DISSERTATION

Titel der Dissertation

Identification of enhancers of A to I editing in functional *in vivo* yeast screen

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Verfasser: **Wojciech Garncarz**

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Betreuer: **Ao. Univ.-Prof. Dr. Michael F. Jantsch**

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Abstract

Adenosine to inosine editing of RNA is a widespread posttranscriptional mechanism increasing the variety of the transcriptome in metazoa. Adenosine deamination is performed by adenosine deaminases that act on RNA (ADARs). A to I editing can change codons or splice patterns and lead to a higher diversity in protein isoforms. Additionally ADARs play an important role in miRNA biogenesis, stability and choice of their silencing target.

Most known coding editing substrates are the transcripts of proteins expressed in the central nervous system like: glutamate receptors, serotonin 2C receptor or gamma-aminobutyric acid type A receptor. Not surprisingly, changes in editing patterns at some of these sites are linked to neurodegenerative diseases like: schizophrenia, depression or ALS (amyotrophic lateral sclerosis) and also to brain tumours. It is important to learn how the activity of ADARs is controlled in attempt to cure some of these diseases.

Although much is known about function and activity of the enzymes, regulation of A to I editing is not well understood so far. Some ADAR isoforms are regulated at the transcriptional level but an increasing amount of evidence suggests other mechanisms of regulation in addition.

The aim of this thesis was answering some of the open questions concerning the regulation of ADAR activity. A functional yeast *in vivo* editing system was developed and successfully employed in identification of cellular enhancers of RNA editing. In this screen the protein DSS1 could be identified as strong enhancer of editing. The properities of this protein were confirmed in the mammalian context on various editing substrates proving the functionality of the developed screening system.

Zusammenfassung

Adenosin Deaminierung von Ribonukleinsäure (RNA) ist ein weitverbreiteter Mechanismus auf posttranskriptioneller Ebene, der zu einer massiven Zunahme der Variabilität des Metazoentranskriptoms führt. Die A zu I Editierung wird von sogenannte Adenosin Deaminasen (ADARs = adenosine deaminases that act on RNA) durchgeführt. Diese Deaminierungen führen zu Veränderungen von Kodons und Spleißmustern und bewirken dadurch eine Steigerung der Zahl von Protein-Isoformen. Zusätzlich spielen ADARs eine wichtige Rolle in der Biogenese, Stabilität und der Zielmolekülauswahl von mikroRNA (miRNA) Molekülen.

Die meisten bekannten kodierenden Substrate für ADARs sind Transkripte von Proteingenen, die im Zentralnervensystem exprimiert werden. Darunter fallen z.B.: Glutamatrezeptoren, der Serotonin 2C Rezeptor oder Gamma-Aminobutiryl-Säure Typ A Rezeptor. Nicht überraschend sind Veränderungen im Editierungsmuster einiger dieser Zielsequenzen mit neurodegenerativen Erkrankungen verbunden, wie z.B. Depression, Schizophrenie oder ALS (Amyotrophe laterale Sklerose) sowie Hirntumoren. Daher ist es entscheidend mehr über die Aktivitätskontrolle von ADARs zu erfahren, um etwaige Therapieansätze zu entwickeln.

Die Funktionen und Aktivitäten von Deaminasen sind sehr gut bekannt, Mechanismen der Regulation von A zu I Editierung sind bisher jedoch noch sehr wenig untersucht. Die Regulation einiger ADAR Isoformen passiert auf transkriptioneller Ebene, es gibt aber zunehmend Hinweise darauf, dass es noch weitere Regulationsmechanismen gibt. Die Aufklärung einiger solcher Regulationsmechanismen war das Hautpziel dieser Dissertationsarbeit.

Zu diesem Zweck wurde ein *in vivo* Editing System in Hefe entwickelt, mit dessen Hilfe es möglich war, zelluläre Verstärkermoleküle im RNA-Editierungsprozess zu identifizieren. Das Protein DSS1 wurde dabei als besonders aktiver Verstärker isoliert. Die Eigenschaften von DSS1 wurden anschließend in einem Expressionssystem im Säugerhintergrund an unterschiedlichen Editierungssubstraten bestätigt, wodurch auch die Funktionalität des neu entwickelten Screening Systems bewiesen werden konnte.

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1 Introduction

1.1 Eucaryotic RNA processing

The beginning of the new millennium has coincided with the completion of the human genome sequence, an event that brought a very surprising outcome. Suddenly a human genome of 100.000 predicted genes shrunk to only to 23.000 confirmed ones (Venter, Adams et al. 2001; 2004). It brought down the amount of protein coding fragments of the genome to 1.5% of its size and rose a few fundamental questions. How is the complexity of the human organism achieved with such a small number of protein coding genes? What makes such a significant differences between organisms if the difference in number of protein-coding genes is not enough to create it? These questions have opened the door for many scientist and many already old but neglected findings to surface. At last the central dogma of genetics stating: one gene = one polypeptide has fallen. The eyes of the scientist worldwide has turned towards non-coding RNAs and RNA modifications as the true agents behind the complexity of the human transcriptome and proteome.

In contrary to prokaryotic messenger RNA eukaryotic mRNA is transcribed from genomic DNA as an unfinished product. It has to undergo three main processes before it can be translated into a protein, which are; 5'-capping, 3' polyadenylation, and RNA splicing. Already these processes being studied in the late 60s and 70s of the last century gave us insights on how a diversity in the transcriptome can be introduced. RNA splicing is the most remarkable of these RNA processing mechanisms discovered already 30 years ago (Chow, Gelinas et al. 1977). It occurs in all eukaryotic pre-mRNA containing coding regions (exons) and non-coding (introns) which are removed during the splicing process. Not every intron is spliced-out with the same efficiency and additionally many times also the exons are being cut out along with them. These occurrences introduce diversity among proteins. In addition to alternative splicing, various promoters and polyadenylation sites generate diversity in protein expression. Moreover, different kinds of single nucleotide modifications, and RNA editing contribute to protein diversity (Karijolich, Kantartzis et al. 2010).

1.2 RNA modifications

Beside the well known and understood post-transcriptional RNA modifications like 7-methylguanosine (m7G) cap and poly-adenosine tail additions to 5' and 3' ends of mRNA,

respectively, or CCA trinucleotide addition to tRNA there are many other chemical nucleotide modifying processes. These kinds of RNA modifications are widespread, very common in cellular RNA and have been known already for decades.

Over 100 different RNA modifications have been characterized to date with the largest number of them found in tRNA, followed by rRNA (Grosjean, Auxilien et al. 1996). A few main characteristics of these modifications are interesting and striking. They are introduced in a very site-specific manner and are widely conserved across phylogenetic lineages. Even 3-10% of all proteins spanning from bacteria to higher eukaryotes are devoted to RNA modifications what would highly suggest an important function of single one of them (Anantharaman, Koonin et al. 2002; Ferre-D'Amare 2003).

RNA particles containing the most nucleotide modifications are tRNAs, where up to 80 different types of modifications affect 25% of all nucleotides. Most abundant are; dihydrouridine (D) and pseudouridine (Ψ) with secondary structures like D loop and TΨC loop named after them respectively (Bjork, Ericson et al. 1987). Most of the modifications present in rRNA particles are methylations at over 100 sites in higher vertebrates, from which 80% are 2´-O-ribose methylations. Additionally, up to 100 uridines in human pre-rRNA are converted into pseudouridines (Ψ). Interesting is that in case of rRNA all modifications are performed by the RNA-guided nucleotide modification mechanism, where snoRNAs (small nucleolar RNAs) containing segments precisely complementary to conserved regions in rRNA. The snoRNAs guide proteins, that are the second constituent of the snoRNP particle, performing the modification reaction (Decatur and Fournier 2003).

1.3   RNA editing

Another posttranscriptional RNA modification is RNA editing. RNA editing alters information transcribed from DNA via a more or less precise change in a single or several nucleotides within an RNA molecule. It is widespread in all eukaryotic organisms and depending on the organism it involves different molecular mechanisms like: insertion or deletion of nucleotides or base modification. Some of the RNA editing function as a repair or correction mechanism of the information encoded in DNA and some simply introduce additional diversity to the pool of the proteins possibly offering organisms a higher complexity. RNA editing may alter codon information, introduce or destroy a stop codon, or

change a translation product of the mature mRNA. By creating or destroying a splice site or regulatory sequence editing can alter splicing or the effectiveness of it. RNA editing may also simply alter the stability of the RNA particle and its tertiary structure (Gott and Emeson 2000; Bass 2002). In addition, RNA editing can affect RNAi pathways in major ways (Nishikura 2006).

1.3.1 Deletion and insertion RNA editing

Deletion and insertion RNA editing was the first type of editing discovered. In the 1980s in the cytochrome oxidase gene *coxII* of *Trypanosoma brucei* the first example of uridine insertion was found, with four of them frame shifting the whole transcript in a way that gives rise to a functional protein (Benne, Van den Burg et al. 1986). Most mitochondrial mRNAs of kinetoplastid protozoa often undergo extensive RNA editing, by which precursor mRNA sequence is changed by the insertion, and less frequently, the deletion of uridine nucleotides (Us). This can create methionine translation initiation codons, correct frameshift mutations, or even give rise to new open reading frames (Feagin, Abraham et al. 1988; Maslov and Simpson 1992; Alfonzo, Thiemann et al. 1997).

The edited mRNAs are translated into components of the oxidative phosphorylation system and are essential for functioning of the mitochondria. To achieve this, the mitochondrial genome consists of 50 identical 22kb large mitochondrial DNA (maxicircle) and of 10000 heterogeneous 1kb small mitochondrial DNA (minicircle). Pre-mRNA is encoded by maxicircles whereas minicircles encode 50-70nt trans-acting guide RNAs (gRNAs) (Stuart, Schnaufer et al. 2005). Guide RNAs encode the proper protein information and by its 5'-anchor complementary sequence binding to pre-mRNA downstream of the editing site guide the process. The information about inserted or deleted uridines is placed in the middle of gRNAs (Blum and Simpson 1990; Simpson, Sbicego et al. 2003). Editing is catalyzed by multiprotein complexes that have been largely characterized. Editing occurs by a series of coordinated catalytic steps: cleavage, insertion or deletion and ligation. Uridines are either added by a TUTase in insertion editing or removed by an ExoUase in deletion editing (Stuart, Schnaufer et al. 2005).

1.3.2 Ribonucleotide deamination

Right after the discovery of editing in *Trypanosoma brucei* evidences of different kind of editing resurfaced in higher eukaryotes. A year later C to U editing was discovered in mice and at the end of the 1980s A to I editing discovery in *Xenopus leavis* eggs followed (Bass and Weintraub 1987; Rebagliati and Melton 1987). Both of these RNA modifications are based on ribonucleotide deamination, cytidine to uracyl in the former and adenosine to inosine in the latter. This type of editing is widespread and common in all higher eukaryotes.

1.3.2.1 Cytidine to Uridine RNA editing

The discovery of transcripts of apoliprotein B (ApoB) being edited more than 20 years ago in the mouse intestine was the first observed example of RNA editing in vertebrates (Chen, Habib et al. 1987; Powell, Wallis et al. 1987). ApoB is a component of the plasma lipoproteins and is necessary for transport of cholesterol and triglycerides in it (Chan 1992). There are two forms of ApoB: long ApoB100 and short ApoB48 isoforms. Expression of ApoB48 is a result of C to U deamination of C^{6666} in the mature transcript which creates a premature stop codon (CAA to UAA).

In humans this deamination occurs only in the small intestine but not the liver and therefore ApoB48 is expressed only in the former tissues (Greeve, Altkemper et al. 1993). ApoB100 is required for assembly of VLDL complex (very low density lipoprotein) required for the transport of endogenously synthesized cholesterol and triglycerides in the blood stream. VLDL is later metabolized to IDL (intermediate-density lipoprotein) and then to LDL particles (low density lipoprotein). Increased plasma level of LDL is coupled with higher risk for coronary heart disease. LDL is removed from blood stream by LDL receptors via the C-terminus of ApoB. ApoB48 generated in small intestine lacks this C-terminus and is necessary for synthesis of chylomicrons, large lipoprotein complexes of the intestines. These huge particles cannot be converted into LDL particles and, therefore, represent lower risk factor for acquiring cardiovascular disease (Herz and Willnow 1995; Chang, Olin et al. 1998).

Additional two known substrates of C to U editing are: neurofibromatosis type 1 (NF1) and eukaryotic translation initiation factor 4 gamma 2 (eIF4G2) mRNAs. NF1 RNA editing generates a translational termination codon at position 3916 that is predicted to truncate the protein product. The translational repressor eIF4G2 undergoes C to U editing at multiple sites,

with the creation of stop codons, that in turn reduce protein abundance (Yamanaka, Poksay et al. 1997; Cichowski and Jacks 2001).

A protein responsible for the deamination of these substrates is a cytidine deaminase APOBEC1 (ApoB mRNA-editing enzyme catalytic polypeptide 1), which performs a very specific reaction, in case of mRNA of ApoB one of the largest proteins known, 1 nucleotide in 14000 (Teng, Burant et al. 1993; Chang, Olin et al. 1998). In order to perform such a task it needs cis-acting sequences and trans-acting factors, a mooring sequence downstream of editing site and 5' and 3' efficiency elements (Backus, Schock et al. 1994; Hersberger and Innerarity 1998). Additionally APOBEC1 is not able to facilitate the reaction alone and needs in addition an auxiliary protein factor and APOBEC1 complementation factor (ACF) to form a functional editosome (Mehta, Kinter et al. 2000; Lellek, Welker et al. 2002). APOBEC1 carries out the same type of reaction as described later for ADARs (adenosine deaminases that act on RNA) but the specificity of it is quite different. Although it is necessary for APOBEC1 deamination for RNA to form a double stranded stem, the deamination still occurs on single stranded RNA (Hersberger, Patarroyo-White et al. 1999) [*Fig. 1.1*]. Additionally, C to U RNA editing of endogenous ApoB *in vivo* is largely confined to spliced and polyadenylated nuclear transcripts (Lau, Zhu et al. 1994).
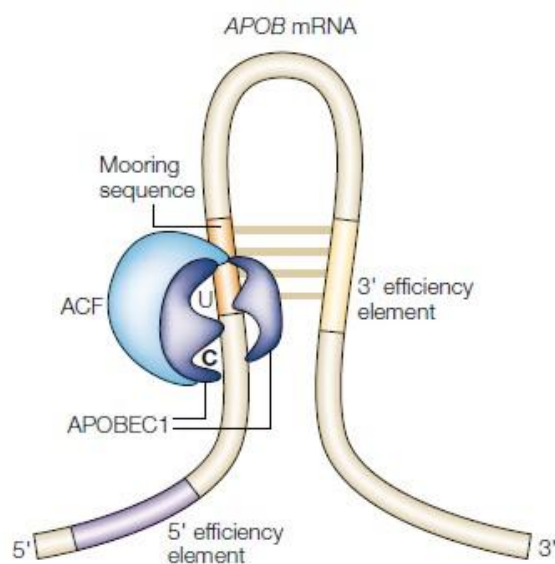
Fig. 1.1: Editing of APOB mRNA by APOBEC1 (from (Keegan, Gallo et al. 2001)). APOBEC1 binds complexed with ACF to folded APOB mRNA. It is not known if the protein binds in monomeric or dimeric state. Essential for editing specificity are: Mooring sequence and 5' and 3' efficiency elements.

APOBEC1 belongs to a large family of cytidine deaminases consisting of 11 members. Most of them are facilitating ssDNA cytidine deamination like: hypermutations introduced in the negative strand of the human immunodeficiency virus (HIV) DNA, which are mediated by

the two APOBEC family members: APOBEC3F and APOBEC3G and mutations introduced in VDJ region of immunoglobulin (Ig) gene by activation-induced (cytidine) deaminase (AID) (Muramatsu, Kinoshita et al. 2000; Chen, Lilley et al. 2006).

1.3.2.2 Adenosine to inosine RNA editing

The second type of RNA-editing by deamination is a similar process to cytidine deamination, which is conversion of adenosine to inosine, an atypical nucleoside [*Fig. 1.4*]. This type of editing is the most common one, found in many RNAs starting from yeast and ending in humans. The substrates of this reaction are mostly imperfect double stranded regions where an adenosine deaminase performs flipping of the target adenosine and its deamination. Inosine does not possess adenosine's base-pairing abilities to uridine on the opposing strand but to cytidine instead. It causes two most important implications of this kind of deamination; unwinding of the double stranded region and reading inosine by cellular machinery as guanosine (Keegan, Gallo et al. 2001; Bass 2002; Nishikura 2009). Up till now editing events have been found in both coding and non-coding regions of protein-coding genes, in viral transcripts, in tRNAs and in miRNAs (Cattaneo, Schmid et al. 1988; Polson, Bass et al. 1996; Morse, Aruscavage et al. 2002; Athanasiadis, Rich et al. 2004; Blow, Futreal et al. 2004; Kim, Kim et al. 2004; Levanon, Eisenberg et al. 2004; Luciano, Mirsky et al. 2004; Li, Levanon et al. 2009).

Two groups of adenosine deaminases editing adenosines on RNA have been discovered. First, ADATs (adenosine deaminases that act on tRNA/tRNA-specific adenosine deaminase) are a protein family responsible for numerous tRNA editing events (Keller, Wolf et al. 1999; Gerber and Keller 2001). These events are often present in the first anticodon position (wobble position 34) in a number of tRNAs in higher eukaryotes and yeast, but also in tRNA^{Arg2} of prokaryotes and chloroplasts. It introduces more flexibility in the process of codon triplet recognition (Sprinzl, Horn et al. 1998). Additionally, another common editing site is known, N^1-methylinosine (m^1I_{37}) found at position 37 of tRNA^{Ala} (A37), adjacent to the anticodon. This atypical base is achieved by adenosine deamination occurring first being followed by the methylation of the inosine (Grosjean, Auxilien et al. 1996).

The first enzyme of this family discovered was ADAT1 performing A37 tRNA^{Ala} site deamination, in *Saccharomyces cerevisiae* (Tad1p/scADAT1) and subsequently in human, mouse and *Drosophila melanogaster* (Gerber, Grosjean et al. 1998; Maas, Gerber et al. 1999;

Keegan, Gerber et al. 2000; Maas, Kim et al. 2000). Another two deaminases from this family, which were able to deaminate adenosine at position A34 of tRNA, were discovered shortly afterwards. These two enzymes form a catalytically active heterodimer in contrast to ADAT1. Unlike ADAT1, ADAT2 and ADAT3 yeast knock-out strains are lethal, indicating the importance of this editing mechanism. Unlike other adenosine deaminases, ADATs do not contain an RNA binding motif, suggesting that the deaminase domain directly recognizes its substrate (Gerber and Keller 1999; Gerber and Keller 2001; Keegan, Gallo et al. 2001). Out of three ADAT family members only ADAT1 is present in mammals. ADAT1 is different in size, structure and mode of interaction with tRNA from ADAT2 and ADAT3. Additionally, only mammalian ADAT1 has the ADAR-like deaminase domain containing a loop insertion in between second and third zinc-chelating residues. A homologue of yeast ADAT2 is also present in *Escherichia coli* called tadA (Keegan, Leroy et al. 2004; Nishikura 2009).

The second family of adenosine deaminases are ADARs (adenosine deaminases that act on RNA) that are thought to have evolved from the ADAT family through the acquisition of double-stranded RNA-binding domains (dsRBDs) and slight alterations of the primary structure in the catalytic (deaminase) domain. These deaminases, their structure and function are described in further chapters of this work.

1.4 Adenosine deaminases that act on RNA (ADARs)

A to I deamination of adenosines in double stranded RNA is catalysed by the ADAR (adenosine deaminase that act on RNA) family of adenine deaminases. Since their first discovery in *Xenopus leavis* they have been characterized in many other metazoa: mammals, birds, frogs, flies and worms (Bass and Weintraub 1987; Rebagliati and Melton 1987; Bass and Weintraub 1988; Kim, Wang et al. 1994; Herbert, Lowenhaupt et al. 1995; Lai, Drakas et al. 1995; O'Connell, Krause et al. 1995; Melcher, Maas et al. 1996; Gerber, O'Connell et al. 1997; Hough and Bass 1997; Palladino, Keegan et al. 2000; Slavov, Clark et al. 2000; Slavov, Crnogorac-Jurcevic et al. 2000).

1.4.1 Discovery of ADARs

ADARs activity was discovered in year 1987 in *Xenopus leavis* independently by two research groups, who described the phenomenon as RNA unwinding or helicase activity (Bass and Weintraub 1987; Rebagliati and Melton 1987). In the performed experiments, silencing in activated eggs failed as an effect of the single-stranded RNA used. It did not reanneal due to conversion of up to half of the adenosines in dsRNA to inosine by an adenosine deaminase acting on RNA. The unwinding observed was an effect of A-U base pairs being converted to I-U mismatches destabilizing RNA duplexes (Bass and Weintraub 1988; Wagner, Smith et al. 1989; Polson, Crain et al. 1991). Shortly thereafter the responsible enzyme was found in *Xenopus leavis* and subsequently in human and in cattle (Hough and Bass 1994; Kim, Wang et al. 1994; O'Connell and Keller 1994). The discovered enzyme was named differently by both laboratories; dsRAD (dsRNA adenosine deaminase) and DRADA (dsRNA adenosine deaminase). In 1996 another two enzymes were cloned, RED1/DRADA2 (dsRNA-specific editase 1/dsRNA adenosine deaminase 2) and catalytically inactive RED2 (Melcher, Maas et al. 1996; Melcher, Maas et al. 1996). To avoid complications names of these adenosine deaminases were unified in mammals to ADAR1, ADAR2 and ADAR3 respectively (Bass 1997).

1.4.2 Members of the ADAR family

As already mentioned ADARs are widespread and highly conserved in all metazoa. In mammals three members of the family are present: ADAR1, ADAR2 and ADAR3. One member homologous to ADAR1 was discovered in frog and chicken and numerous members were found in fishes (Herbert, Lowenhaupt et al. 1995; Hough and Bass 1997; Slavov, Clark et al. 2000; Slavov, Crnogorac-Jurcevic et al. 2000; Jin, Zhang et al. 2009). In invertebrates ADAR members were also characterised. In *Drosophila melanogaster* one member *d*ADAR, in *Caenorhabditis elegans* two: ADR-1 and ADR-2 and also two splicing isoforms of *sq*ADAR2 in squid (Palladino, Keegan et al. 2000; Tonkin, Saccomanno et al. 2002; Palavicini, O'Connell et al. 2009). Additionally, recent findings have shown presence of two ADARs; ADAR1 and ADAR2 in sea urchin and sea anemones (Jin, Zhang et al. 2009). ADARs in contrast to ADATs are absent in Protozoa, Yeast and Plants (Jin, Zhang et al. 2009).

Insects have lost one of ADARs during evolution and that's why *Drosophila melanogaster* has only one member of ADAR family termed dADAR with more similarities to mammalian ADAR2 than to ADAR1 (Palladino, Keegan et al. 2000) [*Fig. 1.2*]. *d*ADAR is nearly exclusively expressed in the central nervous system of embryos and in the brains of adult flies (Palladino, Keegan et al. 2000; Ma, Gu et al. 2001). It consists of two dsRBDs (dsRNA binding domains) and a C-terminal catalytic deaminase domain. It forms dimers on its substrates and the dimerisation occurs through contacts between the N-terminus and the first dsRBD (Gallo, Keegan et al. 2003). A striking majority of its targets localize to the nervous system with mRNAs of voltage- and ligand-gated ion channels genes, as well as the members of the synaptic release complex being edited (Hoopengardner, Bhalla et al. 2003). Like its vertebrate homologue ADAR2, mRNA of this protein is self-edited creating a negative auto-regulation mechanism, leading to the synthesis of the protein isoform with the reduced deamination activity (Palladino, Keegan et al. 2000; Keegan, Brindle et al. 2005). Loss of *d*ADAR activity leads to severe behavioral defects in the adult fly including loss of coordination, seizures, and temperature-sensitive paralysis (Palladino, Keegan et al. 2000; Jepson and Reenan 2009).

In *C. elegans*, two ADAR proteins are present but are so different from vertebrate ADARs, that it's hard to distinguish, how the proteins are related. Both of the enzymes have a C-terminal catalytic domain but while ADR-1 has two dsRBDs, ADR-2 has only a single dsRBD [*Fig. 1.2*]. Both of them are necessary for A to I editing in worms (Hough, Lingam et al. 1999; Tonkin, Saccomanno et al. 2002; Keegan, Leroy et al. 2004). Similar to substrates in *D. melanogaster*, identified substrates are important for the functionality of the nervous system and a knock-out of *adr-1* and *adr-2* leads to defects in behavior in chemotaxis assays (Morse, Aruscavage et al. 2002; Tonkin, Saccomanno et al. 2002). Surprisingly these defects can be rescued by mutations in the *rde-1* or *rde-4* genes that are involved in RNAi, suggesting an interplay of the RNAi and RNA-editing machineries (Tonkin and Bass 2003).

In mammals, there are three proteins that belong to the ADAR family: ADAR1, ADAR2 and ADAR3 [*Fig. 1.2*]. ADAR1 is a long ubiquitously expressed protein consisting of three important structural elements. Z-DNA binding domain (ZBA) composed of the Zα and Zβ moieties is placed N-terminally and is thought to play a role in guiding ADAR1 to the active transcription sites where, due to unwinding, the DNA adopts a Z-like helical conformation (Herbert and Rich 2001). The central part of the proteins comprises three distinctively different dsRNA binding domains (dsRBD) docking the protein to double-

stranded RNA during the deamination reaction. The catalytic deaminase domain responsible for the reaction can be found at the C-terminus of ADAR1. Two isoforms of ADAR1 are known in mammals: a full-length one, 150kDa in mass and a shorter N-terminally truncated one of 110kDa. The shorter version of ADAR1 doesn't contain the Z-DNA binding domain (Patterson and Samuel 1995; George and Samuel 1999). Three promoters driving ADAR1's translation are known (Wang, Zeng et al. 1995; Weier, George et al. 1995; Liu, George et al. 1997). One of them is interferon inducible giving rise to the full-length version (ADAR1-i), that starts at methionine 1. It predominantly localizes to the cytoplasm where the 150kDa protein is implicated in viral RNA editing (Wong, Sato et al. 2003). Beside of its prime target, viral RNA, also transcripts of infected organism are edited more frequently during infection and inflammation (Yang, Luo et al. 2003). Two other promoters are constitutive ones directing synthesis of the shorter isoform (ADAR1-c), which starts translation from downstream methionine at position 296. This version predominantly localizes to the nucleus (Patterson and Samuel 1995; George and Samuel 1999). Additionally two alternative splice variants that are differentially expressed in different tissues have been reported. In comparison to full-length ADAR both splice variants contain a deletion of 26 amino acids at the exon 7-intron 7 junction, which lies between the dsRBD3 motif and the catalytic domain. The third variant has an additional deletion of 19 amino acids at the intron 5-exon 6 junction which lies between the dsRBD2 and dsRBD3 motifs. These splice site variants retain the dsRBDs functionally intact, and all three of the ADAR variants encode active enzymes that possess comparable deaminase activity (Liu, George et al. 1997). The picture may get even more complex if taking into account ADAR1 Intracellular localization studies performed in mouse lymphocytes during acute inflammation. Still unknown inflammatory regulatory mechanism in lymphocytes increases the number of ADAR1 isoforms. More than a dozen different ADAR1 cDNAs were found in spleens harvested from endotoxin-challenged mice. One of the variants is 80kDa ADAR1, in which by alternative splicing the whole exon 2 comprising 1.4 kb is deleted. This event results in a deletion of the entire Z-DNA binding domain, the NLS, and the entire dsRBDI. The translation of p80 starts at a methionine 519 producing a predominantly cytoplasmic protein (Yang, Nie et al. 2003). Additionally numerous C-terminal truncated versions of ADAR1 have been detected (Nie, Zhao et al. 2004).
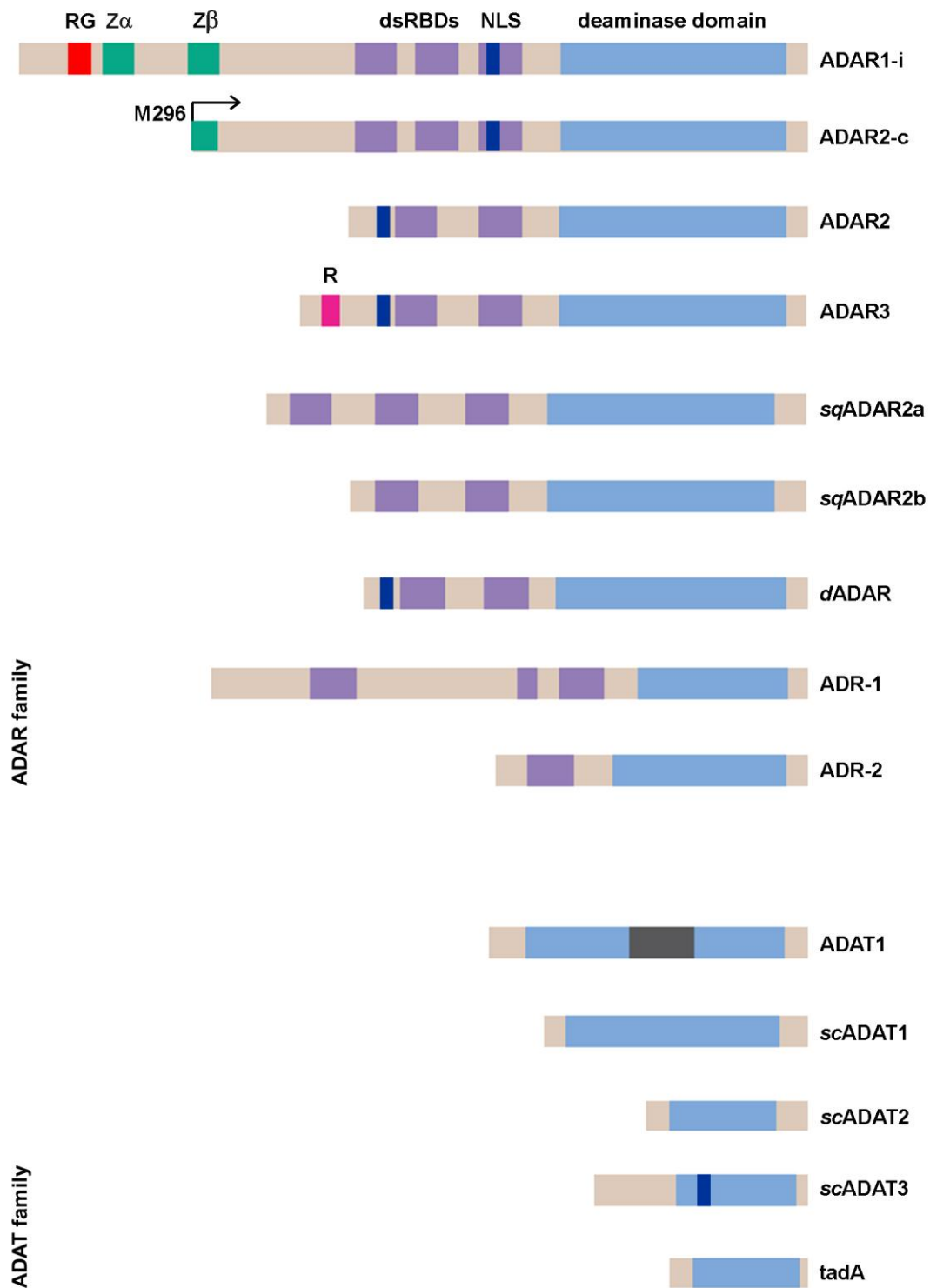
Fig. 1.2: ADAR and ADAT family members (adapted from (Keegan, Leroy et al. 2004; Nishikura 2009)). From ADAR family three mammalian members are shown: ADAR1 in its long and short isoform, ADAR2 and ADAR3. Invertebrate members are represented by two squid ADAR2 isoforms, D. melanogaster ADAR and C elegans ADR-1 and ADR-2. ADAR family shares C-terminal catalytic domain (light blue) and found either N-terminally or centrally dsRNA binding domains (purple). Unique to some members, N-terminal motifs, are Z-DNA binding domain (green) and R- or RG-rich regions (magenta and red

ADAR2 is a smaller than ADAR1 with only 80kDa. It exhibits a high level of structural similarity to ADAR1, 31% sequence identity with the catalytic domain being the most conserved one (Melcher, Maas et al. 1996). ADAR2 lacks the Z-DNA binding domain of ADAR1 and contains only two dsRBDs N-terminally and a catalytic domain C-terminally. ADAR2 exhibits a putative N-terminal NLS which is responsible for the nuclear localization of the protein with its characteristic nucleolar accumulation (Desterro, Keegan et al. 2003; Sansam, Wells et al. 2003). In contrast to ADAR1 being ubiquitously expressed, ADAR2 is present in most tissues but expressed predominantly in the brain (Paul and Bass 1998). Initially, four different splice variants of ADAR2 were detected. All of them were a combination of two alternative splicing events. One of them shows an inclusion/exclusion of an Alu cassette encoding 40-aa in the core of the deaminase domain. The second results in creation of two different C-termini, one long (29-aa) and another short (2-aa). All of them are expressed at different ratios and are active at comparable levels (Gerber, O'Connell et al. 1997; Lai, Chen et al. 1997). Recent findings have introduced more variety to what is known about existing ADAR2 variants. An additional functional promoter was found upstream of ADAR2. A transcript emerging from this promoter adds an additional 49-aa long, highly conserved exon termed exon 0. This new alternative splice form of ADAR2 including exon 0 harbours a stretch of positively charged residues that are highly similar in sequence, length and relative position to the so-called R-domain of ADAR3. This variant of ADAR2 is expressed, at lower levels than ADAR2 lacking exon 0, differently in various tissues with the highest expression rates in hippocampus, colon and the brain (Maas and Gommans 2009). Also a novel alternatively spliced exon, located within intron 7 of ADAR2 was discovered. Exon 7a is highly conserved in the mammalian ADAR2 gene. It has stop codons in all three frames and is down regulated by NMD. The level of exon 7a inclusion differs between different human tissues, with the highest levels of inclusion in skeletal muscle, heart and testis. In the brain, where the level of editing is known to be high, the level of exon 7a inclusion is low (Agranat, Sperling et al. 2010). If the high number of various splicing variants of ADAR2 was not enough, from experiments in rat we know that ADAR2 can edit

its own mRNA at position -1 in intron 4 converting AA into AI and creating in that way a typical 3' splice site (AG). Introduction of a frame shift and a C-terminal truncation directs the protein to NMD, which serves most probably as an auto regulatory negative feedback loop(Rueter, Dawson et al. 1999; Dawson, Sansam et al. 2004).

The last known member of mammalian ADAR gene family is the 81kDa ADAR3 protein. It shows an exclusive brain expression pattern, particularly in the amygdala and the thalamus (Mittaz, Antonarakis et al. 1997). It is 50 % identical to ADAR2 in sequence, with two dsRBDs and a conserved deaminase domain. Interestingly, an additional N-terminal arginine-lysine-rich sequence (R-domain) of ADAR3 has been identified on its N-terminus as a single-stranded RNA binding domain (Chen, Cho et al. 2000). ADAR3 is, however, believed to lack any catalytic activity, firstly because no substrate of it has been identified so far and, secondly, because it is unable to edit any of the known ADAR1 or ADAR2 substrates. Even though it is not capable of performing the catalysis, ADAR3 could bind potential and serve as a negative regulator of the two enzymatically active ADARs (Melcher, Maas et al. 1996; Chen, Cho et al. 2000).

Additional ADAR-like proteins discovered in vertebrates are catalytically inactive ADAD1/TENR (adenosine deaminase domain-containing protein 1/testis nuclear RNA-binding protein) and ADAD2/TENRL (adenosine deaminase domain-containing protein 2/testis nuclear RNA-binding protein-like). TENR is expressed in the male germline and has only one dsRBD. TENR is missing a key glutamate residue in the active site of the deaminase domain and also lacks zinc-chelating residues (Hough and Bass 1997; Connolly, Dearth et al. 2005). TENRL is expressed in the brain (McKee, Minet et al. 2005).

1.4.3   Evolution of the ADAR family

The putative ancestors of ADARs are ADATs. It is believed that ADATs evolved into ADARs by adding dsRBDs to a deaminase domain resembling that from ADARs. ADATs are an older family of proteins, with members present in all eukaryotes, and a bacterial orthologue tadA also discovered. In comparison, ADARs are only present in metazoa. The highest homology between the catalytic domain of ADAR and ADAT is presented by ADAT1. Mammalian ADAT1 acquired additional loop in the catalytic domain, that is not present in yeast ADAT1. That indicates, that ADAT1 is an ancestor of ADARs and itself has evolved from ADAT2, which is older having its homologue already in *E. coli* tadA (Gerber,

Grosjean et al. 1998; Maas, Gerber et al. 1999; Keegan, Gerber et al. 2000; Maas, Kim et al. 2000; Wolf, Gerber et al. 2002; Keegan, Leroy et al. 2004). It appears that the ancestors of ADATs are the cytidine deaminases acting on mononucleotides (CDAs) or on RNAs (CDARs) but not the adenosine deaminases acting on mononucleotides (ADAs) (Kim, Wang et al. 1994; Gerber and Keller 1999; Gerber and Keller 2001). Only the former group of proteins has the ability to dimerize, which is crucial for ADATs and seems to be also important for ADARs (Betts, Xiang et al. 1994; Lau, Zhu et al. 1994; MacGinnitie, Anant et al. 1995; Cho, Yang et al. 2003; Gallo, Keegan et al. 2003). ADARs seem to be present in all metazoa with high conservation levels in all vertebrates (Hough, Lingam et al. 1999; Slavov, Clark et al. 2000; Slavov, Crnogorac-Jurcevic et al. 2000). The recent discovery of homologues of ADAR1 and ADAR2 in sea urchin and sea anemones, primitive metazoa, would suggest an origin of both proteins already in early metazoa. ADAR3 might have evolved more recently by duplication and mutation of *ADAR2* gene in mammals. Although a homologue of ADAR3 is very well conserved in fish genomes suggesting that the protein is older and conserved in all chordate but other groups have lost it on the way. Furthermore also either ADAR1 or ADAR2 might have been frequently lost during the course of evolution. Insects lack ADAR2 for example. Additionally until now only ADAR1 homologues were found in frogs and ADAR2 homologues in squid (Keegan, Leroy et al. 2004; Jin, Zhang et al. 2009).

1.4.4   Domain architecture

All proteins belonging to either ADAR or ADAT family contain a catalytic deaminase domain in their C-terminus, which is the most conserved part of the protein, but show greater variation in their central and N-terminal regions. The deaminase domain is always placed in C-terminal part of the protein and bears high homology to the catalytic centers of DNA methyltransferases and cytidine deaminases that act on RNA (CDARs) (Kim, Wang et al. 1994; Hough and Bass 1997). It consists of all characteristic three deaminase motifs (I - III), with four or five highly conserved amino acids surrounding each of the cysteines and histidines, that bind zinc and are required for the catalysis of hydrolytic deamination (Keegan, Leroy et al. 2004) [*Fig. 1.3*].

All ADARs contain in their central region dsRNA binding domains (dsRBDs) but the number and structure of them differs. While ADAR1 contains three double-stranded RNA-

binding domains, only two dsRBDs can be found in ADAR2 and ADAR3. A typical dsRBD of ADARs is a roughly 65 amino acid long motif forming a characteristic α β β β α formation (Ryter and Schultz 1998). They are involved in the substrate recognition and binding, but also serve as modulators of the intracellular trafficking in case of ADAR1 (Eckmann, Neunteufl et al. 2001; Strehblow, Hallegger et al. 2002; Valente and Nishikura 2007; Fritz, Strehblow et al. 2009).
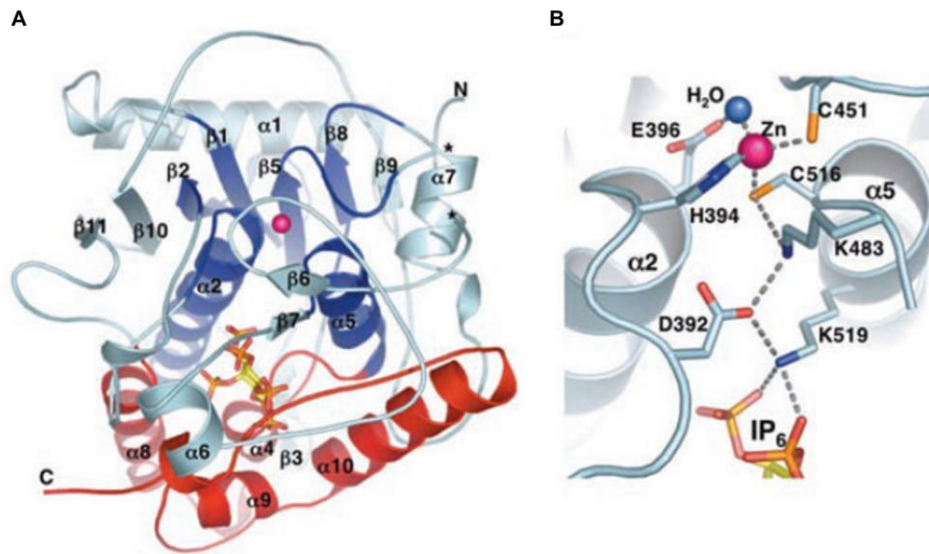
Fig. 1.3: ADAR2 catalytic domain (from (Macbeth, Schubert et al. 2005)). (A) Ribbon model of human ADAR2 catalytic domain. The active-site zinc ion is represented by magenta sphere. Regions that share structural similarity with CDA and tadA are shown (cyan and dark blue). C-terminal helical domain making contacts with IP₆ is also shown (red and IP₆ as sticks under the zinc ion). (B) Residue interactions at the active site. Shown are: the zinc ion, coordinating residues (H394, C451, C516), the nucleophilic water (blue sphere) and the proposed proton shuttling residue, E369. The hydrogen bond, that connects the active site to the IP₆ is also indicated.

