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XXI CICLO

Role of the RNA-binding protein Sam68 in prostate cancer cell proliferation and survival

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Introduction

1. Prostate cancer

1.1 *Signalling pathways in prostate cancer*

Prostate cancer is among the most common neoplastic diseases in the western male population. It originates from the hyperproliferation of the epithelial cells of the gland, which at this stage still require androgens for cell divisions and for inhibition of apoptosis (Feldman and Feldman, 2001; Grossmann and Tindall, 2001). In more advanced stages, cancer cells acquire the ability to invade neighboring tissues, they escape the prostatic envelope and form metastases (Feldman and Feldman, 2001; Grossmann and Tindall, 2001). Development, progression and maintenance of disease are initially dependent on the action of the androgen receptor (AR), a ligand-activated transcription factor. Upon ligand (testosterone or 5-α-dihydrotestosterone, DHT) binding, AR is released from inhibitory heat-shock proteins, translocates to the nucleus and binds to DNA sequences called androgen responsive elements (ARE) within the regulatory regions of target genes (Feldman and Feldman, 2001). Therein, AR induces a program of gene transcription that results in diverse activities that depend on cellular context (Burnstein, 2005; Pienta and Bradley, 2006; Balk and Knudsen, 2008). Prostate specific antigen (PSA) is a typical androgen responsive gene and the best characterized biomarker for disease progression (Lilja *et al*., 2008). Proliferation of PCa cells requires a functional AR; therefore the standard therapies in PCa involve treatments with anti-androgenic compounds to reduce or compete the levels of androgens circulating in the blood and to cause the regression of the tumor mass (Petrylak, 2005). However, in spite of the initial efficacy of these treatments, the majority of prostatic carcinomas acquires with time the ability to grow in a androgen-independent manner and the tumor mass becomes refractory to this kind of therapy

(Petrylak, 2005; Hadaschik and Gleave, 2007). For this reason, it is very important to understand the biological factors and mechanisms that underlie the acquisition of androgen refractoriness in prostate carcinomas.

At the molecular level, multiple mechanisms are involved in androgen refractoriness. Among the events that allow prostate cancer to respond to lower levels of androgens during therapy, or to be stimulated even by the anti-androgens, are: a) AR gene amplification; b) activating mutations in the ligand-binding domain of the AR; c) overexpression or activation of AR-activatory proteins; d) activation of alternative pathways that stimulate AR even in the absence of the ligand (Feldman and Feldman, 2001).

Regarding the activation of AR-downstream pathways, the tyrosine kinase Src seems to play an important role in prostate cancer. Src is the cellular homolog of one of the first oncogenes identified, the Rous sarcoma virus (v-Src), and it is part of a complex network of signal transduction pathways that regulate cell proliferation and differentiation (Thomas and Brugge, 1997). The expression levels and the activity of Src are increased in many kinds of carcinomas, in particular breast cancers, colorectal cancers, pancreatic and prostate cancers. Activation of Src in these kinds of cancers leads to cell proliferation and inhibition of apoptosis (Irby RB and Yeatman TJ, 2000). In prostate cancer cells, stimulation with androgens or with β-estradiol triggers the association of Src with the AR, the activation of the kinase and a proliferative response (Migliaccio *et al*., 2000). Moreover, stimulation of cells with epidermal growth factor (EGF) triggers the assembly of estrogen receptor beta (Erβ) /AR complex with Src and EGF receptor (EGFR) favouring growth and invasiveness of prostate cancer cells (Migliaccio *et al*., 2005). In support of a role for Src in prostate carcinoma, our laboratory has identified an activator of this kinase, the truncated form of c-Kit named tr-Kit (Sette *et al*., 2002), which is aberrantly expressed in human prostate carcinomas at advanced stages of the disease. In these studies, it was also observed that the expression of tr-Kit correlates with the activation of Src in human cancer specimens

(Paronetto *et al*, 2003; Paronetto *et al*., 2004). Among the substrates phosphorylated by Src in prostate cancer cell lines and primary tumors expressing tr-Kit, it was identified Sam68, an RNA binding protein involved in signal transduction pathways and normally phoshorylated by Src in mitosis (Lukong and Richard, 2003).

1.2 *Prostate cancer and post-transcriptional regulation*

As previously mentioned, androgen-refractoriness commonly occurs in patients suffering from PCas and it is associated with poor prognosis. Thus, new biomarkers with diagnostic and prognostic values are urgently required. Several studies indicate that alterations in post-transcriptional regulation events play a key role in gene expression and PCa advancement (Li *et al*., 2006; Zhang *et al*., 2006), and a similar event also occurs in other kinds of cancer, such as breast and ovary carcinomas (Venables *et al*., 2008; Klinck *et al*., 2008; Fischer *et al*., 2004) Distinct mRNA isoforms may be uniquely associated with a disease process, either as products of cellular transformation or as causative factors for a specific disease phenotype, and they could be used as biomarkers for disease diagnosis and prognosis (Brinkman, 2004; Venables, 2004). Recent studies demonstrate that several mutations affect the splicing of oncogenes, tumor suppressors and other cancer relevant genes (Venables, 2006; Grosso *et al*., 2008). However, many splicing abnormalities that have been identified in cancer cells are not associated with mutations in the affected genes. Rather, many evidences indicate that the splicing machinery is an important target for misregulation in cancer. According to recent bioinformatics studies, changes in splicing factor expression might have a key role in the general splicing disruption that occurs in many cancers (Kim *et al*., 2008; Ritchie *et al*., 2008). Moreover, a recent study demonstrate for the first time that over-expression of a splicing factor can trigger malignant transformation (Karni *et al*., 2007). The authors showed that the splicing factor SF2/ASF is upregulated in various human tumors and affects alternative splicing of the tumor suppressor BIN1 and the kinases MNK2

and S6K1, leading to production of new splicing variants with oncogenic properties, which might support malignant transformation. Several additional splicing proteins have been shown to be upregulated in various human cancers; however, the effects that these changes have on splicing regulation is unknown (Grosso *et al*., 2008). These observations highlight the importance to determine what are the RNA-binding proteins that account for the aberrant alternative splicing events that correlate with malignant progression of PCa cells.

We have recently identified one of the splicing factors that is aberrantly expressed in PCa cells. Our studies demonstrated that the RNA-binding protein Sam68 is frequently up-regulated in PCas and sustains PCa cells proliferation and chemotherapy resistance (Busà *et al*., 2007). Thus, it would be very important to understand the post-transcriptional regulatory events modulated by Sam68 in prostate cancer cells and to study the mRNA isoforms pattern that this protein contributes to create.

2. The RNA binding protein Sam68

2.1 *Sam68 structure and functions*

Sam68 belongs to a family of RNA binding proteins called STAR proteins (*S*ignal *t*ransduction and *a*ctivation of *R*NA metabolism), which regulate the metabolism of specific mRNA and play a crucial role in cell cycle and development regulation (Lukong and Richard, 2003). STAR proteins contain a region of homology of about 200 aminoacids called GSG (GRP33/SAM68/GLD1) or STAR domain. This region presents a single KH domain (hnRNP K homology) of about 115 aminoacids, that harbors the RNA binding activity, flanked by two sequences called NK (N-terminal of KH) and CK (C-terminal of KH) regions, of 80 and 30 aminoacids respectively, that confer specificity to the members of

this family and their ability to homodimerize (Lukong and Richard, 2003; Vernet and Artzt, 1997).

In addition to the GSG domain, Sam68 contains proline-rich sequences that mediate interaction with the SH3 domains of proteins involved in signal transduction pathways in response to hormone stimuli like Fyn, PLCγ1, Grb2 and PI3K (Chen *et al*., 1997; Paronetto *et al*., 2003), tyrosine-rich regions, in its carboxyl terminal domain, phosphorylated by tyrosine kinases and bound by SH2 domains, arginine-glycine-rich regions (RG-rich regions) susceptible to methylation and determining the proper localization and function of Sam68, and a nuclear localization signal (NLS) in the C-terminal domain (Lukong and Richard, 2003 as illustrated in Figure 1.

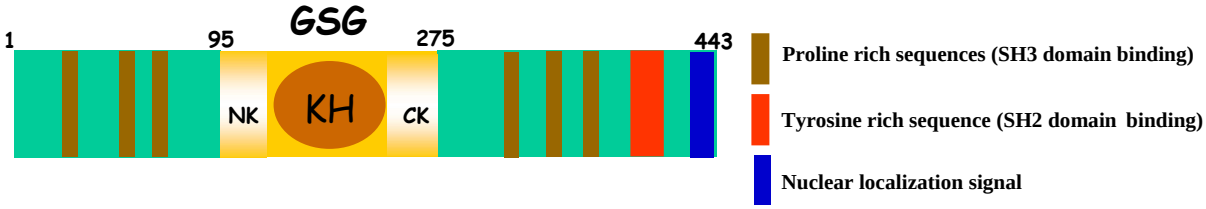

Figure 1 . Schematic representation of the structural/functional domains of Sam68. This protein is composed of the GSG domain, with a single RNA binding domain KH flanked by NK and CK segments, six proline rich sequences (brown bars), a tyrosine rich sequence (red bar) and a nuclear localization signal (blue bar). Relative aminoacid positions are indicated (Adapted from Lukong and Richard, *Bioch. Biophys. Acta* 2003).

The interaction of Sam68 with the SH3 domain of Src promotes the tyrosine-phosphorylation of Sam68 in the carboxyl-terminal region and affects its RNA binding affinity and its sub-cellular localization (Paronetto *et al*, 2003; Lukong and Richard, 2003).

On the other hand, activation of the Ras-Erk pathway triggers serine/threonine phosphorylation of Sam68, that in turn enhances inclusion of exon v5 of the CD44 per-RNA (Matter *et al*., 2002). Changes in the activity, function and localization of the protein have been observed also after methylation, acetylation and sumoylation of specific residues (Cote *et al*., 2003; Babic *et al*., 2004; 2006).

Thus, post-translational modifications are an essential regulatory mechanism of Sam68 RNA-binding activity.

2.2 *Sam68 and tumorigenesis*

A possible role of Sam68 in cancer was proposed after the finding that its depletion of Sam68 was associated with neoplastic transformation of murine NIH3T3 fibroblasts (Liu *et al*, 2000). Consistent with this observation, it had been demonstrated that the overexpression of Sam68 and of a RNA binding defective mutant blocks cell cycle progression (Taylor *et al*., 2004). These initial observations suggested that Sam68 acted as a tumor suppressor and that its depletion caused or favoured neoplastic transformation. However, more recently Sam68 has emerged as a positive regulator of tumorigenesis. Our laboratory has demonstrated that Sam68 is up-regulated in patients affected by PCa (Busà *et al*., 2007) and other types of carcinomas, such as thyroid and liver cancers and testicular seminomas (Figure 2). Remarkably, we have also demonstrated that Sam68 contributes to prostate cancer cell proliferation and that down-regulation of Sam68 by RNAi sensitizes prostate cancer cells to treatments with chemotherapeutic agents like cisplatin and etoposide (Busà *et al*., 2007). In line with our hypothesis for a positive role of Sam68 in cancer, it has now been shown that heterozygous Sam68 female mice, expressing reduced levels of the protein, are protected by polyoma middle T antigen- (MMTV-PyMT) driven tumorigenesis and display a much reduced number of lung metastases (Richard *et al*., 2008). In addition, Sam68 is involved in a common genetic cause of Acute Myeloid Leukaemia (AML). Sam68 is recruited by the SH3 domain of EEN, a gene frequently fused to *Mixed Lineage Leukaemia* (MLL) in patients, and is required for its oncogenic activity by recruiting the methyl transferase PRMT1 and altering gene expression at MLL target sites of the genome. The authors also proved that a fusion between Sam68 and MML could replace the oncogenic potential of the original MLL-EEN fusion, proving that recruitment of Sam68 was the

crucial step in neoplastic transformation. Conversely, specific knockdown of Sam68 expression suppresses MLL-EEN– mediated transformation (Cheung *et al*., 2007).

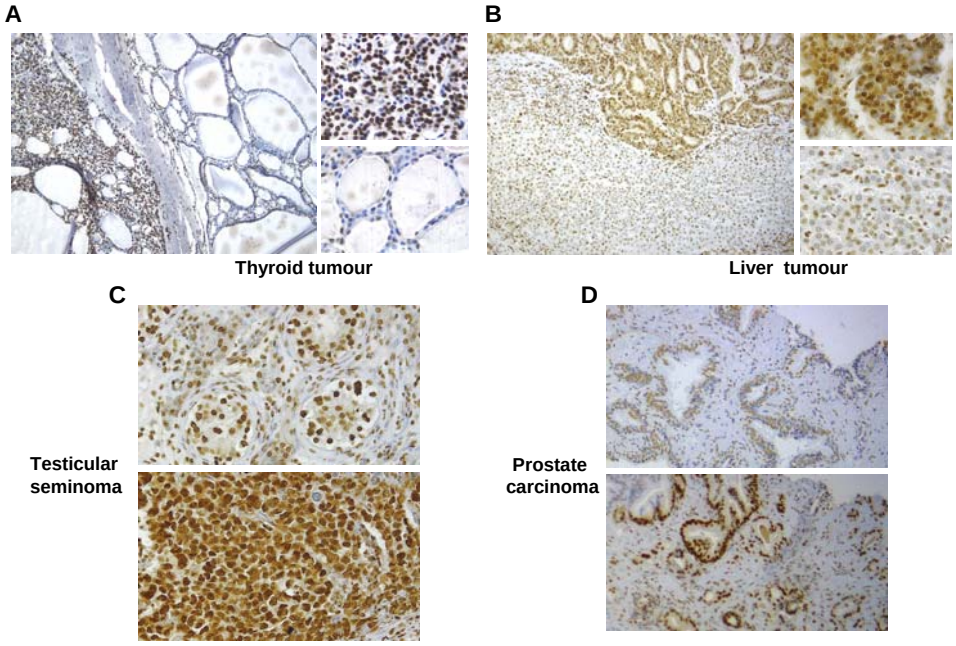

Figure 2. Immunohistochemical analysis of Sam68 expression in human cancer specimens: thyroid, liver, testicular and prostate (A, B, C and D respectively).

Finally, Sam68 has been recently identified as a Vav1 SH3 C-associated protein (Lazer *et al*., 2007). Vav1 is exclusively expressed in the hematopoietic system where it plays a pivotal role in the activation and survival of hematopoietic cells (Katzav *et al*., 1989). It contains several modular motifs known to be involved in protein-protein interactions and signal transduction (Bustelo, 2000; Turner and Billadeau, 2002) and it was recently implicated in several human malignancies (Bartolome *et al*., 2006; Katzav, 2008). The authors showed that Sam68 contributes to cell transformation by oncogenic Vav1 and even enhances its transformation activity in hematopoietic system (Lazer *et al*., 2007).

How can the positive role of Sam68 be reconciled with its cytostatic ad pro-apoptotic effects when overexpressed in cells? A possible explanation comes from the observation that

Sam68 is tyrosine phosphorylated in cancer cells. It was shown that tyrosine phosphorylation of Sam68 by Src kinase correlates with advanced stages of PCas (Paronetto *et al*., 2004). Moreover, in breast cancer cells and primary tumors, the tyrosine kinase BRK phosphorylates Sam68 on Tyr^{440} in the NLS domain (Lukong *et al*., 2005). Since tyrosine phosphorylation of Sam68 affects its RNA affinity and was shown to revert the pro-apoptotic activity of the protein (Paronetto *et al*., 2007), cancer cells might overcome these negative effects of up-regulation of Sam68 by regulating its post-translational modifications.

2.3 *Sam68 and splicing*

Alternative splicing affects 70-90% of all human genes and potentially explains how the mammalian proteome achieves immense complexity from a relatively limited number of genes (Black, 2003; Maitlin *et al*., 2005; Sharp PA, 2005). This regulatory mechanism has been shown to be relevant for many different processes such as apoptosis, sex determination, axon guidance, and cell excitation and contraction (Maniatis and Tasic, 2002; Black, 2003). It arises from the optional use of splice sites, and alternative exon is largely due to the process of splice site selection. The choice between inclusion and exclusion of exons is influenced by *cis*-acting elements known as exon splice ehancers and silencers present inside the exons or in the neighboring introns. Usually, any given region of a pre-mRNA contains, in addition to various potential exon-intron boundaries, several splicing enhancers and silencers that antagonise each other (Black, 2003). Many of these factors are members of serine-arginine-rich (SR) and heteronuclear ribonucleoprotein particle (hnRNP) families of proteins (Black, 2003; Matlin *et al.,* 2005). The SR proteins consist of one or two RNA-binding domains and a domain rich in serine-arginine (SR) dipeptides (Fu, 1995). They bind to splicing enhancers and usually act as positive splicing regulators. The proteins belonging to the hnRNP family , in a wider sense all the mRNA binding proteins that do not contain a

SR domain, often act as negative regulators of RNA processing (Manley and Tacke, 1996; Graveley, 2000).

The nuclear localization of Sam68 suggests a function in post-transcriptional regulation of gene expression. Indeed, it has been shown that nuclear Sam68 integrates signal transduction pathways with pre-mRNA alternative splicing in response to extracellular cues (Matter *et al*., 2002). The first target identified was CD44, a membrane receptor involved in cell proliferation and migration (Ponta *et al*., 2003) that is aberrantly expressed in neoplastic tissues (Naor *et al*., 1997). Sam68 has been shown to regulate CD44 pre-mRNA alternative splicing, mediating the inclusion of the variable exon v5 (Matter *et al*., 2002). Stimulation of mouse thymocytes with phorbol esters triggered activation of the Ras/MAPK/pathway and consequencially determined serine/threonine/ phosphorylation of Sam68 by Erk1/2, which induced the inclusion of v5 exon in a reporter minigene. More recently, it has been observed that Sam68 interacts with the splicing activator SRm160 and they cooperate in the regulation of CD44 v5 and v6 exons alternative splicing (Cheng and Sharp, 2006). Moreover, Sam68 was found to co-transcriptionally associate with Brm, the catalytic subunit of the SWI/SNF chromatin remodelling complex which favours the inclusion of alternative exons in the mRNA of several genes, including CD44 (Batschè *et al*, 2006). Since inclusion of variable exons in CD44 mRNAs causes increased malignancy and invasiveness of some tumors (Naor *et al*., 1997) and the expression of the CD44 v5 and v6 variants has been correlated to poor prognosis in patients (Lee *et al*., 2003; Wu *et al*., 2003; Muller *et al.*, 1997), these data indicate that regulation of Sam68 activity can affect cancer progression.

Another mRNA whose alternative splicing was regulated by Sam68 was identified in our laboratory (Paronetto *et al*., 2007). Sam68 binds the Bcl-x mRNA and changes in the intracellular levels of Sam68 affect the ratio between the pro-apoptotic Bcl-x (s) and the anti-apoptotic Bcl-x (L) mRNA. In particular, an increase in Sam68 levels shifts the balance toward the pro-apoptotic Bcl-x (s) isoform (Paronetto *et al*., 2007). As previously said,

alternative splicing plays a crucial role in apoptosis. Several pre-mRNAs for cell death factors are alternatively spliced and in many cases the different isoforms produced have opposing function during programmed cell death (Schwerk and Schulze-Osthoff, 2005). Advanced PCa cells express high levels of Bcl-x(L) and low levels of the pro-apoptotic Bcl-x(s) (Mercatante *et al*., 2002). Remarkably, our laboratory has shown that this pattern can also be obtained through tyrosine phosphorylation of Sam68 by Src kinases which are often activated in PCa patients (Paronetto *et al*., 2004), while MAPK signalling does not affect Sam68-mediated Bcl-x alternative splicing (Paronetto *et al*., 2007). In this work, it was also shown that Sam68 cooperates with hnRNP A1 in the splice site selection of Bcl-x pre-mRNA and it counteracts the activity of the SR protein ASF/SF2, already reported as a hnRNP A1 antagonist (Eperon *et al.*, 2000), to modulate apoptosis. Moreover, our unpublished data (Paronetto *et al*., manuscript in preparation) suggest that Sam68 has a role in regulating cyclin D1 splicing. Cyclin D1 mRNA is alternatively spliced to give two isoforms: D1a, the most abundant form, and D1b, associated with increased prostate cancer risk (Burd *et al*., 2006). Cyclin D1a is able to interfere with the transcriptional activity of the androgen receptor and limits androgen-dependent PCa cells proliferation. However, in advanced PCas, alternative splicing produces upregulation of the cyclin D1b isoform, which stimulates proliferation of PCa cells (Knudsen, 2006). Our data indicate that Sam68 binds cyclinD1 mRNA (Paronetto *et al*., manuscript in preparation). Moreover, high levels of Sam68 are necessary for cyclin D1b expression in LNCaP cells: depletion of Sam68 in LNCaP cells determines, in fact, a riduction of cyclin D1b mRNA levels and a concomitant increase of cyclin D1a mRNA levels (our unpublished data). These results suggest that Sam68 splicing activity contributes to proliferation and survival of PCa cells.

2.4 *Sam68 and translation*

It has been demonstrated that RNA-binding proteins belonging to SR family, such as ASF/SF2, act as multifunctional regulators of mRNA metabolism with different roles that couple alternative pre-mRNA splicing, mRNA export and translation of specific mRNAs (Sanford *et al*., 2004). A role in promoting translation has been shown also for hnRNP A1 (Bonnal *et al*., 2005).

A similar role in mRNA translation has now also been proposed for Sam68, based on the evidence that this protein promotes nuclear export (Li *et al*., 2002) and cytoplasmic utilization of viral mRNAs (Coyle *et al*., 2003). In addition, it was shown that Sam68 accumulated in granular structures in the dendrites of neurons subjected to membrane depolarization (Ben Fredj *et al*., 2004). Moreover, our laboratory has recently demonstrated that Sam68 localizes in the cytoplasm also in male germ cells undergoing the meiotic divisions, where it associates with the polysomes engaged in translation (Paronetto *et al*., 2006). Translocation correlates with serine/threonine phosphorylation and it is blocked by inhibitors of the mitogen activated protein kinases ERK1/2 and of the maturation promoting factor cyclinB-cdc2 complex. Molecular cloning of the mRNAs associated with Sam68 in mouse spermatocytes reveales a subset of genes that might be post-transcriptionally regulated by this RNA-binding protein during spermatogenesis. This regulation appears relevant because it was found that many Sam68 RNA targets are inefficiently loaded on the polysomes in knockout germ cells. Moreover, since the analysis of the phenotype of knockout mice has revealed that Sam68 is required for spermatogenesis and in male fertility (Paronetto MP, Messina V, Bianchi E, Barchi M, Moretti C, Palombi F, Stefanini M, Geremia R, Richard S and Sette C, manuscript submitted), it is likely that the mRNAs translationally regulated by Sam68 play an essential role during male germ cell differentiation. It will be interesting to investigate if also in cancer cells Sam68 has a role in translational regulation of specific mRNAs.

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Results

1. The RNA-binding protein Sam68 contributes to proliferation and survival of human prostate cancer cells

Abstract

The tyrosine kinase Src is frequently activated in advanced human prostate carcinomas and its activation correlates with tyrosine phosphorylation of the RNA-binding protein Sam68. Here, we have investigated the expression and function of Sam68 in human prostate cancer cells. Analysis of specimens obtained from 20 patients revealed that Sam68 is up-regulated at the protein level in 35% of the samples and by Real time PCR we confirmed the results also at the mRNA level in most patients. It was observed that down-regulation of Sam68 by RNAi in LNCaP prostate cancer cells delayed cell cycle progression and reduced the proliferation rate. Moreover, depletion of Sam68 sensitized cells to apoptosis induced by DNA-damaging agents. Similarly, stable cell lines expressing a truncated GFP-Sam68$_{GSG}$ protein, that interacts with endogenous Sam68 affecting its activity, displayed reduced growth rates and higher sensitivity to cisplatin-induced apoptosis. Finally,microarray analyses revealed that a subset of genes involved in proliferation and apoptosis were altered when Sam68 was knocked down in LNCaP cells. Interestingly, among this subset of genes we found cyclin D1 and Bcl-x (L), two mRNA targets of Sam68, which were respectively up- and down-regulated both in RNA and protein levels following Sam68 depletion.

Together, our results indicate that Sam68 expression supports prostate cancer cells proliferation and survival to cytotoxic agents.

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ORIGINAL ARTICLE

The RNA-binding protein Sam68 contributes to proliferation and survival of human prostate cancer cells

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The tyrosine kinase Src is frequently activated in advanced human prostate carcinomas and its activation correlates with tyrosine phosphorylation of the RNA-binding protein Sam68. Herein, we have investigated the expression and function of Sam68 in human prostate cancer cells. Analysis of specimens obtained from 20 patients revealed that Sam68 is upregulated at the protein level in 35% of the samples. Real-time polymerase chain reaction confirmed the results at the mRNA level in most patients. Downregulation of Sam68 by RNAi in LNCaP prostate cancer cells delayed cell cycle progression and reduced the proliferation rate. Moreover, depletion of Sam68 sensitized cells to apoptosis induced by DNA-damaging agents. Similarly, stable cell lines expressing a truncated GFP-Sam68$_{GSG}$ protein displayed reduced growth rates and higher sensitivity to cisplatin-induced apoptosis. Microarray analyses revealed that a subset of genes involved in proliferation and apoptosis were altered when Sam68 was knocked down in LNCaP cells. Our results indicate that Sam68 expression supports prostate cancer cells proliferation and survival to cytotoxic agents.
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Keywords: prostate cancer; Sam68; cell proliferation; apoptosis; RNA metabolism

Introduction

Prostate carcinoma (PCa) originates as an androgen-dependent hyper-proliferation of the epithelial cells of the gland and it evolves in an androgen-independent, highly aggressive cancer for which no cure is available yet (Feldman and Feldman, 2001). As androgen-refractoriness is associated with poor prognosis, it is of primary importance to identify the molecular pathways that can be targeted by therapies alternative to androgen-depletion. A predominant role in the development of androgen-refractoriness is played by the upregulation of signal transduction pathways that allow prostate cancer cells to autonomously produce their own requirements of growth factors and nutrients (Grossmann *et al*., 2001). In many cases, these autocrine loops trigger the activation of tyrosine kinase pathways. In this regard, it was shown that the tyrosine kinase Src is required for proliferation and migration of prostate cancer cells (Migliaccio *et al*., 2000; Lee *et al*., 2001) and that inhibition of Src blocks their adhesion to the extracellular matrix and invasiveness (Nam *et al*., 2005).

