Title: BART is essential for nuclear retention of STAT3

Authors: Ryuta Muromoto#, Yuichi Sekine#, Seiyu Imoto#, Osamu Ikeda, Taichiro Okayama, Noriko Sato and Tadashi Matsuda*

Affiliation: Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Hokkaido 060-0812, Japan

#These authors equally contributed to this work.

*Correspondence and requests for materials should be addressed to Dr. Tadashi Matsuda, Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-Ku Kita 12 Nishi 6, Sapporo 060-0812, Japan, TEL: 81-11-706-3243, FAX: 81-11-706-4990, E-mail: tmatsuda@pharm.hokudai.ac.jp

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Abstract

Signal transducers and activators of transcription (STATs) mediate cell proliferation, differentiation, and survival in immune responses, hematopoiesis, neurogenesis, and other biological processes. STAT3, for example, is involved in the epithelial-mesenchymal transition during gastrulation, organogenesis, wound healing, and cancer progression. STAT activity is regulated by a variety of mechanisms, including nuclear translocation. To clarify the molecular mechanisms underlying the regulation of STAT activity, we performed yeast two-hybrid screening. Here we identified BART (Binder of ADP-ribosylation factor-like Two) as a novel STAT-binding partner. Importantly, we showed that BART is essential for the transcriptional activity and nuclear retention of STAT3. Furthermore, an effector of BART, ADP-ribosylation factor-like 2 (ARL2) was also involved in nuclear retention of STAT3. These results indicate that BART plays an essential role in the nuclear retention of STAT3 through interaction with ARL2.
**Introduction**

The Jak/STAT pathways are utilized by a wide range of cytokines to regulate gene expression. Cytokines activate members of the Jak family of protein tyrosine kinases, which in turn activate, by tyrosine phosphorylation, one or more members of the STAT family of transcription factors. STATs are unusual among transcription factors in that they have the characteristics of cytoplasmic signaling molecules, such as a Src-homology 2 (SH2) domain and tyrosine phosphorylation sites. Upon tyrosine phosphorylation, the STATs dimerize through their phosphorylated SH2 domains and translocate to the nucleus (1, 2, 3). In recent years, constitutive or dysregulated expression of STATs has been found in cancer cells and oncogene-transfected cells and shown to be involved in a wide range of diseases including autoimmune diseases (4-7). Studies in Dictyostelium, Drosophila, and zebrafish have shown that the Jak/STAT pathway is required for a broad set of developmental processes, including cell proliferation, cell fate determination, cell migration, planar polarity, convergent extension, and immunity (8, 9). These findings imply that STAT activation must be tightly regulated. In fact, STAT activation is regulated by a variety of mechanisms. Cytoplasmic tyrosine phosphatases, such as SH2-containing phosphatase 1 (SHP1), SHP2, and protein-tyrosine phosphatase 1B (PTP1B) prevent further STAT activation in the cytoplasm (10, 11). The importin family proteins are involved in the nuclear translocation of STAT1 (12, 13). Nuclear tyrosine phosphatases like TC45 dephosphorylate nuclear STATs, which allows the STATs to return to the cytoplasm (10).
The PIAS family of proteins decreases STAT-dependent transcription by blocking STAT-DNA binding in the nucleus (10). Suppressor of cytokine signaling (SOCS) is induced by STATs and plays roles in the negative feedback of STAT activation (11). Among these mechanisms, it is especially unclear how the nuclear localization of STATs is regulated.

To investigate the specific regulatory mechanisms of STATs, we sought to identify STAT-interacting proteins using yeast two-hybrid screening with the C-terminal region of STAT4 as bait. We identified, binder of ARL Two, BART, as a novel binding partner of STATs. BART is a 19-kDa protein originally identified as an ARL2-binding protein and purified from bovine brain, whose expression is ubiquitous (14). ARL2 binds BART with high affinity and its binding is dependent on the binding of GTP to ARL2. BART lacks ARL2 GTPase-activating protein activity and, therefore, was the first effector identified for ARL2, although the function of their interaction remains unclear. Here, we show that BART is a STAT binding partner with a critical role in the nuclear retention of STAT3.
Methods

