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Tumor Suppressor Role for the SPOP Ubiquitin Ligase in Signal-Dependent Proteolysis of the Oncogenic Coactivator SRC-3/AIB1

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Abstract

Steroid receptor coactivator-3 (SRC-3/AIB1) is an oncogene that is amplified and overexpressed in many human cancers. However, the molecular mechanisms that regulate 'activated SRC-3 oncoprotein' turnover during tumorigenesis remain to be elucidated. Here we report thatspeckle-type POZ protein (SPOP), a cullin 3 (CUL3)-based ubiquitin ligase, is responsible for SRC-3 ubiquitination and proteolysis. SPOP interacts directly with an SRC-3 phospho-degron in a phosphorylation dependent manner. Casein kinase Iε phosphorylates the S102 in this degron and promotes SPOP-dependent turnover of SRC-3. shRNA knockdown and overexpression experiments substantiated that the SPOP/CUL3/Rbx1 ubiquitin ligase complex promotes SRC-3 turnover. A systematic analysis of the SPOP genomic locus revealed that a high percentage of genomic loss or LOH occurs at this locus in breast cancers. Furthermore, we demonstrate that restoration of SPOP expression inhibited SRC-3-mediated oncogenic signaling and tumorigenesis, thus positioning SPOP as a tumor suppressor.

Keywords

SRC-3/AIB1; oncogene; tumor suppressor; SPOP; Cul3; ubiquitin ligase; steroid receptor; coactivator; breast cancer

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Introduction

Control of differential gene expressions in response to developmental and environmental signals is a central theme in cell biology. Nuclear receptors (NRs) and coregulators play key roles in these processes. Dysregulation of NR and coregulator signaling circuitry can disrupt cell homeostasis and contribute to pathological states, including cancer (Glass and Rosenfeld, 2000; Kumar and O'Malley, 2008; McKenna et al., 1999).

SRC-3/AIB1, also known as ACTR/pCIP/TRAM-1/RAC3, was initially discovered to mediate estrogen receptor (ER) signaling and is frequently amplified or overexpressed in breast cancer (Anzick et al., 1997; Chen et al., 1997; Glass and Rosenfeld, 2000; Halachmi et al., 1994; Li et al., 1997; McKenna et al., 1999; McKenna and O'Malley, 2002; Takeshita et al., 1997; Torchia et al., 1997). Further studies indicated that SRC-3 also is vital to many other signaling pathways containing either androgen receptor, progesterone receptor, or transcription factors such as AP-1, E2F1, NF-κB, STAT, TEF-4 and ER81 (Belandia and Parker, 2000; Goel and Janknecht, 2004; Lee et al., 1998; Louie et al., 2004; Werbajh et al., 2000). Overexpression of SRC-3 elevates oncogenic signaling and promotes tumorigenesis and metastasis in many tissues (Kumar and O'Malley, 2008; O'Malley and Kumar, 2009).

SRC-3 is a member of the SRC family that also includes SRC-1 (Onate et al., 1995) and SRC-2/TIF2/GRIP1 (Hong et al., 1996; Voegel et al., 1996). Studies using mouse models further substantiated SRC-3 as an important oncogene (Kuang et al., 2004); Torres-Arzayus, et al. 2004) and a key regulator of diverse signaling systems (Zhou et al., 2005); (Yu et al., 2007) ; Wu et al. 2002; Louie et al. 2004; Zhou et al. 2005; Yan et al. 2006; Yu et al. 2007). SRC-3 was found to upregulate insulin-like growth factor-1 (IGF-1) and cyclin D1 (Planas-Silva et al., 2001; Torres-Arzayus et al., 2004; Wang et al., 2000; Xu et al., 2000; Yan et al., 2006). Recent studies demonstrated that SRC-3 enhances matrix metalloproteinase (MMP) expression and plays critical roles in cancer cell migration and invasion, a key event during cancer metastasis (Kajiro et al., 2009; Li et al., 2008a; Li et al., 2008b; Qin et al., 2008; Yan et al., 2008). SRC-3 also elevates Smad2 and Twist and promotes tumor metastasis, which can be prevented through targeting SRC-3 for degradation by CHIP (Kajiro *et al*., 2009). Importantly, overexpression of SRC-3/AIB1 and HER-2/neu together contribute to tamoxifen early therapeutic resistance in breast cancer patients (Osborne et al., 2003).

It is known that SRC-3 protein stability can be controlled by the proteasome pathway through both ubiquitin-independent and -dependent mechanisms (Li et al., 2008a; Li et al., 2007a; Li et al., 2007b; Wu et al., 2007; Yi et al., 2008). For instance, GSK3 and Fbw7 promote phosphorylation-dependent SRC-3 ubiquitination of the SRC-3 protein on the promoter in a transcription-linked manner (Wu et al., 2007). Recently we identified an essential SRC-3 phospho-degron which plays a key role in the ubiquitination-mediated degradation of SRC-3 by the 26S proteasome (Li et al., 2008a). However, the molecular mechanism for how this phospho-degron contributes to protein degradation, such as what ubiquitin ligase (complex) recognizes and binds to this phospho-degron has not been identified until now.

Ubiquitin-dependent proteolysis of proteins by the proteasome is responsible for the degradation of most regulatory proteins in the cell. Before being recognized and destroyed by the 26S proteasome, substrates destined for degradation by this pathway are covalently marked with ubiquitins. This process involves the sequential activation of three classes of enzymes: E1 (an ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzymes) and E3 (ubiquitin ligases). The specificity of protein ubiquitination rests in the E3 ubiquitin ligases that physically interact with their target substrates (Hershko and Ciechanover, 1998; Pickart, 2001). Ubiquitin ligases can be classified into two-major groups: the single-subunit forms including RING-finger and HECT-domain E3s, and the multi-subunit ubiquitin ligases that are assembled on a cullin scaffold; the SCF complex (Skp1, Cul1, F-box) is an example of the latter. In the SCF ubiquitin ligase complex, Cul1 serves as a scaffold protein to assemble Skp1/F-box proteins with the small ring finger Rbx1. F-box proteins directly contact their substrates and determine the specificity of the SCF complex (Jin et al., 2004). In addition to Cul1, the human genome encodes six other cullins (2, 3, 4A, 4B, 5, and 7) (Bennett et al.; Petroski and Deshaies, 2005) that also form ubiquitin ligase complexes. Notably, Cul3 interacts with Rbx1 and the BTB/POZ (Bric a brac, Tramtrack and broad Complex/Pox virus and Zinc finger) family of proteins, which merge the functions of the Skp1 and the F-box domains into a single polypeptide (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2003; Xu et al., 2003).

In a search for a SRC-3 phospho-degron interacting ubiquitin ligase we discovered that SPOP, a BTB/POZ protein, directly binds to the SRC-3 degron and promotes its degradation. SRC-3 polyubiquitination and degradation by the SPOP/Cul3/Rbx1 ubiquitin ligase complex occurs in a phospho-degron-dependent manner. Consequently, SPOP inhibits SRC-3-mediated oncogenic signaling and tumorigenesis. Importantly, loss of genomic copy number or LOH at the SPOP locus is significant in human breast cancers. In light of this data, we suggest that SPOP functions as a breast cancer tumor suppressor at least in part by reducing the cellular concentration of SRC-3 protein that is poised for, but not actively engaged in DNA transcription.

Results

The SPOP/Cul3/Rbx1 Ubiquitin Ligase Complex Regulates SRC-3 Oncoprotein Stability

We used the previously identified SRC-3 degron (Li et al., 2008a) to search for proteins that interact with this domain that might promote SRC-3 protein degradation. By yeast two-hybrid screening, using the SRC-3 degron and its flanking PAS region together (96aa) as a bait, we identified a number of interacting proteins. One of these is speckle-type POZ protein (SPOP), an E3 ubiquitin ligase adaptor that contains a Skp1-like domain functioning as both Skp-1 and F-box and is part of a Cul3 ubiquitin ligase complex (Furukawa et al., 2003; Geyer et al., 2003; Jin et al., 2004; Petroski and Deshaies, 2005; Pintard et al., 2003; Xu et al., 2003). In contrast, when we used the same SRC-3 region with the phosphorylation site mutations (S101A/S102A) as a bait in our yeast two-hybrid screening, SPOP was not found in the interacting proteins, suggesting that SRC-3 degron phosphorylation is likely required for its interaction with SPOP.

