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RSK in Tumorigenesis: Connections to Steroid Signaling

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Abstract

The Ser/Thr kinase family, RSK, has been implicated in numerous types of hormone-dependent and -independent cancers. However, there has been little consideration of RSKs as downstream mediators of steroid hormone non-genomic effects or of their ability to facilitate steroid receptor-mediated gene expression. Steroid hormone signaling can directly stimulate the MEK/ERK/RSK pathway to regulate cellular proliferation and survival in transformed cells. To date, multiple mechanisms of RSK and steroid hormone receptor-mediated proliferation/survival have been elucidated. For example, RSK enhances proliferation of breast and prostate cancer cells via its ability to control the levels of the estrogen receptor co-activator, cyclin D1. While in lung and other tumors RSK may control apoptosis via estrogen-mediated regulation of mitochondrial integrity. Thus the RSKs could be important anti-cancer therapeutic targets in many different transformed tissues. The recent discovery of RSK-specific inhibitors will advance our current understanding of RSK in transformation and drive these studies into animal and clinical models. In this review we explore the mechanisms associated with RSK in tumorigenesis and their relationship to steroid hormone signaling.

Keywords

MAPK pathway; p90 ribosomal S6 kinase; estrogen receptor; androgen receptor; tumorigenesis

Introduction

Steroid hormone-activated receptors regulate gene transcription by directly binding to DNA. They can also drive transcription and other cellular processes via “extra-nuclear” non-genomic effects (reviewed in [1]). These non-genomic effects have been shown to regulate cancer cell proliferation via multiple signaling pathways including the phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase 1/2 (ERK1/2) (also called p42/p44 mitogen activated protein kinase (MAPK)) pathways [2]. Estrogen exposure rapidly activates the ERK1/2 pathway in tumor cells via stimulation of p21ras and activation of the tyrosine kinase c-Src [2,3]. Ligand-bound Estrogen Receptor (ER) complexes with c-Src, resulting in phosphorylation of Shc and p190, and promoting interaction with the additional adaptor molecules, MNAR and Cas. Shc phosphorylation engages the upstream components of the ERK1/2 pathway, Grb and SOS, and activates Ras-mediated ERK1/2 signaling [1,3]. The ribosomal S6 kinase (RSK) family of Ser/Thr kinases are downstream effectors of ERK1/2.

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Though not well studied, our emerging knowledge of steroid signaling through the RSKs suggests this pathway may be an important contributor to steroid-mediated tumorigenesis (Figure 1).

The ERK1/2 cascade has long been considered a viable source of cancer treatment targets for both hormone-dependent and independent tumors. Consequently, several MAP/ERK kinase (MEK) and Raf inhibitors have been developed and undergone clinical trial evaluation. As yet, targeting these upstream components of the signaling pathway has not been very successful in the clinic [4]. Additionally, inhibiting these “global regulators” produces significant patient side effects. However, the downstream effectors of the ERK1/2 pathway represent an untapped pool of potential therapeutic targets. These targets control a limited set of downstream effectors compared to master regulators like Raf, MEK, and ERK1/2 and are therefore, less likely to mediate severe side effects. Additionally, because they control fewer downstream pathways it is possible they regulate fewer of the feedback loops shown to reduce the efficacy of Raf and MEK inhibitors [5–9]. The RSKs are one such group of downstream mediators of the ERK1/2 pathway. The RSKs are known to regulate proliferation and survival in a variety of cancer cell lines and are found to be overexpressed or hyperactivated in some human cancers [10–15]. Furthermore, RSK has been found to inhibit apoptosis by protecting mitochondrial integrity [16]. Therefore, further investigation of RSK as a potential anti-cancer target would seem warranted.

There are four RSK isoforms, each the product of a different gene. RSK1-4 possess 73–80% amino acid identity and have the same general structure (reviewed in [11]). The RSKs are unusual in that they possess two distinct functional kinase domains (N-terminal; NTKD and C-terminal; CTKD) connected by a linker region. The sequence differences between the RSK isoforms are found in the extreme termini and the linker region. Thus, the specific functions of the individual isoforms may be due to unique sequences in those regions.

In general, RSK is activated by ERK1/2 phosphorylation, which stimulates autophosphorylation resulting in recruitment of 3′-Phosphoinositide Dependent Kinase 1 (PDK1) and subsequent activation of the NTKD. The NTKD is responsible for the phosphorylation of exogenous substrates (Figure 2). The RSK isoforms are activated by the same general mechanism [11]. However, RSK4 is thought to be constitutively active in most tissues [17]. The isoforms have both unique and overlapping functions [10,11]. RSK1 and RSK2 are the best characterized isoforms. While there are many putative RSK substrates, relatively few have been confirmed using small interfering RNA/short hairpin RNA (siRNA/shRNA) technologies, RSK null-animals or specific inhibitors (Table 1). Confirmed RSK substrate functions can be grouped into three categories—proliferation, survival, and migration (Table 2). The RSKs have diverse functions and substrates in multiple types of human cancer [12,14,18–24]. Based on these observations it is reasonable to hypothesize that increased RSK activity mediates transformation. Recently, Cho et al. tested this hypothesis and observed that RSK2 regulates anchorage-independent growth of mouse epidermal JB6 C141 cells and of Ras-transformed NIH 3T3 fibroblasts [21].

To date, there has been little consideration of the RSKs as downstream mediators of steroid hormone non-genomic effects or of their ability to facilitate steroid receptor-mediated gene expression. Therefore, in this review we will explore the mechanisms associated with RSK in tumorigenesis and their relationship to steroid hormones and their receptors. We will present and discuss the evidence that RSK contributes to hormone-linked tumorigenesis in specific tissues. We will also discuss RSK-mediated tumorigenesis in tissues wherein our understanding of steroid hormone contributions is currently emerging, but is not yet clear. Perhaps by analyzing mechanisms of RSK and steroid hormone signaling in these tumors, we will open up new avenues of investigation and importantly, identify new therapeutic targets.

