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Dissertation Advisor: Professor John Blenis Jamie Michelle Dempsey

SRPK2 phosphorylation by the AGC kinases, and mTORC1 regulation of alternative splicing

Abstract

The mechanisms through which a cell controls its proliferation, differentiation, metabolism, motility, and ultimate survival in response to extracellular cues are largely controlled by the Ras-extracellular signal-regulated kinase (Ras-ERK) and phosphatidylinositol 3-kinase mammalian target of rapamycin (PI3K-mTOR) signaling pathways. Originally delineated as two separate and linear signaling pathways, multitudes of evidence through experimentation have shown that these pathways can co-regulate downstream targets and cellular outcomes. Here, we provide evidence for an additional point of pathway convergence the serine/arginine protein kinase 2 (SRPK2). Originally identified as a target of the mTORC1/S6K signaling pathway, we have shown SRPK2 to be a target of the Ras-ERK-Rsk pathway, as well as the PI3K-AKT. We discovered the S6K, AKT and RSK all phosphorylate SRPK2 at serine 494 in a cell-type, stimulus dependent manner, emphasizing the redundant nature of the AGC kinases. SRPK2 regulates the phosphorylation of the constitutive and alternative splicing factors the SR proteins. This led us to question mTORC1 involvement in splice site selection, and we discovered several alternative splicing events downstream of mTORC1 signaling. We found that the protein levels of the splicing factors ASF/SF2 and hnRNPa2b1 are regulated by mTORC1 signaling, and we hypothesize this is through regulated unproductive splicing and translation (RUST). Interestingly, we found that BIN1, a target of both ASF/SF2 and hnRNPa2b1, is

alternatively spliced, following modulations in mTORC1 signaling. These biochemical studies and knowledge gleaned from them will lead to a better understanding of how the cell can regulate protein expression by controlling alternative splicing.

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Chapter 1 Introduction

A stable isotope labeling of amino acids in cell culture (SILAC) mass spectrometry screen completed by Dr. Yonghao Yu aimed at discovering novel targets of the mTORC1/S6K signaling pathway identified a rapamycin sensitive phosphorylation site on the serine/arginine protein kinase 2 (SRPK2) [1]. This phosphorylation site follows the protein A/protein G/protein C (AGC) canonical basophilic phosphorylation motif (RXRXXS/T), and we hypothesized it to be a target of the AGC kinase family member p70 ribosomal S6 kinase (S6K). During my dissertation studies, in addition to confirming this to be the case, we have also provided evidence that serine 494 of SRPK2 can be phosphorylated by the p90 ribosomal S6 kinase (RSK), as well as the protein kinase AKT depending upon stimulus and cell type. In addition, we have investigated the status of several serine/arginine (SR) proteins, which are known targets of the SRPKs, and have found their stability and cellular concentrations to be regulated by the mTORC1/S6K pathway. Finally, we found that BIN1 is alternatively spliced downstream of mTORC1 signaling. We hypothesize that SRPK2 is a new point of signal convergence for the Ras/ERK/RSK and PI3K/mTORC1 signaling pathways to regulate alternative splicing.

Ras-ERK and PI3K-mTOR pathway integration

The mechanisms through which a cell controls its proliferation, differentiation, metabolism, motility, and ultimate survival in response to extracellular cues are largely controlled by the Ras-extracellular signal-regulated kinase (Ras-ERK) and phosphatidylinositol 3-kinase mammalian target of rapamycin (PI3K-mTOR) signaling pathways. Originally delineated as two separate and linear signaling pathways,

multitudes of evidence through experimentation have shown that these pathways can regulate each other, as well as co-regulate downstream targets and cellular outcomes. Here, we provide evidence for an additional target the serine/arginine protein kinase 2 (SRPK2) originally identified as a target of the mTORC1/S6K signaling pathway, which we show to also be a target of the Ras-ERK-Rsk, as well as the PI3K-AKT pathways. This dissertation describes these important signaling pathways, their crosstalk nodes, in addition to reviewing what is known about the SRPKs and their substrates, the SR proteins. Additionally reviewed is how the SR proteins regulate their own intracellular concentrations, through a process deemed regulated unproductive splicing and translation (RUST). Also included are the biochemical characterizations of the SRPK2 phosphorylation by the AGC kinases, and the downstream alternative splicing events identified in our different cell systems. Discussed are the many faceted directions that this knowledge can be used to further our understanding of how the cell can regulate protein expression by controlling alternative splicing.

PI3K and mTORC1 Signaling

After activation by growth factors, the lipid kinase PI3K generates phosphatidylinositol 3,4,5 triphosphate (PIP3), which in turn recruits the AGC family protein kinase AKT to the plasma membrane. At the membrane, AKT is activated by the 3-phosphoinositide-dependent kinase 1 (PDK1), and the mTOR complex 2 (explained below). In addition to phosphorylating substrates involved in proliferation, survival and mobility, AKT phosphorylates the tuberous sclerosis complex 2 (TSC2) GTPase activating protein (GAP). After phosphorylation of TSC2 at multiple sites, the inhibitory effect of TSC is relaxed, allowing the GTPase Ras homolog enriched in brain (RHEB) to

directly activate mTORC1 (Figure 1.1).

mTOR exists in two distinct protein complexes: mTORC1 and mTORC2 [2-7]. Briefly, mTORC1 contains mTOR, raptor (regulatory-associated protein of mTOR), and mLST8 (also known as GβL) [8]. This complex, directly phosphorylates 4E-BP1 and S6 kinase (S6K) to regulate ribosomal biogenesis, protein synthesis, and cell growth [9] in response to a variety of upstream growth factor, nutrient, and stress signals (Figure 1.1) [10]. Raptor directly interacts with these substrates through the TOR signaling (TOS) motif, an essential phenylalanine followed by four alternating acidic and small hydrophobic residues (FDIDL in S6K1 and FEMDI in 4E-BP1) [18-21].

Rapamycin, which is an antifungal substance isolated from a soil sample of the bacterium *Streptomyces hygroscopicus* on Easter Island, potently inhibits signaling by mTORC1 at nanomolar concentrations. Rapamycin is thought to work by binding the immunophilin FK-506 binding protein of 12 kDa (FKBP12), which then alters the mTOR-Raptor interaction preventing proper signaling to mTORC1 targets [4].

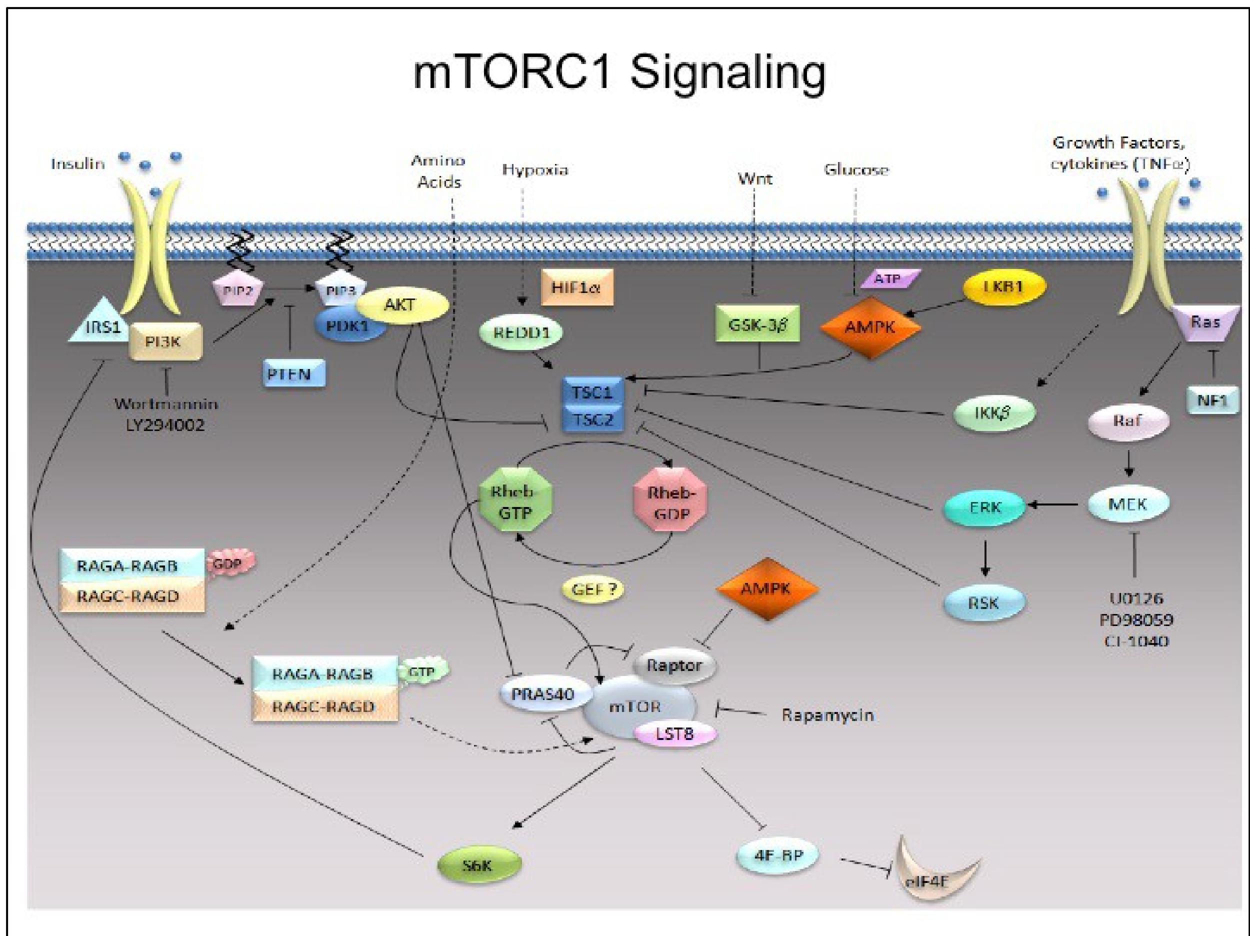

Figure 1.1: mTORC1 signaling pathway activation by growth factors, amino acids and energy signals. mTORC1 is stimulated by the active, GTP-bound form of Rheb, which is in turn regulated by the TSC1–TSC2 complex. TSC2 functions as a GAP for Rheb, converting it to its inactive GDP-bound form. Many different signaling pathways either positively or negatively regulate mTORC1 signaling, including the PI3K–Akt, Ras–ERK–RSK, TNFα–IKKB, AMPK–GSK-3beta, LKB1–AMPK, and Wnt–GSK-3beta pathways after activation by the corresponding stimulus.

Ribosomal protein S6 kinase

In addition to 4E-BP1, the other well-known mTORC1 targets are the S6Ks, which belong to a family of basophilic serine/threonine kinases known as AGC kinases, which phosphorylate targets at basophilic motifs, particularly RXRXXS*/T*. The most well-known S6K target is ribosomal protein S6 (rpS6), although the function of rpS6 phosphorylation remains unclear. Occasionally S6K will phosphorylate non-canonical

basophilic motifs, such as that found in the substrate SKAR [11]. Other targets of S6K that are involved in the regulation of protein translation include the eukaryotic translation initiation factor eIF4B, PDCD4 and eEF2K (explained in detail below).

S6K activation and targets

S6K is phosphorylated at multiple sites (fig. 1. 2). mTORC1 directly phosphorylates the hydrophobic motif at Thr 389, with this phosphorylation creating a docking site for PDK1. PDK1 then phosphorylates Thr229 in the kinase domain's activation loop [12]. Recent work by Sang Oh Yoon a former post-doctoral fellow recently identified GSK-3 as the kinase reponsible for phosphorylating the turn-motif at Ser371, which is required for full S6K activation [13]. There is a pseudo-substrate autoinhibitory domain in the C-terminus of S6K, which contains multiple proline-directed phosphorylation sites. Phophorylation at Ser411, Ser418, Thre421, and Ser424 or deletion of this domain promotes full S6K activation, and ERK1/2, p38-MAPK, cdc2, and mTORC1 have all been implicated in the phosphorylation of these residues *in vivo* [14-16].

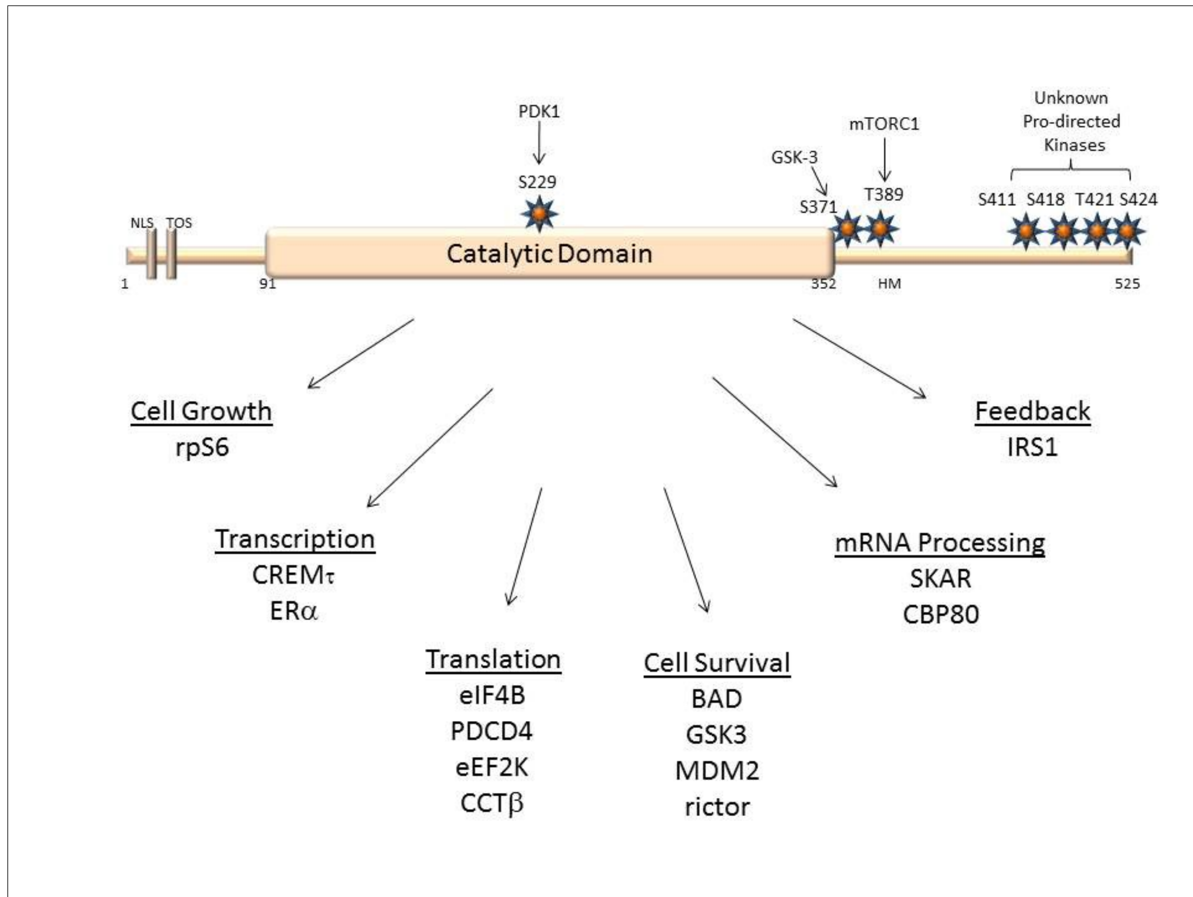

Figure1.2: S6K1 domains, phosphorylation sites, as well as several targets. An alternative translation start site results in two isoforms of S6K1. The 85kDa is mostly-nuclear, while the 70kDa is mostly-cytoplasmic. mTORC1, GSK-3, and PDK1 regulate S6K1 activity through phosphorylation. S6K1 targets are involved in multiple cellular processes including translation, mRNA processing, and cell size. NLS: nuclear localization signal, TOS: TOR substrate motif, HM: hydrophobic motif

Ribosomal protein S6

Upon activation by mTORC1, S6K1 is then able to phosphorylate many proteins at the cap complex to effect different kinds of activities (figure 2). Ribosomal protein (rp) S6, which is a component of the 40S ribosomal subunit, was the first identified target of S6K1. Since rpS6 is a highly abundant protein that can be purified with ribosomes and phosphorylated in a regulated manner at multiple sites [17], it has served as a model for kinase activity and general phosphorylation for many years. Despite the relatively long

and illustrious history of rpS6 phosphorylation as a model for regulated kinase activity, the actual function of rpS6 phosphorylation remains contested, with rpS6 phosphorylation correlating with increased translation under certain stimulation conditions and contexts [18], but not others [19]. Phosphorylated rpS6 has been shown to bind with more affinity to mRNA [20] and the 5' cap [21] *in vitro*. None of the aforementioned studies provide definitive evidence that rates of translation are changed upon introduction of phosphomimetic (aspartate) or nonphosphorylatable (alanine) mutants to cells. Interestingly, alanine mutations in the yeast homologs of rpS6 abolished phosphorylation but gave no discernable effects on growth rate [39], weakening the model that S6Ks regulate translation through phosphorylation of rpS6.

Some of the outstanding questions of the *in vivo* function of rpS6 phosphorylation were cleared up when Ruvinsky and colleagues generated a knock-in/knock-out mouse that effectively abolished the ability of rpS6 to be phosphorylated by substituting the five known C-terminal phosphorylated serines with alanines. Upon investigation, they surprisingly found that some tissues of these mice exhibited a mild *increase* in global translation rates with no significant change in 5'TOP translation, and that MEFs and pancreatic beta-cells were smaller, suggesting a role for rpS6 phosphorylation in cell size regulation and glucose homeostasis [40]. Clearly, more research is necessary to elucidate the biological significance of phosphorylation of rpS6, which may turn out to be cell- and tissue type-specific. Regardless of the function, rpS6 phosphorylation has been and will continue to serve as a valuable, biochemical readout for activation of the mTORC1 pathway.

eIF4B

In 1978, John Hershey's group showed that eIF4B, amongst other translation initiation factors, became phosphoryated *in vitro* in rabbit reticulocytes through activity of a cAMP-independent protein kinase [22]. eIF4B is a critical regulatory subunit of eIF4A helicase, and in combination, these proteins work to unwind complex secondary structures found in the 5'UTR of some mRNAs, allowing for more efficient scanning by the 40S ribosome for the start codon. Later studies indicated that not only was eIF4B phosphorylated, but its phosphorylation status was regulated by serum levels in HeLa cells [23], and upon further investigation into the specific regulators of eIF4B phosphorylation, it was found that phorbol esters, insulin, and EGF could all stimulate the phosphorylation of eIF4B [24-26]. *In vitro* kinase studies showed that several purified kinases, including S6K, could phosphorylate eIF4B, although specific sites were not identified [27].

The early studies of eIF4B phosphorylation showed that multiple kinases could potentially be responsible for phosphorylating eIF4B, but none of them showed a direct effect of phosphorylation on either general or specific translation efficiency. Recently, eIF4B Ser422 was shown to be phosphorylated by S6K1 in cells [28], and this phosphorylation event was shown to promote its association with the translation pre-initiation complex (PIC) [29] (figure 2). Holz and colleagues showed that the overexpressed wild-type eIF4B associated with eIF3 in an insulin- and rapamycin-dependent manner. They also found that a phosphomimetic mutant of eIF4B (Ser422Asp) associated constitutively with eIF3 even in the presence of rapamycin, and that the alanine mutant at this site didn't associate with eIF3, even in stimulated cells [29].

This study also showed a correlation between these mutations and the efficiency of cap-dependent translation in an *in cellulo* luciferase reporter assay.

PDCD4

Programmed cell death 4 (PDCD4) is another target of S6K, and like eIF4B, PDCD4 also binds the helicase eIF4A [30], though instead of activating translation, it inhibits it [31]. Akt was first shown to be able to phosphorylate PDCD4 *in vitro* at Ser67 and Ser457 [32]. Dorrello et al. found later that not only was Ser67 phosphorylated by S6K1 in cells, but that this phosphorylation promoted recruitment of a $SCF^{\beta\text{-TRCP}}$ ubiquitin ligase complex to PDCD4, leading to its ubiquitination and subsequent degradation [33] (figure 2). Since PDCD4 binds to and inhibits eIF4A, this degradation of PDCD4 releases repression of translation at eIF4A [33].

eIF4G

The “scaffold” of the cap complex, eIF4G, provides a docking site for many translation initiation factors such as eIF4E, eIF4A, eIF3, and PABP. There are at least three serine residues in the C-terminal third of eIF4G that are serum- and mitogen-dependent and rapamycin-sensitive. Raught and colleagues suggest that these are not direct mTORC1 target sites, but rather sites whose accessibility may be regulated by mTORC1 signaling [34]. Functions for these phosphorylation events have not yet been determined, although one might speculate that phosphorylation may affect the ability of proteins to dock onto eIF4G.

SKAR, CBP80 and the Pioneer Round of Translation

The "pioneer" round of translation is described as the first passage of the ribosome along the length of a newly transcribed mRNA, and many of the players involved in steady state translation have also been proposed to play a part in this process [35]. During transcription, the pre-mRNA is outfitted with a 5'-m7GpppN cap, which is then bound by the cap-binding complex (CBC) and not eIF4E. The CBC is composed of the cap binding proteins (CBP) 80 and 20, and is required for the proper splicing of the newly transcribed mRNA [36]. During the splicing process, proteins are deposited on the newly processed mRNA near the CBC to form the TREX complex [37], and approximately 20 nucleotides upstream of each exon-exon junction to form exon junction complexes (EJCs) [38]. These complexes are engaged and removed during the pioneer round [39, 40], and it has been suggested that the EJC proteins are involved in regulating the efficiency of this first and subsequent rounds of translation through a mechanism that remains unclear [41].

SKAR, a downstream target of S6K1 involved in cell growth control [11], was recently shown to be a novel EJC-interacting protein, linking mTORC1/S6K1 signaling to the pioneer round of translation. Like the EJC, SKAR contributes to an increase in the translational yield of spliced messages, and serves as a scaffolding protein, recruiting activated S6K1 to the newly generated mRNAs, where it phosphorylates several proteins on the CBP80-bound mRNA species [42]. S6K1 can also phosphorylate CBP80 *in vitro*, and may be involved in cdc42-regulated effects on mRNA splicing [43]. The biological significance of these phosphorylation events and how they affect the pioneer and subsequent rounds of translation will undoubtedly be the subject of future studies.

