SRC Increases *MYC* mRNA Expression in Estrogen Receptor-Positive Breast Cancer via mRNA Stabilization and Inhibition of p53 Function

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ABSTRACT The transcription factor gene *MYC* is important in breast cancer, and its mRNA is maintained at a high level even in the absence of gene amplification. The mechanism(s) underlying increased *MYC* mRNA expression is unknown. Here, we demonstrate that *MYC* mRNA was stabilized upon estrogen stimulation of estrogen receptor-positive breast cancer cells via SRC-dependent effects on a recently described RNA-binding protein, IMP1 with an N-terminal deletion (Δ N-IMP1). We also show that loss of the tumor suppressor p53 increased *MYC* mRNA levels even in the absence of estrogen stimulation. However, in cells with wild-type p53, SRC acted to overcome p53-mediated inhibition of estrogen-stimulated cell cycle entry and progression. SRC thus promotes cell proliferation in two ways: by stabilizing *MYC* mRNA and by inhibiting p53 function. Since estrogen receptor-positive breast cancers typically express wild-type p53, these studies establish a rationale for p53 status to be predictive for effective SRC inhibitor treatment in this subtype of breast cancer.

KEYWORDS SRC, breast cancer, c-myc, estrogen, p53

MYC is important for breast cancer proliferation and progression, and *MYC* mRNA is highly expressed in breast cancer, often independent of gene amplification (reviewed in reference 1). The *MYC* gene product is known to be regulated at the transcriptional (2–4), posttranscriptional (5–8), and posttranslational (9, 10) levels. However, it is unclear which mechanism(s) may be important to maintain high *MYC* mRNA levels in breast cancer.

MYC is known to be an estrogen (E2)-stimulated gene (2, 3, 11–14), and in estrogen receptor-positive (ER⁺) breast cancer, *MYC* is required for E2-dependent breast cancer cell proliferation (13). However, there are conflicting reports on how E2 regulates *MYC* (2, 3, 11–14). One report suggests that E2 stimulates *MYC* transcription although it is unlikely to be a direct ER target because no estrogen-responsive element has been found in the *MYC* promoter (12). On the other hand, in other cell types the *MYC* mRNA half-life is known to be regulated by *cis* elements within its mRNA sequence, including a coding region determinant (CRD) (15–19), as well as the 3' untranslated region (UTR) which contains AU elements and miRNA binding sites (20, 21). Several RNA-binding proteins regulate *MYC* mRNA half-life via these *cis* elements, including stabilization via the CRD by insulin growth factor 2 binding protein 1 (IMP1, IGF2BP1, CRD-BP, and ZBP1) (15, 19, 22) and destabilization by tristetraprolin (TTP) (23, 24). Interestingly, IMP1 is typically expressed during development but is reexpressed during cancer progression

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in several tumor types and cell lines, including breast cancer (15, 25–28). A recent report also suggests that MCF7 cells express an N-terminally truncated form of the protein, Δ N-IMP1, which is required for clonal outgrowth of cells (29). Whether either form of IMP1 is involved in E2-dependent regulation of *MYC* mRNA remains to be tested.

E2 signaling acts via both canonical (genomic) and rapid-action (nongenomic) pathways (reviewed in references 30 to 35). Some evidence exists that the nongenomic pathway is important for E2-dependent proliferation. For example, in cells lacking endogenous ER expression, the expression of ER DNA-binding mutants allowed S-phase entry upon E2 stimulation (36, 37). In addition, E2 stimulation of MCF7 breast cancer cells expressing ER DNA-binding mutants induced *MYC* mRNA expression and proliferation, suggesting that *MYC* induction occurs via nongenomic ER signaling (36).

Previous research in our lab using the model system of platelet-derived growth factor (PDGF)-stimulated fibroblasts has demonstrated that *MYC* mRNA expression is required for cell cycle progression downstream of the tyrosine kinase SRC (38). We have also shown that SRC regulates the stability of several short-lived mRNAs, including *MYC* mRNA (39). These data suggest that SRC promotes *MYC* mRNA expression posttranscriptionally in fibroblasts. Interestingly, overexpression of kinase-dead SRC in fibroblasts engineered to express either wild-type or mutant ER blocked cell cycle progression (37), suggesting that SRC might be a nongenomic E2 signaling mediator. Other reports of interactions between the ER and SRC are also suggestive of a role for SRC in E2 signaling pathways (40–43).

We have also previously demonstrated that the requirement for SRC in PDGF-stimulated cell cycle progression is lost in fibroblasts lacking functional p53, suggesting that SRC may overcome a p53 brake on cell cycle progression (44). Unlike the majority of cancer types, ER⁺ breast cancer cells often retain wild-type p53 (45, 46). Because p53 loss of function seems to be a critical event in cancer development, one hypothesis could be that cancer cells that express wild-type p53 have a mechanism(s) to suppress p53 function. Indeed, several studies have suggested that E2 signaling may inhibit p53 function indirectly (47–49). A direct interaction of the ER with p53 has also been described to modulate its transcriptional activity (50, 51). These data suggest that although ER⁺ breast cancer retains wild-type p53, E2 signaling may suppress p53 function.

The mechanisms by which SRC regulates *MYC* mRNA and p53 function are unknown, as is the relevance of findings from mouse fibroblasts to human cancer cells. We reasoned that ER⁺ breast cancer may be a relevant system to study *MYC* mRNA regulation and SRC involvement in cell cycle progression. Here, we use both *in vitro* and *in vivo* models to study the role of an SRC-p53-*MYC* signaling axis and its importance for proliferation of ER⁺ breast cancer cells.

RESULTS

SRC is required for E2-dependent proliferation. Using the ER⁺ breast cancer cell line MCF7 (Fig. 1), we first tested whether SRC family kinases (SFKs) were required for E2-dependent proliferation. We used a small-molecule inhibitor of SFK activity, SU11333 (52), which is structurally similar to SU6656 (53) but has increased selectivity and potency (52). We serum starved MCF7 cells for 48 h to induce quiescence in the cells. Upon stimulation of cells with E2, we observed an increase in the level of pY418-SRC (a surrogate for activation), which was abolished by pretreatment with SU11333 (Fig. 1A). We next tested whether SFK activity was required for cell cycle progression and proliferation. We pretreated cells with either ICI182870 (an antiestrogen) or SU11333, stimulated them with E2, and then used bromodeoxyuridine (BrdU) incorporation as a surrogate for G₁/S transition. E2 stimulated entry into S phase, which was inhibited by both ICI182870 and SU11333 (Fig. 1B). To test whether this was an acute cell cycle delay or a sustained cell cycle block, we also performed proliferation assays. The ICI182870 and SU11333 inhibitor-treated cells were unable to proliferate, in contrast to E2-stimulated cells (Fig. 1C), providing evidence of a long-term, persistent block to proliferation in the absence of E2 or SFK activity.

