

Figure 2 CHIP suppresses tumour growth in a mouse xenograft model. (a) Expression level of CHIP in the MCF-7 or MDA-MB-231 cells. Protein levels of CHIP in the cells were determined by immunoblotting. (b) Knockdown of CHIP using shRNA (shCHIP). Retrovirus was used to infect MCF-7 cells, which were selected with G418 (1 mg ml^{-1}). CHIP protein levels were assessed by immunoblotting. (c) Tumour growth curves in nude mice inoculated with control or shCHIP MCF-7 cells. Ten nude mice received bilateral subcutaneous injections of control or shCHIP cells. Tumour volume is presented as the mean \pm s.d. ($n = 20$) from 10 mice in each group. (d) Increased tumour size in mice injected with shCHIP cells. Photographs of representative nude mice (left, upper panels) and tumours (left, lower panels) are shown on the right. Bars represent mean \pm s.d. ($n = 20$). (e, f) CHIP knockdown leads to a higher nuclear grade and increased vascularization of tumour cells. Sections of tumours from mice injected with control or shCHIP

cells were stained with haematoxylin and eosin (H&E, e) or labelled with anti-SMA antibodies (SMA, f). Mitotic index (M. I.) values are shown below the H&E images. Arrowheads (f) indicate SMA-positive vessels. (g) Expression of Flag-CHIP in MDA-MB-231 cells. Plasmid vector containing Flag-CHIP (CHIP) or empty vector (control) was transfected into MDA-MB-231 cells, which were selected with G418 (1 mg ml^{-1}). CHIP protein levels in the cells were analysed by immunoblotting. (h) Tumour growth curves in nude mice inoculated with control or CHIP^{OE} cells. Tumour volume is presented as the mean \pm s.d. ($n = 10$). (i) Thirty days after the injections, the mice were killed and tumour tissues were collected. The photographs in the left panel show representative nude mice (upper) and tumours (lower). The scale bars represent 10 mm. The graph in right panel shows the weight of the 10 tumours after 30 days. Bars represent mean \pm s.d. ($n = 10$). See Supplementary Information, Fig. S7 for full scans of blots in a, b and g.

the CHIP expression level was inversely correlated with the malignant phenotype of breast cancer cells, which was characterized by increased anchorage-independent cell growth and metastatic potential.

In human breast cancer tissues, we did not observe an inverse correlation between CHIP expression and ER α positivity. In addition, in ER α -negative MDA-MB-231 cells, anchorage-independent cell growth and metastatic potential were influenced by the CHIP expression level. These results suggest that CHIP is able to suppress tumour progression in the absence of ER α , raising the possibility that besides ER α , other CHIP target proteins may participate in tumour progression.

We then investigated the molecular mechanism by which CHIP protein suppresses the malignant phenotype. Given that CHIP is a U-box E3 ubiquitin ligase¹⁴, it may act by degrading several proteins that contribute to tumour progression. To test this hypothesis, we compared the levels of oncogenic proteins in control and shCHIP MCF-7 cells by immunoblotting (Fig. 3i). The levels of the breast cancer-related

protein SRC-3 (steroid receptor co-activator 3, ref. 13), which is also known as AIB1 (ref. 14), ACTR¹⁵, TRAM-1 (ref. 16), p/CIP¹⁷ and RAC3 (ref. 18), was upregulated in shCHIP cells. The levels of anti-apoptotic proteins, such as Bcl2 (refs 20, 21) and Akt1 (ref. 22), were higher in the shCHIP cells (Fig. 3i), which is related to enhancement of anchorage-independent cell growth in shCHIP cells (Fig. 3b). Expression levels of Smad2, a signal transducer in the TGF- β signalling pathway^{23,24}, as well as Twist, β -catenin and vimentin, which are marker proteins of the epithelial-mesenchymal transition (EMT)^{25,26}, were also upregulated. TGF- β signalling pathways are known to be related to aggressive phenotypes in tumours, including invasion and migration^{23,24}, and the EMT allows benign tumour cells to metastasize²⁶. Thus, this regulation may account for the enhanced migration (Fig. 3c) and invasion (Fig. 3d) of shCHIP MCF-7 cells.

Next, we examined whether the altered protein levels correlated with changes in the corresponding transcript levels using real-time RT-PCRs

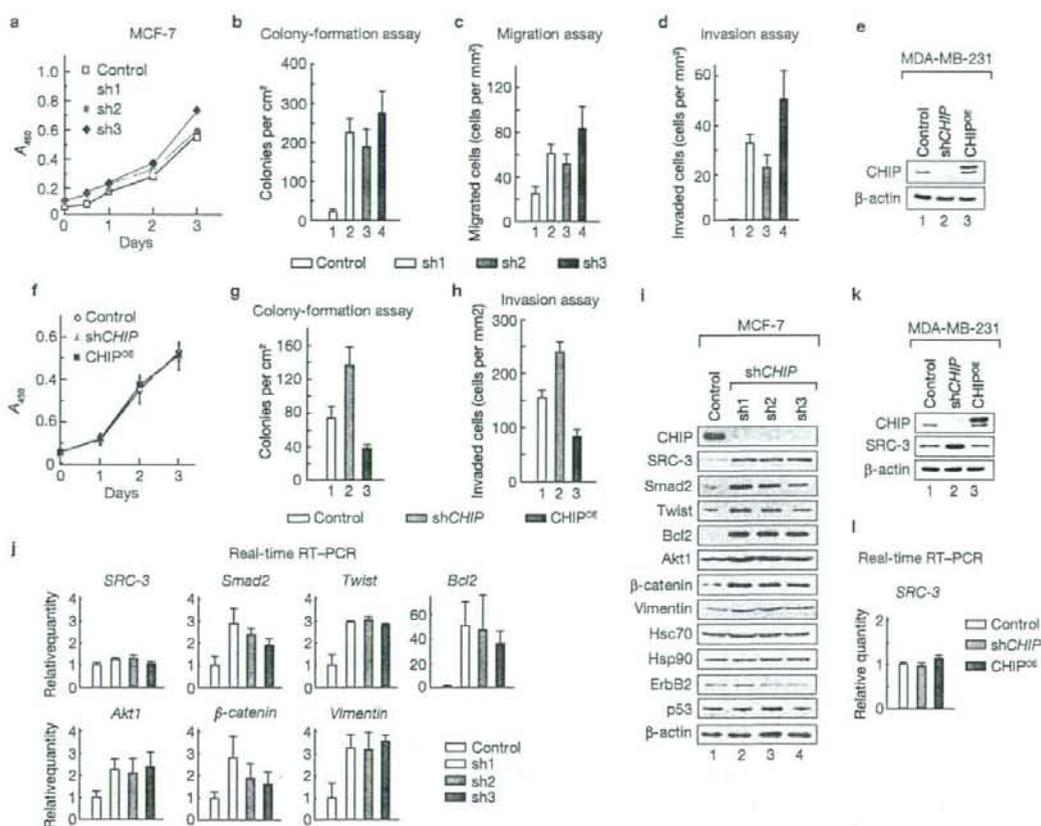


Figure 3 Downregulation of CHIP expression results in the malignant transformation of MCF-7 cells. (a) Proliferation of shCHIP MCF-7 cells. Growth rates of control or shCHIP cells were measured by MTT assay. (b) Increased anchorage-independent cell growth in shCHIP cells. Cells were plated in soft-agar dishes. After incubation for 3 weeks, colonies were examined under a microscope and colonies with a diameter of more than 100 μm were counted. (c, d) Enhanced migration and invasion by shCHIP. Control or shCHIP MCF-7 cells were seeded onto filters with an 8- μm pore size in uncoated (c, migration assay) or Matrigel matrix-coated (d, invasion assay) upper chambers. Both assays were allowed to proceed for 24 h. The average numbers of cells that migrated or invaded per mm^2 are shown. (e) Expression level of CHIP in control, shCHIP, or CHIP^{OE} MDA-MB-231 cells. Protein levels of CHIP were determined by immunoblotting. (f) Proliferation of the MDA-MB-231 cell lines. Growth

rates were measured by MTT assay. (g) Inverse correlation between anchorage-independent cell growth and CHIP expression. (h) Inverse correlation between invasive activity and CHIP expression. (i) Proteins potentially involved in the malignant transformation of shCHIP cells. Total protein levels in shCHIP or control MCF-7 cells were analysed by immunoblotting. (j) Genes potentially involved in the malignant transformation of shCHIP cells. Total RNA was isolated from shCHIP or control cells, and gene expression profiles were analysed using real-time RT-PCRs. (k) Expression level of SRC-3 in shCHIP or CHIP^{OE} MDA-MB-231 cells. The level of SRC-3 protein was determined by immunoblotting. (l) SRC-3 mRNA expression in the cells. Total RNA was prepared from the cells and the SRC-3 mRNA level was quantified using real-time RT-PCRs. Data represent mean \pm s.d., $n = 3$ (a–d, f–h, j, l). See Supplementary Information, Fig. S7 for full scans of blots in e, c and k.