RSK in Breast Cancer

RSK has been shown by numerous groups to be a key regulator of breast cancer proliferation. Our lab was the first to report the importance of RSK in breast cancer proliferation together with the discovery of the first RSK-specific inhibitor, SL0101 [14]. We found that RSK2 is over-expressed in 50% of human breast cancer tissues, compared to normal tissue [14]. Since then many other groups have reported similar findings in breast and other tumor models. Law et al. recently showed that inhibition of the insulin-like growth factor 1 receptor (IGF-1R)/ insulin receptor (IR) reduced proliferation and RSK activity in tamoxifen-resistent MCF-7 cells [13]. In human breast tumors high levels of the phosphorylated RSK substrate ribosomal protein S6 (rpS6), were directly correlated with IGF-1R/IR levels, and were associated with poor patient survival [13]. Thus, high levels of activated RSK promote tumorigenesis. Furthermore, ~56% of human breast tumors showed phosphorylated rpS6 indicating that RSK may be highly active. The ability of RSK to phosphorylate rpS6 has been controversial but recent studies have shown that rpS6 phosphorylation at Ser-235/236 is MAPK-dependent and has been attributed to RSK [25]. RpS6 is one of many RSK substrates associated with proliferation and tumorigenesis (Table 2).

The ability of RSK to regulate survival, anchorage-independent growth, and transformation in breast cancer was recently confirmed by Xian et al [15]. Their findings indicate that Fibroblast Growth Factor Receptor-1 (FGFR1)-mediated transformation of MCF-10A cells is dependent on RSK. FGFR1 is upregulated in many invasive lobular carcinomas (ILC), which, while ER +, may not respond very well to the ER antagonist, tamoxifen [26]. Inhibitors of FGFR1 can suppress growth of the ILC cell line, MDA-MB-134, suggesting that FGFR1 signaling is essential for proliferation of these tumors. Knockdown of RSK1 and inhibition of RSK, with the specific small molecule inhibitor chloromethylketone (CMK), reduced proliferation, suppressed colony formation in soft agar, and decreased survival of FGFR1-expressing cells. Together these data strongly support the hypothesis that the RSKs are important in breast cancer etiology.

RSK4 is a putative tumor suppressor in breast cancer [19,27]. Thakur et al., found that overexpressing RSK4 in MDA-MB-231 cells suppressed colony formation in soft agar, tumor formation, and metastasis. Curiously, RSK4 levels were found to be elevated in MMTV-*c-Myc* transgenic mice [27,28]. Expression of myc, a cell cycle regulator [29,30], is upregulated very quickly following estrogen treatment and is essential for estrogen-mediated proliferation in breast cancer cells [31,32]. Mammary tumors that form in *c-Myc* transgenic mice are neither invasive nor metastatic and it is hypothesized that c-myc overexpression upregulates RSK4, which then suppresses aggressive expansion [27]. Consistent with this hypothesis, c-myc was shown to stimulate RSK4 promoter activity in a luciferase reporter assay [27]. Our knowledge of RSK4 remains limited. RSK4 may have tumor suppressor functions in some cancer types, but the paucity of data on this kinase suggests that further studies are necessary before specific conclusions can be drawn.

The growing body of literature implicating RSK in breast cancer supports the hypothesis that RSK is an important therapeutic target. We have found that treatment with the RSK-specific inhibitor, SL0101 (20 μM; 48hr, reduced proliferation in the immortalized human breast cancer cell line, MCF-7, but did not effect proliferation of the non-tumorigenic breast cell line, MCF-10A (Figure 3A, [14]). Consistent with these findings, silencing RSK2 also reduced proliferation in MCF-7 cells. The mechanism by which RSK2 regulates proliferation in breast cancer cells is not well understood. However, significant evidence is emerging that indicates RSK regulates several key breast cancer-associated proteins. For example, we have found that RSK2 stimulates the transcriptional activity of estrogen receptor α (ERα) [33–36] which is known to be important in the etiology of many breast cancers. Estrogens can stimulate RSK

activity, and RSK2 enhances ERα-mediated transcription by phosphorylation and by physical association [33]. The interaction of ERα and RSK can be disrupted by tamoxifen. This process may be dependent on the ERK1/2 pathway. Additionally, we have found that RSK2 regulates expression of the oncogene, cyclin D1, which is a co-activator of ERα and overexpressed in approximately 50% of human breast tumors [37, 38]. The importance of cyclin D1 as an oncogene is highlighted by the finding that overexpression of the protein is sufficient to induce formation of mammary tumors in transgenic animals [39]. Although the ERK1/2 pathway is known to regulate cyclin D1 levels, we identified that cyclin D1 is a key RSK2 target in breast cancer cells [38]. Consistent with findings in human tissue, we found that MCF-7 cells overexpress cyclin D1 as compared to MCF-10A cells by approximately 5-fold based on normalization to the housekeeping protein, Ran (Figure 3B). SL0101 (50 μM; 4hr reduced cyclin D1 levels in MCF-7 cells by 70% at the protein level and 40% at the mRNA level (Figure 3C, [38]). Importantly, SL0101 did not affect cyclin D1 expression in MCF-10A cells (Figure 3C) suggesting that RSK regulation of cyclin D1 is confined to transformed cells. SL0101 inhibits the kinase activity of RSK1 and RSK2 in *in vitro* kinase assays, but RSK2 is primarily responsible for the regulation of cyclin D1 levels [38]. We also found forced nuclear localization of RSK2 drives cyclin D1 expression in the absence of activation of any other signal transduction pathway [38]. These results suggest that nuclear RSK2 is able to act as an oncogene in breast cancer.

We have also identified a mechanism by which RSK regulates mRNA localization and translation via stress granules in breast cancer cells [38]. Normal mammary and breast cancer cells form cytoplasmic RNA complexes called stress granules under either oxidative stress or serum-deprivation stress. In general, stress granules form under conditions in which translation initiation has been reduced or inhibited [40]. These granules recruit selected mRNAs and associated proteins from polyribosomes, for storage, or for triage through processing bodies [41]. Stress granules are thought to aid cell survival by acting as sites of translational repression and to facilitate post-stress recovery by acting as reservoirs of poly(A)$^+$ RNA. In breast cells subjected to stress, endogenous RSK2 localizes to stress granules and controls recruitment of other key stress granule proteins in the complex [38]. In response to stress, RSK interacts directly with the essential stress granule protein, TIA-1, driving stress granule formation. This regulation is physiologically important, because loss of RSK2, via specific knockdown or inhibition, prevents stress granule formation and decreases cell survival in response to stress. In nutritionally-stressed breast cancer cells, addition of mitogen triggers the dissolution of stress granules. Once released from sequestration in the granules, RSK2 accumulates in the nucleus, where it induces cyclin D1 expression in transformed cell lines, driving entry into the cell cycle. RSK2 has not previously been implicated as a regulatory component in the stress response and RSK2-mediated regulation of translation and cellular stress are understudied. However, there is now a significant amount of evidence suggesting that the involvement of RSK2 in translation may be crucial for understanding its role in tumor cell survival. In addition, several recent studies suggest that stress granule formation protects tumor cells from chemotherapy and radiation-induced stress [42,43]. Interestingly, cyclin D1 mRNA has been found in stress granules [44] suggesting that this mechanism may effectively protect mRNAs necessary for proliferation in breast cancer. RSK2 is an essential regulator of stress granules in breast cancer cells, and therefore RSK2 inhibition may increase tumor cell death in response to standard treatments.

