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An intriguing association between the p14ARF oncosuppressor and TBP-1, a multifunctional protein with a potential role in the control of cell growth.

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INDEX

INTRODUCTION

THE p14ARF TUMOUR SUPPRESSOR.

The ARF tumour-suppressor protein, also known as p14ARF in the human and p19ARF in the mouse, was identified as an alternative transcript of the INK4a/ARF locus that was previously shown to encode for p16INK4a, an inhibitor of the CDK4-CDK6 cyclin dependent kinases (Sharpless et al., 1999). The genetic alteration of this locus occurs at high frequency in a wide spectrum of human cancers such as glioblastoma, melanoma, pancreatic adeno-carcinoma, non-small cell lung cancer and bladder carcinoma (Sherr 1998, reviewed in Sharpless 2005).

Both human (p14ARF) and mouse (p19ARF) ARF are small and highly basic proteins, mainly localized in the nucleolus. They display no homology to other known proteins and share only 50% amino acid identity. The ARF proteins show significant sequence similarity within their 14 amino-terminal amino acids and this region retains many of the known ARF functions, including nucleolar localization, MDM2 binding and ability to induce cell cycle arrest. The carboxy-terminus of ARF also

encodes functional domain; in particular, the ability of ARF to promote the sumoylation of its binding partners involves the C-terminal nucleolar localization sequence of p14ARF (Xirodimas et al., 2002, reviewed in Gallagher et al., 2006). Furthermore, melanoma associated mutations targeting this functional domain impair the nucleolar localization and sumoylation activity of p14ARF (Rizos et al., 2005).

The ARF oncosuppressor is among the most relevant oncogenic stress sensor in mammalian cells. In normal conditions, cells contain low levels of ARF but the expression of a variety of proliferation-promoting proteins, activates ARF as a part of checkpoint response that counters oncogenic signals by promoting cell cycle arrest or apoptosis through both p53-dependent and independent mechanisms.

Interestingly, p19ARF was also found to be upregulated in senescent mouse fibroblast. In contrast, the human p14ARF does not appear to be required for the senescence process; p14ARF expression levels, in fact, remain low as cells near senescence (Sharpless 2005). The differential role of mouse and human proteins in promoting senescence may relate to differences in their regulation.

Expression of p19ARF is induced by many oncogenes including c-Myc, adenovirus E1A, Ras, E2F1 and v-Abl (Sharpless 2004). Much less is known about the regulation of p14ARF expression. p14ARF

transcription is regulated by members of the E2F family (Parisi et al., 2002), a group of transcription factor that control the expression of genes that are involved in cell cycle progression by acting either as transcriptional repressor, in conjugation with members of retinoblastoma family, or as transcriptional activator. The regulation of p14ARF gene by E2F results very particular and is different from that of classical E2F targets. p14ARF is not induced when fibroblasts re-enter the cell cycle following serum addition, as would be expected of an E2F-responsive gene. p14ARF only responds to aberrant levels of E2F ignoring the physiological fluctuations associated with cell-cycle progression (Figure 1). This different regulation is mediated by a novel E2F-responsive element (EREA) in the ARF promoter that varies from the typical E2F site, discriminating abnormal growth signals due to ectopic expression of E2F1 from normal growth signals (Komori et al., 2005). Since E2F3 maintains ARF promoter in repressed state when there is no oncogenic stress signal, the activating E2F must somehow override or negate the repressive function of E2F3 to ensure ARF induction (Aslanian et al., 2004).

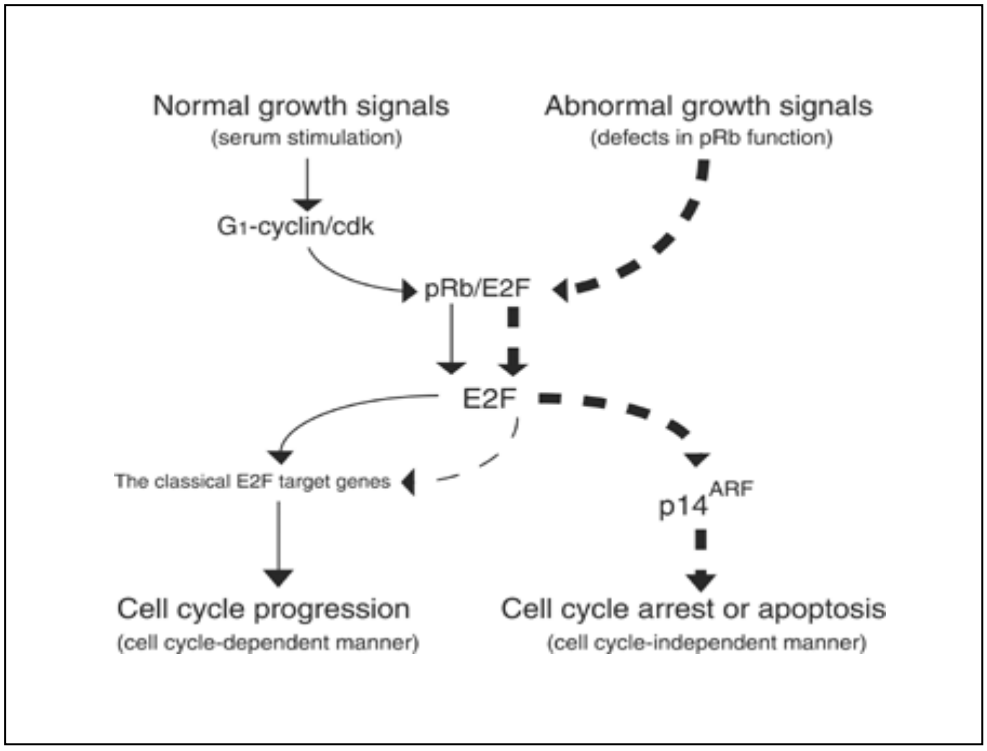

Figure 1 Model for transcriptional regulation of the ARF gene expression by E2F.

Deregulated E2F that arises through defective pRb function activates the ARF promoter through EREA independently of the cell cycle, whereas physiological E2F activity induced during the normal cell cycle cannot activate the ARF promoter.

Recently, it has been described a short form of the ARF tumour suppressor, whose translation begins from the internal methionine codon within both human and mouse (Met48 in p14ARF e Met45 in p19ARF). Also smARF levels were elevated in response to ectopic expression of viral and cellular oncogenes. These unstable proteins localize into mitochondria and are capable to trigger autophagy, a process usually initiated in response to nutrient starvation in which cells digest their own organelles, in an effort to derive energy (Reef et al., 2006). smARF, as well as the full-length version of the protein, does not contain lysines although is quickly degraded by the proteasome.

p53-dependent activity of the p14ARF tumour-suppressor.

ARF expression following aberrant oncogene activation leads to the induction of the p53-pathway. The ARF induction of p53 is mediated through two ubiquitin ligases, MDM2 and ARF-BP1/Mule (ARF-binding protein 1/Mcl-1 ubiquitin ligase E3). Both MDM2 and ARF/BP1 act as specific E3 ubiquitin ligase for p53, are highly expressed in various types of tumours, and have the potential to abrogate the tumour-suppressor function of p53.

MDM2 modulates p53 activity by directly blocking its transcriptional activation functions and by promoting its polyubiquitination and

proteasome-mediated degradation (Kamijo et al., 1998; Pomerantz et al., 1998). ARF interacts with the central acidic domain (CAD) of MDM2 (Bothner et al., 2001) and inhibits MDM2 E3 ligase function, resulting in p53 stabilization and consequent upregulation of p53 target genes, such as BAX and $p21^{Waf1}$ (Midgley et al., 2000). In addition, ARF sequesters MDM2 in nucleoli, thus relieving nucleoplasmic p53 from MDM2-mediated degradation. However, recent data, suggest that nucleolar relocalization of MDM2 is not required for p53 activation and that the redistribuition of ARF into the nucleoplasm enhances its interaction with MDM2 and its p53-dependent growth-suppressive function (Korgaonkar et al., 2005).

In addition to MDM2, ARF-BP1 is a key regulator of the p53 cell cycle regulator pathway; ARF-BP1 directly binds and ubiquitinates p53 in an MDM2-independent manner. Silencing of ARF-BP1 extended the half-life of p53, resulting in the transcriptional activation of p53 targets like $p21^{Waf1}$ and BAX, and activating a p53-dependent apoptotic response (Chen et al., 2005). ARF-BP1 possesses both anti-apoptotic (via p53 degradation) and pro-apoptotic (via Mcl-1 degradation) functions. Following aberrant oncogenic stimuli, ARF is induced and inhibits ARF-BP1 activity toward p53 in the nucleus, thereby leading to p53 dependent cell cycle arrest or apoptosis. In the cytoplasm, where ARF is

not present, oncogene activation may lead to ARF-BP1 mediated Mcl-1 degradation (Figure 2). In this way ARF would act as a regulator keeping the balance between pro-apoptotic and anti-apoptotic functions.

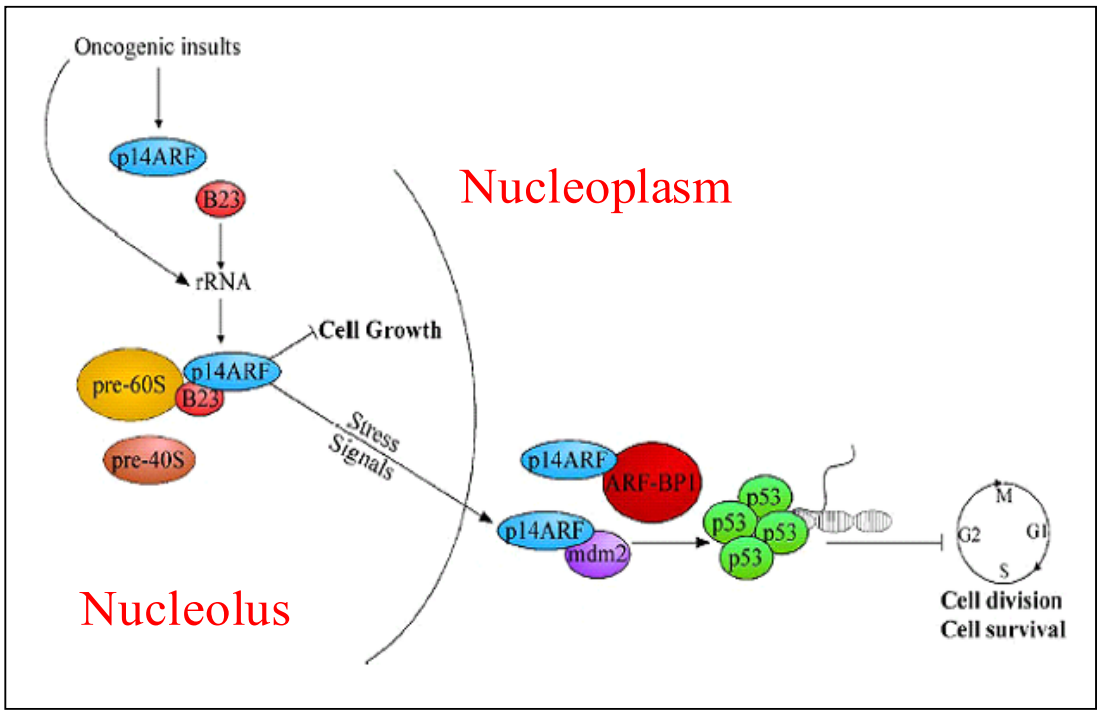

Figure 2 Proposed mechanism of ARF action.

Aberrant oncogenics activation leads to ARF induction and increased rRNA transcription. Under these conditions ARF localizes predominantly in the nucleolus, where it is bound to B23. This complex may allow ARF to influence the programme of cell growth, at least in part, by inhibiting the processing of rRNA. When the cell undergoes to cytotoxic or genotoxic stress, B23 and ARF redistribuite to the nucleoplasm to complex with MDM2 and/or ARF-BP1. These binary complexes inactivate MDM2 and ARF-BP1 ubiquitine-ligase, causing p53 stabilization and leading to cell cycle arrest or cell death.

p53-independent activity of the p14ARF tumour-suppressor.

Although it is generally accepted that much of ARF's tumour-suppressor activity is mediated trough p53, a growing body of evidences suggests that ARF has also p53-independent function.

First evidences came from $ARF^{-/-}$ mice that develop carcinomas and neurogenic tumours rarely seen in $p53^{-/-}$ mice. In addition, c-Myc-induced lymphomas are more aggressive in $ARF^{-/-}$ / $p53^{-/-}$ mice compared to either $p53^{-/-}$ or $ARF^{-/-}$ alone (Eishen et al., 1999). Triple knock out mice nullizygous for ARF, p53 and MDM2 develop multiple tumours at higher frequency than that of mice lacking both p53 and MDM2 or p53 alone (Weber et al., 2000). Recently, it has been reported that tumours emerged significantly earlier in mice lacking both ARF and p53 than in the mice lacking p53 alone (Christophorou et al., 2006).

One of the most streaking evidence of the antiproliferative p53 independent activities of ARF is its involvement in ribosome biogenesis. ARF retards the processing of early 47S/45S and 32S rRNA precursors, as demonstrated in mouse cells (Sugimoto et al., 2003; reviewed in Sharpless 2005) and can inhibit the processing of ribosomal RNA through the direct binding to B23, an abundant nucleo/nucleolar endoribonuclease that plays a key role in the ribosome biogenesis. B23 overexpression induces the cells in mitosis whereas its silencing causes

cell cycle arrest. ARF causes B23 proteasomal degradation by increasing its ubiquitination rate and bringing to an inhibition of rRNA processing (Itahana et al., 2003; Bertwistle et al., 2004).

Several studies also have linked the antiproliferative functions of ARF to its capability to strongly inhibit the HIF-1 (hypoxia indicible factor-1) transcription factor. This effect requires the binding to HIF-1α and its nucleolar relocalization (Fatyol et al., 2001). The alpha subunit of HIF-1 was found to be frequently overexpressed in advanced tumours.

Another p53-independent effect particularly interesting regards the negative regulation of transcription factor such as E2F1 and c-Myc. ARF expression causes relocalization of E2F1 from the nucleoplasm to the nucleolus inducing its degradation through the ubiquitin-proteasome pathway (Martelli et al., 2001; Eymin et al., 2001). Moreover, ARF binds to c-Myc, relocalizes its in the nucleolus and blocks c-Myc ability to activate transcription and induce hyperproliferation and transformation (Datta et al., 2004).

The p53-independent tumour-suppressive function of ARF can be also mediated by its ability to enhance sumoylation of many protein targets. Sumoylation is analogous to ubiquitination, and is the process by which the SUMO (small ubiquitin-like modifier) protein is conjugated to a target protein. Effect of sumoylation are highly diverse and can influence

protein transport, modulation of gene expression, DNA repair and centromeric chromatid cohesion. ARF promotes sumoylation of its binding partners acting as an adaptor molecule, which recruit E2-SUMO conjugative enzyme Ubc9 to their substrates (Tago et al., 2005). Until now, only for B23 it is possible to associate a biological effect of sumoylation mediated by ARF: the increase of sumoylation, in fact, brings to a deep impact on B23 activity inside the cell affecting its subcellular localization, cellular proliferation and survival (Liu et al., 2007). Recent studies have demonstrated that ARF can increase sumoylation of B23 controlling the stability of the nucleolar SUMO-2/3 deconjugating enzyme SENP3 (Kuo et al., 2008). In particular, ARF promotes phosporylation dependent ubiquitination and proteasomal degradation of SENP3. Interestingly, the ability of ARF to accelerate SNP3 turnover also depends on the presence of B23. Conversely, the association of SENP3 with B23 can counteract the effect of ARF by deconjugating SUMO from B23. These observations suggest that ARF and SNP3 antagonize each others' s functions and that B23 acts as a "platform" for both the proteins, bringing in close proximity its two regulators.

Antiviral action of the ARF tumour suppressor.

While it is clear that p53 is involved in the antiproliferative and antiviral effects of IFNs and viral infection (Takaoka et al., 2003), the role of ARF, one of the most important activators of p53, in the viral response, only recently has been highlighted.

The first evidence came from the group of Sandoval (2004). They found that type I interferon up-regulates p53 via ARF, as demonstrated by the absence of p53 induction in ARF-/- mouse embryonal fibroblasts (MEFs) and in ARF null human U2OS cells. Interestingly, they showed an increase in ARF expression following treatment of SAOS cells (p53-/-) with interferon or viral infection, leading to the hypothesis that ARF can act as antiviral also in a p53-independent pathway.

The role of ARF as a sensor of viral stress was also demonstrated by the group of Garcia (2006). They analysed the cytopathic effect caused by VSV (vescicular stomatitis virus) infection of MEFs derived from animals with different ARF gene dosage and observed a clear ARF dosage dependent protection against viral infection. This antiviral action is mediated, at least in part, through the inhibitory effect that ARF exerts on one of its main molecular partner, NPM/B23, involved in the repression of the double-stranded RNA-dependent protein kinase, PKR, one of the principle effectors of the antiviral response. The final effect is

an ARF-induced release and activation of PKR from B23 complexes that leads to VSV attenuation.

ARF regulates the protein turnover and function of most of its interacting partners.

ARF response is exerted through the activation of complex signalling networks accomplished by the interaction with a multitude of different cellular partners. During the last years many efforts have been attempted in search of ARF partners that could partly explain the p53-MDM2 ARF independent functions.

In addition to its first "spouse" MDM2, the ARF interactors "harem" consists of something like 30 different proteins involved in various cellular activities (Figure 3): proteins involved in **transcriptional control**, such as E2Fs, DP1, c-myc, p63, HIF1α, Foxm1b, **nucleolar proteins** such as nucleolin/C23 and nucleophosmin (NPM/B23), **viral proteins** such as HIV-1Tat, proteins involved in copper **metabolism** like COMMD1, proteins involved in **chromosomal stability** and/or chromatin structure such as Topoisomerase I, Tip60, and WRN helicase, **ubiquitin ligases** like Ubc9 (the E2 ligase required for sumoylation), MDM2 and ARF-BP1/Mule, (E3 ubiquitin ligases).

Although the actual mechanism by which ARF affects the function of its partners is still unclear, the functional consequence is, quite invariably, inactivation (Lowe et al., 2003).

For some targets, ARF interaction causes alteration of stability. For example, *B23/NPM* and *E2Fs* become degraded by the proteasome in a ubiquitin-dependent manner, while the *CtBP2* antiapoptotic transcriptional co-repressor and *HIV-1 Tat* become degraded by the proteasome in a ubiquitin-independent manner. Other targets changes their localization like *E2Fs, c-myc, Foxm1b, MDM2, ATR, DP-1, Hif1*α upon ARF expression. Only few others, like *Tip60*, *Topo I* and *COMMD1* become activated or stabilized. Finally, most of the partners become sumoylated although a precise function to this modification has not yet been assigned (Rizos et al., 2005).

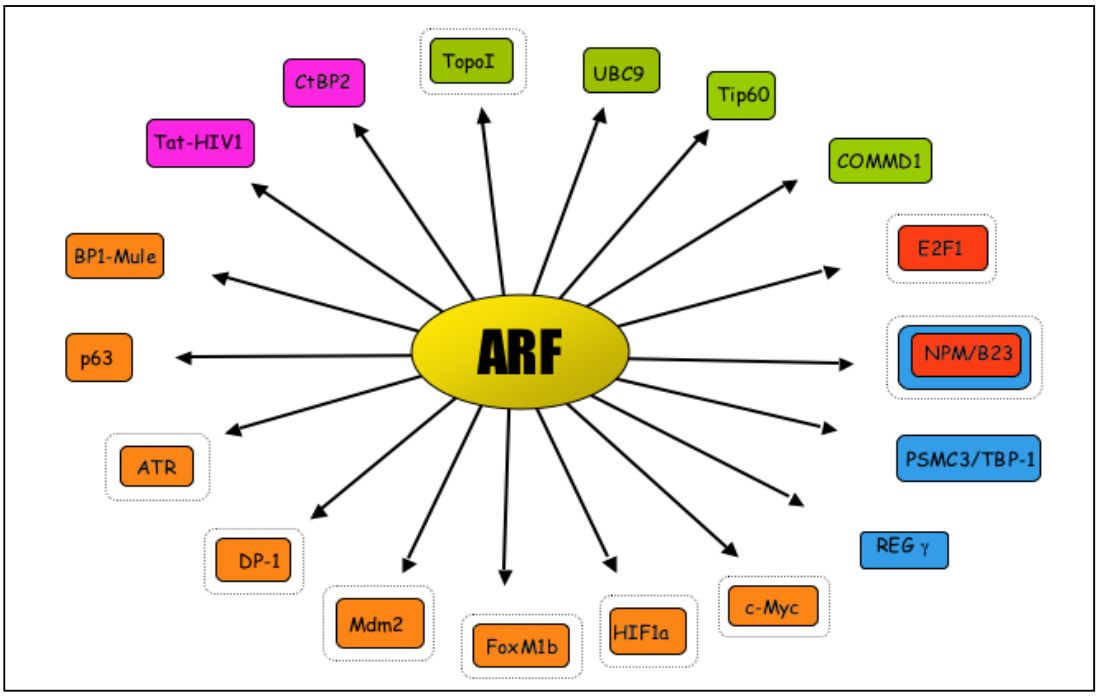

Figure 3 Some of the ARF interactors "harem".

Orange is for partners whose activity is blocked by ARF. **Red** is for partners that are induced to proteasome and ubiquitin dependent degradation by ARF. **Pink** is for partners that are induced to proteasome dependent, ubiquitin independent degradation by ARF. **Green** is for partners whose activity or stability are positively regulated by ARF. **Blue** is for partners that regulate ARF protein turnover. A second black circle indicates nucleolar sequestration.

Particularly interesting is the inhibitory effect that ARF exerts on oncogenes such as members of the E2F family, required for cell-cycle progression and to mediate ARF oncogenic activation, suggesting a potential role of these interactions as being part of a negative feedback loop. In a series of reports ARF was shown to interact with E2F1, and this interaction prevented the formation of active E2F1 transcritional complexes (Datta et al., 2002), inhibited E2F1 transactivation potential (Eymin et al., 2001) and promoted the proteasome dependent degradation of E2F1 (Martelli et al., 2001). The regulation of the ARF-dependent E2F1 turnover by the proteasome appears to involve E2F1 ubiquitination (Rizos et al., 2007), although the genetic context in which this process occur is not yet completely clear.

In line with a role of ARF in promoting ubiquitin dependent degradation of its partners is the observation that NPM/B23, is a molecular target of ARF. The vast majority of ARF appears localized in nucleoli, tightly associated with NPM/B23. Interestingly, the ARF-B23/NPM interaction seems critical in the regulation of both proteins (Korgaonkar et al., 2005; Enomoto et al., 2006). Under normal conditions, B23 appears to retain ARF in the nucleolus, retarding its turnover. Indeed, the stability and nucleolar localization of ARF is markedly reduced in cells lacking NPM and leukemia-associated NPM mutants are unable to stabilize ARF

(Colombo et al., 2006). However, ARF hyper-expression induces B23 proteasomal degradation by increasing its ubiquitination rate and proteasomal degradation bringing to an inhibition of rRNA processing (Itahana et al., 2003). These observations lead to the conclusion of the existence of a regulative loop between ARF and B23, in which degradation and inhibition of both proteins is finely and tightly modulated by external stimuli. In such a situation, ARF serves a dual function to restrain both growth and proliferation.

Interestingly, ARF appears to mediate also ubiquitin-independent degradation of the antiapoptotic transcriptional corepressor C-terminal Binding Protein 2 (CtBP2). CtBP2 is degraded by the proteasome after UV exposure leading to apoptosis in colon cancer cells (Zhang et al., 2003). It has been shown that UV induced CtBP2 degradation by the proteasome is dependent on ARF intracellular protein levels. Moreover, human ARF hyper-expression without UV stimulation, resulted in proteasome mediated degradation of CtBP2, not accompanied by an appreciable increase in CtBP2 ubiquitination (Paliwal et al., 2006) although CtBP2 is ubiquitinated in the cells used. Still open is the possibility that the experimental system used was not enough sensitive to detect changes in the levels of ubiquitination.

p14ARF has also been shown to mediate the proteasome-dependent, ubiquitin independent degradation of the HIV1-Tat protein (Gargano et al., 2008). Interestingly, MDM2 has been shown to ubiquitinate HIV1-Tat, although, in this case, ubiquitination determines an increase of the Tat-mediated transactivation properties (Bres et al., 2003). This lead to the speculation that ARF could act on HIV-1Tat in two ways: directly mediating its degradation and/or inhibiting the MDM2 activity versus Tat, thus blocking viral transcription. This hypothesis would intriguingly fit with the ARF role in viral defence (Garcia et al., 2006).

As mentioned above, in some cases, ARF is able to stabilize its partners from proteasomal degradation. In a quite recent study, it has been described the ARF's ability to induce a non-classical polyubiquitination of a new interacting partner, the COMMD1 factor 60, a multifunctional protein involved in copper metabolism and apoptosis. While in normal conditions COMMD1 is degraded by the proteasome, ARF coexpression leads to a stabilization of the protein through its poly-ubiquitination on K63 lysine of the ubiquitin peptide. K63 is distinct from the classical, K48-linked ubiquitination, usually involved in protein degradation. Interestingly, most neurodegenerative diseases like Parkinson disease, are marked by the presence of ubiquitin-positive protein inclusions that escape proteasomal degradation despite being enriched with ubiquitin: it

has been observed that K63 polyubiquitination plays a role in this stabilization (Lim et al., 2005).

Altogether, although these observations do not explain the molecular mechanisms of ARF effects on many of its targets, they reinforce the idea that its antioncogenic activity could be partly exerted through the cellular degradation machinery.

