Akt deficiency impairs normal cell proliferation and suppresses oncogenesis in a p53-independent and mTORC1-dependent manner

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Summary

Akt contributes to tumorigenesis by inhibiting apoptosis. Here we establish that Akt is required for normal cell proliferation and susceptibility to oncogenesis independently of its antiapoptotic activity. Partial ablation of Akt activity by deleting *Akt1* inhibits cell proliferation and oncogenesis. These effects are compounded by deleting both Akt1 and Akt2. In vivo, *Akt1* null mice are resistant to MMTV-v-H-Ras-induced tumors and to skin carcinogenesis. Thus, partial ablation of Akt activity is sufficient to suppress tumorigenesis in vitro and in vivo. The effect of Akt deficiency on cell proliferation and oncogenesis is p53 independent but mTORC1 dependent. Surprisingly, upon mTORC1 hyperactivation, the reduction in Akt activity does not impair cell proliferation and susceptibility to oncogenic transformation; thus, Akt may mediate these processes exclusively via mTORC1.

Introduction

Akt, also known as protein kinase B (PKB), is an evolutionarily conserved serine/threonine kinase. Mammalian cells express three Akt isoforms (Akt1–3) encoded by three separate genes. The amino acid sequences of the three isoforms are almost identical; relative expression of these isoforms, however, differs in various mammalian tissues. Akt is activated by extracellular signals that activate PI3K. For instance, upon activation, growth factor receptors activate p110 (the catalytic subunit of PI3K) either by recruiting p85 (the regulatory subunit of PI3K) or by activating Ras, which can directly activate p110. Upon activation, p110 phosphorylates phosphoinositides (PI) at the D3 position of the inositol ring to generate PI (3,4,5) P3 (PIP3). The rate-limiting step in Akt activation is the binding of PIP3 to the pleckstrin homology (PH) domain of Akt and subsequent translocation of Akt to the plasma membrane, where it is phosphorylated on a threonine in the catalytic domain and a serine in the C-terminal regulatory domain. These modifications are required for full activation. The kinase that phosphorylates the threonine is PDK1; the kinase that phosphorylates the serine was recently identified as mTORC2, which is a complex containing the mammalian target of rapamycin (mTOR) and Rictor (Sarbassov et al., 2005). Antagonizing PI3K activity can negatively regulate the activity of Akt. PTEN (for "phosphatase and tensin homolog deleted from chromosome 10") is a 3-phosphoinositide phosphatase that negatively regulates Akt activity by reducing the intracellular level of PIP3 produced by PI3K (Parsons and Simpson, 2003).

Akt could phosphorylate a large number of intracellular targets (Lawlor and Alessi, 2001), only a few of which are evolutionarily conserved. mTORC1, a rapamycin-sensitive kinase, comprises mTOR and Raptor and is a conserved downstream effector of Akt, which is considered as a therapeutic target in cancer (Thomas, 2006). The principal functions of mTORC1 are to mediate ribosome biogenesis and mRNA translation via activation of S6 kinase and inhibition of the repressor of mRNA translation, eIF4E binding protein (4E-BP), thereby determining cell mass (Hay and Sonenberg, 2004). Akt can activate mTORC1 through direct phosphorylation of tuberous sclerosis complex 2 (TSC2), which otherwise inhibits mTORC1 activity (Inoki et al., 2002; Manning et al., 2002). Akt can also indirectly inhibit TSC2 via inhibition of AMPK (Hahn-Windgassen et al., 2005). Tuberous sclerosis complex 1 (TSC1) and TSC2 form a

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S I G N I F I C A N C E

Akt is frequently activated in human cancers and therefore is an attractive therapeutic target. Whether partial ablation of Akt activity is sufficient to inhibit tumorigenesis, however, remains unknown. We demonstrate that partial ablation of total Akt activity or selective inhibition of Akt1 could be an efficient general therapeutic approach for cancer. Our results show that mTORC1 is the most critical downstream effector of Akt required for cell proliferation and tumorigenesis. Although inhibitors of mTORC1 have been considered for cancer therapy, they also activate Akt and may not override the antiapoptotic activity of Akt. Because partial ablation of Akt activity may be sufficient to inhibit both mTORC1 and the antiapoptotic effect of Akt, it could be a successful therapeutic approach.

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heterodimer, which functions as a GTPase-activating protein (GAP) that inhibits the activity of Rheb, a small GTPase required for mTORC1 activation (Hay and Sonenberg, 2004). In Tsc2 or Tsc1 null cells, mTORC1 is constitutively activated independently of growth factors and Akt, consistent with an inhibitory role for TSC2. In contrast, Akt activity is markedly reduced in these cells, which is attributed to a negative feedback mechanism (Harrington et al., 2005).

Akt is frequently hyperactivated in human cancers via multiple mechanisms. Inactivating mutations or deletions of the tumor suppressor PTEN, which lead to Akt activation, occur frequently in human cancers with a high incidence in prostate and endometrial cancers, glioblastoma, and melanoma (Sansal and Sellers, 2004). In addition, haplodeficiency of PTEN in mice elicits a wide range of tumors (Di Cristofano et al., 1998; Podsypanina et al., 1999; Suzuki et al., 1998). Amplification and overexpression of the gene encoding p110 isoform also occur in a subset of human cancers (Hennessy et al., 2005). More recently, mutations in one of the genes encoding p110 (p110α) have been observed in a large number of human cancers (Samuels et al., 2004); these mutations likely activate Akt (Kang et al., 2005). Activating mutations of Ras, which can potentially activate Akt, occur in about one-third of epithelial tumors (Downward, 2003). Also, Akt gene amplification has been observed in a subset of human cancers (Hennessy et al., 2005). Thus, Akt appears to be hyperactivated in the majority of human cancers, which thus implies that activation of Akt plays a pivotal role in the genesis of cancer (Hay, 2005; Hennessy et al., 2005). We have recently shown that partial ablation of Akt activity by the deletion of Akt1 is sufficient to inhibit the development of tumors in $Pten^{+/-}$ mice (Chen et al., 2006). However, it is not known whether partial ablation of Akt activity is sufficient to inhibit tumors induced by other mechanisms. Furthermore, the exact mechanisms by which activation of Akt exerts its effect on the genesis of cancer are not well understood, and the most critical downstream effectors of Akt required for the genesis of cancer are largely unknown. The most established function of Akt in mammalian cells is inhibition of apoptosis, which may contribute to the genesis of cancer. However, it is unlikely that this function, by itself, is sufficient to explain the frequent activation of Akt in human cancer.

Here, we provide genetic evidence that Akt is required for cell proliferation and oncogenic transformation, independently of its antiapoptotic function. Partial ablation of Akt is sufficient to inhibit proliferation and oncogenic transformation induced by dominant negative p53 (DN-p53) in combination with activated Ras (H-Ras^{valine12}) or by Myc and activated Ras. These results could be recapitulated in vivo, as Akt1 knockout (KO) mice are resistant to tumors induced by activated Ras and are less susceptible to skin carcinogenesis. The mechanism by which Akt affects cell cycle progression and susceptibility to oncogenic transformation is independent of p53. We show that Akt's functions in cell proliferation and susceptibility to oncogenic transformation are executed primarily or exclusively by mTORC1. In TSC2-deficient cells, mTORC1 is constitutively activated, but Akt activity is impaired to an even greater extent than in Akt1/Akt2 double knockout (DKO) cells. However, the proliferation rate and susceptibility to oncogenic transformation of TSC2 null cells are not impaired; in fact, these aspects are enhanced compared with control cells. The knockdown of Raptor in Tsc2 null cells decreases mTORC1 activity and elevates Akt activity but impairs proliferation and oncogenic transformation.

Likewise, restoring mTORC1 activity in Akt-deficient cells restores normal proliferation and oncogenic transformation. Thus, mTORC1 appears to be the most critical downstream effector of Akt in this respect. Taken together, these results provide insight into the mechanisms by which Akt contributes to the genesis of cancer and thus have important implications for cancer therapy.

Results

Akt-deficient MEFs are impaired in cell proliferation

To assess the role of Akt in normal cell proliferation, we isolated mouse embryo fibroblasts (MEFs) derived from knockout embryos and their wild-type littermates. We found that Akt1 KO MEFs divided significantly more slowly than wild-type cells. This was more pronounced in Akt1/Akt2 DKO MEFs (Figure 1A). This defect in cell proliferation was not due to increased cell death in Akt-deficient cells because, when cultured in 10% FBS, Akt1 KO and Akt1/Akt2 DKO cells did not die significantly more than wild-type cells (Figure 1A).

