

ORIGINAL ARTICLE

Tumor-suppressor role for the SPOP ubiquitin ligase in signal-dependent proteolysis of the oncogenic co-activator SRC-3/AIB1

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Steroid receptor co-activator-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 that speckle-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 I ϵ phosphorylates the S102 in this degron and promotes SPOP-dependent turnover of SRC-3. Short hairpin RNA 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 loss of heterozygosity 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.

Oncogene (2011) 30, 4350–4364; doi:10.1038/onc.2011.151; published online 16 May 2011

Keywords: SRC-3/AIB1; tumor suppressor; SPOP; ubiquitin ligase; steroid receptor co-activator; breast cancer

Introduction

Control of differential gene expressions in response to developmental and environmental signals is a central theme in cell biology. Nuclear receptors and coregulators have key roles in these processes. Dysregulation of nuclear receptor and coregulator signaling circuitry can

disrupt cell homeostasis and contribute to pathological states, including cancer (McKenna *et al.*, 1999; Glass and Rosenfeld, 2000; Kumar and O'Malley, 2008).

Steroid receptor co-activator-3 (SRC-3/AIB1), also known as ACTR/pCIP/TRAM-1/RAC3, was initially discovered to mediate estrogen receptor signaling and is frequently amplified or overexpressed in breast cancer (Halachmi *et al.*, 1994; Anzick *et al.*, 1997; Chen *et al.*, 1997; Li *et al.*, 1997; Takeshita *et al.*, 1997; Torchia *et al.*, 1997; McKenna *et al.*, 1999; Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002). 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, nuclear factor- κ B, STAT, TEF-4 and ER81 (Lee *et al.*, 1998; Belandia and Parker, 2000; Werbajh *et al.*, 2000; Goel and Janknecht, 2004; Louie *et al.*, 2004). 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-Arzuayus *et al.*, 2004) and a key regulator of diverse signaling systems (Louie *et al.*, 2004; Zhou *et al.*, 2005, 2005; Yan *et al.*, 2006; Wu *et al.*, 2007; Yu *et al.*, 2007). SRC-3 was found to upregulate insulin-like growth factor-1 (IGF-1) and cyclin D1 (Wang *et al.*, 2000; Xu *et al.*, 2000; Planas-Silva *et al.*, 2001; Torres-Arzuayus *et al.*, 2004; Yan *et al.*, 2006). Recent studies demonstrated that SRC-3 enhances matrix metalloproteinase (MMP) expression and has critical roles in cancer cell migration and invasion, a key event during cancer metastasis (Li *et al.*, 2008a, b; Qin *et al.*, 2008; Yan *et al.*, 2008; Kajiro *et al.*, 2009). 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).

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Received 10 January 2011; revised 14 March 2011; accepted 20 March 2011; published online 16 May 2011

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.*, 2007a, b, 2008a; 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 has 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 Skp1, Cull1 and F-box (SCF) complex is an example of the latter. In the SCF ubiquitin ligase complex, Cull1 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 Cull1, the human genome encodes six other cullins (2, 3, 4A, 4B, 5 and 7) (Petroski and Deshaies, 2005; Bennett *et al.*, 2010) that also form ubiquitin ligase complexes. Notably, cullin 3 (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 loss of heterozygosity (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

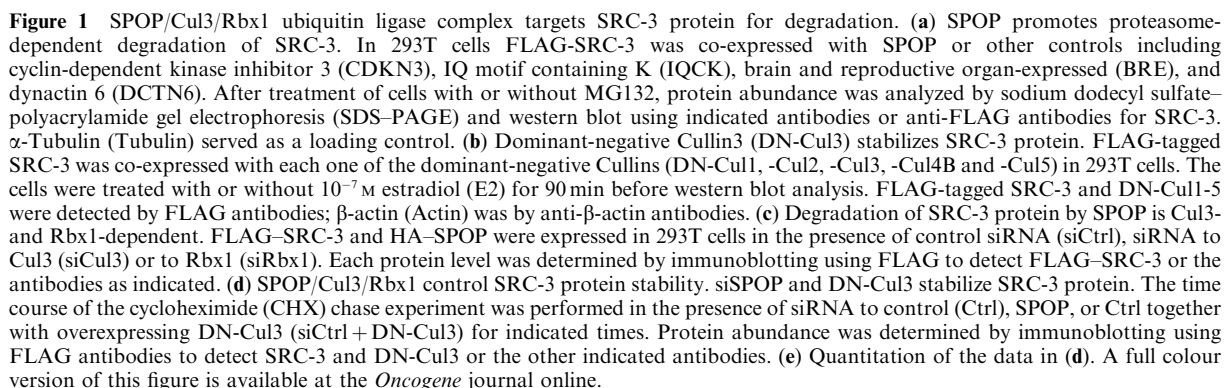
The SPOP/Cul3/Rbx1 ubiquitin ligase complex regulates SRC-3 oncoprotein stability

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 Skp1 and F-box and is part of a Cul3 ubiquitin ligase complex (Furukawa *et al.*, 2003; Geyer *et al.*, 2003; Pintard *et al.*, 2003; Xu *et al.*, 2003; Jin *et al.*, 2004; Petroski and Deshaies, 2005). 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 (Figure 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 1–5 are well-characterized and dominant-negative form mutants (DN-Cul1–5) 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 Figure 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, Fbw7 α 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



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 6 h resulted in a remarkable reduction in the steady-state levels of the SRC-3 protein, whereas knockdown of

The SPOP/Cul3/Rbx1 ubiquitin ligase interacts with the S101/S102 SRC-3 phospho-degron

To validate the physical association between SRC-3 and SPOP, we performed a co-immunoprecipitation experiment, revealing that the SPOP protein binds SRC-3 in