In this sense ARF interaction with the proteasome could serve dual roles: on one side it is necessary to regulate ARF protein turnover, on the other side it could play a role in bringing its interacting partners to the ubiquitin/proteasome machinery (Pollice et al., 2008).

ARF intracellular levels are regulated by the proteasome.

The proteasome is the chief site of protein destruction in eukaryotic cells. It is made by the dynamic, ATP-dependent association of the 20S catalytic proteasome (made by four stacked heptameric rings) with the regulatory 19S subunit that sits as a "collar" on one or both ends of the 20 S proteasome -although “free” 20S as well as 19S caps can be found in the cell (Hershko et al., 1998; Adams et al., 2004). 19S regulatory subunit is composed of a “base” (with ATPase activity) that binds directly to the 20S core particle, and a “lid” that usually contacts substrates that have to be degraded. Protein proteolysis requires

recognition of the substrate, its unfolding and translocation into the cavity of the 20S proteasome. 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). Nonetheless, the mechanism by which proteasomes recognize proteins in the absence of ubiquitination is not understood and appear to mainly regard small, unstructured proteins that can gain direct access to the core proteasome without the need for a specific interaction mechanism.

Activation of the proteasome can be achieved also by binding of the 11S (also called REG or PA28) regulator that, alternatively to the 19S, triggers the "opening of the gate". The REG/11S particle can be found, as the 19S proteasome, independent or associated with 20S proteasomes as a heptameric lid and participate to its activation in an ATP-independent fashion. The REG family of activators has three members: REGα and REGβ subunits, mainly localized in the cytoplasm, enriched in the endoplasmic reticulum, usually involved in the MHC class I antigen presentation, and REGγ primarily residing in the nucleus

(Slaughter et al., 1992; Realini et al., 1997). γ-interferon treatment appears to induce an increase in the levels of REGα and REGβ subunits, but not a change in their localization. The REGγ function 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). Recently it has been isolated a chimerical form of the proteasome composed by both the regulative subunits, whose function is not yet known (Figure 4).

The first evidence of a link between ARF and the proteasome is the observation that both human and mouse ARF accumulate following treatment with proteasome inhibitors suggesting that ARF degradation depends, at least in part, by the proteasome (Rodway et al, 2004; Kuo et al., 2004; Pollice et al., 2007).

For the vast majority of proteins, conjugation of ubiquitin to internal lysines is the initial event in their degradation by the ubiquitin-proteasome system. However, the human p14ARF protein is lysine-less and the murine p19ARF has a single lysine residue whose ubiquitination is not required for its proteasome degradation (Kuo et al. 2004). The same author observed that ARF can be subjected to N-terminal ubiquitination, a process described in engineered lysine-less mutants of certain viral and cellular proteins and that this process appears independent from p53 and MDM2.

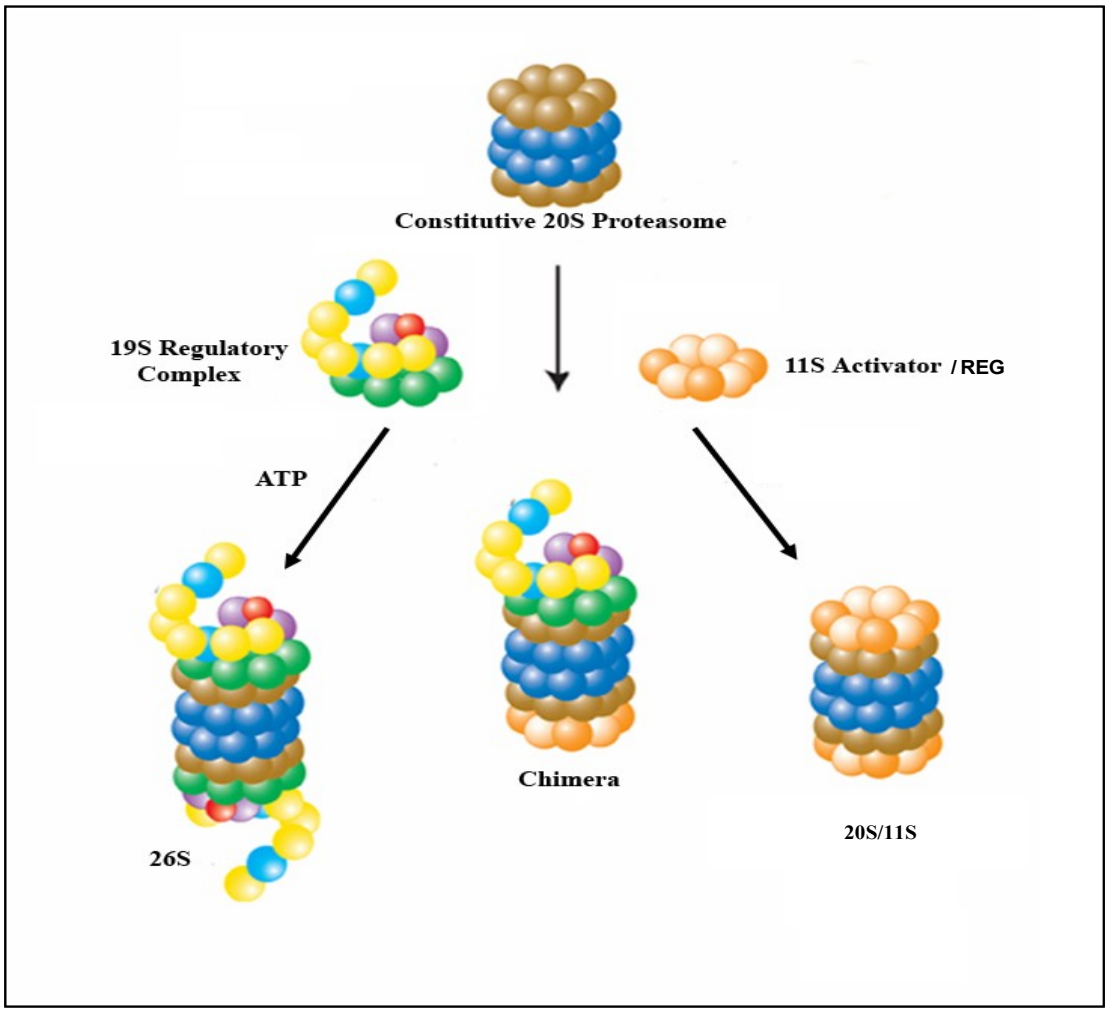

Figure 4 Structure of the proteasome.

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.

The N-terminal ubiquitination is strictly dependent by the sequence of N-terminal amino acids. The vast majority of eukaryotic proteins are acetylated at their N-termini, being acetylation and ubiquitination two processes in competition with each other. Generally, basic residues inhibit acetylation but favour ubiquitination. Examination of the ARF N-termini (human: Met-Val-Arg; mouse: Met-Gly-Arg) predicts that they should be processed by methionine aminopeptidases (methionine is a good substrate for acetylation but when followed by small amino acids it is cut by a specific amino peptidases) and that acetylation of valine and glycine should be strongly inhibited by the penultimate arginine residue. However, other authors do not report ubiquitination of the protein but claim the importance of MDM2 in ARF proteasome degradation. Gordon Peters's group (2004) investigated on the effects of ARF subcellular localization on its turnover and demonstrated that the stability of ARF can be enhanced by redirecting it to the nucleolus, by depleting the cells of MDM2 or by inhibiting proteasome functions, suggesting a model in which ARF associates with MDM2 in the nucleoplasm and is consequently subjected to rapid degradation. The ARF binding to MDM2 could explain why it is difficult to visualize ARF in the nucleoplasm, where it would be vulnerable to MDM2-

mediated degradation. Thus, in binding to MDM2 and blocking its E3 ubiquitin-ligase activity towards p53, ARF committees a "suicide".

Interestingly, a very recent report has described a direct involvement of the REGγ proteasome in a ubiquitin-independent regulation of the ARF turnover (Figure 5). The demonstration that REGγ governs the turnover of ARF and of other important cell-cycle regulators like p21cip and p16INK4a, suggests that the REGγ pathway represents a new important mechanism to control protein turnover (Chen et al, 2007). These observations lead to the hypothesis that this pathway could be specialized for the proteasomal degradation of small unstructured proteins since p19ARF, p21, and p16 are all unstructured when not associated with specific binding partners (such as cyclins and Cdks, for p21 and p16, and nucleophosmin in the case of p19ARF), in agreement with previous data suggesting a role for REGγ only in the degradation of short peptides. However, the REGγ ubiquitin-independent mediated proteolysis of the oncogenic protein SRC-3 (Steroid receptor coactivator-3) challenged the idea that REGγ is involved only in the degradation of substrates with disordered elements (Zhou et al., 2006; Li et al., 2007). An interesting possibility is that the feature of proteins targeted to the REGγ pathway is the lack of ubiquitination, usually due

to the lack of lysine residues. Both p16 and human p14ARF are naturally lysine-less proteins and p21 and SRC-3 can be degraded in the absence of ubiquitination. Interestingly, viral proteins constitute a substantial subset of naturally lysine-less proteins. This raises the hypothesis that the REGγ pathway might play a role in the control of viral pathogenesis. This is very interesting, since ARF activation has been linked to viral response (Garcia et al., 2006).

In this scenario, it is particularly intriguing our observation that one of the ATPases, component of the 19S proteasome, TBP-1, interacts with p14ARF but, unexpectedly, instead of bringing it to degradation, it is involved in its stabilization (Pollice et al., 2004 and 2007).

TBP-1, A MULTIFUNCTIONAL PROTEIN MEMBER OF THE 19S SUBUNIT OF THE PROTEASOME.

TBP-1 (Tat- Binding Protein 1), is transcribed from the PSMC3 locus that maps at the 11p12-13 region. Deletions of this region is present in many types of tumours (Hoyle et al., 1997).

Interestingly, 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 the cellular survival (Sakao et al., 2000).

TBP-1 is a member of a large conserved gene family of six ATPases (ATPAses Associated to a variety of cellular Activities) whose key feature is a highly conserved module of 230 amino acids consisting of an ATPase and a DNA/RNA helicase motif. Despite the sequence conservation, members of this protein family fulfill a large diversity of cellular functions including cell cycle regulation, gene expression, vesicle mediated transport, peroxisome assembly, and proteasome function. Indeed, as other members of the family, (MSS1/Rpt 1, S4/Rpt2, TBP-7/Rpt3, SUG2/Rpt 4, SUG1/Rpt 6 and Rpt5/TBP-1), TBP-1 is associated with the 19S regulatory subunit of the proteasome,

the chief site of protein destruction in eukaryotic cells (Figure 5). Consistent with this assumption, TBP-1, as well as the other 19S subunits, seems to recognize polyubiquitinate substrates and is involved in both the unfolding of target proteins and their subsequent translocation into the 20S proteolytic core.

TBP-1 was originally isolated in a screen for proteins interacting with the HIV1-Tat protein and it has been demonstrated to suppress Tat-mediated transactivation of HIV1 gene expression (Nelbock et al., 1990). More recently, it has been clarified the involvement of all six AAA-ATPases in the control of transcription elongation of the HIV1 transcriptome. TBP-1, although unable to bind DNA, is a strong transcriptional activator when brought into proximity of several promoter elements. Transcriptional activity depends upon the integrity of ATPase and helicase motifs (Ohana et al., 1993).

TBP-1 exerts a more general role on transcription, demonstrated by its activity in stimulating HBx-specific transcription of the HBV virus (Barak et al., 2000).

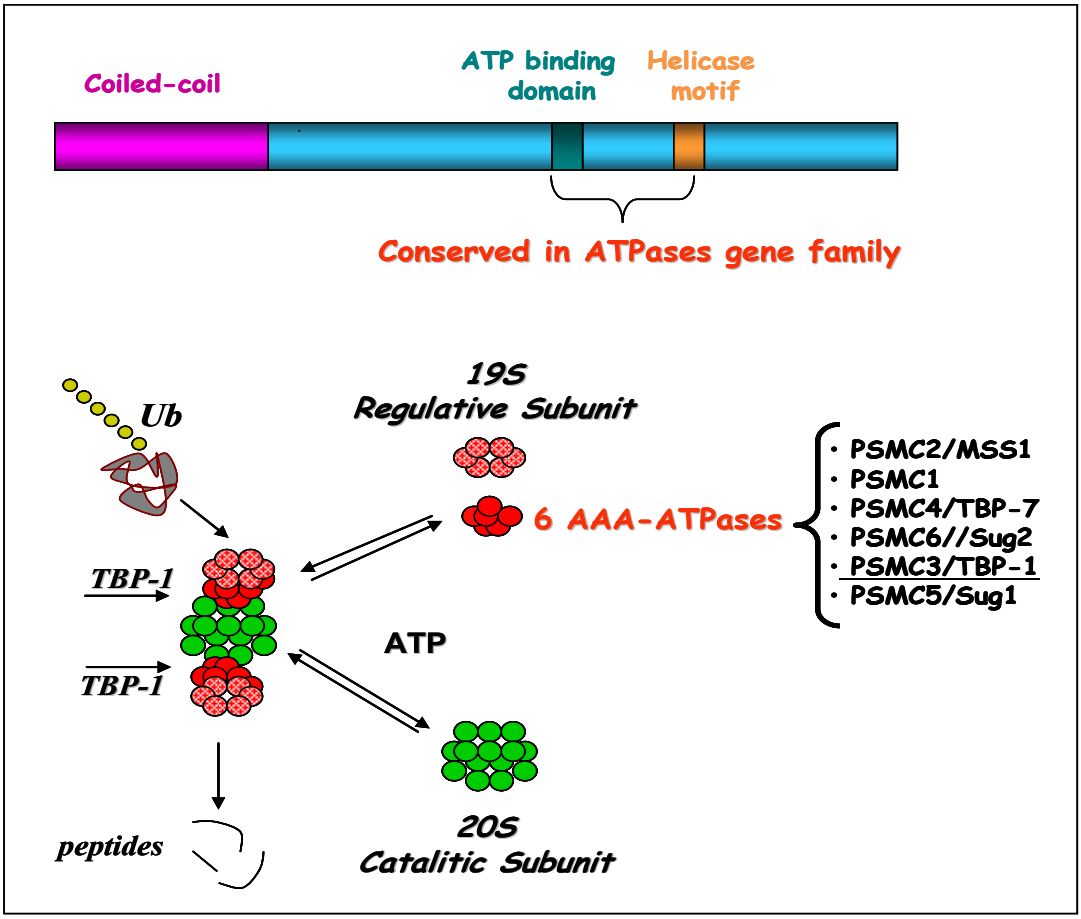

Figure 5 Structure of TBP-1.

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.

Roles other than proteolysis of TBP-1 and of the 19S subunits of the proteasome.

Besides their role in proteolysis of ubiquitinated or non ubiquitinated proteins, many different reports have highlighted the involvement of 19S subunit protein members in cellular events that do not require proteolysis. In particular, it has been largely demonstrated that 19S subunits play a key role in the 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 was demonstrated the association between the Sug1 and Sug2 proteins with the promoters of Gal1/10 genes, upon induction with galactose (Gonzalez et al., 2002).

The group of Ferdous (2002) reported that 19S subunits are critical for efficient elongation of RNA pol II. Antibodies against Rpt6, the mammalian analogue of Sug1 in yeast, abolish activated, but not basal, transcription in HeLa nuclear extract. In addition, immunodepletion of the 19S complex from a crude nuclear extract significantly reduces activated but not basal transcription. Furthermore, experiments using chemical inhibitors of proteasome-mediated proteolysis suggest that 19S requirement in transcription does not reflect a proteolytic event.

Sulahian and co-workers (2006), demonstrated that the proteasomal ATPases Sug1 and Sug2 (respectively Rpt6 and Rpt4 in mammalian) are essential for efficient transcription of several stress-induced genes in yeast. These include both heat shock and oxidative stress-responsive genes. When Sug1 or Sug2 activity was abolished by 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. Furthermore, ChIP assay revealed the induction-dependent recruitment of the proteasomal ATPases, but not of the 20S core complex, to the promoter of these genes. In summary, the proteasomal ATPases are essential for the efficient transcription of many stress response genes through direct association with their promoter.

Evidence that further support the notion that roles other than proteolysis could be feature of the 19S regulatory subunits of the proteasome was the interaction of the proteasome with the ubiquitin-like N-terminus of Rad23, a nucleotide excision repair (NER) protein, in S. cerevisiae (Russell et al., 1999). Deletion of the ubiquitin-like domain causes UV radiation sensitivity. 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*. Surprisingly,

blockage of protein degradation by the proteasome had no effect on the efficiency of NER.

Involvement of the proteasome in cancerogenesis.

Abundant evidences suggest that a unifying principle governing the molecular pathology of cancer is the co-dependent aberrant regulation of core machinery driving proliferation and suppressing apoptosis. These cellular processes are, in part, regulated through the degradation of key regulatory proteins, whose abnormal turnover can contribute to tumour formation. On the other hand, anormal expression levels of proteasome have been described in many tumour cells and proteasome plasma levels appear elevated in neoplastic patients (Lavabre-Bertrand et al., 2001; Bazzaro et al., 2006). Thus, the "proteasome pathway" often appears to be the target of cancer-related deregulation and is involved in processes such as oncogenic transformation, tumour progression, escape from immune surveillance and drug resistance. Recently, it has been reported that gankyrin, an oncogene overexpressed in human hepatocellular carcinoma interferes with the proteasome activity; in fact this oncogene causes the pRB transcription factor degradation and the activation of the E2F family transcription factor with a mechanism similar to that of viral oncoprotein E7, that is, through the interaction with the ATPases

subunits. The gankyrin interacts with the TBP-1 subunit, whereas E7 binds to S4/Rpt2. Both these interactions cause an increase of the pRb degradation rate and subsequent increase of the cellular proliferation (Higashitsuji et al., 2000).

The HEC protein (Highly Expressed in Cancer) specifically interacts with the S7 ATPase and would be able to inhibit its ATPase activity. The result of this interaction causes a modulation of proteasome activity and prevents 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).

TBP-1 and its potential role in the control of cellular proliferation.

A very interesting aspect of TBP-1 regards it possible role as tumour-suppressor. The first evidence underlying the involvement of TBP-1 in the control of cellular proliferation came from Park and its co-workers (1999). They demonstrated that TBP-1 mRNA levels were up-regulated following inhibition of the oncogenic phenotype of transformed cells expressing erbB family receptor. Moreover, TBP-1 overexpression diminished cellular proliferation and reduced the ability of parental cells to forms colonies in Colony Formation Assay (Pollice et al., 2004).

TBP-1 also possesses 46% identity to KAI I, a metastasis tumour 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).

Furthermore, it has been showed that TBP-1 binds to the tumour suppressor pVHL (Von Hippel Lindau), that results inactive in von-Hippel-Lindau disease, an autosomal hereditary cancer syndrome characterized by the development of vasculars tumours in the retina and in the central nervous system and renal cell carcinoma. This association contributes to VHL-E3 ubiquitin ligase function 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 (Figure 6). The interaction between TBP-1 and VHL is crucial since increased expression of TBP-1 resulted in enhanced kinetics degradation of Hif-1α whereas its silencing impairs the VHL-mediated proteasome degradation. Interestingly, tumour derived mutants of VHL, able to ubiquitinate Hif-1α but unable to bind to TBP-1 show delayed degradation rate of Hif-1α suggesting the essential function of TBP-1 in Hif-1α destruction and underlying the potential anti-tumoural activity of TBP-1.

In the last years, we highlighted a very interesting aspect of TBP-1 concerning its role on the regulation of the p14ARF oncosuppressor intracellular levels. TBP-1 interacts with and regulates intracellular protein levels of p14ARF increasing its half life. This effect requires the physical interaction between the two proteins and does not involve the transcriptional properties of TBP-1; in fact, the relative level of ARF mRNA was similar in the presence or absence of TBP-1, and the mutant of TBP-1 that lacks the helicase domain, reported to be essential for the transcriptional activity, is still able to increase ARF levels (Pollice et al., 2004).

Thus, TBP-1 appears to serve a role in the control of cell proliferation either through the increase of degradation of an oncogene or the stabilization of an oncosuppressor.

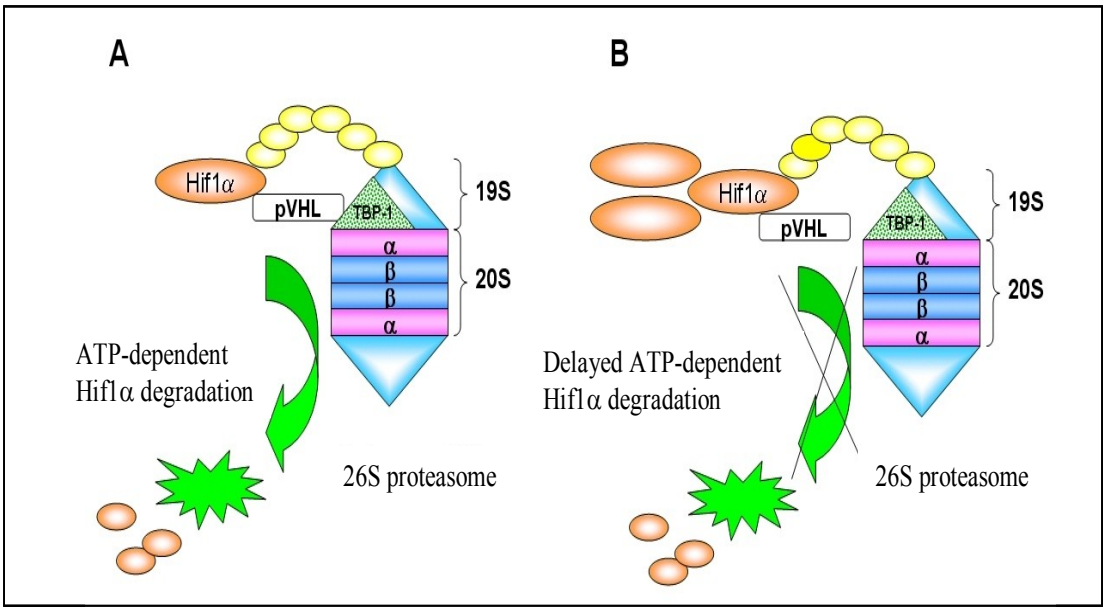

Figure 6 TBP-1 is involved in the degradation of Hif1α.

(A) In cells with wild-type pVHL, pVHL forms a complex with Hif1α and TBP-1.This complex allows for the efficient ubiquitination and degradation of Hif1α by the proteasome.

(B) pVHL mutants that do not interact with TBP-1 are able to bind and ubiquitinated Hif1α but have impaired degradation of Hif1α because of a failure of the pVHL-Hif1α complex to interact with TBP-1 and the proteasome machinery.

PRELIMINARY DATA AND AIM OF THE THESIS

In our laboratory it has been demonstrated that TBP-1 (Tat-Binding Protein 1), one of the six AAA-ATPases, component of the 19S subunit of the proteasome, interacts with and stabilizes the p14ARF oncosuppressor, delaying its turnover. This effect requires the physical interaction between the two proteins and does not involve the transcriptional properties of TBP-1 (Pollice et al., 2004, 2007).

As component of the 19S base of the proteasome, TBP-1 should be involved in the recognition of substrates that have to be degraded, their unfolding and subsequent translocation into the 20S proteasome. Thus, the effect on ARF was somehow unexpected and intriguing at the same time. Importantly, this effect seems to be not dependent on the proteasome functions.

Interestingly, the potential oncosuppressive role of TBP-1 appears not to be restricted to the effect on ARF as we and others (Pollice et al., 2004; Park et al., 1999; Corn et al., 2003) have observed that TBP-1 overexpression can inhibit cell proliferation in various cellular contexts also independently by the presence of ARF. These observations not only further underlie the potential antiproliferative role of TBP-1 but suggest

that it can exert a more general ARF-independent role in the control of cell proliferation and raise the question of what kind of cellular signals modulate TBP-1 expression.

During my thesis I sought to investigate on the potential antioncogenic role of TBP-1, on one side, studying the stabilization effect exerted on p14ARF and, on the other side, analyzing the antiproliferative role exerted by TBP-1 *per se* trying to unravel the molecular pathways involved.

The mechanisms governing ARF' s turnover are not yet completely clarified, being ARF a lysine-less and a relatively stable protein (see Introduction), unstructured and largely disorder in solution (Bothner et al., 2001). I concentrated my efforts to gain more insights into the mechanism involved in the ARF' s turnover, and I studied the involvement of the 20S proteasome in the regulation of ARF protein levels in the absence of ubiquitination and without the requirement of ATP. Furthermore, I investigated on the potential mechanism through which TBP-1 acts on ARF, analyzing the role of the physical interaction between the two proteins -by making use of various deletion mutants- and testing a model in which the TBP-1 chaperone activity causes ARF to fold becoming a poor substrate for proteasome destruction.

In the second part of my thesis I focused my research activity on the potential role of TBP-1 in the control of cell proliferation. I made use of primary human fibroblasts immortalized cell derivatives constitutively expressing an shRNA specifically designed to silence TBP-1 expression. It has been observed that the clones in which TBP-1 expression was silenced proliferate at higher rate respect to the control also in conditions of serum deprivation, display an increase of the S-phase and are more resistant to serum starvation induced apoptosis. These observations led me to look for the possible intracellular pathways involved. I observed that, in agreement with a potential tumour suppressive function, the cellular levels of TBP-1 appear critical to control cell duplication and are tightly regulated by a double-negative feedback loop that is mediated by the activation of the PKB/Akt kinase, one of the major transducers of cell survival and apotosis, that thus acts as a sensor to modulate the TBP-1 levels in actively duplicating cells. Although the players of these molecular pathways have not yet to be identified, these studies open up interesting questions on the intracellular role of TBP-1, a multifunctional protein with yet undiscovered antioncogenic properties.

RESULTS

ROLE OF TBP-1 IN THE CONTROL OF p14ARF LEVELS.

