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Novel roles of Akt and mTOR in suppressing TGF-β/ALK5-mediated Smad3 activation

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Insulin-like growth factor-I inhibits transforming growth factor-β (TGF-β) signaling by blocking activation of Smad3 (S3), via a phosphatidylinositol 3-kinase (PI3K)/Akt-dependent pathway. Here we provide the first report that the kinase activity of Akt is necessary for its ability to suppress many TGF-β responses, including S3 activation and induction of apoptosis. Wild-type and myristoylated Akts (Akt^{WT} and Akt^{Myr}) suppress TGF-β-induced phospho-activation of S3 but not Smad2 (S2), whereas kinase-dead Akt1 ($Akt1^{K179M}$) or dominant-negative PI3K enhances TGF-β-induced phospho-activation of both S2 and S3. Using siRNA, rapamycin (Rap), and adenoviral expression for FKBP12-resistant and constitutively active TGF-β type I receptor (ALK5), we demonstrate that mammalian target of Rap (mTOR) mediates Akt1 suppression of phospho-activation of S3. These and further data on Akt1-S3 binding do not support a recently proposed model that Akt blocks S3 activation through physical interaction and sequestration of S3 from TGF-β receptors. We propose a novel model whereby Akt suppresses activation of S3 in an Akt kinase-dependent manner through mTOR, a likely route for loss of tumor suppression by TGF-β in cancers.

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Introduction

Transforming growth factor-β (TGF-β) is a 25 kDa multifunctional autocrine/paracrine growth regulator belonging to the large TGF-β superfamily (Roberts and Sporn, 1990; Massague, 1992), and may function as either a tumor suppressor in normal or preneoplastic epithelia or a tumor promoter in a variety of late-stage carcinomas (Guo and Kyprianou, 1999; Tang *et al*, 1999; Wakefield and Roberts, 2002; Song *et al*, 2003a; Danielpour, 2005). TGF-β1 signals mainly through two cell surface signaling receptors, TGF-β receptor type I (TβRI) and TβRII, whereby binding of ligand to TβRII promotes receptor heteromerization (Massague, 1992; ten Dijke *et al*, 1996), allowing the TβRII kinase to activate TβRI (Wieser *et al*, 1995). TβRI then activates S2 and S3 by phosphorylating their C-terminal SSXS serines (Abdollah *et al*, 1997), a process shown to involve accessory proteins (Tsukazaki *et al*, 1998). This causes the receptor-activated Smads to multimerize (Wu *et al*, 1997) and then translocate to the nucleus, where they activate gene transcription (Xiao *et al*, 2000).

Akt, which relays signals downstream of phosphatidylinositol 3-kinase (PI3K), is emerging as a central player in the tumorigenesis and pathogenesis of a variety of human cancers (Vivanco and Sawyers, 2002). Akt activation is a multistep process, involving both membrane translocation and phosphorylation. PI3K, which is commonly activated by receptor tyrosine kinases, catalyzes the addition of a phosphate moiety to the D3 position of phosphatidylinositol-4-phosphate. This generates phosphatidylinositol 3,4-diphosphate, which is necessary for the membrane anchor of Akt and PDK1, two interacting PH domain proteins. PDK1 phosphorylates Akt at Thr308, whereas a yet unidentified kinase (PDK2) or a mammalian target of Rap (mTOR) complex phosphorylates Akt at Ser473, leading to full activation of Akt (Downward, 1998; Nicholson and Anderson, 2002; Sarbassov dos *et al*, 2005). Recent studies have unveiled a growing list of Akt substrates (Nicholson and Anderson, 2002) that cooperate to prevent apoptosis and/or promote cell proliferation (Plas and Thompson, 2003; Xu *et al*, 2004).

We previously reported that physiological levels of insulin-like growth factor-I (IGF-I) function through a PI3K-dependent pathway to block several TGF-β-mediated responses, including gene transcription, apoptosis, and Smad3 (S3) activation (Song *et al*, 2003b; Danielpour, 2005), using the well-established NRP-152 non-tumorigenic rat prostate epithelial cell line model developed in our laboratory (Danielpour *et al*, 1994; Danielpour, 1996). The LR^3 analog of IGF-I (LR^3-IGF-I), which binds poorly to IGF-I binding proteins, blocks TGF-β responses by suppressing the phospho-activation of S3 independent of changes in TGF-β receptor expression or changes in the expression of Smad(S)s 2, 3, or 4 (Song *et al*, 2003b). Recently, two other groups also reported that Akt suppresses TGF-β responses, through a mechanism involving the direct binding of Akt to S3, which blocks activation of S3 by sequestering S3 from TβRI (Conery *et al*, 2004; Remy *et al*, 2004). In the current report, we show a different mechanism of Akt suppression of S3, involving the Akt target mTOR.

mTOR is an *in vivo* target for the complex of rapamycin (Rap) with its intracellular receptor, FKBP12 (Fingar and Blenis, 2004). As a member of the PIK-related family of large protein kinases, mTOR controls the phosphorylation of at least two regulators of protein synthesis and cell growth, S6 kinase 1 (S6K) and eIF-4E binding protein (4E-BP1) (Bjornsti and Houghton, 2004; Fingar and Blenis, 2004). Along with PI3K/Akt axis, mTOR pathway is emerging as a

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pivotal regulator of cell growth in response to hormones, nutrition, and growth factors. PI3K-dependent signaling has been implicated in the regulation of mTOR and S6K, and Akt-dependent phosphorylation has also been reported to result in the phosphorylation of common downstream target proteins (Gao *et al*, 2002; Manning and Cantley, 2003; Plas and Thompson, 2003; Tee *et al*, 2003; Fingar and Blenis, 2004).

In our model, Akt blocks phospho-activation of S3 by an Akt kinase-dependent mechanism through mTOR and also blocks TGF-β signals downstream of S3 activation, but through a mechanism that does not require the kinase activity of Akt or mTOR. This is the first direct evidence for roles of Akt kinase and mTOR as suppressors of S3 phospho-activation.

Results

IGF-I, which is strongly implicated in the pathogenesis of prostate cancer (Grimberg, 2003; Renehan *et al*, 2004), is critical to survival and growth of NRP-152 cells, a non-tumorigenic rat prostatic epithelial cell line established in our laboratory (Danielpour *et al*, 1994; Hsing *et al*, 1996; Danielpour, 1999; Hayward *et al*, 1999). Similar to normal prostate epithelium, NRP-152 cells are exquisitely sensitive to the induction of apoptosis by TGF-β (Hsing *et al*, 1996; Stewart *et al*, 2003). Moreover, dominant-negative (DN)-TβRII promotes malignant transformation of these cells, supporting a role of TGF-β in tumor suppression of the prostate (Tang *et al*, 1999). Importantly, IGF-I blocks the ability of TGF-β to induce their apoptosis. We have recently reported that IGF-I specifically blocks TGF-β-induced phospho-activation of Smad3 (S3) but not Smad2 (S2), and we have confirmed roles for PI3K and Akt in mediating this IGF-I effect (Song *et al*, 2003b). We now show that this effect does not require *de novo* protein synthesis, as cycloheximide does not block the ability of IGF-I to suppress TGF-β-induced phospho-S3 (Supplementary Figure 1S).

We have further analyzed the mechanism by which Akt1 kinase inhibits TGF-β responses, using the NRP-152 cell model. We first compared the abilities of wild-type (WT), constitutively active (CA) myristoylated (Myr; N-terminal fusion with src aa 1–11), and kinase-dead (KD) (K179M mutant) Akt1 constructs to control transcriptional responses by TGF-β1. These cells were transiently co-transfected with the above expression constructs along with a plasminogen activator inhibitor-I (PAI-I) promoter reporter construct, 3TP-lux (Figure 1A and B). Enforced expression of both $Akt1^{WT}$ and $Akt1^{Myr}$ inhibited TGF-β-induced PAI-I promoter activity by about two- and seven-fold, respectively, whereas $Akt1^{K179M}$, which functions as a DN of Akt kinase, instead slightly enhanced the response to TGF-β (Figure 1A). Increased expression of $Akt1^{K179M}$ by greater transfection efficiency more effectively enhanced TGF-β-induced 3TP-lux activity (Figure 1B), similar to DN-PI3K (Song *et al*, 2003b), indicating that the Akt1 kinase is required for suppression of this promoter activity by Akt1. Suppression of TGF-β-induced 3TP-lux promoter activity also occurred by the two other Akt isoforms (Figure 1C). Consistent with the dependence on Akt kinase activity, the PI3K inhibitor LY294002 (LY) reversed the suppression of TGF-β-induced 3TP-lux by either $Akt1^{WT}$ or $Akt1^{Myr}$ (Figure 1D). Rap, an mTOR inhibitor, which we previously used to reverse the IGF-I suppression of TGF-β-induced 3TP-lux and S3 activation in NRP-152 cells (Song *et al*, 2003b), also reversed Akt1 suppression of TGF-β-induced 3TP-lux (Figure 1D). Together, these results support a role for the kinase domain of Akt1 in the suppression of TGF-β responses.