The biggest differences between the three proteins are in their N-termini. The interferon-inducible isoform of ADAR1 (ADAR1-i) harbors two left-handed Z-DNA binding domains (Zα and Zβ) in its N-terminus. Structural analyses have shown that each domain builds up a helix-loop-helix structure similar to the one found in DNA-binding proteins, and can bind left-handed, supercoiled DNA Z-helix *in vitro* (Herbert, Alfken et al. 1997; Schade, Turner et al. 1999; Schwartz, Rould et al. 1999). The cocrystal structure of Zα complexed to Z-DNA showed that one monomeric Zα domain binds to one strand of double-stranded DNA

and a second Zα domain binds to the opposite strand with 2-fold symmetry with respect to DNA helical axis. Recent findings support an active B-Z transition mechanism, in which the Zα first binds to B-DNA and then converts it to left-handed Z-DNA, a conformation that is then stabilized by the additional binding of a second Zα (Kang, Bang et al. 2009). Although this type of DNA is not well investigated yet, experimental evidence points to the existence of Z-DNA in living mammalian cells and its conserved role in processes such as gene regulation, nucleosome positioning, chromatin remodeling and recombination (Zhao, Bacolla et al.; Wang and Vasquez 2007). It was speculated that this atypical DNA structure might be present in transcriptionally active sites, which led to the proposition that the ZBDs direct ADAR1-i there but the evidence is still missing to support such a hypothesis (Herbert, Alfken et al. 1997). It has been demonstrated in *Xenopus leavis*, that the ZBDs are dispensable for chromosomal localization (Eckmann and Jantsch 1999). Furthermore, there are hypotheses suggesting the importance of ZBDs for viral dsRNA binding in the cytoplasm, required for hyperediting in the antiviral response(Herbert, Alfken et al. 1997; Brown, Lowenhaupt et al. 2000). In addition to that ADAR1 harbors a RG-rich region in front of the ZBDs (O'Connell, Krause et al. 1995). ADAR3 in contrast contains a N-terminal arginine/lysine-rich sequence (R-domain), which has been identified as a single-stranded RNA binding domain (Chen, Cho et al. 2000).

Each ADAR contains an NLS sequence directing the proteins into nucleus. ADAR2 and ADAR3 exhibit an NLS in the N-terminus in front of dsRBDs. On the other hand the NLS of ADAR1 is a non-canonical one embedded in its third dsRBD with a nuclear localization function independent of RNA-binding activity (Eckmann, Neunteufl et al. 2001). Shuttling of ADAR1-i is performed thanks to a typical leucine-rich N-terminal nuclear export signal (NES), which overlaps with the N-terminal part of the Z-DNA binding domain, Zα (Poulsen, Nilsson et al. 2001). The constitutively expressed version of ADAR1 is also able to shuttle between the nucleus and cytoplasm. This movement is also mediated by dsRBD3, although this time due to RNA-dependent interactions between the dsRBD and exportin 5 (Fritz, Strehblow et al. 2009).

1.4.5   Mechanism of deamination

ADARs, thanks to their catalytic deaminase domain, are able to perform RNA editing, via the well-characterized conversion of adenosine (A) to inosine (I) by hydrolytic

deamination. The reaction mechanism consists of a nucleophilic attack of an activated water molecule at the C6 of the purine ring in an intermediate step. Subsequently, an ammonia molecule is released, resulting in a stable inosine moiety (Bass and Weintraub 1988; Wagner, Smith et al. 1989) [*Fig. 1.4*]. The architecture of the active site of the catalytic domain is defined by highly conserved amino acid residues: histidine H394, glutamic acid E396 and two cysteine residues C451 and C516 obtained from crystallography studies of catalytic domain of ADAR2 and are involved in coordination of a zinc ion (Macbeth, Schubert et al. 2005). Similar to most metalloenzymes also in ADARs, a water molecule is activated by the zinc ion, enabling the nucleophilic attack (Lai, Drakas et al. 1995). This kind of geometry of the catalytic center has also been observed in cytidine deaminases and *E. coli* TadA, a member of the ADAT2 family.

The site of the nucleophilic attack during the ADAR reaction is buried in the major groove of the dsRNA. Due to its inaccessibility, ADARs are thought to employ a base-flipping mechanism to bend the target adenosine out of the dsRNA A-helix (Stephens, Yi-Brunozzi et al. 2000). Interestingly, the dsRBDs of ADARs might facilitate this conformational change through enhancement of the RNA duplex flexibility (Yi-Brunozzi, Stephens et al. 2001). The catalytic zinc centre is located in a deep pocket surrounded by the positively charged residues, making it an ideal binding surface for the negatively charged dsRNA [*Fig. 1.3*].

An essential element of the active site of the enzyme is one molecule of inositol hexakisphosphate (IP_6). This cofactor is deeply buried in the active site [*Fig. 1.7*]. Its presence was confirmed in ADAR2 and ADAT1 and it seem to be necessary for proper folding of the catalytic domain and activity of ADARs (Macbeth, Schubert et al. 2005). Beside of IP_6 no other cofactors are needed for ADARs activity *in vitro* (Polson, Crain et al. 1991; O'Connell, Gerber et al. 1997).

Like for ADATs the formation of homodimers between single ADAR molecules were studied and it seems that like in the case of their distant relatives this process takes place and that dimerisation is required for proper editing (Cho, Yang et al. 2003; Gallo, Keegan et al. 2003). It has been shown that ADAR1 and ADAR2 are the ones that undergo homo and heterodimerisation but not ADAR3, which could explain its inactivity (Gallo, Keegan et al. 2003) Whether dimerisation of the protein molecules requires RNA is still unknown, although the studies suggest, that the interactions take place on a protein-protein level. The region required for dimerisation is still not known even though the dsRBD regions seem to play a

pivotal role (Chilibeck, Wu et al. 2006; Poulsen, Jorgensen et al. 2006; Valente and Nishikura 2007). Although, many studies indicate requirement of dimerisation for deamination reaction there are also some that contradict these findings indicating otherwise. In few experiments it was shown that active ADARs may exist as monomers in solution (O'Connell and Keller 1994; Macbeth, Lingam et al. 2004). Additionally N-terminal deletions and later also expression of catalytic domain fragment of *hsADAR*2, that have shown its functionality leave rather a little maneuver for possible dimer formation (Macbeth, Lingam et al. 2004; Macbeth, Schubert et al. 2005). Also the specific binding of dsRBDs of rADAR2 to GluR-B R/G site would suggest rather a 1 to 1 ratio of the enzyme and the substrate (Stefl, Xu et al. 2006).
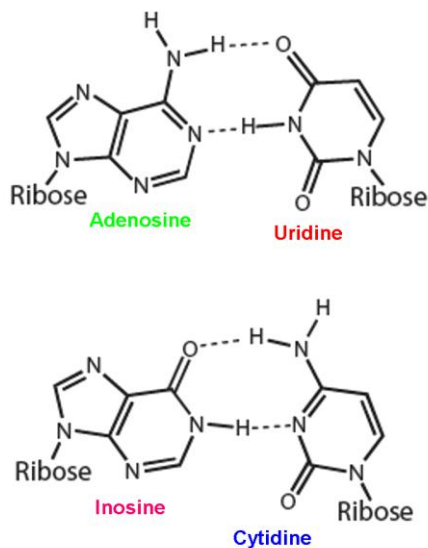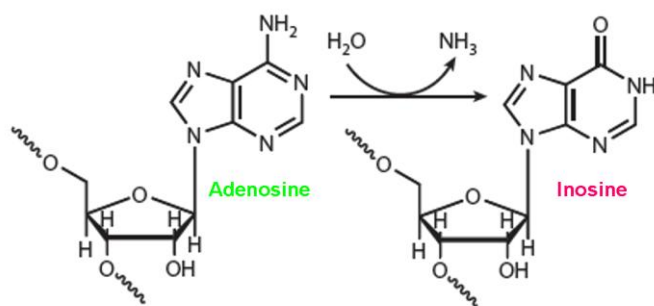

Fig. 1.4: Deamination of adenosine to inosine by ADAR (from (Nishikura 2009)). A simplified hydrolytic deamination of adenosine to inosine is shown. The reaction consists of nucleophilic attack of an activated water molecule at the C6 and an ammonia molecule is release. Adenosine base-pairs with uridine, whereas inosine, as if it was guanosine, with cytidine.

1.4.6 Substrate recognition and specificity of ADARs

ADARs are enzymes binding to dsRNA and edit adenosines within it. Both inter- or intramolecular dsRNAs of >20 base pairs, which are two turns of dsRNA helix, can serve as a substrate (Nishikura, Yoo et al. 1991). ADARs exhibit a wide range of specificity and are able of both quite different tasks; they can edit very specifically, one base in hundreds or lead to random conversion of many adenosines until reaching a state of roughly 50% of them being edited (Nishikura, Yoo et al. 1991; Polson and Bass 1994). An explanation to this remarkably different editing extents lays in the structure of ADARs but also in the RNA conformation.

A typical dsRNA forms an A-form helix, which in contrast to the B-form helix of DNA has a deep and narrow major groove, making the only place where an enzyme could come in contact with bases fairy inaccessible. In fact, dsRBDs are proven to bind the phosphodiester backbone of RNA and are sequence-independent (Bycroft, Grunert et al. 1995; Kharrat, Macias et al. 1995; Nanduri, Carpick et al. 1998; Ryter and Schultz 1998). dsRBDs of ADARs bind tightly to any dsRNA sequence, which can be an explanation for the more random editing events. However, recent findings have shown that in a few cases dsRBDs may bind in very specific way to certain editing substrates including nonhelical regions, that flank dsRNA. This indicates, that some dsRBDs may possess intrinsic binding selectivity. NMR structures of two dsRBDs of ADAR2 have demonstrated, that dsRBD1 recognizes a conserved pentaloop of the GluR-B R/G stem-loop, whereas dsRBD2 recognizes two bulged bases adjacent to the editing site, demonstrating RNA structure-dependent recognition by the ADAR2 dsRBDs (Stephens, Haudenschild et al. 2004; Stefl, Xu et al. 2006).

Still selective editing depends on something different than the specificity of dsRBDs. To understand this kind of deamination one has to remember the specificity of ADARs towards dsRNA and not ssRNA and also that dsRNA fragment is rarely perfectly base-paired (Bass and Weintraub 1987). We also know that by deaminating adenosines in A-U base-pair are converted into a less stable I-U mismatch. Experiments performed on perfect dsRNA have shown that editing is introducing more instability into a dsRNA particle by slowly unwinding it. As the reaction proceeds less and less adenosines are perfect targets for ADAR deamination which causes a halt of hyperediting of perfectly base-paired dsRNA at 50-60% (Nishikura, Yoo et al. 1991; Polson and Bass 1994). Most of the selectively edited substrates are not forming perfectly base-paired dsRNA. They contain internal bulges and loops, which seem to be the answer to the selectivity of ADARs. Even a long dsRNA stretch but

interrupted by bulges may cause ADAR to see RNA as a series of shorter helices, in which maybe only one or two adenosines present themselves as good editing substrates (Lehmann and Bass 1999). These findings led to the conclusion, that the only way to make ADAR bind in register and edit selectively is to make a duplex very short or to enclose editing sites between two mismatches creating only one possible way for the enzyme to bind (Lehmann and Bass 2000; Ohman, Kallman et al. 2000) [*Fig. 1.5*].
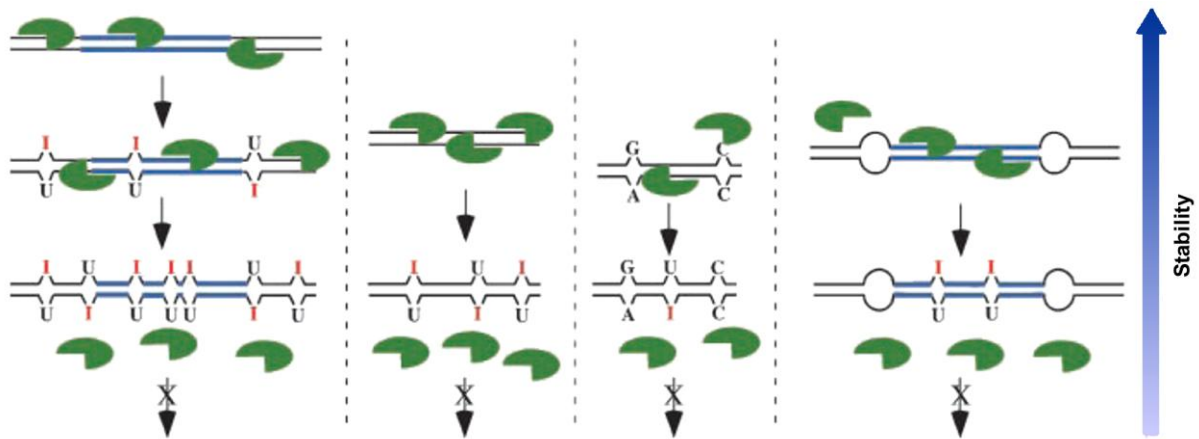
Fig. 1.5: Selectivity of ADAR deamination (from (Bass 2002)). ADARs (green) reacting with four different substrates are shown: perfectly double-stranded long and short fragments and double-stranded fragments interrupted with mismatches or loops. ADAR deaminating dsRNA introduces I-U mismatches decreasing stability of RNA fragment. Reaction stops after editing up to 50-60% of the adenosines shown on hyperedited long RNA fragment. Selective editing is only achieved by editing of shorter fragments or longer ones interrupted with mismatches and loops, which divide long fragment into a series of shorter dsRNA fragments.

Although these results help to understand how ADAR substrate selectivity can be obtained they still do not explain why ADARs exhibit slight preferences of one adenosine over others. Additionally the exchange of the deaminase domains between ADAR1 and ADAR2 resulted in the substrate specificity typical for the enzyme whose deaminase domain was part of the protein chimera (Wong, Sato et al. 2001). So even if targeted binding of dsRBDs to dsRNA may be the major factor positioning proper adenosine in vicinity of the active site, like in the case of the GluR-B R/G site, it seems that also the architecture of the active site dictates the choice of the base being edited (Stefl, Xu et al. 2006). Evidence from *in vivo* and *in vitro* experiments on endogenous and also synthetic substrates have revealed a certain neighbourhood preference of the base adjacent to the edited adenosine and of the

pairing partner on the other strand. These preferences would encompass additional bases that beside the edited adenosine interact with the active site of the enzyme. Studies with *Xenopus* and human ADAR1 show that the enzyme targets adenosines further than three nucleotides from the 5' terminus and eight from the 3' terminus with a 5' neighbour preferences of U = A > C > G (Polson and Bass 1994; Riedmann, Schopoff et al. 2008). ADAR2 on the other hand deaminates as close as three nucleotides from either terminus with 5' and 3' neighbouring preferences, which are; U ≈ A > C =G and U = G > C = A respectively (Lehmann and Bass 2000; Ohman, Kallman et al. 2000; Riedmann, Schopoff et al. 2008). Also the studies concerning preferences of ADARs towards a certain pairing partner in the editing complementary site indicate major role of the active site in this choice. In both mammalian ADAR1 and ADAR2 a mismatch at the editing site is preferred in comparison to A-U base-pairing. Most efficiently edited is adenosine in A-C mismatch followed by A-A and A-G mismatches (Wong, Sato et al. 2001). In the studies on Alu elements the most often base opposite the edited adenosine was uracil, followed by cytosine. This is mostly due to uracil being a usual base-pairing partner of adenosine. Nonetheless, the frequency of C opposite an edited A is about three times higher than expected by the natural occurrence of C residues opposite A residues (Riedmann, Schopoff et al. 2008). Very similar neighbourhood preferences only confirm that ADAR1 and ADAR2 have overlapping but distinct substrate specificities with certain substrates being edited exclusively by one enzyme to some, where the edited state is an effect of the redundancy of the enzymes (Maas, Melcher et al. 1996; Melcher, Maas et al. 1996; Lai, Chen et al. 1997).

1.5   Substrates of ADAR

Initially editing events were detected as A to G changes in cDNA and mostly restricted to coding ones. Only in recent years when more sophisticated computational methods for genome-wide identification were implicated, a real landscape of A to I editing was revealed. A large number of predicted editing sites (~15000 sites in ~2000 genes) was found to be localized in non-coding regions, in inversely oriented Alu and SINE elements in comparison to less than 100 recoding events in human genes (Hoopengardner, Bhalla et al. 2003; Athanasiadis, Rich et al. 2004; Blow, Futreal et al. 2004; Kim, Kim et al. 2004; Levanon, Eisenberg et al. 2004; Ohlson and Ohman 2007; Gommans, Tatalias et al. 2008; Li, Levanon

et al. 2009). Substrates of ADARs obtained in these screens may be divided in three major groups. Firstly, the coding substrates, which in mammals, as well as in flies and worms are located in the central nervous system (CNS) where ADAR-mediated RNA editing regulates important functional properties of neurotransmitter receptors (Maas, Kawahara et al. 2006) [*Fig. 1.6*]. Furthermore, another important role for ADARs in mammals is thought to be editing and hyperediting of viral dsRNA as a defense mechanism against infections. Finally, in recent years the effects of adenosine deamination in the non-coding substrates, most notably the microRNA precursors, emerged as a significant regulatory mechanism in RNA interference (Nishikura 2006; Ohman 2007).

1.5.1 Most known coding substrates

A typical coding editing substrate forms a hairpin structure, ranging from the shortest known stem-loops of ~60-70 nucleotides like in the case of Gabra-3 I/M and GluR-B R/G stem-loops to few with editing site and its editing complementary sequence (ECS) in a distance of a kb in length like in case of GluR-5 and GluR-6 substrates (Seeburg, Higuchi et al. 1998; Ohlson, Pedersen et al. 2007). In most cases the double stranded region is formed between an exonic region with edited adenosines in the vicinity of a splicing site and an intronic ECS like in case of GluR-B and serotonin receptor 2C editing sites [*Fig. 1.5*]. This implies that editing must occur before splicing, when the intron is cut out (Higuchi, Single et al. 1993; Lomeli, Mosbacher et al. 1994; Herb, Higuchi et al. 1996; Burns, Chu et al. 1997; Niswender, Sanders-Bush et al. 1998; Laurencikiene, Kallman et al. 2006; Schoft, Schopoff et al. 2007). Editing in coding regions may lead to a change of the codon and therefore of the information carried by mRNA. The proximity of editing sites to splicing sites may also influence splicing dynamics or even its course (Bass 2002; Schoft, Schopoff et al. 2007) The most prominent and best described targets of ADARs are found in the central nervous system. They are the pre-mRNAs encoding subunits of the glutamate receptor, the serotonin 2C receptor in mammals, potassium, sodium and chloride channels in *Drosophila* or potassium channel in squids (Melcher, Maas et al. 1996; Burns, Chu et al. 1997; Patton, Silva et al. 1997; Seeburg, Higuchi et al. 1998; Lehmann and Bass 2000) (Burns, Chu et al. 1997; Patton, Silva et al. 1997; Palladino, Keegan et al. 2000; Reenan, Hanrahan et al. 2000).
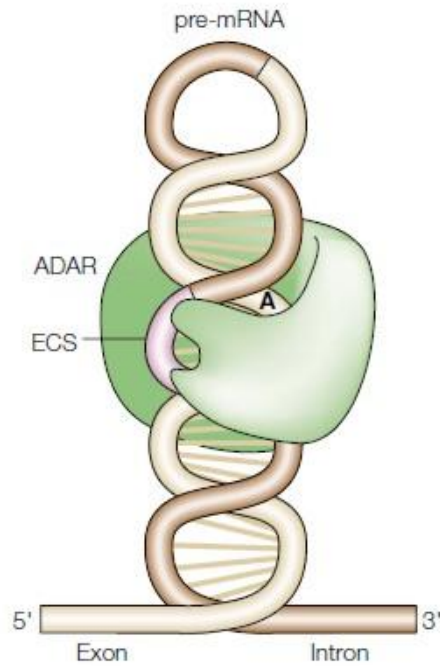
Fig. 1.5: Typical substrate of ADAR editing (from (Keegan, Gallo et al. 2001)). A duplex RNA, that is recognized by ADARs, is formed between the editing site and ECS that is often located in downstream intron. The enzyme binds through the dsRNA binding domains.

L-glutamate is the major excitatory neurotransmitter in the vertebrate central nervous system. It serves the glutamate-gated ion channels in mediating rapid excitatory transmission and synaptic plasticity (Greger and Esteban 2007). Glutamate-gated ion channels belong to heterotetrameric cation channels which consist of three main subtypes: AMPA (α-amino-3-hydroxy-5-methyl-4-isoxasolepropionic acid) receptors, NMDA (N-methyl-D-aspartate) receptors and kainate receptors, all concentrated at postsynaptic sites. AMPA receptors, that are mostly Ca^{2+}-impermeable and displaying extraordinarily fast kinetics, are responsible for the primary depolarization in glutamate-mediated neurotransmission. They exhibit an enormous diversity in their electrophysical properies, that comes from various subunit compositions and the modifications introduced through RNA splicing and RNA editing. Four subunits, termed GluR1-GluR4 or, alternatively, GluR-A–GluR-D in different constellations form the channel. Most studied editing sites are present in the GluR-B subunit pre-mRNA (Seeburg, Higuchi et al. 1998) [*Fig. 1.6*]. Editing of the GluR-B Q/R site is performed by ADAR2 and the binding platform for the enzyme is formed between exon 11 and ECS located in the downstream intron (Higuchi, Single et al. 1993). Editing of this site was shown to be essential. Here, RNA editing leads to conversion of a glutamine (Q) codon (CAG) into an arginine (R) codon (CGG) with 99.9 % efficiency. The biological impact of this conversion is dramatic, with the residue found deep in the ion channel in the second transmembrane domain taking part in the receptor´s central channel formation. Assemblage of Q/R site-edited

24

subunits into the receiver significantly lowers the channel´s Ca^{2+} permeability (Sommer, Kohler et al. 1991). Editing at this site has also been shown to decrease the rate of receptor assembly and its transport to the synaptic membranes in cultured hippocampal neurons (Greger, Khatri et al. 2002; Greger, Khatri et al. 2003). Unlike GluR-B Q/R site in case of R/G site both versions, edited and unedited, coexist introducing more diversity. Editing of this site is not essential and plays a more subtle role in neuronal modulation. Deamination occurs at up to 80 % efficiency in three out of four AMPA receptor subunits (GluR-B, -C and –D). It is located in exon 13, at position -2 with respect to the 5´ splice site of intron 13. Also in this case a vital change in the codon occurs, with an arginine (R) codon (AGA) turned into a glycine (G) codon (GGA). This site exhibits varying editing levels during development and its physiological role is to alter calcium ion permeability and therefore increased recovery rates from desensitization (Kohler, Burnashev et al. 1993; Lomeli, Mosbacher et al. 1994). Besides editing in AMPA receptor subunits B, editing sites are present also in subunits C and D and also in kainate receptor subunits GluR-5 and GluR-6 (Seeburg, Higuchi et al. 1998).

Serotonin (5-hydroxytryptamine) is a neurotransmitter interacting with a large family of receptors to elicit signaling events that are crucial for proper neurotransmission. Serotonin binding to its transmembrane receptor is involved in several physiological functions, like production of cerebrospinal fluid, and behavioral processes such as: sleep regulation, appetite or pain for instance (Maas and Rich 2000). The human 5-hydroxytryptamine receptor subtype 2C (5-HT$_{2C}$R) is ubiquitously distributed throughout the central nervous system and is a member of G protein-coupled receptor superfamily that stimulates phospholipase to produce phosphates and diacylglycerol starting signaling cascades in the cell (Hoyer, Clarke et al. 1994). Five different editing sites have been detected within the *5-HT$_{2C}$R* transcript, which allow five different distinct amino acid changes [*Fig. 1.6*]. All five editing sites (A,B,C,D and E) are situated within the pre-mRNA segment encoding the second intracellular loop of 5-HT$_{2C}$R, a receptor domain important for G-protein coupling (Niswender, Sanders-Bush et al. 1998). While editing at sites A and B is accomplished by ADAR1, site D is deaminated by ADAR2. The C and E sites may be efficiently edited by both proteins (Wang, O'Brien et al. 2000). Experiments have revealed, that out of twenty four possible, at least seven isoforms in rat and twelve in humans are expressed resulting from alternative editing at five different sites by ADARs, which coexist on different levels depending on a part of brain examined (Burns, Chu et al. 1997; Niswender, Copeland et al. 1999). The receptor isoform whose pre-mRNA is edited at all five sites carries amino acid substitutions at positions 156, 158 and 160, where

the genomic DNA-encoded isoleucine, asparagine and isoleucine (I,N and I) are converted into valine, glycine and valine (V,G and V), respectively. Amino acid substitutions result in decreased G-protein coupling and less efficient triggering of the phospholipase C signaling cascade. The ligand affinity is also affected. The unedited version exhibits the highest and the fully edited version the lowest affinity for the neurotransmitter, while partially edited isoforms show intermediate affinity (Burns, Chu et al. 1997; Niswender, Sanders-Bush et al. 1998). Mutant mice with the knocked-in pre-edited (VGV) receptor version were showing elevated energy expenditure and loss of fat mass due to constitutive activation of the sympathetic nervous system (Kawahara, Grimberg et al. 2008).

Recently, editing in a third group of central nervous system receptors was reported in the mouse, namely in the gamma-aminobutyric acid type A (GABA$_A$) receptor subunit α3 (Gabra-3) [*Fig. 1.6*]. This receptor is the member of the Cys-loop ligand-gated ion channel superfamily and conducts chloride ions (Cl⁻) upon GABA-mediated activation. Editing of adenosine found in exon 9 within a short stem-loop structure leads to I/M (isoleucine to methionine) amino acid substitution in the transmembrane domain 3 in the subunit α3 of the five-subunit GABA$_A$ receptor changing its electrophysiological properties. Both ADAR1 and ADAR2 mediate adenosine deamination in this case on developmentally dependent levels; 50 % of transcripts are edited in newborn mice at day 2, while those in adult mouse brain are edited to almost 100% (Ohlson, Pedersen et al. 2007).
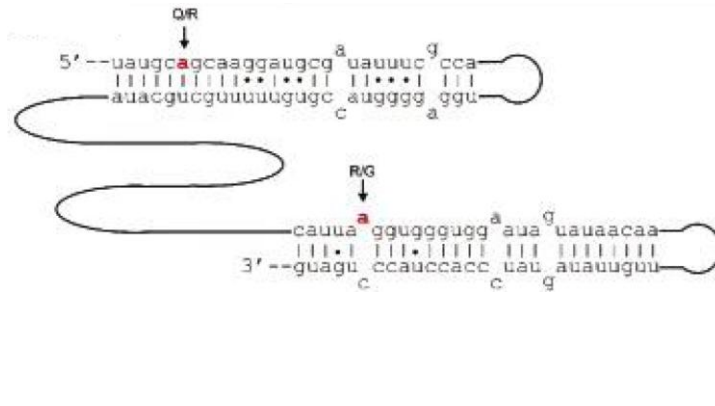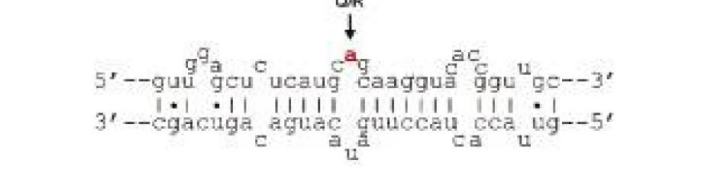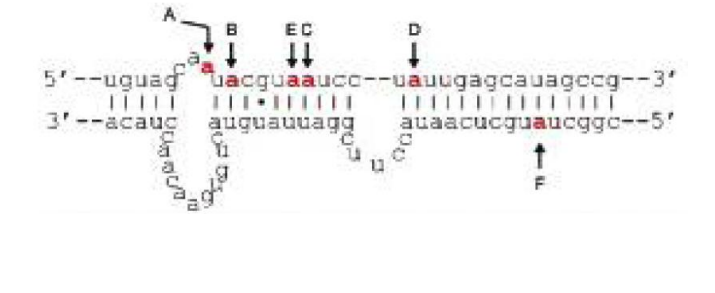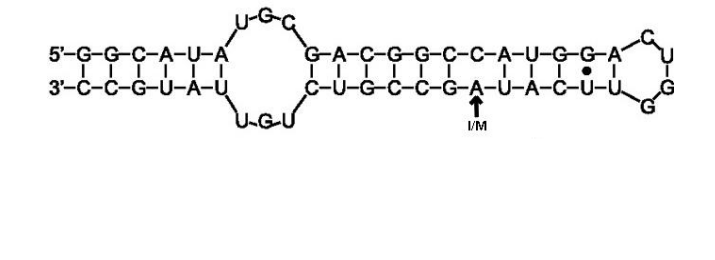
Also mammalian potassium channel mRNAs are edited with Kv1.1 as an example [*Fig. 1.6*]. This editing event leads to expression of an isoform, that recovers faster from inactivation of the receptor and reduces ability of the receptor subunits to tetramerize (Bhalla, Rosenthal et al. 2004).

Most coding substrates known in invertebrates and vertebrates are concentrated in the CNS. In *Drosophila melanogaster* transcripts of three different ion channels are edited: α1 subunit of Na^{2+}-channel (*para*), α1 subunit of Ca^{2+}-channel (*cac*) and α1 subunit of neurotransmitter-gated chloride channel (*GluCl-α1*) with still unknown implications although knock-out of *d*ADAR causes locomotion and behavioral disorders (Smith, Peixoto et al. 1998; Semenov and Pak 1999; Palladino, Keegan et al. 2000; Reenan, Hanrahan et al. 2000; Keegan, Brindle et al. 2005). Editing sites in sodium channel are found throughout the whole mRNA and include codon changes in transmembrane and intracellular domains (Palladino, Keegan et al. 2000).

In squid on the other hand the voltage-gated Kv2 potassium channel mRNA is targeted at multiple sites. Editing of two mostly edited sites affects channel closure upon repolarization (Patton, Silva et al. 1997).

Additional new targets in transcripts of coding genes were identified by different screens over the last years (Levanon, Eisenberg et al. 2004; Levanon, Hallegger et al. 2005; Ohlson and Ohman 2007; Gommans, Tatalias et al. 2008; Li, Levanon et al. 2009). Many of these editing sites were found in receptors, ion channels, and proteins involved in synapse function, trafficking, or membrane organization. The novel targets CyFip2 (cytoplasmic FMR1 interacting protein 2), Filamin A (Flna), and Filamin B (Flnb) are involved in different steps of actin reorganization. CyFip2 is part of WAVE complexes and within these complexes regulates actin nucleation (Takenawa and Suetsugu 2007). Flna and Flnb, however, are key regulators of orthogonal branching of actin filaments (Popowicz, Schleicher et al. 2006). Editing in Flna and Flnb is highly conserved in both proteins in a homologous region located at position -2 relative to a conserved 5`splice site.

Actin reorganization is essential for cell motility and migration and is an important determinant in dendritic spine and synapse formation (Dillon and Goda 2005; Popowicz, Schleicher et al. 2006). Another target protein for RNA editing, IGFBP7, has a proposed function in glioma cell growth and migration (Jiang, Xiang et al. 2008). Additional target proteins with functions in cell migration and/or adhesion are RSU-1, PTK2 (FAK), CRB2 and TRO (Li, Levanon et al. 2009). Numerous editing events were discovered also in proteins of less known function like Blcap (bladder cancer-associated protein) a protein differentially expressed in bladder cancer (Gromova, Gromov et al. 2002; Clutterbuck, Leroy et al. 2005; Levanon, Hallegger et al. 2005) [*Fig. 1.6*].

Editing substrate	Protein product	Editing enzyme	Effect
GluR-B 	glutamate receptor subunit B	Q/R site ADAR2 R/G site ADAR1 and ADAR2	Q/R ion permeability of channel R/G ligand desensitiza-tion recovery rate
GluR-5 	glutamate receptor subunit 5	ADAR2	ion permeability of channel
GluR-6 	glutamate receptor subunit 6	ADAR2	ion permeability of channel
5-HT$_{2C}$R 	Serotonin receptor 2C subtype	sites A, B ADAR1 site D ADAR2 sites C, E ADAR1 and ADAR2	G protein interactions
Gabra-3 	gamma-aminobutyric acid type A receptor subunit α3	ADAR1 and ADAR2	trafficking of the receptor

Kv1.1 5'--gaca u guac c gug caauug a ggca ga a ga u ug --3' |||| |||| |•• |||| |•||| || ||| 3'--cugu c caug cc cgu c guuaac ucgu ag gc cc --5' c c g u u ↑ gg IVV	Kv1.1 potassium ion channel	ADAR2	channel inactivation kinetics
Blcap Y/C ↓ 5'--cagagaucaugu**a**uugccucc--3' |||||||||||||•||||| 3'--gucucuaguacauagcggagg--5'	bladder cancer associated protein	ADAR1	unknown
ADAR2 5'--uuuug c ug g aggau g uu a uaaaug a aaauuu--3' •||•| || ||||| || |||||| |||||| 3'--gaagc ac uccua aa auuuac uuuaaa--5' a g g c g ↑ -1	ADAR2	ADAR2	expression of inactive splice variant
HDV antigenome amber/W ↓ 5'--gga u u ccgg-gga u u ccau a ggau a a cucuuccc--3' ||| ||•| ||| |||| |||| |•|||||| 3'--ccu-gguc ccu ggua ccua gggaaggg--5' aa a a	hepatitis delta virus antigen	ADAR1	expression of large or small antigen

Fig. 1.6: Table of few coding substrates of ADARs (adapted from (Maydanovych and Beal 2006)). Sequences of editing sites of: GluR-B, GluR-5, GluR-6, 5-HT$_{2C}$R, Gabra-3, Kv1.1, Blcap, ADAR2 and HDV antigenome are shown. Additionally data about protein product of each mRNA, enzyme editing particular site and biological effects of editing are provided.

1.5.2 Viral substrates

Shortly after ADARs were discovered a viral RNA isolated from the brain of a patient who died suffering a persistent measles virus infection gave indication of ADAR being responsible for changes in the viral RNA sequence (Cattaneo, Schmid et al. 1988). The editing of viral RNAs occurred promiscuously with up to 50 % of all adenosines modified. One of the pre-mRNA also found nonselectively deaminated is encoding the matrix protein of the measles virus, which is crucial in viral budding. This prevents the progression of the lytic cycle and forces the virus to remain in the persistent infection state (Sheppard, Raine et al. 1985). This alterations in cDNA sequences consistent with the ADAR enzymatic activity and

its neighboring preferences have been reported for many viruses afterwards. Beside of measles virus such hypermutation has been discovered in viral RNA isolates of host cells invaded by parainfluenza virus 3, respiratory syncytial virus and vesicular stomatitis virus (Murphy, Dimock et al. 1991; Rueda, Garcia-Barreno et al. 1994; Kumar and Carmichael 1997; Bass 2002). All of the above mentioned pathogens belong to the group of paramyxoviruses whose perfectly double stranded RNA genome requires an RNA-dependent RNA polymerase for the transcription into an mRNA. Another similarity among them is the exclusively cytoplasmic replication cycle. The obvious candidate to perform editing on such a substrates was (Bass 2002)the full-length isoform of ADAR1, ADAR1-i, which is transcribed from a promoter region containing an interferon-stimulated response element (ISRE) (George and Samuel 1999). Like most genes whose activation is triggered through interferon signaling, from the beginning ADAR1-i has been implicated in the cellular antiviral defense mechanism. Interferon synthesis is induced by dsRNA, a byproduct of the replication cycles of many RNA viruses, as the ones mentioned above, serving as the first line in defense against these viruses (Sen 2001; Sen and Sarkar 2007).

A limited number of viruses, however, have already adapted to ADAR1 activity and take advantage of the ADAR-mediated RNA editing. Most of these viruses use ADARs as a switch between early and late stages in their cycle. For example, during the late stage infection with the polyoma virus, newly synthesized giant multimer transcripts with complementary regions to early short transcripts form double-stranded regions, leading to the hyperediting of the latter. While the hyperedited early transcripts become export-incompetent, the late ones reach the cytoplasm and give rise to proteins fundamental for the transition from early to late stage infection. In the nucleus, such hypermutated RNAs, like the polyoma virus early transcripts, are anchored to the nuclear matrix by the inosine-specific RNA-binding protein p54[nrb], which forms a complex with the splicing factor PSF and the inner nuclear matrix protein matrin 3, thereby preventing escape of the promiscuously edited messages from the nucleus and, subsequently, their translation to the non-functional proteins (Kumar and Carmichael 1997; Zhang and Carmichael 2001).

There are also selective editing events described for viral RNAs, like in hepatitis delta virus (HDV) [*Fig. 1.6*], a subviral human pathogen that requires hepatitis B virus (HBV) for packaging. Its antigenome forms an imperfect double stranded structure interrupted with numerous bulges and loops. It encodes a single open reading frame, giving rise to hepatitis delta antigen (HDAg). Due to ADAR-mediated RNA editing, two forms of this protein are

expressed. The shorter form is expressed constitutively, while the adenosine deamination within the amber (UAG) stop codon gives rise to tryptophane (UGG) codon at the amber/W site, resulting in the extension of the open reading frame by additional 19 amino acids (Polson, Bass et al. 1996). The shorter protein (HDAg-S) plays a role in the viral genome replication, while the longer isoform (HDAg-L) leads to the replication inhibition and promotes the assembly of new viral particles (Ryu, Bayer et al. 1992).

Recently, also ADAR1-i was shown to stimulate human immunodeficiency virus type 1 (HIV-1) replication by using both editing-dependent and editing-independent mechanisms. Over-expression of ADAR1 in HIV-1 producer cells increases viral protein accumulation in an editing-independent manner. ADAR1-i has been shown to reverse the protein kinase R (PKR) inhibition of HIV expression. Additionally, ADAR1 associates with HIV-1 RNAs and edits adenosines in the 5' untranslated region (UTR) and the *Rev* and *Tat* coding sequences. It is believed ,that just like polyoma virus and HDV, HIV-1 has adapted in a way to make use of specific RNA editing activity of the host cell (Clerzius, Gelinas et al. 2009; Doria, Neri et al. 2009).

1.5.3   Non-coding substrates

Recent developments in the field of RNA editing indicate that mRNA recoding at specific sites may not be its sole function. Extensive bioinformatics screening for novel ADAR substrates revealed a completely new set of putative ADAR targets in non-coding regions such as introns and 5'- and 3'UTRs. Strikingly, the majority of editing sites were localized to SINE and LINE elements. Additionally, microRNA precursors Were identified as editing targets (Hoopengardner, Bhalla et al. 2003; Athanasiadis, Rich et al. 2004; Blow, Futreal et al. 2004; Kim, Kim et al. 2004; Levanon, Eisenberg et al. 2004; Ohlson and Ohman 2007; Gommans, Tatalias et al. 2008; Li, Levanon et al. 2009).

In primates, up to 85% of pre-mRNAs may be edited. This can be explained by the 1.4 million copies of Alu elements in the human genome that comprise just over 10,5 % of the entire genome (Jurka 2004). Alu elements rarely occur alone. Presence of two or three Alus in inverted orientation leads to their base-pairing and thus allows their editing (Morse, Aruscavage et al. 2002; Athanasiadis, Rich et al. 2004; Levanon, Eisenberg et al. 2004). It was determined that 90 % of newly discovered editing events reside in one of the members of Alu family indicating more global implication of their editing. These editing events can cause

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creation or elimination of splicing signatures and affect the alternative splicing of Alu-derived exons, but can also serve as a global regulatory mechanism in controlling RNA stability, export, splicing or translation (Athanasiadis, Rich et al. 2004; Levanon, Eisenberg et al. 2004; Lev-Maor, Sorek et al. 2007; Chen and Carmichael 2008; Chen and Carmichael 2009). It is also hypothesized, that RNA editing might influence this evolutionary process (Mattick and Mehler 2008).

miRNAs are synthesized from a dsRNA precursors which can serve as substrates for RNA editing. They are processed in two steps. Firstly a pri-miRNA, a short hairpin structure enclosed in a larger untranslated RNA transcript is cleaved in the nucleus by the microprocessor complex, consisting of Drosha and DGCR8, to 60-70 nucleotide long pre-miRNA. Pre-miRNA is exported to the cytoplasm and cleaved subsequently into a 20 to 22 nucleotide long siRNA-like duplex by Dicer-TRBP (transactivating response RNA-binding protein). One of both strands may serve as a mature miRNA. After integration into the miRNA induced silencing complex (miRISC) and binding to partially complementary targets in 3'UTRs miRNAs may block translation of the transcript or guide it for destruction (Bartel 2004; Kim 2005). Several studies indicate that all steps of miRNA biogenesis and miRNA function may be affected by A to I editing (Nishikura 2006; Ohman 2007; Nishikura 2009). Editing of certain adenosines in miRNA sequence may lead to inhibition of cleavage by Drosha and Dicer or change their substrate specificity (Scadden 2005; Yang, Chendrimada et al. 2006; Kawahara, Zinshteyn et al. 2007; Kawahara, Zinshteyn et al. 2007).

1.5.4   Other implications of editing

Recoding of gene information creating higher diversity in proteome is not the only implication of A to I editing performed by ADARs in the coding genes. One of the possible function of editing is affecting alternative splicing. Firstly, in more direct way a splice site may be destroyed by editing 3' acceptor splice site (AG – IG = GG) or created by editing future 3' acceptor site or 5' donor site (AA – AI = AG or AU – IU = GU respectively). Till now only few examples of this occurrence were discovered. One of them is the self-editing of mammalian ADAR2 pre-mRNA.. Editing converts AA dinucleotide into AI which is read as AG dinucleotide typically found at 3' splice sites. This new event causes incorporation of 47 nucleotide long intronal insertion, a frameshift and a production of only 88aa long polypeptide lacking dsRBDs and the catalytic domain. This process is clearly a negative

feedback loop, which in a tight way controls ADAR2 expression (Rueter, Dawson et al. 1999). Another example is the editing of the same process of creation of 3' splice site in intron of nuclear prelamin A recognition factor (NARF) gene. There the editing site is formed as an effect of folding two inversely repeated Alu elements. Thanks to this event a stop codon is eliminated and an intronic Alu is exonized (Lev-Maor, Sorek et al. 2007).

Less direct influence of ADARs on splicing comes from the fact that most of the platforms for editing are composed of exonic fragment and intronic fragment folding back to form a double-stranded structure. Edited adenosines are present ranging from one to few nucleotides from the splice sites. Such a pattern, which had to have implications on splicing inspired many scientific investigations. Looking only on most known mammalian editing substrates shows the importance of it. Editing at both the Q/R and R/G site is having a crucial influence on splicing of GluR-B pre-mRNA. Reduced editing levels at Q/R site cause aberrant retention of the downstream intron 11 (Higuchi, Maas et al. 2000; Schoft, Schopoff et al. 2007). On the other hand, the R/G site is situated only one nucleotide upstream of the 5´ splice site of intron 13 and influences the mutually exclusive flop and flip incorporation of exon 14 and exon 15, respectively (Schoft, Schopoff et al. 2007; Penn and Greger 2009). Also splicing of intron 5 of serotonin receptor 2C is affected by editing. Three alternative 5' splice sites are known at this junction. Only the first splice site following the editing sites and not the second or the upstream one lead to formation of a functional full-length protein. Completely unedited transcripts tend to be spliced with the upstream splice site resulting in truncated, non-functional protein. Most of the transcripts, which are edited in at least two editing sites are spliced from the proper splice site though. This process may be again an example of a control mechanism, which prohibits expression of the protein from completely unedited transcript (Wang, O'Brien et al. 2000; Flomen, Knight et al. 2004; Marion, Weiner et al. 2004).