(Fig. 3j). The transcript levels of most of the genes, which encode the proteins shown in Fig. 3i, fluctuated in parallel with their protein levels, demonstrating that this regulation occurred at the mRNA level. These results raise the possibility that CHIP regulates transcription factor(s), which in turn regulate the transcription of other oncogenes. Among the candidate CHIP target proteins shown in Fig. 3i, SRC-3 is a strong possibility, because accumulation of SRC-3 protein following CHIP knockdown was not due to the accumulation of mRNA (Fig. 3j). In MDA-MB-231 cells, we also observed that the CHIP level affected the level of SRC-3 protein (Fig. 3k), but not its mRNA level (Fig. 3l). Furthermore, SRC-3 is known to be a co-activator of several transcription factors that show elevated expression levels and copy numbers in

human breast and ovarian cancers^{13–18}. It has also been reported that levels of SRC-3 are correlated with tumour malignancy¹⁹.

Immunoprecipitation and immunoblot analysis showed that SRC-3 and CHIP interact with each other in cells (Fig. 4a, b). CHIP expression resulted in a decrease in the steady-state levels of SRC-3 protein, an effect that was abolished in the presence of the proteasome inhibitor MG132 (Fig. 4c). Pulse-chase experiments showed that the turnover rate of SRC-3 decreased in shCHIP cells ($t_{1/2} >> 12$ h) and increased in CHIP^{OE} cells ($t_{1/2} < 4$ h), compared with that in the control cells ($t_{1/2} = 8$ h) (Fig. 4d; Supplementary Information, Fig. S5). A ubiquitylation assay revealed that CHIP enhanced the ubiquitylation of SRC-3 (Fig. 4e). Taken together, these results indicate that CHIP mediates the ubiquitylation-dependent

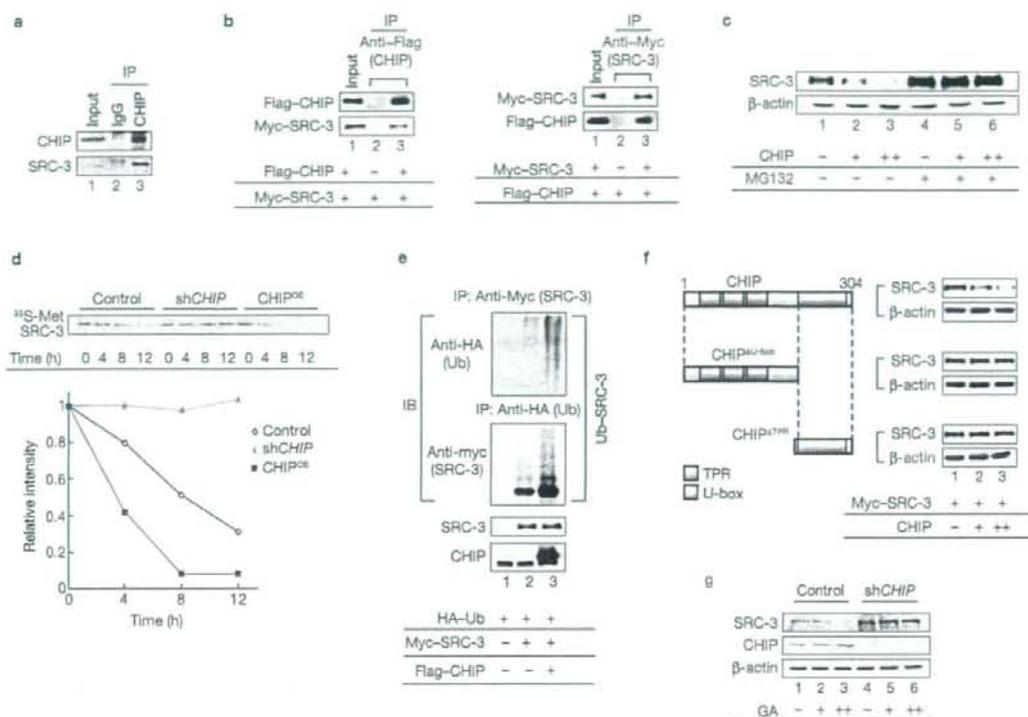


Figure 4 CHIP targets SRC-3 for ubiquitylation and degradation. (a, b) CHIP interacts with SRC-3. Cell lysates from MCF-7 cells were immunoprecipitated using control (IgG) or anti-CHIP antibodies, and immunoblotted using the indicated antibodies (a). Flag-tagged CHIP and 6 \times Myc-tagged SRC-3 were expressed in 293 cells with MG132 (10^{-6} M). Cell lysates were immunoprecipitated using anti-Flag (left) or anti-Myc (right) antibodies, and immunoblotted using the indicated antibodies (b). (c) Reduction of the steady-state SRC-3 protein level due to CHIP expression. A plasmid encoding 6 \times Myc-tagged SRC-3 (150 ng) was co-transfected into 293 cells with various amounts of plasmid encoding Flag-tagged CHIP (0, 15 or 30 ng). The cells were cultured in the absence or presence of MG132 (1 μ M). The level of SRC-3 protein was examined by immunoblotting. (d) CHIP facilitates the turnover of SRC-3. Control, shCHIP, or CHIP^{del} MDA-MB-231 cells were incubated in medium containing 35 S-Met, replaced with label-free medium, and collected at the indicated time-points. SRC-3 was immunoprecipitated, subjected to SDS-PAGE, detected on an autoradiograph (upper panel) and the label intensity was quantified (lower graph). (e) Ubiquitylation of SRC-3 is induced by CHIP. HA-tagged ubiquitin was co-expressed in 293 cells with 6 \times Myc-SRC-3 (lane 2) or 6 \times Myc-SRC-3 and Flag-CHIP (lane 3) with MG132 (10^{-6} M). 6 \times Myc-tagged SRC-3 was immunoprecipitated using anti-Myc antibodies. Ubiquitylation was detected using anti-HA antibodies, or ubiquitylated proteins were immunoprecipitated using anti-HA antibodies and ubiquitylated SRC-3 was detected with anti-Myc antibodies (upper panels). SRC-3 or CHIP was detected in whole-cell lysates using anti-Myc or anti-CHIP antibodies, respectively (lower panels). (f) The TPR and U-box domains are both required for SRC-3 degradation. Various amounts of plasmid encoding CHIP, CHIP^{del}, or CHIP^{TPR} (0, 15 or 30 ng) were transfected into 293 cells expressing 6 \times Myc-tagged SRC-3. The levels of SRC-3 protein were examined by immunoblotting. (g) Geldanamycin (GA) enhanced CHIP-dependent SRC-3 degradation. Control or shCHIP MDA-MB-231 cells were untreated (lanes 1, 4) or treated with GA (1 μ M, lanes 2, 5; 3 μ M, lanes 3, 6) for 24 h. SRC-3 protein levels were examined by immunoblotting. See Supplementary Information, Fig. S7 for full scans of blots in b–g.

degradation of SRC-3. We then created two constructs: CHIP^{del}-box, in which the U-box domain (required for ubiquitylation) was deleted, and CHIP^{TPR}, in which the Hsp-binding TPR domain was deleted⁶. Cells expressing either of the constructs were unable to degrade SRC-3 (Fig. 4f), suggesting that CHIP binds to Hsps to promote the ubiquitylation of their substrate protein, SRC-3. Moreover, SRC-3 protein became unstable when the protein re-folding activity of Hsp90 was inhibited using geldanamycin, consistent with a contribution of Hsps to the CHIP-dependent degradation of SRC-3 (Fig. 4g). SRC-3 is also known to be a target protein of the ubiquitin ligase Fbw7 (ref.27). We found that Fbw7 was not affected by CHIP knockdown in MCF-7 cells (Supplementary Information, Fig. S1a).