PI3K-Akt signaling

As previously discussed, Akt is activated downstream of PI3K signaling in response to insulin, insulin-like growth factor (IGF), as well as other signals. Multiple processes are regulated by the PI3K-Akt pathway including cell survival, proliferation and cell growth. Several disorders, including multiple cancers are resultant from perturbation of this pathway (reviewed in [44]). Following activation of a receptor tyrosine kinase (RTK) by ligand binding, PI3K is recruited to the phosphorylated intracellular domain of the RTK either directly, or via a recruited scaffold protein such as the insulin receptor substrate (IRS). PI3K, which contains two subunits, the catalytic p110 subunit and the regulatory p85 subunit, will then generate phosphatidylinositol-3,4,5-triphosphate (PIP3) upon its recruitment to the plasma membrane by phosphorylating phosphatidylinositol-4,5-biphosphate (PIP2). The tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN) reverses the PIP3 conversion, allowing for pathway inactivation, and is frequently mutated in cancers resulting in hyperactivation of the pathway (reviewed in[44])

AKT Activation

As mentioned above, Akt is phosphorylated by PDK1 and mTORC2. PDK1, which contains a PIP3-binding PH domain is recretuited to the PIP3-enriched plasma membrane along with PH domain-containing Akt, and phosphorylates Akt at Thr308 in the catalytic domain, while mTORC2 phosphorylates Akt at S473 [45, 46]. The protein phosphatase 2A (PP2A) negatively regulates Akt at Thr308, while the PH domain leucine-rich repeat protein phosphatase (PHLPP) negatively regulates Ser473 [47, 48]. Phosphorylation of Akt at Thr308 is sufficient to induce activation to a majority of its

substrates, however, phosphorylation at Ser473 provides full activation and activity towards all known Akt substrates.

AKT Targets

The first identified target of Akt was the glycogen synthase kinase GSK3β, with phosphorylation resulting in its inactivation [49]. GSK is a negative regulator of glycogen synthase, which supports fuel storage in the liver by catalyzing the formation of glycogen polymers from glucose monomers. In spite of the specific name for GSK3, it has been shown to regulate numerous substrates involved in many cellular functions including cytoskeletal rearrangement and cell survival (reviewed in [50]).

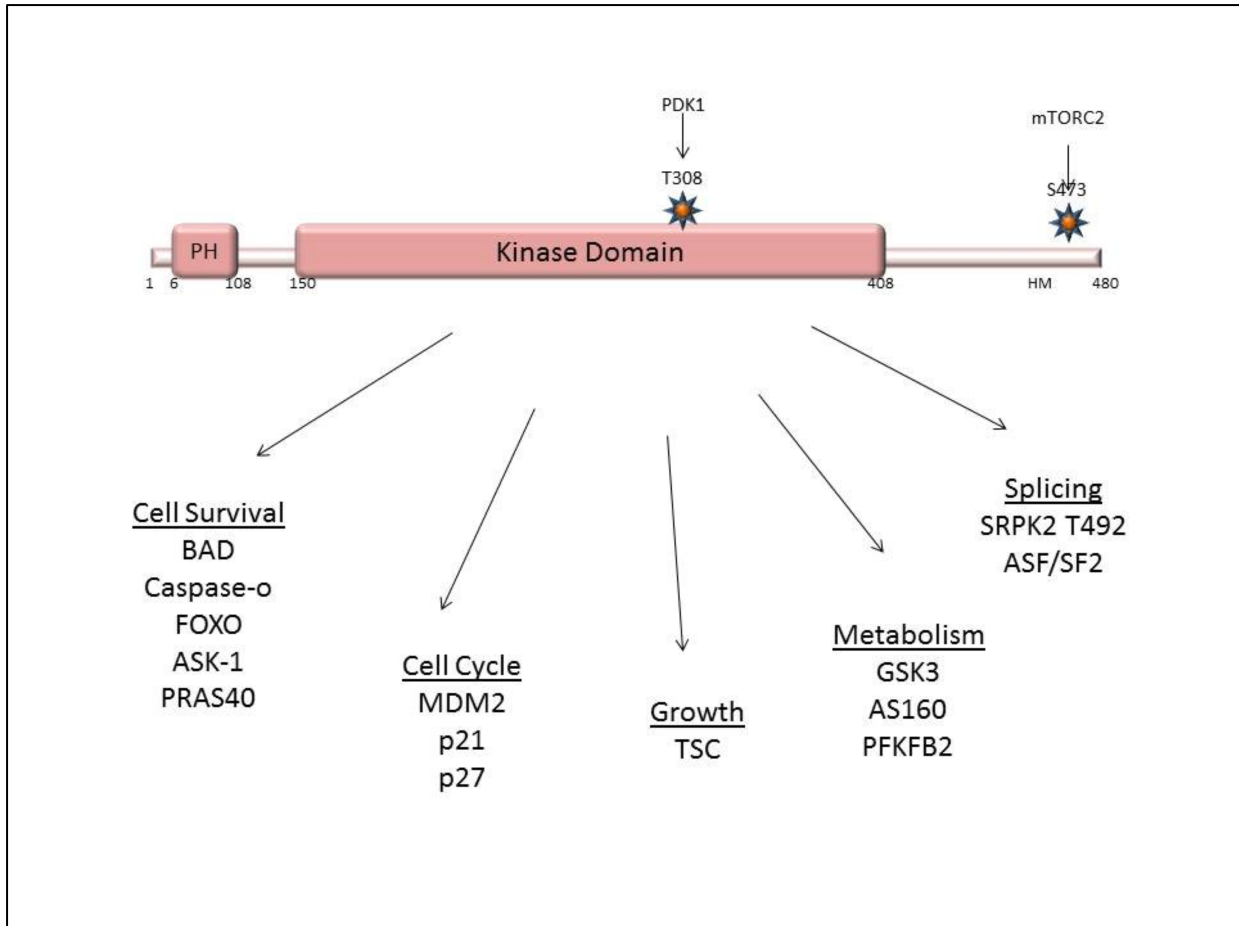

Figure 1.3: Linear depiction of human Akt1 and the downstream targets. Akt is regulated by PDK1 and mTORC2, and contains a pleckstrin homology (PH) domain, a kinase domain, and a hydrophobic motif (HM). The selected targets are an example of the multitude of cellular processes Akt regulates including cell survival, cell cycle, growth, and metabolism.

While Akt was originally identified as the kinase responsible for inactivating GSK3β through phosphorylation, Brendan Manning's group found that in TSC2-/- MEFs S6K can phosphorylate GSK3β [51]. In these cells, mTORC1 activity is hyperactivated, resulting in the upregulation of S6K activity, and the downregulation of Akt activity through phosphorylation of IRS and negative feedback inhibition , providing further evidence of the ability for the AGC kinases to target the same substrates depending upon cell type or stimulus conditions.

Cell cycle regulation

The FOXO family of forkhead transcription factors is another well-known group of Akt targets. These proteins participate in the regulation of the cell cycle, cell death, oxidative stress, as well as cell metabolism. Cell cycle regulation by Akt also occurs by phosphorylation of the cell cycle inhibitor $p27^{Kip1}$, which promotes 14-3-3 binding and cytoplasmic sequestration [52]. Additionally, Akt phosphorylates cyclin-dependent kinase inhibitor $p21^{Cip1/WAF1}$, as well as the inhibitor of p53 MDM2 to regulate the cell cycle [53, 54].

Cell survival

Another example of a substrate that has been shown to be phosphorylated by Akt as well as several other AGC kinases is the Bcl-2 family pro-apoptotic BH3 only protein BAD. Phosphorylation at Ser136 promotes 14-3-3 binding, phosphorylation at Ser155, and disruption of the binding between BAD and the anti-apoptotic Bcl-2 proteins (reviewed in [55]).

The Ras-ERK-RSK Signaling Pathway

A generalized mitogen activated kinase (MAPK) pathway consists of an activating MAP kinase kinase kinase (MAP3K), which is often activated by a small GTP-binding protein. This MAP3K will phosphorylate an intermediary MAP kinase kinase (MAP2K), which will phosphorylate a MAPK at both Tyrosine and Threonine residues in the activation loop. This leads to the MAPK phosphorylation of its targets to elicit a cellular response (reviewed in [56]). In the Ras-ERK pathway, the GTPase Ras activates the MAP3K serine/threonine kinase Raf, which then phosphorylates and activates the MAP2K MAPK/ERK 1/2 (MEK1/2). The duel-specificity kinase MEK1/2 then phosphorylates the MAPKs ERK1/2. Following activation, ERK1/2 phosphorylates

multiple targets involved in cell growth and proliferation, including the 90kDa ribosomal S6 kinase (RSK) family. The Ras/ERK1/2 pathway contains kinases that are mutated frequently in human cancers, and a number of disorders and diseases arise from inappropriate regulation of this pathway (reviewed in [57]).

RSK Activation and Subcellular Localization

There are four isoforms in the vertebrate RSK family termed RSK1, RSK2, RSK3 and RSK4, with all members containing two functional and non-identical kinase domains (reviewed in [58]). Upon mitogen-stimulation, the C-terminal kinase domain (CTKD) is phosphorylated by ERK1/2, which docks to the RSKs via the D docking domain comprised of clusters of positively charged amino acids, surrounded by hydrophobic residues [59]. After the activating input from ERK1/2, the CTKD then phosphorylates and activates the N-terminal kinase domain (NTKD) (Fig 1.4) (reviewed in [60]). There are two separate kinase domains in the RSK proteins that are not the result of gene duplication, and indeed, represent two separate protein kinase families. The NTKD is an AGC protein kinase family member, and the CTKD of the RSKs belongs to the Ca^{2+}/calmodulin-dependent protein kinase (CAMK) family [61]. The only known function of the CTKD is to autophosphorylate the hydrophobic motif and activate the NTKD, which is responsible for the phosphorylation of the downstream substrates (reviewed in [58]).

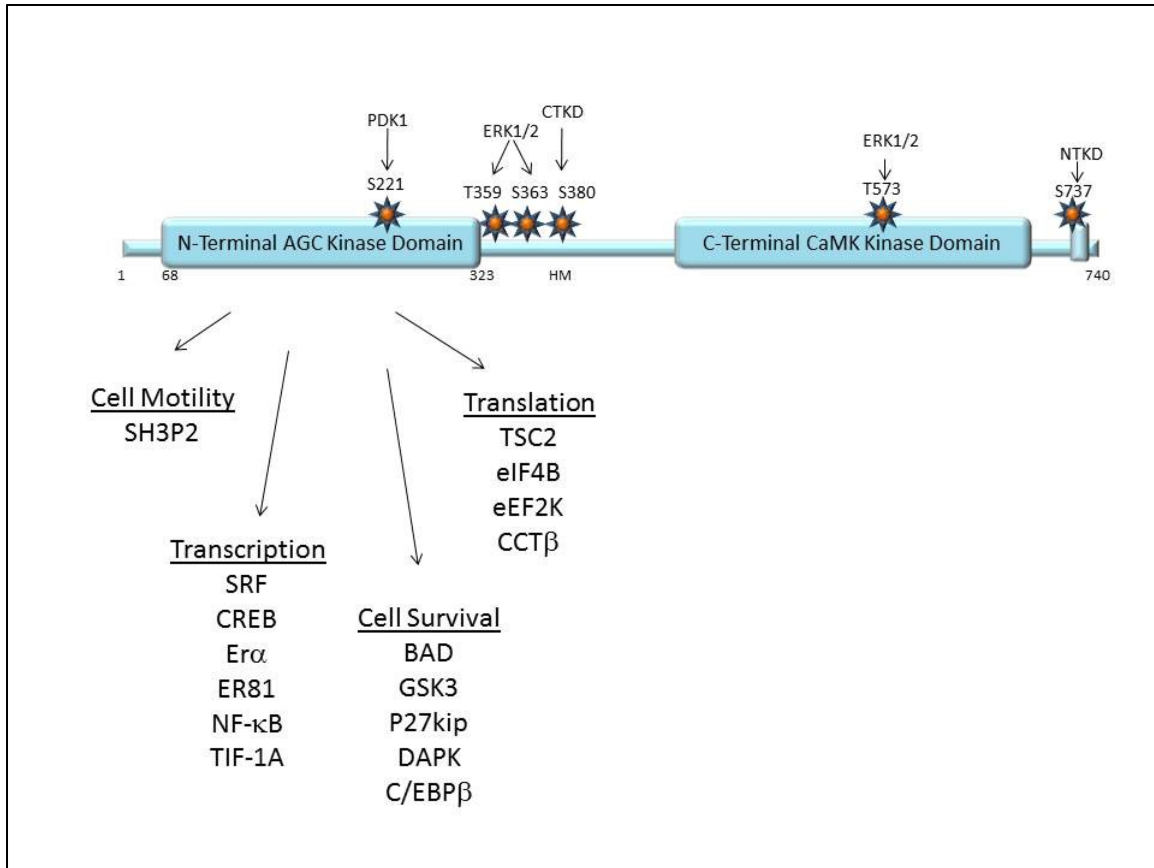

Figure 1.4: Linear depiction of RSK and downstream targets. RSK is regulated by PDK1 and through CTKD autoregulation. RSK contains two kinase domains, and a hydrophobic motif (HM). The N-terminal kinase domain (NTKD) is an AGC kinase family member, while the C-terminal kinase domain (CTKD) is a CaMK kinase domain. The selected targets are an example of the multitude of cellular processes RSK regulates including cell motility, transcription, cell survival and translation.

ERK1/2 phosphorylates RSKs at a number of Ser/Thr residues followed immediately by a proline residue including Thr573, which partially activates the CTKD, as well as at Ser363 in the turn motif of the linker region of the NTKD. The phosphorylation at these residues by ERK1/2 leads to the autophosphorylation of RSK1 at Ser380 at the hydrophobic motif. This phosphorylation allows for full activation following PDK1 docking and phosphorylation at Ser221 (reviewed in [60]).

RSK Targets

A wide range of functions can be attributed to RSK signaling including transcription, cell survival, cell-cycle progression, migration, and protein synthesis. Like AKT, many RSK targets overlap with other AGC kinases further suggesting redundancy in AGC kinase signaling and modes of pathway crosstalk. Reviewed briefly below are the known targets of the RSKs categorized by cellular function.

Transcription

An important function of RSK signaling is the control of transcription of immediate early genes through phosphorylation of transcription factors. These include the serum response factor (SRF), and the orphan nuclear receptor Nur77 [62-64]. RSK also phosphorylates c-fos, which results in its stabilization and an increase in AP-1 activity [65, 66]. Additional transcription factors phosphorylated by RSK are CREB, ER81, ERα and NF-κB [67-72]. In addition, RSK can activate RNA polymerase I transcription by phosphorylating the transcription factor TIF-IA [73].

Cell survival and cell-cycle progression

RSK phosphorylation of substrates that promote cell survival in response to mitogenic stimulation have been identified. RSK1 was reported to phosphorylate the CCAAT/enhancer-binding protein (C/EBPβ) promoting survival of hepatic stellate cells following treatment with the hepatotoxin CCI4. Additionally, RSK1 and RSK2 have been shown to phosphorylate the pro-apoptotic protein Bad at Ser112 prompting 14-3-3 binding and subsequent inactivation [74]. Further evidence of RSK signaling in cell survival is the phosphorylation of the tumor suppressor death associated protein kinase (DAPK) at Ser289, which prevents DAPK pro-apototic activity and enhanced survival in response to mitogen stimulation [75].

Another function where phosphorylation of substrates by RSK is important is the regulation of cell-cycle progression. One notable substrate is the cyclin-dependent kinase 2 inhbitor $p27^{kip1}$that is phosphorylated by RSK at Thr198, which promotes biding by 14-3-3 and prevents its translocation to the nucleus [76]. AKT was also reported to target this site [52]. Another target of RSK involved in cell-cycle progression is the negative regulation of glycogen synthesis kinase 3 (GSK3), which can also be phosphorylated by AKT and S6K depending upon cell-type [49, 51, 77].

Migration

A recent report by Doehn et al, identified RSK1 and RSK 2 as key regulators of Ras/ERK-mediated epithelial-mesenchymal transition (EMT) through activation of a transcriptional program, which coordinates the extracellular environment, the intracellular motility proteins, and the receptors connecting them [78]. They showed a dependence on RSK1 and RSK2 for Raf1-mediated migration in MDCK cells. Additionally, RSK was shown to regulate the integration of multiple migratory stimuli in a genome wide RNAi screen [78]. Lastly, Tanimura et al reported that RSK phosphorylates the Src homology 3 domain-containing protein (SH3P2), which inhibits the proteins ability to act as a negative regulator of cell motility suggesting multiple ways for RSK involvement in migration [79].

Protein synthesis

mTORC1 is a major regulator of protein translation, and RSK phosphorylates both Raptor and TSC2 facilitating further activation of this pathway, and highlighting the importance of pathway crosstalk [80, 81]. Additionally, RSK can phosphorylate targets downstream of mTORC1 that have been previously described as targets of S6K. These

are S6, eIF4B, and eEF2K [28, 82, 83], and provide further evidence of the promiscuous and redundant nature of AGC kinase signaling.

Pathway convergence

Knowing that the AGC kinases share a requirement for PDK1 phosphorylation of their activation loop, as well as the similarities these kinases share in the phosphorylation motifs of their substrates (RXRXXS/T) aids in our understanding of how these kinases signal to the same substrates (reviewed in [84] The redundancy in AGC kinase signaling is exemplified in the fact that BAD, rpS6, eIF4B, GSK3β, YB1, and ERα have all been reported to be *in vivo* targets of these kinases downstream of different signaling pathways and under different conditions (Fig 1.5).

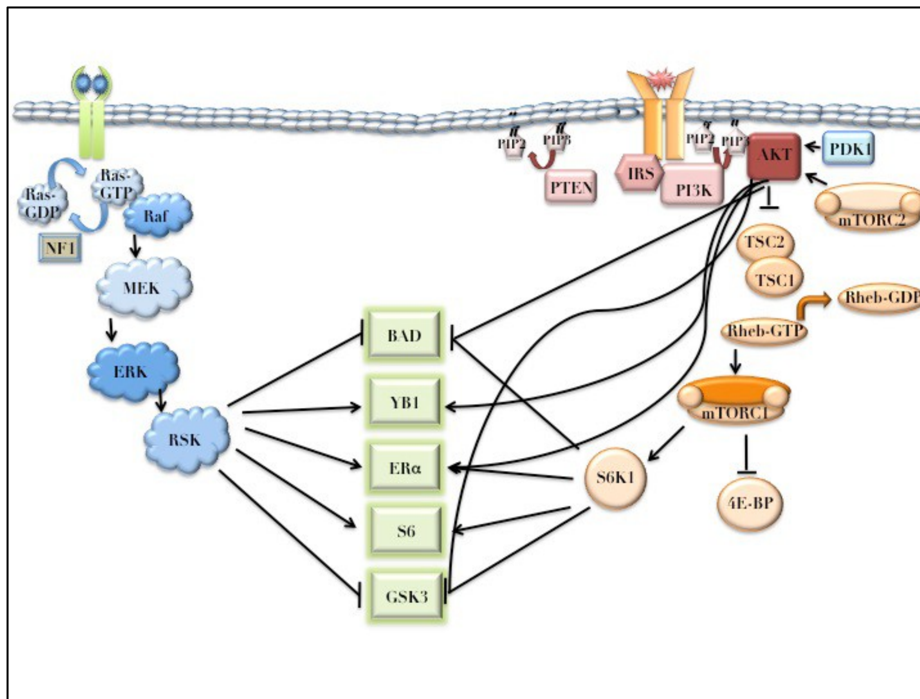

Figure 1.5: The AGC kinase propensity for promiscuity. RSK, Akt and S6K regulate the same residues on GSK3, eIF4B, and ERα. RSK and Akt regulate the same residues on YB1, while RSK and S6K phosphorylate the same residues on rpS6 and MAD1. Figure adapted from Mendoza et al 2011.

As discussed above individually, the AGC kinases phosphorylate many of the same substrates, and these substrates serve as examples of PI3K/mTOR and Ras/ERK pathway convergence. RSK, S6K and Akt phosphorylate GSK3α and β on Ser21/Ser9 [49, 77, 85]. The stimulus and cellular conditions vary for these phosphorylation events, with RSK phosphorylating these sites in response to EGF and PMA stimulation, AKT in response to IGF and insulin stimulation, and S6K phosphorylating these sites in TSC2-/- MEFs. Despite the different conditions, these phosphorylation events all result in the same outcome, by creating pseudo-substrate sites that induce intramolecular inhibition of GSK3[86].

S6K, RSK and/or AKT have been shown to regulate protein synthesis by phosphorylation of eIF4B, eEF2K, and the ribosomal protein S6 [24-26]. All three kinases phosphorylate eIF4B at Ser422, which serves as a co-factor for the eIF4A ATPase/helicase, and promotes association with the pre-initiation complex [29]. S6K and RSK both phosphorylate eEF2K at Ser366, resulting in the activation of translation elongation [82, 87, 88]. Additionally, RSK and S6K phosphorylate the ribosomal protein S6 at Ser235/Ser236, as mentioned previously.

While only a few are highlighted, AGC kinases phosphorylate a vast number and range of proteins that regulate numerous cellular processes [89]. While a full understanding of why there is such a high amount of redundancy in AGC kinase substrates, one can imagine that these kinases serve in a compensatory mechanism in the event of loss of function of a kinase. Additionally, one can postulate that the AGC kinases ability to phosphorylate the same substrates has evolved to allow for the

regulation of the same cellular machinery, eliciting the same cellular response downstream of different signaling pathways in response to different stimuli.

pre-mRNA splicing

Before the sequencing of the human genome, scientific approximations for the number of genes ranged from over 100,000 to 48,000. It is now understood that there are between 20,000 and 25,000 genes. Considering the *Caenorhabditis elegans* genome consists of approximately 19,500 genes, and that of *Arabidopsis thaliana* consists of 27,000, the absolute number of genes does not explain the complexity of the organism.

The vast majority of vertebrate genes contain introns, which are spliced out of primary RNA transcripts (pre-mRNA) to yield mature messenger RNA (mRNA) species. Utilization of different splice-sites during splicing produces different mRNAs from the same pre-mRNA transcript. These alternatively spliced mRNA species often bare unique information [90]. This is one of the most important mechanisms to generate a large number of protein isoforms from the surprisingly low number of human genes, and it is estimated that 75% to 90% of human genes are alternatively spliced [91, 92]. In addition to the creation of functionally distinct protein isoforms being created by the utilization of different splice sites within the open reading frame, gene expression can be influenced through sequence alterations in the 5' and 3' untranslated regions (UTRs) leading to differential translational control, mRNA stability or localization [93, 94].

