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Both Decreased and Increased SRPK1 Levels Promote Cancer by Interfering with PHLPP-Mediated Dephosphorylation of Akt

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SUMMARY

Akt activation is a hallmark of human cancers. Here, we report a critical mechanism for regulation of Akt activity by the splicing kinase SRPK1, a downstream Akt target for transducing growth signals to regulate splicing. Surprisingly, we find that SRPK1 has a tumor suppressor function because ablation of SRPK1 in mouse embryonic fibroblasts induces cell transformation. We link the phenotype to constitutive Akt activation from genome-wide phosphoproteomics analysis and discover that downregulated SRPK1 impairs the recruitment of the Akt phosphatase PHLPP1 (pleckstrin homology (PH) domain leucine-rich repeat protein phosphatase) to Akt. Interestingly, SRPK1 overexpression is also tumorigenic because excess SRPK1 squelches PHLPP1. Thus, aberrant SRPK1 expression in either direction induces constitutive Akt activation, providing a mechanistic basis for previous observations that SRPK1 is downregulated in some cancer contexts and upregulated in others.

INTRODUCTION

Increasing evidence suggests that splicing factors and regulators are multitasking in mammalian cells. In particular, the SR family of splicing factors integrates multiple steps in gene expression, from transcription to mRNA export to translation (Zhong et al., 2009b). Individual heterogeneous nuclear ribonucleoproteins (hnRNPs) seem to target themselves and influence the levels of many other regulatory proteins, resulting in complex responses in gene expression (Huelga et al., 2012). Many of these splicing regulators appear to function beyond the splicing control. For instance, SRSF2, an SR protein, plays a direct role in transcriptional activation at gene promoters (Ji et al., 2013); the abundant U1 small nuclear ribonucleoprotein particle (snRNP) functions to prevent premature Pol II termination (Berg et al., 2012); the classical splicing regulator polypyrimidine tract binding protein (PTB) modulates microRNA targeting during neuronal induction (Xue et al., 2013); and SRSF1 regulates p53 stability by sequestering the p53 E3 ligase Mdm2 (Fregoso et al., 2013). These findings highlight critical contributions of splicing factors and regulators to diverse biological pathways.

We recently demonstrated a central role of SR protein-specific kinases (SRPKs) in transducing growth signals from the cell surface to the nucleus to regulate splicing (Zhou et al., 2012). SR proteins are extensively phosphorylated by two families of kinases, SRPKs and Clks (Zhou and Fu, 2013). SRPKs are mainly sequestrated in the cytoplasm by molecular chaperones (Zhong et al., 2009a; Zhou and Fu, 2013), while Clks are largely distributed in the nucleus; these kinases act together to catalyze consecutive phosphorylation on SR proteins (Velazquez-Dones et al., 2005). Like many signaling molecules, cytoplasmic SRPKs are activated in response to upstream signals and translocate to the nucleus to regulate splicing (Zhou et al., 2012). A key event is induced binding of activated Akt to SRPKs, and global analysis indicates that this Akt-SRPK-SR pathway is responsible for the majority of induced splicing events in EGF-treated cells (Zhou et al., 2012). An outstanding question is how this signal transduction pathway might contribute to a plethora of Akt functions in cell survival, proliferation, and oncogenic transformation.

Akt plays critical roles in diverse cellular signaling pathways (Manning and Cantley, 2007). Akt is cotranslationally phosphorylated at T450 by mammalian target of rapamycin complex 2 (mTORC2) (Oh et al., 2010). In response to EGF or insulin, activated phosphatidylinositol 3-kinase (PI3K) increases the production of phosphatidylinositol-3,4,5-trisphosphate (PI3P), which

recruits Akt to the plasma membrane. This unmasks T308 in its activation loop for phosphorylation by PDK-1, which triggers additional mTORC2-mediated phosphorylation at S473. Akt phosphorylated at both T308 and S473 is maximally activated. The Akt pathway is subjected to various feedback controls, resulting in quick attenuation of growth signals in normal cells, and constitutive activation of Akt is a hallmark of many human cancers (Carracedo and Pandolfi, 2008).

A major mechanism to counteract Akt activation is the conversion of PIP3 back to PIP2 by the lipid phosphatase Phosphatase and tensin homolog (PTEN), a well-known tumor suppressor (Maehama and Dixon, 1999). Activated Akt can also be attenuated by dephosphorylation at critical activation sites. PP2A has been implicated as an Akt phosphatase for T308 (Kuo et al., 2008), and pleckstrin homology (PH) domain leucine-rich repeat protein phosphatases (PHLPP1/PHLPP2) have been shown to function as Akt phosphatases that primarily act on S473 (Brognard et al., 2007; Gao et al., 2005). Interestingly, while PHLPPs appear to also function as tumor suppressors (Chen et al., 2011), little is known about how PHLPP-mediated Akt dephosphorylation might be regulated (O'Neill et al., 2013).

Here, we report an unexpected tumor suppressor function of SRPK1, which is linked to constitutive activation of Akt in *SRPK1* knockout mouse embryonic fibroblasts (MEFs). We show that only activated Akt forms a tight complex with SRPK1, and within the complex, Akt dephosphorylation is potently induced. This turns out to result from SRPK1-dependent recruitment of the Akt phosphatase PHLPP1. Strikingly, overexpression of the splicing kinase also activates Akt by sequestrating the Akt phosphatase. Therefore, either under- or overexpression of SRPK1, as frequently observed in human cancers, leads to constitutive Akt activation. Together, these findings reveal an unusual regulatory paradigm in the well-studied Akt pathway and suggest that SRPK1 may function as an oncogene or tumor suppressor in different cellular contexts, a paradox frequently encountered with other signaling molecules (Feng, 2012).

RESULTS

SRPK1 Is Essential for SR Protein Phosphorylation and Embryonic Development

SRPK1 has been extensively characterized as an SR protein-specific kinase to relay external growth signals to the nucleus to regulate alternative splicing (Zhou and Fu, 2013). To determine its biological function, we generated conditional *SRPK1* knockout mice by using the Cre-LoxP technology (Figure S1A available online). The knockout construct targeted the *SRPK1* locus in mouse 129 embryonic stem cells (ESCs), confirmed by Southern blotting with both 5′ and 3′ primers outside the DNA fragments used to construct the targeting vector (Figure S1B). Germline-transmitted mice could be conveniently genotyped by PCR using a set of primers flanking the targeted exon 3 (Figure S1C). Both hetero- and homozygous floxed mice ($SRPK1^{w/f}$ and $SRPK1^{f/f}$) were phenotypically normal compared to wild-type mice in different genetic backgrounds.

By using an oocyte-specific *ZP3-Cre* transgenic mouse (Wang et al., 2001), conditional knockout *SRPK1* mice were first converted to germline-deleted females, which were further crossed with wild-type males in order to generate heterozygous ($SRPK1^{+/-}$) and null ($SRPK1^{-/-}$) mice. We recovered both wild-type and heterozygous mice in the Mendelian frequency but detected no null mice among live-birth animals. By genotyping mouse embryos at embryonic day 14.5 (E14.5) and E21 stages, we detected a rough 1:2 ratio of $SRPK1^{+/+}$ and $SRPK1^{+/-}$ fetuses, but no $SRPK1^{-/-}$ embryos (Figure 1A). These data indicate that SRPK1 is essential for early embryonic development in the mouse.

The functional requirement of SRPK1 in development is consistent with its central role in phosphorylating SR proteins, important for early steps of spliceosome assembly (Cho et al., 2011). As such lethal phenotype prevented further biological studies at the animal level, we derived MEFs from littermates of conditional knockout mice with different genotypes, which were converted to hetero- and homozygous *SRPK1* knockout by using a Cre recombinase expressed from a retrovirus (Figure 1B). Western blotting analysis showed that SRPK1 protein was reduced in $SRPK1^{+/-}$ MEFs and abolished in $SRPK1^{-/-}$ MEFs, accompanied by a modest induction of SRPK2, a SRPK1 homolog normally expressed in the nervous system (Wang et al., 1998) (Figure 1C). Despite SRPK2 induction, however, multiple SR proteins showed significantly reduced phosphorylation when blotted with a pan-SR protein phospho-antibody (mAb104) (Figure 1D). These data demonstrate unique contribution of SRPK1 to both SR protein phosphorylation and embryonic development in the mouse.

