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A new paradigm for regulation of protein phosphatase 2A function *via* Src and Fyn kinase–mediated tyrosine phosphorylation

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Protein phosphatase 2A (PP2A) is a major phospho-Ser/Thr phosphatase and a key regulator of cellular signal transduction pathways. While PP2A dysfunction has been linked to human cancer and neurodegenerative disorders such as Alzheimer's disease (AD), PP2A regulation remains relatively poorly understood. It has been reported that the PP2A catalytic subunit (PP2Ac) is inactivated by a single phosphorylation at the Tyr307 residue by tyrosine kinases such as v-Src. However, multiple mass spectrometry studies have revealed the existence of other putative PP2Ac phosphorylation sites in response to activation of Src and Fyn, two major Src family kinases (SFKs). Here, using PP2Ac phosphomutants and novel phosphosite-specific PP2Ac antibodies, we show that cellular pools of PP2Ac are instead phosphorylated on both Tyr127 and Tyr284 upon Src activation, and on Tyr284 following Fyn activation. We found these phosphorylation events enhanced the interaction of PP2Ac with SFKs. In addition, we reveal SFK-mediated phosphorylation of PP2Ac at Y284 promotes dissociation of the regulatory Bα subunit, altering PP2A substrate specificity; the phosphodeficient Y127/284F and Y284F PP2Ac mutants prevented SFK-mediated phosphorylation of Tau at the CP13 (pSer202) epitope, a pathological hallmark of AD, and SFK-dependent activation of ERK, a major growth regulatory kinase upregulated in many cancers. Our findings demonstrate a novel PP2A regulatory mechanism that challenges the existing dogma on the inhibition of PP2A catalytic activity by Tyr307 phosphorylation. We propose dysregulation of SFK signaling in cancer and AD can lead to alterations in PP2A phosphorylation and subsequent deregulation of key PP2A substrates, including ERK and Tau.

Regulation of virtually all cellular processes and signal transduction pathways in eukaryotes involves reversible phosphorylation, which is mediated *via* the concerted action of protein kinases and phosphatases. The SFKs are major non-receptor protein tyrosine kinases that function in key signal transduction pathways induced by a large variety of receptor protein tyrosine kinases regulating cell architecture, growth, differentiation, migration, survival, and metabolism (1). Deregulation of the ubiquitously expressed SFKs, c-Src and Fyn, plays an important role in human oncogenesis (2, 3) and Alzheimer's disease (AD) pathogenesis (4, 5). Like SFKs, PP2A enzymes are major multifunctional signaling molecules that are deregulated in cancer (6) and AD (7).

As expected for a key modulator of many diverse cellular processes, PP2A activity, stability, and holoenzyme biogenesis are tightly controlled in cells by multiple regulatory mechanisms that include posttranslational modifications of the catalytic subunit (PP2Ac) and association of PP2Ac with a plethora of regulatory subunits (8). The most studied form of PP2A is the heterotrimeric holoenzyme (ABC), which consists of a structural A subunit, a variable B regulatory subunit, and the catalytic C subunit (PP2Ac). The presence of >20 B subunit isoforms dictates the composition of the PP2A holoenzyme and provides key determinants for substrate selectivity and subcellular localization of the phosphatase (9, 10). Multiple posttranslational modifications have been reported for PP2Ac including ubiquitination of lysine 41, carboxymethylation of leucine 309, and phosphorylation of threonine 304 and tyrosine 307; these modifications are thought to play key regulatory roles in the control of PP2A activity, stability, and holoenzyme biogenesis (11–15).

The phosphorylation of PP2Ac on Y307 was first reported almost 30 years ago (16). In that study, the investigators showed that PP2Ac is phosphorylated by tyrosine kinases (*e.g.*, Src, Lck, and EGFR). However, the Tyr phosphorylated form of PP2A was found to be extremely labile, presumably due to rapid autodephosphorylation. Of particular interest, Src-catalyzed thiophosphorylation of PP2Ac at unidentified Tyr site(s) led to a reduction in phosphatase activity. Based on *in vitro* assays, it was concluded that Y307 is the sole site of phosphorylation on PP2Ac. Consequently, Y307 phosphorylation has been proposed to be an essential mechanism

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involved in catalytic inactivation of PP2A and v-Src-mediated cell transformation (17). However, in a study using a mass spectrometry (MS) shotgun phosphotyrosine (pY) proteomics strategy to obtain global pY profiles of both Src-transformed and nontransformed mouse embryo fibroblasts (MEFs), endogenous PP2Ac was found to be phosphorylated at Y127 and Y284, but not Y307 (18). Likewise, several discovery-mode MS investigations have identified PP2Ac phosphorylation at Y284, but not Y307, in a variety of oncogenic/transformed cells and human cancers (PhosphoSitePlus, https://www.phosphosite.org/proteinAction.action?id=1685&showAllSites=true). Notably, phosphorylation of PP2Ac at Y307 could only be detected at very low levels when using targeted MS studies of HEK293T cells overexpressing both constitutively active Src (Src^{CA}) and PP2Ac (13). To date, most studies assessing PP2A phosphorylation and its regulatory role and function have relied on flawed "anti-pY307 PP2Ac" antibodies (13, 19). Collectively, these findings not only support the paradigm-shifting idea that Y307 is not the major and sole PP2Ac phosphorylation site but also raise important questions on the functional significance of site-specific PP2Ac Tyr phosphorylation.

Here, using PP2Ac phosphosite mutants and newly generated phosphospecific antibodies, we demonstrate in several cell models that PP2Ac is primarily targeted for Src^{CA}-dependent phosphorylation at Y127/284 and Fyn^{CA}-dependent phosphorylation at Y284. These phosphorylation events are required for maximal interaction of pY-PP2A–Src^{CA} and pY-PP2A–Fyn^{CA} complexes and also modulate the association of PP2Ac with the regulatory Bα subunit. The PP2Ac Y127/284F and Y284F mutants inhibit Src^{CA}- and Fyn^{CA}-induced ERK activation and Tau phosphorylation at the AD-like CP13 (pSer202) epitope, respectively. These new findings have important ramifications for both existing and future studies on PP2Ac tyrosine phosphorylation in physiological and pathological conditions, such as cancer and AD.

Results

PP2Ac is phosphorylated by Src^{CA} and Fyn^{CA}, but Y307 is not a major phosphoacceptor site

Given the discovery-mode MS studies that identified PP2Ac pY127 and pY284, but not pY307, peptides in Src-transformed MEFs (18), we first performed experiments to further interrogate PP2Ac Tyr phosphorylation state in this cellular model. To demonstrate that we could detect endogenous Tyr phosphorylation of PP2Ac, we exploited microcystin-Sepharose, an affinity resin for PP2A family members (20), to isolate endogenous PP2Ac from extracts of control and Src^{CA}-transformed MEFs. Src^{CA}-expressing cells were used for these experiments to rule out potential regulatory effects of PP2A and other signaling molecules on Src activity. Western blot analysis of microcystin-bound samples using validated pY and PP2Ac antibodies revealed Src^{CA}-dependent tyrosine phosphorylation of a protein that comigrated precisely with PP2Ac (Fig. 1*A*). To further test that tyrosine phosphorylation of PP2Ac could be promoted upon Src^{CA} expression, we performed immunoprecipitation experiments using MEF-derived SYF cells that lack the SFKs, Src, Yes, and Fyn (21). SYF cells were transiently transfected with a plasmid encoding Src^{CA} or the empty vector (EV), together with vectors encoding wildtype HA3-tagged PP2Ac (WT). This approach was chosen since anti-PP2Ac antibodies cannot quantitatively immunoprecipitate endogenous forms of PP2A (22). Western blot analyses of HA-immunoprecipitates showed that tyrosine phosphorylated

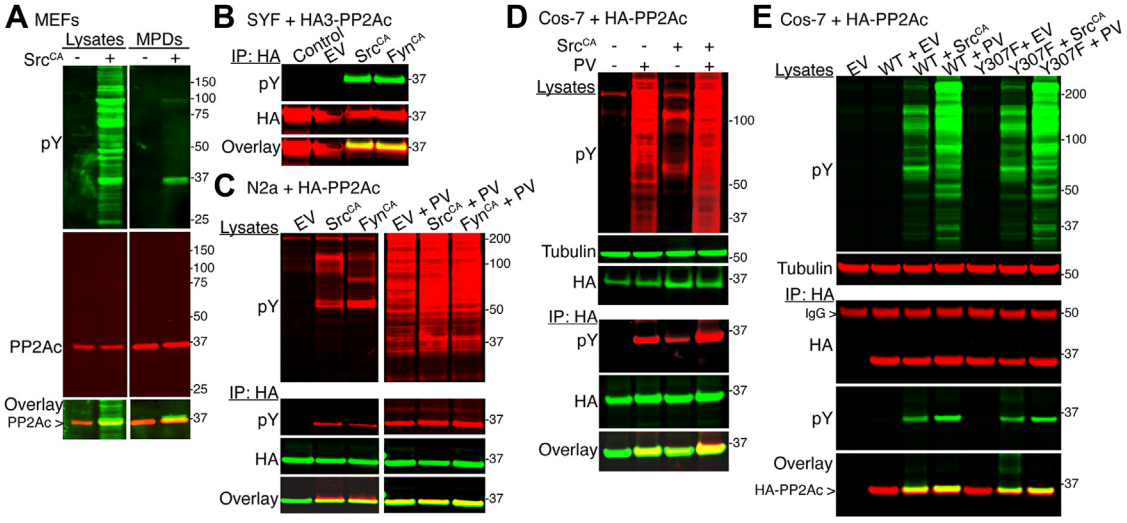

Figure 1. Expression of constitutively active Src or Fyn kinase induces tyrosine phosphorylation of endogenous PP2Ac and HA-tagged WT and Y307F PP2Ac. *A*, lysates from control (–) and Src^{CA}-transformed (+) MEFs were subjected to microcystin-Sepharose pull downs (MPDs). Total cell lysates and MPDs were analyzed by Western with antibodies recognizing phosphotyrosine (pY) and endogenous PP2Ac. Representative Western blots of total cell lysates and HA-immunoprecipitates prepared from SYF (*B*), N2a (*C*), or Cos-7 (*D* and *E*) cells transfected with either empty vector (EV), HA-/HA3-tagged wildtype PP2Ac (WT), or the Y307F phosphomutant of PP2Ac (Y307F) together with plasmids encoding Src^{CA}, GFP-tagged Fyn^{CA}, or EV. Where indicated, a subset of these cells was treated with pervanadate (+PV) prior to harvesting. Well-characterized anti-pY antibodies were used to assess tyrosine phosphorylation of proteins in response to SFK^{CA} and pervanadate. Tubulin antibodies were used to control for protein loading. Similar results were observed in at least four separate experiments.