TBP-1 regulates ARF intracellular levels.

In our laboratory it has been demonstrated that TBP-1 (Tat- Binding Protein-1), a multifunctional protein component of the 19S regulatory subunit of the proteasome, interacts with and regulates ARF protein levels increasing its half-life (Pollice et al., 2004). To further investigate on the importance of TBP-1 in controlling p14ARF steady-state levels, I have reduced endogenous TBP-1 protein levels by making use of RNA interference. To this aim I used human lung carcinoma H1299 cells, that present high levels of both ARF and TBP-1 to transfect them with a siRNA duplex designed to specifically silence TBP-1 expression. At 72 hours after transfection, protein extract were prepared and protein concentrations determined using the Bio-Rad protein assay. Western blot of whole cell extracts and specific immunodetection with anti-TBP1 antibodies and anti-ARF antibodies clearly show that reduction in endogenous TBP-1 protein expression results in a remarkable decrease of ARF intracellular levels, confirming that basal TBP-1 levels are

important in controlling basal p14ARF levels (Figure 1). It is interesting to note that silencing of TBP-1 in cells expressing wild-type pVHL leads to degradation of the Hif1α transcription factor (Corn et al., 2003). On the other hand, I did not observe any change in the basal expression levels of various cellular proteins (p21, Itch, Mdm2, B23/NPM, actin and β-tubulin) (Figure 1). As some of them are reported to be subjected to proteasomal degradation, I concluded that reduction of TBP-1 intracellular levels does not generally affect proteasome function, but rather appears to affect only specific targets.

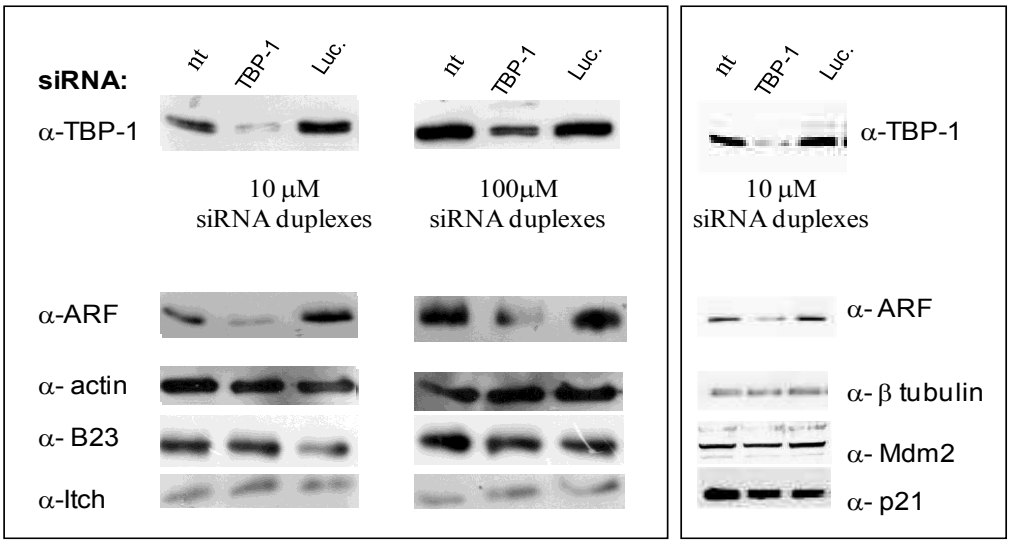

Figure 1 Effect of TBP-1 silencing.

H1299 cells were transfected with 10 μM and 100 μM of a 21 bp TBP-1 siRNA or firefly luciferase siRNA. Western Blot analysis show expression levels of TBP-1, p14ARF and of other endogenous proteins at 72 hours after transfection.

The first N-terminal 39 amino acids in p14ARF are necessary for both interaction with and stabilization by TBP-1.

We have already demonstrated that the interaction between ARF and TBP-1 in yeast requires the first 38 amino acids of ARF (Pollice et al., 2004). To confirm these results also in mammalian cells, I have performed experiments of coimmunoprecipitation using, as a first attempt, a deletion mutant of ARF, lacking the first 39 amino acids (for the construction of the mutant see materials and methods). To this purpose, I have transfected U2OS cells, a human osteosarcoma cell line (devoid of ARF expression), with the pcDNAARF or pcDNA ARF_{39-132} plasmids alone or in combination with the pcDNATBP-1. Cellular extracts were immunoprecipitated with antibodies against either TBP-1 or ARF, run on a SDS-Page, blotted and incubated with anti His (to reveal TBP-1) and anti ARF antibodies. As shown in figure 2, wtARF, as expected, interacts with TBP-1 whereas the deletion of the first 38 amino acids impairs the ability of ARF to bind to TBP-1.

To confirm that the 1-39 N-terminal region of ARF was strictly necessary for the interaction with TBP-1, I used the pCMV 3xFlag ARF_{1-39} in coimmunoprecipitation (CoIP) experiments. As controls, I included the 3xFlag ARF_{1-65} and the 3xFlag ARF_{65-132} plasmids, kindly provided by Prof. Barbara Majello. Cellular extracts were

immunoprecipitated with antibodies against TBP-1, run on SDS-Page, blotted and incubated with anti-His (to reveal TBP-1) and anti-Flag (to reveal ARF). As shown in figure 3 TBP-1 can interact only with the ARF deletion mutants that retain either sequences corresponding to exon 1β (3xFlag ARF_{1-65}) or to the first 39 amino acids (3xFlag ARF_{1-39}). These experiments demonstrate that the first 39 amino acids of p14ARF are necessary and sufficient for the association with TBP-1.

Subsequently, I determined the region of ARF necessary to obtain the stabilization effect exerted by TBP-1. To this purpose I transfected in U2OS cells, fixed amounts of the constructs expressing the different deletion mutants of ARF previously described, together with increasing amounts of TBP-1. As shown in figure 4, coexpression of wtARF with TBP-1 results in a significant increase of ARF levels, as expected. The same effect is observed also on the ARF_{1-39} and ARF_{1-65} mutants. On the contrary, the ARF_{39-132} and ARF_{65-132}, that are unable to interact with TBP-1, do not accumulate following TBP-1 overexpression. These results strongly indicate that the interaction with TBP-1 is strictly required in order to obtain the stabilization of ARF intracellular levels.

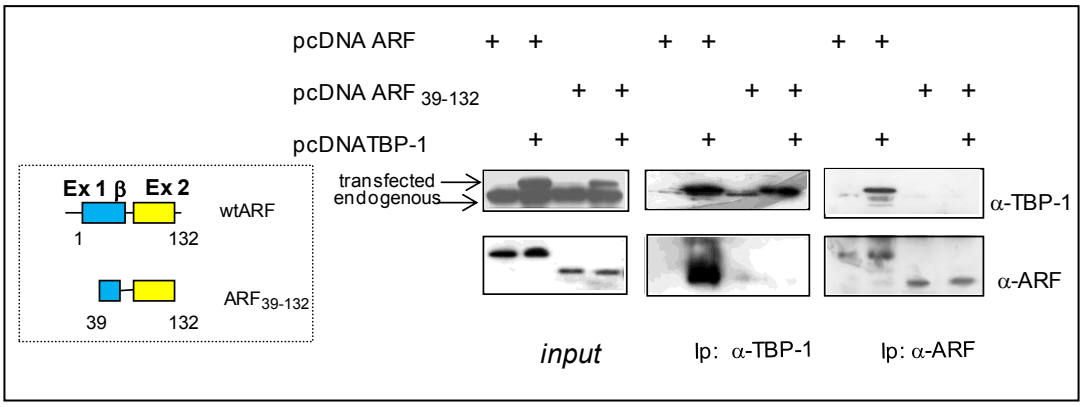

Figure 2 The ARF_{39-132} is unable to interact with TBP-1.

U2OS cells were transfected with 2 µg of the pcDNA ARF or the pcDNA ARF $_{39-132}$ expression vector alone or together with 2 µg of pcDNA TBP-1; 15 µg of the whole extract were analysed with anti-ARF or anti-His antibodies (input), to reveal only transfected TBP-1, whereas 800 µg of the lysate were immunoprecipitated with anti-TBP-1 or anti-ARF and revealed by anti-ARF and anti-TPB-1 antibodies.

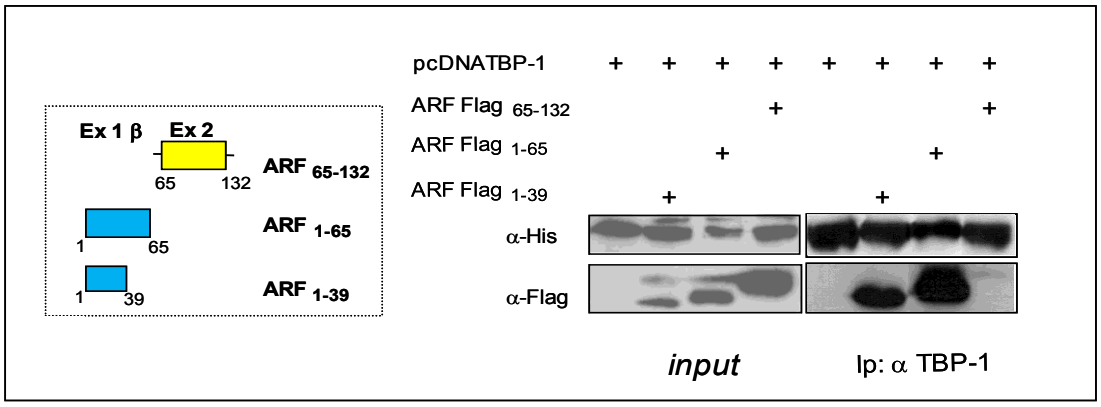

Figure 3 The first 39 amino acids of ARF are necessary for the binding to TBP-1

U2OS cells were transfected with 0.5 µg of the pcDNA TBP-1 expression plasmid alone or together with 0.5 µg of the 3xFlag ARF_{1-39}, 3xFlag ARF_{1-65} or 3xFlag ARF_{65-132} expression vectors; lysates were analysed by anti-His or anti-Flag antibodies (input), immunoprecipitated with anti-TBP-1 antibody and analysed with anti-His and anti-Flag antibodies.

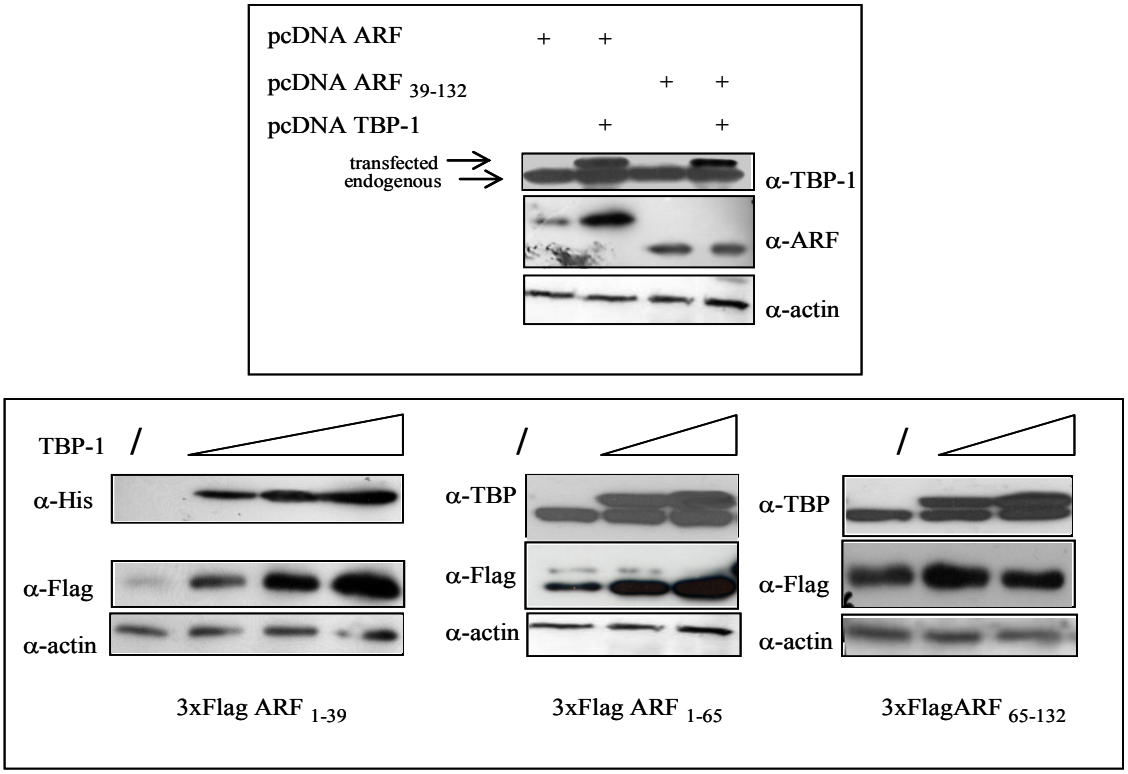

Figure 4 The first 39 amino acids of ARF are necessary for the stabilization effect exerted by TBP-1.

Upper panel: U2OS cells were transfected with pcDN AARF or pcDNA ARF_{39-132} alone or together with pcDNA TBP-1 in a 1:2 ratio; lysates were analysed with anti-ARF, anti-His and anti-actin antibodies.

Lower panel: U2OS cells were transfected with 0.3 µg of the 3xFlag ARF_{1-39}, 3xFlag ARF_{1-65} or 3xFlag ARF_{65-132} expression plasmids alone or with increasing amounts of pcDNA TBP-1; lysates were resolved by SDS-PAGE and analysed with anti-His, anti-Flag and anti-actin antibodies.

Interestingly, TBP-1 overexpression seems to exert a stronger stabilization effect on the ARF_{1-39} mutant respect to the wtARF or to the ARF_{1-65} mutant. I hypothesized that this effect could be due to the fact that the 1-39 peptide is less stable. To go through this point, I measured the ARF_{1-39} half life in the absence and in presence of TBP-1 overexpression. To this aim I transfected U2OS cells with a plasmid encoding ARF_{1-39} with or without pcDNA TBP-1 and 24 hours after transfection, I treated the cells with cycloheximide to inhibit protein synthesis. At the indicated times after exposure to the drug, cells were harvested and the extracts analyzed by Western Blot and probed with anti-His (to revel TBP-1), anti-Flag (to reveal ARF) and anti-Actina antibodies. As indicated in figure 5, ARF_{1-39} half-life is below 2 hours but is shifted to around 4 hours when the cells are cotransfected with TBP-1, confirming that the TBP-1 stabilization effect is exerted at the posttranslational level also on the ARF_{1-39} peptide as on wtARF (Pollice et al., 2004). I also performed a similar experiment to determine the half-life of the ARF_{39-132}. This mutant displays a shorter half-life respect to wtARF, confirming again that the 1-39 N-terminal region plays an important role in regulating the ARF turnover.

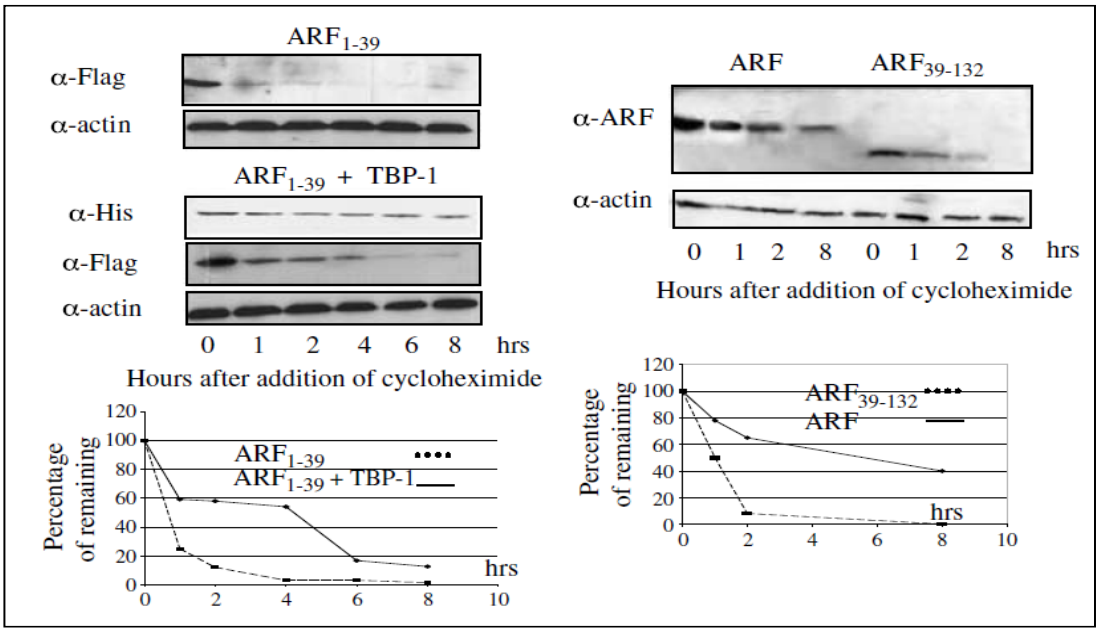

Figure 5 Evaluation of the ARF_{1-39} and ARF_{39-132} half life.

Left panel: U2OS cells were transfected with 0.3 µg of the 3xFlag ARF_{1-39} expression vector in the presence or absence of the pcDNA TBP-1 expression plasmid. Twenty-four hours after transfection, cells were treated with cycloheximide (80µg/ml) and then harvested at the indicated time points. Lysates were analysed with anti-Flag, anti-actin and anti-His antibodies.

Right panel: U2OS cells were transfected with 0.3 µg of a plasmid encoding for pcDNA ARF or pcDNA ARF_{39-132}. Twenty-four hours after transfection, cycloheximide was added and the cells were harvested at the indicated time points. Lysates were analysed with anti-ARF and anti-actin antibodies.

As there is still some disagreement as to whether the p14ARF N-terminal region fully maintains all the biological activity (Lohrum et al., 2000; Rodway et al., 2004), I wanted to get insights into the functionality of the ARF_{1-39} peptide. I examined the ability of this peptide to prevent MDM2-mediated degradation of p53. To this purpose I transfected an expression plasmid coding for MDM2 in U2OS cells (that express high levels of endogenous p53), alone or in combination with increasing amounts of the 3xFlag ARF or 3xFlag ARF_{1-39}. As expected, p53 levels decrease following MDM2 overexpression, but wild-type ARF, as well as the ARF_{1-39} peptide, counteracted this effect, being able to prevent MDM2-mediated p53 degradation (Figure 6). These data indicate that, at least in the p53/MDM2 pathway, the ARF_{1-39} peptide retains its biological function. Many studies pointed to a role of the subcellular localization of ARF in its biological functions, although there is still a debate around this issue (Weber et al., 2000; Rodway et al., 2004). To investigate on this point I analyzed the subcellular localization of the ARF_{1-39} and ARF_{39-132} peptides. Interestingly, I observed that while ARF_{39-132} retains the main nucleolar localization, the ARF_{1-39} shows a more diffuse staining, suggesting that, since the ARF_{1-39} peptide retains its biological activity (see above), the nucleolar localization is not

essential for ARF function, at least in the MDM2/p53 pathway (Figure 7).

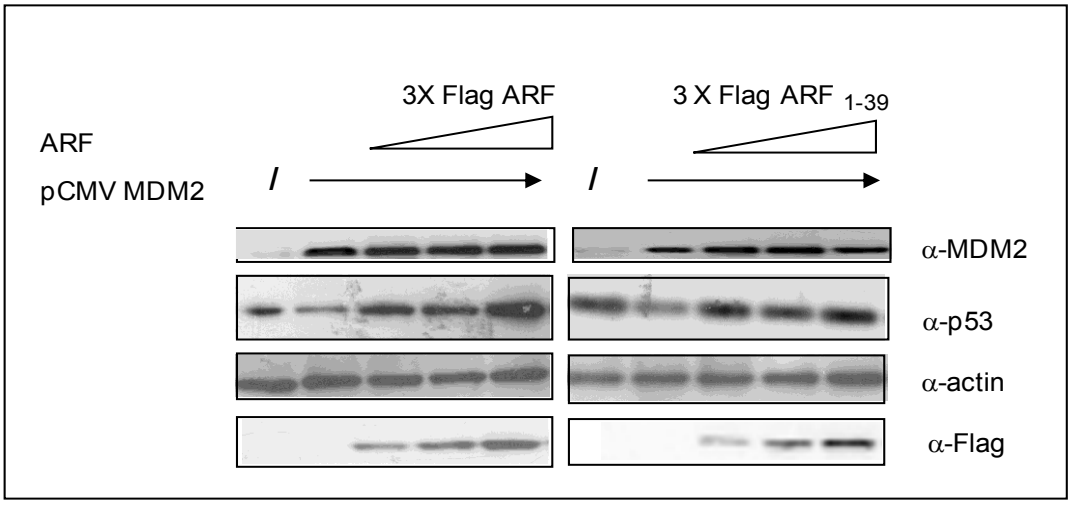

Figure 6 Biological function of the p14ARF1-39 N-terminal peptide.
U2OS cells were transfected with 0.2 µg of pCMV MDM2 plasmid alone or in combination with increasing amounts of 3xFlag ARF or 3xFlag ARF_{1-39} vectors. Lysates were analysed with anti-p53, anti-Flag (to reveal ARF) and anti-actin antibodies.

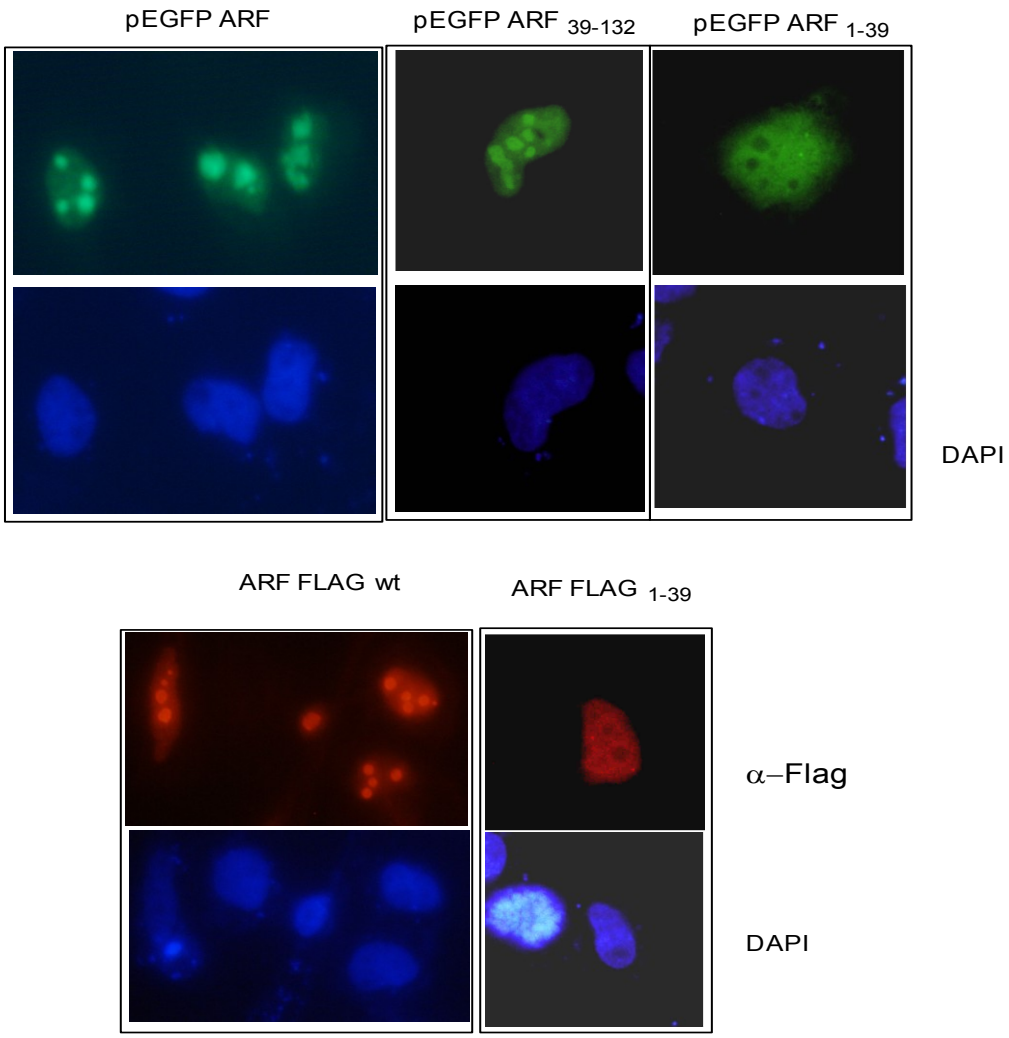

Figure 7 The ARF_{1-39} peptide loses the typical ARF nucleolar localization.

U2OS cells were transfected with GFP ARF, GFP ARF_{1-39} and GFP ARF_{39-132} and fixed 24 hours after transfection. Nuclei were visualized by 4-6-diamidino-2-phenylindole (DAPI) staining.

U2OS cells were transfected with 3xFlag ARF and 3xFlag ARF_{1-39}, fixed 24 hours after transfection and immunostained with anti-Flag antibodies. Nuclei were visualized by DAPI staining.