The spliceosome is a large RNA and protein complex that contains the five small nuclear ribonucleoproteins (snRNPs) U1, U2, U4, U5 and U6 (reviewed in [95]). Spliceosome assembly is a highly ordered and sequential process of events that happens *de novo* on each individual intron to be removed, beginning with U1 snRNP binding to

the 5' splice site of the pre-mRNA and the heterodimeric splicing factor U2 snRNP auxiliary factor (U2AF) binding to the 3' splice site (Fig 1.6). Briefly, splicing is carried out in two steps involving two transesterification reactions, with the branch-point nucleotide defined during spliceosome assembly forming the lariat intermediate with the first nucleotide of the intron at the 5' splice site, followed by the joining of the exons and releasing of the intron lariat.

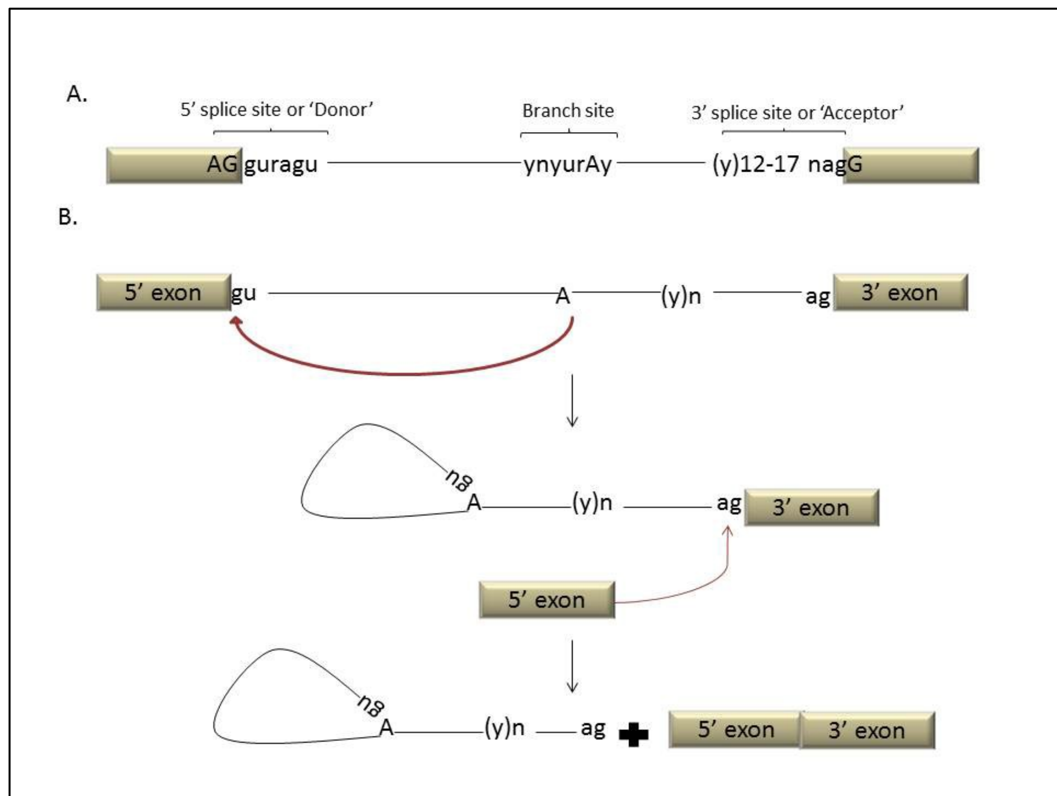

Figure 1.6: pre-mRNA splicing. A. The sequence elements of the 5' splice site, the branch site, as well as the 3' splice site are required for splicing. B. A simplified depiction of the two transesterification reactions of pre-mRNA splicing. The first step yields the intron/3' exon in a lariat structure, as well as the free 5' exon. The second step yields the ligated exons and the intron lariat, which is subsequently degraded.

SR Proteins

From their beginning at transcription to their ultimate degradation, mRNA molecules are dynamically associated with many different proteins in the form of

messenger ribonucleoprotein particles (mRNPs), and these associated proteins, including the SR and the hnRNP family of proteins (discussed below) help determine the eventual fate of the mRNA. The Serine/Arginine rich (SR) proteins are a class of evolutionarily conserved phosphoproteins that were originally identified as being essential for pre-mRNA splicing, but have recently been shown to be involved in many steps of mRNA biogenesis including alternative splice site selection, nuclear export, RNA stability, mRNA quality control, and translation (reviewed in [96]). The modular structure of the SR family of proteins consists of an N-terminal domain that contains one or two copies of an RNA-recognition motif (RRM) and a C-terminal domain rich in alternating arginine and serine residues, known as the RS domain [97]. RNA binding specificity is determined by the RRMs, while the RS domain regulates protein-protein interactions with components of the core splicing apparatus to promote splice site pairing [97].

SR Protein Kinases

Phosphorylation of SR proteins in their RS domain greatly influences splice site selection, as well as subcellular localization [98]. The dominant kinases for SR proteins are the SR protein kinases (SRPK) [99, 100]. Mammalian SRPK1 and SRPK2 processively phosphorylate SR proteins on multiple residues, and are highly related in their kinase domain sequences [101]. The SRPKs specifically phosphorylate serine residues, which follow a serine-arginine/arginine-serine (RS) dipeptide motif [102, 103]. While the SR families of splicing factors are the classical examples for the substrates of the SRPKs, proteins containing scattered RS dipeptides have been shown to be phosphorylated by the SRPKs if they conform to certain limitations [104-106]. Mutation of the Ser to Thr or Arg to Lys in the RS domain will abolish the ability for the SRPKs to

phosphorylate the substrates exemplifying the specificity of these enzymes for the RS motif [102, 107, 108].

SRPKs show conservation throughout evolution from budding yeast to humans [109-111]. Since all SRPK family members are active when expressed in *Escherichia coli,* they have been considered a class of constitutively active kinases, though one paper reports the ability of casein kinase 2 (CK2) to phosphorylate and activate SRPK1 in testis extracts [112]. It is currently thought that the majority of their activity is dependent on their subcellular localization [113]. SRPKs have a conserved serine/threonine kinase domain that is separated into two N- and C- terminal portions by a non-conserved spacer sequence, which appears to be involved in the cytoplasmic sequestering of all SRPK family members [113].

hnRNPs

As mentioned above, the heterogeneous nuclear ribonucleoproteins (hnRNP) are a family of RNA-binding proteins, involved in the processing of heterogeneous nuclear RNAs (hnRNAs) into mature mRNAs. In addition, these proteins also act as *trans*-factors regulating gene expression (reviewed in [114] and[115]). The classical definition of hnRNA was coined by Gideon Dreyfuss as the mRNA precursors that are produced by RNA polymerase II that undergo extensive processing before a functional mRNA is produced. The term hnRNA is often used interchangeably with pre-mRNA. When these hnRNAs/pre-mRNAs are produced by RNA polymerase II and until their maturation into mRNAs, they are associated with large protein complexes. These hnRNA-binding proteins are termed hnRNA proteins (hnRNP) [116].

Alternative splicing

Alternative splicing of pre-mRNAs is an important way to generate protein diversity given the relatively small number of human genes (Fig. 1.7). Many factors, including growth factors, nutrient availability, stress response, and the energy status of the cell all affect splice site selection in a cell-type and developmental stage specific manner. Recent reports indicate that greater than 95% of human genes undergo at least one alternative splicing event (reviewed in [117]). Data compiled from transcriptome profiling using next generation sequencing technology indicate a wide ranging and vast number of variations in normal splicing processes during cellular transformation and inn established tumors (reviewed in [118]). Understanding how these and other aberrant splicing events are regulated downstream of signaling pathways that are hyperactive in cancer is an open question, and of great clinical relevance.

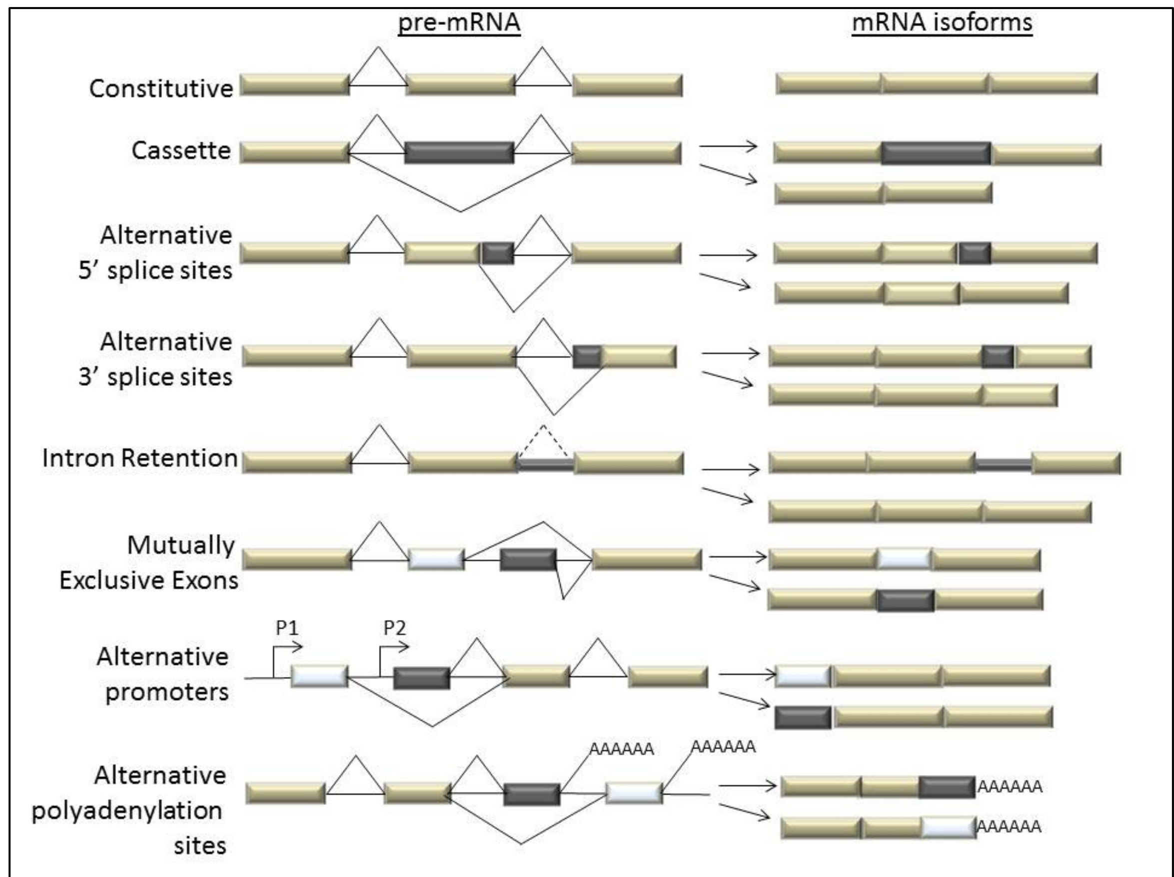

Figure 1.7: Different patterns of alternative splicing. The exons are depicted as boxes, and the introns as solid black lines. The pre-mRNA is shown on the left, with the resultant hypothetical mRNA isoforms on the right.

Many human malignancies are characterized by aberrant patterns of pre-mRNA processing [119], and proteins encoded by splice variants have been shown to affect a variety of tumor-associated processes [120]. SRPK1 overexpression has been reported to occur in breast, colonic, and pancreatic carcinomas [121]. Targeting SRPK1 using siRNA resulted in reduced proliferation and enhanced apoptosis of cancer cell lines suggesting that SRPK1 may serve as a therapeutic target [121]. In addition, the splicing factor SF2/ASF, a known substrate for both SRPK1 and SRPK2, is upregulated in various

tumors, due in part to amplification of its gene *SFRS1.* Overexpression of SF2/ASF is sufficient to transform immortal rodent fibroblasts [122]. Interestingly, SF2/ASF controls the alternative splicing of an oncogenic isoform of S6K1, and its expression recapitulates the transforming activity of SF2/ASF [122].

Nonsense-Mediated mRNA Decay

Nonsense-mediated mRNA decay (NMD) is one of several post-transcriptional mechanisms that eukaryotic cells use to control the quality of mRNA (reviewed in [123]). NMD prevents the production of truncated proteins, arising from abnormal transcripts that prematurely terminate transcription, which could function in a dominant-negative mode of action [124]. These transcripts can arise from routine mistakes in transcription, or be produced from mutated, often times disease-associated, genes. Growing evidence indicates that a substantial amount of alternative splicing events in humans result in mRNA isoforms that harbor a premature termination codon (PTC). While this high number of PTC containing transcripts might indicate a large amount of splicing errors, it is becoming clearer that, at least for certain families of proteins the coupling of alternative splicing and NMD is a way to regulate the abundance of mRNA transcripts and ultimately protein expression levels [125]. This mechanism of coupling alternative splicing and NMD has been deemed Regulate Unproductive Splicing and Translation (RUST), and is frequently employed for the autoregulation of proteins that affect the splicing process itself, namely the SR and hnRNP proteins.

NMD is a translation-dependent process, and is thought to occur during the first, or 'pioneer', round of translation [126]. During the pioneer round of translation, ribosomes will remove and remodel the proteins that have been deposited during the

splicing process (discussed previously). When the ribosomes encounter an EJC situated more than ~25-35 nucleotides downstream of a premature termination codon (PTC), this will trigger the message for degradation [127-129]. The requirement of translation for the process of NMD is well established, and exemplified by the fact that certain antibiotics that inhibit translationally active ribosomes will inhibit NMD [130].

Briefly, during NMD, when translation terminates at a PTC that is then followed by an EJC, the protein complex SURF forms at the PTC. SURF contains the phosphoinositide 3-kinase-related protein kinase SMG1, the regulator of nonsense transcripts 1 UPF1, and the two translation termination factors eukaryotic release factor 1 eRF1 and eRF3 [131]. Following SURF formation at the PTC, SMG1 will phosphorylate UPF1, which will then bind to eIF3 suppressing further translation of the NMD target [132]. Additionally, phosphorylated UPF1 recruits both 5' and 3' endonucleases to mediate mRNA decay [133].

Regulated Unproductive Splicing and Translation

As mentioned above, RUST or the regulated unproductive splicing and translation is the coupling of alternative splicing and NMD (Fig.1.8). It has been postulated that RUST and the regulation of this process is a way to control the amount of mRNA that would go on to direct protein synthesis, ultimately controlling protein expression levels. Originally posited to be a general mechanism with which to regulate all protein expression levels, experiments using microarray analysis of HeLa cells in which the essential NMD factor UPF1 had been knocked down using RNA interference (RNAi) suggested 4.9% of expressed genes are regulated by NMD [134]. While RUST appears to be less prevalent than originally hypothesized, its importance in regulating key

families of proteins mainly the SR and hnRNP proteins is becoming much clearer [135, 136].

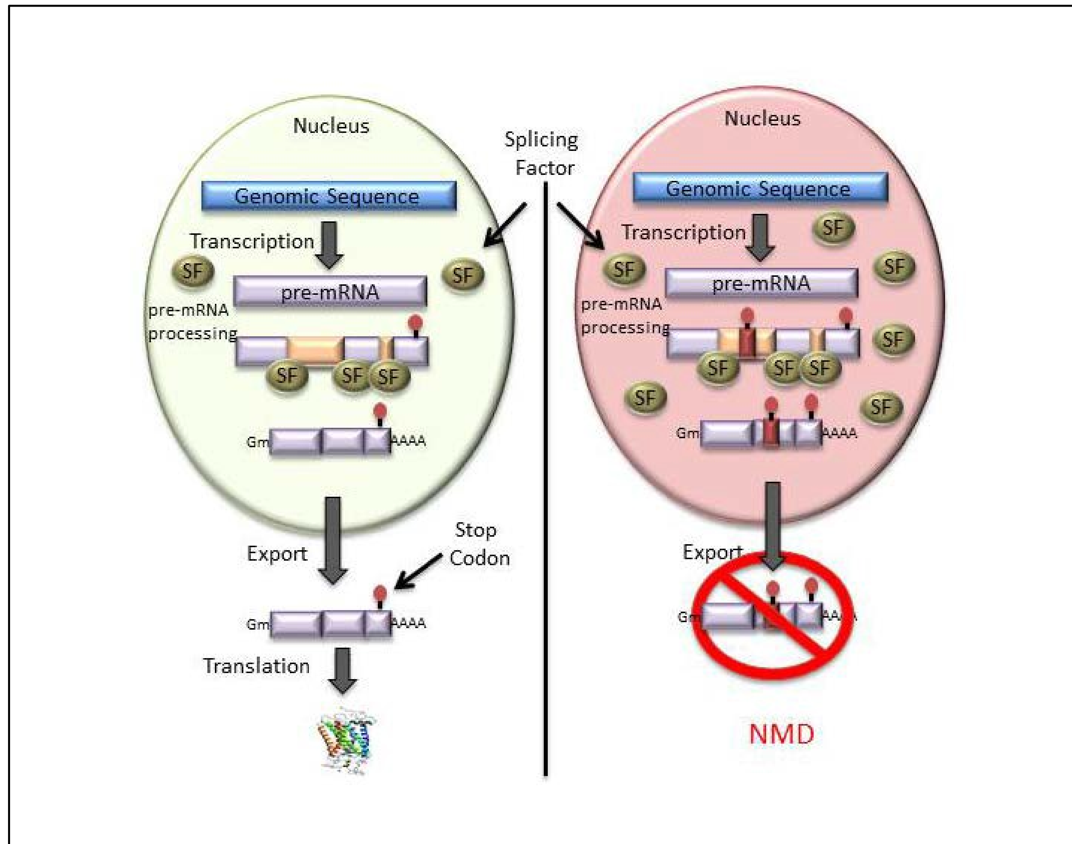

Figure 1.8: Regulated unproductive splicing and translation (RUST). Intracellular concentrations of certain splicing factors are regulated through the coupling of alternative splicing and nonsense mediated decay (NMD). A premature translation termination codon (PTC) triggers the message for degradation by the NMD pathway. The *trans* acting splicing factors are subject to RUST, and have spliced isoforms that have exons that harbor PTCs.

Both the SR and hnRNP families of splicing regulators had been reported to contain single examples of RUST. How important this process is for the regulation of these splicing factors was demonstrated when Lareau and colleagues reported that all members of the human and mouse SR-protein gene family undergo RUST, and that strikingly, these regions that undergo alternative splicing and contain the PTC are highly

or "ultra" conserved [135]. Ultraconserved elements (UCEs) are defined as sequence segments of 200bp or longer that are 100% conserved between *Homo sapiens*, *Mus musculus* and *Rattus norvegicus* [137]. There have been 481 UCEs identified in the human genome, with 111 overlapping the mRNA of known protein-coding genes [137]. RNA binding and AS regulation are the predominant function for these known genes [138]. Two subsequent studies by Ni et al and Saltzman et al confirmed Lareau et al.'s results using a combination of AS-sensitive microarrays and bioinformatics [139-141]. These studies sought to identify cases of cassette-exon RUST that showed a high degree of conservation, and would therefore be predicted to have functional consequences. Ni et al identified a group of 66 exons belonging to both the SR and hnRNP proteins that were enriched for association with UCEs [139]. These results were confirmed by Saltzman et al. who reported RUST with the SR and hnRNP genes, as well as the observation of conserved RUST of many core spliceosomal proteins [141]. This led them to hypothesize that RUST might regulate proteins involved in both constitutive, as well as alternative splicing.

To date, there have been very few studies aimed at identifying the signaling pathways that regulate splice site selection, through regulation of the phosphorylation of the SR proteins by the SRPKs. The experiments in the following chapters aim to provide new data that connect the PI3K/mTORC1 and the Ras/ERK/RSK signaling pathways in the regulation of alternative splice site selection.

Chapter 2 PI3K/mTORC1 signaling inputs to SRPK1/2

Preface

This work is not yet published. Dr. Yonghao Yu performed the SILAC screen that identified the putative phosphorylation site on SRPK2 (Yu, 2010). Dr. Leon Murphy a former post-doctoral fellow in the Blenis now at Novartis identified SRPK1 as a high confidence binding partner of S6K1. Dr. Sarah Mahoney helped with the site directed mutagenesis for SRPK2. Drs Alfredo Csibi and Gina Lee helped with the experiments in figures 2.6 and 2.7.

Introduction

Over the past 20 years of mTORC1 signaling research, many insights into the regulation of and players involved in this pathway have been made, though the functional characterization of this pathway has been held back by the dearth of established substrates. Therefore, in order to identify novel substrates of the mTORC1/S6K signaling pathway, our lab performed two large-scale stable isotope labeling of amino acids in cell culture (SILAC) mass spectrometry screens. In the first screen using TSC2-/- mouse embryonic fibroblasts (MEFs), 4484 and 6832 unique phosphorylation sites on 1615 and 1866 proteins were identified from two biological replicate experiments [1].

Included in this data set was a rapamycin sensitive phosphorylation site on the SR protein kinase 2 (SRPK2). Additionally, a former post-doctoral fellow in the Blenis lab Leon Murphy, who is now a researcher at Novartis, identified the SR protein kinase 1 (SRPK1) as a high confidence, binding partner for S6K1, and contacted the Blenis lab to

establish a collaboration to further explore this protein interaction. Given the prevalence of alternative splicing events in response to growth factor stimulation, as well as changes in nutrient and energy availability, we hypothesized that mTORC1 and S6K signaling could alter splice site selection, and ultimately gene expression in response to the nutrient and energy status of the cell. The identification of SRPK1 and 2 as novel proteins involved in mTORC1 signaling provided a conjectural mechanism for linking this signaling pathway to splice site selection.

The phosphorylation status of the SR proteins affects their many cellular functions, including splice site selection, and subcellular localization (discussed in Chapter 1). Phosphorylation of the SR proteins occurs through the SR protein kinases SRPK1, SRPK2, and the CDC2-like kinase CLK1 and is limited to the serine residues that reside in the arginine/serine (RS) rich domain. The SRPKs have long been thought to be constitutively active kinases since proteins purified in bacteria are able to efficiently phosphorylate their substrates *in vitro.* Recent reports suggest that these kinases are in fact regulated, with subcellular partitioning being the common determinant in regulation of kinase activity. SRPK1 and SRPK2 contain a linker region that interrupts the N-terminal and C-terminal portions of their kinase domains, and has been shown to be responsible for the cytoplasmic sequestering of these kinases. Anchoring of SRPK1 in the cytoplasm has recently been shown to be through interactions with the heat shock proteins hsp40 and hsp90 [142]. Additionally, SRPK2 was reported to be phosphorylated at Thr492, triggering 14-3-3 binding and retention in the cytoplasm [143].