To further examine the impaired cell proliferation, we analyzed the rate of entry into the cell cycle. Wild-type (WT), Akt1 knockout (KO), and Akt1/Akt2 double knockout (DKO) MEFs were serum starved for 48 hr in 0.1% FBS-containing medium followed by stimulation with 10% FBS-containing medium. Cells were pulse labeled with BrdU for 1 hr for each time point, and the percentage of cells with labeled nuclei was quantified (Figure 1B). Loss of Akt1 alone led to a 44% reduction in BrdU incorporation compared to wild-type cells, whereas loss of both Akt1 and Akt2 led to a 61% reduction. These data, together with the reduction in cell division rate, imply that Akt is required for normal G1/S transition.

We immortalized MEFs by infection with a retrovirus encoding GSE56, a genetic suppressor element that functions as a dominant negative suppressor of p53 (DN-p53) (Zou et al., 2002). Following infection with a high-titer virus and selection with puromycin, we established polyclonal cell lines. We analyzed these cell lines for cell cycle progression and BrdU incorporation. All the immortalized cell lines divided faster than the primary cells. Despite p53 inactivation, however, the cell division rate (Figure 1C) and entry into the cell cycle (Figure 1D) were still impaired in the Akt-deficient cells relative to WT. Furthermore, BrdU incorporation was also impaired in asynchronous cell populations (Figure 1E), indicating that DNA synthesis was impaired throughout the cell cycle and not just upon entry. Similar results were obtained with MEFs immortalized with p53 shRNA (Figure 1F). These results imply that the requirement of Akt for normal cell cycle progression is independent of p53. It has been suggested that Akt might mediate its effect via p53 and that Akt decreases the stability of p53 by phosphorylation of MDM2 (Mayo and Donner, 2001; Zhou et al., 2001). In our analysis, however, the half-life of p53 was similar in both WT and Akt1/Akt2 DKO cells (Figure S1 in the Supplemental Data available with this article online).

Akt1 is the predominantly expressed Akt isoform in MEFs (Peng et al., 2003 and Figure 1G), and we estimated that total Akt activity was reduced by about 50% and 80% in Akt1 KO and the DKO MEFs, respectively (Figure 1G). Interestingly, Akt2 KO MEFs were not impaired for proliferation (Figure S2), suggesting that proliferation is impaired only if total Akt activity is reduced beyond a certain threshold.
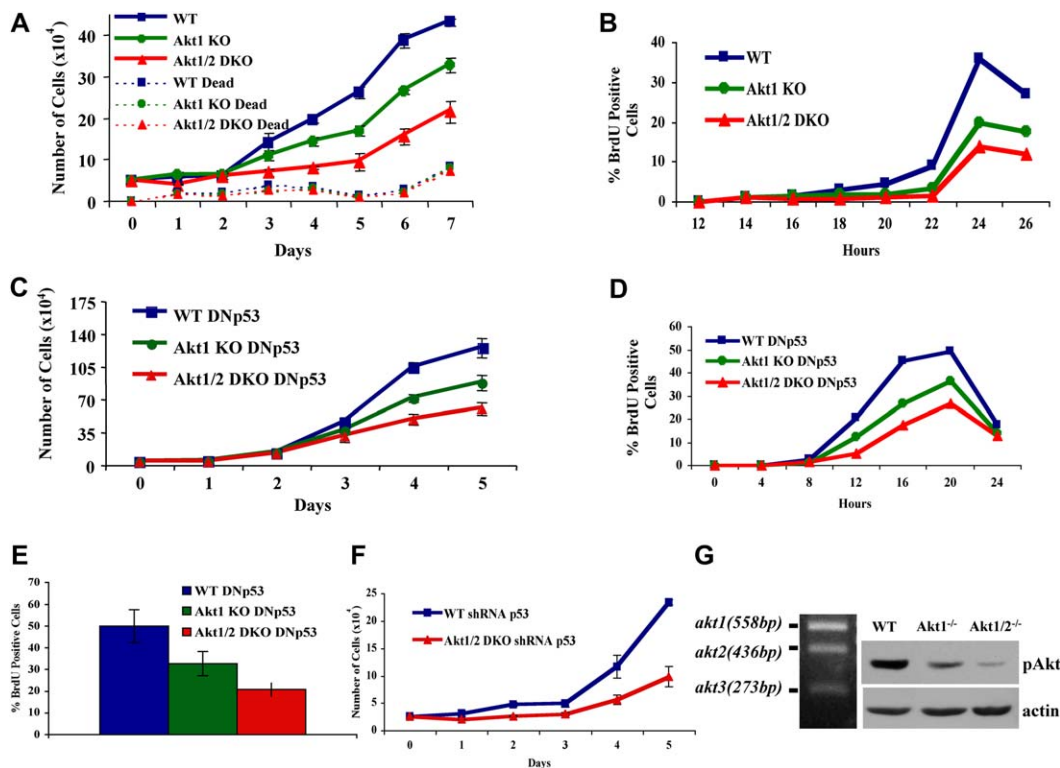
Figure 1. Akt deficiency impairs cell proliferation and cell cycle entry in a p53-independent manner

A: Cell proliferation rates of primary MEFs derived from wild-type, Akt1 KO, or Akt1/2 DKO 13.5 day embryos. Cells (5×10^4) were seeded onto a 6 cm dish, in triplicate, in 10% FBS and allowed to grow. Live and dead cells were counted via trypan blue exclusion using a hemacytometer. Error bars represent ±SD. The experiment shown is representative of three separate experiments with cells derived from different groups of littermates.

B: Entry into the cell cycle of primary MEFs. Cells were cultured in 0.1% FBS for 72 hr followed by release into medium containing 10% FBS. Cells were monitored for BrdU incorporation as described in the Experimental Procedures. Briefly, cells were labeled with BrdU for 1 hr at each time point after release and fixed with ethanol, the DNA was denatured, and the cells were probed with antibodies and then counterstained with DAPI. BrdU incorporation was measured by counting at least 300 cells from at least five fields of view in duplicate plates. These data represent three separate experiments with cells derived from different groups of littermates.

C: Cell proliferation rates of DN-p53-immortalized MEFs. Cells (2.5×10^4) were seeded in a 6 cm dish, in triplicate, in 10% FBS and counted. These data represent three separate experiments with different immortalized MEF cell lines. Error bars represent ±SD.

D: Cell cycle entry of DN-p53-immortalized MEFs. Cells were incubated in 0.1% FBS for 72 hr followed by release into medium containing 10% FBS. BrdU incorporation was monitored as described in the Experimental Procedures. These data represent three separate experiments with different immortalized MEF cell lines.

E: Proliferation rate of asynchronously growing DN-p53-immortalized MEFs was measured by pulse labeling cells for 4 hr with BrdU prior to fixation and staining. BrdU incorporation was carried out as described in the Experimental Procedures and measured by counting at least 300 cells from at least five fields of view in triplicate plates. These data represent three separate experiments with different immortalized MEF cell lines. Error bars represent ±SD.

F: Primary MEFs were immortalized by p53 knockdown via shRNA. Cells (2.5×10^4) were seeded onto a 6 cm dish in 10% FBS and counted. The experiment was performed three times in triplicate. Error bars represent ±SD.

G: Left panel: RT-PCR analysis showing relative expression of the three Akt isoforms in primary MEFs. Right panel: activity of Akt in WT, Akt1 KO, and Akt1/2 DKO primary MEFs as measured by immunoblotting with an antibody that recognizes pSer473 of Akt (anti-pSer473) following 72 hr of serum starvation and 30 min of serum stimulation with 10% FBS.

To further understand the mechanism by which Akt affects cell cycle progression, we studied various cell cycle-regulated proteins in DN-p53 immortalized MEFs upon cell cycle entry. Cells were serum starved in 0.1% FBS, released into 10% FBS, and collected at each time point for immunoblotting. pRb phosphorylation was impaired in Akt1 KO MEFs upon cell cycle entry (Figure 2A). This could be demonstrated by a mobility shift to the hyperphosphorylated forms and by using phospho-specific antibodies directed against sites phosphorylated by CDK4 or CDK2 (Figure 2A). The phosphorylation of pRb was further impaired in Akt1/Akt2 DKO MEFs (data not shown, and also Figure 8C). These results suggest that both CDK4 and CDK2 activities are impaired upon cell cycle entry. Analysis of the expression of proteins that regulate cell cycle progression revealed that cyclin D1 was reproducibly the most affected

protein. The reduction in cyclin D1 expression was mostly observed following cell cycle entry. The expression of cyclins D2 and D3, however, was not impaired in Akt1 KO MEFs (Figure 2B, lanes 3–7 and 10–14). In addition, the level of the cyclin-dependent kinase (CDK) inhibitor p27 was reproducibly elevated in nonsynchronized proliferating Akt1 KO MEFs (Figure 2B, lanes 1 and 8) and Akt1/Akt2 DKO MEFs (data not shown), and following cell cycle entry the reduction in p27 level was slightly delayed in Akt1 KO MEFs (Figure 2B, lanes 3–7 and 10–14).