Ablation of *SRPK1* Induces Premature Cell Senescence and Transformation

Upon *SRPK1* ablation in MEFs, we did not detect obvious cell lethality; instead, we observed retarded cell growth upon Cre viral infection. This prompted us to determine whether *SRPK1* deletion induced cell senescence. Indeed, by staining the cells with the senescence marker β-galatosidase (SA-β-gal), we found that both $SRPK1^{+/-}$ and $SRPK1^{-/-}$ MEFs showed significant β-gal staining compared to $SRPK1^{+/+}$ MEFs (Figure 1E), and the levels of induced premature senescence appeared to depend on the degree of SRPK1 deficiency (Figure 1F). The premature senescent response, a well-known cellular defense mechanism against oncogenic transformation (Prieur and Peeper, 2008), raised an intriguing possibility that SRPK1 might have a tumor suppressor activity.

Since the induction of cell senescence is well known to depend on p53 (Rufini et al., 2013), we explored the potential tumor suppressing property of SRPK1 on immortalized MEFs. By using p53 RNAi (Figure S2) or via expressing the SV40 large T antigen, which is known to downregulate p53 (Ahuja et al., 2005), we found that immortalized MEFs no longer showed cell growth arrest after *SRPK1* removal (Figure 2A). Strikingly, these immortalized *SRPK1* knockout MEFs appeared to be transformed, as indicated by their anchorage-independent growth on soft agar (Figure 2B). Both $SRPK1^{+/-}$ and $SRPK1^{-/-}$ MEFs were transformed, although *SRPK1* null MEFs showed a more potent transformation phenotype, as indicated by the higher number of colony induced (Figure 2B).

To further demonstrate that these transformed MEFs have the capacity to develop into tumors, we subcutaneously injected

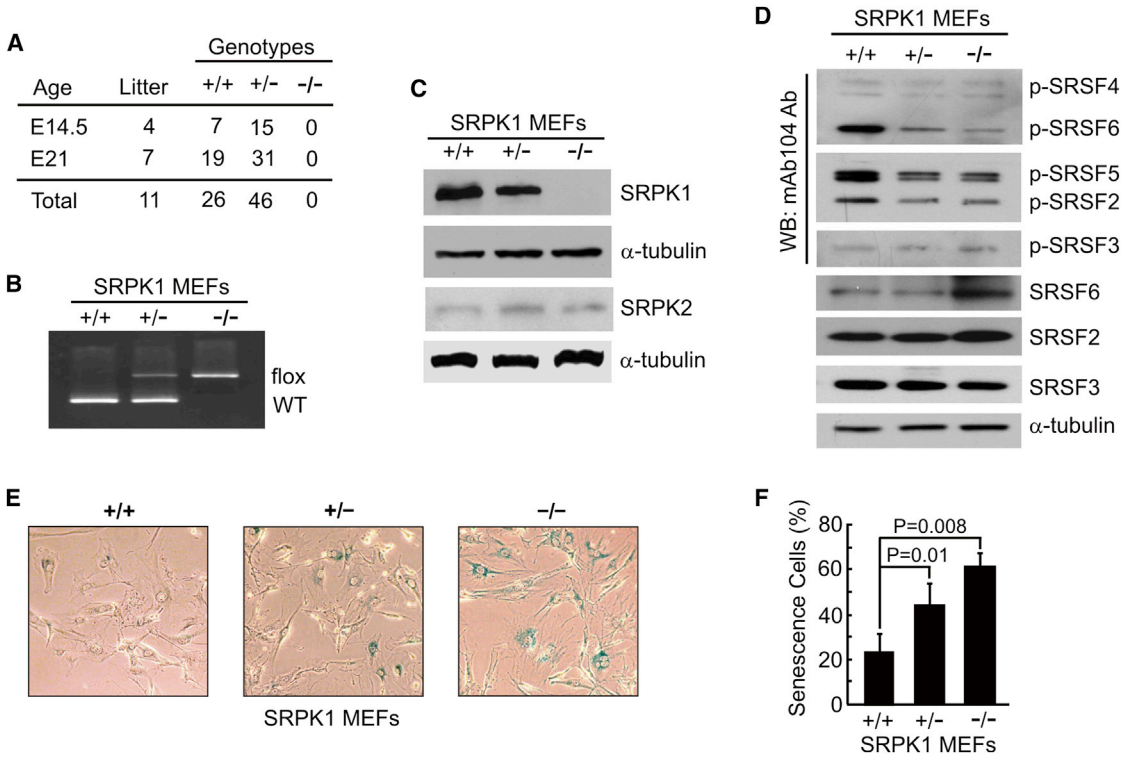


Figure 1. Induction of Premature Cell Senescence in *SRPK1*-Deleted MEFs

(A) Genotypes of progenies from crosses of *SRPK1* heterozygous mice. Genomic DNA from embryos at E14.5 and E21 were genotyped by PCR. No homozygous embryos were detected, indicating that *SRPK1* is essential for embryonic development.

(B) Generation of hetero- and homozygous *SRPK1* deletion MEFs. Embryos of conditional *SRPK1* knockout mice with different genotypes were used to derive MEFs, followed by deletion of the floxed allele(s) with a Cre recombinase-expressing retrovirus. Successful conversion was confirmed by PCR.

(C) Western blot analysis of SRPK1 and SRPK2 expression on wild-type and *SRPK1* hetero- and homozygous MEFs. SRPK2 was expressed at a low level in MEFs and modestly induced in response to SRPK1 knockout.

(D) Analysis of phosphorylation levels of SR proteins in wild-type and *SRPK1*-deleted MEFs by using a pan-SR protein phospho-antibody (mAb104). The assignment of individual SR proteins was based on relative molecular weights. Total levels of three representative SR proteins (SRSF2, SRSF3, and SRSF6) were quantified with specific antibodies reactive to nonphosphorylated regions in these proteins. α-tubulin was probed as a loading control.

(E) Representative images of SA-β-gal staining, showing induced premature cell senescence on $SRPK1^{+/-}$ and $SRPK1^{-/-}$ MEFs (cells in blue) relative to the background on $SRPK1^{+/+}$ MEFs.

(F) The results in (E) were quantified in. Data are shown as mean ± SD, and statistical significance (p value) was determined by Student's t test. See also Figure S1.

MEFs with different genotypes into nude mice. While wild-type MEFs immortalized by the T antigen alone were not tumorigenic, both Tg-immortalized $SRPK1^{+/-}$ and $SRPK1^{-/-}$ MEFs developed full-bloom tumors (Figure 2C). Again, tumor development appeared to depend on the dose of remaining SRPK1 in the MEFs, as tumors from $SRPK1^{+/-}$ MEFs grew much slower in nude mice than those from $SRPK1^{-/-}$ MEFs (Figure 2C, right panel). These data strongly suggest that SRPK1 has a tumor suppressor function in vivo, and the deficiency in SRPK1 expression quantitatively contributes to tumorigenesis in immortalized cells.

Our observation agrees with reduced SRPK1 expression in certain cancer cell lines (Hayes et al., 2006) or tumors (Krishnakumar et al., 2008; Odunsi et al., 2012; Schenk et al., 2004). Examination of a panel of human colon cancers revealed that SRPK1 was undetectable in multiple tumors compared to surrounding noncancerous tissues (Figure 2D). However, similar to other reports of SRPK1 overexpression in different types of human cancers (Hayes et al., 2006, 2007; Wu et al., 2013; Zhou et al., 2013), we also detected SRPK1 overexpression in multiple other colon tumors (Figure 2D). This indicates that SRPK1 expression is dramatically altered in human cancers, which begs the question of what might be the mechanism(s) underlying the potential contribution of aberrant SRPK1 expression to tumorigenesis.

