A search of proteins that interact with Sin3A-associated proteins 30 and 30-like

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Abstract

Background: Histone deacetylase complex (HDAC complex) removes acetyl groups from histones herewith leading to a tighter DNA structure and repression of transcription. A novel member of this complex is a Sin3A-associated protein 30-like (SAP30L) which is 70% identical with a Sin3A-associate protein 30 (SAP30), a protein previously identified to participate in the HDAC complex. Aberrant acetylation is associated with several solid tumours and haematological malignancies. Altered acetylation degree of histones may also play a role in other diseases. In order to better understanding the function of SAP proteins we set out to study their interacting proteins.

Methods: The glutathione-S-transferase pull-down method was used to "fish" the interacting proteins from human cell lysates using SAP30 and SAP30L as baits. The bound proteins were separated on gel and analysed by mass spectrometry. Suspected interactions were ensured by immunoblotting.

Results: Mass spectrometric analysis gave numerous possible interacting proteins, including ribosomal proteins, which were the majority, proteins needed for ribosome biogenesis and cancer related proteins. The interacting proteins were pretty much the same for SAP30 and SAP30L. Interestingly, none of the interacting proteins, found by this experiment, was a member of the HDAC complex. Immunoblotting studies confirmed that SAP30 and SAP30L interact with nucleolin and fibrillarin.

Conclusion: SAP30 and SAP30L interact mainly with the same proteins suggesting that they have similar functions. Mapping of protein interactions suggest a nucleolar role for SAP30 and SAP30L.

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Tiivistelmä

Tutkimuksen tausta: Histonideasetylaasikompleksi (HDAC kompleksi) poistaa histoneista asetyyliryhmiä, jolloin kromatiinin rakenne tiivistyy ja transkriptio estyy. Kompleksin uusin jäsen on Sin3A-associated protein 30-like (SAP30L), joka on 70%:sti identtinen aiemmin löydetyn kompleksin jäsenen, Sin3A-associated protein 30 (SAP30), kanssa. Syövissä geenien ekspressio on poikkeavaa ja on havaittu, että esimerkiksi leukemioissa histonien asetyloitumisaste on muuttunut. Histonien asetylointi/deasetylointi liittynee myös muihin sairauksiin. Tutkimuksen tarkoituksena on selvittää SAP30:n ja SAP30L:n toimintaa ja erityisesti niiden vuorovaikutuksia muiden proteiinien kanssa.

Tutkimusmenetelmät: Pull-down- menetelmää käytettiin "kalastamaan" vuorovaikuttavia proteiineja ihmissolulysaateista käyttäen SAP30- ja SAP30L-proteiineja syötteinä. Sitoutuneet proteiinit eroteltiin geelillä ja analysoitiin massaspektrometrialla. Vuorovaikutuksia varmistettiin immunoblottauksella.

Tutkimustulokset: Pull-down- massaspektrometria- menetelmällä löysimme 28 ja 41 proteiinia, jotka sitoutuvat vastaavasti SAP30:n ja SAP30L:n kanssa. Näiden joukossa oli ribosomaalisia proteiineja, ribosomien biosynteesiin tarvittavia ja syöpään liittyviä proteiineja. SAP30:llä ja SAP30L:llä on vuorovaikutussuhteita jokseenkin samojen proteiinien kanssa. Analyysituloksista ei kuitenkaan löytynyt HDAC kompleksin jäseniä. Immunoblottaus vahvisti, että SAP30 ja SAP30L sitoutuvat nukleoliiniin ja fibrillariiniin.

Johtopäätökset: SAP30 ja SAP30L vuorovaikuttavat useiden tumajyväsproteiinien kanssa, ja niillä näyttää vuorovaikutuksen perusteella olevan samankaltaiset tehtävät solussa. Vuorovaikutusten perusteella SAP-valkuaisilla on tärkeä rooli tumajyväsessä.

CONTENTS

ABBREVIATIONS

aa amino acid

bp base pair

BCC basal cell carcinoma

BSA bovine serum albumin

DNAse deoxyribonuclease

DOC sodium deoxycholate

DTT dithiothreitol

FBS Fetal bovine serum

GST glutathione-S-transferase

HAT histone acetyltransferase

HDAC histone deacetylase

HDACi histone deacetylase inhibitor

HRP horseradish peroxidase

IPTG Isopropyl-β-D-thiogalactopyranoside

kDa kilodalton

LOH loss of heterozygosity

MALDI matrix assisted laser desorption ionisation

PAGE polyacrylamide gel electrophoresis

PBS phosphate-buffered saline

PBST phosphate-buffered saline with Tween20

RbAp Retinoblastoma associated protein

RNAse ribonuclease

SAP30 Sin3A-Associated Protein 30

SAP30L Sin3A-Associated Protein 30 Like

SDS sodium dodecyl sulfate

TCA trichloroacetic acid

TSA trichostatin A

TSG tumor suppressor gene

TOF time of flight

YY1 Ying and Yang 1

1. Introduction

In eukaryotic cells, the ability to express biologically active proteins comes under regulation at several points, like initiation of transcription, processing and stability of the transcript, initiation of translation and stability of the synthetized protein (Alberts et al., 2002). Transcription is the first stage in gene expression and the principal step at which it is controlled. Initiation of transcription is influenced by the manner in which DNA is packaged (Lewin, 2004). Local chromatin architecture is generally recognized as playing a crucial and active role in regulation of gene expression (Wade et al., 1997). The physical structure of the DNA, as it exists compacted into chromatin, can affect the ability of transcriptional regulatory proteins (termed transcription factors) and RNA polymerases to find access to specific genes and to activate transcription from them. (Alberts et al., 2002) The presence of the histones and CpG methylation most affect accessibility of the chromatin to RNA polymerases and transcription factors. DNA is typically methylated by methyltransferase enzymes on cytosine nucleotides in a CpG dinucleotide sequence (also called "CpG islands"). (Albert et al., 2002, Lewin, 2004) Histones can also be methylated at their lysine and arginine residues or acetylated at their lysine residues (Wade, 2001, Grunstain, 1997). Methylation of chromatin or histones inhibits gene expression while acetylation of histones leads to activated gene expression.

As mentioned, the acetylation degree of histones is one of the regulators of gene expression (Wade, 2001). When transcription is active, the histones are acetylated and when the acetyl groups are removed from histones, genes are not expressed (Allfrey et al., 1964, Kuo & Allis, 1998). This acetylation-deacetylation leads to changes in DNA structure. In cells, DNA is wrapped around the histones forming structures called nucleosomes (Strahl & Allis, 2000, Alberts et al., 2002). When histones are acetylated, interaction between DNA and histones is disturbed due to decreasing of the ionic attraction (Hong et al., 1993). As a result, DNA relaxes and become more accessible to transcriptional factors. The opposite happens when acetyl groups are removed from histones (Hebbes et al., 1988, Grunstein M et al., 1997, Wade et al., 1997, Ayer, 1999). The enzymes involved in this process are histone acetyltransferases (HATs) (Roth et al., 2001) and histone deacetylases (HDACs) (de Ruijter et al., 2003), which acetylate and deacetylate histones, respectively.

HDACs form a complex with other proteins. This complex is called histone deacetylase complex and it contains co-repressors required for the proper targeting of HDACs (Qian & Lee, 1995, Zhang et al., 1997, de Ruijter et al., 2003). One of these members is a Sin3-associated protein 30 (SAP30) (Zhang et al., 1998). Recently, a novel nuclear protein was discovered from an in vitro mesenchymal –epithelial cell culture model and it was shown to be 70% identical with SAP30, hence it got the name Sin3-associated protein 30-like (SAP30L) (Lindfors et al., 2003).

In cancer, gene expression is aberrant and because transcriptional activity is related to acetylation degree of histones, HDACs play a role in cancer. Especially in leukaemia, hypoacetylation of histones (hyperactivity of HDACs) has been observed (Lin et al., 1998). This causes silencing of the genes needed for growth control and differentiation of the cell, which in turn speeds up the proliferation and finally may lead to cancer (Li-Zhen et al., 2001). Molecules that prevent HDACs from deacetylating histones, HDAC inhibitors (HDACi), have been used as anti-cancer agents and the results have been promising (Li-Zhen et al., 2001, Minucci et al., 2001, Suenaga et al., 2001). Moreover, HDACs and their co-repressors may be involved in other diseases as well (Barnes et al., 2005, McKinsey & Olson, 2005).

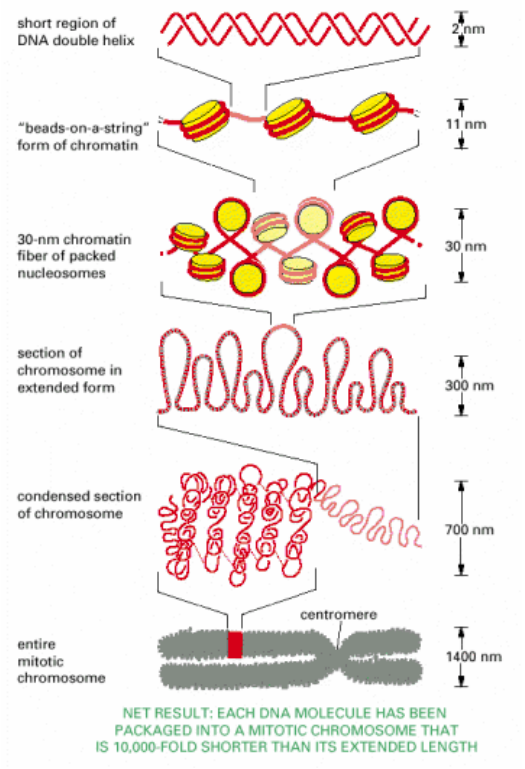
It has been recently shown that SAP30L is a member of Sin3A repressor complex (Viiri et al., in press). To study further the function of SAP30 and SAP30L-proteins, we decided to map their interactions by using GST-pull-downs and MALDI-TOF mass spectrometry. Results suggest a novel, nucleolar role for these proteins.

2. Review of the literature

2.1 Histone deacetylases and DNA condensation

In the nuclei of the cells, genetic material, DNA, is packed into chromatin to form chromosomes. In addition to DNA, chromosomes contain also chromosomal proteins, like histones (see fig. 2.1). Chromatin is composed of small and highly organized subunits, nucleosomes. A nucleosome consists of two molecules of the each core histone H2A, H2B, H3 and H4 surrounded by 146 bp of DNA (Park et al., 2005). Each nucleosome core particle is separated from the next by a region of linker DNA, which can vary in length from a few nucleotide pairs up to about 80. (Strahl & Allis, 2000, Alberts et al., 2002). Histone H1, a histone that is bound to linker DNA between nucleosomes is believed to be involved in condensation of nucleosome chains into higher order structures (Yamamoto & Horikoshi, 1996, Th'ng et al., 2005). The histones have positive charge due to their lysine residues and DNA is negatively charged because of its phosphate groups. Opposite charges attract each other and these bonds allow the structure of DNA to be very condensed.

A



B

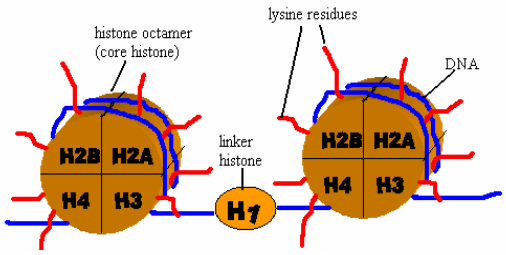


Figure 2.1 A) The structure of chromatin. Nucleosomes form structures (like rosettes) that further shrink the DNA. Thought the total length of DNA in a cell is 2m, this packaging shortens it to 200µm. Figure adapted from The Molecular Biology of the Cell figure 4-55 (Alberts et al., 2002) **B) The structure of the nucleosome.** The nucleosome is built of eight molecules of histones, two of each four core histones. Around the histone octamere, there is 146 bp of DNA. Histones H2A, H2B, H3 and H4 form the core histone, histone H1 is a linker histone between the nucleosomes. This figure is modified from the figure in de Ruijter's et al. (2003) article.

When genes are transcripted, the structure of DNA must loosen so that the genes become accessible to transcription machinery, and vice versa according to the cell's needs. In the area where transcription is not active, DNA is condensed and the genes are silenced (Allfrey et al., 1964, Kuo & Allis, 1998). Compacted nucleosome structure prevents pre-initiation complex formation and reinitation (Sheridan et al., 1997). Condensation-decondensation plays an important role in transcription regulation and it is achieved by many ways. One of the ways is the altering of the acetylation degree of histones (Wade, 2001). As mentioned, the histones have a positive charge but when their lysine residues are acetylated the charge become more neutral because an acetyl group bears a negative charge. The attraction between the histones and the DNA is not as strong as it used to be which causes decondensation of the DNA (Hong et al., 1993).

There are two groups of enzymes, which regulate the acetylation status of histones and thus, the gene expression: Histone acetyltransferases (HATs) and Histone deacetylases (HDACs). HATs transfer acetyl groups to the lysine residues of the histones leading to relaxed DNA structure and active gene transcription (Roth et al., 2001) while HDACs have the opposite function by removing acetyl groups (Hebbes et al., 1988, Grunstein M et al., 1997, Wade et al., 1997, Ayer, 1999) (see fig. 2.2). The HDACs act through the histone deacetylase complex (de Ruijter et al., 2003).

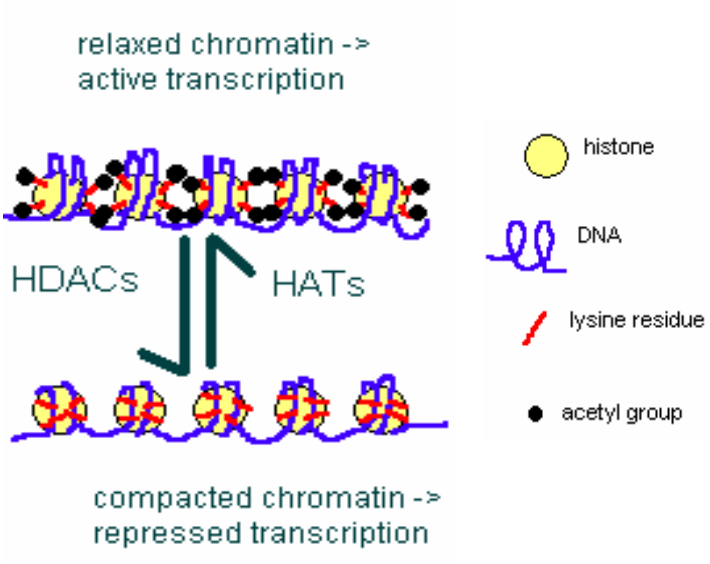


Figure 2.2 Transcriptional activity depends on the acetylation degree of histones. HDACs remove acetyl groups from histones and this leads to condensed DNA structure and inactive transcription while HATs have the opposite effect. This figure is modified from the figure in de Ruijter's et al. (2003) article.

2.2 Histone deacetylase complex Sin3A- members and functions

There are about 18 different HDACs (Gregoretti et al., 2004, Holbert and Marmorstein, 2005) recognized in human and they form at least seven different co-repressor complexes (Taunton et al., 1996, Zhang et al., 1997, de Ruijter et al., 2003). It has been shown that different combinations of proteins in certain complexes lead to silencing of different genes. However, only a set of genes (mainly those that are needed at certain points during the cells life) are regulated by acetylation/ deacetylation. This transcriptional repression is likely position-dependent and its mediated trough the interaction between DNA-binding proteins and components of the HDAC complex.

One of these complexes is called a Sin3 complex. The biggest member of this complex is a protein called mSin3A. It functions as a platform where other members, like Ying and Yang 1 (YY1) (Guo et al., 1995), retinoblastoma associated proteins 46 and 48 (RbAp46, RbAp48) (Qian & Lee, 1995), histone deacetylases 1 and 2 (HDAC1 and HDAC2) (Taunton et al., 1996) and SAPs (Zhang et al., 1997, Zhang et al., 1998, Fleischer et al., 2003, Lindfors et al., 2003), bind either directly or indirectly. (Fig 2.3). Some of the members of this complex are discussed next section of this chapter. SAP30 and SAP30L are presented in their own chapters, 2.3 and 2.4, respectively.