Susceptibility to oncogenic transformation is impaired in Akt-deficient MEFs

Wild-type and Akt KO MEFs were subjected to a cotransformation assay following infection with retroviruses expressing H-Ras^valine12 and Myc or H-Ras^valine12 and DN-p53 (Zou et al.,

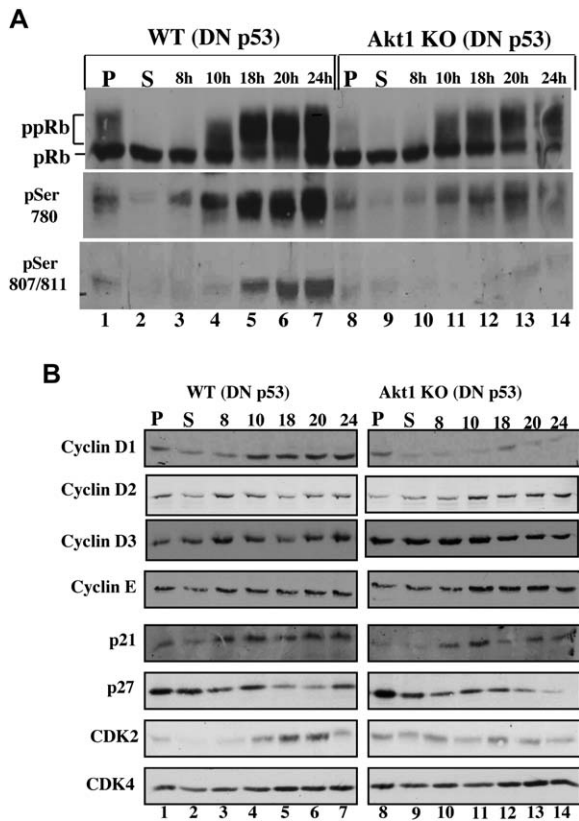**Figure 2.** Analysis of pRb phosphorylation and cell cycle regulatory proteins upon cell cycle entry of DN-p53-immortalized cells

A: pRb phosphorylation following cell cycle entry. DN-p53-immortalized MEFs were incubated in medium containing 0.1% FBS for 72 hr followed by release into medium containing 10% FBS. Cell extracts were collected at the indicated times and were subjected to immunoblotting. Top panel: anti-total pRb, with slower migrating bands representing phosphorylated pRb; middle panel: anti-pSer780 of pRb; bottom panel: anti-pSer807/811 of pRb. Lanes 1 and 8 represent asynchronously proliferating cells (P). Lanes 2 and 9 represent serum-starved cells in 0.1% FBS (S). Lanes 3–7 and 10–14 represent time points following release into medium containing 10% FBS.

B: Expression levels of G1 cyclins, CKIs, and CDKs following cell cycle entry. Cells were treated as in **A**, and cell lysates were collected for immunoblotting. A representative immunoblot is shown. Lanes 1 and 8 represent asynchronously proliferating cells (P). Lanes 2 and 9 represent serum-starved cells in 0.1% FBS. Lanes 3–7 and 10–14 represent time points (in hr) following release into medium containing 10% FBS.

2002). In this assay, transformation is measured by counting the foci that appear on a monolayer. Akt1 deficiency by itself was sufficient to reduce susceptibility to oncogenic transformation with Ras and DN-p53 by about 5-fold and to oncogenic transformation by Ras and Myc by about 7-fold (Figures 3A and 3B). When both Akt1 and Akt2 were deleted, the cells were almost completely resistant to oncogenic transformation. A hallmark of neoplastic cellular transformation is the ability to grow in an anchorage-independent manner in the absence of traditional matrix attachments. To determine if Akt is required for cells to obtain this transformed state, we infected primary MEFs with a retrovirus encoding both DN-p53 and H-Ras^{valine12} followed by plating onto agarose-containing media. The Akt1-deficient cells formed smaller and fewer colonies than WT cells, whereas the Akt1/Akt2 DKO cells were hardly able to form colonies

(Figure 3C). Thus, our data show that loss of Akt1 alone or in combination with Akt2 results in the inability of these cells to acquire a fully transformed state. Furthermore, when DN-p53-immortalized MEFs were subjected to Ras transformation, transformation was still inhibited in Akt-deficient cells (Figure 3D), although to a lesser extent than observed in the primary cells. It is unlikely that the antiapoptotic activity of Akt significantly contributes to susceptibility to oncogenic transformation in the conditions of high serum level that were used. Nevertheless to directly examine the requirement of Akt for susceptibility to oncogenic transformation independently of its antiapoptotic function, we overexpressed Bcl-2 in the cells. Although Bcl-2 overexpression increased the ability of both WT and Akt-deficient cells to grow on soft agarose, the Akt-deficient cells were still impaired, to the same extent, in their ability to be transformed (Figure 3D). In addition, when DN-p53- or p53-shRNA-immortalized WT MEFs were passaged more than ten times, they formed foci on a confluent monolayer, whereas Akt1/Akt2 DKO cells did not (Figure 3E). Taken together, these results imply first that Akt is required for oncogenic transformation in a p53-independent manner and second that even partial inhibition of total Akt activity is sufficient to substantially reduce susceptibility to oncogenic transformation in vitro. Third, Akt is required for oncogenic transformation regardless of its anti-apoptotic activity.

Deletion of Akt1 is sufficient to impair tumorigenesis in vivo

To recapitulate the ability of Akt1 deficiency to suppress oncogenic transformation in vivo, we first crossed Akt1 KO mice with MMTV-v-H-Ras mice, in which activated viral Harvey Ras induces mammary and salivary gland tumors (Sinn et al., 1987). $Akt1^{+/-}/MMTV$-v-H-Ras mice were intercrossed to generate $Akt1^{+/+}/MMTV$-v-H-Ras and $Akt1^{-/-}/MMTV$-v-H-Ras mice. The mice were monitored for tumor appearance, and the percentage of tumor-free mice was calculated (Figure 4A). Deletion of Akt1 was sufficient to markedly delay the onset of tumor appearance from 11 weeks in $Akt1^{+/+}/MMTV$-v-H-Ras mice to 29 weeks in $Akt1^{-/-}/MMTV$-v-H-Ras mice. Overall, $Akt1^{-/-}/MMTV$-vH-Ras mice were dramatically more resistant to tumors induced by activated Ras when compared with $Akt1^{+/+}/MMTV$-v-H-Ras mice. After 50 weeks, we observed that only about 13% of $Akt1^{+/+}/MMTV$-v-H-Ras mice remained tumor free, whereas about 73% of $Akt1^{-/-}/MMTV$-v-H-Ras mice were tumor free. Thus, these results recapitulated the results we obtained ex vivo with cells derived from Akt1 KO mice.

We also subjected 129sv/C57BL/6 (1:1) Akt1 KO and WT littermates to skin carcinogenesis (see Experimental Procedures). As shown in Figure 4B, the number of tumors in Akt1 KO mice was reduced 2-fold. Tumor size was reduced even further in Akt1 KO mice compared with their wild-type littermates, and no tumors larger than 7 mm were observed in Akt1 KO mice 16 weeks after the induction of carcinogenesis (Figure 4B). Thus, we concluded that the deletion of Akt1 is sufficient to inhibit tumorigenesis in vivo, and that partial ablation of Akt activity is not only sufficient to inhibit oncogenic transformation of MEFs but also epithelial tumors in vivo. These results have strong implications for cancer therapy, suggesting that even partial ablation of Akt activity could be an efficient therapeutic strategy.
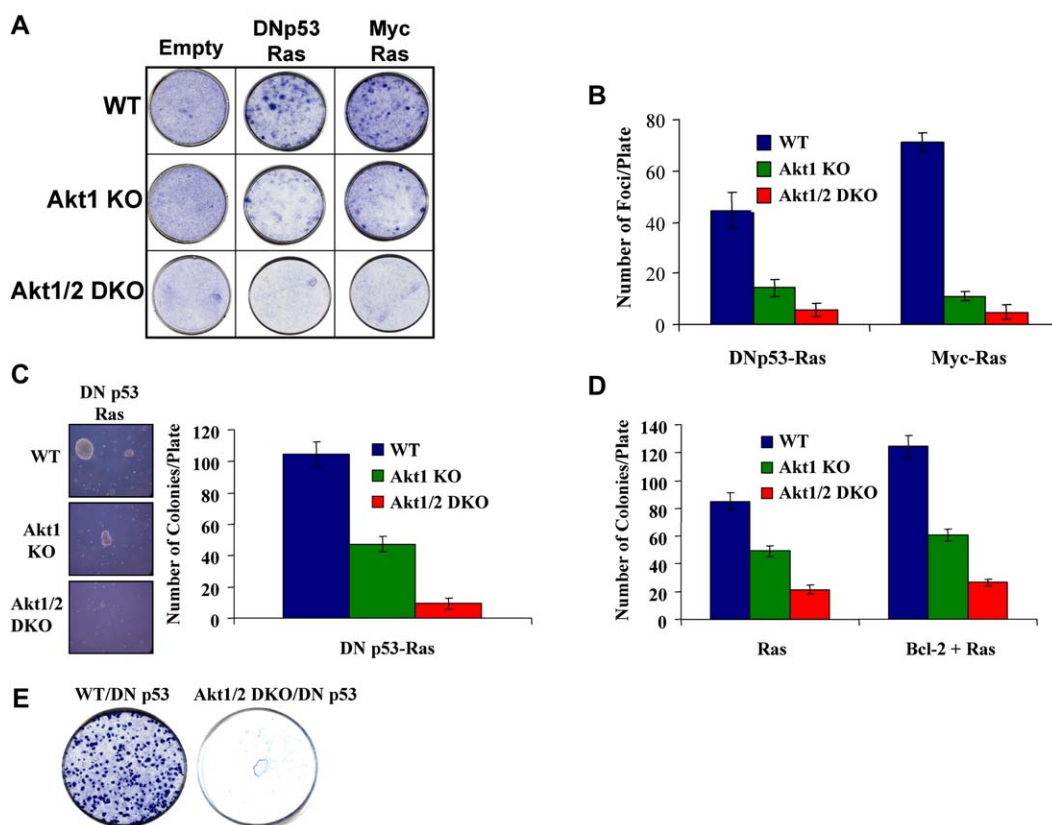
Figure 3. Akt-deficient cells are resistant to oncogenic transformation

