Supplementary Material

1. Supplementary Methods

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Supplementary Methods

Constructs and plasmids

The following expression vectors were described elsewhere: ABL-ΔSH3, ABL, pCEFL Ha-SOS1, ABI1 [1], myc-EPS8 [2], GST-SH3-GRB2 (N-terminal) [3], His-PxxP-SOS1, GST-CRIB [4]. BCR-ABL and BCR-ABL mutant resistant to STI 571 were a gift from Justus Duyster's laboratory, His-DH PH Tiam1 was a gift from John Sondek's laboratory, GFP-BCR-ABL was a gift from Rick Van Etten laboratory, pMFG-HA-mSOS1 (used for the reconstitution of SOS1-/- fibroblasts) was a gift from Egan laboratory, GFP-VAV-DN was a gift from Xosé Bustelo laboratory. SOS1-Y1196F was generated by recombinant PCR. GST-RAC1 and His-RAS were described previously [5]. DH-CDC25-pProEx-Hisb was a gift from J. Kuriyan. GST and Histidine bacterial expression vectors carrying various SOS1 fragments (GST-Histon-fold, GST-DH, GST-DH-PH, GST- H-DH, GST-H-PH, His- H-CDC25) were generated by recombinant PCR and cloned into pGEX-6p1 or pTRC-HisA, respectively.

Reagents and antibodies

The following antibodies were used: anti-SOS1, anti-Hemoglobin and anti-ABL (Santa Cruz Biotechnology, Santa Cruz, CA), anti-ABI1 was generated against the peptide N-PPVDYEDEEAAVVQYNDPYADGDPAWAPKNYI-C [6]; the following

mouse monoclonal antibodies were used: anti-RAC (Transduction Laboratories, Lexington, KY) and anti-Ha (12CA5, Covance, Princeton, NJ), anti-pTyr (Upstate Biotechnology, Lake Placid, NJ), anti-pY412-ABL (Abcam, Cambridge, UK), anti-Myc 9E10 (Babco, Berkeley, CA), anti-Tubulin (Sigma-Aldrich, St. Louis, MO), anti-pAKT, anti-MAPK, anti-pMAPK (Cell Signaling, Boston, MA). Biotinylated SOS1 phosphorylated and non-phosphorylated peptide (KAYSPR(pY)SISDRT) were purchased from Cambridge Research Biochemicals Limited (Cleveland, UK). A monoclonal mouse antibody against pY1196 was generated suing KAYSP(pY)SISDRT conjugated to KLH. The T-Cell protein tyrosine phosphatase, the T-Cell protein tyrosine phosphatase inhibitor and -were from New England Biolabs (Beverly, MA). Nitrocellulose plates were from Merck (Darmstadt, Germany), STI571 was a gift from Novartis Pharma AG (Basel, Switzerland), TRITC-phalloidin was from Molecular Probes (Eugene, OR), PDGF was from Immunological Sciences (Rome, Italy), Heregulin was from R & D Systems (Minneapolis, MN), EGF was from Peprotech (London, UK), Annexin V and Propidium Iodide were from Sigma-Aldrich (St. Louis, MO), 2'-(or-3')-O-(N-methylanthraniloyl)guanosine 5'-diphosphate, disodium salt (MANT-GDP) was from Invitrogen (Milano, Italy), [^{3}H]-GDP was from Amersham. Methylcellulose MethoCult #3434 was from StemCell Technologies (Vancouver, BC, Canada). Recombinant murine Interleukin-3 (IL-3), IL-6 and SCF were purchased from R&D System (Minneapolis, MN, USA). Hanks' Balance Salt Solution (HBSS 1X) was from Sigma-Aldrich (St. Louis, MO). Tamoxifene was from Calbiochem (San Diego, CA, USA).

5-Flouorouracil (5-FU) was from Sigma-Aldrich (St. Louis, MO, USA). Anti B220, anti Tyr 1.2, anti GR-1 and anti Mac-1 were from eBioscience (San Diego, CA,

USA). IMDM (Iscove's Modified Dulbecco's Medium) was from Sigma-Aldrich (St. Louis, MO).

Mass spectrometry

In-gel digestion. Immunoprecipitated SOS1 was *in vitro* phosphorylated and separated by SDS PAGE. The protein was reduced and alkylated by 20 mM DTT for 30 min at RT and 50 mM iodoacetamide for 30 min at RT in the dark, respectively. Digestion was carried out by adding 12.5 ng/µl of trypsin in 25mM NH_4HCO_3 (Proteomics Grade, Sigma). Peptides were acidified to 2% of TFA prior to loading onto a StageTip[30]. Peptides bound to C18 phase were eluted in 10 µl of buffer B (80% acetonitrile, 0.5% acetic acid) and lyophilized. Lyophilized peptides were re-suspended in buffer B containing 300mg/ml DHB to selectively enrich phosphopeptides[31] and loaded onto a self-packed Gelloader tip (Eppendorf, Hamburg, Germany) plugged with C18 material (3M Empore^{TM} C_8 disk, 3M Bioanalytical Technologies, St. Paul, MN), filled with 2mm of TiO_2 beads (GL Sciences Inc. Tokyo, Japan). Eluted peptides with 10 µl of NH_4OH, pH 10.5 were analysed by nanoLC-MS/MS. LTQ-FT mass spectrometer (ThermoElectron, Bremen, Germany) coupled online to an Agilent 1100 binary nano pump (Palo Alto, CA). To prepare an analytical column with a self-assembled particle frit, C_{18} material (ReproSil-Pur C18-AQ 3µm; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) was packed into a spray emitter (75 µm ID, 8 µm opening, 70 mm length; New Objectives, USA) using an air-pressure pump (Proxeon Biosystems, Odense, Denmark). The column was equilibrated with solvent A (2% acetonitrile and 0.1% formic acid). Bound peptides were eluted using a 160-min linear gradient (from 0 to 30% [vol/vol]) of solvent B (80% acetonitrile and 0.085% formic acid) at a 150-nl/min flow rate. The six most