HA-PP2Ac species were readily detected in Src^{CA}-expressing SYF cells but not in control SYF cells (Fig. 1*B*).

Since Fyn is a SFK closely related to Src, and PP2Ac was identified as a putative Fyn substrate in an *in vitro* kinase-interacting substrate screening assay (23), we also assessed whether PP2Ac was tyrosine phosphorylated following expression of a constitutively active Fyn^{CA} mutant. Indeed, like Src^{CA}, Fyn^{CA} promoted the tyrosine phosphorylation of PP2Ac in SYF cells (Fig. 1*B*). To confirm these results, we also examined PP2A tyrosine phosphorylation status in other widely used cell lines that do not lack SFKs. We chose to use fibroblast-like Cos-7 cells for most experiments, due to their high transfection efficiency. In addition, we focused on mouse N2a neuroblastoma cells because there are established models for exploring the role of PP2A in the regulation of tau phosphorylation (24). As observed in SYF cells, transfection of N2a cells with either Src^{CA} or Fyn^{CA} (SFK^{CA}) promoted the tyrosine phosphorylation of PP2Ac (Fig. 1*C*). Additional experiments were performed to determine if incubation of N2a cells with pervanadate, a potent inhibitor of protein tyrosine phosphatases that markedly increases cellular protein tyrosine phosphorylation (25), influences tyrosine phosphorylation of HA-PP2Ac. Not surprisingly, pervanadate treatment alone promoted tyrosine phosphorylation of HA-PP2Ac; the combination of pervanadate and SFK^{CA} further augmented HA-PP2Ac tyrosine phosphorylation in N2a cells (Fig. 1*C*). Synergistic effects of pervanadate and Src^{CA} were also observed in lysates of Cos-7 cells (Fig. 1*D*). Moreover, the tyrosine phosphorylation of PP2Ac was abolished in SFK^{CA}-transfected cells incubated with the SFK inhibitor, PP2 (Fig. S1).

We next comparatively assessed the Tyr phosphorylation state of WT and a point mutant of HA-PP2Ac containing a phenylalanine in place of Y307 (Y307F) in Src^{CA}-expressing or pervanadate-treated Cos-7 cells. We detected very similar levels of tyrosine phosphorylation of WT and Y307F in response to these stimuli (Fig. 1*E*). Similar results were obtained in Src^{CA}-transfected human HEK293 cells (Fig. S2*A*). Together with earlier results from MS studies (13, 18, 23), these findings confirm that cellular pools of PP2As are targeted for tyrosine phosphorylation in response to SFK^{CA} or pervanadate treatment in multiple cell models. However, Y307 is not a predominant tyrosine phosphorylation site of PP2Ac under these experimental conditions.

Differential phosphorylation of PP2Ac Y127 and Y284 in response to pervanadate, Src^{CA}, and Fyn^{CA}

Since PP2Ac pY127 and pY284 peptides were identified in MS studies of Src^{CA}-transformed cells (18), we next performed experiments to assess the contribution of these residues in the phosphorylation of PP2Ac by transiently transfecting our cell models with plasmids encoding HA3-tagged WT or Y→F mutants of PP2Ac (*i.e.*, Y127F, Y284F, and the double Y127/284F mutant (F/F)). Western analysis of HA immune complexes isolated from pervanadate-treated Cos-7 cells revealed that tyrosine phosphorylation of Y127F and Y284F single mutants were decreased and almost absent in F/F, relative to WT (Fig. 2, *A* and *B*). In Src^{CA}-expressing Cos-7 cells, we observed significantly less phosphorylation of Y284F than Y127F (Fig. 2, *C* and *D*). As seen in pervanadate-treated cells, phosphorylation of the double F/F mutant was nearly undetectable in Src^{CA}-expressing Cos-7 cells. In agreement with phosphorylation of the Y127 site, the Y127F and Y127F/Y307F mutants were less phosphorylated than the WT and Y307F mutant in Src^{CA}-expressing HEK293 cells (Fig. S2*A*). Not too surprisingly, based on our prior studies (13), "anti-pY307" antibodies used in numerous studies to assess PP2A Tyr phosphorylation equally recognized WT and the Y127F, Y127F/Y307F, and Y307F mutants in these cells (Fig. S2*B*), thus confirming that these antibodies are not phosphospecific (13).

Interestingly, a phosphorylated Y284 PP2Ac peptide was identified by MS in *in vitro* screens of putative candidate kinase substrates of Fyn (23). Accordingly, the phosphorylation of Y284F was absent in Fyn^{CA}-expressing N2a cells (Fig. 2*E*). Similar results were seen with the F/F mutant, suggesting a minor contribution, if any, of the Y127 site in Fyn-mediated PP2Ac phosphorylation. Together, these findings confirm that Y127 and Y284 are major phosphoacceptor sites in PP2Ac, but their degree of phosphorylation appears to be dependent on the type of tyrosine kinase that is activated in cells.

Generation and validation of phospho-Y127 and phospho-Y284 antibodies

To further confirm the existence of the Y127 and Y284 phosphorylation sites, we generated novel rabbit and alpaca phosphospecific antibodies that recognize the PP2Ac phospho-Y127 and phospho-Y284 epitopes, respectively. The rabbit pY127 and alpaca pY284 antibody specificity was assessed by dot blot assays using purified peptides conjugated to bovine serum albumin (BSA) (Fig. S3). These assays demonstrated that the pY127 and pY284 antibodies were indeed specific and recognized the respective phosphopeptide, but not the nonphosphorylated peptide or other tyrosine phosphorylated epitopes.

The immunoreactivity of the antibodies was next tested by performing Western analyses of HA3-PP2Ac immunoprecipitates prepared from control and SFK^{CA} cells coexpressing WT, Y127F, Y284F, or Y127/Y284F. As observed with generic anti-pY antibodies (Fig. 1*C*), the pY284 antibody recognized WT immunoprecipitated from Fyn^{CA}- but not EV-expressing N2a cells; immunoreactivity was further enhanced by treatment with pervanadate (Fig. 3*A*). In contrast, there was no detectable signal with the pY127 antibody in HA3-PP2Ac immunoprecipitates, in agreement with the earlier observation that the Y127 residue may not be a major site targeted by Fyn^{CA} (Fig. 2*E*). Analyses of HA3-PP2Ac immunoprecipitates prepared from Src^{CA}-expressing Cos-7 cells revealed that the pY127 antibody recognizes WT and Y284F but not Y127F, whereas the pY284 antibody recognizes WT and Y127F but not Y284F (Fig. 3*B*). Neither the pY127 antibody nor the pY284 antibody recognized WT isolated from control cells lacking Src^{CA}, thus demonstrating a Src-dependent phosphorylation of Y127 and Y284 in Src^{CA}-expressing cells