To further confirm that the kinase activity of $Akt1^{WT}$ and $Akt1^{Myr}$ is necessary for the suppression of TGF-β-induced 3TP-lux promoter activity, we developed additional constructs of $Akt1^{WT}$ and $Akt1^{Myr}$ with mutations at the kinase domain (K179M) and/or phosphorylation sites (T308A and S473A). $Akt1^{WT}$ mutated at K179M or at T308A ($Akt1^{K179M}$ or $Akt1^{T308A}$, respectively) were unable to block TGF-β-induced 3TP-lux, whereas the S473A mutant ($Akt1^{S473A}$), similar to $Akt1^{WT}$, suppressed 3TP-lux activity induced by TGF-β1 (Figure 1E). $Akt1^{Myr}$ constructs mutated at K179M ($Akt1^{Myr/K179M}$) or T308A ($Akt1^{Myr/T308A}$) were kinase-inactive (as measured by phosphorylation of an Akt1 substrate, GSK-3α/β) and were unable to suppress TGF-β-induced 3TP-lux, whereas the S473A form ($Akt1^{Myr/S473A}$) was just as active as $Akt1^{Myr}$ in suppressing the induction of 3TP-lux by TGF-β and phosphorylating GSK-3α/β (Figure 1F). $Akt1^{Myr/T308A/S473A}$ was biologically indistinguishable from $Akt1^{Myr/T308A}$, indicating that S473 does not contribute significantly to suppression of this TGF-β response or phosphorylation of GSK-3α/β. Importantly, $Akt1^{K179M}$, $Akt1^{Myr/K179M}$, and $Akt1^{Myr/T308A}$ significantly enhanced this TGF-β activity (Figure 1E–G and Supplementary Figure 2S), in contrast to that of a previous report, where the 'kinase-dead' form of Akt^{Myr} was functionally indistinguishable from kinase-active Akt^{Myr} in Hep3B cells (Conery *et al*, 2004). Our observations that the kinase activity of Akt is required for suppressing this TGF-β response extend to other highly TGF-β responsive cell lines, such as NRP-154 prostatic line (Figure 1G) and Mv1Lu mink lung line (Figure 1H). Together, our data provide strong support that the kinase activity of Akt1 is critical for its suppression of TGF-β responses.

To study the individual roles of PI3K and Akt1 in suppressing the phospho-activation of S3, we used adenoviral constructs for efficient and rapid delivery of $Akt1^{WT}$, $Akt1^{Myr}$, $Akt1^{K179M}$, DN-PI3K, or CA-PI3K in NRP-152 cells. $Akt1^{Myr}$, CA-PI3K, LR^3-IGF-I, and, to a lesser extent, $Akt1^{WT}$ all suppressed the TGF-β1 activation of S3 but not of S2, whereas DN-PI3K and $Akt1^{K179M}$ substantially enhanced both phospho-S3 and phospho-S2 levels (Figure 2A–D). The expression levels of total Akt1, phospho-Akt1 (Ser473), phospho-Akt1 (Thr308), and phospho-GSK-3α/β (Ser21/9) were as expected for each treatment. None of these constructs altered expression of total S2, S3, and S4. The enhanced phospho-activation of S2 by either DN-PI3K or $Akt1^{K179M}$ was unexpected, as Akts did not suppress phospho-S2 levels even at early TGF-β1 treatment times (Figure 2B and C). Neither DN-PI3K nor $Akt1^{K179M}$ alone induced phosphorylation of S3 or S2 (Figure 2D). These results showed that activation of either PI3K or Akt alone is sufficient for suppression of S3 activation, and that the kinase activity of Akt is essential for such suppression. Moreover, S2 is also regulated by the Akt kinase, but likely through a mechanism or stoichiometry different from that of S3. Collectively, these data strongly support that the Akt suppression of the phospho-activation of S3 and possibly that of S2 occurs downstream of the Akt kinase.

Adenoviral-mediated gene delivery was also used to assess the biological end points of PI3K and Akt on TGF-β1-induced

