A RNA Interference Screen Identifies the Protein Phosphatase 2A Subunit PR55γ as a Stress-Sensitive Inhibitor of c-SRC

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Protein Phosphatase type 2A (PP2A) represents a family of holoenzyme complexes with diverse biological activities. Specific holoenzyme complexes are thought to be deregulated during oncogenic transformation and oncogene-induced signaling. Since most studies on the role of this phosphatase family have relied on the use of generic PP2A inhibitors, the contribution of individual PP2A holoenzyme complexes in PP2A-controlled signaling pathways is largely unclear. To gain insight into this, we have constructed a set of shRNA vectors targeting the individual PP2A regulatory subunits for suppression by RNA interference. Here, we identify PR55γ and PR55δ as inhibitors of c-Jun NH₂-terminal kinase (JNK) activation by UV irradiation. We show that PR55γ binds c-SRC and modulates the phosphorylation of serine 12 of c-SRC, a residue we demonstrate to be required for JNK activation by c-SRC. We also find that the physical interaction between PR55γ and c-SRC is sensitive to UV irradiation. Our data reveal a novel mechanism of c-SRC regulation whereby in response to stress c-SRC activity is regulated, at least in part, through loss of the interaction with its inhibitor, PR55γ.

Introduction

The Src family of nonreceptor tyrosine kinases are integral players in the mediation of various physiological processes such as cell motility, adhesion, proliferation, and survival [1]. Members of the Src family share a conserved structure consisting of four Src homology (SH) domains, a unique region, and a short negative regulatory tail. The amino terminal SH4 domain is myristoylated and targets the protein to the membrane, while the carboxy-terminal SH1 domain functions as a tyrosine kinase domain [2]. c-SRC activation is negatively regulated by Carboxy Src Kinase (CSK) or its homologue CHK through Tyrosine 527 (Tyr527) phosphorylation [2]. This inhibitory phosphorylation promotes the assembly of the SH2, SH3, and kinase domains into a closed conformation [2]. Following stimulation by various stresses and growth factors c-SRC activation is initiated by dephosphorylation of the Tyr527 residue by the protein-tyrosine phosphatase PTPα [3] and PTP1B [4]. Alternatively, c-SRC is activated by the binding of tyrosine-phosphorylated proteins to the SH2 domain, resulting in destabilization of the intermolecular interaction between Tyr527 and the SH2 domain [2]. Subsequently, c-SRC is autophosphorylated at Tyrosine 416 (Tyr416), a site within a segment of the kinase domain termed the activation loop, promoting a conformational change that allows the kinase to adopt an open active confirmation [2].

c-SRC is overexpressed or activated in a wide variety of tumors [5,6]. However, overexpression of c-SRC by itself has only minor oncogenic potential [7] and mutations in c-SRC in cancer have only been found sporadically [8]. This led to the hypothesis that c-SRC has a supportive function in tumorigenesis rather than a role in the actual transformation process [9]. Overexpression of v-Src, a constitutively active form of c-SRC lacking the c-terminal part containing the inhibitory Tyr527, is a potent activator of c-Jun NH₂-terminal kinase (JNK), a growth-regulatory enzyme that can control cell proliferation and cell survival both positively and negatively, depending on the stimulus and the cellular context [10,11]. Furthermore, SRC activity is essential for JNK activation following a number of different stress stimuli, including UV irradiation [12–14].

Protein Phosphatase 2A (PP2A) is a serine/threonine phosphatase that can influence the phosphorylation state of many signaling enzymes [15,16], and inhibition of this phosphatase can affect cellular responses such as growth, differentiation, and apoptosis [15,17]. The holoenzyme generally exists as a core dimer, consisting of a 36-kDa catalytic subunit (PP2Ac) and a 65-kDa scaffold subunit (PR65) that associates with a variety of regulatory subunits. These regulatory B subunits can modulate the activity of the PP2Ac/PR65 core unit, thus allowing specific temporal
Author Summary

Protein Phosphatase type 2A (PP2A) represent a family of holoenzyme complexes involved in wide range of activities such as growth, differentiation, and cell death. The PP2A holoenzyme complex is made up of a catalytic, a structural, and one of various “B” subunits. These “B” subunits are thought to provide the substrate specificity required for PP2A activity. Previous work on PP2A has mostly been derived by inhibiting the catalytic subunit through chemical inhibition, as such inhibiting all of the pathways associated with PP2A. To identify individual “B” subunits involved in specific cellular processes we have generated a “B” subunit gene knockdown library, which allows us to inhibit each of the known “B” subunits individually. One of the many pathways regulated by PP2A is the c-Jun NH$_2$-terminal kinase (JNK) kinase pathway, which, depending on stimulus, can affect either cell survival or cell proliferation. Here we report that the “B” subunit PR55$\gamma$ acts as a negative regulator of JNK activity and cell death. We show that PR55$\gamma$ influences JNK activity by inhibiting one of its upstream regulators, the proto-oncogene c-SRC, through dephosphorylation at one of the key residues on c-SRC, a site we show to be critical for c-SRC activation following cell stress. Overall our work describes the novel function of a specific PP2A subunit involved in cell survival and identifies a novel mechanism of c-SRC regulation.

Results

PR55$\gamma$ Is a Modulator of JNK Activity

To identify the specific PP2A holoenzyme complexes involved in pathways known to be modulated by PP2A, we constructed a gene family knockdown library targeting all putative human PP2A regulatory B subunits for suppression. We retrieved the cDNA sequences for each of the PP2A subunit family members from the ENSEMBL database and designed two to four unique 19-mer sequences for each transcript for cloning into pSuper and pRetro-Super [26,28]. In total 61 knockdown vectors were generated, which were then subsequently pooled into 16 sets of two to four vectors per transcript with each set targeting one of the regulatory B subunits or a specific transcript variant (Figure 1A; Table S1). To validate the pooled knockdown vectors, we tested six randomly chosen pools of vectors for their ability to effectively knockdown the target proteins. All pools tested show a notable reduction in target protein expression levels (Figure 1B).