Src is the prototype of a class of tyrosine kinases that have been intensively studied due to their impact on cell tranformation and tumour development (Irby and Yeatman, 2000). The activity of Src-related tyrosine kinases is increased in a multitude of primary tumours and metastatic lesions and Src-specific inhibitors may have a therapeutic application in inhibiting tumour progression and/or metastasis (Nam *et al*., 2005). In line with the role of Src in PCa, the tumour suppressor DOC2/DAB2, an endogenous Src inhibitory protein, is frequently downregulated in this cancer (Zhou *et al*., 2003), whereas an activator of Src, the truncated c-Kit protein tr-Kit, is aberrantly expressed in prostate tumours at advanced stage of the disease. Activation of Src in these PCas correlated with tyrosine phosphorylation of Sam68 (Src substrate in Mitosis, 68 kDa) (Paronetto *et al*., 2004), an RNA-binding protein that acts as a post-transcriptional regulator of gene expression (Lukong and Richard, 2003).

Sam68 belongs to the signal transduction and activation of RNA metabolism (STAR) family of RNA-binding proteins, which appear to link signal transduction pathways with the regulation of RNA metabolism (Lukong and Richard, 2003). They are characterized by a GSG (Gpr33-Sam68-GLD-1) domain, which is required for RNA binding and homodimerization, flanked by regions involved in protein–protein interactions and post-translational modifications, which affect the affinity and specificity of RNA binding. In particular, Sam68 interacts with the SH2 and SH3 domains of several signalling proteins acting as a scaffold molecule in response to different stimuli (Richard *et al*., 1995; Paronetto *et al*., 2003). Physical

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interaction with Src-related kinases and tyrosine phosphorylation of Sam68 (Lukong and Richard, 2003) cause a decreased affinity for RNA. Moreover, it was shown that Sam68 is phosphorylated by the Erk1/2 mitogen-activated protein kinases (MAPK) in response to external cues. As this modification affects alternative splicing of the CD44 receptor pre-mRNA (Matter *et al*., 2002), Sam68 may link growth factors signalling to post-transcriptional modulation of gene expression. The intracellular localization of Sam68 is also regulated by post-translational modifications like methylation (Cote *et al*., 2003), tyrosine phosphorylation (Paronetto *et al*., 2003; Lukong *et al*., 2005) and by its ability to bind to polysomes (Paronetto *et al*., 2006). Although a direct role in translation has not been demonstrated yet, several observations indicate that Sam68 can substitute for the HIV protein Rev and mediate nuclear export and cytoplasmic utilization of viral mRNA (Reddy *et al*., 1999; Soros *et al*., 2001; Coyle *et al*., 2003). Interestingly, a physiological dominant-negative isoform of Sam68, with a deletion in the RNA-binding domain, is expressed by normal cells when they reach confluence and causes cell cycle arrest (Barlat *et al*., 1997), suggesting that Sam68 function is beneficial to cell proliferation.

Recent high-throughput screens have demonstrated that changes in alternative splicing can be more informative than changes in global transcription to classify PCa phenotypes (Li *et al*., 2006; Zhang *et al*., 2006). However, no specific information is available on the aberrant expression or regulation of RNA-binding proteins and splicing factors in prostate cancer cells. Herein, the expression and function of Sam68 in prostate cancer cells was investigated. We report that Sam68 protein is frequently upregulated in the epithelial cells of human PCas and that this protein supports prostate cancer cell proliferation and survival.

Results

Sam68 is upregulated in human prostate carcinomas

The expression of Sam68 was analysed in 20 patients with different stages of PCa lesions (Table 1). Western blot analysis performed on extracts obtained from the neoplastic tissue and from the contralateral part of the gland, indicated that Sam68 protein was upregulated in 35% of the neoplastic tissues tested (Figure 1a; Table 1). Real-time PCR from total RNA obtained from the same tissues confirmed the upregulation of Sam68 at the mRNA level in most of the patients examined (Figure 1b and Table 1). The clinical profile of the patients examined and a summary of the results obtained are described in Table 1.

Neoplastic lesions often correlate with an inflammatory response and with recruitment of blood cells in the prostate gland (Palapattu *et al*., 2005). To determine whether upregulation of Sam68 occurred in the prostate epithelial cells or in the invading inflammatory cells, immunohistochemistry analysis was performed on the subset of samples displaying elevated levels of Sam68. As illustrated in Figure 2a–c, Sam68 was expressed at moderate levels in approximately half of the epithelial cells of normal prostate glands. By contrast, all epithelial cells of neoplastic glands were strongly positive to Sam68 and in some samples these cells had already invaded the surrounding stroma (Figure 2d–f). This result suggests that elevated expression of Sam68 correlates with the neoplastic phenotype of prostate epithelial cells. Sam68 was expressed by cells both in G1 phase and in mitosis, as shown by staining

Table 1 Summary of the results on Sam68 expression in human PCa

Case number	*Age*	*PSA*	*Gleason*	*TNM*	*Sam68 protein*	*Sam68 RNA*
1	68	7.3	2+2	T2cNOMO	+	+
2	67	7.2	4+3	T2cNOMO	+	+
3	70	14.35	3+4	T2cNOMO	+	+
4	68	9.59	4+5	T2aNOMO	=	=
5	69	5.52	3+3	T2aNOMO	=	=
6	64	6.97	3+3	T2aNOMO	=	ND
7	68	11.64	3+4	T2bNOMO	=	=
8	56	5.7	3+3	T2cNOMO	=	+
9	66	6.5	3+3	T2aNOMO	=	=
10	60	14	3+5	T2aNOMO	+	=
11	68	4.5	3+4	T2aNOMO	=	=
12	74	4.11	4+4	T2cNOMO	=	=
13	70	4.55	3+3	T2cNOMO	=	+
14	65	9.49	4+4	T2cNOMO	+	+
15	65	14.86	3+3	T2aNOMO	+	+
16	63	14	4+5	T2cNOMO	=	=
17	58	14	4+3	T2cNOMO	+	+
18	76	5.57	3+2	T2bNOMO	+	+
19	57	7.03	3+3	T2aNOMO	=	=
20	59	3.7	3+3	T2cNOMO	=	=

Clinical and histopatological features of the 20 human PCas examined. Sam68 expression at the mRNA and protein levels are reported as: '+' if they are increased in the tumor versus the contralateral normal part of the same patient; '=' if they are unchanged; '−' if they are decreased. ND means not determined. Real-time PCR data were considered '+' if the fold induction in the tumor was at least 3.

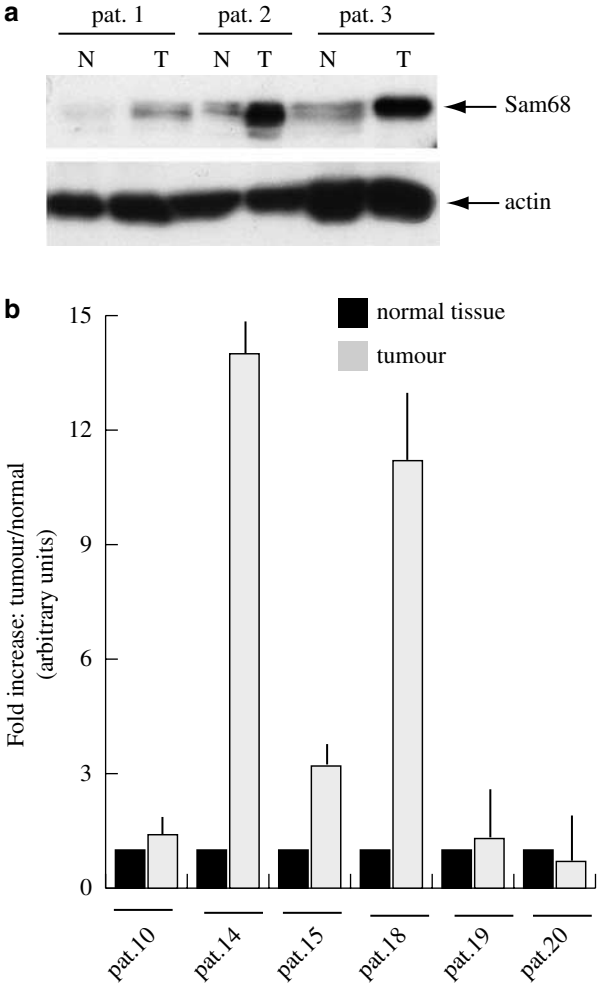

Figure 1 Sam68 is frequently up-regulated in human prostate carcinomas. (**a**) Western blot analysis of Sam68 (upper panel) and actin (lower panel) expression levels in extracts obtained from the neoplastic tissue (T) and from the contralateral part of the gland (N) of three representative patients. (**b**) Analysis of Sam68 mRNA levels by real-time PCR from total RNA obtained from the neoplastic tissue (grey bar) and the contralateral part of the gland (black bar) of six representative patients. Data are expressed as fold induction in the tumour versus the contralateral normal part. Three separate runs for each sample were performed.

of serial sections with cyclin D1 and Ki67, respectively (Figure 2g).

Downregulation of Sam68 in LNCaP prostate cancer cells causes decreased proliferation

To investigate the function of Sam68 in prostate cancer cells, we have attempted to downregulate its expression by RNAi in androgen-responsive LNCaP cells. Sam68 protein is stable in these cells and efficient reduction of its expression levels was obtained after two to three cycles of transfection with either of two different double-strand siRNAs (si1 Sam68 or si2 Sam68 in Figure 3a). Cell number counts and MTS assays indicated that downregulation of Sam68 causes a decrease in the proliferation rate of LNCaP cells (Figure 3b). Similar results were also obtained with the si1 Sam68 RNA (data not shown), indicating that the effect is specific for Sam68. BrdU-labeling showed that reduction of Sam68 obtained with both siRNAs resulted in a slightly decreased number of cells in S phase (Figure 3c). These data indicate that high levels of Sam68 are required for optimal proliferation of LNCaP cells. To determine if cell cycle progression was delayed by depletion of Sam68, transfected LNCaP were treated with mimosine or thymidine, to induce a block in G1/S or with nocodazole to induce a block in G2/M. Scrambled-transfected LNCaP (Figure 3d) were prevalently in G1 (73% of cells) with only a small fraction of cells in G2/M phase (12%). Treatment with mimosine or thymidine caused a further accumulation of cells in G1/S phase and mimosine caused also an increase in the levels of cyclin D1, a marker of the G1/S transition (Figure 3d). Interestingly, we observed that depletion of Sam68 in unsynchronized LNCaP cells augmented the G1 population (77% of cells) and decreased the G2/M (8%), with a concomitant increase in cyclin D1 levels and a decrease in cyclin B1. Mimosine and thymidine did not alter this pattern, but G2/M (21% of cells versus 33% in control transfected cells) and cyclin B1 accumulation induced by nocodozole were less evident. As LNCaP cells are prevalently in G1 in the unsynchronized population, these results indicate that depletion of Sam68 delays entry into G2/M in the 16 h of the treatment.

Downregulation of Sam68 sensitizes LNCaP cells to apoptosis

To test whether Sam68 plays a role in LNCaP cell survival, we analysed apoptosis induced by the chemotherapeutic agents cisplatin and etoposide. Downregulation of Sam68 did not affect activation of caspase 3 or PARP-1 cleavage (Figure 4b–d), which are two well-established late apoptotic events. However, treatment of LNCaP cells with cisplatin (80 μM) or etoposide (85 μM) for 16 h caused a much more dramatic induction of apoptosis in cells silenced for Sam68 than in scrambled siRNA-transfected cells, as determined by cell morphology (Figure 4a), nuclear morphology by Hoechst staining, caspase 3 activation (Figure 4b and c) and PARP-1 cleavage (Figure 4d). These results indicate that Sam68 protects prostate cancer cells from chemotherapeutic agents.

A GFP-Sam68$_{GSG}$ chimeric protein interferes with LNCaP proliferation and survival

Sam68 function is modulated by post-translational modifications that occur on the regions flanking the GSG domain (Chen *et al*., 1999; Lukong and Richard, 2003). In an attempt to interfere with Sam68 function *in vivo*, we established LNCaP cell lines stably transfected with a GFP-Sam68$_{GSG}$ chimeric protein. Although mainly cytoplasmic (Figure 5a), a fraction of GFP-Sam68$_{GSG}$ was nuclear and interacted with the endogenous Sam68, as demonstrated by co-immunoprecipitation experiments from nuclear extracts (Figure 5b). To test whether this interaction interferes with Sam68

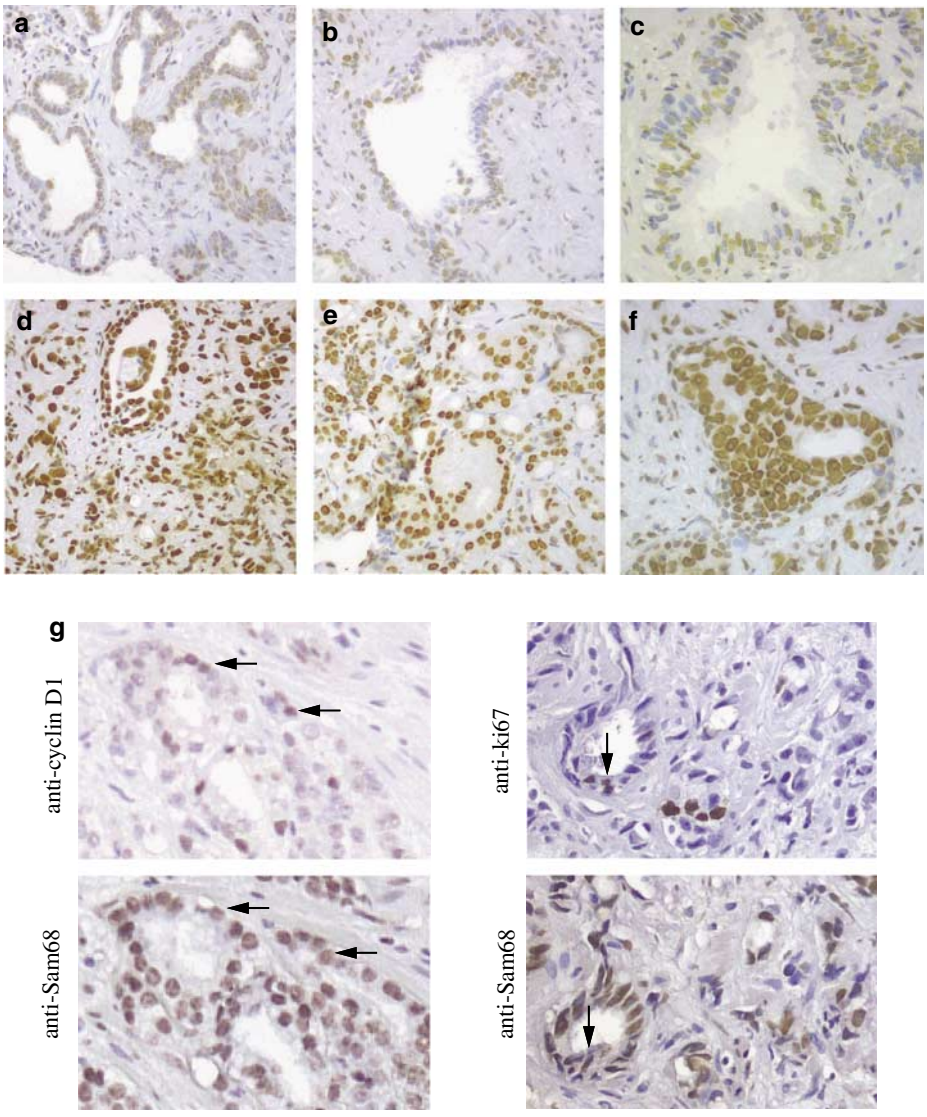

Figure 2 Analysis of Sam68 expression by immunohistochemistry in human normal and neoplastic prostate glands. Specimens from patients were analysed by immunohistochemistry for Sam68 expression. (**a**–**c**) Representative normal glands in the contralateral part of the prostate of three patients affected by neoplastic lesions shown in panels (**d**–**f**). Images in (**a**, **b**, **d** and **e**) were taken with a $\times 20$ objective whereas images in (**c** and **f**) were taken with a $\times 40$ objective. (**g**) Serial sections were stained with anti-Sam68 and either anti-cyclin D1 or anti-Ki67 antibodies. Cells positive to both Sam68 and cyclin D1 or Ki67 are pointed by black arrows. Images were taken with a $\times 40$ objective.

function, we performed an *in vivo* splicing assay using the CD44 v5 minigene (Matter *et al*., 2002). Indeed, v5 exon inclusion induced by Sam68 in the CD44-positive PC3 prostate cancer cells was partially inhibited by the truncated myc-Sam68$_{GSG}$ protein (Figure 5c). Interestingly, GFP-Sam68 was constitutively active in the PC3 prostate cancer cells and did not require stimulation of the MAPK pathway by either EGF or TPA (Supplementary Figure 1). In agreement with its interfering role, the two stable clones expressing GFP-Sam68$_{GSG}$ (GFP-GSG3 and GFP-GSG5) displayed reduced proliferation rate (Figure 5e) and were more sensitive to treatment with cisplatin (Figure 5d). Hence, the effects produced by expression of GFP-Sam68$_{GSG}$ resemble those obtained by downregulation of Sam68 by RNAi and indicate that interference with Sam68 function impairs LNCaP cell proliferation and survival to DNA-damaging agents.

Gene expression profile of Sam68-silenced LNCaP cells

Next, we hybridized RNAs obtained from scrambled siRNA- or siSam68-transfected LNCaP cells onto microarray chips containing a subset of 263 genes with known relevance to prostate cancer cells proliferation and apoptosis (Figure 6a and b). Although silencing of Sam68 did not cause large-spectrum changes in gene expression, selected genes were either upregulated or downregulated between 2- and 10-fold (Table 2). We found that depletion of Sam68 caused the downregulation of genes reported to protect from apoptosis, such as Bcl2L1 (confirmed also at the protein level, Figure 6c)

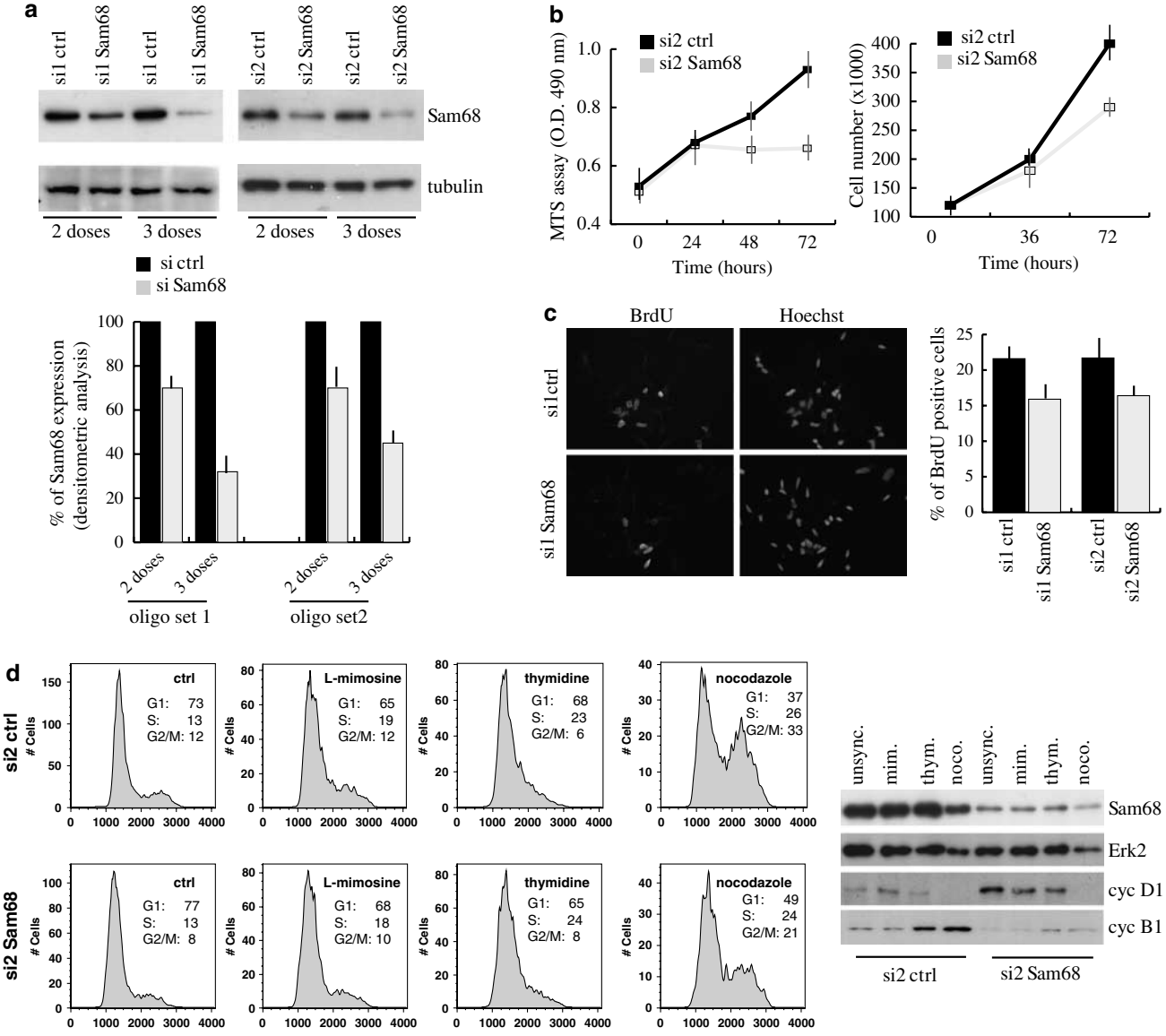

Figure 3 Knock down of Sam68 expression negatively affects LNCaP cell proliferation. (**a**) Western blot analysis of Sam68 and tubulin in LNCaP cells extracts (10 μg) after two or three cycles of transfection with double-strand Sam68 siRNAs (si1 Sam68, si2 Sam68) or scrambled siRNAs (si1 ctrl and si2 ctrl). Densitometric analysis of three separate experiments is shown in the bar graph. (**b**) MTS assay (left panel) and cell count (right panel) on LNCaP transfected with the scrambled siRNA (black squares) or with siSam68RNA (grey squares) as described in (**a**). Data are the mean $\pm$ s.d. of three experiments each performed in triplicate. (**c**) BrdU incorporation (% BrdU-positive nuclei) was measured by immunofluorescence (upper panels) on the fourth day of culture in LNCaP cells transfected as described in (**a**) in three experiments. (**d**) Cell cycle progression of siRNA transfected LNCaP was monitored by Western blot analysis of cyclin D1, B1, Sam68 and Erk2 (left panels) or by FACS analysis of propidium iodide-stained cells.

and Clusterin, or to participate in DNA repair, like Brca1, whereas the pro-apoptotic transcription factor Par-4 was upregulated (Table 2). These results provide support to the higher sensitivity to cisplatin and etoposide of these cells. On the other hand, we found that mRNAs for cdk2 (confirmed also at the protein level, Figure 6c) and cdk3, kinases required for G1/S progression, were downregulated whereas the mRNA for p16INK4, a cdk inhibitor, and cyclin D1 (see also Figure 4) were up-regulated. In addition, depletion of Sam68 led to a decrease in the mRNA levels of growth factors like EGF and IGF-1. The function of other genes altered in Sam68 depleted cells are less clearly implicated with cell proliferation and apoptosis. These data support the hypothesis that Sam68 is beneficial to proliferation and survival of prostate cancer cells.

Discussion

Post-transcriptional regulation of gene expression is often aberrant in cancer cells and changes in both alternative splicing and translational regulation of specific mRNAs have been reported (Ruggero and Sonenberg, 2005; Venables, 2006). Remarkably, changes

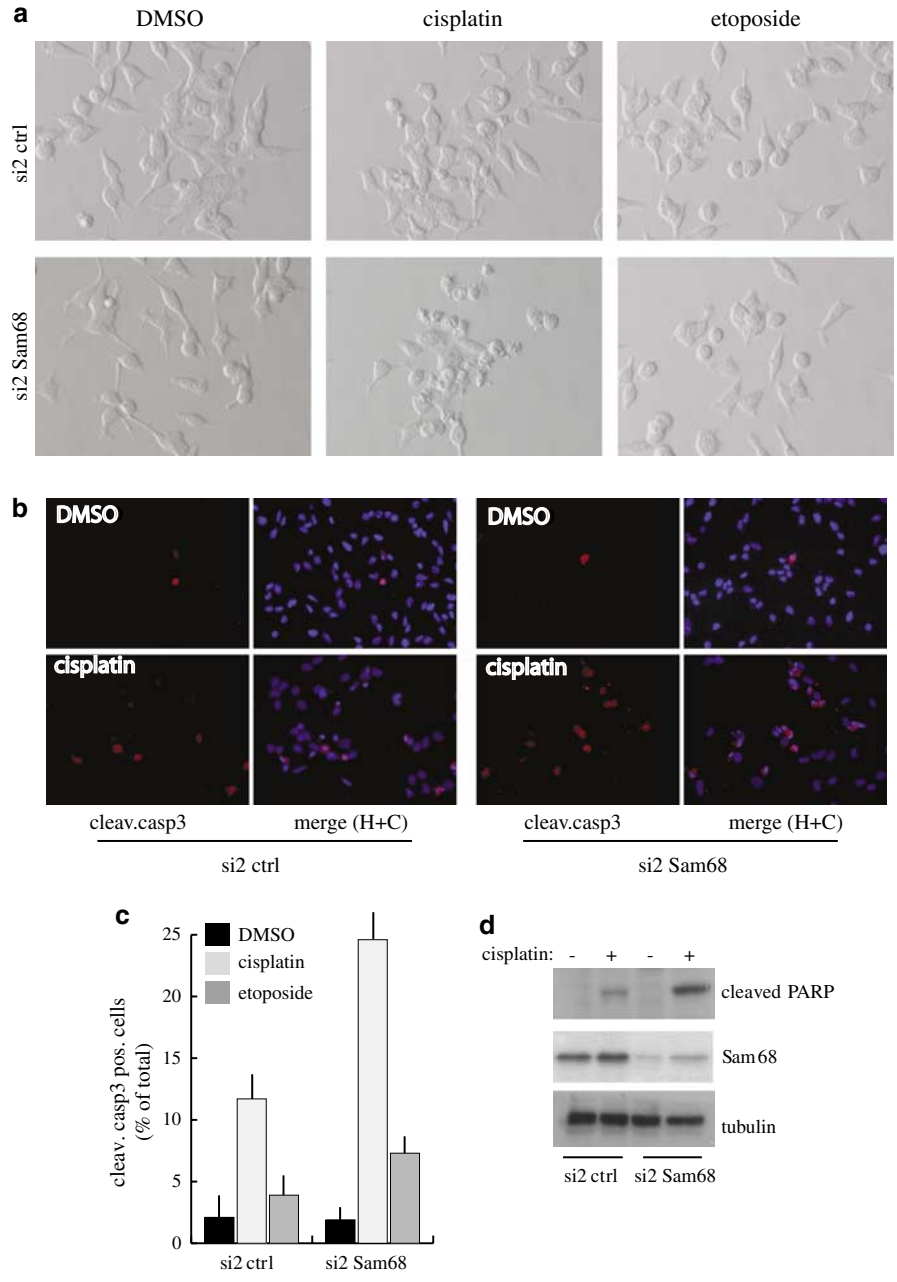

Figure 4 Downregulation of Sam68 by RNAi sensites LNCaP cells to DNA damage-induced apoptosis. (**a**) Phase contrast images of LNCaP cells transfected with either si2 ctrl or si2 Sam68. siRNA and treated with 80 μM cisplatin or 85 μM etoposide. (**b**) LNCaP cells treated as in (**a**) were stained with anti-cleaved caspase 3 (**c**) and Hoechst (**h**). (**c**) Bar graph representation of the percentage of caspase 3-positive apoptotic cells from three experiments. (**d**) Western blot analysis of cleaved PARP expression, Sam68 and tubulin in cells transfected as in (**a**) and incubated $\pm$80 μM cisplatin.

in alternative splicing classify PCa phenotypes more accurately than changes in global transcription (Li *et al*., 2006; Zhang *et al*., 2006). These observations indicate that factors influencing pre-mRNA processing could play a crucial role in determining the neoplastic progression of prostate cancer cells. Herein, we have investigated the role played in human PCas by Sam68, an RNA-binding protein involved in several aspects of mRNA processing (Lukong and Richard, 2003). Our results demonstrate that Sam68 is frequently upregulated in human PCas and that downregulation of its expression or activity affects prostate cancer cell proliferation and survival.