The phosphorylation event on SRPK2 identified in the SILAC screens performed in our lab is found in the linker region, which is found between the N and C terminal lobes of the bifurcated kinase domain, and follows the canonical AGC kinase phosphorylation motif (Fig 2.1). Conservation of this protein sequence is seen in vertebrates.

Fig 2.1: The location of the evolutionarily conserved putative S6K phosphorylation site A. A linear depiction of the SRPK2 protein, which contains a PKC-like bifurcated kinase domain that is interrupted by a linker region. B. The basophilic putative phosphorylation site on SRPK2 is conserved in vertebrates, and not found in any of the other SR protein kinase family members.

Result 2.1: S6K interacts with SRPK1/2

SRPK1 and SRPK2 are about 85% conserved in their kinase domain, but there is little conservation of the linker region, with the putative AGC kinase phosphorylation site identified not conserved between SRPK1 and SRPK2. To confirm the interaction between S6K and SRPK1 and SRPK2 *in cellulo*, a co-immunoprecipitation was

performed (Fig 2.2). We used an antibody to IP S6K1 from 293E cells that were serum starved for 18 hours, insulin stimulated for 60 mins, and/or pretreated with rapamycin for 30 mins before insulin stimulation. After immunoprecipitation, we observed an interaction between S6K1 and SRPK1 in all conditions.

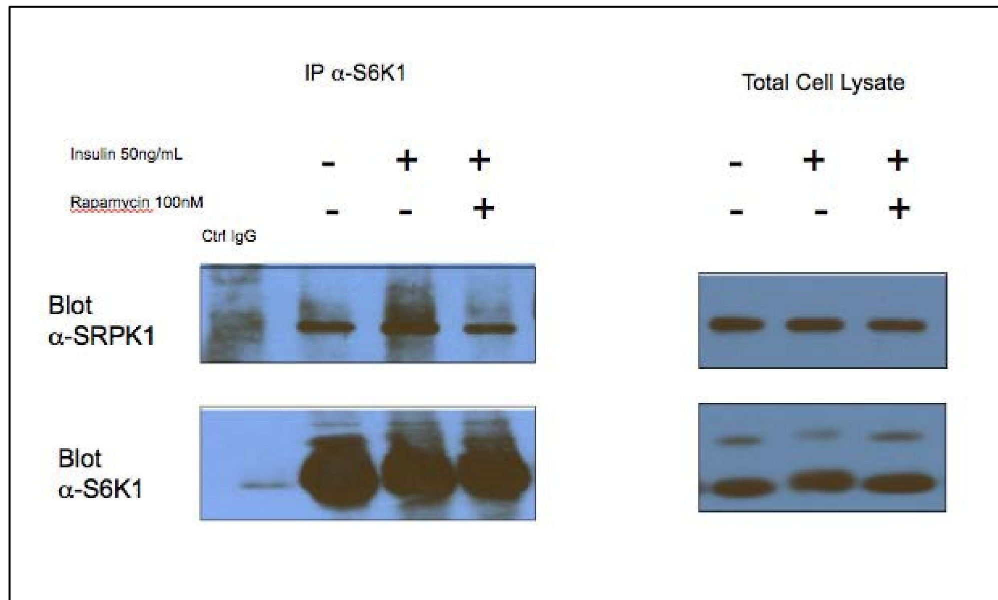

Fig 2.2: Endogenous S6K1 co-Immunoprecipitates with SRPK1 293E cells were serum starved for 18 hours, and treated with stimulants and inhibitors as shown. Immunoprecipitation was performed with a homemade antibody to the C-terminus of S6K1 for 2H, and then Western blot was performed. Treatment conditions: insulin (30', 100nM), rapamycin (30' pretreatment, 20ng/mL) Data representative of experiments performed in duplicate.

Given the interaction seen between, SRPK1 and S6K1, as well as the identification of SRPK2 in the SILAC mass spectrometry screen, we next tested whether SRPK2 and S6K1 interact *in cellulo* by perfoming co-immunoprecipitation using both IPed S6K1 and blotting for SRPK2, as well as the reciprocal experiment IPing SRPK2 and blotting for S6K1 (Figure 2.3). An interaction was observed between S6K1 and SRPK2 under all conditions tested either when S6K1 or SRPK2 were subject to

immunoprecipitation. Interestingly, a mobility shift was observed for SRPK2 when stimulated with insulin, which seemed to be blocked by pretreatment with rapamycin.

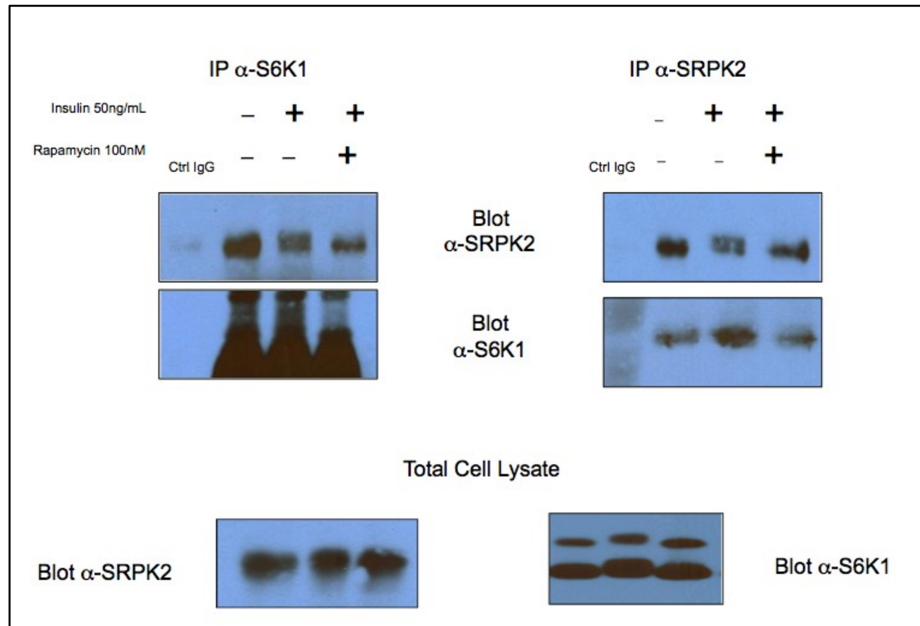

Fig 2.3: Endogenous S6K1 coIPs with SRPK2 293E cells were serum starved for 18 hours, and treated with stimulants and inhibitors as shown. Immunoprecipitation was performed with a homemade antibody to the C-terminus of S6K1, as well as a commercially available SRPK1 antibody for 2H, and then Western blot was performed. Treatment conditions: insulin (30', 100nM), rapamycin (30' pretreatment, 20ng/mL) Data representative of experiments performed in duplicate.

Results 2.2: Insulin induced phosphorylation of SRPK2 at Ser494

Given the recent identification of a putative rapamycin sensitive AGC kinase site in the SILAC mass spec screen discussed previously, we decided to investigate this further, and performed a time course using 293E cells serum starved overnight, and then stimulated with insulin for 15, 30, 60, and 120 minutes (Figure 2.4). We saw an insulin stimulated mobility shift for total SRPK2 clearly after 60 minutes of insulin stimulation, with 120 minutes insulin stimulation resulting in a defined single band. This mobility

shift was blocked in all time points by pretreatment with rapamycin, suggesting a signaling input from mTORC1/S6K1 pathway.

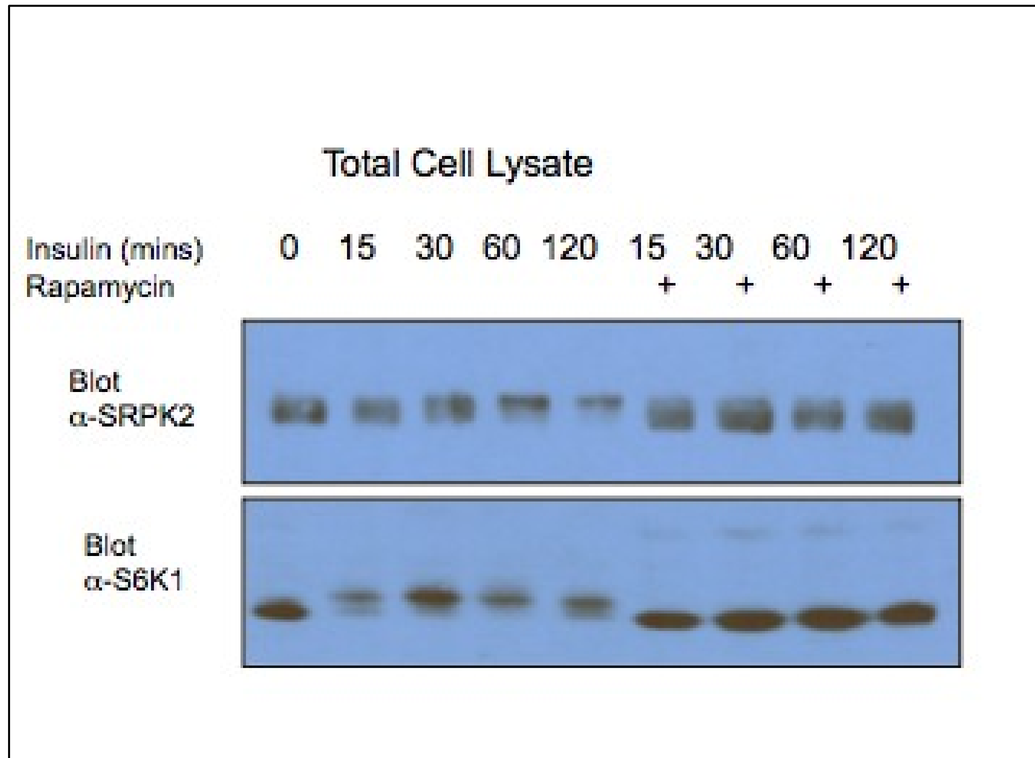

Figure 2.4: SRPK2 displays an insulin induced, rapamycin sensitive mobility shift 293E cells were serum starved for 18 hours, and then stimulated with insulin for the time indicated. A marked shift up following insulin stimulation was observed, and this shift is blocked by pretreatment with rapamycin. Data representative of experiments completed in duplicate.

Working in collaboration with Milipore, we developed a phospho-specific antibody to Ser494 on SRPK2 for use in our studies. This antibody was used to probe total cell lysates prepared following overnight serum starvation, insulin stimulation for 60 minutes, as well as pretreatment with rapamycin before insulin stimulation (Figure 2.5). A clear phosphorylation was observed following insulin stimulation, but was not blocked

by pretreatment with rapamycin, suggesting additional signaling inputs into the phosphorylation of SRPK2.

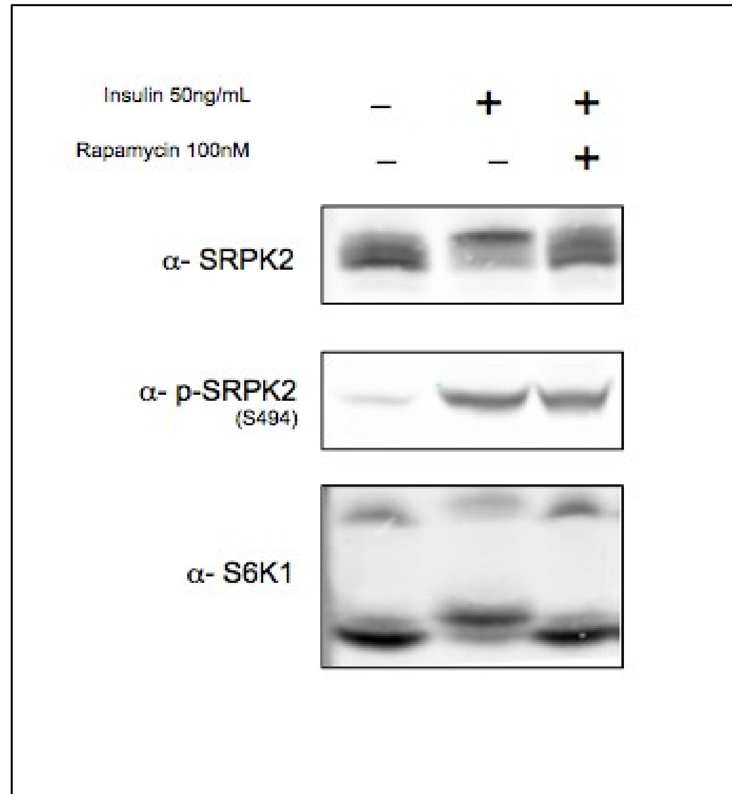

Figure 2.5: SRPK2 phosphorylation responds to insulin stimulation. 293E cells were serum-starved for 18h, and then treated with insulin and rapamycin as indicated. Western blot was performed on whole cell lysates, and data is representative of triplicate experiments. The antibody detecting pSRPK2 was made by Millipore. Treatment conditions: insulin (30', 100nM), rapamycin (30' pretreatment, 20ng/mL)

Since mTORC1/S6K inhibition results in the blocking of IRS2 phosphorylation by S6K and release of the negative feedback loop resulting in the further activation of AKT (discussed in detail in chapter 1), we investigated whether the PI3K/AKT pathway feeds into the phosphorylation of SRPK2 at Ser 494 (Figure 2.5). Again, we used lysates prepared from 293E cells that were serum starved overnight, and then stimulated with

insulin for 60 minutes. We pretreated samples with the mTORC1 inhibitor rapamycin, the PI3K inhibitor LY294002, as well as the dual specificity mTOR/PI3K inhibitor PI103 before stimulation with insulin. We were able to see inhibition of phosphorylation of SRPK2 at Ser494 with pretreatment of cells with both LY294002, as well as PI103 suggesting SRPK2 is phosphorylated by both AKT and S6K.

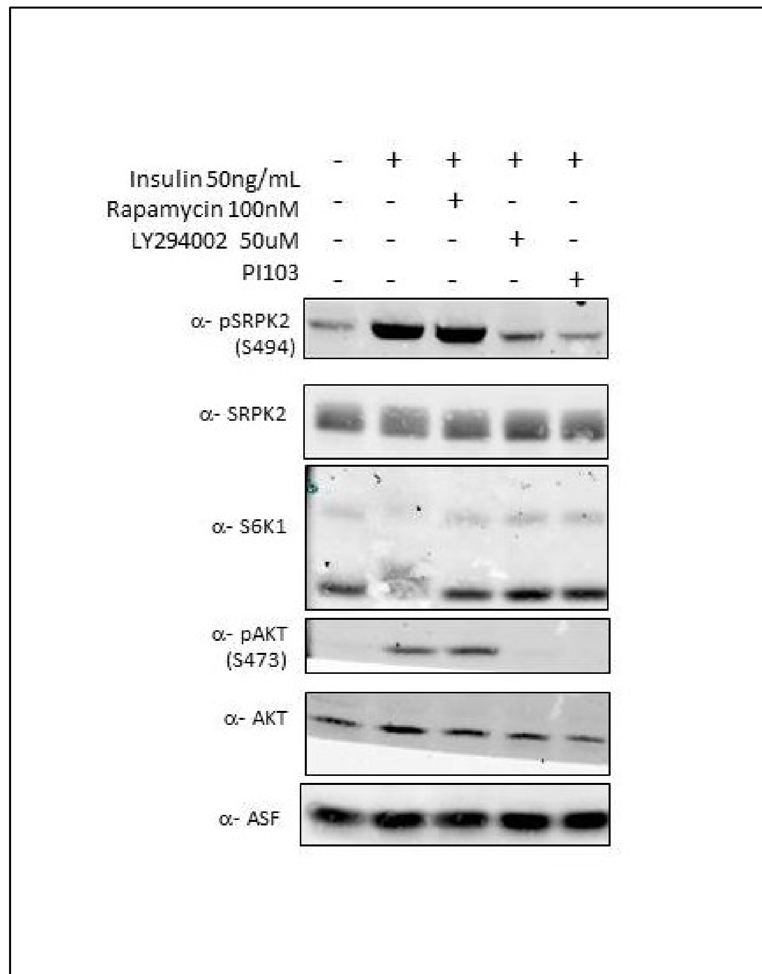

Figure 2.6: SRPK2 phosphorylation responds to PI3K-mTORC1 stimulant and inhibitor treatments. 293E cells were serum-starved for 18h, and then treated with inhibitors and stimulants as indicated. Western blot was performed on whole cell lysates. The antibody detecting pSRPK2 was made by Millipore. Treatment conditions: insulin (30’, 100nM), rapamycin (30’ pretreatment, 20ng/mL), LY294002 (30’ pretreatment, 50uM), PI103 (30’ pretreatment 1uM) Data representative of experiments performed in duplicate.

Results 2.4: S6K phosphorylation of SRPK2 at Ser494

Since Akt and S6K have been reported to phosphorylate the same substrates depending upon cellular context, we investigated whether both S6K and Akt could phosphorylate SRPK2 Ser494. To delineate the signaling inputs, we used the TSC2-/- mouse embryonic fibroblast cell line, which displays hyperactive mTORC1/S6K signaling. We immunoprecipitated transiently transfected HA-SRPK2, as well as a nonphosphorylatable mutant HA-SRPK2 where we substituted an alanine for serine 494. We detected an increase in phosphorylation upon amino acid and growth factor starvation and subsequent stimulation, which was blocked by pretreatment with the S6K specific inhibitor PF4078. Complete abolishment of the phosphorylation was seen in the immunoprecipitated HA-SRPK2S494A (Figure 2.7).

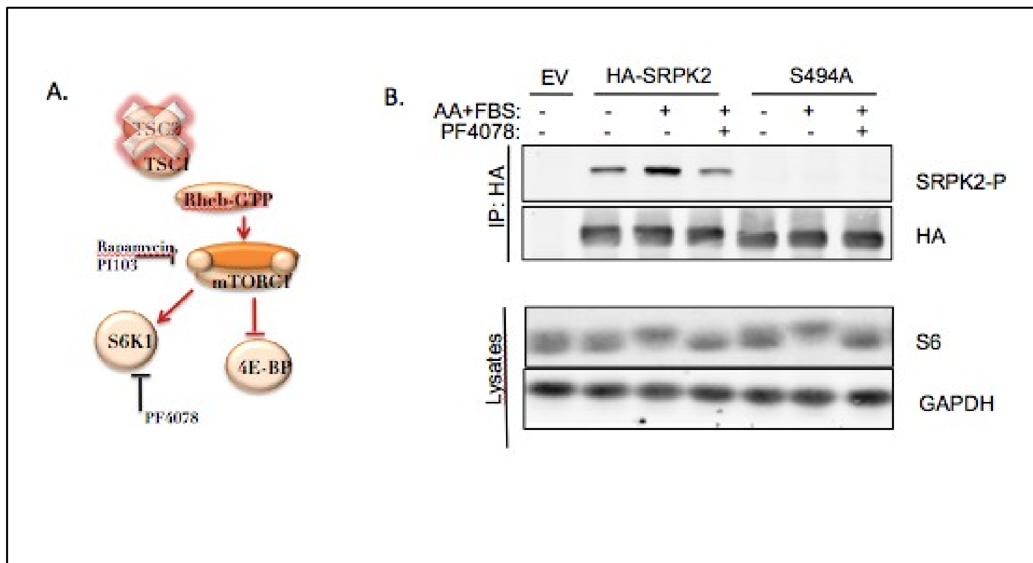

Figure 2.7: S6K phosphorylates SRPK2 in TSC2-/- MEFs A. Schematic showing constitutive activation of mTORC1 in the TSC2-/- MEFS. B. TSC2-/- MEFs were transiently transfected with HA-SRPK2, as well as HA-SRPK2S494A. Cells were serum starved overnight, and then starved of amino acids for 30 minutes before refeeding with both amino acids and FBS. HA-SRPK2 was immunoprecipitated using a homemade HA antibody, and analyzed by Western blot. Data representative of experiments done in duplicate.

Results 2.5: AKT phosphorylation of SRPK2 at Ser494

To determine whether Akt is able to phosphorylate this site, we used PTEN-/- MEFs, which display hyperactivation of the PI3K signaling pathway. We detected an increase in phosphorylation following serum deprivation and then insulin stimulation, which was decreased by pretreatment with the Akt specific inhibitor AktVIII (Figure 2.7).

