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The RhoU/Wrch1 Rho GTPase gene is a common transcriptional target of both the gp130/Stat3 and Wnt-1 pathways

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Abbreviations: STAT, signal transducer and activator of transcription; JAK, Janus kinase; IL-6, interleukin-6; G-CSF, granulocyte colony stimulating factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; EMT, epithelial to mesenchymal transition; PCP, planar cell polarity; JNK, c-Jun N-terminal kinase; qRT-PCR, quantitative real time PCR; EMSA, electrophoretic mobility shift assay; HA-SIE, high affinity SIE; SIE, sis-inducible elements; ChIP, chromatin immunoprecipitation; MEF, mouse embryonic fibroblasts; OSM, oncostatin M; SOCS3, suppressor of cytokine signaling 3; LPS, lipopolysaccharide; TSS, transcription start site; BSs, binding sites

SYNOPSIS

Stat3 is a transcription factor activated by cytokines, growth factors and oncogenes, whose activity is required for cell survival/proliferation of a wide variety of primary tumors and tumor cell lines. Prominent among its multiple effects on tumor cells is the stimulation of cell migration and metastasis, whose functional mechanisms are however not completely characterized. RhoU/Wrch1 (Wnt-responsive Cdc42 homolog) is an atypical Rho GTPase thought to be constitutively bound to GTP. RhoU was first identified as a Wnt-1-inducible mRNA and subsequently shown to act on the actin cytoskeleton by stimulating filopodia formation and stress fibers dissolution. It was in addition recently shown to localize to focal adhesions and to Src-induced podosomes and enhance cell migration. RhoU overexpression in mammary epithelial cells stimulates quiescent cells to re-enter the cell cycle and morphologically phenocopies Wnt-1-dependent transformation. Here we show that Wnt-1-mediated RhoU induction occurs at the transcriptional level. Moreover, we demonstrate that RhoU can also be induced by gp130 cytokines via Stat3 and identify two functional Stat3 binding sites on the mouse RhoU promoter. RhoU induction by Wnt-1 is independent of β -catenin but does not involve Stat3. Rather, it is mediated by the Wnt-Planar Cell Polarity pathway through the activation of JNK. Both the so-called non-canonical Wnt pathway and Stat3 are therefore able to induce RhoU, which in turn may be involved in mediating their effects on cell migration.

INTRODUCTION

Signal Transducers and Activators of Transcription (STAT) are transcription factors playing a major role in the signalling from cytokine receptors. STAT factors become activated via tyrosine phosphorylation by receptor-associated JAK kinases [1], dimerize, concentrate into the nucleus and bind to STAT responsive elements on the promoters of cytokine-induced genes. Stat3 was first isolated as the mediator of acute phase genes induction in the liver in response to IL-6 and subsequently identified as the main STAT factor activated by all gp130 cytokines [2, 3]. Stat3 is also activated by a wide variety of other cytokines and growth factors (e.g. leptin, IL-12, IL-17, IL-10, Interferons, G-CSF, EGF, PDGF) and by a number of oncogenes such as c-Src, c-Abl, v-Sis, v-Fps, v-Ros, HER-2/neu and Met [4]. A Stat3 null mutation in the mouse leads to early embryonic lethality and conditional inactivation has revealed complex roles linked to inflammation, regeneration, proliferation, migration and energy homeostasis [5].

While STATs activity downstream of cytokines and growth factors is normally tightly controlled in both intensity and duration, Stat3 is found to be constitutively active in a vast majority of human and mouse tumors of both hematologic and solid origin, where its inhibition often leads to growth arrest and apoptotic cell death [6]. Stat3 exerts its pro-oncogenic role both directly, inducing the expression of anti-apoptotic and cell-cycle genes in the tumoral cells, and indirectly, through down-modulation of tumor immune surveillance [7]. Stat3 can also regulate cell movement, contributing to cytoskeleton reorganization and controlling cell adhesion properties, and is thought to play a role in tumor invasion and metastasis by inducing the expression of matrix metalloproteinases and promoting the epithelial to mesenchymal transition (EMT) (recently reviewed in Pensa et al.) [8]. In the zebrafish embryo, Stat3 activity is required for cell movements during gastrulation, a defect partially rescued by activation of the Wnt-planar cell polarity (PCP) signaling [9].

The evolutionary conserved Wnt signal transduction pathway plays important roles in development and is strongly implicated in tumorigenesis. Like for Stat3, aberrant Wnt signalling occurs in tumors of diverse origin and is likely to represent an important generic step in tumour development, contributing to functions as diverse as resistance to apoptosis, tissue invasion and metastasis, growth factor-independent growth, evasion from immune response and sustained angiogenesis [10]. Wnt proteins, acting through the frizzled family of seven-pass transmembrane receptors and through recently identified alternative receptors such as Ryk and Ror2 [11], can activate three different pathways: i) the so-called canonical Wnt signalling pathway, leading to the stabilization and nuclear concentration of β -catenin and to the activation of LEF-TCF-mediated transcription. This is the best characterized pathway mediating the proliferative effects of Wnt ligands; ii) the Wnt/PCP pathway, involved in controlling cell polarity and movement, particularly during embryogenesis, and mediated by the activation of the c-Jun N-terminal kinase (JNK) pathway and iii) the Wnt/calcium (Wnt/ Ca^{2+}) pathway, resulting in increased intracellular calcium and activation of protein kinase C (PKC).

Recently, a new Rho family member homologous to Cdc42 named Wrch1 (Wnt-regulated Cdc42 homolog)/RhoU has been isolated as a Wnt-1 responsive mRNA by means of subtraction cloning [12]. RhoU is an atypical Rho GTPase with no detectable GTPase activity and high intrinsic guanine nucleotide exchange, thus likely to be constitutively GTP-bound [13, 14]. Its regulation occurs both at the level of mRNA expression [12] and through release of the inhibitory effects of the N-terminal domain [14]. RhoU acts on the actin cytoskeleton by stimulating the formation of filopodia, stimulates quiescent cells to re-enter the cell cycle and phenocopies Wnt-1-dependent morphological transformation of mouse mammary

epithelial cells [12]. Recently, RhoU was shown to localize to focal adhesions in HeLa cells and fibroblasts and to podosomes in osteoclasts and Src-expressing cells; its depletion causes an increase in focal adhesion numbers and inhibits cell migration [15, 16].

Here we report that RhoU can also be induced by gp130 cytokines and that both its basal and cytokine-induced transcription in MEF cells requires Stat3, which binds to two Stat3 responsive elements on the mouse RhoU promoter. We then show that also Wnt-1-mediated RhoU induction is regulated at the transcriptional level but is independent of Stat3, does not involve the canonical β -catenin-dependent pathway and requires JNK activity instead. Finally, we provide evidence for Stat3-mediated RhoU regulation also in human tumor or non-transformed epithelial cell lines.