To test the hypothesis that the SPOP-containing E3 ubiquitin ligase complex promotes SRC-3 protein degradation, FLAG-tagged SRC-3 was expressed in 293T cells with SPOP. In the absence of the proteasome inhibitor MG132, SRC-3 protein abundance was remarkably reduced when SPOP was expressed; in the presence of MG132 there was virtually no change in SRC-3 protein abundance (Fig 1A). In contrast, a number of other proteins identified in the two-hybrid screen including CDKN3, IQCK, BRE, and DCTN6 had no significant effect on SRC-3 protein levels when overexpressed in the cell. These data suggested that SPOP promotes proteasome-dependent degradation of the SRC-3 protein.

Simultaneously, we studied ubiquitin/proteasome-dependent turnover of the SRC-3 oncoprotein by examining the Cullin-based E3 ubiquitin ligases, the largest known class of ubiquitin ligases. Cullin 1 to 5 are well-characterized and dominant-negative form mutants (DN-Cul1 to 5) have been studied for their ability to stabilize their respective substrates; DN-Culs are truncated versions of Cullins containing the N-terminal regions and therefore still are able to interact with a substrate adaptor such as an F-box protein, but they fail to interact with Rbx1 ubiquitin ligase and are unable to bring ubiquitins onto a substrate (Jin et al., 2005). As shown in Fig 1B, when we transiently expressed each DN-Cul with SRC-3 in 293T cells, we found that DN-Cul3 strongly stabilized the SRC-3 protein; DN-Cul1 showed a relatively weak stabilizing effect, which is consistent with a previous publication indicating a role for the Cul1-associated F-box protein, Fbw7α in SRC-3 turnover during transcription (Wu et al., 2007). Estradiol did not appear to have a significant effect on SRC-3 protein stability. These data indicate that a Cul3-based ubiquitin ligase complex likely is involved in controlling SRC-3 protein degradation.

It has been reported that the SPOP/Cul3/Rbx1 ubiquitin ligase complex targets several proteins for degradation in different cellular contexts (Bunce et al., 2008; Hernandez-Munoz et al., 2005; Kwon et al., 2006). Therefore, we tested the functional requirement for the Cul3 and Rbx1 components in this ubiquitin ligase complex during SRC-3 protein degradation by SPOP. Consistent with the above results, overexpression of HA-tagged SPOP resulted in a marked reduction in the FLAG-tagged SRC-3 protein level; this reduction was not observed when Cul3 or Rbx1 were knocked down by siRNA (Fig 1C), indicating that Cul3 and Rbx1 are indeed required for SPOP's function in SRC-3 protein turnover.

To directly examine the effect of SPOP on SRC-3 protein stability, we performed cycloheximide protein degradation rate experiments. Treatment of cells for up to six hours resulted in a remarkable reduction in the steady-state levels of the SRC-3 protein, whereas knockdown of SPOP by siRNA resulted in an elevation in SRC-3 protein abundance and there was virtually no change in protein levels compared to controls (Fig 1D, E). These data substantiated that SPOP reduces SRC-3 protein stability and as a consequence reduces the steady-state levels of the SRC-3 protein. Overexpression of a dominant negative mutant Cul3 (DN-Cul3) exhibited a similar effect on SRC-3 protein stability as seen for siRNA knockdown of SPOP (Fig 1D, E). This was consistent with the other reports that DN-Culs stabilize substrates targeted by a Cul-based ubiquitin ligase complex (Jin et al., 2005).

The SPOP/Cul3/Rbx1 Ubiquitin Ligase Interacts with the S101/S102 SRC-3 Phosphodegron

To validate the physical association between SRC-3 and SPOP, we performed a Co-IP experiment, revealing that the SPOP protein binds SRC-3 in MCF-7 breast cancer cells (Fig 2A). We then tested whether SRC-3 directly interacts with SPOP. GST-pulldown experiments were performed using purified recombinant FLAG-SRC-3 protein, indicating that SRC-3 indeed binds to GST-SPOP (Fig 2B). Likewise, HA-SRC-3 was observed to bind to GST-SPOP *in vitro* (Fig S1A). In a reciprocal experiment, we found that HA-SPOP binds to the GST-SRC-3 N-terminus (N390) (Fig 2C). To further determine which subdomain of SRC-3 interacts with SPOP, a series of SRC-3 deletion mutants were constructed for GST-pulldown experiments. Because the GST-fusion with the full-length SRC-3 protein was not soluble and HA-tagged small SRC-3 fragments including N390, N180 and N84 were difficult to express, we used a combination of different GST-SPOP and GST-SRC-3 mutants for this assay. In agreement with our yeast two-hybrid screen data above, our results indicate that the SRC-3 phosphodegron region (containing S101 and S102) is required for the interaction between SRC-3 and SPOP (Fig 2D, 2E).

SPOP contains one MATH (meprin and TRAF-homology) and one BTB (Broad complex, Tramtrack, Bric-a-brac) domain as essential parts of the SPOP/Cul3/Rbx1 ubiquitin ligase complex (Bunce et al., 2008; Hernandez-Munoz et al., 2005; Kwon et al., 2006; Petroski and Deshaies, 2005). We used GST-pulldown assays to examine how these three proteins associate with each other. As shown in Fig 2F and Fig 2G, the SPOP N-terminal MATH-containing domain directly bound to SRC-3 while the SPOP C-terminal BTB-containing domain directly bound to Cul3. This result substantiates the finding that SPOP acts as an adaptor for this Cul3-based ubiquitin ligase complex and targets the SRC-3 protein for degradation.

SPOP Promotes SRC-3 Polyubiquitination

We next determined whether the SPOP/Cul3/Rbx1 complex is capable of polyubiquitinating SRC-3. We co-expressed HA-tagged SPOP/Cul3/Rbx1 with FLAG-tagged SRC-3 in 293T cells. Western analysis of lysates from these cells revealed that SPOP/Cul3/Rbx1 promotes polyubiquitination of SRC-3 in the presence of MG132 (Fig 3A). This result was further substantiated by co-expressing HA-Ub with untagged SPOP/Cul3/Rbx1 and FLAG-SRC-3 followed by immunoprecipitation using FLAG antibodies (Fig 3B).

We performed cell-free ubiquitination assays to further study SRC-3 polyubiquitination by SPOP/Cul3/Rbx1. As shown in Fig 3C, purified E1, E2 (UbcH5a) and ubiquitin proteins were added into biochemical ubiquitination reactions containing SPOP, Cul3, Rbx1 and SRC-3 proteins which were transcribed/translated in cell-free TNT reticulocyte systems. Only in the presence of all ingredients (SPOP/Cul3/Rbx1, E1, E2 and ubiquitin), was the formation of polyubiquitinated SRC-3 detected (Fig 3C). These in vitro results demonstrated that SPOP/Cul3/Rbx1 directly promotes SRC-3 polyubiquitination formation.

SPOP Promotes Phosphorylation-Dependent Degradation of SRC-3

Since the SRC-3 degron is phosphorylation-dependent and includes two Ser phosphorylation sites, S101 and S102 (Li et al., 2008a), we explored the role that phosphorylation of these

precise amino acids had in substrate recognition by SPOP. We first tested whether SPOP is able to promote degradation of a phosphorylation-defective SRC-3 mutant at these phosphorylation sites (S101A/S102A). As shown in Fig 4A, overexpression of SPOP in 293T cells resulted in a marked decrease in the protein level of SRC-3; in contrast, it had no effect on the S102A or S101A/S102A mutants. As controls, SPOP deletion mutants with either only the N-terminal MATH or the C-terminal BTB domain did not affect SRC-3 protein levels (Fig 4A). We then asked whether this is due to a defect in the interaction between SPOP and SRC-3 S102A or S101A/S102A. A co-immunoprecipitation experiment was performed and revealed that SPOP binds to SRC-3 wt (FLAG-SRC-3) but not to the S102A or S101A/S102A mutants (Fig 4B). This result was confirmed *in vitro* by GST pulldown experiments (Fig S1B). Our results indicate that phosphorylations at the S101/S102 sites in SRC-3 are necessary for both recognition and degradation by SPOP.

We next examined the function of SPOP in two breast cancer cell lines: MCF-7 and MDA-MB-231. Ectopic expression of SPOP by a lentivirus vector resulted in a significant decrease in the level of the endogenous SRC-3 protein in MCF-7 cells, but there was no change in SRC-1 and SRC-2 levels, suggesting that SPOP specifically targets SRC-3 protein among the SRC/p160 family members (Fig 4C). To study the loss-of-function effect, we used shRNA knockdown of SPOP in both MCF-7 and MDA-MB-231 cells. Consistent with the results above, decreases in SPOP in these breast cancer cells resulted in increases in SRC-3 protein levels (Fig 4D, S1C); whereas the mRNA levels of SRC-3 in these experiments were not changed (Fig 4E, S1D).