The enzymes, HDAC1 and HDAC2 share sequence identity of over 80% (de Ruijter et al., 2003). HDACs remove acetyl moieties from the ε-amino groups of histone lysine residues. 18 HDACs have been identified this far, in humans, and phylogenic analyses have subdivided them into four distinct classes: HDAC1 and 2 both belong to class I (as well as HDAC3 and 8) and they are homologous to yeast Rpd3. Class II HDACs (4-7, 9 and 10) are related to yeast Hda1. Class III contains sirtuins 1–7 that are related to yeast Sir2. Recently identified human HDAC11 constitutes class IV (Gregoretti et al., 2004, Holbert and Marmorstein, 2005). It has been shown, that HDACs have little activity alone and therefore the other members of the complex are required for the silencing the genes.

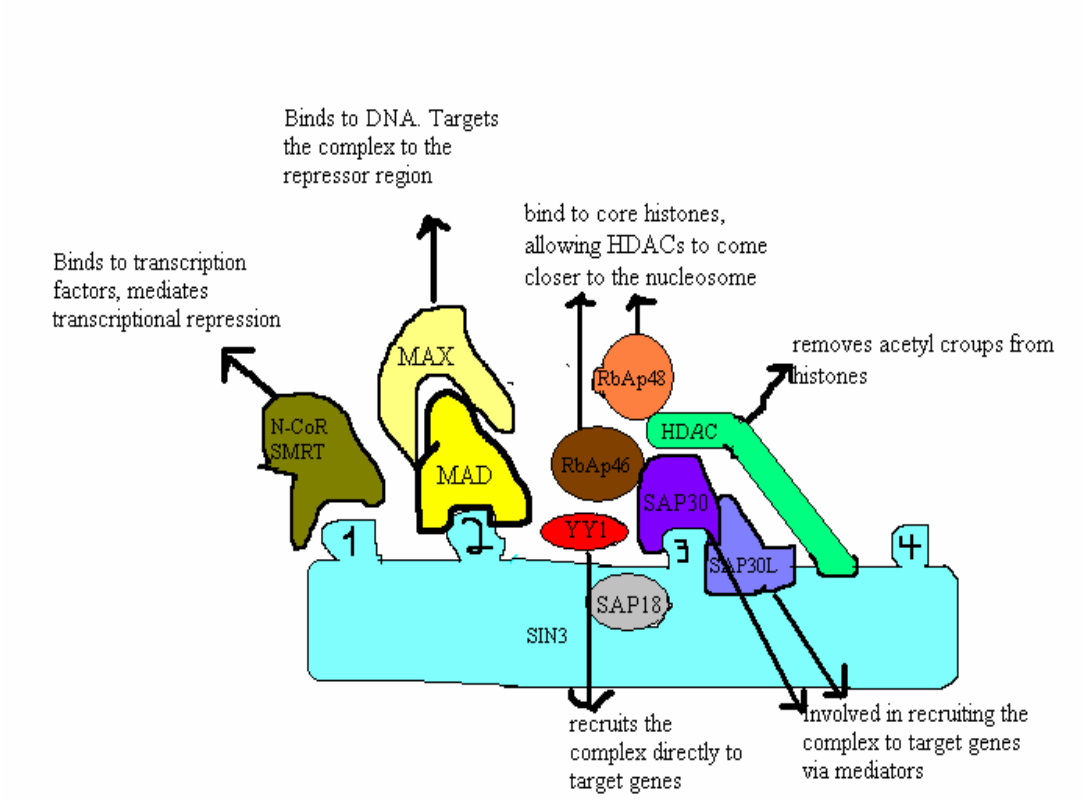


Figure 2.3 The mSin3a complex. In addition to mSin3a and HDAC, the complex contains many more members (not all of them are shown here). Different members have different functions, vital for the function of the complex. The members of the complex are not presented in scale, but giving the idea how they are located in the complex. Numbers 1 to 4 describe the polyamphipathic helix domains (PAHs) of Sin3a. Many members of the complex have been revealed to bind those regions. The figure is modified from figures presented by Laherty et al., (1997) and Zhang et al., (1998).

RbAps interact with core histones and are involved in histone modification, nucleosome assembly and nucleosome remodelling (Verreault et al., 1998). They seem to function as molecular bridges to bring HDACs (histone/nucleosome modifying enzymes) to their targets (Zhang et. al. 1998). They also target the complex to the right site via interactions with DNA-binding transcription factors.

YY1 recruits complex directly to target genes and binds to HDAC1 and 2 (Guo et al., 1995, Yang et al., 1997). It has also been reported to interact with SAP30. Via its binding to SAP30, YY1 recruits HDAC1 containing complex and thus is involved in gene repression (Huang et al., 2003).

Transcription regulators as Mad, Max and p53 bind to DNA and are intermediates between DNA and HDAC complex (Ayer et al, 1995, Manteuffel-Cymborowska, 1999). HDAC complexes are recruited to promoter elements through DNA-binding proteins that interact with Sin3A or other members of the complex. This, in turn, enables RbAps to interact directly with core histones of the remodelled nucleosome, allowing deacetylation of the core histones.

There are also other Sin3 associated proteins than SAP30 and SAP30L. These include SAP18 (Zhang et al., 1997), SAP45, SAP130 and SAP180 (Fleischer et al., 2003) (the numbers denote the size of the protein in kDa). Not much of the function of these proteins is known but all of them seem to bind to mSin3a and act as mediators of transcription repression. And at least SAP45, 130 and 180 are bound to HDAC-interaction domain (HID) of mSin3a. SAP180 is 40% identical to a previously identified mSin3A-associated protein, RBP1 and SAP45 is identical to mSDS3, the human ortholog of the SDS3p component of the Saccharomyces cerevisiae Sin3p-Rpd3p corepressor complex (Fleischer et al., 2003).

2.3 SAP30

Human SAP30 gene is located in chromosome 4q34.1. The gene contains four exons (the exon-intron structure is presented in figure 2.4). SAP30 protein consists of 220 amino acids and its molecular weight is 30 kDa. It was discovered and recognized as a member of Sin3A corepressor complex by Zhang and colleagues (Zhang et al., 1997, Zhang et al., 1998). SAP30 gene was expressed in all eight cell lines and 16 human adult tissues studied by Laherty et al. (1998).

According to Laherty et al. (1998), SAP30 is involved in N-CoR-mediated repression by specific transcription factors. It interacts with N-CoR via its N-terminus and binds to PAH3 region of mSin3a with its C-terminal part. (Laherty et al., 1998) (see also fig. 2.3). YY1 recruits the HDAC repressor complex via binding to SAP30. The interaction domains between YY1 and SAP30 were mapped to the C-terminal segment of YY1 and the C-terminal 91 amino acid region of SAP30 (to the same region as the interaction domain with mSin3a) (Huang et al, 2003). Later SAP30 has

been shown to interact, among other things, with human herpesvirus 8 LANA and CIR proteins (Krithivas et al., 2000).

Figure 2.4 Exon-intron structure of human SAP30. Human SAP30 consists of four exons. The SAP30 gene is located in chromosome 4q34.1. Figure obtained from NCBI database.

2.4 SAP30L

Lindfors et al. (2003) found SAP30L in an *in vitro* mesenchymal-epithelial cell culture model, which was set to study differentiation of intestinal epithelial cells in the crypt villus axis. When transforming growth factor- β (TGF-β) was added into this culture, mRNA levels of certain proteins increased indicating that these proteins may play a role in epithelial cell differentiation. SAP30L mRNA levels increased about two-fold after TGF-β stimulation.

SAP30L got its name because it turned out to be 70% identical with SAP30 by amino acid sequence. Hence the name SAP30L (L in the name comes from like). Human SAP30L gene is located in chromosome 5q33.2. The gene has four exons (the exon-intron structure is presented in figure 2.5).

Figure 2.5 Exon-intron structure of human SAP30L. When compared to the exon-intron structure of SAP30: they both have four exons, the first exon is the longest and the third shortest in the either case, and exons two and four seem to have equal length. Figure obtained from NCBI database.

SAP30L protein contains 183 amino acids and its calculated molecular weight is about 21 kDa. Compared to SAP30, it lacks 38 aa in the N-terminus, otherwise they are highly identical. The sequences are aligned in figure 2.6. SAP30L mRNA was expressed in all 12 human tissues studied by Lindfors et al. (2003).