Reagents and antibodies

Recombinant human IL-6 was a kind gift from Ajinomoto Co. (Tokyo, Japan). Recombinant human LIF was purchased from INTERGEN (Purchase, NY). LMB was a kind gift from Dr. M. Yoshida (Riken, Wako, Japan). Expression vectors for Jak1, STAT3-C, STAT2, STAT1, STAT4, STAT5a, STAT5b, and STAT6 were kindly provided by Dr. J. N. Ihle (St. Jude CRH, Memphis, TN), Dr. J. F. Bromberg (Rockefeller Univ., New York, NY), and Dr. N. Yokosawa (Sapporo Med. Sch., Sapporo, Japan). Expression vectors for Zebrafish BART, STAT3-LUC and YFP-STAT3 were kindly provided from Dr. S. Yamashita and Dr. T. Hirano (Osaka Univ. Med. Sch., Osaka, Japan) (15). Epitope-tagged STAT3 and its mutants were previously described (15, 16). Epitope-tagged BART or ARL2 and its mutants were generated by PCR and sequenced (primer sequences are available upon request). Anti-Myc, anti-GST, and anti-STAT3 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG M2 monoclonal and rabbit polyclonal anti-FLAG were from Sigma (St Louis, MO). Anti-phospho-STAT3 Tyr705 (pSTAT3 Tyr705) was purchased from Cell Signaling Technologies (Beverly, MA). Anti-BART monoclonal antibody was purchased from BD biosciences (San Jose, CA). Anti-ARL2 antibody was purchased from Abgent (San Diego, CA). Guinea pig anti-BART antibody was raised against GST-fused human BART.
**Yeast two-hybrid screen**

Gal4-STAT4 was constructed by fusing the coding sequence for the C-terminal region (amino acids 483-748) of mouse STAT4 in-frame to the Gal4 DNA-binding domain in the pGBK7 vector (Clontech, Palo Alto, CA). *S. cerevisiae* AH109 cells were transformed with pGal4-STAT4, then mated with Y187 cells containing a pretransformed mouse 11-day embryo MATCHMAKER cDNA library (Clontech, Palo Alto, CA) and approximately 2.6 x 106 colonies were screened as previously described (17). Plasmid DNAs derived from positive clones were extracted from the yeast and sequenced.

**Cell culture, transfection, luciferase assays, siRNA, RT-PCR and EMSA**

The human hepatoma cell line Hep3B was cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS). Hep3B cells were treated with an siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA) mixture at 37 °C for 4 h, followed by addition of fresh medium containing 10% FCS as previously described (18). Further transfection into Hep3B transformant cells were performed using JetPEI (Polyplus, Illkirch, France) according to the manufacturer’s instructions. Human cervix carcinoma cell line, HeLa was cultured in DMEM supplemented with 10% FBS. The cells were plated on a 6-well plate and treated with siRNAs as described above. The cells were then transfected with STAT3-LUC using Metafectene (Biontex Laboratories GmbH, München, Germany) according to the manufacturers' protocols. Twenty-four hours after transfection, the cells were treated
with LIF (100 ng/ml) for 8 h. The cells were harvested and assayed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Three or more independent experiments were carried out for each assay. siRNAs targeting human BART and ARL2 used in this study were as follows: siBART, 5’- CUACAGAGCAGAAAAAGAGGCGGA-3’. siARL2, 5’- GAAAGACAAACCACCUAAAGGAGGUU-3’. Primers used for reverse transcription (RT)-PCR were: SOCS3: 5’-ATGGTCACCCACAGCAAGTT-3’ (sense), 5’-TTAAAGCGGGCATCGTACTG-3’ (antisense); G3PDH: 5’-GAAATCCCATCACCATCTCCAGG-3’ (sense), 5’- CAGTAGAGG CAGGGATGATGTTC-3’ (antisense); BART: 5’- ATGGACGCCTTGAAGGA-3’ (sense), 5’-CTAGTGCCGCAGATTGTT-3’ (antisense). Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FBS, and treated with siRNAs as described above. Cell extracts for EMSA were prepared from 293T cells left untreated or treated with LIF (100 ng/ml) for 30 min as previously described (19). To measure STAT3 DNA binding, cell extracts were treated with 5’-biotin-labeled STAT3 consensus oligonucleotide and streptavidin-Sepharose beads (Sigma) conjugate in binding buffer (20 mM HEPES, 0.5 mM EDTA, 1 mM dithiothreitol, 2 µg/ml poly (dI-dC) ) and rotated for 2 h at 4°C. Samples were centrifuged, and the pellets were washed three times with binding buffer. Specifically bound proteins were eluted and subjected to Western blot analysis using anti-STAT3 antibody.
**Immunoprecipitation, immunoblotting and indirect immunofluorescence**