The effects of altering Sam68 expression in prostate cancer cells were determined by RNAi experiments in LNCaP cells. The decrease in Sam68 levels obtained with two different siRNAs reduced the proliferation rate of these cells. BrdU incorporation, FACS and Western blot analyses indicate that cells silenced for Sam68 are delayed in the G1 phase

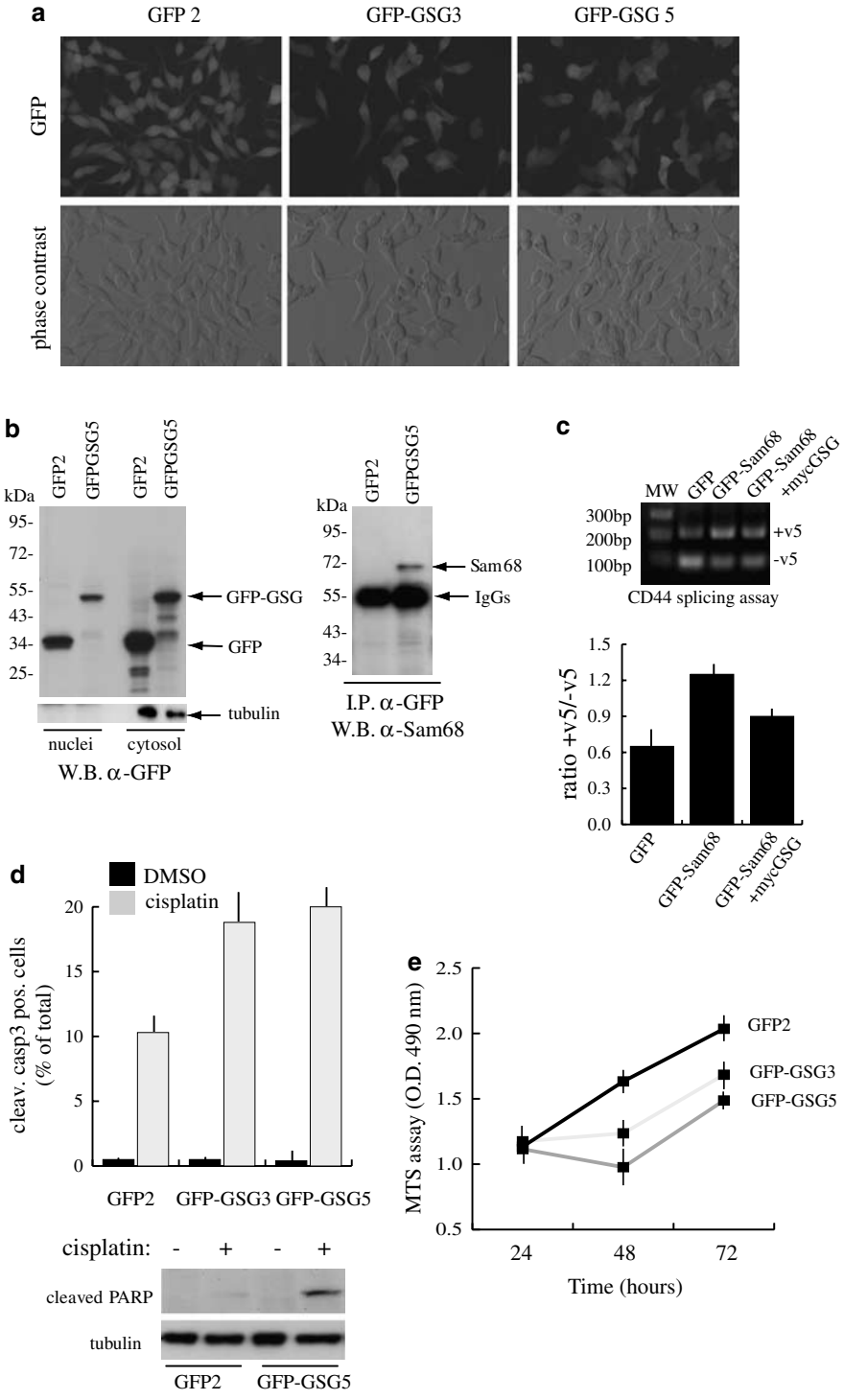

Figure 5 GFP-Sam68$_{GSG}$ chimeric protein interferes with endogenous Sam68 *in vivo* and affects LNCaP proliferation and survival. (**a**) Two GFP-Sam68$_{GSG}$ LNCaP clones (GFP-GSG3 and GFP-GSG5) and one GFP clone were analysed by direct fluorescence *in vivo* for GFP expression (upper panels) and by phase contrast (lower panels). (**b**) Western blot analysis of nuclear and cytosolic extracts (30 μg) from LNCaP stable clones with anti-GFP and anti-tubulin antibodies. Nuclear extracts (600 μg) were immunoprecipitated with anti-GFP antibody and samples were stained with anti-Sam68 antibody (right panel). (**c**) CD44 v5 exon splicing assay after transfection of GFP, GFP-Sam68 and myc-Sam68$_{GSG}$ (myc-GSG) in PC3 cells together with the CD44-v5 minigene. RT–PCR assays determined inclusion (+v5) or exclusion (−v5) of the CD44 exon in three experiments. (**d**) Bar graph representation of the percentage of caspase 3-positive apoptotic cells from three experiments and Western blot analysis of cleaved PARP-1 expression in GFP2 and GFP-GSG5 clones incubated with ±80 μM cisplatin. (**e**) MTS Proliferation assay of LNCaP stable clones in three experiments.

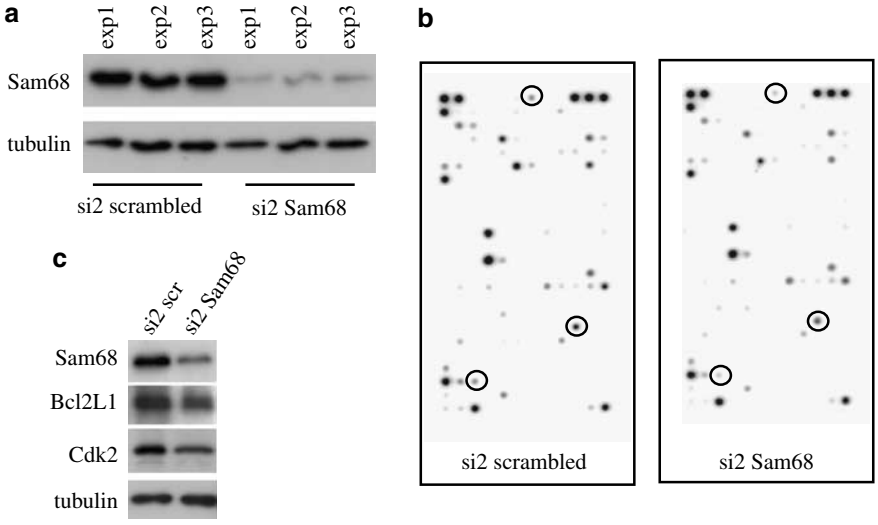

Figure 6 Downregulation of Sam68 in LNCaP affects a subset of genes involved in cell proliferation and survival. Sam68 RNAi was performed in three experiments and verified by Western blot analysis (**a**). Total RNAs from these samples were labelled and analysed on the Superarray Prostate Biomarkers chip. Examples of three genes downregulated in the Sam68 siRNA-transfected cells are marked by circles (**b**). Western blot analysis of Bcl2L1 and cdk2 in LNCaP cells silenced with scramble siRNA or Sam68 siRNA (**c**).

Table 2 Changes in gene expression induced by Sam68 knock down in LNCaP cells

Gene name	*Fold difference*	*Protein function*
Downregulated		
Bcl2L1	4.2±0.5	Apoptotic regulator
Brca1	2.3±0.3	Repair of DNA damage; frequently mutated in breast cancer
Clusterin	2.4±1.0	Cytoprotective chaperone; protects from apoptosis
Cdk2	10.5±3.5	Cell cycle kinase; proliferation
Cdk3	2.2±0.6	Cell cycle kinase; proliferation
EGF	3.0±0.6	Growth factor; cell proliferation
IGF1	3.2±1.1	Growth factor; cell proliferation
Upregulated		
Cyclin D1	3.2±0.8	Marker of G1/S phase; cell proliferation,
IGFbp6	4.6±1.5	IGF-binding protein; inhibits growth
NR2F1	2.4±0.5	Orphan receptor, transcriptional regulation
NR3C1	3.6±0.8	Glucocorticoid receptor
NR4A1	4.1±1.5	Orphan receptor; early gene
Par-4	5.2±1.1	Pro-apoptotic transcription factor, selective for cancer cells
p16INK4	3.3±0.5	Cell cycle inhibitor; up-regulated in quiescent cells
p38beta	3.5±1.0	Stress-activated MAPK; cellular response to various stresses

Total RNA from transfected LNCaP cells were analysed by microarray hybridization onto a chip containing 263 genes with relevance to human PCa. Data are reported as the ratio between the value obtained in cells transfected with scrambled siRNA versus Sam68 siRNA for the downregulated genes and as the opposite ratio for the upregulated ones. Densitometric analysis was performed using the Scanalyze software. Listed are the genes that yield reproducible results (mean±s.d.) in three experiments.

of the cycle. However, they are not blocked and they reach confluence later than cells transfected with control scrambled siRNAs. Interestingly, the delay in cell cycle progression of LNCaP cells silenced for Sam68 affected accumulation in G2/M driven by nocodazole treatment. As unsynchronized LNCaP are prevalently in the G1 phase of the cycle, this result indicates that less LNCaP cells can reach the G2 phase in 16 h when Sam68 levels are reduced. On the other hand, it was previously shown that transient overexpression of Sam68 in nontransformed cells also caused a delay in G1 (Taylor *et al*., 2004). Thus, it is possible that alterations in the cellular levels of Sam68 in either direction affect the normal G1 progression. Alternatively, Sam68 may play a different role in nontransformed and transformed cells. In line with the latter hypothesis, Src activity and tyrosine phosphorylation of Sam68, which affects its cellular function, are frequently increased in human PCas (Paronetto *et al*., 2004). Similarly, phosphorylation of Sam68 on tyrosine 440 has been recently reported in specimens obtained from human breast cancer (Lukong *et al*., 2005). As Src-kinase inhibitors are known to interfere with prostate cancer cell proliferation and invasiveness (Nam *et al*., 2005), it is possible that Sam68 is among the effectors of this pathway in human PCas.

Depletion of Sam68 in LNCaP cells caused accumulation of cyclin D1. Interestingly, in androgen-sensitive prostate cancer cells accumulation of cyclin D1a (the most common isoform) interferes with the transcriptional activity of the androgen receptor thereby inhibiting proliferation (Burd *et al*., 2006). Hence, our observation that cyclin D1 protein is upregulated in Sam68-depleted LNCaP may indicate that the decreased proliferative rate of these cells is due to downregulation of androgen receptor activity.

Perhaps more interesting is the observation that depletion of Sam68-sensitized LNCaP cells to apoptosis induced by DNA damaging agents. It has been previously reported that Sam68 and other splicing factors are re-localized in specific sub-nuclear compartments in response to heat shock of cells, suggesting a function for this protein in the cellular response to a stress (Denegri *et al*., 2001). Indeed, depletion of Sam68 caused the downregulation of mRNAs encoding anti-apoptotic factors (Bcl2L1 and Clusterin) or proteins involved in DNA repair (Brca1), whereas the pro-apoptotic transcription factor Par-4 is upregulated. Thus, our results suggest that maintaining high levels of Sam68 protein could be part of the adaptation mechanisms employed by cancer cells to withstand hostile environments.

The GSG domain of Sam68 is required for RNA binding, protein homodimerization and intracellular localization (Chen *et al*., 1999). However, this domain lacks the motifs required for post-translational modification of Sam68 that affect its activity and localization (Lukong and Richard, 2003). We reasoned that constitutive expression of this domain in cells may influence the activity of the endogenous Sam68 by forming heterodimers that are less susceptible to post-translational control. Indeed, we found that the GFP-Sam68$_{GSG}$ efficiently dimerized with endogenous Sam68 *in vivo* and that LNCaP clones expressing GFP-Sam68$_{GSG}$ strongly resemble cells depleted of Sam68 in terms of reduced proliferation and augmented sensitivity to apoptosis. These observations suggest that expression of the GSG domain alone interferes with Sam68 function in live cells and that this domain could be exploited as therapeutic target to limit the proliferation and survival of prostate cancer cells.

In conclusion, our finding that Sam68 is required for optimal proliferation and survival of prostate cancer cells may represent a first step to understand how these neoplastic cells alter their mRNA isoforms pattern during transformation.

Materials and methods

Human tissue samples
PCa were diagnosed by TRU-CUT biopsy, graded using the Gleason and the TNM grading systems (Table 1) and samples were obtained after informed consent from patients who underwent prostatectomy as previously described (Paronetto *et al*., 2004).

Immunohistochemistry
Immunohistochemical staining was performed on formalin-fixed tissue as described previously (Paronetto *et al*., 2006) with polyclonal rabbit anti-Sam68 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, SC-133 1:800 dilution), rabbit anti-cyclin D1 (Dako, Carpinteria, CA, USA, 1:400 dilution) and rabbit anti-Ki67 (Dako, 1:400). Detection was accomplished using a biotin–streptavidin horseradish peroxides detection kit (Dako EnVision/HRP).

Extraction of RNA and proteins from cultured cells and primary tissue
Total RNA was extracted by homogenization of samples in TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA). Real-time PCR was performed on cDNAs obtained with MMLV reverse transcriptase (Invitrogen). Applied Biosystems gene expression assays for 18S and GAPDH were used for relative expression according to manufaturer's instructions. Sam68 oligonucleotides used can be given upon request. For proteins extraction, LNCaP cells and tissue fragments were homogenized in lysis buffer (100 mM NaCl, 10 mM $MgCl_2$, 30 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, protease inhibitor cocktail), supplemented with 1% Triton-X-100. Soluble extracts from 15 min centrifugation at 12 000 *g* at 4°C, were used for Western blot.

Cell cultures and transfections
LNCaP cells were maintained in RPMI 1640 medium (BioWhittaker Cambrex Bioscience, Belgium) as described (Paronetto *et al*., 2004). Cells at ~50/60% confluency were transfected for 2 or 3 consecutive days with small interfering RNA (siRNAs) (MWG Biotech, Ebersberg, Germany) using Oligofectamine and Opti-MEM medium (Invitrogen). Sam68 siRNAs were: 5′-GGAUCUGCAUGUCUUCAUU-3′ (si1Sam68) and 5′-CUGUCAGGAGCAAUUUCUA-3′ (si2-Sam68). Scrambled siRNAs were: 5′-GUGCUCAAUUGGA UUCUCU-3′ (si1 ctrl) and 5′-GGAGCUUCAUUGCUAUC AA-3′(si2 ctrl).

For cell cycle block in different phases, LNCaP cells were treated with 100 μM L-mimosine (G1/S), or 2 mM thymidine (S) or 500 ng/ml nocodazole (G2/M) for 16 h, collected in PBS, incubated with 70% ethanol for 2 h, then treated with RNAseA (15 min at 37°C) and 10 μg/ml propidium iodide (30 min at 37°C) and analysed on a FACSCalibur Flow Cytometer (Becton Dickinson, San Josè, CA, USA).

Immunoprecipitation assay
Nuclear and cytoplasmic extracts from LNCaP stable clones were prepared by resuspending cells in ipotonic buffer (10 mM Tris/HCl pH7.4, 10 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT, protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). After incubation on ice for 7 min, samples were centrifuged at 700 *g* for 7 min. Pelleted nuclei were resuspended in ipotonic buffer supplemented with 90 mM NaCl and 0.5% Triton, sonicated and centrifuged (5000 *g* for 15′) on 30% sucrose cushion. Nuclear extracts were pre-cleared on Protein A-sepharose beads (Sigma-Aldrich) and immunoprecipitated with 1 μg of anti-GFP for 3 h at 4°C under constant shaking with Protein A-sepharose beads. Absorbed proteins were eluted and analysed by Western blot as described (Sette *et al*., 2002).

LNCaP stable cell lines
The cDNA encoding the GSG domain of Sam68 was amplified by PCR using pCDNA3-GFP-Sam68 as template (Paronetto *et al*., 2003) and PFU polimerase (Roche), cloned in frame

with GFP into pEGFP-C1 (CLONTECH, Mountain View, CA, USA) and sequenced. LNCaP cells were transfected with pEGFPC1 or pEGFP-GSG using Lipofectamine 2000 (Invitrogen) and selected by Geneticin (400 μg/ml, Invitrogen) 24 h after transfection. Geneticin-resistant foci were selected after 8–10 days, picked and expanded. pEGFP-GSG clones could be expanded with more difficulty because of their initial slow growth rate.

CD44 splicing assay
PC3 prostate cancer cells were transfected with CD44 minigene (Matter *et al*., 2002) and constructs encoding GFP, GFP-Sam68 and mycGSG (Paronetto *et al*., 2006). After 20 h cells were collected and RNA was isolated and reverse transcribed. PCR reactions were performed using the following primers: InsF 5′-CCTGGTGTGTGGGGAGCGT-3′ and InsB 5′-CCA CCCAGCTCCAGTTGTGCCA-3′. Samples were separated on a 1.5% agarose gel and bands intensity was quantified.

GeArray prostate cancer biomarkers
Total RNA was isolated using Rneasy Mini Kit (Qiagen Inc., Valencia, CA, USA) from transfected LNCaP cells and used as a template to generate biotin-16-UTP labelled cRNA probes by the True labelling kit (Superarray Inc., Bethesda, MD, USA). The cRNA probes were hybridised at 60°C with the SuperArray Prostate cancer biomarkers membrane and signals were revealed using the SuperArray detection Kit. Data from three experiments were analysed by densitometry using the Scanalyze software (Eisen lab, Stanford University, CA, USA).

Additional Methods
Additional procedures can be found in the Supplementary materials.

Abbreviations

PCa, prostate carcinoma; STAR, signal transduction and activation of RNA metabolism; cdk, cyclin dependent kinase; EDTA, ethilenediaminetetraacetic acid; DMSO, dimethylsulfoxide; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; BrdU, 5-bromo-2-deoxyuridine; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt].

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2. Sam68 and stress response in PCa cells

2.1 Sam68 relocalizes in PCa cells after genotoxic stress

We have previously shown that Sam68-depleted prostate cancer cells are more susceptible to apoptosis induced by genotoxic agents like etoposide and cisplatin (Busà *et al*., 2007). To further investigate this protective function of Sam68 we monitored the intracellular localization of Sam68 in response to cisplatin and to mitoxantrone (MTX), a topoisomerase II inhibitor that is currently used in treatments of androgen-resistant PCa patients and more effective than etoposide in PCa treatment (Petrylak, 2003).

LNCaP cells were treated for 16 hours with 80 μM cisplatin and stained with a Sam68 specific antibody for immunofluorescence (IF) analysis. We observed that, after cisplatin-induced DNA-damage, Sam68 re-localises in nuclear structures surrounding nucleoli (Figure 1B).

We also treated both androgen-dependent LNCaP cells (data not shown) and androgen-independent PC3 cells with 0.1-5 μM mitoxantrone for 2-24 hours and we observed that at the highest dose (5 μM) it induced , like cisplatin, re-localization of Sam68 in large granules that are faintly stained by Hoechst, resembling nucleoli (Figure 1A), which begin to form after 8 hours of MTX treatment. By contrast, lower doses of MTX doses did not appear to affect Sam68 subcellular localization (data not shown). Notably, treatment with MTX caused morphological changes in PC3 cells, which appeared more flattened on the culture plate with larger nuclei than untreated cells, as shown in Figure 1A. Next, we asked if the Sam68 foci induced by MTX co-localized with foci of double strand breaks induced by DNA damage. PC3 cells were treated with 5 μM MTX and were co-stained with Sam68 and the phosphorylated form of H2AX (γH2AX), which is a target of ATM kinase that accumulates to foci of double strand breaks in response to DNA damage (Huang *et al*.,

2003). However, confocal microscopy analyses showed that Sam68 foci and double strand breaks foci are different structures (Figure 1C).

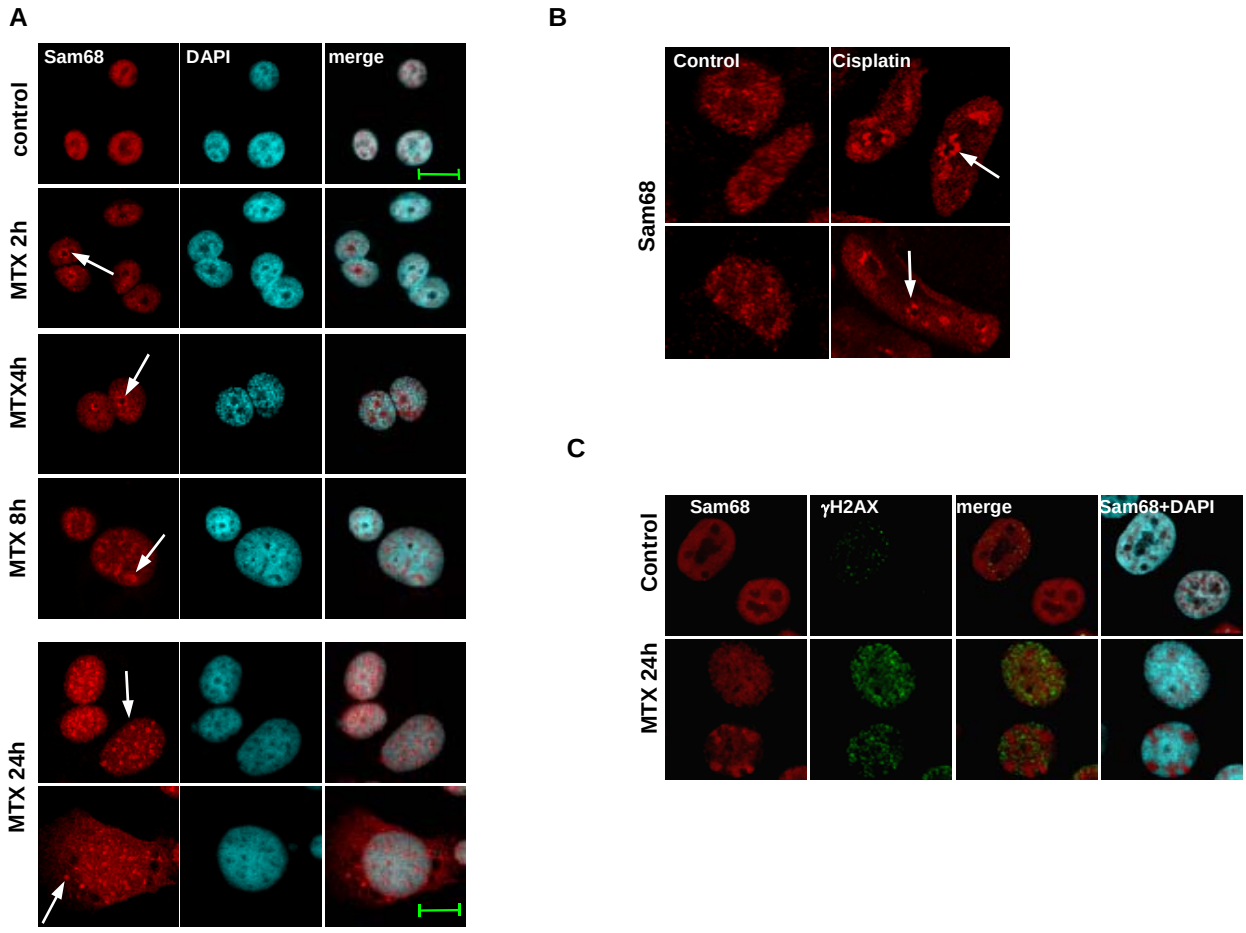

Figure 1. Sam68 relocalizes in subcellular compartments after genotoxic stress. **A)** Time course treatment of PC3 cells with 5μM MTX. Treated cells were stained with an anti-Sam68 specific antibody and analysed by IF. Confocal analysis revealed a time-dependent re-localization of Sam68 at first around nucleoli (2h MTX, as indicated by a white arrow) and later in nucleoli-like granules, more evident after 8h and 24h of treatment (granules are pointed by white arrows). Bar (green), 15 μm. **B)** Sam68 re-localization induced by cisplatin in LNCaP cells. Cells were treated with 80 μM cisplatin for 16 h, stained with anti-Sam68 specific antibody and analysed by confocal miscroscopy. White arrows indicate sites of Sam68 accumulation around nucleoli in treated cells. **C)** Co-staining of Sam68 and γH2AX after 5μM MTX treatment in PC3 cells. Cells were analysed by confocal microscopy.