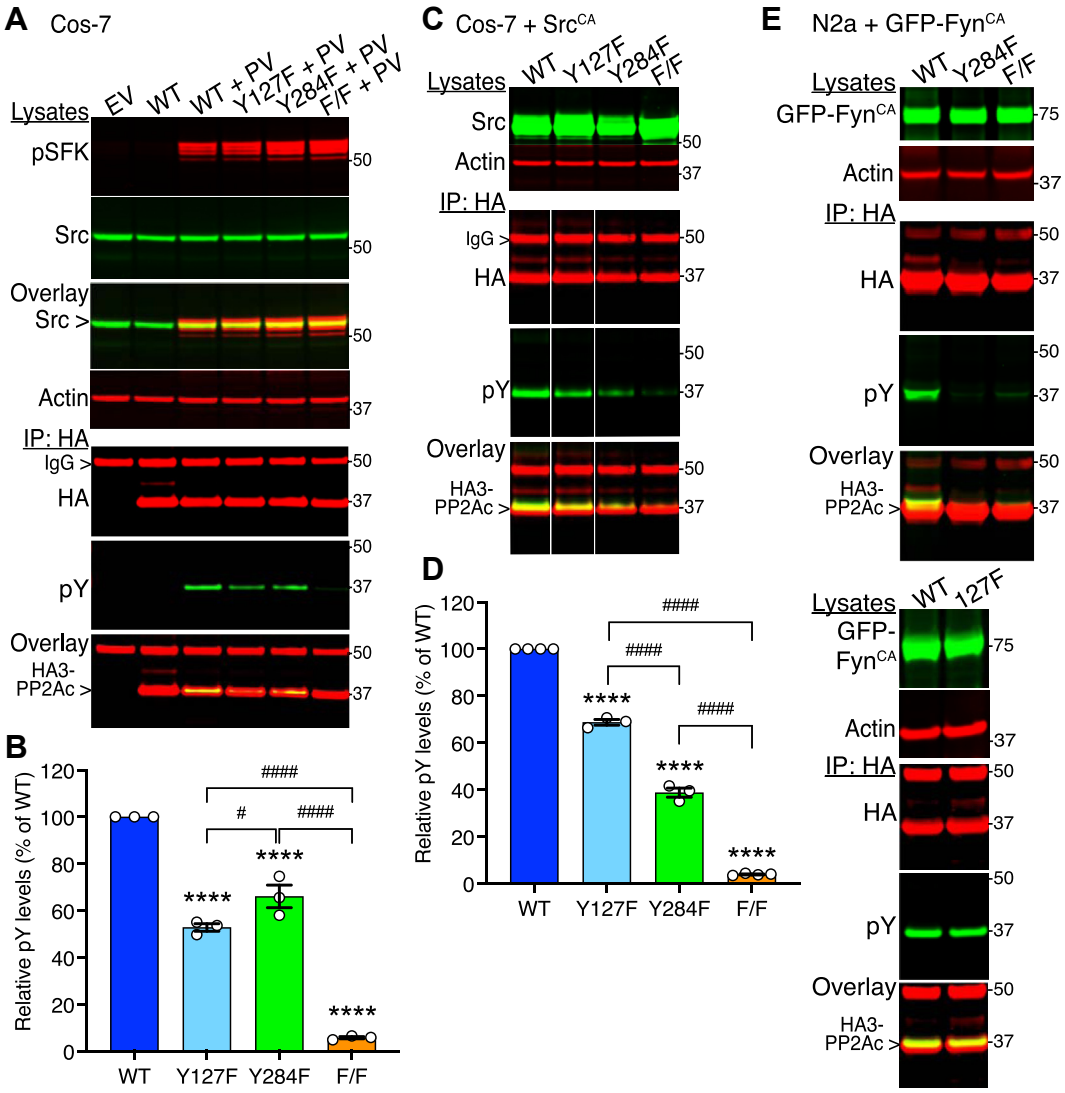

Figure 2. Distinct effects of pervanadate, Scr^{CA}, and Fyn^{CA} on PP2Ac WT, Y127F, Y284F, and Y127/284F tyrosine phosphorylation. Lysates and HA-immunoprecipitates were prepared from control and pervanadate-treated (+PV) Cos-7 cells (*A* and *B*), Src^{CA}-transfected Cos-7 cells (*C* and *D*), and GFP-Fyn^{CA}-transfected N2a cells (*E*) that were cotransfected with plasmids encoding HA3-tagged PP2Ac wildtype (WT), Y127F (Y127F), Y284F (Y284F), Y127/284F (F/F), or the empty vector (EV). Representative Western blots with the indicated antibodies are shown in (*A*), (*C*), and (*E*). Note: the pSFK antibody recognizes Src only when phosphorylated at Y416. *B*, relative levels of pY signal in HA3-PP2Ac immunoprecipitates from (*A*). Data (mean ± SEM from $n = 3$ separate experiments) were appraised using one-way ANOVA ($F = 236$; $p < 0.0001$) with Tukey's post hoc multiple comparisons test. ****$p < 0.0001$, compared with WT; $^{\#\#\#\#}p < 0.0001$, $^{\#}p < 0.05$. *D*, relative levels of pY signal in HA3-PP2Ac immunoprecipitates from (*C*). Data (mean ± SEM from $n = 3$–4 separate experiments) were appraised using one-way ANOVA ($F = 2067$; $p < 0.0001$) with Tukey's post hoc multiple comparisons test. ****$p < 0.0001$, compared with WT; $^{\#\#\#\#}p < 0.0001$. (*E*) similar results were observed in three separate experiments.

(Fig. 3*B*). Treatment of WT/Src^{CA}-expressing Cos-7 cells with PP2 also completely inhibited the Src-dependent activation of ERK and the phosphorylation of Y127 and Y284 (Fig. S4). Furthermore, Western analysis of HA3-PP2Ac immunoprecipitates from Cos-7 cells treated with epidermal growth factor (EGF) +/- PV revealed that EGF alone failed to induce Y127 and Y284 phosphorylation but augmented the PV-induced signals (Fig. S5), consistent with a very transient phosphorylation of these sites in response to EGF that can only be trapped in the presence of PV. Together, these data indicate that the pY127 and pY284 antibodies are indeed highly specific for the respective phosphoepitope and can be utilized to monitor Y127 and Y284 phosphorylation in target cells. Moreover, the data obtained with these antibodies confirm earlier MS results showing that Y127 and Y284 are key phosphoacceptor sites in PP2Ac.

Endogenous PP2Ac in ABαC holoenzymes is phosphorylated on Y284 in response to Src^{CA}

Since we have previously shown that the ABαC holoenzyme copurifies and associates with Fyn in N2a cells (26), we next tested the hypothesis that the PP2Ac subunit in Bα-containing holoenzymes could be phosphorylated in SFK^{CA}-expressing cells. Due to the fact that native PP2A heterotrimers cannot be efficiently immunopurified using existing PP2A subunit antibodies (22), we immunoprecipitated FLAG-Bα from lysates of Cos-7 cells cotransfected with Src^{CA} or EV. Western blot