Inactivation of SRPK1 Leads to Constitutive Akt Activation

Because SRPK1 is largely responsible for transducing growth signals to regulate alternative splicing in the nucleus, thus predicting a critical contribution of kinase-dependent splicing events to cell proliferation (Zhou et al., 2012), it appears counterintuitive to suspect that certain splicing defects in SRPK1-deficient MEFs might account for the transformation phenotype. Furthermore, many SRPK1 substrates are known to exert important dephosphorylation-dependent functions beyond the

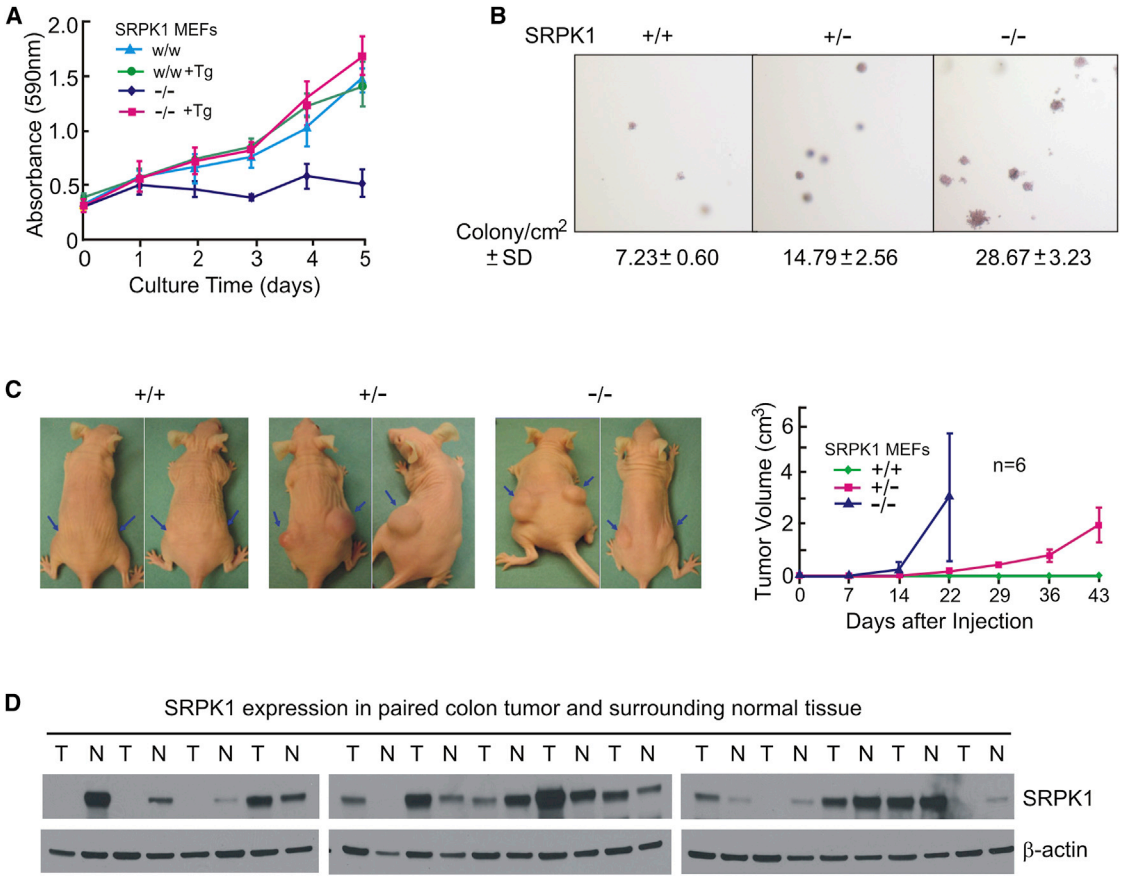


Figure 2. Oncogenic Transformation Induced by *SRPK1* Deletion

(A) Immortalization of wild-type and *SRPK1*$^{-/-}$ MEFs by the large T antigen (Tg). Growth of early passage (passage 2) wild-type and *SRPK1* null MEFs with or without Tg immortalization was monitored by the MTT assay based on absorbance at 590 nm. *SRPK1* deletion retarded cell growth, and Tg immortalization prevented such an effect. See also Figure S2 for p53 RNAi-induced immortalization of *SRPK1*$^{+/+}$ and *SRPK1*$^{-/-}$ MEFs.

(B) Soft agar analysis of anchorage-independent cell growth of Tg-immortalized MEFs of different *SRPK1* genotypes. Colonies on each plate were stained with crystal violet after 16 days in culture. A representative area from each plate is shown, and the colony number under each condition was quantified as an average of nine areas in a grid and presented as number per cm^2 ± SD at the bottom.

(C) Tumor development in nude mice subcutaneously injected with MEFs of different *SRPK1* phenotypes. Tumor formation on a pair of mice with each genotype is shown on the left, and tumor volumes (mean ± SD) from each group of six mice during the period of 6 weeks after injection are plotted on the right.

(D) Western blotting analysis of *SRPK1* expression in multiple pairs of human colon cancer and adjacent normal tissues. T, tumor sample; N, surrounding normal tissue. The data were randomly selected from a large number of tissue pairs surveyed to illustrate aberrant SRPK1 expression in human cancers.

splicing control. For example, dephosphorylated SR proteins appear to be more efficient in enhancing translation in the cytoplasm (Sanford et al., 2004, 2005). A recent study revealed another function of the SR protein SRSF1 in the regulation of p53 stability (Fregoso et al., 2013). For these reasons and others, we decided to focus on characterizing SRPK1 null-induced cellular transformation on MEFs by using unbiased approaches before pursuing specific pathways.

Using the stable isotope labeling by amino acid in cell culture (SILAC) technology (Chen et al., 2010), we labeled Tg-immortalized *SRPK1*$^{+/+}$ and *SRPK1*$^{-/-}$ MEFs with isotopically heavy and light amino acids, respectively, for at least five cell divisions. Cells of different genotypes were next serum starved for 16 hr and then induced with serum for 15 min. Equal numbers of cells were pooled to extract proteins for purification of phosphopeptides by liquid chromatography and tandem mass spectrometry (LC-MS/MS). The initial mass-to-charge ratio (m/z) of individual peptides was measured, and the light (for knockout) and heavy (for WT) chromatographic elution profiles of each peptide pair were used to compute the differences (see representative MS profiles in Figure 3A). We detected various changes in specific signal transduction pathways, some of the most prevalent changes being those associated with the Akt/mTOR pathway, such as Akt1/2-T450, IRS1-S999, Rictor-S1281, etc. (Figures 3A and S3). Some changes in the mitogen-activated protein kinase (MAPK) pathway (i.e., Raf1 and ERK2) were also evident, but the magnitude of such changes appears small and variable between duplicated experiments (Figures 3A and S3).

We paid particular attention to the Akt/mTOR pathway because our previous work established SRPK1 as a downstream target for activated Akt (Zhou et al., 2012). Based on initial clues

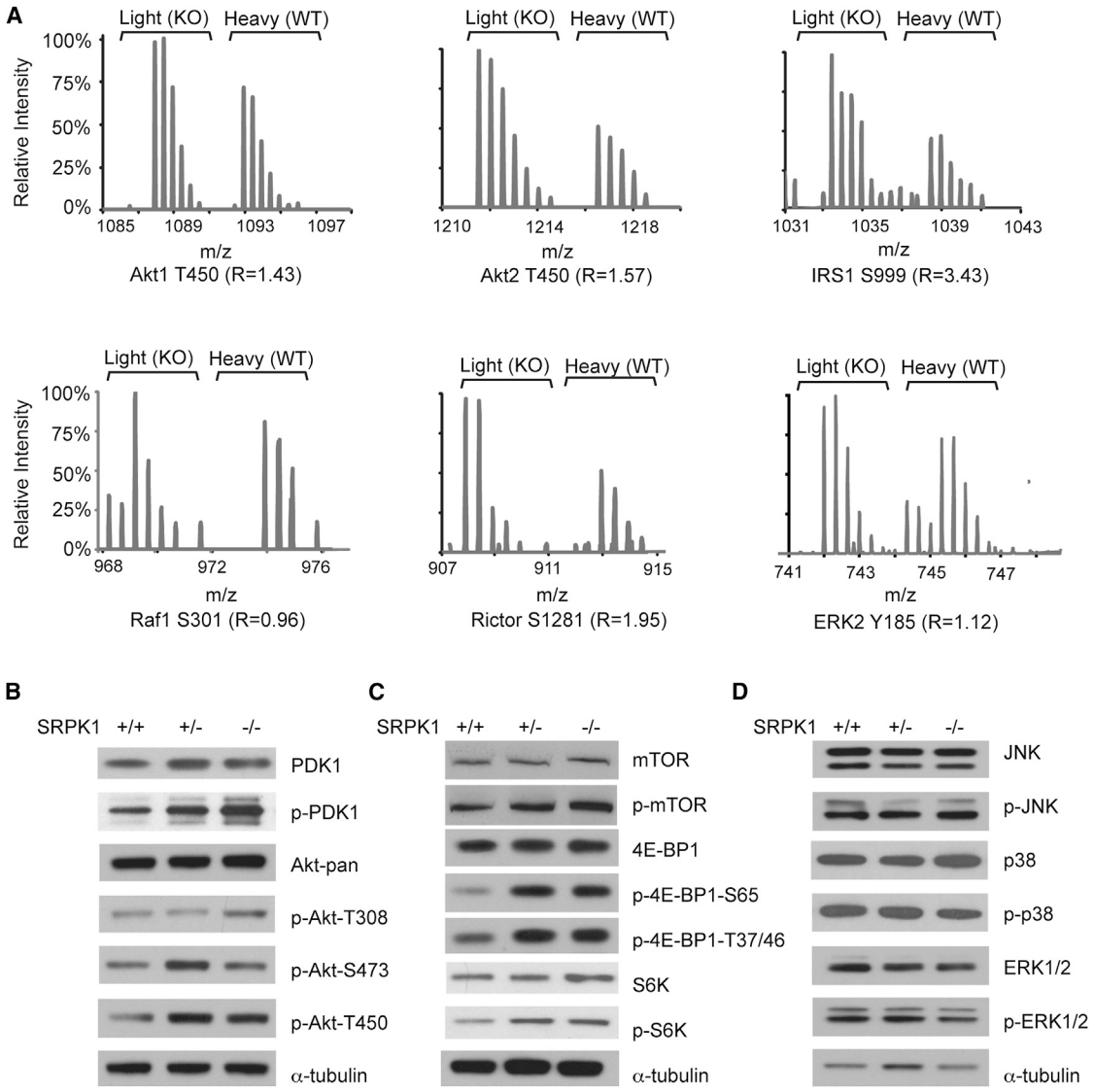


Figure 3. Constitutive Activation of the Akt Pathway in Response to *SRPK1* Deletion