To analyze the N-terminal 39 amino acids in more detail, I decided to use different mutants of ARF bearing small deletions in the first 39 amino acids, kindly provided by Dr. Sherr. They correspond to the pcDNA $ARF_{\Delta 2\text{-}14}$ and the pcDNA $ARF_{\Delta 26\text{-}37}$. As control, I used the pcDNA $ARF_{\Delta 82\text{-}101}$ plasmid. Each plasmid was transfected in U2OS cells alone or together with increasing amounts of pcDNA TBP-1. The protein extract were immunoprecipitated with anti ARF antibodies and revealed with anti-His and anti-ARF antibodies. Figure 8(upper panel) shows that similar levels of the different ARF proteins were recovered from transfected cell lines, but significantly less TBP-1 was coprecipitated from cells expressing $ARF_{\Delta 2\text{-}14}$ and $ARF_{\Delta 26\text{-}37}$ mutants. Finally, I tested the protein levels following TBP-1 overexpression (Figure 8 lower panel). The mutants that fail to interact strongly with TBP-1 do not increase, suggesting that efficient binding and stabilization requires an intact ARF N-terminal portion.

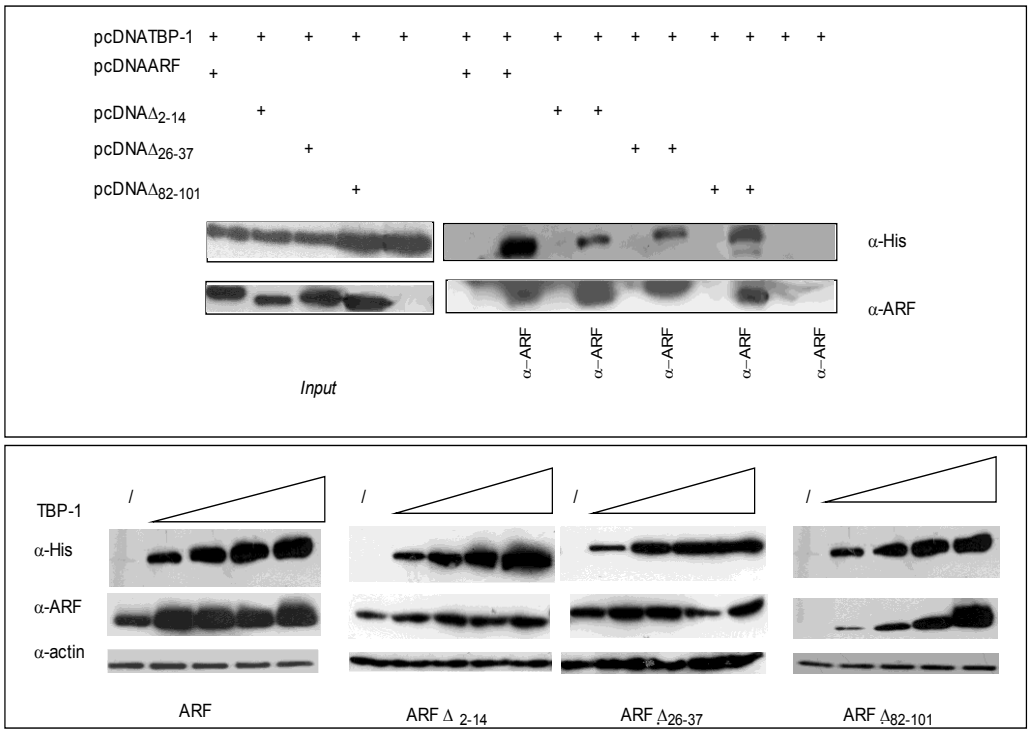

Figure 8 Efficient binding and stabilization requires an intact ARF N-terminal portion.

Upper panel: U2OS cells were transfected with pcDNA TBP-1 alone or together with the ARF plasmids indicated. 48 hours after transfection protein extracts were prepared and immunoprecipitated with anti-ARF antibodies where indicated and revealed with anti-His and anti-ARF antibodies. For negative control, the immunoprecipitation of the same extract was performed without anti-ARF.

Lower panel: U2OS cells were transfected with 0.3 µg of the indicated plasmids alone or together with increasing amounts of pcDNA TBP-1 (0.3; 0.6; 0.9 and 1.2 µg). Cell lysates were resolved by SDS-PAGE and analysed by Western Blot with anti-ARF, anti-His and anti-actin antibodies.

p14ARF is degraded by the proteasome and TBP-1 protects it both in cells and *in vitro*.

The mechanisms governing ARF's turnover are not yet completely clarified being ARF a lysine-less and a relatively stable protein. Recently, it has been reported that ARF protein degradation occurs, at least in part, through the proteasome (Kuo et al., 2004, Rodway et al., 2004), (for more details see Introduction). However, accumulation of endogenous ARF following treatment with proteasome inhibitors is almost undetectable in various cell lines, suggesting that may exist other mechanisms mediating ARF destruction. To go through this point, I checked ARF protein levels after treatment with proteasome and lysosome inhibitors. I exposed HEK293T cells (human embrional kidney) that present good endogenous levels of p14ARF to various lysosomes (Ammonium Chloride, Cloroquine and Bafylomicin) and proteasome (MG132 and ALLnL) inhibitors. As indicated in figure 9, ARF does not accumulate with any of the lysosome inhibitors used but it slightly increases with either MG132 or ALLnL. Furthermore, the $ARF_{39\text{-}132}$ mutant, with a significantly shorter half-life respect to the wild type, clearly accumulate after treatment with proteasome inhibitors confirming that ARF is subjected to degradation by the proteasome.

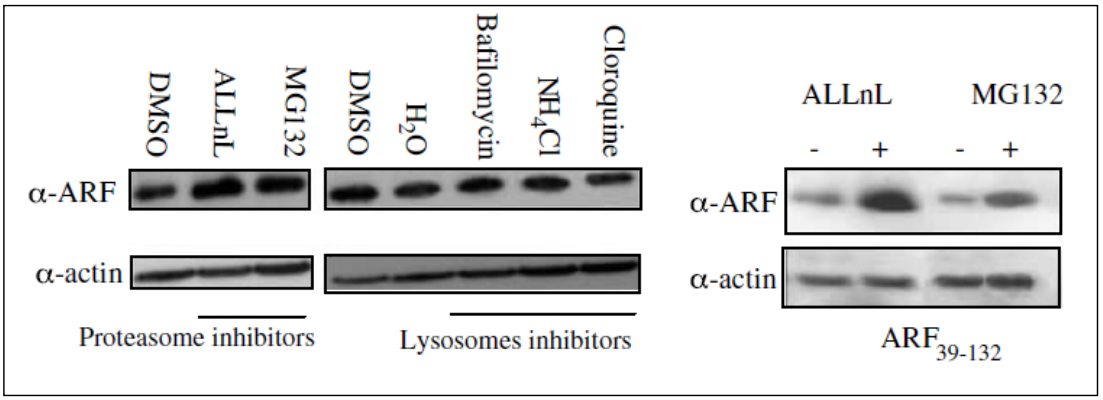

Figure 9 ARF is degraded by the proteasome in mammalian cells.

Twenty-four hours after the plating, 293T cells were treated for 6 hours with proteasome (MG132 25 μM and ALLnL 50 μM) and lysosome inhibitors (Bafilomycin 100 nM, NH_4Cl 25 μM and Cloroquine 200 μM). Lysates were resolved by SDS-PAGE and analysed with anti-ARF and anti-actin.

Right panel: U2OS cells were transfected with the pcDNA ARF_{39-132} plasmid and, 24 hours after transfection, treated with ALLnL or MG132. Lysates were analyzed with anti-ARF and anti-actin antibodies.

Moreover, stabilization of the ARF_{1-39} mutant owing to overexpression of TBP-1 is further increased by addition of MG132 when a 1:1 ratio between TBP-1 and ARF mutant is used in the transfection. However, when a higher amount of TBP-1 is transfected, the ARF_{1-39} mutant accumulates at higher levels that do not further increase after treatment with proteasome inhibitor (Figure 10). These results suggest that TBP-1, as well as proteasome inhibitors, protects ARF from proteasome degradation.

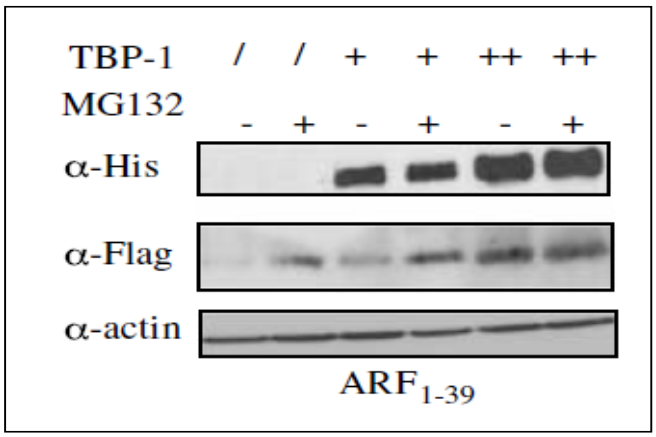

Figure 10 TBP-1 overexpression, as well as proteasome inhibitors MG132, protects ARF_{1-39} from degradation.

U2OS cells were transfected with 3xFlag ARF_{1-39} plasmid alone or with increasing amount of TBP-1 vector and, 24 hours after transfection, treated with MG132 for 10 hours. Lysates were analysed with anti-Flag, anti-His and anti-actin antibodies.

The proteasome plays a central role in the degradation of the majority of cellular proteins in eukaryotes. Recently, it is emerging an involvement of the proteasome also in the degradation of non-ubiquitineted proteins that appear to be physiologically important in higher eukaryotes (Sdek et al., 2005; Kong et al., 2006). In this process, the 20S proteasome subunit and, more recently, the REGγ/11S subunit seem to play a central role (Chen et al., 2007). Since it has been described that ARF is dynamically disordered in acqueous solution (Bothner et al., 2001) and largely unstructured *in vivo*, I wanted to test whether it can be subjected to proteasome degradation in *vitro*, in the absence of ubiquitination. To this purpose, I used in *vitro* translated proteins (for details see materials and methods). In particular I used ARF, TBP-1 and, as control, $p21^{waf}$ since it has already been shown to be naturally unstructured and degraded in *vitro* by the 20S proteasome (Liu et al., 2003). The samples were resolved by SDS-Page and immunodetected with antibodies against ARF, TBP-1 and p21 (Figure 11). As it is possible to observe, ARF is efficiently degraded by the 20S proteasome as well as p21 and accumulates after treatment with the proteasome inhibitor MG132, whereas TBP-1 levels are unchanged. Similarly, the $ARF_{39\text{-}132}$ mutant is degraded in *vitro,* in the same kind of experiments, with a faster kinetic

that probably reflects the shorter half life of this mutant observed in cells (Figure 12).

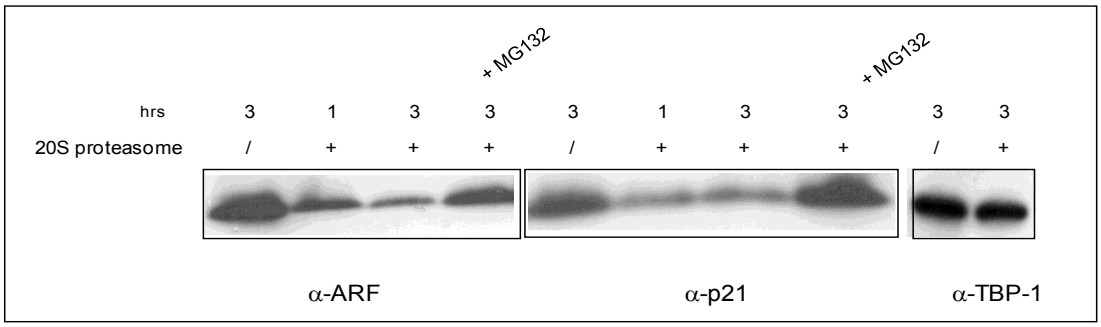

Figure 11 ARF is degraded *in vitro* by the 20S proteasome.
In vitro translated p14ARF, p21 or TBP-1 were incubated with or without 20S proteasome for 1 or 3 hours at 37ºC. MG132 (50 µM) was added to the reaction where indicated. Samples were resolved by SDS-PAGE and immunoblotted with anti-ARF, anti-p21 or anti-TBP-1 antibodies.

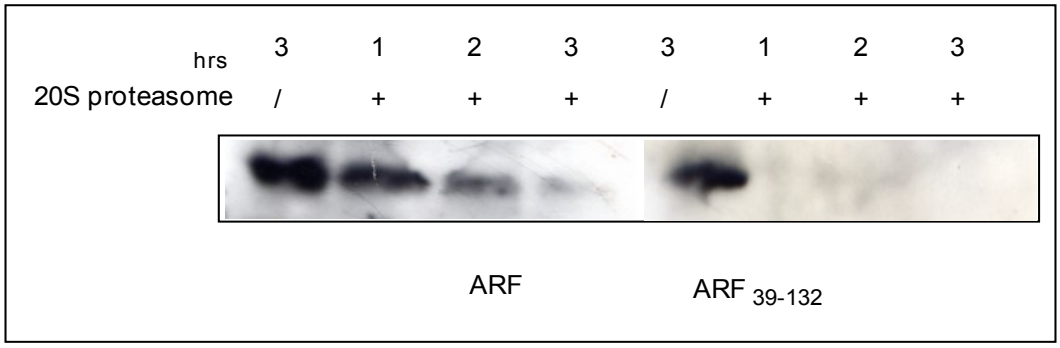

Figure 12 The ARF_{39-132} mutant is degraded *in vitro* by 20S proteasome.
In vitro translated p14ARF or $p14ARF_{39-132}$ were incubated with or without 20S proteasome for the indicated time intervals and analysed with anti-ARF antibodies.

As previously described, TBP-1 effects on ARF protein levels strictly depend on the binding between the two proteins. Thus, I wanted to investigate whether this protection can be exerted also in *vitro*, in the absence of an assembled 19S cap, of which TBP-1 is an integral component. To this purpose, in *vitro* translated TBP-1 was incubated with either in *vitro* translated p14ARF or p14ARF$_{39-132}$ before the degradation assay. Strikingly, I could observe a protective effect of TBP-1 on ARF, which is not exerted on the ARF$_{39-132}$ mutant, that is unable to interact with TBP-1 (Figure 13). The fact that TBP-1 can exert its effect independently from being part of the 19S suggests that a similar mechanism could occur *in vivo*.

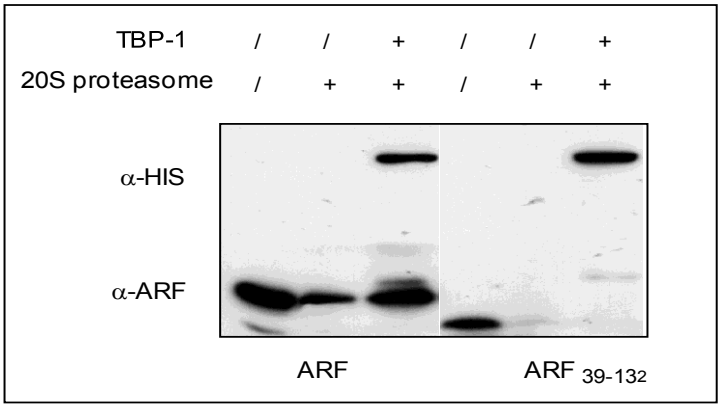

Figure 13 TBP-1 protects ARF from *in vitro* proteasomal degradation.
In vitro translated p14ARF or p14ARF$_{39-132}$ were incubated with or without 20S proteasome. In vitro translated TBP-1 was added 30' before the reaction, where indicated. Samples were resolved by SDS-PAGE and immunoblotted with anti-ARF and anti-TBP-1 antibodies.

Since it has been postulated that 19S proteasome subunits possess chaperone-like activities (residing in the ATPAse module) it is possible that, upon binding, TBP-1 causes ARF to fold becoming a poor substrate for proteasome destruction.

In order to test this hypothesis, I generated a TBP-1 point mutant (for more details see materials and methods) in the ATPAse domain through site directed mutagenesis and I tested in cells its capacity to bind and stabilize p14ARF. U2OS cells were transfected with the pcDNA ARF plasmid alone or in combination with pcDNA TBP-1 ΔGKT. At 24 hours after transfection cellular extracts were prepared and immunoprecipitated with antibodies against ARF, resolved by SDS-Page, blotted and incubated with anti Xpress, to reveal transfected TBP-1, and anti-ARF antibodies. Interestingly, as shown in figure 14, this mutant is able to bind to ARF. However, transfection of U2OS with increasing amounts of TBP-1 ΔGKT and a fixed amounts of p14ARF do not cause a significant increase in ARF protein levels unlike the effect observed with wtTBP-1 (Figure15). These experiments suggest that chaperone activity of TBP-1 could be required for the stabilization effect.

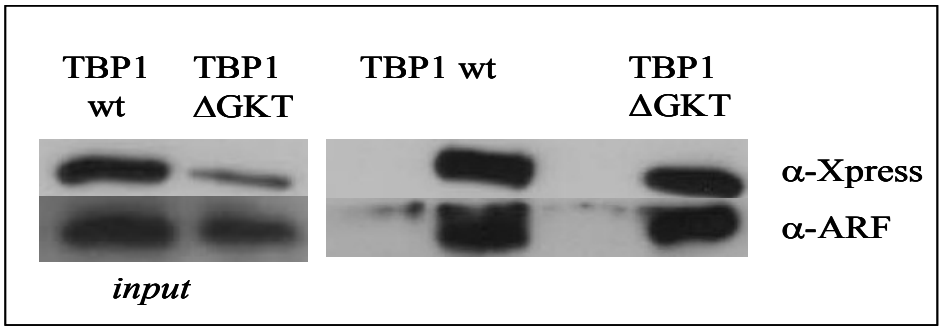

Figure 14 TBP-1 ΔGKT retains the binding to ARF.

U2OS cells were transfected with 1 μg of the pcDNA TBP-1 and pcDNA TBP-1 ΔGKT vectors alone or together with 1 μg of the ARF plasmids. Twenty four hours after transfection protein extracts were immunoprecipitated with anti-ARF antibodies where indicated and revealed with anti-Xpress and anti-ARF antibodies. For negative control, the immunoprecipitation of the same extracts was performed without anti-ARF.

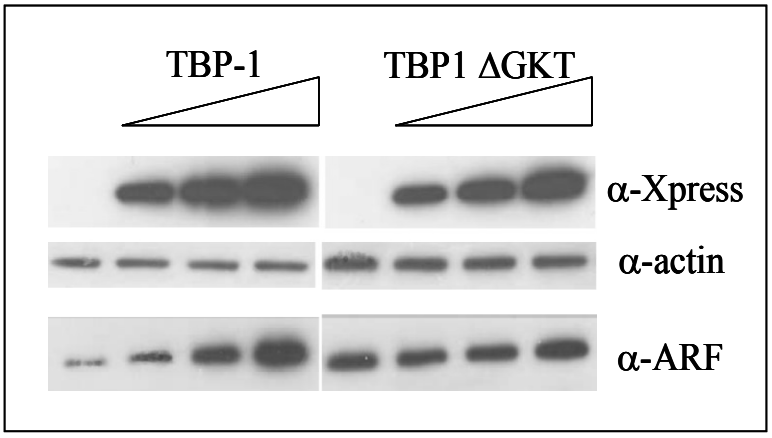

Figure 15 TBP-1 ΔGKT is unable to efficiently increase ARF intracellular levels.

U2OS cells were transfected with 0.2 μg of the pcDNA ARF plasmid alone or in combination with increasing amounts of pcDNA TBP-1. Cell lysates were resolved by SDS-PAGE and analyzed by Western Blot with anti-ARF, anti-Xpress and anti-actin antibodies.

MDM2 is able to regulate p14ARF intracellular levels.

As previously described, some authors (Kuo et al., 2004) reported that ARF could be subjected to N-terminal ubiquitination, a process that requires a free N-terminus and that seems strictly dependent on the first 2–3 amino acids of the protein (see Introduction). Interestingly, it has been shown that TBP-1, as part of the regulatory subunit of the proteasome, is involved in the recognition of the polyubiquitin chains (Lam et al., 2002). As the binding of ARF to TBP-1 requires the N-terminus of the protein, I reasoned that it could occur via the polyubiquitin chain. Therefore, I could expect that changes of the ARF N-terminal amino acids would impair the stabilization effect. However, I have proven that TBP-1 overexpression determines stabilization of ARF proteins independently from the extreme N-terminal sequence (Figure 16 and figure 4 previously shown). Overall, it appears that putative N-terminal ubiquitination does not significantly influence TBP-1 mediated stabilization.

On the other hand, other authors (Rodway et al., 2004) do not observe ubiquitination of ARF but claim the importance of the MDM2 ubiquitin-ligase in the regulation of ARF intracellular levels, although the mechanism through which it occurs is not yet defined. It has also been reported that MDM2 could be involved in the ubiquitin-independent

degradation of some proteins, such as p21 (Jin et al., 2003) an pRb (Sdek et al., 2005). Zhang and coworkers (2004) provided evidences that MDM2 promotes p21 degradation directly binding to it and facilitating its association with the C8 subunit of the 20S proteasome. Sdek and coworkers (2005) described a similar behaviour for pRb. They found that MDM2 promotes Rb degradation in a proteasome-dependent, ubiquitin-independent manner in analogy to p21.

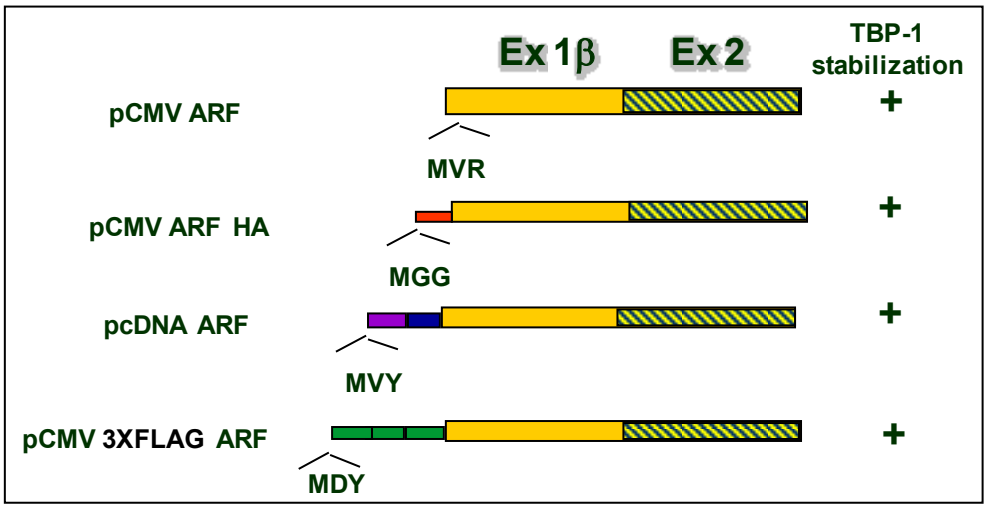

Figure 16 TBP-1 stabilizes ARF independently from its N-terminal sequence.

Since ARF is degraded *in vitro* by the 20S proteasome (Pollice et al., 2007) and is prevalently unstructured I decided to perform experiments to test the potential involvement of MDM2 in the regulation of ARF turnover. I thus transfected U2OS cells with increasing amounts of a vector encoding for wtMDM2 or for two deletion mutants of MDM2, $MDM2_{1\text{-}441}$, lacking the ring finger (the portion of the protein involved in the ubiquitination process) and $MDM2_{\Delta 150\text{-}230}$ (a mutant lacking the NES and NLS sequences, causing relocalization of MDM2 in the cytoplasm) together with a fixed amounts of p14ARF. Western blot of whole extracts and specific immunodetection with anti-MDM2 antibodies and anti-ARF antibodies show that the overexpression of MDM2, as well as of its mutant forms, cause a remarkable decrease of p14ARF protein levels (Figure 17). Interestingly, this effect occurs also on endogenous ARF protein levels in H1299 cells (Figure 18). In order to verify if ARF could be degraded by the 20S proteasome through the binding to C8 in analogy with p21 and Rb, I performed experiments of GST pull-down. Unfortunately the results were not unequivocal.

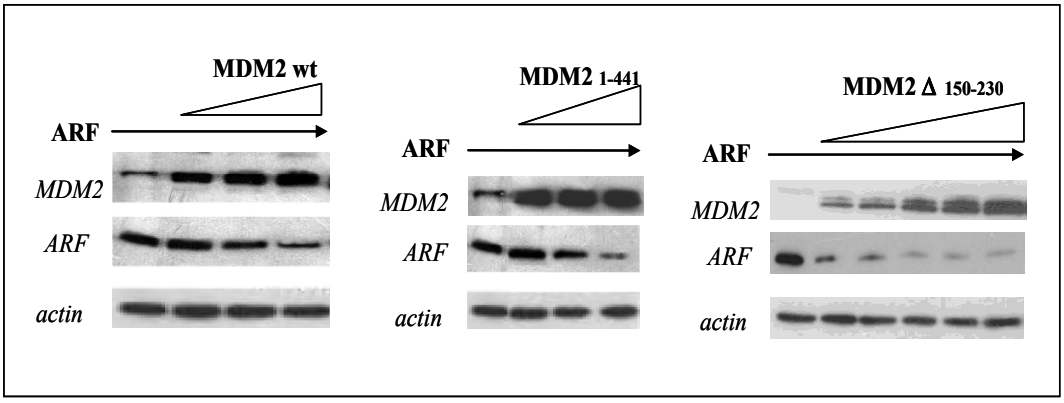

Figure 17 Effect of wt MDM2, MDM2$_{1-441}$ and MDM2$_{\Delta 150-230}$ on ARF.

U2OS cells were transfected with 0.2 µg of the pcDNA ARF plasmid alone or in combination with increasing amounts of the plasmids indicated. Cell lysates were resolved by SDS-PAGE and analysed by Western Blot with anti-MDM2, anti-ARF and anti-actin antibodies.

