

UC Irvine

UC Irvine Previously Published Works

Title

Release of SR Proteins from CLK1 by SRPK1: A Symbiotic Kinase System for Phosphorylation Control of Pre-mRNA Splicing.

Permalink

<https://escholarship.org/uc/item/8067t0cm>

Journal

Molecular cell, 63(2)

ISSN

1097-2765

Authors

Aubol, Brandon E
Wu, Guowei
Keshwani, Malik M
et al.

Publication Date

2016-07-01

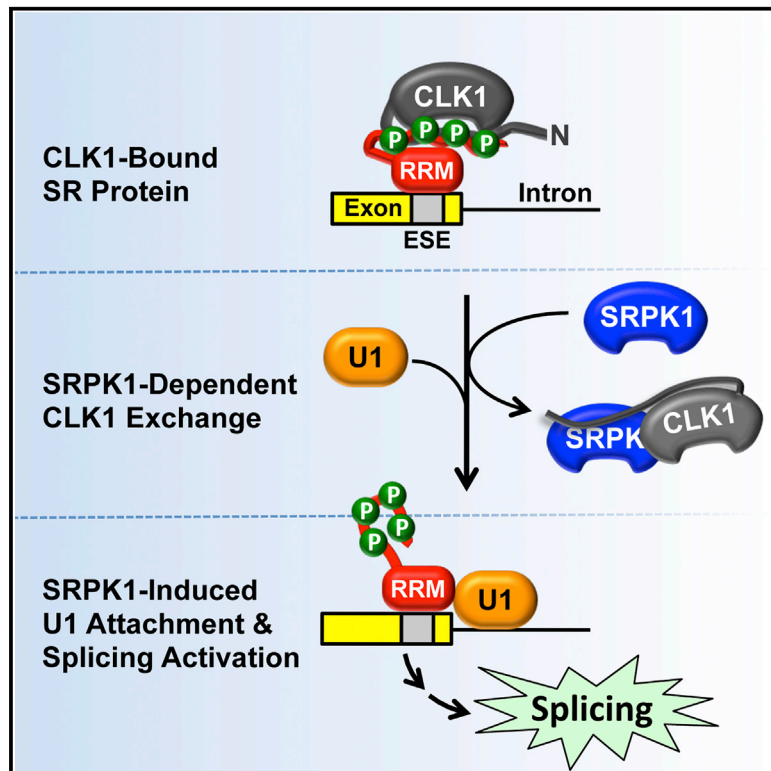
DOI

10.1016/j.molcel.2016.05.034

Peer reviewed

Release of SR Proteins from CLK1 by SRPK1: A Symbiotic Kinase System for Phosphorylation Control of Pre-mRNA Splicing

Graphical Abstract



Authors

Brandon E. Aubol, Guowei Wu, Malik M. Keshwani, ..., Klemens J. Hertel, Xiang-Dong Fu, Joseph A. Adams

Correspondence

j2adams@ucsd.edu

In Brief

Although CLK1 is required for splicing, this protein kinase does not efficiently dissociate its product, phosphorylated splicing factor SRSF1. Aubol et al. show that nuclear SRPK1 strips CLK1 from the tightly bound SRSF1, freeing the splicing factor for engagement with the U1 component of the spliceosome and splicing activation.

Highlights

- SRPK1 and CLK1 form a stable complex in the nucleus of cells
- The kinase domain of SRPK1 interacts with the disordered N terminus of CLK1
- SRPK1 acts as a release factor that strips CLK1 from tightly bound SR proteins
- SRPK1-induced release promotes U1 binding and mRNA splicing



Release of SR Proteins from CLK1 by SRPK1: A Symbiotic Kinase System for Phosphorylation Control of Pre-mRNA Splicing

Brandon E. Aubol,¹ Guowei Wu,² Malik M. Keshwani,¹ Maliheh Movassat,³ Laurent Fattet,¹ Klemens J. Hertel,³ Xiang-Dong Fu,² and Joseph A. Adams^{1,*}

¹Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093, USA

²Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA 92093, USA

³Department of Microbiology and Molecular Genetics, University of California, Irvine, Irvine, CA 92697, USA

*Correspondence: j2adams@ucsd.edu

<http://dx.doi.org/10.1016/j.molcel.2016.05.034>

SUMMARY

Phosphorylation has been generally thought to activate the SR family of splicing factors for efficient splice-site recognition, but this idea is incompatible with an early observation that overexpression of an SR protein kinase, such as the CDC2-like kinase 1 (CLK1), weakens splice-site selection. Here, we report that CLK1 binds SR proteins but lacks the mechanism to release phosphorylated SR proteins, thus functionally inactivating the splicing factors. Interestingly, CLK1 overcomes this dilemma through a symbiotic relationship with the serine-arginine protein kinase 1 (SRPK1). We show that SRPK1 interacts with an RS-like domain in the N terminus of CLK1 to facilitate the release of phosphorylated SR proteins, which then promotes efficient splice-site recognition and subsequent spliceosome assembly. These findings reveal an unprecedented signaling mechanism by which two protein kinases fulfill separate catalytic features that are normally encoded in single kinases to institute phosphorylation control of pre-mRNA splicing in the nucleus.

INTRODUCTION

Alternative mRNA splicing, prevalent in higher eukaryotic genomes, has the potential to singularly magnify the proteome size from a rather limited set of genes in development and disease (Nilsen and Graveley, 2010; Wang and Cooper, 2007). Splicing is tightly coupled with transcription, nuclear export, and mRNA stability, thereby profoundly influencing the expression of gene products (Maniatis and Reed, 2002; Moore and Proudfoot, 2009; Pandit et al., 2008). Despite significant progress in the past two decades, we still have very limited knowledge of the molecular mechanisms underlying regulated splicing (Shin and Manley, 2004). Because up to 60% of disease-causing mutations are linked to defects in alternative splicing (Wang and Cooper, 2007), an understanding of splicing mechanisms and

their regulation continues to represent a major challenge in the post-genome era.

Recent studies have elucidated the role of cellular signaling in the regulation of alternative splicing (Fu and Ares, 2014; Zhou et al., 2012). Most important among different regulatory strategies are the protein kinases and phosphatases that control the activities of various splicing factors through reversible phosphorylation (Stamm, 2008). Protein kinases are key regulators of various cellular processes and have uniquely evolved mechanisms to carry out selective phosphorylation of their substrates. Their substrate selectivity has been attributed, in part, to the catalytic domain that recognizes preferred amino acids (Endicott et al., 2012; Taylor and Kornev, 2011). Protein kinases also encode modular domains in their gene structure that impart secondary binding surfaces for substrate selection as well as serve as signals for their sub-cellular localization and guidance toward physiological substrates (Parsons and Parsons, 2004; Taylor et al., 2012). These modular domains can bind to scaffold proteins that serve as platforms for both the protein kinases and their cognate substrates (Parsons and Parsons, 2004). Additionally, protein kinases have also adopted dimerization strategies for activation and substrate phosphorylation. Some protein kinases form homodimers (e.g., receptor tyrosine kinases), whereas a few form heterodimers (Lavoie et al., 2014; Lemmon and Schlessinger, 2010). The heterodimer assembly between inactivated BRAF and active CRAF activates the latter during MEK-ERK signaling (Poulikakos et al., 2010). RAF heterodimerization suggests a critical disease mechanism, because such elegant arrangement was found in melanoma cells that develop resistance in the background of the most common BRAF (V600E) mutation (Lavoie et al., 2014; Poulikakos et al., 2010).

Alternative splicing is facilitated by the reversible phosphorylation of the SR protein family of splicing regulators (Pandit et al., 2008). Two families of protein kinases specifically phosphorylate these essential splicing factors. The serine-arginine protein kinases (SRPK1–3) strictly phosphorylate Arg-Ser dipeptides, whereas the CDC2-like kinases (CLK1–4) can phosphorylate both Arg-Ser and Ser-Pro dipeptides, common in all SR proteins (Ghosh and Adams, 2011) (Figure 1A). Although SRPKs use a docking groove in the C-terminal lobe of the kinase domain for substrate recognition and phosphorylation, CLKs lack such a

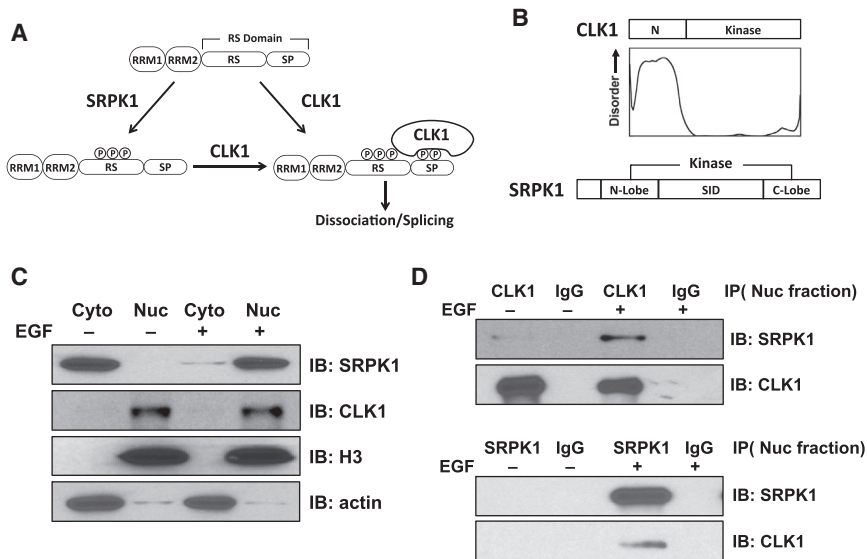


Figure 1. SRPK1 and CLK1 Form a Nuclear Complex

(A) SRPK1 phosphorylates Arg-Ser repeats (RS) and CLK1 phosphorylates these and Ser-Pro dipeptides (SP) in the SRSF1 RS domain.