intense peaks of the MS scan were selected in the ion trap for MS (Normal scan, wideband activation, filling 5e5ions for MS scan, 10^4 ions for MS2, maximum fill time 100 ms, dynamic exclusion for 180 seconds). Raw-files were processed using DTA-supercharge (msquant.sourceforge.net). The generated peak lists were searched against the IPI human database using Mascot 2.0 with the parameters: monoisotopic masses, 5 ppm on MS and 0.5 Da on MS/MS, ESI TRAP parameters, fully tryptic specificity, cysteine carbamidomethylated as fixed modification, phosphorylation on Serine, threonine and Tyrosine, oxidation on methionine and protein N-acetylation as variable modifications. Three missed cleavage sites were allowed. The pY containing peptide was not identified automatically by Mascot search since it was below the automatically-assigned, statistically significant threshold of 20. We than searched the unassigned MS/MS dataset for spectra containing pTyr immonium ion at m/z 216.043[32, 33] and subsequently sequenced manually the peptide of 410.665 (doubly charged) mass corresponding to pYSISDR.

Mice strains

All animal experiments were approved by the OPBA (Organisms for the well being of the animal) of IFOM and Cogentech. All experiments complied with national guidelines and legislation for animal experimentation. All mice were bred and maintained under specific pathogen-free conditions at IFOM animal facility.

The generation of a Tamoxifen inducible *Sos1*-/- mice strain was previously described[34]. Briefly, a strain harboring a floxed version of *Sos1* with LoxP sites flanking exon 10 ($Sos1^{fl/fl}$)[35] was crossed with mice expressing a TAM-inducible Cre recombinase downstream of the RERT (Jackson Laboratories; stock number 017585) This strain expresses an inducible Cre-ERT2, Cre recombinase fused to a triple

mutant form of the human estrogen receptor, from the endogenous and ubiquitous Polr2a locus promoter and was used to generate homozygous *Sos1*$^{fl\text{-}Cre}$/*Sos1*$^{fl\text{-}Cre}$ mice. Experiments with primary cells are described in details in Supplementary Methods

Statistical Analysis.

Differences between experimental groups were examined for statistical significance using the paired Student's *t*-test, unless otherwise indicated. Data are expressed as mean ± s.e.m.

Cell culture and cell biological assays

All cells were provided by IFOM cell culture facility and their identity verified by finger printing. 32D-BCR-ABL cells were a gift from Skorski laboratory.

K562, SOS1-/- MEF, and 293T Phoenix cells were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) supplemented with 10% fetal bovine calf serum (FCS), 100 µg/ml streptomycin, 100 µg/ml penicillin and 2 mM glutamine.

MDA-MB-231, MDA-MB-453 cells were grown in Leibovitz L15 (Leibovitz L15, Gibco) supplemented with 10% fetal bovine calf serum (FCS), 100 µg/ml streptomycin, 100 µg/ml penicillin and 2 mM glutamine. Medium for MDA-MB-435s contained further 10µg/ml Insulin.

Transfections were performed using either calcium phosphate or Lipofectamine (Invitrogen, Carlsbad, CA, USA) reagents, according to manufacturer's instructions. Lipofectamine reagent was used for biochemical studies in MEF cells.

Stable KD of SOS1 was generated in K562 cells by retroviral infection using a pSuperRetro-Puro vector carrying the following short hairpin oligos: 256

(gcaatagctgatgcccaatca), 1640 (ggagtacactggaaaggatgc), 3347 (gcacagcatctgcaccaaact) specific for human SOS1. SOS1 -/- mouse embryo fibroblasts were reconstituted with pMFG-Ha-mSOS1 WT or Y1196F by retroviral infection. Preparation of primary derived BMDC from WT and retroviral infection of with pMig-210-BCR-ABL is described below.

Proliferation assay

Equal amounts of K562 (wild type or SOS1-KD) cells (50.000 cell/ml) were cultured in the absence or presence of 10μl STI571 or 10% of WEHI conditioned medium as a source for Interleukin 3. Cells were counted with standard counting chambers after 48h in the presence of Trypan Blue.

Apoptosis assay

Equal amounts of K562 (wild type or SOS1-KD) cells (50.000 cell/ml) were cultured in the absence or presence of 10 μM STI571 or 10% of WEHI conditioned medium as a source for Interleukin 3. Cells were harvested after 24h and washed with Annexin buffer (10mM Hepes pH 7.5, 150mM NaCl, 1mM $MgCl_2$, 3.6mM $CaCl_2$, 5mM KCl). Cells were re-suspended in 50 μl AnnexinV-FITC (diluted 1:50 with Annexin buffer). After 1h incubation at room temperature in the dark, cells were washed once with Annexin buffer. Cells were re-suspended in 500 μl PBS and 1 μl Propidium Iodide (stock 50 μg/ml) was added and immediately processed for FACS analysis.