Studies using viral proteins that target the regulatory B subunits of the PP2A holoenzyme complex indicate that JNK and the proto-oncogene c-Jun can be regulated by PP2A [29]. This suggests that specific PP2A regulatory B subunits are involved in PP2A-mediated regulation of the JNK pathway. To directly assess the putative role of PP2A in JNK regulation we asked if suppression of one or more PP2A regulatory subunits by RNA interference could affect JNK activity following UV irradiation. U2-OS cells were transfected with the different library pools and then assayed by western blotting for the efficiency of UV induced JNK activation as judged by threonine-183/tyrosine-185 phosphorylation. Unsurprisingly, we found that suppression of a number of the B subunits appeared to enhance the levels of phosphorylated JNK following UV. Of these, PR55$\gamma$ consistently yielded the strongest effect and was chosen for further validation (Figure 1C and unpublished data). To evaluate which of the four individual knockdown vectors in this pool were active against PR55$\gamma$, we transfected cells with HA-tagged PR55$\gamma$ and determined the protein levels of HA-PR55$\gamma$ in lysates of transfected cells in the presence or absence of the individual PR55$\gamma$ knockdown vectors. As depicted in Figure 2A, all four shRNA vectors (A–D) in this pool were able to suppress HA-PR55$\gamma$ expression levels, whereas no effect was detected on a cotransfected green fluorescent protein (GFP) (Figure 2A). A shRNA targeting the mouse-specific B subunit PR59 was used as a negative control in all experiments. Vectors A and C were more efficient in suppressing HA-PR55$\gamma$ protein levels than vectors B and D (Figure 2A). A fifth knockdown vector (E) was designed, which like vector C, induced strong suppression of ectopic PR55$\gamma$ expression (Figure 2A). shRNAs C and E will be referred from here on as shRNA*1 and shRNA*2, respectively.

To test whether these shRNAs #1 and #2 could inhibit endogenous PR55$\gamma$ levels we performed quantitative real-time PCR (QRT-PCR). We found that both shRNAs efficiently suppressed endogenous PR55$\gamma$ mRNA levels (Figure 2B). Furthermore, inhibition of PR55$\gamma$ with both validated knockdown vectors could efficiently enhance the activation of JNK by UV irradiation (Figure 2C), arguing against an off-target effect of the shRNAs. This result underscores the validity of the screen and suggests that endogenous PR55$\gamma$ is a repressor of stress-induced JNK activation.

targeting of a wide range of PP2A substrates. To date 15 genes coding for more than 26 (B) regulatory subunits have been identified that are subdivided into five different subfamilies [17]. The variable PP2A B subunits are targeted by a number of viral oncogenes, which thereby compete for interaction with the PR65/PP2Ac core dimer. This suggests that specific PP2A holoenzymes play a role in viral propagation and oncogenic transformation [18,19], which is further supported by the finding that general inhibitors of PP2A can cause tumor growth on the skin and liver of rodents [20–23]. Understanding the precise manner in which PP2A is involved in the regulation of these different signaling cascades and its role during oncogenic transformation requires the identification of the specific holoenzymes involved in these processes. Interpretation of a large amount of data using general PP2A inhibitors has been limited by the pleiotropic inhibition of all PP2A holoenzyme complexes by the inhibitors used. Furthermore, ectopic expression of the various B subunits can lead to competition with other subunits for binding to the holoenzyme, making it difficult to draw firm conclusions from the data [24,25]. Using a gene family knockdown library targeting all deubiquitinating (DUB) enzymes, we previously identified the familial tumor suppressor gene CYLD as a novel regulator of the NF-kB signaling pathway [26] and USP1 as the deubiquitinating enzyme of the FANCd2 DNA repair protein [27]. To study the role of the various PP2A complexes in specific pathways we have constructed a library of 61 independent vectors expressing short hairpin RNAs (shRNA) targeting the PP2A regulatory B subunits for suppression. Using this knockdown library in a screen for enhancers of JNK activation following cellular stress, we identified a number of PP2A B subunits as novel regulators of JNK activation, most notably PR55$\gamma$ and PR55$\delta$. Furthermore we demonstrate that the PP2A B subunit PR55$\gamma$ negative regulates the JNK effector pathway by acting as a stress sensitive inhibitor of c-SRC activity.
To determine whether the activation of JNK after transfection of PR55γ knockdown vectors was a consequence of the loss of PR55γ expression, we performed an add-back experiment. To do this we restored PR55γ levels to the control situation using a PR55γ construct (ΔPR55γ) containing two noncoding mutations within the region targeted by knockdown vector #2, rendering it refractory to shRNA-mediated suppression (Figure 2D). We found that expression of ΔPR55γ completely abolished the enhanced activation of UV-induced JNK observed with shRNA vector #2, but not with shRNA vector #1, which targets a region that was not mutated in ΔPR55γ (Figure 2E). These results argue that the effects of the knockdown vectors targeting PR55γ for shRNA-mediated suppression on JNK activation are the result of loss of PR55γ.

To investigate whether the enhanced JNK activation upon PR55γ knockdown is specific for UV irradiation, we asked whether other stimuli that lead to the activation of the JNK pathway might also be enhanced by loss of PR55γ. We found that TNFα, insulin, and osmotic stress-mediated JNK activation could all be enhanced by suppression of PR55γ but not EGF-mediated JNK activation (Figure 2F). These results suggest that PR55γ is a regulator of the JNK signaling pathway when activated by diverse stimuli.

It has previously been established that activation of JNK by UV irradiation can enhance apoptosis in cell culture [30]. Since knockdown of PR55γ leads to enhanced JNK activation, we asked whether knockdown of PR55γ could enhance apoptosis following UV irradiation. UV-induced apoptosis was indeed significantly enhanced in PR55γ-depleted cells (Figure 3A) as determined by measuring the mitochondrial membrane potential with a fluorescent dye (3,3′-dihexyloxacarbocyanine iodide, [DiOC6 (3)]). Figure 3B represents three independent DiOC6 experiments demonstrating the percent-
age of apoptosis with or without UV in presence of knockdown vectors targeting PR55γ or a control vector. We also observed an increase in caspase 3 cleavage, a primary executioner of apoptosis, in lysates of cells exposed to UV irradiation, when PR55γ was suppressed (Figure 3C). Similar results were obtained with a second shRNA targeting PR55γ (unpublished data).

Inhibition of JNK Activity by PR55γ Is Dependent on c-SRC

To investigate whether PR55γ regulates the JNK pathway upstream of JNK we asked if loss of PR55γ affected MKK4, the kinase acting directly upstream JNK [31]. We indeed found also that MKK4 activity was significantly enhanced in cells with depleted PR55γ (Figure 4A). These data suggest that PR55γ does not directly affect JNK phosphorylation levels. We therefore asked whether the suppression of PR55γ had an effect on the other MAPK pathways, p38 and ERK. Indeed, western blot analyses indicated that knockdown of PR55γ resulted in increased phosphorylation of both JNK and p38, but not of ERK following UV irradiation (Figure 4B). Thus indicating that PR55γ acts on a key regulatory protein required for activation of both JNK and p38. Of note, no direct interaction was found between PR55γ and components of the MAPK and JNK kinase pathways including the

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Figure 2. PR55γ Regulates c-SRC following UV Irradiation

(A) U2-OS cells expressing pcDNA-HA-PR55γ were cotransfected with the pooled knockdown (PR55γKD) vectors as indicated (A–E) or a control vector. GFP expression serves as a measure of transfection consistency.