A: Primary MEFs were subjected to the cotransformation assay. Subconfluent MEFs were infected with empty retrovirus, retrovirus expressing both DN-p53 and H-Ras^{valine12}, or retrovirus expressing both Myc and H-Ras^{valine12}. Cells were allowed to grow past confluency and were fixed and stained to observe foci formation. These data represent three separate experiments with cells derived from different groups of littermates.

B: Quantitation of foci observed in **A**. The total number of foci per plate was scored by counting. The experiment was performed in triplicate. These data represent three separate experiments with cells derived from different groups of littermates. Error bars represent ±SD.

C: Primary MEFs infected with retrovirus expressing both DN-p53 and H-Ras^{valine12} were plated in 0.35% agarose-containing medium, as described in the Experimental Procedures, and allowed to grow with weekly media changes for approximately 6 weeks. The left panel shows representative fields of soft agarose colonies. The bar graph represents the quantitation of soft agarose colonies. The experiment was performed in triplicate at least three times with cells derived from different groups of littermates. Error bars represent ±SD.

D: DN-p53-immortalized MEFs were infected with retrovirus expressing H-Ras^{valine12} or with both retrovirus expressing H-Ras^{valine12} and retrovirus expressing Bcl-2, as described in the Experimental Procedures. Following selection, polyclonal cell lines were analyzed by soft agarose assays. The bar graph represents the average number of colonies counted from triplicate plates. Experiments were performed with at least three independent cell lines. Error bars represent ±SD.

E: Spontaneous foci formation on a confluent monolayer of late-passage DN-p53-immortalized MEFs. MEFs were passaged at least 15 times and allowed to grow past confluency for 7–10 days. Cells were fixed and stained as described in the Experimental Procedures to visualize foci.

Tsc2 null cells are not impaired in their proliferation rate and susceptibility to oncogenic transformation despite diminished Akt activity

To delineate the mechanism by which Akt affects cell proliferation and oncogenic transformation, we assessed the role of mTORC1 as a potential downstream effector. Inhibition of mTORC1 with rapamycin was shown to inhibit cell cycle progression (Fingar et al., 2004). Akt is required for growth factor-mediated activation of mTORC1, and mTORC1 activity is impaired in Akt-deficient cells (Gingras et al., 1998; Hahn-Windgassen et al., 2005; Peng et al., 2003). Akt activates mTORC1 by inhibiting its upstream negative regulator, TSC2, in the heterodimeric complex TSC1/TSC2 (Inoki et al., 2002; Manning et al., 2002). Thus, in Tsc2 null cells mTORC1 is constitutively active and does not require growth factors and Akt for its activation (Zhang et al., 2003). However, as a result of a feedback inhibitory loop, Akt activity is diminished in Tsc2 null MEFs (Harrington et al., 2005; Zhang et al., 2003). We found that Akt activity in

p53-deficient Tsc2 null MEFs was reduced to an even greater extent than in p53-deficient Akt1/Akt2 DKO MEFs (Figure 5A). Nevertheless, Tsc2 null MEFs proliferated at the same rate as control heterozygous Tsc2 MEFs (Figures 5B and 5C). In addition, following cell cycle entry, pRb phosphorylation was not impaired in Tsc2 KO MEFs despite the marked reduction in Akt activity (data not shown). Moreover, reintroduction of Tsc2 into Tsc2 null cells, by retrovirus infection followed by the generation of polyclonal cell line, elevated Akt activity (Figure 6A) with a concomitant decrease in mTORC1 activity as measured by 4E-BP1 phosphorylation in the absence of serum (Figure 6B) but markedly decreased proliferation rate (Figures 6C and 6D). The proliferation rate was only modestly reduced when an Akt-phosphomimetic mutant of Tsc2 was introduced into the Tsc2 KO MEFs instead of WT Tsc2 (Figure S3C). Furthermore, the reintroduction of Tsc2 dramatically reduced susceptibility to oncogenic transformation by activated Ras (Figure 6E). We also isolated individual clonal cell lines having increasing levels of TSC2
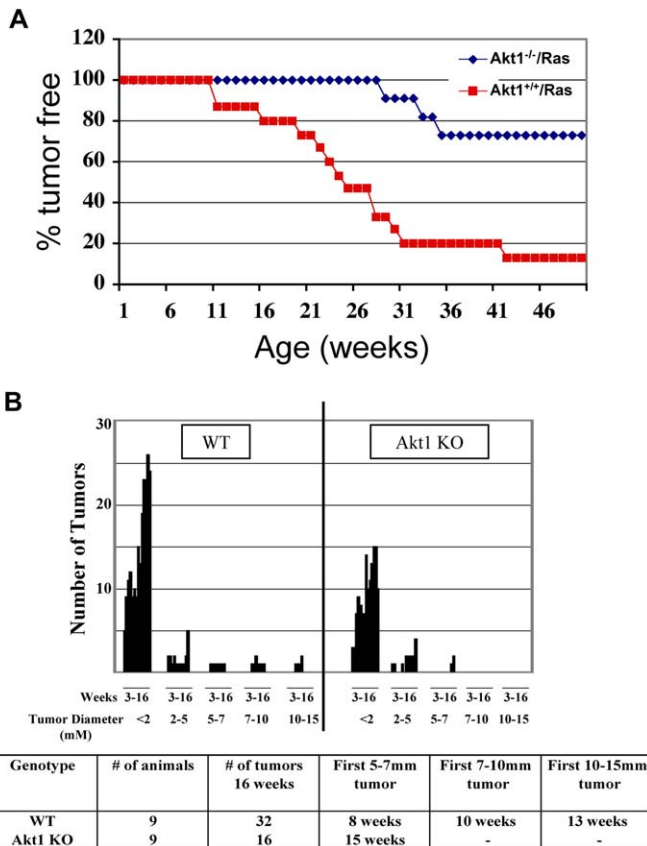
Figure 4. Akt1 null mice are resistant to tumorigenesis

A: *Akt1$^{+/+}$/MMTV-v-H-Ras* (n = 15) and *Akt1$^{-/-}$/MMTV-v-H-Ras* (n = 11) mice were generated as described in the Experimental Procedures. Mice were monitored weekly and scored for tumors; the percentage of tumor-free mice is presented.

B: Wild-type and Akt1 KO littermates were subjected to a two-step skin carcinogenesis protocol as described in the Experimental Procedures. Tumor number and size were monitored weekly. Nine mice per group were treated; animals treated with vehicle alone did not form tumors.

Figure 5. Akt activity is impaired in TSC2 KO cells without impairing cell proliferation

A: Akt activity of DN-p53-immortalized WT and Akt1/2 DKO MEFs, and p53-deficient TSC2 heterozygous and KO MEFs. Immunoblotting of lysates derived from cells prior to and after serum stimulation with anti-pSer473 Akt. Lanes 1, 4, 7, and 10 represent serum-deprived cells; lanes 2, 3, 5, 6, 8, 9, 11, and 12 represent serum stimulation with 10% FBS for 5 min or 50 min.

B: Cell proliferation rate of Tsc2 heterozygous and Tsc2 KO MEFs. Cells (1 × 10^4) were seeded onto a 6 cm dish in 10% FBS and counted.

