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Viral Small T Oncoproteins Transform Cells by Alleviating Hippo-Pathway-Mediated Inhibition of the YAP Proto-oncogene

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SUMMARY

Primary human cells can be transformed into tumor cells by a defined set of genetic alterations including telomerase, oncogenic Ras^{V12}, and the tumor suppressors p53 and pRb. SV40 small T (ST) is required for anchorage-independent growth in vitro and in vivo. Here, we identify the Hippo tumor suppressor pathway as a critical target of ST in cellular transformation. We report that ST uncouples YAP from the inhibitory activity of the Hippo pathway through PAK1-mediated inactivation of NF2. Membrane-tethered activated PAK is sufficient to bypass the requirement for ST in anchorage-independent growth. PAK acts via YAP to mediate the transforming effects of ST. Activation of endogenous YAP is required for ST-mediated transformation and is sufficient to bypass ST in anchorage-independent growth and xenograft tumor formation. Our findings uncover the Hippo tumor suppressor pathway as a final gatekeeper to transformation and tumorigenesis of primary cells.

INTRODUCTION

Tumors are thought to arise from four to six rate-limiting, independent stochastic oncogenic events. The in vitro creation of tumor cells from primary cells by a defined set of genetic elements was an important experimental validation of this concept, identifying a minimal set of pathways that together result in oncogenic transformation (Hahn et al., 1999, 2002). This model offers the possibility to study molecular pathways required for transformation in a minimally disturbed genetic background: all genetic alterations are known. This contrasts with patient-derived cancer cell lines, which carry a diverse array of genetic events that arose during selection for tumorigenesis in vivo and subsequently for growth in cell culture. The complexity of the genetic background complicates the analysis of mechanisms underlying cellular transformation and properties relevant to tumorigenesis, including anchorage-independent growth.

Viral oncogenes have been important tools in the identification of tumor suppressor pathways in human primary cells. The p53 and p16/pRb tumor suppressor pathways were identified as targets of SV40 large T. Hahn's original genetically defined transformation model (Hahn et al., 1999) expressed telomerase, H-Ras^{V12}, and a construct encoding SV40 large T to target the p14^{ARF}/p53 and p16^{INK4A}/pRb pathways. It was subsequently found that SV40 small T (ST) (also encoded in the large T construct) was required independently of large T to support anchorage-independent growth (Hahn et al., 2002). The ability of ST to confer anchorage-independent growth correlates with its ability to inactivate PP2A activity. Inhibition of PP2A can bypass the requirement for ST in cellular transformation models, and mutations inactivating PP2A subunits have been identified in tumors (reviewed in Sablina and Hahn, 2008; Sablina et al., 2010). PP2A has many substrates. Among these, AKT, β-catenin, and c-Myc are known to be activated by ST. However, the combined overexpression of active forms of these proteins only partially replaced ST in cellular transformation (Sablina et al., 2010), suggesting that unidentified PP2A effector pathways might be involved.

The Hippo tumor suppressor pathway is regulated by cell polarity, cell contact, and mechanical forces (Wada et al., 2011; Halder et al., 2012; Aragona et al., 2013). Hippo/MST kinase acts via a membrane-associated complex containing the tumor suppressor NF2/Merlin (Yin et al., 2013). MST kinases activate LATS kinases, which in turn phosphorylate the transcriptional co-activators YAP and TAZ, leading to their inactivation through cytoplasmic sequestration and increased degradation (Dong et al., 2007; Zhao et al., 2007, 2010b; Hao et al., 2008; Lei et al., 2008). Inhibition of YAP and TAZ limits proliferation and induces apoptosis (Barry and Camargo, 2013). Importantly, overexpression of YAP is sufficient to lead to tumorigenesis in mouse

