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DOTTORATO DI RICERCA IN BIOLOGIA CELLULARE E MOLECOLARE

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“Effect of qualitative and quantitative alteration of ribosomal proteins on ribosome synthesis and cell metabolism”.

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SUMMARY

ABSTRACT

Ribosomal biogenesis is important for cell growth and proliferation. Perturbation of the synthesis and assembly of ribosomal components, as for instance inhibition of rRNA synthesis or decrease of ribosomal protein (RP) production, may lead to nucleolar disruption and to release and/or degradation of ribosomal component. In the last years, several studies suggested that p53 is activated in response to such a “ribosomal stress”, leading to growth arrest or apoptosis.Alteration of ribosome synthesis or function is implicated in several diseases, in which mutation in the genes coding for RP or other nucleolar components may cause defect in ribosome biogenesis. We have been studying the molecular mechanisms of Diamond-BlackfanAnemia (DBA), a congenital hypo plastic anemia associated to various physical malformations. Mutations in *rpS19* gene have been identified in about 25% of patients whereas another 2% have mutations in the *rpS24* gene. A decrease in RPS19 and RPS24 level has been shown to cause a defect in the maturation of 18S ribosomal RNA. While the role of RPS19 mutations results well investigated and characterized, less clear is the effect of specific alterations in other RPs such as RPS24 or RPS7. Recently two new mutations of *rpS24* gene: a substitution and a deletion, are emerged from a study on DBA patients in Italy and one mutation has been found in a mouse model from a laboratory studying neuronal diseases. With the aim of analyzing the functional features of these new mutated RPS24, we prepared cDNA constructs. After transfection we analyzed the stability and the ability of exogenous mutated proteins to be assembled into mature ribosomes. Our results indicate that some RPS24 and RPS7 mutations are very unstable when compared with WT proteins. Yet, once stabilized, allelic variants analyzed in this study, even mutated, are able to be assembled into mature ribosome. In the last part of the project, we have studied erythroleukemia cell lines, TF-1C and myelogenous leukemia K562C, infected with a lentiviral vector inducible for the expression of siRNA specific for RPS19 mRNA. Parental cell lines present a fundamental difference: TF-1 express a wild type p53 whereas K562 do not show detectable levels of p53. In these cells we monitored the effect of ribosome alteration on the expression of selected proteins and on cell growth parameters. One of the proteins that we found affected by ribosome alteration is the oncogenic serine/threonine kinase PIM1, highly expressed in cells of hematopoietic origin.

INTRODUCTION

Ribosomal biogenesis depends on the proliferative status of the cells and is finely modulated along the cell cycle. Accordingly, the synthesis of ribosomal proteins (RPs) has been known for a long time as a process strongly linked to the growth status of the cell. Emerging evidences suggest that the perturbation of rRNA processing, synthesis or ribosomal proteins and ribosomal assembly causes the activation of a specific control mechanism in the cell: the "Ribosomal stress". In the last years, several studies suggested that p53 is activated in response to such a cellular activation, leading to growth arrest or apoptosis. Alteration of ribosome synthesis or function is implicated in several diseases, in which mutation in the genes coding for RPs or other nucleolar components may cause defect in ribosome biogenesis, such as Dyskeratosis Congenita (DC), Cartilage Hair Hypoplasia (CHH), Treacher Collins Syndrome (TCS) and Diamond Blackfan Anemia (DBA). For several years, in our laboratory, we studied the molecular mechanisms of Diamond-Blackfan Anemia (DBA), a congenital hypoplastic anemia associated to various physical malformations. Mutations in RPS19 gene have been identified in about 25% of patients whereas another 2% have mutations in the RPS24 gene and a fewer percentages in the ribosomal protein L5, L11, L35a, S17 and S7. A decrease in RPS19, RPS24 and RPS7 levels has been shown to cause a defect in the maturation of 18S ribosomal RNA even if at a different level for the different proteins. While the role of RPS19 mutations results well investigated and characterized, less clear is the effect of specific alterations in RPS24 and RPS7 sequence. Recently two new mutations of *rps24* gene: a substitution and a deletion, have been identified from a study of Irma Dianzani's lab on DBA patients in Italy. In addition, a mutation in the *rps7* gene has been identified in a mouse model for neuronal alterations, the *Montu mouse*, by David Keyes at Oxford University. To study the mechanism underlying Ribosomal stress generated by mutations in RPs, we. we have focused our attention on the study of the two new RPS24 mutations and on the characterization of RPS7 mutation from the *Montu mouse*. In particular we tested the properties of mutated RPS24 and RPS7 with different experimental approaches. In addition we tried to clarify the downstream effects of RPs mutations, both in experimental models and in cells from patients affected by DBA. For this last part of the project, we have studied erythroleukemia cell lines, TF-1C and myelogenous leukemia K562C, infected with a lentiviral vector inducible for the expression of siRNA

specific for RPS19 mRNA. Parental cell lines present a fundamental difference: TF-1 express a wild type p53 whereas K562 do not show detectable levels of p53. In these cells we monitored the effect of ribosome alteration on the expression of selected proteins and on cell growth parameters. One of the proteins that we found affected by ribosome alteration is the oncogenic serine/threonine kinase PIM1, highly expressed in cells of hematopoietic origin. PIM1 kinase has been recently identified as an interactor of RPS19. To facilitate the understanding of the results I will briefly describe the cellular compartments where ribosomal biogenesis takes place and the mechanism that leads to ribosomes' maturation. Then I will describe some human pathologies that are believed to be caused by alteration related to this particular kind of stress and some characteristics of the oncogenic serine/threonine kinase PIM1 that we found affected by ribosome alteration.

1. RIBOSOMAL SYNTHESIS

1.1 Ribosomes and nucleolus

The ribosomes are complexes of RNA and proteins. They are part of the mechanism that translates DNA into the aminoacidic sequence. Ribosomes from bacteria, archaea and eukaryotes, have significantly different structure and RNA. The ribosomes in the mitochondria of eukaryotic cells resemble those in bacteria, reflecting the evolutionary origin of this organelle. The ribosomal subunits of prokaryotes and eukaryotes are quite similar. Prokaryotes have 70S ribosomes, each consisting of a small (30S) and a large (50S) subunit. Their large subunit is composed of a 5S RNA subunit (consisting of 120 nucleotides), a 23S RNA subunit (2900 nucleotides) and 34 proteins. The 30S subunit has a 1540 nucleotide RNA subunit (16S) bound to 21 proteins. Eukaryotes have 80S ribosomes, each consisting of a small (40S) and large (60S) subunit. Their large subunit is composed of a 5S RNA (120 nucleotides), a 28S RNA (4700 nucleotides), a 5.8S subunit (160 nucleotides) and ~49 proteins. The 40S subunit has a 1900 nucleotide (18S) RNA and ~33 proteins. The role that ribosomes play in the cell is fundamental: they decode the message held by mRNA and allow the elongation of the aminoacidic chain forming the peptidic bond. The ribosome consists of two subunits: the small (30S in bacteria and 40S in eukariotes) and the large (50S in bacteria and 60S in eukariotes). While the

decoding functions resides in the small subunit, the duty of catalyzing peptidic bond is up to the large one. In eukariotyc cells the production of mature ribosomes is localized in different cellular compartments, in particular the final steps of maturation happen in the nucleus and in the cytoplasm. The site of ribosomal subunit biogenesis in eukaryotic cells are nucleoli: a non membrane bound structure composed of protein and nucleic acids. Nucleoli form at the end of mitosis around the clusters of ribosomal DNA (rDNA) genes and result in a subnuclear compartment that locally condenses the transcriptional and processing machineries that are involved in the ribosomal subunit genesis (Fig. 1) .The steps in the process of ribosomal subunit assembly requires the initial transcription of rDNA genes by a specific polymerase: RNA polymerase I. The rDNA is arranged in clusters repeated in tandem, termed Nucleolar Organizer Region (NORs). These repeated units comprise internal transcribed spacers (ITS), containing the sequences of mature rRNA (28S, 18S and 5,8S) separated by intergenic spacers (ETS1 and ETS2). In human cells there are approximately 400 transcriptional units for rRNA localized on chromosome 13, 14, 15, 21 and 22 (Raska et al., 2004). The initial product of transcription in mammals is the 47S rRNA, conserved along the whole eukariotyc evolution, that undergoes cleavage to form the mature products: 28S, 18S and 5,8S. These rRNAs are then transcriptionally modified through interaction with small nucleolar ribonucleoproteins (snoRNPs) and others protein processing factors . Finally the mature rRNAs are assembled with the many ribosomal proteins before interacting with the export machinery and to be translocated to the cytoplasm. The processes involved in the formation of ribosome subunits occur in distinct subregions of the nucleolus. These regions, analyzed with electron microscopy and also clearly visible by light microscopy are termed: fibrillar centres (FCs), dense fibrillar components (DFCs) and granular components (GCs). Bibliographic and bioinformatic data from proteomic studies have allowed the classification of nucleolar proteins into functional groups and have suggested potential functions for 150 previously uncharacterized human proteins (Boisvert et al., 2007) (Fig. 1).

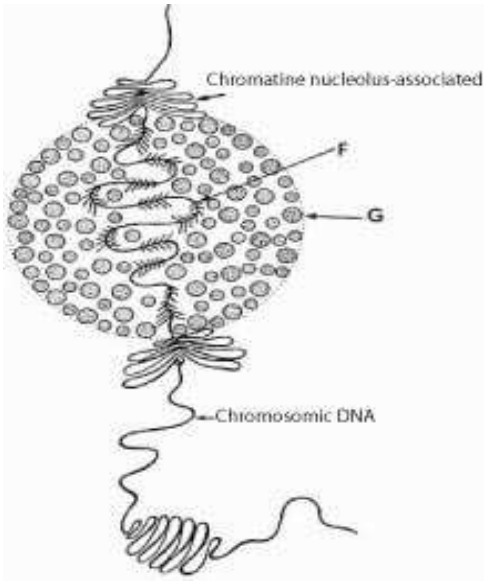


Fig.1) Schematic representation of nucleolus. F= Fibrillar Centres, G= Granular components. From: www.summagallicana.it/Volume2

It has been shown that approximately 30% of these proteins have functions related to the production of the other ribosomal components. However the diverse identities and sites of many of the other nucleolar proteins are consistent with additional processes that occur within the nucleolus, this includes many pre-mRNA processing factors and proteins that are involved in cell cycle control as well as DNA replication and repair. The ability of analyzing the parallel increase and decrease in the levels of many protein component quantitatively and in a hightroughput manner has highlighted just how dynamic the nucleolar proteome could be (Boisvert et al., 2007).