Immunoprecipitation and Western blotting were performed as previously described (16). The cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg /ml each of aprotinin, pepstatin, and leupeptin). The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to an Immobilon filter (Millipore; Bedford, MA), which was then immunoblotted with antibodies. Hep3B cells were left untreated or treated with IL-6 (10 ng/ml) for 30 min, then fixed cells with a solution containing 4% paraformaldehyde, permeabilized with 1% Triton-PBS and reacted with antibodies as previously described (16). Images were obtained using a Zeiss LSM 510 laser scanning microscope with an Apochromat x63/1.4 oil immersion objective and x4 zoom. Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) (Wako, Osaka, Japan).

**Live cell imaging**

293T cells were treated with siRNAs as described the above, and transfected with YFP-STAT3 using the calcium phosphate method. Twenty-four hours after transfection, cells were placed into a perfusion chamber. Stimulation was performed at 37°C with 100 ng/ml of LIF. Five minutes after stimulation a single cell was scanned every 20 sec for 240 min by confocal laser-scanning microscopy. Confocal microscopy was performed using an LSM510 system in conjunction with an Axiovert200 (Carl Zeiss MicroImaging, Inc.).
Results

Molecular interactions between STAT3 and BART

We performed a yeast two-hybrid screen of a mouse embryo cDNA library using the C-terminal region of STAT4 (amino acids 483-748) as bait. From a screen of about 2.6 x 10^6 transformants, we identified several positive clones. Sequence analysis revealed that one of them encoded the entire BART protein (amino acids 1-163). We examined the distribution of BART mRNA in murine tissues using RT-PCR and found that it was ubiquitously expressed, although it was abundant in the brain, heart, lung, bone marrow, and thymus, and was expressed at lower levels in the liver, kidney and spleen (data not shown). This distribution pattern was similar to that of human BART (14), except that human BART expression is lower in the lung.

We first examined whether BART binds STAT4 and/or other STATs in mammalian cells. 293T cells were transfected with a series of FLAG-tagged STAT expression vectors together with Myc-tagged BART. Western blot analysis of the immunoprecipitates using anti-FLAG antibody revealed that BART interacts with STAT2, STAT3 and STAT4, but not with STAT5b or STAT6, although STAT1 and STAT5a showed a faint binding to BART in 293T cells (Fig. 1A). Although STAT3 and STAT4 are more closely related to each other than are any other STATs, STAT4 expression is restricted to the testis, thymus, and spleen, while STAT3 is ubiquitously expressed (20). These results suggested that the STAT3-BART interaction may have a wider range of biological functions than does the STAT4-BART interaction. Therefore,
we focused on the functional association between BART and STAT3.

To further confirm the specificity of interactions between STAT3 and BART, the empty vectors or vectors containing zebrafish BART or human BART was introduced back into *S. cerevisiae* along with the C-terminal domain of STAT3 (amino acids 494-750) fused to the DNA binding domain of Gal4 or empty vector (containing the Gal4 DNA binding domain only). After mating the indicated yeast, growth occurred only in the presence of BART and STAT3 (Fig. 1B), demonstrating that BART interacted with the C-terminal domain of STAT3 in yeast. Next, to delineate the regions of STAT3 involved in the BART-STAT3 interaction, various deletion constructs of GST-fused STAT3 were subjected to pull-down assays in 293T cells. As shown in Fig. 1C, the DNA-binding domain of STAT3 interacted strongly with BART. To further delineate the domains in BART required for the interaction with STAT3, we made two sets of mutant BART proteins. 293T cells were transiently transfected with FLAG-tagged STAT3 and two sets of Myc-tagged BART mutants (BART-N, BART-C). As shown in Fig. 1D, the C-terminal domain of BART, BART-C but not the N-terminal domain, BART-N, interacted with STAT3. Furthermore, Jak1 expression or LIF stimulation enhanced the binding of BART to STAT3, indicating that the activation of STAT3 enhances its binding to BART (Fig. 1E, F). Moreover, an inactive form of STAT3, STAT3 YF mutant showed much less binding to BART than wild-type STAT3 or an active form of STAT3, STAT3-C (Fig. 1G). To exclude the possibility that the interaction between STAT3 and BART was due to overexpression, we examined the direct binding between endogenous BART and STAT3 in untransfected 293T cells by
co-immunoprecipitation. An anti-BART antibody co-immunoprecipitated STAT3 without LIF stimulation (Fig. 1H), indicating that the binding of BART to STAT3 occurs at physiological expression levels without stimulation. However, their endogenous interaction was weak, suggesting that other molecules may be required for their stronger and a ligand-dependent interactions.

