Protein phosphatase 2A inhibits interferon signaling through the Jak STAT pathway and promotes hepatitis C viral replication

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Dedicated to my beloved

great grandparents

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Abbreviations

aa	Amino acids
AP-1	Activator protein 1
AP2	Activator protein 2
Bcl2	B-cell lymphoma 2
Bim	Bcl-2 interacting mediator
BRCA-1	Breast cancer (type) 1
CaMKIV	Ca2+/calmodulin-dependent protein kinase type IV
CBP	CREB binding protein
CD	Cluster of differentiation
cDNA	Complementary DNA
CHC	Chronic hepatitis C
CLDN-1	Claudin 1
CREB	cAMP response element binding protein
dsRNA	Double stranded RNA
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF2α	Eukaryotic initiation factor 2 alpha
EMSA	Electrophoretic mobility shift assay
EphA2	Ephrin receptor A2
ER	Endoplasmic reticulum
ETS-1	E-twenty six-1
FERM	Band 4.1, ezrin, radixin, moesin
GAS	Gamma activated sequence
GT	Genotype
HA	Haemagglutinin
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVcc	Cell-culture derived HCV
HCVpp	HCV pseudo particle
HEAT	Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast kinase TOR1
HIV	Human Immunodeficiency Virus
Huh7	Human hepatoma cell line
I1PP2A	Inhibitor 1 of PP2A
I2PP2A	Inhibitor 2 of PP2A
IFN	Interferon
IFN-α	Interferon alpha
IGBP1	Immunoglobulin-binding protein 1
IFN-AR	Interferon alpha/beta receptor
IFN-γ	Interferon gamma
IFNGR	Interferon gamma receptor
IL	Interleukin

IRES	Internal ribosomal entry site
IRF	Interferon regulatory factor
IRG	Interferon-regulated gene
ISG	Interferon-stimulated gene
ISG15	Interferon-sensitive gene 15
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-stimulated response element
Jak	Janus kinase
JFH	Japanese fulminant hepatitis
JH	Jak homology
JNK	c-Jun N-terminal kinases
kDa	kilo Dalton
LCMV	Lymphocytic Choriomeningitis
LDL	Low density lipoprotein
LDLR	LDL-receptor
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinase
MAVS	Mitochondrial antiviral-signaling
MCM5	Minichromosome maintenance complex component 5
MEF	Mouse Embryonic Fibroblasts
miRNA	Micro RNA
MxA	Myxovirus A
NCp	Nucleocapsid protein
NCR	Non coding region
NF-kB	Nuclear factor kappa B
NK	Natural killer (cell)
NS	Non-structural (protein)
NIH3T3	Mouse embryonic fibroblast cell line
NTPase	Nucleoside triphosphatase
OA	Ocadaic acid
OCLN	Occludin
OAS	2'-5' oligoadenylate synthetase
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
pDCs	plasmocytoid dendritic cells
pegIFN-α	Pegylated interferon alpha
PIAS	Protein inhibitors of activated STATs
PKR	Protein kinase R
PP	Protein Phosphatase
PPM	Metal dependent Protein Phosphatase
PPP	Phosphoprotein Phosphatase
PP2A	Protein phosphatase 2A
PR65	Putative Regulatory 65 (kDa protein)

Prkaa1	Protein kinase, AMP-activated, alpha 1 catalytic subunit
PRMT-1	Protein arginine methyltransferase
PTPA	PTPase activator
Pten	phosphatase and tensin homolog
PTPase	Phosphotyrosyl phosphatase
RdRp	RNA-dependent RNA-polymerase
RIG-I	Retinoic-acid inducible gene-I
RNAi	RNA interference
SCID	Severe combined immunodeficiency
SHP	SH2-containing phosphatase
SH2	*src* homology 2
SIE	Serum inducible element
SOCS	Suppressor of cytokine signaling
SP1	Specificity Protein-1
SR-B1	Scavenger receptor class B type 1
src	Rous sarcoma
ssRNA	Single stranded RNA
STAT	Signal transducer and activator of transcription
STRN	Striatin, calmodulin binding protein
SV40	Simian Vacuolating Virus 40
TBK1	TANK-binding kinase 1
TC-PTP	T cell protein tyrosine phosphatase
TOR	Target of rapamycin
Tyk2	Tyrosine kinase 2
USP18 /UBP43	Ubiquitin-specific peptidase 18
VISA	Virus-induced signaling adaptor
VPR	Viral Protein R
VSV	Vesicular stomatitis virus
WT	Wildtype

1. Introduction

1.1 Protein Phosphatase 2A

1.1.1 Structure and function of Protein Phosphatase 2A

Reversible posttranslational modifications are key mechanisms in regulating various cellular processes such as cell signaling, metabolism, and gene expression. One of the most important posttranslational modifications is the phosphorylation at serine, tyrosine, and threonine. The phosphorylated proteins are subjected to dephosphorylation by phosphatases. These phosphatases are classified into three groups based on their sequences, structures and catalytic mechanisms. The first group contains phosphoprotein phosphatases (PPP) and metal (Mg^{2+} or Mn^{2+}) dependent phosphatases (PPM). PP1, PP2A, PP2B, PP4, PP5, PP6 and PP7 belong to the PPP family and PP2C belongs to the PPM family. The second group is the superfamily of the protein tyrosine phosphatases (PTPs) and the third group is the aspartate based phosphatases (Moorhead et al., 2007).

The protein phosphatase 2A (PP2A) is one of the most widely expressed and extensively studied phosphatases. PP2A expression level represents up to 1.0 % of total cellular proteins and constitutes the major serine/threonine phosphatase in the cell (Virshup, 2000). PP2A is a multimeric holoenzyme composed by a scaffold A (65 kDa), a regulatory B, and a catalytic C (36 kDa) subunit (Janssens and Goris, 2001). Various isoforms of the regulatory B subunit are required for recognition and recruitment to specific substrates. The catalytic C (Green et al., 1987; Stone et al., 1987; Arino et al., 1988) and the scaffold A

(Walter et al., 1989; Hemmings et al., 1990) subunits exist as two isoforms, α and β, encoded by distinct genes.

PP2A-A protein was initially identified as a 61kDa protein, isolated from human 293 cells infected with polyoma virus overexpressing medium tumor antigen. This protein was partially digested with V8 protease and sequenced. Based on sequence information, oligonucleotide probes used for screening a cDNA library from human placenta. This novel protein was characterized as 61kDa protein having 15 HEAT repeats (Walter et al., 1989). Further, two isoforms α and β of the scaffold A subunit were cloned from porcine kidney and skeletal muscles and named PR65 (Hemmings et al., 1990). The PP2A-Aα and PP2A-Aβ isoforms have 86% sequence homology (Hemmings et al., 1990). Their half life is approximately 10h (Zhou et al., 2003). PP2A-Aα is expressed 10 fold more than PP2A-Aβ in H460 cells. PP2A-Aα and PP2A-Aβ have different affinities to protein-protein interactions. For example, PP2A-Aα and PP2A-Aβ both bind to polyoma virus middle tumour antigen whereas only PP2A-Aα binds to SV40 small t antigen (Zhou et al., 2003).

The α and β isoforms of the catalytic subunit (PP2Acα and PP2Acβ) were cloned from different sources like bovine adrenal, porcine kidney, human liver, and plants (Green et al., 1987; Stone et al., 1987; Arino et al., 1988; MacKintosh et al., 1990). Gene transcription from PP2Acα gene promoter is 7-10 fold higher than PP2Acβ (Khew-Goodall et al., 1991)

and this may be the reason of almost 10 fold higher expression of PP2Acα than PP2Acβ at protein level (Khew-Goodall and Hemmings, 1988).

Recently, a shorter spliced variant of the PP2Acα has been reported from fresh peripheral blood mononuclear cells (PBMC) (Migueleti et al., 2011). This newly identified variant, named PP2Acα2, lacks the 5th exon and is catalytically inactive (Migueleti et al., 2011). The regulatory B subunit has at least 18 different isoforms divided into B, B', B'' and B''' family (Diagram 1) (Eichhorn et al., 2009).

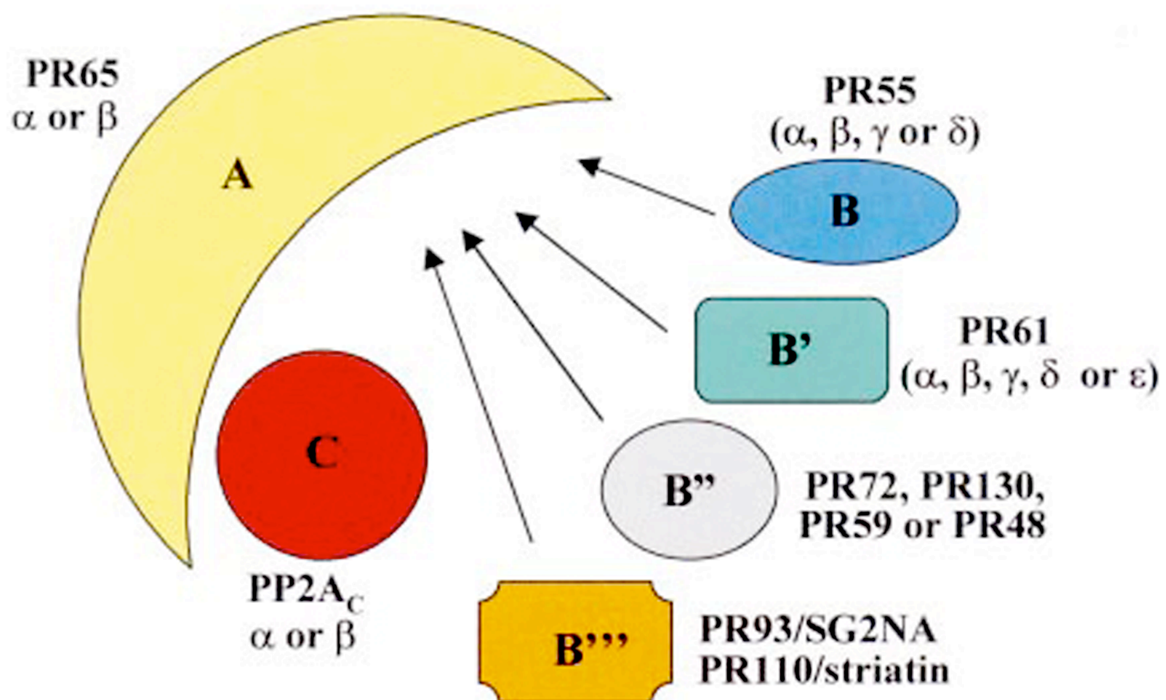

Diagram 1: Hypothetical structure of PP2A holoenzyme complex (Janssens and Goris, 2001)

PP2A subunits coexist as dimeric (A-C) or trimeric (A-C-B) complex. The holoenzyme complex is required for the phosphatase activity on targets (Janssens and Goris, 2001). Although more than 150 targets are identified, the mechanisms by which B subunits mediated PP2A association to the target are not fully understood (Eichhorn et al., 2009). The association of PP2A holoenzyme has been described via the regulatory B subunits. However, several reports have showed that PP2A C and A subunits can also directly associate to proteins such as HIV1 NCp7:vpr, SV40 small t, Bcl2, or CaMKIV (Diagram 2) (Janssens and Goris, 2001).

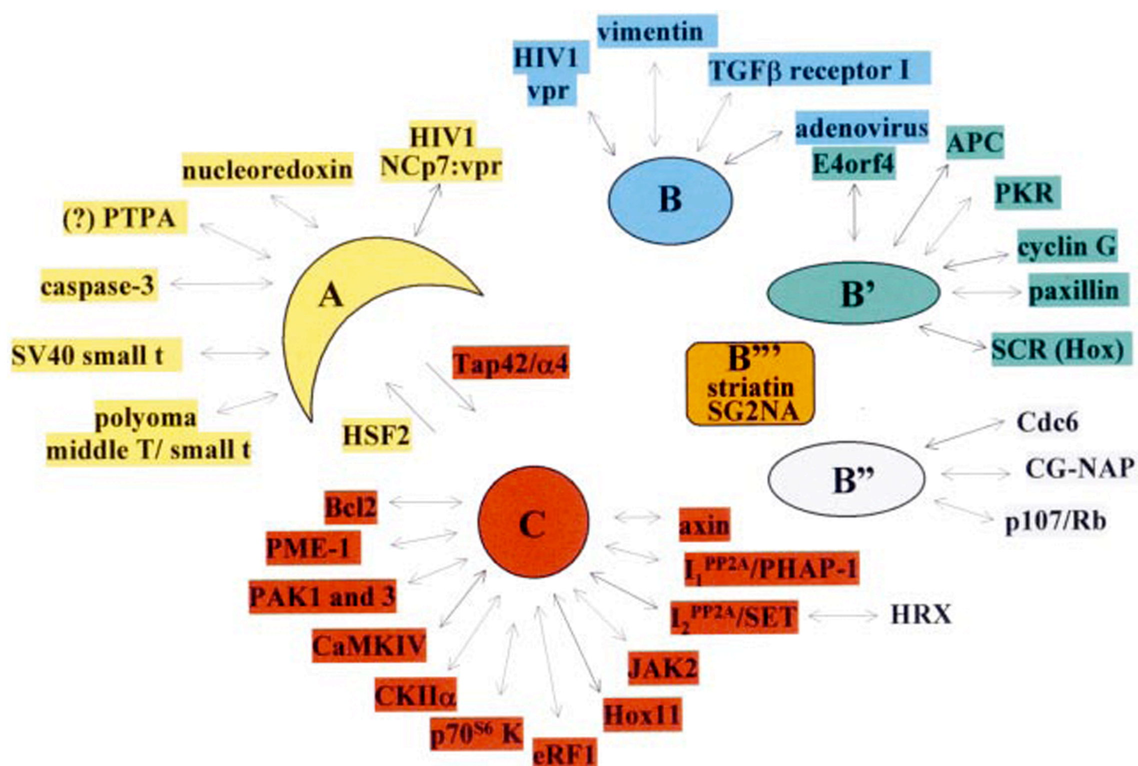

Diagram 2: PP2A interacts with substrates through A, B, or C subunits (Janssens and Goris, 2001).

Generally, PP2A exerts its phosphatase activity through the association with the regulatory B subunits that determine the substrate specificity and the subcellular localization of the enzyme. However, the catalytic activity of PP2A can also be controlled by regulatory molecules such as α4 (see section regulation of catalytic activity of PP2A). The α4 (also termed as IGBP1) is the mammalian orthologue of yeast Tap42 protein which controls the TOR signaling (Onda et al., 1997).

PP2A regulates many cellular functions including metabolism, DNA replication, transcription, RNA splicing, translation, cell cycle progression, cell senescence, apoptosis, cell transformation, morphogenesis, development, and neurotransmission (Cohen, 1989; Stemmer and Klee, 1991; Mumby and Walter, 1993; Hunter, 1995; Olsen et al., 2006; Moorhead et al., 2007).

1.1.2 Regulation of PP2A-A, -B, and -C subunits expression

Several transcription factors like AP1, AP-2α, ETS-1, SP-1, CREB regulate PP2A expression. The promoters of PP2Acα and PP2Acβ are GC rich and lack TATA and CCAAT boxes. PP2Acα promoter has a cAMP-regulatory element (CRE) and several SP-1 binding sites (Khew-Goodall et al., 1991). Incubation of phosphorylated CREB with immunopurified PP2Ac results in a dephosphorylation on serine 133 and consequently the inhibition of the transcriptional activity of CREB (Wadzinski et al., 1993). Additionally, treatment of rat liver extract with OA inhibits dephosphorylation of CREB at serine 133

(Wadzinski et al., 1993). Further, it is reported that PP2Ac associates with autoactivation domain of calcium/calmodulin dependent kinase CaMKIVI and prevents phosphorylation of CREB (Anderson et al., 2004). We have reported that in hepatocytes HCV protein expression induces an ER stress response that leads to serine 133 phosphorylation on CREB and PP2Ac up-regulation (Christen et al., 2007). Moreover, down-regulation of CREB by siRNA did not up-regulate the PP2Ac expression by HCV proteins (Christen et al., 2007). Considering these observations, it is proposed that the expression of PP2Ac could form a feedback loop and inhibit the PP2A expression driven by CREB (Christen et al., 2007). Interestingly, Baharians and Schönthal have shown that PP2Ac protein level is maintained constant through an autoregulatory mechanism in mouse fibroblast cell line NIH3T3 that maintains to constant PP2Ac protein expression irrespective to regulation of transcription or RNA processing. Indeed, partial hepatectomy in mouse shows a constant protein expression level despite a 30 fold increase in PP2Ac mRNA (Kakinoki et al., 1992). Several reports have shown an increased PP2A mRNA expression during G1 phase in mammalian cells (Nakamura et al., 1992; Kikuchi et al., 1997). Consequently to this tight regulation of the protein expression level, it has been observed that PP2A level is constant in various mammalian cells and fission yeast through-out the cell cycle (Virshup et al., 1989; Kinoshita et al., 1990; Ruediger et al., 1991). These data indicates that the accumulation of PP2Ac in the cell is not feasible because of the tight regulation and therefore raise the difficulties of PP2Ac expression experimentally. The half-life of the catalytic C subunit slightly varies from cell line to cell line. It has been reported that the half-life is approximatively 16.5h in mouse fibroblast NIH3T3 cells (Baharians and

Schönthal, 1998).