(A) Representative phosphopeptides detected by SILAC as indicated. The y axis shows the relative intensity of different peaks, and the x axis indicates the mass-to-charge (m/z) ratio. Each graph shows a pair of light (KO) and heavy (WT) peaks. The corresponding phosphorylation site in each specific gene is shown at bottom. The ratio of light versus heavy is indicated in the parentheses in each case. See also Figure S3 for a comprehensive list of SILAC results on some common signaling pathways.

(B and C) Western blotting analysis of multiple components in the Akt pathway with a panel of phosphospecific antibodies on MEFs of different *SRPK1* genotypes. In this analysis, cells were initially maintained in normal growth media, serum starved for 16 hr, and then stimulated with serum for 15 min before harvest for western blotting.

(D) Few effects of *SRPK1* knockout were detected on the major components of the JNK, p38, and MAPK pathways. These analyses were carried out with the same cell lysates as in (B) and (C).

from the phosphoproteomic analysis, we used various available phosphospecific antibodies against key components in the Akt pathway, including those that escaped detection in our SILAC experiments, to determine the activation status of the pathway. We found that multiple key players in the Akt pathway were activated in both *SRPK1*$^{+/-}$ and *SRPK1*$^{-/-}$ MEFs, including PDK1, Akt (at T308, S473, and T450), mTOR, 4E-BP1 (at S65 and T37/46), and S6K (Figures 3B and 3C), but *SRPK1* knockout had little or no effects on JNK, p38, and MAPK pathways (Figure 3D). As the Akt pathway is known to be subject to complex feedback and feedforward regulation (Carracedo and Pandolfi, 2008), our data suggest that SRPK1 deficiency may have somehow induced the activation of the Akt pathway, but may be insufficient to trigger extensive crosstalks with several other major signal cascades in MEFs. As the Akt pathway is central for cell proliferation and survival, the constitutive activation of the

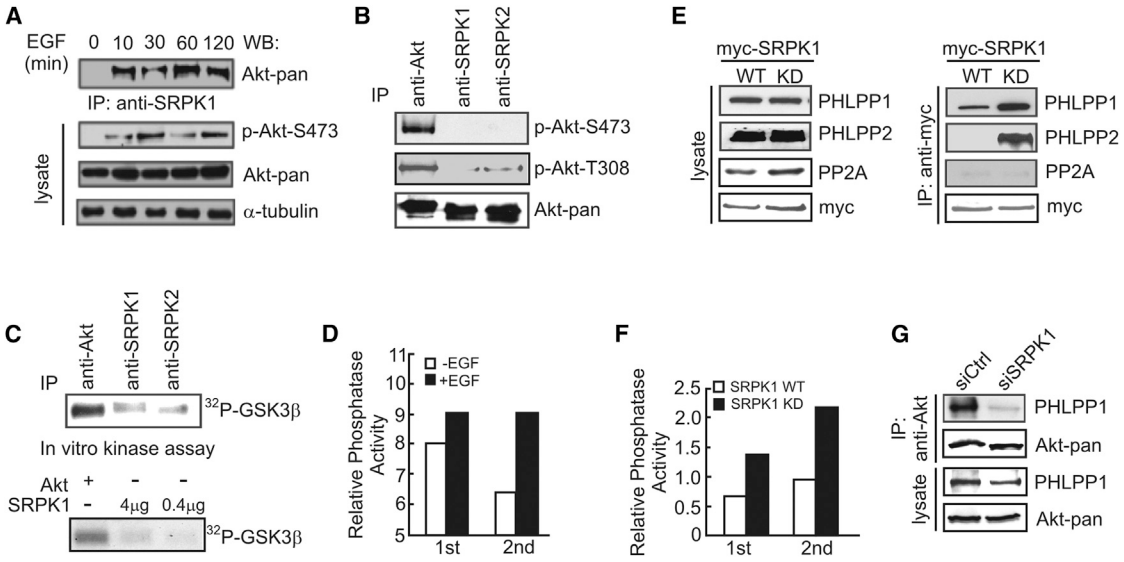


Figure 4. SRPK1-Dependent Recruitment of the Phosphatase PHLPP to Akt

(A) CoIP/western blotting analysis, showing that only EGF-activated Akt becomes associated with SRPK1 in HEK293T cells. A monoclonal anti-SRPK1 was used for IP and polyclonal anti-pan-Akt for western.

(B) Coimmunoprecipitation (coIP)/western blotting analysis of HEK293T cells treated with EGF for 2 hr, showing that Akt is largely inactivated within the complex containing SRPK1 or SRPK2.

(C) In vitro kinase assay using either immunoprecipitated or purified kinases. The top panel shows little activity of SRPK-associated Akt in phosphorylating its canonical substrate GSK3β. The lower panel indicates unappreciable activity of a highly active recombinant SRPK1 on GSK3β.

(D) Relative phosphatase activity associated with immunoprecipitated SRPK1 before and after EGF treatment from two independent experiments, showing increased phosphatase activity associated with SRPK1 from EGF-treated HEK293T cells relative to mock-treated cells.

(E) CoIP/western analysis showing that Myc-tagged SRPK1 was associated with PHLPP1, but not PP2A, and the kinase-dead version of SRPK1 (KD) efficiently trapped both PHLPP1 and PHLPP2 in transfected HEK293T cells cultured under regular growth conditions.

(F) Increased phosphatase activity associated with kinase-dead SRPK1 relative to wild-type SRPK1.

(G) CoIP/western blotting analysis of the association between Akt and PHLPP1 in EGF-treated HEK293T cells with or without knocking down SRPK1, demonstrating that SRPK1 plays a critical role in facilitating PHLPP1 recruitment to Akt.

pathway may directly contribute to SRPK1 deficiency-induced cell transformation.

Akt Is Potently Dephosphorylated within SRPK-Containing Complexes

Both SRPK1 and SRPK2 have been shown to be direct downstream targets of activated Akt (Jang et al., 2009; Zhou et al., 2012). Importantly, these published studies demonstrated that only activated Akt is able to bind SRPKs to induce the activation of the splicing kinases, which we further confirmed on human embryonic kidney 293T (HEK293T) cells in response to EGF treatment (Figure 4A). We next used specific anti-SRPK1 and anti-SRPK2 to detect their association with Akt in different functional states on EGF-stimulated HEK293T cells. Surprisingly, we found that the coimmunoprecipitated Akt was largely dephosphorylated in complex with either SRPK1 or SRPK2 (Figure 4B). Neither anti-SRPK immunoprecipitated Akt nor purified SRPK1 was efficient in phosphorylating its canonical substrate GSK3β in in vitro kinase assays (Figure 4C). These observations indicate that binding of activated Akt to SRPKs appears to be a suicidal mission, leading to its rapid and nearly quantitative inactivation within SRPK-containing complexes. These data suggest that SRPKs may also serve as regulators of Akt in mammalian cells.

SRPK1 Is Required for the Recruitment of Akt-Specific Phosphatases

To understand how Akt might be quickly inactivated upon forming a complex with an SRPK, we suspected that there might be a phosphatase(s) involved. To test this hypothesis, we immunoprecipitated SRPK1 before and after EGF treatment and then measured general phosphatase activity associated using a synthetic phosphatase substrate. We observed from two independent experiments that SRPK1 brought down from EGF-treated HEK293T cells was indeed associated with a higher level of phosphatase activity compared to mock-treated cells (Figure 4D), indicating that an Akt phosphatase(s) might be recruited for inactivating Akt in the complex.