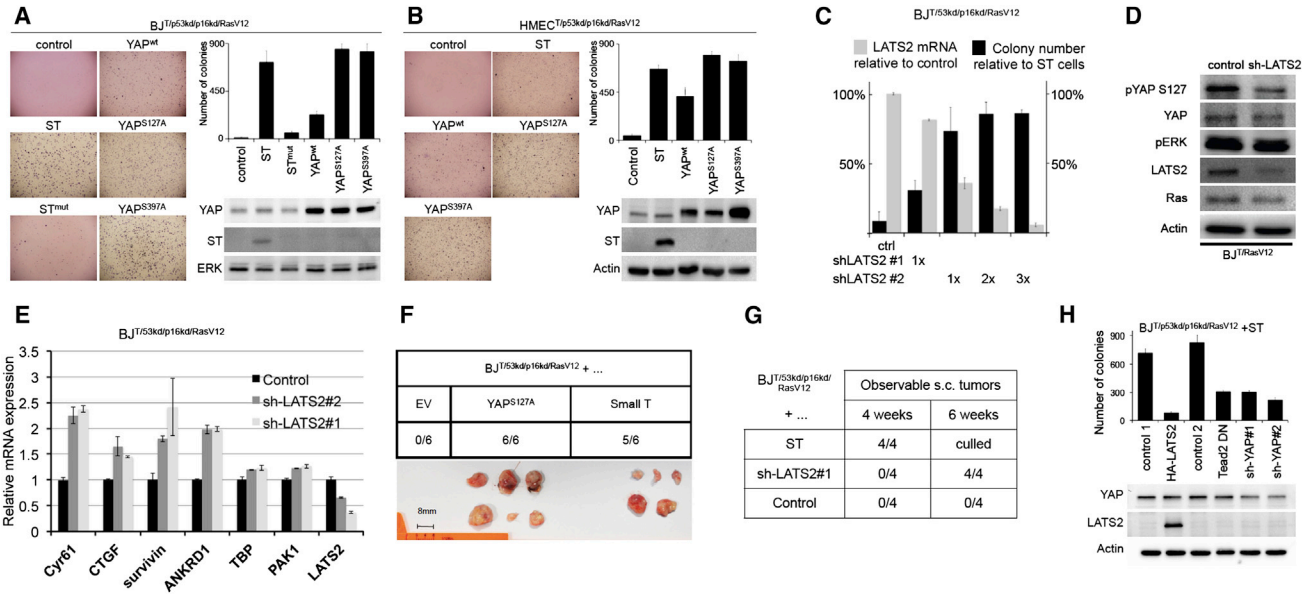

Figure 1. YAP Replaces Small T to Drive Soft Agar Growth

(A) BJ$^{T/p53kd/p16kd/RasV12}$ cells were transduced to express ST; a mutant form of ST unable to bind PP2A (ST$^{mutC97S/E102Q}$); wild-type Flag-YAP, Flag-YAP^{S127A}, or Flag-YAP^{S397A}; or with empty vector as a control. S397 in human YAP corresponds to S381 in mouse. After antibiotic selection, cells were plated in soft agar or harvested for immunoblotting. Left: images of colonies formed after 2 weeks. Right: average colony number ± SD from three independent experiments. ANOVA: $p < 0.0001$ comparing ST, YAP^{S127A}, and YAP^{S397A} with control; $p = 0.001$ YAP^{WT} versus control; mutant form of ST versus control was not significant. Immunoblots show ST and YAP expression. Anti-ERK was used to control for loading.

(B) YAP can replace ST in HMECs. Unmodified HMECs can exhibit epigenetic changes that silence p16 (Dumont et al., 2009; Locke and Clark, 2012). Here, HMECs were transduced to express shRNAs targeting p53 and p16 as well as vectors to express hTert, Ras^{V12} and ST; wild-type Flag-YAP, Flag-YAP^{S127A}, or Flag-YAP^{S397A}; or with empty vector as a control. ANOVA: $p < 0.0001$ comparing all samples versus control. Immunoblots show ST and YAP expression. Anti-actin was used to control for loading. Error bars represent mean ± SD for three independent experiments.

(C) BJ$^{T/p53kd/p16kd/RasV12}$ cells were transduced with LATS2 shRNA vectors (one to three rounds). Gray: LATS2 mRNA levels relative to cells with control vector. Black: soft agar colony formation (normalized to ST transduced cells). Colony number increase was significant for all samples by ANOVA ($p = 0.002$ for sh1; $p < 0.0001$ for all others). Error bars represent mean ± SD for three independent experiments.

