Structural and functional studies of the STAT1 gene from Atlantic salmon

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Abstract

Type I and type II interferons (IFNs) exert their effects mainly through the JAK/STAT pathway, which is presently best described in mammals. A salmon STAT1 homologue, named ssSTAT1a, has been identified and was shown to be ubiquitously expressed in various cells and tissues. The ssSTAT1a has a domain-like structure with functional motifs that are similar to higher vertebrates. These include an amino-terminus that stabilizes interaction between STAT dimers in a promoter-binding situation, a coiled coil domain facilitating interactions to other proteins, a central DNA-binding domain, a SH2 domain responsible for dimerization of phosphorylated STATs and conserved phosphorylation sites within the carboxy terminus. The latter is also the transcriptional activation domain. Endogenous STAT1 were shown to be phosphorylated at tyrosine residues both in salmon leukocytes and in TO cells treated with recombinant type I and type II IFNs. Also ectopically expressed ssSTAT1 was phosphorylated in salmon cells upon *in vitro* stimulation by the IFNs, confirming that the cloned gene was recognized by upstream tyrosine kinases. Treatment with type II IFN also led to nuclear translocation of STAT1 within one hour. The ability of salmon STAT1 to dimerize was also shown.

Keywords: STAT1 (Signal Transducer and Activator of Transcription 1), Atlantic salmon, IPNV, IFN

Introduction

Interferons (IFNs) are cytokines that play a major role in host defense against viral pathogens (Isaacs and Lindenmann, 1957; Samuel, 2001). Mammalian type I IFNs (IFNα/β) are produced by many cell types and confer antiviral activities on them, while type II IFN (IFNγ) is produced mainly by T lymphocytes and natural killer cells when stimulated by macrophage derived cytokines. IFNγ elicits broad effects, particularly on cells of the immune system. The transmission of both type I IFNs and IFNγ signals are dependent on the activation of the transcription factor STAT1 (signal transducer and activator of transcription). STAT family proteins are critical to the action of most cytokines and growth factors, as they are latent cytoplasmic transcription factors that directly activate signaling pathways upon being phosphorylated (Brierley and Fish, 2005; Platanias, 2005; Aaronson and Horvath, 2002).

The activation of STAT is encompassed as part of evolutionary conserved pathways by which signals can be transduced from the membrane to the nucleus rapidly. The classical view is that type I IFN (IFNα/β) signals through STAT1/STAT2 heterodimers, while IFNγ signals through STAT1 homodimers. The binding of secreted type I IFNs to the two subunit receptor (IFNAR1/ IFNAR2) results in activation of the Janus-activated kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which are associated with the cytoplasmic tail of IFNAR1/2. The signal is cascaded further by tyrosine phosphorylation of STAT1 and STAT2 (Brierley and Fish, 2002; Garcia-Sastre and Biron, 2006; Platanias, 2005). The STATs heterodimerize and together with interferon regulatory factor 9 (IRF9) form a complex named ISGF3. This complex enters the nucleus where it associates with specific promoter elements (termed the IFN-stimulated response element or ISRE) to activate the transcription of IFN-stimulated genes (ISGs) (Garcia-Sastre and Biron, 2006). IFNγ signals through an IFNγ-specific receptor (IFNGR1/IFNGR2) to JAK1 and JAK2 resulting in tyrosine phosporylation and homodimerization of STAT1. STAT1 homodimers enter the nucleus and bind the IFNγ-activation site (GAS), which is present in the promoter of certain ISGs.

Facilitated nuclear translocation of such large complexes requires the nuclear pore complex. STAT1 and STAT2 do not contain classical nuclear localization signals (NLS) which is normally necessary to be recognized by the importin receptor, but dimerization of STATs result in conformational changes that establishes NLS activity (McBride et al., 2002). After activation of their target genes, STATs are dephosphorylated, released from the DNA and

shuttled back to the cytoplasm. Consistent with the importance of this pathway in mediating the actions of IFNs, mice with no STAT1 have no innate response to either bacterial or viral infections as a result of dysfunctional IFN signaling (Durbin et al., 1996). Moreover, a number of viruses have the capacity to block the activation of STAT1 by IFN to evade the defense from the host immune system.

Recently, significant progress has been made in identifying and characterizing fish genes related to the IFN system, including several type I IFN genes (Robertsen, 2006; Sun et al., 2009; Zou et al., 2007), IFNγ (Grayfer and Belosevic, 2009; Igawa et al., 2006; Milev-Milovanovic et al., 2006; Zou et al., 2005; Zou et al., 2004) and antiviral genes (Robertsen, 2006). Far less is known about the factors that are involved in IFN-signaling in fish, including the JAK-STAT pathway, although STAT1 homologs have been cloned from several fish species (Collet et al., 2008; Oates et al., 1999; Zhang and Gui, 2004). Additionally, a STAT2 gene was recently identified in salmon (Collet et al., 2009), and TYK2 and JAK1 have been cloned from green pufferfish (*Tetraodon fluviatilis*) (Leu et al., 1998; Leu et al., 2000).

In the present work we describe the identification and characterization of a STAT1 gene from Atlantic salmon. To get insight into the role of STAT1 in response to cytokines and viruses in salmon we have studied the expression and activation of STAT1 in primary leukocyte cultures and in different salmonid cell-lines upon type I and type II IFN treatment and viral infections. The ability of STAT1 to be phosphorylated and to translocate to the nucleus is critical for its role as a transcription factor. By employing a salmon STAT1 antibody the localization of STAT1 in different cells in response to IFN-treatment were studied. Furthermore, STAT1 phosphorylation was detected using a phospho-tyrosine specific antibody after treatment with the same stimulants. Such studies have not been performed in any teleost species earlier. Interestingly, both IFN-a1 and IFNγ treatment led to tyrosine phosphorylation, although STAT1 translocated to the nucleus only after stimulation with IFNγ. We also show, using two different *in vitro* methods, that salmon STAT1 is able to form dimers.

Materials and methods

Fish

For *in vitro* cell-culture studies non-vaccinated Atlantic salmon, *Salmo salar* L., strain Aquagen standard (Aquagen, Kyrksæterøra, Norway), 500–1000 g, was obtained from Tromsø Aquaculture Research Station (Tromsø, Norway). The fish were kept at 6 to 12 °C in tanks supplied with running filtered sea water, and were fed according to appetite on commercial, dry food.

For tissues expression studies, non-vaccinated Atlantic salmon (~30 g), were obtained from SalmoBreed (Norway). The fish were kept in 150 l fresh water at 10 to 13 °C, with an oxygen saturation >65 % and were fed according to appetite on commercial, dry food. The fish were negative for the presence of infectious pancreatic necrosis virus and salmon alphavirus when screened by real-time PCR prior to the experiment

Cell cultures and virus

Atlantic salmon head kidney (HK) or spleen leukocytes were isolated as described by Jørgensen et al. (2001). The density of the leukocyte suspensions was adjusted to 7×10^6 cells/ml. One ml of HK leukocytes was plated per well in 24-well plates in L-15 medium with 5% FBS, whereas one ml splenocytes were plated in RPMI-1640 with 5% FBS. After approximately 24 h of incubation at 14°C, the cells were washed with culture medium prior to stimulation.

Chinook salmon embryo cells (CHSE-214) (Nicholson and Byrne, 1973) were grown as monolayers at 20°C, 5.0% CO_2 in Eagle minimal essential medium with GlutaMAX (EMEM+GlutaMAX, Invitrogen) supplemented with 100 µg/ml streptomycin, 60 µg/ml penicillin, 1% non essential amino acids and 8% fetal bovine serum (FBS, Euroclone). For infection experiments and Western analyses CHSE-214 cells were seeded into 24-well plates (2×10^5 cells/well) and grown to 80% confluence prior to infection.

TO cells originating from Atlantic salmon head kidney (Wergeland and Jakobsen, 2001) were grown as monolayers at 20°C, 5.0% CO_2 in Eagle minimal essential medium with GlutaMAX (EMEM+GlutaMAX, Invitrogen) supplemented with 100 µg/ml streptomycin, 60 µg/ml penicillin, 1% non essential amino acids and 5% fetal bovine serum (FBS, Euroclone).

HEK-293 cells (GP-293; Clonetech) were maintained at 37°C, 5.0% CO_2 in EMEM supplemented with 100 µg/ml streptomycin, 60 µg/ ml penicillin, 4 mM L-glutamine and 10% fetal bovine serum (FBS).

Infectious pancreatic necrosis virus (IPNV) of the N1 strain, serotype Sp (Christie et al., 1988), was used in this study. The experiments were performed with a multiplicity of infection (MOI) of 4 infectious particles in CHSE-214 or TO cells. After absorption of the virus for 3-4 h in serum free culture medium, the medium containing virus was carefully removed from the cells. The infection was then carried out at 17.5°C in the presence of 2 % FBS and cells harvested at different time points. Propagation and titration of virus was performed as described in Pedersen et al. (2007).

Infectious salmon anemia virus (ISAV) of the Norwegian reference strain Glesvaer 2/90 (Mjaaland et al., 1997) (isolate ISAV4, hemagglutinin Genbank accession number AF220607) was kindly provided by Dr. B. Dannevig, National veterinary institute, Oslo, Norway, and used to infect TO cells at a MOI of 4. The infection was carried out at 17.5°C in the presence of EMEM with 2 % FBS and the cells harvested at different time points.

Stimulation of cells

Recombinant Atlantic salmon IFN-a1 (previously named IFN-α1) was produced in HEK293 cells as described elsewhere (Robertsen et al., 2003). The salmon IFN-a1 used in this study had a titer of 24 237 U/ml as estimated by the formula given by Renault et al. (1991). IFN-a1 was administered to the cells at a concentration of 10 U/mL in EMEM containing 2% FBS. Two hundred ng/mL of recombinant rainbow trout IFN-γ (Zou et al., 2005) were used for stimulation of the cells.

Cloning of STAT1 and plasmid constructs

Specific primers for amplifying the salmon STAT1 gene were made based on the rainbow trout (Oncorhynchus mykiss) STAT1 sequence with the GenBank accession number **U60331**. The primers are listed in Table 1. The primers were Gateway compatible allowing the PCR fragment to be inserted into pENTR/D-TOPO vector (Invitrogen). A 2.3 kB fragment was amplified by *Pfu* DNA polymerase (Stratagene) using mixed cDNA from salmon ovary and HK obtained as described earlier (Hansen and Jørgensen, 2007). Constructs were verified by DNA sequencing using the BigDye chemistry and a 3100 Gene Analyzer (Applied

Biosciences). For transfection in cells, inserts were further transferred to the Gateway compatible eukaryotic expression vectors pDEST12.2 (Invitrogen), pDEST-GFP or pDEST-Myc (both provided by Dr. T. Lamark, University of Tromsø) by Gateway recombination using LR clonase II enzyme mix (Invitrogen) following manufacturer's instructions. For yeast two-hybrid analysis modified Clontech vectors (pGADT7 and pGBKT7) were used. The vectors (kindly provided by Dr. O. M. Seternes, University of Tromsø) were made Gateway compatible by insertion of the Gateway polylinker region as described (Lamark et al., 2003) and are named $pDESTGal4_{AD}$ and $pDESTGal4_{DBD}$ respectively. Recombination into $pGal4_{DBD}$ required an intermediate cloning step into the pDONR207 vector (Invitrogen). Control plasmids pTD1-1, pGBKT7-53 and pGBKT7-Lam were purchased from Clontech.