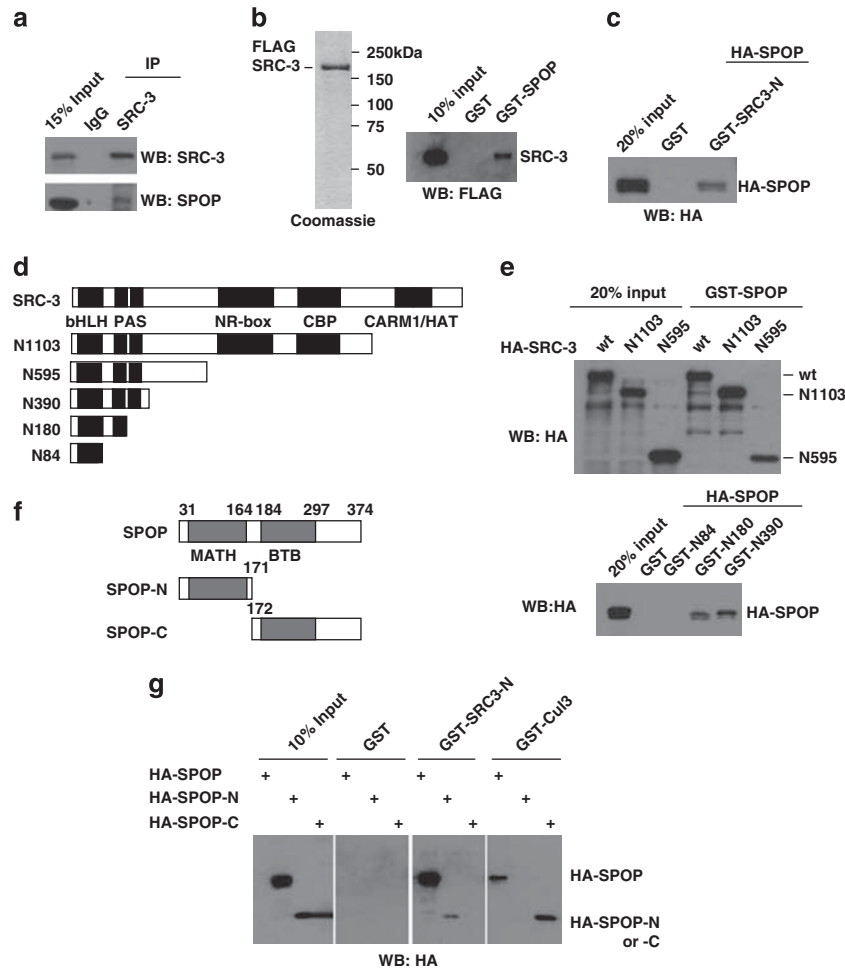


Figure 2 The SRC-3 degenon interacts directly with the N-terminal MATH domain of SPOP. **(a)** SPOP interacts with SRC-3 in MCF-7 cells. Co-immunoprecipitation (Co-IP) was performed using SRC-3 antibodies for IP followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting using SRC-3 and SPOP antibodies as indicated. IP with immunoglobulin G (IgG) served as a control. **(b)** SRC-3 directly binds to SPOP. GST–pull-down 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–pull-down 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. **(e)** Mapping the domain of SRC-3 interacted with SPOP. In GST–pull-down 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–pull-down 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. A full colour version of this figure is available at the *Oncogene* journal online.

Michigan Cancer Foundation-7 (MCF-7) breast cancer cells (Figure 2a). We then tested whether SRC-3 directly interacts with SPOP. Glutathione *S*-transferase (GST)–pull-down experiments were performed using purified recombinant FLAG–SRC-3 protein, indicating that SRC-3 indeed binds to GST–SPOP (Figure 2b). Similarly, HA–SRC-3 was observed to bind to GST–SPOP *in vitro* (Supplementary Figure S1A). In a reciprocal experiment, we found that HA–SPOP binds to the GST–SRC-3 N-terminus (N390) (Figure 2c). To further determine which subdomain of SRC-3 interacts with SPOP, a series of SRC-3 deletion mutants were constructed for GST–pull-down experiments. As 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 phospho-degron region (containing S101 and S102) is required for the interaction between SRC-3 and SPOP (Figures 2d and e).

SPOP contains one MATH (meprin and TRAF-homology) and one BTB domain as essential parts of the SPOP/Cul3/Rbx1 ubiquitin ligase complex (Hernandez-Munoz *et al.*, 2005; Petroski and Deshaies, 2005; Kwon

et al., 2006; Bunce *et al.*, 2008). We used GST-pulldown assays to examine how these three proteins associate with each other. As shown in Figures 2f and g, 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 (Figure 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 (Figure 3b).

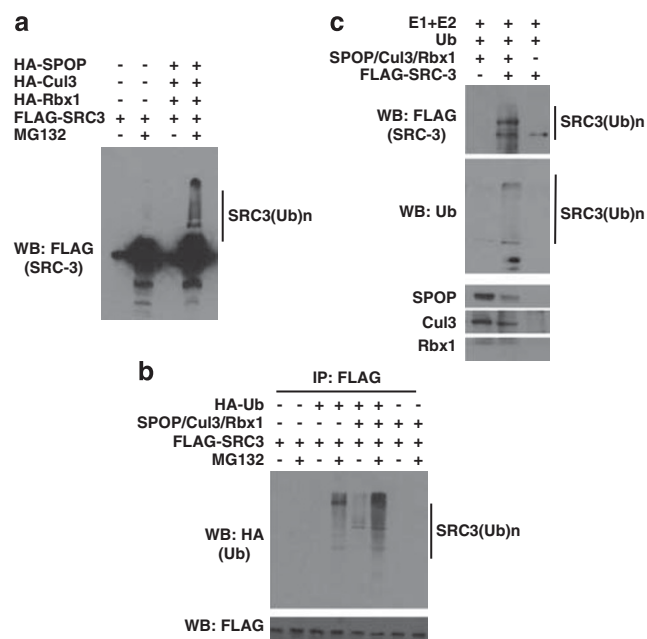


Figure 3 SPOP/Cul3/Rbx1 ubiquitin ligase complex promotes SRC-3 poly-ubiquitination. **(a)** SPOP/Cul3/Rbx1 promote SRC-3 poly-ubiquitination in cells. FLAG-SRC-3 and HA-tagged SPOP/Cul3/Rbx1 were expressed in 293T cells in the presence or absence of MG132. Poly-ubiquitinated SRC-3 was detected in whole 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. A full colour version of this figure is available at the *Oncogene* journal online.

We performed cell-free ubiquitination assays to further study SRC-3 polyubiquitination by SPOP/Cul3/Rbx1. As shown in Figure 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 reticulocyte systems. Only in the presence of all ingredients (SPOP/Cul3/Rbx1, E1, E2 and ubiquitin), was the formation of polyubiquitinated SRC-3 detected (Figure 3c). These *in vitro* results showed that SPOP/Cul3/Rbx1 directly promotes SRC-3 polyubiquitination formation.