(D) Effect of LATS2 shRNA depletion on YAP S127 phosphorylation.

(E) Quantitative real-time RT-PCR to measure YAP target levels in control and LATS2 shRNA-treated cells. LATS2 levels shown at right. Data represent mean ± SD for three independent experiments. ANOVA: upregulation of Cyr61, Survivin and ANKRD1, $p < 0.0001$; CTGF, $p = 0.01$ for sh2, $p < 0.0001$ for sh1. Changes in the PAK and TBP controls were not significant.

(F and G) Tumors from BJ$^{T/p53kd/p16kd/RasV12}$ cells injected subcutaneously into NOD-SCID Il2rg$^{-/-}$ mice. F: cells transduced with ST, YAP^{S127A}, or empty vector control (EV). (G) Cells transduced with ST, LATS2 shRNA#1, or vector control. Tumors were scored at 10 weeks.

(H) BJ$^{T/p53kd/p16kd/RasV12}$+ST cells were transduced to express hemagglutinin-tagged-LATS2, Tead2 dominant-negative (DN), or shRNAs to deplete YAP1 or with relevant empty vector controls. Upper panel: soft agar colony number. $p = 2 \times 10^{-5}$ control 1 versus HA-LATS2 (t test). $p < 0.0001$ comparing TEAD-DN and shYAP with control 2 (ANOVA).

models (Dong et al., 2007) and can stimulate anchorage-independent growth of partially transformed cell lines in vitro (Overholtzer et al., 2006; Zhang et al., 2009). Primary human fibroblasts and epithelial cells fail to divide more than once in anchorage-independent conditions in soft agar unless they also express SV40 ST (Hahn et al., 2002; Voorhoeve and Agami, 2003). We hypothesized that ST expression is required for continued proliferation in soft agar because it alleviates Hippo pathway activity.

Here, we report that SV40 ST and the related oncogenic Merkel cell polyomavirus ST activate endogenous YAP. We present evidence that ST acts though PAK1 to inactivate the NF2 tumor suppressor to alleviate inactivation of YAP. Activation of PAK1 at the cell membrane is sufficient to bypass the requirement for ST. Limiting PAK1 activity blunts the transforming potential of the ST oncoprotein. Thus, ST uncouples YAP from regulation by the Hippo tumor suppressor pathway. Activation of YAP is necessary and sufficient to replace ST in cellular transformation. Thus, the YAP proto-oncogene is a key mediator of the transforming effects of the ST oncoprotein.

RESULTS AND DISCUSSION

YAP Can Replace Small T

To test the hypothesis that the Hippo pathway acts as an effector of SV40 ST in cellular transformation, we asked if YAP activity could replace ST expression to support growth in soft agar. We first confirmed that primary human fibroblasts (BJ) and human mammary epithelial cells (HMECs) expressing Ras^{V12}, hTert, and short hairpin RNAs (shRNAs) to p53 and p16 failed to grow in soft agar unless they also expressed ST (Figures 1A and 1B). Transducing the cells to overexpress YAP stimulated colony growth for both cell types in the absence of ST (Figures

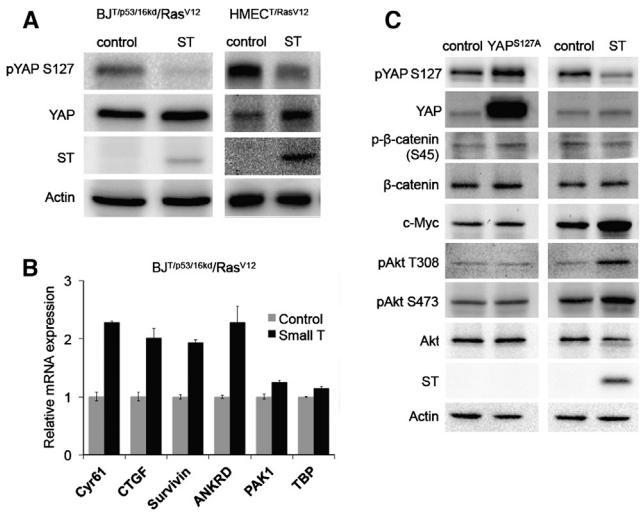

Figure 2. ST Expression Increases YAP Activity

(A) YAP S127 phosphorylation in $HMEC^{T/p53kd/p16kd/RasV12}$ and $BJ^{T/p53kd/p16kd/RasV12}$ cells transduced to express ST.