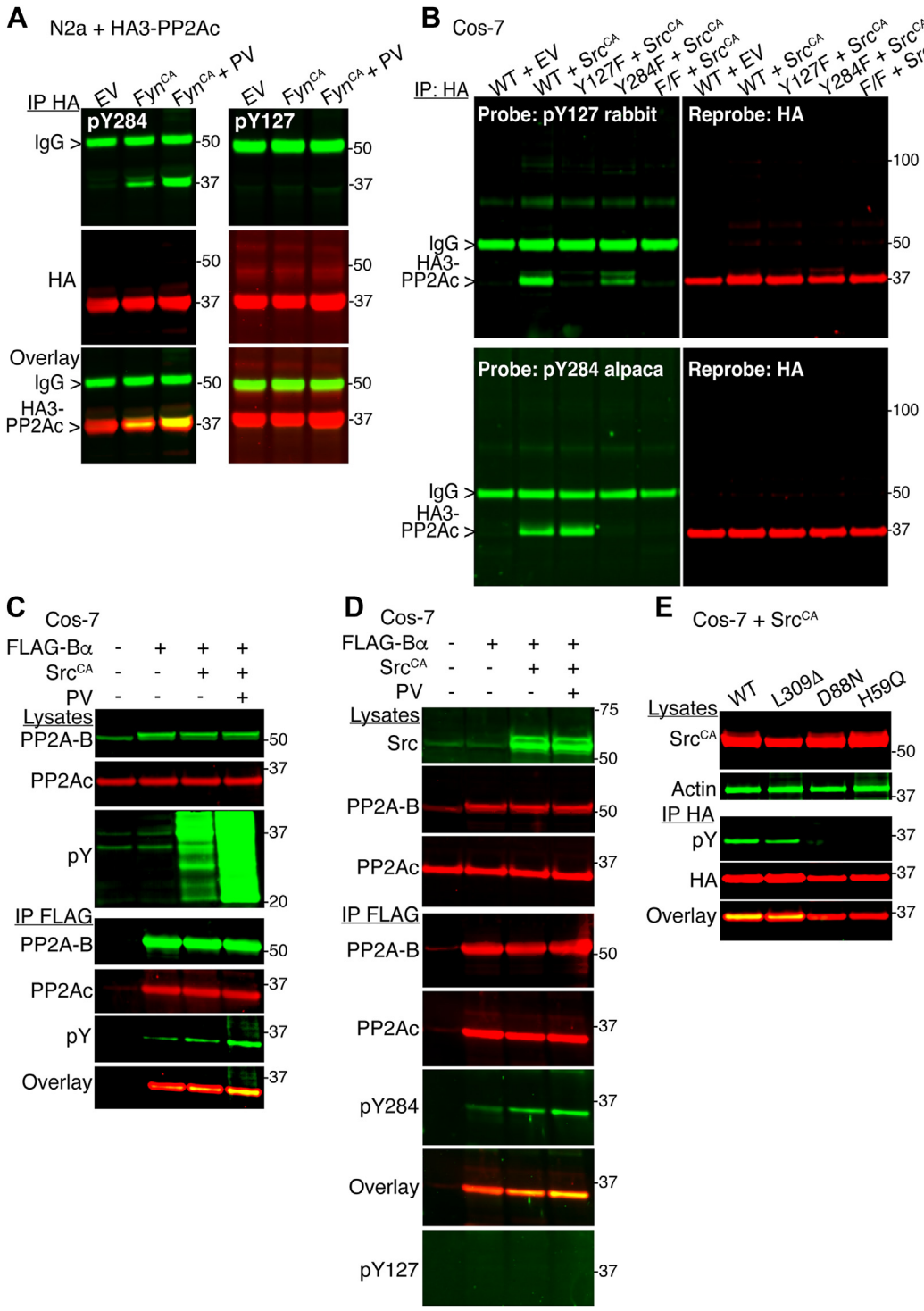

Figure 3. Western analyses using newly generated phosphosite-specific antibodies reveal that PP2Ac is differentially phosphorylated by Fyn^{CA} and Src^{CA} at the Y284 and Y127 epitopes. *A*, representative Western blots of HA-immunoprecipitates prepared from N2a cells coexpressing HA3-tagged PP2Ac and either GFP- Fyn^{CA} or an empty vector (EV) and probed with mouse anti-HA, alpaca anti-pY284 (pY284), or rabbit anti-pY127 (pY127) PP2Ac antibodies. Where indicated, a subset of these cells were treated with pervanadate (+PV) prior to harvesting. *B*, Western analysis of HA-immunoprecipitates prepared from EV- or Src^{CA}-transfected Cos-7 cells that were cotransfected with plasmids encoding HA3-tagged PP2Ac wildtype (WT), Y127F (Y127F), Y284F (Y284F), or Y127/284F (F/F). *C* and *D*, Western analyses of total cell lysates and FLAG-immunoprecipitates prepared from Cos-7 cells that were cotransfected with plasmids encoding Src^{CA}, FLAG-tagged Bα regulatory subunit (FLAG-Bα), and/or the empty vector (-). Blots were probed with anti-PP2Ac, anti-PP2A B subunit, anti-pY (*C*), or the novel PP2Ac phosphospecific Y284 and Y127 (*D*) antibodies. Where indicated, a subset of these cells was treated with pervanadate (PV) prior to harvesting. *E*, Western analyses of HA-immunoprecipitates prepared from Cos-7 cells that were cotransfected with plasmids encoding Src^{CA} and HA-tagged PP2Ac WT or the L309Δ, D88N, and H59Q mutants. Expression of Src^{CA} in the lysates was verified using the pY416-SFK antibody.

analyses confirmed the presence of endogenous pY-PP2Ac (Fig. 3*C*) and pY284-PP2Ac (Fig. 3*D*) associated with FLAG-Bα in Src^{CA}-expressing cells. Enhanced immunoreactivity with the pY and pY284 antibodies was also observed following treatment of Src^{CA}-expressing cells with pervanadate. Low levels of pY- and pY284-PP2Ac species were present in FLAG immunoprecipitates from nonstarved control cells, while being absent in control immunoprecipitates (Fig. 3, *C* and *D*). We were unable to detect endogenous PP2Ac phosphorylated at Y127 (Fig. 3*D*), suggesting that ABαC holoenzymes are preferentially phosphorylated on Y284, but not Y127, under these experimental conditions. Interestingly, the methylation incompetent L309Δ PP2Ac mutant, which is unable to associate with the Bα subunit (27), was tyrosine phosphorylated, but to a lesser extent than WT in Src^{CA}-expressing cells (Fig. 3*E*). This finding suggests that PP2Ac methylation and the presence of the Bα subunit is required for maximal PP2Ac phosphorylation. In contrast, no pY signal was detected with the two catalytically inactive D88N and H59Q PP2Ac mutants (Fig. 3*E*), which are demethylated and stably associate with the protein phosphatase methylesterase-1, PME-1 (28).

Phosphorylation of PP2Ac at Y284 promotes the dissociation of Bα

Having established that PP2Ac in ABαC holoenzymes can be tyrosine phosphorylated, we next tested whether this posttranslational modification influences Bα binding. Indeed, decreased amounts of Bα were present in HA3-PP2Ac immunoprecipitates prepared from Src^{CA}-*versus* EV-transfected Cos-7 cells (Fig. 4, *A* and *B*). In contrast to WT, binding of the Bα subunit to the dephosphorylated F/F mutant was insensitive to the presence of Src^{CA}. Comparison of the results obtained with the Y127F and Y284F mutants in the absence or presence of Src^{CA} indicated that the binding of Bα to PP2Ac was primarily affected by PP2Ac phosphorylation at Y284, and to a lesser extent at Y127. Likewise, phosphorylation of PP2Ac at Y284 in N2a cells in response to Fyn^{CA} also induced a marked decrease in Bα binding (Fig. 4, *C* and *D*). In agreement with Y284 being the primary Fyn-mediated PP2Ac phosphorylation site (Figs. 2*E* and 3*A*), this decrease was completely prevented by expression of the Y284F mutant and, moreover, similar amounts of Bα were found in WT and Y127F immunoprecipitates prepared from N2a cells coexpressing Fyn^{CA} (Fig. 4, *C* and *D*).

Tyrosine phosphorylation of PP2Ac is required for maximal complex formation with SFKs

Since PP2Ac can directly bind to Src (29) and Fyn coimmunoprecipitates with ABαC holoenzymes (26), we next investigated whether tyrosine phosphorylation at Y127 and/or Y284 affects PP2A complex formation with these SFKs. Western blot analyses of HA3-PP2Ac immunoprecipitates

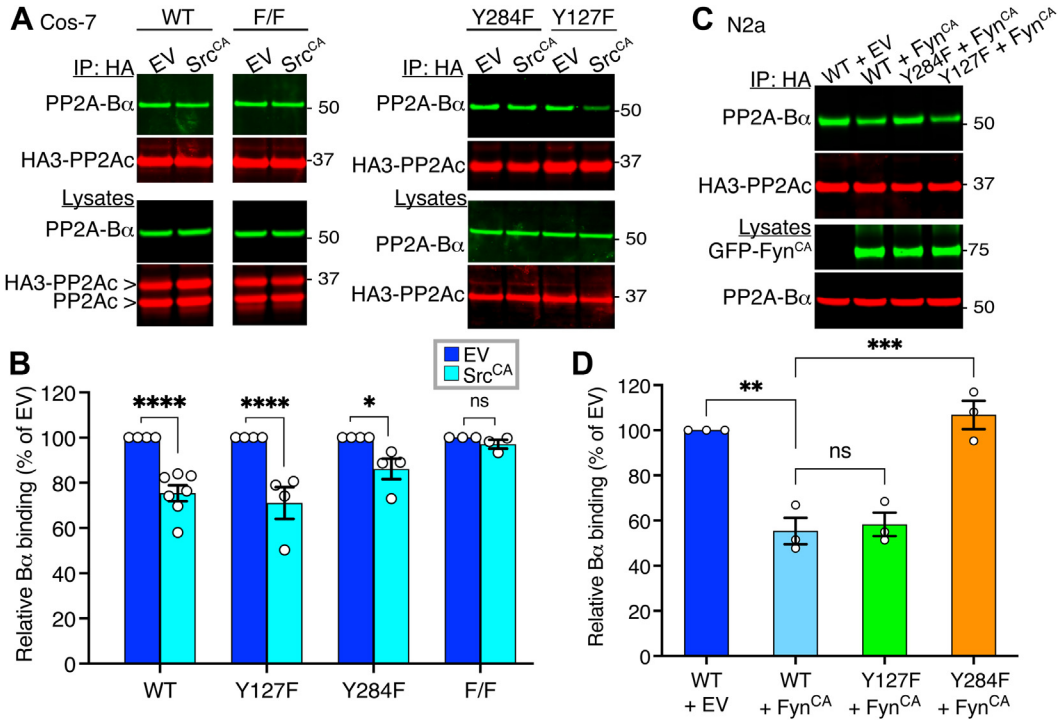

Figure 4. SFK-mediated tyrosine phosphorylation of PP2Ac decreases its binding to the regulatory Bα subunit. *A*, total lysates and HA-immunoprecipitates were prepared from empty vector (EV)- or Src^{CA}-transfected Cos-7 cells that were cotransfected with plasmids encoding either HA3-tagged PP2Ac wildtype (WT), Y127F (Y127F), Y284F (Y284F), or Y127/284F (F/F). Blots were analyzed using anti-Bα, anti-PP2Ac, or anti-HA antibodies. *B*, relative levels of Bα were quantified in HA3-PP2Ac immunoprecipitates. Data are mean ± SEM from at least $n = 4$ separate experiments and were appraised using unpaired *t*-tests as indicated. *$p < 0.05$; ****$p < 0.0001$, Src^{CA} *versus* EV; ns, not significant. *C*, Western blots of total lysates and HA-immunoprecipitates prepared from empty vector (EV)- or GFP-Fyn^{CA}-transfected N2a cells that were cotransfected with plasmids encoding either HA3-tagged PP2Ac wildtype (WT), Y284F (Y284F), or Y127F (Y127F). Levels of GFP-Fyn and Bα in the input used for immunoprecipitation (lysates) are shown for reference. *D*, relative levels of Bα binding in HA3-PP2Ac immunoprecipitates. Data are mean ± SEM from $n = 3$ separate experiments and were appraised using one-way ANOVA ($F = 29.02$; $p = 0.0001$) with Tukey's post hoc multiple comparisons test. **$p < 0.01$, ***$p < 0.001$; ns, not significant.

from lysates of Cos-7 cells coexpressing Src^{CA} and WT or mutant PP2Ac revealed that Src^{CA} associates with WT; however, lower levels of Src^{CA} were present in the Y284F immunoprecipitates (Fig. 5, *A* and *B*). While the Y127F mutation alone had only a small effect on the ability of Src^{CA} to coimmunoprecipitate with PP2Ac, it exerted a synergistic effect with the Y284F mutation, resulting in a ∼60 to 70% decrease in Src^{CA} amounts recovered in the F/F immunoprecipitates. As observed with Src^{CA}, reduced levels of Fyn^{CA} were found in Y284F relative to WT PP2Ac immunoprecipitates from N2a cell lysates (Fig. 5, *C* and *D*). However, the levels of Fyn^{CA} recovered in HA-PP2Ac immunoprecipitates were not affected by expression of the Y127F mutant, in agreement with our earlier data suggesting that Y127 is not a major Fyn^{CA} phosphorylation site. Together, these findings indicate that tyrosine phosphorylation at Y284 (for Fyn^{CA} and Src^{CA}) and Y127 (for Src^{CA}) is likely required for maximal SFK-PP2A complex formation.