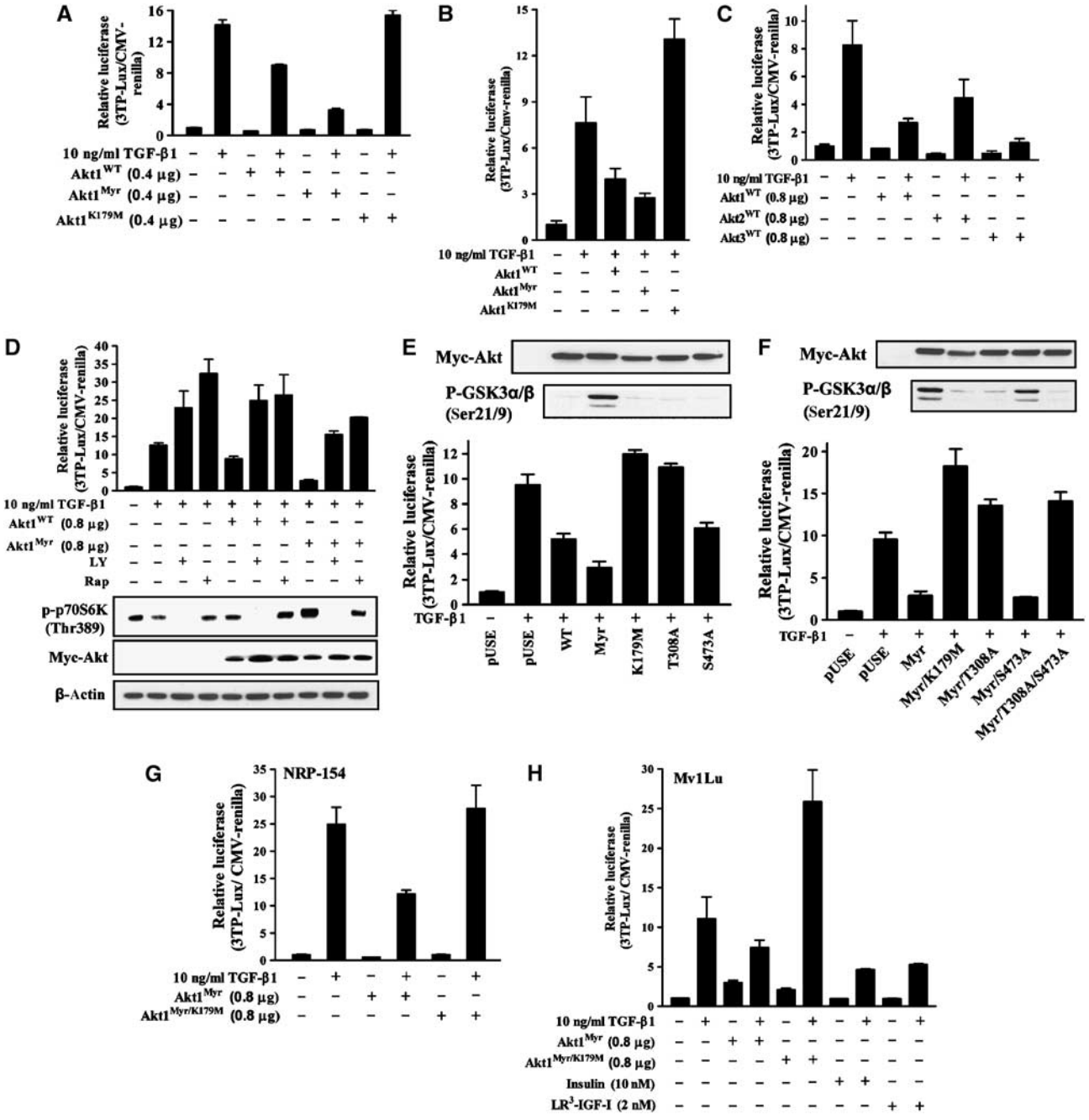


Figure 1 Akt kinase activity is essential for suppression of TGF-β signaling by Akt in NRP-152, NRP-154, and Mv1Lu cells. (**A–F**) NRP-152 cells were transiently transfected in 12-well plates with 1 μg of DNA, which included 3TP-lux, CMV-renilla plasmids, and either pUSE empty vector, pUSE-Myc-Akt1s (WT, Myr, or kinase-dead (K179M)) (A, B, D), pCDNA3 empty vector, or pCDNA3-Myc-Akts 1–3 (C), followed by treatment with LY (10 μM) or Rap (200 nM) (D). Expression of Myc-Akt1s and phospho-p70S6 kinase (Thr389) is shown in panel D. Separately, cells were transfected with $Akt1^{WT}$, $Akt1^{Myr}$, $Akt1^{WT}$ mutants (K179M, T308A, S473A) (E), or $Akt1^{Myr}$ mutants (Myr/K179M, Myr/T308A, Myr/S473A, Myr/T308A/S473A) (F). Expression of these Akt mutants and their kinase activity were confirmed by immunoblotting for Myc and phospho-GSK-3α/β (Ser21/9), respectively. (**G, H**) NRP-154 (G) and Mv1Lu (H) were co-transfected with pUSE empty vector, pUSE-$Akt1^{Myr}$, or pUSE-$Akt1^{Myr/K179M}$. For the Mv1Lu cells, IGF-I or insulin pretreatments were added after transfection (H). Cells were then incubated overnight and cultured for an additional 24 h in the presence or absence of TGF-β1 (10 ng/ml) before assay. A dual luciferase assay was performed, and data shown are averages (±s.d.) of triplicate independent measurements of Firefly/*Renilla* luciferase readings normalized to untreated controls. Results are representative of two to three different experiments (A–H).

growth suppression and apoptosis. Impressively, CA-PI3K and $Akt1^{Myr}$ blocked TGF-β1-induced downregulation of cyclin D2 (Figure 2E), cell death (Figure 2F), and apoptosis (by intranucleosomal DNA fragmentation; Figure 2G), whereas $Akt1^{K179M}$ was ineffective in suppressing the above responses of TGF-β1 (Figure 2). Rather, $Akt1^{K179M}$ alone killed cells, especially >24 h of infection, consistent with Akt kinase being antiapoptotic (Yamaguchi and Wang, 2001; Jetzt *et al*, 2003). The ability of Akt to reverse the suppression of cyclin D2 expression by TGF-β suggests that Akt also reverses growth suppression by TGF-β. We used flow cytometry to analyze how Akt affected TGF-β responses on cell cycle

distribution. However, under the above growth conditions, NRP-152 cells are essentially growth arrested (G1/G0), and these cells require both high serum and insulin ($\geqslant 1\,\mu M$) or IGF-I ($\geqslant 2$ nM) to proliferate (Hsing *et al*, 1996). Thus, to test the effect of Akt on TGF-β-induced growth suppression, NRP-152 cells were cultured in GM3 containing 5% fetal bovine serum (FBS). Under these conditions, only 8% of the viable cells were in G2/M + S. TGF-β suppressed the G2/M + S population by 60%, whereas $Akt1^{Myr}$ partially reversed this suppression (52% reversal at S; 40% reversal of G2/M + S) and the TGF-β-induced sub-G1 (35% reversal) (Figure 2H). The above results are consistent with our previous report that IGF-I suppresses TGF-β responses downstream of Akt (Song *et al*, 2003b).

We next determined whether the ability of the Akt1 to suppress TGF-β1 responses is limited to inhibition of S3 activation or also acts downstream of active S3. To address the latter possibility, we studied the ability of $Akt1^{Myr}$ or CA-PI3K to suppress 3TP-lux activity induced by CA-S3 (C-terminal DDVD; Chipuk *et al*, 2002) versus that induced by TGF-β1 or by transfection of $S3^{WT}$. CA-PI3K suppressed 3TP-lux activity induced by TGF-β1 or to a lesser extent by CA-S3 (Figure 3A and B). In confirmation of the biological significance of these results, we showed that IGF-I similarly inhibits the activation of 3TP-lux by CA-S3, but not by $S3^{WT}$ (not activated by TGF-β) (Figure 3C). Overexpressed $S3^{WT}$ activates transcription by nuclear import of S3 without its phospho-activation. Thus, our data suggest that $Akt1^{Myr}$ or CA-PI3K blocks not only phospho-activation of S3 but also the biological responses of S3 downstream of this activation. However, neither LY nor Rap reversed Akt suppression of CA-S3, as indicated by both 3TP-lux and SBE4-luc reporters (Figure 3D and E). This contrasts with the reversal of Akt's effect on the above response by TGF-β1 (Figure 1), suggesting that Akt suppresses CA-S3 by an Akt kinase-independent mechanism. To test this, we compared the effects of $Akt1^{K179M}$, $Akt1^{Myr/K179M}$, and $Akt1^{Myr}$ on 3TP-lux by CA-S3 (Figure 3F). Whereas the kinase-dead Akt mutants did not suppress this activity of TGF-β1 (Figure 1), they suppressed that of CA-S3 (Figure 3F). In contrast, kinase-active or kinase-dead Akts did not suppress 3TP-luciferase activated by transfection of $S3^{WT}$ (Figure 3G), which is consistent with our demonstration that the overexpression of $S3^{WT}$ does not increase phospho-S3 levels (Figure 3H and I). However, the enhanced 3TP-lux activity by co-transfection of S4 with $S3^{WT}$, over that by $S3^{WT}$ alone, was reduced by Akt (Supplementary Figure 3S). Together, the above experiments suggest that suppression of TGF-β signaling by Akt1 occurs not only through suppression of S3 activation via an Akt kinase-dependent pathway, but also by transcriptional suppression of TGF-β-activated S3 by means of an Akt kinase-independent mechanism.

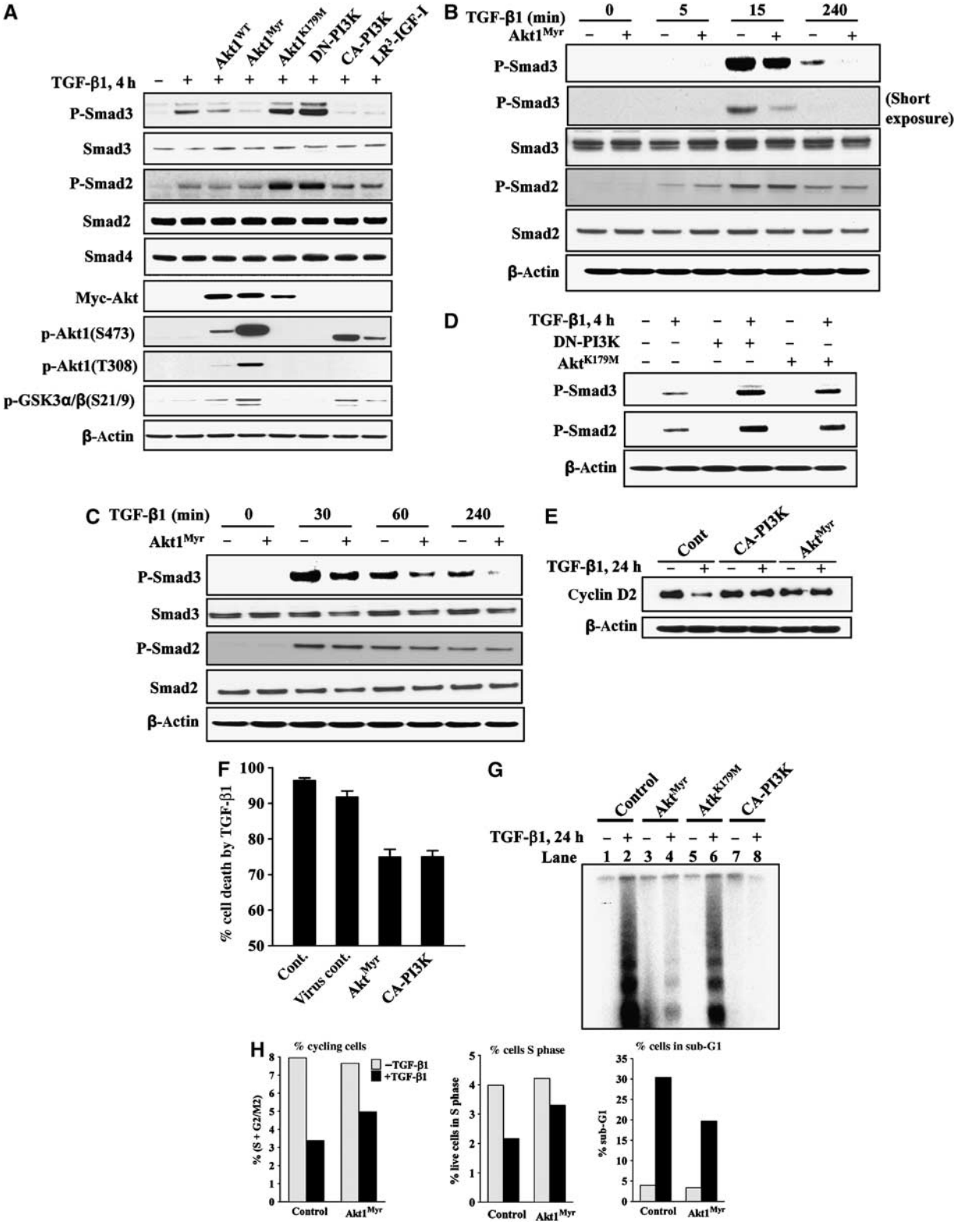
The kinase independence of Akt1 on the suppression of CA-S3 suggests that Akt1 may associate with S3, although in a kinase-independent manner. To examine this possibility, NRP-152 cells were infected with an adenovirus for Flag-$S3^{WT}$ and various forms of Akt1, either alone or with DN-PI3K, followed by treatment with TGF-β, IGF-I, LY, or Rap. Co-immunoprecipitation (Co-IP) assays showed that $S3^{WT}$ physically associates with WT, Myr, and KD forms of Akt1 (Figure 4A). Moreover, these physical interactions are not suppressed by TGF-β1, LY, or Rap, and not enhanced by IGF-I, supporting that the association of Akt1 with S3 is not sufficient for IGF-I or Akt1 to suppress phospho-activation of S3 (Figure 4B), in contrast to results of another group (Conery *et al*, 2004). Interestingly, DN-PI3K slightly reduced the amount of the complex formed between Akt1 and S3, whereas LY enhanced such complex formation (Figure 4B). On close inspection of inputs, however, changes in complex formation actually reflected changes in the expression of S3 and/or Akt1 by the above treatments (Figure 4B). Similarly, although lesser $Akt1^{Myr}$ and $Akt1^{K179M}$ were immunoprecipitated than $Akt1^{WT}$, these differences also reflected their relative expression levels (input, Figure 4A).

