Dsk1p kinase phosphorylates SR proteins and regulates their cellular localization in fission yeast

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Evolutionarily conserved SR proteins (serine/arginine-rich proteins) are important factors for alternative splicing and their activity is modulated by SRPKs (SR protein-specific kinases). We previously identified Dsk1p (*dis1*-suppressing protein kinase) as the orthologue of human SRPK1 in fission yeast. In addition to its similarity of gene structure to higher eukaryotes, fission yeast *Schizosaccharomyces pombe* is a unicellular eukaryotic organism in which alternative splicing takes place. In the present study, we have revealed for the first time that SR proteins, Srp1p and Srp2p, are the *in vivo* substrates of Dsk1p in *S. pombe*. Moreover, the cellular localization of the SR proteins and Prp2p splicing factor is dependent on *dsk1*⁺: Dsk1p is required for the efficient nuclear localization of Srp2p and Prp2p, while it promotes the cytoplasmic distribution of Srp1p, thereby differentially influencing the destinations of these proteins in the cell. The present

study offers the first biochemical and genetic evidence for the *in vivo* targets of the SRPK1 orthologue, Dsk1p, in *S. pombe* and the significant correlation between Dsk1p-mediated phosphorylation and the cellular localization of the SR proteins, providing information about the physiological functions of Dsk1p. Furthermore, the results demonstrate that the regulatory function of SRPKs in the nuclear targeting of SR proteins is conserved from fission yeast to human, indicating a general mechanism of reversible phosphorylation to control the activities of SR proteins in RNA metabolism through cellular partitioning.

Key words: alternative splicing, cellular localization, green fluorescent protein (GFP)-fusion protein, protein kinase, protein phosphorylation, serine/arginine-rich protein (SR protein).

INTRODUCTION

SR proteins (serine/arginine-rich proteins) are a class of evolutionarily conserved factors important for alternative splicing. They contain structural features with one or two N-terminal RBDs (RNA-binding domains) and a C-terminal RS (arginine/serine-rich) domain. SR proteins play a pivotal role in both splice site selection and constitutive pre-mRNA splicing (for a review, see [1–3]). Moreover, the functions of SR proteins extend beyond pre-mRNA splicing and they act at different steps of post-transcriptional gene expression in eukaryotes [4]. SR proteins serve as multifunctional adaptors to couple splicing with transcription [5] and RNA export [6] in a spatially and temporally regulated manner.

The versatile functions of SR proteins are modulated by reversible phosphorylation. Two protein kinase families are key to SR protein regulation: SRPK (SR protein-specific kinase) and Clk/Sty (Cdc2p/Cdc28p-like kinase or serine/threonine/tyrosine kinase). SRPK and Clk/Sty kinases phosphorylate SR splicing factors, predominantly on serine residues in the RS domain. The kinase families are conserved through evolution from yeast to human [7–14]. Members of the kinase families are involved in various cellular processes as diverse as cytokinesis, apoptosis, viral infection, drug-sensitivity, oxidative-stress response, differentiation, and development [12,14–19], thus lending themselves to important, yet complex functions. Very little is understood about whether and how the kinases function in these processes through their action on SR proteins.

Reversible phosphorylation of SR proteins not only is necessary for spliceosome assembly and the transesterification reaction to occur, but also plays an important role in the regulation of pre-mRNA splicing [20–22]. A previously identified mammalian SR protein, SRp38, acts as a potent repressor of splicing when specifically dephosphorylated in M-phase cells [23]. In addition, human SRPK1-mediated SR protein phosphorylation is down-regulated as a result of the infection by HSV-1 (herpes simplex virus 1), and this decrease in SR protein phosphorylation contributes to host shut-off by inhibiting pre-mRNA splicing [24]. The biological significance of SR protein phosphorylation is manifested by the observation that the phosphorylation changes during early development coincide with major zygotic gene activation in the nematode *Ascaris lumbricoides* [25]. Phosphorylation may modulate the activity of an SR protein by influencing its interactions with RNA molecules or other proteins [21].

Phosphorylation also governs the proper localization of SR proteins to perform their functions in the cell. Newly synthesized SR proteins in the cytoplasm need to be transported into the nucleus for RNA processing. Interestingly, the RS domain of SR proteins serves as a NLS (nuclear localization signal) and its phosphorylation by human SRPK1 facilitates the *in vitro* interaction of the proteins with TRN-SR2 (transportin-SR2; hMtr10), an SR protein import receptor [26,27]. After entering the nucleus, serine phosphorylation of SR proteins is required for their interaction with the C-terminal domain of the RNA polymerase II large subunit and their subsequent recruitment from the nuclear speckles to the transcription sites, so that splicing takes place

Abbreviations used: Clk/Sty, Cdc2p/Cdc28p-like kinase or serine/threonine/tyrosine kinase; DAPI, 4',6-diamidino-2-phenylindole; Dsk1p, *dis1*-suppressing protein kinase; EMM2, Edinburgh minimal medium 2; ESE, exonic splicing enhancer; GFP, green fluorescent protein; HRP, horseradish peroxidase; Kic1p, kinase in Clk family; mAb, monoclonal antibody; NES, nuclear export signal; NLS, nuclear localization signal; *nmt*⁺, no message in thiamine; NOR, nucleolar organizing region; RBD, RNA-binding domain; RS domain, arginine/serine-rich domain; SR protein, serine/arginine-rich protein; SRPK, SR protein-specific kinase; TRN-SR2, transportin-SR2; ts mutant, temperature-sensitive mutant; U2AF, U2 auxiliary factor; wt, wild-type.

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co-transcriptionally in the cell [5]. Both human SRPK1 and mammalian Clk/Sty function to control the intranuclear trafficking of SR proteins between nuclear speckles/IGCs (interchromatin granule clusters) and nucleoplasm in response to signals for transcriptional activation and the interphase–M-phase transition during the cell cycle [7,13]. Previously, hypophosphorylated SR proteins were found to co-localize with Clk/Sty in the patches around NORs (nucleolar organizing regions), so-called NAPs (NOR-associated patches) in telophase before they are destined for nuclear speckles in G₁, implying a novel distribution pathway of SR proteins separated from that of snRNPs (small nuclear ribonucleoproteins) within the nucleus [28]. Finally, serving as adaptors for nuclear export of spliced mRNA, dephosphorylation of the RS domain of SR proteins enables them to interact with a nuclear export factor, TAP (Tip-associated protein) [6]. Therefore the dynamic localizations and functions of SR proteins are altered in a phosphorylation-dependent fashion.