Exonic and intronic regions of the pre-mRNA fold together to form a substrate for ADAR editing and splicing. This gives the possibility to interfere between these two processes. It also suggests a possible interplay between the two events and also a system that regulates it. *In vitro* studies on GluR-B editing sites have shown that editing can both stimulate and repress splicing efficiency. Lack of editing inhibits splicing at the Q/R site. Editing of both the Q/R nucleotide and an intronic editing hotspot are required to allow efficient splicing (Schoft, Schopoff et al. 2007). On the other hand the presence of an edited nucleotide at the R/G site reduces splicing efficiency of the adjacent intron facilitating

alternative splicing events occurring downstream of the R/G site (Schoft, Schopoff et al. 2007). However, the same site was found *in vivo* to efficiently edit and splice without interference, suggesting that the two RNA processing events are somehow coordinated (Bratt and Ohman 2003). The answer to this dilemma may lie in the interaction of ADAR2 with CTD of RNA polymerase II. CTD may work as a landing pad for different factors depending on the nature of the transcript coordinating the RNA processing events, enhancing or inhibiting RNA editing or splicing (Laurencikiene, Kallman et al. 2006).

Also evidence for nuclear retention due to A to I editing has been reported with p54[nrb] protein playing a pivotal role. A model, based on the 3'UTR of the mouse cationic amino acid transporter 2 (CAT2) transcript postulates, that p54[nrb] binds to edited inverted SINE repeats present in the 3'UTR leading to their sequestration to nuclear speckles. During stress *CAT2* mRNA (CAT-RNA) is posttranscriptionally cleaved and *de novo* polyadenylated to allow the production of a functional protein (Zhang and Carmichael 2001; Prasanth, Prasanth et al. 2005). Discovery of abundance of editing events in Alu repeats in human genes rose a possibility of a global regulation system directing all 3'UTR edited transcripts for nuclear retention. However, while some reports confirm this theory others show that the presence of double stranded structure in 3'UTRs and their editing has no effect on nuclear export. This raises doubt that inversely oriented repeats may influence nuclear retention of edited transcripts (Chen, DeCerbo et al. 2008; Hundley, Krauchuk et al. 2008). It was also reported that inosine-containing dsRNAs are reduced due to a lower abundance of the transcript and that such transcripts are likely to be found in a stress-granule-like-complex. This suggests that ADARs may take part in stress induced gene silencing via extensive mRNA editing and formation of stress granules in the cytoplasm (Scadden 2007).

A possible involvement of ADARs in heterchromatic gene silencing has also been proposed. ADAR1 and inosine containing RNA were found to form complexes with vigillin, the Ku86-Ku70 heterodimer, and RNA helicase A. Vigillin localizes to heterochromatin and the whole complex is involved in the phosphorylation of a set of targets important for heterochromatin silencing. Although these findings may hint to a role of ADAR1 in this kind of silencing a definitive proof is still missing (Wang, Zhang et al. 2005).

Besides ADARs editing miRNA there seems to be a more global interplay between A to I editing and RNAi (Nishikura 2006; Nishikura 2009). This revelation comes as no surprise considering that both processes rely on the presence of dsRNA. Studies of *adr-1* and *adr-2* null mutant worms indicate an equilibrium between ADAR and Dicer activities.

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Caenorhabditis elegans lacking A to I editing activity shows chemotaxis defects. These defects can be rescued by the absence of RNAi components. Although the precise process has not been discovered it seems, that expression of the genes involved in chemotaxis are controlled by the balance between editing and dicing (Tonkin and Bass 2003). Since dsRNAs are both substrates of ADARs and Dicer an antagonistic competition for substrate scenario was suggested in which editing of dsRNA would reduce the RNAi efficacy (Bass 2000; Nishikura 2006). It seems that Dicer is able to distinguish dsRNA containing I − U wobble pairs. dsRNA extensively edited (up to 50%) becomes resistant to Dicer cleavage (Scadden and Smith 2001). Inosine-containing dsRNA may be later degraded in the cytoplasm by a RISC-associated component, Tudor staphylococcal nuclease (Tudor-SN), which shows specificity towards A to I edited RNAs (Scadden and Smith 2001). However, the moderately edited dsRNA may be processed by Dicer to esiRNA (endoribonuclease-prepared siRNAs) the substitution of only one adenosine with inosine could reduce the efficacy of RNAi. Editing of targets of siRNA leads to generation of less esiRNA and also nonfunctional edited siRNA (Zamore, Tuschl et al. 2000). Additionally, an editing-independent way of antagonizing RNAi has been reported. Cytoplasmic ADAR1-i is induced by the presence of viral, dsRNA. Also introduction of siRNAs to the cell induces increased ADAR1-i expression as a response (Hong, Qian et al. 2005). It was reported that cytoplasmic ADAR1-i binds very tightly to siRNAs sequestering the RNA and therefore limiting its potency by decreasing its functional concentration (Yang, Wang et al. 2005).

Recently it was shown that ADAR1-i takes part in the regulation of gene expression. It binds to NF90/ILF3 (nuclear factor 90/NFAR/Interleukin enhancer-binding factor 3), which is a protein known to stimulate gene expression at the transcription and translation levels in vertebrates. ADAR1-i stimulates and regulates NF90-mediated gene expression and may be involved in translation control by facilitating interactions between the NF90 proteins and the dsRNA components of highly structured mRNAs (Nie, Ding et al. 2005).

1.6   Biological implications of A to I editing

Changes in the information stored in the genome can be achieved in few several ways: through gene mutations, deletions, duplications, but also through alternative splicing and RNA-editing. However, the former group represents permanent changes hardwired in the

genome while the latter occurs posttranscriptionally, at the RNA level. Alternative splicing and RNA editing has an advantage of a regulation possibility, causing its occurrence and therefore its implications ranging from low to high levels. This kind of control allows many different isoforms of one protein to exist simultaneously enriching its possible function, what seems to be most important in central nervous system where most known coding substrates of A to I editing were discovered. Different editing levels of diverse substrates differentially distributed in various brain subregions varying throughout developmental stages contribute in fine-tuning of neurotransmission and evolution of higher functions. It is naturally anticipated that malfunctions in editing would lead to diseases in human connected with learning, memory, thinking and behavior. In order to elucidate syndromes connected to ADARs malfunction knock-out animals in several species were obtained and studied showing, that the role of ADAR-mediated RNA editing became increasingly important during the course of evolution. Not surprisingly also some neurodegenerative diseases were linked to ADAR malfunctions. ADARs also seem to play an important role in tumorogenesis (Bass 2002; Maas, Kawahara et al. 2006; Nishikura 2009; Skarda, Amariglio et al. 2009).

1.6.1 Phenotype of mutant animals

Inactivation of ADAR genes has severe consequences in all tested systems. However it seems that the importance of A to I editing varies between invertebrates and vertebrates. In the former loss of ADAR activity leads to behavioral defects while loss of ADAR is lethal in mice for example(Bass 2002). Despite their viability, knock-outs of both *adr-1 and adr-2* genes in *C. elegans* show aberrant chemotaxis (Tonkin, Saccomanno et al. 2002; Ohta, Fujiwara et al. 2008). A knock-out of the *Drosophila melanogaster dADAR* gene causes mostly behavioral and motoric phenotype in flies. The adult specimens lacking dADAR exhibit various defects including: temperature-sensitive paralysis, locomotor discoordination and tremors and, finally, changes in grooming and mating behavior, all intensifying with age. Detectable anatomical defects are: changes observed in retina and liaisons in all areas of the brain (Palladino, Keegan et al. 2000). These findings suggest important but non-essential role of ADARs in invertebrates affecting integrity and function of central nervous system.

In contrast to invertebrate ADAR deletions, mammals show more severe phenotypes. In the case of *ADAR1* locus deletion, heterozygosity leads to birth of apparently completely

healthy mice. On the other hand, homozygous deletion leads to early embryonic death around day E11.5. Death occurs as an effect of halted developmental processes, distorted fetal liver structure and widespread programmed cell death the liver and hematopoietic lineage (Wang, Khillan et al. 2000; Wang, Miyakoda et al. 2004). This phenotype is most probably due to inappropriate induction of interferon signaling pathways and apoptosis of hematopoietic progenitor cells. This role of ADAR1 is attributed to the editing of as yet unidentified target involved in the interferon-induced cellular response (Hartner, Schmittwolf et al. 2004; Hartner, Walkley et al. 2009).

Also heterozygous *ADAR2* knock-out mice are viable and appear normal. Their homozygous mutant counterparts, however, die few weeks after birth, between postnatal days 0 and 20, exhibiting frequent epileptic seizures. This phenotype is a consequence of underediting at exclusively ADAR2-targeted Q/R site of the *GluR-B* pre-mRNA. This underedited site, normally edited up to 99,9%, is giving rise to a subunit incorporated in receptors with increased Ca^{2+} permeability in hippocampal neurons, leading to hippocampal neurodegeneration. Interestingly ADAR2 null mice mutants can be rescued by introducing mutation in GluR-B Q/R site mimicking its 'edited' state, which suggests that ADAR2 knock-out lethality is only due to underediting of this site (Higuchi, Maas et al. 2000).

Additionally, mutant animal models with permanently altered editing patterns of specific substrates have been created. Heterozygous mutant mice harboring an editing incompetent *GluR-B* allele that lacks editing complementary sequence (ECS) for GluR-B Q/R site were created. Importance of editing at this site was presented by increased Ca^{2+} permeability leading to epileptic seizures and premature death by 3 weeks of age (Brusa, Zimmermann et al. 1995). Mutant mice harboring hyper and hypo-edited alleles of serotonin receptor 2C (5-HT$_{2C}$R) were generated. Surprisingly not the latter, editing-blocked allele-expressing mice have shown a abnormal growth but the former, fully-edited allele-expressing mice. These mice had drastically reduced fat mass in spite of compensatory hyperphagia connected with constant excitation of sympathetic nervous system and consequent increase in energy expenditure. This finding show an importance of all editing variants of 5-HT$_{2C}$R and additionally would indicate a possibility of existence of yet unknown mechanism controlling fat metabolism and energy expenditure via *5-HT$_{2c}$R* mRNA editing (Kawahara, Grimberg et al. 2008).

1.6.2 Neurodegenerative diseases linked to ADAR

Most known and well studied examples of diseases linked to ADAR dysfunction are neuronal and neurodegenerative diseases. A possible connection between such a diseases and the most known editing substrates was made: amyotrophic lateral sclerosis (ALS) and epilepsy to underediting at the GluR-B Q/R and schizophrenia and depression to reduced or disregulated editing at $5\text{-HT}_{2C}R$ editing sites (Maas, Kawahara et al. 2006).

Amyotrophic lateral sclerosis is a progressive neurodegenerative disease mostly fatal 1-5 years after the onset (Cleveland and Rothstein 2001). In ALS motor neurons degenerate causing characteristic symptoms: muscle weakness, muscle atrophy, spasticity and eventually paralysis and respiratory failure. Glutamate excitotoxixity contribute to the pathology of ALS with elevated glutamate levels in 40% of sporadic ALS cases suggesting prolonged activation of glutamate gated channels, to which GluR-B belong. Prolonged exposure to Ca^{2+} ions is causing damage to motor neurons end eventually leads to their death (Spreux-Varoquaux, Bensimon et al. 2002). There may be many reasons of increased level of synaptic glutamate but increased permeability of GluR-B receptors due to underediting of GluR-B Q/R site is one of them. Decreased levels of editing at that site, ranging from 60-100% has been shown among few ALS patients (Kawahara, Kwak et al. 2003; Kawahara, Ito et al. 2004; Kawahara and Kwak 2005; Kwak and Kawahara 2005).

A role of underediting of GluR-B Q/R site in epilepsy has also been proposed. Epilepsy is a neurological disorder characterized by recurrent, unprovoked seizures (Delorenzo, Sun et al. 2005). Changes in receptor subunit expression, channel density and posttranscriptional regulation of AMPA-type glutamate receptors have been observed in epilepsy patients and animal models (Feldmeyer, Kask et al. 1999). As mentioned before phenotype of GluR-B editing deficient mouse models exhibit seizures resembling epilepsy (Brusa, Zimmermann et al. 1995; Feldmeyer, Kask et al. 1999; Higuchi, Maas et al. 2000). Comparison of models not expressing GluR-B, also resulting in higher permeability, and expressing unedited version of it show epileptic phenotype only in case of the second one suggesting that this phenotype may be connected with gating kinetics, channel conductance or its assembly and trafficking (Greger, Khatri et al. 2002; Greger, Khatri et al. 2003).

Until now no specific A to I editing target has been connected to a psychiatric disorder. such as schizophrenia or depression (Abdolmaleky, Thiagalingam et al. 2005). However, considering the role that serotonin plays in the central nervous system, it is believed that

reduced or disrupted ADAR activity could play a role in this kind of psychiatric disorders. Members of serotonin receptor (5-HTR) family are believed to play a major role in psychological and behavioral processes such as: emotion, appetite and sexual behavior. 5-HT$_{2c}$R is the only G protein-coupling receptor that is edited (Burns, Chu et al. 1997). There are few evidences of changes in editing pattern among suicide victims with documented chronic major depression. In comparison with healthy individuals, these patients had overedited E and C sites and an underedited D site. Since all of the affected sites are ADAR2 targets, altered function of this protein seems to be a likely cause of the symptoms (Gurevich, Tamir et al. 2002). Moreover, studies on mice and rat models have shown that the treatment with the antidepressant, serotonin-selective reuptake blocker, fluoxetine leads to exactly reverse changes in editing frequency at E, C and D sites when compared to the suicide victims, implying one of the possible mechanisms of action for this type of medication (Iwamoto, Nakatani et al. 2005). Unfortunately results regarding schizophrenia are not that clear with few reports claiming no difference in editing levels in patients diagnosed witch the disease and one showing increased level of expression of unedited version (INI) of 5-HT$_{2c}$R (Niswender, Herrick-Davis et al. 2001; Sodhi, Burnet et al. 2001; Dracheva, Elhakem et al. 2003; Iwamoto and Kato 2003).

In addition to studying glutamate R-B and serotonin 2C receptors expression levels of ADAR2 and RNA editing were studied in trisomy 21 (Down's syndrome) since Human ADAR2 is located on this chromosome. However, no effect of gene dosage was observed on the expression level of ADAR2 and consequently no impact on RNA-editing levels was observed (Kawahara, Ito et al. 2004).

1.6.3   ADAR in cancer

It was clear that ADAR, proteins that may change information carried by mRNA and affect many other processes, may take part in tumorigenesis. Misregulation of the editing pattern or editing levels of many vital substrates could lead to cellular instability and therefore to formation of tumors. Most of the tumors connected with misregulation of ADAR editing are the tumors of the brain (Skarda, Amariglio et al. 2009).

Underediting of GluR-B Q/R editing site was found in glioblastoma multiforme (astrocytoma grade IV), a common and most aggressive type of primary brain tumor in humans, involving glial cells and accounting for 52% of all parenchymal brain tumor cases

and 20% of all intracranial tumors (Maas, Patt et al. 2001; Iacob and Dinca 2009). Overexpression of Ca^{2+}-impermeable (R) versions of the receptors in the tumor cells inhibited cell locomotion and induced apoptosis. In contrast overexpression of Ca^{2+}-permeable (Q) versions of the receptor facilitated migration and proliferation of the tumor cells. Formation of this type of malignant gliomas may be due to downregulated expression of ADAR2 in proliferating cells (Ishiuchi, Tsuzuki et al. 2002). It was additionally demonstrated that high calcium permeability of AMPA receptors influences migration by activating the Akt pathway, that plays a key role in multiple cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcription and cell migration (Ishiuchi, Yoshida et al. 2007).

Also another type of malignant gliomas has been studied, many types of pediatric astrocytomas (type I-IV) and also here a correlation between ADAR2 activity and tumor mailgnancy has been detected (Cenci, Barzotti et al. 2008). Underediting of GluR-B Q/R site in pediatric astrocytomas (92-100%) is not as severe as in their adult counterparts (70-95%). Overexpression of ADAR2 leads to a decrease in malignant cell behavior. Indeed ADAR2 overexpression slows down the cell cycle during S-G_2 transition. In this type of tumor cells ADAR2 is expressed at normal level but ADAR1-c and ADAR3 are overexpressed leading to the assumption that higher concentrations of ADAR1 and 3 may influence the activity of ADAR2 (Cenci, Barzotti et al. 2008).

Beside the tumors originating from glial cells in the CNS other examples of cancerogenic processes connected to ADAR malfunctions are known. In patients with acute myeloid leukemia (AML) an aberrant editing event has been discovered. In comparison to healthy patients a transcript of tyrosine phosphatase PTPN6 (SHP-1), a tumor suppressor, isolated from $CD34^+$ and $CD117^+$ lymphocytes is edited at one of the splice branch points. This event leads to an expression of a non-functional protein. The alternative splicing due to this editing event decreases also in patients with remissions (Beghini, Ripamonti et al. 2000).

Recent bioinformatic approaches discovering editing in Alu element have also shown a global hypoediting process occurring in numerous tumor tissues. Beside brain tissues editing levels were decreased in prostate, lung, kidney and testicular tumors. Downregulation of all three, ADAR1, ADAR2 and ADAR3, was detected, which correlated significantly with malignancy of the tumors. However one of the known editing substrates of ADAR1, Blcap (bladder cancer-associated protein) Y/C editing site, was at the same time edited at higher level in the brain (Paz, Levanon et al. 2007).

1.6.4   Other diseases

Another disease has been found associated with ADAR1 malfunction: Dyschromatosis symmetrica hereditaria is a pigmenting genodermatosis (reticulate acropigmentation of dohi, symmetrical dyschromatosis of the extremities) present primarily in Japan and China, but has also been found to affect individuals from Europe, India, and the Caribbean, characterized by progressively pigmented and depigmented macules, often mixed in a reticulate pattern, concentrated on the dorsal extremities (Tomita and Suzuki 2004). The disease is an effect of numerous mutations occurring in front of methionine 296 of ADAR1, which cause expression of aberrant or truncated versions of ADAR1-i but don't influence expression of ADAR1-c. Twelve missense, nine frameshifts, eight nonsense and three splice site mutations discovered up to date cause only a mild heterozygous dominant phenotype not affecting mental or physical condition of the patients. It is speculated that numerous expressed aberrant variants of ADAR1 may quench wild-type ADAR1 activity by heterodimerizing with it forming a non-functional complex (Miyamura, Suzuki et al. 2003; Zhang, He et al. 2004; Cui, Wang et al. 2005; Gao, Wang et al. 2005).

Also a possible link between RNA editing and type 2 diabetes has been proposed. Mice chronically fed a high-fat diet exhibit an elevated level of ADAR2, but not ADAR1, transcripts and therefore elevated editing levels of primary ADAR2 substrates, such as: *ADAR2* and *GluR-B* transcripts in pancreatic islets and β-cell lines. These insulin-secreting cells play a central role in glucose metabolism and homeostasis responding to fluctuations of blood sugar levels by adequate insulin secretion. Additionally expressed in this cell line ionotropic glutamate receptors are responsible for insulin secretion. It still remains to be determined whether ADAR2 has any influence on insulin secretion (Gan, Zhao et al. 2006).

1.7   Regulation of ADAR

A poorly understood chapter of A to I editing is the regulation of ADAR activity. Activity of ADARs is regulated throughout developmental and in a tissue specific manner (Paul and Bass 1998; Jacobs, Fogg et al. 2009; Nishikura 2009; Tan, Huang et al. 2009). The activity of the enzyme depends on substrate specificity of it and is controlled by: alternative splicing, localization and dimerisation (Gerber, O'Connell et al. 1997; Lai, Chen et al. 1997;

Liu, George et al. 1997; Tan, Huang et al. 2009). Also sumoylation of ADAR1, a cofactor of ADAR2 and interaction between ADAR and the C-terminal domain (CTD) of polymerase II were discovered. (Desterro, Keegan et al. 2005; Macbeth, Schubert et al. 2005; Laurencikiene, Kallman et al. 2006) Until now no other factors that influence ADAR activity were found and it is not known whether such factors exist.

1.7.1 Developmental und tissue-specific expression of ADARs

All three members of ADAR family are developmentally controlled. Experiments in mice have shown that ADAR1 and ADAR2 are expressed at embryonal stage day 10 (E10.0) with the highest editing level in embryonic heart (Wang, Khillan et al. 2000; Reymond, Marigo et al. 2002; Wang, Miyakoda et al. 2004). At this stage expression of ADAR1 is downregulated by miRNA-1 (Lim, Lau et al. 2005). Start of expression of ADARs at this early developmental stage perfectly coincides with embryonal lethality of homozygous knock outs of ADAR1 in mice at E12.5 (Hartner, Schmittwolf et al. 2004). From that point on the expression level of ADAR1 mRNA stays constant while ADAR2 expression increases in the brain to reach its peak in the adult, as shown in the rat model (Hang, Tohda et al. 2008). However a better studied mouse model shows increase of expression of both proteins through development, with both enzymes widely distributed in brain around birth (Jacobs, Fogg et al. 2009).

Analyses of expression of ADAR1 and ADAR2 have also shown a tissue specific expression of ADARs (Kim, Wang et al. 1994; O'Connell, Krause et al. 1995; Melcher, Maas et al. 1996; Paul and Bass 1998). Measured total inosine level in most tissues perfectly correlate with ADARs expression, that follows the pattern: brain>lung>heart>skeletal muscle (Paul and Bass 1998). Many studies performed on specific editing substrates have confirmed this finding (Nishimoto, Yamashita et al. 2008; Riedmann, Schopoff et al. 2008). Expression of ADARs in the brain appears at E15.0, with expression of ADAR1 higher than ADAR2, and reaches its peak at P21 in the adult mouse. The highest levels of RNA editing have been detected in the thalamus and hypothalamus and the lowest levels in cortex and hippocampus with editing levels about 50% lower than on the average in the rest of the brain. ADARs are not only differentially expressed in different tissues but also cell specific. Both ADAR1 and ADAR2 are detectible in neurons but not in astrocytes, for example (Paupard, Friedman et al. 1997; Jacobs, Fogg et al. 2009).

Not surprisingly also editing levels of most known coding substrates change during development. Editing in mice starts to be detectible at low levels at day E15 followed by a rapid increase until just after birth (P0-P2). This is followed by a slow increase until P21 when editing levels of most of the substrates reach their maximum. On the other hand there are some editing sites that stay at the same editing level throughout development from day E15 till the adult mouse. The most extreme example is the GluR-B Q/R site with editing at E15 already over 90% and increasing only minimally in development. Also C and E editing sites of 5-HT$_{2C}$R show this pattern (Bass 2002; Du, Davisson et al. 2006; Ohlson, Pedersen et al. 2007; Daniel and Ohman 2009; Jacobs, Fogg et al. 2009).

However in many cases expression of ADARs does not correspond to editing of certain substrates. GluR-B Q/R site and D site of 5-HT$_{2C}$R are edited at embryonal day 15 of mouse embryos to 90% and 50% respectively, when ADARs are barely detectable (Jacobs, Fogg et al. 2009). Additional studies have shown that expression of ADAR2 does not correspond temporally and spatially throughout development with its prime substrates (Hang, Tohda et al. 2008) (Liu, Emeson et al. 1999; Seeburg 2000). Also editing of the K/E site of CyFip2 nicely correlates with ADAR2 expression and in many tissues. However, in lung and leukocytes editing is not detectable although ADAR2 levels are high (Nishimoto, Yamashita et al. 2008). In malignant gliomas hypoediting of GluR-B Q/R and 5-HT$_{2C}$R sites was detected with no significant change in expression of ADAR2 (Maas, Patt et al. 2001). Similar results were obtained from pediatric astrocytomas, also suggesting that abnormal overexpression of ADAR1 and ADAR3 may form heterodimers with ADAR2 sequestering it (Cenci, Barzotti et al. 2008). A global decrease of Alu element editing was shown in different types of tumors due to a general decrease of ADAR expression. However Blcap that is exclusively edited by ADAR1 showed a higher editing activity in tumors versus controls in brain, oral cavity and lung (Paz, Levanon et al. 2007).

1.7.2 Regulation of ADAR activity

Today, already a few ways are known how expression and activity of ADARs can be regulated (Tan, Huang et al. 2009).

One mode of regulation is represented by self-editing of ADAR2 transcript. The editing of rat ADAR2 occurs in 6 different positions in intron 4 and exon 5. Editing in intron 4 converts AA dinucleotide into AI, which is read as AG a canonical 3' splice site [*Fig. 1.6*]. It

results in addition of 47 nucleotides to exon 5 generating a frameshift. This results in synthesis of a truncated non-functional protein (Rueter, Dawson et al. 1999). Transgenic mice lacking ADAR2 self-editing activity have elevated editing levels of some ADAR2 substrates indicating editing of ADAR2 mRNA being a negative feedback loop mechanism, in which ADAR2 regulates its own expression (Feng, Sansam et al. 2006). Interestingly in *Drosophila melanogaster* editing occurs in the catalytic domain changing the properties of the *d*ADAR (Palladino, Keegan et al. 2000).

Widespread regulation of ADARs occurs on a level of alternative splicing, as an effect all of them occur in many isoforms. Existence of alternative splicing variants results in differential tissue- and developmental-specific expression of them and also different activity. Two additional ADAR1 variants contain deletions, either one or two, between catalytic domain and dsRBD3 and dsRBD2 and dsRBD3 changing distances between them. All variants are ubiquitously expressed, but splice variant containing both deletions at the same time is expressed at the lower level that the variants with only one deletion (Liu, George et al. 1997). The various splice variants of ADAR1 are active enzymes, with comparable specific activity. All three human ADAR1 variants possess editing activity for the A, B, and C editing sites of $5-HT_{2C}R$ but were inactive for the D site. These results indicate that the two deletions in ADAR1 mRNA do not significantly affect the site-selectivity for editing the $5-HT_{2C}R$ RNA substrate. However, they displayed differential activities towards these sites resulting in different editing levels (Liu, Emeson et al. 1999). Also editing of GluR-B R/G site shows differences in activity (Liu and Samuel 1999).

Also four known classical human ADAR2 splicing variants are expressed in a different manner. The variants with Alu cassette inclusion in the catalytic domain are expressed at the higher level (Lai, Chen et al. 1997). Additionally the presence of Alu cassette in the sequence in human version of the protein reduces catalytic activity of ADAR2 by 50% (Gerber, O'Connell et al. 1997). Also newly discovered splicing variant containing exon 0 is expressed in a tissue-specific manner, with the highest level in mouse cerebellum (Maas and Gommans 2009) Also an inclusion of a novel alternatively spliced exon 7a, located within intron 7 of the human ADAR2 gene, is more prominent in some tissues with the highest levels of inclusion in skeletal muscle, heart and testis. In the brain, where the level of editing is known to be high, the level of exon 7a inclusion is low (Agranat, Sperling et al. 2010). Overall the evidence presented indicate, that different splice variants of ADAR1 and 2 are differentially expressed and possess different activities.

As presented earlier the evidence about homodimerisation of ADARs is not clear and sometimes contradictory (O'Connell and Keller 1994; Cho, Yang et al. 2003; Gallo, Keegan et al. 2003; Macbeth, Lingam et al. 2004; Macbeth, Schubert et al. 2005). Although it is known, that homodimerisation happens but most probably is not essential for the editing reaction it is still not sure if ADARs could heterodimerise. However, some evidence could indicate this process. For example, an overexpression of ADAR1 in pediatric astrocytoma could inhibit editing activity of ADAR2. This and the finding that ADAR1 can be precipitated along with ADAR2 suggest that this kind of complexes could be formed (Cenci, Barzotti et al. 2008). Unfortunately these results were obtained without RNAse treatment and one cannot dismiss RNA acting as a bridge between the proteins. A reduction in ADAR activity was also observed in *Drosophila* when active and inactive versions of dADAR were expressed (Gallo, Keegan et al. 2003). Although this findings may be still an effect of competitive binding of ADARs to substrate the possibility of yet another ADAR regulation system exist. One could imagine various splice variants of ADARs with different editing activities affecting each other via formation of dimers. Also catalytically inactive ADAR3 could form heterodimer sequestering active ADARs.

Also cellular localization has a great influence on ADAR activity. Both ADAR1 isoforms, ADAR1-i and ADAR1-c can shuttle from nucleus to the cytoplasm with the former being predominantly cytoplasmic and the latter nuclear. ADAR2 and ADAR3 are predominantly nuclear (Eckmann, Neunteufl et al. 2001; Poulsen, Nilsson et al. 2001; Desterro, Keegan et al. 2003; Fritz, Strehblow et al. 2009). ADARs are localized to the compartments where they are needed most. ADAR1-i in the cytoplasm as a response to viral infection and ADAR1-c and ADAR2 in the nucleus to perform post-transcriptional editing on mRNA. It was shown that high abundance of RNA in the nucleolus may tether ADAR1 and ADAR2 to this compartment. That would explain characteristic nucleolar localization of ADAR2. The process is reversed via inhibition of rRNA synthesis or overexpression of an editing substrate. Only when editing substrates accumulate in the nucleoplasm ADAR2 is released from the nucleoli (Desterro, Keegan et al. 2003; Sansam, Wells et al. 2003). It was also demonstrated that although both ADAR2 and ADAR1 are present in nucleolus, only ADAR2 is able to edit a substrate expressed from Pol I in this compartment (Vitali, Basyuk et al. 2005).

One of the most obvious processes affecting enzyme activity are also post-translational modifications. Human ADAR1 has been found to be sumoylated. An ubiquitin-like protein

SUMO, which can affect protein localization, stability and activity modifies ADAR1 at lysine 418, which lies between the Z-domain and the first dsRBD. Upon this modification, activity of the enzyme is substantially reduced. This suggest an important role of SUMO in control of ADAR1 activity (Desterro, Keegan et al. 2005). Although many possible phosphorylation sites for ADARs have been predicted until now no such modification was reported (Blom, Sicheritz-Ponten et al. 2004).

Additionally any change in structure of dsRNA and modification to RNA in vicinity of the editing site could affect editing activity. Possibilities are infinite starting with proteins binding to RNA, changing allosteric properties of the editing site, unwinding of RNA by helicases to modification of the RNA backbone or bases. Since both A to I editing and splicing are taking part post-transcriptionally and most of the known editing substrates are formed in between an exon and an intron this two processes compete for the same substrate. The moment splicing occurs cutting out the intron harboring the ECS editing cannot occur (Bratt and Ohman 2003). A single modification at the editing site can also influence editing level of this site. A 2'-O-methylation C editing site of 5-HT$_{2C}$R is guided by C/D snoRNA MBII-52 and results in complete inhibition of editing at that site without affecting a nearby D editing site (Yi-Brunozzi, Easterwood et al. 1999; Vitali, Basyuk et al. 2005).

1.7.3   Interactors of ADARs

Even less is known about RNA, proteins or small molecules that would bind to ADARs modulating their activity. A crystallographic study of the catalytic domain of ADAR2 has revealed the presence of a small molecule buried deep in the active site of the enzyme. Inositol hexakisphosphate (IP$_6$) serves as a cofactor of the protein and is essential for its function, which was proven by expressing ADAR2 in a yeast strain deficient in the last step of IP$_6$ synthesis. It seems that this molecule is acquired during ADAR2 folding. Since IP$_6$ was also found bound to ADAT1, but not ADAT2 and ADAT3, it seems that its function is conserved and it will be found also bound to other ADARs (Macbeth, Schubert et al. 2005) [*Fig. 1.7*]. IP$_6$ is an abundant molecule implicated in many cellular functions including: RNA export, DNA repair, endocytosis and chromatin remodeling (York, Odom et al. 1999; Hanakahi and West 2002; Hoy, Efanov et al. 2002; Shen, Xiao et al. 2003; Steger, Haswell et al. 2003). It also serves as a cofactor of deoxyhemoglobin and clathrin adaptor complex (AP2) (Arnone and Perutz 1974; Collins, McCoy et al. 2002). Interestingly IP$_6$ is affecting AMPA

receptors via increasing presence of GluR-A and GluR-B subunits in the functional receptor (Valastro, Girard et al. 2001). Also connection of IP_6 to serotonin receptor 2C allows us to raise some speculations about its role in regulation of ADAR editing. This type of receptor upon excitation activates a phospholipase C a first agent in the cellular response cascade. A cell membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) is cleaved into diacylglycerol and inositol 1,4,5-triphosphate (IP_3), which subsequently is phosphorylated to form IP_6. Since $5\text{-}HT_{2c}R$ is a substrate of editing it poses a possibility of a feedback loop. In presence of increased stimulation by serotonin more IP_6 and therefore ADAR2 is produced, which by more active editing of the receptors transcript lets only receptors less sensitive to the ligand to be expressed (Macbeth, Schubert et al. 2005).
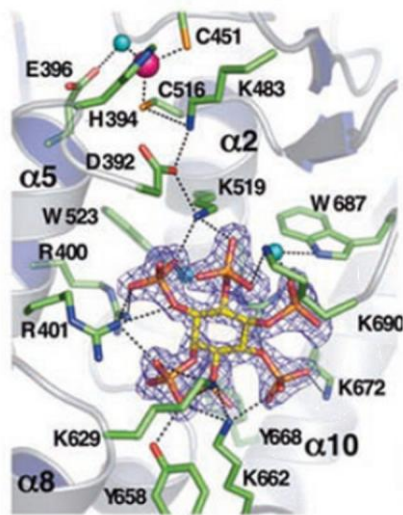

Fig. 1.7: Stereo image of IP_6 buried in binding site of ADAR2. (from (Macbeth, Schubert et al. 2005)) The zinc ion (magenta sphere) and E396 residue are coordinating the nucleophilic water (aqua sphere). Underneath Ip_6 molecule is visible (yellow sticks) interacting with conserved residues (green) of active site via hydrogen bonds, which in case of W523 and W687 are mediated by water.

It was also reported that ADAR2 interacts with the C-terminal domain (CTD) of polymerase II. Thus changes in pol-II phosphorylation and associated factors could act as developmental modulators of editing. Although the CTD is required for successful splicing in general it showed not to be the case for splicing of ADAR2 intron 4 at either the normal 3' splice site or the alternative site created by editing. Surprisingly, the CTD is required for efficient co-transcriptional auto-editing of ADAR2 intron 4, which would implicate an important role of the CTD in site-selective RNA editing by ADAR2 and in coordination of editing with alternative splicing. Expression of polymerase II without a CTD has gravely reduced ADAR2 editing. The CTD of polymerase II could serve as a landing pad for different factors depending on the nature of the transcript. Either CTD directly binds ADAR2 or one of CTD-associated factors is (Laurencikiene, Kallman et al. 2006).

Also the already known relationship between RNA editing and life cycles of few viruses presented some candidates of molecules, that interact with A to I editing. In the life cycle of previously mentioned hepatitis B virus (HBV) ADAR1 induces a switch between expression of two variants of hepatitis delta antigen (HDAg). The editing at this site cannot occur before replication of the genome is not completed because the expression of HDAg-L leads to the replication inhibition. It was discovered that HDAg inhibits editing at amber/W site not allowing this site to be edited completely. However since it is believed that HDAg can bind to HDV RNA the inhibition of editing occurs most probably via interference by binding near or at the editing site (Polson, Ley et al. 1998).

Another viral molecule inhibiting ADAR1 activity is a small and highly structured regulatory RNA expressed by Adenovirus, VAI RNA (Ma and Mathews 1996). It accumulates in high concentrations at the late times after viral infection and antagonizes IFN-induced antiviral response (Soderlund, Pettersson et al. 1976; Kitajewski, Schneider et al. 1986). Its main target is RNA-dependent protein kinase PKR (Samuel 1991). It was shown that VAI RNA can also inhibit the activity of endogenous and overexpressed ADAR1 (Lei, Liu et al. 1998). Most probably VAI RNAis competing with other substrates for dsRBDs of ADAR1 or binds to ADAR changing its conformation to a less favorable one.

Another factor, this time a protein, inhibiting ADAR1 activity is E3L protein expressed from DNA of vaccinia virus. Vaccinia virus (VACV or VV) is a large, complex, enveloped virus belonging to the poxvirus family. E3L is a short 190 amino acid-long protein bearing a Z-DNA binding domain and one dsRBD important for viral replication and inhibition of IFN-induced antiviral response (Beattie, Tartaglia et al. 1991; Chang, Watson et al. 1992; Chang and Jacobs 1993; Davies, Chang et al. 1993; Chang, Uribe et al. 1995; Patterson and Samuel 1995; Herbert, Alfken et al. 1997). A-to-I RNA editing activity of ADAR1-i is impaired by the product of the vaccinia virus E3L. It weakly inhibits the site-selective editing activity by ADAR1 at the GluR-B R/G site and the A site of serotonin 2C receptor. The C-proximal dsRBD of E3L was essential for antagonism of ADAR1. The mechanism of ADAR1 inhibition by E3L is still unknown (Liu, Wolff et al. 2001).

Beside of endogenously discovered interactors of ADARs, attempts are made to influence ADAR activity via creation of synthetic compounds. Already after the discovery of ADAR1 from *Xenopus leavis* attempts to find inhibitors of ADAR activity were made. Not surprisingly most of the active molecules (iodoacetic acid, N-ethylmaleimide or p-hydroxymercuriphenylsulfonic acid) were reagents known to interact with cysteine residues,

which in the active site of ADARs functions in coordinating the zinc ion (Hough and Bass 1994) Inhibition occurred also upon treatment with basic proteins, intercalators and high salt conditions but not acidic proteins, ATP, GTP, Mg^{2+}, Ca^{2+}, EDTA or EGTA. Also high doses of RNA, not allowing the formation of homodimers, inhibited the activity (Hough and Bass 1994). Studies with nucleoside analogues have also been performed. One of the candidates is 8-azanebularine (8-azaN), which is characterized by a very high propensity to undergo covalent hydration of the C6-N1 double bond. After this reaction this analogue would perfectly mimic a transition state of adenosine deamination, in that way trapping ADAR in unfinished reaction. Inhibition was obtained only at the high concentrations of the analogue (Veliz, Easterwood et al. 2003). In order to make a perfect substrate for ADARs for base-trapping inhibition this analogue has to be incorporated into RNA since ADARs preferentially deaminate adenosines in dsRNA. Attempts to create a phosphoramidite containing this nucleoside for RNA synthesis were also made (Haudenschild, Maydanovych et al. 2004). Also studies with molecules binding next to editing sites were performed. Recently a helix-threading peptide binding near editing sites of 5-$HT_{2c}R$ has been designed and proven to perform a substrate-specific inhibition of these sites (Schirle, Goodman et al. 2010).

1.8   DSS1

DSS1 is a short 70 amino acid long and very acidic protein. This polypeptide contains three α-helical folds and two β sheets following first α helix [*Fig. 4.40*]. Three long aspartic acid/glutamic acid-rich stretches contribute to its acidity, 37% acidic residues. Also its content of aromatic amino acids is high (Yang, Jeffrey et al. 2002).

The gene was originally identified as a gene deleted in patients with SHFM (split hand/foot malformation) type 1, a developmental disorder in humans (Scherer, Poorkaj et al. 1994; Crackower, Scherer et al. 1996). Although a tissue-specific expression profile of DSS1 was detected, in limb bud, craniofacial primordia and skin, deletions in mice in other genes in the cluster, *DLX5* and *DLX6* have lead to the phenotype similar to SHFM, which may indicate, that they, and not *DSS1*, are responsible for this disease (Merlo, Paleari et al. 2002; Lo Iacono, Mantero et al. 2008).

DSS1 is a very abundant protein present in the nucleus and the cytoplasm. DSS1 has been shown to be a part of 26S proteasome particle where it binds to the 19S regulatory

particle both in mammalian cells and in fission and budding yeast. This binding is possibly mediated by lid components Rpn3 and Rpn7, with separate binding sites in DSS1 for both proteins (Coux, Tanaka et al. 1996; Sone, Saeki et al. 2004). The function of DSS1 on the proteasome was connected in regulating the proteasome interactions and utilizing a specific subset of poly-ubiquitinylated p53 protein as a substrate (Funakoshi, Li et al. 2004; Krogan, Lam et al. 2004; Gudmundsdottir, Lord et al. 2007; Wei, Williams et al. 2008). Accumulation of poly-ubiquitinylated proteins occurs in *sem1* mutants, the *DSS1* homologue in *Saccharomyces cerevisiae*. These Sem1 deleted cells are temperature-sensitive, ceasing to grow at elevated temperatures. In addition, loss of Sem1 triggers or enhances a cell differentiation process in diploid *S. cerevisiae* cells but has no influence on viability of the organism (Jantti, Lahdenranta et al. 1999; Marston, Richards et al. 1999; Funakoshi, Li et al. 2004; Sone, Saeki et al. 2004).

DSS1 also plays a role in the control of DNA repair and therefore in tumorogenesis. Several studies show DSS1 interacting with BRCA2 (breast cancer type 2 susceptibility protein) responsible for homologous recombination of double strand breaks in DNA. Amino acids in DSS1 responsible for interactions with BRCA2 are highly conserved from yeast to mammals. Loss of either BRCA2 or DSS1, both in mammalian and fungal cells, results in defects in homologous recombination. DSS1 has been shown to influence affinity of BRCA2 to ssDNA, therefore affecting its activity. A model based on the crystal structure of DSS1 bound to BRCA2 proposes DSS1 mimicking oligonucleotides due to its acidic and aromatic content and in that way binding to BRCA2, changing conformation of the protein and the affinity to ssDNA (Marston, Richards et al. 1999; Yang, Jeffrey et al. 2002; Zhou, Kojic et al. 2009). Additionally proteasomes have been shown to bind to double-stranded breaks in DNA, and specifically BRCA2 binds the proteasome lid components Rpn3 and Rpn7, similar to DSS1. However, DSS1 does not regulate BRCA2 affinity for the proteasome (Krogan, Lam et al. 2004; Gudmundsdottir, Lord et al. 2007).

The knock-out animal model for the gene encoding the proteasome subunit DSS1 in *Caenorhabditis elegans* 1/Rpn15/Sem1 has been studied. In *C. elegans dss-1* encodes an essential protein, which is required for embryogenesis, larval growth, and oogenesis. The most similar phenotype to *dss-1* knock-out worms have *kgb-1* mutants. In *kgb-1* mutants an increased level of the GLH-1 RNA helicase appears to result in a defective germ line. In *dss-1* mutants GLH-1 levels were normal suggesting that KGB-1 and DSS-1 are not involved in the same regulatory process. It is possible that the oogenesis defects in *dss-1* mutants might be

caused by the aberrant regulation of degradation of another essential protein required for oogenesis, in combination with errors in homologous recombination (Orsborn, Li et al. 2007; Pispa, Palmen et al. 2008).

Recently a polymorphism in human DSS1 was associated to skin squamous cell carcinoma. Guanosine at the position 143 is found more frequent as an adenosine substitution in this type of tumor. This substitution leads to less effective expression and more cytoplasmic localization of the protein (Venza, Catalano et al.).