Another mechanism by which RSK may regulate breast cancer cell proliferation is through phosphorylation of the transcription factor YB-1 at Ser-102 [45]. YB-1 regulates expression of numerous proteins associated with tumorigenesis via direct interaction with the promoter regions of target genes [46,47]. Not only is YB-1 overexpressed in breast and other cancers, but overexpression of YB-1 is sufficient to drive mammary tumor formation in mice [45,48–51]. Conversely, knockdown of YB-1 suppresses tumor cell proliferation [49]. Phosphorylation

of YB-1 at Ser-102 is essential for nuclear translocation of the protein as well as the interaction of YB-1 with target genes [52]. Therefore, RSK-dependent phosphorylation at Ser-102 may be essential for YB-1 function. Interestingly, YB-1 nuclear localization and ERα regulation appear to be connected [53]. However, the mechanism and physiological outcome of this relationship are not yet clear. The observation that RSK participates in ERα and YB-1 signaling suggests that the potential connection between YB-1 and ERα may be mediated by RSK.

RSK2 and Prostate Cancer

We found that RSK2 is over-expressed in ~50% of prostate cancer tissues compared with normal tissue and benign prostate hyperplasia [12]. RSK inhibition via SL0101 (20 μM; 48hṛ reduced proliferation in LNCaP and PC3 prostate cancer cell lines but not in the untransformed prostate cell line, RWPE1 (Figure 4A). SL0101 (50 μM; 4hṛ also reduced cyclin D1 levels in prostate cancer cell lines compared to untransformed cells (Figure 4B). These results are consistent with those observed in breast cell lines. An isoform of cyclin D1 has been found to be overexpresed in prostate cancer and has been shown to stimulate proliferation in prostate cancer cells [54]. We have also found cyclin D1 to be overexpressed in LNCaP and PC3 prostate cancer cells as compared to RWPE1 cells (Figure 3B). Thus cyclin D1 appears to be implicated in the transformation of prostate and breast cells. RSK2 indirectly regulates androgen receptor (AR)-mediated transcription in LNCaP cells [12]. The AR is known to be important in the etiology of prostate cancer [55,56]. Therefore, RSK regulates both AR and ERα in cancer suggesting a relationship between RSK and general steroid-receptor signaling.

RSK3 and Ovarian Cancer

Ovarian cancers are among the most lethal malignancies in women [57]. Interestingly, 40%–60% of ovarian tumors express ERα but less than 20% of tumors respond to anti-estrogen treatments in the clinic [58–60]. These observations suggest that growth factor-activated pathways like the ERK1/2-RSK pathway, rather than genomic hormone signaling, may be important in some ovarian tumors [61,62]. Non-genomic hormone-mediated signaling is thought to play a role in growth factor pathways via the orphan G-protein coupled receptor 30 (GPR30) [63]. In ovarian cancer cells estrogens and G1, the GPR30 agonist, were shown to activate the EGFR pathway, resulting in upregulation of c-Fos expression. RSK is a key regulator of c-Fos levels and function [64,65], which suggests a link between estrogen signaling and RSK-mediated c-Fos activity in ovarian cancer.

In contrast to standard view of RSKs as tumor promoters, Bignone et al. found that RSK3 suppresses growth in multiple ovarian cancer cell lines [18]. Overexpression of RSK3 reduced proliferation and colony formation in soft agar compared to a control. RSK3 levels were found to be high in normal ovarian tissue and decreased in cancer cell lines and in $\geq$ 50% of the sporadic human tumors of various stages and grades. These findings suggest that RSK3 may function as a tumor suppressor in some ovarian cancer. However, given our limited knowledge of RSK3, more studies analyzing RSK3 function are necessary.

RSK2 and Multiple Myeloma

ERα and ERβ are both expressed in multiple myeloma cell lines [66]. The exact role of these receptors in multiple myeloma is not known, but interestingly, these tumor cells undergo apoptosis following exposure to anti-estrogens like tamoxifen [66–68], suggesting a role for estrogen signaling in multiple myeloma. Furthermore, a recent analysis of gene expression in plasma cells from 74 multiple myeloma patients showed a significant increase in *CCND1* levels [69]. *CCND1*, which encodes the oncogene cyclin D1, is regulated, in part, by estrogens [70] suggesting that estrogen signaling may promote myeloma via cyclin D1 expression. As

discussed above, RSK2 is a key regulator of cyclin D1 expression in some cancer cell lines. In addition to elevated *CCND1* levels, increased expression of the fibroblast growth factor receptor 3 (FGFR3) was found in plasma cells of multiple myeloma patients [69]. Mutations in FGFR3, a receptor-tyrosine kinase, occur in approximately 15% of multiple myeloma cases [71]. Constitutively active FGFR3 was shown to phosphorylate RSK2 at Tyr-529 in Ba/F3 cells, a murine pro-B cell line, enhancing RSK interaction with inactive ERK1/2 [72]. This association is thought to increase the subsequent activation of RSK2 by ERK1/2 [73]. Inhibition of RSK with the inhibitor fluoromethyl ketone (fmk) induced apoptosis in FGFR3- expressing primary and immortalized human myeloma cells. In subsequent studies this group has shown that FGFR3 interacts with RSK2 at Trp-332, enhancing both Tyr-529 and Tyr-707 phosphorylation and promoting RSK activation [74,75] (Figure 2). In FGFR3 transformed bone marrow transplantation studies, survival of animals with RSK2-depleted marrow was prolonged compared with control animals. Together, these findings suggest that RSK2 plays a significant role in hematopoietic transformation. The contribution of estrogen signaling in multiple myeloma is not clear. However, the role of estrogens in bone cells is becoming increasingly well understood [76]. Estrogen signaling has a protective effect on osteoblasts, mediated in part via regulation of essential cytokines, thus supporting healthy bone formation [77]. Additionally, RSK2-mediated phosphorylation of activating transcription factor 4 (ATF-4) promotes osteoblast proliferation [78]. Perhaps, some of the RSK-dependent transformation effects in hematopoetic cells are mediated by hormone signaling.