We further studied the physical interaction of S3 with Akt1, by defining the domains of S3 required for such binding. For this, we used truncations of S3 containing MH1, MH1 + middle linker, MH2, and MH2 + middle linker. Co-IP experiments revealed that S3 MH2 domain bound to $Akt1^{WT}$ with the highest affinity, whereas the S3 MH1 domain associated also with $Akt1^{WT}$, albeit with much lower affinity. The middle linker was inhibitory to the interaction of Akt1 with MH1 or MH2 domains (Figure 4C). Thus, the suppression by the middle linker may negatively regulate S3's interaction with Akt.

To examine the association of other Smad proteins with Akt1, HEK293 cells were transiently co-transfected with Flag-tagged Smads (S2, S3, S4, S7, CA-S3, or CA-S2), and either Myc-$Akt1^{WT}$ or Myc-$Akt1^{Myr}$. Co-IP assays showed that all these Smads physically interact with Akt1, with the CA Smads (S3* and S2*) having slightly less affinity than WT Smads to Akt1 (Figure 4D). These data suggest that Akt may regulate TGF-β signaling through interaction with multiple Smads, in contrast to the reports that Akt1 binds only the S3 isoform in HEK293T cells (Conery *et al*, 2004; Remy *et al*, 2004). We therefore compared the ability of transfected S2 and S3 to co-IP Akt1 in HEK293T cells grown in either serum-containing or serum-free conditions (Figure 4E). Under both conditions, S2 and S3 each comparably pulled down Akt1. Moreover, we also showed that endogenous S2 and S3 could co-IP endogenous Akt in NRP-152 cells (Figure 4F).

In our previous report, we showed that Rap reverses the IGF-I inhibition of TGF-β responses including the phospho-activation of S3 (Song *et al*, 2003b). Here, we show that Rap can also reverse the ability of either CA-PI3K or $Akt1^{Myr}$ to inhibit TGF-β-induced phospho-activation of S3 (Figure 5A and B), suggesting a role for mTOR in this mechanism. However, Rap has also been implicated in activation of TGF-β signaling through reversing the inhibitory action of FKBP12 on activation of TβRI by TβRII (Stockwell and Schreiber, 1998). To test whether the above results with Akt1 and Rap could instead be explained by suppression of TβRI by FKBP12, we tested the effects of Akt1 and Rap on activation of phospho-S3 and 3TP-lux promoter activity induced by $T\beta RI^{L193A/P194A/T204D}$ (LPT), which is a constitutively active and FKBP12-dead ALK5 (Charng *et al*, 1996). Adenoviral transduction of LPT led to phospho-activated S3 that was inhibited by coinfection with $Akt1^{Myr}$, and this $Akt1^{Myr}$ suppression was reversed in cells pretreated with Rap (Figure 5C). Similarly, activation of 3TP-lux by LPT was significantly suppressed by both $Akt1^{WT}$ and $Akt1^{Myr}$ (Figure 5D) and such suppression was Rap reversible (Figure 5E), suggesting that such suppression by Akt1 is independent of the interaction of FKBP12 with TβRI.

Interestingly, shorter treatment times of Rap or LY enhanced the induction of 3TP-lux by LTP, indistinguishable from that by $T\beta RI^{T204D}$ (Supplementary Figure 4S). Together, these results support that Rap reverses the Akt suppression of receptor-mediated S3 activation by blocking mTOR. We further tested this model by silencing mTOR with siRNA (si-mTOR). Consistent with the dependence of Akt kinase activity, silencing of mTOR by siRNA reversed the Akt1 suppression of LPT-induced 3TP-lux activity (Figure 5F), but not that induced by CA-S3 (Figure 5G). We next examined whether the activation of S3 by LPT could be blocked by $Akt1^{Myr}$ in an mTOR-dependent manner. The activation of S3 with LPT was clearly suppressed by $Akt1^{Myr}$, and silencing of mTOR by siRNA completely reversed the Akt block on such S3 activation (Figure 5H). mTOR expression was downregulated about three-fold by si-mTOR, and TGF-β-induced S3



activation was slightly enhanced by silencing mTOR (Figure 5H). Lastly, we showed that $Akt1^{Myr}$ can block the induction of apoptosis by LPT, and that the suppression of LPT-induced apoptosis was reversed by Rap (Figure 5I). These results firmly support a role of mTOR as a key mediator of Akt kinase-dependent effects on a number of TGF-β responses, including S3 activation, transcription, and apoptosis.

We propose a novel and unifying model by which Akt/mTOR suppresses TGF-β signaling (Figure 5J), whereby Akt1 inhibits S3 activity through both Akt kinase-dependent and Akt kinase-independent mechanisms. Our data support that mTOR is the downstream target of Akt kinase, which mediates the suppression of S3 phospho-activation. These findings further suggest that enhanced activation of mTOR may be pivotal to loss of TGF-β responses during tumor progression. The mechanism by which mTOR is able to suppress S3 activation may occur either by an interaction with mTOR or via downstream signals. In the kinase-independent mechanism, Akt blocks downstream of S3 activation but not phospho-activation of S3. Thus, our data do not support the mechanism proposed by a recent study (Conery *et al*, 2004) that Akt blocks S3 activation by sequestering it from TGF-β receptors. Rather, this molecular association may indirectly inhibit signals downstream of receptor-activated S3.