Expression of PP2Ac phosphomutants interferes with SFK^{CA}-dependent ERK activation

Both Src (30) and Fyn (31–33) are linked to upstream activation of ERK signaling, and PP2A inactivation can induce Src-dependent ERK activation in various cellular models (34–36). PP2A ABαC holoenzymes are also important regulators of ERK signaling (37–39) and Fyn-dependent function (26). Therefore, we performed experiments to explore whether SFK-mediated Tyr phosphorylation of PP2Ac contributes to the regulation of SFK-dependent ERK activation. We coexpressed WT PP2Ac, or its phosphomutants, together with Src^{CA}, Fyn^{CA}, or EV in target cells, and then probed the lysates for active phosphorylated forms of ERK1/2. Of note, due to autoregulation of PP2Ac expression levels, transfected PP2Ac species replace a portion of endogenous PP2Ac (24); transfected HA3-tagged PP2Ac WT and mutant subunits represented 47 ± 2% and 41 ± 4% (mean ± SD; $n = 4$) of total PP2Ac expression levels in Cos-7 and N2a cells, respectively. As expected, under basal conditions, low levels of active ERK1/2 were detected in unstimulated control Cos-7 (Fig. 6*A*) and N2a (Fig. 6*D*) cells. Likewise, low levels of active ERK1/2 were seen following expression of either WT, Y127F, Y284F, or F/F, suggesting that all PP2Ac species were catalytically active. Relative to EV-transfected controls, an ∼1.75-fold increase in p-ERK1/2 was observed in Cos-7 coexpressing WT and Src^{CA} (Fig. 6, *B* and *C*) and N2a cells coexpressing WT and either Src^{CA} or Fyn^{CA} (Fig. 6, *D* and *E*). However, compared with WT, the Y284F mutant significantly inhibited Src^{CA}- or Fyn^{CA}-mediated ERK1/2 phosphorylation. As noted earlier, the F/F mutant most efficiently blocked Src^{CA}-dependent ERK1/2 phosphorylation. As observed in Src^{CA}-expressing cells, expression of the Y284F mutant inhibited Fyn^{CA}-mediated ERK1/2 activation (Fig. 6, *D* and *E*). These findings suggest that phosphorylation of PP2Ac at Y284 is a major contributor to Src^{CA}- or Fyn^{CA}-induced ERK activation.

SFK^{CA}-induced Tau phosphorylation at the AD-like Ser 202 phosphoepitope is dependent on PP2Ac tyrosine phosphorylation

Besides modulating ERK-dependent signaling, both Src and Fyn can affect Tau phosphorylation. It has been reported that

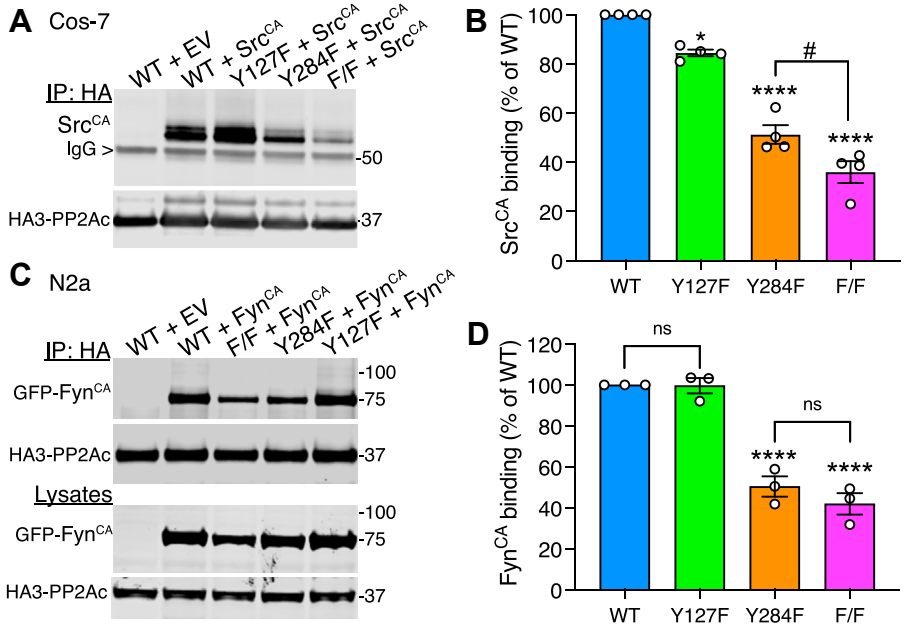

Figure 5. PP2Ac phosphomutants exhibit decreased interactions with SFK^{CA}. *A*, duplicate HA3-PP2Ac immunoprecipitates from the same transfected Cos-7 cells analyzed in Figure 2*C* were subjected to Western analysis using anti-pY416 SFK and anti-HA antibodies. *B*, relative levels of Src^{CA} in HA3-PP2Ac immunoprecipitates. Data are mean ± SEM from $n = 4$ separate experiments and were appraised using one-way ANOVA (F= 97.17; $p < 0.0001$) with Tukey's post hoc multiple comparisons test. *$p < 0.05$, ****$p < 0.0001$, compared with WT; #$p < 0.05$. *C*, representative Western blots of total lysates and HA-immunoprecipitates prepared from empty vector (EV)- or GFP-Fyn^{CA}-expressing N2a cells that were cotransfected with plasmids encoding either HA3-tagged PP2Ac wildtype (WT), Y284F (Y284F), Y127F (Y127F), or Y127/284F (F/F). *D*, relative levels of Fyn^{CA} in HA3-PP2Ac immunoprecipitates. Data are mean ± SEM from $n = 3$ separate experiments and were appraised using one-way ANOVA (F = 59.07; $p < 0.0001$) with Tukey's post hoc multiple comparisons test. ****$p < 0.0001$, compared to WT; ns, not significant.