Activation of RSK via Tyr-529 may be mediated by estrogen signaling in some cells. In 293T and COS7 cells, which do not express FGFR3, RSK2 is phosphorylated at Tyr-529 by Src family kinases, Src and Fyn, as determined by *in vitro* kinase assay [79,80]. Src family kinases have been implicated in the development of multiple human cancers including those associated with RSK, which include breast, prostate, lung, melanoma, ovarian, and gastric cancers [81]. In response to estrogen, Src is activated via its interaction with ERα and the scaffold protein MNAR [2]. Thus, activation of Src by estrogen may enhance RSK2 activity in some tumors. Activation of RSK by Src has yet to be investigated in any tumor type.

RSK in Non Small Cell Lung Cancer (NSCLC)

Expression of ERα and ERβ have been shown, in multiple studies, to be elevated in human lung tumors [82–85]. These observations suggest that estrogens might play a role in lung cancer development and may explain why female non-smokers have a higher risk of developing lung adenocarcinomas than male non-smokers [82]. Estrogen as a proliferative driving force in lung cancer is supported by observations that estrogen promotes tumor progression in mouse models of lung adenocarcinoma [86].

RSK has been implicated in lung cancer cell survival, via an anti-apoptotic mechanism. Several groups have shown that RSK activation or overexpression inhibited cell death via inactivation of the Bcl-2 homology 3-only proapoptotic protein, Bad [16,87,88]. RSK directly phosphorylated Bad at Ser-75 (Ser-75; human, Ser-112; mouse), which resulted in the sequestration of Bad by 14-3-3 and inhibition of its association with the apoptosis-inducing Bcl-xl [87,89]. Estrogen signaling may play a role in RSK-mediated phospho-Bad induced survival. Fernando et al. showed that estradiol-activated RSK1, immunoprecipitated from MCF-7 cells, phosphorylated Bad by *in vitro* kinase assay [90]. Supporting this finding, estrogen treatment has also been shown to induce Bad phosphorylation in skeletal muscle cells [91]. Additionally, in NSCLC cells Amphiregulin and Insulin like-growth factor type 1 (IGF1) stimulated RSK-mediated Bad phosphorylation [22]. These cells often secrete Amphiregulin and IGF1 [92,93], which cooperate to prevent serum starvation-induced apoptosis [94]. Silencing RSK1, or overexpression of a catalytically inactive RSK2, in the NSCLC cell line H322 inhibited the ability of AR/IGF1 to prevent apoptosis due to serum-starvation. Thus,

RSK-mediated Bad phosphorylation is a well-established anti-apoptotic mechanism that may be regulated by estrogen signaling in NSCLC.

There are other signaling events, in addition to Bad phosphorylation, by which RSK regulates apoptosis in response to estrogens. Mitochondrial regulation of apoptosis is ultimately facilitated by activation of the pro-apoptotic proteins Bcl2 homologous antagonist killer protein (Bak) and Bcl2-associated X protein (Bax). Bak expression and therefore, mitochondrial integrity and survival, may be regulated by estrogen signaling [95]. Upon activation, Bak and Bax permeabilize the mitochondrial membrane permitting the release of cytochrome c into the cytosol and apoptosis [96]. Bak and Bax are regulated through interactions with binding partners that sequester them and prevent them from compromising the mitochondria. Dehan et al. showed that RSK phosphorylates and regulates the stability of the pro-apoptotic protein Bcl-2 interacting mediator of death- extra long (BimEL) [23]. BimEL is one of three Bim splice variants that are thought to regulate Bax-mediated apoptosis [23,96]. RSK-dependent phosphorylation promotes BimEL interaction with the F-box potein bTrCP, facilitating BimEL degradation and inhibition of apoptosis. Knockdown of RSK1/2 was found to stabilize BimEL levels and induce apoptosis in the lung cell lines, HCC87 and H1650 cells. Consistent with these findings, downregulation of BimEL by siRNA suppressed apoptosis in RSK1/2 knockdown cells, confirming that RSK1/2 can regulate apoptosis via BimEL in lung cancer cells [23]. Estrogen activation of RSK in lung cancer cells could therefore inhibit apoptosis by protecting mitochondrial activity.

RSK and Melanoma

Several studies have shown that estrogen receptors are expressed in melanoma tumors and cell lines [97–99] and a small study of 14 patients has shown that ERα and ERβ mRNA and ERβ protein are expressed in neoplastic skin cells [100]. However, the role of steroid-hormone activated receptors in melanocytes and melanoma is controversial. Tamoxifen treatment reduces growth of some melanoma cell lines [101], and overall survival was increased for melanoma patients receiving tamoxifen in initial clinical trials [102,103]. Subsequent clinical testing did not confirm these findings. Thus, the role of steroid hormone signaling in melanoma is not clear. RSK1 has been implicated in melanoma proliferation suggesting that estrogen signaling in melanoma may be connected to RSK activation. In melanoma cells, Eisenmann et al., showed that RSK1 was hyperactivated leading to the phosphorylation of Bad at Ser-75 and increased cell survival [24]. Thus RSK may regulate apoptosis via Bad phosphorylation in skin cancer.

RSK has also been implicated in other mechanisms of melanoma formation and metastasis. RSK1 phosphorylation and inactivation of the tumor suppressor Tuberin at Ser-1798 led to activation of mTOR and enhanced proliferation [104]. In contrast to these findings, RSK1 has been proposed to have tumor suppressor activity in some melanoma cells via phosphorylation of the Ser/Thr kinase tumor suppressor Liver Kinase B1 (LKB1) at Ser-431. RSK may be able to both suppress and promote tumorigenesis in melanoma; its function is likely dependent on additional signaling inputs that are not yet known.

RSK has also been implicated in regulation of migration in melanoma cells. Filamin A, a RSK substrate, is a membrane localized cytoskeletal protein essential for migration in some melanoma cell lines[105–107]. Additionally, RSK1-mediated phosphorylation of p27 at Thr-198 stimulates migration of melanoma cells [108]. RSK-mediated migration may promote tumor cell invasion in melanoma and other cancer cells via regulation of pro-invasion genes; such as matrix metallo-proteinases and protease receptor complex proteins [109]. These findings suggest that RSK may play an important role in metastases of melanoma and other

cancer cells, but it is not clear if RSK-mediated metastases occur in response to estrogen signaling.

