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"TBP-1 (TAT BINDING PROTEIN-1) IN THE CONTROL OF CELL PROLIFERATION: FUNCTIONAL RELATIONSHIP WITH THE AKT/PKB SIGNALING PATHWAY"

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TBP-1 (Tat Binding Protein-1) in the control of cell proliferation: functional relationship with the Akt/PKB signaling pathway

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LIST OF PUBLICATIONS RELATED TO THE THESIS

Sepe M, **Festa L**, Tolino F, Bellucci L, Sisto L, Alfano D, Ragno P, Calabr V, de Franciscis V, La Mantia G, Pollice A. A regulatory mechanism involving TBP-1/Tat-Binding Protein 1 and Akt/PKB in the control of cell proliferation. PLOsOne 2011: 6:1-13.

Abstract

TBP-1/Tat-Binding Protein 1 (also named Rpt-5, S6a or PSMC3) is a multifunctional protein, originally identified as a regulator of HIV-1-Tat mediated transcription.
It is an AAA-ATPase component of the 19S regulative subunit of the proteasome and, as other members of this protein family, fulfils different cellular functions including proteolysis and transcriptional regulation. Consistent with the role in protein destruction, TBP-1 has been shown to enhance the ubiquitin ligase function of the VHL (Von-Hippel-Landau) tumour suppressor toward Hif1α and to be involved in the degradation of Fra-1 (Fos related antigen -1), both overexpressed in a variety of human cancers. Moreover, TBP-1 binds to and stabilizes the p14ARF human oncosuppressor, likely avoiding its entrance into the proteasome and increasing its anti-oncogenic functions.
Intriguingly, TBP-1 overexpression can inhibit cell proliferation in a different celllular contexts suggesting that it may function as a negative regulator of cell proliferation. Infact, inhibition of the oncogenic phenotype of erb-B transformed cells was accompanied by an increase of TBP-1 intracellular levels and, accordingly, its overexpression in erb-B transformed cells strongly inhibited tumour formation in athymic mice.
The results presented here demonstrate that stable reduction of TBP-1 intracellular levels increases cell proliferation, renders cells able to grow also in absence of serum, and increases resistance to serum-deprivation induced apoptosis of primary human fibroblasts immortalized by h-TERT expression. Moreover, TBP-1 silencing determines the activation of the Akt/PKB serin-threonin kinase, one of the major transducers of growth signals mediating proliferative and pro-survival effects. TBP-1 itself turned out to be a downstream target of Akt/PKB and MDM2, a known Akt target, plays a major role in this regulation. Altogether, these results suggest the existence of a negative feedback loop involving Akt/PKB that might act as a sensor to modulate TBP-1 levels in proliferating cells.

1. BACKGROUND

1.1 TBP-1, A MULTIFUNCTIONAL PROTEIN COMPONENT OF THE 19S REGULATORY SUBUNIT OF THE PROTEASOME 26S.

TBP-1/Tat Binding Protein-1 (also named Rpt-5, S6a or PSMC3) is a multifunctional protein, originally identified as a regulator of HIV-1-Tat mediated transcription (Nelbock et al. 1990).
The gene coding for TBP-1/PSMC3 mapped to human chromosome 11, in the 11p12-p13 region (Hoyle et al. 1997). It is ubiquitously expressed in a variety of human cell lines and in rodent tissues (Nelbock et al. 1990; Nakamura et al. 1998). Deletions of this region are present in many types of tumors (Bepler et al. 1994; Hoyle et al. 1997).
Mouse embryo knock out for the PSMC3 gene die before implantation and display defective blastocyst development suggesting the importance of the TBP-1 protein in the early phases of the embryogenesis and more in general for cellular survival (Sakao et al. 2000).
TBP-1 is a member of a large highly conserved gene family of six AAA ATPases (ATPAses Associated to a variety of cellular Activities) and contains two conserved domains, one resembling a nucleotide-binding motif (ATP-binding site), the other resembling a motif common to proteins with helicase activity.
TBP-1, as other five members of the family (MSS1/Rpt-1, S4/Rpt-2, TBP-7/Rpt-3, SUG2/Rpt-4, SUG1/Rpt-6), is associated with the 19S regulatory subunit of the 26S proteasome (Schnall R. et al. 1994) (Figure 1).
As component of the 19S base of the proteasome, TBP-1, as well as other 19S proteins, is involved in the recognition of substrates that have to be degraded, their unfolding and subsequent translocation into the 20S proteolytic core.
In fact, it has been demonstated that 19S proteasome subunits (six AAA ATPases) possess chaperone-like activities residing in the ATPase module (Braun et al. 1999).

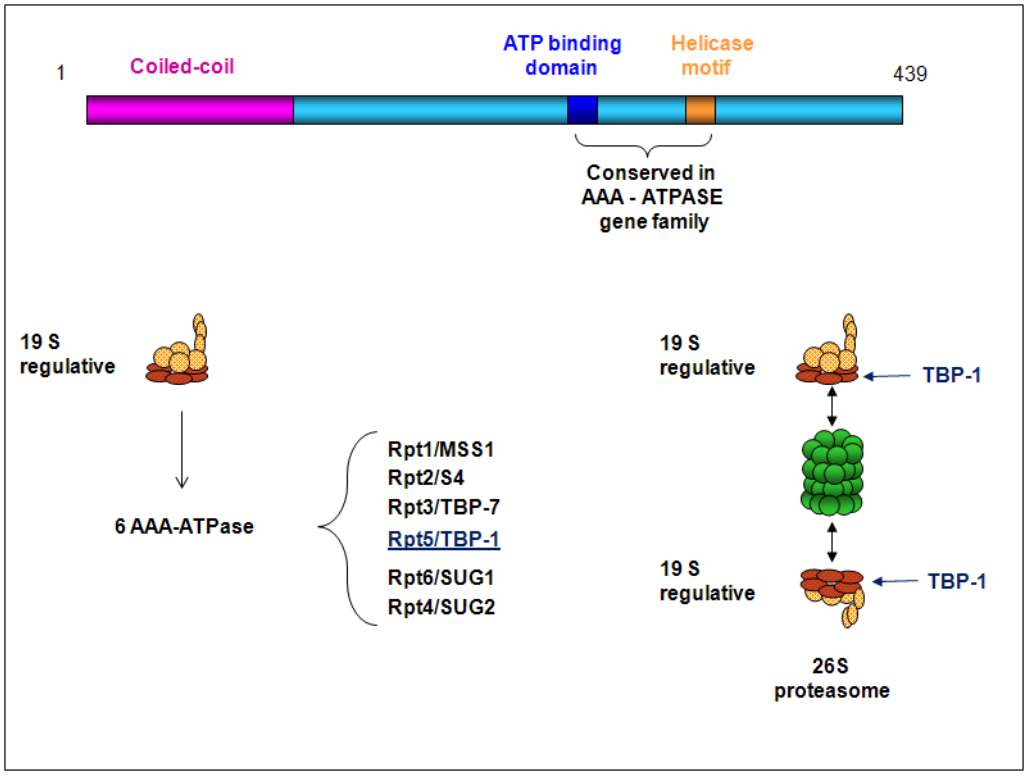

Figure 1 Structure of TBP-1.

TBP-1 belongs to the AAA-ATPases gene family and, as well as other members of this family, is part of the 19S subunit of the proteasome.

1.1.1 The 26S proteasome complex.

The 26S protesome is the chief site of regulatory protein turnover in eukaryotic cells. It is a multisubunit complex responsible for the non-lysosomal protein degradation in both the nucleus and cytosol (Pickart and Cohen 2004).
The 26S proteasome is composed of a 20S catalytic core and a 19S regulatory subunit that sits as a "collar" on one or both ends of the 20S proteasome (Hershko et al. 1998).
The 20S core complex, which is highly conserved from archea to higher eukaryotes, is composed of at least 14 subunits. On the basis of sequence similarities, these subunits can be divided into α and β subfamilies, which assemble into four stacked hepatameric rings in αββα order (Lowe et al. 1995; Groll et al. 1997).
The 19S regulatory particle (RP_s) is composed of at least 18 different subunits, which are assembled into two main subcomplexes: a “base” that contains six ATPase (AAA-ATPases: TBP-1/Rpt-5, MSS1/Rpt-1, S4/Rpt-2, TBP-7/Rpt-3, SUG2/Rpt-4, SUG1/Rpt-6) and two non-ATPase, and a “lid” containing at least 10 non-ATPase subunits that sit on top of the base (Voges et al. 1999).
The regulatory complex carries out a number of biochemical functions including recognition of the polyubiquitinated substrates (Verna et al., 2004), its unfolding and translocation into the cavity of the 20S proteasome (Pickart and Cohen 2004). Ubiquitinated proteins are sent to the 20S catalytic barrel-shaped proteasome core by ubiquitin dependent binding to the 19S particles whose function is to open the barrel and allow protein entry.
On the other hand, non-ubiquitinated proteins can also be degraded by the 20S proteasome, as well as by the 26S proteasome itself (Kalejta et al. 2003; Asher et al. 2005).
In addition to binding to the 19S regulatory complex, the 20S core can be capped by two other activator protein complexes, the 11S (also called PA28 or REG) (Hoffman et al. 1992) and PA200 (Ustrell et al. 2002).
Different from the 26S proteasome, the resulting activated proteasome complexes do not recognize ubiquitinated protein substrates, and their activities and assembly are ATP-independent.
There are three 11S homologues, REG α, β and γ. The REG α and β subunits form a heteroheptameric complex that activate the 20S proteasome and are mainly cytoplasmic. This complex can be induced by γ-interferon treatment suggesting that REGαβ is involved in the MHC class I antigen presentation (Slaughter et al. 1992; Realini et al. 1997). The REGγ subunit forms homoheptameric activator complex and is localized in the nucleus.
The function of this complex is still largely elusive although knock out mice suggest a role in the regulation of mitosis and apoptosis (Barton et al. 2004; Zannini et al. 2008).

The PA200 proteasome activator is a broadly expressed nuclear protein. Although how PA200 normally functions is not fully understood, it has been suggested to be involved in the repair of DNA double-strand breaks (DSBs) (Ustrell et al. 2002). It has been recently isolated a chimerical form of the proteasome composed by both the regulative subunits, whose function is not yet known (Figure 2).

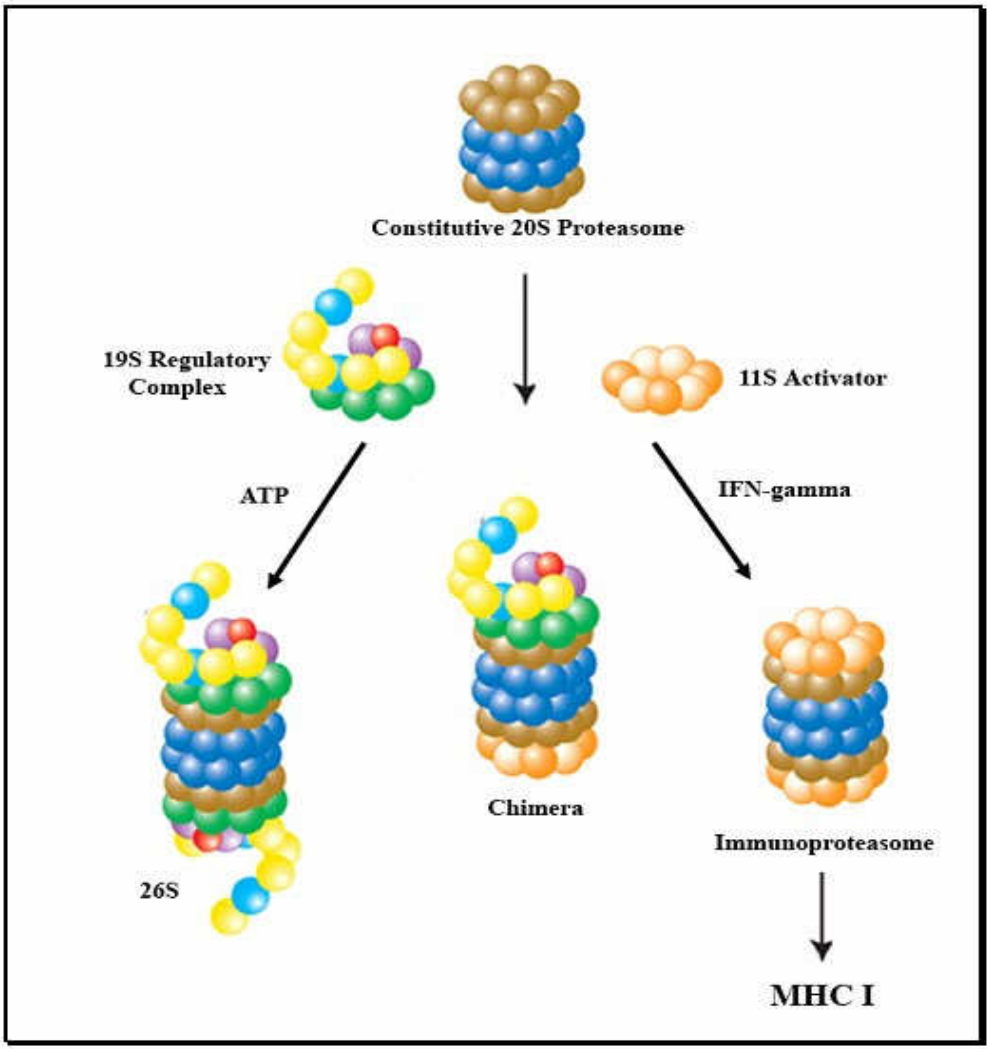

Figure 2 Structure of the proteasome.

The 20S catalytic proteasome can associate with one or two 19S regulative subunit giving rise to the 26 proteasome, mainly involved in the degradation of ubiquitinated proteins or with the 11S/REG subunit.

1.1.2 The 19S regulatory subunits have roles other than proteolysis.

There are different evidences suggesting the involvement of 19S subunit protein members in cellular events that do not require proteolysis.
These proteins play a key role in transcriptional regulation, acting at different levels: co-activators recruitment, mRNA elongation, initiation of transcription.
The first evidences were obtained in yeast, where, by chromatin immunoprecipitation assay, it was demonstrated the association between the Sug1 and Sug2 proteins with the promoters of the Gal1/10 genes upon induction with galactose (Gonzalez et al. 2002).
The involvement of the 19S subunits in mRNA elongation was reported by Ferdous and co-workers (2002). They demonstrated that antibodies against Rpt6, the mammalian analogue of Sug1 in yeast can abolish activated, but not basal, transcription in HeLa nuclear extract. Moreover, experiments using chemical inhibitors of proteasome-mediated proteolysis suggest that 19S requirement in transcription does not reflect a proteolytic event (Ferdous et al. 2002). Furthermore, the proteasomal ATPases Sug1 and Sug2 (respectively Rpt-6 and Rpt-4 in mammalian) is required for efficient transcription of several stress-induced genes in yeast. In particular, Sug1 and Sug2 are essential for the efficient transcription of both heat shock and oxidative stress-responsive genes. When Sug1 or Sug2 activity was abolished shifting temperature-sensitive strains to the restrictive temperature, transcription of these genes was crippled. In contrast, inactivation of temperature-sensitive 20S core proteins had little or no detectable effect on expression of these genes (Sulahian et al. 2006).
ChIP assay revealed the induction-dependent recruitment of the proteasomal ATPases, but not of the 20S core complex, to the promoter of these genes.
Lee and coworkers (Lee et al. 2005) reported that 19S subunits can facilitate SAGA (Spt-Ada-Gcn5-Acetiltransferase) targeting to promoters by transcriptional activators and that there is a functional link between SAGA and the proteasomal ATPases.
Moreover, in S. cerevisiae it was demonstrated the interaction of the 19S subunit of the proteasome with the ubiquitin-like N-terminus of Rad23, a nucleotide excision repair (NER) protein. (Russell et al. 1999).
Rad23 is required for optimal activity of an *in vitro* NER system. Inhibition of proteasomal ATPases diminishes NER activity *in vitro* and increases UV sensitivity *in vivo*.

1.1.3 TBP-1 has a role in transcriptional regulation.

As mentioned before, there are different evidences suggesting the involvement of 19S subunit protein members in cellular events that do not require proteolysis.
In particular, TBP-1 was originally isolated in a screen for proteins interacting with the HIV-1-Tat protein and was reported to inhibit Tat-mediated transactivation of HIV-1 LTR promoter (Nelbock et al. 1990).
More recently, Lassot and co-workers demosrated the involvement of all six AAA-ATPases in the control of HIV-1 transcription by both proteolytic and non-proteolytic mechanism (Lassot et al. 2007).
TBP-1, although unable to bind DNA, is a strong transcriptional activator when brought into proximity of several promoter elements and transcriptional activity depends upon the integrity of the ATPase and helicase motifs (Ohana et al. 1993). Consistent with this assumption, TBP-1 interacts and enhances the HBx-specific transcription of the HBV virus (Barak et al. 2000).
It was demonstrated that TBP-1 is also involved in transcriptional activation of the thyroid hormone (TR)-responsive gene.
In particular, it directly interacts with the TR-binding protein-1 (Trip-1), a putative transcriptional mediator for TR that binds to the AF-2 domain of TR in a ligand dependent manner.
These findings demonstrated that TBP-1 interacts not only with Tat but also with TR and might function as a novel DNA binding domain coactivator specifically involved in the TR-mediated gene activation (Ishizuka et al. 2001).
Recently, it has been reported that TBP-1 could directly interact with androgen receptor (AR) *in vivo* and *in vitro*, and functionally potentiated the AR-mediated transcriptional activation in cooperation with TBPIP (TBP-1-interacting protein) (Satoh et al. 2009).
Truax and co-workers (2010) demonstrated that TBP1 is crucial for regulating cytokine-inducible transcription of Class II Transactivator (CIITA).
They observed that decreased expression of TBP1/S6a significantly reduces the recruitment of transcription factors to the CIITA interferon-γ-inducible promoter (CIITA promoter IV (pIV)) (Truax et al. 2010).

1.1.4 TBP-1 roles in the control of cell proliferation and oncosuppression.

The acquisition of a transformed phenotype is a quite complex stepwise accumulation of genetic changes and requires the acquisition of different hallmark traits.
Arguably one of the most fundamental traits of cancer cells involves their ability to sustain chronic proliferation (Hanahan and Weinberg 2000).

Abundant evidences suggest that TBP-1 may function as a negative regulator of cell proliferation.
The first evidence came from Park and its co-workers (1999).
They demonstrated that the inhibition of the oncogenic phenotype of *erb*-B transformed cells was accompanied by an increase of TBP-1 intracellular levels and, accordingly, its overexpression in *erb*-B transformed cells strongly inhibited tumor formation in athimic mice (Park et al. 1999).
TBP-1 also possesses 46% identity to KAI I, a metastasis tumor suppressor gene for human prostate cancer (Dong et al. 1995) and maps to chromosome 11p12-13, a region frequently deleted in cancers (Hoyle et al. 1997).
Moreover, TBP-1 overexpression in different cellular contexts diminished cell proliferation (Pollice et al. 2004; Park e al. 1999). Furthermore, consistent with its role in protein destruction, TBP-1 has been shown to bind the tumor suppressor pVHL (Von Hippel Lindau), involved in the Von-Hippel-Lindau disease, an autosomal hereditary cancer syndrome characterized by the development of vascular tumors in the retina, in the central nervous system and in renal cell carcinoma. The TBP-1-VHL binding enhances the VHL-E3 ubiquitin ligase activity toward the Hif1-α factor, highly overexpressed in cancer cells that have to sustain growth in low oxygen concentration. In normoxic conditions, TBP-1 binds to VHL and stimulates its ubiquitin ligase activity towards Hif1-α that is rapidly degraded by the proteasome.
The increased expression of TBP-1 resulted in enhanced kinetics degradation of Hif1α whereas its silencing impairs the VHL-mediated proteasome degradation suggesting the essential function of TBP-1 in Hif1α destruction (Corn et al. 2003).
More recently, TBP-1 has been shown to be directly involved in the negative regulation of intracellular levels of Fos related antigen-1(Fra-1). Fra-1 is the member of the Activator Protein-1 (AP-1) transcription factor superfamily that is overexpressed in a variety of human cancers and its overexpression is associated with enhanced cell proliferation, survival, migration and invasion.
It was demonstrated that TBP-1 associates with and downregulates Fra-1 intarcellular levels in cancer cells expressing oncogenic components of the RAS-ERK pathway that regulate Fra-1 synthesis and stability.
In particular, TBP-1 overexpression reduces endogenous Fra-1 protein levels in three cancer cell lines expressing oncogenic ERK pathway components, while its silencing increases Fra-1 endogenous levels (Pakay et al. 2011).
My research group highlighted a very interesting aspect regarding the role of TBP-1 in the control of cell proliferation. In fact it has been demonstrated that TBP-1 interacts with the p14ARF human oncosuppressor increasing its half life. This effect requires the physical interaction between TBP-1and the first 39 amino acids of p14ARF and does not involve the transcriptional properties of TBP-1 (Pollice et al. 2004). Moreover, p14ARF is degraded *in vitro* by the 20S proteasome and TBP-1 protects it from proteasomal degradation. Interestingly, a point mutation in the ATPase domain of TBP-1 that destroys its chaperone-like activity, impairs TBP-1's ability to stabilize ARF.

This observation suggests that this effect requires the chaperone-like activity of TBP-1 and leads to the hypothesis that, upon binding, TBP-1 could cause ARF to fold, rendering it a poor substrate for proteasome destruction (Pollice et al. 2007). On the other hand, the observation that TBP-1 overexpression can inhibit cell proliferation also in ARF minus contexts (Pollice et al. 2004; Park e al. 1999) suggests an ARF-independent role of TBP-1, raising the question of what molecular pathways may be involved.
In this scenario, it is particularly intriguing our observation that the cellular levels of TBP-1 are modulated by the activation of Akt/PKB serine-threonine kinase, one of the major transducers of the growth signals mediating pro-proliferative and pro-survival effects (Sepe et al. 2011).

1.2 THE SERINE-THREONINE KINASE AKT/PKB.

Akt/PKB is a 63 kDa serine/threonine kinase involved in the promotion of cell survival, proliferation and metabolic responses downstream the phosphoinositide-3-kinase (PI3K) signaling pathway. In 1991, the group of Philip Tsichlis identified v-Akt as the gene transduced by rodent retrovirus AKT8 (Bellacosa et al. 1991), and subsequently showed that its cellular homolog, then named c-Akt encoded the serine-threonine protein kinase Akt (Bellacosa et al. 1993). Akt/PKB was also identified by two other research teams that independently cloned the same sequence using approaches designed to identify new protein kinases related to protein kinases A and C (PKA and PKC) (Coffer et al. 1991).
In mammals, three closely related isoforms of Akt are encoded by distinct genetic loci: Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ, located respectively at chromosomes 14q32, 19q13, and 1q44.
The three known mammalian Akt family members are differentially expressed at both the mRNA and protein levels (Bellacosa et al. 1993). Akt orthologs have been cloned from a number of species, including Drosophila melanogaster and Caenorhabditis elegans (Franke et al. 1994).
Each AKT family member contains an amino-terminal pleckstrin homology (PH) domain (Franke et al. 1994), a short α-helical linker, and a carboxyl-terminal kinase domain (Ahmed et al. 1993) (Figure 3).
The pleckstrin homology (PH) domain of AKT kinases has affinity for the 3'-phosphorylated phosphatidylinositol 3,4,5-triphosphate (PIP_3) produced by phosphatidylinositol 3 kinase (PI3K) (Franke et al. 1997).
The kinase domain of Akt, located in the central region of the molecule, with specificity for serine or threonine residues in substrate proteins, shares a high similarity with other AGC kinases such as PKA and PKC (Peterson et al. 1999).

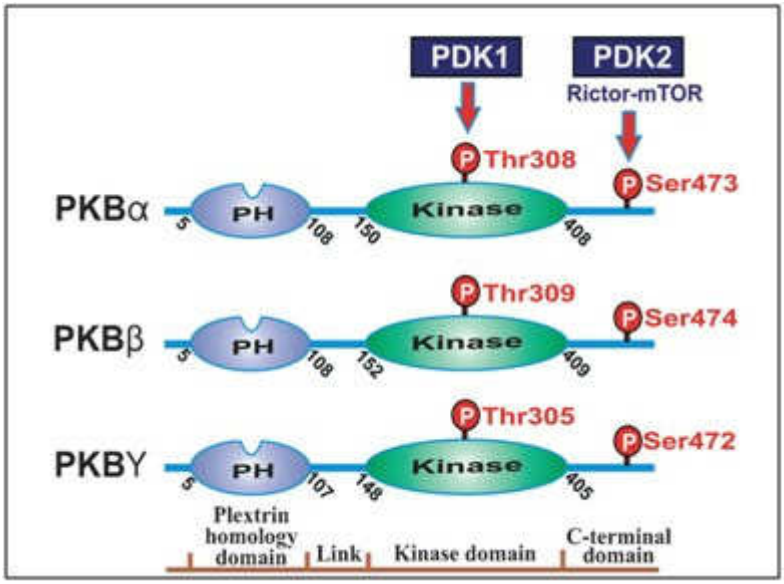

Figure 3 Akt1,2,3/PKBα,β,γ Structure.