EXPERIMENTAL

Cell lines and treatments

Stat3^{+/+}, Stat3^{-/-}, Stat3Rev immortalized MEFs [17], HEK293, HEK293-Wnt1, HeLa, PC3, DU145 and SKBR3 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-BRL). T47D, SKOV3, MDA-MB-231 and MDA-MB-468 were grown in RPMI 1360 (Gibco-BRL). Both media were supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Gibco-BRL), 2 mM L-glutamine, 100 U/ml of penicillin and 100 μ g/ml of streptomycin. MCF10A cells were grown in DMEM F12 (Gibco-BRL) supplemented with 5% (v/v) Horse Serum (Gibco-BRL), 20 ng/ml EGF, 0.5 μ g/ml hydrocortizone and 10 μ g/ml insulin. All cell lines were maintained at 37°C in a 5% CO₂ atmosphere. The HEK293-Wnt1 stable transfectants were generated by stable transfection with a Wnt-1 expression vector. Cells were treated with 20 ng/ml Oncostatin M (OSM) (R&D Systems Inc.) or with recombinant IL-6 (500 ng/ml) plus soluble receptor (250 ng/ml) [18, 19] for different time lengths. MEF cells were treated with the following inhibitors: SP600125 (Sigma-Aldrich) 50 μ M, Y-27632 (Sigma-Aldrich) 10 μ M and Myristoylated Protein Kinase C Peptide Inhibitor (Promega) 100 μ M for the indicated times. DU145 and SKBR3 were treated with 50 mM Stat3 inhibitor S3I-201 for the indicated times.

RNA extraction, retrotranscription and quantitative Real Time PCR (qRT-PCR)

Total RNA was prepared with the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen) according to manufacturer's protocol. Samples were treated with Dnase I Amp Grade (Invitrogen) before retrotranscription to eliminate genomic DNA contamination. To produce template cDNA, 125 ng of total RNA were reverse-transcribed using the RT High Capacity kit (Applied Biosystems) according to manufacturer's instructions.

Quantitative PCR reactions were performed with the ABI Prism 7300 Real-time PCR System (Applied Biosystems) using Platinum Quantitative PCR SuperMix-UDG with ROX (Invitrogen). The reactions were carried out in a total volume of 25 μ l using a TaqMan assay (Mm00505976_m1, Applied Biosystems) specific for murine RhoU. Human RhoU was detected by using the Universal Probe Library system (Roche) with the following primers and probe: 5'-GACTCCAACCTCTGTGACACTGC-3'; 5'-ATGAGGGGCTCACGACACT-3'; Probe #4. Data were analyzed with the 2^{- $\Delta\Delta$ Ct} method using the 18S rRNA pre-developed TaqMan assay (Applied Biosystem) as an internal control.

EMSA Competition Assays.

EMSA probes/competitors consisted in double stranded DNA oligonucleotides formed by a 9 bp long Stat3 binding site flanked by 3 bp on both sides and by a GATC protruding sequence on the 5' end. Labelling and EMSA were performed as previously described [2]. Briefly, the HA-SIE probe (1 pmole) was labeled using ³²P-dCTP and Klenow and purified from free nucleotides on a Sephadex spin column. 4 μ g of nuclear extracts were pre-incubated on ice for

10 minutes in KCl 80 mM, MgCl₂ 2 mM, EDTA 2 mM, HEPES 40 mM, Glycerol 20% v/v, Poly dI/dC at 3 µg/reaction, DTT 5 mM, and BSA 1 µg/µl. 1 µl of competitor, corresponding to concentration ratios 10, 50, or 100 fold higher with respect to that of the labeled probe, was added and let interact for 10 minutes on ice followed by incubation with the labeled probe (10,000 cpm) for 10 minutes at room temperature. Samples were fractionated on a 5% v/v polyacrylamide gel in 0.25x TBE buffer pH 8.3 (Tris 4.65 µg/µl, Boric Acid 30 µg/µl, EDTA 60.5 µg/µl) at room temperature. Gels were dried and autoradiographed. The oligo nucleotide sequences used are indicated below:

Site1, Upper 5'-GATCCATTTCCAAGACAGC-3', Lower 5'-GATCGCTGTCTTGGAAATG-3'; Site2, Upper 5'-GATCATATTAAGGAATTC-3', Lower 5'-GATCGAATTCCTTAAATAT-3'; Site3, Upper 5'-GATCGGTTGCTGGGAATTG-3', Lower 5'-GATCCAATTCAGCAACC-3'; Site4, Upper 5'-GATCCTTTTCAGATAATAC-3', Lower 5'-GATCGTATTATCTGAAAAG-3'; Site5, Upper 5'-GATCAGGAGCCGGGAAAGG-3', Lower 5'-GATCCCTTTCCCGGCTCCT-3'; Site6, Upper 5'-GATCCGTTTCGAGGCGCCG-3', Lower 5'-GATCCGGCGCCTCGAAACG-3'; HA-SIE, Upper 5'-GGGCATTTCCCGTAAATCGT-3', Lower 5'-CCCACGATTTACGGGAAATG-3', Unrel., Upper 5'-GATCGGTTTCCGGACCAGC-3', Lower 5'-GATCGCTGGTCCGGAAACC-3'.

Western Blots.

Total protein extracts were obtained in 50 mM TRIS pH 7.4, 10% (v/v) glycerol, 1% (v/v) NP-40, 150 mM NaCl, 2 mM EDTA, 2 mM DTT, 0.4 mM Na₂VO₄, 10 mM NaF, 0.5 mM PMSF, and 40 µg/ml protease inhibitor cocktail (Sigma-Aldrich, St.Louis MO, USA). Proteins were cleared by centrifugation and concentration measured by the Bradford assay (Bio-Rad, Hercules CA, USA). Samples were fractionated on SDS-PAGE and transferred to PVDF membrane (Millipore, Billerica MA, USA) for immunoblotting. The following antibodies were used: anti-phospho-Stat3 (Tyr705), (Cell Signaling Technology, Danvers MA, USA), total Stat3 and Actin, (Santa Cruz Biotechnology, Santa Cruz CA, USA), Gapdh (Millipore, Billerica, MA, USA).

Generation of Luciferase Reporter Plasmids

RhoU promoter fragments were generated by PCR using a genomic BAC vector as a template. The following primers were used: -1765 forward (5'-ACTAGGTACCGAATTCAGGTGGCCCAAGGGC-3'); -1300 forward (5'-GGCCGGTACCGCATCTCTGTACTTCAGGTTGTCC-3'); -770 forward (5'-GGCCGGTACCGCTAACAAAGGGTGGTATCCTGC-3'); -200 forward (5'-GGCCGGTACCGGTCTCGGATAGAAGAAGGAGCC-3'); Reverse (5'-TATCTCGAGGCCGCGAGACCAGCTGCC-3'). Amplified fragments were digested with KpnI/XhoI, cloned into pBlueScript (Stratagene, La Jolla, CA), sequenced and subcloned into the promoterless luciferase reporter vector pGL3basic (Promega, Madison, WI). Mutant constructs were made by site directed mutagenesis using the QuickChange Mutagenesis Kit (Stratagene) according to manufacturer's instructions. The following primers were used for mutagenesis (mutated bases are underlined): -1300 M2 sense (5'-GGGTATAAGTAATGCTAATATTAAGCCCTTCGTTTTGACAGAATGGTTA-3'); -1300 M2 antisense (5'-TAACCATTCTGTCAAAACGAAGGGCTTTAATATTAGCATTACTTATACCC-3'); -1300 M3 sense (5'-CGTGAGTCACCATGTGGTTGCTGGCCCTTGAACTCAGGACC-3'); -1300 M3 antisense (5'-GGTCCTGAGTTCAAGGGCCAGCAACCACATGGTGACTCACG-3'); -1300 M5/-770 M5 sense (5'-GGAAGCTTGGGAAGGAGCCGGCCAGGGGGGTGG-3'); -1300 M5/-770 M5 antisense (5'-CCACCCCCCTGGGCCGGCTCCTCCCAAGCTTCC-3'); -1300 MA sense (5'-CAACAACATCTGGGACATAAACAGGCTCAGTATTTAACTAGCTCTTGTC-3'); -1300 MA antisense (5'-GACAAGAGCTAGTTAAATACTGAGCCTGGTTTATGTCCCAGATGTTGTTG-3'); -770 MB sense (5'-CCAA

AGAGAGGGAGCGCTAAACCAGGGTGGTATCCTGCATC-3'); -770 MB antisense (5'-GATGCAGGATAACCACCCTGGTTTAGCGCTCCCTCTCTTTGG-3'); -770 MC sense (5'-CAAAGGGTGGTATCCTGCATCCAAATGAGAATACCTGATTTTTAACAG-3'); -770 MC antisense (5'-CTGTTAAAATCAGGTATTCTCATTGGATGCAGGATAACCACCCTTTG-3'). All mutant constructs were controlled by sequencing.

