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PhD Thesis

Characterization of a novel p63/p73 interacting protein

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Alla mia mamma
e al mio papà per
tutti i loro
sacrifici

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Abstract

Tumors are one of the main causes of death in the occidental population. Developmental abnormalities are not as frequent, but demand consistent investments in terms of cures and attentions, both from families and institutions. A deep understanding of the molecular mechanisms at the base of these pathologies is therefore of crucial importance for medicine. A large amount of evidence demonstrates how many genes and signals involved in regulating embryonic development and differentiation are also involved in cancer. In particular members of the p53 family of tumor suppressors play a fundamental role in cellular homeostasis, and altered function of these transcription factors leads to tumor development and/or severe developmental abnormalities. The activity of these proteins is finely regulated by post-translational modifications and protein-protein interactions, so their modulators and modifiers are to be considered as potential pharmacological targets.

Here we present the characterization of human C16orf35, a novel p63 and p73 interacting protein that was isolated during a screen for interactors of Drosophila p53.C16orf35 is a highly conserved, widely expressed nuclear protein, which is able to associate with cytoplasmic RNA processing compartments such as P bodies and Stress granules. Overexpression of C16orf35 induces formation of stress granules and inhibits proliferation of transformed cells in culture, suggesting a possible involvement in growth-regulatory cellular pathways.

List of papers not directly relevant to the thesis

F.Mauri, L.M.Mcnamee, A. Lunardi, F. Chiacchiera, G. Del Sal, M.H. Brodsky, L.Collavin (2007). Modification of drosophila p53 by sumo modulates its transactivation and pro-apoptotic functions. *J. Biol. Chem. (submitted)*

INTRODUCTION

p53 family proteins: crucial player in cancer and development

p53 family proteins is a small family of transcription factors involved in tumor suppression and embryonic development. It is composed by three members: p53, p63 and p73. They shared some structural and biological features, essentially as tumor suppressors.

p53

The first member, and the most studied ones, is the tumor suppressor p53. This protein play key roles in apoptosis induction and cell cycle arrest, and results mutated in 50% of tumors. Moreover, germ line mutations in p53 gene are the basis of the Li-Fraumeni syndrome type 1, a genetic disorder characterized by early onset of multiple tumors, including principally soft tissue sarcomas and osteosarcomas, breast cancer, brain tumors, leukemia, and adrenocortical carcinoma (Petitjean et al., 2007). In human, p53 is a 393 aa long protein mainly organized into three different structural domains: a transactivation domain (TAD aa 1-83), a DNA-binding domain (DBD aa 102-292) and an oligomerization domain (OD aa 324-355) (Murray-Zmijewski et al., 2006). p53 activity and stability is finely regulated at post translational level by a complex series of protein-protein interactions (Bode and Dong, 2004). p53 is a sequence specific transcription factor that binds DNA in a tetrameric form (Laptenko and Prives, 2006). Several different genes involves in cell cycle regulation, apoptosis, senescence,(Amundson et al., 1998; Vogelstein et al., 2000) cell metabolism (Green and Chipuk, 2006) and autophagy (Crighton et al., 2007) were found to be p53 transcriptional targets. The most important players in p53 pathway (Fig.1) are the ubiquitin ligases MDM2 (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997), Cop1 (Dornan et al., 2004) and PIRH2 (Leng et al., 2003) that target p53 for proteasomal-dependent degradation, and several stress-activated kinases which activate p53. These kinases - p38, JNK,CK1, ATM, ATR - are activated upon different kind of cellular insults (DNA damage, oxidative and metabolic stress…) and phosphorylates p53 on S/T residues (Fig.2). These phosphorylations promote p53 stabilization and increase its DNA-binding capacity thus activating a specific

transcriptional response(Bode and Dong, 2004; Brooks and Gu, 2003). Other important p53 post-translational modification includes acetylation of different lysines residues, mainly localized into the carboxy-terminous that modulate p53-dependent transcriptional response (Barlev et al., 2001; Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998), sumoylation (Gostissa et al., 1999) and NEDDylation (Xirodimas et al., 2004). Beside its role as tumor suppressor p53 plays also important role in embryonic development. Indeed p53 is involved in Tgf-β signaling pathway by interacting with SMAD-2 and SMAD-3 and is required for proper embryonic development (Cordenonsi et al., 2003).

Therefore all these interactions and the subsequent modifications modulate p53-dependent apoptosis induction or cell cycle arrest thus indirectly influencing cellular fate. It is therefore evident how the complex pattern of protein interactions influence important functions of this fundamental transcription factor.

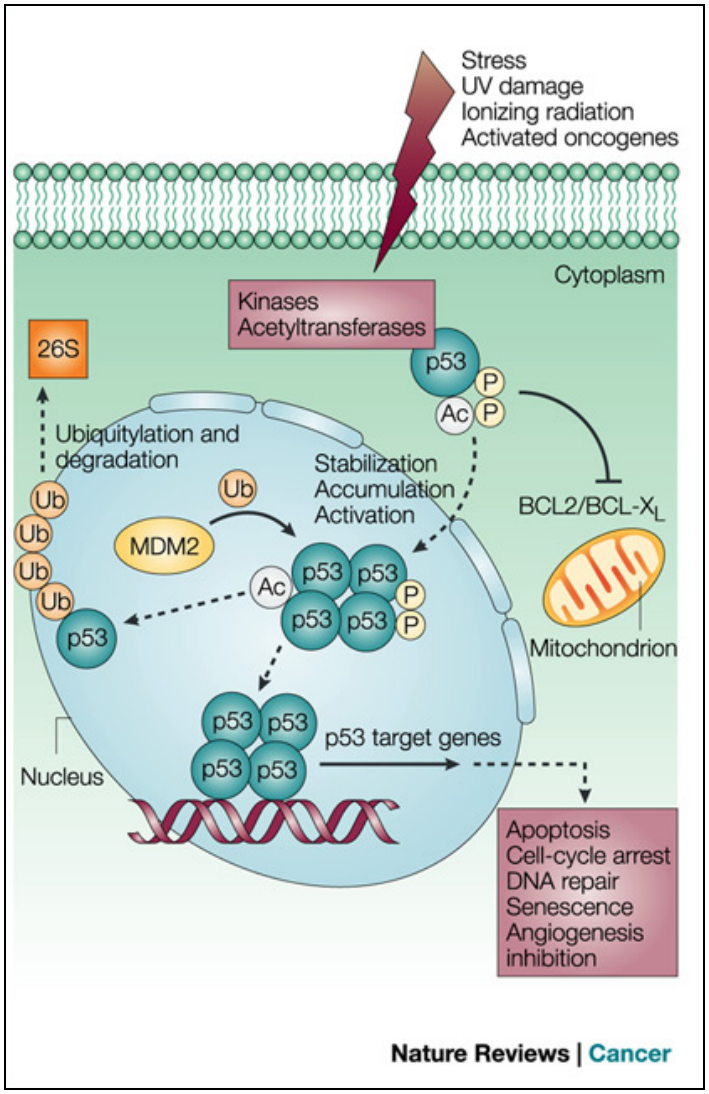

Fig.1 p53 signaling pathway (from (Bode and Dong, 2004)

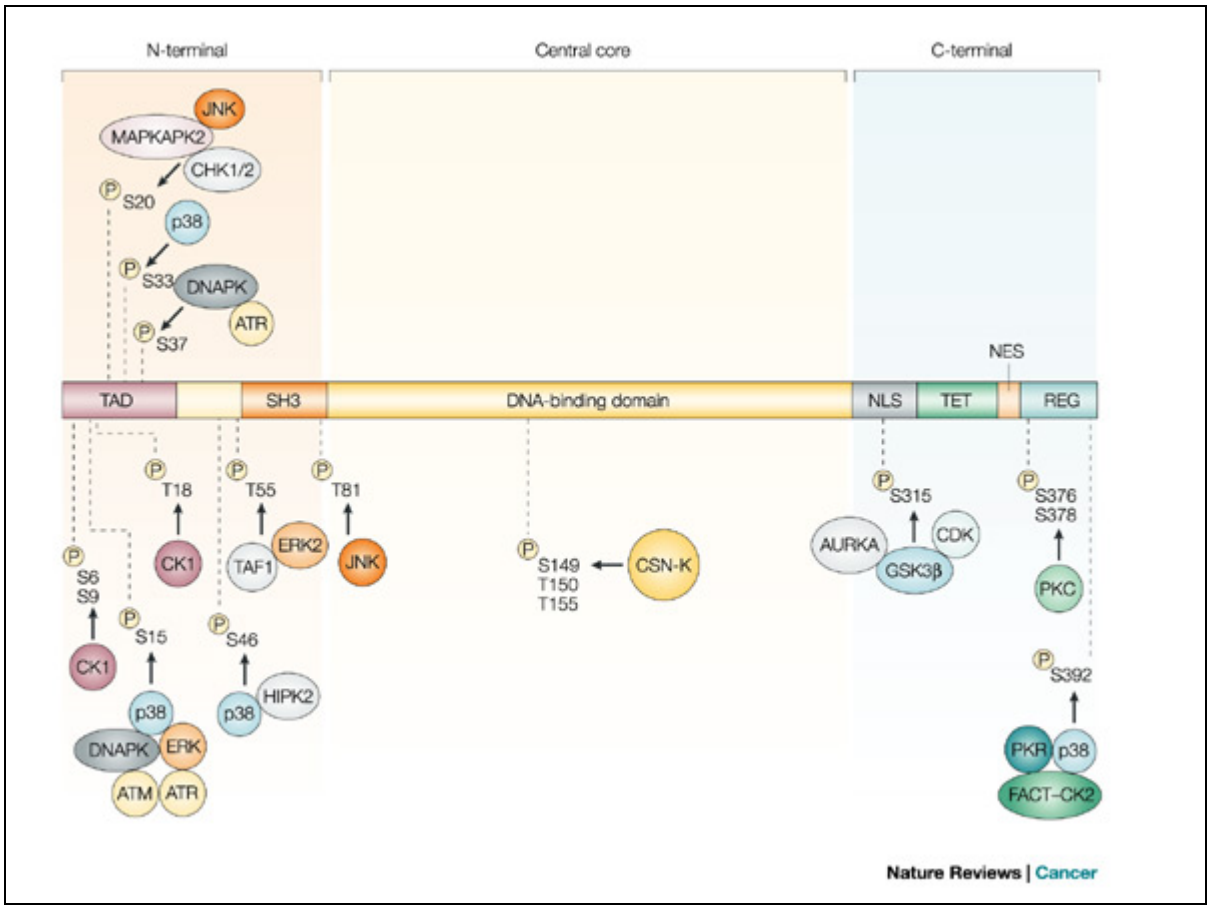

Fig.2 Several kinases modulate p53 activity (Bode and Dong, 2004).

p63

Another members of the p53 family is p63. It was characterized quite recently and it is now evident that it play major roles in epithelial development, morphogenesis and maintenance. Similarly to p53, p63 is an oligomeric transcription factor. The presence of alternative promoters and alternative splice sites in p63 gene determine the presence of six isoforms. Through the use of alternative splice sites three different isoforms (α,β,γ) that differs in the structure of their carboxi-termini are generated. For each one of these isoforms, by the use of alternative promoters an isoform containing a transactivation domain (TAp63 forms) and another isoform, that lack the N-terminal tranactivation domain (ΔN isoforms), are generated (Fig.3) (Irwin and Kaelin, 2001).

p63 localizes mainly in the basal layer of different multilayered epithelia and in particular in multipotent cells that maintained them (Candi et al., 2007; McKeon, 2004; Perez and Pietenpol, 2007). p63 knock-out mice display strong cranio-facial

abnormalities. They completely lack epidermis and the different structures derived from epidermis such as hair follicles, mammary, lachrymal and salivary glands. Due to the absence of the apical ectodermal ridge they display limb truncation and die at birth for rapid and severe dehydration (Mills et al., 1999; Yang et al., 1999). Germ line mutations (Fig. 4) in p63 gene correlate in human with the EEC (ectrodactyly, ectodermal dysplasia, and cleft lip/palate) syndrome (Ianakiev et al., 2000). This is well studied genetic pathology characterized by developmental abnormalities such as ectrodactyly of both hands and one foot, ectodermal dysplasia with severe keratitis, and cleft lip/palate (Gershoni-Baruch et al., 1997).

Given the structural similarity between p63 and p53, it was reasonable to hypothesize that also p63 acts as a sensor to DNA damage. Indeed, multiple studies have shown that p63 can induce apoptosis and is upregulated in cells that have been treated with DNA damaging agents (Flores et al., 2002; Suh et al., 2006). Anyway in human cancer p63 is not frequently mutated. Moreover several reports suggest a role for p63 in tumor progression and invasiveness (Flores, 2007). Therefore is still a matter of debate whether p63 could be considered a tumor suppressor or an oncogene (Finlan and Hupp, 2007). This is probably due to the presence of different isoforms with different and specific roles that in several reports are not distinguished (Flores, 2007). Even if poorly investigated also p63 is exposed to post-translational modification. Its stability is regulated by the E3 ligase Itch that target p63 for proteasomal-mediated degradation (Rossi et al., 2006a; Rossi et al., 2006b). It is also phosphorylated upon DNA damage in oocytes (Suh et al., 2006) and p38-dependent ΔNp63 phosphorylation induces transcriptional downregulation and degradation (Papoutsaki et al., 2005).

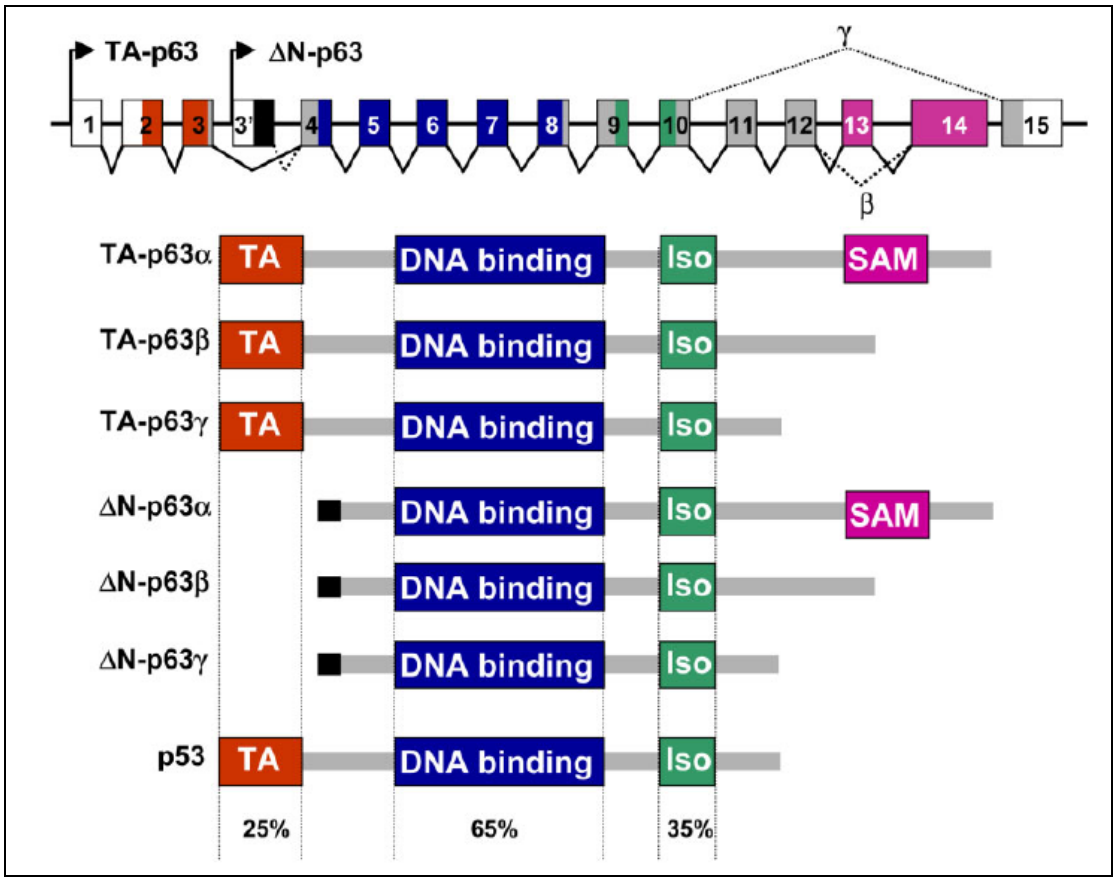

Fig.3 p63 gene structure and transcripts. The top panel is a schematic of the intron-exon structure, showing the two transcription initiation sites and alternative splicing routes, yielding at least six different p63 isotypes.

p73

p73 is the third member of the p53 related transcription factors family. By the use of alternative splice sites p73 gene encode six different isoforms with different carboxi-termini (α,β,γ,δ,ε,ζ) and two different isoforms that starts from alternative promoters and differs for the presence of the transactivation domain at NH_2-terminous (ΔNp73α and Δnp73β). Jost and collegues found that p73 can activate transcription of p53-responsive genes and inhibit cell growth in a p53-like manner by inducing apoptosis through the binding of the same p53-resposive elements (Jost et al., 1997). p73 stability is increased upon chemotherapy-induced DNA damage and this upregulation correlates with apoptosis induction in p53 deficient cells (Gong et al., 1999). Moreover loosing p73 in a p53-deficient background sensitize cells to genomic instability and severely induce aneuploidy (Talos et al., 2007). Beside its tumor

suppressor role p73 plays essential roles in development of nervous system (Pozniak et al., 2000; Yang et al., 2000). This is highlighted by the phenotype of p73-null mice. These animals are not tumor-prone but display several developmental abnormalities. In particular they exhibit profound neurologic defects, including hippocampal dysgenesis, hydrocephalus, chronic infections and inflammation, as well as abnormalities in pheromone sensory pathways (Yang et al., 2000). As the other p53 family members also p73 activity is modulated by a series of post-translational modifications (Fig.4) determined by a multiple protein-protein interactions. DNA-damage induced p73 activation is achieved through the binding of c-Abl kinase. c-Abl null fibroblasts are unable to upregulate p73 levels and were resistant to cysplatin treatment (Gong et al., 1999). Agami and colleagues shown that p73-dependent apoptosis induction requires functional c-Abl (Agami et al., 1999). Upon DNA damage it phosphorylates p73 on a tyrosine residue at position 99 and stimulate its proapoptotic activity (Yuan et al., 1999). Activation of p73 by c-Abl has been found to involve the p38 MAP kinase pathway (Sanchez-Prieto et al., 2002). Upon c-Abl-dependent phosphorylation, p73 associates with the prolil-isomerase Pin1 that in turn mediate the binding to the acetyl-transferase p300 on proapoptotic gene promoters (Mantovani et al., 2004). p300-dependent p73 acetylation increase its affinity for specific promoters and transcriptional activity(Costanzo et al., 2002; Mantovani et al., 2004). Another important player in p73 pathway is Yes-associated protein (Yap1 or Yap65) that binds the SH3 domain of the Src family kinase c-Yes. Yap1 binds to p73 through its WW domain and the PPPY motif of p73 to serve as a p73 transcription coactivator (Strano et al., 2001). This interaction is also required for p73 stabilization. Indeed it promote dissociation of the E3 ubiquitin ligase Itch that target p73 for proteasomal-dependent degradation (Levy et al., 2007; Rossi et al., 2005).