PH, Pleckstrin Homology domain; Kinase, catalityc domain

1.2.1 Upstream regulation of Akt kinase activity.

The AKT kinases are major downstream targets of growth factor receptor tyrosine kinases that signal via phosphatidylinositol 3-kinase (PI3K). The Akt/PKB kinase has emerged as a critical mediator of signal transduction pathways downstream of growth factors such as platelet derived growth factor (PDGF) (Franke et al. 1995), insulin and insulin-like growth factor (IGF) (Cross et al. 1995; Kohn et al. 1995), epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) (Burgering and Coffer 1995).
The PI3K-dependent activation of Akt requires two consecutive events: the binding of the Akt PH domain to PIP_3 that induces the relocalization of the cytoplasmic Akt protein to signaling complexes at the plasma membrane (Franke et al. 1997), and the phosphorylation of two key residues, Thr-308 and Ser-473, that allow full activation of the kinase domain (Figure 4) (Alessi et al. 1996).
The Thr-308 residue is located in the activation loop and the kinase responsible for this phosphorylation is the phosphoinositide-dependent kinase (PDK1) (Alessi et al. 1997).
More controversial is the identity of the kinase(s) responsible for Ser-473 phosphorylation (Chen et al. 2001).
At least 10 kinases have been proposed to function as the hydrophobic motif domain protein kinase (HMD-PK) that phosphorylates Akt on Ser-473, including protein kinase Cα (PKCα) (Partovian et al., 2004), double-stranded DNA-dependent protein kinase (DNA-PK), and ataxia telangiectasia mutated (ATM) gene product (Viniegra et al. 2005).
In most cell types and conditions the predominant kinase is the mammalian target of rapamycin complex 2 (mTORC2). It was recently demonstrated that the target of rapamycin (TOR) kinase and its associated protein Rictor are necessary for Ser-473 phosphorylation and a reduction in Rictor or mammalian TOR (mTOR) expression inhibited an Akt/PKB effector (Sarbassov et al. 2005; Hresko et al. 2005). The activation of Akt/PKB is prevented if the cells are pretreated with inhibitors of PI3K (wortmannin or LY 294002) or by overexpression of a dominant negative mutant of PI3K (Burgering and Coffer 1995). The PI3K/Akt axis is inactivated through the action of Phosphatase and tensin homolog (PTEN) protein. PTEN is the main negative regulator of AKT activation and inhibits phosphoinositide 3-kinase (PI3K)-dependent signaling dephosphorylating phosphatidylinositol 3,4,5-triphosphate (PIP_3) (Maehama et al. 1998).
Another mechanism through which the PI3K/Akt axis is inactivated acts through dephosphorylation of the two key residues of Akt kinase.
The PH-domain leucine-rich repeat protein phosphatase (PHLPP) has recently been proposed to dephosphorylate Ser-473 (Gao et al. 2005), while the protein phosphatase 2A (PP2A) is known to dephosphorylate Thr-308 (Millward et al. 1999).

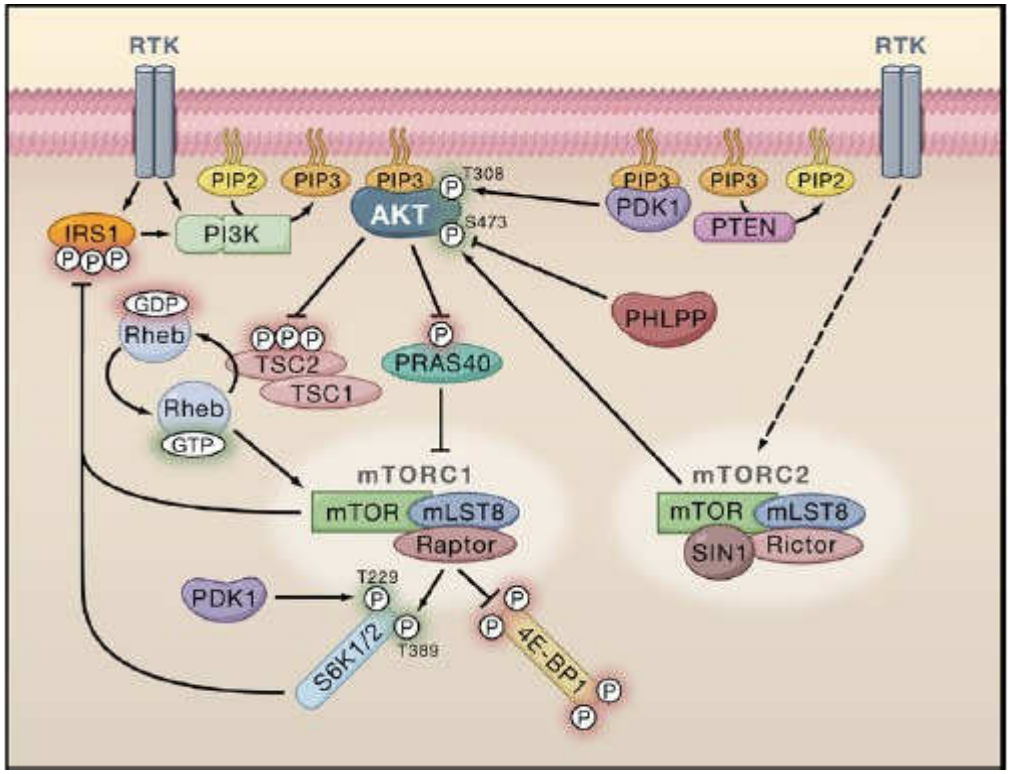

Figure 4 Upstream activation of PI3K-Akt pathway.

This figure shows the upstream activation of Akt by Growth Factors and also depicted is the complex relationship between Akt signaling and mTOR complex 1 and 2. Activated receptor tyrosine kinases (RTKs) activate PI3K. PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP_3), in a reaction that can be reversed by the PIP_3 phosphatase PTEN. AKT and PDK1 bind to PIP_3 at the plasma membrane, and PDK1 phosphorylates the activation loop of AKT at Thr-308.

1.2.2 Akt targets and cellular functions.

The multiplicity of Akt functions might be due to the variety and specificity of its substrates involved in different cellular functions. The minimal substrate consensus sequence for Akt, RXRXXS/T, where X is any amino acid and S/T is the phosphorylation site, is based on the sequence around the phosphorylation site on glycogen synthase kinase 3 beta (GSK3β), the first identified substrate (Datta et al. 1999).
Akt-mediated phosphorylation leads to the activation or inhibition of protein targets contributing to the activation of various interrelated cellular processes (cell survival, cell proliferation, growth (size), glucose uptake, metabolism, and angiogenesis) (Figure 5).

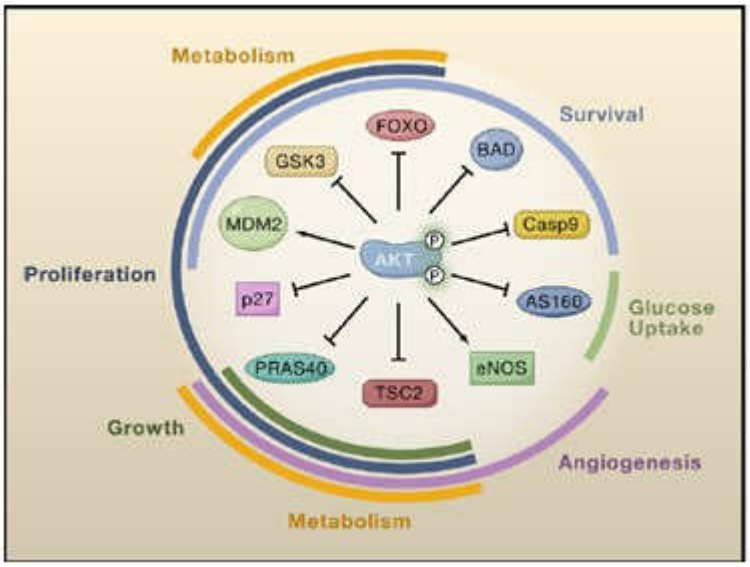

Figure 5 Cellular functions of ten Akt substrates.

Akt-mediated phosphorylation of these proteins leads to their activation (arrows) or inhibition (blocking arrows). Regulation of these substrates by Akt contributes to activation of the various cellular processes shown (i.e., survival, growth, proliferation, glucose uptake, metabolism, and angiogenesis).

- **Akt-dependent control of cell survival**.

Several groups have independently demonstrated a critical role for Akt in promoting cell survival downstream of growth factors, oncogenes, and cell stress, by blocking the function of pro-apoptotic proteins and processes.
Protein database analysis reveals a large number of mammalian proteins that contain Akt consensus phosphorylation sites (RXRXXS/T). Among these proteins there are several components of the apoptotic machinery.
It was demonstrated that Akt negatively regulates the function or expression of several Bcl-2 homology domain 3 (BH3)-only proteins, which exert their pro-apoptotic effects by binding to and inactivating pro-survival Bcl-2 family members. Akt directly phosphorylates and inhibits the BH3-only protein BAD (Datta et al. 1997). Survival factors stimulate Akt-mediated phosphorylation of BAD on Ser-136, creating a binding site for 14-3-3 proteins, triggering release of BAD from its target proteins (Datta et al. 2000).
Akt has also been found to directly phosphorylate Ser-196 on human procaspase-9, and this phosphorylation correlates with a decrease in the protease activity of caspase-9 in vitro (Cardone et al. 1998).
Moreover, Akt promotes cell survival by phosphorylating and restricting the nuclear entry of transcription factors of the Forkhead family (FOXO), preventing transcription of pro-apoptotic genes such as the Fas ligand (Brunet et al. 1999).
It is also possible that Akt exerts some of its cell-survival effects through crosstalk with other pathways. For instance, it has been reported that, under some conditions, the PI3K-Akt pathway activates NFkB survival signaling or inhibits JNK/p38 apoptotic signaling (Kim et al. 2001; Ozes et al. 1999).
Furthermore, AKT counteracts apoptosis promoting phosphorylation of the murine double minute 2 (MDM2) oncoprotein, the E3 ubiquitin ligase that triggers p53 degradation.
In 2001, Mayo and Donner demonstrated that mitogen-induced activation of PI3K and its downstream target, the Akt kinase, results in a phosphorylation of MDM2 on two serine residues (S166 and S186) (Mayo and Donner 2001). These Akt phosphorylation sites in MDM2 are close to the nuclear localization signal (NLS) and nuclear export signal (NES), which are required for MDM2 to shuttle between the nucleus and the cytoplasm.
Phosphorylation promotes translocation of MDM2 from the cytoplasm to the nucleus, where it negatively regulates the p53 function (Mayo and Donner 2001; Zhou et al. 2001a). There are still disagreements on which are the target residues important for MDM2 phosphorylation and translocation from the cytoplasm to the nucleus. In fact another group has shown that Akt phosphorylates MDM2 at Serine 186 and this phosphoryation does not affect the subcellular localization of MDM2 (Ogawara et al. 2002).

- **Akt-dependent control of cell proliferation.**

The proliferative effect of Akt is mediated by multiple mechanisms. Several groups have independently found that Akt phosphorylates the $p27^{Kip1}$ cyclin-dependent kinase inhibitor on threonin 157 (Liang et al. 2002; Shin et al. 2002; Viglietto et al. 2002). This phosphorylation leads to cytosolic sequestration of p27 preventing its localization to the nucleus (Sekimoto et al. 2004).
Akt also inhibits p27 expression through phosphorylation and inhibition of the FOXO transcription factors (Medema et al. 2000).
Consistently, Akt has also been found to phosphorylate the cyclin-dependent kinase inhibitor $p21^{Cip1/WAF1}$**.** In breast cancer cells exhibiting Akt activation due to HER-2/neu overexpression, phosphorylation by Akt prevents nuclear localization of $p21^{Cip1/WAF1}$, separating this cell cycle inhibitor from its cyclin/cyclin-dependent kinase targets (Zhou et al. 2001b).
Furthermore, Akt-dependent phosphorylation of targets such as the glycogen synthase kinase 3 beta (GSK3β), also affected cell proliferation through regulation of the stability of proteins involved in cell-cycle entry.
Akt prevents degradation of the cyclins D1 and E that play a central role in the G1-S phase cell-cycle transit, by phosphorylating and inhibiting GSK3β kinase activity (Diehl et al. 1998).
Akt signaling also controls the translation of proteins important for cell-cycle progression, predominantly through the activation of mammalian target of rapamycin complex 1 (mTORC1).
The predominant cellular effect of mTORC1 inhibitors, such as rapamycin, is to cause a G1 cell-cycle arrest, and Akt mediated cell proliferation and oncogenic transformation has recently been shown to be dependent on mTORC1 activation (Skeen et al. 2006).
In some cell lines, Akt activity has been shown to be elevated during the G2/M phase of the cell cycle, and Akt activation promotes progression through mitosis, even in the presence of DNA damage (Kandel et al. 2002).
One mechanism explaining this observation is that Akt directly phosphorylates the DNA damage checkpoint kinase Chk1 (King et al. 2004).
This phosphorylation blocks checkpoint function by stimulating Chk1 translocation to the cytosol, where it is sequestered from the DNA damage-sensing kinases ATM and ATR (Puc et al. 2005).

- **Akt-dependent control of cell growth.**

One of the best-conserved functions of Akt is its role in promoting cell growth (an increase in cell mass). The predominant mechanism appears to be through activation of Mammalian target of rapamycin complex 1 (mTORC1) which is regulated by both nutrients and growth factors signaling.

MTORC1 is a critical regulator of translation initiation and ribosome biogenesis and plays an evolutionarily conserved role in cell growth control. Akt phosphorylates and inhibits the function of tuberous sclerosis complex (TSC2), a critical negative regulator of mTORC1 signaling (Inoki et al. 2002).

- **Akt-dependent control of angiogenesis.**

Akt plays an important role also in both physiological and pathological angiogenesis through effects in both endothelial cells and cells producing angiogenic signals, such as tumor cells. Akt activates endothelial nitric oxide synthase (eNOS) through direct phosphorylation of Ser-1177 (Dimmeler et al. 1999; Fulton et al. 1999). The release of nitric oxide (NO) produced by activated eNOS can stimulate vasodilation, vascular remodeling, and angiogenesis.
Akt signaling also leads to an increased production of the hypoxia-inducible factor α (HIF1-α and HIF2-α) transcription factors (Toschi et al. 2008). HIF-α activation in endothelial and other cells leads to expression and subsequent secretion of VEGF and other angiogenic factors, thereby stimulating angiogenesis.

- **Akt-dependent control of glucose uptake.**

One of the most important physiological function of Akt is to acutely stimulate glucose uptake in response to insulin. Akt2, the primary isoform in insulin-responsive tissues, has been found to associate with glucose transporter 4 (Glut4)-containing vesicles upon insulin stimulation of adipocytes (Calera et al. 1998), and Akt activation leads to Glut-4 translocation to the plasma membrane (Kohn et al. 1996).

- **Akt-dependent control of cell migration and invasion.**

AKT has also been shown to contribute to tumor invasion and metastasis by promoting the secretion of matrix metalloproteinases (Thant et al. 2000). More recently, Grille and co-workers show that Akt activation determines Epithelial-mesenchymal transition (EMT) characterized by down-regulation of numerous epithelial cell-specific proteins, including E-cadherin and β-catenin, and upregulation of the mesenchymal cell-specific protein vimentin. Interestingly, they showed that EMT was accompanied by increased *in vivo* cell motility on

fibronectin-coated surfaces and increased invasiveness in animals, incresing even more the spectrum of Akt biological activities (Grille et al. 2003).

1.2.3 Activation of Akt plays a central role in tumorigenesis.

Akt signaling regulates cell proliferation and survival, cell growth (size), glucose metabolism, cell motility and angiogenesis. Aberrant regulation of these processes results in cellular perturbations considered hallmarks of cancer, and several studies suggest the frequent alteration of AKT gene in many human cancers (**Table 1**).

In 1992, the group of Cheng reported the first recurrent involvement of an AKT gene in a human cancer, demonstrating amplification and overexpression of AKT2 in ovarian tumors and cell lines (Cheng et al. 1992).

Subsequent studies documented AKT2 amplification and/or mRNA overexpression in 10–20% of human ovarian and pancreatic cancers (Bellacosa et al. 1995; Cheng et al. 1996) and activation of the AKT2 kinase in 40% of ovarian cancers (Yuan et al. 2000).

Unlike AKT2, amplification of AKT1 has not been reported as a frequent event in any tumor type. AKT1 amplification was initially detected in a human gastric carcinoma (Staal et al. 1987), and a more recent investigation of 103 malignant glial tumors revealed a single case (a gliosarcoma) with amplification and overexpression of AKT1 (Knobbe and Reifenberger 2003). Likewise, AKT1 protein levels have been reported to be elevated in some types of cancer, even though the gene is rarely amplified. An immunohistochemical analysis of a series of breast cancers revealed elevated AKT1 staining in 24% of tumors, while strong AKT2 staining was evident in only 4% of tumors (Stal et al. 2003).

AKT1 kinase activity is often increased in prostate and breast cancers and is associated with a poor prognosis (Sun et al. 2001).

AKT3 mRNA is upregulated in estrogen receptor-negative breast tumors, increased AKT3 enzymatic activity was found in estrogen receptor-deficient breast cancer and androgen-insensitive prostate cancer cell lines (Nakatani et al. 1999). More recently, an AKT3 gene amplification in estrogen receptor-positive breast carcinomas has been described (Kirkegaard et al. 2010).

Several oncoproteins and tumor suppressors intersect in the AKT pathway, finely regulating cellular functions at the interface of signal transduction and classical metabolic regulation. This careful balance is altered in human cancer by a variety of activating and inactivating mechanisms that target both AKT and interrelated proteins. Various mechanisms contribute to activation of the AKT pathway in human tumors, including loss or down-regulation of PTEN function and amplifiacation/up-regulation of the PI3KCA gene (which encodes the catalytic subunit of PI3K) (Shayesteh et al. 1999).

Loss of PTEN function leads to an elevated concentration of the PIP_3 substrate, and consequent constitutive activation of downstream components of the PI3K pathway, including the AKT and mTOR kinases. Somatic mutation and biallelic inactivation of PTEN occur frequently in high-grade glioblastoma, melanoma, and cancers of the prostate and endometrium (reviewed in Sansal and Sellers 2004).
Other mechanisms resulting in activation of the AKT pathway in human cancer, include activation of PI3K due to autocrine or paracrine stimulation of receptor tyrosine kinases (Tanno et al. 2001) and overexpression of growth factor receptors such as the epidermal growth factor receptor in glioblastoma multiforme (Schlegel et al. 2002) or HER-2/neu in breast cancer (Bacus et al. 2002).

TABLE 1. Hallmarks of cancer and multiple roles of AKT.

Table 1. Hallmarks of cancer and the multiple roles of AKT

Cancer hallmarks (46)	Akt functions/substrates (in bold)
Acquired growth signal autonomy	Overexpression of AKT may mediate hyper-responsiveness to ambient levels of growth factors
Insensitivity to antigrowth signals	Promotes nuclear entry of **Mdm2**, thus inhibiting p53 pathway
	Induces cytoplasmic localization of **$p21^{WAF1}$** and **$p27^{Kip1}$**, promoting cell growth
	Stabilizes cyclin D1/D3
Inhibition of programmed cell death	Inactivates pro-apoptotic factors **BAD** and **(pro)caspase-9**
	Activates **IKK**, resulting in NF-κB transcription of anti-apoptotic genes
	Inactivates **forkhead transcription factors**, thereby inhibiting expression of Fas ligand
Unlimited replicative potential	Enhances telomerase activity by phosphorylation of **hTERT**
Sustained angiogenesis	Activates **eNOS**, thus promoting angiogenesis
Tissue invasion and metastasis	Contributes to invasiveness by stimulating secretion of MMP

IKK, IκB kinase; hTERT, human telomerase reverse transcriptase; eNOS, endothelial nitric oxide synthase; MMP, matrix metalloproteinase.

Testa J. R. and. Bellacosa A. "AKT plays a central role in tumorigenesis"PNAS 2001;98: 10983–10985.

2. PRELIMINARY DATA AND AIM OF THE STUDY

Several observations point to the involvement of TBP-1 in the control of cell proliferation and oncosuppression. Among these, my research group highlighted a very interesting aspect regarding TBP-1 that turned out to be a regulator of the p14ARF human oncosuppressor (Pollice et al. 2004; Pollice et al. 2007). In particular, this effect requires the physical interaction between ARF and TBP-1 and does not involve its transcriptional properties.
Actually, TBP-1 is able to counteract the degradation of p14ARF by the 20S proteasome *in vitro*, independently from the presence of an assembled 19S particle.
Interestingly, a point mutation in the ATPase domain of TBP-1 that destroys its chaperone-like activity, impairs TBP-1's ability to stabilize ARF, suggesting that this effect requires the chaperone-like activity of TBP-1 (Fabio Tolino, personal communication).
We and other groups (Park et al. 1999; Corn et al. 2003; Pollice et al. 2004 and 2007; Pakay et al. 2011; Sepe et al. 2011) reported that TBP-1 may function as a negative regulator of cell proliferation in various cellular contexts also independently by the presence of ARF.
These observations not only further underlie the antiproliferative role of TBP-1 but suggest that it can exert a more general ARF-independent role in the control of cell proliferation raising the question of what kind of molecular pathways may be involved.
My research project is focused on the antiproliferative role exerted by TBP-1 and the analysis of molecular pathways involved.
To this aim I used, as a model, a primary human fibroblast cell line expressing undetectable levels of p14ARF, the T11hT cell line, immortalized by h-TERT (human telomerase catalytic subunit) expression. TBP-1 intracellular levels were reduced in these cells by the constitutive expression of a specifically designed shRNA.
My results show that cellular levels of TBP-1 are critical in the control of cell proliferation, according to its potential tumor suppressive functions, and suggest the existence of a functional relationship between TBP-1 and the Akt/PKB serine-threonine kinase, one of the major transducers of growth signals, crucial in development of cancer.

3. MATERIALS AND METHODS

Constructs

pSUPERIORshTBP-1 has been obtained from pSUPERIOR.retro.neo (Oligoengine) by cloning into BglII-HindII sites a duplex oligonucleotide obtained by MWG-Biotech that could give rise to a short hairpin RNA specifically designed to silence TBP-1 expression.
Oligoseq:$^{5'}$GATCCCCAACAAGACCCTGCCGTACCTTCAAGAGAGGTACGGCA GGGTCTTGTTTTTTTA$^{3'}$
pCA-Akt plasmid was kindly provided by Prof. G. Condorelli.

The $MDM2_{1\text{-}441}$ and $MDM2_{\Delta 150\text{-}230}$ expression plasmids were previously described (Jin et al., 2003).
Plasmids $MDM2_{S166A}$ and $MDM2_{S166A/S186A}$ mutant were generated by Quick Change Site Direct Mutagenesis Kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instruction.
Briefly, pCMV MDM2 plasmid (with target site for mutation) was denatured.
After the annealing of the mutagenic primers:

S166A (F) $^{5'}$GGAGAGCAATTGCTGAGACAGAAG $^{3'}$,
S166A (R) $^{5'}$CTTCTGTCTCAGCAATTGCTCTCC $^{3'}$
S166A/S186A (F) $^{5'}$ CGCCACAAAGCTGATAGTATTTCCC $^{3'}$
S166A/S186A (R) $^{5'}$GGGAAATACTATCAGCTTTGTGGCG$^{3'}$

a PCR reaction was performed with a 2720 Thermo Cycler Applied Biosystem, resulting in a nicked circular strands.
The addition of DpnI to the reaction causes the digestion of the methylated, non mutated DNA template.
DpnI treated DNA from sample reaction was transferred to 50 µl of super-competent cells. After transformation, the super-competent cells repair the nicks in the mutated plasmid.

Cell culture and transfection.

T11hT (human primary fibroblasts immortalized by costitutive expression of the telomerase catalytic subunit h-TERT) human cell line was kindly provided by dr. Eric Gilson. Both T11hT and TBP-1 silenced clones were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine serum and 1 mg/ml puromycin (to maintain selection for h-TERT). U2OS and MEFs p53-/MDM2- cells were grown in Dulbecco's Modified EagleMedium supplemented with 10% Fetal Bovine serum.
TBP-1 silenced cells derived by retroviral infection of T11hT: briefly, $3x10^6$ HEK 293-LinX packaging cells (kindly provided by Prof. Nicola Zambrano)

were transfected with ARREST-IN (Open Biosystems, Huntsville, AL, USA) with pSUPERIOR.shTBP-1. 24 hrs after transfection, virus containing supernatant was filtrated through 0,45 mm cellulose acetate syringe filter, supplemented with 5 mg/ml polybrene, and used to infect recipient T11hT cells, previously plated at 50% confluence. Twenty-four hours following infection, 1 mg/ml G418 was applied to select stably infected cells. After four weeks, resistant cells were collected, expanded and analyzed. Transfection of the T1 clone was performed by the use of a Microporator MP-100 (Digital Bio Technology) using either $2x10^6$ cells with 2 μg of either pcDNA empty vector or pcDNATBP-1. Transfection of U20S and MEF cells was performed by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Protein extraction, Western Blotting and antibodies.

After transfection, cells were lysed in RIPA Buffer (50 mMTris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, and protease inhibitors). Cell lysates were incubated on ice for 40 minutes, and the extracts were centrifuged at 13,000 rpm for 15 minutes to remove cell debris.
Protein concentrations were determined by the Bio-Rad protein assay.
After the addition of 2x Laemmli buffer (SIGMA), the samples were boiled at 100□C for 5 minutes and resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) probed with the primary antibodies described in the results (2 hours at room temperature or over night at 4□C), followed by incubation with the horseradish peroxidase secondary antibodies (1 hour at room temperature).
Antibodies to Akt (used in 1:1000 dilution), Phospho-Akt Ser473 (used in 1:1000 dilution), Phospho-GSK-3b/pSer21/9 (used in 1:1000 dilution), Caspase-3 (1:1000), PTEN (1:1000) and PARP-1 (1:1000) were purchased from Cell Signalling Technologies, Boston, MA, USA. Antibodies to MDM2 (used in 1:500 dilution) was purchased from Calbiochem, to Rpt-1 (PSMC2) (used in 1:6000 dilution), Rpt6 (PSMC5) (used in 1:6000 dilution) and C8 (used in 1:6000 dilution) were purchased from BioMol. Anti-Xpress antibody (used in 1:1000 dilution) was purchased from Invitrogen. Secondary antibodies for Western Blot analysis (goat anti-rabbit IgG-HRP 1:3000 dilution) were purchased from Santa Cruz Biotechnology, CA, USA. Proteins were visualized with an enhanced chemiluminescence detection system (Amersham ECL TM) and images were taken with ChemiDoc XRS System (Bio-Rad Laboratories) and analysed with the QuantityONE software.

