Kep1 antibody

(A) Ovaries of the appropriate genotype were dissected from 2–3-day-old flies fixed and stained with anti-Kep1 antibody. (B) Increasing amounts of ovary extracts from *w¹¹¹⁸* (lanes 1, 3, 5 and 7) or *kep1^{-/-}* (lanes 2, 4, 6 and 8), were separated on polyacrylamide gels and transferred on to a PVDF membrane prior to Western-blot analysis. The arrow indicates the position of the endogenous Kep1 protein. (C) HEK-293 cells were either mock transfected or transfected with Myc–Kep1. Cells were lysed 24 h after transfection, and extracts were subjected to Western-blot analysis. Kep1 antibody was used at a 1:500 dilution.

GGTCT-3' specific to the mouse CD44v5 exon. All quantitative PCR experiments were carried out in duplicate on two independently isolated samples using the Stratagene Mx3000P system.

RESULTS

Kep1 is present during all stages of *Drosophila* oogenesis

In order to understand the mechanism of action of the Kep1 protein, we generated a Kep1 antibody and examined its specificity using immunohistochemistry on wild-type (*w¹¹¹⁸*) and *kep1^{-/-}* mutant ovaries. We focused on ovaries for two reasons; first, we have previously observed a decrease in apoptotic activity in *kep1^{-/-}* ovary extracts as assessed by a caspase-8 cleavage assay, and secondly, eggs laid by *kep1^{-/-}* homozygous females display the presence of both ventralized egg shell and small egg phenotypes [7].

In wild-type ovaries, Kep1 is restricted to the nuclei of nurse and follicle cells at all developmental stages (Figure 1A). This localization is consistent with the egg phenotypes previously observed for *kep1^{-/-}* homozygotes. Ovaries isolated from *kep1^{-/-}* homozygotes showed no immunoreactivity in both the nurse and follicle cells (Figure 1A).

We next characterized the antibody using Western-blot analysis. Western blots carried out using *Drosophila* ovary extracts showed the loss of the Kep1 signal in the mutant sample in addition to the presence of a non-specific band in all the samples examined (Figure 1B). This cross-reacting band is specific to *Drosophila* extracts since Western-blot analysis on Kep1 transfected mammalian cells (HEK-293, COS-7 and NIH 3T3) only detect the transfected protein (Figure 1C and results not shown). Since members of the STAR family are capable of forming protein–protein complexes [6,14–16], the cross-reacting band observed in

Figure 2 Kep1 post-translational modifications

HEK-293 cells were transfected with different *kep1* constructs in the presence or absence of activated Src. (A) Cell lysates were subjected to a phosphotyrosine immunoprecipitation (I.P.) (Py99) followed by a GFP Western blot. Lanes 1–3, kinase dead Src; lanes 4–6, activated Src. (B) Cell lysates were subjected to a Kepl immunoprecipitation followed by a phosphotyrosine Western blot. TCL (total cell lysate), 5% of the cleared transfected cell lysates. IgG, mouse immunoglobulin used as a negative control for immunoprecipitations. NRS, normal rabbit serum obtained from the preimmune rabbit. Arrows indicate the position of the transfected Kepl proteins. The asterisk indicates the position of the IgG heavy chain.

the ovary extracts may be due to one of the other quaking-related proteins present in the *Drosophila* genome which have a high homology to Kepl [17] but whose epitope is masked in the native form.

Kep1 is phosphorylated in the presence of activated Src

STAR family members can be phosphorylated by a number of non-receptor tyrosine kinases including Src, Fyn and PTK6. The primary amino acid sequence of the Kepl protein shows the presence of 16 amino acids in the last 70 amino acids of the protein that are potential substrates for phosphorylation. We carried out a BLASTP analysis using the human PTK6 sequence (GenBank® accession no. NP_005966) to identify potential *Drosophila* homologues. The closest *Drosophila* proteins identified were all non-receptor tyrosine kinases closely related to the mammalian Src kinase including Src42A (Src at 42A), Src64B and Src29A as well as the *Drosophila* homologue of the mammalian Abl kinase.