Phylogenetic analyses

Alignment of different STAT1 protein sequences from Atlantic salmon (ssSTAT1a, **EU016199** and **BT045567**), Rainbow trout (**U60331**), Snakehead (**EF079868**), Green pufferfish (**AF307105**), Japanese flounder (**EF491182**), human (**NM_007315),** rat (**NM_032612**), African clawed frog (Xenopus, **AY101602**), Zebrafish (**NM131480**), Crucian carp (**AY242386**), mouse (**NP_033309**), pig (**NP_998934**), cow (**NP_001071368**), and chicken **(NP_001012932**). In addition other STATs (STAT2, STAT3, STAT4, STAT5 and STAT6 from some of these species were included in the analysis done by BioEdit and Clustal W version 1.81. A phylogenetic tree of STAT1 proteins was constructed using the neighbour-joining algorithm in clustal W. Bootstrap values were set to 1000.

Semiquantitative RT-PCR

Total RNA was extracted from salmon organs using TRIzol® (Invitrogen). The cDNA was synthesized from 5 µg of total RNA with Superscript III RT polymerase (Invitrogen) using poly dT primers. Two µl of cDNA was used in the 25 µl PCR reaction with primers specific to Atlantic salmon STAT1 (sSTAT1fw and sSTAT1rev, see table 1) and DyNAzyme™ II DNA Polymerase (Finnzymes). The amplification was done in a GeneAmp® 2700 thermal cycler (Applied Biosystems). Samples were denatured for 2 min at 94°C, followed by 35 cycles of denaturing at 94°C for 15 s, annealing at 55°C for 20 s and extension at 72°C for 20 s. A linear range for the STAT1 PCR product was determined to be reached with 35 cycles and 30 cycles for the actin gene. PCR was performed separately for target and housekeeping genes.

Real-time RT-PCR quantification

Total RNA was extracted from HK leukocytes and splenocytes using Pure Link RNA Mini Kit® (Invitrogen). RNA (300 ng in a 20µl reaction) was reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen), which includes a treatment that, according to the manufacturer, will eliminate contaminating genomic DNA. A volume of 2.5 µl of cDNA (6.25 ng of reverse-transcribed RNA) per 20 µl PCR reaction was used with primers for sSTAT1 (described above) together with an sSTAT1 probe (5'-6FAM-ACCACCAAGGAATGTTC-3'). While 6.25 pg of reverse-transcribed RNA per 20 µl PCR reaction was used to estimate 18S rRNA and EF1AB levels. The expression levels of mRNA was measured in an ABI Prism 7500 FAST Cycler (Applied Biosystem) using custom TaqMan assays designed by Applied Biosystem and FAST PCR mastermix (Applied Biosystem). The amplification profile was 95 °C for 20 s followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. All cDNA samples were performed in triplicates. The expression was normalized against EF1AB and presented as relative expression compared with the non-treated control sample. Relative expression was calculated using the Pfaffl's mathematical model (Pfaffl, 2001). The expression profiles from non-treated control fish organ samples were presented as expression of STAT1/ EF1AB.

Two-hybrid analysis

Both rich and selective yeast growth media were made from commercially available powders (BD Biosciences Clonetech). Yeast cells were grown at 30°C for 2 – 4 days. Plasmid constructs based on the $pGal4_{DBD}$ vector or the $pGal4_{AD}$ vector were transformed using Frozen-EZ Yeast Transformation II kit (Zymo Research) into competent yeast cells of strains *S. cerevisiae* Y187 (MATα) or PJ69-2A (MATa) by selecting for growth on medium lacking leucin or tryptophan, respectively. At least ten of each transformants carrying STAT1 were mated to each other. Ten diploid yeast cells from the mating were plated and scored for growth on a triple drop out medium (TDO) lacking leucin, tryptophan and histidine or a quadruple drop out medium (QDO) lacking leucin, tryptophan, histidine and adenine. Growth on TDO plates indicates a weak interaction, whereas growth on QDO plates indicates a stronger interaction. SV40 T-antigen (pTD1-1) and p53 (pGBKT7-53) served as positive controls, LaminC (pGBKT7-Lam) and empty vectors as negative controls.

Transfection

For transfection, HEK-293, CHSE-214 or TO cells were seeded into 24-well plates with a density of 2×10^5 cells/well for HEK293 and CHSE-214, and $1x10^5$ cells/well for TO, while in 6-well plates the densities were $1x10^6$ or 5×10^5 cells/well, respectively. The cells were transfected the next day at 80 – 90% confluence. Transfection of the HEK-293 and CHSE-214 cells was performed by using the Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer's protocol. For each well, a total of 0.8 µg of plasmid DNA was incubated with 2 µl Lipofectamine 2000 in 100 µl serum-free EMEM for 20 min at room temperature before added to the cells. Three hours post transfection, FBS was added to a total concentration of 2%. For transfection of the TO cells the transfection reagent FuGENE HD (Roche Applied Science) was used according to the manufacturer's protocol. A total of 0.6 µg of plasmid DNA was mixed with 1.25 µl FuGENE HD in 50 µl EMEM and incubated 15 min before added to the cells with medium containing 2% FBS.

Gel electrophoresis, Western blotting and antibodies

Cells were lysed in 50 µl sodium dodecyl sulfate (SDS) sample buffer (160 mM Tris-HCl [pH 6.8], 10% β-mercaptoethanol, 2% SDS, 20% glycerol, 0.1% bromophenol blue), transferred from the culture well into a microcentrifuge tube and boiled for 5 min. Then, typically 15-20 µl of the samples were loaded in each well of a precast 4-12% gradient NuPAGE Novex Bis-Tris gel and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with MES buffer and Western blotting using the Invitrogen NuPAGE system. Gel electrophoresis, blotting, blocking and antibody incubation were performed as described by the manufacturer. A polyclonal antibody against STAT1 (α-STAT1) was custom made from a C-terminal peptide retrieved from the salmon STAT1 sequence: RSVAPVFQCWTGPKE. The peptide was cross-linked by BSA to glutaraldehyde and two rabbits were injected with antigen every 14 days. The terminal bleed of the rabbits took place after day 68 (5 injections). The antiserum was antigen-purified and reactivity against the peptide was confirmed by dot blot analysis against the peptide. The specificity of the antiserum was checked by transfection and expression of a STAT1-GFP fusion construct in different cell-types and the results are presented as supplementary data. A dilution of 1:2000 of the anti-STAT1 antibody (α-STAT1) was found to be appropriate for Western blotting. A polyclonal Mx antibody (α-Mx, 1:1000 dilution) (Trobridge et al., 1997) was applied as primary antibody for detection of Mx protein, and a GFP antibody (1:10000 dilution)(Abcam) for detection of GFP-tagged proteins.

An actin antibody (1:1000 dilution) produced in rabbit (Sigma) was used as loading control in most Western blots. Goat anti-rabbit-Horseradish Peroxidase (HRP) antibody or goat anti-mouse-HRP antibody (Santa Cruz Biotechnology) diluted 1:25000 were used as secondary antibodies. Detection was performed by using SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology Inc.). Stripping of the membranes was performed in 0.2 M NaOH for 10 min followed by washing, blocking and new antibody incubation.

Immunoprecipitation (IP) of STAT1

TO cells or Atlantic salmon spleen and HK leukocytes were stimulated with IFN-a1 or IFNγ for 1 and 3 h before washed two times with ice-cold PBS and harvested in buffer A (20 mM Tris-acetate, pH 7.0; 0.27 M sucrose; 1 mM EDTA; 1 mM EGTA; 1 mM orthovanadate; 10 mM β-glycerophosphate; 50 mM sodium fluoride; 5 mM sodium pyrophosphate; 1% [vol/vol] Triton X-100; 0.1% [vol/vol] 2-mercaptoethanol) and 'Complete' protease inhibitor cocktail (one tablet / 50 ml, Roche). Lysates were cleared by centrifugation at 4 ºC for 15 min at 18000 x g. Lysates were then subjected to IP by incubating for 1 h at 4ºC with αSTAT1(1:100), before addition of 10 µl protein A-agarose (50% slurry preequilibrated in buffer A) and incubation at 4ºC for 1 h. The immunoprecipitated material was washed four times in ice-cold buffer A with 0.5 M NaCl and resuspended in 40 µl 2x LDS-sample buffer. STAT1 was detected by the tyrosine phospho-specific antibody 4G10 Anti-Phosphotyrosine (Millipore) after SDS-PAGE and Western blotting.

Co-IP analyses

HEK-293 cells co-transfected with the eukaryotic expression vectors pEXP12.2-STAT1 and pEXP-GFP-STAT1 or pEXP12.2-STAT1 and pEXP-GFP were washed two times with ice-cold phosphate-buffered saline (PBS) and harvested in HA-lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100) with a protease inhibitor cocktail added (Complete EDTA-free, Roche). Cell lysates were incubated on ice for 15 min and cleared by centrifugation for 15 min at 18000 x g in a microfuge. Lysates were then subjected to IP with either α-STAT1 or α-GFP together with pre-blocked Protein A/G PLUS-Agarose beads (Santa Cruz biotechnology). The agarose beads were then washed four times with HA-lysis buffer, and all traces of buffer removed with a pipette tip before elution in 50 µl 2X SDS sample buffer. Eluted proteins were subjected to SDS-PAGE and visualized by Western blotting and antibody detection.

Immunofluorescence microscopy

To examine localisation of STAT1 in cells, primary cells from salmon HK were seeded on 14 mm coverslips. Non-adherent cells were washed away 1 day after seeding, and adherent monocyte/macrophages were stimulated with recombinant IFNs as described above. The cells were rinsed with 1X PBS (phosphate-buffered saline), before fixed using 4% paraforaldehyde for 20 min at room temperature. Cells were washed three times with PBS, and permabilized with 0.3% Triton X-100 for 15 min, before blocked for 30 min with 7.5% FBS in PBS. The cells were then incubated with the primary antibody (α-STAT1) at a 1:500 dilution in PBS 7.5% FBS for 1 h washed three times with PBS and incubated with secondary antibodies conjugated to Alexa Fluor 546 at a 1:2000 dilution (Invitrogen) for 45 min away from light. Cells were washed and stained with DAPI (4',6'-diamidino-2-phenylindole, 1:300, Invitrogen) and mounted on slides with an antifade mounting medium. Confocal laser scanning microscopy was performed using a Leica TCS SP5 confocal microscope with LAS AF software.