Discussion

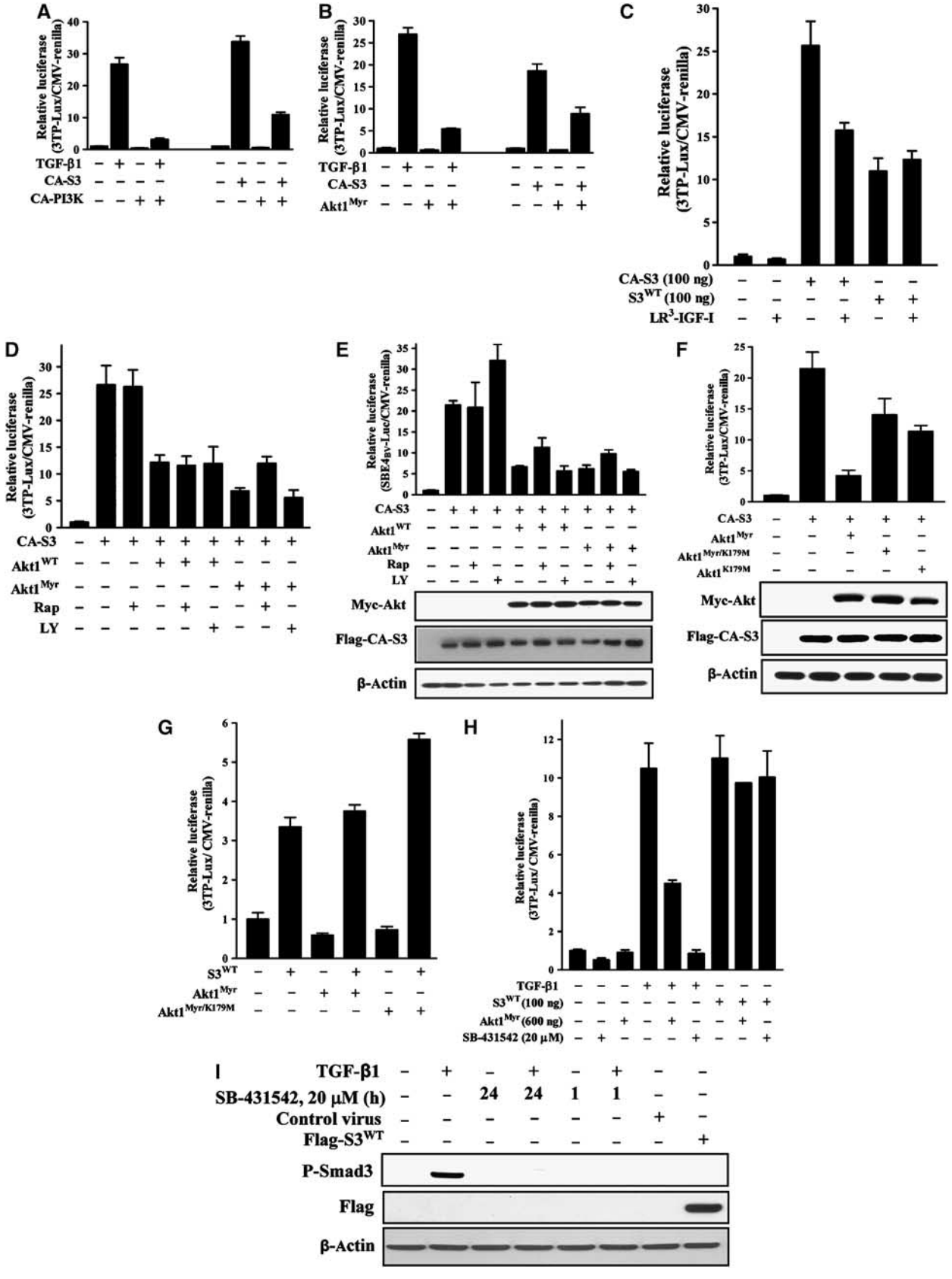
We published the first report that IGF-I, acting through the IGF-I receptor/PI3K/Akt signaling pathway, selectively blocks the ability of TGF-β to activate S3 but not S2, induce apoptosis, and mediate gene expression (Song *et al*, 2003b). Two subsequent publications reported similar suppression of S3 activation in other cell lines including the Hep3B hepatocarinoma cell line (Conery *et al*, 2004; Remy *et al*, 2004), using insulin, but only at super-physiological concentrations, levels that are well established to cross-activate IGF receptors (Megyesi *et al*, 1975; Rosenfeld and Hintz, 1980). To specifically focus on the IGF-IR signaling, our approach was to use physiologically relevant doses of IGF-I and an analog of this growth factor (LR^3-IGF-I) that is essentially unable to bind to IGF-I binding proteins (Hsing *et al*, 1996). We found that physiological concentrations of insulin or IGF-I were not able to suppress such TGF-β-induced responses in Hep3B cells (data not shown), in contrast to NRP-152, DP-153, and Mv1Lu cells. Insulin receptor signaling was reported to block the phospho-activation of S3 by inducing the membrane localization of Akt, which directly binds to and sequesters S3 away from TβRI (Conery *et al*, 2004; Remy *et al*, 2004). In their model, the Akt kinase is not involved in suppression of S3 activation, as they reported that Akt^{K179A} was just as effective as Akt^{Myr} in suppressing S3 activation. However, we show that Akt^{K179M} and Akt^{Myr} have opposite effects on both biological responses of TGF-β, and these Akts or IGF-I are ineffective in suppressing transcriptional responses of overexpressed $S3^{WT}$ (without TGF-β), evidence against the above sequestration model. Moreover, our data (not shown) with Hep3B cells, which are weakly responsive to TGF-β, suggest that the underlying discrepancies result from differences in experimental design rather than cell type. For example, in our study, we measured changes in the level of phospho-S3 by direct Western blot analysis. In contrast, the other studies detected phospho-S3 only indirectly in the material precipitated with anti-S3 antibodies, without assessing input levels of phospho-S3. Moreover, cell lines that stably express KD-Akt were derived without the use of an inducible expression system (Conery *et al*, 2004). As DN-Akt blocks the downstream survival pathways of Akt (Jetzt *et al*, 2003), stable overexpression of DN-Akt in this way would be selected against. Neither group showed data to confirm suppression of Akt kinase activity in KD-Akt-expressing cells, leaving open the possibility of compensation by activation of endogenous Akt kinase or a downstream target. To avoid such secondary phenotypic changes, our approach was to use adenoviral gene delivery for rapid and efficient gene expression. We also clearly show that cells expressing KD-Akt are actually kinase dead, by the absence of GSK-3β phosphorylation (Ser21/9).

Consistent with the role of S3 as a major mediator of TGF-β responses, our results suggest that Akt1 suppresses multiple TGF-β signals, including apoptosis, PAI-1 induction, growth suppression, and downregulation of cyclin D2. Recent studies with neuroepithelial and glioblastoma cells suggest that Akt kinase may more directly reverse the growth suppressive effects of TGF-β by blocking the induction of $p21^{Cip1}$ promoter activated by the association of S3 and S4 with FoxO Forkhead transcription factors (Seoane *et al*, 2004). In contrast, Conery *et al* (2004) reported that Akt's suppression of TGF-β responses is more limited, as they exclude TGF-β-mediated growth suppression, c-myc suppression, or induction of $p21^{Cip1}$ promoter activity.

The role of the physical interaction of S3 with Akt1 in the ability of Akt1 to block phospho-activation of S3 is not clear. Our results show that the ability of Akt1 to block S3 activation does not correlate with the strength of its association to S3. For example, Akt^{WT}, Akt^{K179M}, and Akt^{Myr} similarly associate with S3; however, only Akt^{WT} and Akt^{Myr} block phospho-activation of S3. The PI3K inhibitor, LY, enhances

Figure 2 Akt abolishes TGF-β-induced S3 phosphorylation and apoptosis and reverses growth arrest in NRP-152 cells. (**A**) Cells were infected with control virus, Admax-Myc-tagged Akt1 ($Akt1^{WT}$, $Akt1^{Myr}$, $Akt1^{K179M}$), Admax-DN-PI3K, or Admax-Myc-CA-PI3K, and incubated overnight in the presence or absence of 2 nM LR^3-IGF-I, followed by treatment with 10 ng/ml of TGF-β1 for 4 h. (**B, C**) The ability of $Akt1^{Myr}$ to block the phospho-activation of S2 and S3 at various time points after TGF-β1 addition was examined. (**D**) Effect of $Akt1^{K179M}$ or DN-PI3K alone or together with TGF-β1 on phospho-activation of S2 and S3. (**E**) TGF-β1 failed to downregulate cyclin D2 expression in NRP-152 cells overexpressing $Akt1^{Myr}$ or CA-PI3K. Whole-cell lysates (50 μg protein) were subjected to Western blot analysis (A–E). (**F**) Cells were infected with control virus, Admax-Myc-$Akt1^{Myr}$, or Admax-Myc-CA-PI3K for 2 h and incubated overnight followed by 48 h with TGF-β1. Cell numbers were determined with a Coulter Counter. Data shown are averages (±s.d.) of triplicate independent measurements. (**G**) The intranucleosomal DNA fragmentation induced in NRP-152 cells by 24 h of TGF-β1 treatment was blocked by preinfection with adenovirus delivering $Akt1^{Myr}$ or CA-PI3K, but not by $Akt1^{K179M}$. (**H**) Cell cycle analysis was performed on NRP-152 cells infected with control virus or Admax-Myc-$Akt1^{Myr}$ following treatment with vehicle or TGF-β1 for 48 h. Cells fixed with 90% methanol were stained with propidium iodide and analyzed by flow cytometry, based on a reference count of 20 000 cells at G1/G0. The distributions of cycling cells (S + G2/M), undergoing DNA synthesis (S), and dead cells (sub-G1) are shown. Results are representative of two to three experiments per treatment.

the formation of a complex between S3 and Akt1^{WT} by elevating the levels of Akt1, whereas DN-PI3K reduces the level of Akt1 precipitating with S3 (Figure 4B), and PTEN neither affects the levels of Akt or S3 nor alters complex formation between S3 and Akt1 (data not shown). Unexpectedly, our results show that Akt1^{K179M} and DN-



PI3K each enhances the level of phospho-S2 induced by TGF-β, although kinase-active Akt1 does not suppress S2 activation, in contrast to S3 (Figure 2A–D). These results suggest that S2 activation may be fully suppressed by basal levels of Akt1 activity, unlike S3, which can be suppressed further or completely by induced levels of Akt1 kinase. It is thus likely that different mechanisms are involved in the Akt suppression of S3 versus S2.