Two protein kinases, Dsk1p (*dis1*-suppressing protein kinase) and Kic1p (kinase in Clk family), are the fission yeast *Schizosaccharomyces pombe* orthologues of human SRPK1 and mouse Clk1/Sty1 respectively, based on sequence analyses and assays of cross-species genetic complementation [7,9,14]. Dsk1p promotes metaphase–anaphase transition, whereas Kic1p influences cytokinesis and filamentous growth [14,29,30]. These two related kinases constitute a critical function in cell growth [9,14]. Besides the functional conservation of the kinases in *S. pombe*, Srp1p and Srp2p are considered bona fide SR proteins according to the homology of their RBD sequences to mammalian SR proteins [31,32]. Srp2p is not only essential for viability, but is also probably the mediator of ESEs (exonic splicing enhancers) in fission yeast [33]. Other indispensable *trans*-acting factors that recognize and respond to ESEs are also conserved in *S. pombe*: both subunits of U2AF (U2 auxiliary factor), Prp2p/Mis11p (spU2AF^{LG}) and spU2AFSM [34–36]. Another *prp2* mutant allele, *mis11-453*, affects chromosome segregation and leads to minichromosome loss [37]. In addition, Srp2p and spU2AFSM specifically interact with each other, as do their mammalian homologues [36].

The genome of *S. pombe* harbours a total of 4730 introns distributed in 43 % of 4972 annotated protein-encoding sequences. Nearly 25 % of *S. pombe* genes have multiple introns [38]. Moreover, alternative splicing does occur in fission yeast via intron retention with continuous open reading frames [39,40], making it a useful genetically tractable model to study the process.

We previously showed that Dsk1p displayed high specificity in phosphorylating Srp1p, Srp2p and Prp2p *in vitro* [9,41]. All three proteins contain RBD and RS regions. Dsk1p not only phosphorylates Prp2p *in vitro* but also genetically interacts with the splicing factor [9,41]. In addition, Srp1p and Srp2p interact with each other, and Srp1p phosphorylation by Dsk1p inhibits Srp complex formation [42]. However, it remains unclear whether Srp1p and Srp2p are actually the *in vivo* substrates of Dsk1p and/or Kic1p and how this Dsk1p- or Kic1p-mediated phosphorylation influences the functions of the SR proteins. Taking advantage of the conserved SRPKs and SR proteins in this single-cellular organism, we investigated whether Srp1p and Srp2p are the *in vivo* targets of Dsk1p or Kic1p. We also determined the effect of the kinases on the cellular localizations of Prp2p, Srp1p and Srp2p by ectopic expression of the corresponding GFP (green fluorescent protein) fusion molecules in *S. pombe* wt (wild-type), *dsk1*-deletion and *kic1*-deletion strains. Here, we report that Dsk1p phosphorylates both Srp1p and Srp2p *in vivo* and affects the cellular localization of the SR proteins. Our studies provide the first biochemical and genetic evidence for the *in vivo* targets of Dsk1p, the SRPK1 orthologue in fission yeast,

Table 1 *S. pombe* strains used

Strain	Genotype	Reference/source
1913	<i>h⁻ leu1-32</i>	William Dunphy (Caltech)
B8	<i>h⁻ leu1-32 ura4 dsk1::ura4⁺</i>	Takeuchi et al. [29]
2A5	<i>h⁺ leu1-32 ura4 kic1::ura4⁺ his2</i>	Tang et al. [14]
2D4	<i>h⁺ leu1-32 ura4 kic1::ura4⁺ dsk1::ura4⁺ his2</i>	Tang et al. [14]
<i>mis11/prp2</i>	<i>h⁻ leu1-32 mis11-453</i>	Takahashi et al. [37]

and the consequences of Dsk1p-mediated phosphorylation on the cellular localization of the SR proteins.

MATERIALS AND METHODS

S. pombe strains and cell culture

The strains used in the present study are listed in Table 1. Cell culture and maintenance were carried out as described by Alfa et al. [43].

Plasmid construction

The *srp1⁺*, *srp2⁺* and *prp2⁺* genes were subcloned into pREP81GFP and pREP41GFP (gifts from Dr Kathleen Gould, Howard Hughes Medical Institute and Vanderbilt University Medical School, Nashville, TN, U.S.A.) so that the N-terminal GFP-tagged fusion proteins were produced under the control of thiamine-repressible *nmt1⁺* (no message in thiamine) promoter derivatives with reduced strength [44]. A 0.9 kb NdeI–SmaI DNA fragment containing the *srp1⁺* gene was isolated from pREP1*srp1⁺* and inserted into the same sites of vector pREP81GFP to construct pREP81GFP*srp1⁺*. The pREP81GFP*prp2⁺* was constructed by using a 1.5 kb NdeI–BamHI DNA fragment carrying the *prp2⁺* gene from pREP1*prp2⁺*. Plasmid pREP1*srp2⁺* was digested with restriction enzymes SalI and BamHI and the resultant 1179 bp fragment was inserted into pREP41GFP/pREP81GFP to generate pREP41/81GFP*srp2⁺*.

Transformation of *S. pombe*

The vectors with GFP gene alone or the plasmids encoding GFP-fusion proteins were transformed into fission yeast wt, *dsk1*-null (Δ *dsk1*), *kic1*-null (Δ *kic1*) and *dsk1kic1* double-deletion (Δ *dsk1* Δ *kic1*) strains. These strains are auxotrophic for leucine (with *leu1-32* allele) and transformants harbouring the plasmids were selected in EMM2 (Edinburgh minimal medium 2) without leucine. Transformation of fission yeast was accomplished using the lithium acetate method [43] with modifications [9,14]. Transformed cells were spread on to EMM2 plates in the presence of 2 μ M thiamine with appropriate supplements, if needed, and incubated at 30 °C until colonies appeared. For functional assay of GFP–Prp2, *mis11/prp2* [34,37] *ts* (temperature-sensitive) mutant cells were grown at 25 °C for transformation and at 36 °C after transformation.

Ectopic expression of GFP-fusion proteins in *S. pombe*

The expression of genes under the control of the *nmt1⁺* promoter in *S. pombe* was induced as described in [9,14]. The fission yeast strains harbouring the vectors with GFP gene alone or the plasmids encoding GFP-fusion proteins were grown at 30 °C to mid-exponential phase in EMM2. Repression of the *nmt1⁺* promoter derivatives was achieved by adding thiamine to a

concentration of 2 μ M in the growth medium. Cells were then washed three times with water and resuspended in EMM2 lacking thiamine. In order to induce the expression of the GFP-fusion proteins, the cells were cultured for 20–22 h in thiamine-depleted medium to a suitable cell density, 1×10^6 – 1×10^7 cells/ml.