SPOP promotes phosphorylation-dependent degradation of SRC-3

As 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 Figure 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 (Figure 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 (Figure 4b). This result was confirmed *in vitro* by GST-pulldown experiments (Supplementary Figure 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 (Figure 4c). To study the loss-of-function effect, we used short hairpin RNA (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 (Figure 4d, Supplementary Figure S1C); whereas the mRNA levels of SRC-3 in these experiments were not changed (Figure 4e, Supplementary Figure 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

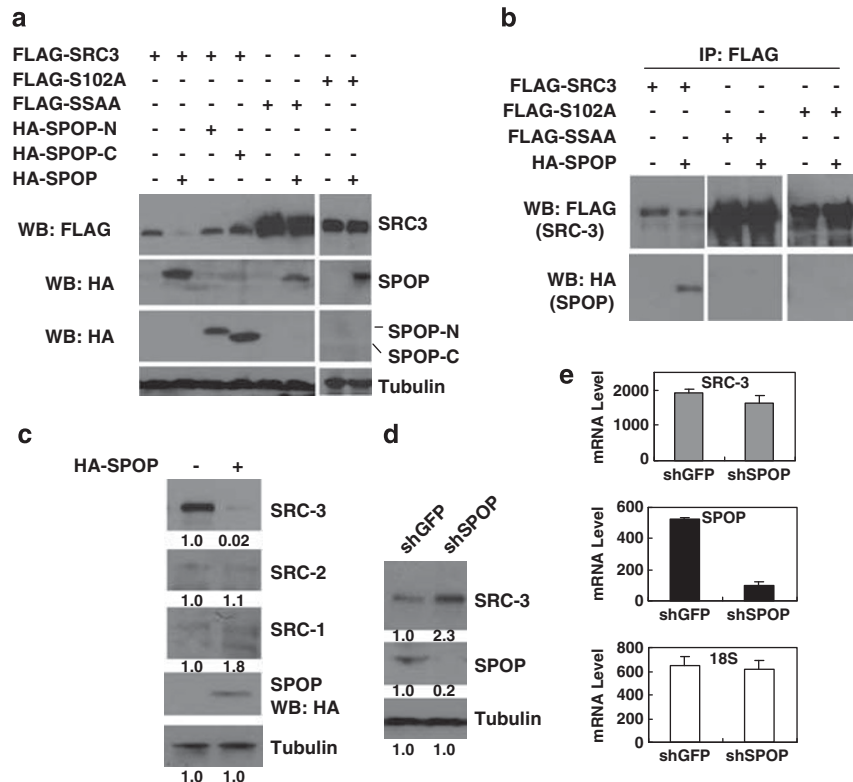


Figure 4 Phospho-degron-dependent regulation SRC-3 protein stability by SPOP. (a) SPOP promotes phospho-degron-dependent degradation of SRC-3. SRC-3 wt (FLAG-SRC-3) or its phospho-degron mutants S102A (FLAG-S102A) or S101A/S102A (FLAG-SSAA) were expressed in 293T cells with HA-SPOP. The SPOP N-terminal MATH-containing domain (SPOP-N) or C-terminal BTB-containing domain (SPOP-C) also were examined in the experiment. Protein levels were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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-immunoprecipitation (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 downregulates the SRC-3 protein level in MCF-7 breast cancer cells. Lentiviral vectors expressing HA-SPOP were transduced into MCF-7 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 upregulation of the SRC-3 protein level in MCF-7 breast cancer cells. Lentiviral vectors expressing shRNA against SPOP were transduced into MCF-7 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 MCF-7 cells. In the same experiment as in (d), total RNAs were isolated and the mRNA levels were analyzed by real-time reverse transcriptase (RT)-PCR using each gene-specific Taqman probe as indicated. Error bars indicate s.e.m. A full colour version of this figure is available at the *Oncogene* journal online.

unknown (Li *et al.*, 2008a). Using a bioinformatic approach (Sakanaka, 2002; Luo *et al.*, 2007), the candidate kinase, casein kinase I ϵ (CKI ϵ), was identified as a potential kinase. To test whether CKI ϵ phosphorylates SRC-3 at the S101 or S102 sites, we performed *in vitro* kinase assays. First, by using purified recombinant SRC-3 (Figure 2b) and CKI proteins, we found that CKI is able to phosphorylate SRC-3 (Figure 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 (Figure 5b), indicating that SRC-3 S102 is the primary phosphorylation site targeted by CKI ϵ . 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* (Supplementary Figure S1E).

As the S101/S102 SRC-3 degron phosphorylation is crucial for its protein stability, we examined whether the CKI ϵ enzyme itself destabilizes the SRC-3 protein. Overexpression of CKI ϵ 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 (Figure 5c). CKI inhibitors IC261 and D4476 increased SRC-3 protein levels (Figure 5d), supporting that CKI ϵ indeed destabilizes the SRC-3 protein. As both SPOP and CKI ϵ 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 CKI ϵ on the interaction between SPOP and SRC-3. After FLAG-SRC-3, HA-SPOP and HA-CKI ϵ were expressed in 293T cells, co-immunoprecipitation experiments were performed which showed that binding of