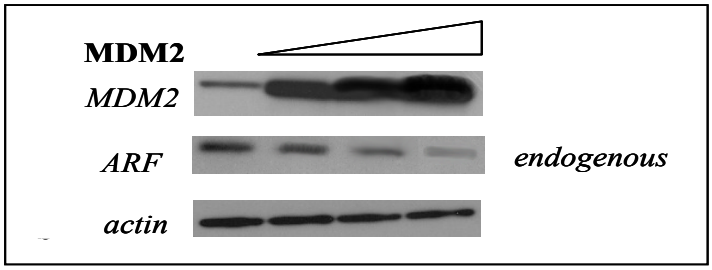

Figure 18 Effect of wtMDM2 on endogenous ARF in H1299.

H1299 cells were transfected with increasing amounts of wtMDM2 plasmid. Whole cell extract was run on a SDS-Page and immunorevealed by Western Blot with anti-MDM2, anti-ARF and anti-actin antibodies.

To determine which portion of ARF was required for the degradation effect exerted by MDM2, I transfected U2OS cells with plasmids coding for different deletion mutants of ARF together with increasing amounts of wtMDM2. They corresponded to $ARF_{\Delta 2\text{-}14}$ (lacking a nucleolar localization signal in exon 1β), $ARF_{\Delta 82\text{-}101}$ (lacking a localization signal present in the exon 2), $ARF_{\Delta 2\text{-}14/\Delta 82\text{-}101}$, $ARF_{1\text{-}65}$ (that retains only exon 1β and $ARF_{65\text{-}132}$ (that retains exon 2). Interestingly, protein levels of all the ARF mutants, except that of the $ARF_{65\text{-}132}$, were reduced following MDM2 overexpression suggesting that the first 65 amino acids of ARF are important for the degradation effect (Figure 19).

I then evaluated the capability of the different ARF mutants to bind to MDM2. To this purpose, whole cell extract of U2OS cells transfected with $ARF_{\Delta 2\text{-}14/\Delta 82\text{-}101}$ and, as control, with the wtARF plasmid, alone or in presence of MDM2, were subjected to immunoprecipitation with anti-ARF antibody, run on a SDS-PAGE, blotted and immunodetected with anti-ARF and anti-MDM2 antibodies. Figure 20 shows that the $ARF_{\Delta 2\text{-}14/\Delta 82\text{-}101}$ is able to bind to MDM2. Similarly, the $ARF_{1\text{-}65}$ mutant is able to bind to MDM2 unlike the $ARF_{65\text{-}132}$.

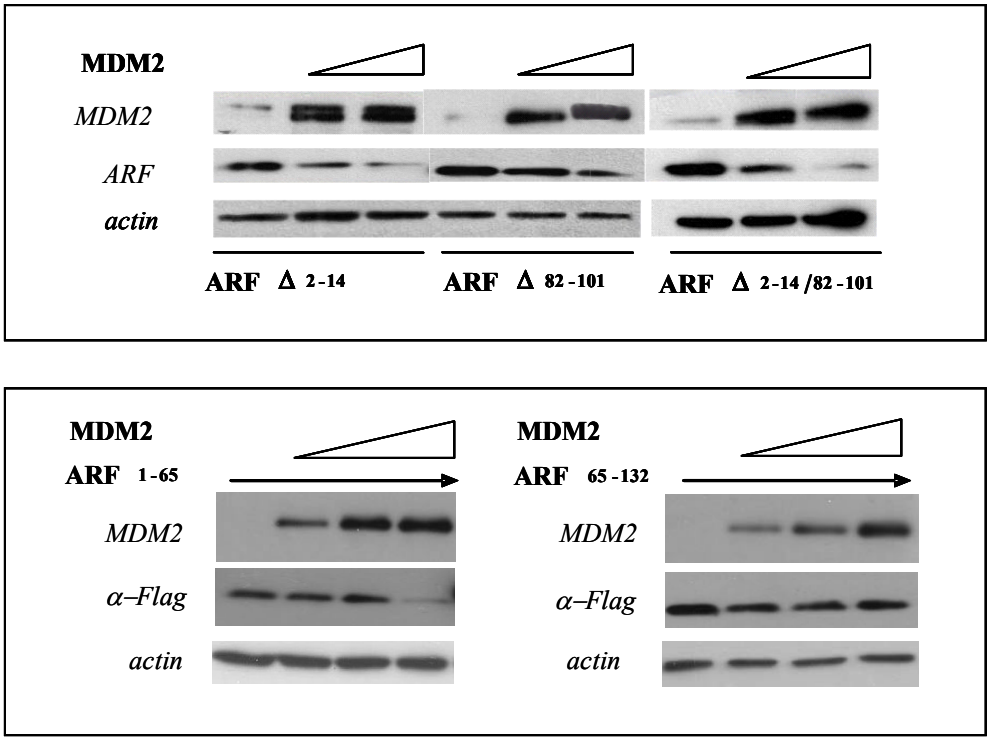

Figure 19 The first 65 amino acids of ARF are important in order that MDM2 exerted its degradation effect on ARF.

(Upper) U2OS cells were transfected with 0.3 µg of the ARF mutants alone or in combination with increasing amounts of wtMDM2 (0.6 and 0.9µg).Cell lysates were resolved by SDS-PAGE and analysed by Western Blot with anti-MDM2, anti-ARF and anti-actin antibodies.

(Lower) U2OS cells were cotransfected with 0.3 µg of the pCMV ARF $Flag_{1-65}$ and pCMV ARF $Flag_{65-132}$ plasmids and increasing amounts (0.3; 0.6 and 0.9 µg) of MDM2 expression vector. Twenty four hours after transfection cell extracts were analyzed by Western blot and immunorevealed with anti MDM2, anti Flag and anti actin antibodies.

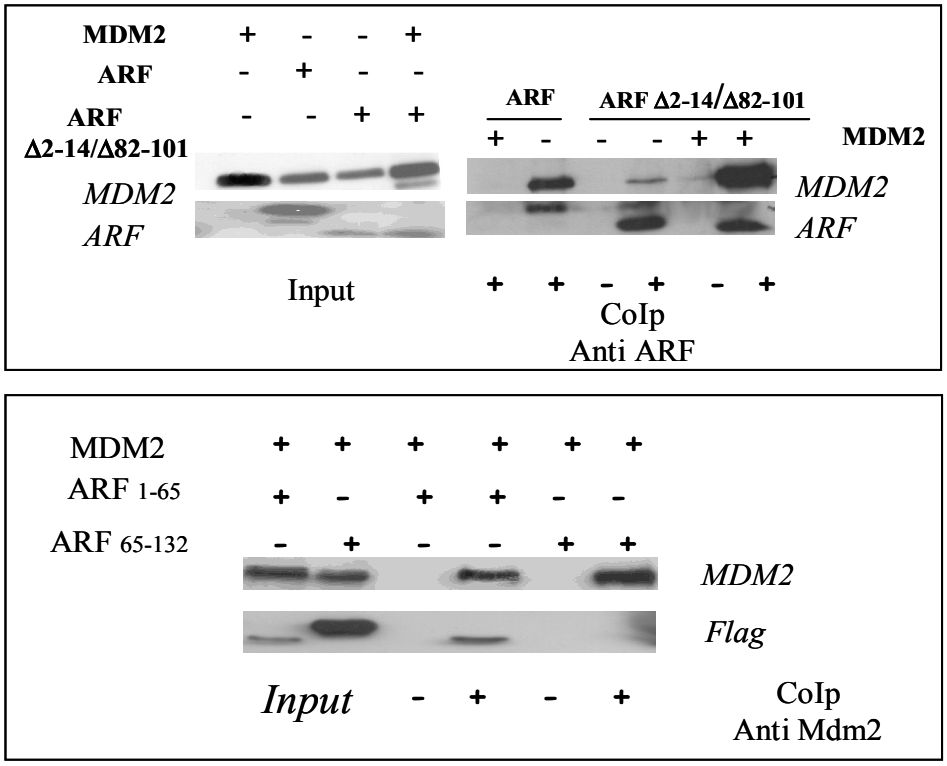

Figure 20 ARF Flag$_{65-132}$ that is not degraded by MDM2 loses its capability to bind to it.

(Upper) U2OS cells were transfected with 1 µg of pcDNA ARF and pcDNA ARF$_{\Delta 2-14/\Delta 82-101}$ vectors alone or together with the MDM2 plasmids. Twenty four hours after transfection protein extracts were immunoprecipitated with anti-ARF antibodies where indicated and revealed with anti-MDM2 and anti-ARF antibodies. For negative control, the immunoprecipitation of the same extracts was performed without anti-ARF.

(Lower) U2OS cells were transfected with 0.5 µg of pCMV ARF Flag$_{1-65}$ and pCMV ARF Flag$_{65-132}$ vectors alone or in combination with the MDM2 plasmids. Twenty four hours after transfection cell extracts were immunoprecipitated with anti-MDM2 antibodies where indicated and revealed with anti-MDM2 and anti-ARF antibodies. For negative control, the immunoprecipitation of the same extracts was performed without anti-MDM2.

Consistently, the MDM2 mutants that are able to degrade ARF (see above) bind to it (Figure 21). Overall, these results led to the hypothesis that the interaction between ARF and MDM2 is crucial for ARF degradation following MDM2 overexpression.

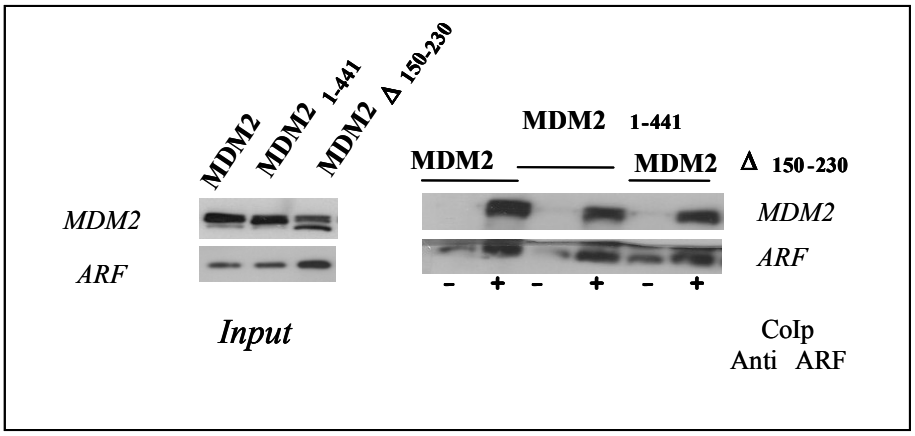

Figure 21 All the MDM2 mutants bind to ARF.

U2OS cells were transfected with 1 μg of the pcDNA ARF expression plasmid alone or together with 1μg of the wtMDM2, $MDM2_{1-441}$ and $MDM2_{\Delta 150-230}$ expression vectors; lysates were analysed by anti-MDM2, or anti-ARF antibodies (input), immunoprecipitated with anti-ARF antibody and immunocomplex were analyzed with anti-MDM2 and anti-ARF antibodies.

Interestingly, I observed a decrease of ARF protein levels, in response to MDM2 overexpression, also in p53-/- MDM2-/- context (Mouse Embryo Fibroblasts), indicating that the observed effect, is p53 independent (Figure 22).

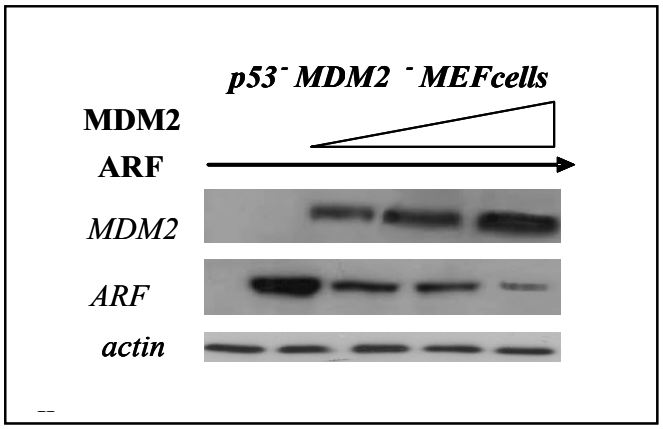

Figure 22 MDM2-mediated ARF degradation occurs in p53 independent manner.

Mouse Embryo Fibroblasts (p53-/- MDM2-/-) were transfected with 0.1 µg of pcDNA ARF alone or together with increasing amounts of MDM2 expression vector. At 24 hours after transfection cell extracts were analyzed by Western blot and immunodetection with anti MDM2, anti ARF antibodies. Anti actin antibody was used as loading control.

ROLE OF TBP-1 IN THE CONTROL OF CELL GROWTH.

During the last ten years many reports highlighted a very interesting aspect regarding the potential role of TBP-1 in the control of cell proliferation, supported by various evidences. Among these, our observation that TBP-1 increases the p14ARF oncosuppressor intracellular levels well fits with the proposed antioncogenic role of TBP-1 (Pollice et al., 2004; 2007). Interestingly, the potential oncosuppressive role of TBP-1 appears not to be restricted to the effect on ARF as we and others (Park et al., 1999; Corn et al., 2003) have demonstrated that TBP-1 overexpression can inhibit cell proliferation in various cellular contexts also independently by the presence of ARF. These observations not only further underlie the potential antiproliferative role of TBP-1 but suggest that it can exert a more general ARF-independent role in the control of cell proliferation and raise the question of what kind of cellular signals modulate TBP-1 expression.

In our laboratory the potential role of TBP-1 as a regulator of cell proliferation has been addressed. To this aim primary human fibroblasts immortalized cell derivatives, constitutively expressing an shRNA

specifically designed to silence TBP-1 expression, were used. The obtained clones show the following features:

- The clones express different levels of TBP-1 whereas the protein levels of other proteasome subunits, as well as the proteasome activity measured by cleavage of synthetic substrates, are unaffected by the silencing of TBP-1.
- the clones display a higher proliferation rate respect to the control and are able to sustain growth in the absence of serum.
- the clones display an increase of the S-phase and are more resistant to serum starvation induced apoptosis.

Overall, these data indicate that high TBP-1 levels contributes to control cell proliferation.

TBP-1 inhibits PKB/Akt activation.

The observations that clones in which TBP-1 expression is silenced present a higher proliferation rate respect to the control and can sustain growth in the absence of serum, raise the question of which are the potential intracellular pathways involved.

To go trough this point, I wondered whether 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 cycle progression. To this aim, by western blot, I evaluated the levels of phospho-Akt, a protein kinase controlling the balance between cell survival and apoptosis, in three silenced clones (T1, T10C, and T10E). As shown in figure 23, the level of pAkt seems to be inversely correlated to the extent of silencing, being lower in the parental T11HT and higher in the T1 clone. Furthermore, a corresponding reduction in the $p21^{Waf}$ and in the phospho-cyclinD1 levels appears to be consistent with the observed higher proliferation rate and with the Akt enhanced activation.

To further confirm that depletion of TBP-1expression results in the increase of activation of PKB/Akt, I treated the T1 clone and parental cells with Wortmannin and LY294002, two different inhibitors of the upstream Akt regulator PI3K.

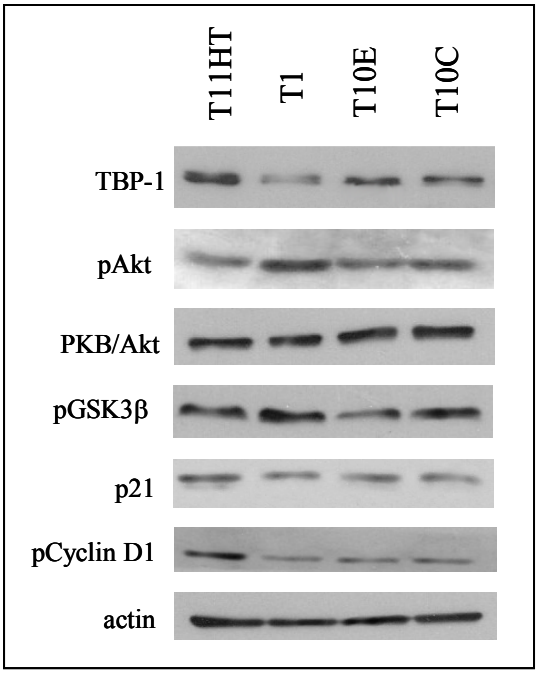

Figure 23 TBP-1 silenced clones show higher levels of activated PKB/Akt.
Wild-type T11hT, T1, T10E and T10C cells were plated in 100 mm dishes. 48 hours upon plating cellular extracts were prepared, resolved by SDS-PAGE and immunoblotted with the antibodies indicate.

As it is possible to observe in figure 24 the steady state level of pAkt in the T1 clone is around twice that present in the parental cells. Furthermore, the drug concentration necessary to inhibit pAkt level is almost the double as compared to that needed to inhibit pAkt level in the parental cells.

Since TBP-1 silencing causes an increase of the steady state levels of pAkt, I supposed that TBP-1 could control the extent of PKB/Akt activation. Consequently, I would expect that high levels of TBP-1 may exert a negative control either directly or indirectly on the activation of Akt. To this purpose I performed overexpression experiments of TBP-1 in U2OS cells and 24 hours upon transfection cell extracts were analyzed with antibodies either against TBP-1 or pAkt. Unfortunately the results were not unequivocal (data not shown). Therefore, I decided to evaluate the extent of PKB/Akt activation following insulin stimulation, a well known inducer of PKB/Akt. As shown in Figure 25, when TBP-1 is overexpressed, insulin stimulation causes a reduced activation of Akt, confirming that TBP-1 may contribute to pAkt regulation. Taken together these data suggest that TBP-1 may exert an antiproliferative effect by negatively regulating the Akt pathway.

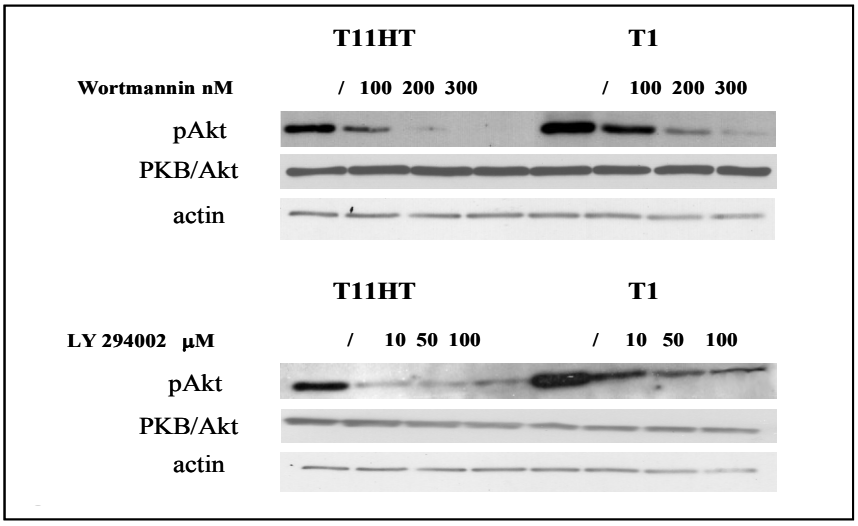

Figure 24 TBP-1 downregulation in the T1clone renders the cells more resistant to Wortmannin and LY294002 inhibitors.

T11hT cell line and T1 clone were plated in 35 mm dishes. Twenty four hours after the plating, the cells were treated with Wortmannin for 1 hour and LY2940002 for 15 minutes. Then, the cells were harvested and analyzed by Western Blot with anti pAkt, anti PKB/Akt and anti Actin antibodies.

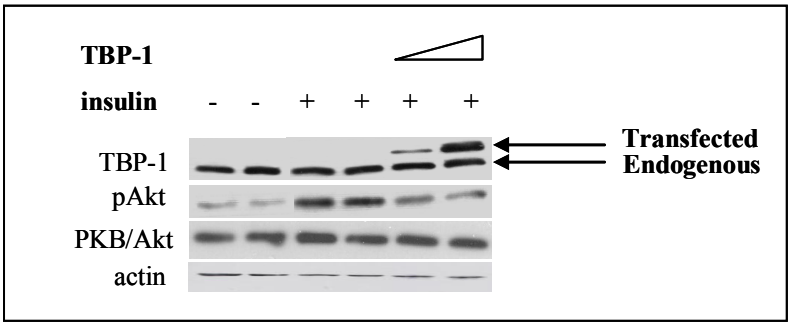

Figure 25 Overexpression of TBP-1 downregulates pAkt levels.

U2OS cells were transfected with 0.2 and 0.5 µg of the pcDNA TBP-1 expression vector. At 24 hours after transfection, cells were 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 PKB/Akt and anti Actin antibodies.

TBP-1 is a downstream target of Akt activation.

Since I have demonstrated that TBP-1 can inhibit the activation of pAkt following insulin stimulation I wondered if Akt could exert a down regulation of TBP-1, as well. U2OS osteosarcoma cells were starved for four hours and then stimulated by insulin addition at the concentration of 10 ng/ml 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. This effect results specific to TBP-1 as other proteasome subunits protein levels (PSMC5 and C8) remain unchanged following insulin treatment (Figure 26). Consistently, treating cells with the PI3K inhibitors Wortmannin and LY294002, I observed a slight but reproducible increase in TBP-1 endogenous levels (Figure 27).

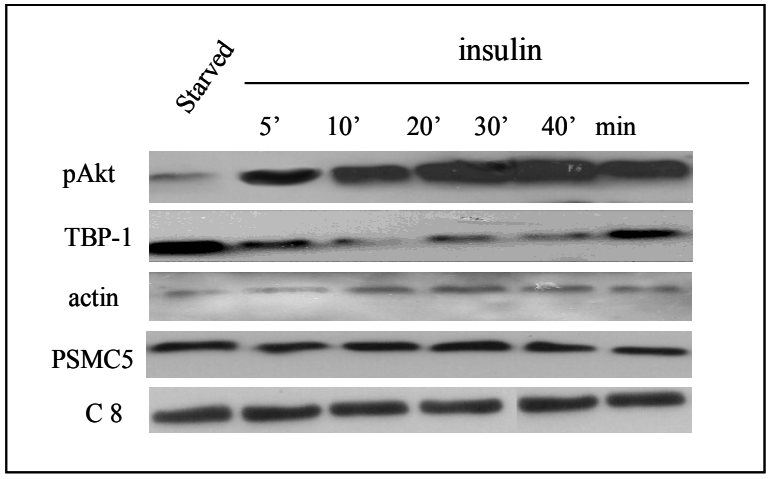

Figure 26 TBP-1 levels decrease following treatment with insulin.

U2OS cells were 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.

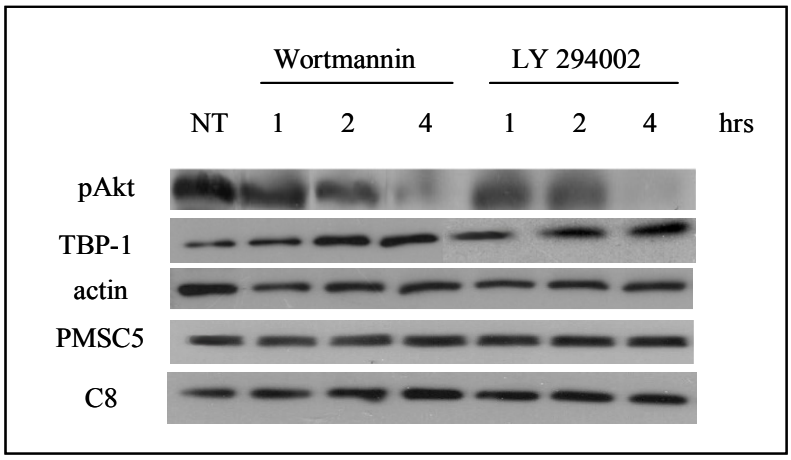

Figure 27 PI3K inhibitors upregulate TBP-1 levels.

U2OS cells were treated with 200 nM of Wortmannin and 50μM of LY294002 for the time indicated. Whole extracts were analyzed by Western Blot and immunorevealed with anti pAkt, anti TBP-1, anti Actin, anti PSMC5 and anti C8 antibodies.

Overall these data indicate that insulin down regulation of TBP-1 levels is mediated by the PI3K/Akt pathway. Further, the rapidity of such decrease, strongly suggests that insulin likely acts by inducing a drastic reduction in TBP-1 protein stability rather than by interfering with its biosynthesis. On the other hand, the low efficiency of PI3K inhibitors in increasing the TBP-1 steady state levels well fits with the hypothesis of a further small increase in its stability.

To further confirm the importance of the PI3K/Akt pathway in controlling TBP-1 levels I performed overexpression experiments using the Constitutively Active mutant of Akt (CA Akt), which is brought to the plasma membrane independently from the PI3K activity. U2OS cells were transfected with increasing amount of CA Akt and then analyzed by Western Blot with antibodies against TBP-1 and pAkt. As indicated in figure 28, the overexpression of CA Akt causes a remarkable reduction in TBP-1 endogenous levels. Furthermore, a corresponding increase in the pGSK3β levels, a direct downstream target of Akt, resulted consistent with the observed Akt activation. Interestingly, the other proteasome subunits analyzed do not change following Akt CA overexpression suggesting that this effect is specific to TBP-1.

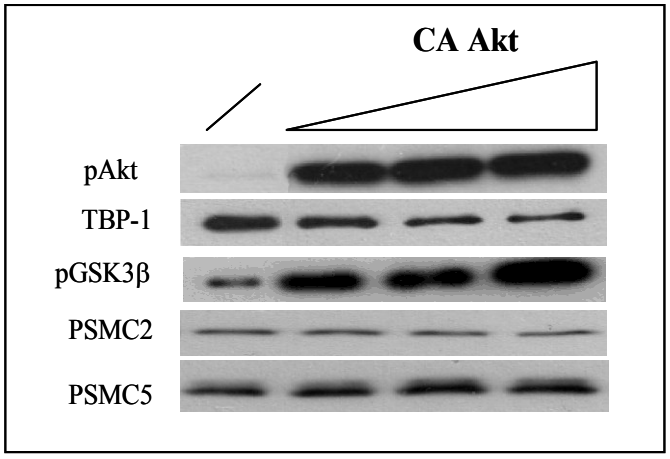

Figure 28 Effect of CA Akt mutant on TBP-1.