(B) SRPK1 and CLK1 domain structures. CLK1 N terminus is predicted to be disordered using DISOPRED.

(C) Cytoplasmic and nuclear fractionations of SRPK1 and CLK1 in HeLa cells with and without EGF stimulation.

(D) Co-immunoprecipitation of SRPK1 and CLK1 in nuclear lysates of HeLa cells.

groove and instead use a disordered N-terminal domain to bind SR proteins with very high affinity (Figure 1B).

Although SRPKs are located in both the cytoplasm and nucleus, their function in the cytoplasm is best understood. SRPKs support phosphorylation-dependent transport of SR proteins from the cytoplasm to the nucleus via an SR-specific transport protein, TRN-SR2 (Kataoka et al., 1999; Lai et al., 2001; Yun et al., 2003). Unlike SRPKs, CLKs possess a nuclear localization signal in their N termini and are thus localized strictly to the nucleus, where they play a vital role in mobilizing SR proteins from speckles to sites of active gene splicing via Ser-Pro phosphorylation (Keshwani et al., 2015a). This has led to a simple model in which SRPKs generate basal SR protein phosphorylation levels in the cytoplasm and CLKs enhance phosphorylation in the nucleus for splicing regulation (Ding et al., 2006; Keshwani et al., 2015a). However, this simplistic relay mechanism fails to explain two long-standing issues regarding the role of phosphorylation in alternative splicing. First, while phosphorylation is thought to enhance the activity of SR proteins in splice-site recognition, high levels of CLK paradoxically inhibit this function (Prasad et al., 1999). Second, as both SRPKs and CLKs are also present in the nucleus, this sets up a potential competitive rather than a cooperative relationship, because we recently found that although SRPK has built-in sequences that assist in the release of SR proteins after phosphorylation (Aubol et al., 2014; Koizumi et al., 1999), CLK does not release fully phosphorylated SR proteins (Aubol et al., 2014). These findings also suggest that CLKs may require a release factor to generate functional SR proteins for splicing function.

Epidermal growth factor (EGF) signaling increases nuclear levels of SRPKs and enhances bulk SR protein phosphorylation (Zhou et al., 2012). This mechanism involves the Akt-dependent release of several chaperones (Hsp70/90) from the spacer insert domain (SID) in SRPK1 that pins the kinase in the cytoplasm (Zhong et al., 2009) (Figure 1B). Such signaling affects the alternative splicing of numerous genes and hints to a role for SRPKs in the nucleus. Furthermore, osmotic stress increases nuclear

of SRPKs in the nucleus. We here report that endogenous SRPK1 and CLK1 interact in the nucleus, forming a complex. Surprisingly, rather than acting in a competitive manner, the dual kinase complex acts symbiotically to associate with and phosphorylate their substrates with SRPK1, functioning as a release factor for CLK1, unleashing phosphorylated SR proteins for the promotion of spliceosome assembly. These findings solve some major puzzles from previous studies and, more importantly, reveal a signaling paradigm where two protein kinases are required to catalyze a full phosphorylation cycle (from binding to release) of their substrates.

RESULTS

SRPK1 Forms a Complex with CLK1 in Cells

SRPKs and CLKs have separate functions in the cell with regard to SR proteins. Whereas cytoplasmic SRPKs facilitate phosphorylation-dependent transport of SR proteins to the nucleus, CLKs perform additional phosphorylation steps that mobilize SR proteins for splicing activity in the nucleus (Keshwani et al., 2015a). What has gone unappreciated is that SRPKs are present not only in the cytoplasm but also in the nucleus (Colwill et al., 1996; Naylor et al., 1997; Wang et al., 1998). We showed in a previous study that, upon EGF stimulation, SRPK1 levels can be greatly increased in the nucleus, where CLKs are localized (Zhou et al., 2012), an observation that we confirmed through fractionation studies (Figure 1C). This raises the question of how the two kinases function in the same compartment. To begin to address this, we determined whether these kinases can interact with one another in the nucleus by co-immunoprecipitation using specific monoclonal antibodies. We found that anti-CLK1 efficiently co-immunoprecipitates with SRPK1 (Figure 1D, top) and, similarly, anti-SRPK1 can capture CLK1 (Figure 1D, bottom) in EGF-treated HeLa cells. These findings indicate that CLK1 and SRPK1 have the capacity to form a stable complex in the nucleus.

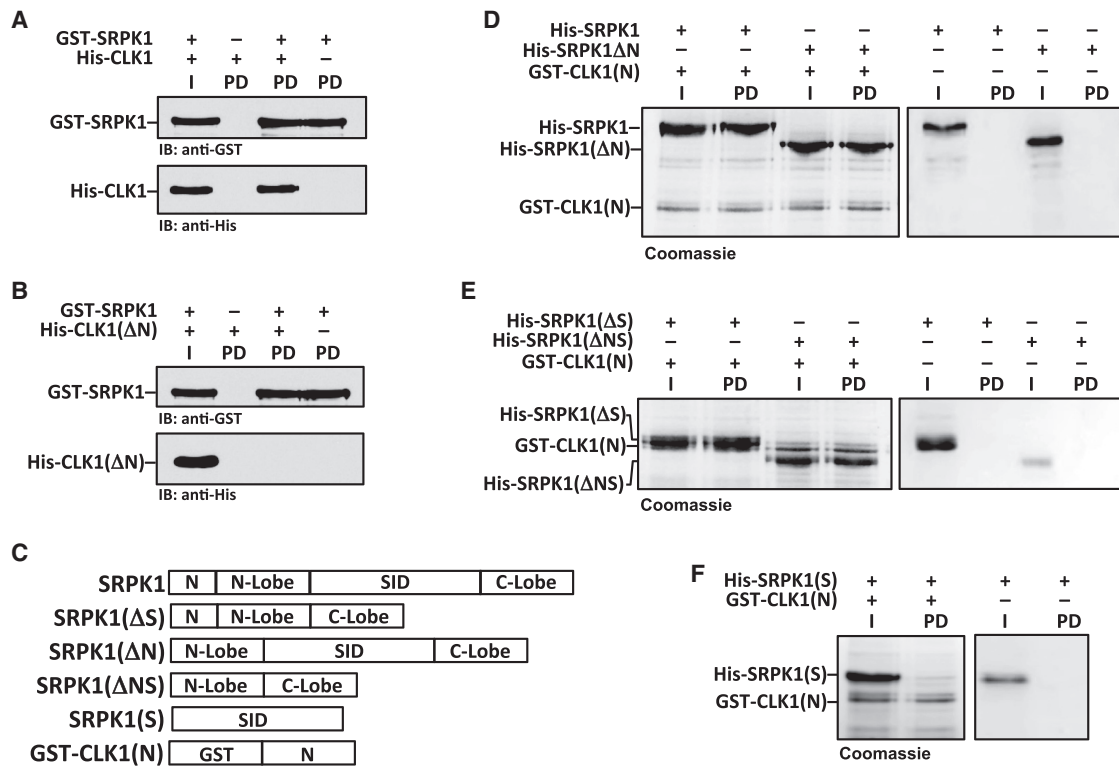


Figure 2. The CLK1 N Terminus Interacts with the SRPK1 Kinase Domain

(A and B) Pull-downs of GST-SRPK1 and His-CLK1 (A) or His-CLK1(ΔN) (B). I, input; PD, pull-down.

(C) Deletion constructs of His-SRPK1 and GST-CLK1(N).

(D–F) Pull-downs of His-SRPK1 deletions using GST-CLK1(N). I, input; PD, pull-down.