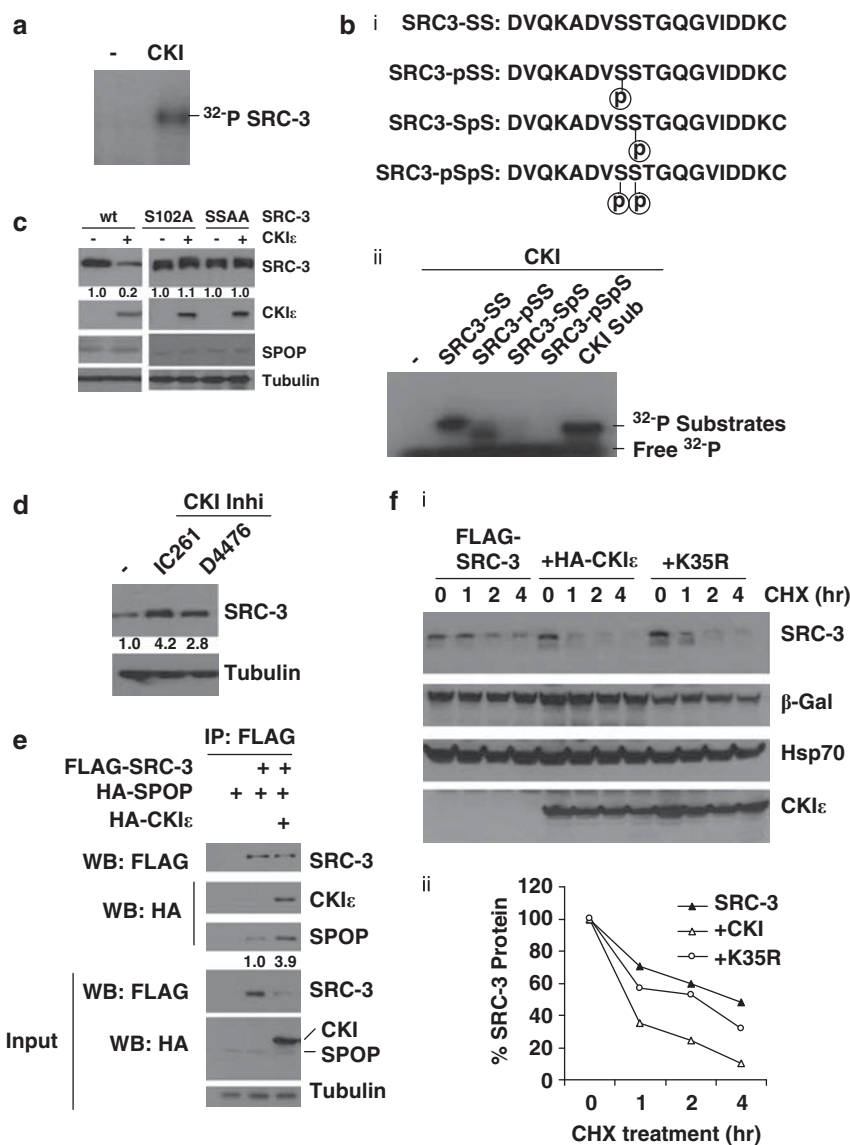


Figure 5 CKIε phosphorylates the S102 site in the SRC-3 degron and enhances SPOP-mediated degradation of SRC-3. (a) CKI phosphorylates SRC-3 protein *in vitro*. CKI protein was incubated with purified recombinant SRC-3 protein in an *in vitro* kinase assay. Phosphorylated SRC-3 was detected by ³²P autoradiography. (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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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 (SRC-3), HA-tagged SPOP and CKIε were expressed in 293T cells followed by co-immunoprecipitation (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. (f) (i) Cycloheximide (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. A full colour version of this figure is available at the *Oncogene* journal online.

SPOP to SRC-3 was increased when CKIε was present (Figure 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, cycloheximide 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 (Figure 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;

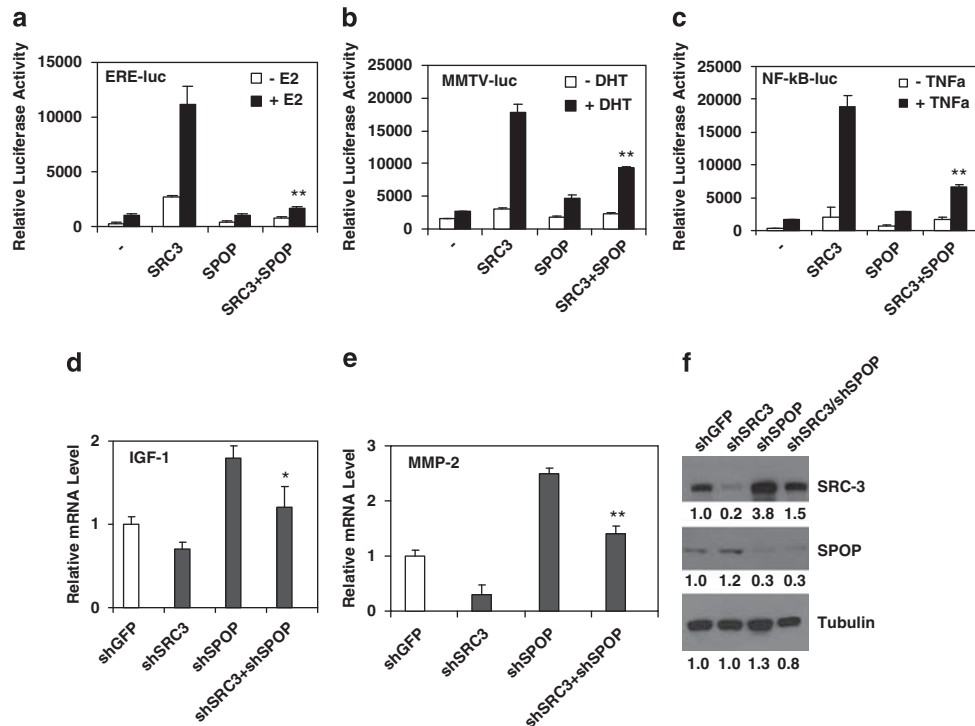


Figure 6 SPOP inhibits SRC-3-mediated oncogenic signaling. (a) SPOP inhibits SRC-3-enhanced estrogen receptor (ER) reporter gene activity. ERE-luc luciferase assays were carried out with ER treated with (+ E2) or without (–E2) estradiol in the presence of SRC-3 or SPOP only, or SRC-3 and SPOP together, as indicated. Error bars indicate s.e.m. 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 panel a, except using AR, MMTV-luc reporter gene and DHT hormone. Error bars indicate s.e.m. Statistically significant differences were indicated (** $P < 0.001$, SRC-3 versus SRC-3 + SPOP). (c) SPOP inhibits SRC-3-enhanced nuclear factor (NF)-κB-luc reporter gene activity. Similar to panel a, except using NF-κB-luc and tumor necrosis factor (TNF)α. Error bars indicate s.e.m. 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. Quantitative reverse transcriptase (qRT)-PCR was performed to measure mRNA levels as indicated. Error bars indicate s.e.m. 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 s.e.m. 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. A full colour version of this figure is available at the *Oncogene* journal online.

O'Malley and Kumar, 2009). As SPOP is able to reduce SRC-3 protein levels, we examined the potential role for SPOP in suppressing SRC-3-mediated signaling. As SRC-3 is a co-activator for estrogen receptor and androgen receptor, we used estrogen response element (ERE)-luciferase and mouse mammary tumor virus (MMTV)-luciferase as reporters for estrogen receptor and androgen receptor signaling, respectively. Overexpression of SRC-3-enhanced estrogen- and androgen-dependent activation of each reporter gene and these gene activations were markedly reduced on overexpression of SPOP; SPOP alone had little effect on reporter gene activities (Figures 6a and b). Similarly, SRC-3-enhanced tumor necrosis factor-α-stimulated nuclear factor-κB-luciferase activity was dramatically decreased by SPOP overexpression (Figure 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 (Wang *et al.*, 2000; Xu *et al.*, 2000; Qin *et al.*, 2008; 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 (Figures 6d and e). With shRNA knockdown of both SRC-3 and SPOP, IGF-1 and MMP-2 mRNA levels were similar to the control small hairpin RNA green fluorescence protein (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 (Figures 6d–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 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 (Figure 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 (Figure 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 MCF-7 cell growth, while ectopic overexpression of SPOP resulted in a slower proliferation rate compared with the control cells; this inhibitory effect was substantially rescued by additional expression of the SRC-3 S101A/S102A mutant, suggesting that inhibition of MCF-7 cell growth by SPOP is largely due to a reduction in SRC-3 levels (Figure 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, small hairpin RNA SPOP (shSPOP)-treated MDA231 cells revealed a marked increase in invasiveness; this effect was compromised when both SRC-3 and SPOP were knocked down (Figure 7d). In addition, we also observed that shSPOP increased MCF-7 cell colony formation in soft agar assays (Supplementary Figure S2) and increased cell size (Supplementary Figure 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 with 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 (Figure 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 particu-

larly, LOH of the chromosome 17q21 region have been described in up to 30% of primary human breast cancers (Orsetti *et al.*, 1999; De Marchis *et al.*, 2004). It also has been reported that LOH in the 17q21 region is frequently associated with inflammatory breast cancer (Lerebours *et al.*, 2002). In pre-invasive breast ductal carcinoma *in situ*, 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 single-nucleotide polymorphism microarray genotype data in a cancer genome data set (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 (Figure 7a). We also found that LOH at the SPOP locus is remarkably high in multiple cancers, particularly in breast cancers (Figure 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 (Figures 7c–e, Supplementary Figure S2). We also found that shRNA knockdown of SPOP in MDA-MB-231 cells increases cancer cell size (Supplementary Figure S3), consistent with previous publications (Zhou *et al.*, 2003; Torres-Arzuayus *et al.*, 2004). 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 PIPKII β (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.*, 2010). 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 MCF-7 cell growth; additional overexpression of degron-mutated SRC-3 rescued significantly cell growth (Figure 7c). We reason that restoration of SRC-3 did not fully rescue the cell growth rate because of the existence of other growth-promoting targets of SPOP. In a specific cancer cell, SPOP may target one or more of its oncogenic