Transient Transfections, Co-culture and Luciferase Assays

MEF and HeLa cells were seeded in 24-well plates and transfected 24h later with Lipofectamine Reagent (Invitrogen). Each promoter construct was mixed at a 10:1 ratio with the pSEAP vector [20] as an internal control for transfection efficiency for a total amount of 0.4 µg of DNA and incubated with 5 µl of Lipofectamine reagent according to the manufacturer's instructions. In co-transfection experiments, promoter constructs were mixed at a ratio 5:5:1 (reporter : GTPase vector : pSEAP). 24h after transfection the cells were treated with OSM or detached and re-seeded with an equal amount of Wnt1-expressing HEK293 or wild-type control cells for co-culture experiments. 48h after transfection the cells were washed twice with phosphate-buffered saline and lysed in 50 µl of Passive Lysis Buffer (Promega). Luciferase assays were performed using a Luciferase Assay System (Promega) according to manufacturer's instructions. SEAP assays were performed using the Phospha-Light System (Applied Biosystems) according to manufacturer's instructions. Luciferase and SEAP chemiluminescent activity were quantified using a Mithras LB940 Multilabel Reader (Berthold Technologies, Bad Wildbad, Germany). SEAP values were used to normalize for differences caused by unequal transfection efficiency.

Chromatin Immunoprecipitation (ChIP) Assay

Stat3^{+/+} and Stat3^{-/-} MEFs were treated or not with OSM as described above. ChIP assays were performed with the fast ChIP method [21] with some modifications. Briefly, cross-linking was performed with 1% (v/v) formaldehyde for 10 min at room temperature. The reaction was quenched by the addition of glycine to a final concentration of 125 mM and incubation for 10 min at room temperature. Cells were lysed and sonicated at 4°C for six 10 sec rounds followed by a 60 sec pause, at an amplitude of 40% (Ultrasonic Processor VCX-750, Sonics and Materials).

Immunoprecipitations were performed by incubating overnight at 4°C 1 ml of sheared chromatin with anti-Stat3 serum (R&D Systems, 5 µl), anti-acetyl-Histone H3 (Upstate Cell Signaling Solutions, 2 µg) or negative control IgG (ChIP-IT Control Kit-Mouse, Active Motif, 2 µg). Primers for PCR amplification were designed using the program Primer3 [22] to obtain an amplicon size of about 150 bp. PCR products were fractionated on a 2% (w/v) agarose gel and stained with Ethidium Bromide. PCR reactions were performed using 5 µl of immunoprecipitated DNA using the following primers: RhoU site3, Forward 5'-GGCACCAGATCCCATTACAG-3', Reverse 5'-TTTTGTGCTGCCCTTTCTCT-3'; RhoU site 5, Forward 5'-ATCGCAAGGTCAGCTTTCAC-3', Reverse 5'-CTTGCTTGCCATTTACCAT-3'; Socs3⁻⁶⁵, Forward 5'-GGAGAGACAGCGGTCGTAAG-3', Reverse 5'-CACAGCCTTTCAGTGCAGAG-3'; β-globin, Forward 5'-CTCCCCCTCACTCTGTTCTG-3', Reverse 5'-AGGAGGAGGGGAAGCTGATA-3'.

RESULTS

RhoU is a Stat3 transcriptional target induced by gp130 cytokines.

Gene expression profiling of Stat3^{+/+} or Stat3^{-/-} MEF cells either untreated or stimulated with different cytokines has led us to identify, among others, the atypical Rho GTPase RhoU/Wrch-1 as a Stat3-dependent target of gp130 cytokines stimulation (unpublished observations). Moreover, RhoU displayed a striking degree of conserved coexpression with

Stat3 across hundreds of human and mouse microarray experiments [23], further suggesting a close functional correlation. Quantitative PCR analysis of RhoU mRNA levels showed indeed profoundly defective expression in Stat3^{-/-} MEFs already under basal conditions (Fig. 1a). In addition, while both IL-6 and OSM treatment induced RhoU mRNA in Stat3^{+/+} MEFs, they were completely ineffective in the absence of Stat3. As a result, RhoU expression levels were 4-5 folds lower in the Stat3^{-/-} MEFs following cytokine stimulation. These defects are indeed Stat3-dependent since reintroducing Stat3 by stable transfection in the Stat3^{-/-} MEFs (revertant MEFs) completely rescued both RhoU basal expression and cytokine inducibility (Fig. 1a). RhoU mRNA induction in response to OSM stimulation was already appreciable 30' after OSM treatment, peaked at 6 hours and decreased to basal levels 24 hours later (Fig.1b).

RhoU promoter contains two functional Stat3 binding sites fully responsible for OSM inducibility.

Since RhoU expression is induced by gp130 cytokines/Stat3, we searched the mouse RhoU promoter region for potential Stat3 regulatory elements. Bioinformatics analysis [24] identified six potential Stat3 binding sites (Site 1-Site 6) that were assessed for *in vitro* binding capacity by EMSA competition assays (Fig. 2a). Liver nuclear extracts from LPS-treated mice were used as a source for activated Stat3, with a labeled high affinity sis-inducible element (HA-SIE) [25] double-stranded oligonucleotide as a probe. Stat3 binding to the HA-SIE probe was then competed with cold double-stranded oligonucleotides based on the sequences of the six predicted Stat3 binding sites. Site 3 showed the greatest *in vitro* binding activity, with full competition at a ratio of 100:1 between cold competitor and labeled probe and partial competition at the ratios of 50:1 and 10:1. Sites 5 and 2 were also able to compete, albeit with lower efficiency/affinity, while sites 1, 4 and 6 did not show any significant competing activity. Self-competition with a cold HA-SIE oligonucleotide or competition with an unrelated sequence were used as positive and negative controls, respectively.

In order to analyze RhoU promoter activity, mouse RhoU promoter deletion fragments were fused to a luciferase reporter and transiently transfected in Stat3^{+/+} and Stat3^{-/-} MEF cells (Fig. 2b). Transfected cells were either stimulated with OSM or left untreated for 24 hours before measuring luciferase activity. In keeping with the mRNA expression data, transcriptional activity of all promoter fragments was drastically reduced in Stat3-deficient MEFs as compared to their wild-type controls already under basal conditions. In addition, the constructs -1765, -1300 and -770 were induced by OSM treatment in the Stat3^{+/+} MEFs, confirming that cytokine-dependent RhoU mRNA induction occurs transcriptionally. In contrast, OSM-dependent induction of all constructs was completely defective in the Stat3-deficient cells. Both basal and OSM-stimulated activity of RhoU promoter constructs were comparable to those of the mouse SOCS3 promoter [26], a well recognized Stat3 target. RhoU-200, the shortest promoter fragment tested, displayed reduced activity in both Stat3^{-/-} and ^{+/+} MEFs and was unresponsive to OSM treatment, suggesting that the region(s) required for OSM induction must be located upstream of the -200 position. Interestingly, and only in the presence of Stat3, both basal transcription levels and OSM inducibility increased with the progressive shortening of the promoter up to position -770, before dropping at -200. This might suggest the presence of repressor element(s) in the distal portions of the promoter.

