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RNA editing and autophagy in *Drosophila melanogaster*

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Abstract

Post-transcriptional regulation of gene expression involves a diverse set of mechanisms such as RNA splicing, RNA localization, and RNA turn-over. Adenosine to Inosine (A-to-I) RNA editing is an additional post-transcriptional regulatory mechanism. Temporally, it occurs after transcription and before RNA splicing and has been shown in some instances to possibly modulate alternative splicing events. This is the case for example, with the pre-mRNA encoding the *GluR-2* subunit of AMPA receptor, a glutamate-activated ion channel.

ADAR (Adenosine deaminase acting on RNA) proteins bind to double-stranded regions in pre-messenger RNAs. They deaminate specific adenosines, generating inosines; if the editing event occurs within the coding region, inosine is then interpreted as guanosine by the ribosomal translational machinery, changing codon meaning. These editing events can increase the repertoire of translated proteins, generating molecular diversity and modifying protein function.

In mammals there are four *ADAR* genes: *ADAR1*, *ADAR2*, *ADAR3* and *TENR*. ADAR3 and TENR are enzymatically inactive. All the proteins have two types of functional domains: (i) the catalytic deaminase domain at the carboxyl-terminus and (ii) the double stranded RNA binding domains, dsRBDs, at the amino terminus. ADAR1 and ADAR2 differ significantly at the amino terminus, by the number of the dsRNA binding domains (three and two dsRBDs for ADAR1 and ADAR2 protein, respectively). The differences observed between ADAR1 and ADAR2 are likely to reflect the different repertoires of substrates edited by these two enzymes.

Data concerning the conservation of *Adar* genes throughout evolution suggest that *Drosophila melanogaster* has a unique *Adar* gene which is a true ortholog of human *ADAR2* rather than an invertebrate gene ancestral for both vertebrate genes. Flies that are null mutants for *Ada*r (*Adar*^{5G1} mutants) display profound behavioral and locomotive deficits. Impairment in motor activity of the mutants is succeeded by age-dependent neurodegeneration, characterized by swelling within the *Adar*-null mutant fly brain.

The initial focus of my thesis was to elucidate what causes *Adar* mutant phenotypes or, whether it is possible, to suppress them. I took advantage of *Drosophila* genetics to establish a forward genetic screen for suppressors of reduced $Adar^{5G1}$ viability which is approximately 20-30% in comparison to control flies at eclosion. The results from an interaction screen on Chromosome 2L were further confirmed using Exelixis *P*-element insertion lines. The screen revealed that decreasing *Tor* (Target of rapamycin) expression suppresses *Adar* mutant phenotypes.

TOR plays a role in maintaining cellular homeostasis by balancing the metabolic processes. It controls anabolic events by phosphorylating eukaryotic translation initiation factor 4E-binding protein (4E-BP) and p70 S6 kinase (S6K) and inducing cap-mediated translation. However, different types of stress, signals or increased demand in catabolic processes, converge to reduce TOR enzymatic activity. This results in long-lived proteins and organelles being engulfed in double-membrane vesicles and degraded; this bulk degradation process is called (macro)autophagy.
The second aim of my thesis was to clarify which pathway, downstream to TOR, was responsible for the suppression of *Adar*-null phenotypes. I mimicked the effect of reduced *Tor* expression by manipulating genetically the cap-dependent translation and the autophagy pathways. Interestingly, boosting the expression of *Atg* (*autophagy specific genes*) genes, such as, *Atg1* and *Atg5*, thereby increasing the activation rate of the autophagy pathway, suppresses $Adar^{5G1}$ phenotypes. Finally, I found that $Adar^{5G1}$ mutant flies have an increased level of autophagy that is observable from the larval stage.

I investigated possible stresses affecting our mutants; *Adar*-mutant larval fat cells show ER stress triggering an unfolded protein response as indicated by expression of *XbpI-eGFP* reporter. Thus, ER stress might induce increased autophagy and it can lead to locomotive impairments and neurodegeneration in *Adar*-null mutants. These results suggest a function for the *Adar* gene in regulating cellular stress.

Declaration

I declare that the work in this PhD is my own, unless otherwise stated.