The CLK1 N Terminus Interacts with the SRPK1 Kinase Domain

We showed previously that the CLK1 N terminus binds with high affinity to the RS domain of its substrate SRSF1 (Aubol et al., 2014). Furthermore, we showed that the N terminus also interacts with its kinase domain but is not a substrate, suggesting that it can bind different proteins in an intra- and intermolecular fashion (Aubol et al., 2014; Colwill et al., 1996). To determine whether the CLK N terminus also interacts with SRPK, we performed *in vitro* pull-down experiments using glutathione S-transferase (GST)-tagged SRPK1 with either full-length His-tagged CLK1 or a form lacking its N terminus (CLK1(ΔN)). We found that GST-SRPK1 pulled down full-length CLK1, but not CLK1(ΔN) (Figures 2A and 2B). As controls, we showed that CLK1 and CLK1(ΔN) did not interact with the glutathione-agarose resin. These findings suggest that the N terminus is the principle domain that stabilizes the CLK-SRPK complex.

To determine how the N terminus interacts with SRPK1, we performed a series of pull-down experiments using a GST-tagged form of the CLK1 N terminus (GST-CLK1(N)) and several SRPK1 truncation mutants (Figure 2C). We found that GST-CLK1(N) interacts strongly with SRPK1 and all truncations that include the kinase domain (Figures 2D and 2E). In comparison, GST-CLK1(N) did not pull down SRPK1(S), suggesting that the N terminus does not form a stable complex with the SID (Figure 2F). As controls, we showed that all SRPK1 forms did not

interact with glutathione-agarose resin without GST-CLK1(N) (Figures 2D–2F). Together, these results suggest that the CLK1 N terminus interacts with the SRPK1 kinase domain.

CLK1 Regulates Nuclear Levels of SRPK1

Although prior studies showed that SRPKs are maintained in the cytoplasm through chaperone binding (Hsp70/90) (Zhong et al., 2009), it is unclear whether there are nuclear constituents that help maintain nuclear pools of the kinase. We addressed whether complex formation with CLK anchors SRPK1 in the nucleus. By monitoring endogenous SRPK1 in HeLa cells using confocal microscopy, we found SRPK1 predominantly in the cytoplasm with a small fraction in the nucleus (Figure 3A), consistent with our fractionation studies and previous findings (Gui et al., 1994). We then transfected an RFP-tagged CLK1 (CLK1-RFP) that expressed to an observed level six times that of the endogenous kinase and found that it increased the nuclear SRPK1 levels relative to the cytoplasmic pools (Figures 3B and 3E). This result suggests that the SRPK1 cytoplasmic/nuclear levels are controlled by an equilibrium set by chaperones in the cytoplasm and CLK1 in the nucleus. Expressed CLK1 shifts this equilibrium toward greater levels of nuclear SRPK1 in accordance with mass action. To determine whether this phenomenon is dependent on catalytic activity, we expressed a kinase inactive CLK1 (kdCLK1-RFP) and found that it also induced nuclear SRPK1 (Figures 3C and 3E). To determine whether the

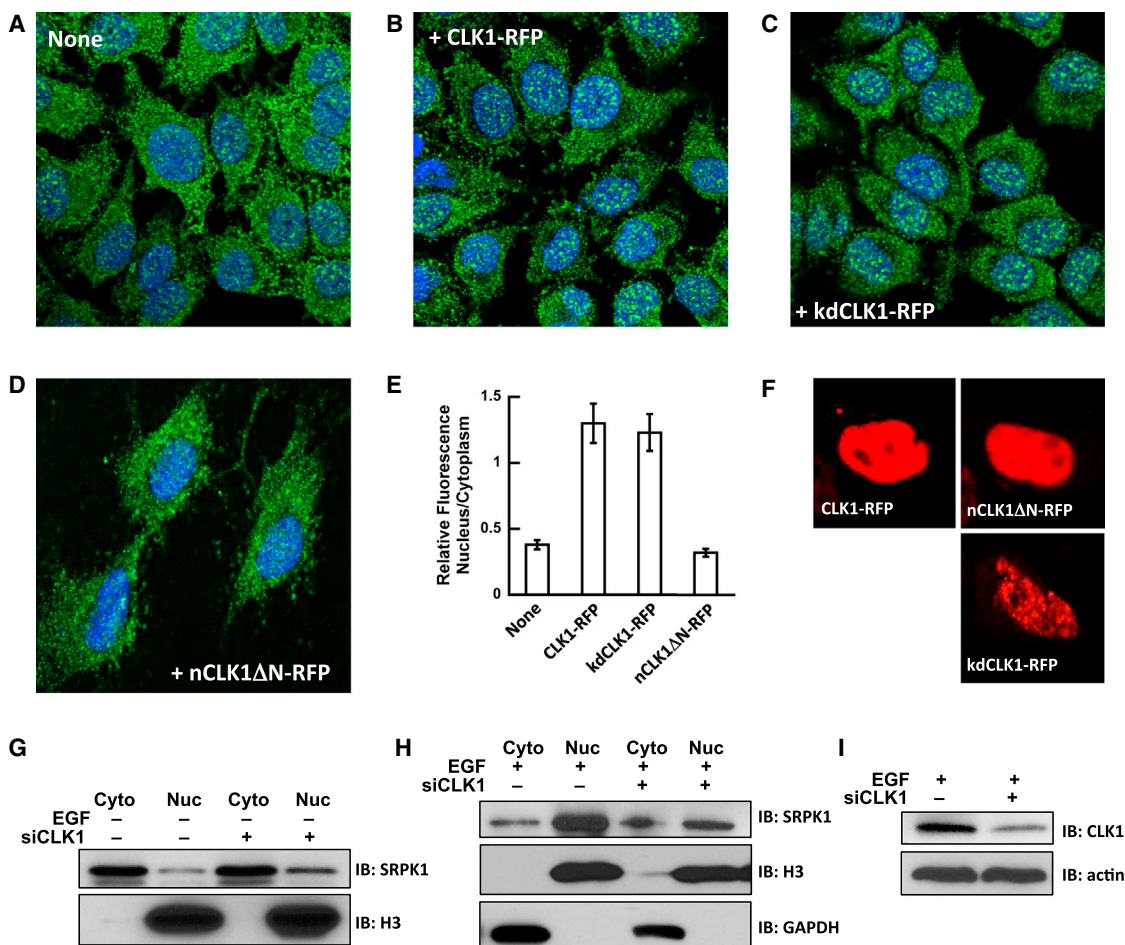


Figure 3. CLK1 Expression and SRPK1 Subcellular Localization

(A–D) Confocal imaging of endogenous SRPK1 (green) in HeLa cells transfected with mock (A), CLK1-RFP (B), kdCLK1-RFP (C), and nCLK1ΔN-RFP (D). Nuclei are stained with DAPI (blue).

(E) Relative amounts of nuclear and cytoplasmic SRPK1 with and without CLK1 expression. Fractional amounts are calculated using ImageJ. The data represent an average of $n = 3$, and error bars indicate \pm SD.

(F) Confocal imaging of CLK1-RFP, kdCLK1-RFP and nCLK1ΔN-RFP showing nuclear localization.

(G and H) Nuclear and cytoplasmic SRPK1 levels in HeLa cells treated with CLK1 siRNA in the absence (G) and presence (H) of EGF stimulation.

(I) CLK1 levels in HeLa cells treated with CLK1 siRNA.

N-terminal RS-like domain of CLK1 plays a role in nuclear retention of SRPK1, we expressed a version of CLK1 that replaces the N terminus with a nuclear localization sequence from nucleoplasmin 2 (KRLVPQKQASVAKKKK) and found that this new construct, nCLK1ΔN-RFP, did not increase nuclear SRPK1 levels (Figures 3D and 3E). CLK1-RFP, kdCLK1-RFP, and nCLK1ΔN-RFP are all exclusively present in the nucleus, indicating that the increased nuclear SRPK1 is due to increased nuclear CLK1 (Figure 3F).

We next addressed whether CLK1 is required for SRPK1 nuclear localization by disrupting CLK1 using small interfering RNA (siRNA) methods. CLK1 depletion did not affect SRPK1 levels in unstimulated cells, where most of the kinase is present in the cytoplasm (Figure 3G). However, in EGF-stimulated cells when SRPK1 is mostly in the nucleus, CLK1 depletion had little effect on the levels of SRPK1 in the cytoplasm but prevented

the accumulation of SRPK1 in the nucleus (Figure 3H), likely due to unstable SRPK1 in the nucleus in the absence of CLK1. In control experiments, we showed that siRNA-treated cells displayed a significant decrease in CLK1 (Figure 3I). These observations suggest that CLK1 acts as a nuclear anchor for SRPK1 through complex formation and that the CLK1 N terminus is necessary for this function.