We show that Akt1 can also inhibit TGF-β signals downstream of receptor-activated S3, as shown by the induction of 3TP-lux activity following transfection with CA-S3, a disparity with Conery *et al* (2004), in which Akt1 was reported to not suppress 3TP-luciferase activity induced by CA-S3. We show that Akt1 can suppress signals downstream of S3 activation through a mechanism that is independent of the kinase activity of Akt1, in contrast to its suppression of S3 activation

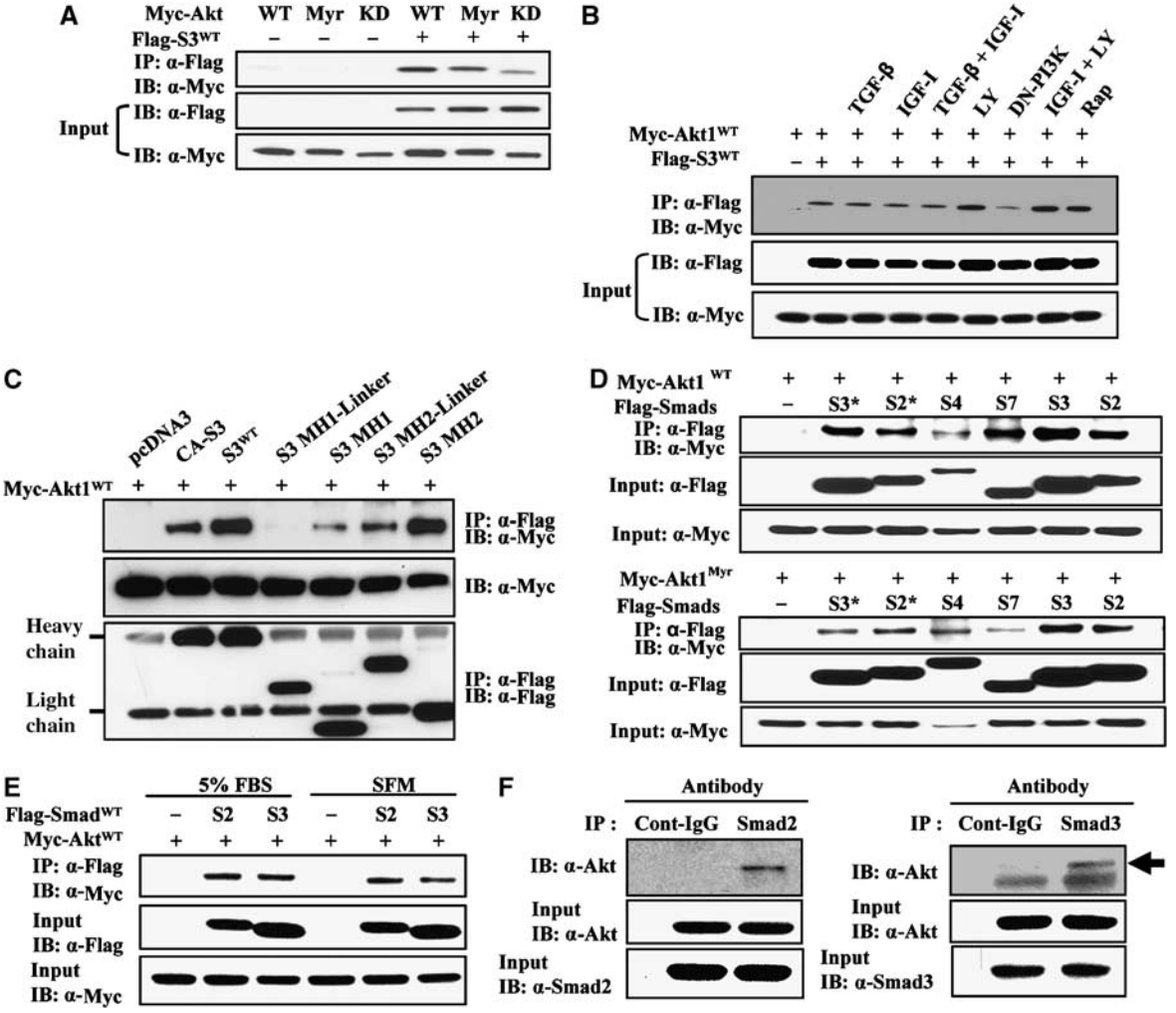


Figure 4 Akt1 binds to multiple Smad proteins in an Akt-kinase-independent mechanism. (**A, B**) NRP-152 cells were coinfected with Admax-Myc-Akts (WT, Myr, KD), Admax-Flag-S3, and Admax-DN-PI3K, followed by washing with PBS. Cells were then incubated for 24 h with/without LY (10 μM) or Rap (200 nM) in GM3 medium followed by treatment with LR^3-IGF-I (2 nM, 5 h), TGF-β1 (10 ng/ml, 4 h), or both LR^3-IGF-I and TGF-β1 before cell lysis. (**C, D**) Akt1s associate with not only S3 (at the MH2 domain) but also with S2, S4, S7, CA-S2 (S2*), and CA-S3 (S3*) in HEK293 cells. (**E**) Akt1 associates with $S2^{WT}$ and $S3^{WT}$ in HEK293T cells cultured in serum-free DMEM/F12 medium (SFM) or in DMEM/F12 containing 5% FBS. (**F**) Endogenous Akt1 associates with endogenous Smads 2 and 3 in NRP-152 cells. Whole-cell lysate (500 μg (A–E) or 1 mg (F)) was subjected to immunoprecipitation with mouse anti-Flag M2 IgG (A–E) or with either anti-Smad2 or anti-Smad3 IgGs (F), and expression of co-immunoprecipitated Akts was detected with rabbit anti-Myc IgG (A–E) or with anti-Akt1 IgG (F). Results are representative of two to three different experiments.

Figure 3 Transcriptional activity of CA-S3 but not $S3^{WT}$ is blocked by an Akt-kinase-independent mechanism in NRP-152 cells. (**A–C**) CA-PI3K, LR^3-IGF-I, and $Akt1^{Myr}$ block 3TP-Lux transcriptional activity induced by TGF-β and CA-S3 in NRP-152 cells. (**D, E**) Inhibition of CA-S3-induced 3TP-Lux (D) and $SBE4_{BV}$-Luc (E) by Akt was not reversed by LY (10 μM) or Rap (200 nM). (**F**) $Akt1^{Myr}$, $Akt1^{Myr/K179M}$, and $Akt1^{K179M}$ inhibit 3TP-Lux induced by CA-S3 activity. (**G**) $Akt1^{Myr}$ and $Akt1^{Myr/K179M}$ do not block 3TP-Lux induced by $S3^{WT}$ activity, similar to the absence of a suppressive effect of LR^3-IGF-I on $S3^{WT}$ activity. (**H, I**) Demonstration that activation of 3TP-lux by $S3^{WT}$ does not involve the phospho-activation of S3 (H), and that overexpression of $S3^{WT}$ does not enhance its phospho-activation, by Western blot analysis (I). Cells were pretreated with the TβRI kinase inhibitor SB-431542 (20 μM) to specifically block the phospho-activation of receptor Smads. Firefly luciferase values were normalized to *Renilla* luciferase. Each bar represents the average of triplicate determinations ± s.e. (A–H). Results are representative of two to three experiments.