Simona Paro

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Abbreviations

General abbreviation

aa	amino acids
α	alpha
β	beta
ε	epsilon
bp	base pairs
°C	degrees centigrade
CNS	central nervous system
CTD	carboxyl terminal domain
DD	deaminase domain
dsRBD	double stranded RNA-binding domain
ECS	exon coding sequence
FITC	fluorescein isothiocyanate
g	gram
kb	kilo base
l	litre
m	metre
M	molar
µl	microlitre
ml	millilitre
mM	millimolar
mol	moles
n	nano
NES	nuclear export signal
nm	nanometre
nmol	nanomolar
NLS	nuclear localization signal
nt	nucleotide
UPR	unfolded protein response
UTR	un-translated region

Amino Acid code

A	Ala	Alanine	L	Leu	Leucine
R	Arg	Arginine	K	Lys	Lysine
N	Asn	Asparagine	M	Met	Methionine
D	Asp	Aspartate	F	Phe	Phenylalanine
C	Cys	Cysteine	P	Pro	Proline
Q	Gln	Glutamine	S	Ser	Serine
E	Glu	Glutamate	T	Thr	Threonine
G	Gly	Glycine	W	Trp	Tryptophan
H	His	Histidine	Y	Tyr	Tyrosine
I	Iso	Isoleucine	V	Val	Valine

Cellular molecules abbreviation

A	Adenosine
AMP	adenosine monophosphate
ATP	adenosine triphosphate
C	Cytosine
cAMP	cyclin adenosine monophosphate
DAG	Diacylglycerol
DNA	deoxyribonucleic acid
G	Guanosine
gRNA	guide RNA
GDP	guanosine diphosphate
GTP	guanosine triphosphate
I	Inosine
IFN	Interferon
IMP	inositol monophosphate
Ins	Inositol
IP6 or Ins P_6	inositol hexakisphosphate
mRNA	messanger RNA
PE	phosphatidylethanolamine
PIP2	phosphatidylinositol (4,5)-bisphosphate
PIP3 or IP3	phosphatidylinositol (3,4,5)-trisphosphate
PtdIns	phosphatidylinositol
pre-mRNA	pre-messanger RNA
RNA	ribonucleic acid
rRNA	ribosome RNA
SUMO	small ubiquitin-like modifier
T	Thymine
tRNA	transfer RNA
U	uracil
ubl	ubiquitin-like molecule

Cellular component abbreviation

CMA	chaperone-mediated autophagy
CRE	cAMP response element
e-MI	endosomalmicroautophagy
ER	endoplamic reticulum
IRE-1	Inositol requiring 1, ER stress-responsive element
MVBs	multivesicular bodies
PAS	pre-autophagosomal structure
PMN	piecemeal microautophagy of the nucleus
RER	rough ER
SER	smooth ER
SR	sarcoplasmic ER
UAS	Upstream Activation Sequence
UPRE	UPR promoter element

Protein and gene abbreviation

4E-BP	Eukaryotic translation initiation factor 4E-binding protein
Aβ	amyloid beta
ADAR	adenosine deaminases that act on RNA
ADAT	adenosine deaminases acting on tRNA
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AMPK	AMP activated protein kinase
AMPKK	AMP activated protein kinase kinase
APOBEC	apolipoprotein B mRNA-editing enzyme catalytic polypeptide
APP	amyloid precursor protein
ATG	autophagy related gene
BSK	basket
CaM	calmodulin
CaMKK	calcium/calmodulin kinase kinase
CD4	cluster of differentiation 4
CREB	cAMP response element-binding protein
eIF4E	Eukaryotic translation initiation factor 4E-binding protein 1
FKBP12	FK506 binding protein
FOXO	Forkhead Box O factor
FUS	Fused-In-Sarcoma
GFP	green fluorescent protein
HEP	hemipterous
HSC70	Heat Shock Protein 70 kDa
Htt	huntingtin protein
IMPase	inositol monophosphatase
IGFR	Insulin growth factor receptor
IR	Insulin Receptor
IRS	Insulin receptor substrate
JNK	Jun-N-terminal kinase
JNKK	Jun-N-terminal kinase kinase
JRA	Jun-related antigen
KAY	Kayak
LAMP2A	lysosome-associated membrane protein type-2A
LC3	light chain 3 protein
MHC	major histocompatibility complex
ND75	complex I protein NaDH Dehydrogenase subunit 75
Nf-kB	nuclear factor kappa-light chain-enhancer of activated B cells
PI3K	phosphatidylinositol 3-OH kinase
PERK	dsRNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase
PLCε	phospholipase C type ε
PTEN	Phosphatase and tensin homolog
PUC	Puckered
Raptor	regulatory associated protein of TOR
Rictor	rapamycin-insensitive companion of TOR
S6K	translational regulators p70 S6 kinase
SNARE	SNAP (Soluble NSF Attachment Protein) Receptor
SOD	superoxide dismutase