The transcription of the PP2A-A subunit is regulated by AP-2α, ETS-1, CREB, and SP-1 transcription factors (Chen et al., 2009). It has been demonstrated that the inhibition of PP2A catalytic activity by okadaic acid leads to an activation of JNK, and consequently enhanced c-Jun phosphorylation. Phosphorylated c-Jun forms a complex with c-Fos, AP-1 and initiates PP2A-A transcription (Shanley et al., 2001). EGF exposure to rat fibroblasts 208F cells activates AP-1 transcription factors leading to an increased transcription of PP2A-B subunit (Ozanne et al., 2000). These observations indicate an inter-regulatory effect of PP2A subunits i.e. an alteration of the activity and the expression level of one subunit can alter the expression of another one.

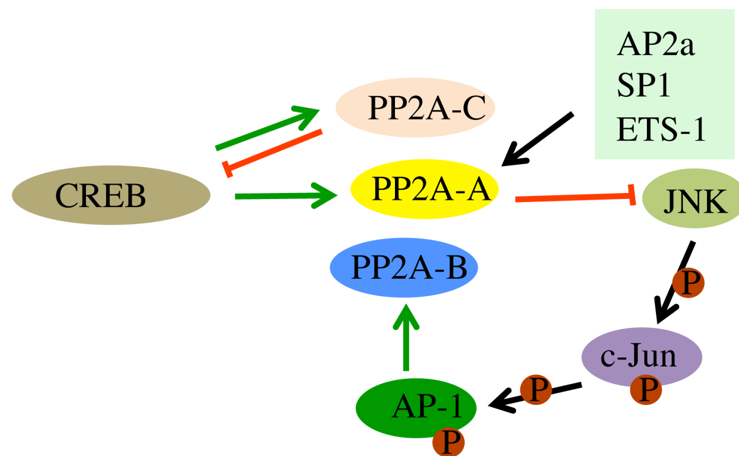

Diagram 3: A simplified model showing regulation of gene transcription of PP2A subunits by activation and inhibition of transcription factors by itself.

Another possibility of inter-regulation of PP2A subunits is through miRNA. Recent studies showed that miRNA-19 have multiple target genes as Bim (Bcl2L11), AMP-activated kinase (Prkaa1), phosphatases Pten and PP2A-B56ε (Mavrakis et al., 2010). Another report showed that the overexpression of miRNA-1 down-regulates PP2A-B56α leading to an enhanced Ca^{2+} release and an increased cardiac arrhythmogenesis (Terentyev et al., 2009). miRNA-34b is known to suppress the expression of α4 in transformed human embryonic kidney (HEKTER) and human lung cells. Chen et al., (Chen et al., 2011) have shown in HEKTER and human lung cells that down-regulation of miRNA-34b leads to an up-regulation of α4 and consequently to a reduction of PP2A activity (Chen et al., 2011).

1.1.3 Regulation of catalytic activity of PP2A

1.1.3.1 Regulation of PP2A activity by posttranslational modifications on C-terminal of the catalytic subunit

The six amino acid (^{304}TPDYFL^{309}) residues on the C-terminus of the C subunit are conserved across species. The threonine 304, the tyrosine 307, and the leucine 309 residues are known to undergo reversible posttranslational modifications. Phosphorylation at tyrosine 307 inhibits phosphatase activity (Chen et al., 1992). Recently, Wu et al. (Wu and Wilson, 2009) have reported that cotreatment of skeletal muscle microvascular endothelial cells with LPS and IFN-γ leads to nitration on tyrosine 307. This nitration increases the phosphatase activity by preventing tyrosine 307 from phosphorylation. In line with these observations, several research groups have reported that exposure of cells with exogenous

peroxynitrite donor substances increases nitration of tyrosine 307 and subsequently decreases phosphatase activity (Guner et al., 2009; Kohr et al., 2009). Similarly, casein kinase 2α has been shown to inhibit PP2A activity by phosphorylating the catalytic subunit at Y307 (Hériché et al., 1997). The catalytic activity of PP2A could be enhanced by treatment of cells with urate, a specific peroxynitrite scavenger, (Kohr et al., 2009). The phosphorylation on threonine 304 residues by auto-phosphorylation-activated protein kinases has been reported to inactivate the phosphatase activity (Guo and Damuni, 1993; Guo et al., 1993). An another posttranslational modification, the carboxymethylation on leucine 309 by methyltransferase (Favre et al., 1994; Floer and Stock, 1994; Xie and Clarke, 1994; Turowski et al., 1995) has been shown to enhance the interaction of the catalytic subunit to the PP2A-B55α subunit and activates the phosphatase activity (Bryant et al., 1999).

1.1.3.2 Regulation of activity by varying holoenzyme complex

PP2A activity also depends on the interaction of the C subunit with B subunits or proteins other than PP2A components. For example, the interaction of the catalytic subunit with different isoforms of PP2A-B regulatory subunits forms different holoenzymes and delivers activity at different physiological level (Csortos et al., 1996; McCright et al., 1996). The complex mechanism behind substrates selection is still poorly understood. However, the selection and the association to the substrates are dependent on posttranslational modifications, as well as on single or multiple mutations in the catalytic region of the

PP2Ac subunit. Indeed, the carboxymethylation at leucine 309 on the C subunit is required for the association to the regulatory PP2A-B55α subunit (Bryant et al., 1999). Moreover the single mutation at tyrosine 307 or leucine 309 of the C subunit forms mostly A-C dimer whereas the double mutations at tyrosine 307 and leucine 309 favours association to the α4 subunit instead of the A subunit (Chung et al., 1999). The regulation of PP2A activity by α4 is ambiguous. Several reports suggest that α4 inhibits PP2A activity while others showed an enhanced phosphatase activity. Indeed, Yoo et al. (Yoo et al., 2008) have reported that α4 forms a complex with PP2Ac in fetal as well as adult liver and inhibits the catalytic activity. In line with this observation other reports suggest an association of α4 with PP2Ac and therefore an inhibition of the catalytic activity in HEK293 cells (Nanahoshi et al., 1998, 1999) and MEFs (Kong et al., 2009). In contrast, Nien et al., (Nien et al., 2007) have shown that the over-expression of mTOR together with α4 protein increases PP2A activity leading to increased STAT1α-PIAS1 binding. Other reports confirm α4-mediated PP2A increased activity (Murata et al., 1997; Inui et al., 1998; Chung et al., 1999). Additional studies, using purified endogenously expressed protein inhibitors I1PP2A (Li et al., 1996a) and I2PP2A (Li et al., 1996b), have reported a direct association of the catalytic subunit to these inhibitors and consequently an inactivation of PP2A. An oncogenic transcription factor Hox11 has been shown to interact with PP2Ac and inhibits the phosphatase activity (Kawabe et al., 1997).

Despite the constant protein expression level of PP2Ac, there are examples of altered enzymatic activity of PP2A. Indeed, using purified proteins, Yang et al. (Yang et al., 1991)

have shown that simian virus 40 small t antigen (SV40ST) does not bind to either C subunit or A-B-C complex but rather binds to the A subunit of the A-C complex in the absence of B subunit and leads to decreased phosphatase activity. Therefore, the ectopic expression of SV40ST in the CV-1 cells leads to an increased phosphorylation of MAPK through inhibition of PP2A activity (Sontag et al., 1993).

1.1.4 **PP2A activity modulation by viruses and toxins**

PP2A subunits are the target of various molecules including drugs, viruses and toxins (Millward et al., 1999). For instance, protein kinase R (PKR) phosphorylates PP2A-B56α subunit at multiple serine and threonine residues. Phosphorylation of the PP2A-B56α subunit enhances PP2A phosphatase activity (Xu and Williams, 2000). Various viral particles associate to PP2A and modify its substrate specificity and activity. For instance, the SV40 small t antigen can associate to the C subunit and displaces the B subunits from A-C complex inhibiting the phosphatase activity (C. Kamibayashi, M. Mumby, Adv. Prot. Phosphatases, 9 (1995), pp. 195–210). Cayla et al. (Cayla et al., 2005) have shown that polyoma virus small t and middle T antigens bind to PP2A catalytic subunit and impart the tyrosine phosphatase activity in polyoma virus transformed cells. E4orf4, an adenovirus protein, associates to the PP2A-B55α and downregulates PP2A expression by modulating the transcriptional activity of AP-1 during late stages of viral infection (Kleinberger and Shenk, 1993). HIV proteins NCp7 and Vpr directly associate to the PP2A-B'δ subunit and initiate PP2A catalytic activity (Tung et al., 1997).

Various chemicals such as PP2A inhibitors are used in laboratories to study PP2A activity. Okadaic acid (from marine sponge) is one of the most widely used among them. The hydrophobic end of the okadaic acid binds to the hydrophobic cage within the binding pocket of PP2Acα (Xing et al., 2006). This hydrophobic cage is constituted by 4 amino acids (Q122, I123, H191, and W200). Other chemicals like calcyculin A (also from marine sponge), tautomycin (an antibiotic from streptomyces spiroverticillatus), microcystin, nodularins (hepatotoxins from cynobacteria) cantharidin (from blister beetles) (Millward et al., 1999) are known as well to inhibit the activity of PP2A.

1.1.5 Model for PP2A over-expression

Various attempts have been made with only minor success to express PP2Ac. Microinjection of purified PP2Ac in fibroblast F9 cells potentiated the activity of promoters containing binding site for AP-1 leading to increased c-Jun expression and therefore an increased expression of PP2Ac (Alberts et al., 1993). However, this approach was successful but only in a small number of cells (Fernandez et al., 1990). In another approach, transient transfection of HA tagged PP2Ac at the N-terminus in NIH3T3 cells resulted in over-expression of PP2A and an increased phosphatase activity (Jaramillo-Babb et al., 1996). Additionally, Ruediger and colleagues have generated an N-terminal PP2A-A mutant that binds specifically to C subunit, therefore, allowing the accumulation of core protein (A-C) in COS cells (Ruediger et al., 1997). Generation of a mouse model of PP2Ac over-expression is shown to be a challenging and frustrating task. Indeed, PP2Acα gene deleted mice die at embryonic day 6.5. Moreover, Götz et al. (Götz et al., 1998) found that

normal and degenerated embryos from PP2Acα gene deleted mice express comparable level of total PP2Ac (PP2Acα and PP2Acβ) suggesting that the absence of PP2Acα cannot be compensated by PP2Acβ. Very recently, conditional null alleles of PP2Acα and PP2Acβ flanking *loxP* have been generated (Gu et al., 2012). PP2Acα$^{fl/fl}$ mice did not show any visible phenotype while Cre-mediated PP2Acα deleted homozygous mice die in the embryonic stage. PP2Acβ deleted homozygous mice did not show any physiological or morphological change.

1.1.6 Clinical significance of PP2A

Although, the expression of PP2A is tightly regulated (Baharians and Schönthal, 1998)' the down-regulation of PP2Ac has been found during all-transretinoic acid induced differentiation of HL-60 cells (Nishikawa et al., 1994), during peroxisome proliferator-activated receptor-γ induced adipocyte differentiation (Altiok et al., 1997) in hippocampus of patients with Alzheimer's disease (Vogelsberg-Ragaglia et al., 2001). Over-expression of PP2Ac has been reported in response to colony-stimulating factor 1 in macrophages (Wilson et al., 1999), in HCV harboring cells, mice and human individuals (Duong et al., 2004).

1.2 Interferon signaling

1.2.1 The Interferon family

Isaacs and Lindenmann discovered interferon five decades ago during their studies on viral interference (Isaacs A, Lindenmann J. Virus interference. I. The interferon. Proceedings of the Royal Society of London. Series B, Containing papers of a Biological character 1957; 147 (927): 258–67). Interferons (IFNs) exercise their role in modulating antiviral and antiproliferative responses. They are grouped into three types: type I, type II and type III. Type I IFNs consists of IFNαs, IFNβ, IFNε, IFNϰ, IFNω and IFNν (Heim, 2012). Presently, 13 different IFNαs have been reported in humans. All subtypes are encoded by distinct genes (Pestka et al., 2004). Interferon-γ is the sole member of type II IFNs. A recently discovered type III IFN is composed by IFN-λ2, IFN-λ3 and IFN-λ1, also known as IL28A, IL28B and IL29, respectively (Kotenko et al., 2002; Sheppard et al., 2002).

Type I and type III IFNs are produced by human plasmacytoid dendritic cells (pDCs) and monocyte-derived dendritic cells (MDDCs) through a common mechanism involving RIG-I, IPS1 (also termed as MAVS, VISA and Cardif), TBK1, IRF3 and NF-κB signaling molecules in response to viral infection (Siegal et al., 1999; Coccia et al., 2004; Österlund et al., 2007; Onoguchi et al., 2007). In response to antigens or mitogens type II IFNs are produced by T lymphocytes and natural killer (NK) cells (Dorman and Holland, 2000).

1.2.2 The Jak-STAT signaling pathway

Type I IFNs exert their effect by binding to the interferon alpha/beta receptor (IFNAR) that is composed of two subunits - IFNAR1 (also known as alpha) and IFNAR2 (known as beta). Type II IFNs (IFN-γ) binds to distinct receptor IFNGR consisting two subunits IFNGR1 and IFNGR2 (Aguet et al., 1988; Hemmi et al., 1994; Soh et al., 1994). Type III IFNs signal through the IFN-λ receptor comprising a unique IFN-λ chain and IL-10 receptor common with IL-10R2 chain. (Kotenko et al., 2002; Donnelly et al., 2004) (Diagram 4).

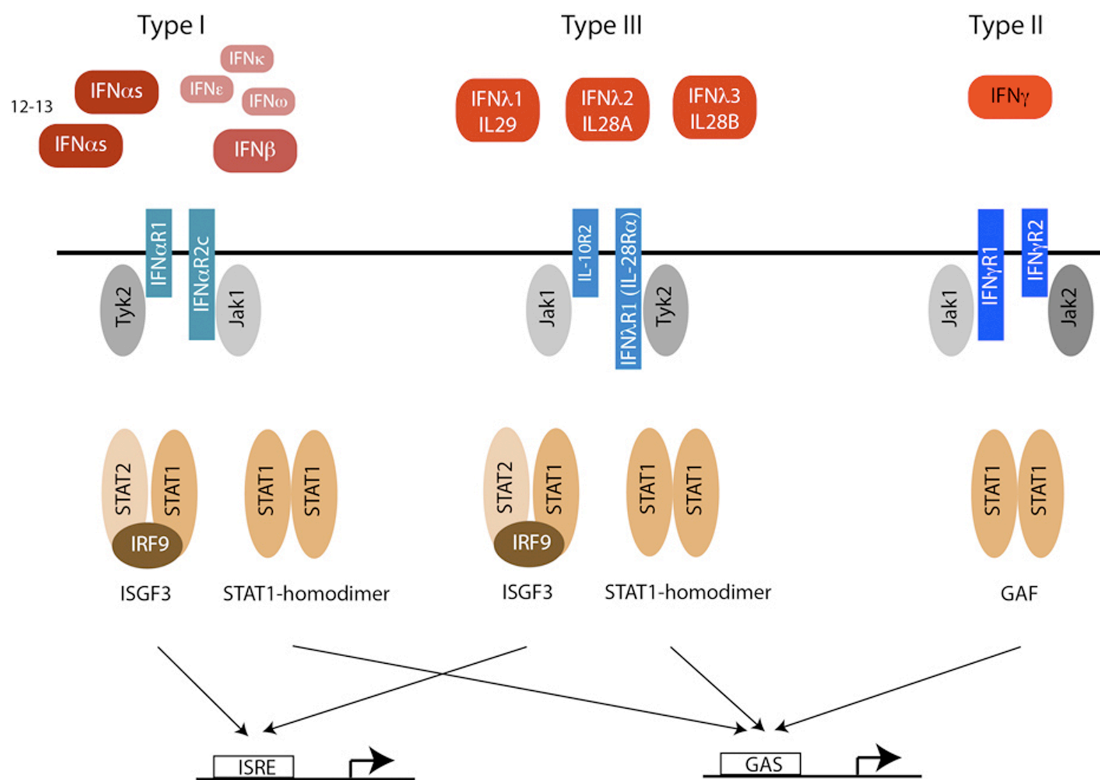

Diagram 4: IFN signaling through the Jak-STAT pathway (Heim, 2012).

Upon association of IFNs to their cognate receptors, the Jak kinases are phosphorylated and activated. Jaks (Janus Kinases) are cytoplasmic kinases. There are 4 known human Jaks, namely – Jak1-3 and Tyk2. Their molecular weight ranges from 120-140 kDa. Jaks are made up of 7-conserved JH (Jak Homology) domains characterized as JH1-JH7. JH1, located at the C-terminal end, is the kinase domain. JH2 is pseudokinase domain, responsible for recruiting substrates (Wang et al., 1995). The JH3-JH7 domains on N-terminus recognize and bind to the receptor. JH3 and JH4 possess structural features of a potential SH2 (*src* homology 2)-like domain (Bernards, 1991). A fraction of JH4 and JH5-JH7 region termed as FERM domain, bind to the cytoplasmic tail of transmembrane receptors (Hilkens et al., 2001) (Diagram 5).

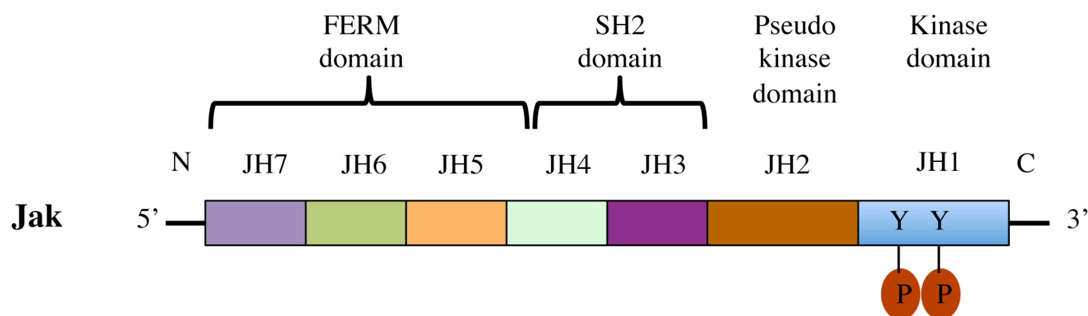

Diagram 5: Structure of Jaks. JH - Jak Homology, SH2 – *src* homology 2, FERM - 4.1, ezrin, radixin, moesin.