Immunoprecipitation.

For immunoprecipitation in U2OS cells, $1x10^6$ cells were seeded in 100 mm dishes and transfected with the plasmids indicated in the figure legend. Cells were harvested 24 hours post-transfection, lysed in IBP buffer (150 mM NaCl, 50 mM Tris-HCl, 0.5% Nonidet P-40, , 5mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors). 800 μg of whole cell extract were incubated overnight at 4^0C with anti-TBP1 (BioMol) or anti-MDM2 C-18 (Santa Cruz). Controls of immunoprecipitations were perceived with mouse anti-GFP (Roche) or rabbit anti-Flag (Sigma). Immunocomplexes were collected by incubation with 30 ml of protein A-agarose (Roche Applied Science) at 4^oC for 4 hrs. The beads were collected by centrifugation, and the immunoprecipitates were washed three times with IBP buffer (4 □C), solubilized in Laemmli buffer (Sigma) and loaded on a SDS-8% polyacrylamide gel.

siRNA of TBP-1.

A duplex siRNA oligomer designed to target human TBP-1 was obtained by MWG Biotech according to Corn et al. (2003).
The siRNA sequence for TBP-1 used is :
5'-AACAAGACCCUGCCGUACCUU-3', corresponding to position 204 in the PSMC3 mRNA. As negative control a siRNA targeting a sequence firefly luciferase mRNA was used. U20S and T11HT cells were transfected with Hyperfect (Quiagen) according to the manufacturer's instructions applying siRNA duplex at final concentrations of 10 μM. Western blots were performed with anti-TBP-1 and anti-pAkt antibodies.

siRNA of MDM2.

A duplex siRNA oligomer designed to target human MDM2 was obtained by MWG Biotech according to Jin et al. (2003).
The siRNA sequence for MDM2 used is:
5'- AAGCCAUUGCUUUUGAAGUUA-3'.
U20S cells were transfected with Hyperfect (Quiagen) according to the manufacturer's instructions applying siRNA duplex at final concentrations of 10 nM. Western blots were performed with anti-MDM2, anti-TBP-1 and anti-pAKT Ser473 (Cell Signalling 9271).

Treatment with proteasome inhibitor.

2,5x10^5 U2OS cells were seeded into 35-mm plates and transfected with pcDNA TBP-1 either alone or in combination with incresing amount of pcMVMDM2 expression plasmids. The following day, each plate was treated for 5 hours with 10 μM MG132 (Sigma) and with 0.1% dimethyl sulfoxide (Sigma) as control. Cell lysates were collected and probed with anti-TBP-1, anti-MDM2 and anti-actin antibodies.

Treatment of the cells with PI3K inhibitors.

2,5x 10^5 T11HT and U20S cells were plated in 35 mm dishes.
24 hours after plating, cells were treated with Wortmannin (Calbiochem) at the concentration of 200 nM for 1, 2 and 4 hours. Total cell extracts were prepared as described above and samples were analyzed by Western blot. LY294002 at the concentration of 50mM for 1, 2 and 4 hours.

Treatment of the cells with insulin.

U2OS and T11HT cells were plated in 35 mm dishes. 24 hours after plating, cells were starved for four hours in serum free medium and then stimulated with insulin at the concentration of 10 ng/ml for 5, 10, 20, 30 and 40 minutes. Cells were harvested and analyzed by Western Blot with antibodies indicated in the Results.

Cell growth analysis.

For cell growth analysis, T11hT parental cell line, T1 and T10C clones, or control pool and sh-TBP-1 pool, were plated in 100 mm dishes in presence of 10% FBS at the cell density of 1x10^5 cells/plate. Cells were cultured for 24, 48 and 72 hrs, collected, and counted in a Burker chamber.
For growth in the absence of serum, after 6 hrs from plating, medium was removed and replaced with medium without serum.
As above, cells have been grown for 24, 48 and 72 hrs, collected and counted. Each point is the result of triplicate samples.

MTS assay.

Cell viability was evaluated using the MTS [3-(4,5-dimethylthiazhol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium,inner salt] (Cell Titer 96AQueous assay G358purchased from Promega) colorimetric assay. Briefly, T11hTparental cell line or cells derived from the T1 clone were plated atdifferent cell densities as indicated in 96 well plates ($2.5x10^3$/well,10^3/well, $3x10^2$ /well) either in DMEM or in DMEM+10%FBS. After six hrs from plating, 1:5 MTS solution was added to each well and the cells were incubated for 30' at 37^0C. Plates were read on a Microplate Reader (BIO-TEK Instruments, Model Elx800) at 492 nm. Survival was expressed as the percentage of viable cells in treated samples relative to non-treated control cells. All the experiments were repeated in quintuplicate.

Flow cytometry.

For flow cytometry analysis, T11hT and T1 cells were counted and seeded at 210^5cells/35 mm plate. At 24 hours after plating the medium was replaced and the cells treated with DMEM containing 10% Fetal bovin serum or DMEM without serum. At the indicated time points, cells were collected, centrifuged, washed twice with PBS 1x and then fixed with ice-cold 70% ethanol. Fixed cells were incubated with staining buffer solution (50 µg/ml PI and 50 µg/ml RNase A in PBS pH 7.4) for 20' at room temperature in a dark box. Stained cells were analysed in a fluorescence-activated cytometer (DakoCytomation). Data on DNA cell-content were acquired on 20,000 events at a rate of 150+/-50 events/second and the percentages of cells in the SubG1, G0–G1, S and G2–M phases were quantified with Summit v4.1 software.

4. RESULTS AND DISCUSSION

4.1 TBP-1 stable silencing determines an increase in the growth properties.

As a first attemp to analyze the role of TBP-1 in the control of cell proliferation we studied, in collaboration with Maria Sepe, the effects of its long term silencing in an human primary fibroblast cell line (T11hT) immortalized by the stable expression of the catalytic subunit of human telomerase.
T11hT cells were either transfected with the pSUPERIOR.shTBP-1.neo or pSUPERIOR.retro.neo empty vector (see Materials and Methods).
Twenty-four hours following transfection, 1 mg/ml G418 was applied to select cells. After four weeks of selection, resistant cells were collected and expanded; the extent of TBP-1 silencing was then evaluated in cell pools.
As it is shown in Figure 6A, TBP-1 is efficiently silenced in the T11hT cell pool containing the pSUPERIORshTBP-1 vector.
The growth rate of the T11hT cell pool was then compared to that of the control pool and to that of the T11hT parental cells.
To this purpose T11hT cells, control and *sh*-TBP-1 pools were plated in the presence of serum for 24, 48 and 72 hrs.
In parallel, after 6 hrs from plating, the medium was removed, replaced with medium without serum and cultured for 24, 48 and 72 hrs.
Figure 6B shows that TBP-1 silenced cells proliferate at higher rate respect to the parental T11hT and respect to control cells (Figure 6B).
Importantly, serum deprivation doesn't appreciably alter the growth rate of the TBP-1 silenced cells that continue to proliferate, although at a less extent, while control cells tend to stop proliferating (Figure 6C).
We also derived single clones resistant to neomycin selection. Six stable clones were analyzed and in all of them TBP-1 is silenced, with an extent of silencing ranging from 80% to 48% (Figure 7A). In order to exclude that reduced expression of TBP-1 may have altered the proteasome composition, as it was described in *Drosophila melanogaster* (Wojcik and De Martino 2002), intracellular protein levels of proteasome subunits other than TBP-1 was analysed by Western Blot in three of the silenced clones (T1, T10E and T10C). Figure 7B shows that the levels of expression of three different proteasome subunits (Rpt-6, Rpt-1 of the 19S subunit and C8 of the 20S subunit) do not change significantly as compared to that of the parental T11hT.

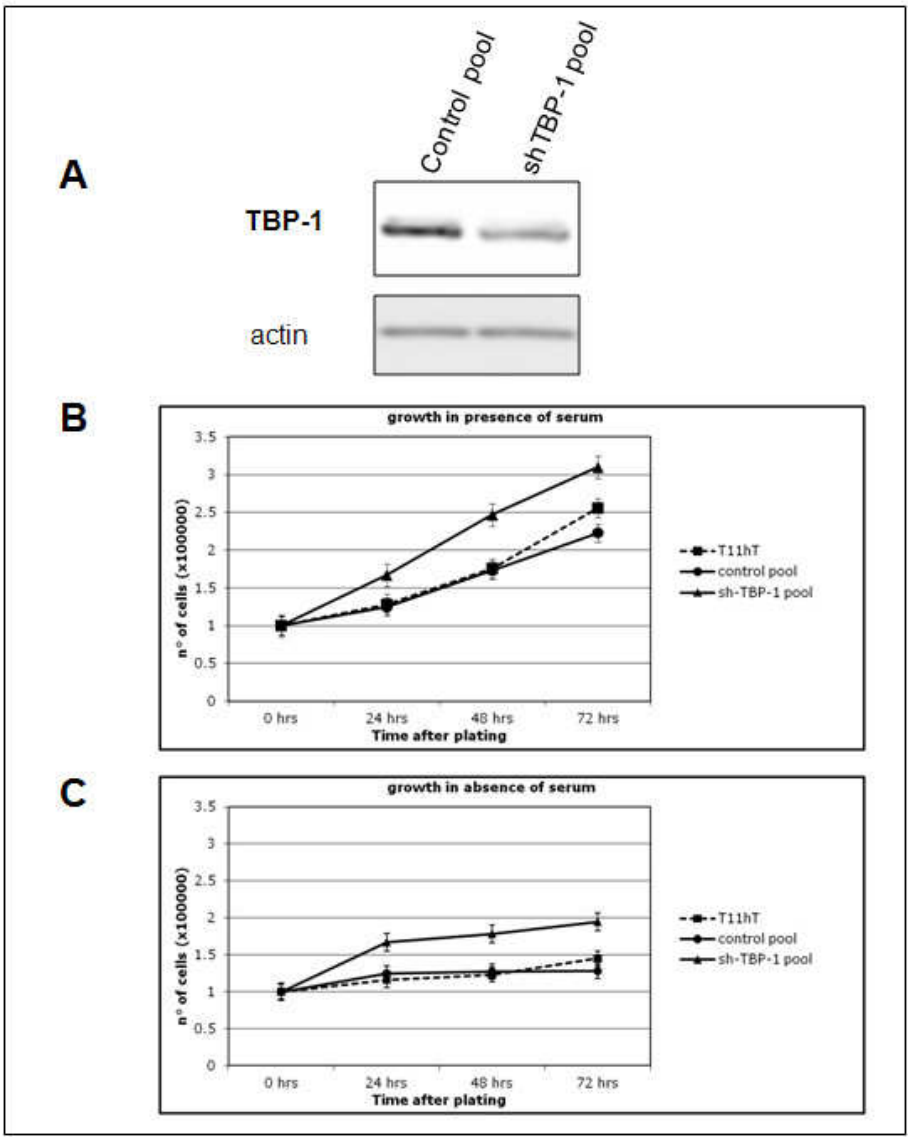

Figure 6 TBP-1 stable silencing determines an increase in the growth properties.

(**A**) T11hT cells stably transfected with pSUPERIOR.shTBP-1 or pSUPERIOR.retro.neo vector were cultured in DMEM +10%FBS for 24 hrs. Levels of TBP-1 expression were evaluated with anti-TBP-1 antibody on whole protein lysates. (**B,C**) wt T11hT cells, cells from control or from the *sh*-TBP-1 cell pool were cultured in DMEM either in the presence (**B**) or in absence (**C**) of serum. Cells were collected at the time points indicated and counted in a Burker chamber. The values are the mean $\pm$ SE of three experiments performed in triplicate.

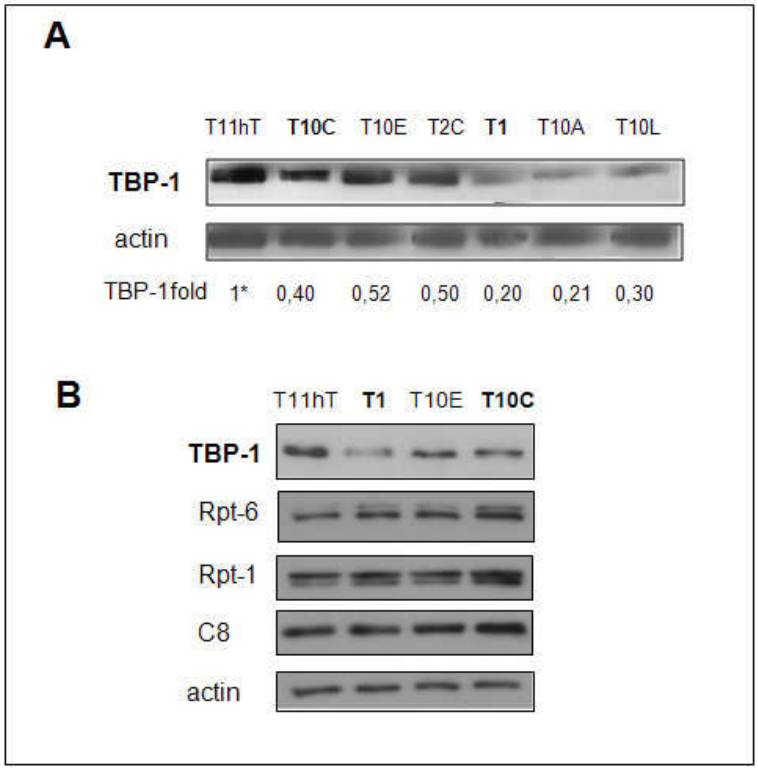

Figure 7 Characterization of TBP-1 silenced clones.

A, B: Cells stably transfected with TBP-1 *sh*-RNA vector or control cells (wt T11hT) were cultured in DMEM +10%FBS for 24 hrs. Levels of TBP-1 expression were evaluated by Western Blot with anti-TBP-1 on whole protein lysates. **B**: As control, protein levels of other proteasome components (two 19S-ATPases, Rpt-1 and Rpt-6, and a 20S component, C8) was evaluated in the clones T1, T10C and T10E. Bands intensity was evaluated by ImageQuant analysis on at least two different expositions to assure the linearity of each acquisition, each normalised for the respective actin values. Asterisk, fold value is expressed relative to the reference point (i.e. TBP-1 levels in T11hT cells), arbitrarily set to 1. Representative of at least four independent experiment.

The growth profile of the T1 and T10C clones, with different TBP-1 residual expression levels, was then analyzed and compared to that of the T11hT parental cells. As shown in Figure 8, TBP-1 silenced clones proliferate at higher rate respect to the T11hT cells. In particular, the T1 clone, expressing very low TBP-1 levels, grows at a rate that is roughly twice that of the parental cell line (Figure 8A). Moreover, TBP-1 silenced cells display the same growth profile, both in the presence (A) and in the absence of serum (Figure 8B).

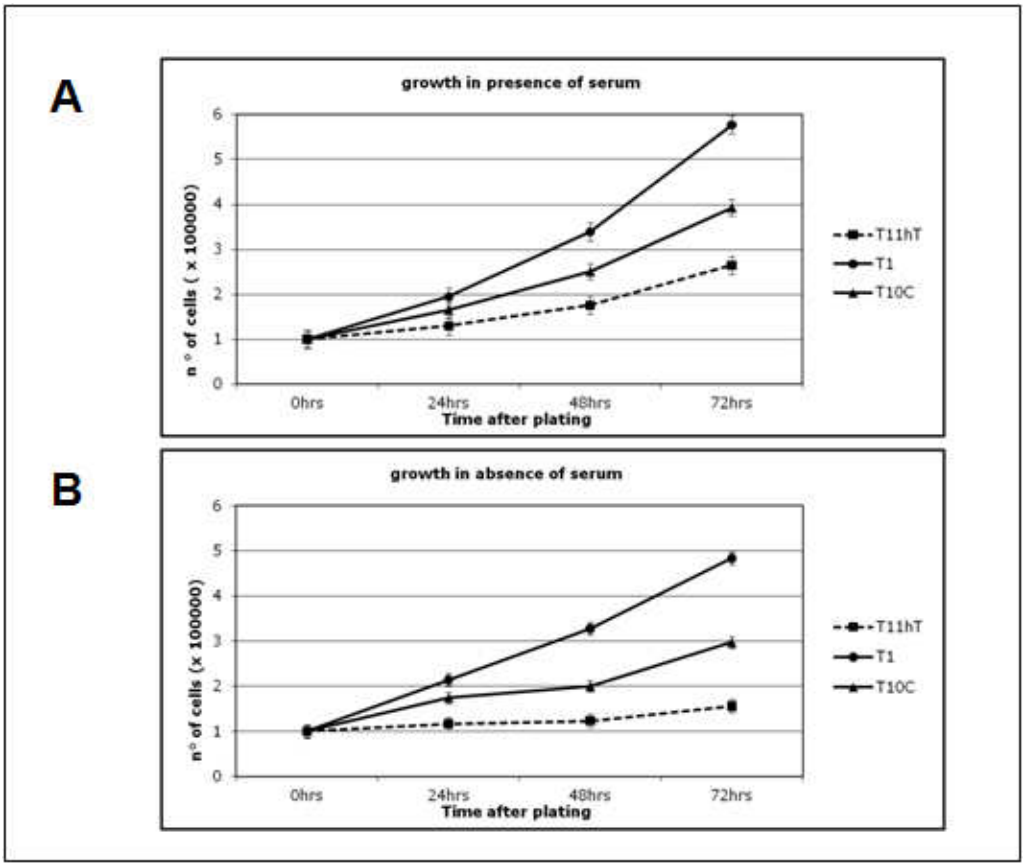

Figure 8 TBP-1 knockdown determines an increase in the growth properties.

A, B: Cells from the T1, T10C and control cells (wt T11hT) were cultured in DMEM either in the presence (**A**) or in absence (**B**) of serum. Cells were collected at the time points indicated and counted in a Burker chamber. The values are the mean $\pm$ SE of three experiments performed in triplicate.

4.2 TBP-1 silencing determines resistance to serum deprivation induced apoptosis.

Normal cells require mitogenic growth signals before they can move from a quiescent state into an active proliferative state.
These signals are transmitted into the cell by transmembrane receptors that bind distintive classes of signaling molecules: diffusible growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction molecules.
Normal cell can not proliferate in the absence of such stimulatory signals.
The ability to sustain chronic proliferation even in the absence of growth factors is one of the fundamental features of cancer cells that generate many of their own growth signals, thereby reducing their dependence on stimulation from their normal tissue microenvironment (Hanahan and Weinberg 2000).
For this reason, the capacity of the *sh*-TBP-1 cell pool and of TBP-1 silenced clones to actively proliferate even in the absence of serum, prompted us to measure the cell viability of the T1 clone by a MTS assay (see Material and Methods).
T11hT parental cell line or cells derived from the T1 clone were plated at different cell densities, (2.5 x 10^3/well, 10^3/well, 3 x 10^2 /well) as indicated in Figure 9A and B, either in presence or absence of serum.
After six hrs from plating, the assay was performed and survival was expressed as the percentage of viable cells relative to non-treated control cells.
The Figure shows that the cell viability of the T1 clone is almost unaffected after 6 hrs of serum withdrawal at the higher cell density, being reduced only up to 60% at the lowest cell density (Fig. 9B).
T11hT cell viability was reduced to 10% at the lowest cell density (Figure 9A).
Serum withdrawal represents a potent trigger to induce caspase-dependent apoptosis in several cellular system including mouse fibroblast (Simm et al. 1997; Sch□fer et al. 2002).
For this reason T11hT parental cells and T1 clone cells were grown for 24 hours in presence or in absence of serum and the protein lysates were analyzed by Western Blot.
According to the previous observation, the T1 clone is more resistant to serum-deprivation induced apoptosis respect to the parental cells, as assessed by the very faint amounts of both Caspase-3 and PARP-1 cleavage (Figure 10A).
Subsequently, the cell growth profile was analyzed by flow cytometry analysis.
To this purpose, T11hT and T1 cells were plated in the presence or absence of serum for 24 hours. In Figure 10B, the histogram from one representative experiment is shown.
On the right are shown the ratios between cells in sub-G1 or S phase in the absence and in the presence of serum, that highlight the different behaviour of T1 cells respect to control cells, when grown in absence or presence of serum.
This analysis indicates that serum starvation only slightly affects the percentage of T1 cells in S phase (8% reduction), while more drastically

reduces that of the parental cell line (55% reduction) (Figure 10B). Furthermore, the increase of the sub-G1 population (of around 1.8 fold for the T1 clone and 3,14 fold for the parental cell line) is consistent with PARP-1 and Capase-3 cleavage data (Figure 10A).

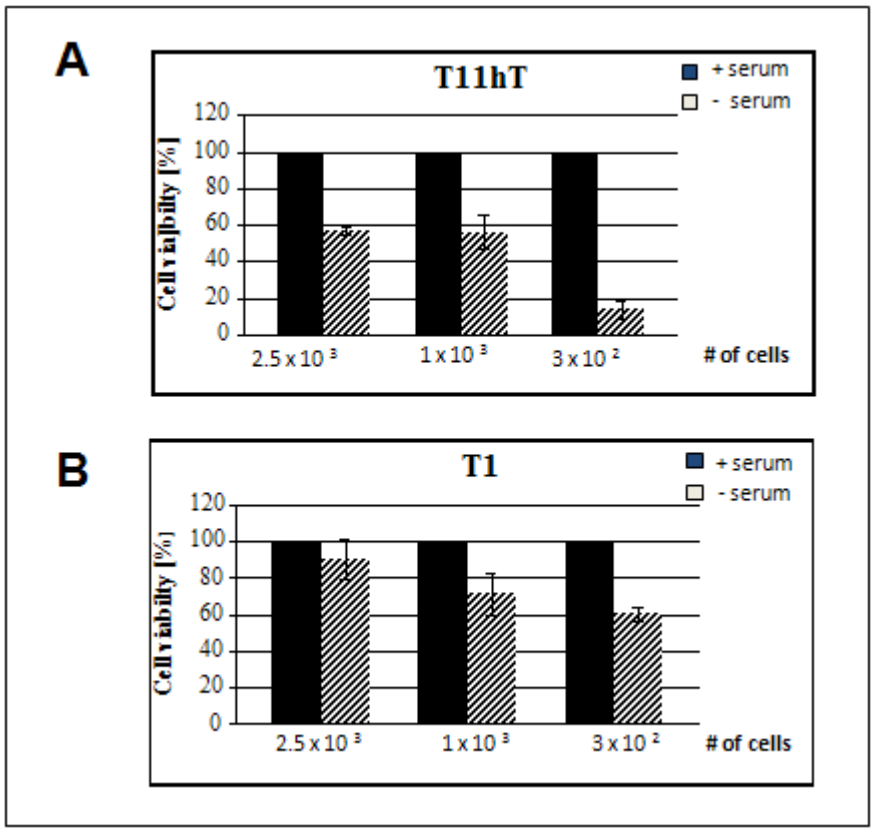

Figure 9 TBP-1 silencing reduces sensitivity to serum starvation.

A,B: Cells from the T1 clone **(B)** and control cells **(A)** (wt T11hT) were plated at different cell densities as indicated, either in the presence or absence of serum. After six hrs from plating, cell viability was measured by MTS assay. In the histograms, cell viability is expressed as relative to controls, arbitrarily set to 100 (%). The values are the mean $\pm$ SE of three experiments performed in quintuplicate.

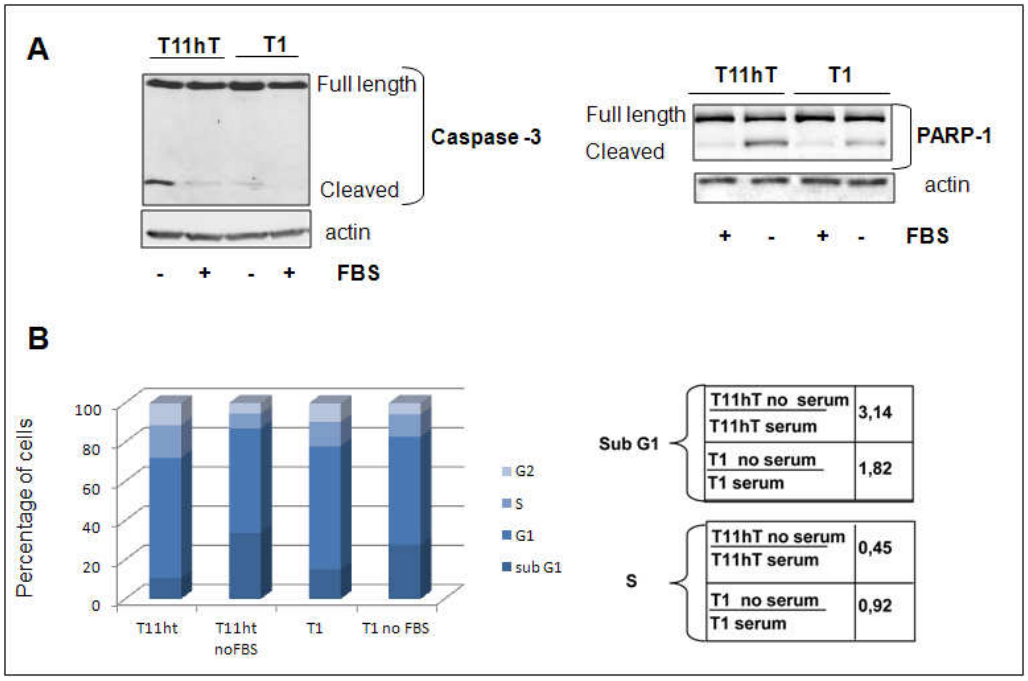

Figure 10 TBP-1 silencing determines resistance to serum deprivation induced apoptosis.

A: 1.8 x 10^5 cells/35 mm plates from the T1 clone and control cells (wt T11hT) were plated in presence of 10% FBS. 24 hours after plating were cultured in presence (+FBS) or absence(-FBS) of serum. Apoptosis was checked by detection of Caspase-3 and PARP-1 cleavage (**A**) in Western Blot.