SP-1	Specificity Protein 1
TDP43	TAR DNA binding protein 43
TENR	testis-expressed nuclear RNA-binding protein
TOR	Target of rapamycin
TSC1/2	tuberous sclerosis protein 1 and 2 complex of proteins
Vsp34	vacuolar protein sorting 34

Chemical abbreviation

AM	Acetoxymethyl
Ca^{2+}	Calcium ion
CBZ	Carbamazepine
CO_2	carbon dioxide
DHE	Dihydroethidium
FCS	fetal calf serum
H2DCF:	2',7'-dichlorofluorescein
HCl	hydrochloric acid
H_2O	Water
H_2O_2	hydrogen peroxide
HE	haematoxylin and eosin
K^+	Potassium ion
KCl	potassium chloride
Na^+	Sodium ion
NaCl	sodium chloride
NH_4	Ammonium
O_2	oxygen
O_2^-	Superoxide
PBS	phosphate saline buffer
pH	potential of hydrogen
ROS	reactive oxygen species
Tris	Aminotris(hydroxymethyl)methane
VPA	valproic acid

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CHAPTER 1: Introduction

1.1 RNA editing

RNA editing is an essential biological process that modifies the genetic information encoded in an RNA molecule. There are many different types of RNA editing however in mammals only base conversion by hydrolytic deamination occurs.

RNA editing was first discovered into *Trypanosome brucei* (Benne *et al.*, 1986), and in this organism, the mitochondrial pre-mRNA encoding cytochrome oxidase subunit II editing is targeted by base-pairing of the unedited primary transcript with a guide RNA (gRNA) which contains complementary sequence around the insertion/deletion point. The editing event in this double-stranded (ds) region is then catalyzed by the editosome, a large multi-protein complex that inserts/deletes U bases in mRNA, resulting in a translated protein that differs from what is encoded in the genome.

Base conversion is the most common RNA editing event in mammals. The modification deaminates either a cytosine (C) or adenosine (A). In the case of cytosine, the apolipoprotein B mRNA-editing enzyme catalytic polypeptide (APOBEC) family of enzymes deaminates cytosine into uracil. Different members of the APOBEC family can modify RNA or DNA or both. This event is known as C-to-U editing. In the case of A-to-I RNA editing, adenosine deaminases that act on RNA (ADARs) family recognize target adenosines (A) within dsRNAs and convert them into inosines (I). Both families of enzymes belong to the cytidine deaminase (CDA) superfamily, based on sequence conservation around the active site.

The consequences of these base conversions vary. RNA editing can (i) change coding capacity of the transcript, (ii) create start or stop codons, (iii) influence alternative splicing, generating alternative splice donor or acceptor sites, or (iv) modulate mRNA stability and localization through editing of regulatory sequences at the 5'- or 3'-untranslated region (UTR). Thus, the editing event potentially increases the repertoire of

the translated proteins generating molecular diversity and modifying protein function through recoding of genomic information, especially within the immune and the central nervous system (CNS). C-to-U editing can rearrange chromatin structure, as the case of immunoglobulins class switch recombination (Honjo *et al.*, 2002).