*BART is required for STAT3-mediated transactivation*

To clarify the molecular mechanisms underlying how BART affects STAT3 activity, we used small interfering RNA (siRNA) to reduce the endogenous expression of BART in Hep3B cells. We first examined whether reduction of endogenous BART expression affects STAT3-mediated gene expression using a BART-specific siRNA. As shown in Fig. 2A, IL-6/STAT3-mediated SOCS3 mRNA expression was markedly reduced in BART siRNA-treated Hep3B cells. The LIF-induced STAT3-mediated transcriptional response of the $\beta$2-macroglobulin promoter was also suppressed in the human cervix carcinoma cell line, HeLa cells treated with BART siRNA (Fig. 2B). These results indicate that BART plays a role in the regulation of STAT3 activity *in vivo*. Next, we examined whether reduction of BART expression affects phosphorylation of STAT3. As shown in Fig. 2C, tyrosine-phosphorylation of STAT3 was decreased by a reduction in BART expression in Hep3B cells. We then tested the effect of a protein tyrosine phosphatase (PTPase) inhibitor, vanadate, on the reduced tyrosine-phosphorylation of STAT3 in BART siRNA-treated Hep3B cells. Importantly, vanadate treatment induced sustained tyrosine-phosphorylation of STAT3 even when
cells were treated with BART siRNA (Fig. 2D), suggesting that BART plays a role in the maintenance of the phosphorylation state of STAT3. We also observed the effect of a reduction in BART expression by confocal microscope images. Coincident with the above data, IL-6-induced nuclear accumulation of STAT3 and the amount of phosphorylated STAT3 in the nucleus was decreased in BART siRNA-treated Hep3B cells (Fig. 2E). Furthermore, vanadate treatment induced enhanced accumulation of STAT3 and phosphorylated STAT3 in the nucleus. However, treatment with BART siRNA together with vanadate resulted in a diffused pattern of phosphorylated STAT3 in the cytoplasm and nucleus (Fig. 2F), indicating that BART may participate in keeping phosphorylated STAT3 within the nucleus. Moreover, transfection of siRNA for BART in HeLa cells suppressed LIF-induced nuclear accumulation of STAT3 (Fig. 2G). These results also demonstrated that nuclear localization of STAT3 protects phosphorylated STAT3 from dephosphorylation by PTPases.