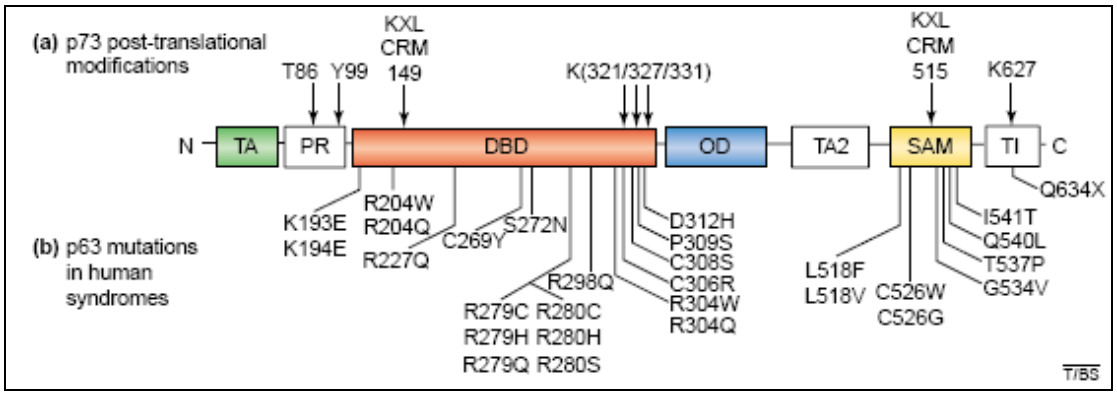

Fig.4 Post-translational modifications of p73 and mutations of p63 in human diseases. (a) The post-translational modifications that control p73 protein stability and activity. (b) Location of p63 mutations described in different human diseases.

Of note also cellular compartimentalization influence p53, p63 and p73 transcriptional activity (Dobbelstein et al., 2005). For example, their transcitpional activity is influenced by recruitment into PML nuclear bodies (Bernassola et al., 2005; Bernassola et al., 2004; Fogal et al., 2000). Nuclear bodies are subnuclear compartments, assembled by promielocytic leukemia protein (PML), where chromatin loops converge to be engaged by transcriptional multiprotein complexes. Here transcription factors met their target promoters and cofactors that positively or negatively regulate their activity (Bernardi and Pandolfi, 2007).

Relevance of protein-protein interactions in the regulation and function of p53-related proteins.

Among p53 related proteins, the most studied is clearly p53. Actually more than 150 p53-binding proteins have been described (source: BioGrid and Mint databases). p53 interactors can be schematically divided into two large groups: regulators or effectors. The network that regulate p53 stabilization and activation appear quite complex (Lavin and Gueven, 2006). Regulatory proteins act upstream of p53 and, essentially, directly modify p53 structure. As mentioned, several upstream kinases such as ATM/ATR, JNK, p38, and HIPK2 phosphorylate p53 upon cellular stress, and induce its stabilization and activation (Bode and Dong, 2004). Ubiquitin ligases such as Mdm2, Cop1 and PirfH2 are also of fundamental importance in p53 function, because they warrant a rapid turnover of this proapoptotic transcription

factor in the absence of cellular stress (Brooks and Gu, 2006). Another important class of p53 interacting proteins is represented by enzymes that control its acetylation: HATs and HADACs. These proteins regulate the attachment of acetyl groups on a series of carboxy terminal lysines, and modulate the transcriptional activity of p53 downstream of activator kinases (Barlev et al., 2001; Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998). Other p53 interacting proteins can act as transcriptional cofactors to target p53 on selected promoters; for instance Smad proteins (Cordenonsi et al., 2003). Finally, p53 interaction with the peptidyl-prolyl isomerase Pin1 affects conformation, other post-translational modifications, and its transcriptional activity (Zacchi et al., 2002).

In addition to modifiers/regulators, p53 interacting proteins can act as effectors of specific p53 functions: this class of molecules are responsible for transcription-independent p53 functions. In particular BclX and Bcl2 are two important mitochondrial-associated proteins with which p53 makes contact to induce apoptosis (Moll et al., 2005). Other proteins are in fact recruited by p53 on multi-protein complexes, to modify other proteins. This is the case of SWI/SNF chromatin remodeling complexes, that mediate histone acetylation and contribute to p53 dependent transcription (Oh et al., 2008; Xu et al., 2007), or methyl transferases that mediate p53-dependent transcriptional repression (Esteve et al., 2007). Proteins that localize to DNA-damage foci are another important class of p53 interactors. These proteins, such as RAD51, accumulate on sites of DNA damage, and bind p53 during DNA repair process (Buchhop et al., 1997).

When compared to the large number of studies on p53 interactions, much fewer studies were conducted to isolate p73 or p63 binding proteins. Among various p73 interactors Rossi and colleagues identified the protein Itch as an important p73 ubiquitin ligase (Rossi et al., 2005). An important upstream regulator of p73 is the protein kinase c-Abl, that phosphorylates p73 and promotes its binding with p300, which in turn acetylates p73 thus promoting transcription (Mantovani et al., 2004). Recently Levy and colleagues reported that YAP-1 is a c-Abl substrate and that c-Abl-dependent YAP-1 phosphorylation is required for recruiting p73 and p300 on promoters of pro-apoptotic genes (Levy et al., 2007).

At the moment, even fewer proteins have been described that interact with p63.

In some cases, protein partners are shared among all p53-related proteins (i.e Daxx; (Gostissa et al., 2004), but most of the numerous identified p53 interactors have not been specifically tested for binding to p63 or p73.

Given the current data on the protein interaction profile of p53, it is conceivable that many more p63 and p73 (and p53) binding proteins exist, that can be involved in regulating specific functions of these important transcription factors, under different conditions.

An *in vitro* approach identifies novel evolutionarily conserved interactors of the p53 family of proteins

All these considerations highlight the importance of understanding the various protein interactions established by fundamental proteins such as p53. To identify new p53 binding proteins, an *in vitro* protein-protein interaction screening was performed in the lab. The novelty of this approach is the use of the *Drosophila melanogaster* p53 homologue as a bait. From an evolutionary point of view this protein is closely related to the common ancestor of all three mammalian paralogs: p53, p63 and p73 (Lu and Abrams, 2006). It is structurally related to all p53 family members and lacks the C-terminal SAM domain (Ou et al., 2007) Although some authors consider Dmp53 more similar to p63 in virtue of primary sequence conservation, and accurate phylogenetic analysis, Dmp53 has marked functional homologies to mammalian p53: in fact, it is activated by DNA damage and it induces apoptosis in damaged cells (Brodsky et al., 2000).

In addition, the Drosophila system offers an additional advantage: the relatively small size of the Drosophila transcriptome, and the availability of a Unigene collection of about 12,000 non-redundant full-length cDNAs (the Drosophila Gene Collection, DGC (Rubin et al., 2000) - make it possible to efficiently screen a large portion of the entire proteome of this organism.

Using baculovirus expressed Dmp53 as a bait, the first two releases of the Drosophila Gene Collection were screened by *in vitro* pull-down (see Fig.5).

From this screening, 95 Dmp53-interacting proteins were isolated, of which 64 have a defined human ortholog. Among them, we focused our attention on human C16ORF35, a novel protein of unknown function, which is extremely conserved in evolution.

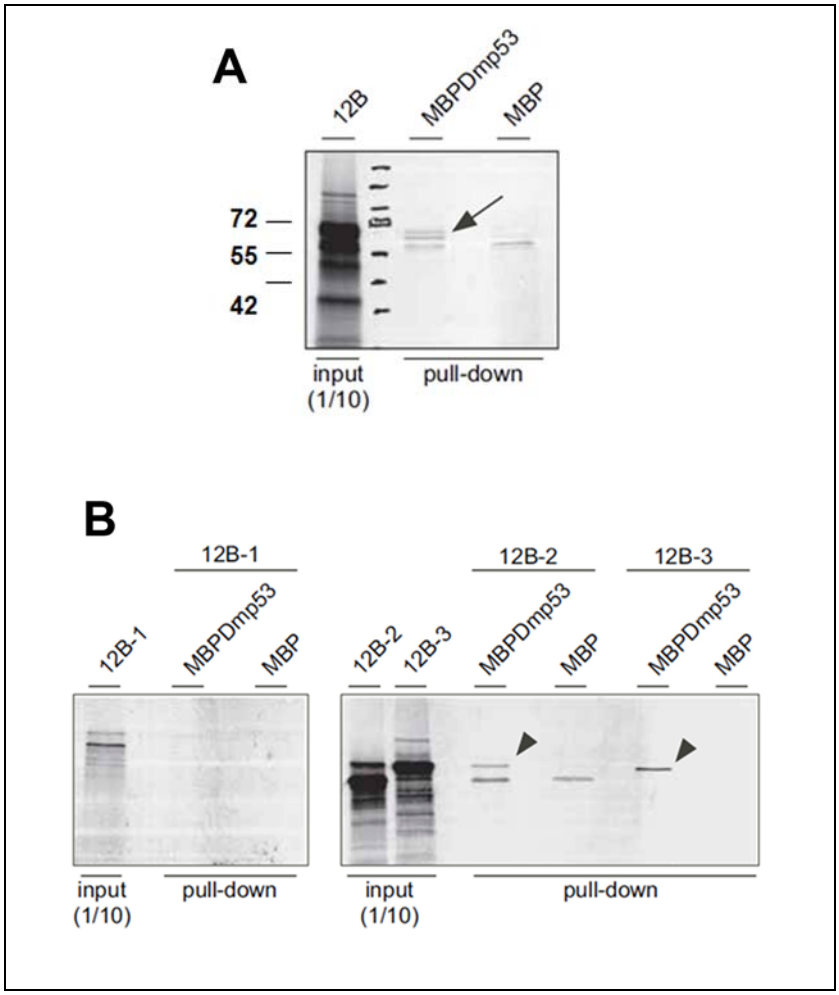

Fig. 5 In vitro pull-down screening of the Drosophila Gene Collection. The passages that led to isolation of the Drosophila ortholog of c16orf35. A) The primary pool 12B contained a doublet that bound specifically to Dmp53 beads. B) When the primary pool was split into smaller pools (sib selection), two independent Dmp53 binding proteins were identified (arrowheads). One of them is the Drosophila ortholog of human c16orf35.

C16ORF35

In 1995 Vyas and colleagues published the first characterization of the C16ORF35 gene (Vyas et al., 1995). They showed that it lies on chromosome 16, in position 16p13.3, immediately upstream to the alpha globin gene cluster. Considered its position respect to the cluster, they named it "-14".

C16orf35 is organized into 15 exons that span approximately 55kbp of genomic DNA from position -14 to -69 (Fig.6). They reported the presence of a TATA-less, GC-rich promoter underling CpG island and a DNaseI hypersensitive site at -14 position (respect to alpha globin genes). Of note, another highly conserved DNaseI hypersensitive site resides into c16orf35 intron 5 in position -40. This was characterized as the HS-40 upstream alpha globin regulatory element (Craddock et al., 1995; Higgs et al., 1990). Even if the presence of HS-40 is required for an efficient alpha globin transcription it does not appear to be involved in c16orf35 transcriptional regulation (Bernet et al., 1995; Craddock et al., 1995). Of note C16orf35 display a very high level of conservation among vertebrates (Fig.7) and is also syntenic, respect to the alpha globin cluster, in human, mouse, chicken and zebrafish. It was estimated to be conserved for at least 270 million years. C16orf35 is a widely expressed gene (Vyas et al., 1992). It is transcribed in opposite direction respect to the alpha globin gene cluster and the presence of active HS-40 or the simultaneous expression of alpha globin does not influence its expression (Bernet et al., 1995; Craddock et al., 1995; Zhou et al., 2006). Indeed c16orf35 transcription is warranted both in erythroid, were the chromatin in its proximity is relaxed and actively transcribed, and in non-erithroid cells, where chromatin is tightly condensed. This is achieved through the binding of specific insulators around the c16orf35 promoter (Zhou et al., 2006). Patients hemizygous for this chromosomal region are rare but have no apparent abnormalities apart from α-thalassemia (Vyas et al., 1995). No homozygotic deletion of c16orf35 has been described to date; this probably means that homozygous deletion of this gene results in embryonic lethality (Daniels et al., 2001). Interestingly, this is supported by *in vivo* studies on a mouse model. In particular, in 2002 Anguita and colleagues described the effect of c16orf35 knock-down in mice (Anguita et al., 2002). To shed light on the importance of HS-26 hypersensitve site (the mouse homolog of human HS-40) in alpha globin transcriptional regulation, they generated transgenic mice in which a neomycin-resistance cassette was inserted in place of the HS-26 sequence.

When homozygous, these mice die *in utero*, between day 13.5 and 15.5 after conception (Anguita et al., 2002). Homozygous fetuses were recognizable from their obvious pallor, resulting from severe anemia. Blood films from these mice showed marked abnormalities, and the fetal livers were reduced in size, with considerable dyserythropoiesis. Remarkably, recombinase-mediated removal of the neomycin cassette totally rescued the lethal phenotype, leaving only a mild form of anemia. The authors therefore checked expression of the genes neighboring the -26 region in these transgenic animals. Analysis of c16orf35 expression revealed that the presence of the neomycin cassette completely abrogated its transcription: c16orf35 expression was in fact restored by the removal of cassette. These data were obtained serendipitously, and can only correlate the c16orf35 knock-down with a lethal phenotype. Anyway, they strongly suggest that c16orf35 expression is necessary for embryonic development and recommend further investigations.

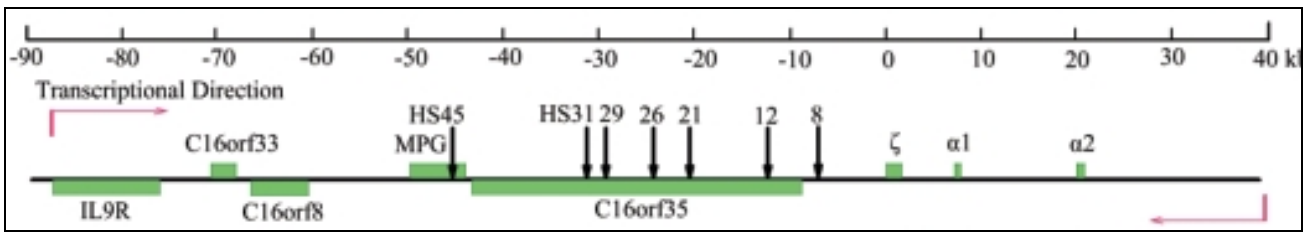

Fig.6 Schematic presentation of the mouse α-globin locus and the flanking region. Black arrows indicate erythroid cell-specific hypersensitive sites, and green boxes indicate genes. The transcription direction toward centromere (above the line) or telomere (below the line) is indicated by red arrows (modified from (Zhou et al., 2006)

Despite its extreme evolutionary conservation, the function of the protein encoded by the C16ORF35 gene is currently unknown. The EST, mRNA, and deduced aminoacid sequences available in public databases suggest the presence of at least two protein isoforms, one of 569aa and a predicted MW of 63kDa and the other of 390aa and a predicted MW of 43kDa, generated by an internal, downstream ATG codon. No putative structural/functional domains can be identified in the primary sequence of C16orf35 isoforms; indeed, this protein identifies a conserved domain of unknown function (Pfam03666: UPF0171).

This Thesis describes a set of studies aimed at understanding the function of the C16orf35 protein, both as a novel highly conserved gene, and as a putative interaction partner of the p53-family of tumor suppressor proteins. The experiments revealed that C16orf35 interacts with p63 and p73 but not with p53, and that it strongly inhibits the proliferation of transformed cells in culture. We also found that C16orf35 has a peculiar nuclear-cytosolic distribution, and interacts with cytoplasmic mRNA "triage centers" such as P-bodies and Stress granules. Finally, C16orf35 overexpression induces formation of Stress granules in human osteosarcoma cells, suggesting a possible mechanism for its growth suppressive function.