Cell lysates, immmunoprecipitations and immunofluorescence

Whole Cell Lysates: After washing with PBS, cells were lysed in lysis buffer [50 mM HEPES PH 7.5, 50 mM NaCl, 1% glycerol; 1% Triton X-100, 1.5 mM $MgCl_2$. 5 mM EGTA plus protease inhibitor cocktail (Roche, Basel, Switzerland), 1 mM DTT, 20 mM Na pyrophosphate pH 7.5, 50 mM NaF, 0.5 M Na-vanadate in HEPES pH 7.5 ti nhibit phosphatases] directly on plates using a cell-scraper. About 200 μl of lysis

buffer buffer/10 cm plates was used. Lysates were incubated on ice for 10 minutes and spun at 12000 rpm for 10 min at 4ºC. Total protein concentration was measured by the Bradford assay (Biorad, Berkely, CA, USA), following manufacturer's instructions.

Immunoprecipitation: Protein A Sepharose beads (Sigma, for rabbit polyclonal antibodies) or protein G Sepharose beads (Zymed, for mouse monoclonal antibodies) were added, and samples were left for an additional hour at 4ºC, rocking. Immunoprecipitates were then washed four times in lysis buffer. After washing, beads were re-suspended in 1:1 volume of 2x SDS-PAGE Sample Buffer, boiled for 5 minutes at 95ºC, and loaded onto polyacrylamide gels.

Western blotting was carried out as described [1]. Immunofluorescence: Cells plated on glass coverslips were pre-incubated with 0.5% gelatin in PBS at 37˚C for 30 minutes and fixed in 4% paraformaldehyde (in PBS) for 10', permeabilised in PBS 0,1% Triton X-100 for 10 minutes at room temperature, blocked with 2% BSA for 30 min, and then incubated with primary and secondary antibodies for 45 and 30 min, respectively. F-actin was detected by staining with FITC-conjugated phalloidin (Sigma-Aldrich) at a concentration of 6.7 U ml^{-1}.

Immunohistochemistry: Formalin-fixed and paraffin-embedded of agarose cell-blocks prepared with ΔSH3ABL with SOS1 or ΔSH3ABL with SOS1Y1196F co-overexpressing Phoenix cells was performed as described [7]. Epitope unmasking was carried out in 0.25 mM EDTA, slides were incubated overnight at +4°C with primary anti-SOS1pY1196 antibody diluted 1:25 followed by detection HRP-conjugated secondary antibodies were used. Samples were developed with DAB and were counterstained with hematoxylin.

Soft agar assay

Difco Agar (3 g) was dissolved in 100 ml water and kept at 50°C. DMEM containing 25% fetal bovine calf serum (FCS) was kept at 40°C. Two volumes of Agar were mixed with 8 volumes of DMEM+FCS to a final concentration of 0.6% Agar and 20%FCS in DMEM. 2 ml were poured in a 6well plate and allowed to solidify. 100.000 cells in a volume of 0.9 ml DMEM (with or without STI571 or 10% WEHI conditioned medium as a source for Interleukin3) were mixed with 0,1 ml of Agar and poured over the already solidified Agar basis. The final concentration of Agar in the cell containing top layer is 0.3%. After the solution has been cooled down to room temperature, the plate was transferred to the 37°C incubator. After 14 days Colonies were photographed.

Methyl-cellulose, instead of Agar, was used for colony formation assays of primary BMCs infected with BCR-ABL [8] (see also below).

Biochemical assays

***In Vitro* Kinase assay**

The in vitro kinase assay was performed according to the manufactures protocol (New England Biolabs). Briefly, immunoprecipitated SOS1 or purified proteins (2μM) were incubated with 22nM ABL and 100μM ATP for 30min at 30°C. The final reaction volume was 50μl. After the reaction mixture was subjected to SDS-Page and immunoblotting.

Phosphopeptide mapping

The phosphopeptide mapping was performed as described [9]. Briefly, SOS1 or SOS1Y1196F were transfected in Phoenix, immunoprecipitated and in vitro phosphorylated with [^{32}P]-ATP by ABL. The reaction mixture was subjected to SDS-PAGE and Western blot. After Ponceau-S staining the corresponding SOS1 bands

were cut out and placed in 0.5% Polyvinylpyrrolidone360 in 100mM Acetic Acid for 30min at 37°C. After washing the membrane extensively with H_2O and with 50mM NH_4HCO_3, the proteins were digested with Trypsin. Dried peptides were oxidized in freshly made performic acid and dried again. After resuspension in H_2O, the labeled peptides were spotted onto a cellulose thin layer plate. The peptides were resolved in an electric field (1kV) for 20min in a pH8.9 ammoniumcarbonate buffer. After air-drying the plate, the peptides were separated in the second dimension by subjecting the plates to a thin layer chromatography in phosphochromobuffer (75 vol n-Butanol, 50 vol Pyrridine, 15 vol Acetic Acid and 60vol H_2O). The dried plates were analyzed by Phosphoimager standard procedures.