Moreover, we tested whether Sam68 co-localized with promyelocytic leukemia nuclear bodies (PML) after genotoxic stress. PML bodies are site of posttranscriptional modification of nuclear proteins such as HIPK2 and CBP (D'Orazi *et al*., 2002; Hofmann *et al*., 2002)

and act as a dynamic subnuclear compartment implicated in tumor suppression, as well as transcription and DNA repair. Indeed, PML bodies are considered DNA damage sensors that respond to DNA double-strand breaks and are regulated by ATM kinase and its target Chk2. The activation of the DNA-damage pathway determines phosphorylation of PML proteins and increases PML bodies number in the cell (Dellaire *et al*., 2006). To investigate whether Sam68 nuclear granules induced by MTX are sites of accumulation of nuclear proteins associated with PML bodies, we treated PC3 cells with 5μM MTX for 24 hours and we co-stained cells with specific antibodies for Sam68 and PML. However, also in this case we did not see a co-localization between Sam68 and PML (data not shown), indicating that Sam68 granules are different subnuclear structures induced by DNA damage.

2.2 Sam68 co-localizes with other RNA binding proteins both in nuclear and cytoplasmic granules

In addition to the nuclear re-localization of Sam68, 16-24 hours of treatment with MTX (Fig. 1A lower panels) and cisplatin (data not shown) caused the translocation of Sam68 in the cytoplasm in 5-10% of cells, where it accumulated in cytoplasmic granules. These structures resembled the cytoplasmic stress granules (SG) in which splicing factors, such as the RNA-binding proteins hnRNP A1 (Guil *et al*., 2006) and TIA-1 (Kedersha and Anderson, 2002), re-localize as a stress-adaptation response of the cell. Thus, we tested whether upon genotoxic stress Sam68 was recruited to the same granules as these stress-response RNA-binding proteins.

PC3 cells were treated with 5 μM MTX for 24 hours and then stained with specific antibodies for hnRNP A1 or TIA-1 for immunofluorescence analysis. We found that genotoxic stress induced a subcellular re-distribution of both hnRNP A1 and TIA-1 in the nucleus and in the cytoplasm similar to that observed for Sam68 (data not shown and see also Figure 2). As previously mentioned, relocalization of hnRNP A1 to the cytoplasm and

SG after cellular stresses, such as UV, heat shock, osmotic and oxidative stress, regulate the export of specific mRNAs and might ensure cell viability during the response to stress (Guil *et al*., 2006). Our results indicate that Sam68 might be part of this stress reponse to DNA damaging agents. To test directly whether Sam68 co-localized with these stress-response RNA-binding proteins, we performed a time course treatment with 5 μM MTX in PC3 cells and co-stained cells with Sam68 and hnRNP A1 or TIA-1 specific antibodies. Confocal analysis revealed that Sam68 co-localized to the same granules with hnRNP A1 and TIA-1 both in the nucleus and, albeit in a small percentage of cells, in the cytoplasm (Figure 2A, C and B, D respectively).

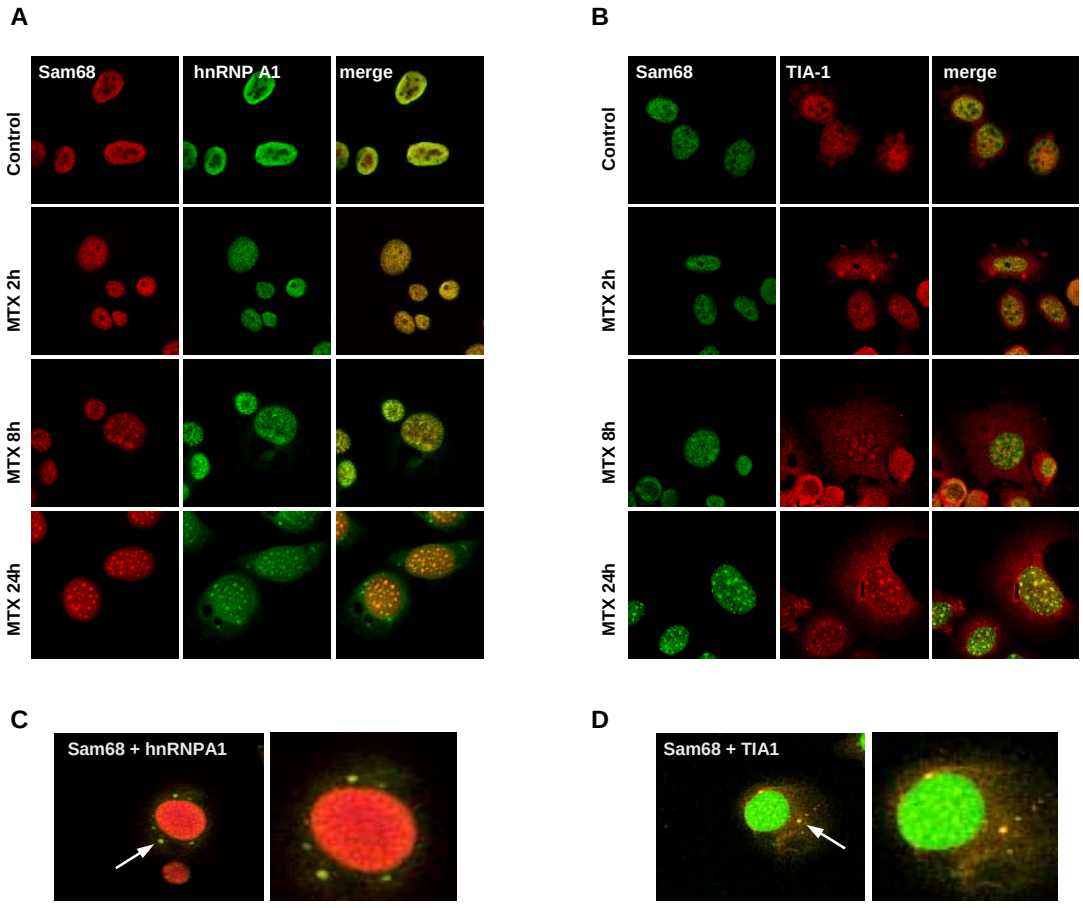

Figure 2. Sam68 co-localizes with stress-response RNA-binding proteins after genotoxic stress. PC3 cells were treated with 5 μM MTX (2-24h) and co-stained with Sam68 and hnRNP A1 or TIA-1 specific antibodies. Sam68 co-localized with hnRNP A1 and TIA-1 to the same granules both in the nucleus and in the cytoplasm (panels **A**, **C** and **B**, **D** respectively. Cytoplasmic granules are indicated by white arrows in panels **C** and **D**).

Our laboratory has previously demonstrated that hnRNP A1 and Sam68 interact in a functional splicing complex (Paronetto *et al*., 2007). The results presented here indicate that in response to DNA damage Sam68 still co-localizes with hnRNP A1 and it also co-localizes with TIA-1. These data strongly suggest that Sam68 is part of a RNA-mediated stress response of the cell and that it may exert an active role in genotoxic stress response altering its subcellular localization. To test whether other splicing factors are also implicated in this response, we analysed the subcellular localization of SC35 and ASF/SF2, two RNA-binding proteins belonging to SR family, which act antagonistically to hnRNP proteins in the regulation of alternative splicing (Matlin *et al*., 2005). We performed a time course treatment with 5 μM MTX and co-stained PC3 cells with Sam68 and SC35 or ASF/SF2 specific antibodies. Confocal microscopy analyses showed that, after genotoxic stress, both SR proteins re-localize in nuclear granules, but not in cytoplasmic granules, and they both relocalize with Sam68 in nuclear granules (Figure 3 A, B).

These results allowed us to hypothesize that Sam68 relocalization in nuclear granules after DNA damage is a process involving several RNA-binding proteins that regulate mRNA alternative splicing and translation. This response might elicit post-transcriptional regulation of specific RNA targets, similarly to what occurs after heat shock, which induces a nuclear relocalization in the so called "nuclear stress granules" of hnRNP proteins (hnRNP M and SAFB) and SR proteins (SRp30c and ASF/SF2) (Biamonti, 2004). Moreover, in line with the hypothesis of post-trascriptional regulation of mRNAs by DNA damage, several reports have shown that changes in alternative splicing of specific transcripts occurs in cells treated with cisplatin or etoposide. Some of these transcripts encode for proteins regulating apoptosis, such as Caspase 2 (Montecucco and Biamonti, 2007; Solier *et al*., 2004) and Bcl-2 related genes (Wotawa *et al*., 2002), or cell cycle and proliferation, like the p53 negative modulators MDM2 and MDM4 (Chandler *et al*., 2006) or cyclin D1b, a splicing variant aberrantly expressed in prostate and breast cancer cells that confers resistance to androgen

depletion therapies and chemotherapeutic drugs (Wang *et al*., 2008). Thus, alternative splicing is emerging as a novel mechanism by which cancer cells gain drug resistance.

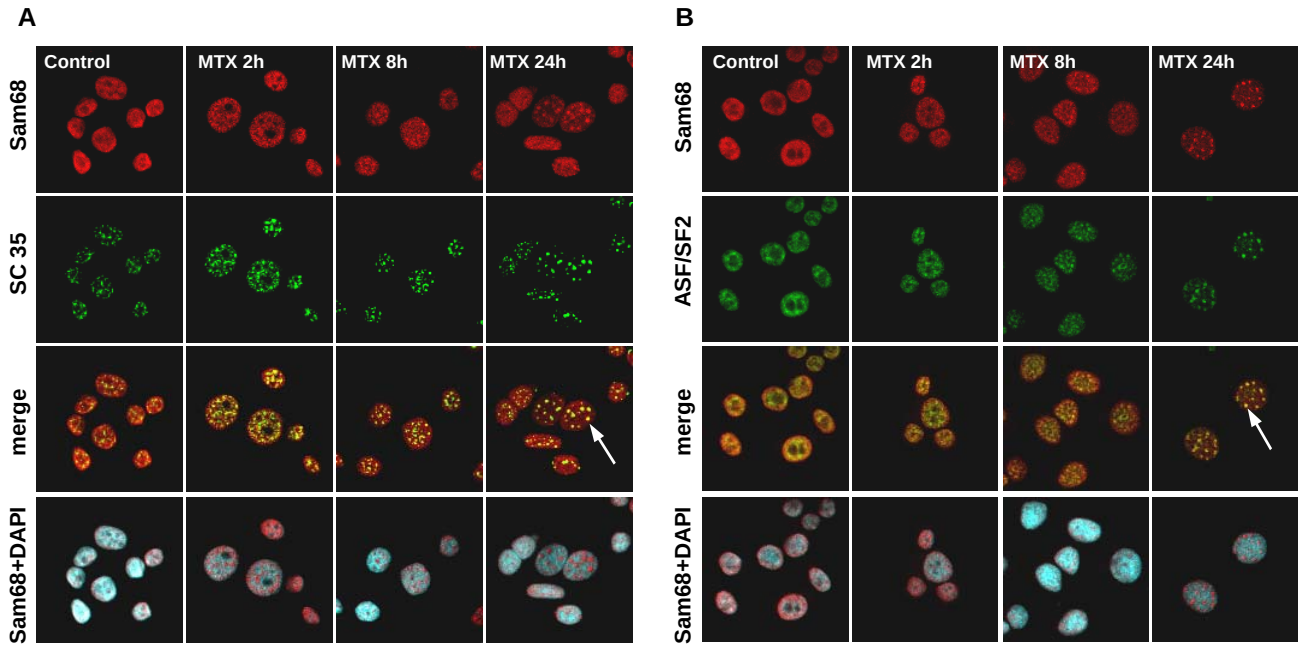

Figure 3. SR-proteins co-localize with Sam68 in nuclear granules after genotoxic stress. PC3 cells were treated with 5 μM MTX (2-24h) and co-stained with Sam68 and SC35 or ASF/SF2 specific antibodies. Confocal analysis revealed that Sam68 co-localized to the same stress-induced nuclear granules both with SC35 and ASF/SF2 (panels **A** and **B**, respectively. Stress-induced nuclear granules are pointed by white arrows).

2.3 Genotoxic stress induced by MTX affects CD44 alternative splicing profile

Sam68 regulates alternative splicing of specific pre-mRNAs (Matter *et al*., 2002; Paronetto *et al*., 2007; Chawla *et al*., 2009). The first pre-mRNA target identified was CD44, a membrane receptor involved in cell proliferation and migration (Ponta *et al*., 2003). The CD44 gene contains 10 constitutive exons and 10 variable exons that can be differentially assembled in the mature mRNA (Figure 4A, lower panel). Inclusion of several variable exons (v5, v6, v8 and v10) correlates with increased cell motility, invasion of neighbouring tissues and malignancy (Naor *et al*., 1997) and the expression of the CD44 v5 variant has been correlated to poor prognosis in patients (Lee *et al*., 2003; Wu *et al*., 2003).

Moreover, recent data have implicated a change in CD44 splicing isoforms as an adaptive response of some cancer cells to genotoxic stress (Filippov *et al.*, 2007). Sam68 is one of the main regulators of the inclusion of several CD44 variable exons and its activity is modulated by signal transduction pathways (Matter *et al.*, 2002; Cheng and Sharp, 2006). Other splicing factors involved in CD44 alternative splicing regulation are hnRNP A1 ad SRm160, two proteins that have been demonstrated to associate with Sam68 in splicing compexes (Matter *et al.*, 2000; Paronetto *et al.*, 2007; Cheng and Sharp, 2006).

Thus, we have begun to investigate whether the change in subcellular localization of Sam68 induced by genotoxic drugs as an effect on CD44 alternative splicing. We have used PC3 cells, which express detectable levels of endogenous CD44 mRNA. To avoid a general block in transcription, a process tightly coupled with alternative splicing (Kornblihtt, 2007), we have used sub-maximal doses of MTX (0, 0.1 and 0.5 μM) in a time-course study and analysed the changes in CD44 alternative splicing by RT-PCR. As shown in Figure 4A, MTX influences alternative splicing of the CD44 pre mRNA. The inclusion of some variable exons is stimulated (variable exons v5 and v6) whereas inclusion of other exons is decreased (exon v9), as indicated by the densitometric analyses reported in Figure 4B.
Since alternative splicing of the CD44 v5 and v6 exons is regulated by Sam68 activity, it is likely that this protein takes part to this process. Current studies are aimed at determining whether downregulation of Sam68 by RNAi affects this modifications of CD44 alternative splicing caused by MTX and whether this has an impact on PC3 cell survival, proliferation or motility.

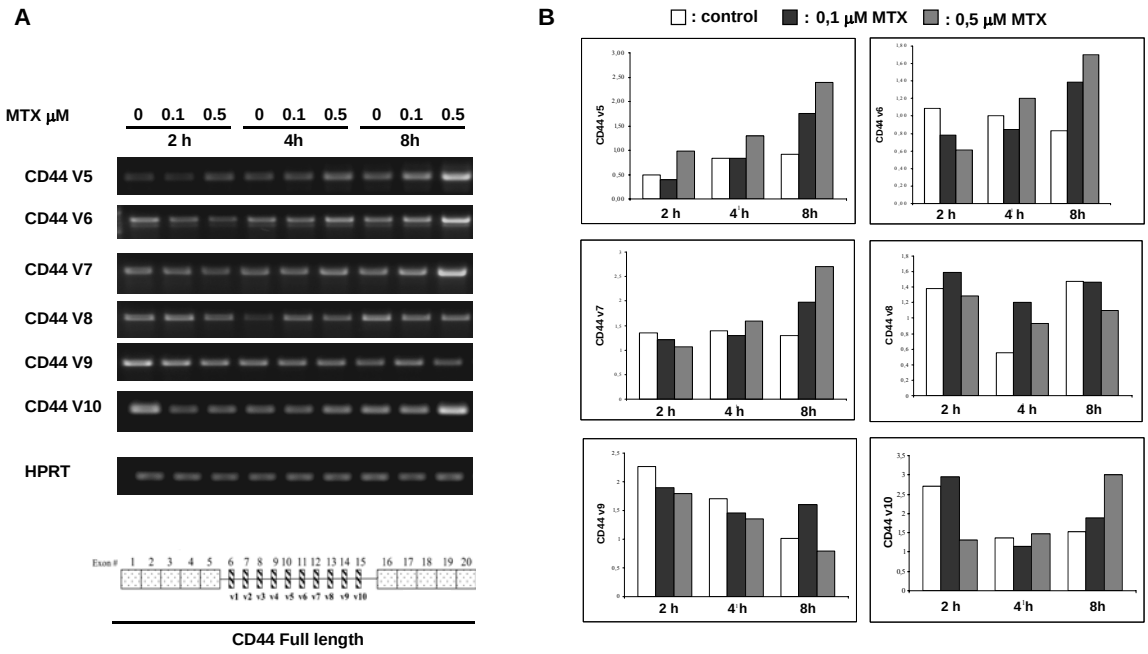

Figure 4. Genotoxic stress induced by MTX affects CD44 alternative splicing profile. **A)** PCRs analysis showing expression of CD44 variants in PC3 cells after DNA damage induced by MTX in a time and dose dependent manner. PCRs were performed using 5' primers specific for variant exons v5,v6,v7,v8,v9 ,v10 and a 3' primer pairing in the costant region. Samples were normalized with hprt. **B)** Densitometric analysis of CD44 alternative splicing after MTX treatment in PC3 cells.

2.4 Signal transduction pathways activated after genotoxic stress in PCa cells

Sam68 is known to link signal transduction pathways to RNA metabolism (Lukong and Richard, 2003). We asked whether the MTX-induced change in Sam68 subcellular localization was determined by activation of specific signal transduction pathways. PC3 cells were treated with MTX in a time- and dose-dependent manner and extracts were analysed by Western blot. We observed that MTX treatment induced a rapid phosphorylation of Chk2, a substrate of the ATM kinase, which is activated by DNA double strand breaks (Bartek and Lukas, 2003; Shiloh, 2003), and a slower activation of stress induced MAPKs like JNK1/2 and p38 (Figure 5A). These kinases are known to be activated in response to varius stimuli, such as genotoxic stress (Stadheim and Kucera, 2002) for

JNK1/2 and cytokines, hyperosmolarity, UV irradiation (Roux and Blenis, 2004; Guil *et al*., 2006) and DNA-damaging agents (Reinhardt *et al*., 2007) for p38 kinase. We noticed that the re-localization of Sam68, induced by the highest dose of MTX (5μM) (Figure 5B), is temporally correlated more with the late activation of MAPKs than with the early activation of ATM (Figure 5A).

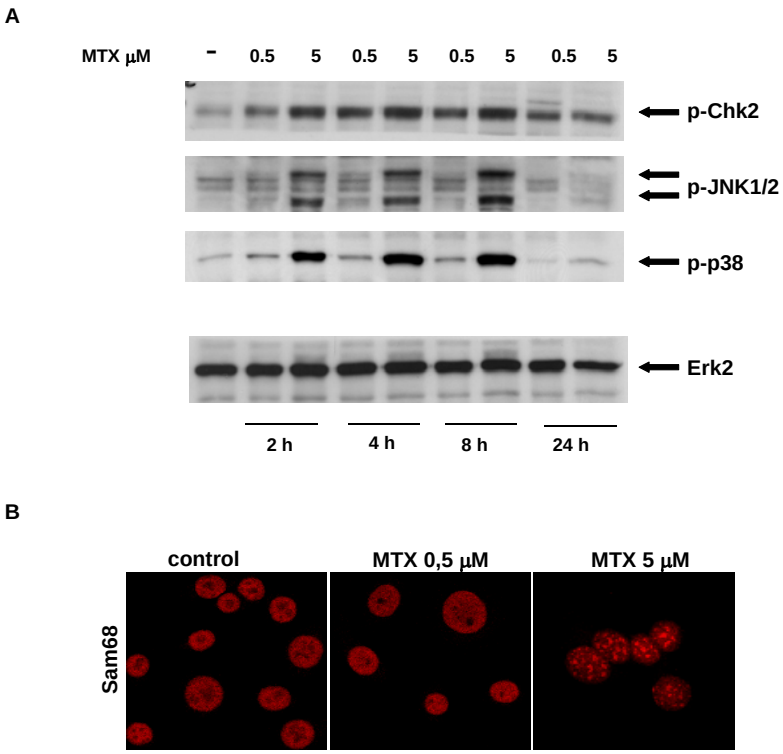

Figure 5. A) Western blot analysis of signal transduction pathways activated after genotoxic stress in PC3 cells. Cells were treated with MTX in a time and dose dependent manner, cell extracts (60 μg) were loaded and stained with specific antibodies as indicated. Sample loading was normalized with anti-Erk2 antibody. **B)** Immunofluorescence analysis of Sam68 re-localization in a MTX 24h dose-response.

In order to understand which of the pathways activated by genotoxic stress was required for the subcellular relocalization of Sam68, we used kinase-specific inhibitors such as SB202190 for p38, JNK-inhibitor (JNK in.) for JNK1/2 and KU3353 for ATM. PC3 cells were treated with 5 μM MTX ± specific kinase inhibitors in a time-dependent manner (2-8 hours with MTX ± SB202190 or JNK in. and 24 hours with KU3353), then were fixed and stained with a Sam68 specific antibody and analyzed by confocal microscopy. We observed

that Sam68 re-localization was not affected in the presence of such inhibitors (Figure 6A, B). We analysed cell extracts by Western blot analysis to confirm the inhibition of the signal transduction pathways, using c-Jun and eIF4E, targets of JNK1/2 and p38 respectively, to test MAPKs inhibition (Figure 6C), and pChK2 to confirm inhibition of ATM by KU3353 (Figure 6D).

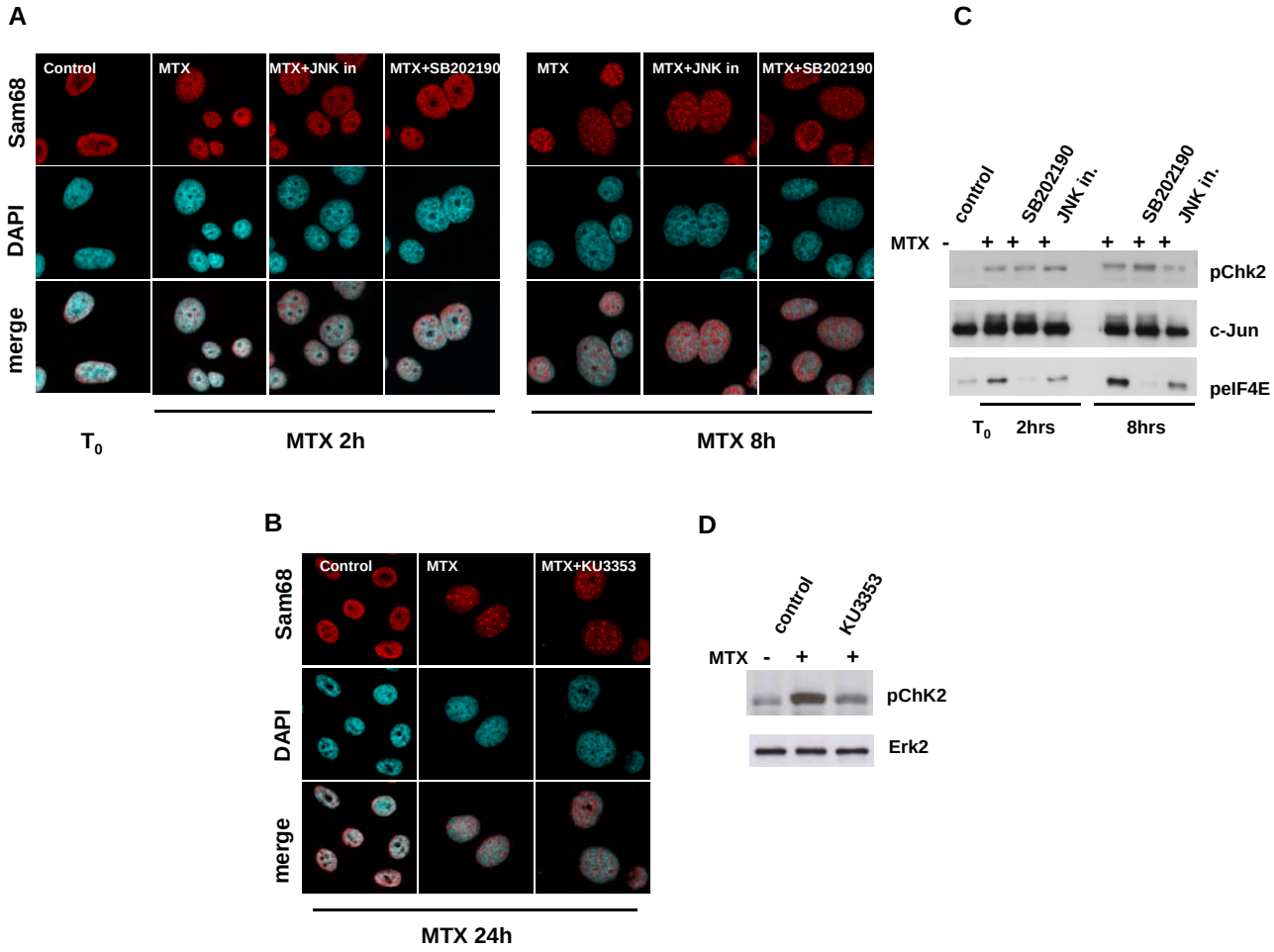

Figure 6. Inhibition of signal transduction pathways activated after genotoxic stress in PC3 cells. Cells were treated with 5μM MTX ± 10μM JNK inhibitor (JNK in.), 10μM SB202190 (as p38 kinase inhibitor) or 10μM ATM kinase inhibitor KU3353 in a time-dependent manner and stained with anti-Sam68 specific antibody. Confocal microscopy analysis revealed that stress-induced Sam68 re-localization is not inhibited either in presence of JNK in. and SB202190 (panel **A**) or KU3353 (panel **C**). Cell extracts (40 μg) were analysed by Western blot analysis with specific antibodies as indicated, to confirm inhibition of p38, JNK/SAPK (panel **B**) and ATM (panel **D**) pathways.