FIG 1 SRC is required for E2-stimulated cell cycle progression and proliferation. (A) Representative immunoblot analysis of phospho-SFK (pSFK) and total SRC expression of quiescent MCF7 cells pretreated with vehicle or 1 μ M SU11333 and stimulated with vehicle or 5 nM E2. (B and C) BrdU incorporation assay or proliferation assay of quiescent MCF7 cells pretreated with 10 nM ICI182870 (ICI) or 1 μ M SU11333 (SU) and treated with vehicle or 5 nM E2 and BrdU. (D) Representative immunoblot analysis of SRC expression of either shCtrl or shSRC stable knockdown MCF7 lines. Results of a BrdU incorporation assay are also shown. (E) Proliferation assay of quiescent shCtrl or shSRC stable knockdown MCF7 lines. **, $P < 0.01$.

SU11333 inhibits a number of SFKs (52), and MCF7 cells express SRC at high levels and YES (another SFK) at low to moderate levels (Fig. 2A). To determine whether the cell cycle block was due specifically to SRC, we repeated the cell cycle progression and proliferation assays using two different SRC-targeting short hairpin RNAs (shRNAs) (Fig. 1D and E and 2A and B). In cells expressing an shRNA targeting SRC (shSRC), both BrdU incorporation and proliferation were inhibited relative to levels in shCtrl cells similarly using either shRNA (Fig. 1D and E). However, shYES cells showed no statistical difference in BrdU incorporation or proliferation levels from the level in shCtrl cells (Fig. 2). These data suggest that SRC is the primary SFK required for cell cycle progression and proliferation upon E2 stimulation and that its kinase activity is required for these functions.

Estrogen stimulates SRC-dependent expression of MYC mRNA. Previous studies had suggested that MYC expression is required for proliferation of an ER⁺ cell line (54),

FIG 2 YES is not required for E2-dependent proliferation. (A) Representative immunoblot analysis of YES expression in either shCtrl or shYES stable knockdown MCF7 lines. A BrdU incorporation assay is also shown. (B) Proliferation assay of quiescent shCtrl or shYES stable knockdown MCF7 lines.

and we have previously shown that in mouse fibroblasts stimulated with PDGF, SFK activation increases *MYC* mRNA (39). To test whether *MYC* was required for E2-dependent cell cycle progression in the MCF7 cells, we used shRNA-mediated knock-down of *MYC* mRNA followed by BrdU incorporation and proliferation assays. The sh*MYC* cells grew in complete growth medium. However, in quiescent cells, E2 failed to stimulate the *MYC* knockdown cells, compared to the level for shCtrl cells, to enter S phase in either the presence or absence of SU11333 (Fig. 3A). Downregulation of *MYC* also led to a decrease in E2-dependent proliferation over several days (Fig. 3B).

Since both ER and SRC have been implicated in regulating expression of *MYC* mRNA, we next tested whether E2 stimulated *MYC* mRNA expression, and, if so, whether SRC is required. Indeed, E2 stimulated *MYC* mRNA levels in a dose- and time-dependent manner (Fig. 3C and D). To test whether SRC was required for this stimulation, we again used either SU11333 or shSRC knockdown cells. *MYC* mRNA levels were not induced upon any concentration of E2 stimulation or at any time after E2 stimulation in either the inhibitor-treated or the shSRC cells (Fig. 3C and D). These data suggest that SRC activity is required for *MYC* mRNA accumulation after E2 stimulation.

SRC mediates E2-dependent expression of *MYC* mRNA by stabilizing its mRNA levels. We next tested possible mechanisms of *MYC* mRNA accumulation after E2 stimulation. As E2 has previously been reported to transcriptionally activate *MYC* mRNA (2), we first performed a nuclear run-on assay to test whether E2 induced *MYC* mRNA transcription (Fig. 3E). We stimulated quiescent cells with E2 and then paused transcription at various time points. We isolated intact nuclei, incubated them with 5-ethynyl uridine (EU), and allowed elongation of initiated transcripts and incorporation of the EU for various time periods. The EU was then biotinylated and captured using streptavidin beads (to collect only actively transcribed mRNAs), and quantitative PCR (qPCR) analysis was performed. The housekeeping gene *PPIA* was analyzed as a negative control since E2 should not stimulate its transcription (Fig. 3E, right). As a positive control, we measured the transcription of *PS2*, a known transcriptional target of the ER (55, 56), and observed an increase in *PS2* transcription after E2 stimulation (Fig. 3E, middle). However, we detected no increase in transcription of *MYC* mRNA after E2 stimulation (Fig. 3E, left). For each time point, we had also collected the cytoplasmic fractions of mRNA. Interestingly, we observed an increase in both *PS2* and *MYC* mRNA but not *PPIA* mRNA in the cytoplasm (Fig. 3F). These data suggest that in this system, the predominant mechanism of *MYC* regulation is posttranscriptional.

There is evidence that E2 may regulate *MYC* mRNA posttranscriptionally by stabilizing the transcript (3). We tested this by stimulating cells with E2 to allow for an increase in *MYC* mRNA, treating them with actinomycin D to block transcription, and then isolating RNA for qPCR analysis. We detected an increase in stabilization of *MYC* mRNA in E2-stimulated cells compared to the basal level of decay of *MYC* mRNA observed in vehicle-treated cells (Fig. 3G, left). Calculated half-lives for all of the mRNA stability assays can be found in Table 1. *PS2* mRNA was not stabilized (Fig. 3G, right), suggesting that these two genes are regulated by different mechanisms following E2 stimulation.

We previously reported that in fibroblasts stimulated with PDGF, SRC stabilizes *MYC* mRNA (as well as other short-lived RNAs such as those of *MCP-1*, *MCP-3*, and *ADM*) (39). We hypothesized that E2 may also stabilize *MYC* mRNA in an SRC-dependent manner in ER⁺ breast cancer cells. To test this, we performed an mRNA stability assay on SU11333-treated and shSRC knockdown cells. E2-stimulated *MYC* mRNA stabilization was abrogated in both cases (Fig. 3G and H, left panels), but there was no effect on *PS2* half-life (Fig. 3G and H, right panels).