STATs (signal transducers and activator of transcription) act as signal carrier as well as activator of transcription. In unstimulated cells, they predominantly localize in the cytoplasm, whereas, upon activation, they translocate to the nucleus and activate

transcription. There are 7 mammalian STATs known so far – STAT1-4, STAT5a, STAT5b, and STAT6. They are ubiquitously expressed except STAT4, which is mainly expressed in thymus and testes (Darnell et al., 1994; Heim, 1999).

STATs have 6 domains. The transactivation domain at C-terminal end associates to the transcriptional co-activators CBP and p300 (Bhattacharya et al., 1996). The SH2 domain is involved in the recruitment of the STATs to activated receptors (Heim et al., 1995) and dimerization of activated STATs (Shuai et al., 1994). The DNA binding coiled-coil domain interacts with IRF9 and forms the ISGF3 complex. The N-terminus, containing a protein-protein interaction domain, has been reported in regulation of STAT tyrosine dephosphorylation, CBP/p300 interaction, and nuclear transportation regulation (Zhang et al., 1996) (Diagram 6).

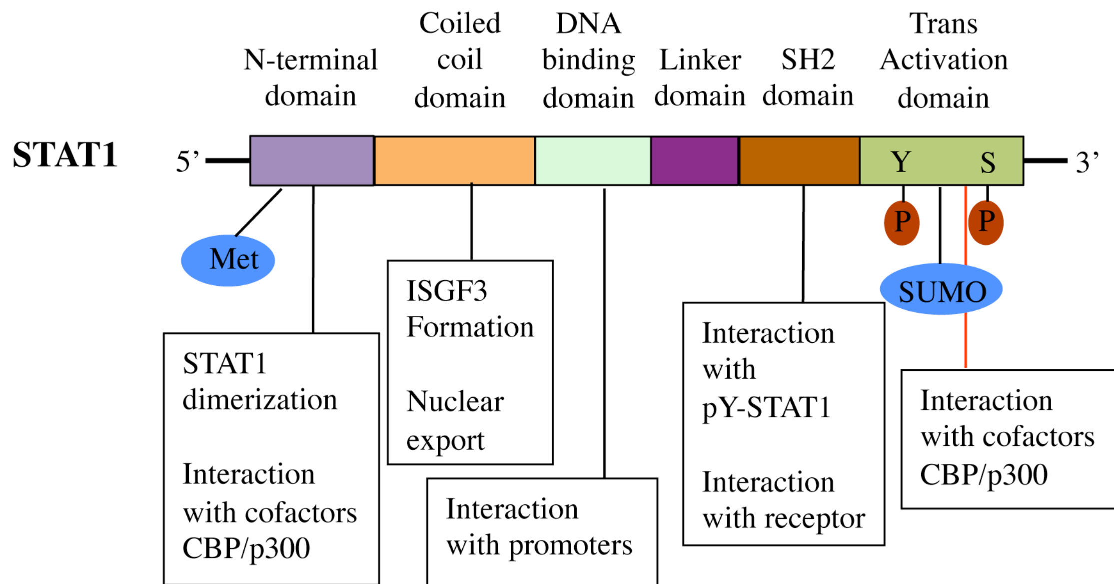

Diagram 6: Structural organization of domains of STAT1 and their functions.

1.2.3 Activation of the Jak-STAT signaling by IFN-α

Cells exposed to IFN-α activate a cascade of events such as cross-linking of receptor chains (IFNAR1 and IFNAR2), activation of kinases (Jak1 and Tyk2), and phosphorylation of STAT1, STAT2 and STAT3 (Shuai et al., 1993; Heim et al., 1995). Activated STATs dissociate from receptor-kinase-substrate complex, form homo- or/and heterodimers, translocate to the nucleus, bind to promoter elements, and induce ISGs transcription (Horvath, 2000) (Diagram 7). This ISGs transcription includes an array of genes up-regulation including antiviral genes (PKR, OAS1, MxA, ISG15), signaling genes (STAT1, STAT2, IRF9) and IFN-α itself (Lehtonen et al., 1997).

STAT1 was first defined based on its role in mediating gene activation in response to interferon-γ (IFN-γ) (Darnell, 1997; Horvath and Darnell Jr, 1997). STAT1 contains a C-terminal trans-activation domain, which possesses phosphorylation sites at tyrosine 701 and serine 727. Tyrosine phosphorylation of STAT1 on C-terminus is important for dimerization, DNA binding, and transcriptional activation whereas serine phosphorylation leads to maximal transcriptional activity (Wen et al., 1995; Decker and Kovarik, 2000; Kovarik et al., 2001). STAT1 activity is also regulated through methylation on arginine 31 residue by protein arginine methyl transferase 1 (PRMT1) (Mowen et al., 2001). Indeed, methylation of arginine 31 leads to a decrease STAT1-PIAS1 association, thereby, enhances STAT1 binding to promoters of target genes and consequently increased ISGs transcription.

Recently, it has been reported that several ISGs can be induced by STAT1 in a tyrosine 701 independent manner (Chatterjee-Kishore et al., 2000; Ramana et al., 2001). Indeed, tyrosine to a phenylalanine mutation of STAT1 does not alter the enhancement of the activity of Fas and FasL promoters. In contrast, a STAT1 serine 727 to an alanine mutation is less efficient than wild-type STAT1 in enhancing Fas and FasL promoters, as well as in inducing apoptotic cell death in cardiac myocytes (Stephanou et al., 2001). Moreover, phosphorylation on serine 727 has been shown to be critical for the C-terminal region of STAT1 to act as a transcriptional co-activator by binding to molecules such as MCM5 (Zhang et al., 1998) and BRCA-1 (Ouchi et al., 2000).

1.2.4 Negative regulation of the Jak-STAT pathway

The constitutive activation of interferon signaling is harmful to the cell. Therefore, it is essential to regulate the interferon signal transduction cascade. As reported before, STATs are constitutively activated in many hematological malignancies (Gouilleux-Gruart et al., 1996). The regulation of the Jak-STAT signaling is attributed to its various negative regulators at different levels. For instance, the inhibition of IFNs signaling by the Suppressors of Cytokine Signaling (SOCS) which constitutes the first level of inhibition at the receptor-kinase-STATs complex, occurs either by inhibiting Jaks activation or by preventing STATs binding to the receptor (Krebs and Hilton, 2001; Larsen and Röpke, 2002). The SOCS family consists of 8 members SOCS1 – SOCS7 and CIS. SOCS1 and SOCS3 are induced by type I IFNs (Song and Shuai, 1998). SOCS1 deficient mice develop

a complex fatal neonatal disease (Alexander et al., 1999) and die during neonatal period due to hepatotoxicity and multi-organ failure (Starr et al., 1998). SOCS1 deleted mice are resistant to viral infection due to an enhanced type I IFN signaling (Fenner et al., 2006). SOCS3 binds and inhibits the kinase domain of Jak1, Jak2 and Tyk2 (Babon et al., 2012).

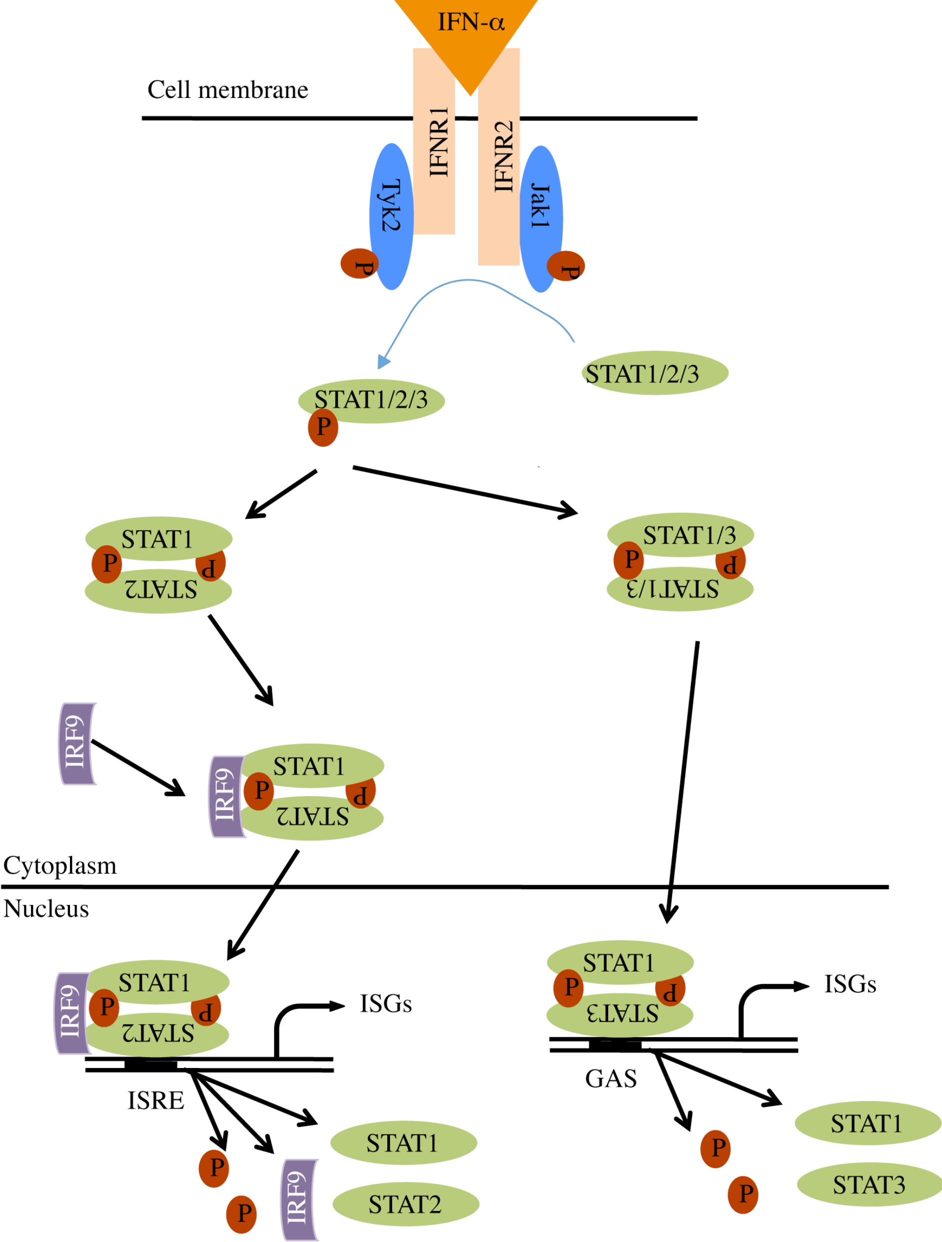

Diagram 7: IFN-α induced Jak-STAT signaling pathway. IFN-α binding to receptors causes their dimerization. Kinases (Jak1/Tyk2) are recruited to receptors and auto-activated. Activated kinases phosphorylate STATs leading to their dimerization and ISGF3 formation by association with IRF9, translocation to nucleus and activation of gene transcription.

Downstream to the SOCSs inhibition, the protein inhibitor of activated STAT (PIAS) binds to hypomethylated STAT dimers and inhibits STAT-DNA interaction (Liu et al., 1998; Shuai, 2000; Mowen et al., 2001). PIAS1 and PIAS3 bind to STAT1 and STAT3, respectively. PIAS1 selectively inhibits interferon inducible gene and is essential for innate immunity. PIAS1 deleted mice show an enhanced protection against viruses and microbes (Liu et al., 2004).

Another important negative regulator of the Jak-STAT pathway is the ubiquitin specific peptidase 18 (USP18/UBP43). Initially, USP18/UBP43 was considered as protease cleaving ubiquitin-like modifier ISG15 (Liu et al., 1999). USP18/UBP43 was recently found to exert a negative regulatory role independent to its ISG-deconjugating ability (Malakhov et al., 2002). USP18/UBP43 inhibits the activation of Jak1 by disrupting Jak1 interaction with IFNAR2 (Malakhova et al., 2006). USP18/UBP43 is induced by IFN-α (Der et al., 1998; Malakhova et al., 2002) and provides a negative feedback loop that restricts IFN-α signals. It has been reported that UBP43 deficient mice display a critical phenotypic characteristic as brain cell injury, hypersensitivity to poly-(I:C) and premature

death (Ritchie et al., 2002; Malakhova et al., 2003). Interestingly, these animals are resistant to fatal cerebral infections caused by VSV and LCMV (Ritchie et al., 2004). We and others have shown that USP18/UBP43 is up-regulated in liver of CHC non-responders to pegIFN-α therapy (Chen et al., 2005a; Sarasin-Filipowicz et al., 2008) and contributes to the refractoriness to IFN-α stimulation in mouse liver (Sarasin-Filipowicz et al., 2009). Furthermore, USP18/UBP43 knockdown in cells enhances the antiviral activity of interferon against hepatitis C virus infection (Randall et al., 2006).

STATs are dephosphorylated by a 45kDa isoform nuclear T-cell protein tyrosine phosphatase (TC-PTP), followed by nuclear export (ten Hoeve et al., 2002; Zhu et al., 2002). It has been reported that SHP-2, a SH2 containing phosphatase, dephosphorylates at both tyrosine and serine residues on STAT5 and STAT1 in the nucleus (Wu et al., 2002; Chen et al., 2003). Recently, TC-PTP has been reported as a negative regulator of cytokine signaling. TC-PTP deleted mice display immune defects and developed systemic inflammatory diseases like sialadenitis, gastritis, chronic myocarditis and nephritis. These animals have an elevated serum of IFN-γ causing death of animals within five weeks. (Heinonen et al., 2004) (Diagram 8). Additionally, we have demonstrated that PP2A inhibits the Jak-STAT pathway at the level of STAT-DNA interaction by altering the methylation status of STAT1 (Duong et al., 2004).

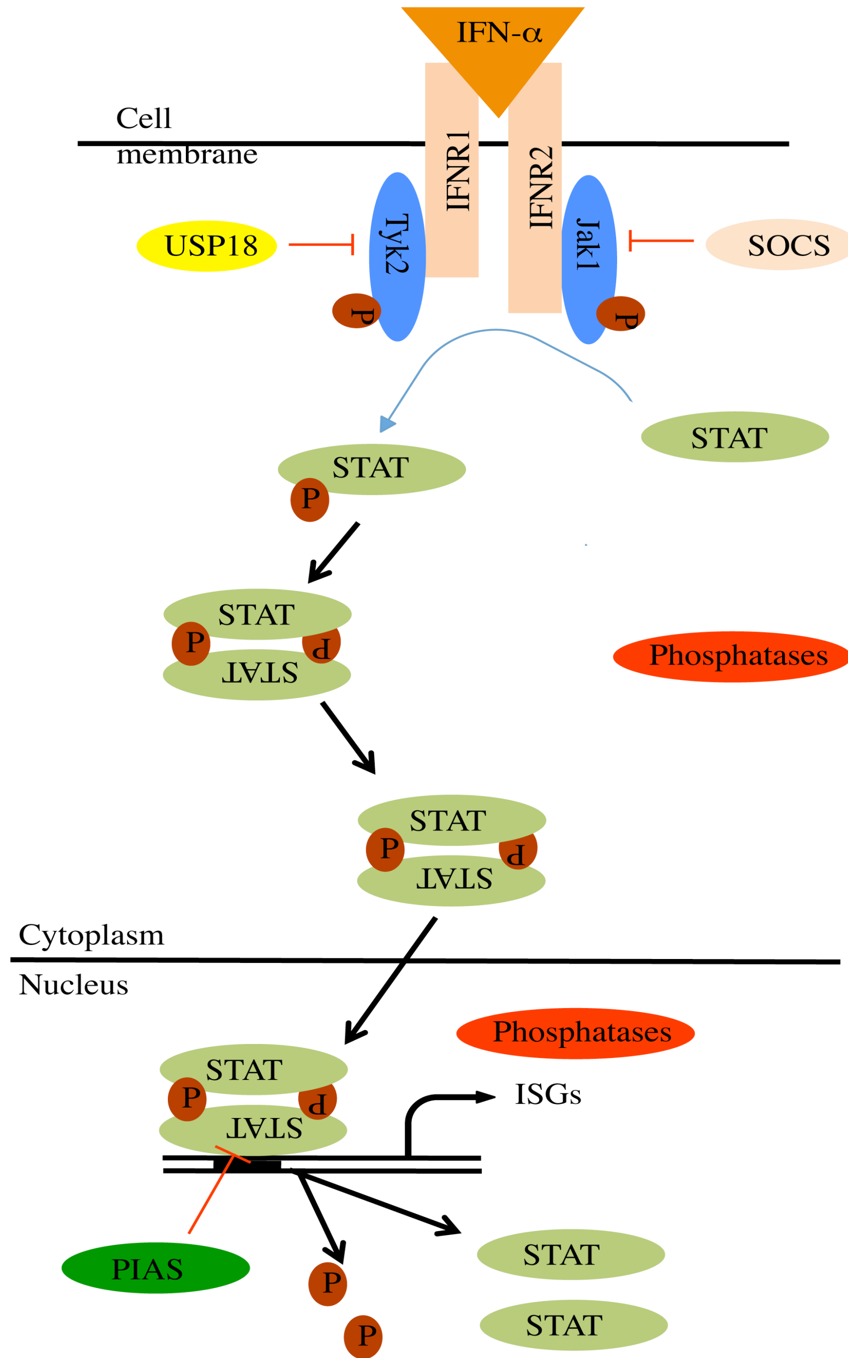

Diagram 8: Negative regulation of IFN-α induced Jak-STAT signaling pathway. Jak-STAT pathway is negatively regulated in cytoplasm (by USP18, SOCSs, Phosphatases) as well as in the nucleus (by PIAS, Phosphatases).