3. A role for Sam68 in rRNA metabolism

3.1 Characterization of nucleoli-like structures induced by genotoxic stress

Sam68 is predominantly localized in the nucleoplasm but a small quote, under physiological conditions, is also present in nucleoli where it is known to be recruited together with several other proteins after a transcriptional block elicited by actinomycin D (Andersen *et al*., 2005). We asked if a similar recruitment to the nucleoli of Sam68 could also be elicited by genotoxic stress induced by MTX. Indeed, it is known that the ATM repair pathway blocks RNA polimerase I transcription in response to double strand breaks (Kruhlak *et al*., 2007) suggesting that DNA damage may affect nucleoli function.

In order to understand the nature of these Sam68 nucleoli-like granules induced by DNA damage, we stained PC3 cells with nucleolar markers such as UBF (Upstream binding factor), which binds to the ribosomal RNA (rRNA) promoter and interacts with the RNA polimerase I (Jordan and Carmo-Fonseca, 1998; Grummt, 2003; Russell and Zomerdijk, 2005), and Nucleolin, a nucleolar RNA binding protein necessary for pre-rRNA transcription and maturation (Rickards *et al*., 2007). PC3 cells were treated with 5 μM MTX, co-stained with Sam68 and Nucleolin or UBF specific antibodies and analysed by a confocal microscopy (Figure 7 A, B). We observed that both Nucleolin and UBF dispersed to nucleoplasm after MTX treatment, ruling out their use as nucleolar markers after genotoxic stress.

3.2 Interaction between Sam68 and Nucleolin

To try to understand what are the proteins associated with Sam68 in PCa cells and investigate how these interactions are modulated by exposure of cells to DNA damage, we performed a co-immunoprecipitation. LNCaP cell extracts were immunoprecipitated with an anti-Sam68 antibody or non specific abbit IgGs as control and immunocomplexes were

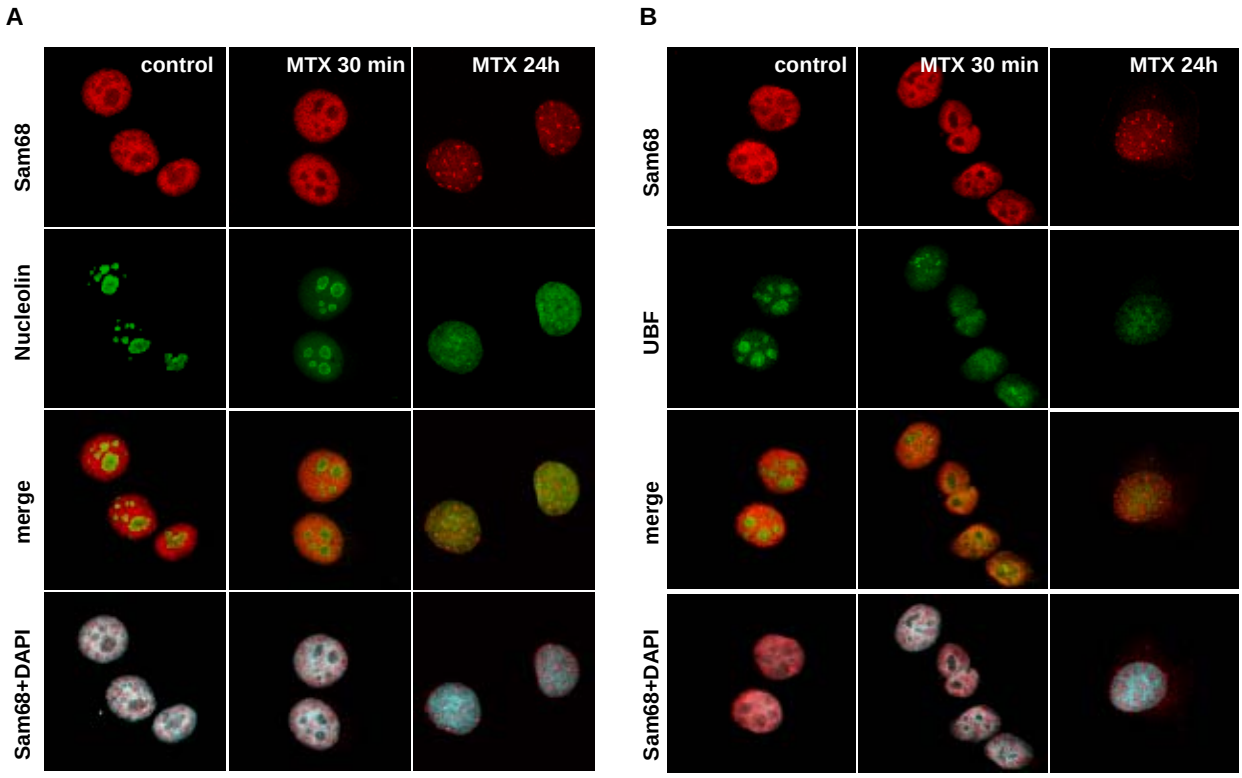

Figure 7. Nucleolin and UBF dispersed to nucleoplasm after MTX treatment. Confocal analysis of co-stainings for Sam68 with nucleolar markers, Nucleolin (**A**) and UBF (**B**) in untreated and 5μM MTX treated cells

revealed by Silver staining and analysed by mass spectrometry. Among other interacting proteins, we found Nucleolin (Busà R., Paronetto MP, Bielli P, and Sette C., manuscript in preparation) (Figure 8A, Nucleolin is indicated by an arrow). A co-immunoprecipitation experiment in PC3 cells confirmed the interaction between Sam68 and Nucleolin under normal growth conditions. However, this interaction was impaired after genotoxic stress induced by 5μM MTX (Figure 8B), possibly due to the relocalization of Nucleolin outside of nucleoli (Figure 7A).

Previously, in our laboratory was identified the C-terminal region of Sam68 (last 93 aminoacids, from aminoacid (aa) 351 to aa 443 as sufficient to interact with hnRNP A1 and for the splicing activity of Sam68 toward Bcl-x pre-mRNA (Paronetto *et al.*, 2007). To confirm the interaction between Sam68 and Nucleolin and to map the critical domains of

Sam68 involved in this interaction, we performed a pull-down assay with Hek293

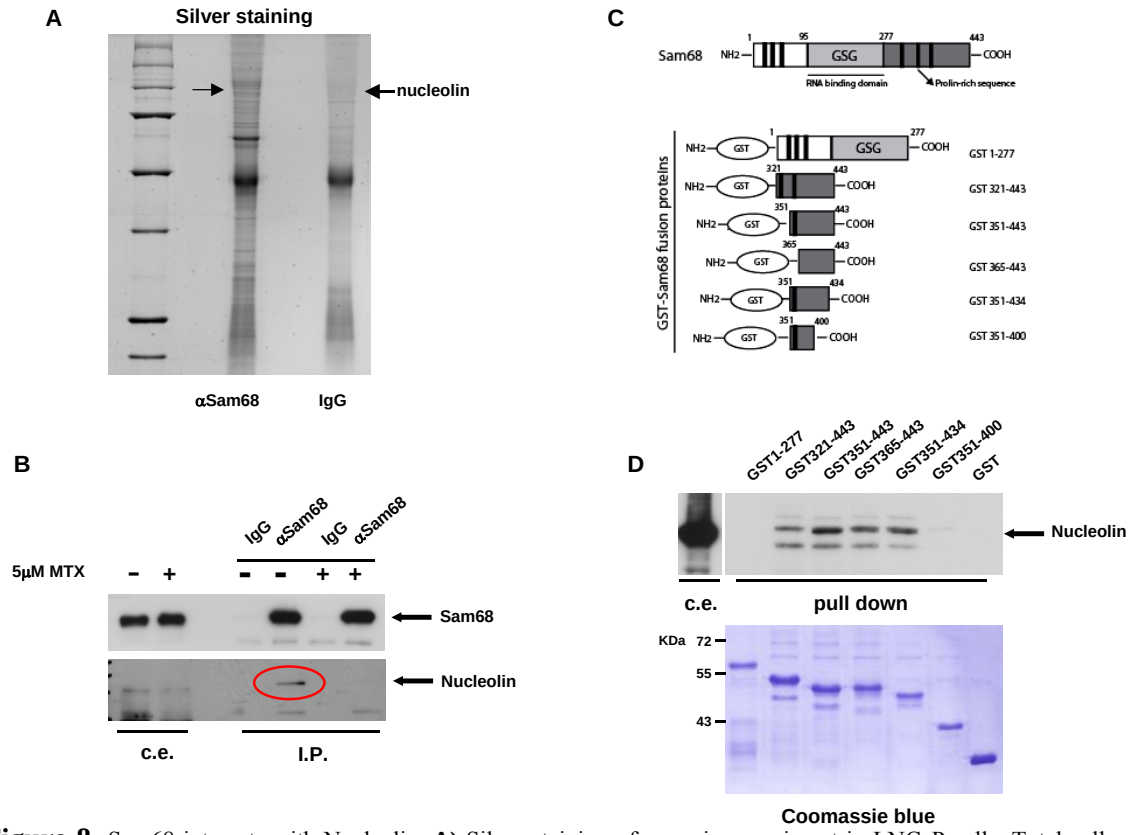

Figure 8. Sam68 interacts with Nucleolin. **A)** Silver staining of a co-ip experiment in LNCaP cells. Total cell extracts were immunoprecipitated with anti-Sam68 specific antibody and loaded on a SDSPAGE gel. Specific bands, detected by Silver staining, were analysed by mass-spectrometry. The arrow indicates the band corresponding to Nucleolin. **B)** PC3 cells were treated with 5μM MTX for 12 hours, and cell extracts were immunoprecipitated for Sam68 or IgG as control. Samples were loaded and stained as indicated. **C**) Schematic representation of Sam68 GST-proteins. Numbers on the right indicate the amino acid regions present in each mutant. (**D**). Pull down assay of GST Sam68 proteins with Hek293 cell extracts. Upper panel shows anti-Nucleolin Western blot; lower panel shows a Coomassie blue staining of the same samples.

cell extracts by using GST-Sam68 deletion proteins. The Sam68 GST fusion proteins consisted of Sam68 N-terminal domain (aa 1-277) and various portions of Sam68 C-terminal domain (spanning from aa 321 to aa 443): GST 321-443 (containig two proline rich sequences); GST 351-443 (containing one proline rich sequence); GST 365-443 (without proline rich sequences); GST 351-434 (without a portion of the NLS domain) and GST 351-400 (without the NLS domain) (see Figure 8C for a schematic representation of Sam68 GST-proteins and Figure 8D, lower panel, for a coomassie gel of purified GST proteins). Detection for Nucleolin among the proteins pulled down with Sam68 fusion

proteins showed interaction between Nucleolin and all the portions of Sam68 C-terminal domain, with the exception of GST-Sam68 351-400 (Figure 8D, upper panel) and of GST-Sam68 1-277. This observation underscores the importance of the C-terminal Tyrosine-rich region of Sam68 for the interaction with Nucleolin. Since this region is phosphorylated by Src-related kinases in cancer cells, it is likely that these interactions are modulated by signal transduction pathways involving these soluble tyrosine kinases.

3.3 Sam68 is involved in pre-rRNA metabolism

Although Sam68 is predominantly diffused in the nucleoplasm, a small amount is present in nucleoli, as shown by a hightroughput characterization of nucleolar proteins (Andersen *et al*., 2005). Moreover, a RNA-protein co-immunoprecipitation experiment analysed by microarray revealed that Sam68 associates with the 18S rRNA among the RNAs bound by Sam68 (Figure 9).

Together with the evidence that Sam68 interacts with Nucleolin (Busà R., Paronetto MP, Bielli P, and Sette C., manuscript in preparation), these preliminary observations lead us to investigate whether the nucleolar pool Sam68 plays a role in rRNA metabolism under normal and stress conditions.

As first step, we performed a fluorescence-in-situ hybridization (FISH) experiment for 18S pre-rRNA in PC3 cells stably silenced with a siSam68-plasmid (plko 527) or transfected with control siRNA-plasmid (pSuper). We treated these cells with MTX to test whether Sam68 can relocalize with stalled pre-rRNA after genotoxic stress and if depletion of Sam68 can alter rRNA transcription. PC3 cells were treated with 5μM MTX in a time-course (1-2 hours), fixed for I.F./FISH and stained with an anti-Sam68 specific antibody and a probe labelled with a cy3 fluorophore at 3', specific for pre-rRNA 18S (Rouquette *et al*., 2005).

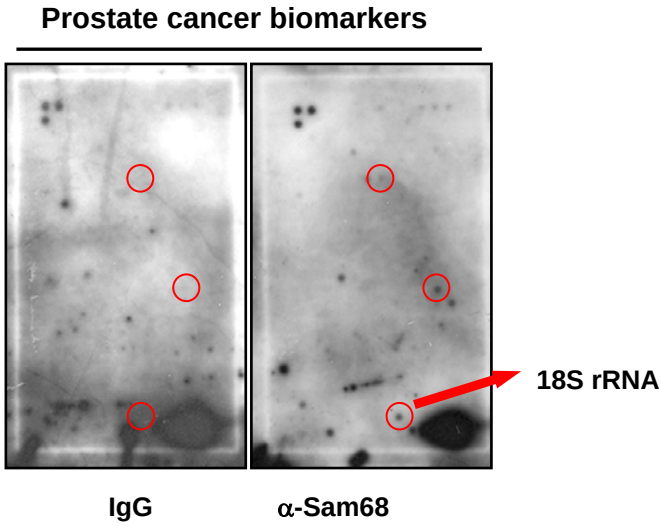

Figure 9. Sam68 binds 18S rRNA. RNA-protein co-immunoprecipitation experiment performed in LNCaP cell extracts. Sam68 was immunoprecipitated with a specific antibody and IgGs were used as mock reaction. RNAs associated were extracted, labelled and analysed on a Superarray prostate Biomarkers chip (specific spots are indicated by red circles). 18S rRNA has been found among the RNAs bound by Sam68 (as indicated).

Confocal analysis showed that after 1 hour of MTX treatment the transcription of pre-rRNA began to switch off and it was not possible to follow both Sam68 re-localization and changes in pre-rRNA foci. However, the intensity of signal suggested that depletion of Sam68 caused an increase in the amount of pre-rRNA 18S (Figure 10B). To determine whether Sam68 has a function in regulating rRNA transcription, we measured the levels of the 45S pre-rRNA by quantitative real time PCR using a set of primers specific for pre-rRNA, which anneal in 5' external transcribed spacer (ETS) region. PC3 cells were interfered for Sam68, total RNA was extracted and analyzed by real time PCR. Western blot analysis of cell extracts confirmed depletion of Sam68 (Figure 10D). Surprisingly, depletion of endogenous Sam68 caused a significant increase in the expression of pre-rRNA compared with control siRNA treated cells (Figure 10C).

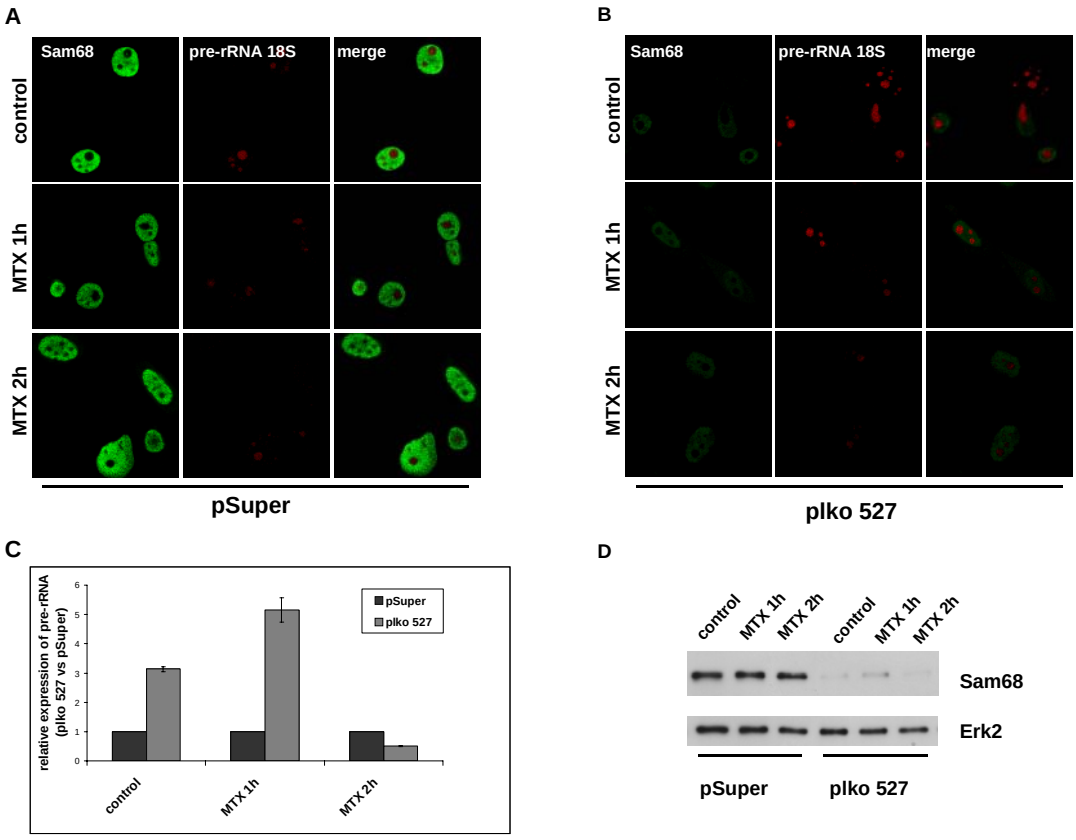

Figure 10. Down-regulation of Sam68 leads to an increase of pre-rRNA levels. Confocal analysis of IF/FISH stainings in PC3 cells stably silenced with a siSam68-plasmid (plko 527, panels **B**) or transfected with control siRNA-plasmid (pSuper, panels **A**). Cells were treated with 5μM MTX (1-2 h), fixed and stained with anti-Sam68 specific antibody and a probe labelled with a cy3 fluorophore at 3', specific for pre-rRNA 18S. **C)** Total RNAs from these samples were analysed by real time PCR with sets of primers specific for pre-rRNA. The value given for the amount of pre-rRNA PCR product present in pSuper cells was set as 1. Data are reported as the mean ± SD of three independent experiments. **D)** Sam68 depletion was verified by western blot analysis (α-Erk2 was used as a loading control)

These results suggest that Sam68 can act either as a repressor of rDNA transcription or as an activator of pre-rRNA processing. This second hypothesis is also supported by the interaction of Sam68 with Nucleolin, a protein that has a positive role in regulating pre-rRNA maturation (Rickards *et al.*, 2007). In this case, Sam68 depletion could lead to accumulation of pre-rRNA uncleaved form.

3.4 A role for Sam68 in rRNA transcription?

To address the question of the regulation of pre-rRNA by Sam68 we started from its interaction with Nucleolin, a nucleolar protein implicated in pre-rRNA transcription and processing. It has been proposed that Nucleolin associates with nascent pre-rRNA in vivo (Herrera and Olson, 1986) and that through this association it initiates the assembly of a pre-rRNA processing complex, thus coordinating transcription of rRNA genes with pre-rRNA processing (Ginisty *et al*., 1998).

Moreover, a recent study demonstrates that Nucleolin associates with chromatin containing the rRNA gene and that is essential for RNA polymerase I transcription, unwinding ribosomal chromatin (Rickards *et al*., 2007). Thus, we asked whether Sam68 can also associate with rDNA to regulate rRNA transcription or processing. As first step, we performed a chromatin immunoprecipitation (ChIP) assay for Sam68 to identify the site of binding (if any) on the rDNA. Nucleolin was used as positive control. Cross-linked PC3 cell extracts were immunoprecipitated with anti- Sam68 or anti-Nucleolin antibodies and associated chromatin was analysed by quantitative real time PCR. We used two pairs of primers, H1 and H4 (Frescas *et al*., 2007), annealing respectively to the region around rDNA promoter and to the 18S rRNA coding region. We found that neither Sam68 nor Nucleolin bound to the ribosomal promoter (data not shown). However, we observed that both proteins bound internal regions in the rDNA corresponding to the coding sequence for the 18S rRNA (Figure 11).

Since it was known that Nucleolin does not bind the ribosomal promoter while it binds the 18S rRNA coding region (Rickards *et al*., 2007), our results appear to validate these observations and suggest a possible role also for Sam68 in pre-rRNA metabolism together with Nucleolin.

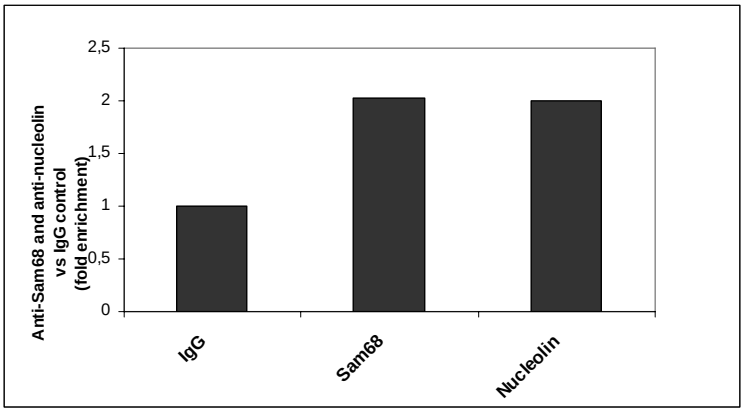

Figure 11. Sam68 binds to rDNA. ChIP experiment for rDNA performed in PC3 cells. Cross-linked cell extracts were immunoprecipitated with anti- Sam68 or anti-Nucleolin antibodies and IgGs as negative control. The associated chromatin was analysed by quantitative real time PCR by using primers annealing to the 18S rRNA coding region.. The value of control IgGs was set as 1 and values from Sam68 and Nucleolin ChIPs were reported as fold enrichment.

Discussion and future directions

The studies presented in the last part of this PhD Thesis stemmed from our previous observation that Sam68 played a protective role in PCa cells subjected to genotoxic stress induced by chemotherapeutic drugs, such as cisplatin, a DNA alkylating agent, and etoposide (Busà *et al*., 2007). In the current work we have used cisplatin and mitoxantrone (MTX), a topoisomerase II inhibitor, like etoposide, that is used in treatment of patients affected by PCa (Petrylak, 2003).

As a first approach, we monitored the subcellular localization of Sam68 in response to treatment with these drugs. Interestingly, both cisplatin and MTX caused a subcellular re-localization of Sam68 from nucleoplasm (where it is diffused under normal conditions) to nuclear granules. It is known that DNA damage and stress response alter protein redistribution in subcellular compartment as a cellular defence to external cues. Genotoxic drugs that induce double strand breaks, such as cisplatin, trigger the recruitment of proteins involved in the DNA damage response to sites of double strand breaks and the relocalization of nucleolar components belonging to the rRNA transcription machinery, such as UBF, TATA-binding protein (TBP), TBP-associated factors for RNA polymerase I (TAF_Is) and rRNA polymerase I to the periphery of the nucleolus (Jordan and Carmo-Fonseca, 1998). Moreover, cisplatin-induced proteotoxic stress and a mild heat shock determine subcellular redistribution and nucleolar accumulation of RNA recognition motif (RRM)-containing proteins, such as RDM1 isoforms, that modulate the cellular response to genotoxic agents (Messaoudi *et al*., 2007). Finally, various stresses, like UV and heat shock, induce accumulation of several RNA binding proteins in nuclear stress bodies, which might play a role in the post-transcriptional regulation of gene expression (Biamonti, 2004) . We have attempted to better characterize the sites of accumulation of Sam68 through a series of co-staining experiments and confocal microscopy analyses. Our results indicate that Sam68-

positive nuclear granules are different entities than the nuclear foci induced by DNA damage, both the γH2AX DNA-repair foci and the PML bodies (Hofmann *et al*., 2002). However, we found that these granules are sites of accumulation of several RNA-binding proteins involved in alternative splicing and other steps of post-transcriptional regulation of mRNAs. Among the proteins co-localized with Sam68 in the nuclear granules induced by MTX we found the SR proteins SC35 and ASF/SF2, two positive regulators of alternative splicing, and TIA-1 and hnRNP A1, two proteins generally involved in cellular stress responses to various stimuli (Guil *et al*., 2006; Kedersha and Anderson, 2002). Notably, in addition to nuclear localization, Sam68 also accumulated in cytoplasmic granules that were also co-stained with hnRNP A1 and TIA-1, suggesting that these structures are the well described cytoplasmic stress granules (SGs) involved in the stress-adaptation response of the cell (Guil *et al*., 2006; Kedersha and Anderson, 2002). These data strongly suggest that Sam68 is part of a RNA-mediated stress response of the cell and that it may exert an active role in genotoxic stress response altering its subcellular localization.

The DNA damage-induced relocalization of splicing factors in nuclear granules suggested that is a response process involving regulation of mRNA alternative splicing. Notably, recent data indicate that up to 90% of the human genes undergo alternative splicing and produce at least two mRNA variants. In many cases, a single gene can encode several mRNA variants that often play slightly or completely different roles within the cell. This remarkably complex process amplifies the complexity and plasticity of the genome (Black, 2003; Matlin *et al.*, 2005; Sharp PA, 2005). Since changes in alternative splicing correlate with cell and tissue differentiation and can be regulated by external and internal cues, this process of gene regulation allows a finer adaptation of the cell to different environments. In line with this adaptation role, it has been recently shown that changes in alternative splicing occur during neoplastic transformation and that specific signatures of mRNA variants can be used to classify the stage of advancement of prostate, breast and ovarian carcinomas (Li *et*

al., 2006; Zhang *et al*., 2006; Venables *et al*., 2008; Fischer *et al*., 2004). Thus, we have begun to investigate whether changes in subcellular localization of Sam68 induced by genotoxic drugs affect alternative splicing of Sam68 target mRNAs, such as CD44 (Matter *et al*., 2002). Preliminary experiments have shown that MTX treatment in PC3 cells induces changes in alternative splicing of CD44 pre-mRNA**.** In particular, inclusion of variable exons v5 and v6 was stimulated, while inclusion of v9 was decreased. It has been shown that ectopic expression of CD44 v4-v7 variants confers metastatic potential to nonmetastatic cells (Gunthert *et al*., 1991). Conversely, the expression of the CD44 v5 and v6 variants has been correlated to poor prognosis in patients (Lee *et al*., 2003; Wu *et al*., 2003; Muller *et al*., 1997).