We examined whether the Kepl protein is a substrate for phosphorylation employing transient transfections in HEK-293 cells. We co-transfected GFP-Kepl with either activated Src or kinase dead Src constructs. Following transfection, we carried out immunoprecipitations using the phosphotyrosine-specific antibody (Py99) followed by GFP Western blotting. Figure 2(A) shows that in the presence of kinase dead Src, we obtain basal phosphorylation of Kepl. This phosphorylation was increased when we carried out the same experiment in the presence of an activated Src construct. These results suggest that Kepl is tyrosine-phosphorylated following co-transfection that activated Src. However, we could not rule out the possibility that we are co-immunoprecipitating a phosphoprotein that interacts with Kepl. We repeated the same experiment using HEK-293 cells transfected with Myc-Kepl or GFP-Kepl and immunoprecipitated with either an anti-Myc or anti-GFP antibody followed by a phosphotyrosine Western blot. Similar to the results obtained in Figure 2(A), we detected a tyrosine-phosphorylated protein migrating at the molecular mass of Kepl (Figure 2B and results not shown). Taken together, the two experiments strongly suggest that the Kepl protein is phosphorylated following transfection with activated Src.

Figure 3 *In vivo* status of the Kepl protein

Ovaries from *w¹¹¹⁸* *Drosophila* were dissected into Schneider S2 medium and left untreated or treated with camptothecin for 6 h prior to a phosphotyrosine immunoprecipitation (IP). The arrow indicates the position of the endogenous Kepl protein. TCL, total cell lysate.

Kep1 is phosphorylated in ovaries undergoing apoptosis

Since the Kepl protein is present during all stages of ovary development (Figure 1A) and transfection of *kep1* in both mammalian and insect cells is sufficient to induce apoptosis [6], we were interested in determining what the *in vivo* phosphorylation status of the Kepl protein was in cells undergoing apoptosis. We dissected wild-type ovaries into Schneider S2 medium and incubated them in the presence or absence of the topoisomerase I inhibitor camptothecin, a potent inducer of apoptosis [18], for 6 h prior to a phosphotyrosine immunoprecipitation. Under these conditions, we were able to immunoprecipitate a greater amount of phosphorylated Kepl protein in apoptotic samples relative to our untreated control (Figure 3).

Kep1 interaction with the ASF splicing factors

The presence of an alternative isoform of the caspase-8-like gene *dredd* in *kep1^{-/-}* mutant ovaries [7] led us to investigate the mechanism of action of the Kepl protein. The closest mammalian homologue to Kepl is SLM2 [19]. Rat SLM2 was shown to interact with various proteins involved in alternative splicing, including SRp30c and YT521-B [20]. In addition, SLM2 can also influence the splicing pattern of the CD44v5, human transformer-2 β and tau minigenes in co-transfection experiments in a concentration-dependent manner [20].

Confocal microscopy of NIH 3T3 transfected cells shows the GFP-Kepl fusion protein to have a nuclear speckled distribution (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/400/bj4000091add.htm>), reminiscent of the nuclear localization of splicing factors. We next examined whether the Kepl protein co-localizes with different splicing factors using monoclonal antibodies raised against a class of splicing factors, the SR proteins. Antibody 16H3 recognizes an epitope common to the SR protein family members SRp20 (RBP-1 and RBP-1-like in *Drosophila*), SRp40, SRp55 and SRp75 (all B52 in *Drosophila*) but not ASF [12]. In contrast, antibody 103 is specific to the ASF family member [13]. Both of the above antibodies detected epitopes that co-localized with the transfected GFP-Kepl protein in NIH 3T3 and COS-7 cells (Supplementary Figure S1 and results not shown).