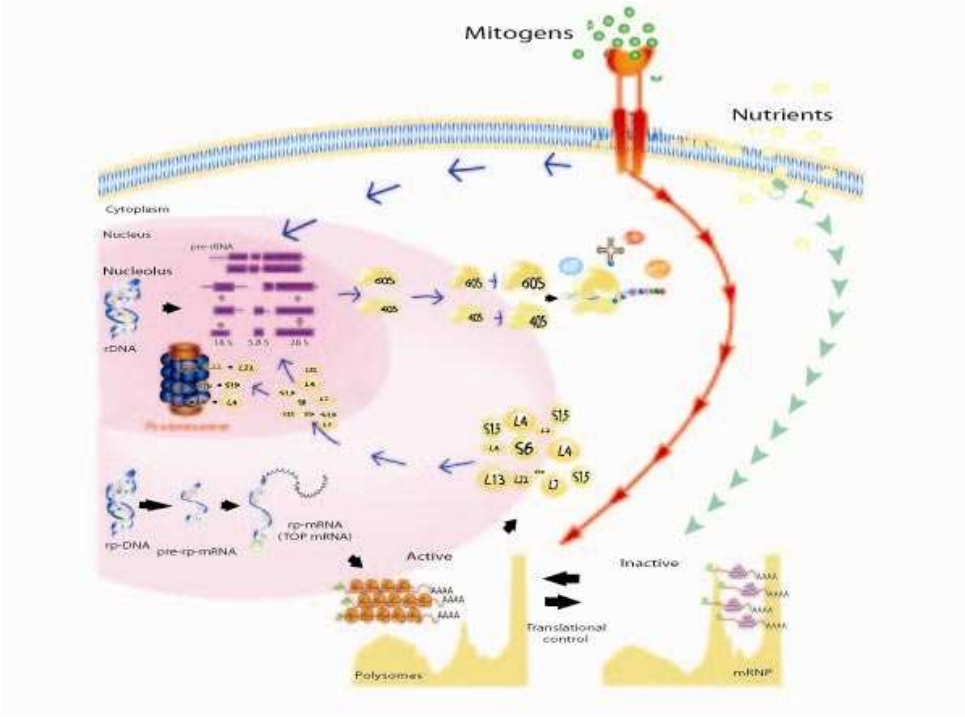


Fig.2) The control of ribosome biogenesis and translation is fundamental for cell survival.

1.2.The rRNA maturation.

The majority of the ribosomal subunits production takes place within the nucleolus: here pre-rRNAs are processed and assembled with the ribosomal proteins imported from the cytoplasm. At some point during this process, the newly synthesized ribosomal subunits must exit the nucleus and reach the cytoplasm to ensure translation of mRNAs. Nuclear export must be tightly coordinated with pre-rRNA maturation. The mechanism underlying the nuclear export and its coordination with pre-ribosome maturation remains largely unknown. Pre-rRNA maturation starts with the large 80-90S RNP particle which splits into the pre-40S and pre-60S particles after cleavage within the internal transcribed spacer 1 (ITS1) at site A2 (Fig.3). (Rouquette et al., 2005).

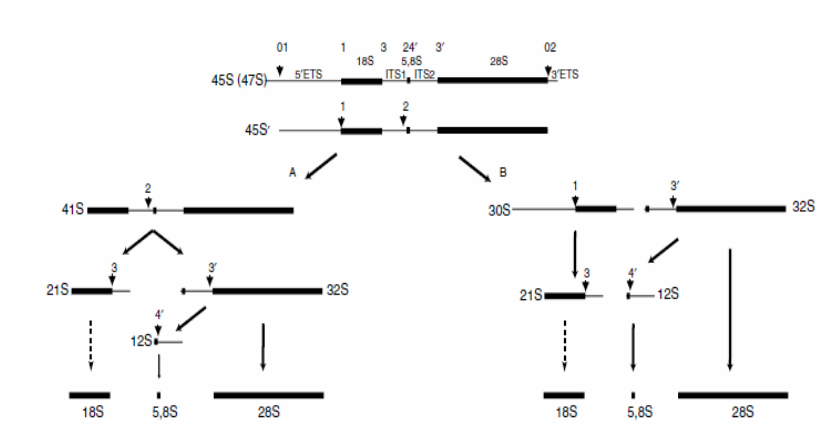


Fig 3) Pre-rRNA processing: schematic nomenclature according to Hadjiolova et al. (1993). Two alternative pathways are presented.

Analysis of rRNPs precursors in yeast has revealed that their composition is highly dynamic and involves roughly 150 non ribosomal proteins including nucleases, RNA helicases, GTPases, kinases etc (Fatica et al., 2002; Milkereit et al., 2003). Most striking was the discovery that the two subunits follow independent biogenesis pathways, with very little overlap (Leger-Silvestre et al., 2004). During rDNA transcription, a large complex containing the U3 snoRNP and proteins involved in the cleavage of the ETS1 binds to the 5'-end of the neo-synthesized pre-RNA (Léger-Silvestre et al., 2004). This complex, the small subunit processome, seems to be released after cleavage of the pre-rRNA at site A2, from which point the fate of the two subunits becomes distinct (Léger-Silvestre et al., 2004). Many of the factors involved in ribosome biogenesis have homologues in vertebrates and some have found in mammalian pre-ribosomes (Rouquette et al., 2005). The main steps that lead to the production of the mature 18S, 5.8S and 28S rRNAs in vertebrated have been defined. Some of these steps have been thoroughly studied using both *in vitro* and *in vivo* assays, others are still poor characterized (Borovjagin and Gerbi, 2004). The overall scheme of the process is comparable to that established in more detail for yeast *Saccharomices cerevisiae* (Venema and Tollervey, 1999). However the sequences of the ETS and ITS diverge greatly from one eukariot to the other so that it is difficult to predict the cleavage sites by direct sequence comparison (Rouquette et al., 2005). In vertebrates the order of endo- and exonucleolytic cleavages, that eliminate the transcribed spacers, seems to vary according to species, cell type or physiological conditions (Gerbi and Borovjagin, 2004). In vertebrates, although the assembly of the ribosomal subunits is only completed in the cytoplasm by addition of the last ribosomal proteins, it is widely assumed that processing of the pre-ribosomal transcripts into mature 18S, 5,8S, 28S rRNAs takes place entirely into the nucleolus (Rouquette et al., 2005). In contrast, in *S. cerevisiae*, the final maturation process of the small subunit occurs in the cytoplasm and includes the last cleavage at the 3' end of the 18S rRNA (Vanrobays et al., 2001). This step, whose mechanism remains to be defined, requires the participation of various factors associated with the pre-40S particles, including the "Rio" proteins (Vanrobays et al., 2001). In 2005 the group of Gleizes addressed the nuclear transport of the pre-40S particles and the coordination of this process with the final steps of pre-18S rRNA maturation in mammalian Hela cells. They showed that the cytoplasmic maturation of the 18S rRNA is not an

exception restricted to yeast but also takes place in mammalian cells. Nuclear export of these pre-40S particles depends mainly on the human ribosomal protein Rps15 and other related factors. These data suggested that despite a strong divergence in the sequence of the transcribed spacers and differences in pre-rRNA processing, the localization of the final cleavage of the pre-18S rRNA within the cytoplasm is a common feature in eukariotes, probably to prevent the premature translation initiation on pre-mRNAs within the nucleus (Rouquette et al., 2005).

2. RIBOSOMAL PATHOLOGIES

2.1 The Diamond-Blackfan Anemia (DBA)

While there is a high level of conservation between the components of ribosome biogenesis in yeast and mammals, studies of mammalian ribosome production are still in their infancy. However the interest in mammalian ribosome biosynthesis has been recently stimulated by the finding that pathogenesis of a number of inherited and acquired diseases involves mutations in genes encoding components of the ribosome or proteins involved in ribosome maturation, assembly or export. Diamond Blackfan Anemia (DBA; OMIM #205900) was first reported by Josephs (1936) and refined as a distinct clinical entity by Diamond and Blackfan in 1938 (Vlachos et al., 2008). DBA is a member of a rare, genetically and clinically heterogeneous, inherited cell aplasia (Vlachos et al., 2008). It is now accepted that the disorder results from a cellular defect in which erythroid progenitors and precursors are highly sensitive to death by apoptosis, leading to erythropoietic failure (Miyake et al., 2008). The syndrome is caused by mutation in several genetic loci, all of them coding for r-proteins in both the small and large ribosomal subunits (Cmejlova et al., 2006; Draptchinskaia et al., 1999; Farrar et al., 2008; Gazda et al., 2006) (Tab.1). The first DBA gene, mutated in approximately 25% of patients, has been cloned and was identified as *rps19*, which codes for a ribosomal protein located at chromosome 19q13.2 (Gustavsson et al., 1997; Draptinchinskaia et al., 1999). The function of RPS19 protein in ribosome biogenesis is poorly understood. The identification of a ribosomal protein as the cause of DBA was unexpected given that the clinical symptoms of most patients are

confined to erythropoiesis and in some cases to some organs during embryogenesis. However the involvement of RPS19 gene in DBA has been verified sequencing the DNA of different affected individuals. Several studies have detected more than 60 different types of mutations in this gene (missense, nonsense, insertions, deletions and splicing defects), (Proust et al., 2003; Ramenghi et al., 2000; Willig et al., 1999). Missense mutations altering the nucleolar localization sequence of the protein not only lead to the loss of localization of the protein in the nucleolus but also to a drastic decrease in the cellular protein levels (Da Costa et al., 2003). A decrease in the levels of ribosomes into the cell or a diminished ribosome synthesis or ribosomes defective in their function have been proposed as possible consequences of such a failure in nucleolar localization of RPS19. It has been further demonstrated that RPS19 is required for a specific step in the maturation of 40S ribosomal subunit in yeast. Removal of RPS19 caused a decrease of the 40S subunit and thereby affected the translational capacity of the yeast cells (Leger-Silvestre 2005) . What these results do not clarify yet is how deletion of RPS19 does affect selectively the hematopoietic cell lineage. One possible explanation is that the erythroid precursors are more sensitive to defects that alter the translational apparatus because of their high demand for protein synthesis (Flygare et al., 2005). Other studies have demonstrated the repair of defective hematopoiesis by increased RPS19 protein expression in both RPS19-deficient patient-derived progenitors (Hamaguchi et al., 2002) and in RPS19 knockdown cellular models (Flygare et al., 2005). To identify other gene(s) involved in DBA, Gazda et al in 2006 performed a genomewide linkage screen and subsequently sequenced candidate genes (Gazda et al., 2006). They reported that another RP gene: RPS24 was mutated in 2% of probands with DBA. A total of 215 families participated in the study. They found evidence favouring linkage of DBA phenotype to a region on chromosome 8q and to regions on chromosomes 10 and 6. They focused their attention on RP genes *rps20* and *rpl7,* present in the critical region on chromosome 8q and on *rps24* located in the linked region on chromosome 10. Sequence results for *rps20* an *rpl7* were normal, in contrast, they found heterozygous nonsense mutation in exon 4 of *rps24.*The human *rps24* gene includes six exons that encode an RP that is a component of the 40S ribosomal subunit (14). Xu and colleagues found that human *rps24* encodes RPS24 protein isoforms a and c, of length 130 and 133 aa, respectively, as a result of alternative 3’-end splicing into mRNA variants 1 and 2 (fig. 2a) (Xu and Roufa, 1996). These variants show tissue-specific differences in expression pattern. In 2006 Gazda found a third