Fluorescence and confocal microscopy

Cells were fixed and stained with DAPI (4',6-diamidino-2-phenylindole) and calcofluor. Methods for DAPI and calcofluor staining were based on the published protocol [43] and the *Fission Yeast Handbook* by Frans Hochstenbach (<http://www.bio.uva.nl/pombe/handbook/>) with modifications [9,14]. In order to stain cells with both DAPI and calcofluor, 1 $\times$ DAPI and 1 $\times$ calcofluor solutions were mixed at a ratio of 20:1–10:1, and 5–10 μ l of the mixture was used to stain the fixed cells. An Olympus IX81 fluorescence microscope with a magnification of $\times 960$ and a Nikon fluorescence microscope with a magnification of $\times 400$ were used to capture the cell images. For confocal microscopy, cells were prepared in the same way as for fluorescence microscopy and observed under a Zeiss LSM 510 confocal microscope with a magnification of $\times 630$. Cells were observed at a single plane generated by Z-sections of 17 planes with an interval of 0.45 μ m between consecutive planes of a given sample.

Immunological analyses of proteins with antibodies

Fission yeast cell lysates with or without the ectopically expressed GFP and GFP-fusion proteins were prepared using the protocol in [45] with modifications as described previously [14]. Approx. 5×10^8 or 1.25×10^9 cells were collected to make 1 ml of lysate containing 5 mg/ml or higher total protein concentration, depending on whether the proteins under study were ectopically or endogenously expressed. Usually 10–15 μ l of individual lysates were loaded on to 10% (w/v) SDS gel for PAGE. The intensity of protein bands was visualized by using a HRP (horseradish peroxidase)-linked ECL (enhanced chemiluminescence) detection system. mAb (monoclonal antibody) against GFP was purchased from Roche Diagnostics and used with 4000 times dilution in immunoblotting. mAb3C5 was obtained from mouse ascites (a gift from Dr Adrian Krainer, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.) and used at a 1000 times dilution. The sera containing anti-Srp1p and anti-Srp2p polyclonal antibodies were produced as described in [42], and were diluted 1000 times in the immunoblotting step. HRP-conjugated rabbit anti-mouse IgG, goat anti-mouse IgM or swine anti-rabbit IgG was used at a 5000 or 7000 times dilution as a secondary antibody for anti-GFP, mAb3C5 or polyclonal antibodies respectively.

In vitro dephosphorylation of endogenous Srp1p and Srp2p

The dephosphorylation procedure is the same as described previously by Tang et al. [46] except that fission yeast cell extracts prepared in the presence of protease inhibitor mix rather than immuno-isolated proteins were incubated with alkaline phosphatase (New England Biolabs) at 37 °C for 1 h in a total volume of 25 μ l reaction mixture. Na_2HPO_4 (100 mM) was used to inhibit the phosphatase.

RESULTS

In order to investigate whether the SR proteins, Srp1p and Srp2p, are *in vivo* targets of Dsk1p and/or Kic1p, and whether the phosphorylation by the kinases results in changes in the cellular localization of these proteins in fission yeast, we expressed these

proteins as the N-terminal GFP-tagged fusions and determined their phosphorylation and distribution *in vivo* (see the Materials and methods section). Since Prp2p is a defined pre-mRNA splicing factor in fission yeast [34], and it is known to genetically interact with *dsk1*⁺ [9], we used it as a reference for nuclear proteins and for the kinase-specific effect on the cellular localization of SR proteins in our analyses. The ectopic expression system with attenuated *nmt* promoters (pREP81 and pREP41) permits the selection of an optimal condition for adequate fusion-protein production and GFP signal detection with relatively less perturbation to the cell [44].

Prior to the biochemical and cytological analyses, we ensured that the GFP-fusion proteins in testing strains were produced. Cell lysates were prepared after GFP-fusion proteins were induced in wt (1913), Δ *kic1* (2A5) and Δ *dsk1* (B8) strains, which contain plasmid pREP81GFP, pREP81GFPsrp1⁺, pREP41/81GFPsrp2⁺ or pREP81GFPprp2⁺. The GFP-fusion proteins were actually expressed at a sufficient level for biochemical studies (results not shown). Adequate green fluorescent signal was also detected cytologically (Figures 3–5).

Both Srp1p and Srp2p are *in vivo* substrates of Dsk1p

We previously showed that Srp1p and Srp2p are phosphorylated by Dsk1p *in vitro* [9]. An important question to address is whether the SR proteins are phosphorylated *in vivo* by Dsk1p and/or Kic1p. Since the mAb, mAb3C5, reacts with a phosphoepitope commonly shared between mammalian [47] and fission yeast SR proteins phosphorylated by SRPK1 or Dsk1p *in vitro* [9] respectively, we used mAb3C5 to assess the phosphorylation of Srp1p and Srp2p by either Dsk1p or Kic1p *in vivo*. We also carried out phosphatase assays to examine the changes in the phosphorylation state of the SR proteins under the influence of the two kinases.

Phosphorylation of Srp1p by Dsk1p *in vitro* inhibits the Srp1p–Srp2p complex formation [42]. To examine the *in vivo* phosphorylation of Srp1p, we induced the expression of GFP–Srp1p in fission yeast wt strain, Δ *kic1*, Δ *dsk1* and Δ *dsk1* Δ *kic1* mutants. The resultant lysates from these cells containing the fusion protein were analysed by immunoblotting with the anti-GFP antibody (Figure 1A, top panel) and mAb3C5 (bottom panel). The same amount of total protein (~ 40 μ g) was loaded into each lane of the gel. Comparable levels of GFP–Srp1p were produced in all strains tested (Figure 1A, top panel, lanes 1–4). However, mAb3C5-reactive epitope was detected mainly in wt and Δ *kic1* cells (bottom panel, lanes 1 and 2), but very little in Δ *dsk1* and Δ *dsk1* Δ *kic1* mutants (lanes 3 and 4). Weak signals of two additional bands with apparent molecular mass lower than the full-length Srp1p was seen in the mAb3C5 blot (Figure 1A, bottom panel, lanes 1–3). The faster migrating band probably resulted from the degradation of GFP–Srp1p (Figure 1A, bottom panel, lanes 1 and 2), as a longer exposure of the GFP blot (top panel) showed a band in the same position (results not shown), while the other lower mobility band was due to some non-specific reaction of the antibody (lanes 1–3). Overall, deletion in *dsk1* abolishes the cell's ability to produce mAb3C5-specific phosphoepitope in GFP–Srp1p.