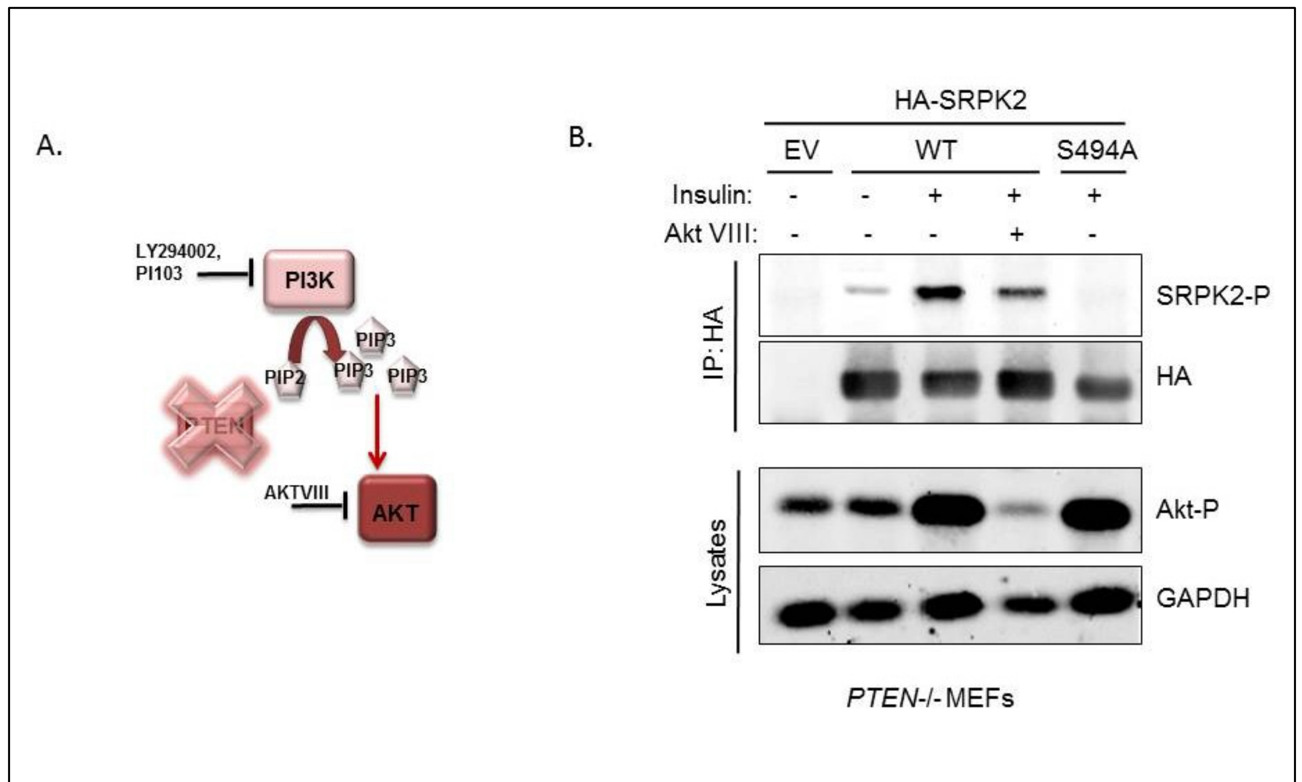

Figure 2.8: AKT phosphorylates SRPK2 in PTEN-/- MEFs A. Schematic showing hyperactivation of PI3K signaling in PTEN-/- MEFS, as well as which proteins the inhibitors target. B. PTEN-/- MEFs were transiently transfected with HA-SRPK2, as well as HA-SRPK2S494A. Cells were serum starved overnight, and then stimulated with insulin for one hour, or pretreated with the Akt inhibitor AktVIII for 30 minutes. HA-SRPK2 was immunoprecipitated, and analyzed by Western blot. Duplicate experiments were performed, and data is representative of both experiments.

Again, complete abolishment of the phosphorylation signal was seen in the immunoprecipitated HA-SRPK2S494A, suggesting that AKT phosphorylates SRPK2 at Ser494.

Conclusions and future directions

Phosphoproteomic and interaction screens aimed at identifying novel proteins involved in mTORC1/S6K signaling led us to study the SR protein kinases 1 and 2. In our studies, we confirmed that S6K interacts with SRPK1 and SRPK2 in a non-growth factor mediated manner. Additionally, we found that S6K can phosphorylate SRPK2 at Ser494 in TSC2-/- MEFs, which display hyperactive mTORC1 signaling. Interestingly, we found that another AGC kinase AKT can phosphorylate SRPK2 at Ser494 in PTEN-/- MEFS, which display hyperactive PI3K signaling. We suggest that SRPK2 can be phosphorylated by S6K and AKT in a cell-type stimulus specific manner. While the results are preliminary and therefore not included in this dissertation, we have evidence that S6K and AKT can phosphorylate GST-SRPK2 *in vitro* as well as *in cellulo*. These results will be repeated, and will hopefully provide further evidence that S6K and AKT phosphorylate SRPK2 at Ser494.

Our work with SRPK2 will be continued, and Ser494 phosphorylation will be investigated for its potential role in SRPK2 kinase activity towards its substrates the SR proteins. Additionally, we will investigate how this phosphorylation influences the subcellular localization of SRPK2. The hypothesis that this phosphorylation event affects subcellular localization is attractive since previous data suggests that the linker region

where Ser494 is located has previously been shown to be responsible for the cytoplasmic sequestering of both SRPK1 and SRPK2. Our initial studies using transiently transfected, tagged SRPK2 were inconclusive, and future immunofluorescent studies are planned using stably expressed wild type and phospho-site mutant SRPK2.

Chapter 3 Ras/ERK/RSK signaling inputs into SRPK2

Preface:

This work is not yet published. Dr. Gina Lee performed the RT-PCR and quantification of CD44 mRNA isoforms in figure 3.6. Didem Ilter was generous in providing the Ras/Vector stable cell lines for experiments in figures 3.5 and 3.6.

Introduction:

The links between splicing and human disease have been extensively studied, and the importance of alternative splicing in cancer is beginning to receive more attention [118]. Multiple genes have been reported to be alternatively spliced in cancers, with the resultant protein product contributing to the oncogenic phenotype. These include the genes that code for the following proteins; the recepteur d'origine nantais (RON) receptor tyrosine kinase, fibronectin, vascular endothelial growth factor (VEGF), pyruvate kinase M1/M2 (PKM2), and the cell-surface glycoprotein CD44 (reviewed in [118]). Given the fact that there have been no mutations observed in the *cis*-acting splicing elements within the genes that code for these proteins suggests that the alternative splicing could be attributed to alterations in the regulation of the trans-acting splicing factors.

An example of a well-studied alternative splicing event in breast cancer is for the gene encoding *CD44*. CD44 is a cell surface protein that forms coreceptor complexes with several receptor tyrosine kinases, resulting in modulations in cellular signaling [144, 145]. Inclusion of variable exons in CD44 correlates with tumor development and metastasis [146-148], and the Ras signaling pathway has been implicated in the regulation of the alternative splicing of these exons[149]. Several splicing factors have been implicated in the splicing of the variable exon 5 including the RNA binding protein

Src-associated in mitosis (Sam68) in T-lymphoma cells. Matter et al found that PMA activation of ERK1/2 leads to phosphorylation of Sam68 [150]. A study by Cheng and Sharp identified SRm160 as another Ras-regulated splicing co-activator that is responsible for the inclusion of the variable exon 5 in CD44 [145]. A more recent study by Brown et al provided evidence that alternative splicing of CD44 is essential for the epithelial-mesenchymal transition, in addition to accelerating breast cancer progression

[151].

We identified a phosphorylation event on SRPK2 in the linker region of the protein, which we found to be a target of S6K and AKT at Ser494 (chapter 2). Given the redundant nature of AGC kinase signaling, we investigated whether the p90 ribosomal S6 kinase (RSK) can phosphorylate SRPK2 downstream of Ras/ERK signaling, as well as sought to identify genes that are alternatively spliced following HRasV12 infection.

Results 3.1: SRPK2 is phosphorylated at Ser494 In cellulo following activation of the ERK1/2 MAP kinase pathway

For this study, we chose to focus on the activation of the MAP kinase pathway induced phosphorylation of SRPK2. As shown in figure 3.1, the phorbol ester phorbol-12-myristate-13-acetate (PMA) and the epidermal growth factor (EGF) activate ERK1/2, as well as stimulate phosphorylation of SRPK2. Phosphorylation of SRPK2 following PMA stimulation is sensitive to the RSK inhibitor BID-1870, as well as MEK inhibitor UO126 when used before stimulation with EGF, suggesting it to be a downstream target of RSK.

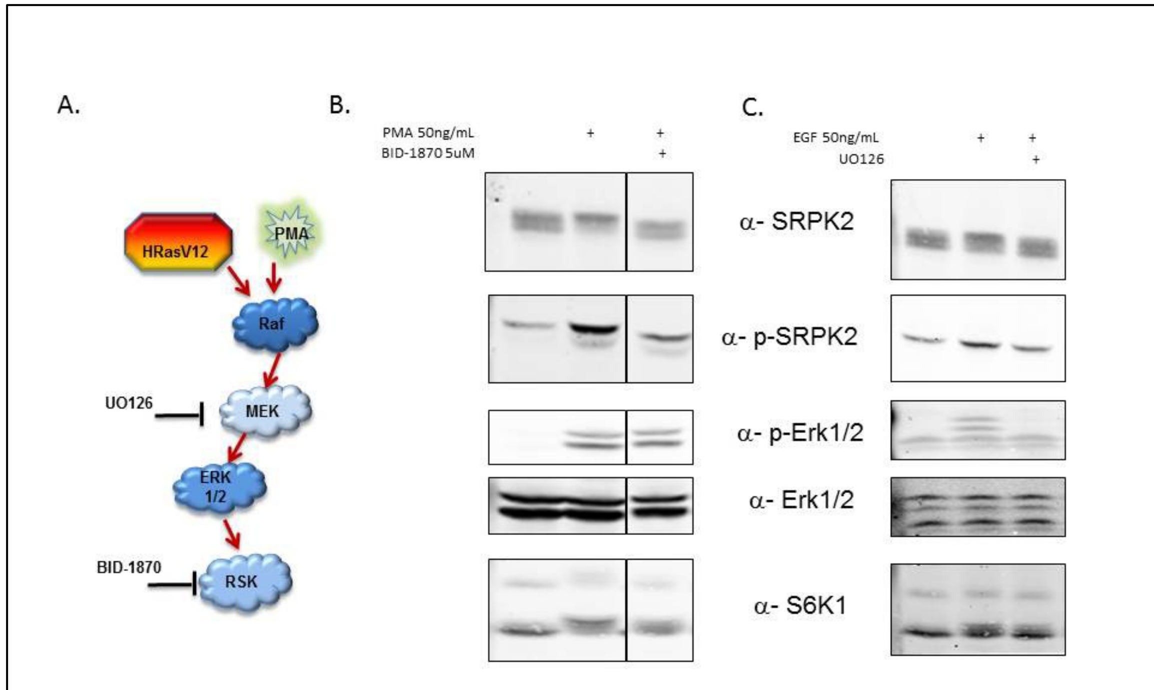

Figure 3.1: SRPK2 phosphorylation responds to ERK1/2 MAP kinase pathway stimulant and inhibitor treatments. 293E cells were serum starved for 18h, and then treated with inhibitors and stimulants as indicated. Whole cell lysates were subjected to Western blot. Treatment conditions: PMA (20’, 50ng/mL), epidermal growth factor (EGF, 10’, 20ng/mL), U0126 (30’ pretreatment, 10μM), BI-D1870 (30’ pretreatment, 5μM) Data representative of experiments performed in duplicate.

To confirm that the antibody is recognizing the phosphorylated Ser494 of SRPK2, we mutated this site to alanine. We used transient overexpression and immunoprecipitation of the HA-tagged wild type and nonphosphorylatable mutant of SRPK2 from total cell lysates prepared from 293E cells that were serum starved overnight, stimulated with PMA for 20 minutes, as well as pretreated with the MEK inhibitor UO126 for 30 mins. We confirmed that SRPK2 is phosphorylated at Ser494 following PMA stimulation, and that this phosphorylation is sensitive to inhibition of the ERK1/2 MAP kinase pathway using the MEK inhibitor UO126 (Fig 3.2).

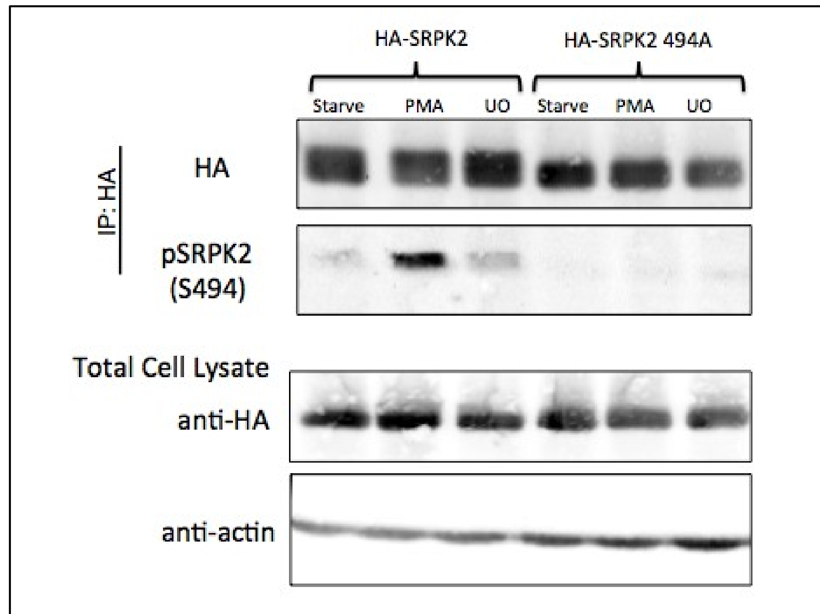

Figure 3.2: SRPK2 is phosphorylated at Ser494 following PMA stimulation. 293E cells were transfected with HA-tagged wild type SRPK2, as well as the nonphosphorylatable alanine mutants S494A. Following immunoprecipitation with an anti-HA antibody, samples were run on a gel, and visualized by western blotting. . Treatment conditions: PMA (20', 50ng/mL), U0126 (30' pretreatment, 10μM) Data representative of experiments performed in duplicate.

Results 3.3: RSK phosphorylates SRPK2 *in vitro* at serine 494

Since pretreatment of 293E cells with the RSK inhibitor BID-1870 blocked PMA stimulation of phosphorylation of SRPK2, and we suspected that RSK was the primary kinase for SRPK2 *in vivo*, we wanted to ensure that RSK could phosphorylate SRPK2 in a regulated manner *in vitro.* We performed *in vitro* kinase assays using bacterially purified GST-fusion proteins as the substrate, and immunoprecipitated N-terminally HA-tagged RSK1. We cloned the linker region of both SRPK2 and SRPK1, which serves as a negative control since the phosphorylation event on SRPK2 is not conserved in SRPK1. Indeed, we were able to confirm that RSK phosphorylates GST-SRPK2 in a PMA stimulated, UO126 sensitive manner (Fig 3.3).

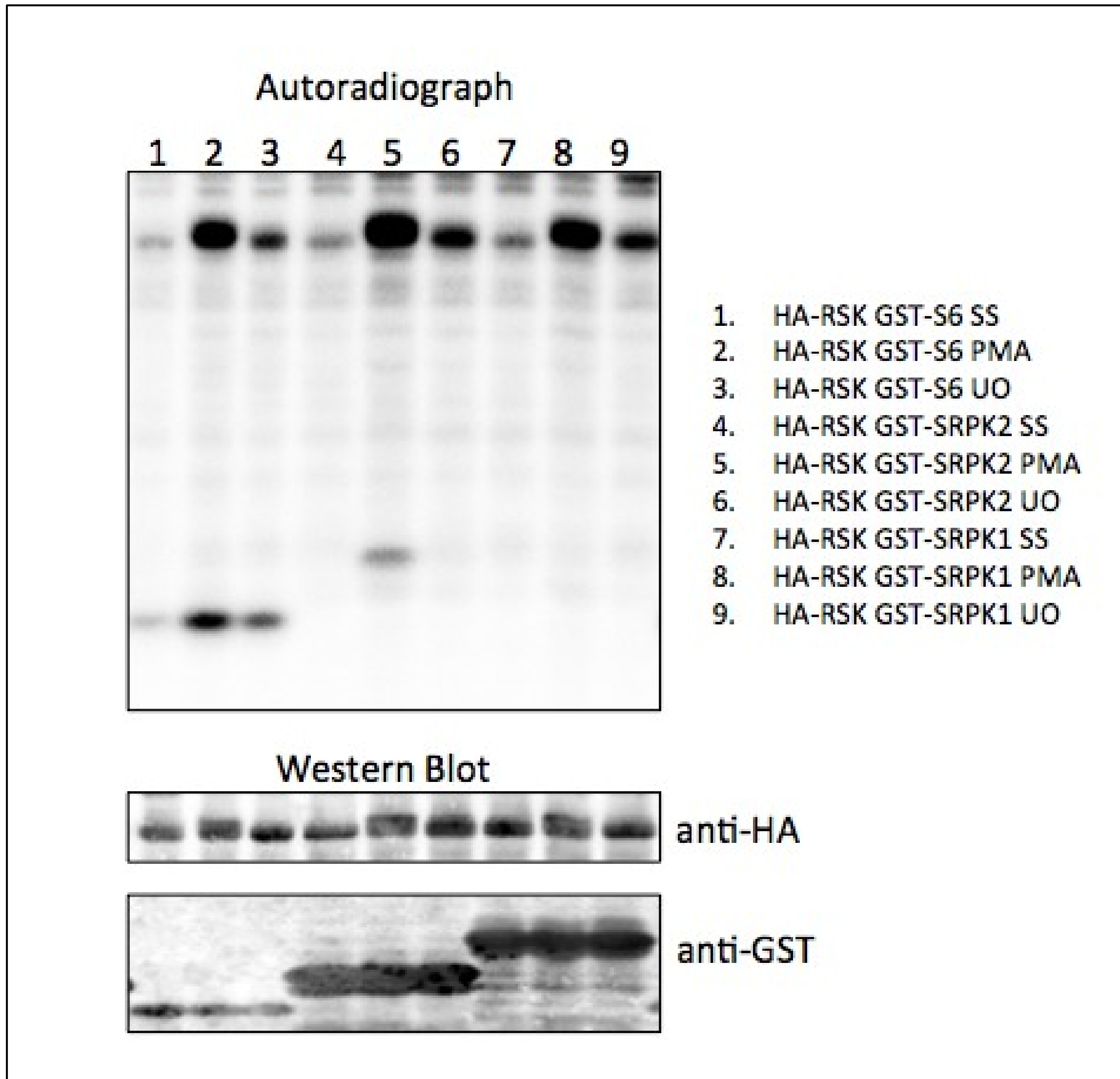

Figure 3.3: SRPK2 can be phosphorylated by RSK *in vitro*. 293E cells were transfected with HA-RSK1, serum starved, and IP kinase assay performed as described in material and methods. GST-S6 was used as a positive control, and GST-SRPK1 was used as a negative control. Treatment conditions: PMA (20', 50ng/mL), UO126 (30' pretreatment, 10uM). Data representative of experiments performed in duplicate.

To confirm that RSK phosphorylates SRPK2 at Ser494, we mutated Ser494 to the nonphosphorylatable alanine. Using this GST-fusion protein, we were able to completely abolish RSK's ability to phosphorylate GST-SRPK2 *in vitro* further confirming that this is the phosphorylation site as determined by the phospho-specific antibody studies (Fig 3.4).

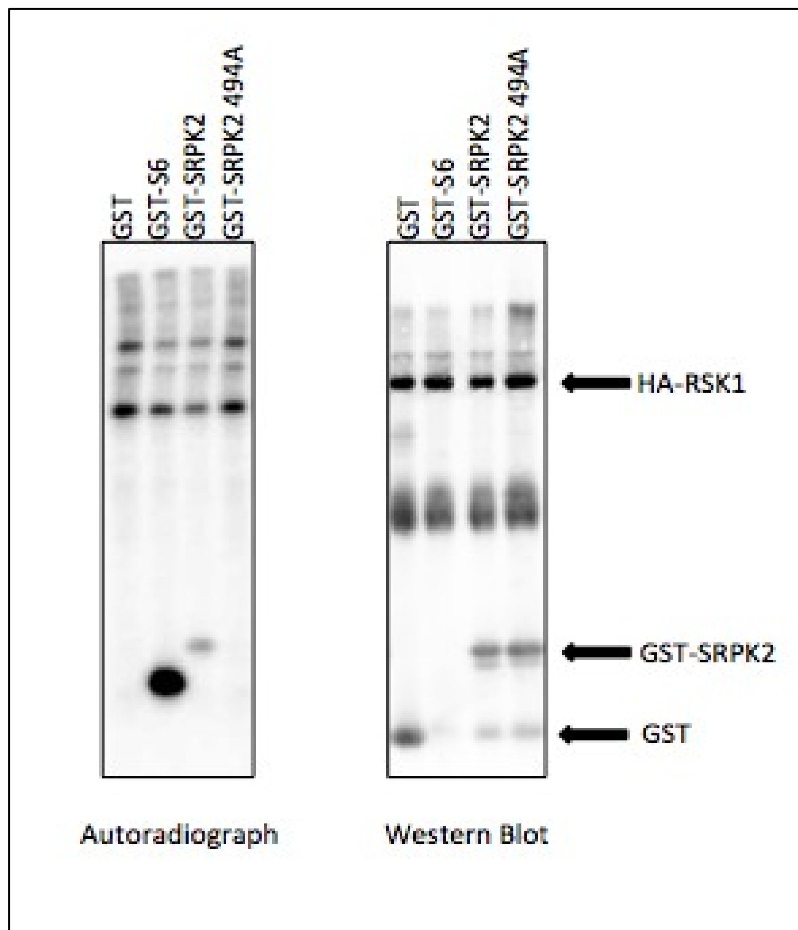

Figure 3.4: Rsk phosphorylates SRPK2 *in vitro* at serine 494. 293E cells were transfected with HA-RSK1, serum starved, and IP kinase assay performed. GST-S6 was used as a positive control, and GST-SRPK2 wild type and GST-SRPK2S494A were used as substrates. Treatment conditions: PMA (20', 50ng/mL) Data representative of experiments performed in duplicate.

Results 3.4: SRPK2 is hyperphosphorylated following cellular transformation with the Ras oncongene HRasV12

The development of many human malignancies is dependent upon the deregulation of the Ras-ERK pathway. Our lab has established a cell culture system using infection of the normal human mammary epithelium cell line MCF10A, with a retrovirus encoding oncogenic HRasV12. This infection results in Ras-induced epithelial-to-mesenchymal transformation (EMT). Given the hyperactivation of the ERK1/2 MAP kinase pathway in these transduced cells, we wanted to assess the status of SRPK2 phosphorylation in this system. Lysates from these cells were probed with an antibody to total SRPK2, as well p-SRPK2 Ser494. Following transformation with HRasV12, SRPK2 displays a marked mobility shift up, as well as an increase in phosphorylation (Fig 3.5). To determine whether the hyperphosphorylation of SRPK2 was due to ERK1/2 signaling, HRasV12 infected cells were treated with the MEK inhibitor UO126 for 2 hours, and lysates were analyzed by western blot (Fig 3.5). We observed a reduction in the amount of p-SRPK2 Ser494 detected, as well as an abolishment of the band shift observed following HRasV12 infection. Given the previous data showing that RSK is the dominant kinase phosphorylating SRPK2 at Ser494, we suspect that this is the candidate kinase downstream of HRasV21 infection responsible for phosphorylation in this cell system, and studies in the near future will hopefully confirm this hypothesis.