To demonstrate that the above observations were not a cell line-specific effect, we also performed similar *in vitro* assays using another ER⁺ breast cancer cell line, ZR-75-1, and observed similar effects in both BrdU incorporation and proliferation assays (Fig. 4A and B) as well as *MYC* mRNA accumulation and stability assays (Fig. 4C to E).

ΔN-IMP1, a truncated form of the RNA-binding protein IMP1, is required for *MYC* mRNA stability. We hypothesized that a candidate RNA-binding protein, IMP1,

FIG 3 E2 induces MYC mRNA expression via SRC-dependent stabilization of mRNA and not via transcriptional activation. (A) Representative immunoblot analysis of MYC expression in shCtrl or shMYC MCF7 stable knockdown cell lines. A BrdU incorporation assay of quiescent shCtrl or shMYC stable knockdown MCF7 lines pretreated with 1 μ M SU11333 (SU) and treated with vehicle or 5 nM E2 and BrdU is shown. (B) Proliferation assay of quiescent shCtrl or shMYC stable knockdown MCF7 lines treated with vehicle or 5 nM E2. (C) qPCR analysis of quiescent MCF7 cells that were pretreated with vehicle or 1 μ M SU and treated with vehicle or 5 nM E2. (Continued on next page)

TABLE 1 Calculated mRNA half-lives

Fig. no. and mRNA	Cell line modification	Treatment	Mean half-life [min (SEM)]	
3G				
MYC	None	Vehicle	39 (8)	
		E2	104 (12)	
		E2/SU	35 (11)	
PS2	None	Vehicle	34 (9)	
		E2	51 (15)	
		E2/SU	43 (12)	
3H				
MYC	shCtrl	Vehicle	36 (11)	
		E2	124 (21)	
	shSRC	Vehicle	35 (9)	
PS2	shCtrl	E2	29 (7)	
		Vehicle	31 (9)	
	shSRC	E2	35 (11)	
		Vehicle	43 (8)	
		E2	37 (11)	
4E: MYC				
None	None	Vehicle	31 (9)	
		E2	107 (14)	
		E2/SU	37 (12)	
6C: MYC				
shCtrl	shCtrl	Vehicle	36 (13)	
		E2	129 (19)	
		E2/SU	39 (14)	
	sh8079	Vehicle	43 (13)	
		E2	106 (12)	
		E2/SU	38 (9)	
	sh5149	Vehicle	42 (8)	
		E2	57 (13)	
		E2/SU	61 (17)	
6F: MYC				
sh5149/Empty	sh5149/Empty	Vehicle	34 (12)	
		E2	39 (10)	
	sh5149/ Δ N-IMP1	Vehicle	36 (11)	
		E2	117 (16)	
6H: MYC				
sh5149/Empty	sh5149/Empty	Vehicle	38 (9)	
		E2	44 (11)	
	sh5149/ Δ N-IMP1	Vehicle	47 (10)	
		E2	118 (19)	
	sh5149/Y260F	Vehicle	36 (8)	
		E2	39 (13)	
	8F: MYC			
	shCtrl	shCtrl	Vehicle	33 (12)
E2			139 (23)	
E2/SU			39 (15)	
shTP53		Vehicle	63 (12)	
		E2	81 (24)	
		E2/SU	74 (11)	

could be the cause of *MYC* mRNA stabilization. IMP1 is a developmentally expressed RNA-binding protein which is reexpressed in many cancers (15, 27, 57) and stabilizes *MYC* mRNA in a variety of cell types (19, 28, 29, 57). We did not detect *IMP1* mRNA in the MCF7 or ZR-75-1 cell lines although we did in 293T cells, the positive control (Fig. 5A). However, a recent report suggested that there is also a truncated form of the

FIG 3 Legend (Continued)

increasing concentrations of E2 (left) or with 5 nM E2 over 90 min (right). (D) qPCR analysis of quiescent shCtrl or shSRC MCF7 lines that were pretreated with vehicle or 1 μ M SU and treated with increasing concentrations of E2 (left) or with 5 nM E2 over 90 min (right). (E) Nuclear run-on assay of MCF7 cells treated with E2 over a time course. (F) Cytoplasmic fractions from the same samples as the nuclear run-on assay shown in panel E. (G and H) *MYC* mRNA stability assays in MCF7 cells (G) or shCtrl and shSRC MCF7 cells (H) pretreated with vehicle or 1 μ M SU and stimulated with vehicle or 5 nM E2 were performed. After 60 min, cells were treated with 5 μ M actinomycin D to block transcription. *, $P < 0.05$; **, $P < 0.01$; ns, not significant. Calculated mRNA half-lives can be found in Table 1.

FIG 4 ZR-75-1 cells require SFKs for E2-dependent proliferation, *MYC* mRNA accumulation, and *MYC* mRNA stabilization. (A and B) A BrdU incorporation assay (A) or proliferation assay (B) of quiescent ZR-75-1 cells pretreated with 10 nM ICI182870 or 1 μ M SU11333 (SU) and treated with vehicle or 5 nM E2 and BrdU was performed. (C and D) qPCR analysis of quiescent ZR-75-1 cells that were pretreated with vehicle or 1 μ M SU11333 and treated with increasing concentrations of E2 (C) or with 5 nM E2 over 90 min (D) was performed. (E) *MYC* mRNA stability assay in ZR-75-1 cells pretreated with vehicle or 1 μ M SU and stimulated with vehicle or 5 nM E2. After 60 min, cells were treated with 5 μ M actinomycin D to block transcription. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Calculated mRNA half-lives can be found in Table 1.

protein, Δ N-IMP1, which is expressed in some cancer cell lines, including MCF7 cells (29). We used primers specific for the truncated form and, indeed, detected Δ N-IMP1 mRNA in both MCF7 and ZR-75-1 cells as well as at low levels in 293T cells (Fig. 5A). We next analyzed protein lysates from 293T, MCF7, and ZR-75-1 cells by immunoblotting using two IMP1 antibodies. The Cell Signaling (CS) antibody detects an epitope present only in full-length IMP1 (FL-IMP1), while the Sigma antibody can detect both FL-IMP1 and Δ N-IMP1. In keeping with the qPCR results, we found that the MCF7 and ZR-75-1 cells expressed only the short form, while the 293T cells expressed primarily FL-IMP1 but also Δ N-IMP1 at low levels (Fig. 5B). We extended this analysis to several ER⁺ breast cancer cell lines. We detected Δ N-IMP1 in all of the cell lines tested, but none expressed the full-length form (Fig. 6A). To test the specificity of the antibodies as well as generate knockdown cell lines for functional analyses, we generated stable knockdown in either 293T cells (Fig. 5C) or MCF7 cells (Fig. 6B) using shRNAs targeting FL-IMP1 only (sh8079) or both forms of IMP1 (sh5149). Immunoblot analysis demonstrated specific knockdown.