B: T11hT and T1 cells were counted and seeded at 2 x 10^5 cells/35 mm plate in presence of 10% FBS. Cells were cultured in presence or absence of serum for 24 hours. Subsequently were collected and treated for analysis of cellular DNA content by flow cytometry. Percentages of cells in the SubG1, G0–G1, S and G2–M phases were quantified with Summit 4.1 software. The numerical ratios reported on the right is the ratio between cells in sub G1-S phase in the absence of serum and cells in the presence of serum.

4.3 TBP-1 intracellular levels affect the activation status of Akt/PKB kinase.

The observation that TBP-1 stable depletion increases cells proliferation and resistence to apoptosis raises the question of which are the potential intracellular pathways underlying the observed phenomenons. To investigate this point we wondered if the extent of TBP-1 expression may regulate the activation status and/or the levels of expression of proteins involved in the control of cell proliferation.

In particular, as a first attempt, was assessed the activation status of one of the major transducers of growth signals, critical for cell proliferation and apoptosis.

To this aim, the levels of activation of the Akt (phospho-Akt)/PKB kinase was evaluated both in cell pool and in two of the silenced clones (T1 and T10C) under actively growth conditions (i.e. in the presence of serum).

As shown in Figure 11A, pAkt levels are inversely correlated to the extent of TBP-1 silencing, being lower in the parental T11hT and in control pool and higher in the TBP-1 silenced cells (T1, T10C and shTBP-1 pool).

Consistently, there is an increase in the extent of phosphorylation of GSK3β, a well known Akt/PKB direct target.

Interestingly, TBP-1 reduction appears to specifically affect Akt activation but not that of other important transducers of growth signals, like $ERK_{1/2}$.

Importantly, we could reproduce the same effect after transient reduction of TBP-1 levels by siRNA duplex designed to specifically silence TBP-1 expression (see Materials and Methods).

Figure 11B and C shows that silencing of TBP-1 is accompanied by a concomitant increase in the steady-state level of pAkt in both T11hT and U2OS cells, indicating that this effect was not cell-specific.

This data suggest the existence of a causal relationship between TBP-1 levels and Akt activation.

Since TBP-1 silencing determines an increase in the activation status of the Akt/PKB kinase, we asked whether TBP-1 depletion could be causative of this effect.

To this aim we set up a “rescue” experiment in the T1 clone cells in wich we re-established high TBP-1 levels by transient overexpression. T1 cells were transfected with a fixed amount of either pcDNA empty vector or pcDNATBP-1 and cultured in presence or in absence of serum for 24, 48 and 72 hrs.

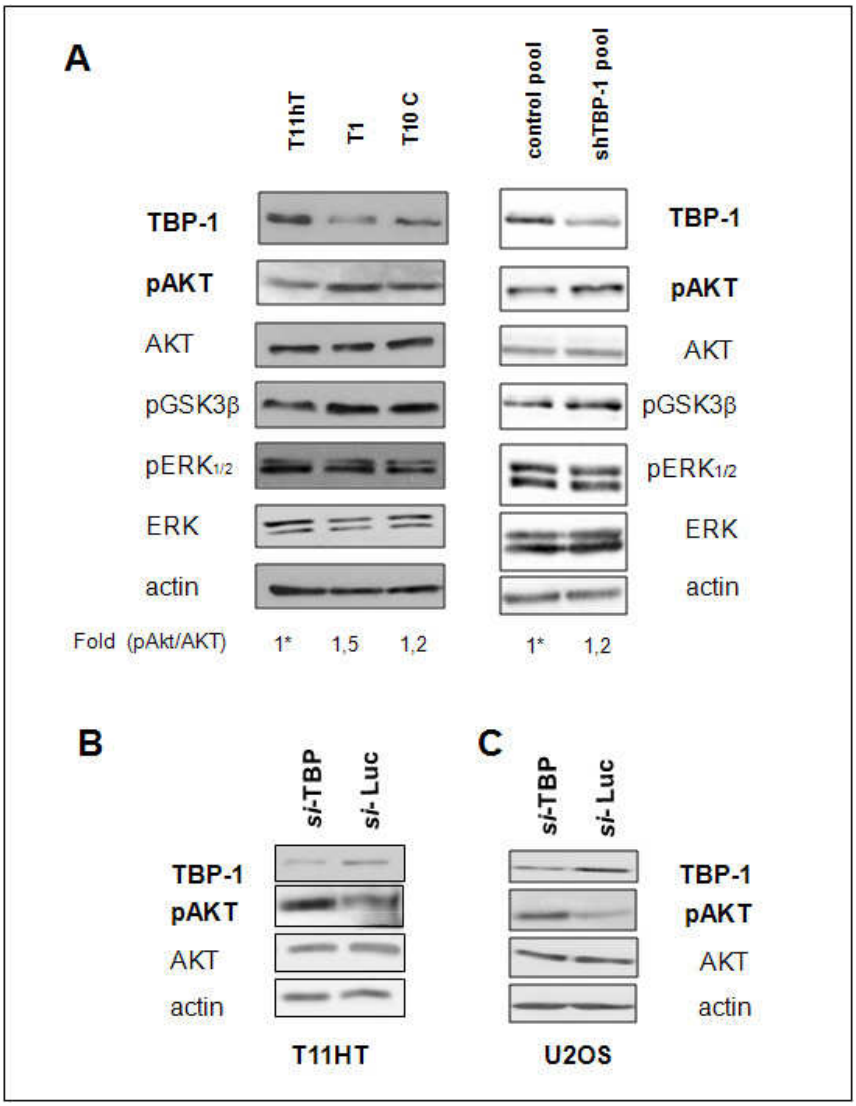

Figure 11 TBP-1 knockdown determines activation of the Akt/PKB kinase.

A: Cells from the T1, T10C, shTBP-1 pool and control cells (wt T11hT and control pool) were cultured in DMEM + 10%FBS for 24 hrs. Activation of Akt/PKB was revealed with anti-Phospho-Akt (Ser-473) antibody. As control, extracts were also probed with anti-Akt, anti-Phospho-GSK-3β (Ser-219), anti-pERK$_{1/2}$, anti-ERK$_{1/2}$ and anti-actin antibodies. Bands Intensity was measured by ImageQuant analysis. Asterisk, fold value is expressed relative to the reference point, arbitrarily set to 1.
T11hT **(B)** or U2OS cells **(C)** were transfected with a *si*RNA directed against TBP-1 or Luciferase. Whole extracts were probed with antibodies against Phospho-Akt Ser473, Akt and actin.

As shown in Figure 12, transient re-expression of TBP-1 in the faster proliferating T1 clone dramatically reduces its proliferation rate, both in presence (A) and absence (B) of serum.
Western Blot of whole cell extracts and specific immunodetection with anti-TBP1 and anti-pAkt antibodies clearly shows that the overexpression of TBP-1 in the T1 clone determines a strong reduction in the Akt activation (Figure 12A and B, lower panels). Interestingly, when the expression of exogenous TBP-1 was reduced, after 48 hours from the transfection, cells start to proliferate faster (compare upper and lower panels in Figure 12A and B), strongly indicating that Akt activation is linked to TBP-1 intracellular levels.

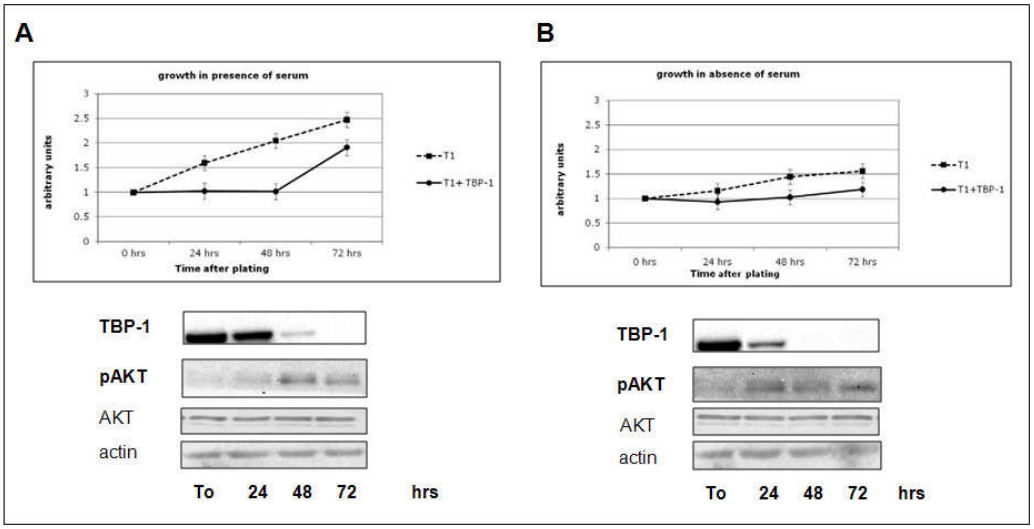

Figure 12 TBP-1 rescue in the T1 clone reduces the proliferation rate.

A, B: Cells from the T1 clone were transfected by electroporation with empty vector (indicated just as T1) or TBP-1 expression plasmid (indicated as T1 + TBP-1); cells were then cultured either in the presence (**A)** or in absence (**B**) of serum and collected at the time points indicated (being T_0 the time at 24 hours after transfection). Cells from each time point have been counted in a Burker chamber. Values are mean $\pm$ SE of two experiments performed in triplicate and are indicated as values relative to the reference point (T_0).
Lower panels: TBP-1 expression and Akt activation have been evaluated by Western Blot with anti-TBP-1, anti-Phospho-Akt (Ser-473), anti-Akt and anti-actin, as loading control, on whole protein lysates of cells collected at each time point, as indicated.

We confirmed that TBP-1 overexpression counteracts Akt activation also in another cellular context (U2OS) and in a different kind of experiment. We evaluated the extent of Akt activation following insulin stimulation, a well known inducer of Akt kinase, in condition of TBP-1 overexpression. To this purpose, U2OS cells were transfected with a fixed amount of pcDNA empty vector or increasing amounts (0.2 and 0.5 μg) of pcDNA TBP-1 expression vector. 24 hours after transfection the cells were stimulated by insulin addition for ten minutes and cell lysates were analyzed by Western Blot.
As shown in Figure 13, TBP-1 overexpression counteracts Akt activation by insulin stimulation.
This observation further proves the existance of causal relationship between TBP-1 levels and Akt activation and strongly suggests that TBP-1 can act upstream of the Akt kinase.

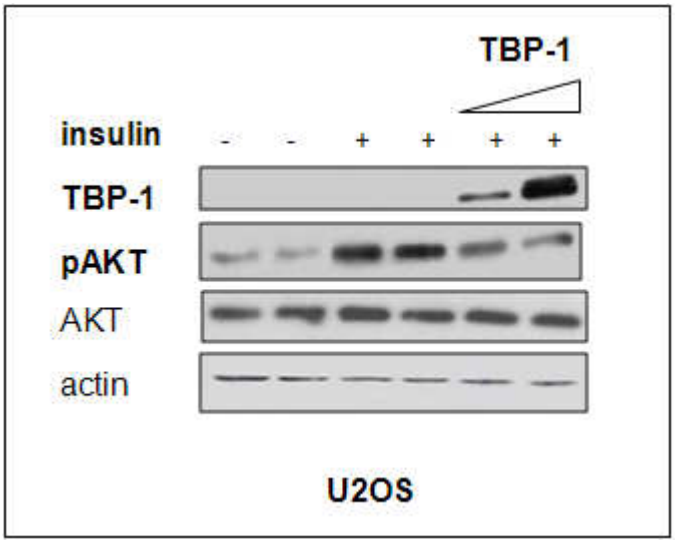

Figure 13 TBP-1 overexpression counteracts akt activation.

U2OS cells were transfected with 0.2 and 0.5 μg of the pcDNA TBP-1 expression vector. 24 hours after transfection, cells were serum starved for 4 hours and then treated with 10 ng/ml insulin for 10 minutes. Extracts were analyzed by Western blot and immunorevealed with anti TBP-1, anti pAkt, anti-Akt/PKB and anti-actin antibodies.

4.4 TBP-1 and AKT are linked in a reciprocal regulatory loop.

We next decided to investigate whether TBP-1 protein levels are regulated by activation or inhibition of PI3K/Akt pathway.
To this purpose either T11hT or U2OS cells were stimulated by insulin addition for the indicated time periods. Protein lysates were prepared and analyzed by Western Blots with anti-TBP-1 and anti-pAkt antibodies. Strikingly, insulin treatment results in a rapid drop of TBP-1 intracellular levels: TBP-1 levels are reduced of around two times in 5 minutes and remain low up to 40 minutes with a kinetic that mirrors that of activation of Akt/PKB (Figure 14A and B). On the other hand, other proteasome subunits (Rpt-6 and C8) protein levels remain stable or, at least, slightly increased, following insulin treatment. These experiments suggest that TBP-1 responds to acute insulin stimulation with a decrease of its intracellular levels in two different cellular contexts. Conversely, when we inhibited the PI3K/Akt pathway (Wortmannin and LY294002) to analyze the effects on TBP-1 protein levels, we observed an increase in TBP-1 endogenous levels in both U2OS and T11hT cells. This suggest that TBP-1 is, indeed, either directly or indirectly regulated by PI3K/Akt pathway (Figure 15A,B).

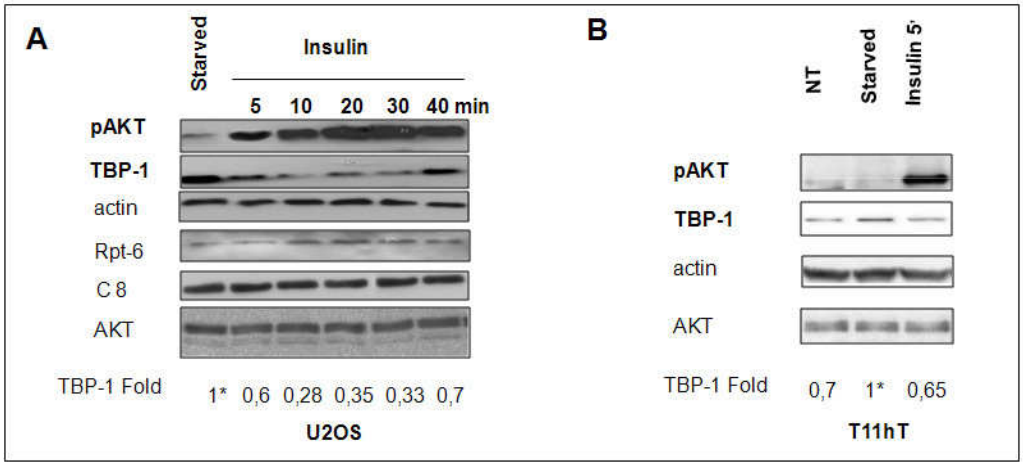

Figure 14 TBP-1 levels decrease following insulin treatment.
U2OS (**A**) and T11hT (**B**) cells were serum starved for 4 hours and then treated with 10 ng/ml of insulin for the time indicated. Cellular lysates were analyzed by Western Blot and revealed with anti pAkt, anti TBP-1, anti PSMC5 and anti C8 antibodies. Anti actin was used as loading control. TBP-1 bands intensity was measured by ImageQuant analysis on two different expositions to assure the linearity of each acquisition, each normalised for the respective actin values. Asterisk, fold value is expressed relative to the reference point, (i.e. TBP-1 levels in starved cells) arbitrarily set to 1.

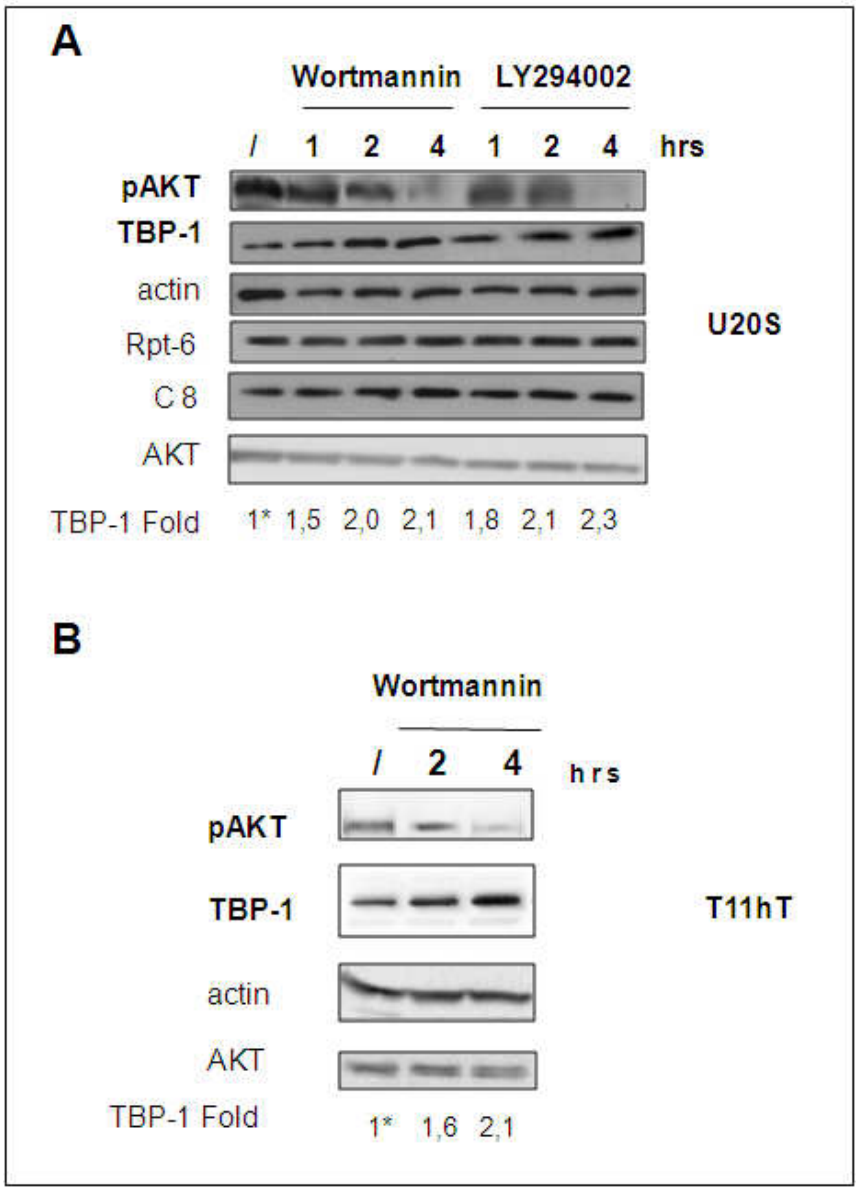

Figure 15 PI3K inhibitors upregulate TBP-1 levels.

U2OS **(A)** or T11hT **(B)** cells were treated, 24 hrs after plating, either with DMSO (/) or with 200nM Wortmannin or 50 μM LY294002 for the times indicated. Cells were then lysed and Western Blot analysis was performed by using specific antibodies against phospho-Akt (Ser-473), anti-Akt, anti-TBP-1, anti-C8 and anti-Rpt-6. TBP-1 bands intensity was calculated as in figure 14.

To verify the direct involvement of the Akt kinase in controlling TBP-1 levels, we analyzed the effects of a Constitutively Active mutant of Akt (CA-Akt) (see Materials and Methods) on TBP-1 intracellular levels.
U2OS cells were transfected with increasing amounts of CA-Akt expressing vector (kindly provided by Prof. G. Condorelli) and cell lysates were analyzed by Western Blot.
As indicated in Figure 16, overexpression of CA-Akt was accompanied by a reduction of endogenous TBP-1 levels, while other proteasome subunits (Rpt1, Rpt6) protein levels remain unchanged suggesting that this effect is specific to TBP-1. Levels of expression of pGSK3β, a direct downstream target of Akt, resulted consistent with the observed Akt activation.
Taken together these data strongly indicate the existence of a reciprocal regulatory loop in which Akt and TBP-1 balance and control each other.
We didn't observe any physical interaction between the two proteins, suggesting the existence of mediator(s) of these effects.

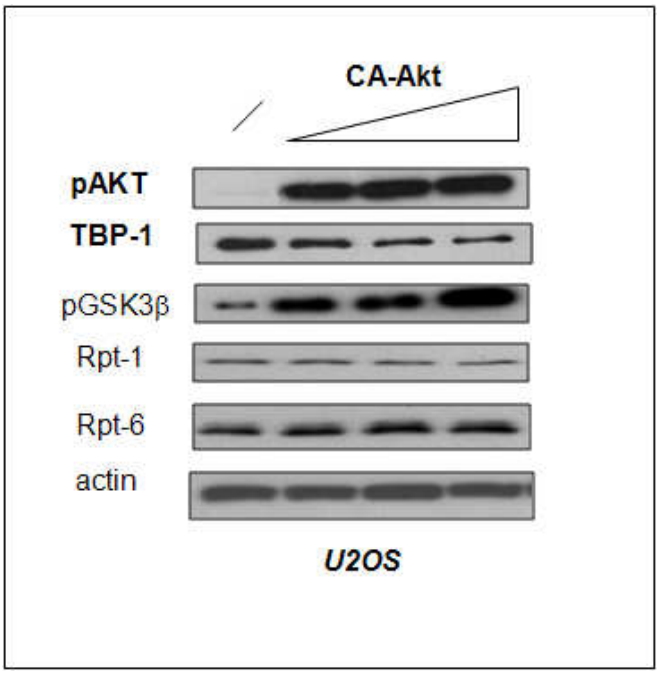

Figure 16 CA-AKT overexpression reduces TBP-1 levels.

U2OS cells were transfected either with empty vector (lane 1) or increasing amounts (0,2; 0,5; 1μg) of the constitutive active mutant of the Akt kinase (CA-Akt) expression plasmid. After 24 hrs cells were lysed and whole cell lysates probed with anti-phospho-Akt (Ser-473), anti-Akt, anti-TBP-1, anti-Rpt-1, anti-Rpt-6, and anti-phospho-GSK3β.

As a first attempt, we wondered if PTEN that is among the main regulators of Akt activation could be involved in TBP-1 action on Akt activation. Infact, as mentioned in the introduction, PTEN inhibits phosphoinositide 3-kinase (PI3K)-dependent signaling dephosphorylating phosphatidylinositol 3,4,5-triphosphate (PIP_3) (Maehama et al. 1998). We repeated the same kind of experiment shown in Figure 13 in U2OS cells either treated with a PTEN specific siRNA or with a luciferase siRNA and evaluated the extent of Akt activation following insulin stimulation, in conditions of TBP-1 overexpression. U2OS cells were co-transfected with a fixed amount of pcDNA empty vector or increasing amounts (0.2 and 0.5 μg) of pcDNA TBP-1 vector either with a PTEN siRNA or with a luciferase siRNA. 24 hours after transfection, cells were stimulated by insulin addition at the concentration of 10 ng/ml for five minutes and cell lysates were analyzed by Western Blot. Interestingly, as shown in Figure 17, PTEN silencing inhibits the effect of TBP-1 overexpression on Akt activation.
This preliminary result suggests the involvement of PTEN although the mechanism by which TBP-1 prevents Akt/PKB activation will be the subject of further studies.

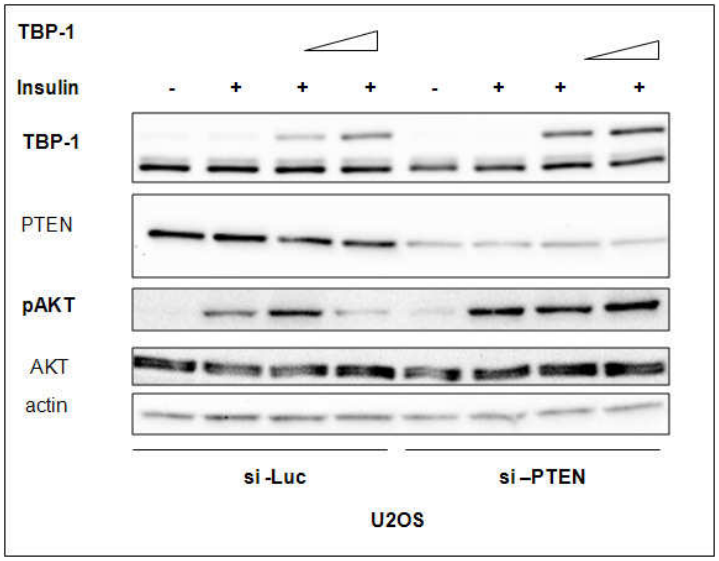

Figure 17 TBP-1 overexpression does not interfere with AKT activation in the absence of PTEN.

U2OS cells were transfected with the pcDNA TBP-1 expression vector either with 10nM siRNA PTEN or with a luciferase siRNA. 24 hours after transfection, cells were serum starved for 4 hours and treated with 10 ng/ml insulin for 10 minutes. Extracts were analyzed by Western Blot and immunorevealed with anti-TBP-1, anti-pAkt, anti PKB/Akt and anti-PTEN antibodies.

We then wondered which could be the mediator of Akt/PKB action on TBP-1 intracellular levels. As mentioned in the introduction, among the known Akt/PKB effectors, is the MDM2 (Mouse Double Minute) protein that increases its activity following phosphorylation by Akt/PKB (Mayo and Donner 2001; Zhou et al. 2001a).
We asked whether MDM2 mediates the functional relationship between TBP-1 and Akt/PKB. We stimulated U2OS osteosarcoma cells, that were previously either treated with a MDM2 specific siRNA or, as control, with a luciferase siRNA, by insulin treatment for the indicated time periods and analyzed protein lysates by Western Blots with anti-TBP-1, anti-pAkt and MDM2 antibodies. Interestingly, MDM2 silencing prevented the reduction of TBP-1 intracellular levels following insulin treatment, suggesting that MDM2 could be the mediator of Akt/PKB action on TBP-1 intracellular levels (Figure 18).