mRNA variant encoding a 131-aa isoform identical to the murine RPS24 isoform 3. The results showed the presence of this novel variant in several human tissues (fetal and adult brain, skeletal muscle and heart) and the correspondent lack of variant 2. Moreover Choesmel and collaborators showed that cells from patients carrying mutations in RPS24 have defective pre-rRNA maturation, as in the case of RPS19 ones (Choesmel et al., 2008). However, in contrast with RPS19 involvement in maturation of the internal transcribed spacer 1 (ITS1), RPS24 is required for processing of the 5' external transcribed spacer (5'-ETS). Remarkably, epistasis experiments with siRNAs indicate that the functions of RPS19 and RPS24 in pre-rRNA processing are connected. Resolution of the crystal structure of RPS24e from the archeon *Pyroccocus abyssi* reveals domains of RPS24 potentially involved in interactions with pre-ribosomes. Robledo in 2008 investigated the role of 15 r-proteins in ribosome biosynthesis and rRNA processing, including six r-proteins that are currently known to be affected in DBA (RPS17, RPS19, RPS24, RPL5, RPL11, and RPL35a). This group found that depletion of r-proteins involved in the pathogenesis of DBA may affect either the subunit but always associates with a decrease in the production of ribosomes (Robledo et al., 2008). They concluded that any of the r-proteins, when mutated, compromises ribosome biogenesis and represents a potential candidate for DBA. The common factor is the decreased production of ribosomes rather than a specific step in ribosomal biogenesis or the accumulation of a specific rRNA or ribosome subunit precursor (Robledo et al., 2008). In 2008 Gazda and collaborators, reported the results of a large scale screening in a cohort of DBA probands. They retained having identified probable new pathogenic mutations in four of these genes: RPL5 (MIM603634), RPL11 (MIM 604175), RPS7 (MIM 603658) and RPS17 representing rughly 16% of their patient cohort. In addition they reported possible single mutations in three other RP genes, RPL36, RPS15 (MIM 180535) and RPS27 (MIM 191343). In contrast to previously data on patients with RPS19 mutations, mutations in RPL5 are associated with multiple physical abnormalities, including craniofacial, thumb and heart anomalies. Isolated thumb abnormalities are predominantly present in patients carrying mutations in RPL11. They also demonstrated defective rRNA maturation in RPL5, RPL11 and RPS7 mutated DBA cells from patients (Gazda et al., 2008).

2.2 Other ribosomal diseases

DBA is not the only ribosomal disease known so far but it fits into a framework of diseases associated with mutations of genes involved at various levels in the biogenesis of the ribosome (Tab. 1). Many of the genes identified play a role in the rRNA maturation. These diseases have as their common feature the presence of physical malformations caused by defects in development.

The Dyskeratosis Congenita is a hereditary syndrome that affects the bone marrow and shows a significant clinical and genetic heterogeneity (Dokal and Luzzatto, 1994). Two forms of pathology are known: X-linked recessive and autosomal dominant or recessive. The gene responsible for the X-linked recessive variant encodes a nuclear protein highly conserved call dyskerin (DKC1) (Meier and Blobel, 1994; Phillips et al., 1998). The dyskerin is associated with both telomere complex and with the class of snoRNA H / ACA that guide pseudourylidation of ribosomal RNA. Experimental data show that mutant mice hypomorphic for DKC1 have a defect in rRNA pseudourylidation indicating that the deregulation of ribosome biogenesis is involved in the onset of the disease (Gu et al., 2009; Ruggero and Pandolfi, 2003).

The Treacher Collins syndrome (TCS) is a congenital disorder of craniofacial development arising from mutations of TCOF1, which encodes the nucleolar phosphoprotein Treacle. Haplinsufficiency of TCOF1 perturbs mature ribosome biogenesis, resulting in stabilization of p53 and the cyclin G1- mediated cell cycle arres that underpins the specificity of neuroepithelial apoptosis and neural crest cell hypoplasia characteristic of TCS (Jones et al., 2008).

Patients with the Chromosome 5q deletion syndrome (or 5q Syndrome) present the loss of part of the long arm (q arm, band 5q31.1) of human chromosome 5.In particular has been demonstrated haploinsufficiency of the ribosomal gene RPS14, which is required for the maturation of 40S ribosomal subunits and maps to the commonly deleted region, in the 5q-syndrome Related to this genetic defect are alterations in the expression of genes involved in ribosome biogenesis and in the control of translation suggesting that the 5q syndrome represents a disorder of aberrant ribosome biogenesis (Pellagatti et al., 2008).

Cartilage-hair hypoplasia (CHH) is an autosomal recessive inherited disorder. A rarely encountered genetic phenomenon, known as uniparental disomy (a genetic circumstance where a child inherits two copies of a chromosome from one parent, as opposed to one copy from each parent) has also been observed with the disorder. Cartilage-hair hypoplasia has an autosomal recessive pattern of inheritance. An association between mutations near or within the ncRNA component of RNase MRP, RMRP, has been identified (Ridanpaa et al., 2001).

Disease	Gene	Main clinical feature	Other clinical phenotype
Diamond-Blackfan anemia (DBA)	RPs	Hypoplastic anemia	Physical anomalies
Dyskeratosis congenita (DC)	DKC1 (Dyskerin)	Abnormal skin pigmentation, nail dystrophy	Bone marrow failure
Cartilage-Hair Hypoplasia (CHH)	RMRP (RNA component of MRP)	Short stature, hypoplastic	Hypoplastic anemia
Shwachman-Diamond syndrome (SDS)	SBDS (nucleolar protein)	Bone marrow failure, pancreatic insuff	Bone defects
Treacher Collins syndrome (TSC)	TCOF1 (nucleolar protein)	Disorder of craniofacial development	Other deformities

Tab1) Ribosomal diseases. Listed are the main known ribosomal syndromes in humans.

3. PIM1

To clarify the molecular mechanism of DBA some experiments have been conducted in order to identify putative interactors of RPS19 protein. Among these studies, in particular, in our laboratory, in collaboration with the laboratory of prof. Irma Dianzani at University of Eastern Piedmont, by yeast two-hybrid screening we identified Pim kinase as an interactor of RPS19 (Chiocchetti et al., 2005). PIM1 belongs to the family of calcium-dependent calmodulin with PIM2 and PIM3. All three isoforms of the protein are highly conserved in vertebrates and show each other 53% homology in amino acid sequence. *Pim 1* gene (for MuLV proviral integration site 1) that was originally identified as a preferred site of proviral insertion of Moloney murine leukemia virus (MoMuLV) (Cuypers et al., 1984), maps at the fragile site on chromosome 6p21. As a result of viral insertion were found high levels of expression of PIM1, consequently to this evidence it was suggested a role for PIM1 in malignant transformation predisposing to this form of leukemia. The exogenous and constitutive expression of Pim1 in lymphocyte cells of transgenic mice, predisposes the animal to the development of lymphomas. This transformation is particularly evident in the presence of activation of a second oncogene such as c-Myc (van Lohuizen et al., 1989).

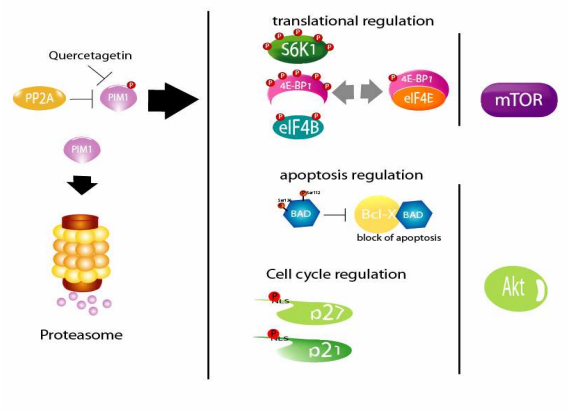


Fig 4). PIM1 pathway

3.1 Structural features.

The gene coding for PIM1 in humans is composed of six exons and five introns. The sequence of the promoter is rich in GC and does not contain a TATA box, characteristic of the promoters “house keeping” (Meeker et al., 1987). The transcript shows, in regions not translated to the 3’ UTR, an AU rich sequence that causes destabilization of mRNA. The messenger of PIM1 encodes two proteins of molecular weight of 34 kDa and 44 by using an alternative translation start site upstream of the canonical CUG codon AUG (Saris et al., 1991). On the other hand the role of 44 kDa isoform remains to be clarified, since it was detected only in some tissues and in a small proportion in comparison to the other isoform. The kinase catalytic domain includes the region between amino acids 38 and 290. The replacement of lysine 67 with methionine (L67M) inactivates the kinasic activity of PIM1. The average life of the kinase is very short: in peripheral blood leukocytes is approximately 10 minutes and up to 20 minutes in K562 cells (cells of chronic myeloid lukemia). Biological function and regulation of PIM1 is expressed at high level in the thymus, the spleen, the bone marrow but also in a large number of tumors of hematopoietic origin and in oral epithelial tumors of the pancreas, lung, prostate and colon. PIM is present in 38 cell lines showing high levels of expression in myeloid lines such as K562, U937, BV173, and lower in B cells while is not detectable in T lymphocytes. Several substrates of PIM1, involved in the regulation of growth and cell cycle (p21, p27, cdc25a, cdc25c) in apoptosis (Bad) and in the regulation of protein synthesis (S6K1, 4EBP1), have been identified. All these evidence suggest Pim1 as an important regulator of development and cellular homeostasis by acting directly on the survival and development of the cell. One of the first identified substrates of PIM1 was the adapter protein p100, transcriptional coactivator of c-Myc (Leverson et al., 1998). Different experimental evidences document the PIM1 cooperation with c-Myc in tumor transformation. Moreover the double-transgenic mice for c-Myc and PIM1 develops lymphoma already in utero and is not vital (Moroy et al., 1993). Among the substrates known to be phosphorylated by PIM1 there are cell cycle proteins cdc25a and cdc25c (Mochizuki et al., 1999), the protein p21 (Wang et al., 2001) and p27 (Morishita et al., 2008) (Fig. 5). Therefore the action of PIM1 could lead to cell cycle regulation both at the checkpoint G1 / S phosphorylating p27 and cdc25a and at the G2 / M checkpoint acting on cdc25c. For example when phosphorylated by PIM1 on residues T157 and T198, p27 undergoes export in the nucleus via 14-3-3 proteins. Once in the cytoplasm, p27 protein is degraded through proteasome (Fig. 5). Consistent with these findings, the overexpression of PIM1 in K562

cells is correlated with a decrease of half-life of the p27 protein and acceleration of the transition G1 / S. Several literature data indicate that the regulation of protein synthesis is under the control of the signal transduction pathway starting from the nutrient and leading to activation of PI3K, AKT and mTOR and to phosphorylation of S6K and 4E-BP1. In the Kraft's group has been highlighted that overexpression of PIM2 determines the phosphorylation of 4E-BP1 in the presence of rapamycin, a drug known to cause the inactivation of mTOR (Chen et al., 2005). PIM1 also seems to phosphorylate, after overexpression, the S6K1 kinase necessary for the activation of eEIF4B translational factor and the ribosomal protein RPS6. In this context PIM appears acting as a promoter of protein synthesis in substitution or in parallel with mTOR. In addition, phosphorylating different substrates the PIM kinase would be able to regulate and promote the general protein synthesis in the cell.