Last but not least, budding and fission yeast homologues of DSS1 Sem1 were shown to be involved in splicing and in mRNA export pathways. Studies in *S. pombe* show involvement of this protein in crucial interactions with export factor Rae1p FG nucleoporins in vivo and in vitro indicating its essential role in mRNA export (Thakurta, Gopal et al. 2005). Also studies in *S. cerevisiae* indicate a similar function of Sem1. Building an interaction map using the Epistatic MiniArray Profile (E-MAP) approach helped to link this protein to mRNA export and splicing (Wilmes, Bergkessel et al. 2008). Like in case of fission yeast Sem1 was shown to be a part of mRNA export pathway in budding yeast, of TREX-2 (nuclear pore bound TREX-2 complex). *sem1* deletion mutants negatively influence targeting of Thp1, an essential subunit of TREX-2, to the complex. Additionally these mutants show an impairment in transcription of long GC-rich genes, that leads to higher susceptibility to mutations (Faza, Kemmler et al. 2009).

2   Specific aims

Regulation of ADAR activity is a poorly understood topic in A to I editing. Still many questions remain unanswered. With this work a search for endogenous molecules that modulate ADAR activity was made.

1. Designing and performing a functional editing screen.

The main goal of this work was to design an *in vivo* yeast editing system, that could be employed in a screen for endogenous molecules modulating the activity of ADARs. This system would be based on changes in ADAR activity and not only on protein-protein interactions broadening in this way the spectrum of potential candidates. The next step in this work was, to employ this system in a genome-wide screen.

2. Verifying and confirming results of the screen.

Candidates obtained in the screen would be verified for the false positives and afterwards tested in the mammalian system in order to confirm their relevance in an organism, that unlike yeast possesses *ADAR* genes.

3. Investigating mode of action of positive candidates.

If any of the candidates from the screen were also confirmed to have a positive effect in the mammalian system, the mode of action of it would be investigated.

3   Materials and Methods

3.1 In vivo yeast editing system

3.1.1 Basic strain

Yeast strain used in the editing system was haploid *Saccharomyces cerevisiae* strain W303 (MATa, *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15).*

3.1.2 Editing substrate vector

A vector expressing *HIS3* as a auxotrophy marker gene containing amber stop codon, which served as an editing site at the same time was created. All the procedures were carried out on pUC18 leu2::HIS3 vector. Firstly a unique XhoI restriction site was introduced via *in vitro* mutagenesis in the 5' translated region of the *HIS3* gene. Thymine, the 101st nucleotide of *HIS3* was substituted by guanosine. For selection of integrants the *KanMX4* gene was introduced. *KanMX4* was cut out of pFA6-KanMX4 vector using SmaI and SacI restriction sites and after blunting introduced into previously blunted NsiI restriction site downstream of *HIS3* gene. Amber editing substrate was obtained by cloning of annealed and filled complementary oligonucleotides containing XhoI restriction sites into the XhoI site of *HIS3*, creating pUC18 HIS3amber construct. Vector for a negative selection carrying *URA3* as a auxotrophy marker was constructed on the base of vector carrying *HIS3*. In order to do that *URA3* was amplified from YCplac33 expression vector and cloned into *HIS3* gene using MscI and NheI sites removing at the same time 296 nucleotides of the gene [*Fig. 3.1*].
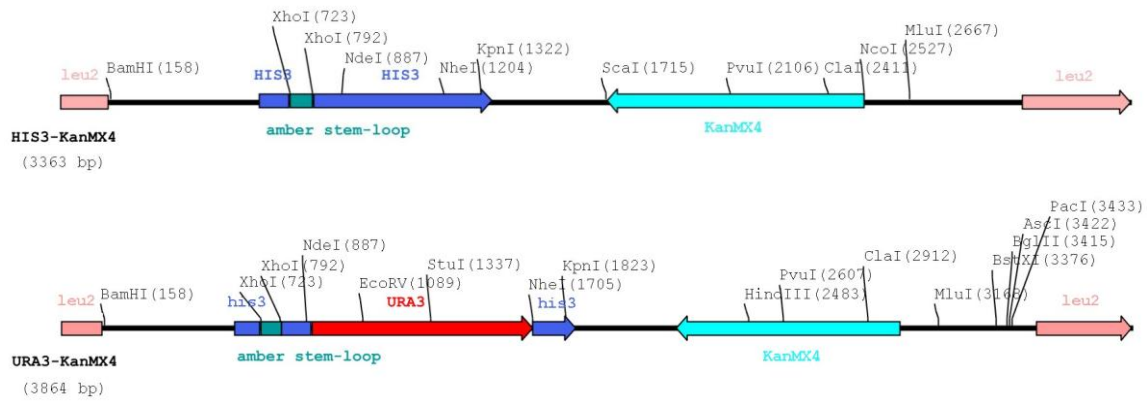
Fig. 3.1: HIS3-amber and URA3-amber integrative constructs. An architecture of a integrative fragment, consisting of editing substrate in the yeast marker gene and accompanied by KanMX4 resistance gene, both disrupting LEU2 gene, is shown. Fragment was carried on pUC18 plasmid and was cut with SacI and XbaI restriction enzymes prior to genomic integration.

3.1.3   ADAR expressing vectors

Human *ADAR1* and rat *ADAR2* were cloned into a centromeric tetracycline-inducible expression vector for *Saccharomyces cerevisiae*. Construct pCM251 containing two copies of tetO boxes and *TRP1* as a yeast marker gene, that have been developed in the group of Enrique Herrero (Lleida, Spain), is utilizing a reversible gene expression system, where a repressor halting gene expression is released after an induction with tetracycline or doxycycline (Arino and Herrero 2003; Yen, Gitsham et al. 2003).

3.1.3.1 Human ADAR1 expression construct

Human *ADAR1* has been cut out of pEGFP-C3-hsADAR1 vector with XhoI, blunted and subsequently with ClaI and inserted into ClaI and blunted PstI restriction sites of pCM251 [*Fig. 3.2*].
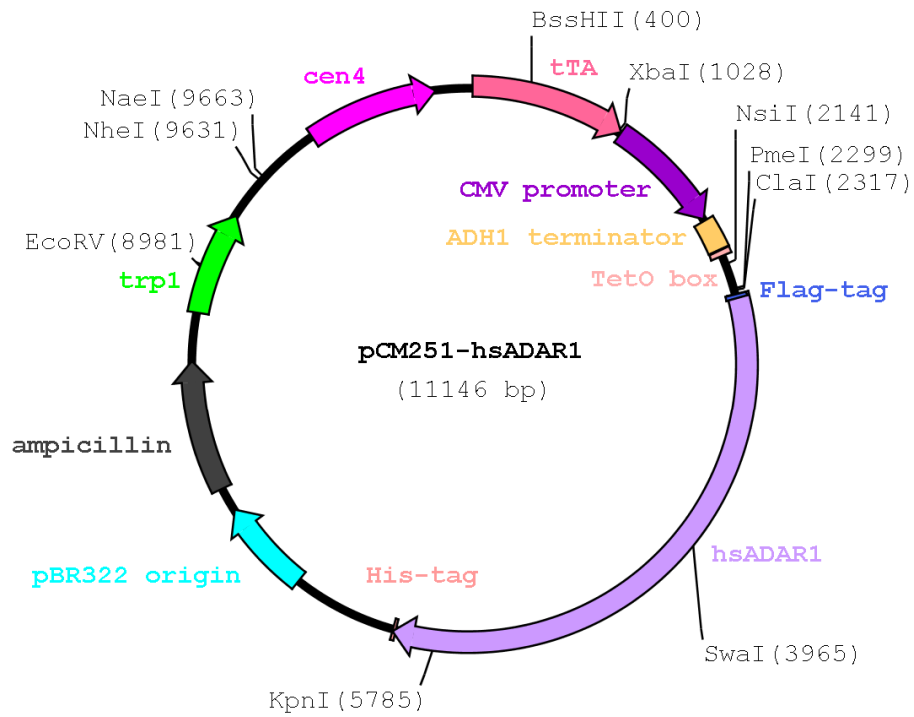
Fig. 3.2: Yeast expression vector carrying hsADAR1 gene. A human Flag/His-tagged ADAR1 version was cloned into a yeast centromeric vector with 2 TetO box tetracycline inducible system.

3.1.3.2 Rat ADAR2 expression construct

Rat *ADAR2* has been amplified from pCDNA3.1-rADAR2 vector with ClaI and EcoRV bearing oligos and inserted into ClaI and blunted PstI restriction sites of pCM251 [*Fig. 3.3*].
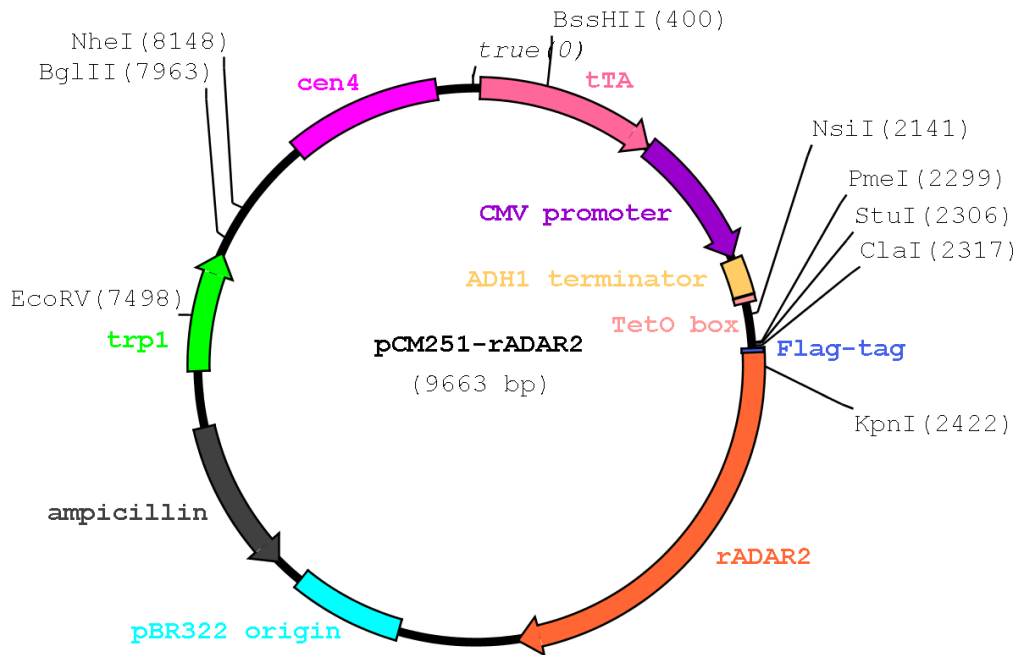
Fig. 3.3: Yeast expression vector carrying rADAR2 gene. A rat Flag-tagged ADAR2 version was cloned into a yeast centromeric vector with 2 TetO box tetracycline inducible system.

3.1.4   Yeast growth assays

Yeast cultures were grown in 28-30°C conditions. Depending on a growth purpose yeast were grown in full medium YPD (1% yeast extract, 2% tryptone, 2% glucose, pH 7.0) or in synthetic complete selective medium, SC (0.67% bacto-yeast nitrogen base w/o amino acids and with ammonium sulphate, 2% dropout mix (consisting of appropriate amino acids), 2% glucose, pH 7.0). Solid media were prepared with an addition of bacto agar (20% in case of YPD and 15% in case of SC). SC FOA (5-fluoroorotic acid) solid medium was prepared by mixing a solution consisting of: autoclaved water (¼ of end volume), 0.67% bacto-yeast nitrogen base w/o amino acids and with ammonium sulphate, 2% dropout mix (always supplemented with uracyl), 2% glucose, which was warmed up to 55-60°C and supplemented with 1% 5-FOA. After thorough mixing, already autoclaved and still warm (50-60°C) 15% agar/water solution (¾ of end volume), was added, mixed and the plates were poured. Very acidic pH, needed for permeability of 5-FOA, was never adjusted. Liquid SC FOA medium was prepared without agar. Additional possible supplements in the media were: 200µg/ml geneticin (G418) and 5µg/ml doxycycline (DOX).

Yeast for growth comparison on solid media were grown in 5ml culture overnight and in the morning brought to $OD_{660} = 0.01$ in deionized autoclaved water. A water based dilution series was, consisting of a sample with concentration of $OD_{660} = 0.01$ and another four, diluted each time in 1:2 ratio, was patched, in form of 3-5µl droplets, onto a Petri dish containing selective medium. Growth comparison in liquid media was performed in sterile 96-well round bottom titer plates. To each well containing 200µl of appropriate medium 10µl of water suspension of yeast was added. For the growth curves yeast strains were first grown in 5ml overnight cultures to be diluted to $OD_{660} = 0.1$-0.2 in 20ml of selective medium in the morning. Cultures were incubated with vigorous shaking for 24h, each time taking 1ml sample for OD measurements.

3.1.5 Yeast competent cells transformation

A 5ml yeast overnight culture was grown in YPD (1% yeast extract, 2% tryptone, 2% glucose, pH 7.0) and on the next day 1ml of the culture was transferred to 50ml YPD and incubated another 3-4 hours until OD_{660} reached 0.6. Cells were harvested by centrifugation (5min, 2000rpm), washed once and then resuspended in 1ml of 1xTE, 0.1M LiAc. The competent cells were used either immediately or in between of a week. For the transformation 100µl of competent cells were mixed with either 10µg of linearized DNA for genomic integration (HIS3amber–KanMX4 or URA3amber-KanMX4 substrates) or 1µg of vector DNA (*hs*ADAR1 and *r*ADAR2 expressing vectors), 25µg of sonicated and boiled carrier salmon sperm DNA and 700µl of 40% PEG4000 in 1xTE, 0.1M LiAc. For genomic integration pUC18 HIS3/URA3amber vectors were restricted with SacI and XbaI restriction enzymes and the longer band (~3700bp in case of *HIS3*) eluted from the gel. The mixture was incubated for 30-60 minutes at 37°C and afterwards kept 15 minutes at 42°C. The transformed cells were recovered after centrifugation in water and plated on appropriate selective medium (SC –his G418 (200µg/ml) and SC -ura G418 for editing substrate integration and SC -his-trp G418 and SC -ura-trp G418 for ADAR expressing vectors transformation).

3.1.6 Yeast genomic DNA extraction

A 5ml overnight culture in appropriate medium was spun down (5min, 1000rpm), pellet resuspended in 200µl lysis buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0) and transferred to an Eppendorf tube. 200µl of phenol:chloroform:isoamyl alcohol (25:24:1) and 200µl of acid etched glass beads has been added and vortexed at the maximum speed for 10min and after adding another 200µl TE still vortexed for another 5min. Aqueous phase was transferred to 1ml 96% ethanol, incubated 10-20min at -20°C and spun at full speed for 5min. RNA contamination was digested with 5µl of 10mg/ml RNAse A solution in 200µl TE at 37°C. Digested sample was purified again with equal amount of chloroform:isoamyl alcohol (24:1) and after centrifugation aqueous phase was precipitated with 20µl of 5M KAc and 800µl of 96% ethanol for 30min to overnight at -20°C. After precipitation DNA was washed with 70% ethanol and resuspended in 50µl of deionized water.

3.1.7 Yeast RNA extraction

A 5ml overnight culture was diluted to OD_{660}=0.1-0.2 and incubated in 5ml selective medium (with DOX induction 5µg/ml when needed) for another 6 hours. The cultures were spun down at 1000rpm and washed with DEPC treated deionized water once. Yeast pellet was resuspended in 500µl of the AE lysis buffer (50mM NaOAc pH 5.5, 10mM EDTA pH 8.0) and 100µl of 10% SDS was added. The suspension was added to hot (65°C) RNA phenol (pH 4.0) and after mixing vigorously rapidly frozen in liquid nitrogen. The samples were then thawed on 65°C, vortexed for 30s and incubated with shaking for 4min at this temperature. The whole freezing and thawing procedure was still repeated two times. Samples were centrifuged for 5min at the full speed, extracted with 700µl phenol:chloroform:isoamyl alcohol (25:24:1) and then with chloroform:isoamyl alcohol (24:1) once. The aqueous phase was precipitated with 2.5x volume 96% ethanol and 0.1x volume 3M NaOAc pH 5.0 for 30min at -80°C or overnight at -20°C. After precipitation DNA was washed with 70% ethanol and resuspended in 50µl of DEPC water. In that way obtained RNA was DNaseI digested twice, first with 0.5µl of 50u/µl DNaseI for 30min-1h at 37°C in 100µl followed with PCI/CI extraction and 96% ethanol/3M NaOAc precipitation and second with 10µl of 1u/µl DNaseI for 30min at 37°C in 100µl followed by heat inactivation at 65°C for 10min with addition of

25mM EDTA and 96% ethanol/3M NaOAc precipitation. After washing isolated RNA was resuspended in 50µl DEPC treated water.

3.1.8 Yeast protein extracts

A 5ml culture in an appropriate medium was grown overnight and in the morning diluted to OD_{660} = 0.1-0.2 and incubated in 5ml selective medium (with DOX induction 5µg/ml when needed) for another 6 hours. Cultures were centrifuged for a minute at full speed and then the pellet resuspended in YEX lysis buffer (1.85M NaOH, 7.5% β-mercaptoethanol) and incubated 10min on ice, 150µl of cold 50% trichloroacetic acid was added and incubated another 10min on ice. The extracts were centrifuged 5min at full speed and resuspended in 2x SDS loading buffer (220mM Tris-HCl pH 6.8, 1.8% SDS, 29% glycerol, 0.03% bromophenol-blue, 0.03% β-mercaptoethanol). The colour suppose to be blue, if it turned yellow 20-50µl 1M Tris-HCl pH 8.8 were added until the colour turned blue. The extracts were boiled for 5min and centrifuged at full speed for 5min.

3.2   Mating assay

3.2.1   Mating assay cDNA library

For mating assay with editing yeast strain a pretransformed human fetal brain Matchmaker cDNA library in a *Saccharomyces cerevisiae* strain Y187 (MATα, *ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met-URA3::GAL1_{UAS}-GAL1_{TATA}-LacZ MEL1*) has been used . The library was developed by BD Biosciences Clontech (cat. no. 638831). cDNA in this library have been synthesized on a mRNA template obtained from whole brains of spontaneously aborted male/female fetuses using an XhoI-$(dT)_{15}$ primer and after ligation to EcoRI adaptor cloned via this restriction sites to pACT2 AD mammalian expression vector. Library contained 3.5×10^6 independent clones.

3.2.2   Mating assay procedure

A 50ml culture of the editing strain (AHA2) was set up in SC-trp medium with addition of G418 (200µg/ml) for overnight incubation. Next morning the culture suspension of more than 1×10^7 has been joined with 1×10^8 of frozen stock of cDNA library containing strain Y187 in 50ml of 2x YPDA (2% yeast extract, 4% tryptone, 4% glucose, 0.01% adenine, pH 7.0) and incubated at 30°C for 20-24 hours in a 2l flask with gentle swirling (30-50rpm). The culture was spun down, resuspended in water, number of cells counted and ~1×10^6 cells were spread on a certain number of 100mm Petri dishes, containing SC -his-leu-trp medium with addition of 40mM 3-amino-1,2,4-triazole decreasing His3 expression, to cover desired number of cDNA library clones. After few days the colonies growing the fastest were picked up and transferred onto plates with appropriate selective medium and also frozen in YPD, 15% glycerol and kept on -80°C.

A negative control diploid strain was also obtained in the same procedure. Y187 yeast strain, pretransformed with pTD1-1 vector, was mated with the editing strain. pTD1-1 encodes an AD/SV40 large T antigen fusion protein and uses *LEU2* as a yeast marker auxotrophy gene.

3.2.3   Efficiency of mating assay

Viability of strains taking part in the mating, of the diploid strain effecting from the process and mating efficiency were calculated according to Matchmaker cDNA library manual. In case of performed screening calculated viability of mated strains was as followed: AHA2 - 1×10^8 and Y187 - 5.7×10^6 and diploids strain - 7.4×10^6, the mating efficiency was calculated to be of 129%. Using viability of limiting strain, in this case Y187, and amount of the mating culture used for plating the number of cDNA library clones covered has been calculated, which was 3.33×10^6. The amount of the clones screened was slightly lower than the number of the independent clones of the library, which was 3.5×10^6. The amount of colonies that have appeared on dishes with SC –his-leu-trp 40mM 3-AT medium was roughly 2×10^3. As a first step of selection only the biggest colonies were picked up and streaked out for further growth.

3.2.4   Removal of the expression vector from yeast strains

A single colony of desired diploid yeast strain, containing pCM251-rADAR2 and cDNA library vectors, was grown overnight in 2-5ml medium selecting only for auxotrophy marker gene found on cDNA library vector (*LEU2*) and *KanMX4* being SC –leu G418. After overnight incubation at 30°C the culture was diluted 1:1000 to the same medium and again incubated overnight. Passaging was repeated still 2 times and after the third passage the culture was streaked out on solid medium again with tryptophan. Grown single colonies were patched on either SC –leu or SC –trp plates for phenotype determination and only the colonies growing on SC –leu but not SC –trp were picked up.

3.3 Confirmation of mating assay results in tissue culture

3.3.1 Tissue culture expression vector

The vector was based on the pcDNA3.1(-) (Invitrogen), which was restricted with XhoI and EcoRI enzymes, where a 6 x Myc-tag fragment cut out with the same enzymes from KS 7myc no Ochre vector was placed. The vector obtained in that way was used as a template to clone in NLS sequence from NLS GFP vector via PCR using MfeI and EcoRI restriction sites into cleaved with EcoRI pcDNA3.1(-)-6Myc vector. In order to clone various candidates in three possible frames three different versions of the NLS bearing vectors were prepared; with EcoRI site downstream of NLS being in frame, with one nucleotide in front of it and with two [*Fig. 3.4*].
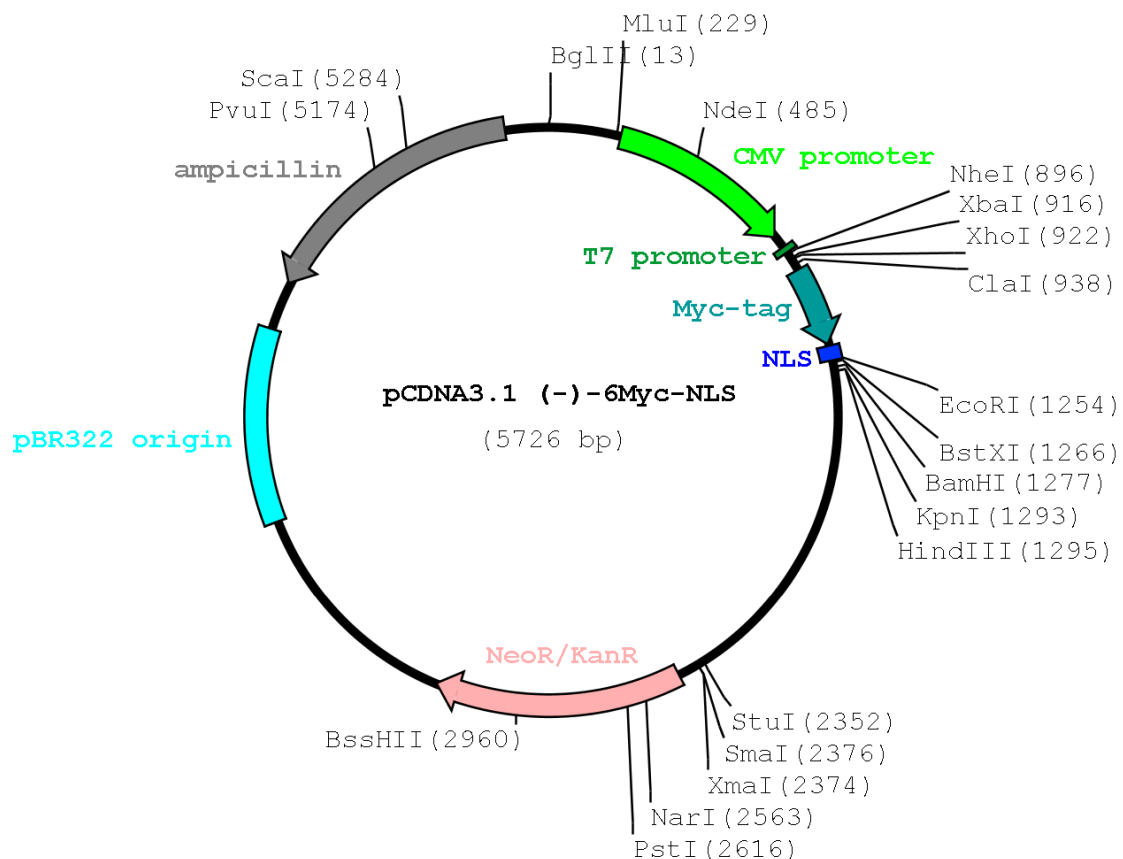
Fig. 3.4: Vector for expression of Myc-tagged screening candidates in tissue culture. pCDNA3.1 (-) vector was equipped with 6x Myc-tag and a NLS sequence and prepared in three different, frame-dependent versions for cloning at EcoRI site.

3.3.2 Cloning of yeast screen candidates into tissue culture expression vector

Yeast screen candidates were cloned into one of the pcDNA3.1(-)-6Myc-NLS vectors using already preexisting EcoRI site from the yeast expression vector. Since this restriction site was in second frame according to yeast expression frame to correct it in mammalian expression vector the frame had to be shifted by one nucleotide more than it would come out from the frame of the gene in the yeast expression vector. Genes in the first frame were cloned to a vector with one additional nucleotide, ones in the second frame into vector with EcoRI site in frame and ones in the third frame into a vector with two additional nucleotides. Cloning was performed according to the frameshift via PCR using 5' primer annealing further upstream used previously for amplifying the library hits for sequencing or specific primer containing EcoRI restriction site and various 3' primers depending on the presence of the restriction sites in the amplified sequence (KpnI, HindIII, BamHI, BglI). Additionally a bare sequence of DSS1 gene or truncated versions of the protein sequence were cloned into pcDNA3.1(-)-6Myc, pcDNA3.1(-)-6Myc-NLS and pCaggs vector, bearing Myc-tag sequence on the 3' end, via PCR using EcoRI and BamHI and EcoRI and XhoI restriction sites respectively.

3.3.3 Tissue culture

Tissue culture cell lines; human embryonal kidney cells (Hek 293), HeLa cervical cancer cells and Hek 293 stably transformed with Flag-tagged *r*ADAR2 were cultivated in cell culture dishes at 37°C, 5% CO_2 and constant humidity. The medium used was DMEM (Dulbecco's modified Eagle's Medium), high glucose (4,5g/l) with addition of heat inactivated fetal bovine serum (10%), 2mM L-glutamine and 1x penicillin/streptomycin solution (100x stock = 10000 Units/ml penicillin, 10mg/ml streptomycin). *r*ADAR2 stable cell lines were grown in a presence of 300µg/ml G418 (geneticin). Adhesive cell culture was passaged by exposing the cells to the digestion of 1x trypsin EDTA in PBS solution (10x stock = 5mg/ml trypsin, 2,2mg/ml EDTA) for 5-10 minutes at room temperature and

afterwards inactivated with serum containing medium. For longer maintenance of the cell lines the cells were kept at -80°C in 10% DMSO (dimethyl sulfoxide) and 90% fetal bovine serum. All media and reagents used for tissue culture were produced by PAA, Austria.

3.3.4   Transfection

Tissue culture cell lines were transfected using Nanofectin reagent (PAA, Austria) according to the manufacturer's instructions. After 32-72 hours of incubation the cells were processed to obtain RNA, protein extracts or were stained to visualize protein expression. Cotransfections of RNAG and vectors expressing screen candidates were performed in 1:4 ratio with the latter in an optimal amount suggested by the manufacturer.

3.3.5   Creation of cell line stably expressing *r*ADAR2

Stable cell lines expressing rADAR2 were prepared by Aamira Tariq in our laboratory on the base of Hek 293 cell line, transformed transiently with pCDNA3.1-rADAR2 and selected for positive integration using 400µg/ml G418.

3.3.6   Tissue culture RNA isolation

The layer of cells growing on a dish was washed with 1xPBS and RNA was isolated directly from the dish using guanidinium thiocyanate-phenol-chloroform extraction with TRIzol or TriFast reagents (Invitrogen or Peqlab respectively) according to manufacturer's protocols and after 96% ethanol/3M NaOAc precipitation and 70% ethanol washing step were kept in deionized water treated with DEPC at -20°C. Before RT-PCR, RNA was treated, depending on a purpose, once or twice with DNaseI, 10µl of 1u/µl DNaseI for 30min at 37°C in 100µl followed by heat inactivation at 65°C for 10min with addition of 25mM EDTA and 96% ethanol/3M NaOAc precipitation. In case of RNAs expressed from plasmids (RNAG) additional treatment with DpnI, an enzyme cutting only Dam methyleted DNA or with frequent cleaving restriction enzyme like AluI was applied.

3.3.7   Tissue culture protein extracts

Tissue culture cells were washed with 1xPBS, detached from the surface with a cell scraper, transferred to an Eppendorf tube and collected via short centrifugation (10s at full speed). Cells resuspended in an appropriate amount (depending on a size of the dish) NET-2 buffer (150mM NaCl, 80mM Tris pH 7.4, 0.05% NP-40) were sonicated 3x 15s with 30% output and 50% pulse keeping in between on ice for another 15s. The cell lysate was spun down twice at 4°C for 10 minutes at full speed to get rid of insoluble proteins transferring the supernatant to a new tube each time. The remaining lysate was mixed with 2× SDS sample buffer (220mM Tris-HCl pH 6.8, 1.8% SDS, 29% glycerol, 0.03% bromophenol-blue, 0.03% β-mercaptoethanol), boiled for 5 minutes, centrifuged for 5 minutes and kept at -20°C until use.

3.3.8   Immunoprecipitation assay

The cell extracts for immunoprecipitation were prepared the same way as described above but only a small aliquot of it was mixed with 2xSDS sample buffer as an input control. Concentration of the extract was measured using the Bradford reagent (Biorad) and the equal amounts of them has been added to already coupled with proper antibody Protein A Sepharose beads (GE Healthcare). 3-5mg of the beads were used per sample, swollen in NET-2 buffer and washed twice in it before adding 500µl of the antibody solution. The beads were incubated on a rotating wheel for 2h at the room temperature or over night at 4°C and washed three times in 500µl NET-2 buffer. Immunoprecipitation lasted 1-2h incubated on a rotating wheel at the room temperature. Afterwards the unbound extract was washed off again with 3 washings with NET-2 buffer. After last washing the excess of the buffer was removed and the beads were resuspended in 30-40µl of 2x SDS sample buffer, boiled for 5 minutes, centrifuged for 5 minutes and kept at -20°C until use.

3.3.9   *In situ* staining

Transfected cells grown on acid-etched coverslips after 32 to 48 hours were washed once with 1×PBS and fixed for 5 minutes with a fixing solution (2% paraformaldehyde,

$1\times$PBS, 0.05% Triton X-100). The next washing step was followed by a one minute methanol-permeabilisation-step and another washing step. In order to visualize expressed proteins an immunocytology was performed. The fixed cells were blocked with 10% horse serum solution in 1xPBST (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.76mM KH_2PO_4, pH 7.4, 0.2% Tween-20). The cells were kept in primary antibody for 2 hours at room temperature or over night at 4°C in a moist chamber, washed 3 times with 2.5% horse serum solution in 1xPBST, kept in secondary antibody for 1 hour at room temperature in a moist chamber and after that still washed 3 times with 2.5% horse serum 1xPBST solution. For visualization of chromatin, preparations were mounted with Antifade (1g p-phenylenediamine/100ml phosphate buffer (150mM NaCl, 10mM KH_2PO_4, 10mM Na_2HPO_4), pH to 8.0 with carbonate buffer (500mM $NaHCO_3$, 500mM Na_2CO_3); mix 1 part with 8 parts of glycerol) containing DAPI.

3.3.10 Microscopical analysis

Microscopic images were captured on a Zeiss fluorescence microscope equipped with an ORCA cooled charged-coupled device camera (Hamamatsu Middlesex, CA). Images were imported into Photoshop 4 (Adobe Systems, Mountain View, CA) with the help of a QED plug-in module (QED-Imaging, Pittsburgh, PA).

3.3.11 FACS analysis

The flow cytometry data was collected on a FACScalibur (Becton & Dickinson) using the CellQuest 3.3 software at the BioOptics department of the Institute of Molecular Pathology, Vienna, Austria. The data was further analysed with the FlowJo 6.3.1 software.

3.3.12 Cloning of FACS reporter system

In order to investigate influence of ADAR2 activators in the tissue culture an A to I editing substrate was cloned between red fluorescent protein (RFP) and green fluorescent protein (GFP), used as a constitutively expressed control and as an editing efficiency marker respectively, in RNLG. (Schoft, Schopoff et al. 2007) Modified GluR-B R/G sequence,

flanked with XhoI sites and with addition of two nucleotides to keep GFP in frame with it, was amplified via PCR and cloned into polylinker of RNLG using HindIII and BamHI restriction sites. Additionally to RNAG vector a construct with 'pre-edited' version of the editing substrate containing tryptophan (W) codon as a positive control named RNWG has been prepared [*Fig. 3.5*].
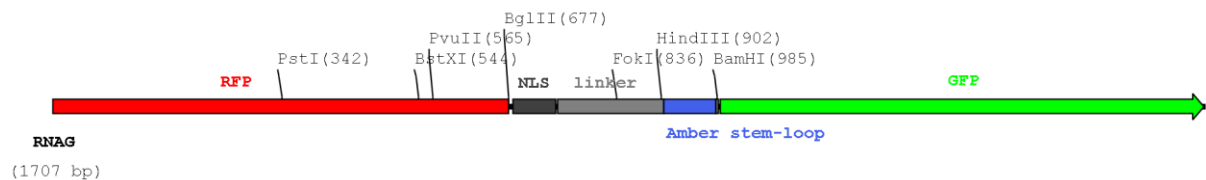

Fig. 3.5: FACS reporter system construct RNAG. A fusion protein sequence consisting of RFP, NLS, linker, Amber stem-loop and GFP was expressed from pDsRedExpress-C1 vector (Clontech) carrying kanamycine resistance gene for bacterial propagation.

3.3.13 FACS data analysis

Each time a readouts of 30000 cells were taken. The red and green fluorescence readouts of each cell were in effect plotted with help of FlowJo 6.3.1 software of on a graph with logarithmic scale with red fluorescence on y axis and green fluorescence on x axis. For statistical purposes 6 different gates were taken with very broad window for the green fluorescence and very narrow for the red one to observe changes of the green fluorescence compared to fairly stable red fluorescence. The gates were chosen above a region of untransfected cells background and below a region where cell density started to drop down. The mean green florescence values from each gate calculated using Excel software (Microsoft) were divided by mean red fluorescence values to become normalized relative fluorescence values, which were plotted again on the graph on x axis against the chosen gates on y axis.

3.4   Other techniques used

3.4.1   Polyacrylamide gel electrophoresis (SDS PAGE)

Protein separation by molecular weight was performed in a polyacrylamide gel matrix. A SDS gel is composed of a resolving gel (x% 30:1 acrylamide/bis-acrylamide according to the size of the proteins to be resolved, 375mM Tris-HCl pH 8.8, 0.1% SDS) and a stacking gel (concentrates negatively charged proteins to a thin boundary; 3.6% acrylamide (30:1 acrylamide/bis-acrylamide), 110mM Tris-HCl pH 6.8, 0.1% SDS, 13% glycerol). The proteins were mixed with $2\times$ SDS sample buffer (220mM Tris-HCl pH 6.8, 1.8% SDS, 29% glycerol, 0.03% bromophenol-blue, 0.03% β-mercaptoethanol), boiled for 5 minutes, centrifuged for 5 minutes and loaded onto the gel. Electrophoresis was carried out in $1\times$ SDS running buffer (25mM Tris-HCl, 190mM glycine, 0.1% SDS). 20mA electric current was applied per gel.

3.4.2   Western Blotting

After electrophoresis, the proteins were transferred to a polyvinylidene fluoride membrane (GE Water & Process Technologies) via tank-blotting. For that, the membrane activated in methanol and equilibrated in transfer buffer (20mM Tris-HCl, 150mM glycine) was placed on the gel and all air bubbles were removed. The gel and membrane were placed between two Whatman papers and two sponges, and that sandwich was then put into the tank, which was filled up with transfer buffer. The transfer was carried out at room temperature with icepacks in the apparatus at 360-450mA for 80 minutes. Afterwards the membrane was blocked with 5% dry skim milk in TBST (25mM Tris, pH 7.4, 3.0mM KCl, 140mM NaCl and 0.05% Tween 20) for 20 minutes at room temperature. The primary antibody was diluted in 0.5% milk in TBST and applied to the membrane. The membrane was shaken in the antibody solution for one to two hours at room temperature or overnight at 4°C. It was then washed three times for 10 minutes with 0.5% milk in TBST. The secondary antibody was diluted in 0.5% milk and the blot was again incubated for one hour. This was followed by three washing steps (10 minutes each, 0.5% milk in TBST). The proteins on the blot were either detected by

chemiluminescence or NBT/BCIP chromogen precipitation. For the chemiluminescence, the secondary antibody had to be coupled to horseraddish peroxidase. For the detection the PIERCE supersignal solution was used according to the manufacturers instruction. For the chromogen detection, the secondary antibody had to be coupled to alkaline phosphatase. In the presence of an alkaline phosphatase enzyme, the chromogen NBT/BCIP produces a purple precipitate. The detection was carried out as recommended by the manufacturer.

3.4.3 Reverse transcription

The cDNA was synthesized with RevertAid M-MuLV Reverse Transcriptase (Fermentas) according to the manufacturer's instructions using sequence specific oligos. 1/4 of the heat inactivated RT-reactions were used as templates for PCR reactions.

3.4.4 Cycle sequencing

Sequencing was performed with use of BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to manufacturer's instructions and afterwards by the Department of Systematic and Evolutionary Botany at the Faculty of Life Sciences, University of Vienna.

3.4.5 Poisoned primer reaction

To certain amount of RNA (0.5-1µg in case of *in vitro* transcribed and 5-100µg in case of total RNA) 4pmol of radioactively labelled primer (P^{32}) was added and resuspended in 4µl of the hybridisation buffer (225mM Hepes pH 7.0, 450mM KCl). After 1 minute of boiling the mixture was slowly cooled down. Afterwards 2.2µl of the extension mix (0.6µl 10x extension mix (1.3 Tris-HCl pH 8.5, 100mM DTT, 100mM $MgCl_2$), 0.3µl poisoned mix (1mM dATP, dCTP, dGTP, 4mM dTTP), 0.3µl AMV reverse transcriptase (4u/µl New England Biolabs), 1µl DEPC treated water) was added and the mixture was incubated for an hour. The reaction was stopped by addition of 30µl of 96% ethanol, 1µl of 3M NaOAc pH 5.0, 1µl 0.5M EDTA and following precipitation for 30min at -80°C or overnight at -20°C. After precipitation the samples were washed with 70% ethanol, resuspended in 10µl of DEPC

treated deionized water and separated on 10-12% native acrylamide gel (29:1 acrylamide/bis-acrylamide, 8M urea, 1x TBE (0.89M Tris-HCl, 0.89M boric acid, 20mM EDTA set to pH 8.3)).