RSK2 and Osteosarcoma

ERα, ERβ, and Progesterone Receptor (PR) expression and signaling have been linked to osteosarcoma proliferation [110,111]. The majority of human osteosarcomas possess elevated levels of the transcription factor c-Fos. ERα can stimulate c-Fos expression via an estrogen dependent mechanism [112,113]. There is a substantial body of evidence suggesting c-Fos is a protooncogene capable of initiating RSK2-dependent transformation of osteoblasts [114–117]. *c-Fos* transgenic mice crossed with RSK2 null mice (H2-*c-fos*LTR/*Rsk2*$^{-/y}$ mice) produce offspring whose tumors have increased levels of apoptosis and decreased proliferation compared to *c-Fos* transgenic animals expressing wild-type RSK2. [65]. These findings suggest that RSK2 is essential for survival of c-Fos-induced osteosarcomas. Both expression of total c-Fos protein and phosphorylation of c-fos at Ser-362 were abolished in the H2-*c-fos*LTR/*Rsk2*$^{-/y}$ mice. Phosphorylation of Ser-362 by RSK2 is essential for c-Fos transactivation because it stabilizes c-fos protein [64,65,118]. Thus, RSK2 may regulate osteosarcoma development via control of c-Fos activation and stability. Furthermore, the expression of ERα and ERβ in osteosarcomas suggests that hormone signaling enhances RSK-mediated tumor formation via induction of c-Fos expression.

Interestingly, mutations in the human *Rsk2* gene result in truncated forms of RSK2 protein, causing diverse skeletal and cognitive defects collectively known as Coffin-Lowry Syndrome (CLS) (reviewed in [119,120]). These findings suggest that RSK2 may be an important regulator of bone development whose hyperactivation could contribute to bone tumors. The transcriptional activity of ATF-4, a critical regulator of bone formation [121,122], was significantly impaired in osteoblasts isolated from *Rsk2*-deficient mice [78]. Thus, RSK2 may control osteoblasts via ATF4. Importantly, the skeletal deformities observed in *ATF4*$^{-/-}$ and *Rsk2*$^{-/-}$ mice are nearly identical [78]. These data suggest that RSK2 phosphorylation of ATF-4 is crucial for normal skeletal development. Though this mechanism is untested in osteosarcomas, it is possible that overexpression or hyperactivation of RSK may drive tumor formation via ATF-4. ATF-4 is upregulated in primary human breast cancer tissue compared with paired normal samples [123]. ATF-4 levels were increased specifically near necrotic areas of the tumor, as shown by immunostaining, and could be induced in breast cancer cell lines under anoxic conditions. ATF-4 may also be regulated by estrogen [124]. A recent microarray analysis of mouse uterine tissue showed a 2-fold increase in ATF-4 mRNA following 12hr of estradiol treatment. Further study of estrogen signaling in RSK-mediated regulation of ATF-4 in breast cancer would be of interest.

RSK and Big MAPK1/Extracellular Regulated Kinase 5 (BMK1/ERK5) in Angiogenesis

Angiogenesis is essential for tumor progression as tumors must develop neovasculature to procure oxygen and nutrients for survival [125]. Recent studies indicate that angiogenesis might be under the control of steroid hormone receptors in cancer [126–128]. Numerous investigations have shown that the only adult human system to undergo angiogenesis during homeostasis is the female reproductive tract, suggesting a connection between estrogens and angiogenic potential [129,130]. There are several putative mechanisms for estrogen-mediated angiogenesis. Estrogen treatment increases expression of vascular endothelial growth factor (VEGF), a master regulator of angiogenesis, in mouse mammary tumors [131,132]. Estrogens can also mediate Nitric oxide production, which is essential for VEGF-dependent angiogenesis [129]. Interestingly, RSK regulation of angiogenesis is thought to occur downstream of ERK5 (also known as BMK1), a key regulator of tumor vascularization [133–135] and a member of

the MAPK superfamily. Deletion of *ERK5* in Lewis lung carcinoma or in B16F10 melanoma xenografts resulted in smaller and fewer blood vessels than the control xenografts [136]. Inhibition of ERK5 signaling prevented bFGF-mediated RSK activation in endothelial cells. Loss of ERK5 in endothelial cells decreased phosphorylation of the RSK substrate, rpS6 and reduced tumor growth and angiogenesis [136]. Similarly, re-expression of ERK5 in the area of tumor injection in ERK5 knockout mice restored rpS6 phosphorylation and angiogenesis. These findings suggest that RSK-mediated phosphorylation of rpS6 and related tumor angiogenesis are dependent on ERK5 signaling. The possibility that estrogen signals promote angiogenesis via activation of ERK5 and RSK has not yet been explored, but the hypothesis is consistent with current observations.

Conclusion

Steroid hormone-activated receptors are now well established in the etiology of many cancers; including classical hormone-dependent tumors like breast and prostate, and unexpected malignancies like melanoma. It is likely that the role steroid receptors play will vary between tumor types, acting in some cases through non-genomic effects and in other cases through genomic/transcriptional regulation of key proteins. Steroid hormone receptor signaling can activate the ERK1/2-RSK pathway via a non-genomic mechanism (Figure 5). Activation of RSK inhibits mitochondrial-mediated apoptosis and increases proliferation. Thus, non-genomic steroid hormone signaling to RSK may promote transformation in multiple tumor types. Importantly, active RSK can stimulate higher steroid receptor transcriptional activity as has been shown for the androgen and estrogen receptors Therefore, we postulate that in some RSK-mediated tumors hormone signaling may drive proliferation and survival by stimulating both genomic and non-genomic pathways. This dual signaling response may increase expression and/or activity of oncogenic target proteins like c-Fos and cyclin D1, resulting in tumor formation and progression.

The recent discovery of RSK-specific inhibitors has the potential to dramatically advance our knowledge of RSK-mediated mechanisms in cancer and to test the effects of RSK inhibition in pre-clinical studies. There are currently no isoform-specific RSK inhibitors, but development of these tools is the next logical step in the process of understanding RSK function and the clinical implications of RSK. Given the preponderance of data linking steroid hormone signaling to RSK-associated cancers, these inhibitors would be of particular value in the study and treatment of hormone-dependent tumors.