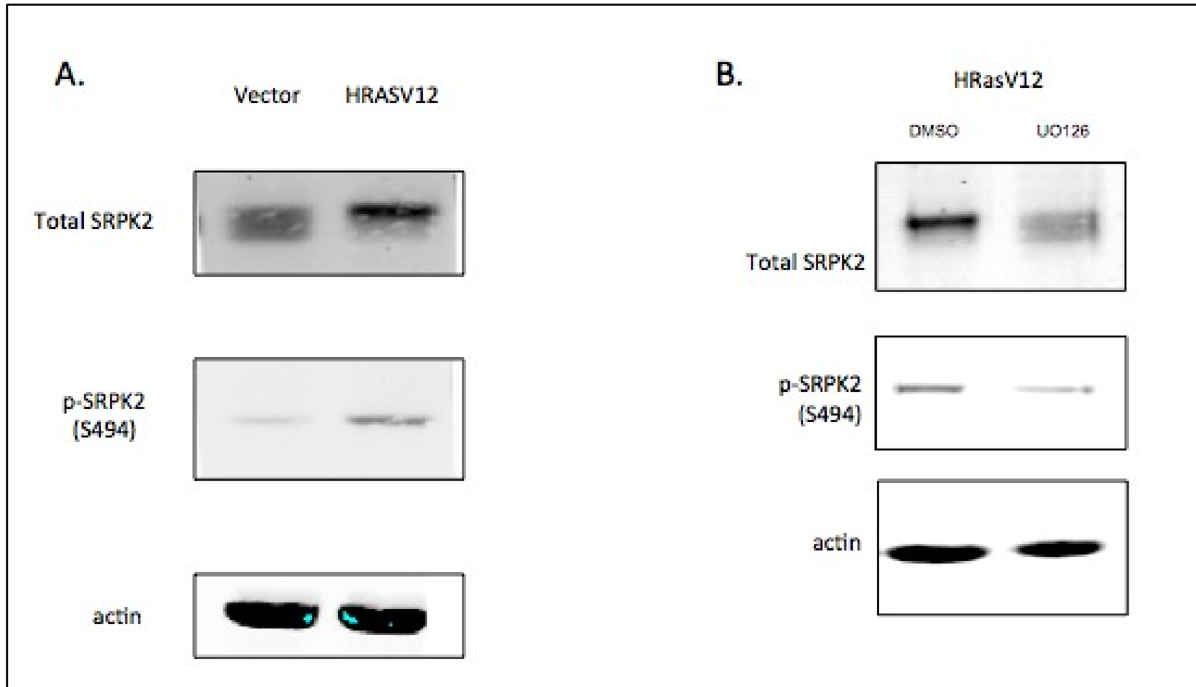

Figure 3.5: A. SRPK2 is hyperphosphorylated in HRasV12 transformed cells. MCF10A cells were infected with the HRASV12 oncogenic virus. Total cell lysates were visualized by western blot, and increase in SRPK2 phosphorylation was observed. B. Cells were treated with the MEK1/2 inhibitor UO126 for two hours before lysis and western blot, and a decrease in phosphorylation was observed. Data representative of experiments performed in duplicate.

Result 3.5: CD44 is alternatively spliced following HRasV12 transformation in MCF10A mammary epithelium cells

Given previous studies implicating the Ras/ERK1/2 MAP kinase pathway in the regulation of the alternative splicing of *CD44 [144]*, we wanted to investigate the splicing patterns for this gene in our cell system. We used previously published primers that detect multiple isoforms of CD44, which differ in the inclusion or exclusion of the variable exons v2 to v10 [152]. Previous reports indicate an increase in the amount of shorter CD44 isoforms following activation of Ras.

We performed RT-PCR on cDNA prepared from three biological replicates of asynchronously growing MCF10A cells that were infected with either HRasV12 or the control pBABE vector (Fig 3.6). We confirmed that Ras infection induced alterations in the splice site selection for CD44. A statistically significant decrease in the amount of the long CD44 isoforms following stable expression of HRasV12 was observed.

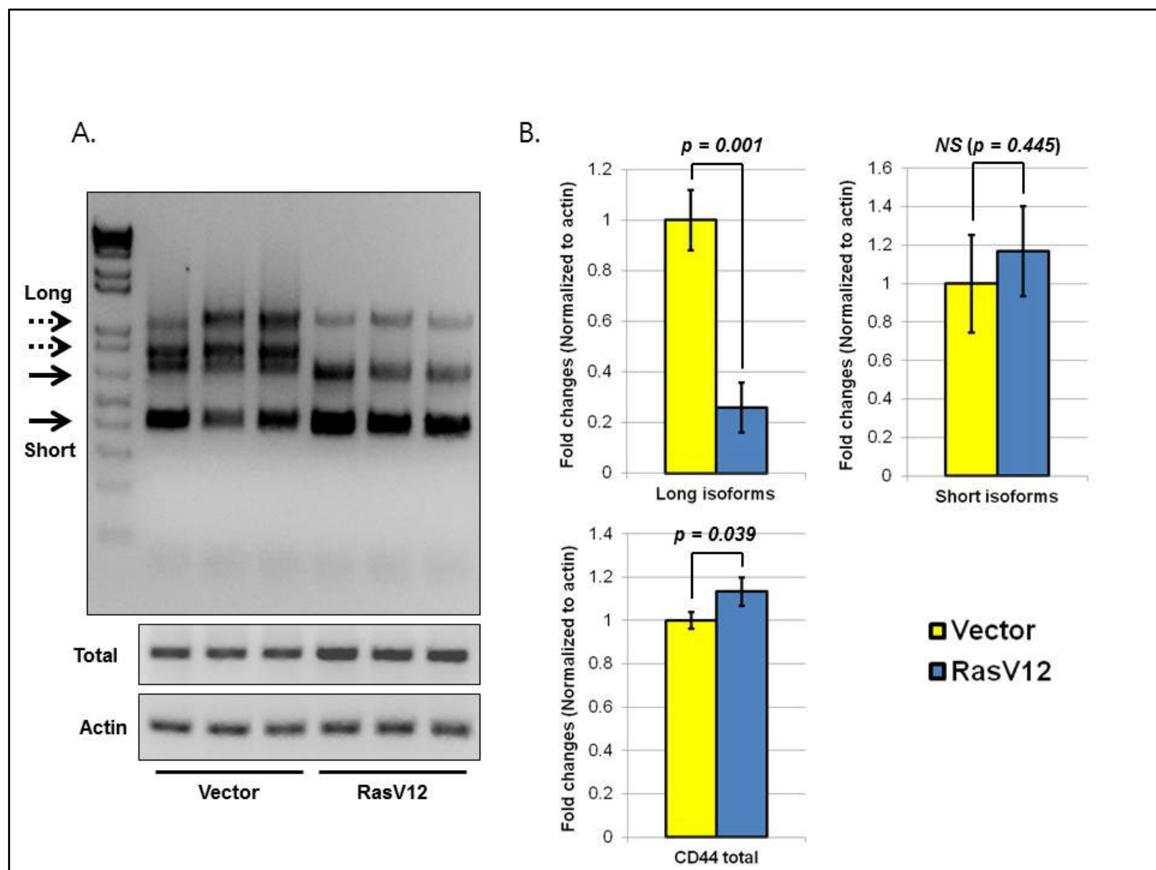

Figure 3.6: CD44 is alternatively spliced to the CD44v6 following HRasV12 stable expression. cDNA from three sets of biological replicates of HRasV12 and pBABE vector control infected MCF10A cell samples were prepared according to details in Chapter 6 (materials and methods). A. RT-PCR was performed using 1ul of cDNA mixture, and primers specific for the constitutive exons flanking the variable exons. B. Fold changes for the long and short isoforms were compared between the vector and HRasV12 infected cells, and were determined by comparing the average intensities of the biological triplicates, which was then normalized to actin.

Conclusions and future directions

Here we provide evidence that RSK phosphorylates SRPK2 at Ser494 both *in vivo* and *in vitro*. We also observed hyperphosphorylation of SRPK2 following transduction with the oncogenic HRasV12 in the mammary epithelium MCF10A cell line. In correlation, we observed a difference in the alternative splicing of CD44. We hope to use this cell system to delve deeper into the Ras/ERK/RSK signaling inputs to SRPK2, and how that affects splice site selection of the variable exons of CD44. We will use pharmacologic inhibition of the ERK1/2 MAP kinase pathway, as well as siRNA mediated knockdown of proteins in this pathway. In addition, we will examine how knockdown of SRPK2 alone, as well as in combination with SRPK1 affects splice site selection of CD44. It will be of interest to see if SRPK2 hyperphosphorylation affects the phosphorylation status of the SR proteins previously reported to be responsible for the splice site selection of CD44 Sam68 and SRm160. These experiments are planned for the very near future, and will hopefully clarify if Ras-mediated signaling to SRPK2 mediates alternative splice site selection for CD44.

Chapter 4 Regulated unproductive splicing of the SR protein ASF/SF2 and hnNRPa2/b1 and regulation of the alternative splicing of Bin1 downstream of mTORC1/SRPK2 signaling

Preface

This work is not yet published, and has been done in complete collaboration with Dr. Alfredo Csibi. Any publication resulting from this data will be a co-first author publication with Dr. Csibi and I sharing first authorship.

Introduction

The Serine/Arginine rich (SR) family of proteins participates in many aspects of mRNA metabolism, including splice site selection, mRNA export and translation efficiency, and can be phosphorylated by the SR protein kinases (SRPK) affecting their activity. The SR family of proteins, as well as the heterogeneous nuclear ribonucleoproteins (hnRNPs) are themselves subject to alternative splicing, and the cellular concentrations of these proteins are controlled through the inclusion of a premature termination codon (PTC), which targets the message for degradation through the nonsense mediated decay (NMD) pathway. This coupling of alternative splicing and NMD has been termed regulated unproductive splicing and translation (RUST).

McGlincy et al showed that knockdown of the NMD machinery by targeting the core protein Up-frameshift suppressor 1 (UPF1) using siRNA resulted in an increase in hnRNPa2b1 protein levels. They identified a highly conserved alternative splicing event that triggers NMD within the 3' untranslated region (UTR) of hnRNPa2b1. Additionally, ASF/SF2 has been reported to be subject to RUST also through inclusion of an intron in

the 3'UTR that triggers the message for degradation by NMD. This intron is located in an ultraconserved element region.

Also of importance, numerous reports of alterations in the cellular concentrations of splicing factors during different stages of cellular transformation have been published, with ASF/SF2 receiving the most attention as a proto-oncogene [122]. hnRNPa2b1 was reported to be overexpressed in glioblastoma, and hypothesized to be an oncogenic driver. These studies have examined the effect of overexpression these splicing factors, and alterations in splice site selection of known tumor suppressor and/or oncogenes. One common tumor suppressor reported to be targeted by both ASF/SF2 and hnRNPa2b1 is the gene coding for bridging integrator-1 (*BIN1*).

BIN1 is a nucleocyoplasmic adaptor protein that interacts with and inhibits the c-MYC transcription factor. This interaction occurs through the BIN1 MYC-binding domain (MBD). Inclusion of exon 12a disrupts this domain, resulting in a loss of tumor suppressor activities. Previous reports show that overexpression of both ASF/SF2 and hnRNPa2b1 results in an increase in the inclusion of exon 12a. A different study showed that inclusion of exon 12a in BIN1 abolished the ability of BIN1 to inhibit malignant transformation by c-MYC in melanoma cells (Ge 1999).

Results 4.1 ASF/SF2 and hnRNPA2B1 protein levels are decreased following rapamycin treatment

Given that S6K1 phosphorylates SRPK2 at Ser494, and a protein interaction with SRPK1 was observed (Chapter 2), we wanted to see if modulations in mTORC1/S6K/SRPK signaling affected the migration and/or amount of the SRPK substrate ASF/SF2. Using the TSC2-/- MEFS, which display hyperactive mTORC1 signaling, we treated asynchronously growing cells with rapamycin for varying time points up to 24 hours, and performed Western blot (Fig 4.1). Following rapamycin treatment, we observed a reduction in total ASF/SF2 protein levels.

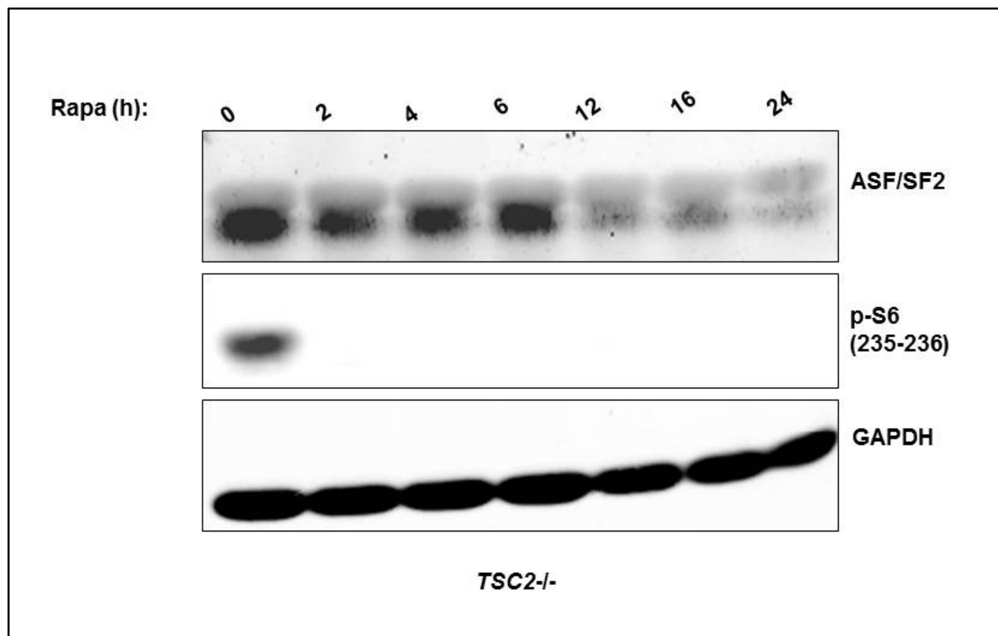

Figure 4.1: ASF/SF2 protein levels are reduced following long term rapamycin treatment. Asynchronously growing TSC2-/- MEFs were treated with rapamycin for 0, 2, 4, 6, 12, 16 and 24 hours. Total cell lysates were run for analysis by Western blot using commercially available antibodies. Treatment conditions: rapamycin (time points indicated; 20ng/mL) Data representative of duplicate experiments.

We next wanted to determine if the loss of ASF/SF2 protein amount after rapamycin treatment was specific to the SR proteins, or if the stability of other splicing factors were affected by inhibition of mTORC1/S6K signaling. We chose to examine the total protein levels of hnRNP A2/B1 following rapamycin treatment (Fig 4.2). Surprisingly, we also observed a reduction in total protein level of hnRNP A2/B1.

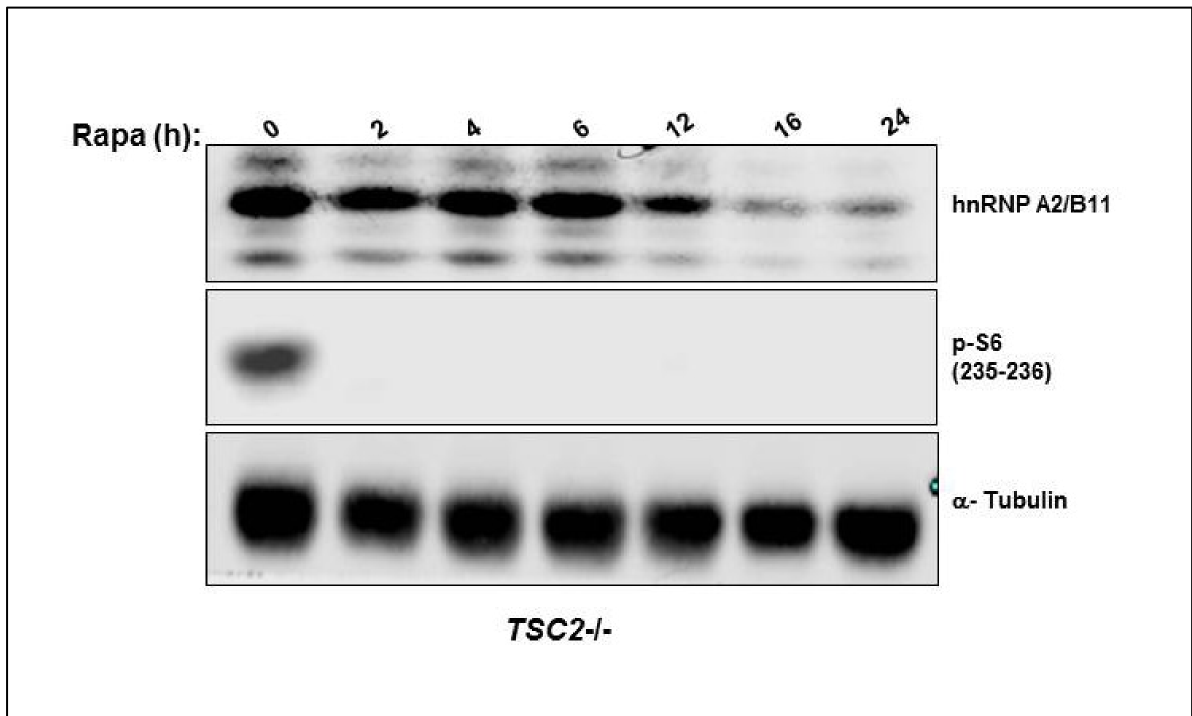

Figure 4.2: hnRNP A2/B1 protein levels are reduced following long term rapamycin treatment. Asynchronously growing TSC2-/- MEFs were treated with rapamycin for 0, 2, 4, 6, 12, 16 and 24 hours. Total cell lysates were run for analysis by Western blot using commercially available antibodies. Treatment conditions: rapamycin (time points indicated; 20ng/mL) Data representative of duplicate experiments.

Results 4.2 ASF/SF2 and hnRNPA2B1 protein levels are decreased following knockdown of SRPK1/2

Following long term inhibition of mTORC1/S6K signaling, we saw a marked reduction in total protein amounts of the splicing factors ASF/SF2 and hnRNP A2/B1. We wanted to next determine if knockdown of SRPK1/2 also resulted in a reduction of total ASF/SF2 and hnRNP A2/B1, we transiently transfected siRNAs against SRPK1, SRPK2, and ASF. While a small decrease in ASF/SF2 and HNRNPA2B1 was seen following knockdown of SRPK1 and SRPK2 individually (data not shown), a greater reduction was seen following knockdown of SRPK1 and SRPK2 in combination (Fig 4.3).

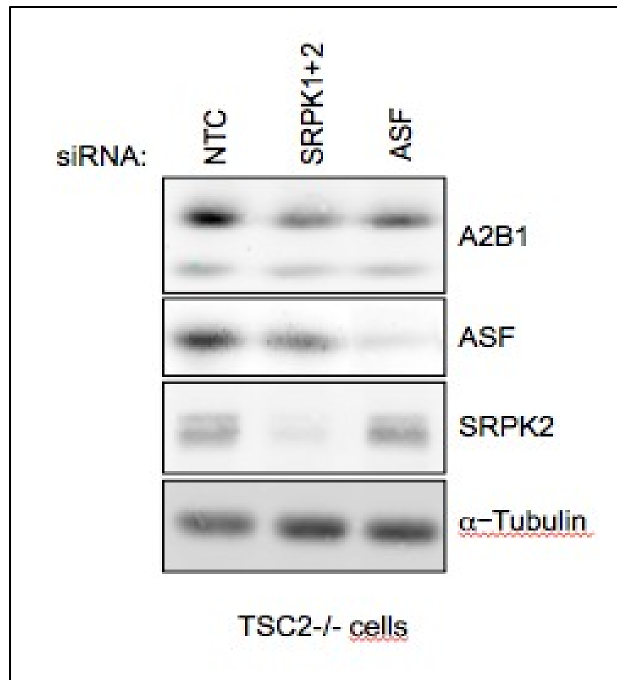

Figure 4.3: siRNA mediated knockdown of SRPK1/2 reduces hnRNP A2/B1 and ASF/SF2 protein levels. Whole cell lysates were analyzed by Western blot. TSC2-/- MEFS were transiently transfected with 10nM siRNAS as indicated. Data representative of experiments performed in duplicates.

Results 4.3 UPF1 knockdown rescues rapamycin mediated protein reduction of ASF/SF2 and hnRNPa2b1

Recent papers have described the mechanisms by which the SR family of proteins as well as the hnRNP proteins controls their own intracellular concentrations of proteins through regulated unproductive splicing and translation (RUST) (detailed in chapter 1). To determine if the loss of protein that we observed following inhibition of mTORC1 signaling was through RUST, we used siRNAs against the core NMD machinery protein UPF1. siRNA mediated knockdown of UPF1 has been previously reported to inhibit NMD of the SR protein ASF/SF2 and HNRNPA2B1, resulting in an increase in protein level. Again, we saw a decrease in total hnRNP A2/B1 and ASF/SF2 protein levels following 24h rapamycin treatment, as well as 24h treatment with the S6K inhibitor PF4708. Following knockdown of the core NMD protein UPF1, we were able to rescue the loss in protein that was seen following mTORC1/S6K signaling inhibition, suggesting the loss of protein following inhibition of mTORC1 signaling is due to NMD (Fig 4.4).

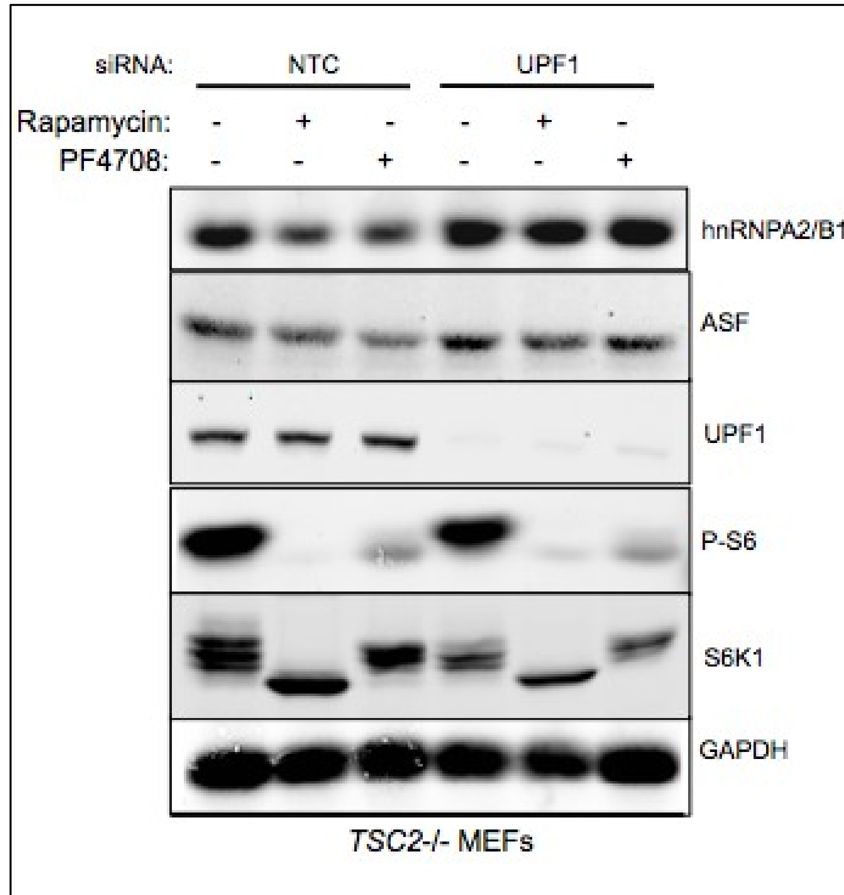

Figure 4.4: Inhibiting the NMD machinery rescues the decrease in ASF/SF2 and hnRNP A2/B1 loss of protein. TSC2-/- MEFs were transiently transfected with either a nontargeting control siRNA or siUPF1, and then treated with DMSO vehicle control, rapamycin, or PF4708 as indicated. Antibodies used are commercially available, and listed in Chapter 6 (materials and methods). Treatment conditions: rapamycin (24 hours, 20ng/mL), PF4708 (24 hours, 10uM) Experiments were performed in duplicates.