Purification of phosphorylated pY1196 SOS1

Lysates (200 mg) of 293T Phoenix cells transfected with HaSOS1 and ΔSH3-ABL were incubated with GST-SH3-Grb2 (190nmol). Bound proteins were eluted with 240 nmol of His-PxxP-SOS1. Soluble, phosphorylated SOS1 was immunoprecipitated with 900 μg (5,4 nmol) of antibody against pY1196 SOS1 and eluted with 100 nmol of phosphorylated pY1196-containing peptide. See also **Fig. 3.**

GEF Assays with ^{3}H-GDP

In vitro GEF activity of SOS1 purified from mammalian cells toward H-Ras or Rac1 was performed in the presence of ^{3}H-GDP, exactly as described [1]. Data are averages (with standard deviations) of at least three independent experiments performed in triplicate. Results are expressed as ^{3}H-GDP released after 20 min relative to time zero. Control reactions were obtained by incubating ^{3}H-GDP-loaded Rac either in exchange buffer or in the presence of the non-related protein, bovine serum album (BSA).

GEF assay with Fluorescent Mant-GDP

The reaction was performed as described [10]. Briefly, the reaction conditions were 25mM Tris HCl pH 7.6, 100mM NaCl, 10mM $MgCl_2$, 1mM DDT, 2μM MANT-GDP, 5μM GST-RAC and the DH-PH of Tiam1 (1μM) or purified pY1196-SOS1 (30 and 120 μl purified protein corresponding to 2nM or 8nM, respectively) in a final reaction volume of 200μl. The reaction containing all reagents except for the GEFs was allowed to equilibrate for about 500s. Fluorescence emission was monitored using a Safax flx fluorimeter using the following conditions: λex: 360nm, λem: 440nm, slits 5/5nm. Data are representative of at least three independent experiments.

RAC-GTP Crib Assay

The crib assay was performed as described [11]. Briefly, 1 mg of cell lysate was incubated with 30ug of purified GST-CRIB for 1h at 4°C. Beads were washed 3 times with lysis buffer and subjected to SDS-Page with following immunoblotting for RAC.

Transduction of murine bone marrow derived cells and methylcellulose re-plating assays

C57BL/6 or TAM-inducible Cre recombinase $SOS1^{fl/fl}$ mice [12], 12 weeks of age, were treated with 5-FU (150mg/Kg). After 4 days the mice were sacrificed, and bone marrow (BM) was collected by flushing as described [13]. Briefly, BM cells were pre-stimulated overnight in BBMM medium (IMDM, 30% fetal calf serum, 1% bovine serum albumin, 10ng/ml mIL-3, 10ng/ml mIL-6, 50ng/ml mSCF) were infected by four rounds of spin infection with pMigBCR-ABL^{p210} IRES GFP and plated in methylcellulose MethoCult #3434 (StemCell Technologies, Vancouver, BC, Canada) in the absence or presence of tamoxifen (100 nM) to induce SOS1 deletion. $EGFP^+$ BMCs ($0.5–1 \times 10^3$) in 1 ml of methylcellulose medium per 12 wells were plated in duplicates or quadruplicates and the colonies were photographed and counted on day

9. For Re-plating assays, Colonies were washed out of the MC, counted and 10.000 or 5.000 cells were re-plated in duplicates and the colonies were photographed and counted on day 10 after seeding.

Transplantation assays

After transduction of BMDCs with pMigBCR-ABL^{p210} 20.000 sorted GFP^{+} and 180.000 Support BMDC/ per mice cells were transplanted into lethally irradiated recipient mice. For Homing/Engraftment assays, BMDC of recipient mice were harvested 24 hours after transplantation and analyzed for GFP-positive cells by FACS analysis. Mice were treated with TAM (1 mg in peanut oil/ 25 g body weight) or solvent at day 1 and 2 after intra-peritoneal transplantation. Peripheral blood of mice was taken at the indicated time points and WBCs were measured automatically (ABC scil vet) to monitor leukemia induction. Leukemic burden was measured by GFP-FACS analyses of peripheral blood at indicated time points. For Hematopoietic/Leukemic stem cell analyses, spleen cells of leukemic mice were harvested and stained for lineage marker (CD4, CD8, CD3e, B220, Ter119, Gr1, CD11b), cKit, Sca1, CD150 and CD48 and analyzed by FACS. Stem cells were expected to be alive, EGFP+Lin-Sca1+cKit+ or EGFP+Lin- and/or CD150+CD48- cells.

Xenotransplantation of K562 cells into immunocompromised mice

An equal number (5×10^5) of control and SOS1-KD K562 cells were injected into the tail vein of immunocompromised NOD/SCID (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice. After injection mice were weighed and monitored daily for sickness and tumor formation. Mice were monitored for up to 60 days and were euthanized when

approximately 20% of total body weight was lost or display impairment in eating or walking. Single cell suspensions were stained for FACS with glyA PE (GA-R2) (BD biosciences) to detect tumour cells (not shown).

Supplementary Figures and Movie Legends

Figure S1. Mapping of the minimal region of SOS1 tyrosine phosphorylated by ABL kinase.