Our results raise the possibility that upregulation of SRC-3 protein expression following CHIP knockdown may in turn lead to the altered gene expression shown in Fig. 3i. To test this possibility, we generated MCF-7 cell lines in which expression of SRC-3, CHIP or both CHIP and SRC-3 was suppressed using shRNAs (shSRC-3, shCHIP or shCHIP/shSRC-3). In shCHIP/shSRC-3 cells, the levels of Smad2 and Twist proteins, which were elevated in the shCHIP cells, were reduced to control levels, whereas the level of Akt1 protein was unchanged and that of Bcl2 protein was slightly reduced (Fig. 5a). Similar results were obtained when the expression profiles of these genes were examined (Fig. 5b). IGF-1 has been previously reported to be a target of SRC-3 and to contribute to tumorigenic phenotypes induced by SRC-3 (ref.28). However,

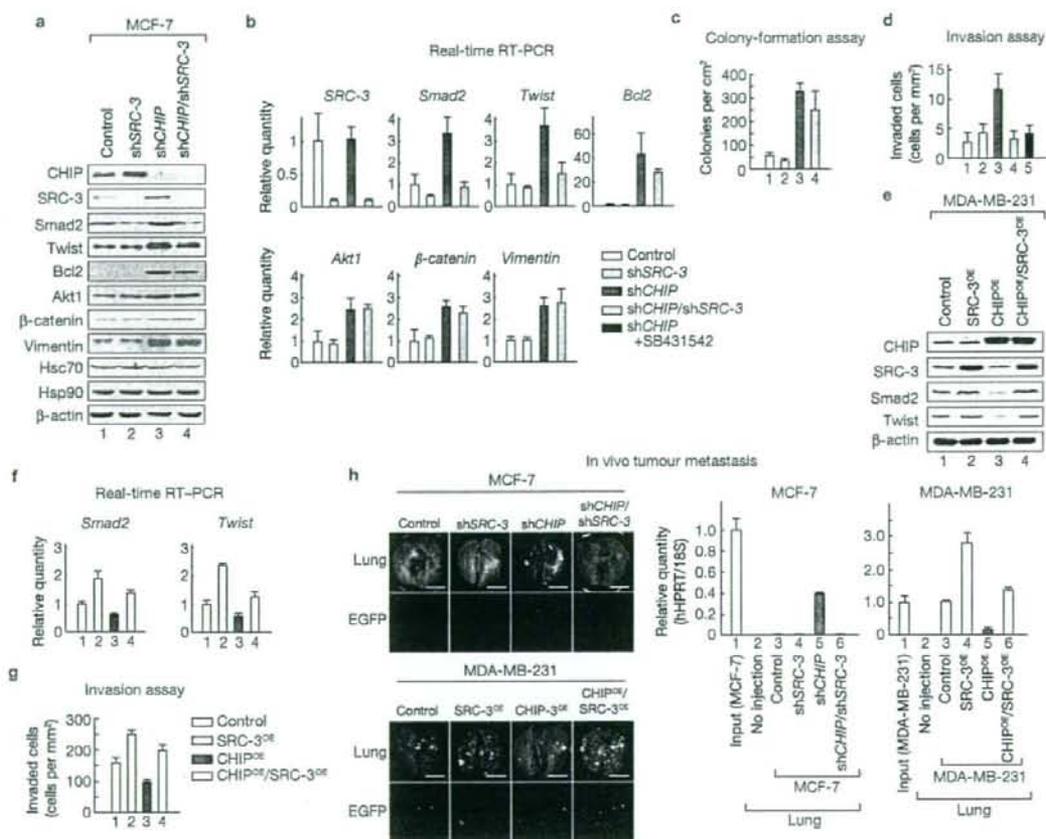


Figure 5 SRC-3 is the downstream regulator of CHIP during cancer metastasis. (a, b) Effect of downregulating SRC-3 on protein or transcription levels. Total proteins in the control, shSRC-3, shCHIP, or shCHIP/shSRC-3 MCF-7 cells were analysed by immunoblotting (a). Total RNA was isolated from the indicated cells, and gene expression profiles were analysed using real-time RT-PCR (b). (c) A slight decrease in anchorage-independent cell growth following SRC-3 knockdown. Colony-formation assays were performed as described in Fig. 3b. (d) Reduced invasiveness following SRC-3 knockdown. Invasion assays were performed as described in Fig. 3d. shCHIP cells were treated with the TGF- β inhibitor SB431542 (10 μ M; lane 5). The numbers of invasive cells per mm² are shown. (e, f) Effect of SRC-3 on protein or transcription levels in MDA-MB-231 cells. Total protein in control, SRC-3^{OE}, CHIP^{OE}, or CHIP^{OE}/SRC-3^{OE} MDA-MB-231 cells were examined by immunoblotting (e). Total RNA

was prepared from the indicated cells and expression of *Smad2* or *SRC-3* was analysed using real-time RT-PCR (f). (g) SRC-3 rescues the CHIP^{OE}-induced reduction in invasive activity. Invasive activities of the indicated cell lines were assayed as described in Fig. 3d. (h) Effect of SRC-3 on tumour metastasis *in vivo*. EGFP-labelled control; shSRC-3, shCHIP or shCHIP/shSRC-3 MCF-7 cells were injected into the tail veins of nude mice ($n = 5$). Alternatively, EGFP-labelled control, SRC-3^{OE}, CHIP^{OE}, or CHIP^{OE}/SRC-3^{OE} MDA-MB-231 cells were injected into the tail veins of nude mice ($n = 5$). Six weeks after the injections, the mice were killed and lung metastasis was detected by fluorescence microscopy (left panel) and quantified by real-time RT-PCR (right graph). Specific primers for human HPRT were used. Scale bars represent 5 mm (left). Data are mean \pm s.d., $n = 3$ (b–d, f, g) and mean \pm s.d., $n = 5$ (h, right). See Supplementary Information, Fig. S7 for full scans of blots in a and e.

altering SRC-3 expression did not affect the IGF-1 signalling pathway (Supplementary Information, Fig. S1c, d).

We next investigated whether *Smad2* and *Twist* are direct targets of SRC-3. Chromatin immunoprecipitation and transcription assays revealed that *Smad2* is a direct target of SRC-3 (Supplementary Information, Fig. S2). We also showed that *Smad2* bound to the promoter region of *Twist*, in agreement with previous reports²⁹. These observations demonstrate that SRC-3 increases the level of *Twist* expression by enhancing *Smad2* expression.

We then examined the anchorage-independent cell growth and invasiveness of the shCHIP/shSRC-3 MCF-7 cells. Consistent with

the expression profiles of *Bcl2* and *Akt1*, anchorage-independent cell growth was similar in shCHIP/shSRC-3 and shCHIP MCF-7 cells (Fig. 5c). In contrast, the invasiveness of shCHIP MCF-7 cells was significantly reduced by SRC-3 knockdown (shCHIP/shSRC-3; Fig. 5d). Given that the TGF- β /Smad pathway is known to regulate *Twist* transcription and enhance cancer invasion^{23,24,29}, our results suggest that the enhanced metastatic activity in shCHIP MCF-7 cells was a result of the enhanced *Smad2* and *Twist* expression mediated by SRC-3. This idea was supported by the inhibition of shCHIP cell invasiveness after treatment with the TGF- β inhibitor SB431542 (Fig. 5d). Moreover, in MDA-MB-231 cells, CHIP^{OE} reduced levels of *Smad2*

and *Twist* mRNA and SRC-3 protein. *Smad2* and *Twist* mRNA levels were restored by co-overexpression of SRC-3 with CHIP (CHIP^{OE}/SRC-3^{OE}) (Fig. 5e, f). As expected, the repression of invasive activity by CHIP^{OE} was restored in CHIP^{OE}/SRC-3^{OE} cells (Fig. 5g).

Finally, to investigate whether CHIP knockdown in MCF-7 cells enhanced *in vivo* metastatic activity, we injected control and shCHIP MCF-7 cells into the tail veins of nude mice. Lung metastasis was enhanced in mice given the shCHIP MCF-7 cells (Fig. 5h; Supplementary Information, Fig. S3) but SRC-3 knockdown (shCHIP/shSRC-3) suppressed this effect. In contrast, CHIP^{OE} in MDA-MB-231 cells reduced lung metastasis, which was restored by co-overexpression of SRC-3 (CHIP^{OE}/SRC-3^{OE}).

Our observations indicate that when CHIP expression is reduced, SRC-3 accumulates, leading to enhanced tumour migration and invasion through increased *Smad2* and *Twist* gene transcription (Supplementary Information, Fig. S4). In agreement with this model, we observed a parallel correlation between tumour grade and the SRC-3 protein level (Supplementary Information, Fig. S6). A recent report showed that when CHIP was exogenously expressed, it induced the degradation of Smad proteins⁹. In our experiment, however, accumulation of Smad following CHIP knockdown was due to enhancement of transcription rather than inhibition of degradation. Previous reports indicate that Smad proteins are ubiquitinated by other E3 ubiquitin ligases, such as Smurf and SCF/Roc1 (ref. 3). Therefore, CHIP may make a minor contribution to Smad protein degradation, at least in breast cancer cells.

ERα is one of the targets of ubiquitylation and degradation by CHIP⁹. However, we did not observe an inverse correlation between CHIP expression and ERα positivity in human breast cancer tissues. ERα protein levels in breast cancers are determined by both transcription and protein degradation, which may explain why ERα protein levels do not correlate with CHIP expression levels. Moreover, CHIP preferentially ubiquitylates misfolded forms of ERα, and functional ERα protein is not a CHIP target⁹, which may also account for lack of the inverse correlation between CHIP expression and ERα positivity. Given that SRC-3 is a co-activator of ERα, however, the CHIP/SRC-3/ERα signalling pathway may also participate in ERα-positive breast cancer progression.

Our results also indicate that CHIP suppresses the expression of other oncogenic proteins that enhance anchorage-independent tumour growth (Supplementary Information, Fig. S4). CHIP is known to be involved in degrading misfolded forms of several oncogenic proteins, including p53 and ErbB2. In breast cancer cell lines, the levels of these proteins were not markedly affected by the CHIP level, although we cannot rule out the possibility that CHIP functions in tumour growth suppression by maintaining the overall quality of these proteins.