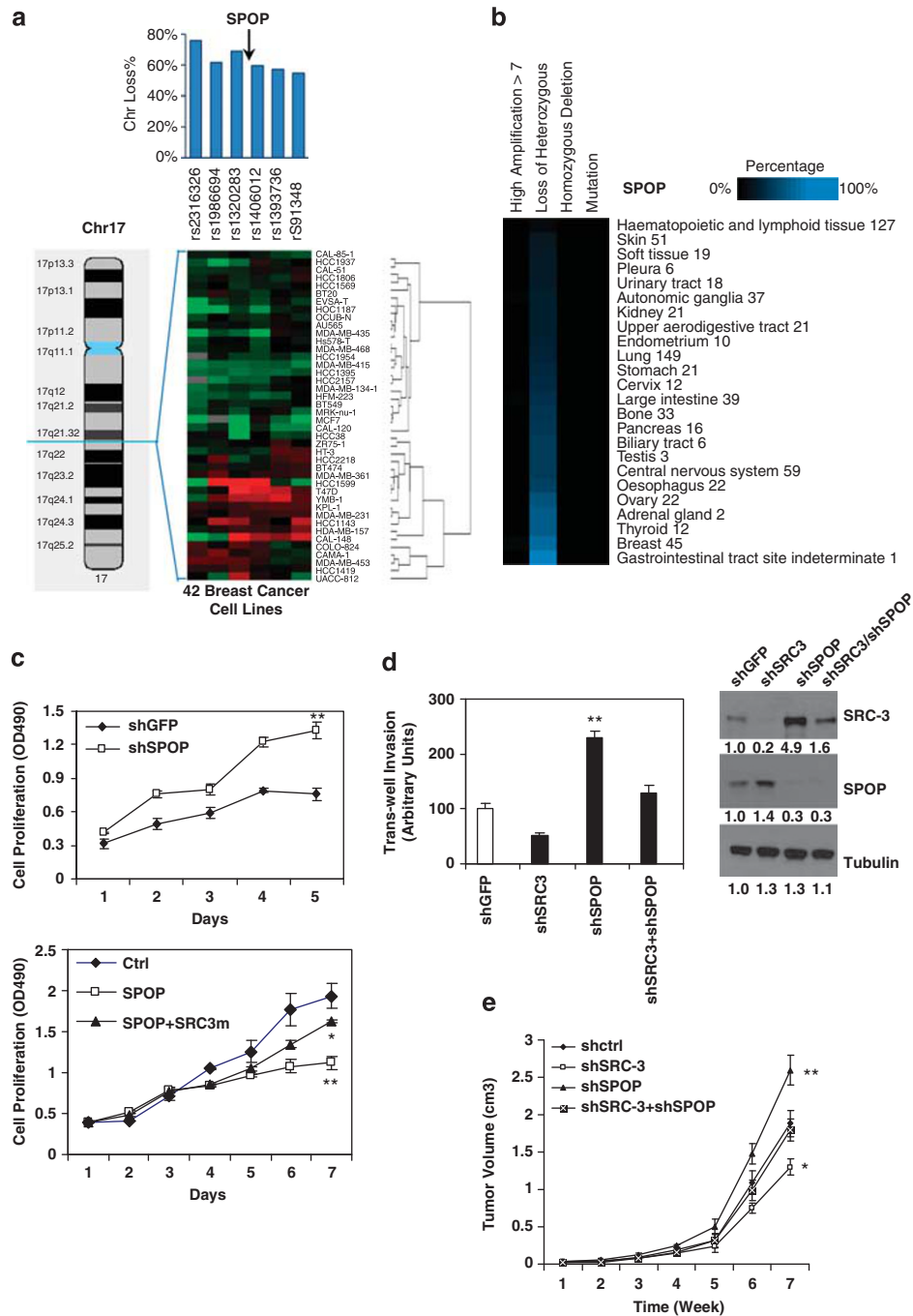


Figure 7 Tumor-suppressor role for SPOP. **(a)** Genomic copy number losses of SPOP loci in breast cancer cell lines. Analysis of the SPOP loci by 10K single-nucleotide polymorphism (SNP) arrays in 42 breast cancer cell lines. Colograms represent SNP copy numbers. Red represents allelic gain; green indicates 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 s.e.m. 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 s.e.m. 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 s.d. Statistically significant differences were indicated (** $P < 0.001$, shctrl versus shSPOP; * $P < 0.05$, shctrl versus shSRC-3).

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 (Figures 4 and 5). We further demonstrated that casein kinase CKI ϵ is an enzyme that phosphorylates the S102 site, enhances the binding between SPOP and SRC-3, and destabilizes the SRC-3 protein (Figure 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 CKI ϵ -dependent phosphorylation (Eide *et al.*, 2005; Shirogane *et al.*, 2005). In this 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 CKI ϵ and protein phosphatase 1, respectively (Li *et al.*, 2008a). Consequently, the balance of CKI ϵ and protein phosphatase 1 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) SPOP-binding consensus 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 with SPOP-binding consensus. Importantly, we identified that this motif is phosphorylated in SRC-3.

A three-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, before 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 regulatory subunit gamma (REG γ)-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 (Supplementary Figure S4). Applications that harness the extraordinary power and specificity of these regulatory enzymes and pathways could hold future promise for therapeutic interventions.

Materials and methods

Yeast two-hybrid screen

Yeast two-hybrid screening was performed by using Matchmaker Gal4 Two-Hybrid System 3 and a Matchmaker Pretransformed Normalized HeLa Complementary DNA (cDNA) Library (Clontech Laboratories, Mountain View, CA, USA). 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 *Bam*HI/*Not*I and *Xho*I/*Not*I 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 isopropyl beta-D-1-thiogalactopyranoside in BL21 bacteria for 2 h and purified by glutathione sepharose 4B (Amersham, Piscataway, NJ, USA). The GST-protein-containing beads were further incubated with *in vitro* transcribed/translated HA-tagged proteins, which were generated by the *in vitro* transcription/translation Coupled Reticulocyte Lysate System (Promega, Madison, WI, USA). 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 h with rotation followed by washes using the lysis buffer. Finally, the bound proteins were resolved in sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by western blot.

