The role of STAT3 signaling in carcinogenesis

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"Colui che conosce il proprio obiettivo, si sente forte: questa forza lo rende sereno;
questa serenità assicura la pace interiore;
solo la pace interiore consente la riflessione profonda;
la riflessione profonda è il punto di partenza di ogni successo"
(LAO-TSE)

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1. INTRODUCTION

The biology of a cancer cell is largely driven by the pattern of gene expression within that cell. Since a key point of control of gene expression is the activation state of transcription factors, it is likely that a small number of transcription factors may represent a central convergence point in cancer pathogenesis. One such protein is STAT3, a member of a family of seven genes thet encode proteins coupling signaling events at cell surface with gene regulation (Frank D. A., 2007).

The STAT family of cytoplasmic latent transcription factors consists of seven members: STAT-1-4, STAT-5a, STAT-5b, and STAT-6. STAT-3 activation results in expression of genes that control cell proliferation, survival, differentiation, and development. It was first identified in 1994 as a DNA-binding factor that selectively binds to the IL-6-responsive element in the promoter of acute-phase genes from IL6-stimulated hepatocytes. The Stat3 cDNA was cloned in the same year and encodes an open reading frame of 770 amino acids resulting in a protein of 88 kDa (Akira et al., 1994).STAT-3 was also independently identified as a DNA-binding protein in response to epidermal growth factor (Bharat B. Aggarwal et al, 2009).

1.1 Domain structure of STAT-3 protein

STAT proteins are 750–850 aminoacids long and have a common structure of six functionally conserved domains.

The N-terminal domain (NTD) was shown to be important for dimerization of unphosphorylated STATs monomers and for the tetramerization between STAT1, STAT3, STAT4 and STAT5 on promoters with two or more tandem STAT binding sites. Xu and colleagues (Xu et all,

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1996) proposed that these tetramer complexes are able of transcribing selective target genes to which they bind weakly through recognition of non-consensus regions. This domain also plays a role in promoting interactions with other proteins, namely transcriptional co-activators such as CREB binding protein (CBP)/p300. Additionally, it is important for nuclear import and deactivation of STATs involving tyrosine de-phosphorylation (C. Mertens, 2006).

The four α-helices coiled–coil domain (CCD) is known to be involved in interactions with other proteins, such as IFN regulatory protein 9 (IRF-9/p48) with STAT1 (Zhang X., 1999) and c-JUN with STAT3 (Horvath C.M., 1996). STATs coiled–coil domain is also thought to participate in the negative regulation of these proteins. The STAT3 coiled–coil domain has been implicated in the recruitment and binding of STAT3 to the interleukin 6 (IL-6) and epidermal growth factor (EGF) receptors (T. Zhang et all, 2000) and in its nuclear translocation upon treatment with EGF, or IL-6 (Ma J., 2003).

The DNA-binding domain (DBD) is highly conserved. Small differences in the amino acid sequences of this domain across different STAT molecules often determine binding to and transcription of distinct genes by each STAT (Boucheron C., 1998). This domain is also involved in nuclear translocation of activated STAT1 and STAT3 phosphorylated dimers upon cytokine stimulation.

The α-helical linker domain (LD) links the DNA-binding and SH2 domains and is important for the transcriptional activation of STAT1 in response to IFN-γ (Yang E., 1999). It also plays a role in protein–protein interactions, for instance, between STAT3 and gene associated with retinoid-IFN-induced mortality 19 (GRIM19), a negative regulator of transcription

(Lufei C., 2003; Zang Y., 2003). This STAT domain has also been implicated in constitutive nucleocytoplasmic shuttling of unphosphorylated STATs in resting cells, an effect that is mediated through interactions with nuclear pore proteins (Marg A., 2004). The most well conserved domain in the structure of STATs, the SH2 domain (SH2D), is necessary for receptor association and tyrosine phosphodimer formation. This domain precedes the transactivation domain located on the C-terminus. Some residues within this domain may be particularly important for some cellular functions mediated by the STATs.

The C-terminal domain is the one that differs the most among STATs and functions as a transactivation domain (TAD) (Levy D.E., 2002). The conserved tyrosine residue present on all STAT proteins in the C-terminus is crucial for dimerization. In addition, with exception of STAT2 and STAT6, all other STATs have in the TAD a conserved serine residue. Phosphorylation of a single serine (S727) in the C-terminal transactivation domain of STAT3 allows for the maximal activation of transcription of responsive genes. (Z. Wen, 1995; Santos C.I., 2011). In the canonical STAT3 signaling pathway, activation of cell surface receptors by growth factors and cytokines induces the specific phosphorylation of receptor tyrosine residue to create docking sites for the recruitment of latent cytoplasmatic STAT3. STAT3 docking to receptor phosphotyrosine (pY) sites is mediated through its SH-2 domain. The binding of STAT3 at receptor pY sites leads to the phosphorylation of a specific tyrosine residue of STAT3 (Y^{705}) in the C-terminal domain, and this phosphorylation activates STAT3. The phosphorylation of STAT3, in turn, promotes homodimerization, or heterodimerizes with STAT-1, wherein the SH-2 domain of each monomer interacts with the Y705 residue of its partner. STAT3 dimers translocate to the nucleus where they bind to specific DNA response elements, such as the

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TTN4-5AA sequence (where N refers to any nucleotide base), in the promoter regions of responsive target gene to regulate their transcription (Emily C. Brantley 2008).

1.1.1 *The STAT3 isoforms α and β*

An important factor determining STAT3 functional heterogeneity is probably the existence of two alternatively spliced isoforms: the fulllength STAT3α and the truncated STAT3β, which lacks the C-terminal activation domain and is generally considered a dominant negative form (Caldenhoven E., 1996, Schaefer T.S, 1995) (Fig.1). To assess the specific functions of STAT3α and STAT3β, Maritano D. et al. generated mice that can produce only one isoform or the other. They confirmed that STAT3β is not required for viability but is involved in inflammation. In contrast, STAT3α-deficient mice died within 24 h of birth, showing that this isoform is required for postnatal function(s) but that STAT3β can rescue the embryonic lethality of a complete STAT3 deletion. Indeed, their data indicate that STAT3β is not a dominant negative factor, as it can activate specific STAT3 target genes. However, STAT3α has nonredundant roles in modulating IL-6 signaling and mediating IL-10 functions. A question was how does STAT3β, which lacks the transcription activation domain responsible for recruiting coactivators such as p300-CBP, activate transcription? This isoform was first isolated by a two-hybrid screen using c-Jun as a bait and cooperates with this factor to activate transcription of the acute-phase α2-macroglobulin and α1-antichymotrypsin gene promoters through STAT3 and transcription factor AP-1 binding sites. Interaction with c-Jun requires the STAT3 Nterminal coiled-coil region, present in both the STAT3α and the STAT3β isoforms (Zhang X., 1999; Zhang X., 2001). In addition, STAT3α and STAT3β can partecipate equal

well in the formation of a ternary complex (enhanceosome) involving the STAT3 and AP-1 sites plus two downstream AT-rich regions on the α2-macroglobulin promoter33. Therefore, although only STAT3α might be able to activate specific target genes on its own through the direct recruitment of coactivators, both STAT3α and STAT3β may be able to activate a common subset of target genes by participating in the formation of higher hierarchy complexes, which in turn provide the optimal surface to recruit coactivators and the basal transcriptional machinery. Depending on promoter composition, specific subsets of target genes might then be solely responsive to STAT3β. Global expression profiling coupled with promoter composition analysis may lead to understanding of the rules dictating activation by the two isoforms (Maritano D. et al, 2004).
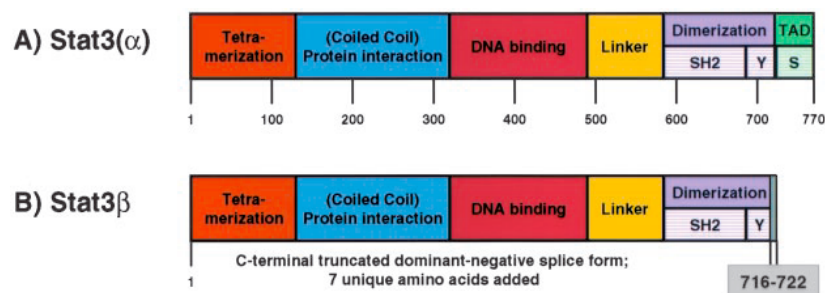
Fig.1: Schematic structure of STAT proteins. A) The domain structure of full-length Stat3 protein (designated either Stat3 or Stat3α). B) as compared with Stat3α, the Stat3β splice variant has an COOH-terminal deletion, resulting in an altered open reading frame with the addition of seven new amino acids downstream of the deletion, and loss of the TAD domain. This naturally occurring truncated isoform still dimerizes and binds to DNA. Y, critical phosphotyrosine in all STATs (amino acid 705 in Stat3); S, critical phosphoserine in some STATs (amino acid 727 in Stat3). (*Buettner R., 2002*).

1.2 STAT3 activation pathways

Various growth factors that have been linked to tumor cell

proliferation have been found to activate STAT3. EGF signaling, which has been linked with proliferation of almost 30% of all tumor cells, has been shown to mediate its effect through activation of STAT3. Similarly, IL-6, which has been linked with proliferation of multiple myeloma, renal cell carcinoma, prostate cancer, and other cancers, has been shown to mediate its effect through activation of STAT3. Moreover, several other cytokines have been shown to activate STAT3. These include growth hormone, transforming growth factor-α (TGF-α), oncostatin M, thrombopoietin, platelet-derived growth factor (PDGF), IL-5, IL-6, IL-9 IL-10, IL-12, IL-22 and leptin. Certain chemokines, such as macrophage inflammatory protein 1α and RANTES (regulated upon activation of normal T cell expressed and secreted) also have been shown to activate STAT3. Besides growth factors and cytokines, other factors that activate STAT3 include oxidative stress, tobacco chewing, hepatitis C virus, ultraviolet B, lipopolysaccharide, osmotic shock and progestins. Cytokines and growth factors initiate STAT-3 signal transduction when they bind to their receptors and activate intracellular kinases. STAT-3 is tyrosine phosphorylated by three types of kinases (Fig. 2):

- receptor tyrosine kinases such as EGFR, FGF receptor (FGFR), or platelet-derived growth factor receptor

- Janus kinase (JAK) family members, which are constitutively bound to the cytoplasmic tails of cytokine receptors

- non-receptor associated tyrosine kinases, including Ret, Src family (hck, src), or the Bcl-Abl fusion protein.

JAK2 is one of the major mediators of STAT3 phosphorylation. JAK proteins are autophosphorylated and transphosphorylated and phosphorylate one of four tyrosine residues on the intracellular portion of gp130 subunit.

JAK protein activation results in downstream signal transduction through both the MAPK and STAT-3 pathways.
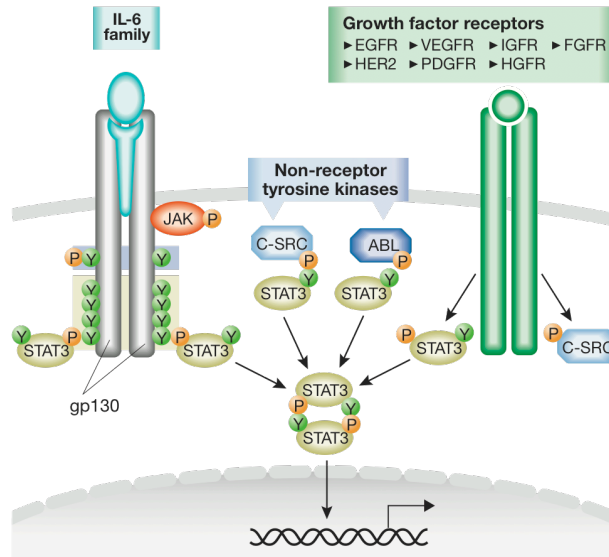
Fig. 2: STAT3 signaling (*Bollrath J., 2009*)

1.3 Negative regulators of STAT3

Under normal conditions, both the amplitude and duration of receptor-induced STAT3 activation are tightly controller by a variety of endogenous protein regulators. Peak STAT3 phosphorylation occurs within approximately 30-60 min of exposure to cytokine, and even in the presence of continuous cytokines, STAT3 activation decreases over several hours. Since continuous STAT3 activation can lead to neoplastic transformation, it is clear that the feedback mechanisms attenuating STAT3 function must be

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efficient and multi-layered.

1.3.1 *STAT-3 inhibition by SOCS-3*

Suppressors of cytokine signaling (SOCS) proteins down-regulate the upstream kinase activity responsible for STAT3 phosphorylation. SOCS-3 is a member of the SOCS protein family, which comprises seven SOCS proteins (SOCS-1 through SOCS-7) and cytokine inducible SH2 domain containing protein. The cytoplasmatic, inducibly expressed SOCS proteins attenuate STAT activity by inhibiting upstream JAK activation in a classic negative feedback loop. Activated STAT-3 induces the expression of SOCS-3, which subsequently inhibits STAT-3 signaling by binding to and attenuating the signal transduction of gp130-related cytokine receptors and their associated JAK kinases.

1.3.2 *STAT-3 inhibition by PIAS Proteins and Protein Tyrosine Phosphatases.*

The protein inhibitors of activated STATs (PIAS) proteins and protein tyrosine phosphatases target STAT proteins directly. Several protein tyrosine phosphatases (PTPs) have been implicated in the termination of STAT3 signaling, including SHP-2, PTP1B, PTPεC, TC45, and SHP-1. SHP-2 is ubiquitously expressed, and like SHP-1, contains two N-terminal SH-2 domains and a C-terminal PTP domain. SHP-2, by virtue of its two SH-2 domains, associates with pY-sites of growth factor receptors, cytokine receptors, gp130, JAKs, members of the Src family of tyrosine kinases (SFKs), and STAT3. SHP-2 may in turn become phosphorylated by RTK activities, JAK1, JAK2, and SFKs. All of the pY components of STAT3 signaling pathways are potential substrates for PTPs. Because only STAT3 dimers bind to DNA, the nuclear PTP TC45 may be important in the

termination of STAT3-mediated transcriptional activation. The highly conserved PIAS family of proteins includes PIAS1, PIAS3, PIASx, and PIASy. Except for PIAS1, each protein has two isoforms, and PIAS3 regulates STAT-3. In contrast to the inducibly expressed SOCS proteins, PIAS proteins are constitutively expressed in the nucleus and mediate transcriptional repression by directly interfering with the binding of STAT proteins and other transcription factors to their target DNA sequenze. All PIAS proteins contain a zinc ring finger domain, an NH2-terminal LXXLL motif, a COOH-terminal acidic domain, a serine/threonine–rich domain, and a recently discovered PINIT motif that is involved in nuclear retention. PIAS proteins inhibit STAT transcriptional activity by interfering with DNA binding via their NH2-terminal domains. PIAS proteins also recruit transcriptional corepressors such as histone deacetylases to target gene promoters to inhibit transcription. Furthermore, PIAS proteins influence the activation status of transcription factors by directly modifying the proteins themselves. Some PIAS proteins, including PIAS3, exhibit E3-SUMO (Small Ubiquitin-like MOdifier) ligase activity and SUMOylate a variety of transcription factors, including STAT3, but it is unclear whether sumoylation contributes to the inhibition of STAT3 function.

1.4 STAT3 target genes

STAT-3 is an oncogenic protein that is constitutively activated in many human cancers. For instance, in 30–60% of primary breast cancers, STAT-3 is constitutively active. Constitutive activation of STAT-3 has also been reported in several other primary cancers, in tumor cell lines, and in many oncogenetransformed cells. Inactivation of STAT-3 in most of these

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cell lines leads to inhibition of cell proliferation. The evidence below shows that STAT-3 activation is intimately connected with all aspects of tumorigenesis. Stat3 target genes have been identified and include regulators of crucial steps in oncogenesis such as proliferation and survival of tumor cells, their migration and invasion properties, their potential to induce angiogenesis and to evade the host immune response. Stat3 is a transcriptional activator of the *Mcl-1, Bcl-XL* and *Survivin* genes, which act as suppressors of apoptosis. Stat3 regulates expression of *c-Myc* and *Cyclin D1* which are proliferation-promoting genes as well as vascular endothelial growth factor (VEGF), which favor angiogenesis. *P53* is negatively regulated by Stat3, inhibiting its tumor suppressor activity. Tumor immune evasion is enhanced through Stat3-mediated reduction of the secretion of pro-inflammatory cytokines and chemokines. STAT-3 activation regulates the expression of matrix metalloproteinase (*MMP-2* and *MMP-1*), which then mediate tumor invasion and metastasis. STAT-3, however, is also known to upregulate tissue inhibitors of metalloproteinase (*TIMP-1*), a cytokine known to block metalloproteinases and decrease invasiveness in certain cancer cell types. STAT-3 also controls the expression of the *MUC1* gene, which can mediate tumor invasion. However, genome-wide analysis of the effects of STAT3 activation reveal a potentially more complex transcriptional network. A large number of genes that are up-regulated early following STAT3 activation are transcription factors themselves including junB, egr1, KLF4, bcl-6, and NFIL3, suggesting that STAT3 can regulate broad programs of gene expression. Interestingly, some of the previously reported STAT3 targets such as cyclin D1 were not up-regulated during this time frame. This observation suggests that STAT3 may act upstream of several transcription programs such that different targets may be activated or repressed at distinct

time points following STAT3 activation. Such interplay of transcription factors and the potentially transient activation of specific targets may significantly complicate the identification of key targets. Despite this potential difficulty, microarray analysis of STAT3-mediated transcriptional changes after longer periods of activation identified several genes involved in several important pathways implicated in oncogenesis. The validation of such targets represents an important new avenue of research on a relatively under-explored aspect of STAT3 biology.

1.5 STAT3 and its Coregulators

One important question to be resolved is what molecular basis governs STAT3 gene activation, which cofactors are associated with the transcription factor on its target genes, and how is the coregulator exchange process used to integrate dynamic changes in the signaling network and transmit these changes to the chromatin? It was established an essential role of NH2 -terminal acetylation of STAT3 in protein-protein interaction resulting in stable formation of the enhanceosome complex, target gene expression, and nucleoplasmic shuttling (Giraud S. et al, 2004). Among the most coactivators described in the literature (Fig. 3) there is the CBP/p300 protein, requied by many different transcription factors for its ability to acetylate histones and other proteins. The histone acetylases allow greater access to DNA but also function as bridging factors to the transcriptional machinery. In the table below are show the most known coactivators were reported in the literature, but many question are still unclear: are the interactions between two proteins the same in all enhanceosomes? Stated another way, how many enhanceosomes can be built from a limited repertoire of participants?

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Type	Name	Functions	Description
Co-activator	CBP/p300	Histone acetylases	Wang R. et al [2005]
	NcoA/SRC1a	Histone acetylases	Giraud S. et al [2002]
	BRG1	Component of the SWI/SNF chromatin-remodeling complex	Giraud S. et al [2004]
	Cdk9	Component of the elongation factor PTEFb	Giraud S. et al [2004]
	c-Jun/c-Fos	Transcription factor	Lerner L. et al [2003]
	Oct-1	Transcription factor	Lerner L. et al [2003]
	APE-1/Ref-1	Protein in the DNA base-excision repair	Sutapa R. et al [2010]
	ERp57	Protein disulfide isomerase	Chichiarelli S. et al [2010]
	AR	Androgen Receptor	Miguel de F. [2003]
Co-repressor	Tip60	HAT activity	Xiao H. et al [2003]
	PPAR-γ	Peroxisome Proliferator-Activated Receptor-γ	Wahn H. et al [2005]
	KAP1/TIF1b	Corepressor for the Kruppel-associated box-domain-containing zinc- finger proteins	Tsuruma R. et al [2008]

Fig.3 : Coactivators and Corepressor of STAT3 reported in literature

1.6 Nuclear import of STAT3

The presence of STAT3 in the nucleus and the cytoplasm under basal conditions suggests a constant shuttling of STAT3 between the two cellular compartments. Unlike other STATs, such as STAT1 and STAT2, which accumulate in the nucleus only following their phosphorylation, STAT3 can enter the nucleus independently of its phosphorylation. The mechanism underlying these differences relates to the involvement of distinct importins

used by STATs for their nuclear import. For instance, the phosphorylation of the nuclear localization signal of STAT1 is a prerequisite for its interaction with importin α-5 and subsequent nuclear import. In contrast, STAT3 binds constitutively to importin α-3 and α-6. Although the shuttling of STAT3 in and out of the nucleus seems independent of its phosphorylation, the strict requirement for phosphorylation for the transcriptional activation of STAT3 remains a controversial topic. Initially, the finding that introducing cysteine residues into the COOH terminus of STAT3 (generating the STAT3C mutant) is sufficient to promote the constitutive activation of STAT3 led to the hypothesis that these cysteines promote the formation of disulfide bonds between unphosphorylated monomers. This suggested that although phosphorylation could trigger dimerization, it was not necessary for activation. However, because mutation of the critical tyrosine residues within STAT3C abolished its transcriptional activity, it was suggested that STAT3 may be phosphorylated and dephosphorylated at a low rate in the absence of cytokine and that introducing cysteine residues simply acts to trap spontaneously phosphorylated dimers. Conversely, another study reported that STAT3 with a mutation of Tyr705 retains transcriptional activity but affects a distinct subset of genes compared with wild-type STAT3. Nevertheless, the nuclear translocation of STAT3 in the absence of phosphorylation raises the question as to whether unphosphorylated STAT3 has a role in the nucleus. The finding that the nuclear tyrosine kinase breast tumor kinase phosphorylates STAT3 raises the possibility that such nuclear rather than cytoplasmic phosphorylation of STAT3 acts as a rapid activation mechanism and may contribute to its oncogenic effect (Doris Germain, 2007).