CKIε Phosphorylates the S102 Site in the SRC-3 Degron and Destabilizes SRC-3

Although we demonstrated previously that the SRC-3 S101/S102 phospho-degron promotes degradation of SRC-3, the kinases targeting these two sites were unknown (Li et al., 2008a). Using a bioinformatic approach (Luo *et al*., 2007; Sakanaka, 2002), the candidate kinase, casein kinase I ε (CKIε), was identified as a potential kinase. To test whether CKIε phosphorylates SRC-3 at the S101 or S102 sites, we performed *in vitro* kinase assays. First, by using purified recombinant SRC-3 (Fig 2B) and CKI proteins, we found that CKI is able to phosphorylate SRC-3 (Fig 5A). Second, we tested whether CKI targets S101 or S102 in SRC-3 by peptide kinase assays. We synthesized four different peptides spanning the SRC-3 phospho-degron with either no phosphorylation (SS), phosphorylation at S101 (pSS), phosphorylation at S102 (SpS), or phosphorylations at both S101 and S102 (pSpS). Kinase assays revealed that the peptides with no phosphorylation or phosphorylation at S101 were phosphorylated by CKI; in contrast, all the others were not (Fig 5B), indicating that SRC-3 S102 is the primary phosphorylation site targeted by CKIε. As a positive control in this experiment, CKI phosphorylated its known substrate peptide. These SRC-3 peptides were not sufficiently long to bind SPOP *in vitro* (Fig S1E).

Since the S101/S102 SRC-3 degron phosphorylation is crucial for its protein stability, we examined whether the CKIε enzyme itself destabilizes the SRC-3 protein. Overexpression of CKIε decreased the SRC-3 protein level in the presence of SPOP, but this effect was not seen for SRC-3 S102A or S101A/S102A phospho-mutants (Fig 5C). CKI inhibitors IC261 and D4476 increased SRC-3 protein levels (Fig 5D), supporting that CKIε indeed

destabilizes the SRC-3 protein. Since both SPOP and CKIε promoted phospho-degron-dependent turnover of SRC-3, we wished to understand the underlying mechanism linking these two events. To address this question, we studied the effect of CKIε on the interaction between SPOP and SRC-3. After FLAG-SRC-3, HA-SPOP, and HA-CKIε were expressed in 293T cells, co-immunoprecipitation experiments were performed which showed that binding of SPOP to SRC-3 was increased when CKIε was present (Fig 5E). This result suggests that destabilization of SRC-3 protein by CKIε is at least in part due to increased association between SPOP and SRC-3 in the presence of CKIε. To substantiate the effect of CKIε on SRC-3 stability, CHX degradation rate experiments were performed. In the presence of CKIε, SRC-3 protein was more unstable and its half life was reduced more than in the absence of CKIε, whereas the CKIε kinase deficient (partial) mutant K35R was less effective in promoting the rate of SRC-3 degradation (Fig 5F).

SPOP Inhibits SRC-3-Mediated Oncogenic Signaling

In human cancers, SRC-3 is frequently overexpressed and activates multiple oncogenic signaling pathways and promotes tumorigenesis (Kumar and O'Malley, 2008; O'Malley and Kumar, 2009). Since SPOP is able to reduce SRC-3 protein levels, we examined the potential role for SPOP in suppressing SRC-3-mediated signaling. Since SRC-3 is a coactivator for ER and AR, we used ERE-luciferase and MMTV-luciferase as reporters for ER and AR signaling, respectively. Overexpression of SRC-3 enhanced estrogen- and androgen-dependent activation of each reporter gene and these gene activations were markedly reduced upon overexpression of SPOP; SPOP alone had little effect on reporter gene activities (Fig. 6A and B). Likewise, SRC-3-enhanced TNFα-stimulated NF-κB-luciferase activity was dramatically decreased by SPOP overexpression (Fig. 6C). These results indicate that SPOP inhibits SRC-3 activities in multiple signaling pathways.

Next, we examined SRC-3 target genes in breast cancer cells. Using shRNAs to knockdown SRC-3, SPOP, or both SRC-3 and SPOP in MCF-7 cells, we determined the effects on IGF-1 and MMP-2 gene expression, two SRC-3 responsive genes (Qin *et al*., 2008; Wang *et al*., 2000; Xu *et al*., 2000; Yan *et al*., 2008). Both IGF-1 and MMP-2 mRNA levels were significantly reduced when SRC-3 expression was knocked down; in contrast, their mRNA levels were increased when SPOP was knocked down (Fig 6D, E). With shRNA knockdown of both SRC-3 and SPOP, IGF-1 and MMP-2 mRNA levels were similar to the control (shGFP), suggesting that the increases in IGF-1 and MMP-2 mRNAs by shRNA against SPOP were largely due to increases in SRC-3 protein levels (Fig 6D, E, F).

Genomic Loss of the SPOP Locus Occurs Frequently in Human Cancers

Using cancer genomic approaches, we further investigated whether the SPOP-CUL3-RBX1-SRC-3 axis is dysregulated in human breast and other cancers, which generally show overexpression of SRC-3. It has been demonstrated that the CUL3 and RBX1 genomic loci exhibit a high percentage of genome loss in human cancers (Lee et al., 2009). The human SPOP gene is located in Chromosome 17q21.33. In an evaluation of 42 breast cancer cell lines using single-nucleotide polymorphism (SNP) arrays, the copy numbers at the SPOP locus (between rs1320283 and rs1406012) revealed that recurrent copy number losses are observed (60-70%) involving both the upstream and downstream regions of SPOP (Fig 7A).

Moreover, in 24 different cancer types, copy number analyses of amplification, LOH, deletion and mutation revealed LOH at high percentages in the SPOP locus (Fig 7B). Specifically, in 45 breast cancer samples, SPOP represented one of the highest loci for LOH (57.8%). These data suggest that genome loss of the E3 ubiquitin ligase complex at the SPOP, CUL3 and RBX1 loci could lead to overexpression of SRC-3 and contribute to several types of cancer development, particularly in a subset of breast cancers.

SPOP Functions as a Tumor Suppressor

We next asked whether SPOP directly influences cancer cell growth and invasion. shRNA knockdown of SPOP increased MCF7 cell growth, while ectopic overexpression of SPOP resulted in a slower proliferation rate compared to the control cells; this inhibitory effect was substantially rescued by additional expression of the SRC-3 S101A/S102A mutant, suggesting that inhibition of MCF7 cell growth by SPOP is largely due to a reduction in SRC-3 levels (Fig. 7C). To examine whether the invasive potential of the cancer cells is affected by SPOP, we performed *in vitro* cell invasion assays. Using shRNA to reduce SRC-3 expression decreased the number of cells that penetrated a Matrigel-coated membrane as reported previously (Li et al., 2008b; Yan et al., 2008). In contrast, shSPOP-treated MDA231 cells revealed a marked increase in invasiveness; this effect was compromised when both SRC-3 and SPOP were knocked down (Fig. 7D). In addition, we also observed that shSPOP increased MCF-7 cell colony formation in soft agar assays (Fig. S2) and increased cell size (Fig.S3). Finally, using a tumor xenograft mouse model, we found that tumor growth occurred at a faster rate in shSPOP cells, while it was slower in shSRC-3 cells, compared to a control cell line; in contrast under both shSPOP and shSRC-3 conditions, tumor growth was similar to the control. These results again substantiated that the SPOP tumor suppressive function is largely mediated through its ability to reduce the cellular concentration of the SRC-3 oncoprotein (Fig 7E). Taken together, these data indicate that SPOP can function as a tumor suppressor in breast, and potentially other cancers.

Discussion

A Specific Tumor Suppressor Role for SPOP

The SPOP gene is located in the 17q21 locus where a number of reports about allelic imbalance, and particularly, LOH of the chromosome 17q21 region have been described in up to 30% of primary human breast cancers (De Marchis et al., 2004; Orsetti et al., 1999). It also has been reported that LOH in the 17q21 region is frequently associated with inflammatory breast cancer (Lerebours et al., 2002). In preinvasive breast ductal carcinoma in situ (DCIS), 17q LOH has an incidence of 15.9%, which is among the highest percentages (Radford et al., 1995). Although the well known tumor suppressor BRCA-1 is located in this relative large chromosomal region and is involved in breast and ovarian cancers, additional genes in this tumor suppressor locus also may be involved (Durocher et al., 1996). Consistent with these previous publications, using currently available SNP microarray genotype data in a cancer genome dataset (Lee et al., 2009), we were able to more precisely define that there is significant copy number loss at the SPOP genomic locus in 42 breast

cancer cell lines (Fig 7A). We also found that LOH at the SPOP locus is remarkably high in multiple cancers, particularly in breast cancers (Fig 7B).