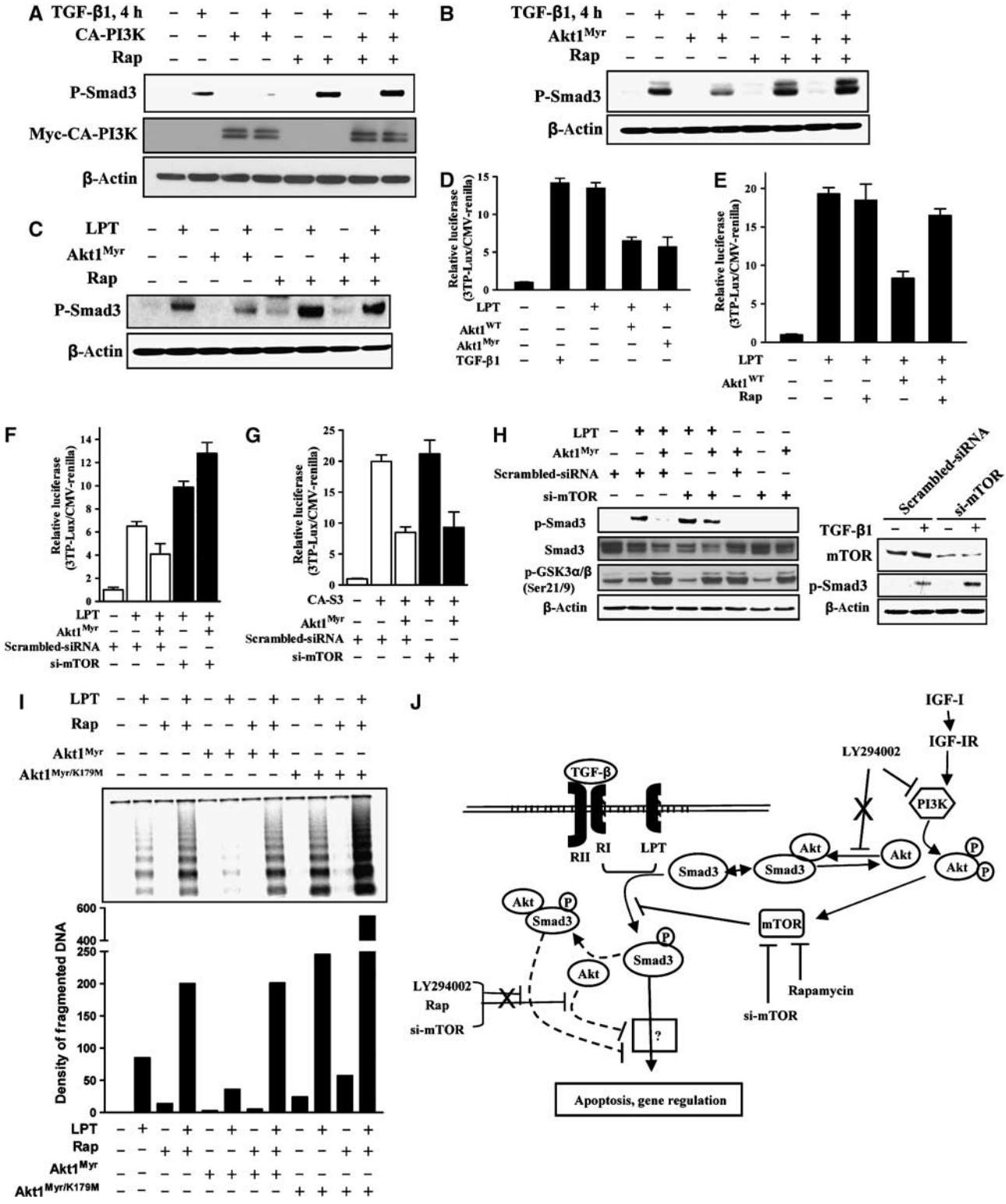


Figure 5 mTOR is the critical mediator of inhibition of TGF-β signaling by Akt in NRP-152 cells. (**A–C**) Effect of Rap on suppression of either TGF-β-induced or LPT-induced phospho-Smad3 by either CA-PI3K or Akt1^{Myr}. (**D, E**) 3TP-lux activity induced by LPT in NRP-152 cells was inhibited by Akt and this inhibition was Rap (200 nM)-reversible. (**F, G**) Silencing mTOR expression by siRNA reverses the Akt suppression of LPT-induced 3TP-lux. Cells were transiently transfected with siRNA against mTOR (si-mTOR, 80 nM), scrambled control siRNA (80 nM), pUSE-Myc-Akt1^{Myr}, 3TP-Lux reporter construct, and either pCMV5-LPT or pCMV5-CA-S3. Data shown are relative values of Firefly luciferase normalized to *Renilla* luciferase. Each bar represents the average of triplicate determinations ± s.e. (D–G). (**H**) si-mTOR (80 nM) reversed the inhibition by Akt of phospho-activation of S3 induced by LPT, and phosphorylation of S3 by TGF-β was enhanced by blocking mTOR expression with si-mTOR. Results are representative of two to three experiments. (**I**) Rap reverses the ability of Akt1^{Myr} to block apoptosis induced by LPT (upper panel, DNA ladder; lower panel, DNA ladder was quantified by a phosphoimager). Adenoviral transduction was used to deliver CA-PI3K, Akt^{Myr}, and LPT (A–C, H, I). (**J**) A schematic representation of our model showing that TGF-β signaling is blocked by both Akt kinase-dependent and Akt kinase-independent mechanisms, with mTOR as the downstream substrate of Akt that mediates suppression of S3 phospho-activation. The kinase-independent function of Akt inhibits gene transcription by activated S3, but does not block the activation of S3 by TGF-β.

that requires Akt1 kinase activity. Although the mechanism behind this kinase-independent suppression is not known, our results suggest that this may occur through a physical association of Akt1 with phospho-S3. We also show novel associations of $Akt1^{WT}$ or $Akt1^{Myr}$ with Smads 2, 3, 4, and 7 (Figure 4D), in contrast to the other groups (Conery *et al*, 2004; Remy *et al*, 2004), who claim that S3 is the only Smad that binds Akt1. These discrepancies cannot be due to the variant of HEK293 cells used (we used HEK293 cells versus HEK293T), as we showed that S2 interacted with Akt1 in HEK293T cells (used by the other groups) by co-IP experiments, and endogenous S2 and S3 can also interact with endogenous Akt1 as shown by co-IP experiments with NRP-152 cells.

Our data strongly support a new model of S3 suppression by Akt, in which mTOR is essential for the ability of Akt to suppress biological responses of TGF-β and the phospho-activation of S3. Our studies using LPT indicate that such suppression is independent of the association of FKBP12 to ALK5. There has been increased interest in understanding the role of mTOR in cancer, particularly in mediating Akt effects on tumor growth and angiogenesis (Humar *et al*, 2002; Chan, 2004). Rap, which has gained broad interest as a useful drug for therapeutic intervention of a number of late-stage cancers, may function by preventing the Akt survival signals activated during cancer (Rao *et al*, 2004). Thus, a better understanding of the function of mTOR in TGF-β signaling is likely to impact on the therapeutics of a variety of cancers.

Materials and methods

Materials

The following were used: recombinant human TGF-β1 (R&D Systems); phospho-Akt1 (Ser473, #9271), anti-Akt1 (#9272), phospho-GSK-3α/β (#9331, Ser21/9), phospho-S3 (Ser433/435, #9514), phospho-S2 (#3101), and mTOR (#2972) antibodies (Cell Signaling); anti-Smad3 (sc-8332), anti-cyclin D2 (sc-593), and anti-Myc (sc-789) antibodies (Santa Cruz); anti-Flag M2 (F-3165), anti-Flag M1 (F-3040), and anti-β-actin (A-5441) antibodies (Sigma); DMEM/F12 (1:1, v/v) and calf serum (Invitrogen); LR^3-IGF-I, insulin (BioSource International); dexamethasone (Sigma); LY and Rap (BioMol); pCMV5-Flag-CA-S3 (Chipuk *et al*, 2002; Song *et al*, 2003b), pSG5-DN-PI3K ($p85\alpha\Delta SH_2N$) and pSG5-CA-PI3K (p110αCAAX) (Downward, 1998), $pUSE\text{-}Myc\text{-}Akt1^{WT}$, $pUSE\text{-}Myc\text{-}Akt1^{Myr}$, and $pUSE\text{-}Myc\text{-}Akt1^{K179M}$ (Upstate), LPT ($T\beta RI^{L193A/P194A/T204D}$) (Charng *et al*, 1996); pcDNA3-Flag-S3 truncations (Wang *et al*, 2005); characterized FBS (HyClone); SB-431542 (Tocris).