Using co-immunoprecipitations, we determined that the co-localization of Kepl with SR proteins was concomitant with a protein–protein interaction. We were able to co-immunoprecipitate

Figure 4 *In vitro* interaction between GST-Kepl and *Drosophila* ASF

cDNAs for different members of the SR protein family were transcribed and translated *in vitro* in the presence [35 S]methionine. Reactions were incubated with either GST or GST-Kepl and subjected to a pull-down. TCL lane is the input control for the splicing factors ASF, Srp54, Sc35 and B52 containing 10% of the rabbit reticulocyte lysate.

GFP-Kepl with ASF from NIH 3T3 cell transfected with GFP-Kepl, but none of the other members of the SR protein family (Supplementary Figure S2 at <http://www.BiochemJ.org/bj/400/bj4000091add.htm> and see Figure 5 below).

An essential step in the proper localization and functioning of the mammalian ASF protein involves its interactions with and phosphorylation of its RS (arginine/serine-rich) domain by the SRPK (SR protein kinase) [21]. The *Drosophila* ASF protein does not contain an extensive RS domain, therefore cannot be phosphorylated by SRPK, yet it still retains the ability to be properly localized to the nucleus and is functional in splicing reactions [22]. We examined whether the *Drosophila* ASF could also interact with the Kepl protein. We *in vitro* transcribed and translated the *Drosophila* ASF cDNA and subjected the labelled protein to a GST pull-down. Similar to the results obtained in the immunoprecipitation experiments, ASF was pulled-down in the presence of GST-Kepl but not GST alone (Figure 4). Sequence analysis reveals the presence of seven SR proteins, including ASF, in the *Drosophila* genome [23]. We *in vitro* transcribed and translated all seven *Drosophila* SR proteins and examined their ability to interact with GST-Kepl. Consistent with the above immunoprecipitation data, we observed no interaction between Kepl and the other SR protein family members (Figure 4 and results not shown). Therefore Kepl only interacts with the ASF/SF2 member of the SR family.

Phosphorylation of Kepl alters its affinity towards ASF

Having determined that the Kepl protein could be phosphorylated, we next asked whether the phosphorylation status of Kepl has any influence on its ability to interact with ASF/SF2. In Figure 5(A), we observed a basal interaction between the Kepl protein and ASF (lanes 3 and 9). This interaction was increased when we co-transfected the cells with an activated Src construct (lanes 6 and 12).

Since both ASF and Kepl are RNA-binding proteins, we next determined whether the interactions observed above were a result of a protein complex formation or were the result of the proteins being tethered to the same RNA and thus the immunoprecipitation and GST pull-down results arise through an RNA bridge. We utilized two different approaches in order to determine whether the protein interactions observed are independent of the presence of RNA. We introduced into Kepl the equivalent KH domain

Ile367Asn (I to N) substitution found in a fragile-X patient, which results in a more severe phenotype than the complete loss of the fragile-X gene itself [24]. We tested the RNA-binding ability of the mutant Kepl protein and found that it no longer binds homopolymeric RNA *in vitro* (results not shown). Repeating the above experiments with the Kepl protein containing the I to N mutation and the addition of RNase A to wild-type or mutant protein extracts prior to immunoprecipitations did not affect the ASF-Kepl protein interaction (Figure 5B). We observed basal interactions between Kepl and Kepl I to N with ASF/SF2 (lanes 3 and 9), and these interactions were increased in the presence of activated Src (lanes 6 and 12). Therefore the Kepl-ASF interaction is independent of the presence of RNA or of the ability of Kepl to bind RNA.