A. *Left Panels:* full length SOS1 was immunopurified with anti-SOS1 abs from lysates of mammalian cells ectopically expressing SOS1. Irrelevant IgG-beads were used as control (Ctr). *Middle and right panels*: SOS1fragments were produced in bacteria and purified by affinity chromatography with either Nickel-NTA (His-1-CDC25, H-CDC25; and His-PxxP, PxxP,) or with Glutathione-sepharose beads (Histone-fold, H; DH, DH-PH, H-DH-PH). SOS1 full length and various fragments were subjected to ABL kinase assay or mock treatment (Ctr). Typically, 2 μM of each recombinant protein was used. After the kinase reaction, the proteins were separated by SDS-Page and detected either with anti-SOS1 antibody, in the case of full length SOS1 (*Left panels*), or by PonceauS staining. Membranes were immunoblotted with anti-pY abs (IB). As positive control (Control) of ABL kinase reaction a GST-fused-fragment (aa: 1-285) of ABI1 (GST-ABI1), a known interactor of ABL[57], was used.
B. A scheme of the various SOS1 fragments with their amino acid boundaries and domain organization and their tyrosine phosphorylation status after ABL kinases is shown.
C. Lysates (1 mg) of 293T cells transfected with WT-SOS1 or Y1196F-SOS1 together with ABL-ΔSH3 were immunoprecipitated (IP) using an anti-SOS1 antibody. Lysates (20 μg) and IP were separated by SDS-Page and analyzed by immunoblotting with the indicated antibodies (Ab).

Figure S2. Development and characterization of mouse monoclonal anti-pY1196 SOS1 antibodies

A. Summary table of pY1196 SOS1 monoclonal antibody production**.** We obtained 43 different hybridomas. Supernatants from either the mass population (First Fusion on the right), or from individual clones (Clones, on the left) of hybridomas were initially tested by Elisa, using either Y1196-phosphorylated or non-phosphorylated-containing biotinylated peptides. Antibodies displaying specific binding to phosphorylated peptides (color labeled) were further tested by immunoblotting (IB on lys) and immunoprecipitation (IP) on lysates of cells expressing either SOS1, or SOS1 and ABL-ΔSH3, or Y1996F-SOS11 and ABL-ΔSH3 (See also Figure 2B-C). Selected hybridomas were further tested for their ability to immunoprecipitate phosphorylated pY1196-SOS1, but not unphosphorylated WT-SOS1 or Y1196F-SOS1 (See Fig. 2B-D). Finally, some fusions were tested in immunofluorescence (IF on Oex) and immunohistochemistry on cell expressing various combination of WT-SOS1, Y1196F-SOS1 together with activated ABL-ΔSH3. The most promising mass populations of hybridomas were sub-cloned (color labeled, but not yellow) (BS12, BI20, BD7, AFG12, VP20, TR16, TR54, TF7). Only TR16 (grey) and TF7 (light orange) clones remained positive during the cloning procedure. TR16 clones produced monoclonal antibody efficient for immunohistochemistry. TF7 clones produced antibodies for immunoblotting, immune-precipitations (see also Fig. 2B-D) and immunofluorescence analysis. The specific strength of binding of the antibodies to the phosphorylated peptide is indicated with + (+ = low binding, ++++ = high binding, - = no specific binding).

B. Example of the efficacy of the monoclonal antibody obtained from clone TR16 in detecting pY1196-SOS1 by immunohistochemistry (Left images) and immunofluorescence (Right images). Left images, Phoenix cells were transfected with either WT-SOS1 or Y1196F-SOS1 together with ABLΔSH3, paraffin embedded and

processed for immunohistochemistry analysis using an anti-pY1196 SOS1 ab (TR16 clone) and an ABL ab. Bar, 50 μm. Right images, mouse embryo fibroblasts were transfected with WT-SOS1 or Y1196F-SOS1 together with GFP-BCR-ABL. Cells were fixed and stained with an anti-p1196Y-SOS1 or anti-total SOS1 ab. Bar, 20 μm.

Figure S3. SOS1 is tyrosine phosphorylated *in vivo* on Y1196

A. Lysates of 293T cells, transfected with ABL-ΔSH3, HA-WT-SOS1 and HA-Y1196F-SOS1 as indicated, were immunoblotted with anti-ABL, anti-SOS1 and anti pY1196-SOS1 Abs. HA-immunoprecipitates were immunoblotted with anti-SOS1 and the phosphospecific anti-pY1196 ab. pY1196-SOS1 was immunoprecipitated with anti-pY1196-SOS1 ab. The immunoprecipitated tyrosine phosphorylated SOS1 was detected by immunoblotting using an anti-HA ab, to prevent the detection of endogenous phosphorylated SOS1.

B. Serum starved *Sos1*-/- Mouse embryo Fibroblasts (MEFs), reconstituted with murine HA-SOS1, were stimulated with 30 ng/ml PDGF for 7 minutes. Lysates and SOS1 immunoprecipitates were immunoblotted with the indicated abs.

Breast cancer cells, MDA-MB-353 and MDA-MB231, were starved overnight and treated with 10 μm of STI571 or vehicle as a control for 1h, before adding the indicated ligands [Heregulin (Hrg_ = 160 ng/ml) or Epidermal growth factor (EGF = 100 ng/ml)] for 7 minutes. Lysates (20 μg) and SOS1 immunoprecipitates were immunoblotted with the Abs indicated on the right.

Figure S4. Purification scheme of phosphorylated pY1196F-SOS1 (related to Figure 2).