We hypothesized that Δ N-IMP1 might be required for *MYC* mRNA stabilization, which we tested using the MCF7 sh8079 or sh5149 knockdown or scrambled control (shCtrl) cells in the RNA stability assay to measure *MYC* mRNA half-life (Fig. 6C). The sh8079 cells behaved similarly to shCtrl cells in this assay (Fig. 6C). However, sh5149 cells (targeting both forms of IMP1) were unable to stabilize *MYC* mRNA upon E2 stimulation (Fig. 6C). This suggested that Δ N-IMP1 is required for *MYC* mRNA stabilization. We next determined whether the loss of IMP1 also affected cell cycle progression and proliferation. Loss of only FL-IMP1 had no effect, whereas sh5149 cells were unable to enter S phase and also had a proliferative defect (Fig. 6D and E). We also tested whether Δ N-IMP1 was specifically required for *MYC* mRNA stability due to E2 stimulation. To test this, we expressed nontargetable Δ N-IMP1 (or an empty vector as a control) in sh5149 cells (Fig. 6F). Δ N-IMP1 expression rescued *MYC* mRNA stabilization whereas empty-vector-expressing cells did not (Fig. 6F).

Phosphorylation of tyrosine 260 of Δ N-IMP1 is required for *MYC* mRNA stability. In the context of regulating *ACTB* mRNA levels, SRC regulates IMP1 function through phosphorylation of tyrosine 396 (58). We hypothesized that phosphorylation of this residue was also required for Δ N-IMP1 to stabilize *MYC* mRNA. To test this, we

FIG 5 ΔN -IMP1 mRNA expression and antibody and shRNA validation. (A) Reverse transcription-PCR analysis of RNA expression in 293T, MCF7 and ZR-75-1 cells. FL, FL-IMP1; ΔN , ΔN -IMP1. (B) Representative immunoblot analysis of 293T, MCF7, and ZR-75-1 cell lysates and probing for FL-IMP1 (CS) or FL-IMP1/ ΔN -IMP1 (Sigma). (C) Validation of shRNA knockdown in 293T cells of FL-IMP1 (sh8079) or FL-IMP1/ ΔN -IMP1 (sh5149).

generated an ΔN -IMP1 mutant with tyrosine 260 (corresponding to the Y396 residue in FL-IMP1) mutated to phenylalanine to mimic a nonphosphorylated residue (Fig. 6G). We then expressed either wild-type ΔN -IMP1 or the Y260 mutant in sh5149 knockdown cells. Introduction of a wild-type version of ΔN -IMP1 resulted in an increased *MYC* mRNA half-life compared to that with the empty vector (Fig. 6H). However, expression of the ΔN -IMP1-Y260F mutant was unable to rescue stabilization of *MYC*, suggesting that phosphorylation of the tyrosine residue at 260 is required for increased *MYC* mRNA stability (Fig. 6H). The lack of immunoprecipitating antibodies with selectivity for ΔN -IMP1 prevented us from analyzing the tyrosine phosphorylation of the endogenous proteins. We therefore generated either C- or N-terminally Flag-tagged ΔN -IMP1 expression constructs; however, we were unable to express these constructs in the sh5149 cells. We hypothesize that adding a tag to either end of the protein interferes with ΔN -IMP1 function, leaving us unable to directly test the role of tyrosine 260 at this time.

We next tested the effect of the Y260F mutant on cell cycle progression and proliferation. Using a BrdU incorporation assay, the sh5149 knockdown cells were unable to progress in the cell cycle, in contrast to shCtrl cells (Fig. 6I), whereas reexpressing wild-type ΔN -IMP1 rescued this defect. However, in the cells where we expressed ΔN -IMP1-Y260F, we were unable to observe cells entering S phase or proliferating compared with results with the wild-type ΔN -IMP1 rescue cells, and they resembled more the sh5149 knockdown cells (Fig. 6I and J). These data suggest that the

FIG 6 ΔN-IMP1 is required for E2- and SRC-dependent stabilization of MYC mRNA. (A) Representative immunoblot analysis of ER⁺ breast cancer cell lines for FL-IMP1/ΔN-IMP1 expression. (B) Representative immunoblot of shCtrl, sh8079 (FL-IMP1), or sh5149 (FL-IMP1/ΔN-IMP1) knockdown in MCF7 cells. (C) MYC mRNA stability assay. shCtrl, sh8079, and sh5149 MCF7 cells were pretreated with vehicle or 1 μM SU11333 and stimulated with vehicle or 5 nM E2. After 60 min, cells were treated with 5 μM actinomycin D to block transcription. (D and E) BrdU incorporation assay and proliferation assay in shCtrl,

(Continued on next page)

phosphorylation of Y260 is required for *MYC* mRNA stability and, ultimately, cell cycle progression.

SRC is required to relieve a p53 cell cycle block after E2 stimulation. Earlier we demonstrated a requirement for SRC for E2-dependent cell cycle progression and *MYC* mRNA stabilization, consistent with our previous observations in PDGF-stimulated mouse fibroblasts (59, 60). In this model system we also observed that SRC relieves a p53 cell cycle block to allow cell cycle entry (44). To test whether this also held true in the ER⁺ breast cancer model, particularly because ER⁺ breast cancer cells typically retain wild-type p53, we generated stable p53 (shTP53) and scrambled control (shCtrl) knockdown in MCF7 cells and performed a BrdU incorporation assay after stimulating quiescent MCF7 cells with E2, with and without SU11333 treatment (Fig. 7A). As before, E2-stimulated cell cycle entry was blocked in cells pretreated with SU11333. However, in shTP53 knockdown cells, SU11333