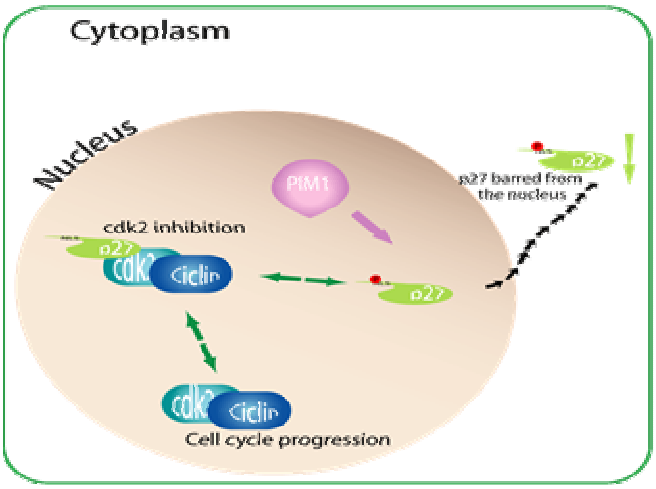
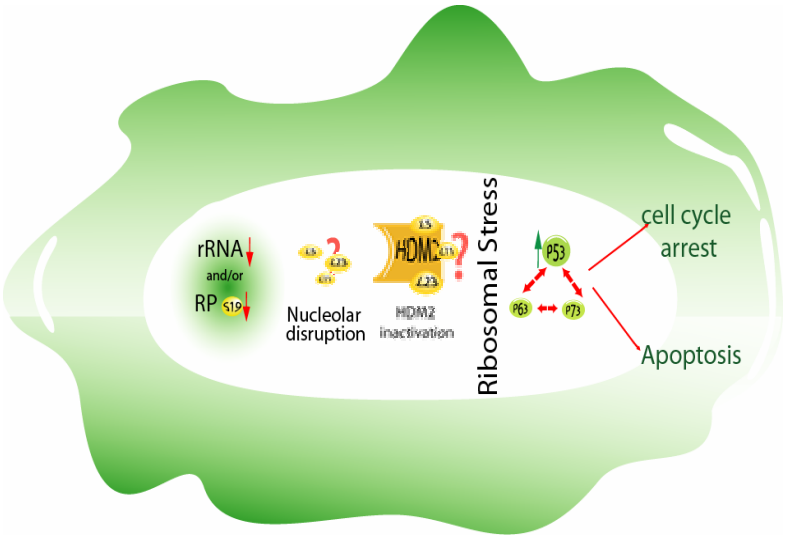


Fig. 5) Schematic representation of Pim 1 target p27/Kip1 pathway

4. THE RIBOSOMAL STRESS



Tab. 2). The proposed model for the Ribosomal Stress.

As previously asserted the nucleolus is the site of ribosomal biogenesis. Different evidences from experimental model systems have pointed on the possibility that the nucleolus integrity may act as a stress sensor able to provoke the cellular response blocking the proliferation and the cell cycle, and costimulate apoptosis. According to this model, after nucleolar disruption, some ribosomal proteins, (maybe RPL11, RPL5 and RPL23) would bind and inactivate HDM2: the protein responsible for p53 ubiquitination. This particular interaction could trigger the p53 activation followed by cell cycle arrest and/or apoptosis. In addition, the analysis of *in vivo* systems such as *Zebrafish* or mice with mutations of specific ribosomal proteins shows that their particular phenotype was associated to p53 overexpression. On the other hand, p53 inhibition both with genetic or pharmacological approaches, is able to soften or completely recover the effects due to ribosomal alterations. For instance mice carrying heterozigous mutations in *rps20* gene show haematologic phenotype and legs'

hyperpigmentation while the offsprings of RPS20 X p53 -/- imbreeding lead to a complete recovery of the phenotype (McGowan et al., 2008). Glaser and collaborators identified in 2004 belly spot and tail (*Bst*) in mouse, a semidominant mutation disrupting pigmentation, somitogenesis and retinal cell fate determination, as a deletion within the *rpl24* riboprotein gene. They assessed that *Bst* significantly impaired RPL24 splicing and ribosome biogenesis. In their cellular model *Bst*/+ cells possessed decreased rates of protein synthesis and proliferation, and were outcompeted by wild type cells in C57BLKS↔ROSA26 chimeras. Bacterial artificial chromosome (BAC) and cDNA transgenes corrected the mutant phenotypes (Oliver et al., 2004). The model for a p53-dependent Ribosomal stress is also supported by experiments in cultured cells treated with inhibitors known to cause disintegration of the nucleolus. Following the treatment the activation of p53 and the formation of the complex HDM2-RPs have been shown (Zhang et al., 2006). Also RPL11-deficient embryos from *Zebrafish* (morphants) displayed developmental abnormalities related to altered p53 activation. In this animal model the effect of RPL11 deficiency affected primarily the brain and it was reported to lead to embryonic lethality within 6–7 days post fertilization. In the head region of the morphants extensive apoptosis and also a dramatic decrease in total abundance of genes involved in neural patterning of the brain was observed, suggesting a reduction in neural progenitor cells. At the same time in this context, the researchers described the upregulation of the genes involved in the p53 pathway. Simultaneous knockdown of the p53 gene rescued the developmental defects and apoptosis in the morphants. These results once more give clues to the model that ribosomal dysfunction due to the loss of a RP activates a p53-dependent checkpoint response that, in this case, prevents improper embryonic development (Chakraborty et al., 2009). Understanding the regulation of genes encoding ribosomal proteins and the role that stress may have in the ribosomal phenotype of these animal models is helping researchers to clarify the mechanisms driving the cellular activation associated to Ribosomal stress. Maybe these findings will be exploited for the comprehension of the mechanism underlying human etiopathogenesis.

AIM OF THE PROJECT:

Since 2001 different clues have been accumulated about the existence of a molecular response specifically activated by the damage to components of ribosomal machinery. The whole of possible endogenous and/or exogenous factors causing impairment to the ribosomes or to their related components has been called: Ribosomal stress. The aim of this work has been to clarify, considering different point of views, the relationships among the ribosomal proteins and related factors, and the effect of their mutations on cellular metabolism, with a glance at the postulated Ribosomal stress. As a basis for our working model we have considered Diamond Blackfan Anemia (DBA), known to be the only human disease (together with 5q Syndrome) caused by mutation in a ribosomal protein (25% of patients with mutations in *rps19* gene) and studied since several years in our laboratory. By now it is ascertained that RPS19 is not the only ribosomal protein that, once mutated, may cause DBA: recent investigations have highlighted that roughly 2% of DBA cases are attributable to RPS24 mutations and another 2% are very rare mutations found in a lot of ribosomal proteins such as RPS7, RPS17, RPS19, RPS24,RPL5, RPL11, and RPL35a. Moreover it is interesting to note that some of mutated proteins found in the few percentage of DBA patients (RPL5 and RPL11) have been described as mediators of the hypothetical Ribosomal stress signalling pathway. In fact in physiological conditions the E3 Ubiquitin ligase HDM2 binds to p53 causing its degradation via proteasome. Yet in the hypotesis of nucleolar stress it has been speculated that some RPs may physically interact with HDM2 avoiding its inhibition on p53. However an exact and convincing model for the induction and the action of Ribosomal stress is quite far to be depicted. With the aim of characterizing some aspects of this cellular response, we followed different experimental approaches:

1) In collaboration with Irma Dianzani's laboratory (Università del Piemonte Orientale), we tried to understand the impact of two new RPS24 mutations found in a recent screening on Italian patients, on cellular metabolism of RPS24 possibly tracing evidences useful for a more detailed

knowledge of the syndrome's pathogenesis. Our studies evidenced that the mutations analyzed can affect protein stability resulting in a lower protein amount. However we found that the mutated proteins we can detect are correctly assembled into mature ribosomes.

2) Another branch of this research came from the collaboration of our lab with Dr. D. Keyes (University of Oxford), which is studying neural diseases and found its animal model to carry a substitution in *rps7* gene. This has been a further opportunity to investigate the alteration caused by a mutation of an RP (moreover found in some DBA cases). We found in brain and liver from this mouse model a marked alteration of rRNA processing in terms of precursor's accumulation in mutant mouse tissues.

3) In the last part of this project we have studied the downstream effects of alteration in the synthesis of ribosomal components. The current model predicts the binding and inactivation of HDM2, the p53 inhibitor, after nucleolar disruption. This particular interaction could trigger the p53 activating cell cycle arrest and/or apoptosis. This last line of research gave us the possibility to broaden the characterization of ribosomal stress pathway and the eventual involvement of p53. In particular we investigated the role of Pim1, a protein that interacts with RPS19 and that we demonstrated to act as a sensor for Ribosomal stress independently from or in concert with the known p53-mediated response.

RESULTS

1. ANALYSIS OF MUTATIONS IN RPS24 GENE.

In collaboration with our laboratory, Irma Dianzani and collaborators, in a screening of 92 Italian DBA patients negative for RPS19 mutations, have found two new heterozygous changes in RPS24 protein (2/92). A deletion of three nucleotides in exon 2 (64_66delCAA) resulting in the loss of the highly conserved glutamine 22 was identified in a patient who was transfusion-dependent at last follow up, without somatic malformations. This mutation was not found in other family members, nor reported as a polymorphism (dbSNP at www.ncbi.nlm.nih.gov/SNP). A missense mutation (c.371A>G; p.Asn124Ser) in exon 4 causing a substitution of an Asparagine with a Serine at codon 124 was found in a steroid-dependent female patient with cleft palate, short stature and increased eADA level. This mutation was found in her healthy father and unaffected sister: the sequence change did not cosegregate with the DBA phenotype.

1.1 Expression and stability of mutated RPS24.

As indicated in the introduction, *rps24* gene undergoes alternative splicing to produce three mRNA isoforms. To study the impact of mutations on the functionality of RPS24, among the different isoforms, we initially considered the isoforms 1 and 2, selectively expressed in haematological lineage, where the main pathological features in human have been observed. However, since in preliminary analysis they showed identical behavior (not shown), we focused our attention on one protein variant (var1). First of all we have characterized the capability of mutant RPS24 proteins to be expressed into the cell. The substitution (N124S) and the deletion (codon 22) identified in DBA patients were introduced into Flag-tagged RPS24 isoform 1 or isoform 2 cDNA by site-directed mutagenesis. DNA constructs

encoding for i) wild type RPS24 (WT), ii) RPS24 with codon 22 deletion (M1) and iii) RPS24 with the N124S mutation (M2) were used in transient trasfection experiments into human embryonic kidney (HEK) 293 cells. The expression of the mutated RPS24 proteins, distinguishable from the endogenous by size, was monitored by western blot analysis using Flag polyclonal antibody (rAb). As a control for transfection efficiency, we used the product of the Neomycin Phosphotransferase II (NPT) gene present in the cloning vector (pcDNA3) and therefore expressed at a level proportional to the amount of plasmids inserted into the cells (Fig. 6). As shown in Fig. 4 the level of both M1 and M2 is clearly lower compared to WT RPS24. This indicates that both mutations affect protein stability. Consistent with the instability of the mutated RPS24, a possible degradation product appeared sometimes on Western blot as a faster-migrating band of variable intensity. Next, we separated nuclear (N) and cytoplasmic (C) fractions from the extract of the transfected cells. Western analysis, reported in Fig. 7, showed that mutated RPS24 (M1 and M2) accumulate into the nucleus more evidently compared to the WT.