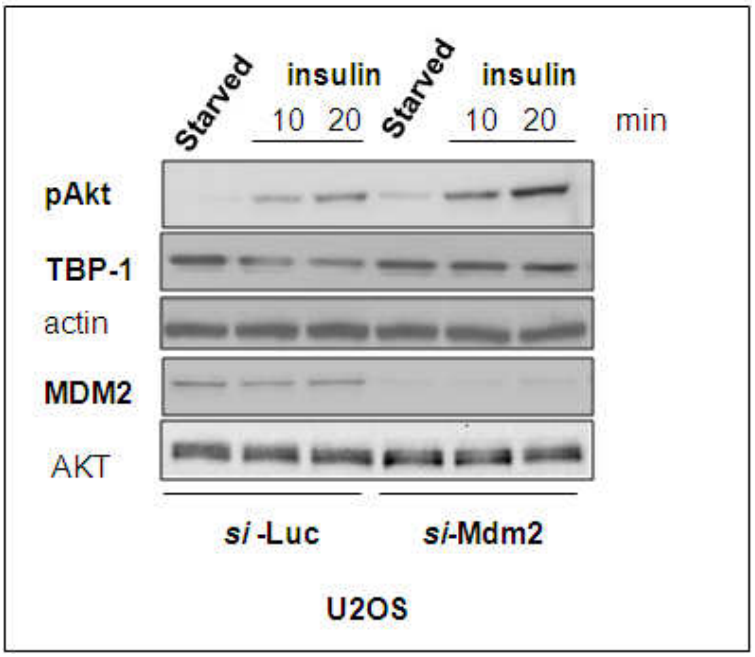

Figure 18 Activation of PI3K/AKT pathway does not interfere with TBP-1 intracellular levels in the absence of MDM2.

U2OS cells were transfected with a *si*RNA directed against MDM2 or Luciferase, as control, at the final concentration of 10nM. After 24 hrs, cells were starved for 4 hrs and then treated with 10 ng/ml insulin for the times indicated. Cells were then lyzed and Western Blot analysis was performed by using specific antibodies against Phospho-Akt Ser473, TBP-1, MDM2, Akt, and actin.

4.5 TBP-1 is a downstream target of MDM2

To further analyze the role of MDM2 on TBP-1 protein levels, we transfected U2OS cells either with pcDNATBP-1 and increasing amounts of pCMVMDM2 expression plasmid. Cell lysate were analyzed by Western Blot with antibodies against TBP-1, MDM2 and actin. As shown in Figure 19, MDM2 overexpression in U2OS cells reduces TBP-1 levels, both endogenous and transfected.

The next question was whether TBP-1 and MDM2 could interact in coimmunoprecipitation experiments. To this purpose, U2OS cells were either transfected with the MDM2 expression plasmid or with the empty vector. 24 hrs after transfection, cell extracts were prepared, subjected either to immunoprecipitation with anti-TBP-1 (Fig. 20A) or anti-MDM2 (Figure 20B) antibodies and revealed with either anti-MDM2 or anti-TBP-1 antibodies.

The results show that endogenous TBP-1 is a binding partner of MDM2 (Figure 20A and B). We noted that the amount of co-immunoprecipitated protein (either MDM2 or TBP-1) is fewer when cells are transfected with MDM2, further confirming the effect of MDM2 on TBP-1.

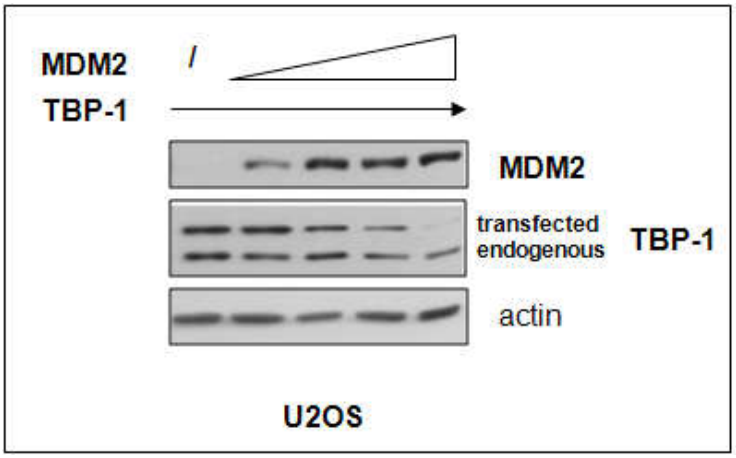

Figure 19 MDM2 overexpression reduces TBP-1 levels, both endogenous and transfected.

U2OS cells were transfected with a fixed amount of TBP-1 expression plasmid and increasing amounts of MDM2 expression plasmid. After 24 hrs, cells were lysed and whole cell extracts probed with anti-TBP-1, anti-MDM2, and anti-actin, for loading control.

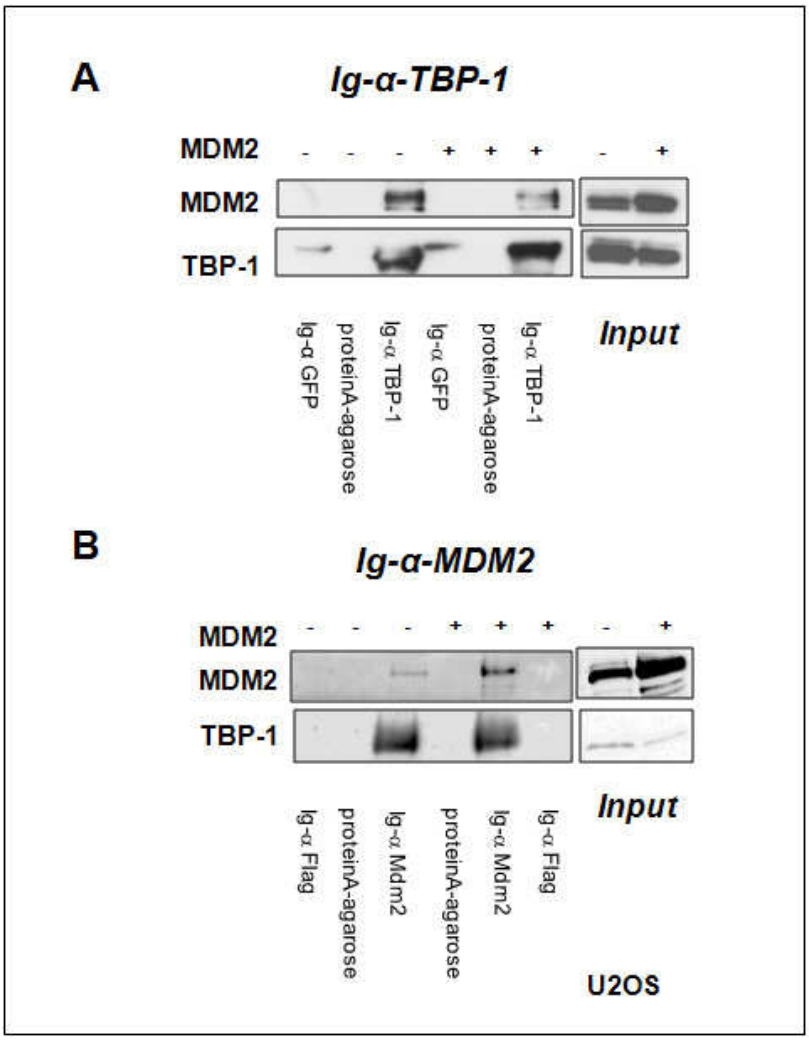

Figure 20 A,B. TBP-1 interacts with MDM2.

A: U2OS cells were either transfected (lanes +) or untransfected (lanes 2) with the MDM2 expression plasmid. 24 hrs after transfection cell extract was subjected either to immunoprecipitation with anti-TBP-1 antibody where indicated or, with anti-GFP antibody as negative control. Cell extracts were also incubated with protein A-agarose as control, where indicated. Immunoprecipitated extracts were analyzed by Western Blot with anti-MDM2 or anti-TBP-1 antibody. Aliquots of cell extracts were analyzed by Western Blot before immunoprecipitation (input). **B:** U2OS cells were either transfected (lanes +) or untransfected (lanes 2) with the MDM2 expression plasmid. 24 hrs after transfection cell extract was prepared and subjected either to immunoprecipitation with anti-MDM2 antibody where indicated or, with anti-Flag antibody as negative control. Cell extracts were also incubated with protein A-agarose as control, where indicated. Immunoprecipitated extracts were analyzed by Western Blot with anti-MDM2 or anti-TBP-1 antibody. Aliquots of cell extracts were analyzed by Western Blot before immunoprecpitation (input).

Since the observed effect occurs both on the endogenous and on the exogenous protein, it is likely that MDM2 acts on TBP-1 at the post-transcriptional level. Consistently, we observed that treatment of U2OS cells with the proteasome inhibitor MG132 counteracts the MDM2 effect on TBP-1intracellular levels, indicating the proteasome as the final effector of the MDM2 action on TBP-1 (Figure 21).

As mentioned before, MDM2 is a direct downstream target of Akt/PKB kinase. In fact it has been shown that Akt phosphorylates MDM2 at Serine 166, 186 and 188 (Zhou et al. 2001; Mayo et al. 2001; Feng et al. 2004).

We thus asked if point mutations in MDM2 that render it less responsive to Akt/PKB stimulation (Zhou et al. 2001; Ogawara et al. 2002; Feng et al. 2004; Mayo et al. 2001) reduces, as well, its ability to downregulate TBP-1 levels.

To test this hypothesis we generated the $MDM2_{S166A}$ and $MDM2_{S166A/186A}$ point mutants (for details see Materials and Methods) through site directed mutagenesis and analyzed the effect of this mutant on TBP-1 intracellular levels.

U2OS cells were transfected either with pcDNATBP-1 and increasing amount of $pCMVMDM2_{S166A}$ or $pCMVMDM2_{S166A/186A}$ expression plasmids.

Cell lysate were analyzed by Western Blot with antibodies against TBP-1 and MDM2. As shown in Figure 22 (A,B), MDM2 mutants appear almost unable to mediate TBP-1 degradation, indicating that only a functionally Akt-responsive MDM2 molecule, could regulate TBP-1 levels.

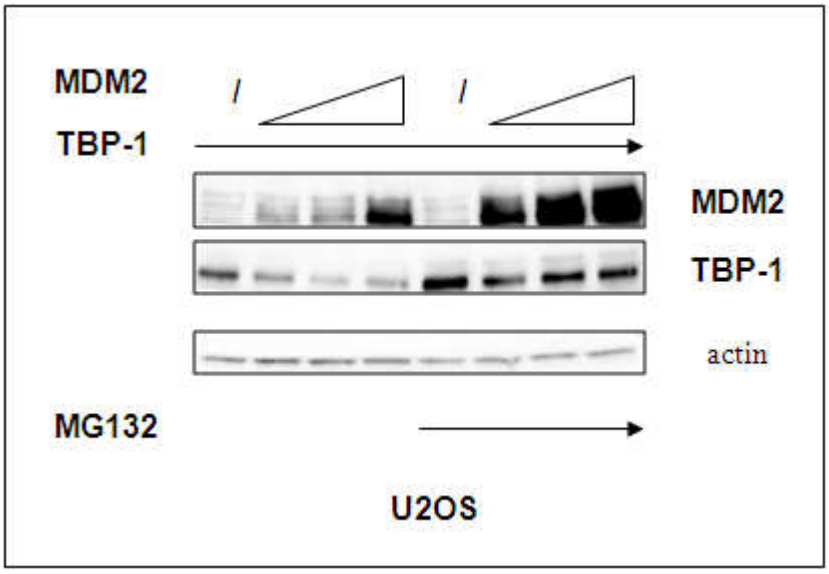

Figure 21. MDM2 downregulates TBP-1 protein levels acting in a proteasome dependent manner.

U2OS cells were transfected with TBP-1 expression plasmid and increasing amounts of MDM2 expression plasmid. After 24 hrs cells were treated either with DMSO (first four lanes) or with 10 μM MG132 where indicated. Cell extracts were analyzed by Western Blot with anti-Xpress (to reveal transfected TBP-1), anti-MDM2, and anti-actin, for control.

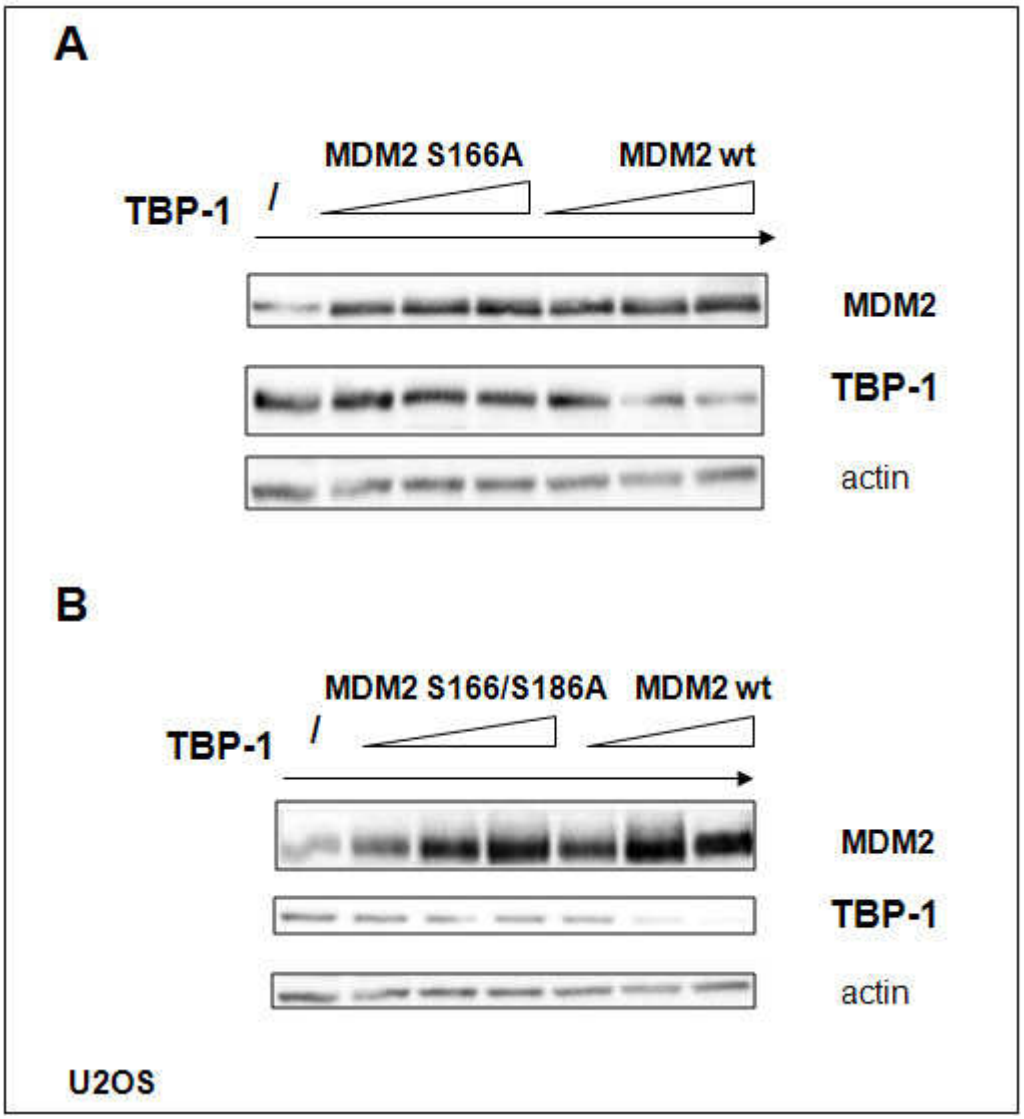

Figure 22. $MDM2_{S166A}$ and $MDM2_{S166A/186A}$ mutants are unable to downregulate TBP-1 protein levels.

A, B: U2OS cells were transfected with TBP-1 expression plasmid and increasing amounts of either MDM2wt, $MDM2_{S166A}$ or $MDM2_{S166A/S186A}$ expression plasmids. After 24 hrs cells were lysed and whole cell extracts were analyzed by Western Blot with anti-Xpress (to reveal transfected TBP-1), anti-MDM2, and anti-actin, for control.

We thus asked if phosphrylation mutant of MDM2 ($MDM2_{S166A/186A}$), that is unable to downregulate TBP-1 protein levels, is still able to bind TBP-1.
To this purpose, MEF (Murine Emrionic Fibroblast) p53-/- MDM2-/- cells were either untransfected or transfected with pcDNATBP-1 in combination with pCMVMDM2 or pCMV$MDM2_{S166A/186A}$ expression plasmids.
Cellular extracts were subjected to immunoprecipitation with anti-X-press antibody, run on a SDS-Page, blotted and incubated with anti-X-press (to reveal transfected TBP-1) and anti-MDM2 antibodies.
The result shows that MDM2 phosphrylation mutant ($MDM2_{S166A/186A}$) is still able to bind TBP-1 (Figure 23).
It is known that MDM2 has multifaceted roles in protein degradation.
The well described role of MDM2 is that of an E3-ubiquitin ligase which mediates the ubiquitination and proteasome degradation of the p53 tumor suppressor protein (Honda et al. 1997; Momand et al. 2000).
Furthermore, MDM2 has been shown to mediate proteasome-dependent but ubiquitin-independent degradation of p21Waf1/Cip1 (Jin et al. 2003) and of Retinoblastoma Protein (Sdek et al. 2005) through direct binding with the C8 subunit of the 20S proteasome.
Since the effect of MDM2 on TBP-1 levels occurs both on the endogenous and on the transfected protein, it is likely that MDM2 acts on TBP-1 at the post-transcriptional level. We asked whether this could occur through the ubiquitination domain of MDM2 (*ring finger*).
For this reason we analyzed the effect of the $MDM2_{1–441}$ deletion mutant (Jin et al. 2003), that lacks the *ring finger* domain, on TBP-1 intracellular levels.
The results indicated that $MDM2_{1–441}$ deletion mutant mediates the downregulation of TBP-1 intracellular levels, suggesting that ubiquitine ligase activity of MDM2 is not necessary (Figure 24).
It has been reported that MDM2 can act as a molecular cargo and shuttle p63 protein to the cytoplasm mediating its interaction with proteins specifically involved in its turnover (Galli et al. 2010).
Morover, it has been shown that MDM2 phosphorylation by Akt determines shuttling of MDM2 from the cytoplasm to the nucleus (Mayo et al. 2001; Zhou et al. 2001).
Accordingly, we noticed that $MDM2_{S166A}$ and $MDM2_{S166A/186A}$ phosphorylation mutants are unable to mediate TBP-1 degradation suggesting that cellular localization plays a role in this effect.

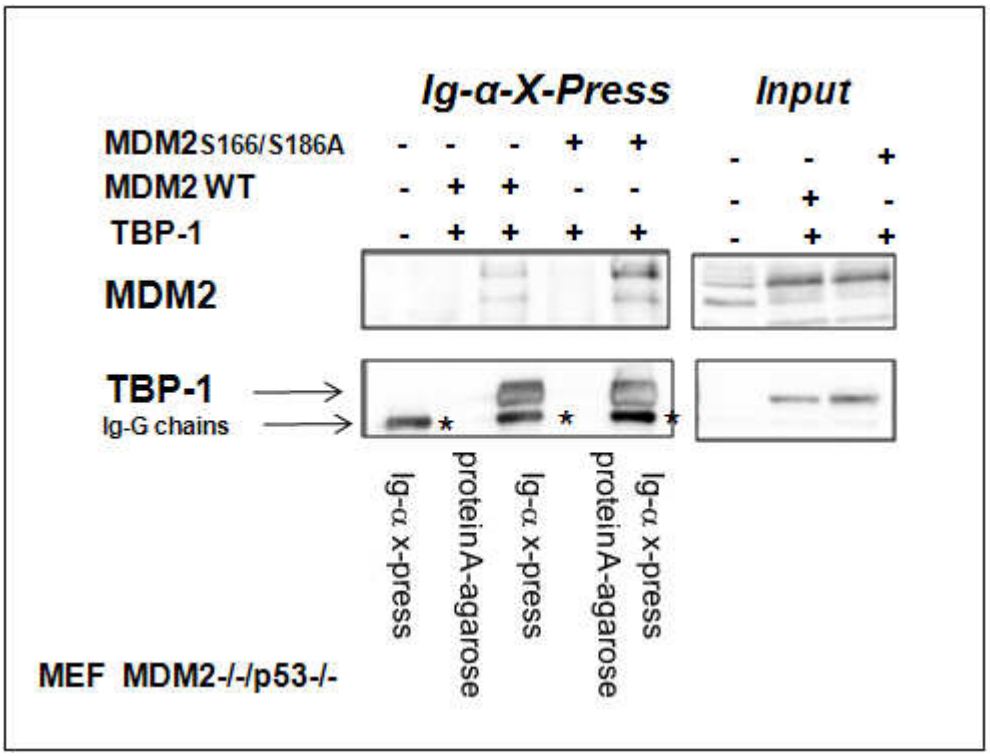

Figure 23. The MDM2$_{S166A/186A}$ phosphrylation mutant is still able to bind TBP-1.

MEF p53-/- MDM2-/- cells were either transfected (lanes +) or untransfected (lanes -) with the pcDNATBP-1, pCMVMDM2 or pCMVMDM2 $_{S166A/186A}$ expression plasmids. 24 hrs after transfection cell extract was prepared and subjected to immunoprecipitation with anti-X-press antibody where indicated. Cell extracts were also incubated with protein A-agarose as control, where indicated. Immunoprecipitated extracts were analyzed by Western Blot with anti-MDM2 or anti-x-press (to reveal transfected TBP-1) antibodies. Aliquots of cell extracts were analyzed by Western Blot before immunoprecipitation (Input).

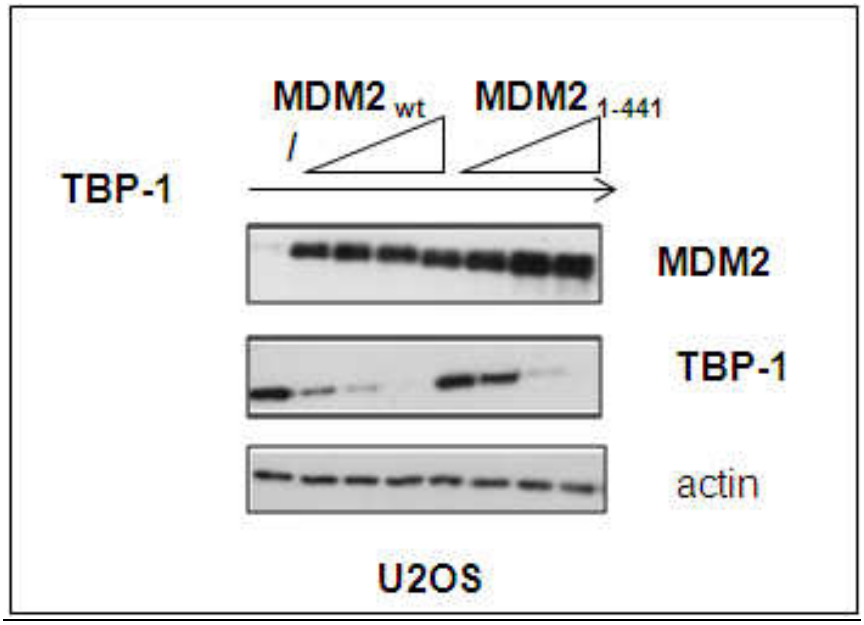

Figure 24. The downregulative effect of MDM2 on TBP-1 does not require the ubiquitine ligase activity of MDM2.

U2OS cells were transfected with 0.1 μg of the TBP-1 plasmid alone or together with increasing amounts of the MDM2 plasmids indicated. Cellular extracts were resolved by SDS PAGE and analyzed by Western Blot with anti MDM2 and anti-TBP-1 antibodies. Actin was checked as loading control.

We thus analyzed the effect of the MDM2 deletion mutant ($MDM2_{\Delta 150–230}$) on TBP-1 intracellular levels. This mutant lacks all the Akt target sites in MDM2 and it was described to be unable to shuttle between the nucleus and the cytoplasm, displaying a predominant cytoplasmic localization, because lacks nuclear localization and nuclear export signals (Jin et al. 2003).
U2OS cells were transfected either with pcDNATBP-1 and increasing amounts of $pCMVMDM2_{\Delta 150–230}$ expression plasmids. As shown in Figure 25, this mutant appears unable to act on TBP-1 levels, suggesting that probably the MDM2 action on TBP-1 intracellular levels requires its nuclear localization that is described to occur following phosphorylation by Akt. Overall these data provide clear evidence that TBP-1 is a downstream target of the Akt/PKB-MDM2 axis, even though the molecular mechanisms through which MDM2 acts on TBP-1 intracellular levels remain to be understood.

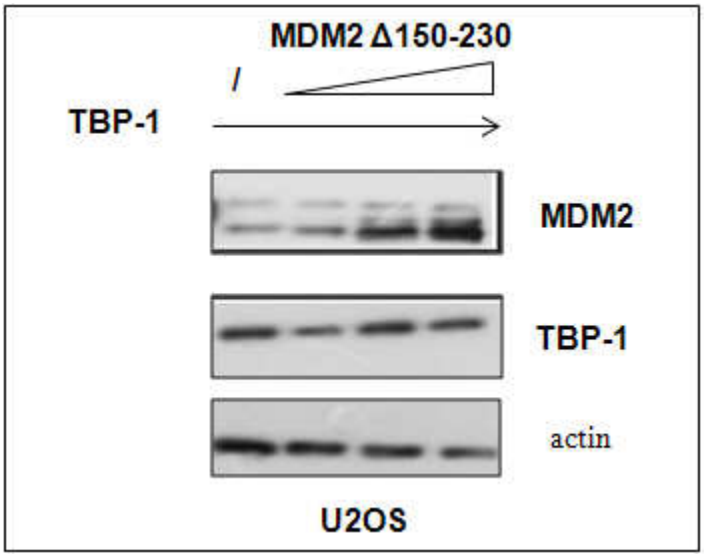

Figure 25. The $MDM2_{\Delta 150–230}$ is unable to downregulate TBP-1 protein levels.
U2OS cells were transfected with 0.1 μg of the TBP-1 plasmid alone or together with increasing amounts of the MDM2 plasmids indicated. Cellular extracts were resolved by SDS PAGE and analyzed by Western Blot with anti MDM2 and anti-TBP-1 antibodies. Actin was checked as loading control.