(B) U2-OS cells were cotransfected with PR55γKD vectors #1 or #2, pcDNA-PR55γ serves as a positive control. pSuper vector targeting a mouse PP2A subunit PR59 served as an shRNA control. mRNA levels relative to the control are shown as evaluated by quantitative real-time PCR.

(C) U2-OS cells were cotransfected with PR55γKD vectors as indicated (#1 or #2) or control vector. Selected cells were exposed to UV irradiation (100 J/m2) and incubated for a further 60 min. Protein samples were analyzed by immunoblotting with antibodies targeting phosphorylated JNK (α-pJNK) or JNK1 and JNK2 (α-JNK).

(D) U2-OS cells expressing pcDNA-HA-PR55γ or pcDNA-HA-PR55γ (Δ) were cotransfected with PR55γKD vector #2. Protein samples were analyzed by immunoblotting with antibodies targeting HA.

(E) U2-OS cells expressing pcDNA-HA-PR55γ, pcDNA-HA- PR55γ(Δ), or a control vector were cotransfected with PR55γKD vectors #1 or #2. A pSuper vector targeting a mouse PP2A subunit PR59 served as an shRNA control. Selected cells were exposed to UV irradiation (100 J/m2) and incubated for a further 60 min. Protein samples were analyzed by immunoblotting with antibodies targeting phosphorylated JNK (α-pJNK), total JNK (α-JNK), or haemoglutinin (α-HA, reprobe).

(F) U2-OS cells expressing PR55γKD vector or a control vector exposed to TNF-α, EGF, NaCl, or insulin for 5 min and incubated for a further 30–60 minutes. pJNK relative to total JNK levels are shown.

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previously described PP2A interacting proteins JNK, MKK4, p38, or c-RAF as determined by coimmunoprecipitation assays (unpublished data) [32–35].

One of the major contributors to the activation of the JNK pathway is the nonreceptor tyrosine kinase c-SRC [12–14]. It was previously shown that the polyoma middle t (MT) antigen, which binds to c-SRC and has been suggested to compete with the PP2A regulatory B subunit for binding to the holoenzyme complex [36–38], is also a potent activator of the JNK signaling cascade [38]. It has recently been described that of the ubiquitously expressed SRC family members only c-SRC [39] and LYN [40] play decisive roles in UV-induced JNK activation. Consistent with this, only c-SRC and Lyn have putative PKC sites in the N-terminal region. However, LYN appears to exclusively regulate JNK kinase but not p38 or ERK. Since knockdown of PR55γ in our system regulates not only JNK but also the MAPK p38 (Figure 4B), it would suggest that c-SRC may be the critical target of PR55γ in negatively regulating the JNK pathway in U2-OS cells following stress.

To test whether the enhanced activation of JNK after suppression of PR55γ is dependent on c-SRC, we cotransfected a dominant negative version of c-SRC, which has a lysine 295 to methionine mutation, resulting in a kinase deficient c-SRC (Src295M) [41]. UV irradiation-induced JNK phosphorylation was attenuated in the presence of Src295M, in agreement with the earlier finding showing that JNK is activated by both c-SRC independent and c-SRC dependent pathways [39]. However, the enhancing effect of PR55γ knockdown was completely abolished upon coexpression of Src295M (Figure 4C). Likewise inhibition of c-SRC by the generic Src family inhibitor PP2 also inhibited the enhanced JNK activity caused by suppression of PR55γ (Figure 4D). Similarly, cotransfection of a hairpin targeting PR55γ with an shRNA targeting c-SRC completely abolished the enhancing effects of PR55γ on JNK activity (Figure 4E).

To further investigate whether PR55γ can influence the levels of phosphorylated JNK by a non c-SRC family kinase–associated stimulus, we cotransfected a constitutively active form of the GTPaseCdc42 (Cdc42V12), which functions upstream of MKK4 in the JNK pathway, in the presence or absence of PR55γ. As expected, transfection of Cdc42V12

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**Figure 3.** PR55γ is a Regulator of Apoptosis following UV Irradiation

(A) U2-OS cells expressing PR55γ shRNAs as indicated. Selected cells were exposed to UV irradiation (50 J/m²) and treated 18 h later with fluorescent dye measuring mitochondria membrane potential (DiOC6(3)). FACS scan analysis of treated cells is shown. Right and left peaks reflect living and apoptotic cells, respectively. Top panel graph represents breakdown of the mitochondrial membrane potential in the absence of UV. Bottom panel graph represents breakdown of the mitochondrial membrane potential in the presence of UV.

(B) Shown is the percentage of apoptotic cells treated with or without UV. Figure represents three independent experiments.

(C) U2-OS cells were cotransfected with PR55γKD vector #2 or control vector. Selected cells were exposed to UV irradiation (50 J/m²) and incubated for a further 3 h. Protein samples were analyzed by immunoblotting with antibodies targeting phosphorylated cleaved caspase 3. A background band denotes loading control.

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resulted in activation of JNK [42]. However, the knockdown of PR55γ had no significant effect on JNK activation, whereas it did enhance phosphorylation of JNK following exposure to UV, which served as a control (Figure 4F). Since suppression of a number of the B subunits appeared to enhance the levels of phosphorylated JNK following UV (Figure 1C), we wanted to determine whether the increased levels of phosphorylated JNK observed with knockdown of the other B subunits are dependent on c-SRC. As expected knockdown of PR55γ enhanced the activity of JNK following UV irradiation. Furthermore, like PR55γ, the increased JNK activity was completely attenuated upon cotransfection with kinase dead Src295M (Figure 4G). Together these results suggest that PR55γ and PR55δ negatively regulate JNK signaling in a c-SRC-dependent manner. Since PR55γ is primarily expressed in neuronal tissues and PR55δ is more ubiquitously expressed, it may be that PR55γ and PR55δ mediate the same biochemical responses to stress in different tissues.