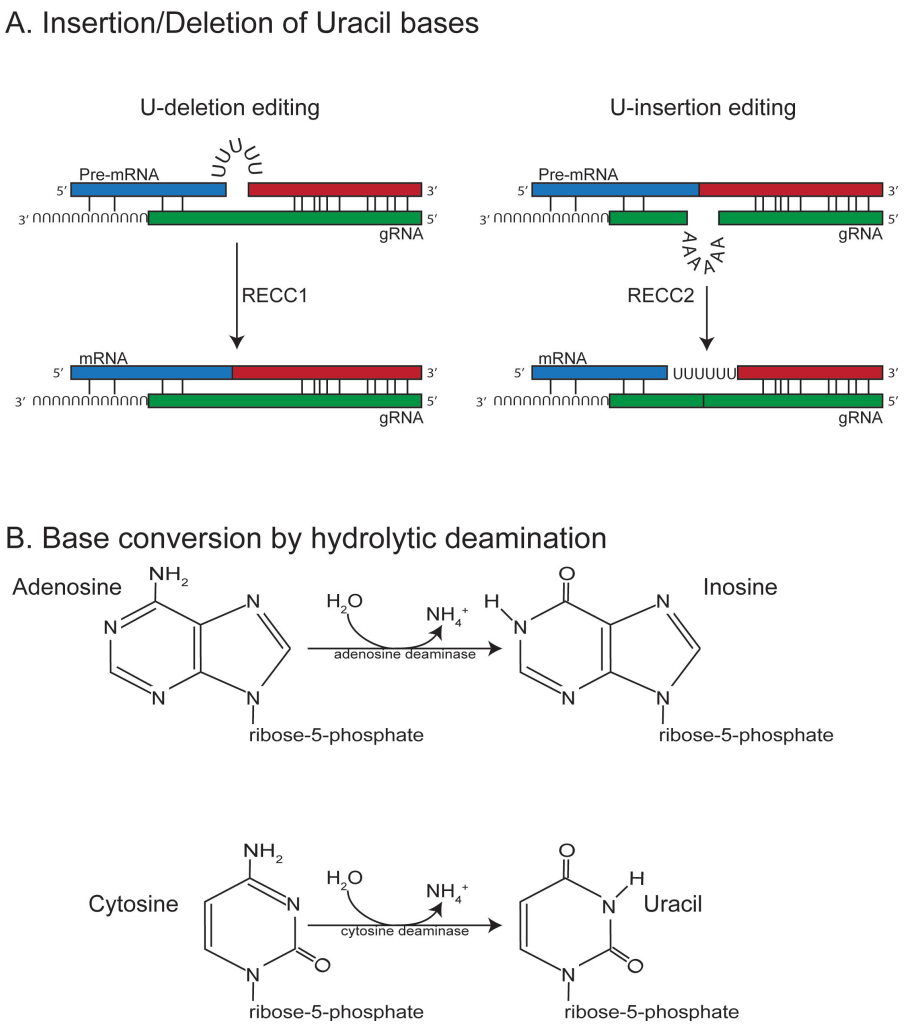


Figure 1.1: RNA editing. (A) A schematic representation of deletion (left) and insertion (right) of uracil bases by RNA editing which was first identified in *Trypanosome brucei*. Pre-mRNA (blue and red bars) base-pairs with guide RNAs (gRNA, in green). Two different large multi-protein complexes, editosome RECC1 and RECC2, catalyze the deletion or the insertion of uracil bases in the pre-mRNAs, respectively. (B) A diagram depicting the two possible types of base conversion RNA editing. Adenosine is converted into inosine by ADAR enzymes. This mechanism occurs in pre-mRNA molecules as well in tRNAs where it is catalyzed by ADATs (Adenosine deaminases acting on tRNA). On the bottom it is shown the hydrolytic removal of the amino group of cytosine to generate a uracil base. This reaction is catalysed by the APOBEC family of enzymes, whose members can edit RNA as well as DNA.