C: Proliferation rate of asynchronously growing Tsc2 heterozygous cells and Tsc2 KO MEFs, as measured by pulse labeling cells for 4 hr with BrdU prior to staining as described in the Experimental Procedures. BrdU incorporation was measured by counting at least 300 cells from at least five fields of view. The average of triplicate plates is presented. Error bars represent ±SD.

and examined their ability to overcome contact inhibition. As shown in Figure 6F, Tsc2 null MEFs formed foci on a confluent monolayer, which was diminished with increasing TSC2 expression despite increasing Akt activity. Finally, expression of activated Akt fully restored susceptibility to oncogenic transformation by activated Ras of *Tsc2$^{-/-}$* cells expressing WT Tsc2 but not of *Tsc2$^{-/-}$* cells expressing a Tsc2 mutant that cannot be phosphorylated by Akt (Figure S4). Together these results demonstrate that, when mTORC1 is constitutively active, Akt activity is no longer required to promote cell proliferation and susceptibility to oncogenic transformation. Because inhibition of TSC2 by Akt leads to the activation of mTORC1 and because mTORC1 activity is impaired in Akt-deficient cells, our results strongly suggest that mTORC1 is the most critical and possibly the exclusive downstream effector of Akt required for cell proliferation and susceptibility to oncogenic transformation.

Inhibition of mTORC1 in Tsc2 null cells via knockdown of Raptor elevates Akt activity but impairs cell proliferation and susceptibility to oncogenic transformation

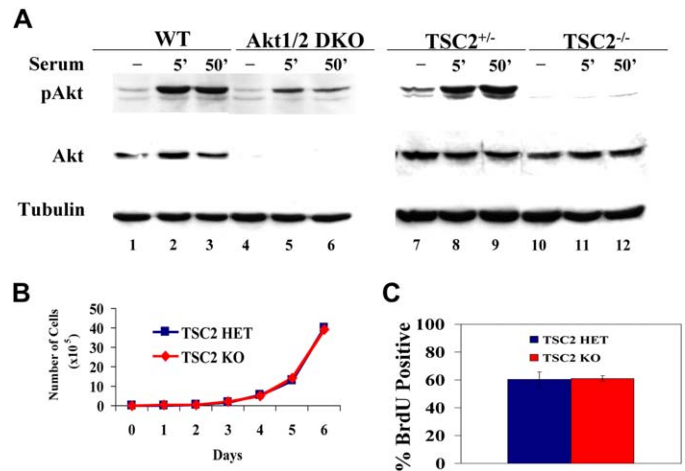To further substantiate the notion that mTORC1 mediates Akt's requirement for cell proliferation and susceptibility to oncogenic transformation, we first knocked down Raptor in Tsc2 null cells. Compared with control Tsc2 null cells, Raptor knockdown diminished mTORC1 activity but markedly elevated Akt activity (Figure 7A) due to recovery from the inhibitory feedback loop. Despite this elevated Akt activity, cell proliferation (Figure 7B) and susceptibility to Ras-mediated transformation (Figure 7C) were impaired. In a complementary experiment, we used rapamycin to inhibit mTORC1 activity in WT MEFs and found that it elevated Akt activity (Figure 7D) but reduced proliferation to the level observed in Akt1/Akt2 DKO MEFs (Figure 7E). In addition, rapamycin treatment inhibited the ability of WT MEFs to be transformed by Ras (Figure S3A). Thus, these results provide further compelling evidence that mTORC1 is the predominant downstream effector of Akt-mediated proliferation and oncogenic transformation.

Overexpression of activated Rheb restores normal cell proliferation and oncogenic transformation of Akt1/Akt2 DKO MEFs

The above results, together with our previous observation that mTORC1 activity is impaired in Akt1/Akt2 DKO MEFs (Hahn-Windgassen et al., 2005; Peng et al., 2003), suggest that the restoration of mTORC1 activity in Akt1/Akt2 DKO MEFs should also restore proliferation and susceptibility to oncogenic transformation. Because Rheb acts downstream of TSC2 to activate mTORC1, we have generated a polyclonal Akt1/Akt2 DKO MEF line ectopically expressing an activated form of Rheb (Li et al., 2004) to restore mTORC1 activity in these cells (Figure 8A). Although this form of Rheb did not constitutively activate mTORC1 as in TSC2-deficient cells (data not shown), its
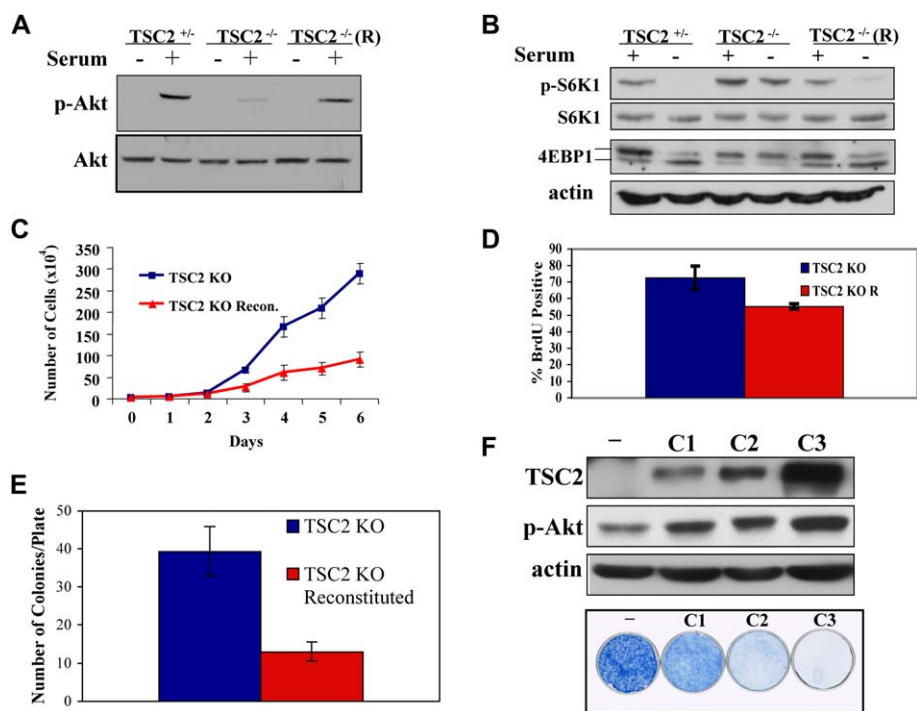
Figure 6. Reexpression of TSC2 in Tsc2 KO MEFs reduces mTORC1 activity with concomitant decrease in cell proliferation and oncogenic transformation capacity, despite elevated Akt activity

A: Akt activity in Tsc2 KO MEFs or Tsc2 KO MEFs expressing exogenous Tsc2. Immunoblot showing Akt activity as measured by the phosphorylation of Ser473 of Akt in $Tsc2^{+/-}$ MEFs, control $Tsc2^{-/-}$ MEFs, or $Tsc2^{-/-}$ MEFs expressing exogenous Tsc2 [$Tsc2^{-/-}$ (R)] after serum deprivation (−) followed by serum stimulation with 10% FBS for 30 min. The generation of the polyclonal Tsc2 KO cell line expressing exogenous Tsc2 or vector control is described in the Experimental Procedures.

B: mTORC1 activity in Tsc2 KO cells and Tsc2 KO cells expressing exogenous Tsc2. Immunoblot showing mTORC1 activity, as measured by S6K1 phosphorylation and 4EBP1 phosphorylation via mobility shift, in $Tsc2^{+/-}$ MEFs, control $Tsc2^{-/-}$ MEFs, or $Tsc2^{-/-}$ MEFs expressing exogenous Tsc2 [$Tsc2^{-/-}$(R)] in the presence or absence of serum.

C: Cell proliferation rates of Tsc2 KO MEFs or Tsc2 KO MEFs expressing exogenous Tsc2. Cells (2.5×10^4) were seeded onto a 6 cm dish in 10% FBS and counted. The experiment was conducted at least three times in triplicate. Error bars represent ±SD.

D: BrdU incorporation rate of Tsc2 KO MEFs and reconstituted Tsc2 KO MEFs. Asynchronously growing cells were pulse labeled for 4 hr with BrdU prior to fixation and staining. BrdU incorporation was measured by counting at least 300 cells from at least five fields of view in triplicate plates. Error bars represent ±SD.

E: Soft agarose assays for Tsc2 KO MEFs and reconstituted Tsc2 KO MEFs after transformation by H-Ras^{valine12}. Cells were infected with retrovirus expressing H-Ras^{valine12} and were subjected to soft agarose assay as described in Figure 3D. The experiment was performed at least three times in triplicate. Error bars represent ±SD.