**PR55γ Interacts with c-SRC**

Several studies have suggested a role for PP2A in the regulation of c-SRC [43–45]. For instance, both polyoma MT

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**Figure 4.** PR55γ Regulates JNK Upstream of MKK4 and at the Level or Upstream of c-SRC

(A) pJNK and pMKK4 in relation to the total level of unphosphorylated protein in UV irradiated U2-OS cells followed over time (0–60 min) in the presence or absence of PR55γ KD vector.

(B) U2-OS cells expressing PR55γ KD or a control vector were treated with UV and incubated for 60 min. Whole cell extracts were probed with the indicated antibodies.

(C) PR55γ KD vector or control vector were cotransfected as indicated in the presence or absence of c-SRC295M (dominant negative). Selected cells were exposed to UV irradiation and whole-cell extracts were analyzed by immunoblotting with antibodies targeting phosphorylated JNK (a-pJNK) or JNK1 and JNK2 (a-JNK).

(D) U2-OS cells were cotransfected with PR55γ KD vector or control vector as indicated and incubated with PP2 for 2 h and UV for 1 h. Whole cell extracts were analyzed by immunoblotting with antibodies targeting phosphorylated JNK (a-pJNK) or JNK1 and JNK2 (a-JNK).

(E) PR55γ KD vector or control vector were cotransfected as indicated in the presence or absence of pSuper-c-SRC. Selected cells were exposed to UV irradiation and whole cell extracts were analyzed by immunoblotting with antibodies targeting phosphorylated JNK (a-pJNK), JNK1 and JNK2 (a-JNK), or SRC (a-SRC).

(F) PR55δ KD vector or control vector were cotransfected as indicated in the presence or absence of CDC42V12 (dominant active). Selected cells were exposed to UV irradiation and whole cell extracts were analyzed by immunoblotting with antibodies targeting phosphorylated JNK (a-pJNK) or JNK1 (a-JNK).

(G) Cells were transfected with either the PP2A pool targeting PR55δ vector or control vector and cotransfected as indicated in the presence or absence of c-SRC295M (dominant negative). Selected cells were exposed to UV irradiation and whole cell extracts were analyzed by immunoblotting with antibodies targeting phosphorylated JNK (a-pJNK) or JNK1 and JNK2 (a-JNK).

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antigen and adenovirus E4orf4 were previously shown to interact with both c-SRC and PR55γ independently, but the relevance of these interactions remained elusive [46–48]. To further address the functional relationship between PR55γ and c-SRC, we asked whether PR55γ could physically interact with c-SRC. To investigate this we performed coimmunoprecipitation experiments. We found that immunoprecipitation of c-SRC from lysates of cotransfected cells resulted in coprecipitation of PR55γ (Figure 5A). We also detected this interaction reciprocally by immunoprecipitating GFP-tagged PR55γ with a GFP antibody and then probing the blotted precipitate with a c-SRC antibody (Figure 5B). Importantly, endogenous c-SRC also coimmunoprecipitated with GFP-PR55γ (Figure 5B). Together, these data suggest that PR55γ and c-SRC can form a complex in vivo.

Since PR55γ binds to c-SRC we asked if PR55γ could form...
physical complexes with other SRC family members. We cotransfected FLAG-PR55γ with either the c-SRC family kinases LYN or FYN. We found that LYN and FYN do not share the ability of c-SRC to interact with PR55γ in coimmunoprecipitation assays (Figure 5C and 5D).

To test if the PR55γ/c-SRC interaction was specific for the B subunit PR55γ we cotransfected c-SRC with PR55γ or the PP2A B subunit PR72. As shown in Figure 5E, c-SRC physically associated with PR55γ but failed to coimmunoprecipitate with PR72.

Moreover, the specific binding of c-SRC to the B subunit PR55γ suggests that PR55γ is able to recruit the holoenzyme complex to c-SRC. We therefore asked if PR55γ could mediate binding of the PR65/PP2Ac core dimer to c-SRC. We transfected HEK293 cells with constructs expressing FLAG-SRC, HA-PR65, and HA-PP2Ac in the presence or absence of GFP-PR55γ and performed coimmunoprecipitation assays for FLAG-SRC. We found that c-SRC formed a complex with the PP2A holoenzyme exclusively in the presence of PR55γ, indicating that PR55γ is required as bridging factor between c-SRC and the PR65/PP2Ac core dimer (Figure 5F). These observations demonstrate that PR55γ specifically interacts with c-SRC and mediates the recruitment of the PR65/PP2Ac core dimer to c-SRC.

The physical interaction between PR55γ and c-SRC suggests a role as a modulator of c-SRC activity. Since c-SRC activity is increased following UV irradiation, we asked whether UV irradiation could affect the interaction between PR55γ and c-SRC. We followed the interaction between PR55γ and c-SRC after UV irradiation by performing immunoprecipitation experiments. We found that the interaction between PR55γ and c-SRC was gradually lost over time (Figure 5G) demonstrating that the interaction between c-SRC and PR55γ is sensitive to UV irradiation.

PR55γ Modulates c-SRC Activation

Since PR55γ appears to regulate JNK activation at the level of c-SRC, we examined the role of PR55γ on c-SRC-activated transcription of a JNK responsive luciferase reporter. We found that suppression of PR55γ enhanced the ability of c-SRC to activate this reporter (Figure 6A). Conversely, overexpression of PR55γ represses the ability of c-SRC to activate this reporter (Figure 6B). Consistent with these results, western blot analyses demonstrate that overexpression of c-SRC causes an increase in JNK phosphorylation after UV (Figure 6C). Moreover, when we cotransfected short hairpins targeting PR55γ in the presence of c-SRC, we observed that suppression PR55γ enhanced the levels of phosphorylated JNK compared to c-SRC alone (Figure 6D). Consistent with this, ectopic expression of PR55γ inhibited the synergistic activation of JNK mediated by c-SRC and UV (Figure 6E). These results demonstrate that PR55γ is able to influence c-SRC-mediated signaling to the JNK pathway.

To assess whether PR55γ directly modulates c-SRC kinase activity, we evaluated c-SRC Tyr416 phosphorylation, a hallmark of its activity [2], by western blot analyses. We found that knockdown of PR55γ could further enhance c-SRC Tyr416 phosphorylation following stimulation by UV irradiation (Figure 6F). In contrast overexpression of PR55γ could reduce c-SRC Tyr416 phosphorylation following stimulation by UV irradiation (Figure 6G). Together, these results suggest that PR55γ is an inhibitor of c-SRC activity.