The fact that this protein interacts with p73 and p63, suggests the intriguing hypothesis that these important transcription factors may also play a role in the regulation of gene expression at the post-transcriptional level.

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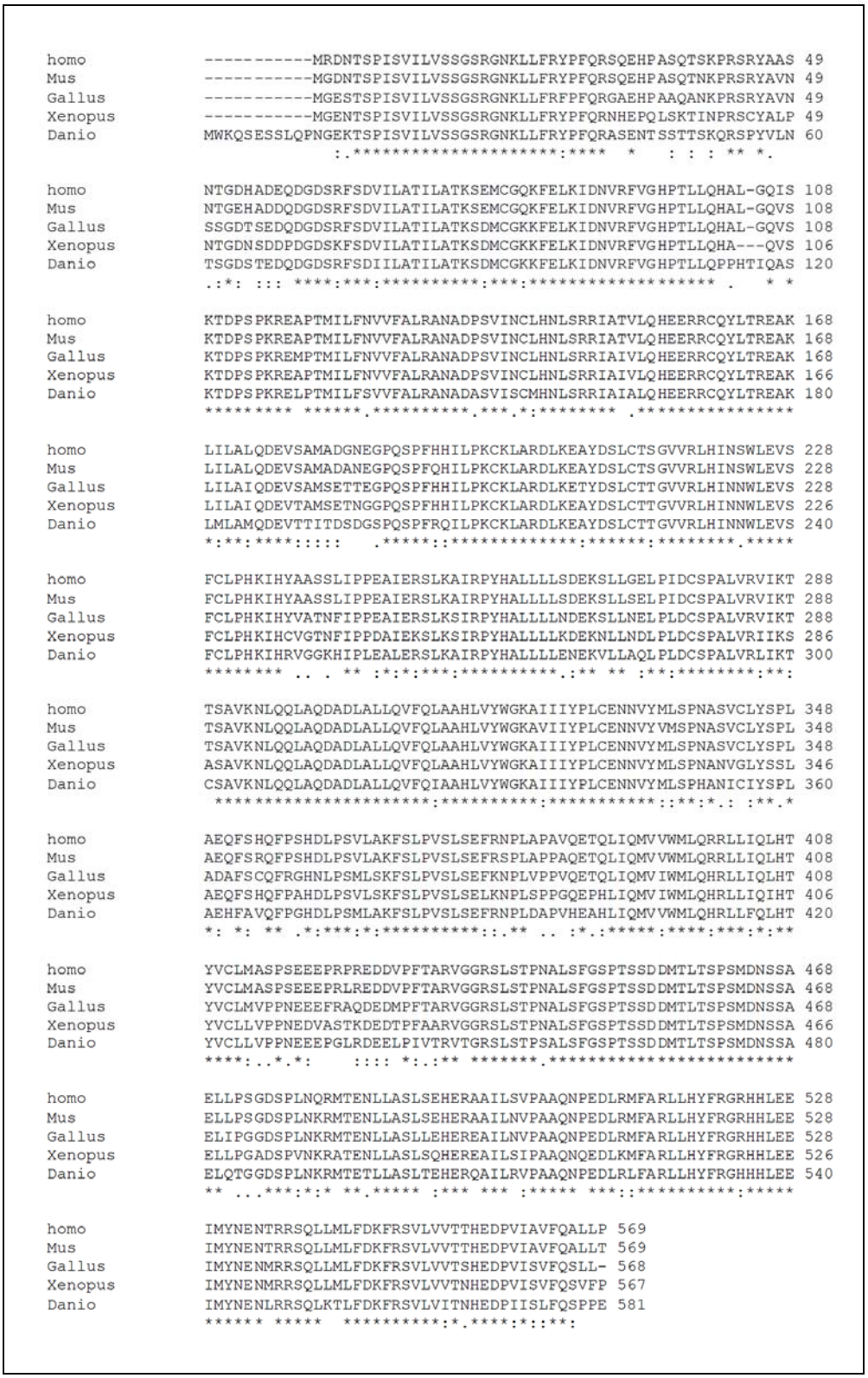
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ELLPGADSPVNKRATENLLASLSQHEREAILSIPAAQNQEDLKMFARLLHYFRGRHHLEE 526
ELQTGGDSPLNKRMTETLLASLTEHERQAILRVPAAQNPEDLRLFARLLHYFRGHHHLEE 540
IMYNENTRRSQLLMLFDKFRSVLVVTTHEDPVIAVFQALLP 569
IMYNENTRRSQLLMLFDKFRSVLVVTTHEDPVIAVFQALLT 569
IMYNENMRRSQLLMLFDKFRSVLVVTSHEDPVISVFQSLL- 568
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B

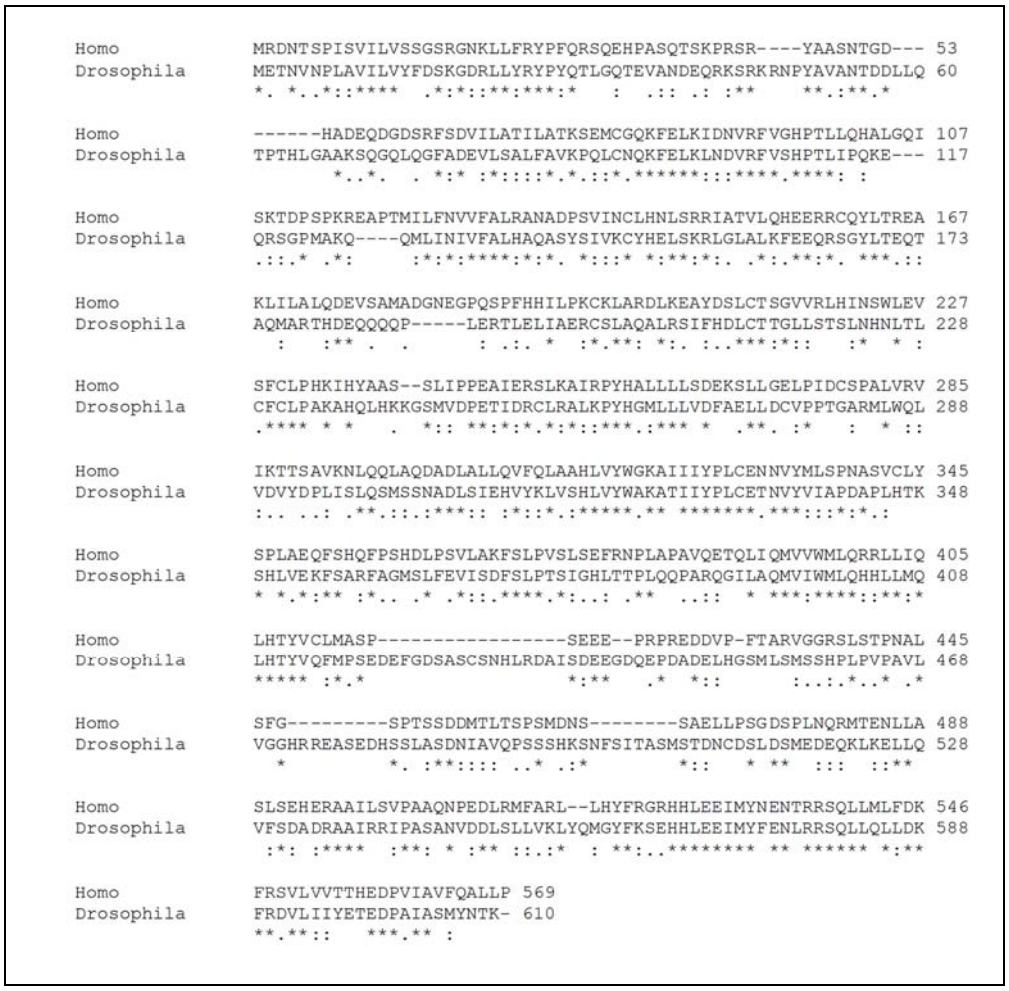

Fig. 7 c16orf35 is highly evolutionary-conserved protein. Here is shown the alignment of protein sequences from different animal species. Human c16orf35 protein sequence was compared with vertebrates (A) in particular *Mus musculus*, *Gallus gallus*, *Xenopus tropicalis* and *Danio rerio* proteins, and with invertebrates (B), in particular *Drosophila melanogaster* protein.

AIM OF THE STUDY

Important molecules often represent the center of complex signaling pathways, a sort of "focal nodes" into an intricate web. In particular p53 family proteins dictate cellular destiny by modulating a variety of fundamental processes ranging from apoptosis to cell differentiation and renewal, independently from cell type. It is conceivable that several other proteins contribute to their activity modulating their functions. This is principally achieved through post-translational modifications and cellular compartmentalization. The characterization of novel proteins that interact with p53, p63 or p73 therefore represents a crucial point to understand several aspects of their biology. Moreover, as these transcription factors are involved in important pathologies, from cancer to developmental abnormalities, any novel interacting partner is a novel potential pharmacological target that could modulate their functions. Accordingly, there is a strong interest to identify p53, p63 and/or p73 interacting proteins. We believe that among p53-family interactors, particularly interesting are those that are most conserved in evolution, since they are likely involved in pathways of crucial importance for basic aspects of cell/organism function.

The aim of this study is to characterize a novel mammalian protein, named "c16orf35", whose Drosophila ortholog interacts with Drosophila p53. C16orf35 protein is highly conserved in metazoans, and widely expressed, but nothing is currently known about its function. Our aim is to gain insight into the biological role of this protein *per se,* and to dissect the functional relevance of its possible interaction with members of the p53 family of tumor suppressors.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

The human tumor cell lines U2OS, H1299, HEK293T were used. Cells were cultured in 5% CO2 in DMEM or RPMI medium supplemented with 10% fetal bovine serum, 10% penicillin 100 U/ml, streptomycin 100mg/ml and L-glutamin 2mM.

U2OS cells were transfected using the FuGene6 (Roche) reagent. H1299 were transfected using Lipofectamine 2000 (Invitrogen). Cells were transfected according to the manifacture indications. HEK293T were transfected using calcium-phospate method.

U2OS cells were transfected using siRNA MAX (invitrogen) following manufacturer instructions with 20nM double-stranded siRNA oligonucleotides specific for c16orf35 (5'-GACCACAUCUGCUGUGAAG(dTdT)-3) or with the same amount of a scrambled control ds RNA.

For biochemical fractionation the ProteoExtract® Subcellular Proteome Extraction Kit (Calbiochem) were used following manufacturer instructions.

PLASMIDS

The following plasmids were used: pLPC-hc16orf35; pcDNA3-hc16orf35; peGFP-SATB1; pcDNA3-GATA1; pcDNA3-p53; pcDNA3-Myc-TAp63; pcDNA3-HA-TAp73; pRCCMV-Flag-HA-Hdac1; pRCCMV-Flag-HDAC3; pcDNA3-Myc-mSin3A; peGFP-G3BP1 and peGFP-G3BP1 S149A (kindly provided by dr. Jamal Tazi); pIRESneo-HA-Ago1 and pIRESneo-HA-Ago2 (Thomas Tuschl lab).

ANTIBODY PRODUCTION

The C-terminal 390 aminoacids of C16orf35 were cloned in frame with MBP in the pMAL-c2 vector. MBP-c16orf35 was produced in competent BL-21 cells and purified on amylose resin following manufacturer's instruction. The protein was eluted using

1% Maltose in PBS. The eluted protein was dialyzed and concentrated on Microcon Y30 columns (Millipore).

Two rabbits were injected intra dermis with 200 μg of purified MBP-c16orf35 protein mixed 1:l with complete Freund's adjuvant (Sigma). The same amount of MBP-c16orf35 was subsequently injected intra muscular in incomplete Freund's adjuvant (Sigma) every 2 weeks until a significant immunoreactivity was detectable by Western blot analysis. The two resulting polyclonal c16orf35 antibodies (C16A and C16B) were purified by affinity chromatography on a column carrying recombinant GST-C16orf35 protein.

WESTERN BLOT

Whole cellular lysates where prepared in Laemmli sample buffer. The lysates were run in SDS-polyacrilamide gels and transferred with the trans-blot semi-dry transfer cell (Biorad). In addition to the rabbit anti-c16orf35 polyclonal (C16A), the following primary antibodies were used to detect endogenous or overexpressed proteins: anti-HA (12CA5), anti-Myc (9E10); anti-Hsp90, anti pan-cadherin, anti-GATA1(N9) and anti Vimentin were purchased from Santa Cruz Biotechnology; anti-actin and anti-FLAG (Sigma). Primary antibodies were incubated in PBS 0,1% tween-20 supplemented with 5% non fat dry milk. HRP-conjugated secondary antibodies were diluited 1:2000 in PBS 0,1% tween-20, 5% milk. The secondary antibodies were visualized with the ECL PLUS (Amersham) chemioluminescent kit following the manufacturer's instruction.

COLONY FORMATION

U2OS cells were plated at low density and transfected with pLPC-hc16orf35 or pLPC alone using calcium-phosphate method. After 48h the medium was changed and replaced with puromycin-conditioned medium. Cells were kept in selection medium until the end experiment. Medium were replaced every two days. After 12 days cells were washed in PBS and stained with Giemsa solution.

IMMUNOFLUORESCENCE AND BrdU ASSAY

Cells were plated on glass coverslips and transfected as indicated. After 24h cells were harvested and fixed with 4% paraformaldehyde. Cells were treated with glycine

0,1 M solution in PBS and permeabilized for 5 minutes with PBS plus 0,1% TritonX-100.

Single or double fluorescence were performed with the following primary Ab: anti-TIA1, anti-HA(12CA5), anti-GATA1(N9) (Santa Cruz Biotechnology), and anti-c16orf35 (16CA). We used TRITC or FITC-conjugated goat anti-mouse and anti rabbit IgG (Jackson Immunoresearch) to detect the primary Ab as described in every single experiment. Nuclei were visualized by Hoechst 33342 (2ug/ml in PBS).

For DNaseI digestion cells were treated as in (Yeh et al., 2005) than subjected to standard immunofluorescence.

For cytoplasmic extraction, cells were trated with CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES (pH 6.8), 3 mM MgCl2, 1 mM PMSF) on ice for 2 minutes than fixed in 4% paraformaldehyde and used for standard immunofluorescence analysis.

In the BrdU assay cells were pulsed with 50uM BrdU for 18 hours just before the fixation. The detection of BrdU incorporation was visualized with an anti BrdU antibody (Sigma) after fixation, permeabilitation and after two washes with NaOH 50mM.

Images were acquired using a Leica DM4000B microscopy or a Zeiss Axiovert 100M confocal microscope.

TRANSCRIPTIONAL ACTIVITY ASSAY

This kind of assay was performed with the commercial kit Dual Luciferase reporter assay (Promega), by co-transfecting two luciferase expression vector, one constitutive expressed (Renilla) and the other under the control of the promoter whose activity is under investigation. In particular this assay was used to test the possible effect of c16orf35 on the transcriptional activity of GATA, p53, TAp63 and TAp73. The vector used were pcDNA3-GATA1, pcDNA3-p53, pcDNA3-MycTAp63, pcDNA3-HATAp73 transfected alone or in combination with pcDNA3-hc16orf35 as transcription factors and GATA-luc BAX-luc p21-luc and PG13-luc as reporter vector. The assay was performed according to the manufacturer instruction.

CO-IMMUNOPRECIPITATION

Cells were transfected as indicated. 24 hours after transfection cells were harvested and lysed in hypotonic buffer (150mM NaCl, 50mM Tris HCl pH7,5, 10% w/v

glicerolo, 0,1-0,5% NP-40) supplemented with CLAP (chimatostatin, leupeptin, antipain and pepstatin A), PMSF 250 mM, NaF and Na_3VO_4, DTT 1mM. After 30 minutes of lysis at 4°C cell lysates were cleared by centrifugation. Supernatants were pre-cleared with protein A/G beads to avoid non-specific binding during the immunoprecipitation step. After pre-clearing, a fraction was saved as input. Precleared lysates were incubated for 1 hour with the antibody crosslinked to protein G beads. After 3-4 washes with lysis buffer, beads were dired and bound proteins were eluted in 2x Laemmli buffer, separated by SDS-PAGE and visualized by western blot.

PULL-DOWN

MBP-c16orf35 was produced in BL-21 cells according to manufacturer's instructions**.** GATA-1 was translated in vitro using the TNT® T7 Coupled Transcription/Translation System (Promega) and ^{35}S-methyonine, following the manufacturer's instructions**.** Radioactively labeled GATA1 was incubated for 1 hour at 4°C with MBP-c16orf35 or MBP beads in pull-down buffer. Resins were then washed three times, bound proteins were eluted in Laemmli sample buffer and separated by SDS-PAGE. After electrophoresis, the gel was dried and exposed to autoradipgraphy overnight.

RESULTS

***C16orf35* is a nucleo-cytoplasmic protein.**

c16orf35 was picked up from an *in vitro* protein-protein interaction screening carried on in our laboratory. The major goal of this screening was to isolate new evolutionary conserved p53-binding proteins using *Drosophila melanogaster* p53 as a bait. To investigate the role of *c16orf35* we have sub-cloned the entire coding sequence into a specific expression vector and over-expressed the protein in U2OS osteosarcoma cell line. As shown in Fig.8 the protein localizes both in nucleus and cytoplasm with two discrete patterns suggesting that *c16orf35* could plays discrete nuclear and cytoplasmic functions.