The SPOP/Cul3/Rbx1 complex is one of the major Cullin-based E3 ubiquitin ligases. Genetic allelic loss of the *CUL3* and *RBX1* loci occurs frequently in both breast cancer cell lines and tissue specimens (Lee et al., 2009). Moreover, our experimental results from breast cancer cell proliferation, invasion, anchorage-independent growth, and tumor growth in nude mice indicate that SPOP inhibits cancer cell functions (Fig 7C, D, E, S2). We also found that shRNA knockdown of SPOP in MDA-MB-231 cells increases cancer cell size (Fig S3), consistent with previous publications (Torres-Arzayus *et al*., 2004; Zhou *et al*., 2003). Taken together, these data suggest that the SPOP/Cul3/Rbx1 ubiquitin ligase complex functions as a breast cancer tumor suppressor.

As a component of a Cul3-based ubiquitin ligase complex, SPOP has been shown to target several proteins in different signaling pathways for ubiquitination and degradation, including the Polycomb group protein BMI1, the histone variant MacroH2A1 (Hernandez-Munoz et al., 2005), the death domain-associated protein Daxx (Kwon et al., 2006), the phosphatidylinositol phosphate kinase PIPKIIβ (Bunce et al., 2008), the transcription factor Gli (Chen et al., 2009), the Jun-kinase phosphatase Puckered (Liu et al., 2009) and Pdx1 (Claiborn *et al*.). Notably, while it was initially identified as an apoptotic factor, Daxx has also been reported to be anti-apoptotic (Michaelson et al., 1999; Tang et al., 2006). It has been reported that overexpression of SPOP as well as Cul3 and Rbx1 increases cell apoptosis (Kwon et al., 2006). Thus, it is likely that by destroying one or more potent growth-promoting oncoproteins in multiple signaling pathways of breast cancer, SPOP acts to suppress cancer cell functions. Indeed, overexpression of SPOP inhibited MCF7 cell growth; additional overexpression of degron-mutated SRC-3 rescued significantly cell growth (Fig 7C). We reason that restoration of SRC-3 did not fully rescue the cell growth rate due to existence of other growth-promoting targets of SPOP. In a specific cancer cell, SPOP may target one or more of its oncogenic substrates in addition to SRC-3 for degradation, resulting in an additive or synergistic effect on inhibition of tumorigenesis. A number of E3 ubiquitin ligases have been well-characterized to function as tumor suppressors through their ability to degrade oncoprotein substrates. One of the better known examples is the SCF ubiquitin ligase adaptor Fbw7 that functions as a tumor suppressor through its ability to degrade proto-oncoproteins including Myc, cyclin E, Notch and Jun (Welcker and Clurman, 2008).

Role of SPOP in the Regulation of Phosphorylation-Dependent Degradation of SRC-3

A common feature of SCF and SCF-like E3 ubiquitin ligases is that their recognition of substrates often involves posttranslational modifications of substrates, and the modification frequently is phosphorylation. Phosphorylated degrons bind to E3 ubiquitin ligase adaptors such as F-box proteins or BTB domain-containing proteins (Jin et al., 2004; Petroski and Deshaies, 2005). Consistent with these findings, binding and degradation of SRC-3 by SPOP is SRC-3 phospho-degron dependent, requiring phosphorylation at S101 and S102 residues (Fig 4 and 5). We further demonstrated that casein kinase CKIε is an enzyme that phosphorylates the S102 site, enhances the binding between SPOP and SRC-3, and

destabilizes the SRC-3 protein (Fig 5). In another example, it has been shown that the F-box protein β-TrCP controls degradation of circadian regulatory protein PER1 and PER2 via CKIε-dependent phosphorylation (Eide et al., 2005; Shirogane et al., 2005). In the present and previous studies, we demonstrated that phosphorylation critically determines SRC-3 protein stability. We now have completed identification of this reversible pathway by showing that the steady-state phosphorylation level of SRC-3 is controlled by phosphorylation/dephosphorylation targeted by CKIε and protein phosphatase 1 (PP1), respectively (Li et al., 2008a). Consequently, the balance of CKIε and PP1 activity in a specific cellular setting can determine SRC-3 phosphorylation, ubiquitination, protein half-life and its oncogenic activity.

Additionally, recent studies of the structures of SPOP-substrate complexes have revealed that a five residue ϕ-π-S-S/T-S/T (ϕ, nonpolar; π, polar) *S*POP-*b*inding *c*onsensus (SBC) motif is present in known SPOP substrates such as Puc, MacroH2A, Ci and Daxx (Zhuang et al., 2009). This is consistent with our finding that the SRC-3 degron also resembles a SST consensus sequence (Li et al., 2008a) and is required for binding to SPOP. The 5′-end flanking sequence located before SST in SRC-3 is DV, which are polar and non-polar residues respectively, although they occur in a reversed order compared to SBC. Importantly we identified that this motif is phosphorylated in SRC-3.

A 3-Step Mechanism for Regulation of SRC-3 Oncoprotein Turnover

SRC-3 is a master gene regulator and potent oncoprotein, critically involved in a number of tumor growth promoting signaling pathways. Its protein turnover is rapid and very tightly controlled so that the cell is able to respond selectively to different hormones, growth factors and environmental signals. Consequently, SRC-3 protein turnover is controlled at multiple levels. We hypothesize that (1) at the first step of SRC-3 protein regulation, prior to phosphorylation, the SRC-3 protein exists in an inactive state in a cellular reservoir where its degradation is controlled in an ubiquitin- and ATP-independent manner by the REGγ-proteasome (Li et al., 2006). (2) During its activation, SRC-3 protein is phosphorylated at a number of important sites including the S101 and S102. When SRC-3 becomes an activated oncoprotein, its turnover is mainly controlled by the SPOP/Cul3/Rbx1 ubiquitin ligase complex. (3) In the third step, functioning SRC-3 is actively engaged in transcription on the promoters of its target genes; on the promoter, SRC-3 protein turnover is regulated by the Fbw7 ubiquitin ligase in a different phosphorylation-dependent manner via GSK3 phosphorylation of serine residues S505 and S509 (Wu et al., 2007). These three distinct levels of regulations contribute to the highly regulated turnover of SRC-3 and to its function as a potent oncogenic molecule (Fig S4). Applications that harness the extraordinary power and specificity of these regulatory enzymes and pathways could hold future promise for therapeutic interventions.

Methods and Materials

Yeast Two-Hybrid Screen

Yeast two-hybrid screening was performed by using Matchmaker Gal4 Two-Hybrid System 3 and a Matchmaker Pretransformed Normalized HeLa cDNA Library (Clontech

Laboratories, Mountain View, CA). The SRC-3 degron and part of the PAS region (SRC3-PASC) was subcloned into the pGBKT7 vector as bait and subsequently transformed into the yeast AH109 strain growing on SD/-Trp medium according to the company's protocol. *Saccharomyces cerevisiae* strain Y187 containing the cDNA library was used to mate the yeast AH109 containing SRC3-PASC. Positive blue colonies growing on SD/-Ade/-His/-Leu/-Trp/X-α-Gal medium were selected, and PCR using the T7 and 3′ AD sequencing primers was performed for the positive cDNA clones followed by sequencing to identify genes.

GST Pulldown

GST-SPOP and GST-Cul3 vectors were made by subcloning SPOP and Cul3 into the BamHI/NotI and XhoI/NotI sites, respectively, of the pGEX-4T-1 vector. GST-SRC3-N390 was also constructed similarly into pGEX-4T-1, while the GST-SRC3-N180 and –N84 each were generated by introducing a stop-codon in GST-SRC3-N390. All the GST-fusion proteins were induced by 0.2 mM IPTG in BL21 bacteria for 2 hrs and purified by Glutathione Sepharose 4B (Amersham). The GST-protein-containing beads were further incubated with *in vitro* transcribed/translated HA-tagged proteins, which were generated by the TNT Coupled Reticulocyte Lysate System (Promega). Additionally, GST-SPOP was incubated with purified FLAG-SRC-3 protein, which was expressed by baculoviruses in Sf9 cells. Subsequently the binding assays were carried out in the mammalian cell lysis buffer with protease inhibitors for 2 hr with rotation followed by washes using the lysis buffer. Finally the bound proteins were resolved in SDS-PAGE and analyzed by Western Blot.