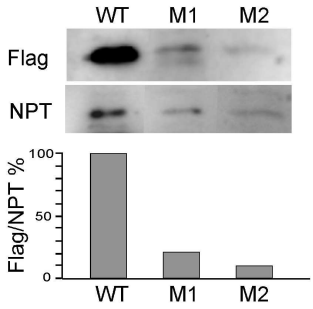


Fig.6) Western analysis of Flag-RPS19 proteins. HEK293 cells were transiently transfected with the RPS24 constructs indicated in each lane. About 50 micrograms of cell extract was separated on SDS-PAGE gel and transferred onto membrane. Immunodetection was performed with antibodies specific for Flag, RPS19 and neomycin phosphotransferase II (NPT). Signals relative to Flag-RPS24 and NPT are indicated.

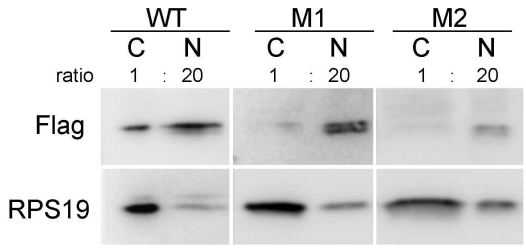


Fig.7) Different accumulation of mutated proteins in nuclei of transfected cells. Nuclear (N) and cytoplasmic (C) fractions were obtained from the extract of the transfected cells and analyzed by western blot with antibodies specific for Flag and RPS19. Nuclear and cytoplasmic extracts were loaded on the gel in a ratio 20:1.

1.2 RPS24 Ribosome association

The other property of the mutated Flag-RPS24 proteins we analyzed is their capability to be assembled into ribosomal subunits. For this purpose, after transient transfection into HEK293 cells, cytoplasmic extracts were separated by ultracentrifugation for 3 hours at 100,000g on 15% sucrose cushion. This allows the isolation of two fractions: 1) pellet (P) which includes polysomes and ribosomal subunits and 2) supernatant (S) which includes free cytoplasmic proteins. As shown in Fig 8, WT and mutant Flag-RPS24 localize in P fraction appearing able to associate with ribosomes. As an additional control for this experiment, we transfected a plasmid expressing an RPS19 construct (RPS19flag) with the Flag epitope at the N terminus. RPS19flag fusion protein was previously shown to poorly assemble into the ribosome (Angelini et al., 2007), possibly as a consequence of the position of the Flag epitope (N terminus). Western analysis, reported in Fig. 8, confirmed that RPS19flag can be observed mostly in the free cytoplasmic fraction. On the contrary, both the WT and the mutated RPS24 (M1 and M2) appear to be mainly associated to ribosome.

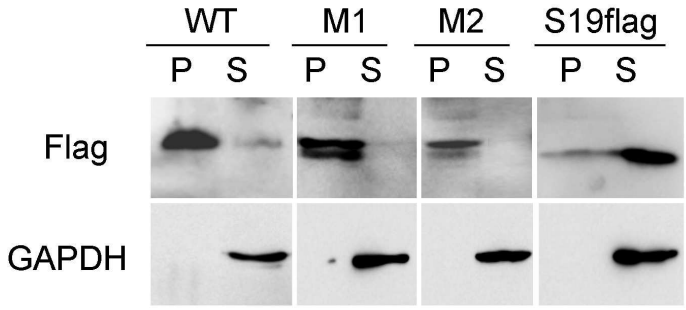


Fig.8) Extracts from HEK293 cells transiently transfected with Flag-RPS24 constructs were centrifuged at 100,000 g for 90 min. Cells were transfected with the Flag-RPS24 constructs indicated in each lane (the first number indicates the kind of mutation, the second one the variant). The pellet (P), containing polysomes and ribosomal subunits, and the supernatant (S), containing free cytosolic proteins, were analyzed by western blot with antibodies specific for Flag and GAPDH.

To further confirm this observation, we performed a separation of cytoplasmic extracts from transfected cells by sucrose gradient centrifugation. Polysome fractionation allows the polysome/subpoysome distribution of a messenger and has been used to monitor possible differences among WT and mutated proteins. The gradients were collected into fractions while monitoring the absorbance at 260 nm. As shown in the absorbance profile of Fig. 7, translating polysomes are localized in fractions 1–5, which were pooled for further analysis. Ribosome monomers (80S) and 60S and 40S ribosomal subunits are also indicated in the absorbance profile. Proteins isolated from the different fractions were subjected to SDS–PAGE and immunoblot analysis with antibodies against Flag, RPS19 and GAPDH. The results, relative to the transfection of Flag-RPS24 WT, M1 and M2 are reported in Fig. 9 A, B and C. Endogenous RPS19 is present, as expected, in all the ribosomal fractions including 40S subunits (fraction 1–9) whereas GAPDH is visible only in light cytosolic fractions (fractions 10–12). The Flag-tagged WT RPS24 protein is absent in the free cytoplasmic fractions and can be clearly seen to be associated with ribosomes, including polysomes. The experiment was repeated for all RPS24 constructs and, as

shown in Fig. 7B and 7C, produced essentially the same result: mutated RPS24 is found associated with ribosomes.

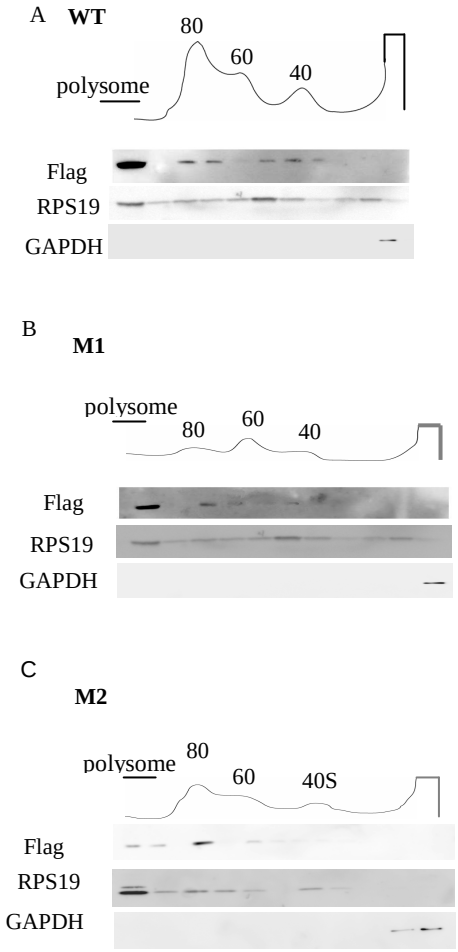


Fig.9) Ribosome association of Flag-RPS24 proteins. **(A)** Extracts from HEK293 cells transiently transfected with WT Flag-RPS24 were fractionated onto a 10%-30% sucrose gradient.
Polysomal fractions and the position of 80S, 60S and 40S are indicated in the absorbance profile in the upper part of the figure. Collected fractions were precipitated by trichloroacetic acid and analyzed by Western blot using antibodies specific for Flag, RPS19 and GAPDH.
For fractions 11 and 12 only 1/10 of the extract was loaded on the gel.
(B) and **(C)** Same analysis as in A) on HEK293 cells transiently transfected with the indicated RPS24 constructs.

2. ANALYSIS OF A RPS7 MUTATION IN MOUSE STRAIN WITH MILD HAEMATOLOGICAL PHENOTYPE.

A British research group has recently isolated a mouse strain with a mutation in RPS7 protein (V156G) showing a mild hematological phenotype similar to that observed in DBA. In collaboration with Dr. David Keyes at Oxford Univeristy we have addressed the effect of the mutation on the function of RPS7 and ribosomal biogenesis. Our experimental strategy has been based mostly on transient transfection of wild type (WT) and mutated (V156G) constructs of RPS7. In particular as we did previously, we analyzed the capability of the exogenous proteins to be integrated in the mature ribosome (Fig. 10). After this we followed the same experimental procedure we adopted for RPS24, performing ultracentrifugation on sucrose cushion to analyze the polysomal association of RPS7 mutated protein (Fig. 11)

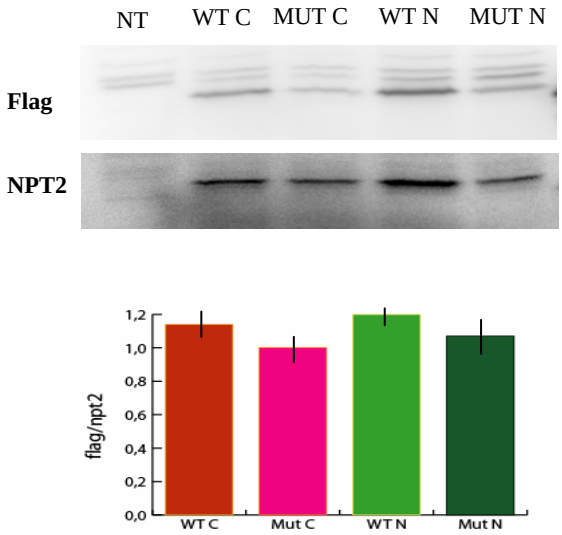


Fig.10) Western analysis of Flag-RPS19 proteins. (A) HEK293 cells were transiently transfected with the RPS7 constructs indicated in each lane. WTC = cells transfected with wild type construct flagged at C terminus, MUT C= cells transfected with V156G mutant construct flagged at C terminus, WT N= cells transfected with wild type construct flagged at N terminus, MUT N= cells transfected with V156G mutant construct flagged at N terminus. About 50 micrograms of cell extract was separated on SDS-PAGE gel and transferred onto

membrane. Immunodetection was performed with antibodies specific for Flag and neomycin phosphotransferase II (NPT).

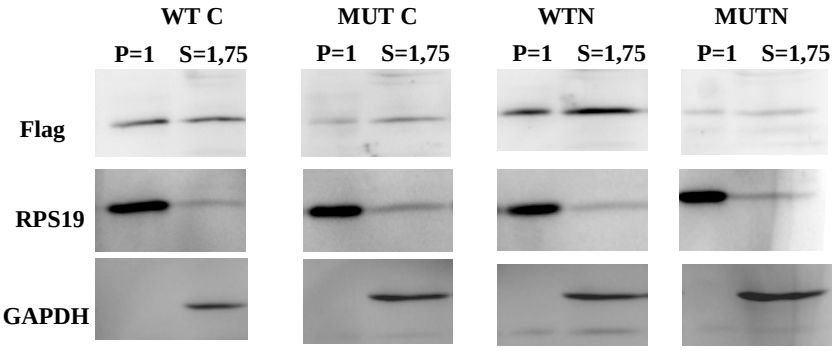


Fig 11) Extracts from HEK293 cells transiently transfected with Flag-RPS24 constructs were centrifuged at 100,000 g for 90 min. Cells were transfected with the Flag-RPS24 constructs indicated in each lane (the first number indicates the kind of mutation, the second one the variant). The pellet (P), containing polysomes and ribosomal subunits, and the supernatant (S), containing free cytosolic proteins, were analyzed by western blot with antibodies specific for Flag, RPS19 and GAPDH. WTC = cells transfected with wild type construct flagged at C terminus, MUT C= cells transfected with V156G mutant construct flagged at C terminus, WT N= cells transfected with wild type construct flagged at N terminus, MUT N= cells transfected with V156G mutant construct flagged at N terminus.

