universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Establishing stable cell lines for the generation of interaction profiles of proteins involved in RNA-editing

(Etablierung von stabilen Zelllinien zur Generierung von Interaktionsprofilen von Proteinen, die in RNA-Editierung involviert sind)

Verfasserin

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Zusammenfassung

Adenosin zu Inosin Editierung von Ribonukleinsäuren (RNA) ist eine konservierte post-transkriptionelle Modifikation in höheren Metazoen. Die hydrolytische Desaminierung von Adenosin zu Inosin wird von einer Enzymfamilie durchgeführt, welche als Adenosin-Deaminasen bekannt sind (ADARs) und doppelsträngige RNA desaminieren. Durch RNA-Editierung wird die Sequenz eines primären Transkriptes verändert, was dramatische Auswirkungen haben kann: Editierung der mRNA eines kodierenden Transkriptes kann zu alternativem Splicing und Aminosäurensubstitutionen im translatierten Protein führen. Abgesehen davon gehören nicht-codierende RNAs und repetitive Sequenzen, wie Alu-Elemente und micro RNAs, zu den Hauptsubstraten von ADARs. Editierung dieser Substrate kann Einfluss auf die Expression von Genen haben, weil die Sequenz regulatorischer Elemente und kleiner RNAs verändert wird. Durch aktuelle Studien wurden bereits viele Substrate für ADAR-vermittelte Editierung identifiziert. Jedoch sind Auswirkungen der Editierung auf die Funktion vieler prozessierter Substrate noch nicht bekannt. Zusätzlich zu den unbekannten Konsequenzen der RNA-Editierung gibt auch die Regulation dieses Mechanismus Rätsel auf.

In den vergangenen Jahren wurden in unserer Arbeitsgruppe einige Kandidaten identifiziert, welche als mögliche Regulatoren für RNA-Editierung in Frage kommen. In diesem Projekt haben wir versucht, diese Kandidaten stabil in Säugerzellen zu exprimieren, um durch Aufreinigungsmethoden und anschließende massenspektrometrische Analyse Interaktionsnetzwerke dieser Kandidaten aufzuklären. Der zweite Teil dieser Arbeit behandelt zwei Substrate für RNA-Editierung: das zytoskelettale Protein Filamin A und BLCAP, ein Protein, assoziiert mit der Entstehung von Blasenkrebs. Editierte und nicht editierte Versionen dieser Proteine wurden stabil in Säugerzellen exprimiert. Durch Aufreinigungsexperimente unter nativen Bedingungen und anschließender massenspektrometrischer Analyse konnten einige Proteine identifiziert werden, welche mit diesen ADAR-Substraten interagieren.

Abstract

Adenosine to inosine RNA editing is a posttranscriptional modification highly conserved in higher metazoa. The hydrolytic deamination of adenosine to inosine is catalysed by a family of enzymes, known as adenosine deaminases that act on double-stranded RNA (ADARs). Changing the sequence of a primary transcript by RNA editing can have dramatic consequences: Editing of the pre-mRNA of a coding transcript can lead to alternative splicing events and may cause amino acid substitutions in the translated protein, as the triplet codon becomes changed. Apart from that, most of the known substrates of RNA editing are non-coding RNAs and repetitive elements, as Alu elements in untranslated regions of the transcript, and micro RNAs. These editing events can influence gene expression, as the sequence of regulatory elements or the target specificity of small RNAs is altered. To date, on-going studies have identified many targets of ADAR editing. However, very little is known about the consequences of the editing events. In addition to the consequences of editing on its targets, mechanisms of regulation of A to I editing are still unclear.

In the last few years several candidates for regulators of ADAR activity have been identified in our lab. In this thesis we stably expressed these candidates in mammalian cell lines for purification assays. Subsequent mass spectrometric analysis of purified complexes led to the identification of proteins interacting with editing regulator candidates, what may help to clarify regulatory networks involved in A to I RNA editing. The second part of this project deals with two protein-coding targets of RNA editing: The effect of RNA editing on the interaction profiles of the cytoskeletal cross-linker Filamin A, and the bladder cancer associated protein BLCAP is investigated. Edited and unedited versions of both proteins were stably expressed in mammalian cell lines. Purification of the two targets of RNA editing under native conditions led to the identification of interacting proteins after mass spectrometric analysis.

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Abbreviations

RNA…ribonucleic acid

mRNA…messenger RNA

ADAR...Adenosine deaminase that act on RNA

DsRBD ... double-stranded RNA binding motif

A...Adenosine

I...Inosine

U...Uracil

G...Guanosine

C...Cytosine

miRNA...micro RNA

rRNA...ribosomal RNA

snRNA...small nucleolar RNA

HDV...Hepatitis delta virus

Tudor-SN...Tudor staphylococcal nuclease

RISC...RNA induced silencing complex

RNAi...RNA interference

Pre-mRNA...precursor messenger RNA

FLNA...Filamin A

F-actin...filamentous actin

GBM...Glioblastoma multiforme

AMPA-receptor... α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor

GluR-B...Glutamate receptor B subunit

ALS...Amyotrophic lateral sclerosis

DSH...Dyschromatosis symmetrica hereditaria

IgG…Immunglobulin G

HRP...horseradish peroxidase

AP...alkaline phosphatase

1. Introduction

After eukaryotic transcription the produced RNA transcript is still immature and has to undergo several processing steps to fulfil its destined function. The primary transcript of a coding region gets modified by 5' capping and 3' processing (e.g. polyadenylation) what stabilizes the transcript (Darnell, 1979; Darnell et al., 1971; Shatkin, 1976). Splicing removes introns from a primary transcript (Konarska and Sharp, 1987; Kruger et al., 1982). Lastly, RNA editing induces sequence changes (Benne, 1992; Cattaneo et al., 1988; Melcher et al., 1996b; Visomirski-Robic and Gott, 1997). These processing steps together with posttranslational mechanisms are thought to be very important for the complexity of higher organisms, to generate a diversity of products from a minimal set of coding regions. The mechanism I focus on in this thesis is RNA editing, a posttranscriptional mechanism, where single nucleotides get selectively inserted, deleted or chemically modified (Aphasizhev et al., 2003; Kapushoc and Simpson, 1999; Navaratnam et al., 1995; Polson et al., 1991; Visomirski-Robic and Gott, 1995).

1.1. RNA editing

Several subtypes of RNA editing exist where bases are changed (C to U/ A to I deamination), deleted or inserted. Nucleotide insertion or deletion editing generally affects mitochondrial and plastid RNA in lower eukaryotes. This subtype of RNA editing was discovered in Trypanosomes. These protozoa insert and delete uridines extensively in mitochondrial messenger RNAs by an editing mechanism, involving guide RNAs (Aphasizhev et al., 2003; Kapushoc and Simpson, 1999; Visomirski-Robic and Gott, 1995). Another subtype of RNA editing is the modification type found in nuclear-encoded RNA. One of these modifications, which occurs in plants and animals, is the deamination of cytidine to form uracil. The first C to U editing reaction identified in mammals was found in the Apolipoprotein B transcript. Here the primary mRNA gets edited to convert a CAA codon into a UAA stop codon to generate the two versions of Apolipoprotein B, type 48 and type 100, which both play

important roles in cholesterol metabolism (Navaratnam et al., 1995; Takenaka et al., 2008).

The conversion of adenosine into inosine in double-stranded RNAs is catalysed by a family of enzymes called adenosine deaminases that act on double-stranded RNA (ADARs) (Bass et al., 1997). During this editing reaction the C-6 position of an adenine base becomes hydrolytically deaminated to generate inosine (Polson et al., 1991; Wagner et al., 1989). As depicted in figure 1, inosine is recognized as guanidine by cellular machineries, because it forms Watson-Crick base pairs with cytosine (Polson et al., 1991). RNA editing may be a powerful mechanism for the generation of protein diversity, because the edited and the unedited version of the transcript are present within a cell, regulated in a tissue- and temporal-specific manner. The first member of the ADAR family was discovered and isolated from *Xenopus laevis* in 1987 as a double-stranded RNA unwinding activity (Bass and Weintraub, 1987; Rebagliati and Melton, 1987). Later it was found, that the enzyme does not actively unwind RNA but the duplexes are destabilized by inosine : uracil base pairs (Wagner et al., 1989). To date several other members of ADARs have been identified and isolated from many metazoa, like from mammals, birds, flies and worms (Herbert et al., 1995; Melcher et al., 1996b; O'Connell et al., 1995; Palladino et al., 2000b; Tonkin et al., 2002).

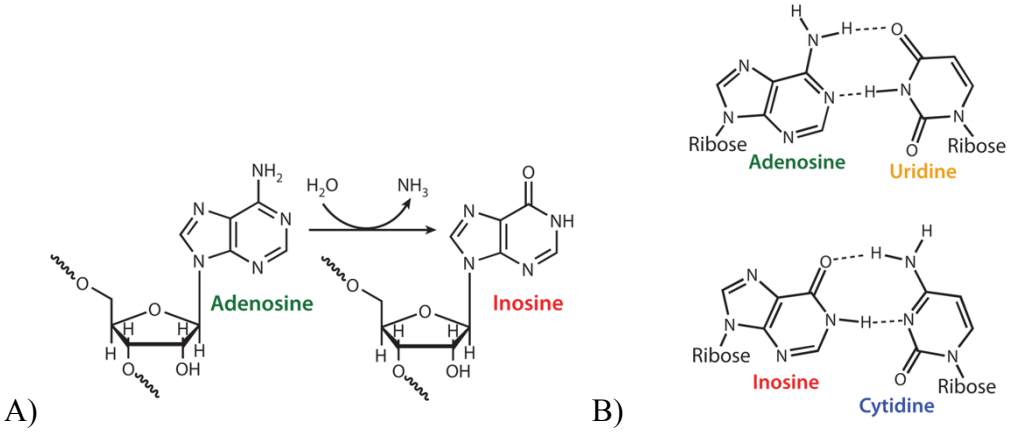


Figure 1) RNA editing. A) Hydrolytic deamination of adenosine into inosine. B) Base-pairing properties before and after RNA editing (Nishikura, 2010).

Due to the fact that the conversion of adenosine into inosine alters the primary sequence and structural features of a RNA transcript, RNA editing may also effect

functional activities of the mature RNA. Several cellular mechanisms affected by RNA editing are depicted in figure 2 and discussed in the following section.

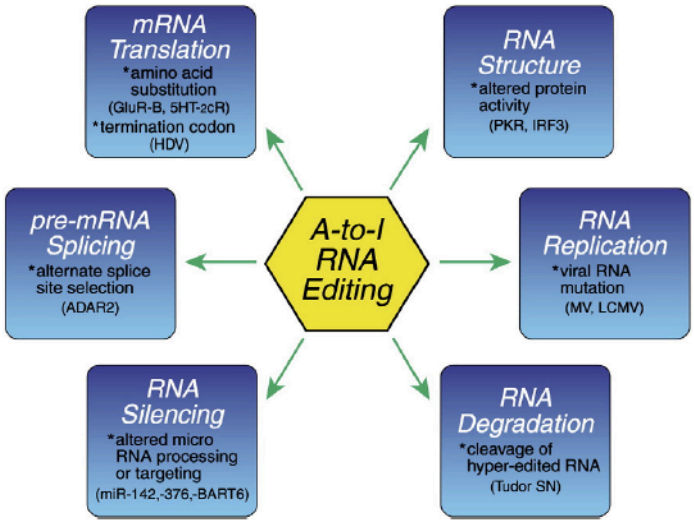


Figure 2) Mechanisms affected by RNA editing. Since inosine forms Watson-Crick base pairs with cytosine, RNA editing leads to a nucleotide substitution in the primary RNA transcript. A to I RNA editing can alter the meaning of a triplet codon and therefore lead to an amino acid substitution of the translated product. Pre-mRNA splicing is also affected by RNA editing if a conserved A of a splice site recognition sequence becomes edited. Editing of miRNA can influence the action of miRNA processing proteins. Additionally the target specificity of the mature miRNA can be altered if an A within the seed region becomes edited. Also RNA stability and structure are manipulated by RNA editing because I:U base pairs generate bulges within the RNA duplex and destabilize the molecule (Samuel, 2011).

The change of the primary sequence of protein coding transcripts may lead to a substitution of the encoded amino acid since inosine is interpreted as guanosine by cellular machineries, like the translation machinery. Thus the translated product may show alternative activity features as was shown for example for HDV viral proteins (Chen et al., 2010) and several neuronal receptors as described in 1.5. Since RNA editing alters the primary transcript, also conserved RNA-encoded recognition sites can be altered, such as AU dinucleotides, which are highly conserved splice site recognition sites and intronic or exonic splicing enhancer or silencer sequences respectively. Thus RNA editing may be involved in regulating RNA processing. To mention one example, ADAR2 was found to autoregulate its own expression. By autoediting of ADAR2 pre-mRNA, an alternative 3' splice site is generated what causes the insertion of 47 nucleotides into the mature transcript (Rueter et al., 1999). In addition to the effects of RNA editing on mRNA translation and splicing it also

affects replication of viral RNA, since A to G and U to C transitions are generated (Cattaneo and Billeter, 1992). A to I RNA editing leads to mutations in the viral genome after RNA-dependent RNA replication. As mentioned earlier, RNA editing was initially discovered as an RNA unwinding activity (Bass and Weintraub, 1987; Rebagliati and Melton, 1987). The reduced stability of RNA duplexes is due to the conversion of a A:U Watson-Crick base pair into a less stable I:U wobble base pair (Serra et al., 2004; Strobel et al., 1994). Micro RNAs are also known to be targets of ADARs (Kawahara et al., 2008; Kawahara et al., 2007a; Kawahara et al., 2007b). Thus by RNA editing the action of miRNA processing proteins, such as Drosha/DGCR8 or Dicer, can be inhibited (Kawahara et al., 2007a; Yang et al., 2006). Additionally, the target specificity of the mature miRNA can be altered, if an A within the seed region becomes edited (Kawahara et al., 2007b). As mentioned before, RNA editing reduces the stability of the RNA by changing the base pairing properies of the transcript. Additionally, hyperediting leads to efficient degradation of the inosine containing RNA. The RISC subunit Tudor staphylococcal nuclease (Tudor-SN) is known to cleave hyperedited inosine-containing RNA, thus linking RNA editing to RNAi pathways (Scadden, 2005).

1.2. The family of ADARs

Enzymes with deaminase activity have been identified in several organisms (Herbert et al., 1995; Melcher et al., 1996c; O'Connell et al., 1995; Palladino et al., 2000a; Tonkin et al., 2002). These proteins, belonging to the family of ADAR enzymes, show a common domain structure. The deaminase domain is located at the C-terminus and highly conserved. The N-terminal structure by contrast varies in the amount of double-stranded RNA binding motifs (dsRBDs) (St Johnston et al., 1992) and several additional nucleotide binding domains, such as the Z-domain of ADAR1 isoforms, which enables them to bind DNA, and the arginine-rich R-domain in ADAR3, which is known to be a common single-stranded RNA binding motif (Chen et al., 2000; Herbert et al., 1997). Though, the functional significance of these additional DNA/RNA-binding domains remains largely unknown. Three variants of ADARs are highly conserved among vertebrates (Slavov et al., 2000). In humans, ADAR1 is expressed in two isoforms. The expression of the larger 150 kDa ADAR1 isoform is induced by interferon signalling, stimulated by cellular stress or viral infection and is

known to be present within the cytoplasm and nucleus. The shorter p110-ADAR1 isoform is constitutively expressed and exclusively nuclear located. Both ADAR1 isoforms are able to bind double-stranded RNA through three dsRBDs (Patterson and Samuel, 1995). ADAR2 was found to be alternatively spliced to yield isoforms containing distinct C-termini and 3' UTRs expressed abundantly in brain and heart tissues (Gerber et al., 1997). A third member of the human ADAR enzymes, ADAR3, was identified in 1996 (Melcher et al., 1996a). ADAR3 is expressed exclusively in brain tissues (Melcher et al., 1996a) and contains both, double- and single-stranded, RNA binding motifs (Chen et al., 2000). To date there is no evidence for the enzymatic activity of ADAR3. However, in vitro editing assays have shown inhibited editing activity of ADAR1 and ADAR2 in the presence of ADAR3 indicating a regulatory role of ADAR3, for the editing process (Chen et al., 2000).

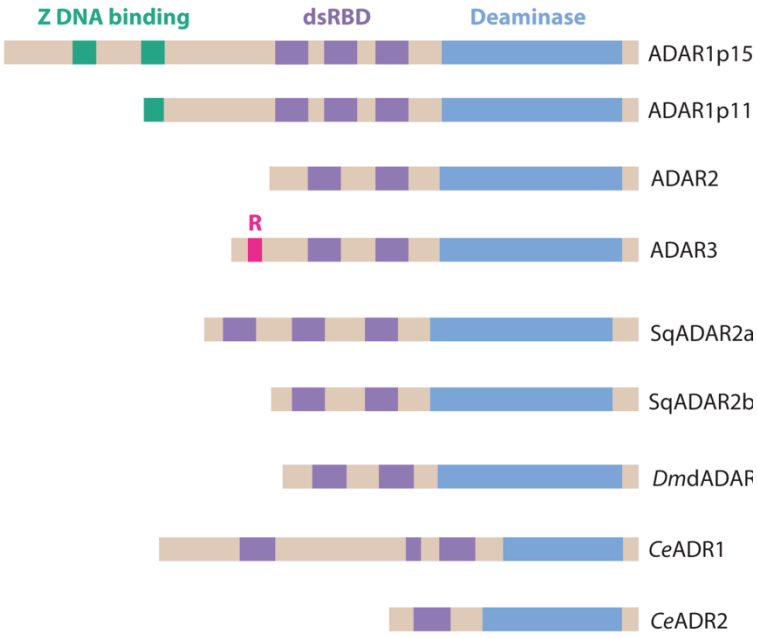


Figure 3) The family of ADARs. ADAR1, ADAR2 and ADAR3 are the three known vertebrate family members, where ADAR1 is expressed in two isoforms. Two squid, two *C. elegans* ADAR enzymes and one *D. melanogaster* ADAR enzyme are known. These ADAR family members show a highly conserved deaminase domain at their C-termini and variable numbers of dsRBDs. Some members comprise additional nucleotide binding motifs such as a DNA-binding Z-motif in ADAR1 and a single-stranded RNA binding motif in ADAR3. ADAR: Adenosine deaminase that act on RNA; Sq: *squid*; Dm: *Drosophila melanogaster*; Ce: *Caenorhabditis elegans*; blue: deaminase domain; violet: dsRBD; green: Z-DNA binding domain; pink: R-motif (Nishikura, 2010).

1.3. Regulation of RNA editing

RNA editing has a dramatic effect on protein function and the regulation of RNA meditated cellular mechanisms. However, little is known about the regulation of the editors, the ADARs. Nevertheless, some facts about ADAR expression pattern and known posttranscriptional and posttranslational modifications are discussed in this section.

Expression of ADAR proteins is highly regulated during development and in a tissue specific manner (Gerber et al., 1997; Jacobs et al., 2009; Melcher et al., 1996a; Melcher et al., 1996c; O'Connell et al., 1995). The two active enzymes, ADAR1 and ADAR2, are expressed in many tissues. However, their expression was found to be highest in brain tissues (Melcher et al., 1996c). A different set of proteins becomes edited during brain development with increasing rates, where editing levels are highest in the adult brain. The target specificity and rate of editing during development cannot simply be explained by altered expression levels of ADARs (Wahlstedt et al., 2009). ADAR1 is expressed in two isoforms, a full-length ADAR1p150, driven by an interferon-inducible promoter, and a N-terminally truncated ADARp110, which is constitutively expressed (George and Samuel, 1999; Patterson and Samuel, 1995). The ADAR1 gene comprises three alternative promoters, whereas, one of them is induced by interferon signalling. The produced transcripts comprise alternative first exons (George and Samuel, 1999; Kawakubo and Samuel, 2000). Both isoforms of ADAR1 contain three dsRBDs and the C-terminal deaminase domain (O'Connell et al., 1995). However, ADAR1p110 contains just one DNA-binding motif, the Z-binding domain, whereas ADAR1p150 encodes two Z-binding domains, Zα and Zβ, as a N-terminal extension of the short isoform, where only the Zβ domain is found (Herbert et al., 1997). The p150 isoform of ADAR1 comprises nuclear export and nuclear import sequences (Eckmann et al., 2001), which enable it to shuttle between nucleus and cytoplasm, regulated by the binding of transportin-1 and exportin-5 respectively, where binding of dsRNA would interfere with transportin-1 binding (Fritz et al., 2009). Additionally it was found earlier, that another nuclear export signal is located within one Z-binding domain of ADAR1p150, which is bound by the nuclear export receptor Crm1, what is another indication for a shuttling activity of ADAR1p150 (Poulsen et al., 2001). Also

posttranslational modifications of the editing enzymes have been shown to be important for their enzymatic function. Sumoylation of lysine residues of ADAR1 by SUMO-1 reduces the editing activity of ADAR1 in vitro and in vivo. ADAR1 co-localizes with ADAR2 in the nucleolus. However, only ADAR1 gets modified by SUMO-1, because ADAR2 lacks the N-terminal sumoylation site (Desterro et al., 2005).

ADAR2 is predominantly located within the nucleus, where it accumulates and co-localizes with ADAR1 within defined compartments of the nucleolus (Desterro et al., 2003). Sansam et al. have shown in 2003, that ADAR2 is able to shuttle rapidly from the nucleolus to other nuclear compartments. This concentration of ADAR2 within the nucleolus is rRNA-dependent, where ADAR2 translocation is targeted by direct interactions with rRNA. In order to become enzymatically active ADAR2 translocates out of the nucleolus to the nucleoplasm to edit its nuclear encoded RNA substrates, suggesting an important impact of localization of the enzyme on the regulation of editing (Sansam et al., 2003). Various isoforms of ADAR2 are generated by alternative splice events in a tissue-specific manner (Agranat et al., 2010; Gerber et al., 1997; Maas and Gommans, 2009). ADAR2 selectively modifies its own pre-mRNA and thereby auto-regulates its expression by generating various alternatively spliced products. This editing event happens as a co-transcriptional process (Laurencikiene et al., 2006). One of these editing events converts an intronic AA into an AI dinucleotide, generating an alternative proximal 3' splice site acceptor. Alternative splicing of the ADAR2 pre-mRNA leads to an alternative transcript, including additional nucleotides and thus results in a frame shift. By changing the reading frame of the ADAR2 mRNA the translated protein lacks the dsRBDs and zinc-coordination motifs required for deaminase activity. Hence RNA editing is a possible mechanism for the regulation of alternative splicing and protein expression (Rueter et al., 1999). Another alternative splice event within the ADAR2 primary transcript leads to the generation of an ADAR3-like nuclear localization sequence bound by importins. This splicing event alters the binding specificity of importins and may play an important role during localization of the protein, thus could be another way of regulating ADAR activity (Maas and Gommans, 2009). Structural studies of the ADAR2 deaminase domain have illustrated the requirement of one zinc atom and one molecule of inositol hexakisphosphate in the catalytically active centre of the ADAR2 deaminase domain. The zinc atom may help the enzyme to localize the

adenosine to be edited, located in the major groove of the dsRNA. The inositol hexakisphosphate molecule is required as cofactor for the catalytic activity of the deaminase and for protein folding. However, ADAR1 does not need inositol hexakisphosphate for its enzymatic activity (Macbeth et al., 2005). In addition it was found that ADAR1 and ADAR2 both predominantly form homodimers. Functional protein-protein interactions between the molecules seem to be important for their enzymatic activity and for choosing the adenosine to be edited (Cho et al., 2003; Jaikaran et al., 2002). The formation of homodimers is RNA-independent, but two ADAR monomers with functional dsRBDs are required for the editing reaction to occur (Valente and Nishikura, 2007). However, recombinant expressed ADAR3 does not form homodimers, what may explain its enzymatic inactivity. Whether brain specific posttranslational modifications or oligomerization of ADAR3 might lead to an enzymatically active enzyme is still not proven (Cho et al., 2003). As mentioned before, no enzymatic activity of the third member of the vertebrate ADAR family, ADAR3, has been shown so far. Though it contains a homologous deaminase domain, similar dsRBDs and an additional R-domain for binding of single-stranded RNA (Chen et al., 2000), no editing activity on known editing substrates could be demonstrated. ADAR3 also differs from its family members in its expression pattern since it its exclusively expressend in brain tissues (Melcher et al., 1996a). One nuclear localization signal was found within the arginine-rich R-domain of ADAR3, which is bound by importin alpha 1. Nevertheless, identification of this binding partner of ADAR3 does still not elucidate the functional role of this ADAR family member (Maas and Gommans, 2009).

In the last few years several candidates for regulators of ADAR-mediated editing have been identified in our lab. In a new developed yeast screen the small acidic protein DSS1 (yeast ortholog SEM1) was found to be an activator of ADAR2 activity. In addition, some editing inhibiting candidates have been identified: The RNA helicase DDX15, the splice factor SRSF9 and the ribosomal protein RPS14. The regulatory role of these proteins was also confirmed in mammalian cells (Aamira Tariq, unpublished data).

DSS1 is of 70 amino acids in length and seems to be a ubiquitous protein, present in the nucleus and the cytoplasm. This short protein is comprised of three alpha-helices

separated by two beta-sheets, containing a high number of acidic amino acids (Yang et al., 2002). As part of the small subunit of the 26S proteasome in yeast and mammals, the evolutionarily conserved DSS1 may play an important role in protein degradation (Coux et al., 1996; Funakoshi et al., 2004; Josse et al., 2006; Sone et al., 2004). Additionally DSS1 may be involved in DNA repair, as it forms a stoichiometric complex with the repair protein BRCA2 (breast cancer associated protein 2). DSS1 is required for BRCA2 stability and regulates its ability to bind single-stranded DNA (Li et al., 2006). Other studies have indicated an important role of DSS1 during the recruitment of BRCA2-RAD51 complexes to sites of DNA damage (Dray et al., 2006; Gudmundsdottir et al., 2004). DSS1 seems to be a multifunctional player within the cell, as in addition to its role during protein degradation and DNA repair, studies in yeast indicated also an important role of DSS1 during splicing and mRNA export through interactions with nucleoporins (Thakurta et al., 2005; Wilmes et al., 2008).

The DExD/H-box RNA helicase DDX15 (Imamura et al., 1997), the human ortholog of the yeast Prp43, has been identified as a possible negative regulator of ADAR activity. DEAH-box RNA helicases are involved in several cellular processes where they unwind double-stranded RNA in an energy dependent manner, reviewed in Rajkowitsch et al., (Rajkowitsch et al., 2007). DDX15 was found to localize to the nucleus, nuclear speckles and nucleoli, where it co-localizes with U snRNAs, and may play important roles during splicing of primary transcripts as its yeast ortholog Prp43 does. The N- and C-terminal regions of DDX15 contain localization signals. The N-terminal part of the protein is involved in localization of DDX15 to the nucleus and nucleolus, whereas C-terminal signal sequences are responsible for the localization to nuclear speckles. It was also shown that parts of the DDX15 DEAH-box domain interact with the LA antigen, a putative RNA chaperone (Fouraux et al., 2002).

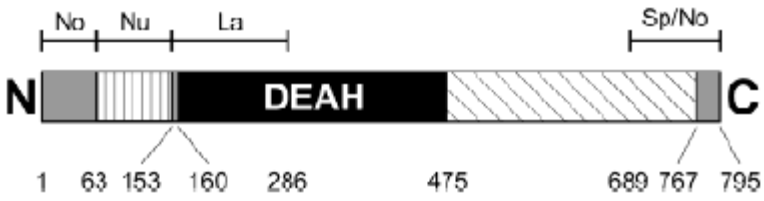


Figure 4) Scematic domain structure of DDX15. The highly conserved DEAH domain of DDX15 (black) is flanked by N-and C-terminal extensions, which contain signal sequences important for DDX15 subcellular localization. At the N-terminus sequences for localization to the nucleus (Nu) and to the nucleolus (No) are found. The C-terminal signal sequences are involved in localization to nuclear speckles. The LA antigen binds within the helicase domain (La) (Fouraux et al., 2002).

Another candidate, identified in an ADAR-inhibiting activity screen, is the ribosomal protein RPS14. RPS14 is a subunit of the 40S ribosomal part and is required for 18S pre-rRNA processing (Ebert et al., 2008; Ferreira-Cerca et al., 2005) and 40S ribosomal subunit formation (Chen et al., 1986; Rhoads et al., 1986)**.** Heterozygous deletion of RPS14 leads to a decrease in the production of erythroid progenitors and blocks the production of terminally differentiated erythroid cells. The phenotype of this haploinsufficiency of RPS14 correlates with the 5q- syndrome or myelodysplastic syndrome, where heterozygous deletion of RPS14 was identified as a major cause of the disease (Ebert et al., 2008).

The last putative candidate for a negative regulator of ADAR activity identified in the editing screens is the arginine/serine-rich splice factor SRSF9. SR proteins are characterized by their ability to interact simultaneously with RNA and protein components via an RNA recognition motif (RRM) and through a domain rich in arginine and serine residues, the RS-domain. Their functional roles in gene expression are surprisingly diverse, ranging from their classical involvement in constitutive and alternative pre-mRNA splicing to various post-splicing activities, including nuclear export of mRNA, nonsense-mediated decay, and mRNA translation, reviewed in Shepard et al., (Shepard and Hertel, 2009). Our candidate, SRSF9, also known as SRp30, is involved in alternative splicing of pre-mRNA (Screaton et al., 1995).

Figure 5) Scematic domain structure of SRSF9. The arginine/serine rich splice factor SRSF9 was initially named as SRp30c. It contains a RRM, RNA recognition motif, RRMH, RNA recognition motif homology, and an arginine/serine-rich domain (RS), taken and modified from Shepard et al, 2009 (Shepard and Hertel, 2009).

1.4. Site-selectivity and substrate specificity of ADARs

Several studies on A to I RNA editing have indicated that an RNA duplex, completely double-stranded or comprising single-strand bulges and loops, is required for the editing reaction to occur, whereat inter- and intra-molecular double-stranded regions can serve as substrates for editing. However, the modification efficiency is dependent on the length of the double-stranded region (Morse et al., 2002; Nishikura et al., 1991). Long double-stranded viral RNAs become extensively edited during replication, presumably as mechanism for adaptation (Cattaneo et al., 1988). By contrast, site selectivity of RNA editing is dependent on the RNA secondary structure, where mismatched bases, bulges or loops may determine which adenosine becomes edited (Lehmann and Bass, 1999). Overall, RNA editing seems not to be sequence specific. Nevertheless, in addition to the importance of the secondary structure of editing targets, several studies on specific substrates of ADARs have shown site-selective editing events dependent on the sequence context of the edited RNA. Thus, structural elements and the sequence of the editing substrate determine the extent of editing in a given double-stranded RNA (Dawson et al., 2004; Maas et al., 1996). ADAR1 has a 5' neighbour preference (A = U > C > G) but edits independent of the adenosine 3' neighbour. Also the distance of the edited adenosine to strand termini was found to impact the probability of this adenosine to be edited (Barraud et al., 2011; Eggington et al., 2011; Polson and Bass, 1994). ADAR2 has a similar 5' neighbour preference as ADAR1 (A = U > C = G), but was found to have a 3' neighbour preference too (U = G > C = A). In general, the nearest 5' neighbour highly

influences the choice of the A to be edited (Eggington et al., 2011). Dependent on the structure and sequence of the substrate, the adenosine to be edited, may be flipped out of the helix (Hough and Bass, 1997). Studies on editing selectivity of both enzymes have shown an overlap in substrate specificity of ADAR1 and ADAR2 (Lehmann and Bass, 2000; Riedmann et al., 2008).

1.5. Targets of ADARs

Messenger RNAs expressed in the central nervous system have been the first identified substrates for A to I RNA editing (Sommer et al., 1991). Though on-going bioinformatical and biochemical studies have identified thousands of new editing sites within non-coding RNAs, untranslated highly repetitive regions and in some protein-coding RNAs, little is known about the effect of RNA editing on the organism (Athanasiadis et al., 2004; Blow et al., 2006; Hundley and Bass, 2010; Kim et al., 2004; Levanon et al., 2004; Levanon et al., 2005; Li et al., 2009; Riedmann et al., 2008; Xia et al., 2005). However, knock out studies on worms, flies and mice have revealed a fundamental role for ADAR enzymes for the living organism. The expression of the *Drosophila melanogaster* homolog of ADAR, dADAR, is highly regulated during development (Chen et al., 2009). Flies lacking dADAR show a severe behavioural phenotype and have impaired coordinated locomotion (Palladino et al., 2000a, c). If the *Caenorhabditis elegans* homologs of ADAR, ceADAR1 and ceADAR2, are homozygously deleted, the worms show defects regarding chemotaxis and in development of the vulva (Tonkin et al., 2002). Mice carrying a homozygous deletion of ADAR1 die early during embryonic development between day E11.0 and E12.5. This severe ADAR1 knock out phenotype is accompanied by elevated levels of apoptosis (Hartner et al., 2004; Wang et al., 2004) liver disintegration and defects in haematopoiesis (Hartner et al., 2004; Hartner et al., 2009). Additionally, conditional knock-out studies with mice have indicated an essential role of ADAR1 during fetal and adult hematopoietic stem cell maintenance and as a suppressor of interferon signalling in hematopoietic stem cells and fetal liver progenitor cells (Hartner et al., 2009). ADAR2 deficiency does not interfere with embryonic development, but mice lacking ADAR2 die early after birth due to recurrent epileptic

seizures (Brusa et al., 1995; Higuchi et al., 2000). This severe phenotype is due to underediting of one particular position in a protein-coding ADAR2 substrate, the Q/R-site of glutamate receptor B (GluR-B), what leads to an increased Ca^{2+} permeability of the AMPA receptor channel. Thus, the GluR-B Q/R-site may be the most important physiological substrate of ADAR2 (Higuchi et al., 2000).

Non-coding RNAs and repetitive elements, like Alu elements, within transcripts are the most abundant substrates for RNA editing (Greenberger et al., 2010; Kim et al., 2004; Levanon et al., 2004; Morse et al., 2002; Neeman et al., 2006). Nevertheless, also some protein-coding targets have been identifed, where editing of the pre-mRNA can lead to recoding of the edited transcript. Several proteins involved in neurotransmission have been some of the first identified editing substrates of this target group (Hoopengardner et al., 2003; Seeburg and Hartner, 2003). As mentioned earlier, A to I RNA editing of the AMPA receptor GluR-B subunits leads to an amino acid substitution from a glutamine into an arginine, what regulates Ca^{2+} influx into the cell (Sommer et al., 1991). Editing of potassium channels within the ion-conducting pore leads to fast inactivation of the channel (Bhalla et al., 2004). Also one G-protein coupled seven-transmembrane-spanning receptor, the 5-HT_{2C} serotonine receptor, has been found to be an ADAR target (Niswender et al., 1998). In this case RNA editing also leads to amino acid substitutions, what decreases G-protein coupling interactions to the receptor (Burns et al., 1997) and thus silences constitutive activity of the serotonine receptor (Niswender et al., 1999). Anyhow, except from the lack of editing of the GluR-B subunit in ADAR2-deficient mice, to date there is no explanation for the embryonic lethal phenotype of ADAR1 knock-out mice on the molecular level. Hence several groups have developed new biochemical and bioinformatical methods to identify other targets of RNA editing (Hoopengardner et al., 2003; Levanon et al., 2005; Maas et al., 2011; Riedmann et al., 2008). Up to now many other protein-coding targets have been identified, but the effect of the amino acid substitutions happening in some of these proteins is unclear. In this thesis I focus on two protein-coding targets of RNA editing identified in 2005: the cytoskeletal cross-linker Filamin A and the bladder cancer associated protein BLCAP (Levanon et al., 2005).

1.5.1. Filamin A

Filamin A (FLNA) was initially identified as actin filament cross-linker in non-muscle cells (Hartwig and Stossel, 1975; Stossel and Hartwig, 1975). Three highly conserved mammalian Filamins are known, Filamin A, Filamin B and Filamin C, and are expressed in a developmental specific pattern (Sheen et al., 2002; Thompson et al., 2000). FLNA is a multifunctional high molecular weight protein, of 280 kDa, and is involved in many cellular processes. The structure of FLNA is comprised of a N-terminal actin-binding domain, containing two calponin homology (CH) domains, followed by 24 beta-sheets folding into immunglobulin-like domains (Fucini et al., 1997; Gorlin et al., 1990; van der Flier and Sonnenberg, 2001). The IgG-like repeats form two rod domains, separated by two calpain-sensitive hinge regions (van der Flier and Sonnenberg, 2001). The N-terminal actin binding domain plus the IgG-like repeats 9 to 15 are responsible for FLNA interactions to F-actin. IgG-like repeats located at the C-terminus of FLNA are docking sites for other FLNA-interacting proteins (Nakamura et al., 2007). The last IgG-like repeat, repeat 24 at the C-terminus of FLNA, is important for FLNA dimerization. FLNA dimers form a V-shaped structure, what mediates branching of actin filaments (Janmey et al., 1990; Pudas et al., 2005; Seo et al., 2009).