Results

Cloning of STAT1

With primers derived from a rainbow trout STAT1 sequence we obtained a 2.3 kB DNA fragment by PCR using cDNA from salmon ovaries and HK. Sequencing of the cloned fragment revealed a 2 274 base pairs long open reading frame which translated into an amino acid sequence with strong homology to the rainbow trout STAT1 sequence (97.6% identity, 98.8% similarity). This salmon sequence was 3 amino acids longer than the rainbow trout sequence, and had a predicted molecular mass of 87.5 kDa. While preparing this manuscript two other STAT1 sequences from Atlantic salmon were submitted to the GenBank database (accession numbers **EU016199** and **BT045567**). These two sequences shared 96.3% and 98.3% identity, respectively, to our clone. Our sequence was named ssSTAT1a (GenBank accession number **GQ325309**). The major differences found between the three salmon sequences were located in the C-terminal end (Fig. 1), where **EU016199** has two single nucleotide deletions when compared to ssSTAT1a (T in position between 2331/2332, and T in position between 2341/2342), which leads to frame shifts and subsequently a premature stop. In **BT045567** is found an insertion of 39 nucleotides (13 amino acids) in the C-terminus when

compared to ssSTAT1a (Fig. 1). Two pairs of PCR primers were designed in an attempt to pick up, and to distinguish between the ssSTAT1/EU016199 variants. One primer pair (ssSTAT1 2204 fw + rtSTAT1rev, Table 1) would amplify PCR products with different sizes, whereas the other primer pair (rtSTAT1fw + ssSTATgap rev, Table 1) would detect only the **BT045567** variant. In all our attempts to amplify the different STAT1 variants, only the ssSTAT1a isoform was detected in cDNA from both stimulated and unstimulated salmon leukocytes (results not shown).

The amino acid sequence of ssSTAT1a was compared to known STAT1 sequences from other species (Table 2) and revealed high homology, also with human and rat STAT1 (>60% identity, >80% similarity). The non-salmonid teleost species that was found to share the highest homology to salmon STAT1 was snakehead (*Channa argus*) STAT1 (80.1% identity, 90.4% similarity), whereas crucian carp (*Carassius auratus*) had the lowest identity of the compared species (58.4% identity, 75.7% similarity). In the presented phylogenetic tree ssSTAT1a is located in the same clade as other piscine STAT1 sequences when high bootstrap values were applied (Fig. 2).

The amino acid sequence of the ssSTAT1a clone contained several prototypic features and conserved domains crucial for STAT1 functions (Fig. 1). In the most C-terminal part of the sequence, the transcriptional activation domain (TAD), a conserved serine phosphorylation site was found in position 719 (homologous to serine 727 in human STAT1). This phosphorylation site is known to be crucial for the activation of transcription (Horvath, 2000). A conserved tyrosine residue was located in position 695 (701 in human). Phosphorylation of this tyrosine residue is crucial for activating the STAT molecule and thereby enabling it to interact with the phosphotyrosine binding pocket located in the Src homology 2 (SH2) domain of other STAT molecules (Shuai et al., 1993). An analysis of this conserved domain (using NCBI CDD (Marchler-Bauer et al., 2007)) revealed that a phosphotyrosine binding pocket was located in SH2 of ssSTAT1a, where all four of the residues composing this feature was present, which of the conserved arginine at position 597 is believed to be the most crucial for forming H-bonds with phosphate oxygens of the phosphotyrosine side chain (Sawyer, 1998). In addition residues important for DNA-binding (DNAB), nuclear import (NLS) and export (NES) (Horvath et al., 1995; McBride et al., 2002; McBride et al., 2000; Reich and Liu, 2006) were found to be conserved between mammalian and teleost STAT1. Residues crucial for these functions are marked with asterisks in Fig. 1.

Tissue distribution of STAT1 mRNA

A semiquantitative PCR was undertaken to examine expression of STAT1 mRNA in different salmon tissues. A uniform distribution of ssSTAT1a mRNA was observed in all tissues tested, including ovaries, liver, kidney, head kidney, spleen, heart and gills (Fig. 3A). This even distribution of STAT1 was confirmed by real-time PCR on some of these organs (head kidney, spleen, heart and gills), and additionally in intestines (Fig. 3B) derived from 10 unvaccinated and healthy salmon controls.

Induction and expression of STAT1 protein in salmon primary cells

For detection of STAT1 protein in salmon cells, a custom made peptide antibody was applied for Western blotting. Primary cells from salmon were subjected to Western blotting upon stimulation with both type I and type II IFN (Fig. 4). In unstimulated adherent HK leukocytes (mostly monocytes and macrophages) (Fig. 4A) the STAT1 protein was barely detectable, while the STAT1 levels were increased upon both IFN-a1 and IFNγ stimulation, showing maximum levels at 96 h post stimulation. Individual variations among the fish tested were observed, as exemplified in Fig. 4, where increased STAT1 levels upon stimulation were induced earlier in Fish 2 compared to Fish 1. In splenocytes (mostly lymphocytes) (Fig. 4B) a more even STAT1 expression pattern was seen when comparing non-treated cells with the stimulated cells, indicating that STAT1 expression levels in splenocytes were less affected by IFN treatment, but with a slight up-regulation after IFNγ stimulation. Mx protein was detected on the same membranes after stripping and reprobing with an Mx antibody. For HK leukocytes the relative increase in Mx expression occurred earlier than the increase in STAT1 expression, while the splenocytes showed a constitutive Mx expression which paralleled the STAT1 expression.

Transcription levels of salmon STAT1 mRNA in salmon HK leukocytes

To determine whether the increase in STAT1 protein expression upon type I and type II IFN treatment of HK leukocytes were induced at transcriptional levels, the amount of mRNA was measured at various times after stimulation by real-time RT-PCR. A representative experiment is shown in Fig. 5. Both types of IFNs induced STAT1 expression levels slightly above unstimulated cells. In cells isolated from two fish, IFN-a1 caused an average increase in transcription of STAT1 ranging from a 1.5-fold at 4 h through a 4-fold induction at 12 h to nearly a 6-fold induction at 24 h. The levels of STAT1 transcripts after stimulation with IFNγ

did barely change relative to the unstimulated control at the time points investigated, with a peak at 2-fold increase at 12 h (Fig. 5A). To verify the activity of the IFNs used, the levels of Mx transcripts were recorded in the same samples. At 24 h the Mx transcripts were induced to a level 60 times above the unstimulated cells when IFN-a1 was added to the cells, whereas the IFNγ induced transcription to a 3.5-fold level at the same time point (Fig. 5B).

STAT1 protein expression levels in TO and CHSE-214 cells upon IFN-treatment and viral infections

Two salmonid cell-lines, TO and CHSE-214 cells, known to be permissive for several viruses, were tested for expression of STAT1 protein upon stimulation with different cytokines or virus. In unstimulated CHSE-214 cells expression of STAT1 protein was barely detectable, while a modest induction upon IFN-a1 or IFNγ stimulation was found (Fig. 6A). Infection with IPNV at MOI of 4 for 12, 24 or 48 h, did not increase protein expression above the level of uninfected cells (Fig. 6B). Also infections with Norwegian field isolates of highly virulent IPNV strains showed the same results (results not shown). In TO cells STAT1 was constitutively expressed and exposure to IFN-a1 or IFNγ for 24 h did not induce any further expression. Neither did infection with IPNV after 12, 24 and 48 h nor ISAV for 12, 24 , 48 , 72 and 96 h. Mx protein was, however, induced after 24 h and 48 h of ISAV infection.

Subcellular localization of STAT1

The subcellular localization of STAT1 after type I and II IFN stimulation in salmon primary leukocytes and salmonid cell-lines was examined by confocal laser scanning microscopy. For this purpose cells were seeded on coverslips and stimulated for 1 h and 4 h with 10 U/ml IFN-a1 and 400ng/ml of IFNγ. The cells were then fixed and immunostained for STAT1 as described in Material and Methods. In all cell types studied, STAT1 (red dye) localized exclusively to the cytoplasm in unstimulated cells (Fig. 7 A, B and C). In primary cells from Atlantic salmon HK, a relocalization of STAT1 from the cytoplasm to the nucleus took place after stimulation with IFNγ for 4 h. A slight shift from cytoplasmic to perinuclear distribution was observed already at 1 h with IFNγ treatment, although individual variations occurred among the 3 fish studied (Fig. 7A). In TO cells the response to IFNγ was faster than in the primary cells. At 1 h after IFNγ stimulation, some of the nuclei in the TO cells had already been stained with STAT1 and after 4 h STAT1 had relocated to the cytoplasm (Fig. 7B).

CHSE-214 cells did not respond to IFNγ in a similar way (Fig. 7C). IFN-a1 stimulation did not cause relocalization of STAT1 in any of the cell types at the time points chosen in this study.

Salmon STAT1 is phosphorylated upon IFN treatment.

Tyrosine phosprylation is a key step in STAT1 mediated IFN-signal transduction. To address whether the conserved tyrosine residue found in the salmon STAT1 sequence is phosphorylated upon stimulation, an antibody specific to phosphorylated tyrosine was used. IFNγ-stimulated primary leukocytes were harvested and the STAT1 antibody applied in order to immunoprecipitate STAT1 molecules. The pulled-down material was subjected to SDS-PAGE and Western blotting, and the membrane incubated with a tyrosine phospho-specific antibody. By IFNγ-stimulation phosphorylated STAT1 was detected in adherent HK cells and splenocytes after 1 h and 3 h (Fig.8A), although cells from different individuals responded differently to this treatment (results not shown). Similarly, STAT1 was phosphorylated upon stimulation with IFN-a1 at the same time-points (Fig.8A). Phosphorylation of STAT1 was also confirmed in TO cells (Fig. 8 B). A time-course study in these cells showed that after IFNγ treatment STAT1 was phosphorylated already after 5 min and peaked at 15 min, while in IFN-a1 treated cells a weak band was detected at 5 min, increased at 15 min and then remained relatively constant up to 60 min After 120 min phosphorylation was regressing for both types of IFN. Phosphorylated ectopically expressed ssSTAT1a was detected after 30 min by the tyrosine phospho-specific antibody (Fig. 8C). This experiment confirms that the cloned ssSTAT1a can be tyrosine phosporylated upon IFN treatment, which is an important characteristic of this protein.

Salmon STAT1 homodimerisation

Tyrosine phosphorylation of STAT1 induces a conformational change that generates STAT1 dimers via reciprocal phosphotyrosine and SH2 domain interaction. In mammals, phosphorylated STAT1 form either homodimers or heterodimers with STAT2. By employing the yeast two-hybrid (Y2H) system we were able to demonstrate a strong salmon STAT1-STAT1 interaction (Table 3). Interacting proteins were assessed by the ability of growth on complete medium deficient in histidine (TDO) or histidine and adenine (QDO). Growth was recorded at day 4, when the positive control expressing p53 fused to $Gal4_{DBD}$ and SV40 T-antigen fused to $Gal4_{AD}$ showed massive growth on QDO (++++). The specificity of the

interaction was tested with negative controls, and confirmed negative (no growth on TDO nor QDO) by co-expressing the $Gal4_{AD}$-ssSTAT1a fusion protein both with $Gal4_{DBD}$ alone and with $Gal4_{DBD}$ fused to the human LaminC protein, which is known to be a non-interacting protein (Bartel et al., 1993; Hughes et al., 1996).