We further determined the targets of Dsk1p and Kic1p in fission yeast by examining the phosphorylation of the endogenous Srp1p from the corresponding strains without harbouring the GFP-fusion plasmids (Figure 1B). The immunoblots were probed with polyclonal anti-Srp1p sera and multiple protein bands of 40–45 kDa, as predicted for the molecular mass of Srp1p, were detected (Figure 1B, top panel, lanes 1–4). Interestingly, the mobility of the bands on the gel recognized by the anti-Srp1p

Figure 1 Srp1p is phosphorylated at the mAb3C5-specific site by Dsk1p

(A) Deletion in *dsk1* abolishes the cell's capability to produce mAb3C5-specific phosphoepitope in GFP-Srp1p. GFP-Srp1p was expressed in fission yeast strains. The cell lysates containing GFP-Srp1p were analysed by immunoblotting with the anti-GFP antibody (top panel) and mAb3C5 antibody (bottom panel). The expected position of GFP-Srp1p and its mAb3C5-recognized form on the gel are indicated on the right. (B) Endogenous Srp1p is differentially modified in the cells with different genetic backgrounds of the two kinases and is also reactive to mAb3C5 in a *dsk1*⁺-dependent manner. Cell lysates were prepared from fission yeast wt, *Δkic1*, *Δdsk1* and *Δdsk1Δkic1* strains without any GFP-encoding plasmids. Immunoblots were probed by using polyclonal anti-Srp1p sera (top panel) and mAb3C5 antibody (bottom panel). The expected position of Srp1p and its mAb3C5-recognized form on the gel are indicated on the right. (C) Dsk1p is the major kinase to mediate Srp1p phosphorylation. Cell lysates of 10 μl prepared from different strains as in (B) were mock-treated (-), phosphatase-treated (+) with 5 units of alkaline phosphatase (CIP) in the presence (+) or absence (-) of a phosphatase inhibitor, Na₂HPO₄ (INH), or treated with the inhibitor alone. The immunoblots were analysed by using polyclonal anti-Srp1p sera. The expected position of Srp1p on the gel is indicated on the right.

sera differed among the four strains: the primary fraction of Srp1p from wt and *Δkic1* migrated the most slowly in gel (Figure 1B, top panel, lanes 1 and 2), while most of the Srp1p molecules from *Δdsk1Δkic1* migrated the fastest (lane 4); moreover, the Srp1p counterpart from *Δdsk1* appeared as three bands, including one with intermediate mobility (Figure 1B, top panel, lane 3), indicating various modification states of Srp1p in these strains. Based on the different apparent molecular masses observed on the gel, Srp1p is modified at the lowest level in *Δdsk1Δkic1* (Figure 1B, top panel, lane 4) compared with that in the wt and either single kinase-deletion strains (lanes 1–3), revealing an additive role of both kinases in the modification of this protein. On the other hand, Srp1p is less modified in *Δdsk1* cells than that in wt and *Δkic1* strains (Figure 1B, top panel, compare lanes 1, 2 and 3), suggesting that Dsk1p exerts a bigger effect on Srp1p modification than Kic1p. To check whether the levels of Srp1p modification correlate with its differential phosphorylation states in the cells varying with the genetic background of the kinases, we analysed the immunoblots of identical samples with mAb3C5.

Similar to the *dsk1*⁺-dependent phosphorylation of GFP-Srp1p *in vivo* (Figure 1A), the endogenous Srp1p in wt and *Δkic1* lysates reacted strongly to mAb3C5 (Figure 1B, bottom panel, lanes 1 and 2), whereas little mAb3C5-positive Srp1p was detected in the lysates of *Δdsk1* and *Δdsk1Δkic1* (lanes 3 and 4). Therefore the generation of mAb3C5 epitope corresponds to the more extensively modified state of Srp1p. Importantly, consistent with the ectopic expression result, the endogenous Srp1p is phosphorylated at the mAb3C5 epitope only when Dsk1p is functional in the cell.

In order to address whether the gel mobility shift of Srp1p is due to phosphorylation, the lysates from different fission yeast strains were incubated with alkaline phosphatase (calf intestinal, 'CIP') in the presence or absence of a phosphatase inhibitor ('INH'), Na₂HPO₄ (Figure 1C). Plain lysates without any treatment (Figure 1C, lanes 17–20) confirmed the gel mobility pattern of Srp1p from these strains (Figure 1B, top panel). Incubation of the wt lysate with alkaline phosphatase eliminated the upper band of Srp1p (Figure 1C, lane 2), whereas the band remained in the mock-treated sample (lane 1) and the samples with the phosphatase inhibitor (lanes 3 and 4), indicating that the low-mobility band is a result of hyperphosphorylation. Similar dephosphorylation by the phosphatase also occurred under the same condition to Srp1p from *Δkic1* (Figure 1C, lanes 5–8) and *Δdsk1* (lanes 9–12). The weak signal of the hyperphosphorylation band in lane 8 of a *Δkic1* sample may be attributed to protein degradation. Again, note the Srp1p band(s) of intermediate mobility in *Δdsk1* samples (Figure 1C, lanes 9, 11 and 12) compared with that in wt and *Δkic1* samples (lanes 1, 3–5 and 7). This reflects the hypophosphorylated state of Srp1p in *dsk1*-deletion cells. In contrast, the treatment gave rise to little change in the mobility of Srp1p from *Δdsk1Δkic1*, as only a high-mobility band of the protein was present in all samples with and without the phosphatase (Figure 1C, lanes 13–16), demonstrating its dephosphorylated status in the double-deletion strain of the kinases. The results revealed different phosphorylation states of Srp1p, given the altered genetic background of the kinases. Taken together, Dsk1p is the primary kinase responsible for phosphorylating Srp1p in the cell, although Kic1p also acts on it.

We are also interested in the *in vivo* phosphorylation of Srp2p because of its potential role in splicing regulation involving ESE [33]. We conducted parallel experiments to determine the substrate specificity of Dsk1p for GFP-Srp2p by using immunoblotting with the anti-GFP antibody (Figure 2A, top panel) and mAb3C5 antibody (bottom panel). GFP-Srp2p was expressed in all four strains (Figure 2A, top panel, lanes 1–4); nevertheless, the mAb3C5-specific phosphoepitope in GFP-Srp2p was mainly produced in *dsk1*⁺ strains, wt and *Δkic1* (bottom panel, lanes 1 and 2), although the signal detected was weaker compared with that for Srp1p (Figure 1A, bottom panel, lanes 1 and 2). Fewer GFP-Srp2p molecules of full length were observed in *Δdsk1Δkic1* (Figure 2A, top panel, lane 4) than in other strains (lanes 1–3), as the protein in this strain tends to be degraded into lower molecular-mass peptides. However, neither the full-length (Figure 2A, top panel, lane 4) nor the partial (results not shown) peptides of GFP-Srp2p from the double-deletion strain reacted to mAb3C5. Therefore, similar to GFP-Srp1p (Figure 1A), the generation of mAb3C5-specific phosphoepitope in GFP-Srp2p is *dsk1*⁺-dependent as well.

