HOKKAIDO UNIVERSITY

Title	STAP-2 interacts with and modulates BCR-ABL-mediated tumorigenesis
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Supplementary information

Supplementary Methods

RT-PCR and quantitative real-time PCR

Cells were harvested, total RNAs from the transfected cells were prepared by using Iso-Gen (Nippon Gene, Tokyo, Japan) and used in RT-PCR. RT-PCR was performed using RT-PCR high -Plus- Kit (TOYOBO, Tokyo, Japan). Primers used for RT-PCR were: STAP-2: 5'-GGAGAATGGCGAGAATGTGT-3' (sense), 5'- GCAACTTCTTTGGCTTCAGG-3' (antisense); CCR1: 5'- TTTGGTGTCATCACCAGCAT-3' (sense), 5'-GCCTGAAACAGCTTCCACTC-3' (antisense); CCR7: 5'-GATGCGATGCTCTCTCATCA-3' (sense), 5'- TGTAGGGCAGCTGGAAGACT-3' (antisense); CXCR1: 5'- TTTGTTTGTCTTGGCTGCTG-3' (sense), 5'-AGTGTACGCAGGGTGAATCC-3' (antisense); CXCR4: 5'-CTTCTACCCCAATGACTTGTGG-3' (sense), 5'- AATGTAGTAAGGCAGCCAACAG-3' (antisense); ccr1: 5'- AGGGCCCGAACTGTTACTTT-3' (sense), 5'-TTCCACTGCTTCAGGCTCTT-3' (antisense); ccr7: 5'- GTGTGCTTCTGCCAAGATGA-3' (sense), 5'- CCACGAAGCAGATGACAGAA-3' (antisense); cxcr1: 5'-TCAGTGGTATCCTGCTGCTC-3' (sense), 5'- CTGGCGGAAGATAGCAAAAG-3' (antisense); CXCR4: 5'- TCAGTGGCTGACCTCCTCTT-3' (sense), 5'-TTTCAGCCAGCAGTTTCCTT-3' (antisense); G3PDH: 5'-GAAATCCCATCACCATCTTCCAGG-3' (sense), 5'-CAGTAGAGGCAGGGATGATGTTC-3' (antisense). Quantitative real-time PCR analyses of STAP-2, STAT5b, BCL-2, BCL-xL, CYCLIN D1, CCR1, CCR7, CXCR1, CXCR4, as well as the control G3PDH mRNA transcripts were carried out using the assay-on-demandTM gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system (Applied Biosystems, Tokyo, Japan).

Chemotaxis assays

In vitro cell migration assay was conducted as previously described (Sekine et al 2009). Briefly, the transwell filters (5 μM pore filter; BD Falcon, Franklin lakes, NJ, USA) were placed in the lower chamber containing 500 μl of complete medium with or without SDF-1α. Ba/F3 cells ($5x10^5$) were resuspended in 200 μl of RPMI1640 medium and allowed to migrate toward the underside of the top chamber. After 2 hrs of incubation at 37°C in 5% CO_2, the numbers of migrated cells of the lower chamber were counted with a phase contrast microscope.

Immunoprecipitation, immunoblotting and pull-down assay

The immunoprecipitation and Western blotting assays were performed as described previously (Sekine et al 2005). Briefly, cells were harvested and lysed in a lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride). The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to PVDF transfer membrane (PerkinElmer; Boston, MA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore; Bedford, MA). Pull-down assay using glutathione S-transferase (GST) fusion proteins was performed as previously described (Sekine et al 2007).

Cell proliferation assay

The numbers of viable Ba/F3 and K562 cells after the indicated treatments were measured using a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay (Cell Counting Kit-8; Wako Pure Chemicals).(Ikeda et al 2010) Briefly, 10 μl of WST-8 solution was added to the cells in each well and incubated for 2 h. The absorbances were measured at a test wavelength of 450 nm and a reference wavelength of 595 nm using a microplate reader (Bio-Rad, Hercules, CA).

Cell cycle analysis

Ba/F3-p210 and Ba/F3-p210/STAP-2 (#1 and #2) cells were cultured without WEHI-3B conditioned medium for 24 hrs. The cells were then washed once with ice-cold PBS, suspended with 100 μL of PBS and then fixed by the addition of 900 μL of ethanol. Cells were incubated at -20°C for 20 min, pelleted, resuspended with 300 μL of staining buffer (1 mg/mL RNase, 20 μg/mL propidium iodide, 0.01% NP-40 in PBS) and incubated at 37°C for 20 min. The DNA content of nuclei was analyzed using an FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA, USA).

Animal tumorigenesis

Ba/F3-pcDNA, Ba/F3-p210 and Ba/F3-p210/STAP-2 #2 cells ($1x10^7$) were injected s.c. into BALB/c nude mice aged 4 weeks. After 4 weeks, the animals were sacrificed and the weighs of the tumor, liver, lymph node, and spleen were measured. K562 control shRNA #1 and K562 STAP-2 shRNA #1 cells ($1.5 x10^7$) were injected s.c. into BALB/c nude mice aged 4 weeks. Tumor sizes were monitored daily. Tumor volume was calculated using the following formula: length × width × height × 0.5236 (Xie et al 2009). After 4 weeks, the animals were sacrificed and the weighs of the tumor were measured.

Statistical methods

The significance of differences between group means was determined by Student's t-test.

Supplementary References

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Supplementary Figure legend

Supplementary Figure 1 Treatment with Imatinib influences physical and functional interactions between STAP-2 and BCR-ABL.

(a) Ba/F3-p210 and Ba/F3-p210/STAP-2#2 cells ($1x10^6$ cells/well) in a 12-well plate were treated or untreated with STI571 (0, 1, 3, 5 μM) for 6 hrs. Cells were lysed, and an aliquot of total cell lysate (TCL) was immunoblotted with anti-PY, anti-ABL or anti-Myc or anti-Actin antibody. **(b)** 293T cells ($1x10^7$) were transfected with p210BCR-ABL (10 μg) with Myc-tagged STAP-2 (8 μg). At 36 h after transfection, the cells were treated or untreated with STI571 (0, 1, 3, 5 μM) for 12hrs. Cells were lysed, and immunoprecipitated with anti-Myc antibody and blotted with anti-ABL or anti-Myc antibody (upper panels). An aliquot of TCL was also blotted with anti-PY, anti-ABL or anti-Myc antibody (lower panels).