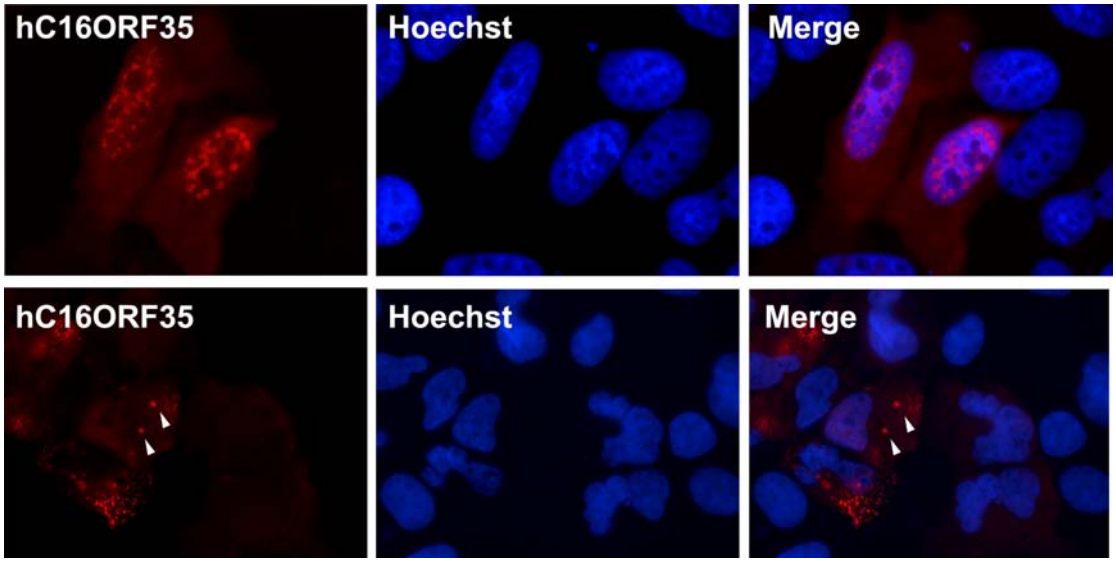

Fig.8 Cellular distribution of overexpressed *c16orf35.* U2OS cells were transfected with a vector encoding human c16orf35 and processed for immunofluorescence analysis 24h later. As shown, c16orf35 localizes in the nucleus (upper panels) with a "web-like" or dotted pattern. In the cytoplasm (lower panels) c16orf35 localizes in small round dots (see arrowhead) variable in number and size.

ANALYSIS OF NUCLEAR C16ORF35.

Nuclear *c16orf35* localizes on matrix attachment regions

Nuclear *c16orf35* displays a typical web-like or dotted pattern. In particular it localizes in interchromatin spaces surrounding eterochromatin, the dense regions of Hoechst staining. To test if the protein associates with the nuclear matrix, *c16orf35* transfected cells were permeabilized and treated with DNAseI before immunofluorescence. As shown in Fig.9A nuclear C16orf35 is resistant to extraction and DNase-I treatment, suggesting that *c16orf35* is associated to the nuclear matrix rather than being a soluble DNA binding protein. To confirm that *c16orf35* localizes to MARs, we used SATB1 as a marker of such domains. SATB1 is a matrix associated protein involved also in transcriptional regulation. It organizes chromatin into large transcriptional loops were multiprotein complexes assemble to regulate transcription (Alvarez et al., 2000; Cai et al., 2006; Dickinson et al., 1997; Gondor and Ohlsson, 2006; Ramakrishnan et al., 2000; Seo et al., 2005; Yasui et al., 2002). Therefore it also represent a sort of "landing platform" for transcriptional complexes such as N-COR. As shown in fig.9C *C16orf35* perfectly co-localizes with SATB-1 in double immunofluorescence experiments. We next asked if *C16ORF35* might physically interact with SATB1. To this aim we co-transfected HA tagged *c16orf35* and GFP-SATB1 in U2OS cells, and showed by co-immunoprecipitation that the two proteins effectively bind to each other (Fig. 9B).

Recently Kumar and colleagues shown that SATB-1 is also required to organize chromatin loops into specific transcriptional domain called "nuclear bodies" (Kumar et al., 2007). Nuclear bodies are subnuclear domains were chromatin loops converge to be engaged by several multiprotein complexes that regulate transcription. p53 family proteins are also recruited into nuclear bodies under specific conditions (Bernassola et al., 2005; Bernassola et al., 2004; Fogal et al., 2000). Nuclear bodies assembly require the presence of PML protein and PML overexpression is sufficient to induce nuclear bodies formation (Zhong et al., 2000). We asked if *C16ORF35* might be also recruited to PML-NBs under certain conditions. Similar to what has

been described for SATB1, when *c16orf35* is co-transfected with human PML-IV, it is strongly recruited into PML-nuclear bodies (Figure 10).

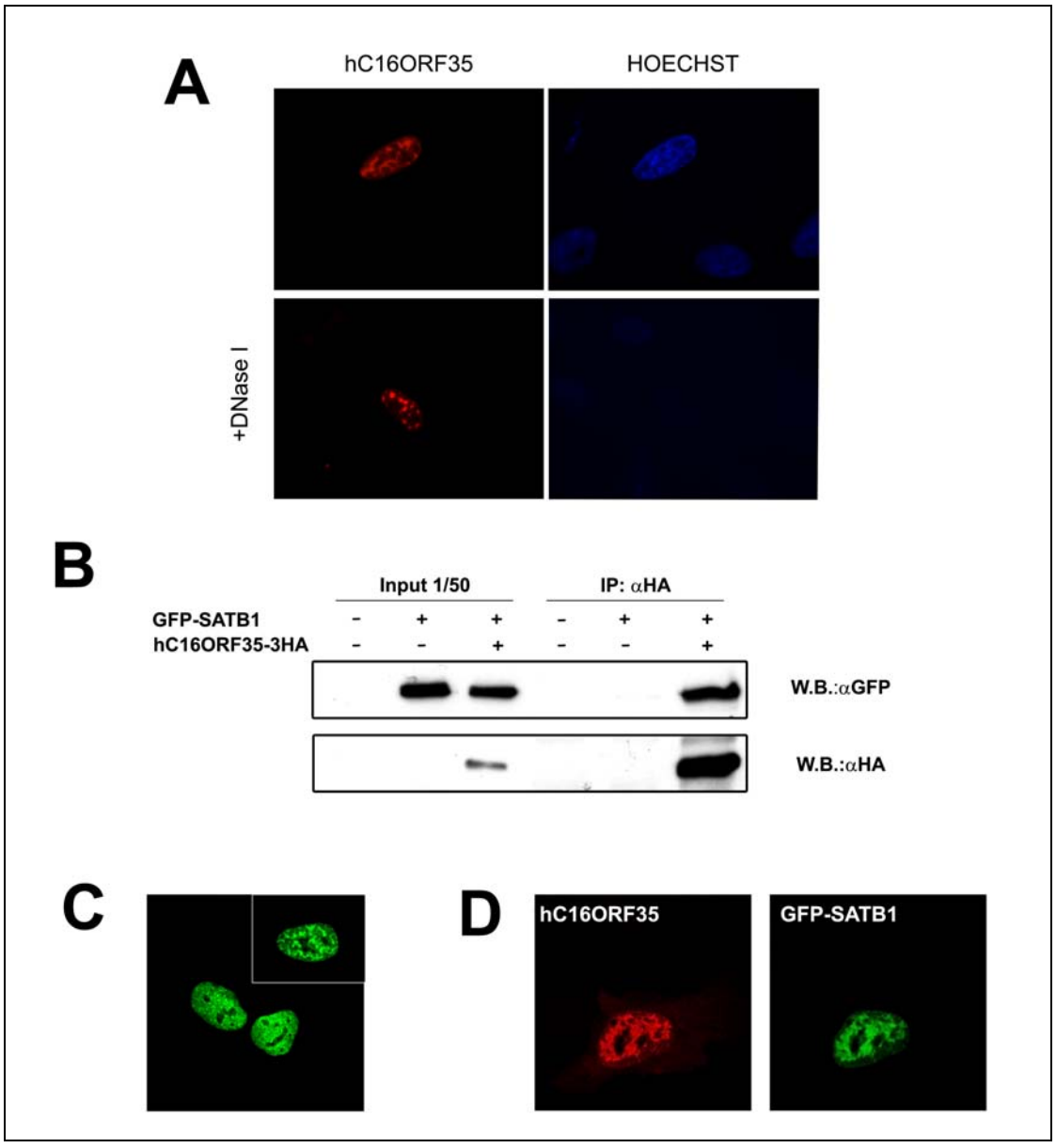

Fig.9 Nuclear c16orf35 associates with the nuclear matrix and binds SATB-1, a marker of MAR/SAR regions. A) Nuclear C16orf35 is bound to insoluble, DNase-resistant nuclear marix. U2OS cells were transfected with c16orf35. After 24h they were permeabilized and treated with DNase-I before immunofluorescence analysis. Lack of Hoechst staining confirms removal of genomic DNA. B) C16orf35 interacts with a marker of matrix attachment regions. HA-tagged C16orf35 was co-transfected with GFP-SATB1 in U2OS cells. Interaction of the two proteins was determined by co-immunoprecipitation. C) Typical nuclear localization pattern of GFP-SATB1. D) Nuclear co-localization of C16orf35 and SATB1. U2OS cells were co-transfected with the indicated constructs. Proteins were analyzed by double-immunofluorescence.

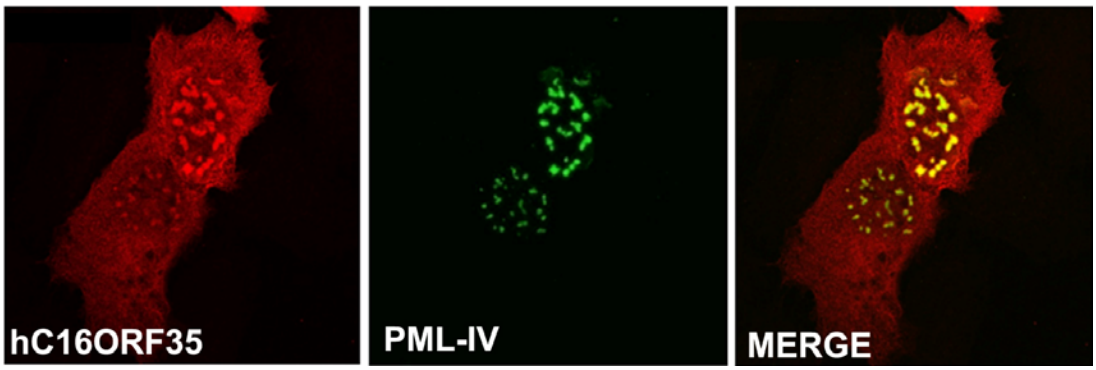

Fig.10 c16orf35 is recruited into PML nuclear bodies. U2OS cells were co-transfected with c16orf35 and human PML-IV, and proteins were detected by immunofluorescence. As shown, PML overexpression induces accumulation of nuclear C16orf35 into Nuclear Bodies.

***c16orf35* binds p63 and p73 but not p53.**

Since *c16orf35* was isolated during an *in vitro* protein-protein interaction screening using *Drosophila melanogaster* p53 as a bait, we asked if this interaction is evolutionary conserved between the human orthologs. In particular, since the single p53 gene in Drosophila has evolved into three paralogs in mammals, we tested *c16orf35* binding to all three members of the p53 family.

To this aim U2OS cells were transfected with *c16orf35* alone or in combination with p53, HA tagged TAp73α or Myc tagged TAp63α and the interaction of *C16ORF35* with p53-family proteins was assayed by co-immunoprecipitation. Interestingly *c16orf35* clearly binds both TAp73α and TAp63α but does not interact with p53 at least in these conditions (Fig. 11). Lack of binding to p53 was also observed in a variety of other experimental conditions (not shown).,

Given the peculiar nuclear staining of *c16orf35*, we asked if overexpression of *C16ORF35* might affect the localization of p53-realted proteins, Confocal immunofluorescence experiments performed in U2OS cells confirmed that *c16orf35*

is able to recruit into its sub-nuclear domains both TAp73α and TAp63α, but not p53 (Fig.12).

As the recruitment of promoters regions and transcription factors into MAR or nuclear bodies were previously shown to influence transcription positively and negatively (Ben-Yehoyada et al., 2003; Jiang et al., 2001; Okorokov et al., 2002; Seo et al., 2005; Yasui et al., 2002) we tested whether *c16orf35* could influence TAp73α or TAp63α–dependent transcription using a p21 promoter-driven and a BAX promoter-driven luciferase reporter constructs. These experiments showed that TAp73α and TAp63α transcriptional activity is not influenced by *c16orf35* overexpression, at least in this kind of assay (Data not shown).

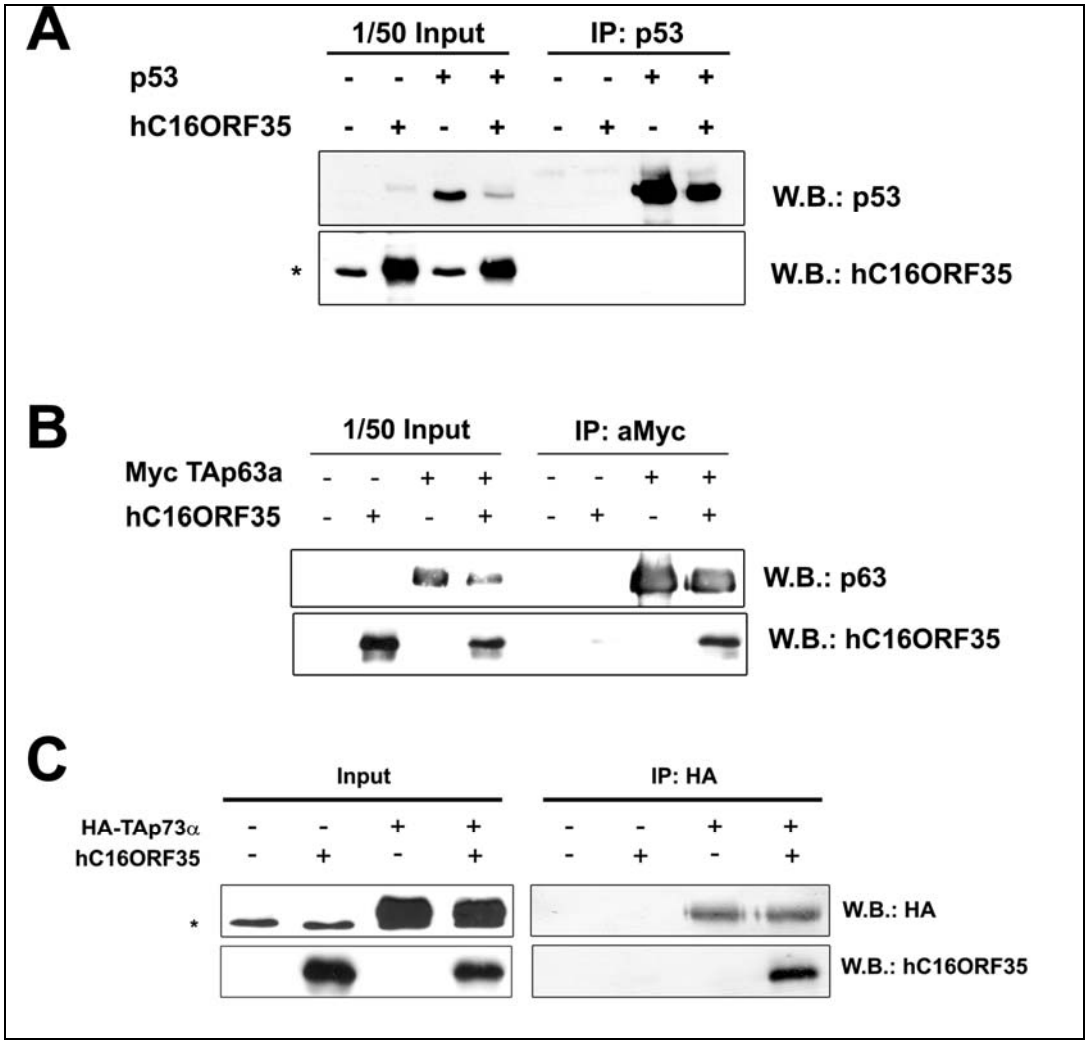

Fig.11 c16orf35 binds p63 and p73, but not p53. U2OS cells were transfected with the indicated constructs. After 24h, p53-related proteins were immunoprecipitated with the indicated antibodies cross-linked to protein-G beads. The presence of C16orf35 in the immunprecipitate was determined by western blotting with a polyclonal anti-C16orf35 antibody. Asterisks indicate non-specific bands.