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1.7 Cytoplasmic role of STAT3.

Although most attention has been focused on the transcriptional function of STAT3, the recent discovery of cytoplasmic partners of STAT3 has raised the possibility of unsuspected new roles for this protein in the cytoplasm. One of the first clues was the observation that p130CAS, an adaptor protein that localizes to focal adhesion sites and which assembles with focal adhesion kinase and its partner paxillin, was hyperphosphorylated in STAT3- deficient keratinocytes. Further, phosphorylated STAT3 was found to localize to the migrating protrusions and focal adhesions in migrating cells. These observations therefore suggested a transcription-independent function of STAT3 in regulating cell migration. More recently, stathmin, a tubulin binding protein, which promotes microtubule depolymerization, was identified as a binding partner of STAT3. Although stathmin did not seem to regulate STAT3 transcriptional activity, unphosphorylated STAT3 was found to prevent stathmin from binding to microtubules, thereby promoting microtubule polymerization and cell migration. Thus, STAT3 can affect migration both via its transcriptional regulation of genes involved in cell migration and also through its transcription-independent interactions with focal adhesion molecules and its inhibition of stathmin.

1.8 STAT3 in the mitochondria

Recently, STAT3 was shown to play an unconventional role in mitochondria, although it does not contain a mitochondrial targeting

sequence. Reconstitution of STAT3- deficient cells with different STAT3 mutants specifically targeted to the mitochondria revealed that mitochondrial STAT3 is sufficient to modulate respiratory chain activity and that phosphorylation on serine 727 and a monomeric conformation play a crucial role in this process (Nicole Büchner, 2010). Mitochondrial STAT3 (mSTAT3) appears to sustain Electron Transport Chain basal activity under normal or RAS-transformed conditions, supporting RAS-dependent oncogenic transformation, and it was suggested to act as a negative regulator of the Mitochondrial Permeability Transition Pore (MPTP), thus mediating cardioprotection after ischemia/reperfusion (Demaria M., 2011; Figure 4). Ras mediated transformation in vitro and tumor growth in mice were impaired in STAT3-deficient cells. Mutational analysis demonstrated that the N-terminal DNA binding domain, the Src homology 2 (SH2) domain, phosphorylation on tyrosine 705, and nuclear localization of STAT3 are dispensable for supporting malignant transformation by Ras. In contrast, tyrosine phosphorylation and presence in the nucleus are required for transformation by the tyrosine kinase oncogene v- Src. This newly discovered function of STAT3 was ascribed to its mitochondrial localization accompanied by augmentation of respiratory chain activity, particularly that of complex II and V, and a dependence on phosphorylation of serine 727.

Fig. 4: Converging roles of nuclear (n) and mitochondrial (m) STAT 3
(*Demaria M. and Poli V., 2011*).
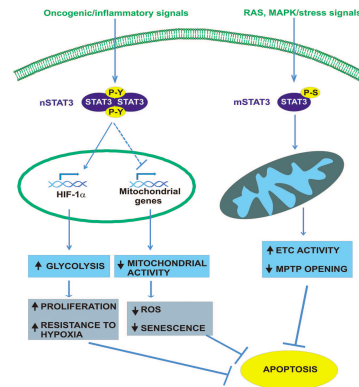
1.9 Unphosphorylated STAT3

The presence of STAT3 in the nucleus and the cytoplasm under basal conditions suggests a constant shuttling of STAT3 between the two cellular compartments. For a long time, the tyrosine phosphorylation of STATs by ligand activated receptors was thought to be an obligatory requirement for dimerization in an active conformation, nuclear import, and transcriptional activation. More recently it has been shown that nonphosphorylated STATs shuttle between the cytoplasm and the nucleus at all times in a costitutive manner and that also these nonphosphorylated STATs (U-STATs) can be transcriptionally active, either as homodimers or in a complex with other transcription factors. However, these nonphosphorylated STATs regulate a different set of target genes than their phosphorylated counterparts. Major mechanisms of STAT3 activation in tumor cells are autocrine production of IL-6 and paracrine activation by IL-6 from stroma and infiltrating inflammatory cells. Indeed, circulating IL-6 levels are usually high in cancer patients (Giannitrapani et al. 2002). STAT3 activation provides an important link between inflammation and cancer. There are several reports that STAT3 and NFκB interact with each other (Battle and Frank 2002). The expression of U-STAT3 is greatly increased in response to their activation, since the

STAT3 gene is strongly activated by P-STAT3 dimers, which are formed in response to IL-6 and other ligands that activate the gp130 common receptor subunit. U-STAT3, induced to a high level due to activation of STAT3 gene, binds to unphosphorylated NFκB (U-NFκB), in competition with IκB, and the resulting U-STAT3/p65/p50 complex accumulates in the nucleus with help of importin-α3, activating a subset of κB-dependent genes (Yang J., 2008). Since the Y705F mutant of STAT3, which cannot be phosphorylated on tyrosine, also activates RANTES expression, this function of U-STAT3 is clearly distinct from the absolute requirement for tyrosine phosphorylation that enables STAT3 dimers to bind to GAS (gamma-activated site) sequences (Wen et al. 1995; Kaptein et al. 1996; Zhang et al. 1999). The full biological relevance of the ability of P-STAT3 to increase the intracellular concentration of U-STAT3 remains to be established. In the contest of cancer, the costitutive tyrosine phosphorylation of STAT3 in many different tumori is likely to lead to increased expression of U-STAT3, which in turn drives the expression of oncogenes such as MET and MRAS.

1.10 STAT3 activation in cancer

Reflecting the potentially important role for STAT3 in cellular function, in recent years, a large series of publications has demonstrated the costitutive activation of STAT3 in nearly every major human cancer (Frank DA., 2003). One question that arises is the mechanism by which constitutive STAT activation occurs in a tumor. Under normal physiologic conditions, STAT3 activation in response to growth factors is a transient response. This reflects the fact that a variety of negative feedback systems exist to rapidly

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dampen signals leading to STAT activation. Thus, constitutive STAT3 activation seen in tumors likely reflects not only enhanced activity of a tyrosine kinase that can mediate the phosphorylation of STAT3, but also a loss or repression of the normal mechanisms that dampen the STAT3 response. STAT3 can be activated in tumors through increased signaling through cytokine receptors, such as the receptor for interleukin-6, which can occur through autocrine or paracrine release of this factor. This is likely a significant feature in tumors such as multiple myeloma, in which IL-6 has long been known to play a pivotal role (Kawano M., 1988; Catlett-Falcone R., 1999). However, autocrine activation of STAT3 is also important in epithelial cancers such as breast cancer (Li L., 2002) and pancreatic cancer (DeArmond D., 2003). In many forms of cancer, increased levels of IL-6 are associated with a poor prognosis, and this may reflect enhanced STAT3 phosphorylation within the tumor cell, although it may also be a marker of the physiological stress induced by the presence of an advanced cancer. Activation of STAT3 in tumor cells may also obviate the need for external growth signals. Thus, costitutive STAT3 activation is associated with IL-6 independence in animal models of myeloma (Rawat R., 2000) and androgen independence in prostate cancer (Lee S.O., 2003). However, as noted, several feedback mechanisms are in place to dampen or interrupt STAT activation. These findings provide insight into the pathogenesis of these tumors, and they also have implications for therapeutic interventions targeting this pathway. It is becoming increasingly clear that activating mutations in tyrosine kinases are a common occurrence in a range of human cancers, and many of these lead directly to STAT3 activation. However, other mechanisms of cellular transformation, such as viral carcinogenesis, may also make use of STAT3. For example, the core protein of the epatitis C virus,

which is associated with the development of hepatocellular carcinoma, mediates the tyrosine phosphorylation of STAT3, which is necessary for malignant cellular transformation (Yoshida T., 2002). While the mechanism for this is unclear, this finding emphasizes the central role played by this transcription factor in malignancy. Although tyrosine phosphorylation is clearly necessary for the ability of STAT3 to mediate transcriptional activation, in many systems maximal activity of STAT3 requires phosphorylation of STAT3 on serine 727 as well. There is evidence that activation of the EGF receptor through mutation can maximally enhance the serine phosphorylation of STAT3, and that this event may be critical to the transforming ability of these mutations (Alvarez J.V., 2006)s. In chronic lymphocytic leukemia, constitutive serine phosphorylation of STAT3, in the absence of tyrosine phosphorylation has been found (Frank D.A., 1997) , although it is unclear how this may alter the biology of these cells. It could be argued that there are multiple intracellular pathways that can lead to malignancy, only one of which involves STAT3. However, increasing studies on primary human samples has revealed that a majority of patients with common tumors such as cancer of the breast, prostate, and pancreas display STAT3 activation (Alvarez J.V., 2005; Wei D., 2003). Furthermore, given the central role that STAT3 target genes play in promoting proliferation and survival, it is perhaps not surprising that activation of STAT3 is associated with a poor prognosis in cancers such as acute myeliod leukemia (Figure 5).

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1.11 STAT3 and its Post-Translational Modifications (PTMs)

STAT3 protein is regulated by multiple tyrosine kinase and serine kinase pathways, and thus sit at a key convergence point where they can integrate the effects from different stimuli. Although less well understood currently, there is evidence that STAT3 cas also be modulated by post-translational modifications such as acetylation, glutathionylation, poli-ADP ribosylation and SUMOylation. Post-translational modifications (PTMs) of nuclear proteins are involved in activating and inactivating critical signaling pathways in cellular processes of malignant transformation and progression. Thus, the model of diverse pathways converging on STAT3 may be even broader than currently appreciated.

1.11.1 Acetylation.

Function-related posttranslational modifications of STAT proteins in response to treatment with cytokine or growth factor include phosphorylation of a single tyrosine residue in the short segment and a single serine residue in the TAD. However, unphosphorylated or tyrosine-mutated STAT proteins can still form dimers and induce transcription (5–8), suggesting that another type of regulation contributes to the formation of stable STAT dimers. The C-terminal region of STAT interacts with other proteins during signaling or transcription. For instance, members of the CREB-binding protein (CBP)/p300 family have intrinsic histone acetyltransferase (HAT) activity, associate with various STAT family members within both the C-terminal TAD and N-terminal domain, and increase STAT activity in transcription. There are evidence of Stat3 acetylation on Lys685 site. It resides in the highly hydrophilic region between the SH2 domain and TAD and is highly

conserved in Stat1, Stat3, Stat4, Stat5a, and Stat5b of different species. Use of a prostate cancer cell line (PC3) that lacks Stat3 and PC3 cells expressing wildtype Stat3 or a Stat3 mutant containing a Lys685-to-Arg substitution revealed that Lys685 acetylation was critical for Stat3 to form stable dimers required for cytokine-stimulated DNA binding and transcriptional regulation, to enhance transcription of cell growth–related genes, and to promote cell cycle progression in response to treatment with oncostatin M (Zheng-long Yuan, 2005).

1.11.1 Redox modification.

Several reports have highlighted an effect of reactive oxygen species (ROS) on STAT3 activity. Although ROS scavengers and inhibitors of NOX enzymes generally inhibited STAT3 activity, the otherwise divergent reports indicated that the effects of ROS may be tissue specific and manifested at several levels (e.g., by their actions on tyrosine phosphatases and Janus kinases). The susceptibility of reactive cysteines in proteins to ROS and nitrosative stress render them vulnerable to oxidative modification. In most cases, low-molecular mass thiols such as glutathione (GSH) can form mixed disulfides with oxidized cysteine forms in protein. This process, known as S-glutathionylation, has emerged as a redox-sensitive posttranslational modification that can modulate enzymatic activities, protein folding and functions, and protein-protein interactions. STAT3 itself is susceptible to oxidation in cells under oxidative stress and was shown to be modified and repressed by cysteine glutathionylation. Xie et al (Xie Y, 2009) showed that a small but detectable S-glutathionylation of STAT3 occurred in intact cells under basal conditions, and that exogenous oxidant diamide elicited an increase in STAT3 S-glutathionylation, which was accompanied by reduction in IL6-induced tyrosine phosphorylation of this transcription factor, with

subsequent impairment in the retention of STAT3 to the nucleus and transcriptional activation of target genes. STAT3 contains 14 cysteines, of which 5 are conserved in STAT1. The DNA-binding domain (DBD) of STAT3 contains up to three reactive cysteines (C418, C426, and C468) with one other (C765) present within the C-TAD (Li Li, 2010). It appear that this modification hampers the access of the Tyr-705 region to the STAT3 recognition site on JAK2. The modifications in a cluster of cysteines in the DNA binding domain may down-regulate the transcriptional potency of STAT3 in response to diverse stimuli. It is possible that essential transcriptional coactivators of STAT3 may also be the target of dynamic redox control through S-glutathionylation.

Constitutive STAT3	Activators	Genes	Kinases	Inhibitors
Hematopoietic tumors	EGF[3]	**Antiapoptosis**	**Nonreceptor tyrosine kinases**	**Synthetic**
Multiple myeloma[37]	IL-6[3]	Bcl-x$_L$[95]	JAK[152]	AG490[32]
HTLV-1-dependent leukemia[29]	IL-5[60]	Bcl-2[110]	JAK2[6]	Sodium salicylate[117]
CLL[33]	IL-9[61]	Mcl-1[110]	JAK3[152]	Atiprimod[119]
CML[49]	IL-10[63]	cIAP-2[110]	TYK2[152]	BMS-354825[154]
AML[36]	IL-12[64]	Survivin[97]	Src[5]	Ethanol[116]
Large granular lymphocyte leukemia[40]	IL-22[65]			Nelfinavir[155]
Erythroleukemia[46]	TNF-α[104]	**Cell cycle progression**	**Receptor tyrosine kinases**	PS-341[120]
Polycythemia vera[39]	MCP-1[144]	Cyclin D1[42]	EGFR[57]	R115777[156]
EBV-related/Burkitt's[30]	GCSF[62]	c-Myc[148]	ErbB-2[6]	WP-1034[157]
Mycosis fungoides[32]	GMCSF[60]	c-Fos[25]	Gp130[153]	Platinum compound[115]
Cutaneous T cell lymphoma[35]	CSF[145]	p21[101]	Grb2[27]	15-Deoxy-delta 12, 14-PGJ2[158]
HSV saimiri-dependent (T cell)[34]	LIF[146]			UCN-01[159]
Hodgkin's disease[41]	OSM[4]	**Tumor invasion and metastasis**	**Serine kinases**	Statin[121]
Anaplastic lymphoma[48]	IFN-γ	MMP-2[149]	JNK[14]	
Hematopoietic tumors	MIP-1α[67]	MMP-9[99]	P38MAPK[15]	**Peptides**
Multiple myeloma[37]	RANTES[67]	β-catenin[20]	ERK[13]	SOCS3[82]
HTLV-1-dependent leukemia[29]	SLF[147]	VEGF[100]		PIAS[160]
	UVB[72]	hTERT[150]	**Tyrosine**	GRIM-19[161]
	Osmotic shock[74]	IRF-1[106]	**Phosphatase**	Adiponectin[162]
Solid tumors	Progestin[75]	NLK[151]	SHP2[77]	Duplin[163]
Breast cancer[31]	LPS[73]	MyD88[105]		SSI-1[102]
Brain tumor[47]	Tobacco[69]	RANKL[103]		α-Thrombin[164]
Colon carcinoma[54]	HCV[70,71]	TNF[104]		Lipoxin A4[165]
Ewig sarcoma[55]	EGF[3]	β-macroglobulin[107]		DIF-1[166]
Gastric carcinoma[52]		SOCS[102]		PTPεC[167]
Lung cancer[51]				STAT3-DN[77]
Nasopharyngeal cancer[50]				
Ovarian caricinoma[38]				

Fig. 5: Tumor that express constitutively active STAT3, activators of STAT3, genes regulated by STAT3, and inhibitors of STAT3.

1.12 STAT3 in human prostate cancer

Constitutive STAT activation occurs frequently in a variety of human tumor cell lines and primary human tumors including leukemias, lymphomas, multiple myeloma, head and neck cancer, and breast cancer because signaling by Stat3 protein participates in regulating the processes of cell growth and survival during oncogenesis. Stat3 is constitutively activated with high frequency in prostate adenocarcinomas compared with matched adjacent nontumor prostate tissues. Prostate cancer (PCa) cell growth was stimulated by IL-6, accompanied by activation of STAT3. Dhir et al demonstrated that cells derived from both rat and human prostate cancer have costitutively activated STAT3, and that STAT3 activation is associated with malignant potential (Ni Z., 2000). In the initial stage, PCa is dependent on androgens for growth, which is the basis for androgen-deprivation therapy (ADT). However, in most cases, prostate cancer progresses to a hormone refractory phenotype for which there is no effective therapy available at present. The development of androgen escape is therefore one of the most significant events in the progression of prostate cancer, resulting in a greater risk of morbidity, and reduced survival. The androgen receptor (AR) is required for prostate cancer growth in all stages, including the relapsed, "androgen-indipendent" tumor in the presence of very low levels of androgens (Kaarbø M., 2007). Failure of ADT, as in hormone-resistant prostate cancer, was therefore logically linked to modification of the action of the AR, as mutations, AR gene amplifications, post-translational modification of the AR, via phosphorylation at specific sites. In recent years it has become increasingly apparent that androgen escape may also involve mechanisms that do not directly modify the AR, but may also involve the action of AR coactivators or pathways independent of the AR. A way by which AR

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cofactors can influence the development of androgen escape is not as well established and involves binding of cofactors to the AR, resulting in AR translocation to the nucleus. An example of this is STAT3. *In vitro* studies show that interleukin-6 activation of the JAK/STAT3 pathway is accompanied by transition from androgen-sensitive to -insensitive prostate cancer cell growth (Fig. 6). Levels of activated STAT3 are significantly higher in the hormone-refractory prostate cancer cell lines (DU145 and PC3) than in hormone-sensitive cell lines (LNCaP cells). In LNCaP cells the activated dimer of STAT3 binds ligand-free AR before entering the nucleus, therefore facilitating the translocation of AR to the nucleus in the absence of androgens. The AR/STAT-3 complex can activate androgen regulated gene transcription, and PSA (kallikrein-like serine protease) expression is elevated even in the absence of androgens. This mechanism is supported by data showing that interleukin-6 can activate the AR in a ligand-independent manner. However, the oncogenic role of STAT3 in prostate cancer is not clearly established and STAT3 has also been correlated with interleukin-6-induced growth arrest in cell lines, including LNCaP cells (Edwards J., 2005).
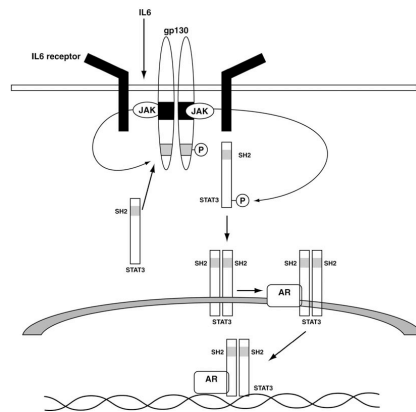

Fig. 6: How the JAK/STAT pathways affects prostate cancer growth (*Edwards J., 2005*).