Co-immunoprecipitation 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, USA) (Li *et al.*, 2008a). Cell lysates were immunoprecipitated with EZview red anti-FLAG M2 affinity gel for 2 h at 4 °C with constant rotation and then the beads were washed four times with the above buffer. Finally, the beads were directly boiled in Laemmli sample buffer before separation by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. 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, Santa Cruz, CA, USA) and protein G-plus agarose. Western blot was performed by first blocking nitrocellulose membranes with 5% nonfat milk in phosphate-buffered saline-T buffer before adding antibodies as indicated. Image J software (NIH, Bethesda, MD, USA) 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–horseradish peroxidase (Sigma, St Louis, MO, USA), anti- β -actin–horseradish peroxidase (Sigma), anti- β -Gal (Roche, Basel, Switzerland), anti-Hsp70 (BD Biosciences, Franklin Lakes, NJ, USA), anti- α -tubulin (Millipore, Billerica, MA, USA), anti-SRC-3/AIB1 (BD Biosciences) anti-Ub (Santa Cruz), anti-SRC-1 and anti-SRC-2 (BD Biosciences). Rabbit polyclonal were anti-Cul3 (Bethyl, Montgomery, TX, USA), and anti-Rbx1 (Millipore), while anti-HA–horseradish peroxidase (Roche) was rat monoclonal.

Cycloheximide protein stability assays

Protein decay was studied by cycloheximide treatment experiments as described previously (Li *et al.*, 2008a). 293T cells were transfected with either siRNA control or small interfering RNA SPOP (siSPOP), and 1 day later FLAG–SRC-3 or together with FLAG–DN–Cul3 were transfected for an additional 2 days. Afterward, 0.5 mM cycloheximide (Sigma) was added for different times as indicated, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western analysis. FLAG–SRC-3 expressed alone or together with HA–CKI ϵ was also transfected into 293T cells and analyzed in these cycloheximide-based experiments.

siRNA and shRNA knockdown

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

Lentiviral overexpression

Lentiviral Expression Systems were purchased from System Biosciences (SBI, Mountain View, CA, USA). 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 2 days before each virus-containing medium was collected. The resultant lentiviruses were transduced into breast cancer cells in the presence of polybrene (5 μ g/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 *in vitro* transcription/translation Coupled Reticulocyte Lysate Systems in the presence of E1, E2 (UbcH5a), Ubiquitin and ATP (Boston Biochem, Boston, MA, USA). The biochemical reactions were conducted at 37 °C for 1.5 h followed by SDS–polyacrylamide gel electrophoresis 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, USA) using PCR primers containing an HA tag at the N-terminal. PCR amplification was performed by the plaque-performing unit (Pfu) polymerase (Stratagene, Santa Clara, CA, USA). 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, USA). FLAG–DN–Cul3 was purchased from Addgene (Cambridge, MA, USA) deposited by Wade Harper (Jin *et al.*, 2005). HA–CKI ϵ 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, USA). Kinase Assays were performed using CKI, a positive control peptide for the CKI known substrate and buffer systems (New England Biolabs, Ipswich, MA, USA). 32 P–ATP was purchased from MP-Biomedicals (Solon, OH, USA). Full-length SRC-3 protein was expressed by baculoviruses and purified from Sf9 cells as described previously (Li *et al.*, 2008a).

Real-time reverse transcriptase–PCR

The basic procedure was described previously (Li *et al.*, 2008a). Total RNA was isolated using TRIzol (Invitrogen). TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA, USA) 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, Billerica, MA, USA). A total of 10 000 cells were suspended in complete

medium containing 0.4% top agar layered on 0.8% solidified base agar in each of a six-well plate. Feed cells 1–2 times per week with cell culture media. After 3–4 weeks, colonies with a diameter of > 100 μ m 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 the manufacturer's protocols. In all, 20 000 cells were placed in each insert chamber containing 0.1% bovine serum albumin medium while in the lower chamber containing 10% fetal bovine serum. Cells were allowed to migrate and invade through the matrigel membrane for 1 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 single-nucleotide polymorphism microarray genotype data for 42 breast cancer cell lines from the Cancer Genome Project of the Wellcome Trust Sanger Institute. We analyzed the single-nucleotide polymorphism 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, Stanford University, CA, USA), and presented the results using heat maps. CONAN (Copy Number Analysis) 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 fourth mammary glands on both sides of 5–6 weeks old female athymic Ncr-Nu/Nu mice (NCI) after being anesthetized with 2.5% Avertin (0.1 ml/10 g, intraperitoneal). For each site, 5×10^6 cells in 100 μ l Dulbecco's modified Eagle's medium were injected. Tumor length (*L*) and width (*W*) were measured once a week and tumor volume was calculated by $(\pi L \times W^2)/6$.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Ming-Jer Tsai and Sophia Y Tsai for their suggestions and Dr Kevin White (University of Chicago) for the monoclonal antibodies against SPOP. This work was supported by funding from State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute and grants (81072163) from National Natural Science Foundation of China (CL), and NIH HD-08188 and HD-07857 grants (BWO).