Co-Immunoprecipitation (Co-IP) and Western Blot

293T cells were transfected with pcDNA3.1-HA-SPOP and pXF2F-FLAG-SRC-3. Two days later, the cells were lysed in lysis buffer supplemented with protease inhibitor cocktails (Roche, Indianapolis, IN) (Li et al., 2008a). Cell lysates were immunoprecipitated with EZview red anti-FLAG M2 affinity gel for 2 hrs at 4 °C with constant rotation and then the beads were washed 4 times with the above buffer. Finally the beads were directly boiled in Laemmili sample buffer prior to separation by 10% SDS-PAGE. Western blot was carried out using FLAG or HA antibodies. HA-SPOP and FLAG-SRC3-S101A/S102A in 293T cells were analyzed in the same method described above. Interaction of SRC-3 and SPOP in MCF-7 cells was examined using SRC-3 antibodies (Santa Cruz) and protein G-plus agarose. Western blot was performed by first blocking nitrocellulose membranes with 5% nonfat milk in PBS-T buffer prior to adding antibodies as indicated. Image J software (NIH, US) was used for quantification of Western blots.

The antibodies against SPOP were either affinity-purified rabbit polyclonal generated by Abgent or monoclonal antibodies obtained from Dr. Kevin White. The other mouse monoclonal antibodies used in the experiments were: anti-FLAG-HRP (Sigma), anti-β-Actin-HRP (Sigma), anti-β-Gal (Roche), anti-Hsp70 (BD Biosciences), anti-α-tubulin (Millipore), anti-SRC-3/AIB1 (BD Biosciences), anti-Ub (Santa Cruz), anti-SRC-1 and anti-SRC-2 (BD Transduction). Rabbit polyclonal were anti-Cul3 (Bethyl), and anti-Rbx1 (Millipore), while anti-HA-HRP (Roche) was rat monoclonal.

Cycloheximide Protein Stability Assays

Protein decay was studied by cycloheximide (CHX) treatment experiments as described previously (Li et al., 2008a). 293T cells were transfected with either siRNA control or siSPOP, and one day later FLAG-SRC-3 or together with FLAG-DN-Cul3 were transfected for an additional 2 days. Afterwards, 0.5 mM CHX (Sigma) was added for different times as indicated, followed by SDS-PAGE and Western analysis. FLAG-SRC-3 expressed alone or together with HA-CKIε was also transfected into 293T cells and analyzed in these CHX-based experiments.

siRNA and shRNA Knockdown

The siRNAs for SPOP, Cul3, Rbx1 and control were purchased from Dharmacon as ON-TARGET plus SMART pools. siRNAs were transfected using TransIT-TKO Transfection Reagent (Mirus) according to manufacturer's instructions. Lentiviral shRNAs for SPOP and control GFP were purchased from Sigma and transduced into breast cancer cells followed by puromycin (5ug/ml) selection.

Lentiviral Overexpression

Lentiviral Expression Systems were purchased from System Biosciences (SBI, Mountain View, CA). Each cDNA for SPOP or SRC-3 was subcloned into pCDH-CMV-MCS-EF1-Puro vector, which was subsequently co-transfected with pPACKH1 Lentiviral Packaging Plasmid Mix (SBI) into 293T cells for two days before each virus-containing medium was collected. The resultant lentiviruses were transduced into breast cancer cells in the presence of polybrene (5ug/ml) followed by puromycin selection.

Ubiquitination Assays

In vivo ubiquitination assays of SRC-3 were performed by co-transfection of FLAG-SRC-3 and either HA-tagged or untagged SPOP, Cul3, Rbx1 into 293T cells in the presence or absence of HA-Ub. After immunoprecipitation by FLAG antibodies, ubiquitinated SRC-3 was detected by HA antibodies in Western Blot. *In vitro* ubiquitination assays were performed by *in vitro* transcribed/translated FLAG-SRC-3, and HA-tagged SPOP, Cul3 and Rbx1 proteins produced by TNT Coupled Reticulocyte Lysate Systems in the presence of E1, E2 (UbcH5a), Ubiquitin and ATP (Boston Biochem). The biochemical reactions were conducted at 37°C for 1.5 hrs followed by SDS-PAGE and Western Blot analysis.

Expression Plasmids

HA-tagged SRC-3 and its deletion mutants were constructed by pcDNA3.1 Directional TOPO Expression Kit (Invitrogen, Carlsbad, CA) using PCR primers containing an HA tag at the N-terminal. PCR amplification was performed by the pfu polymerase (Stratagene). HA-N84 and HA-N180 were generated by introducing a stop codon in HA-595. HA-SPOP, HA-SPOP-N and-C were generated using pCMV-SPORT6-SPOP (ACCESSION: NM_001007226) as a template in the same manner as HA-SRC-3 (Open Biosystems, Huntsville, AL). FLAG-DN-Cul3 was purchased from Addgene deposited by Wade Harper (Jin et al., 2005). HA-CKIε also was from Addgene deposited by David Virshup (Rivers et al., 1998).

In Vitro Kinase Assays

Biotin-labeled phosphorylated or non-phosphorylated peptides were synthesized by Abgent (San Diego, CA). Kinase Assays were performed using CKI, a positive control peptide for the CKI known substrate and buffer systems (New England Biolabs). ^{32}-γ-ATP was purchased from MP-Biomedicals (Solon, OH). Full-length SRC-3 protein was expressed by baculoviruses and purified from *Sf*9 cells as described previously (Li et al., 2008a).

Real-Time RT-PCR

The basic procedure was described previously (Li et al., 2008a). Total RNA was isolated using TRIzol (Invitrogen). TaqMan Gene Expression Assays (Applied Biosystems) were performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems) to analyze each gene expression level. mRNA levels were normalized to 18S as the endogenous control. Each TaqMan probe for its corresponding target gene mRNA and One-Step RT-PCR master mix also were from Applied Biosystems. Each target was measured in triplicate.

Soft Agar Assay

This assay was performed according to Cell Transformation Detection Assay (Chemicon International). 10,000 cells were suspended in complete medium containing 0.4% top agar layered on 0.8% solidified base agar in each of a 6-well plate. Feed cells 1-2 times per week with cell culture media. After 3-4 weeks, colonies with a diameter of more than 100 μm were counted.

Trans-well Matrigel Invasion Assay

As basically described previously (Li et al., 2008a), the shRNA knockdown stable cells were detached and transferred to Trans-well Matrigel Invasion chambers (BD Biosciences) following manufacturer's protocols. 20,000 cells were placed in each insert chamber containing 01% BSA medium while in the lower chamber containing 10% FBS. Cells were allowed to migrate and invade through the matrigel membrane for one day before being fixed with 4% formaldehyde and stained with crystal violet. The cells on the apical side of each insert were scraped off by Q-tips. The number of cells that had migrated through the matrigel membrane was counted under a microscope.

SPOP Genome Structure Analyses

For *SPOP* copy number analysis, we obtained Affymetrix 10K SNP microarray genotype data for 42 breast cancer cell lines from the Cancer Genome Project of the Wellcome Trust Sanger Institute. We analyzed the SNP data of the *SPOP* loci (47,617,000-47,815,000 bp) locating between rs1320283 (46,806,920-46,807,350 bp) and rs1406012 (48,292,440-48,293,020 bp) in chromosome 17, visualized them using the Cluster and TreeView software (Eisen, MB), and presented the results using heat maps. CONAN program was applied to analyze genomic high amplification, LOH, homozygous deletion, and mutation in multiple cancer types and the summarized percentages of genome alternations were shown in heat maps.