To assess the functional contribution of the tested Stat3 binding sites (see Fig. 2a), sites 2, 3 and 5 were mutagenized in the context of the RhoU-1300 construct and the transcriptional activity of the resultant RhoU -1300 M2, M3 or M5 constructs tested in wild type MEFs (Fig. 2c). Site 2 mutation did not have any effect on either basal or OSM-induced expression, suggesting that this site is not involved in Stat3-mediated regulation. Indeed, site 2 was the

one with the lowest *in vitro* binding activity (Fig. 2a). Disruption of sites 3 or 5 significantly decreased transcriptional activation in response to OSM stimulation, and the compound double mutation (-1300 M3+M5) completely abolished promoter inducibility and decreased its basal activity (Fig. 2c). Thus, both Stat3 binding sites 3 and 5 cooperate to confer full promoter activation in response to OSM stimulation. On the other hand, site 5 plays also a prominent role to confer OSM inducibility to the RhoU-770 promoter construct, since its mutation in this context (-770 M5) almost completely abolished OSM inducibility (Fig. 2c). Site 6 did not appear to contribute to this residual induction since neither a site 6 single mutation nor a compound site 5/site 6 double mutation were able to abolish it (-770 M6 and M5+M6 mutants, Fig. 2c).

To further analyze the *in vivo* functionality of sites 3 and 5 we assessed Stat3 *in vivo* occupancy by chromatin immunoprecipitation (ChIP). ChIP assays were performed with chromatin samples prepared from Stat3^{+/+} and Stat3^{-/-} MEFs, either untreated or stimulated with OSM for 30 minutes. With respect to the negative IgG controls, both sites showed weak Stat3 binding already in untreated cells (Fig. 2d). Similar to what observed for the well characterized SOCS3-65 Stat3 responsive element [26], Stat3 binding to both site 3 and site 5 was strongly induced by OSM, confirming their *in vivo* functional role in mediating RhoU promoter activity. Importantly, no Stat3 binding was ever detected with chromatin from Stat3^{-/-} MEFs, thus confirming the specificity of our ChIP assay.

RhoU induction by Wnt-1 occurs at the transcriptional level but does not require Stat3.

Since RhoU was initially isolated as a Wnt-1-regulated gene but its transcriptional regulation was never characterized, we decided to investigate the mechanisms involved in RhoU up-regulation downstream of Wnt-1 and the potential cooperation with the Stat3 pathway. Thus, RhoU mRNA expression levels were measured in Stat3^{+/+} and Stat3^{-/-} MEFs stimulated with OSM and Wnt-1 either alone or in combination (Fig. 3a). Wnt-1 stimulation was obtained by co-culturing MEF cells with HEK293 cells stably expressing Wnt-1, while control cells were co-cultured with wild-type HEK293. In keeping with the previous observations, RhoU mRNA expression was dependent on Stat3 already under basal conditions and failed to be induced by OSM stimulation in Stat3^{-/-} cells. In contrast, Wnt-1 stimulation could similarly induce RhoU mRNA in both Stat3^{+/+} and ^{-/-} MEFs. Likewise, Wnt-1 could induce RhoU promoter transcription at similar levels in the presence or absence of Stat3 (Fig. 3b). These data suggest that Wnt-1-mediated RhoU induction occurs at the transcriptional level and is independent of Stat3. This was further supported by the observation that the RhoU -770 M5 construct, which has lost OSM-inducibility, is still fully responsive to Wnt-1 stimulation (Fig. 4a). Thus, both the Wnt-1 and the gp130/Stat3 pathways can activate transcription of the RhoU promoter, but they act through independent mechanisms. Of note, RhoU induction could not be obtained using the supernatants of the Wnt-1-expressing HEK293 cells (data not shown), suggesting that specific stimulation by membrane-bound Wnt-1 and not secretion of soluble mediators by the Wnt-1-expressing cells is involved.

Similar to what observed for OSM, Wnt-1-mediated induction progressively increased with promoter shortening, suggesting that the inhibitory sequence(s) already postulated in the case of OSM induction may be active also in the context of Wnt-1 stimulation (Fig. 3c). As in the case of OSM responsiveness, the RhoU-200 construct was not anymore able to respond to Wnt-1 stimulation indicating that the Wnt-1-responsive element(s) must lie in the region between positions -770 and -200. The reporter vector Super8X-TOPFlash and its negative control Super8X-FOPFlash [27] were used in all co-culture experiments to monitor Wnt pathway activation (not shown).

RhoU promoter induction by Wnt-1 does not involve the canonical β -catenin pathway.

The molecular mechanisms leading to RhoU gene activation downstream of Wnt-1 are currently unknown. Among the many signalling events activated by Wnt ligands, the canonical Wnt/ β -catenin pathway which leads to the up-regulation of several target genes mediated by the LEF/TCF transcription factor is the best characterized [28, 29]. Mouse RhoU promoter analysis using the TRANSFAC open-source program [30] identified three consensus sequences for LEF/TCF (Sites A, B, C, Fig. 2b). However, none of the three sequences appears to play a role in RhoU promoter activity, as shown by transient transfection assays of the relative promoter mutants -1300 MA, -770 MB or MC (Fig. 4a). Moreover, downstream activation of the Wnt-1/ β -catenin pathway by expression of a stabilized mutant form of β -catenin did not have any effect on RhoU promoter transcription while it strongly activated the TOPFlash control construct (Fig. 4b). Thus, the canonical Wnt/ β -catenin pathway does not appear to be involved in mediating RhoU transcriptional induction.

A JNK-mediated non-canonical Wnt pathway is responsible for RhoU activation.

Besides the canonical β -catenin pathway, Wnt ligands are known to activate the Wnt/ Ca^{2+} and the planar cell polarity (PCP) non-canonical pathways, as illustrated in Figure 7. In order to assess their potential involvement in RhoU induction by Wnt-1, we have used available specific chemical inhibitors to interfere with the activities of the main mediators involved.

First, RhoU mRNA levels were measured by Real Time PCR in wild-type MEF cells treated or not with specific inhibitors for PKC (Wnt/ Ca^{2+} pathway), ROCK and JNK (PCP pathway) in the presence or absence of Wnt-1 stimulation (fig. 5a). Wnt-1-mediated RhoU mRNA induction was unaffected by PKC and ROCK inhibitors, while it was completely abolished by inhibiting JNK activity (Fig. 5a). A similar effect was also observed at the level of RhoU promoter activation, since Wnt-1-mediated induction of the -770 promoter construct was strongly reduced in the presence of the JNK inhibitor (Fig. 5b).

Thus, RhoU transcriptional activation by Wnt-1 appears to be mainly mediated by the non-canonical PCP pathway, with a central role played by the MAP kinase JNK. Since the activation of JNK downstream of Wnt is known to be mediated by the small GTPases Cdc42 and Rac (see Fig. 7), we performed co-transfection experiments of the RhoU promoter with constitutively active Rac1 and Cdc42 mutants (Fig. 5c). Both Rac1-Val12 and Cdc42-Val12, but not the control RhoA-Val14, were able to significantly activate RhoU promoter and this induction was abolished by JNK inhibition, strengthening the idea that Wnt-1 can activate the PCP pathway leading to the activation of the RhoU promoter through Rac1 and Cdc42-mediated JNK activation.