References

- Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY *et al.* (1997). AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* **277**: 965–968.
- Belandia B, Parker MG. (2000). Functional interaction between the p160 coactivator proteins and the transcriptional enhancer factor family of transcription factors. *J Biol Chem* **275**: 30801–30805.
- Bennett EJ, Rush J, Gygi SP, Harper JW. (2010). Dynamics of cullin-RING ubiquitin ligase network revealed by systematic quantitative proteomics. *Cell* **143**: 951–965.
- Bunce MW, Boronenkov IV, Anderson RA. (2008). Coordinated activation of the nuclear ubiquitin ligase Cul3-SPOP by the generation of phosphatidylinositol 5-phosphate. *J Biol Chem* **283**: 8678–8686.
- Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L *et al.* (1997). Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell* **90**: 569–580.
- Chen MH, Wilson CW, Li YJ, Law KK, Lu CS, Gacayan R *et al.* (2009). Cilium-independent regulation of Gli protein function by Sufu in Hedgehog signaling is evolutionarily conserved. *Genes Dev* **23**: 1910–1928.
- Claiborn KC, Sachdeva MM, Cannon CE, Groff DN, Singer JD, Stoffers DA. (2010). Pcf1l modulates Pdx1 protein stability and pancreatic beta cell function and survival in mice. *J Clin Invest* **120**: 3713–3721.
- De Marchis L, Cropp C, Sheng ZM, Bargo S, Callahan R. (2004). Candidate target genes for loss of heterozygosity on human chromosome 17q21. *Br J Cancer* **90**: 2384–2389.
- Durocher F, Tonin P, Shattuck-Eidens D, Skolnick M, Narod SA, Simard J. (1996). Mutation analysis of the BRCA1 gene in 23 families with cases of cancer of the breast, ovary, and multiple other sites. *J Med Genet* **33**: 814–819.
- Eide EJ, Woolf MF, Kang H, Woolf P, Hurst W, Camacho F *et al.* (2005). Control of mammalian circadian rhythm by CKIepsilon-regulated proteasome-mediated PER2 degradation. *Mol Cell Biol* **25**: 2795–2807.
- Furukawa M, He YJ, Borchers C, Xiong Y. (2003). Targeting of protein ubiquitination by BTB-Cullin 3-Roc1 ubiquitin ligases. *Nat Cell Biol* **5**: 1001–1007.
- Geyer R, Wee S, Anderson S, Yates J, Wolf DA. (2003). BTB/POZ domain proteins are putative substrate adaptors for cullin 3 ubiquitin ligases. *Mol Cell* **12**: 783–790.
- Glass CK, Rosenfeld MG. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev* **14**: 121–141.
- Goel A, Janknecht R. (2004). Concerted activation of ETS protein ER81 by p160 coactivators, the acetyltransferase p300 and the receptor tyrosine kinase HER2/Neu. *J Biol Chem* **279**: 14909–14916.
- Halachmi S, Marden E, Martin G, MacKay H, Abbondanza C, Brown M. (1994). Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. *Science* **264**: 1455–1458.
- Hernandez-Munoz I, Lund AH, van der Stoep P, Boutsma E, Muijers I, Verhoeven E *et al.* (2005). Stable X chromosome inactivation involves the PRC1 Polycomb complex and requires histone MACROH2A1 and the CULLIN3/SPOP ubiquitin E3 ligase. *Proc Natl Acad Sci USA* **102**: 7635–7640.
- Hershko A, Ciechanover A. (1998). The ubiquitin system. *Annu Rev Biochem* **67**: 425–479.
- Hong H, Kohli K, Trivedi A, Johnson DL, Stallcup MR. (1996). GRIP1, a novel mouse protein that serves as a transcriptional

- coactivator in yeast for the hormone binding domains of steroid receptors. *Proc Natl Acad Sci USA* **93**: 4948–4952.
- Jin J, Ang XL, Shirogane T, Wade Harper J. (2005). Identification of substrates for F-box proteins. *Methods Enzymol* **399**: 287–309.
- Jin J, Cardozo T, Lovering RC, Elledge SJ, Pagano M, Harper JW. (2004). Systematic analysis and nomenclature of mammalian F-box proteins. *Genes Dev* **18**: 2573–2580.
- Kajiro M, Hirota R, Nakajima Y, Kawanowa K, So-ma K, Ito I *et al*. (2009). The ubiquitin ligase CHIP acts as an upstream regulator of oncogenic pathways. *Nat Cell Biol* **11**: 312–319.
- Kuang SQ, Liao L, Zhang H, Lee AV, O'Malley BW, Xu J. (2004). AIB1/SRC-3 deficiency affects insulin-like growth factor I signaling pathway and suppresses v-Ha-ras-induced breast cancer initiation and progression in mice. *Cancer Res* **64**: 1875–1885.
- Kumar RPD, O'Malley BW. (2008). *NR Coregulators and Human Diseases*. World Scientific: Singapore; London, xi, 602 p.
- Kwon JE, La M, Oh KH, Oh YM, Kim GR, Seol JH *et al*. (2006). BTB domain-containing speckle-type POZ protein (SPOP) serves as an adaptor of Daxx for ubiquitination by Cul3-based ubiquitin ligase. *J Biol Chem* **281**: 12664–12672.
- Lee DF, Kuo HP, Liu M, Chou CK, Xia W, Du Y *et al*. (2009). KEAP1 E3 Ligase-mediated downregulation of NF-kappaB signaling by targeting IKKbeta. *Mol Cell* **36**: 131–140.
- Lee SK, Kim HJ, Na SY, Kim TS, Choi HS, Im SY *et al*. (1998). Steroid receptor coactivator-1 coactivates activating protein-1-mediated transactivations through interaction with the c-Jun and c-Fos subunits. *J Biol Chem* **273**: 16651–16654.
- Lerebours F, Bertheau P, Bieche I, Driouch K, De The H, Hacene K *et al*. (2002). Evidence of chromosome regions and gene involvement in inflammatory breast cancer. *Int J Cancer* **102**: 618–622.
- Li C, Liang YY, Feng XH, Tsai SY, Tsai MJ, O'Malley BW. (2008a). Essential phosphatases and a phospho-degron are critical for regulation of SRC-3/AIB1 coactivator function and turnover. *Mol Cell* **31**: 835–849.
- Li C, Wu RC, Amazit L, Tsai SY, Tsai MJ, O'Malley BW. (2007a). Specific amino acid residues in the basic helix-loop-helix domain of SRC-3 are essential for its nuclear localization and proteasome-dependent turnover. *Mol Cell Biol* **27**: 1296–1308.
- Li H, Gomes PJ, Chen JD. (1997). RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2. *Proc Natl Acad Sci USA* **94**: 8479–8484.
- Li LB, Louie MC, Chen HW, Zou JX. (2008b). Proto-oncogene ACTR/AIB1 promotes cancer cell invasion by up-regulating specific matrix metalloproteinase expression. *Cancer Lett* **261**: 64–73.
- Li X, Amazit L, Long W, Lonard DM, Monaco JJ, O'Malley BW. (2007b). Ubiquitin- and ATP-independent proteolytic turnover of p21 by the REGgamma-proteasome pathway. *Mol Cell* **26**: 831–842.
- Li X, Lonard DM, Jung SY, Malovannaya A, Feng Q, Qin J *et al*. (2006). The SRC-3/AIB1 coactivator is degraded in a ubiquitin- and ATP-independent manner by the REGgamma proteasome. *Cell* **124**: 381–392.
- Liu J, Ghanim M, Xue L, Brown CD, Iossifov I, Angeletti C *et al*. (2009). Analysis of Drosophila segmentation network identifies a JNK pathway factor overexpressed in kidney cancer. *Science* **323**: 1218–1222.
- Louie MC, Zou JX, Rabinovich A, Chen HW. (2004). ACTR/AIB1 functions as an E2F1 coactivator to promote breast cancer cell proliferation and antiestrogen resistance. *Mol Cell Biol* **24**: 5157–5171.
- Luo W, Peterson A, Garcia BA, Coombs G, Kofahl B, Heinrich R *et al*. (2007). Protein phosphatase 1 regulates assembly and function of the beta-catenin degradation complex. *EMBO J* **26**: 1511–1521.
- McKenna NJ, Lanz RB, O'Malley BW. (1999). Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev* **20**: 321–344.
- McKenna NJ, O'Malley BW. (2002). Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* **108**: 465–474.
- Michaelson JS, Bader D, Kuo F, Kozak C, Leder P. (1999). Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development. *Genes Dev* **13**: 1918–1923.
- O'Malley BW, Kumar R. (2009). Nuclear receptor coregulators in cancer biology. *Cancer Res* **69**: 8217–8222.
- Onate SA, Tsai SY, Tsai MJ, O'Malley BW. (1995). Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270**: 1354–1357.
- Orsetti B, Courjal F, Cuny M, Rodriguez C, Theillet C. (1999). 17q21–q25 aberrations in breast cancer: combined allelotyping and CGH analysis reveals 5 regions of allelic imbalance among which two correspond to DNA amplification. *Oncogene* **18**: 6262–6270.
- Osborne CK, Bardou V, Hopp TA, Chamness GC, Hilsenbeck SG, Fuqua SA *et al*. (2003). Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst* **95**: 353–361.
- Petroski MD, Deshaies RJ. (2005). Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol* **6**: 9–20.
- Pickart CM. (2001). Mechanisms underlying ubiquitination. *Annu Rev Biochem* **70**: 503–533.
- Pintard L, Willis JH, Willems A, Johnson JL, Srayko M, Kurz T *et al*. (2003). The BTB protein MEL-26 is a substrate-specific adaptor of the CUL-3 ubiquitin-ligase. *Nature* **425**: 311–316.
- Planas-Silva MD, Shang Y, Donaher JL, Brown M, Weinberg RA. (2001). AIB1 enhances estrogen-dependent induction of cyclin D1 expression. *Cancer Res* **61**: 3858–3862.
- Qin L, Liao L, Redmond A, Young L, Yuan Y, Chen H *et al*. (2008). The AIB1 oncogene promotes breast cancer metastasis by activation of PEA3-mediated matrix metalloproteinase 2 (MMP2) and MMP9 expression. *Mol Cell Biol* **28**: 5937–5950.
- Radford DM, Fair KL, Phillips NJ, Ritter JH, Steinbrueck T, Holt MS *et al*. (1995). Allelotyping of ductal carcinoma *in situ* of the breast: deletion of loci on 8p, 13q, 16q, 17p and 17q. *Cancer Res* **55**: 3399–3405.
- Rivers A, Gietzen KF, Vielhaber E, Virshup DM. (1998). Regulation of casein kinase I epsilon and casein kinase I delta by an *in vivo* futile phosphorylation cycle. *J Biol Chem* **273**: 15980–15984.
- Sakanaka C. (2002). Phosphorylation and regulation of beta-catenin by casein kinase I epsilon. *J Biochem* **132**: 697–703.
- Shirogane T, Jin J, Ang XL, Harper JW. (2005). SCFbeta-TRCP controls clock-dependent transcription via casein kinase 1-dependent degradation of the mammalian period-1 (Per1) protein. *J Biol Chem* **280**: 26863–26872.
- Takeshita A, Cardona GR, Koibuchi N, Suen CS, Chin WW. (1997). TRAM-1, a novel 160-kDa thyroid hormone receptor activator molecule, exhibits distinct properties from steroid receptor coactivator-1. *J Biol Chem* **272**: 27629–27634.
- Tang J, Qu LK, Zhang J, Wang W, Michaelson JS, Degenhardt YY *et al*. (2006). Critical role for Daxx in regulating Mdm2. *Nat Cell Biol* **8**: 855–862.
- Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK *et al*. (1997). The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* **387**: 677–684.
- Torres-Arzayus MI, Font de Mora J, Yuan J, Vazquez F, Bronson R, Rue M *et al*. (2004). High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene. *Cancer Cell* **6**: 263–274.
- Voegel JJ, Heine MJ, Zechel C, Chambon P, Gronemeyer H. (1996). TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J* **15**: 3667–3675.
- Wang Z, Rose DW, Hermanson O, Liu F, Herman T, Wu W *et al*. (2000). Regulation of somatic growth by the p160 coactivator p/CIP. *Proc Natl Acad Sci USA* **97**: 13549–13554.
- Welcker M, Clurman BE. (2008). FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. *Nat Rev Cancer* **8**: 83–93.
- Werbajh S, Nojek I, Lanz R, Costas MA. (2000). RAC-3 is a NF-kappa B coactivator. *FEBS Lett* **485**: 195–199.
- Wu RC, Feng Q, Lonard DM, O'Malley BW. (2007). SRC-3 coactivator functional lifetime is regulated by a phospho-dependent ubiquitin time clock. *Cell* **129**: 1125–1140.
- Xu J, Liao L, Ning G, Yoshida-Komiya H, Deng C, O'Malley BW. (2000). The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty,