There are two major classes of characterized Akt phosphatases, PP2A and PHLPP1/PHLPP2 (Beaulieu et al., 2005; Gao et al., 2005). To determine which was specifically associated with the SRPK1-containing complex, we used a myc-tagged SRPK1 to perform immunoprecipitation followed by western blotting in transfected HEK293T cells, identifying PHLPP1, but little PP2A, in the complex (Figure 4E). We next took advantage of the previous observation that Akt induces SRPK1 autophosphorylation, which is required for releasing the splicing kinase from activated Akt (Zhou et al., 2012), by testing a kinase-dead (KD) SRPK1 for its ability to

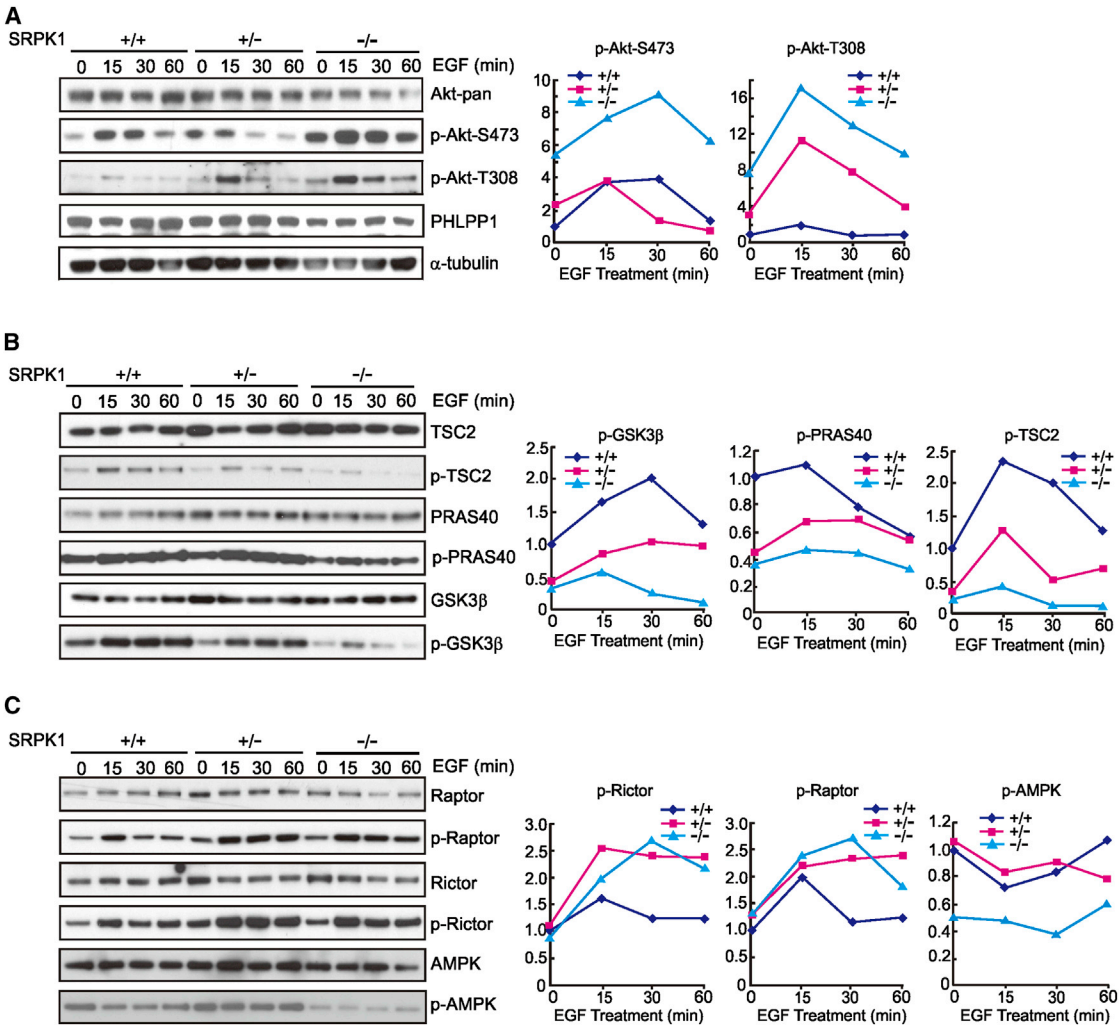


Figure 5. Time Course Analysis of the Akt Pathway on MEFs

(A) Time course analysis of Akt activation in response to EGF treatment on MEFs, showing that SRPK1 deficiency induced the basal levels of activated Akt and prolonged Akt activation in response to EGF, while the total level of PHLPP1 remained constant in the cell. Quantified data are presented on the right.

(B) Similar analysis of the immediate Akt substrates (TSC2, PRAS40, and GSK3β) in the same time course experiment as in (A), showing reduced basal levels of all three Akt substrates examined before EGF treatment (0 point). Their induction was compromised in *SRPK1*$^{+/-}$ and more so in *SRPK1*$^{-/-}$ MEFs relative to wild-type MEFs. Quantified data are presented on the right.

(C) Similar analysis of critical components of mTORC1 (Raptor), mTORC2 (Rictor), and AMPK in the same time course experiment as in (A), showing that while none of these targets were activated before EGF treatment (0 point), both mTORC1 and mTORC2 were induced by EGF treatment on *SRPK1*$^{+/-}$, and more so in *SRPK1*$^{-/-}$ MEFs relative to wild-type MEFs. AMPK phosphorylation was significantly reduced in *SRPK1*$^{-/-}$ MEFs. Quantified data are presented on the right.

retain PHLPP1 in the complex. The kinase-dead mutant indeed showed increased association with PHLPP1, and interestingly, the mutant kinase also efficiently trapped PHLPP2 (Figure 4E). Consistently, the kinase-dead SRPK1 brought down a higher level of the phosphatase activity from two independent experiments (Figure 4F). Finally, to directly demonstrate that SRPK1 is required for the recruitment of PHLPP1 to activated Akt, we showed that Akt was efficiently associated with PHLPP1, but upon SRPK1 RNAi, such association was lost (Figure 4G). We conclude from these experiments that SRPK1 is required for the recruitment of PHLPP1 to activated Akt.

SRPK1 Dynamically Modulates the Akt Pathway

To demonstrate the physiological relevance of SRPK1-dependent recruitment of the Akt phosphatase PHLPP1, we analyzed the kinetics of Akt activation and attenuation in response to EGF treatment on wild-type and *SRPK1* knockout MEFs. Relative to wild-type MEFs, both *SRPK1*$^{+/-}$ and *SRPK1*$^{-/-}$ MEFs displayed elevated basal levels of activated Akt before EGF stimulation, and SRPK1 deficiency significantly prevented attenuation of activated Akt after the EGF treatment (Figure 5A, see quantified data on the right). The effect of *SRPK1*$^{+/-}$ was less potent compared to *SRPK1*$^{-/-}$. The level of PHLPP1 remained relatively constant under these conditions.

We next examined the status of a set of immediate Akt downstream targets, such as TSC2, GSK3β, and PRAS40. Surprisingly, relative to wild-type MEFs, these Akt targets were somewhat suppressed in *SRPK1*$^{+/-}$ and *SRPK1*$^{-/-}$ MEFs before EGF treatment, and furthermore, they showed a much reduced response to EGF stimulation, which is opposite to the induction of Akt activation in these cells (Figure 5B). These data, while initially puzzling, indicate that SRPK1 deficiency-induced Akt activation in the cytoplasm may be quite distinct from EGF-activated Akt at the plasma membrane, the latter of which is responsible for phosphorylating multiple immediate Akt substrates. Additionally, activated Akt in the cytoplasm likely trigger various feedback pathways, resulting in weakened responses of various immediate Akt targets to EGF treatment.

The question is what happened to more downstream targets in the Akt pathway. Our phosphoproteomic analysis and subsequent confirmation with phosphospecific antibodies had detected activation of multiple components in the Akt pathway (see Figure 3). To carefully examine the status of these further downstream Akt targets under serum-starved followed by EGF-stimulated conditions, we analyzed the induction kinetics of Raptor and Rictor. We found that, while their basal levels in serum-starved MEFs were similar, they became quite resistant to decay after EGF treatment (Figure 5C).