1.2.5 **Refractoriness of the IFN signaling**

It is well known that the IFNs induction becomes rapidly refractory. Indeed, maximal induction of the IFN signaling pathways occurs within the initial 2 hours of IFN stimulation and continuous exposure to IFNs leads to a “desensitization” and consequently to an absence of response to further stimulation for the next 2-3 days (Larner et al., 1986). Refractoriness has been also reported in the liver of mice injected with mouse IFN-α (Sarasin-Filipowicz et al., 2009). *In vivo,* subcutaneous injection of IFN-α make hepatocytes refractory within hours after the first injection and remain so for at least 2 days.

Studies on negative regulator of IFN-α have revealed SOCSs as early inhibitors of STAT phosphorylation within 2-4 hours of IFN-α stimulation - whereas USP18/UBP43 is involved in long lasting refractoriness. Interestingly, IFN-β induced signaling in mice liver and IFN-γ induced signaling in intestine are not subjected to refractoriness (Makowska et al., 2011).

1.2.6 **IFN-α induced antiviral activity**

The Jak-STAT signaling cascade plays a major antiviral role by inducing the ISGs such as double-stranded RNA (dsRNA) activated protein kinase R (PKR), 2'-5' oligoadenylate synthetase (OAS), and Mx proteins (Liu et al., 2004). In virally infected cells, viral dsRNA

triggers the phosphorylation of PKR resulting in the phosphorylation of the α subunit of eukaryotic initiation factor (eIF2α). The phosphorylation of eIF2α leads to the inhibition of translation of both viral and cellular proteins, providing the antiviral and antiproliferative effect of IFNs (Sadler and Williams, 2008). OAS-dependent activated RNase L catalyses the cleavage of viral and cellular single-stranded RNAs (ssRNAs) (Carroll et al., 1996). RNase L plays an important role in positive feedback mechanisms and enhancement of the innate immunity in the cells. Presence of small RNA in the virally infected cells induces the antiviral innate immunity. Indeed, small self-RNA generated by RNase L amplifies the antiviral innate immunity by producing antiviral cytokines like IFN-β (Malathi et al., 2007). Marschall and colleagues have reported that the multiplication of negative-stranded RNA viruses such as influenza C viruses was suppressed in cells expressing Mx proteins, an IFN-α inducible GTPases (Marschall et al., 2000).

1.2.7 Clinical significance of alteration of the Jak-STAT signaling

Defect of Jak-STAT1 signaling is beneficial for HCV to escape from the host antiviral activity and to establish persistent infection. HCV infection results in an inhibition of the IFN-induced antiviral effector system through the phosphorylation of PKR and eIF2α, and a decrease of IFN-α receptor expression in the liver through disruption of the IFN-α signaling (Yatsuhashi et al., 1999). The expression of the entire HCV ORF in cell lines inhibits the formation of ISGF3 and its subsequent DNA binding (Heim et al., 1999). Lack of functional Tyk2 in melanoma cell reduces tyrosine phosphorylation of STAT1 and STAT3 upon IFN-α induction (Pansky et al., 2000). STAT1 deficient mice do not show

any developmental defects but have compromised innate immunity to viral disease (Durbin et al., 1996). Impaired Jak-STAT signaling due to mutation in IL-2 receptor and Jak3 has been reported in human X-Linked Severe Combined Immunodeficiency (SCID) (Puck et al., 1997) syndrome. Moreover, a V617F mutation has been reported in the coding sequence of Jak2 in human myeloproliferative disorders, which leads a gain of function causing expansion of the myeloproliferative disorder (Kralovics et al., 2005).

1.2.8 PP2A and the Jak-STAT signaling

We have reported previously that the expression of HCV proteins in osteosarcoma cell lines or in transgenic mice liver leads to an up-regulation of PP2A catalytic subunit and inhibits the IFN-α signaling at the STAT-DNA interactions level (Heim et al., 1999; Blindenbacher et al., 2003). We have consistently observed an up-regulation of PP2Ac in human cell lines that inducibly express HCV proteins, HCV transgenic mice, and human liver biopsy from chronic hepatitis C (CHC) patients (Duong et al., 2004). The over-expression of PP2Acα in Huh7 cells results in a reduction of PRMT1 activity, a hypomethylation of STAT1, an increased STAT1-PIAS1 association, and consequently, an inhibition of STAT1-DNA binding (Duong et al., 2006). The PP2A-induced inactivation of PRMT1 can be reversed *in vitro* by treatment with a methyl-group donor S-adenosyl-L-methionine (SAMe) (Duong et al., 2006). It is reported that treatment of cellular extracts from U973 cells stimulated with IFN-γ with purified PP2Ac resulted in a decreased GAF-DNA binding (Eilers et al., 1995). Further, Fielhabler and colleagues (Fielhaber et al., 2009) have shown that PP2A forms a macromolecular complex with STAT1, α4, mTOR in HEK 293T and silencing of PP2Ac

increases the activated STAT1 content into the nucleus and up-regulates of Jak-STAT signaling in response to IFN-γ. Additionally, it is shown that inhibition of PP2A in human $CD4^+$ T or T lymphoma or Jurkat T cells increases the serine phosphorylation on STAT6 and STAT3 (Woetmann et al., 1999, 2003).

1.3 The Hepatitis C virus

1.3.1 Structure of HCV

Hepatitis C virus is a member of *Hepacivirus* genus of the *Flaviviridae* family. (Moradpour et al., 2007). HCV is small (55–65 nm in size) (Shimizu et al., 1996), enveloped, positive-sense single-stranded RNA virus. HCV was identified as a causative agent of a liver disease and CHC. It was previously defined as a non-A, non-B hepatitis (Choo et al., 1989). CHC can lead to liver cirrhosis and hepatocellular carcinoma. Six distinct genotypes (GT) of HCV with 65-70 % sequence homology have been reported so far (Simmonds et al., 2005).

The HCV genome is approximately 9.6kb long and is composed of a 5'-non-coding region (NCR), an open reading frame (ORF) for polyprotein and a 3'-NCR. The HCV ORF encodes approximately 3000 amino acids (aa) (Suzuki et al., 1999) long precursor polyprotein which is processed into at least ten proteins by viral and cellular proteases. These proteins are grouped into structural (core, E1 and E2) and non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins. The core protein forms a nucleocapsid

whereas E1 and E2 are embedded into lipid and are responsible for recognition of receptor and entry of the virus into the host cell (Grakoui et al., 1993b). The p7 is an ion channel protein. It has been shown using an expression plasmid encoded for NS2 and NS3 that the major part of NS2 and the N-terminal part of NS3 constitutes a zinc-dependent metalloproteinase and cleaves the NS2/NS3 junction. The cleavage of the remaining non-structural polyprotein to generate individual protein is performed by NS3-4A protease. (Grakoui et al., 1993a; Hijikata et al., 1993). NS3 contains a serine protease and a NTPase/RNA helicase activity. The NS4A is a cofactor for NS3 proteases. It is shown that the processing of the polyprotein occurs at ER membranous web which is formed by NS4B (Egger et al., 2002; Gosert et al., 2003). The function of NS5A is not clear though it has been proposed that NS5A (hyper)phosphorylation plays an important role in the regulation of NS5A interaction to the host protein (hVAP-A) as well as to viral proteins (NS3, NS4B). The hyperphosphorylation of NS5A disrupts the interaction with hVAP-A leading to a decrease viral replication (Evans et al., 2004) In line with this observation, substitution of serine by alanine residue within the central cluster 1 led to a reduction of phosphorylation and an enhance RNA replication (Appel et al., 2005). Furthermore, NS5A interacts with IFN-α inducible host protein PKR and inhibits its activity leading to a decrease of IFN-α mediated antiviral activity (Gale et al., 1998). NS5B contains an RNA-dependent-RNA-polymerase activity (RdRp) and is essential for HCV replication (Behrens et al., 1996; Ishii et al., 1999).

The highly conserved NCRs are required for viral replication. The 5’-NCR contains an internal ribosomal entry site (IRES) that is responsible for the initiation of a cap independent translation of viral protein. Recently, it has been reported that the liver specific miRNA-122 associates to the 5’-NCR of HCV RNA and positively modulates HCV replication (Jopling et al., 2005). The 3’-NCR consists of three regions. A poorly conserved 40 bases among various genotypes, a variable length poly(U)/poly-pyrimidine tract, and an element of highly conserved 98 bases (Tanaka et al., 1995, 1996; Yamada et al., 1996; Kolykhalov et al., 2000). Similarly to the 5’-NCR, the 3’-NCR is required for HCV replication (Diagram 9).

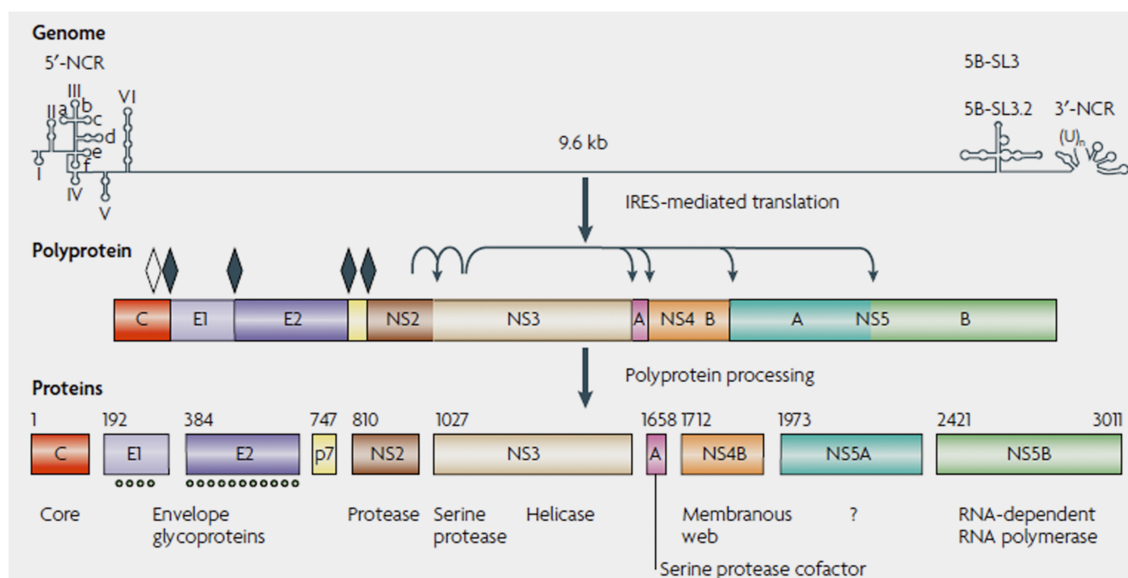

Diagram 9: Structural organization of HCV genome (Moradpour et al., 2007).

1.3.2 Pathogenesis

The primary target cell for HCV infection is the hepatocyte, and potentially additional targets are B-lymphocytes and dendritic cells Since the discovery of HCV in 1989 (Choo et al., 1989) much has been learned about HCV replication. Most of the knowledge obtained on HCV replication are based on genomic and subgenomic replicon systems (Bartenschlager, 2002). So far, only humans are known as host as well as reservoir of HCV (Chisari, 2005). Indeed, chimpanzees have been infected experimentally but no HCV transmission from one chimpanzee to another has been reported. Efforts are being made to generate humanized mouse models, but until now only chimpanzees exist as immuno-competent animal model.

Viral entry is the first step of replication and needs cooperative interaction between several viral and host factors. HCV entry is internalized in a clathrin-dependent endocytosis that requires various host factors engaging viral E2 protein and channelizing the process. Several reports have shown that HCV is internalized through various host factors. Indeed, the HCV virus envelope protein E2 interacts with tetraspanin CD81, expressed on various cells including hepatocytes and B lymphocytes, and together with cellular factors induces HCV internalization (Pileri et al., 1998; Cormier et al., 2004; Molina et al., 2008). HCV is inable to interact with cells when human low-density lipid receptor (LDLR) is expressed at normal level. The ectopic expression of LDLR in Cos7 cells enables the HCV to bind and infect the cells (Monazahian et al., 1999). A correlation study of LDLR expression and

HCV entry in primary human hepatocytes has shown that the down-regulation of LDLR by 25-hydroxycholesterol or the up-regulation of LDLR by squalestatin leads to a decrease or to an increase viral RNA, respectively (Molina et al., 2007). A recent report suggests that the LDLR is not essential for HCV entry, but the physiological function of LDLR is important for optimal replication of the HCV genome (Albecka et al., 2012).

HCV can also associate to an another receptor termed as scavenger receptor class B type I (SR-BI), which then initiates the internalization (Scarselli et al., 2002), The SR-BI-based viral entry is mediated by Apo-B containing lipoprotein in which HCV E2 protein first interacts with Apo-B containing lipoprotein and then this complex interacts with SR-BI receptor (Maillard et al., 2006). A tight junction component, claudin-1 (CLDN1) was found to be a co-receptor for the HCV entry. Unlike CD81 and SR-BI that are required at the initial stage of HCV attachment to the receptors, CLDN1 is required at late stage of viral entry (Evans et al., 2007). Recently another tight junction protein, occludin (OCLN) has been found as an entry factor. Indeed, silencing of OCLN led to a decrease HCVpp infection in Hep3B cells as well as a decrease HCVpp and HCVcc infection in Huh7.5 cells. And, inversely, the over-expression of OCLN in NIH-3T3 cells enhances the HCVpp infection by approximately 120 fold (Ploss et al., 2009). Eventually, the ephrin receptor A2 (EphA2) and epidermal growth factor receptor (EGFR) have been identified as viral entry host factors (Lupberger et al., 2011). It has been hypothesized that EGFR activation helps in HCV binding to cells by modulating intracellular or cell surface trafficking of CLDN1, CD81 or both (Lupberger et al., 2011)

2. Aims of the PhD thesis project

The hepatitis C virus is a major pathogen that causes liver cirrhosis and hepatocellular carcinoma. Current standard IFN-α based therapies are costly and elicit severe side effects. Moreover, existing IFN-α based therapies are not effective in all HCV infected individuals. We have previously reported that HCV infected patients who had a constitutive up-regulation of ISGs do not respond to IFN-α based therapies (Sarasin-Filipowicz et al., 2008). We have also reported that HCV infection induces PP2Acα over-expression in human individuals (Duong et al., 2004) which encourages us to study further.

The aims of this PhD thesis are:

2.1 Analysis of the molecular mechanism used by PP2Ac to inhibit IFN-α induced antiviral activity.

2.2 Assessment of the role of PP2Ac over-expression on HCV replication

2.3 Identification of B subunits that modulate the dual effect of PP2Ac

3. Materials and methods

Materials and methods for the aims (section 2.1 and 2.2) - ‘‘Analysis of the molecular mechanism used by PP2Ac to inhibit IFN-α induced antiviral activity’’ and ‘‘Assessment of the role of PP2Ac over-expression on HCV replication’’ is incorporated with the manuscript.

Materials and methods for the aim (section 2.3) ‘‘Identification of B subunits that modulate the dual effect of PP2Ac’’ is described in further sections 3.1 and 3.2.

3.1 Silencing of PP2A-A and -B subunits

The sh-RNA sequence for various subunits were designed using online software from Promega. The short-hairpin oligos targeting PP2A-A, -B subunits and scrambled oligos were cloned into psiSTRIKE-Puromycin vector (Promega) that allows contitutive expression of inserts. Plasmids encoding sh-RNA against various B and A subunits were transfected into Huh7 cells and clones were selected into puromycin containing medium. Details of sh-RNA oligos for silencing of PP2A-A and -B subunits are listed below.