SRPK1 Releases Phospho-SRSF1 from CLK1

Although SRPK1 forms a highly stable complex with its substrate, SRSF1, multi-site RS-domain phosphorylation induces dissociation (Aubol and Adams, 2011; Ma et al., 2010). We verified these results in pull-down assays by incubating GST-SRSF1 with SRPK1 in the absence and presence of ATP. GST-SRSF1, bound to glutathione beads, strongly pulls down SRPK1 in the absence, but not in the presence, of ATP (Figure 4A). In

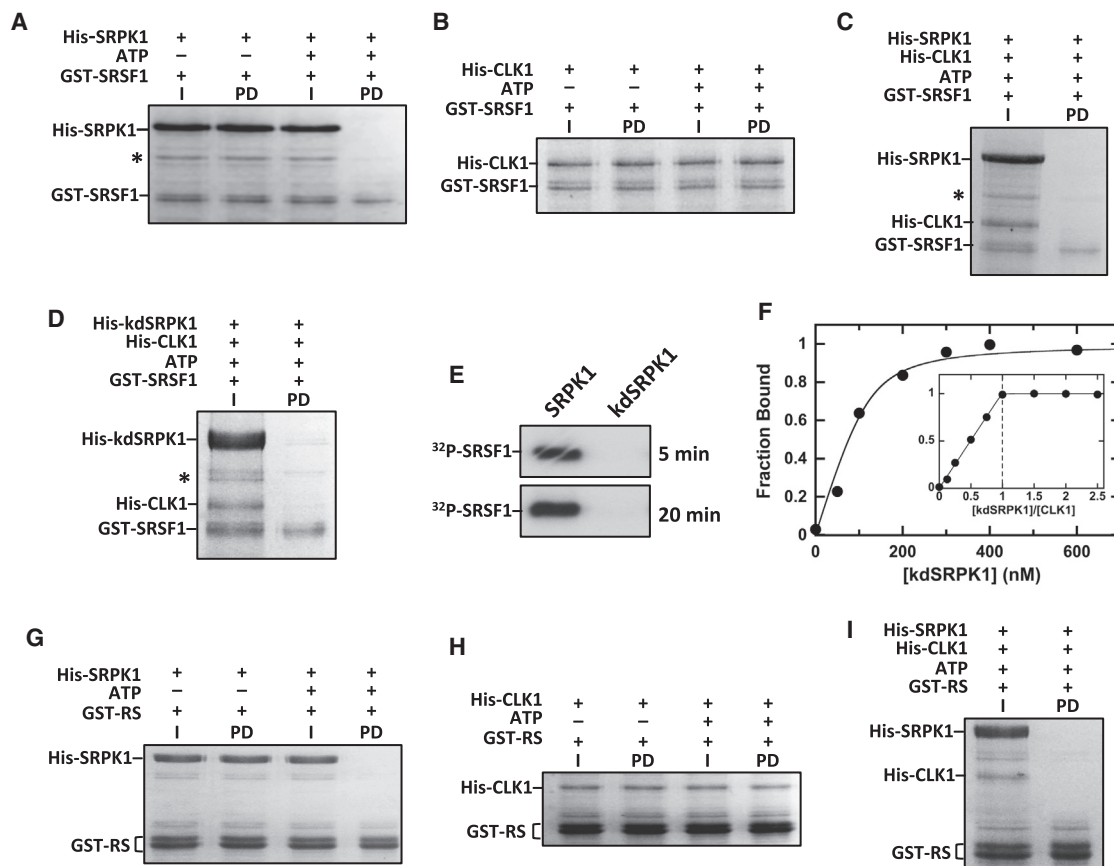


Figure 4. SRPK1 Releases CLK1 from Phosphorylated SRSF1

(A and B) Phosphorylation disrupts SRSF1 interaction with SRPK1, but not CLK1. Immobilized GST-SRSF1 is incubated with His-SRPK1 (A) or His-CLK1 (B) with ATP, washed, and run on SDS-PAGE. I, input; PD, pull-down.

(C and D) Binding of phosphorylated SRSF1 to His-CLK1 is disrupted by His-SRPK1 (C) and His-kdSRPK1 (D). Immobilized GST-SRSF1 is incubated with His-CLK1 and ATP and treated with His-SRPK1 or His-kdSRPK1. I, input; PD, pull-down.

(E) SRSF1 (0.2 μ M) is phosphorylated by SRPK1 (1 μ M), but not kdSRPK1 (1 μ M).

(F) Complex affinity and stoichiometry. His-CLK1 fraction bound versus His-kdSRPK1 using 100 nM His-CLK1 is fit to Equation 1 to obtain a K_d of 16 ± 6 nM and R_0 of 120 ± 17 nM. Fraction bound using 400 nM His-CLK1 is plotted as a ratio of total His-kdSRPK1 to His-CLK1, establishing a complex stoichiometry of 1:1.

(G and H) Phosphorylation disrupts RS domain interactions with His-SRPK1, but not His-CLK1. GST-RS is incubated with His-SRPK1 (G) or His-CLK1 (H) in the presence of ATP, bound to glutathione-agarose resin, washed, and run on SDS-PAGE. I, input; PD, pull-down.

(I) Binding of phosphorylated RS domain to His-CLK1 is disrupted by His-SRPK1. GST-RS is incubated with His-CLK1 and ATP, treated with His-SRPK1, bound to glutathione-agarose resin, washed, and run on SDS-PAGE. I, input; PD, pull-down.

comparison, although CLK1 also forms a very stable complex with SRSF1 similar to that observed with SRPK1, phosphorylation of the RS domain does not reduce the stability of the CLK1-SRSF1 complex (Figure 4B). This suggests that, when acting alone, CLK1 would behave as an inhibitor by titrating free SR proteins in the nucleus.

Given the ability of SRPK1 to interact with CLK1, we were intrigued by the possibility that SRPK1 might serve as a release factor for phospho-SRSF1 from CLK1. To this possibility, we performed pull-down experiments using phosphorylated GST-SRSF1 in the presence of SRPK1. We showed that CLK1-phosphorylated GST-SRSF1 readily dissociates CLK1 in the presence of SRPK1 (Figure 4C). This function does not depend on catalytic activity, because the kinase-inactive SRPK1 (kdSRPK1) also leads to dissociation (Figure 4D). In control experiments, we

showed that kdSRPK1 is inactive and does not phosphorylate SRSF1 in the time frame of the exchange reaction (Figure 4E). Thus, while CLK1 forms a complex with phosphorylated SRSF1, SRPK1 facilitates the release of the splicing factor from CLK1. This process is not a simple competitive event with regard to the SR protein, because SRPK1 does not bind phosphorylated SRSF1 under these conditions (Figure 4A).

To measure the affinity of the SRPK1-CLK1 complex, we used the ability of SRPK1 to displace phospho-SRSF1 from CLK1 as a reporter for the kinase-kinase complex. We phosphorylated GST-SRSF1 (500 nM) with His-tagged CLK1 (100 nM) and 32 P-ATP, bound the kinase to the Ni-resin, and then added varying kdSRPK1 to displace the phospho-SR protein (Figure 4F). We used kdSRPK1 to avoid any potential additional phosphorylation of the substrate. The fraction bound of CLK1 is plotted against

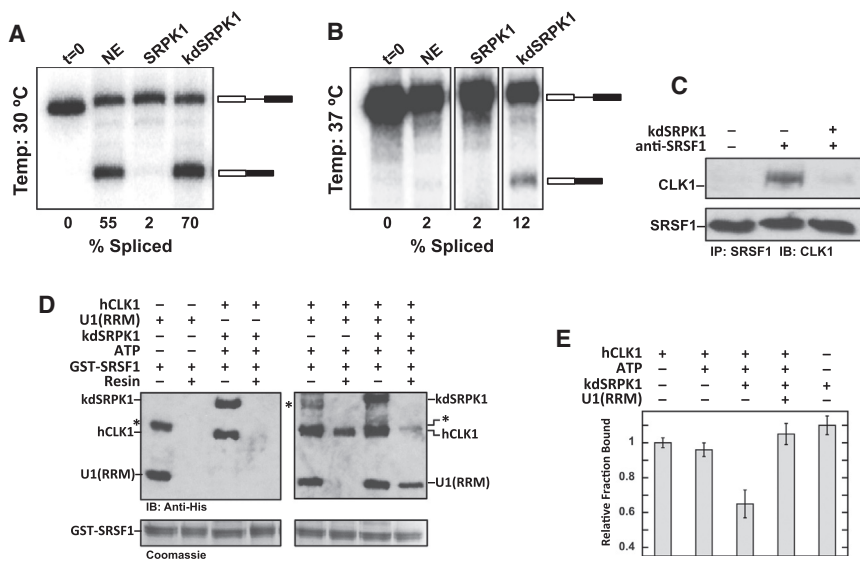


Figure 6. SRPK1-Induced Release Affects Splicing of β -Globin Pre-mRNA

(A and B) Splicing of the β -globin gene in nuclear extracts in the absence and presence of exogenous SRPK1 and kdSRPK1 at 30°C (A) and 37°C (B). Several unrelated lanes were removed from the gel in (B).