2. AIM OF RESEARCH

STAT3 plays a central role in a range of physiologic process, and when subverted in cancer, can be a central mediator of malignant cellular function. The high incidence of STAT3 activation in human tumor cells of diverse origins (Migone et al., 1995; Gouilleux-Gruart et al., 1996; Weber-Nordt et al., 1996; Garcia et al., 1997; Takemoto et al., 1997) suggest an important role for aberrant activation of STAT3 signaling in regulanting fundamental cellular processes associated with malignant transformation. Consistent with an indispensabile function during devlopment (Takeda et al., 1997), STAT3 signaling has been implicated in regulation of apoptosis, differentiation, and cell proliferation (Fukada et al., 1996; Ihara et al., 1997; Bromberg et al., 1998; Turkson et al., 1998). We predicted that this persistent STAT3 signaling affects a wide range of cellular function, many of which still remain to be characterized such as the mitotic process. Literature screening data indicating that TPX2 protein has multiple function during mitosis, including microtubule nucleation around the chromosomes and the targeting of Xklp2 and Aurora A to the spindle; TPX2 is also overexpressed in many tumor types. Then the present research has intended to investigate, through in vitro and in vivo methods, the role of STAT3 as transcription factor in the regulatory region of *TPX2* gene coding for a protein involved in the complex process of mitosis, and identified as one of the microtubule-associated proteins. Potential STAT3-binding sites obtained by consulting databases as MatInspector and ElDorado have been tested by EMSA and Chomatin-Immunoprecipitation experiments. To confirm our results in vivo

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reporter gene assay has been conducted in M14 cells treated with specific phosphopeptidic inhibitor of STAT3 (Turkson et al., 2001).

In variety of human tumor STAT3 has been descrive as molecular hub to link extracellular signals to transcriptional control (Brantley E. and Benveniste E.N., 2008). Whereas STAT3 is tyrosine phosphorylated by three types of kinases and regulated broad programs of gene expression, it uses a precise sequence of functional actions by multiple coactivator complexes and post-translational modifications for mediating gene activation. This observation suggests that STAT3 may act upstream of several transcription programs such that different targets may be activated or repressed at distinct time point following STAT3 activation. A dynamic interplay between coactivator/corepressor complexes as "sensors" to integrate signaling inputs to generate precise programs of gene expression (Rosenfeld M.G., 2006) was investigated in several tumor cell lines, particularly in melanoma cell lines M14 stimulated with EGF and IL-6 to simulate different pathways of STAT3 activation. The cells have been subjected to co-immunoprecipitation and chromatin-immunopurification to study STAT3-interacting proteins and to demonstrate the requirement of specific coactivators in transcriptional activation of STAT3-regulated genes.

Furthermore, activated STAT3 has been implicated as an important signaling molecule for IL-6 to activate AR (androgen receptor) in androgen-dependent and −independent cell lines (Edwards J., 2005; Kaarbø M. 2007). The aim of last part of this study was to explore the relation between the immunochemical expression of p-Tyr705 STAT3 and its post-traslational modificazions (PTMs), as acetylation and glutathionylation, in normal and malignant human prostate tissues (FFPE) and clinicopathological features. Parallel to investigate how differences in PTMs of STAT3 can influence gene

expression and interactions with coactivators, immunoblottig analysis, Co-IP and ChIP experiments have been conducted in LNCaP and PC3 cells treated with IL-6 and H_2O_2, to simulate a physiological response to citokines or to oxidative stress.

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3. MATERIALS AND METHODS

3.1 Cell lines and cell culture

Human cervical adenocarcinoma cells (HeLa) were grown in Dulbecco's minimal essential medium (DMEM). Human melanoma cells (M14) and human prostate cancer cells (LNCaP and PC3) were maintained in RPMI-1640 (Roswell Park Memorial Institute) medium, supplemented with 10% v/v fetal bovine serum (FBS), 100 units/ mL penicillin and 100 μg/mL streptomycin (Invitrogen, Burlington, ON, Canada), 1% w/v sodium pyruvate, 2mM glutamine. All cells were grown to 70-80% confluence at 37°C with 5% CO_2.

3.2 Cells treatment with IL-6, EGF, H_2O_2

All cell lines were grown in 25 cm^2 flasks for cell fractionation and treated with IL-6 at final concentration of 50ng/ml, EGF (Epidermal growth factor, Sigma Aldrich) 100 ng/ml, H_2O_2 1mM for 5, 15, 30, 60, 90, 120, 240 minutes. For the inhibitions, cells were treated with anacardic acid 100μM for 40 minutes at 37°C and 3-ABA (3-aminobenzamide) at final concentration of 3 mM for 24h.

3.3 Cell fractionation

Cells, treated and untreated, were washed with serum-free medium

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and scraped with cold PSB. Cells were harvested by centrifugation and washed twice with PBS. Cells were than lysed by pi petting in hypotonic ELB buffer (10mM Hepes pH 8, 10 mM NaCl, 3 mM $MgCl_2$, 1mM DTT or orthovanadate) supplemented with proteases inhibitors. The homogenate was cwntrifuged at 600 g for 3 minutes at 4°C and the supernatant was again centrifuged at 15,000 g for 15 minutes at 4°C to abtain the clarified cytoplasm. The crude nuclear franction obtained in low-speed centrifugation (pellet) was further purified by resuspending the homogenate with a hypodermic needle in lysis buffer (10 mM Hepes pH 8, 10mM KCl, 1,5 mM $MgCl_2$, 30 mM sucrose, 0,5 mM DTT or 1 mM orthovanadate and proteases inhibitors) containing 0,05% v/v Triton X-100, incubating for 10 min in ice. The suspention was centrifuged at 14000 g for 10 min at 4°C and the nuclear pellet was washed with lysis buffer containing 1 mM $CaCl_2$. The purified nuclei, after quantification performer by measuring spectrophotometrically the absorbance at 260 nm, were resuspended in 1X Laemmli Buffer (2% SDS, 62,5 mM Tris-HCl pH 6.8, 10% v/v glycerol, 5 mM DTT, 0.02% bromophenol blue) and sonicated with Labsonic M (Sartorius) (3 x 30 seconds strokes) or further manipulated to obtain the nuclear extracts.

3.4 Nuclear extracts preparation

Nuclear extracts were obtained by resuspending the purified nuclei in a hypertonic buffer (20 mM Hepes ph 8, 20% v/v glycerol, 0.4 M KCl, 1 mM EDTA, 1mM DTT), supplemented with proteases inhibitors, all in 1:100 diluition. After a 30 minutes incubation in ice with the buffer, the suspention was centrifuged at 14000 rpm for 30 minutes at 4°C. The supernatant

containing the nuclear extracts was collected, while the pellet was again resuspended in the hypertonic buffer and incubated in ice for 15 minutes twice. The suspention was then centrifuged at 14000 rpm for 10 minutes at 4°C and the second and the third supernatant, containing the nuclear extracts, were added to the first one.

3.5 Western blotting

Samples in 1X Laemmli buffer were boiled for 3 minutes and than loaded on 7.5% or 10% SDS-acylamide gel. After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride (PVDF, Immobilon, Millipore) membranes using a semi-dry blotting apparatus (Pharmacia) in 10 mM CAPS ph 11, containing 20% v/v methanol. Membranes were extensively blocked with TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% v/v Tween 20) containing 0.2% w/v I-Block (Tropix) for colorimetric detection. Membranes were incubated with the primary antibodies, apprpriately diluited, for 1 hour or overnight at 4°C, washed and incubated with anti-mouse or anti-rabbit IgG conjugated with alkaline phosphatase or horseradish peroxidase (Jackson Immunoresearch) for 1 hour. After washing in TBST, membranes were subjected to colorimetric detection using alkaline phosphatase substrates (Roche). Alternatively, after electrophoresis the gels were stained with Coomassie R-250 or Bio-Safe colloidal coomassie (BioRad) to visualize total protein.

3.6 Chromatin-Immunoprecipitation (ChIP)

3.6.1 ChIP for TPX$_2$ experiment

A chromatin immunoprecipitation experiment was carried out on M14 cells (Chichiarelli et al., 2002). DNA- proteins cross-linkages were formed by treating intact cells with cis-diamminedichloroplatinum. This cross-linking reagent was chosen for its high efficiency and for its capacity to identify the protein directly interacting (Lemaire et al., 1991). DNA-protein cross-linked complexes were purified by gel filtration and subjected to immunoprecipitation with a polyclonal anti-Stat3 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). The DNA was purified and enriched, by PCR, with the same 150-base pairs fragment used for the experiment *in vitro*:

5'-CTAAAAAATTAGCTGGGCGTC-3' (forward)

biotin-5'-GACAAAATTTCGCTCTTTCAC-3 (reverse)

3.6.2 ChIP for Results 4.2 and 4.3

DNA-protein cross-linked complexes were formed in cell lines using of 1% formaldehyde (5 min at 37°C) that has propensity to form protein-protein cross-linkages. The immunoprecipitation procedure was carried out by anti-STAT3 antibody immobilized on IgG rabbit magnetic beads (Dynabeads, Invitrogen). The DNA was then purified by phenol-chloroform extraction and ethanol precipitation, and amplified with the primers listed in Table 1. The amount of immunoprecipitated DNA subjected to amplification was measured by means of a QUBIT^{TM} Fluorometer (Invitrogen) following the manufacturer's instructions.

TABLE 1. Promoter-specific primer sequences used for ChIP assays

Gene	**Promoter-specific primers**
DNMT1	5'-CAAAGTGCTGGGATG-3' (forward) 5'-CATGCCACTTCTATAC-3' (reverse)
c-Myc	5'-CTCCTGCCTCGAGAAG-3' (forward) 5'-CTTTTATACTCAGCGCGATC-3' (reverse)
P21	5'-GATCAGGTTGCCCTTTTTTG-3' (forward) 5'-GAAATTGAGGTCCACTGAAC-3' (reverse)
CDC25A	5'-GTTTGGCGCCAACTAGGA-3' (forward) 5'-GAAAACCAAGCCGACCTAC-3' (reverse)
KLK3I	5'-GATCCATGAGGTTCTCAC-3' (forward) 5'-GAGTGGAAGGATATACTTAG-3' (reverse)
SRD5A2	5'-CAAGATGGAGTCGCTCTG-3' (forward) 5'-GTAAGCCTTAACTTGGCCTC-3' (reverse)
EME-1	5'-CAAGCTCTGTGGATGGTAAC-3' (forward) 5'-CTCTCCTCAGGGCTATTAC-3' (reverse)
CRP	5'-CATCTTAGTCTAATAGGACTG-3' (forward) 5'-CTAGTGTCTGTATCGTAATATAG-3' (reverse)
A$_2$-Macroglobuline	5'-GCTTATTAGCTGCTGTAC-3' (forward) 5'-GTCAAGGTTAATTCCTGG-3' (reverse)
MMP-2	5'-GAAGTCAGGGTAGTGGTG-3' (forward) 5'-GATTATCAGCATATCAGGATCTG-3' (reverse)
MMP-9	5'-GTATCCTTGACCTTCTTTC-3' (forward) 5'-GTGGTGATTGGTTTTAATTAG-3' (reverse)
Survivine	5'-GTTGCAGTGAGCTGAGATC-3' (forward) 5'-GTGCCTCCACTGTCTTTTTC-3' (reverse)
hTERT	5'-CTTTGCAGGTGTGATCTC-3' (forward) 5'-CTTTTCAGCCTCAGACTG-3' (reverse)

3.7 Total RNA extraction

Cells were harvested and total RNA was isolated with TRIzol$^{®}$ Reagent (Invitrogen) following the manufacturer's instructions. RNA was precipitated by adding isopropanol and subsequently centrifuged at 12,500 g for 10 minutes at 4°C. The precipitated RNA was washed with 75% ethanol and air dried. Total RNA was than resuspended in RNase-free water and subjected to reverse transcription.

3.8 cDNA synthesis and quantitative Reverse Transcription-PCR

The reverse transcription of RNA was conducted with cDNA Synthesis Kit (Bioline), according to the manufacturer's instructions. The reaction mixture was prepared incubating total RNA with oligo dTs at 65°C for 10 minutes, then the primed RNA was incubated with the reverse transcriptase at 42°C for 1 hour. The reaction was terminated by incubating at 70°C for 15 minutes. Gene expression was evaluated with specific primers (Quanti-Tect QIAGEN) by Real-Time PCR using a MJ MiniOpticon Detection System (BioRad Laboratories) with SYBR green fluorophore using Brilliant SYBR Green QPCR Master Mix (Stratagene). All reactions were carried out at least in duplicate. GAPDH (QuantiTect QIAGEN) gene was used as reference for normalization and the relative quantification was analyzed using Gene Expression analysis for iCycler iQ real-time PCR detection

3.9 Electophoresis Mobilty Shift Assay (EMSA)

An EMSA experiment was performed using, as probes, the ^{33}P-end labeled double-stranded oligonucleotides corresponding to the four potential Stat3-binding sites present in the region between the *BCL2L1* and the *TPX2* genes. The probes were treated with the nuclear extracts prepared from M14 cells. Protein-DNA complexes were resolved by non-denaturing 5% polyacrylamide gel electrophoresis and specific Stat3/DNA complexes were detected by autoradiography. Poly (dI-dC).poly (dI-dC) was present as a competitor in a 500-, 1000- or 2000-fold excess.

Effect of mutation of the Stat3 consensus sequence. The gel-retardation was performed with a similar DNA fragment, mutated within the Stat3 consensus sequence #3 (TGCAGGAGAATCACTTCC**ATG**T**T**GGGGGAG GTTCA, the mutated bases are indicated in bold characters).

3.9.1 EMSA analysis with radiolabeled probe #3

Nuclear extracts from M14 and ARO cells were preincubated for 30 min with or without 1 mM phosphopeptide PpYLKTK-mts (Tukson et al., 2001; Rojas et al., 1998) before the addition to the probe. The probe was tested with the nuclear extracts prepared from M14 cells treated or untreated with the inhibitor peptide. M14 cells were grown in plastic dish in RPMI supplemented with 10% calf serum and were incubated for 48 hours with or without 1mM phosphopeptide inhibitor.

3.10 In vitro DNA-affinity

A biotinylated DNA fragments from the 5'-flanking region (-4379/-4229) of the human *TPX2* gene was prepared by PCR using the following primers: 5'-CTAAAAAATTAGCTGGGCGTC-3' and biotin-5'-GACAAAATTTCGCTCTTTCAC-3'. The other biotinylated oligonucleotides of 100 bp, homologous to the promotes of the genes that spanning STAT3 sites, were prepared by PCR using the primers listed in the table above. These probes were immobilized on magnetic Dynabeads (Dynal, InVitrogen, Carlsbad, CA, USA) M-280-Streptavidin (200 pmol/mg beads) and incubated with nuclear extracts of M14, PC3 and LNCaP cell in the presence of a 1000-fold excess poly(dI-dC).poly(dI-dC). The specifically bound proteins were eluted with Laemmli buffer and analyzed by SDS-gel electrophoresis in 10% polyacrylamide and Western blotting using specific antibodies (Cell Signaling Technology, Beverly, MA, USA). A control experiment was carried out by using streptavidin beads saturated with biotin.

3.11 Reporter plasmid and luciferase assay

The reporter gene constructs bearing the fragment of the human 5'-flanking region (-4379/-4229) of the human *TPX2* gene were generated by PCR amplification of human genomic DNA. The primers for the PCR reaction were as follows:

tpx2-rg**sacI** 5'-GG**GAGCTC**GTCTGTCTTTTTAC -3' (forward)

tpx2-rg**bglII** 5'-GGG**AGATCT**GAAATTTGTAAGGTAAC-3' (reverse).

The bp of the 5'-flanking fragment were then cloned into the SacI/BglII site

of firefly luciferase pGL3-promoter vector (Promega) and verified by sequencing. For luciferase assays, cells (5 x 10^4 cells/well) grown in a standard culture medium containing FBS in 24-well plates were transiently transfected with the reporter plasmids using TurboFect^{TM} *in vitro* Transfection Reagent (Fermentas) according to the manufacturer's instructions. After treatment, cells were lysed, and luciferase activities were determined by a chemiluminescence assay using the luciferase assay kit (Promega) and a Appliskan luminometer.

3.12 TISSUE SPECIMENS

3.12.1 Immunohistochemical Stained

Briefly, formalin fixed, paraffin embedded tumor and normal sections (5 μm) were deparaffinized in xylene and rehydrated through graded ethanols. The slides were placed in citrate buffer (10 mM., pH 6.0) and microwaved for 60 minutes for antigen retrieval. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide for 30 minutes. The sections were incubated overnight at 4°C with rabbit polyclonal anti-p-STAT3 at Tyr 705 (p-STAT3) antibody or rabbit polyclonal anti-total STAT3 antibody (Cell Signaling Technology). The secondary antibody is with FITC goat-anti-Rabbit-IgG (Jackson Immunoresearch). The two antibodies were used at a dilution of 1:25. The sections also were counterstained with DAPI in PBS (0,03μg/ml) and mounted with glass coverslips. Formalin fixed, paraffin embedded sections of prostate cancer specimens served as a positive control in each experiment. For the negative

control the primary antibody was omitted during the procedure (Horiguchi A. et all, 2002).

3.12.2 Protein Extraction From FFPE (Formalin-Fixed Paraffin Embedded)

Before protein extraction, 5 μm tissue section were weighed (about 3 approximately equals 30 mg) (Ilona Gräntzdörffer, 2010). The deparaffinization was carried out incubating twice with xylene for 5 minutes at room temperature before rehydration in a graded series of ethanol (100%, 90% and 70%) for 10 min each. The eppendorf were put in vacuum dryer for 15'. Afterwards, the samples were mixed with FFPE extraction buffer (150 μl per sample), sonicate for about 7-8 times, incubated at 100 °C for 20' h and 80°C for 2 hours, than centrifuged for 30 min at 14.000 rpm. The supernatant was supplemented with 1/10 volume of Thiourea 1M and stored at −20 °C. The protein extraction of the FFPE tissue slides was similar. Protein concentrations were determined by the BCA assay (Bio-Rad, Munich, Germany).

3.12.3 Extraction of total RNA from formalin-fixed paraffin-embedded tissue

Total RNA was extracted from three 10 μm thick FFPE sections (corresponding about 30 mg of tissue). RNA was purified with WAXFREE[TM] paraffin kit (TrimGen) according to the manufacturer's

instructions. RNA was quantified spectrophotometrically, and its quality was ossesse by 1.5% agarose gel electrophoresis and staining with ethidium bromide. To rule out the possibilità of DNA contamination, the resolved RNA was incubated with 10 μm/ml RNase-free DNase at 37°C for 30 min.

3.12.4 Reverse Transcription and RT^2-PCR

The reverse transcription was carried out with PrimeScript^{TM} RT reagent Kit (Takara). Gene expression was evaluated with specific primers (Quanti-Tect QIAGEN) by Real-Time PCR using a MJ MiniOpticon Detection System (BioRad Laboratories) with SYBR green fluorophore using Brilliant SYBR Green QPCR Master Mix (Stratagene).

4. RESULTS

4.1 IDENTIFICATION OF A NOVEL STAT3 TARGET GENE.

An inspection of the 5'-region of human *TPX2* gene on chromosome 20 revealed the presence of many consensus sequences for transcription factors, among which are some putative sites for STAT factors. Among the genes whose expression is favoured by STAT3 and which may contribute to the oncogenic potential of STAT3 are those coding for the anti-apoptotic proteins Bcl-2, Mcl-1, survivin, and Bcl-x_L. However, the binding sites of STAT3 on the promoters of these genes have not always been experimentally demonstrated. In particular, the 5'-flanking region of the human *BCL2L1* gene, coding for Bcl-x_L, has many potentially binding sites for STAT proteins, but none of these correspond closely to the consensus sequence of Stat3. An inspection of this region on human chromosome 20 has revealed that the gene for TPX2 is in proximity with that for Bcl-x_L, the two genes being transcribed in opposite direction on DNA, so that they have in common a 5'-flanking region of more than 16,000 base pairs (Figure 7).
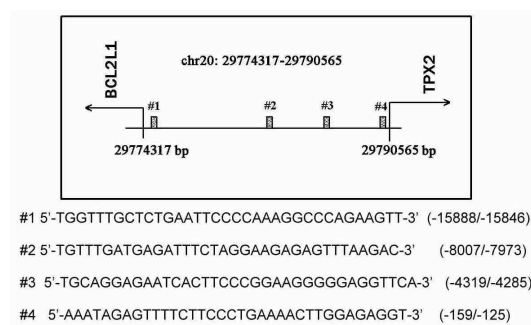

#1 5'-TGGTTTGCTCTGAATTCCCCAAAGGCCCAGAAGTT-3' (-15888/-15846)

#2 5'-TGTTTGATGAGATTTCTAGGAAGAGAGTTTAAGAC-3'   (-8007/-7973)

#3 5'-TGCAGGAGAATCACTTCCCGGAAGGGGGAGGTTCA-3' (-4319/-4285)

#4 5'-AAATAGAGTTTTCTTCCCTGAAAACTTGGAGAGGT-3'   (-159/-125)

Fig. 7: Sequences and positions of putative Stat3-binding elements on the 5'-flanking region of the TPX2 gene.