3.5 Primers

The following primers were used in this investigation:

Name	Sequence 5' – 3'	Purpose
leu2-1	AGGATATACCATTCTAATGT	5' primer for amplification of disrupted sequence of *LEU2* gene
leu2-2	AGCAGACAAGATAGTGGCGA	3' primer for amplification of disrupted sequence of *LEU2* gene
MJ1136	GGTGGAGTTCAAGTCCATCTACATGG	5' primer for RT-PCR of RNAG (binds to 3`end of *RFP*)
MJ1137	GTGCAGATGAACTTCAGGGTCAGC	3' primer for RT-PCR of RNAG (binds to 5` end of *GFP*)
MJ1421	CTATTCGATGATGAAGATCCACCAAACC	5' primer for amplification of the pACT2 (cDNA library) content
MJ1422	GTGAACTTGCGGGGTTTTTCATCTACGA	3' primer for amplification of the pACT2 (cDNA library) content
MJ1715	CACTCGAGCTTCCCAGAAAAAGAGGCAG	Forward primer used to introduce a XhoI restriction side in a *HIS3*
MJ1716	AGCTCGAGTGCTCTATCGCTAGGGGAC	Reverse primer used to introduce a XhoI restriction site in a *HIS3*
MJ1717	GGTGAGCGCTAGGAGTCAC	Forward primer containing EcoR47III restriction site used to cut pUC18 leu2:HIS3 plasmid upstream of *HIS3*
MJ1718	AATGATGCATTACCTTGTCATC	Reverse primer containing NsiI restriction site used to cut pUC18 leu2:HIS3 plasmid downstream of *HIS3*
MJ1745	TCGATTTAGGTGGGGTATATGTGCTCAATGTATAGCCTACCCAGATCA	Forward primer for creation of short version of GluR-B amber stem-loop substrate
MJ1746	TCGATGATCTGGGTAGGCTATACATTGAGCACATATACCCCACCTAAA	Reverse primer for creation of short version of GluR-B amber stem-loop substrate
MJ1747	TCGATTTGGGTGGGGTATATGTGCTCAATGTATAGCCTACCCAGATCA	Forward primer for creation of short version of GluR-B W stem-loop substrate
MJ1748	TCGATGATCTGGGTAGGCTATACATTGAGCACATATACCCCACCCAAA	Reverse primer for creation of short version of GluR-B W stem-loop substrate
MJ1787	ATGTGTATATATGTATACCTATG	Forward sequencing primer of KanMX4 insert put into 3' flanking region of *HIS3* gene in pUC18-leu2:HIS3 plasmid
MJ1788	GCTTCATCGGAGATGATTCG	Reverse primer used to sequence KanMX4 insert put into 3' flanking region of *HIS3* gene in pUC18-leu2:HIS3 plasmid
MJ1832	GCGGAATACCCTCGCCAG	Reverse sequencing primer placed after start codon of *rADAR2*
MJ1833	GTCGCTGGATGCGGGTAC	Forward sequencing primer placed before the end of *rADAR2*
MJ1845	GCTCATCGATATGGACTACAAAGACGATGAC	5' primer for amplification of *rADAR2* gene out of pCDNA3.1-rADAR2 containing ClaI
MJ1846	GCTCGATATCCTATAGAATAGGGCCCTCTAG	3' primer for amplification of *rADAR2* gene out of pCDNA3.1-rADAR2, contains EcoRV
MJ2073	GATCTCGAGTTAGGTGGGTGGAATAGTATAAC	Forward primer to amplify GluR-B R/G stem-loop with XhoI sticky ends to create amber stem-loop (additional preliminary stop codon was created)
MJ2074	GATCTCGAGTTGGGTGGGTGGAATAGTATAAC	Forward primer to amplify GluR-B R/G stem-loop with XhoI sticky ends to create W stem-loop loop

		(additional preliminary stop codon was created)
MJ2075	TAACTCGAGAGCTGGGTAGGTGGGATACTATAA	Reverse primer to amplify GluR-B R/G stem-loop with XhoI sticky ends to create amber and W stem-loops loop (additional preliminary stop codon was created)
MJ2080	GTAATTCTGCTAGCCTCTG	Reverse primer downstream of amber/W stem-loop containing NheI site
MJ2102	TTCTACGTAGGCTCCAATG	Primer for internal sequencing of *rADAR2*
MJ2103	TTGCTGATACCGCTGAGC	Primer for internal sequencing of *rADAR2*
MJ2139	GTAGAACAATATGCTCAATGTTGTTCTAGTATCCCAC	Forward primer to get rid of preliminary stop codon from stem-loop
MJ2140	CTAGAACAACATTGAGCATATTGTTCTACTATTCCAC	Reverse primer to get rid of preliminary stop codon from the stem-loop
MJ2256	ATCATGGCCAAAGCTACATATAAGGAACGTG	Forward primer to amplify *URA3* without start codon and clone it in stem-loop-*HIS3* constructs, contains BalI (MscI) site.
MJ2257	ATACGCTAGCTTAGTTTTGCTGGCCGCAT	Reverse primer to amplify *URA3* with stop codon and clone it in stem-loop-*HIS3* constructs, contains NheI restriction site.
MJ2263	ATGACAGAGCAGAAAGCCCT	Forward primer for RT-PCR of *HIS3*
MJ2264	CTACATAAGAACACCTTTGGT	Reverse primer for RT-PCR of *HIS3*
MJ2274	CATACTCGAGCATCAGGGGAAACCAGGGATTTCCATAGGATATACGC	5' primer for formation of hepatitis delta type 1 amber editing substrate for *in vivo* yeast editing system
MJ2275	CATACTCGAGCATCAGGGGAAACCAGGGATTTCCATGGGATATACGC	5' primer for formation of hepatitis delta type 1 control 'pre-edited' version of editing substrate for *in vivo* yeast editing system
MJ2276	CATACTCGAGAGCACCGGGGACCAGTGGAGCCATGGGATGCGTATATC	3' primer for formation of hepatitis delta type 1 editing substrate for *in vivo* yeast editing system
MJ2384	AGCACTCGAGAAAGTGGCATATGCGACGGCCATGGACTGGTTC	5' primer for formation of *Gabra-3* amber editing substrate for *in vivo* yeast editing system
MJ2385	GAAGCTCGAGAAAGGCATAACAGACGGCTATGAACCAGTCCAT	3' primer for formation of *Gabra-3* amber editing substrate for *in vivo* yeast editing system
MJ2386	GAAGCTCGAGAAAGGCATAACAGACGGCCATGAACCAGTCCAT	3' primer for formation of *Gabra-3* control 'pre-edited' editing substrate for *in vivo* yeast editing system
MJ2420	GCAACGTGCTGGTTATTGTG	pCaggs forward sequencing primer
MJ2431	CGCAAATGGGCGGTAGGCGTG	Forward primer for sequencing of pcDNA3.1 vectors
MJ2577	TCCCAATTGGACCATGGGGGTGGCCCAC	5' primer containing MfeI restriction site for NLS cloning from NLS GFP in pGEX2T into pcDNA3.1(-)-6Myc expression vector
MJ2578	CATGAATTCGTCTTCTACCTTTCTCTTCTT	5' primer containing EcoRI restriction site for NLS cloning from NLS GFP in pGEX2T into pcDNA3.1(-)-6Myc expression vector
MJ2579	CATGAATTCCGTCTTCTACCTTTCTCTTCTT	5' primer containing EcoRI restriction site for NLS cloning from NLS GFP in pGEX2T into pcDNA3.1(-)-6Myc expression vector with addition of one nucleotide upstream of restriction site
MJ2580	CATGAATTCGCGTCTTCTACCTTTCTCTTCTT	5' primer containing EcoRI restriction site for NLS cloning from NLS GFP in pGEX2T into pcDNA3.1(-)-6Myc expression vector with addition of two nucleotides upstream of restriction site
MJ2581	CCGGTACCCAGTATCTACGATTCATAGAT	3' primer for cDNA library amplification from pACT2 containing KpnI
MJ2582	CCAAGCTTCAGTATCTACGATTCAT	3' primer for cDNA library amplification from

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	AGAT	pACT2 containing HindIII
MJ2584	GCGAAGCTTGGCTCGAGTTAGGTGGGTGG	5' primer for GluR-B R/G stem-loop with amber stop codon cloning to RNLG vector containing HindIII
MJ2585	GCGAAGCTTGGCTCGAGTTGGGTGGGTGG	5' primer for GluR-B R/G stem-loop with Trp codon cloning to RNLG vector containing HindIII
MJ2586	AGGGATCCCTCGAGAGCTGGGTAGGT	3' primer for cloning GluR-B R/G stem-loop with BamHI
MJ2648	CCAAGCTTCAGTATCTACGATTCATAGAT	3' primer for cDNA library amplification from pACT2 containing AflII (BspTI)
MJ2649	TCCCAATTGGCGGCCGCGTCGAC	5' primer for cDNA library amplification from pACT2 containing MfeI instead of EcoRI
MJ2679	TCGAATTCATGATTATCTACCGGGACCT	A primer containing EcoRI site to amplify candidate no. 60 omitting stop codon in 5'UTR
MJ2726	TCTACCTAATGGATGGAAATGTCAGTAAC	Forward RT-PCR *CyFip2* primer
MJ2727	ATCCCGGATCTGAACCATCTG	Reverse RT-PCR *CyFip2* primer
MJ2729	ATGAATTCATGGTGGAGGCTTTCTGTG	5' primer to amplify candidate no. 02 without stop codon in 5'UTR
MJ2730	ATGAATTCATGGTGGATTACTATGAAGTT	5' primer to amplify candidate no. 48 without stop codon in 5'UTR
MJ2734	CCGGATCCCAGTATCTACGATTCATAGAT	3' primer for cDNA library amplification from pACT2 containing BamHI
MJ2774	ATGAATTCATGTCAGAGAAAAAGCAGC	5' primer with EcoRI restriction site to clone *DSS1* into 3' 3xMyc containing pCaggs
MJ2775	ATCTCGAGTGAAGTCTCCATCTTATAAC	3' primer with XhoI restriction site to clone *DSS1* into 3' 3xMyc containing pCaggs
MJ2783	TACTCGAGATCTTCATCTTCATCTAAGC	A reverse primer containing XhoI restriction site to clone 1st 35 amino acids of *DSS1* into 3' 3xMyc containing pCaggs
MJ2784	TAGAATTCATGGCACATGTCTGGGAGGAT	A forward primer containing EcoRI restriction site to clone 2nd 35 amino acids of *DSS1* into 3' 3xMyc containing pCaggs (with start codon)
MJ2785	TAGGATCCCTAATCTTCATCTTCATCTAAGC	A reverse primer containing BamHI restriction site to clone 1st 35 amino acids of *DSS1* into pCDNA3.1-6Myc-NLS0 vector (with stop codon)
MJ2786	TAGGATCCCTATGAAGTCTCCATCTTATAAC	A reverse primer containing BamHI restriction site to clone 2nd 35 amino acids of *DSS1* into pCDNA3.1-6Myc-NLS0 vector (with stop codon)
MJ2811	ATGAATTCAATGTCAGAGAAAAAGCAGC	A forward primer containing EcoRI restriction site to clone *DSS1* into pCDNA3.1-6Myc vector

3.6 Antibodies

The following antibodies were used in this study:

Name	Target	Source	Dilution	Company
309	*hs*ADAR1	Rabbit	1:500 (WB)	homemade
9E10	Myc-tag	Mouse	1:3 (WB), 1:1 (IP, Cyt)	homemade
Anti-FLAG	Flag-tag	Rabbit	1:1000 (WB), 1:300 (Cyt)	Sigma
Anti-Rabbit	rabbit (secondary antibody, coupled to horseradish peroxidase)	Goat	1:10000 (WB)	Axell
Anti-Rabbit	Rabbit (secondary antibody, coupled to alkaline phosphatase)	Goat	1:1000 (WB)	Pierce
Anti-Mouse	Mouse (secondary antibody, coupled to alkaline phosphatase)	Goat	1:1000 (WB)	Sigma
Alexa Fluor 568 Anti-Mouse	Mouse (secondary antibody)	Goat	1:400 (Cyt)	Invitrogen
Alexa Fluor 488 Anti-Rabbit	Rabbit (secondary antibody)	Goat	1:400 (Cyt)	Invitrogen

(WB = Western Blotting, Cyt = *in situ* staining)

4   Results

4.1 Basic yeast strain

In order to screen for enhancers and inhibitors of ADAR activity a yeast model was designed. Two different auxotrophy marker genes were employed: *HIS3* was used for positive selection, while *URA3* was used for negative one. A canonical editing site, which is at the same time a stop codon, was introduced in the sequence of these genes. Expression of the gene was only possible after editing of the stop codon by ADARs. The substrate-reporters fusions were introduced into the genome of *Saccharomyces cerevisiae* strain W303. At the same time ADAR expressing vector constructs were introduced. With this system one can screen for activators of editing, as this results in an increase of His3 expression and in turn of yeast growth. In addition, one can screen for inhibitors of editing, as this causes an increase in Ura3 expression and in turn to a decreased growth in the presence of 5-fluoroorotic acid (5-FOA) being converted by this protein into toxic 5-fluoroacil.

4.1.1 Creation of basic strains

The strain and needed controls were created by integrating a marker gene with the editing substrate into the genome of the auxotroph *Saccharomyces cerevisiae* strain W303 in order to obtain a stable, one copy expression. The introduced fragment consisted of *HIS3* or *URA3* genes containing an insert derived from the glutamate receptor subunit B R/G site and kanamycin resistance cassette (*KanMX4* gene) all integrated into the *leu2* locus in the genome. For creation of the editing stem-loop, not the original coding frame of GluR-B was used but it was shifted by one nucleotide upstream. A nucleotide change of an adenosine in front of the one being edited into thymine changed AAG codon encoding lysine into TAG codon encoding amber stop codon. This codon was used as a switch between unedited state, which meant a preliminary stop in translation and edited state (TTG − tryptophan) when the translation proceeded. Compensatory mutation was introduced also on the 3' part of the stem-loop sequence. Additionally a 'pre-edited' version of the editing substrate containing tryptophan (W) codon as a positive control has been prepared [*Fig. 4.1*].
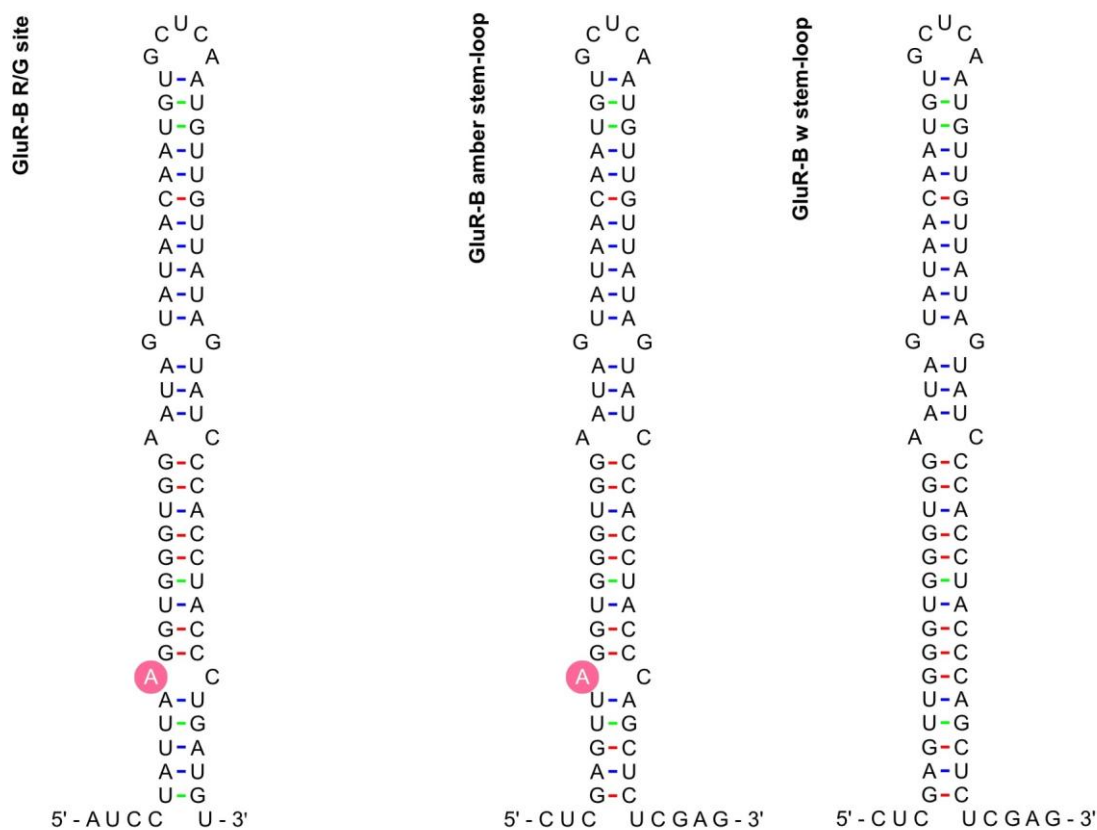
Fig. 4.1: RNA folding predictions of glutamate receptor subunit B R/G editing site, editing substrate used in yeast system based on GluR-B R/G site with amber stop codon as an editing site and a 'pre-edited' version of this substrate with edited adenosine in a circle. Editing substrates are shown in their XhoI restriction site context.

Accordingly, two strains were obtained for each selection and both contained marker genes with an amber stop codon as an editing site (from now on AH and AU for *HIS3* and *URA3* respectively). In addition, the strains with a 'pre-edited' variant of the editing substrate were constructed, with a tryptophan (W) codon instead of the amber stop codon (from now on WH and WU respectively). Integrants were selected on YPD G418 medium and verified by PCR [*Fig. 4.2 and Fig. 4.3*].
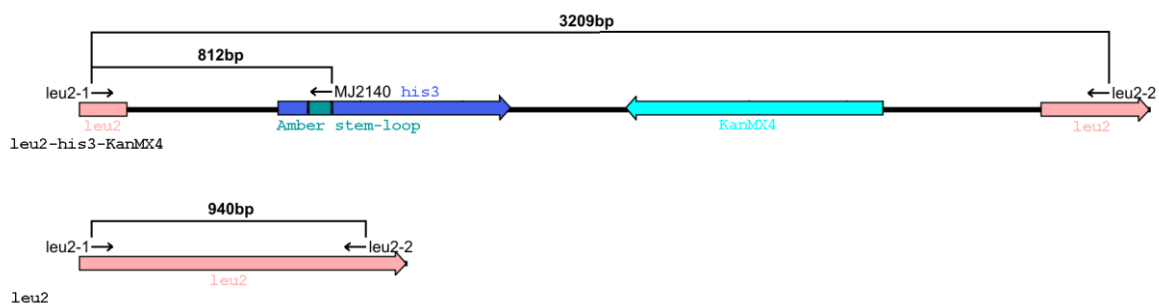
Fig. 4.2: Amplification products of editing substrate for determination of homologous integration. Proper integration gives rise to 3209bp fragment amplified using leu2-1 and leu2-1 primers and 812bp fragment using MJ2140 as a reverse primer instead. In case of non-homologous integration also a wild type LEU2 gene would be amplified with a product of 940bp in size.
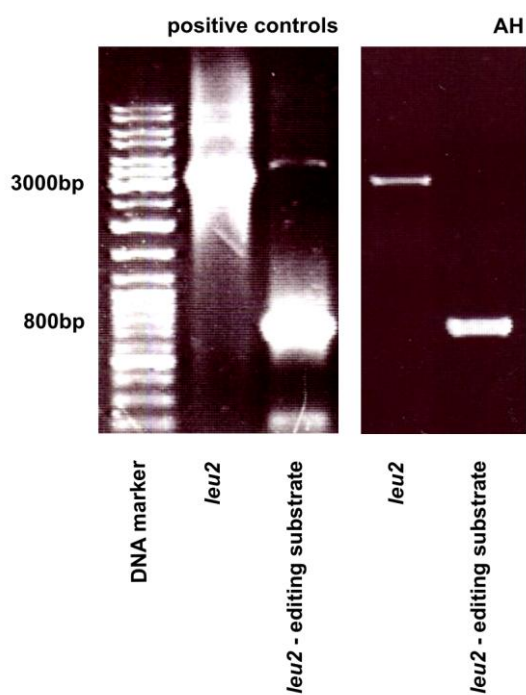
Fig. 4.3: Determination of homologously integrated HIS3amber-KanMX4 editing substrate into leu2 locus. The first panel shows Gene Ruler DNA Ladder Mix marker (Fermentas) and bands amplified from pUC18 HIS3amber-KanMX4 vector as positive controls with leu2-1 and leu2-2 primers in the second lane and leu2-1 and MJ2140 in the third. The next panel shows an evidence of the homologous integration with yeast total DNA amplification products of 3209bp in case of the leu2 with properly integrated editing substrate in the first lane and 812bp in case of 5' leu2-editing substrate in the second lane.

Basic strains were transformed with the tetracycline inducible centromeric vector pCM251, which carries either *hsADAR1* fused to a Flag- and His-tags or *rADAR2* with Flag-tag. As a control, a strain transformed with an empty pCM251 vector was created. Since the selection marker on the centromeric vectors was *TRP1* gene the positive transformants were selected on a SC –trp G418 medium. Strains containing an empty centromeric vector were termed AHE, WHE, strains expressing *hs*ADAR1 were termed AHA1, WHA1 and strains expressing *r*ADAR2 were termed AHA2, WHA2.

Expression of ADARs and the editing substrate was tested by RT-PCR. This assay also showed a leaky expression of *ADAR* in the absence of DOX [*Fig. 4.4*].
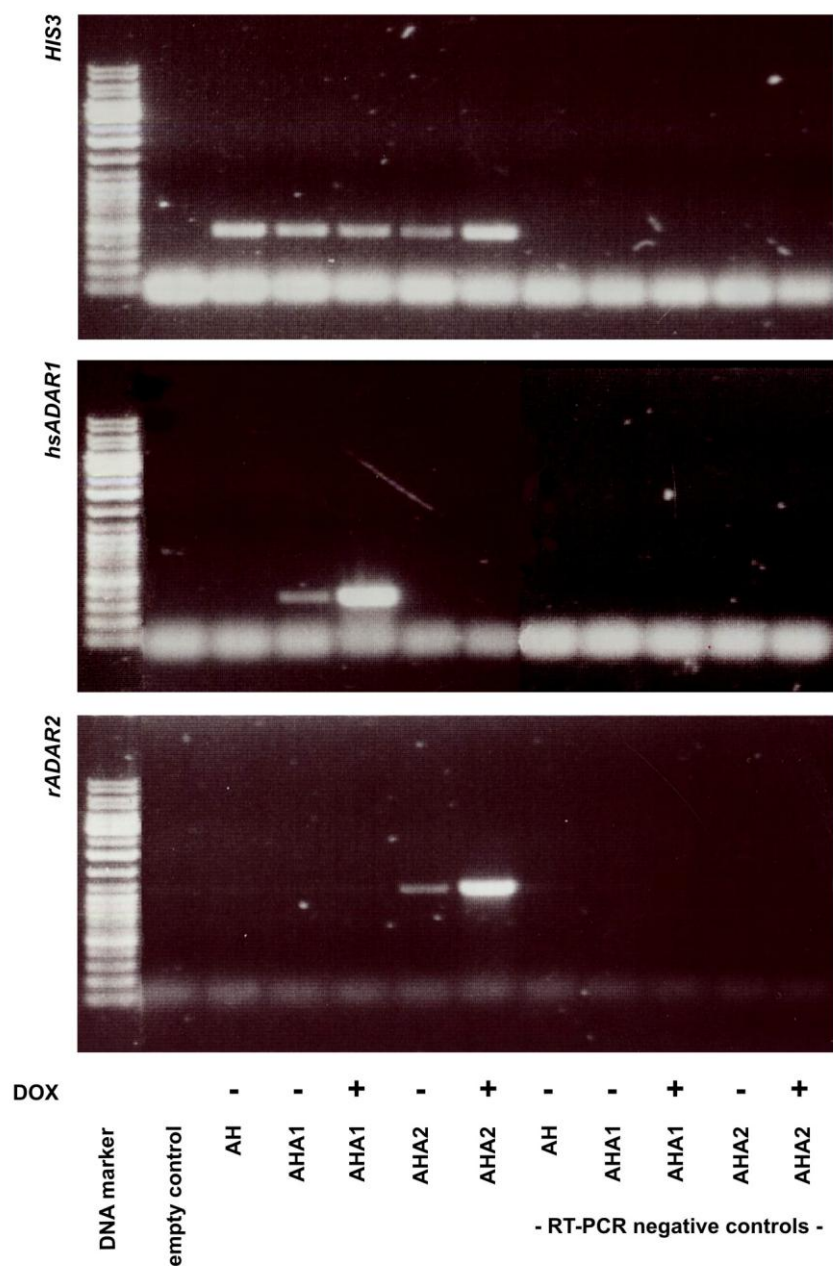
Fig. 4.4: Confirmation of mRNA transcribed from HIS3-editing substrate, hsADAR1 and rADAR2 genes. The indicated strains were grown on appropriate media in the presence or absence of DOX. RT-PCR was performed with MJ2139 and MJ2080 for HIS3, MJ317 and MJ532 for hsADAR1 and MJ2102 and MJ2103 for rADAR2. On the first panel expression of HIS3-editing substrate is shown in all examined strains. Second shows expression of hsADAR1 mRNA only in AHA1 strain and increase in amplification product in case of DOX induction. The same effect can be seen in case of rADAR2 mRNA in AHA2 strain.

Expression of ADARs in the obtained strains was also shown via Western Blotting using antibodies against *hs*ADAR1 or against Flag-tag epitope in case of *r*ADAR2. Surprisingly expression was only detected upon addition of DOX [*Fig. 4.5*].
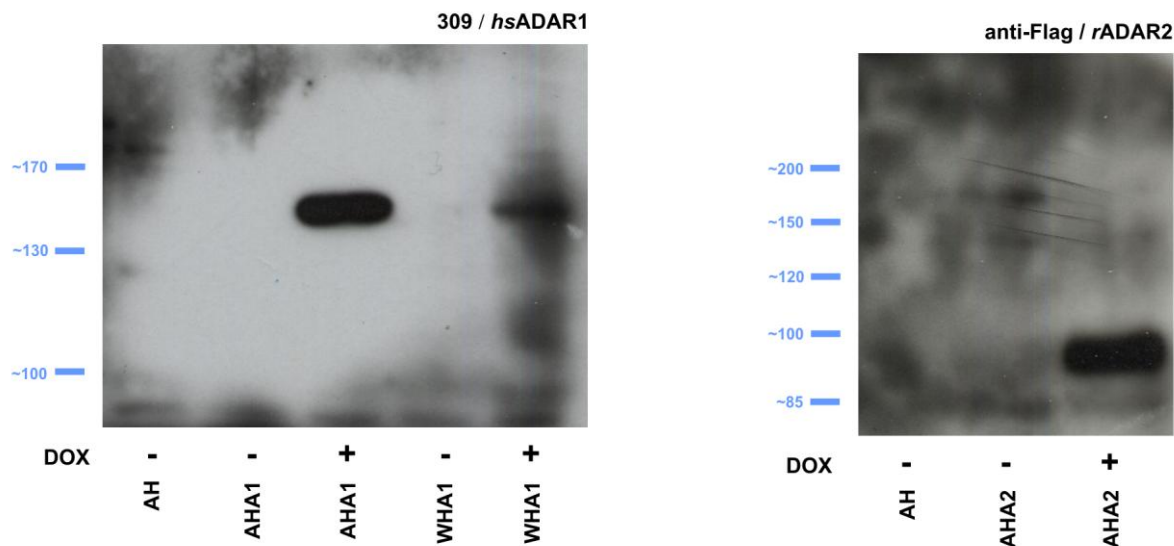
Fig. 4.5: Picture of blots stained with anti-hsADAR1 primary antibody (309), with anti-Flag primary antibody and with horse radish peroxidase conjugated secondary antibodies. AH, AHA1, WHA1 and AHA2 yeast strains were grown on selective media without and with addition of doxycycline induction. Protein extracts isolated from the cultures were separated via SDS-PAGE. In both cases, hsADAR1 and rADAR2, the protein expression is only detectible in ADAR expressing strains and only upon DOX induction of the yeast culture.

Although both of the proteins, *hs*ADAR1 and *r*ADAR2 seemed to be properly expressed, only *r*ADAR2 in yeast had the predicted effect on the growth of the developed strains on the selective media. Additionally, leaky expression previously visible in RT-PCR experiment leads to growth on selective media even in the absence of DOX induction [*Fig. 4.6*].
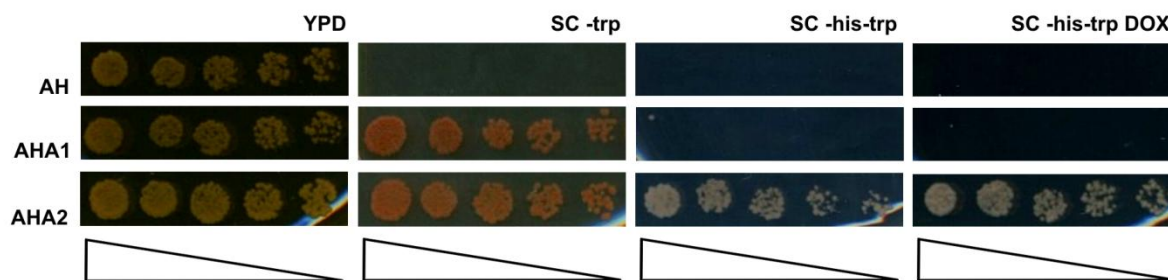
Fig. 4.6: Growth comparison of indicated strains on different selective media for phenotypical comparison of the strains expressing hsADAR1 and rADAR2. Strain expressing hsADAR1 in contrast to one expressing rADAR2 did not grow on medium lacking histidine and even induction via DOX didn't induce growth.

To understand, why yeast expressed ADAR1 did not lead to editing of the reporter, several assays were performed. First, *hs*ADAR1 was expressed in HeLa cells from its original vector and pulled down via immunoprecipitation with an anti-*hs*ADAR1 antibody. The immunoprecipitated material was able to edit a *hs*ADAR1 editing substrate (FlnA) *in vitro* [data not shown]. Second, HeLa cells expressing *hs*ADAR1 from the same vector were used later in FACS experiments together with a fluorescence editing reporter (GluR-B R/G amber stem-loop). A major effect on editing was observed [*Fig. 4.30*].

Even though the *hs*ADAR1 is properly expressed in yeast, it seems to be inactive, as the designed substrate is not edited [*Fig.4.7*]. Importantly, the same ADAR1 construct is active when expressed in mammalian cells, indicating, that the protein is not properly folded or lacks some important cofactors in yeast.
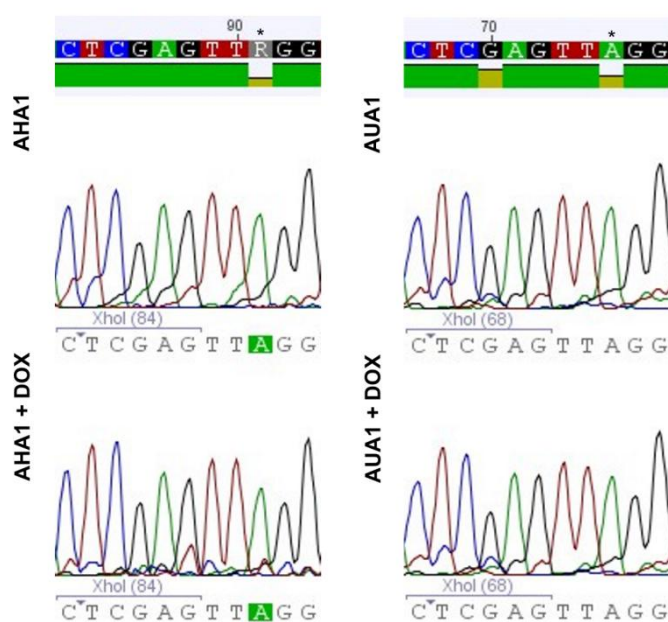
Fig. 4.7: Editing level comparison of the editing substrate expressed in the developed yeast strains expressing hsADAR1 gene. (edited adenosine marked with an asterisk) Both AHA1 and AUA1 strains were grown on the selective liquid media without or with an addition of DOX induction. cDNA obtained via reverse transcription was amplified using MJ2263 and MJ2080 primers and sequenced with MJ2263 primer. There is no visible effect on editing in case of expression of hsADAR1. (for rADAR2 editing levels Fig. 4.10 and Fig. 4.16)

4.1.2 Yeast strain for enhancer screening

An appropriate strain was developed for positive selection, to allow isolation of enhancers of editing. Enhancement in ADAR activity resulted in increased growth. To achieve this a *HIS3*-editing substrate was integrated into *Saccharomyces cerevisiae* W303 as a marker gene [*Fig. 3.1 and Fig. 4.1*]. rADAR2 gene was expressed from tetracycline inducible centromeric yeast expression vector [*Fig. 3.3*]. Induction by doxycycline and expression of His3 was tested on liquid and solid selective media [*Fig. 4.8 and Fig. 4.9*]. All strains showed the expected phenotype reflecting the auxotorphy markers used for introduction of transgenes. Interestingly, leakiness of the tetracycline inducible promoter can be observed when the AHA2 strain is growing on media lacking histidine. Even in the absence of doxycycline growth can be observed. DOX induction significantly increases the growth on the selective medium. The leakiness is even more apparent on solid media. Here the uninduced AHA2 strain is growing almost as good as WHA2 on medium lacking histidine. DOX induction improves the growth only marginally.
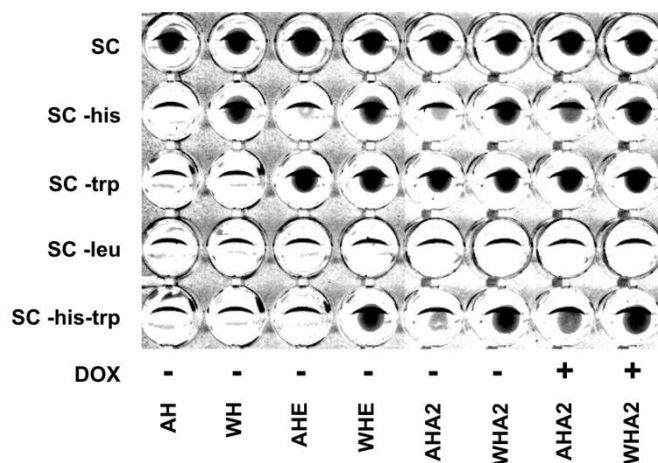
Fig. 4.8: Growth comparison of the developed strains on different selective liquid media for phenotypical comparison. AH, WH, AHE, WHE, AHA2 and WHA2 strains were grown on different liquid selective media in 96-well microtiter plate.
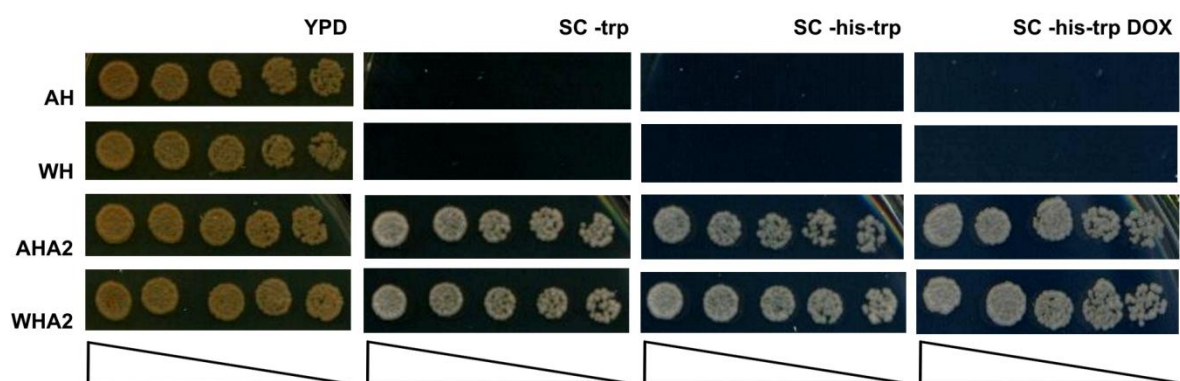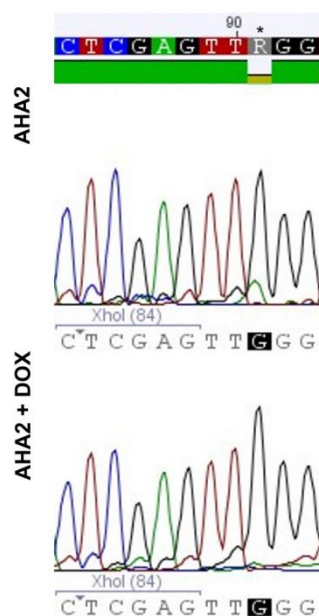

Fig. 4.9: Growth comparison of the developed strains on different selective solid media for phenotypical comparison. AH, WH, AHA2 and WHA2 strains were grown on different solid selective media in a dilution series.

Direct proof of editing of the substrate by *r*ADAR2 in yeast was provided by bulk sequencing of RT-PCR products obtained from the *HIS3*-editing substrate mRNA. A high level of editing was visible even without DOX induction due to the leakiness of the system. Induction by DOX led to 100% editing [Fig. 4.10].

Fig. 4.10: Comparison of the editing level of the substrate expressed in the developed yeast strain expressing rADAR2 gene (edited adenosine marked with an asterisk). AHA2 strain was grown on the selective liquid media without or with an addition of DOX. cDNA obtained via reverse transcription was amplified using MJ2263 and MJ2080 primers and sequenced with MJ2263 primer.

The yeast strain AHA2 together with control strains has been used for screening for enhancers of *r*ADAR2's editing activity. Since the leakiness of the system reduces the stringency of the screen, a 3-amino-1,2,4-triazole (3-AT) selection was applied. This compound is a competitive inhibitor of the *HIS3* gene product, imidazoleglycerol-phosphate dehydratase, and should reduce the background expression of *HIS3*. Thus, only the clones that have elevated expression of *HIS3* were expected to survive. Different concentrations of 3-AT were tested. Concentrations higher than 20mM 3-AT reduced background growth to undetectable levels [*Fig. 4.11*]. In the screen a concentration of 40mM 3-AT was used.
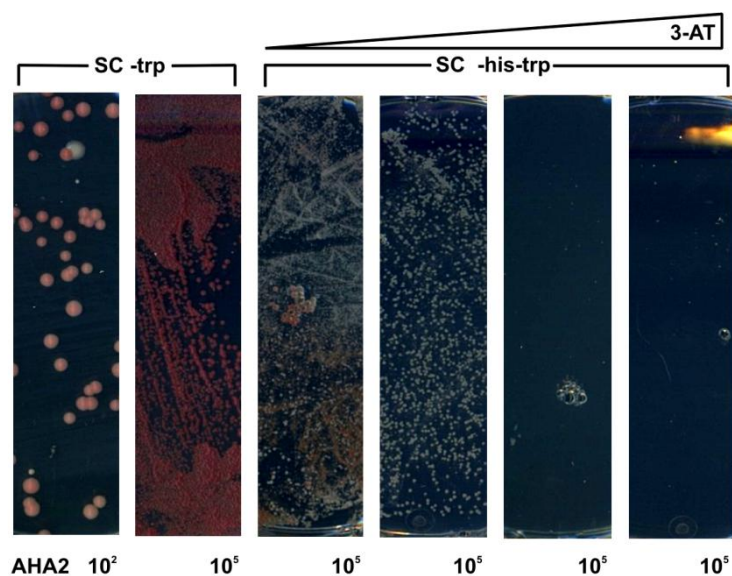
Fig. 4.11: A growth comparison of AHA2 yeast strain grown in the SC-his-trp medium containing an increasing concentration of 3-AT. 10^5 cells of AHA2 yeast strains were incubated on 10cm dishes for 3 days. On the picture growth on medium with different concentrations of 3-AT is shown (0, 5, 10 and 15mM). From 20mM on no growth could be observed.

4.1.3 Yeast strain for inhibitor screening

To allow for a negative selection on editing activity a strain containing an editable stop codon upstream of *URA3* was created [*Fig. 3.1*]. Additionally, the *rADAR2* gene was introduced on a tetracycline inducible centromeric expression vector [*Fig. 3.3*]. The drug 5-fluoroorotic acid (5-FOA) was added to the media. Ura3 converts FOA to 5-fluoro-uracil which is toxic to cells. Thus, editing leads to expression of Ura3 which in turn is toxic. Only cells that are inhibited in their editing activity are therefore able to grow on this medium. Transformation of this starting strain with an expression library, therefore allows to screen for factors that inhibit ADAR activity. The proper phenotype of strains and also induction of ADAR by doxycycline was tested on liquid and solid selective media [*Fig. 4.12 and Fig. 4.13*]. All strains show the expected phenotypes corresponding to the auxotrophic markers introduced. Again, leakiness of the tetracycline inducible promoter was observed. DOX induction significantly increases the growth on the selective medium. In comparison to His3 expressing strain [Fig. 4.8 and Fig. 4.9] the leakiness is not that extent and the DOX induction is more visible.
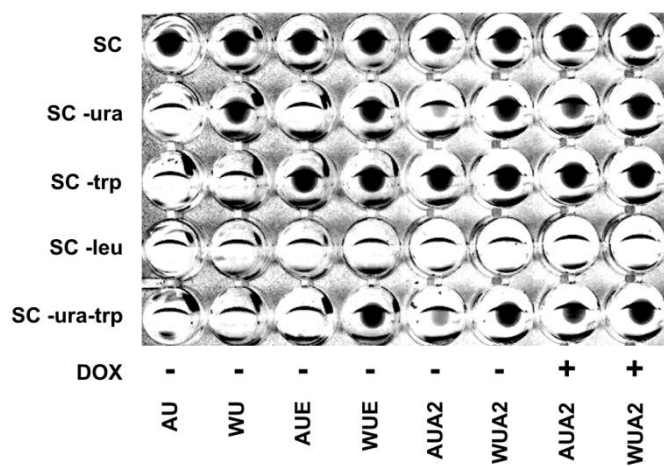
Fig. 4.12: Growth comparison of the developed strains on different liquid selective media for phenotypical comparison. AU, WU, AUE, WUE, AUA2 and WUA2 strains were grown on different liquid selective media in 96-well microtiter plate.
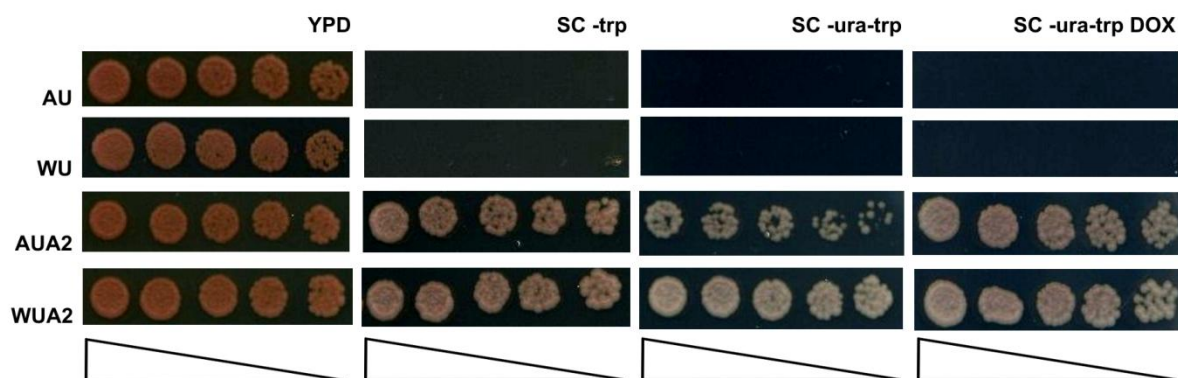

Fig. 4.13: Growth comparison of the developed strains on different solid selective media for phenotypical comparison. AU, WU, AUA2 and WUA2 strains were grown on different solid selective media in a dilution series.

In order to utilize 5-FOA in screens its influence on growth was tested. DOX increases the growth in medium without Ura, while it decreases the growth on medium with 5-FOA as seen on liquid media [*Fig. 4.14*]. On solid media 5-FOA prohibits the growth of strains expressing Ura3, but due to leakiness of the system and the smaller difference between uninduced and induced strain, also the uninduced AUA2 strain fails to grow on 5-FOA containing media [*Fig. 4.15*].
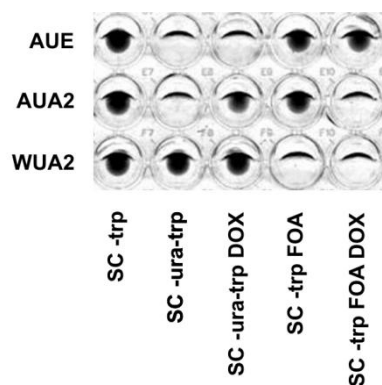
Fig. 4.14: Study of 5-FOA effect on URA3 expressing strains on liquid media. AUE, AUA2 and WUA2 strains were grown in different liquid selective media in 96-well microtiter plate.
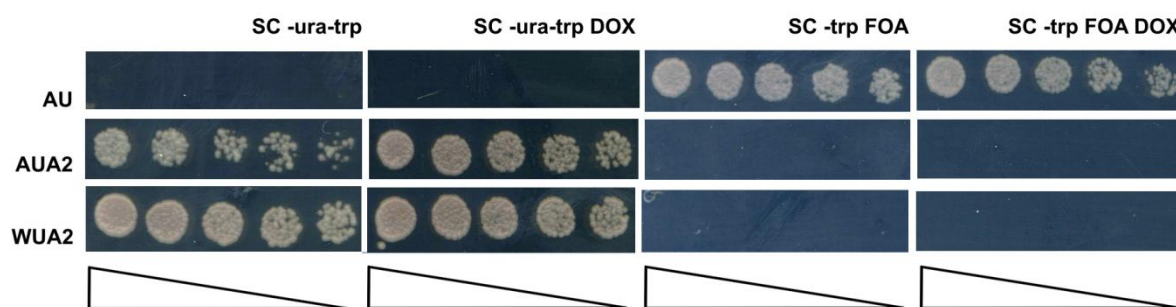

Fig. 4.15: Study of 5-FOA effect on URA3 expressing strains on solid media. AU, AUA2 and WUA2 strains were grown on different solid selective media in a dilution series.