As these responses correlated to Akt activation, but not to TSC2 activation, we suspected some TSC2-independent mechanisms that might contribute to mTOR activation. One of these is AMP-activated protein kinase (AMPK), a separate pathway involved in the regulation of mTOR activity through sensing the AMP/ATP ratio in the cell (Hahn-Windgassen et al., 2005; Inoki et al., 2006). Indeed, we found that AMPK phosphorylation was greatly reduced in *SRPK1*$^{-/-}$ MEFs under both serum starvation and EGF induction conditions (Figure 5C). As AMPK is an established inhibitor of mTOR, reduced AMPK activity likely caused mTOR activation and induced feedback responses in *SRPK1*$^{-/-}$ MEFs. We noted, however, that the AMPK activity remained largely unaltered in both wild-type and *SRPK1*$^{+/-}$ MEFs, indicating that additional mechanisms, including less-potent feedback responses that might not have met a required threshold, could contribute to increased mTOR activity when SRPK1 was only partially compromised. In aggregate, these data suggest that SRPK1 deficiency induces dynamic and differential responses of various components in the Akt pathway, which appear to be quite distinct from those induced by growth factors at the cell surface.

SRPK1 Overexpression Squelches PHLPP from Akt

It has been reported that SRPK1 was downregulated in a subset of human cancers while overexpressed in others (Hayes et al., 2006, 2007; Zhou et al., 2013). Using two large published data sets on normal human tissues and cancers, we found that SRPK1 expression is more tightly controlled than β-actin in most normal human tissues but becomes highly variable with general overexpression in diverse cancer types (Figure S4). In fact, overexpressed SRPK2 has been shown to enhance cell proliferation on a leukemia model (Jang et al., 2008). While an overexpressed SRPK likely causes complex changes at the levels of alternative splicing (Amin et al., 2011), our current finding of SRPK-dependent recruitment of an Akt phosphatase suggests yet another intriguing possibility that overexpression of the splicing kinase might squelch such Akt phosphatase, thus serving as a synergistic event with other oncogenic activities. This possibility would also provide a unifying mechanism to explain the paradox where both under and overexpression of the splicing kinase might be oncogenic.

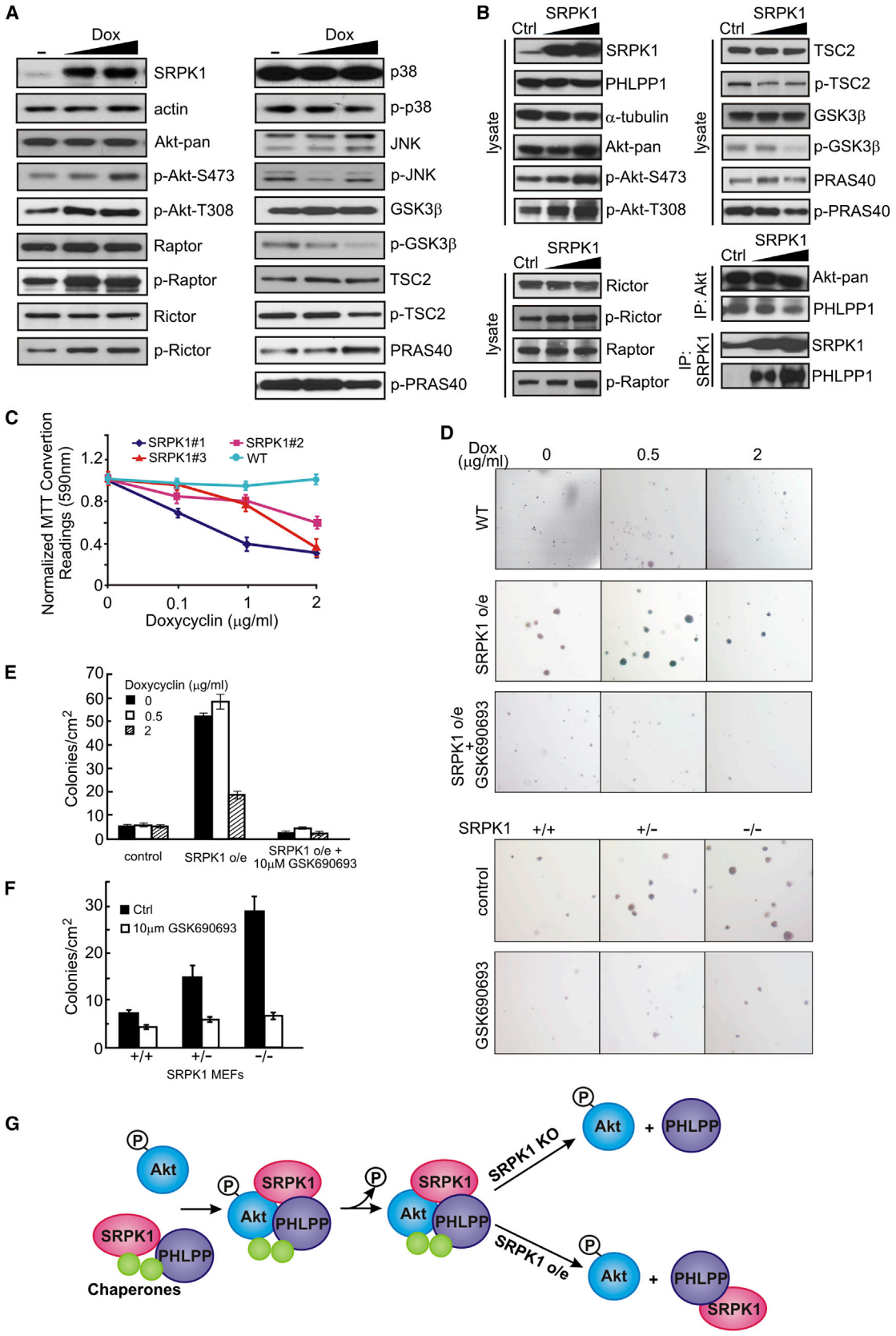
To test this possibility, we engineered an *SRPK1* expression unit under a tetracycline-inducible promoter. Indeed, SRPK1 overexpression was also able to induce Akt phosphorylation at T308 and S473 as well as activation of Raptor and Rictor (Figure 6A, left). Interestingly, while there was no effect on the p38 and JNK pathways, the immediate Akt targets were progressively suppressed, a response similar to that after SRPK1 knockout (Figure 6A, right panel). We made similar observations on HEK293T cells, suggesting a general response regardless of cell type (Figure 6B). We next asked whether overexpressed SRPK1 could sponge PHLPP1, thereby preventing the association of the Akt phosphatase with Akt. We found that, while the overall level of PHLPP1 remained constant in transfected HEK293T cells, the association of PHLPP1 with Akt was progressively reduced, and conversely, the association of the Akt phosphatase with SRPK1 was gradually increased (Figure 6B, both right panel). These data strongly suggest the ability of overexpressed SRPK1 to titrate PHLPP1 away from Akt.

SRPK1 Overexpression Promotes Anchorage-Independent Cell Growth

Our previous studies showed that transient overexpression of SRPK1 alone was inhibitory to cell growth, especially after translocation of an excessive amount of the kinase to the nucleus (Ding et al., 2006), which we also confirmed on MEFs with three separate SRPK1 overexpression MEF lines in the presence of increasing doses of Dox (Figure 6C). Notably, however, the engineered MEFs appear to express an elevated level of SRPK1 even in the absence of Dox, and a modest increase in SRPK1 expression with low levels of Dox did not seem to severely compromise cell growth. We took this window of opportunity to determine whether SRPK1 overexpression (from line #2) was able to generate some recordable oncogenic effects. By placing the cells on soft agar, we indeed detected a significant increase in anchorage-independent cell growth, although cell growth was inhibited when treated with a higher dose of Dox (Figures 6D and 6E).

Having observed the induction of anchorage-independent growth by both *SRPK1* knockout and overexpression and linked both to Akt activation, we next asked whether Akt activation is essential for the detected oncogenic property. We repeated the soft agar assay on both *SRPK1* knockout and overexpressing MEFs in the presence of the Akt inhibitor GSK690693, finding that the drug potently inhibited the induced anchorage-independent growth under both conditions (Figures 6D–6F). We thus conclude that Akt activation is essential for conferring the oncogenic property in both *SRPK1* knockout and overexpressing MEFs.

Collectively, our results suggest a model where SRPK1 provides a key regulatory function in Akt activation (Figure 6G): the Akt-specific phosphatase PHLPP1 may be dynamically partitioned among various protein complexes, including those



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associated with an SRPK, which is also known to contain various molecular chaperones (Zhong et al., 2009a; Zhou et al., 2012). When activated Akt is engaged with an SRPK in such a complex, a PHLPP is responsible for Akt inactivation within the complex. SRPK deficiency would cause inefficient recruitment of a PHLPP, whereas excessive SRPK would titrate the PHLPP away from Akt. Therefore, aberrant SRPK expression in either direction would induce constitutive Akt activation and cellular transformation.