F: Tsc2 level, Akt activity, and contact inhibition in clonal cell lines expressing exogenous Tsc2. Individual clonal cell lines expressing varying levels of Tsc2 were isolated as described in the Experimental Procedures. Upper panels: immunoblots showing relative expression of exogenous Tsc2 and Akt activity as measured using an antibody against pSer473 of Akt, with anti-B-actin serving as a control. Lane 1, $TSC2^{-/-}$; lane 2, clone #1 has a low level of TSC2 expression; lane 3, clone #2 has a moderate level of TSC2 expression; and lane 4, clone #3 has a high level of TSC2 expression. Bottom panel: contact inhibition and spontaneous foci formation of the clonal cell lines following 7–10 days of growth past confluency.

expression restored the proliferation of Akt1/Akt2 DKO MEFs to the level observed in WT cells and increased the proliferation rate of WT cells (Figure 8B). The restoration of cell proliferation by Rheb is inhibited by rapamycin (Figure S3D). Rheb also restored pRb phosphorylation upon the entry of Akt1/Akt2 DKO MEFs into the cell cycle (Figure 8C). Finally, Rheb restored the ability of Akt1/Akt2 DKO MEFs to be transformed by activated Ras (Figure 8D). Ectopic expression of Rheb in WT MEFs increased their ability to grow on soft agarose, as both the size and the number of colonies were increased, whereas Rheb expression in Akt1/2 DKO MEFs increased their ability to grow on soft agarose to the same level observed for WT MEFs (Figures 8D and 8E). Thus, we conclude that Akt promotes cell proliferation and oncogenic transformation predominantly through the activation of mTORC1.

Discussion

Lesions leading to the hyperactivation of Akt are probably the most frequent lesions occurring in human cancers (Hay, 2005; Hennessy et al., 2005). However, it is not completely understood why hyperactivation of Akt is required and how it contributes to the genesis of cancer. It is well established that activation of Akt inhibits apoptosis. However, the antiapoptotic activity of Akt, by itself, is unlikely sufficient to explain its frequent activation in human cancers, particularly since hyperactivated Akt alone is sufficient to transform cells in culture (Aoki et al., 1998) and to

induce tumorigenesis in vivo in transgenic mice (Majumder et al., 2003). Although activation of Akt has been implicated in increased cell proliferation, it is not known whether Akt is in fact required for cell proliferation and susceptibility to oncogenic transformation. How Akt affects these processes and the identity of critical downstream effectors also remain largely unknown. As such, we undertook genetic approaches to address these issues.

We first demonstrated that partial ablation of Akt activity by the knockout of Akt1 or both Akt1 and Akt2 genes in MEFs is sufficient to markedly impede cell cycle progression and to inhibit oncogenic transformation. Akt1 KO MEFs were significantly more resistant than WT MEFs to transformation induced by activated Ras together with Myc or with DN-p53. Akt1/2 DKO MEFs were much more resistant to oncogenic transformation than Akt1 KO MEFs, and this correlates directly with the relative decrease in total Akt activity. Interestingly, the proliferation rate of Akt2 KO MEFs was not impaired compared with WT MEFs. Akt2 deletion by itself reduced total Akt activity to a lesser extent than Akt1 deletion, suggesting that, although partial ablation of total Akt activity was sufficient to impair cell proliferation and oncogenic transformation, this reduced Akt activity needs to be above a certain threshold. Alternatively, it is possible that Akt1 and Akt2 are functionally distinct with respect to cell proliferation and oncogenic transformation. However, we do not have any experimental evidence to support this alternative, and our results suggest that the effect of Akt1 and Akt2 on cell proliferation
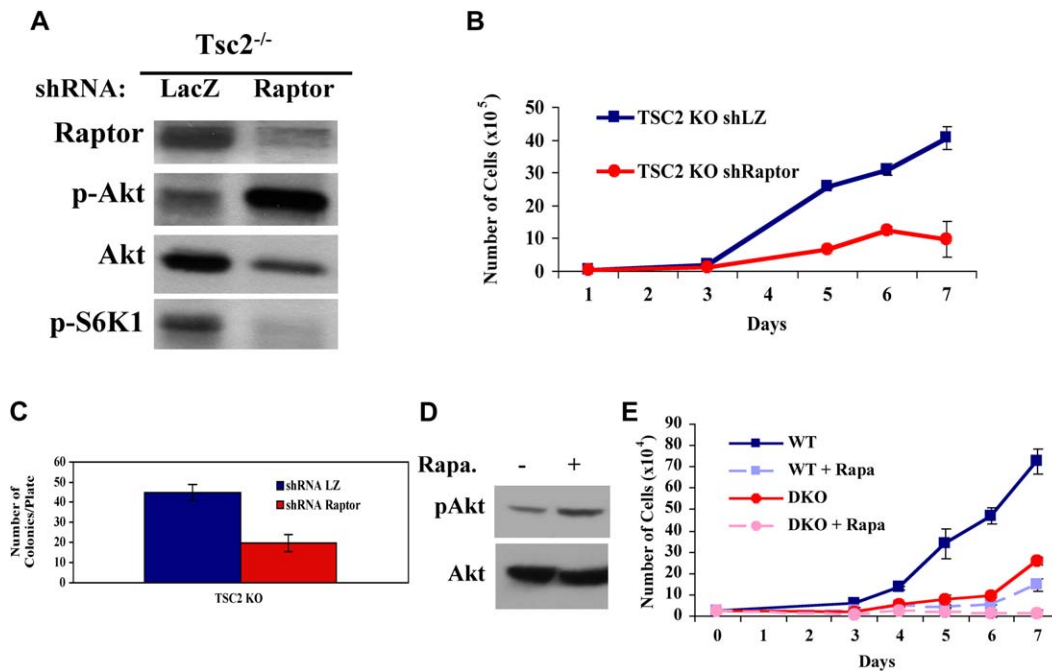
Figure 7. Reducing mTORC1 activity by Raptor knockdown, or rapamycin treatment, decreases cell proliferation and oncogenic transformation despite elevated Akt activity

A: The knockdown of Raptor in Tsc2-KO MEFs following infection with lentivirus expressing Raptor shRNA is described in the Experimental Procedures. Cell lysates derived from Tsc2-KO MEFs expressing Raptor shRNA or control Tsc2-KO MEFs expressing LacZ shRNA were subjected to immunoblotting using anti-Raptor, anti-pSer473 Akt, anti-total Akt, and anti-pS6K1.

B: Proliferation rate of LacZ shRNA- or Raptor shRNA-expressing Tsc2 KO MEFs. Cells (2.5×10^4) were seeded in a 6 cm dish in 10% FBS and counted. The experiment was conducted at least three times in triplicate. Error bars represent ±SD.

C: Soft agarose assays for Tsc2 KO MEFs expressing LacZ shRNA or Raptor shRNA following transformation by H-Ras^{valine12}. Cells were infected with retrovirus expressing H-Ras^{valine12} and were subjected to the soft agarose assay as described in Figure 3D. The experiment was performed at least three times in triplicate. Error bars represent ±SD.

D: Akt activity following rapamycin treatment. DN-p53-immortalized WT MEFs were treated with vehicle or 20 nM rapamycin for 72 hr and then analyzed by immunoblotting using anti-pSer473 Akt or anti-total Akt.

E: Growth rate following rapamycin treatment. Wild-type or Akt1/2 DKO immortalized MEFs (2.5×10^4) were seeded onto a 6 cm dish in the absence or presence of 20 nM rapamycin and counted. The experiment was performed at least three times in triplicate. Error bars represent ±SD.

and oncogenic transformation is additive. Nevertheless, because our results are limited to MEFs in vitro, we cannot exclude the possibility that Akt1 and Akt2 are functionally distinct in other cell types or during tumorigenesis in vivo.

Because Akt phosphorylates MDM2 and enhances its ability to degrade p53 (Mayo and Donner, 2001; Zhou et al., 2001), we analyzed the steady-state level and half-life of p53 in WT versus Akt1/2 DKO MEFs and found no significant difference. Thus, the remaining Akt3 activity may be sufficient to phosphorylate MDM2. Indeed, we found that the requirements of Akt activity for cell proliferation and for transformation with activated Ras are independent of p53 because Akt1 KO or Akt1/Akt2 DKO MEFs immortalized by expressing DN-p53 or p53 shRNA were still impaired in their ability to proliferate and to be transformed with activated Ras. Interestingly, these effects of Akt on cell proliferation and susceptibility to oncogenic transformation appear to be independent of its antiapoptotic activity. First, we found no significant spontaneous increase in apoptosis in Akt-deficient MEFs when they were cultured in the presence of 10% FBS. Second, Akt-deficient MEFs were still resistant to oncogenic transformation even when Bcl-2 was ectopically expressed. We therefore conclude that, in addition to its antiapoptotic activity, Akt is required for cell proliferation and oncogenic transformation regardless of p53 status.