U2OS cells were transfected with increasing amounts of the CA Akt plasmid (0.2; 0.5; 1 μg). Twenty four hours after transfection cells were harvested, run on a SDS-PAGE and immunoblotted with the antibodies indicated in figure.

Regulation of TBP-1 intracellular levels by Akt occurs through MDM2.

The involvement of PI3K/Akt pathway in the regulation of MDM2 levels has been largely demonstrated. When activated, Akt is able to phosphorylate a vast number of proteins, MDM2 among them. Although there are still disagreements around which are the target residues important for MDM2 phosphorylation (Mayo et al., 2001; Ogawara et al., 2002; Feng et al., 2004) seems quite clear that MDM2 phosphorylation by Akt results in its stabilization with consequent protection from proteasomal degradation. Since TBP-1 levels are regulated by Akt I wondered if MDM2 could be a mediator in this pathway. To test this hypothesis I used the Akt inhibitor Wortmannin on U20S cells in which the MDM2 expression has been silenced with specific siRNA. As expected, treatment of cells with the PI3K inhibitor results in an increase of TBP-1 levels (figure 29 left) that is abolished when MDM2 levels are reduced by siRNA(figure 29 right), suggesting that MDM2 could be a mediator of the TBP-1 increase following Akt downregulation.

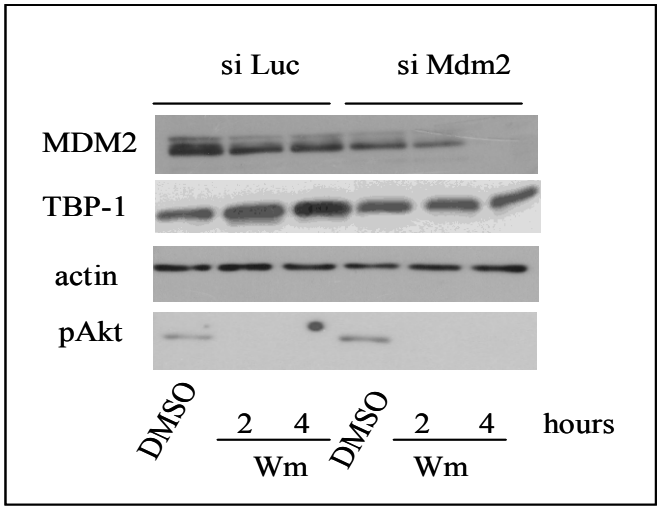

Figure 29 Treatment of cells with Wortmannin causes TBP-1 accumulation only in the presence of high levels of MDM2.

U2OS cells were transfected with 10 nM MDM2 siRNA or firefly luciferase siRNA. 48 hours after transfection cells were treated with Wortmannin for 2 and 4 hours, cell extracts were prepared, resolved by SDS Page and immunorevealed with the antibodies indicated in figure.

To keep more insights into the regulation of TBP-1 levels by MDM2, I tested if TBP-1 is a direct target of MDM2. To this purpose, I performed coimmunoprecipitation experiments to check the binding between the two proteins. U2OS cells were immunoprecipitated with anti-MDM2 antibody and revealed with either anti-MDM2 or anti-TBP-1 antibodies. As showed in figure 30 TBP-1 results a binding partner of MDM2. Interestingly, in U2OS cells, I observed that MDM2 overexpression causes a strong decrease in TBP-1 levels, both endogenous and transfected (Figure 31).

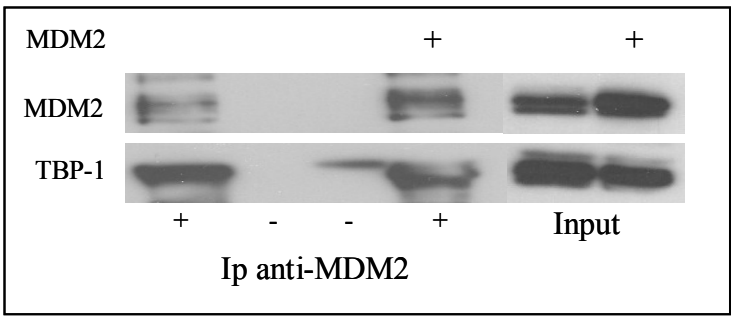

Figure 30 TBP-1 interacts with MDM2.

U2OS cells were transfected with 1 μg of the pCMV MDM2 expression plasmid where indicated. Lysates were analysed by anti-MDM2 or anti-TBP-1 antibodies (input), immunoprecipitated with anti-MDM2 antibody and revealed with anti-MDM2 and anti-TBP-1 antibodies.

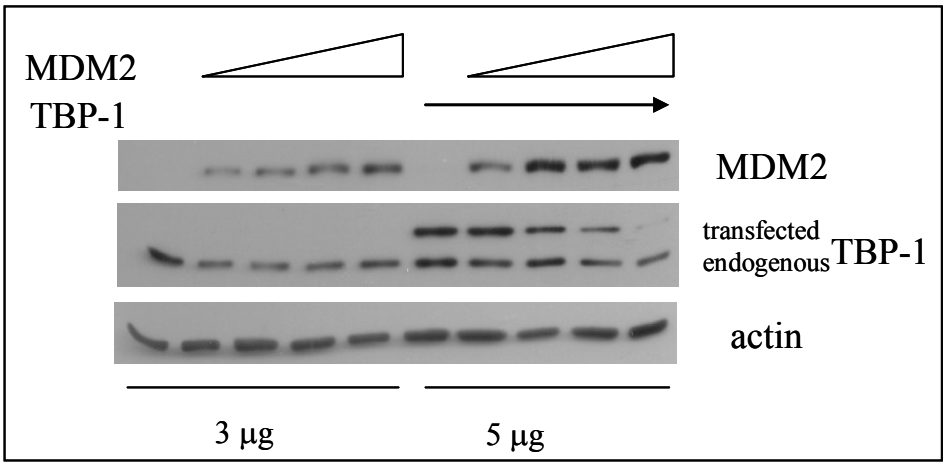

Figure 31 MDM2 overexpression causes decrease of TBP-1 levels, both endogenous and transfected.

U2OS cells were transfected with increasing amounts of the MDM2 plasmid to evaluate the effect on the endogenous TBP-1 levels and cotransfected with a fixed amount of the TBP-1 plasmid and increasing amounts of the MDM2 vector. Cell lysates were analyzed by Western Blot with anti MDM2, anti TBP-1 and anti actin antibodies.

Importantly, this effect does not require the ubiquitine ligase activity of MDM2, since the $MDM2_{1-441}$ (lacking the ring finger) is still able to degrade TBP-1. Conversely, the $MDM2_{\Delta 150-230}$ mutant, that displays a cytoplasmic localization, is unable to degrade TBP-1 suggesting that the observed effect requires the translocation of MDM2 in the nucleus (Figure 32), although further experiment are needed to elucidate this point.

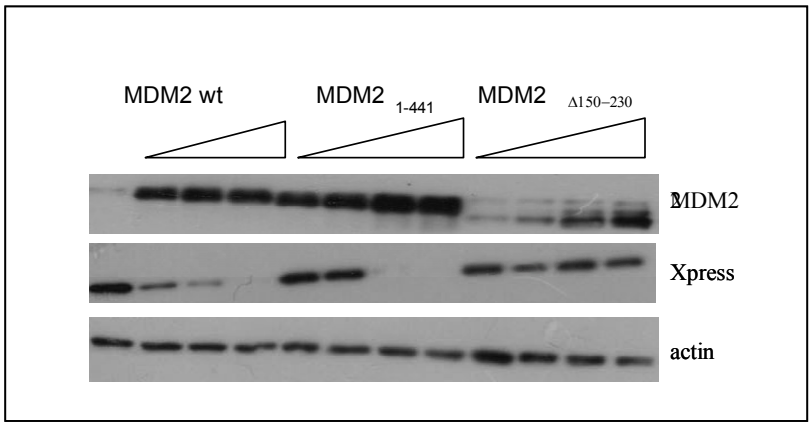

Figure 32 Effect of the MDM2 mutants on TBP-1.

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 Xpress (to reveal TBP-1) antibodies. Actin was checked as loading control.

Finally, to gain more insights into the mechanism responsible of the TBP-1 degradation, I performed the same kind of experiments also in (p53-/-mdm2-/-) Mouse Embryo Fibroblasts. MEFs cells were transfected with a fixed amount of TBP-1 and increasing amounts of the MDM2 vector. Western blot of whole extracts and specific immunodetection with anti-MDM2 antibodies and anti-Xpress antibodies show that also in this cellular context, MDM2 is able to degrade efficiently TBP-1 suggesting that this effect is p53 independent (Figure 33).

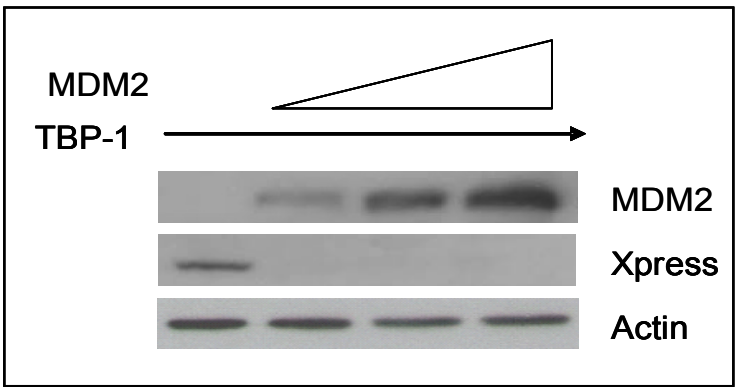

Figure 33 MDM2 downregulates TBP-1 in a p53 independent manner.
Mouse Embryo Fibroblasts (p53-/- MDM2-/-) were transfected with 0.1 μg of the pcDNA TBP-1 alone or together with increasing amounts of the MDM2 expression vector. At 24 hours after transfection cell extracts were analyzed by Western blot and immunodetection with anti MDM2, anti Xpress (to reveal TBP-1) and anti Actin antibodies.

DISCUSSION

TBP-1 belongs to the AAA-ATPases gene family (ATPAses Associated to a variety of cellular Activities) and, as well as other members of this family, is a component of the 19S subunit of the proteasome**.** Interestingly, during the last years, 19S proteasome subunits and TBP-1 itself turned out to be involved also in cellular events that do not require proteolysis, such as the regulation of transcription (Gonzalez et al., 2002; Ferdous et al., 2002; Sulahian et al., 2006), the involvement in N.E.R. (Nucleotide Excision Repair) (Russell et al., 1999) and in the mitotic process (Chen et al., 1997).

Furthermore, several evidences support a potential tumour-suppressive role of TBP-1: its expression was found to be elevated following inhibition of the oncogenic phenotype of *erb-B* transformed cells suggesting that its activation could be necessary to counteract oncogenic signals (Park et al., 1999). Consistently, forced expression of TBP-1 in different human tumour cells diminished cell proliferation, reduced the ability of the parental cell line to form colonies *in vitro* and strongly inhibited the transforming efficiency in the athymic mice (Park et al., 1999). Moreover, TBP-1 has been shown to bind to the tumour

suppressor VHL (Von Hippel Landau) gene product (Corn et al., 2003) contributing to its E3-ubiquitin ligase function toward the Hifl α factor, thus acting as a *bona fide* tumour suppressor.

Our observation that TBP-1 interacts with and stabilizes the p14ARF oncosuppressor delaying its turnover, well fits with its proposed antioncogenic role (Pollice et al., 2004). Interestingly, the fact that TBP-1 overexpression diminished cellular proliferation also in ARF minus contexts (Pollice et al, 2004) suggests that its potential oncosuppressive role is not restrict to the effect on ARF but can exert a more general ARF-independent role in the control of cell proliferation.

ARF is among the most relevant tumour suppressors in mammalian cells, sensing hyperproliferative stimuli and acting to restrict cell proliferation through both p53-dependent and independent pathways (Lowe et al., 2003). The discovery of multiple ARF interactors and the observation that, aside oncogenic stimuli, also viral, genotoxic, hypoxic and oxidative stresses activate an ARF dependent response, suggest that ARF could exert a wider role to protect the cell (Fatyol et al., 2001; Menendez et al., 2003; Garcia et al., 2006).

It is becoming clear that the ARF response is quite complex and is likely accomplished by the interaction with a multitude of cellular partners that makes difficult the formulation of a unique model that could depict the

ARF role in the cell. On the other side, the regulation of ARF intracellular levels itself is not yet completely clear and the mechanisms regulating its turnover appear still controversial. Given its strong ability to block both growth and proliferation, cells must develop mechanisms that promptly reduce ARF protein levels when its activity is no more required. ARF is a relatively stable protein, although prevalently unstructured and largely disordered in solution (Bothner et al., 2001). For long time it has been thought that it could not be degraded by the proteasome since its sequence lacks lysine residues (the mouse protein presenting only one lysine residue) that can allow ubiquitination in a canonical way. Recently, we and others (Kuo et al., 2004, Pollice et al., 2004, 2007) demonstrated that ARF is degraded, at least in part, by the proteasome, although the mechanisms governing its delivery into the proteasome still remain to be completely clarified. Furthermore, it has been reported that ARF can be subjected to N-terminal ubiquitination, a process independent from p53 and MDM2 (Kuo et al., 2004) whose physiological role is still elusive (see Introduction).

It has to be noted that, although most cellular proteins that are directed to the proteasome are previously ubiquitinated, a growing body of evidence underline the existence of proteasome-dependent but ATP and ubiquitin-independent mechanisms of degradation (ornithine decarboxylase, the

Cdk inhibitor p21, α-synuclein, Hifα, members of the Rb family of tumour suppressors, p53 and p73 (Sdek et al., 2005; Kong et al., 2006). In many cases, if a protein can be delivered to the proteasome in a denatured or partially unfolded state, ubiquitination should not be required for its degradation. In fact, p21 and α-synuclein that are considered "naturally unstructured" proteins can be degraded *in vitro* by the proteasome, in the absence of ubiquitination (Liu et al., 2003). Accordingly to its native unstructured nature, ARF can be degraded *in vitro* by the 20S proteasome, in ATP and ubiquitin-independent manner (Pollice et al., 2007).

However, a very recent report give a different explanation of the ubiquitin-independent and ATP-independent degradation of important cell-cycle regulators like ARF, p21Cip/WAF1 and p16INK4a. In this process there is the direct involvement of the REGγ proteasome, a regulative subunit of the proteasome that is alternative to the 19S with yet uncovered functions. (Chen et al., 2007). It has been proposed that the REGγ pathway is specialized for the proteasomal degradation of small unstructured proteins since p19ARF, p21, and p16 are all unstructured when not associated with specific binding partners (such as cyclins and Cdks, for p21 and p16, and nucleophosmin in the case of p19ARF).

Strikingly, my data show that TBP-1 is able to counteract the degradation of ARF by the 20S proteasome *in vitro*, independently from the presence of an assembled 19S particle, highlighting that the TBP-1 protective effect on ARF is proteasome-independent. On the other hand, we have already demonstrated that a similar effect occurs also *in vivo*, since TBP-1 overexpression in various cell lines delays ARF turnover (Pollice et al 2004). Furthermore, I have demonstrated that silencing of TBP-1 expression causes a concomitant reduction of ARF intracellular protein levels, strongly suggesting that basal TBP-1 levels controls basal ARF levels. Interestingly, a point mutation in the ATPAse domain of TBP-1 that destroys its chaperone-like activity, impairs TBP-1's capacity to stabilize ARF, leading us to postulate the hypothesis that, upon binding, TBP-1 could cause ARF to fold, rendering it a poor substrate for proteasome destruction.

Thus, the discovery that p14ARF can directly interact with regulative components of the proteasome multi-protein complex, such as TBP-1 of the 19S subunit (Pollice et al., 2004, 2007) and REGγ of the 11S lid (Chen et al., 2007) has offered a new key to interpret the mechanisms through which ARF is regulated and regulates cell growth and proliferation. It is possible that alternative binding of ARF to different proteasome subunits, TBP-1 or REGγ, could dictate its fate, mediating

either its stabilization or its degradation (Figure 1). It would be of great interest to investigate on the hypothesis of a direct competition between REGγ and TBP-1 for the binding to ARF and to explore the stimuli and molecular pathways involved.

Overall my data on ARF result relevant in the comprehension of the regulation of its turnover. Furthermore, it is interesting to underline that TBP-1 is excluded from the nucleolus and binds to ARF mainly in the nuclear compartment (Pollice et al., 2007), where probably determines its folding, that is necessary to ARF for its biological function. A possibility still to be explored is that the interaction between ARF and TBP-1 is important not only to control ARF levels but could mediate the drag of ARF partners into the proteasome cavity. The reported observations that both TBP-1 and ARF exert a negative effect on Hif1α (Fatyol et al., 2001; Corn et al., 2003) seem to support the idea that a synergy of action between ARF and the proteasome could occur.

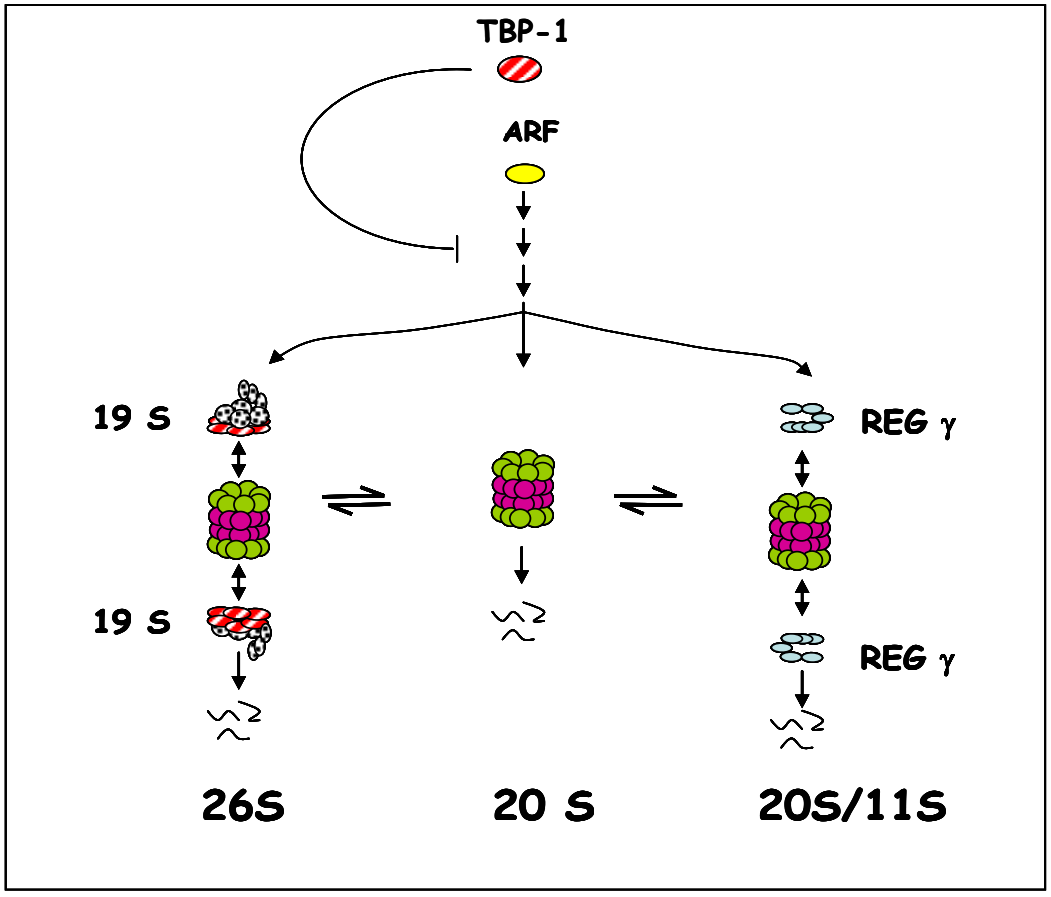

Figure 1 A model for the regulation of ARF turnover.

ARF can be degraded by the proteasome through ubiquitin independent (by 20S or 20S/REGγ complex) or dependent (by 26S complex) mechanisms. Binding to 19S subunit TBP-1 protects ARF from degradation both in vitro and in cells.

Other sets of data accumulated in the laboratory and by myself pointed to a very interesting ARF-independent tumour suppressive role of TBP-1. In fact human fibroblasts immortalized cell clones that constitutively express an shRNA designed to silence TBP-1 expression proliferate at higher rate respect to the control also in condition of serum deprivation, display an increase of the S-phase of the cell cycle and are more resistant to serum starvation induced apoptosis. TBP-1 silenced clones exhibit higher levels of activated pAkt, a protein kinase controlling the balance between cell survival and apoptosis, leading to the hypothesis that TBP-1 could exert a down-modulation of activated Akt levels and activity. Consistently, transient overexpression of TBP-1 causes a reduced activation of pAkt following insulin stimulation.

Moreover, my data demonstrate that TBP-1 is likely a downstream target of Akt activation. Acute insulin stimulation of U2OS osteasarcoma cell line causes a rapid drop in TBP-1 intracellular levels. Conversely, the inhibition of the PI3K/Akt pathway by making use of specific drugs (Wortmannin and LY) determine a reproducible increase of TBP-1 intracellular levels. Importantly, this effect is specific to TBP-1 as other proteasome subunits protein levels (PSMC5 and C8) remain unchanged. Interestingly, it has been described that another AAA-ATPase component of the 19S proteasome (S4/Rpt2 ATPase) also respond to

growth factor stimulation (in this case by heregulin β1) but with an opposite effect, i.e., an increase of its intracellular levels (Barnes et al., 2005), strongly suggesting that the effect seen on TBP-1 is specific.

Taken together, these data demonstrate that TBP-1 intracellular levels are critical to control cell duplication and are tightly regulated by a double-negative feedback loop that is mediated by the activation of the PKB/Akt kinase that thus seems to act as a sensor that modulate the TBP-1 levels in actively duplicating cells. As I did not observe a direct binding between TBP-1 and Akt, I supposed that TBP-1 is an indirect target of Akt activation.

Interestingly, I accumulated preliminary data suggesting that MDM2 could be a mediator of the PI3K/Akt signalling on TBP-1. In fact MDM2 is among the main direct targets of PKB/Akt activation (Mayo et al., 2001, Ogawara et al., 2002, Feng et al., 2004), and in other sets of experiments I demonstrated that it is a partner of TBP-1. Furthermore, MDM2 overexpression can cause a reduction of both endogenous and transfected TBP-1 intracellular levels indicating that TBP-1 is indeed a target of MDM2. Strikingly, blocking the PI3K activation by the addition of Wortmannin to cells previously transfected with a siRNA designed to silence MDM2 expression, prevents the accumulation of

TBP-1, strongly suggesting the involvement of MDM2 in the "PI3K/Akt –TBP-1 axis".

It is interesting to note that we and others (Rodway et al., 2004) have observed an involvement of MDM2 also in the regulation of ARF protein levels. In fact MDM2 overexpression can cause a significant and reproducible decrease of both endogenous and transfected ARF intracellular levels. Interestingly, by using a mutant of MDM2 that lack the RING Finger domain, I demonstrated that the ubiquitin ligase activity is not required for the MDM2-induced degradation of both ARF and TBP-1. However, these results are somehow not new since it has already been described that proteins like p21 and pRb (Jin et al., 2003; Sdek et al., 2005) can be directed to proteasome-mediated degradation by MDM2 without preventive ubiquitination. The molecular mechanism through which this occurs is far to be comprised.

These observations lead to the suggestion of a unique model of action of MDM2 toward targets that can be degraded without being ubiquitinated. However, the use of an MDM2 mutant that lacks both NES and NLS sequences (the $MDM2_{\Delta 150\text{-}230}$) and displays only a cytoplasmic localization, pointed out that the existence of a unique model of action of MDM2 both on ARF and TBP-1 cannot be assumed. In fact, the $MDM2_{\Delta 150\text{-}230}$ mutant is capable to degrade ARF, while it is not respect

to TBP-1. To unravel this mechanism represents an interesting subject to study. In any case, aside the mechanisms that have still to be clarified, the MDM2 action on ARF underlies the existence of a negative feedback loop in which ARF regulates and is regulated by MDM2 and viceversa. On the other hand, the same could be true for the MDM2 effect on TBP-1, in which the MDM2 phosphorylation/activation, following insulin stimulation through PI3K/Akt signalling causes MDM2 to translocate in the nuclear compartment (Mayo et al., 2001, Ogawara et al., 2002) where it could act dowregulating TBP-1 levels; in turn, TBP-1 can counteract PKB/Akt activation that is likely reflected in a reduced activation of MDM2. How TBP-1 prevents PKB/Akt activation is, up to now, completely unknown. However, given the TBP-1 capability to stabilize p14ARF, one of the most important human oncosuppressors, a possibility to explore is that it could exert a more general role in the cell through the stabilization of other oncosuppressors, like, for example, PTEN, that is among the most important negative regulator of the PI3K activation (Hirsch et al., 2007; Sale et al., 2008),

In conclusion my results suggest that TBP-1 can exert antioncogenic properties through different molecular mechanisms still to be explored. Further studies are needed for the comprehension of the mechanisms through which TBP-1 is regulated and regulated cell proliferation.

MATERIALS AND METHODS

Plasmids

pcDNA ARF_{39-132} was obtained from NarI/XbaI cut of pcDNA ARF, fill in at the NarI site and ligated into the pcDNA3.1His (Invitrogen) cut with EcoRV/XbaI.

pcDNA ARF_{1-39} was obtained from EcoRI/NarI cut of pcDNA ARF, fill in of the NarI site, and cloning in pcDNA3c.1 EcoRI/EcoRV digested.