Homologous studies show that SAP30L is evolutionary older than SAP30 because only mammals have both of these proteins and SAP30 of evolutionary lower animals resembles more SAP30L than SAP30 of mammals. (Lindfors et al., 2003)

```
SAP30L      MNGFSTEEDSR-----------------------------------
SAP30       MNGFTPEEMSRGGDAAAAVAAVVAAAAAAASAGNGNAAGGGAEVPGAGA
            ****: ** **

SAP30L      EGPPAAPAAAAPGYGQSCCLIEDGERCVRPAGNASFSKRVQKSISQKKLK
SAP30       VSASGPPGAAGPGPGQLCCLREDGERCGRAAGNASFSKRIQKSISQKKVK
                : *:**:** ** *** ******:* *********:********:*

SAP30L      LDIDKSVRHLYICDFHKNFIQSVRNKRKRKTSDD-GGDSPEHDTDIPEV
SAP30       IELDKSARHLYICDYHKNLIQSVRNRRKRKGSDDDGGDSPVQDIDTPEV
            :::***:*******:*** ********** *** *****  * * ***

SAP30L      DLFQLQVNTLRRYKRHYKLQTRPGFNKAQLAETVSRHFRNIPVNEKETLA
SAP30       DLYQLQVNTLRRYKRHFKLPTRPGLNKAQLVEIVGCHFKSIPVNEKDTLT
            ** ************* ** **** *****:* *  **: ******:**

SAP30L      YFIYMVKSNKSRLDQKSEGGKQLE
SAP30       CFIYSVRNDKNKSDLKADSGVH-—
             *** *:  * : * * : *
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Figure 2.6 Alignment of human SAP30L with human SAP30 amino acid sequences. They are highly identical except for the 38 aa in the N-terminus, which is lacking in SAP30L (indicated with -). Asterisks mark identical, colons similar (e.g. hydrophobic, like leusine and isoleusine) amino acids. Adapted from the article by Lindfors et al. (2003).

SAP30L recently been found to interact with mSin3A with the region between amino acids 120 and 140, and to bind to the PAH3/HID-region of mSin3a (SAP30 binds to PAH3 region of mSin3a). It has been shown to interact with HDAC1 and 2. SAP30L has also been identified to have repression activity. (Viiri et al., in press). However, SAP30L does not seem to interact with N-CoR (data not published) like SAP30 does. Probably because SAP30- N-CoR interaction is mediated by N-terminal region of SAP30 which lacks from SAP30L.

Immunocytochemical studies indicate that both SAP30 and SAP30L are localized into the nucleolar region. SAP30 and SAP30L got, in addition to nuclear localizing signal (NLS) also the signal that targets them to the nucleolus, nucleolar localizing signal or NoLS (Viiri et al., in press). When the NoLS is mutated, SAP30L “leaks” out from the nucleolus tough it is not completely removed. Because the nucleolus is a membraneless organelle, it has been proposed that a protein does not need the NoLS

to get into the nucleolus but to be able to stay there. There is also some evidence that SAP30L would target Sin3a to the nucleolus.

Beausoleil et al., (2004) determined phosphorylation sites of proteins from HeLa cell lysates (there were almost 1000 proteins in their study) using tandem mass spectrometry. Interestingly, both SAP30 and SAP30L were identified to have phosphorylation sites in their serine residues: serines 131 and 138 for SAP30 and serine 99 for SAP30L, respectively. This finding may give some evidence that the activity of SAPs could be regulated by phosphorylation.

2.5 Diseases related to histone deacetylases

One of the ways gene expression is regulated by is the acetylation status of histones (as already mentioned). In many diseases, gene expression is altered compared to normal cells. That is why it is not surprising that altered acetylation degree of histones seems to be behind a bunch of diseases. The relationship of HDACs and cancer, e.g. in leukaemia, has been known for a long time. Later it became obvious that HDACs can play a role in other diseases as well, like cardiovascular and respiratory diseases.

2.5.1 HDACs and cancer

In cancer cells, the gene expression is aberrant. Compared to normal cells, different genes are expressed in cancer cells and in different amounts. Because the acetylation status of histones is a key component in the regulation of gene expression, HDACs and their relation to cancer have been studied. There is some evidence that HDACs have a role in cancer development although no direct alteration in the expression of HDACs has yet been demonstrated in human oncogenesis. The results have shown that HDACs associate with a number of well-characterised cellular oncogenes and tumour suppressor genes (e.g. MAD and retinoblastoma protein (Rb)) leading to aberrant recruitment of HDAC activity, which in turn results in changes in gene expression. (Marks et al., 2001)

In leukaemias (like acute promyelocytic leukaemia and acute myelocytic leukaemia), chromosome translocations lead to fusion of genes and production of oncoproteins.

These oncoproteins appear to suppress the transcription of specific genes through the recruitment of HDAC (Minucci et al., 2001). Without the expression of appropriate genes, the cell is unable to undergo differentiation. This leads to excessive proliferation of the cell and finally to cancer (He et al., 2001). One of these oncoproteins is LAZ3 (BLC-6) which is disrupted by chromosome translocation in non-Hodgkin lymphomas (Kerckaert et al., 1993). LAZ3 is a transcriptional repressor and it associates with both mSin3a and HDAC1 so it may be involved in recruiting the HDAC complex (Dhordain et al., 1998).

Many different types of molecules have been developed to inhibit HDACs (called HDAC inhibitors, HDACi). Among these inhibitors are trichostatin A (TSA) (Yoshida et al., 1990), which is the most common HDACi and trapoxin (Kijima et al., 1993) and butyrate (Della Ragione et al., 2001). Inhibition of HDACs results in hyperacetylation of histones followed by the transcriptional activation of certain genes through relaxation of the DNA conformation (Lin et al., 1998, Suenaga et al., 2002). These inhibitors have been shown to induce growth arrest, differentiation and apoptosis of cancer cells both *in vitro* and *in vivo*.

He and colleagues showed that, in transgenic mice, HDACi induced apoptosis and growth inhibition leading to remission of therapy- resistant acute promyelocytic leukaemia and prolonged survival. (He et. al, 2001). Suenaga et al. (2002) have studied HDACi and their role in telomerase activity. Telomerase activity is involved in cellular immortality: cells stop dividing when their telomeres are too short. Normal cells have no telomerase activity and thus limited live span, while cancer cells have active telomerase and thus continue dividing (Kim et al., 1994, Alberts et al., 2002). They found out that in prostate cancer cells, HDAC inhibitors are able to suppress cell proliferation by down-regulating telomerase activity via suppression of reverse transcriptase mRNA expression. (Suenaga et. al., 2002).

There is some evidence that SAP30 might be a tumor suppressor gene (TSG): Sironi and colleagues studied basal cell carcinoma (BCC) and identified a significant loss of heterozygosity (LOH) in the chromosomal region 4q32-35 (remember that SAP30 is located in 4q34.1). Identification of LOH at specific loci in a tumoral sample suggests the presence of a TSG within the deleted region (Sironi et al., 2004).

2.5.2 HDACs play a role also in other diseases

HDAC complexes are involved in other diseases as well. In these diseases, there have been established either hypoacetylation of histones (silenced transcription) or inactivity of HDACs/hyperactivity of HATs (the cell is unable to switch off genes).

The former is true in some cardiac diseases. It has been shown that hyperactivity of HDACs leads to cardiac muscle hypertrophy by silencing of anti-growth genes and activation of pro-growth genes and finally to heart failure (McKinsey & Olson, 2005).

There are also HDACs that repress transcription of pro-growth genes of heart cells. Mutations in these HDAC genes cause hypertrophy because the expression of pro-growth genes is no longer controlled. However, these HDACs (like HDAC5 and 9) belong to a different group (class II) than HDACs involved in the Sin3a repressor complex (class I).

The latter effect is observed in inflammatory lung diseases, like asthma, where expression of multiple inflammatory genes is increased because hyperactivity of HATs and inactivity of HDACs (especially HDAC1). (Barnes et al., 2005). If the patient with asthma smokes, the reduction of HDAC activity is even higher (Murahidy et al., 2005). In chronic obstructive pulmonary disease, HDAC2 activity is decreased and the reduction in the activity is correlated with severity of the disease and resistance to corticosteroids (To et al., 2004, Ito K et al., 2005).

As HDACs seldom operate alone, also the other members of the complex, like SAPs (hopefully), may be possible targets of inhibitors and are worth considering when designing new (cancer) drugs.

2.6 The Nucleolus

2.6.1 The structure of the nucleolus

Inside the nucleus, there is an area called the nucleolus, which is seen concentred in microscope because of the condensed chromatin (see figure 2.7 A) (Alberts et al., 2002). The nucleolus is a site of ribosome biogenesis (Lewis and Tollervey, 2000).

The nucleus is enclosed by the nuclear envelope, but nucleolus itself, unlike many other organelles, is not bound by any membrane. The nucleolus is a dynamic structure in the cell: it disappears during mitosis and is reassembled during G1-phase of the cell cycle (Anastassova-Kristeva, 1997). Cells with active protein syntheses and thus ribosome production, have prominent nucleoli.

The nucleolus is formed around the ribosomal DNA (rDNA) repeats. These repeats are clustered in five different chromosomes (13, 14, 15, 21 and 22) at loci called nucleolar organizing regions (NORs) (Anastassova-Kristeva, 1997). In addition to rRNA genes, rRNA precursors, ribosomal proteins and partly assembled ribosomes, the nucleolus consist of also many proteins and enzymes like nucleolin, fibrillarin, small nuclear and nucleolar ribonucleoproteins (snRNPs and snoRNP) needed for ribosome biosynthesis (Fatica and Tollervey, 2002). The nucleolus can structurally be divided into three distinct areas (see figure 2.7 b): fibrillar centres (FC) surrounded by a dense fibrillar component (DFC), the rest of the nucleolus is called granular component (GC) (Alberts et al., 2002). These three areas are believed to have different functions and many nucleolar proteins are localized in one of the three areas (e.g. fibrillarin is found mainly in a dense fibrillar component).

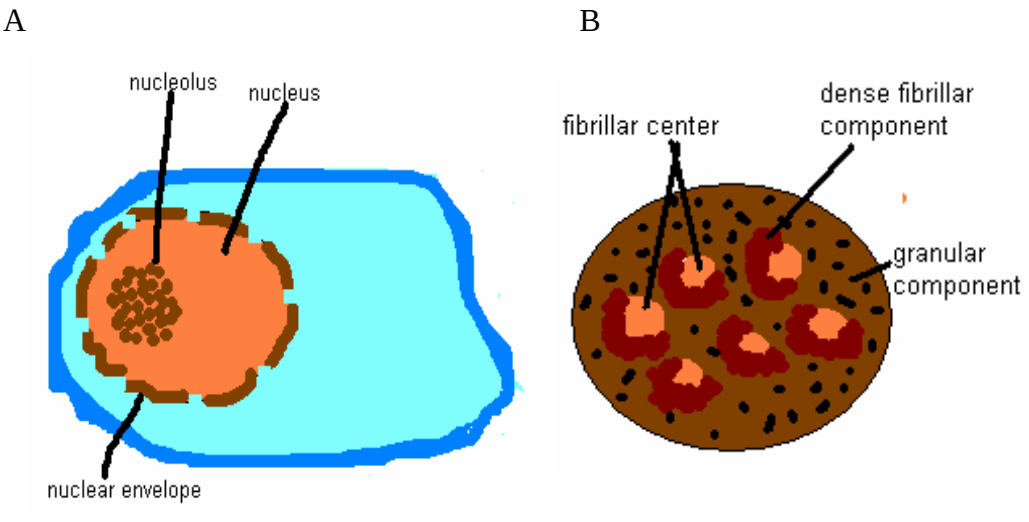


Figure 2.7 The nucleolus and its structure. A) The nucleolus is an area inside the nucleus. Depending on the cells activity, it can occupy almost 20% of the nucleus B) The three different areas of the nucleolus. Each area is believed to have different functions.

2.6.2 Functions of the nucleolus

The nucleolus is the organelle where ribosome biogenesis (ribosomal DNA transcription, rRNA processing and ribosome assembly) takes place (see figure 2.8) (Lewis & Tollervey, 2000). There are four types of eukaryotic rRNAs. Three of them, 5.8S, 18S and 28S are transcribed in the nucleolus as a larger precursor RNA which is then chemically modified and cleaved to form these rRNAs. The fourth rRNA, 5S, is synthesized in nucleoplasm. In addition to rRNAs, ribosomes are composed also of ribosomal proteins. 18S rRNA forms the smaller ribosomal subunit (40S) together with 33 ribosomal proteins. The larger subunit (60S) consists of 49 ribosomal proteins and the 5, 5.8 and 28S rRNAs. Ribosomes are needed for protein synthesis and thus nucleolus has a role in cell growth. (Fatica & Tollervey, 2002, Alberts et al., 2002).

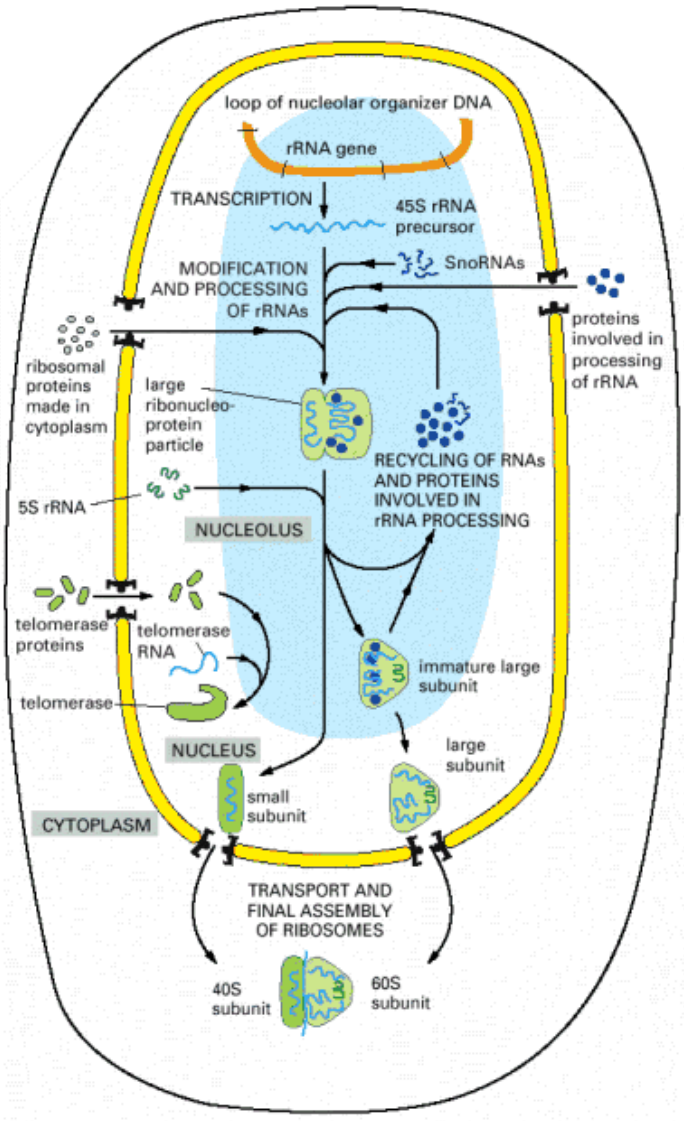


Figure 2.8 The function of the nucleolus in ribosome synthesis. Ribosomes are composed of ribosomal RNA which are mainly transcribed in the nucleolus (only 5S rRNA is transcribed in the nucleus) and ribosomal proteins made in cytoplasm. These are then assembled into small and large subunits which are combined together in cytoplasm forming the functional ribosome. These processes need a lot of enzymes and proteins. Adapted from The Molecular Biology of the Cell, Figure 6-47 (Alberts et al., 2002).