METHODS

Expression vectors. DNA fragments encoding full-length and various truncated forms of CHIP were amplified by PCR and subcloned into the pcDNA3 vector containing sequences coding for Flag. Similarly, the SRC-3 full-length cDNA was cloned into the pcDNA3 vector containing 6xMyc sequence.

Cell culture and transfection. MCF-7, MDA-MB-231 and 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The proliferation of cultured cells was measured by MTT assay using an MTT cell count kit (Nakarai Tesque). Transfection was performed with PerFectin transfection reagent (Gene Therapy Systems) or Transfast reagent (Promega).

Co-immunoprecipitation and immunoblotting. Cells were lysed in TNE buffer (10 mM Tris-HCl (pH 7.8), 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 1 μM phenylmethylsulphonyl fluoride (PMSF) and 1 μg ml⁻¹ aprotinin). Extracted proteins were immunoprecipitated with the appropriate antibodies. Bound proteins were separated by SDS-PAGE, transferred onto PVDF membranes (Millipore) and immunoblotted with the appropriate antibodies. The antibodies used in this study were: mouse monoclonal antibodies specific for Flag (1:1000; M2; Sigma), c-Myc (1:2000; Nakarai Tesque), β-catenin (1:1000; BD Biosciences), Bcl2 (1:1000; BD Biosciences), Vimentin (1:1000; Sigma), Hsp90 (1:5000; BD Biosciences), p53 (1:5000; Santa Cruz), rat monoclonal antibodies against HA (1:500; Roche) and human CHIP (1:1000; Green Space Biomed, Japan), rabbit monoclonal antibody against Smad3 (1:1000; Cell Signaling), rabbit polyclonal antibodies against Twist (1:1000; Sigma), Akt1 (1:1000; Cell Signaling), Hsc70 (1:3000; Streegen), ErbB2 (1:1000; Cell Signaling), IGF-1 (1:250; Abcam), Fbw7 (1:1000; Invitrogen), IGF-1R (1:500; Cell Signaling), phospho-IGF-1R (1:500; Cell Signaling) and goat polyclonal antibodies against SRC-3 (1:500; Santa Cruz) and Smad2 (1:1000; Santa Cruz).

Ubiquitylation assay. 293 cells expressing Myc-tagged SRC-3 and/or Flag-tagged CHIP were lysed with radioimmunoprecipitation buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with complete protease inhibitor mixture (Roche) and placed on ice for 20 min. The extracts were cleared by centrifugation and immunoprecipitation reactions were carried out with anti-Myc agarose beads for 3 h at 4°C. After washing the resin with radioimmunoprecipitation buffer, the bound proteins were immunoblotted with the appropriate antibodies.

Pulse-chase experiments. MCF-7 or MDA-MB-231 cells were incubated with Met- and Cys-free medium (Gibco) for 2 h, replaced with medium containing ³⁵S-Met and incubated for 2 h. Cells were then washed three times with phosphate-buffered saline and incubated with medium supplemented with 1 mM non-radioactive Met and Cys for 0, 4, 8 or 12 h. Cells were collected at the indicated times and SRC-3 was immunoprecipitated from cell lysates using anti-SRC-3 antibody. The precipitated SRC-3 was subjected to SDS-PAGE and detected with an autoradiograph.

RNA interference. For stable RNA interference, we used a retroviral expression system. GP2-293 cells (Clontech) were co-transfected with the p10A1 vector and either pSIN6-hU6 (Takara) or pRETRO-SUPER (OligoEngine) vector containing either the CHIP, SRC-3 or luciferase (control) target sequence. MCF-7 or MDA-MB-231 cells were incubated with the retroviral supernatant in the presence of 8 μg ml⁻¹ polybrene. Twenty-four hours after infection, the viral supernatant was replaced with fresh DMEM containing 10% FBS. The infected cells were selected with 1 mg ml⁻¹ G418 or 1 μg ml⁻¹ puromycin. The target sequences were 5'-gcacgacagatcatggc-gga-3' for CHIP, 5'-ggcttactcagctgggaa-3' for SRC-3 and 5'-gaagctgcgggtgtgt-3' for luciferase.

Soft agar colony-formation assay. For soft agar assays, 10,000 cells were suspended in DMEM containing 0.35% agar and layered on top of 1 ml of DMEM solidified with 0.6% agar in each well of a 6-well plate. After growing at 37 °C for 2–3 weeks, colonies with a diameter of more than 100 μm were counted. The reported results represent the averages of three independent experiments.

In vitro assays of cell migration and invasion. The invasive potential of the MCF-7 or MDA-MB-231 cells was tested with Matrigel invasion chambers (24-well format, 8-μm pore size; BD Biosciences). Briefly, 150,000 cells suspended in DMEM containing 1% FBS were transferred into an insert chamber. In the lower chamber, DMEM containing 10% FBS served as the source of the chemoattractants. After incubation for 24 h, the cells on the upper surface of the filter were wiped away with a cotton swab. The cells on the lower surface of the filters were fixed with methanol for 10 min, stained with Giemsa for 3 h and counted. For each replicate, cells in five randomly selected fields were counted. Migration assays were performed using the same procedure, except that the insert chambers were not coated with Matrigel and DMEM containing 10% FBS was used for the cell suspensions.

In vivo tumour formation assay. BALB/cA|cl-nu female mice at 5 weeks of age were purchased from CLEA Japan. MDA-MB-231 or MCF-7 cells were cultured as monolayers, trypsinized and resuspended in an equal volume of Matrigel

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at 5.0×10^7 or 1.0×10^8 cells per ml, respectively. Female nude mice were given bilateral subcutaneous injections of 5.0×10^6 or 1.0×10^7 (0.1 ml) MDA-MB-231 or MCF-7 cells. The mice were kept in a pathogen-free environment. Tumour growth was monitored twice each week by measuring the tumour size using calipers; tumour volume was determined using the formula $V = 1/2 \times \text{larger diameter} \times (\text{smaller diameter})^2$. The mice were killed and the tumour tissues were collected, weighed and fixed with 20% formalin. All animal experiments were in accordance with institutional guidelines.

In vivo tumour metastasis assay. BALB/cAJcl-nu/nu female mice at 5 weeks of age were purchased from CLEA Japan. The mice were injected with 1.5×10^6 MDA-MB-231 or MCF-7 cells which stably expressed EGFP, administered through the tail vein. Lungs were collected on day 42 and analysed by fluorescence microscopy. After analysis, the lungs were snap-frozen for RNA extraction. For metastasis quantification, total mRNA from the lung was extracted and reverse transcribed. Complementary DNA was quantitatively analysed by real-time PCR. Specific primers for human *HPRT* that do not cross-react with its mouse counterpart were designed. 18S ribosomal RNA was used for normalization.

Patients and tissue samples. Twenty-seven breast tumour samples were surgically obtained at the Saitama Cancer Center Hospital (Saitama, Japan) after informed consent was obtained from the patients. The Saitama Cancer Center Ethics Committee approved this study. For RNA extraction, tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C . The clinico-pathological parameters are summarized in Supplementary Information, Table S1.

Real-time RT-PCR. Tissues and cells were homogenized in 1 ml of isogen and total RNA was extracted according to the manufacturer's instructions (Nippon Gene). cDNA was synthesized from total RNA using RevtraAce reverse transcriptase (Toyobo) and oligonucleotides or Random primers. Real-time PCR was performed to amplify DNA fragments. Primer sequences can be found in Supplementary Information Methods.

Immunohistochemistry. Formalin-fixed mouse and human tumour tissues were embedded in paraffin and sectioned (5 μm). The paraffinized sections were treated with 0.3% hydrogen peroxidase/methanol and incubated with the monoclonal antibodies in 0.01 M citrate buffer (pH 6.0) followed by incubation with secondary antibodies (peroxidase-labelled EnVision reagent; Dako) for 30 min at room temperature. Samples were developed using either AEC or DAB as substrates (Dako).

Statistical analysis. Data are representative of at least three different experiments. Statistical analysis was performed using *t*-test.

Note: Supplementary Information is available on the Nature Cell Biology website.