Since alternative splicing of the CD44 v5 and v6 exons is regulated by Sam68 activity (Matter *et al*., 2002; Cheng and Sharp, 2006) it is likely that this protein takes part to this process. We are current extending these studies to determine whether downregulation of Sam68 by RNAi affects these modifications of CD44 alternative splicing caused by MTX and whether this has an impact on PC3 cell survival, proliferation or motility under basal conditions or in response to light genotoxic stress.

It has been recently shown that in response to various stresses, like heat shock, UV irradiation and genotoxic drugs, cells adopt diverse defence mechanisms. Among these, there is the transcriptional activation of specific sets of genes, including non-coding RNA molecules of various length composed of Satellite III (SatIII) sequences (Rizzi *et al*., 2004; Valgardsdottir *et al*., 2005). SatIII RNAs remain associated with sites of transcription (Valgardsdottir *et al*., 2005) and are bound by several RNA-binding factors, like SAFB, thus leading to the formation of nuclear stress bodies (nSBs). SAFB is a RNA binding protein that interacts with Sam68 after heat shock (Denegri *et al*., 2001). Moreover, recently it has been shown that transcription of SatIII DNA is triggered by a wide range of stress treatments, including genotoxic agents, such as etoposide (Valgardsdottir *et al*., 2008), a

topoisomerase II inhibitor analogous to MTX. We are currently investigating whether MTX treatment also induces transcription of SatIII RNAs in PCa cells and whether Sam68 co-localizes with both SatIII RNAs and SAFB in nuclear granules and is required for this response.

Since Sam68 is known to link signal transduction pathways to RNA metabolism (Lukong and Richard, 2003), we asked whether changes in Sam68 subcellular localization induced by MTX are determined by activation of specific signal transduction pathways. Our data show that although MTX triggers activation of DNA damage pathway, through ATM kinase, and stress-induced MAPKs p38 and JNK1/2 pathways, specific inhibition of none of these pathways affected the subcellular relocalization of Sam68 or hnRNP A1. Thus, it is possible that direct changes in the chromatin structure or function trigger the observed accumulation of Sam68 and splicing factors in nuclear granules.

The protein trafficking in response to DNA damage and stress is well documented and involves different organells, such as mitochondria and nucleoli (Tembe and Henderson, 2007). For instance, genotoxic stresses are known to affect nucleolar structure and compromise nucleolar function (Rubbi and Milner, 2003). Since the Sam68-positive granules are faintly stained with DAPI or Hoechst like nucleoli, we have hypothesized that Sam68 re-localization to nucleoli-like granules was similar to cellular response to transcription inhibitors, such as actinomycin D, which triggers changes of nucleolar morphology and nucleolar proteome (Andersen *et al*., 2005). This work showed that Sam68, which is predominantly localized in the nucleoplasm, under normal conditions is also present as a small pool in the nucleoli, where it sharply accumulates after a transcriptional block induced by actinomycin D (Andersen *et al*., 2005). Our attempts to identify the MTX-induced Sam68 granules as nucleoli have failed so far because the treatment caused the dispersion of both Nucleolin and UBF, two well described nucleolar markers (Rickards *et al*., 2007; Jordan and Carmo-Fonseca, 1998), in the nucleoplasm after genotoxic stress.

Nevertheless, other sets of observations performed during our studies further implicate Sam68 in nucleolar functions. In a co-immunoprecipitation experiment aimed at the identification of Sam68-interacting proteins in LNCaP cells we found Nucleolin (Busà R., Paronetto MP, Bielli P, and Sette C., manuscript in preparation). This interaction has been confirmed and mapped to the carboxyterminal region of Sam68 by in vitro studies. Moreover, a RNA-protein co-immunoprecipitation experiment revealed that Sam68 associates with the 18S rRNA among the RNAs bound by Sam68. These preliminary observations lead us to investigate whether the nucleolar pool Sam68 plays a role in rRNA metabolism under normal and stress conditions. First, we have analysed the effects of Sam68 depletion on rRNA metabolism. Surprisingly, downregulation of Sam68 caused a significant increase in the levels of pre-rRNA compared with control siRNA treated cells. These results suggest that Sam68 can act either as a repressor of rDNA transcription or as an activator of pre-rRNA processing. However, ChIP assays aimed at determining the site of the association of Sam68 with rDNA in PC3 cells revealed that Sam68, like Nucleolin (Rickards *et al*., 2007), does not bind the ribosomal promoter while it binds the 18S rRNA coding region. This result indicates that Sam68 could play a role in pre-rRNA processing and than in its absence the pre-rRNA accumulates because its processing is delayed or stopped. Indeed, since proteins acting as transcription inhibitors or coactivator, such as the the nucleolar protein JHDM1B/FBXL10 and c-Myc respectively, usually bind to DNA promoters (Frescas *et al*., 2007; Grandori *et al*., 2005), the finding that Sam68 does not bind this region seems to rule out the alternative hypothesis. Thus, the results presented herein strongly suggest a novel role of Sam68 in the regulation of pre-rRNA maturation in conjunction with the well known regulator Nucleolin. Our current studies are aimed at investigating this hypothesis further. We will address this question by performing Northern blot assays to follow the time-course of pre-rRNA processing in control and Sam68-depleted

PC3 cells. Moreover, we will perform pre-rRNA FISH experiments after 8 hours of MTX, when Sam68 nuclear granules are more evident, to test whether Sam68 can bind pre-rRNA that is still transcribed after a long-term DNA damage. Finally, we will perform a FISH for ribosomal DNA and see if Sam68 binds to ribosomal chromatin in nucleoli-like granules induced by MTX.

Materials and Methods

Cell cultures and transfections

LNCaP cells were mantained in RPMI 1640 medium (Lonza) supplemented with 10 % fetal bovine serum, glucose (4,5 g/L), gentamycin, and penicillin/streptamycin. PC3 cells were cultured in DMEM-Glutamax (Invitrogen) supplemented with 10% fetal bovine serum, gentamycin and penicillin/streptamycin. PC3 cells at 70 % of confluency were transfected with a siSam68-plasmid (plko 527) or a control siRNA-plasmid (pSuper), purchased from Sigma-Aldrich**,** using Lipofectamine 2000 and Opti-MEM medium (Invitrogen) according to manufacturer's instructions. First, 24 h after transfection cells were selected for 24 h with 3 μg/ml puromycin (Sigma-Aldrich) and then mantained with 0,5 μg/ml puromycin for 4 days.

Cell treatments

LNCaP cells at 80% of confluency were treated with 80 μM cisplatin (Sigma-Aldrich) for 16 h, fixed and stained for immunoflorescence analysis. For mitoxantrone (MTX) (Sigma-Aldrich) treatment, PC3 cells at 80 % of confluency were incubated with different doses (0.1-5 μM) of MTX at increasing times as indicated in the text. For MAPKinase inhibition in presence of genotoxic stress, PC3 cells were incubated for 15 min with 10 μM JNKinhibitor (Alexis Biochemicals) or 10 μM SB202190 (Calbiochem) and then treated with 5μM MTX for increasing times, as indicated in the text. For ATM kinase inhibition, PC3 cells were incubated for 1 h with KU3353 (generous gift from Dott.ssa Barilà) and then treated with 5μM MTX for 24 h.

Plasmid constructs

The GST-(1-277)Sam68 pGEX4-T1, GST-(321-443)Sam68 pGEX4-T1 and GST-(351-443)Sam68 pGEX4-T1 were previously described in Paronetto *et al*., 2007. Sam68 351-434, Sam68 351-400 and Sam68 365-443 sequences were amplified by PCR from GST Sam68 pGEX4-T1 by using the primers listed in the table1 below, and cloned by using Eco RI-Sal I restricyion enzymes in pGEX4-T1 plasmid (Clontech).

Plasmid	Primers
GST-(365-443)Sam68 pGEX4-T1	Fw AGGAATTCACATACGAAGATTATGGATATG Rv AGGTCGACTTAATAACGTCCATATGGATGCTC
GST-(351-434)Sam68 pGEX4-T1	Fw AGGAATTCGGCATCCAGAGGATACCT Rv AGGTCGACTTATGCTCCCTTCACTGGC
GST-(351-400)Sam68 pGEX4-T1	Fw AGGAATTCGGCATCCAGAGGATACCT Rv AGGTCGACTTATCCATAGTCATAATACTCT

Table1. GST-Sam68 constructs

RNA extraction, RT-PCR and Real time PCR

Total RNA was extracted from PC3 cells using cold Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was then treated with DNAse/RNAse free (Roche) for 20 min at 37°C and stored at –80°C for further analysis. For CD44 alternative splicing analysis, 1 μg of total RNA of MTX treated PC3 cells was used for RT-PCR using M-MLV reverse transcriptase (Invitrogen) according to manifacturer's instructions. 10% of the reverse transcription reaction was used as template together with the following primers: CD44 1 (forward) and CD44 3 (reverse) for CD44 v5; CD44 v6, v7, v8, v9, v10 (forward) and CD44 c (reverse) for the other variable exons. PCR conditions were the followings: 94 °C for 30 sec, 52 °C for 30 sec and 72°C for 30 sec (30 cycles) for CD44 v5; 94°C for 30 sec, 56°C for 30 sec and 72°C for 45 sec (27 cycles for v6-v9 and 25 cycles for v10).

Samples were normalized with HPRT and analysed on 1.5% agarose gel.. For pre-rRNA analysis by real time PCR, 200 ng of RNA/each sample was reverse transcribed as previously described. An aliquot (1/20th) of the reaction was then used in a quantitative real time PCR with Sybr Green mix (for Light-Cycler480 - Roche) according to manufacturer's instructions. Primers used for pre-rRNA were: 5'ETS pre-rRNA (forward) and 5'ETS pre-rRNA (reverse). The housekeeping gene GAPDH was used to obtain the ΔΔct values for the calculation of fold increases. The expression of pre-rRNA was measured in at least three independent experiments.

Western blot analysis

Cell extracts (obtained as previously described in Busà *et al*., 2007) or immunoprecipitated proteins were diluted in SDS sample buffer and boiled for 5 minutes. Proteins were separated on either 10% or 8% SDS-PAGE gels and transferred to PVDF Transfer Membrane Hybond^{Tm}-P (Amersham Bioscience, UK). Membranes were saturated with 5% nonfat dry milk in PBS containing 0.1 %Tween-20, or with TBS containing 0.1 Tween-20 and 5% bovine serum albumine (BSA) (Sigma-Aldrich) for 1 h at RT, and incubated with primary antibodies. Primary antibodies were the followings: (1:1000 dilution; overnight at 4°C) rabbit anti-Sam68 and rabbit anti-Erk2 (Santa Cruz Biotechnology); rabbit p-ChK2, p-p38 (Cell Signaling); and mouse c-Jun (BD biosciences); rabbit anti-pJNK1/2 and rabbit p-eIF4E (Biosource), mouse anti-Nucleolin (Abcam). Secondary anti-mouse or anti-rabbit IgGs conjugated to horseradish peroxidase (Amersham) were incubated for 1h at room temperature (1:10000 dilution in PBS containing 0.1% Tween 20). Immunostained bands detected by chemiluminescent method (Santa Cruz Biotecnology).

Co-Immunoprecipitation and GST-pull-down

PC3 or LNCaP cells were homogenized in lysis buffer (100 mM NaCl, 10 mM MgCl2, 30 mM Tris/HCl, 1 mM DTT, protease inhibitor cocktail (Sigma-Aldrich) supplemeted with 0.5% Triton X-100. Soluble extracts were separated by centrigugation at 10,000 g for 10 min, and they were precleared for 1h on protein A-Sepharose beads (Sigma-Aldrich) at 4°C under rotation. Cell extracts (11 mg of total proteins for co-ip from PC3 cells and 1 mg of total proteins for co-ip from LNCaP cells) were then precleared for 1h 30 min on proteinA-Sepahore beads (Sigma-Aldrich) in the presence of rabbit IgGs, 0.05% BSA. After centrifugation for 1 min at 1,000 g, supernatants were incubated with anti-Sam68 (10 μg for PC3 cell extracts and 2 μg for LNCaP cell extracts) or rabbit IgGs for 3 h at 4°C under costant rotation. Beads were washed three times with lysis buffer, and were eluted in SDS sample buffer for Western Blot analysis or silver staining (Sigma-Aldrich) and mass-spectrometry (co-ip from LNCaP cells).

The GST fusion proteins were purified from 100 ml of LB E.coli cultures, incubated for 3 h at 37°C in presence of 0.5 mM IPTG (Sigma-Aldrich). Bacterial cells were centrifuged at 4.000x rpm for 15 min. The cells were resuspended in 10 ml of PBS supplemented with protease inhibitor, 0.1% Triton X-100 and 1mM DTT. The bacteria cells were lysated by three cycles of 30sec of sonication following 30sec of ice incubation. Then the bacterial total extracts were centrifuged at 20.000xg for 30min at 4°C. The supernatants were filtered (0,45μm) and incubated at 4°C for 3 h with 200 μl of GSH-Sepharose beads. The beads were then washed with PBS. The GST proteins bound to the GSH-Sepharose beads were resuspended in PBS supplemented with protease inhibitor, 0.05% NaN_3 and 1mM DTT and stored at 4 °C.

Pull down assays were carried out as previously described (Paronetto et al., 2007), with Hek293 cell extracts. In details, Hek293T cell extracts were precleared for 1 h at 4°C by rotation on GSH-Sepharose beads. Meanwhile, GST fusion proteins bound to the GSH-

Sepharose beads were blocked with 0.5% BSA for 1 h at 4°C under rotation. Precleared cell extracts were incubated with the GST fusion proteins bound to the GSH-Sepharose beads after washing with PBS to remove excess of BSA. The incubation was carried out for 3 h at 4°C under rotation. After incubation, the beads were washed three times with PBS and resuspended in SDS sample buffer. Samples were boiled for 5 min and the supernatants were loaded in SDSPAGE gels for Western Blot analysis or Coomassie Blue staining.

Chromatin immunoprecipitation (ChIP)

Proteins were cross-linked to the DNA in PC3 cells by the addition of 1% (vol/vol) formaldehyde to the culture medium for 10 minutes at RT. Cells were then washed twice with cold phosphate-buffered saline, harvested and lysed to isolate nuclei in a ipotonic buffer containing 5mM Pipes pH 8.0, 85 mM KCl and NP-40 0.5%. Nuclei were then resuspended and lysed in a buffer containing 1% SDS, 10 mM EDTA and 50 mM Tris/HCl pH 8.1. Lysates were sonicated with five pulse (1' - 80% Amplitude) to achieve a chromatin size of 700 bp and the insoluble debris was removed by centrifugation. The lysates were diluted 1:10 with diluition buffer (0.01% SDS, 1.1% Triton X100, 1.2 mM EDTA, 16.7 mM Tris/HCl pH 8.0, 16.7mM NaCl) and precleared by rotation with protein A/G beads that had been blocked by incubation with 1μg/ml bovine serum albumine (BSA) (Sigma Aldrich) and 1μg/ml sonicated salmon sperm (SSS) DNA 2h at 4°C. After preclearing, lysates were centriguged at 600 g for 15 min. The cross-linked DNA was then quantified with nanodrop: 100 μg of chromatin for each sample were incubated with 1μg of anti-Sam68 rabbit (Santa Cruz, SC-333) or anti-Nucleolin mouse (Abcam) and protein A/G beads were blocked with 1μg/ml BSA and 1 μg/ml SSS., by rotation at 4°C overnight. Next, pre-saturated protein A/G beads were incubated with lysates and antibodies by rotation at 4°C for 3 hours. Protein A/G beads were then washed and cross-links were reversed adding 16 μl 5M NaCl and incubating at 65°C overnight. The immunoprecipitated DNA was recovered with

phenol/clorophorme and resuspended in 30 µl of TE (10 mM Tris pH 8.0, 1 mM EDTA). In parallel, an input DNA sample was prepared and resuspended in 100 µl TE. Immunoprecipitated DNA was normalized with a standard curve built by real time PCR with 1 µl of input dilutions. The fold enrichment was calculated relative to the background detected with no-antibody for each primer set. Quantitative real time PCRs were performed with Sybr Green mix (for Light-Cycler480 - Roche) according to manufacturer's instructions. Primers (H1, around rDNA promoter, data not shown, and H4, in the coding region of 18S rRNA) were derived from Frescas *et al*., 2007.

Immunofluorescence and FISH

LNCaP cells and PC3 cells were fixed and stained for immunofluorescence analysis as previously described (Paronetto *et al*., 2007).

Primary antibodies were the followings: rabbit anti-Sam68 1:1000 (Santa Cruz, Biotechnology); mouse anti-γH2AX 1:10.000 (Cell signaling); mouse anti-hnRNP A1 1:500 (Sigma); goat anti-TIA-1 1:200 (Santa Cruz Biotechnology); mouse anti-Nucleolin 1:500 (Abcam); mouse anti-UBF 1:500 (Santa Cruz, Biotechnology); mouse anti-SC35 1:500 (Sigma); mouse anti-SF2/ASF 1:200 (USBiological). Secondary antibodies were: Alexa Fluor 488 and 568 goat anti-rabbit 1:500; Alexa Fluor 488 goat anti-mouse 1:500; FITC donkey anti-rabbit and cy3 rabbit anti-goat 1:500 (Jackson Immunoresearch). Cells were analysed using a confocal microscope (Leica). For IF/FISH analysis cells grown on coverslips were fixed in 4% paraformaldehyde (PFA) 10 min at RT and washed twice with sterile PBS. Cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) supplemented with 2 mM Vanadyl Ribonucleoside complex (VRC) (Sigma-Aldrich) for 5 min on ice. Cells were washed three times with PBS and blocked with 1% BSA for 30 min at RT. Cells were washed twice with PBS and incubated at 4°C overnight with primary antibody (Sam68, 1:1000), diluted in 1% BSA and supplemented with RNAse out

(Invitrogen). Cells were then washed three times with PBS and incubated 1 h at RT with secondary antibody (Alexa Fluor 488 goat anti-rabbit, Molecular probe) diluted 1:500 in 1% BSA (Sigma) supplemented with RNAse out (Invitrogen). Cells were washed three times with PBS and fixed again in PFA for 10 min at RT. Cells were washed twice with 2 X SSC (prepared as in Maniatis et al.) for 5 min at RT and processed for FISH (protocol from Rouquette *et al*., 2005). Two hybridization solutions (A and B) were prepared. The following conditions are for one 20 mm x 20 mm square coverslip. Solution A: 5µl formamide (Sigma-Aldrich), 2.5 µl 2x SSC, 2.5 µl tRNA (10 mg/ml), 8,75 µl H20 RNAse free (Sigma-Aldrich) and 2.5 µl probe (10 ng/ml) (to be added last). Solution B: 25 µl dextran sulfate (Sigma-Aldrich) 20% in 4X SSC, 1.25 µl BSA (10 mg/ml) (Sigma-Aldrich) and 2.5 µl VRC (Sigma-Aldrich, stock 200 mM). Solution A was denatured at 95°C for 5 min and then mixed with solution B. Coverslips were flipped over 50 µl drops of the mixture on parafilm in a humid chamber an incubate for 3h at 37°C. Cells were then washed twice for 30 min at RT with 2X SSC + 10% formamide (Sigma-Aldrich), once with PBS and mounted with Mowiol (Calbiochem) for observation on a confocal microscope (Leica). The probe used for FISH was 5'-ITS1 (sequence from Rouquette *et al*., 2005) labelled with Cy3 at 3' end. It hybridizes with the last nucleotides of the 18S rRNA and with the 5' part of the ITS1 in human pre-rRNAs.

Image acquisition and manipulation

The confocal images in Figure 1-3, 5-7 and 11 were taken from a confocal microscope (Leica) using a Plan-Neofluar HCX 40.0x/1.25 oil UV objective (except for Figure 1, panel C, taken using a HCX PL APO 63.0x1.40 oil UV objectiveA) and IAS AF Lite software (Leica Microsystems). Images were acquired as TIFF files, and Photoshop (Adobe) and PowerPoint (Microsoft) were used for composing the panels.

GeArray prostate cancer biomarkers

RNAs purified from a Sam68-RNAs co-immunoprecipitation in LNCaP cells (protocol as previously described, Paronetto *et al*., 2007) were used as a template to generate biotin-16-UTP labelled cRNA probes by the True Labelling kit (Superarray Inc., Bethesda, MD, USA). The cRNA probes were hybridised at 60°C with the SuperArray Prostate cancer biomarkers membrane and signals were revealed using the SuperArray detection Kit.

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Appendix

Phosphorylation of eIF4E by MNKs supports cell cycle progression and proliferation in prostate cancer cells

Abstract

Deregulation of signal transduction pathways that modulate mRNA translation is a frequent feature of different types of tumors, including human prostate carcinomas (Pcas). Two pathways are often deregulated: the AKT/mTOR and the MAPK/MNK pathways.

We studied the contribution of each pathway on translational control of Pca cell proliferation. Our experiments provide four main conclusions: a) the activity of the mTOR and MNKs pathways are under a controlled balance in PCa cells and the downregulation of the former has a positive feedback on the latter; b) translational control of mRNAs encoding for ribosomal proteins is under control of both the signalling pathways; c) concomitant inhibition of the mTOR and MNKs pathways strongly suppresses protein synthesis, cell cycle progression and proliferation of PCa cells; d) inhibition of the MNK pathway leads to translational repression of mRNAs encoding for proteins involved in cell cycle.

Together, our results indicate that a fine balance between these two pathways is required for a proper translational control of specific mRNAs, involved in ribosome biogenesis, cell cycle and stress response.

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Phosphorylation of eIF4E by MNKs supports protein synthesis, cell cycle progression and proliferation in prostate cancer cells

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Deregulation of the phosphatidyl inositol trisphosphate kinase/AKT/mammalian target of rapamycin (mTOR) and RAS/mitogen-activated protein kinase (MAPK)/MNK pathways frequently occurs in human prostate carcinomas (PCas) and leads to aberrant modulation of messenger RNA (mRNA) translation. We have investigated the relative contribution of these pathways to translational regulation and proliferation of PCa cells. MNK-dependent phosphorylation of eIF4E is elevated in DU145 cells, which have low basal levels of AKT/mTOR activity due to the expression of the tumor suppressor *PTEN*. In contrast, eIF4E phosphorylation is low in PC3 and LNCaP cells with mutated *PTEN* and constitutively active AKT/mTOR pathway, but it can be strongly induced through inhibition of mTOR activity by rapamycin or serum depletion. Remarkably, we found that inhibition of MNKs strongly reduced the polysomal recruitment of terminal oligopyrimidine messenger RNAs (TOP mRNAs), which are known targets of mTOR-dependent translational control. Pull-down assays of the eIF4F complex indicated that translation initiation was differently affected by inhibition of MNKs and mTOR. In addition, concomitant treatment with MNK inhibitor and rapamycin exerted additive effects on polysomal recruitment of TOP mRNAs and protein synthesis. The MNK inhibitor was more effective than rapamycin in blocking proliferation of PTEN-expressing cells, whereas combination of the two inhibitors suppressed cell cycle progression in both cell lines. Microarray analysis showed that MNK affected translation of mRNAs involved in cell cycle progression. Thus, our results indicate that a balance between the activity of the AKT/mTOR and the MAPK/MNK pathway in PCa cells maintains a defined translational level of specific mRNAs required for ribosome biogenesis, cell proliferation and stress response and might confer to these cells the ability to overcome negative insults.

Introduction

Prostate cancer (PCa) remains the second leading cause of death in the western male population. Although initially counteracted by androgen deprivation therapy, PCa cells often evolve to become independent of androgens for their growth (1). At this stage, no effective cure is available for the patients, rendering the discovery of new therapeutic approaches a clinical priority. A common feature in PCa is represented by inactivating mutations in *PTEN* (2). This gene encodes for the phosphatase that hydrolyses the phosphatidyl inositol trisphosphate and turns off the proliferative signal exerted by the phosphatidyl inositol trisphosphate kinase (PI3K) in response to growth factors (3). Mouse models have shown that haploinsufficiency in the *PTEN* locus predisposes to the onset and the progression of PCa (4,5). Mechanistically, inactivation of *PTEN* leads to increased basal levels of phosphatidyl inositol trisphosphate in the cell and constitutive activation of the AKT pathway.

The oncogenic potential of the AKT pathway relies on the downstream serine/threonine kinase mammalian target of rapamycin (mTOR) (6,7). The involvement of mTOR in neoplastic transformation appears to depend on its regulatory activity toward the translation initiation complex eIF4F. By this way, mTOR stimulates ribosome biogenesis in response to nutrients and growth factors (8,9). The eIF4F complex consists of eIF4E, the protein that binds to the 5′-cap of messenger RNAs (mRNAs), eIF4G, a scaffold protein and eIF4A, a helicase that unwinds the 5′-UTR of mRNAs allowing recognition of the first AUG codon. mTOR phosphorylates the eIF4E-binding proteins (4E-BPs) causing their dissociation from eIF4E and promoting the assembly of eIF4F (8). Additional mTOR substrates are the ribosomal protein S6 kinase and eIF4B (8,10), which stimulates the RNA-unwinding activity of eIF4A. Due to its central role in cell proliferation and oncogenesis, inhibitors of mTOR, such as rapamycin and its derivatives CCI-779 and RAD001, have been enrolled in clinical trials for several neoplastic diseases, including PCa (7,11). However, in spite of their potent activity against various tumor cell types, these inhibitors often elicit activation of additional pathways that support cancer cell survival. Prolonged treatment of cancer cells or patients with mTOR inhibitors causes a positive feedback on PI3K activity that leads to phosphorylation of AKT and eIF4E (12,13). While activation of AKT has been described to elicit anti-apoptotic responses in cancer cells through several mechanisms (14), the role of eIF4E phosphorylation is less understood. Mouse models have shown that eIF4E phosphorylation on serine 209 depends on the activity of two kinases, MNK1 and 2, that are not essential during development (15). Nevertheless, eIF4E phosphorylation correlates with cancer cell proliferation (16) and eIF4E protein levels are upregulated in many tumors thereby favoring the translation of mRNAs for proteins involved in cell proliferation and survival (17). Remarkably, a recent report demonstrated that the oncogenic potential of eIF4E strictly depends on phosphorylation of serine 209 by MNK in a mouse lymphoma model (18). Thus, phosphorylation of eIF4E appears to be pivotal to neoplastic transformation rather than a consequence of the aberrant regulation of signaling pathways in cancer cells. Although these results highlight the relevance of the MNK/eIF4E pathway in lymphoma, no information on the occurrence and relevance of this regulatory phosphorylation event in PCa is available. Moreover, although phosphorylation of eIF4E was hypothesized to affect translational control, only one potential target, the anti-apoptotic protein Mcl-1, was identified in the former study (18).