**BART is essential for the nuclear retention of STAT3**

To further identify the molecular mechanisms underlying the effect of BART on the nuclear localization of STAT3, we perfomed time-lapse live cell imaging using yellow fluorescent protein (YFP)- fused STAT3 (YFP-STAT3). We transiently transfected YFP-STAT3 into 293T cells and observed nuclear translocation of YFP-STAT3 after LIF stimulation (Fig. 3A). YFP-STAT3 was predominantly present in the cytoplasm without stimuli. After 5 min of LIF stimulation, YFP-STAT3 was accumulated in the nucleus. Interestingly, YFP-STAT3 could be observed in dot-like structures in the
nucleus between 15 min and 60 min after LIF stimulation as recently described (21). The dot-like structures disappeared following the addition of STAT3 consensus oligonucleotides (data not shown), indicating that these structures may represent DNA-bound STAT3 complexes. Additional expression of zebrafish BART (zBART) resulted in no alteration in appearance. Importantly, treatment of cells with BART siRNA decreased the nuclear accumulation and dot-like structure formation of STAT3 after LIF stimulation. Furthermore, LIF-induced nuclear accumulation and dot-like structure formation of STAT3 were recovered in BART siRNA-treated cells by overexpression of zBART. These results indicate that BART is involved in the accumulation of STAT3 in the nucleus. However, this may be caused by BART interfering in the nuclear export of STAT3. To examine whether BART affects the nuclear import or export of STAT3, we treated transfected cells with Leptomycin B (LMB), which inhibits chromosome region maintenance I(CRM1)-dependent nuclear export (22). Surprisingly, LIF-induced nuclear import or accumulation of STAT3 was recovered in BART siRNA-treated cells by pretreatment of cells with LMB, indicating that BART is not involved in CRM-dependent nuclear export of STAT3 and may act as a nuclear retention factor for STAT3. The above results also indicate that BART may affect the DNA binding activity of STAT3, which keeps STAT3 in the nucleus. We further examined whether a reduction in BART expression affects the DNA binding activity of STAT3. As shown in Fig. 3B, LIF-induced the DNA binding activity of STAT3 was abolished by BART siRNA treatment. However, this activity was recovered by additional treatment of cells with vanadate or LMB, suggesting that
BART has no effect on STAT3’s DNA binding activity. However, there is the possibility that BART may stabilize the STAT3-DNA interactions to keep STAT3 in the nucleus, because BART interacts with STAT3 through the DNA-binding domain of STAT3. Taken together, these results indicated that BART functions as a nuclear retention factor for STAT3.

*ARL2 T30N but not Q70L inhibits nuclear accumulation of STAT3*

BART was first identified as the effector for an ARL family GTPase, ARL2 (14). ARF-like proteins (ARLs) are a functionally distinct group of incompletely characterized members of the ARF family of Ras-related GTPases (22). The sequence analysis of BART did not reveal previously identified domains that might have provided insight into its function or other binding partners. Furthermore, the lack of a conserved GTPase or other, more general, protein-binding motifs is quite common among ARF family effectors. BART binds specifically to ARL2-GTP and with high affinity, and it does not interact with ARL2·GDP or activated ARF or Rho proteins (14). To first examine whether ARL2 is involved in interaction between STAT3 and BART, we estimated the binding potential of BART to STAT3 in the presence or absence of ARL2. Expression of ARL2 significantly enhanced interactions between STAT3 and BART (Fig. 4A). Thus, ARL2 effectively bridges BART and STAT3. To examine whether activated ARL2 played a role in nuclear retention of STAT3, we generated ARL2 mutants Q70L and T30N (the numbers refer to the amino acids in ARL2), corresponding to the classical Ras mutations Q61L and T17N, and examined their binding to BART. Ras Q61L- or T17N-type mutations have the same
effect on many small G proteins: Q61L-type mutations are GTPase-defective (GTP remains bound), whereas T17N are defective in GTP binding and, when expressed in vivo, act in a dominant-negative manner, sequestering guanine nucleotide exchange factors, so that both mutant and endogenous proteins remain primarily GDP-bound. As shown in Fig 4B, a weak interaction between BART and ARL2 T30N was observed, whereas BART interacted strongly with wild-type ARL2 and ARL2 Q70L in 293T cells. We then examined whether overexpression of ARL2 T30N had any effect on the nuclear accumulation of STAT3 in Hep3B cells. Interestingly, when ARL2 T30N but not wild-type ARL2 was expressed together with STAT3, STAT3 did not accumulate in the nucleus even after IL-6 treatment (Fig. 4C, D). Moreover, transfection of siRNA for ARL2 in HeLa cells suppressed LIF-induced nuclear accumulation of STAT3 (Fig. 4E). These results suggest that the BART-ARL2·GTP system plays an important role in the nuclear translocation of STAT3.
Discussion

Our findings led us to conclude that BART is indispensable for STAT3’s nuclear retention. Reduction of endogenous BART inhibited STAT3-mediated transactivation. We also provide the first evidence that BART is a nuclear retention factor for STAT3 using time-lapse live cell imaging. We further demonstrate the involvement of an ARL family GTPase, ARL2, in nuclear accumulation of STAT3. The interaction between STAT3 and a classical small GTPase, Rac1, has been reported previously (23); however, the biological significance of this interaction in relation to STAT3 function was not studied.