A. Phosphorylation of Y1196 triggers RAC, but not RAS GEF activity. Lysates (1mg) from 293T cells transfected with WT- or Y1196F-SOS1 together with ΔSH3-ABL were immunoprecipitated using an anti-SOS1 antibody (See Supplementary Figure S3A). Equal amounts of immunopurified SOS1 were subjected to either *in vitro* RAC1 (A^{i}) or H-RAS filter binding GEF assays using radiolabelled 3[H]-GDP (A^{ii}). The assays were performed in triplicates. Data are the mean ± s.e.m. (n = 4). Student T-test, * = $P < 0.001$. The RAC1 or H-RAS GEF reactions mix were also resolved on SDS-PAGE (See S3A, bottom panel) and stained with PonceauS to compare the amounts of SOS1 used in each assay.

B**.** Purification of tyrosine phosphorylated SOS1 and Y1996F mutant from cells co-expressing activated ABL. Lysates (1mg) from 293T cells transfected with WT- or Y1196F-SOS1 together with ΔSH3-ABL were immunoprecipitated using an anti-SOS1 antibody. Equal amounts of immunopurified SOS1 were subjected to either *in vitro* GEF assays for RAC1 and H-RAS as described in Figure 3. The lysates (20 μg) and immunoprecipitates were immunoblotted with the Abs indicated on the right or stained with PonceauS. On the bottom panel, the RAC1 or H-RAS GEF reactions mix were also resolved on SDS-PAGE and stained with PonceauS to compare the amounts of SOS1 used in each assay

C. On the right, a double-affinity purification scheme of tyrosine phosphorylated SOS1 is shown. Lysates (200 mg) of 293T cells transfected with HA-SOS1 and ABL-ΔSH3 were incubated with GST-SH3-GRB2 (190 nmol). Bound proteins were eluted with 240 nmol of His-PxxP-SOS1. Soluble, phosphorylated SOS1 was immunoprecipitated with 900 μg (5.4 nmol) of immobilized antibody against pY1196-SOS1 and eluted with 100 nmol of phosphorylated peptide. 0.01% of the lysates before GRB2 binding (Input), 0.5% of the GRB2 pull down (SH3-GRB2),

0.5% of the His-PxxP-SOS1 eluted fraction (PxxP-SOS1), 0.5% of the His-PxxP-SOS1 not eluted fraction (not eluted), 0.5% of the fraction that didn't bind to the pY1196 antibody and 20 % of the final eluted pY1196 SOS1 were immunoblotted with the indicated Abs.

Figure S5. SOS1 proline-rich region interacts directly with the DH-PH domain and mediates an inter-molecular interaction controlled by Y1196 phosphorylation

A. SOS1 proline rich region (PxxP) interacts with the DH-PH tandem motif. Equal amounts (10 pmol or 30 pmol) of various recombinant SOS1 fragments (Left panel: H, DH, DH-PH, DH-CDC25, H-CDC25, Right panel: DH-PH, PH, DH see also the schematic representation of the various fragments used in B) or BSA, as a control, were spotted onto nitro cellulose membrane. After incubation with 0.5 µM recombinant His-PxxP in solution, the binding of the PxxP to the fragments was determined by immunobloting using the anti-SOS1 antibody, which recognizes the proline-rich region of SOS1.

B. Scheme of the various SOS1 fragments and their binding ability to the proline rich region (PxxP) of SOS1. "+" indicates binding; "-" indicate that no binding was detected.

C. SOS1 proline-rich (PxxP) region and its DH-PH domain interact with an apparent micromolar affinity. *Left panels*: Equal molar amounts (20 pmol) of DH-PH domain or BSA (control) were spotted onto nitrocellulose membrane. After the incubation with increasing concentrations of recombinant His-PxxP, the fraction of bound PxxP to the DH-PH domain was determined by immunoblotting using an anti-SOS1 antibody. *Right graph*: the apparent dissociation constant was determined (Kd) by

blotting the concentration of total PxxP against the bound fraction to the DH-PH domain. The half maximum binding was at ~ 2.8 μM. Data are the mean ± s.e.m. (n = 3 independent experiments).

D. Phosphorylation on Y1196 reduced the affinity of the PxxP of SOS1 for its DH-PH domain. *Left panels*, equal molar amounts (30 pmol) of SOS1 DH-PH domain or BSA, as a control, were spotted onto nitrocellulose membrane. Following incubation with increasing concentrations of phosphorylated or non-phosphorylated biotinylated peptides [amino acid sequence KAYSP(pY1196)SISDRT]. The amount of peptides bound to the DH-PH domain was determined using horseradish peroxidase conjugated to streptavidin. *Right graph*, the binding curves of the peptides are represented in the graph. The concentrations of the peptides were plotted against the amount of the peptides bound to the DH-PH domain. The maximum binding was set to 1. Data are the mean ± s.e.m. (n = 3 independent experiments).

E. SOS1 form inter-molecular interaction that are disrupted by activated ABL. Lysates (3 mg) from 293T cells transfected with GFP-SOS1, HA-SOS1 and activated together with ΔSH3-ABL were immunoprecipitated using an anti-GFP antibody. Lysate and IPs were immunoblotted with the indicated antibodies.

F. SOS1-Y1196F forms ABL-resistant dimers. Lysates (3 mg) from 293T cells transfected with GFP-SOS1-Y1196F, HA-SOS1-Y1196F and activated together with ΔSH3-ABL were immunoprecipitated using an anti-GFP antibody. Lysate and IPs were immunoblotted with the indicated antibodies.