- female reproductive function, and mammary gland development. *Proc Natl Acad Sci USA* **97**: 6379–6384.
- Xu L, Wei Y, Reboul J, Vaglio P, Shin TH, Vidal M *et al.* (2003). BTB proteins are substrate-specific adaptors in an SCF-like modular ubiquitin ligase containing CUL-3. *Nature* **425**: 316–321.
- Yan J, Erdem H, Li R, Cai Y, Ayala G, Ittmann M *et al.* (2008). Steroid receptor coactivator-3/AIB1 promotes cell migration and invasiveness through focal adhesion turnover and matrix metalloproteinase expression. *Cancer Res* **68**: 5460–5468.
- Yan J, Yu CT, Ozen M, Ittmann M, Tsai SY, Tsai MJ. (2006). Steroid receptor coactivator-3 and activator protein-1 coordinately regulate the transcription of components of the insulin-like growth factor/AKT signaling pathway. *Cancer Res* **66**: 11039–11046.
- Yi P, Feng Q, Amazit L, Lonard DM, Tsai SY, Tsai MJ *et al.* (2008). Atypical protein kinase C regulates dual pathways for degradation of the oncogenic coactivator SRC-3/AIB1. *Mol Cell* **29**: 465–476.
- Yu C, York B, Wang S, Feng Q, Xu J, O'Malley BW. (2007). An essential function of the SRC-3 coactivator in suppression of cytokine mRNA translation and inflammatory response. *Mol Cell* **25**: 765–778.
- Zhou G, Hashimoto Y, Kwak I, Tsai SY, Tsai MJ. (2003). Role of the steroid receptor coactivator SRC-3 in cell growth. *Mol Cell Biol* **23**: 7742–7755.
- Zhou HJ, Yan J, Luo W, Ayala G, Lin SH, Erdem H *et al.* (2005). SRC-3 is required for prostate cancer cell proliferation and survival. *Cancer Res* **65**: 7976–7983.
- Zhuang M, Calabrese MF, Liu J, Waddell MB, Nourse A, Hammel M *et al.* (2009). Structures of SPOP-substrate complexes: insights into molecular architectures of BTB-Cul3 ubiquitin ligases. *Mol Cell* **36**: 39–50.

Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)