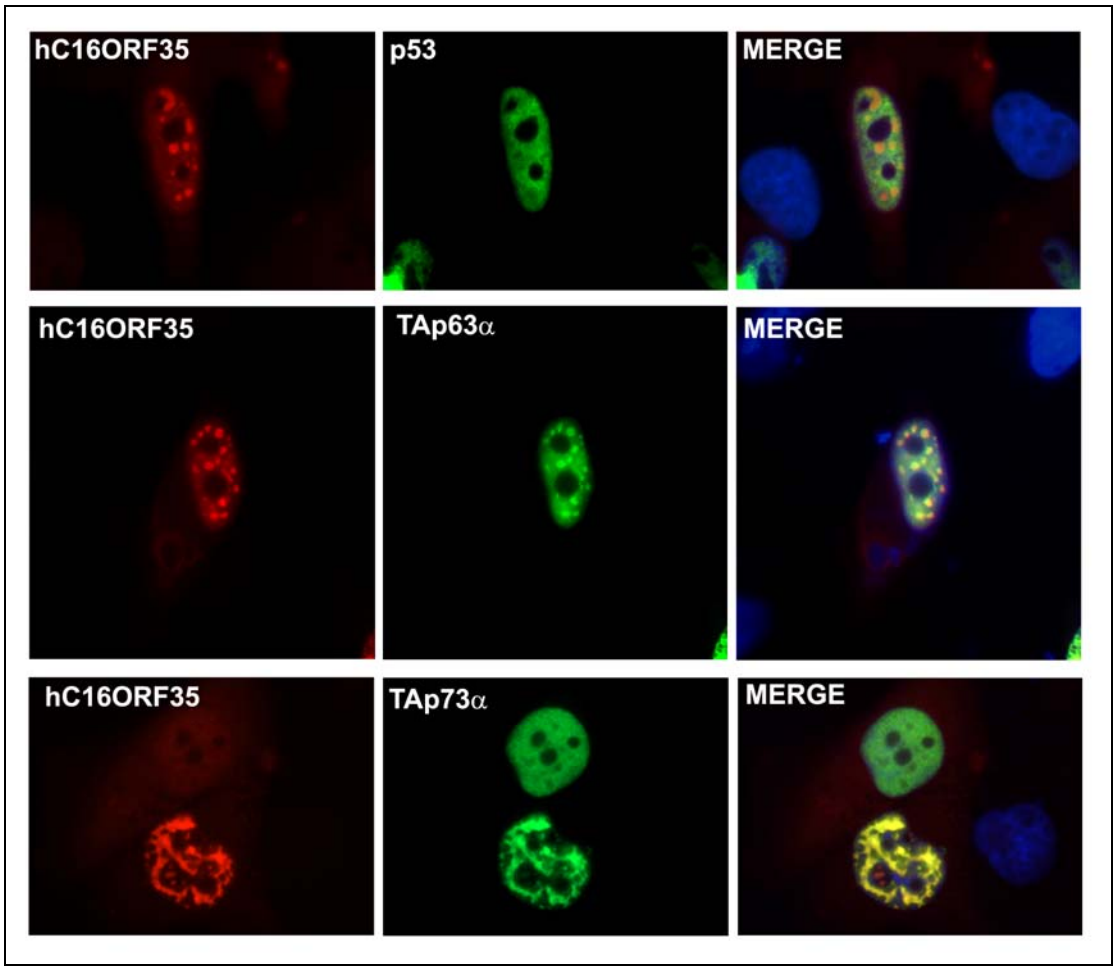

Fig.12 c16orf35 recruits p63 and p73 into its nuclear domains. U2OS cells were co-transfected with c16orf35 together with each one of the p53 family members (as indicated). After 24h cells were processed for immunofluorescence. As shown, c16orf35 is able to influence the nuclear staining of p63 and p73 by recruiting them into its subnuclear domains.

c16orf35 binds GATA-1 and repress its transcriptional activity

Considered the data presented by Anguita and colleagues (Anguita et al., 2002) on the accidental knock-down of *C16ORF35* in transgenic mice (see introduction for review) we decided to explore if the transcriptional activity of the master regulator of erithopoyesis GATA-1 could be affected by *c16orf35*.

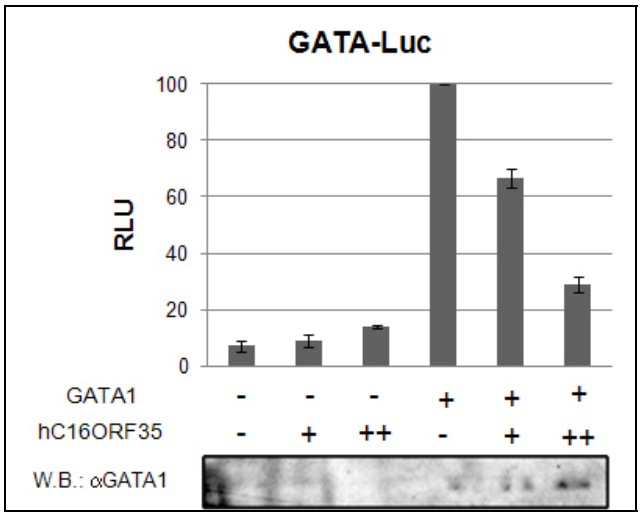

Fig.13 c16orf35 affects GATA-1 transcritpional activity. U2OS cells were transfected with a GATA1-reporter plasmid together with a fixed amount of GATA-1 expression vector, and increasing amounts of human c16orf35. A plasmid expressing renilla luciferase was included to normalize for transfection efficiency. Protein levels of GATA-1 were checked by western blot of the same lysates.

By transient luciferase reporter assays we were able to show that *c16orf35* is able to repress GATA-1 transcriptional activity in a dose-dependent manner (Fig. 13). Next, we explored the possibility that the two proteins could bind each other. By using *in vitro* radioactive pull-down we were able to show that *c16orf35* can bind GATA-1 (Fig.14A) and this was confirmed by co-immunoprecipitation experiments in HEK-293T cells (Fig 14B). We also analyzed the localization of the two proteins when expressed in U2OS cells. As shown in Fig. 14C, the two proteins not only perfectly co-localize, but are also able to re-localize each other.

These data might explain at least in part the lethal anemic phenotype observed when *c16orf35* was accidentally knocked-down by Anguita and colleagues, since both the over-expression (or over-activation) and the knock-down of GATA-1 activity can potently affect erithropoyesis *in vivo*.

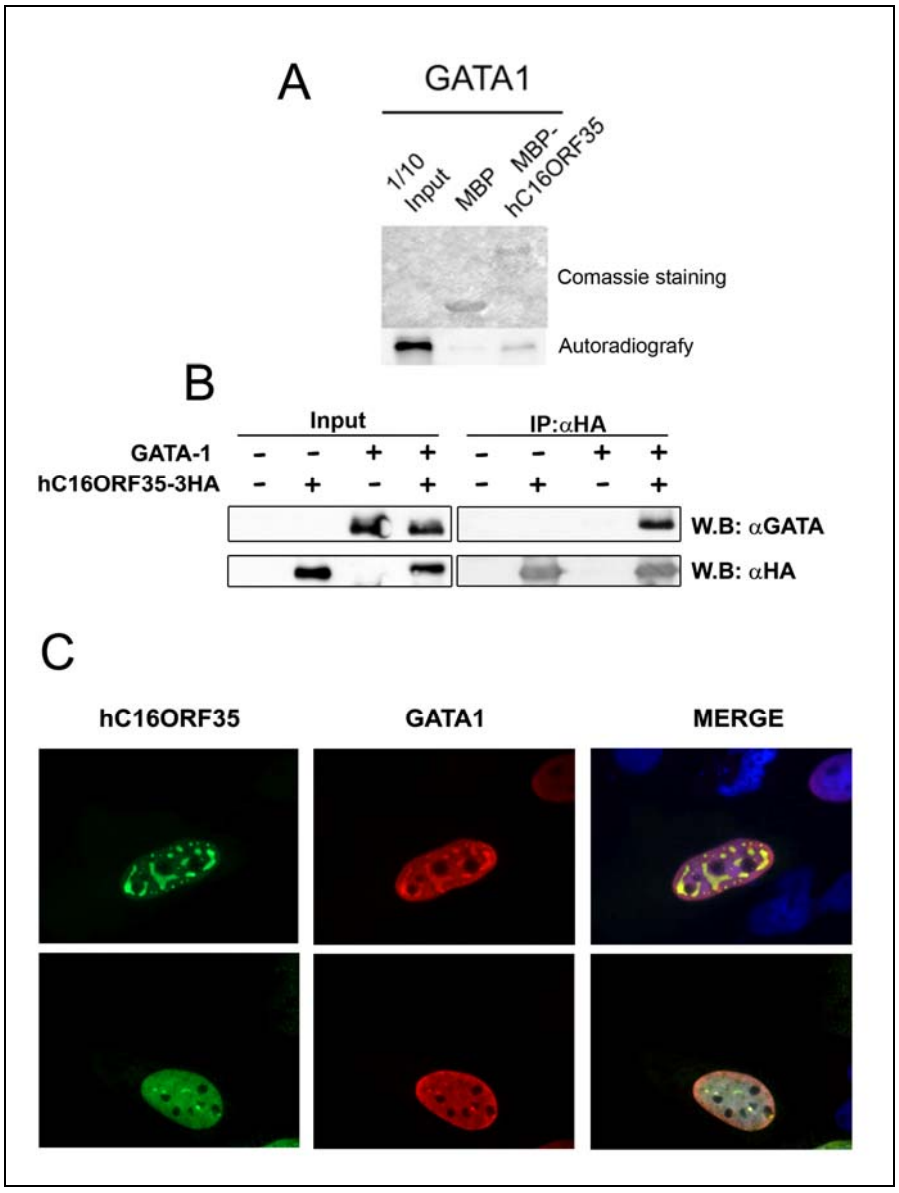

Fig.14 C16orf35 interacts with GATA-1. A) C16orf35 binds GATA-1 in vitro. GATA-1 was translated in vitro and bound to bacterially expressed MBP-C16orf35 resin. B) C16orf35 binds GATA-1 in cells. U2OS were co-transfected with the indicated constructs, and interaction between the two proteins was determined by co-immunoprecipitation. C) C16orf35 co-localizes with GATA-1 into nuclear domains. The proteins were co-transfected in U2OS cells and analyzed by immunofluorescence, In a subset of positive cells, C16orf35 clearly re-localizes GATA-1 into discrete nuclear domains.

c16orf35 binds members of transcriptional repressor complexes

With the aim to find a possible molecular mechanism for *c16orf35*-dependent GATA1 transcriptional repression we performed a series of co-immunoprecipitation experiments with members of repressor complexes such as the histone deacetylases.

As shown in fig.15 *c16orf35* is able to bind HDAC1 and HDAC3 and the HDAC1 "landing platform" mSin3A. Even if these data only correlate the transcriptional repression activity of *c16orf35* and its ability to bind histone deacetylases they shows that *c16orf35* could play different roles in cytoplasm and nucleus by forming different protein complexes.

All these data suggest that *c16orf35* is a novel Nuclear matrix associated protein that is able to associate with subnuclear structures like PML-nuclear bodies, with transcription factors such as p63, p73 and GATA-1, and with transcriptional modulators such as HDACs. Moreover, its ability to repress GATA-1 transcriptional activity suggest that it could play a direct role in transcription modulation.

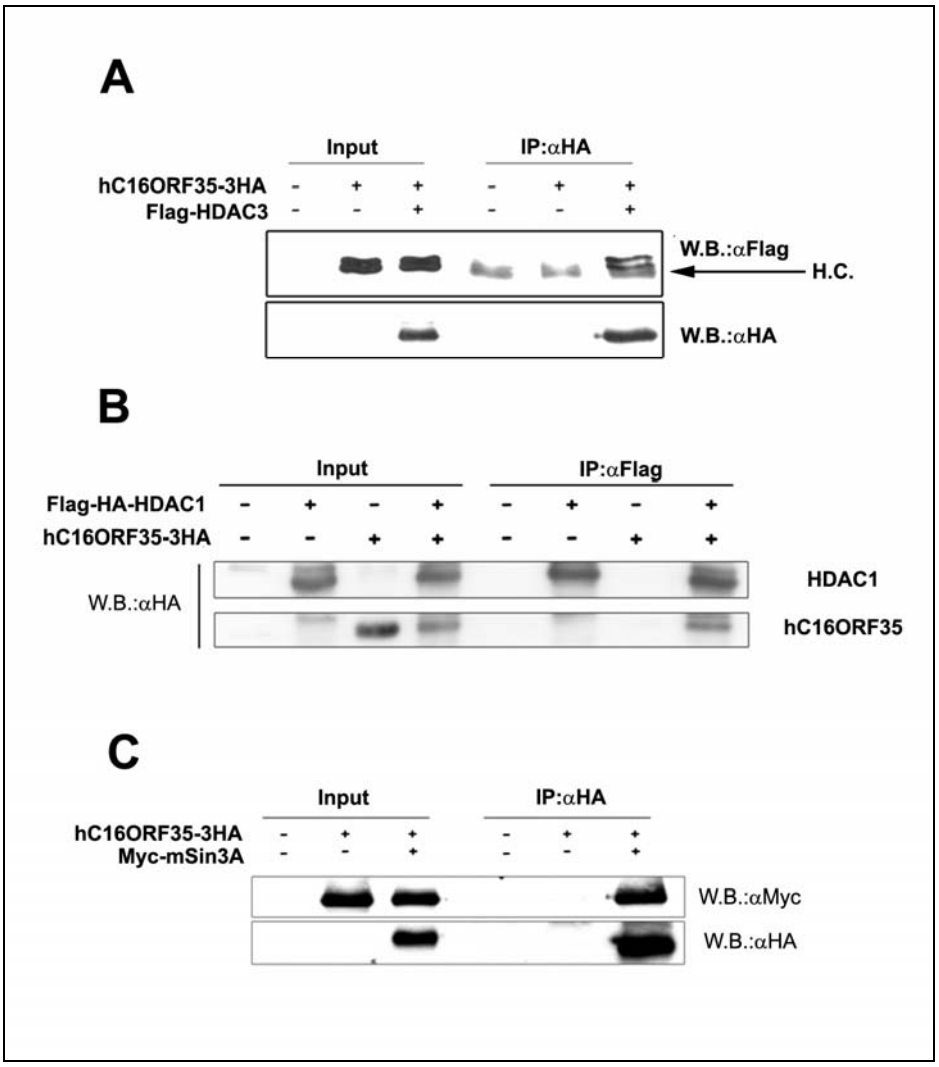

Fig.15 C16orf35 interaction with components of transcriptional co-repressor complexes. U2OS cells were co-transfected with the indicated constructs. Co-immunoprecipitation experiments were performed 24 hrs after transfection. Under these conditionzs, c16orf35 is able to efficiently bind histone deacetilases, in particular (A) HDAC3 and (B) HDAC1, and the HDAC binding protein mSin3A (C).

ANALYSIS OF CYTOPLASMIC C16ORF35.

As mentioned before (see fig 8 lower panel) in addition to the punctate nuclear staining, overexpressed *c16orf35* also displays cytoplasmic localization. In the cytosol, *c16orf35* is often distributed homogeneously, but in approximately 30% of the cells, it forms discrete round dots variable in number and size. When soluble cytoplasmic proteins are extracted by permeabilization of the cell membrane before fixation, the fraction of *c16orf35* associated with cytoplasmic dots is not affected, indicating that they are associated to insoluble cytoplasmic structures (Fig.16).

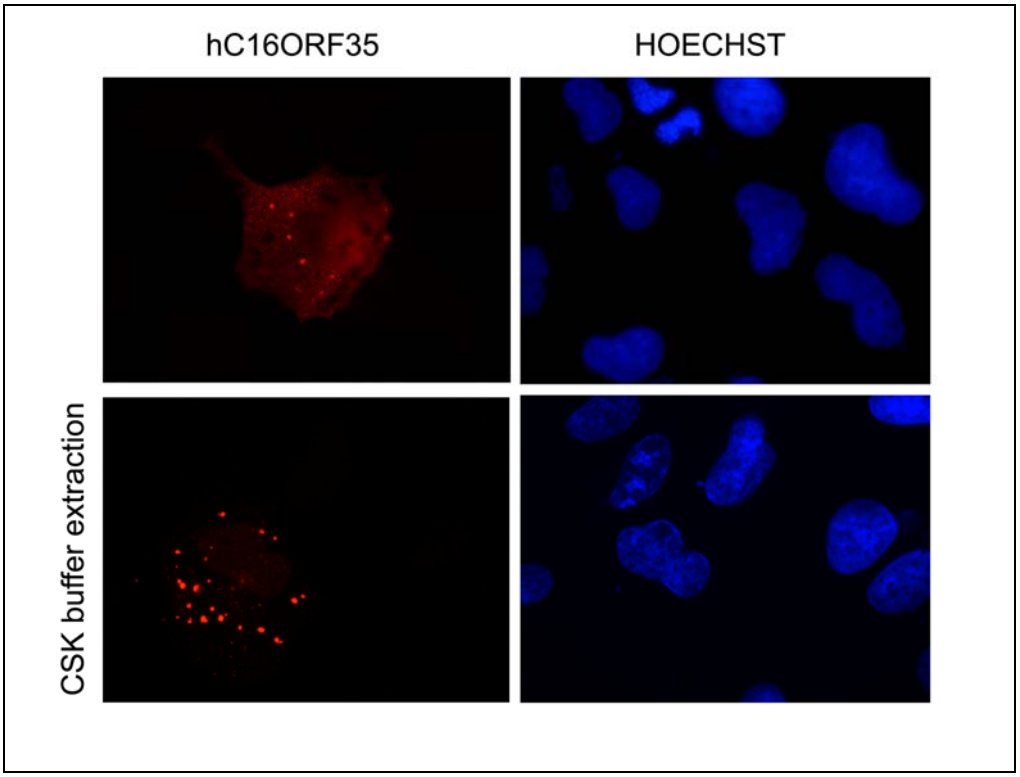

Fig.16 C16orf35 cytoplasmic dots are insoluble structures. U2OS cells were transfected with human c16orf35. After 24h they were permeabilized to extract soluble proteins prior to fixation, then processed for standard immunofluorescence. As shown, c16orf35 cytoplasmic dots are resistant to strong cytoplasm extraction.