A co-IP analysis of over-expressed ssSTAT1a and ssSTAT1a-GFP fusion constructs in HEK-293 cells verified the ssSTAT1a-ssSTAT1a interaction further. The Western blot in Fig. 9 was probed with α-STAT1 and showed expression of both ssSTAT1a and ssSTAT1a-GFP in the lysate (lane 2, ~ 85 kD and ~ 110 kD respectively). ssSTAT1a was co-precipitated along with ssSTAT1a-GFP when the GFP antibody was applied for the IP (lane 3) and α-STAT1 precipitated both ssSTAT1a and ssSTAT1a-GFP (lane 4). As a negative control ssSTAT1a was co-transfected with the pDEST-GFP vector. The GFP antibody precipitated only GFP (25 kD, lane 6, lower panel) whereas α-STAT1 precipitated ssSTAT1a (lane 7).

Discussion

IFN induced immune responses in which STATs are required are among the best understood signaling systems in mammals. Although a number of proteins involved in the JAK/ STAT signaling pathway have been cloned from fish, less is known about their function and whether the signaling resembles mammalian systems.

We have here cloned a cDNA that corresponds to the salmon STAT1 gene. A clustal W alignment confirmed that the cloned sequence was a STAT1 homolog sharing extensive amino acid identity with other salmon STAT1 isoforms and trout STAT1 (Table 1 and Fig.1). The 2 274-amino-acid open reading frame of the cloned cDNA has a structural arrangement of functional motifs that is similar to mammalian STAT1 suggesting that the salmon cDNA encode a functional protein. These include an amino terminus (ND) that plays a role in stabilizing bonds between dimers, a coiled coil domain that can be involved in interactions with other proteins, a central DNA binding domain, a SH2 domain with a phospotyrosine binding pocket, and a carboxyl transcriptional activation domain with a conserved serine residue and a tyrosine residue that is phosporylated in response to stimuli.

So far STAT1 has been found in multiple fish species, including pufferfish, zebrafish, rainbow trout, Atlantic salmon and Japanese flounder. Previous expression data, along with data presented here, has revealed that piscine STAT1 is widely expressed in many tissues (Collet et al., 2008; Collet et al., 2007; Herrada and Wolgemuth, 1997; Kileng et al., 2009; Oates et al., 1999; Park et al., 2008). However data concerning functional activity in lower vertebrates, such as STAT1 phosporylation and cellular localization upon stimulation of cells, is scarce. The presented data demonstrates for the first time that a teleost STAT1 protein is being activated by IFNs. Salmon STAT1 was tyrosine phosphorylated upon IFN-a1 and IFNγ stimulation of leukocytes, and additionally in TO cells. In addition, relocalization of STAT1 into the nucleus of leukocytes and TO cells was observed following IFNγ stimulation. Our data show a more evident response for IFNγ than for IFN-a1 when studying nuclear translocation of STAT1. This was also consistent with the levels of phosphorylated STAT1 observed upon the different IFN-stimulations where IFNγ consistently gave higher levels compared to IFN-a1. STAT1 is believed to be involved in both type I and type II IFN signaling, and their distinct responses could be due to unequal concentrations or activity of the cytokines used, or be dependent on differences in the kinetics of forming the complexes that enter the nuclei. We also showed that ssSTAT1a is able to form homodimers which is thought to be a prerequisite for entering the nucleus due to lack of a functional nuclear localization signal in its monomeric form. Although nuclear import and export normally occur over the nuclear pore complex, it has been shown that nuclear import can occur by receptor mediated endocytosis in mammals (Bild et al., 2002). In unstimulated cells STAT proteins can exist as stable unphosphorylated dimers or monomers which are also shuttled over the nuclear membrane (Braunstein et al., 2003; Marg et al., 2004; Pranada et al., 2004) and are able to regulate gene expression in unconventional manners (Brierley and Fish, 2005).

The TO cell line is derived from salmon HK and it consists of heterogeneous cell types (Wergeland and Jakobsen, 2001). The CHSE-214 cells are embryo cells derived from Chinook salmon (Nicholson and Byrne, 1973). Both these cell lines are widely used as experimental systems to study immune responses in salmon (Hansen et al., 2008; Johansen et al., 2004; Jørgensen et al., 2007; Kileng et al., 2009; Kileng et al., 2007; Pettersen et al., 2008; Skjæveland et al., 2009). According to our results, the expression of STAT1 protein seems to be equally sensitive to stimulation with type I as compared to type II IFN as shown in both the cell lines and in primary HK leukocytes. However, at the transcription level, STAT1 was

stimulated by type I IFN to a greater extent (6-fold) then by type II IFN (2-fold), and also by different kinetics, where the response to type II IFN peaked at an earlier time-point. Unlike the HK leukocytes, the splenocytes, which are mostly lymphoid–like cells, did not show any increase in STAT1 abundance upon IFN-stimulation. The up-regulation of salmon STAT1 after IFN-a1 stimulation of TO cells is reported by others (Collet et al., 2008; Kileng et al., 2009), additionally the type I IFN inducer poly (I:C) has been shown to induce STAT1 mRNA expression in RTG-cells (Collet et al., 2007).

Following encounter with viral pathogens, CHSE-214 and TO cells did not seem to boost the levels of STAT1 protein, but stayed relatively constant. Mx-protein was induced 24-48 h after infection with ISAV, but did not respond to IPNV infection, which is in compliance with results reported earlier (Jensen and Robertsen, 2002; Kileng et al., 2007; Skjesol et al., 2009). The uniform expression patterns in salmon tissues and in different cell-types treated in various ways indicate that it is likely that STATs are present in the cytoplasm in most resting tissues, alert and prepared to be activated upon cellular receptor signaling. The signal transduction and activation of ISGs may lead to a feedback loop that amplifies IFN-responses and induces STAT1 in a secondary manner, STAT1 itself being an ISG. This is shown in human cells-lines where IFNγ-induced IRF-1 in concert with CREB binding protein acts as key up-regulator of STAT1 mRNA transcription by binding to a combined IRF-E/GAS element in the STAT1 promoter (Wong et al., 2002). Mutual regulation of STAT1 and IRF-1 indicates an intracellular amplifying circuit in response to IFN.

Unlike TO cells and primary leukocytes, no relocalization of STAT1 from the cytoplasma to the nucleus was found upon IFNγ treatment of CHSE-214 cells. This could due to the apparent low levels of endogenously expressed STAT1 protein in these cells. The expression levels were evaluated by Western blotting and may have been caused by a lower affinity of the STAT1 antibody to Chinook salmon STAT1. However, the levels of STAT1 in CHSE-214 were induced by both types of IFN, but considerably later (24 h, Fig. 6) than the timepoints used for the confocal microscopy examination. The presence of the IFN receptors (IFNAR and IFNGR) in the different cell-lines is also uncertain although an IFNγ-receptor was recently cloned from rainbow trout (Gao et al., 2009). The differences in response endorse the assumption that there are distinct proteins involved in signaling from type I and type II IFNs, although in fish there are examples of genes such as Mx that are up-regulated upon stimulation by both IFN types (Jørgensen et al., 2007; Martin et al., 2007), which is

unlike the (most common) action of human IFNγ (Schindler et al., 1992; Zou et al., 2005). The presence of a STAT2 gene in salmon was recently reported (Collet et al., 2009), and the first teleost importin alpha gene was recently cloned from red seabream (*Pagrus major*) (Gen et al., 2008), both findings adding to the assumption that IFN signaling in fish resembles that of mammals.

The ability of IFNγ to activate Mx might be a consequence of indirect stimulation, as fish IFNγ can activate type I IFN ((Martin et al., 2007) own unpublished data). Interestingly, recombinant IFNγ activates an ISRE-containing reporter-construct in a dose dependent manner whereas constructs containing only GAS elements give no response to either type of IFNs as shown by Castro et al. (2008). This finding suggests that cross-talk between IFN signaling pathways occur. It has also been reported that IFNγ induces both STAT1 and STAT2 tyrosine phosphorylation and formation of ISGF3 complex in murine embryonic fibroblasts (Matsumoto et al., 1999) and that STAT1 homodimers can associate with IRF9 and activate ISRE elements in human cells (Bluyssen et al., 1996; Reid et al., 1989). A heterodimer of STAT1 and STAT2 can bind weakly to a GAS element, resulting in transactivation (Li et al., 1998). In the absence of exogenously administered IFNs, overexpression of ssSTAT1a by transfection in TO cells did not activate the Mx-promoter by itself (unpublished data), probably due to lack of factors that activate the overexpressed STAT1 molecules.

As many as 11 type I IFN genes, of which many encode similar IFNs, have recently been identified in salmon (Sun et al., 2009). In general different cytokines have preferences as to which STAT they activate. Whether the different type I IFNs found in salmon differ in their ability to activate STAT1 is an interesting question to be addressed in future studies.

Naturally occurring truncated forms of STATs can act as competitors of functional STATs and inhibit transcriptional activation (Wang et al., 1996). We have cloned an Atlantic salmon STAT1 gene that differs from two other salmon STAT1s published in GeneBank. The presence of more than one STAT1 isotype in salmon implies distinct functions for the different STAT1s, possibly at the level of transcription activation as the main differences are located in the TAD. The presented ssSTAT1a is longer than EU016199 (ssSTAT1 (Collet et al., 2008)) while shorter than BT045567. Our attempt to detect both ssSTAT1a and BT045567 transcripts in salmon primary cells failed, and only ssSTAT1a was detected

independent of the type of treatment the cells were subjected to. Other cell-types, signals or cellular conditions might favor BT045567 expression. All three isoforms are likely to be recognized by the STAT1 antibody used in this study and the differences in molecular mass are minimal, thus the STAT1 antibody could not be used to distinguish between the three.

One of the properties possessed by IPNV in order to secure its own reproduction is to counteract the IFN-signaling pathway (Skjesol et al., 2009). Several viruses target STAT or JAK proteins for degradation (Didcock et al., 1999; Parisien et al., 2001; Parisien et al., 2002) or otherwise inhibit their activation (Polyak et al., 2001). Neither of the five virally encoded proteins VP1-VP5 of IPNV were able to interact directly with ssSTAT1a in a yeast two-hybrid assay (results not shown), predicting that the target for the antagonistic effect of this virus is either an intermediate binding partner to STAT1 or another molecule acting downstream of IFN production. The ability of IPNV to impair STAT1 activation/phosphorylation is currently being examined, and the interacting ability of activated STAT1 needs to be elucidated. Employment of the viral proteins in an Y2H library screening might reveal other interacting molecules involved in this pathway.

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Figure legends

Fig. 1. Salmon STAT1 protein harbors conserved domains and sequences. The NCBI conserved domains database and ClustalW alignment was combined to depict the schematic presentation of STAT1. *Abbreviations:* ND = amino-terminal domain, DNAD = DNA-binding domain, SH2 = Src Homology 2 domain, TAD = transcriptional activation domain, NES = nuclear export signal, NLS = nuclear localization signal, ss = Atlantic salmon, rt = rainbow trout, hs = human, asterisks indicate conserved residues with importance for the functional activity within the domains.

Fig. 2. An un-rooted phylogenetic tree of STAT1 and other STAT proteins based on sequences aligned by ClustalW was constructed using the neighbor-joining algorithm. Bootstrap value = 1000.