(B) Quantitative PCR of YAP targets in $BJ^{T/p53kd/p16kd/RasV12}$ cells ± ST. Target increases were significant in ST-expressing cells (ANOVA $p < 0.0001$). Changes in the TBP and PAK controls were not significant. Error bars represent mean ± SD for three independent experiments.

(C) Immunoblots comparing the effect of ST and YAP^{S127A} expression on Myc, β-catenin and Akt. The two sets of samples were run on the same gel, with an intervening marker lane removed.

1A and 1B). Mutant forms of YAP that are resistant to LATS kinases (Zhao et al., 2010a) were more potent in supporting colony formation. ST increased the expression of YAP targets, including Cyr61, CTGF, Survivin/Birc5, and ANKRD1 (Figure S1A). These results indicate that the requirement for ST to support anchorage-independent growth can be overcome by providing YAP in these two primary human cell types.

YAP is overexpressed in some human tumors (e.g., Zhang et al., 2009), so we considered the possibility that YAP might bypass ST in the preceding experiments only because it was overexpressed. To test this, we asked whether increasing the activity of endogenous YAP could also support anchorage-independent growth. Cells were transduced to express shRNAs to deplete LATS2 (the major LATS kinase in these cells). LATS2 depletion was monitored by quantitative PCR (Figure 1C). As expected, LATS2 depletion reduced YAP S127 phosphorylation, indicating elevated YAP activity (Figure 1D). Expression of the YAP targets Cyr61, CTGF, Survivin/Birc5, and ANKRD1 increased, reflecting elevated YAP activity (Figure 1E). Reducing LATS2 levels gave BJ cells only a small growth advantage on plastic (Figure S1B) but conferred a robust capacity for growth in soft agar (Figure 1C). The effect of LATS2 depletion was dose dependent (Figure 1C), suggesting that YAP activity is limiting for soft agar growth in these cells. Activation of endogenous YAP accounted for ~85% of the activity of ST in colony formation in these experiments. LATS2 depletion also supported growth of HMECs in soft agar (Figure S1C). BJ cells expressing YAP^{S127A} or with activation of endogenous YAP by LATS2 depletion produced tumors in xenograft assays (Figures 1F and 1G). These findings provide evidence that activation of endogenous YAP can bypass the requirement for SV40 ST in a genetically defined model of cellular transformation.

To ask whether YAP activity is required for ST-mediated transformation, we used three methods to lower YAP activity in fully transformation-competent cells. First, LATS2 was overexpressed to reduce YAP activity. Second, a dominant-negative form of TEAD (Liu-Chittenden et al., 2012) was used to lower YAP activity. Third, two different shRNAs were used to deplete YAP (Figure S1D). Each of these treatments reduced YAP luciferase reporter activity (Figure S1E) and significantly reduced colony formation in soft agar (Figure 1H). Comparable results were obtained following YAP depletion in HMECs (Figure S1F). Thus, YAP activity is required for anchorage-independent growth in these primary cells. YAP activity is both necessary and sufficient for full transformation of cells expressing oncogenic forms of Ras and ST.

ST Acts on YAP

Since activation of endogenous YAP was sufficient to replace ST, we asked whether YAP is a molecular target of ST. Activation of the Hippo pathway leads to phosphorylation of LATS1/2 by MST1/2. LATS1/2 in turn phosphorylate YAP on multiple sites, including S127, which promotes cytoplasmic retention of YAP (Zhao et al., 2007; Hao et al., 2008; Lei et al., 2008), and S381 (S397 in human YAP1), which promotes YAP turnover (Zhao et al., 2010b). Expression of ST decreased YAP^{S127} phosphorylation in BJ fibroblasts and in HMECs (Figure 2A). Consistent with the decrease in YAP^{S127} phosphorylation, ST increased expression of the YAP target genes (Figure 2B). Thus, ST upregulates YAP activity.