(C) SRPK1 induces release of SRSF1 from CLK1 in nuclear extracts. SRSF1 is immunoprecipitated and probed for CLK1 with and without exogenous kdSRPK1. Top (first lane): nuclear lysate with only protein G beads and no SRSF1 antibody.

(D) CLK1 release promotes binding of U1(RRM) to SRSF1. Interaction of U1(RRM) with GST-SRSF1 bound to glutathione-agarose beads is probed using an anti-His antibody. An asterisk represents an impurity in the U1(RRM) preparation.

(E) Binding of the Ron ESE to SRSF1 with and without CLK1 and kdSRPK1. The data are an average of $n = 3$. Error bars indicate \pm SD.

and active site of SRPK1 and thus cannot permit simultaneous association of both SRPK1 and CLK1 (Ngo et al., 2008). We showed that GST-RS1 binds well to both SRPK1 and CLK1 in separate pull-down experiments (Figure 5C). However, SRPK1 and CLK1 binding was not affected when both were added, a result that is not consistent with a competitive relationship between the kinases. Instead, these findings suggest that both kinases can bind simultaneously to RS1. Given the limited binding surface of RS1, these observations suggest that CLK1 and SRPK1 form a stable complex that fully phosphorylates the RS domain of SRSF1.

SRPK1 Is the Primary Catalyst in the SRPK1-CLK1 Complex

Having shown that SRPK1 and CLK1 phosphorylate the SRSF1 RS domain as a complex, we next wished to determine which of the kinases take the principle role in phosphorylating the RS domain. To address this, we performed single-turnover experiments in which a CLK-specific inhibitor, TG003, was added to the SRPK1-CLK1 complex prior to reaction initiation. If SRPK1 is the principle kinase that binds the RS domain and rapidly phosphorylates the Arg-Ser dipeptides whereas CLK1 mostly modifies the Ser-Pro dipeptides, then we expect that TG003 addition will not affect the rapid, initial kinetic phase in the progress curve for the complex. Indeed, we observed that TG003 did not impact the fast phase for the complex but instead slowed down later phosphorylation events, suggesting that SRPK1 is the primary kinase that binds the RS domain and performs rapid Arg-Ser phosphorylation (Figure 5D). To confirm inhibitor efficacy, we showed that TG003 addition to CLK1 by itself significantly inhibited SRSF1 phosphorylation (Figure 5D). These data suggest that SRPK1 takes on a dominant role in binding the RS domain in the SRPK1-CLK1 complex, rapidly phosphorylating Arg-Ser dipeptides. By itself, CLK1 can also phosphorylate Arg-Ser dipeptides, but in the complex, is likely to take on the specialized function of phosphorylating Ser-Pro dipeptides.

SRPK1-Induced Release Enhances Splicing of β -Globin Pre-mRNA

Having demonstrated that SRPK1 is a release factor for CLK1 in vitro, we wished to determine whether SRPK1 could also serve a similar role in nuclear extracts to regulate splicing. To address this possibility, we studied splicing of β -globin pre-mRNA in HeLa cell nuclear extracts. We found that the addition of SRPK1 to the extracts significantly inhibited β -globin pre-mRNA splicing (Figure 6A). This result was previously ascribed to a misbalance in the phosphorylation-dephosphorylation cycle necessary for spliceosome assembly and subsequent catalysis (Gui et al., 1994). As a control, we added kdSRPK1 to the nuclear extracts and, unexpectedly, found that it enhanced splicing of β -globin pre-mRNA (Figure 6A).

In vitro splicing assays are typically performed at 30°C, an optimum temperature for nuclear extracts (Movassat et al., 2014; Xiao and Manley, 1998). Given that SRPKs are part of the spliceosome (Mathew et al., 2008), we wished to determine whether the addition of exogenous SRPK1 could stabilize the spliceosome, thus enabling splicing at non-permissible temperatures. We showed that while nuclear extracts are not capable of facilitating splicing at 37°C, kdSRPK1 addition indeed stimulated splicing function (Figure 6B). These findings indicate that SRPK1 can facilitate splicing in a phosphorylation-independent manner. To determine whether the general splicing enhancement at either temperature could be linked to SRPK1-dependent substrate release, we monitored the interaction of CLK1 and SRSF1 in nuclear extracts. We found that although endogenous CLK1 interacts with endogenous SRSF1 in nuclear extracts, the addition of recombinant kdSRPK1 dissociates this complex (Figure 6C). These findings strongly suggest that SRPK1-induced release of SRSF1 from CLK1 may account for enhanced splicing.

CLK1 Dissociation Induces U1 RRM Binding to SRSF1

Previous studies from the Ghosh lab showed that a phosphomimetic form (poly-serine-to-glutamate mutant) representing the

CLK-phosphorylated state of SRSF1 associated favorably with the RRM from the 70K subunit of U1 small nuclear ribonucleoprotein particle (snRNP) (U1(RRM)) whereas the unphosphorylated, wild-type SRSF1 did not (Cho et al., 2011). These studies suggest that CLK-phosphorylated SRSF1 supports establishment of the 5' splice site through interactions between the RRMs from SRSF1 and U1 snRNP. Given the pivotal role for CLK1 in establishing this interaction, we wished to determine whether SRPK1-induced release of SRSF1 could play a role in this key step of splicing initiation. We performed pull-down assays and found that U1(RRM) did not interact with unphosphorylated GST-SRSF1, consistent with the previous study (Cho et al., 2011) (Figure 6D, left, second lane). Surprisingly, we found that CLK1-phosphorylated GST-SRSF1 also did not interact with U1(RRM), suggesting that phosphorylation alone is not sufficient to initiate binding (Figure 6D, right, second lane). Strikingly, kdSRPK1 addition dissociates CLK1 and strongly promotes U1(RRM) binding to SRSF1 (Figure 6D, right, last lane). These findings indicate that SRPK1-induced release of CLK1-phosphorylated SRSF1 is needed to support the binding of U1(RRM) to SRSF1.

U1 RRM Binding and SRPK1-Induced Release Alters RNA Binding to SRSF1

SR proteins promote pre-mRNA splicing by binding to exonic splicing enhancers (ESEs) in pre-mRNA. We wished to define the potential role of CLK1 phosphorylation and SRPK1-induced release on this recognition mechanism. To accomplish this, we monitored SRSF1 binding to the ESE from the Ron proto-oncogene (5'-AGGCGGAGGAAGC-3') using a filter-binding assay (Aubol et al., 2014). We found that SRSF1 binds the ESE similarly in the presence of CLK1 whether or not the RS domain was phosphorylated (Figure 6E, first two columns), indicating that CLK1 does not interfere with the RNA binding property of the SR protein. In contrast, kdSRPK1 addition to the CLK1-pSRSF1-RNA complex reduced RNA binding by ~35%, suggesting that CLK1 release causes the splicing factor to adopt a less productive conformation for RNA binding (Figure 6E, third column). As control, we showed that kdSRPK1 alone does not reduce ESE affinity to SRSF1 in the absence of phosphorylation (Figure 6E, last column). Interestingly, U1(RRM) addition to CLK1-phosphorylated SRSF1 lacking bound CLK1 led to restored high-affinity binding of the SR protein to the ESE (Figure 6E, fourth column). Together, these findings suggest that, while SRPK1-induced release generates a form of SRSF1 that binds poorly to RNA in isolation, the released SR protein favors the formation of the ternary complex with U1 and ESE, which likely reflects the active process in 5' splice site recognition in the cell.

DISCUSSION

Enzymes have highly evolved active sites containing residues that specifically bind substrates with high affinity and position select functional groups for the ensuing catalytic steps. Indeed, the front end and internal portion of the enzymatic reaction is typically so fine-tuned that the product is oftentimes not easily released, thus leading to buildup of an inhibitory

complex. Enzymes have accordingly developed strategies to destabilize the product so that catalytic cycling can proceed unimpeded. The splicing kinase SRPK1 binds the RS domain of the SR protein SRSF1 with unusually high affinity and then initiates a series of fast phosphoryl transfer steps. During these modifications, significant negative charge develops that clashes with the negatively charged docking groove in the kinase domain. The result is a progressive destabilization of the phospho-RS domain and rapid release of the SR protein. Transient-state kinetic studies showed that the binding affinity of the product is reduced by about two orders of magnitude after eight phosphate additions, the minimum number required for translocation into the nucleus (Aubol and Adams, 2011). In contrast, CLK1 incorporates an alternative strategy for SR protein recognition that uses a disordered N-terminal extension rather than a docking groove in the kinase domain (Aubol et al., 2014). This thematic variation is highly effective for binding SR proteins with very high affinity but does not offer a discriminatory mechanism between substrate and product. This observation raises the question of how CLKs release phospho-SR proteins for splicing.