Protein localization is a highly dynamic biological process. To ensure a proper cellular function, the spatial distribution of different proteins needs to be tightly regulated and coordinated. Therefore, upon extracellular stimuli, STATs in the cytoplasm move to the nucleus as transcription factors, they need access to the nucleus. Although the STAT proteins show structural similarity features, their nuclear–cytoplasmic localization has been shown to be differently regulated. After the importin-STAT1 complex enters the nucleus, the importin is released from the complex and STAT1 can then bind to DNA. Mutagenic studies showed that the DNA-binding domain of STAT1 is also required for its nuclear translocation (12,13). Recently, in contrast to STAT1, STAT3 has been shown to accumulate in the nucleus independent of its phosphorylation state (24). Moreover, a reduction of importin-3 expression by siRNA inhibits the nuclear localization of STAT3. Therefore, STAT3
continuously shuttles between nuclear and cytoplasmic compartments and can thereby respond to extracellular stimuli. The nuclear retention of unphosphorylated STAT3 might assist a rapid response of activated STAT3 by extracellular or intracellular stimuli. This might be one reason that STAT3 is a pleiotropic transcription factor that is activated by several growth factors, hormones and intracellular tyrosine kinases.

Here we propose a novel scenario in which a STAT3-BART-ARL2 complex serves as machinery for STAT3 nuclear retention. Discovering every step of STAT3-specific nuclear translocation is important, since STAT3 may be a key player in the pathogenesis of diverse human diseases and a prime target for novel therapies. Specific inhibitors for the STAT3-BART-ARL2 pathway would be good candidates for STAT3-related human disease therapies.
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Abbreviations

IL-6, interleukin-6

LIF, leukemia inhibitory factor

STAT, signal transducers and activators of transcription

SH, Src-homology

Jak, Janus kinase

BART, Binder of ADP-ribosylation factor-like Two

ARL, ADP-ribosylation factor-like

CRM1, chromosome region maintenance 1

LMB, Leptomycin B

YFP, yellow fluorescent protein
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Figure legends

Figure 1. Molecular interactions between STAT3 and BART
(A) 293T cells \((1 \times 10^7)\) were transfected with Myc-tagged BART \((10 \mu g)\) and/or FLAG-tagged STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, or STAT6 \((10 \mu g)\). The cells were lysed 48 h later, immunoprecipitated with FLAG antibody, and immunoblotted with anti-Myc (upper panel) or anti-FLAG antibody (middle panel). Total-cell lysates (TCL)(1%) were blotted with anti-Myc or FLAG antibody (lower panels). (B) Growth of transformed \(S.\ cerevisiae\), demonstrating an interaction between either zebrafish BART (zBART) or human BART (hBART) and mouse STAT3 (mSTAT3). pGBK7-mSTAT3, pGBK7-p53, or empty pGBK7 in AH109 were mated with pACT2-zBART, pACT2-hBART, pACT2-SV40 T-antigen (T), or empty pACT2 in Y187 as indicated. Colonies were then re-streaked onto high stringency plates. (C) Domain structure of STAT3 and GST-fused mutant fragments are shown schematically. 293T cells \((1 \times 10^7)\) were transfected with GST or GST-fused STAT3 deletion mutants \((10 \mu g)\) and/or Myc-tagged BART \((10 \mu g)\). The cells were lysed 48 h later, pulled down with Glutathione (GSH)-Sepharose and blotted with anti-Myc (upper panel) or anti-GST (middle panel) antibody. TCL (1%) was blotted with anti-Myc antibody (bottom panel). (D) Domain structure of BART and Myc-tagged mutant fragments (BART-N, BART-C) are schematically shown. 293T cells \((1 \times 10^7)\) transfected with FLAG-tagged STAT3 \((10 \mu g)\) and/or Myc-tagged BART-N or BART-C \((10 \mu g)\). The cells were lysed 48 h later, immunoprecipitated with anti-FLAG antibody,
and immunoblotted with anti-Myc (upper panel) or anti-FLAG antibody (middle panel). TCL (1%) were blotted with anti-Myc antibody (bottom panel). (E) 293T cells (1x10^7) transfected with FLAG-tagged STAT3 (5 μg) and/or Myc-tagged BART (10 μg) with or without Jak1 (2.5 μg). The cells were lysed 48 h later, immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-Myc (upper panel) or anti-FLAG (middle panel). TCL (1%) was blotted with anti-Myc or anti-pSTAT3 (lower panels). (F) 293T cells (1x10^7) transfected with FLAG-tagged STAT3 (10 μg) and/or Myc-tagged BART (10 μg). Forty-eight hours later, the cells were left untreated or treated with LIF (100 ng/ml) for 30 min. The cells were lysed, immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-Myc (upper panel), anti-FLAG (middle panel). TCL (1%) was blotted with anti-Myc or anti-pSTAT3 antibody (lower panels). (G) 293T cells (1x10^7) transfected with FLAG-tagged STAT3 WT, STAT3 YF or STAT3-C (10 μg) with Myc-tagged BART (10 μg). The cells were lysed 48 hrs later, immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-Myc (upper panel) or anti-FLAG antibody (middle panel). TCL (1%) was blotted with anti-Myc antibody (lower panel). (H) 293T cells (2x10^7) were lysed, immunoprecipitated with control or anti-BART antibody (Guinea pig), and immunoblotted with anti-STAT3 (upper panel) or anti-BART antibody (middle panel). TCL (1%) was blotted with anti-pSTAT3, anti-STAT3 or anti-BART antibody (left panels).