Sh-RNA oligos

1. **sc-RNA_Aα**

5'-ACCGCGTGTGCGGCGCTACTTAAAGTTCTCTTAAGTAGCGCCGCACACGCTTTTTC -3'

5'-TGCAGAAAAAGCGTGTGCGGCGCTACTTAAGAGAACTTTAAGTAGCGCCGCACACG -3'

2. **sh-RNA_Aα (binding position 1254-1272) (19 bases)**

5'-ACCGGTGCGGCTGGCCATCATTAAGTTCTCTAATGATGGCCAGCCGCACCTTTTTC -3'

5'-TGCAGAAAAAGGTGCGGCTGGCCATCATTAGAGAACTTAATGATGGCCAGCCGCAC -3'

3. **sh-RNA_Bα (B55α) (binding position 589-607) (19 bases)**

5'-ACCGATTTGCGGATTAATCTTTAAGTTCTCTAAAGATTAATCCGCAAATCTTTTTC -3'

5'-TGCAGAAAAAGATTTGCGGATTAATCTTTAGAGAACTTAAAGATTAATCCGCAAAT -3'

4. **sh-RNA_Bβ (B55β) (binding position 137-155) (19 bases)**

5'-ACCGTCGGGTTGTAATATTTCAAAGTTCTCTTGAAATATTACAACCCGACTTTTTC -3'

5'-TGCAGAAAAAGTCGGGTTGTAATATTTCAAGAGAACTTTGAAATATTACAACCCGA -3'

5. **sh-RNA_Bγ (B55γ) (Binding region 1043-1061) (19 bases)**

5'-ACCGGAGCGACAGCGTCATCATAAGTTCTCTATGATGACGCTGTCGCTCCTTTTTC -3'

5'-TGCAGAAAAAGGAGCGACAGCGTCATCATAGAGAACTTATGATGACGCTGTCGCTC -3'

6. **sh-RNA _Bδ (B55δ) (Binding region 94-112) (19 bases)**

5'-ACCGCCGAAGCGGACATCATTTAAGTTCTCTAAATGATGTCCGCTTCGGCTTTTTC -3'

5'-TGCAGAAAAAGCCGAAGCGGACATCATTTAGAGAACTTAAATGATGTCCGCTTCGG -3’

7. **sh-RNA_B`α (B56α) (Binding region 1347-1366) (20 bases)**

5'-ACCGGAACGTGAAGAATTATGGAAAGTTCTCTTCCATAATTCTTCACGTTCCTTTTTC -3'

5'-TGCAGAAAAAGGAACGTGAAGAATTATGGAAGAGAACTTTCCATAATTCTTCACGTTC -3

8. **sh-RNA_B\`β (B56β) (Binding region 382-400) (19 bases)**

5'-ACCGACATCATCCGCATGATCTAAGTTCTCTAGATCATGCGGATGATGTCTTTTTC -3'

5'-TGCAGAAAAAGACATCATCCGCATGATCTAGAGAACTTAGATCATGCGGATGATGT -3'

9. **sh-RNA_B\`γ (B56γ) (binding position 294-312) (19 bases)**

5'-ACCGTTTGCAGTTAACATGTTTAAGTTCTCTAAACATGTTAACTGCAAACTTTTTC -3'

5'-TGCAGAAAAAGTTTGCAGTTAACATGTTTAGAGAACTTAAACATGTTAACTGCAAA -3'

10. **sh-RNA_B\`δ (B56δ) (Binding position 699-717) (bases 19)**

5'-ACCGTACATCGACCAGAAGTTTAAGTTCTCTAAACTTCTGGTCGATGTACTTTTTC -3'

5'-TGCAGAAAAAGTACATCGACCAGAAGTTTAGAGAACTTAAACTTCTGGTCGATGTA -3'

11. **sh-RNA_B\`ε (B56ε) (binding position 1351-1369) (19 bases)**

5'-ACCGAGGATCTGGAGTTAAAGAAAGTTCTCTTCTTTAACTCCAGATCCTCTTTTTC -3'

5'-TGCAGAAAAAGAGGATCTGGAGTTAAAGAAGAGAACTTTCTTTAACTCCAGATCCT -3'

12. **sh-RNA_B\`\`α (B72α) (binding position 507-525) (19 bases)**

5'-ACCGCTGAATAACCATCATGATAAGTTCTCTATCATGATGGTTATTCAGCTTTTTC -3'

5'-TGCAGAAAAAGCTGAATAACCATCATGATAGAGAACTTATCATGATGGTTATTCAG -3'

13. **sh-RNA_B\`\`β (B72β) (binding position 33-52) (20 bases)**

5'-ACCGTTCGAGCTCGAGTACTTCTAAGTTCTCTAGAAGTACTCGAGCTCGAACTTTTTC -3'

5'-TGCAGAAAAAGTTCGAGCTCGAGTACTTCTAGAGAACTTAGAAGTACTCGAGCTCGAA -3'

14. **sh-RNA_B\`\`γ (B72γ) (binding position 340-358) (19 bases)**

5'-ACCGAGGAAGCGATGATCAATTAAGTTCTCTAATTGATCATCGCTTCCTCTTTTTC -3'

5'-TGCAGAAAAAGAGGAAGCGATGATCAATTAGAGAACTTAATTGATCATCGCTTCCT -3'

3.2 qRT-PCR

For details please see the qRT-PCR section in the manuscript incorporated.

	Genes	Fwd (5`-3`)	Rev (5`-3`)
1	PPP2R1A (PP2A-Aa)	CTGGACAACGTCAAGAGTGAGATC	CCGAGTCCTGCTCGTCAGA
2	PPP2R2A (PP2A-B55α)	TCAGTGGTGTTTTTCTCAGGTGAA	GACAACTCTACCACCTTTATCTCCTGTT
3	PPP2R5A (PP2A-B56α)	AACACTGGAATCCGACCATTG	AAAGCTTGCCATTCATTTCCAT
4	PPP2R5B (PP2A-B56β)	CACCTGCAGCTGGTATATGAGTTT	CACGGAGGGCTGGAAGTCT
5	PPP2R2B (PP2A-B55β)	CCCGCAAAATCAACAACAGTT	TAATGTCAGCTTCGGTCGCATA
6	PPP2R2C (PP2A-B55γ)	CGGGAACCAGAGAGTAAAAATGC	TGGAAAGTGCTGTACACGTCGTA
7	PPP2R2D (PP2A-B55δ)	TTCGGATAGCGCCATCATG	CGTGTCTCTATCAAACATCCTGAAG
8	PPP2R5C (PP2A-B56γ)	CAAAGCCAATCCCCAGTACAC	CCATTGCAACCGGAATGC
9	PPP2R5D (PP2A-B56δ)	CCATCATGTTCCCTGCACTCTA	GTCCATGGATTGTCTTGTTCCA
10	PPP2R5E (PP2A-B56ε)	GATCCTTCACTCACAGAACCAGTTATT	CCCAAGGAACATGACCTCTTTT
11	PPP2R3A (PP2A-B72α)	GCCCAACTGCAGCTCTCTAGA	GGGTATCCACCACATCCTGAA
12	PPP2R3B (PP2A-B72β)	GACTGCCTCTGCCAGATGCT	GCAGCGTGATCTTCCCTTCA
13	PPP2R3C (PP2A-B72γ)	TGGAGCAAAGTGCAAGCAAT	CATATGAATCTGTATGAAGGAGTTTAGCA

Table 1: List of forward and reverse primers used. All primers used were designed across the exon-exon junction to avoid genomic DNA amplifications.

4. Results

4.1 Protein Phosphatase 2A impairs IFNα-induced antiviral activity against the hepatitis C virus through the inhibition of STAT1 tyrosine phosphorylation

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Protein phosphatase 2A impairs IFNα-induced antiviral activity against the hepatitis C virus through the inhibition of STAT1 tyrosine phosphorylation

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SUMMARY. Mammalian cells have developed several mechanisms to sense viruses and initiate adequate responses such as production of interferons. Interferons activate the antiviral response through the Jak-STAT signalling pathway. To establish a chronic infection, viruses need to counteract this barrier of defence. The hepatitis C and hepatitis B viruses are known to up-regulate the expression of protein phosphatase 2A (PP2A). In this study, we show that PP2Ac associates with Jak1/Tyk2/STAT1 and reduces Jak1/Tyk2/STAT1 phosphorylation resulting in an impairment of the IFNα-induced HCV antiviral response. Using the fully infectious HCV cell culture system (HCVcc), we demonstrate that the PP2A catalytic activity is not required to block the antiviral effect of IFNα, although it is needed to support HCVcc replication. Our data suggest an important contribution of virus-induced PP2Ac up-regulation in the establishment of a chronic infection.

Keywords: antiviral activity, HCV replication, interferon α, protein phosphatase 2A, STAT1.

INTRODUCTION

The innate immune system is the first response of the host against invasion by pathogens. Cells of the immune system have developed detection methods to sense viruses and activate the production of interferons (IFNs) [1,2]. IFNs associate with their cognate receptors and initiate the Jak-STAT signalling pathway. The binding of IFNs to the receptor leads to receptor dimerization and activation of the Janus kinases (Jaks) [3]. Signal transducer and activator of transcription (STATs) are then recruited and phosphorylated by the Jaks. Activated STATs regulate transcription by association with the promoters of IFN-stimulated genes (ISGs) [4,5]. IFNα activates important antiviral effector systems such as double-stranded RNA (dsRNA), activated protein kinase R (PKR), 2′-5′ oligoadenylate synthetase (OAS) and Mx proteins [6–8]. The regulation of the Jak-STAT pathway occurs at various levels and is crucial to switch off the interferon signal. Several inhibitors are involved such as suppressor of cytokine signalling (SOCS) [9], protein inhibitor of activated STAT (PIAS) [10], T-cell protein tyrosine phosphatases (TC-PTPases) [11], and ubiquitin-specific peptidase 18 (USP18) [12].

The major serine/threonine phosphatase, protein phosphatase 2A (PP2A) is ubiquitously expressed in all cell types. PP2A is a trimeric enzyme composed of a scaffolding subunit A, a regulatory subunit B and a catalytic C subunit [13]. The C subunit associates with the A subunit to form the catalytic core [14]. Heretofore attempts to overexpress the fully active PP2A catalytic subunit (PP2Ac) failed because of the autoregulatory mechanism that maintains PP2A expression level constant [15]. However, addition of a peptide sequence derived from the influenza haemagglutinin protein (HA) at the N-terminal end of the PP2Ac sequence has been reported to increase the phosphatase activity [16] and resulting in an impairment of the enzymatic activity of the protein arginine methyl transferase 1 (PRMT1) [17,18]. This autoregulatory mechanism is disrupted in chronic hepatitis C (CHC) and chronic hepatitis B (CHB) viral infection leading to an over-expression of the catalytic subunit [19,20]. The regulatory B subunit recognizes a similar sequence of the A subunit and determines the subcellular localization of the heterotrimeric enzyme as well as the substrate specificity [21]. The activity of PP2A is regulated by post-translational modifications on the C subunit. Indeed, the phosphorylation on tyrosine 307 inhibits PP2A activity [22], and the carboxymethylation on leucine 309 controls the interaction of the catalytic

Abbreviations: CHC, chronic hepatitis C; ISGs, IFN-stimulated genes; OAS, oligoadenylate synthetase; PKR, protein kinase R.

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subunit with the Bα subunit [23]. Previously, we have reported that PP2Ac over-expression impairs the IFNα signalling at the STAT1-DNA-binding level by a direct association of PP2Ac with PRMT1 leading to the inhibition of PRMT1-induced STAT1 methylation [18].

We show here that PP2Ac associates with Jak1/Tyk2/STAT1 upon IFNα stimulation and blocks Jaks/STAT1 phosphorylation. Interestingly, this inhibitory effect is independent of the catalytic activity and is abolished by high concentrations of IFNα. Using a functional assay based on the HCV fully infectious system, we demonstrate that the over-expression of a PP2Ac mutant lacking the catalytic activity is sufficient to impair the antiviral activity of IFNα. Our data reveal a dual role for PP2Ac; first to support HCV replication and second to impair the IFNα-mediated antiviral activity favouring the establishment of a chronic infection.

EXPERIMENTAL PROCEDURES

Reagents, antibodies and plasmids

Human IFNα and the protease inhibitor cocktail were obtained from Roche (Hoffmann La Roche, Basel, Switzerland). Mouse IFNα was a generous gift from Prof. Radek Skoda. Lipofectamine 2000 was from Invitrogen (LuBio-Science GmbH, Buchs, Switzerland). Protein A-Sepharose 4B beads, okadaic acid and Actin antibody were from Sigma-Aldrich (Fluka Chemie GmbH, Lucerne, Switzerland). PP2Ac and pY1022/23-Jak1 antibodies were from Millipore (Millipore AG, Zug, Switzerland). HCV NS3A and Tyk2 antibodies were from Abcam (Cambridge, UK). HCV core antibody was from Thermo Scientific (Perbio Science, Lausanne, Switzerland). STAT1 antibody was from Santa-Cruz Biotechnologie (LabForce AG, Nunningen, Switzerland). pS473-Akt, Akt, pS727-STAT1, pY1054/55-Tyk2, pY701-STAT1 and PRMT1 antibodies were from Cell Signalling (BioConcept, Allschwil, Switzerland). H4 and MetR3H4 antibodies were purchased from Abcam. HRP-conjugated secondary antibody (NA931V) for HCV detection was from GE Healthcare (GE Healthcare Europe GmbH, Glattbrugg, Switzerland).

Wild type (pDE3-HA-Cα-WT) and mutated PP2Acα plasmids (pDE3-HA-Cα-D88N and pDE3-HA-Cα-H118N) tagged with single HA coding sequence immediately downstream to the human PP2Acα initiation codon were a gift from Dr. Brian Hemmings. pSIH1-puro-shPP2Ac and pSIH1-puro-shPRMT1 were generated by digestion of the pSIH1-puro-STAT3 shRNA plasmid (Addgene, no. 26596) and insertion of short-hairpin RNA sequence against PP2Acα or PRMT1, respectively. The control plasmid was purchased from Addgene (no. 26597). Lentiviral packaging plasmids were a gift from Prof. Martin Stern. Plasmid Luc-JC1 containing codons 1-846 of J6/CF and 847-3033 of JFH1 was kindly provided by Prof. Ralf Bartenschlager.

Mice

Eight weeks old male C57BL/6 mice were used for the experiment. The animals were bred and maintained in the University Hospital Basel animal facility. They were injected (n = 2) subcutaneously with 1000 IU/mL mIFNα or PBS for 1 h and the liver was collected. All animal experiments were conducted with the approval of the animal care committee of the canton Basel-Stadt (Switzerland).

Cell lines

Huh7, Huh7.5.1, UPP2A-C8 and stable silencing PP2Acα cell lines are described elsewhere [17].

SDS-PAGE, immunoblotting and immunoprecipitation

Cells were lysed in whole-cell lysis buffer containing 50 mM Tris HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10 mM PMSF and 1 mM orthovanadate. Lysates were cleared by centrifugation and quantified by Bradford (Bio-Rad Protein Assay, Bio-Rad Laboratories AG, Reinach, Switzerland). Proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membrane and probed with specific antibodies. Immunoprecipitation was performed as described previously [18]. Densitometry analysis of protein bands was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Electrophoretic mobility shift assay

Nuclear extracts and mobility shift assay (EMSA) were performed as described previously [18].

Lentiviral particle production and transduction

pSIH1-puro-control shRNA, pSIH1-puro-shPP2Ac and pSIH1-puro-shPRMT1 were cotransfected with packaging vectors into HEK293T cells using Lipofectamine 2000 according to the manufacturer's instructions. Supernatants containing lentiviral particles were cleared by centrifugation and stored at −70 °C.

Huh7.5.1 cells were transduced with 1MOI of lentiviral particles for 3 days, and PP2Ac or PRMT1 expression was analysed by immunoblotting and quantitative PCR.

PP2Ac primers were 5′-CCACAGCAAGTCACACATTGG-3′ and 5′-CAGAGCACTTGATCGCCTACAA-3′. *PRMT1* primers were 5′-GACATCCAAAGATTACTACTTTGACTCCTA-3′ and 5′-GCGCACCTCGTCCTTCAG-3′.

Hepatitis C virus particle production in cell culture (HCVcc) and infection of Huh7.5.1 cells

RNA preparation and electroporation were performed as described [24]. Huh7.5.1 cells were infected with 1MOI of

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HCVcc strain JC1 particles for 3 days, and replication was analysed by immunoblotting, qPCR and immunostaining [24]. *HCV* primers were 5′-AGGAGGCCCGCACTGCCATA-3′ and 5′-CTGGCGCGGCAACGTCTGTA-3′.

Analysis of interferon-stimulated gene expression

Total RNA extraction, cDNA synthesis and SYBR-based quantitative PCR were performed as described elsewhere [17]. Primers were designed across exon–exon sequences to avoid genomic DNA amplification. *STAT1* primers were 5′-TCCCCAGGCCCTTGTTG-3′ and 5′-CAAGCTGCTGAAGT TCGTACC-3′. *IP10* primers were 5′-CGATTCTGATTTGC TGCCTTATC-3′ and 5′-GCAGGTACAGCGTACGGTTCT-3′. *GBP1* primers were 5′- TGAACAAGCTGGCTGGAAAGA-3′ and 5′-ACATCCAGATTCCTTTAGTGTGAGACT-3′. *SOCS1* primers were 5′- CCCCTTCTGTAGGATGGTAGCA-3′ and 5′-TGCTGTGGAGACTGCATTGTC-3′. *GAPDH* primers were 5′- GCTCCTCCTGTTCGACAGTCA-3′ and 5′- ACCTTCCCCA TGGTGTCTGA-3′.

Cell proliferation assays

Viable cell quantification was performed using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega AG, Düdendorf, Switzerland), according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Continuous data are expressed as the mean ± standard error of the mean (SEM) and were analysed using a Fisher's *t*-test. A two-tailed *P* value < 0.05 was considered statistically significant.

RESULTS

Alteration of PP2Ac expression modulates STAT1 tyrosine phosphorylation

We have previously reported that PP2Ac is up-regulated in patients with CHC and impairs IFNα-mediated Jak-STAT signalling through the alteration of PRMT1-induced STAT1 arginine methylation [18]. We now show that in the mouse liver, PP2Ac is bound to STAT1 upon IFNα stimulation suggesting a potential role of PP2A in the direct regulation of STAT1 activation (Fig. 1a). PP2A is a major serine/threonine phosphatase, however, its autodephosphorylation capacity on tyrosine residues suggests that it could be a tyrosine phosphatase under particular conditions [25]. We therefore investigated the effect of PP2Ac over-expression on STAT1 phosphorylation using UPP2A-C8 cells that allow the inducible over-expression of PP2Ac (Fig. 1b). We first performed a dose-response experiment. Control (Tet+) and PP2Ac over-expressing (Tet-) cells were exposed to increasing doses of IFNα and pY-STAT1 signal was analysed by immunoblotting. Figure 1c shows enhanced STAT1 phosphorylation that reached the maximal level with 500 IU/mL of IFNα in control and PP2Ac over-expressing cells (lanes 5 *vs* 11 and 6 *vs* 12). We did not observe any difference of the pY-STAT1 signal between control and PP2Ac over-expressing cells at 500 and 1000 IU/mL IFNα. However, we noticed a reduction in pY-STAT1 signal in PP2Ac over-expressing cells treated with 50 and 100 IU/mL IFNα compared with control cells (Fig. 1c; lanes 3 *vs* 9 and 4 *vs* 10). Thus, to investigate further the effect of PP2Ac on STAT1 phosphorylation, we decided to perform further experiments using 100 UI/mL of IFNα.