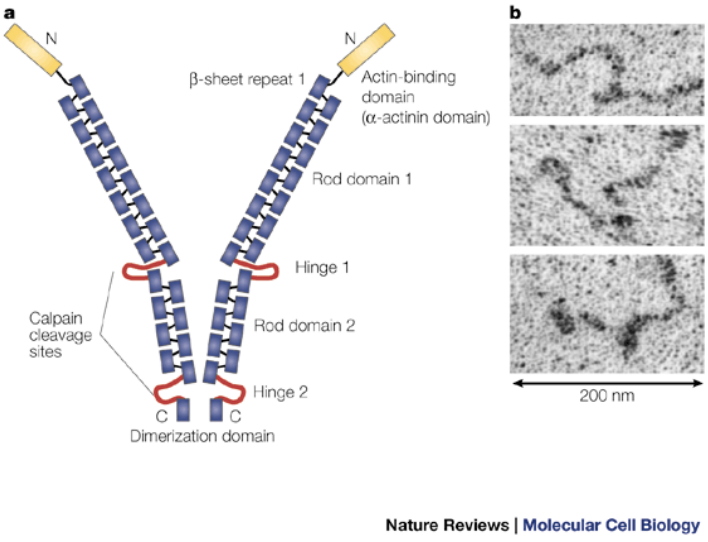


Figure 6) Schematic representation of the overall structure of a FLNA dimer (Stossel et al., 2001). a) This figure shows the V-shaped dimer structure of two FLNA monomers. Dimerization is mediated via the IgG-like repeat 24 at the C-terminus. A filamentous actin-binding domain (yellow) is present at the N-terminus, followed

by 24 IgG-like repeats (blue). Repeats 1 to 15 form rod 1, repeats 16 to 23 form a more globular rod 2. The rod-structures are separated by two hinge regions (red). b) Electron micrographs of tantalum-tungsten-cast rabbit macrophage FLNA dimers (Hartwig and Stossel, 1981; Tyler et al., 1980)

By high-angle orthogonal branching of actin filaments, FLNA plays an important role for cell locomotion, as this cortical actin network is found at the leading edge of migrating cells (Matsudaira, 1994; Weihing, 1988). The V-shaped structure of FLNA allows it to be flexible and stiff at the same time, what is important for the maintenance and rearrangements of the cortical actin network (Gorlin et al., 1990; Hartwig and Stossel, 1981). Since the identification of FLNA as actin filament cross-linker, many other FLNA interacting proteins, apart from F-actin, have been discovered. Among these proteins are trans-membrane receptors, such as integrin beta (Travis et al., 2004), but also cytoplasmic signalling molecules, like small GTPases (Bellanger et al., 2000; Ohta et al., 1999), and transcription factors (Sasaki et al., 2001; Yoshida et al., 2005). Additionally, some studies have referred, that in some cases FLNA is also found within the nucleus and thereby maybe actively regulates transcription factors (Berry et al., 2005). Thus FLNA is involved in the regulation of many different cellular mechanisms, such as cell migration, polarization and stability, cell signalling and transcriptional regulation, reviewed in Zhou et al., (Zhou et al., 2010).

The importance of FLNA as a cytoskeletal cross-linker and signalling scaffold is highlighted in FLNA A null mice. The FLNA knockout phenotype is embryonic lethal, due to abnormal epithelial and endothelial cell junctions in developing blood vessels, heart and brain tissues (Feng et al., 2006). Apart from the studies on model organisms, several human diseases are linked to FLNA mis-function. Periventricular heterotopia is a X-linked brain malformation, caused by loss-of-function mutations of FLNA, where in most cases, truncated versions of FLNA are expressed (Eksioglu et al., 1996; Fox et al., 1998; Moro et al., 2002). This neuronal disease manifests as late-onset epilepsy (Eksioglu et al., 1996). Since FLNA is important for cell migration, a lack of FLNA constricts migrating cerebral neurons from wandering out of the ventricular zone (Fox et al., 1998). A group of FLNA-associated disorders are otopalatodigital spectrum disorders (OPD), as OPD type 1, OPD type 2, frontometaphyseal dysplasia (FMD) and Melnik-Needles syndrome (MNS). In this

cases, point mutations within the actin-binding domain, or localized miss sense mutations in other regions of the protein lead to a high range of pathological manifestations (Robertson et al., 2003). Nevertheless, extended information about the FLNA interaction network will be necessary to declare these FLNA-associated syndromes.

As mentioned earlier, FLNA was identified as one of the protein coding targets of RNA editing. ADAR1 and ADAR2 edit the FLNA transcript at one particular position, which is encoded in the IgG-like repeat 22. This editing event leads to an amino acid substitution from a glutamine into an arginine, what possibly changes the electrostatic features of the protein (Levanon et al., 2005; Riedmann et al., 2008). The IgG-like repeat 22 of FLNA is known to interact with integrin beta (Travis et al., 2004) and small GTPases involved in signalling networks (Ohta et al., 1999). However, the biological effect of FLNA editing on the protein function is still unclear.

1.5.2. BLCAP

As described earlier, the bladder cancer associated protein (BLCAP) has been identified as human substrate for ADAR-mediated editing (Clutterbuck et al., 2005; Galeano et al., 2010; Levanon et al., 2005). BLCAP is also called BC10 (bladder cancer 10 kDa protein) and consists of 87 amino acids, generating two hypothetical membrane-spanning regions. Initially, BC10 was identified in a screen for proteins involved in bladder cancer tumour progression, where its expression was highly down regulated (Gromova et al., 2002; Moreira et al., 2010). In addition to its down regulation in bladder cancer tissues, BLCAP expression was also found to be reduced in renal cell carcinoma (Rae et al., 2000) and cervical carcinoma (Zuo et al., 2006). Very little is known about the function of this small protein. Nevertheless, BLCAP was found to be highly conserved among various species (Galeano et al., 2010; Makalowski and Boguski, 1998).

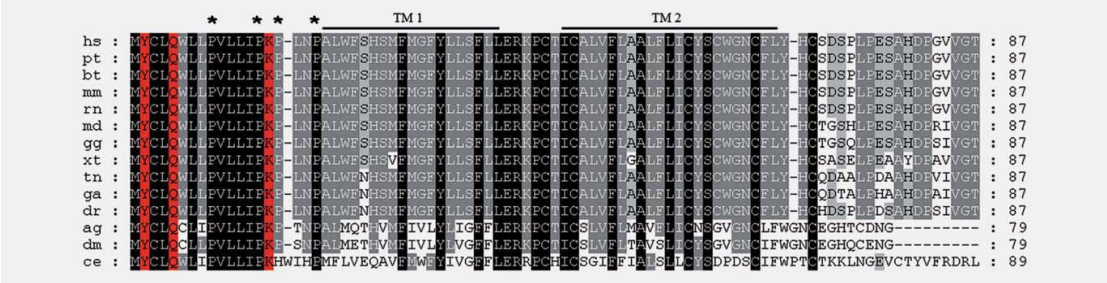


Figure 7) Alignment of BLCAP protein from mutliple species. Identical amino acids among all investigated species are indicated in black. Amino acids identical in most of the samples are gray. Red are edited amino acids. (hs, *Homo sapiens*; pt, *Pan troglodytes*; bt, *Bos taurus*; mm, *Mus musculus*; m, *Rattus norvegicus*; md, *Monodelphis domestica*; gg, *Gallus gallus*; xt, *Xenopus tropicalis*; tn, *Tetraodon nigroviridis*; ga, *Gasterosteus aculeatus*; dr, *Danio rerio*; ag, *Anopheles gambiae*; dm, *Drosophila melanogaster*; ce, *Caenorhabditis elegans (Galeano et al., 2010)*

The BLCAP gene locus shows brain-specific imprinting, thus various transcripts are generated in a tissue-specific manner (Schulz et al., 2009). The gene encoding BLCAP consists of two exons separated by one single intron. Exon 1 encodes parts of the 5' UTR and exon 2 comprises the remaining 5' sequence, the coding region plus the 3' UTR (Galeano et al., 2010).

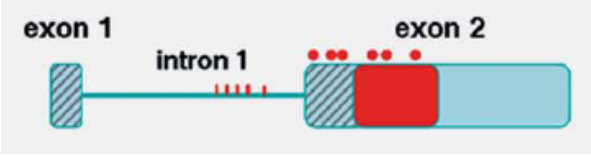


Figure 8) Schematic representation of the BLCAP pre-mRNA structure. Exon1 encodes parts of the 5' UTR (lined box). Exon 1 and exon 2 are separated by one single intron (green line). The remaining parts of the 5' UTR, the coding region (red box) plus the 3' UTR are encoded by exon 2. Red dots indicate editing sites within the coding region of BLCAP. Red lines show editing sites within the intron (Galeano et al., 2010).

It is known that multiple editing events occur within the coding region of the BLCAP exon 2, the 5' UTR in exon 2 and within intronic sequences. In the non-coding parts of BLCAP, 11 editing events have been reported. Within the coding region 3 editing events occur within the highly conserved N-terminal part of BLCAP, that lead to amino acid substitutions, Y/C, Q/R, K/R. Additionally three editing sites have been identified to occur in the exon 2 encoded 5' UTR (Clutterbuck et al., 2005; Galeano et

al., 2010; Levanon et al., 2005). Both enzymes, ADAR1 and ADAR2 have been found to act cooperatively on the editing sites in the BLCAP transcript where the level of editing is tissue-specific (Galeano et al., 2010). Due to the clustering of editing events within the pre-mRNA, a secondary structure formed between the intron and the coding regions has been predicted. The multiple editing positions of BLCAP are edited to different extents in different human tissues, whereby malignant tissues show less editing events in addition to a down regulated expression of BLCAP. Each of the editing events within the coding region of the protein leads to a dramatic change of the originally encoded amino acid. For example, the Q/R event substitutes an uncharged glutamine into a positively charged arginine whereas the K/R site lies within a hypothetical proline-rich region (Galeano et al., 2010). However, the particular function of this highly conserved and ubiquitously expressed protein is still unknown.

1.6. RNA editing and disease

As mentioned before, RNA editing can alter the coding potential of a triplet codon (Chen et al., 2010; Rueter et al., 1995), it can alter or generate a splicing recognition site (Rueter et al., 1999), or change splicing enhancer and silencer sites respectively (He et al., 2011). Other RNA processing mechanisms like the generation of mature miRNA (Yang et al., 2006) and the process of RNAi (Wu et al., 2011) can also be manipulated by RNA editing. In the following section, some examples are described how changes of RNA editing could possibly be involved in disease development.

1.6.1. RNA editing and cancer

A to I RNA-editing is known to occur frequently in the central nervous system (Jacobs et al., 2009; Melcher et al., 1996b; Sommer et al., 1991). Thus on-going studies focus on altered editing patterns in cancerous tissues of the brain. Astrocytomas are classified in four grades, where grade four, the glioblastoma multiforme (GBM), is one of the most dangerous forms of human tumours (Maher et al., 2001). Studies on cancerous tissues of patients suffering from malignant gliomas

have shown a decrease in editing of the GluR-B subunit of AMPA receptors (Maas et al., 2001). As described earlier, this editing event is important for the control of Ca^{2+} influx into the cell (Seeburg et al., 1998). As GluR-B is known to be the physiologically most important substrate of ADAR2 (Higuchi et al., 2000), analysis of paediatric and adult glioblastoma tissues have shown an overall down regulation of ADAR2 (Cenci et al., 2008; Paz et al., 2007). The long isoform of ADAR1 was found to be alternatively spliced in these tissues, leading to the expression of mainly the ADAR1p110 isoform. Excess of ADAR1p110 may be a main cause for ADAR2 down regulation, as ADAR1 and ADAR2 form an inactive heterodimer (Cenci et al., 2008). Further studies on A to I editing substrates were done in many cancerous tissues (Cenci et al., 2008; Galeano et al., 2010; Paz et al., 2007). In 2007, Paz et al. did a bioinformatic investigation on some human cancer tissues. They found a global hypoediting of Alu elements in brain cancer and in malignant tissues of prostate, lung, kidney and testis tumours. This overall reduction of A to I RNA editing was again found to be due to a highly reduced expression of ADAR1 and ADAR2 in the investigated tissues (Paz et al., 2007). However, it was also reported that over-expression of ADAR1 in breast tumours leads to A to I editing of new RNA targets (Shah et al., 2009). An increase in substrate editing was also detected in neuroblastoma samples, where some miRNAs are more often edited than in normal healthy tissues (Schulte et al., 2010). These are just some observations gained from studies on cancerous tissues. Nevertheless, an overall deregulation of ADAR expression and subsequent changes in editing levels of important substrates may play a role during cancer development and tumour progression, reviewed in Gallo et al., (Gallo and Locatelli, 2011).

1.6.2. RNA editing and diseases of the central nervous system

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease, caused by a gradual loss of functional motor neurons. Little is known about the pathological mechanisms leading to the disease. A major cause for the development of familiar ALS is a mutation in the gene of the radical scavenger SOD1 (superoxide dismutase). Progressive loss of motor neurons may be caused by excessive accumulation of SOD1 and other neurofilament proteins (Rosen, 1993). However, there are several other hypotheses that may explain the development of the disease.

One of these hypotheses, leading to death of motor neurons, concerns α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, a subtype of ionotropic glutamate receptors. Increased Ca^{2+} influx through AMPA receptors was repeatedly shown to play a pivotal role during motor neuron death (Lu et al., 1996). The Ca^{2+} permeability of these AMPA-receptors is dependent on the presence and properties of the GluR-B subunit (Hollmann et al., 1991). The properties of the GluR-B subunit are determined by RNA-editing, leading to an amino acid substitution where glutamine gets changed into arginine (Burnashev, 1992). The incorporation of unedited GluR-B into AMPA receptors causes high Ca^{2+} permeability of the receptors whereas the presence of the edited version of GluR-B blocks the Ca^{2+} influx into the cell (Swanson et al., 1997). It was found, that patients suffering from sporadic ALS show defects in editing the glutamate receptor GluR-B subunit. Editing of the Q/R position was dramatically decreased in diseased neurons (Kawahara et al., 2004). In 2005 it was shown that the expression of a transgenic Ca^{2+} permeable GluR-B (N) version of the AMPA receptor in mice leads to the development of late-onset degeneration of motor neurons and neurons of the spinal cord, mimicking a neurodegenerative disorder. If those genetically modified mice express a mutated version of the superoxide dismutase gene (SOD1) additionally, disease progression and severity is enhanced (Kuner et al., 2005). Nevertheless, the defect in RNA editing of the glutamate receptor subunit may be just an additional cause for neuronal death, as mainly spinal motor neurons show deficient RNA editing of the GluR-B (Kawahara et al., 2004).

Recurrent unprovoked seizures are a main characteristic of a neurological disorder, called epilepsy. Hyperexcitability of neurons, caused by changes in AMPA receptor composition, expression, channel density and subsequent imbalanced intracellular levels of Ca^{2+}, is thought to be one of the main causes of several epileptic phenotypes. These changes in glutamate receptor excitability can be caused by brain injuries, like stroke and traumatic brain damages, what eventually leads to neuronal death (Delorenzo et al., 2005). Efficiency of membrane conductance mediated by AMPA receptors is highly influenced by the subunit composition of the receptor. As mentioned earlier, the mRNA encoding glutamate receptor subunit GluR-B gets posttranscriptionally modified by RNA editing (Melcher et al., 1996c; Rueter et al., 1995), where the conversion of adenosine into inosine leads to an amino acid substitution from glutamine into arginine. This editing event was shown to be critical

for the Ca^{2+} permeability of the AMPA receptor (Burnashev, 1992; Sommer et al., 1991). There are several indications for the impact of deregulated RNA editing at the GluR-B Q/R site for the development of at least some epileptic pathologies. Mice expressing a genetically altered editing-incompetent version of the GluR-B receptor subunit die early after birth caused by epileptic seizures (Brusa et al., 1995). Mice defecient in the RNA editing enzyme ADAR2 are also prone to seizures and die a view days after birth. Studies on the ADAR2 knock-out phenotype have illustrated, that the GluR-B pre-mRNA may be the physiologically most important substrate of ADAR2 as the lethal phenotype can be rescued by the expression of a transgenic AMPA receptor containing an arginine at the Q/R site (Higuchi et al., 2000). However, more studies on tissue specimen of diseased patients have to be done, to link deficient RNA editing to human disease development.

Several missense, frame-shift, nonsense and splice site mutations in the human ADAR1 gene have been linked to the autosomal dominant inherited pigmenting disease Dyschromatosis symmetrica hereditaria (DSH) (Cui et al., 2005; Gao et al., 2005; Miyamura et al., 2003; Zhang et al., 2004). One of these mutations leads to a frame-shift upstream of the ADAR1p110 translation initiation site thus eliminating the expression of the large p150 isoform of ADAR1 (Gao et al., 2005). Another mutation in the ADAR1 gene generates a premature stop codon, which leads to the production of a truncated ADAR1 protein lacking the deaminase domain (Xing et al., 2005). However, more information on the development of the disease is still missing, because the wide range of mutations in the ADAR1 gene lead to phenotypes of different severity suggesting an important role for environmental factors (Zhang et al., 2004).

2. Aims and project proposal

The aim of this project is on the one hand to define the interaction pattern of four newly identified regulators of ADAR activity: DSS1, DDX15, RPS14 and the SRSF9 protein. Little is known about the cellular function of these four proteins. By tandem affinity purification and following mass spectrometric analysis we want to generate an interaction profile of the possible regulators of ADAR activity on the one hand and on

the other hand we want to define a model how these proteins could possibly regulate ADAR activity.

In the second part of this thesis we investigate the effects of RNA editing on the interaction behaviour of the two editing targets Filamin A and BLCAP. Several constructs of Filamin A and BLCAP, edited and unedited, were stably expressed in various cell lines. Immunoprecipitations and subsequent mass spectrometric analysis of pulled down interaction partners will bring new insights into the functional effects of RNA editing on these two investigated targets.

3. Material and Methods

3.1. Expression vectors

To investigate interaction proteomics of factors that modulate RNA editing, we had to generate appropriate expression vectors for stable expression of the identified candidates of activators and inhibitors of editing in mammalian cell lines. To improve the overall yield of protein purification, proteins of interest were tagged with a 6x c-MYC-TEV-HA TAP tag, 6x c-Myc, TEV-protease cleavage site, Hemagglutinin, for tandem affinity purification. A TEV-HA fragment was designed *in silico* and cloned downstream to a 6x MYC-tag into a pcDNA 3.1 (-) (*Invitrogen*) expression vector. This 6x c-MYC-TEV-HA tag was cloned into a similar pcDNA vector containing a selection marker, hygromycin, for selection of stable transfectants, pcDNA 3.1 (-), Hygro (*Invitrogen*). The full-length versions of ADAR-affecting candidates were cloned into the generated TAP-tag expression vector by either BamHI or NheI/XhoI digestion.

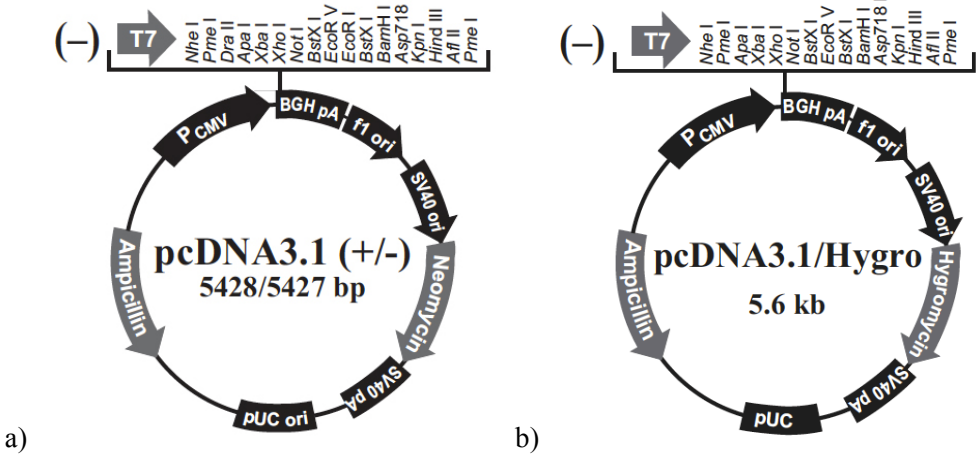


Figure 9) pcDNA vectors. For the generation of an appropriate expression vector for tandem affinity purification the two listed vectors were used. The pcDNA (-) vector already contained a 6x myc tag, which was fused to an additional TEV-HA sequence N-terminally. a) pcDNA (-) containing Neomycin resistance gene. b) pcDNA (+) Hygro containing a Hygromycin resistance gene[1]. T7 = T7 RNA polymerase promoter; P CMV = CMV promoter; BGH pA = BGH polyadenylation signal; f1 ori = f1 origin; SV40 ori = SV40 promoter and origin; SV40 pA =

[1] Invitrogen™ (http://www.invitrogen.com/, 2012)

SV40 polyadenylation signal; pUC = pUC origin; Ampicillin = Ampicillin resistance gene; Hygromycin = Hygromycin B resistance gene.

For transient and stable expression of 3x c-Myc-tagged full length Filamin A, edited and unedited, we used a pREP4 expression vector[2] containing a Hygromycin resistance gene. Truncated versions of edited and unedited Filamin A, comprising immunoglobulin-like repeats 20 to 24, were kindly provided by Christina Godfried, Lehigh University of Pennsylvania, cloned into pCeMM expression vectors. For generation of stable transfected cells we co-transfected the pCeMM vectors with a helper plasmid containing a Hygromycin resistance gene[3].

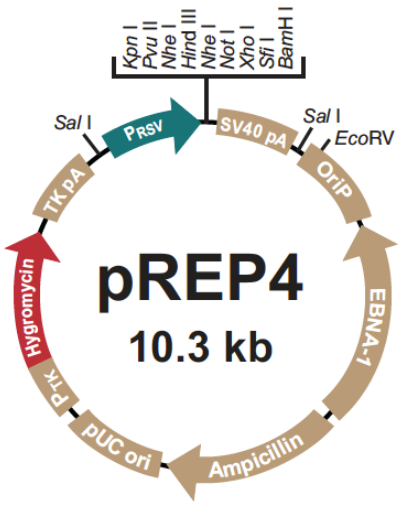


Figure 10) pREP4 mammalian expression vector (*Invitrogen*) for transient and stable expression of full length edited and unedited Filamin A. Full length versions of edited and unedited Filamin A were cloned into a pREP4 expression vector to be tagged with a 3x c-MYC tag. P_{RSV} = RSV LTR promoter; SV40 pA = SV40 polyadenylation signal; OriP = origin; EBNA-1 = Epstein-Barr virus nuclear antigen; Ampicillin = Ampicillin resistance gene; pUC ori = origin; P_{TK} = Tyrosine Kinase promoter; Hygromycin = Hygromycin B resistance gene; TK pA = Tyrosine Kinase polyadenylation signal.

[2] Invitrogen™ (http://www.invitrogen.com/, 2012)
[3] MYC-TEV-HA TAP-tag pcDNA 3.1 (-) expression vector containing Hygromycin resistance gene

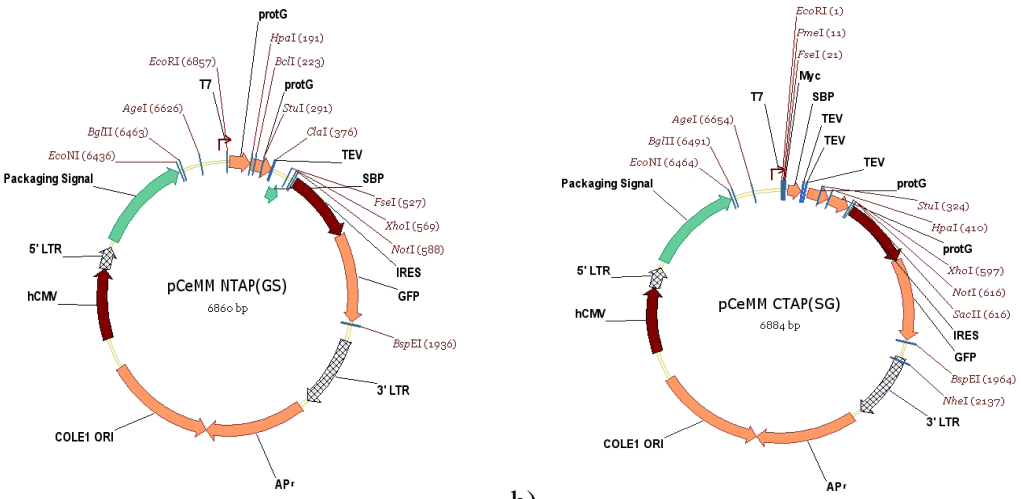


Figure 11) Expression vectors for stable and transient expression of full-length and truncated versions of edited and unedited Filamin A. a) pCeMM expression vector containing protein G plus a streptavidin tag separated by a TEV protease cleavage site for tandem affinity purification of N-terminally tagged inserts. b) pCeMM expression vector containing a c-Myc tag plus a protein G tag separated by a TEV-protease cleavage site for tandem affinity purification of C-terminally tagged inserts (*C-e-M-M, Research Center for Molecular Medicine of the Austrian Academies of Sciences*). protG = protein G; TEV = TEV-protease cleavage site; SBP = Streptavidin binding protein; IRES = internal ribosomal entry site; GFP = green fluorescent protein; 3' LTR = 3' long terminal repeat; AP^r = Alkaline phosphatase reporter gene; COLE1 ori = origin of replication of *Escherichia coli*; hCMV = human cytomegalovirus antigen; 5' LTR = 5' long terminal repeat; MYC = c-Myc.

3.2. Tissue culture

3.2.1. Cell lines

- HeLa: derived from cervical cancer (*Homo sapiens*)
- Hek293: human embryonic kidney cells (*Homo sapiens*)
- U2OS: osteosarcoma (*Homo sapiens*)
- SCaBER: squamous cell carcinoma (*Homo sapiens*)
- STANB: St. Anna Neuroblastoma (*Homo sapiens*)
- cN2A: neuroblastoma (*Mus musculus*)
- C2C12: myoblasts (*Mus musculus*)

3.2.2. Cultivating mammalian cells

All cell lines were cultivated in DMEM (Dulbecco's modified eagle medium, *PAA Laboratories GmbH*) containing 10% FCS (foetal calf serum, *PAA Laboratories*

GmbH), 1% L-glutamine (*PAA Laboratories GmbH*) and 1% penicillin/streptomycine (*PAA Laboratories GmbH*) on 3 to 10 cm dishes (*PAA Laboratories GmbH*) at 37°C, 5% CO_2, 95% humidity. Dense plates were split by washing with 1x PBS to remove all medium and incubation with 1x Trypsin/EDTA (*PAA Laboratories GmbH*) at 37°C for 5'-10'. The reaction was stopped by dilution of the cells in DMEM. The cells were seeded on new plates.

3.2.3. Mammalian Cell Transfection

Material

- 60% confluent cells on acid-edged cover slip (*VWR®*) on 3 cm/6 cm dish (*PAA Laboratories GmbH*), (seeded 24h to 48 h before transfection)
- Nanofectin cell transfection Kit (*PAA Laboratories GmbH*)
- Preparation for different cell culture formats: (*Protocol for use: Nanofectin Kit*[4])

Table 1) Nanofectin complex preparation for different cell culture formats (www.paa.com)

Culture vessel	DNA (µg]	Diluent (µl)	Nanofectin (µl)	Diluent (µl)	Final volume (µl)
96 well	0.25	10	0.8	10	20
48 well	0.5	25	1.6	25	50
24 well	1	50	3.2	50	100
12 well	2	50	6.4	50	100
6 well	3	100	9.6	100	200
25 cm^2	5	250	16	250	500
75 cm^2	8	500	25	500	1000

Method

Cells were seeded on 3 cm dishes 24 h before transfection. Before use, DNA was cleaned by adding Chloroform, mixing, and spinning down for 5' at maximum speed. The aqueous phase can directly be used for transfection. DNA and Nanofectin were mixed with diluents (according to the table above). As a next step the Nanofectin dilution was added to the DNA mix and incubated for > 30' at RT. Afterwards the DNA-Nanofectin-Mix was added dropwise to the cells. 24 h later the medium was changed. 48 h after transfection the cells were fixed and stained or selection was started respectively.

[4] Manufacturers' protocol: www.paa.com

3.3. Immunofluorescence staining

Material

- 1x PBS (phosphate buffered saline)
- 2% PFA/0.05% Triton X-100 (*Fluka*) in 1x PBS
- Methanol (*Riedel-de Haën*)
- 10% horse serum (*PAA Laboratories GmbH*) in 1x PBS-T (0.05% Tween 20, *Fluka*)
- 2.5% horse serum in 1x PBS-T
- Primary antibodies: 9E10 (anti-myc antibody from tissue culture supernatant)
- Secondary antibodies: goat anti-mouse coupled to Alexa 568 (*Invitrogen*) / Alexa 488 (*Invitrogen*), Atto-565 Biotin (*ATTO-TEC GmbH*)
- DAPI 1:1000 in Antifade (1 g p-phenylenediamine/100ml phosphate buffer (150mM NaCl, 10mM KH2PO4, 10mM Na2HPO4), pH 8.0, 1:8 in glycerol)

Method

After washing the cells with 1x PBS (on ice) they were incubated for 5' with ice cold 2% PFA / 0.05% Triton X-100 in 1x PBS, to fix proteins. The cells were washed again with 1x PBS and incubated with ice-cold methanol for 1'. Before blocking for 10' with 10% horse serum in 1x PBS-T the cells were again washed with 1x PBS. When the blocking was done the cover slips were incubated on microscopy slides with a dilution of the primary antibody (9E10 1:1 in 2.5% horse serum in 1xPBST) at 37°C in a humidity chamber for 1h. Before incubation with the secondary antibody the cover slips were washed three times for 5' with 2.5% horse serum in 1x PBS-T. After incubation with the secondary antibody for 1h at 37°C the cover slips were again washed for three times with 2.5% horse serum, stained with DAPI-Antifade and stored at -20°C[5]. For microscopic analysis of the immunofluorescence stained samples a Zeiss fluorescence microscope was used. Images were imported into Photoshop 4 (Adobe Systems, Mountain View, CA) with the help of a QED plug-in module (QED-Imaging, Pittsburgh, PA).

3.4. Immunoprecipitation

Material

- Sepharose A beads (*GE Healthcare*), (2.5-5 mg beads per IP)
- NET-2 lysis buffer: 150mM NaCl, 80mM Tris pH 7.4, 0.05% NP-40 (*AppliChem GmbH*)

[5] Method taken and modified from *Current Protocols in Molecular Biology*

- For membrane-spanning BLCAP a solubilisation buffer was used containing 0.1% Triton-X 100, 140 mM NaCl, 10 mM KCl, 20 mM Hepes pH 7.4
- TAP-Lysis Buffer, for purification of proteins expressed from pCeMM vectors: 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 7.5% Glycerol, 25 mM NaF, 0.2% NP-40 (*AppliChem*), 1 mM Na_3VO_4 (Burckstummer et al., 2006)
- 9E10 anti-c-Myc antibody (2mg / 10-15 µl per reaction or undiluted tissue culture supernatant)
- 0.2 M Sodium borate pH 9 (*neoLab*)
- Dimethylpimelimidate (*Sigma*), (final concentration 20 mM)
- 0.2 M Ethanolamine pH 8
- 1x PBS
- 2x SDS sample buffer (220mM Tris-HCl pH 6.8, 1.8% SDS, 29% glycerol, 0.03% bromophenol-blue, 0.03% β-mercaptoethanol)

Method

For 10 immunoprecipitations 25mg Sepharose A beads were incubated with NET-2 lysis buffer for 1‘, spinned briefly and the supernatant was removed. The beads were dissolved in 5 ml of 9E10 tissue culture supernatant and incubated on a rotation wheel at 4°C o/n. On the next day the antibody-coupled beads were spinned and washed 4 times with NET-2 before a next washing step with 10 vol. of 0.2 M sodium borate pH 9. After this washing step a first aliquot was taken. Finally the beads were suspended in 20 mM dimethylpimelimidate in 10 vol. of 0.2 M sodium borate and incubated for 30’ at RT on a rotation wheel. The cross-linking reaction was stopped by washing the beads with 0.2 M ethanolamine, pH 8, and incubating in 0.2 M ethanolamine for 2 h. The beads can be stored in PBS containing sodium azide (0.1%).

For immunoprecipitation the cells were prepared by scraping off of the tissue culture plate, washing with 1x PBS (2000 rpm) and diluting in the appropriate lysis buffer (1ml per 10 cm dish). Cell lysis was done by sonication (30% output, 50% pulse) 3 times for 15’’ on ice. The lysate was spinned at full speed at 4°C for 10’. The supernatant was spinned a second time for 3’ at full speed. An aliquot of total cell lysate was taken. The gained supernatants were aliquoted to the cross-linked sepharose A beads and incubated for 1-2 h on a rotation wheel at 4°C. Finally the beads were washed 4 to 8 times with ice cold lysis buffer and resuspended in 2x SDS sample buffer. After heating the samples at 95°C for 5’ the samples can be loaded on a SDS PAGE gel or stored at -20°C.

3.5. Tandem Affinity Purification

Material

- 1-3 10 cm dishes (*AppliChem GmbH*) of stable cells of 80% confluency
- Lysis buffer: Purification of regulators of editing/Filamin A constructs:
  0.5x RIPA-buffer: 150 mM NaCl, 50 mM Tris/HCl pH 8.0, 1% NP-40, 1mM MgCl2, 0.05% SDS, 0.25% DOC
- NET-2 lysis buffer: 150mM NaCl, 80mM Tris pH 7.4, 0.05% NP-40
- TAP-Lysis buffer: 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 7.5% Glycerol, 25 mM NaF, 0.2% NP-40 (*AppliChem*), 1 mM Na_3VO_4 (Burckstummer et al., 2006)
- 1x PBS (Phosphate buffered saline)
- Sepharose A beads (*Invitrogen, Life Technologies Corporation*) crosslinked with 9E10 (anti-c-Myc antibody), Pan anti-mouse IgG Dynabeads (*Invitrogen, Life Technologies Corporation*), Sepharose A beads (*Invitrogen, Life Technologies Corporation*) crosslinked with 12CA5 (anti-HA antibody)
- TEV protease (self-made)
- TEV protease cleavage buffer: 10 mM Tris/HCl pH 7.4, 100 mM NaCl, 0.2% NP-40
- 2x SDS sample buffer: 50 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercapto-ethanol, 12.5 mM EDTA, 0.02% bromphenole blue

Method

Cells of one 80%-90% confluent 10cm petri dish were scraped off in 1x PBS and pelleted by centrifugation at 2000 rpm. After washing one time in 1x PBS the cells were resuspended in the adequate lysis buffer and lysed by sonication for 3 times 15 seconds, 50% pulse. The lysate was spinned for 10’ at 14 000 rpm at 4°C. After spinning the supernatant for another 3’ at 14 000 rpm at 4°C an aliquot (10 µl) of the total lysate was saved. The cell lysate was incubated with IgG Dynabeads (constructs expressing truncated Filamin A), (25 µl/IP) or antibody-cross-linked beads (9E10 coupled Sepharose A beads), (2.5 mg/IP) for 1-2h at 4°C. The beads were washed four times with lysis buffer and two times with TEV protease cleavage buffer. After resuspending the beads in 100µl reaction volume of TEV protease cleavage buffer an aliquot (10µl) was saved for analysis of 9E10 precipitation. The cleavage reaction with 1 µl TEV protease was done for 1h at 16°C. After the cleavage reaction the bead-supernatant was saved. Additionally the beads were washed another two times with lysis buffer, whereby the supernatants again were saved. After taking an aliquot of the cleaved product (10µl) the supernatant was loaded onto the second beads (9E10 anti-MYC antibody coupled Sepharose A beads/ 12CA5 coupled Sepharose A beads), (2.5 mg - 5 mg/IP) and incubated for 1h at 4°C. The second beads were washed four to eight times with lysis buffer before resuspending in 2x SDS sample buffer and

heating for 5' at 95°C. This protocol was modified from Burckstummer et al., 2006 (Burckstummer et al., 2006). Samples from immunoprecipitations and tandem affinity purifications were analysed on 7.5%-17% gradient SDS PAGE gels and subsequent silver staining.

3.6. SDS PAGE / Western Blotting

Material

- Separation gel (mix has to be prepared according to the size of proteins to be separated):
  30% acrylamide-mix (30:1 acrylamide/bis-acrylamide) (*AppliChem GmbH*), 1M Tris-HCl, pH 8.8, dH_2O, 10% SDS, 10% APS, TEMED (*AppliChem GmbH*)
- 2-propanol
- Stacking gel stock: 3.6% acrylamide-mix, 110mM Tris-HCl pH 6.8, 0.1% SDS, 13% glycerol) + 10% APS, TEMED
- 2x SDS sample buffer (220mM Tris-HCl pH 6.8, 1,8% SDS, 29% glycerol, 0.03% bromphenol-blue, 0.03% β-mercaptoehtanol)
- 1x SDS running buffer (25mM Tris-HCl, 190mM glycine, 0.1% SDS)
- Coomassie staining: Coomassie brilliant blue, destaining solution (10% acetic acid, 20% MetOH)
- Western blotting:
  Nylon membrane (*Whatman*), 5% milk in 1x TBS-T, 0.5% milk in 1x TBS-T, 2.5% milk in 1x TBS-T
  Primary antibodies: 9E10 tissue culture supernatant, diluted 1:1 in 0.5% milk / TBS-T
  Secondary antibodies diluted 1:10 000 in 0.5% milk/TBS-T (goat anti-mouse HRP, *Axell*/ goat anti-mouse AP, *Sigma*)
  TBS-T (Tris buffered saline, *AppliChem GmbH,* containing 0.05% Triton X-100, *Fluka*)
  - Detection: AP-detection: Alkaline phosphatase buffer (100 mM NaCl, 100 mM Tris pH 9.5, 50 mM $MgCl_2$, 1% Tween 20)
    NBT (Nitro blue tetrazolium chloride)
    BCIP (5-Bromo-4-chloro-3-indolyl phosphate)
    HRP-detection: ECL reagent (*Pierce*)

Method

Protein separation by molecular weight was performed by denaturing-polyacrylamide gel electrophoresis (SDS-PAGE). The gel matrix consists of a separation gel, containing different percentages of 30:1 acrylamide/bis-acrylamide mix according to the size of the proteins to be separated, and a stacking gel which concentrates negatively charged proteins to a thin boundary. Before loading the samples were dissolved in 2x SDS sample buffer and heated for 5' at 95°C. Electrophoresis was carried out in 1x SDS running buffer at an electric current of 20 mA per gel.