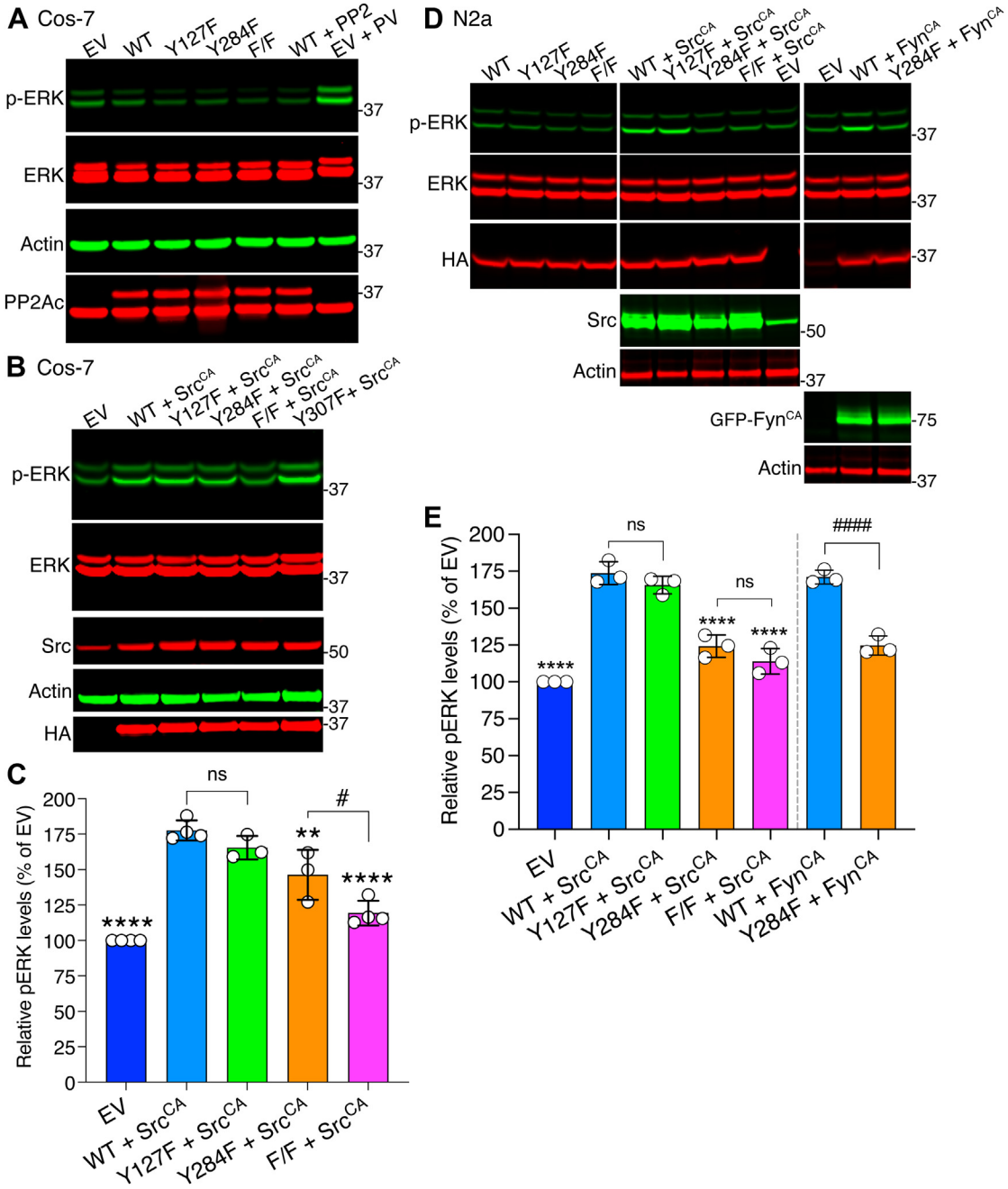

Figure 6. The Y127/284F and Y284F phosphoincompetent mutants inhibit Src^{CA}- and Fyn^{CA}-mediated ERK activation, respectively. *A*, Cos-7 cells were transfected with plasmids encoding HA3-tagged PP2Ac WT, Y127F, Y284F, or the empty vector (EV). Total cell homogenates from serum-starved cells were analyzed by immunoblotting for active ERK using validated antibodies recognizing the phosphorylated forms of ERK2 (or p42-MAPK, lower band) and ERK1 (or p44-MAPK, upper band), and total ERK1/2. PP2Ac expression levels in the cells are shown as reference. Pervanadate-treated control cells and WT-transfected cells incubated with the SFK inhibitor, PP2, were used as controls. *B*, Western analyses of active ERK in Cos-7 cells that were cotransfected with plasmids encoding Src^{CA} and HA3-tagged WT, Y127F, Y284F, F/F or Y307F PP2Ac, or the empty vector (EV). Extracts from EV-transfected cells served as controls. HA3-PP2Ac and Src expression levels in these cells are shown as reference. *C*, relative phosphorylated ERK (pERK) levels in Cos-7 cells transfected as in *B*. Data are mean ± SEM from at least three separate experiments and were appraised using one-way ANOVA ($F = 45.13$; $p < 0.0001$) with Tukey's post hoc multiple comparisons test. $^{**}p < 0.01$, $^{****}p < 0.0001$, compared to WT + Src^{CA}; $^{\#}p < 0.05$; ns, not significant. *D*, representative Western blots of cell homogenates prepared from control Src^{CA}-, or Fyn^{CA}-transfected N2a cells that were cotransfected with an empty vector (EV), HA3-tagged PP2Ac wildtype (WT), Y127F, Y284F, or F/F. Duplicate aliquots of the cell lysates were analyzed for active ERK, HA-PP2Ac, Src, and GFP-Fyn expression levels. Note that top ERK panels were assembled from the same blot. *E*, relative phosphorylated ERK (pERK) levels in N2a cells transfected as in *D*. Data are mean ± SEM from n = 3 separate experiments and were appraised using one-way ANOVA ($F = 66.78$; $p < 0.0001$) with Tukey's post hoc multiple comparisons test. $^{****}p < 0.0001$, compared with WT + Src^{CA}; $^{\#\#\#\#}p < 0.0001$; ns, not significant.

inactivation of PP2Ac following Src-mediated Y307 phosphorylation leads to the accumulation of highly phosphorylated Ser/Thr Tau species (40, 41), a pathological hallmark of AD and other tauopathies (42). However, the conclusions drawn from these studies are questionable due to the use of flawed Y307 antibodies (13, 19). Expression of Fyn^{CA} in

transgenic mice can also indirectly enhance phosphorylation of Tau at S202/T205 *via* an unknown mechanism (43). Since the Bα-containing PP2A holoenzyme is the primary brain Tau Ser/Thr phosphatase (44), we tested the hypothesis that SFK^{CA} can indirectly promote Tau phosphorylation at Ser/Thr residues *via* phosphorylation of PP2Ac at Y284/Y127. We chose to assess the role of PP2Ac phosphomutants in the regulation of Tau phosphorylation at pS202, an AD-like phosphoepitope strongly phosphorylated by Fyn (43) and directly dephosphorylated by PP2A *in vitro* (45). As expected from our earlier studies in N2a cells (24), Tau was dephosphorylated at S202 (*i.e.*, the CP13 phosphoepitope) in unstimulated, starved N2a cells expressing either the empty vector or WT PP2Ac (Fig. 7*A*). As observed with ERK1/2 (Fig. 6), the Y127F, Y284F, or F/F mutants appeared to be catalytically active toward Tau, as illustrated by the absence of CP13 immunoreactivity in these cells. In contrast to WT-expressing N2a cells, very strong CP13 immunoreactivity was observed in N2a cells coexpressing WT and either Src^{CA} or Fyn^{CA} (Fig. 7, *A* and *B*). Expression of Y127F induced a modest inhibitory effect on Src^{CA}- but not Fyn^{CA}-dependent Tau phosphorylation. In contrast, p-Tau levels were reduced by ∼40% in N2a cells coexpressing the Y284F mutant and either Src^{CA} or Fyn^{CA}. Once again, the F/F mutant maximally inhibited Src^{CA}-mediated Tau phosphorylation.

While these data suggest that the F/F mutant can directly promote Tau dephosphorylation by being resistant to SFK^{CA}-mediated phosphorylation, PP2A also indirectly regulates Tau phosphorylation *via* its ability to regulate major Tau protein Ser/Thr kinases such as glycogen synthase kinase-3β (GSK-3β)

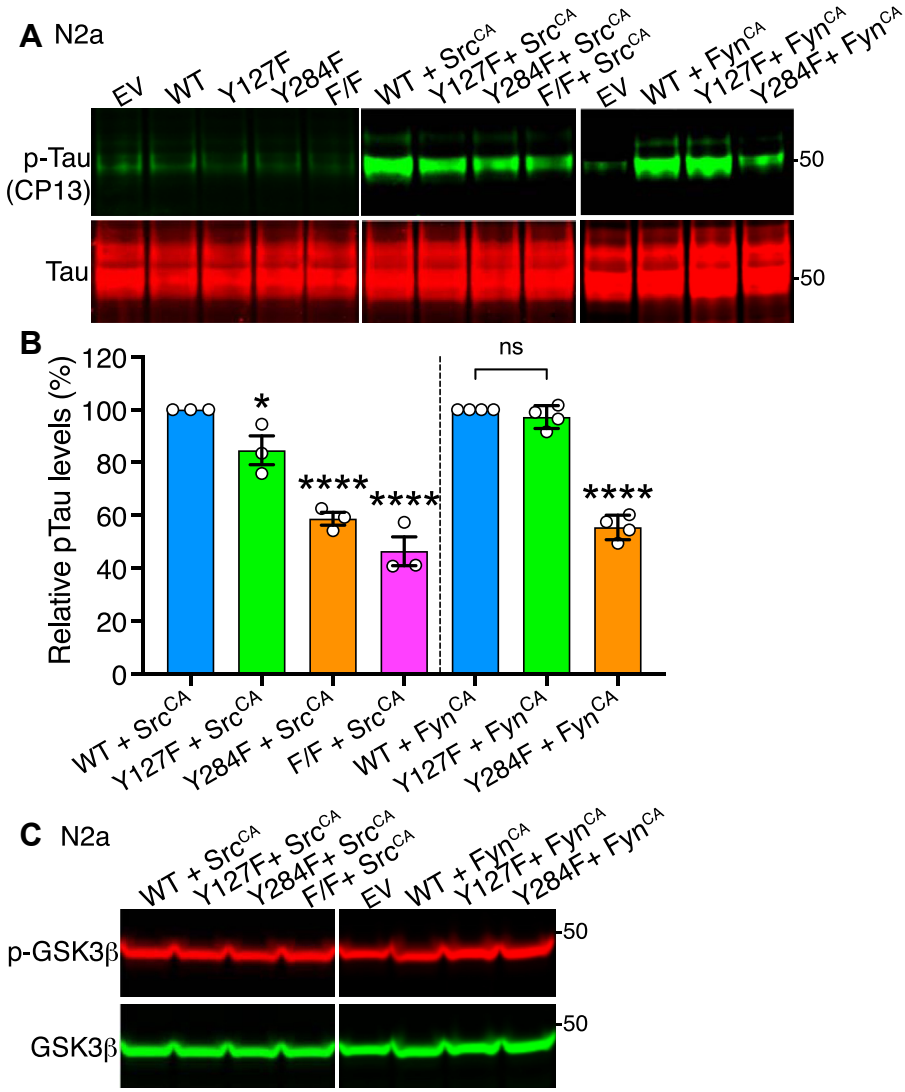

Figure 7. PP2Ac phosphomutants inhibit Src^{CA}- and Fyn^{CA}-mediated Tau phosphorylation. *A*, the same total cell homogenates from control (Fig. 6*D*, *left panel*), Src^{CA}- (Fig. 6*D*, *center panel*), or Fyn^{CA}- (Fig. 5*C*, *right panel*) transfected N2a cells that were cotransfected with EV or HA-PP2Ac species were reanalyzed by Western for endogenous Tau phosphorylation at the CP13 phosphoepitope (pSer202) and for total Tau. *B*, relative Ser 202-phosphorylated Tau (p-Tau) levels in N2a cells transfected as in *A*. Data are mean ± SEM from n = 3 separate experiments and were appraised using one-way ANOVA ($F = 61.67$; $p < 0.0001$) with Tukey's post hoc multiple comparisons test. *$p < 0.05$, ****$p < 0.0001$, compared with WT + SFK^{CA}; ns, not significant. *C*, representative immunoblots of total and Ser 9-phosphorylated GSK-3β from the same cell lysates analyzed in *A*. Similar results were obtained in two other experiments.