Stat3/gp130-dependent regulation of RhoU in human epithelial cell lines.

In order to extend our results to more biologically relevant experimental systems, we analyzed RhoU mRNA expression and its correlations with the gp130/Stat3 pathway in human transformed and non-transformed epithelial cell lines. OSM treatment led to RhoU mRNA up-regulation also in HeLa cells, albeit with a different kinetics as compared to MEF cells (Supplementary Fig. 1a). In the same cells, additionally, both OSM and Wnt1 stimulation were able to induce RhoU promoter activity (fragment -770) to a similar extent as observed in MEF cells (Supplementary Fig. 1a).

Since RhoU is thought to mediate Wnt-1 oncogenic properties and might therefore play a similar role downstream of Stat3, we decided to correlate Stat3 activation and RhoU expression levels in a panel of human tumor cell lines well characterized for Stat3 constitutive activation. RhoU expression levels (Fig. 6 c) were always elevated in cell lines with high (DU145, SKOV3, MDA-MB-231, MDA-MB-468) or moderate (SKBR3) levels of constitutively active Stat3 (Fig. 6 a,b), all of which, with the exception of SKOV3 cells, have

been reported to be addicted to Stat3 activity for survival/proliferation [31-33]. Conversely, cell lines with no detectable phospho-Stat3 showed variable RhoU mRNA levels (Fig. 6 a-c). In particular, RhoU mRNA was almost undetectable in the breast cancer cells T47D, while its levels were comparable to those of the other cell lines in the prostate cancer PC3 cells. Of note, these are known to display constitutive Wnt-1 expression, which may be responsible for RhoU up-regulation in the absence of Stat3 activity [34].

In order to assess the functional correlation between Stat3 activity and RhoU expression, DU145 and SKBR3 cells were then treated with the specific Stat3 inhibitor S3I-201 [33]. Both phospho- and total Stat3 levels were drastically reduced in both cell lines after S3I-201 treatment (Fig. 6d), correlating with a dramatic decrease of RhoU mRNA levels as determined by qRT-PCR (Fig. 6e, 78% and 93% reduction at 24 hours in DU145 and SKBR3 cells, respectively).

Finally, IL-6 stimulation triggered Stat3 phosphorylation correlating with a strong up-regulation of RhoU mRNA expression in MCF10A cells, a non-transformed human mammary cell line displaying almost undetectable levels of Stat3 phosphorylation under basal conditions. (Fig. 6f,g).

DISCUSSION

The atypical Rho GTPase RhoU was first identified as a Wnt-1-responsive gene by subtractive cloning [12]. RhoU over-expression can trigger cellular transformation and phenocopies the effects of Wnt-1 in mammary epithelial cells, suggesting a potential role in Wnt-mediated tumorigenesis [12]. Rather than being regulated by GDP-GTP exchange like most Rho GTPases, RhoU activity can be modulated by acting on its N-terminal domain, which exerts a partial inhibitory effect on RhoU ability to activate PAK and to cause cellular transformation [14]. This inhibition can be relieved by interaction of the PxxP motif in the N-terminal domain with SH3-containing protein adaptors such as Grb2 and Nck β , suggesting functional interactions with receptor tyrosine kinases-activated pathways. Moreover, unlike most Rho GTPases that are constitutively expressed, RhoU expression is tightly controlled [35, 36]. In addition to being induced by Wnt-1, RhoU mRNA has been recently shown to accumulate in osteoclasts in response to RANKL (Receptor Activator for Nuclear Factor κ B Ligand)-induced differentiation. However, the molecular mechanisms for either inductions have not been characterized [37]. Here, we demonstrate for the first time that RhoU induction by Wnt-1 occurs at the transcriptional level. Moreover, we show that RhoU is also a prominent target for Stat3-mediated transcription downstream of the gp130 cytokines IL-6 and OSM. Intriguingly, while both *in vivo* functional Stat3 binding sites 3 and 5 are required to confer full OSM inducibility to the -1300 RhoU promoter construct, site 5 alone is sufficient in the context of the shorter -770 promoter fragment, which lacks site 3. Both sites might indeed be required both *in vivo* and in the longer promoter construct to overcome the observed inhibitory effect of sequence(s) located between the -1300 and -770 positions. Interestingly, Stat3 was required not only for RhoU cytokine-mediated induction, but also for its basal expression, and both functionally tested Stat3 binding sites, site 3 and site 5, displayed a low level of constitutive binding following ChIP analysis. Constitutive Stat3 binding to a subset of sites was recently shown in NIH 3T3 cells [38], although a correlation with basal gene expression was not tested. Low amounts of Tyr⁷⁰⁵-phosphorylated Stat3 are present in growing MEFs and could be responsible for the constitutive binding observed. Alternatively, non-canonical mechanisms recruiting unphosphorylated Stat3 may be involved. At any rate, Stat3 ability to transcriptionally regulate a subset of target genes under basal conditions may have important implications for its physiology.

Both Wnt ligands and Stat3 play surprisingly similar multiple roles in tumorigenesis. In particular, both are involved in stimulating cell movements in tumoral cells as well as during development [8, 39]. RhoU is known to act on the actin cytoskeleton, triggering filopodia formation and stress fibers dissolution. In addition, it was recently shown to localize to focal adhesions and podosomes and to regulate their genesis promoting cell migration [15, 16]. Intriguingly, also Stat3 was shown to localize to focal adhesions in HeLa and ovary tumor cells, where it interacted with activated Focal Adhesion Kinase and paxillin and promoted cell migration [40], and we could demonstrate that activation of the gp130/Stat3 pathway induces both RhoU mRNA and promoter activity also in HeLa cells. Thus, RhoU transcriptional induction might be one of the mechanisms through which Stat3 acts on focal adhesions and ultimately triggers cell migration. Indeed, the same Stat3^{-/-} MEF cells used in this study, exhibiting low basal RhoU levels, also display impaired migration in a wound healing assay [41]. In addition, the convergence of both Wnt-1 and Stat3 signalling on RhoU may represent a point of crosstalk between the two pathways, which has been proposed in several systems. Stat3 expression was induced by the Wnt/beta-catenin pathway in esophageal cancer cells and in ES cells [42, 43]. Stat3 was also proposed to act upstream of the Wnt/PCP pathway during the gastrulation of zebrafish embryos [9] and to promote β -catenin nuclear accumulation in colorectal cancer cells [44]. Moreover, the negative regulator of Wnt signalling Duplin was recently shown to suppress also Stat3 activity [45]. Indeed, both Stat3 and Wnt-1 aberrant activation are often observed in the same tumors, suggesting that the two pathways can cooperate in different ways depending on the cellular context.

However, the convergence of the Wnt-1 and gp130/Stat3 pathways on RhoU induction must involve distinct mechanisms, since Stat3 is not required for Wnt-1-mediated induction of the RhoU promoter. In addition, as previously suggested by A. Levine and co-workers [12], RhoU induction by Wnt-1 appears also to occur independently of β -catenin, suggesting the involvement of one of the non-canonical pathways. We could identify this pathway with the Wnt/PCP pathway, since Wnt-1-mediated RhoU induction is abolished by an inhibitor of JNK activity. Interestingly, RhoU itself was reported to be able to activate JNK [12], suggesting the existence of a positive feedback loop whereby activated RhoU may enhance its own transcription by means of JNK activation. Supporting and extending this observation, we could demonstrate that both constitutively active Rac1 and Cdc42, which lie upstream of JNK in the Wnt/PCP pathway, can activate RhoU transcription in a JNK-dependent way. Thus, at least in MEF cells, Wnt-1 can activate both the canonical β -catenin pathway and the non-canonical PCP pathway at the same time, as shown by the simultaneous activation of RhoU and TOPFlash in our system. It is intriguing that the Wnt/PCP pathway is best characterized as regulating cell movements during embryonic gastrulation and that RhoU main functions appear to be linked to focal adhesions and cell migration.