Next, we studied the effect of PP2Ac over-expression on IFNα-induced *STAT1* and *IP10* expression. Our results show a significant diminution of *STAT1* and *IP10* expression in PP2Ac over-expressing cells (Fig. 1d).

We have previously reported that PP2Ac negatively modulates the IFNα-induced ISG expression by reducing the methylation on STAT1 via the inhibition of PRMT1 activity [18]. Therefore, to distinguish the effect of PP2Ac over-expression on STAT1 phosphorylation from its inhibitory effect on PRMT1, we performed experiments knocking down PRMT1. UPP2A-C8 were silenced for PRMT1 using a lentiviral expression system (Fig. 1e) and cultured in the presence or absence of tetracycline to induce PP2Ac over-expression. The functional effect of shPRMT1 knockdown was evaluated by analysis of methylation on histone H4 at arginine 3, a known substrate for PRMT1 [26]. Figure 1e shows that PRMT1 silencing leads to a decrease in methylation on H4. The cells were then stimulated with 100 IU/mL of IFNα, and the ISG expression was quantified by RT-qPCR. Figure 1f shows a significant diminution of *STAT1* and *IP10* expression upon IFNα stimulation in PP2Ac over-expressing cells in the absence of PRMT1 demonstrating that the reduction in ISG expression in PP2Ac over-expressing cells was caused by the inhibitory effect of PP2Ac on STAT1 phosphorylation.

We further validated these results on a cell line with a stable silencing of PP2Ac. The decrease in PP2Ac expression level was verified by immunoblotting (Fig. 2a). We then analysed STAT1 tyrosine and serine phosphorylation upon IFNα stimulation. The reduction in PP2Ac expression enhanced STAT1 phosphorylation on serine and tyrosine residues in response to IFNα stimulation (Fig. 2b; lanes 2, 3, 4 *vs* 6, 7, 8, respectively). The increased STAT1 activation in PP2Ac-silenced cells resulted in a stronger STAT1-probe binding shown by EMSA (Fig. 2c; lanes 2, 3, 4 *vs* 6, 7, 8 respectively). Consequently, we observed a significant enhanced expression of the ISGs in PP2Ac-silenced cells upon IFNα stimulation (Fig. 2d).

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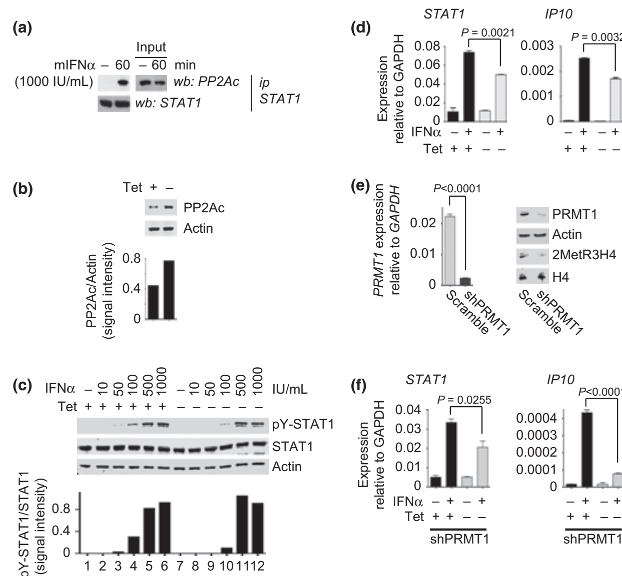

Fig. 1 PP2Ac over-expression impairs IFNα-mediated STAT1 activation and ISGs expression. (a) C57BL/6 mice ($n = 2$) were injected subcutaneously with 1000 IU of IFNα per g of body weight for 1 h and 500 μg of total protein from mouse liver homogenate were used to immunoprecipitate STAT1. The PP2Ac signal was detected by immunoblotting. The experiment was performed in duplicate. A representative blot is shown. (b) UPP2A-C8 cells were cultured in the presence or absence of tetracycline for 24 h and the expression level of PP2Ac analysed by immunoblotting. (c) PP2Ac over-expression was achieved by removing tetracycline from the culture medium for 24 h prior to stimulation with increasing concentrations of IFNα for 15 min. pY-STAT1 and STAT1 signals were visualized by immunoblotting and then quantified using ImageJ. Shown is a representative result from 2 independent experiments. (d) Total RNA from cells unstimulated or stimulated with 100U/mL IFNα for 4 h were extracted and cDNA prepared. *STAT1* and *IP10* expression levels were measured by qPCR. Results ($n = 3$) are expressed as means ± SEM. The statistical significance was assessed using Fisher's exact *t*-test. Shown are the representative results from 3 independent experiments. (e) PRMT1 was silenced in UPP2A-C8 cells using a lentiviral system. PRMT1 expression was then analysed by quantitative RT-PCR and by immunoblotting. (f) UPP2A-C8 cells were silenced for PRMT1 and then cultured in the presence or absence of tetracycline for 24 h. Cells were stimulated with 100 IU/mL IFNα for 4 h and the expression of *STAT1* and *IP10* was measured by qPCR. Results ($n = 3$) are expressed as means ± SEM. The statistical significance was assessed using Fisher's exact *t*-test.

PP2Ac does not modulate the dephosphorylation rate of STAT1 on tyrosine residues

Next, we analysed whether the enhanced STAT1 tyrosine phosphorylation observed in PP2Ac silenced cells is caused by a slower dephosphorylation due to the absence of PP2Ac. We exposed PP2Ac silenced and control cells for different durations to IFNα and quantified the pY-STAT1 signal. It is well known that the IFNα signalling becomes refractory after an initial activation inhibiting re-activation of STAT1 by IFNα [27]. Indeed, cells exposed to IFNα rapidly showed strong STAT1 phosphorylation followed by a gradual decrease in pY-STAT1 until complete disappearance of the signal at 8-h postexposure due to a refractory period (Fig. S1). Interestingly, the time-course analysis of pY-STAT1 after IFNα stimulation revealed a comparable decrease rate of the pY-STAT1 signal intensity between control and PP2Ac-silenced cells (Fig. S1; lanes 1, 2, 3 *vs* 7, 8, 9, respectively) suggesting that the dephosphorylation process of tyrosine residues was not affected by PP2Ac silencing. Therefore, the enhanced pY-STAT1 signal observed in PP2Ac-silenced cells upon IFNα stimulation (Fig. S1; lane 1 *vs* 7) was presumably caused by increased phosphorylation of STAT1.

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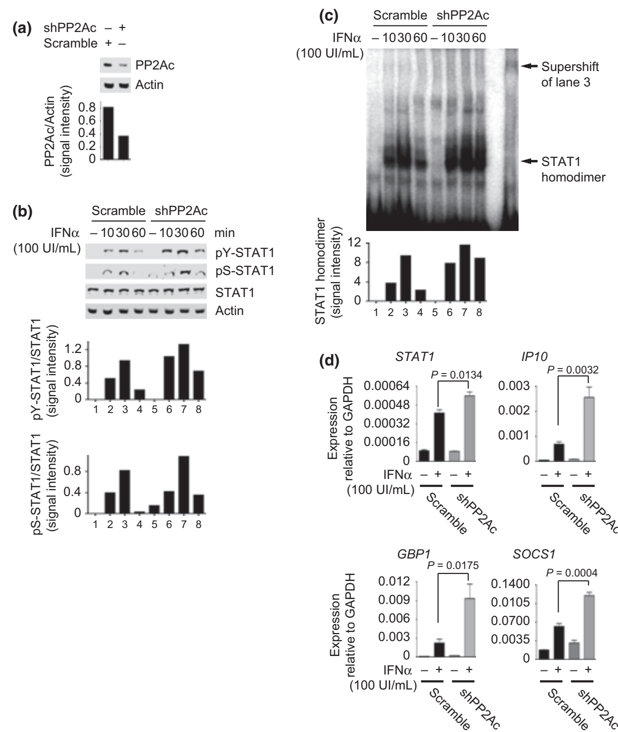

Fig. 2 PP2Ac silencing enhances pY-STAT1 signals, DNA binding and ISGs expression. (a) PP2Ac silencing was obtained by stable transfection of Huh7 with scrambled or shPP2Ac plasmids. The expression of PP2Ac was analysed by immunoblotting. (b) A kinetic of stimulation with 100U/mL IFNα was performed. pY-STAT1 and pS-STAT1 signals were visualized by immunoblotting and then quantified using ImageJ. Results are representative of 3 independent experiments. (c) Nuclear extracts from scramble and shPP2Ac were prepared, and STAT1-DNA binding was visualized by EMSA using GAS probe. Cells were treated with 100U/mL IFNα for the indicated time. STAT1 homodimer bands were quantified using ImageJ. Shown is a representative result from 2 independent experiments. (d) *STAT1*, *IP10*, *GBP1* and *SOCS1* expression levels were analysed by qPCR upon stimulation with 100U/mL IFNα for 4 h. Results (n = 3) are expressed as means ± SEM. Shown are representative results from 4 independent experiments.

PP2Ac associates with Jak1/Tyk2/STAT1 in response to IFNα stimulation and modulates Jak1/Tyk2 phosphorylation

We further investigated the mechanism by which PP2Ac controls IFNα-mediated STAT1 phosphorylation by performing a coimmunoprecipitation assay. Our results show that PP2Ac associates with STAT1 upon IFNα stimulation in Huh7 cells (Fig. 3a). This binding was also observed with Jak1 and Tyk2 (Fig. 3b) suggesting that PP2Ac might have a direct regulatory role on Jak1/Tyk2 and STAT1 activation.

As we have observed that PP2Ac associates with Jak1 and Tyk2, we analysed the effect of PP2Ac on Jak1 and Tyk2 activation. Time-course analysis of IFNα-treated cells revealed a stronger phosphorylation of Jak1 and Tyk2 in cells silenced for PP2Ac compared with control cells (Fig. 3c; lane 2 *vs* 6).

PP2A catalytic activity is not required to inhibit Jak and STAT1 tyrosine phosphorylation

Next, we investigated if the catalytic activity of PP2Ac is required to inhibit Jak and STAT1 phosphorylation. We treated Huh7 cells with okadaic acid (OA) to block PP2A activity. The inhibition of the phosphatase activity was confirmed by a strong pS-Akt signal (Fig. 4a, asterisk), a known substrate for PP2A [28]. Our data show that the

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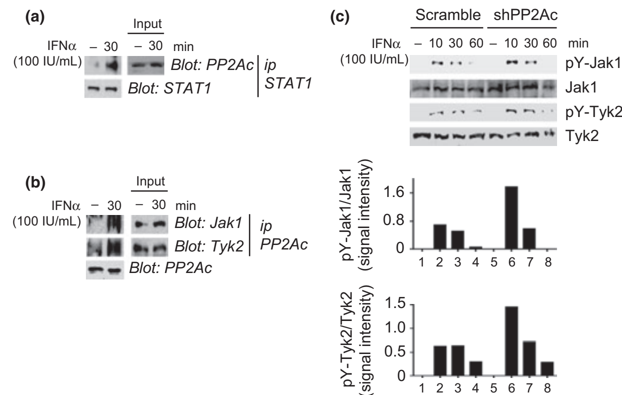

Fig. 3 PP2Ac associates with Jak1/Tyk2/STAT1 upon IFNα stimulation and impairs Jak1/Tyk2 activation. (a) Huh7 cells were stimulated with 100 IU/mL IFNα and STAT1 was immunoprecipitated. The PP2Ac signal was then detected by immunoblotting. Results are representative of 2 independent experiments. (b) Huh7 cells were stimulated with 100 IU/mL IFNα and PP2Ac was immunoprecipitated. Jak1 and Tyk2 signals were detected by immunoblotting. Results are representative of 2 independent experiments. (c) Scrambled and PP2Ac-silenced cells were treated with 100U/mL IFNα for the indicated time and then analysed for pY-Jak1 and pY-Tyk2 signals. Protein bands were quantified using ImageJ. Results are representative of 2 independent experiments.

inhibition of PP2A activity by OA does not alter Jak1/Tyk2/STAT1 phosphorylation (Fig. 4a; lane 2 *vs* 3).

Because OA can affect multiple phosphorylation events simultaneously, we confirmed our observations by transfecting Huh7 cells with two PP2Ac mutant plasmids (D88N and H118N) that abolish PP2A catalytic activity [29] (Fig. 4b) and analysed pY-STAT1 upon IFNα stimulation. The expression level of mutant PP2Ac was analysed by immunoblotting (Fig. 4b,c). We found that both constructs significantly reduced tyrosine phosphorylation of STAT1 demonstrating that the phosphatase activity is not required for the inhibition of IFNα-induced pY-STAT1 signal (Fig. 4c).

PP2Ac modulates IFNα-mediated antiviral activity against HCV

Our finding that PP2A negatively modulates the pY-STAT1 strength suggests an effect of PP2A on IFNα-mediated antiviral activity. We therefore performed a functional assay using an HCV fully infectious system. We silenced PP2Ac in Huh7.5.1 cells prior infection with HCVcc and studied the antiviral effect of IFNα. Silencing of PP2Ac for 4 days did not significantly affect cell proliferation (Fig. S2). However, we observed a significant impairment of HCVcc replication (Fig. 5a–c). To investigate if the catalytic activity of PP2A is involved in the modulation of HCV replication, we treated cells with OA and infected them with HCVcc. We found that the core protein signal is weaker in OA-treated cells (Fig. S3; lane 3 *vs* 4) suggesting that HCV replication is dependent on the phosphatase activity. We confirmed our results by infecting Huh7.5.1 cells transfected with wild type (WT) or mutant (H118N) PP2Ac. Ectopic expression of WT-PP2Ac significantly increased core expression after 3 days of infection (Fig. 5d; lane 3 *vs* 2). As expected, HCV replication in H118N–PP2Ac-transfected cells was similar to mock-transfected cells (Fig. 5d; lane 4 *vs* 5) demonstrating that the phosphatase activity supports HCVcc replication.

Next, we wanted to study the effect of PP2Ac on IFNα-mediated antiviral activity. Because PP2Ac catalytic activity positively modulates HCVcc replication, we performed the experiment using a PP2Ac mutant (H118N) that allows increased expression of PP2Ac without up-regulating the phosphatase activity. Huh7.5.1 cells transfected with H118N-PP2Ac or mock plasmid were infected with HCVcc and then treated with IFNα. Our results showed that the expression of the mutant impairs the IFNα-induced antiviral response (Fig. 5e,f; lane 4 *vs* 2). Because the inhibition of pY-STAT1 by PP2Ac is independent of the catalytic activity and the effect of PP2Ac on HCV replication requires the phosphatase activity to reduce NS3 methylation [30], we can conclude that the effect observed with the H118N mutant results mainly from the inhibition of STAT1 phosphorylation by PP2Ac.

DISCUSSION

The rapid production of IFN is a crucial step to limit viral propagation. Therefore, to establish a persistent infection

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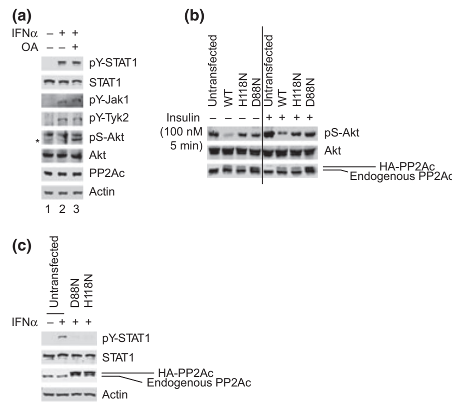

Fig. 4 The phosphatase activity of PP2A is not required to inhibit the IFNα-mediated STAT1 tyrosine phosphorylation. (a) Huh7 cells were exposed to 100 nM Okadaic acid for 3 h prior to been treated with 100U/mL IFNα for 30 min. Indicated proteins were detected by Western blotting. Asterisk shows pS-Akt specific band. Shown is a representative result from 3 independent experiments. (b) Huh7 cells were transiently transfected for 24 h with plasmid for HA-PP2Ac WT, H118N or D88N. Cells were then stimulated with PBS or 100 nM insulin for 5 min and pS-Akt signal was monitored. (c) Huh7 cells were transiently transfected for 24 h with plasmid for HA-PP2Ac D88N, or H118N. Cells were stimulated with 100U/mL IFNα for 1 h and the pY-STAT1 signal was visualized. Shown is a representative result from 3 independent experiments.

viruses have to develop a strategy to counteract this first line of defence of the host cell. We have reported that HCV infection leads to an activation of the ER stress response in the host cell resulting in an up-regulation of PP2Ac [20]. Several viruses are known to alter the expression of PP2A. Indeed, we have previously demonstrated that HBV also induces an up-regulation of the PP2A catalytic subunit [19]. Furthermore, it has been reported that PP1 and PP2A expression are increased during cytomegalovirus infection demonstrating that the deregulation of PP2Ac expression is not restricted to hepatotropic viruses [31]. PP2Ac associates with PRMT1 and inhibits the methyltransferase activity in a PP2A catalytic activity dependent manner, resulting in a reduction in methylation levels of STAT1 and of the NS3 helicase. Hypomethylated STAT1 associates to Pias1 preventing the binding of activated STAT1 to the promoter of target genes [18]. Hypomethylated NS3 has increased helicase activity favouring HCV replication [30]. In the present study, we report an interesting observation that PP2Ac associates with Jak1/Tyk2/STAT1 upon IFNα stimulation and impairs phosphorylation of tyrosine residues independently of the catalytic activity. Together with our previously published results, these data suggest that the over-expression of PP2Ac in the context of viral infection modulates the IFNα-induced Jak-STAT signalling pathway at two distinct levels. We believe that this particularity confers an important role to PP2Ac in the establishment of chronic infection. Indeed, STAT1 is continuously methylated by PRMT1 in the cell [32]. Presently, only two enzymes are described, based on histone work, to remove methyl groups from arginine residues. The peptidylarginine deiminase 4 is known to convert the methyl group to citrulline [33] and the Jumonji domain-containing proteins are identified to regenerate arginine residues from methylated histones [34]. It is unknown whether methylated arginine residues on STAT1 could be removed via these mechanisms. Therefore, we hypothesize that the only way to reverse the methylation effect on STAT1 would be to degrade the protein. Thus, the negative regulation of PP2Ac on STAT1 transcriptional activity via PRMT1 is effective only on newly transcribed STAT1. STAT1 protein is stable and has a half life of over 24 h [35]; therefore, the inhibitory effect of PP2Ac on STAT1 phosphorylation would allow the virus to immediately block the early host antiviral response induced by IFNα.