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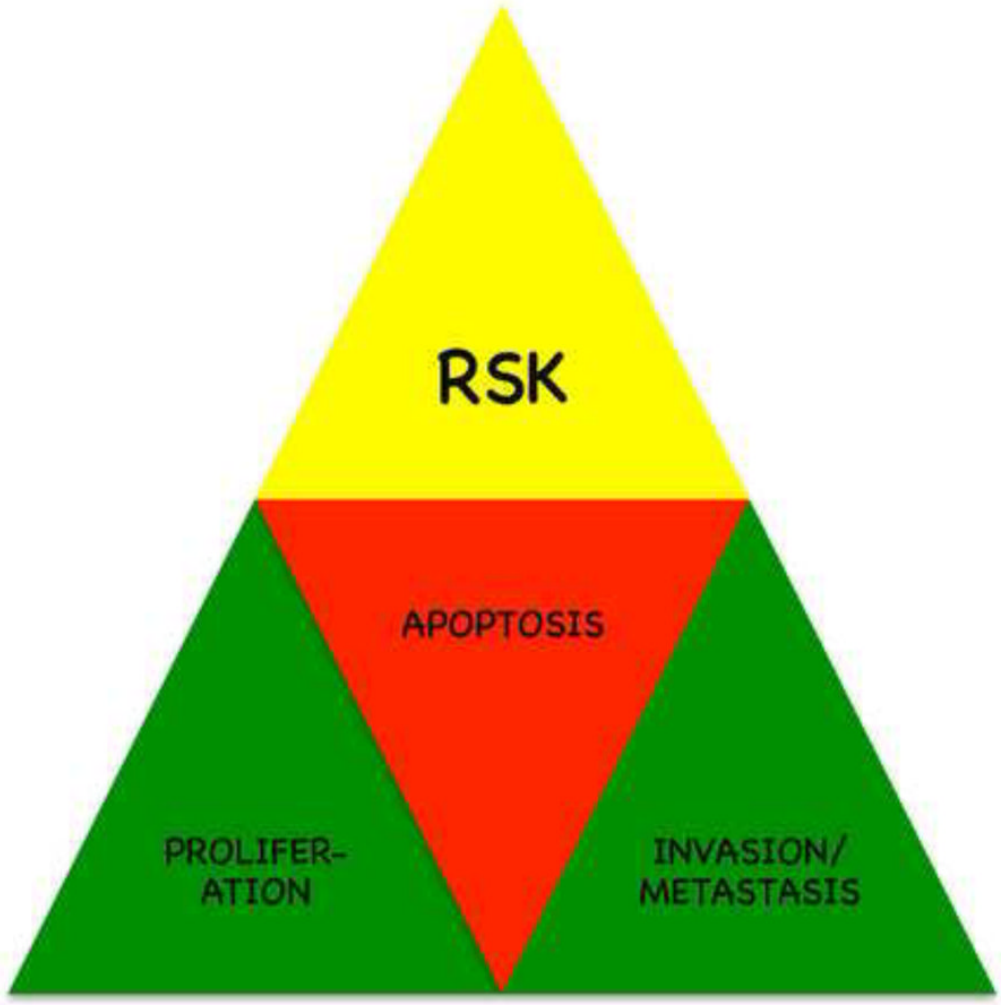

Figure 1. RSKs are downstream mediators of the ERK1/2 pathway that regulate proliferation in a variety of cancer cell lines and are overexpressed or hyperactivated in many human cancers

The RSKs can increase proliferation, inhibit apoptosis and promote the invasive phenotype by increasing migration. The participation of RSK in these three major pathways that promote tumorigenesis argues that this kinase family has potential as therapeutic targets.