The ST oncoprotein has been reported to act via the cellular proto-oncogenes AKT, β-catenin, and c-Myc (Sablina et al., 2010). Given that YAP can replace ST, we considered the possibility that YAP might indirectly activate the other ST effectors. We confirmed that ST expression increased c-Myc levels and increased phosphorylation of AKT on S437 and Thr308 in BJ cells (Figure 2C; β-catenin was not affected). BJ cells expressing YAP^{S127A} in place of ST showed no increase in Myc expression or AKT phosphorylation (Figure 2C). Yet, these cells were fully capable of anchorage-independent growth (Figure 1A). Thus, Myc and AKT do not need to be hyperactivated in the presence of active YAP. ST expression normally activates all these pathways concurrently, and it is likely that their combined activity contributes to cellular transformation. Our findings provide evidence that activating endogenous YAP can account for much of the transforming activity of ST, independent of previously described ST effector pathways.

Merkel Cell Carcinoma

To explore the relevance of the relationship between ST and YAP in human cancer, we turned to Merkel cell carcinoma, an aggressive skin cancer that depends on Merkel cell polyomavirus (MCV) ST activity (Feng et al., 2008; Shuda et al., 2011). Like SV40 ST, MCV ST is required for anchorage-independent growth of MCV-transformed cells (Shuda et al., 2011). MCV ST reduced phosphorylation of YAP on Ser127 (Figure 3A) and was able to support anchorage-independent growth (Figure 3B). MCV ST

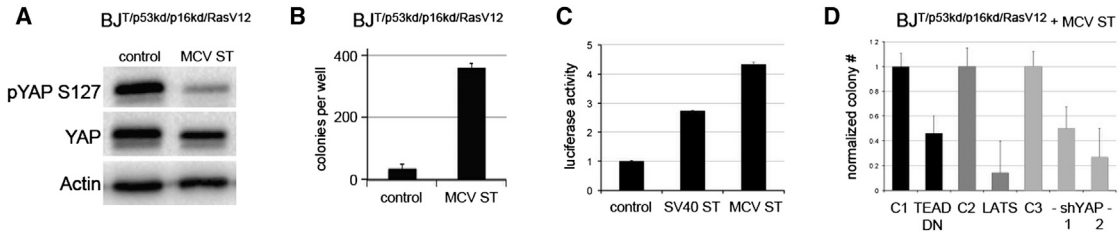

Figure 3. Merkel Cell Carcinoma ST Increases YAP Activity

$BJ^{T/p53kd/p16kd/RasV12}$ cells were transduced to express MCV ST or with empty vector control.

(A) Immunoblots showing the effect of MCV ST on YAP S127 phosphorylation.

(B) Soft agar colony formation: mean ± SD for three independent experiments. $p < 0.001$ comparing MCV-ST with control (t test).

(C) Cells were transfected with 8xGTIIC-luciferase reporter and transduced to express SV40 ST or MCV ST. Data represent the average of three independent experiments ± SD. ANOVA: $p < 0.001$ for SV40ST; $p < 0.0001$ for MCV-ST.

(D) Effects of reducing YAP activity on colony formation by MCV-ST. ANOVA: $p < 0.0001$ experiments versus their respective controls. Error bars represent mean ± SD for three independent experiments. LATS denotes LATS2. C denotes control.

also increased YAP activity in a luciferase reporter assay (Figure 3C). To assess the contribution of YAP to MCV-ST activity, we used three methods to lower YAP activity in fully transformation-competent cells expressing MCV-ST: expression of dominant-negative TEAD, expression of LATS2, and expression of shRNAs to deplete YAP (shRNA efficiency is shown in Figure S2). Each of these treatments significantly reduced colony formation in soft agar (Figure 3D). Thus, activation of endogenous YAP is required for the transforming activity of oncogenic MCV ST.