(44). Under any of our experimental conditions, we were unable to detect any significant changes in the phosphorylation state of GSK-3β at the PP2A sensitive-Ser 9 site, which regulates GSK-3β activity (Fig. 7*C*). Collectively, our findings indicate that the tyrosine kinases Src^{CA} and Fyn^{CA} can promote Tau phosphorylation at Ser 202 by inducing tyrosine phosphorylation of PP2Ac at Y127/Y284.

Discussion

PP2A plays a central role in cellular signal transduction and homeostasis. As such, PP2A activity and substrate specificity are tightly controlled in cells by sophisticated processes involving protein–protein interactions and posttranslational modifications. Among the latter, phosphorylation of PP2Ac on Y307 has been proposed as a key regulatory mechanism that leads to transient inactivation of PP2A (16). Y307 was identified as the site of phosphorylation within PP2Ac based solely on *in vitro* data showing that a C-terminal truncated mutant of purified PP2Ac lacking the Y307 residue is not phosphorylated. Unfortunately, the focus on Y307 as the sole site of phosphorylation has led to subsequent (mis)use of poorly characterized commercial "pY307 antibodies" to assess PP2A phosphorylation and activity status in a variety of cell lines and human diseases (13, 19). To complicate matters, Y307F has been reported to block Src-mediated Tau phosphorylation, leading the authors to propose that Y307 is a physiological phosphosite within PP2Ac (40). However, this interpretation is also misleading because Y307F and Y307E mutants display significantly altered binding to PP2A regulatory B subunits (27, 46, 47), which could interfere with PP2A regulation and block B subunit-dependent substrate dephosphorylation in the absence or presence of Src. In marked contrast to prior reports, our studies indicate that Src activation can regulate PP2A function *via* phosphorylation of Y127 and Y284, two PP2Ac phosphosites identified in multiple MS studies (PhosphoSitePlus; (18)), and not *via* phosphorylation of Y307. Sequence analysis of human and mouse PP2Ac using PhosphoNET (Kinexus Bioinformatics) also predicts that Y127/Y284 are Src phosphorylation sites. We demonstrate that Fyn, like Src, can phosphorylate PP2Ac on Y284, a site also predicted to be phosphorylated by Fyn (PhosphoNET). However, the Y127 site does not appear to be a major PP2Ac site targeted by Fyn, in agreement with an MS study identifying Y284, but not Y127, as a putative PP2Ac phosphorylation site in *in vitro* Fyn kinase assays (23).

We have highlighted a mechanism by which tyrosine kinases can regulate the phosphorylation status of Ser/Thr residues by presenting the first cellular evidence that Fyn-mediated phosphorylation of PP2Ac on Y284 results in enhancement of Tau phosphorylation at the AD-like pS202 phosphoepitope. These findings provide a plausible explanation for how the tyrosine kinase Fyn not only phosphorylates Tau at Tyr sites but also promotes phosphorylation at a serine residue (S202) *in vivo* (43). Likewise, Src-mediated phosphorylation of PP2Ac at Y127/Y284 likely contributes to the Src-induced phosphorylation of Tau at Ser/Thr epitopes reported in earlier studies (40, 41). Notably, increased phosphorylation of Tau at AD-like Ser/Thr residues enhances its interaction with Fyn, while reducing its binding to and dephosphorylation by PP2A (44, 48). Thus, overstimulation of Fyn in AD (5) has the potential to induce aberrant phosphorylation of PP2A at Y284, resulting in pathological increase in Tau phosphorylation and deregulation of PP2A/Bα-dependent signaling cascades that ultimately contribute to neurodegeneration.

Constitutive Src-dependent ERK activation is a common event associated with deregulation of cell proliferation in cancer (3), and persistent activation of the Fyn/ERK signaling cascade has been linked to cancer progression and drug resistance (2, 31, 32). Significantly, we found that expression of PP2Ac Y127/284F and Y284F mutants interfered with Src- and Fyn-dependent ERK activation, respectively, thereby suggesting an important role for PP2A tyrosine phosphorylation at this site in SFK-mediated cell transformation. Owing to the complex and multifaceted regulation of ERK signaling by numerous PP2A isoforms (49), additional studies will be required to determine the exact role of Y127 and Y284 phosphorylation of PP2A in the regulation of ERK and/or upstream modulators of ERK.

Prior *in vitro* assays have shown that PP2Ac tyrosine phosphorylation at unidentified sites is associated with its inactivation (16). Our findings indicate that PP2Ac phosphoincompetent mutants are enzymatically active and resist SFK^{CA}-mediated PP2A phosphorylation/inactivation (Figs. S6 and S7). Despite numerous attempts, and in agreement with the reported rapid autodephosphorylation of tyrosine phosphorylated PP2Ac (16), we were unable to reliably and comparatively measure PP2A activity in immunoprecipitates prepared from our cells without concomitantly losing PP2Ac phosphorylation.

While earlier MS studies indicate that endogenous PP2Ac is tyrosine phosphorylated at Y127/284, we were unable to detect by Western blotting pY127/284-PP2Ac in total lysates from SFK^{CA}-expressing cells unless these species were enriched by immunoprecipitation. This observation suggests that, while collectively PP2A enzymes are abundant cellular proteins (50), the endogenous pool of phosphorylated PP2Ac may be comparatively rather small and/or phosphorylation of PP2Ac is highly labile (16). There are currently no PP2Ac subunit antibodies that can reliably be used for quantitative immunoprecipitation of endogenous PP2A holoenzymes (22). Likewise, we found that our pY127 and pY284 antibodies were unsuitable for immunoprecipitation of endogenous PP2A enzymes. These technical hurdles prevent a reliable assessment of the precise stoichiometry of endogenous PP2Ac phosphorylation, as reported in earlier studies (17). Nevertheless, if Tyr phosphorylation of PP2A inhibits its catalytic activity (16) and complete inhibition of PP2A activity causes cell death (51, 52), it is likely that only a subset of the total cellular pool of PP2Ac is subject to inhibitory Y127/284 phosphorylation. Additional support for this hypothesis comes from our analyses of existing PP2A structures (53–56), which suggest that only a few of the PP2A oligomeric complexes can be targeted for Y127 and/or Y284 phosphorylation (Fig. S6).

Phosphorylation of Y127 likely plays a role in PP2Ac inactivation as this residue resides within the catalytic cleft and is important for substrate binding (57). However, mutation of Y127 to a Phe is unlikely to affect PP2Ac activity because the corresponding residue in another active PPP family member, namely, PP2B (calcineurin), is a Phe. Structural studies also suggest that Y284 phosphorylation could involve alterations in the binding of the structural A subunit, in addition to potentially regulating PP2Ac-SH2 interactions.

Using our newly generated antibodies, we were able to show for the first time in cells that endogenous PP2Ac in ABαC holoenzymes can be phosphorylated on Y284, and this phosphorylation event partially decreases the association of the regulatory Bα subunit with PP2Ac. Reduced binding of Bα to the core enzyme could mediate the inhibition of PP2A catalytic activity toward Bα-sensitive substrates, such as Tau and ERK. In contrast to pY284-PP2Ac, we were unable to detect pY127-PP2Ac in FLAG-Bα immunoprecipitates. Structural analyses suggest that binding of the Bα subunit interferes with phosphorylation of PP2Ac at Y127 in the core enzyme (Fig. S6). Moreover, Src^{CA}-mediated Tyr phosphorylation of the Bα-binding incompetent L309Δ mutant was still detectable, albeit decreased relative to WT. Thus, it is possible that phosphorylation on Y284 and detachment of Bα is required for subsequent phosphorylation on Y127, and/or other PP2A isoforms are subject to phosphorylation on Y127/284. Surprisingly, we found no pY signal in immunoprecipitates from cells coexpressing Src^{CA} and catalytically inactive PP2Ac mutants. It is possible that the stable association of PME-1 with these mutants (28) prevents proper access of Src to the phosphorylation sites on PP2Ac (Fig. S6). Thus, the subunit composition of PP2A, which controls its protein–protein interactions, subcellular targeting, and substrate specificity, likely has a critical influence on PP2Ac phosphorylation. Additional studies and novel tools will be required to fully elucidate the precise contribution and relationship of Y127 and Y284 phosphorylation in the regulation of specific PP2A holoenzymes. In any case, our findings clearly indicate that phosphorylation of PP2Ac cannot be readily utilized as a readout of PP2A catalytic activity in total cell lysates and cast doubt on the validity of numerous published studies (listed in (13)).

In conclusion, the studies presented herein represent a paradigm shift in our understanding of PP2Ac regulation by Tyr phosphorylation and demonstrate that PP2Ac is primarily targeted for phosphorylation on Y127/284 by Src^{CA}, and on Y284 by Fyn^{CA}. Our studies suggest that PP2Ac Y127/284 phosphorylation is a key molecular determinant that the cell uses to convert a "phospho-Tyr signal" into changes in protein Ser/Thr phosphorylation (*e.g.*, growth factors → activation of protein tyrosine kinases → increased Tyr phosphorylation [Y127/284] of a specific subcellular pool of PP2Ac → elevated serine/threonine phosphorylation of localized PP2A substrates). We have also uncovered significant differences in Y127 and Y284 phosphorylation. Finally, the recent identification of Y127C mutations in neurodevelopmental disorders (58) implicates an important role of PP2Ac tyrosine phosphorylation in disease.