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In order to identify high-affinity binding sites for Stat3 possibly present in this region, we carried out an EMSA experiment with nuclear extracts from M14 melanoma cells, where Stat3 has already been shown to be constitutively activated (Eufemi et al., 2004). Protein-DNA complexes were resolved by non-denaturing 5% polyacrylamide gel electrophoresis and specific Stat3/DNA complexes were detected by autoradiography. Only one, the probe #3, of the four potential binding sites examined, showed in Figure 1A, appeared to bind Stat3 with high affinity, since the binding was not affected by the presence of a 1000-fold excess of competitor Poly (dI-dC).poly (dI-dC) (Figure 8A).
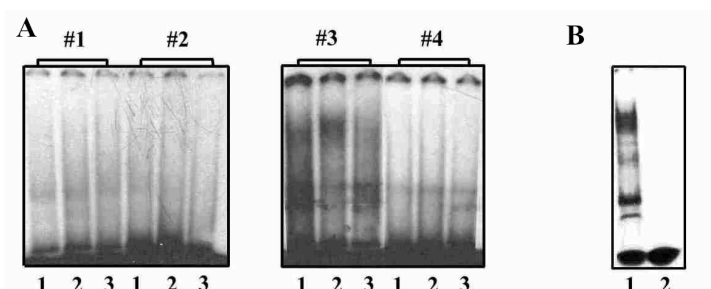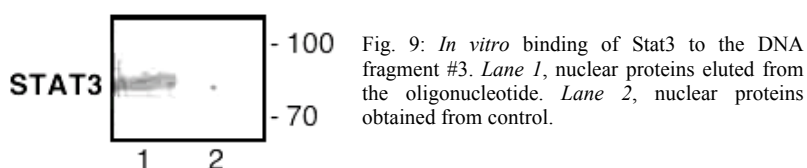

Fig. 8: **A**) Stat3 DNA-binding activity as measured by EMSA in nuclear extracts prepared from M14 cells treated with poly(dI-dC).poly (dI-dC) as a competitor in a 500-, 1000- or 2000-fold excess in *lanes 1, 2* and *3* respectively. **B**) Effect of mutation of the Stat3 consensus sequence. An EMSA experiment with a mutated probe #3. *Lane 1*: probe #3. *Lane 2*: mutated probe #3.

This site is located at -4305/-4297 base pairs from the transcription start of TPX2 and has a TTCCCGGAA sequence, which is identical to the sequence bound by activated Stat3 in the promoter of gene *CDKN1A*, coding for the protein p21$^{WAF1/CIP1}$. Instead no complex was formed when the gel-retardation was performed with a mutated probe #3 TGCAGGAGAATCACTTCC**A**TG**TT**GGGGGAGGTTCA (with, the mutated bases are in indicated in bold characters). (Figure 8B)

Since other members of the Stat family might have been responsible for the formation of this DNA-protein, a 150-base pairs DNA fragment (-4379 to -4229 in the 5'-flanking region of the TPX2 gene, containing the same consensus sequence, was immobilized on magnetic beads and tested for the binding of proteins from the M14-nuclear extract in the presence of a 1000-fold excess of aspecific DNA. The proteins that were specifically bound were eluted and analyzed by gel electrophoresis and Western blotting, and Stat3 was clearly identified among them, as shown in Figure 9.

Fig. 9: *In vitro* binding of Stat3 to the DNA fragment #3. *Lane 1*, nuclear proteins eluted from the oligonucleotide. *Lane 2*, nuclear proteins obtained from control.

These results demonstrate that the examined region in the 5'-flanking sequence of TPX2 gene is able to bind Stat3 *in vitro* with high affinity. To detect the binding of Stat3 *in vivo* to the same sequence, a ChIP experiment was carried out on M14 cells. The DNA-Stat3 complexes were immunoprecipitated with an anti-Stat3 antibody and the DNA was purified and analyzed by PCR. As shown in Figure 9, the immunoprecipitated DNA was enriched with the same 150-base pairs fragment used for the experiment *in vitro*, containing the consensus sequence for Stat3 as described before.

Fig. 10: *In vivo* binding of Stat3 to the DNA fragment #3.

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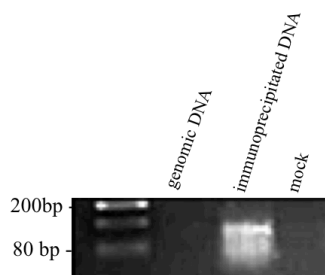
Thus, constitutively activated Stat3 is bound in vivo to this site found in the 5'-region of the *TPX2* gene. This finding supported the possibility that Stat3 is involved in the regulation of *TPX2* expression. To verify this hypothesis, the effect of a specific inhibitor of Stat3 on gene expression was tested. Tukson et al. (2001) have shown that a phosphopeptide able to interact with the SH2 site of STAT3 inhibits the binding of STAT3 to DNA in vitro and in vivo, probably by disrupting the active STAT3 dimers. When used in vivo, it might also associate with non-phosphorylated cytoplasmic STAT3 monomers and form STAT3-peptide complexes that would be incapable of binding to the docking sites of receptors for subsequent *de novo* phosphorylation and activation. By adding a membrane translocating sequence (Rojas et al., 1998) the peptide acquires a full cell-membrane permeability. In the first place this peptide was tested for its inhibitory activity on the formation of the STAT3-DNA complex as detected by gel retardation using the nuclear extracts of M14 cells and the previously described consensus sequence. As expected, the presence of the peptide in the nuclear extracts abolished the formation of the complex (Figure 11).
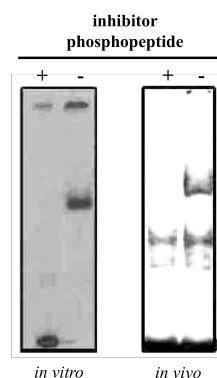

Fig. 11: EMSA analysis with radiolabeled probe #3 to verify the effects of Stat3 inhibition by the phosphopeptide PpYLKTK-mts in M14

Next, the M14 cells in culture were treated with the peptide and then assayed for the presence of TPX2 by gel electrophoresis and Western blotting. Figure

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12 shows that treatment with the inhibitory peptide decreased significantly the amount of TPX2, while leaving essentially unaltered the total proteins of whole nuclear extracts as evidenced by Coomassie staining.
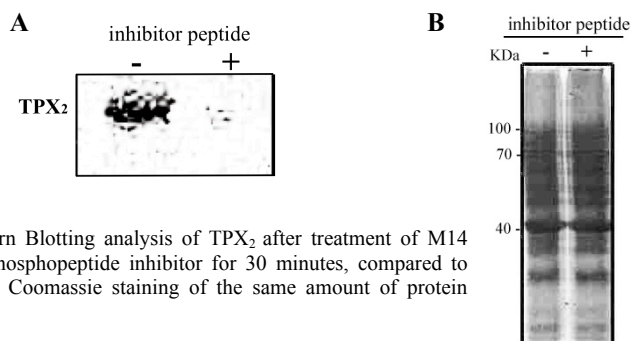

Fig. 12: **A**) Western Blotting analysis of TPX_2 after treatment of M14 cells with 1mM phosphopeptide inhibitor for 30 minutes, compared to untreated cells. **B**) Coomassie staining of the same amount of protein showed in A).

Furthermore, the nuclear extracts of the cells treated with the peptide lost their capacity to form the protein-DNA complex with the consensus sequence for STAT3 (Figure 13). The effect of the peptide on the transcription of the *TPX2* gene was analyzed by RNA isolation, reverse transcription and amplification. As shown in Figure 13, *TPX2* expression was strongly decreased by the action of the inhibitory peptide in comparison to the untreated cells, as showed in RT-PCR assay for *TPX2* expression.
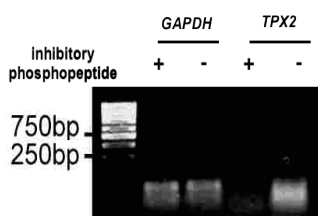

Fig. 13: RT-PCR for *TPX2* expression in M14 cells treated and non with inhibitory phosphopeptide

To demonstrate that Stat3 activation regulated in vivo TPX_2 expression, M14 cells were transiently transfected with a luciferase reporter construct containing the -4379 to -4229 region containing the putative Stat3 binding

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site for reporter activity assay, in the presence or in the absence phosphopeptide inhibitor. Whereas in untreated condition M14 cells showed an increase of luciferase expression eightfold around basal levels, this effect was repressed in M14 treated with STAT3 inhibitor (Figure 14). This result suggests that STAT3 is recruited on the *TPX₂* gene promoter and is involved in its activation.

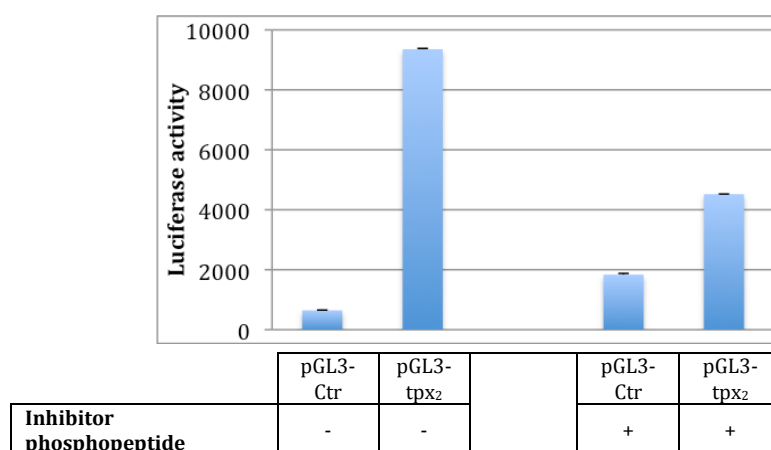|	pGL3-Ctr	pGL3-tpx₂		pGL3-Ctr	pGL3-tpx₂
Inhibitor phosphopeptide	-	-		+	+

Fig. 14: M14 cells, treated and not with inhibitor phosphopeptide, were transfected with a vector expressing a luciferase reporter gene containing a STAT3 consensus binding site for *TPX2* gene. As a control, the same experiments were performed in parallel using a pGL3-control plasmid.

This results and the effect of the same peptide were confirmed on another cell line, the ARO cells, which are human anaplastic thyroid cancer cells with constitutively activated Stat3. As a whole, these results indicate that STAT3 contributes to the activation of *TPX2* expression.

4.2 STUDY ON DYNAMIC INTERPLAY OF STAT3-ASSOCIATED PROTEINS IN RESPONSE TO MULTIPLE ACTIVATION PATHWAYS.

STAT3 has been shown to be constitutively activated in many melanoma cell lines. However, the characterization of the STAT3-containing complexes in these cell lines is still lacking. We proceeded therefore to characterize such complexes in the well characterized M14 melanoma cell line. The activation of STAT3 may occur in through different parhways, we hypotized an interplay between PARP-1 STAT3-associated proteins and CBP/P300, a protein with histone acetyl transferase activity and transcriptional coactivator of STAT3, in response to different stimulations.

The specific binding in vitro of nuclear proteins to a DNA fragment containing a known STAT3 consensus sequence was analyzed by incubating the nuclear extracts of M14 cells stimulated with IL6 (100ng/ml) for 5 min, EGF (100ng/ml) for 10 min and non stimulated with a double stranded fragment of DNA, present in the 5'-regulatory region of the *CDKN1A* gene, which codes for the protein p21$^{WAF1/CIP1}$. This is a 100 base-pair sequence, containing the STAT3-consensus TTCCGGGAA. The DNA fragment, biotinylated at its 5'-end, was immobilized on streptavidin-Dynal-magnetic beads and incubated with the nuclear extract in the presence of a 500-fold excess of unspecific competitor DNA. The specifically bound proteins were then eluted in high salt and analyzed by SDS-PAGE. This was followed either by Coomassie staining or by Western blotting. The Coomassie staining revealed the presence of many protein bands, one of which in the 110-kDa region did not correspond to any other known STAT3-interacting protein. This band was eluted, and its tryptic hydrolyzate examined by mass-spectroscopy, which allowed to identify it as PARP-1 (Figure 15). When the

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specifically bound proteins were analyzed by Western blotting, STAT3 was found to be present, as expected, and also PARP1 (Figure 17).

NAME	SWISS PRONT ACCESSION	MW kDa
54 kDa nuclear RNA and DNA binding protein	Q 15233	54
Poly ADP-Ribose Polymerase-1	P 09874	113
APEX-1	P 27695	116
Ku 80	P 13010	80
ERp57	P 30101	57
Splicing Factor, Proline and Glutamine Rich	P 23246	76
Mitf	O 75030	55-58
Rb	P 06400	105

Fig. 15: STAT3-interacting proteins in 5'-regulatory region of the *CDKN1A* gene obtained by mass-spectroscopy analysis from M14 stimulated and non with EGF. Same results has been found in A431 cells.

This confirmed the identification provided by the mass-spectroscopy analysis and also the in vitro affinity of PARP1 to the STAT3-containing enhanceosome of the *CDKN1A* gene. The binding of PARP-1 to this particular DNA region was tested also by the use of nuclear extracts from HepG2 hepatoma cells, where STAT3 is normally non-activated unless they are subjected to the stimulatory action of a cytokine or a growth factor. In these cell lines we found a different composition of STAT3-dependent enhanceosome of the *CDKN1A* gene. Our attention was then been focused on protein P300 (Figure 16).

NOME	SWISS PRONT ACCESSION	MW kDa
54 kDa nuclear RNA and DNA binding protein	Q 15233	54
P300	Q 92831	300
Brg-1	P 51532	45
Scr-1	P A4H5H2	75
Splicing Factor, Proline and Glutamine Rich	P 23246	76
Mitf	O 75030	55-58
Rb	P 06400	105

Fig. 16: STAT3-interacting proteins in 5'-regulatory region of the *CDKN1A* gene obtained by mass-spectroscopy analysis from M14 stimulated with IL6. Same results has been found in HepG2 and HeLa cells.
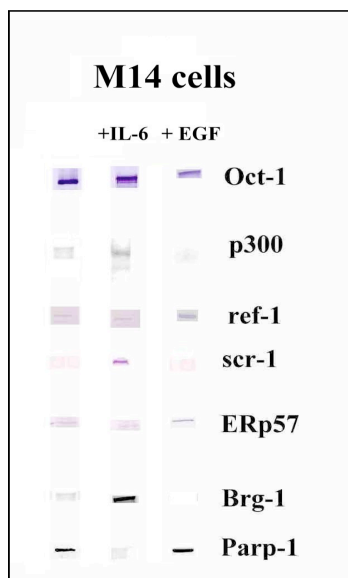
Fig. 17: Western blotting of STAT3-interacting proteins on regulatory region of *CDKN1A* in M14 cells stimulated with IL6 and EGF.

In order to verify if such PARP-1-STAT3 and P300-STAT3 associations take place also in the regulatory regions of other genes, we exploited the same procedure described above to analyze the interactions of nuclear extracts from M14 and HepG2 cells stimulated with IL6 with two other DNA fragments containing well characterized STAT3-consensus sequences, i.e. those of the *α2-macroglobulin* gene and the *MMP9* gene. PARP-1 was found to be present among the proteins specifically bound to DNA in M14 cells, but not in HepG2 cells treated with citokynes, where instead the P300 protein was observed (Figures 18, 19).
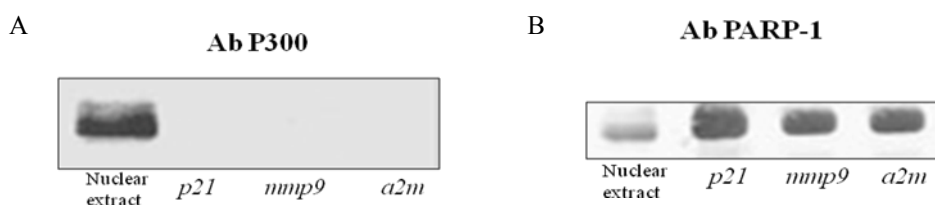

Fig. 18: Western blotting (A) Ab P300 and (B) Ab PARP-1 of total nuclear extracts and proteins specifically bound to of the *p21*, *α2-macroglobulin* and the *MMP2* genes in **M14** cells.
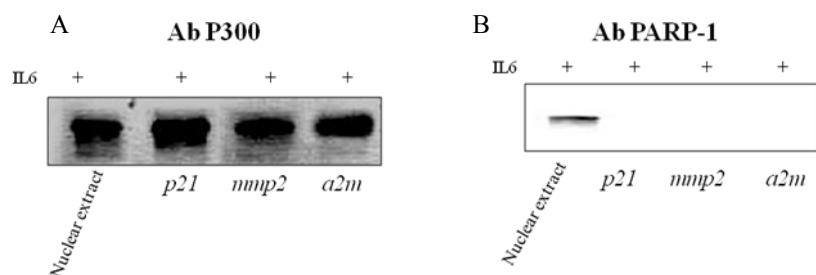
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Fig. 19: Fig. : Western blotting (A) Ab P300 and (B) Ab PARP-1 of total nuclear extracts and proteins specifically bound to of the *p21*, *α2-macroglobulin* and the *MMP2* genes in **HepG2** cells stimulated with IL6.

It is evident that the presence of proteins, PARP-1 or CBP/p300, as components of nucleoproteins complexes depends on specific pathways of STAT3 activation. Our attention was focused in melanoma cell (M14), cellular model of choice, because STAT3 is constitutively activated by src but is also responsive to stimulation by cytokine (IL-6 100ng/ml), and growth factors (EGF100ng/ml). To test whether the interactions between PARP-1 or CBP/p300 and DNA-bound STAT3 is present also *in vivo,* were performed ChIP and CoIP experiments. The nuclear extracts from M14 cells stimulated with EGF and IL6 were treated with formaldehyde, the immunoprecipitation with Ab STAT3 was carried out as described in Materials and Methods. An analysis of the immunopurified material by Western blotting revealed the presence of PARP-1 when STAT3 was activated by SCR/EGF. (Figure 20) On the other hand, treatment with anti-PARP-1 antibodies revealed the presence of STAT3 in the immunopurified fraction (data not shown). After treatment of IL6, we found the presence of CBP/P300 instead of PARP-1.This result shows that the two proteins are associated even in the absence of DNA, either through a direct association or through an interaction mediated by another protein.
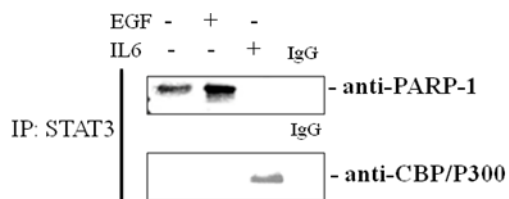
Fig. 20: Anti-STAT3 immunoprecipitates from M14 cells untreated and stimulated with EGF and Il6 analyzed by Western blot with antibodies raised against PARP-1 (top panel) and P300 (bottom panel). Immunoprecipitation in the last lanes refer to rabbit IgG shows no signal indicating assay specificity.

The same experiments performed after pretreatment of the cell with specific inhibitors: 3-ABA for PARP-1 and anacardic acid for CBP/p300, showed that interactions STAT3-PARP-1 and STAT3-CBP/p300 were abolished (Figure 21).
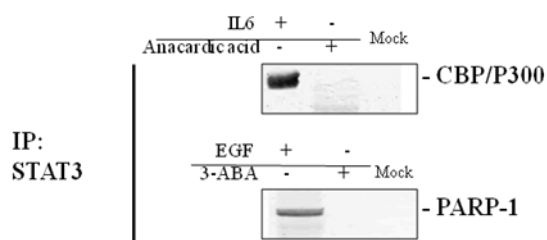

Fig. 21: Anti-STAT3 immunoprecipitates from M14 cells stimulated with EGF and Il6 and treated with anacardic acid, as P300 inhibitor, and 3-ABA, as PARP-1 inhibitor, analyzed by Western blot with antibodies raised against P300 (top panel) and PARP-1 (bottom panel). Immunoprecipitation in the last lanes refer to rabbit IgG shows no signal indicating assay specificity.