Previously, it was reported that okadaic acid, a specific inhibitor of PP2A, induces pS727-STAT3 in human antigen-specific CD4+ T-cell lines and cutaneous T-cell lymphoma lines [36]. Furthermore, PP2Ac has been shown to physically associate with a macromolecular protein complex composed by mTOR, STAT1 and Tap42 suggesting a potential regulatory effect of PP2Ac on STAT1 phosphorylation [37]. We show in this study that PP2Ac associates with Jak1/Tyk2/STAT1 and impairs their activation. However, the mechanism used by PP2A to reduce Jak1/Tyk2/STAT1 phosphorylation remains unclear. It might be through a physical interaction of PP2Ac to Jak1/Tyk2/STAT1 disrupting the phosphorylation events. Additionally, PP2A could also be a tyrosine phosphatase under particular conditions [25]. Indeed, the catalytic subunit can be phosphorylated on tyrosine 307 leading to the inactivation of the enzyme [22]. This phosphatase can be re-activated through autodephosphorylation suggesting a tyrosine phosphatase activity [25]. However, our findings that PP2Ac silencing did not affect the STAT1 dephosphorylation rate suggest that the inhibitory effect of PP2Ac on STAT1 phosphorylation is not caused by a tyrosine phosphatase activity of PP2A. The PP2A catalytic subunit is phosphorylated on tyrosine residues by epidermal growth factor (EGF), insulin or p60v-src leading to the inactivation of the phosphatase [22,38]. Furthermore, we have observed that IFNα induces PP2Ac phosphorylation (data not shown) suggesting an inhibition of PP2Ac activity by IFNα. Therefore, a blockade of STAT1 activation in a PP2Ac catalytic activity independent manner would be

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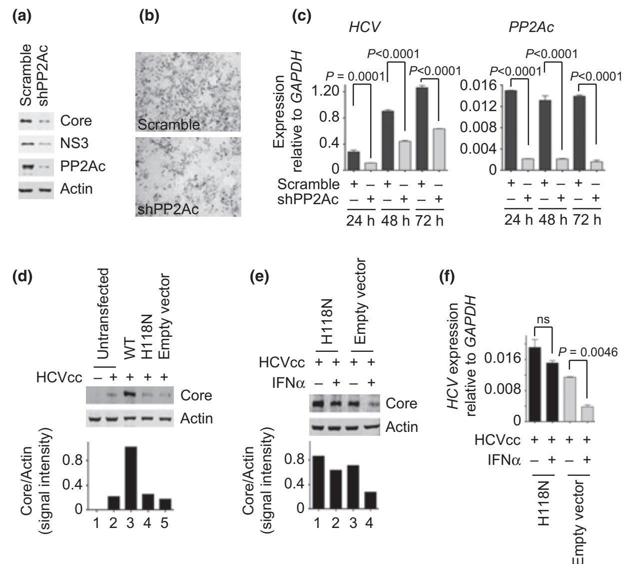

Fig. 5 PP2A negatively modulates the IFNα-mediated HCV antiviral activity. (a) PP2Ac was silenced in Huh7.5.1 cells using short-hairpin technology and then infected with JC1 HCVcc particles. HCV core and NS3 proteins were analysed by immunoblotting. (b) Immunostaining of HCV core after 3 days of infection with 1MOI HCVcc. (c) Analysis of HCV and PP2Ac expression in PP2Ac silenced cells by qPCR (n = 3). Shown is a representative result from 2 independent experiments. (d) Huh7.5.1 cells were transfected with wild type (WT) or the mutant H118N PP2A plasmid for 10 h prior to being infected with HCVcc. HCV core was visualized by immunoblotting. Shown is a representative result from 3 independent experiments. (e) Huh7.5.1 cells were transfected with H118N-PP2Ac or mock plasmids and then treated with 100U/mL IFNα for 24 h. HCV core was visualized by immunoblotting. Shown is a representative result from 2 independent experiments. (f) Analysis of HCV expression 24 h after IFNα treatment by qPCR in Huh7.5.1 cells transfected with H118N-PP2Ac or mock plasmids (n = 2). A representative result from 2 independent experiments is shown in this figure.

beneficial for a virus to immediately and efficiently counteract the IFNα antiviral response.

Heretofore, it is unclear whether PP2Ac exists as a free subunit in the cell. Considering that the C subunit forms a dimer with the A subunit [39], we have silenced PP2Aa and investigated the effect of PP2Aa knockdown on IFNα-induced pY-STAT1 signals. We did not observe any alteration of the pY-STAT1 signal in PP2Aa silenced cells (data not shown) suggesting that the A subunit does not participate in the inhibitory effect of the C subunit on STAT1 phosphorylation.

During viral infection, the protein kinase R (PKR) and the eukaryotic translation initiation factor 2 (eIF2) are phosphorylated on threonine and serine residues, respectively, leading to an inhibition of *de novo* protein synthesis [40]. This reduction in translation initiation limits the synthesis of antiviral proteins but also perturbs the virus life cycle [41,42]. Therefore, it is important that the virus develops a strategy to block the host antiviral response without impairing its replication. Our data demonstrate that PP2A could be that key player. Indeed, by inhibiting the Jak-STAT signalling pathway through PP2Ac up-regulation, HCV could counteract the early immune response. Additionally, PP2Ac over-expression blocks PRMT1 activity leading to hypomethylation of STAT1 and NS3 helicase. The reduction in methylation of the NS3 helicase enhances the unwinding activity of the helicase favouring viral replication [30], whereas the hypomethylation of STAT1 inhibits the late IFNα-induced antiviral response [18]. Our data provide evidence that the protein expression level and the catalytic activity of PP2A inhibits the interferon α signalling pathway and favours viral replication, suggesting that PP2A could be a key player in the establishment of a chronic infection (Fig. S4).

PP2A is highly expressed in the cell and is frequently targeted by viral proteins to subvert selectively important cellular pathways leading to enhanced viral replication and tumour formation. For instance, PP2A targeting by

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the MCV small T-Antigen is required for the accomplishment of the virus life cycle [43]. Furthermore, it has been reported that the small t antigens and the polyomavirus middle T proteins can associate with the PP2A core and induce cell transformation [44]. These observations identify PP2A as a potential key target for therapeutic developments. Presently, two natural antitumour molecules, cantharidin and fostriecin that specifically bind and inhibit PP2A have been tested for hepatoma and oesophageal carcinomas [45,46]. Although these compounds are potent antitumour molecules, their use has severe side effects probably due to the inhibition of PP2A that is the major phosphatase in the cell. Therefore, a precise mechanistic analysis of PP2A will permit the development of better drugs improving the benefit to side effects ratio.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. PP2Ac does not alter the rate of pY-STAT1 dephosphorylation.

Fig. S2. PP2Ac silencing does not modulate cell proliferation.

Fig. S3. Inhibition of PP2A catalytic activity alters HCVcc replication.

Fig. S4. PP2Ac up-regulation favours the establishment of a chronic infection.

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Figure S1

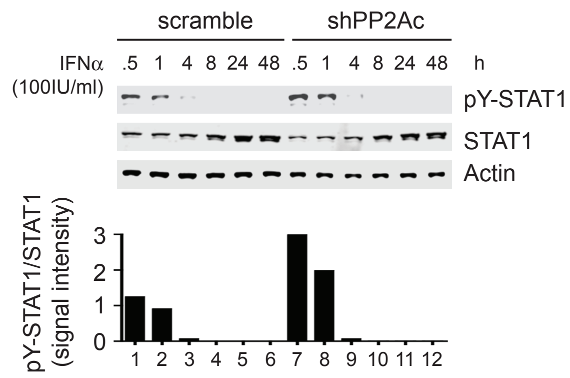


Figure S2

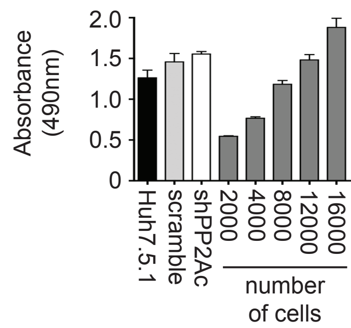


Figure S3

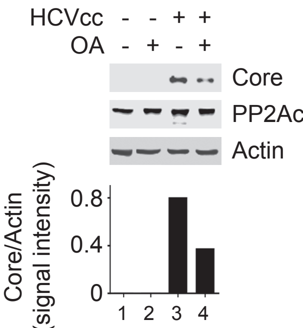
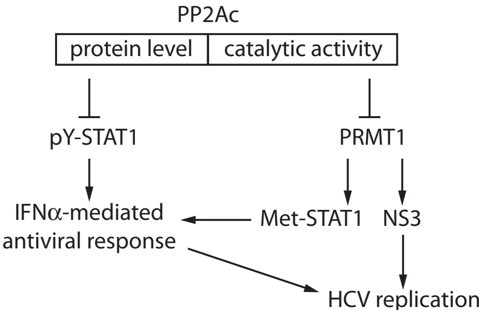


Figure S4



4.2 The role of PP2A-A and -B subunits in the regulation of the Jak-STAT signaling pathway.

Because PP2A is a multimeric holoenzyme and the substrates recognition is determined through the association to specific regulatory B subunits, we have tried to identify which B subunit could potentially be an important component in PP2Ac-mediated inhibition of the IFN-α induced Jak-STAT signaling. For that purpose, we have serially silenced several B subunits using short-hairpin technology (Figure 1).

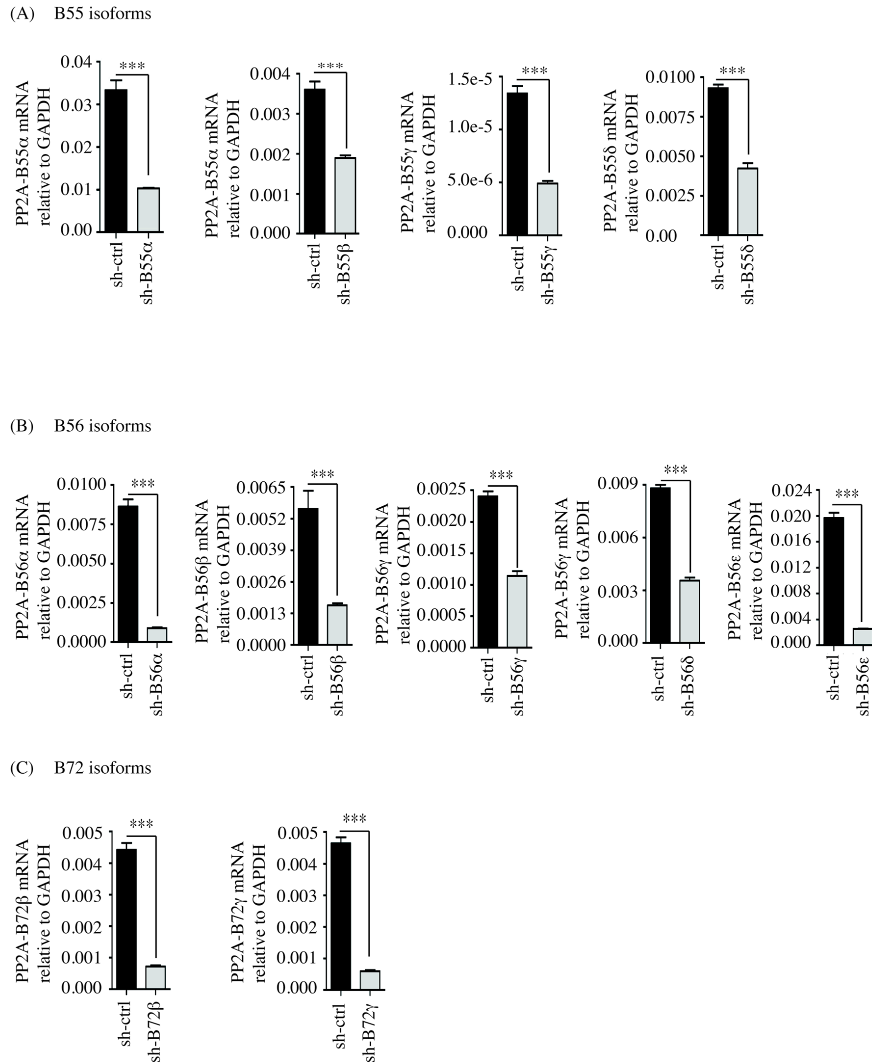


Figure 1: Silencing of various PP2A-B isoforms. Silencing of PP2A-B55 or B isoforms (A), B56 or B` isoforms (B) and B72 or B`` isoforms. Huh7 cells were stably transfected with sh-scramble and sh-RNA against particular isoforms as indicated. The mRNA expression of silenced isoforms (grey color bar) against sh-scramble (black color bar) is shown. Data are representative of at least 2 independent experiments.

4.2.1 Effect of silencing of a specific PP2A subunit on the expression of the other subunits

The phosphatase activity is mainly provided by the α isoform of the catalytic C subunit in specific association with B subunits. Therefore, it is likely that alteration of a particular B subunit could modify the proportion of holoenzyme complexes and localization into subcellular compartments. This could affect the various cellular processes including genes transcription, RNA/protein stability. Therefore, in order to identify which specific PP2A-B subunits regulate INF-α signaling and HCV replication we serially silenced various isoforms of PP2A subunits. Interestingly, qRT-PCR results show that silencing of a particular isoform of B subunit leads to a down-regulation of majority of the other isoforms. Additionally, this impairment is not only restricted to A, B or C subunits but also deregulates the expression of other regulatory proteins such as α4. As shown in figure 2, we observed a down-regulation of PP2A-Aα in cells silenced for PP2A-cα, B55α, and B56β. We also observed that PP2A-Aα expression was up-regulated in PP2A-B56α silenced cells (figure 2A). Furthermore, PP2A-B55α expression was unchanged in PP2A-Aα silenced cells though we observed significant down-regulation of PP2A-B55α in PP2A-B56α/β cells (figure 2B). Interestingly, we found that silencing of PP2A-B55α resulted in a decrease in PP2A-Aα expression (figure 2A) whereas silencing of PP2A-Aα did not impair PP2A-B55α expression (figure 2B). Our observation revealed that, silencing of PP2A-B55α and B56β down-regulates the expression of PP2A-B56α (figure 2C). Interestingly, silencing of PP2A-B56α led to an increased expression of PP2-Aα (figure

2A) and vice-versa (figure 2C). Finally, silencing of PP2A-Aα, B55α, B56α, and B56β resulted in a decreased α4 expression (figure 2d).

Additionally, silencing of the α isoform of the catalytic subunit attenuated the mRNA expression of various isoforms of PP2A including B56α-ε and B72α-γ (figure 3). This reduction is ranging from slight to moderate. These observations are summarized in table 2. Earlier, Sablina and colleagues have reported that silencing of PP2Acα leads to a significant reduction of the basal level of B55α, B56β, B56γ, PR93, and PP2A-A subunits whereas silencing of PP2A-Cβ exerts marginal effect on the expression of the other subunits (Sablina et al., 2010). Furthermore, it has been reported that an association of the PP2A-A with Cα subunit is required for their stability (Silverstein et al., 2002; Chen et al., 2005b). Our observations are in the line with previous observations and show inter-regulatory mechanisms governing the expression of various PP2A subunits. Such regulatory mechanism could be obtained through miRNAs that mediate mRNA degradation or/and activation/inhibition of various transcription factors. Because of the lack of commercially available antibodies against all the B subunits, we were not able to analyze the effect of silencing at protein level.