1.2 A-to-I RNA editing by ADAR

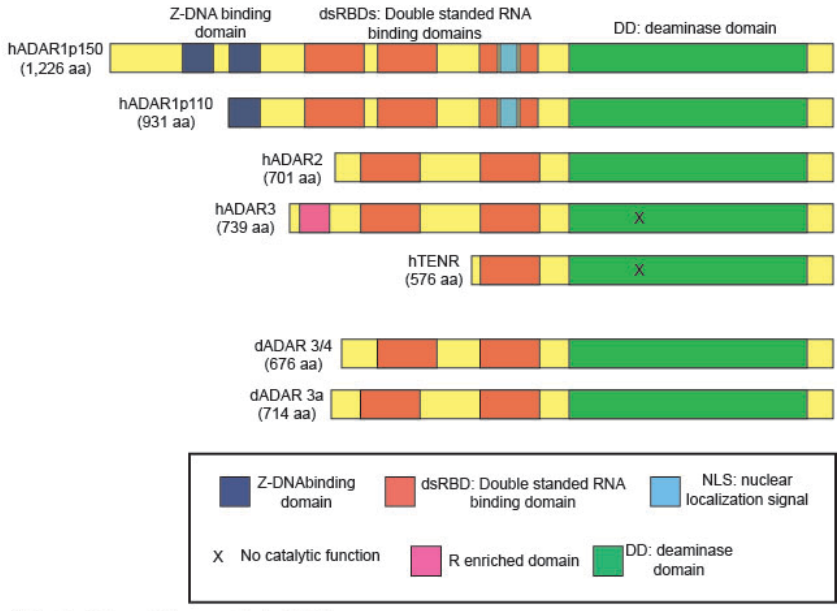
A-to-I RNA editing was firstly reported in *Xenopus laevis* where it was observed that RNA:RNA hybrids undergo an unwinding process (Bass and Weintraub, 1987; Rebagliati and Melton, 1987). Originally, it was thought that a new RNA helicase had been identified. Later on it was found that up to 50% of the adenosine residues in the dsRNA were actually deaminated to inosines (Bass and Weintraub, 1988). The first mammalian gene encoding an adenosine deaminases acting on RNA enzyme, *ADAR1*, was purified from bovine extract and the human gene later cloned (Kim *et al.*, 1994a; Kim *et al.*, 1994b; O'Connell and Keller, 1994; O'Connell *et al.*, 1995). Subsequently ADAR2 was also purified and the gene was cloned (Melcher *et al.*, 1996c; Gerber *et al.*, 1997; Lai *et al.*, 1997; O'Connell *et al.*, 1997; Chen *et al.*, 2000). ADAR3 was initially identified in rat (Melcher *et al.*, 1996a) More recently, another ADAR-like gene, named *TENR*, was identified as a testis nuclear RNA-binding protein (Schumacher *et al.*, 1995). In 2000 it has been found that *Drosophila melanogaster* has only one *Adar* gene that encodes for an adenosine deaminases acting on RNA enxyme (Palladino *et al*., 2000a).

ADAR enzymes have a common structure that consists of different numbers of 65-amino acid dsRNA-binding domains (dsRBDs) at the amino-terminus and the highly conserved deaminase domain, DD, at the carboxyl terminal (Figure 1.2). The crystal structure of the deaminase domain of the murine free-nucleotide adenosine deaminase (Wilson *et al.*, 1991) and *Escherichia coli* free-nucleotide cytidine deaminase (Betts *et al.*, 1994) were determined and revealed that a zinc atom is essential for the catalytic activity of these enzymes (Hogg *et al.*, 2011). The conserved residues responsible for chelating the zinc and therefore for the catalytic activity are organized into three motifs:

- Motif I, H/CxE: a cysteine (C) or a histidine (H) residue followed by a glutamic acid (E) residue which acts as a proton donor during the nucleophilic deamination reaction
- Motif II, PC: a proline (P) followed by one conserved cysteine
- Motif III, C: cysteine residue

In mammals only *ADAR1* and *ADAR2* are catalytically active. It is likely that they have a common ancestor before a gene duplication event in early metazoan evolution (Keegan *et al.*, 2011; Jin *et al.*, 2009). While *ADAR3* and *TENR* might originate only after Urochordata-Vertebrata divergence (Jin *et al.*, 2009), they do not show any catalytic activity in the tissue where they are expressed, which is the brain for *ADAR3* (Melcher *et al.*, 1996b; Melcher *et al.*, 1996d) and the testis for *TENR* (Schumacher *et al.*, 1995; Connolly *et al.*, 2005). Recently it has been shown that *Drosophila Adar* might be a true orthologue of human *ADAR2* rather than an invertebrate ancestor of both human *ADAR1* and *ADAR2* (Keegan *et al.*, 2011). However the enzyme is catalytically active.