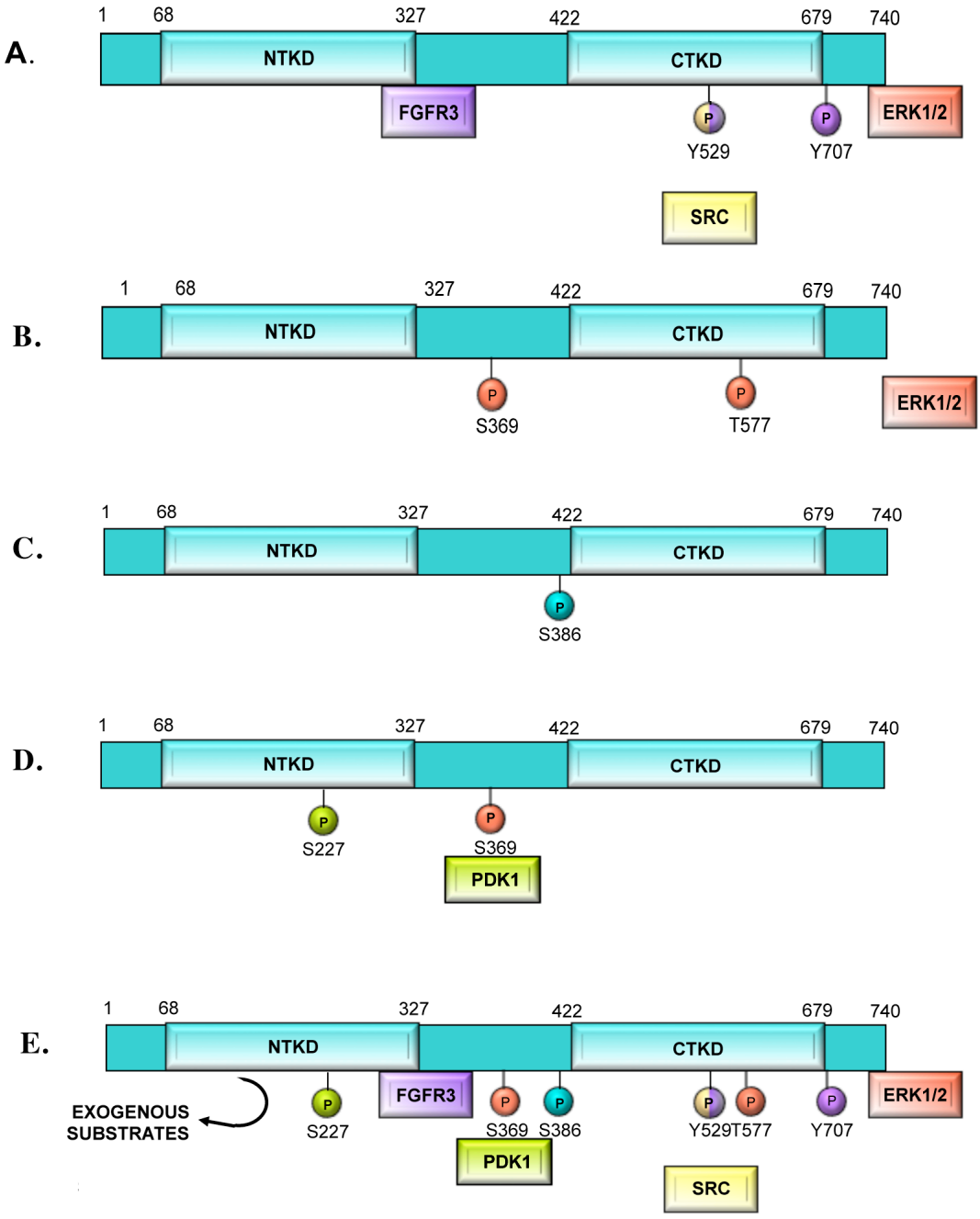

Figure 2. RSK activation

The RSK isoforms are activated by the same general mechanism. That mechanism is summarized in this figure using amino acid numbering corresponding to human RSK2. Color coding identifies the kinase with its associated phosphorylation. A) Inactive ERK1/2 binds to the extreme C-terminus of inactive RSK. In some cell types inactive RSK is also phosphorylated at Tyr-707 and Y529 by FGFR3 or SRC. B) In response to mitogen, ERK1/2 phosphorylates RSK on Ser-369 and Thr-577, activating the CTKD. After phosphorylating its target sites, ERK1/2 disassociates. C) The active CTKD autophosphorylates Ser-386. D) PDK1 is recruited to phospho-Ser-369 and then phosphorylates Ser-227 in the NTKD, completing

activation of the NTKD. E) Summary of RSK activation steps leading to NTKD-mediated phosphorylation of exogenous substrates.

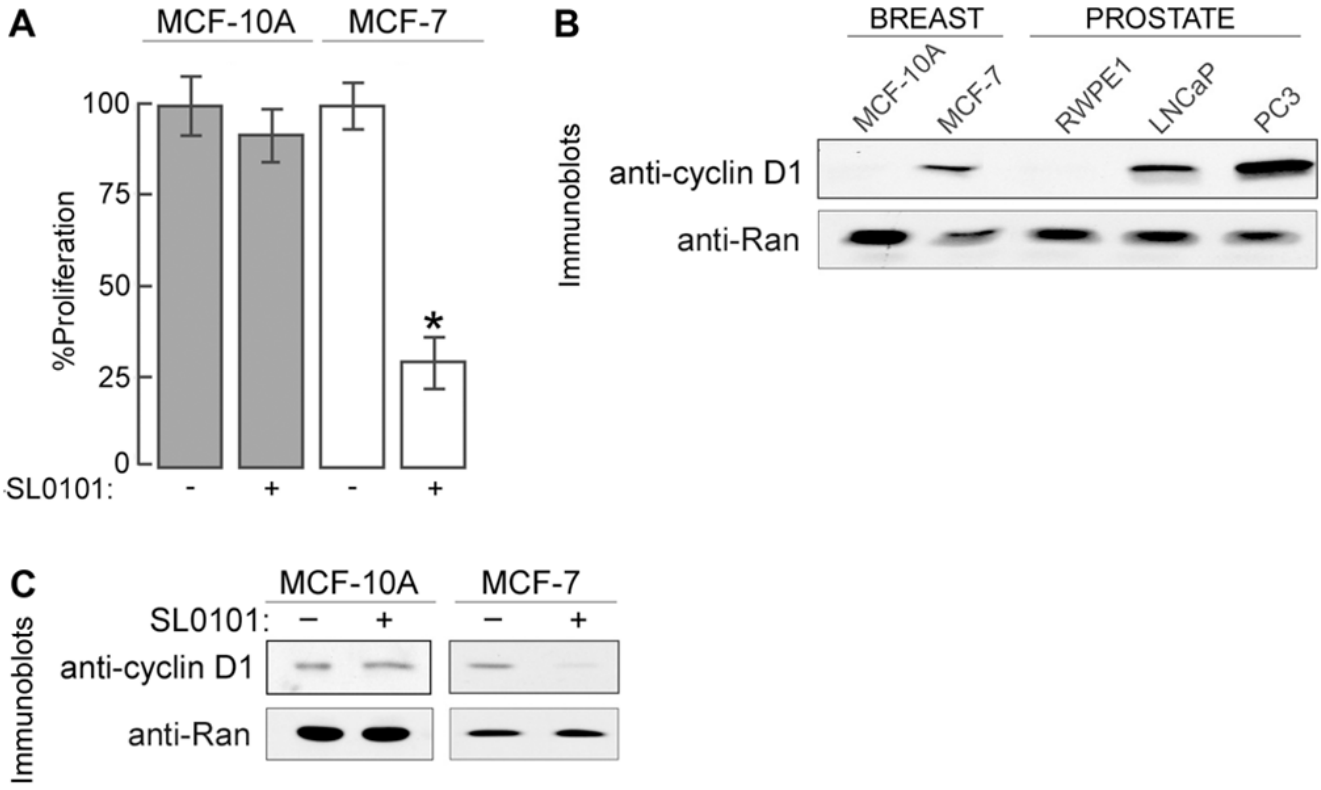

Figure 3. RSK regulates proliferation and cyclin D1 levels in breast cancer cell lines

A) Cells were treated with vehicle (−) or 20 μM SL0101, and cell viability was measured after 48 hr of treatment. Values are % of the growth observed in vehicle-treated cells. *Columns*, mean (n=2, in triplicate); *bars*, SD. *, p=0.005, Student's t test B) Lysates of the normal human cell lines, MCF-10A and RWPE1, and of the human cancer cell lines, MCF-7, LNCaP and PC-3 were prepared from cells grown in the appropriate media as recommended by ATCC. C) Cells were treated with vehicle (−) or 50 μM SL0101 for 4 hr before lysis. To permit detection of cyclin D1 the total protein loaded differed between cell lines. Equal loading of the lysate within a cell line is shown by the anti-Ran immunoblot.

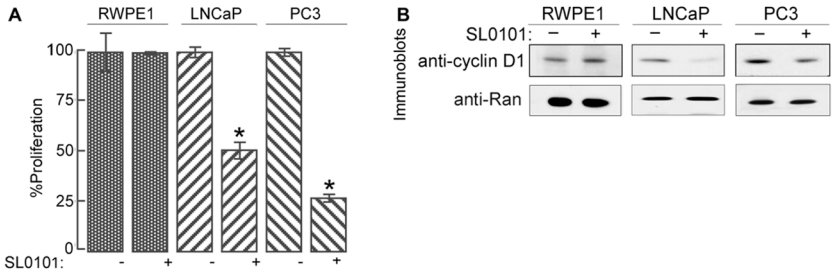

Figure 4. RSK regulates proliferation and cyclin D1 levels in prostate cancer cell lines

A) RWPE1, LNCaP, and PC3 cells were treated as in Figure 3A. *Columns*, mean (n = 2 in quadruplicate); *bars*, SD. *, p = 0.005, Student's t test. B) RWPE1, LNCaP, and PC3 cells were treated as in Figure 3C.