Figure 2. BART is required for STAT3-mediated transactivation

(A) Hep3B cells in a 6-well plate were transfected with control or BART siRNA, and
cells were stimulated with IL-6 (10 ng/ml) for the indicated periods. Total RNA samples isolated from these cells were subjected to RT-PCR analysis using SOCS3, G3PDH or BART primers. (B) HeLa cells in a 6-well were transfected with control or BART siRNA. The cells were then transfected with STAT3-LUC (1 µg). Twenty-four hours after transfection, the cells were stimulated with LIF (100 ng/ml) for 6 h. The cells were then harvested, and luciferase activities were measured. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D. (C) Hep3B cells in a 6-well plate were transfected with control or BART siRNA, and cells were stimulated with IL-6 (10 ng/ml) for the indicated periods. The cells were lysed, and TCL was immunoblotted with anti-pSTAT3 (upper panel), anti-STAT3 (middle panel) or anti-BART antibody (bottom panel). Densitometric quantification of the above results was also shown. Relative intensity of pSTAT3 was normalized to the STAT3 protein of the same sample. (D) Hep3B cells in a 6-well plate were transfected with control or BART siRNA, and cells pretreated with vanadate (100 µM) for 30 min and stimulated with IL-6 (10 ng/ml) for the indicated periods. The cells were lysed, and TCL was immunoblotted with anti-pSTAT3 (upper panel), anti-STAT3 (middle panel) or anti-BART antibody (bottom panel). Densitometric quantification of the above results was also shown. Relative intensity of pSTAT3 was normalized to the STAT3 protein of the same sample. (E) Hep3B cells in a 6-well plate were transfected with control or BART siRNA, and untreated or treated with IL-6 (10 ng/ml) for the indicated periods. The cells were fixed and stained with anti-STAT3 or anti-pSTAT3 antibody, then Alexa350 conjugated
anti-rabbit IgG (blue) or Alexa555-conjugated anti-mouse IgG2b antibody (red). (F) Hep3B cells in a 6-well plate were transfected with control or BART siRNA, and left untreated or treated with vanadate (100 μM) for 30 min. The cells were then untreated or treated with IL-6 (10 ng/ml) for 30 min. The cells were fixed and stained with anti-STAT3 or anti-pSTAT3 antibody, then Alexa350 conjugated anti-rabbit IgG (blue) or Alexa555-conjugated anti-mouse IgG2b antibody (red). (G) HeLa cells in a 6-well were transfected with control or BART siRNA, and then untreated or treated with LIF(10 ng/ml) for 30 min, 48 hrs later. Cells were fixed and stained with anti-STAT3 antibody followed by FITC-conjugated anti-rabbit IgG (green). Quantitative analysis of the subcellular localization of STAT3. Approximately 100 cells were classified according to fluorescent signals in the cytoplasm or nucleus.