To test whether the interaction between PARP-1, P300 and the DNA-bound STAT3 is present also *in vivo*, chromatin immunoprecipitation experiments were performed. Intact M14 cells were stimulated with EGF or IL6 and after treated with the specific inhibitors, 3-ABA in cells with EGF, anacardic acid

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in cells with IL6. The cross-linking was carried out with formaldehyde, and immunoprecipitated with antibody against STAT3. The immunoprecipitated DNA was analyzed for enrichment of the *CDKN1A (P21)*, the *α2-macroglobulin* and the *MMP9* enhancers by PCR amplification with specific primers. As shown in Fig. 22, cross-linking with formaldehyde led to the enrichment of the three enhancers sequences in the immunoprecipitated DNA in both conditions of stimulation. After the treatment with specific PARP-1 and P300 inhibitor we observed a down-regulation of same genes.
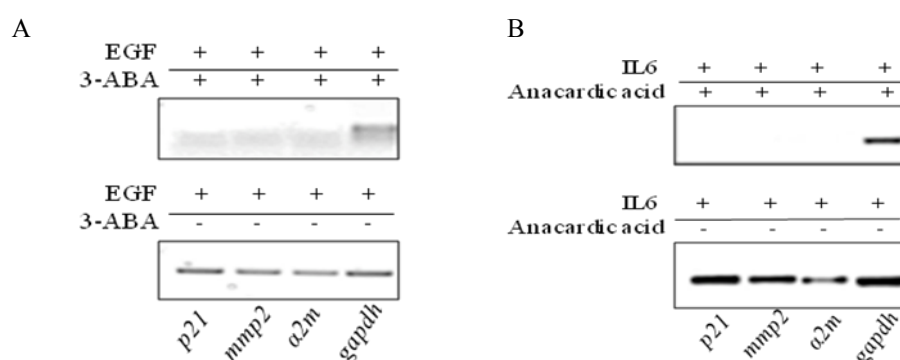

Fig. 22: PCR analysis of the enrichment of STAT3-binding sites in the immunoprecipitated DNA of *p21, mmp2 and α2macroglobulin* genes in M14 cells. (A) Cells were stimulated with EGF and after treated with 3-ABA (top panel) or not (bottom panel). (B) Cells were stimulated with IL6 and after treated with Anacardic acid (top panel) or not (bottom panel). *Gapdh* gene was used as reference gene.

To study the functional effects of the interaction between PARP-1/STAT3 and P300/STAT3 we performed experiments of RT-PCR in the same conditions descrive above. Figure 23 shows that in both cases the transcription of the three genes was significantly repressed.

A

B

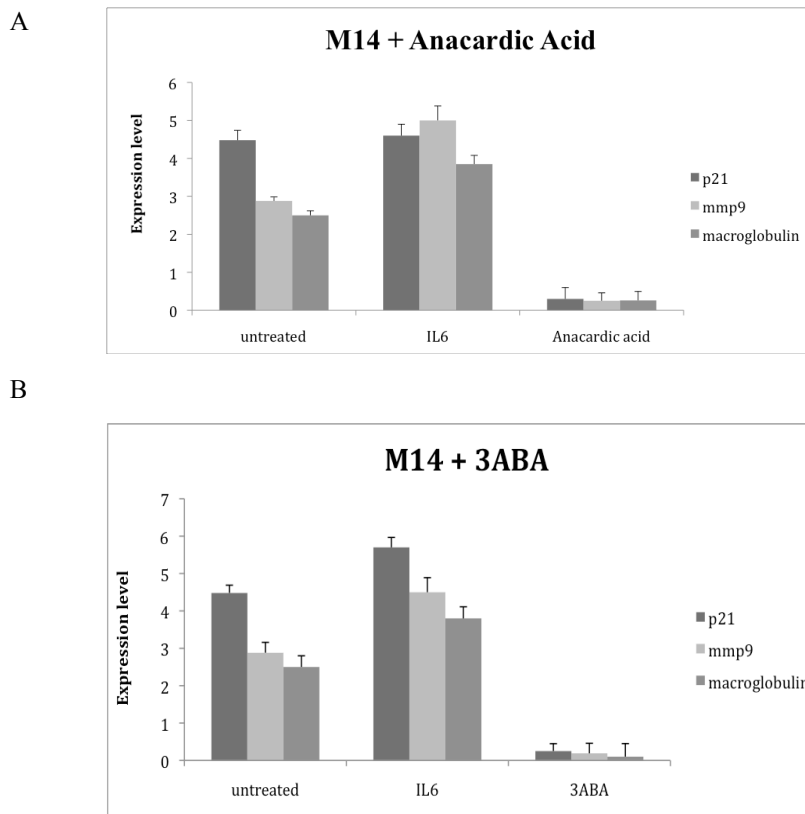Fig. 23: Expression profile of *p21, mmp-9, α2M,* in M14 cells. (**A**) M14 treated with EGF and 3-ABA; (**B**) M14 treated with IL6 and Anacardi acid.

These results support the hypothesis that either PARP-1 or CBP/p300 appears to be specific co-activators of STAT3, in different pathways of activation, and their enzymatic activity seem necessary for this role.

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4.3 STAT3 IN PROSTATE CANCER

4.3.1 *A) Studies on human prostate cancer: LNCaP hormone-responsive and PC3 hormone refractory.*

4.3.1.1 Immunoblotting analysis of Stat3 protein and its Post-Translational Modifications (PTMs).

To study of involvement of STAT3 in prostate cancer we analyzed two well-characterized human cell lines, LNCaP and PC3. To simulate the different physiological conditions in which STAT3 is involved, cells were stimulated with IL-6 and hydrogen peroxide for 15 min. Nuclear extract were prepared and subjected to Western blotting to verify the presence of STAT3, phospho-Y^{705}STAT3, acetyl-STAT3 and STAT3 glutathionylated (Figure 24). In LNCaP untreated there was evidence of STAT3 and pY-STAT3 in all conditions, while the acetylation evidence after cytokines stimulation and the glutathionylation after oxidative stress. Surprisingly in untreated PC3 cell line we found STAT3 unphosphorylated and glutathionylated, instead the modification of STAT3 protein by acetylation was observed also after IL-6 stimulation.
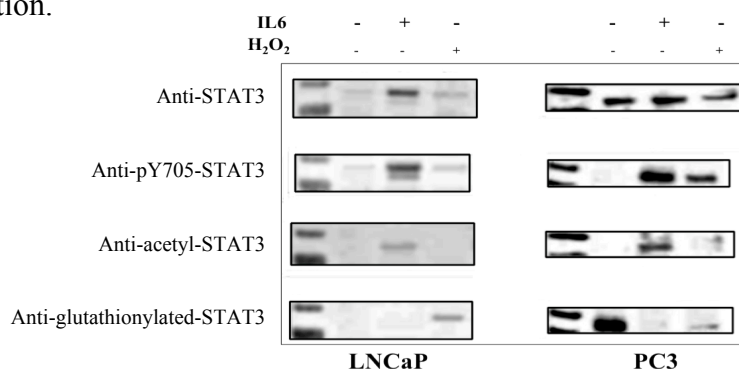

Fig 24. LNCaP and PC3 cell lines were stimulated with IL6 (50ng/ml) and H$_2$O$_2$ (100mM) for 15 min at 37°C. Nuclear extracts from untreated and stimulated cells were analyzed by Western blot with antibody raised against STAT3, pY^{705}-STAT3, acK^{685}-STAT3. For the glutathionylation we performed a double staining with a monoclonal antibody against protein-bound GSH and Ab STAT3.

4.3.1.2 STAT3 nuclear protein interactors complex in PC3 cell line.

To identify protein interactors of STAT3 in PC3 cell we performed coimmunoprecipitation assays using wild-type and stimulated cells with IL-6 and H_2O_2. Anti-STAT3 antibodies conjugated beads were incubated with nuclear lysates of this cells and the immune complexes were fractionated on SDS-PAGE and proteins were detected by immunoblot. We focused attention on specific proteins previously described as coactivators of pY-Stat3 and U-Stat3 (Chichiarelli S., 2010; Eufemi M., 2004; Grillo C. et al., 2006; Yang J., 2007). Western blot analysis was performed with antibodies raised against p50 and p65 subunits of NFκB, p300, ERp57 and Ref1 (Figure 25). We observed that p50 and p65 subunits of NFκB were pulled down with STAT3 in unstimulated conditions. Instead STAT3 coimmunoprecipitated with p300 protein in IL-6 stimulated cells and with ERp57 and APE1/Ref-1 in response to H_2O_2 stimulation. We did not observe any complex formation using IgG as the immunoprecipitanting Ab, demonstrating assay specificity. AbSTAT3-Ref-1 and STAT3-ERp57 complexes in IL-6 and H_2O_2 stimulated PC3 cells indicate that the interactions are primarily stimulus-dependent.
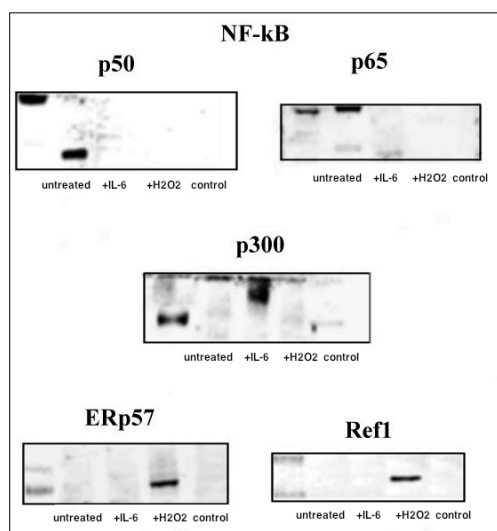
Fig. 25. Western blot of p50 and p65 subunits of NFκB, p300, ERp57 and Ref-1 in PC3 cells untreated and stimulated with IL6 and H_2O_2. Immunoprecipitation in the lanes control refer to rabbit IgG shows no signal indicating assay specificity.

4.3.1.3 STAT3-DNA binding activity in the promoter of selected genes.

Our next aim was to determine how the differences in post-translational modifications and in cofactors could affect STAT3 protein in its function as transcription factors under physiological conditions. Therefore chromatin immunoprecipitation (ChIP) experiments were carried out with the Ab STAT3 in LNCaP and PC3 cell lines untreated and stimulated as described before. Chromatin was prepared using formaldehyde cross-linking protocol, and occupancy of the promoters was analyzed by PCR using specific pairs of primers in the 5'-flanking regions of STAT3-binding sequences, representing the main functions under STAT3 control (see "Materials and Methods"). To ensure the specificity of the reaction, all immunoprecipitations were subjected to one round of preclearing with an excess of nonrelated IgG. Figure 26 shows that the fragments examinated

were enriched in the immunoprecipitated in different manner in both cell lines and in response to two stimulations.
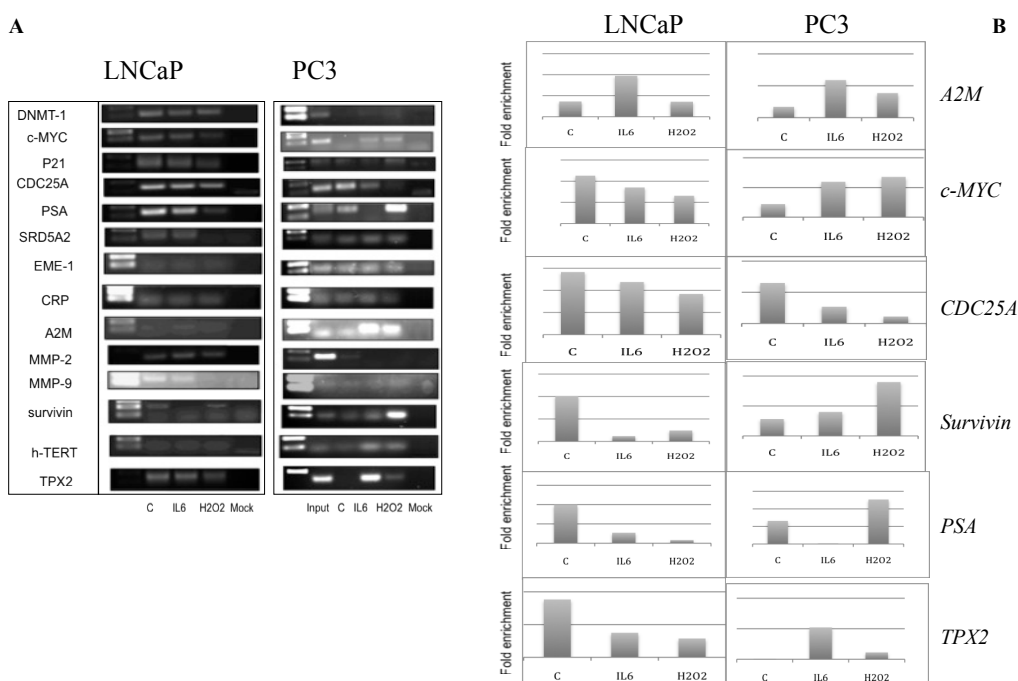

Fig. 26: (A) PCR analysis of the enrichment of STAT3-binding sites in the immunoprecipitated DNA in comparison with the mock immunoprecipitation (performed with preimmune IgGs). The DNA fragments present in the 5'-region of the genes indicated, and containing the STAT3 consensus sequence, were amplified with the primers shown in Table , as described in "Materials and Methods". (B) Enrichment of STAT3-binding site of α2-Macroglobulin, c-MYC, CDC25A, Survivin, PSA and TPX$_2$ primers obtained from densitometric analysis of panel A.

Particularly, we observed a significant variation in the level of enrichment in six fragments out of fourteen examinated: *c-MYC, CDC25A, PSA, A2M, Survivin, TPX$_2$,* coding respectively for a transcription factor, for a cell-cycle protein phosphatase, for a kallikrein-related peptidase3, for α2-macroglobulin, for an inhibitor of apoptosis and for a microtubule-associated protein.

To study the functional effect of STAT3 on its target gene in this cell lines, we extracted total RNA from treated and control cells and mRNA was

converted to DNA by reverse transcription. The expression levels of genes tested by ChIP experiments was measured by real-time PCR. In Figure 27 reports a graphical dispaly of the expression profile of the *A2M, c-MYC, CDC25A* and *Survivin* genes in IL-6 and H_2O_2 stimulated and control cells. The expression levels of the genes was normalized with the expression of GAPDH in the same sample, as reference gene. The expression profiles of this genes showed some differences in two cell lines and in response to stimulations and was compared to results obtained from densitometric anlaysis of the same genes. Only for A2M expression level, we did not observe any expression, but this can be explained by negative control that STAT3 can have on the promoter of this gene.
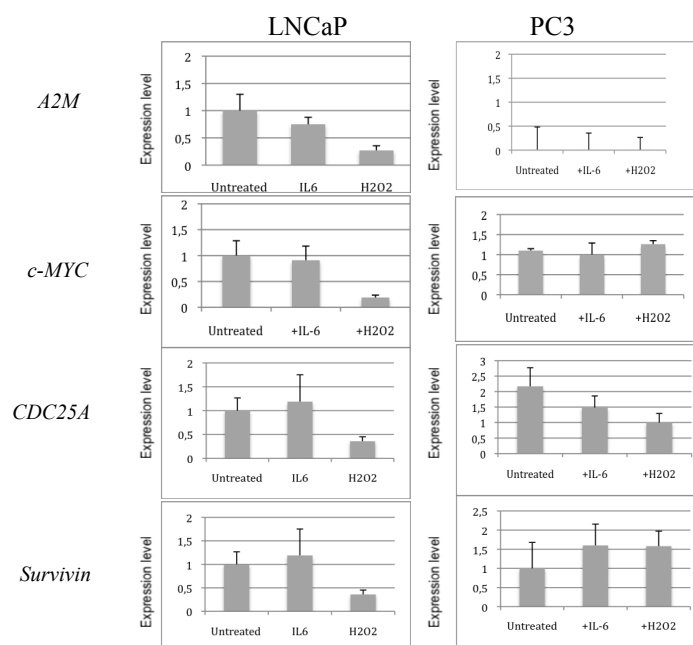

Fig .27: Expression profile of *A2M, c-MYC, CDC25A, Survin* in LNCaP and PC3 cells untreated and stimulated with IL-6 and H_2O_2.

4.3.1.4 Correlation between STAT3 PTMs and expression genes.

Post-translational modifications (PTMs) of nuclear proteins play essential roles in the regulation of gene transcription and signal transduction pathways. We evaluated the phosphorylation, the acetylation and the glutathionylation with STAT3 transcriptional activity in wild-type prostate cancer cells and in response to cytokines and to oxidative stress. In table (Figure 28) we correlated STAT3 PTMs and genes that showed greater differences in expression profiles obtained from ChIP and RT^2-PCR experiments. Stat3 activation regulates genes involved in cell growth and cell survival, including *c-Myc*, *Survivin*, *Cdc25A*. Lys685 acetylation was found to be critical for cell cycle–related genes expression, *Cdc25A* and *Survivin*, in hormone-responsive LNCaP cell line. By contrast in PC3 cell line the glutathionylation of STAT3 was involved in the the regulation of genes. However, neither Tyr705 phosphorylation alone nor Lys685 acetylation alone seems to be sufficient for Stat3 activation, contrary to glutathionylation that occurs in intact PC3 cells under basal conditions and is critical for up-regulation of *Cdc25A*. The simultaneus presence of both the phosphorylation and glutathionylation, that occurs in response to oxidative stress, had two different effects: in LNCaP cells all genes were down-regulated and particulary the *c-Myc*; in PC3 cells these modifications promoted the up-regulation of *Survivin* compared to untreated cells.

LNCaP

STAT3- glutC	
STAT3 pY^{705}-acK^{685}	*CDC25A, Survivine*
STAT3 pY^{705}-glutC	All genes down-regulated, in particular *c-MYC*

PC3

STAT3- glutC	*CDC25A*
STAT3 pY^{705}-acK^{685}	α_2*-Macroglobuline*
STAT3 pY^{705}-glutC	*Survivine*

Fig. 28: Correlations between PTMs of STAT3 and STAT3 transcriptional activity on genes that showed greater differences in expression profiles obtained from previous ChIP and RT^2-PCR experiments in LNCaP and PC3 cell lines.

4.3.2 B) FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUES

4.3.2.1 Stat3 and its PTMs correlate with High Gleason Score.

Forty-six specimens from 23 matched prostate tumor were evaluated. The sample FFPE analyzed consisted of one sample each from area of prostatic adenocarcinomas and "normal adjacent to tumors" from patients with different Gleason grades and TNM stage tumors. The levels of activated phospho-Stat3 protein and the main post-translational modifications in prostate tumors were detected by immunoblotting analysis, normalized with actin, and were correlated with more malignant tumor specimens exhibiting Gleason scores 6, 7 and 8 (Figures 29, 30).

Gleason Score 6

STAT3

T N T N T N T N T N T N T N T N T N T N

STAT3-TYR705-P

T N T N T N T N T N T N T N T N T N T N

STAT3 Acetilata

T N T N T N T N T N T N T N T N T N T N

STAT3 Gutationilata

T N T N T N T N T N T N T N T N T N T N

Gleason Score 7

STAT3

T N T N T N T N T N T N T N T N T N

STAT3-TYR705-P

T N T N T N T N T N T N T N T N

STAT3 acetilata

T N T N T N T N T N T N T N T N T N

STAT3 glutationilata

T N T N T N T N T N T N T N T N

Gleason Score 8

STAT3

T N T N T N T N

STAT3-TYR705-P

T N T N T N T N

STAT3 acetilata

T N T N T N T N
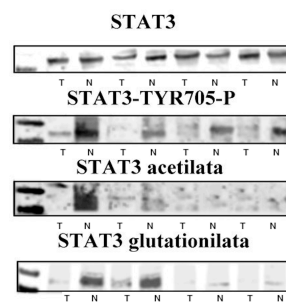
STAT3 glutationilata

T N T N T N T N

Fig. 29: Western blot analysis of protein extracted from FFPE for Gleason Scores 6, 7, 8 of STAT3, pY[705]-STAT3, acK[685]-STAT3. For the glutathionylation we performed a double staining with a monoclonal antibody against protein-bound GSH and Ab STAT3. T: prostate carcinomas; N: matched normal adjacent to tumor.