Fig. 3. A. Semi-quantitative RT-PCR showing expression of STAT1 mRNA in different tissues from adult healthy fish. Salmon tissues tested all show uniform expression of STAT1 mRNA. *Abbreviations:* O (ovaries), L (liver), K (kidney), HK (head kidney), S (spleen), H (heart) and G (gills). The housekeeping gene β-actin was used as an internal control. **B.** An even distribution of STAT1 in salmon tissues was confirmed by quantitative realtime-PCR and expression levels presented as change in expression relative to EF1AB. Samples from 10 fish were tested.

Fig. 4. SDS-PAGE followed by Western blot showing expression of STAT1 protein in adherent head kidney (**A**) leukocytes and splenocytes (**B**) from two individuals upon stimulation with IFN. The samples were harvested 12, 24, 48, and 96 h after stimulation with IFN-a1 (10 U/mL) and IFNγ (200 ng/mL). The unstimulated control (C) was harvested at the 48 h time-point. STAT1 protein was detected simultaneously with actin which was used as a loading control. The membranes were stripped and reprobed with an anti-Mx-antibody as a control for the IFN-activity. M = MagicMark molecular weight marker.

Fig. 5. QPCR showing expression of STAT1 and Mx mRNA in IFN-stimulated adherent head kidney leukocytes. The cells were stimulated with 10 U/mL of recombinant Atlantic salmon IFN-a1 or 200 ng/mL of recombinant rainbow trout IFNγ. The mRNA levels were normalized against EF1AB. **A.** STAT1 mRNA expression after 4, 12 and 24 h of stimulation **B.** Mx

mRNA expression after 4, 12 and 24 h of stimulation. The results are an average of samples from two fish and presented as fold increase relative to unstimulated head kidney cells.

Fig. 6. SDS-PAGE followed by Western blot showing expression of STAT1 protein in salmonid cell-lines. Antibodies against salmon STAT1 (α-STAT1 1:2,000), actin (α-actin, 1:1,000) and Mx (α-Mx, 1:1,000) were used. **A.** CHSE-214 and TO cells were harvested 24 h after stimulation with IFN-a1 (10 U/mL) and IFNγ (200 ng/mL) along with an unstimulated control (C). **B**. CHSE-214 and TO cells were infected with IPNV (MOI = 4) and harvested at 12, 24 and 48 h p.i. TO cells were also infected with ISAV (MOI = 4) and harvested at 12, 24, 48, 72 and 96 h p.i. Actin was used as a loading control and Mx protein detected after the membrane was stripped and reprobed. M = MagicMark molecular weight marker.

Fig. 7. Subcellular localization of STAT1 in different cell-types after stimulation with IFN-a1 and IFNγ. The cells were treated with IFN-a1 (10 U/mL) or IFNγ (200 ng/mL) for 1 or 4 h or left unstimulated before fixed in 4% parafomaldehyde and stained for STAT1 (red). Nuclei were stained with DAPI (blue). **A.** Salmon adherent head kidney leukocytes. Translocation of STAT1 to the nucleus took place 4 h after stimulation with IFNγ as indicated by arrows. **B.** TO cells. Translocation of STAT1 to the nucleus took place 1-4 h after stimulation with IFNγ as indicated by arrows. **C.** CHSE-214 cells. No translocation of STAT1 to the nucleus was observed. A no α-STAT1 control is included for each of the cell types.

Fig. 8. STAT1 is phosphorylated in response to IFN-1a and IFNγ. Cells were either treated with IFN-1a (10 U/ml) or IFN-γ (200 ng/ml) or left untreated. Cells were harvested at indicated time points and endogenous STAT1 were immunoprecipitated with α-STAT1 from the whole cell extracts. Tyrosine phosphorylated STAT1 was detected by immunoblotting using anti-phosphotyrosine antibody (pY, upper panel). The total amount of immunoprecipitated STAT1 was detected with α-STAT1 antibody (lower panel). **A.** Head kidney leukocytes. **B**. TO cells. **C**. TO cells were transfected with a GFP-ssSTAT1a construct. After 48 h, the cells were treated with IFNγ (200 ng/ml) for 30 min, or left untreated. Cells were lysed and GFP-tagged proteins were immunoprecipitated with α-GFP. Phosphorylated GFP-STAT1 was detected by immunoblotting using anti-phosphotyrosine antibody (upper panel). The total amount of immunoprecipitated GFP-STAT1 was detected with α-GFP antibody (lower panel).

Fig. 9. Co-IP analyses of the ssSTAT1a-ssSTAT1a interaction. ssSTAT1a was co-expressed with GFP-ssSTAT1a or pEXP-GFP (negative control) in HEK-293 cells and the lysed cells subjected to IP with a STAT1 antibody (α-STAT1) or a GFP antibody (α-GFP). Samples were analyzed along with the total cell lysate (sup) by SDS-PAGE followed by Western blot using α-STAT1 and subsequently, after stripping, the α-GFP.

Table 1. Primer sequences and their applications in this study.

Table 2. Percent amino acids sequence identities (top right triangle) and similarities (bottom left triangle) of STAT1 proteins. The accession numbers for the STAT1 from each species are given in parentheses.

Table 3. Interaction between ssSTAT1a and ssSTAT1a detected in the yeast two-hybrid system. ++++ strong interaction, - no interaction, QDO quadruple drop out medium, TDO tipple dropout medium, ND not determined.

Supplementary Fig 1. The specificity of the STAT1 peptide antibody was checked by transfection and expression of a GFP-ssSTAT1a fusion construct in different cell-types followed by SDS-PAGE and Western blotting. GFP-ssSTAT1a was expressed and recognized by the STAT1 antibody in HEK-293 cells and CHSE-214 cells, whereas in TO cells the level of transfected GFP-ssSTAT1a was undetectable while endogenous expression of STAT1 was detected in these cells.

Table 1

Primer name	Sequence 5' – 3'	Application
rtSTAT1fw	CAC CAT GGC CCA GTG GTG CCA GCT GCA	Gene cloning/ sequencing
rtSTAT1rev	CTA CTA TCA GTT GCA GTC CGA GTC AGG TG	Gene cloning/ sequencing
ssSTAT1 2204 fw	AGTGTTGGACTGGTCCTAAGGA	Isoform detection (PCR)
ssSTAT BT045567 rev	TGA AAT TCT TCA GCT AAA AAC TCT C	Isoform detection (PCR)
ssSTAT1fw	CGGGCCCTGTCACTGTTC	Real-time RT-PCR/ semiquantitative RT-PCR
ssSTAT1rev	GGCATACAGGGCTGTCTCT	Real-time RT-PCR/ semiquantitative RT-PCR
ssSTAT1 probe	ACCACCAAGGAATGTTC	Real-time RT-PCR

Table 2

	ssSTAT1a	Atlantic salmon (EU016199)	Atlantic salmon (BT045567)	Rainbow trout (U60331)	Snakehead (EF079868)	Green pufferfish (AF307105)	Japanese flounder (EF491182)	Human (NM_007315)	Norway rat (NM_032612)	African clawed frog (AY101602)	Zebrafish (NM_131480)	Crucian carp (AY242386)
ssSTAT1a		96.3	98.3	97.6	80.1	76.4	77.9	66.1	64.7	65.8	63.3	58.4
Atlantic salmon (EU016199)	96.7		94.6	94.9	79.9	76.2	78.0	66.3	65.1	65.7	63.6	59.5
Atlantic salmon (BT045567)	98.3	95.1		95.9	79.1	75.8	76.8	65.4	64.1	65.2	62.6	57.4
Rainbow trout (U60331)	98.8	96.4	97.1		80.1	76.5	78.1	66.8	65.4	66.2	63.2	58.6
Snakehead (EF079868)	90.4	90.0	89.3	90.5		86.1	88.4	66.5	66.0	65.7	62.8	58.3
Green pufferfish (AF307105)	87.5	86.9	87.1	87.2	92.9		83.1	65.2	64.8	63.8	60.8	57.1
Japanese flounder (EF491182)	89.2	89.0	88.0	89.5	94.0	92.2		66.0	65.7	63.8	63.0	57.9
Human (NM_007315)	82.4	82.3	81.7	82.8	83.9	81.9	82.9		93.6	79.0	59.9	58.4
Norway rat (NM_032612)	81.5	81.6	80.8	81.8	83.2	81.4	82.8	97.2		78.2	59.8	59.1
African clawed frog (AY101602)	82.0	82.0	81.3	82.6	83.1	81.2	82.2	90.6	89.5		59.8	57.1
Zebrafish (NM_131480)	79.9	80.1	79.1	80.2	79.8	79.8	80.7	79.2	78.9	79.1		52.8
Crucian carp (AY242386)	75.7	77.1	74.4	75.9	76.2	75.2	76.5	76.8	76.5	76.1	71.5	

Table 3

$Gal4_{DBD}$-fusion	$Gal4_{AD}$-fusion		
	ssSTAT1b	SV40 T-antigen	No insert
ssSTAT1b	++++ (TDO) +++ (QDO)	ND	-
P53	ND	++++ (TDO) ++++ (QDO)	-
LamC	-	-	-
No insert	-	-	-