4.6 Discussion

In this work we provide evidences that stable reduction of TBP-1 expression levels in primary human fibroblasts immortalized by h-TERT, affects cell proliferation and resistance to serum-deprivation induced apoptosis. Furthermore, TBP-1-silenced cells appear capable to survive in absence of serum much better than control as judged by a MTS assay.
TBP-1 reduction causes an increase in the activation status of the Akt/PKB serin-threonin kinase, known to control the balance between cell survival and apoptosis. It has to be underlined that this can be directly ascribed to TBP-1 depletion, rather than to clonal secondary effects, as it also occurs after transient silencing of TBP-1 and irrespective of the cell type. Remarkably, transient expression of TBP-1 in one of the silenced clones restores phospho-Akt basal levels and drastically reduces the proliferation rate. Furthermore, TBP-1 overexpression in other cellular systems prevents Akt/PKB activation by insulin, thus confirming that TBP-1 can act upstream of Akt.
In principle, the increase of Akt/PKB activity could account for all the changes induced by TBP-1 silencing (i.e. proliferation, cell viability, escape from apoptosis, migration capabilities).
Activation of the Akt/PKB pathway plays a central role in tumorigenesis. Indeed, Akt is overexpressed in many different tumour cell types, with a burgeoning list of substrates implicated in oncogenesis (Datta et al. 1997). Cellular transformation is a stepwise process, and several successive genetic or epigenetic changes are required for the development of a neoplastic phenotype. It seemed plausible to hypothyze that, by acting on Akt/PKB, down-modulation of TBP-1 intracellular levels might contribute to the acquisition of a transformed phenotype. In this context, we wondered whether TBP-1 silenced clones could have acquired the ability to grow in soft agar. However, we were unable to show any growth of both the T1 and T10C clones, suggesting that other genetic lesions are needed in order to acquire a full transformed phenotype (Fabio Tolino, personal communication). An intriguing possibility to explore is the introduction of “key” cellular lesions to cause cell transformation in these clones.
The mechanism by which TBP-1 prevents Akt/PKB activation remains an open question. Like the other AAA-ATPases of the 19S base of the proteasome, TBP-1 is supposed to act by conferring specificity to the proteasome (Voges et al. 1999). In fact, it has been shown to be directly involved in negative regulation of intracellular levels of HIF-1α (Corn et al. 2003) and Fra-1 (Pakay et al. 2011), two transcriptional factors overexpressed in a variety of human cancers. Moreover, my research group has already observed that TBP-1 stabilizes p14ARF avoiding its entrance into the proteasome and enhancing its oncosuppressive functions (Pollice et al. 2004; Pollice et al. 2007).
Thus, TBP-1 appears to serve a role in the control of cell proliferation either through the increase of degradation of two oncogenes or in the stabilization of an oncosuppressor. On the other hand, an increase in the proliferation rate is

frequently associated to an increase of proteasome levels needed to guarantee high metabolic activity. Proteasome subunits often appear to be the target of cancer-related de-regulation and are involved in tumour progression. Despite evidences that dysregulation of the catalytic processes mediated by the proteasome system is associated with tumorigenesis and tumor progression, the relationship between proteasome expression and cancer progression is still poorly understood. Abnormal expression levels of proteasome subunits have been described in many tumour cells and proteasome plasma levels appear elevated in neoplastic patients underlying the involvement of the proteasome in cancer developement (Lavabre-Bertrand et al. 2001; Bazzaro et al. 2006). On the other hand, proteasomal inhibition causes increased cell death in lymphoma cell lines when compared with normal lymphoblasts (Soligo et al. 2001). Moreover, it has been reported that gankyrin, an oncogene overexpressed in human hepatocellular carcinoma, interferes with the proteasome activity.
In particular, gankyrin interacts with the TBP-7 subunit increasing the degradation of the pRB transcription factor and subsequent increase of the cellular proliferation (Higashitsuji et al., 2000). Another evidence suggests that the HEC protein (Highly Expressed in Cancer) specifically interacts with the S7 ATPase and would be able to inhibit its ATPase activity, preventing the degradation of the A and B cyclin, necessary for the progression of the cell cycle from the G2phase to the M phase (Chen et al. 1997).
However, various observations suggest that TBP-1 may act, as well, in a proteasome independent manner (Gonzales et al. 2002; Lassot et al. 2007; Pollice et al. 2004; Pollice et al. 2007; Satoh et al. 2009). Indeed, the proteasome seems very unlikely involved in the modulation of the Akt/PKB activity by TBP-1, as TBP-1 seems not to act on Akt intracellular levels. Moreover, in TBP-1 silenced clones, both proteasome composition and activity remain unaffected. Infact, the level of expression of other protesaome subunits do not change , as well as proteasome activity measured by an Elisa assay (Fabio Tolino, personal communication).

Intriguingly, our results reveal the existence of a reciprocal regulatory loop where Akt/PKB activation leads to TBP-1 reduction and, in turn, TBP-1 overexpression prevents Akt/PKB activation. In fact, insulin treatment of T11hT or U2OS cells results in a downregulation of TBP-1 intracellular levels, while inhibition of the PI3K/Akt pathway by Wortmannin determines an increase in TBP-1 endogenous levels. Accordingly, the transient overexpression of increasing amounts of a constitutively active mutant of the Akt kinase (CA-Akt) in U2OS cells determines a reduction of TBP-1 endogenous levels.
In search of the molecular mechanisms through which TBP-1 and AKT are linked each other, we hypothesized the existence of mediators of these effects as the two proteins appear not to interact in coimmunoprecipitation experiments. Our preliminar results suggest that the mechanism by which TBP1 prevents Akt/PKB activation could require the involvement of the PTEN

phosphatase, one of the main negative regulators of Akt/PKB activation although this will be the subject of further studies.
On the other hand, we demonstrated that the PI3K/Akt signaling effect on TBP-1 is prevented in cells in which MDM2 is silenced. We thus proposed the MDM2 protein, one of the main direct targets of Akt/PKB activation (Zhou et al. 2001; Ogawara et al. 2002; Feng et al. 2004; Mayo et al. 2001), as mediator of the PI3K/Akt signaling on TBP-1. The data show that MDM2 can bind to TBP-1 and its overexpression causes a reduction of TBP-1 intracellular levels. Moreover, the $MDM2_{S166A}$ and the $MDM2_{S166A/S186A}$ phosphorylation mutants, lacking Akt responsive sites, are unable to act on TBP-1 protein levels although are still able to bind TBP-1, likely placing TBP-1 downstream of the Akt/PKB-MDM2 axis.
It is known that MDM2 has multifaceted roles in protein degradation. In fact, aside its well-described role as E3-ubiquitin ligase, under appropriate stimuli, MDM2 can shuttle p63 to the cytoplasm mediating its interaction with proteins specifically involved in its turnover (Galli et al. 2010). Furthermore, MDM2 has been shown to mediate proteasome-dependent but ubiquitin-independent degradation of p21Waf1/Cip1 (Jin et al. 2003) and of Retinoblastoma Protein (Sdek et al. 2005) through direct binding with the C8 subunit of the 20S proteasome. On the other hand, it has very recently been reported that MDM2 interacts with components of the 19S proteasome in a ubiquitylation independent manner (Kulikov et al. 2010) claiming a wider view of its mechanism of action. Interestingly, the $MDM2_{\Delta 150–230}$ mutant that lacks all the Akt target sites in MDM2 and it was described to be unable to shuttle between the nucleus and the cytoplasm, displaying a predominant cytoplasmic localization (Jin et al. 2003), is not able to downregulates TBP-1 levels.
This could imply that the MDM2 action on TBP-1 levels requires its nuclear localization that, indeed, is described to occur following phosphorylation by Akt (Ogawara et al. 2002; Mayo et al. 2001). Moreover, the fact that the $MDM2_{1–441}$ deletion mutant, that lacks the *ring finger* domain, is still able to act on TBP-1, indicates that MDM2 is not acting on TBP-1 levels through its ubiquitination activity, supporting the possibility that it rather acts as a molecular cargo and should plausibly act in concert with other pAkt effector molecule(s) needed to direct TBP-1 for degradation.
Altogether this data provide further insights on the proposed antiproliferative role of TBP-1 (Park et al. 1999; Pollice et al. 2004; 2007) indicating the involvement of the Akt/PKB kinase. It is possible that, under standard growing conditions, TBP-1 contributes to balance Akt/PKB up-regulation; whilst, under growth factor acute stimulation, activation of the Akt/PKB signaling pathway lowers TBP-1 levels and initiate a feedback loop.
Thus, although far from being elucidated, the relationships among the different pathways that TBP-1 controls (degradation of oncogenes as Fra-1 and Hif-1α, stabilization of oncosuppressor as p14ARF, negative control on the activation of Akt/PKB), place TBP-1 at the cross road of different cellular pathways involved in the control of cancerogenesis.

5. CONCLUSIONS

The data described in this thesis reveal the existence of a reciprocal regulatory loop in which TBP-1 and Akt balance and control each other.
The consequence of this regulatory relationship could be that, under standard growing conditions, TBP-1 contributes to balance Akt/PKB up-regulation, whilst, under growth factor acute stimulation, activation of the Akt/PKB signaling pathway downregulates TBP-1 intracellular levels.

Our results provide further insights on the proposed potential oncosuppressive role of TBP-1 (Park et al. 1999; Pollice et al. 2004; 2007) and place TBP-1 at the cross road of different cellular pathways involved in the control of cancerogenesis. Infact, TBP-1 has already been described to controls degradation of oncogenes as Fra-1 and Hif-1α and stabilization of oncosuppressor as p14ARF. Our findings add a new point linking TBP-1 to Akt/PKB pathway, a main regulator of cellular signals that control balance between cell survival and apoptosis.

In conclusion, we hypothyze that, by acting on Akt/PKB, down-modulation of TBP-1 intracellular levels might contribute to the acquisition of a transformed phenotype, although other genetic lesions are needed in order to acquire a full transformed phenotype.

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PLoS one

A Regulatory Mechanism Involving TBP-1/Tat-Binding Protein 1 and Akt/PKB in the Control of Cell Proliferation

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Abstract

TBP-1 /Tat-Binding Protein 1 (also named Rpt-5, S6a or PSMC3) is a multifunctional protein, originally identified as a regulator of HIV-1-Tat mediated transcription. It is an AAA-ATPase component of the 19S regulative subunit of the proteasome and, as other members of this protein family, fulfils different cellular functions including proteolysis and transcriptional regulation. We and others reported that over expression of TBP-1 diminishes cell proliferation in different cellular contexts with mechanisms yet to be defined. Accordingly, we demonstrated that TBP-1 binds to and stabilizes the p14ARF oncosuppressor increasing its anti-oncogenic functions. However, TBP-1 restrains cell proliferation also in the absence of ARF, raising the question of what are the molecular pathways involved. Herein we demonstrate that stable knock-down of TBP-1 in human immortalized fibroblasts increases cell proliferation, migration and resistance to apoptosis induced by serum deprivation. We observe that TBP-1 silencing causes activation of the Akt/PKB kinase and that in turn TBP-1, itself, is a downstream target of Akt/PKB. Moreover, MDM2, a known Akt target, plays a major role in this regulation. Altogether, our data suggest the existence of a negative feedback loop involving Akt/PKB that might act as a sensor to modulate TBP-1 levels in proliferating cells.

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Introduction

TBP1/Tat-Binding Protein 1 (also named Rpt-5, S6a or PSMC3) is a member of a large highly conserved gene family of ATPases (ATPAses Associated to a variety of cellular Activities) whose key feature is a highly conserved module of 230 aa consisting of an ATPase and a DNA/RNA helicase motif. This protein family fulfils a large diversity of cellular functions including cell cycle regulation, gene expression, vesicle mediated transport, peroxisome assembly and proteasome function [1]. Indeed, as other members of the family, TBP-1 is associated with the 19S regulatory subunit of the proteasome, the chief site of protein destruction in eukaryotic cells [2]. The last 10 years have highlighted the essential role of proteolysis in governing cell physiology. Protein breakdown is required not only for removal of abnormal or aged proteins, but also to control most biological pathways through the regulated degradation of key cellular factors. Moreover, abnormal proteasome expression levels have been described in many tumor cells and proteasome plasma levels appear elevated in neoplastic patients, underlying the involvement of the proteasome in cancer development [3,4]. Consistent with the role in protein destruction, TBP-1 has been shown to bind the tumour suppressor VHL (Von-Hippel-Landau) gene product [5] contributing to its E3-ubiquitin ligase function towards the Hif1-a factor, thus acting as a *bona fide* tumor suppressor.

On the other hand, 19S protein components (TBP-1 among them) behave as multifaceted proteins, being implicated in different cellular events that do not require proteolysis like transcriptional initiation and elongation, [6,7,8] Nucleotide Excision Repair [9] and regulation of mitosis [10].

We and others have reported that TBP-1 may function as a negative regulator of cell proliferation: inhibition of the oncogenic phenotype of *erb-B* transformed cells was accompanied by an increase of TBP-1 intracellular levels and, accordingly, its overexpression in *erb-B* transformed cells strongly inhibited tumour formation in athymic mice [11]; furthermore, TBP-1 overexpression in different cellular contexts diminished cell proliferation [11,12]. Our reported results [12,13] showing that TBP-1 enhances the levels of the p14ARF oncosuppressor well fit with TBP-1 proposed antioncogenic role [11]. On the other hand, the observation that TBP-1 overexpression can inhibit cell proliferation also in ARF minus contexts [11,12] suggests an ARF-independent role of TBP-1, raising the question of what molecular pathways may be involved.

In this paper, we address the role of TBP-1 in the control of cell proliferation. To this aim we used, as model, a primary human fibroblast cell line immortalized by h-TERT (human telomerase) expression where p14ARF levels are undetectable and in which we have silenced the expression of TBP-1. Our results show that cellular levels of TBP-1 are critical in the control of cell

proliferation pointing to a functional relationship between TBP-1 and the Akt/PKB serine-threonine kinase, one of the major transducers of growth signals mediating proliferative and pro-survival effects.

Results

TBP-1 depletion determines an increase in the growth properties

We decided to first study the effects of long term silencing of TBP-1 in an immortalized human fibroblast cell line (T11hT). To this purpose, by retrovirus infection, we generated stable T11hT-derived cell clones that constitutively express a *sh*-RNA specifically designed to silence TBP-1 expression (see Materials and Methods). As shown in Figure 1A, TBP-1 is efficiently silenced in six stable clones analyzed, with an extent of silencing ranging from 80% to 48%. To exclude that reduced expression of TBP-1 may have altered proteasome assembly and function [14], we analyzed intracellular levels of proteasome subunits other than TBP-1 in three of the silenced clones (T1, T10E and T10C). In all cases we observed that the levels of expression of three different proteasome subunits (Rpt-6, Rpt-1 of the 19S subunit and C8 of the 20S subunit) do not change significantly as compared to parental T11hT (Figure 1B). Furthermore, we didn't observe any variation of the *in vitro* proteasome activity of cell extracts obtained from TBP-1-silenced clones and parental cells on two different peptide substrates (data not shown).

We then measured the growth rate of the T1 and T10C clones as compared to that of the T11hT cells. Figure 2A shows that both the TBP-1 silenced clones analysed proliferate at higher rate respect to the parental T11hT cell line. In particular, the T1 clone, expressing very low TBP-1 levels (see Figure 1), grows at a rate that is roughly twice that of the parental cell line. Moreover, serum deprivation doesn't appreciably alter the growth rate of the silenced clones (Figure 2B). To exclude any clonal secondary effect due to the selection process, we also generated, by stable transfection, T11hT cell pools either containing the *sh*-TBP-1 vector or the empty vector. As it is shown in Figure 2C and D, TBP-1 silenced cells, although in a less pronounced way respect to single clones, display the same growth profile, both in presence and absence of serum.

The enhanced growth rate in TBP-1 silenced cells seems to be dependent on TBP-1 silencing. In fact, transient expression of TBP-1 in the faster proliferating T1 clone dramatically reduces its proliferation rate, both in presence (Figure 2E) and absence (Figure 2F) of serum; however, after 48 hrs, when the expression of exogenous TBP-1 was greatly reduced (see Figure 2E and F, lower panels), cells start to proliferate faster suggesting that slow proliferating TBP-overexpressing cells were selected against.

Consistently with the ability of TBP-1 silenced clones to actively proliferate even in the absence of serum, the cell viability of the T1 clone, measured after 6 hrs of serum withdrawal, remains high. In particular, T11hT cell viability was reduced from 60% to 10% (depending on cell density), while that of the T1 clone is reduced only up to 60% at the lowest cell density (Figure 3A and B). We thus investigated whether TBP-1 silencing may increase cell resistance to serum withdrawal-induced apoptosis. As shown in Figure 3C and D, the T1 clone behaves more resistant to serum-deprivation, respect to the parental cells, as assessed by the very faint amounts of both Caspase-3 and PARP-1 cleavage. Accordingly, flow cytometry analysis indicates that serum starvation only slightly affects the percentage of T1 cells in S phase (8% reduction), while more drastically reduces

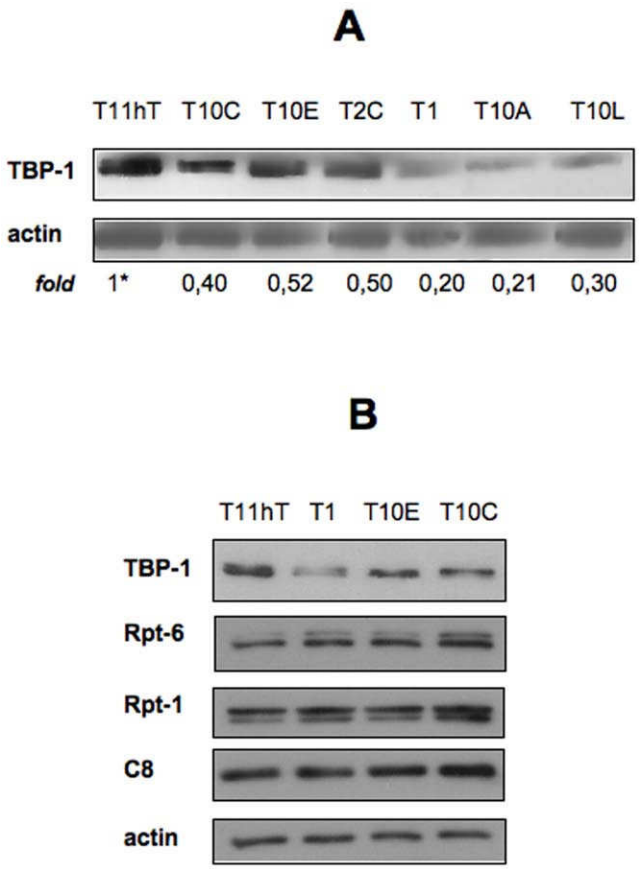

Figure 1. Characterization of TBP-1 silenced clones. A, B: Cells stably transfected with TBP1 *sh*-RNA plasmid or control cells (wt T11hT, Human Primary Fibroblasts Immortalized by hTERT) were cultured in DMEM+10%FBS for 24 hrs. Levels of TBP-1 expression was evaluated by Western Blot with anti-TBP-1 on whole protein lysates. **B**: As control, protein levels of other proteasome components (two 19S-ATPases, Rpt-1 and Rpt-6, and a 20S component, C8) was evaluated in the clones T1, T10C and T10E. Bands intensity was evaluated by ImageQuant analysis on at least two different expositions to assure the linearity of each acquisition, each normalised for the respective actin values. Asterisk, fold value is expressed relative to the reference point (i.e. TBP-1 levels in T11hT cells), arbitrarily set to 1. Representative of at least four independent experiments.
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that of the parental cell line (55% reduction) (Figure 3E). Furthermore, the increase of the sub-G1 population (of around 1.8 fold for the T1 clone and 3,14 fold for the parental cell line) is consistent with PARP-1 and Capase-3 cleavage data (Figure 3E and see 3C and D).

Next, we analyzed the invading capability of the T1 clone respect to control cells by a chemoinvasion assay in which cells were plated on Matrigel coated filters and allowed to migrate. As shown in Figure 4A, as compared to parental cells, T1 cells possess a moderate but significant higher ability to migrate through Matrigel. Interestingly, similar results are also obtained when cells were allowed to migrate toward a generic chemoattractant as EGF (Epidermal Growth Factor) (Figure 4B). To further prove that the difference in invasion ability could be ascribed to the reduction of TBP-1 protein levels and not to any clonal secondary effects, making use of a specific *si*RNA, we transiently silenced TBP-1 in parental T11hT cells. Consistently, transient silencing of TBP1 is even more effective than stable silencing in T1 cells in inducing a

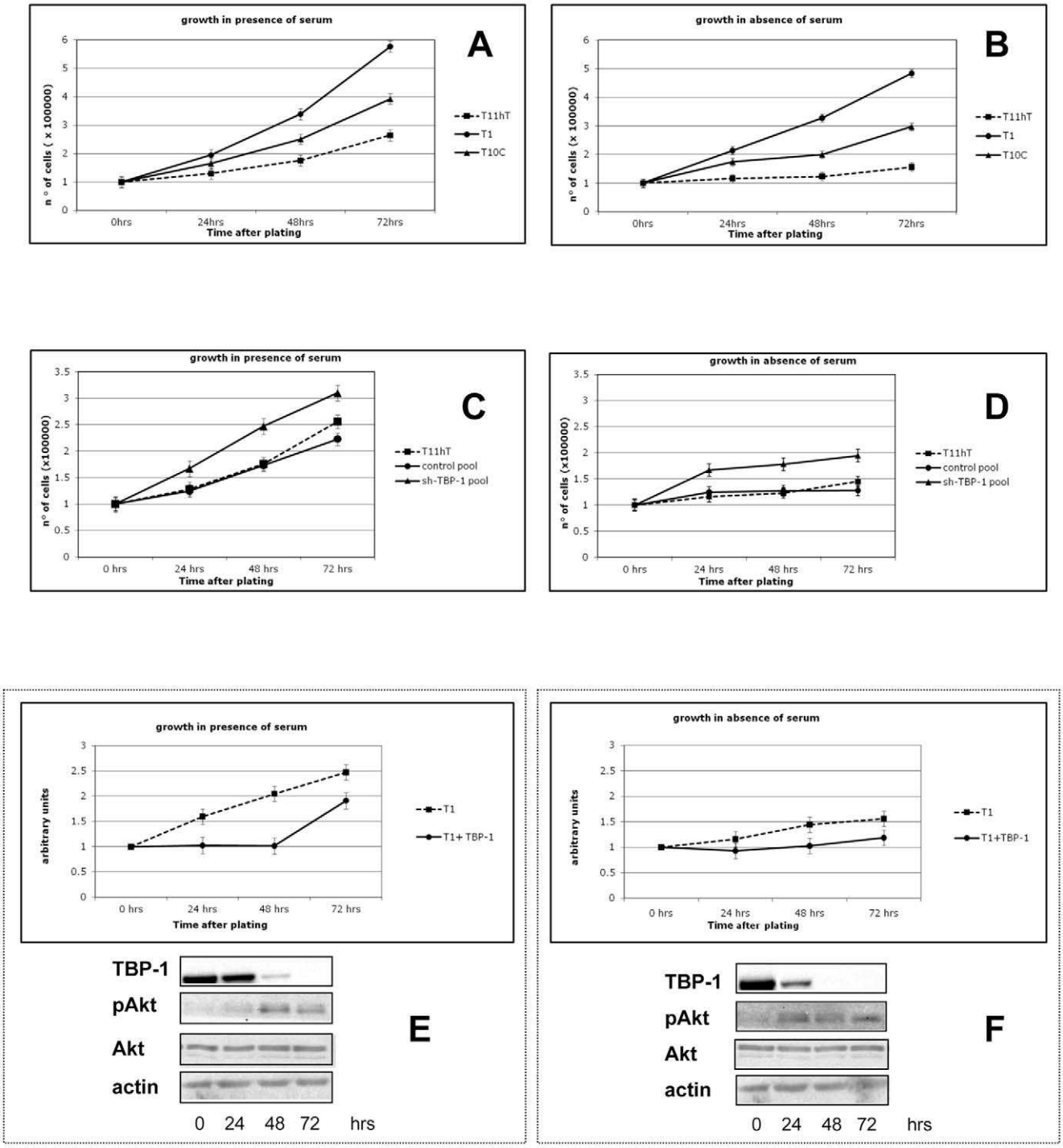

Figure 2. TBP-1 knockdown determines an increase in the growth properties. A, B: Cells from the T1, T10C and control cells (wt T11hT) were cultured in DMEM either in the presence (**A**) or in absence (**B**) of 10% FBS. Cells were collected at the time points indicated and counted in a Burker chamber. The values are the mean ± SE of three experiments performed in triplicate. **C, D**: wt T11hT cells, cells from control cell pool or from the *sh*-TBP-1 cell pool were cultured in DMEM either in the presence (**C**) or in absence (**D**) of 10% FBS. Cells were collected at the time points indicated and counted in a Burker chamber. The values are the mean ± SE of three experiments performed in triplicate. **E, F:** Cells from the T1 clone were transfected by electroporation with empty vector (indicated just as T1) or TBP-1 expression plasmid (indicated as T1+TBP-1); cells were then cultured either in the presence (**E**) or in absence (**F**) of 10%FBS and collected at the time points indicated (being T_0 the time at 24 hours after transfection). Cells from each time point have been counted in a Burker chamber. Values are mean ± SE of two experiments performed in triplicate and are indicated as values relative to the reference point (T_0). **E, F lower panels**: TBP-1 expression and Akt activation have been evaluated by Western Blot with anti-TBP-1, anti-Phospho-Akt Ser473, anti-Akt and anti-actin, as loading control, on whole protein lysates of cells collected at each time point, as indicated.