These RNA editing genes are not present in protozoa, yeast, and plants (Jin *et al.*, 2009). The ADAR family evolved from tRNA adenosine deaminases (Tad2/Tad3) which have smaller deaminase domains by the acquisition of dsRNA-binding domains (dsRBDs) at the amino terminus of the protein (Gerber and Keller, 2001) and by enlargement of the deaminase domain.



Adapted from Hogg, *et al.*, 2011

Figure 1.2: Protein structure of adenosine deaminases that act on RNA, ADAR, enzymes. ADAR proteins are characterized by a catalytic deaminase domain (DD: in green). The X within the catalytic domain of hADAR3 and TENR depicts catalytically inactive proteins. The proteins are characterized by dsRNA binding motifs, dsRBM, (in orange) that are involved in RNA binding. The RNA binding specificity is dependent on the distance between the different dsRBMs. ADAR1 contain a Z-DNA binding domains (in blue). hADAR3 has a R-enriched domain (pink) in its amino-terminus region that is thought to bind single-stranded RNA.

1.3 Human ADARs

1.3.1 ADAR1

The human *ADAR1* gene spans approximately 30 kilobases and it lies at cytogenetic position 1q21.3 (Weier *et al.*, 1995). There are three alternative first exons; exon 1A-C, and consequently there are three different pre-mRNA generated for *ADAR1* that differ in their 5'-untranslated regions (UTRs). The exon 1A transcript is transcribed from an interferon-(IFN)-inducible promoter and contains the AUG codon for the first initiator methionine, Met1. IFN-α, IFN-β and IFN-γ can induce the expression of exon 1A-containing *ADAR1* to generate a 150 kDa protein referred to as ADAR1p150 (Wang *et al.*, 1995; George and Samuel, 1999b, a). ADAR1p150 is involved in the interferon-responses such as antibacterial- and antiviral-responses. Whereas the expression of ADAR1p150 is interferon-induced, a constitutive isoform of *ADAR1* is also expressed. *ADARp110* encoding pre-mRNAs contain exon 1B and 1C and the initiating methionine resides in exon 2, Met296. ADAR1p110 expression is driven by three constitutive promoters present in exon 1B, 1C and exon 2, before Met296 (Kawakubo and Samuel, 2000). The ADAR1p110 and ADAR1p150 isoforms contain an NLS within the third dsRBD that is recognised by transportin-1 that mediates the nuclear localization of ADAR1 that is distributed between nucleus and nucleolus (Fritz *et al.*, 2009). The three dsRBDs are preceded by a Z-DNA binding domain, Zβ (Figure 1.2). ADARp150 has another Z-DNA binding domain, Zα, within the additional 296 amino acids at the amino end (Figure 1.2). These Zα and Zβ domains can bind nucleic acids such as DNA and structured RNA in the Z-form (Herbert *et al.*, 1997; Herbert and Rich, 1999, 2001). Zα domain contains an NES sequence that is recognized by exportin 5 (Fritz *et al.*, 2009); this evidence suggests that ADAR1p150 might have additional cytoplasmic substrates from those of ADAR1p110 within the nucleus.

Interestingly, the RNA editing activity of ADAR1 is altered by addition of a small ubiquitin-like modifier 1, SUMO-1, on Lys418, between Zβ domain and the first RBD (Desterro *et al.*, 2005).