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AUTHOR CONTRIBUTIONS

M. Kajiro and R.H. performed most of the experiments; K.K., Y.Y., Y.K., Y.S., H.T., S. H. and M. Kurosumi, analysed human tissue samples; Y.N., K.S., I.L., S.O. and M. Kawano contributed to the animal experiments; K.K. and J.Y. planned the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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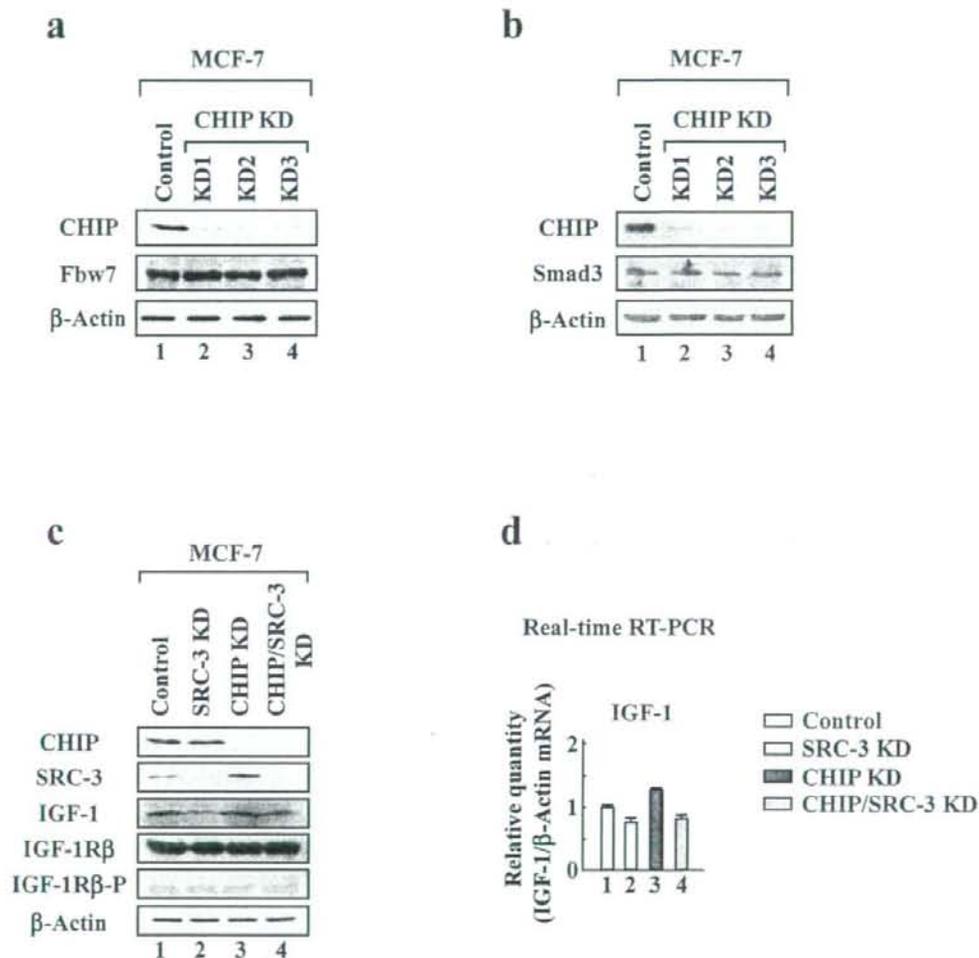


Figure S1 Effect of CHIP KD on the indicated protein and mRNA expression. The level of Fbw7 protein in the CHIP KD or control MCF-7 cells was analyzed by immunoblotting with anti-Fbw7 antibodies. (b) Protein level of Smad3 in the CHIP KD or control MCF-7 cells was analyzed by immunoblotting using anti-Smad3 antibodies. (c) Level of IGF-1 signaling

factors in control, SRC-3 KD, CHIP KD and CHIP/SRC-3 KD MCF-7 cells. Total protein levels in the cells were analyzed by immunoblotting using the indicated antibodies. (d) IGF-1 mRNA expression in the cells. Total RNA was prepared from the cells and the IGF-1 mRNA level was quantified using real-time RT-PCRs. Bars represent mean + s.d. (n = 3).

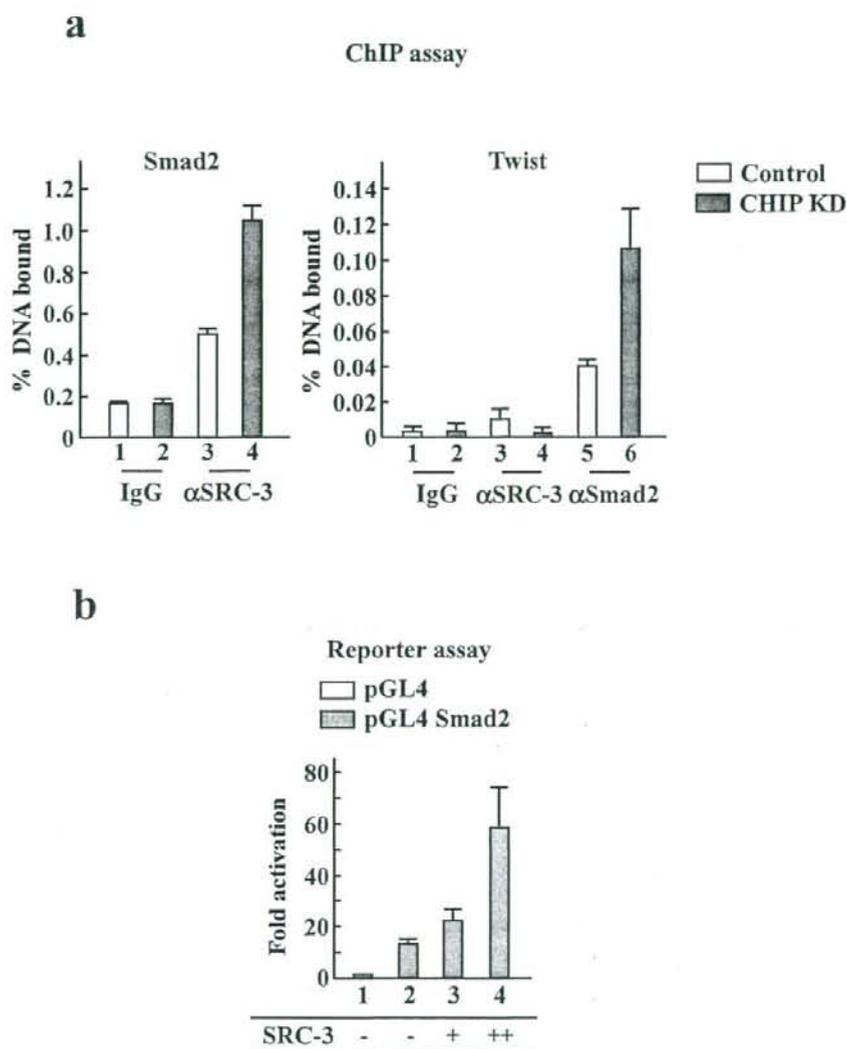


Figure S2 (a) Direct binding of SRC-3 to the upstream region of the Smad2 gene. Chromatin immunoprecipitation analysis was performed to examine the binding of SRC-3 or Smad2 proteins to the upstream regions of the Smad2 (-1040 to -937; left graph) and Twist (-130 to -20; right graph) genes in both control and CHIP KD cells. Bars represent mean + s.d. (n = 3). (b) SRC-3 mediated enhancement of

transcription from the Smad2 gene promoter by SRC-3. MCF-7 cells were co-transfected with various amounts of SRC-3 expression plasmid and either a luciferase reporter plasmid (pGL4) or a plasmid containing the upstream region of the Smad2 gene (pGL4 Smad2; -1500 to +34). Cell extracts were analyzed using a luciferase assay. Bars represent mean + s.d. (n = 3).