Protein detection was done by either Coommassie brilliant blue staining or by Western blot analysis. For staining with Coomassie brilliant blue the gel was incubated for ca. 30' with the staining solution and destained for 1h at room temperature.

For Western blot analysis the separated proteins were transferred onto a nylon membrane by blotting for 1 h at 450 mA. After blotting, the membrane was incubated for 10' with blocking solution (5% milk in TBS-T) and with the primary antibody, diluted in 0.5% milk in TBS-T (tissue culture supernatants were diluted 1:1, other primary antibodies were diluted following the manufacturers' protocols) for 1h at RT. Before incubation with the secondary antibody (HRP coupled: 1:10 000 in 0.5% milk/TBS-T; AP coupled: 1:400) for 1h at RT, the membrane was washed three times for 5' with 2.5% milk/TBS-T. For detection of the anti-c-MYC primary antibody, developed from mouse, secondary antibodies, developed from goat, were used, coupled with either horseradish peroxidase (HRP) or alkaline phosphatase (AP). After the secondary antibody incubation the membrane was again washed three times 5' with TBS-T. For protein detection the membrane was incubated for 5' with ECL reagent (HRP detection). Detection by alkaline phosphatase was done by incubation for 10' with BCIP (1:300) and NBT (1:150) in alkaline phosphatase buffer.

3.7. 7.5%-17% gradient SDS PAGE

Material

Acrylamide (29:1) (*AppliChem GmbH*), 1 M Tris/HCl, pH 8.8, dH_2O, 50% Glycerol, 10% SDS, 10% APS, TEMED (*AppliChem GmbH*)

Table 2) Reaction mix for big gradient SDS PAGE:

7.5%	*Chemical*	*17%*
3.62 ml	30% Acrylamide	8.18 ml
5.44 ml	Tris/HCl pH 8.8	5.44 ml
5.18 ml	dH_2O	0
0.074 ml	50% Glycerol	0.738 ml
0.132 ml	10% SDS	0.132 ml
0.06 ml	10% APS	0.06 ml
0.004 ml	TEMED	0.004 ml

3.8. Blum silver staining (Helmut Blum, 1987)[6]

Material

- 40% EtOH/ 10% acetic acid
- 30% EtOH
- dH_2O
- 0.02 % Sodium thiosulfate (*Riedel-deHaën*)
- 0.1% Silver nitrate (*SIGMA-ALDRICH®*)
- 3% Sodium carbonate/0.05% formaldehyde
- 5% acetic acid (*AppliChem*)
- 1% acetic acid (*AppliChem*)

Method

In this staining protocol the proteins separated on a polyacrylamide gel were fixed by shaking the gel for at least 1 h in 40% EtOH/10% acetic acid. After fixing the gel was washed two times with 30% EtOH and one time with dH_2O, each time for 20'. After sensitizing with 0.02% sodium thiosulfate, the gel was incubated at 4°C with 0.1% silver nitrate for 20'. To develop the staining, the gel was incubated with 3% sodium carbonate containing 0.05% formaldehyde for 3-5 minutes. The reaction was stopped by washing for 5' with 5% acetic acid and 3 times for 10' with dH_2O. Bands were cut out and sent for mass spectrometric analysis to the Max. F. Perutz Laboratories Mass Spectrometry Facility.

3.9. Other methods used

3.9.1. PCR (Polymerase Chain Reaction)

Material

PCR-reaction mixes:

Taq-Polymerase:	*GoTaq-Polymerase:*	*Dream Taq Polymerase:*
1 µl Primer 1 (100 ng)	1 µl Primer 1 (100 ng)	1 µl Primer 1 (100 ng)
1 µl Primer 2 (100 ng)	1 µl Primer 2 (100 ng)	1 µl Primer 2 (100 ng)
1.5 µl dNTPs (10 mM)	1.5 µl dNTPs (10 mM)	1 µl dNTPs (10 mM)
1.25 / 2.5 µl $MgCl_2$	5 µl 5x buffer	5 µl 10x Dream Taq buffer
2.5 µl 10x buffer $(NH_4)_2SO_4$	1 µl DNA	1 µl DNA
0.5 / 1 µl DNA	15 µl dH_2O	40 µl dH_2O
17.25 / 16.75 / 16 / 15.5 µl dH_2O	0.5 µl GoTaq polymerase (*Promega*)	1 µl Dream Taq polymerase
1 µl Taq polymerase (*Fermentas*)		

[6] protocol obtained from the Max. F. Perutz Mass Spectrometry Facility

All used primers were designed in Geneious Pro 5.1.7 and synthesized by Microsynth AG[7], Switzerland. To generate N-terminally tagged proteins the sequence of interest was cloned downstream to the MYC-TEV-HA tag by BamHI restriction digestion. Sequences that had to be cloned upstream of the MYC-TEV-HA tag were flanked by a 5' XhoI and a 3' NheI restriction site to generate C-terminally tagged versions of the expressed protein.

- Oligos, designed for cloning of TEV-HA tag into 6x myc containing vector (pcDNA):

MJ2948 5'-AATTTAGAAAATCTCTATTTCCAGGGTTACCCATACGACGTCCCAGACTACGCGG-3' (F)[8]
MJ2949 5'-AATTCCGCGTAGTCTGGGACGTCGTATGGGTAACCCTGGAAATAGAGATTTTCTA-3' (R)[9]

- Amplification of DSS1 for N-terminal tagging (MYC-TEV-HA tag):

MJ2999 5'-GAGGGGATCCAATGTCAGAGAAAAAGCAGCCGGTA-3' (F)
MJ3000 5'-GAGGGGATCCCTATGAAGTCTCCATCTTATAACC-3' (R)

- Amplification of DSS1 for C-terminal tagging (MYC-TEV-HA tag):

MJ2968 5'-GATTGCTAGCATGTCAGAGAAAAAG-3' (F)
MJ2969 5'-GCTCCTCGAGTTGAAGTCTCCATCTTAT-3' (R)

- Amplification of DDX15 for N-terminal tagging (MYC-TEV-HA tag):

MJ3001 5'-GAGGGGATCCAATGTCCAAGCGGCACCGGTTG-3' (F)
MJ3002 5'-GAGGGGATCCTCACCAAACCAGTACAAAGGA-3' (R)

- Amplification of DDX15 for C-terminal tagging (MYC-TEV-HA tag):

MJ2970 5'-ATTCGCTAGCATGTCCAAGCGGCAC-3' (F)
MJ2971 5'-AGTCCTCGAGTCCAAACCAGTACAA-3' (R)

- Amplification of RPS14 for N-terminal tagging (MYC-TEV-HA tag):

MJ3003 5'-GAGGGGATCCCATGGCACCTCGAAAGGGGAAGGAA-3' (F)
MJ3004 5'-GAGGGGATCCTCACAGACGGCGACCACGGCG-3' (R)

[7] http://www.microsynth.ch/
[8] (F)...forward primer for PCR amplification
[9] (R)...reverse primer for PCR amplification

- Amplification of RPS14 for C-terminal tagging (MYC-TEV-HA tag):

MJ3005 5'-GATTGCTAGCATGGCACCTCGAAAGGGGAAGGAA-3' (F)
MJ3006 5'-GATTCTCGAGGCAGACGGCGACCACGGCGAC-3' (R)

- Amplification of SRSF9 for N-terminal tagging (MYC-TEV-HA tag):

MJ3025 5'-GCGGGGATCCCATGTCGGGCTGGGCGGACGAGCGC-3' (F)
MJ3026 5'-CCTGGGATCCTCAGTAGGGCCTGAAAGGAGAGAA-3' (R)

- Amplification of SRSF9 for C-terminal tagging (MYC-TEV-HA tag):

MJ3027 5'-GCTGGCTAGCCATGTCGGGCTGGGCGGACGAG-3' (F)
MJ3028 5'-GCTCCTCGAGGGTAGGGCCTGAAAGGAGAGAA-3' (R)

- Control primer for pcDNA 3.1 (-) Hygromycine vector:

MJ2712 5'-GACCCAAGCTGGCTAGCGT-3' (F)
MJ2431 5'-CGCAAATGGGCGGTAGGCGTG-3' (F)
MJ2782 5'-TAGAAGGCACAGTCGAGG-3' (R)
MJ2747 5'-CACAGTCGAGGCTGATCAGC-3' (R)

3.9.2. Cycle Sequencing

Material

DNA (500 ng/µl):	1 µl
Primer (0.8 pMol/µl):	4 µl
2.5x Buffer:	1 µl
Big Dye® Terminator v3.1 cycle sequencing kit (Applied Biosystems):	1 µl
dH_2O:	3 µl

Method

PCR program (25 cycles): rapid thermal ramp to 96°C
96°C for 10''
rapid thermal ramp to 50°C
50°C for 5''
rapid thermal ramp to 60°C
60°C for 4'

The cloned DNA constructs were sequenced using BigDye® Terminator v3.1 cycle sequencing kit according to manufacturers' instructions. The Department of Systematic and Evolutionary Botany at the Faculty of Life Sciences, University of Vienna, performed analysis of prepared DNA samples.

3.9.3. Restriction analysis

DNA oligomers were designed coding for a TEV-HA tag. After primer alignment the insert was cloned into a pcDNA 3.1 (-) vector downstream of a 6x MYC tag by EcoRI restriction sites while the 5' EcoRI site got destroyed. The 6x MYC-TEV-HA tag was cloned into a pcDNA 3.1 (-) Hygromycin vector by PmeI-digestion. The different amplified inserts (candidates for ADAR impact factors, Dss1, Rps14, Ddx15, Srsf9) were cloned into the completed expression vector by BamHI (tagging the insert N-terminally) and NheI/XhoI (tagging the insert C-terminally). Restriction digestions were done in a total reaction volume of 20 µl containing 1/10 reaction buffer plus RNAse A (20µg/ml per reaction) and BSA (bovine serum albumin) (100 µl/ml per reaction) for 1 h at 37°C. An aliquot of 5µl was analysed by DNA Gel electrophoresis (see DNA Gel electrophoresis).

Used restriction enzymes:

EcoRI (*Fermentas GmbH, Thermo Fisher Scientific Inc.*),
restriction site[10]: 5' ...G^AATTC... 3'
3' ...CTTAA^G... 5'

PmeI (*New England BioLabs® Inc.*),
restriction site[11]: 5' ...GTTT^AAAC...3'
3' ...CAAA^TTTG...5'

BamHI (*Fermentas GmbH, Thermo Fisher Scientific Inc.*)
restriction site[3]: 5' ...G^GATCC... 3'
3' ...CCTAG^G...5'

XhoI (*Fermentas GmbH, Thermo Fisher Scientific Inc.*)
restriction site[3]: 5' ...C^TCGAG... 3'
3' ...GAGCT^C... 5'
NheI (*Fermentas GmbH, Thermo Fisher Scientific Inc.*)
restriction site[3]: 5' ...G^CTAGC... 3'
3' ...CGATC^G... 5'

3.9.4. DNA Gel electrophoresis

Material

- Agarose
- 1x TAE buffer
- Etidiumbromide (*Roth*)
- 10x loading dye

[10] http://www.fermentas.de/ product information
[11] http://www.neb.com/ products

Method

According to the size of the DNA fragments to be analysed the DNA gels were casted with different concentrations of agarose (*peqlab*) to maintain efficient separation (see table 1.). The agarose was mixed with the desired buffer, 1x TAE (Tris-acetate-EDTA) or 1x TBE (Tris-borate-EDTA), (*AppliChem*), and heated up to solve the agarose. After cooling down the gel, Etidium bromide (*Roth*) was added (0.1 – 0.5 µl/ml) to stain DNA fragments.

Fragment length	Agarose concentration (w/v)
1 - 30 kb	0.5%
0.8 – 12 kb	0.7%
0.5 – 7 kb	1.0%
0.4 – 6 kb	1.2%
0.2 – 3 kb	1.5%
0.1 – 2 kb	2.0%

Table 3. Used agarose concentrations according to DNA fragment length [12]

3.9.5. DNA transformation into bacteria

Material

- 30µl – 50µl competent bacteria (E.coli: XL-1 blue)
- 1-10 ng DNA
- SOB media
- Antibiotic plates

Method

The competent bacteria were thawn on ice and mixed with 1-10 ng of DNA. After 15' on ice the cells were heat shocked by incubating them for 90'' at 42°C and returning them back on ice for another 5'. In 300 µl of SOB medium the transformed cells shook at 37°C for > 1h. Finally 100-300 µl were plated on agar plates already containing the appropriate antibiotic.

3.9.6. DNA preparations

DNA preparations were done following standard methods [13] .

[12] Der Experimentator: Molekularbiologie/Genomics; 6. Auflage (Mülhardt, 2009)
[13] *Current Protocols in Molecular Biology, Chapter 2, Section I, Unit 2.1A*

4. Results

4.1. ACTIVATORS AND INHIBITORS OF ADAR

4.1.1. Generation of Expression Vectors for Tandem Affinity Purification[14]

Primers have been designed *in silico* using Geneious Pro 5.1.7 for the amplification of the ADAR regulator candidates, DSS1, RPS14, DDX15 and SRSF9, from existing vectors or from cDNA generated from total RNA extracts (see 3.). The primers were designed to flank the inserts with restriction sites for cloning into the designed mammalian expression vector pcDNA 3.1 (-) Hygromycin containing a TAP-tag (6x c-MYC/TEV/HA) and a Hygromycin resistance gene. To generate C-terminally tagged inserts the PCR-product was cloned upstream of the c-MYC-TEV-HA tag by NheI/XhoI restriction digestion. For N-terminally tagged inserts the PCR-product was cloned downstream of the tag by BamHI restriction digestion. DSS1, DDX15 and RPS14 were successfully cloned to be C- and N-terminally tagged. For cloning of the SR protein SRSF9 cDNA was prepared from the mouse neuroblastoma cell line cN2A and from the human osteosarcoma cell line U2OS, using Trizol® Reagent (*Invitrogen*). The mouse N2A sequence differs from the human sequence in transitions in the wobble position of some amino acids, but in two cases the amino acid differs from the human sequence: at the N-terminus, at position V147, there appeared a Valine/Methionine substitution after sequencing. A second transition near the C-terminus, at position R197, leads to an Arginine/Glutamine substitution within the SR-rich domain.

[14] For sequence details see Appendix

4.1.2. DSS1

4.1.2.1. Transient Immunofluorescence staining: DSS1

Dss1 is a small acidic protein known to be a subunit of the 26S proteasome complex (Funakoshi et al., 2004; Sone et al., 2004). After cloning of full-length Dss1 (GenomeNet Accession number: NP_006295) in the generated pcDNA 3.1 (-) Hygro / 6x c-MYC/TEV/HA TAP expression vector the DNA preparation was tested on its transfection efficiency by transient transfection of 3 µg DNA into the human cell line Hek293 and subsequent immunofluorescence staining, detecting the c-Myc antigen tag.

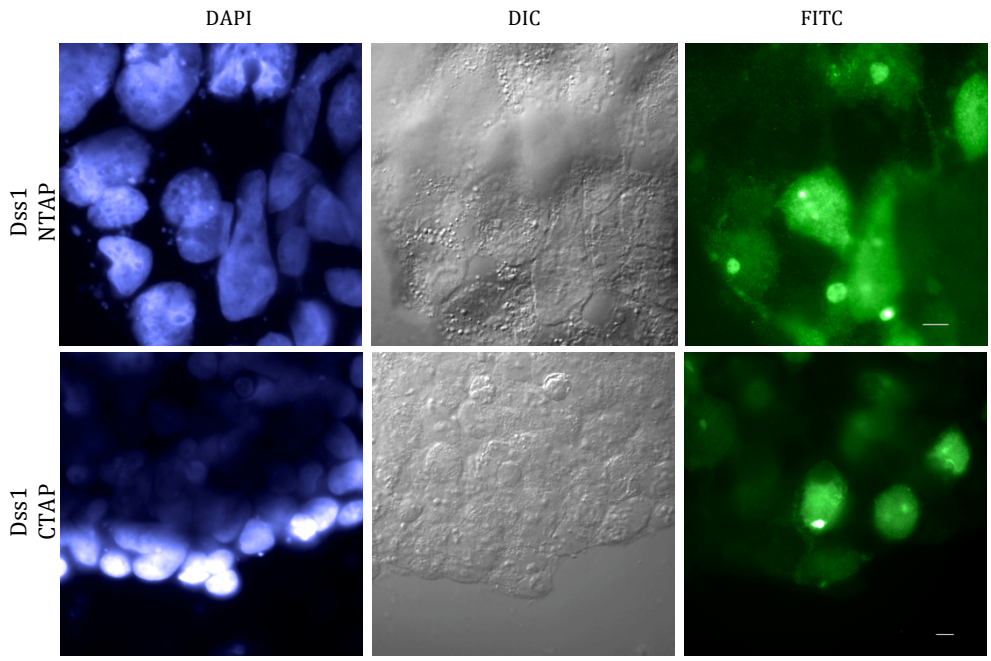


Figure 12) Transient transfection of Dss1 N- and C-terminally tagged with a c-MYC-TEV-HA tag in Hek293. For testing the transfection efficiency of the generated mammalian expression vectors the plasmid preparations were transiently transfected into the human cell line Hek293. Detection of transiently transfected Dss1 by Alexa 488-coupled antibodies shows mainly nuclear localization of both variants, the C- and the N-terminally tagged Dss1. Dss1 NTAP: Dss1 N-terminally tagged with a c-MYC-TEV-HA tag. Dss1 CTAP: Dss1 C-terminally tagged with a c-MYC-TEV-HA tag. DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescence staining. Scale bar: 20 µm.

In addition to an important function during protein degradation, DSS1 was also found to be involved in DNA repair, as it forms a stoichiometric complex with the DNA repair protein BRCA2 (Li et al., 2006). Thus, the transiently expressed tagged protein shows mainly nuclear localization pattern. Similar subcellular localization pattern was found for both versions of Dss1, C- and N-terminally tagged. Transfection efficiencies of about 30% to 40% were obtained with both generated vectors, encoding C- and N-terminally tagged versions of Dss1.

4.1.2.2. Stable expression of DSS1: Immunofluorescence staining

After testing the Dss1 expression vector on it's transfect ability the generation of stable expressing cell lines was started. Several cell lines were transfected with 5 -8 µg of DNA. Twenty four to forty eight hours after transfection, selection was started, by growing the cells in DMEM medium containing 50 µg/ml – 200 µg/ml of the antibiotic Hygromycin B. During selection the transfected DNA gets randomly integrated into the genome. After ten days to three weeks of selection transfected cells start to form colonies. Picked stable clones were tested on their expression of Dss1 by immunofluorescence staining and western blot analysis.

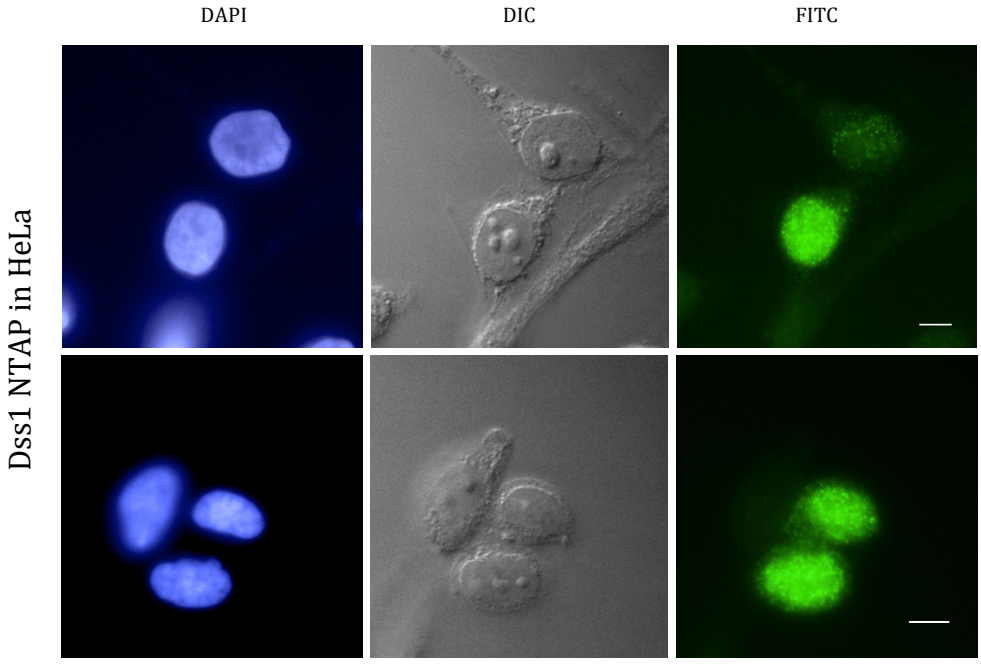


Figure 13) HeLa cell line stably expressing Dss1 N-terminally tagged with a c-MYC-TEV-HA TAP-tag. After selection for stable random integration of the transfected DNA construct, the picked clones were tested on their homogeneity of stable protein expression by immunofluorescence staining. Tagged Dss1 was detected by an anti-c-MYC primary antibody and subsequent labelling with a goat anti-mouse secondary antibody, coupled to Alexa 488. In stable expressing cells, Dss1 was also mainly detected within the nucleus. About 50% of analysed cells show positive expression of Dss1 in immunofluorescence staining analysis. Dss1 NTAP: Dss1 N-terminally tagged with a MYC-TEV-HA tag. DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescence staining. Scale bar: 20 µm.

Two picked HeLa clones were found to stably express N-terminally tagged DSS1. In immunofluorescence staining analysis about 50% of the cells show DSS1 expression, where DSS1 was mainly detected within the nucleus. The N-terminally tagged version of DSS1 was also stably expressed in the human osteosarcoma cell line U2OS. This clone shows inhomogeneity of DSS1 expression. About 30% of cells stained by immunofluorescence staining were found to express the protein. However, in this case DSS1 again shows nuclear localization pattern in positive cells.

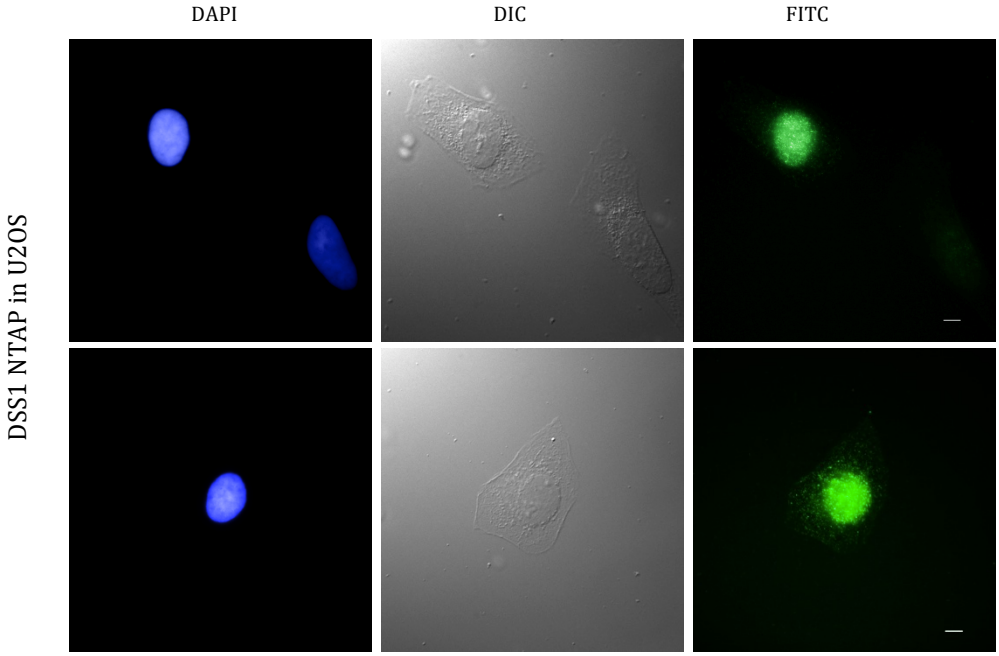


Figure 14) U2OS cell line stably expressing Dss1 N-terminally tagged with a c-MYC-TEV-HA TAP-tag. After selection for stable random integration of the transfected DNA construct, the picked clones were tested on their homogeneity of stable protein expression by immunofluorescence staining. Tagged Dss1 was detected using an anti-MYC primary antibody and subsequent labelling with a goat anti-mouse secondary antibody coupled to Alexa 488. In U2OS cells, stable expressed Dss1 shows nuclear localization in immunofluorescence staining

analysis. Dss1 NTAP: Dss1 N-terminally tagged with a c-MYC-TEV-HA tag. DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescence staining. Scale bar: 20 µm.

The pcDNA 3.1 (-) Hygro TAP expression vector expressing C-terminally tagged DSS1 was also used for transfection and selection of stable cell lines. But to date, no stable expressing clones have been gained.

4.1.2.3. Stable expression of DSS1: Western blot analysis

To further test the level of protein expression of the picked stable clones, Western blot analysis was done, using an anti-c-Myc primary antibody for detection of TAP-tagged DSS1. Visualization of the tagged protein was achieved by using a goat anti-mouse secondary antibody coupled to horseradish peroxidase. The human full-length DSS1 shows a molecular weight of 8.27 kDa. Fused to a 6x c-Myc/TEV/HA tag, the protein has a molecular weight of about 35 kDa.

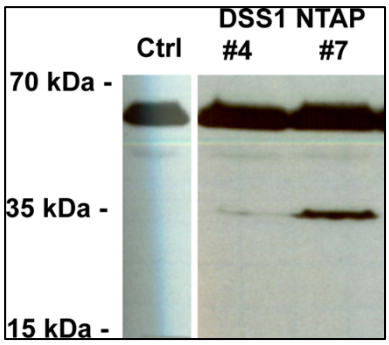
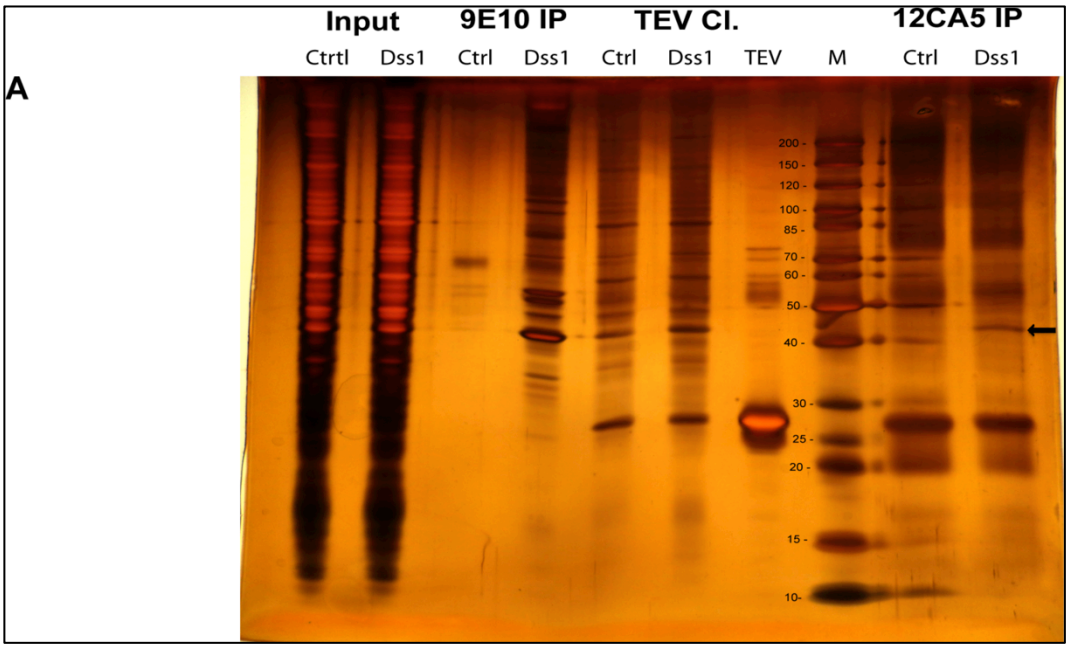


Figure 15) Stable expression of Dss1: Western blot analysis. To check the expression level of N-terminally tagged Dss1 in HeLa clones, found to express Dss1 by immunofluorescence staining, Western blot analysis of the positive clones was done. Dss1 was detected by an anti-c-Myc primary antibody and visualization with a goat anti-mouse secondary antibody coupled to HRP. TAP-tagged Dss1= 35 kDa. Ctrl = plane HeLa cell lysate; #4 = lysate of Dss1 in HeLa clone 4; #7 = lysate of Dss1 in HeLa clone 7.

4.1.2.4. Immunoprecipitation / Tandem Affinity Purification: DSS1

After testing the generated stable expressing DSS1 cell lines, anti-c-Myc Immunoprecipitation and Tandem Affinity Purification respectively was done. DSS1

expressing cells and control cells (empty) were lysed using 0.5x RIPA lysis buffer. After sonication the lysates were incubated with 9E10 anti-c-Myc cross-linked Sepharose A beads. The obtained samples were analysed on a 7.5% to 17% gradient SDS PAGE gel and subsequent silver staining. Protein bands specifically found in lysates prepared from DSS1 expressing cells were cut and sent for mass spectrometric analysis. After two rounds of tandem affinity purification two Dss1-specific protein bands, at a molecular weight of 30 kDa and 50 kDa, were obtained from stable expressing HeLa cells. Anti-c-Myc immunoprecipitation of Dss1 from stable expressing U2OS cells yielded a Dss1-specific protein band at a molecular weight of 67 kDa.


A
Input
9E10 IP
TEV Cl.
12CA5 IP
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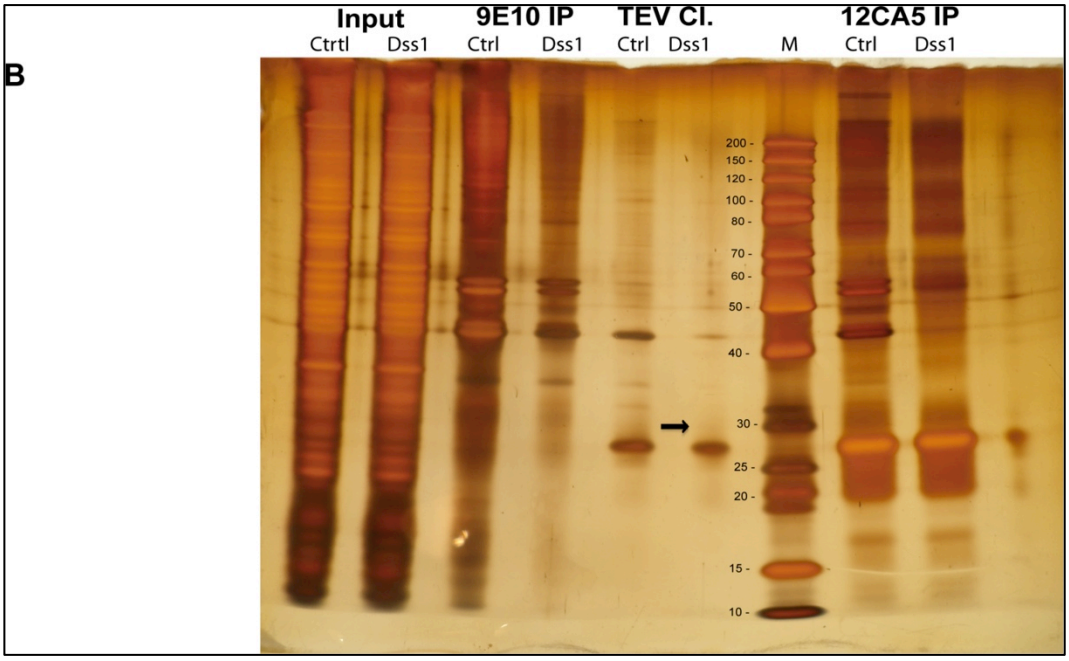
B
Input
9E10 IP
TEV Cl.
12CA5 IP
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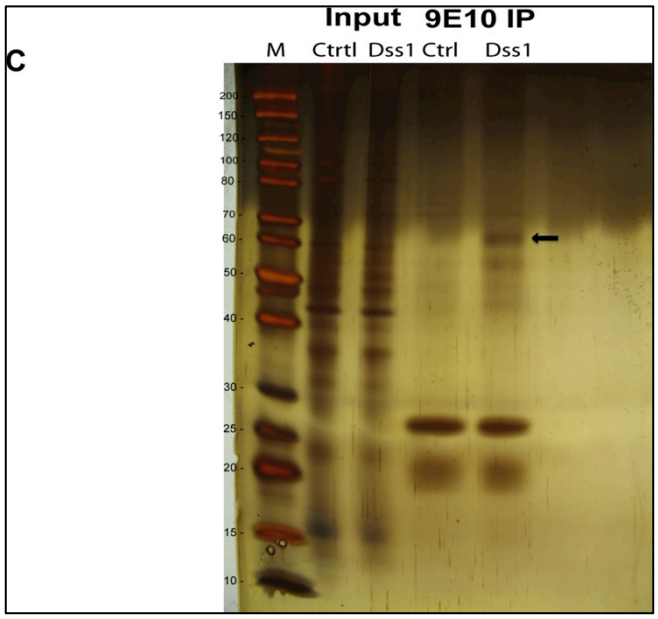


Figure 16) Silver gels: Purification of Dss1. The N-terminally tagged version of Dss1 was stable expressed in the human cell lines HeLa (clone 3, clone 7) and U2OS. Tandem Affinity Purification was done with HeLa cells expressing Dss1. The first IP was done with Pan anti-mouse IgG Dynabeads coupled with an anti-c-Myc antibody (9E10). After TEV-cleavage from the first beads a second IP was done with Sepharose A beads coupled with an anti-HA antibody (12CA5). The samples were separated on a 7.5%-17% gradient SDS PAGE gel and stained by Blum's silver staining protocol. A) TAP of Dss1, N-terminally tagged, expressed in HeLa, clone 7. Black arrow: band at 50 kDa B) TAP of Dss1 N-terminally tagged expressed in HeLa, clone 3. Black arrow: band at 30 kDa C) Anti-c-Myc-IP of Dss1, N-terminally tagged, expressed in U2OS. Black arrow: band at 67 kDa. Black arrows mark protein bands cut out and sent for mass spectrometric analysis. Input = total lysate; 9E10 IP = c-Myc immunoprecipitation; TEV Cl. = TEV cleavage reaction; 12CA5 IP = HA immunoprecipitation; TEV = TEV protease loading control; M = PAGE Ruler unstained protein ladder; Ctrl = lysate of empty cells; Dss1 = lysate of Dss1 expressing cells.

4.1.2.5. Mass spectrometry: DSS1

Cut protein samples were digested and analysed by members of the mass spectrometry facility of the Max F. Perutz Laboratories. The following tables list proteins, pulled down specifically with N-terminally tagged DSS1 from the human cell lines HeLa and U2OS. Data labelled in bold was found to interact with DSS1. Not highlighted data are proteins, also detected in other analysed samples. Many ribosomal subunits and proteins involved in RNA-processing, like hnRNPs, were detected in the analysed samples (see Table 4). These proteins, acting on RNA, could be the missing link in the network of the stimulatory role of Dss1 on RNA-editing. Candidates for these linkers are highlighted in green.

Table 4) Dss1-interacting proteins detected by mass spectrometric analysis. This table lists all proteins, detected in protein bands after precipitation of Dss1 from stable exressing HeLa and U2OS cells by mass spectrometric analysis. The identified protein, the number of analysed peptides and the percentage of coverage of the annotated sequence is listed. Proteins specifically pulled down with Dss1 are highlighted in bold. Green data marks possible candidates in the network of linking Dss1 to RNA-editing. Mw = molecular weight of identified protein in kDa. Sample = molecular weight of cut protein band.