3xFlag ARF_{1-39} was obtained by PCR amplification using the primers ARFup (AAGAATTCAATGGTGCGCAGG) and ARFdown (AAAAGATCTCCCTGGCGCTGCCCA) and subsequent cloning in p3xFlagCMV10 cut EcoRI/BglII.

pEGFP ARF_{1-39} and pEGFP ARF_{39-132} were obtained from EcoRI/XbaI cut of respectively, pcDNAARF_{1-39} and pcDNA ARF_{39-132} and subsequent cloning in EcoRI/XbaI of pEGFPc2.

pCMV3xFlag ARF, pCMV3xFlag ARF_{1-65} and pCMV3xFlag ARF_{65-132} were kindly provided by prof. Majello.

pcDNA $ARF_{\Delta 2-14}$, pcDNA $ARF_{\Delta 26-37}$, pcDNA $ARF_{\Delta 82-101}$ and pcDNA$ARF_{\Delta 2-14/\Delta 82-101}$ plasmids were kindly provided by CJ Sherr.

pcDNA TBP-1ΔGKT deletion mutant was obtained using Quick Change Site Direct Mutagenesis (Stratagene) following the manufacturer's instruction. Briefly, pcDNA TBP-1 plasmid (with target site for mutation) was denatured. After the annealing of the mutagenic primers (ΔATPf 5'-TGGGCCCCCAGGGACGCTCCTGGCCCGGGCCTG-3', ΔATPr 5'-CAGGCCCGGGCCAGGAGCGTCCCTGGGGGCCCA-3') the addition of the Taq enzyme leads to the extension and incorporation of the primers, 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.

H1299, U2OS, HEK293T, MEFs p53-/MDM2-, T11HT and T1 cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and grown at 37°C in humidified atmosphere of 5% CO_2.

Transfections were performed as described below.

Cell Line	Plate diametr	# Cells	DNA	Reagent	DNA/Reagent
H1299	35 mm	$2.5x10^5$	2µg	Lipofectamine	1:3
	60 mm	$5x10^5$	5µg		
U20S	35 mm	$2.5x10^5$	2µg	Lipofectamine 2000	1:1,5
	60 mm	$5x10^5$	5µg		
HEK 293T	35 mm	$3.3x10^5$	2µg	Lipofectamine	1:3
	60 mm	$6.5x10^5$	5µg		
MEFs	35 mm	$2.5x10^5$	2µg	Lipofectamine 2000	1:3
	60 mm	$5x10^5$	5µg		

The cells were transfecting following the manufacturer' s instructions. The total amount of transfected DNA was kept constant by using the "empty" expression vector when necessary.

SDS-Page and Western Blot analysis.

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). Proteins were visualized by enhanced chemiluminescence method (ECL) (GE-Healthcare).

Coimmunoprecipitation Assay.

Coimmunoprecipitations were carried out in U2OS cells. 5.0 x10^5 cells were plated in 60 mm dishes and transfected with 1:1 ratio of TBP-1 and various ARF constructs. 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) and then incubated overnight at 4°C with 2 μg of anti-ARF (Santa Cruz sc-8613) or with 2μg of monoclonal anti-TBP-1 (BIOMOL PW8770). The following day protein-A agarose (Roche) or protein G-sepharose (Amersham Biosciences) beads were added for 3 hours at 4^o C. The beads were collected by centrifugation,

and the immunoprecipitates were washed three times with IBP buffer (4 °C), solubilized in Laemmli buffer, loaded on 8 or 12% SDS-Page and analyzed by immunoblotting with anti-TBP-1 antibody or anti-ARF antibody.

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. H1299 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-MDM2 (Calbiochem OP115), anti-Itch (BD-Clontech 611198), anti β-tubulin (Santa Cruz sc-9104), anti-B23 (Zymed FC61991), anti-p21 (Santa Cruz sc-397), anti actin (Santa Cruz sc-1616), anti-ARF and anti-TBP-1.

siRNA of MDM2.

A twenty-one nucleotide RNA targeting human MDM2 mRNA 5'-AAGCCAUUGCUUUUGAAGUUA-3' and a siRNA targeting a sequence firefly luciferase-mRNA, used as control, were chemically synthesized by MWG Biotech. U2OS cells were transfected with Hyperfect (Quiagen) according to the manufacturer's instructions using 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).

Subcellular localization assay.

U2OS were plated in 35mm dishes and grown on micro cover glasses (BDH). Twenty four hours after transfection with the appropriate vectors (see Results), cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (Sigma-Aldrich), for 15 minutes. Cells were then washed twice with PBS and permeabilized in 0.1% Triton X-100 for 10 minutes at room temperature. After the permeabilization cells were incubated in blocking buffer solution for 15 minutes (PBS containing 5% of FBS) and then incubated in blocking

buffer supplemented by 0.5% Tween-20, containing the primary antibody (anti-FLAG 1:2000 (M2-Sigma) (to determine subcellular localization of ARF FLAG and ARF $FLAG_{1-39}$) for 30 minutes at room temperature. Following extensive washing in PBS, fixed cells were incubated with the appropriate fluorochrome-conjugated secondary antibody for a further 30 minutes at room temperature. In details, anti-Flag was visualized with anti-Cy3-conjugated anti-mouse (ImmunoResearch). After PBS washing, the cells were incubated with DAPI (4',6'-diamidino-2-phenylindole) 10 mg/ml [Sigma]) for 3 minutes. Glasses were then washed in PBS, mounted with Moviol (Sigma) and examined under a fluorescence microscope (Nikon). Images were digitally processed by Adobe Photoshop software.

Treatment with proteasome and lysosomes inhibitors.

U2OS cells were seeded into two 60-mm plates and transfected with pcDNA ARF_{39-132}. The following day, each plate was split into two aliquots to obtain four 35 mm plates and incubated to allow the adhesion. Subsequently, each plate was treated for 6 hours with 50 μM ALLnL (Sigma), 50 μM MG132 (Sigma) and with 0.1% dimethyl sulfoxide

(Sigma) as control. Cell lysates were collected and probed with anti-ARF and anti-actin antibodies.

293T cells were treated for 6 hours with proteasome (50 μM ALLnL, 25 μM MG132) and lysosome inhibitors (100 nM Bafilomycin, 25 μM NH_4Cl, 200 μM Cloroquine). Cell lysates were probed with anti-ARF and anti-actin antibodies.

Decay rate analysis.

U2OS cells were transfected with the plasmids decribed in the Results. Twelve hours after transfection, the cells were trypsinized and reseeded in smaller wells corresponded to the different time points. Twenty-four hours after transfection, cycloheximide (Sigma) was added to the medium at a final concentration of 80 μg/ml, and cells were harvested at the indicated time points. Total cell extract were prepared as described above. Cell extracts were probed, in Western blot, with anti-ARF antibody and, as control, with anti-actin antibody.

In vitro protein degradation assay.

Reticulocyte lysate translated proteins (kit Promega), were treated for 1 or 3 h at 37ºC with or without 1 mg of 20S proteasome (Sigma) in degradation buffer (20mM Tris-Hcl pH 7, 0.2M. NaCl, 10mM MgCl2, 1mM dithiothreitol) with or without 50 μM MG132.

Translated p14ARF or $p14ARF_{39-132}$ were also pre-incubated for 30 minutes on ice, with *in vitro* translated TBP-1 before the addition of the 20S proteasome. Reaction was stopped by the addition of Laemmli Buffer and samples were analysed by Western blot as previously described.

Treatment of the cells with PI3K inhibitors.

T11HT and T1 cells were plated in 35 mm dishes. 24 hours after the plating, cells were treated with Wortmannin (Calbiochem) at the concentration of 100, 200 and 300 nM for 1 hour and with LY294002 (Calbiochem) at the concentration of 10, 50 and 100 μM for 15 minutes. Total cell extracts were prepared as described above and samples were analyzed by Western blot.

Treatment of the cells with insulin.

U2OS 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.

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ORIGINAL ARTICLE

TBP-1 protects the human oncosuppressor p14ARF from proteasomal degradation

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The p14ARF tumor suppressor is a key regulator of cellular proliferation, frequently inactivated in human cancer. The mechanisms that regulate alternative reading frame (ARF) turnover have been obscure for long time, being ARF a relatively stable protein. Recently, it has been described that its degradation depends, at least in part, on the proteasome and that it can be subjected to N-terminal ubiquitination. We have previously reported that ARF protein levels are regulated by TBP-1 (Tat-Binding Protein 1), a multifunctional protein, component of the regulatory subunit of the proteasome, involved in different cellular processes. Here we demonstrate that the stabilization effect exerted by TBP-1 requires an intact N-terminal 39 amino acids in ARF and occurs independently from N-terminal ubiquitination of the protein. Furthermore, we observed that ARF can be degraded *in vitro* by the 20S proteasome, in the absence of ubiquitination and this effect can be counteracted by TBP-1. These observations seem relevant in the comprehension of the regulation of ARF metabolism as, among the plethora of cellular ARF's interactors already identified, only NPM/B23 and TBP-1 appear to be involved in the control of ARF intracellular levels.

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Keywords: p14ARF; TBP-1; proteasome; N-terminal ubiquitination

Introduction

The alternative reading frame (ARF) protein induces potent growth arrest or cell death in response to hyperproliferative oncogenic stimuli. ARF can activate the p53 tumor surveillance pathway by interacting with and inhibiting the p53 antagonist, mouse double minute 2 (Mdm2) (Sherr, 2001). Nonetheless, ARF can exert antiproliferative, p53-independent activity including the ability to inhibit ribosomal RNA processing (reviewed by Sharpless, 2005) and different effects on gene expression (reviewed by Gallagher *et al*., 2006; Paliwal *et al*., 2006). Very recently, a shortened, unstable form of the ARF tumor suppressor protein, localizing to mitochondria and triggering autophagy, has been described (Reef *et al*., 2006).

Normally, ARF protein localizes in the nucleolus, where undergoes a variety of interactions, including that with B23/NPM a multifunctional nucleolar phosphoprotein (Bertwistle *et al*., 2004). On the other hand, ARF binds to many different cellular proteins besides Mdm2 and B23 and, in few cases, such interactions regulate ARF's stability and/or activity (Gallagher *et al*., 2006), whereas B23 and TBP-1 (Tat-Binding Protein 1) affects ARF protein stability (Colombo *et al*., 2005; Pollice *et al*., 2004).

Interestingly, the ARF-B23/NPM interaction seems critical in the regulation of both proteins: ARF causes B23 polyubiquitination and degradation, whereas B23 overexpression protects ARF from degradation, bringing it into the nucleolus and antagonizing its ability to inhibit cell division (Itahana *et al*., 2003; Korgaonkar *et al*., 2005).

The mechanisms that regulate ARF turnover have been obscure for long time, being ARF a relatively stable protein. Recently, it was shown that its degradation depends, at least in part, on the proteasome (Kuo *et al*., 2004, Rodway *et al*., 2004), but the mechanisms governing ARF's delivery into the proteasome still remain to be clarified. It has also been described that ARF can undergo N-terminal ubiquitination (Kuo *et al*., 2004).

We previously reported that TBP-1, a multifunctional protein, component of the regulatory subunit of the proteasome, interacts with and regulates ARF protein levels increasing its half-life (Pollice *et al*., 2004).

Most known protein substrates of the proteasome are covalently modified with a polyubiquitin chain as a prerequisite for their proteolysis. Recognition of this signal is followed by substrate unfolding and translocation; these processes are presumably catalysed by one or more of the six non-redundant ATPases located at the base of the 19S regulatory subunit. Actually, TBP-1 is a member of this protein family and is likely to be involved in this process (Voges *et al*., 1999).

However, an interesting aspect of the 19S regulatory subunits is their apparent involvement in cellular events that do not require proteolysis. In fact, 19S protein

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members are involved in transcription regulation (Gonzalez *et al*., 2002), in Nucleotide Excision Repair (Russell *et al*., 1999) and in the regulation of mitosis (Chen *et al*., 1997). Interestingly, we and others have observed that TBP-1 overexpression can reduce cell proliferation in certain cellular contexts (Park *et al*., 1999; Pollice *et al*., 2004). Furthermore, TBP-1 is involved in the von Hippel–Lindau (VHL)-dependent degradation of Hif1α, frequently overexpressed in tumors (Corn *et al*., 2003). Taken together, these observations suggest that TBP-1 could act as tumor suppressor in human cells.

In this paper we sought to investigate more precisely on the stabilization effect exerted by TBP-1 on p14ARF. Consistent with a physiological role of the interaction between the two proteins, we observed that reduction of TBP-1 intracellular levels by siRNA causes a drop down in the ARF endogenous levels. Furthermore, we demonstrate that the stabilization effect exerted by TBP-1 requires an intact N-terminal 39 amino acids in ARF and occurs independently from N-terminal ubiquitination of the protein. Finally, we observed that ARF can be degraded *in vitro* by the 20S proteasome and that this effect can be counteracted by TBP-1.

Results

TBP-1 silencing reduces p14ARF protein levels

We previously demonstrated that TBP-1 is a partner of p14ARF and that its overexpression results both in an increase in p14ARF half-life and activation of Mdm2/p53 pathway (Pollice *et al*., 2004).

To further confirm the importance of TBP1 in controlling p14ARF steady-state levels, we reduced endogenous TBP-1 protein levels by making use of RNA interference. H1299 cells, that present detectable levels of both ARF and TBP-1 were transfected with two different concentrations of a siRNA duplex designed to silence TBP-1 expression (Corn *et al*., 2003). At 72 h after transfection, protein lysates were analysed with anti-TBP-1 and anti-ARF antibodies. Figure 1 clearly shows that reduction in endogenous TBP-1 protein expression resulted in a reproducible decrease of ARF intracellular levels, confirming that basal TBP-1 levels are important in controlling basal p14ARF levels.

It is interesting to note that silencing of TBP-1 in cells expressing wild-type pVHL delays degradation of the Hif1α transcription factor (Corn *et al*., 2003). On the other hand, we did not observe any change in the basal expression levels of various cellular proteins (p21, Itch, Mdm2, B23/NPM, actin and β-tubulin) (Figure 1). As some of them are reported to be subjected to proteasomal degradation, we conclude, according to Corn *et al*., that reduction of TBP-1 intracellular levels does not generally affect proteasome function, but rather appears to affect only specific targets.

The first N-terminal 39 aa in p14ARF are necessary for both interaction with and stabilization by TBP-1

Given that TBP-1 is a widely expressed protein, with a preferential cytoplasmic localization (Pollice *et al*., 2004), we wanted to define the subcellular compartment in which the interaction among the two proteins takes place. To this purpose, nuclear or cytoplasmic extracts from H1299 cells were immunoprecipitated with anti-TBP-1 antibodies. Figure 2a shows that, in this experimental setting, ARF is mainly immunoprecipitated in the nuclear fraction. Western blot by anti-poly-ADP-ribose polymerase (PARP) (specific for nuclei) and anti-αtubulin (specific for cytoplasm) represents the control of the extract fractionation.

By two-hybrid assays in yeast, we already narrowed the ARF region necessary for the binding to TBP-1 to the first 38 amino acid (aa) (Pollice *et al*., 2004). To confirm our results in mammalian cells, we transfected U2OS cells (devoid of ARF expression), with pcDNAARF or pcDNAARF$_{39-132}$ alone or in combination with pcDNATBP-1 and immunoprecipitated protein extracts with either anti-TBP-1 or anti-ARF antibodies (Figure 2b). Only wtARF, as expected, interacts with TBP-1.

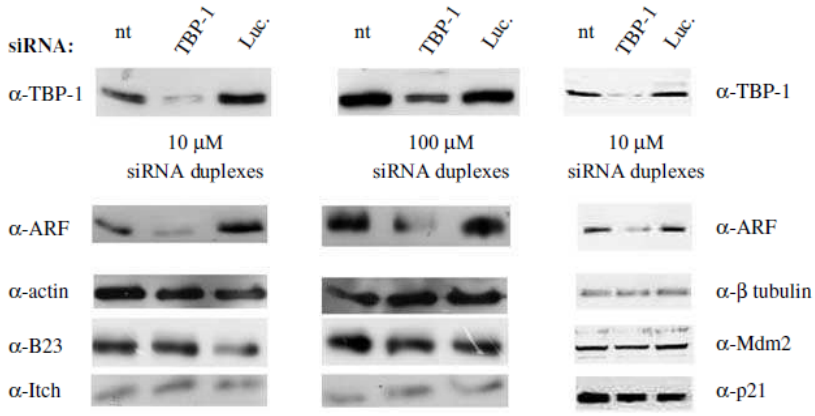

Figure 1 Effect of TBP-1 siRNA. H1299 cells were transfected with 10 and 100 μM of 21 bp TBP-1 siRNA or firefly luciferase siRNA. Levels of TBP-1, p14ARF and of other endogenous proteins at 72 h after transfection are shown.

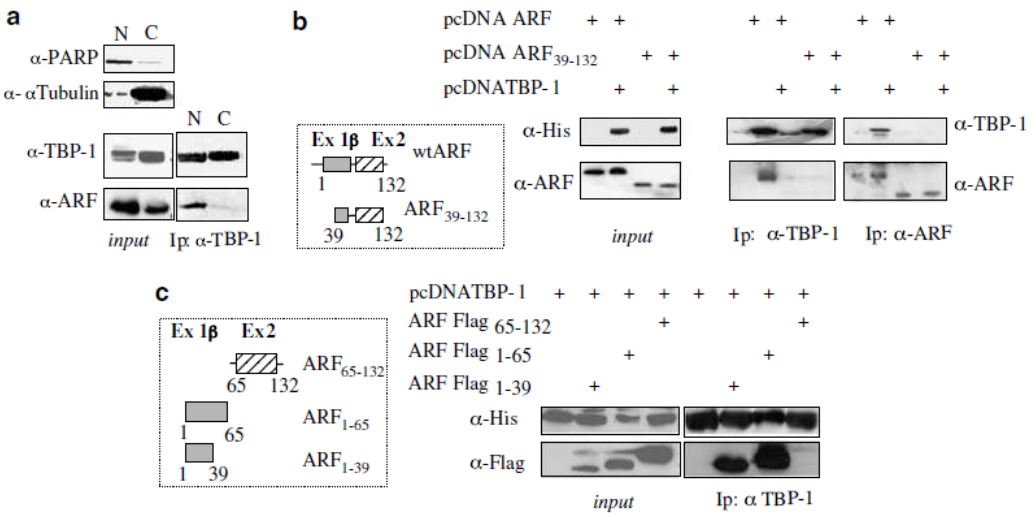

Figure 2 The p14ARF$_{1-39}$ N-terminal peptide is necessary for the interaction with TBP-1. (**a**) Fractionated nuclear (N) or cytoplasmic (C) extracts from H1299 cells were analysed with anti-ARF, anti-TBP-1, anti-PARP and anti-α tubulin or coimmunoprecipitated by anti-TBP-1 and revealed by anti-ARF and anti-TBP-1. (**b**) U2OS cells were transfected with pcDNAARF, or pcDNAARF$_{39-132}$ alone or together with pcDNATBP-1; lysates were analysed with anti-ARF or anti-His antibodies (input), to reveal only transfected TBP-1, immunoprecipitated with anti-TBP-1 or anti-ARF and revealed by anti-ARF and anti-TPB-1. (**c**) U2OS cells were transfected with pcDNATBP-1 alone or together with 3xFlagARF$_{1-39}$, 3xFlagARF$_{1-65}$ or 3xFlagARF$_{65-132}$; lysates were analysed by anti-His, or anti-Flag antibodies (input), immunoprecipitated with anti-TBP-1 and revealed by anti-His or anti-Flag.

To confirm that the 1–39 N-terminal region of ARF was strictly necessary for the interaction with TBP1, we used a 3xFlagARF$_{1-39}$ in coimmunoprecipitation (CoIP) experiments (Figure 2c). As controls, we included the 3xFlagARF$_{1-65}$ and the 3xFlagARF$_{65-132}$. TBP-1 can interact only with the ARF deletion mutants that retain either sequences corresponding to exon 1β (3xFlagARF$_{1-65}$) or to the first 39 aa (3xFlag ARF$_{1-39}$).

Given that TBP-1 overexpression results in an increase in ARF protein levels, we wanted to determine which part of p14ARF is required for this effect. Figure 3a and b shows the protein levels of the different ARF deletion mutants following TBP-1 overexpression. ARF$_{39-132}$ does not accumulate, whereas wtARF is greatly increased (Figure 3a); similarly, overexpression of TBP-1 resulted in a sharp, linear increase in the expression of ARF$_{1-39}$ and ARF$_{1-65}$ but not of ARF$_{65-132}$ (Figure 3b). These results clearly suggest that the interaction with TBP-1 is a prerequirement in order to obtain the stabilization effect. Interestingly, TBP-1 overexpression seems to have a more dramatic effect on ARF$_{1-39}$ respect to wtARF or ARF$_{1-65}$. To go through this point, an evaluation of the ARF$_{1-39}$ half-life was performed: U2OS cells were transfected with 3xFlagARF$_{1-39}$ alone or together with pcDNATBP-1; 24 h after transfection, cells were treated with cycloheximide for the indicated times. ARF$_{1-39}$ half-life is below 2 h but is shifted to around 4 h when cells are co-transfected with TBP-1 (Figure 3c), confirming that the TBP-1 stabilization effect is exerted at the posttranslational level also on the ARF$_{1-39}$ peptide as on wtARF (Pollice *et al*., 2004). ARF$_{39-132}$ also displays a shorter half-life respect to wtARF (Figure 3d), suggesting again that the 1–39 N-terminal region plays an important role in regulating the ARF turnover.

As there is still some disagreement as to whether the p14ARF N-terminal region fully maintains all the biological activity (Lohrum *et al*., 2000; Rodway *et al*., 2004), we wanted to get insights into the functionality of the ARF$_{1-39}$ peptide. We examined the ability of this peptide to prevent Mdm2-mediated degradation of p53. U2OS cells were transfected with an expression plasmid coding for Mdm2 (Figure 4a) alone or in combination with increasing amounts of 3xFlagARF or 3xFlagARF$_{1-39}$. p53 levels decrease following Mdm2 overexpression, but wtARF, as well as the ARF$_{1-39}$ peptide, is able to prevent Mdm2-mediated p53 degradation.

Many studies pointed to a role of the subcellular localization of ARF in its biological functions, although there is still a debate around this point (Weber *et al*., 2000; Rodway *et al*., 2004). Human ARF possesses two different nucleolar localization signals (NoNLrS), one in the first exon (aa 2–14) and the second in exon 2 (aa 82–101) (Weber *et al*., 2000). The ARF$_{1-39}$ peptide retains the first NoNLrS, whereas the ARF$_{39-132}$ retains the other. GFPARF$_{39-132}$ and GFPARF fusion proteins display a nucleolar localization (Figure 4b) in almost 80% of the cells although the GFPARF$_{39-132}$ displays a more diffuse staining in the nucleoplasm. On the contrary, the GFPARF$_{1-39}$ loses the nucleolar localization in almost all of the cells. We confirmed these data for the ARF$_{1-39}$ peptide using the 3xFlagARF$_{1-39}$ in immunofluorescence with anti-Flag antibodies (Figure 4c).

Overall these data indicate that, although the 1–39 peptide does not accumulate in the nucleolus, it retains the capacity to stabilize p53.

To analyse the N-terminal 39 aa in more detail, we used ARF mutants bearing deletions in the first 39 aa.

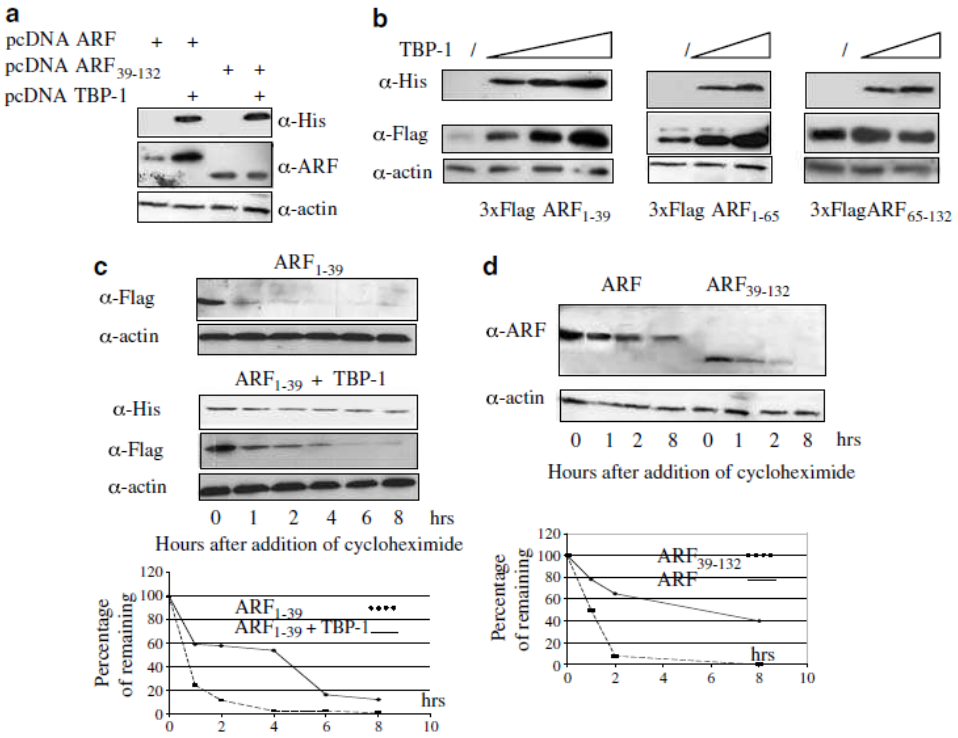

Figure 3 The p14ARF$_{1-39}$ N-terminal peptide is the target of the stabilization induced by TBP-1. (**a**) U2OS cells were transfected with pcDNAARF, or pcDNAARF$_{39-132}$ alone or together with pcDNATBP-1 in a 1:2 ratio; lysates were analysed with anti-ARF, anti-His and anti-actin antibodies (**b**) U2OS cells were transfected with 3xFlagARF$_{1-39}$, 3xFlagARF$_{1-65}$ or 3xFlagARF$_{65-132}$ alone or with increasing amounts of pcDNATBP-1; lysates were analysed with anti-His, anti-Flag or anti-actin antibodies. (**c**) U2OS cells were transfected with 3xFlagARF$_{1-39}$ in the presence or absence of pcDNATBP-1. Twenty-four hours after, cycloheximide was added and the cells were harvested at the indicated time points. Lysates were analysed with anti-Flag, anti-actin and anti-His (**d**) U2OS cells were transfected with pcDNAARF or pcDNAARF$_{39-132}$. Twenty-four hours after, cycloheximide was added and the cells were harvested at the indicated time points. Lysates were analysed with anti-ARF and anti-actin antibodies.