To verify whether Akt deficiency is also sufficient to confer resistance to tumorigenesis in vivo, we first determined whether Akt1 KO mice were resistant to tumors induced by activated Ras. We therefore crossed Akt1 KO mice with transgenic MMTV-v-H-Ras mice, which develop salivary and mammary gland tumors. Akt1 deletion was sufficient to substantially delay the onset of tumors and to profoundly increase the resistance to tumors induced by activated Ras. Additionally, Akt1 KO mice were markedly more resistant to skin carcinogenesis than wild-type mice. Thus, the conclusions based on the ex vivo results with MEFs could be extended to epithelial tumors in vivo in mice.

It was recently shown that Akt1 deficiency is sufficient to significantly attenuate tumor development induced by PTEN deficiency (Chen et al., 2006). However, the results presented here suggest that partial Akt ablation could be used as a general approach to inhibit tumorigenesis induced by lesions, which do not directly activate Akt. Furthermore, because Akt1 KO mice are not impaired in their life span and may possibly live longer than wild-type mice (Chen et al., 2006), these results suggest that partial ablation of Akt could be used as a therapeutic approach for cancer without eliciting severe physiological consequences. Notably, it was recently suggested that Akt1 suppresses metastasis (Wyszomierski and Yu, 2005). However,
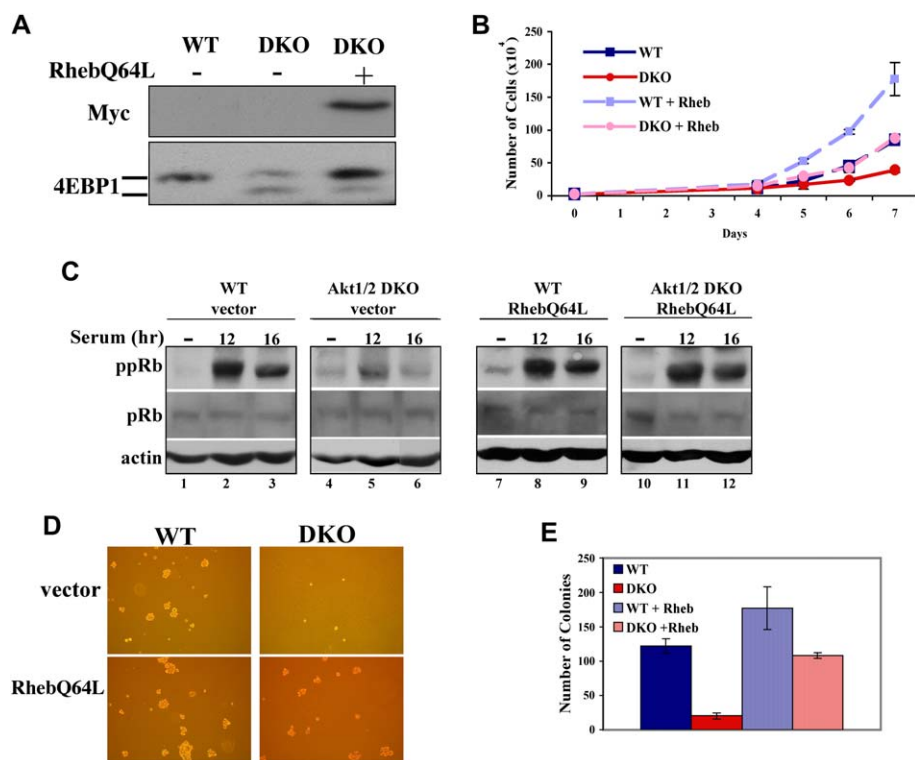
A

WT DKO DKO

RhebQ64L − − +

Myc

4EBP1

B

Number of Cells (×10⁴)

250
200
150
100
50
0

0 1 2 3 4 5 6 7 Days

- WT
- DKO
- WT + Rheb
- DKO + Rheb

C

WT vector | Akt1/2 DKO vector | WT RhebQ64L | Akt1/2 DKO RhebQ64L

Serum (hr) − 12 16 − 12 16 − 12 16 − 12 16

ppRb

pRb

actin

1 2 3 4 5 6 7 8 9 10 11 12

D

WT DKO

vector

RhebQ64L

E

Number of Colonies

250
200
150
100
50
0

- WT
- DKO
- WT + Rheb
- DKO +Rheb

Figure 8. Proliferation and transformation defects in Akt-deficient cells are rescued by overexpressing Rheb

A: mTOR activity in Akt1/2 DKO MEFs expressing RhebQ64L. Cell lysates of asynchronously growing DN-p53-immortalized WT MEFs expressing empty vector, Akt1/2 DKO MEFs expressing empty vector, or Akt1/2 DKO MEFs expressing Myc-tagged RhebQ64L were subjected to immunoblotting with anti-Myc and with anti-4EBP1 to determine the mobility shift.

B: Proliferation rate of DN-p53-immortalized WT and Akt1/2 DKO MEFs expressing empty vector or Myc-tagged RhebQ64L. Cells (2.5×10^4) were seeded onto a 6 cm dish in triplicate in 10% FBS and counted. The experiment was conducted at least three times on three independently derived cell lines. Error bars represent ±SD.

C: Phosphorylation of pRb upon cell cycle entry. Immunoblotting of cell lysates derived from WT and Akt1/2 DKO immortalized MEFs expressing empty vector or Myc-tagged RhebQ64L using antibodies against pSer780 of pRb, total pRb, and β-actin. Lanes 1, 4, 7, and 10 represent cells depleted of serum for 72 hr. Lanes 2, 3, 5, 6, 8, 9, 11, and 12 represent time points (in hr) following release into 10% FBS.

D: Soft agarose assays following H-Ras^valine12 expression in wild-type and Akt1/2 DKO immortalized MEFs expressing empty vector or Myc-tagged RhebQ64L. Cells were infected with retrovirus expressing H-Ras^valine12 and subjected to soft agarose assay as described in Figure 3D. Shown are representative fields from each cell line at the time of quantitation.

E: Quantitation of colonies counted in triplicate. The experiment was performed three times on independently derived cell lines. Error bars represent ±SD.

our preliminary results suggest that the deficiency of Akt1 inhibits not only tumor development but also metastasis because, thus far, every MMTV-v-H-Ras mouse that was examined developed lung metastasis, whereas in *Akt1⁻/⁻MMTV-v-H-Ras* mice, which developed tumors, we did not observe metastasis.

To elucidate the mechanism by which Akt affects cell cycle progression, we analyzed proteins that regulate entry into and progression of the cell cycle. We found that pRb phosphorylation by both CDK2 and CDK4 is significantly impaired in Akt-deficient cells. However, among the cell cycle regulators that were tested only the induction of cyclin D1 was impaired upon cell cycle entry, and the p27 level was slightly elevated in proliferating Akt-deficient cells. Because the reduction in cyclin D1 level was only observed upon cell cycle entry, it cannot explain the reduced proliferation and the resistance to oncogenic transformation of Akt-deficient cells. Furthermore, cyclin D1 KO MEFs are not impaired in their ability to be transformed by activated Ras (Yu et al., 2001). Interestingly, two evolutionarily conserved downstream effectors of Akt, FOXO and mTORC1, have been shown to affect the expression of both p27 and cyclin D1 by different mechanisms. Activation of FOXO transcription factors, which is inhibited by Akt, or the inhibition of mTORC1, which is activated by Akt, elevate p27 expression and decrease cyclin D1 expression (Gera et al., 2004; Luo et al., 1996; Schmidt et al., 2002; Stahl et al., 2002).

The interrelationships between Akt and mTOR (Hay, 2005), and the observations that the inhibition of mTORC1 attenuates

cell cycle progression and inhibits pRb phosphorylation (Luo et al., 1996) and that mTORC1 activity is impaired in Akt-deficient cells (Hahn-Windgassen et al., 2005; Peng et al., 2003), led us to examine mTORC1 as a potential downstream effector of Akt required for cell proliferation and oncogenic transformation. We provided strong evidence, through a series of experiments, that Akt could mediate cell proliferation and oncogenic transformation exclusively through mTORC1. We took advantage of the feedback inhibition of Akt activity in Tsc2 KO MEFs to show that, when mTORC1 was hyperactivated, Akt activity was no longer required for cell proliferation or oncogenic transformation, in contrast to its requirement when mTORC1 is not hyperactivated. Thus, despite a marked reduction in Akt activity and FOXO activation (Manning et al., 2005), Tsc2-deficient cells and Tsc2-proficient cells are equivalent with regard to proliferation and susceptibility to oncogenic transformation. In contrast, Akt could not promote cell proliferation and oncogenic transformation when mTORC1 was inhibited, as ectopic expression of TSC2 or knockdown of Raptor in Tsc2-deficient cells inhibited mTORC1, with concomitant reduction in cell proliferation and oncogenic transformation, despite elevated Akt activity. Finally, ectopic expression of activated Rheb rescued the phenotype of Akt-deficient cells by restoring mTORC1 activity in these cells.