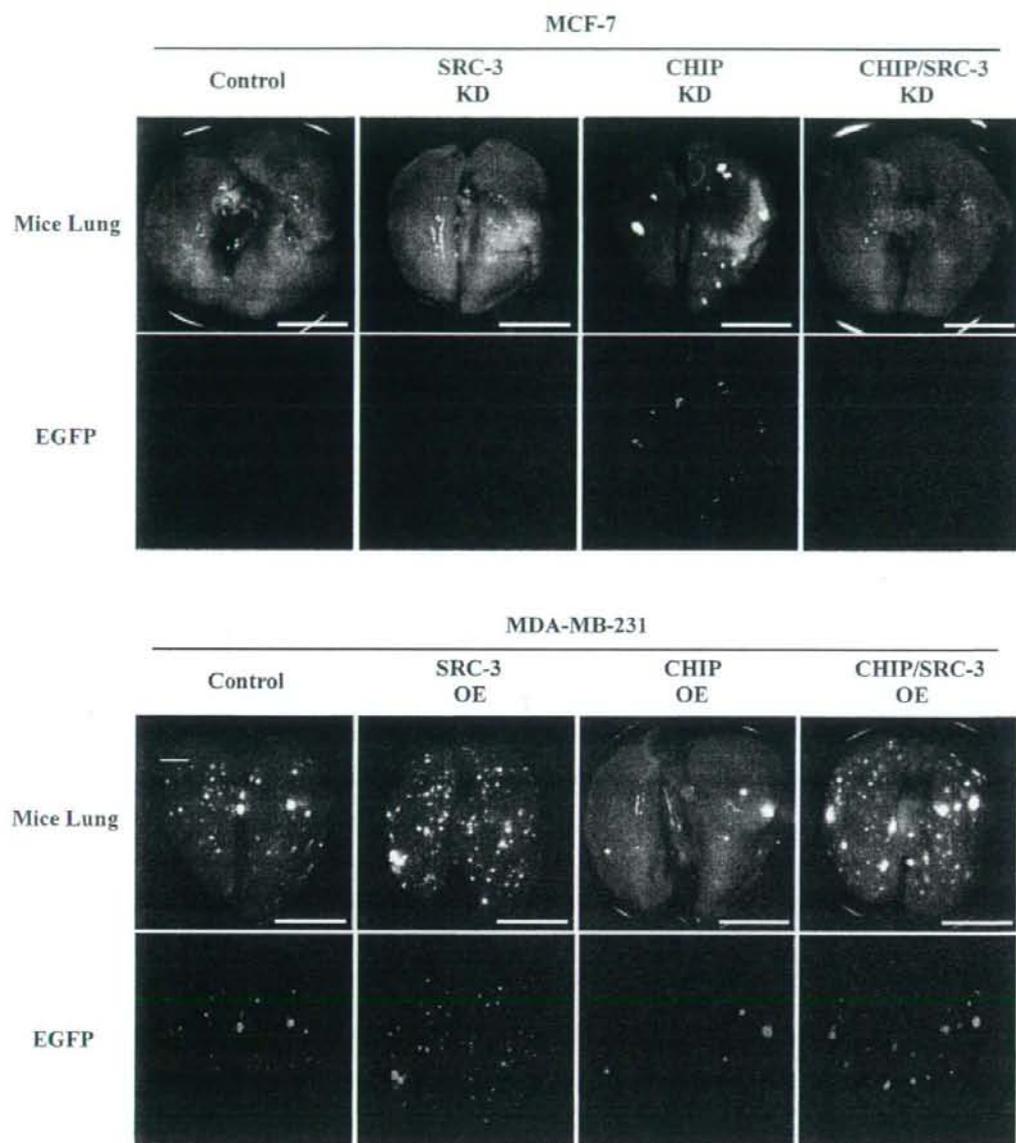


Figure S3 Enlarged images of Fig. 5h. EGFP labeled control, SRC-3 KD, CHIP KD, or CHIP/SRC-3 KD MCF-7 cells were injected into tail vein of nude mice. Alternatively, EGFP labeled control, SRC-3 OE, CHIP OE, or

CHIP/SRC-3 OE MDA-MB-231 cells were injected into tail vein of nude mice. Six weeks after the injections, the mice were sacrificed, and the lung metastasis was analysed by fluorescence microscopy. Scale bar = 5 mm

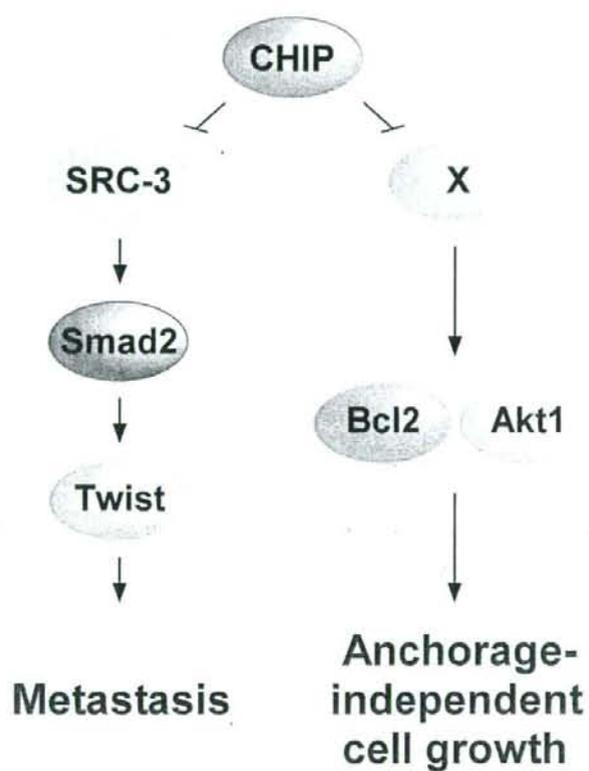


Figure S4 A model for the functions of CHIP in suppression of breast cancer progression.

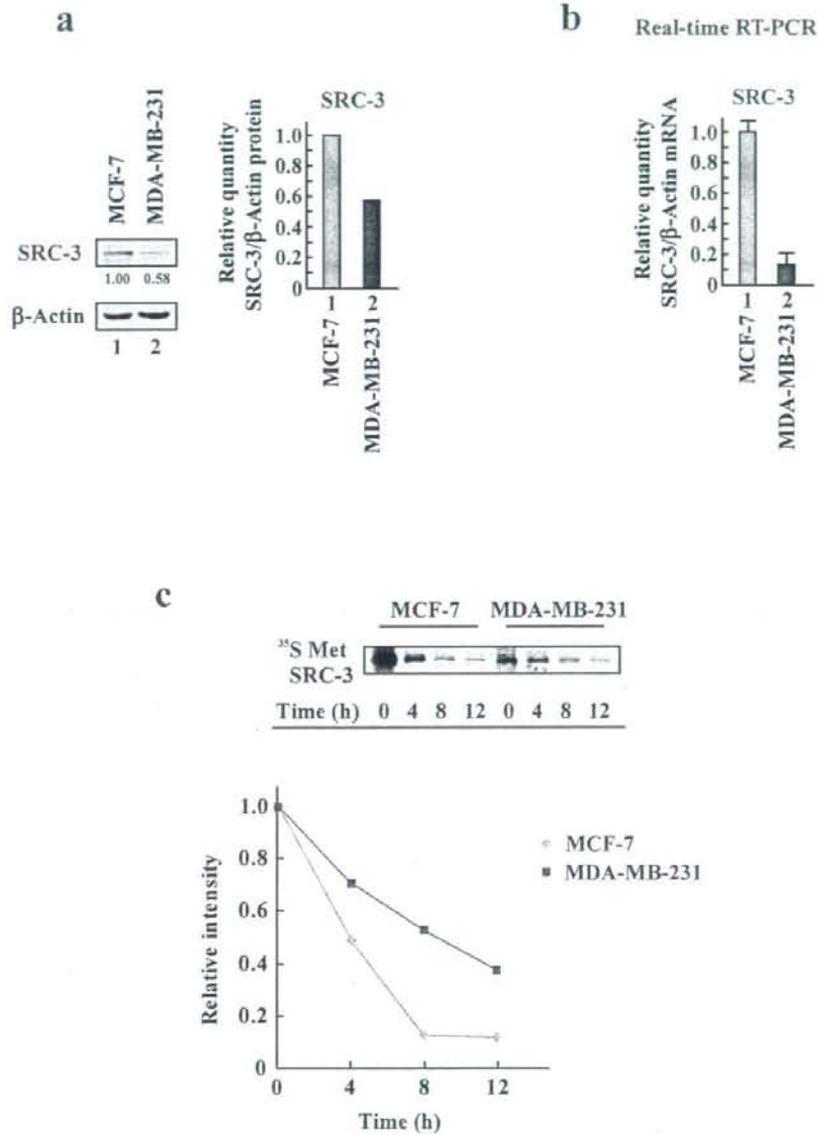


Figure S5 (a) SRC-3 protein level in MCF-7 and MDA-MB-231 cells. The level of SRC-3 protein in MCF-7 or MDA-MB-231 cells was analysed by immunoblotting with anti-SRC-3 antibody. (b) SRC-3 mRNA level in MCF-7 and MDA-MB-231 cells. The level of SRC-3 mRNA in MCF-7 or

MDA-MB-231 cells was analysed by real-time RT-PCR. Bars represent mean + s.d. (n = 3). (c) Turnover rate of SRC-3 protein in MCF-7 and MDA-MB-231 cells. SRC-3 protein turnover in MCF-7 and MDA-MB-231 cells were analysed by ³⁵S labeling chase.

SUPPLEMENTARY INFORMATION

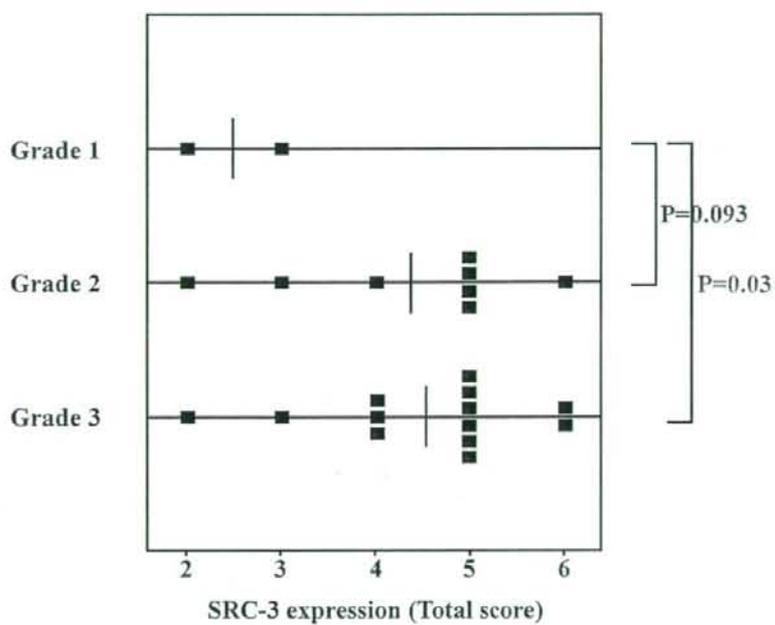


Figure S6 Positive correlation between SRC-3 protein level and histological grade. The level of SRC-3 protein in human breast tumor tissues was

analysed by immunostaining with anti-SRC-3 antibody (Becton, Dickinson and Company; BD 611105).