Direct proof of editing of the substrate by *r*ADAR2 could be provided by bulk sequencing of RT-PCR products obtained from the *URA3*-editing substrate mRNA, which had been isolated from the yeast strain AUA2 (expressing *r*ADAR2). There is a moderate level of editing visible in the absence of DOX due to the leakiness of the system but the extent of editing increased after induction with DOX [*Fig. 4.16*]. Editing levels of AUA2 strain are comparably lower than the ones of AHA2 which would explain the differences in growth between them.
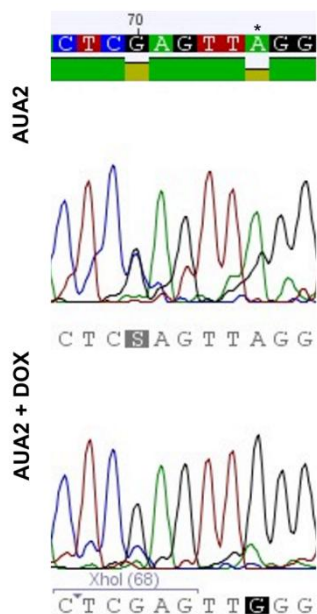
Fig. 4.16: Comparing the editing level of the substrate expressed in the developed yeast strain expressing rADAR2 gene (edited adenosine marked with an asterisk). The AUA2 strain was grown on the selective liquid media without or with an addition of DOX. cDNA obtained via reverse transcription was amplified using MJ2263 and MJ2080 primers and sequenced with MJ2263 primer.

The yeast strain AUA2 together with control strains has not been used for screening for inhibitors of *r*ADAR2's editing activity in this study.

4.1.4   Additional substrates tested

Besides the editing substrate derived from GluR-B Q/R site additional substrates were tested. For example, a shortened version of the GluR-B-derived substrate was tested in an attempt to interfere as little as possible with the mRNA structure and with folding and function of protein translated from this mRNA. However, this shortened version has not been a good substrate for *r*ADAR2. Other substrates that were tested for editing in yeast were derived from the hepatitis delta virus type 1 amber/W site present in the antigenome of the virus or from the Gabra-3 I/M editing site. Also, these substrates were known to be edited by both ADAR enzymes (Sato, Wong et al. 2001; Ohlson, Pedersen et al. 2007). The hepatitis delta virus substrate was adapted from the minimal U23/D5 substrate (Sato, Wong et al. 2001) [*Fig. 4.17*].
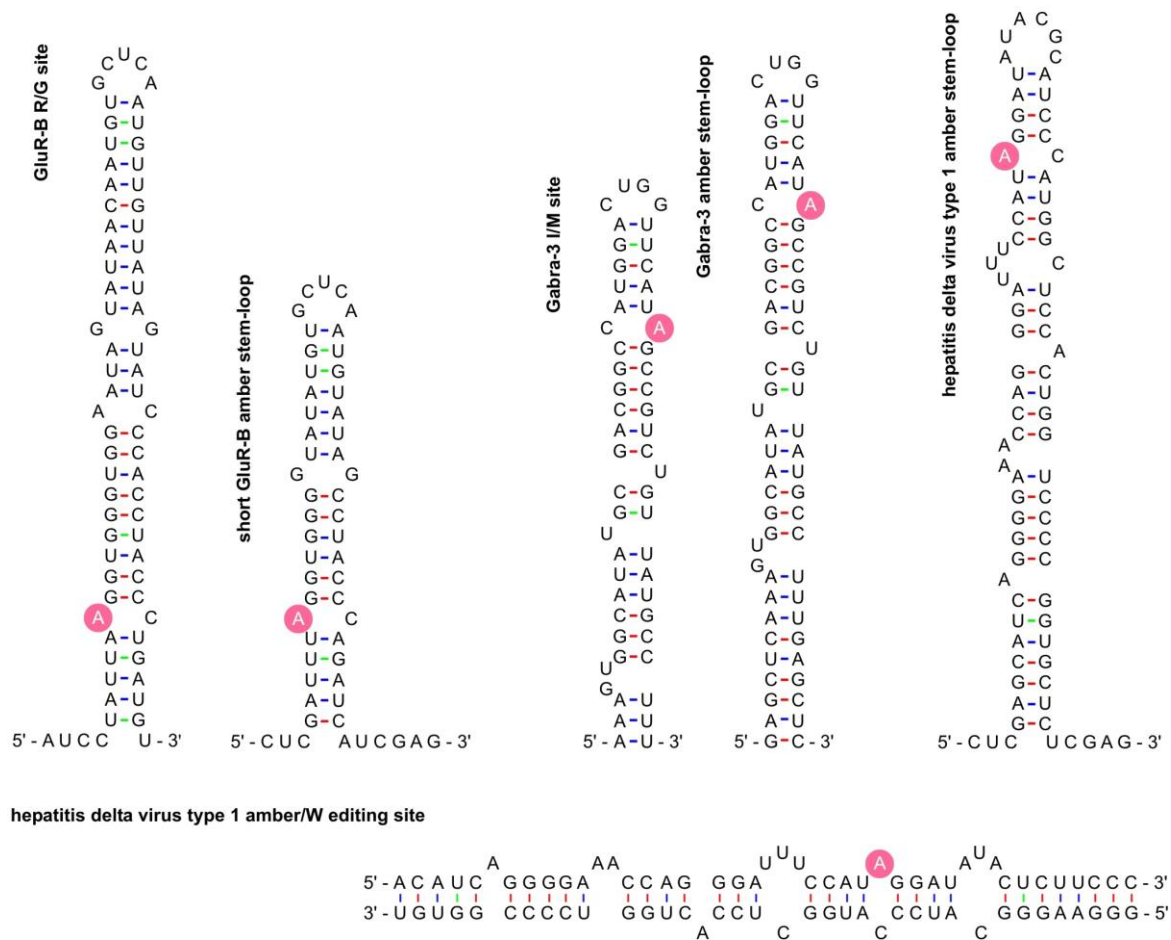
Fig. 4.17: RNA folding predictions of editing substrates tested in yeast system based on GluR-B R/G, hepatitis delta virus type 1 amber/W and Gabra-3 I/M editing sites with edited adenosine in a circle. Editing substrates are shown in their XhoI restriction site context.

The strains were tested on liquid or solid selective media [*Fig. 4.18 and Fig. 4.19*]. None of the strains containing editable version of the substrate and expressing either *hs*ADAR1 or *r*ADAR2 seemed to be editing the substrate, which resulted in lack of expression of *HIS*3 and no growth on histidine-lacking medium. Also, the addition of DOX didn't improve the growth. A possible change in editing was not investigated further than phenotype comparison.
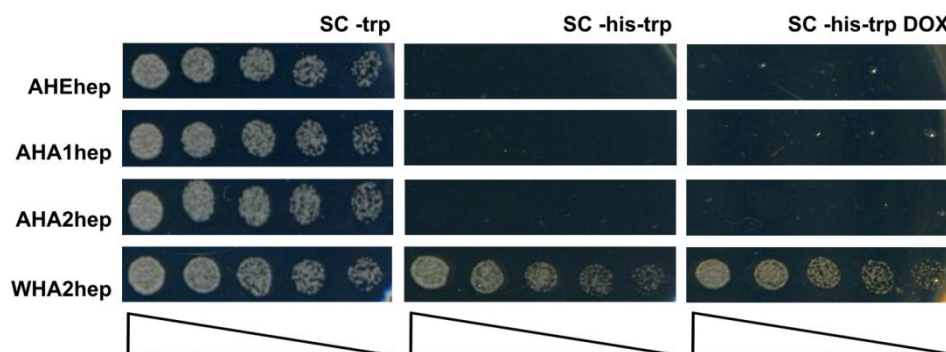
Fig. 4.18: Growth comparison of yeast strains bearing hepatitis delta virus type 1 based substrate on solid media. AHEhep, AHA1hep, AHA2hep and WHA2hep strains were grown on different solid selective media in a dilution series.
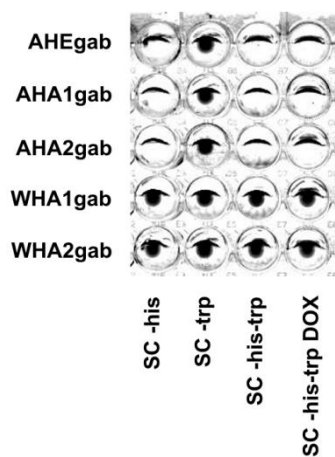

Fig. 4.19: Growth comparison of yeast strains bearing Gabra-3-derived substrate on liquid media. AHEgab, AHA1gab, AHA2gab, WHA1gab and WHA2gab strains were grown in different liquid selective media in 96-well microtiter plate.

4.2 Results of mating assay

To screen for activators of editing the test strain AHA2 described above was mated with strain Y187, expressing a human fetal brain cDNA library from a *LEU2* vector. $7.4x10^6$ mated colonies were screened on 40mM 3-AT medium. Approximately $2x10^3$ colonies appeared on the selective plates. The fastest colonies (a total of 61) were picked for further analysis.

4.2.1 Confirmation of yeast strains obtained in the screen

To exclude false positive colonies and to confirm that expression of *r*ADAR2 was essential for growth on medium lacking histidine the *rADAR2* bearing vector was selectively lost from the strains by growth under non-selective conditions. As expected diploids that had lost the *r*ADAR2 plasmid failed to grow on *his⁻* plates [*Fig. 4.20*].
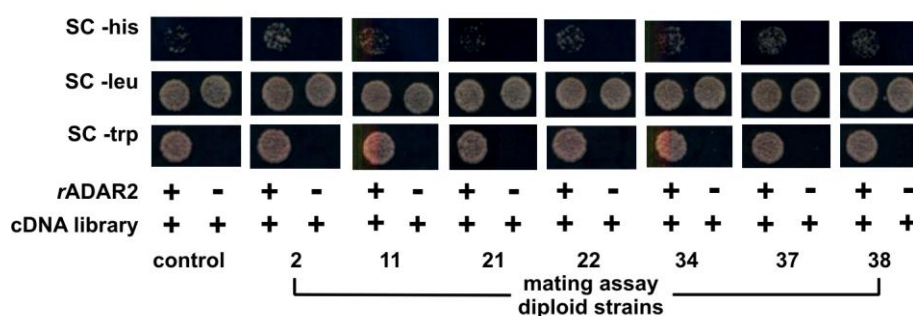

Fig. 4.20: Phenotype comparison of the strains obtained in the screen expressing or not expressing rADAR2. In this case a selection of 7 diploid strains with an additional diploid control was grown on different solid selective media for 2 days.

4.2.2 Hits obtained in the screen

no	protein	function	frame	gene length (bp)	Coding	repetitive elements
1	chromosome 5 contig					SINEs (ALU, MIR)
2	FABP7	transport of a hydrophobic ligand	2	997	Coding	none
3	chromosome 7					none

	contig					
4	chromosome 15 contig					SINEs (ALU, MIR)
5	short stretch					
6	RPS17L4	small subunit ribosomal protein	2	502	Coding	none
7	chromosome 12 contig					LINEs
8	PDPR	pyruvate dehydrogenase		7999	3'UTR	SINEs (ALU)
9	CAMKV	kinase like protein		3028	3'UTR	none
10	STK38	serine/threonine kinase	3	3569	Coding	none
11	SSRP1	transcriptional elongation factor	2	2818	Coding	none
12	chromosome 15 contig					
13	NPDC1	oncogene, transcription factor	2	1511	Coding	none
14	CytC oxidase sub II	oxidase	2	684	Coding	none
15	short stretch					none
16	chromosome 16 contig					SINEs (ALU, MIR)
17	HN1	androgen regulated protein	3	1584	Coding	none
18	no significant hits					
19	COF1	actin-modulating protein	2	1234	Coding	none
20	CytC oxidase sub III	oxidase	2	781	Coding	none
21	KAT5/HTATIP	histone acetyltransferase	3	2313	Coding	none
22	DYRK2	tyrosine kinase		6159	3'UTR	none
23	WIPF2	organisation of the actin cytoskeleton		5525	3'UTR	SINEs (ALUs)
24	chromosome 5 contig					LTR
25	no amplification					
26	chromosome 15 contig					SINEs (ALU)
27	MARCKSL1/MRP	calmodulin transduction	3	1545	Coding	none
28	RPS3	small subunit ribosomal protein	3	840	Coding	none
29	chromosome 8 contig					SINEs (ALU)
30	chromosome 5 contig					none
31	PSMB4	proteasome protein	3	925	Coding	none
32	SOX1	embryonic transcription factor		4108	3'UTR	none
33	IGFBP4/IPB4	growth factor binding protein		2235	3'UTR	none
34	chromosome 1 contig					none
35	GPX4	glutathione peroxidase	2	594	Coding	none
36	DSS1/SHFM1	oncogene, proteasome protein	3	494	Coding	none
37	no amplification					
38	chromosome 17					SINE

	contig					(ALU)
39	**chromosome X contig**					SINE (ALU)
40	**HNRNPA2B1**	hnRNP	2	1714	Coding	none
41	**chromosome 13 contig**					SINE (MIR) LINE
42	**ZBTB4**	transcription regulation		5852	3'UTR	none
43	short stretch					
44	**chromosome 17 contig**					SINE (ALU)
45	**chromosome 1 contig**					LINE
46	no hits					
47	**chromosome X contig**					none
48	**DNAJB6**	chaperone	3	2484	Coding	none
49	**STMN2**	membrane/cytoskeleton interaction	2	1814	Coding	
50	no significant hits					
51	**NBPFs**	neuroblastoma breakpoint family proteins		4337	3'UTR	none
52	**Q5TGE2**	ubiquitin conjugated enzyme		1880	3'UTR	LINE, SINE (ALU)
53	**chromosome 10 contig**					none
54	short stretch					
55	**CytB**	oxidase	1	1135	Coding	
56	**chromosome 20 contig**					LINE, SINE (ALU)
57	**EEF1A1**	translation elongation factor		3510	3'UTR	none
58	no amplification					
59	**chromosome 22 contig**					none
60	**TCTP**	tumour protein	1	1127	Coding	
61	no significant hits					SINE (ALU)

- **transcription factors**

- **mitochondrial proteins**

- **oncogenes**

- **kinases**

- **cytoskeleton associated proteins**

- **proteasome associated proteins**

- **ribosomal proteins**

- **RNA binding proteins**

- **chaperones**

- **translation factors**

Fig. 4.21: A table listing sequencing results and a short description of genes from all 61 yeast strains obtained in the screen. The names of the proteins, their function, the frame, in which the cDNA sequence was cloned in yeast expression vector, and the length of the whole gene are listed. Also, the information if the query sequence aligns to the coding regions and the presence of the repetitive elements in the sequence is provided.

Out of 61 hits the plasmid DNA of 58 diploid strains from the screen were sequenced successfully. Of these 58 hits 4 sequences were too short to yield clear results and another 4 sequences did not align with the human genome. The remaining 50 hits were divided into two major groups: i) 30 candidates aligned to annotated protein coding sequences and ii) 20 candidates aligned to non-annotated, unresolved DNA contigs. The biggest subgroups among the candidates aligning to the coding sequences are as follows: transcription factors – 5 hits, mitochondrial proteins – 5, oncogenes – 4, kinases – 3, cytoskeleton associated proteins – 3 and proteasome associated proteins - 3. Only three proteins were RNA binding proteins, out of which two are ribosomal proteins.

4.2.3   Non-coding result of the screen

Further investigation revealed that 10 out of 30 hits that aligned to mRNAs were located in the respective 3'UTRs. Moreover, 18 out of 20 candidates were not in frame with the upstream Gal4 activation domain of the expression plasmid. Additionally, the remaining 2 candidates were either a mitochondrial protein sequence with a different genetic code, which cannot be translated in the cytoplasm or a stop codon containing 5'UTR fragment raising the possibility of the proteins also not being properly expressed from these sequences.

4.2.4   Assays for selection of the best candidates

To further select the best out of 61 candidate strains we wanted to compare the strains via growth on selective media. However, this turned out to be inconclusive as too small differences occurred between the strains to choose the ones that grow the fastest [*Fig. 4.22*].
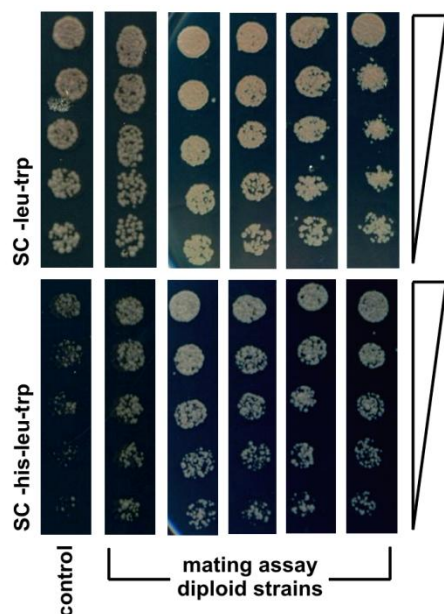
Fig. 4.22: Growth comparison between few diploid strains obtained in the screen on selective media. Diploid control strain and chosen candidate strains were grown on different solid selective media in a dilution series. Candidate strains show faster growth on SC –his-leu-trp medium than the control strain but not much difference between themselves.

Different candidate strains were also tested on selective liquid media. In order to do that a diploid strain lacking ADAR2 was created for comparison. On medium containing histidine all strains grow with comparable velocity. As expected, diploid strains expressing both *r*ADAR2 and hits showed different growth on medium without histidine. Strains containing hit #10 and #36 grew faster than the control strain and strain #36 grows faster than strain #10. The last graph shows growth on medium lacking histidine of the *r*ADAR2 expressing strains normalized to empty strains [*Fig. 4.23*].
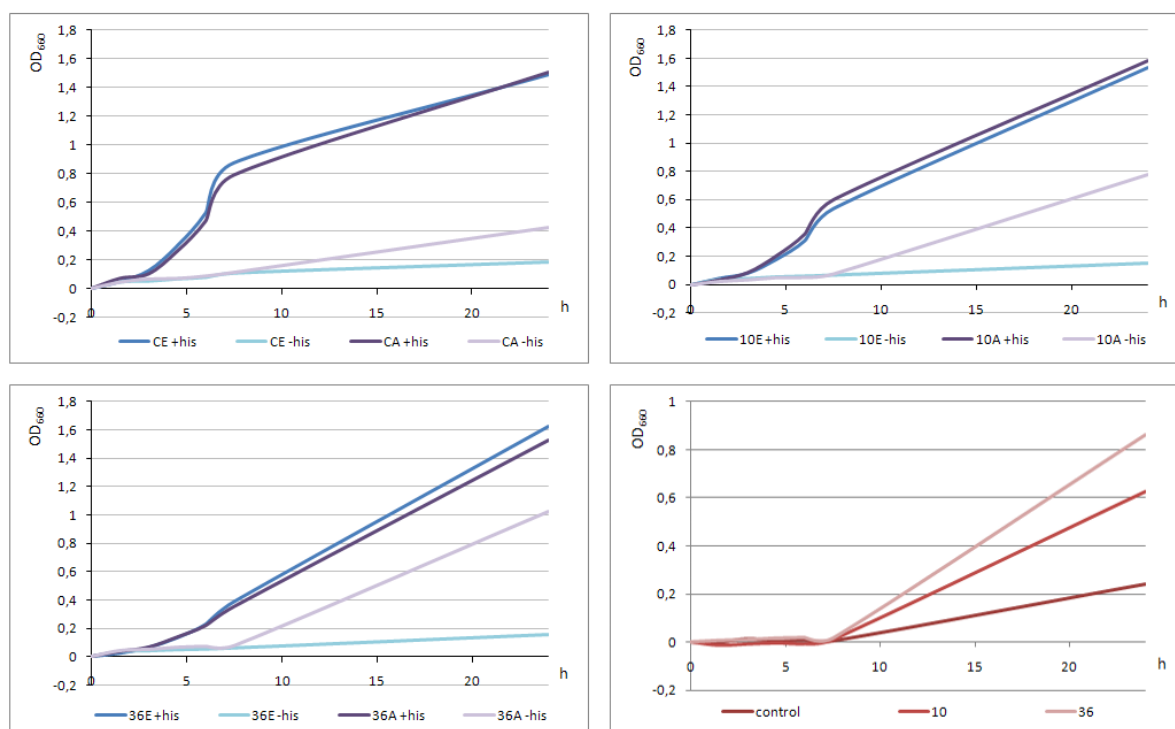
Fig. 4.23: Picture of the growth curves of the three strains: diploid control strain (C) and two candidate strains; 10 and 36. The diploid strains expressing rADAR2 (A) and with an empty vector (E) were grown for over 24h in selective media selecting only for trp1and leu2 and in media selecting for his3, trp1 and leu2.

Although growth curves in liquid media showed clear differences a more direct way to determine editing levels was sought. The first choice would have been a poisoned primer reaction (Keegan, Rosenthal et al. 2007). Several primers situated downstream of the editing site were tested. While clear stops were observed on *in vitro* transcribed RNA no products were obtained on total RNA. This might be due to low abundance of the *HIS3* transcript and/or low purity of the RNA [*Fig. 4.24*].

Fig. 4.24: Picture of primer extension reaction of the editing substrate separated on 10% polyacrylamide gel. On RNA a reaction with AMV Reverse Transcriptase and a radiolabeled primer was performed. Shown are: radiolabeled primer only, in vitro transcribed RNA from pUC18 HIS3amber and pUC18 HIS3w vectors and total RNA from liquid cultures of AHE, AHA2 and WHA2 strains. One can amplify shorter and longer fragments from in vitro transcribed RNA but not from the total RNA.

To assess the differences in editing levels between different candidates editing levels were directly compared by sequencing of the substrate site. A diploid strain carrying an empty cDNA library vector and expressing *r*ADAR2 served as a negative control. Heights of adenosine and guanosine peaks in the electrophorograms at the editing site were measured. The height of the guanosine peak was divided by the combined heights of guanosine and adenosine peaks. The method was tested in biological replicas. This indicated quite some variability amongst replicas [*Fig. 4.25*]. However, replicas showed the same trend with little standard deviation [*Fig. 4.26*].
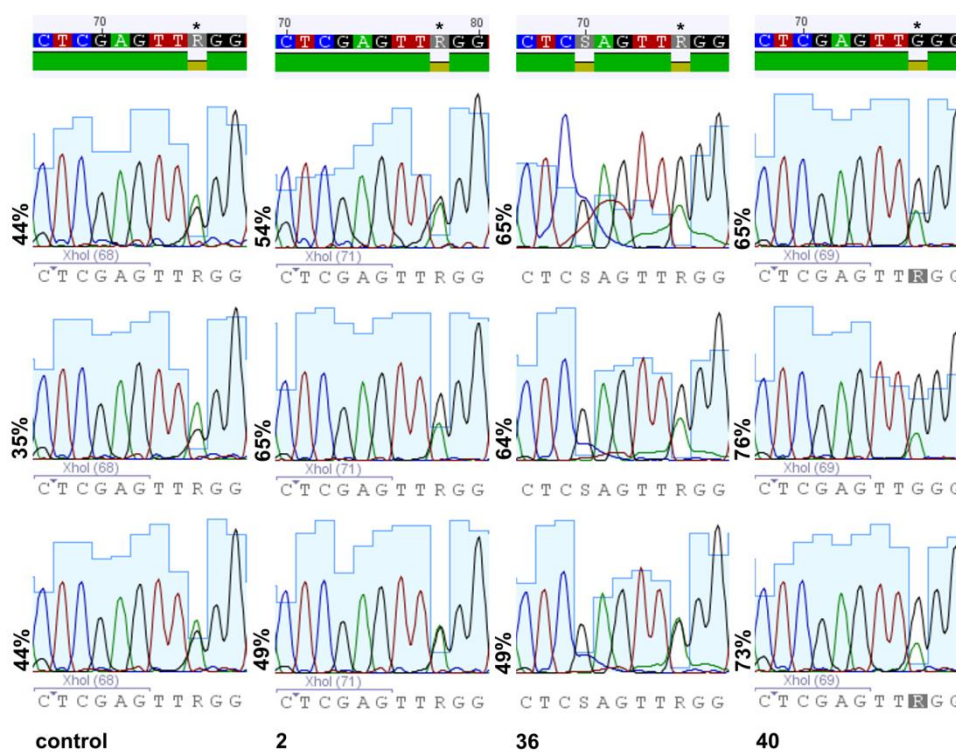
Fig. 4.25: Sequence comparison between RT-PCR amplification products of his3-editing substrate of different candidates (edited adenosine marked with an asterisk). The strains were grown in selective liquid media containing DOX (5μg/ml). cDNA obtained via reverse transcription was amplified using MJ2263 and MJ2080 primers and sequenced with MJ2263 primer. The electrophorograms are showing slight differences in editing between different repeats for each candidate and also that all chosen candidates show higher editing levels than in case of the control strain carrying empty cDNA library vector.
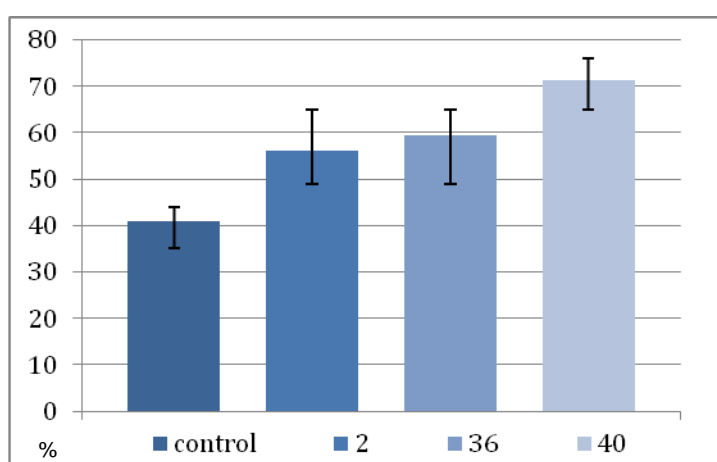
Fig. 4.26: Difference in editing levels between triplicates of few chosen screen candidates. The mean value with the standard deviation is shown. The graph shows a control strain showing editing activity at ~40% and few chosen candidates with editing levels exceeding it.

The same RT-PCR procedure was applied to all isolated candidates. However, 11 could not be amplified and were thus not considered in this assay. Of the remaining clones, 32 candidates showed higher editing levels than the control strain while 17 candidates even suggested a decrease in editing [*Fig. 4.27 A*]. Four candidates showed editing levels higher than 80-90% which corresponds to a 50% increase in editing compared to the control strain. About half of the candidates showed an increase of more than 14% in editing [*Fig. 4.27A*].

Verification by sequencing was repeated in a second run this time including all candidates except candidate #22, which could not be amplified. In this round the results of editing are slightly different from the first round. Only 2 candidates showed lower editing levels than the control in this run. All but 2 candidates exceeding 14% threshold in the first run exceeded the it also this time [*Fig. 4.27B*]. While repeats of the sequencing showed some variation, most of candidates displaying high editing did so in both rounds of the experiment.
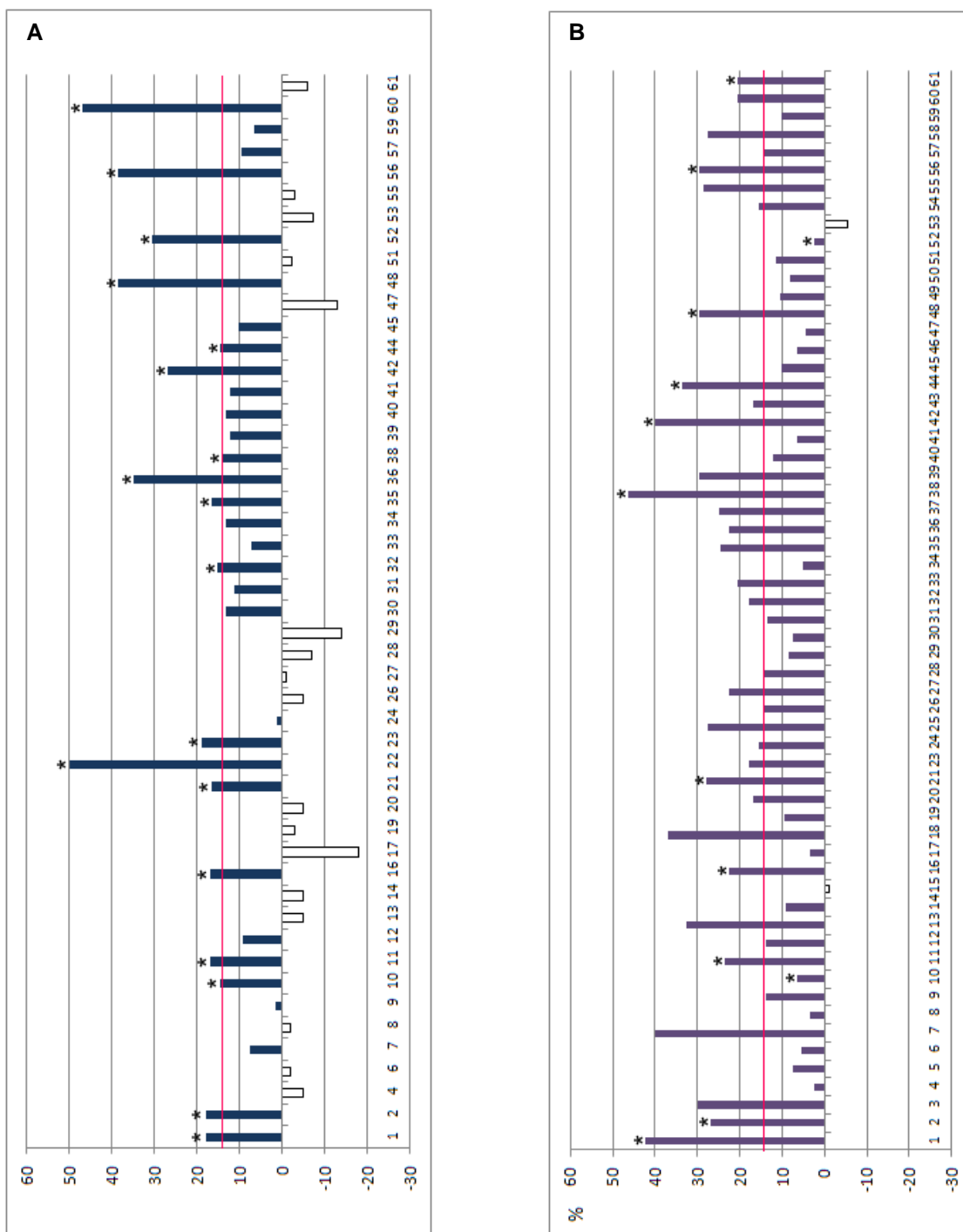
Fig. 4.27: Difference in editing levels between all screen candidates. Values are normalized to the control showing % of editing above or below editing level of the control, which was 43% and 36% in the first and the second run respectively. Both graphs show also 14% threshold and candidates exceeding it in the first run indicated with asterisks.

4.3   Confirmation in tissue culture

Candidate clones leading to highest editing levels had to be verified in a mammalian system. Therefore, 15 best hits were cloned into a tissue culture expression vector and transfected in a HeLa cell line for indirect and direct assessment of their ability to enhance editing.

4.3.1   Hits expressed in mammalian system

HeLa cells express ADAR1 and ADAR2 at moderate levels leading to detectable editing of endogenous substrates (Rueter, Burns et al. 1995; Yang, Sklar et al. 1995). The 15 hits were cloned and expressed from a vector containing a Myc-tag and a NLS directing the proteins to the nucleus [*Fig. 4.28*]. Candidates that were initially out of frame in the yeast expression vector were cloned in two versions: in the original out of frame version, and the corrected one allowing translation of the proper polypeptide. Non-coding candidates were cloned only in the original frame.

#	protein	editing level – control [%]	sequence
1	chromosome 5 contig	18	
2	FABP7	18	coding
10	STK38	14.5	coding
11	SSRP1	17	coding
21	KAT5/HTATIP	16.5	coding
22	DYRK2	50	3'UTR
23	WIPF2	19	3'UTR
32	SOX1	15	3'UTR
35	GPX4	16.5	coding
36	DSS1/SHFM1	35	coding
42	ZBTB4	27	3'UTR
44	chromosome 17 contig	14.5	
48	DNAJB6	38.5	coding
52	Q5TGE2	30.5	3'UTR
60	TCTP	47	coding

Fig. 4.28: A table listing the candidates chosen for confirmation in the tissue culture. The editing levels from the first run normalized to the editing level of control strain, which was 43%, are listed. Also coding character of the candidates is indicated.

Successfull expression was tested by Western Blotting. Sequences cloned out of frame only expressed polypeptides slightly larger than the Myc- and NLS sequence [*Fig. 4.29*]. Moreover, cytological staining of HeLa cells expressing the Myc-tagged candidates proved their predominant nuclear localisation. Transfection efficiency was ranging from 20 to 50% and varied slightly throughout the experiments [*Fig. 4.29*].
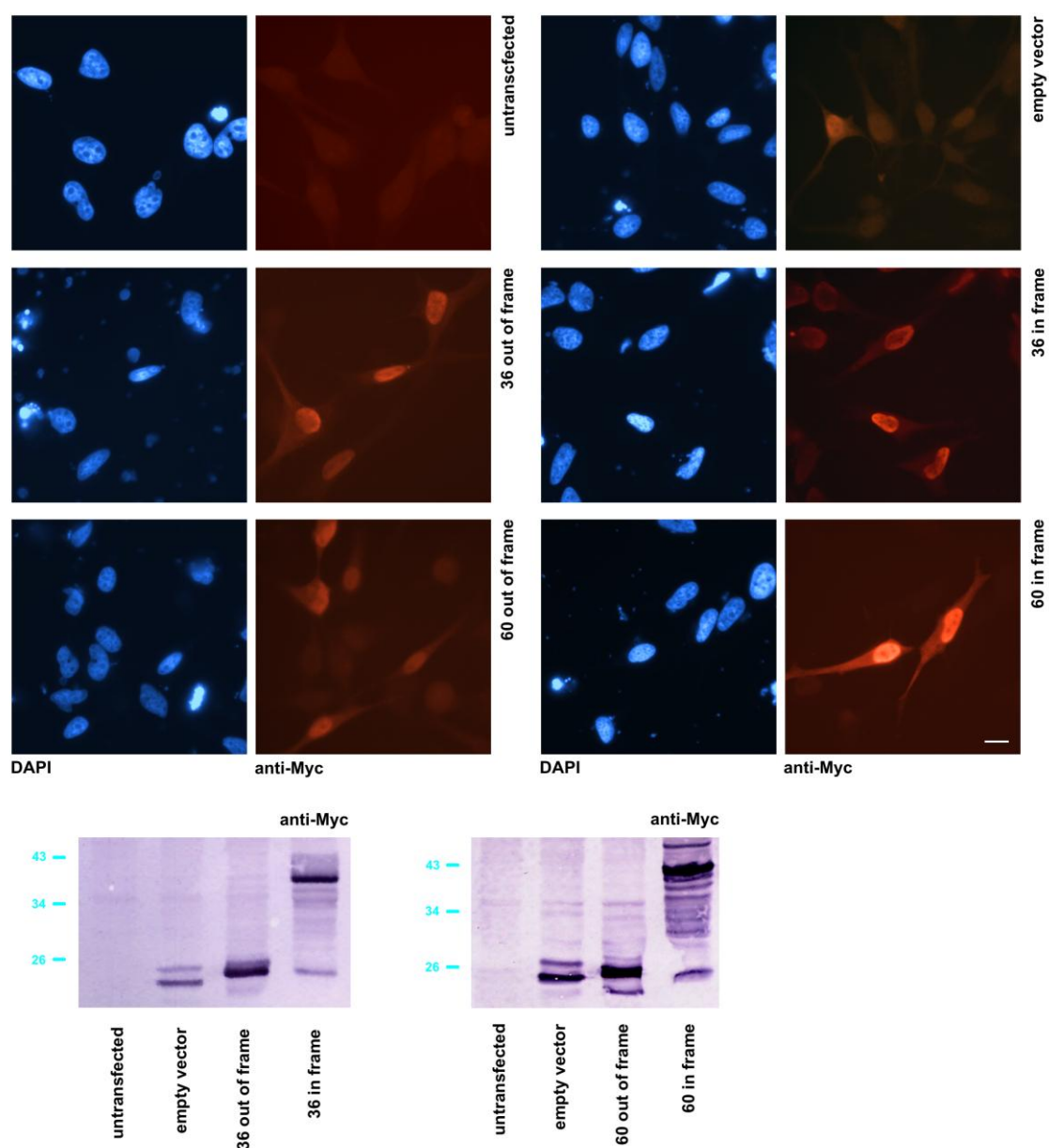

Fig. 4.29: Western Blotting and cytological staining of two candidates. As an example expression of candidates #36 and #60 are shown. Candidates were cloned out of frame and in frame and these two variants along with the empty vector control were transfected into HeLa cell line. Western Blotting and detection with an anti-Myc antibody showed proper expression of the polypeptides. Shown with staining

in the red channel the polypeptides are visible in the nucleus due to the presence of NLS. Scale bar: 10μm.

4.3.2 A FACS reporter system for quantification of editing levels

For quantitative assessment of editing in tissue culture cells a GluR-B amber editing site was introduced in a vector expressing RFP and GFP separated by a short linker (Schoft, Schopoff et al. 2007). The editing site was a part of a stop codon. Introduction downstream of RFP allows constitutive expression of RFP and editing-dependent expression of GFP. The fluorescence has been measured by flow cytometry and in particular by fluorescence-activated cell sorting flow cytometer (FACS). The cells were transfected with both the editing substrate (RNAG) and the candidates.

For graphic depiction, the red fluorescence was plotted on a logarithmic scaled x axis, while the green fluorescence was plotted on the y axis. If both proteins are expressed in equal amounts the readout of each single cell would lie on the diagonal of the graph. This is the case in a 'pre-edited' version of RNAG vector (RNWG). HeLa cells with moderate ADAR1 and ADAR2 expression readouts are placed more towards the x axis. Increased editing would shift the readouts towards the diagonal of the graph. Maximal editing is achieved upon cotransfection of *hs*ADAR1 or *r*ADAR2, shifting most cells to the diagonal [*Fig. 4.30*]. The same changes could also be visualized upon microscopic inspection [*Fig. 4.31*].
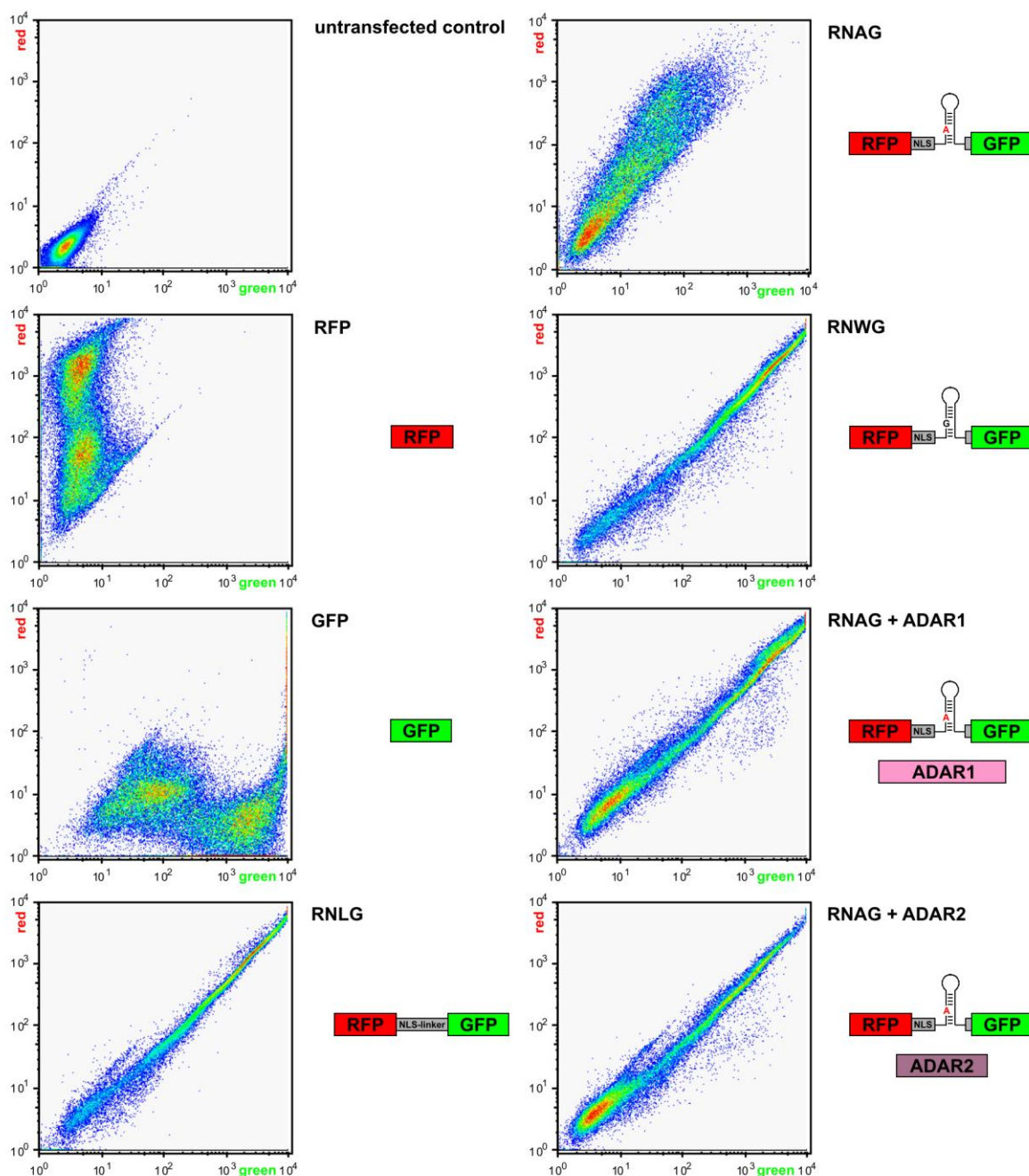
Fig. 4.30: FACS graphs of editing substrate and various controls. The untransfected control, RFP and GFP only expressing vectors, RNLG, the vector the system was based on, RNAG, the editing substrate containing vector, RNWG, the 'pre-edited' version of RNAG and cotransfections of RNAG with hsADAR1 and rADAR2 expressing vectors from strong promoters as controls are shown to confirm functionality of the system.
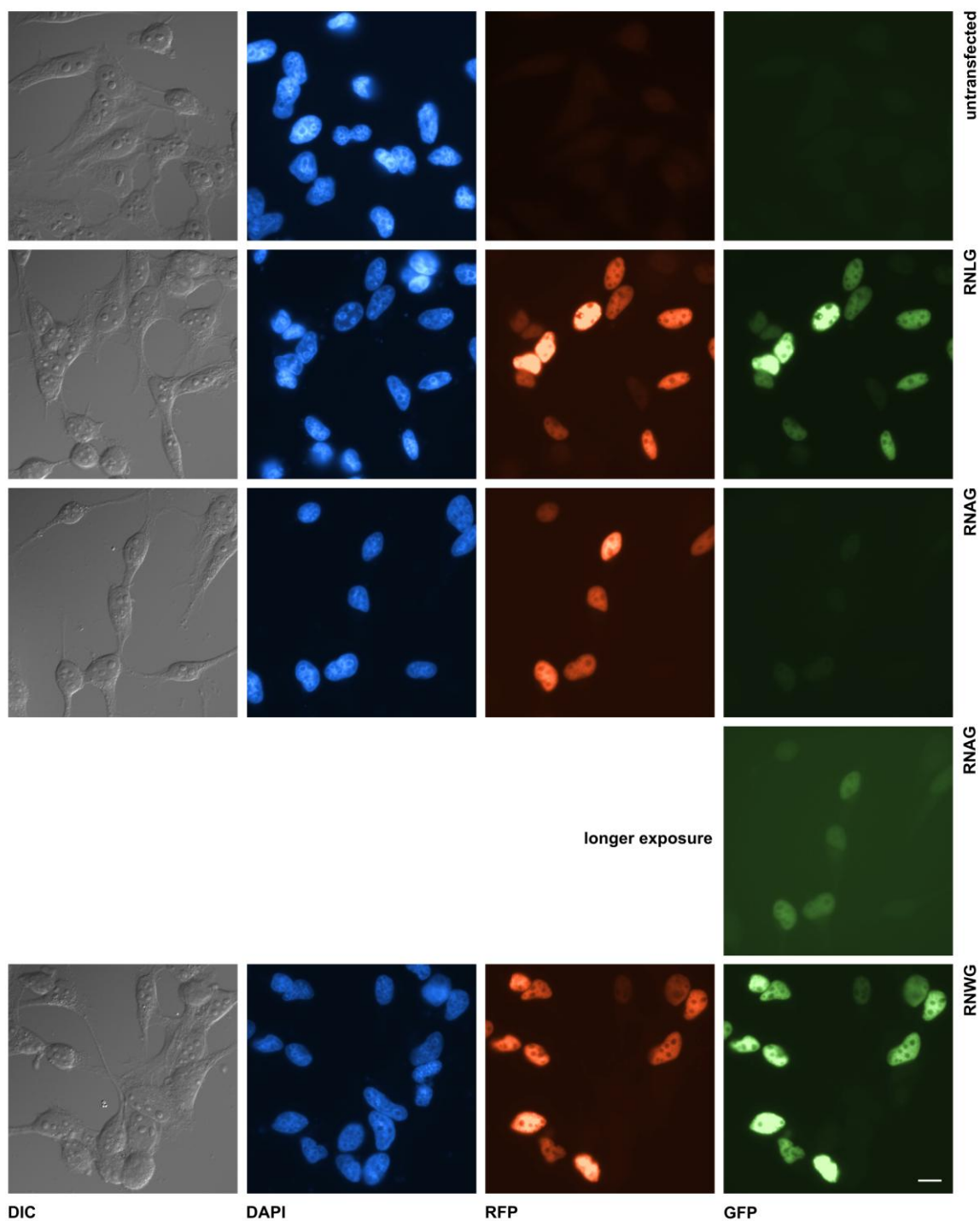
Fig. 4.31: Microscopic pictures showing HeLa cells transfected with vectors constructed for FACS analysis expressing RFP and GFP. After transfection of HeLa cells with appropriate constructs the cells were incubated 36-48 hours before fixing. Expression of RFP and GFP in case of RNLG and RNWG are at comparable levels but in case of RNAG, where expression of GFP depends on editing by ADARs, it is at the level visibly lower than RFP. To help in visualization of GFP expression in that case a photo of GFP expression made under longer exposure was added. Scale bar: 10μm.