2.1 Alteration of pre-rRNA processing in RPS7 mutant mouse.

Moreover, by using tissue (liver and brain) from the mouse with the V156G RPS7 substitution, we checked for possible alterations of rRNA processing. Total RNAs were submitted to northern-blotting and analyzed with a probe complementar to the internal transcribed spacer 1 (5'-ITS1 probe) specific for the 18S rRNA precursors (Fig. 12 A). Cells with mutations in RPS7 showed increased levels of 30S pre-rRNA when compared to control cells. This phenotype, characterized by high 41S/30S ratios, corresponds to delayed maturation of the pre-40S subunits (Fig. 12 B e C). These data indicate that the mutations in RPS7 found in our mouse model affect maturation of the 18S rRNA.

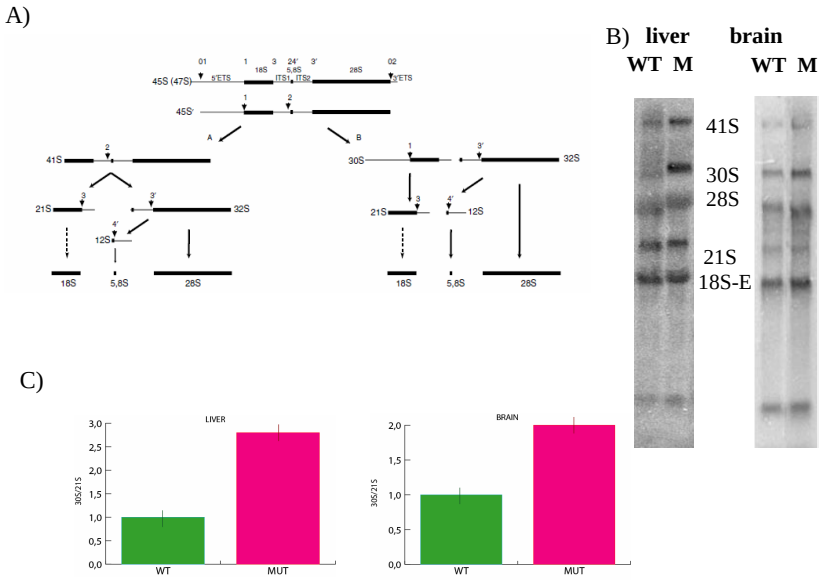


Fig.12 A) Pre-rRNA processing: schematic nomenclature according to Hadjiolova et al. (1993). Two alternative pathways are presented**. B)** About 3 micrograms of total RNA, extracted from liver and brain of wild type (WT) and mutated RPS7 mice (M), was fractionated on formaldehyde-agarose gels and transferred to membrane. After hybrydization the signals were evaluated PhosphorImager analysis. **C)** Quantitation of the signals of Northern experiments reported as ratio between 30S and 21S rRNA precursors. The experiments were performed at least in triplicate on tissues from wt and mutant littermate mice (2 wt and 2 mutants for liver, 3 wt and 3 mutants for brain). The average of the values is reported in the bar graphs with S.E.M..

3. POSSIBLE ROLE FOR PIM1 KINASE TO THE RIBOSOMAL STRESS.

To define the implications of ribosomal proteins deficiency in Ribosomal stress activation, we used two erythroid cell line inducible for RPS19 interference that mimic this specific stress condition (Miyake et al., 2005): TF1C (erythroleukemic cell line from human blood) and K562C (human myelogenous leukemia line). These cell lines can be induced, by giving doxycycline, for the expression of a siRNA that causes the decrease of RPS19 levels. These inducible cell lines were created in the laboratory of Prof. S. Karlsson in Sweden, for studying DBA ethiogenesis and in particular to characterize the effects achieving from the lack of ribosomal protein S19 (Fig.13). Cell counting and FACS analysis showed that the induction of RPS19 deficiency causes an evident reduction of cell proliferation and an accumulation of cells in the G0/G1 phase of the cell cycle. It is important to notice that alteration of cell cycle and proliferation can be also observed in the p53-null K562C cells, suggesting that a p53-independent mechanism is activated in these cells (Fig. 14). Western analysis of both TF-1C and K562C cells showed that, during induction, in parallel to RPS19 decrease, there is a comparable reduction of PIM1 level (as shown in the next section of the Results), whereas the level of the CDK inhibitor $p27^{Kip1}$ (known to be one of PIM1 targets) increases.

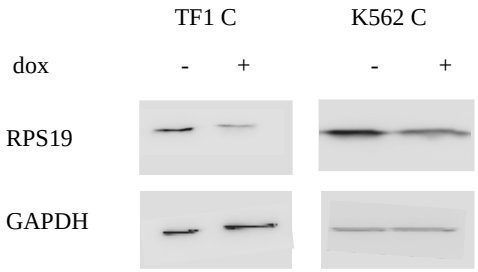


Fig.13) Characterization of experimental model. TF1 C (Human Erythroleukemic cell line) and K562 C (Human Myelogenous leukemia) cells were treated or not with doxicycline for four days to induce RPS19 interference. Total protein extracts were analyzed with antibodies specific for RPS19 or GAPDH

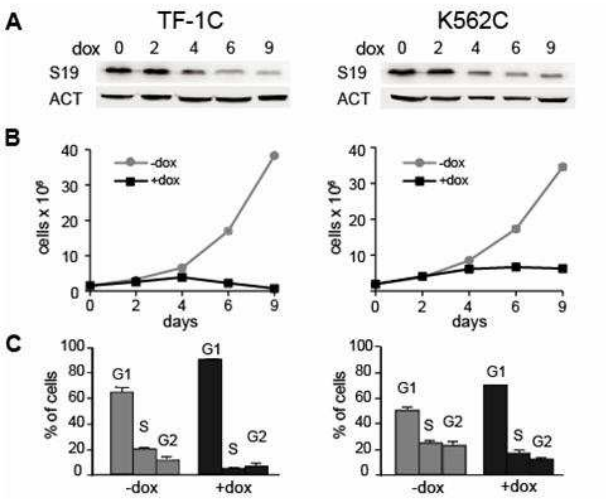


Fig.14 Effects of RPS19 deficiency. **A**; TF-1C and K562C cells were treated for the indicated number of days with doxycycline (dox). Total protein extracts were separated on SDS-PAGE and transferred onto nitrocellulose membrane. Blots were decorated with primary antibodies against RPS19 (S19) and beta-actin (ACT). **B**; Aliquots of cells treated as in A were counted in triplicate and the results are reported in a linear plot with s.e.m. barely visible. **C**; TF-1C and K562C cells untreated or treated for four days with doxycycline were analyzed by FACS in triplicate. The percentage of cells in the different cell cycle phases G0/G1 (G1), S (S) and G2/M (G2) is reported in a column plot +/- s.e.m..

3.1 Stimulation of Ribosomal stress by RPS19 downregulation causes decreasing of Pim1 kinase expression.

A current hypotesis on the mechanism of DBA and other ribosomal pathologies is that the defect in ribosome synthesis (Ribosomal stress) would activate growth arrest in early hematopoiesis. Given the involvement of PIM1 in hematopoietic cell metabolism and its recently discovered binding with the ribosome, in our laboratory has been decided to test whether this oncogenic kinase could play a role in the response to Ribosomal stress and in

particular in the downstream effects of this cellular activation pathway. This project was carried out in collaboration with Dr. V. Iadevaia from our laboratory. First of all we demonstrated that in our cells the reduction of RPS19 induces a rapid decrease of PIM1 expression (Fig. 15).

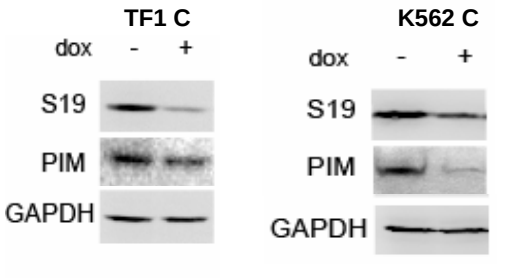


Fig. 15). PIM1 protein level during interference for RPS19. PIM1 decreases during the interference for RPS19. Total protein extracts were analyzed with antibodies specific for RPS19, Pim1 or GAPDH (from V. Iadevaia).

3.2 Stimulation of Ribosomal stress by specific drug-treatments.

A decrease of PIM1 level is also observed during treatment with drugs known to induce Ribosomal stress such as Actinomycin D, Camptothecin and Cisplatin. Infact, in order to test the direct linkage of PIM1 protein expression with the Ribosomal stress response we carried out a number of treatments with drugs that interfere with nucleolar function. Our hypothesis was that these drugs should affect PIM1 protein levels similarly to RPs deficiency. Cells were treated for 16 hours with Actinomycin D, Camptothecin and Cisplatin all known to affect rRNA synthesis at different steps. As a control cells were also treated with the cell cycle inhibitor Nocodazole and Hydroxyurea that do not affect the nucleolar function. Western blot analysis showed that all three inhibitors of nucleolar function provoke the decrease of PIM1 level while Nocodazole and Hydroxyurea treatments do not (Fig. 16).

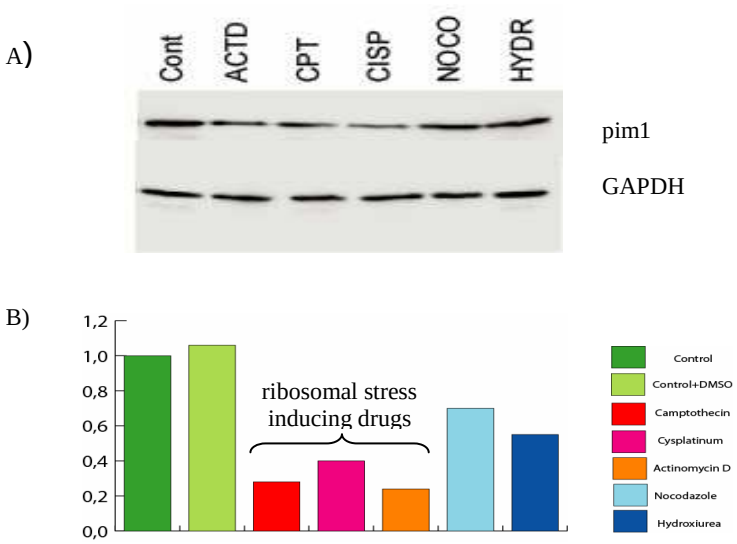


Fig 16). PIM1 and Ribosomal stress. K562 C cells not treated (Cont) or treated with Camptothecin (-CPT-5 uM), Cisplatin (-CISP-20 uM), Actinomycin D (-ACTD-50 ng / ml), Nocodazole (-NOCO-100 ng / ml), Hydroxyurea (-HYDR-300 uM) for 15 hours. A) Western blotting of total protein extracts. Protein extracts were analyzed with antibodies specific for Pim1 or GAPDH. B) Histogram of the quantitation of Pim1 and GAPDH related signals with Image Quant 5.1 (Molecular Dynamics). The error reported was obtained from three different experiments.