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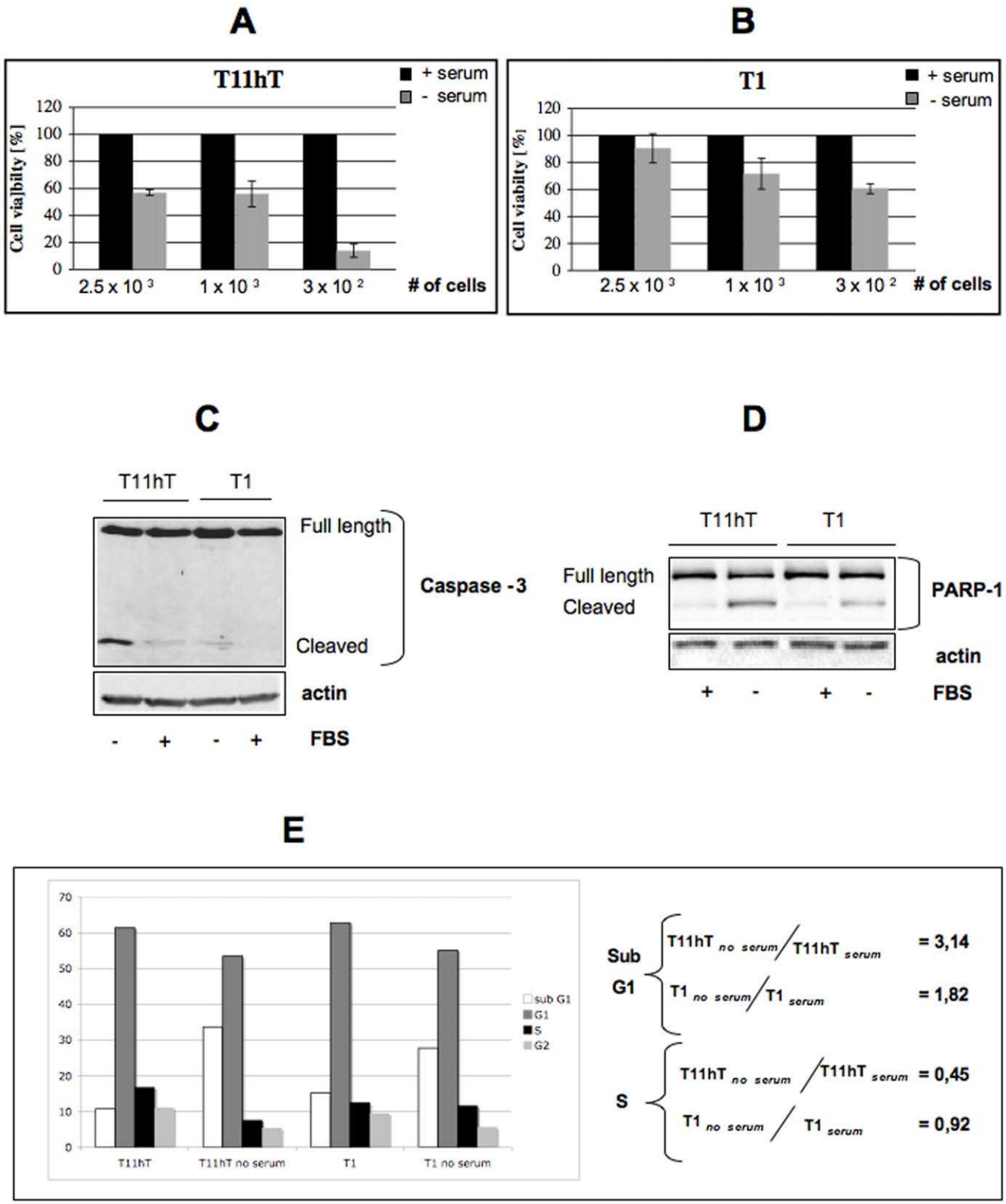

Figure 3. TBP-1 knockdown reduces sensitivity to serum starvation. A, B: Cells from the T1 clone and control cells (wt T11hT) were plated at different cell densities as indicated, either in the presence or absence of 10% FBS. After six hrs from plating, cell viability was measured by MTS assay. In the histograms, cell viability is expressed as relative to controls, arbitrarily set to 100 (%). The values are the mean ± SE of three experiments performed in quintuplicate. **C, D:** 1.8×10^5 cells/35 mm plates from the T1 clone and control cells (wt T11hT) were grown for 24 hrs, in the presence or absence of 10% FBS. Apoptosis was checked by detection of Caspase-3 (**C**) and PARP-1 (**D**) cleavage in Western Blot. Detection with anti-actin was included for control of equal loading. Bands Intensity was measured by ImageQuant analysis on at least two different expositions to assure the linearity of each acquisition. Representative of at least four independent experiments. **E:** T11hT and T1 cells were counted and seeded at 2×10^5cells/

35 mm plate. At 24 hrs cells were collected and treated for analysis of cellular DNA content by flow cytometry. Percentages of cells in the SubG1, G0–G1, S and G2–M phases were quantified with Summit 4.1 software. Representative of three different experiments. The numerical ratios reported on the right highlight the different behaviour of T1 cells when grown in absence or presence of serum. Table 1 provides the mean values (and standard deviations in parentheses) relative to this analysis.
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high per cent of migrating cells (Figure 4C). Thus, the difference in Matrigel invasion was likely due to an increased invading capability of TBP1 silenced cells, as also suggested by the fact that we don't observe any difference both in the expression and activation status of the EGF receptor (not shown).

Taken together these data show that TBP-1 sensitizes cells to apoptosis induced by serum withdrawal and interferes with cell growth and migration.

TBP-1 inhibits Akt/PKB activation

The observation that TBP-1 depletion allows cells to grow in a serum-independent manner, prompted us to ask whether TBP-1 expression levels may control, in some way, the activity of the Akt/PKB serine-threonine kinase, one of the major transducers of growth signals, critical for cell proliferation and apoptosis. We thus evaluated the levels of phospho-Akt in our TBP-1 depleted clones, under actively growth conditions (i.e. in the presence of serum). As

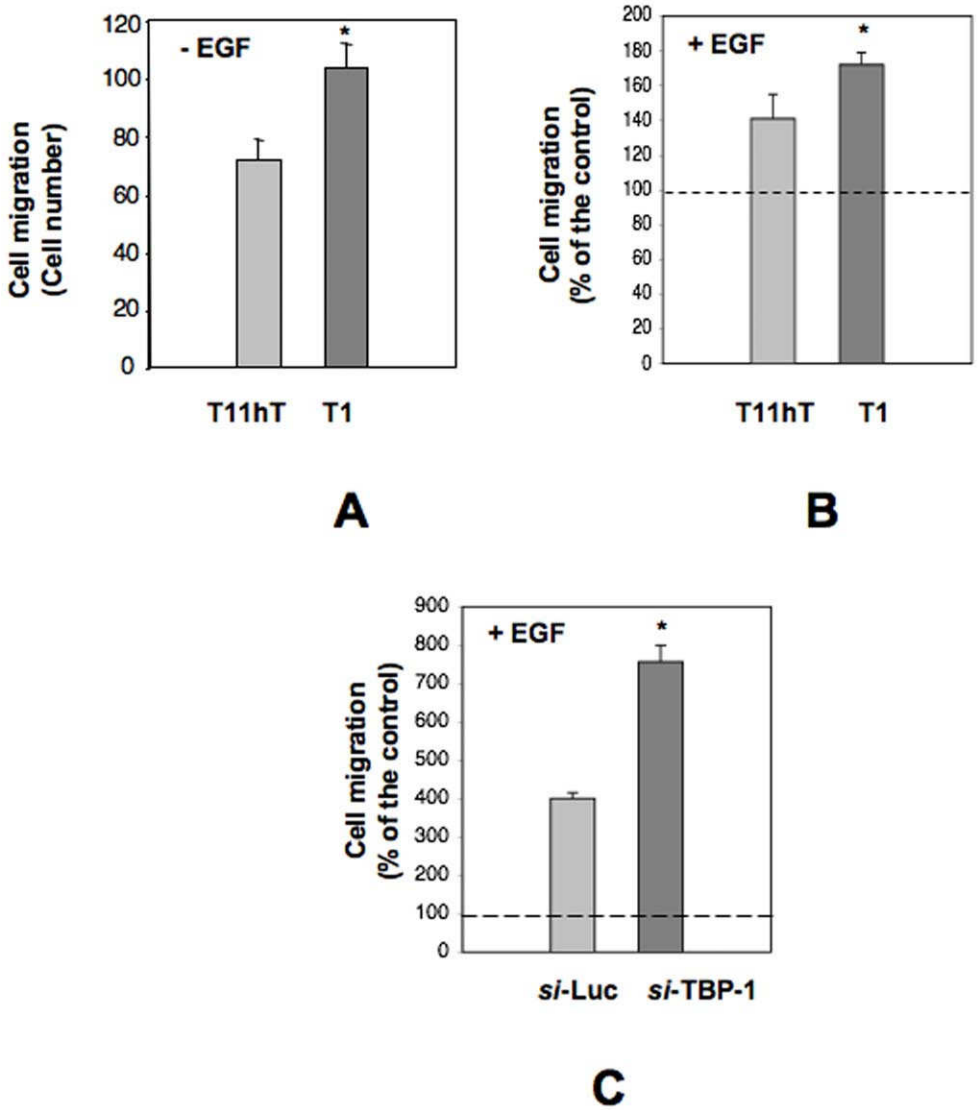

Figure 4. Silencing of TBP-1 determines an increase of the invading capability. A: Cells from the T1 clone or control cells (wt T11hT) were plated in Boyden chambers and allowed to migrate on filters coated with Matrigel. The values are the mean ± SE of three experiments performed in triplicate. (*) $p = 0.046$ as determined by the Student's t test. **B:** Cells from the T1 clone or control cells (wt T11hT) were plated in Boyden chambers and allowed to migrate toward EGF on Matrigel filters. 100% values represent cell migration in the absence of chemoattractants. The values are the mean ± SE of three experiments performed in triplicate. (*) $p = 0.027$ as determined by the Student's t test. **C:** Cells transiently transfected with TBP1 *si*-RNA or with the control si-RNA (*si*-Luc) were plated in Boyden chambers and allowed to migrate toward EGF on filters coated with Matrigel. 100% values represent cell migration in the absence of chemoattractants. The values are the mean±SE of three experiments performed in triplicate. (*) $p = 0.016$ as determined by the Student's t test.
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Table 1. Mean values (and standard deviations in parentheses) relative to the flow cytometry analysis described in Figure 3.

	T11hT +serum	T11hT −serum	T1 +serum	T1 −serum
Sub-G1	**12,69** (+/−2,35)	**39,79** (+/−5,02)	**13,02** (+/−1,8)	**29,05** (+/−3,12)
S	**14,87** (+/−1,2)	**6,44** (+/−0,9)	**13,94** (+/−1,75)	**14,13** (+/−2,49)

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shown in Figure 5A, pAkt/PKB levels are inversely correlated to the extent of silencing of TBP-1, being the lowest in the parental T11hT and the highest in the T1 clone. Consistently, we observed an increase in the extent of phosphorylation of GSK3β, a well characterized Akt/PKB direct target. TBP-1 reduction appears to specifically affect Akt activation but not that of other important transducers of growth signals, like $ERK_{1/2}$. Furthermore, in agreement with the observed higher proliferation rate of the clones, we observed a reduction of phospho-cyclin D1 protein levels (data not shown). Both in parental cells and in TBP-1 silenced clones, Akt activation appears to be dependent on the upstream phosphatidylinositol 3-kinase activity (PI3K) as evidenced by Wortmannin and LY294002 treatment that block PI3K activity (Figure 5B).

Importantly, we could reproduce, in T11hT cells, the same effect after transient reduction of TBP-1 levels by *si*RNA: silencing of TBP-1 was accompanied by a concomitant increase in the steady-state level of pAkt, suggesting the existence of a causal relationship between TBP-1 intracellular levels and Akt activation (Figure 5C). This effect was not cell-specific since we could reproduce it in the U2OS osteosarcoma-derived cells (Figure 5D).

Further, we set up a rescue experiment in which we re-established high TBP-1 levels in the T1 clone by transient overexpression. In these conditions we observed a strong reduction of pAkt levels (Figure 5E and see also Figure 2E, F). Consistently, insulin-mediated activation of Akt in a different cellular context (U2OS cells) is counteracted by TBP-1 overexpression (Figure 5F). Altogether these data suggest that TBP-1 levels modulate the extent of Akt/PKB activation.

TBP-1 is a downstream target of Akt activation

The new insights into the role of TBP-1 in the control of cell growth prompted us to investigate whether TBP-1 protein levels are sensitive to acute growth factors stimulation. We thus stimulated either T11hT or U2OS osteosarcoma cells by insulin treatment for the indicated time periods and analyzed protein lysates by Western Blots with anti-TBP-1 antibodies. Figure 6A clearly shows that insulin treatment results in a rapid, transient drop of TBP-1 intracellular levels; indeed, TBP-1 levels are reduced of around two times in 5 min and remain low up to 40 minutes with a kinetic that mirrors that of the activation of Akt/PKB (Figure 6A, left panel). On the other hand, other proteasome subunits (Rpt-6 and C8) protein levels remain almost stable or, at least, slightly increased, following insulin treatment (Figure 6A). To test the effects of inhibition of the PI3K/Akt pathway on TBP-1 protein levels, U2OS cells or T11hT cells were treated for the indicated time periods with PI3K inhibitors and protein lysates analyzed by Western Blots. As shown in Figure 6B, inhibition of the PI3K/Akt pathway determines a slight though reproducible increase in TBP-1 endogenous levels, suggesting that they are either directly or indirectly regulated by PI3K activity. Again, in these conditions, other proteasome subunits (Rpt-6 and C8) protein levels remain stable (Figure 6B, left panel). To further confirm these observations, we transiently transfected increasing amounts of a constitutively active mutant of the Akt kinase (CA-Akt) in U2OS cells. Overexpression of CA-Akt was accompanied by a reduction of endogenous TBP-1 levels, while other proteasome subunits protein levels remain unchanged (Figure 6C). Taken together, these data strongly indicate that TBP-1 protein levels are modulated by the Akt/PKB activity.

On the other hand, by immunoprecipitation experiments we were unable to observe any physical interaction between TBP-1 and Akt/PKB (data not shown), suggesting that TBP-1 levels are indirectly modulated by Akt activation. We thus wondered which could be the mediator of Akt/PKB action on TBP-1.

Among the known Akt/PKB effector is the MDM2 protein that, following phosphorylation by Akt/PKB, increases its activity [15,16,17]. We thus determined whether MDM2 mediates the functional relationship between TBP-1 and Akt/PKB. In order to obtain Akt activation, we treated with insulin U2OS cells that were previously either treated with a MDM2 specific *si*RNA or, as control, with a luciferase *si*RNA (Figure 6D). Interestingly, MDM2 silencing prevented the reduction of TBP-1 intracellular levels following treatment with insulin, although has no effects on TBP-1 basal levels. Consistently, the increase in TBP-1 levels following treatment with PI3K inhibitors, is prevented in cells in which MDM2 is silenced (Figure 6E), suggesting that, indeed, silencing of MDM2 renders Akt activation/inactivation ineffective on TBP-1 levels. These data strongly implicate MDM2 to be needed, even not sufficient, for TBP-1 regulation by Akt/PKB. Involvement of MDM2 is supported by co-immunoprecipitation experiments in U2OS cells. As shown in Figure 7A and 7B we found TBP-1 in complex both with endogenous and with transfected MDM2. Furthermore, we observed that overexpression of MDM2 causes a decrease of TBP-1 intracellular levels (Figure 7A and B, see *input*). We confirmed this observation transfecting U2OS cells with fixed concentration of pcDNA-TBP-1 and increasing amounts of the MDM2 expression plasmid (Figure 7C). Since the observed effect occurs both on the endogenous and on the exogenous protein, it is likely that MDM2 acts on TBP-1 at the post-transcriptional level. Moreover, treatment of U2OS cells with the proteasome inhibitor MG132 counteracts the MDM2 effect on TBP-1, indicating the proteasome as the final effector of the MDM2 action on TBP-1 (Figure 7D).

We thus asked if mutations in MDM2 that render it less responsive to Akt/PKB stimulation [15,16,17,18] reduces, as well, its ability to downregulate TBP-1 levels. The $MDM2_{S166A}$ and $MDM2_{S166A/186A}$ mutants appear almost unable to mediate TBP-1 degradation (Figure 7E), indicating that only a functionally Akt-responsive MDM2 molecule, could regulate TBP-1 levels. Accordingly, a MDM2 deletion mutant that lacks all the Akt target sites in MDM2 ($MDM2_{\Delta 150-230}$) [19] appear unable to act on TBP-1 levels (Figure 7F). Interestingly, a MDM2 mutant, lacking the ring finger domain ($MDM2_{1-441}$), is still able to act on TBP-1 (Figure 7F), indicating that MDM2 is not acting on TBP-1 levels through its ubiquitination activity.

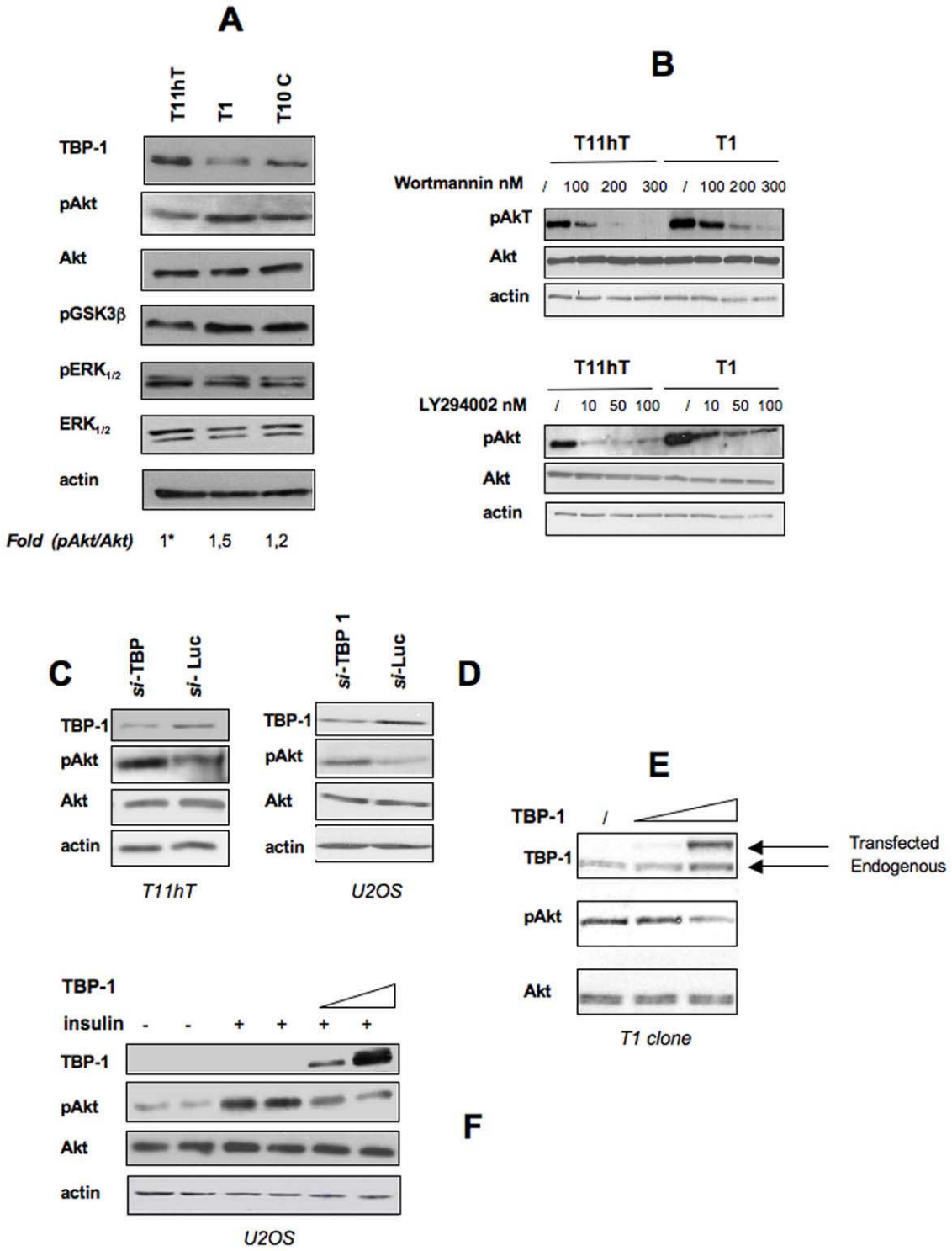

Figure 5. TBP-1 knockdown determines activation of the Akt/PKB kinase. A: Cells from the T1, T10C and control cells (wt T11hT) were cultured in DMEM+10%FBS for 24 hrs. Activation of Akt/PKB was revealed by Western Blot with anti-Phospho-Akt Ser473 antibody. As control, extracts were also probed with anti-Akt, anti-Phospho-GSK-3β/pSer219, anti-pERK$_{1/2}$, anti-ERK$_{1/2}$ and anti-actin antibodies. Bands Intensity was measured by ImageQuant analysis on at least two different expositions to assure the linearity of each acquisition, each normalised for the respective actin values. Asterisk, fold value is expressed relative to the reference point, arbitrarily set to 1. Representative of at least four independent experiments. **B:** Cells from the T1 clone or control cells (wt T11hT) were plated at the cell density of 2.5×10^5 in DMEM+10%FBS in six wells. After 24 hrs, either DMSO (/) or with Wortmannin or LY294002, where indicated, were added to the cells at the concentrations indicated and left for either 1 hour (with Wortmannin) or 15′(with LY294002). Extracts were then probed in Western Blot with antibodies against Akt, Phospho-Akt Ser473 and actin. **C, D:** T11hT cells (**C**) or U2OS cells (**D**) were transfected with an *si*RNA directed against TBP-1 or Luciferase. Extracts were probed with antibodies against Phospho-Akt Ser473, Akt and actin. **E:** Cells from the T1 clone were transfected with empty vector (first lane) or increasing amounts of TBP-1 expression plasmid. Activation of Akt/PKB was evaluated by Western Blot on whole protein lysates probed with anti-Phospho-Akt

Ser473 and, as control, with anti-Akt and anti-actin. **F:** U2OS cells were transfected with empty vector (lanes 1–4) or TBP-1 expression plasmid (lanes 5, 6). After 24 hrs cells were starved for 4 hrs and treated with 10 ng/ml insulin for 10′ where indicated. Activation of Akt/PKB was evaluated by Western Blot on whole protein lysates probed with anti-Phospho-Akt Ser473. Extracts were also probed with anti-Akt, anti-actin and anti-Xpress (to reveal transfected TBP-1).
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These data provide clear evidence that TBP-1 is a downstream target of the Akt/PKB-MDM2 axis, even though the molecular mechanisms through which MDM2 acts on TBP-1 remain to be elucidated.

Discussion

Herein we report data showing that reduction of TBP-1 intracellular levels affects cell proliferation, invading capabilities and resistance to apoptosis of human fibroblasts immortalized by h-TERT expression. Interestingly, unlike the parental cells, proliferation of TBP-1 silenced clones appears to be serum-independent. Our data indicate that TBP-1 modulates the extent of activation of the Akt/PKB kinase, a critical effector of intracellular signaling. In fact, we demonstrate that reduction of TBP-1 intracellular levels causes the activation of the Akt signaling pathway. It has to be underlined that this can be directly ascribed to TBP-1 depletion rather than to clonal secondary effects as it also occurs after transient silencing of TBP-1 and irrespective of the cell type. Remarkably, transient expression of TBP-1 in one of the silenced clones restores phospho-Akt basal levels and drastically reduces the proliferation rate. Furthermore, TBP-1 overexpression in other cellular systems prevents Akt/PKB activation thus confirming that TBP-1 can act upstream of Akt.

Activation of the Akt/PKB pathway plays a central role in tumorigenesis. Indeed, Akt is overexpressed in many different tumour cell types, with a burgeoning list of substrates implicated in oncogenesis [20]. In principle, the increase of Akt/PKB activity could account for all the changes induced by TBP-1 silencing (i.e. proliferation, cell viability, escape from apoptosis, migration capabilities) [21,22,23,24]. On the other hand, the acquisition of a transformed phenotype is a quite complex stepwise accumulation of genetic changes [25,26]. In this context, it seems plausible to predict that, by acting on Akt/PKB, down-modulation of TBP-1 intracellular levels might contribute to the acquisition of a transformed phenotype thus cooperating with other genetic lesions. Since TBP-1 silenced clones are normal fibroblasts that only bear h-TERT overexpression to guarantee immortalization, an intriguing possibility to explore is the introduction of "key" cellular lesions to cause cell transformation in these clones.

The mechanism by which TBP-1 prevents Akt/PKB activation remains an open question. Even though, like the other AAA-ATPases of the 19S base of the proteasome, TBP-1 is supposed to act by conferring specificity to the proteasome [27,28] various observations suggest that TBP-1 may act, as well, in a proteasome independent manner [6,7,8,12,13,29]. Indeed, the proteasome seems very unlikely involved in the modulation of the Akt/PKB activity by TBP-1. In fact, an increase in the proliferation rate is frequently associated to an increase of proteasome levels needed to guarantee high metabolic activity. Here we show that TBP-1 silenced clones don't display a significant alteration in proteasome composition and activity. Furthermore, unlike other proteasome components (C8 and Rpt-6), TBP-1 responds to acute insulin stimulation with a decrease of its intracellular levels. In a different context, other proteasome subunits respond to growth factor stimulation with an increase of intracellular levels [30].