Experimental procedures

Reagents and antibodies

Unless indicated, all chemicals were purchased from Sigma/Merck Millipore. Antibodies used in this study included mouse anti-HA (clone 16B12, Covance); rabbit anti-HA (clone C29F4, Cell Signaling Technology); rabbit anti-GFP (clone D5.1, Cell Signaling Technology); mouse anti-Bα (clone 2G9, Merck Millipore); mouse anti-$PP2Ac_{\alpha/\beta}$ (BD Transduction); goat anti-pY307 PP2Ac (Santa Cruz Biotechnology, Inc; denoted SC); rabbit anti-phospho-SFK (Y416) (clone 100F9, Cell Signaling Technology); rabbit anti-phospho-p44/42 MAPK (clone D13.14.4E, Cell Signaling Technology); mouse anti-p44/42 MAPK (ERK1/2) (L34F12, Cell Signaling Technology); rabbit anti-phospho-GSK-3β (Ser 9) (Cell Signaling Technology) and mouse anti-GSK3β (clone 3D10, Cell Signaling Technology); rabbit anti-Src (clone 32G6, Cell Signaling Technology); mouse anti-Src (clone GD11, Merck Millipore); rabbit anti-Fyn (clone EPR5500, Merck Millipore); mouse anti-Fyn (BD Transduction and Cell Signaling Technology); mouse and rabbit anti-p-Tyr (P-Tyr-100 and P-Tyr-1000, Cell Signaling Technology); rabbit anti-Tau (rPeptide), mouse anti-pSer202 Tau (CP13) (59) (a gift from Peter Davies). Mouse (clone C4, Merck Millipore) or rabbit (Cytoskeleton) anti-actin, and mouse (clone 236-10501, Life Technologies Australia Pty Ltd) or rabbit (Epitomics) anti-α-tubulin antibodies were used for protein normalization.

Generation of pY127 and pY284 PP2Ac antibodies

Peptides encompassing the nonphosphorylated/phosphorylated Y127 (ESRQITQVYGFYDEC; ESRQITQVpYGFYDEC) and Y284 (CDDTLKYSFLQ; CDDTLKpYSFLQ) epitopes were synthesized with a C- or N-terminal Cys residue and conjugated to Imject Maleimide-Activated mcKLH (pY127-KLH and pY284-KLH), Imject Maleimide-Activated BSA (Y127-BSA, pY127-BSA, Y284-BSA, and pY284-BSA), and SulfoLink Coupling Resin following the manufacturer's recommended protocol (ThermoFisher Scientific). The pY127-KLH and pY284-KLH conjugates were used for antibody production in rabbits (GL Biochem Shanghai) and alpacas (Turkey Creek Biotechnology, Waverly, TN), respectively. Phosphospecific antibodies recognizing the pY127 and pY284 epitopes were purified from the rabbit and alpaca antisera, respectively, by differential affinity chromatography. Briefly, 5 ml of pY127 and pY284 antisera was passed over the respective nonphosphorylated peptide column (2 ml), and the not bound material was then applied to the respective phosphorylated peptide column (2 ml). After washing with 40 to 50 ml PBS, bound antibodies were eluted with 8.5 ml of 100 mM glycine, pH 2.5 and collected in tubes containing 1.5 ml of 1.5 M Tris-HCl, pH 8. Finally, to remove any potential nonspecific phospho-Tyr antibodies, the enriched pY127 and pY284 antibodies were passed over pY284 and pY127 columns, respectively. The specificity of the not bound material (pY127 antibodies and pY284 antibodies) was then assessed by dot blot analysis using Y127-BSA, pY127-BSA, Y284-BSA, and pY284-BSA conjugates (Fig. S2).

Plasmids

Plasmids used in this study included pcDNA 3.1 encoding HA-tagged WT, L309Δ or Y307F mutants of PP2Ac (27), HA3-tagged WT PP2Ac (60), pGRE5-2 encoding HA-tagged H59Q and D88N PP2Ac mutants (28, 61), and Bα/pcDNA5/TO plasmid encoding FLAG-tagged PP2A Bα (38). The HA3-tagged Y127F, Y284F, and Y307F point mutants of PP2Ac were generated using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene) and WT HA3-PP2Ac/pcDNA-5TO as a template. The PP2Ac double mutants Y127/284F and Y127/307F were generated by site-directed mutagenesis of the Y127F construct. The plasmid encoding constitutively active c-Src kinase (Src^{CA}) was previously described (18). The plasmid expressing the constitutively active Fyn Y531F mutant (Fyn^{CA}) was generated after mutagenesis using the Transformer Site-Directed mutagenesis kit (Clontech) and pAcCMV Fyn–GFP plasmid encoding GFP-tagged human Fyn (26) as a template. All plasmids were verified by sequencing.

Cell culture and transfection

The Src-transformed and counterpart nontransformed MEFs (62) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. SYF, Neuro-2a (N2a), and Cos-7 cell lines (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (Life Technologies Australia Pty Ltd) containing 25 mM Hepes, pH 7.4, 10% fetal bovine serum (Bovogen), and 10 μg/ml gentamycin (Life Technologies Australia Pty Ltd); these cells were transiently transfected with the indicated plasmids using Metafectene Pro reagent following the manufacturer's instructions (Biontex Laboratories). Cells mock-transfected with empty vectors (EVs) were used as controls and behaved like nontransfected cells in our experiments.

Cell treatment

Unless otherwise indicated, experiments were performed in ∼90% confluent cells cultured in regular cell culture medium. When indicated, cells were serum-starved overnight in medium containing 0.5% fetal bovine serum. Cells were treated for 15 min with 100 μM pervanadate, prepared according to a published protocol (63). When indicated, cells were incubated for ∼18 h with 5 μM PP2, an SFK inhibitor (Merck Millipore). In some experiments, serum-starved cells were treated for 15 min with 40 ng/ml recombinant human EGF (#E-100, Alomone Labs) alone, or in combination with 100 μM pervanadate.

Cell lysis, microcystin affinity purification, and immunoprecipitations

Total SYF, N2a, and Cos-7 cell homogenates were prepared in Buffer 1 (25 mM Tris, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol [DTT], 0.5 μM okadaic acid [Merck Millipore], 5 mM PMSF [Sigma], 1% NP-40 [Sigma], Sigma Protease Inhibitor Cocktail, and Sigma Phosphatase Inhibitor Cocktail) and clarified by centrifugation at 4 °C for 5 min at 13,000*g*. Src-transformed and control MEFs were lysed in Buffer 2 (25 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, and 0.5% Triton X-100) for microcystin-Sepharose experiments. Protein concentration was determined in diluted aliquots of cell homogenates using the Bradford protein assay kit (Bio-Rad).

For microcystin-Sepharose affinity purifications, clarified lysates from two ∼90% confluent 100-mm dishes of Src-transformed or control MEFs were incubated with 50 μl of microcystin-Sepharose (prewashed in Buffer 2). Following overnight incubation at 4 °C, the beads were washed 3 times with Buffer 2 and bound proteins were eluted with 200 μl of SDS sample buffer. A 15-μl aliquot of the eluted proteins was subjected to Western analysis. For immunoprecipitation (IP) assays, cells (one ∼90% confluent 100-mm dish per condition) were lysed in IP buffer (Buffer 1 without DTT and containing 0.5% sodium deoxycholate [Sigma]) and clarified by centrifugation at 4 °C for 10 min at 13,000*g*. The clarified lysates were divided into two equal aliquots that were incubated overnight with anti-HA-coupled magnetic beads (Cell Signaling Technology) following the manufacturer's instructions. Beads were then extensively washed in IP buffer, and immune complexes were resuspended in gel loading buffer.

Western analyses

SDS-solubilized immune complexes, microcystin-Sepharose bound proteins, and total cell lysates (∼50 μg) were resolved on 4 to 12% Bis-Tris gels (ThermoFisher Scientific) or homemade 10% Tris-glycine gels. Prestained Protein Standards (Bio-Rad) were used as molecular weight standards. Western blotting was performed using the indicated primary antibodies, followed by Infrared IRDye-labeled secondary antibodies and visualization using the Odyssey Infrared imaging system (LI-COR Biosciences). Band intensity was determined using the associated Image Studio Lite version 5.0 Software (LI-COR Biosciences) to accurately quantify protein expression levels. Data were analyzed for statistical significance using GraphPad Prism 9. Differences with $p < 0.05$ were considered statistically significant.

Data availability

All data are available in the main text or the supplementary materials. All materials are available upon reasonable request.

Supporting information—This article contains Supporting Information (46, 53–57).

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writing – review & editing; J.-M. S., M. D. M., B. W. S., E. S., B. E. W. visualization; J.-M. S., E. S., B. E. W. supervision; E. S., B. E. W. project administration; J.-M. S., E. S., B. E. W. funding acquisition.

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Conflicts of interest—B. E. W. and B. W. S. are cofounders of Turkey Creek Biotechnology LLC and have equity ownership. The authors declare no other competing conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: BSA, bovine serum albumin; EGF, epidermal growth factor; EV, empty vector; GSK-3β, glycogen synthase kinase-3β; IP, immunoprecipitation; MEF, mouse embryo fibroblast; MS, mass spectrometry; PP2A, protein phosphatase 2A; pY, phosphotyrosine; SFK, Src family kinase; Src^{CA}, constitutively active Src.

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