Herein, we have investigated the functional interaction between the AKT/mTOR and MNK/eIF4E pathways in PCa cells. We found that upregulation of eIF4E phosphorylation occurs under conditions in which the AKT/mTOR pathway is stably or transiently downregulated. Both pathways are required for efficient cap-dependent translation and for cell proliferation. Finally, by using the PTEN-positive DU145 PCa cells, we show that, similarly to the mTOR pathway, the MNK/eIF4E pathway influences translation of mRNAs for proteins required for cell cycle transitions and cell growth. Thus, our results indicate that the AKT/mTOR and MNK/eIF4E pathways are linked by a compensatory feedback that supports PCa cell proliferation.

Abbreviations: 4E-BP, eIF4E-binding proteins; FBS, fetal bovine serum; IEG, immediate early gene; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; mTOR, mammalian target of rapamycin; PCa, prostate carcinoma; PCR, polymerase chain reaction; PI3K, phosphatidyl inositol trisphosphate kinase; TOP mRNA, terminal oligopyrimidine messenger RNA.

Materials and methods

Cell culture and treatments

PC3 cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) (BioWhittaker Cambrex

Bioscience). LNCaP and DU145 cells were grown in RPMI medium (BioWhittaker Cambrex Bioscience) supplemented with 10% FBS. Cells were seeded at $5 \times 10^4/cm^2$. After 30–36 h, growing cells (50–70% confluence) were starved in medium without FBS for 16 h unless specified. The MNK inhibitor 4-amino-5-(4-fluoroanilino)-pyrazolo[3,4-d]pyrimidine (Calbiochem), rapamycin (Alexis), U0126 and LY294002 (Calbiochem) were preincubated 15 min before the addition of FBS. After stimulation, cells were washed twice with ice-cold phosphate-buffered saline and extracted as described below.

For the *in vivo* labeling experiments, 2×10^5 cells per 35 mm well were incubated in the presence of FBS and/or inhibitors as indicated. In the last 30 min, [^{35}S] cell labeling mix (PRO-MIX, Amersham, >1000 Ci/mmol) was added to a final concentration of 10 μCi/ml. Cells were lysed in phosphate-buffered saline–sodium dodecyl sulfate buffer (150 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.4 mm KH_2PO_4 and 0.1% sodium dodecyl sulfate) and proteins were precipitated in 10% trichloroacetic acid. After three washes with 5% cold trichloroacetic acid, the insoluble material was collected on GFC filters (Whatman) and the incorporated radioactivity was measured in scintillation fluid. For the cell cycle analysis, treated cells were collected in phosphate-buffered saline, incubated with 70% ethanol for 2 h, then treated with RNAseA (15 min at 37°C) and 10 μg/ml propidium iodide (30 min at 37°C) and analyzed on an FACSCalibur Flow Cytometer (Becton Dickinson, San Josè, CA).

Preparation of cell extracts and western blot analysis

Cells were lysed directly on the plate by the addition of lysis buffer as described previously (19). Protein concentration was determined by using Bradford reagent (Bio-Rad). Cell extracts were used for western blot analysis as described previously (20) with the following primary antibodies (1:1000 dilutions): rabbit anti-eIF4E, rabbit anti-pSer^{473}-AKT, mouse anti-p Thr^{389}S6K1, rabbit anti-4E-BP1, rabbit anti-p44/42 mitogen-activated protein kinase (MAPK) (Thr^{202} and Tyr^{204}), rabbit anti-eIF4G and rabbit anti-rpS6 (Cell Signaling Technology); rabbit anti-extracellular regulated kinase 2 (Santa Cruz Biotechnology); rabbit anti-pSer$^{235/236}$ rpS6 and rabbit anti-pSer^{209} eIF4E (BioSource International); mouse anti β-tubulin (Sigma–Aldrich); mouse anti-c-JUN (BD Biosciences). After incubation with secondary anti-mouse or anti-rabbit IgGs conjugated to horseradish peroxidase (Amersham), immunostained bands were detected by chemiluminescent method (Santa Cruz Biotechnology).

Polysome separation and extraction of RNA

Whole-cell extracts were prepared in the presence of 10 μg/ml cycloheximide and 30 U/ml of RNase inhibitor as described previously (19). An aliquot of each fresh lysate was used for western blot analysis and another aliquot was extracted with TRIZOL reagent (Invitrogen) as a source for total RNA. The remaining extracts were centrifuged at 13 000 r.p.m. for 10 min in a microfuge at 4°C and the clarified supernatants (2 mg of total proteins) were loaded onto a 15–50% linear sucrose gradient as described (19). The absorbance at 254 nm of the collected fractions was monitored and polyribosome-containing fractions were pooled. RNA was isolated with RNeasy Mini Kit (Qiagen), resuspended in RNase-free water (Sigma–Aldrich) and immediately frozen at −80°C for further analysis.

Polymerase chain reaction analysis

RNA (0.5–1 μg) from total or polysomal preparation was used for reverse transcription–polymerase chain reaction (PCR) using M-MLV reverse transcriptase (Invitrogen) and random examers (Roche). Ten percent of the reverse transcription reaction was used as a template. All primer sequences are listed in supplementary Table 1 (available at *Carcinogenesis* Online). Amplification of β-actin was used as an internal control. Quantitative real-time PCR amplification reactions were carried out in triplicate using Biorad iQ™ SYBR-green Supermix according to the manufacturer's instruction. At least two housekeeping genes among HPRT, GAPDH and β-actin were used to obtain the ΔΔCt values for the calculation of fold increases. In order to obtain a measure of the ribosomal loading efficiencies, we calculated the ratio between polysomal mRNA and total mRNA. For each drug treatment, the value of this ratio is expressed as a percentage of that in FBS-stimulated sample.

GeArray prostate cancer biomarkers

Total and polysomal RNAs were isolated as described above and used as a template to generate biotin16-UTP-labeled cRNA probes using the True Labelling Kit (Superarray, Bethesda, MD). The cRNA probes were hybridized at 60°C with the SuperArray Prostate Cancer Biomarkers membranes and signals were revealed using the SuperArray Detection Kit. Data were analyzed by densitometry using the Scanalyze software and following the manufacturer's instruction.

7-Methyl-GTP-Sepharose chromatography

For the isolation of eIF4E and associated proteins, cells were lysed in buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid, pH 7.4, 75 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 8 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid, 10 mM β-glycerophosphate, 0.5 mM Na_3VO_4, 0.5% Triton-X-100 and protease inhibitor cocktail. Cell extracts were incubated for 10 min on ice and centrifuged at 12 000*g* for 10 min at 4°C. The supernatants were precleared for 1 h on Sepharose beads (Sigma–Aldrich). After centrifugation for 1 min at 1000*g*, supernatants were recovered and incubated for 2 h at 4°C with 7-methyl-GTP-Sepharose (Amersham) under constant shaking. Beads were washed three times with lysis buffer and absorbed proteins were eluted in sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer.

Cell proliferation assay

The CellTiter A96 MTS (Promega) assay was used to monitor cell viability and proliferation as described previously (21).

Results

Low levels of activity of the PI3K/AKT/mTOR pathway are correlated with eIF4E phosphorylation in prostate cancer cells

PTEN is frequently mutated in PCa cells, leading to constitutive activation of the PI3K/AKT/mTOR pathway (3). Since cap-dependent translation is regulated by the AKT/mTOR and MAPK/MNK pathways through regulation of their common target eIF4E, we investigated whether eIF4E phosphorylation levels were affected by the status of PTEN in PCa cells. In LNCaP and PC3 cells, which are PTEN null, AKT is constitutively active and phosphorylated, whereas eIF4E is weakly phosphorylated (Figure 1A). In contrast, in DU145, which express wild-type PTEN, we observed lower levels of AKT phosphorylation and higher levels of eIF4E phosphorylation. To further test whether low levels of AKT activity trigger eIF4E phosphorylation in PCa cells, we set out to interfere with the PI3K/AKT/mTOR pathway in PC3 and LNCaP cells (Figure 1B). Treatment with the mTOR inhibitor rapamycin (1 or 4 h) or with serum-depleted medium (6 or 16 h) reduced AKT phosphorylation and mTOR activity, as demonstrated by decreased phosphorylation of its direct (4E-BP1, note the increase in the α isoform) and indirect (rpS6) substrates. Remarkably, both treatments strongly induced phosphorylation of eIF4E at all time points analyzed. These results support the hypothesis that a transient or stable decrease in PI3K/AKT/mTOR activity induces a response that causes eIF4E phosphorylation in PCa cells.

Next, we checked whether stimulation with growth factors induced activation of the AKT/mTOR and MNK/eIF4E pathways in similar fashion in cells with different PTEN status. Serum-deprived cells were treated with FBS for up to 1 h and analyzed by western blot. As shown in Figure 1C, although both cell lines readily responded to growth factors, the kinetic of phosphorylation of several proteins differed. Unlike in PC3 cells, the AKT/mTOR pathway was completely inhibited in starved DU145 cells and its activation did not lead to complete phosphorylation of the mTOR target 4E-BP1 even after 1 h, as shown by the persistence of the non-phosphorylated α isoform (Figure 1C). In contrast, we observed that eIF4E was constitutively phosphorylated in DU145 cells, whereas its phosphorylation slowly increased upon time in PC3 cells. These results highly suggested that the relative activity of the AKT/mTOR and of the MNK/eIF4E pathways are under a controlled balance in PCa cells.

The AKT/mTOR and MNK/eIF4E pathways stimulate the polysomal recruitment of terminal oligopyrimidine messenger RNAs *in PCa cells*

The AKT/mTOR and MNK/eIF4E pathways converge on the regulation of cap-dependent mRNA translation (8). For instance, growth factor-induced polysomal recruitment of specific mRNAs containing a polypyrimidine tract in their 5′-UTR, the terminal oligopyrimidine messenger RNAs (TOP mRNAs), is mostly dependent on the mTOR pathway (22–25). It is currently unknown, however, whether the MNK/eIF4E pathway also plays a role in this rapid mitogenic response. To answer this question, cell extracts from serum-deprived PC3 cells or from cells stimulated for 1 h with FBS were fractionated on sucrose gradients to separate the heaviest actively translating polysomes from

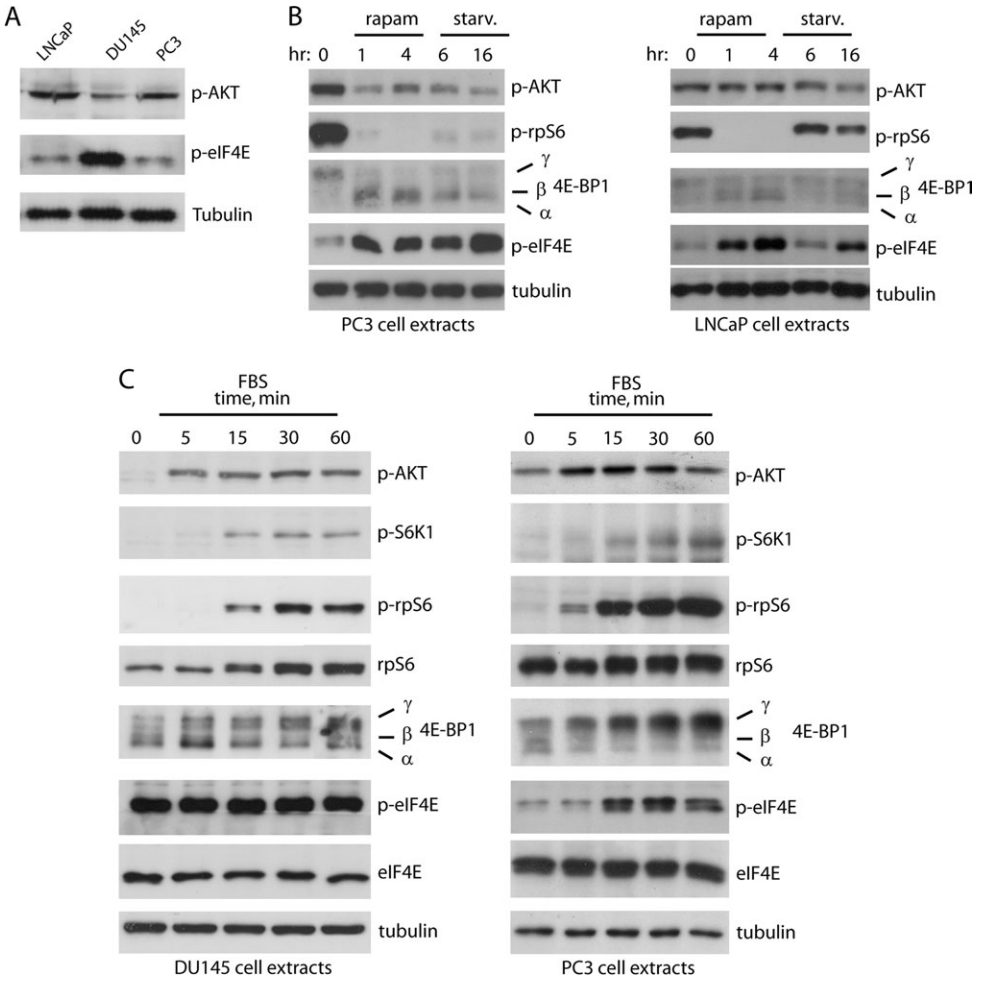

Fig. 1. Low levels of activity of the PI3K/AKT/mTOR pathway stimulate eIF4E phosphorylation in prostate cancer cells. (**A**) Western blot analysis of extracts from PC3, LNCaP or DU145 cells grown in medium supplemented with 10% FBS. Antibodies used are indicated on the right side of each panel. (**B**) Western blot analysis of time-dependent effects of rapamycin (10 nM) and serum depletion on the AKT/mTOR and MNK/eIF4E pathways in PC3 and LNCaP cells. (**C**) Western blot analysis of time-dependent activation of AKT/mTOR and MNK/eIF4E pathways following stimulation with 10% FBS in PC3 and DU145 cells. The antibodies used are indicated on the right. Cell extract loading was normalized with anti-tubulin (lower panel).

monosomes and from ribonucleoprotein particles fractions (supplementary Figure 1A is available at *Carcinogenesis* Online). PC3 cells were selected because in this cell line we could observe an increase in both AKT/mTOR-dependent phosphorylations and eIF4E phosphorylation (Figure 1C). In addition to TOP mRNAs, we also checked the polysomal recruitment of immediate early genes (IEGs), whose transcription is strongly induced upon mitogenic stimulation. Semiquantitative and quantitative real-time PCR demonstrated that FBS induced a 2-fold increase in polysomal recruitment of the TOP mRNAs rpL32 and rpS19, without significant increase in the total mRNA levels. In contrast, accumulation of mRNAs for IEGs (c-FOS, c-JUN, NR4A1 and EGR1) on the polysomes followed their transcriptional increase (supplementary Figure 1B and C is available at *Carcinogenesis* Online). Hence, FBS induce polysomal recruitment of TOP mRNAs from a preexisting pool and of newly transcribed IEG mRNAs.

Next, we set out to selectively interfere with AKT/mTOR and MAPK/MNK signaling pathways to determine their role on polysomal recruitment of TOP and IEG mRNAs. We observed that the mTOR inhibitor rapamycin specifically inhibited phosphorylation of rpS6 and 4E-BP1 (γ isoform) (supplementary Figure 2A is available at *Carcinogenesis* Online), without affecting the transcriptional activation or stability of IEG mRNAs (supplementary Figure 2B and C is available at *Carcinogenesis* Online and data not shown). On the other hand, the MNK inhibitor inhibited exclusively phosphorylation of eIF4E, without affecting the AKT/mTOR pathway (supplementary Figure 2A is available at *Carcinogenesis* Online) or mRNA transcription or stability (supplementary Figure 2B and CM is available at *Carcinogenesis* Online and data not shown). In contrast, inhibition of PI3K by LY294002 or MEK1/2 by U0126 either affected IEG mRNA transcription or incompletely abolished eIF4E phosphorylation (supplementary Figure 2A and B is available at *Carcinogenesis* Online). Thus, to determine the contribution of the AKT/mTOR and MNK/eIF4E pathways on polysomal recruitment of IEG and TOP mRNAs, we selected rapamycin and MNK inhibitor that had a pathway-specific effect without affecting *de novo* transcription.

PC3 cells were stimulated with FBS in the presence or absence of rapamycin or MNK-4inhibitor and the polysomal or total RNA fractions were purified from each sample. The ribosomal loading efficiencies were calculated as the ratios between polysomal and total mRNA. As expected, rapamycin decreased the recruitment of TOP mRNAs (rpS19 and rpL32) on polysomes (Figure 2A). Interestingly, a similar effect was also exerted by treatment with MNK inhibitor, which blocks eIF4E phosphorylation without affecting phosphorylation of 4E-BP1 and rpS6 (Figure 2B). This result indicates that phosphorylation of eIF4E is also required for cap-dependent translation of TOP mRNAs in PCa cells. On the other hand, polysomal loading of IEG mRNAs was not impaired by these inhibitors (Figure 2A), demonstrating that their translation in response to growth factors completely escapes the control exerted by the AKT/mTOR and MAPK/MNK pathways. Western blot analyses showed that rapamycin and MNK

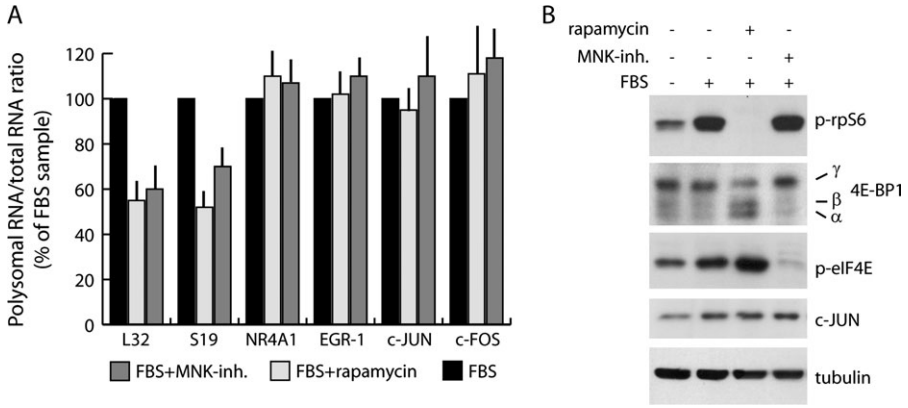

Fig. 2. Effect of rapamycin and MNK inhibitor on polysomal recruitment of TOP and IEG mRNAs. (**A**) Analysis by real-time PCR of the effect of 50 nM rapamycin or 10 μM MNK inhibitor on FBS-induced polysomal recruitment of IEGs (NR4A1, EGR-1, c-JUN and c-FOS) and TOP (rpL32 and rpS19) mRNAs. The values of samples treated with FBS and inhibitors are expressed as percentage of the FBS stimulated. Data represent mean ± SD from three independent experiments. At least two different housekeeping genes were used for each determination. (**B**) Western blot analysis of the effects of the inhibitors treatment on AKT/mTOR and MAPK pathways. Cell extract loading was normalized with anti-tubulin (lower panel).

inhibitor did not affect the accumulation of c-JUN protein induced by FBS stimulation (Figure 2B), confirming at the protein level the lack of effect on posttranscriptional regulation of this transcription factor.

Rapamycin and MNK inhibitor exert additive effects on polysomal recruitment of TOP mRNAs

Since both rapamycin and MNK inhibitor affected the polysomal recruitment of TOP mRNAs, we asked whether they influence the same step in the assembly of the eIF4F complex by pull-down assays with 7-methyl-GTP-Sepharose beads. FBS caused an increase in the association eIF4G with eIF4E (Figure 3A). This effect was suppressed by rapamycin, which increased the binding of hypophosphorylated 4E-BP1 to eIF4E and its competition with eIF4G (Figure 3A). In contrast, pretreatment with the MNK inhibitor did not inhibit the association of eIF4G with eIF4E. Similar amounts of eIF4E were bound to 7-methyl-GTP-Sepharose under all conditions. These results indicate that, although phosphorylation of 4E-BP1 by mTOR and eIF4E by MNK are both required for the polysomal recruitment of TOP mRNAs in PC3 cells, they probably affect different biochemical steps.

To test this hypothesis, we investigated whether rapamycin and MNK inhibitor exert additive effects on polysomal recruitment of TOP or IEG mRNAs. First, we determined the effect of mTOR or MNK inhibition on the polysomal profile of PC3 cells. After starvation, the absorbance profile of sucrose gradient fractionations revealed that the ribosomal subunits 40 and 60 S, the monosome 80 S and the polysomes were all detected in PC3 cell extracts (Figure 3C). Stimulation with FBS for 1 h caused a decrease in the 80 S peak and an increase in the polysomal peaks. This shift was abolished by pretreatment with rapamycin, which caused the maintenance of the profile of starved cells even in the presence of FBS. Treatment with the MNK inhibitor caused an even more dramatic increase in the 80 S monosomal peak than rapamycin. Moreover, cotreatment with both inhibitors induced an additive effect on the monosomal fractions and a larger decrease of the polysomal peaks. Western blot analyses of the phosphorylation status of target proteins confirmed the specificity of effect of the two inhibitors (Figure 3B). In line with the effect on the polysomal profile, the analysis of the distribution of the rpL32 and rpS19 by quantitative real-time PCR indicated that cotreatment with MNK inhibitor and rapamycin exerted an additive effect also on the polysomal recruitment of TOP mRNAs (Figure 3D). In contrast, the mRNA for NR4A1, and for other IEGs (data not shown), was not affected even in the presence of this stronger inhibition of translation initiation, demonstrating that these mRNAs are completely independent from the activation of the AKT/mTOR or MAPK/MNK pathways in PCa cells.

Rapamycin and MNK inhibitor exert additive effects on protein synthesis

Since both rapamycin and MNK inhibitor affected polysomal recruitment of mRNAs for proteins involved in ribosome biogenesis, we tested whether they displayed long-term effects in PCa cells. First, we measured the rate of protein synthesis by ^{35}S-methionine incorporation in starved cells treated for either 1 or 16 h with FBS. The short-term treatment did not significantly affect protein synthesis in both PC3 and DU145 cells (Figure 4A and B). Under these conditions, rapamycin mildly reduced the rate of synthesis, whereas MNK inhibitor had no effect in PC3 cells (Figure 4A). In contrast, in DU145 cells, inhibition of MNK was more effective than rapamycin in decreasing protein synthesis (Figure 4B). Prolonged incubation with FBS (16 h) was able to weakly stimulate ^{35}S-methionine incorporation in both cell lines. Both rapamycin and MNK inhibitor prevented this increase in protein synthesis at this time point. Again, we observed a different effect in PCa cells, possibly dependent on the PTEN status. In the PTEN-null PC3 cells, rapamycin exerted a stronger inhibition than MNK inhibitor (Figure 4A), whereas the opposite was observed in the PTEN-expressing DU145 cells (Figure 4B). Remarkably, combined treatment with the two inhibitors exerted an additive effect in PC3 cells, confirming the results obtained with polysomal loading of TOP mRNAs. Under these conditions, the rate of protein synthesis was lowered well below the levels observed in cells deprived of serum (Ctrl white bar in Figure 4). The inhibitors maintained the specificity of effect on protein phosphorylation after 16 h treatment in both cell lines (Figure 4C and D). These results suggest that the mTOR/4EBP1 and the MNK/eIF4E pathways are both required to sustain protein synthesis in PCa cells. Moreover, the relative contribution of each pathway is affected by the status of PTEN.