G. SOS1-Y1196F acts as dominant negative. Lysates (3 mg) from 293T cells transfected with empty vector (-), or wild type SOS1, or SOS1-Y1196, or a DH—deficient mutant of SOS1 devoid of the proline rich region used as control were either immunoblotted (*top two panels*) with the abs indicated on the left or subjected

(*bottom panel*) to CRIB binding assays to detect RAC-GTP levels. Lysates and bound GTP-loaded RAC were immunoblotted with anti-RAC1.

Figure S6. Effect of reconstitution of *Sos1*-/- MEF with WT- and pY1196F-SOS1 (Related to Figure 3)

A. Abl-mediated RAC1 activation requires SOS1**.** *Left panels*: lysates (1mg) from 293T cells transfected with HA-WT- or HA-Y1196F-SOS1 in combination with activated ΔSH3-ABL were either immunoblotted (IB) with the Abs indicated on the left or subjected (*Right panels*) to CRIB binding assays to detect RAC-GTP levels. Lysates and bound GTP-loaded RAC were immunoblotted with anti-RAC.

B. *Sos1*-/- MEF were reconstituted with HA-WT-SOS1 or HA-Y1196F-SOS1 (YF) or with the empty vector (Ctr), as control. Lysates (20 μg) were immunoblotted with the indicated Abs.

C. Removal of SOS1 has no major impact on PDGF-induced MAPK or AKT signaling. Serum starved, *Sos1*-/- fibroblasts, reconstituted with either HA-WT- and HA-pY1196F-SOS1 or the empty vector (Ctr) were stimulated with 10 ng/ml of PDGF for the indicated time points. Lysates (20 μg) were immunoblotted with the antibodies indicated on the right.

D. Total and Y1196 phosphorylated SOS1 localize to PDGF-induced circular dorsal ruffles. Serum starved SOS1 -/- MEFs, reconstituted with HA-WT-SOS1 were stimulated with 10ng/ml of PDGF for 7 min. Cells were fixed and stained with an anti-pY1196 SOS1 abs (BD7), or anti-total SOS1 (anti-SOS1) or Tritc-Phalloidin to detect F-actin. Bar, 20 μm.

E. VAV proteins are not involved in PDGF-induced circular dorsal ruffle (CDR) formation. SOS1-/- MEF were transfected with a dominant negative form of VAV (GFP-VAV DN) or an empty vector (GFP). Cells were starved for 12h, stimulated with 10 ng/ml of PDGF for 7 min, fixed and stained Tritc-Phalloidin to detect F-actin. The % of GFP-positive cells with CDR were counted. The expression of GFP or GFP-VAV DN did not affect the % of CDR formation. At least 100 cells were counted for each condition. The data represents the mean ± s.e.m (n = 4 experiments). Right panel: Representative pictures of CDRs are shown. n.s. non-significant. Bar, 40 μm.

F. Phosphorylation of SOS1 on Y1996 is required for directional migration. Serum starved *Sos1*-/- MEFs, reconstituted with control or HA-WT-SOS1 or HA-Y1196F-SOS1 or the empty vector (Ctr), were monitored by time lapse light field microscopy for 3.5 h, before adding 10 ng/ml of PDGF and further analyzed for 15 more hours. *Left grap,* velocity of cells was determined before and after the addition of PDGF by manually tracking cells using Image J software. The mean ± s.e.m. velocity of at least 50 cells is shown. The statistical relevance was determined by t-test and is indicated with * (*=0.0028; **= 0.0005). *Right graph,* the circularity factor of cells treated with PDGF was determined with the help of Image J software by determining the ratio between long and short axis. To determine the mean value of circularity at least 50 cells were measured. The statistical relevance was determined by t-test and is indicated with * (*=0.0326; **=0.0009; ***=1,.8320E-07; ****=1.3919E-05). See also Movie S1.

Figure S7. SOS1 is phosphorylated in BCR-ABL leukemic blast (related to Figure 4)

A. BCR-ABL expression in hematopoietic K562 and 32D cells. Total lysates (20μg) of BCR-ABL-expressing cells, K562, 32D–BCR-ABL and control 32D cells were immunoblotted with anti-ABL antibody. The slower-migrating band represents the isoform p210 BCR-ABL, while the faster-migrating band is endogenous ABL.

B. BCR-ABL mediates SOS1 phosphorylation on Y1196. SOS1 was immunoprecipitated from 1 mg of total cellular lysates of K562 and 32D–BCR-ABL, treated with 10 μM STI571 or vehicle for 1h. Lysates (20 μg) or immunoprecipitates were immunoblotted with the indicated Abs.

C. SOS1 loss does not affect ERK1/2 activity in K562 cells. K562 cells infected with pSuperRetroPuro Control vector (Ctr) or pSuperRetroPuro SOS1 interfering vector (SOS1-KD) were treated for 10 min with 10 μM STI571 or vehicle as control (Ctr). Lysates (20μg) from vehicle-treated or STI571-treated control cells and SOS1 interfered K562 cells were immunoblotted to detect ERK1/2 phosphorylation.

D. shRNA-mediated knocked down of SOS1 in K562 cells does not cause apoptosis. Equal number (50.000 cells/ml) of K562 control cells (Ctr), STI571-treated, NSC2376-treated or SOS1-KD cells (SOS1 KD) were seeded into 6 well plates. After 24 hours, cells were collected, fixed and stained with Annexin V. Cells were then analysed by FACS analysis. The percentage of cells, which is positive for Annexin V (Proapoptotic marker), was calculated. Data represents the mean±s.e.m of three independent experiments. ** $P < 0.005$.