PR55γ Mediates Dephosphorylation of Serine 12 on c-SRC

Several studies have indicated that pretreatment with the PP2A inhibitor okadaic acid (O.A.) induces the phosphorylation of the PKC phosphorylation site, Ser12 on c-SRC, while simultaneously stimulating c-SRC kinase activity [45,49]. To further investigate if PR55γ alters the phosphorylation status of SRCSer12, we stimulated U2-OS cells with UV irradiation in the presence of [32P] orthophosphate and performed a 2D tryptic phospho-peptide analysis of phosphorylated c-SRC. Indeed, comparisons with trypsin phosphopeptide maps indicate that overexpression of PR55γ decreased the levels of the phosphorylated Ser12 peptide while okadaic acid slightly increased the phosphorylation levels of the Ser12 peptide, when compared to control samples (Figure 7A–7E). Of note peptide maps showed similar patterns to those performed by Moyers et al. [47]. Similar results were observed when the cells were treated with phorbol 12-myristate 13-acetate (PMA) and Forskolin, potent activators of PKC and PKA, respectively (unpublished data).

From these data we conclude that treatment with either UV or PMA induces the phosphorylation of the PKC site Ser12 on c-SRC and that this specific phosphorylation event is significantly diminished in cells overexpressing PR55γ. Of note, no direct interaction was observed between PR55γ and PKCα, the kinase that directly phosphorylates Ser12 of c-SRC, as determined by coimmunoprecipitation assays (unpublished data).

Since phosphorylation of a specific target sequence has been suggested to be one of the requirements for the targeting of the B regulatory subunit and the subsequent recruitment of the PP2A holoenzyme to the substrate, we wanted to address whether recruitment of PR55γ to c-SRC is dependent upon the phosphorylation status of residue Ser12. To answer this question we generated a gain-of-function (SRCSer12D) and a loss-of-function (SRCSer12A) mutant of this phospho-site. To test if phosphorylation of Ser12 was required for the binding of PR55γ we cotransfected HA-PR55γ with either: FLAG-SRC, FLAG-SRCSer12D, or FLAG-SRCSer12A in the presence or absence of UV irradiation and performed coimmunoprecipitation assays. As shown in Figure 7F, mutation of serine 12 to an alanine decreased the association of PR55γ compared to wild-type c-SRC, while the association of PR55γ with SRCSer12D significantly increased. However, the association of PR55γ with either wild-type c-SRC or the mutant forms of SRCSer12 were completely abolished following UV irradiation. Together these results suggest that phosphorylation of Ser12 is one of the factors determining PR55γ affinity towards c-SRC and demonstrates that the interaction between PR55γ and c-SRC is sensitive to UV irradiation regardless of the presence of a phospho-moity on Ser12.

To determine whether the effects of PR55γ on SRCSer12 phosphorylation were the cause of PR55γ-mediated c-SRC regulation, we examined the role of SRCSer12 on a JNK-responsive luciferase reporter. We found that overexpression of the SRCSer12A mutant significantly inhibited c-SRC’s ability to activate a JNK-responsive luciferase promoter (Figure 7G). Conversely, overexpression of SRCSer12D enhanced c-SRC’s ability to activate the JNK-responsive luciferase promoter (Figure 7G).

Since SRCSer12D and SRCSer12A significantly enhanced and
diminished c-SRC’s ability to activate the JNK-responsive luciferase promoter respectively, we wanted to determine whether phosphorylation of this site affects c-SRC kinase activity. Kinase activity was assayed by monitoring the levels of phosphorylated enolase as an exogenous substrate. As shown above (Figure 6F and 6G), exposure to UV irradiation increases the activation of c-SRC compared to nonstimulated cells (Figure 7H). However, the c-SRC kinase activity was severely crippled in cells expressing SRCS12A. Furthermore, c-Src kinase activity was significantly enhanced in SRCS12D cells compared to controls in unstimulated cells (Figure 7H).

Similar results were detected when measuring the autophosphorylation of c-SRC at Tyr416 (α-pTyr<sup>416</sup>) (Figure 7I). Taken together these results demonstrate that phosphorylation of Ser12 on c-Src is one of the requirements for full activation of the protein following stress.

**Inhibition of JNK Activity by PR55γ Is Dependent on c-SRC Ser12**

To determine if the observed effect on JNK activity by PR55γ following UV stimulation is dependent on Ser12, we cotransfected hairpins targeting PR55γ with wild-type c-SRC or the SRC<sup>S12A</sup> mutant and measured the levels of phosphorylated JNK by western blotting following UV irradiation. As expected knockdown of PR55γ intensified the effect on JNK phosphorylation compared to c-SRC alone. However, the enhancing effect of PR55γ knockdown on JNK phosphorylation was completely attenuated upon coexpression on the SRC<sup>S12A</sup> mutant (Figure 8A). In agreement with this result, cotransfection of SRC<sup>S12D</sup> interfered with PR55γ’s ability to inhibit JNK phosphorylation following exposure to UV (Figure 8B). Our data collectively demonstrate that modu-
lation of SRC\(^{S12}\) phosphorylation by PR55\(\gamma\) is critical for PR55\(\gamma\)'s effects on JNK activation.

We next asked whether SRC\(^{S12A}\) could abrogate the enhanced apoptosis observed with knockdown of PR55\(\gamma\). We cotransfected hairpins targeting PR55\(\gamma\) with either SRC\(^{S12A}\) or wild-type c-SRC and quantified apoptosis using DiOC\(_6\) staining following exposure to UV irradiation. In line with results above (Figure 3A), knockdown of PR55\(\gamma\) increased UV-induced apoptosis in the presence of wild-type c-SRC. However, coexpression of SRC\(^{S12A}\) completely curtailed the enhancing effect of PR55\(\gamma\) suppression (Figure 8C). In contrast, coexpression of PR55\(\gamma\) and c-SRC repressed UV-induced apoptosis compared to c-SRC alone, while these effects were completely abolished when PR55\(\gamma\) was coexpressed with SRC\(^{S12D}\) (Figure 8D). Taken together, these results demonstrate that the regulation of c-SRC by PR55\(\gamma\) and its subsequent effects on cell survival are mediated through regulation of Ser12 phosphorylation on c-SRC.

**Discussion**

It has previously been proposed that the stress response to environmental stimuli mediated by the JNK pathway is regulated by PP2A protein phosphatase activity [33,50,51], although the way in which this pathway is regulated and the specific PP2A holoenzyme responsible for this regulation have not been identified. Via an RNAi-mediated gene family–knockdown screen of regulatory PP2A B subunits, we now
identify PR55γ as a specific negative regulator of stress-induced JNK activation. We find that PR55γ regulates the JNK pathway through negative regulation of c-SRC kinase activity. Importantly, we identify here c-SRC serine 12 as a critical residue for the regulation of the c-SRC kinase activity during stress signaling. We show that PR55γ physically interacts with c-SRC and has a higher affinity for c-SRC when it is phosphorylated on serine 12. Since the interaction of the PP2A holoenzyme complex with c-SRC is dependent on PR55γ, this would suggest a transient interaction between PR55γ-containing PP2A holoenzyme and c-SRC, which is reduced as soon as serine 12 dephosphorylation has occurred.