Phosphorylation status of Kepl affects the splicing pattern of a reporter construct

We next wished to determine if changes in the phosphorylation status of the Kepl protein would be sufficient to modulate the alternative splicing of a reporter construct. We employed the CD44v5 reporter construct [10] that has been previously demonstrated to be regulated by two members of the single maxi KH domain-containing family, Slm2 and Sam68 [20,25]. We transfected the CD44v5 construct, in which exon 5 is alternatively spliced, either alone or with *myc-kepl* in different cell lines and examined the levels of the different alternatively spliced constructs utilizing real-time RT-PCR. Transfection of CD44v5 in NIH 3T3 cells resulted in 15% of the generated transcripts having exon 5 included (Figure 6A). The level of exon 5 inclusion decreased to 7.5% following co-transfection of the reporter construct with *myc-kepl* (Figure 6A). Western-blot analysis of the protein extracts from the above experiments showed the presence of the transfected Myc-Kepl protein (Figure 6B). We next repeated the above experiments in a NIH 3T3 cell line that constitutively expresses the v-Src kinase [26]. Under these conditions, we observed 13.5% exon 5 inclusion for the CD44v5 reporter construct co-transfected with an empty expression plasmid. In contrast, we obtained almost complete exon 5 inclusion when we co-transfected the reporter construct with *myc-kepl* (Figure 6C).

DISCUSSION

We have previously demonstrated that the *Drosophila kepl* gene plays a role during oogenesis with a null mutation resulting in reduced caspase-8-like activity in ovary extracts. Kepl transfection into mammalian and *Drosophila* cells is sufficient to induce apoptosis without the presence of any additional signals [6].

Utilizing a Kepl antibody, we have now shown that the Kepl protein is present in both the follicle cells and nurse cells during all stages of *Drosophila* oogenesis. Although the Kepl protein is ubiquitously expressed, both ovary cell populations survive and develop normally pointing to the presence of an inhibitory mechanism in place to protect the cells against premature apoptosis due to the presence of Kepl. We have examined the phosphorylation status of the Kepl protein in ovaries. In our untreated samples, we observe basal amounts of phospho-Kepl. In contrast, ovaries that were induced to undergo apoptosis displayed an increase in the amount of phosphorylated Kepl protein. The basal amount of phosphorylated Kepl in untreated ovaries probably arises from the population of late stage nurse cells, which are normally cleared by apoptosis following their transfer of cytoplasmic content to the developing oocyte [27–30].

The prototypic member of the STAR family is the Sam68 protein. Numerous studies have demonstrated the dependence

Figure 5 Kep1-ASF interactions in the presence of activated Src

(A) COS-7 cells were co-transfected with GFP-Kep1 and either kinase dead Ser (lanes 1–3 and 7–9) or activated Src (lanes 4–6 and 10–12). Total cell lysates (lanes 1, 4, 7 and 10; TCL), IgG controls (lanes 2, 5, 8 and 11), ASF immunoprecipitations (lanes 3 and 6) and GFP immunoprecipitations (lanes 9 and 12) are shown. Open arrow indicates the position of GFP-Kep1, whereas the closed arrow indicates the position of ASF. (B) COS-7 cells were transfected with GFP-Kep1 (lanes 1–6) or GFP-Kep1 I to N which no longer binds homopolymeric RNA (lanes 7–10). To examine the effect of phosphorylation, samples were co-transfected with activated Src (lanes 4–6 and 10–12). For RNase A treatment, cell lysates were incubated with 10 μ g of RNase A for 30 min at 37 °C prior to performing the immunoprecipitation (IP) experiments. Open arrow denotes the position of the different GFP-Kep1 fusion proteins. *, heavy chain; and <, light chain.

of phosphorylation of Sam68 for its biological function. Phosphorylation of Sam68 by BTK (Bruton's tyrosine kinase) induces its relocalization in human breast tumour cell lines [31]. In addition, tyrosine phosphorylation of Sam68 abolishes its ability to bind homopolymeric RNA [32,33] and inhibits the utilization of intron-containing RNAs containing the Mason-Pfizer monkey virus constitutive transport element [34]. In contrast, activation of the ERK (extracellular-signal-regulated kinase) pathway generates ERK-threonine-phosphorylated Sam68, which enhances inclusion of exon v5 of the CD44 pre-mRNA [25]. These results point to a complex regulation of Sam68 by following different phosphorylation events. Similar to the case above, the closely related Sam68 members Slm1 and Slm2 [19] also show a dependence on phosphorylation for their biological activities [35,36].