3.3 Effect of Pim1 kinase on cellular proliferation.

Interestingly we noted that, accordingly to previously described decrease of PIM1 protein levels, the proliferation rate in our cellular model was slowed down. In order to explain whether this effect was related to PIM1 decrease we tried a rescue experiment by transiently transfecting PIM1-expressing plasmids into RPS19 deficient cells. TF1C and K562C cells were treated along 5 days with doxycycline to induce RPS19 downregulation and, where indicated, transfected with PIM1 constructs (Fig. 17).

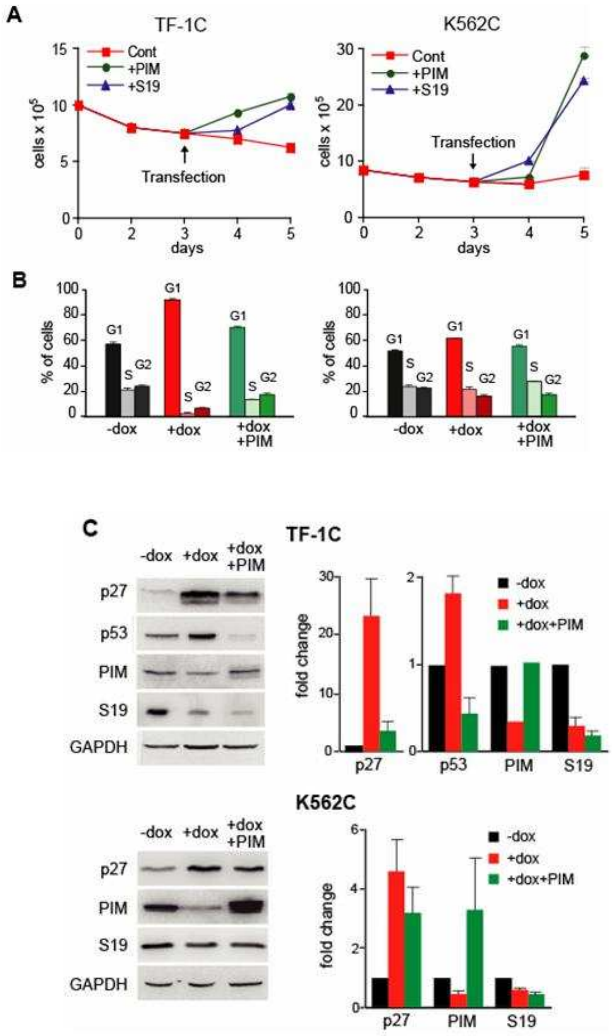


Fig 17) A. Analysis of cell growth. K562 C cells and TF1 C cells induced or not induced with doxycycline were counted every 24 hours for 5 days. On the third day some of the induced cells were transfected with pcDNA3 constructs: PIM1-HA or RPS19-flag. To the initial number of cells has been assigned a value of 1 10^{6}. The graph results from the average of three different experiments. **B**; TF-1C and K562C cells untreated (-dox), treated for five days with dox (+dox) or treated for five days with dox and transfected with PIM1 at day 3 (+dox+PIM), were analyzed by FACS in triplicate. The percentage of cells in the different cell cycle phases, G0/G1 (G1), S (S) and G2/M (G2) is reported in a column plot of the mean +/- s.e.m. **C)** total extracts from TF-1C and K562C cells treated as in B were analyzed by western blot with the indicated primary antibodies. An example of western blot is shown on the left. Quantification (at least three blots from two independent experiments) is reported on the right as a column plot of the mean +/- s.e.m. of the values normalized for GAPDH and considering untreated sample as 1.

The growth curves showed that restoring PIM1 level during RPS19 interference restarts cellular proliferation (Fig. 17 A) and cell cycle (Fig. 17 B). In addition, given that several regulators of cell cycle progression were identified as PIM 1 target and in particular p27/Kip1 protein, we also analyzed in this experimental conditions, the effect of Pim1 on p27/Kip1 both at transcriptional and translational level (Fig.17C and 18). We reported that even if p27/Kip1 mRNA levels analyzed by Real Time PCR assay were not significantly altered, the restoring of PIM1 level during RPS19 interference causes the reduction of p27/Kip1 levels (Fig. 17C). Interestingly, the level of p53, present only in TF1C cells, is also affected by PIM1 transfection (data do not shown).

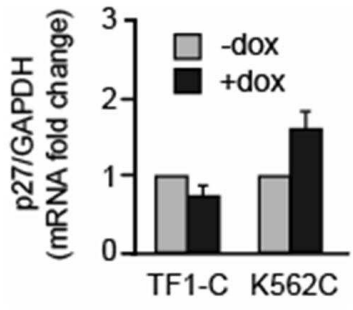
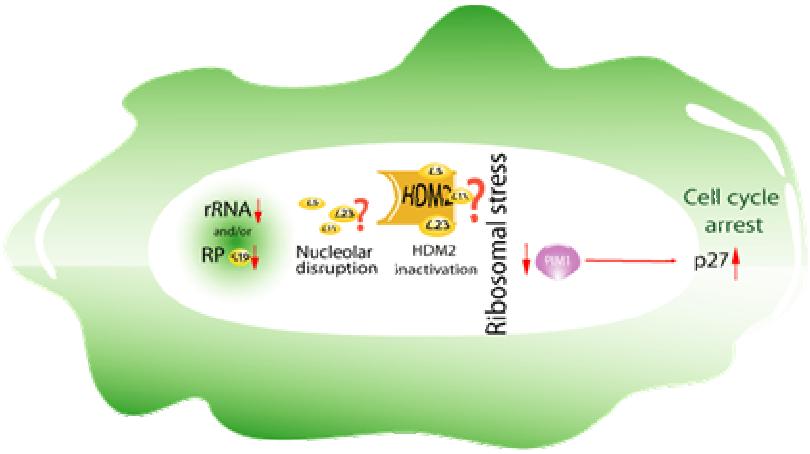


Fig. 18). Total RNA extracted from TF1 C and K562 C cells untreated or treated 4 days with doxycycline were analyzed by Real Time PCR with primers specific for p27/Kip1 or GAPDH. The histograms report the mean of three experiments with three different RNA extracts. mRNA levels in uninduced cells were cosidered as 1.



Tab.3). Our proposed model for Ribosomal Stress response activation.

DISCUSSION

Ribosome biogenesis is a complex process that requires the transfer of multiple proteins in and out of the nucleolus. Maturation of the rRNA and its assembly into ribosomal subunits involves roughly 150 accessory proteins and about as many as small nucleolar ribonucleoprotein particles (Cmejla et al., 2007). The role of the RPs in this context has not been clarified yet. In the last few years several reports supplied the hypothesis that some pathologies may be caused by alteration of ribosome structure and or function. These include Dyskeratosis Congenita, Cartilage Hair Hipoplasia and Diamond Blackfan Anemia. Starting from these evidences ribosome function has been hypotesized to be a critical step during development (and in particular it is erytropoiesis for the mentioned diseases). Any decrease of ribosomal activity could compromise the ontogenetic process.
Yet this hypothesis does not explain, so far, the tissue-specific effect of ribosome synthesis alteration. Suggestive evidence came from a number of studies that showed how nucleolar or Ribosomal stress activate the tumor suppressor p53 causing growth arrest and apoptosis (Anderson et al., 2007; Danilova et al., 2008; Fumagalli et al., 2009; McGowan et al., 2008; Panic et al., 2007; Pestov et al., 2001; Rubbi and Milner, 2003). Moreover DBA erythroid precursors systems or animal models mutated for specific RPs have shown an increased sensitivity to apoptosis (Perdahl et al., 1994). It is by now ascertained that depletion of RPS19 protein causes defects in the processing of 18S rRNA precursor and interfers with the maturation of 40S subunit (Kuramitsu et al., 2008; Miyake et al., 2008). Lymphoblastoid cells derived from DBA patients with mutated RPS24 also display a clear defect in pre-rRNA maturation, although distinct from the phenotype observed in cells derived from patients with mutations in RPS19 (Cmejla et al., 2007). Recently other ribosomal proteins such as RPS17 or RPL35a mutated in a few percentage of DBA patients (Angelini et al., 2007; Cmejla et al., 2007; Dai and Lu, 2004). Here we discuss our finding about three proteins related with different aspects of Ribosomal stress.

1. To investigate on the two new RPS24 mutations (one substitution and one deletion) found in the screening on Italian patient by Irma Dianzani's Laboratory, we used RPS24 cDNA. The first observation we made was that in transient transfection experiments into HEK 293 cells, the level of the mutated RPS24, after normalization for transfection efficiency, is variable among the different proteins. In fact we could detect a different stability of

the wt RPS24 protein compared with the RPS24 allelic variants. Moreover, among the two RSP24 the ones with codon 22 deletion appear more stable then those carrying substitution N124S. A higher stability of the transfected RPS24 (comparable with the endogenous RPS19) could be observed when the transfected protein successfully participates in ribosome assembly (WT) and is exported into the cytoplasm.
Further analysis of the RPS24 allelic variants showed that they were associated with ribosomes or 40S subunits. This result indicates that, although the RPS24 mutants are less stable and accumulate into the nucleus, a small fraction of them is incorporated into the small subunit and exported into the cytoplasm, thus, forming a functional ribosome.
In conclusion, the two RPS24 variants analyzed in this study cause a decrease in protein stability, with deletion mutations having a stronger effect. However, both mutated RPS24 alleles appear to be able to be assembled into the ribosome. It could be speculated that the subtle defect observed is sufficient to cause the disease. Alternatively, these mutations play a contributory role in the disease that is due also to other mutations. Indeed, the patient showing the substitution in RPS24 (N124 we considered in this work) has subsequently been found to have also a *de novo* five nucleotide deletion in RPL5 exon 3 (c.134_138delACACA). The mutation in RPS24 gene was inherited from the healthy father and also carried by a healthy sister suggesting that N124S could not be the cause of the disease.
Implications of our findings for the understanding of molecular mechanism of DBA should be considered with caution. In fact our experimental setup consists in overexpression of tagged RPS24 constructs in standard cell lines (HEK 293). However considering the ubiquitous and constitutive expression of RPS24, it could be that the effect of mutations on the function of the protein is the same in all cell types. In addition a previous report showed that mutations of another ribosomal protein (RPS19), in the same experimental conditions, caused severe consequences easily detectable such as the inability of this protein to be assembled in the ribosome (Angelini et al., 2007).

2. In the second part of this study we extended our investigation to the RPS7 mutant (V156G) found in the *Montu mouse* animal model of our collaborator D. Keyes. This investigation highlighted that expression of RPS7 mutant alleles does not alter the capacity of the protein to be assembled into the ribosomes as well as mutated constructs for RPS24. However it must be mentioned and it is interesting to note that the presence of a single allele of

the mutated protein causes alteration of the rRNA maturation pathway in the mouse tissues we considered (liver and brain). Our results suggest that RPS7 may impair the cellular metabolism, so exhibiting the characteristics of a DBA gene.

Yet under a wider perspective these results give indication just upon upstream events triggering the activation cascade that blocks cell growth and, in some cases, leads to apoptosis in cells.
So that to make more complete our point of view on Ribosomal stress we observed the downstream effects inducted by a ribosome alteration, as mutation of RPs is.