Results 4.4 BIN1 is alternatively spliced following long term rapamycin treatment and knockdown of S6K

Since we observed a dramatic difference in the cellular concentrations of the splicing factors ASF/SF2 and hnRNP A2/B1 following manipulations of the mTORC1/S6K/SRPK signaling pathway, we hypothesized this difference in cellular concentration of splicing factors would affect splice site selection in other genes. We

searched for known targets of ASF/SF2 and hnRNP A2/B1, and found reports detailing the involvement of these proteins in splice site selection for the tumor suppressor BIN1 [160]. Separate reports show that these two proteins control the inclusion of an exon that when present results in the inability of BIN1 to suppress the activity of c-MYC, suggesting these proteins to be proto-oncogenic [161].

We performed qPCR on cDNA made from TSC2-/- MEFs that were treated with rapamycin or DMSO vehicle control for 24 hours. To control for total message levels, a primer pair specific for the constitutive exon 13 was used. Using primers specific for exon 12a, we were able to detect a decrease in the inclusion of this exon in BIN1 messages following rapamycin treatment (Fig 4.5). Total message levels did not change, as assayed by using primers to exon 13, suggesting that inhibition of mTORC1 signaling causes an increase in the amount of BIN1 that is able to bind to and inhibit c-MYC.

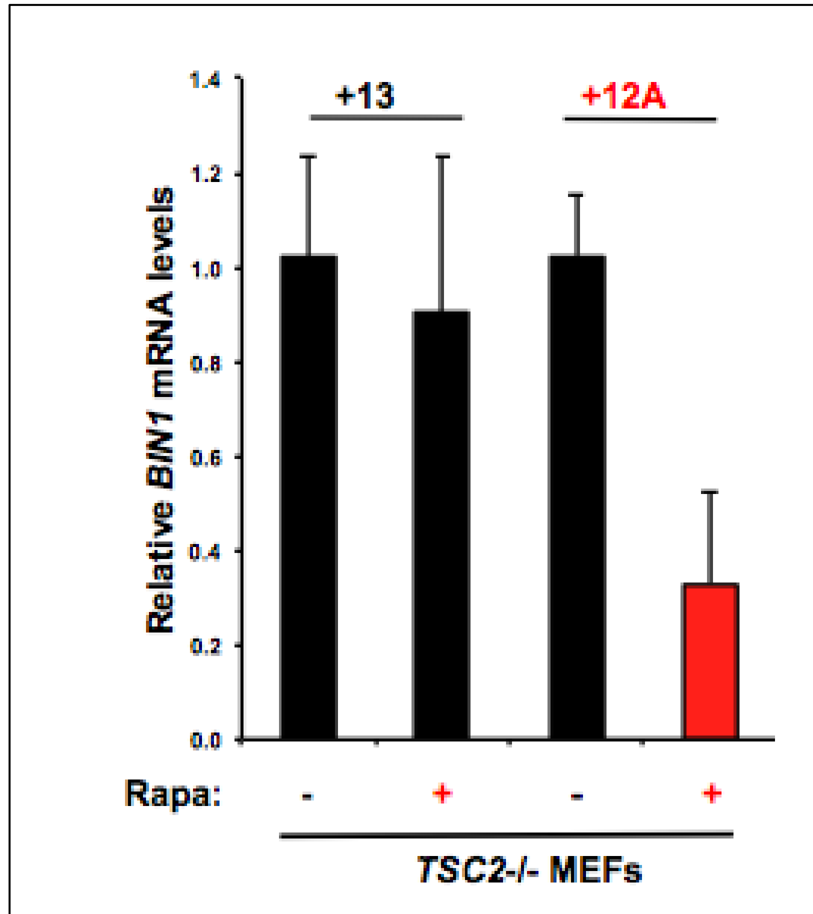

Figure 4.5: BIN1 is alternatively spliced following rapamycin treatment. cDNA was made from TSC2-/- MEFS treated with DMSO or rapamycin as indicated. qPCR analysis was performed using primers specific for exons 12A and 13 in the BIN1 message. Treatment conditions: rapamycin (24h, 20ng/mL)

We wanted to confirm this alternative splicing of BIN1 was through S6K signaling downstream of mTORC1. Therefore, we used siRNAs targeting both S6K1 and S6K2. Again, we observed a decrease in the inclusion of exon 12a in BIN1 mRNA messages (Fig 4.6)

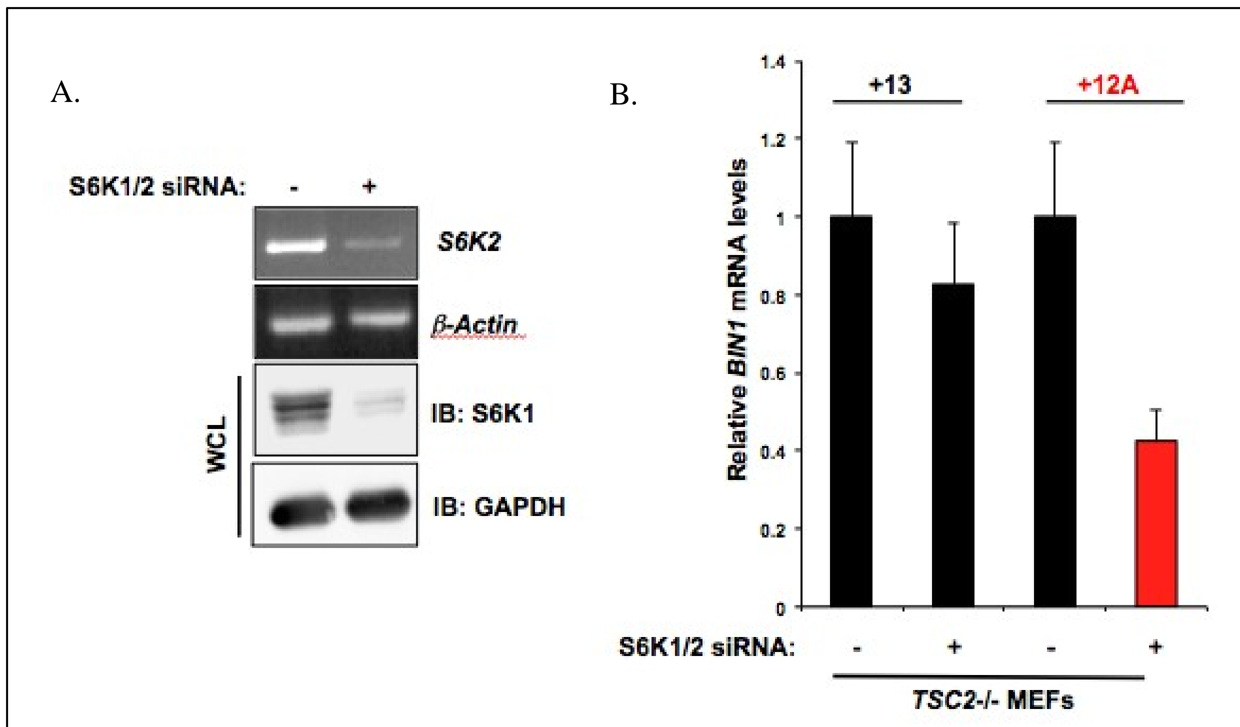

Figure 4.6: BIN1 is alternatively spliced following knockdown of S6K1/2. A. TSC2-/- MEFs were transiently transfected with non-targeting control siRNA, or siRNAs targeting S6K1/2. B. cDNA was made from TSC2-/- MEFS treated with DMSO or rapamycin as indicated. qPCR analysis was performed using primers specific for exons 12A and 13 in the BIN1 message.

Results 4.5 BIN1 is alternatively spliced following knockdown of SRPK1/2, ASF/SF2 and hnRNPa2b1

Since we saw a decrease in ASF/SF2 protein levels, as well as a difference in the alternative splicing of BIN1 following rapamycin treatment, we wanted to see if knockdown of ASF/SF2 as well as SRPK1 and SRPK2 resulted in alternative splicing of BIN1 in our cell system. Using an antibody specific for exon 12A of the BIN1 protein, we performed Western blot on lysates prepared from separate experiments using TSC2-/- MEFs that had been transiently transfected with siRNAs targeting SRPK1, SRPK2, and

ASF/SF2 (Fig 4.7). While we saw a decrease in exon 12A of BIN1 at the protein level after knockdown of SRPK1, SRPK2 and in combination, a more dramatic effect was seen following knockdown of ASF/SF2.

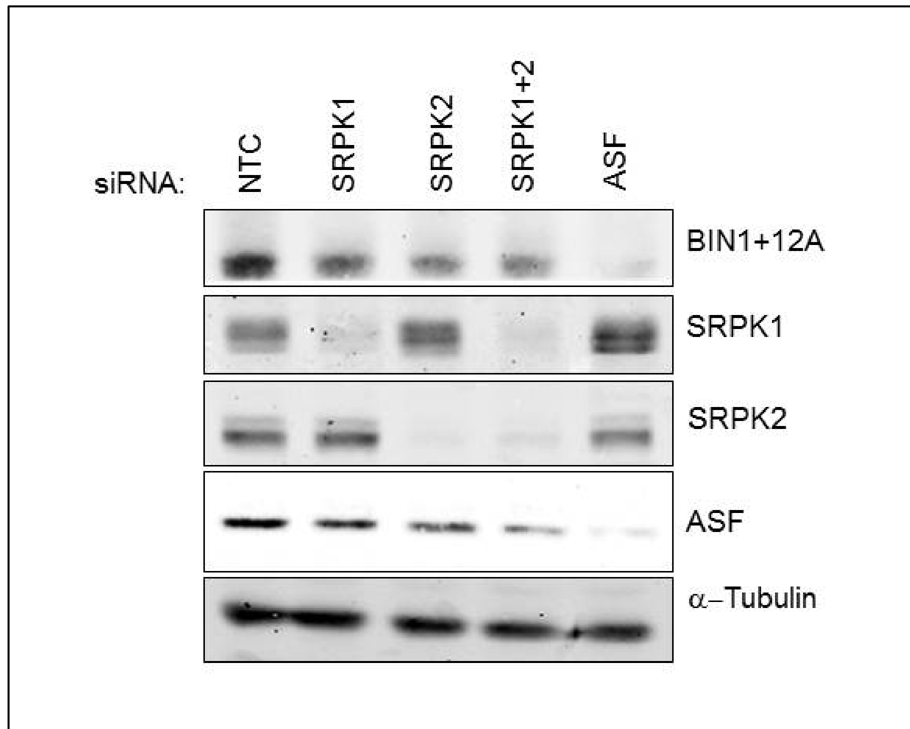

Figure 4.7: Exon 12A of BIN1 is reduced following knockdown of SRPK1/2 and ASF/SF2. TSC2-/- MEFs were transfected with siRNAs targeting SRPK1, SRPK2 alone and in combination, as well as ASF/SF2, and SDS-PAGE was performed. An antibody specific for exon 12A of BIN1 was used. Duplicate experiments were performed.

Since we also saw a decrease in the total protein levels of the splicing factor hnRNP A2/B1 following rapamycin treatment, we wanted to see if siRNA mediated knockdown of this gene would result in alternative splicing of BIN1 exon 12A.

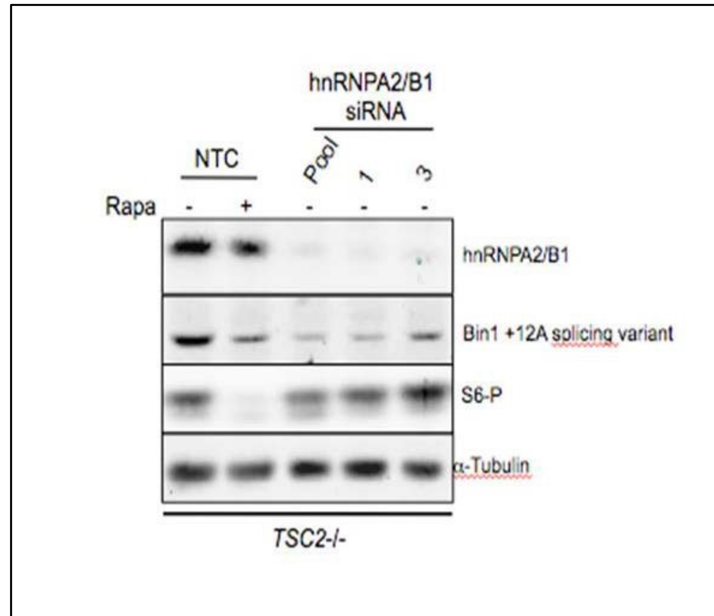

Figure 4.8: Exon 12A of BIN1 is reduced following knockdown of hnRNP A2/B1. TSC2-/- MEFs were transfected with siRNAs targeting hnRNP A2/B1, and SDS-PAGE was performed. Additionally, lysates were prepared from cells transfected with the non-targeting control siRNAs that had been treated with either DMSO or rapamycin for 24h. An antibody specific for exon 12A of BIN1 was used. Treatment conditions: rapamycin (24h, 100ng/mL). Data representative of duplicate experiments.

As a control, we used a non-targeting control siRNA, and treated these cells with either DMSO vehicle control or rapamycin for 24hrs. We confirmed the decrease of hnRNP A2/B1 protein, as well as saw a decrease in the exon 12A at the protein level following mTORC1 inhibition. Additionally, we saw a dramatic decrease in the amount of BIN1 exon 12A after siRNA mediated knockdown of hnRNP A2/B1.

Conclusions and future directions

Here we provide evidence for the first example of the mTORC1/S6K signaling pathway regulating alternative splicing. Additionally, we hypothesize that mTORC1/S6K is controlling the intracellular concentration of certain splicing factors through RUST. Highlighted below are the observations we've made, as well as the

experiments that are planned for the near future, which we hope will further enhance our understanding of mTORC1 mediated regulation of alternative splicing.

We showed that long term rapamycin treatment caused a decrease in the amount of ASF/SF2 and hnRNP A2/B1 proteins (Fig 4.1 and 4.2), and that knockdown of the NMD machinery by targeting the core factor UPF1 rescues protein expression levels (Fig 4.4). While this observation is indicative of an alternative splicing event that triggers NMD, we must confirm the inclusion of the PTC containing exon following rapamycin treatment in these mRNA messages. Preliminary results suggest a decrease in total message levels for ASF/SF2 and hnRNP A2/B1 following rapamycin treatment. We will knockdown the NMD machinery, stabilizing all transcripts before assaying for the amount of PTC containing messages.

Additionally, we provided evidence that BIN1 is alternatively spliced downstream of mTORC1 signaling (Fig 4.5 and 4.6), and we hypothesize that this is through the splicing factors ASF/SF2 and hnRNP A2/B1. Given the data in Chapter two showing S6K phosphorylation of SRPK2, we postulate that mTORC1 is regulating the splicing factors through SRPK2. We have preliminary evidence suggesting that phosphorylation of SRPK2 inhibits its ability to phosphorylate substrates (data not included).

Phosphorylation of the SR proteins promotes nuclear translocation. Therefore, we hypothesize that long term inhibition of mTORC1 signaling results in an increase in phosphorylation of the SR proteins, and accumulation of these factors in the nucleus, ultimately affecting splice site selection. We will test this hypothesis by performing

immunofluorescence of endogenous ASF/SF2 in TSC2-/- MEFs with and without rapamycin treatment.

We are also in the process of making virus for the stable expression of the phosphosite mutants, as well as wild type SRPK2. Experiments planned with these cells include qPCR of BIN1 exon 12A, as well qPCR of the PTC containing exons of ASF/SF2 and hnRNP A2/B1. We hope these experiments will support the data we provided showing knockdown of SRPK1/2 on the stability of ASF/SF2 and hnRNP A2/B1 (Fig 4.3), as well as the alternative splicing of BIN1 (Fig 4.7).

Chapter 5 Discussion and Future Directions

Phosphorylation of SRPK2 by the AGC kinases

As discussed in detail in Chapter 2, there are multiple examples of the AGC kinases phosphorylating the same target. Here we provide evidence for an additional site of pathway convergence. We have shown that S6K, AKT and RSK all phosphorylate Ser494 on SRPK2.

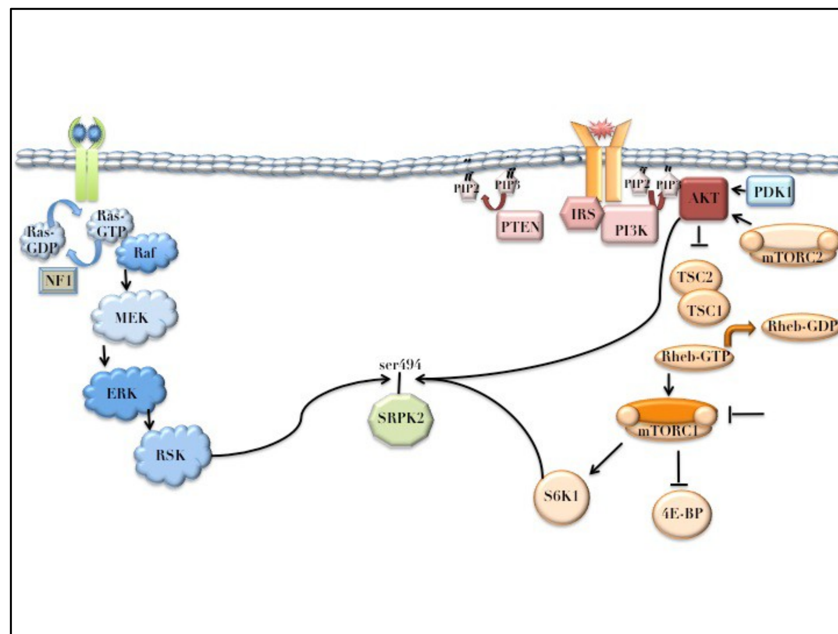

Figure 5.1: The Ras-ERK and PI3K/mTORC1 pathway converge on SRPK2. AKT, S6K and RSK all phosphorylate SRPK2 at Ser494, in a cell-type, stimulus dependent manner.

Based upon the ability of rapamycin to block the insulin induced mobility shift of total SRPK2 but not the phosphorylation (Fig 2.4 and 2.5), as well as the mobility of the HA-SRPK2S494A mutant in comparison to the HA-SRPK2 WT (Fig 3.5), we believe that phosphorylation at Ser494 is necessary for the mobility shift, but not sufficient. There are many proteins, whose phosphorylation status can be monitored by mobility

shift of the total protein on an SDS-PAGE gel including S6K and ERK1/2. It is generally thought that the phosphorylation sites that will induce an observable mobility shift are proline directed, or result from the phosphorylation of multiple residues. Given that Ser494 follows the basophilic phosphorylation motif, we believe that the insulin stimulated, rapamycin sensitive mobility shift is a result of multiple phosphorylations.

In support of the hypothesis that SRPK2 is extensively phosphorylated and that phosphorylation of Ser494 is necessary for these post-translational modificaitons, two additional phosphorylation sites were identified on SRPK2 in our proteomic studies, and will likely be the subject of future studies. These sites are Ser490 and Ser497 (Fig 5.2). Interestingly, additional phosphorylation sites on SRPK2 have been identified in separate mass spectrometry proteomic studies, and are reported on phosphosite.org.

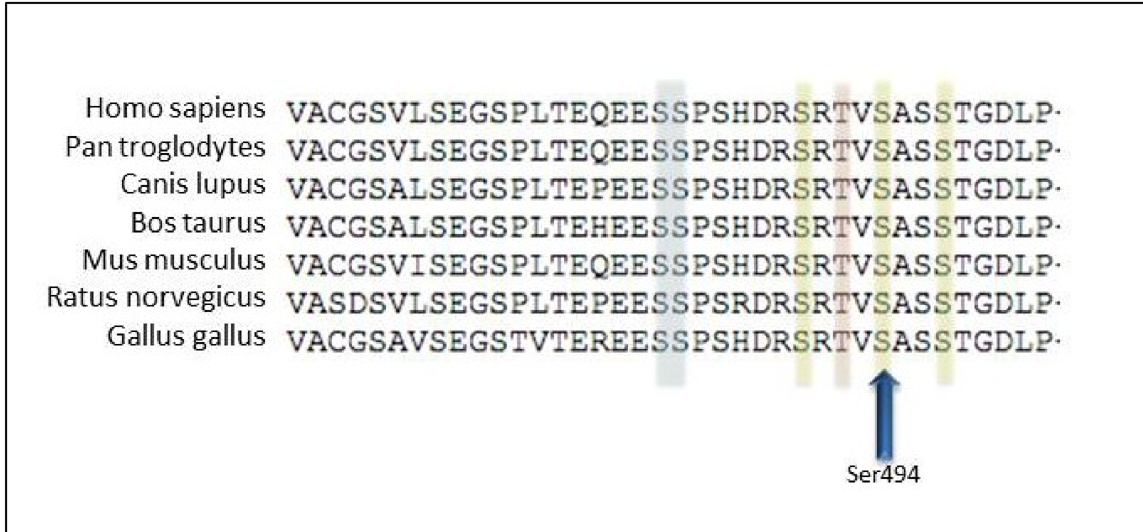

Figure 5.2: SRPK2 phosphorylation sites. The serines identified in our proteomic studies are highlighted in yellow. Threonine 492 highlighted in pink has been previously reported to be a target of AKT. The Ser483 and Ser484 highlighted in blue were reported to be phosphorylated in mass spectrometry screens on phosphosite.org.