Although a number of reports have established links between phosphorylation and RNA binding and/or changes in biological functions, only one report has demonstrated that a phosphorylation event inhibits Sam68 self-association [14]. Similar to other STAR proteins, we have shown that Kep1 can be tyrosine-phosphorylated in the presence of activated Src. We have also demonstrated that a phosphorylation event on Kep1 results in an increase in affinity for one of its protein partners ASF/SF2.

Following co-transfection of *myc-kep1* with the CD44v5 reporter construct in NIH 3T3 cells, we observe an overall decrease in exon 5 inclusion relative to the control (7.5 % versus 15 %). The Kep1 protein is capable of forming homodimers [6] and heteromeric complexes (the present study). It is conceivable that both the unphosphorylated and phosphorylated forms of the Kep1 proteins have affinities for different cellular protein partners. In its unphosphorylated form, the Kep1 protein would bind to and sequester one or several cellular proteins, resulting in the inhibitory effects on exon 5 inclusion observed. Once the appropriate cellular signal is sent, in this case co-transfection with activated Src, the phosphorylation status of the Kep1 protein is altered resulting in a change in its binding partners thus lifting the inhibition in exon 5 inclusion. The presumed sequestering of protein partners by Kep1 could also explain why the presence of Kep1 during all stages of oogenesis does not result in massive apoptosis. This sequestering of protein binding partners has been previously observed for the tra2- β 1 splicing factor [37]. We propose that the overall effects induced by phosphorylation are more important in protein complex formation than previously thought for members of the STAR family. The phosphorylation status is a key mediator in establishing which proteins are most likely present in a given complex by modulating their binding

Figure 6 Modulation of CD44 exon 5 inclusion in cells transfected with Kep1

(A) NIH 3T3 cells were co-transfected with the CD44v5 reporter construct and either an empty expression plasmid, Myc-pCDNA, or with Myc-Kep1. Isolated RNA was subjected to real-time RT-PCR. (A) shows the ratio of exon 5 included over exon 5 excluded from the amplified transcripts (I/E). (B) Western blotting of the above cell lysates demonstrating the presence of Myc-Kep1 expression. (C) Src 3T3 cells were transfected as above and the ratio of exon 5 included over exon 5 excluded was determined by real-time RT-PCR.

affinities. These changes in affinity translate to different proteins being exchanged in the complex resulting in a loss of RNA binding and/or a shift in biological function.

From the above data, we propose a model in which post-translational modifications are important in maintaining the Kep1 protein in a non-apoptotic inducing form. Following the appropriate cellular signal, post-translational modifications of the Kep1 protein will result in a change in affinity for its protein binding partners. This change will impinge on the RNA metabolism of Kep1 mRNA targets allowing for the apoptotic programme to proceed.

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REFERENCES

- Schwerk, C. and Schulze-Osthoff, K. (2005) Regulation of apoptosis by alternative re-mRNA splicing. *Mol. Cell* **19**, 1–13
- Wang, L., Miura, M., Bergeron, L., Zhu, H. and Yuan, J. (1994) Ich-1, an Ice/ced-3-related gene, encodes both positive and negative regulators of programmed cell death. *Cell* **78**, 739–750
- Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G. and Thompson, C. B. (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **74**, 597–608
- Shaham, S. and Horvitz, H. R. (1996) An alternatively spliced *C. elegans* ced-4 RNA encodes a novel cell death inhibitor. *Cell* **86**, 201–208
- Srinivasula, S. M., Ahmad, M., Guo, Y., Zhan, Y., Lazebnik, Y., Fernandes-Alnemri, T. and Alnemri, E. S. (1999) Identification of an endogenous dominant-negative short isoform of caspase-9 that can regulate apoptosis. *Cancer Res.* **59**, 999–1002