1.3.2 ADAR2

The human *ADAR2* gene is located at cytogenetic position 21q22.3 and it spans approximately 153 kb (Mittaz *et al.*, 1997a). Its expression is driven by cAMP response element-binding (CREB), which binds upstream cAMP response elements, CREs (Peng *et al.*, 2006). Maas and Gommans have reported a new *ADAR2*-alternative promoter that drives the expression of exon 0 which is located 18 kilobases upstream of the canonical first coding exon. The alternative promoter includes a TATA box sequence and a consensus binding sites for the nuclear factor kappa-light chain-enhancer of activated B cells (Nf-kB) and for the Specificity Protein 1 (SP1) (Maas and Gommans, 2009a). ADAR2 has two dsRBDs at the amino terminus (Figure 1.2). Its NLS signal has not been precisely identified (Desterro *et al.*, 2003). ADAR2 is imported and localized in the nucleus by the activity of importin α family member, particularly α4 and α5. The inclusion of the recently identified exon 0 in the *ADAR2*-encoding pre-mRNA introduces an arginine-enriched domain and an NLS upstream of the dsRBDs. This new NLS is preferentially recognized by importin α1 (Maas and Gommans, 2009). ADAR2 is usually sequestered in the nucleolus probably by binding to abundant dsRNA structures associated with nucleolar RNAs (Desterro *et al.*, 2003). ADAR2 as well as ADAR1 actively enter and exit the nucleoli in living cells (Desterro *et al.*, 2003). Recently our group has observed that ADAR2 sub-cellular localization is controlled by the interaction with phosphorylation-dependent peptidyl-prolyl *cis*/*trans* isomerase Pin1 (peptidyl-prolyl isomerase *N*IMA interacting protein 1**)** (Lu *et al.*, 2007; Marcucci *et al.*, 2011). Pin1 recognises pSer26-Pro27 and pSer31/pThr32-Pro33 motifs at the amino terminus of ADAR2 and it mediates its retention within the nucleus. Pin1 is therefore a positive regulator of ADAR2 editing activity as ADAR2 has to be in the nucleus to be able to edit pre-mRNA (Marcucci *et al.*, 2011). Furthermore WWP2, a HECT (homologous to the E6-AP C terminus) E3 ubiquitin ligase (Pirozzi *et al.*, 1997) has been identified as a negative regulator of ADAR2 activity. WWP2 binds to two conserved PPxY motifs in ADAR2, one between the dsRBDs and the other within the

deaminase domain. This interaction results in the ubiquitination and subsequent degradation of ADAR2 (Marcucci *et al.*, 2011).

Macbeth and co-workers have crystallized the deaminase domain of ADAR2. They demonstrated that the catalytic activity of this enzyme is dependent on the presence of inositol hexakisphosphate (IP6) within the catalytic domain, identifying amino acids that have been demonstrated previously to be essential for activity as being involved in its coordination of IP6 (Macbeth *et al.*, 2005).

1.3.3 ADAR3

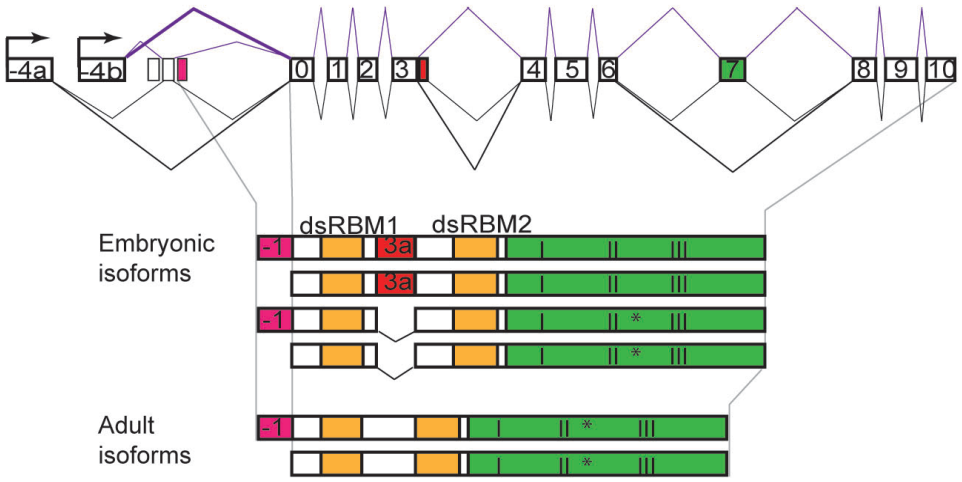
The *ADAR3* gene resides at cytogenetic position 10p5 in close proximity to the telomere and spans 10 exons (Mittaz *et al.*, 1997a). ADAR3 is mostly expressed in the brain and has two dsRBDs and an upstream arginine-enriched domain that binds to single-stranded RNA, ssRNA (Figure 1.2) (Melcher *et al.*, 1996b; Chen *et al.*, 2000) although no editing activity has yet been associated with this enzyme (Chen *et al.*, 2000). The nuclear localization of ADAR3 is mediated by importin α1, which specifically recognizes the arginine-enriched domain (Maas and Gommans, 2009a, b). Despite having no editing activity this protein is evolutionarily conserved in mammals.