We then assessed the modification of endogenous Srp2p in the cells with different genetic background of the kinases by its mobility shift on gel (Figure 2B). Multiple mobility bands of 50–55 kDa, as the molecular mass expected for Srp2p, were specifically recognized by anti-Srp2p sera in each testing strain (Figure 2B, lanes 1–4). The *dsk1* deletion resulted in more Srp2p

Figure 2 Srp2p is phosphorylated *in vivo* in a *dsk1*⁺-dependent manner

The GFP-Srp2p expression, lysate preparation and immunoblotting were carried out as in Figure 1. **(A)** The mAb3C5-specific phosphoepitope in GFP-Srp2p is produced at a higher level in *dsk1*⁺ strains than in *dsk1*-deletion strains. Lysates were processed for immunoblotting and analysed with the anti-GFP antibody (top panel) and mAb3C5 (bottom panel). GFP-Srp2p and its mAb3C5-reactive version are indicated on the right. **(B)** Endogenous Srp2p is differentially modified in the cells with different genetic background of Dsk1p and Kic1p. Cell extracts were prepared from fission yeast strains as in Figure 1(B). Immunoblots were probed by using polyclonal anti-Srp2p sera. The expected position of Srp2p is indicated on the right. **(C)** Dsk1p is responsible for the hyperphosphorylation of Srp2p. Cell lysates were prepared and the phosphatase assays were carried out as in Figure 1(C), except that the immunoblots were analysed by using polyclonal anti-Srp2p sera instead of anti-Srp1p sera. Two blots from separate experiments are presented. The expected position of Srp2p on the gel is indicated on the right. A non-specific band is indicated by an arrow.

molecules of high mobility (Figure 2B, lanes 3 and 4), compared with that in wt and *Δkic1* strains (lanes 1 and 2), while *kic1* deletion has little effect on Srp2p modification. To examine whether the nature of the modification is phosphorylation, we conducted the same phosphatase assay for Srp2p (Figure 2C) as for Srp1p (Figure 1C). Blots of two separate experiments are presented. The phosphatase treatment dephosphorylated the higher molecular mass versions of Srp2p from all four strains, increasing their mobility in gel, which displayed a smear-like pattern with decreased amounts or complete loss of the upper-most band(s) (Figure 2C, lanes 2, 6, 10 and 14). Relatively, more Srp2p bands of lower mobility were present in the samples of wt and *Δkic1* (Figure 2C, lanes 1–8) than that of *dsk1*-deletions (lanes 9–16), indicating that Srp2p is hypophosphorylated without Dsk1p in the cell. In agreement with the lower phosphorylation level of Srp2p, higher mobility molecules of the protein were observed in the double-deletion strain; consequently, the phosphatase effect on Srp2p was less extensive in the *Δdsk1Δkic1* samples (Figure 2C, lanes 13–16) than that in other testing strains. However, deletions in both kinases did not completely abrogate the higher molecular

Figure 3 Deletion in *dsk1* alters the cellular localization of GFP-Srp2p

GFP-Srp2p was expressed in different fission yeast strains as in Figure 2. Cells were fixed and stained with DAPI alone or with DAPI/calcofluor as indicated. **(A)** GFP-Srp2p is concentrated in the nucleus of the wt strain but is diffuse throughout the *Δdsk1* cells. Cell images were obtained by using fluorescence microscopy for DAPI/calcofluor (left panels) and GFP (middle panels) as indicated. Merged images (right panels) were generated by overlapping the images of DAPI alone or DAPI/calcofluor, GFP and phase contrast. Original magnification is $\times 960$, and the scale bar represents 10 μm. **(B)** GFP-Srp2p accumulates in the nucleus of the *Δkic1* mutant as in the wt cells. DAPI and calcofluor staining (left panel), GFP signal (middle panel) and merged (right panel) images are indicated. The upper panels show the images obtained by conventional fluorescence microscopy as in **(A)**. The lower panels show confocal microscopic images of a Z-section plane. Original magnification is $\times 630$, and the scale bar represents 10 μm.

mass counterparts of Srp2p (Figure 2B, lanes 3 and 4; Figure 2C, lanes 19 and 20), implying that another kinase besides Dsk1p may exist in the cell for the *in vivo* phosphorylation of Srp2p. Taken as a whole, we conclude that both Srp1p and Srp2p are *in vivo* targets of Dsk1-mediated phosphorylation in fission yeast.

The efficient nuclear localization of GFP-Srp2 requires a functional Dsk1p protein kinase

To address the *in vivo* consequences of Dsk1p-dependent phosphorylation of the SR proteins, we determined the cellular distribution of the SR proteins in wt and mutant cells with altered genetic background of the Dsk1p and Kic1p kinases by visualizing the corresponding GFP-fusion proteins within the cell using fluorescence microscopy. Lutzberger et al. [32] previously reported that GFP-Srp2p expressed under the control of the *nmt1*⁺ promoter was localized in the nucleus of a *ts* mutant *prp4*. In agreement with that study, GFP-Srp2p was concentrated in the nucleus of the wt strain (Figure 3A, top panels). Interestingly, deletion of *dsk1*⁺ led to the distribution of the protein throughout

Figure 4 GFP-Srp2p is located in the cytoplasm of *dsk1kic1* double-deletion mutant

(A) Cellular localization of GFP-Srp2p in $\Delta dsk1\Delta kic1$ cells. Plasmid pREP81GFPsrp2⁺ was transformed into $\Delta dsk1\Delta kic1$ cells. The GFP-Srp2p protein expression, cell fixation and staining, as well as the cell image production by using fluorescence microscopy, were conducted as in Figure 3, except the original magnification is $\times 400$. (B) Quantitative analysis of the nuclear localization of GFP-Srp2p in wt, $\Delta dsk1$, $\Delta kic1$ and $\Delta dsk1\Delta kic1$ strains. The cells with GFP-Srp2p expression were observed under a microscope to monitor the cellular localization of the fusion protein. The percentage of cells within the area of 1000×1000 pixels that exhibited nuclear localization of GFP-Srp2p was computed.

$\Delta dsk1$ cells in both the nucleus and cytoplasm (Figure 3A, bottom panels). In contrast, the localization pattern of GFP-Srp2p remained the same in $\Delta kic1$ mutants as in the wt strain, that is, in the nucleus (Figure 3B, top panels). To ensure that GFP-Srp2p was not merely piled up on top of the nuclear envelope, the accumulation of GFP-Srp2p in the nucleus was verified by confocal microscopy in *kic1*-deletion cells (Figure 3B, bottom panels). Note the appearance of the yellow colour regions in the merged image and the similar staining pattern of the merge (Figure 3B, bottom right panel) to that of the individual images (bottom left and middle panels), which were captured from the same Z-section plane by using a confocal microscope. The data demonstrate again the overlapping between DAPI/calcofluor staining and GFP signals, confirming the nuclear localization of GFP-Srp2p.