Previous work has indicated that PP2A might play a role in the regulation of c-SRC activity, since treatment of cells with okadaic acid, a chemical inhibitor of PP2A [22], resulted in enhanced c-SRC activity [45], and PP2A can inactivate c-SRC in vitro [43]. Interestingly, polyoma MT is able to compete with the PP2A B regulatory subunit for interaction with the PR65/PP2Ac core dimer [38], and overexpression of polyoma MT is able to activate c-Jun kinase by virtue of its interaction with PP2A [29]. Furthermore, polyoma MT was also found to interact with c-SRC [36] leading to its activation [37]. Moreover, it was reported that adenovirus E4orf4 can interact with both c-SRC and PR55α independently and that the interaction with c-SRC is required for E4orf4 to induce apoptosis [46,52]. Overexpression of E4orf4 phenocopies loss of PR55α in yeast [53], allowing the possibility that inhibition of PR55α is a prerequisite for E4orf4-induced apoptosis in mammalian cells. Our data identifying PR55γ as a negative regulator of c-SRC are in agreement with these studies and could suggest that these viral proteins may function to displace PR55γ from c-SRC.

It has previously been reported that JNK is activated by both c-SRC independent and c-SRC dependent pathways [39]. This present study confirms and extends these results by demonstrating that the inhibition of SRC by PR55γ does not completely inhibit JNK activation but rather results in an overall decrease, similar to the effects observed with a kinase dead mutant of c-SRC. In contrast, knockdown of PR55γ increases SRC kinase activity following UV resulting in enhanced levels of phosphorylated JNK. These results suggest that modulation of one of the upstream activator pathways may result in a prolonged and amplified JNK effect. It has previously been shown that the majority of c-SRC is present in the perinuclear region where it was found to be inactive as judged by Tyr 416 phosphorylation [54]. c-SRC was

Figure 8. Inhibition of JNK Activity by PR55γ Is Dependent upon Ser12 of c-SRC
(A) PR55γKDO vector or control vector were cotransfected as indicated in the presence of FLAG-SRC or FLAG- SRC512A. Selected cells were exposed to UV irradiation and whole cell extracts were analyzed by immunoblotting with antibodies targeting phosphorylated JNK (α-pJNK), JNK1 and JNK2 (α-JNK), or FLAG (α-FLAG).
(B) GFP-PR55γ vector or control vector were cotransfected as indicated in the presence of FLAG-SRC or FLAG- SRC512D. Selected cells were exposed to UV irradiation and whole cell extracts were analyzed by immunoblotting with antibodies targeting phosphorylated JNK (α-pJNK), JNK1 and JNK2 (α-JNK), FLAG (α-FLAG), or GFP (α-GFP).
(C) U2-OS cells coexpressing PR55γ shRNAs and FLAG-SRC or FLAG- SRC512A as indicated. Selected cells were exposed to UV irradiation (50 J/m²) and treated 18 h later with fluorescent dye measuring mitochondria membrane potential (DiOC6(3)). Figure represents three independent experiments.
(D) U2-OS cells expressing PR55γ and FLAG-SRC or FLAG- SRC512D as indicated. Selected cells were exposed to UV irradiation (50 J/m²) and treated 18 h later with fluorescent dye measuring mitochondria membrane potential (DiOC6(3)). Figure represents three independent experiments.

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also found in the cytoplasm at lower levels correlating with increasing activity and moved to the membrane in response to various stimuli where it was fully active [54]. Similarly we found PR55γ to be primarily expressed in the perinuclear region indicating that PR55γ may colocalise with c-SRC (unpublished data). These data suggest that PR55γ may interact with c-SRC within the perinuclear region inhibiting the induction of c-SRC by PKC by limiting the phosphorylation status of Ser12. Since PR55γ did not decrease the overall levels of other phosphorylation sites within the unique region of c-SRC primarily SER17, phosphorylation of which has been shown to be involved in SRC dependent ERK signaling [55], it suggests that the selective response of c-SRC following PKC phosphorylation at Ser12 may reflect the restricted activation of the JNK downstream effector pathway through either, phosphorylation dependent changes in subcellular localization, as suggested by Liebenhoff et al. who demonstrated that cytoskeletal association of pp60c-src is dependent on phosphorylation of pp60c-src at Ser12 by PKC [56] or by regulation of the binding of proteins that may function to regulate the activity of c-SRC towards JNK.

One of the intriguing findings of this study is that upon treatment of cells with UV irradiation the interaction between c-SRC and PR55γ is lost. We propose a model in which we suggest that in response to stress c-SRC is activated in part by losing the interaction with its inhibitor allowing c-SRC to be localized to the plasma membrane and subsequent activation of the downstream JNK effector pathways (Figure 9). Similarly, it was described for PR55γ that its interaction with the PP2A2c/PR55γ dimer is sensitive to gamma irradiation [57]. Further work will be required to reveal the mechanism of UV-induced dissociation of the c-SRC/PR55γ in response to stress.

Materials and Methods

Plasmids and antibodies. pcDNA3-FLAG-SRC, pEGFP-SRC, pMT-SRC, pMT-SRC(527), pMT-SRC(295), pcDNA-LYN, and pcDNA-FYN (Table S2) were kindly provided by P. Stork, G. Superti-Furga, W. Molenar, and J. Borst. All other Flag-, GFP-, and HA-coding constructs were generated using pcDNA (Invitrogen). Detailed cloning information will be provided upon request. PP2A knockdown library vectors were generated by annealing the individual oligonucleotide primer pairs and cloning them into pSuper as described in [58]. The bacterial colonies of each B subunit were then pooled and used for plasmid preparation. The extra shRNA (E) that gave the most efficient knockdown against PR55 was from Cell Signaling; anti-SRC, anti-SRC knockdown sequence was obtained from Gonzalez et al. and cloned into pSuper [58]. Antibodies anti-p-JNK, anti-p-MKK-4, anti-p-Src(416), and cleaved caspase-3 were from Cell Signaling; anti-SRC, anti-JNK (C-17), anti-MKK4, HA (Y11), anti-GFP, and anti-FYN were purchased from Santa Cruz Biotechnology Inc. The anti-LYN antibody was a kind gift from J. Borst.