4.3.3 Testing screen candidates in FACS reporter system

Chosen candidates were tested three times via FACS to allow statistical evaluation. Since cotransfection of RNAG with the candidate vectors reduced transfection efficiency an empty 'candidate' vector was used as control. Transfection efficiencies were in the range of 20-50%. Of all tested hits the in frame variant of hit #36 showed the most obvious difference in the FACS assay [*Fig. 4.32*].
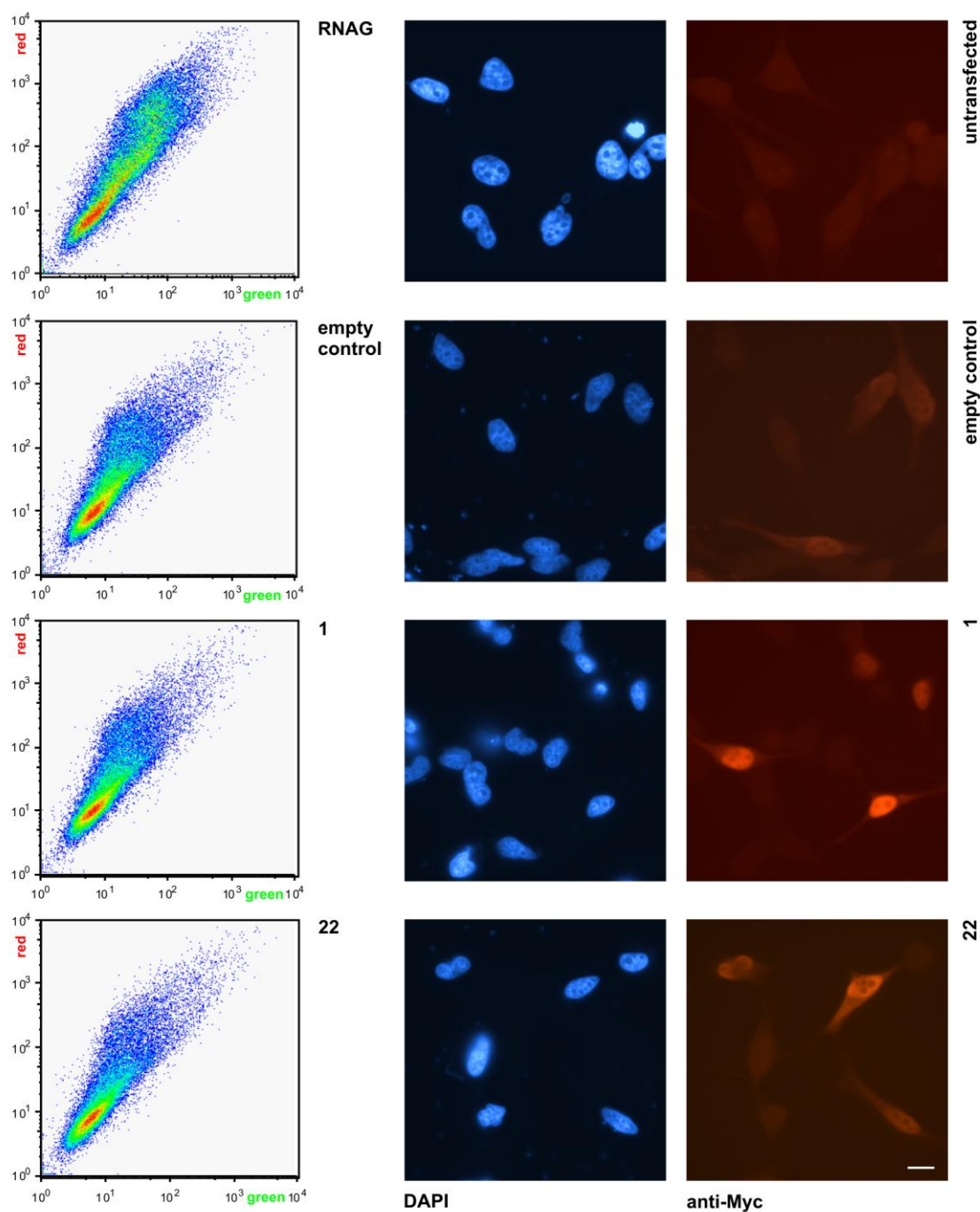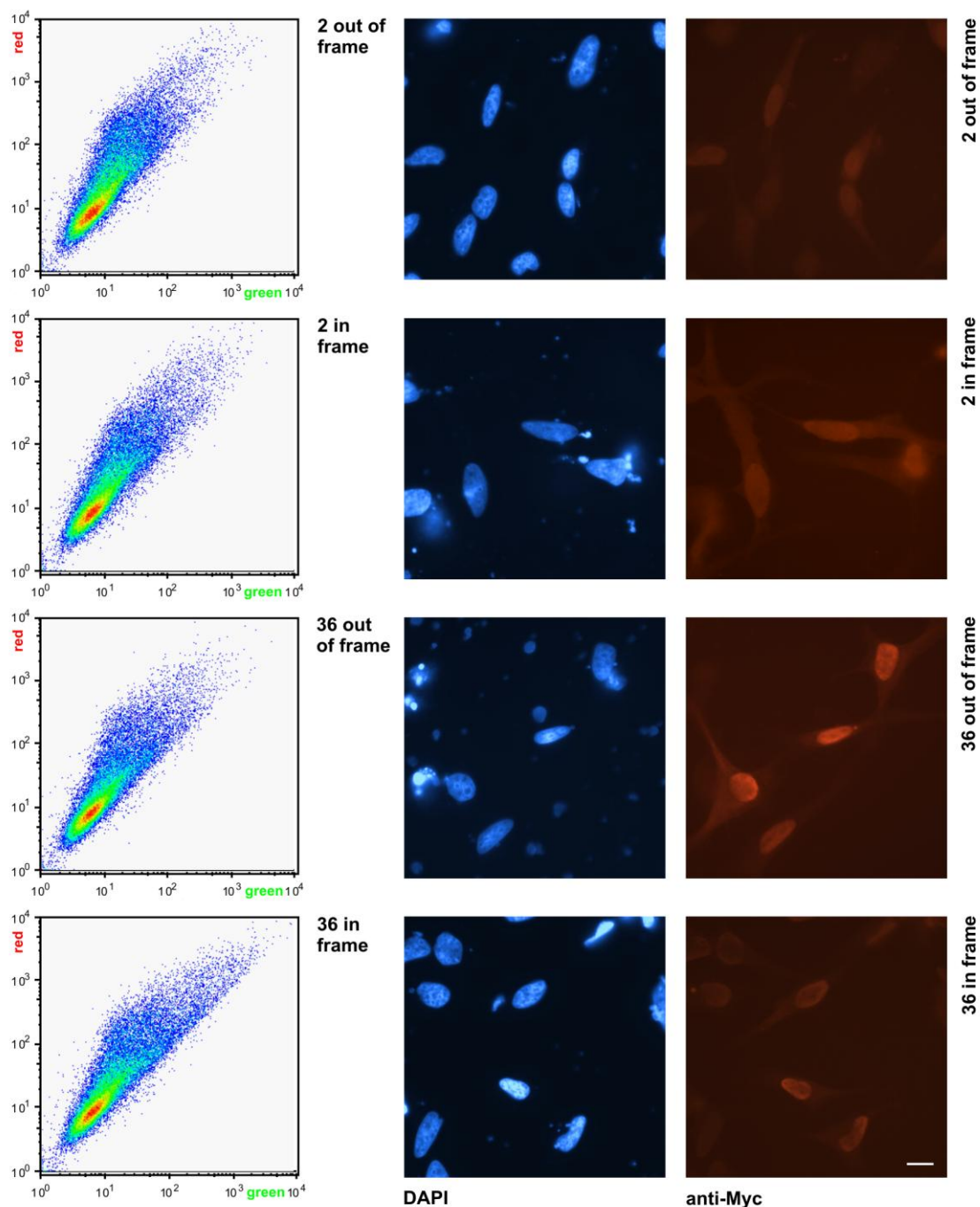
Fig. 4.32: Influence of different candidates on editing of the RNAG substrate shown via FACS assay immunostainings showing efficiency of transfection. After transfection cells were incubated 36-48 hours for immunostaining and 72h for FACS analysis. Cells transfected with hits are stained with anti-Myc and Alexa Fluor 568 antibodies. RNAG only, empty vector control and hits #1, #22 and #2, which had no visible effect on editing, and hit #36, which has a visible effect on editing, are shown. Scale bar: 10μm .

To evaluate the FACS results a statistical approach was applied. The area of each graph above the readouts from untransfected cells and excluding cells at the very top of the graph was divided into six equal gates [*Fig. 4.33*]. This way narrow pools in red fluorescence were compared to wide changes in green fluorescence intensity. For each cell in a gate green fluorescence values were divided by the red fluorescence values. The mean values of this calculation together with the standard deviation were plotted for each gate on the x axis of a two dimensional graph. This more sensitive method allowed to identify #2 and #22 as two additional candidates, showing only a slight increase when compared to #36 [*Fig. 4.33*].
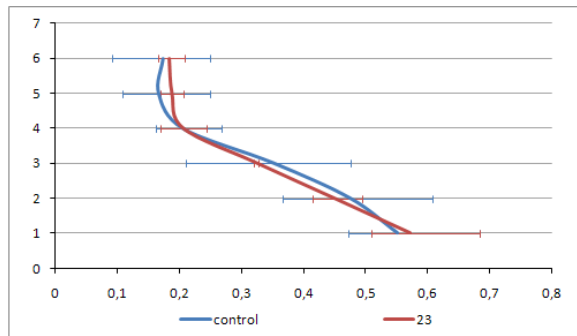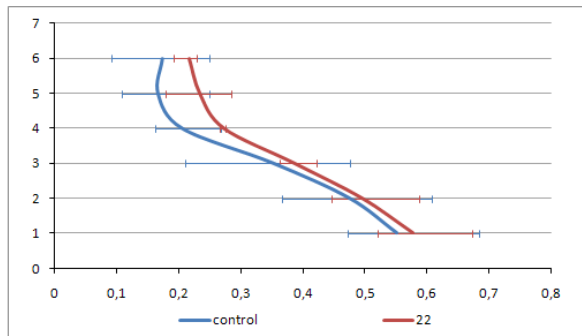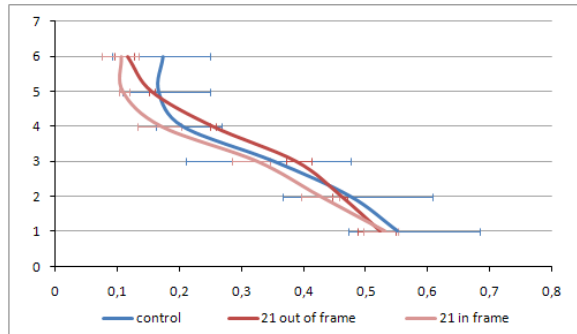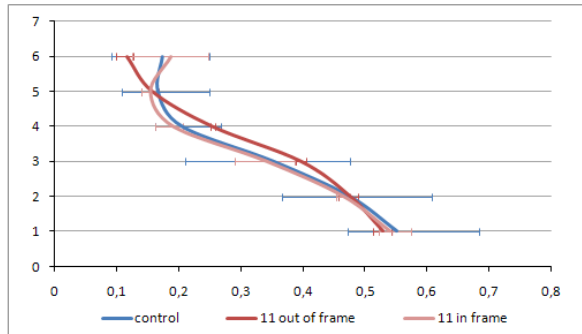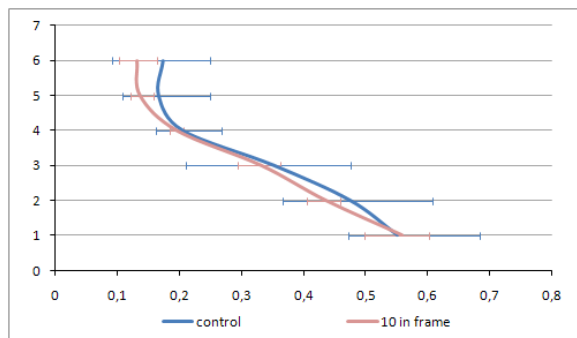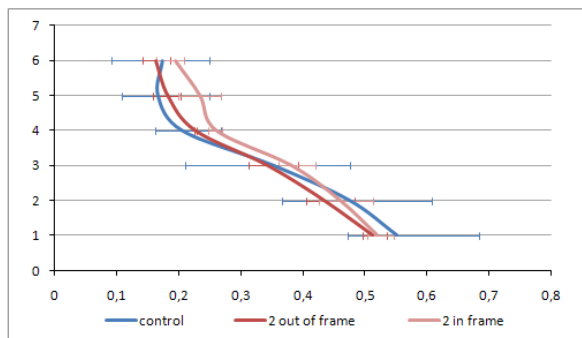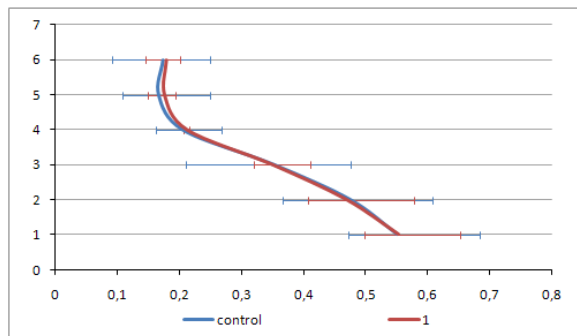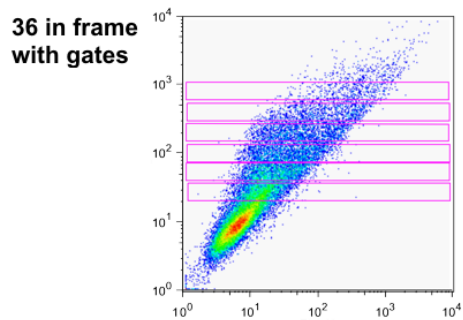
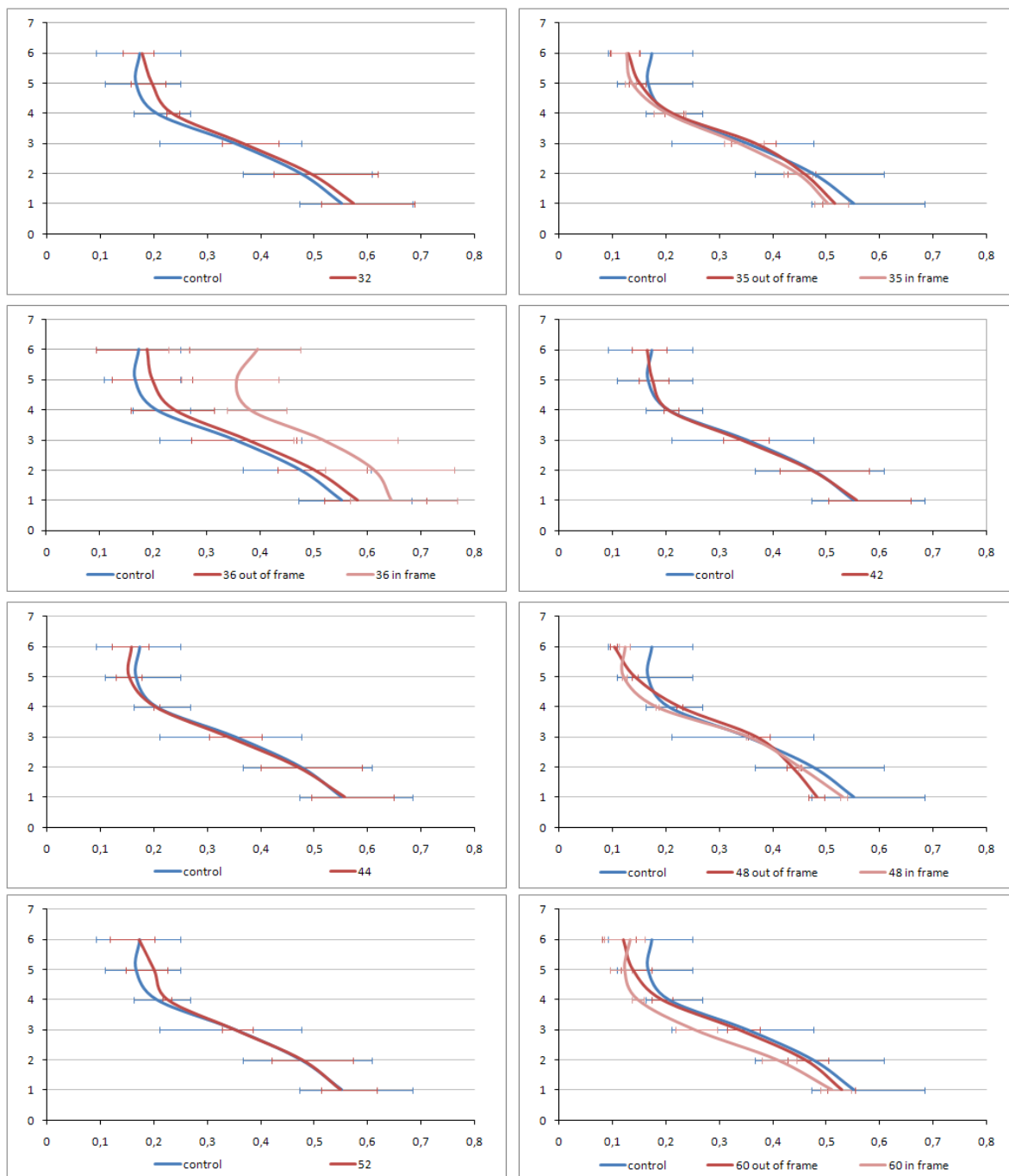*Fig. 4.33: Mean values of red versus green fluorescence of all candidates in six different gates. In each case the values of candidates are compared to the ones the control. In case of coding hits readouts between out of frame and in frame versions of them were compared. Additionally, one graph from FACS analysis with chosen gates is shown.*

For this study candidate #36, showing the strongest effect, was further investigated.

4.4   DSS1, an enhancer of RNA editing

Candidate #36 contains a full length mRNA encoding *DSS1*. To confirm the indirect results obtained using the FACS reporter system also direct influence of this protein on editing substrates was investigated. Editing levels of both; RNAG and endogenous CyFip2 editing substrates after DSS1 overexpression were studied. Additionally attempts to elucidate the mode of action of this enhancer were made.

4.4.1   Editing of RNAG in HeLa

To verify an increase of editing induced by DSS1, editing levels of the amber site in RNAG was investigated. RNA isolated from transfected HeLa cells was reverse transcribed and afterwards the editing site was amplified and sequenced. Sequencing results showed an increase in editing upon coexpression of DSS1 [*Fig. 4.34*].

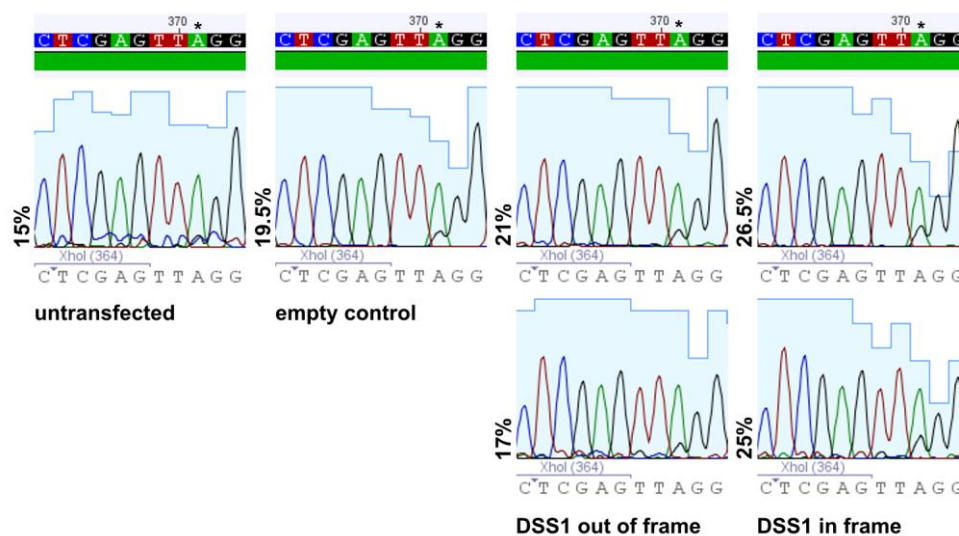*Fig. 4.34: Sequence comparison of the RNAG transcript edited in HeLa cells with or without the expression of candidate DSS1 (edited adenosine marked with an asterisk) The cDNA obtained via reverse transcription was amplified using MJ1136 and MJ1137 primers and sequenced with MJ1136 primer. The electrophorograms show two separate repeats of editing of RNAG in HeLa cells via endogenous ADARs.*

4.4.2   Editing of CyFip2 in the cell lines stably expressing *r*ADAR2

Since transiently transfected substrates and enhancers of editing rely on the cotransfection of both plasmids into the same cell the effect of DSS1 on endogenous editing substrates should be determined. HeLa and U2OS cell lines routinely used in our lab showed no detectable editing of any previously reported ADAR2 transcripts. Thus, to confirm the influence of DSS1 on ADAR2 activity several Hek 293 cell lines stably expressing *r*ADAR2 were generated in the lab (courtesy of Aamira Tariq). A number of cell lines with different levels of *r*ADAR2 expression were tested on CyFip2, a known ADAR2 substrate. Of these, clone 6 and 11 show low editing levels while clones #5 and #10 show high levels reaching up to 70% of CyFIP2 editing [*Fig 4.35 and Fig. 4.36*].
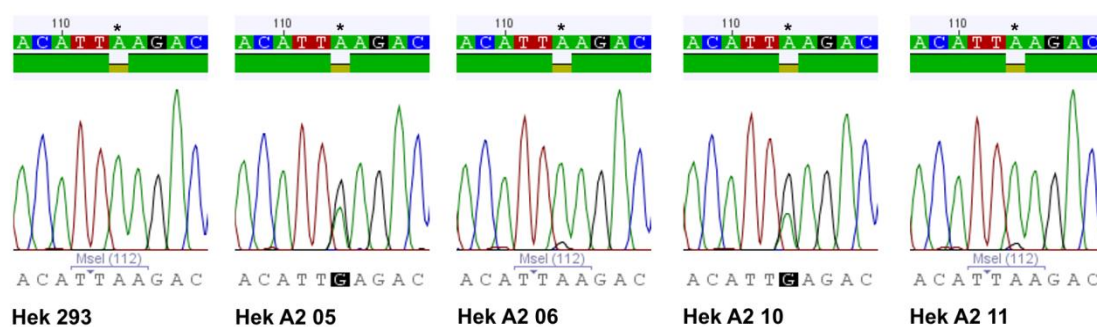
Fig. 4.35: Comparison of editing levels of CyFip2 K/E editing site in Hek 293 cell line and few examplatory rADAR2 expressing strains based on Hek 293 (edited adenosine marked with an asterisk).

The best choice for studying the influence of DSS1 on editing would be a cell line with low or moderate editing levels, for example cell lines 6 and 11. Immunostainings and FACS analysis indicated that low editing levels of some cell lines are caused by a variable number of cells lacking ADAR2 expression [*Fig. 4.36*]. To exclude inhomogeneous cell populations we decided to use the stable Hek line #10 which shows the highest editing level of CyFip2 and consists of a homogeneous ADAR2 expressing population [*Fig. 4.36*].

Hek 293

Hek A2 05

Hek A2 06

Hek A2 10

Hek A2 11
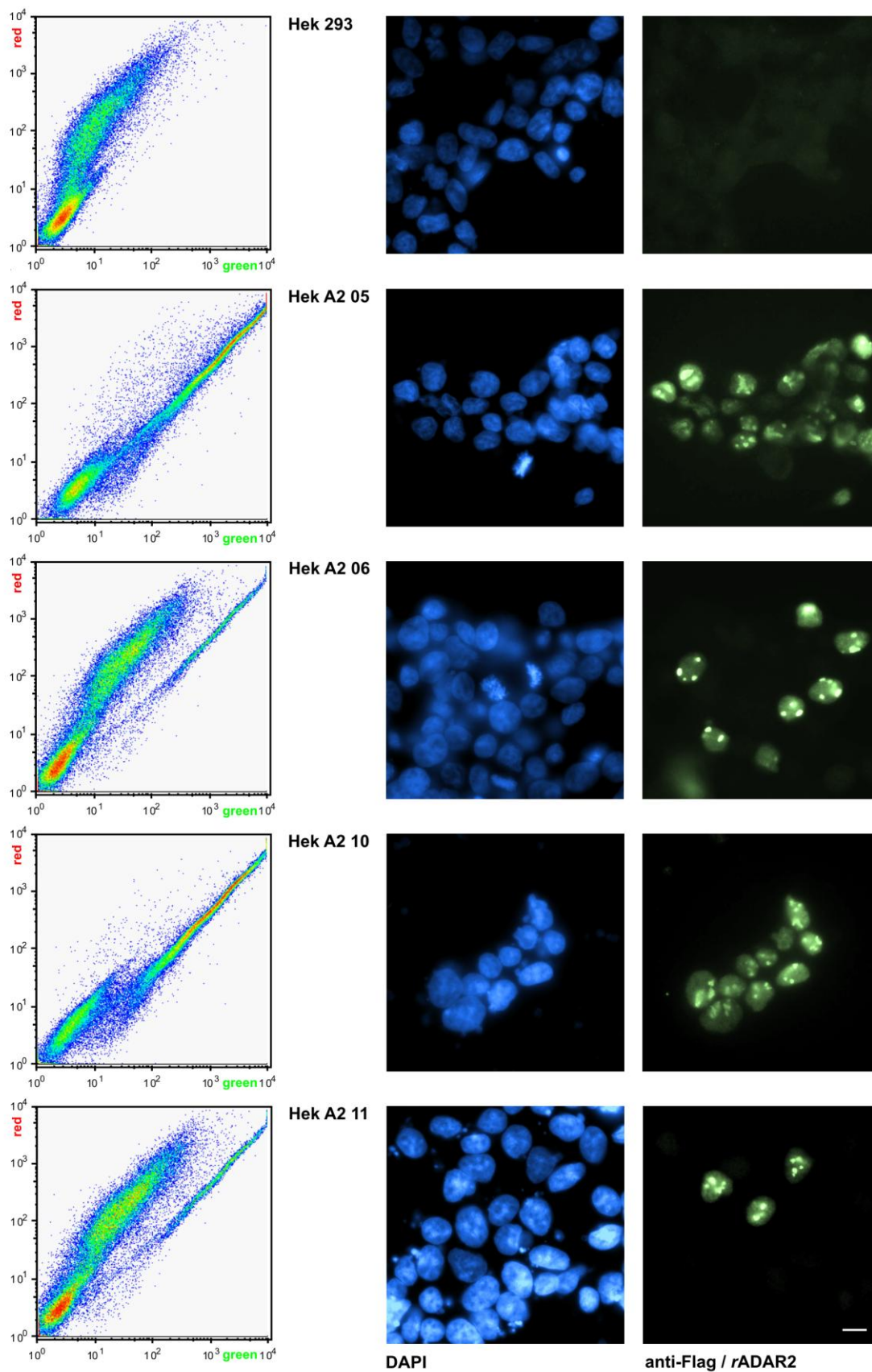
DAPI

anti-Flag / *r*ADAR2

Fig. 4.36: FACS graphs of RNAG editing in Hek 293 and stably expressing rADAR2 cell lines and the immunostaining of these cell lines. After transfection the cells were incubated 36-48 hours for immunostaining and 72 for FACS analysis. The cells were stained for the presence of rADAR2 with anti-Flag and Alexa Fluor 488 antibodies. On FACS graphs are two different populations of cells visible; one resembling Hek 293 and one fully edited. The first population of cells is visible in clones 6 and 11 and slightly in 5, but completely disappears in clone 10 in favour of the fully edited. The FACS data correlates nicely with the percent of cells expressing rADAR2 visible in the immunostaining. Scale bar: 10µm .

Using cell line Hek #10 the influence of DSS1 on CyFip2 editing was tested. Transfection of the cell line with DSS1 expressing vector was performed [*Fig. 4.37*].
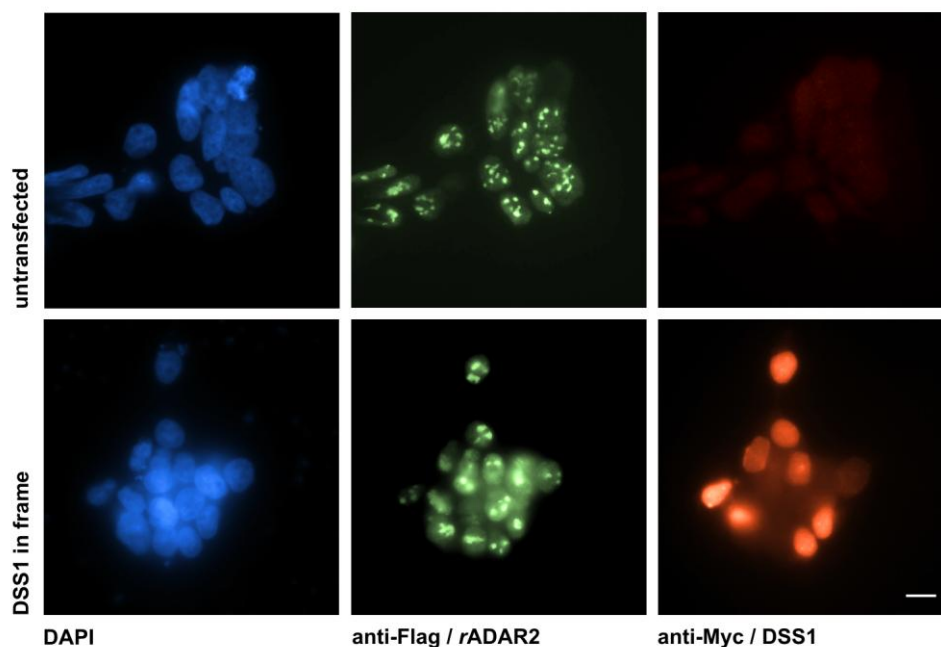
Fig. 4.37: Transfection efficiency of DSS1 expressing vector in the Hek A2 10 stable cell line. After transfection the cells were incubated 36-48 hours and stained for the presence of rADAR2 with anti-Flag and Alexa Fluor 488 antibodies and for the presence of hits with anti-Myc and Alexa Fluor 568 antibodies. Transfection of DSS1 in frame expressing vector is shown in comparison to untransfected control. Scale bar: 10µm.

Sequencing of RT-PCR products of CyFip2 demonstrated a statistically significant increase in editing upon cotransfection of in frame *DSS1*, while all controls displayed the same editing levels. Coexpression of DSS1 resulted in an increase in editing by ~7%.

Cotransfecting a vector, which contained only the *DSS1* ORF, lacking its untranslated regions and flanking vector sequences had an even stronger effect of up to 8 % on editing [*Fig. 4.38*].
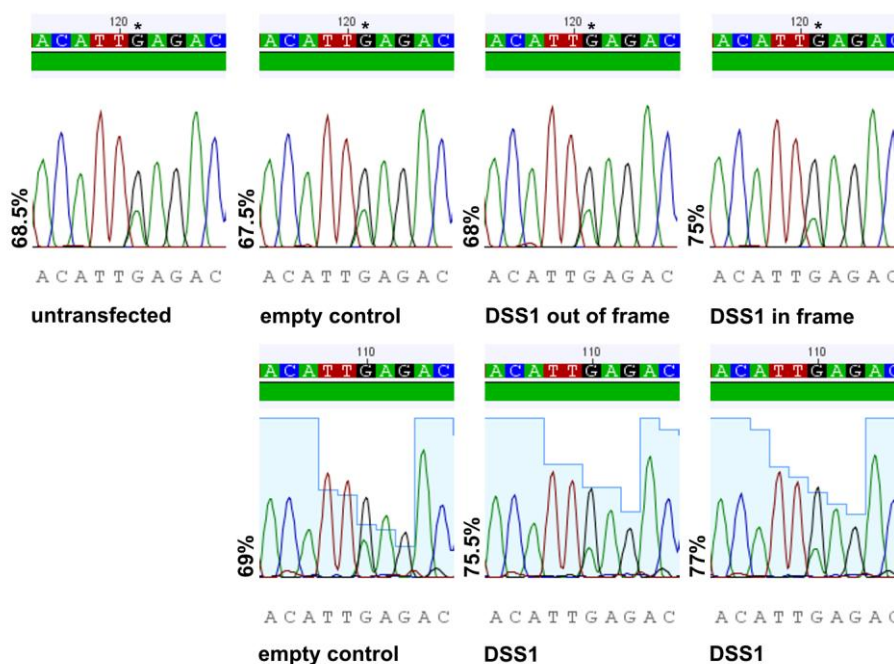
Fig. 4.38: CyFip2 editing site sequence comparison after transfection a cell line stably expressing rADAR2 with different versions of DSS1 expressing vectors (edited adenosine marked with an asterisk). The cDNA obtained via reverse transcription was amplified using MJ1136 and MJ1137 primers and sequenced with MJ1136 primer. The electrophorograms show two experiments: first a comparison of out of frame and in frame DSS1 variant is shown and second the vector expressing only the translated part of DSS1 gene is shown in two repeats.

4.4.3   Interactions of DSS1

To elucidate the mode of action by which DSS1 affects activity of *r*ADAR2, we tested whether there is a direct interaction between the two proteins. Hek A2 10 stable cell lines transiently expressing Myc-tagged DSS1 were used for immunoprecipitating Myc-DSS1. However, no coprecipiation of rADAR2 could be detected in these experiments [*Fig. 4.39*]. UV crosslinking, use of mild buffers or shortened incubation times failed to detect an interaction between ADAR2 and DSS1. Also using an anti Flag antibody directed against Flag-DSS1 failed to pull down *r*ADAR2.
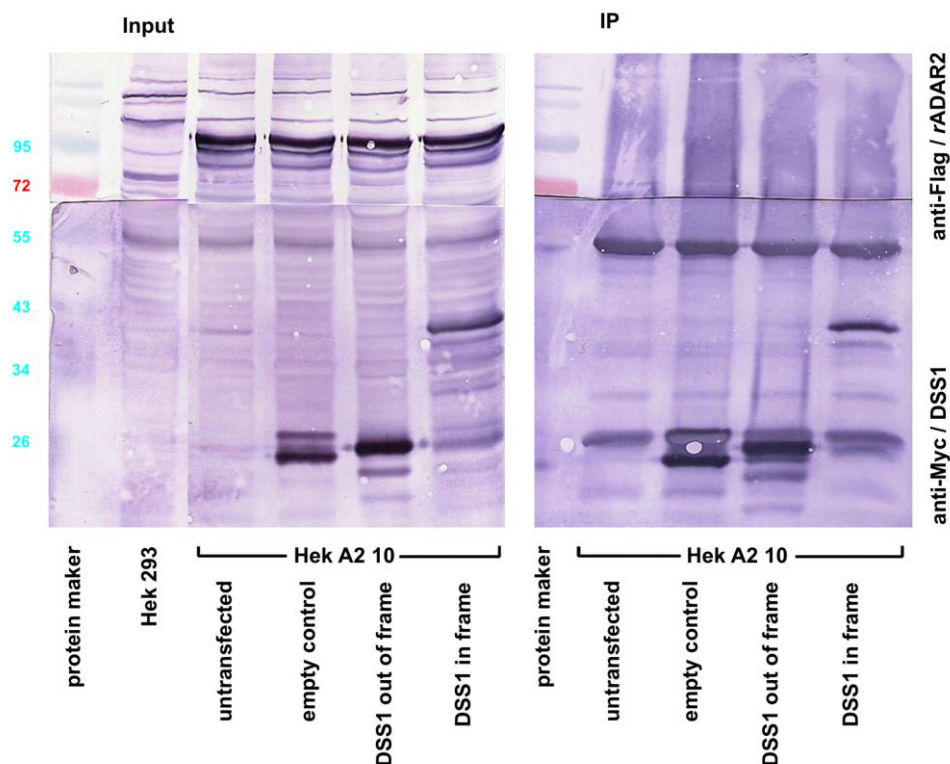
Fig. 4.39: IP experiment to pull down rADAR2 with Myc-tagged DSS1. The cells transfected with appropriate constructs were cultured for 72 hours and afterwards the extracted proteins were used for the IP experiment. The Western Blot was performed with anti-Myc antibody detecting hits and anti-Flag antibody detecting Flag-tagged rADAR2. Two Blots are shown: the input with additional Hek 293 extract to visualize presence of rADAR2 in stable cell lines and output blot with IP results. Although all three Myc-tagged polypeptides are visible after the IP, there is no sign of rADAR2 being pulled down along with them.

4.4.4 Domain structure of DSS1

To learn whether the full length DSS1 or fractions of it would be sufficient to stimulate editing, truncated variants of it were cloned and expressed. Since DSS1 is a small, 70aa long protein it was only divided into two halves of 35 amino acids each [*Fig. 4.40*]. DSS1 contains three α-helices and two β-sheets, which nicely overlap with conserved BRCA2 binding sites (Yang, Jeffrey et al. 2002). Division of DSS1 into two halves was performed without disrupting the protein's secondary structure. Also Asp/Glu-rich regions, that could take part in binding were left intact.
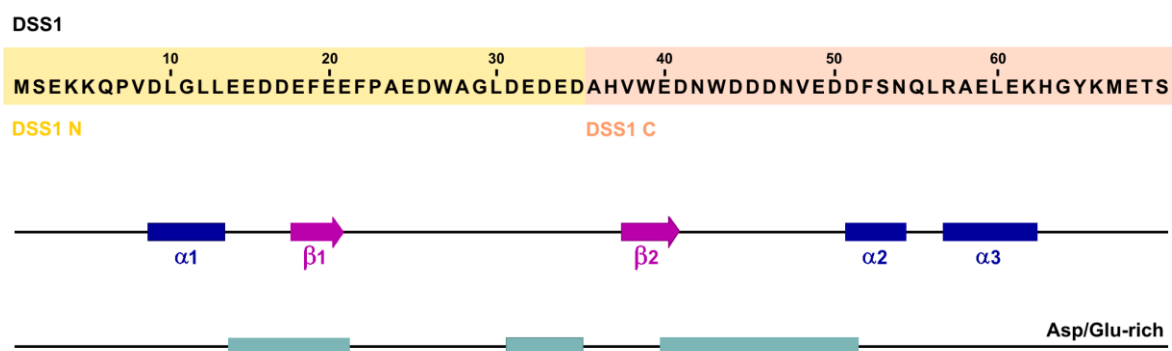
Fig. 4.40: The amino acid sequence of DSS1. Shown is the whole sequence of DSS1 and how the protein was divided into two shorter N- and C-terminal versions of it for further experiments. Additionally, secondary structure of the protein is depicted as well as localization of acidic regions of it.