Our results describe a unique paradigm in which two splicing kinases (CLK and SRPK) develop a symbiotic relationship to functionally compensate for their limitations and cooperatively facilitate the release of phospho-SRSF1. In the nucleus, SRPK1 strips the N terminus from the phospho-RS domain, thereby destabilizing its interaction with CLK1 to generate free SR protein (Figure 7A). This mechanism bears some resemblance to a classic exchange process akin to the guanine exchange factors (GEFs) that destabilize and promote GDP release from the Rho GTPases (Rossman et al., 2005). However, unlike a simple inert exchange factor, SRPK1 is also a catalyst and works symbiotically with CLK1 to yield phosphorylated SR proteins in the nucleus. This symbiosis generates an active SRPK1-CLK1 complex that sheds the limitations of each individual kinase by incorporating the favorable attributes of both kinases (Figure 7B). Although SRPK1 is a very agile kinase that rapidly phosphorylates Arg-Ser dipeptide repeats in a C-to-N-terminal direction, it cannot modify Ser-Pro dipeptides that are common in SR proteins and whose phosphorylation mobilizes the splicing factors and alters splicing patterns (Keshwani et al., 2015a). CLK1 can phosphorylate Ser-Pro dipeptides along with Arg-Ser repeats, but only at very reduced rates. In the hetero-kinase complex, SRPK1 takes on a superior position by binding and rapidly phosphorylating the lengthy Arg-Ser repeats in SRSF1. With its N terminus re-purposed for interaction with SRPK1, the kinase domain of CLK1 can freely pivot within the complex and modify the individual Ser-Pro dipeptides in the RS domain. We believe that Ser-Pro dipeptide modification occurs later in the multisite reaction, because a CLK1-specific inhibitor does not affect the rapid initial phase of the progress curves. There is likely to be some allosteric effects in the complex, as the second reaction phase is much faster than the net rate of SR phosphorylation by CLK1 alone. In all, the heterocomplex avoids a competitive roadblock by melding the speed and dissociative mechanism of SRPK1 with the dipeptide specificity of CLK1 to achieve a fully phosphorylated SR protein that participates in spliceosome assembly.

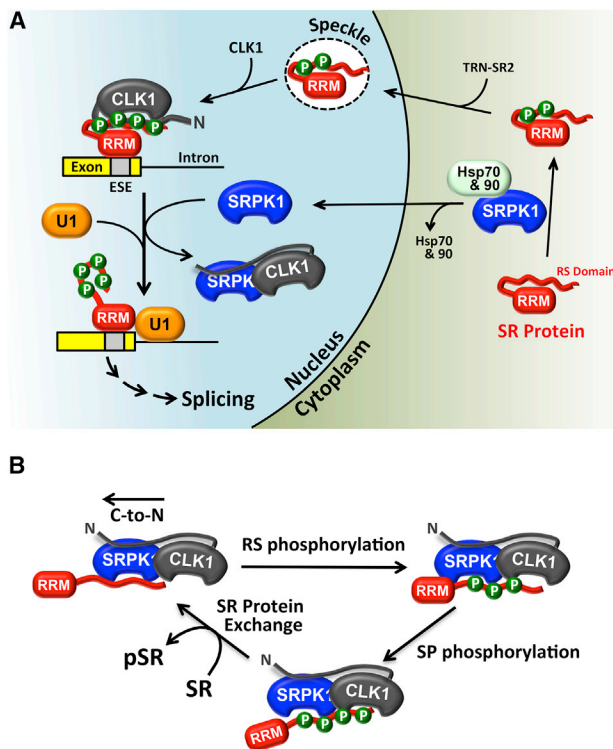


Figure 7. Role of Nuclear SRPK1 for SR Protein Phosphorylation and Splicing

(A) SRPK1 is drawn into the nucleus through contacts with CLK1. The SRPK1-CLK1 complex releases the SR protein from CLK1 and promotes splicing activity through enhanced contacts with U1.

(B) Catalytic cycle for SR protein phosphorylation by the SRPK1-CLK1 complex. The complex is held together by interactions between the N terminus of CLK1 and the kinase domain of SRPK1. SRPK1 is the primary catalyst that phosphorylates Arg-Ser repeats in a C-to-N-terminal direction, and CLK1 primarily phosphorylates Ser-Pro dipeptides. SRPK1 induces SR protein release by prohibiting contacts with the RS domain.

In addition to demonstrating how the dual-kinase mechanism solves the biochemical release problem for CLK1, the SRPK1-CLK1 complex also provides a framework for understanding the biological role of these kinases in splicing regulation. Although we showed previously that SRPKs localize in the cytoplasm through interactions with molecular chaperones (Zhong et al., 2009), we could not explain why SRPKs are also found in the nucleus without a localization sequence. Furthermore, we could not explain why removal of the SID that interacts with the cytoplasmic chaperones leads to complete nuclear localization of SRPK1 (Ding et al., 2006). These phenomena can now be explained by the ability of CLK1 to retain SRPK1 in the nucleus, balancing the cytoplasmic tethering of the chaperones (Figure 7A).

Once in the nucleus, SRPK1 serves a vital function in splicing. Prior studies using phospho-mimics of SRSF1 suggest that CLK1 phosphorylation promotes interaction of a component of the 70K subunit of U1 snRNP with the SR protein and subsequent formation of the 5' splice site in pre-mRNA (Cho et al., 2011). We now show that CLK1-dependent phosphorylation is

not sufficient for this process. Instead, CLK1 behaves as an inhibitor of splicing when bound to phosphorylated SRSF1. This explains the early observation that overexpressed CLK1 gave rise to similar functional consequences to SR protein depletion in transfected cells (Prasad et al., 1999). Moreover, formation of the SRPK1-CLK1 heterocomplex shifts internal binding contacts and promotes the association of the U1 RRM with phospho-SRSF1, a key step in establishing the 5' splice site. Signals that re-attach SRPK1 to cytoplasmic chaperones (e.g., downregulation of Akt) are then expected to increase cytoplasmic SRPK1 levels, leading to dissociation of the SRPK1-CLK1 complex and resumption of CLK1-bound SR proteins.

Traditionally, SRPKs and CLKs have been viewed in light of their spatial orientation in eukaryotic cells. SRPKs phosphorylate Arg-Ser dipeptides in RS domains, releasing phospho-SR proteins for attachment to TRN-SR and nuclear import. Partially phosphorylated SR proteins are further phosphorylated at Ser-Pro dipeptides by nuclear CLKs, a modification that shunts the splicing factors toward the splicing machinery. We have now uncovered an arrangement of these two kinases in the nucleus that helps explain the mechanism of SR protein-dependent splicing that needs both kinases in the nucleus. The SRPK1-CLK1 complex brilliantly avoids the dilemma of establishing two kinases in a single compartment that, owing to their similar high affinities, would compete for the SR protein pool. Such elegant intracellular symbiosis between kinases in the nucleus explains how the inhibitory effects of CLK1 are removed to facilitate spliceosome assembly and the splicing reaction upon signaling-induced nuclear translocation of SRPK1.

EXPERIMENTAL PROCEDURES

Materials

ATP, Mops, Tris, MgCl₂, NaCl, EDTA, glycerol, sucrose, acetic acid, lysozyme, DNase, RNase, Phenix imaging film, BSA, Protein G agarose, Ni-resin, and liquid scintillant were obtained from Fisher Scientific. ³²P-ATP was obtained from NEN Products. RNA (5'-AGGCGGAGGAAGC-3') was purchased from Integrated DNA Technologies. siRNA for CLK1 was obtained from Bioneer. Protease inhibitor cocktail was obtained from Roche, and TG003 was obtained from Sigma. Anti-CLK1 monoclonal antibody was purchased from Aviva Systems Biology, and anti-SRPK1 monoclonal antibody was purchased from BD Biosciences. InstantBlue was purchased from Expedon, Hybond ECL nitrocellulose blotting membrane was purchased from Amersham, and the KinaseMax Kit was purchased from Ambion.

Expression and Purification of Proteins

SRPK1, SRSF1, and CLK1(ΔN) (residues 148–484) were expressed and purified from pET19b vectors with an N-terminal His tag, and GST-SRSF1, GST-RS1, and GST-CLK1(N) (residues 1–160) were expressed and purified from a pGEX vector as previously described (Aubol et al., 2014). kdSRPK1, inactive SRPK1 containing K109M, was expressed and purified from a pET19b vector. Deletion constructs SRPK1(ΔN), SRPK1(ΔS), and SRPK1(S) (residues 222–492) were expressed and purified as previously described (Aubol et al., 2012, 2014). CLK1 virus was transfected and expressed in Hi5 insect cells, and CLK1 was purified with a nickel resin and a previously described procedure (Keshwani et al., 2015b).