Ribosome assembly needs many proteins and enzymes. Nucleolin, for example, is involved in the first processing step of precursor ribosomal RNA. It catalyses the first cleavage of pre-rRNA (45S) from which three rRNAs are formed (Ginisty et al., 1998, Ginisty et al 1999). Fibrillarin is needed in pre-rRNA processing. If the gene is mutated, progressive impairment of all pre-rRNA processing steps is seen and rRNA is not methylated effectively (Tollervey et al., 1991).

Ribosome biogenesis involves also nucleotide modification of pre-RNA, like Decatur and Fournier described in their article (2003). The most common modifications are formation of 2'-O- methylated nucleosides and conversion of uridine to pseudouridine. These modifications have been shown to be important for the activity and function of the ribosome. The modifications are mediated by complex, called snoRNP, which contains small nucleolar RNA and ribonucleoproteins. The methylation process involves at least nucleolar proteins 56 and 58 (Nop56 and Nop58, respectively) and fibrillarin (accepted to be methyltransferase). (Decatur & Fournier, 2003). In figure 2.9, the modifications are shown in more detail.

Recent studies have suggested that the nucleolus is involved in other important functions like cell cycle control (remember that the nucleolus disappears during mitosis). Also other RNAs are produced and other RNA-protein complexes (snRNPs and snoRNPs) are assembled in the nucleolus (see figure 2.8). The nucleolus is also believed to have a role in tumorgenesis and aging. (Olson et al., 2000) Maybe because telomerase, an enzyme related to elongation of telomeres at the end of chromosomes, is assembled in the nucleolus. Normal cells loose their telomeres gradually when they divide, thought some telomeric repeats are added after every cell division. And when their telomeres are short enough, they stop dividing. In contrast, all immortal cells (cancer cells) show no net loss of telomere when they divide. It has been shown that maintenance of telomeres is required for cells to escape from replicative senescence and proliferate indefinitely. (Kim et al., 1994)

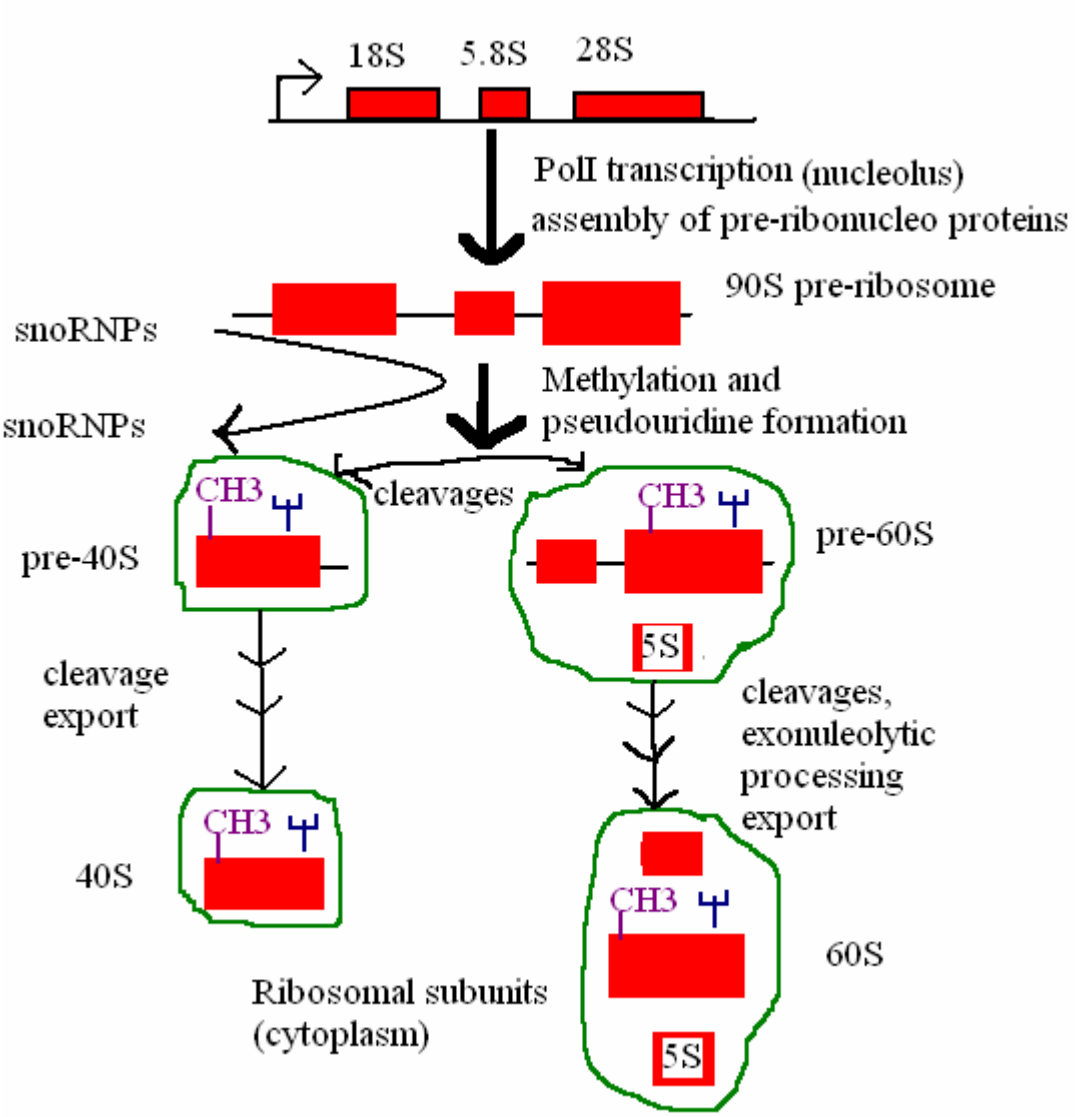


Figure 2.9 Modification of rRNA by snoRNPs. Ribosome biogenesis involves in addition to cleavages and processing also methylation at 2' carbon of the ribose and conversion of uridine to pseudouridine. Figure modified from the figure presented in Decatur & Fournier's article (2003).

2.7. Theory behind the methods

2.7.1 GST- Pull-down

In the cell, proteins form extensive interaction networks via which they participate in cellular pathways. The function of a protein is determined by its interactions with other proteins or other molecules. Revealing the function of a protein helps us to understand its role in the cell and consequences of an abnormal protein (Alberts et al.,

2002, Golemis, 2002). The pull-down techniques have become invaluable tools for studying these pathways via protein: protein interactions.

The GST fusion protein pull-down technique is an *in vitro* method used to determine physical interaction between two or more proteins. The method uses the high binding affinity of GST for glutathione. Glutathione –coupled beads (e.g. glutathione sepharose 4 beads) are used to purify interacting proteins from a protein solution (Einarson, 2001). The assay needs a purified and tagged protein, the bait (GST fusion protein), which will be used to capture and 'pull-down' a protein-binding partner, the prey, from cell lysate. GST-pull-down assay is useful for both confirming the existence of a predicted protein: protein interaction and identifying previously unknown interactions (Einarson & Orlinick, 2002, Schantl et al., 2003, Vikis & Guan, 2004). In the former case, the cDNA of the prey protein is transfected into cells and the protein is expressed in large amounts. These proteins are recognized by antibodies, either against themselves or a tag (e.g. His or myc) they contain. In the latter case, cell lysate proteins are used as preys and interacting proteins are identified by mass spectrometry.

The principle of the method is following (see fig. 2.10): The cDNA of the bait protein is cloned into a vector after GST gene. Then the GST-fusion protein is expressed in bacteria and purified from other bacterial proteins with glutathione -coupled beads (bacteria have not got GST). Then cell lysate is added and if there are some proteins that interact with the fusion protein they bind to it. The unbound proteins are washed away and the fusion protein- cell lysate protein complexes are collected by centrifugation with help of glutathione sepharose beads. Then these complexes are run on the SDS gel and interesting bands are either cut out and sent to the MALDI-TOF to be identified or the Western blotting followed by antibody staining is performed. Plain GST with the cell lysate is used as a negative control to exclude those proteins from the analysis that interact with GST (to prove that the interaction isn't mediated by GST but the protein fused to it. It is also good to put fusion proteins with no cell lysate as a control to exclude the bands coming from the fusion protein itself (important if mass spectrometric analysis is performed).

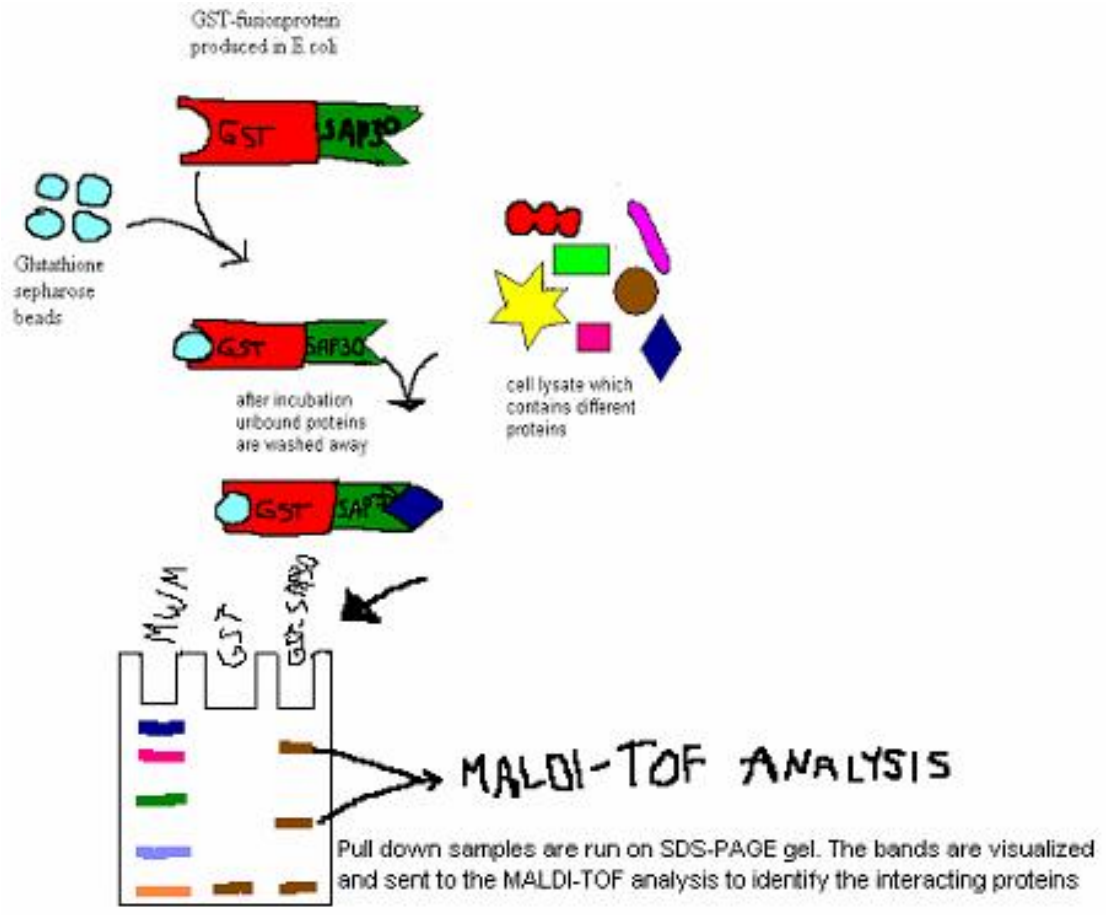


Figure 2.10 The principle of the GST pull-down. The fusion protein presented here is GST-SAP30. Here unknown proteins are the interest of the study. In the case of known proteins, antibodies are used to identify them. The blue protein (diamond) binds to GST-SAP30 fusion protein while the yellow protein (star) does not interact with SAP30 and does not bind. The band found also in the GST lane (at the orange marker) is excluded from MALDI-TOF analysis because it interacts with GST (or more probably is GST itself).

2.7.2 MALDI-TOF

The history of mass spectrometry began over a decade ago based on early experiments of Sir J. J. Thomson (Borman et al., 1998). After that, it rapidly became an important tool for researchers and lots of applications have been developed since then. Currently, mass spectrometry is used in proteomics in three major areas (Mann et al., 2001): for characterization and quality control of recombinant proteins, detection and characterization of posttranslational modifications and identification of proteins. One of the most important methods based on mass spectrometry is MALDI-TOF.

MALDI-TOF means Matrix-assisted Laser Desorption Ionization Time-of-Flight. The instruments needed to perform a mass spectrometric analysis are a chamber, where the ionization can take place, a matrix, where analyte is placed, a laser source that produces ionization, a detector, which collects the ions and an analyzer, which produces the spectra. The analyzer is usually a TOF-analyzer though other analyzers exist. Between the matrix and the detector there is a field free region (meaning there is no electric field, which could trap the ions or change their movement) through which the ions travel (Yates, 1998). MALDI was developed by Karas & Hillenkamp in the 1980s and it begun a new era in mass spectrometric research. (Karas et al., 1987, Karas & Hillenkamp, 1988). MALDI is very sensitive and accurate: only about a femtomole of peptide material is enough to produce signal and the masses of analyzed peptides are given in the range of a few ppm (parts per million) accuracy (Huang L et al., 2002, Mann et al., 2001).

MALDI-TOF is based on the bombardment of sample molecules with a laser light to bring about sample ionisation (Yates, 1998, Mann et al., 2001). The ionized sample fragments move through the field free region to the detector. The detector monitors the ion current, amplifies it and the signal is then transmitted to the analyser. The TOF analyser separates ions according to their mass-to-charge (m/z) ratios by measuring the time it takes for ions to travel through a field free region. The heavier ions move slower than the lighter ones (see fig. 2.11). The m/z values of the ions are plotted against their intensities to show the number of components in the sample, the molecular weight of each component, and the relative abundance of the various components in the sample and the results are recorded in the form of mass spectra. (see fig 2.12).

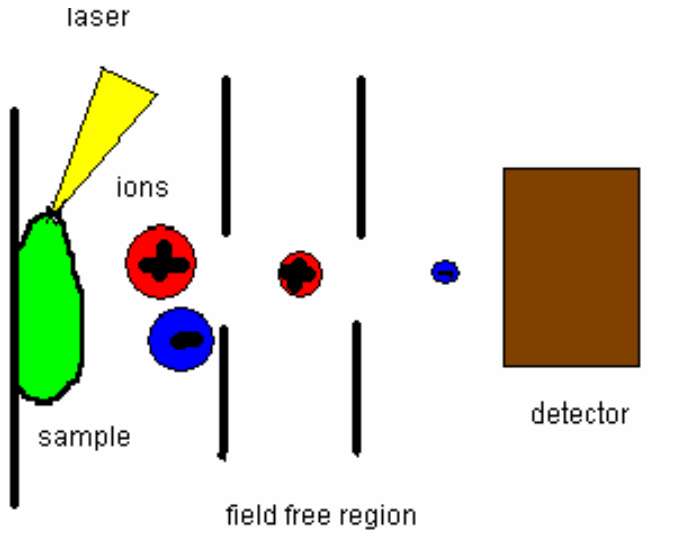


Figure 2.11 The principle of the MALDI. Protein sample is digested with appropriate enzyme e.g. trypsin to produce peptide fragments. Laser causes ionization in the sample. The ions move through the field free region to the detector, which monitors the ion current. This data is then transformed to the TOF analyser, which separates the ions according to their mass-to-charge ratio.

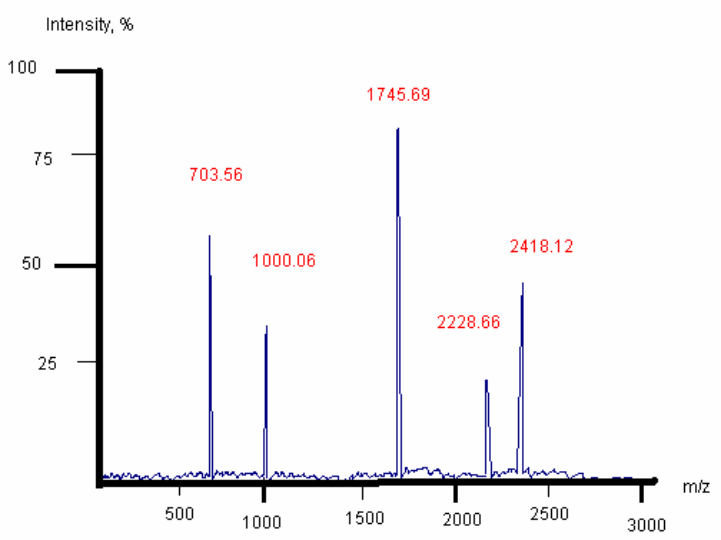


Figure 2.12 The spectra from the TOF analyzer. The m/z ratios identify the ions (each ionized sample fragment, e.g. oligopeptides, has a different m/z ratio) and the intensity tells their number in the sample. Different proteins give different spectras and with help of mass spectrometric tools (e.g. Mass-Fit) they can be identified. The spectra presented here is from a fictitious protein.

There are many proteomic tools, like Mascot (Perkins et al., 1999), which was used in this research project, ProteinProspector (Clauser et al., 1999) and PepIdent (Wilkins & Williams, 1997), which provide protein identification service. The values of the peaks of the spectra are used to identify the sample. In the case of a protein, each peak corresponds to an oligopeptide. Programs compare these values to theoretical spectras

of known proteins in the database (Yates, 1998). If there are proteins that have corresponding peaks they are listed according to their scores (see fig.2.13). The program shows oligopeptide(s) that have matched to the database protein and gives their P-values, which tells the probability that the oligopeptide is from other protein. The P-value of the peptide depends on the uniqueness of the sequence and it should be <0.05 to be significant (less than a 1 in 20 chance that the observed match is a random event). Score is number of amino acids in matched peptides, the higher the score the better. Other important value is coverage, which is the number of aa in matched peptides (score) divided by the number of aa in the whole protein. Coverage is usually given as percentage and it should be >15% to be valid. Programs give also molecular masses of the predicted proteins that can also be used to evaluate the data (if mass of the protein is near to the mass of the band on the gel it may be the right protein). None of these values alone is enough but the results should evaluate as entirety.

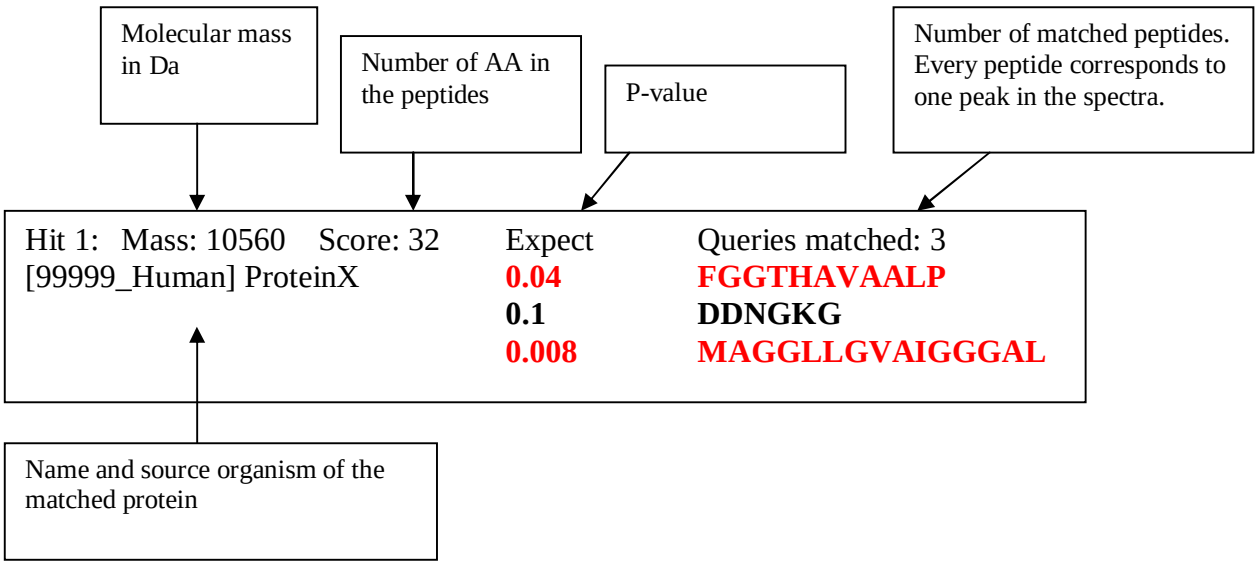


Figure 2.13 An example of MALDI-TOF results. Significant hits are in red, others in black. This fictitious protein contains 96 aa, so the peptide coverage is 33%. The score is low but it is because the protein is so short not because there is not enough matched peptides. This could be correct match because it contains two significant matches, the coverage-% is high and it was found at the 10 kDa marker. Usually the program gives more matched proteins among which one selects the most probable according to their parameters.

Though a mass spectrum of the protein digested by an enzyme provides a fingerprint of great specificity there are also some disadvantages: Because MALDI-TOF is very