FIG 6 Legend (Continued)

sh8079, and sh5149 knockdown MCF7 cells pretreated with vehicle or 1 μ M SU11333 (SU) and stimulated with vehicle or 5 nM E2. (F) Representative immunoblot of sh5149 knockdown MCF7 cells also expressing either empty vector or Δ N-IMP1 (left). The tubulin blot was run on a separate gel with the same samples as the Δ N-IMP1 blot. An *MYC* mRNA stability assay with empty vector- or Δ N-IMP1-expressing sh5149 knockdown MCF7 cells pretreated with vehicle or 1 μ M SU and stimulated with vehicle or 5 nM E2 is shown (right). (G) Representative immunoblot of shCtrl, sh5149, Δ N-IMP1/sh5149, or Δ N-IMP1-Y260F/sh5149 MCF7 cells. (H) *MYC* mRNA stability assay in sh5149 cells expressing empty vector, Δ N-IMP1, or Δ N-IMP1-Y260F pretreated with vehicle or 1 μ M SU11333 and stimulated with vehicle or 5 nM E2. After 60 min, cells were treated with 5 μ M actinomycin D to block transcription. (I and J) BrdU incorporation assay and proliferation assay in shCtrl, sh5149, Δ N-IMP1, and Δ N-IMP1-Y260F MCF7 cells pretreated with vehicle or 1 μ M SU11333 and stimulated with vehicle or 5 nM E2. *, $P < 0.05$. Calculated mRNA half-lives can be found in Table 1.

was unable to block E2-dependent BrdU incorporation (Fig. 7A). We also performed a proliferation assay to assess the long-term effects of SFK inhibition in shTP53 cells (Fig. 7B). The vehicle-treated shCtrl and shTP53 cells grew at nearly the same rates. Pretreatment with SU11333 blocked E2-stimulated proliferation in the shCtrl cells but not in the shTP53 cells (Fig. 7A and B). To complement the SFK inhibitor experiment, we also generated individual shSRC or shTP53 knockdown cells as well as double shSRC/shTP53 knockdown cells and assessed both BrdU incorporation and proliferation (Fig. 7C). Consistent with the inhibitor experiments, SRC knockdown blocked S-phase entry and proliferation in control cells but was unable to block BrdU incorporation or proliferation in shTP53 cells (Fig. 7C and D). We also introduced mouse p53 in the shTP53 cells and showed that it restored the cell cycle block and proliferation upon SU11333 treatment (Fig. 7E and F). Taken together, these experiments suggest a role for SRC kinase activity to overcome a p53 block to E2-stimulated S-phase entry and proliferation.

p53 regulates E2-dependent MYC mRNA expression. Our earlier results demonstrated a requirement for MYC in proliferation downstream of E2 and SRC (Fig. 4), and we also demonstrated that SRC overcame p53 function downstream of E2 signaling. Given these data, we hypothesized that p53 may also regulate MYC mRNA. We observed an increase in the basal level of MYC mRNA (Fig. 8A), as well as in MYC and Δ N-IMP1 protein levels (Fig. 8B), in TP53 knockdown cells compared to levels in controls. However, E2 stimulation caused the same fold increase in MYC mRNA in shTP53 and control cells (Fig. 8C and D). Importantly, SU11333 blocked E2 induction of MYC mRNA in shCtrl cells but had no effect in p53 knockdown cells (Fig. 8E). These data suggested that p53 is likely upstream of MYC mRNA expression and downstream of SRC.

We next tested how p53 knockdown affected MYC mRNA expression. We observed neither changes in transcription of MYC nor MYC cytoplasmic mRNA accumulation compared to levels in shCtrl cells (Fig. 9). We therefore tested whether MYC mRNA stability was changed by p53 knockdown. Surprisingly, we did not see an increase in half-life upon E2 stimulation compared to that of the shCtrl cells (Fig. 8F). However, we did see an increased half-life of basal MYC mRNA compared to that of the shCtrl cells, and a similar half-life was observed in the SU11333-treated cells (Fig. 8F). Upon knockdown of p53, it appears that the cells lose the ability to stabilize MYC mRNA upon E2 stimulation but, instead, maintain a higher basal half-life of MYC mRNA. We hypothesize that increased Δ N-IMP1 levels in shTP53 cells may increase the basal half-life of MYC mRNA independent of E2. In this scenario, E2 stimulation may increase the accumulation of MYC mRNA potentially via regulating Δ N-IMP1 mRNA binding. These data are consistent with a model that increased expression of MYC mRNA upon p53 loss is due to an increase in the basal half-life of MYC mRNA.

MCF7 xenografts require SRC kinase expression for tumor initiation and growth in the mouse mammary fat pad. Finally, we wanted to determine the physiological significance of our *in vitro* findings. We therefore tested whether SRC was required to inhibit p53 function *in vivo* using an orthotopic MCF7 xenograft model. We injected shCtrl cells, shSRC cells, shTP53 cells, or double shSRC/shTP53 knockdown cells into the mouse mammary fat pad and measured tumor volume over time. The shTP53 cells grew at nearly the same rate as shCtrl cells, generating large tumors during the course of the experiment (Fig. 10A). However, mice injected with the shSRC cells did not develop tumors at any point during the experiment, and only 2 of 7 mice inoculated with the double shSRC/shTP53 knockdown cells developed tumors, which were very small (Fig. 10A). We repeated this experiment, injecting cells after they had entered quiescence prior to injection (Fig. 10B), and observed delayed tumor initiation but similar overall results. From these data, we conclude that SRC is likely required for grafting of the MCF7 orthotopic tumors, independent of p53 expression.

To overcome the grafting defect of the xenografts in the absence of SRC, we generated cells expressing doxycycline (Dox)-regulated control (TOshCtrl) or SRC (TOsh-

FIG 8 Loss of p53 leads to increases in MYC and ΔN-IMP1 expression independent of E2 or SFK activity. (A and B) qPCR analysis and representative immunoblot analysis of shCtrl and shTP53 cells. (C and D) qPCR analysis of quiescent shCtrl or shTP53 knockdown MCF7 lines that were treated with increasing concentrations of E2 (C) or with 5 nM E2 over 90 min (D). (E) qPCR analysis of quiescent shCtrl or shTP53 knockdown MCF7 lines that were pretreated with vehicle or 1 μM SU11333 and treated with 5 nM over 90 min. (F) MYC mRNA stability assay in shCtrl and shp53 knockdown MCF7 cells pretreated with vehicle or 1 μM SU11333 and stimulated with vehicle or 5 nM E2. After 60 min, cells were treated with 5 μM actinomycin D to block transcription. *, $P < 0.05$. Calculated mRNA half-lives can be found in Table 1.