Nude Mice Tumor Xenograft

Mouse protocols were approved by the Baylor College of Medicine Animal Care and Use Committee. Stable MDA-MB-231 cell pools expressing shRNAs for SRC-3, SPOP and both, or control shRNA were injected into the 4th mammary glands on both sides of 5-6 weeks old female athymic Ncr-Nu/Nu mice (NCI) after being anesthetized with 2.5% Avertin (0.1ml/10g, i.p.). For each site, 5×10^6 cells in 100 μl DMEM were injected. Tumor length (L) and width (W) were measured once a week and tumor volume was calculated by $(\pi L \times W^2)/6$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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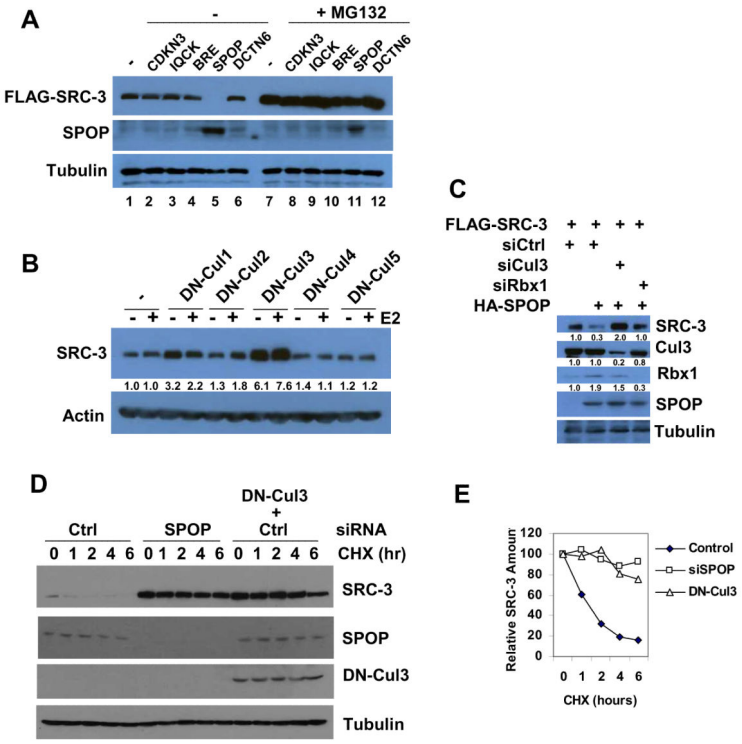

Figure 1. SPOP/Cul3/Rbx1 ubiquitin ligase complex targets SRC-3 protein for degradation

(A). SPOP promotes proteasome-dependent degradation of SRC-3. In 293T cells FLAG-SRC-3 was co-expressed with SPOP or other controls including Cyclin-dependent kinase inhibitor 3 (CDKN3), IQ motif containing K (IQCK), Brain and reproductive organ-expressed (BRE), and Dynactin 6 (DCTN6). After treatment of cells with or without MG132, protein abundance was analyzed by SDS-PAGE and Western blot using indicated antibodies or anti-FLAG antibodies for SRC-3. α-tubulin (Tubulin) served as a loading control.

(B). Dominant negative Cullin3 (DN-Cul3) stabilizes SRC-3 protein. FLAG-tagged SRC-3 was co-expressed with each one of the dominant negative Cullins (DN-Cul1, -Cul2, -Cul3, -Cul4B and -Cul5) in 293T cells. The cells were treated with or without 10^{-7}M estradiol (E2) for 90 min before Western blot analysis. FLAG-tagged SRC-3 and DN-Cul1-5 were detected by FLAG antibodies; β-actin (Actin) was by anti- β-actin antibodies.

(C). Degradation of SRC-3 protein by SPOP is Cul3- and Rbx1-dependent. FLAG-SRC-3 and HA-SPOP were expressed in 293T cells in the presence of control siRNA (siCtrl), siRNA to Cul3 (siCul3) or to Rbx1 (siRbx1). Each protein level was determined by immunoblotting using FLAG to detect FLAG-SRC-3 or the antibodies as indicated.

(D). SPOP/Cul3/Rbx1 control SRC-3 protein stability. siSPOP and DN-Cul3 stabilize SRC-3 protein. The time course of the cycloheximide (CHX) chase experiment was performed in the presence of siRNA to control (Ctrl), SPOP, or Ctrl together with overexpressing DN-Cul3 (siCtrl+DN-Cul3) for indicated times. Protein abundance was determined by immunoblotting using FLAG antibodies to detect SRC-3 and DN-Cul3 or the other indicated antibodies.

(E). Quantitation of the data in Fig 1D.

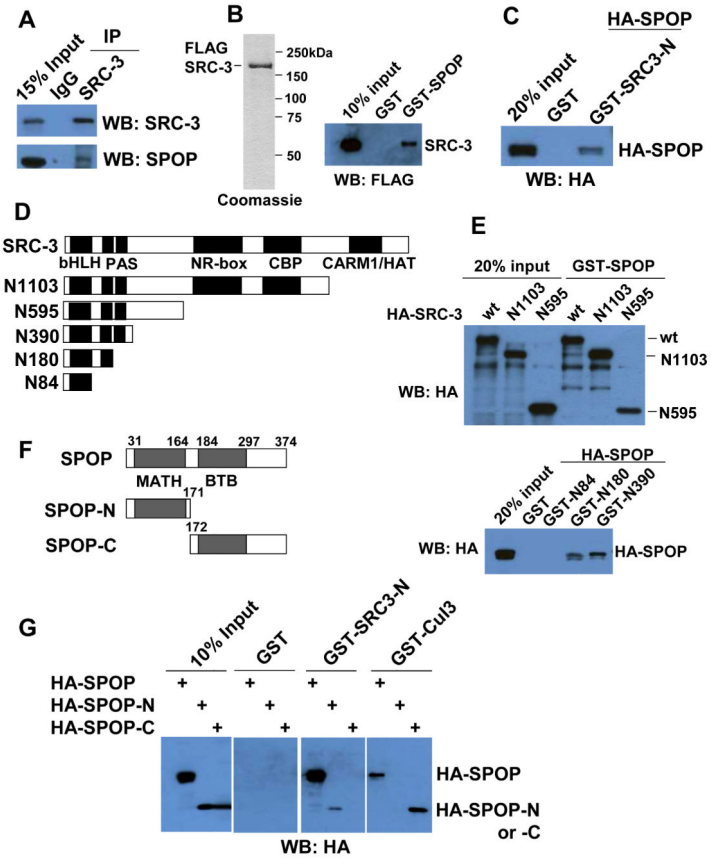

Figure 2. The SRC-3 degron interacts directly with the N-terminal MATH domain of SPOP

(A). SPOP interacts with SRC-3 in MCF7 cells. Co-IP was performed using SRC-3 antibodies for IP followed by SDS-PAGE and Western blotting using SRC-3 and SPOP antibodies as indicated. IP with IgG served as a control.

(B). SRC-3 directly binds to SPOP. GST-pulldown was performed using GST-SPOP and purified recombinant FLAG-SRC-3 protein, which was expressed from baculoviruses (Coomassie staining). The bound protein was resolved by SDS-PAGE and Western blotting using anti-FLAG antibodies. GST alone served as a negative control.

(C). SPOP directly binds to the SRC-3 N-terminal region. GST-pulldown was performed using GST-SRC-3 N-terminus N390 (GST-SRC3-N) and *in vitro* translated HA-SPOP followed by SDS-PAGE and Western blot using HA antibodies.

(D). Schematic representation of SRC-3 deletion mutants was shown.

(E). Mapping the domain of SRC-3 interacted with SPOP. In GST-pulldown experiments, GST-SPOP was assayed with HA-tagged SRC-3 wt, N1103, and N595 (upper panel); GST-SRC-3 N390, N180 and N84 were assayed with HA-SPOP (lower panel). Western blots were performed using HA antibodies.

(F). Schematic representation of SPOP deletion mutants containing the MATH or BTB domain.

(G). The SPOP MATH-containing domain binds to SRC-3 and the SPOP BTB-containing domain binds to Cul3. In GST-pulldown experiments, GST-SRC3-N or GST-Cul3 were assayed with HA-tagged SPOP wt (HA-SPOP), SPOP N-terminal MATH-containing (HA-

SPOP-N) or its C-terminal BTB-containing domain (HA-SPOP-C). Western blot was performed using HA antibodies to detect SPOP wt or its mutants.