To test the cellular localization of DSS1, the full length gene was cloned into pCDNA vector containing an additional NLS sequence and also into pCDNA (Invitrogen) and pCaggs vectors without artificial NLS (BCCM/LMBP plasmid catalogue) (Niwa, Yamamura et al. 1991). The pCDNA vector carries a Myc-tag N-terminally and pCaggs C-terminally. Also, the truncated versions of *DSS1* were cloned into these two vectors. The immunostaining has shown that DSS1 expressed from the pCDNA vector without NLS sequence still localizes predominantly in the nucleus. DSS1 and its truncation variants expressed from the pCaggs vector without NLS sequence were also mostly located in the nucleus as well, but the cytoplasmic signal was stronger than observed for the N-terminal Myc-tagged DSS1 [*Fig. 4.41*]. This might indicate a negative influence of C-terminal Myc-tag on nuclear localization.
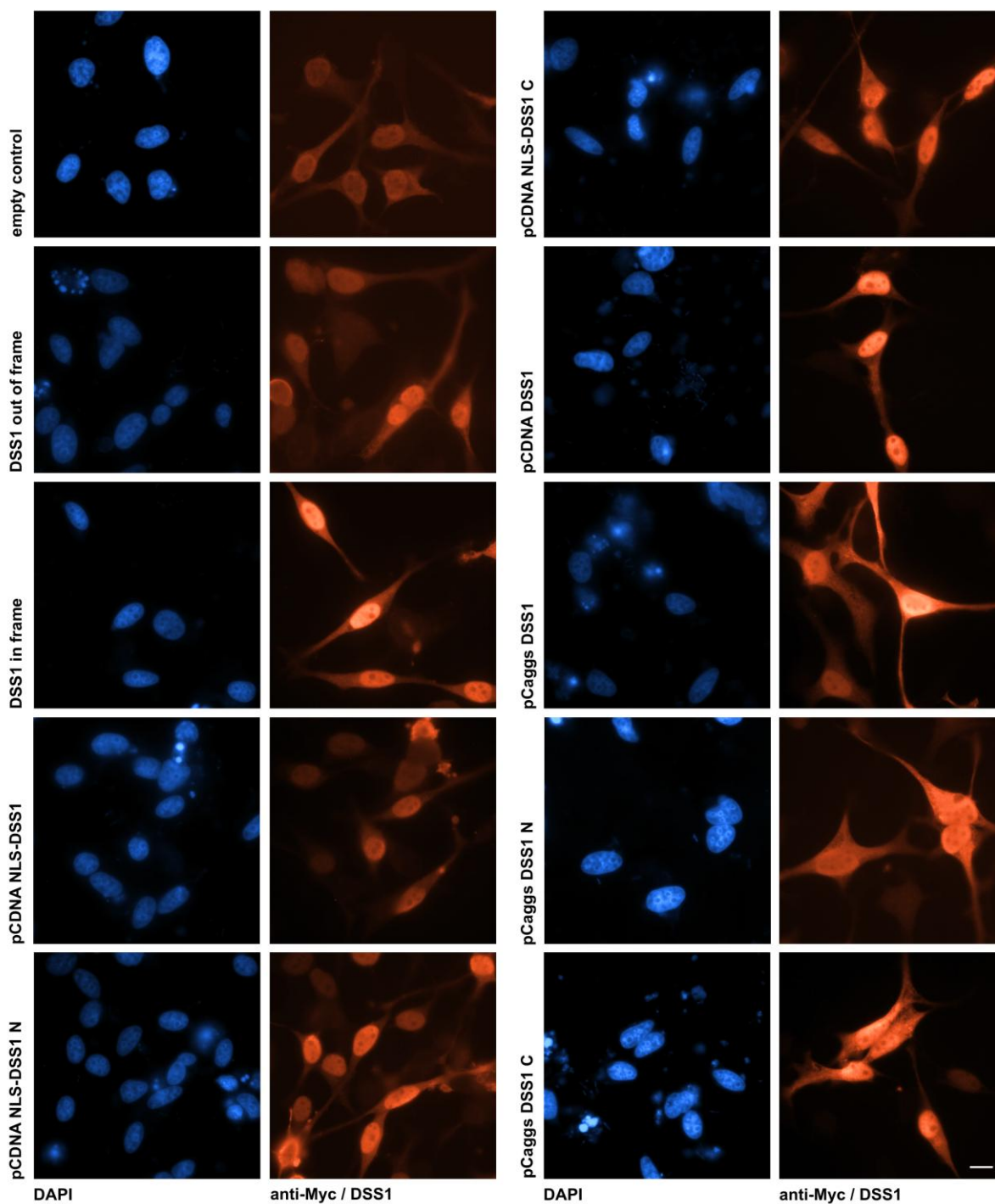
Fig. 4.41: Immunostaining of HeLa cells transfected with vectors expressing different variants of DSS1. The cells transfected with appropriate constructs were cultured for 72 hours and afterwards the immunostaining was performed with anti-Myc antibody detecting Myc-tagged DSS1 and Alexa Fluor 568 antibody for detection. Scale bar: 10µm.

The same constructs were tested via FACS for their influence on RNAG editing. Firstly, HeLa cells expressing either full-length DSS1 or its truncated variants displayed an increase in editing of the RNAG substrate compared to the empty vector control. Secondly, DSS1 and its deletion mutants expressed from pCDNA vectors seem to have a stronger influence on editing than the ones expressed from pCaggs. This could relate to the reduced nuclear localization of C-terminal Myc-tagged DSS1. A similar explanation might hold true for the observed weaker effect in case of DSS1 expressed from pCDNA vector without NLS sequence. Independent of the vector backbone, all truncated versions of DSS1 have less influence on editing than the full-length protein. Of the full-length DSS1 the protein expressed from pCDNA with an artificial NLS enhances editing even more efficiently that the DSS1 in frame construct. In both cases the C-terminal fragment of DSS1 enhances editing to a larger extent than the N-terminal DSS1 fragment, which is nearly comparable to the empty vector control [*Fig. 4.42*]. Although the whole protein is responsible for the enhancing effect, it seems that the C-terminal part is more essential in this process.
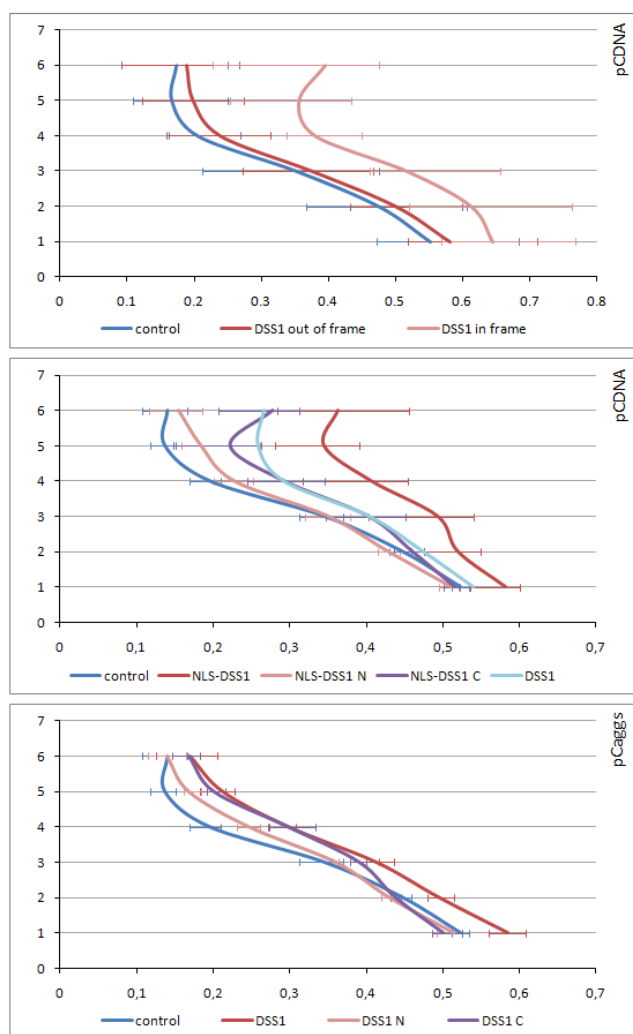
Fig. 4.42: Mean values of the FACS results of all tested variants of DSS1. On the different graphs the following DSS1 variants are shown: out of frame and in frame versions of DSS1, full version of DSS1 and its truncated versions expressed from pCDNA vector with or without NLS and the same polypeptides expressed from NLS-less pCaggs vector. The effect on editing is higher in case of the pCDNA vector and the full-length variants have the highest impact, followed by the C-terminal fragment and then N-terminal fragment.

4.4.5   Specific localization of overexpressed DSS1

At the same time the localization of the overexpressed DSS1 was investigated. In particular we were interested in whether DSS1 localizes in a specific pattern within the nucleus. Indeed, overexpressed DSS1 localizes to nuclear structures while the polypeptide expressed from Myc-NLS construct with out-of-frame sequence of *DSS1* is homogenously dispersed in the whole nucleus. These strongly stained structures are often observed in pairs and localize in the vicinity of nucleoli, but mostly do not colocalize with them [*Fig.4.43*].

DSS1 out of frame

DSS1 out of frame

DSS1 in frame

DSS1 in frame

DIC   DAPI   anti-Flag / rADAR2   anti-Myc / DSS1
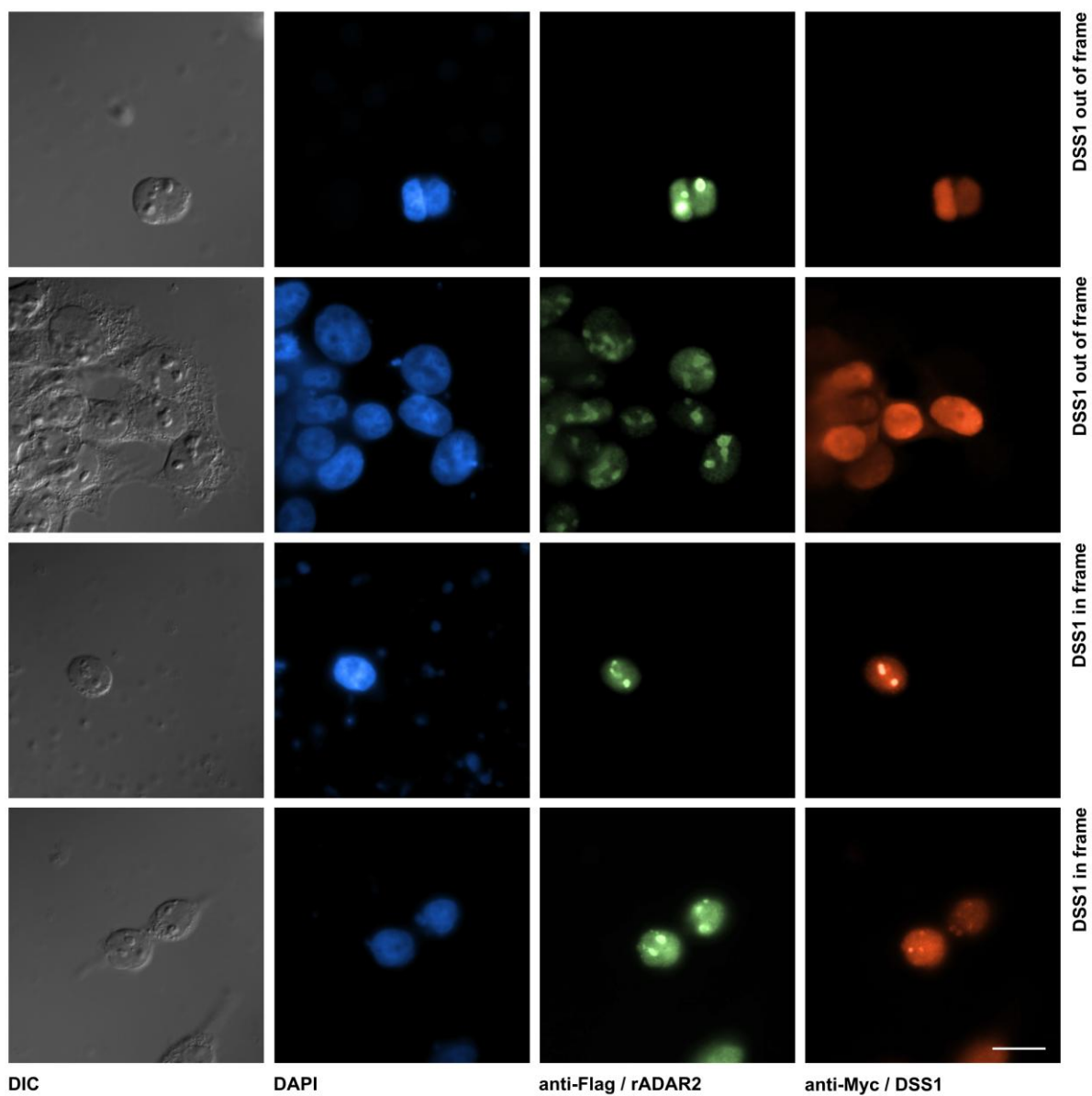
Fig. 4.43: Immunostaining of Hek A2 10 stable cell line transfected with vectors expressing DSS1 for localization studies. The cells transfected with appropriate constructs were cultured for 72 hours and afterwards the immunostaining was performed with anti-Flag antibody detecting rADAR2, anti-Myc antibody detecting Myc-tagged DSS1 and Alexa Fluor 488 antibody and Alexa Fluor 568 antibodies for detection. Scale bar: 10μm .

5   Discussion

5.1   Regulators of ADAR activity

Although there is a lot known how A to I editing is performed and what might regulate the process, the precise picture of ADARs cellular interactions holds many mysteries. Little is known about factors that would directly affect the editing activity of ADARs. Direct enhancers or inhibitors of editing are yet to be identified.

In light of the fact that ADARs don't need any partners to perform editing (Bass 2002; Nishikura 2009), this raises the question of whether there is any evidence for the existence of molecules interacting with and acting on ADARs? As ADARs are involved in co- and post-transcriptional processes, this suggests that these enzymes are part of a very complex RNP architecture that forms on nascent transcript, which have to be edited, spliced and in the end exported. Recently, a direct interaction between ADAR2 and the C-terminal domain (CTD) of RNA polymerase II has been shown. The CTD is necessary for ADAR2 to auto-edit its transcript (Laurencikiene, Kallman et al. 2006). This would indicate a possible existence of factors influencing co-transcriptional RNA editing. A to I editing has recently become a factor, that might be involved in controlling important processes, like alternative splicing and miRNA biogenesis, the products and efficiency of them both (Higuchi, Maas et al. 2000; Schoft, Schopoff et al. 2007; Penn and Greger 2009). Thus, it would be only illogical to believe that editing itself is not controlled.

Our hypothesis is further supported by fact, that many discrepancies between the expression levels of ADARs and the editing levels of main substrates, already mentioned earlier in this work, have been reported. Although one still cannot sufficiently explain these findings, few plausible theories exist. One of the explanation may lay in developmental- and tissue-specific expression of the various isoforms and splice variants of ADARs, that possess different editing efficiency and substrate specificity. Another explanation may be still highly controversial possibility of ADARs to homo- and heterodimerise. Again, different ADAR proteins, including ADAR3, could influence their activity by interacting with each other.

Although both the existence of various splice variants and dimerisation of ADARs may even cover all explanations for all discrepancies in ADAR expression and activity, it is hardly believable that there are no factors that could affect the enzymatic activity of ADARs in a direct or indirect manner. With that in mind we designed and performed a screen in search for endogenous molecules that might enhance editing of ADARs. Until now only two attempts to

perform similar screens were reported. First, the Peter A. Beal laboratory performed a screen in yeast, employing the same principle, as in ours: the editing site is part of a stop codon located in front of a reporter gene, in their case a β-galactosidase. This system was used for high-throughput screening for mutations in ADAR2 and the substrate that support editing (Pokharel and Beal 2006). The laboratory of Stefan Maas has set up a luciferase tissue culture assay based on a comparable principle as well (Gommans, McCane et al.). Up to now molecules interacting with ADAR have not been reported.

5.2   *r*ADAR2 efficiently edits in yeast system

The basic yeast strain used in the screen was a mutant strain, in which an auxotrophy gene, preceded by an editing site, was expressed as a marker. It was based on the idea that editing of an adenosine, which was a part of a stop codon, would allow this gene to be expressed and therefore let this strain to grow on selective medium. The advantage of using yeast for our screen over a mammalian system was a rather quick and inexpensive way to screen millions of genes in various cDNA libraries. The possibility of having such a library pretransformed in a opposite mating type yeast strain also helped to get rid of tiresome transformation efficiency problems that one faces when working with mammalian cells. Additional convenience of this model system was the absence of endogenous ADAR homologues, which helped to avoid possible redundancy. Each of this advantage had unfortunately its dark side. Using *Saccharomyces cerevisiae* as a model would help to quickly screen a high number of genes, but these genes had to be later confirmed in the mammalian system to avoid yeast-borne false positives. Also, the absence of ADAR meant a possible absence of other molecules interacting with them posing a threat, that even a true ADAR interactor, expressed in this screen, could remain undetectable. Despite all these drawbacks and knowing that no system is perfect and able to detect everything, we decided to proceed.

The editing screen was based on a common two-hybrid mating assay screen. The goal was to adapt it in purpose to detect differences in editing via increase in growth rate of the prepared editing yeast strain. This aspect already turned out to be difficult showing discrepancies in growth rates between obtained yeast, which were sometimes hard to explain. One had to learn to cope with contamination, differences in growth rate depending on age of the plates the single colonies were picked from or growth conditions, which all would have an

impact on editing levels. Even transforming the W303 yeast strain with two different constructs (HIS3amber or URA3amber), which were integrated into its genome, repeatedly resulted in strains of a different growth rate. The strain carrying URA3amber was always the one growing faster and after rADAR2 transformation this strain accumulated inosines slower [Fig. 4.9, 4.10, 4.13 and 4.16]. This could be the result of a faster synthesis of Ura3 or of a less structured mRNA this gene. In brief, the yeast metabolism is still quite complex and with a screen based on most basic and essential functions like cell growth and proliferation, these kind of problems may not be trivial. One had to understand that some of the screen results may be false positives, since there would be a heavy pressure on the cell to promote the cells boosting up cell cycle and counteracting the selection. Additionally, because the screen was not based on protein-protein interaction, there was no fail-safe control ensuring that all positives are the result of such an interaction.

Out of three substrates, which were based on the GluR-B R/G, Gabra-3 I/M and hepatitis delta type 1 amber/W sites, only the first one was edited in yeast. These substrates have been chosen because all of them were proven to be edited by both ADAR1 and ADAR2 (Sato, Wong et al. 2001; Wong, Sato et al. 2001; Ohlson, Pedersen et al. 2007). There are numerous reasons that might explain for the lack of editing of the other substrates. It is possible that a yeast cell presenting still a good environment for GluR-B R/G editing wasn't so favorable for others. On the other hand, it is also possible the mRNA molecules weren't in fact folding *in vivo* according to the predictions made *in silico*. Also, additional cofactors might be needed for these substrates. Lastly, an undesired signal for NMD might have been created. A definitive answer cannot be provided, since these substrates were not investigated further. Additionally, a shortened version of GluR-B R/G based substrate was prepared. This substrate was not edited, which is in line with the later finding that each part of GluR-B R/G stem-loop structure is necessary for proper ADAR2 binding, since both dsRBDs of ADAR2 bind to its distinctly different motifs (Stefl, Xu et al. 2006). This might also be the case for ADAR1.

In this investigation attempts to use both ADAR1 and ADAR2 were made. As more or less expected, it was only possible to succeed in case of ADAR2. It is known in A to I editing field that ADAR1 is the one of these two causing the most problems. ADAR1 is the much larger and more complex protein. All crystalographical studies were made on ADAR2 for a reason (Macbeth, Schubert et al. 2005; Stefl, Xu et al. 2006). Also, large-scale expression of ADAR1 has proven to be difficult. Only low yield ADAR1 expression from *Pichia pastoris*

and only of ADAR2 from *Saccharomyces cerevisiae* were reported (Keegan, Rosenthal et al. 2007; Macbeth and Bass 2007). Also, another yeast screen based on the similar principle was performed also only with human ADAR2 (Pokharel and Beal 2006). In this case the expression of *hs*ADAR1 seemed to be successful judging from the presence of *hs*ADAR1 mRNA and Western Blot detection, although it wasn't as strong as in case of expressed rADAR2 [*Fig. 4.4 and Fig. 4.5*]. Also, functional protein was expressed from the original vector encoding *hs*ADAR1 and edited a GluR-B R/G-derived substrate in tissue culture experiments [*Fig. 4.30*]. This could only suggest that either the *hs*ADAR1 expressed in *Saccharomyces* was inactive, not in sufficient amount, or the yeast cellular environment wasn't the right one for proper editing.

The expression system used for *r*ADAR2 expression was a tetracycline inducible one. For the purpose of the screen, which is the identification of enhancers of ADAR editing activity, a low basal ADAR expression was essential. This was achieved by a controlled DOX induction of rADAR expression. To my surprise, a very tight system in mammalian cells has proven to be leaky in yeast. The levels of the leakiness were above acceptable ones, as growth and editing levels of uninduced and induced yeast cultures were comparable. Unfortunately, all attempts to reduce the leaky expression, such as reducing the number of TetO boxes and coexpression of a repressor, were unsuccessful. To increase the stringency of the screen, it was performed in presence of 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the product of the *HIS3* gene. In order to achieve a better control over ADAR expression in the future, a different expression system would have to be employed.

5.3   The screen gives rise to enhancers of rADAR2 activity

For the screen a yeast strain already pretransformed with human fetal brain cDNA library was used. This kind of library was used knowing that ADARs are predominantly expressed in the CNS. The performed screen has covered 3.33×10^6 clones of the cDNA library, which was roughly the amount of the clones in the library. Since the manufacturer suggested to double the number of library clones, this result could prevent the screen from efficiently covering of the whole library. In the end 61 clones, which were showing the highest growth rate, were retrieved. All these clones were still expressing His3 and *r*ADAR2

and removal of *rADAR2* bearing vector caused lack of growth on histidine deficient medium. All this indicated that the system went through the mating procedure successfully.

Sequencing of the candidates has brought many surprises. Firstly, among the candidates, there were no striking candidates, that would immediately fit in one ADAR regulation theory. Also, the fact that none of the hits was enriched, was discouraging. An explanation for this observation could be the lack of proper cDNA library coverage. In this case only part of the genome that is expressed in the human brain would be covered and many of maybe essential genes would be missed in the process. Also, duplicates of genes having an effect on editing would be rarer. One should also not underestimate the bias created by using GluR-B R/G-derived editing substrate and expression of *r*ADAR2. Although ADARs are able to edit this site by themselves, purely hypothetically, some of the factors affecting ADAR activity might be also site-specific, discriminating against those that cannot bind the site. Also, many binding studies were made on ADAR1 and may not apply to the obtained *r*ADAR2 results.

The second surprise was an abundance of 3'UTR fragments and not annotable sequences among obtained candidates. Also, coding sequences were found to be expressed from the yeast vector out of the coding frame. This situation shouldn't be a surprise concerning the way this Matchmaker cDNA library was cloned, by random, not reading frame dependent, amplification. It would still leave 1/3 of the clones that should be expressed in frame, but this was not the case. A possible explanation would be that many human proteins lack a yeast ortholog and might thus act in a destabilizing, toxic way on the yeast metabolism and decrease the 'in frame' pool to a minimum. This still doesn't answer the question why these candidates emerged from the screen and what made them grow faster. It may be possible that the screen failed, but several other explanations exist as well. For example, the RNA component, mRNA and hidden repetitive element in it, may be responsible for the increase in editing and therefore in yeast growth. Overexpression of another binding platform for *r*ADAR2 could have been a reason for concentrating the protein and rendering the editing process more efficient. Alternatively, the proteins are in fact still expressed correctly. However, most of the expressed genes were found to be out of frame, there are processes in eukaryotic organisms that allow the expression of a protein not from the first start codon. One of these processes is reinitiation, where translation starts at a start codon downstream of the preliminary stop codon. Although these processes are not common in higher eukaryotes, yeast is known to allow more flexibility in protein expression (Kozak 1999). Also, experience

gathered in our laboratory led us to think that this may be happening. Perhaps a low expression of the protein was needed to enhance editing.

In order to confirm results on editing level, a comparison of editing of HIS3amber fragment via bulk sequencing was performed. This method was chosen over single clone sequence comparison due to less time invested in it. A more time- and work-consuming method wouldn't be applicable with this amount of candidates. A big drawback of bulk sequencing emerged while performing it: the obtained results varied significantly for the same candidate. Although initially few independent repeats of the same clone have shown a relatively high standard deviation, the overall trend did however not change for the individual candidates. Afterwards the editing level for all candidates was measured twice, showing two different results in many cases. It has only shown that bulk sequencing, which may be a great method to study editing levels in tissues, may not be the perfect one for the yeast, the metabolism of which is less stable and highly depends on given conditions. In the end, the bulk sequencing runs of all candidates confirmed that the screen was a success. Most of the candidates displayed elevated editing levels and indicating a few candidates that should be further characterized in mammalian tissue culture.

5.4   DSS1 is an enhancer of A to I editing

The chosen candidates were confirmed to enhance editing activity of ADAR in mammalian system as well. A red and green fluorescent protein with GluR-B R/G-derived editing site in between was utilised and measured via FACS. This method was used to obtain statistical data about the influence of chosen candidates on editing in a very fast way. Although the FACS reporter system has proven to be a nice tool to distinguish between candidates influencing editing, a few drawbacks of this method have emerged. Firstly, it was highly dependent on transfection efficiency, and even with stable transfection rate of 30-50% many candidates could have been lost in this way. Transfection rate of DSS1 expressing vector below 20-30% equalled mostly no detectible difference of editing level in bulk sequencing. [data not shown] Also, the discrepancy between the FACS results and bulk sequencing data had to be taken into consideration. The enhancement of editing was better visible in case of FACS graphs than in case of bulk sequencing of RNAG construct or CyFip2 mRNA. In case of DSS1 an increase in relative fluorescence ranging from 20% up to 100%

depending on a gate was measured, while only a 5-8% increase in bulk sequencing of RNAG mRNA was detected [*Fig. 4.33 and 4.34*]. Also, as for Hek 293 stably expressing *r*ADAR2 clone #10 nearly all cells were found on the diagonal axis of the FACS plot suggesting complete editing of the substrate, but only 66% editing of CyFip2 K/E editing site was determined by sequencing [*Fig. 4.35 and 4.36*]. The lack of a precise correlation between these two experimental strategies may be due to a different pool of cells taking part in both experiments or less copies of GFP needed to saturate the FACS system. Also in case of RNAG and CyFip2 substrates one has to take into consideration the differences in editing between them and possible role of ADAR1 in HeLa-based FACS system, that does not edit CyFip2 site. These results show that both methods are not fully proportional and candidates that show lower effect in FACS reporter system would not be detected by bulk sequencing of CyFip2 K/E editing site.

As a result only one strong candidate, DSS1, emerged and was studied further. Additional two possible ones; FABP7, a transport protein and 3'UTR of DYRK2, a tyrosine kinase, presented a lot lower effect but may still be interested to investigate in the future. One out of fifteen surprises at first glance but it was expected that some of the candidates from yeast screens are indeed artefacts and have no influence on editing. Additionally, from the reasons mentioned above some of the candidates may be true enhancers of editing, but these weren't detected in the mammalian system due to the low sensitivity of FACS reporter system. Moreover, even if they were showing lower enhancement in editing, bulk sequencing wouldn't detect it most probably, considering the data obtained for DSS1. A way to improve the sensitivity of FACS system would be increasing the transfection efficiency by using a more efficient method. Applying such a solution could help in testing candidates, that have shown a lesser effect.

Studying DSS1 in a mammalian system directly confirmed that DSS1 enhances editing by rADAR2. Comparing the editing levels of the RNAG and CyFip2 substrates has shown that DSS1 increases editing of both substrates by rADAR [*Fig. 4.34 and 4.38*]. Since the studies concentrated on stable expression of rADAR2 to prove the influence of DSS1 on the protein activity, one cannot say much about the possible influence of ADAR1, which could also contribute in FACS results. Additionally, different DSS1 variants were analyzed for their cellular localization and their ability to enhance editing, enabling us to gain a few interesting insights. Although DSS1 is a very small protein, the editing enhancing function of it seems to be localized at its C-terminus, as the C-terminal fragment showed a notable enhancement of

editing [*Fig. 4.41*]. Also, nuclear localization of the protein seems to be important. Overexpressed DSS1 localizes to nuclear structures, which don't colocalize with nucleoli where most of ADAR2 resides, but stay in their vicinity [*Fig. 4.43*]. This observation could already suggest that both these proteins do not form any physical contact although it doesn't discriminate this possibility considering the pool of both proteins present in the rest of the nucleus.

The question that still remains open is the role that DSS1 plays in RNA editing. It's not easy to answer, since DSS1 is an abundant protein taking part in many cellular processes, but one can speculate. DSS1 is a very small, acidic and aromatic in content protein, and is therefore predicted to rather bind as a cofactor to other proteins than to RNA. Although one cannot discard the thought of DSS1 binding next to editing site and recruiting ADAR, it would be more likely to believe that it binds to ADAR changing its properities, like it was shown in case of BRCA2, where DSS1 facilitates ssDNA binding affinity of the protein (Yang, Jeffrey et al. 2002). Unfortunately, the lack of success in proving a direct interaction between DSS1 and rADAR2 disproves this theory [*Fig. 4.39*]. It's possible that DSS1 binds in a weak manner or, what is more likely, affects editing via binding to another protein interacting with ADAR/RNA or affecting yet another process controlling A to I editing.

At first sight from all reports about DSS1 function, the less likely fitting our scenario is about DSS1 being a part of 26S proteasome particle, where it regulates the proteasome interactions and utilizes a specific subset of poly-ubiquittinated p53 protein as a substrate (Coux, Tanaka et al. 1996; Funakoshi, Li et al. 2004; Krogan, Lam et al. 2004; Sone, Saeki et al. 2004; Gudmundsdottir, Lord et al. 2007; Wei, Williams et al. 2008). Although one could imagine an inhibitor of RNA editing, the proteolysis of which would be promoted by DSS1, like in case of p53. This theory, however, is still far-fetched and hard to prove or disprove without knowing such an inhibitor.

More promising are reports from fission yeast, where the homologue of DSS1, Sem-1, was shown to be involved in splicing and in TREX-2 mRNA export pathway, which is already closer to what we know about ADARs (Thakurta, Gopal et al. 2005; Mannen, Andoh et al. 2008; Wilmes, Bergkessel et al. 2008; Faza, Kemmler et al. 2009). Since ADARs are a part of mRNP, intertwined in splicing and transport of mRNA, one could postulate a theory connecting both proteins. Maybe DSS1 is an essential factor needed for proper architecture of mRNP and therefore helping ADAR to take its proper place on mRNA particle and perform

its task. On the other hand the lack of DSS1 may also inhibit export of mRNA and lead export-impaired mRNPs for destruction.

Also reports from *Caenorhabditis* are promising. Quite a severe phenotype of *dss-1* knock out was very similar to phenotype of *kgb-1* mutant worms. The *kgb-1* knockout leads to increased levels of the GLH-1 RNA helicase. In *dss-1* mutants GLH-1 levels were normal, but there is a chance that a similar protein to GLH-1 is also regulated by DSS-1 (Orsborn, Li et al. 2007; Pispa, Palmen et al. 2008). It would be possible that this helicase unwinds A to I editing substrates, thereby inhibiting editing. Surprisingly, that brings us again to proteasome with DSS-1 and KGB-1, both being a part of it. Therefore, DSS-1 at the proteasome could indeed control abundance of this hypothetical helicase that acts as a inhibitor of ADARs.

Although it was shown in this study, that DSS1 is enhancing rADAR2 activity a mode of action of this process remains yet to be uncovered.

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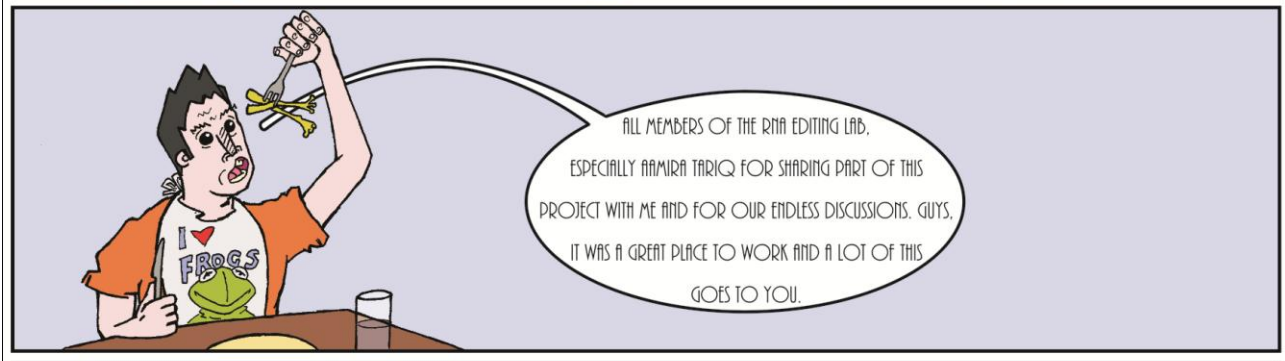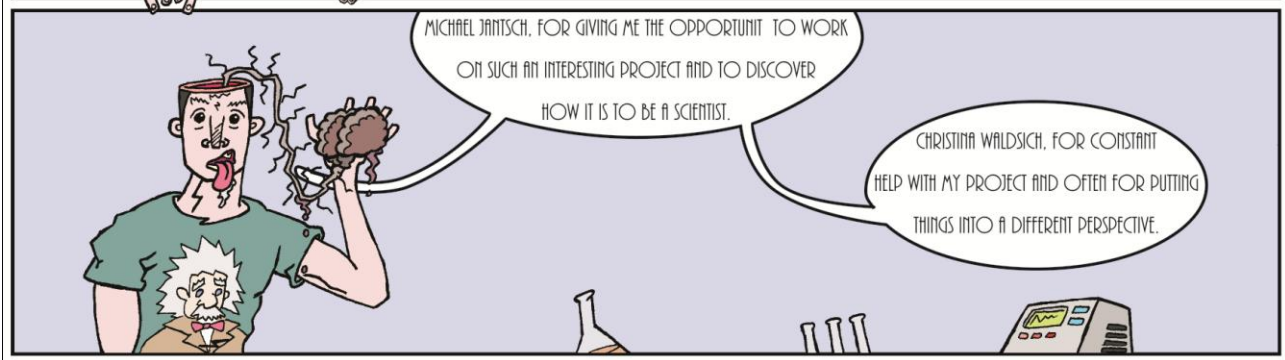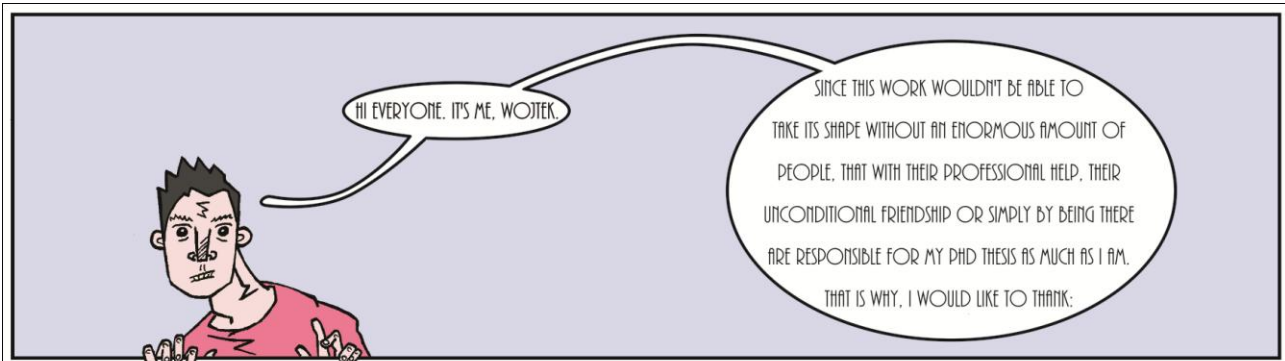
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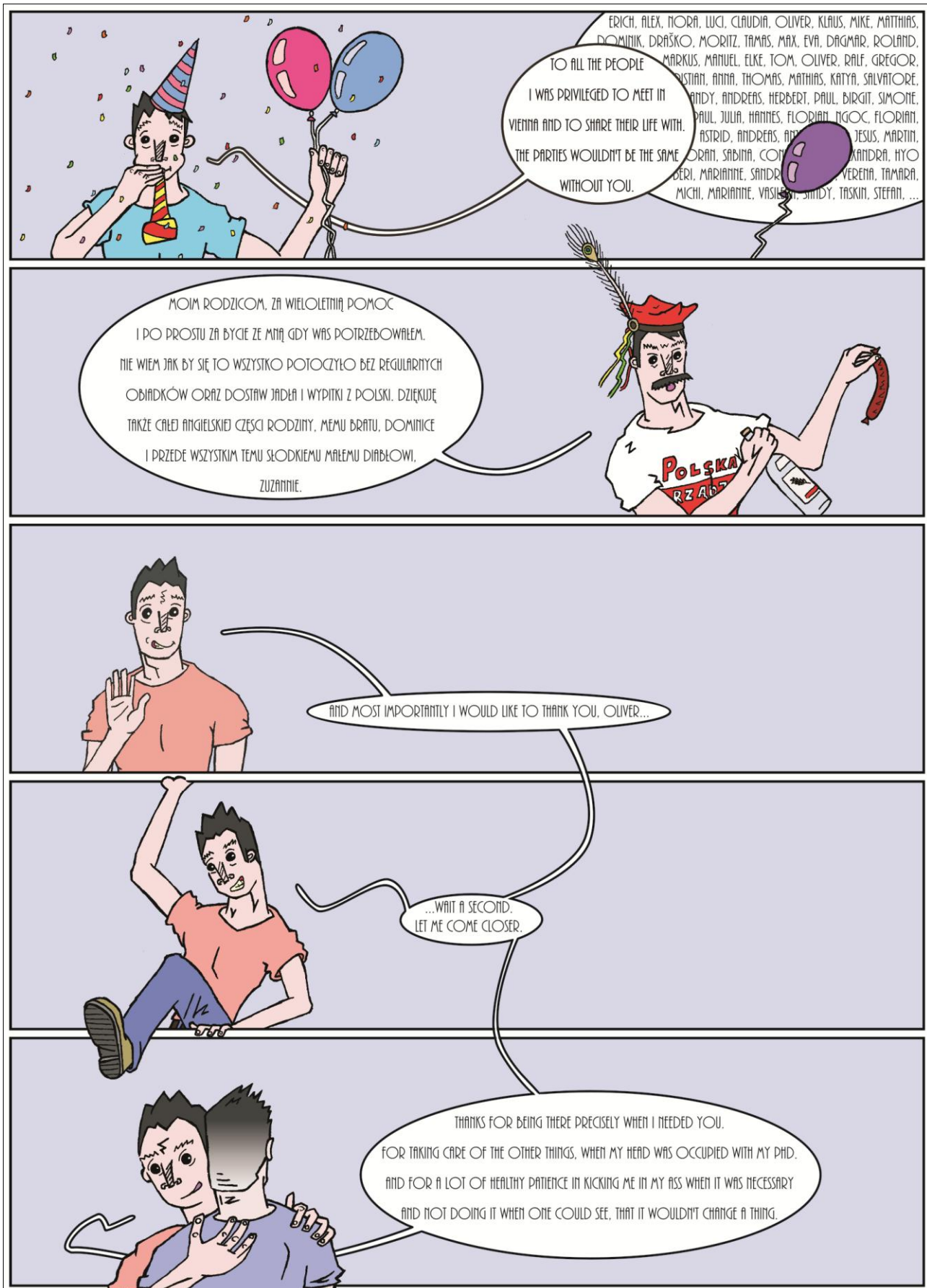
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7 Acknowledgments

8   Curriculum vitae

Mag. Wojciech Garncarz

Halbgasse 15/22

1070 Vienna

Tel.: +43-650-581-05-69

E-Mail: wojciech.garncarz@gmx.at

born on April 8th, 1980 in Gliwice (Poland)

unmarried

Studies

07/2005 – 05/2010

PhD Studies in Molecular Biology

Center of Molecular Biology, University of Vienna, Dept. of Chromosomal Biology

Research group of Univ. Prof. Dr. Michael F. Jantsch

10/2004 – 05/2005

Traineeship in Structural and Computational Biology

Center of Molecular Biology at University of Vienna, Dept. of Structural and Computational Biology

Research group of Univ. Prof. Dr. Gottfried Köhler

07/2002 – 07/2004

Diploma Thesis

Cancer Center and Institute of Oncology in Gliwice (Poland), Dept. of Experimental and Clinical Radiobiology

Research group of Prof. Dr. Joanna Rzeszowska

10/1999 – 07/2004

Diploma Studies

University of Silesia, Katowice (Poland)

Studies of Microbiology and Biochemistry

Education

09/1987 – 06/1999

Grammar school in Gliwice, Poland

Elementary school in Gliwice, Poland

Scientific Skills

Standard molecular biology and biochemistry procedures including cell culture, immunobiological analysis, FACS analysis

Self dependent establishing and standardisation of novel screening approaches

Experience in lab management

Teaching and supervising undergraduate students

Teaching assignment at the University of Vienna, lectureships and organisation of undergraduate courses

Additional Skills

Languages: Polish (mother tongue), English (fluent), German (fluent), Swedish (basic)

Computer skills: Office applications, graphic applications, scientific software

Teaching: University of Vienna (practical courses and seminars)

Economics and quality management Course in Processes and Methods in the Life Science Industry

Publications

Isolation of enhancers of I to I editing. Garncarz W, Tariq A, Jantsch M
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Regulation of the human apoptotic DNase/RNase endonuclease G: involvement of Hsp70 and ATP. Kalinowska M, Garncarz W, Pietrowska M, Garrard WT, Widłak P. Apoptosis. 2005 Aug;10(4):821-30.

Oral presentations

SFB RNA lecture series, 2007, 2009 and 2010

MFPL seminar series, WS 2009

University of Vienna, Newest Developments in Chromosomal Biology, SS 2008

Posters

RNA 2008: Thirteenth Annual Meeting of the RNA Society, Berlin, Germany
Yeast system to identify activators and inhibitors of ADARs
Wojciech Garncarz , Aamira Tariq and Michael F. Jantsch

Gordon Research Conference – RNA Editing 2007, Ventura, California, USA
Interfering with ADAR activity
Wojciech Garncarz and Michael F. Jantsch

Gliwice Scientific Meetings 2004, Gliwice, Poland
Regulation of the human apoptotic nuclease Endonuclease G
Magdalena Kalinowska, Wojciech Garncarz, Monika Pietrowska and Piotr Widłak