At present, the transcription factors mediating the effects of the PCP pathway are unknown, and our system may provide a relatively straightforward way to identify at least some of them. However, bioinformatic analysis of the RhoU promoter region involved in Wnt-1 responsiveness did not disclose any obvious candidate, and co-transfection experiments with transcription factors potentially activated by JNK such as c-fos, c-jun and CREB did not modify promoter activity (D.S. and V.P., unpublished results). More detailed promoter analysis will therefore be required in order to identify the PCP-activated transcription factors. The identification of RhoU as a common transcriptional target of both Stat3 and Wnt1, together with the known roles of both pathways in stimulating cell migration in normal and tumoral cells, suggests the intriguing idea that RhoU may represent a common mediator of Stat3 and Wnt effects on cell motility under both physiological and pathological conditions, being potentially a common therapeutic target. Interestingly, we have observed a

positive correlation between Stat3 phosphorylation and RhoU expression levels in all tested tumor-derived cell lines. Indeed, phosphorylated Stat3 was undetectable in T47D cells, the only ones displaying very low RhoU mRNA levels. The metastatic prostate cancer cell line PC3 in contrast, also lacking detectable Stat3 activation, displayed high RhoU mRNA expression. In these cells Wnt-1 expression was reported to be extremely elevated, and could thus play a dominant role in RhoU induction bypassing the need for Stat3 activity [34]. A direct correlation between Stat3 activity and RhoU transcription in tumor cell lines is also supported by our observation that specific Stat3 inhibition triggered a dramatic reduction of RhoU mRNA in DU145 and SKBR3 cells. Accordingly, acute Stat3 activation by IL-6 treatment could efficiently up-regulate RhoU mRNA in the non-transformed mammary epithelial cell line MCF10A. Taken together, these data strengthen the hypothesis that RhoU may represent a common transcriptional and functional target of both the Wnt-1 and Stat3 pathways in physiological as well as pathological contexts. Further studies addressing the role played by RhoU in mediating specific features of Stat3 and Wnt-mediated cell transformation will be required to address this issue.

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FIGURE LEGENDS

Fig.1 RhoU mRNA expression is defective in Stat3^{-/-} MEFs.

Total RNA was extracted from Stat3^{+/+}, Stat3^{-/-} and revertant (rev) MEFs either untreated or stimulated with IL-6 or OSM for the indicated times. RhoU mRNA was measured by quantitative PCR (qRT-PCR). Expression was normalized to 18S, and results are shown as mean \pm SEM of two independent experiments performed in double. Asterisks indicate statistically significant differences between selected groups (*p<0.05, **p<0.01).

Fig.2 Functional Stat3 binding sites in the RhoU promoter.

(a). EMSA competition assays. A radioactively labeled HA-SIE probe was incubated with liver nuclear extracts containing activated Stat3 and the binding was competed with increasing amounts of unlabeled oligonucleotides based on the sequence of the six predicted Stat3 binding sites on the mouse RhoU promoter, whose sequence and position from the TSS (Transcription Start Site) are indicated. Unlabelled unrelated or HA-SIE oligonucleotides were used as negative or positive competing controls, respectively. (b,c). Transient transfection assays. The indicated constructs were co-transfected with a pSEAP expression vector as an internal control. 24h later cells were stimulated or not with OSM and cultured for further 24h. Luciferase activity was normalized to SEAP activity as control for transfection efficiency. Data are shown as mean \pm SEM of at least two independent experiments performed in double. (b). Transfections were performed with Stat3^{+/+} and Stat3^{-/-} MEFs. Numbers over the bars indicate the induction fold of stimulated over unstimulated cells. The empty pGL3 vector and the previously characterized SOCS3 promoter-luciferase construct [26] were used as controls. (c). Stat3^{+/+} MEFs were transfected with the indicated wild type or mutant (M) RhoU promoter constructs. (d). Chromatin Immunoprecipitation (ChIP) assays were performed with Stat3^{+/+} and Stat3^{-/-} MEFs, either unstimulated (NT) or treated with OSM for 30 minutes. Immunoprecipitations were performed with antibodies against Acetylated Histone H3, Stat3 antisera or control IgG. Non-immunoprecipitated chromatin was used as total input control (T.I.). Specific primers were used to amplify regions of about 150 bp containing the indicated Stat3 binding sites (see Materials and Methods section). Primers amplifying the second intron of the silent β -globin gene (shown) or the non-coding transcript Xist (inactive X specific transcript) (not shown) were used as negative controls.

Fig.3 RhoU induction downstream of the Wnt-1 and Stat3 pathways.

(a). qRT-PCR. Stat3^{+/+} and Stat3^{-/-} MEFs were co-cultured with wild type or Wnt-1-expressing HEK293 cells for 24 hours with or without OSM stimulation. RhoU mRNA levels were measured by qRT-PCR and normalized to 18S RNA. Results are shown as mean \pm SEM of two independent experiments performed in double. (b). Transient transfection assays. Stat3^{+/+} and Stat3^{-/-} MEFs were transfected with the RhoU -770 construct and the pSEAP vector. 24 hours after co-transfection, cells were treated as in (a) for other 24 hours. Data are shown as mean \pm SEM of normalized luciferase activity from at least two independent experiments performed in double. (c) Stat3^{+/+} MEFs were transfected with the following vectors: the indicated RhoU promoter deletion fragments and TOPFlash and FOPFlash reporters. Transfected MEFs were Wnt-1-stimulated as in (a). Data are shown as mean \pm SEM of normalized luciferase activity from at least two independent experiments performed in double.

Fig.4 RhoU induction downstream of Wnt-1 is independent from Stat3 and β -catenin.

(a) Stat3^{+/+} MEFs were transfected with the RhoU -1300 and -770 vectors either wild-type or mutated (M) in the indicated LEF/TCF or Stat3 binding sequences. Wnt-1 stimulation and data analysis were performed as described for figure 3. (b) The RhoU -770 construct and TOPFlash and FOPFlash reporters were co-transfected with a stable mutant β -catenin (S33A) or with an empty control vector in Stat3 wild-type MEFs. Luciferase activity was normalized and reported as above.

Fig.5 Wnt-1-mediated RhoU activation follows a non-canonical pathway involving JNK.

(a). qRT-PCR. Wild-type MEFs were stimulated or not with Wnt-1 as described for Figure 3 and treated with specific inhibitors for PKC (myristoylated peptide inhibitor, 100 μ M), ROCK (Y-27632, 10 μ M) and JNK (SP600125, 50 μ M) for 24 hours. RhoU expression levels were measured by qRT-PCR and normalized to 18S RNA. Means \pm SEM of two independent experiments performed in double are shown. (b,c). Transient transfection assays. Wild-type MEFs were transfected with the promoter fragment -770 alone (b) or in combination with plasmids encoding the constitutively active GTPases RhoA (RhoA V14), Rac1 (Rac1 V12), Cdc42 (Cdc42 V12), or a control empty vector (c). 24 hours later cells were stimulated or not with Wnt-1 as described for Figure 3 and treated with 50 μ M SP600125 (b) or simply treated or not with 50 μ M SP600125 (c) and cultured for further 24 hours. Luciferase activity was quantified and normalized as described for Figure 2.