SRPK1 Overexpression Is Tightly Linked to Akt Activation in Human Colon Cancers

Through basic research on cell lines and on nude mice, our data establish that dysregulated SRPK1 expression is oncogenic. To obtain such evidence on human cancers, we again turned to colon cancers, as a previous study showed that ~80% of this cancer type is linked to Akt activation, and interestingly, activated Akt is largely distributed in the cytoplasm, rather than at the cell surface, suggesting multiple mechanisms that may be responsible for Akt activation (Rychahou et al., 2008). Strikingly, from a total of 47 colon cancer tissues, we found that 19 (40%) showed both strong SRPK1 expression and Akt activation (based on p-Akt-S473 signals), as exemplified in Figures 7A–7C. If some modest, but still significant, signals were also considered, this number went up to 83% (Figure 7G). In comparison, little SRPK1 overexpression or Akt activation was evident in normal sigmoid colon tissues (Figure 7E) and normal rectum tissues (Figure 7F). Interestingly, we also identified one strong (Figure 7D) and three modest (Figure 7G) cases for negative correlation, i.e., Akt activation linked to little SRPK1 expression. These observations are entirely consistent with the induction of Akt activation by either SRPK1 overexpression or downregulation, although SRPK1 overexpression appears quite predominant among colorectal cancers.

DISCUSSION

While largely unexpected in the initial phase of this study, the findings in the end tie together diverse biological phenotypes and biochemical responses to a unified molecular mechanism. As SRPK1 is either downregulated or overexpressed in human cancers, our results provide critical insights into its tumor suppressing and promoting functions, which is converged on constitutive Akt activation. The Akt kinase system is known to be widely involved in the regulation of cell proliferation, transformation, and apoptosis in mammalian cells. While Akt activation by upstream signaling events has been well elucidated, relatively little is known about its attenuation in normal cells and sustained activation in cancer cells. Our data now elucidate a key role of SRPK1 in the recruitment of the Akt phosphatase PHLPP1 for Akt attenuation.

A Suicidal Mission for Akt to Activate the Splicing Kinase

SRPK1 and SRPK2 have been shown to act as downstream targets for activated Akt to transduce growth signals to the nucleus to activate key cell-cycle genes (Jang et al., 2009) and induce widespread changes in alternative splicing (Zhou et al., 2012). Only activated Akt is able to form complexes with an SRPK in this process. For SRPK1, the interaction with Akt triggers its autophosphorylation, thereby inducing rearrangement with various molecular chaperones to facilitate its subsequent nuclear translocation. We now show that the formation of such complexes is also a key event for Akt inactivation. If Akt were not released fast enough from an SRPK-containing complex, a recruited PHLPP would rapidly inactivate Akt within the complex. The elucidated mechanisms for both SRPK1 activation and Akt inactivation within such complex thus suggest a strategy for Akt to transduce a signal, and in the same time receive a feedback control, which is fundamentally distinct from typical kinase-substrate interactions.

At this point, we do not know how exactly a PHLPP is recruited to the Akt-SRPK1 complex. Preliminary immunoprecipitation (IP)-mass spectrometric (MS) experiments suggest the association of numerous cellular proteins with a hemagglutinin (HA)-tagged SRPK1, including multiple molecular chaperones we characterized earlier (Zhong et al., 2009a). As the kinase-dead SRPK1 appears to be able to attract more PHLPP (both PHLPP1 and PHLPP2) to the complex, the recruitment of a PHLPP likely occurs before SRPK1 autophosphorylation-induced rearrangement of the complex. Future experimentation will address whether PHLPP interacts with SRPK1 directly or via its associated molecular chaperones by characterizing the SRPK1 proteome and protein-protein interaction networks within the SRPK1-containing complex.

Multiple Feedback Control Mechanisms in Response to Altered SRPK1 Expression

The Akt pathway is notorious for complex feedback controls in mammalian cells (Carracedo and Pandolfi, 2008). It is well known that activated mTOR, a key downstream effector in the Akt

Figure 6. PHLPP1 Squelched from Akt by Overexpressed SRPK1

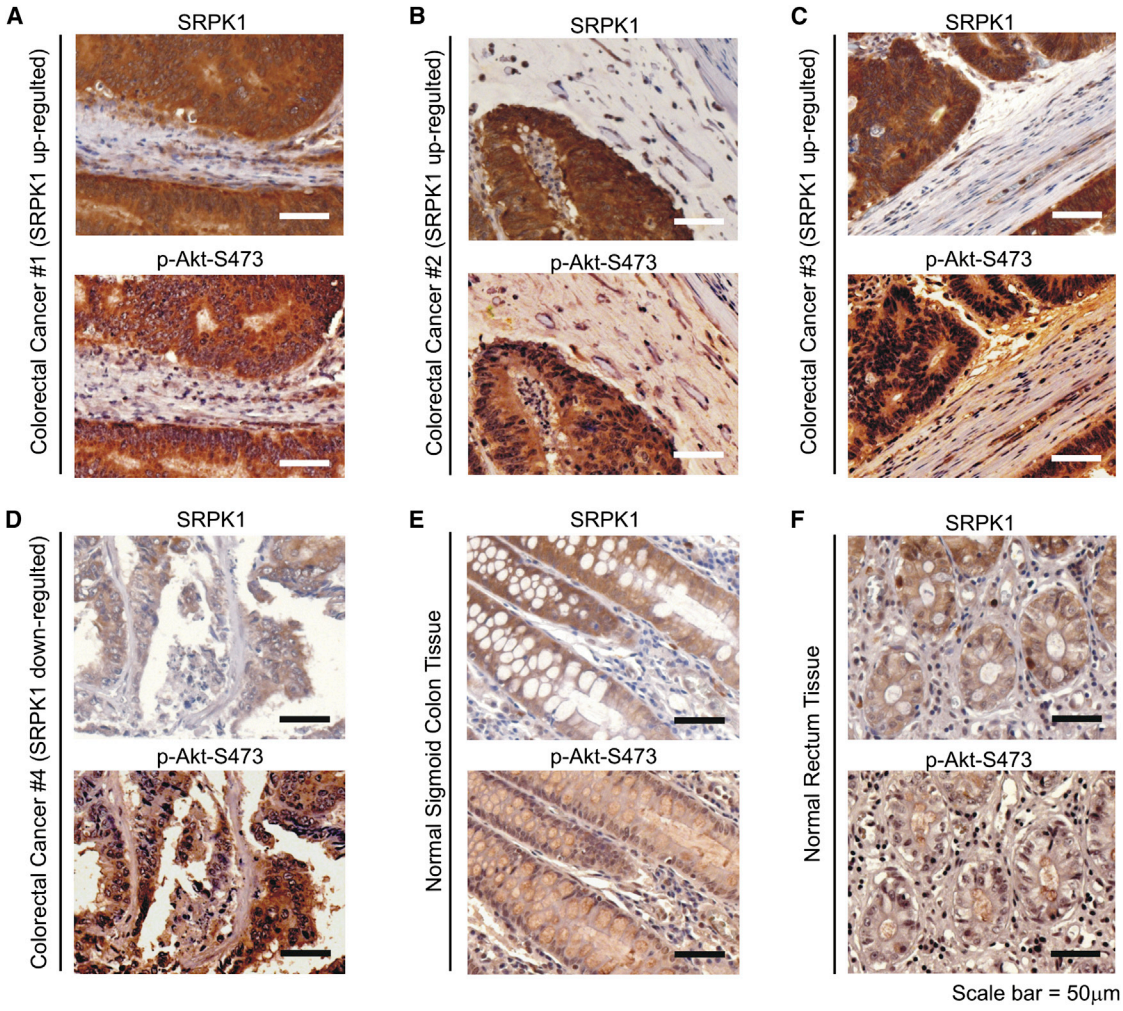
(A) Overexpression of SRPK1 in doxycycline (Dox)-treated MEFs, resulting in the activation of Akt, Raptor, and Rictor (left). In contrast, no effects were detected on the p38 and JNK pathways, and the immediate Akt substrates were somewhat repressed, similar to the responses to *SRPK1* knockout, as shown in Figure 5B.

(B) Similar analysis and responses on HEK293T cells as in (A) on MEFs. Importantly, PHLPP1 showed a progressive decrease in the Akt immunoprecipitant but an increase in the SRPK1 immunoprecipitant (bottom right panel).

(C) Cell growth detected by MTT assays on wild-type MEFs and three independent SRPK1-overexpressing MEF lines, showing progressive inhibition of cell proliferation in response to increasing doses of doxycycline (DOX).

(D–F) Anchorage-independent cell growth induced by either SRPK1 overexpression or knockout. Relative to mock-treated MEFs (row 1), modest SRPK1 overexpression (row 2) induced colony formation on soft agar. The Akt-specific inhibitor GSK690693 abolished the induction of anchorage-independent growth induced by SRPK1 overexpression (row 3), which also suppressed colony formation with SRPK1 null MEFs (compared between rows 4 and 5). The data in (D) for rows 1–3 were quantified and shown as mean ± SD in (E), and the data in (D) for rows 4 and 5 were quantified and shown as mean ± SD in (F).