- Di Fruscio, M., Chen, T., Bonyadi, S., Lasko, P. and Richard, S. (1998) The identification of two *Drosophila* K homology domain proteins. Kep1 and SAM are members of the Sam68 family of GSG domain proteins. *J. Biol. Chem.* **273**, 30122–30130
- Di Fruscio, M., Styhler, S., Wikholm, E., Boulanger, M. C., Lasko, P. and Richard, S. (2003) Kep1 interacts genetically with dredd/caspase-8, and kep1 mutants alter the balance of dredd isoforms. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 1814–1819
- Wang, S. L., Hawkins, C. J., Yoo, S. J., Muller, H. A. and Hay, B. A. (1999) The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. *Cell* **98**, 453–463
- Vernet, C. and Artzt, K. (1997) STAR, a gene family involved in signal transduction and activation of RNA. *Trends Genet.* **13**, 479–484
- Konig, H., Ponta, H. and Herrlich, P. (1998) Coupling of signal transduction to alternative pre-mRNA splicing by a composite splice regulator. *EMBO J.* **17**, 2904–2913
- Guan, K. L. and Dixon, J. E. (1991) Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.* **192**, 262–267
- Neugebauer, K. M., Stolk, J. A. and Roth, M. B. (1995) A conserved epitope on a subset of SR proteins defines a larger family of pre-mRNA splicing factors. *J. Cell Biol.* **129**, 899–908
- Hanamura, A., Caceres, J. F., Mayeda, A., Franza, B. R. and Krainer, A. R. (1998) Regulated tissue-specific expression of antagonistic pre-mRNA splicing factors. *RNA* **4**, 430–444
- Chen, T., Damaj, B. B., Herrera, C., Lasko, P. and Richard, S. (1997) Self-association of the single-KH-domain family members Sam68, GRP33, GLD-1, and Qk1: role of the KH domain. *Mol. Cell. Biol.* **17**, 5707–5718
- Chen, T. and Richard, S. (1998) Structure-function analysis of Qk1: a lethal point mutation in mouse quaking prevents homodimerization. *Mol. Cell. Biol.* **18**, 4863–4871
- Zorn, A. M. and Krieg, P. A. (1997) The KH domain protein encoded by quaking functions as a dimer and is essential for notochord development in *Xenopus* embryos. *Genes Dev.* **11**, 2176–2190
- Lasko, P. (2000) The *Drosophila melanogaster* genome: translation factors and RNA binding proteins. *J. Cell Biol.* **150**, F51–F56
- Morris, E. J. and Geller, H. M. (1996) Induction of neuronal apoptosis by camptothecin, an inhibitor of DNA topoisomerase-I: evidence for cell cycle-independent toxicity. *J. Cell Biol.* **134**, 757–770
- Di Fruscio, M., Chen, T. and Richard, S. (1999) Characterization of Sam68-like mammalian proteins SLM-1 and SLM-2: SLM-1 is a Src substrate during mitosis. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 2710–2715
- Stoss, O., Olbrich, M., Hartmann, A. M., König, H., Memmott, J., Andreadis, A. and Stamm, S. (2001) The STAR/GSG family protein rSLM-2 regulates the selection of alternative splice sites. *J. Biol. Chem.* **276**, 8665–8673
- Koizumi, J., Okamoto, Y., Onogi, H., Mayeda, A., Krainer, A. R. and Hagiwara, M. (1999) The subcellular localization of SF2/ASF is regulated by direct interaction with SR protein kinases (SRPKs). *J. Biol. Chem.* **274**, 11125–11131
- Allemand, E., Gattoni, R., Bourbon, H. M., Stevenin, J., Caceres, J. F., Soret, J. and Tazi, J. (2001) Distinctive features of *Drosophila* alternative splicing factor RS domain: implication for specific phosphorylation, shuttling, and splicing activation. *Mol. Cell. Biol.* **21**, 1345–1359
- Mount, S. M. and Salz, H. K. (2000) Pre-messenger RNA processing factors in the *Drosophila* genome. *J. Cell Biol.* **150**, F37–F44
- De Boulle, K., Verkerk, A. J., Reyniers, E., Vits, L., Hendrickx, J., Van Roy, B., Van den Bos, F., de Graaff, E., Oostra, B. A. and Willems, P. J. (1993) A point mutation in the FMR-1 gene associated with fragile X mental retardation. *Nat. Genet.* **3**, 31–35
- Matter, N., Herrlich, P. and König, H. (2002) Signal-dependent regulation of splicing via phosphorylation of Sam68. *Nature* **420**, 691–695
- Johnson, P. J., Coussens, P. M., Danko, A. V. and Shalloway, D. (1985) Overexpressed pp60c-src can induce focus formation without complete transformation of NIH 3T3 cells. *Mol. Cell. Biol.* **5**, 1073–1083
- Giorgi, F. and Deri, P. (1976) Cell death in ovarian chambers of *Drosophila melanogaster*. *J. Embryol. Exp. Morphol.* **35**, 521–533
- Foley, K. and Cooley, L. (1998) Apoptosis in late stage *Drosophila* nurse cells does not require genes within the H99 deficiency. *Development* **125**, 1075–1082
- Nezis, I. P., Stravopodis, D. J., Papassideri, I., Robert-Nicoud, M. and Margaritis, L. H. (2000) Stage-specific apoptotic patterns during *Drosophila* oogenesis. *Eur. J. Cell Biol.* **79**, 610–620
- McCall, K. and Steller, H. (1998) Requirement for DCP-1 caspase during *Drosophila* oogenesis. *Science* **279**, 230–234
- Lukong, K. E., Larocque, D., Tyner, A. L. and Richard, S. (2005) Tyrosine phosphorylation of sam68 by breast tumor kinase regulates intranuclear localization and cell cycle progression. *J. Biol. Chem.* **280**, 38639–38647