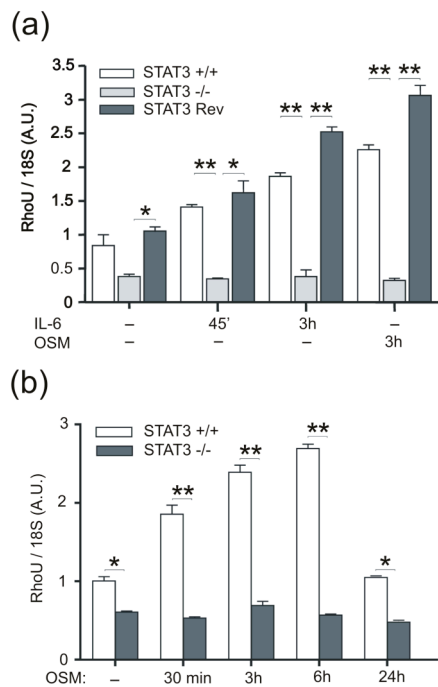
Fig.6 RhoU mRNA regulation by Stat3 in human tumor cell lines.

(a) Total and phospho-Stat3 were measured by Western blot along with Gapdh as a loading control in the indicated tumor cell lines. (b) Quantification of the phospho-Stat3 signal in (a) normalized to Gapdh and reported as mean \pm SEM of two replicates. (c) RhoU mRNA from the same samples analyzed in (a) was measured by qRT-PCR and normalized to 18S. Results are shown as mean \pm SEM of two independent experiments performed in double. (d,e,f,g) Total and phospho Stat3 protein levels (d,f) and RhoU mRNA (e,g) were measured by Western blot and qRT-PCR, respectively, in DU145 and SKBR3 cells treated or not with the Stat3 inhibitor S3I-201 (d,e) or in MCF10A cells treated with IL-6 plus soluble IL-6 receptor.

Fig.7 Schematic representation of the best known Wnt signal transduction pathways.

Wnt signal branches into three main pathways: the canonical β -catenin-dependent and the non-canonical planar cell polarity (PCP) and Wnt-Ca²⁺. All these pathways are thought to occur through the Frizzled receptor and Dishevelled. Other receptors have recently been shown to be able to activate additional non-canonical pathways. In particular the Ror2 receptor, depicted here, converges onto JNK like the Wnt/PCP pathway. In our work we have demonstrated that RhoU is transcriptionally induced both by Wnt-1, through the PCP pathway involving the Rho GTPases Rac1/Cdc42 and the MAP kinase JNK, and by gp130-cytokines through the JAK/Stat3 pathway (thick lines).

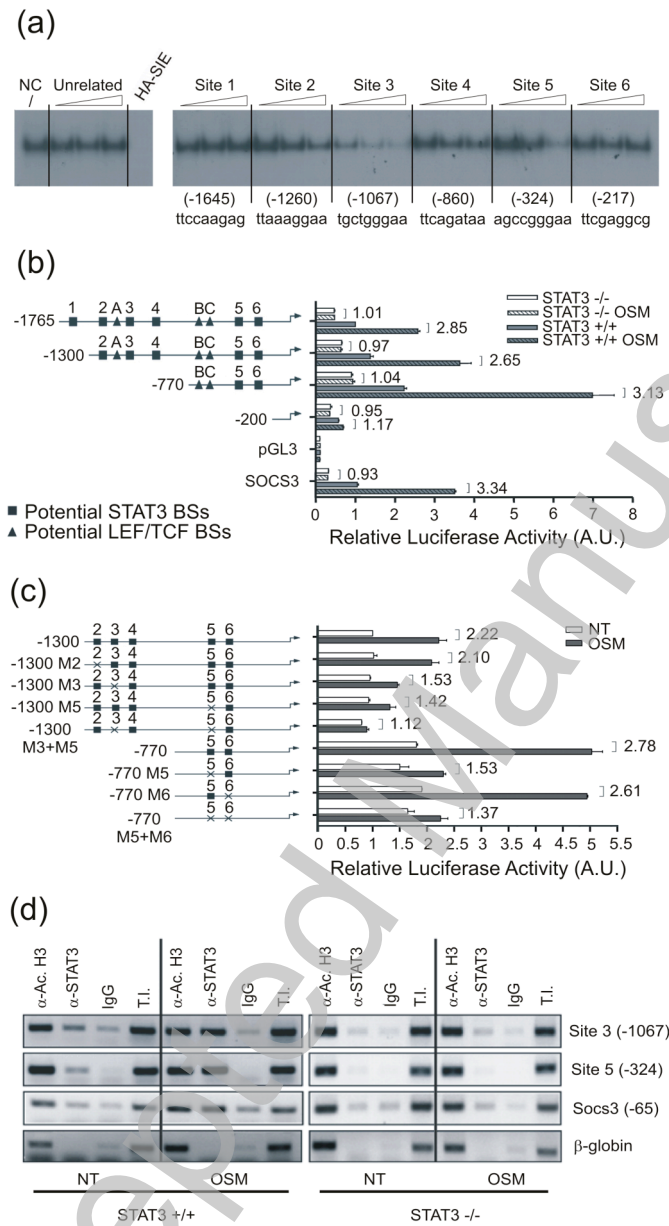
Figure 1



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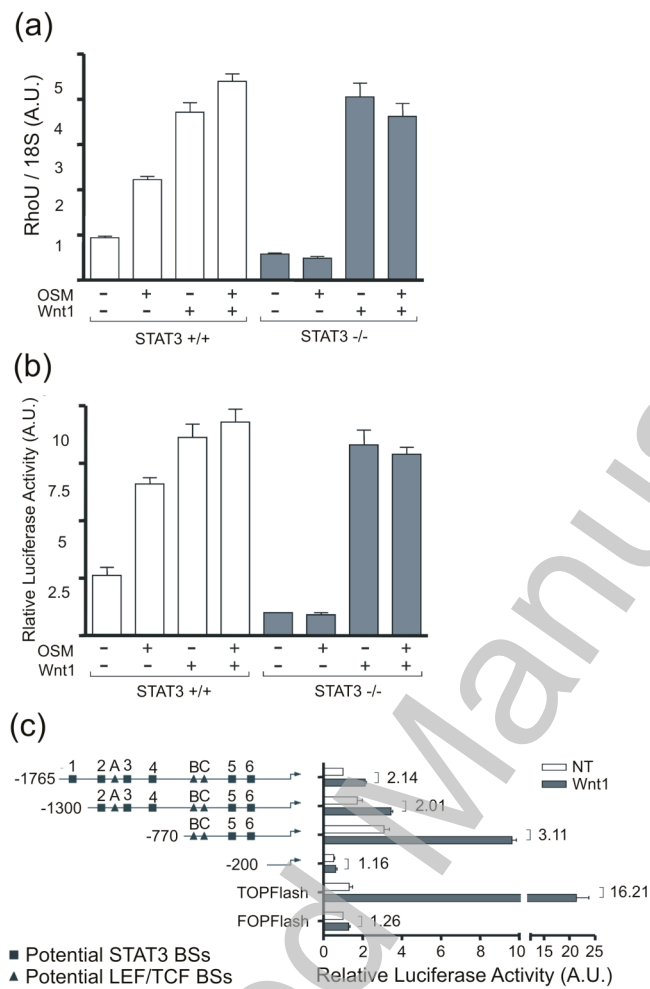
Figure 2