E. shRNA-mediated stable SOS1 interference of K562 leads to the acquisition of erythrocytes features. Control (Ctr) or SOS1-KD K562 cells were extensively washed with PBS and pelleted by centrifugation. Control K562 cells have a white appearance, while the SOS1-interfered cells have a red appearance (*Upper panel*).

Lysates (20 μg) of control (Ctr) or SOS1-KD K562 cells were immunoblotted with the Abs indicated on the right (*Lower panels*).

Figure S8. Re-expression of shRNA-resistant WT, but not of Y1196F-SOS1 into SOS1-KD K562 cells restores proliferation, transformation and RAC activation

A. RNAi-resistant WT-, but not Y1196F-SOS1 restores the levels of hemoglobin expression and RAC activation of SOS1-KD K562 cells. Control and SOS1-KD cells were stably infected with lentiviral vectors expressing either GFP or RNAi-resistant WT or Y1196F-SOS1. Lysates of the various K562 cells were immunoblotted with the Abs indicated on the right.

B. Lysates (20μg) from parental control K562 and SOS1-KD K562 cells re-expressing GFP or RNAi-resistant WT or Y1196F-SOS1 were incubated for 1h with 20 μg of GST-CRIB to pull down GTP-loaded RAC. Lysates (20 μg) of the various K562 cells and bound proteins were immunoblotted with the Abs indicated on the right.

C. RNAi-resistant WT-, but not Y1196F-SOS1 restores cell proliferation of SOS1-KD k562 cells. An equal number (50.000 cells/ml) of control (Ctr), or SOS1-KD K562 cells re-expressing either GFP or shRNA-resistant WT or Y1196F-SOS1 were seeded. After various time points, cells were counted. Data are the mean±s.e.m. of 4 independent experiments. *** $P < 0.001$ Student's t-test.

D. RNAi-resistant WT-, but not Y1196F-SOS1 restores K562 transformation. An equal number (100.000 cells/ml) of control (Ctr), or SOS1-KD K562 cells re-expressing either GFP or shRNA-resistant WT or Y1196F-SOS1 were plated in soft agar. Quantification of colony number is shown. At least 50 colonies were counted.

Data are the mean ± s.e.m (n=3 independent experiments). ** $P < 0.005$ Student's t-test.

Figure S9. Characterization of control and *Sos1*$^{fl/fl}$ BMDC infected with BCR-ABL (related to Figure 5)

A. *Sos1* loss impairs transformation. A^{i}. 5-FU-enriched BM-derived progenitor cells from Sos1$^{fl/fl}$ and control mice were infected with pMigBCR-ABL^{p210} retrovirus and plated in methylcellulose (MC) without growth factors in the absence or presence of tamoxifen to induce Sos1 deletion as indicated. Representative photographs of methylcellulose colonies of Sos1$^{fl/fl}$ and control BMC infected with pMigBCR-ABL^{p210}. Bar, 200 µm. A^{ii}. CFUs were quantified nine days after plating, results are shown from two independent experiments performed in duplicates and expressed relative to control SOS1$^{fl/fl}$. P values were determined by Student t-test, *** $P < 0.001$. A^{iii}. PCR-Analyses revealed correct deletion of Sos1 in TAM-treated samples. DNA was prepared from BMC washed out of the MC 12 after seeding and PCR performed as described.

B. FACS analyses of BCR-ABL-infected bone marrow cells nine days after seeding in methylcellulose demonstrated mainly myeloid (Mac-1 and Gr-1-positive cells), but also some lymphoid cells (Thy1.2 and B220-positive cells) independently of Sos1 deletion.

C. PCR-analyses revealed correct deletion of *Sos1* in TAM-treated animals. DNA was prepared from spleen cells of diseased animals.

D. Homing efficiency of BMDC with and without *Sos1* deletion in vivo. BMDC were harvested 24 hours after transplantation from 3 representative mice and subjected to flow cytometry. EGFP-positive cells from harvested BMDCs were compared in

relation to injected EGFP-positive BMDCs at transplantation. P-values were determined by Students t-test; n.s. non-significant

E-F. FACS analyses of BCR-ABL-positive spleen cells revealed no differences in leukemic stem cells (D, LSC) and CD150+/CD48$^-$ (E) stem cell level upon *Sos1* deletion. EGFP-positive spleen cells were harvested from leukemic mice and stained for lineage-markers c-kit, sca-1 (not shown) and CD150 and CD48. n.s. non-significant, Student t-test.

G. Re-plating efficiency was similar in *Sos1* deleted and control BMDCs after BCR-ABL infection. 5.000 or 10.000 BCR-ABL-infected BMCDs were re-plated nine days after first seeding in methylcellulose. Colonies were counted 10 days after re-plating and p-values were determined by Students t-test; n.s. non-significant

Movie S1

Serum starved *Sos1* -/- MEFs, reconstituted with control or HA-WT-SOS1 or HA-Y1196F-SOS1 or the empty vector (Ctr), were monitored by time lapse light field microscopy for 3.5 h, before adding 10 ng/ml of PDGF and further analyzed for 15 more hours. Images were taken every 5 min.

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