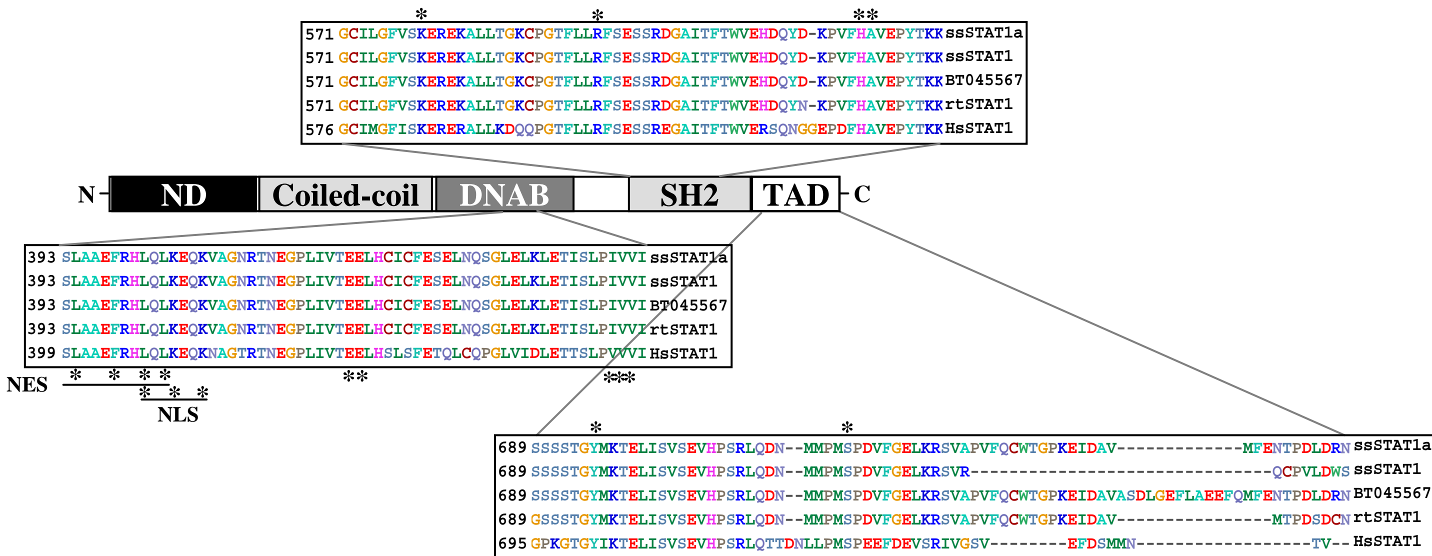

Figure 1

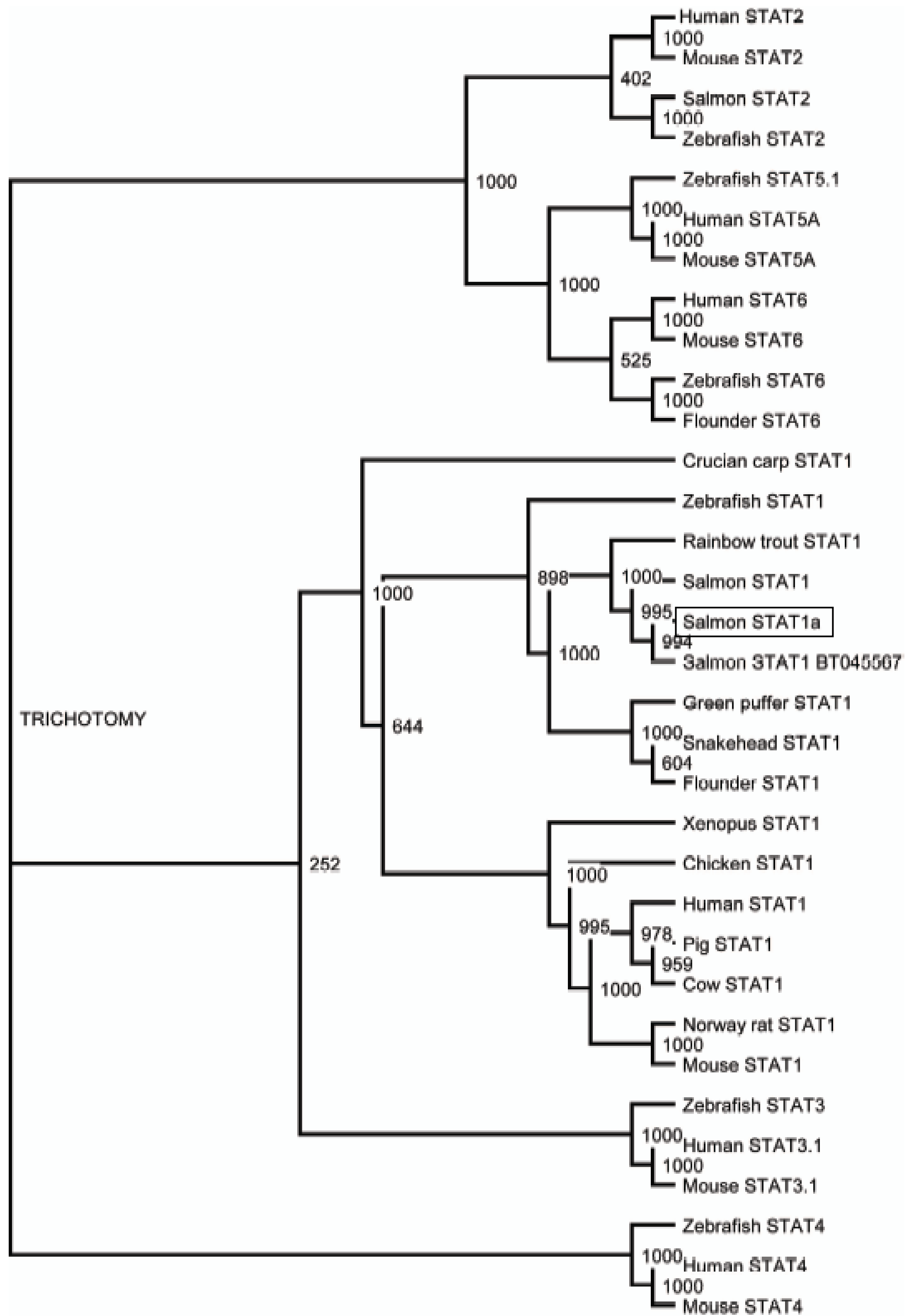

Figure 2

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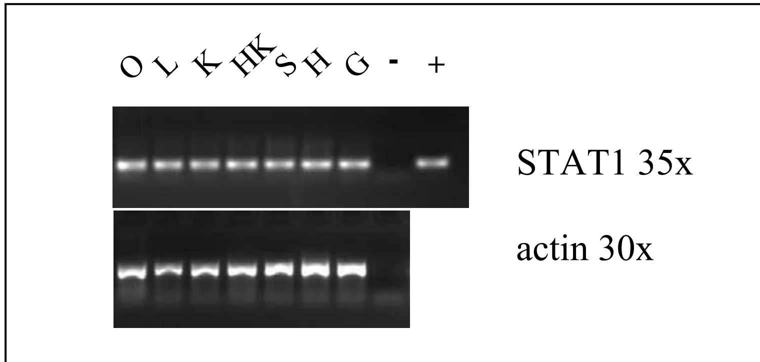

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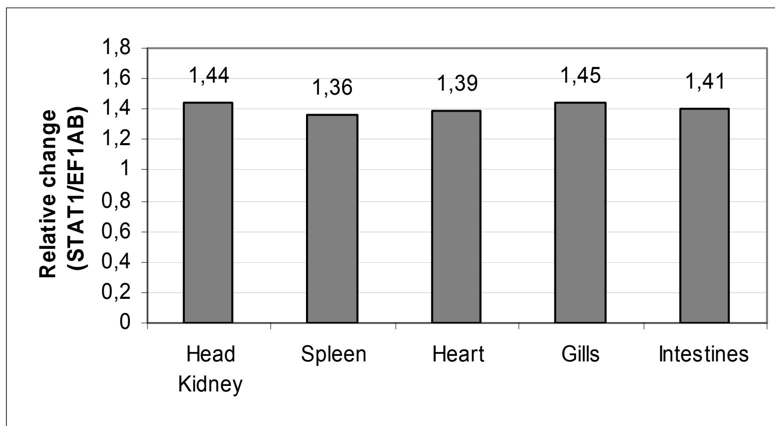

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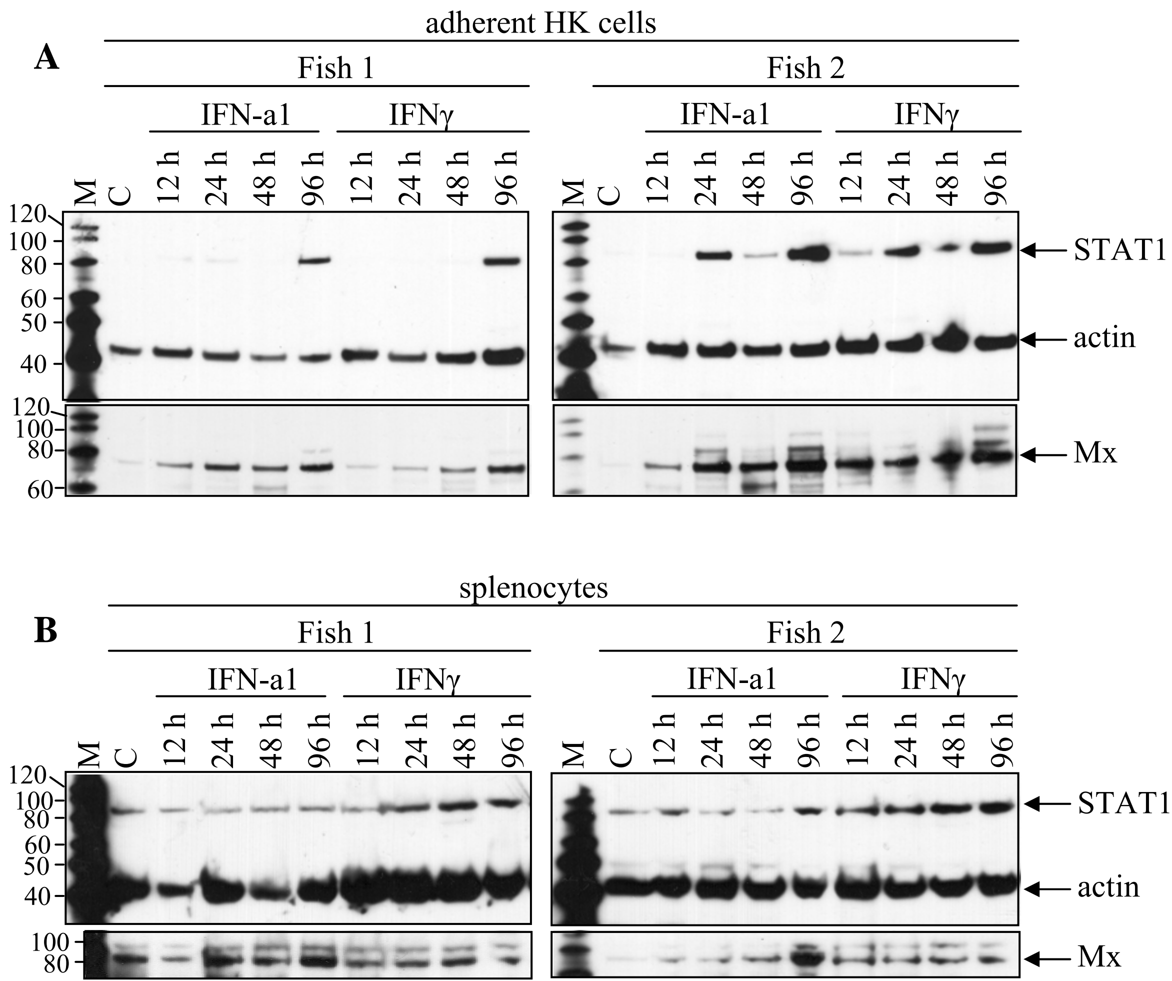
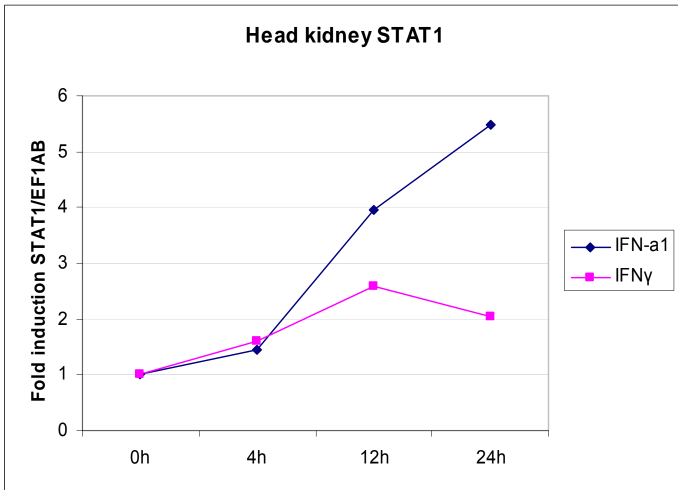