Dss1 interacting protein	peptides	coverage	Mw (kDa)	Sample (Mw, source)	comments	function	references
vimentin	20	45%	54	50 kDa HeLa	also comes with DDX15	Class-III intermediate filament; binding and stabilization of collagen mRNAs	(Ferrari et al., 1986); (Challa and Stefanovic, 2011)
hnRNP G	18	39%	47	50 kDa HeLa/U2-OS	also comes with DDX15	RNA-binding protein; associates with Pol II transcripts; regulates splice site selection	(Soulard et al., 1993); (Heinrich et al., 2009)
26S proteasome non-ATPase regulatory subunit 6	**12**	**26%**	**46**	**50 kDa HeLa**		**regulatory 26S proteasome subunit (ATP-dependent degradation of ubiquitinated proteins)**	**(Ewing et al., 2007)**
ribosomal protein L8	**2**	**8,10%**	**24**	**50 kDa HeLa**		**rRNA binding, part of 60S ribosome**	**(Hanes et al., 1993)**
proteasome subunit p42	**7**	**16%**	**44**	**50 kDa HeLa**		**proteasome regulatory protein**	**(Fujiwara et al., 1996)**
26S protease regulatory subunit 8 isoform 2	**10**	**29%**	**45**	**50 kDa HeLa**		**antigen processing to generate class I MHC binding peptides**	**(Akiyama et al., 1995)**
40S ribosomal protein S3a	**7**	**28%**	**30**	**50 kDa HeLa**		**Eukaryotic ribosomal protein**	**(Vladimirov et al., 1996) (Ewing et al., 2007)**

interleukin enhancer-binding factor 2	**3**	**9,70%**	**43**	**50 kDa HeLa**		**regulates transcription of IL2 in T-cell activation/ formation of stable DNA-dependent protein kinase holoenzyme**	**(Ting et al., 1998) (Satoh et al., 1999) (Reichman et al., 2002)**
ribosomal protein S8	**3**	**14%**	**27**	**50 kDa HeLa**		**Ribonucleoprotein of 40S ribosome**	**(Vladimirov et al., 1996)**
TDP43	**3**	**10%**	**45**	**50 kDa HeLa**		**neurofilament mRNA binding protein**	**Strong et al (unpublished data)**
ribosomal protein S4	**2**	**5,70%**	**43**	**50 kDa HeLa**		**Ribonucleoprotein of 40S ribosome**	**(Zinn et al., 1994)**
erlin-2 isoform 1	2	7,10%	38	50 kDa HeLa	also comes with edited BLCAP	mediates ER-associated degradation of activated IP_3-receptors	(Browman et al., 2006)
hnRNP C isoform 1	**2**	**7,30%**	**32**	**50 kDa HeLa**		**Nucleation of 40S hnRNP particles**	**(Huang et al., 1994)**
hnRNP K	**2**	**5,60%**	**51**	**67 kDa U2OS**		**Transcription factor**	**(Michelotti et al., 1996)**
mitochondrial elongation factor Tu	**2**	**6,20%**	**50**	**50 kDa HeLa**		**promotes binding of amino-acyl-tRNA to A site of ribosomes**	**(Woriax et al., 1995)**
hnRNP D	**2**	**8,50%**	**41**	**50 kDa HeLa**		**AU-rich element binding**	**(Nagata et al., 1999)**
ubiquinol-cytochrome c reductase core protein II	**3**	**17%**	**28**	**50 kDa HeLa**		**ubiquinol-cytochrome c reductase complex assembly**	**(Hosokawa et al., 1989)**
heat shock 60kDa protein 1 (chaperonin)	**2**	**5,50%**	**41**	**67 kDa U2OS**		**Mitochondrial protein import**	**(Singh et al., 1990)**
26S proteasome subunit 9	**2**	**4,50%**	**47**	**50 kDa HeLa**		**Chaperone during 26S proteasome assembly**	**(Kaneko et al., 2009)**

4.1.3. RPS14

4.1.3.1. Transient Immunofluorescence staining: RPS14

The second candidate for a regulator of ADAR activity was the ribosomal protein RPS14. After cloning of RPS14 (GenomeNet Accession number: NP_001020242) into the newly generated TAP-tag expression vector pcDNA 3.1 (-) Hygromycin with a c-MYC-TEV-HA tag, the plasmids containing C- and N-terminally tagged versions of RPS14 were tested on their transfection ability by transient transfection into the human cell line Hek293. The cells were transfected as described in 3.3. After 24 to 48 hours, the transfection efficiency of the RPS14 expression vector DNA-preparation was tested by immunofluorescence staining. For detecting the expressed protein of interest a primary anti-c-MYC antibody was used. Detection of the primary antibody was conducted using a goat anti-mouse secondary antibody coupled with a fluorescent dye, Alexa 488.

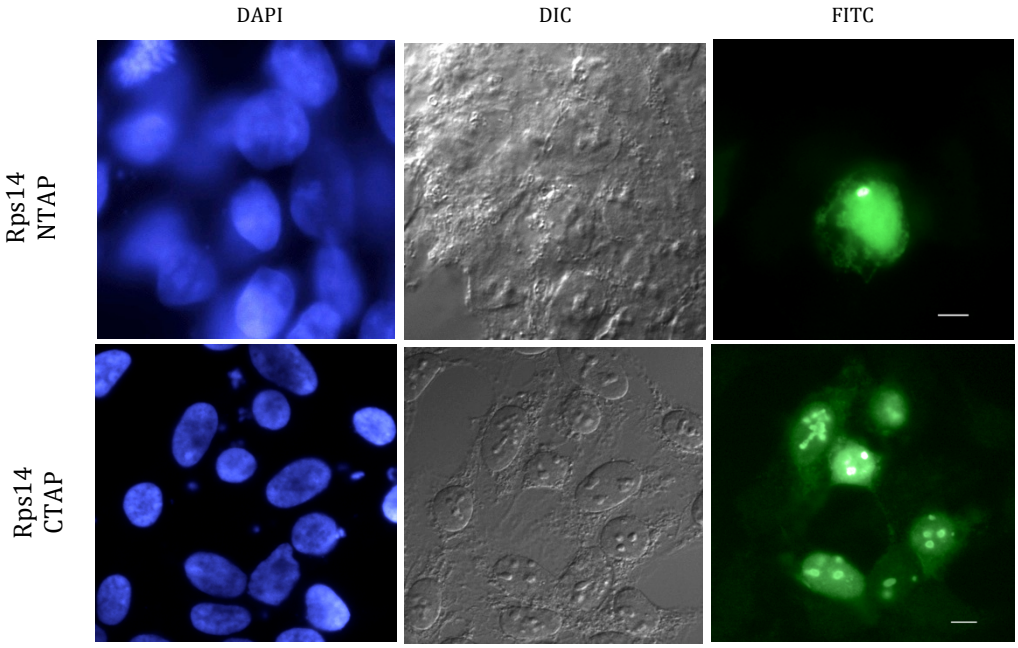


Figure 17) Transient transfection of Rps14 N- and C-terminally tagged with a c-MYC-TEV-HA tag in Hek293. For testing the transfection efficiency of the generated mammalian expression vectors the plasmid preparations were transiently transfected into the human cell line Hek293. Transient transfected N-terminally tagged Rps14 shows nuclear localization, while C-terminally tagged Rps14 was mainly detected in nucleoli. Rps14 NTAP: Rps14 N-terminally tagged with a c-MYC-TEV-HA tag. Rps14 CTAP: Rps14 C-terminally tagged with a c-MYC-TEV-HA tag. DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescence staining. Scale bar: 20 µm.

The ribosomal protein RPS14 (Chen et al., 1986; Rhoads et al., 1986) is involved in the processing of ribosomal RNA (Ferreira-Cerca et al., 2005), thus was found to localize mainly in the nucleolus after transient transfection into the human cell line Hek293, if it was C-terminally tagged. N-terminally tagged Rps14 shows nuclear subcellular localization. With the N-terminally tagged Rps14 very low transfection efficiency was detected. However, the vector encoding the C-terminally tagged version of the ribosomal protein showed a transfection efficiency of about 40%, where the protein mainly localized to the nucleolus and nucleoplasm.

4.1.3.2. Stable expression of RPS14: Immunofluorescence staining

Following the evaluation of transfection efficiency of the generated expression vectors expressing the ribosomal protein Rps14, various cell lines were transfected. Twenty four to forty eight hours later, selection for stable integration of the transfected DNA into the genome was started by adding 50 µg/ml to 200 µg/ml of the antibiotic Hygromycin B to the growth medium. After ten days up to three weeks surviving cells started growing in colonies. These colonies were picked and analysed for their expression of Rps14 by immunofluorescence staining and Western blot analysis.

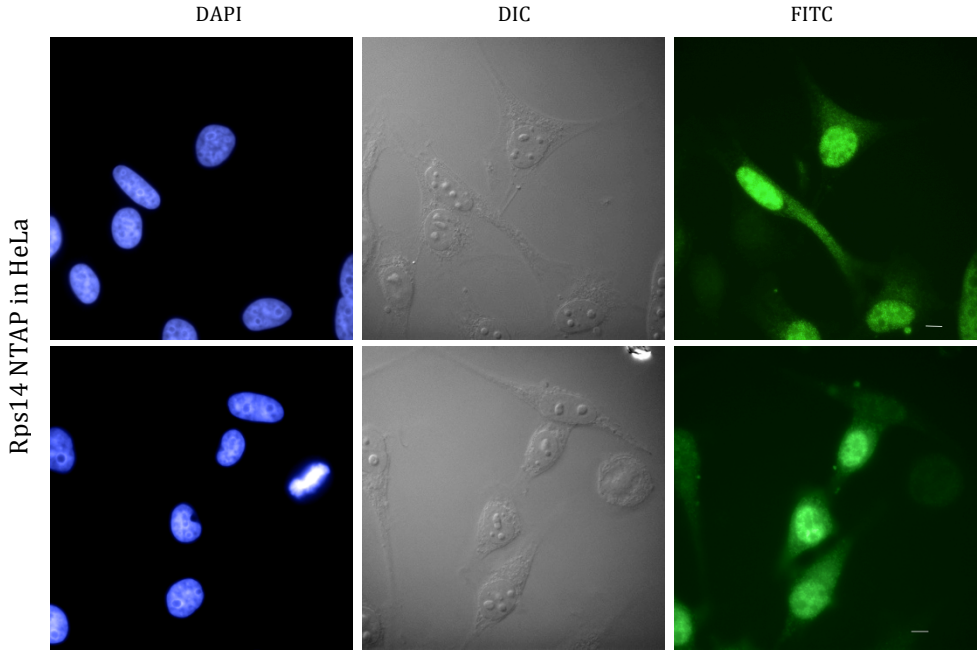


Figure 18) HeLa cell line stably expressing Rps14 N-terminally tagged with a c-MYC-TEV-HA TAP-tag. After selection for stable random integration of the transfected DNA construct, the picked clones were tested on their homogeneity of stable protein expression by immunofluorescence staining. Tagged Rps14 was detected by an anti-c-MYC primary antibody and adjacently labelling with a goat anti-mouse secondary antibody coupled to Alexa 488. N-termnally tagged Rps14 expressed in HeLa cells shows nuclear subcellular localization, but can also be found in the cytoplasm at a low level. Rps14 NTAP: Rps14 N-terminally tagged with a c-MYC-TEV-HA tag. DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescent staining. Scale bar: 20 µm.

Also the C-terminally TAP tagged version of the ribosomal protein RPS14 was successfully expressed in the human osteosarcoma cell line U2OS. One stable

expressing clone was obtained. About 50% to 60% of the cells show stable expression of Rps14 in immunofluorescence staining analysis. Interestingly, like in transient immunofluorescence staining assays, the C-terminally tagged Rps14 shows a different subcellular localization pattern than the N-terminally tagged version does. While N-terminally tagged Rps14, expressed in HeLa cells, shows nuclear localization, C-terminally tagged Rps14 in U2OS cells shows a promintent nucleolar localization pattern. This observation is supposedly due to the TAP tag, as the position of the tag may influence protein folding and function.

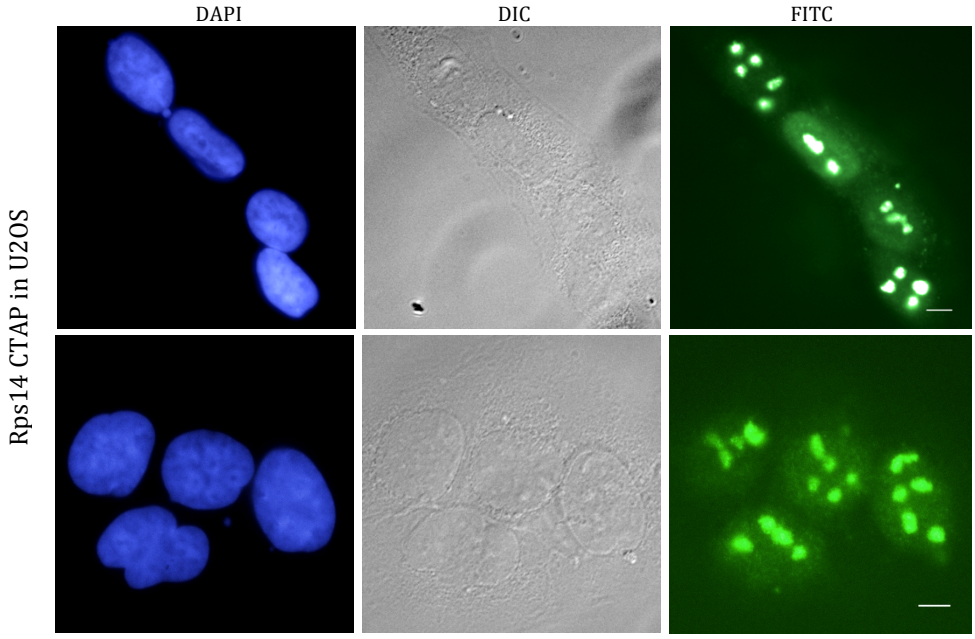


Figure 19) U2OS cell line stably expressing Rps14 C-terminally tagged with a c-MYC-TEV-HA TAP-tag. After selection for stable random integration of the transfected DNA construct, the picked clones were tested on their homogeneity of stable protein expression by immunofluorescence staining. Tagged Rps14 was detected using anti-c-MYC primary antibody and subsequent labelling with a goat anti-mouse secondary antibody coupled to Alexa 488. C-terminally tagged Rps14 expressed in U2OS cells shows a prominent nucleolar localization pattern in positive cells. Rps14 CTAP: Rps14 C-terminally tagged with a c-MYC-TEV-HA tag. DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescence staining. Scale bar: 20 µm.

4.1.3.3. Stable expression of RPS14: Western blot analysis

To further analyse the expression level of obtained RPS14 expressing clones, Western blot analysis was done. TAP-tagged RPS14 was detected using an anti-c-MYC primary antibody. The primary antibodies were visualized using goat anti-mouse secondary antibodies coupled to either alkaline phosphatase (RPS14 NTAP in HeLa) or horseradish peroxidase (RPS14 CTAP in U2OS).

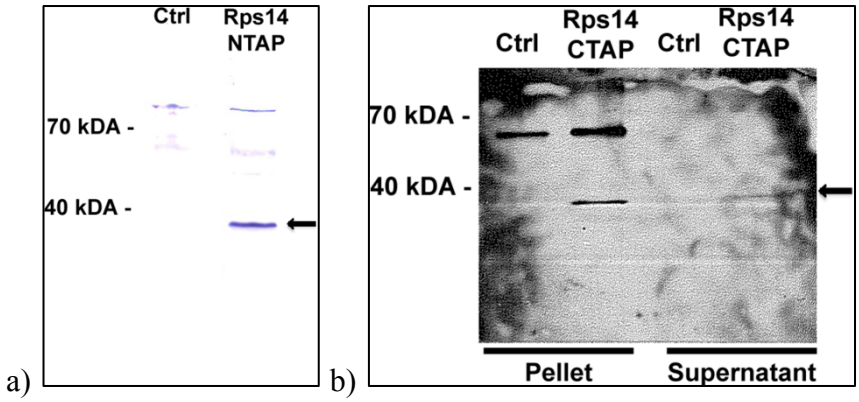


Figure 20) Stable expression of RPS14, C- and N-terminally tagged: Western blot analysis. C- and N-terminally tagged RPS14, expressed in HeLa and U2OS cells, was detected by Western blot analysis. The TAP-tagged protein was detected using an anti-c-MYC primary antibody and secondary antibodies coupled to either alkaline phosphatase or horseradish peroxidase. a) N-terminally TAP-tagged RPS14 expressed in HeLa cells. Cell lysates were prepared, using 2x SDS sample buffer. N-terminally tagged RPS14 was detected using a goat anti-mouse secondary antibody coupled to alkaline phosphatase. b) C-terminally TAP-tagged RPS14 expressed in U2OS cells. Cell lysates were prepared, using NET-2 lysis buffer. Samples from precipitated pellets and supernatants were analysed using an anti-c-Myc primary antibody and a goat anti-mouse secondary antibody coupled to horseradish peroxidase. Full-length RPS14 is of a molecular weight of 16.3 kDa. TAP tagged RPS14 is detected at a molecular weight of about 38 kDa. Ctrl = lysate of empty HeLA/U2OS cells; RPS14 NTAP = N-terminally tagged RPS14; RPS14 CTAP = C-terminally tagged RPS14.

N-terminally tagged RPS14 expressed in HeLa cells was detected using a goat anti-mouse secondary antibody coupled to alkaline phosphatase after preparing the cell lysate, using 2x SDS sample buffer. U2OS cells, expressing C-terminally tagged RPS14, were lysed using NET-2 lysis buffer. Samples from precipitated pellets and supernatants of C-terminally tagged Rps14 expressing cells were analysed by Western blot analysis, using goat anti-mouse secondary antibodies coupled to horseradish peroxidase. This analysis showed that most of the C-terminally tagged protein was not

solubilized and precipitated in the pellet. However, at a low level RPS14 was also detected in the supernatant.

4.1.3.4. Immunoprecipitation / Tandem Affinity Purification: RPS14

Positive clones of HeLa and U2OS cells, that have shown adequate expression levels of C- and N-terminally tagged RPS14 in immunofluorescence staining tests and Western blot analysis, were expanded for immunoprecipitation and Tandem Affinity Purification of RPS14. So far most of the obtained precipitated protein samples have been obtained by anti-c-MYC immunoprecipitation assays as the Tandem Affinity Purification protocol for c-MYC-TEV-HA tagged proteins still needs to be improved, because the second purification step using anti-HA crosslinked Sepharose A beads yields high levels of background signal. N-terminally TAP tagged RPS14 expressed in HeLa cells, was purified by anti-c-MYC immunoprecipitation and Tandem Affinity Purification. By these methods, NTAP-Rps14-specific bands of a molecular weight of 107 kDa and 52 kDa were purified. To date RPS14- specific protein bands (120 kDa, 107 kDa and 41 kDa) after purification of C-terminally tagged RPS14 from U2OS cells were gained by immunoprecipitation, using anti-c-MYC cross-linked Sepharose A beads. Obtained samples were analysed on 7.5% to 17% gradient SDS PAGE gels and subsequent silver staining. Protein bands exclusively found in RPS14 containing lysates had been cut out and were sent for mass spectrometric analysis.

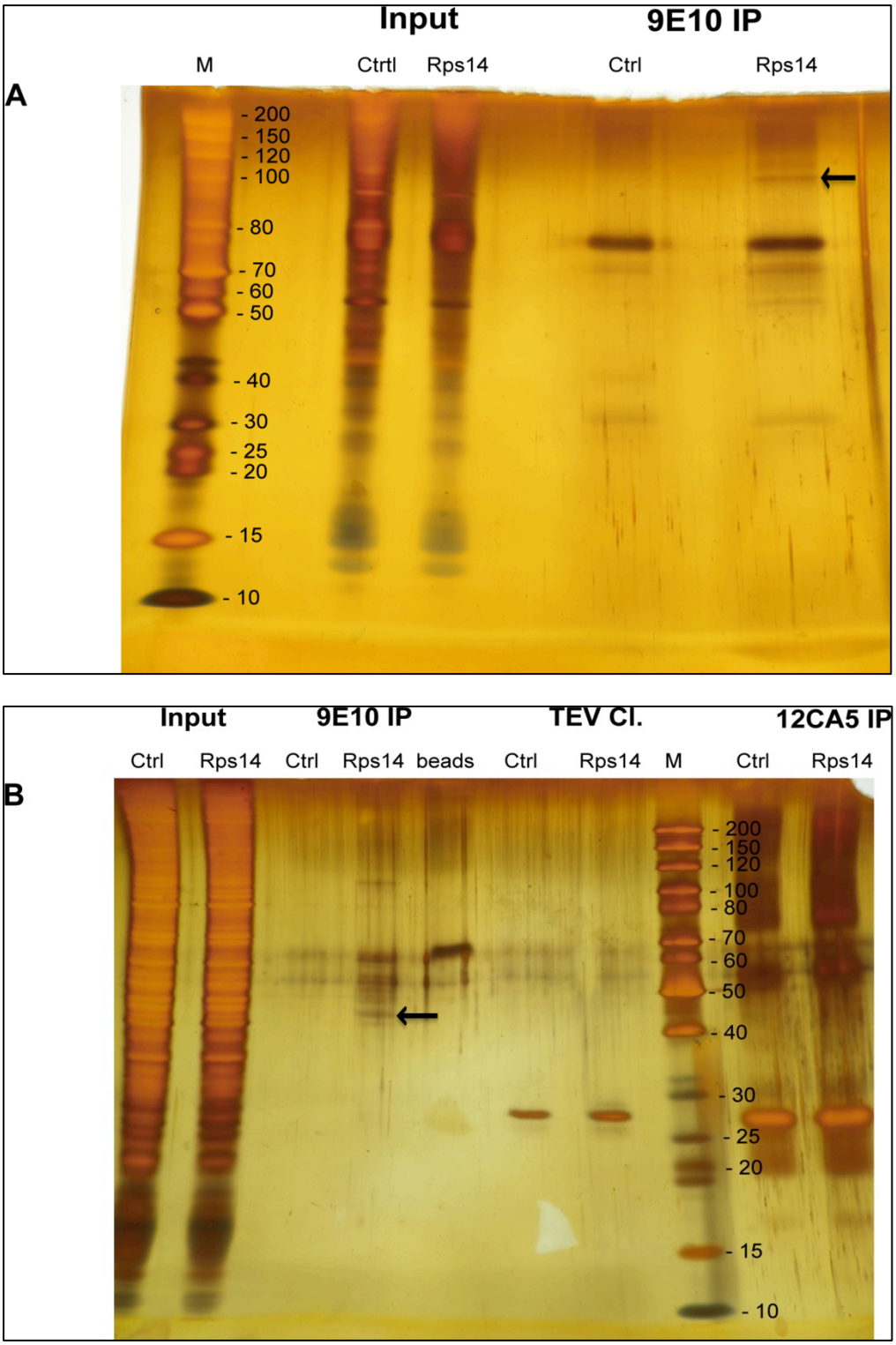


Figure 21) Silver gels: Purification of Rps14 I. The N-terminally tagged version of Rps14 was stable expressed in the human cell line HeLa. Anti-c-Myc immuno-precipitation and Tandem Affinity Purification were done with HeLa cells expressing Rps14. The samples were separated on a 7.5%-17% gradient SDS PAGE gel and stained by Blum's silver staining protocol.

A) Anti-c-Myc IP of Rps14, N-terminally tagged, expressed in HeLa. Black arrow: band at 107 kDa B) Initial try of a TAP of Rps14, N-terminally tagged, expressed in HeLa. Black arrow: band at 47 kDa. Black arrows mark protein bands cut out and sent for mass spectrometric analysis. Input = total lysate; 9E10 IP = c-Myc immunoprecipitation; TEV Cl. = TEV cleavage reaction; 12CA5 IP = HA immunoprecipitation; Ctrl = lysate of empty cells; Rps14 = lysate of Rps14 expressing cells; M = PAGE Ruler unstained protein ladder.

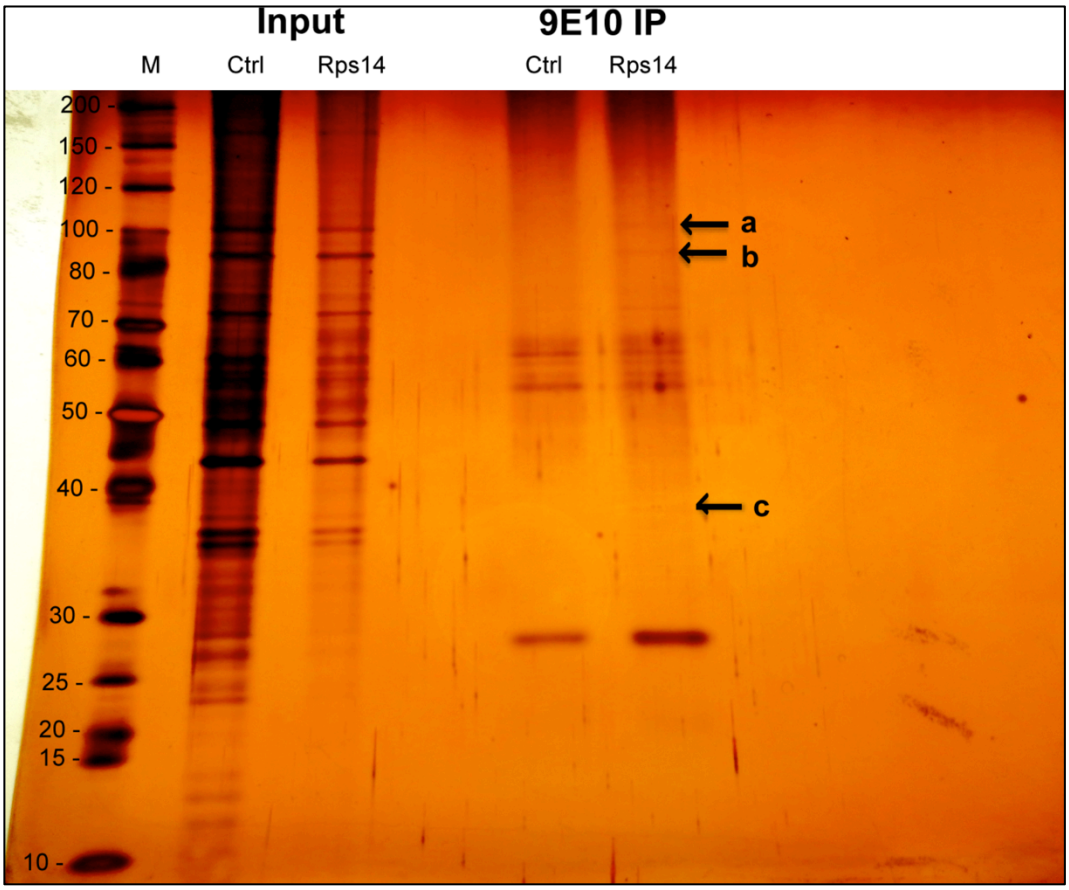


Figure 22) Silver gels: Purification of Rps14 II. The C-terminally tagged version of Rps14 was stable expressed in the human cell line U2OS. Anti-c-Myc immunoprecipitation was done with U2OS cells expressing Rps14. The samples were separated on a 7.5%-17% gradient SDS PAGE gel and stained by Blum's silver staining protocol. a) Protein band at a molecular weight of 120 kDa. b) Protein band at a molecular weight of 100 kDa. c) Protein band at a molecular weight of 40 kDa. Black arrows mark cut protein bands sent for mass spectrometric analysis. Input = total lysate; 9E10 IP = c-Myc immunoprecipitation; Ctrl = lysate of empty cell line; Rps14 = lysate of Rps14 expressing cell line; M = PAGE Ruler unstained protein ladder.

4.1.3.5. Mass spectrometry: RPS14

The following tables list proteins, which were pulled down with RPS14 from HeLa and U2OS cell lysates by purification assays and subsequent mass spectrometric analysis. For each protein the number of unique peptides, the percentage of coverage with the full-length protein, the molecular weight and protein function is mentioned in the mass spectrometry data. Bold highlighted data depicts proteins, which were found to interact only with RPS14 in our data. Non-highlighted data indicates proteins, which have also been detected in other samples.

Table 5) Rps14-interacting proteins, detected by mass spectrometric analysis I. This table lists all proteins, detected in protein bands after precipitation of N-termnally tagged Rps14 from stable exressing HeLa cells by mass spectrometric analysis. The identified protein, the number of analysed peptides and the percentage of coverage of the annotated sequence is listed. Proteins specifically pulled down with Rps14 are highlighted in bold. Mw = molecular weight of identified protein in kDa. Sample = molecular weight of cut protein band.

RPS14 interacting protein	unique peptides	percent coverage	Mw (kDa)	Sample: Mw source	comments	function	References
desmocollin-1 isoform Dsc1a preproprotein	7	8,50%	100	52 kDa HeLa	also comes with short edited FLNA	Epidermal barrier function	(Toulza et al., 2007)
protein S100-A8	**2**	**19%**	**11**	**52 kDa HeLa**		**Ca-binding protein**	**(Lagasse and Clerc, 1988)**
small proline rich protein	2	31%	8	100 kDa HeLa	also comes with short edited FLNA	Keratinocyte differentiation	(Kartasova et al., 1988)
arginase-1	**2**	**6,80%**	**35**	**100 kDa HeLa**		**Arginine metabolism/ urea cylcic enzyme**	**(Haraguchi et al., 1987), (Takiguchi et al., 1988)**
apolipoprotein D precursor	**2**	**9,50%**	**21 kDa**	**100 kDa HeLa**		**Lipid transporter**	**(Drayna et al., 1986)**

The mass spectrometry data obtained from N-terminally tagged RPS14 purification did not yield any suitable information about RPS14 interaction partners. To begin with, no ribosomal proteins were pulled down with RPS14 although it is a known ribosomal protein and functions in the complex formation of the 40S ribosome (Rhoads et al., 1986). Secondly none of the identified proteins correlates with the cut protein bands as they were cut at a different molecular weight height.

Mass spectrometry data after purification of C-terminally tagged Rps14 from U2OS cells is listed in table 6. Proteins, found only in one of the Rps14 cut samples are highlighted in bold. Identified proteins, which may be interesting for further analysis, are stained in green.

Table 6) Rps14-interacting proteins detected by mass spectrometric analysis II. This table lists all proteins, detected in protein bands after precipitation of C-termnally tagged Rps14 from stable exressing U2OS cells by mass spectrometric analysis. The identified proteins, the number of analysed peptides, the molecular weight of the identified protein and of the cut sample are listed. Proteins specifically pulled down with Rps14 are highlighted in bold. Data highlighted in green are possible candidates for linking Rps14 to RNA-editing. Mw = molecular weight of identified protein in kDa. Sample = molecular weight of cut protein band.

RPS14 interacting protein	number of assigned spectra	Mw (kDa)	sample: Mw, source	function	reference
nucleophosmin isoform 1	**8**	**32,7**	**41**	**ribosome biogenesis, chaperone during ribosome nuclear export, histone chaperone**	**(Okuwaki et al., 2001) (Maggi et al., 2008) (Swaminathan et al., 2005)**
HIST1H4H protein	**3**	**11,3**	**41**	**core component of nucleosomes**	**RuleBase RU000528**
hnRNP A2/B1	**3**	**37**	**41**	**hnRNP complex formation**	**(Kozu et al., 1995)**
caspase-14 precursor	**3**	**27,6**	**120**	**cysteine-type endopeptidase**	**InterPro**

In this run of purification of C-terminally tagged RPS14 just very low amounts of protein were detected by mass spectrometric analysis. Thus, high levels of keratin contamination may mask other interacting factors. Nevertheless, at least two of the identified proteins match the cut bands in their molecular weight, the nucleolar protein nucleophosmin and the hnRNP A2/B1.These two identified Rps14-interactors may be interesting for further analysis of Rps14 function in RNA-editing.

4.1.4. DDX15

4.1.4.1. Transient Immunofluorescence staining: DDX15

The next candidate for an ADAR regulator was the RNA helicase DDX15 (Imamura et al., 1997). This RNA helicase (GemoneNet Accession number: NP_001349) was also cloned into the generated pcDNA TAP expression vector to be C- and N-terminally tagged. By mistake, only a truncated version of DDX15 was cloned into the expressing vector. Cloning of full-length DDX15 is still in progress. DDX15

contains nuclear and nucleolar localization signal peptide sequences, which may help the protein traffic between different subcellular compartments (Fouraux et al., 2002). Transient transfection studies on the human cell line Hek293 have shown both, nuclear and in a high number of cells also nucleolar localization of the C- and N-terminally tagged protein. However, transfection efficiency of both vectors was less than 30%.

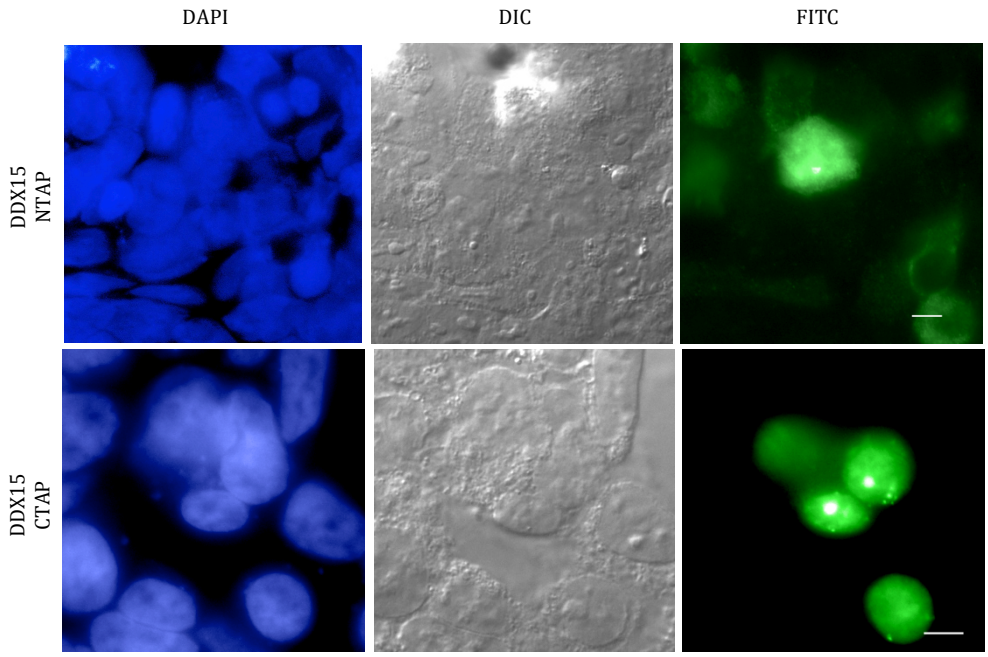


Figure 23) Transient transfection of DDX15 N- and C-terminally tagged with a c-MYC-TEV-HA tag in Hek293. For testing the transfection efficiency of the generated mammalian expression vectors the plasmid preparations were transiently transfected into the human cell line Hek293. Nuclear localization of C- and N-terminally tagged truncated DDX15 was detected by Alexa 488 immunofluorescence staining of an anti-c-Myc primary antibody. Most of the analysed cells got damaged during the fixation procedure. DDX15 NTAP: DDX15 N-terminally tagged with a c-MYC-TEV-HA tag. DDX15 CTAP: DDX15 C-terminally tagged with a c-MYC-TEV-HA tag. DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescence staining. Scale bar: 20 µm.

4.1.4.2. Stable expression of DDX15: Immunofluorescence staining

Though the transfection efficiency of the RNA helicase DDX15 in transient transfection tests was very low, the two versions of the protein, the C- and the N-terminally tagged DDX15, were used for the generation of stable expressing cell lines. We failed to stably express the C-terminally tagged version of DDX15 in any of the used cell lines. Nevertheless, we obtained one U2OS clone stably expressing N-terminally tagged DDX15 although expression levels of DDX15 were found to be very low.

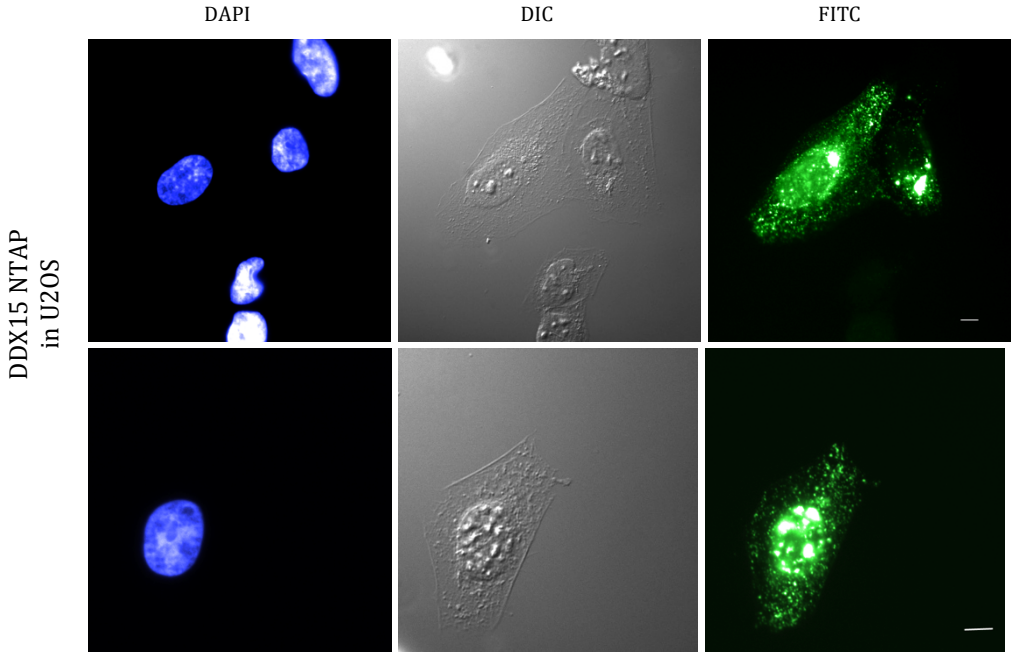


Figure 24) U2OS cell line stably expressing DDX15 N-terminally tagged with a c-MYC-TEV-HA TAP-tag. After selection for stable random integration of the transfected DNA construct, the picked clones were tested on their homogeneity of stable protein expression by immunofluorescence staining. Tagged DDX15 was detected by an anti-c-MYC primary antibody and labelling with a goat anti-mouse secondary antibody coupled to Alexa 488. Stable expressed truncated DDX15 shows nucleolar/nucleoplasmic and granular cytoplasmic localization. DDX15 NTAP: DDX15 C-terminally tagged with a c-MYC-TEV-HA tag. DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescence staining. Scale bar: 20 µm.

As mentioned before, very few cells of the picked clones have shown positive expression of N-terminally tagged DDX15 in immunofluorescence staining tests. Like

in transient transfection assays, using the N-terminally tagged DDX15 expression vector, also stable expressing U2OS cells show mainly nuclear and nucleolar localization of the RNA helicase.