We have been unable as of yet to determine what the function of phosphorylation of Ser494 on SRPK2 is for. As stated in Chapter 2, the hypothesis that phosphorylation affects the subcellular localization, thereby regulating either substrate availability or substrate binding is guiding the experiments currently planned. The SRPKs were originally thought to be constitutively active kinases because they are able to phosphorylate their substrates after expression in bacteria [153]. Additionally, co-expression of SRPK1 and ASF/SF2 in *Excherichia coli* results in phosphorylation of ASF/SF2 [154]. There have been structural and biochemical studies undertaken with SRPK1 and the *Sacchoaromyces cerevisiae* SR protein kinase Sky1p, which show that the core kinase domains can adopt an active conformation when their linker regions are truncated [155]. The linker region is required for the cytoplasmic sequestration of the SRPKs, and deletion of this region for the *S. pombe* and *S. cerevisiae* SRPKS Dsk1 and Sky1p, as well as SRPK1/2, results in nuclear accumulation.

In addition to the requirement for the linker region for subcellular localization of the SRPKs, the cytoplasmic anchoring of these kinases has been shown to be regulated by association with molecular chaperones. SRPK1 was shown to directly interact with the cochaperones Aha1 and the heat shock protein Hsp40, which facilitates the formation of a complex with the Hsp70/Hsp90 machinery [156]. SRPK2 was shown to bind -β and −ε 14-3-3 following phosphorylation of Thr492 by AKT [157]. We plan on investigating the possibility that Ser494 phosphorylation promotes association with molecular chaperones, in particular the 14-3-3 class of proteins.

mTORC1 regulation of RUST

We showed that long term inhibition of mTORC1/S6K signaling by rapamycin and PF4078 treatment caused a decrease in the total protein levels for ASF/SF2 and hnRNP A2/B1, and that this decrease could be rescued by knockdown of the NMD machinery (Chapter 4). This provides evidence that mTORC1 signaling is regulating the recently described phenomenon of the coupling of alternative splicing and NMD for these specific splicing factors (Fig. 5.3).

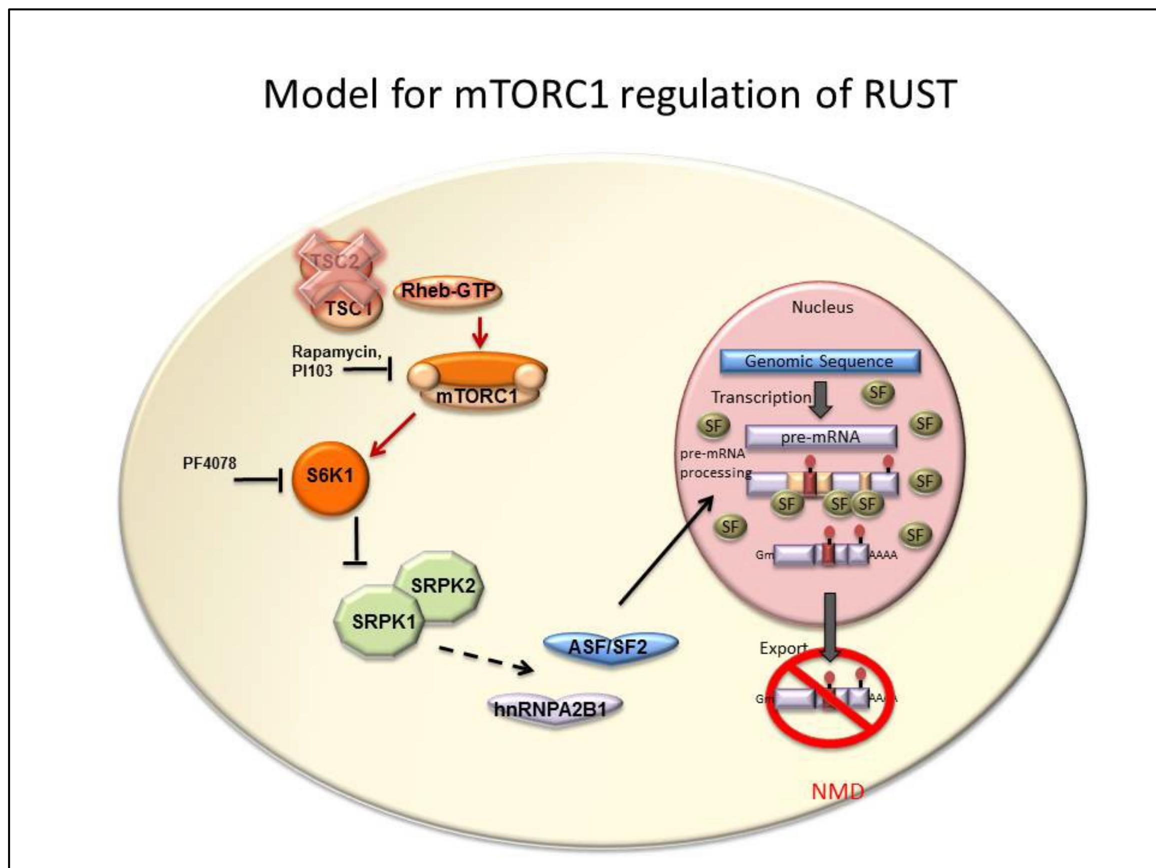

Figure 5.3: Model for mTORC1 regulation of RUST. Hyperactivation of the mTORC1 signaling pathway by loss of TSC2 results in phosphorylation of SRPK2 by S6K. Inhibition of mTORC1/S6K signaling results in a decrease in total protein amount for the splicing factors hnRNPA2B1, as well as ASF/SF2. Concurrent inhibition of the nonsense mediated decay (NMD) pathway rescues expression of these splicing factors, indicating an alternative splicing event following mTORC1 inhibition that results in more messages being degraded by the NMD pathway.

We are in the process of doing qPCR to confirm that rapamycin treatment causes an increase in the premature termination codon (PTC) containing exons for ASF/SF2 and hnRNP A2/B1. In studies exploring the ultraconserved elements (UCEs) in the human genome, it was reported that all SR proteins have UCEs that contain putative PTC containing exons. We are excited to explore the idea that mTORC1 signaling is regulating the intracellular concentrations of other splicing factors, and have primers specific for these exons for use in future qPCR studies. We plan on using cDNA from cells treated with rapamycin and vehicle control, as well as knockdown of the NMD machinery to examine if mTORC1 is regulating the inclusion of these exons.

mTORC1 regulation of alternative splicing

Given the correlation between up-regulation of the mTORC1 signaling pathway in multiple types of cancer with aberrations in the alternative splicing of tumor suppressors and oncogenes, we postulate that mTORC1 is contributing to the oncogenic phenotype by altering splice site selection (Fig. 5.4).

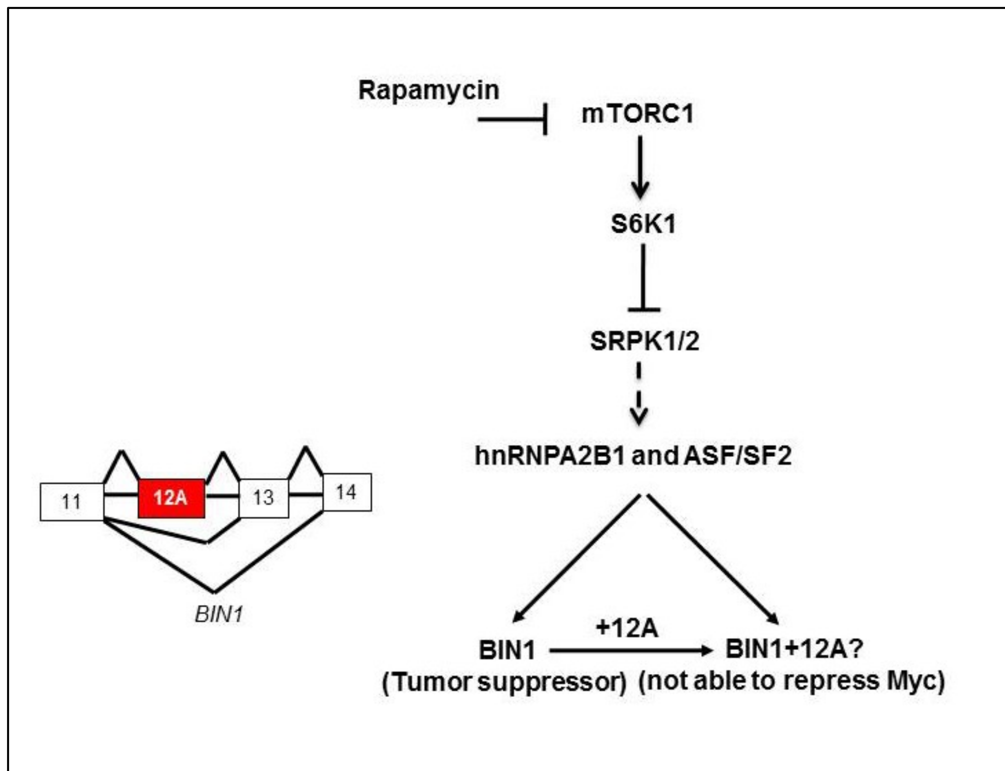

Figure 5.4. Putative model for mTORC1 regulation of BIN1 alternative splicing. Inhibition of mTORC1 signaling in the TSC2-/- mouse embryonic fibroblasts (MEFs) by rapamycin treatment or knockdown of S6K results in a decrease in the amount of exon 12A included in the BIN1 transcript. Additionally, knockdown of the splicing factors ASF/SF2 and hnRNPA2B1 decrease the amount of exon 12A in the BIN1 protein that is expressed.

In addition to the evidence we provided about the alternative splicing of the tumor suppressor BIN1, we've shown mTORC1 regulation of the splicing factors ASF/SF2 and hnRNP A2/B1. These proteins have been reported to regulate the alternative splicing of numerous targets in cancer. Specifically, ASF/SF2 has been shown to regulate splice site selection for the receptor tyrosine kinase RON, which is involved in cell dissociation, motility and matrix invasion [158]. An alternatively spliced isoform of RON termed ΔRON was shown to be the result of skipping exon 11 rendering the protein

constitutively active, and was shown to induce an invasive phenotype in transfected cells [158]. ASF/SF2 was reported to control the inclusion of exon 11, and it will be interesting to see if mTORC1 regulates the splicing of this exon by regulating ASF/SF2.

ASF/SF2 has also been reported to upregulated in a variety of different types of cancer. Specifically, it has been shown to be altered in colon, thyroid, kidney, lung, breast and small intestine cancers [159]. Additionally, hnRNP A2/B1 was shown to be overexpressed in both lung cancer and glioblastomas [160]. It will be interesting to investigate mTORC1 contributions to alternative splicing in these types of cancer.

PI3K-AKT regulation of alternative splicing

In Chapter 2, we provided evidence that AKT phosphorylates SRPK2 at Ser494. AKT has been previously reported to regulate the alternative splicing of several genes including caspase-9 and fibronectin. Shultz et al reported that the gene *CASP9* was alternatively spliced in non-small cell lung cancers (NSCLC), and that AKT regulated the exclusion of exons 3, 4, 5, and 6 via phosphorylation of the splicing factor SRp30a. It will be interesting to see if this occurs through phosphorylation of SRPK2 at Ser494.

Fibronectin (FN) is a ubiquitously expressed glycoprotein found in two forms. The circulating fibronectin that is found in the plasma compartment (plasma or pFN) is produced in hepatocytes, while the cellular fibronection (cFN) is made by fibroblasts (reviewed in White 2008 JPath). The PI3K-AKT pathway was shown to regulate the alternative splicing of FN. Dr. Gina Lee, a post-doctoral fellow involved in this project is investigating the role of PI3K-AKT-SRPK2 regulation of the alternative splicing of FN.

Ras-ERK regulation of alternative splicing

As described in Chapter 1, activation of the Ras-ERK signaling pathway has been associated with multiple types of cancer [161]. There have been several reports that implicate this pathway in the regulation of the alternative splicing of genes in cancer. In addition to CD44 alternative splicing (Chapter 3), the Ras-ERK pathway has also been shown to regulate the alternative splicing and inactivation of the tumor suppressor Krüppel-like factor 6 (KLF6). KLF6, a zinc finger transcription factor, inhibits cellular growth by transcriptional activation of p21, and is inactivated in several human cancers [162]. It will be interesting to look at the alternative splicing pattern of this gene in our HRasV12 cell system, and to investigate the involvement of SRPK2 Ser494 phosphorylation on this alternative splicing event.

Perspective

This dissertation outlines key new concepts for how the PI3K-mTORC1 and Ras-ERK signaling pathway could regulate alternative splicing. Here we provide evidence that mTORC1 signaling is controlling the levels of splicing factors resulting in the alternative splice site selection for a known tumor suppressor. We are excited about the questions and experiments that one can ask from this knowledge, and hope that it will lead to a better understanding of how signaling pathways regulate alternative splice site selection.

Materials and Methods

Cell culture methods

Human embryonic kidney (HEK) 293E, TSC2-/- mouse embryonic fibrobrasts (MEF), and PTEn-/- MEF cells were grown in DMEM and 10% fetal bovine serum, at 37°C and 5% CO_2. Cells were lysed on ice in the standard Blenis lab buffer: fill in 1mM DTT, 1mM Na_3VO_4, and a cocktail of 1mM PMSF, 5ug/mL Pepstatin A, and 5ug/mL Leupeptin. Lysate was generally cleared of solids by centrifugation for 5' at 4°C at 8,000g.

Transfection using Lipofectamine 2000 (Invitrogen) was carried out on cells seeded 24 hours before transfection, and was carried out according to the manufacturer's recommendations for both cDNA and siRNA.

Retroviral infection for stable overexpression in MCF10A cells was done using the virus-producing 293T cell lines that stably expressed the VSVg and gag/pol. Transfection of the constructs for HRasV12 stable expression was done using Lipofectamine 2000 and according to manufacturers recommendations. Virus was collected 4, 5, and 6 days post-transfection. MCF10A cells were infected using a mix of 50% virus-containing media and 50% fresh media. Infected cells were selected with 2ug/mL puromycin for 2 days.

Cell stimulant treatments are at 37 °C as follows, unless otherwise noted:

- PMA (Enzo/Biomol): 20’ treatment, 400 ug/mL stock in DMSO, working concentration 50 ng/mL (1:8000)
- EGF (PeproTech): 10’ treatment, 20 ug/mL stock in ddH_2O, working concentration 20 ng/mL (1:1000)
- Insulin (Sigma): 30’ treatment, 100 µM stock in PBS, working concentration 100 nM (1:1000)

Appropriate vehicle controls were used for serum-starved or control conditions in all experiments.

Cell inhibitor pretreatments are 30’ at 37°C as follows, unless otherwise noted:

- Rapamycin (NIH): 20 ug/mL stock in DMSO, working concentration 20 ng/mL (1:1000)
- U0126 (Biomol/Enzo OR Selleck): 20 mM stock in DMSO, working concentration 10 µM (1:2000)
- LY294002 (Enzo): 30’ pretreatment, stock concentration 20 mM in DMSO, working concentration 50 µM (1:400)
- BI-D1870 (gift from Nathanael Gray OR Symansis): 30’ pretreatment, 10mM stock in DMSO, working concentration 5 µM (1:2000)

Again, appropriate vehicle controls were used for control conditions in all experiments, and conditions where inhibitors were not used. For those inhibitors with

multiple sources (BI-D1870 and U0126), new aliquots were compared with old ones for activity.

Western blot

Western blots were performed by first running protein samples on homemade SDS-PAGE gels. For enhancement of the mobility shift of SRPK2, SDS concentration was increased from .1% to 1%. Proteins were transferred to either 0.45µm or 0.2µm nitrocellulose membranes (Protran) in transfer buffer. Membranes stained with Ponceau-S stain (0.5% Ponceau-S w/v, 1% glacial acetic acid v/v), then blocked in 50% Aquablock (East Coast Bio) in TBS-T (150mM NaCl, 10mM Tris pH 7.5, 0.05% Tween-20) for 30', then placed in primary antibody (listed below) in 50% Aquablock/TBS-T at 4°C overnight. Membranes were then washed for 2 x 5' with TBS-T, then incubated with secondary antibody (LiCor) for 30', then washed for 3 x 5' with TBS-T. Detection was performed with an Odyssey infrared scanner (LiCor).

Primary antibodies used:

- pSRPK2 S494: Millipore (not yet released), 1:1000
- SRPK2: BD Biosciences #ab, 1:100
- rpS6 mAb: Cell Signaling #2317, 1:1000
- p-rpS6 S235/S236: Cell Signaling #2211, 1:1000
- p-rpS6 S240/S244: Cell Signaling #4838, 1:1000
- HA: Santa Cruz sc-805, 1:2000
- S6K1: Cell Signaling #2708, 1:1000,
- pS6K1 T389: Cell Signaling #9206, 1:1000

- Akt1: Cell Signaling # 2967, 1:1000
- pAkt S473 - Cell Signaling # 9271 rabbit polyclonal antibody) (60 kDa)
- actin: Santa Cruz (C-11) sc-1615, 1:3000
- ERK1/2: homemade, 1:5000
- pERK1/2 (T202/Y204 and T183/Y185): Sigma M8159, 1:5000
- pRSK T359/S363: Cell Signaling #9344, 1:1000
- RSK1 (total): Santa Cruz sc-231, 1:300

Preparation of GST fusion proteins

Truncated GST-fusion proteins were generated as follows: first, constructs were transformed into BL-21 cells. Small cultures were grown in 2xYT overnight at 37°C, and then large cultures were inoculated from the smaller ones and grown at 28°C until A_{600} reached 0.8 OD. Cultures were induced with 100 nM IPTG for an additional 2h at 28°C.

Samples were then spun down and resuspended in ice-cold PBS + 1mM PMSF in 1/20 the volume of the culture. Bacterial cells were sonicated 4 x 30” per sample in a Heat Systems - Ultrasonics sonicator, model W-385 (output level 5, duty cycle 100%) at 4°C. Triton X-100 was added to a final concentration of 1% w/v, and samples were rocked at 4°C for 30’. Samples were centrifuged 12,000g, 4°C for 10’, then the lysates were precleared over a column of sepharose beads. The samples were incubated with PBS-washed Glutathione Fast-Flow Sepharose beads overnight at 4°C with rotation for a batch binding.

The beads and lysates were placed in a column and the flow-through was allowed to pass through. Beads were then washed with cold PBS until A_{280} was less than 0.01. The GST-fusion proteins were then eluted off the bead with Glutathione Elution buffer (50mM Tris-HCl, 10mM reduced glutathione, pH 8.0, + 1mM PMSF). Fractions with protein were combined and dialyzed in 25mm dialysis tubing (Spectra/Por) in 15% glycerol in PBS overnight at 4°C. Aliquots were made and snap-frozen in liquid nitrogen before storage at -80°C.

In vitro kinase assay

In vitro kinase assays were performed as follows: first, 293E cells were plated, transfected with pKH3-S6K1, -Rsk1, and -Akt serum-starved, inhibitor- and stimulant-treated, and lysed in BLB buffer (see above). HA-S6K1, Akt and Rsk were immunoprecipitated for 2h using a slurry of combined Protein A and Protein G beads (GE Healthcare) and a homemade antibody to the HA tag. Beads were washed 2x with 1% NP-40 in PBS, then 2x in kinase assay buffer (25mM Tris pH 7.4, 2mM DTT, 10mM $MgCl_2$, 5mM β-glycerophosphate, 1mM PMSF, 1mM $NaVO_4$). Supernatant was aspirated down to the beads. Reaction solution (1-5μg substrate protein as indicated, 50mM β-glycerophosphate, and 100μM cold ATP in kinase assay buffer) was prepared on ice then added to lyophilized ATP-γ-^{32}P (Perkin Elmer), 5μCi/reaction. The reaction solution was added to each of the HA IP bead samples in turn, and incubated at 30°C for 10-30', as indicated. Reactions were terminated by adding 2x SDS-PAGE sample loading buffer to them.

For visualization, kinase reactions were run on 12% SDS-PAGE gels, which were then stained with co-omassie stain and dried down on Whatman paper. The gels were

exposed to an autoradiography screen and counts were read on a Personal FX Phosphorimager (BioRad) and analyzed using QuantityOne software.

For *in vitro* kinase assays other than S6K1, cells were first transfected with the indicated kinase plasmid 72h before cell lysis, and IP was done using homemade ascites antibody against the HA tag.

Site-directed mutagenesis

Site-directed mutagenesis was done by first having a primer and its reverse complement made containing the mutation sequence plus about 19nt on each side. Amplification was done as with PCR for 15-20 cycles, and then the samples were digested with DpnI (NEB), transformed, and sequenced.

RT-PCR

Total RNA was extracted with the RN-easy RNA Isolation Kit (Qiagen) per manufacturer specifications and 2 μg of total RNA was reverse transcribed with SuperScript II (Invitrogen). PCR was performed on 1/10 (2 μl) of the cDNA, in 50- μl reactions containing 0.2 mM dNTP mix, 10× PCR buffer with 15 mM $MgCl_2$, 2.5 units of Taq (Invitrogen) and 0.2 μM of each primer

qPCR

For quantitative real-time PCR (qRT-PCR), RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The resulting cDNA was analyzed by qRT-PCR using the QuantiTect SYBR Green qPCR System (Qiagen). A QuantiTect Primer Assay for mouse Grb10 was used to amplify the target gene, while the β-actin primers (β-actin forward, ACCCAGATCATGTTTGAGACCT; and β-actin reverse,

GCAGTAATCTCCTTCTGCATCC) were used as a normalization control. All reactions were run on an ABI 7900HT Fast Real-Time PCR instrument with a 15 min hot start at 95°C followed by 40 cycles of a 3-step thermocycling program: denaturation: 15 s at 94°C, annealing: 30 s at 55°C and extension: 30 s at 70°C. Melting curve analysis was performed at the end of every run to ensure that a single PCR product of the expected melting temperature was produced in a given well. A total of 3 biological replicates × 4 technical replicates were performed for each treatment group. Data was analyzed using the comparative Ct method (ΔΔCt method).

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