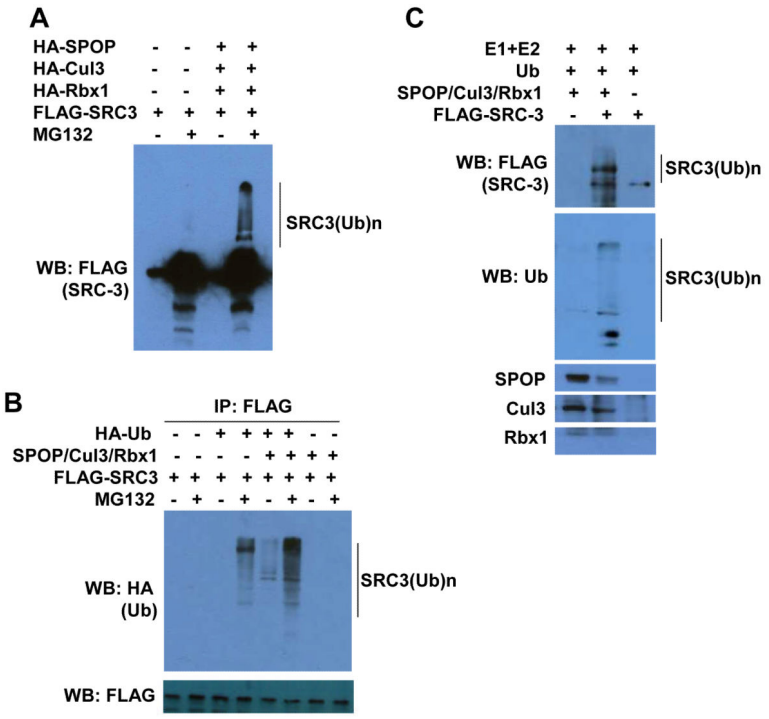

Figure 3. SPOP/Cul3/Rbx1 ubiquitin ligase complex promotes SRC-3 poly-ubiquitination

(A). SPOP/Cul3/Rbx1 promote SRC-3 poly-ubiquitination in cells. FLAG-SRC-3 and HA-tagged SPOP/Cul3/Rbx1 were expressed in 293T cells in the presence or absence of MG132. Poly-ubiquitinated SRC-3 was detected in whole cell lysates by Western blot using FLAG antibodies.

(B). SPOP/Cul3/Rbx1 promote SRC-3 poly-ubiquitination. FLAG-SRC-3 and HA-Ub were co-expressed with SPOP/Cul3/Rbx1 in 293T cells in the presence or absence of MG132. Immunoprecipitation using FLAG antibodies was performed, followed by immunoblotting using HA antibodies to detect poly-ubiquitinated SRC-3. Western blot using FLAG antibodies to detect SRC-3 was shown as a loading control.

(C). SPOP/Cul3/Rbx1 poly-ubiquitinate SRC-3 in a cell-free system. Purified recombinant E1, UbcH5a (E2) and ubiquitin (Ub) were incubated in ubiquitination reactions with SPOP, Cul3, Rbx1 and FLAG-SRC-3 proteins, which were *in vitro* transcribed/translated. Poly-ubiquitinated forms of SRC-3 were detected by Western blotting using either anti-FLAG antibodies or anti-Ub antibodies.

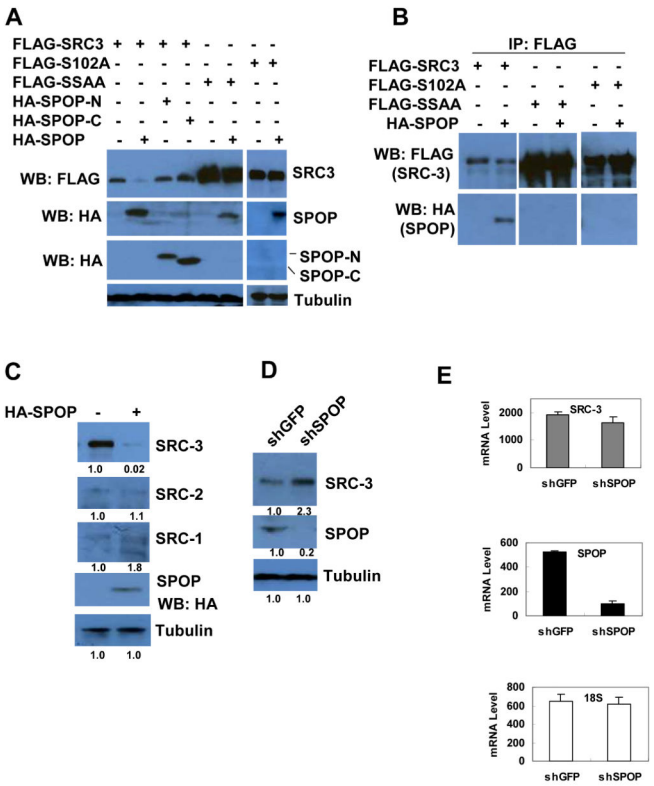

Figure 4. Phosphodegron-Dependent regulation SRC-3 protein stability by SPOP

(A). SPOP promotes phosphodegron-dependent degradation of SRC-3. SRC-3 wt (FLAG-SRC-3) or its phospho-degron mutants S102A (FLAG-S012A) or S101A/S102A (FLAG-SSAA) were expressed in 293T cells with HA-SPOP. The SPOP N-terminal MATH-containing domain (SPOP-N) or C-terminal BTB-containing domain (SPOP-C) also were examined in the experiment. Protein levels were detected by SDS-PAGE and Western blot using the indicated antibodies.

(B). Interaction between SPOP and SRC-3 is phospho-degron dependent. In the experiment shown in (A), Co-IP was performed using FLAG antibodies followed by SDS-PAGE and Western blot using FLAG or HA antibodies to detect SRC-3 or SPOP, respectively.

(C). Overexpression of SPOP down-regulates the SRC-3 protein level in MCF7 breast cancer cells. Lentiviral vectors expressing HA-SPOP were transduced into MCF7 cells. After puromycin selection, these cells were analyzed for SRC-1, -2, -3, SPOP and tubulin protein levels by immunoblot using each antibody indicated.

(D). shRNA knockdown of SPOP leads to up-regulation of the SRC-3 protein level in MCF7 breast cancer cells. Lentiviral vectors expressing shRNA against SPOP were transduced into MCF7 cells. After puromycin selection, these cells were analyzed for SRC-3 and SPOP protein levels by immunoblot using each antibody indicated.

(E). shRNA knockdown of SPOP does not change the SRC-3 mRNA level in MCF7 cells. In the same experiment as in (D), total RNAs were isolated and the mRNA levels were analyzed by real-time RT-PCR using each gene-specific Taqman probe as indicated. Error bars indicate SEM.

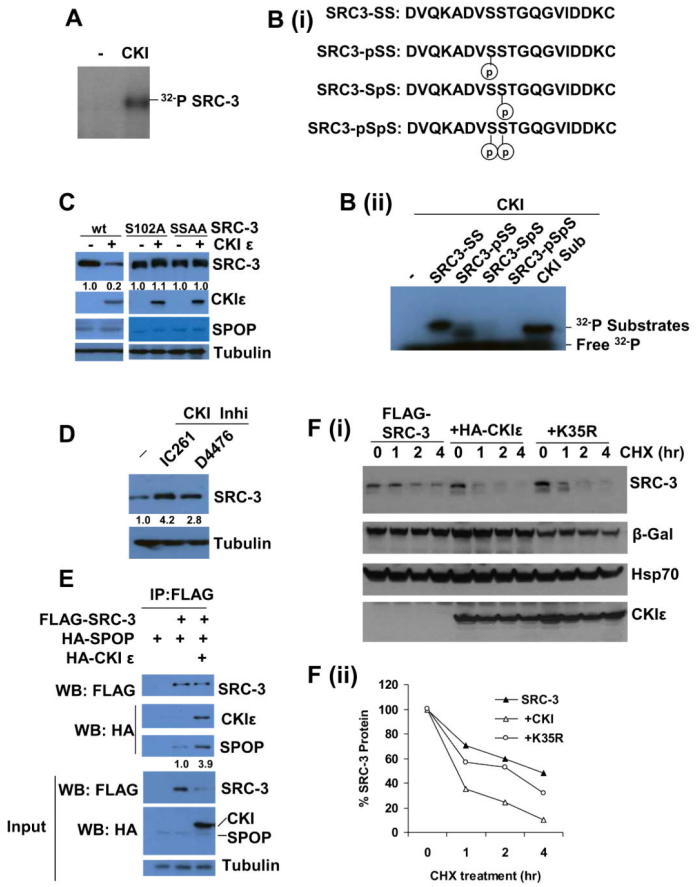

Figure 5. CKIε phosphorylates the S102 site in the SRC-3 degron and enhances SPOP-mediated degradation of SRC-3

(A). CKI phosphorylates SRC-3 protein *in vitro*. CKI protein was incubated with purified recombinant SRC-3 protein in an *in vitro* kinase assay. Phosphorylated SRC-3 was detected by ^{32}P autoradiography.

(B). CKI phosphorylates SRC-3 at Ser 102. Four peptide sequences spanning the SRC-3 degron with phosphates at S101 and S102 in different combinations are shown in (i). *In vitro* kinase assays were performed using these peptides as indicated and CKI's known substrate (CKI Sub) as a positive control. Assayed products were analyzed by SDS-PAGE and autoradiography (ii).