4.1.4.3. Stable expression of DDX15: Western blot analysis

Western blot analysis of the DDX15 expressing U2OS clone did not yield proper information about the expression of the truncated, N-terminally tagged, DDX15. The human full-length protein has a molecular weight of about 90 kDa. Tagged with a 6x c-Myc-TEV-HA tag, DDX15 is of about 120 kDa. The molecular weight of tagged truncated DDX15 is not known. Detected bands are due to a high background level after visualization of DDX15 with an anti-c-Myc primary antibody and a horseradish peroxidase coupled secondary antibody.

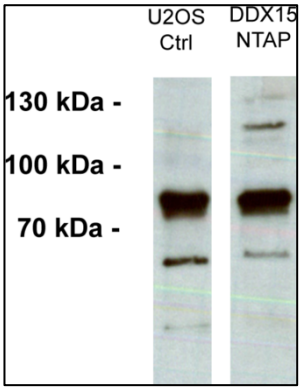


Figure 25) Stable expression of truncated DDX15 in U2OS cells: Western blot analysis. To check the expression of N-terminally tagged truncated DDX15 in the U2OS clone, found to express the protein by immunofluorescence staining, Western blot analysis of the positive clone was done. DDX15 was detected by an anti-c-Myc primary antibody and subsequent visualization with a goat anti-mouse secondary antibody coupled to HRP. The protein band detected at a molecular weight of about 128 kDa is a background band. Thus the size of the truncated version of DDX15 is still unknown. TAP-tagged DDX15 = 120 kDa. Ctrl = plane U2OS cell lysate. DDX15 NTAP = U2OS clone expressing DDX15 tagged with a N-terminal tag for tandem affinity purification.

4.1.4.4. Immunoprecipitation: DDX15

Although the overall expression levels of truncated DDX15 in the obtained U2OS clone were very low in immunofluorescence staining tests and not detectable in Western blot analysis, an initial experimental trial of anti-c-Myc immunoprecipitation was done with Sepharose A beads cross-linked with a 9E10 anti-c-Myc antibody. The samples were analysed on a 7.5% to 17% gradient SDS PAGE gel and subsequent silver staining. Data gained by DDX15 immunoprecipitation from U2OS cells is not significant, as the molecular weight of the truncated version of DDX15 itself was not known. However, one specific protein band was detected at a molecular weight of 65 kDa. Additionally a prominent double band at 56 kDa and 58 kDa was detected. All three bands were sent for mass spectrometric analysis.

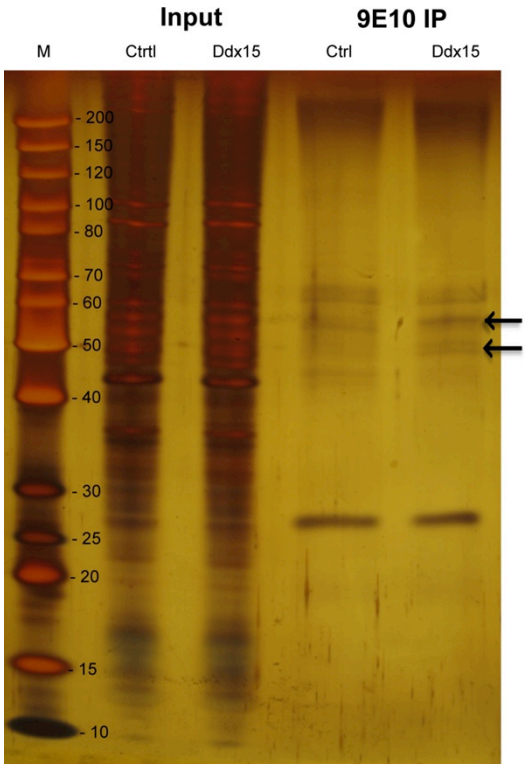


Figure 26) Silver gels: Purification of truncated DDX15. The N-terminally tagged, truncated version of DDDX15 was stable expressed in the human cell line U2OS. Anti-c-Myc immunoprecipitation was done. The samples were separated on a 7.5%-17% gradient SDS PAGE gel and stained by Blum's silver staining protocol. a) Protein band at a molecular weight of 65 kDa. b) Protein double band at a molecular weight of 58 kDa and 56 kDa. Black arrows mark cut protein bands stent for mass spectrometric analysis. The molecular weight of the expressed

truncated version of DDX15 is not known. This was an initial experimental trial. Input = total lysate; 9E10 IP = c-Myc immunoprecipitation; M = PAGE Ruler unstained protein ladder; Ctrl = empty U2OS lysate; Ddx15 = DDX15 expressing U2OS lysate.

4.1.4.5. Mass spectrometry: DDX15

Table 7 lists proteins detected after immunoprecipitation using U2OS cells expressing a truncated version of DDX15. As the size of this protein is not known, this was just an experimental trial. It was also not known which localization signals are comprised in the sequence of the truncated protein. Very few proteins were found in the cut gel bands, what may be due to the truncation and low expression level of the DDX15 itself in the cell line used for purification studies. Data labelled in bold was identified only in lysates, prepared from DDX15 expressing cells.

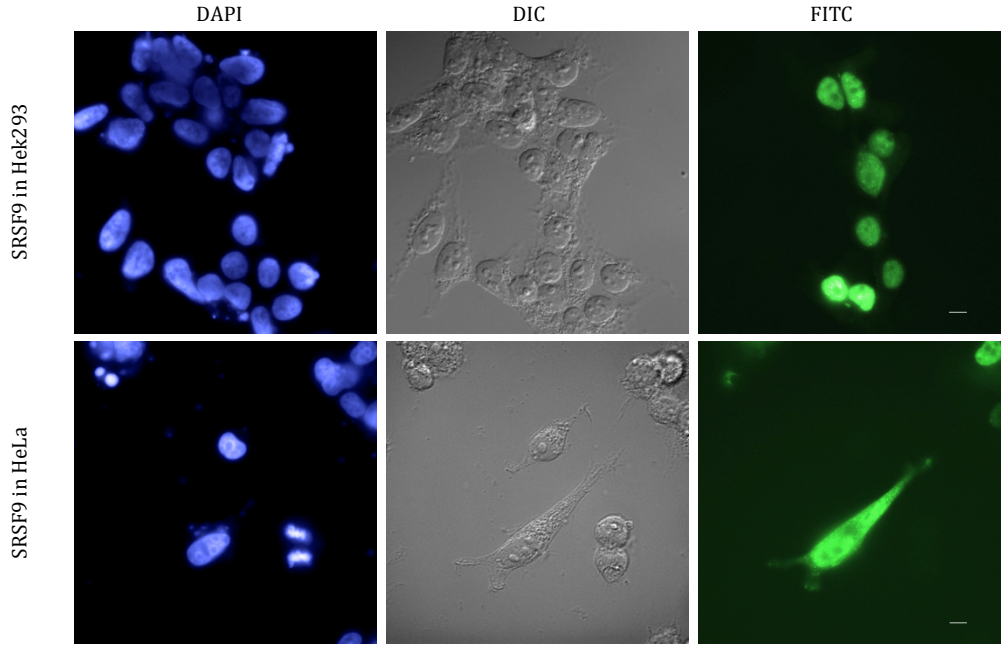
Table 7) DDX15 interacting proteins detected by mass spectrometric analysis. This table lists all proteins, detected in protein bands after precipitation of N-termnally tagged truncated DDX15 from stable exressing U2OS cells by mass spectrometric analysis. The identified protein, the number of analysed peptides and the percentage of coverage of the annotated sequence is listed. Proteins specifically pulled down from the DDX15 lysate are highlighted in bold. Mw = molecular weight of identified protein in kDa. Sample = molecular weight of cut protein band.

DDX15 interacting protein	unique peptides	percent coverage	Mw (kDa)	Sample: Mw source	comments	function	references
alpha-2-glycoprotein 1	**2**	**11%**	**23**	**50 kDa U2OS**		**MCH class I protein**	**(Kennedy et al., 2001)**
suprabasin isoform 1 precursor	2	6,10%	61	65 kDa U2OS	also comes with unedited BLCAP	epidermal barrier function	(Toulza et al., 2007)
Tubulin, beta	**2**	**6,10%**	**50**	**50 kDa U2OS**		**microtubule subunit**	**(Lee et al., 1983)**
vimentin	34	65%	54	65 kDa U2OS	also comes with DSS1	Class-III intermediate filament; binding, stabilization of collagen mRNAs	(Ferrari et al., 1986), (Challa and Stefanovic, 2011)

4.1.5. SRSF9

4.1.5.1. Transient Immunofluorescence staining: SRSF9

The last identified putative regulator of ADAR activity investigated in this thesis was the arginine/serine- rich splice factor SRSF9. For cloning of the SR protein, cDNA from the mouse neuroblastoma cell line N2A was prepared. Primers for amplification of the full-length SR sequence were designed *in silico* (UniProt Accession number: Q13242). The amplified sequence was cloned into the pcDNA (-) Hygro vector, designed for Tandem Affinity Purification. A C-terminally tagged version of SRSF9 was obtained which showed adequate levels of transfection efficiency during transient transfection studies in the human cell line Hek293. No positive signals could be detected after transfection of N-terminally tagged SRSF9, raising the question on the suitability of the used construct.



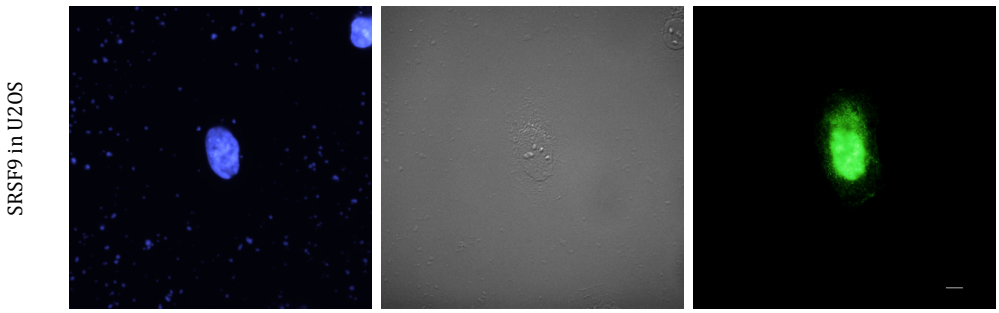


Figure 27) Transient transfection of SRSF9 C-terminally tagged with a c-MYC-TEV-HA tag in Hek293, HeLa and U2OS. For testing the transfection efficiency of the generated mammalian expression vector expressing SRSF9, the plasmid preparations were transiently transfected into the human cell lines Hek293, HeLa and U2OS. In most of the cells nuclear localization of the TAP-tagged SRSF9 was observed. Though, in some of the positive cells SRSF9 was also detected within the cytoplasm. A similar subcellular localization pattern of C-terminally tagged SRSF9 was observed in all three investigated cell lines. SRSF9 CTAP: SRSF9 C-terminally tagged with a c-MYC-TEV-HA tag. DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescence staining. Scale bar: 20 µm.

SRSF9 is an arginine/serine- rich splice factor involved in constitutive and alternative splicing (Screaton et al., 1995). Pre-mRNA splicing is a post-transcriptional processing mechanism, taking place in the nucleus (Misteli et al., 1997). Thus in transient transfection tests with SRSF9 we observed mainly nuclear localization pattern in three different human cell lines. However, in some transfected cells of all three investigated cell lines, SRSF9 was also found to localize within the cytoplasm.
The generation of stable expressing cell lines was started with the C-terminally TAP-tagged SRSF9. But to date no SRSF9 expressing positive clone was sustained.

4.1. FILAMIN A

The cytoskeletal cross-linker Filamin A (FLNA) is a large cytoplasmic protein involved in many cellular processes (Weihing, 1985). FLNA forms a high molecular weight dimer, mediated by its C-terminus, and cross-links actin filaments (Hartwig et al., 1980; Nakamura et al., 2007). In addition to the high avidity binding of FLNA to F-actin many other proteins have been identified to be interaction partners of Filamin. These partners can be grouped into cytoskeletal proteins, proteins involved in cell signalling and motility, and regulators of cellular mechanic stability, for detailed

information see following reviews: (Feng and Walsh, 2004; Popowicz et al., 2006; Stossel et al., 2001). A N-terminal actin binding domain (Hartwig, 1995) is followed by 24 antiparallel beta-barrels, forming IgG-like domains, separated by two calpain-sensitive hinge regions (Gardel et al., 2006). This highly versatile protein was identified as one of the protein-coding targets of RNA editing. Here, editing of the Filamin A transcript leads to an amino acid substitution of a glutamine into an arginine within IgG-like repeat 22 (Levanon et al., 2005). This domain of the protein is known to interact with integrins (Travis et al., 2004) and small GTPases (Ohta et al., 1999). As mentioned in the introduction, the effect of this editing event, which changes the electrostatic properties of the protein, is not known. Thus mammalian expression vectors (pREP4 containing a Hygromycin resistance gene) were established in our lab, encoding the full-length versions of edited and unedited Filamin A tagged with a 3x c-Myc tag. Additionally, we were provided by Christina Godfried (Lehigh University, PA) with pCeMM expression vectors, encoding edited and unedited Filamin A IgG-like domains 20 to 24, tagged by a Protein G-TEV-MYC tag, C-terminally tagged Filamin, and a Protein G-TEV-SBP tag, N-terminally tagged Filamin, for tandem affinity purification. Using the listed expression vectors, we wanted to generate stable cell lines for purification studies under native conditions to investigate interaction profiles of edited and unedited Filamin A.

4.2.1. Transient Immunofluorescence staining: Filamin A

The pREP4 vectors and pCeMM vectors were transiently transfected into several mammalian cell lines to explore potential differences regarding localization pattern of edited and unedited Filamin A. In the following section pictures taken after transient transfections are shown. The c-Myc-tagged Filamins were detected using a 9E10 anti-c-Myc primary antibody and goat anti-mouse secondary antibodies coupled to Alexa568/488 fluorescent dyes. We failed to detect short N-terminally tagged Filamins in transient transfection tests. Goat anti-mouse secondary antibodies coupled to different fluorescent dyes have been tried to detect the protein G-tag. Additionally, fluorescently labelled biotin was used to detect the SBP-tag without success.

Due to the actin-cross-linking nature of Filamin, it shows cytoplasmic, filament-associated localization pattern in all cell lines. However, in some of the transfected cell lines the edited version of full-length Filamin A seems to be more stress fibre

associated, e.g. in HeLa cells. No difference in localization pattern of edited and unedited Filamin A was found in transiently transfected SCaBER cells.

Furthermore, we also used mouse cell lines for investigating subcellular localization pattern of Filamin A. Edited Filamin A was found to be stress fibre associated in the mouse myoblast cell line C2C12, similar to the localization pattern in HeLa cells. Transient transfection of Filamin A in the mouse neuroblastoma cell line cN2A revealed no obvious differences in localization of edited and unedited Filamin A.

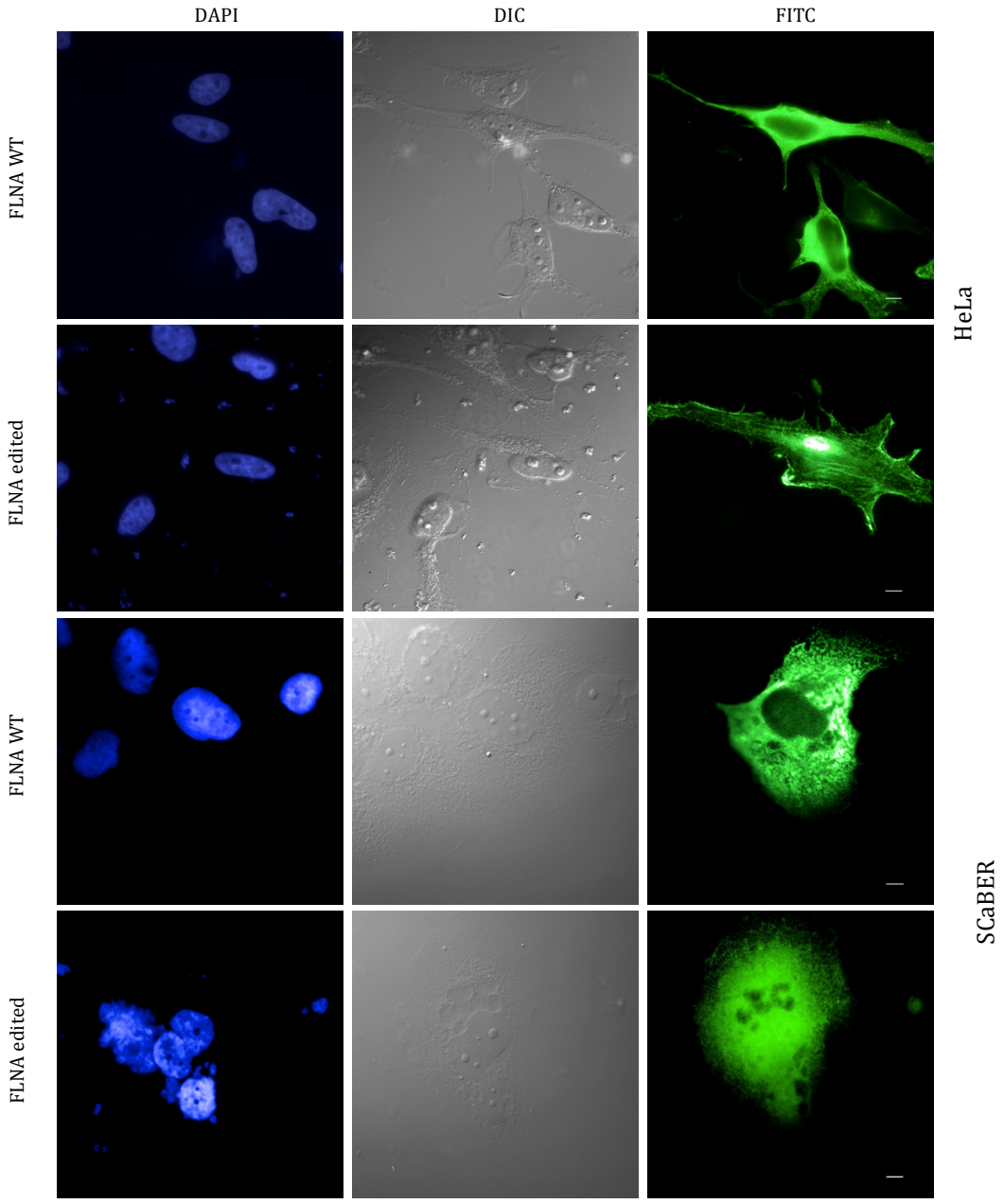


Figure 28) Transient transfection of edited and unedited full-length Filamin A, in the human cell lines HeLa and SCaBER. For testing the transfection efficiency of the mammalian expression vector pREP4 expressing full-length edited and unedited versions of Filamin A, the plasmid preparations were transiently transfected into the human cell lines HeLa and SCaBER. C-Myc-tagged FLNA was detected using a 9E10 antibody and a goat anti-mouse secondary antibody coupled to Alexa 488. Due to FLNA's role as a cytoskeletal cross-linker, transiently transfected FLNA shows cytoplasmic localization. In general, both versions of FLNA, the edited and the unedited protein, show filament-associated localization. Though, in transiently transfected HeLa cells, a more stress-fibre associated localization pattern of edited FLNA was observed, while in SCaBER cells, no obvious differences in localization behaviour of edited and unedited FLNA was found. FLNA = Filamin A, WT = unedited version of Filamin. DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescent staining. Scale bar: 20 µm.

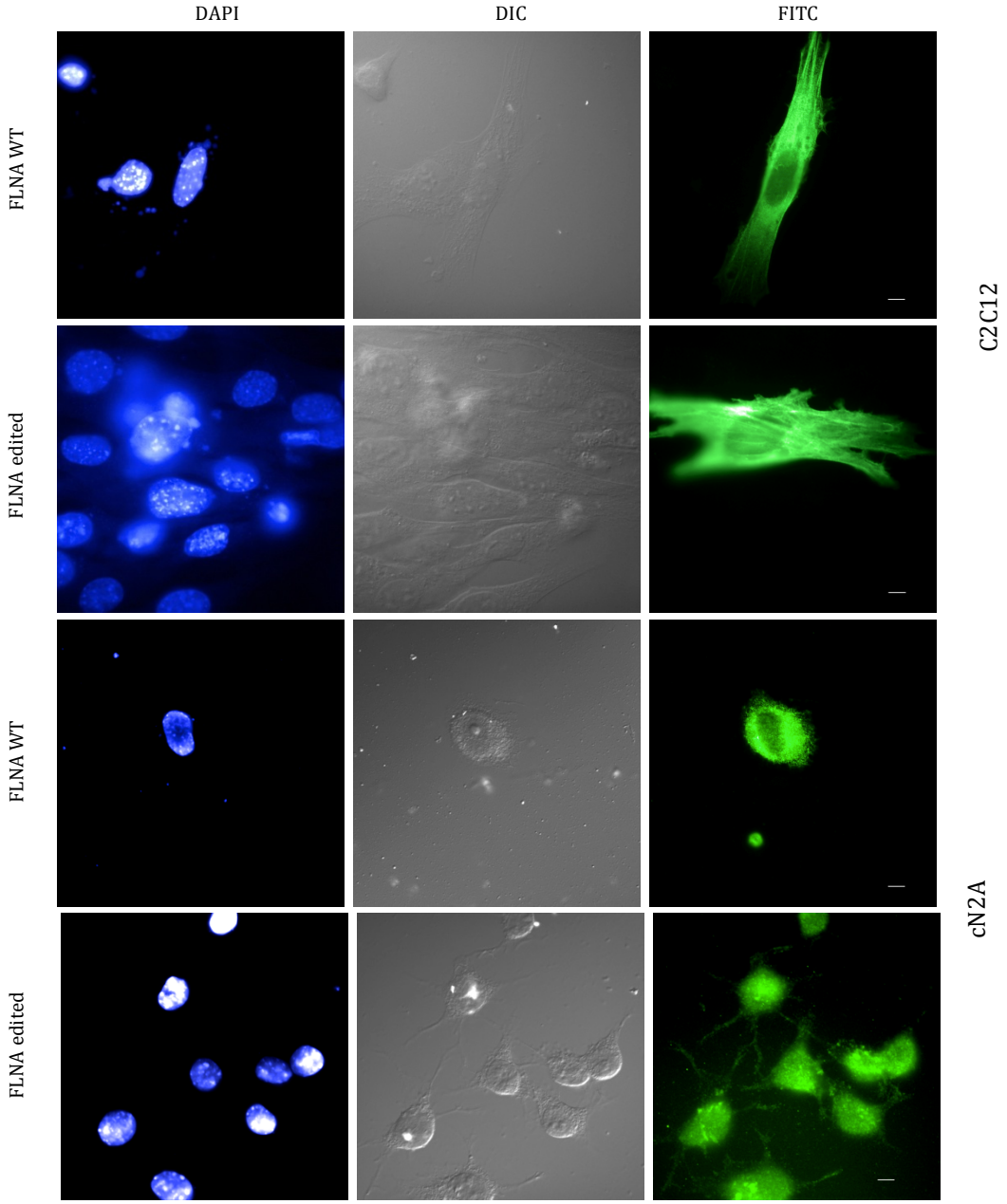


Figure 29) Transient transfection of edited and unedited full-length Filamin A in the mouse cell lines C2C12 and cN2A. For testing the transfection efficiency of the mammalian expression vector pREP4 expressing full-length edited and unedited versions of Filamin A, the plasmid preparations were transiently transfected into the mouse myoblasts C2C12 and neuroblastoma cN2A. C-Myc-taged FLNA was detected using a 9E10 primary antibody and a goat anti-mouse secondary antibody coupled to Alexa 488. As a cytoskeletal cross-linker, transiently transfected FLNA shows cytoplasmic localization also in the mouse cell lines. In general, both versions of FLNA, the edited and the unedited protein, show filament-associated localization. Though, in transiently transfected C2C12 myoblasts, a more stress-fibre associated localization pattern of edited FLNA was observed, while in the neuroblastoma N2A, no obvious differences in localization behaviour of edited and unedited FLNA was found. FLNA = Filamin A, WT = unedited version of Filamin. DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescent staining. Scale bar: 20 µm.

The pCeMM vectors for expression of edited and unedited Filamin A IgG-repeats 20 to 24 are designed to be transfected into mammalian cells via virus-mediated transfection methods. These transfection procedures cannot be carried out in our laboratory facility. Thus we did transient transfection tests with the short Filamin A encoding expression vectors to investigate the transfection efficiencies obtained by nanoparticle mediated transfection techniques. This method was quite promising for the generation of stable cell lines expressing these pCeMM encoded short versions of Filamin A, as an transfection efficiency of about 30% to 40% in several cell lines was gained. Similar to the localization pattern of full-length Filamins, no obvious differences were found in the subcellular localization of short edited and unedited Filamin A in the squamous cell carcinoma cell line SCaBER. Nevertheless, in mouse myoblasts, C2C12, again a more stress fibre associated behaviour of edited truncated Filamin A was observed.

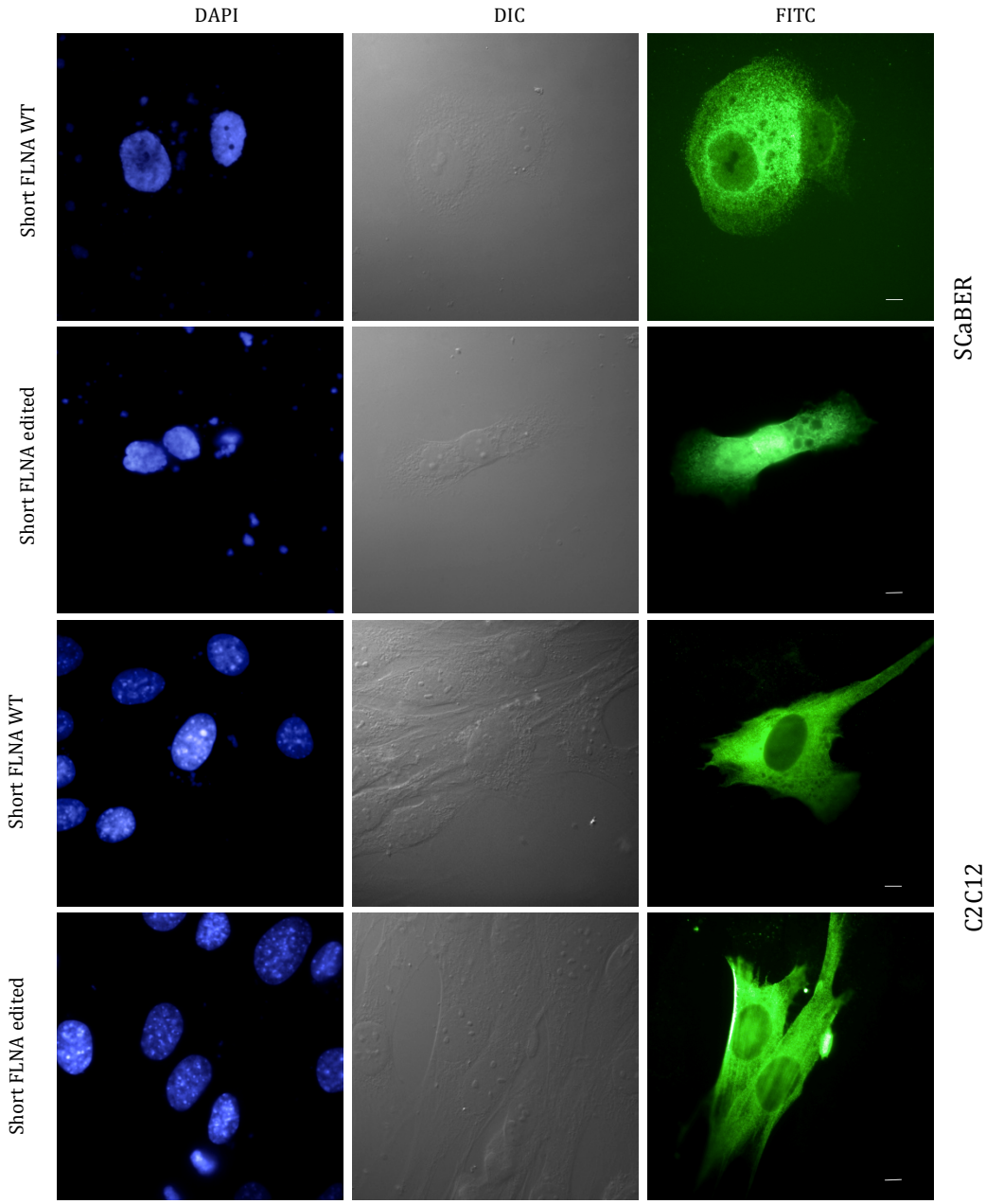


Figure 30) Transient transfection of truncated Filamin A (IgG-like repeats 20 to 24), edited and unedited, in the human cell line SCaBER and the mouse myoblast cell line C2C12. For testing the transfection efficiency of the mammalian expression vector pCeMM expressing C-terminally tagged truncated edited and unedited versions of Filamin A, the plasmid preparations were transiently transfected into the human cell line SCaBER and the mouse myoblasts C2C12. TAP-tagged truncated FLNA was detected, using an anti-c-Myc 9E10 primary antibody and a goat anti-mouse secondary antibody coupled to Alexa 488. Also the truncated versions of FLNA show a cytoplasmic staining pattern. Thoug no obvious differences in subcellular localization of short edited and unedited FLNA could be detected in transiently transfected SCaBER cells, in the mouse cell line C2C12 edited short FLNA again shows a more stress-fibre associated localization. FLNA = Filamin A, WT = unedited version of Filamin A. DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescent staining. Scale bar: 20 µm.

4.2.2. Stable expression of Filamin A: Immunofluorescence staining

After investigating transfection efficiencies of the available Filamin A expression vectors, we started to generate stable cell lines, expressing full-length and truncated versions of edited and unedited Filamin A. The pREP4 vectors for the expression of full-length Filamin A, encodes a Hygromycin resistance gene. Two days after transfection, selection was started by adding the antibiotic Hygromycin B. After 10 days up to three weeks of selection the cells started growing in colonies. These clones were picked and tested on their expression level of Filamin A by immunofluorescence staining and Western blot analysis. Up to now we were able to generate several stable cell lines expressing full-length versions of Filamin A. However, only in the human cell line Hek293 we were able to express both variants of the protein. Edited full-length Filamin A was also stable expressed in the human cell lines U2OS and HeLa cells and in the mouse neuroblastoma cell line cN2A. Two clones of HeLa and U2OS were generated which show a highly heterogenous expression level of unedited full-length Filmain A in immunofluorescence staining tests. Nevertheless, they were useless for purification studies as their Filamin A expression level was much lower than in the clones expressing the edited Filamin A. In order to complete the localization studies on Filamin A, the immunofluorescence pictures of several generated Filamin A expressing cell lines are depicted in the next section.

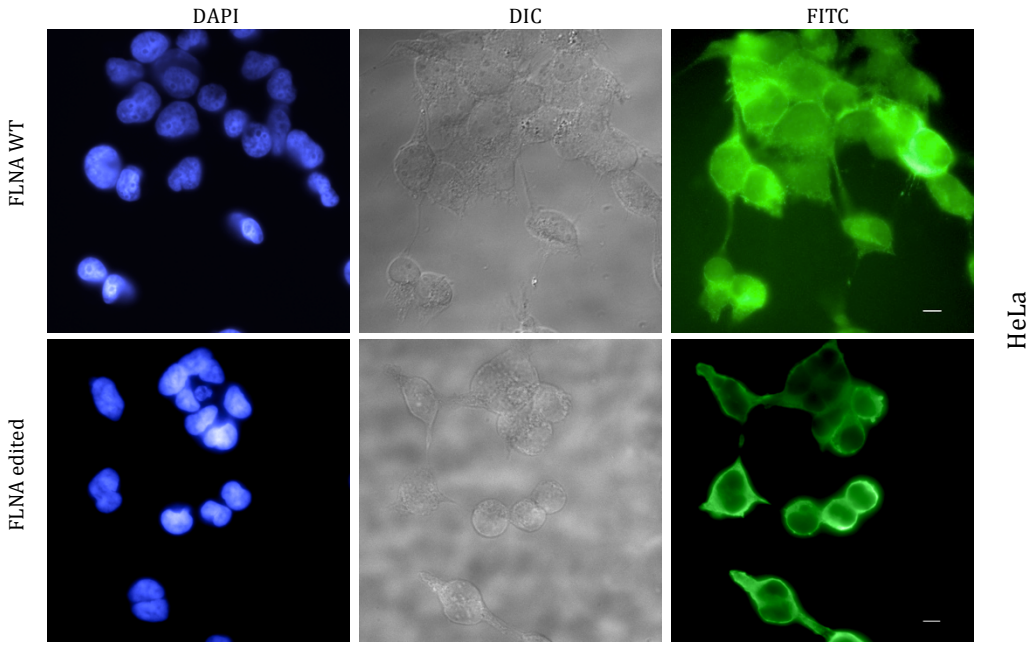


Figure 31) Stable expression of full-length Filamin A in the human cell line HeLa. After selection for stable random integration of the transfected DNA construct, the picked clones were tested on their homogeneity of stable protein expression by immunofluorescence staining. C-Myc-tagged full length Filamin A was detected by an anti-MYC primary antibody and subsequent labelling with a goat anti-mouse secondary antibody coupled to Alexa 488. The full-length versions of edited and unedited FLNA show a cytobplasmic localization pattern in stable expressing HeLa cells. In contrast to transient transfection assays of full-length FLNA in HeLa cells, no obvious differences in filament-association of edited and unedited FLNA were observed in stable expressing HeLa cells. FLNA = Filamin A; WT = unedited Filamin A; DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescence staining. Scale bar: 20 µm.

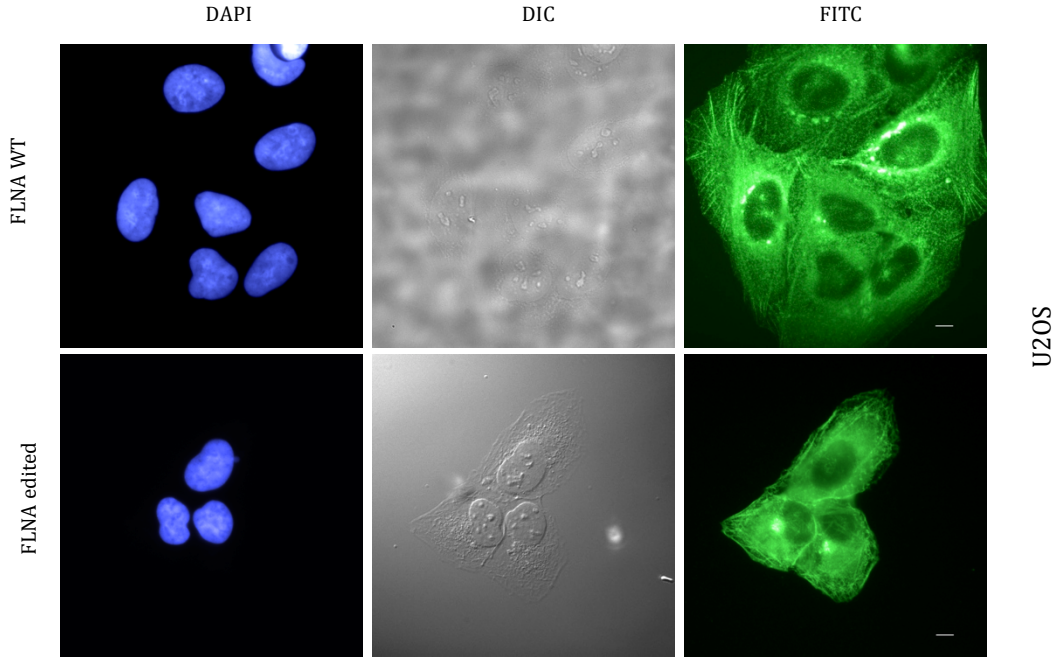


Figure 32) Stable expression of full-length Filamin A in the human cell line U2OS. After selection for stable random integration of the transfected DNA construct, the picked clones were tested on their homogeneity of stable protein expression by immunofluorescence staining. C-Myc-tagged full length Filamin A was detected by an anti-MYC primary antibody and subsequent labelling with a goat anti-mouse secondary antibody coupled to Alexa 488. Also in the human cell line U2OS full-length FLNA is localized in the cytoplasm. Due to the spreading of these cells the association of FLNA with cortical actin fibres is easy to detect and nicely visible after immunofluorescence staining. No obvious differences in localization of edited and unedited FLNA are visible in stable expressing U2OS cells. FLNA = Filamin A; WT = unedited Filamin A; DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescent staining. Scale bar: 20 µm.

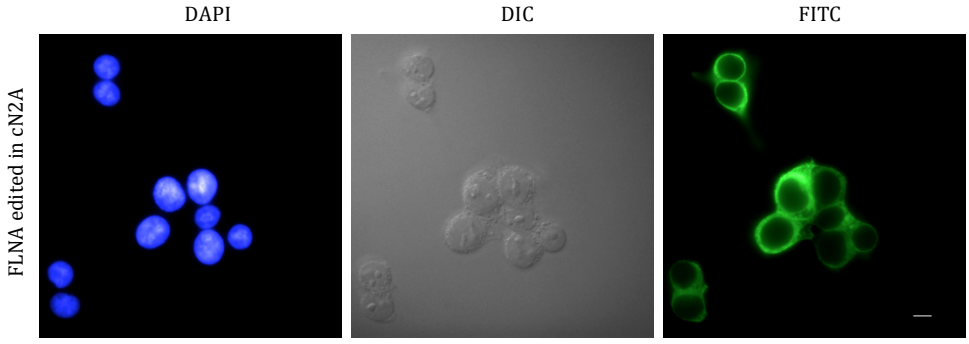


Figure 33) Stable expression of edited full-length Filamin A in the mouse cell line cN2A. After selection for stable random integration of the transfected DNA construct, the picked clones were tested on their homogeneity of stable protein expression by immunofluorescence staining. C-Myc-tagged full-length Filamin A was detected by an anti-MYC primary antibody and subsequent labelling with a goat anti-mouse secondary antibody coupled to Alexa 488. In undifferentiated mouse N2A cells edited full-length FLNA was again detected in the cytoplasm by

immunofluorescence staining. FLNA = Filamin A; DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescent staining. Scale bar: 20 μm.