sensitive, impurity of the sample causes problems and the samples should be handled with care that no contaminants (e.g. keratin from skin) enter to the sample. It is limited to the identification of proteins which sequences are already known. Non-specific cleavages and unpredictable posttranslational modifications produce fragments not found in the database. And if the band digested contains mixture of proteins, number of insignificant hits increases. (Huang et al., 2002). The MALDI-TOF device is also quite expensive.

3. Aims of the research

The aim of this project was to find out proteins interacting with SAP30L and SAP30. We used the GST pull-down method for “fishing” and MALDI-TOF for the identification of proteins. Two interacting proteins identified in this screening, nucleolin and fibrillarin, were further analysed by using immunoblotting and immunocytochemistry.

4. Materials and methods

4.1 Production and purification of the GST-fusion proteins

Human SAP30 and SAP30L were produced in *E. coli* as GST-fusion proteins. The cDNAs of SAP30 and SAP30L (SAP30 cDNA was obtained from IMAGE clone 4074154 and SAP30L cDNA was purified from mRNA (Lindfors et al., 2003)) were cloned into the pGEX-4T-1 vector (Amersham Biosciences) into EcoRI -XhoI and BamHI -EcoRI restriction sites, respectively (Fig. 4.1). Then the plasmids were transformed to the *E. coli* BL21 strain, according to manufacturer's procedure.

Overnight cultures, grown in LB-medium and 50µg/ml ampicillin at +37°C, were grown from above mentioned bacterial stocks. An aliquot (1:50) of the overnight cultures was added to fresh LB-medium with 50µg/ml ampicillin and grown at +37°C until the logarithmic growth phase (optical density at 600 nm 0.6). Fusion protein production was induced by adding IPTG to final concentration of 1mM and grown at +37°C for three hours. The bacteria were harvested by centrifugation at 5000xg at +4°C for 15 min. The pellets were resuspensed in PBS with freshly added protease inhibitors (Roche) and lysozyme, and incubated on ice for 30 min. Lysates were sonicated 4x20 sec to degrade genomic DNA. PBS containing 0.2% TritonX-100 and freshly added protease inhibitors (Roche) and DNAse and RNAse to final concentrations of 5µg/ml were added to the solutions and the tubes were shaked at +4°C at 100 rpm for 10 min. Then the proteins were collected by sedimentation at +4°C at 3000xg for 30 min. The supernatants were collected and 1M DTT was added to final concentration of 1mM. The fusion proteins were cleared from the other proteins with glutathione sepharose 4B beads (Pharmacia Biotech, Amersham Biosciences) at +4°C end over end rotation overnight. The beads were collected by sedimentation at 500xg for 5 min at +4°C, washed by resuspension and sedimented once with PBS containing 1M NaCl and 0.05% Igepal-CA630 and three times with PBS containing 200mM NaCl and 0.05% Igepal-CA630. (modified from Sambrook and Russel, 2001).

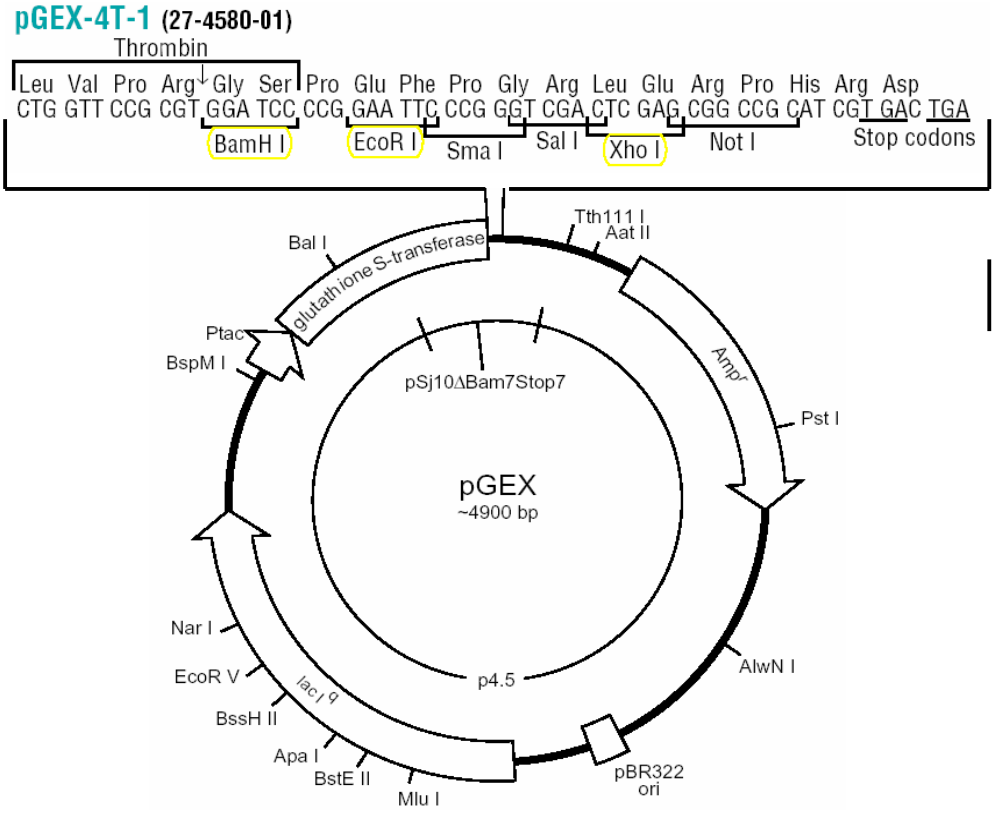


Figure 4.1 pGEX4T-1 vector. The cloning sites of SAP30 and SAP30L are circulated, EcoRI-XhoI and BamHI-EcoRI, respectively. The cloning sites are right after the GST gene. The vector has an ampicillin resistance gene as a selection marker. This vector contains also a thrombin cleavage site in front of the cloning site. Figure adapted from http://www4.amershambiosciences.com/pdfs/970004M2-01.pdf

4.2 Preparing the cell lysates

Daudi cells (human Burkitt lymphoma cell line) were cultured in RPMI 1640 (Gibco) supplemented with penicillin antibiotic, 10% fetal bovine serum (FBS) and L-glutamine. The nuclei from those cells were extracted as follows: Cell suspension was poured to Falcon tubes and the tubes were centrifuged at 400xg for 3 min. The supernatants were discarded and the pellets were resuspensed in hypotonic lysis buffer (25mM HEPES, 10mM $MgCl_2$, 10mM KCl, 0.625% Igepal-CA630 and freshly added protease inhibitors (Roche)). Tubes were incubated on ice for 20 min and then centrifuged in 800xg at +4°C for 10 min. The supernatants were discarded and the pellets were resuspensed in D-buffer (50mM HEPES, 20% glycerol, 10% sucrose, 0.42M KCl, 5mM $MgCl_2$, 0.1mM EDTA, freshly added DTT to 1mM and protease

inhibitors (Roche)). This solution was sonicated for 4x20 sec and then centrifuged at 5000xg in +4°C for 20 min. The supernatant was taken into a new tube and bovine serum albumin (BSA) was added to final concentration of 0.10mg/ml to stabilize proteins in the solution.

4.3 GST- pull down

11ml of the cell lysate and 200µl of the purified GST-SAP30 or GST-SAP30L fusion proteins beads (bead vol. 100µl) were incubated end-over-end rotation overnight in a 15ml Falcon tube at +4°C. GST with the cell lysate and GST-SAP30 and GST-SAP30L with D-buffer were used as negative controls. The samples were centrifuged at 500xg at +4°C for 5min and the supernatant was removed. The beads were washed five times with PBS containing 250mM NaCl and 0.05% Igepal-CA630 and once with PBS. Between the washes the samples were incubated at +4°C end over end rotation for 10 min and centrifuged at 500xg at +4°C for 5min. The supernatants were removed.

Bound proteins were eluted using 1.5 M NaCl in PBS and incubated for 1 h at RT vortexing every 10 min. The tubes were centrifuged at full speed for 2 min. The pellets were resuspensed in 30µl of Laemmli and stored at -20°C. The supernatants were collected and concentrated with trichloroacetic acid/sodium deoxylate precipitation (TCA/DOC). 1/100 vol of 2% DOC (Sodium deoxylate, detergent) was added to the tube, vortexed and incubated at +4°C for 30 min. Then 1/10 vol of 100% TCA (Trichloroacetic acid, prepared by adding 454ml water per 1 kg of TCA) was added, vortexed and incubated overnight at +4°C. Then samples were centrifuged at maximum speed (15000xg) at +4°C for 15 min. The supernatant was discharged. The pellet was washed twice with cold acetone. The samples were vortexed and repelletted at full speed for 5 min at +4°C. Pellets were dried and resuspensed in 30µl of reducing sample buffer (Laemmli).

4.4 SDS-PAGE and silver staining

The proteins were resolved by SDS-PAGE (10% gel, Hoefer gel (Bio-Rad)). 26µl of the samples a control input (Daudi cell lysate) and 5 µl of the molecular weight markers (Kaleidoscope Prestained Standard, Bio-Rad) were pipetted into the wells. The proteins were visualised with Silver Staining (Bio-Rad Silver Staining Plus Kit): At first the proteins were blocked with Fixative Enhancer solution (50% methanol, 10% acetic acid, 10% Fixative Enhancer Concrete) for 20 min after which the gel was rinsed twice with distilled water for 10 min. Next the staining solution containing 5 ml of Silver Complex Solution, 5 ml of Reduction Moderator solution and 5 ml of Image Development Solution in 35ml of distilled water and 50 ml of freshly added Development accelerator solution was poured onto the gel. The stain was allowed to develop for 15 min after which the reaction was stopped by adding 5% acetic acid for 15 min. The gel was stored in distilled water.

A total of 40 protein bands were selected from the gel and cut out. The gel pieces were put into 1.5 ml eppendorf tubes, vacuum dried and sent to Turku Centre for Biotechnology for identification. (Proteomics and Mass spectrometry Unit University of Turku).

4.5 Protein Identification by Peptide Mass Fingerprinting

Proteins were in-gel digested for identification as described in previous works by Rosenfeld et al. (1992), Shevchenko et al. (1996), Nyman et al. (2000) and Havliš et al. (2003), with slight modifications. In brief, dried gel bands were re-hydrated with MilliQ water (20 µl), washed twice with 0.2 M NH_4HCO_3, then reduced and alkylated. These were subsequently incubated on ice for thirty minutes with 0.4 µg Sequence Grade Modified trypsin (Promega, Madisson, WI, USA), after which 0.1 M NH_4HCO_3 in 10 % acetonitrile was added such that the gel pieces were completely covered, then incubated for 30 minutes on ice, followed by digestion over night at 37^{o}C. Peptides were extracted from the gel pieces with 5 % formic acid/50 % acetonitrile (2 x 20 µl), and dried in a vacuum centrifuge. These were re-dissolved in 2% formic and divided into two aliquots for analysis and/or storage (-20 ^{o}C).

Analyses were made with automated on-line desalting and pre-concentration coupled with nanoscale capillary LC-MS/MS . An UltimateTM capillary LC system was used with a Famos autosampler, and a Switchos Micro column switching module (LC Packings, The Netherlands), coupled to a quadrupole time-of-flight mass spectrometer (Q-Star Pulsar, ABI/MDS-SCIEX, Toronto, Canada). A 300 µm × 1 mm PepMapTM -pre-column (LC Packings) was used at a flow rate of 40 µL/min for desalting and pre-concentration. Reversed phase separations were performed at 200 nl/min with a 75 µm × 15 cm C-18 column (packed in-house). A separations gradient from 95 % solution A (5% acetonitrile: 95% water: 0.1% formic acid) to 20 % solution B (95% acetonitrile: 5% water: 0.1% formic acid) in 5 min, then to 60 % B in 20 min was employed.

Peak lists were created for the Mascot Server (ver. 2.0, Matrix Science Ltd.) using AnalystQS software (Service Pack 8, Applied Biosystems) with the AnalystQS Mascot script search parameters such that MS/MS data was centroided with a height percentage of 50 and a merge distance of 0.01 Da. Searches were made against the Swiss-Prot -database with the following parameters specified: mass accuracy 0.3 Da and 0.3 Da for the parent and fragment ions respectively, trypsin digestion with up to one missed cleavage, carbidomethylated cysteine as a fixed modification, and oxidised methionine as a variable modification.

4.6 Western blotting

K-562 (human erythromyeloblastoid leukaemia cells) cells were cultured in RPMI 1640 (Gibco) supplemented with penicillin antibiotic, 10% fetal bovine serum (FBS) and L-glutamine. The cell lysate was prepared according to the same protocol as Daudi cell lysate (see 4.2). This lysate was used in pull-down experiments similar to those done by Daudi cells.

The protein samples were boiled in SDS-PAGE sample buffer and resolved on 10% Novex® pre-cast gels (InVitrogen). Proteins were transferred to a nitrocellulose membrane (Amersham Biosciences) and blotted with the indicated primary antibodies and HRP-conjugated secondary antibodies. Detection was performed with the ECL Plus Western blotting detection system (Amersham Biosciences). Primary antibodies

used were anti-nucleolin (sc-17826/sc-8031) from Santa Cruz and anti-fibrillarin from Novus Biologicals® (Immunodiagnostic). Both antibodies were produced in mice. Anti-mouse HRP-conjugated secondary antibodies were from DAKO (p0260).

4.7 Confocal microscopy

For immunofluorescence experiments, HEK-293T (human embryonic kidney epithelial cells) and MCF-7 cells (breast adenoma cells) were grown on BD Falcon™ Culture Slide (BDBiosciences), 2 x 10^4 cells seeded into 1 cm^2 surface area. HEK-293T cells were cultured in DMEM (Gibco) supplemented with penicillin-streptomycin antibiotics, 5 % FBS, 1mM sodium pyruvate, and 50 µg/ml of uridine. MCF-7 cells were cultured in RPMI 1640(GIBCO) supplemented with 10% FBS, non-essential amino acids 1 mM sodium pyruvate and 10 µg/ml human insulin. When the dishes were 50% confluent, HEK-293T cells were transfected: 3µl of FuGENE 6 Transfection Reagent (Roche) was added to 100µl of serum free media, mixed gently by tapping the tube and incubated at room temperature for 5 min. 1µg of DNA (SAP30 and SAP30L in pcDNA3.1–myc-his vector) was added and the solution was incubated at RT for 30 min. Then the reagent was added to the cells in a drop-wise manner and the plates were incubated at 37°C (5% CO_2) for 24h. MCF-7 cells were not transfected.