Cell Fractionation Studies

HeLa cells were harvested and lysed in 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, 0.05% Triton, and protease inhibitor cocktail. The lysates were centrifuged at 228 × g for 10 min to pellet nucleus, and the supernatant was retained as the cytoplasmic fraction. The pellet was washed with

lysis buffer and re-suspended in 200 μ l of 0.25 mM sucrose and 10 mM $MgCl_2$. The nuclear suspension was layered on 0.88 mM sucrose and 0.5 mM $MgCl_2$ and spun at 2,800 \times g to obtain a nuclear pellet. The pellet was re-suspended in 1X RIPA buffer, spun at 2,800 \times g, and the supernatant was retained as nuclear fraction.

Immunoprecipitation Experiments

HeLa cell nuclear lysates (200 μ l) were pre-cleared with Protein A beads and incubated overnight in the cold room with 25 μ l Protein A beads and either 3 μ l rabbit anti-CLK1 (Aviva systems) or mouse anti-SRPK1 antibody (BD Biosciences). The beads were spun at 2,052 \times g and washed with 200 μ l lysis buffer followed by the addition of 2 \times SDS loading buffer. The heated slurry was run on a 12% SDS-PAGE followed by immunoblot analysis. SRSF1 was immunoprecipitated from nuclear lysates (100 μ l) using 3 μ l SRSF1 antibody with and without 5 μ M kdSRPK1 and probed using anti-CLK1 antibody.

Confocal Imaging

HeLa cells were plated on 2.5-cm² MatTek poly-D-lysine plates and transfected with mock, CLK1-RFP, kdCLK1-RFP, and nCLK1 Δ N-RFP DNA constructs (2 μ g) for 24 hr. The cells were washed with PBS and fixed with 2% paraformaldehyde in PBS for 20 min followed by a 2 \times PBS wash. Cell permeabilization was done with 0.25% Triton in PBS for 10 min followed by a 3 \times PBS wash. Cells were then blocked in 10% donkey serum PBS for 1 hr at room temperature followed by overnight incubation with SRPK antibody at 4°C. Cells were washed three times with PBS, incubated with secondary fluorescent anti-mouse antibody conjugated with Alexa 488 (Life Technologies) for 1 hr at room temperature, followed by 3 \times PBS washes for 5 min. Cells were mounted with DAPI-containing mounting medium (Vector Laboratories). For live-cell imaging, transfected HeLa cells were analyzed using an Olympus FV1000 as described previously (Keshwani et al., 2015a).

Pull-Down Assays

GST- and His-tagged proteins (4 μ M) were incubated in 40 μ l binding buffer (0.1% NP40 [Nonidet P40], 20 mM Tris/HCl [pH 7.5], and 75 mM NaCl) for 30 min before incubating with 25 μ l glutathione-agarose resin for 30 min at room temperature. Where phosphorylation was performed, GST- and His-tagged enzymes (4 μ M) were mixed with and without 100 μ M ATP in 10 mM Mg^{2+} , 20 mM Tris/HCl (pH 7.5), and 75 mM NaCl at 37°C for 30 min, followed by incubation with 25 μ l glutathione-agarose resin for 30 min at room temperature. In some cases, 4 μ M kdSRPK was added and incubated at room temperature for 10 min prior to wash steps. Resin was washed four times with 200 μ l binding buffer, and the bound proteins were eluted with SDS quench buffer and boiled for 5 min. Retained protein was resolved by SDS-PAGE (12% gel) and visualized by Instant Blue Coomassie stain or western blotting with a mouse anti-His antibody.

Phosphorylation Assays

Single-turnover assays were performed in assay buffer (100 mM Mops [pH 7.4], 10 mM Mg^{2+} , and 5 mg/mL BSA), at 37°C using 3 μ M enzyme, 300 nM SRSF1 and 100 μ M ³²P-ATP (4,000–8,000 cpm/pmol) with and without 50 μ M TG003 and 5% DMSO. Steady-state assays using 5 nM SRPK1 or 25 nM CLK1 were performed in assay buffer with 100 μ M ³²P-ATP (4,000–8,000 cpm pmol⁻¹) at 23°C (SRPK1) and 37°C (CLK1) and quenched with 10 μ l SDS/PAGE loading buffer at 2 and 20 min, respectively. Phosphorylated SR proteins were cut from a dried 12% SDS-PAGE and counted in liquid scintillant. The amount of kdSRPK1 bound to CLK1 was measured by phosphorylating GST-SRSF1 (500 nM) with CLK1 (100 or 400 nM) and 100 μ M ³²P-ATP for 90 min at 37°C and binding to the Ni-resin. Varying kdSRPK1 (0–1,000 nM) was added to CLK1-bound resin and washed two times with 400 μ l binding buffer. The CLK1 fraction bound (FB) was measured from the CPMs on the resin and fit to Equation 1:

$$FB = \frac{R_o + L_o + K_d - \sqrt{(R_o + L_o + K_d)^2 - 4K_dL_o}}{2R_o} \quad (\text{Equation 1})$$

where R_o is the total CLK1, L_o is the total kdSRPK1, and K_d is the complex dissociation constant.

RNA Preparation

In vitro transcription was performed using 0.5 μ g/ μ l linear β -globin DNA in a reaction buffer containing 0.67 μ M (α -³²P) UTP, 0.4 mM ATP, 0.4 mM CTP, 0.1 mM UTP, 0.1 mM GTP, 2 mM m7G(5')ppp(5'G) (cap analog), 2 mM DTT, 10 U/ μ l ribonuclease inhibitor, 7.5 U/ μ l T7 RNA polymerase (Promega), and 1 \times transcription buffer (Promega). Reactions were incubated at 37°C for 2 hr, gel purified using denaturing PAGE, eluted from the gel (elution buffer: 0.5 mM NaOAc [pH 5.6], 0.1% SDS, 10 mM Tris [pH 7.5], and 1 mM EDTA), ethanol precipitated, and resuspended in nuclease-free water.

In Vitro Splicing

Splicing reactions were performed using α -³²P-labeled β -globin RNA in 30% HeLa nuclear extract with 500 nM SRPK1 or kdSRPK1 in reaction buffer as previously described (Movassat et al., 2014) with final 70 mM NaCl and incubated at 30°C for 90 min. Reactions were digested using proteinase K, extracted with phenol chloroform, precipitated with ethanol, and separated on denaturing PAGE. The gel was exposed to BaFBr:Eu screen and imaged on PhosphorImager (Bio-Rad). Appearance of final spliced product was determined by taking the percent of the sum of the adjusted intensity for the spliced band divided by the total signal for the spliced and unspliced product band (Movassat et al., 2014).

RNA Binding Assays

Pull-down experiments were performed with a ³²P-labeled RNA oligomer based on the Ron ESE (5'-AGGCGGAGGAAGC-3'). Labeling was carried out using the KinaseMax kit from Ambion and confirmed by 12% urea PAGE. GST-tagged SRSF1 proteins (4 μ M) were incubated with 3 pmol ³²P-labeled Ron ESE at 23°C for 30 min in 20 μ l binding buffer and mixed with 25 μ l glutathione-agarose resin for 30 min at room temperature. Where phosphorylation was required, GST- and His-tagged enzymes (4 μ M) were incubated with and without 100 μ M ATP and 10 mM Mg^{2+} in 20 mM Tris/HCl (pH 7.5) and 75 mM NaCl at 37°C for 30 min prior to the RNA addition step. In some cases, 4 μ M kdSRPK or buffer blank was added and incubated at room temperature for 10 min prior to the RNA addition step. The resin was washed three times with 200 μ l binding buffer, and the retained RNA was counted in liquid scintillant.

AUTHOR CONTRIBUTIONS

B.E.A., G.W., M.M.K., L.F., and M.M. performed all experiments. K.J.H. and X.-D.F. helped with data analysis and manuscript editing. J.A.A. planned the experiments and wrote the paper.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health (NIH) grants GM67969 (to J.A.A.), GM52872 (to X.-D.F.), and GM62287 (to K.J.H.). We would like to thank Dr. Gourisankar Ghosh for providing U1(RRM).

Received: October 9, 2015

Revised: April 26, 2016

Accepted: May 25, 2016

Published: July 7, 2016

REFERENCES

- Aubol, B.E., and Adams, J.A. (2011). Applying the brakes to multisite SR protein phosphorylation: substrate-induced effects on the splicing kinase SRPK1. *Biochemistry* 50, 6888–6900.
- Aubol, B.E., Plocinik, R.M., McGlone, M.L., and Adams, J.A. (2012). Nucleotide release sequences in the protein kinase SRPK1 accelerate substrate phosphorylation. *Biochemistry* 51, 6584–6594.
- Aubol, B.E., Plocinik, R.M., Hagopian, J.C., Ma, C.T., McGlone, M.L., Bandyopadhyay, R., Fu, X.D., and Adams, J.A. (2013). Partitioning RS domain phosphorylation in an SR protein through the CLK and SRPK protein kinases. *J. Mol. Biol.* 425, 2894–2909.