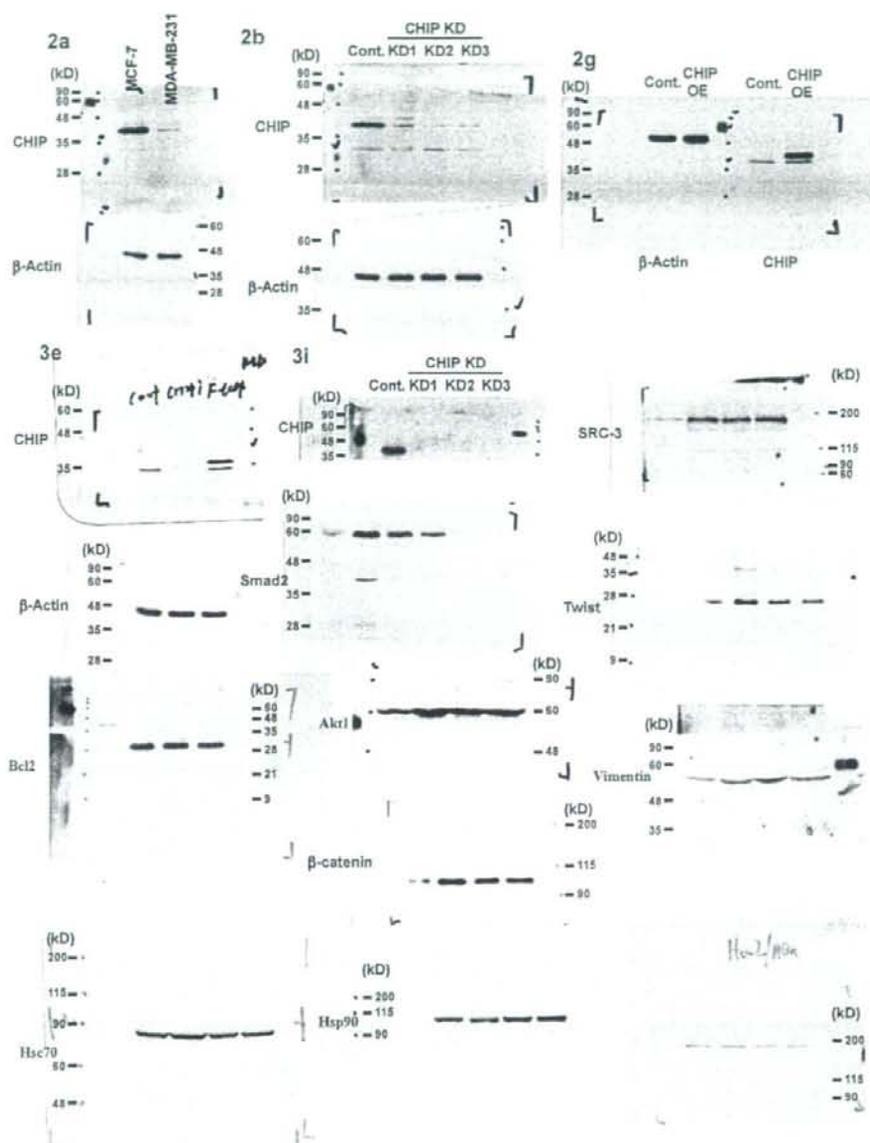


Figure S7 Full blots/scans of original Western data shown in the regular Figures 2a, 2b, 2g, 3e, 3i, 3k, 4a, 4b, 4c, 4d, 4e, 4f, 4g, 5a, and 5e.