The cells were fixed with 4 % paraformaldehyde in PBS for 20 min and then washed with PBS and permeabilized for 10 min with 0.2 % Triton X-100 in PBS. Unspecific binding of the antibodies was blocked by 1 % BSA in PBS for 60 min before incubation of the cells with primary antibody at 1:200 dilutions in humid chamber for 60 min at 37°C. The primary antibodies used were rabbit anti-c-myc (sc-789; Santa Cruz) mouse anti-nucleolin (Santa Cruz) mouse anti-fibrillarin (Novus Biologicals®, Immunodiagnostic) and rabbit anti-VKS (GeneTex®, GTX77650) (recognizes an 18 amino acid sequence (VKSNKSRLDQKSEGGKQLE) at the C-terminus of SAP30L). After washed three times with PBS (mild rocking, 5 min each), the cells were incubated with secondary antibody at 1:1000 dilutions in humid chamber for 60 min. The secondary antibodies used were Alexa® Fluor conjugated anti-mouse (A11031) and anti-rabbit (A11034) IgG. Then the cells were washed four times with PBS (mild rocking, 5 min each) and mounted on a DAPI-mount (VectaShield®). The slides were

analyzed and photographed with a confocal microscope (Ultraview Confocal Imaging System, Perkin Elmer Life Sciences Inc., Boston, MA).

5. Results

5.1 Silver staining and mass spectrometry

A number of possible interacting proteins was found (as seen in fig. 5.1). A total of 40 bands deemed specific for either SAP30 or SAP30L, and were selected for MALDI-TOF analysis. Of the 28 proteins interacting with SAP30, 14 (50%) were ribosomal and of the 41 proteins interacting with SAP30L, 17 (41%) were ribosomal proteins. 10 ribosomal and 4 other proteins were found to interact with both SAP30 and SAP30L. Half of the proteins which interacted with SAP30 interacted also with SAP30L and 34% of those proteins interacted with SAP30L also interacted with SAP30. The percentages were 71% and 59% for ribosomal and 29% and 17% for other proteins respectively. However, the exclusion of particular proteins from one set does not rule out an interaction with the other because of the experimental setup.

We excluded from the analysis proteins from other species than human (e.g. pig trypsin which was used for digestion), keratins (deemed contaminations from the skin and hair), proteins with low statistical scores and whose calculated molecular mass differed greatly with their molecular weight in the gel.

We have classified proteins into five separate groups: ribosomal proteins, ribosome biogenesis associated proteins (other than ribosomal proteins), histones, transcription associated and other proteins. The MALDI-TOF results are presented in the table 5.1 for SAP30 and in the table 5.2 for SAP30L, respectively. The MALDI-TOF analysis program used was Mascot.

In figure 5.2 we show the identified proteins and their respective bands on the gel. Most of the proteins had molecular weights under 40 kDa.

In tables 5.3 and 5.4 localization and function(s) of the interacting proteins are shown. Figures 5.3 and 5.4 show diagrams where the all interacting proteins are presented according to their cellular localization and function, respectively. The majority of the proteins were nucleolar (51%). Most of the proteins were ribosomal

(38%) and there were also many proteins involved in ribosome biogenesis (21%). These numbers are got from diagrams made according to tables 5.3 and 5.4.

Figures 5.3 and 5.4 show also the same information for those proteins that interacted with SAP30 or SAP30L. Here the values were a bit different but still the distribution into different classes was similar.

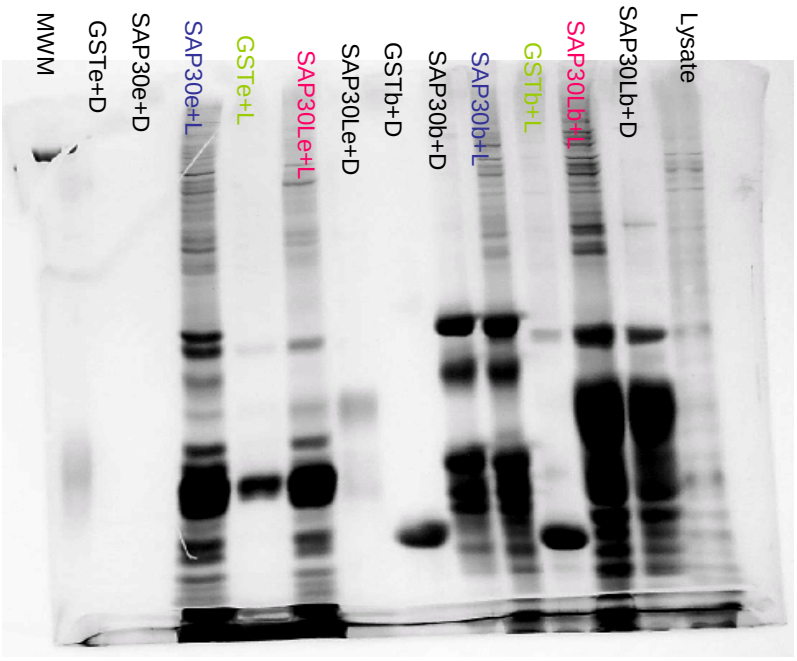


Figure 5.1 Silver staining of the pull-down samples. The result suggests several proteins that may interact with SAP30 and SAP30L. Proteins that appeared both in SAP30/30L + cell lysate lanes and in GST or SAP30/30L + D-buffer lanes were excluded from the analysis because these bands were from GST or SAPs themselves and not from real interacting proteins. MWM= molecular weight marker, e/b=eluted/beads sample, D=pull-down with D-buffer, L= pull-down with cell lysate, Lysate=Nuclear extraction lysate from Daudi cells (see also section 4.3 to refer how the samples were made).

		Table 5.1 MALDI-TOF results for SAP30				
	Protein code	**Protein name**	**Size (kDa)**	**Score**	**N. pep. (p<0.05)**	**Goverage (%)**
		Ribosomal proteins				
	P46777	60S ribosomal protein L5	34.523	239	8(3)	19,19
**	Q02878	60S ribosomal protein L6 (TAX-responsive enhancer element binding protein 107)	32.634	255	12(3)	20,14
	P18124	60S ribosomal protein L7	29.264	112	5(1)	12,1
**	P27635	60S ribosomal protein L10 (QM protein) (Tumor suppressor QM) (Laminin receptor homolog)	24.888	114	3(1)	13,08
	P26373	60S ribosomal protein L13	24.173	295	10(4)	30,33
**	P40429	60S ribosomal protein L13a (23 kDa highly basic protein)	23.488	158	6(1)	20,69
**	P50914	60S ribosomal protein L14 (CAG-ISL 7)	23.258	182	3(2)	15,49
**	P18621	60S ribosomal protein L17 (L23)	21.480	68	2(1)	11,96
**	Q02543	60S ribosomal protein L18a	21.034	106	3(2)	18,75
**	Q07020	60S ribosomal protein L18	21.604	175	6(3)	21,81
**	P23396	40S ribosomal protein S3	26.842	174	8(1)	31,69
**	P62753	40S ribosomal protein S6 (Phosphoprotein NP33)	28.834	231	5(5)	18,88
**	P62241	40S ribosomal protein S8	24.344	232	7(5)	27,88
**	P46781	40S ribosomal protein S9	22.504	107	3(1)	11,34
		Ribosome biogenesis				
		associated proteins				
**	O00567	Nucleolar protein Nop56 (Nucleolar protein 5A)	66.594	80	4(1)	8,05
**	P29728	2'-5'-oligoadenylate synthetase 2	84.074	67	3(1)	3,85
	P07910	Heterogeneous nuclear ribonucleoproteins C1/C2 (hnRNP C1 / hnRNP C2)	33.725	123	3(2)	10,13
	O60812	ribonucleoprotein C-like dJ845O24.4 (hnRNP core protein C-like)	32.180	122	3(2)	10,58
	P19338	Nucleolin (Protein C23)	76.224	127	3(2)	4,38
**	P17844	Probable RNA-dependent helicase p68 (DEAD-box protein p68)	69.618	221	7(3)	9,12
	Q9NVP1	ATP-dependent RNA helicase DDX18 (DEAD-box protein 18)	75.702	103	3(3)	3,28
	Q9NR30	Nucleolar RNA helicase II (Nucleolar RNA helicase Gu) (RH II/Gu) (DEAD-box protein 21)	87.816	321	8(4)	11,62
		Histones				
	P16401	Histone H1.5 (Histone H1a)	22.435	184	5(3)	21,24
		Transcription associated				
	P26641	Elongation factor 1-gamma (EF-1-gamma) (eEF-1B gamma)	50.298	218	6(2)	13,27
		Other proteins				
**	P62988	Ubiquitin				
	P20700	Lamin B1	66.522	48	2(0)	3,92
	Q13310	Polyadenylate-binding protein 4 (Poly(A)-binding protein 4)	71.080	70	2(1)	3,26
	Q14444	GPI-anchored protein p137 (p137GPI)	72.935	48	1(1)	1,69
		** Came down also with SAP30L				

		Table 5.2 MALDI-TOF results for SAP30L				
	Protein code	**Name**	**Size (kDa)**	**Score**	**N. pep. (p<0.05)**	**Goverage (%)**
		Ribosomal proteins				
**	Q02878	60S ribosomal protein L6 (TAX-responsive enhancer element binding protein 107)	32.634	236	8(2)	21,18
	P62424	60S ribosomal protein L7a (Surfeit locus protein 3) (PLA-X polypeptide)	30.017	206	19(3)	17,29
	P62917	60S ribosomal protein L8	28.104	130	12(1)	9,73
	P32969	60S ribosomal protein L9	21.964	61	2(1)	8,85
**	P27635	60S ribosomal protein L10 (QM protein) (Tumor suppressor QM) (Laminin receptor homolog)	24.888	53	2(0)	8,88
**	P40429	60S ribosomal protein L13a (23 kDa highly basic protein)	23.488	93	4(1)	14,78
**	P50914	60S ribosomal protein L14 (CAG-ISL 7)	23.258	64	1(1)	5,16
**	P18621	60S ribosomal protein L17 (L23)	21.480	87	2(1)	13,04
**	Q02543	60S ribosomal protein L18a	21.034	86	2(2)	15,34
**	Q07020	60S ribosomal protein L18	21.604	175	10(4)	17,02
	P47914	60S ribosomal protein L29 (Cell surface heparin binding protein HIP)	17.667	34	1(0)	9,43
	P05388	60S acidic ribosomal protein P0 (L10E)	34.423	214	9(1)	20,82
	P15880	40S ribosomal protein S2 (S4) (LLRep3 protein)	31.590	119	6(1)	13,65
**	P23396	40S ribosomal protein S3	26.842	261	15(3)	37,04
**	P62753	40S ribosomal protein S6 (Phosphoprotein NP33)	28.834	305	21(7)	24,5
**	P62241	40S ribosomal protein S8	24.344	177	5(2)	21,63
**	P46781	40S ribosomal protein S9				
		Ribosome biogenesis associated proteins				
	P22087	Fibrillarin (34 kDa nucleolar scleroderma antigen)	33.877	52	2(0)	5,92
	Q9Y2X3	Nucleolar protein NOP5 (Nucleolar protein 5) (NOP58) (HSPC120)	60.054	80	5(1)	6,81
**	P29728	2'-5'-oligoadenylate synthetase 2	84.074	139	7(1)	8,8
**	O00567	Nucleolar protein Nop56 (Nucleolar protein 5A)	66.594	157	4(2)	8,05
	Q08211	ATP-dependent RNA helicase A (Nuclear DNA helicase II) (NDH II) (DEAH-box protein 9)	142.099	209	5(4)	4,09
**	P17844	Probable RNA-dependent helicase p68 (DEAD-box protein p68)	69.618	188	6(2)	9,12

		Histones				
	P17317	Histone H2A.z (H2A/z)	13.414	77	2(1)	14,84
	P62807	Histone H2B.c (H2B/c)	13.767	86	3(0)	20,63
	Q93081	Histone H3/b	15.315	52	2(0)	10,29
	Q02539	Histone H1.1	21.698	51	2(1)	7,91
	P16403	Histone H1.2 (Histone H1d)	21.221	192	30(7)	32,39
		Transcription associated proteins				
	O60264	SWI/SNF related matrix associated actin dependent regulator of chromatin subfamily A, member 2 (Sucrose nonfermenting protein 2 homolog)	122.513	275	11(1)	10,46
	Q92900	Regulator of nonsense transcripts 1 (Nonsense mRNA reducing factor 1)	125.578	44	1(1)	0,8
	O75533	Splicing factor 3B subunit 1 (Spliceosome associated protein 155)	146.464	195	4(3)	3,6
	P11388	DNA topoisomerase II, alpha isozyme	175.017	174	6(1)	4,05
	Q7KZF4	containing protein 1 (p100 co-activator)	102.618	138	4(2)	4,4
		Other proteins				
	Q9P0W8	Spermatogenesis associated protein 7 (Spermatogenesis associated protein HSD3)	68.190	41	1(1)	1,17
**	P62988	Ubiquitin	8.560	69	3(2)	40,79
	P05109	Calgranulin A (Migration inhibitory factor-related protein 8)	10.885	88	4(1)	24,73
	P62736	Actin, aortic smooth muscle (Alpha-actin 2)	42.381	61	3(0)	8,22
	Q8WUM0	Nuclear pore complex protein Nup133 (Nucleoporin Nup133)	129.958	53	3(0)	3,34
	Q9Y262	Eukaryotic translation initiation factor 3 subunit 6 interacting protein (HSPC021/HSPC025)	66.912	96	5(0)	7,45
	Q07666	KH domain containing, RNA binding, signal transduction associated protein 1 (p21 Ras GTPase--activating protein-associated p62)	48.311	64	3(0)	4,97
	P11142	Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8)	71.082	108	3(0)	6,04
		** Came down also with SAP30				

The score, given by the Mascot program, tells the number of amino acids identified to the certain protein. The higher the score the better. Next there are given the number of peptides found to correspond to the protein. Number of significant peptides ($p<0.05$) are given in parenthesis. The more significant peptides, peptides specific to the protein, the higher reliability of the results. The coverage tells the number of the amino acids identified by the analysis divided by the number of amino acids in the protein. The higher the coverage percent the better (>15 is the limit of high reliability).

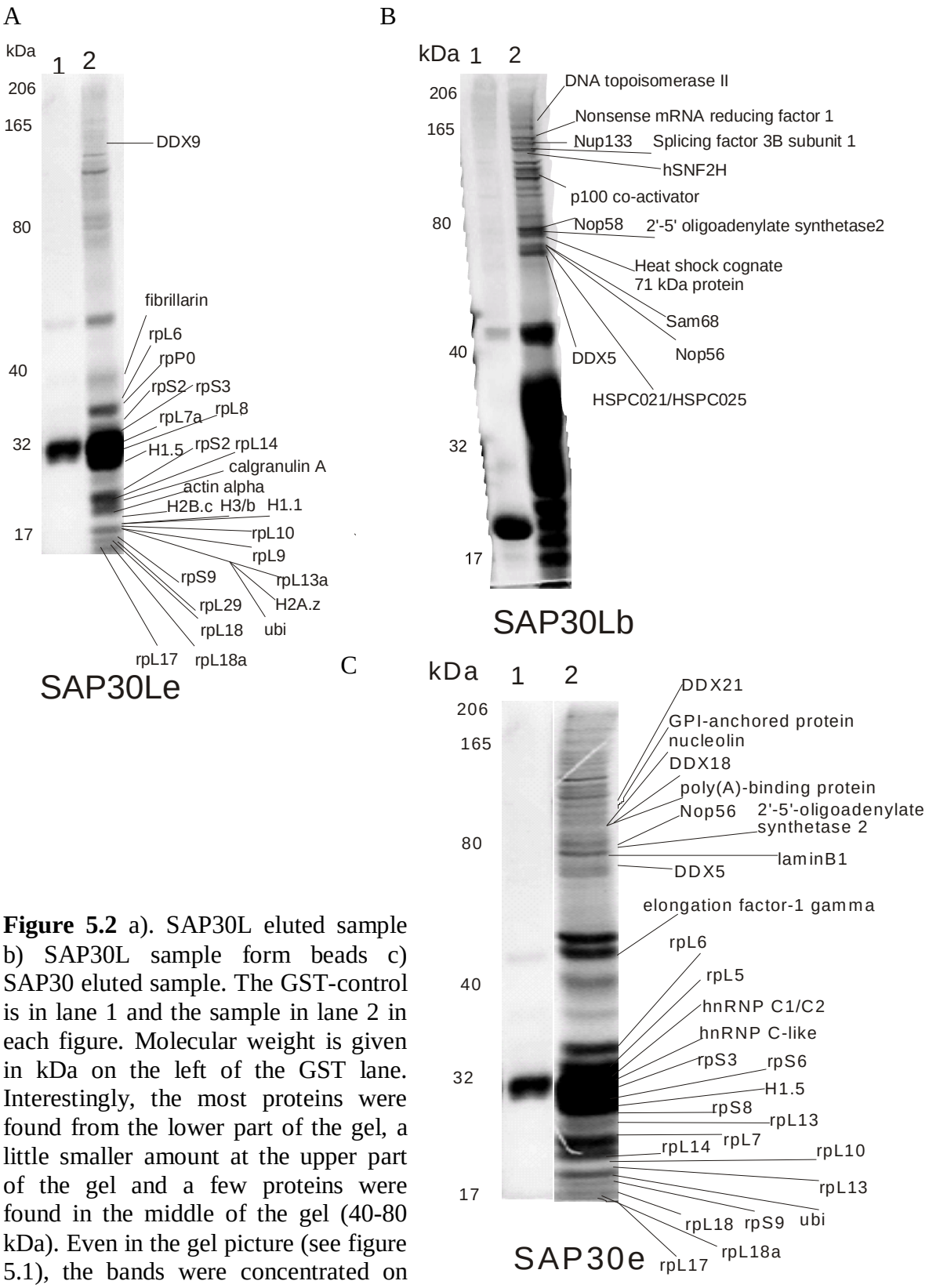


Figure 5.2 a). SAP30L eluted sample b) SAP30L sample form beads c) SAP30 eluted sample. The GST-control is in lane 1 and the sample in lane 2 in each figure. Molecular weight is given in kDa on the left of the GST lane. Interestingly, the most proteins were found from the lower part of the gel, a little smaller amount at the upper part of the gel and a few proteins were found in the middle of the gel (40-80 kDa). Even in the gel picture (see figure 5.1), the bands were concentrated on the top and the bottom of the gel. Abbreviations used: DDX=DEAD-box protein, rpL/S= ribosomal protein large/small subunit, ubi=ubiquitin, H=histone, the other protein names are found in the tables.

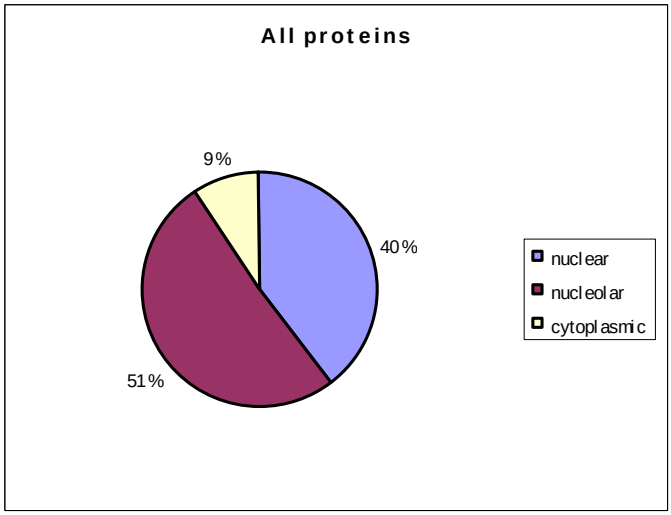
Table 5.3 Ribosomal proteins interacting with SAP30, SAP30L or both
The known functions in mammals were extracted from the NCBI database (proteins with multiple functions only some of them are shown).

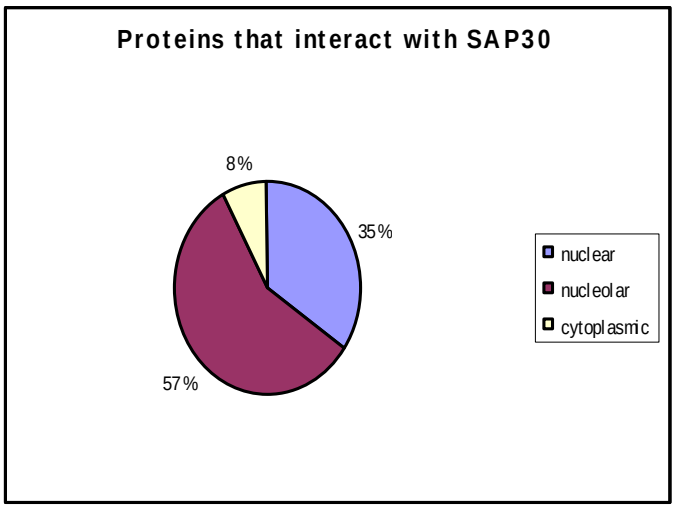
bait	Protein	Known function in mammal	Cellular localization*	References.
SAP30	60S ribosomal protein L5	Binds 5S RNA	No/C	Trotta et al., 2003, Odintsova et al., 2003