There was a significant correlation between levels of acetylated and glutathionylated Stat3 in different clinical stage (Table A). In fact we observed a decrease of acetylation as post-translational modification with more malignant tumor, by contrast an increase of glutathionylation with more Gleason Score. It was not possible to assess the correlation between levels of Stat3 and its PTMs and established parameters of disease progression (*e.g.,* PSA levels after prostatectomy) because of the short time of follow-up after

treatment of this cohort of patients. Thus, more different PTMs of Stat3 levels correlate well with more aggressive disease in prostate cancer as determined by Gleason score at the time of radical prostatectomy.

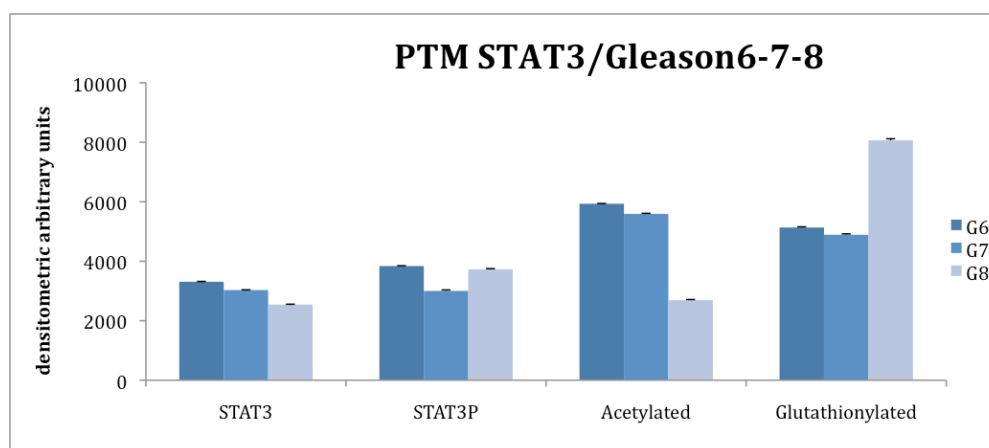| TABLE A. STAT3 Activity and Gleason Score			
	Gleason score = 6	Gleason score = 7	Gleason score = 8
Sample no.	**10**	**8**	**4**
STAT3 total	3309	3031	2541
pY-STAT3	3838	3002	3727
acK-STAT3	5932	5591	2691
Glut-STAT3	5133	2691	8062

Fig. 30: Cumulative results of STAT3 levels in tumoral prostate tissues from 23 matched prostate carcinomas. The data were obtained from densitometric analysis of Immunoblotting in Fig. Each bar is the average of all tumor samples for each Gleason Score normalized with actin, used as a reference to normalize the amount of protein extracts loaded.

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5. DISCUSSION

STAT3 transcription factor resides at the critical junction between intracellular signaling events and the regulation of genes involved in many key cellular processes (Frank D.A., 2007). In addition, its effect mediated through interactions with other proteins allows it to control cellular function on multiple levels (German D., 2007).

In our research we have demonstrated that STAT3 contributes to the activation of a novel target gene involved in oncogenic process. The high-affinity binding site for at –4305/-4297 bp from the transcription start in *TPX2* regulatory region is likely to be involved in this regulation. In fact, this site is bound in vivo by STAT3 and its sequence is identical to the well-characterized STAT3-binding site in the *CDKN1A* gene. Furthermore an examination of the corresponding 5'-region of the mouse *Tpx2* gene on chromosome 2 revealed the existence of a STAT consensus sequence at -3965/-3957 base pairs. The similarity of sequences in gene-flanking regions in genomes of different species supports the existence of similar regulatory mechanisms and the intervention of the same transcription factors. The regulatory binding sites of the STAT proteins are often found at a short distance from the promoter. However, binding at high distance has also been described. Thus, for example, a site for STAT3 at -1093 has identified in the human perforin gene, and for STAT5 at -4285 in the human IL-2Rα gene. Considering that the *BCL2L1* and the *TPX2* genes have the 5'-region in common, it could be argued that the STAT3 site that we described might also regulate the expression of *BCL2L1*. However, it appears that constitutively activated STAT3 produces an increase in the transcription of the gene,

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contributing to the over-expression of TPX2 observed in a variety of tumours. In this regard it should be mentioned that highly expressed TPX2 has been detected by in hepatocellular carcinoma and in lung, prostate, ovarian, pancreatic and colon cancer cell lines. In all these cancer cells STAT3 has been found constitutively activated (Yu and Jove, 2004; Sanchez et al., 2003; Lin et al., 2005).

Activated STAT3 is involved in the regulation of expression of a multitude of genes, but the cellular effects elicited can vary greatly depending on cell type and on the mechanism of activation of STAT3 itself. This variety of effects might be explained by many factors, such as the particular composition of the STAT3-multiprotein complex binding to DNA, the post-translational modifications of STAT3. Our finding add PARP-1 to the list of proteins which can associated in the nucleus with STAT3. PARPs constitute a family of 18 distinct enzymes catalyzing the sequential transfer of ADPr units, by using nicotinamide adenine dinucleotide (NAD^+) as substrate, on to protein acceptors to produce linear and/or branched polymers of ADPr. The mammalian PARP-1 is the major isoform of the PARP family, an abundant and ubiquitous nuclear protein, that plays important roles in a variety of genomic processes, including the regulation of chromatin structure and transcription in response to specific cellular signals (Kraus W.L., 2008). The nuclear PARP (type I) is a 113 kDa protein and appears to be the major poly(ADP-ribosyl)ating activity in higher eukaryotes after DNA damage. In fact in the past the main interest about PARP-1 was directed to its role on sensing, and binding to, the damaged DNA, thus being involved in the process of DNA repair. But another important role of PARP-1 is its participation in transcription regulation, either by means of poly(ADP-ribosyl)ation of histones with a consequent decondensation of chromatin, or

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by its association with protein complexes which bind to enhancers or promoter, which can lead to an activation or repression of transcription. Interestingly, the some function, but in different way, is carried out by CBP/P300 protein, an histone acetylases protein able to modifie histone subunits within the core nucleosome, described as coactivator for many transcription factor. The association of STAT3 with the P300 coctivator it was largely described (Ray S., 2005; Yuan Z., 2005). Phosphorylated STAT3 dimers recruit CBP/P300 protein, which, in turn, acetylates the NH2 terminus of STAT3. Finally, deacetylation of AcSTAT3 by HDACs 1 and 4 reduced the affinity of STAT3 for the P300 coactivators, leading to subsequent STAT3 dephosphorylation, ubiquitination, and/or cytoplasmatic redistribution. The existence of a specific order of the action of the histone/factor-modifying complexes implies a signaling pathway for mediating gene activation/repression events, and for temporal-specific "sensor" responding to additional signalling pathway activated/extinguished during the periodic time interval of coregulator exchange (Rosenfeld M.G., 2009). Considering that we found a different set of STAT3-associated proteins in tumoral cell lines in which the activation of STAT3 can be mediated by different pathways, we can speculate not only a tumor-specific, but a signal-dependent composition of enhanceosome STAT3-specific. Particularly PARP-1 interacted with STAT3 when the transcription factor was phosphorylated by Src-kinases or after EGF stimulation, instead the association with CBP/P300 is IL-6 inducted. We have shown that PARP-1 and P300 can affect STAT3 activity in the regulation of some target genes. Using ChIP we found that PARP-1 and P300 were recruited to the promoter of the *P21^{Waf1}*, *α2macroglobulin* and *MMP-9* genes depending on the stimulus. Moreover the two proteins directly regulated STAT3's

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transcriptional activity because a specific inhibition of PARP-1 and P300 decreased its target genes expression by modulating its binding to the promoter. However, the exact mechanism by which P300 and specially PARP-1 regulates STAT3's-DNA binding is still not clear, but may be correlated with different PTMs that these proteins can bring not only on the chromatin, but also on the different components of macromolecular complexes. In the study of STAT3 partners that affect its function, we demonstrated the importance of PTMs of STAT3 in prostate cancer (CaP). This work evaluated in parallel, by immunoblotting analysis, the variation of phosphorylation, acetylation and gluthationylation of STAT3 in cell lines and in human prostate tumor (FFPE). Our analysis demonstrates that inverse levels of acetylation and gluthationylation of Stat3 are associated with higher Gleason scores (6, 7, 8), which is indicative of more aggressive and poorly differentiated tumors. This observation may be valuable for identification and management of high-risk prostate cancer patients, especially those with high Gleason scores. In LNCaP and PC3 cell lines we observed how a change in PTMs could affect in different interaction with coactivator protein and then in gene expression. It can be speculate that S-gluthationylation of STAT3 and the recruitment, as coactivators, of ERp57 and Ref-1, two protein involved in redox modification, is a response to oxidative stress, associated with a more advanced state of disease. Moreover, recent studies indicate that IL-6 induces androgen receptor-mediated gene regulation through the STAT3 protein, raising the possibility that IL-6-induced STAT3 activation may contribute to the development of hormone-refractory prostate cancer. More studies would be required on PTMs of STAT3 and its coregulators to extend the knowledge about its oncogenic potential to find a viable strategy for delaying or preventing progression of PCa to androgen refractory state.

REFERENCES

Aggarwal B.B., Kunnumakkara A.B., Harikumar K.B., Gupta S.R., Tharakan S.T., Koca C., Sanjit Dey, and Sung B. Signal Transducer and Activator of Transcription-3, Inflammation, and Cancer. Natural Compounds and Their Role in Apoptotic Cell Signaling Pathways 2009. *Ann. N.Y. Acad. Sci*. 1171: 59–76.

Aggarwal B.B., Sethi G., Ahn K.S., Sandur S.K., Pandey M.K., Kunnumakkara A.B., Sung B., and Ichikawa H. Targeting Signal-Transducer-and-Activator-of-Transcription-3 for Prevention and Therapy of Cancer. Modern Target but Ancient Solution. 2006; *Ann. N.Y. Acad. Sci*. 1091: 151–169.

Akira S., Nishio Y., Inoue M., Wang X.J., Wei S., Matsusaka T., Yoshida K., Sudo T., Naruto M., Kishimoto T. Molecular cloning of APRF, a novel IFN-stimulated gene factor involved in the gp 130-mediated signaling pathway. *Cell* 1994; 77(1): 63-71.

Alvarez J.V., Greulich H., Sellers W.R., Meyerson M., Frank D.A. STAT3 is required for the oncogenic effects of nonsmall- cell lung cancer-associated mutations of the EGF receptor. *Cancer Res*. 2006; 66: 3162–3168.

Alvarez J.V., Frank D.A. Genome-wide analysis of STAT target genes: elucidating the mechanism of STAT-mediated oncogenesis. *Cancer Biol. Ther*. 2004; 3: 1045–1050.

Battle T.E., Frank D.A. The role of STATs in apoptosis. *Curr Mol Med.* 2002 Jun; 2(4): 381-92.

Boucheron C., Dumon S., Santos S.C., Moriggl R., Hennighausen L., Gisselbrecht S., Gouilleux F. A single amino acid in the DNA binding regions of STAT5A and STAT5B confers distinct DNA binding specificities. *J. Biol. Chem*. 1998; 273: 33936–33941.

Brantley E. C. and Benveniste E. N. Signal Transducer and activator of Transcription 3: a molecular hub for signaling pathways in gliomas, *Mol. Cancer Res.* 2008; 6: 675-684.

Bromberg J.F., Horvath C.M., Besser D., Lathem W.W., Darnell J.E. Jr. Stat3 activation is required for cellular transformation by v-src. *Mol Cell Biol* 1998; 18: 2553-8.

Büchner N., Altschmied J., Jakob S., Saretzki G., and Haendeler J. Well-Known Signaling Proteins Exert New Functions in the Nucleus and Mitochondria. *Antioxidant and redox signaling* 2010;13 (4): 551-558.

Caldenhoven, E. et al. STAT3beta, a splice variant of transcription factor STAT3, is a dominant negative regulator of transcription. *J. Biol. Chem* 1996; 271: 13221-13227.

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Catlett-Falcone R., Landowski T.H., Oshiro M.M., Turkson J., Levitzki A., Savino R., Ciliberto G., Moscinski L., Fernandez-Luna J.L., Nunez G., Dalton W.S., Jove R. Constitutive activation of Stat3 signaling confers resi stance to apoptosis in human U266 myeloma cells. *Immunity* 1999; 10: 105–115.

Chichiarelli S., Coppari S., Turano C., Eufemi M., Altieri F., Ferraro A. Immunoprecipitation of DNA-protein complexes cross-linked by cis-diamminedichloroplatinum. *Anal. Biochem*. 2002; 302(2): 224-9.

Chichiarelli S., Gaucci E., Ferraro A., Grillo C., Altieri F., Cocchiola R., Arcangeli V., Turano C., Eufemi M. Role of ERp57 in the signaling and transcriptional activity of STAT3 in a melanoma cell line. *Archives of Biochemistry and Biophysics* 2010; 494:178–183.

DeArmond D., Brattain M.G., Jessup J.M., Kreisberg J., Malik S., Zhao S., Freeman J.W. Autocrine-mediated ErbB-2 kinase activation of STAT3 is required for growth factor independence of pancreatic cancer cell lines. Oncogene 2003; 22: 7781–7795.

Demaria M., Poli V. From the nucleus to the mitochondria and back. The odyssey of a multitask STAT3. *Cell Cycle* 2011; 10 (19): 3221-3222.

Doris G. and David A. F. Targeting the Cytoplasmic and Nuclear Functions of Signal Transducers andActivators of Transcription 3 for Cancer Therapy. *Clin Cancer Res* 2007; 13 (19):56-65.

Edwards J. and Barlett J.M.S. The androgen receptor and signal-transduction pathways in hormone-refractory prostate cancer. Part 1: modifications to the androgen receptor. *B J U International* 2005; 95: 1320-1326.

Edwards J. and Barlett J.M.S. The androgen receptor and signal-transduction pathways in hormone-refractory prostate cancer. Part 2: androgen-receptor cofactors and bypass pathways. *B J U International* 2005; 95: 1327-1335.

Frank D. A. STAT3 as a central mediator of neoplastic cellular transformation. *Cancer Letters* 2007; 251:199-210.

Frank D.A., Mahajan S., Ritz J. B lymphocytes from patients with chronic lymphocytic leukemia contain signal transducer and activator of transcription (STAT) 1 and STAT3 constitutively phosphorylated on serine residues. *J. Clin. Invest*. 1997; 100: 3140–3148.

Fukada T., Hibi M., Yamanaka Y., Takahashi-Tezuka M., Fuijtani Y., Yamaguchi T., Nakajima K., Hirano T. Two signals are necessary for cell proliferation induced by a cytokine recepito gp130: involvment of STAT3 in anti-apoptosis. *Immunity* 1996; 5: 79-85.

Garcia R., Yu C.L., Hudnall A., Catlett R., Nelson K.L., Smithgall T., Fujita D.J., Ethier S.P., Jove R. Constitutive activation of STAT3 in fibroblast transformed by diverse

oncoproteins and in breast carcinoma cells. *Cell Growth Diff.* 1997; 8: 1267-1276.

Giannitrapani L., Cervello M., Soresi M., Notarbartolo M., La Rosa M., Virruso M., D'Alessandro N., Montalto G. Circulating IL-6 and sIL-6R in patients with hepatocellular carcinoma. *Ann. N.Y. Acad. Sci.* 2002; 963: 46-52.

Giraud S., Hurlstone A., Avril S. and Coqueret O. Implication of BRG1 and cdk9 in the STAT3-mediated activation of the p21waf1 gene. *Oncogene* 2004; 23: 7391–7398.

Giraud S., Bienvenu F., Avril S., Gascan H., Heery D.M., and Coqueret O. Functional Interaction of STAT3 Transcription Factor with the Coactivator NcoA/SRC1a. *The Journal of Biological Chemistry* 2002; 277 (10): 8004–8011.

Gouilleux-Gruart V., Gouilleux F., Desaint C., Claisse J.F., Capiod J.C., Delobel J., Weber-Nordt R., Dusanter-Fourt I., Dreyfus F., Groner B., Prin L. STAT-related trandcription factors are constitutively activated in peripheral blood cells from acute leukemia patients. *Blood* 1996; 87: 1692-1697.

Gräntzdörffer I., Yumlu S., Gioeva Z., Wasielewski R., Ebert M.P.A., Röcken C. Comparison of different tissue sampling methods for protein extraction from formalin-fixed and paraffin embedded tissue specimens. *Experimental and Molecular Pathology* 2010; 88:190–196.

Horiguchi A., Mototsugu O., Shimada T., Uchida A., Marumo K. And Murai M. Activation of signal transducer and activator of transcription 3 in renal cell carcinoma: a study of incidence and its association with pathological features and clinical out come. *The Journal of Urology* 2002; 168: 762-765.

Horvath C.M., Stark G.R., Kerr I.M., Darnell J.E. Jr. Interactions between STAT and non-STAT proteins in the interferon-stimulated gene factor 3 transcription complex. *Mol. Cell. Biol.* 1996; 16: 6957–6964.

Ihara S., Nakaijma K., Fukada T., Hibi M., Nagata S., Hirano T., Fukui Y. Dual control of neurite outgrowth by STAT3 and MAP kinase in PC12 cells stimulated with interleukin-6. *EMBO J.* 1997; 16: 5345-5352.

Kaptein A, Paillard V, Saunders M. Dominant negative stat3 mutant inhibits interleukin-6-induced Jak-STAT signal transduction. *J Biol Chem*. 1996; 271(11):5961-4.

Kawano M., Hirano T., Matsuda T., Taga T., Horii Y., Iwato K., Asaoku H., Tang B., Tanabe O., Tanaka H., Kishimoto T. Autocrine generation and requirement of BSF-2/ IL-6 for human multiple mieloma. *Nature* 1988; 332: 83-85.

Lee S.O., Lou W., Hou M., Miguel F., Gerber L., Gao A.C. Interleukin-6 promotes androgen-independent growth in LNCaP human prostate cancer cells. *Clin. Cancer Res.* 2003; 9: 370–376.

Lemaire M.A., Schwartz A., Rahmouni A.R., Leng M. Interstrand cross-links are preferentially formed at the d(GC) sites in the reaction between cis-

71

diamminedichloroplatinum (II) and DNA. *Proc Natl Acad Sci USA*. 1991; 88(5): 1982-5.

Lerner L., Henriksen M.A., Zhang X., and Darnell Jr J.E. STAT3-dependent enhanceosome assembly and disassembly: synergy with GR for full transcriptional increase of the α2-macroglobulin gene. *Genes & Development* 2003; 17: 2564–2577.

Levy J.D.E., Darnell Jr.J.E. Stats: transcriptional control and biological impact. *Cell Biol.* 2004; 165: 823–833.

Li L., Cheung S., Evans E.L., and Shaw P.E. Modulation of Gene Expression and Tumor Cell Growth by Redox Modification of STAT3. Cancer Res 2010; 70 (20): 8222-8232.

Li L., Shaw P.E. Autocrine-mediated activation of STAT3 correlates with cell proliferation in breast carcinoma lines. J. Biol. Chem. 2002; 277: 17397–17405.

Lin Q.,Lai R., Chirieac L.R., Li C., Thomazy V.A., Grammatikakis I., Rassidakis G.Z., Zhang W., Fujio Y., Kunisada K., Hamilton S.R., Amin H.M. Constitutive activation of JAK3/STAT3 in colon carcinoma tumors and cell lines: inhibition of JAK3/STAT3 signaling induces apoptosis and cell cycle arrest of colon carcinoma cells. *Am. J. Pathol.* 2005; 167(4): 969-80.

Lufei C., Ma J., Huang G., Zhang T., Novotny-Diermayr V., Ong C.T., Cao X. GRIM-19, a death-regulatory gene product, suppresses Stat3 activity via functional interaction. *EMBO J.* 2003; 22: 1325–1335.

Ma J., Zhang T., Novotny-Diermayr V., Tan A.L., Cao X.. A novel sequence in the coiled–coil domain of Stat3 essential for its nuclear trans location. *J. Biol. Chem.* 2003; 278: 29252–29260.

Marg A., Shan Y., Meyer T., Meissner T., Brandenburg M., Vinkemeier U. Nucleocytoplasmic shuttling by nucleoporins Nup153 and Nup214 and CRM1-dependent nuclear export control the subcellular distribution of latent Stat1. *Nat. Rev. Mol. Cell Biol.* 2002; 3: 651–662.