Figure 3. BART is essential for nuclear retention of STAT3

(A) YFP-STAT3 nuclear translocation analyzed by live cell imaging. 293T cells were treated with control (upper panels) or BART siRNA (lower panels), and then transfected with YFP-STAT3. The cells were also transfected with zBART (upper middle, lower middle) or pretreated with LMB (10μM) for (upper bottom, lower bottom). Transfected 293T cells were placed in a perfusion chamber at 37°C, and analyzed by confocal laser scanning microscopy. Cells were stimulated with LIF (100 ng/ml) and pictures of a single cell were taken every 20 sec. Images taken at the timepoints indicated are depicted. (B) 293T cells were treated with control or BART siRNA, and then cells
were stimulated with LIF (100 ng/ml) for 30 min. The cells were also left untreated or treated with vanadate (100\(\mu\)M) or LMB (10\(\mu\)M) for 30 min. Pulldown assays were performed using biotin-labeled STAT3 oligonucleotides bound to streptavidin-sepharose beads. Specifically bound proteins were eluted and subjected to Western blot analysis using anti-STAT3 antibody.

Figure 4. ARL2 T30N but not Q70L inhibits nuclear accumulation of STAT3

(A) 293T cells (1x10^7) transfected with HA-tagged STAT3 (10 \(\mu\)g) and/or Myc-tagged BART or FLAG-tagged ARL2 (10 \(\mu\)g). The cells were lysed 48 h later, immunoprecipitated with anti-HA antibody, and immunoblotted with anti-Myc (upper panel) or anti-HA antibody (middle panel). TCL (1%) were blotted with anti-FLAG, anti-Myc or anti-HA antibody (bottom panels). (B) 293T cells (1x10^7) were transfected with FLAG-tagged BART (10 \(\mu\)g) and/or Myc-tagged ARL2 wild-type, Q70L, or T30N (10 \(\mu\)g). The cells were lysed 48 h later, immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-Myc (upper panel) or anti-FLAG antibody (middle panel). TCL (1%) were blotted with anti-Myc antibody (bottom panel).

(C) Hep3B cells (2x10^5) were transfected with Myc-tagged ARL2 Q70L or T30N (1 \(\mu\)g) and FLAG-tagged STAT3 (1 \(\mu\)g), then untreated or treated with IL-6 (100 ng/ml) for 30 min, 48 hrs later. Cells were fixed and stained with anti-Myc or anti-FLAG antibody followed by FITC-conjugated anti-mouse IgG (green) or rhodamine-conjugated anti-rabbit IgG (red). Nuclei were counterstained with DAPI (blue). (D) Quantitative analysis of the subcellular localization of STAT3. Approximately 100 cells were
classified according to rhodamine signals in the cytoplasm or nucleus. When ARL2 T30N but not wild-type was expressed with STAT3, STAT3 did not accumulate in the nucleus even after IL-6 treatment in more than 60% of transfected cells. (E) HeLa cells in a 6-well were transfected with control or ARL2 siRNA, and then untreated or treated with LIF (10 ng/ml) for 30 min, 48 hrs later. Cells were fixed and stained with anti-STAT3 antibody followed by FITC-conjugated anti-rabbit IgG (green). Quantitative analysis of the subcellular localization of STAT3. Approximately 100 cells were classified according to fluoresceine signals in the cytoplasm or nucleus.
Figure 1
## Figure 2

### A

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### B

- **Fold induction**
- **Graph showing fold induction**

### C

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### D

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### E

- **IL-6 STAT3 pSTAT3**
  - **Control siRNA**
  - **BART siRNA**

### F

- **IL-6 STAT3 pSTAT3**
  - **Control siRNA**
  - **BART siRNA**

### G

- **LIF stimulation**
  - **Control siRNA**
  - **BART siRNA**

- **TCL IB: BART**
- **IB: Actin**

- **Graph showing percentage distribution**
- **Bar chart showing percentage distribution**
Figure 3
Figure 4