It has to be underlined that we have already observed that TBP-1 stabilizes p14ARF [12,13] avoiding ARF entrance into the proteasome. We retain that TBP-1 could play a role in ARF folding, rendering it a poor substrate for degradation by the 20S as well as by the 20S/11S proteasome [31,32]. The existence of a similar mechanism that permits to TBP-1 to increase the intracellular levels of proteins that regulate Akt/PKB activity is the subject of further studies.

Furthermore, our results reveal the existence of a reciprocal regulatory loop where Akt/PKB activation leads to TBP-1 reduction and, in turn, TBP-1 overexpression prevents Akt/PKB activation. In this scenario, the Akt/PKB kinase thus might act as a sensor that modulates TBP-1 levels in actively duplicating cells. On the other hand, based on the fact that the PI3K/Akt signaling effect on TBP-1 is prevented in cells in which MDM2 is silenced, we propose, as mediator of the PI3K/Akt signaling on TBP-1, the MDM2 protein, one of the main direct targets of Akt/PKB activation [15,16,17,18]. Actually, MDM2 can bind to TBP-1 and its overexpression causes a reduction of TBP-1 intracellular levels. Strikingly, the $MDM2_{S166A/S186A}$ mutant and the $MDM2_{\Delta 150–230}$, lacking Akt responsive sites, are unable to act on TBP-1 protein levels, likely placing TBP-1 downstream of the Akt/PKB-MDM2 axis.

Even though the specific mechanism for MDM2-dependent depression of TBP-1 levels remains to be understood, it has to be noted that MDM2 has multifaceted roles in protein degradation. In fact, aside its well-described role as E3-ubiquitin ligase, under appropriate stimuli, MDM2 can shuttle p63 to the cytoplasm mediating its interaction with proteins specifically involved in its turnover [33]. Moreover, MDM2 has been shown to mediate proteasome-dependent but ubiquitin-independent degradation of $p21^{Waf1/Cip1}$ [19] and of Retinoblastoma Protein [34] through direct binding with the C8 subunit of the 20S proteasome. On the other hand, it has very recently been reported that MDM2 interacts with components of the 19S proteasome in a ubiquitylation independent manner [35] claiming a wider view of its mechanism of action.

Interestingly, the $MDM2_{\Delta 150–230}$ mutant was described to be unable to shuttle between the nucleus and the cytoplasm, displaying a predominant cytoplasmic localization [19]. This could imply that the MDM2 action on TBP-1 levels requires its nuclear localization that, indeed is described to occur following phosphorylation by Akt [15,18].

Moreover, the fact that the $MDM2_{1–441}$ deletion mutant, that lacks the ring finger domain, is still able to act on TBP-1 (Figure 7F), indicates that MDM2 is not acting on TBP-1 levels through its ubiquitination activity, supporting the possibility that it rather acts as a molecular cargo and should plausibly act in concert with other pAkt effector molecule(s) needed to direct TBP-1 for degradation.

Altogether our observations provide further insights on the proposed antiproliferative role of TBP-1 [11,12,13], indicating the involvement of the Akt/PKB kinase. Indeed, we could speculate that, under standard growing conditions, TBP-1 contributes to balance Akt/PKB up-regulation; whilst, under growth factor acute stimulation, activation of the Akt/PKB signaling pathway lowers TBP-1 levels and initiate a feedback loop. Further, it's interesting to underline that the human oncosuppressor p14ARF that is stabilized by TBP-1 overexpression [12,13] is itself able to antagonize the activity of Akt/PKB [36] with yet unknown

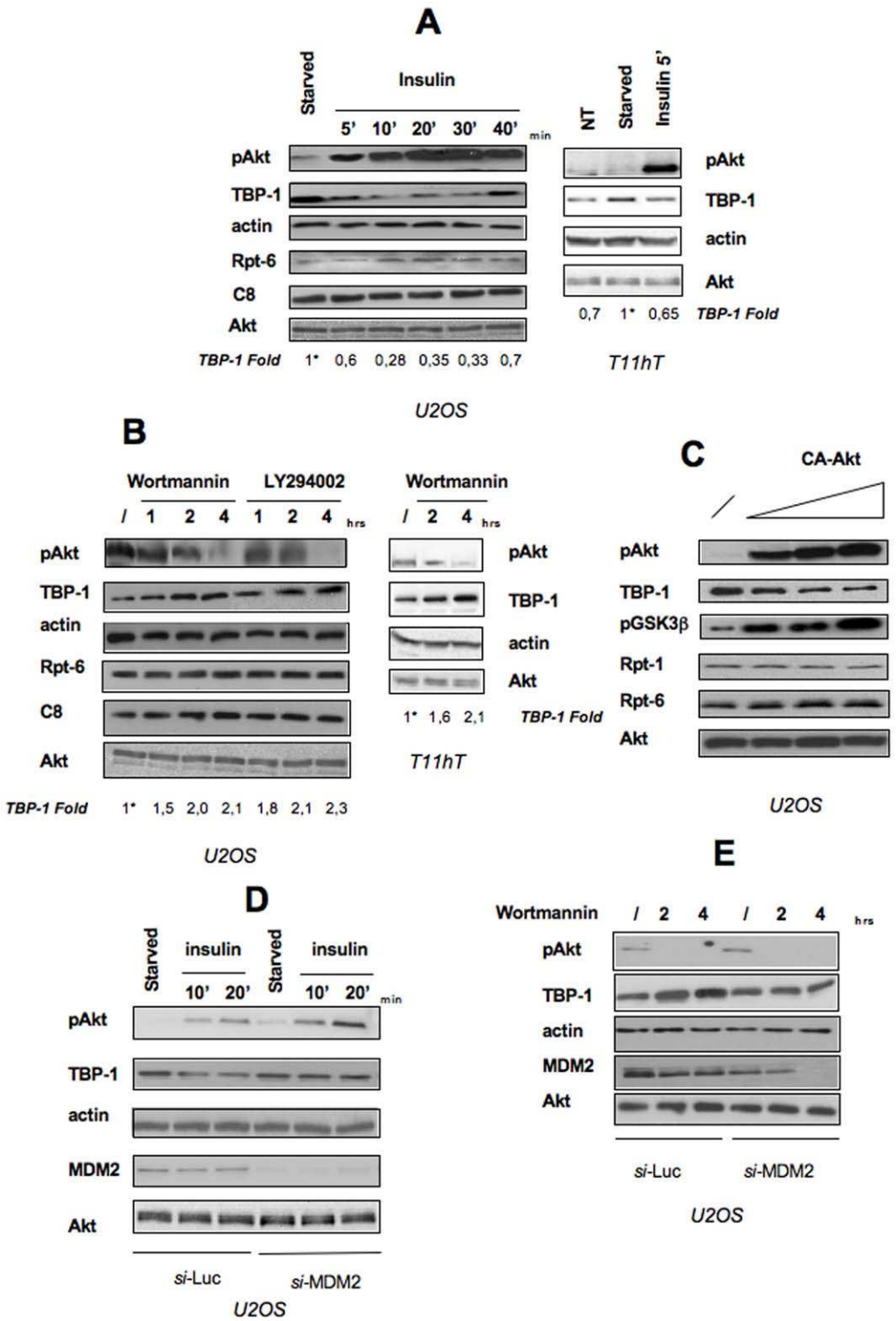

Figure 6. TBP-1 is a downstream target of Akt activation. A: U2OS cells or T11hT cells were starved for 4 hrs and then treated with 10 ng/ml insulin for the times indicated. Activation of Akt/PKB was evaluated by Western Blot on whole protein lysates probed with anti-Phospho-Akt Ser473 and anti-Akt. Levels of endogenous TBP-1 and of two proteasome components (C8 and Rpt-6) were analyzed where indicated. TBP-1 bands intensity was measured by ImageQuant analysis on two different expositions to assure the linearity of each acquisition, each normalised for the respective actin values. Asterisk, fold value is expressed relative to the reference point, (i.e. TBP-1 levels in starved cells) arbitrarily set to 1. Representative of three independent experiments. **B:** U2OS cells or T11hT cells were treated, 24 hrs after plating, either with DMSO (/) or with 200 nM Wortmannin or 50 mM LY294002 for the times indicated. Cells were then lysed and Western Blot analysis was performed by using specific antibodies against Phospho-Akt Ser473, anti-Akt, anti-TBP-1, anti-C8 and anti-Rpt-6. TBP-1 bands intensity was calculated as in A. Representative of three independent experiments. **C:** U2OS cells were transfected with empty vector (lane 1) or increasing amounts of the constitutive active mutant of the Akt kinase (CA-Akt). After 24 hrs cells were lysed and whole cell lysates probed with anti-Phospho-Akt Ser473, anti-Akt, anti-TBP-1, anti-Rpt-1, anti-Rpt-6, and anti-

phospho-GSK3b. **D:** U2OS cells were transfected with a *si*RNA directed against MDM2 or Luciferase, as control, at the final concentration of 10 nM. After 24 hrs, cells were starved for 4 hrs and then treated with 10 ng/ml insulin for the times indicated. Cells were then lysed and Western Blot analysis was performed by using specific antibodies against Phospho-Akt Ser473, TBP-1, MDM2, Akt, and actin. **E:** U2OS cells were transfected with a *si*RNA directed against MDM2 or Luciferase, as control. After 48 hrs, either DMSO (/) or 200 nM Wortmannin was added to the cells and left for the times indicated. Cells were then lysed and Western Blot analysis was performed by using specific antibodies against Phospho-Akt Ser473, TBP-1, MDM2, Akt and actin.
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mechanism. On the other hand, other reports [37] underline an *in vivo* requirement of ARF for full activation of PTEN, one of the major negative regulators of Akt activity.

In conclusion, our data well support a role for TBP-1 in the attenuation of Akt/PKB activity and place this protein with a key role in the control of cell proliferation. Even though, further studies are necessary to understand the potential cross-talks linking TBP-1 action on p14ARF and on Akt/PKB regulation.

Materials and Methods

Cell cultures, viral infection, transfections

T11hT (human primary fibroblasts immortalized by constitutive expression of the telomerase catalytic subunit h-TERT) human cell line was kindly provided by dr. Eric Gilson. T1, T10C and T10E (TBP-1 silenced clones) derived by retroviral infection of T11hT: briefly, 3×10^6 HEK 293-LinX packaging cells (kindly provided by Prof. Nicola Zambrano) were transfected with ARREST-IN (Open Biosystems, Huntsville, AL, USA) with pSUPERIOR.shTBP-1. 24 hrs after transfection, virus containing supernatant was filtrated through 0,45 μm cellulose acetate syringe filter, supplemented with 5 μg/ml polybrene, and used to infect recipient T11hT cells, previously plated at 50% confluence. Twenty-four hours following infection, 1 mg/ml G418 was applied to select stably infected cells. After three weeks, 23 individual single G418 resistant clones were picked up and expanded. Six neomycin resistant colonies from 5 different plates, were screened by Western Blot with anti-TBP-1.

Both T11hT and TBP-1 silenced clones were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine serum and 1 mg/ml puromycin (to maintain selection for h-TERT). U2OS cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine serum.

To obtain *sh*-TBP-1 pool and control pool, 2×10^6 T11hT cells were transfected by electroporation by making use of a Microporator MP-100 (Digital Bio Technology) either with 3 μg of pSUPERIOR.shTBP-1 or 3 μg of pSUPERIOR.retro.neo; twenty-four hours following transfection, 1 mg/ml G418 was applied to select cells. After four weeks, resistant cells were collected, expanded and analyzed.

Transfection by Lipofectamine2000 were performed as described [12].

Transfection of the T1 clone was performed by the use of a Microporator MP-100 (Digital Bio Technology) using either 2×10^6 cells with 2 μg of either pcDNA empty vector or pcDNATBP-1 (rescue of cell proliferation, Figure 2E and F) or 1×10^6 cells with either pcDNA empty vector or pcDNATBP-1 (0.3 or 0.6 μg) (rescue of Akt activation, Figure 5 E). Cells were then plated in DMEM+10% FBS for 24 hrs at 37°C or DMEM without FBS at 37°C and collected for subsequent analysis.

For transient silencing experiments, the duplex siRNA oligomer designed to target human TBP-1 is described in [12]; the duplex siRNA oligomer targeting human MDM2 has the following sequence: 5′- AAGCCAUUGCUUUUGAAGUUA-3′ and was designed as described in [19]. *si*RNA were all synthesized by MWG Biotech, Germany. Either U2OS, T11hT or T1 cells were transfected by Hyperfect (Quiagen, GmBH, Germany) according to the manufacturer's instructions.

Cell growth analysis, MTS assay, Flow cytometry analysis, Chemoinvasion assay

For cell growth analysis, T11hT parental cell line, T1, T10C and T10E clones, or T11hT, control pool and *sh*-TBP-1 pool, were plated in 100 mm dishes in presence of 10% FBS at the cell density of 1×10^5 cells/plate. Cells were cultured for 24, 48 and 72 hrs, collected, and counted in a Burker chamber. For growth in the absence of serum, after 6 hrs from plating, medium was removed and replaced with medium without serum. As above, cells have been grown for 24, 48 and 72 hrs, collected and counted. Each point is the result of triplicate samples.

Cell viability was evaluated using the MTS [3-(4,5-dimethylthiazhol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium,inner salt] (Cell Titer 96AQueous assay G358 purchased from Promega) colorimetric assay. Briefly, T11hT parental cell line or cells derived from the T1 clone were plated at different cell densities as indicated in 96 well plates (2.5×10^3/well, 10^3/well, 3×10^2 /well) either in DMEM or in DMEM+10%FBS. After six hrs from plating, 1:5 MTS solution was added to each well and the cells were incubated for 30′ at 37°C. Plates were read on a Microplate Reader (BIO-TEK Instruments, Model Elx800) at 492 nm. Survival was expressed as the percentage of viable cells in treated samples relative to non-treated control cells. All the experiments were repeated in quintuplicate.

For flow cytometry analysis, T11hT and T1 cells were counted and seeded at 2×10^5cells/35 mm plate. At 24 hours after plating the medium was replaced and the cells treated with DMEM containing 10% Fetal bovin serum or DMEM without serum. At the indicated time points, cells were collected, centrifuged, washed twice with PBS 1× and then fixed with ice-cold 70% ethanol. Fixed cells were incubated with staining buffer solution (50 μg/ml PI and 50 μg/ml RNase A in PBS pH 7.4) for 20 minute at room temperature in a dark box. Stained cells were analysed in a fluorescence-activated cytometer (DakoCytomation). Data on DNA cell-content were acquired on 20,000 events at a rate of 150±50 events/second and the percentages of cells in the SubG1, G0–G1, S and G2–M phases were quantified with Summit v4.1 software.

Chemoinvasion assays were performed in Boyden chambers using 8 μm pore size PVPF polycarbonate filters coated with 50 μg/ml of Matrigel. 1×105 cells were plated in the upper chamber in serum-free medium. 100 ng/ml EGF or serum free medium was added in the lower chamber. Cells were allowed to migrate for 4 h at 37°C, 5% CO2. To examine basal migration, serum free medium was added to both upper and lower chamber, and migration was allowed for 12 h at 37°C, 5% CO2, in the absence of chemoattractants.

The cells on the lower surface of the filter were then fixed in ethanol, stained with hematoxylin, and counted at 200× magnification (10 random fields/filter).

Western Blotting, Immunoprecipitations, Insulin treatments, MG132 treatment

Western Blots were performed as described [12]. Antibodies to Akt (used in 1:1000 dilution), Phospho-Akt Ser473 (used in 1:1000 dilution), Phospho-GSK-3β/pSer21/9 (used in 1:1000 dilution),

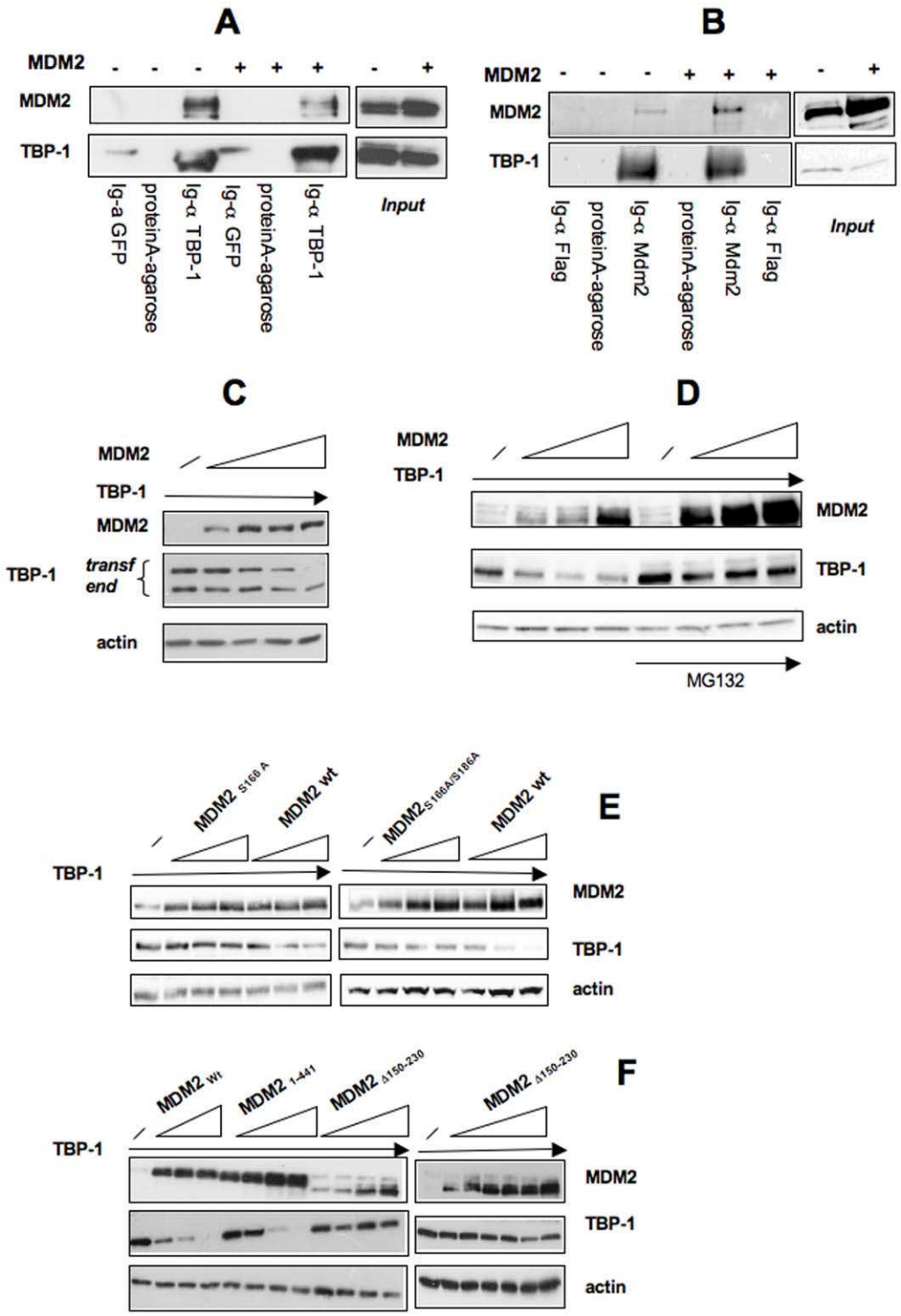

Figure 7. TBP-1 is a downstream target of MDM2 activation. A: U2OS cells were either transfected (lanes +) or untransfected (lanes −) with the MDM2 expression plasmid. 24 hrs after transfection cell extract was prepared and subjected either to immunoprecipitation with anti-TBP-1 antibody where indicated or, with anti-GFP antibody as negative control. Cell extracts were also incubated with protein A-agarose as control, where indicated. Immunoprecipitated extracts were analyzed by Western Blot with anti-MDM2 or anti-TBP-1 antibody. Aliquots of cell extracts were analyzed by Western Blot before immunoprecipitation (*input*). **B:** U2OS cells were either transfected (lanes +) or untransfected (lanes −) with the MDM2 expression plasmid. 24 hrs after transfection cell extract was prepared and subjected either to immunoprecipitation with anti-MDM2 antibody where indicated or, with anti-Flag antibody as negative control. Cell extracts were also incubated with protein A-agarose as control, where indicated. Immunoprecipitated extracts were analyzed by Western Blot with anti-MDM2 or anti-TBP-1 antibody. Aliquots of cell extracts were analyzed by Western Blot before immunoprecpitation (*input*). **C:** U2OS cells were transfected with TBP-1 expression plasmid and increasing amounts of MDM2 expression plasmid. After 24 hrs, cells were lysed and whole cell extracts probed with anti-TBP-1, anti-MDM2, and anti-actin, for loading control. **D:** U2OS cells were transfected with TBP-1 expression plasmid and increasing amounts of MDM2 expression plasmid. After 24 hrs cells were treated

either with DMSO (first four lanes) or with 10 μM MG132 where indicated. Cell extracts were analyzed by Western Blot with anti-Xpress (to reveal transfected TBP-1), anti-MDM2, and anti-actin, for control. **E:** U2OS cells were transfected with TBP-1 expression plasmid and increasing amounts of either $MDM2_{wt}$, $MDM2_{S166A}$ or $MDM2_{S166A/S186A}$ expression plasmids. After 24 hrs cells were lysed and whole cell extracts were analyzed by Western Blot with anti-Xpress (to reveal transfected TBP-1), anti-MDM2, and anti-actin, for control. **F:** U2OS cells were transfected with TBP-1 expression plasmid and increasing amounts of either $MDM2_{wt}$, $MDM2_{1–441}$ or $MDM2_{\Delta150–230}$ expression plasmids. After 24 hrs cells were lysed and whole cell lysates analyzed by Western Blot with anti-Xpress (to reveal transfected TBP-1), anti-MDM2, and anti-actin, for control.
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Caspase-3 (1:1000) and PARP-1 (1:1000) were purchased from Cell Signalling Technologies, Boston, MA, USA. Antibodies to MDM2 (used in 1:500 dilution) was purchased from Calbiochem, to Rpt-1 (PSMC2) (used in 1:6000 dilution), Rpt6 (PSMC5) (used in 1:6000 dilution) and C8 (used in 1:6000 dilution) were purchased from BioMol. Anti-Xpress antibody (used in 1:1000 dilution) was purchased from Invitrogen. Secondary antibodies for Western Blot analysis (goat anti-rabbit IgG-HRP 1:3000 dilution) were purchased from Santa Cruz Biotechnology, CA, USA. Proteins were visualized with an enhanced chemiluminescence detection system (Amersham ECL ^{TM}) and images were taken with ChemiDoc XRS System (Bio-Rad Laboratories) and analysed with the QuantityONE software.

For insulin treatment, U2OS cells were transfected by Lipofectamine with 0.2 and 0.5 μg of the pcDNATBP-1 plasmid. At 24 hrs after transfection, cells were starved for 4 hrs and then treated with 10 ng/ml insulin for 10′.

To analyze TBP-1 levels following insulin treatments, either U2OS cells or T11hT cells were starved for 4 hrs and then treated with 10 ng/ml insulin for the times indicated.

For immunoprecipitation in U2OS cells, 1.0×10^6 cells were seeded in 100 mm dishes and transfected with the plasmids indicated in the figure legend. Cells were harvested 24 hours after transfection and cell lysates were prepared as described [12]:

800 μg of whole cell extract were incubated overnight at 4°C with anti-TBP1 (BioMol) or anti-MDM2 C18 (Santa Cruz). Controls of immunoprecipitations were perceived with mouse anti-GFP (Roche) or rabbit anti-Flag (Sigma). Immunocomplexes were collected by incubation with 30 μl of protein A-agarose (Roche Applied Science) at 4°C for 4 hrs. The beads were washed with Co-Ip buffer (50 mM tris-HCL pH 7.5; 150 mM NaCl; 5 mM EDTA; 0,5% Np40), resuspended in 2× loading buffer (Sigma) and loaded on a SDS-8% polyacrylamide gel.

Treatment with proteasome inhibitor was performed as follows: U2OS cells were treated either with DMSO or 10 μM MG132 for five hours. Cells were harvested and total extracts prepared for subsequent analysis as described.

Constructs

pSUPERIORshTBP-1 has been obtained from pSUPERIOR.-retro.neo (Oligoengine) by cloning into BglII-HindII sites a duplex oligonucleotide obtained by MWG-Biotech that could give rise to a short hairpin RNA specifically designed to silence TBP-1 expression.

Oligoseq: $^{5'}$GATCCCCAACAAGACCCTGCCGTACCTT-CAAGAGAGGTACGGCAGGGTCTTGTTTTTTTA$^{3'}$

pCA-Akt plasmid was kindly provided by Prof. G. Condorelli. The $MDM2_{1–441}$ and $MDM2_{\Delta150–230}$ expression plasmids were previously described [19].

Plasmids $MDM2_{S166A}$ and $MDM2_{S166A/S186A}$ mutant were generated by Quick Change Site Direct Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and amplified using the following primers:

S166A (F) $^{5'}$GGAGAGCAATTGCTGAGACAGAAG $^{3'}$,

S166A (R) $^{5'}$CTTCTGTCTCAGCAATTGCTCTCC $^{3'}$

S166A/S186A (F) $^{5'}$ CGCCACAAAGCTGATAGTATTTCC-C $^{3'}$

S166A/S186A (R) $^{5'}$GGGAAATACTATCAGCTTTGTGG-CG$^{3'}$

PCR was performed with a 2720 Thermo Cycler Applied Biosystem.

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Author Contributions

Conceived and designed the experiments: AP Vdf PR GLM. Performed the experiments: MS LF FT LB LS DA. Analyzed the data: MS LF FT LB LS DA PR VC VdF GLM AP. Contributed reagents/materials/analysis tools: PR VdF. Wrote the paper: AP GLM VdF. MS and LF equally contributed to this work.

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