(G) Model for constitutive activation of Akt in response to under- or overexpression of SRPK1. When SRPK1 is low, PHLPP may not be efficiently recruited to Akt, but when SRPK1 is high, PHLPP may be titrated away from Akt, thus resulting in Akt activation when SRPK1 is aberrantly expressed in either direction.



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		SRPK1 ++	SRPK1 +	SRPK1 –
p-Akt-S473	++	19	0	1
	+	12	8	3
	–	2	1	1

Figure 7. SRPK1 Expression and Akt Activation in Human Colon Cancer Tissues

(A–C) Three representative pairs of human colon cancers (Colorectal Cancer #1, A; Colorectal Cancer #2, B; and Colorectal Cancer #3, C), showing significant elevation of both SRPK1 overexpression and Akt activation in cancer cells. Immunohistochemistry was carried out with the VECTASTAIN ABC Kit and HRP-conjugated secondary antibody in the presence of the peroxidase substrate DAB and counterstained by hematoxylin.

(D) An example of colon cancer with strong Akt activation but little SRPK1 expression.

(E and F) Examples of SRPK1 expression and p-Akt-S473 levels in normal sigmoid colon tissue (E) and normal rectum tissue (F).

(G) Summary of colorectal cancer tissue array results from a total of 47 colon cancer tissues examined. ++, strong positive; +, positive; –, weak or negative.

pathway, is able to induce suppression of multiple Akt downstream targets (Shah et al., 2004). Indeed, we observed the same responses in both SRPK1 knockout and overexpressing cells. Interestingly, the mTOR downstream targets were more resistant to such feedback regulation. We provided initial evidence that compromised AMPK might be one of the key pathways for maintaining active mTOR in *SRPK1* null MEFs, but this ATP-sensing kinase still showed comparable activity in wild-type and *SRPK1*$^{+/-}$ MEFs. However, we noted early on that the effect of heterozygous *SRPK1* deletion is more variable

in SR protein phosphorylation, as were the induced feedback responses. In any case, it is likely that additional feedback and feedforward mechanisms might be triggered in SRPK1-deficient cells to account for sustained mTOR activation after active TSC2 was quickly attenuated during EGF treatment.

In fact, based on our findings, we may envision one of such positive feedforward mechanisms for Akt activation in SRPK1-deficient cells where elevated SRPK1 expression may first induce Akt activation by interfering with PHLPP recruitment, after which activated Akt then gains the ability to bind and activate more SRPK1. This mechanism may account for such a tight correlation between SRPK1 overexpression and Akt activation in human colon cancers.

Oncogenic and Tumor Suppressing Function of SRPK1

Similar to many signal molecules that appear to have both tumor suppressing and promoting functions in different cellular contexts (Feng, 2012), our results suggest that SRPK1 also has such dual roles, but within the same cells. Given its essential function for embryonic development, we were initially surprised to see such a tumor suppressor phenotype on MEFs, especially when heterozygous deletion of SRPK1 showed partial activation of Akt activation, cellular transformation, and tumor growth in nude mice. This raises the potential for coupling partial loss of function of SRPK1 with defects in other signal transduction pathways to jointly promote tumorigenesis, a possibility to be systematically investigated on animal models in the future.

The ability of SRPK1 to squelch an Akt-specific phosphatase also provides mechanistic insights into the biological consequence of SRPK1 overexpression in many human cancers. Although augmented expression of SRPK1 in primary cells (MEFs) is inhibitory to cell growth, which may be related to the observed premature mitosis induced by overexpressed SRPK2 in neurons (Jang et al., 2009), we were able to detect a significant gain of anchorage-independent growth with modest SRPK1 overexpression, suggesting a degree of cellular transformation. In real tumors, SRPK1 overexpression may be coupled with other defects in cell-cycle checkpoints, thus synergistically promoting tumorigenesis. Once such interdependency is established, SRPK1 may even become essential for multiple oncogenic properties of the tumor, which may even include Akt activation, as indicated by a recent SRPK1 overexpression/knockdown study on a human hepatocellular carcinoma cell line (Zhou et al., 2013).

Synergizing Aberrant SRPK1 Expression with Other Tumorigenic Events

Although Akt activation is essential for some oncogenic properties of SRPK1-deficient cells, it is likely that this is also coupled with other distinct activities induced by under- and overexpression of SRPK1 to promote tumorigenesis. For example, SRPK1 deficiency causes hypophosphorylation of SR proteins, which is known to enhance translation in the cytoplasm (Sanford et al., 2004, 2005). This may synergize with activated mTORC1 to increase protein synthesis in cancer cells.

Compared to SRPK1 deficiency-induced tumorigenic events, SRPK1 overexpression may be coupled with a different set of cellular pathways. In fact, Akt activation has been long suggested to induce SR protein hyperphosphorylation to promote cellular transformation (Liu et al., 2003). More recently, SRPK1 was found to be overexpressed in Wilms' tumors, where SRPK1 is transcriptionally repressed by the tumor suppressor gene *WT1*, and derepressed SRPK1 in *WT1* mutant cells induces SRSF1 phosphorylation and nuclear translocation, leading to the increased production of proangiogenic $VEGF_{165}$ (Amin et al., 2011). Therefore, dysregulation of SRPK1 may fundamentally alter diverse pathways in RNA metabolism, which may synergize with activated Akt to induce cellular transformation and promote tumorigenesis.

EXPERIMENTAL PROCEDURES

Generation of Conditional SRPK1 Knockout Mice and MEFs

Specific restriction fragments containing *SRPK1* genomic sequences were isolated from a mouse 129SV/J λ clone and cloned into the pBKSII vector (Figure S1A) as previously described (Wang et al., 2001; Ding et al., 2006). Characterization of knockout mice, development of corresponding MEFs, and various biochemical and computational assays, including western blotting, immunoprecipitation, RNAi, measurements of kinase and phosphatase activities, and analysis of published gene expression profiling data, were detailed in Supplemental Experimental Procedures.

Assays for Cell Senescence, Anchorage-Independent Cell Growth, and Tumor Development in Nude Mice

SRPK1 MEFs (~1×10^4) with different genotypes (+/+, +/−, or −/−) were seeded in 12-well plates in triplicates, and cells were stained 8 days post-transduction for senescence-associated β-gal activity using X-gal solution, as described previously (Serrano et al., 1997). For anchorage-independent growth, ~5,000 cells were resuspended in the culture media containing 0.32% low-melting-point agarose and plated onto a solidified bottom layer containing 0.53% agarose in 6-well plates. Colonies were stained with crystal violet, photographed, and counted after 2–4 weeks. For tumor formation assay, 2×10^6 MEFs of different genotypes were subcutaneously injected into the flanks of 6-week-old female HSD:athymic nude mice in 100 μl of serum-free Dulbecco's modified Eagle's medium (DMEM). Tumor growth was monitored weekly for 6 weeks.

SILAC Analysis

To analyze changes in global phosphorylation profile, MEFs were cultured in DMEM plus 10% dialyzed fetal bovine serum (FBS) supplemented with either isotopically heavy (on WT MEFs) or light (on *SRPK1*$^{-/-}$ MEFs) lysine and arginine, respectively, for at least five doublings to ensure complete isotopic labeling of all cellular proteins. Afterward, cells were serum starved for 16 hr followed by serum stimulation for 15 min. After the treatment, cells were lysed to extract proteins, followed by extensive trypsin digestion to yield peptides. Phosphopeptides were purified using immobilized metal affinity column and analyzed by the LC-MS/MS method as described (Chen et al., 2010). MS/MS data were analyzed using SEQUEST to identify phosphopeptides, which were quantified using the XPRESS software based on the human protein database, as previously detailed (Chen et al., 2010).

Analysis of Primary Tumor Samples

Pairs of colon tumors and adjacent nontumorous tissues were obtained from anonymized excess colon tissues from the University of Maryland Medical Center and Baltimore Veterans Affairs Medical Center that were no longer needed for diagnostic or clinical purposes. Colorectal cancer and normal colon tissue slides were purchased from Imgenex. Immunohistostaining was performed with the VECTASTAIN ABC Kit (Vector Laboratories) and HRP-conjugated secondary antibody in the presence of the peroxidase substrate DAB. Use of these tissues was approved by the University of Maryland Medical Center and Baltimore Veterans Affairs Medical Center Human Studies Committees and by the NIH Office of Human Subjects Research.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.03.007.

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