Cell culture, transient transfections, and luciferase assays. All cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, D-Glucose, and Penicillin/Streptomycin. U2-OS cells were divided in 10-cm dishes 1 d prior to transfection. Subconfluent cells were transfected using the calcium phosphate transfection method [60]. Cells were incubated overnight, washed in PBS, and puromycin selected (1.5 µg/ml) for 48 h. When required cells were serum starved for 48 h prior to stimulation. The cells were not allowed to reach confluency. For the screen and subsequent knockdown experiments, U2-OS cells were cotransfected with 20 µg of pooled PP2A shRNAs and 1 µg of pBabe-puro. After 72 h, selected cells were trypsinized and 5 × 10⁶ cells were plated out in a 10-cm dish. After incubating overnight cells were exposed to UV irradiation (100 J/m²) and incubated for a further 60 min in the same medium. The following agents were used to stimulate cells: 50 ng/ml EGF (Upstate), 10 ng/ml TGF (Sigma), 10 ng/ml Insulin (Sigma), 500 mM NACL, or UV-C (254 nm, 100 J/m²). Luciferase assays were performed using the Dual luciferase system (Promega). AP1 luciferase vector (300 ng) was transfected in the presence of CMV-c-SRC (0.5 µg) or a control vector and CMV-Renilla (0.25 µg). For loss of function, 2.5 µg of pSuper vector [58] was cotransfected, and luciferase counts were measured 72 h post-transfection using a TD-20/20 Luminocounter (Promega). For gain-of-function assays, 0.5 µg of CMV construct or control vector (empty CMV) was cotransfected, and luciferase counts were measured 48 h post-transfection.

Apoptosis assay. For detection of apoptotic cells, selected cells were incubated for 72 h, trypsinized, and incubated for another 10–12 h in new media. The cells were washed twice in PBS and incubated for 18–24 h following UV treatment (50 J/m²). Trypsinized, washed once with PBS, and resuspended for 10–15 minutes in 250 µl PBS containing 40 nM DiOC6 (3). After incubation the cells were analyzed by FACs analysis.

Western blotting and coimmunoprecipitation. Cells were lysed in solubilizing buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 1 mM Sodium Vanadate, 1 mM pyrophosphate, 50 mM sodium fluoride, 100 mM β-glycerophosphate), supplemented with protease inhibitors (Complete; Roche). Whole cell extracts were then separated on 7%–12% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked with bovine serum albumin and probed with specific antibodies. Blots were then incubated with an HRP-linked second antibody and resolved with chemiluminescence (Pierce). For coimmunoprecipitations, cells were lysed in ELB (0.25 M NaCl, 0.1% NP-40, 50 mM HEPES [pH 7.3]) supplemented with protease inhibitors. Lysates were then incubated for 2 h with 2 µg of the indicated antibodies conjugated to Protein A or Protein G. Western blotting for c-SRC was performed using the Dual luciferase system (Promega). AP1 luciferase vector (300 ng) was transfected in the presence of CMV-c-SRC (0.5 µg) or a control vector and CMV-Renilla (0.25 µg). For gain-of-function assays, 0.5 µg of CMV construct or control vector (empty CMV) was cotransfected, and luciferase counts were measured 48 h post-transfection.

2D tryptic phospho-peptide analysis and phospho-labeling. For tryptic phospho-peptide analysis U2-OS cells were cotransfected with 4 µg Flag-Src or 4µg Flag-Src (12A) and 20 µg PR55γ or control vector. Cells were phospho-starved for 45 min and 2 µCi of [³²P]orthophosphate was then added to the cells and incubated an additional 3 h. PMA at a final concentration of 200 nM was added for 30 min at 37 °C or the cells were treated with UV irradiation (100 J/m²) and incubated for a further 30 min at 37 °C. c-SRC was immunoprecipitated with Flag antibody (Sigma) as described above. The entire sample was loaded onto an SDS-PAGE gel, run, and then dried. The film was then exposed for 3 h at room temperature. The radioactive bands were then isolated, proteins eluted, digested with trypsin, and phosphopeptide mapping was performed as described.
previously [61,62]. For phospholabeling analysis HEK 293 cells were cotransfected with 4 μg Flag-PR55γ, 10 μg HA-PR65, and 10 μg HA-PP2Aγ. Cells were phospho-starved for 45 min, UV stimulated as above, and then 2 μCi of [32P] orthophosphate was added to the cells and incubated for a further 2 h in the same medium. c-SRC was immunoprecipitated with Flag antibody (Sigma) and an HA antibody (Y11, Santa Cruz) as described above. The entire sample was loaded onto an SDS-PAGE gel, run, and then dried.

**Supporting Information**

**Table S1.** Gene Names and Primer Sequences

Found at doi:10.1371/journal.pgen.0030218.s001 (114 KB DOC).

**Table S2.** Gene Names and GenBank Numbers

Found at doi:10.1371/journal.pgen.0030218.s002 (26 KB DOC).