In order to examine further the effect of Dsk1p and Kic1p on the destination of Srp2p in the cell, the localization of GFP-Srp2p in *dsk1kic1* double-deletion mutant was studied by fluorescence microscopy (Figure 4). Consistently, most of the $\Delta dsk1\Delta kic1$ cells expressing the GFP fusion displayed a diffuse cellular localization (Figure 4A), confirming the cytoplasmic distribution of GFP-Srp2p observed in *dsk1* single-deletion mutant lacking a functional Dsk1 kinase (Figure 3A, bottom panels). However, some GFP-Srp2p molecules accumulated in dots of various sizes in the cytoplasm (Figure 4A, middle panel), different from the relatively homogeneous distribution observed in $\Delta dsk1$ cells (Figure 3A, bottom middle panel). To compare the localization of GFP-Srp2p in wt cells and the deletion mutants quantitatively, the cells that exhibited the nuclear localization of GFP-Srp2p

Table 2 Quantitative analysis of nuclear localization of GFP-Srp2p in fission yeast strains with different genetic backgrounds of Dsk1p and Kic1p kinases

Strain	Sample size (cell no.)	Nuclear localization (cell no.)	Cells with nuclear localization (%)
1913 (wt)	212	210	99.1 %
B8 ($\Delta dsk1$)	267	17	6.37 %
2A5 ($\Delta kic1$)	143	141	98.6 %
2D4 ($\Delta dsk1\Delta kic1$)	169	12	7.10 %

were counted and the numbers were expressed as a fraction of the total cells producing the GFP fusion (Figure 4B). Approximately 99 % of the cells in the wt and $\Delta kic1$ population displayed nuclear localization of GFP-Srp2p, whereas only 6–7 % of the cells in the $\Delta dsk1$ and the $\Delta dsk1\Delta kic1$ samples had GFP-Srp2p trafficked to the nucleus (Table 2). In conclusion, the localization of GFP-Srp2p is altered by *dsk1* deletion but not by *kic1* deletion.

The cellular localization of Prp2p is also regulated by Dsk1p

The nuclear distribution of Prp2p was reported based on a large-scale screening of protein cellular localization in fission yeast by using a GFP-fusion genomic DNA library [48]. Here we have examined whether the localization of the splicing factor is regulated by Dsk1p *in vivo*. As anticipated, based on the role of Prp2p in pre-mRNA splicing [34], GFP-Prp2p accumulated in the nuclear region of wt cells (Figure 5A, top panels). Deletion in *dsk1*, however, altered GFP-Prp2p localization from the nucleus to the cytoplasm (Figure 5A, bottom panels). GFP-Prp2p again was retained predominantly in the nucleus of $\Delta kic1$ cells (results not shown) as it was in the wt strain (Figure 5A, top panels). Therefore the efficient nuclear localization of both GFP-Srp2p and GFP-Prp2p requires a functional Dsk1p protein kinase, while deletion in *kic1* has no effect on the cellular distribution of these proteins. In addition, Dsk1 influences the localization of Srp2p in the same fashion as it does that of Prp2p splicing factor in fission yeast; yet in the absence of Dsk1p, Prp2p seems to be excluded from the nucleus (Figure 5A, bottom panels), and Srp2p appears in both the nucleus and the cytoplasm (Figure 3A, bottom panels).

The nuclear localization of GFP-Srp1 is inhibited by Dsk1p activity

We next investigated the cellular localization of Srp1 using the same assay system. As shown in Figure 5(B), the GFP-Srp1p signal was diffuse throughout wt cells in both the cytoplasm and nucleus (Figure 5B, top panels), but deletion in *dsk1*⁺ abrogated the cytoplasmic localization of GFP-Srp1. Instead, GFP-Srp1p in most of the cells became confined to the nucleus of $\Delta dsk1$ cells (Figure 5B, bottom panels), thereby exhibiting a distribution pattern opposite to that of GFP-Srp2p and GFP-Prp2p with respect to the effect of Dsk1p. Table 3 provides a summary of the results obtained in our studies on the cellular localization of fission yeast SR proteins and Prp2p splicing factor in the form of their GFP fusions in wt (1913), $\Delta dsk1$ (B8) and $\Delta kic1$ (2A5) cells.

GFP-Prp2p is functional to complement the growth phenotype of *ts* mutant *mis11/prp2*

To make sure that the GFP-tagged fusion proteins are functional and the results obtained using this ectopic expression system reflect the physiological condition, we tested the ability of GFP-Prp2p to rescue the *ts* mutant *mis11/prp2* [34,37] as an example of a functional assay for the GFP-fusion constructs. According to previous studies, *mis11/prp2* mutant cells are arrested in

Figure 5 Deletion in *dsk1* affects the localization pattern of GFP-Prp2p oppositely to that of GFP-Srp1p

GFP-fusion protein expression and fluorescence microscopy were carried out as in Figure 3. (A) Dsk1p is essential for the nuclear localization of GFP-Prp2p as it is for GFP-Srp2p. Cells with GFP-Prp2p induced were fixed and stained with DAPI or DAPI/calcofluor (left panels) as indicated. (B) Dsk1p negatively influences the nuclear localization of GFP-Srp1p. Cells expressing GFP-Srp1p were fixed and stained with DAPI/calcofluor (left panels). (C) GFP-Prp2p-fusion protein can rescue the growth defect of *mis11/prp2* *ts* mutants. pREP81GFP, pREP81GFPkic1⁺ and pREP81GFPprp2⁺ plasmids were transformed into *mis11/prp2* *ts* mutants and the cells were incubated at 36°C for 4 days (1–3). Colonies of *mis11/prp2* mutant cells rescued by pREP81GFPprp2⁺ were re-streaked out on an EMM2 plate with thiamine and incubated at 36°C for approx. 3 days (4).

interphase and fail to form colonies at the restrictive temperature, 36°C [37]. To complement the cell growth and division phenotype, pREP81GFP, pREP81GFPkic1⁺ and pREP81GFPprp2⁺ plasmids were transformed into the *mis11/prp2* mutant and the cells were incubated at 36°C for 4 days (Figure 5C). Only the transformants harbouring pREP81GFPprp2⁺ grew into colonies and thus rescued the *ts* phenotype of the *mis11/prp2* mutant at the restrictive temperature (Figure 5C, panel 3). No colonies appeared on the plates with the transformants containing either

Table 3 Cellular localization of SR proteins and Prp2p splicing factor in *S. pombe*

GFP-fusion protein	<i>S. pombe</i> strains		
	wt (1913)	$\Delta dsk1$ (B8)	$\Delta kic1$ (2A5)
GFP-Srp1	Cytoplasmic/nuclear	Nuclear	Mixed*
GFP-Srp2	Nuclear	Cytoplasmic/nuclear	Nuclear
GFP-Prp2	Nuclear	Cytoplasmic	Nuclear

* GFP-Srp1 accumulated in the nucleus in approximately half of the cell population and in the cytoplasm in the other half.

pREP81GFP (Figure 5C, panel 1) or pREP81GFPkic1⁺ (panel 2). Further, healthy colonies formed after the rescued *mis11/prp2* mutants containing pREP81GFPprp2⁺ were re-streaked on to an EMM2 plate with thiamine and incubated at 36°C for approx. 3 days (Figure 5C, panel 4). Therefore GFP-Prp2p produced from the recombinant plasmid is functional to complement the *ts* phenotype of the *mis11/prp2* mutant even under the repressed condition (with thiamine). Similar experiments have not been done for GFP-Srp1p and GFP-Srp2p, due to the lack of suitable mutants for functional assays.