SUPPLEMENTARY INFORMATION

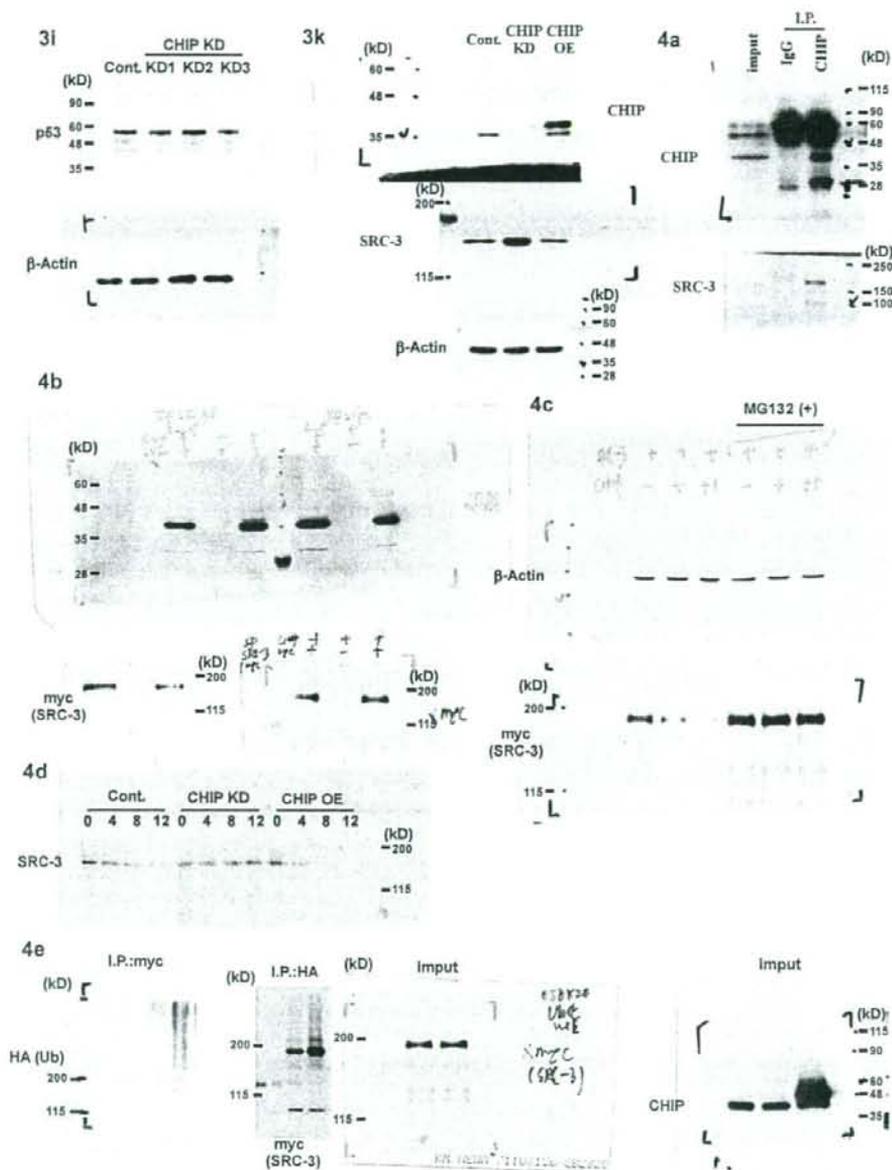


Figure S7 continued

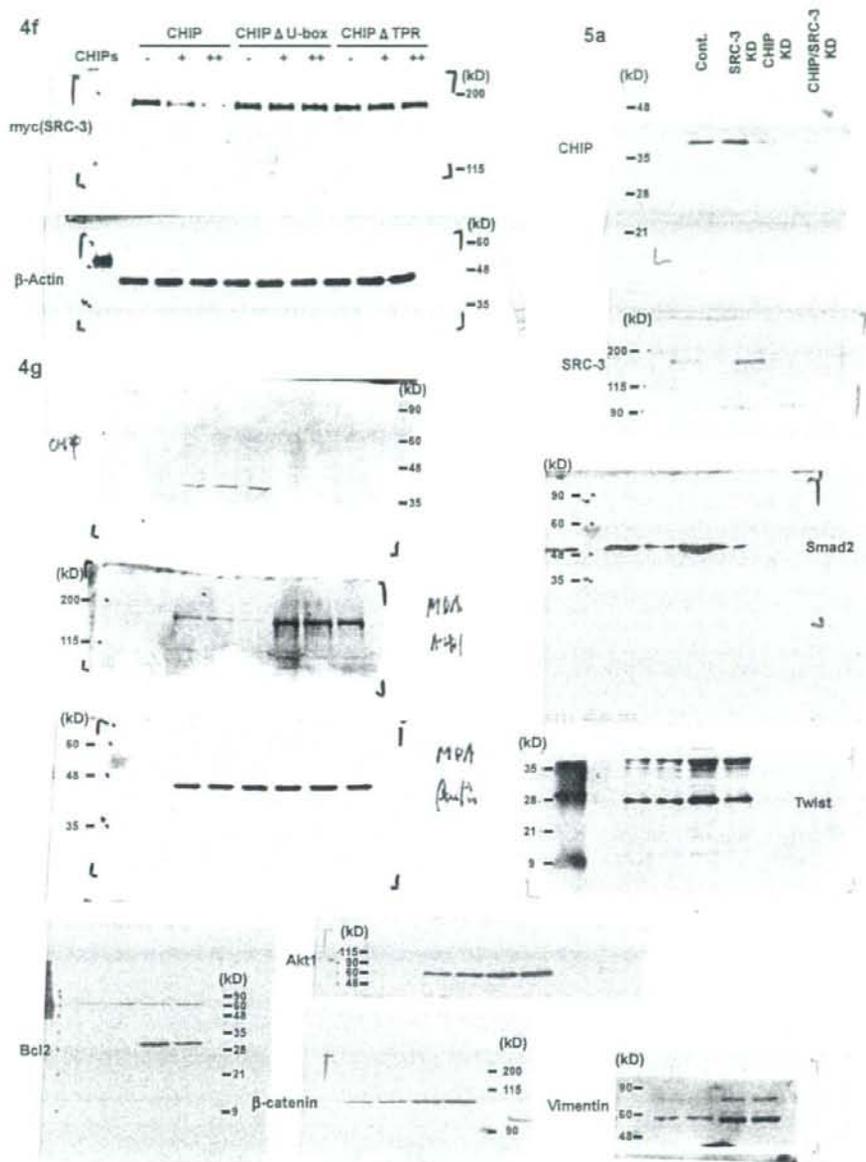
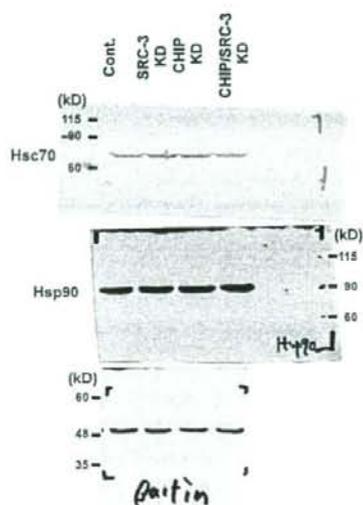


Figure S7 continued

SUPPLEMENTARY INFORMATION

5a



5e

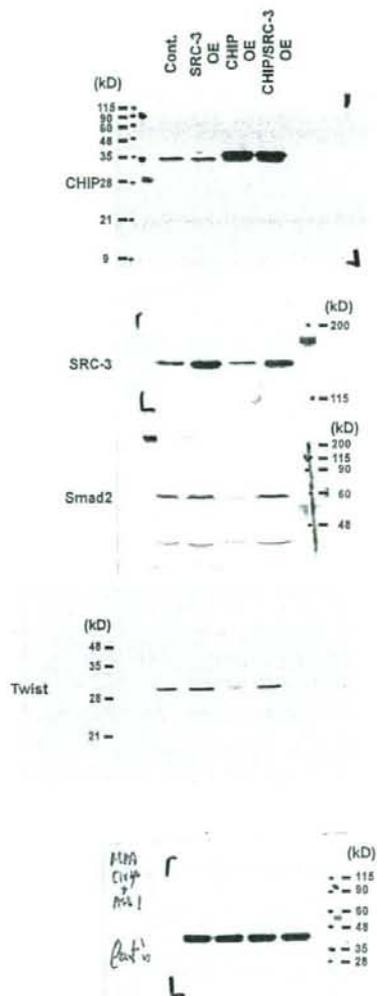


Figure S7 continued

Table S1. Clinicopathological parameters of the breast tumors

		Total No.
Age (yr)	≤ 50	6
	> 50	21
Menopausal status	Pre	11
	Post	16
Tumor diameter	≤ 2 cm	15
	> 2 cm	12
ER ^a	Positive	19
	Negative	7
	Unknown	1
PgR ^a	Positive	22
	Negative	4
	Unknown	1
Stage ^b	I	12
	II	14
	III	1
Histology	Invasive ductal	23
	Special	4
Histological grade	1	2
	2	8
	3	13
	Unknown	4
Nodal status ^b	Negative	16
	Positive	11

^a Cases were considered positive for ER or PgR expression when nuclear staining was observed in at least 10% of the examined tumor cells tested.

^b Stage and nodal status were determined according to the TMN classifications. Nodal status was interpreted as positive when n score was one or more.

Supplementary Information Methods

Chromatin immunoprecipitation and real-time PCR detection.

Formaldehyde was added to culture medium to a final concentration of 1% to cross-linked nuclear proteins with genomic DNA. After centrifugation, the cell pellets were resuspended in lysis buffer [1% SDS, 10 mM EDTA, protease inhibitors and 50 mM Tris-HCl (pH 8.1)], and sonicated. The lysates were diluted 1:10 in ChIP dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM NaCl, protease inhibitors and 16.7 mM Tris-HCl (pH 8.1)], and incubated with indicated antibodies overnight at 4°C with rotation. The immuno-complexes were collected with 30 µl of protein G agarose beads (Upstate Biotechnology) for 1 hours at 4°C with rotation. The beads were washed with the following buffers: low salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris-HCl (pH 8.1)], high salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl and 20 mM Tris-HCl (pH 8.1)] and LiCl wash buffer [0.25 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA and 10 mM Tris-HCl (pH 8.1)]. Finally, the beads were washed twice with 1 ml TE buffer [1 mM EDTA and 10 mM Tris-HCl (pH 8.0)]. The immuno-complexes were then eluted by adding 150 µl elution buffer (10mM DTT, 1%SDS, 100mM NaHCO₃). The elute was collected and the cross-linking was reversed by adding NaCl to final concentration of 200 mM and incubating overnight at 65°C. The remaining proteins were digested by adding proteinase K. The DNA was phenol precipitated, ethanol precipitated, and amplified by realtime PCR using the Thermal Cycler Dice™ TP800 (Takara) and Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The primers for real-time PCR are as follows: 5'-CCTCCTGTAGGGCAGATGTG-3' and 5'-tAatccatgtcccgaagggtc-3' for Smad2

gene upstream region (-1040 bp to -937 bp), 5'-GGTTTGGGAGGACGAATTGTTAG-3' and 5'-CGTGCAGGCGGAAAGTTTGG-3' for Twist gene upstream region (-130 bp to -20 bp).

Luciferase reporter assay.

DNA fragments encoding Smad2 gene upstream region (-1500 bp to +13 bp) was amplified by PCR from MCF-7 genome and cloned into a pGL4 reporter plasmid (Promega). For luciferase assays, MCF-7 cells were co-transfected with a pGL4 empty plasmid or a pGL4 containing the upstream region of Smad2 plasmid and pcDNA3 SRC-3 expression plasmid. pRL-CMV, a reference plasmid used to normalize transfection efficiency. Twenty-four hours later, the cells were harvested and cell extracts were prepared. Luciferase assays were performed according to the manufacturer's protocol (Promega). Individual transfections, each of which was performed in three wells, were repeated at least three times. The primers for Smad2 upstream region cloning are as follows: 5'-CTGAAGGCACCCTCCTCTGGGA-3' and 5'-TGCTGGCTGGCGAGCTGCTTCT-3'

Primer sequences for real-time RT-PCR.

β -Actin fw primer: 5'-atcgccaccgcaaatgcttcta -3'

β -Actin rv primer: 5'-agccatgccaatctcatctgtt -3'

CHIP fw primer: 5'-aggccaagcagcagacaagtacat-3'

CHIP rv primer: 5'-ctgatcttgccacacaggtagt-3'

SRC-3 fw primer: 5'-cagtgattcagaaaacgca-3'

SRC-3 rv primer: 5'-cagctcagccaattcttcaat-3'

Smad2 fw primer: 5'-accgaaggcagacggtaacaag-3'
Smad2 rv primer: 5'- atgacatgcttgagcaacgcac-3'
 β -catenin fw primer: 5'-gaaacggcttcagttgagctg-3'
 β -catenin rv primer: 5'-taggatcatcctggcgatatcc-3'
Twist fw primer: 5'-tgcagctatgtggtcagcag-3'
Twist rv primer: 5'-aatgacatctaggtctccggcc-3'
Bcl2 fw primer: 5'-agcatggctcaaagtcagct-3'
Bcl2 rv primer: 5'-cagatgtccctaccaaccaga-3'
Akt1 fw primer: 5'-aagcaccgctgacccatgaac-3'
Akt1 rv primer: 5'-ctggccacgatgactcctc-3'
Vimentin fw primer: 5'-ggaacccagatgcgtgaaatg-3'
Vimentin rv primer: 5'-caggtcttggtattcacgaagg-3'.
IGF-1 fw primer: 5'-cctctcgcacatcttctactg-3'
IGF-1 rv primer: 5'- ctgctggagccataccctgtg-3'.
HPRT fw primer: 5'-ttccttggtcaggcagtataatcc-3'
HPRT rv primer: 5'-agtctggcttatatccaacacttcg-3'.
18S fw primer: 5'-cgacgaccattcgaacgtct -3'
18S rv primer: 5'-ctctccggaatcgaaccctga -3'.