To generate stable cell lines expressing the truncated versions of edited and unedited Filamin A, the pCeMM expression vectors were co-transfected with a helper plasmid containing a Hygromycin resistance gene. After 10 days up to three weeks of selection, stable Filamin A expressing clones were picked and analysed by immunofluorescence staining and Western blot analysis. Up to now we successfully expressed edited and unedited truncated Filamin A in the human cell line Hek293. Additional U2OS clones have been obtained expressing the unedited form of Filamin A repeats 20 to 24. We failed to express both versions of the protein in other cell lines. Future studies on edited and unedited Filamin A will focus on the generation of stable expressing Filamin A clones in cell lines, where major differences in localization pattern of edited and unedited Filamin A have been detected in this study, like in HeLa cells and C2C12 mouse myoblasts.

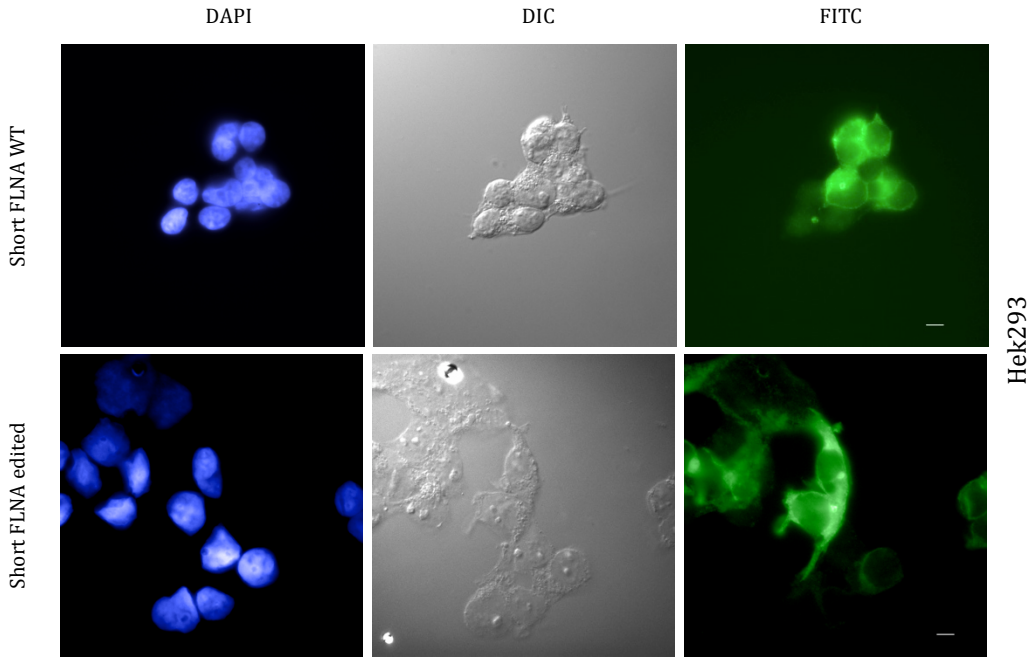


Figure 34) Stable expression of edited and unedited Filamin A IgG-repeats 20 to 24 in the human cell line Hek293. After selection for stable random integration of the transfected DNA construct, picked clones were tested on their homogeneity of stable protein expression by immunofluorescence staining. C-terminally TAP-tagged short Filamin A (IgG-like repeats 20 to 24) was detected by an anti-c-MYC primary antibody and subsequent labelled with a goat anti-mouse secondary antibody coupled to Alexa 488. Due to the harming action of fixation reagent on Hek293 cells, detected positive cells are miss-shaped. Though, again an overall cytoplasmic localization of stable expressed short FLNA is visible FLNA = Filamin A; WT = unedited Filamin A; DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescent staining. Scale bar: 20 µm.

4.2.3. Stable expression of Filamin A: Western blot analysis

Western blot analysis of clones expressing full-length FLNA is not shown, as a high level of protein fragmentation was detected. Hek293 clones expressing edited and unedited FLNA were lysed using 2x SDS sample buffer and subsequent sonication. TAP-tagged C-terminally tagged truncated edited and unedited FLNA was detected using a 9E10 anti-c-MYC primary antibody and a goat anti-mouse secondary antibody coupled to horseradish peroxidase.

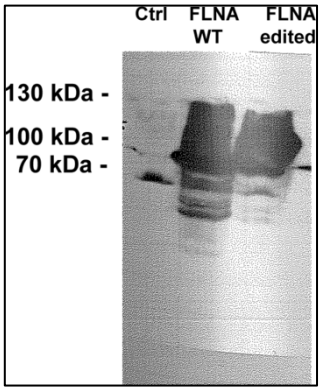


Figure 35) Stable expression of truncated C-terminally tagged edited and unedited FLNA: Western blot analysis. Hek293 cells, found to express truncated FLNA C-terminally tagged by immunofluorescence staining, were lysed in 2x SDS sample buffer and sonication. Samples were analysed using an anti-c-MYC primary antibody and a goat anti-mouse secondary antibody coupled to horseradish peroxidase. Truncated edited and unedited FLNA tagged by a Protein G/TEV/MYC TAP-tag shows a molecular weight of about 75 kDa. Ctrl = lysate of empty Hek293 cells; FLNA WT = lysate of Hek293 cells expressing truncated unedited FLNA, C-terminally TAP-tagged; FLNA edited = lysate of Hek293 cells expressing truncated edited FLNA, C-terminally TAP-tagged.

4.2.4. Immunoprecipitation / Tandem Affinity purification: Filamin A

After testing the picked clones on their Filamin A expression immunoprecipitation assays were done using Sepharose A beads cross-linked to a 9E10 anti-c-Myc antibody. To date no specific protein bands have been obtained in purification assays of full-length Filamin A. Due to high levels of protein fragmentation and high background signal after 9E10-immunoprecipitation, sustained protein bands may mask Filamin A specific bands, thus the purification procedure for full-length Filamin A still has to be improved. The Hek293 cell line expressing edited and unedited Filamin A IgG- like repeats 20 to 24 was used for purification studies. Immunoprecipitation using 9E10 anti-c-Myc antibody coupled Sepharose A beads and Tandem Affinity Purification was done. The first purification step in the Tandem Affinity Purification procedure for Filamin A precipitation was conducted using pan anti-mouse IgG-magnetic beads. After the TEV cleavage reaction c-Myc tagged truncated Filamin A was pulled down by anti-c-Myc coupled Sepharose A beads. So far no FLNA-specific protein bands have been obtained by tandem affinity purification. IP-Obtained samples were examined by 7.5% to 17% SDS PAGE and

subsequent silver staining. Filamin A was purified under several conditions, using different lysis buffers to improve the overall yield of purified protein.

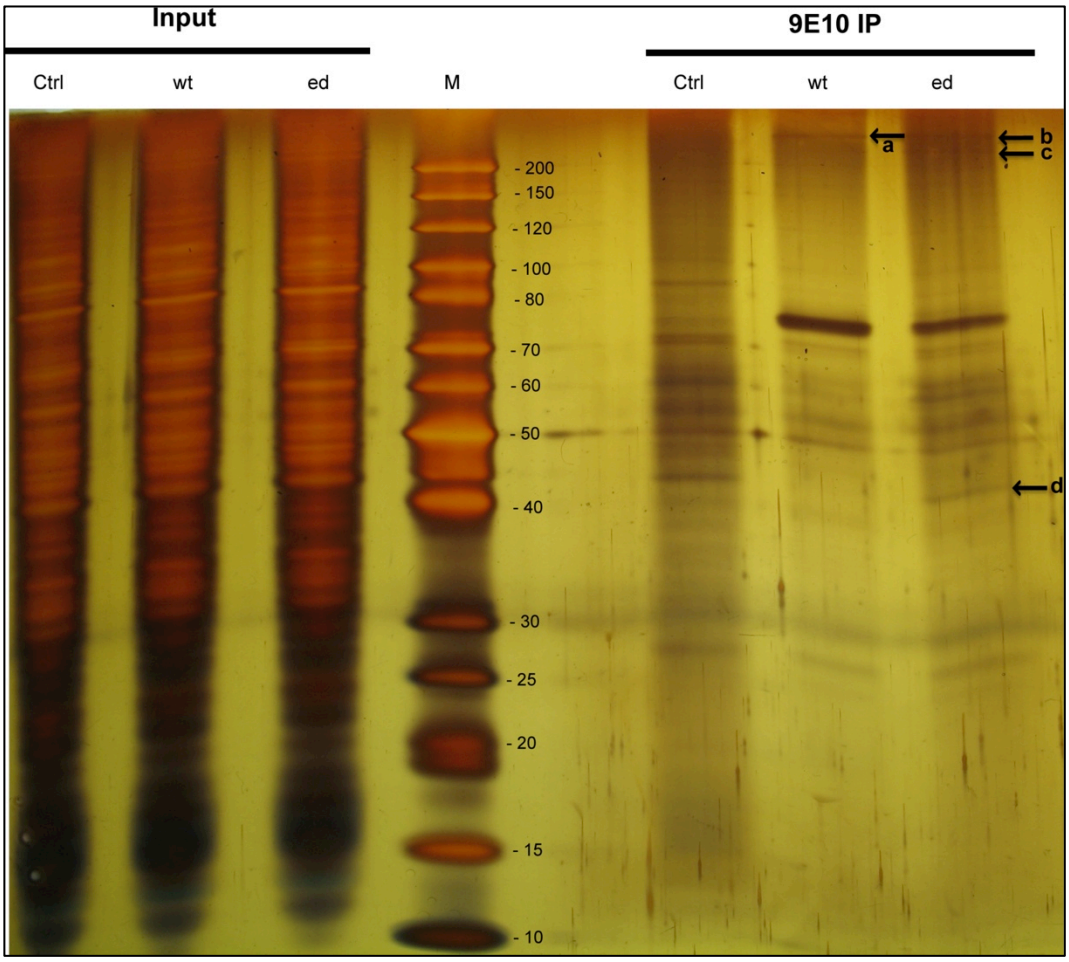


Figure 36) Silver gels: Purification of short Filamin A tagged with a C-terminal TAP-tag. The C-terminally tagged edited and unedited versions of Filamin A IgG-like repeats 20 to 24 were stable expressed in the human cell line Hek293. Anti-c-Myc immuno-precipitation was done with Hek293 cells expressing edited and unedited Filamin A IgG-repeats 20-24. The samples were separated on a 7.5%-17% gradient SDS PAGE gel and stained by Blum's silver staining protocol. a) Protein band at a molecular weight of > 200 kDa after IP of unedited short Filamin A. b) Protein band at a molecular weight of > 200 kDa from IP of short edited Filamin A c) Protein band at a molecular weight of 200 kDa from IP of short edited Filamin A. d) Protein band at a molecular weight of 47 kDa. Black arrows mark cut protein bands sent for mass spectrometric analysis. Ctrl = Lysate of plane Hek293; wt = lysate of Hek293 expressing unedited short Filamin A; ed = lysate of Hek293 expressing edited short Filamin A; Input = total lysate; 9E10 IP = c-Myc immunoprecipitation; M = PAGE Ruler unstained protein ladder.

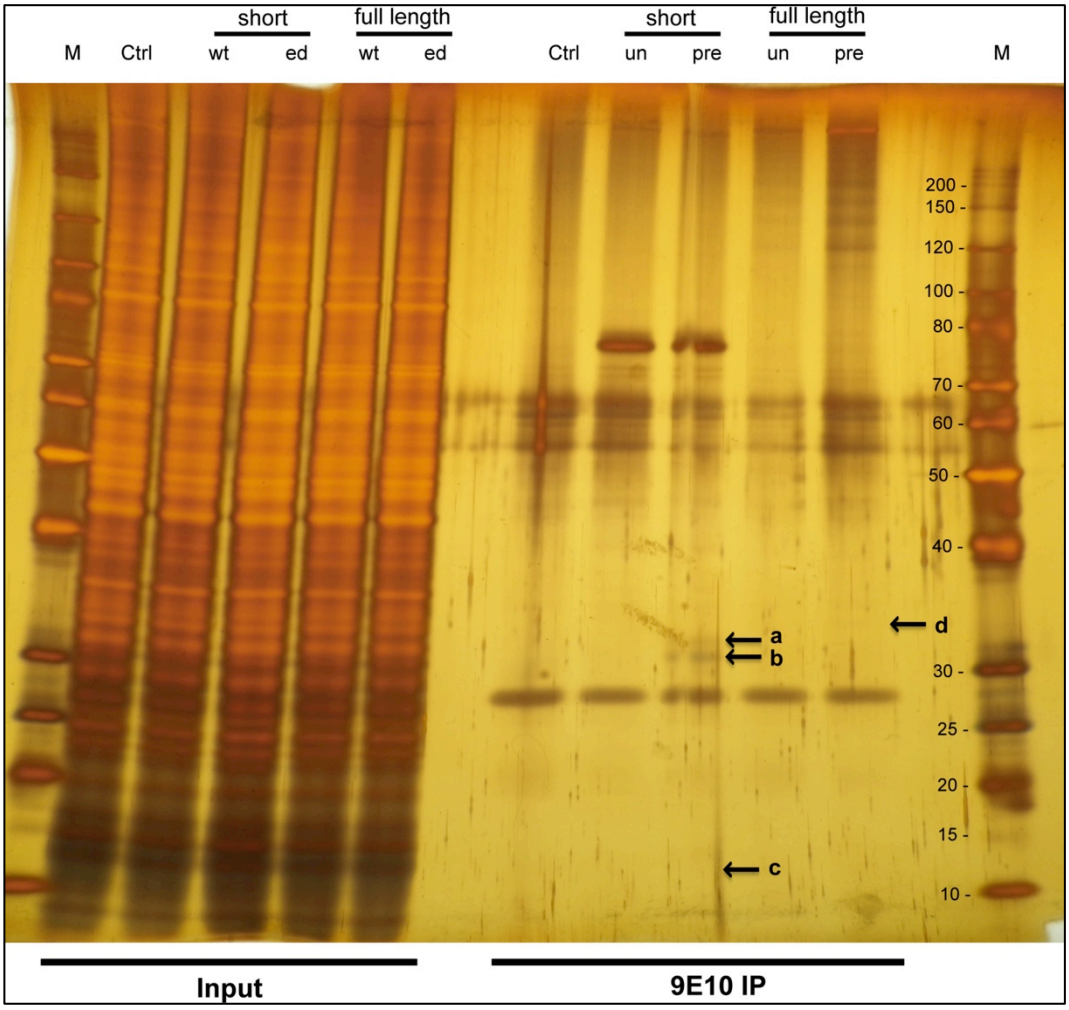


Figure 37) Silver gels: Purification of full length FLNA and short FLNA tagged with a C-terminal TAP-tag. The c-Myc tagged edited and unedited full length FLNA and the C-terminally tagged edited and unedited versions of FLNA IgG-like repeats 20 to 24 were stable expressed in the human cell line Hek293. Anti-c-Myc immunoprecipitation using the lysis buffer NET-2 was done with Hek293 cells expressing edited and unedited full-length FLNA and FLNA IgG-repeats 20-24. The samples were separated on a 7.5%-17% gradient SDS PAGE gel and stained by Blum's silver staining protocol. a) Protein band at a molecular weight of > 24 kDa after IP of edited short FLNA b) Protein band at a molecular weight of > 22 kDa from IP of short edited FLNA c) Protein band at a molecular weight of 11 kDa from IP of short edited FLNA d) Protein band at a molecular weight of 26 kDa from IP of full length edited FLNA. Black arrows mark cut protein bands sent for mass spectrometric analysis. Ctrl = Lysate of plane Hek293; wt = lysate of Hek293 expressing unedited full length and short Filamin A; ed = lysate of Hek293 expressing edited full-length and short Filamin A; Input = total lysate; 9E10 IP = c-Myc immunoprecipitation; M = PAGE Ruler unstained protein ladder.

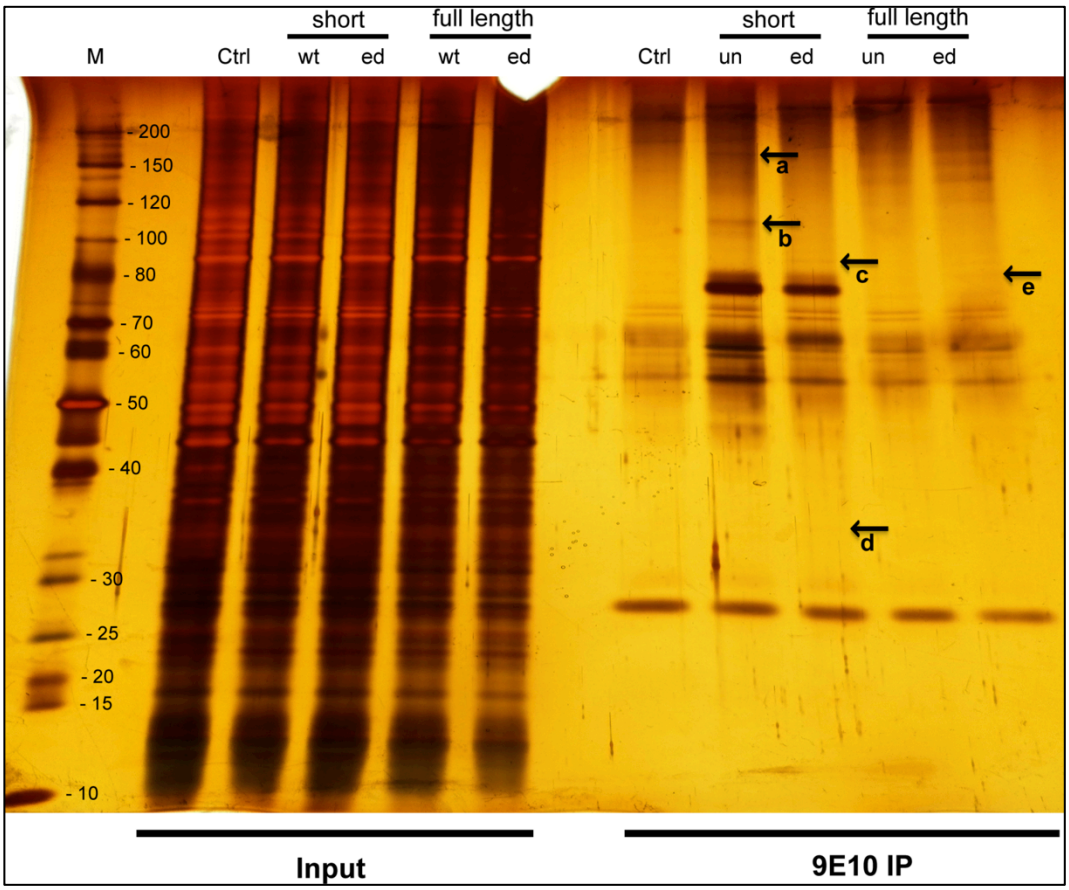


Figure 38) Silver gels: Purification of full length FLNA and short FLNA tagged with a C-terminal TAP-tag. The c-Myc tagged edited and unedited full length FLNA and the C-terminally tagged edited and unedited versions of FLNA IgG-like repeats 20 to 24 were stable expressed in the human cell line Hek293. Anti-c-Myc immunoprecipitation using 0.5x RIPA buffer for cell lysis was done with Hek293 cells expressing edited and unedited full length FLNA and FLNA IgG-repeats 20-24. The samples were separated on a 7.5%-17% gradient SDS PAGE gel and stained by Blum's silver staining protocol. a) Protein band at a molecular weight of > 156 kDa after IP of unedited short FLNA b) Protein band at a molecular weight of > 22 kDa from IP of short edited FLNA c) Protein band at a molecular weight of 11 kDa from IP of short edited FLNA d) Protein band at a molecular weight of 26 kDa from IP of full length edited FLNA. Black arrows mark cut protein bands sent for mass spectrometric analysis. Ctrl = Lysate of plane Hek293; wt = lysate of Hek293 expressing unedited full-length and short Filamin A; ed = lysate of Hek293 expressing edited full-length and short Filamin A; Input = total lysate; 9E10 IP = c-Myc immunoprecipitation; M = PAGE Ruler unstained protein ladder.

4.2.5. Mass spectrometry: Filamin A

We tried to purify full-length and truncated edited and unedited Filamin A in complex with its interaction partners under different conditions. Tables below list proteins pulled down by anti-c-Myc immunoprecipitation of truncated edited and unedited Filamin A. Proteins found to interact only with Filamins are listed in bold in two tables, separating interaction partners of unedited and edited Filamin A. In this sudy several proteins have been identified which only interact with short edited or unedited Filamin A. These interaction partners and other interesting identified proteins, like several histone variants, are highlighted in green. Furhter studies need to be done to verify mass spectrometry data gained in this project.

Table 8) Interaction partners of unedited FLNA identified by mass spectrometric analysis I. Proteins, detected in protein bands after precipitation of C-termnally tagged truncated unedited FLNA from stable exressing Hek293 cells are listed in this table. The identified proteins, the numbers of analysed peptides, the molecular weight of the identified protein and of the cut sample are shown. Proteins specifically pulled down with unedited short FLNA are highlighted in bold. Data highlighted in green are proteins either specifically interacting with unedited FLNA (spectrin alpha) or nuclear proteins (histone H1.3), which may lead to new insights on FLNA localization and function in the cell. Mw = molecular weight of identified protein in kDa. Sample = molecular weight of cut protein band.

WT FLNA-interacting protein	unique peptides	percent coverage	Mw (kDa)	Sample: Mw (kDa)	comments	function	reference
filamin-A isoform 1	34	16%	280	> 200	comes with edited FLNA	cytoskeletal cross linker, signalling scaffold	(Stossel et al., 2001), (van der Flier and Sonnenberg, 2001)
filamin-B isoform 2	9	5,10%	278	> 200	comes with edited FLNA	links actin cytoskeleton to membrane constituents	(Stossel et al., 2001), (van der Flier and Sonnenberg, 2001)
filamin-C isoform a	2	2,20%	291	> 200	comes with edited FLNA	actin filament cross linker; muscle-specific filamin	(van der Ven et al., 2000)
histone H1.3	2	9,50%	22	> 200	comes with edited FLNA	condensation of nucleosome DNA	(Albig et al., 1991)
spectrin, alpha, non-erythrocytic 1	**3**	**1,30%**	**279**	**> 200**		**Ca2+ dependent movement of cytoskeleton**	**(Moon and McMahon, 1990)**

Table 9) Interaction partners of edited FLNA identified by mass spectrometric analysis I. Proteins, detected in protein bands after precipitation of C-termnally tagged truncated edited FLNA from stable exressing Hek293 cells are listed in this table. The identified proteins, the numbers of analysed peptides, the molecular weight of the identified protein and of the cut sample are shown. Proteins specifically pulled down with edited short FLNA are highlighted in bold. Data highlighted in green are proteins either specifically interacting with edited FLNA (spectrin beta) or nuclear proteins (histone H1.3), which may lead to new insights on FLNA localization and function in the cell. Mw = molecular weight of identified protein in kDa. Sample = molecular weight of cut protein band.

Edited FLNA-interacting protein	unique peptides	percent coverage	Mw (kDa)	Sample: Mw (kDa)	comments	function	references
filamin-A isoform 1	22	11%	280	> 200	comes with unedited FLNA	cytoskeletal cross linker, signalling scaffold	(Stossel et al., 2001; van der Flier and Sonnenberg, 2001)
desmoglein-1 preproprotein	3	4,00%	114	47	comes with Rps14 and DDX15	component of intercellular desmosome junctions	(Nilles et al., 1991)
filamin-B isoform 2	7	3,00%	278 kDa	> 200	comes with unedited FLNA	links actin cytoskeleton to membrane constituents	(Stossel et al., 2001), (van der Flier and Sonnenberg, 2001)
glyceraldehyde-3-phosphate dehydrogenase	**3**	**12%**	**36 kDa**	**47**		**Dehydrogenase and nitrosylase activities**	**(Hanauer and Mandel, 1984)**
histone H2A type 1-B/E	**2**	**12%**	**14 kDa**	**47**		**core component of nucleosomes**	**(Bonenfant et al., 2006)**
filamin-C isoform a	5	3,00%	291 kDa	> 200	comes with unedited FLNA	actin filament cross linker; muscle-specific filamin	(van der Ven et al., 2000)
small proline rich protein	2	31%	6 kDA	47	comes with Rps14	envelope protein of keratinocytes	(Kartasova and van de Putte, 1988)
spectrin beta chain, brain 1 isoform 1	**2**	**0,89%**	**275 kDa**	**> 200**		**cytoskeletal protein, crosslinks actin**	**(Byers and Branton, 1985)**
histone H1.3	5	15%	22 kDa	> 200	comes with edited FLNA	condensation of nucleosome DNA	(Albig et al., 1991)
band-6-protein	**2**	**2,80%**	**80 kDa**	**47**		**constituent of desmosomes**	**(Hatzfeld et al., 1994)**
PELOTA	**2**	**6,20%**	**43 kDa**	**47**		**Mitosis/genomic stability/ degradation of damaged mRNA**	**(Shamsadin et al., 2000)**

After anti-c-Myc immunoprecipitation of C-terminally tagged short edited and unedited FLNA from Hek293 cells, lysed by either NET-2 or RIPA lysis buffer, another set of cut protein bands was sent for mass spectrometric analysis. This time just a few peptides were detected. Nevertheless, most of these peptides were assigned to annotated protein sequences and are listed in the tables below. For these runs of

short Filamin A purification different lysis buffers were used, thus a different set of proteins was identified than in the first Filamin A purifications. This time several components of cell adhesion contacts, like subunits of desmosomes, have been identified to interact either with edited or unedited short Filamin A. Again one histone variant was identified to be an interacting partner of Filamin A (Histone H2B type 1-K).

Table 10) Interaction partners of unedited FLNA identified by mass spectrometric analysis II. Proteins, detected in protein bands after precipitation of C-termnally tagged truncated unedited FLNA from stable exressing Hek293 cells are listed in this table. Different lysis buffers have been used for this run of FLNA purification. The identified proteins, the numbers of analysed peptides, the molecular weight of the identified protein and of the cut sample are shown. Proteins specifically pulled down with unedited short FLNA are highlighted in bold. Data highlighted in green are proteins either specifically interacting with unedited FLNA (Desmoplakin) or nuclear proteins (Histone H2B type 1-K), which may lead to new insights on FLNA localization and function in the cell. Mw = molecular weight of identified protein in kDa. Sample = molecular weight of cut protein band.

WT FLNA-interacting protein	Nr. of assigned spectra	Mw (kDa)	Sample: Mw (kDa)	function	reference
Desmoplakin	**2**	**330**	**120**	**component of desmosomes**	**(Green et al., 1990)**
Histone H2B type 1-K	**2**	**14**	**120**	**histone binding protein**	**(Lorain et al., 1998)**

Table 11) Interaction partners of edited FLNA identified by mass spectrometric analysis II. Proteins, detected in protein bands after precipitation of C-termnally tagged truncated edited FLNA from stable exressing Hek293 cells are listed in this table. The identified proteins, the numbers of analysed peptides, the molecular weight of the identified protein and of the cut sample are shown. Proteins specifically pulled down with edited short FLNA are highlighted in bold. Data highlighted in green are proteins either specifically interacting with edited FLNA (spectrin beta) or nuclear proteins (histone H1.3), which may lead to new insights on FLNA localization and function in the cell. Mw = molecular weight of identified protein in kDa. Sample = molecular weight of cut protein band.

Edited FLNA-interacting protein	Nr. of assigned spectra	Mw (kDa)	Sample: Mw (kDa)	function	reference
Desmoglein-1	**3 / 1**	**114**	**24 / 10**	**cadherin family protein**	**(Wheeler et al., 1991)**
Serpin A12	**3**	**47**	**24**	**adipocatokine**	**(Hida et al., 2005)**
Serpin B3	**2**	**44,6**	**24**	**squamous cell carcinoma antigen**	**(Suminami et al., 2000)**
Annexin A2	**1 / 2**	**38,6**	**24 / 26**	**Ca2+ dependent membrane binding protein**	**(Emans et al., 1993)**
Desmocollin-1	**2**	**100**	**24**	**component of desmosomes**	**(King et al., 1991)**

Gamma-glutamylcyclotransferase	2	21	24	cytochrome c releasing factor	(Masuda et al., 2006)
Four and a half LIM domains protein 3	2	31,2	22	Zink-finger binding protein in sceletal muscle development	(Morgan and Madgwick, 1999)
Vinculin	2	123,8	24	actin binding protein	(Le Clainche et al., 2010)
Small proline-rich protein 2G	2	8,2	10	regulation of barrier function	(Cabral et al., 2001)
14-3-3 protein epsilon	2	29	22	involved in cell cycle regulation	(Conklin et al., 1995)
Heat shock protein HSP 90-beta	5	83	103	involved in progesteron signalling	(Chadli et al., 2006)

4.3. BLCAP

The bladder cancer associated protein BLCAP has been identified as another target for RNA editing (Levanon et al., 2005). Up to 11 editing events in the primary transcript have been reported where three of them lead to amino acid substitutions within the coding region. *In silico* studies have predicted two trans-membrane spanning regions in the primary sequence of BLCAP (Galeano et al., 2010). However, little is known about the function of this ubiquitously expressed protein.

In our lab, vectors have been generated for stable expression of one edited and unedited version of BLCAP comprising 9x c-Myc tagged full-length variants of the protein and a Neomycin resistance gene.

4.3.1. Transient Immunofluorescence staining: BLCAP

To investigate the subcellular localization of BLCAP, transient transfection studies have been done in various mammalian cell lines. Edited and unedited BLCAP was transiently transfected and stained by a 9E10 anti-c-Myc primary antibody, visualized by a goat anti- mouse secondary antibody coupled to the fluorescence dye Alexa 488. An overall granular localization pattern of BLCAP was detected in all cell lines investigated. No differences were observed between unedited and edited BLCAP in any of the cell lines.

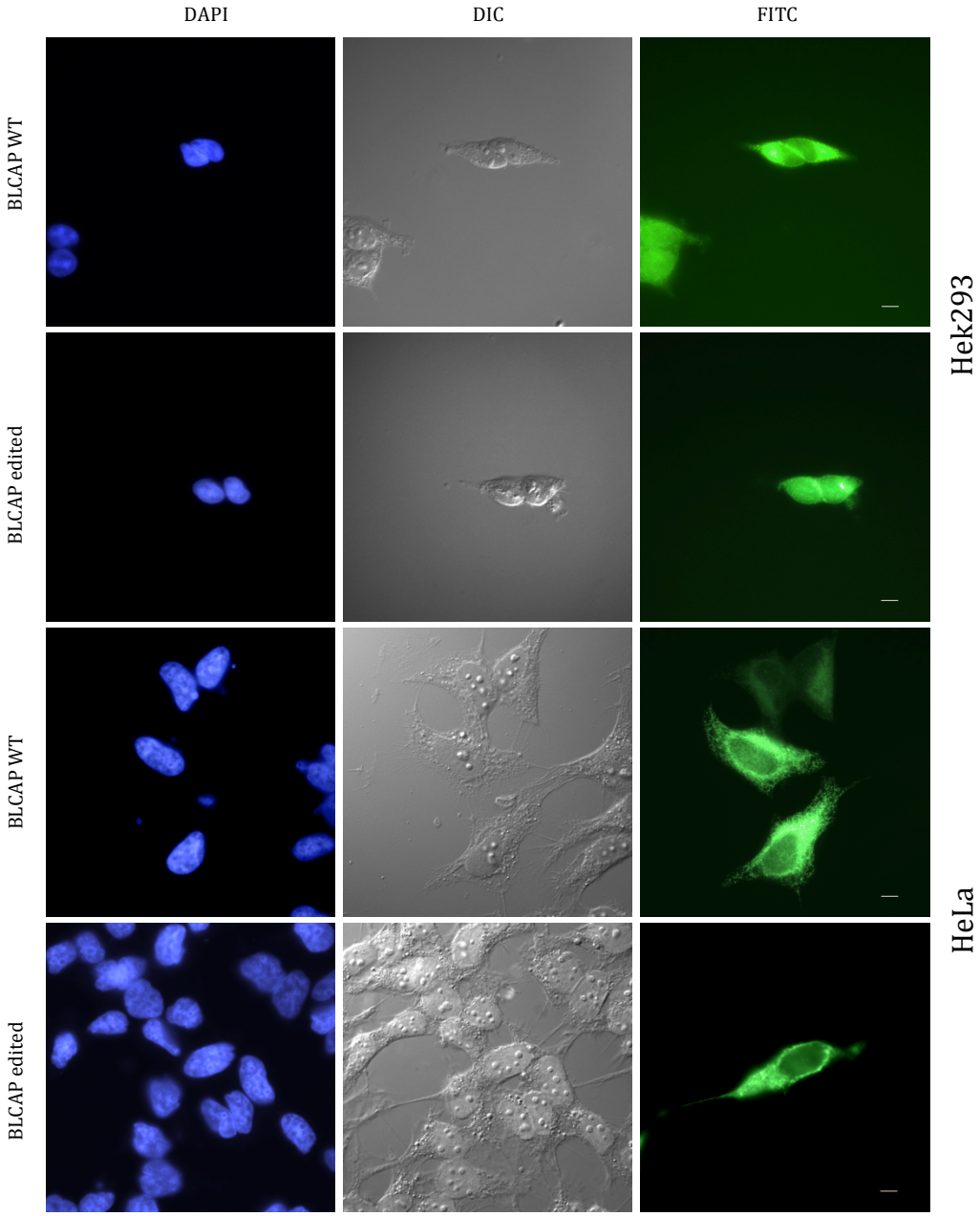


Figure 39) Transient Immunofluorescence staining of edited and unedited BLCAP in the human cell lines Hek293 and HeLa. 9x c-Myc tagged edited and unedited BLCAP, transiently transfected into Hek293 and HeLa cell lines, was detected by a 9E10 anti-c-Myc primary antibody from mouse. For visualization a goat anti-mouse secondary antibody was used coupled to the green fluorescence dye Alexa 488. In Hek293 and HeLa cells, edited and unedited BLCAP show cytoplasmic localization. Additionally, what can be seen from HeLa cells, transfected with edited and unedited BLCAP, an overall granular localization pattern can be observed. No differences in localization pattern between edited and unedited BLCAP are observed in immunofluorescence staining assays of transiently transfected BLCAP versions. DAPI = nuclear staining; DIC = phase contrast; FITC = localization of BLCAP, Alexa 488; Scale bar: 20 µm.

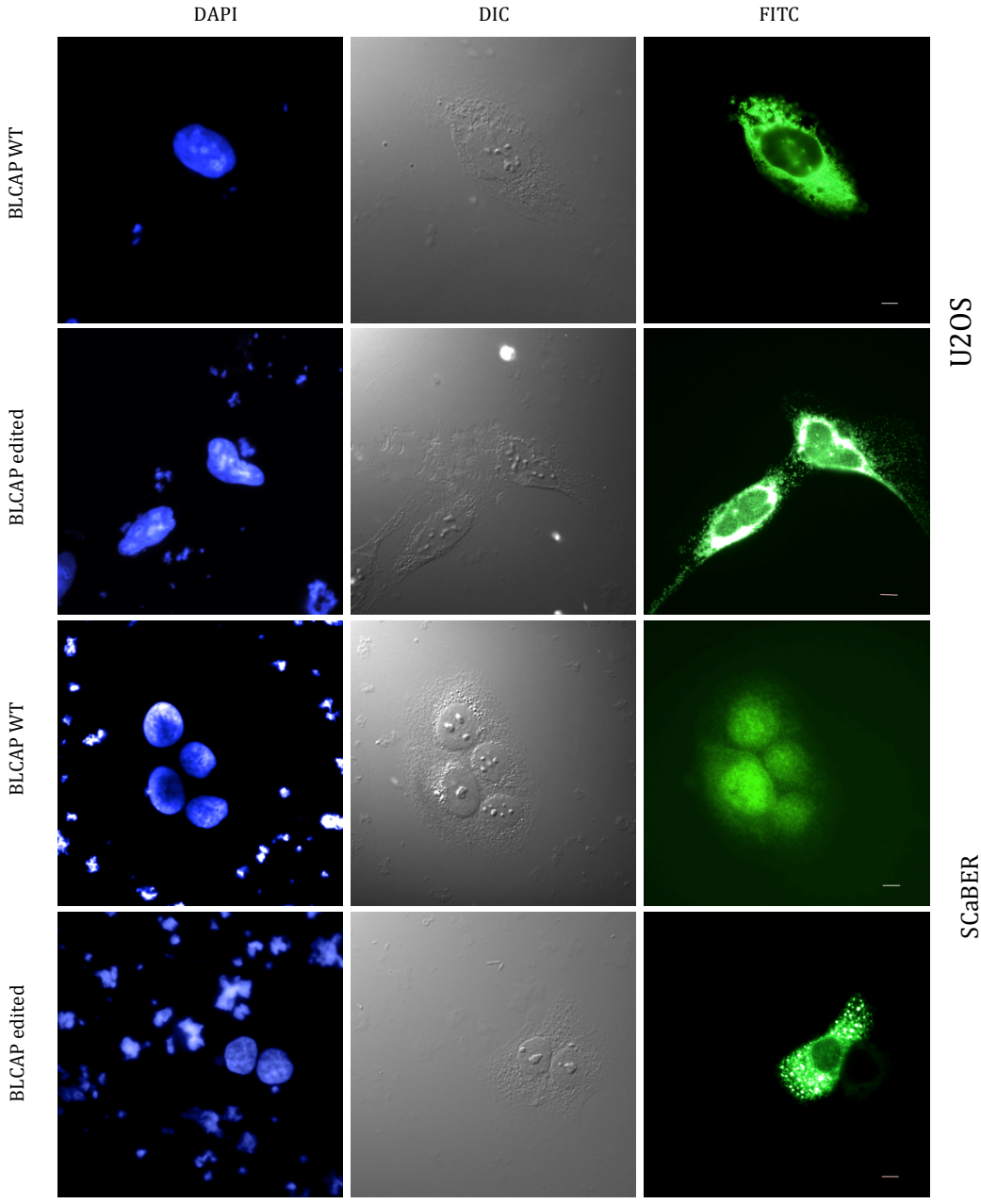


Figure 40) Transient Immunofluorescence staining of edited and unedited BLCAP in the human cell lines U2OS and SCaBER. 9x c-Myc tagged edited and unedited BLCAP, transiently transfected into U2OS and SCaBER cell lines, was detected by a 9E10 anti-c-Myc primary antibody from mouse. For visualization a goat anti-mouse secondary antibody was used, coupled to the green fluorescence dye Alexa 488. In both tested cell lines, edited and unedited BLCAP show cytoplasmic localization. Again an overall granular localization pattern can be observed, like in tests on other mammalian cell lines. No differences in localization pattern between edited and unedited BLCAP are observed in immunofluorescence staining assays of transiently transfected BLCAP versions in the investigated cell lines. DAPI = nuclear staining; DIC = phase contrast; FITC = localization of BLCAP, Alexa 488; Scale bar: 20 µm.