SAP30L	60S ribosomal protein L6	Ribosomal complex. Specifically binds to domain C of the Tax-responsive enhancer element in the long terminal repeat of HTLV-I.	No/C	Uchiumi et al., 1999, Ulbrich et al., 1988, Trotta et al., 2003, Odintsova et al., 2003
SAP30	60S ribosomal protein L7	Ribosomal complex	No/C	Trotta et al., 2003, Odintsova et al., 2003
SAP30L	60S ribosomal protein L7a	Ribosomal complex	No/C	Trotta et al., 2003, Odintsova et al., 2003
SAP30L	60S ribosomal protein L8	Ribosomal complex	No/C	Trotta et al., 2003, Odintsova et al., 2003
SAP30L	60S ribosomal protein L9	Ribosomal complex	No/C	Trotta et al., 2003, Odintsova et al., 2003
both	60S ribosomal protein L10	Ribosomal complex	No/C	Trotta et al., 2003, Odintsova et al., 2003
SAP30	60S ribosomal protein L13	Ribosomal complex	No/C	Trotta et al., 2003, Odintsova et al., 2003
both	60S ribosomal protein L13a	Ribosomal complex	No/C	Trotta et al., 2003, Odintsova et al., 2003
both	60S ribosomal protein L14	Ribosomal complex	No/C	Trotta et al., 2003, Odintsova et al., 2003
both	60S ribosomal protein L17	Ribosomal complex	No/C	Trotta et al., 2003, Odintsova et al., 2003
both	60S ribosomal protein L18a	Ribosomal complex	No/C	Trotta et al., 2003, Odintsova et al., 2003
both	60S ribosomal protein L18	Ribosomal complex	No/C	Trotta et al., 2003, Odintsova et al., 2003
SAP30L	60S ribosomal protein L29	Ribosomal complex	No/C	Trotta et al., 2003, Odintsova et al., 2003
SAP30L	60S acidic ribosomal protein P0	Ribosomal complex	No/C	Trotta et al., 2003, Odintsova et al., 2003
SAP30L	40S ribosomal protein S2	Ribosomal complex	No/C	Vladimirov et al., 1996
both	40S ribosomal protein S3	Ribosomal complex	No/C	Vladimirov et al., 1996
both	40S ribosomal protein S6	Ribosomal complex. May play an important role in controlling cell growth and proliferation through the selective translation of particular classes of mRNA.	No/C	Vladimirov et al., 1996
both	40S ribosomal protein S8	Ribosomal complex	No/C	Vladimirov et al., 1996
both	40S ribosomal protein S9	Ribosomal complex	No/C	Vladimirov et al., 1996
	* No=nucleolus, C=cytoplasm			

	Table 5.4 Non-ribosomal proteins interacting with SAP30, SAP30L or both			
	The known functions in mammals were extracted from the NCBI database.			
	(proteins with multiple functions only some of them are shown)			
bait	**Protein**	**Known function in mammal**	**Cellular localization***	**References.**
	Ribosome biogenesis associated proteins			
SAP30L	Fibrillarin	A component of a nucleolar small nuclear ribonucleoprotein particle thought to participate in the first step in processing preribosomal RNA. It is associated with the U3, U8 and U13 small nuclear RNAs.	No/N	Aris and Blobel, 1991, Tollervey et al., 1991
SAP30L	Nucleolar protein NOP5	Required for 60S ribosomal subunit biogenesis.	No/N	Lyman et al., 1999, Gautier et al, 1997
both	Nucleolar protein Nop56	Required for 60S ribosomal subunit biogenesis.	No/N	Gautier et al, 1997
both	2'-5'-oligoadenylate synthetase 2	May play a role in mediating resistance to virus infection, control of cell growth, differentiation, and apoptosis. Binds double-stranded RNA and polymerizes ATP into PPP(A2'P5'A)N oligomers, which activate the latent RNase L that, when activated, cleave	N	Behera et al., 2002, Ghosh et al., 1991, Rogozin et al., 2003
SAP30	Nucleolin	The major nucleolar protein of growing eukaryotic cells. It is found associated with intranucleolar chromatin and preribosomal particles. It induces chromatin decondensation by binding to histone H1. It is thought to play a role in pre-rRNA transcription.	No/N	Lapeyre et al., 1987, Khurts et al., 2004
SAP30	Heterogeneous nuclear ribonucleoproteins C1/C2	May play a role in ribonucleosome assembly by neutralizing basic proteins such as A and B core hnRNP. May associate with telomerase.	N	Ford et al., 2000
SAP30	Heterogeneous nuclear ribonucleoprotein C-like	May play a role in ribonucleosome assembly by neutralizing basic proteins such as A and B core hnRNP. May associate with telomerase.	N	Ford et al., 2001
both	Probable RNA-dependent helicase p68	RNA-dependent ATPase activity. The rate of ATP hydrolysis is highly stimulated by single-stranded RNA. A novel transcriptional coactivator of the p53 tumour suppressor.	N	Bates et al., 2005, Huang and Liu, 2002
SAP30L	ATP-dependent RNA helicase A	Unwinds double-stranded DNA and RNA in a 3' to 5' direction. Generates multiple secondary structures that influence RNA-binding proteins. May play a role in X-linked gene expression.	N	Aratani et al., 2001
SAP30	ATP-dependent RNA helicase DDX18	Probable RNA-dependent helicase.	N	Krishnan and Zeichner, 2004, Rocak and Linder, 2004
SAP30	Nucleolar RNA helicase II	Can unwind double-stranded RNA (helicase) and can fold or introduce a secondary structure to a single-stranded RNA (foldase).	No/N	Krishnan and Zeichner, 2004, Rocak and Linder, 2004

	Histones			
SAP30L	Histone H2A.z	The nucleosome is an octamer containing two molecules each of H2A, H2B, H3 and H4. The octamer wraps approximately 146 bp of DNA.	N	Rangasamy et al., 2003, Park et al., 2005
SAP30L	Histone H2B.c	The nucleosome is an octamer containing two molecules each of H2A, H2B, H3 and H4. The octamer wraps approximately 146 bp of DNA.	N	Park et al., 2005
SAP30L	Histone H3/b	Histone H3, along with histone H4, plays a central role in nucleosome formation.	N	Park et al., 2006
SAP30L	Histone H1.1	Histones H1 are necessary for the condensation of nucleosome chains into higher order structures.	N	Yamamoto and Horikoshi, 1996, Th'ng et al., 2005
SAP30L	Histone H1.2	Histones H1 are necessary for the condensation of nucleosome chains into higher order structures.	N	Yamamoto and Horikoshi, 1996, Th'ng et al., 2005
SAP30	Histone H1.5	Histones H1 are necessary for the condensation of nucleosome chains into higher order structures.	N	Yamamoto and Horikoshi, 1996, Th'ng et al., 2005
	Transcription associated proteins			
SAP30L	SWI/SNF related matrix associated actin dependent regulator of chromatin subfamily A, member 2	Possesses intrinsic ATP-dependent nucleosome remodelling activity. Complexes containing SMARCA5 are capable of forming ordered nucleosome arrays on chromatin in vitro; this may require intact histone H4 tails. Also required for replication of pericentric	N	Collins et al, 2002
SAP30L	Regulator of nonsense transcripts 1	Part of a post-splicing multiprotein complex. Involved in nonsense-mediated decay (NMD) of mRNAs containing premature stop codons. Essential for embryonic viability.	No/C	Lejeune and Maquat, 2005, Lejeune et al., 2003
SAP30L	Splicing factor 3B subunit 1	Subunit of the splicing factor SF3B required for 'A' complex assembly formed by the stable binding of U2 snRNP to the branchpoint sequence (BPS) in pre-mRNA. Sequence independent binding of SF3A/SF3B complex upstream of the branch site is essential.	N	Das et al., 1999, Boudrez et al., 2002
SAP30L	DNA topoisomerase II, alpha isozyme	Control of topological states of DNA by transient breakage and subsequent rejoining of DNA strands. Topoisomerase II makes double-strand breaks. ATP-dependent breakage, passage and rejoining of double-stranded DNA.	C	Hochhauser et al., 1992
SAP30L	Staphylococcal nuclease domain containing protein 1	A bridging factor between STAT6 and the basal transcription factor. Plays a role in PIM1 regulation of MYB activity. Functions as a transcriptional coactivator for the Epstein-Barr virus nuclear antigen 2 (EBNA2).	N	Paukku et al., 2003, Välineva et al., 2005
SAP30	Elongation factor 1-gamma	This gene encodes a subunit of the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome. Probably plays a role in anchoring the complex to other cellular components.	N	Janssen and Moller, 1988

	Other proteins			
both	Ubiquitin	Involved in the ATP-dependent selective degradation of cellular proteins, the maintenance of chromatin structure, the regulation of gene expression, the stress response, and ribosome biogenesis.	N/C	Baker and Board, 1987
SAP30L	Spermatogenesis associated protein 7	Spermatogenesis		Zhang et al, 2003
SAP30L	Calgranulin A	Expressed by macrophages in chronic inflammations. Also expressed in epithelial cells constitutively or induced during dermatoses. May interact with components of the intermediate filaments in monocytes and epithelial cells.	No/C	Lagasse and Clerc, 1988
SAP30L	Actin, aortic smooth muscle	Involved in cell motility, structure and integrity	C	Miwa et al., 1991, Ueyama et al., 1984
SAP30L	Nuclear pore complex protein Nup133	Form aqueous channels to regulate the flow of macromolecules between the nucleus and the cytoplasm.	N	Pemberton et al., 1995
SAP30L	Eukaryotic translation initiation factor 3 subunit 6	Translation initiator	C	Sette et al., 1999
SAP30L	KH domain containing, RNA binding, signal transduction associated protein	Role in G2-M progression in the cell cycle. Represses CBP-dependent transcriptional activation. Also acts as a putative regulator of mRNA stability and/or translation rates and mediates mRNA nuclear export.	N	Wong et al., 1992, Barlat et al., 1997
SAP30L	Heat shock cognate 71 kDa protein	Chaperone. Isoform 2 may function as an endogenous inhibitory regulator of HSC70 by competing the cochaperones.	N	Dworniczak and Mirault, 1987
SAP30	Lamin B1	Component of the nuclear lamina	N	Panorchan et al., 2004
SAP30	Polyadenylate-binding protein 4	Binds mRNA	C	Grange et al., 1987
SAP30	GPI-anchored protein p137	May play a role in transporting nutrient from the gut lumen across the gutlining epithelial cell layer.	C	Gessler et al., 1996
	* No=nucleolus, N=nucleus, C=cytoplasm			





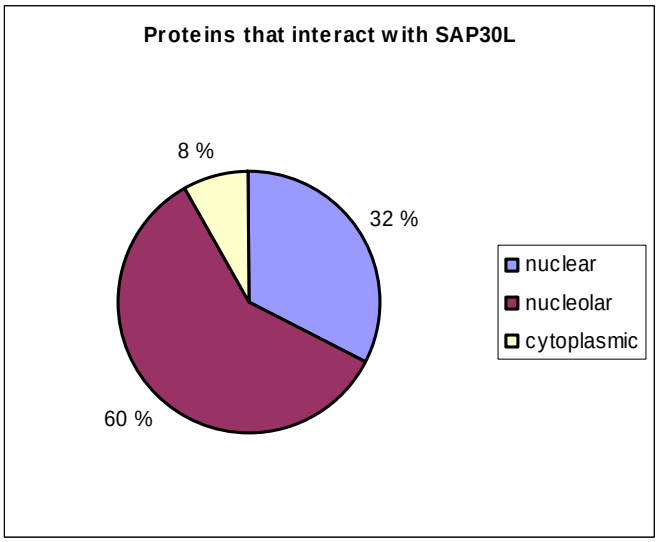


Figure 5.3 The interacting proteins and their localization in the cell. Most of the proteins were nucleolar.

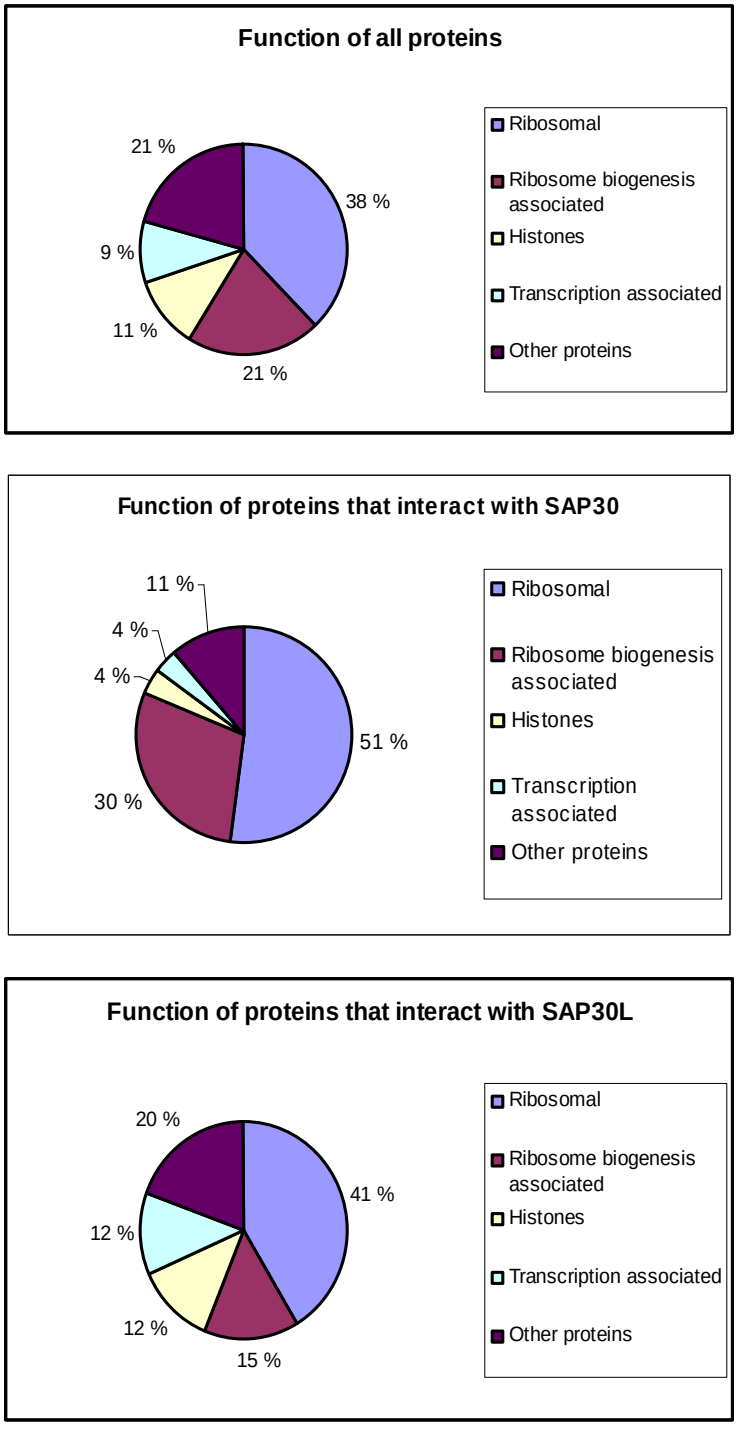


Figure 5.4. Function of the interacting proteins. The identified proteins were classified into five different groups as described previously. These diagrams show proportion of proteins in different groups for those interacting with SAP30 and SAP30L. Also the distribution of all identified proteins into these groups is shown. The diagrams are not identical but the majority of proteins are ribosomal and also proteins involved in ribosome biogenesis are also well represented.

5.2 SAP30 and SAP30L interact with endogenous nucleolin

Nucleolin is a major nucleolar phosphoprotein. It's very abundant protein in the cells. Nucleolin has been reported to involve in pre-RNA processing, and recently has shown to interact with telomerase (Khurts et al., 2004). In this experiment K-562 cell lysate was used and the pull-down was performed as descriped in 4.6. Figure 5.5 shows that endogenous nucleolin associates with GST-SAP30 and GST-SAP30L but not with GST alone.

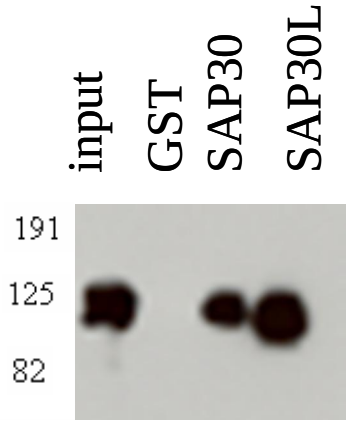


Figure 5.5 SAP30 and SAP30L interact with endogenous nucleolin. Both SAP30 and SAP30L but not GST alone can pull nucleolin down. Here SAP30L seems to have a little higher affinity.

5.3 SAP30 and SAP30L interact with endogenous fibrillarin

Fibrillarin is a component of a nucleolar small nuclear ribonucleoprotein (snRNP) particle thought to participate in the first step in processing preribosomal RNA. It is associated with the U3, U8, and U13 small nuclear RNAs and is located in the dense fibrillar component (DFC) of the nucleolus (Tollervey et al., 1991).

Pull-down was performed similarly (from K-562 cell lysate) as previously described for nucleolin. As shown in figure 5.6, GST-SAP30 and GST-SAP30L associate with endogenous fibrillarin whereas GST does not.

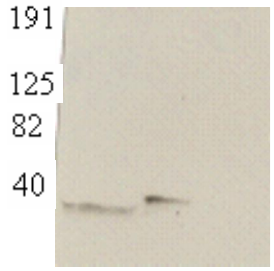
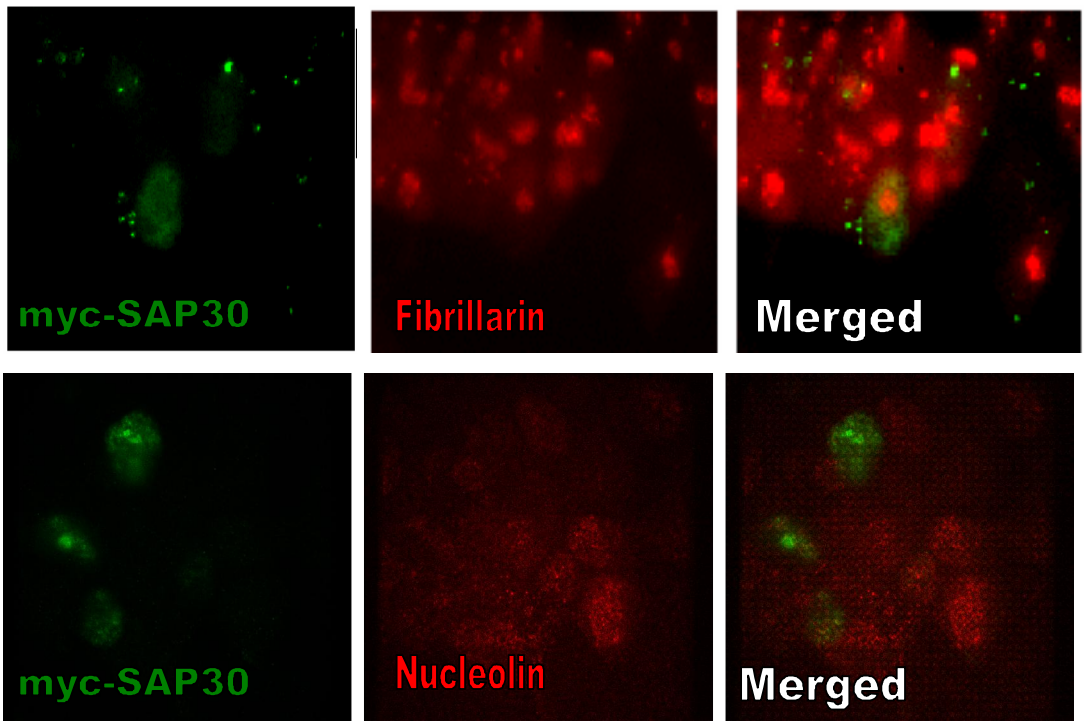


Figure 5.6 SAP30 and SAP30L interact with endogenous fibrillarin.

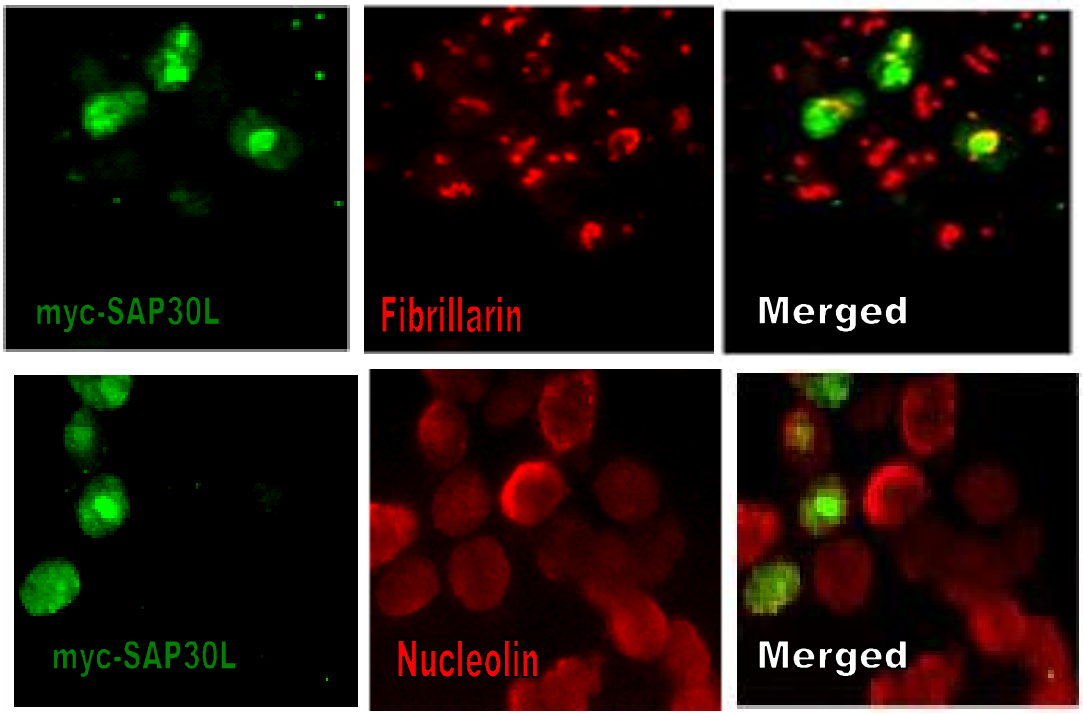
5.3 Colocalization of SAPs with nucleolin and fibrillarin