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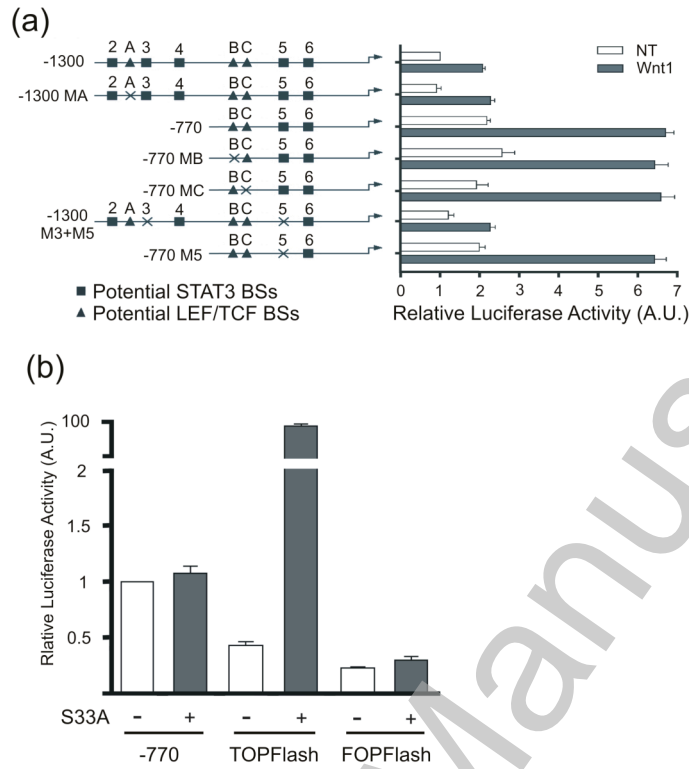
Figure 3



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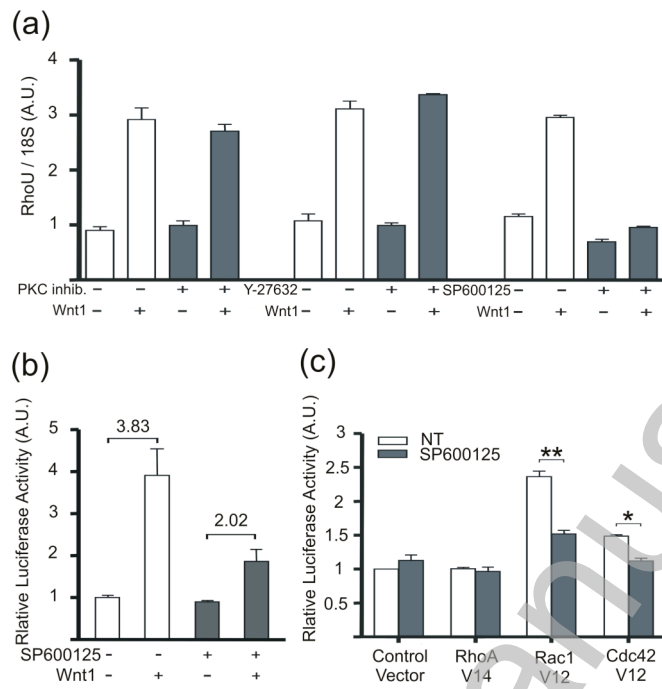
Figure 4



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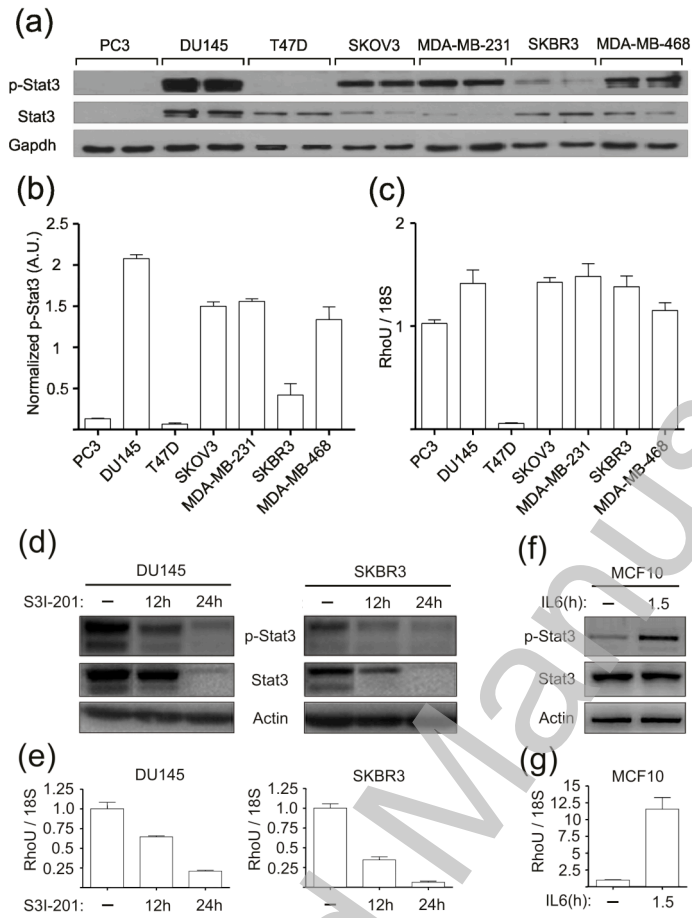
Figure 5



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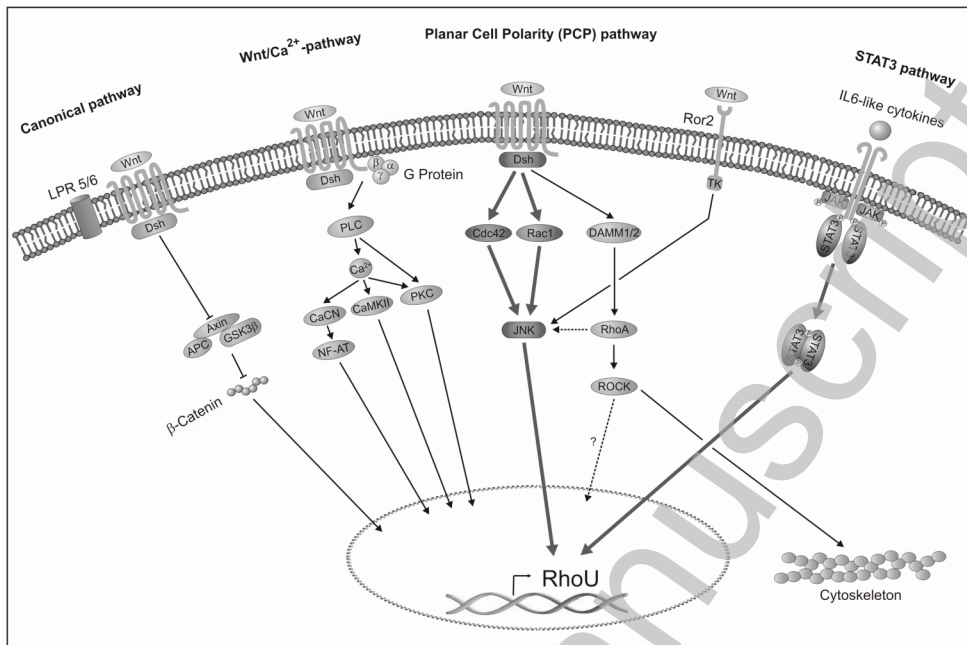
Figure 6



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Figure 7



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