SRC) shRNA in the presence of stable shCtrl or shTP53 constructs, giving rise to the following cells: TOshCtrl/shCtrl, TOshSRC/shCtrl, TOshCtrl/shTP53, and TOshSRC/shTP53. To test the inducible knockdown in the cells, we first performed an *in vitro* time course. At 24 h after Dox treatment, we observed some expression of SRC; however, by 48 h and persisting at 72 h, we observed efficient knockdown of SRC (Fig. 10C). For the *in vivo* experiments, cells were injected and allowed to grow until tumors reached ~100 mm³, and then mice were randomized into chow groups without (–) or with (+) Dox. To control for potential basal expression of the shRNAs, some mice were not given Dox (Fig. 10D, top). All groups of mice in the –Dox group grew at the same rate, suggesting that the inducible shRNA vectors had no basal leaky expression. The Dox-treated TOshCtrl/shCtrl and the TOshCtrl/shTP53 cells grew at nearly the same rate as the untreated cells (Fig. 10D, bottom). However, upon doxycycline treatment, the TOshSRC/shCtrl tumors regressed until no measurable tumors were present by day 25 (Fig. 10D,

FIG 9 Loss of p53 does not affect MYC mRNA transcription. For panels A and B as indicated, a nuclear run-on assay was performed in MCF7 shCtrl- and shTP53-expressing cells treated with E2 over a time course (top rows). Respective cytoplasmic fractions from the same samples as those of the nuclear run-on assay are also shown (bottom rows).

bottom). Interestingly, the T0shSRC/shTP53 tumors neither grew nor regressed following Dox treatment (Fig. 10D, bottom). We conclude that loss of SRC in tumors with wild-type p53 leads to regression but to tumor stasis in those lacking p53 expression.

DISCUSSION

Here, we show that in ER⁺ breast cancer, SRC was required for E2-dependent stabilization of MYC mRNA via a mechanism that involves the RNA-binding protein Δ N-IMP1. Little is known about Δ N-IMP1; it lacks the first two RNA-binding domains (RNA recognition motifs [RRMs]) found in IMP1 but retains the four KH domains (29). This loss of RNA-binding domains may result in changes in the specificity and affinity for target mRNAs that Δ N-IMP1 can bind relative to IMP1 binding. The full-length form of IMP1 has been reported to have an oncofetal expression pattern, but the tissue expression of Δ N-IMP1 is also largely unknown. Detailed analysis is limited by lack of antibodies specific for this isoform. Indeed, the interpretation of several immunohistochemistry expression studies must be tempered by uncertainty about isoform expression (15, 25–27, 61). In future studies, it would be interesting to conduct global analysis of E2-dependent genes regulated by mRNA stabilization and determine whether these genes are dependent upon Δ N-IMP1.

FIG 10 SRC is required for tumor initiation and growth *in vivo*. (A) Mice were injected with shCtrl, shSRC, shTP53, or shSRC/shTP53 double knockdown MCF7 cells at day 0. Tumors were measured every 3 days. (B) The same experiment as described for panel A except that cells entered quiescent phase *in vitro* 2 days before injection in the fat pad. (C) Representative immunoblot analysis of TOshSRC/shCtrl and TOshSRC/shp53 double knockdown MCF7 cells after *in vitro* treatment with doxycycline. (D) Mice were injected with TOshCtrl/shCtrl, TOshCtrl/shTP53, TOshSRC/shCtrl, and TOshSRC/shp53 double knockdown MCF7 cells. Once tumors reached 100 mm³, mice were randomly selected and either not treated with Dox or treated with Dox every 3 days, and tumors were measured at the same time.

Loss of p53 increased the basal half-life of *MYC* mRNA independent of both E2 and SRC. We also observed that accumulation of *MYC* mRNA was still E2 dependent in these cells but no longer dependent upon SRC. This finding suggests that E2-dependent *MYC* mRNA accumulation in cells lacking p53 may act through other pathways involved in nongenomic E2 signaling. The observed increase in expression of the Δ N-IMP1 protein may provide an explanation for this. An alternative, but not mutually exclusive, possibility is that p53 regulates expression of microRNAs (miRNAs) that themselves regulate the stability of certain mRNAs. In colon cancer cells, p53 represses miRNA 145 (miR-145), a direct inhibitor of *MYC* mRNA stability (62). Additionally, p53 regulates miR-34a which also targets *MYC* mRNA (63, 64). We have previously shown that miR-145 is suppressed via SRC inhibition of p53 in vascular smooth muscle cells (65), hinting that SRC may also regulate RNA stability via p53-regulated miRNAs. However, in our initial analysis, we did not see changes in these microRNAs upon loss of p53 (data not shown), suggesting that further analysis of miRNAs is needed or that other means of regulating basal *MYC* mRNA stability may exist.

In addition to understanding how *MYC* mRNA is regulated in ER⁺ breast cancer, we also gained insight into the role of SRC in ER⁺ breast cancer cell proliferation. We show that SRC is required for cell cycle progression *in vitro* as well as for tumor initiation and growth *in vivo*. This is consistent with previous literature implicating SRC as an important mediator in breast cancer. SRC plays an important role during mammary gland development and breast cancer progression in several mouse models (41, 66–68). Furthermore, in human breast cancer, SRC is highly expressed in more than 70% of patient samples (69–71). In particular, ER⁺ breast cancer patients with increased SRC levels have reduced survival (72) and more frequent recurrence after treatment (73). These data suggest that SRC may be clinically relevant in the ER⁺ subtype, and, indeed, several studies provide hints that this may be the case. Clinical trials using multitarget-

geting kinase inhibitors that inhibit SRC, dasatinib (74, 75) and bosutinib (76), have been largely disappointing; however, there were some hints of stable disease in ER⁺ breast cancer patients. While these trials did not take into account the p53 status of the ER⁺ patients, many primary ER⁺ breast cancers retain wild-type p53 (45, 46).