- Aubol, B.E., Plocinik, R.M., Keshwani, M.M., McGlone, M.L., Hagopian, J.C., Ghosh, G., Fu, X.D., and Adams, J.A. (2014). N-terminus of the protein kinase CLK1 induces SR protein hyperphosphorylation. *Biochem. J.* *462*, 143–152.
- Cho, S., Hoang, A., Sinha, R., Zhong, X.Y., Fu, X.D., Krainer, A.R., and Ghosh, G. (2011). Interaction between the RNA binding domains of Ser-Arg splicing factor 1 and U1-70K snRNP protein determines early spliceosome assembly. *Proc. Natl. Acad. Sci. USA* *108*, 8233–8238.
- Colwill, K., Pawson, T., Andrews, B., Prasad, J., Manley, J.L., Bell, J.C., and Duncan, P.I. (1996). The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution. *EMBO J.* *15*, 265–275.
- Ding, J.H., Zhong, X.Y., Hagopian, J.C., Cruz, M.M., Ghosh, G., Feramisco, J., Adams, J.A., and Fu, X.D. (2006). Regulated cellular partitioning of SR protein-specific kinases in mammalian cells. *Mol. Biol. Cell* *17*, 876–885.
- Endicott, J.A., Noble, M.E., and Johnson, L.N. (2012). The structural basis for control of eukaryotic protein kinases. *Annu. Rev. Biochem.* *81*, 587–613.
- Fu, X.D., and Ares, M., Jr. (2014). Context-dependent control of alternative splicing by RNA-binding proteins. *Nat. Rev. Genet.* *15*, 689–701.
- Ghosh, G., and Adams, J.A. (2011). Phosphorylation mechanism and structure of serine-arginine protein kinases. *FEBS J.* *278*, 587–597.
- Gui, J.F., Lane, W.S., and Fu, X.D. (1994). A serine kinase regulates intracellular localization of splicing factors in the cell cycle. *Nature* *369*, 678–682.
- Kataoka, N., Bachorik, J.L., and Dreyfuss, G. (1999). Transportin-SR, a nuclear import receptor for SR proteins. *J. Cell Biol.* *145*, 1145–1152.
- Keshwani, M.M., Aubol, B.E., Fattat, L., Ma, C.T., Qiu, J., Jennings, P.A., Fu, X.D., and Adams, J.A. (2015a). Conserved proline-directed phosphorylation regulates SR protein conformation and splicing function. *Biochem. J.* *466*, 311–322.
- Keshwani, M.M., Hailey, K.L., Aubol, B.E., Fattat, L., McGlone, M.L., Jennings, P.A., and Adams, J.A. (2015b). Nuclear protein kinase CLK1 uses a nontraditional docking mechanism to select physiological substrates. *Biochem. J.* *472*, 329–338.
- 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.
- Lai, M.C., Lin, R.I., and Tam, W.Y. (2001). Transportin-SR2 mediates nuclear import of phosphorylated SR proteins. *Proc. Natl. Acad. Sci. USA* *98*, 10154–10159.
- Lavoie, H., Li, J.J., Thevakumaran, N., Therrien, M., and Sicheri, F. (2014). Dimerization-induced allostery in protein kinase regulation. *Trends Biochem. Sci.* *39*, 475–486.
- Lemmon, M.A., and Schlessinger, J. (2010). Cell signaling by receptor tyrosine kinases. *Cell* *141*, 1117–1134.
- Ma, C.T., Ghosh, G., Fu, X.D., and Adams, J.A. (2010). Mechanism of dephosphorylation of the SR protein ASF/SF2 by protein phosphatase 1. *J. Mol. Biol.* *403*, 386–404.
- Maniatis, T., and Reed, R. (2002). An extensive network of coupling among gene expression machines. *Nature* *416*, 499–506.
- Mathew, R., Hartmuth, K., Möhlmann, S., Urlaub, H., Ficner, R., and Lührmann, R. (2008). Phosphorylation of human PRP28 by SRPK2 is required for integration of the U4/U6-U5 tri-snRNP into the spliceosome. *Nat. Struct. Mol. Biol.* *15*, 435–443.
- Moore, M.J., and Proudfoot, N.J. (2009). Pre-mRNA processing reaches back to transcription and ahead to translation. *Cell* *136*, 688–700.
- Movassat, M., Mueller, W.F., and Hertel, K.J. (2014). In vitro assay of pre-mRNA splicing in mammalian nuclear extract. *Methods Mol. Biol.* *1126*, 151–160.
- Nayler, O., Stamm, S., and Ullrich, A. (1997). Characterization and comparison of four serine- and arginine-rich (SR) protein kinases. *Biochem. J.* *326*, 693–700.
- Ngo, J.C., Giang, K., Chakrabarti, S., Ma, C.T., Huynh, N., Hagopian, J.C., Dorrestein, P.C., Fu, X.D., Adams, J.A., and Ghosh, G. (2008). A sliding docking interaction is essential for sequential and processive phosphorylation of an SR protein by SRPK1. *Mol. Cell* *29*, 563–576.
- Nilsen, T.W., and Graveley, B.R. (2010). Expansion of the eukaryotic proteome by alternative splicing. *Nature* *463*, 457–463.
- Pandit, S., Wang, D., and Fu, X.D. (2008). Functional integration of transcriptional and RNA processing machineries. *Curr. Opin. Cell Biol.* *20*, 260–265.
- Parsons, S.J., and Parsons, J.T. (2004). Src family kinases, key regulators of signal transduction. *Oncogene* *23*, 7906–7909.
- Poulikakos, P.I., Zhang, C., Bollag, G., Shokat, K.M., and Rosen, N. (2010). RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature* *464*, 427–430.
- Prasad, J., Colwill, K., Pawson, T., and Manley, J.L. (1999). The protein kinase Clk/Sty directly modulates SR protein activity: both hyper- and hypophosphorylation inhibit splicing. *Mol. Cell. Biol.* *19*, 6991–7000.
- Rossmann, K.L., Der, C.J., and Sondek, J. (2005). GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat. Rev. Mol. Cell Biol.* *6*, 167–180.
- Shin, C., and Manley, J.L. (2004). Cell signalling and the control of pre-mRNA splicing. *Nat. Rev. Mol. Cell Biol.* *5*, 727–738.
- Stamm, S. (2008). Regulation of alternative splicing by reversible protein phosphorylation. *J. Biol. Chem.* *283*, 1223–1227.
- Taira, K., and Benkovic, S.J. (1988). Evaluation of the importance of hydrophobic interactions in drug binding to dihydrofolate reductase. *J. Med. Chem.* *31*, 129–137.
- Taylor, S.S., and Kornev, A.P. (2011). Protein kinases: evolution of dynamic regulatory proteins. *Trends Biochem. Sci.* *36*, 65–77.
- Taylor, S.S., Ilouz, R., Zhang, P., and Kornev, A.P. (2012). Assembly of allosteric macromolecular switches: lessons from PKA. *Nat. Rev. Mol. Cell Biol.* *13*, 646–658.
- Wang, G.S., and Cooper, T.A. (2007). Splicing in disease: disruption of the splicing code and the decoding machinery. *Nat. Rev. Genet.* *8*, 749–761.
- Wang, H.Y., Lin, W., Dyck, J.A., Yeakley, J.M., Songyang, Z., Cantley, L.C., and Fu, X.D. (1998). SRPK2: a differentially expressed SR protein-specific kinase involved in mediating the interaction and localization of pre-mRNA splicing factors in mammalian cells. *J. Cell Biol.* *140*, 737–750.
- Xiao, S.H., and Manley, J.L. (1998). Phosphorylation-dephosphorylation differentially affects activities of splicing factor ASF/SF2. *EMBO J.* *17*, 6359–6367.
- Yun, C.Y., Velazquez-Dones, A.L., Lyman, S.K., and Fu, X.D. (2003). Phosphorylation-dependent and -independent nuclear import of RS domain-containing splicing factors and regulators. *J. Biol. Chem.* *278*, 18050–18055.
- Zhong, X.Y., Ding, J.H., Adams, J.A., Ghosh, G., and Fu, X.D. (2009). Regulation of SR protein phosphorylation and alternative splicing by modulating kinetic interactions of SRPK1 with molecular chaperones. *Genes Dev.* *23*, 482–495.
- Zhou, Z., Qiu, J., Liu, W., Zhou, Y., Plocinik, R.M., Li, H., Hu, Q., Ghosh, G., Adams, J.A., Rosenfeld, M.G., and Fu, X.D. (2012). The Akt-SRPK-SR axis constitutes a major pathway in transducing EGF signaling to regulate alternative splicing in the nucleus. *Mol. Cell* *47*, 422–433.