Figure 4

A



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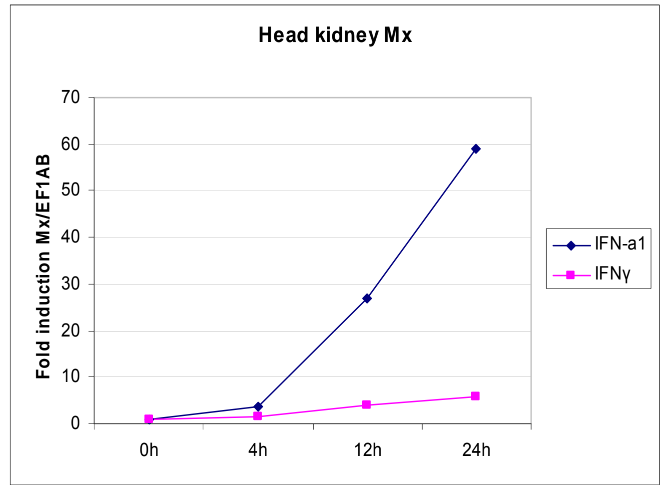

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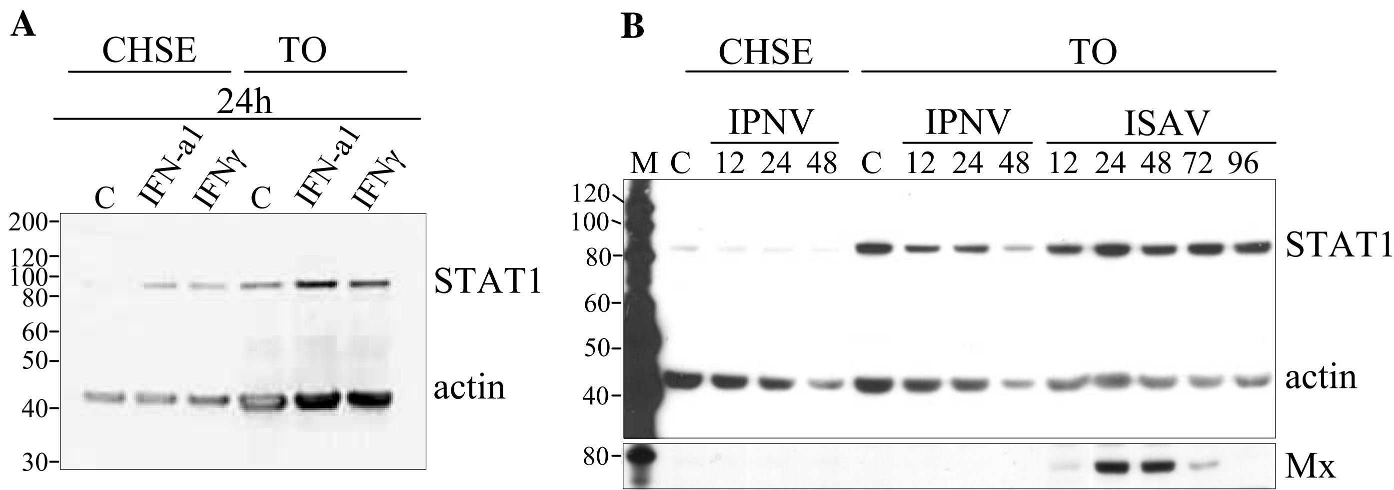

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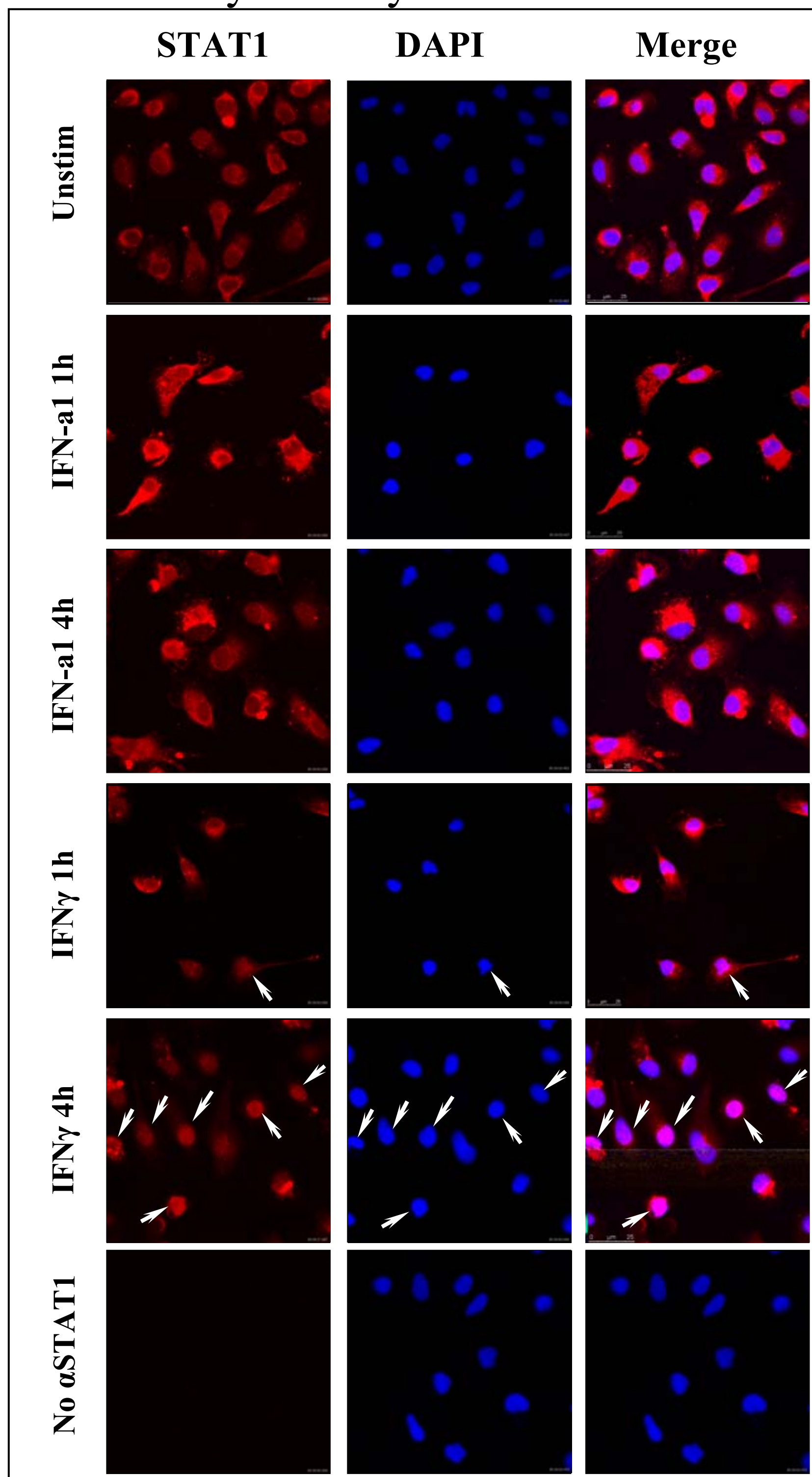