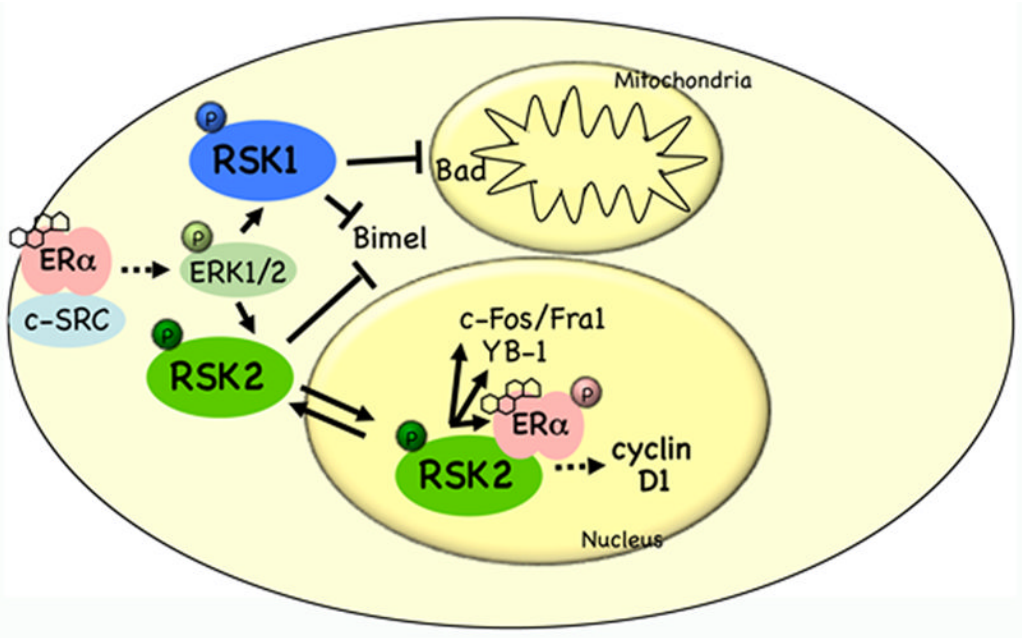

Figure 5. Major mechanisms proposed to regulate hormone-dependent transformation in some tumors

Estrogens stimulate ERα complex formation with cytoplasmic signaling proteins like MNAR, cas, and c-SRC. This complex activates the ERK1/2 signal transduction pathway, and phosphorylation of RSK. RSK1 signaling inhibits apoptosis via phosphorylation and inactivation of the pro-apoptotic protein Bad. RSK2 can translocate to the nucleus where it regulates nuclear targets that drive proliferation. In the nucleus RSK directly phosphorylates ERα stimulating its transcriptional activity.

Table I

RSK substrates are grouped under the phosphorylating isoform(s). RSK substrates are considered "validated" if they have been tested using siRNA/shRNA, RSK-specific inhibitors, or RSK null animals/cells. Substrates that have been tested in overexpression systems or *in vitro* are considered "unconfirmed". RSK4 is absent from this table because there are no known RSK4 substrates.

RSK1 substrates	shRNA/siRNA	Specific Inhibitors	Knockout Animals/Cells	In vitro/overexpression	Refs.
AS160		X		X	[1]
Bad		X		X	[2, 3]
CCTB	X	X		X	[4]
C/EBP		X		X	[5, 6]
DAPK	X			X	[7]
EF2K				X	[8, 9]
eIF4B	X	X		X	[10, 11]
ERα		X (unpublished data)		X	[12]
ER8				X	[13]
Filamin A	X			X	[14]
IkBa	X			X	[15]
IKBb				X	[16]
LKB1				X	[17]
MAD1	X			X	[18]
Myt1				X	[19]
Mi				X	[20]
NHE1				X	[9]
nNos	X			X	[21]
P27kip		X		X	[22, 23]
Raptor	X	X		X	[24]
RanBP3	X			X	[25]
rpS6	X	X		X	[26]

RSK1 substrates	shRNA/siRNA	Specific Inhibitors	Knockout Animals/Cells	In vitro/overexpression	Refs.
Tuberin				X	[27]
YB-1	X	X	X	X	[28, 29]
RSK2 substrates					
ATF-4			X	X	[30]
Bad	X			X	[31–33]
CCTB	X	X		X	[4]
c-fos			X	X	[34, 35]
Emi2				X	[36]
ERμ		X (unpublished data)		X	[37]
Filamin A	X			X	[14]
MEF2c				X	[38]
NFAT3				X	[39]
NHE1		X		X	[9, 40]
Nur-77		X		X	[41, 42]
RanBp3	X			X	[25]
rpS6	X	X		X	[26]
STAT-3			X	X	[43]
TIF1A				X	[44]
YB-1	X	X	X	X	[28]
RSK3 substrates					
Bad				X	[31]
NHE1				X	[9]
rpS6				X	[45]
H2B				X	[45]

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Table II

RSK1, RSK2, and RSK3, substrates are grouped according to their cellular functions; proliferation, migration, and survival. Substrates of a given RSK isoform are indicated with an "X". Many RSK substrates are phosphorylated by multiple isoforms and are therefore labeled with more than one "X". Some RSK substrates have been identified using reagents that were not isoform-specific. In these cases the "X" is placed in the unknown column because the phosphorylating RSK isoform is unknown. RSK4 is absent from this table because there are no known RSK4 substrates.

Proliferation	**Unknown**	**RSK1**	**RSK2**	**RSK3**
RanBP3		X	X	
YB1		X	X	
Transcription				
ER8		X		
H2B				X
IkBa		X		
IKBb		X		
NFATc4	X			
NFAT3			X	
TIF1A			X	
MAD1		X		
C/EBP		X		
ERa		X	X	
Stat3			X	
C-fos			X	
MEF2c			X	
Mi		X		
Translation				
EF2K		X		
eIF4B		X		
rps6		X	X	X
Cell Cycle Regulation				
Bub1	X			
Myt1		X		
Emi2			X	
Migration				
L1	X			
Filamin A			X	
P27 kip		X		
Survival				
DAPK		X		
nNos		X		
ATF-4			X	
Nutrient Signaling				
Raptor		X		

Proliferation	**Unknown**	**RSK1**	**RSK2**	**RSK3**
LKB1		X		
CCTB		X	X	
Tuberin		X		
Metabolism				
GSK3	X			
AS160		X		
Ion transport				
NHE1			X	X
Mitochondrial Integrity				
Nur-77			X	
Bad		X	X	X