DISCUSSION

One of the main questions necessary to understand phosphorylation-based signalling is to identify the substrates of a relevant kinase. We previously showed that as the orthologue of human SRPK1 in fission yeast, Dsk1p specifically phosphorylates SR proteins, Srp1p and Srp2p, as well as splicing factor Prp2p *in vitro* [9,41]. The interaction between Srp1p and Srp2p is inhibited by Dsk1p-mediated phosphorylation [42]. In the work presented here, we investigated the *in vivo* phosphorylation of Srp1p and Srp2p by Dsk1p or Kic1p, and the effect of the phosphorylation on their cellular localization by using the kinase knockout strains of *S. pombe*. Consistent with previous *in vitro* results [9], we have demonstrated that Srp1p and Srp2p are indeed authentic targets of Dsk1p in fission yeast, establishing the first physiological kinase–substrate relationship between Dsk1p and the two SR proteins. Moreover, the *in vivo* phosphorylation of Srp1p and Srp2p by Dsk1p correlates with their distribution pattern in the cell in a *dsk1*⁺-dependent manner, providing biochemical and genetic evidence for the biological significance of Dsk1p function in *S. pombe*.

Our study shows the first successful detection of the *in vivo* antigen of mAb3C5 in fission yeast, although the *in vitro* phosphorylated epitope in *S. pombe* SR proteins has been reported [9]. Combining the phosphopeptide recognition, gel mobility shift and phosphatase treatment of Srp1p and Srp2p, we determined their *in vivo* phosphorylation by Dsk1p and Kic1p. Interestingly, the phosphatase treatment revealed different phosphorylation states of both endogenous Srp1p and Srp2p (Figures 1C and 2C). Multiple bands of distinct mobility were observed in strains with different genetic background of the kinases Dsk1p and Kic1p (Figures 1B and 2B, lanes 1–4). Clearly, the deletion in *dsk1*⁺ changes the phosphorylation status of Srp1p more than the deletion in *kic1*⁺. Therefore Dsk1p is the principal kinase to phosphorylate Srp1p. Further, Kic1p does act on Srp1p, as deletions of both kinases lead to the highest mobility band in gel and thus the completely dephosphorylated state of Srp1p (Figure 1B, top panel, lane 4 and Figure 1C, lane 20). According to the effect of the kinases on the phosphorylation status of Srp1p, Dsk1p and Kic1p perhaps are the only two kinases in the cell responsible for

Srp1p phosphorylation. In contrast, Srp2p is an *in vivo* substrate of Dsk1p but may not be a target for Kic1p, since deletion of *kic1⁺* does not significantly affect the mobility in gel and phosphorylation state of Srp2p in the cell (Figure 2B, lane 2 and Figure 2C, lane 18). Moreover, deletions of both kinases do not result in complete dephosphorylation of Srp2p (Figure 2B, lane 4 and Figure 2C, lane 20), indicating the existence of another kinase(s) besides Dsk1p for Srp2p phosphorylation in fission yeast. The identity of the potential kinase(s) also responsible for the *in vivo* phosphorylation of Srp2p remains to be determined. Supporting this notion, a lack of both Dsk1p and Kic1p in the cell reduced the production of mAb3C5-reactive epitope in Srp2p but did not eliminate the generation of the phosphoepitope (results not shown). Intriguingly, Srp1p reacts more strongly than Srp2p to mAb3C5 (compare Figure 1A with Figure 2A, bottom panels), no matter whether it is GFP-tagged or not. The varying specificities of mAb3C5 for these two proteins may reflect the difference between Srp1p and Srp2p in either the phosphorylation level of the epitope or the sequence/structure surrounding the epitope, or both.

Evidence has been obtained for phosphorylation-dependent nuclear import of SR proteins and RS domain-containing splicing factors in budding yeast and mammalian systems. Phosphorylation of an SR-like protein in *Saccharomyces cerevisiae*, Npl3p, by the orthologue of human SRPK1, Sky1p, is necessary for the efficient binding of Npl3p to its nuclear import receptor Mtr10p [49]. Similar to the cellular localization mechanism in budding yeast, phosphorylation of human SR proteins by SRPK1 is essential for their nuclear import mediated by hMtr10/TRN-SR2 [27].

Our study is the first attempt to determine SR protein distributions in the cell and to assess the *in vivo* effect of the kinases on the cellular localization in fission yeast. To commence their roles as a potential mediator of ESE [33] and as an essential splicing factor [34] respectively, Srp2p and Prp2p must be transported into the nucleus following their synthesis in the cytoplasm. If the role of SRPKs in nuclear import of SR proteins were conserved in eukaryotes, a prediction would be the requirement of Dsk1p for the nuclear localization of Srp2p and Prp2p in *S. pombe*. As anticipated, disrupting the *dsk1⁺* gene in the cell abolishes the nuclear restriction of Srp2p (Figure 3A, bottom panels) and Prp2p (Figure 5A, bottom panels) in fission yeast. Since splicing is partially blocked in *dsk1*-deletion strains, and Prp2p genetically interacts with Dsk1p [9,14], we propose that Dsk1p may accomplish parts of its function in pre-mRNA splicing by controlling the nuclear transport of Prp2p and Srp2p.

Strikingly, the distribution of Srp1p in the cell is differently affected in the *dsk1*-null background. Contrary to the known RS domain-containing factors [27,49], Srp1p is diffuse throughout wt cells but becomes concentrated in the nucleus when *dsk1* is disrupted (Figure 5B). This implicates Dsk1p in the negative modulation of Srp1p nuclear targeting. One scenario may be that Dsk1p-mediated phosphorylation of Srp1p masks or inactivates its NLS, and consequently sequesters most of the molecules in the cytoplasm. An appealing alternative is that non-phosphorylated Srp1p is associated with Srp2p, as reported previously [42], and thus transported into the nucleus as an Srp complex (Figure 6). In this scheme, Dsk1p-mediated Srp1p phosphorylation inhibits the interaction between Srp1p and Srp2p, leading to the retention of Srp1p alone in the cytoplasm. Since Srp2p phosphorylation is required for its efficient nuclear targeting, therefore, to accomplish the Srp1p import with this mechanism, the two SR proteins need to be differentially regulated by Dsk1p under the same condition: Srp1p non-phosphorylated, whereas Srp2p phosphorylated. This may be achieved by higher specificity of Dsk1p for

Figure 6 Cellular localization of SR proteins and Prp2 splicing factor is regulated by Dsk1p-mediated phosphorylation in fission yeast

The efficient nuclear localization of both Srp2p and Prp2p, which allows their function in pre-mRNA splicing, requires phosphorylation by Dsk1p. Phosphorylated Srp2p may be imported into the nucleus and subsequently associated with ESEs to play a role in modulating pre-mRNA splicing in fission yeast. On the other hand, phosphorylation of Srp1p by Dsk1p may facilitate its retention in the cytoplasm by inhibiting the nuclear import or stimulating the nuclear export, or alternatively, disrupting its interaction with Srp2p. SR-specific phosphatases antagonistic to Dsk1p action may exist to dephosphorylate Srp1p, Srp2p and Prp2p. See more details in the Discussion section.