The clinical trial data are particularly interesting given that we found a second role for SRC in ER⁺ breast cancer, by which SRC inhibits p53 function. Loss of p53 overcame a requirement for SRC for E2-dependent cancer cell proliferation. Our model is that SRC provides a means of suppressing p53 activity in the ER⁺ breast cancers which express wild-type p53 (45, 46). The mechanism remains to be fully uncovered, but one report has suggested that SRC phosphorylates the p53 inhibitor MDM2 to inhibit p53 function (77). Knockout of MDM2 in mice is embryonic lethal unless combined with p53 knockout (78, 79). Consistent with this, we were unable to knock down MDM2 in MCF7 cells unless p53 levels were also reduced (data not shown), preventing us from testing this mechanism directly.

In addition to our *in vitro* findings, we also supported these studies with a xenograft model of ER⁺ breast cancer wherein loss of SRC led to tumor regression while combined loss of both SRC and p53 resulted in marked impairment of tumor growth but no regression. Unfortunately, there are no suitable mouse models in which to extend these findings. Current genetically engineered mouse models of ER⁺ breast cancer often have mutated or inactivated p53, express the ER at relatively low frequency, and typically have a very long (10 to 24 months) latency period before tumor development (reviewed in references 80 and 81). Patient-derived xenografts (PDXs) can be attractive models to conduct preclinical experiments; however, very few ER⁺ PDXs have been generated thus far (82, 83), and it remains to be determined whether these tumors retain wild-type p53. Nevertheless, suppression of wild-type p53 function by SRC has important clinical implications. Several preclinical and clinical trials in breast cancer have suggested that inhibition of SRC using the multitargeting kinase inhibitors dasatinib (74, 75) and bosutinib (76, 84) resulted in stable disease in a fraction of the ER⁺ cohort. We postulate that these patients may have expressed wild-type p53 although p53 status was not reported in these studies. We suggest that therapeutic inhibition of SRC may be effective in early-stage ER⁺ breast cancer expressing wild-type p53 and, more generally, also in other cancer types that retain wild-type p53.

MATERIALS AND METHODS

Cell lines. MCF7 cells (ATCC) were grown in Iscove's modified Eagle's medium (IMEM) supplemented with 10% fetal bovine serum (FBS). ZR-75-1 (ATCC), T47D (ATCC), ZR-75-30, and SUM44PE (gift from Joe Gray) cells were grown in RPMI medium supplemented with 10% FBS. 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. For starvation prior to estrogen stimulation in the assays described below, cells were washed once in phosphate-buffered saline (PBS) and starved for 48 h in starvation medium (phenol red-free IMEM, 0.5% charcoal, dextran-stripped FBS [Sigma-Aldrich]). All experiments were performed in the absence of antibiotics. MCF7, ZR-75-1, and T47D cells were authenticated by the Oregon Health and Science University (OHSU) DNA Services Core.

Mice and animal housing. Female, athymic nude mice (nude Nu/J) at 6 to 7 weeks of age were purchased from The Jackson Laboratory. Mice were grouped by 5 animals in large plastic cages and were maintained under pathogen-free conditions. All animal experiments were performed with the approval of OHSU's Institutional Animal Care and Use Committee (IACUC).

Small-molecule inhibitor studies. For all small-molecule inhibitor studies, cells were starved for 48 h in starvation medium prior to estrogen stimulation in the assays described below. For inhibitor pretreatment, inhibitors were diluted into starvation medium and incubated for 2 h prior to stimulation. Estrogen (Sigma-Aldrich), ICI182780 (Tocris Biosciences), actinomycin D (Sigma-Aldrich), and SU11333 (Sanford Burnham Prebys Medical Research Institute) were used in pretreatment of cells during starvation as described above.

shRNA transduction. Lentiviral preparations were made by the viral core facilities at the Sanford Burnham Prebys Medical Discovery Institute (La Jolla, CA) and Oregon National Primate Research Center (Molecular and Cell Biology, Lentivirus Service) at Oregon Health and Science University (Portland, OR). Cells were plated at ~60% confluence prior to addition of lentivirus. Cells were transduced for 24 h before removal of viral supernatant. Cells were then selected by puromycin or neomycin to generate stable cell lines. Sequences for the shRNA lentiviral vectors used for these studies are from the RNAi Consortium and are as follows (clone number in parentheses): shSRC1 (TRCN0000195339), shSRC2 (TRCN0000038149), shYES (TRCN0000001609), shMYC (TRCN0000039640), shIMP1 (sh5149) (TRCN0000075149), and shIMP1 (sh8079) (TRCN0000218079).

TABLE 2 Primers for qPCR and cloning

Primer name or gene ^a	Strand ^b	Function (reference)	Sequence
EcorI-ΔN-IMP1	F	For cloning ΔN-IMP1 into pCDH vector	GGAATTCGGCAAGCCATCATGAAGCTGA
BAMHI-IMP1	R	For cloning ΔN-IMP1 into pCDH vector	TTGGATCCCGTTGTTGCTCGGACTCGAAG
MYC	F	qPCR (19)	CAGCTGCTTAGACGCTGGATT
MYC	R	qPCR (19)	GTAGAAATACGGCTGCACCGA
PS2	F	qPCR	TTGTGGTTTTCTCTGGTGTCA
PS2	R	qPCR	GCAGATCCCTGCAGAAGTGT
PPIA	F	qPCR (19)	GTCAACCCACCGTGTCTT
PPIA	R	qPCR (19)	CTGCTGTCTTTGGACCTTGT
RPLP0	F	qPCR (19)	GGCGACCTGGAAGTCCAAC
RPLP0	R	qPCR (19)	CCATCAGCACACAGCCTTC
FL-IMP1	F	qPCR (29)	TGAAGGCCATCGAACTTTCTC
FL-IMP1	R	qPCR (29)	GATTTGAATTTCCGGCTCCTTG
ΔN-IMP1	F	qPCR (29)	AAGCTGCCGAAGACAGATTG
ΔN-IMP1	R	qPCR (29)	GATTTGAATTTCCGGCTCCTTG

^aqPCR primers were used for human genes.^bF, forward; R, reverse.