Next we wanted to study if SAPs colocalize with fibrillarin and nucleolin. As shown in left panel of figure 5.7 A, SAP30 is localized in the nucleus and concentrated into the nucleolus. Fibrillarin is localized in the dense fibrillar component of the nucleolus and nucleolin seems to occupy the whole nucleus (middle panel). Right panel present a merged image of the first two pictures. Figure 5.7 B presents the same experiment done to SAP30L. Here it seems that SAP30L is in the nucleolus more clearly than SAP30. Here also fibrillarin is stained better. Nucleolin stained the whole nucleolus. In both merged images, fibrillarin seems to be on the edges of the area where SAPs are localized and nucleolin, because it is present evenly in the nucleolus, is also there where SAPs. SAP30L has a little better colocalization than SAP30. Endogenous SAP30L (fig. 5.7 C) is localized mostly in the cytoplasm and thus do not colocalize with fibrillarin or nucleolin. The reason for this may be that our anti-SAP30L antibody recognizes cytoplasmic splicing variant of SAP30L (data not shown).

A


myc-SAP30
Fibrillarin
Merged
myc-SAP30
Nucleolin
Merged

B


myc-SAP30L
Fibrillarin
Merged
myc-SAP30L
Nucleolin
Merged

C

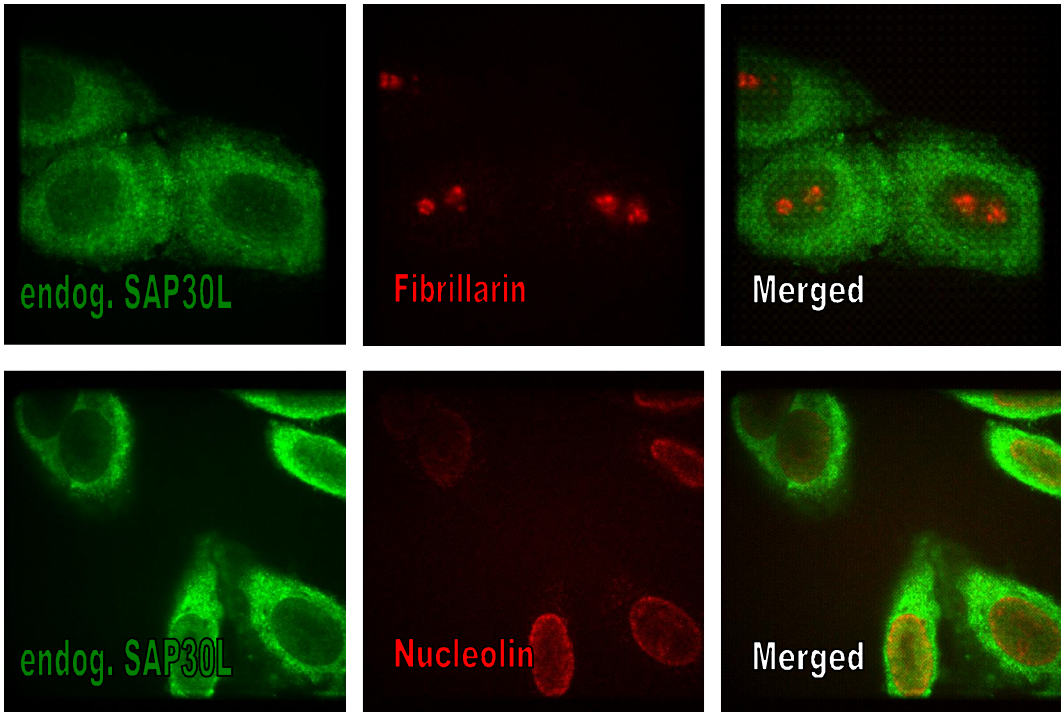


Figure 5.7 Colocalization of SAP30 and SAP30L with fibrillarin and nucleolin. a) SAP30 is concentrated into the nucleolus. Fibrillarin seems to localize to the nucleolus, into the region called dense fibrillar component (DFC), as expected. The merged picture shows that fibrillarin localizes on the edges of the area where SAP30 is present. Nucleolin on the other hand seems to localize not only to the nucleolus but also all over the nucleus. b) This experiment is similar to that done in picture a except that the transfected protein was SAP30L instead of SAP30. The same observations as in A were made also for SAP30L. However, here fibrillarin seems to have more fibrous structure and is more clearly in the DFC area. c) In these pictures, SAP30L is endogenous and stained with anti-VKS antibody. Here SAP30L isn't concentrated to the nucleolus as it was in figure b. Fibrillarin is again localized to the certain area of the nucleolus while anti-nucleolin stains the whole nucleus.

6. Discussion

Altogether, 54 different proteins were identified from MALDI-TOF analysis and they were divided into five groups as described previously (tables 5.1 and 5.2). The majority of them were ribosomal. They include both the members of the smaller subunit (40S) and the larger subunit (60S) (5 and 15 proteins, respectively). Some ribosomal proteins have also other functions, like S6, which may be involved in cell growth control (Vladimirov et al., 1996). Ribosomal protein L5 has been shown to bind 5S RNA but for the others it was only mentioned that they belong to ribosomal complex (Trotta et al., 2003, Odintsova et al., 2003). Proteins involved in ribosome biogenesis included, for example, nucleolar proteins Nop56 and Nop58, DEAD-box proteins, which have RNA helicase activity, nucleolin and fibrillarin. Nop56 and Nop58 are required for 60S ribosomal subunit biogenesis (Lyman et al., 1999, Gautier et al., 1997). Histones were also found and majority of them were linker histones (different forms of histone H1). Core histones H2A, H2B and H3 were found but not histone H4. There were also proteins that are associated to transcription and translation, like elongation factor 1- gamma (Janssen & Moller, 1988) and translation initiator factor (Sette et al., 1999).

In figure 5.2, which shows the identified proteins on the gel, is seen that many proteins had quite low molecular weights (<40 kDa) and most of those were ribosomal proteins. Comparing the place of the proteins on the gel to the molecular weight marker is seen that they correspond quite well to their calculated (true) molecular weights. Also when the same protein was found from the two lists it was found approximately on the same place on the gel. This means that MALDI-TOF analysis has worked (it has identified real proteins) and that the results are more reliable.

The scores and other MALDI-TOF parameters measuring the quality of the results varied from protein to protein (tables 5.1 and 5.2). As mentioned before, none of the parameters is valid alone. Ribosomal proteins were well expressed in both tables and their mass spectrometric values were excellent compared to other proteins. For

example, 60S ribosomal protein L13 (table 5.1) got the score 295, coverage percent of 30.33% and four of its 10 peptides recognized from MALDI-TOF, were significant. In addition to this, it was found from the gel approximately at the place corresponding to its molecular weight (see figure 5.2c, the molecular weight of rpL13 is about 24kDa). The scores for ribosomal protein in the other table (5.2) were a little bit lower but still very good.

If we then look at the other proteins (those that were not ribosomal), we see that they have lower scores and coverage percents than the ribosomal proteins. Some histone proteins, like H1.5 (table 5.1) and H1.2 (table 5. 2) were found and they, however, got quite high mass spectrometric parameters (almost as good as ribosomal proteins).

Thought the other proteins did not have as good values as ribosomal proteins and histones, they might still interact with SAPs. Among them, were very interesting proteins, like DEAD-box protein p68, a RNA helicase, which has been shown to be a transcriptional co-activator of p53 (p53 is a tumor suppressor gene) (Bates et al., 2005). There were proteins involved in ribosome biogenesis, like nucleolar proteins Nop5 (Nop58) and Nop56, nucleolin and fibrillarin. Nucleolin has also shown to interact with telomerase (Khurts et al., 2004). The members of DEAD-box protein family can also be involved in ribosome biogenesis because they are RNA helicases. Also proteins involved in transcription were found, like DNA topoisomerase II and elongation factor- 1-gamma. If we compare these results to similar work performed by Hayano et al (2003) where they studied Nop56 (Nop56 was found from both SAP30 and SAP30L lists) we see that many of the proteins found by our study were also found in theirs and from the same place of the gel.

To our surprise, no members of the mSin3a repressor complex were found expect a sucrose non-fermenting protein homolog 2, which probably recruits Sin3/HDAC1 corepressor complex to the rDNA promoter (Collins et al., 2002), although both SAP30- and SAP30L-GST fusion proteins have been shown to interact with members of the histone deacetylase complex (mSin3a and HDAC1 and 2) (Laherty et al., 1997, Zhang Y et al., 1998, Viiri et al (submitted)). The only thing related to histone deacetylation (in addition to that sucrose non-fermenting protein homolog 2, mentioned above) were histones themselves. The reason for this might be that being

in the complex is not the only thing SAPs do. Interaction could also be cell line dependent or happen at certain point during the cell cycle. Also the amount of protein in the cell may not be enough to see the interactions in the experiments we performed.

All in all, silver staining and MALDI-TOF are very sensitive methods. If there was enough protein to be visible on the gel, it should be enough for MALDI-TOF analysis. But as mentioned in their article by Huang et al. (2002), multiple peptides found from the same sample have an effect on the results. Contamination of keratin or other proteins from skin might mask the real interacting proteins especially if there is not much protein in the sample. And if there are (unexpectable) post-translational modifications in a protein or nonspecific cleavages, analyse programs may not recognize the protein. And if there was not much of certain protein in the cell lysate, thought it could bind to SAPs, the change that it and a fusion protein met is low.

The three diagrams in figure 5.3, which show the localization of the identified proteins, are quite similar. Over half of the proteins were nucleolar and almost all the rest nuclear. Only a minority (< 10%) of proteins were cytoplasmic. In figure 5.4 the diagrams differ a bit. All identified proteins show the similar distribution to the five classes as those that interacted with SAP30L but those that interacted with SAP30 are distributed a bit differently. This is due to the experimental set up where not all of the proteins were allowed to send to the MALDI-TOF analysis (thus the proteins expressed in the diagrams are not the same), which also caused the number of identified proteins to be lower in SAP30 list. Ribosomal proteins were anyway in majority in each diagram. They constituted 38% of all proteins, and 51% and 41% of those that interacted with SAP30 and SAP30L respectively (see figure 5.4). Together with proteins associated in ribosome biogenesis they occupied more than half of the pie diagram.

The diagrams in figures 5.3 and 5.4 suggest a nucleolar role for SAPs. Most of the identified proteins were nucleolar and many of them had something to do with ribosomes: they were either ribosomal proteins or proteins needed in ribosome biogenesis. Finding of so many ribosomal proteins, many of which were same for both SAP30 and SAP30L, suggests that SAPs might participate in ribosome biogenesis. This assumption is further supported by the fact that snRNP, snoRNP and

RNA helicases, needed for ribosome assembly, were found from mass spectrometric analysis (tables 5.1 and 5.2).

When the pull-down experiments were performed using nucleolin as bait both SAP30 and SAP30L were found to interact with it (see fig. 5.5). This happened though nucleolin was not found from SAP30 pull- down and it did not get good parameters from MALDI-TOF (table 5.2). The same was true for fibrillarin (fig. 5.6), still it interacted with both SAPs. These results support the assumption that SAPs have something to do with ribosomes maybe being involved in ribosome biogenesis with nucleolin and fibrillarin. In this experiment, nucleolin and fibrillarin were endogenous, thus making the results more reliable than using transfected cDNAs.

Transfected, myc-tagged SAPs are localized into the nucleolus (see figures 5.7 A and B) while endogenous SAP30L has mainly cytoplasmic localization and thus is not colocalized with nucleolin or fibrillarin (fig. 5.7 C). The reason for this might be that there are different splicing variants for SAP30L and our antibody recognizes the form that is localized in the cytoplasm (data not shown). Nucleolin and fibrillarin are present on the same area as SAPs, though they do not have perfect colocalization (meaning they both are present or absent on the same area as SAPs). SAP30L is colocalized a little better with both nucleolin and fibrillarin than SAP30. The colocalization studies are not against the assumption that SAPs interact with nucleolin and fibrillarin.

7. Conclusion

The aim of this study was to find the proteins SAP30 and SAP30L interact with. SAP30 and SAP30L are highly identical and they interacted mainly with the same proteins suggesting that they have similar functions. The MALDI-TOF results suggest that they may be involved in ribosome biogenesis because they interact with many ribosomal proteins and proteins involved in ribosome assembly and ribosomal RNA modification pathways. This is also supported by the fact that in immunoblotting studies they were found to interact with endogenous nucleolin and fibrillarin, proteins needed for ribosomal RNA processing. According to the mentioned interactions they have a nucleolar role, which is supported by the fact that they both have been shown to localize into the nucleolus.

According to these results, it seems that SAP and SAP30L may have also in other function(s) in the cell than regulation of gene expression via recruiting the HDAC complex.

8. References

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