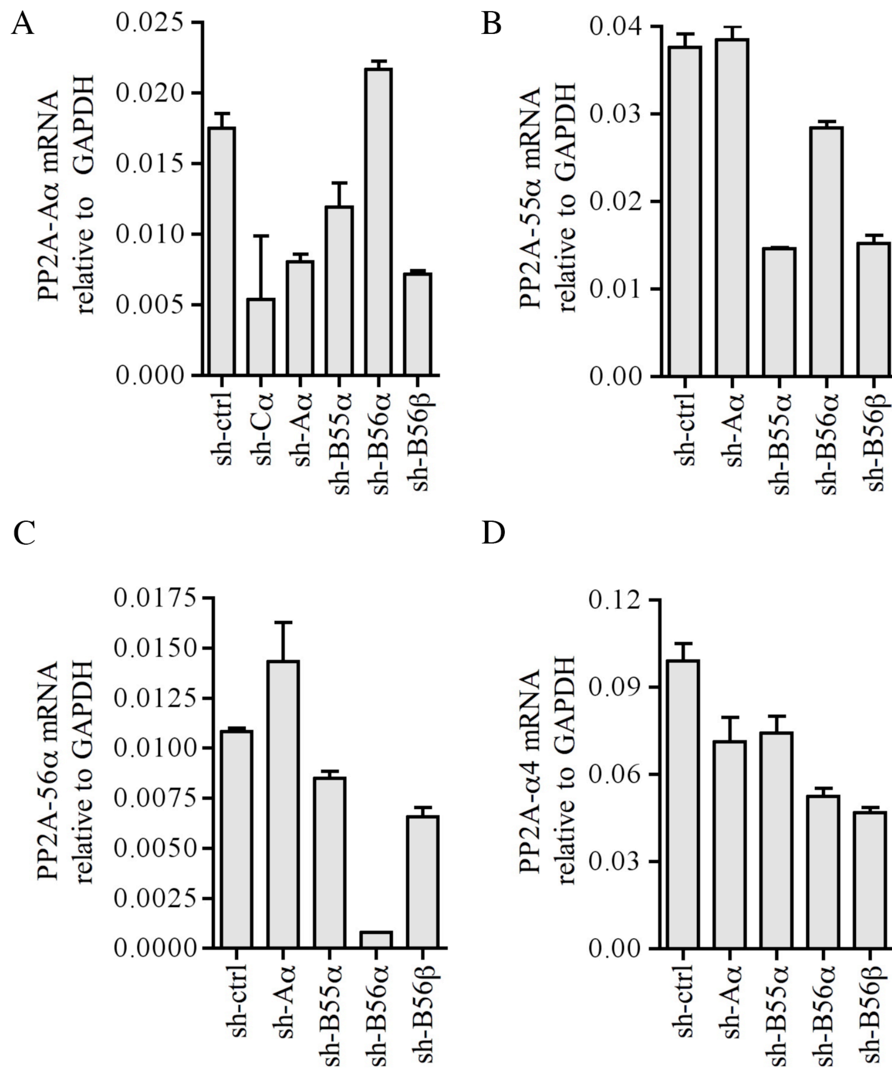


Figure 2: Inter-regulation of different isoforms of PP2A. The X-axis represents the stably silenced cells while the Y-axis represents mRNA expression from indicated PP2A subunit coding genes and α4 gene.

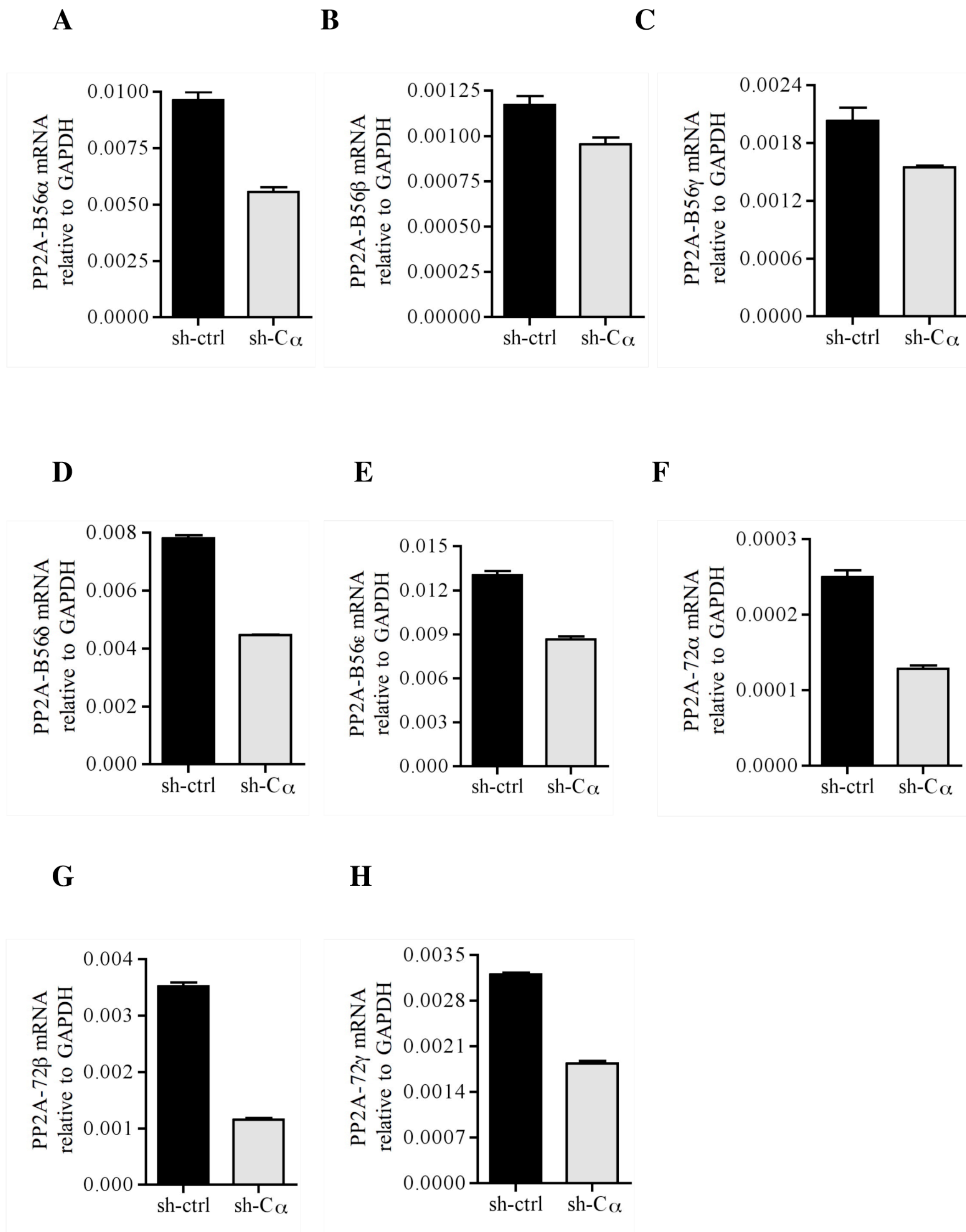


Figure 3: mRNA level of various isoforms of PP2A in PP2A-Cα silenced cells.

A

Subunits	Silencing	mRNA expression of other isoforms/subunits
PP2A–A	PP2A-Aα	→ PP2A-B55α ↑ PP2A-B56α ↓ PP2A-B56β ↓ PP2A-Cα
PP2A–B	PP2A-B55α	↓ PP2A-Aα ↓ PP2A-B56α ↓ PP2A-B56β ↓ PP2A-Cα
	PP2A-B56α	↑ PP2A-Aα ↓ PP2A-B55α ↓ PP2A-B56β ↓ PP2A-Cα
	PP2A-B56β	↓ PP2A-Aα ↓ PP2A-B55α ↓ PP2A-B56α ↓ PP2A-Cα
PP2A–C	PP2A-Cα	↓ PP2A-Aα ↓ PP2A-B55α ↓ PP2A-B55β ↓ PP2A-B55γ ↓ PP2A-B55δ ↓ PP2A-B56α ↓ PP2A-B56β ↓ PP2A-B56γ ↓ PP2A-B56δ ↓ PP2A-B56ε ↓ PP2A-B72α ↓ PP2A-B72β ↓ PP2A-B72γ

Legends: → = no change; ↓ = decreased expression; ↑ = increased expression

B

Subunits	Silencing	mRNA expression of α-4
PP2A–A	PP2A-Aα	Decreased
PP2A–B	PP2A-B55α	Decreased
	PP2A-B56α	Decreased
	PP2A-B56β	Decreased

Table 2: Summary of the effect of silencing of one subunit on other

4.2.2 **Predication of miRNA targeting PP2A subunits**

One potential mechanism to explain the inter-regulation of the subunits expression could be through miRNAs. We, therefore, performed a miRNA targeting PP2A subunits analysis using online bioinformatics softwares such as miRBase (http://www.mirbase.org/index.shtml) or miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/). Using this approach potential miRNAs was predicted against each subunit. We found that many subunits have multiple miRNA target sites. Interestingly, our miRNA and target genes data analysis revealed that miRNA-19 targets 4 PP2A isoforms including B56α, B56ε, Aβ, and STRN3. Furthermore, we found that miRNA-34b targets Aβ and STRN isoforms. The role of various miRNA including miRNA-19, miRNA-1, and miRNA-34b involved in expression of PP2A subunits have been previously described (Terentyev et al., 2009; Mavrakis et al., 2010; Chen et al., 2011).

5. Discussion

PP2A is known to regulate several signaling pathways via dephosphorylation of serine/threonine residues (Eichhorn et al., 2009). It has been shown that inhibition of PP2A increases serine phosphorylation on STAT6 and STAT3 (Woetmann et al., 1999, 2003) and also increases IFN-γ mediated serine and tyrosine phosphorylation of STAT1 in mTOR dependent fashion (Fielhaber et al., 2009). Recently, it has been reported that PP2A inhibits the Jak-STAT1 signaling by forming a macromolecular complex with mTOR, STAT1 and α4 and (Fielhaber et al., 2009). Moreover, it has been also shown that PP2A associates to Jak2 in myeloid progenitor cells and modulates the Jak2-STAT5 signaling pathway in response to IL-3 (Yokoyama et al., 2003). Presently, it is not clear how PP2Ac associates to Jak1 and Tyk2 and inhibits their activation. One could hypothesize that PP2Ac-Jak1/Tyk2 binding shares similar molecular mechanism like PP2Ac-Jak2 association (Yokoyama et al., 2003).

Production of IFNs by cells in response to viral infection leads to initiation of antiviral activity. For the establishment of persistent infection, viruses develop strategies to counteract this first line of defense. We have reported that HCV proteins inhibit the IFN-α signaling (Heim et al., 1999; Blindenbacher et al., 2003). The inhibition is mediated through an up-regulation of PP2Ac that leads to a hypomethylation of STAT1 (Duong et al., 2004). Hypomethylated STAT1 associates with PIAS1 and prevents activated STAT1 binding to promoter elements of the ISGs (Mowen et al., 2001; Liu et al., 2004).

In this work, we demonstrated that the phosphatase activity of PP2A is not required for the inhibition of IFN-α induced Jak-STAT signaling. However, it is required to promote HCVcc replication. Indeed, using different approaches to reduce PP2A catalytic activity (pharmacological inhibitors, catalytic dead or dominant negative mutants, siRNA against PP2Ac), we have observed a reduction of HCV replication. The mechanism underlying regulation of viral life cycle by PP2A remains unclear. Three hypothesis could be proposed: (1) PP2A reduces the methylation of NS3 helicase by inhibiting PRMT1 leading to an enhanced unwinding activity and therefore viral replication (Duong et al., 2005); (2) PP2A dephosphorylates NS5A and induces viral replication (Evans et al., 2004); (3) PP2A dephosphorylates eIF2α (Groskreutz et al., 2010) and releases the inhibitory effect of eIF2α on protein translation thus favoring HCV replication. Further experiments are needed to clarify this issue.

We have made an interesting observation that tyrosine phosphorylation of Jak1/Tyk2 and STAT1 is independent of PP2A phosphatase activity while it is needed to positively modulate HCV replication. This phenomenon can be explained by the complexity of substrate selection by PP2A. Indeed, depending on the B subunit that associates to the A-C core complex, PP2A holoenzyme could preferentially target the IFN-α signaling pathway or the HCV replication process. We have made an attempt to identify these B subunits using short-hairpin siRNA technology. Unfortunately, we found that silencing of a specific B subunit alters the expression of the C, A, or B subunits showing the difficulties to

identify the B subunits that are required to direct PP2A towards IFN-α signaling inhibition or towards HCV replication.

The mechanism of subunits inter-regulation is not known. One possibility is that down-regulation of a specific subunit alters the expression of some so far undiscovered miRNAs that then mediate RNA degradation of the other subunits. We have performed a PP2A and miRNA target gene analysis and observed that several subunits can be targeted by the same miRNA. Indeed, we found that miRNA-19 targets PP2A-B56ε subunits therefore one can hypothesize that silencing of one specific subunit increases the proportion of miRNA-19 which then down-regulates the expression of the other subunits. Further experimentations are needed to clarify the mechanism underlying the inter-regulation of the expression of PP2A subunits. Another possible mechanism of inter-regulation of PP2A subunits could be via the regulation of transcription through epigenetic changes. Indeed, we have previously reported that PP2Ac can modify the post-translational modification status on histones such as methylation/acetylation/phosphorylation (Duong et al., 2010). There are several reports showing that modifications on histone can alter gene transcription by modifying the chromatin structure. These chromatin structure rearrangements lead to open/close chromatin status throughout the nucleosome facilitating/inhibiting the accessibility of transcription factors to the promoters (Berger, 2010). Therefore, the alteration of the expression of a specific PP2A subunit can change the modification status on histones and thus induces a change in the chromatin structure leading to an impairment of the expression of the other subunits.

6. Summary

The first part of the work demonstrate the role of PP2A in IFN-α induced Jak-STAT1 signaling and HCV replication. We show here that PP2Ac activity is not required for IFN-α induced tyrosine phosphorylation of Jak1/Tyk2 and STAT1. In response to IFN-α induction, PP2Ac associates with Jak1/Tyk2 and STAT1. This association modulates the Jak1/Tyk2 and STAT1 tyrosine phosphorylation. However, this study shows that PP2A activity is required for the HCV replication.

The selective behavior of PP2A for Jak-STAT1 signaling pathway inhibition and promotion of HCV replication could be due to the targeted substrate selection by PP2A holoenzyme complex. In the second part of this work, attempts were made to determine the specific regulatory B subunits involved in IFN-α induced Jak-STAT1 signaling inhibition and HCV replication. We observed the inter-regulatory behavior of PP2A subunits. During the course of this study, due to unavailability of specific antibodies for various isoforms of B subunit, the aim to determine specific holoenzyme complex involved in regulation of Jak-STAT1 signaling and HCV replication was not achieved.

Further studies are required to investigate the specific B subunits and thereby holoenzyme complex responsible for IFN-α induced Jak-STAT signaling inhibition and HCV replication by PP2A.

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Current Position

Postdoctoral fellow at ETH, Zurich / DBSSE, Basel, Nov 2012 – Sept 2014.
Working on High throughput cloning, expression & screening of antibodies for the establishement of antibodyone

Higher Education

PhD in Biochemistry, University of Basel, Switzerland, 2012
MSc in Biotechnology, University of Hyderabad, India, 2004
BSc in Zoology (Honors), Veer Kunwar Singh University, India, 2001

Fellwoships

Qualified Junior Research Fellowship, Department of Biotechnology, Govt. Of India, 2004
Scholarship for MSc by Department of Biotechnology, Govt. Of India, 2002-2004

Work Experince

PhD Biochemistry (2008 –2012)

Worked with bacteria, hepatitis C virus (HCV), mammalian cells, and mice.

Generated numerous lentiviral constructs that were used for gene silencing or over-expression by transient or stable transfection.

Established hepatitis C virus (HCV) infectious system (bio safety level 3) and performed HCV infection experiments on cellular level

Actively participated in generation of Alb-Cre and Mx-Cre mouse models for the silencing and over-expression of PP2Ac protein

Predicted and analyzed the micro-RNAs for various PP2A subunits

Studied the role of PP2A on interferon-α induced Jak-STAT signaling and HCV replication

Evaluated small (siRNA) and large (plasmid) molecules embedding & carrying capacity of biocompatible novel peptide beads and analyzed the release of encapsulated molecules into the cells

Project Assistant (2006 –2007)

Cloned and expressed numerous CCR5 and RANTES promoters from human and monkey

Studied polymorphism and functional activities of CCR5 and RANTES promoters

Master's dissertation and project student (2003 –2005)

Studied bio-transformation of aromatic toxic commpound – anilines by photosynthetic bacteria

Extracted and purified the secondary metabolites by organic solvents based column chromatography

Actively played role in characterization of purified compounds by Mass spectroscopy, NMR

Patent applications and Publications

de Bruyn Ouboter D, Schuster T, Shanker V, Meier W. European patent application (EP11172558) - "Peptide Beads"

Shanker V, Trincucci G, Heim HM, and Duong HT, Protein phosphatase 2A impairs IFNα-induced antiviral activity against the hepatitis C virus through the inhibition of STAT1 tyrosine phosphorylation. J Viral Hepat. 2013 Sep;20(9):612-21.

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Participations and Presentations at Scientific meetings

SGG/SSG meeting Interlaken, Switzerland, 20-21, September 2012. Oral

Schauinsland Retreat. May-June 2012, Schauinsland, Germany. Oral

Jak-Stat Signaling: From Basics to Diseases, FEBS-Special Meeting. Feb. 2010, Vienna, Austria. Oral

Hepatology Gastrointestinal research retreat, Jan 2010, Vulpera, Switzerland. Oral

Conference on Human Viruses and Translational medicine, November 2008, National Institute of Immunology, New Delhi, India. Poster

Attended various conference relating Gastroenterology / Hepatology /HCV at Vulpera, Switzerland; Schauinsland, Germany; Strasbourg, France

Attended many science conferences and congress in India druing 2002 to 2006