3. In our study we used an experimental model exploited for the study of the DBA: erythroid cell lines TF1C and K562C.
Parental cell lines present a fundamental difference: TF1C express a wild type p53 whereas K562C do not show detectable levels of p53. In these cells we monitored the effect of ribosome alteration on the expression of selected proteins and on cell growth parameters.
We and others noted that cell counting and FACS analysis showed that an evident reduction of cell proliferation and an accumulation of cells in the G0/G1 phase of the cell cycle, in conditions of RPS19 downregulation. It is important to notice that alteration of cell cycle and proliferation can be also observed in the p53-null K562C cells, suggesting that a p53-independent mechanism is activated in these cells. Western analysis of both TF-1C and K562C cells showed that, during induction, in parallel to RPS19 decrease, there is a comparable reduction of PIM1 level, whereas the level of the CDK inhibitor and PIM1 target $p27^{KIP1}$ increases.
This work characterizes the effect of transfection of PIM1 WT constructs on cell proliferation after RPS19 silencing and analyzes the transcriptional levels of cell cycle inhibitor $p27^{KIP1}$ in this experimental context. Literature data indicate that this protein is phosphorylated by PIM1 *in vitro* and *in vivo* and that phosphorylation will cause degradation in the cytoplasm. Therefore, the decrease of PIM1 could lead to the stabilization of $p27^{KIP1}$ resulting in cell cycle arrest (see also Morishita et al., 2008). The data obtained suggested that the decrease of PIM1 may be the cause of the proliferative effect since after transfection cell proliferation is restored. As regards in our lines RPS19-deficient the mRNA levels of cell cycle inhibitors do not significantly change.

Finally, it has been shown that a p53 dependent stress response may occur as a result of alteration of nucleolar function, as for instance, inhibition of rRNA synthesis. Accordingly, a number of inhibitors that induce nucleolar disruption cause p53 activation. We decided to test if the treatment with drugs that interfere with nucleolar function would also may affect PIM1 levels similarly to RPs deficiency. Effectively we verified that all the three used inhibitors of nucleolar function (Camptothecin, Cisplatin and Actinomycin D) cause a decrease of PIM1 level, whereas drugs not affecting cell cycle (such as Nocodazole and Hydroxyurea) and not related with a specific damage of nucleolar function, do not. The data presented, thus, indicate a role of PIM1 in the Ribosomal stress as a trigger of some effects of this response, also given to its direct contact with ribosomes (by physical interaction with RPS19). In addition the documented function of PIM1 as a regulator of cell cycle and cell growth fits well with the well described role as coordinator of the stress signals to various key cellular components. Among the PIM1 interactors that may be involved in the response to the stress we have tested p27 and also p21 (data not shown) but there are other targets such as cdc25a, cdc25c and so on. In conclusion with this work we postulate PIM1 as a sensor of certain Ribosomal stress events, even if its relationship with the p53-dependent response remains unclear.

MATERIALS AND METHODS

PATIENTS

One hundred twenty-eight unrelated DBA families were studied. Fourteen had more than one clinically affected individual. Diagnosis was always based on normochromic, often macrocytic anemia, reticulocytopenia, erythroid bone marrow aplasia or hypoplasia, and, in some patients, congenital malformations and elevated eADA. We excluded short stature because it was difficult to evaluate in the context of severe anemia, iron overload and chronic corticosteroid use.
Informed consent was obtained from all patients and/or their family members participating in the study. RPS19 mutations were found in 36/128 (28%) unrelated DBA patients using both sequencing and MLPA technique as reported elsewhere (Campagnoli, 2008; Quarello, 2008).

MOLECULAR ANALYSIS

Genomic DNA from 92 unrelated Italian DBA probands negative for RPS19 mutations was isolated from peripheral blood leukocytes using a commercial kit (Gentra Systems, Inc., Minneapolis, MN). We analyzed six RP genes (RPS14, RPS16, RPS24, RPL5, RPL11, and RPL35A) by direct sequencing of the coding exons and intron-exon boundaries. Primers for amplification were designed with the Primer 3 software (primer sequences available on request). PCR products were purified with the QIAquick purification kit (QIAGEN GmbH, D-40724 Hilden), and sequenced on both strands with the ABI PRISM BigDye Terminator kit (Applied Biosystems, Foster City,
CA) on an Applied Biosystems 3100 DNA Sequencer (Applied Biosystems). When sequence changes were found, independent PCR products were sequenced to confirm the mutations identified in the probands.
Subsequently, we sequenced DNA samples from available family members to determine whether the mutation cosegregated with the DBA phenotype within the pedigree. To determine whether these sequence changes were polymorphic variations, we sequenced DNA samples from at least 100 control individuals, and verified that none was reported in the Single Nucleotide Polymorphism database (dbSNP at www.ncbi.nlm.nih.gov/SNP).

GENOTYPE-PHENOTYPE CORRELATION and STATISTICAL ANALYSIS

Genotyope-phenotype correlations were evaluated in the whole survey of patients.
Differences between two independent samples were checked with Student's t-test or Mann-Whitney test as appropriate, whereas the Kruskal-Wallis test was used to assess the differences between more than two groups. Associations between categorical variables were assessed with Fisher's exact test or with odds ratio and 95% confidence interval (95% CI). For categorical variables with more than two categories simple logistic regression was used to calculate odds ratio and 95% CI.
All tests are two-sided and significant for $p<0.05$. Data were analyzed with the SPSS 16 software (SPSS Inc., Chicago, IL).

CELL LINES, CULTURE CONDITIONS AND TRANSFECTIONS

HEK293 (ATCC #CRL-11268) cell line was cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C with 5% CO_2. For transient transfection, cells were plated at 90% of confluence and transfected with Lipofectamine 2000 (Invitrogen, Milan, Italy) according to the manufacturer's instructions.

DNA CONSTRUCTS

RPS24 expression plasmids were constructed by inserting cDNA, tagged with the Flag epitope at the C terminus, into the mammalian expression vector pcDNA3.1 (Invitrogen, Milan, Italy).

ANTIBODIES AND WESTERN BLOT ANALYSIS.

For western blot analysis, cells were washed twice with phosphate-buffered saline (150 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 1.4 mM KH_2PO_4) and were treated with lysis buffer (150 mM NaCl, 50mM Tris–HCl (pH 7.5), 1% NP40, 0,5 % sodium deoxycholate, 0,1 % SDS, Aprotinin 1 mg/ml, Leupeptins 1 mg/ml, Pepstatin A 1 mg/ml, PMSF 100 mg/ml). After 1 min of incubation on ice, the extract was centrifuged for 40 min at maximum speed in a microcentrifuge at 4 °C. Proteins were separated on 12% SDS–PAGE, transferred on Nitrocellulose membrane and incubated with a mouse mAb specific for RPS19, rabbit anti-Flag (Sigma), rabbit anti-NPT II (Upstate, 06-747), mouse monoclonal anti-Pim1 (Santa Cruz) mouse monoclonal anti-p27 (BD) Detection of immunoblots was carried out with Ablot Plus (Euroclone).

EXTRACT FRACTIONATION

For ribosome isolation, cells (~$10*10^6$) were lysed in 300µl of 10 mM Tris HCl (ph 7,5), 10 mM NaCl , 3mM $MgCl_2$, 0,05% NP40 with proteases' inhibitors and centrifuged for 5 minutes at 1000 g. The supernatant was collected and represents the cytoplasmic extract, the pellet is the nuclear fraction. The cytoplasmic extract was layered onto 1 ml of 15% sucrose, 30 mM Tris–HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl2 and centrifuged in a Beckman type 70.1 rotor for 90 minutes at 100,000g. The pellet (P, ribosomal fraction) was resuspended directly in SDS–PAGE loading buffer. The supernatant (S, free cytoplasmic proteins) was precipitated with 10% TCA and the pellet, washed with acetone, was resuspended in SDS–PAGE loading buffer. The proteins were then analyzed by western blot.
For sucrose gradient fractionation, cytoplasmatic extracts, prepared as described above, were layered onto 5%-65% linear gradient containing 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10 mM MgCl2 and centrifuged for 3h at 37,000 rpm in a Beckman SW41 rotor. Twelve fractions were collected while monitoring the absorbance at 260 nm. Proteins from each fraction were precipitated with 10% TCA. The pellet was washed with acetone, dried, and resuspended in SDS-PAGE Loading Buffer (63 mM Tris-HCl pH 6.8, 5% Glycerol, 1% SDS, 2.5% bromophenol-blue) .

The first five fraction (polysome) were pooled and loaded entirely on a single well whereas only part (1/10) of fraction 11 and 12 was loaded on the gel.

NUCLEI PURIFICATION

The pellet obtained after the 1000 g centrifugation, was used for further analysis. It was resuspended in 1,2 ml of 10 mM Tris HCl (ph 7,9), 10 mM NaCl, 5 mM $MgCl_2$, 0,3 M Sucrose. Nuclei were then layered onto a 2ml cushion of 10 mM Tris HCl (ph 7,9), 10 mM NaCl, 5 mM $MgCl_2$, 0,6 M Sucrose and centrifuged 10 minutes at 2000 g at 4°C.

RIBOSOMAL BIOGENESIS

RNA was extracted from mouse tissues with Trizol (Invitrogen) according to manufacturers protocol. Total RNA was fractionated on formaldehyde-agarose gels and transferred to Gene Screen Plus membrane (NEN). ITS1 probe was prepared by 5' end-labeling of a 28-mer oligonucleotide (GCTCCTCCACAGTCTCCCGTTAATGATC) with 25 μCi of γ[32P]-ATP and T4 polynucleotide kinase according to standard protocols. Hybridization was carried out overnight at 42 °C in 6X SSPE, 1% SDS, 0,25 mg/ml ssDNA and 5X Denhardt solution. After hybridization, the blot was washed twice with 1% SDS in 2X SSPE for 30 min at 37 °C. Quantitation of signals was obtained by phosphor screen scanning with a STORM PhosphorImager and ImageQuant software analysis (Molecular Dynamics).

REAL TIME PCR

For Real-time RT-PCR, total RNA was extracted from cells using Eurogold Trifast (Euroclone), according to the manufacturer's protocol. RNA (2.5 μg) was treated with DNAse I (Promega), then reverse transcribed into single strand cDNA, using Moloney murine leukemia virus (MMLV) Reverse Transcriptase (Promega) and random primers (Invitrogen). cDNA was diluted at a concentration of 80 ng/μl in nuclease-free water and stored in

aliquots at –80°C until used. Real-time PCR was performed with the LightCycler (Roche Diagnostics) using SYBR green detection (Kapa SYBR Fast qPCR kit -Kapa Biosystems-), the cDNA as the template and the primer mix of interest: (GAPDH NM_002046.3: sense primer 5'-accagggctgcttttaactctggt-3'; antisense primer 5'-gcaaatttccatggcaccgtcaagg-3'; $p27^{Kip1}$ NM_004064: sense primer: 5'-acgtgagagtgtctaacgg-3'; antisense primer: 5'-agtgcttctccaagtccc-3'; $p21^{Waf1}$ NM_000389 sense primer 5'-cggcagaccagcatgacagattt-3' antisense primer: 5'-tcaaaggcccgctctacatctt-3'). The amount of mRNA transcripts encoding these genes was determined using the expression $2^{-\Delta\Delta Ct}$, considering the threshold cycle (Ct) of the sample relative to the internal reference GAPDH (ΔCt) and to untreated cells (ΔΔCt).

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