Our results showing that mTORC1 activation was sufficient to recapitulate the functions of Akt in regards to cell proliferation and oncogenic transformation are consistent with genetic analysis in *Drosophila* showing that Tsc1 and Tsc2 are epistatic to

Pten and that cells doubly deficient for *Pten* and *Tor* are indistinguishable from cells lacking *Tor* alone (Zhang et al., 2000). The results are also consistent with the observation that most changes in gene expression upon induction of glioblastoma in mice by activated Ras or Akt occur at the level of mRNA translation (Rajasekhar et al., 2003). Nevertheless, the exact mechanism(s) by which mTORC1 affects cell proliferation and oncogenic transformation and the mechanism by which it compensates for Akt activity are not clear. The reported effects of mTORC1 on p27 and cyclin D1 (Gera et al., 2004; Luo et al., 1996) could, at least in part, provide a mechanistic explanation. It is likely, however, that mTORC1 may have a much more global effect by accelerating general protein synthesis, as protein synthesis determines cell mass, which in turn could determine entry into the cell cycle and consequent proliferation. Additionally, although speculative, it is possible that hyperactivation of S6K1 by mTORC1 could compensate at least to some extent for the loss of Akt activity. Given that proteins targeted by the S6K1 and Akt kinases have similar consensus phosphorylation motifs, hyperactivation of S6K1 could lead to the phosphorylation of certain proteins that are normally phosphorylated by Akt. For instance, both Akt and S6K1 phosphorylate serine 136 of Bad (Harada et al., 2001).

Interestingly, as we have previously shown, Akt can activate mTORC1, at least in part, by maintaining cellular energy metabolism (Hahn-Windgassen et al., 2005). Since the ability of Akt to inhibit apoptosis is coupled to its effect on cellular energy metabolism (Robey and Hay, 2005), it is possible that the major impact of Akt on tumorigenesis is via its effect on energy metabolism.

Finally, our results could have important implications for cancer therapy. First, the results suggest that even partial ablation of Akt activity may be sufficient to inhibit cancer development. Second, the results could imply that the use of rapamycin derivatives for cancer therapy should be sufficient to inhibit excess proliferation and tumorigenesis induced by the activation of Akt as well as by other oncogenes, even in the absence of functional p53. However, rapamycin treatment, unlike the inhibition of Akt, alone might not be sufficient to overcome the antiapoptotic activity of Akt, as rapamycin elevates rather than inhibits Akt activity.

Experimental procedures

Cells and viruses

Akt1 KO, Akt2 KO, and $Akt1^{+/-}Akt2^{+/-}$ mice (Chen et al., 2001; Peng et al., 2003) were backcrossed to a C57BL/6 background for six generations. WT, Akt1 KO, Akt2 KO, and Akt1/2 DKO primary MEFs were harvested from E13.5 embryos as previously described (Chen et al., 2001; Peng et al., 2003). Experiments with primary cells were performed no later than passage 5. To immortalize MEFs, primary cells were infected with a retrovirus (pBabePuro-GSE56) expressing dominant-negative p53 (Zou et al., 2002). Following infection and selection with 2 μg/ml puromycin, stable polyclonal cell lines were generated. Immortalization by p53 knockdown was done following infection with a lentivirus, Plenti6/blockitdest (Invitrogen), expressing p53 shRNA. Following infection, selection with 7 μg/ml of blasticidin was done to generate polyclonal cell lines. $Tsc2^{+/-}$ and $Tsc2^{-/-}$ MEFs were previously described (Hahn-Windgassen et al., 2005). Cells were routinely cultured in DMEM containing 10% FBS.

Retroviruses were generated following transient transfection of retroviral vector into Phoenix ecotropic packaging cell line. Retrovirus infection was carried out for 6–12 hr in the presence of 8 μg/ml polybrene. Antibiotic selection was carried out 48 hr later until complete loss of uninfected cells, to generate polyclonal cell lines. To generate clonal cell lines expressing different levels of Tsc2, individual clones were isolated and propagated. Lentiviruses were generated using ViraPower Lentiviral Expression System (Invitrogen). The individual retroviral and lentiviral vectors used are described in the Supplemental Data.

Cell proliferation rate

Primary (5×10^4) or immortalized (2.5×10^4) MEFs were plated on 6 cm dishes and were counted daily. Cells were trypsinized for counting using a hemacytometer. Primary MEFs were stained with a 0.4% trypan blue solution and counted with a hemacytometer; positive and negative cells were scored to distinguish between dead and live cells, respectively. Experiments were conducted on cells isolated from independent sets of littermates. At least three experiments, in triplicate, were performed for each cell line.

BrdU incorporation

Cells (2.5×10^5) were plated in each well of a 6-well dish. Cells were starved in 0.1% FBS in DMEM for 48–72 hr and then were stimulated to reenter the cell cycle by addition of 10% FBS in DMEM. At each time point, cells were pulse labeled with 3 μg/ml BrdU for 1 hr and then fixed with 70% ethanol. For the proliferation assay, asynchronously growing MEFs were pulse labeled with 3 μg/ml BrdU for 4 hr and then fixed with 70% ethanol. Cells were permeabilized, and DNA was denatured for 1 hr with 2 M HCl containing 0.5% Triton X-100, followed by neutralization with 0.1 M sodium borate and rinsing with PBS. Cells were then incubated overnight with mouse anti-BrdU (Dako; diluted 1:200). Cells were rinsed with PBS and then incubated for 2 hr with FITC-conjugated anti-mouse IgG (Vector; 1:200) and were counterstained with DAPI prior to counting.

Cotransformation focus formation assays

Primary MEFs (1×10^5) were plated in a 6 cm dish for infection with pL56-Ras or LMIRMyc-Ras retrovirus. Following infection, cells were allowed to grow 7–10 days past confluency. Plates were rinsed with PBS, fixed with methanol, dried, and then stained with a 2% methylene blue solution (in 50% methanol in H_2O). The total number of foci per plate was scored. Experiments were performed in triplicate with cells derived from three different groups of littermates.

Soft agarose assays

Cells (2.5×10^5) were resuspended in 10% FBS in DMEM containing 0.35% agarose and plated onto a layer of 0.7% agarose-containing medium in a 6-well dish. Once the medium was solidified, 10% FBS in DMEM was added and was changed weekly. For Ras transformation, polyclonal cell lines were generated by infecting immortalized MEFs with pBabe-hygro or pBabe-hygro-H-Ras^{valine12} retrovirus and were selected with 150 μg/ml hygromycin until all uninfected cells were dead. For coexpression of Bcl-2, cells were reinfected with high-titer pBabeGFP-Bcl-2 or pBbabeGFP retroviruses. Colonies were quantitated by counting every colony on a plate or colonies from ten random fields. Experiments were performed three times, in triplicate, using independently derived cell lines.

Protein analysis

Protein cell lysates were prepared as previously described (Hahn-Windgassen et al., 2005). SDS-PAGE gels (6%, 8%, 10%, or 12%) were used depending on the protein of interest. Anti-cyclin D1, anti-cyclin D2, anti-cyclin D3, and anti-cyclin B were from Neomarkers. Anti-total pRb was from BD Pharmingen or Neomarkers. Phosphorylation-specific antibodies for pan-Akt, pSer473 Akt, S6K1, pT389S6K1, pSer780pRb, and pSer807/811pRb were from Cell Signaling Technology. Anti-p27 was from BD Biosciences. Anti-CDK4, anti-cyclin E, and anti-tuberin (TSC2) were purchased from Santa Cruz Biotechnology. Anti-p53 was from Calbiochem (Oncogene Research), and anti-β-actin was from Sigma. Anti-Raptor and anti-4EBP1 were kindly provided by Nahum Sonenberg (McGill University). HRP-conjugated antibodies (rabbit anti-mouse, goat anti-rabbit, and rabbit anti-sheep) were from Zymed.

Skin carcinogenesis protocol

Seven-week-old littermates, WT or Akt1 KO C57BL/6:129sv (1:1) mice, were shaved on the dorsal surface and allowed to recover for 48 hr. A one-time dose of 25 μg DMBA (Sigma) in 200 μl of ethanol was applied to the shaved

surface. After 1 week, 3.7 μg of TPA (LC Laboratories) in 200 μl of ethanol was applied twice weekly for the duration of the experiment. Each week, calipers were used to determine the size of each tumor. Nine mice per genotype were subjected to this protocol. Mice of each genotype that received vehicle alone did not develop tumors.

Monitoring mammary and salivary gland tumors in MMTV-v-H-Ras transgenic mice

MMTV-v-H-Ras mice (Sinn et al., 1987) were purchased from Charles River. *Akt1*^{+/−}/*MMTV-v-H-Ras* mice were intercrossed to generate *Akt1*^{+/+}/*MMTV-v-H-Ras* and *Akt1*^{−/−}/*MMTV-v-H-Ras* mice. Mice were monitored weekly for tumor appearance.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures and four supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/10/4/269/DC1/.

Acknowledgments

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