**References**

1. Parsons SJ, Parsons JT (2004) Src family kinases, key regulators of signal transduction. Oncogene 23: 7906–7919.
2. Boggon TJ, Eck MJ (2004) Structure and regulation of Src family kinases. J Biol Chem 279: 41395–41406.
3. Zhang WM, Yang P, Pallan CJ (1992) Cell transformation and activation of pp60src by overexpression of a protein tyrosine phosphatase. Nature 359: 336–339.
4. Bjorge JD, Pang A, Fujita DJ (2000) Identification of protein-tyrosine phosphatase 1B as the major tyrosine phosphatase activity capable of dephosphorylating and activating c-Src in several human breast cancer cell lines. J Biol Chem 275: 41439–41446.
5. Irby RB, Yeatman TJ (2000) Role of Src expression and activation in human cancer. Oncogene 19: 5656–5662.
6. Yeatman TJ (2004) A renaissance for SRC. Nat Rev Cancer 4: 470–480.
7. Biocardi JR, Ishizawar RC, Silva CM, Parsons SJ (2000) Tyrosine kinase signalling in breast cancer: epidermal growth factor receptor and c-Src interactions in breast cancer. Breast Cancer Res 2: 203–210.
8. Irby RB, Mao W, Coppola D, Kang J, Loubeau JM, et al. (1999) Activating SRC mutation in a subset of advanced human colon cancers. Nat Genet 21: 187–190.
9. Ishizawar R, Parsons SJ (2004) c-Src and cooperating partners in human cancer. Cancer Cell 6: 209–214.
10. Nature (2000) Signal transduction by the JNK group of MAP kinases. Cells 103: 259–252.
11. Xie W, Hershman HR (1995) v-src induces progestagen synthease 2 gene expression by activation of the c-jun N-terminal kinase and the c-jun protooncogene. J Biol Chem 270: 27622–27628.
12. Chen K, Vita JA, Berk BC, Keaney JF Jr (2001) c-jun N-terminal kinase activation by hydrogen peroxide in endothelial cells involves SRC-dependent epidermal growth factor receptor transactivation. J Biol Chem 276: 16045–16050.
13. Devary Y, Gottlieb RA, Smeal T, Karin M (1992) The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. Cell 107: 1081–1091.
14. Sun Y, Yuan J, Liu H, Shi Z, Baker K, et al. (2004) Role of Gab1 in UV-induced c-jun NH2-terminal kinase activation and cell apoptosis. Mol Cell Biol 24: 1531–1539.
15. Sontag F (2001) Protein phosphatase 2A: the Trojan Horse of cellular signalling. Cell Signal 13: 7–16.
16. Millward TA, Zohnerwicz S, Hemmings BA (1999) Regulation of protein kinase cascades by protein phosphatase 2A. Trends Biochem Sci 24: 186–191.
17. Janssens V, Goris J (2001) Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem J 355: 417–439.
18. Hahn WC, Counter CM, Landberg AS, Beijersbergen RL, Brooks MW, et al. (1999) Creation of human tumour cells with defined genetic elements. Nature 400: 464–468.
19. Pallas DC, Shahrk LK, Martin BL, Jaspers S, Miller TB, et al. (1999) Polymy small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A. Cell 90: 167–176.
20. Suganuma M, Fujiki H, Furuya-Suguri H, Yoshizawa S, Yamaoto S, et al. (1999) Calycin A, an inhibitor of protein phosphatases, a potent tumor promoter on CD-1 mouse skin. Cancer Res 59: 3521–3525.
21. Fujiki H, Suganuma M (1993) Tumor promotion by inhibitors of protein phosphatases 1 and 2A: the okadaic acid class of compounds. Adv Cancer Res 61: 143–194.
22. Bialojan C, Takai A (1988) Inhibitory effect of a marine sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. Biochem J 253: 299–309.
23. Suganuma M, Fujiki H, Suguri H, Yoshizawa S, Hirota M, et al. (1988) Okadaic acid: an additional non-phorbol-12-tetradecanoyl-13-acetate-type tumor promoter. Proc Natl Acad Sci U S A 85: 1768–1771.

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ylation of pp60c-src is stimulated by a serine/threonine phosphatase inhibitor. Oncogene 9: 1947–1955.

46. Champagne C, Landry MC, Gingras MC, Lavoie JN (2004) Activation of adenovirus type 2 early region 4 ORF4 cytoplasmic death function by direct binding to Src kinase domain. J Biol Chem 279: 25905–25913.

47. Glover HR, Brewster CE, Dilworth SM (1999) Association between src-kinases and the polyoma virus oncogene middle T-antigen requires PP2A and a specific sequence motif. Oncogene 18: 4364–4370.

48. Pallas DC, Fu H, Haehnel LC, Weller W, Collier RJ, et al. (1994) Association of polyomavirus middle tumor antigen with 14-3-3 proteins. Science 265: 535–537.

49. Moyers JS, Bouton AH, Parsons SJ (1993) The sites of phosphorylation by protein kinase C and an intact SH2 domain are required for the enhanced response to beta-adrenergic agonists in cells overexpressing c-src. Mol Cell Biol 13: 2391–2400.

50. Thevenin C, Kim SJ, Kehrl JH (1991) Inhibition of protein phosphatases by okadaic acid induces AP1 in human T cells. J Biol Chem 266: 9563–9566.

51. Al-Murrani SW, Woodgett JR, Durnin Z (1999) Expression of I2PP2A, an inhibitor of protein phosphatase 2A, induces c-Jun and AP-1 activity. Biochem J 341 (Pt 2): 293–298.

52. Shtrichman R, Sharf R, Bitt H, Dobner T, Kleinberger T (1999) Induction of apoptosis by adenovirus E4orf4 protein is specific to transformed cells and requires an interaction with protein phosphatase 2A. Proc Natl Acad Sci U S A 96: 10680–10685.

53. Roopchand DE, Lee JM, Shahinian S, Paquette D, Bussey H, et al. (2001) Toxicity of human adenovirus E4orf4 protein in Saccharomyces cerevisiae results from interactions with the Cdc55 regulatory B subunit of PP2A. Oncogene 20: 5279–5290.

54. Sandilands E, Cans C, Fincham VJ, Brunton VG, Mellor H, et al. (2004) RhoB and actin polymerization coordinate Src activation with endosome-mediated delivery to the membrane. Dev Cell 7: 855–869.

55. Schmitt J, Stork PJ (2002) PKA phosphorylation of Src mediates cAMP's inhibition of cell growth via Rap1. Mol Cell 9: 85–94.

56. Liebenhoff U, Greenacher A, Preseki P (1994) The protein tyrosine kinase pp60c-src is activated upon platelet stimulation. Cell Mot Biol (Noisy-le-grand) 40: 645–652.

57. Guo CY, Brautigan DL, Lerner JM (2002) ATM-dependent dissociation of B55 regulatory subunit from nuclear PP2A in response to ionizing radiation. J Biol Chem 277: 4839–4844.

58. Brummelkamp TR, Bernards R, Agami R (2002) A system for stable expression of short interfering RNAs in mammalian cells. Science 296: 550–553.

59. Gonzalez L, Aguillo-Ortuno MT, Garcia-Martinez JM, Calcabrini A, Gamallo C, et al. (2006) Role of c-Src in human MCF7 breast cancer cell tumorigenesis. J Biol Chem 281: 20851–20864.

60. van der Eb AJ, Graham FL (1980) Assay of transforming activity of tumor virus DNA. Methods Enzymol 65: 826–839.

61. van der Geer P, Hunter T (1989) Mutation of Tyr697, a GRB2-binding site, and Tyr721, a PI 3-kinase binding site, abrogates signal transduction by the murine CSF-1 receptor expressed in Rat-2 fibroblasts. Embo J 12: 5161–5172.

62. Wilhelmson K, Burkhalter S, van der Geer P (2002) C-Cbl binds the CSF-1 receptor at tyrosine 973, a novel phosphorylation site in the receptor’s carboxy-terminus. Oncogene 21: 1079–1089.