(C). CKIε enhances SPOP-mediated degradation of SRC-3. FLAG-SRC-3 (SRC-3), its mutants S102A (S102A) or S101A/S102A (SSAA), HA-CKIε (CKIε) and HA-SPOP (SPOP) were expressed as indicated in cells followed by immunoblot analysis.

(D). CKI inhibitors stabilize SRC-3 protein. SRC-3-overexpressing cells were treated with CKI inhibitors IC261 or D4476 followed by Western blot analysis using FLAG antibodies to detect SRC-3.

(E). CKIε enhances the binding of SPOP and SRC-3. FLAG-SRC-3, HA-tagged SPOP and CKIε were expressed in 293T cells followed by Co-IP and immunoblot analyses using the indicated antibodies. SRC-3 immunoprecipitates were loaded in the same amount into the gel.

(F). CKIε destabilizes SRC-3 protein. CHX chase experiments were performed with FLAG-SRC-3 alone, or in the presence of CKIε (+HA-CKIε) or its kinase deficient mutant (+K35R). β-gal and Hsp70 were also included as the exogenous and endogenous controls in the experiment. F (ii) panel shows the quantitative analysis of this immunoblot.

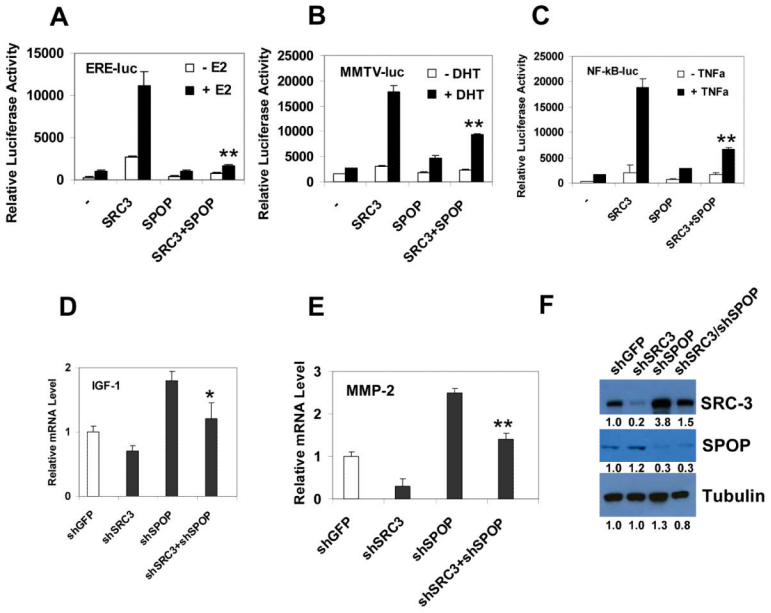

Figure 6. SPOP inhibits SRC-3-mediated oncogenic signaling

(A). SPOP inhibits SRC-3-enhanced estrogen receptor (ER) reporter gene activity. ERE-luc luciferase assays were carried out with ER treated with (+E2) or without (−E2) estradiol in the presence of SRC-3 or SPOP only, or SRC-3 and SPOP together, as indicated. Error bars indicate SEM. Statistically significant differences were indicated (***P*<0.001, SRC-3 versus SRC-3 + SPOP).

(B). SPOP inhibits SRC-3-enhanced androgen receptor (AR) reporter gene activity. Experiments were performed similar to Fig 6A, except using AR, MMTV-luc reporter gene and DHT hormone. Error bars indicate SEM. Statistically significant differences were indicated (***P*<0.001, SRC-3 versus SRC-3 + SPOP).

(C). SPOP inhibits SRC-3-enhanced NF-kB-luc reporter gene activity. Similar to Fig 6A, except using NF-kB-luc and TNFα. Error bars indicate SEM. Statistically significant differences were indicated (***P*<0.001, SRC-3 versus SRC-3 + SPOP).

(D). SPOP inhibits SRC-3 target gene IGF-1. IGF-1 mRNA levels were analyzed in MCF-7 cells with indicated shRNA knockdown of target gene expression. qRT-PCR was performed to measure mRNA levels as indicated. Error bars indicate SEM. Statistically significant differences were indicated (**P*<0.05, shSRC-3 versus shSRC-3 + shSPOP).

(E). SPOP inhibits SRC-3 target gene MMP-2. MMP-2 mRNA levels were analyzed in MCF-7 cells with indicated shRNA knockdown of target gene expression. qRT-PCR was performed to measure mRNA levels as indicated. Error bars indicate SEM. Statistically significant differences were indicated (***P*<0.001, shSRC-3 versus shSRC-3 + shSPOP).

(F). Western blot analysis of SRC-3 and SPOP in MCF-7 cells in panel D and E. Lentiviral shRNAs were used to knockdown SRC-3, SPOP or both as indicated.

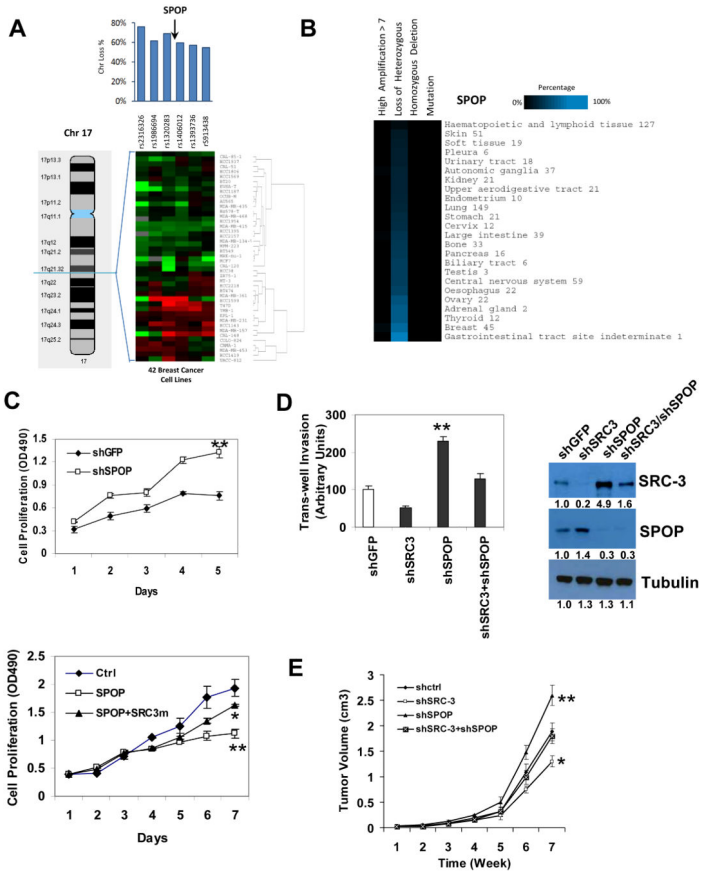

Figure 7. Tumor suppressor role for SPOP

(A). Genomic copy number losses of SPOP loci in breast cancer cell lines. Analysis of the *SPOP* loci by 10K SNP arrays in 42 breast cancer cell lines. Cologrames represent SNP copy numbers. *Red* represents allelic gain; *green* indicates allelic loss. Bar chart shows the percentage of chromosome loss.

(B). LOH of SPOP in human cancers. CONAN (Copy Number Analysis) resulted from Sanger Cancer Genome Project. There are high percentages of distinct cancers containing LOH of SPOP.

(C). Inhibiting cancer cell proliferation by SPOP. Cell proliferation was increased in MCF-7 cells with shSPOP knockdown (upper panel). Cell proliferation was inhibited in MCF-7 cells overexpressing SPOP (SPOP) by lentiviruses and this inhibition was substantially rescued by additional expression of SRC-3 S101A/S102A degradation mutant (SPOP +SRC3m) (lower panel). Error bars indicate SEM. Statistically significant differences were indicated (**P<0.001, control versus SPOP; *P<0.05, SPOP versus SPOP+SRC3m).

(D). SPOP inhibits breast cancer cell invasion. Trans-well invasion assays were performed using MDA-MB-231 breast cancer cells after shRNA knockdown of the indicated gene expression (Left panel). Each indicated protein level was measured by Western blot (Right panel). Error bars indicate SEM. Statistically significant differences were indicated (**P<0.001, shGFP versus shSPOP).

(E). SPOP inhibits tumor growth in nude mice. The nude mice were inoculated with MDA-MB-231 cells after shRNA knockdown of the genes indicated. Tumor volumes were

measured each week. Error bars indicate SD. Statistically significant differences were indicated (**P<0.001, shctrl versus shSPOP; * P<0.05, shctrl versus shSRC-3).