ST Acts via PAK1 on YAP Activity

ST inhibits PP2A activity (Cho et al., 2007). A mutant form of ST that is unable to bind to PP2A was severely compromised in its ability to induce colony formation (Figure 1A). PP2A has been reported to complex with Mob1/Mats to regulate phosphorylation of the Hippo pathway core kinases (Ribeiro et al., 2010; Couzens et al., 2013). Inhibition of PP2A in this context should increase MST and LATS phosphorylation, leading to increased LATS-mediated phosphorylation of YAP. Instead, we observed a decrease in YAP phosphorylation upon ST expression (Figures 2A and 3A). Thus, the activation of YAP by ST is unlikely to be mediated directly via PP2A on Hippo pathway core components.

This prompted us to explore other potential Hippo regulators with known PP2A associations. PAK1 kinase has been linked to PP2A (Westphal et al., 1999; Taglieri et al., 2011). PAK1 can phosphorylate and inactivate the tumor suppressor neurofibromin 2 (NF2; Shaw et al., 2001; Xiao et al., 2002). Depletion of NF2 leads to activation of YAP (Zhang et al., 2010) by preventing recruitment of LATS kinases to cytoplasmic membranes, where they are activated by MST kinases (Yin et al., 2013). We reasoned that ST might act on the Hippo pathway via activation of PAK1.

ST expression led to an increase in PAK1 protein and active phospho-$PAK1^{T423}$ (Figure 4A) while having little or no effect on PAK1 mRNA (Figure 2B). Interestingly, this led to an increase in the amount of PAK1 associated with cellular membranes (Figure 4B), raising the possibility that membrane association of PAK1 might mediate the effect of ST on the Hippo pathway. Expression of membrane-tethered $PAK1^{CAAX}$ (Manser et al., 1997) carrying the activating mutation T423E proved to be sufficient to replace ST, whereas wild-type $PAK1^{CAAX}$ was not (Figures 4C and S3B). Expression of ST or $PAK1^{CAAX\text{-}T423E}$ led to reduced YAP^{S127A} phosphorylation, consistent with an increase in YAP activity; other forms of PAK were less effective (Figure S3A). Like ST, expression of membrane-tethered $PAK1^{T423E}$ increased the phosphorylation of NF2 (Figure 4D). Thus, activated PAK1 was able to replace ST in blocking NF2 activity and was sufficient to support anchorage-independent growth when localized to the membrane.

To ask whether PAK1 is required for ST-mediated transformation, we made a membrane-tethered kinase-inactive form of PAK1 (K299A), which acts as a dominant negative (He et al., 2008). Expression of $PAK1^{CAAX\text{-}K299A}$ did not compromise proliferation under normal culture conditions (cytoplasmic PAK^{K299A} impaired proliferation). $PAK1^{CAAX\text{-}K299A}$ strongly reduced soft agar growth of cells expressing Ras^{V12} and ST (Figure 4E). Interestingly, membrane-tethered $PAK1^{K299A}$ was much less effective in reducing colony formation when expressed in cells where ST had been replaced by YAP^{S127A} (Figure 4E). PAK1 can also activate ERK, independent of PAK kinase activity (Wang et al., 2013). We examined ERK phosphorylation in cells expressing membrane-tethered forms of PAK, activated PAK and kinase-inactive PAK. The level of ERK phosphorylation was similar, suggesting that ERK activation was independent of PAK kinase activity in these cells. However, the ability of these PAK-CAAX proteins to support soft agar growth correlated with their effect on YAP^{S127} phosphorylation (Figure S3A). Thus, the effects of membrane associated PAK on anchorage-independent growth appear to be independent of the ERK pathway. In the absence of other methods to selectively manipulate PAK activity at the cell membrane, we cannot exclude the possibility that membrane-tethered kinase-inactive PAK might have additional effects.

As an independent means to lower PAK activity in colony-formation assays, we used the inhibitor IPA-3 (Deacon et al., 2008). Interestingly, IPA-3 treatment compromised colony formation by ST-expressing cells at concentrations that did not impair colony formation by YAP^{S127A}-expressing cells (Figure 4F). Thus, inhibiting PAK blunts the transforming potential of ST.