Figure 7A

B TO-cells

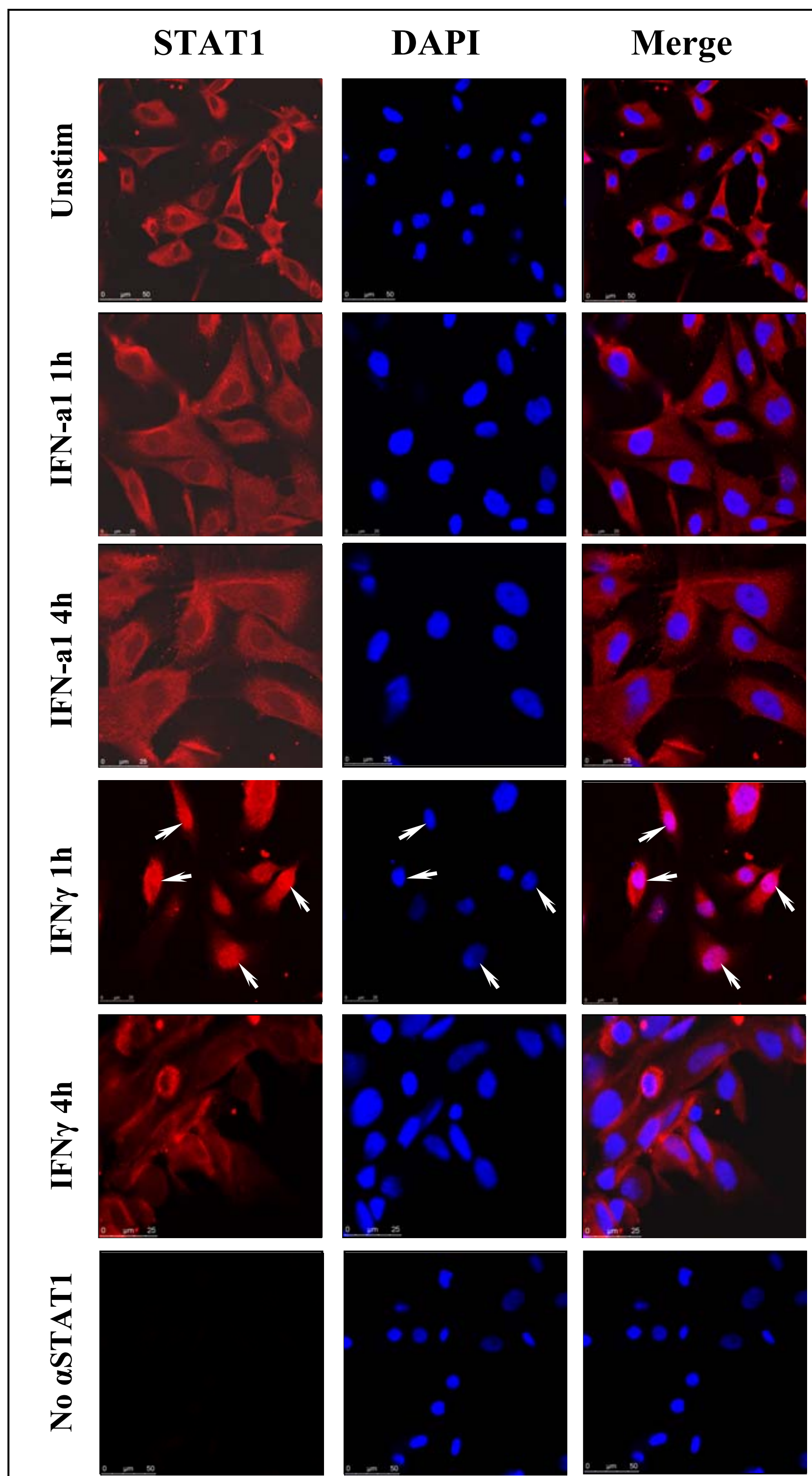


Figure 7**B**

C CHSE-214 cells

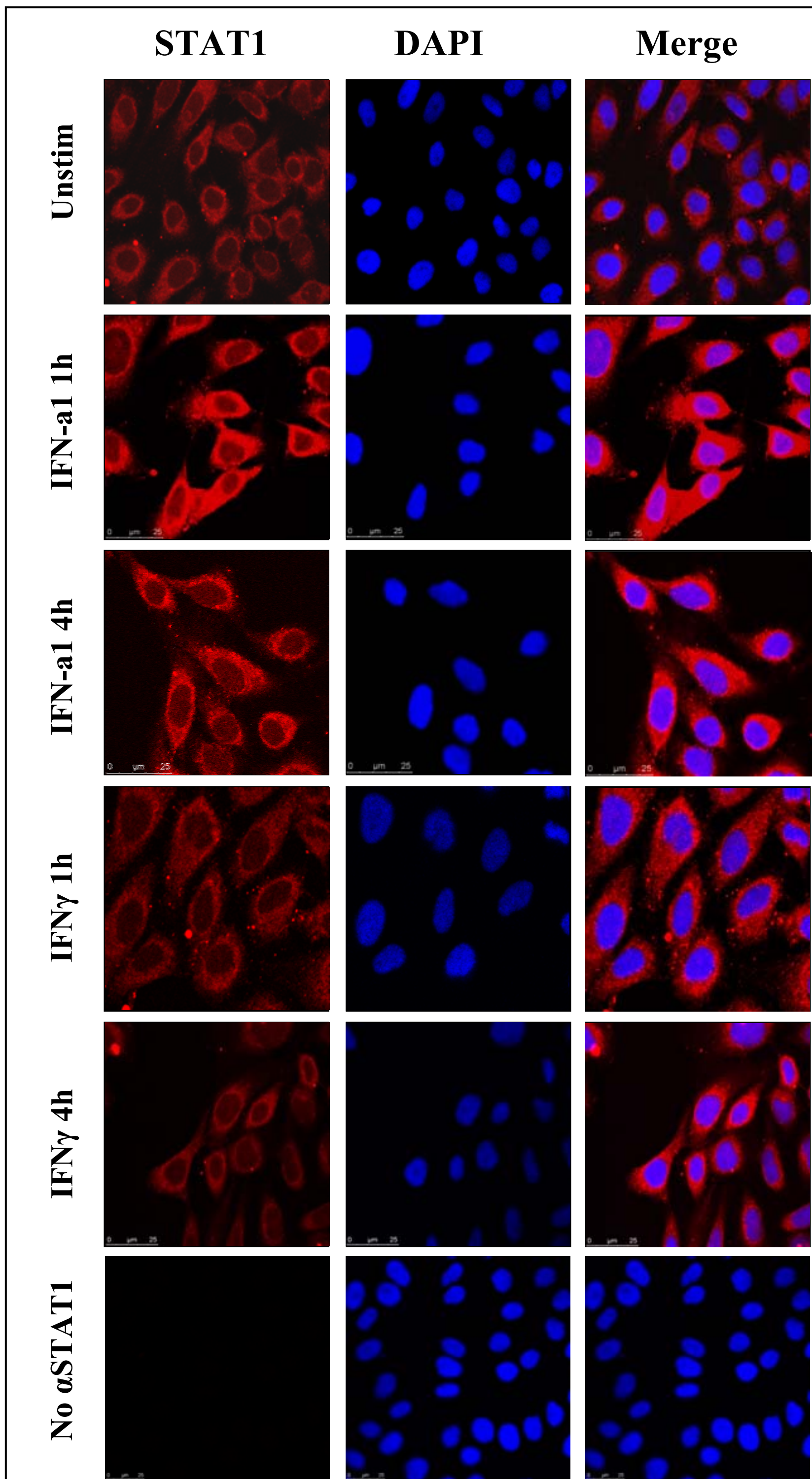
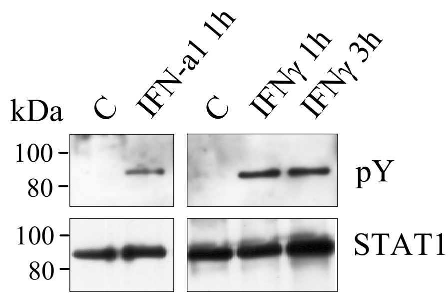


Figure 7C

A



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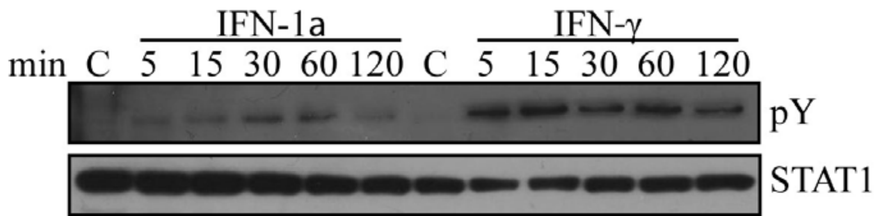

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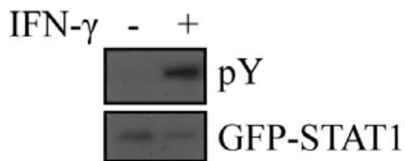

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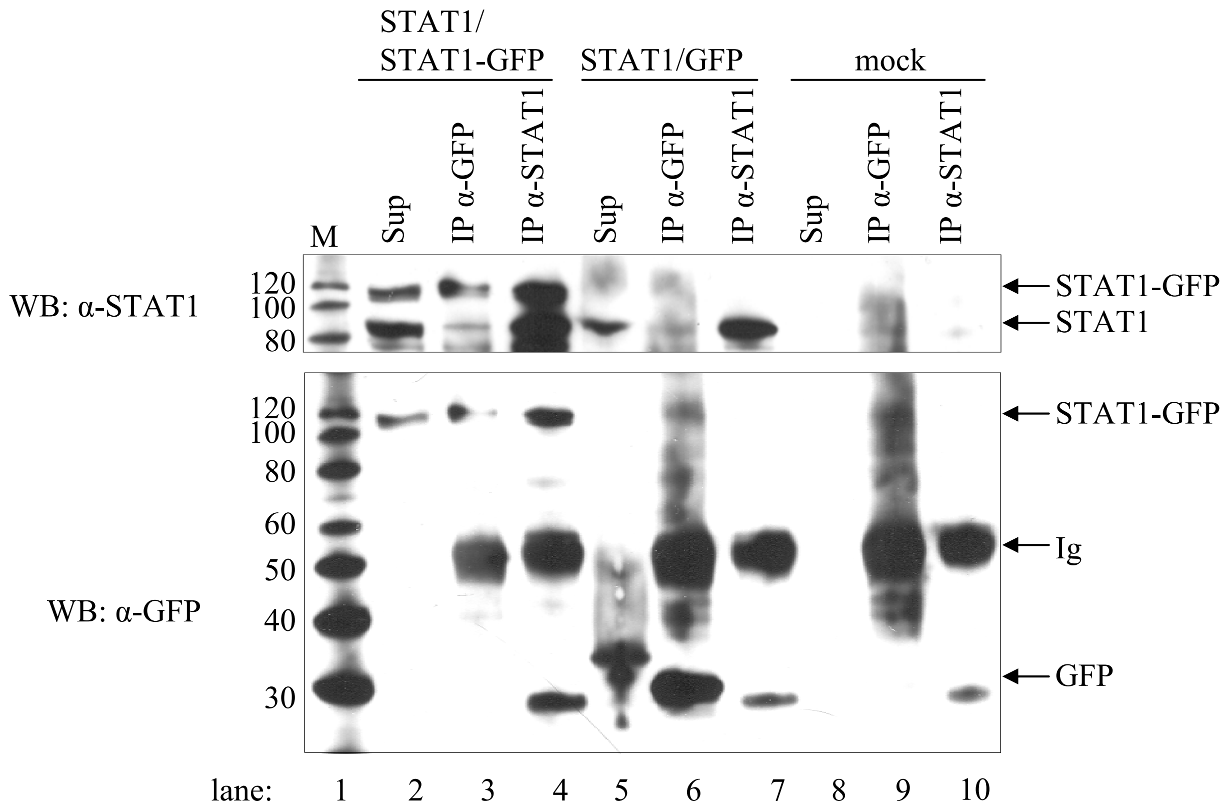

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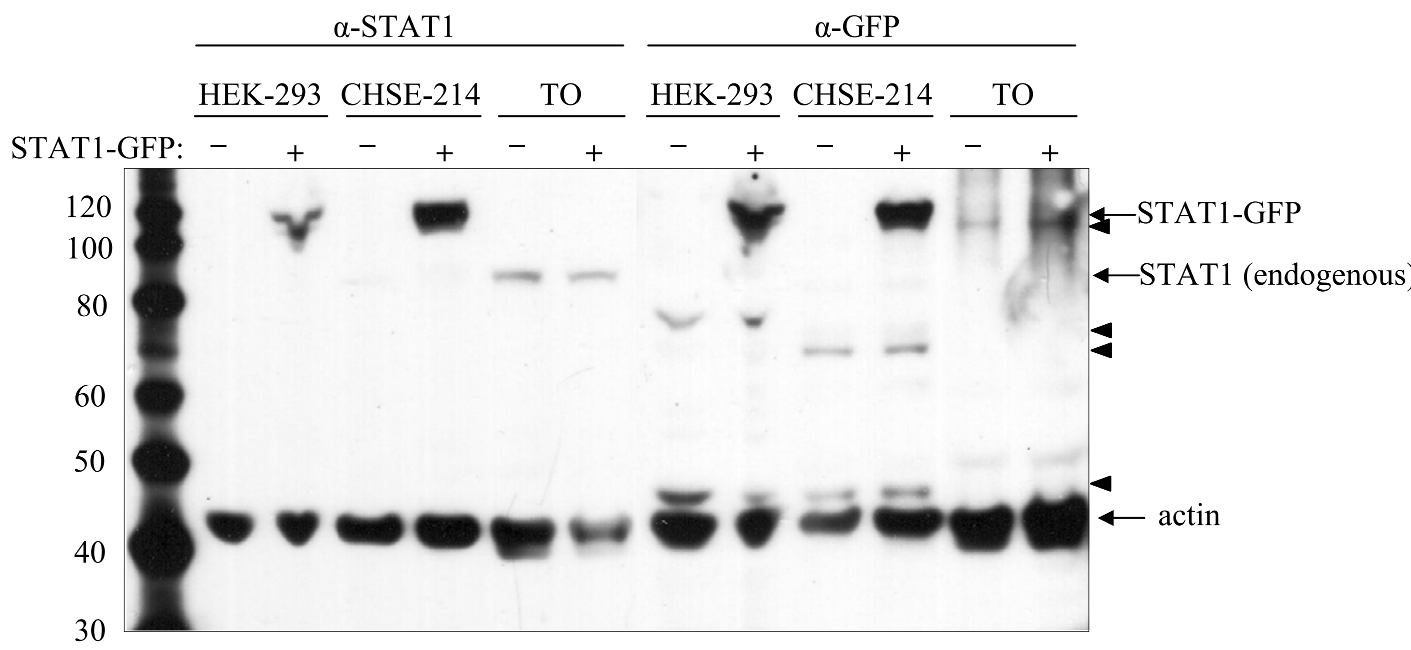

Supplementary Figure 1