-
- 32 Wang, L. L., Richard, S. and Shaw, A. S. (1995) P62 association with RNA is regulated by tyrosine phosphorylation. *J. Biol. Chem.* **270**, 2010–2013
- 33 Derry, J. J., Richard, S., Valderrama Carvajal, H., Ye, X., Vasioukhin, V., Cochrane, A. W., Chen, T. and Tyner, A. L. (2000) Sik (BRK) phosphorylates Sam68 in the nucleus and negatively regulates its RNA binding ability. *Mol. Cell. Biol.* **20**, 6114–6126
- 34 Coyle, J. H., Guzik, B. W., Bor, Y. C., Jin, L., Eisner-Smerage, L., Taylor, S. J., Rekosh, D. and Hammariskjold, M. L. (2003) Sam68 enhances the cytoplasmic utilization of intron-containing RNA and is functionally regulated by the nuclear kinase Sik/BRK. *Mol. Cell. Biol.* **23**, 92–103
- 35 Stoss, O., Novoyatleva, T., Gencheva, M., Olbrich, M., Benderska, N. and Stamm, S. (2004) p59(fyn)-mediated phosphorylation regulates the activity of the tissue-specific splicing factor rSLM-1. *Mol. Cell. Neurosci.* **27**, 8–21
- 36 Haegebarth, A., Heap, D., Bie, W., Derry, J. J., Richard, S. and Tyner, A. L. (2004) The nuclear tyrosine kinase BRK/Sik phosphorylates and inhibits the RNA-binding activities of the Sam68-like mammalian proteins SLM-1 and SLM-2. *J. Biol. Chem.* **279**, 54398–54404
- 37 Stoilov, P., Daoud, R., Nayler, O. and Stamm, S. (2004) Human tra2- β 1 autoregulates its protein concentration by influencing alternative splicing of its pre-mRNA. *Hum. Mol. Genet.* **13**, 509–524
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