Cytoplasmic *c16orf35* localizes to stress granules and processing bodies

To better characterize *c16orf35* sub-cellular distribution we have analyzed the cytoplasmic dots observed in a subset of over-expressing cells. Such structures resemble morphologically both "stress granules" and "processing bodies". These structures are particular sites were mRNAs are processed or stored, and finally are degradated or reentered into translation machinery (Anderson and Kedersha, 2006). These two compartments are tightly linked each other and the processed mRNA can sequentially shuttle from stress granules to processing bodies. Processing bodies are physiologically present into every cells, as they also participate in miRNA processing.

On the contrary, stress granules are induced upon different kinds of environmental stresses from heat shock to virus infection (Anderson and Kedersha, 2006). The core of stress granules are components of a noncanonical, translationally silent 48S pre-initiation complex and RNA binding proteins that regulate mRNA translation and stability such as TIA-1. Indeed, the TIA-1 protein can be used as a marker of SG (Kedersha et al., 1999). As shown in Fig. 17 a subset of cytoplasmic *c16orf35* dots effectively colocalize with endogenous TIA-1 into stress granules (SG), while remaining cytoplasmic dots reside in the close proximity of these structures (inset, arrows in Fig 17).

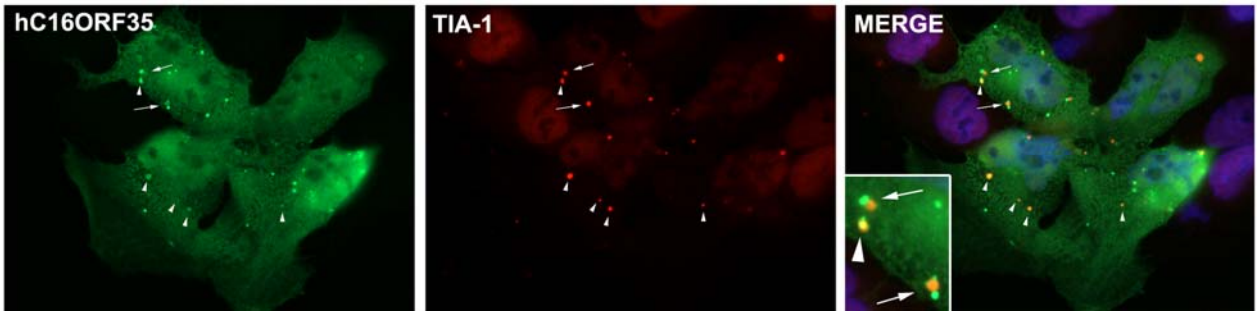

Fig.17 c16orf35 associates with TIA1-positive stress granules. U2OS cells were transfected with human c16orf35. After 24h the cells were fixed and stained with anti-c16orf35 and anti TIA-1 antibody. Several c16orf35 cytoplasmc dots perfectly colocalize with endogenous TIA-1 (inset, arrowhead). A subset of cytoplasmic c16orf35 dots reside in proximity of stress granules (inset, arrows).

Stress granules are induced upon stress-induced eIF2α phosphorylation: this prevents the assembly of 48S pre-initiation complex and polysome disassembly. Non-polysomal transcripts are then reorganized into stress granules by a process mediated by different RNA binding proteins (Kedersha et al., 2002). Independently from this mechanism, the over-expression of constituent RNA-binding proteins such as TIA-1 or G3BP1 (Ras-gap SH3-binding protein) are sufficient to induce formation of SGs, and are commonly used as SG inducers, instead of generic stresses such as heat shock or arsenite (Gilks et al., 2004; Tourriere et al., 2003). Considered these data, we asked

if the over-expression of G3BP-1 or its constitutively active mutant G3BP-1S149A was able to reclutate *c16orf35* into stress granules.

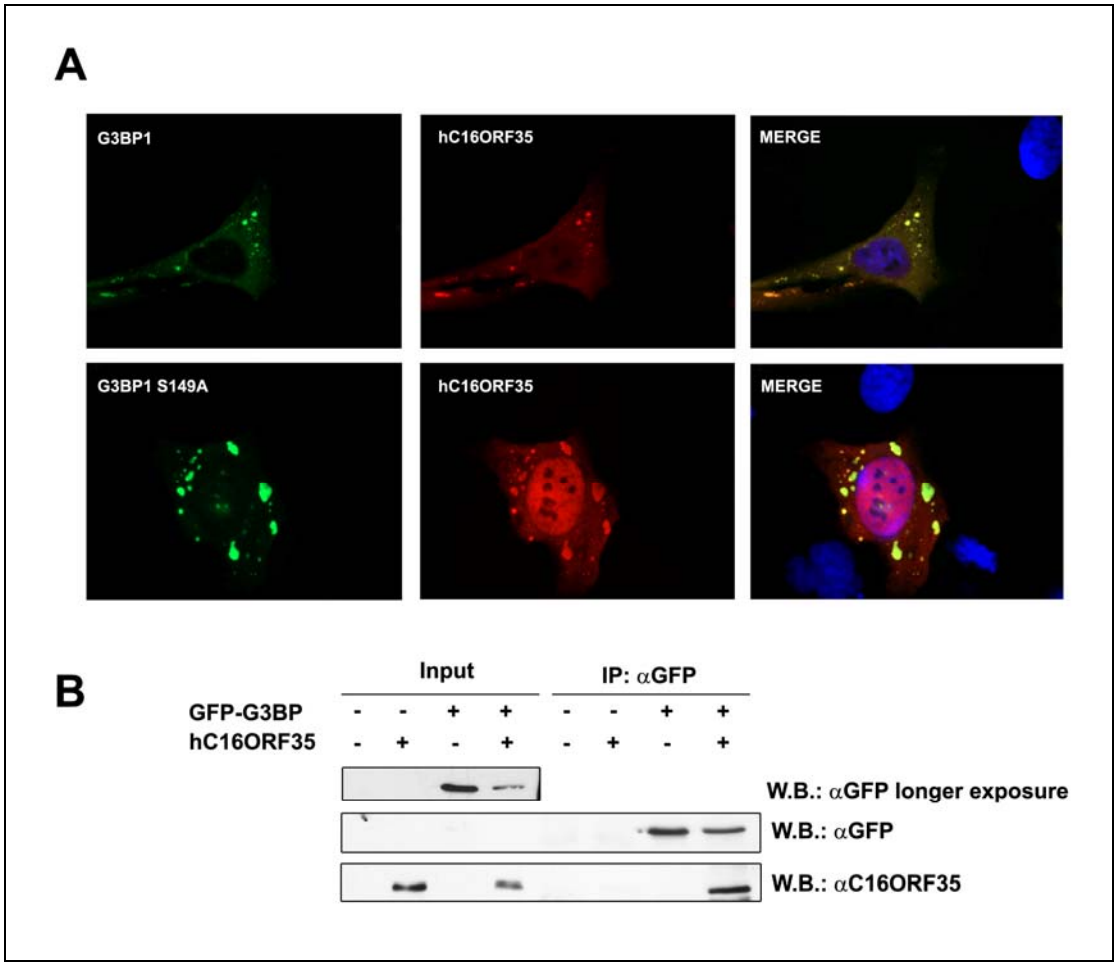

Fig.18 c16orf35 can be recruited into Stress Granules (SG) and binds G3BP1, an essential SG component and inducer. A) C16orf35 co-localizes with G3BP1. U2OS cells were co-transfected with human c16orf35 and GFP-tagged G3BP1 or its costitutively active mutant G3BP1(S149E). Overexpression of G3BP1 induces formation of SGs and recruits C16orf35 to these structures. B) C16orf35 binds G3BP1. The indicated constructs were co-transfected in U2OS cells; protein interaction was analyzed by co-immunoprecipitation.

As shown in Fig.18A *c16orf35* is efficiently recruited into stress granules formed by overexpression of G3BP-1. As stress granules are in fact multiprotein complexes assembled on stalled RNAs, we investigated if *c16orf35* was able to enter this complexes by physically interacting with G3BP-1. To this aim we co-transfected G3BP-1 and *c16orf35* in U2OS cells and performed co-immunoprecipitation experiments. As shown in fig.18B *c16orf35* does indeed binds G3BP-1.

As not all cytoplasmic dots were colocalized into stress granules (see Fig.17 inset) we tested the possibility that these structures could be processing bodies (P-bodies), which are frequently associated to stress granules (Kedersha et al., 2005). To test if *c16orf35* was able to associate also with these structures, we co-transfeìected *c16orf35* with two different Argonaute proteins (AGO-1 and AGO-2), members of the RNA-induced silencing machinery (RISC) and resident markers of P bodies (Sen and Blau, 2005). As shown in fig.19A, *c16orf35* co-localizes with both AGO-1 and AGO-2, and therefore can be recruited into cytoplasmic P bodies.

We completed this analysis by asking whether *c16orf35* can physically interact with Argonaute proteins: as shown in Fig.19B, co-immunoprecipitation experiments confirmed that *c16orf35* can binds to both AGO-1 and AGO-2.

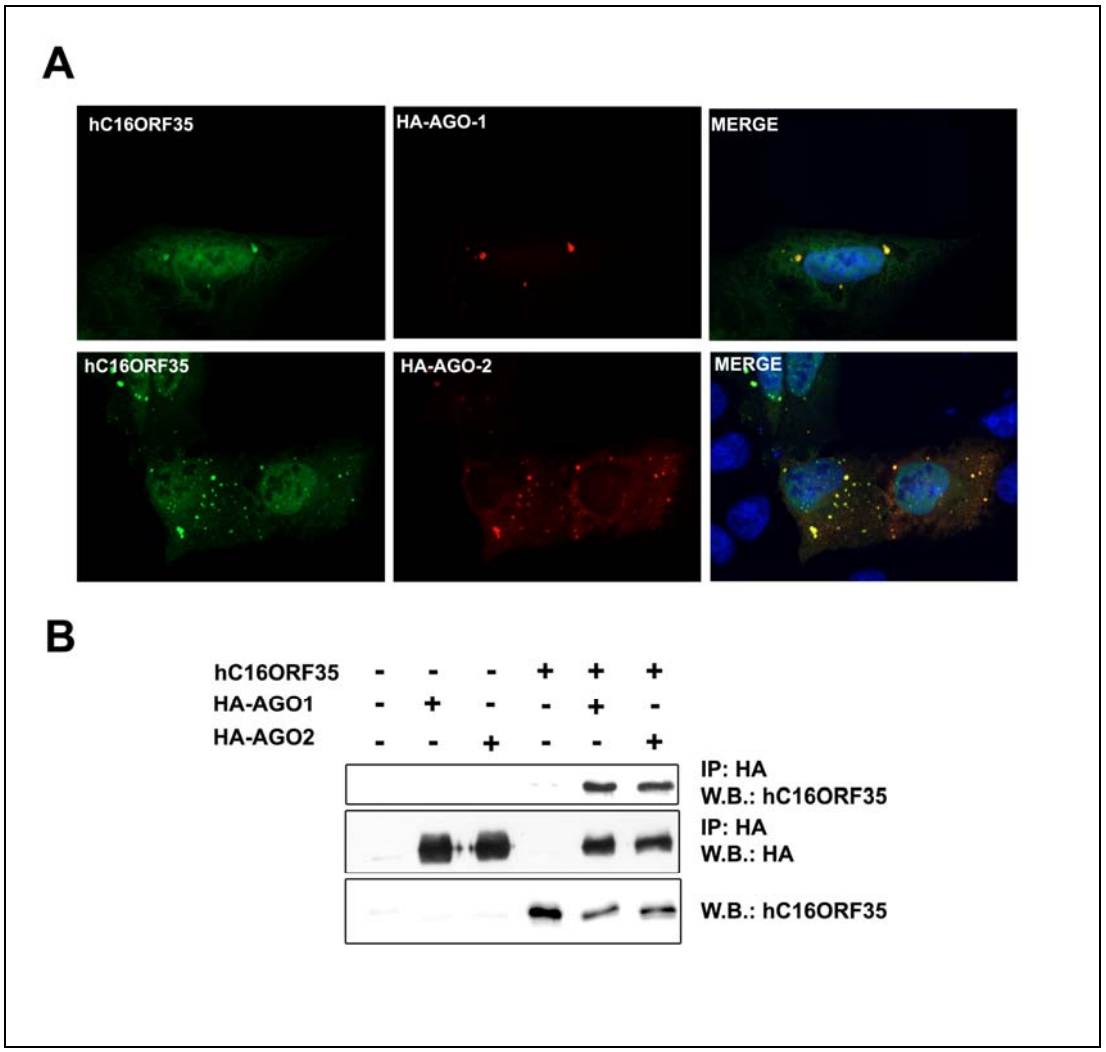

Fig.19 c16orf35 can be recruited to RNA processing bodies (P bodies), and physically interacts with RISC components. A) C16orf35 co-localizes with Argonaute-1 (AGO1) and Argonaute-2 (AGO2). The indicated proteins were co-transfected in U2OS cells for analysis by double immunofluorescence. B) C16orf35 interacts with AGO-1 and AGO2. The indicated proteins were co-transfected in U2OS cells; interaction was assayed by co-immunoprecipitation.

Overexpression of *c16orf35* induces Stress Granules formation

As previously mentioned, stress granules are stress-dependent structures normally absent in healthy cells. However, *c16orf35* overexpressing cells tend to show cytoplasmic dots co-localized with TIA-1 in the absence of any specific stress treatment. Therefore we decided to test if *c16orf35* overexpression might induce stress granules formation, similarly to G3BP1. To this aim *c16orf35* was transfected in U2OS cells, and stress granules were scored by immunofluorescence, using

endogenous TIA-1 as marker of these structures. Stress granules can be induced by several different stresses and this must be taken into consideration when choosing experimental conditions, and in particular a suitable negative control. For instance, we found that GFP overexpression induces formation of stress granules (data not shown), probably by affecting proteasomal-dependent protein degradation (Baens et al., 2006): indeed, block of proteasomal activity is one of the stimuli able to induce these structures (Mazroui et al., 2007).We therefore used a vector expressing MBP (**M**altose **B**inding **P**rotein) as a control. As shown in Fig.20 *c16orf35* overexpression is able to efficiently induce stress granules formation in U2OS cells.

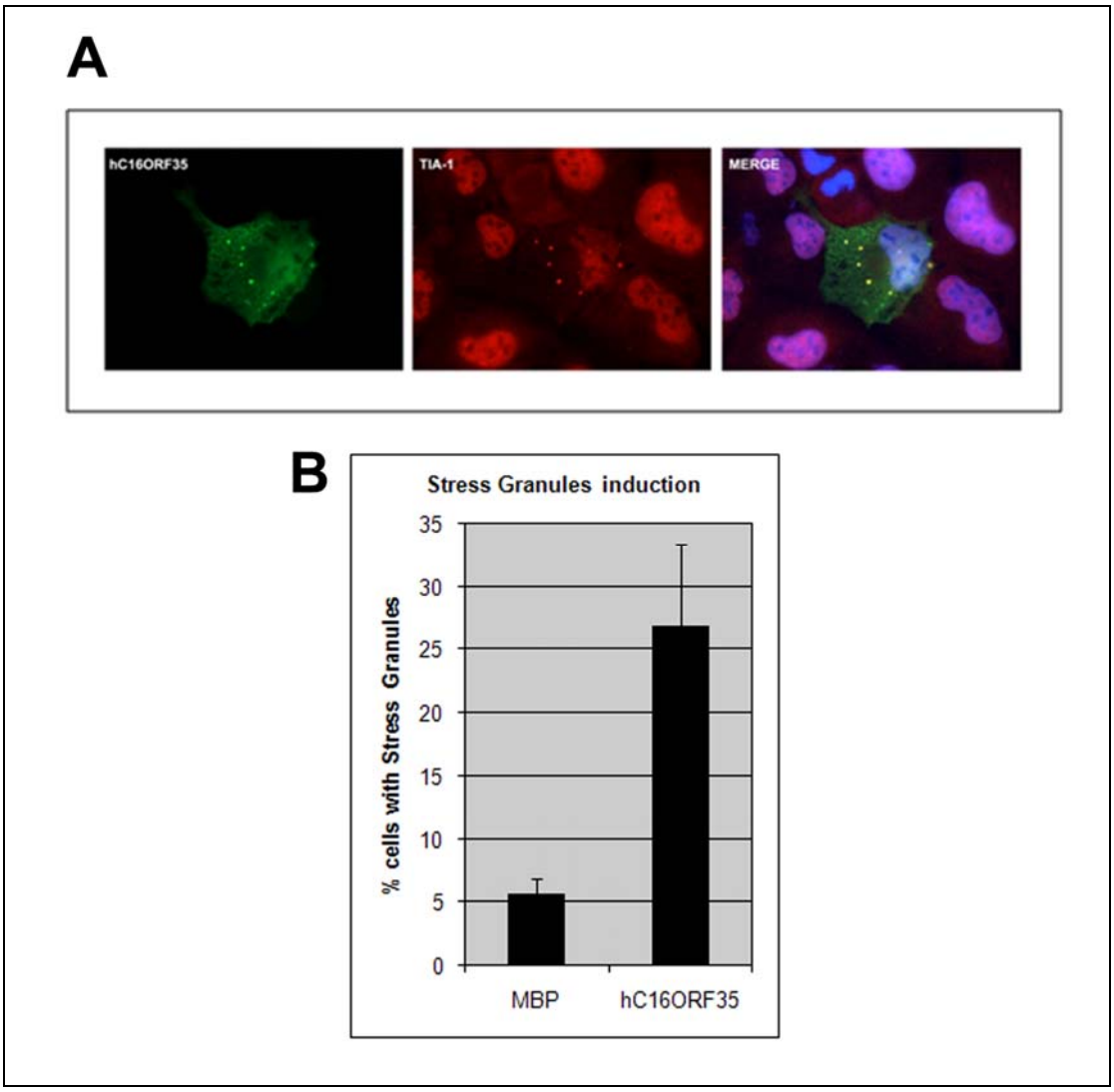

Fig.20 Overexpression of c16orf35 induces formation of stress granules. U2OS cells were transfected with a plasmid encoding human c16orf35 or a plasmid expressing Maltose Binding Protein (MBP) as a control. After 24h cells were fixed and C16orf35, MBP and endogenous TIA-1 were visualized by immunofluorescence (A). The fraction of C16orf35 or MBP overexpressing cells displaying TIA-1 positive cytoplasmic dots (SGs) was counted (B).