Next, we asked whether YAP acts downstream of membrane-tethered activated PAK. Expression of shRNAs to deplete YAP reduced colony formation by cells expressing ST or

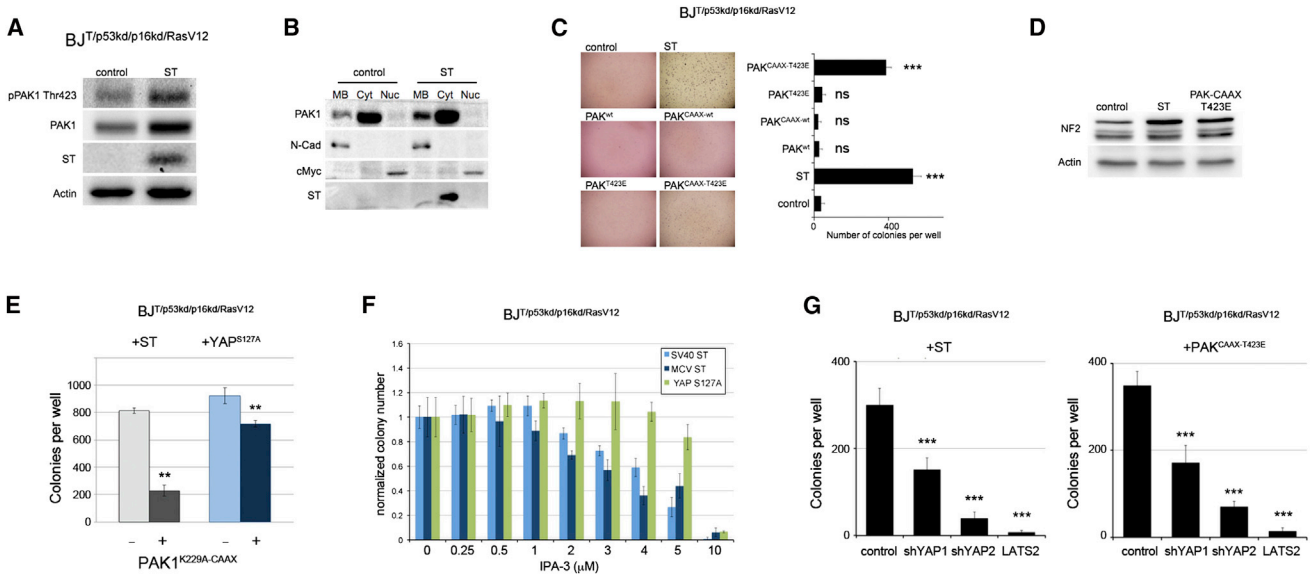

Figure 4. PAK1 Is Required to Mediate the Transforming Effect of ST

(A) Immunoblots showing the effect of ST on PAK1 and PAK1 phosphorylation in $BJ^{T/p53kd/p16kd/RasV12}$ cells ±ST.

(B) Immunoblots showing the subcellular localization of PAK1 and control proteins. Cells were fractionated into membrane (MB) cytoplasmic (Cyt) and nuclear (Nuc) fractions. N-Cadherin, membrane marker; cMyc, nuclear marker. Note the relative increase of PAK1 in the membrane fraction of the ST-expressing cells.

(C) Soft agar colony formation by $BJ^{T/p53kd/p16kd/RasV12}$ cells transduced to express ST or variants of PAK1. Data represent the mean ± SD for three independent experiments. ANOVA: ***$p < 0.0001$ versus control. ns: not significant.

(D) $BJ^{T/p53kd/p16kd/RasV12}$ cells were transduced to express ST or activated $PAK1^{L107F\text{-}CAAX}$. Immunoblots show the expression of NF2. Actin was used as a loading control. The phosphorylated form of NF2 is inactive and migrates slower in SDS-PAGE.

(E) Soft agar colony formation by $BJ^{T/p53kd/p16kd/RasV12}$ cells transduced to express ST or YAP^{S127A} ± $PAK1^{K229A\text{-}CAAX}$. Data represent the mean ± SD for three independent experiments. **$p < 0.01$ (t test).