Srp2p than for Srp1p. It is also possible that Srp2p becomes a better substrate for Dsk1p upon binding Srp1p and is thus phosphorylated subsequent to complex formation. In agreement, Dsk1p-mediated Srp2p phosphorylation does not inhibit the Srp complex formation as does Srp1p phosphorylation [42]. The opposing localization pattern of Srp1p compared with that of Prp2p and Srp2p may reflect some unique feature of Srp1p function awaiting determination. Since Srp1p is distributed in the cytoplasm/nucleus in wt cells, it is likely to be an SR protein that shuttles between the two cellular compartments and may thus play a role in mRNA export, as illustrated for SR proteins in mammalian systems [6].

The RS domain of human SR proteins that contain a single RBD/RRM (RNA-recognition motif), such as SC35, is both necessary and sufficient for targeting the proteins to the nucleus and the speckles, thereby acting as a localization signal [50]. Neither the NLS nor the NES (nuclear export signal) of the fission yeast SR proteins has been identified. Reminiscent of the SR protein NLS in mammalian cells, the two short SR repeat elements in Srp2p are indispensable for its nuclear localization and for cell viability [32]. However, without further molecular dissection of the mechanisms, we cannot discern whether Dsk1p facilitates the nuclear distribution of SR proteins by promoting their import into or by inhibiting their export from the nucleus. It is conceivable that Dsk1p may phosphorylate the SR elements of Srp2p to

enhance the ability of the NLS to be recognized by a potential nuclear import receptor yet to be identified. The active role of SRPKs in nuclear import explains why most of these kinases studied, including Dsk1p, Sky1p, SRPK1 and SRPK2, are restricted primarily to the cytoplasm during interphase [7,29,51]. Our study provides further *in vivo* evidence for the conserved function of the SRPKs in governing the cellular localization of SR proteins in eukaryotes. Determining the NLS and NES of the fission yeast SR proteins will offer an insight into the mechanisms of the regulated nuclear targeting process.

As a schematic representation, Figure 6 summarizes the possible effects of Dsk1p-mediated phosphorylation on the cellular localization of newly synthesized SR proteins and Prp2p, combining our previous *in vitro* data and the present *in vivo* results. Phosphorylation of Prp2p and Srp2p by Dsk1p may aid their targeting into the nucleus, thereby facilitating their participation in the spliceosome assembly and ESE activity respectively. The phosphorylation of Srp1p, on the other hand, may restrict Srp1p to the cytoplasm by masking its NLS or by preventing it from association with Srp2p.

One model envisions that Dsk1p affects the cellular localization of the SR proteins through direct phosphorylation. Alternatively, Dsk1p may phosphorylate other factors to invoke the cellular pathways or signalling required for the specific localization of these proteins. Our findings that the *in vivo* phosphorylation of Srp1p and Srp2p by Dsk1p correlates with their cellular localization in a Dsk1p-dependent fashion, in conjunction with our previous reports on the *in vitro* phosphorylation of these proteins by Dsk1p [9,41,42], support the notion that direct phosphorylation of the SR proteins by Dsk1p regulates their distribution in fission yeast. The negative modulation of Srp complex formation by Dsk1p-mediated phosphorylation of Srp1p [42] still requires validation *in vivo*. It will be interesting to investigate whether distinct sites of Srp1p and Srp2p are phosphorylated for the regulation of their nuclear import, Srp complex formation, and their engagement in splicing such as the interaction of Srp2p with ESE.

Individual distributions of Srp1p, Srp2p and Prp2p are differentially affected by Dsk1p; nevertheless, the localization of all three proteins is Dsk1p-dependent and the effects are Dsk1p-specific. As shown previously, *dsk1*-null and *kic1*-null mutations exacerbate each other in growth defect, and expression of the *dsk1*⁺ gene rescues the cell aggregation phenotype of the *kic1*-deletion mutant [9,14]. Conversely, Dsk1p and Kic1p strongly contrast with each other regarding their effect on the cellular localization of the SR proteins and Prp2p splicing factor in fission yeast. Dsk1p alters the distribution of the SR proteins and Prp2p in the cell, while Kic1p has only a partial influence on the cellular localization of Srp1p (Table 3). The overlapping functions of Dsk1p and Kic1p underscore the physiological significance of the study: it reveals the distinctive *in vivo* functions of Dsk1p and Kic1p in *S. pombe*.

Based on our results, neither the mAb3C5-specific phosphorylation of Srp2p nor the cellular localization of Srp2p and Prp2p is sensitive to the absence of Kic1p. However, we cannot rule out the possibility that Kic1p is also able to phosphorylate Srp2p and Prp2p, acting on some sites different from the mAb3C5-reactive sequence to modulate the activity of these proteins. Given the multifaceted roles of SR protein phosphorylation in RNA metabolism, together with the reported functions of Kic1p in cytokinesis, filamentous growth and oxidative-stress response in fission yeast [14,18,30], it will be interesting to identify the potential phosphorylation sites for Kic1p in Srp1p, Srp2p and Prp2p, as well as to discover its new targets other than SR proteins and splicing factors.

Alternative splicing is a highly dynamic and complex process. Because of its similarity of gene structure to metazoans and its possession of splicing regulators, fission yeast is an attractive unicellular model for deciphering the complex mechanism of alternative splicing critical to the regulation of gene expression in eukaryotes. Our study not only demonstrates the conservation of the regulatory role of SR protein kinases in the nuclear localization of SR proteins from fission yeast to human through evolution, but also presents the first biochemical and genetic evidence for the *in vivo* targets of Dsk1p in *S. pombe*, as well as the significant correlation between Dsk1p-mediated phosphorylation and the cellular localization of the SR proteins, thus offering information about the physiological functions of the SRPK1 orthologue. Further investigation of Dsk1p and Kic1p in fission yeast will provide more insight into the mechanisms of SRPK and Clk/Sty kinase functions in all eukaryotes.

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