Concomitant inhibition of mTOR and MNK suppresses PCa cell cycle progression and cell proliferation

The additive effect observed on TOP mRNA translation and protein synthesis in PC3 cells suggested that inhibition of eIF4E phosphorylation might reinforce the cytostatic effect of rapamycin in PCa cells. To test this hypothesis, we measured proliferation in the presence of both inhibitors. Indirect MTS assays (upper panels) and direct cell counts (lower panels) showed that rapamycin and MNK inhibitor only partially decreased the rate of PC3 proliferation when administered alone (Figure 4E and F). However, the effect was much more pronounced when the inhibitors were supplied together. Indeed, concomitant inhibition of mTOR and MNKs almost suppressed the growth of PC3 cells in 72 h of treatment (Figure 4E). On the other hand, inhibition of MNK alone strongly affected proliferation of DU145 cells,

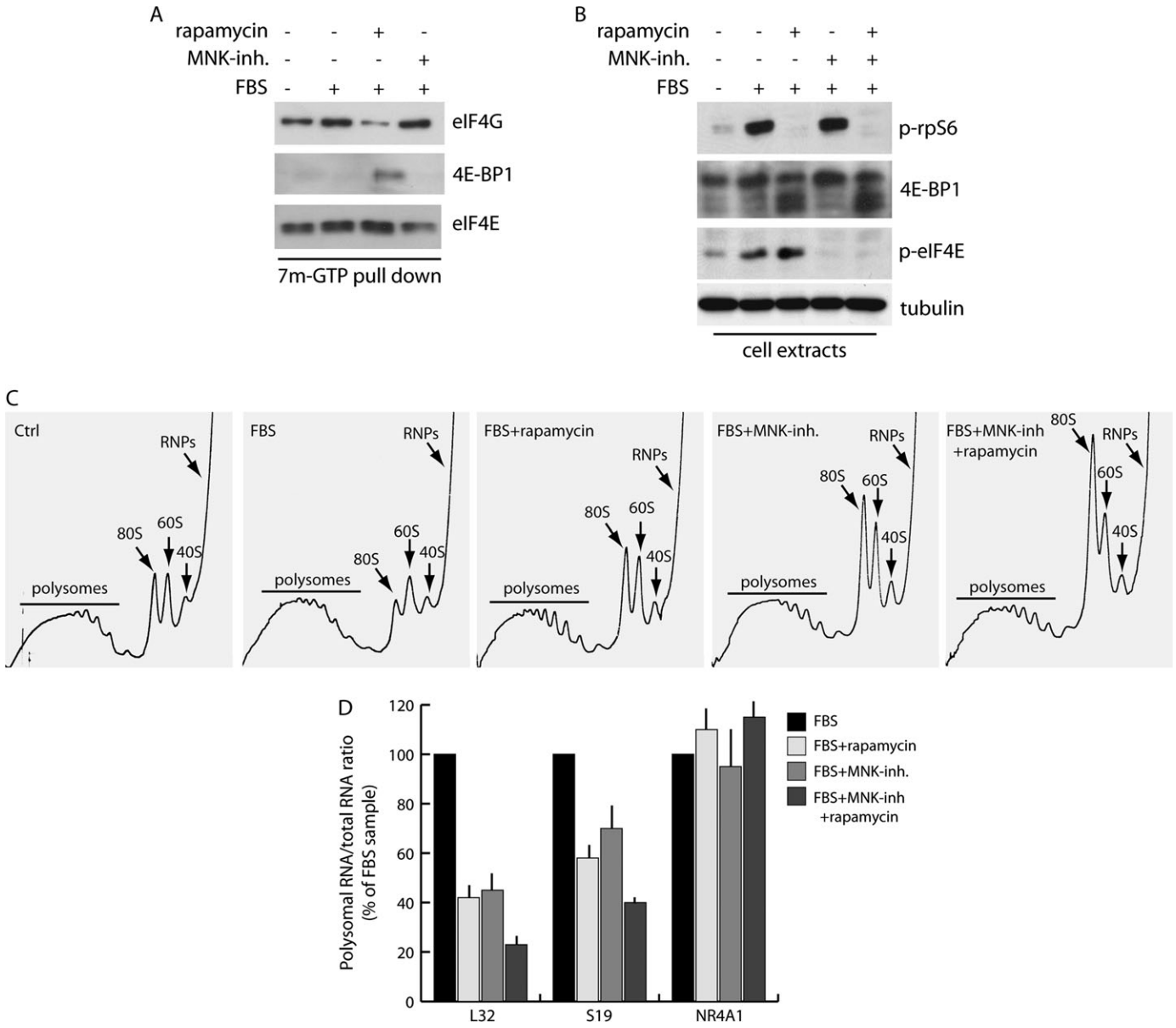

Fig. 3. Effect of rapamycin and MNK inhibitor on eIF4F complex assembly and TOP mRNAs translation. (**A**) 7-methyl-GTP-Sepharose pull-down assay of the effect of 50 nM rapamycin or 10 μM MNK inhibitor on FBS-induced eIF4F complex assembly. The proteins adsorbed to 7-methyl-GTP-Sepharose beads were analyzed in western blot with the antibodies listed to the right of each panel. (**B**) Western blot analysis of the effect of the inhibitors treatment on Akt/mTOR and MAPK pathways. Cell extract loading was normalized with anti-tubulin. (**C**) Absorption profiles (A_{254}) from sucrose gradient fractionations of FBS-stimulated PC3 cell samples pretreated or not with 50 nM rapamycin and/or 10 μM MNK inhibitor. The polysomes, the ribonucleoprotein particles-containing fractions and the three different ribosomal peaks are indicated. (**D**) Analysis by real-time PCR of cotreatment of rapamycin and MNK inhibitor on polysomal recruitment of rpL32, rpS19 and NR4A1 mRNAs. Data are expressed as percentage of the FBS-stimulated sample ± SD from three independent experiments. At least two different housekeeping genes were used for each determination.

and it almost completely suppressed growth when it was combined with rapamycin (Figure 4F). These results confirm that in PCa cells with normal PTEN expression, proliferation is mainly sustained by the MNK/eIF4E pathway.

Next, we tested whether the AKT/mTOR and MAPK/MNK pathways specifically affected cell cycle progression in PCa cells. FACS analysis indicated that rapamycin caused accumulation of PC3 cells in the G_1 phase, whereas MNK inhibitor alone did not exert any effect. However, cotreatment with rapamycin and MNK inhibitor reinforced the block in G_1/S transition exerted by the mTOR inhibitor alone (Figure 5A). This effect could be explained by the increased phosphorylation of eIF4E in PC3 cells treated with rapamycin and the complete reversion of this response in the presence of MNK inhibitor (Figure 4C; compare lanes 5 and 9). In fact, in DU145, where eIF4E is constitutively phosphorylated (Figure 1C), both inhibitors affected the G_1/S transition cells and the MNK inhibitor exerted a slightly stronger effect than rapamycin (Figure 5C). Moreover, concomitant inhibition of the AKT/mTOR and MNK/eIF4E pathways almost completely suppressed cell cycle progression of DU145 cells, causing accumulation of ∼90% of the cells in the G_1 phase and virtually no cells in S phase. Consistently, the effects observed by FACS analysis were mirrored by the expression levels of specific cyclins (Figure 5B and D). Western blot analyses showed that cotreatment with rapamycin and MNK inhibitor strongly reduced the levels of cyclin D1, cyclin A and cyclin B, indicating that PCa cells exit cell cycle progression under these conditions. Our results suggest that, in addition to mTOR

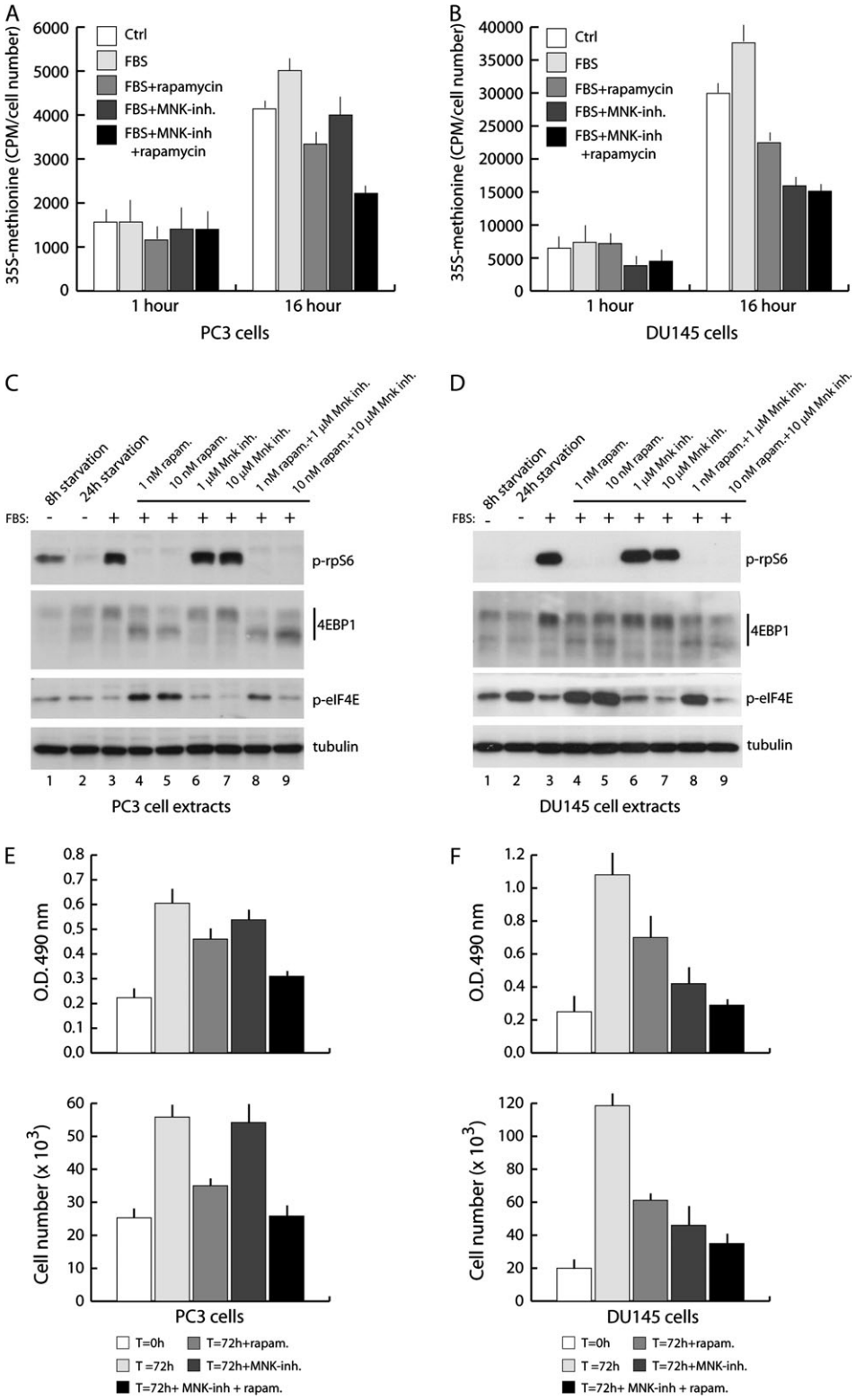

Fig. 4. Concomitant inhibition of mTOR and MNKs limits protein synthesis and cell proliferation. Protein synthesis was measured by ^{35}S-amino acid incorporation in PC3 cells (**A**) and DU145 cells (**B**). Cells were starved for 16 h before treatment with FBS and kinase inhibitors for either 1 or 16 h as indicated in the figure and explained in the text. Rapamycin was used at 10 nM, MNK inhibitor at 10 μM. Results of ^{35}S-amino acid incorporation are illustrated in the left panels and represent the mean ± SE of three experiments. Western blot analyses of extracts from PC3 (**C**) or DU145 cells (**D**) stimulated with FBS and kinase inhibitors for 16 h are illustrated in the right panels. Western blots were performed with antibodies specific for the proteins indicated on the right side of each panel. Rapamycin was used at 1 or 10 nM, MNK inhibitor at 1 or 10 μM, as indicated. Representative results of three experiments are shown. Cell proliferation assays were performed using the MTS assay (upper graph) or by counting the cells (lower graph) in PC3 (**E**) and DU145 (**F**) prostate cancer cells. $T = 0$ h and $T = 72$ h represent the samples grown in 10% FBS at the beginning and at the end of the experiment, respectively. The other samples represent cell growth for 72 h in the presence of FBS and the indicated kinase inhibitors. Results are the mean ± SD of three experiments.

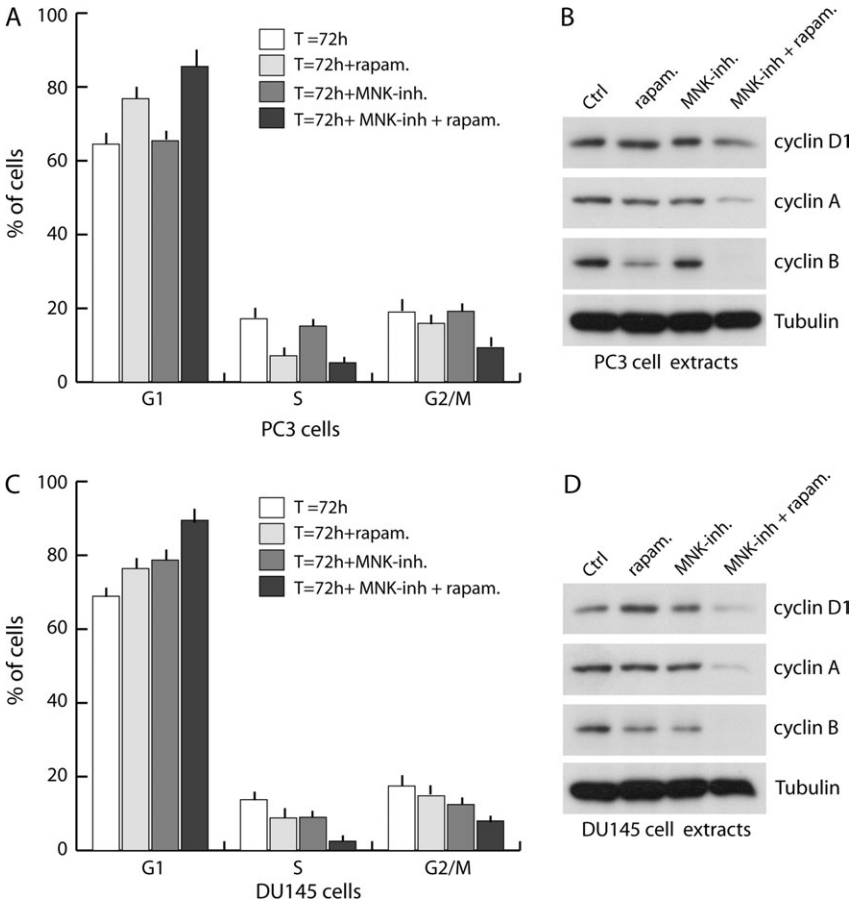

Fig. 5. Cotreatment with rapamycin and MNK inhibitor causes cell cycle exit in PCa cells. PC3 (**A** and **B**) and DU145 (**C** and **D**) prostate cancer cells were treated with the indicated inhibitors for 72 h. Cells were collected and stained with propidium iodide for FACS analysis (A and C) or lysed for western blot analysis (B and D) with antibodies for cyclin D1, cyclin A or cyclin B. Ten micrograms of total extracts were loaded on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Results are the mean ± SD (A and C) or representative (B and D) of three experiments.

inhibitors, pharmacological inhibition of MNK is a valuable tool to regulate PCa cell proliferation and its efficacy might depend on the level of activity of the PI3K/AKT pathway.

The MNK/eIF4E pathway is involved in translation of cell cycle proteins in DU145 cells

mTOR activity regulates translation of specific mRNAs in different cell types (25–27). On the other hand, with the exception of *MCL-1* (18), no information is available on the requirement of eIF4E phosphorylation for translational regulation of specific genes. To determine whether, in addition to TOP mRNAs, eIF4E phosphorylation also affected other mRNAs, we performed a microarray analysis. DU145 cells were chosen because of their higher sensitivity to the MNK inhibitor. Polysomal mRNAs were purified from cells cultured for 16 h in the presence or absence of MNK inhibitor and hybridized to a microarray chip containing 263 genes with known relevance to PCa (21). We observed that treatment with MNK inhibitor did not affect the overall pattern of mRNAs present on the polysomes (Figure 6A). Nevertheless, several mRNAs were selectively downregulated (Figure 6B). Most of these mRNAs encode for proteins involved in cell cycle progression (CDK2, CDK8, CDK9 and KAP1), which might explain the strong effect on cell proliferation observed in DU145 cells by the MNK inhibitor. In addition, RASSF1 is a regulator of cell cycle and apoptosis and its locus is often aberrantly methylated in PCa (28), whereas PCNA is a cofactor of DNA polymerases that recruits crucial players to the replication fork (29). PIAS1 regulates protein sumoylation and affects checkpoints of the exit from G_1 and G_2 phases of the cell cycle (30). HIF1α is a transcription factor that induces angiogenesis in PCa and its expression is regulated by the AKT/mTOR pathway in the PTEN-negative PC3 cells (31). The microarray results were validated by real-time PCR analysis for three of the targets: KAP1, CDK2 and PIAS1. These RNAs were decreased in the polysomal pool after treatment with MNK inhibitor, whereas they were only marginally affected at the total level (Figure 6C). Moreover, inhibition of MNK also caused a detectable decrease in CDK2 protein levels (Figure 6D), confirming the data obtained at the mRNA level. These results indicate that phosphorylation of eIF4E is required for efficient translation of specific mRNAs involved in cell cycle progression in PCa cells.

Discussion

In this study, we have investigated the activation and function of signaling pathways involved in the regulation of mRNA translation in PCa cells (Figure 6E). Our experiments provide four major conclusions: (i) the activity of the mTOR and MNK pathways is under a controlled balance in PCa cells and downregulation of the former is correlated with activation of the latter; (ii) translation of TOP mRNAs encoding for ribosomal proteins depends on activation of both the mTOR and MNK pathways by growth factors; (iii) concomitant inhibition of the mTOR and MNK pathway strongly suppresses protein synthesis, cell cycle progression and proliferation of PCa cells and (iv) inhibition of the MNK pathway leads to translational repression of mRNAs for proteins involved in cell cycle.

The rationale of this study stems from the widely accepted notion that deregulation of the PI3K/AKT/mTOR signaling pathway is one

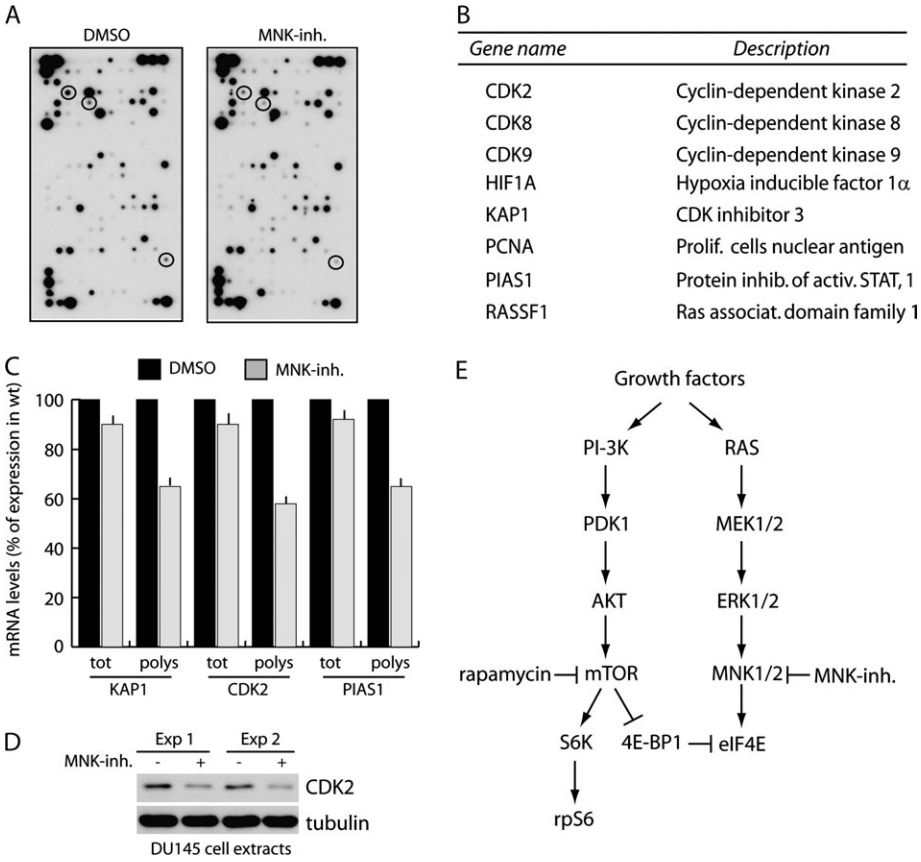

Fig. 6. MNK activity is required for polysomal recruitment of mRNAs encoding proteins involved in cell proliferation. (**A**) Examples of Superarray Prostate Biomarkers chip: circles indicate representative examples of mRNAs downregulated on the polysomes after treatment of DU145 cells with MNK inhibitor for 16 h. (**B**) List of the mRNAs whose polysomal loading was dependent on MNK activity in DU145 cells. Description of the known function of the proteins encoded is listed on the right side of the table. (**C**) Real-time PCR analysis of mRNA levels of three selected target of MNK activity. Total or polysomal RNA from DU145 were analyzed. Data represent the mean ± SD of three experiments. (**D**) Western blot analysis of CDK2 expression in DU145 treated with dimethyl sulfoxide (DMSO) or MNK inhibitor. Extracts from two separate experiments are shown. Tubulin was analyzed as loading control. (**E**) Schematic representation of the signal transduction pathways involved in the regulation of mRNA translation mediated by growth factors. Only some mediators of PI3K/Akt/mTOR and MAPK pathways are indicated. The targets of the inhibitors used in this study are indicated.

of the major causes of increased malignancy in different types of tumors, including PCa (3). A frequent feature in PCa patients is the loss of function of PTEN, which leads to constitutive activation of AKT and mTOR (5). The effects of mTOR were ascribed to its role in cap-dependent translation through the regulation of the eIF4F complex. Since eIF4E is frequently phosphorylated in cancer cells and this event is required for the oncogenic potential of eIF4E in lymphoma cells (18), we asked whether the MNK/eIF4E pathway plays a role in mRNA translation and proliferation in PCa cells. Our results strongly suggest that the MNK/eIF4E pathway is particularly relevant in PCa cells expressing wild-type PTEN, such as DU145. In these cells, AKT activity is low and it does not suffice for full activation of mTOR, as demonstrated by the persistence on unphosphorylated α isoform of 4E-BP1 even after acute stimulation with growth factors (Figure 1C). However, the MNK/eIF4E pathway can be activated also in PTEN-null cells after inhibition of the predominant PI3K/AKT/mTOR pathway, such as after serum depletion or by pharmacological treatments (Figure 1B). These results highlight a controlled balance between the relative activity of the mTOR and MNK pathways in PCa cells.

One possibility is that the AKT/mTOR and the MAPK/MNK pathways regulate translation of the same targets or of targets involved in the same biological processes. The best-characterized targets of translational regulation by mTOR are a class of mRNAs containing short 5′- and 3′-UTRs named TOP mRNAs (22–25). Our experiments showed that the polysomal accumulation of TOP mRNAs in response to growth factors strongly depends on mTOR activity also in PCa cells. Remarkably, we found that specific inhibition of MNK alone strongly suppressed polysomal recruitment of TOP mRNAs. Moreover, concomitant inhibition of mTOR and MNK caused an even stronger inhibition of TOP mRNA translation. This effect was specific because inhibition of eIF4E phosphorylation had no effect on IEG mRNAs translation. To our knowledge, this is the first evidence that phosphorylation of eIF4E is directly linked to the translational activation of TOP mRNAs. A recent report has demonstrated that forced induction of eIF4E in fibroblasts caused translational regulation of genes involved in ribosome biogenesis, including several TOP mRNAs (17). Our results demonstrate that a similar effect can be rapidly achieved by growth factors in PCa cancer cells through modulation of eIF4E phosphorylation rather than by its upregulation. Although rapamycin and MNK inhibitor acted similarly on inhibition of polysomal recruitment of TOP mRNAs, the molecular mechanisms underlying their actions appeared different. In the m^7-GTP pull-down experiments, rapamycin inhibited eIF4F complex assembly and stabilized the 4E-BP1/eIF4E interaction, whereas the MNK inhibitor had no effect. Together with the additive effect of the two inhibitors on TOP mRNAs recruitment and monosome/polysome distribution, our results suggest that mTOR and MNK affect different biochemical steps in translation initiation.

Since the mTOR inhibitor rapamycin and its derivatives CCI-779 and RAD001 exhibited potent activity against various tumor cell types, they have been enrolled in clinical trials for several neoplastic diseases (7). However, prolonged treatment of cancer cells or patients

with mTOR inhibitors causes a positive feedback on PI3K activity that leads to phosphorylation of AKT and eIF4E (12,13). This elicits an anti-apoptotic response that may confer resistance to rapamycin treatment. Herein, we have observed that upregulation of eIF4E phosphorylation occurs also in PCa cells treated with mTOR inhibitors and it was completely reverted by cotreatment with the MNK inhibitor, indicating a role for MNK in this event. A similar inhibition of eIF4E phosphorylation could be achieved by concomitant inhibition of extracellular regulated kinase 1/2 and p38 activity, but not by either kinase alone (data not shown), suggesting that both upstream MAPKs can fuel MNK activity in PCa cells. In line with the additive effects of rapamycin and MNK inhibitor on TOP mRNA translation, we found that overall protein synthesis was also strongly suppressed by concomitant treatment of PCa cells with these inhibitors. Moreover, inhibition of both pathways was required to cause proliferation arrest and exit from the cell cycle, as indicated by the dramatic reduction in the levels of mitotic cyclins. Remarkably, the MNK inhibitor alone had a stronger effect than rapamycin on proliferation in DU145 cells, which express functional PTEN, indicating that this inhibitor might be particularly effective in cancer cells in which AKT is not constitutively active. Since eIF4E was highly phosphorylated even in serum-depleted DU145, our results suggest that the constitutive activation of this pathway in PTEN-expressing PCa cells is an attempt to compensate for the lower levels of activity of the AKT/mTOR pathway. Nevertheless, our observations in PC3 cells strongly suggest that cotreatment with mTOR and MNK inhibitors also limits the growth potential of PTEN-null PCa cells, especially under conditions in which the AKT/mTOR pathway is inhibited. An important advantage of targeting MNK activity in cancer cells is given by the absence of phenotype in mice deleted of the *MNK1* and *MNK2* genes (15), indicating that MNK activity might be more stringently required for cancer cells than normal cells. Thus, the concomitant use of rapamycin and MNK inhibitors might be beneficial to PCa patients in which the PI3K/AKT or MAPK/MNK pathways are deregulated.

Intriguingly, phosphorylation of eIF4E is turned on under conditions that are clearly opposed, such as after growth factors stimulation or after various types of stresses (16,32). Although not required during development (15), phosphorylation of eIF4E on serine 209 correlates with cancer cell proliferation (16). eIF4E is upregulated in many tumors and its elevated expression promoted cell transformation (17,33). Furthermore, a recent report demonstrated that the oncogenic potential of eIF4E depends on phosphorylation of serine 209 by MNK and that a similar efficiency in neoplastic transformation could be obtained by upregulation of a constitutively active form of MNK1 instead of eIF4E (18). These results strongly indicate that phosphorylation of eIF4E plays a crucial role in neoplastic transformation and it is not a side effect caused by altered signaling pathways in the transformed cells. Phosphorylation of eIF4E was hypothesized to affect translational control in lymphoma cells, but only one potential target, the anti-apoptotic protein Mcl-1, was identified in that study (18). In addition to TOP mRNAs, using the highly MNK-sensitive DU145 cell line, we have now identified eight new translationally regulated targets of the MNK/eIF4E pathway. Many of these mRNAs encode for proteins involved in cell cycle transitions or cell proliferation, such as cyclin-dependent kinases or inhibitors. An interesting target of MNK activity is the HIF1α mRNA, which encodes for a transcription factor that modulates angiogenesis and confers adaptation to hypoxic conditions leading to increased resistance to therapies (34). HIF1α expression is regulated by the AKT pathway in PC3 cells (31). However, under hypoxic conditions, when the AKT/mTOR pathway is inhibited, HIFα is one of the few mRNAs that remains associated to actively translating polysomes in PC3 cells (35). Our results suggest that activation of the MNK pathway might sustain HIF1α translation under these conditions and compensate for the inhibition of the mTOR pathway. Indeed, hypoxia induces a cellular stress and eIF4E phosphorylation strongly increases upon various stresses in most cancer cells (16,32).

In conclusion, our results indicate that a fine balance between the activity of the AKT/mTOR and the MAPK/MNK pathway in PCa cells maintains translation of specific mRNAs required for ribosome biogenesis, cell proliferation and stress response and might confer them the ability to overcome negative insults.

Supplementary material

Supplementary Table 1 and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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