Maritano D., Sugrue M.L., Tininini S., Dewilde S., Strobl B., Fu X., Murray-Tait V., Chiarle R., Poli V. The STAT3 isoforms α and β have unique and specific functions. *Nature Immunology* 2004; 5(4): 401-409

Mertens C., Zhong M., Krishnaraj R., Zou W., Chen X., Darnell Jr E. J.. Dephosphorylation of phosphotyrosine on STAT1 dimers requires extensive spatial reorientation of the monomers facilitated by the N-terminal domain. *Genes Dev*. 2006; 20: 3372–3381.

Migone T.S., Lin J.X., Cereseto A., Mulloy J.C., O'Shea J.J, Franchini G, Leonard W.J. Constitutively activated Jak-STAT pathway in T cells transformed with HTLV-I. *Science* 1995; 269: 79-81.

References

Rawat R., Rainey G.J., Thompson C.D., Frazier- Jessen M.R., Brown R.T., Nordan R.P. Constitutive activation of STAT3 is associated with the acquisition of an interleukin-6 independent phenotype by murine plasmacytomas and hybridomas. *Blood* 2000; 96: 3514-3521.

Ray S., Lee C., Hou T., Kishor K.K., and Brasier A.A. Regulation of Signal Transducer and Activator of Transcription 3 Enhanceosome Formation by Apurinic/Apyrimidinic Endonuclease 1 in Hepatic Acute Phase Response. *Mol Endocrinol* 2010; 24(2): 391–401.

Rojas M., Donahue J.P., Tan Z., Lin Y.Z. Genetic engineering of proteins with cell membrane permeability. *Nat Biotechnol*. 1998; 16(4): 370-5.

Sanchez A., Nagy P., Thorgirsson S.S. STAT3 activity in chemically-induced hepatocellular carcinoma. *Eur J. Cancer* 2003; 39(14): 2093-8.

Santos C. I., Costa-Pereira A. P. Signal transducers and activators of transcription- from cytokine signalling to cancer biology. *Biochimica et Biophysica Acta* 2011; 1816: 38-49.

Schaefer T.S., Sanders L.K. & Nathans D. Cooperative transcriptional activity of Jun and Stat3β, a short form of Stat3. *Proc. Natl. Acad. Sci. USA* 1995; 92: 9097–9101.

Shen F., Kirmani K. Z., Xiao Z., Thirlby B. H., Hickey R. J., and Malkas L. H. Nuclear Protein Isoforms: Implications for Cancer Diagnosis and Therapy. *Journal of Cellular Biochemistry* 2011; 112:756–760.

Takeda K., Noguchi K., Shi W., Tanaka T., Matsumoto M., YoshidaN., Kishimoto T., Akira S. Targeted disruption of the mouse STAT3 gene leads to early embryonic lethality. *Proc. Natl. Acad. Sci USA* 1997; 94: 3801-3804.

Takemoto S., MulloyJ.C., Cereseto A., Migone T.S., Patel B.K., Matsuoka M., Yamaguchi K., Takatsuki K., Kamihira S., White J.D. Proliferation of adult T cell leukemia/lymphoma cells is associated with the costitutive activation of Jak-STAT proteins. *Proc. Natl. Acad. Sci. USA* 1997; 94: 13897-13902.

Tsuruma R., Ohbayashi N., Kamitani S., Ikeda O., Sato N., Muromoto R., Sekine Y., Oritani K. and Matsuda T. Physical and functional interactions between STAT3 and KAP1. *Oncogene* 2008; 27: 3054-059.

Turkson J., Ryan D., Kim J.S. Phosphotyrosyl peptides block Stat3-mediated DNA binding activity, gene regulation, and cell transformation. J Biol Chem 2001; 276(48): 45443–55.

Wang L.H., Yang X.Y., Zhang X., Farrar W.L. Nuclear Receptors as Negative Modulators of STAT3 in Multiple Myeloma. *Cell Cycle* 2005; 4 (2): 242-245.

Weber-Nordt R.M., Egen C., Wehinger J., Ludwig W., Gouilleux-Gruart V., Mertelsmann R., Finke J. Constitutive activation of STAT proteins in primary lymphoid and

73

myeloid leukemia cells and in Epstein-Barr virus (EBV)-related lymphoma cell lines. *Blood* 1996; 88: 809-816.

Wei D., Le X., Zheng L., Wang L., Frey J.A., Gao A.C., Peng Z., Huang S., Xiong H.Q., Abbruzzese J.L., Xie K. Stat3 activation regulates the expression of vascular endothelial growth factor and human pancreatic cancer angiogenesis and metastasis. *Oncogene* 2003; 22: 319–329.

Wen Z., Zhong Z., Darnell Jr J.E.. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell 1995; 82: 241–250.

Xiao H., Chung J., Kao H.Y., and Yang Y.C. Tip60 Is a Co-repressor for STAT3. *The Journal of Biological Chemistry* 2003, 278 (13): 11197–11204.

Xie Y., Kole S., Precht P., Pazin M. J., and Bernier M. S-glutathionylation impairs signal transducer and activator of transcription 3 activation and signaling. *Endocrinology* 2009; 150: 1122-31.

Xu, Sun Y.L., Hoey T.. Cooperative DNA binding and sequence-selective recognition conferred by the STAT amino-terminal domain. *Science* 1996; 273:794–797.

Yang E., Wen Z., Haspel R.L., Zhang J.J, Darnell J.E. Jr. The linker domain of Stat1 is required for gamma interferon-driven transcription. *Mol. Cell. Biol*. 1999; 19: 5106–5112.

Yang J., Stark G.R. Roles of unphosphorylated STATs in signaling. *Cell Res*. 2008; 18(4): 443-51.

Yoshida T., Hanada T., Tokuhisa T., Kosai K.-i, Sata M., Kohara M., Yoshimura A. Activation of STAT3 by the hepatitis C virus core protein leads to cellular transformation. *J. Exp. Med.* 2002; 196: 641–653.

Yu H. and Jove R. The STATs of cancer- new molecular targets come of age. *Nat Rev Cancer* 2004; 4(2): 97-105.

Yuan Z., Guan Y., Chatterjee D., Chin E.Y. Stat3 Dimerization Regulated by Reversible Acetylation of a Single Lysine Residue. *Science* 2005; 307:269-273.

Zhang J., Yang J., Roy S.K., Tininini S., Hu J., Bromberg J.F., Poli V., Stark G.R., Kalvakolanu D.V. The cell death regulator GRIM-19 is an inhibitor of signal transducer and activator of transcription 3. *Proc. Natl Acad. Sci. U.S.A.* 2003; 100: 9342–9347.

Zhang T., Kee W.H., Seow K.T., Fung W., Cao X.. The coiled–coil domain of Stat3 is essential for its SH2 domain-mediated receptor binding and subsequent activation induced by epidermal growth factor and interleukin-6. *Mol. Cell. Biol* 2000; 20: 7132–7139.

Zhang T., Kee W.H , Seow K.T., Fung W., Cao X. The coiled-coil domain of Stat3 is

essential for its SH2 domain-mediated receptor binding and subsequent activation induced by epidermal growth factor and interleukin-6. *Mol Cell Biol.* 2000; 20(19): 7132-9.

Zhang X., Wrzeszczynska M.H., Horvath C.M., Darnell J.E. Interacting regions in Stat3 and c-Jun that participate in cooperative transcriptional activation, *Mol. Cell. Biol.* 1999; 19: 7138–7146.
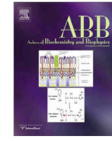
Zhang X. & Darnell J.E., Jr. Functional importance of Stat3 tetramerization in activation of the α2-macroglobulin gene. *J. Biol. Chem.* 2001; 276: 33576–33581.

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Role of ERp57 in the signaling and transcriptional activity of STAT3 in a melanoma cell line

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ABSTRACT

Chromatin immunoprecipitation in M14 melanoma cells showed that the protein ERp57 (endoplasmic reticulum protein 57) binds to DNA in the proximity of STAT3 in a subset of STAT3-regulated genes. In the same cells, IL-6 induced a significant increase of the expression of one of these genes, i.e. CRP. Upon depletion of ERp57 by RNA interference, the phosphorylation of STAT3 on tyrosine 705 was decreased, and the IL-6-induced activation of CRP expression was completely suppressed. *In vitro* experiments showed that ERp57 is also required for the binding of STAT3 to its consensus sequence on DNA. Thus ERp57, previously shown to associate with STAT3 in the cytosol and in the nuclear STAT3-containing enhanceosome, is a necessary cofactor for the regulation of at least a subset of STAT3-dependent genes, probably intervening both at the site of STAT3 phosphorylation and at the nuclear level.

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STAT3, a member of the STAT family of transcription factors, participates in the regulation of expression of genes involved in the immune response, in inflammation, in cell survival and in cell proliferation. Its activation, which takes place at the level of cell membrane, either by receptor or non-receptor protein kinases, is induced by a variety of cytokines and growth factors and promotes its nuclear import and the binding to its consensus sequences at the promoters or enhancers of the corresponding regulated genes [1]. While the function of STAT3 is important in physiological conditions for the biological processes mentioned before, and is even essential for the embryonic development, the occurrence of its deregulated activation, i.e. its constitutive activation, is a contributing factor for oncogenesis and has been detected in various types of cancer and transformed cell lines [2,3]. In fact, STAT3 has been proposed as a target for anti-tumor therapy. However, understanding its activity is complicated by the variety of effects for which it is responsible, since even opposite effects have been described, depending on the cell types or different tissues which have investigated. Thus, for example, pro-apoptotic and anti-apoptotic effects have been described [4]. A more detailed understanding of the activity of STAT3 is most likely dependent on the knowledge of the multi-protein nuclear complexes in which it takes part, which might differ in different cell types and might contribute to activating or repressory effects on gene expression. The nuclear STAT3 has been found associated with various co-regulators such as CBP/p300, Oct-1, c-jun, NcoA1/SRC-1a,

Brg-1 and STAT1 [5–9]. More recently ERp57/GRP58, a member of the protein disulfide isomerase family of proteins, has also been found associated with STAT3 in the nucleus [10]. ERp57 is mainly localized in the endoplasmic reticulum, where it participates in the folding and the disulfide groups shuffling of the proteins [11] and in the assembly of the major histocompatibility complex I [12]. However, ERp57 has been found also in other subcellular locations, such as the cell surface, the cytosol and the nucleus [13].

The STAT3/ERp57 interaction was first detected in the cytosol of HepB2 cells as part of a large multiprotein complex which was called the statosome [14]. The two proteins were subsequently found associated also in the nucleus of M14 melanoma cells and in IL-6-stimulated HepG2 hepatoma cells [10]. However, the role of ERp57 for the signaling and transcriptional activity of STAT3 has not yet been established, nor has its presence been demonstrated in the DNA–STAT3-complexes other than that bound to the *A2M* enhancer. In view of the importance of the functional and structural characterization of the multi-protein complexes formed by STAT3, we investigated ERp57 for its involvement in the transcriptional activity of STAT3 by RNA interference and analyzed its presence in a variety of STAT3-associated complexes by chromatin immunoprecipitation (ChIP)[1].

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[1] *Abbreviations used:* ERp57, endoplasmic reticulum protein 57; CRP, C-reactive protein; ChIP, chromatin immunoprecipitation; GST, glutathione S-transferase; siRNA, small interfering RNA; IL-6, interleukin-6; *cis*-DDP, *cis*-diamminedichloroplatinum; PAO, phenyl-arsenoxide; S/MAR, nuclear scaffold/matrix associated regions; *PDIA3*, the gene for protein ERp57; pSTAT3, STAT3 phosphorylated on tyrosine 705; PBS, phosphate buffered saline.

Experimental

Materials

Human recombinant ERp57 was obtained as previously described [15]. The anti-ERp57 antibody was prepared as an antiserum by Eurogentec (Seraing, Belgium) using as an antigen the human recombinant protein prepared in our laboratory, and partially purified on immobilized protein G (Sigma). Its specificity has been verified, as previously reported [16]. For some experiments a commercial polyclonal antibody was employed (Stressgen). The anti-STAT3 antibody was obtained from Santa Cruz, the antibody for STAT3 phosphorylated on tyrosine 705 was from Cell Signaling, IL-6 from Relia and vancomycin and phenyl-arsenoxide from Sigma. Unmodified or 5′-biotinylated oligonucleotides to be used as primers for PCR were purchased from Primm (Milano, Italy).

Cell culture

Human melanoma M14 cells were grown to 80% confluence at 37 °C in 5% CO_2 in RPMI 1640 medium, added with 1% sodium pyruvate, 10% fetal bovine serum, 2 mM glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin. For IL-6 stimulation the cells were left in serum-free RPMI for at least 4 h, and then added with IL-6 at a final concentration of 25 ng/ml.

Chromatin immunoprecipitation (ChIP)

DNA–protein cross-linked complexes were formed in M14 cells by the use of cis-diamminedichloroplatinum II (cis-DDP, 0.1 mM for 2 h at 37 °C). The immunoprecipitation procedure was carried out by the use of an anti-ERp57 or an anti-STAT3 antibody as previously described [17]. The DNA was recovered from the complexes cross-linked with cis-DDP by a treatment with 1.5 M thiourea at 50 °C for 30 min. The DNA was then purified by phenol–chloroform extraction and ethanol precipitation, and amplified with the primers listed in Table 1. The amount of immunoprecipitated DNA to be subjected to amplification was measured by means of a Spex FluoroMax spectrofluorimeter with the use of Pico Green (Invitrogen) following the manufacturer's instructions.

RNA Interference (siRNA)

siRNA for ERp57 and scrambled RNA were purchased from QIAGEN, and Lipofectamine™ RNAiMAX from Invitrogen. The day before transfection, 8×10^4 M14 cells per well were seeded in a 24-well plate in 500 µl of a complete culture medium. The cell cultures were grown under normal growth conditions (typically 37 °C and 5% CO_2).

The transfection was carried out according to the manufacturer's instructions and the siRNA was used at 100 nM final concentration. The M14 cells were incubated with the transfection complexes under their normal growth conditions and gene silencing was monitored at the mRNA level after 24, 48 and 72 h.

Table 1
Primers used in ChIP assay for the promoters/enhancers containing STAT3-binding sequences.

CRP	CATCTTAGTCTAATAGGACTG	CTAGTGTCTGTATCGTAATATAG
MMP9	GTATCCTTGACCTTCTTTC	GTGGTGATTGGTTTTAATTAG
A2M	GCTTATTAGCTGCTGTAC	GTCAAGGTTAATTCCTGG
CDKN1A	GATCAGGTTGCCCTTTTTTG	GAAATTGAGGTCCACTGAAC
CDC25A	GTTTGGCGCCAACTAGGA	GAAAACCAAGCCGACCTAC
MMP2	CTGTTCAAGATGGAGTCG	GTAAGCCTTAACTTGGCCTC
NOS2A	CTGTGAGGGAAACCTTTG	CATAAATATGAGTGGGGTAG

Total RNA preparation and real-time PCR

The treated cells were harvested after 24, 48 and 72 h and total RNA was isolated with TRIzol (Invitrogen) following the manufacturer's instructions. The reverse transcription of total RNA was carried out with SideStep™ II QPCR cDNA Synthesis Kit (Stratagene).

Gene expression was evaluated with specific primers (Quanti-Tect® QIAGEN or RT²-PCR primers from SuperArray) by Real-Time-PCR, which was performed using a MJ MiniOpticon Detection System (BioRad Laboratories) with SYBR green fluorophore by means of Brilliant® SYBR® Green QPCR Master Mix (Stratagene). The protocol used was: denaturation (95 °C for 5 min), amplification repeated 40 times (95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s). PCR fluorophore acquisition temperatures were set from 40 °C to 95 °C, reading every 0.5 °C. All reactions were carried out at least in duplicate. A melting curve analysis was performed following every run to ensure a single amplified product for every reaction.

RPS27A (QuantiTect® QIAGEN) gene was used as reference for normalization and the relative quantification was analyzed using Gene Expression analysis for iCycler iQ real-time PCR detection system software, Version 1.10 (BioRad Laboratories, Ltd.).

Immunofluorescence

Cells grown on glass coverslips were treated with siRNA or left untreated for a control. The cells on coverslips, after 48 h of treatment, were washed three times with PBS, fixed in Carnoy solution and then air dried for 30 min. The samples were permeabilized with 0.1% Triton X-100 in PBS for 15 min, washed with 0.5% BSA in PBS and incubated first with 1% BSA in PBS for 1 h and then for 90 min with anti-pSTAT3 in 1% BSA and PBS at 37 °C. After washing, the cells were incubated, in the dark, with FITC-goat-anti-Rabbit-IgG (Jackson Immunoresearch) for the next 90 min. The cells were then washed and stained with DAPI in PBS (0.03 µg/ml) for 2 min at room temperature. Finally the cells were mounted in VECTASHIELD (Vector Laboratories) and analyzed by a Leica DM5000B fluorescence microscope. Images were recorded by digital camera and processed by ImageJ 1.37v.

In vitro binding of STAT3 and ERp57 to DNA

A biotinylated DNA fragment spanning the STAT3-binding site of the CDKN1A gene enhancer was prepared by PCR using the same primers indicated in Table 1. The nuclear extracts from M14 cells were prepared as described before [10]. The in vitro binding of STAT3 and ERp57 from the nuclear extracts to the biotinylated DNA fragment, which had been immobilized on streptavidin-coated magnetic beads (Dynabeads, Invitrogen), was tested as previously described in the presence of 500-fold excess of poly-(dI–dC)·poly(dI–dC) [10]. The effect of phenyl-arsenoxide or vancomycin on the binding of STAT3 and ERp57 to DNA was assayed by adding these inhibitors to the nuclear extracts before the incubation with the immobilized DNA.

Results

Chromatin immunoprecipitation

To verify the presence in vivo of ERp57 in the proximity of the STAT3-binding sites on DNA, chromatin immunoprecipitation was carried out on intact M14 cells, using both an anti-ERp57 and an anti-STAT3 antibody. The immunoprecipitated DNA was analyzed for the possible enrichment of DNA fragments containing the STAT3-binding sites in the promoters or enhancers of selected
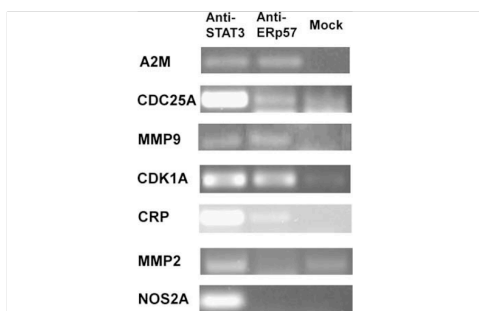
Fig. 1. PCR analysis of the enrichment of ERp57 and STAT3-binding sites in the immunoprecipitated DNA in comparison with the mock immunoprecipitation (performed with preimmune IgGs). The DNA fragments present in the 5′-region of the genes indicated, and containing the STAT3 consensus sequence, were amplified with the primers shown in Table 1.

Fig. 3. Effects of IL-6 and RNA interference on the expression of the *PDIA3* and *CRP* genes. The expression of these genes after treatment of the cells with scrambled RNA (control) and siRNA for ERp57 (sil) were measured, and here they are shown as their increase or decrease relative to the values (set to one) observed with scrambled RNA and without IL-6 treatment. The M14 cells were treated with the scrambled or siRNA and after 48 h were left untreated or treated with IL-6 for 60 min, before extracting the RNA for the RT-PCR measurement. The expression of the genes was normalized with that of the housekeeping gene *RPS27A*. Each value is the average of three experiments ± standard deviation.

genes. It should be noted that this ChIP procedure was performed by the use of *cis*-DDP as a DNA–protein cross-linking reagent [18], which, contrary to formaldehyde, does not usually induce protein–protein cross-linking [19]. Therefore, we detected only a direct binding of STAT3 and ERp57 to DNA.

We tested the binding of ERp57 and of STAT3 on the regulatory regions of the following STAT3-dependent genes: *A2M*, *CDC25A*, *CDKN1A*, *CRP*, *NOS2A*, *MMP9* and *MMP2*, coding respectively for α2-macroglobulin, a cell-cycle protein phosphatase, the p21$^{WAF1/CIP1}$, the C-reactive protein, the inducible NO-synthase and two matrix metalloproteinases. These genes were chosen for having well-characterized STAT3-binding sequences in their 5'-flanking regions.

Fig. 1 shows that five fragments out of seven examined were enriched in the immunoprecipitated DNA when either anti-ERp57 or anti-STAT3 antibodies were used, although a significant variation can be observed in the level of enrichment of the five DNA fragments. This can be explained by differences in the yield of the cross-linking reaction, which requires a proximity of sulfhydryl or imidazolyl groups of the protein with guanine or adenine moieties of DNA. An alternative explanation will be proposed later.