Cell culture

NRP-152 and NRP-154 prostatic epithelial cell lines (Danielpour *et al*, 1994; Danielpour, 1996) were maintained in GM2.1 culture medium (DMEM/F12 supplemented with 5% FBS, 5 μg/ml insulin, and 0.1 μM dexamethasone) in 100-mm Falcon culture dishes. The cells were kept at 37°C in a 95% air/5% CO_2 environment and passaged every 3–4 days (at subconfluence), plating at a seeding density of 1:40. All experiments were performed in GM3 medium (DMEM/F12 supplemented with 1% calf serum, 15 mM HEPES (pH 7.5), and 0.1 μM dexamethasone). HEK293, HEK293T, Hep3B, and Mv1Lu cells were cultured in DMEM/F12 medium supplemented with 5% FBS.

Western blot analysis

Western blot analyses were performed essentially as described (Song *et al*, 2003a, b). In brief, NRP-152 cells were plated overnight at a density of 4×10^5 cells/2 ml of GM3 media/well in six-well plates and infected for 2 h by adenovirus expressing Akt, DN-PI3K, or CA-PI3K, and washed once with PBS followed by addition of 2 ml GM3 medium. Following treatment, cells were washed with PBS and lysed at 4°C with ice-cold RIPA buffer (PBS, 1% Nonidet P-40, 0.1% sodium dodecylsulfate, 0.5% sodium deoxycholate) containing 1 mM sodium orthovanadate, Complete Mini-EDTA-free Protease Inhibitor Mixture™, and 1 mM phenylmethylsulfonyl fluoride. Lysates were clarified at 16 000 r.c.f. for 20 min (at 4°C) and supernatants were quantified by the BCA protein assay (Pierce). Lysates were boiled for 5 min in SDS–PAGE loading buffer containing 5% 2-mercaptoethanol and electrophoresed through 4–12% NuPAGE BisTris gel (Invitrogen) and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked for 1 h in TBS (10 mM Tris–HCl, pH 8.0, and 150 mM NaCl) containing 5% nonfat dry milk and 0.05% thimerosal, and incubated with the indicated primary antibodies and the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000; Jackson Laboratory). The SuperSignal Chemiluminescence Substrate System (Pierce) was used to visualize protein bands.

Immunoprecipitation

All these procedures were performed as described (Chipuk *et al*, 2002; Wang *et al*, 2005). NRP-152 cells were infected by adenovirus for 2 h to introduce the indicated genes and cultured in GM3 medium. Separately, HEK293 and HEK293T cells were plated overnight in 5% FBS-DMEM/F12 in six-well plates at a density of 5×10^5 cells/2 ml/well and transfected with 2 μg of DNA per well by a calcium phosphate precipitation method (Chipuk *et al*, 2002). The tagged proteins were co-immunoprecipitated with anti-Flag M2 IgG1 monoclonal antibody (Sigma) and subjected to Western blot analysis.

Transient transfection and luciferase assay

All the procedures were performed essentially as described (Chipuk *et al*, 2002; Song *et al*, 2003b). In brief, NRP-152, NRP-154, or Mv1Lu cells were plated overnight at a density of 1×10^5 cells/1 ml/well in 12-well plates. A total of 1 μg of DNA including reporter construct was transfected using a standard calcium phosphate co-precipitation method, and 3 h later cells were glycerol-shocked (15% glycerol in 1 × HEPES-buffered saline) for 90 s. Cells were washed twice and incubated overnight in GM3 medium either in the presence or absence of signaling inhibitors and then cultured with or without TGF-β1 for 24 h. Luciferase activity was measured using the Promega's Dual Luciferase Assay Kit and an ML3000 Microtiter Plate Luminometer.

siRNA

Rat mTOR siRNA oligonucleotide (si-mTOR), 5′-CCCAGCCUUUGU CAUGCCUTT-3′, and its complementary RNA strand harboring TT at the 3′ end were synthesized by Dharmacon, annealed *in vitro*, and co-transfected into NRP-152 cells (80 nM per well, six-well plates) with indicated plasmids by either Lipofectamine 2000 reagent (Invitrogen) or calcium phosphate precipitation method. siRNA pair of identical length and design of a scrambled sequence not found in the rat genome, 5′-AGCUGUGCCCUUCAUCCCUTT-3′, was used as a negative control. Cell extracts were prepared 24 or 48 h after siRNA transfection and subjected to Western blot analysis or dual luciferase assay.

Adenovirus

Adenovirus vectors that direct the expression of $Myc\text{-}Akt1^{WT}$, $Myc\text{-}Akt1^{Myr}$, $Myc\text{-}Akt1^{K179M}$, DN-PI3K, CA-PI3K, and $Flag\text{-}Smad3^{WT}$ ($AdMax\text{-}Myc\text{-}Akt1^{WT}$, $Admax\text{-}Myc\text{-}Akt1^{Myr}$, $Admax\text{-}Myc\text{-}Akt1^{K179M}$, Admax-DN-PI3K, Admax-Myc-CA-PI3K, and $Admax\text{-}Flag\text{-}Smad3^{WT}$) were constructed using the AdMax system (Microbix Biosystems). The regions corresponding to the coding sequence of each gene were subcloned from $pUSE\text{-}Myc\text{-}Akt1^{WT}$, $pUSE\text{-}Myc\text{-}Akt1^{Myr}$, $pUSE\text{-}Myc\text{-}Akt1^{K179M}$, pSG5-DN-PI3K, pSG5-Myc-CA-PI3K, and $pCMV5\text{-}Flag\text{-}Smad3^{WT}$ into the adenovirus shuttle vector pDC515. Constructs were confirmed by DNA sequencing (Cleveland Genomics) and protein expression (in HEK293 cells) by Western blot analysis with two different primary antibodies (epitope-tagged antibody and one against expressed protein). $pDC515\text{-}Myc\text{-}Akt1^{WT}$, $pDC515\text{-}Myc\text{-}Akt1^{Myr}$, $pDC515\text{-}Myc\text{-}Akt1^{K179M}$, pDC515-DN-PI3K, pDC515-Myc-CA-PI3K, or $pDC515\text{-}Flag\text{-}Smad3^{WT}$ (1 μg) was co-transfected with 1 μg of the genomic vector pBHGfrtDE1,3FLP into HEK293 cells in six-well plates using the calcium phosphate precipitation method. Transfected cells were maintained in DMEM/F12 containing 2% FBS. Approximately 10 days after transfection, when viral cytopathic effect was observed, the cells were lysed by freeze–thaw and

then expanded according to the manufacturer's protocol. The ability of viral preparations to deliver the desired functional protein was evaluated by overnight infection of NRP-152 and NRP-154 cells followed by Western blot analysis of cell lysates and, in certain cases, by direct immunostaining of the infected cells. To assay viral titers, aliquots of virus stocks were diluted 100-fold in lysis solution (0.1% sodium dodecylsulfate, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA) and incubated for 10 min at 56°C. The optical density of the samples at 260 nm was used to calculate the virus content using the relationship of 10^{12} virus particles/ml/OD_{260} unit.

DNA fragmentation assay

All the procedures were performed essentially as described (Hsing *et al*, 1996; Danielpour, 1999). Internucleosomal DNA fragmentation was detected using the TACS apoptosis DNA ladder kit (Trevigen). NRP-152 cells were infected for 2 h with control virus, Admax-Myc-Akt1^{Myr}, Admax-Myc-Akt1$^{Myr/K179M}$, Admax-Myc-Akt1^{K179M}, or Admax-Myc-CA-PI3K in the absence or presence of LPT. Cells were then washed once with 1 × PBS and cultured for 24 or 48 h in GM3 medium, with the inclusion of 10 ng/ml TGF-β1 or vehicle control for another 24 h. Cells were then lysed and DNA was purified according to the manufacturer's instructions. The nicked ends of 0.5 μg of DNA were labeled using 2.5 U of Klenow fragment of DNA polymerase I with 0.5 μCi [α-^{32}P]dCTP (NEN) for 30 min at room temperature. The labeled DNA was electrophoresed through a 1.5% Trevigel 500 gel in 1 × TAE at 85 V for 2 h. The gel was dried and directly exposed to an X-Omat AR film (Kodak).

Cell cycle distribution with propidium iodide

In brief, NRP-152 cells infected with Admax-Myc-Akt1^{Myr} were treated with TGF-β1 or vehicle for 48 h and then fixed with 90% methanol (final concentration) at −20°C. Fixed cells were washed free of methanol and incubated with RNase A for 20 min at 37°C. Cells were placed on ice for 10 min followed by staining with propidium iodide solution (final concentration 50 μg/ml) for 30 min at 4°C and analyzed by flow cytometry using Coulter XL (Coulter Electronics).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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