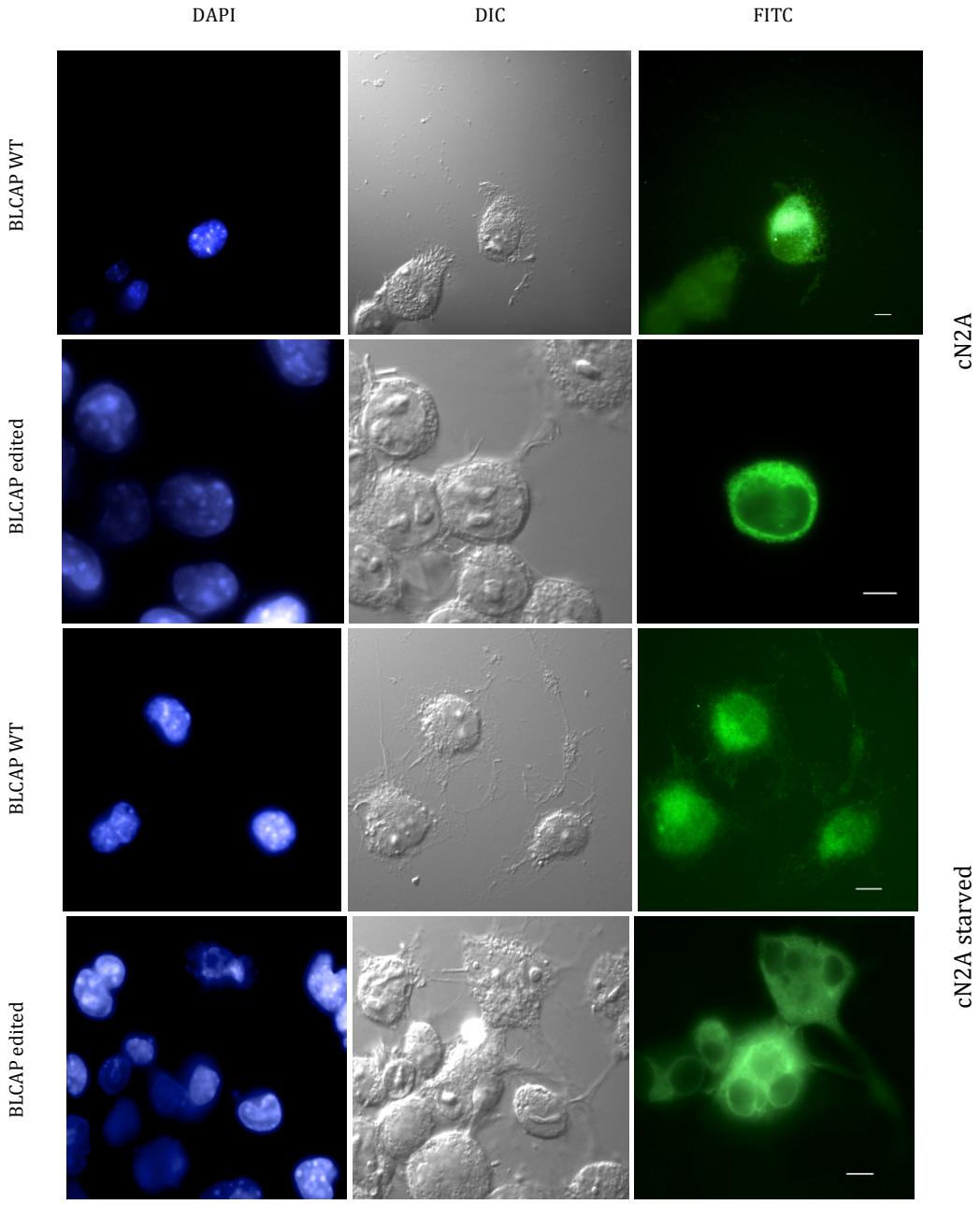


Figure 41) Transient Immunofluorescence staining of edited and unedited BLCAP in the mouse neuroblastoma cell line cN2A. 9x c-Myc tagged BLCAP transiently transfected into the mouse neuroblastoma cell line N2A was detected by a 9E10 anti-c-Myc primary antibody from mouse. For visualization a goat anti-mouse secondary antibody was used coupled to the green fluorescence dye Alexa 488. To investigate if and how the expression of BLCAP changes during differentiation, the cells have been starved after transfection culturing them over night in DMEM medium containing no FCS. Also in the murine cell line N2A BLCAP shows an overall granular, cytoplasmic localization pattern. After inducing differentiation in the neuroblastoma cell line, the cells start forming growth cones. Upon differentiation BLCAP seems to stay within the cell body, though also a low level of BLCAP is found in growing extensions. No obvious differences in localization pattern of edited and onedited BLCA were detected. DAPI = nuclear staining; DIC = phase contrast; FITC = localization of BLCAP, Alexa 488; Scale bar: 20 µm.

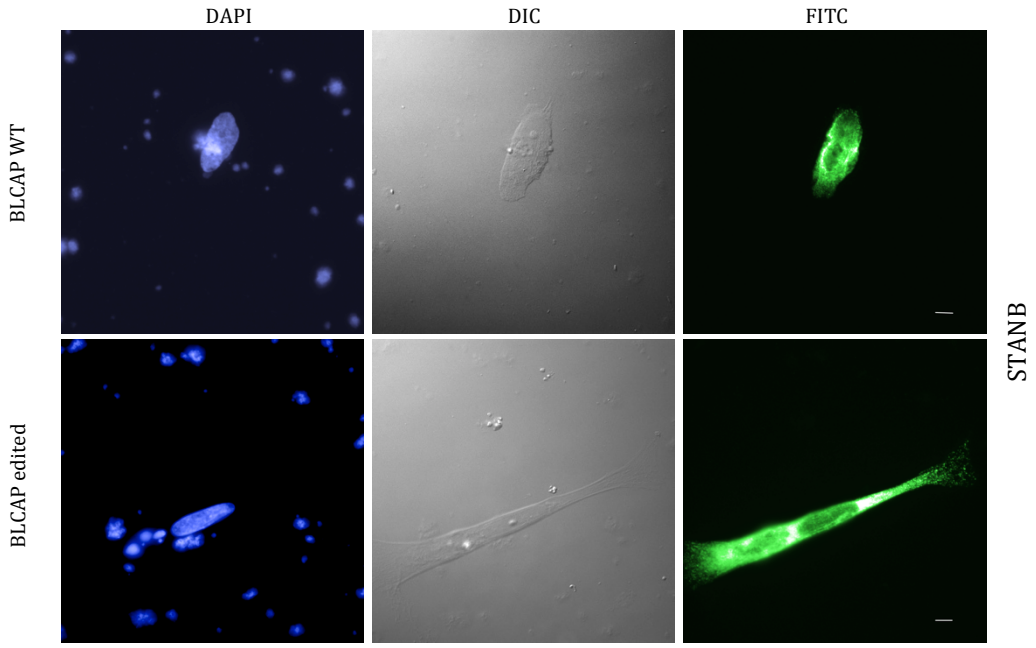


Figure 42) Transient Immunofluorescence staining of edited and unedited BLCAP in the human neuroblastoma cell line STANB (St. Anna Neuroblastoma). 9x c-Myc tagged BLCAP, transiently transfected into the human neuroblastoma cell line STANB, was detected by a 9E10 anti-c-Myc primary antibody from mouse. For visualization a goat anti-mouse secondary antibody was used, coupled to the green fluorescence dye Alexa 488. Also in the human neuroblastoma cell line BLCAP shows a cytoplasmic, granular localization, while no differences were detected in localization of edited and unedited BLCAP. DAPI = nuclear staining; DIC = phase contrast; FITC = localization of BLCAP, Alexa 488.

4.3.2. Stable expression of BLCAP: Immunofluorescence staining

After testing the BLCAP expression vectors on their transfection efficiency, the constructs were transfected into several mammalian cell lines for the generation of stable expressing clones. To select for stable random integration the cells were cultivated in DMEM medium containing 100 µg/ml to 300 µg/ml of the antibiotic G418 (Geneticin). After one week to ten days of selection stable expressing clones were picked and tested on their BLCAP-expression level by immunofluorescence staining using 9E10 anti-c-Myc primary antibody and goat anti-mouse secondary antibodies coupled to Alexa fluorescence dyes. Both, the edited and the unedited version of the BLCAP protein were successfully expressed at high levels in two mammalian cell lines, the human cell line HeLa, and the mouse neuroblastoma cell line cN2A. Resembling the results of transient transfection studies, BLCAP shows an overall granular localization pattern in stable expressing cell lines. Again no obvious differences were detected between edited and unedited versions of the protein.

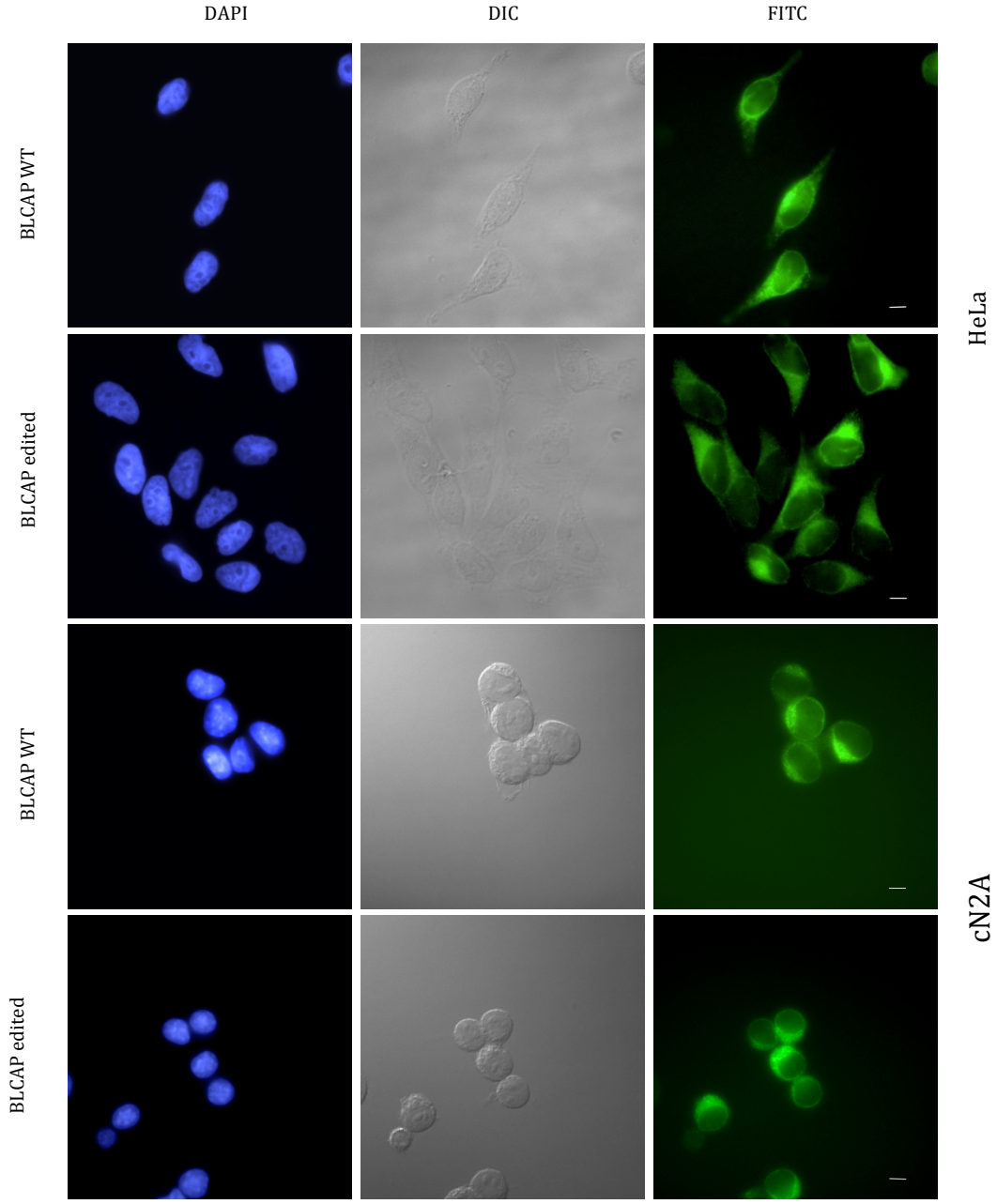


Figure 43) Stable expression of 9x c-Myc tagged BLCAP in the human cell line HeLa and the mouse neuroblastoma cell line cN2A. After selection for stable random integration of the transfected DNA construct, the picked clones were tested on their homogeneity of stable protein expression by immunofluorescence staining. C-Myc-tagged BLCAP was detected by an anti-MYC primary antibody and subsequent labelling with a goat anti-mouse secondary antibody coupled to Alexa 488. In both generated stable BLCAP expressing cell lines BLCAP was found to localise within the cytoplasm. Similar to observations from immunofluorescence staining assays of transiently transfected cell lines, a granular pattern of BLCAP localization was found in stable expressing cells. Additionally, no obvious differences in localization of edited and unedited BLCAP can be detected. BLCAP WT = unedited version of BLCAP; BLCAP edited = edited version of BLCAP. DAPI: nuclear staining. DIC: phase contrast. FITC: Alexa 488 fluorescence staining. Scale bar: 20 µm.

4.3.3. Stable expression of BLCAP: Western blot analysis

As nothing is known on the protein function and properties of BLCAP, Western blot analysis was done after lysing stable expressing cells in lysis buffers containing different reagents for solubilisation. The cells were lysed by NET-2, Tris-Buffer containing 0.1% of deoxycholic acid DOC and one Tris-buffer containing 0.1% of the detergent Triton X-100. Treating the cells with Tris-buffer containing Triton X-100 yielded high levels of BLCAP in solution after sonication. BLCAP was detected using a 9E10 anti c-Myc primary antibody and a goat anti-mouse secondary antibody coupled to alkaline phosphatase.

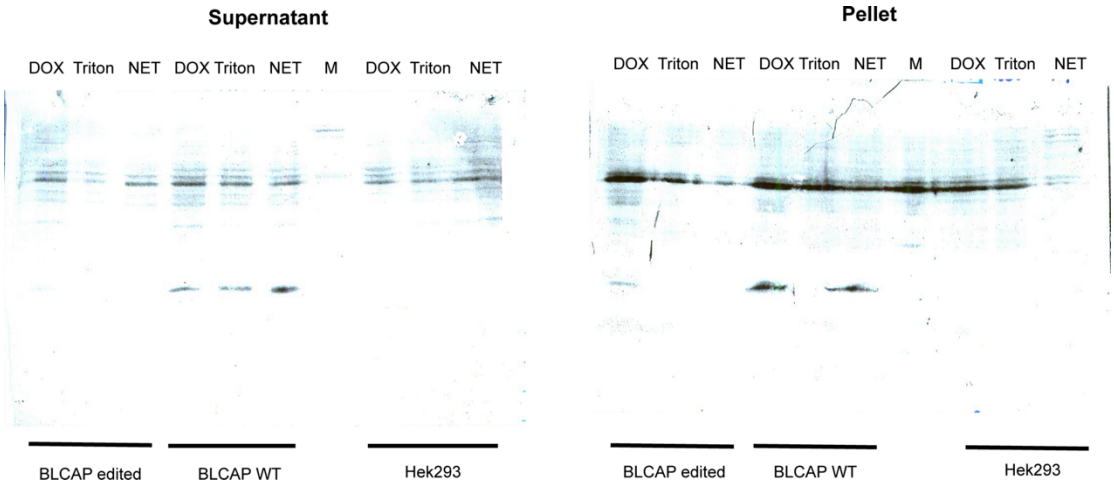
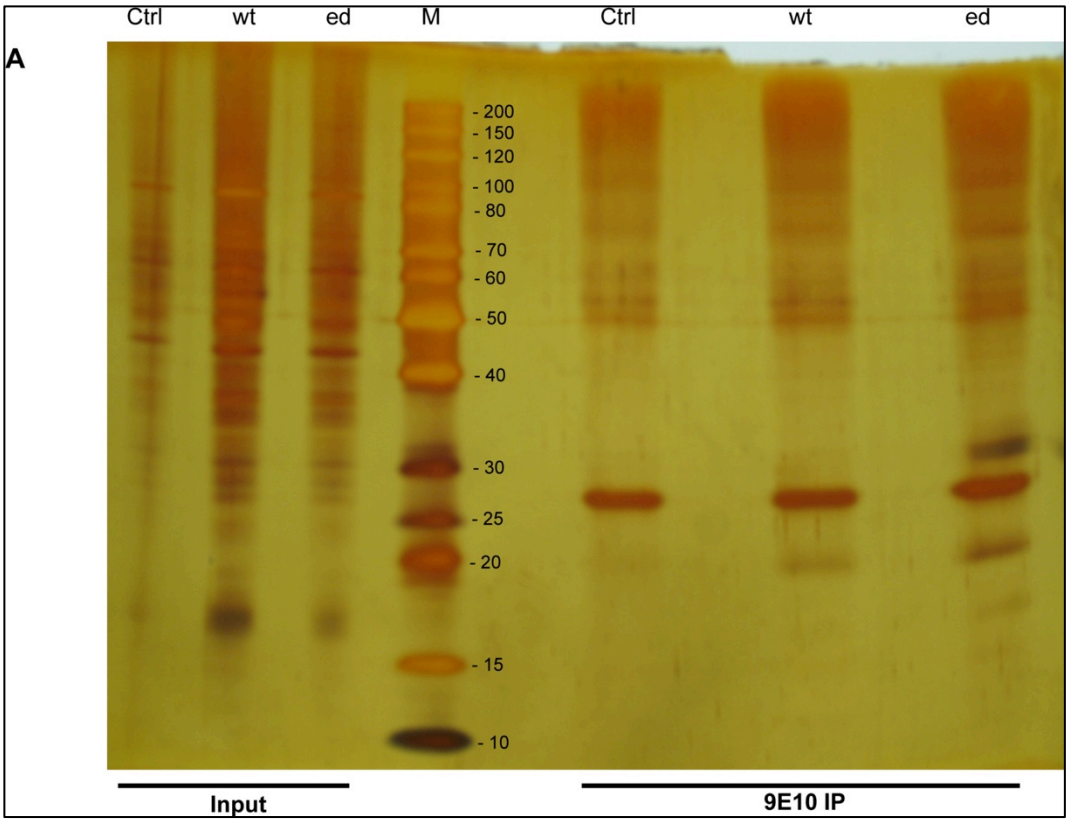


Figure 44) Western Blot: BLCAP expression in Hek293. Hek293 cells expressing edited and unedited BLCAP were lysed using buffers of different compositions. After lysing the cells, cell debris were precipitated by centrifugation. Samples from total lysate and pellet were analysed by Western blot analysis, using anti-c-Myc primary antibodies and secondary antibodies coupled to alkaline phosphatase. DOX = Tris buffer containing 0.1% DOX, Triton = Tris buffer containing 0.1% Triton X-100, NET = NET-2 lysis buffer containing 0.02% Tween-20. BLCAP edited = lysate of Hek293 cells expressing edited BLCAP, BLCAP WT = lysate of Hek293 cells expressing unedited BLCAP, Hek293 = lysate of empty Hek293 cells; M = PAGE Ruler pre-stained protein ladder.

This Western blot analysis shows, that most of the BLCAP protein is solubilized best after using a lysis buffer, containing 0.1% Triton X-100, as no protein was detected in the pellet.

4.3.4. Immunoprecipitation: BLCAP

After testing the obtained stably expressing clones on their BLCAP-expression level, the cells were expanded for BLCAP-purification assays. Edited and unedited BLCAP was precipitated using Sepharose A beads cross-linked with 9E10 anti-c-Myc antibody. Gained samples were analysed on 7.5% to 17% gradient SDS PAGE gels and subsequent silver staining. To date BLCAP-specific pulled down bands had been obtained by immunopreciptiation of BLCAP expressed in HeLa cells. Protein bands were cut out from silver gels and sent for mass spectrometric analysis.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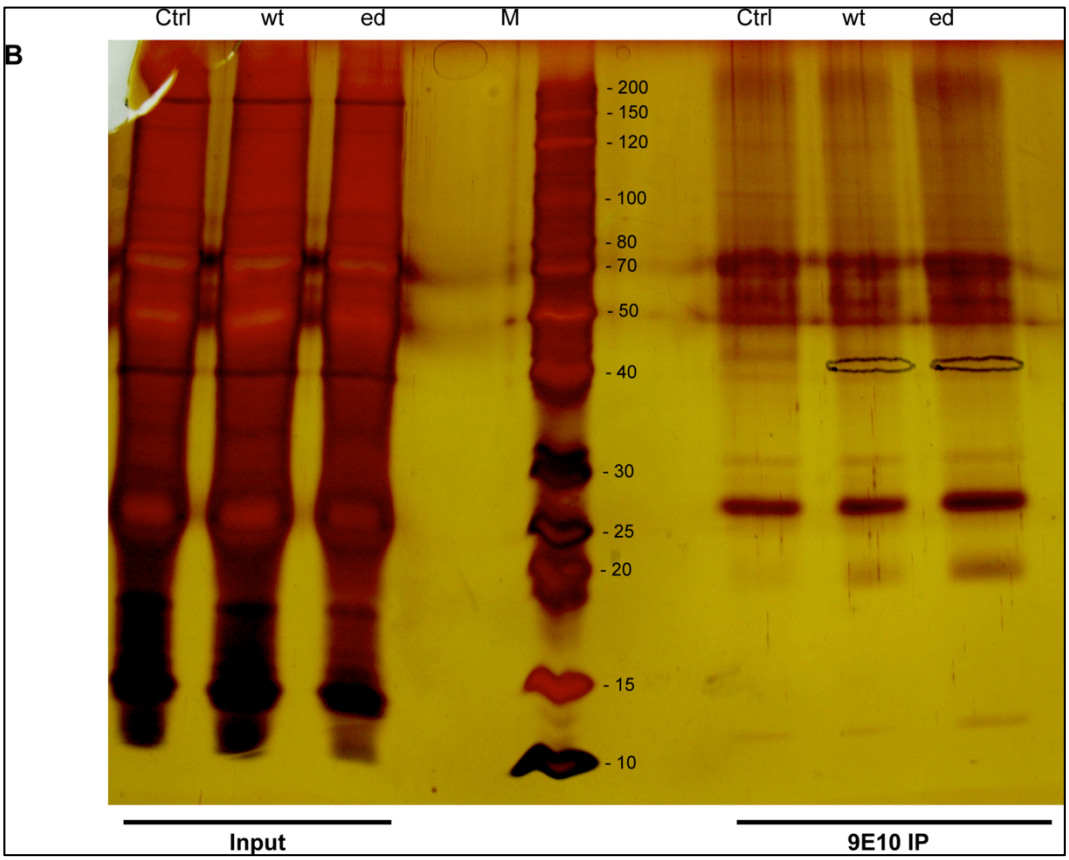
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9E10 IP

Figure 45) Silver gels: Immunoprecipitation of edited and unedited BLCAP from HeLa. Stable expressing HeLa cells were lysed using a Tris-lysis buffer containing 0.1% Triton X-100. 9E10 anti-c-Myc immuno-precipitation was done using sepharose A beads cross-linked to 9E10 antibodies. Samples were analysed on 7.5% to 17% gradient SDS PAGE gels and subsequent silver staining. BLCAP specific bands were cut out and sent for mass spectrometric analysis. A) anti-c-Myc immunoprecipitation of 9x c-Myc tagged edited and unedited BLCAP from HeLa cells. Black arrow indicates cut protein band of 32 kDa from edited BLCAP pull down. B) anti-c-Myc immunoprecipitation of 9x c-Myc immunoprecipitation of 9x c-Myc tagged edited and unedited BLCAP from HeLa cells. Black arrows indicate cut protein bands from edited and unedited BLCAP pull down at a molecular weight of 47 kDa. Black arrows mark protein bands sent for mass spectrometric analysis. Input = total cell lysate; 9E10 IP = anit-c-Myc immunoprecipitation; Ctrl = lysate of empty HeLa cells; wt = lysate of HeLa cells stably expressing unedited BLCAP; ed = lysate of HeLa cells stably expressing edited BLCAP; M = PAGE Ruler unstained protein ladder.

4.3.5. Mass spectrometry: BLCAP

Three BLCAP-specific protein bands have been pulled down by anti-c-Myc immunoprecipitation from BLCAP expressing HeLa cells. Mass spectrometry data of the gained BLCAP-interacting proteins is listed in the tables below. Little information about protein interactions of unedited BLCAP was gained from anti-c-Myc immunoprecipitation from HeLa cells. Nevertheless, some mitochondrial proteins were found to interact with edited BLCAP. Proteins, which interact only with BLCAP are highlighted in bold. Identified proteins, which give insights on BLCAP protein function are stained green.

Table 12) Proteins interacting with unedited BLCAP identified by mass spectrometry. Proteins, detected in protein bands after precipitation of unedited BLCAP from stable expressing HeLa cells are listed in this table. The identified proteins, the numbers of analysed peptides, the percentage of coverage with annotated sequences, the molecular weight of the identified protein and of the cut sample are shown. Due to a high level of sample contamination with keratin, no bands were found specifically interacting with unedited BLCAP. It cannot be excluded, that the found protein, suprabasin isoform, is also a contamination. Mw = molecular weight of identified protein in kDa. Sample = molecular weight of cut protein band.

Unedited BLCAP-interacting protein	unique peptides	percent coverage	Mw (kDa)	Sample: Mw (kDa)	comments	function	references
suprabasin isoform 1 precursor	2	6,10%	61	47	comes with DDX15	epidermal barrier function	(Toulza et al., 2007)

Table 13) Proteins interacting with edited BLCAP identified by mass spectrometry. Proteins, detected in protein bands after precipitation of edited BLCAP from stable expressing HeLa cells are listed in this table. The identified proteins, the numbers of analysed peptides, the percentage of coverage with annotated sequences, the molecular weight of the identified protein and of the cut sample are shown. Several mitochondrial proteins have been detected in this study by mass spectrometry. All mitochondrial proteins identified, match cut sample size and thus are stained in green. Proteins, which interact only with edited BLCAP, are highlighted in bold. Mw = molecular weight of identified protein in kDa. Sample = molecular weight of cut protein band.

Edited BLCAP-interacting protein	unique peptides	percent coverage	Mw (kDa)	Source: Mw (kDa)	comments	function	references
coiled-coil domain-containing protein 124	**8**	**30%**	**26**	**32**		**DNA-binding protein**	**uniprot-database Q96CT7**
polyubiquitin	**5**	**7,70%**	**68**	**32**		**posttranslational modification**	**(Komander et al., 2009)**
erlin-2 isoform 1	2	7,10%	38	47	also comes with Dss1	mediates ER-associated degradation of activated IP_3 receptors	(Yildirim et al., 2011)
7-de-hydro-cholesterol reductase 7	**2**	**3,80%**	**54**	**47**		**cholesterol metabolism**	**(Moebius et al., 1998)**
solute carrier family 25, member 5	**2**	**7,20%**	**28**	**32**		**ATP hydrolysis across mitochondrial membrane**	**(De Marcos Lousa et al., 2002)**
voltage-dependent anion channel 2	**2**	**7,80%**	**34**	**32**		**mitiochondrial anion channel**	**(Yu et al., 1995)**
mitochondrial glutamate carrier 1	**3**	**9,00%**	**34**	**32**		**mitochondrial glutamate transporter**	**(Fiermonte et al., 2002)**

4.3.6. BLCAP, a mitochondrial protein?

According to the gained information after mass spectrometric analysis of BLCAP interacting proteins, BLCAP seems to be a mitochondrial protein. To confirm the mass spectrometry data, co-staining assays were done, detecting c-Myc-tagged BLCAP and a mitochondrial marker, cytochrome c. U2OS cells were transiently transfected with constructs expressing edited and unedited BLCAP. Twenty four to forty eight hours later the cells were fixed and stained by immunofluorescence staining. The c-Myc tagged BLCAP versions were detected using a 9E10 anti-c-Myc primary antibody developed from rabbit. The anti-c-Myc primary antibody was visualized using a goat anti-rabbit secondary antibody coupled to the fluorescence dye Alexa 488. As a control for mitochondrial localization of BLCAP an antibody binding the mitochondrial marker protein cytochrome c, developed from mouse, was used for

co-staining assays. This mitochondrial marker was visualized using a goat anti-mouse secondary antibody coupled to the fluorescence dye Alexa 568. Microscopy pictures from BLCAP localization (FITC) and mitochondrial cytochrome c (TRITC) were merged using Photoshop CS5. These immunofluorescence-staining assays showed, that localization of BLCAP almost completely overlaps with mitochondrial markers as cytochrome c.

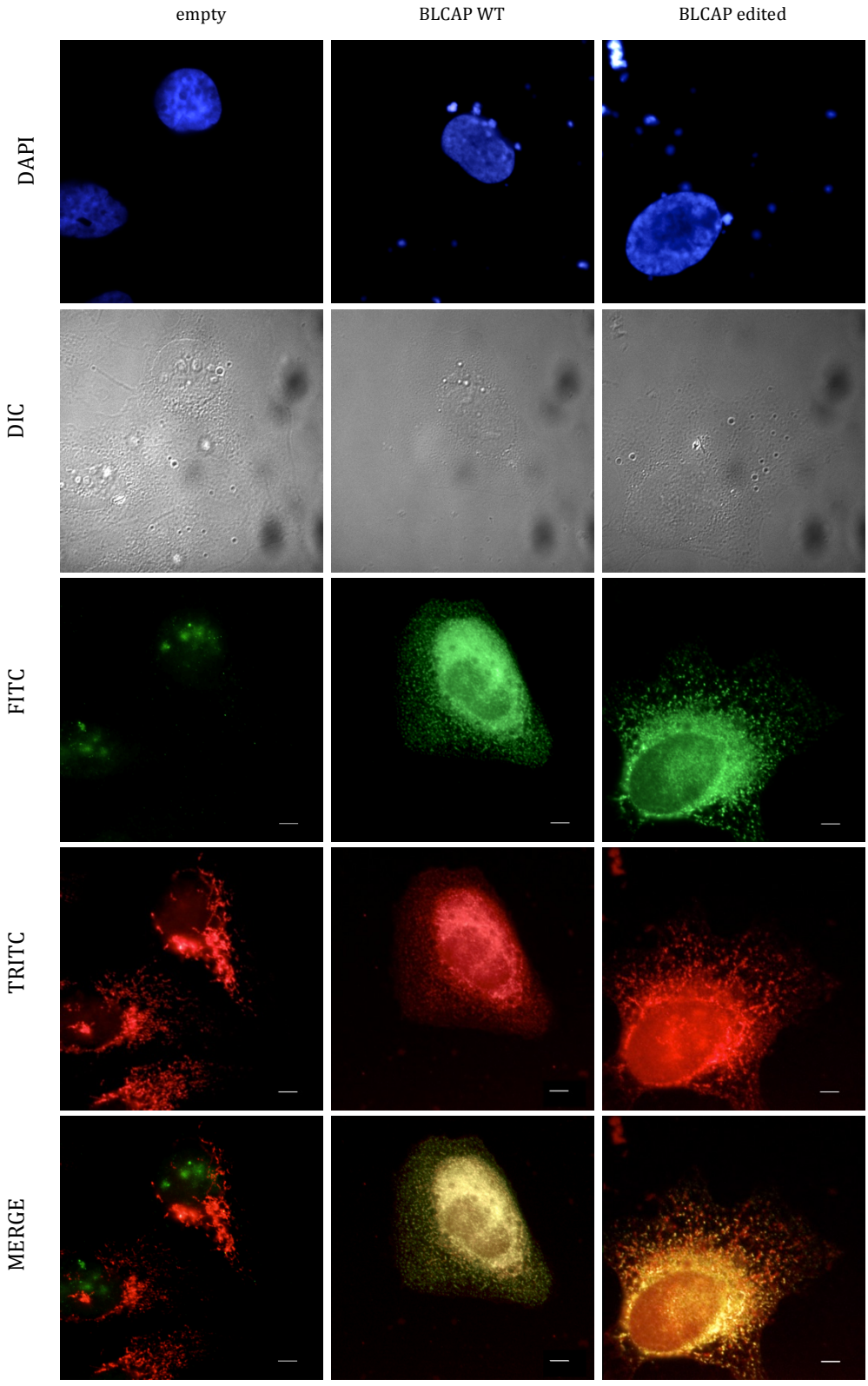

Figure 46) Transient co-staining: BLCAP vs. Cytochrome C. The human cell line U2OS was transiently transfected with edited and unedited BLCAP. C-Myc-tagged BLCAP was visualized using a 9E10 anti-c-Myc primary antibody from rabbit, detected by a goat anti-rabbit Alexa 488 coupled secondary antibody. Cytochrome

C, a mitochondrial marker protein, was detected using a goat anti-mouse antibody, coupled to Alexa 568. In untransfected U2OS cells a high nuclear background staining of the used goat anti-rabbid secondary antibody was detected in FITC. Edited and unedited BLCAP show a similar granular, cytoplasmic localization pattern (FITC). Additionally, staining of the mitochondrial marker, Cytochrome c, shows a similar granular localization. Thus, merging the FITC of BLCAP figures and the TRITC of mitochondria localization, yields pictures where an almost completely colocalization of BLCAP with mitochondria was observed. Empty = untransfected cell line; BLCAP WT = U2OS cells expressing transiently transfected unedited BLCAP; BLCAP edited = U2OS cells expressing transiently transfected edited BLCAP; DAPI = nuclear staining; DIC = phase contrast; FITC = Alexa 488 green fluorescence staining; TRITC = Alexa 568 red fluorescence staining; Scale bar = 10 μm.

5. Discussion

During this thesis we tried to establish stable cell lines expressing several proteins identified to be involved in RNA editing. These proteins can be separated into two groups. On the one hand we wanted to express regulators of ADAR activity, identified earlier in our lab by screening assays, in several mammalian cell lines. The second group of proteins involved in RNA editing, investigated in this thesis, were two protein-coding targets of ADAR, the cytoskeletal cross-linker Filamin A and the bladder cancer associated protein BLCAP. The aim of this project was to purify the listed proteins as complexes with interacting proteins by immunoprecipitation assays and Tandem Affinity Purification. Mass spectrometric analysis of these co-purified proteins should yield further insights into regulatory networks of RNA editing on the one hand. On the other hand, we wanted to explore effects of RNA editing on interaction profiles of the two investigated ADAR targets, Filmain A and BLCAP, as RNA editing leads to amino acid changes in their primary sequence.

The first aim of this thesis was to generate expression vectors containing a TAP-sequence for Tandem Affinity Purification, and an appropriate selection marker for stable expression of cloned, TAP-tagged inserts in mammalian cell lines. The candidate proteins for regulators of RNA editing were the small acidic protein DSS1, the RNA helicase DDX15, the ribosomal subunit RPS14 and the arginine/serine-rich splice factor SRSF9. Full-length versions of all four ADAR regulators were cloned into the TAP-expression vector to be C- and N-terminally tagged with a 6x c-Myc/TEV/HA tag. We obtained stable clones of HeLa cells and U2OS cells, expressing N-terminally tagged DSS1. These clones have shown adequate expression levels of DSS1 in immunofluorescence staining assays and Western blot analysis. Till now, we failed to express the C-terminally tagged version of DSS1 in any of the tested cell lines. Immunoprecipitaion and following silver staining yielded three DSS1-specific protein bands with a molecular weight of 30 kDa, 50 kDa and 67 kDa. DSS1 is a regulatory subunit of the 26S proteasome (Funakoshi et al., 2004; Josse et al., 2006; Wilmes et al., 2008). As expected, we could identify several proteasome subunits as interaction partners of DSS1. What was astonishing is, that according to our mass spectrometry data, DSS1 also interacts with several ribosomal proteins and

heterogenous ribonucleoprotein particles (hnRNPs). On trial, we tried to co-purify Dss1 with ribosomes in an immunoprecipitation assay, but so far without success. However, our data may provide another indication for DSS1 to be involved in RNA processing mechanisms, because other groups already have linked this multifunctional protein to mRNA export processes (Wilmes et al., 2008). By binding to hnRNPs, ribosomes and other RNA-processing factors, Dss1 might destabilize their binding to the target RNA, what in turn would allow or facilitate binding of ADAR2. Thus, our data from mass spectrometric analysis might give hints for the missing link in the role of Dss1 as an activator of ADAR2 activity.