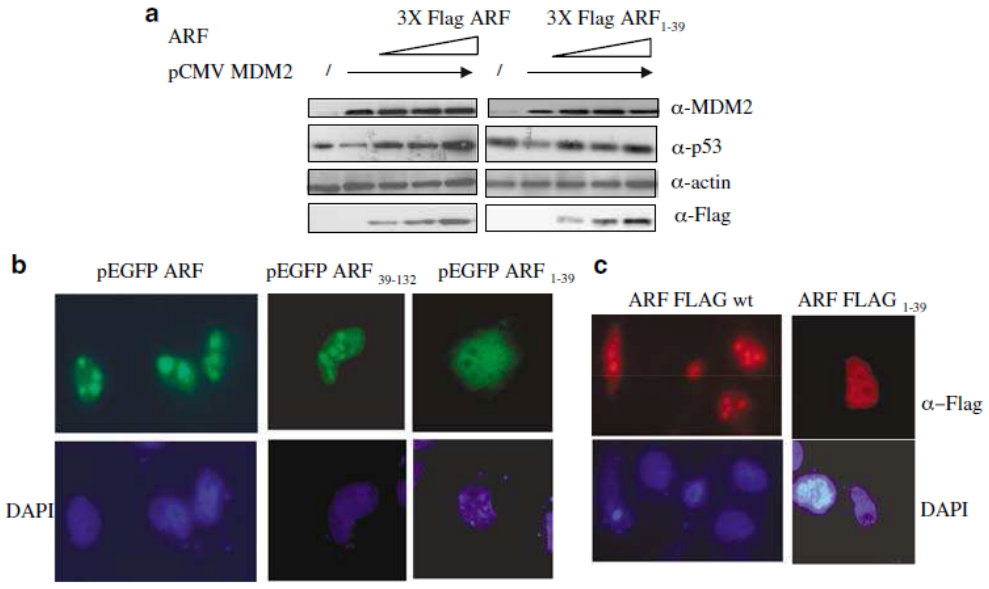

Figure 4 Biological function of the p14ARF$_{1-39}$ N-terminal peptide. (**a**) U2OS cells were transfected with pCMVMdm2 alone or in combination with increasing amounts of 3xFlagARF or 3xFlagARF$_{1-39}$. Lysates were analysed with anti-p53, to reveal the endogenous protein, anti-Flag and anti-actin antibodies. (**b**) U2OS cells were transfected with GFPARF, GFPARF$_{1-39}$ and GFPARF$_{39-132}$ and fixed 24 h after transfection. Nuclei were visualized by 4′-6-diamidino-2-phenylindole (DAPI) staining. (**c**) U2OS cells were transfected with 3xFlagARF and 3xFlagARF$_{1-39}$, fixed 24 h after transfection and immunostained with anti-Flag antibodies. Nuclei were visualized by DAPI staining.

They correspond to the pcDNAARFΔ_{2-14} and the pcDNAARFΔ_{26-37}. As control, we used pcDNAARFΔ_{82-101}. Figure 5a shows that similar levels of the different ARF proteins were recovered from transfected cell lines, but significantly less TBP-1 was coprecipitated from cells expressing ARFΔ_{2-14} and ARFΔ_{26-37} mutants. Finally, we tested the protein levels following TBP-1 overexpression (Figure 5b). The mutants that fail to interact strongly with TBP-1 do not increase, suggesting that efficient binding and stabilization requires an intact ARF N-terminal portion.

It has been reported that ARF can be subjected to N-terminal ubiquitination, a process that requires a free N-terminus and that seems strictly dependent on the first 2–3 amino acids of the protein. The vast majority of eukaryotic proteins are acetylated at their N-termini and this process depends on the chemical nature of the first amino acids. A protein whose N-terminal sequence inhibits acetylation has a better chance to become ubiquitinated at its N-terminus (Kuo *et al*., 2004).

Interestingly, it has been shown that TBP-1, as part of the regulatory subunit of the proteasome, is involved in the recognition of the polyubiquitin chains (Lam *et al*., 2002). In principle, it is possible that, as the binding of ARF to TBP-1 requires the N-terminus of the protein, it could occur via the polyubiquitin chain. Therefore, we could expect that changes of the ARF N-terminal amino acids would impair the stabilization effect. However, we have proven that TBP-1 overexpression determines stabilization of ARF proteins differing in their N-terminal sequence, like the fusion protein synthesized by 3xFlagARF starting with the sequence methionine aspartic acid tyrosine (MDY) that should promote acetylation (Figures 3a and b, and 5b; data not shown). Overall, it appears that putative N-terminal ubiquitination does not significantly influence TBP-1 mediated stabilization.

p14ARF is degraded by the proteasome both in cells and in vitro

Recently, it has been reported that ARF protein turnover depends, at least in part, on proteasome function (Kuo *et al*., 2004, Rodway *et al*., 2004). However, accumulation of endogenous ARF following treatment with proteasome inhibitors is almost undetectable in various cell lines, suggesting that there may exist other mechanisms mediating ARF destruction. Therefore, we exposed 293T cells that present good endogenous levels of p14ARF to various lysosomes and proteasome inhibitors. Figure 6a shows that ARF does not accumulate with any of the lysosome inhibitors used, but it slightly increases with either MG132 or ALLnL. Consistently, the ARF$_{39-132}$ and the ARF$_{1-39}$ mutants, with a significantly shorter half-life respect to the wild type, clearly accumulate after treatment with proteasome inhibitors (Figure 6b and c, lanes 1 and 2) confirming that ARF is subjected to degradation by the proteasome. Moreover, stabilization of the ARF$_{1-39}$ mutant owing to overexpression of TBP1 is further increased by addition of MG132 when a 1:1 ratio between TBP1 and ARF mutant is used in the transfection (see Figure 6c, lanes 3 and 4). However, when a higher amount of TBP1 is transfected, the ARF$_{1-39}$ mutant accumulates at higher levels that do not further increase after treatment with proteasome inhibitor (Figure 6c, lanes 5 and 6). These results suggest that TBP-1, as well as proteasome inhibitors, protect ARF from proteasome degradation.

The proteasome plays a central role in the degradation of the majority of cellular proteins in eukaryotes.

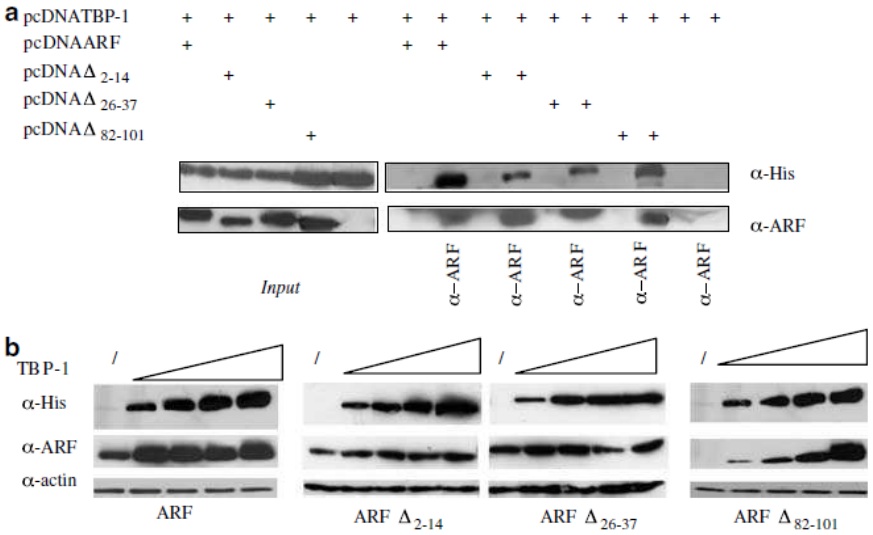

Figure 5 The integrity of the p14ARF$_{1-39}$ N-terminal peptide, is required both for interaction with and stabilization by TBP-1. (**a**) U2OS cells were transfected with pcDNATBP-1 alone or together with the ARF plasmids indicated. Forty-eight hours after transfection protein extracts were immunoprecipitated with anti-ARF antibodies where indicated and revealed with anti-His and anti-ARF antibodies. For negative control, the immunoprecipitation of the same extracts was performed without anti-ARF. (**b**) U2OS cells were transfected with the indicated plasmids alone or together with increasing amounts of pcDNATBP-1. Cell lysates were analysed with anti-ARF, anti-His and anti-actin antibodies.

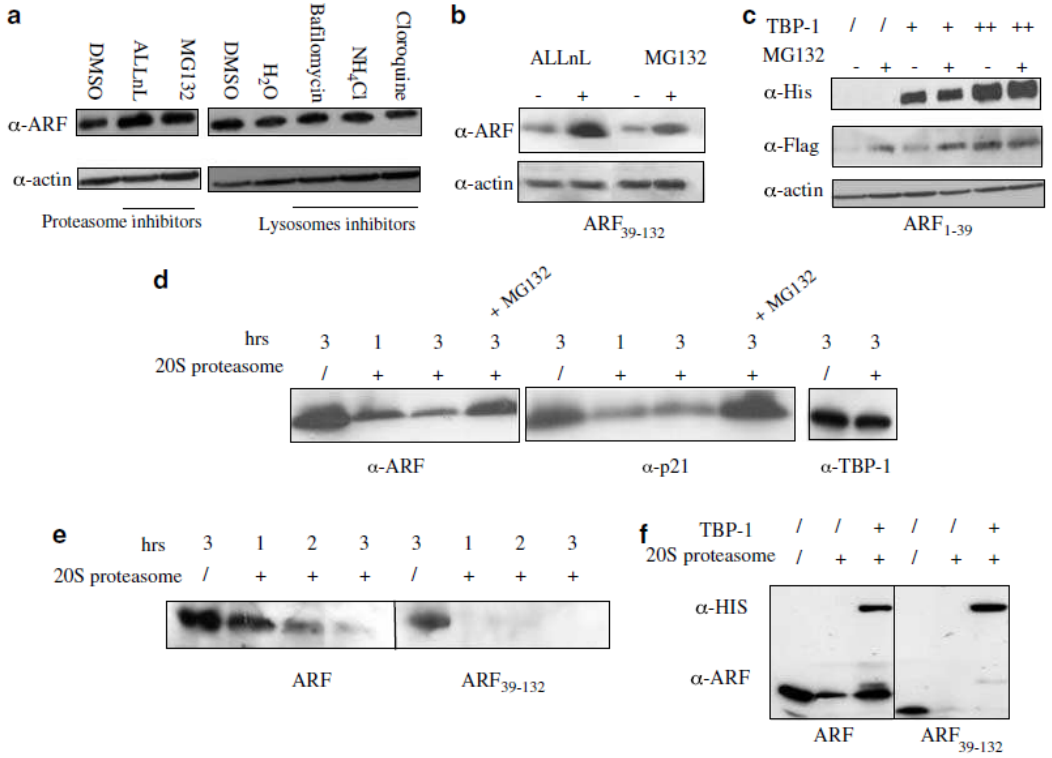

Figure 6 ARF is degraded by the proteasome both in cells and *in vitro*. (**a**) 293T cells were treated with the drugs indicated. Lysates were analysed with anti-ARF and anti-actin. (**b**) U2OS cells were transfected with pcDNAARF$_{39-132}$ and, 24 h after transfection, treated with ALLnL or MG132 for 10 h. Lysates were analysed with anti-ARF and anti-actin. (**c**) U2OS cells were transfected with 3xFlagARF$_{1-39}$ alone or with increasing amount of TBP1 and, 24 h after transfection, treated with MG132 for 10 h. Lysates were analysed with anti-Flag, anti His and anti-actin (**d**) *In vitro* translated p14ARF, p21 or TBP-1 were incubated at 37°C without or with 20S proteasome for 1 or 3 h at 37°C. MG132 (50 μM) was added to the reaction where indicated. Samples were analysed with anti-ARF, anti-p21 or anti-TBP-1 antibodies. (**e**) *In vitro* translated p14ARF or p14ARF$_{39-132}$ were incubated without or with 20S proteasome for the indicated time intervals and analysed with anti-ARF antibodies. (**f**) *In vitro* translated p14ARF or p14ARF$_{39-132}$ were incubated without or with 20S proteasome. *In vitro* translated TBP-1 was added 30′ before the reaction where indicated.

The accepted physiological form of the proteasome is composed by the 20S core particle (the catalytic subunit) and two 19S regulatory caps that can dynamically associate in an assemble/disassembly cycle. A growing body of evidences is pointing to mechanisms of ubiquitin-independent proteasome degradation that appear to be widespread and physiologically important in higher eukaryotes (Sdek *et al*., 2005; Kong *et al*., 2006). In this context, the 20S proteasome subunit plays the major role being clearly involved in degradation of unubiquitinated proteins.

Reasoning that ARF is dynamically disordered in acqueous solution (Bothner *et al*., 2001) and largely unstructured *in vivo*, we tested whether it can be subjected to proteasome degradation *in vitro*, in the absence of ubiquitination. *In vitro* translated p14ARF was incubated with 20S proteasome at 37°C for the indicated time intervals (Figure 6d). As controls, we used *in vitro* translated p21, that has been shown to be naturally unstructured and degraded *in vitro* by the 20S proteasome (Liu *et al*., 2003) and TBP-1. As shown, ARF is degraded by the 20S proteasome as efficiently as p21 and accumulates after treatment with proteasome inhibitor, whereas TBP-1 levels are unchanged. Interestingly, the ARF$_{39-132}$ mutant that in cells shows a reduced half-life has a faster kinetic of degradation respect to ARF (Figure 6e), suggesting that, also *in vitro*, it is less stable.

We observed that TBP-1 effects on ARF protein levels strictly depend on the binding between the two proteins. Actually, we wanted to test whether this protection can be exerted also *in vitro*, in the absence of an assembled 19S cap, of which TBP-1 is an integral component. To this purpose, *in vitro* translated TBP-1 was incubated with either *in vitro* translated p14ARF or p14ARF$_{39-132}$ before the degradation assay. Strikingly, we could observe a protective effect of TBP-1 on ARF, which is not exerted on the ARF$_{39-132}$ mutant, unable to interact with TBP-1 (Figure 6f). The fact that TBP-1 can exert its effect independently from being part of the 19S suggests that a similar mechanism could occur *in vivo*.

Discussion

Herein, we report data showing that changes in TBP-1 intracellular levels either by overexpression or by siRNA

affect ARF expression levels, uncovering an important role for TBP-1 in controlling ARF turnover. Moreover, our results indicate that the 1–39 aa N-terminal region is essential for interaction with TBP-1 and its consequent stabilization. By making use of various deletion mutants, we could also demonstrate the importance of the integrity of this region for the stabilization effect. On the other hand, our mutant lacking the 1–39 region is much less stable than the wild type, in agreement with a previous observation showing that a mutant ARF lacking aa 2–14 shows a decreased half-life (Kuo *et al*., 2004). All together, these data suggest a crucial role of the N-terminal portion in the regulation of ARF steady-state levels.

It is important to underline that the ARF N-terminal domain plays a fundamental role in its biological functions: mouse p19ARF N-terminal 37 aa are necessary and sufficient for binding to Mdm2, localization to nucleoli and p53-mediated cell-cycle arrest (Weber *et al*., 2000). Despite the high similarity of the two proteins in the N-terminal segments, for p14ARF the situation was less clear, as efficient nucleolar localization and Mdm2 binding seem to require also sequences present in exon 2 (Weber *et al*., 2000). However, our experiments show that the 1–39 peptide, although being highly unstable and not localized to nucleoli, is biologically active, at least in the p53-Mdm2 pathway, confirming other reports (Lohrum *et al*., 2000; Rodway *et al*., 2004).

Normal human cells contain low levels of p14ARF, but the expression of a variety of proliferation-promoting proteins upregulates ARF as part of a checkpoint response that limits cell-cycle progression in response to hyperproliferative signals (Sharpless, 2005). Interestingly, p19ARF was also found to be upregulated in senescent mouse fibroblasts (Sharpless, 2004). In contrast, the human p14ARF does not appear to be required for the senescence process, as p14ARF expression levels remain low as cells near senescence (Sharpless, 2004; Gallagher *et al*., 2006). The differential role of mouse and human ARF proteins in promoting senescence may relate to differences in their regulation.

ARF is a relatively stable protein, and we and others (Kuo *et al*., 2004; Pollice *et al*., 2004) estimated its half-life approximately 6–8 h. On the other hand, the mechanisms governing the p14ARF degradation pathway are field of intensive studies: it has been described that ARF is subjected to more dynamic controls than previously thought, being degraded, at least in part, by the proteasome, but the mechanisms governing its delivery into the proteasome still remain to be clarified.

However, our results show that ARF endogenous levels are influenced by addition of proteasome inhibitors, whereas various drugs, known to inhibit lysosomal function, have no effect, indicating that ARF is not subjected to lysosomal degradation. Moreover, levels of less stable p14ARF mutants, displaying a shorter half-life respect to the wild type, are more markedly affected by the addition of proteasome inhibitors, confirming the involvement of the proteasome in the regulation of ARF turnover.

TBP-1 overexpression seems not to cause general effects on proteasome function, but rather it appears to affect only specific targets, that is Hif1α and ARF, and with opposite effects, as it promotes degradation of Hif1α (Corn *et al*., 2003). On the other hand, it has to be noted that TBP-1 is one of the ATPases of the 19S proteasome subunit that are apparently involved also in cellular events that do not require proteolysis. Furthermore, the entire 19S subunit seems to exist in a dynamic equilibrium with the 20S catalytic subunit that possesses the ubiquitin-independent proteolytic functions and is clearly involved in the degradation of unubiquitinated proteins. Although most cellular proteins degraded in the proteasome are ubiquitinated, proteins such as ornithine decarboxylase, the Cdk inhibitor p21, Hif1α, members of the Rb family of tumor suppressors, p53 and p73 (Sdek *et al*., 2005; Kong *et al*., 2006) can be directed to the proteasome without prior ubiquitination. In many cases, if a protein can be delivered to the proteasome in a denatured or partially unfolded state, ubiquitination should not be required for its degradation. In fact, p21 and α-synuclein that are considered 'naturally unstructured' proteins can be degraded *in vitro* by the proteasome, in the absence of ubiquitination (Liu *et al*., 2003). It has been reported that ARF can be subjected to N-terminal ubiquitination, a process independent from p53 and Mdm2 (Kuo *et al*., 2004). On the other hand, Rodway *et al*. (2004), postulated a role of Mdm2 in mediating ARF delivery to the proteasome without any requirement for ubiquitination.

The TBP-1-mediated stabilization does not depend on the sequence of the first 2–3 amino acids, as the protein levels of many different p14ARF proteins, differing for their N-terminal portion, are equally well increased upon TBP-1 overexpression (data not shown). Therefore, we suggest that eventual posttranslational modifications (acetylation, ubiquitination) occurring at the N-terminus of ARF do not influence the effect exerted by TBP-1. ARF is dynamically disordered in acqueous solution and becomes highly structured upon binding to Mdm2 (Bothner *et al*., 2001); it has also been postulated that *in vivo* ARF is intrinsically unstructured although it can fold upon binding to its biological substrates. Interestingly, we have observed that ARF can be degraded *in vitro* by the 20S proteasome in the absence of ubiquitination, and that TBP-1 can counteract its degradation. The evidence that TBP-1 can exert its effect *in vitro*, independently from being part of the 19S, suggests that a similar mechanism could occur *in vivo*. Accordingly, in Figure 6 we show that TBP-1 and proteasome inhibitors cooperate in order to protect ARF from proteasome degradation. In principle, it is possible that TBP-1 binding causes ARF folding and renders it a poor substrate for 20S proteasome degradation.

Regardless of the underlying molecular mechanisms, these observations are relevant in the comprehension of the regulation of ARF metabolism as, among the plethora of already identified ARF's interactors, only TBP-1 and NPM/B23 appear to regulate ARF's intracellular levels.

Materials and methods

Plasmids

The ARF_{39-132} fragment was retrieved by *Nar*I/*Xba*I cut of pcDNAARF (Pollice *et al*., 2004), filled in at the *Nar*I site and ligated into the pcDNA3.1His (Invitrogen Corporation, Carlsbad, CA, USA) cut with *Eco*RV/*Xba*I to give $pcDNAARF_{39-132}$. $pcDNAARF_{1-39}$ was obtained from *Eco*RI/*Nar*I cut of pCDNAARF, fill in of the *Nar*I site, and cloning in pcDNA3c.1 *Eco*RI/*Eco*RV digested. $3xFlagARF_{1-39}$ was obtained by PCR amplification using the primers ARFup(AAGAATTCAATGGTGCGCAGG) and ARFdown(AAAAGATCTCCCTGGCGCTGCCCA) and subsequent cloning in p3xFlagCMV10 cut *Eco*RI/*Bgl*II. $pEGFPARF_{1-39}$ and $pEGFPARF_{39-132}$ were obtained from *Eco*RI/*Xba*I cut of respectively, $pcDNAARF_{1-39}$ and $pcDNAARF_{39-132}$ and subsequent cloning in *Eco*RI/*Xba*I of pEGFPc2. p3xFlagARF, $p3xFlagARF_{1-65}$ and $p3xFlagARF_{65-132}$ were kindly provided by B Majello. $pcDNAARF\Delta_{2-14}$, $pcDNAARF\Delta_{26-37}$ or $pcDNAARF\Delta_{82-101}$ were kindly provided by CJ Sherr. The other plasmids were already described (Pollice *et al*., 2004).

Cell culture, transfection, CoIP and subcellular fractionation

U2OS, H1299, 293T cell lines were maintained in Dulbecco's modified Eagle media supplemented with 10% fetal calf serum. Transfections were performed as described (Pollice *et al*., 2004).

CoIPs were performed as described (Pollice *et al*., 2004). Briefly, lysates from cells transfected with 1:1 ratio of TBP-1 and various ARF constructs were incubated with anti-ARF (Santa Cruz Biotechnology Inc., 8613, Santa Cruz, CA, USA), or with monoclonal anti-TBP-1 (Affinity Research BIOMOL Internat LP Mamhead, Exeter, UK) for 4 h at 4°C, followed by addition of protein-A agarose (Roche Applied Science, Manneheim, Germany) or protein G-sepharose (Amersham Biosciences, Uppsala, Sweden) beads. Immunoprecipitates were analysed by immunoblotting as indicated. Other antibodies used for detection were: anti-Flag (SIGMA F-3165, Saint Louis, MO, USA) and anti-His (BD-Clontech Laboratorie Inc., 631212, Mountain View, CA, USA). Subcellular fractionation of H1299 cells was carried out as by Colucci-D'Amato *et al*., 2000. To verify the purity of the subcellular fractionation, anti-PARP (Cell Signaling Technologies 9542, Boston, MA, USA) and anti-α tubulin (SIGMA T 9026) have been used in Western blot (WB) with 6 μg of nuclear and 18 μg of cytoplasmic extracts. CoIP has been performed using 100 μg of nuclear and 300 μg of cytoplasmic extracts.

Treatment with proteasome and lysosomes inhibitors

293T cells or transfected U2OS cells were treated for 10 h with either 0.1% dimethyl sulfoxide, 50 μM ALLnL, 50 μM MG132, 100 nM Bafilomycin, 25 mM NH_4Cl, 100 nM Cloroquine or water. Cell lysates were probed with anti-ARF and anti-actin (Santa Cruz 1616).

Decay rate analysis

Decay rate analysis was already described (Pollice *et al*., 2004) Briefly, U2OS cells were transfected, pooled and replated into six plates or four plates. Twenty-four hours from transfection, cycloheximide was added at a final concentration of 80 μg/ml, and cells were harvested at the indicated time points.

Immunofluorescence analysis

For imaging analysis, the same experimental procedure described by Pollice *et al*. (2004) was followed.

siRNA of TBP-1

A duplex siRNA oligomer designed to target human TBP-1 was obtained by MWG Biotech Martinsried (Germany) according to Corn *et al*. (2003). H1299 cells that were transfected with Hyperfect (Quiagen, GmbH 400724, Hilden, Germany) according to the manufacturer's instructions. Western blots were performed with anti-Mdm2 (Santa Cruz 965), anti-Itch (BD-Clontech 611198), anti- β-tubulin (Santa Cruz 9104), anti-B23 (Zymed Laboratorie, Inc., FC61991, South Francisco, CA, USA), anti-p21 (Santa Cruz 397), anti-actin, anti-ARF and anti-TBP-1.

In vitro *protein degradation assay*

Reticulocyte lysate translated proteins were treated for 1 or 3 h at 37°C with or without 1 μg of 20S proteasome (SIGMA) in degradation buffer (20 mM Tris-Hcl pH 7, 0.2 M. NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol) with or without 50 μM MG132. Translated p14ARF or $p14ARF_{39-132}$ were also incubated for 30′ on ice, with *in vitro* translated TBP-1 before the addition of the 20S proteasome. Samples were analysed by Western blot.

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