RNA interference

The relevance of ERp57 for the STAT3-dependent gene expression was investigated by means of an RNA interference procedure to decrease the content of ERp57 in the cell.

The treatment of M14 cells with siRNA specific for *PDIA3* gene, (which is the gene of ERp57) resulted in a lowered expression of this gene, which was 25% and 12% of the initial after 24 h and 48 h, respectively. The reduction of the ERp57 content of M14 cells brought up by RNA interference was confirmed by comparing the amount of protein present in a whole cell lysate before and after the siRNA treatment by means of Western blots (Fig. 2).
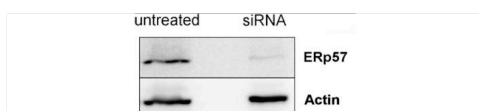

Fig. 2. Western blot with anti-ERp57 antibody of a M14 cell lysate before and 48 h after treatment with siRNA for ERp57. Actin is shown as a control for the amount of total proteins.
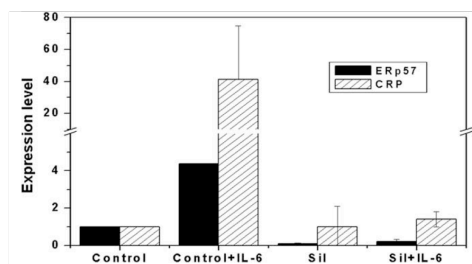
The expression of the seven aforementioned genes was measured after 48 h of siRNA treatment and compared to the effect of scrambled RNA. Although their expression was generally slightly decreased, the differences between the cells treated with the two RNA types were too small to be significant (data not shown).

However, although STAT3 is constitutively activated in M14 cells [10], the level of phosphorylated STAT3 and the expression of the tested genes are both rather low. Looking for a higher level of STAT3 activation and gene expression, we stimulated the M14 cells with IL-6 and measured the expression of the same genes. Only the CRP gene was found to be activated by more than one order of magnitude, and was therefore chosen for the measurement of the effect of the ERp57 protein on its activation.

The M14 cells were treated with siRNA specific for *PDIA3* or with a scrambled RNA. Forty-eight hours from the treatment, IL-6 was added and after 60 min the expressions of *PDIA3* and of *CRP* were measured. The expression of both genes was dramatically decreased by the siRNA-treatment, and the activating effect of IL-6 on *CRP* was entirely suppressed, as shown in Fig. 3.

The effects of RNA interference on the amount of STAT3 and ERp57 in IL-6-stimulated cells were then investigated.

Upon RNA interference, the amount of ERp57 was decreased, as expected, both in the cytosol and in the nucleus. However, while in the cytosol ERp57 was nearly suppressed, it decreased only slightly (approximately 50%) in the nucleus (Fig. 4A). This can be explained by the fact that nuclear ERp57 is mainly a nuclear-matrix protein, and as such has a lower turnover and/or a lower mobility than the cytosolic counterpart.

Total STAT3 was essentially unchanged in the cytosol but decreased in the nucleus, while, unexpectedly, the tyrosine-phosphorylated form of STAT3 was decreased in both subcellular compartments (Fig. 4A and B). This diminished activation/phosphorylation of STAT3 might represent a contributing factor, although probably not the only one, to the decrease of CRP gene expression.

To confirm the involvement of ERp57 in the process of the phosphorylation of STAT3 on tyrosine 705, we carried out an immunofluorescence experiment with an anti-pSTAT3 antibody on M14 cells treated or untreated with siRNA. As shown in Fig. 5, a strong decrease in cellular pSTAT3 was observed following the RNA interference.

In vitro binding of STAT3 and ERp57 to DNA

Considering the decrease of ERp57 in the nucleus of IL-6-treated cells observed after RNA interference, our aim was to verify if
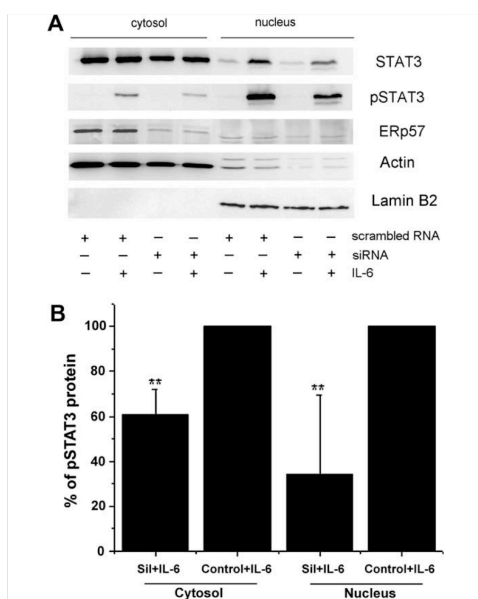
Fig. 4. (A) Western blots of ERp57, total STAT3 or phosphorylated STAT3 in the cytosol and in the nuclei of M14 cells, treated as described in Fig. 3. (B) Relative amounts of phosphorylated STAT3 in cells treated with scrambled- or siRNA, after IL-6 stimulation. The averages of three experiments are shown ±SD. Each value of pSTAT3, obtained by densitometric analysis, was corrected for the corresponding value of its loading control (actin for cytosol, lamin B2 for nuclei). $**p < 0.05$.

ERp57 has a role in the function of STAT3 also in the nucleus. For this purpose, the interaction of ERp57 to the well-characterized binding site of STAT3 on the *CDKN1A* promoter was tested *in vitro*. A double stranded-DNA fragment of the promoter of the *CDKN1A* gene, containing the consensus sequence of STAT3, was prepared, end-labeled with biotin on one strand and immobilized on streptavidin-bound magnetic beads. Nuclear extracts from M14 cells were added in the presence of an excess of non-specific competitor DNA [10], and the specifically-bound proteins were eluted and examined by Western blotting. As shown in Fig. 6, both proteins were present among the proteins displaying a high affinity for the selected promoter. We also found (data not shown) other well-known components of the DNA-binding STAT3 complexes, such as Oct-1, NcoA1/SRC-1a and Brg-1, thus validating the method described.

This *in vitro* binding was then assayed in the presence of two inhibitors of ERp57, i.e. phenyl-arsenoxide (PAO) and vancomycin. Vancomycin has recently been shown to bind reversibly to ERp57 with a K_D of 6.7×10^{-6} M, and to inhibit the reductase and the DNA-binding activities of the protein [20]. The addition of vancomycin inhibited the binding of STAT3 and ERp57 to DNA (Fig. 6, lane 2).

PAO is known to bind specifically to vicinal thiol groups, and is often used to inhibit the protein disulfide isomerases. ERp57 contains two cys-gly-his-cys sequences in its first and fourth domain, respectively. PAO abolished of the binding of STAT3 and ERp57 to the DNA (Fig. 6, lane 3). The addition of an excess of recombinant ERp57 to the PAO-treated nuclear extract reversed the inhibition of STAT3-binding (Fig. 6, lane 4). It could be argued that PAO might

act through the inhibition of proteins other than ERp57. However, considering the reversal of inhibition brought up by the addition of ERp57, this possibility is highly unlikely, since in order to dissociate the PAO-protein complexes, relatively high concentrations of small dithiols are needed, forming stable five-member ring structures with PAO. This supports the specificity of PAO inhibitory action towards ERp57.

Discussion

The functions of ERp57 in its main localization, i.e. the endoplasmic reticulum, are well understood [21]. However in other subcellular locations this protein appears to be involved in a variety of other processes. On the sperm surface, ERp57 is required for the process of gamete fusion [22]; on the surface of colon carcinoma cells, ERp57, together with calreticulin, is required to determine an immunogenic cell death [23,24]; on the surface of epithelial intestinal cells, it has been shown to bind calcitriol and is thought to be responsible for the fast non-genomic response to this hormone [25]. In the cytosol it is associated with phosphorylated STAT3 in hepatoma cells [14] and with a sodium-chloride cotransporter in renal cells [26].

Some previous findings suggested that ERp57 is involved, directly or indirectly, in transcription regulation. Johnson et al. [27] found that ERp57 alters the formation of complexes between nuclear proteins and regulatory regions of interferon inducible genes. ERp57 and its isoform PDI may have a reduction-dependent regulatory role on transcription, since they are able to alter the dimerization state of E2A transcription factors, with a consequent modulation of their activities [28]. ERp57 has been found associated with Ref-1/APE1 [29], which is a multiple-function protein, acting both as an endonuclease involved in DNA repair and as an activator for a number of transcription factors. Finally, ERp57 binds in HeLa cells to specific DNA sites, some of which present the typical features of gene regulatory sequences, such as DNase hypersensitivity or proximity to S/MAR regions [17].

Our experiments presented here demonstrate that ERp57 binds to DNA in the proximity of STAT3 consensus sequences in a variety of STAT3-binding promoters, in addition to that of the *A2M* gene which was previously detected. However, ERp57 is absent in a subset of STAT3-binding promoters, as shown by the ChIP data. This indicates that the structure and/or composition of the DNA-bound STAT3 multi-protein complexes are not necessarily the same in the various promoters/enhancers, even in the same cell type. This might be explained by different chromatin structures present in the various regulatory sites, so that different protein complexes can be recruited. An alternative explanation is that ERp57 and another protein bind to STAT3 in competition for the same site on the STAT3 molecule. When STAT3 binds to its consensus sequence on DNA, the flanking DNA sequence would determine which STAT3 partner prevails, favoring the protein with a higher DNA affinity. This explanation is compatible with the results of the ChIP experiments, which suggest that the stoichiometry of the STAT3-ERp57 complexes varies among the different binding sites.

The importance of ERp57 for the signaling and transcriptional activities of STAT3 has been demonstrated by silencing the expression of ERp57 by means of RNA interference. Regarding the mechanism by which ERp57 cooperates with STAT3 to elicit its activity, it should be noted that the STAT3–ERp57 interaction has been detected at the cell membrane level [30], in the cytosol [14] and in the nucleus [10]. For this reason, in principle it is not obvious to assess at which level the presence of ERp57 is necessary for the function of STAT3.

Our present experiments do unexpectedly show that silencing the expression of ERp57 impairs the activation of STAT3, which
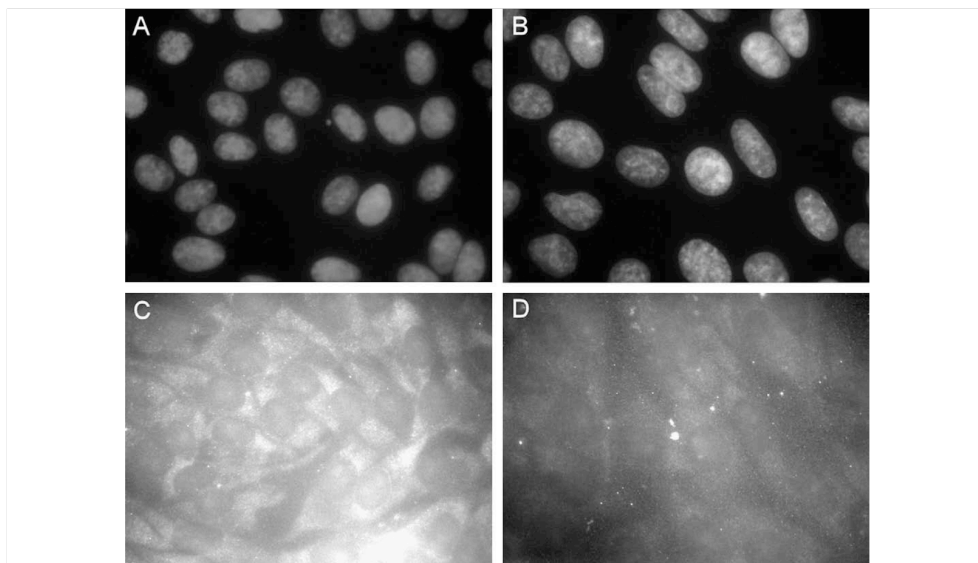
Fig. 5. Detection of pSTAT3 in M14 cells by immunofluorescence. (A and C) Untreated cells. (B and D) Cells treated with siRNA for ERp57, after 48 h. (A and B) Staining with DAPI. (C and D) Staining for pSTAT3.
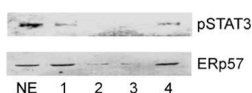

Fig. 6. Effects of ERp57 inhibitors on the *in vitro* binding of pSTAT3 and ERp57 to a DNA fragment present in the 5′-region of the human *CDKN1A* gene (from −737 to −635), containing the STAT3 consensus sequence. The proteins bound to DNA with high affinity were dissociated from DNA, separated by SDS–PAGE and subjected to Western blotting. pSTAT3 and ERp57 are shown in total nuclear extract (NE), or among the proteins specifically bound to DNA in the absence (1) or in the presence of 50 μM vancomycin (2) or 1 μM PAO (3). In lane 4 an excess of ERp57 was added after the treatment with PAO.

consists in the phosphorylation of tyrosine 705. The decrease of phosphorylated STAT3 in the nucleus cannot originate from an inhibition of its nuclear import, considering that this decrease can already be observed in the cytosol. However, since this phosphorylation is not entirely suppressed but only diminished, even with a robust silencing of ERp57 expression, it is unlikely that this impairment is entirely responsible for the dramatic inhibition of the CRP expression that we observe following the RNA interference. On the other hand, the *in vitro* experiments show that inhibitors of ERp57 hinder the formation of STAT3–DNA complex. Therefore, the hypothesis of a role of ERp57 also at the nuclear level is legitimate, although it awaits confirmation by further experiments.

Overall, our data demonstrate that ERp57 is required, at least for some genes, for the signaling and transcriptional activity of STAT3. The probable sites of action of ERp57 reside both in the activation reaction of STAT3 and in the DNA binding of STAT3 at the nuclear level. The mechanism by which ERp57 contributes to the phosphorylation of tyrosine of STAT3 remains to be ascertained. In this connection, Guo et al. [30] have shown that ERp57 interacts with STAT3 already at the level of cell membrane, where the phosphorylation reaction takes place. However, a recent report leads to speculation about the intervention of ERp57 in the process of STAT3 phosphorylation and in its binding to DNA. In fact a glutathionylation of STAT3 has been described, and its consequences are an inhibition of STAT3 phosphorylation and of STAT3–DNA interaction [31]. Since ERp57 appears to be an acceptor of glutathione, with which it easily forms a disulfide bond [32], a glutathione exchange between the two proteins might explain the involvement of ERp57 in the signaling and transcriptional activities of STAT3 described here.

With regard to the action of ERp57 in the nucleus, it is interesting to note that another member of the protein disulfide isomerase family, the PDI, i.e. the first one to be investigated in detail, is involved at the nuclear level in the transcriptional regulation, in this case intervening in the activity of the estrogen receptor by influencing its conformation and by modulating its affinity for the estrogen-responsive element [33].

ERp57 is a redox-sensitive and stress-responsive protein, whose expression is regulated by a variety of stimuli. One of these is the oxidative stress, which is known to lead also to the activation of STAT3 by an increased phosphorylation [34]. It is conceivable that the functional interaction of ERp57 with STAT3 could reinforce the response of STAT3 to the oxidative stress conditions.

References

[1] J.E. Darnell Jr., Science 277 (1997) 1630–1635.
[2] T. Bowman, R. Garcia, J. Turkson, R. Jove, Oncogene 19 (2000) 2474–2488.
[3] J. Bromberg, J. Clin. Invest. 109 (2002) 1139–1142.
[4] J. Bromberg, J.E. Darnell Jr., Oncogene 19 (2000) 2468–2473.
[5] S. Ruff-Jamison, Z. Zhong, Z. Wen, K. Chen, J.E. Darnell Jr., S. Cohen, J. Biol. Chem. 269 (1994) 21933–21935.
[6] M. Paulson, S. Pisharody, L. Pan, S. Guadagno, A.L. Mui, D.E. Levy, J. Biol. Chem. 274 (1999) 25343–25349.

[7] S. Giraud, F. Bienvenu, S. Avril, H. Gascan, D.M. Heery, O. Coqueret, J. Biol. Chem. 277 (2002) 8004–8011.

[8] L. Lerner, M.A. Henriksen, X. Zhang, J.E. Darnell Jr., Genes Dev. 17 (2003) 2564–2577.

[9] S. Giraud, A. Hurlstone, S. Avril, O. Coqueret, Oncogene 23 (2004) 7391–7398.

[10] M. Eufemi, S. Coppari, F. Altieri, C. Grillo, A. Ferraro, C. Turano, Biochem. Biophys. Res. Commun. 323 (2004) 1306–1312.

[11] R.B. Freedman, T.R. Hirst, M.F. Tuite, Trends Biochem. Sci. 19 (1994) 331–336.

[12] J.A. Lindquist, O.N. Jensen, M. Mann, G.J. Hämmerling, EMBO J. 17 (1998) 186–2195.

[13] C. Turano, S. Coppari, F. Altieri, A. Ferraro, J. Cell Physiol. 193 (2002) 154–163.

[14] M.I. Ndubuisi, G.G. Guo, V.A. Fried, J.D. Etlinger, P.B. Sehgal, J. Biol. Chem. 274 (1999) 25499–25509.

[15] C. Grillo, S. Coppari, C. Turano, F. Altieri, Biochem. Biophys. Res. Commun. 295 (2002) 67–73.

[16] S. Coppari, F. Altieri, A. Ferraro, S. Chichiarelli, M. Eufemi, C. Turano, J. Cell Biochem. 85 (2002) 325–333.

[17] S. Chichiarelli, A. Ferraro, F. Altieri, M. Eufemi, S. Coppari, C. Grillo, V. Arcangeli, C. Turano, J. Cell Physiol. 210 (2007) 343–351.

[18] Z.M. Banjar, L.S. Hnilica, R.C. Briggs, J. Stein, G. Stein, Biochemistry 23 (1984) 1921–1926.

[19] R.B. Ciccarelli, M.J. Solomon, A. Varshavsky, S.J. Lippard, Biochemistry 24 (1985) 7533–7540.

[20] E. Gaucci, S. Chichiarelli, C. Grillo, E. Del Vecchio, M. Eufemi, C. Turano, J. Antibiot. (Tokyo) 61 (2008) 400–402.

[21] E.M. Frickel, P. Frei, M. Bouvier, W.F. Stafford, A. Helenius, R. Glockshuber, L. Ellgaard, J. Biol. Chem. 279 (2004) 18277–18287.

[22] D.A. Ellerman, D.G. Myles, P. Primakoff, Dev. Cell 10 (2006) 831–837.

[23] M. Obeid, J. Immunol. 181 (2008) 2533–2543.

[24] T. Panaretakis, N. Joza, N. Modjtahedi, A. Tesniere, I. Vitale, M. Durchschlag, G.M. Fimia, O. Kepp, M. Piacentini, K.U. Froehlich, P. van Endert, L. Zitvogel, F. Madeo, G. Kroemer, Cell Death Differ. 15 (2008) 1499–1509.

[25] I. Nemere, M.C. Farach-Carson, B. Rohe, T.M. Sterling, A.W. Norman, B.D. Boyan, Proc. Natl. Acad. Sci. USA 101 (2004) 7392–7397.

[26] B. Wyse, N. Ali, D.H. Ellison, Am. J. Physiol. Renal Physiol. 282 (2002) F424–430.

[27] E. Johnson, W. Henzel, A. Deisseroth, J. Biol. Chem. 267 (1992) 14412–14417.

[28] M. Markus, R. Benezra, J. Biol. Chem. 274 (1999) 1040–1049.

[29] C. Grillo, C. D'Ambrosio, A. Scaloni, M. Maceroni, S. Merluzzi, C. Turano, F. Altieri, Free Radic. Biol. Med. 41 (2006) 1113–1123.

[30] G.G. Guo, K. Patel, V. Kumar, M. Shah, V.A. Fried, J.D. Etlinger, P.B. Sehgal, J. Interferon, Cytokine Res. 22 (2002) 555–563.

[31] Y. Xie, S. Kole, P. Precht, M.J. Pazin, M. Bernier, Endocrinology 150 (2009) 1122–1131.

[32] C.E. Jessop, N.J. Bulleid, J. Biol. Chem. 279 (2004) 55341–55347.

[33] J.R. Schultz-Norton, W.H. McDonald, J.R. Yates, A.M. Nardulli, Mol. Endocrinol. 20 (2006) 1982–1995.

[34] M. Carballo, M. Conde, J. Biol. Chem. 274 (1999) 17580–17586.

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