Overexpression of *c16orf35* affects tumor cells growth

To gain more insight into a possible role of *c16orf35* in cellular homeostasis, we have monitored the proliferative ability of osteosarcoma cells overexpressing *c16orf35*.The ability of *c16orf35* to inhibit cell growth was initially explored by colony formation assay. A vector expressing *c16orf35* and carrying Puromycin resistance was transfected in U2OS cells. After 12 days in selection, significantly fewer puromycin-resistant colonies were formed by cells transfected with *c16orf35*

expressing vector compared to control empty vector (Fig.21A). These data indicate that *c16orf35* overexpression inhibits cell proliferation.

To understand if this growth inhibition is due to a block of the cell cycle, we transiently trasfected *c16orf35* in U2OS cells, and monitoredentry in S-phase by BrdU incorporation over a period of 18h. As shown in Fig.21B *c16orf35* strongly inhibits BrdU incorporation in U2OS cells when compared to eGFP, transfected as a control. Importantly, no signs of cell death were observed, as assessed by overall morphology and condensation of the DNA in transfected cells. Of note, GFP overexpression is also able to affect cell cycle to some extent (Fig. 8B). This is probably due to its negative effect on ubiquitin-proteasomal system that led to p53 stabilization and activation (Baens et al., 2006).

Taken together, these data shows that *c16orf35* is a novel component of processing bodies and stress granules and that its over-expression is able to significantly induce stress granules assembly thus generating a condition of cellular stress. This condition could be the cause of the *c16orf35*-dependent growth arrest observed.

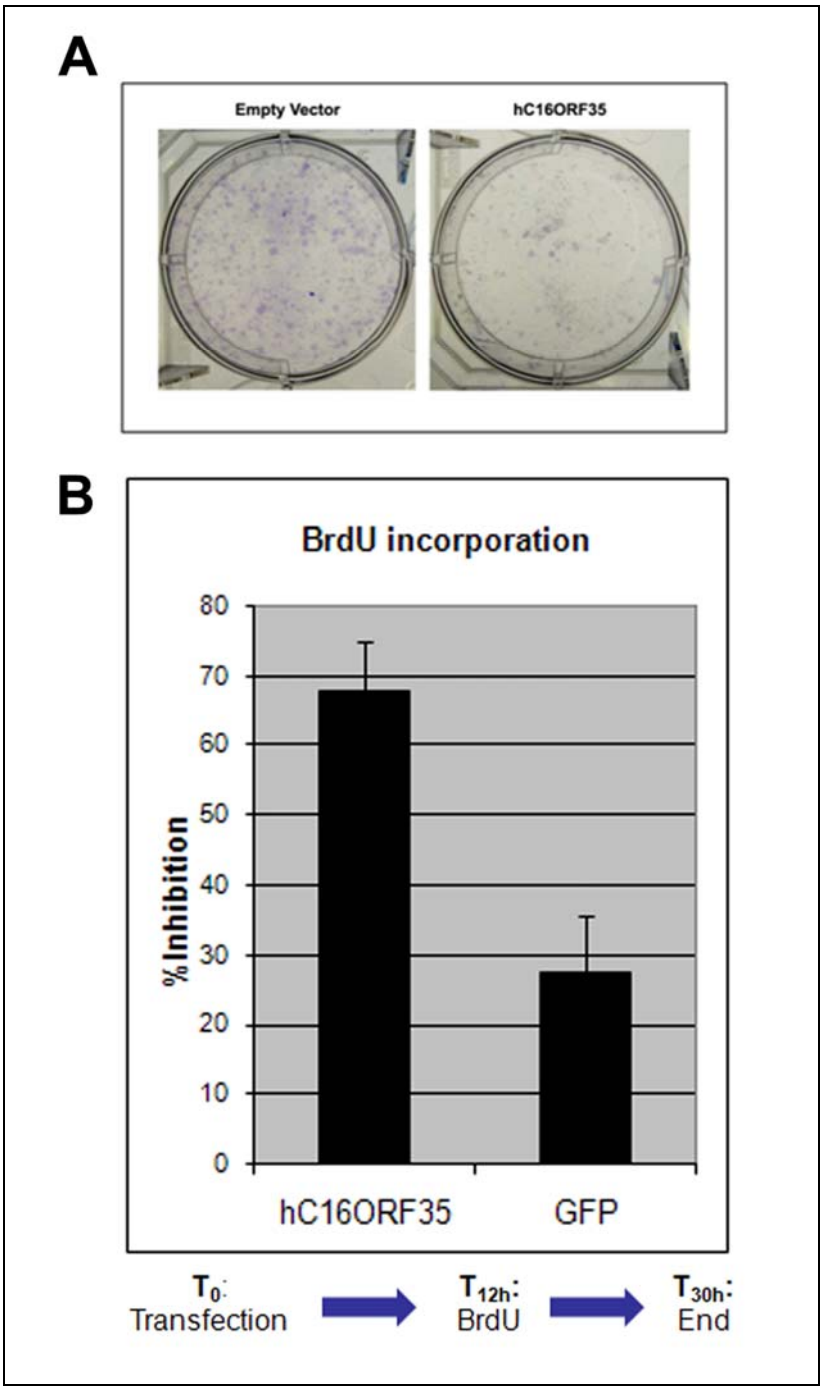

Fig.21 c16orf35 overexpression inhibits cell proliferation. A) C16orf 35 inhibits colony formation. U2OS cells were transfected with pLPC-hC16orf35 or pLPC empty vector. After 12 days of selection, puromycin resistant colonies were visualized by Giemsa staining. B). C16orf35 overexpression inhibits BrdU incorporation. *C16orf35* was transiently transfected in U2OS cells, and entry in S-phase was monitored by BrdU incorporation over a period of 18h. A vector expressing eGFP was transfected as a control. Percent inhibition is measured as the *difference in BrdU positive cells among the transfected versus untransfected background population (on the same glass slide*). The percent of relative inhibition of DNA sysnthesis in transected cells was calculatedby the formula: % = [%BrdU-positive cells (c16orf35 or GFP **negative**) - % BrdU-positive cells (c16orf35 or GFP **positive**)] / % BrdU-positive cells (c16orf35 or GFP **negative**).

ANALYSIS OF ENDOGENOUS C16ORF35

Endogenous *c16orf35* is a widely expressed nucleo-cytoplasmic protein

To characterized endogenous *c16orf35* we produced a polyclonal antibody raised against the C-terminal half of the protein. We produced a recombinant MBP fusion protein, comprising aminoacids 180 to 569. This was inoculated in two different rabbits, to obtain two independent antisera (C16A and C16B)..

Polyclonal antibodies were affinity purified and tested for their ability to recognize the overexpressed protein. Both detect exogenous *C16ORF35* very efficiently, and were used for most of the immunofluorescence experiments described in this thesis. In an affort to detect endogenous *C16ORF35*, we have used our polyclonal antibody to immunoprecipitate endogenous *c16orf35 form U2OS cells*. As shown in Fig.22A the antibody recognizes two bands of apparent Mw of about 50-60kDa in the total lysate. However, only the upper band is immunoprecipitated. To gain more insight into this phenomenon we used an siRNA specifically designed to knock-down all putative *c16orf35* isoforms. As shown in fig.22B, only the upper band is knocked-down by this reagent. Finally, the upper band migrates identically to exogenous transfected full-length *C16ORF35*.

Taken together, these observations strongly suggest that the lower band detected by our antibody in total lysates might be a non-specific, cross-reacting protein.

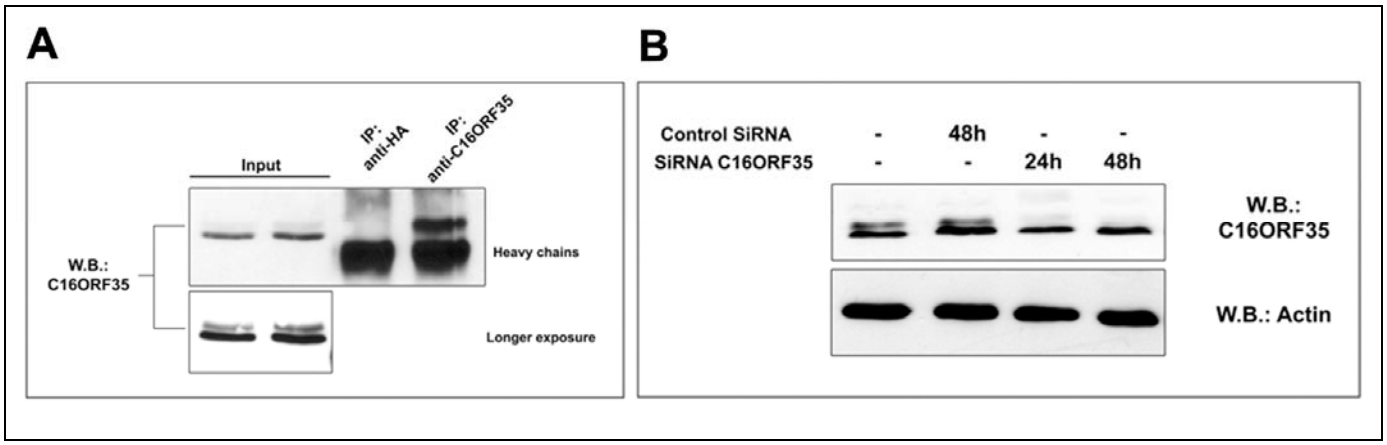

Fig.22 A polyclonal antibody raised against recombinant c16orf35 recognizes the endogenous protein. A) The affinity purified C16A antibody immunoprecipitates the endogenous protein. The reagent detects two bands in lysates of U2OS cells (see input panel), but immunoprecipitates only the higher molecular weigth band of approximately 60kDa. B) Only the higher molecular weight band is affected by transfection of specific siRNAs against C16orf35.

Endogenous *C16orf35* isoforms

As mentioned in the introduction, alternative splicing of the first exons can give rise to a variety of transcripts for *C16orf35*. In particular, a number of mRNAs are predicted to translate a shorter *c16orf35* protein, starting form an internal AUG and corresponding to the 390 C-terminal aminoacids of *C16ORF35*, with an expected MW of about 43 kDa (GenBank acc. NP_001034565). Using our polyclonal antibody, we have analyzed *c16orf35* expression in different tumor cell lines and in naturally immortalized human keratinocytes (HaCaT). As shown in fig.23 *c16orf35* is expressed at different levels in different cell lines. Importantly, we never observed bands of apparent molecular weight compatible with the shorter isoform (theoretical Mw: 43kDa). These data indicate that the antibody is able to recognize endogenous *c16orf35* in different cell lines and that the shorter isoform *c16orf35*s is not expressed at detectable levels in the cell lines analyzed.

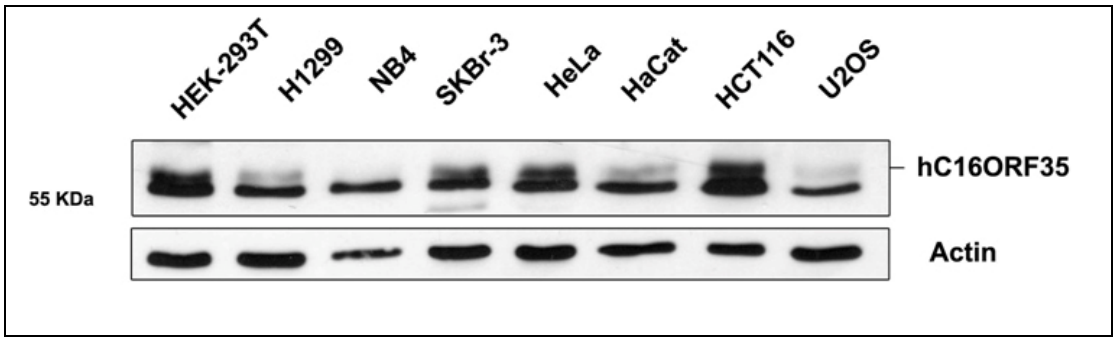

Fig.23 Differential expression of the c16orf35 protein in human cell lines. Different human tumor cell lines were used to analyze endogenous c16orf35 by western blotting using the C16A antibody.

As the antybody was able to efficiently recognize *c16orf35* in its native form (as shown by the immunoprecipitation experiment Fig.22A) we decided to use it to immunolocalize endogenous *c16orf35* in U2OS cells. As shown in Fig. 24A, our polyclonal antibody detects an endogenous *protein which* localizes in the nucleus, were it is uniformly distributed with some dotted regions intensively stained. This protein has also some cytoplasmic staining, were it localizes in small rounded dots. As control we used preimmune serum that gave a uniform faint staining in the cytoplasm and no signal into the nucleus. This localization is clearly similar to what observed with overexpressed *c16orf35*, suggesting that endogenous *c16orf35* has indeed both nuclear and cytosolic localization.

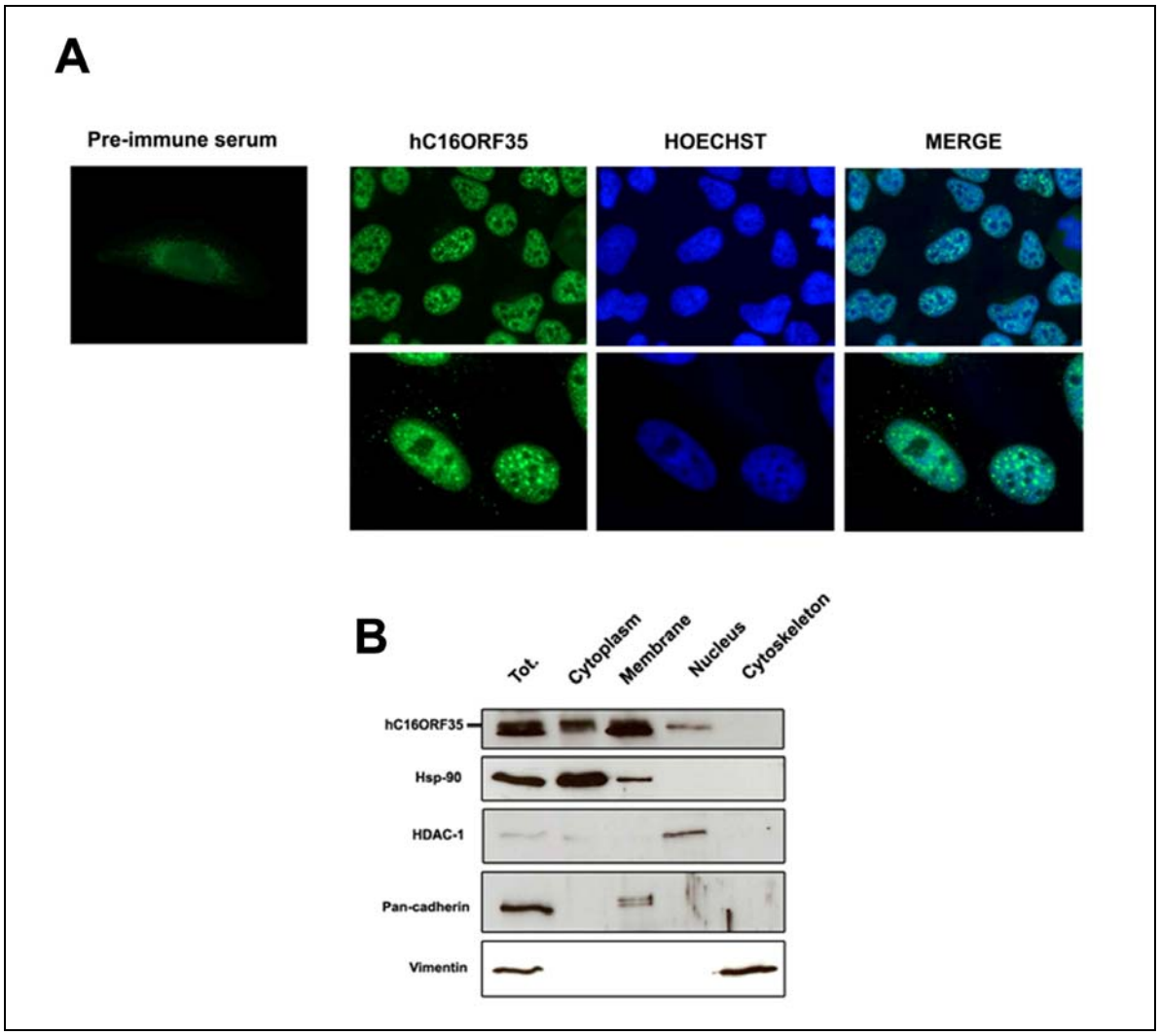

Fig.24 Analysis of the endogenous c16orf35 protein confirms its dual nucleo-cytoplasmic localization. A) Endogenous C16orf35 has a localization similar to the exogenous protein. The C16A antibody was used for immunofluorescence on U2OS cells. The antibody detects a nuclear speckled staining, and few cytoplasmic dots. Only a faint bakground is detected using the pre-immune serum, confirming specificity of the signal. B) Biochemical analysis confirms the nuclear-cytoplasmic localization of endogenous C16orf35. U2OS cells were fractionated into the indicated components. The efficiency of the fractionation was confirmed by the use of specific markers. We blotted Hsp90 as a marker for cytosolic fraction, HDAC-1 for the nucleus, cadherin for the plasma membrane, and vimentin as a marker of the cytoskeletal fraction.