(F) Soft agar colony formation by $BJ^{T/p53kd/p16kd/RasV12}$ cells transduced to express SV40 or MCV ST or YAP^{S127A}. Cells were treated with the PAK inhibitor IPA-3. Data represent the mean ± SD for three independent experiments.

(G) Soft agar colony formation by $BJ^{T/p53kd/p16kd/RasV12}$ cells transduced to express ST (left) or $PAK1^{T423E\text{-}CAAX}$ (right). Cells were transduced to express shRNAs to deplete YAP or to overexpress LATS2. Data represent the mean ± SD for three independent experiments. ANOVA: ***$p < 0.0001$ versus control.

membrane-tethered activated PAK^{T423E} (Figure 4G). Similarly, blocking Hippo pathway activity by overexpressing LATS2 reduced colony formation by ST- or PAK^{T423E}-expressing cells (Figure 4G).

The experiments in Figures 4E–4G provide evidence that the ST oncoprotein acts via PAK1 at the cell membrane to regulate the Hippo pathway. The importance of this mechanism is shown by the finding that inhibition of PAK1 can compromise ST-mediated transformation while having relatively little effect on YAP-mediated transformation. We have provided evidence that PAK contributes to oncogenic transformation through activation of the YAP proto-oncogene and that activation of YAP is a key requirement for ST in transformation of primary human cells. YAP activity is required downstream of ST and activated PAK1, serving as a gatekeeper for anchorage-independent growth.

EXPERIMENTAL PROCEDURES

Antibodies, plasmids, primers, and standard methods are described in Supplemental Experimental Procedures.

Viral Transduction

Ecotropic and amphotropic retroviruses were made as described previously (Brummelkamp et al., 2002). Supernatant from transfected Ecopack2 and Phoenix-Ampho cells was harvested at 36–48 hr and frozen in aliquots. Cells were plated to reach 70% confluence when infected with virus overnight in the presence of 8 μg/ml polybrene. Antibiotic selection was started at 36 hr. HMECs and BJ cells were first retrovirally transduced to express the ecotropic receptor for subsequent transductions by ecotropic retroviruses (Voorhoeve and Agami, 2003).

Soft Agar Assay

A total of 4×10^4 cells were resuspended in 2 ml 0.375% low-gelling-temperature agarose (Sigma-Aldrich, A-9045) in Dulbecco's modified Eagle's medium (DMEM), 10% serum, seeded in triplicate in six-well plates coated with 1% low-gelling agarose in DMEM, 10% serum. A total of 1 ml of DMEM, 10% fetal calf serum (BJ cells), or MGEM bullet kit medium (HMECs) was added weekly. Colonies were stained with 0.75 mg/ml 3-(4,5-dimethythiazol)-2,5-diphenyl tetrazolium bromide after 2–3 weeks and imaged. Colony counting was automated (MATLAB). Data were analyzed using ANOVA or a two-tailed t test (unequal variance), as indicated.

Luciferase Assays

8xGTIIC (Dupont et al., 2011) was used to monitor YAP/TEAD activity in HEK293 cells. Firefly luciferase activity was normalized to a Renilla luciferase transfection control and to empty vector controls. Dual luciferase assays were performed (Bio-Rad).

Tumorigenic Growth in NOD-SCID Mice

Animal work was carried out according to institutional guidelines (Institute of Molecular and Cell Biology [IMCB] Institutional Animal Care and Use

Committee number 120768). Approximately 2 × 10^6 cells were injected subcutaneously in each flank of immunocompromised NOD-SCID *Il2rg*$^{-/-}$ mice (Chen et al., 2009). Tumor growth was monitored every 3–4 days. Mice were sacrificed when tumors reached 2 cm in diameter.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.06.062.

AUTHOR CONTRIBUTIONS

H.T.N., X.H., S.T., Q.C., and L.C. performed experiments. M.F. wrote the MATLAB script. H.T.N., X.H., P.M.V., and S.M.C. designed experiments, analyzed data, and wrote the paper.

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