Bromodeoxyuridine (BrdU) incorporation assay. Cells were plated at 60% confluence on glass coverslips in normal growth medium and allowed to grow overnight. The next morning cells were washed once with PBS and then placed in starvation medium. Cells were pretreated for 2 h with 1 μ M SU11333 or dimethyl sulfoxide (DMSO) vehicle prior to stimulation. Cells were then treated with either ethanol or 5 nM estrogen (E2) with 5 μ M BrdU for 18 h. After treatment, cells were washed twice with cold PBS and fixed with ice-cold 1:1 methanol-acetone. Coverslips were then rehydrated in PBS for 15 min. Cells were permeabilized in 0.1% Triton X-100 in PBS for 10 min. Cells were then treated with 2 N hydrochloric acid at 37°C for 15 min. Coverslips were then washed three times in PBS with rocking. Coverslips were blocked with 1% goat serum in PBS for 1 h with rocking. Anti-BrdU (Millipore) antibody was used to detect incorporated BrdU (Millipore). Alexa Fluor 488–anti-mouse 488 secondary antibody was used to visualize staining via immunofluorescence analysis. Vectashield containing 4',6'-diamidino-2-phenylindole (DAPI) was used to mount the coverslips and stain nuclei.

Proliferation assays. Cells were plated at 25 by 103, and viable cells were counted every 2 days using a Countess II automated cell counter (Thermo Fisher Scientific).

Immunoblotting. Cell lysates were prepared by washing cells twice with cold Tris-buffered saline (TBS) containing 100 μ M Na₃VO₄ and then lysed in 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1% Triton X-100, 50 mM NaF, 100 μ M Na₃VO₄, and 1 mM EDTA lysis buffer containing a dissolved protease EDTA-free inhibitor cocktail (Roche). Supernatant of cell lysates was assayed for total protein content using a detergent-compatible (DC) protein assay (Bio-Rad), and 50 μ g of total protein per sample was separated in a polyacrylamide gel. Membranes were scanned using an infrared imaging system (Odyssey; Li-Cor Biosciences). The following commercial antibodies were used: anti-SRC (pY418) antibody (Invitrogen), anti-SRC (327) antibody (Abcam), anti-YES antibody (Cell Signaling Technology), anti-MYC (Y69) antibody (Abcam), anti-IMP1 antibody (Cell Signaling Technology), anti-IGF2BP1 antibody (Sigma-Aldrich), anti- γ -tubulin antibody (Sigma-Aldrich), anti-beta-actin antibody (Sigma), and anti-BrdU (Millipore). For secondary antibodies, Alexa Fluor 680–goat anti-rabbit IgG (Invitrogen) or IR800 (Rockland Immunochemicals) was used for immunoblotting.

RNA isolation and cDNA generation. TRIzol extraction of RNA was performed per the manufacturer's instructions. cDNA was generated using a SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer's instructions.

MYC mRNA accumulation assay. Cells were starved and stimulated as described above. Post-E2 stimulation, RNA was isolated at the times indicated on the figures and cDNA was generated. To quantify RNA accumulation, qPCR analysis was performed. For each sample, MYC mRNA was normalized independently to two different housekeeping genes (*PPIA* and *RPLP0*). Primers for analysis were used as previously described (19) (Table 2). Time points for each condition were all normalized to time zero for each condition. Representative experiments are shown.

Transcriptional run-on assay. To label transcripts, a Click-iT Nascent RNA Capture kit (Invitrogen) was adapted and used for this assay. Cells were starved and stimulated as described above. Transcription was then paused by placing samples on ice at various time points poststimulation. Intact nuclei were isolated at each time point for analysis. Labeled 5-ethyl uridine (EU) was added, and samples were incubated at 37°C to allow elongation of initiated transcripts. EU was incorporated into only these initiated transcripts. The EU was then biotinylated and collected using streptavidin beads. RNA was isolated off the beads, and cDNA was generated for qPCR analysis. For each sample, MYC, PS2, and PPIA mRNA levels were measured via qPCR.

RNA stability assay. Cells were starved and stimulated as described above. At 60 min poststimulation with E2, cells were treated with 5 μ M actinomycin D (Invitrogen) to block transcription. At the time points indicated in the figures, RNA was isolated, and cDNA was generated for each condition. For each

sample, the MYC mRNA level was normalized independently to the levels of two different housekeeping genes (*PPIA* and *RPLP0*). These genes were selected so that actinomycin D treatment did not alter their expression. Time points were all normalized to time zero for each condition. The MYC mRNA half-life was then calculated for each. To determine significance, one-way ANOVA and Tukey's *post hoc* tests were performed on the MYC mRNA half-life at the endpoint of the experiment to determine statistical significance.

Orthotopic mouse xenograft experiments. Female, athymic nude mice were injected with a slow-release 17 β -estradiol pellet (0.72 mg/pellet, 60-day release; Innovative Research of America) using a trocar. One week later, mammary fat pad injections in the number 4 mammary gland were carried out without clearing of the fat pad. Briefly, cells were harvested by trypsinization and resuspended in PBS (Invitrogen). For each cell line, the mice were injected in a noncleared mammary fat pad with 1×10^6 cells per animal in a volume of 100 μ l (1:1 ratio with phenol red-free Matrigel [BD Biosciences]), and tumors were allowed to form with a diameter of up to 1 cm. Tumor onset was determined by physical palpation. Tumor growth was measured every 3 to 4 days using calipers; both the longest (L) and shortest (S) measurements were recorded. Using these values, tumor volumes were calculated as follows: $(L \times S^2) \times 0.5$; results are expressed as mean volume $\pm$ standard error of the mean (SEM). Mice were sacrificed when the tumors reached a diameter of 1 cm, according to the Animal Care and Use Policy of OHSU. These experiments were repeated at least three times, using 5 to 10 mice per tumor group. One-way ANOVA, Student's *t* test, or Tukey's *post hoc* test was performed on the tumor volume at the endpoint of the experiment to determine statistical significance, as appropriate. For the doxycycline-inducible shRNA-expressing cells, tumors were allowed to grow to either 100 or 200 mm³ before mice were placed on chow with 0.2% (2,000 ppm) doxycycline (test diet). Tumors were then measured as described above until those in control animals reached a diameter of 1 cm.

Quantification and statistical analysis. The numbers of samples (*n*) are indicated in each figure legend or in Materials and Methods. All qPCR data represent three replicates performed for each experiment. All data points and error bars represent the means and SEMs. GraphPad Prism software was used to calculate Student's *t* test or ANOVA with Tukey's *post hoc* test to determine significance. Statistical significance was determined by calculating the *P* value, and a *P* value of <0.05 was considered to be statistically significant.

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We declare that we have no conflicts of interest.

C.A. designed and performed experiments, analyzed data, and wrote the manuscript; H.K. designed and performed pilot experiments. S.I. cloned constructs and assisted on *in vivo* experiments. S.A.C. planned and designed experiments, wrote the manuscript, and had overall responsibility for the study.

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