The RNA helicase DDX15 was also stably expressed in the human cell line U2OS, as N-terminally TAP-tagged truncated version. But due to the low expression level of DDX15 in the obtained clone, no significant protein interactions have been identified after immunoprecipitation and following mass spectrometric analysis of gained protein bands. This lack in interaction partners may also be due to the truncation, as in the absence of nucleolar or nuclear localization peptide stretches, DDX15 may not localize correctly and thus a complete interaction profile cannot be provided. In the case of DDX15 we again failed to express the C-terminally tagged version of the protein in any of the tested cell lines, though both versions of the protein, the N- and C-terminally tagged construct, were successfully transfected transiently. Nevertheless, though we could not detect interaction partners of DDX15, that may explain its inhibitory action on ADAR2 activity, possibly just its RNA helicase activity may be a cause for a decrease in editing. Namely, if an RNA helicase, like DDX15, unwinds double-stranded RNA structures, ADAR2 may no longer be able to recognize its substrates.

Another candidate for an ADAR regulator was the ribosomal protein RPS14. In this case we successfully generated cell lines stably expressing the C- and the N-terminally tagged versions of the protein. Observations from immunofluorescence staining assays have shown that these two versions of RPS14 show a different subcellular localization pattern. N-terminally tagged RPS14 localizes mainly within the nucleoplasm of stable expressing HeLa cells, whereas the C-terminally tagged protein expressed in U2OS cells was detected in nucleoli. Initial purification studies were done by immunoprecipitation assays and Tandem Affinity Purification of N-terminally tagged RPS14 expressed in HeLa cells. Mass spectrometric analysis of the cut protein bands, at a molecular weight of 107 kDa and 52 kDa, yielded no valuable

information about RPS14 interacting proteins. No ribosomal proteins were detected in N-terminally tagged RPS14 precipitates, though it is known to be a ribosomal protein (Chen et al., 1986; Ferreira-Cerca et al., 2005; Rhoads et al., 1986). Nevertheless, we also did anti-c-Myc immunoprecipitation of C-terminally tagged RPS14 expressed in U2OS cells. In this round of purification of RPS14 we could obtain just very low amounts of protein after the samples were analysed by gradient SDS PAGE and silver staining. Just few proteins were detected in these samples by mass spectrometry. Though one identified interaction partner from RPS14 precipitation seems to be interesting: we detected the histone chaperone nucleophosmin. Beside its function in chromatin remodelling (Swaminathan et al., 2005), nucleophosmin is also known to be involved during nuclear export of ribosomes (Maggi et al., 2008). Another hint for nucleophosmin to be a real RPS14 interacting protein is, that its deregulation is involved in the development of several variants of leukemia. On the one hand, aberrant localization of nucleophosmin was detected in a high number of patients suffering from acute myelogenous leukemia (AML) and myeloid sarcoma (Bolli et al., 2006; Falini et al., 2005). In other studies a chromosomal translocation, fusing parts of nucleophosmin to the retinoic acid receptor alpha, was detected in samples from patients suffering from acute promyelocytic leukemia (APL) (Redner et al., 1996). Another chromosomal translocation was detected in non-Hodgkin's lymphoma, where nucleophosmin is fused to the gene of the tyrosine kinase ALK, what may have a strong impact on transformation of these lymphomas (Morris et al., 1994). What links these molecular causes for leukemic diseases to our project is, that another translocation of the nucleophosmin gene, encoded on chromosome 5, to chromosome 3, leading to a fusion of nucleophosmin with a gene named myelodysplasia/myeloid leukemia factor 1 (MLF1) was linked to the development and progression of the myelodysplastic syndrome and acute myeloid leukemia (Yoneda-Kato et al., 1996). As mentioned in the introduction, haploinsufficiency of RPS14 was identified to be a main cause for the development of the myelodysplastic syndrome or 5q- syndrome where parts of chromosome 5 are deleted (Ebert et al., 2008). However, we need to repeat this purification experiments to get more hints about interaction partners of RPS14 to declare its role during RNA editing.

The last putative candidate for an inhibitor of RNA editing was the SR-protein SRSF9. As mentioned earlier the full-length sequence of SRSF9 amplified from mouse cDNA was cloned into the generated TAP-expression vector to be C- and N-

terminally tagged. We failed to transfect the N-terminally tagged SRSF9 into mammalian cell lines in transient transfection tests, what may be due to cloning mistakes, leading to frame shifts. The C-terminally tagged version has shown a nuclear localization pattern in these tests. However, up to now we failed to stably express this version of SRSF9 in any of the tested cell lines.

In the second part of this thesis we tried to establish stable cell lines expressing two targets for RNA editing, Filamin A and BLCAP. By immunoprecipitation assays and Tandem Affinity Purification under native conditions we tried to detect differences in protein interactions due to the editing events.

Hek293 clones were obtained expressing edited and unedited versions of full-length Filamin A. Additionally we got clones of U2OS, HeLa and N2A cells, expressing either the edited or the unedited version of Filamin A. The method for purification of full-length Filamin A still has to be improved as high protein fragmentation and degradation was detected in samples obtained after anti-c-Myc precipitation.

In addition to the full-length versions of Filamin A we were provided with TAP-expression vectors encoding for truncated versions of Filamin A, comprising IgG-like repeats 20 to 24. We failed to conduct transient transfection studies and subsequent immunofluorescence staining with the constructs encoding for edited and unedited versions of N-terminally tagged Filamin A, as we were not able to detect the streptavidin binding protein tag. C-terminally tagged, edited and unedited Filamin A was successfully detected by anti-c-Myc antibodies. We also obtained two Hek293 clones stably expressing C-terminally tagged edited and unedited Filamin A. Tandem affinity purification of short Filamin A was conducted. But, though after the second immunoprecipitation, TEV-cleaved Filamin A was detected, no Filamin A-specific protein bands were found in these precipitations. Different interacting proteins of Filamin A may bind under different conditions. Thus, the use of other lysis buffers may yield specific protein bands after Filamin A TAP. However, immunoprecipitation assays were done using beads coupled to anti-c-Myc antibodies. Mass spectrometric analysis of proteins co-purified with Filamin A have shown, that both versions, the edited and the unedited Filamin A, interact with Filamin A, Filamin B and Filamin C and the histone core protein H1.3. In addition to that, unedited truncated Filamin A was found to interact with non-erythrocytic spectrin alpha, whereas edited Filamin A interacts with spectrin beta. To date, this purification protocol, that yielded the listed mass spectrometry data was not repeated, thus the

spectrin-interaction pattern of edited and unedited Filamin A still needs to be proved. Additionally, very few peptides have been detected in some of the cut gel bands. Due to a high level of keratin contamination, some of the Filamin A-interacting proteins may be masked by this contamination. Several other proteins have been found to be pulled down with edited Filamin A, like the band-6-protein, a component of desmosomes, and nuclear proteins, like Histone H2A and the PELOTA protein. This data was quite surprising, because just few cytoskeletal and cell signalling proteins have been pulled down with Filamin A in the initial rounds of purification assays. In contrast to these purifications, in subsequent immunoprecipitation assays, other lysis buffers have been used to purify Filamin A. In these runs, several proteins, which are known to be involved in cell adhesion and cell-cell interactions, like subunits of desmosomes, were found in samples from either edited of unedited short Filamin A. Interestingly, many nuclear proteins were found to interact with the C-terminal part of Filamin A. There is some evidence for a nuclear localization of Filamin A. In studies on human skin fibroblasts and HeLa cells, Filmain A was found in the nucleus, where it is involved in DNA damage response by interacting with the DNA repair protein BRCA2 (Yuan and Shen, 2001). Additionally, could play a role during transcriptional regulation of FOXC1, as it was found to translocate into the nucleus as a complex with PBX1 (Berry et al., 2005). Nevertheless, a convincing proof for a translocating activity of Filamin A into the nucleus, where it may influence nuclear specific processes, is still missing. Our data may provide another hint for subsequent studies on Filamin A function in the nucleus. Till now, the Filamin A purifications have not been repeated. Further purification studies on several other cell lines expressing Filamin A are necessary to verify the obtained interaction maps of edited an unedited Filamin.

The second protein-coding target of RNA editing, investigated in this project, is the bladder cancer associated protein, BLCAP. Very view is known about the function of this highly conserved protein. But, BLCAP is known to be edited at many positions (Galeano et al., 2010; Gromova et al., 2002; Levanon et al., 2005). During this work we successfully expressed one edited and one completely unedited version of BLCAP in the mammalian cell lines HeLa and N2A. First purification studies have been done on Hek293 clones also expressing both versions. So far, results after anti-c-Myc immunoprecipitation of BLCAP from HeLa cells were obtained. Our mass spectrometry data lists several mitochondrial proteins as interacting proteins of edited

BLCAP, the member 5 of the mitochondrial solute carrier family 25, the voltage-dependent anion channel 2 and the mitochondrial glutamate carrier 1. To proof the obtained data, we did co-staining assays on cells, transiently transfected with edited and unedited BLCAP. In these tests we found, that localization of BLCAP in the cell is associated with mitochondria as a nearly completely overlap of BLCAP with the mitochondrial marker cytochrome C was detected by immunofluorescence staining. Thus, we conclude that BLCAP is a mitochondrial protein.

In this thesis we successfully established stable cell lines expressing proteins involved in RNA editing. Data obtained from purification assays and subsequent mass spectrometric analysis provides some hints on the effect of editing on the two investigated proteins, Filamin A and BLCAP. Additionally DSS1, a putative activator of RNA editing, was found to interact with ribosomal proteins, what may be another link for a role of DSS1 as an activator of editing activity. However, we came up with several issues. To date, we still do not have stable clones, expressing the SR-protein, SRSF9, or the C-terminally tagged versions of Dss1 and DDX15, what may be due to cloning mistakes. Additionally, mass spectrometric analysis of pulled down proteins yielded high levels of keratin contamination in the samples. These high levels of keratin peptides may mask peptides of other interacting proteins. Thus, several control experiments need to be done to verify the obtained information on protein interactions of the investigated candidates.

6. Literature

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Appendix

Detailed Sequence information

pcDNA 3.1 (-) Hygromycin MYC-TEV-HA TAP vector:
TEV/HA sequence was cloned after 6xMYC tag by EcoRI, destroying the 5' restriction site after cloning. MYC-TEV-HA tag was cloned by PmeI.

GACGGATCGGGAGATCTCCCGATCCCCTATGGTGCACTCTCAGTACAATCTGCTCTGATGC
CGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCG
AGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGACAATTGCATGAAGAATCTGCTT
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TATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGA
GTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCG
CCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGA
CGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATA
TGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCC
AGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTAT
TACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACG
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CGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGT
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TGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTGGCTAGCGTTTAAAC
GGGCCCTCTAGACTCGAGGTCGACGGTATCGATCAGCCATGGAGCAAAAGCTCATTTCTG
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GGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAG
CTGATTTAACAAAAATTTAACGCGAATTAATTCTGTGGAATGTGTGTCAGTTAGGGTGTGG
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CTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAG
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TGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACC
GCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGG
CTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTAT
CGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGA
CGCCCAACCTGCCATCACGAGATTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTT
CGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGA
GTTCTTCGCCCACCCCAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGC
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CATCAATGTATCTTATCATGTCTGTATACCGTCGACCTCTAGCTAGAGCTTGGCGTAATCA
TGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAG
CCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTG
CGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAAT
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ATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCA
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ACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTT
ATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATC
CGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAAT
AGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTA
TGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTG
CAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGT
GTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGA
TGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGAC
CGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAA
AAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTT
GAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTC
ACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAG
GGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTAT
CAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATA
GGGGTTCCGCGCACATTTCCCCGAAAAGTG

6 x MYC HA PmeI restriction site

DSS1 C-terminally tagged in pcDNA 3.1 (-) Hygro/ MYC-TEV-HA: cloned by NheI/XhoI:

CTGGCTAGCATGTCAGAGAAAAAGCAGCCGGTAGACTTAGGTCTGTTAGAGGAAGACGAC
GAGTTTGAAGAGTTCCCTGCCGAAGACTGGGCTGGCTTAGATGAAGATGAAGATGCACAT
GTCTGGGAGGATAATTGGGATGATGACAATGTAGAGGATGACTTCTCTAATCAGTTACGA
GCTGAACTAGAGAAACATGGTTATAAGATGGAGACTTCAACTCGAGGTCGACGGTATCGA
TCAGCCATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTC
ATTTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAAT
GAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATT
TCTGAAGAGGACTTGAATGAAATGGAGAGCTTGGGCGACCTCACCATGGAGCAAAAGCTC
ATTTCTGAAGAGGACTTGAATTTAGAAAATCTCTATTTCCAGGGTTACCCATACGACGTCC
CAGACTACGCGGAATTCCAC

NheI restriction site XhoI restriction site DSS1 full-length sequence 6x MYC HA

DSS1 N-terminally tagged in pcDNA 3.1 (-) Hygromycin/ MYC-TEV-HA, cloned by BamHI:

GCGTTTAAACGGGCCCTCTAGACTCGAGGTCGACGGTATCGATCAGCCATGGAGCAAAAG
CTCATTTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTG
AATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCT
CATTTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAA
TGAAATGGAGAGCTTGGGCGACCTCACCATGGAGCAAAAGCTCATTTCTGAAGAGGACTT
GAATTTAGAAAATCTCTATTTCCAGGGTTACCCATACGACGTCCCAGACTACGCGGAATTC
CACCACACTGGACTAGTGGATCCAATGTCAGAGAAAAAGCAGCCGGTAGACTTAGGTCTG
TTAGAGGAAGACGACGAGTTTGAAGAGTTCCCTGCCGAAGACTGGGCTGGCTTAGATGAA
GATGAAGATGCACATGTCTGGGAGGATAATTGGGATGATGACAATGTAGAGGATGACTTC
TCTAATCAGTTACGAGCTGAACTAGAGAAACATGGTTATAAGATGGAGACTTCATAGGGA
TCCGAGCTCGGTACCAAGCTTAAGTTTAAAC

BamHI restriction site Dss1 full-length sequence 6x MYC HA

DDX15 C-terminally tagged in pcDNA 3.1 (-) Hygromycin/ MYC-TEV-HA, cloned by NheI, XhoI:

AAGCTGGCTAGCATGTCCAAGCGGCACCGGTTGGACCTAGGGGAGGATTACCCCTCTGGC
AAGAAGCGTGCGGGGACCGATGGGAAGGATCGAGATCGAGACCGGGATCGTGAAGATCG
GTCTAAAGATCGAGACCGAGAACGTGATAGAGGAGATAGAGAGCGAGAGAGGGAGAAA
GAAAAGGAGAAGGAGTTGCGAGCTTCAACAAATGCTATGCTTATCAGTGCTGGATTACCA
CCTTTGAAAGCTTCCCATTCAGCTCACTCAACCCACTCAGCACATTCAACGCATTCAACAC
ATTCTGCTCATTCAACGCATGCCGGACATGCAGGTCACACGTCACTTCCACAGTGCATTAA
TCCGTTCACCAACTTACCCCATACTCCTCGATACTATGATATTCTAAAGAAACGTCTTCAG
CTCCCTGTTTGGGAATACAAGGATAGGTTTACAGATATTCTGGTTAGACATCAGTCCTTTG
TACTGGTTTGGCTCGAGGTCGACGGTATCGATCAGCCATGGAGCAAAAGCTCATTTCTGA
AGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAATGG
AGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAG
AGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAATGGAG
AGCTTGGGCGACCTCACCATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATTTAGAA
AATCTCTATTTCCAGGGTTACCCATACGACGTCCCAGACTACGCGGAATTCCACC

NheI restriction site XhoI restriction site DDX15 N-terminal truncation 6x MYC HA

DDX15 N-terminally tagged in pcDNA 3.1 (-) Hygromycin/ MYC-TEV-HA, cloned by BamHI:

AAACGGGCCCTCTAGACTCGAGGTCGACGGTATCGATCAGCCATGGAGCAAAAGCTCATT
TCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAA

ATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCT
GAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAAT
GGAGAGCTTGGGCGACCTCACCATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATTT
AGAAAATCTCTATTTCCAGGGTTACCCATACGACGTCCCAGACTACGCGGAATTCCACCA
CACTGGACTAGTGGATCCAATGTCCAAGCGGCACCGGTTGGACCTAGGGGAGGATTACCC
CTCTGGCAAGAAGCGTGCGGGGACCGATGGGAAGGATCGAGATCGAGACCGGGATCGTG
AAGATCGGTCTAAAGATCGAGACCGAGAACGTGATAGAGGAGATAGAGAGCGAGAGAGG
GAGAAAGAAAAGGAGAAGGAGTTGCGAGCTTCAACAAATGCTATGCTTATCAGTGCTGG
ATTACCACCTTTGAAAGCTTCCCATTCAGCTCACTCAACCCACTCAGCACATTCAACGCAT
TCAACACATTCTGCTCATTCAACGCATGCCGGACATGCAGGTCACACGTCACTTCCACAGT
GCATTAATCCGTTCACCAACTTACCCCATACTCCTCGATACTATGATATTCTAAAGAAACG
TCTTCAGCTCCCTGTTTGGGAATACAAGGATAGGTTTACAGATATTCTGGTTAGACATCAG
TCCTTTGTACTGGTTTGGTGAGGATCCGAGCTCGGT

BamHI restriction site DDX15 N-terminal truncation 6x MYC HA

RPS14 C-terminally tagged in pcDNA 3.1 (-) Hygromycin/ MYC-TEV-HA, cloned by NheI, XhoI:

GGGAGACCCAAGCTGGCTAGCATGGCACCTCGAAAGGGGAAGGAAAAGAAGGAAGAAC
AGGTCATCAGCCTCGGACCTCAGGTGGCTGAAGGAGAGAATGTATTTGGTGTCTGCCATA
TCTTTGCATCCTTCAATGACACTTTTGTCCATGTCACTGATCTTTCTGGCAAGGAAACCATC
TGCCGTGTGACTGGTGGGATGAAGGTAAAGGCAGACCGAGATGAATCCTCACCATATGCT
GCTATGTTGGCTGCCCAGGATGTGGCCCAGAGGTGCAAGGAGCTGGGTATCACCGCCCTA
CACATCAAACTCCGGGCCACAGGAGGAAATAGGACCAAGACCCCTGGACCTGGGGCCCA
GTCGGCCCTCAGAGCCCTTGCCCGCTCGGGTATGAAGATCGGGCGGATTGAGGATGTCAC
CCCCATCCCCTCTGACAGCACTCGCAGGAAGGGGGGTCGCCGTGGTCGCCGTCTGCCTCG
AGGTCGACGGTATCGATCAGCCATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGA
AATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTC
TGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAAT
GGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAATGGAGAGCTTGGGCGACCTCAC
CATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATTTAGAAAATCTCTATTTCCAGGGT
TACCCATACGACGTCCCAGACTACGCGGAATTCCACCACACTGGACTAGTGGATCCGAGC
TCGGTACCAAGCTTAAGTTT

NheI restriction site XhoI restriction site RPS14 full-length sequence 6x MYC HA

RPS14 N-terminally tagged in pcDNA 3.1 (-) Hygromycin/ MYC-TEV-HA, cloned by BamHI:

GCCCTCTAGACTCGAGGTCGACGGTATCGATCAGCCATGGAGCAAAAGCTCATTTCTGAA
GAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAATGGA
GCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGA
GGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAATGGAGA
GCTTGGGCGACCTCACCATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATTTAGAAA
ATCTCTATTTCCAGGGTTACCCATACGACGTCCCAGACTACGCGGAATTCCACCACACTGG
ACTAGTGGATCCCATGGCACCTCGAAAGGGGAAGGAAAAGAAGGAAGAACAGGTCATCA
GCCTCGGACCTCAGGTGGCTGAAGGAGAGAATGTATTTGGTGTCTGCCATATCTTTGCATC
CTTCAATGACACTTTTGTCCATGTCACTGATCTTTCTGGCAAGGAAACCATCTGCCGTGTG
ACTGGTGGGATGAAGGTAAAGGCAGACCGAGATGAATCCTCACCATATGCTGCTATGTTG
GCTGCCCAGGATGTGGCCCAGAGGTGCAAGGAGCTGGGTATCACCGCCCTACACATCAAA
CTCCGGGCCACAGGAGGAAATAGGACCAAGACCCCTGGACCTGGGGCCCAGTCGGCCCTC
AGAGCCCTTGCCCGCTCGGGTATGAAGATCGGGCGGATTGAGGATGTCACCCCCATCCCC
TCTGACAGCACTCGCAGGAAGGGGGGTCGCCGTGGTCGCCGTCTGTGAGGATCCGAGCTC
GGTACCAAGCTTAAGTTTAAA

BamHI restriction site RPS14 full-length sequence 6x MYC HA

SRSF9 C-terminally tagged in pcDNA 3.1 (-) Hygromycin/ MYC-TEV-HA, cloned by NheI, XhoI:

AGCTGGCTAGCCATGTCGGGCTGGGCGGACGAGCGCGGCGGCGAGGGCGACGGGCGCAT
CTACGTGGGGAACCTTCCGACCGACGTGCGCGAGAAGGACTTGGAGGACCTGTTCTACAA
GTACGGCCGCATCCGCGAGATCGAGCTCAAGAACCGGCACGGCCTCGTGCCCTTCGCCTT
CGTGCGCTTCGAGGACCCCCGAGATGCAGAGGATGCTATTTATGGAAGAAATGGTTATGA
TTATGGCCAGTGTCGGCTTCGTGTGGAGTTCCCCAGGACTTATGGAGGTCGGGGTGGGTG
GCCCCGTGGTGGGAGGAATGGGCCTCCTACAAGAAGATCTGATTTCCGAGTTCTTGTTTCA
GGACTTCCTCCGTCAGGCAGCTGGCAGGACCTGAAGGATCACATGCGAGAAGCTGGGGAT
GTCTGTTATGCTGATGTGCAGAAGGATGGAGTGGGGATGGTCGAGTATCTCAGAAAAGAA
GACATGGAATATGCCCTGCGTAAACTGGATGACACCAAATTCCGCTCTCATGAGGGTGAA
ACTTCCTACATCCGAGTTTATCCTGAGAGAAGCACCAGCTATGGCTACTCACGGTCTCGGT
CTGGGTCAAGGGGCCGTGACTCTCCATACCAAAGCAGGGGTTCCCCACACTACTTCTCTCC
TTTCAGGCCCTACCCTCGAGGTCGACGGTATCGATCAGCCATGGAGCAAAAGCTCATTTCT
GAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAAT
GGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGA
AGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAATGG
AGAGCTTGGGCGACCTCACCATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATTTAG
AAAATCTCTATTTCCAGGGTTACCCATACGACGTCCCAGACTACGCGGAATTCCACCACAC
TGGA

NheI restriction site XhoI restriction site SRSF9 full-length sequence 6x MYC HA

SRSF9 N-terminally tagged in pcDNA 3.1 (-) Hygromycin/ MYC-TEV-HA, cloned by BamHI:

AAACGGGCCCTCTAGACTCGAGGTCGACGGTATCGATCAGCCATGGAGCAAAAGCTCATT
TCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAA
ATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCT
GAAGAGGACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAAT
GGAGAGCTTGGGCGACCTCACCATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATTT
AGAAAATCTCTATTTCCAGGGTTACCCATACGACGTCCCAGACTACGCGGAATTCCACCA
CACTGGACTAGTGGATCCCATGTCGGGCTGGGCGGACGAGCGCGGCGGCGAGGGCGACG
GGCGCATCTACGTGGGGAACCTTCCGACCGACGTGCGCGAGAAGGACTTGGAGGACCTGT
TCTACAAGTACGGCCGCATCCGCGAGATCGAGCTCAAGAACCGGCACGGCCTCGTGCCCT
TCGCCTTCGTGCGCTTCGAGGACCCCCGAGATGCAGAGGATGCTATTTATGGAAGAAATG
GTTATGATTATGGCCAGTGTCGGCTTCGTGTGGAGTTCCCCAGGACTTATGGAGGTCGGGG
TGGGTGGCCCCGTGGTGGGAGGAATGGGCCTCCTACAAGAAGATCTGATTTCCGAGTTCT
TGTTTCAGGACTTCCTCCGTCAGGCAGCTGGCAGGACCTGAAGGATCACATGCGAGAAGC
TGGGGATGTCTGTTATGCTGATGTGCAGAAGGATGGAGTGGGGATGGTCGAGTATCTCAG
AAAAGAAGACATGGAATATGCCCTGCGTAAACTGGATGACACCAAATTCCGCTCTCATGA
GGGTGAAACTTCCTACATCCGAGTTTATCCTGAGAGAAGCACCAGCTATGGCTACTCACG
GTCTCGGTCTGGGTCAAGGGGCCGTGACTCTCCATACCAAAGCAGGGGTTCCCCACACTA
CTTCTCTCCTTTCAGGCCCTACTGAGGATCCGAGCTCGGTACCAAGCTTAAGTTTAAA

BamHI restriction site SRSF9 full-length sequence 6x MYC HA

FLNA in Expression vector pCeMM-CTAP(GS)

Start codon
Protein G
TEV cleavage site

SBP
myc
Restriction sites
Insert (FLNA)

atggaattcggtttaaaccaggatatgacagcccaggtgaccagcccatcgggcaagacc
M E F G L N Q D M T A Q V T S P S G K T
catgaggccgagatcgtggaaggggagaaccacacctactgcatccgctttgttcccgct
H E A E I V E G E N H T Y C I R F V P A
gagatgggcacacacacagtcagcgtgaagtacaagggccagcacgtgcctgggagcccc
E M G T H T V S V K Y K G Q H V P G S P
ttccagttcaccgtggggcccctaggggaaggggagcccacaaggtccgagctgggggc
F Q F T V G P L G E G G A H K V R A G G
cctggcctggagagagctgaagctggagtgccagccgaattcagtatctggacccgggaa
P G L E R A E A G V P A E F S I W T R E
gctggtgctggaggcctggccattgctgtcgagggccccagcaaggctgagatctctttt
A G A G G L A I A V E G P S K A E I S F
gaggaccgcaaggacggctcctgtggtgtggcttatgtggtccaggagccaggtgactac
E D R K D G S C G V A Y V V Q E P G D Y
gaagtctcagtcaagttcaacgaggaacacattcccgacagccccttcgtggtgcctgtg
E V S V K F N E E H I P D S P F V V P V
gcttctccgtctggcgacgcccgccgcctcactgtttctagccttcA/Ggagtcagggcta
A S P S G D A R R L T V S S L Q/R E S G L
aaggtcaaccagccagcctcttttgcagtcagcctgaacggggccaaggggggcgatcgat
K V N Q P A S F A V S L N G A K G A I D
gccaaggtgcacagcccctcaggagccctggaggagtgctatgtcacagaaattgaccaa
A K V H S P S G A L E E C Y V T E I D Q
gataagtatgctgtgcgcttcatccctcgggagaatggcgtttacctgattgacgtcaag
D K Y A V R F I P R E N G V Y L I D V K
ttcaacggcacccacatccctggaagccccttcaagatccgagttggggagcctgggcat
F N G T H I P G S P F K I R V G E P G H
ggaggggacccaggcttggtgtctgcttacggagcaggtctggaaggcggtgtcacaggg
G G D P G L V S A Y G A G L E G G V T G
aacccagctgagttcgtcgtgaacacgagcaatgcgggagctggtgccctgtcggtgacc
N P A E F V V N T S N A G A G A L S V T
attgacggcccctccaaggtgaagatggattgccaggagtgccctgagggctaccgcgtc
I D G P S K V K M D C Q E C P E G Y R V
acctatacccccatggcacctggcagctacctcatctccatcaagtacggcggcccctac
T Y T P M A P G S Y L I S I K Y G G P Y
cacattgggggcagcccccttcaaggccaaagtcacaggcccccgtctcgtcagcaaccac
H I G G S P F K A K V T G P R L V S N H
agcctccacgagacatcatcagtgtttgtagactctctgaccaaggccacctgtgccccc
S L H E T S S V F V D S L T K A T C A P
cagcatggggccccgggtcctgggcctgctgacgccagcaaggtggtggccaagggcctg
Q H G A P G P G P A D A S K V V A K G L
gggctgagcaaggcctacgtaggccagaagagcagcttcacagtagactgcagcaaagca
G L S K A Y V G Q K S S F T V D C S K A
ggcaacaacatgctgctggtgggggttcatggcccaaggacccccctgcgaggagatcctg
G N N M L L V G V H G P R T P C E E I L
gtgaagcacgtgggcagccggctctacagcgtgtcctacctgctcaaggacaaggggggag
V K H V G S R L Y S V S Y L L K D K G E
tacacactggtggtcaaatgggggggacgagcacatcccaggcagccccctaccgcgttgtg
Y T L V V V K W G D E H I P G S P Y R V V
gtgccctggccggccgagcagaagcttatctccgaggaggatctcatggacgagaagacc
V P W P A E Q K L I S E E D L M D E K T
accgggtggcggggcggccacgttgtggagggtctcgctggcgagctggagcagctcagg
T G W R G G H V V E G L A G E L E Q L R
gcccgcttggagcaccatccccaggggcaacgcgagcctatcgatgaaaatctgtacttc
A R L E H H P Q G Q R E P I D E N L Y F
cagggggatcgatgagaacctgtactttcagggggtagtaccccggccgtgacgacctac

Q G I D E N L Y F Q G G S T P A V T T Y
aagctggtgatcaacggcaagaccctgaagggcgagaccacaaccaaggccgtggacgct
K L V I N G K T L K G E T T T K A V D A
gaaaccgcagagaaggccttcaagcagtacgccaacgacaatggcgtggacggagtgtgg
E T A E K A F K Q Y A N D N G V D G V W
acctacgacgatgccaccaagaccttcaccgtgaccgaggttaacacccccgcagtcacc
T Y D D A T K T F T V T E V N T P A V T
acatacaaactggtgatcaacggaaaaacgctgaagggcgaaactacgaccaaggctgtg
T Y K L V I N G K T L K G E T T T K A V
gacgcggagacggccgaaaaggcgttcaaacagtatgccaacgataacggtgtagacggc
D A E T A E K A F K Q Y A N D N G V D G
gtctggacatatgacgacgcgacaaagactttcactgtcactgaatagctcga
V W T Y D D A T K T F T V T E -

601 gggccggcgc gccgcggccg ctacgtaaat tccgcccctc tccctcccc ccccctaacg
661 ttactggccg aagccgcttg gaataaggcc ggtgtgcgtt tgtctatatg ttattttcca
721 ccatattgcc gtcttttggc aatgtgaggg cccggaaacc tggccctgtc ttcttgacga
781 gcattcctag gggtctttcc cctctcgcca aaggaatgca aggtctgttg aatgtcgtga
841 aggaagcagt tcctctggaa gcttcttgaa gacaaacaac gtctgtagcg acccttttgca
901 ggcagcggaa ccccccacct ggcgacaggt gcctctgcgg ccaaaagcca cgtgtataag
961 atacacctgc aaaggcggca caaccccagt gccacgttgt gagttggata gttgtggaaa
1021 gagtcaaatg gctctcctca agcgtattca acaaggggct gaaggatgcc cagaaggtac
1081 cccattgtat gggatctgat ctggggcctc ggtgcacatg ctttacatgt gtttagtcga
1141 ggttaaaaaa cgtctaggcc ccccgaacca cggggacgtg gttttccttt gaaaaacacg
1201 atgataatat ggccacaacc atggtgagca agggcgagga gctgttcacc ggggtggtgc
1261 ccatcctggt cgagctggac ggcgacgtga acggccacaa gttcagcgtg tccggcgagg
1321 gcgagggcga tgccacctac ggcaagctga ccctgaagtt catctgcacc accggcaagc
1381 tgcccgtgcc ctggcccacc ctcgtgacca ccctgaccta cggcgtgcag tgcttcagcc
1441 gctaccccga ccacatgaag cagcacgact tcttcaagtc cgccatgccc gaaggctacg
1501 tccaggagcg caccatcttc ttcaaggacg acggcaacta caagacccgc gccgaggtga
1561 agttcgaggg cgacaccctg gtgaaccgca tcgagctgaa gggcatcgac ttcaaggagg
1621 acggcaacat cctggggcac aagctggagt acaactacaa cagccacaac gtctatatca
1681 tggccgacaa gcagaagaac ggcatcaagg tgaacttcaa gatccgccac aacatcgagg
1741 acggcagcgt gcagctcgcc gaccactacc agcagaacac ccccatcggc gacggccccg
1801 tgctgctgcc cgacaaccac tacctgagca cccagtccgc cctgagcaaa gaccccaacg
1861 agaagcgcga tcacatggtc ctgctggagt tcgtgaccgc cgccgggatc actcacggca
1921 tggacgagct gtacaagtaa agcgccagca cagtggtcga gatccggatt agtccaattt
1981 gttaaagaca ggatatcagt ggtccaggct ctagttttga ctcaacaata tcaccagctg
2041 aagcctatag agtacgagcc atagataaaa taaaagattt tatttagtct ccagaaaaag
2101 gggggaatga aagaccccac ctgtaggttt ggcaagctag cttaagtaac gccattttgc
2161 aaggcatgga aaaatacata actgagaata gagaagttca gatcaaggtc aggaacagat
2221 ggaacagctg aatatgggcc aaacaggata tctgtggtaa gcagttcctg ccccggctca
2281 gggccaagaa cagatggaac agctgaatat gggccaaaca ggatatctgt ggtaagcagt
2341 tcctgccccg gctcagggcc aagaacagat ggtccccaga tgcggtccag ccctcagcag
2401 tttctagaga accatcagat gtttccaggg tgccccaagg acctgaaatg accctgtgcc
2461 ttatttgaac taaccaatca gttcgcttct cgcttctgtt cgcgcgcttc tgctccccga
2521 gctcaataaa agagcccaca acccctcact cggggcgcca gtcctccgat tgactgagtc
2581 gcccgggtac ccgtgtatcc aataaaccct cttgcagttg catccgactt gtggtctcgc
2641 tgttccttgg gagggtctcc tctgagtgat tgactacccg tcagcggggg tctttcacac
2701 atgcagcatg tatcaaaatt aatttggttt tttttcttaa gtatttacat taaatggcca
2761 tagtattgaa aaaggaagag tatgagtatt caacatttcc gtgtcgccct tattcccttt
2821 tttgcggcat tttgccttcc tgtttttgct cacccagaaa cgctggtgaa agtaaaagat
2881 gctgaagatc agttgggtgc acgagtgggt tacatcgaac tggatctcaa cagcggtaag
2941 atccttgaga gttttcgccc cgaagaacgt tttccaatga tgagcacttt taaagttctg
3001 ctatgtggcg cggtattatc ccgtattgac gccgggcaag agcaactcgg tcgccgcata
3061 cactattctc agaatgactt ggttgagtac tcaccagtca cagaaaagca tcttacggat
3121 ggcatgacag taagagaatt atgcagtgct gccataacca tgagtgataa cactgcggcc
3181 aacttacttc tgacaacgat cggaggaccg aaggagctaa ccgctttttt gcacaacatg
3241 ggggatcatg taactcgcct tgatcgttgg gaaccggagc tgaatgaagc cataccaaac
3301 gacgagcgtg acaccacgat gcctgtagca atggcaacaa cgttgcgcaa actattaact
3361 ggcgaactac ttactctagc ttcccggcaa caattaatag actggatgga ggcggataaa

3421gttgcaggac cacttctgcg ctcggccctt ccggctggct ggtttattgc tgataaatct
3481ggagccggtg agcgtgggtc tcgcggtatc attgcagcac tggggccaga tggtaagccc
3541tcccgtatcg tagttatcta cacgacgggg agtcaggcaa ctatggatga acgaaataga
3601cagatcgctg agataggtgc ctcactgatt aagcattggt aactgtcaga ccaagtttac
3661tcatatatac tttagattga tttaaaactt cattttaat ttaaaaggat ctaggtgaag
3721atccttttg ataatctcat gaccaaaatc ccttaacgtg agttttcgtt ccactgagcg
3781tcagaccccg tagaaaagat caaaggatct tcttgagatc ctttttttct gcgcgtaatc
3841tgctgcttgc aaacaaaaaa accaccgcta ccagcggtgg tttgtttgcc ggatcaagag
3901ctaccaactc tttttccgaa ggtaactggc ttcagcagag cgcagatacc aaatactgtc
3961cttctagtgt agccgtagtt aggccaccac ttcaagaact ctgtagcacc gcctacatac
4021ctcgctctgc taatcctgtt accagtggct gctgccagtg gcgataagtc gtgtcttacc
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ORIGIN
Start codon
Protein G
TEV cleavage site
SBP
Restriction sites
Insert (FLNA)

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Wahlbeispiel Zellbiologie
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R&D, Department of Pharmacology
Betreuung durch Frank Hilber, PhD

Wahlbeispiel Molekulare Medizin
Institut für Pharmakologie, Medizinische Universität Wien
Arbeitsgruppe von Dr. Michael Freissmuth

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Standard molekularbiologische und biochemische Methoden, inklusive Zellkultur

- ***Sonstige Kenntnisse***

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