Nucleo-cytoplasmic distribution were also confirmed by biochemical fractionation. The presence of endogenous *c16orf35* in nuclear, cytosolic, membrane, and insoluble fractions prepared from U2OS cells was analyzed by western blotting. As shown in Fig. 24B, endogenous *c16orf35* is detected in both cytoplasm and nucleus. Interestingly, the lower non-specific band which strongly cross-reacts with

our antibody is detected mainly in the membrane fraction but no membrane staining was observed in immunoflurescence experiments corroborating its non-specific nature. Of note the overexpression of Argonaute-1 is able to recruit endogenous *c16orf35* into AGO-1 positive processing bodies (Fig.25) as observed with the overexpressed *c16orf35*.

We conclude that the preliminary analysis of the endogenous protein confirms that *C16orf35* is indeed a nucleo-cytoplasmic protein, with a very specific nuclear localization to interchromatininc domains, and cytoplasmic localization to important mRNA triage sites such as P bodies.

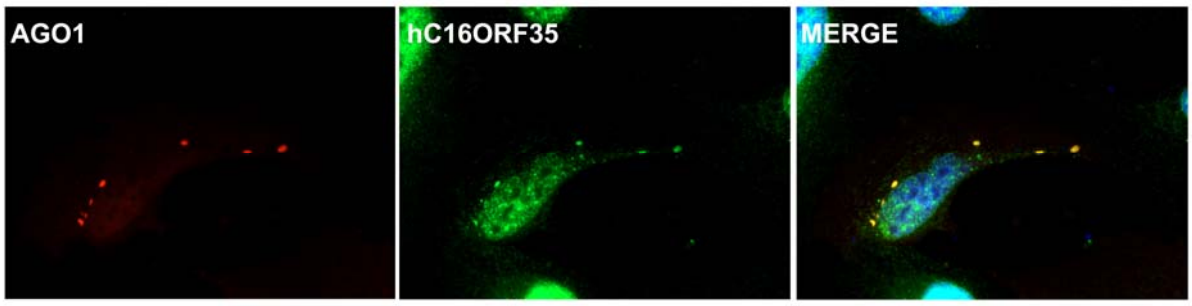

Fig.25 Endogenous c16orf35 is recruited by AGO-1 into processing bodies. U2OS cells were transfected with AGO-1 and fixed after 24h for immunofluorescence analysis. Endogenous c16orf35 was detected with the C16A polyclonal antibody. Similar to what observed with the exogenous protein, endogenous c16orf35 is recruited into AGO-1 positive cytoplasmic dots (P bodies).

DISCUSSION

In this work, we describe the characterization of *C16ORF35* protein product. This widely expressed gene lies on chromosome 16 immediately upstream to the α-globin cluster in man, mouse and chicken. It was first described by Vyas and colleagues in 1995 and named "-14 gene" from its position respect to the α-globin gene cluster. The gene comprises 15 exons spanning coordinates -14, -69 of the human α-globin gene locus. The authors mapped a constitutive DNase I hypersensitive site and a CpG island at position -14. Interestingly another DNaseI hypersensitive site, the major erithroid-specific regulatory region, was mapped in position -40 into the intron 5 of *C16ORF35* and was shown to be involved in α-globin gene cluster transcriptional regulation (Vyas et al., 1995). The coding region of *C16orf35* starts in exon 2 and continues to exon 14. Both the coding sequence and its position respect to the α-globin gene locus are evolutionary conserved and *C16ORF35* is actually considered an housekeeping gene based on Northern blot analysis and ESTs and microarray data available in public databases. Despite the initial efforts in *C16ORF35* characterization, no other work has been published on this gene. In particular, nothing is known on its protein product.

The protein sequence, inferred from nucleic acid sequence, displays no homology with other characterized proteins and no known structural domain can be identified. It is a highly evolutionary conserved protein. The highest sequence conservation is between vertebrates, with 96% aminoacid identity and 98% similarity between *Homo sapiens* and *Mus musculus* and with 79% identity and 87% similarity between *Homo sapiens* and *Danio rerio.*

In our laboratory an *in vitro* protein-protein interaction screening aimed to identify new evolutionary conserved p53 interacting proteins identified *Drosophila melanogaster c16orf35* as new *Drosophila melanogaster* p53 interactor. Considered its evolutionary conservation and its putative role as housekeeping gene, we have decided to gain insight into the role of *c16orf35* both as a new putative interactor of p53-family proteins, and as a novel protein of unknown function.

Characterization of nuclear *C16ORF35* protein product. We show that both endogenous and overexpressed *c16orf35* localizes in nucleus and cytoplasm. In the nucleus it associates with the nuclear matrix. In particular it is able to bind SATB-1, a protein that associates with particular DNA regions called matrix attachment regions (MAR). SATB-1 organizes chromatin into distinct loops by periodic anchoring of MARs to the nuclear matrix. SATB-1 is able to modulate gene expression by modulating chromatin architecture and by interacting with chromatin modifiers (such as corepressor complexes) (Alvarez et al., 2000; Cai et al., 2006; Dickinson et al., 1997; Kumar et al., 2007; Ramakrishnan et al., 2000; Seo et al., 2005; Yasui et al., 2002). Another important *c16orf35* feature is its ability to associate with PML nuclear bodies. Indeed the overexpression of PML IV is able to strongly relocalize *c16orf35* into these particular regions. Nuclear bodies are subnuclear regions where chromatin loops converged and are transcriptional regulated (Bernardi and Pandolfi, 2007). The composition of PML nuclear bodies is highly heterogeneous and PML itself is a dynamic component of PML nuclear bodies and SATB-1 itself is able to associate with these structures, where it is required for the correct assembly of DNA loops into nuclear bodies (Kumar et al., 2007). Therefore also nuclear bodies are nuclear matrix associated structures. Of note both MARs and PML-NBs are sites where multiprotein complexes assemble into transcriptional factories to finely regulate the transcriptional process (Bernardi and Pandolfi, 2007; Cai et al., 2006; Seo et al., 2005; Yasui et al., 2002). It is possible that *C16ORF35* might interact with p53-family members within these nuclear compartments. In particular we found that *c16orf35* can bind TAp63α and TAp73α, and this is reflected by its ability to relocalize these two proteins into subnuclear domains. Of note *c16orf35* was not able to bind nor relocalize the tumor suppressor p53. As recruitment into nuclear bodies or MAR was shown to be sufficient to modulate transcription factors activity (Ben-Yehoyada et al., 2003; Jiang et al., 2001; Okorokov et al., 2002; Seo et al., 2005; Yasui et al., 2002), it is possible that *C16ORF35* might affect the activity of p63 or p73 on specific promoter targets. We tested this hypothesis using transient luciferase assays, but were unable to detect any *c16orf35*-dependent alteration of p63 or p73 transcriptional activity. This, of course, doesn't means that *c16orf35* is unable to modulate p63 and p73 response. Indeed there could be several technical bias in this kind of assay. During transient transfection experiments, plasmids may not be fully wrapped into nucleosomes, nor

entered into a proper chromatin tridimensional structure. Therefore chromatin remodeling and the assembly of large multiprotein complexes could be inefficient and transcriptional modulation abilities could be not revealed. It will be necessary to generate a stable *c16orf35*-inducible cell line, to evaluate if p73 or p63 dependent gene expression is altered by *c16orf35* overexpression and how this might influence cell fate under conditions of p73/p63 activation. Our BrdU incorporation and colony formation experiments showed that *c16orf35* overexpressing cells undergo cell cycle arrest and do not proliferate. At the moment we cannot know whether this proliferation block could be p73 or p63-dependent; specific experiments will be required to test this possibility.

A transcriptional role of *c16orf35* is anyway suggested by its effect on GATA-1 activity. As *c16orf35* knock-out mice die *in utero* for severe anemia and display strong dyserythropoiesis (Anguita et al., 2002), we have tested its role as GATA-1 transcriptional modulator. GATA-1 is the master regulator of erythropoiesis, and perturbing GATA-1 activity or stability induces strong developmental defects that lead to a fatal anemia (Pevny et al., 1991; Whyatt et al., 2000). Here we found that *c16orf35* can bind GATA-1, and can strongly repress its transcriptional activity in transient luciferase assays. It is tempting to speculate that this repression is mediated through the recruiting of repressor complexes , histone deacetylases, with which *c16orf35* efficiently interacts (see Fig.15). It will be interesting to investigate more deeply if *c16orf35* affects GATA1 activity during erythropoyesis, and if *c16orf35* is able to influence GATA-1 acetylation and/or ubiquitination, two processes that are tightly linked each other (Hernandez-Hernandez et al., 2006).

Characterization of cytoplasmic *C16ORF35* protein product.

Beside its nuclear localization and the ability to interact with a variety of transcriptional modulators, *c16orf35* displays a peculiar cytoplasmic distribution. In approximately 30% of the cells where the protein is cytosolic, it forms regular round dots variable in number and size. A subset of these cytoplasmic dots colocalize with TIA-1, indicating in fact that they are stress granules (SG). Stress granules are particular cytoplasmic regions that function as “triage centers” that sort, remodel, and export specific mRNAs for reinitiation, decay or storage (Anderson and Kedersha, 2006). Stress granules are not present in the cytosol of healthy cells growing under favorable conditions. They are rapidly induced by a variety of environmental stress

included UV, heat shock and hypoxia (Anderson and Kedersha, 2006). Stressed cells respond to environmental stresses by adapting mRNAs metabolism. During this process the translation of mRNAs for housekeeping genes is stalled whereas the translation of mRNAs encoding chaperones is enhanced. Stress granules are induced upon stress-activated kinases (PKR, GCN-2) dependent eIF2α phosphorylation. This prevents the assembly of the 48S pre-initiation complex, and polysome disassembly. Non-polysomal transcripts are then reorganized into stress granules, a process mediated by different RNA binding proteins (Anderson and Kedersha, 2006; Kedersha et al., 2002). Several RNA binding proteins act downstream of eIF2a. In particular, TIA-1 and the ras-gap SH3-binding protein G3BP-1 promote assembly of SG by combining their RNA binding activity with a process of self-aggregation (Kedersha et al., 1999; Tourriere et al., 2003). Accordingly, overexpression of one of these - proteins is a specific way to induce stress granules assembly in the absence of cellular stress. We show that *c16orf35* is strongly recruited into SGs induced by G3BP-1 overexpression. Moreover *c16orf35* is able to bind G3BP-1. These data show that *c16orf35* can enter stress granules, and can interact with the RNA-binding proteins that assemble these structures.

More importantly, we found that overexpression of *c16orf35* is able to induce stress granules formation in U2OS cells. even if reduced in shape and size compared with G3BP-1overexpressing cells (data no shown).

It is important to emphasize that not all *c16orf35* cytoplasmic dots are stress granules. There are in fact round *c16orf35*-positive cytoplasmic dots that are negative for TIA-1 but are closely associated with SGs. This staining resembles that of processing bodies, cytoplasmic structures involved in mRNA degradation and miRNA processing that are able to associate with stress granules (Anderson and Kedersha, 2006). Processing bodies contain components of the 5′-3′ mRNA decay machinery, the non-sense mediated decay pathway and the RNA-dependent silencing complex (Anderson and Kedersha, 2006). Processing bodies are physiologically present in healthy cells, where they behave as highly motile cytoplasmic structures whereas stress granules are relatively fixed (Kedersha et al., 2005). In stressed cells processing bodies can interact with stress granules. They are engaged and fixed around stress granules and mRNAs can be delivered from stress granules to processing bodies for degradation (Kedersha et al., 2005). No reagents are currently available to detect endogenous components of P bodies; therefore, to mark

processing bodies we transfected two proteins involved in micro RNA processing: Argonaute-1 and Argonaute-2.
Immunofluorescence experiments demonstrated that overexpressed *c16orf35* does indeed co-localize with overexpressed Ago-1 and Ago-2. Immunoprecipitation experiments showed that *c16orf35* can bind both AGO proteins, providing a mechanism for co-localization into these structures.

Considering these data, we can conclude that *c16orf35* is able to interact with two cytoplasmic compartments that play essential roles in translation, processing and stability of messenger RNAs, both during healthy and stressed conditions.

Perhaps the most relevant observation is that *c16orf35* can induce formation of stress granules when overexpressed in human cells. We ignore if *c16orf35* induces stress granules directly or indirectly. It is possible that overexpression of *c16orf35* indirectly induces some kind of cellular stress that ultimately leads to SG assembly. However *c16orf35* can localize to these structures and binds G3BP-1, one of the main proteins involved in stress granules assembly. We therefore favored the hypothesis that *c16orf35* is a novel component of both stress granules and processing bodies and that it is able to assemble these structures by interacting with other resident proteins.

Given the relevance in controlling mRNA translation and turnover, it is conceivable that formation of SGs influences cell homeostasis. Very intriguingly the strong reduction of BrdU incorporation observed in *c16orf35*-overexpressing cells could be dependent on its role as stress granules inducer. By inducing SGs, *c16orf35* could modulate expression of cell-cycle related or cytokines genes at the post-transcriptional level thus influencing cell growth. Indeed it was just observed that some stress granules components, such as TIA-1, are able to repress mRNA translation (Piecyk et al., 2000).

Analysis of endogenous *c16orf35*. According to the sequences deposited in public databases, the *C16orf35* gene gives rise to a variety of differently spliced mRNAs; in particular, at least two different translation products are predicted form these cDNAs. The two different protein isoforms have predicted molecular weight of 63kDa and 43kDa respectively. The shorter 43kDa isoform starts from an internal AUG and lacks approx. 100 N-terminal aminoacids with respect to the putative 63kDa isoform.

We have generated a polyclonal antibody that recognizes all predicted isoforms. Using this reagent we have noted that in several human cell lines the 43kDa isoform cannot be detected. On the contrary, the 60kDa isoform can be detected in most cell lines tested.

Our results using this antibody fully support the notion that endogenous *c16orf35* localizes in nucleus and cytoplasm. In particular, cytoplasmic *c16orf35* localizes in small rounded dots and is efficiently recruited into AGO-1 positive processing bodies, demonstrating its ability to enter this structures

In conclusion, our data suggests that, at least in the cell lines analyzed, only one *c16orf35* isoform of apparent molecular weight of 60kDa exists. In U2OS cells, endogenous *c16orf35* localizes in sites were mRNAs are either synthesized, or post-transcriptionally modified

CONCLUSIONS AND PERSPECTIVES

In this work we have presented several novel findings that characterize the *c16orf35* protein product as a p63 and p73 interacting protein that is also able to enter specific cellular structures were RNAs are stored, modified or degraded. In particular its ability to associate with the nuclear matrix and histone deacetylases, as well as with cytoplasmic stress granules and processing bodies suggests that it may be able to associate with RNAs along their entire maturation process from nucleus to cytoplasm. There are no defined RNA-binding domains in the primary sequence of *c16orf35*, but it will be interesting to investigate if *c16orf35* is per se an RNA-binding protein and if it would co-purify with active or stalled polysomes.

c16orf35 overexpresion is able to inhibit proliferation of osteosarcoma-derived U2OS cells. It is tempting to speculate that *c16orf35* inhibits cell cycle by inducing formation of SGs, and affecting global mRNA translation. At the same time, it is possible that *c16orf35* overexpression activates a p73 or p63 dependent pathway which in turn affects cell cycle progression. These are the two primary hypothesis that will drive future experiments.

We will shed light on these alternative hypothesis by performing siRNA experiments. It will be also interesting to evaluate if translation of specific mRNAs is perturb and also better characterize *c16orf35*-dependent cell growth inhibition.

C16orf35 ability to modulate also GATA-1 dependent transcriptional activity suggests that it is able to enter nuclear multiprotein complexes and modulate transcription as just shown in yeast for Argonaute-1. Indeed AGO-1 is able to associate with specific DNA regionsand inhibit transcription depending on its ability to associate with specifics RNAs (Irvine et al., 2006; Janowski et al., 2006; Kim et al., 2006; Sigova et al., 2004). The evolutionary conservation level observed for *c16orf35* argue for an important role in cell homeostasis and favor an "ancient" way to modulate transcription/translation such as that observed for AGO-1. Indeed it is tempting to speculate that before the appearance of sequence-specific transcription factors a way to redundantly regulate both transcription and translation was tethering (RNA binding) protein complexes on specific promoters/mRNAs by using specific small RNAs. Therefore RNA binding proteins could represent a foundamental connection to control both transcription and translation.

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Considering that several peoples that I want to acknowledge are not "familiar" with foreign languages, the coming lines will be written in Italian. I apologize for that.

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