1.3.4 TENR

TENR, testis-expressed nuclear RNA-binding protein, is only expressed in the round spermatids in the testis. It contains only one dsRBM and has been identified as an interacting protein with the 3'UTR of the protamine 1 (Prm1) RNA (Schumacher *et al.*, 1995). Interestingly it is likely to be catalytically inactive as it lacks crucial and conserved residues involved in catalysis (Connolly *et al.*, 2005). The biological role is still unclear. However *TENR*-null male mice are sterile due to high incidence of sperm morphological defects and low counts of sperm suggesting a key role of TENR in sperm morphogenesis.

1.4 *Drosophila Adar* (Paro *et al.*, 2011)

The single *Adar* gene in *Drosophila melanogaster* lies at cytogenetic position 2B6-7, near the centromere distal end of the X chromosome (Palladino *et al.*, 2000c). Its expression is higher in the CNS but it has also been reported outside the CNS albeit at a lower expression. It has been proposed that two different promoters, 4A and 4B, control the transcription of the *Adar* gene (Figure 1.3). The constitutive 4A promoter is active through all fly development and its transcription increases at the pupal stage. The 4B promoter was proposed to be approximately 1 kb downstream, within a large intron of transcripts from the 4A promoter (Palladino *et al.*, 2000c). Annotation of *Drosophila* genes and chromosomes has been greatly facilitated by the addition of new information recently provided by the Model Organisms component of the Encyclopedia of DNA Elements project (ModENCODE), which covers the entire fly genome (Roy *et al.*, 2010). Developmental transcription data from the *Drosophila* ModENCODE project does not show a dramatic increase in transcripts corresponding to the first exon-4B of the adult-specific transcript in adult flies (see *Adar* data at FlyBase at http://flybase.org and GBrowse links to data for *Adar* on their mirror site for ModENCODE at http://modencode.oicr.on.ca/fgb2/gbrowse/fly/?name=Adar), though some exons may be underrepresented in the RNA-Seq data. Other data from the ModENCODE project shows that the *Adar* locus lies in an open chromatin region, actively transcribed, with expected enrichments of histone H3K4Me1, H3K4Me3 and H3K27Ac modifications at the constitutive promoter, as well as RNA Polymerase II accumulation in both embryos and adults. Upstream of the constitutive promoter there is a very strong prediction for a chromatin insulator based on CTCF protein binding in embryos and adults. Insulators may establish chromatin loops and form boundaries between regions of gene regulation. Other insulator predictions are about 60 kb downstream and 130 kb upstream of the *Adar* promoter.



Adapted from Paro *et al.*, 2011

Figure 1.3: The *Drosophila Adar* gene transcripts and encoded protein isoforms. The expression of *Adar* gene is driven by two upstream promoters; -4a and -4b. The first promoter is active throughout *Drosophila* development and expresses 4 different protein isoforms from the embryonic stage. -4b is activated only after metamorphosis during pupa stage and adulthood. The catalytic domain is shown in green. Exon 7 contains the editing site (shown with an asterisk). The inclusion of exon 3a mutually excludes the presence of exon 7 and so the possibility to detect auto-edited *Adar*-transcript.

The promoter region also binds Origin Recognition Complex (ORC) proteins in embryo stage and at metamorphosis. Evidence suggests that the promoter region contains an origin of replication, which is active at these stages. A possible enhancer immediately upstream of the constitutive promoter is suggested to be bound by the transcriptional coactivator P300/CBP, which is encoded by the *Drosophila* gene *Nejire* (Akimaru *et al.*, 1997a; Akimaru *et al.*, 1997b). This protein has been extremely valuable in identifying non-conserved enhancers in human and vertebrate genomes (Visel *et al.*, 2009). CREB-binding protein, CBP, is a transcriptional coactivator that binds to the DNA-binding cAMP response element binding (CREB) protein as well as to many other transcription activators bound at enhancers (Vo and Goodman, 2001). The CBP coactivator has histone acetyltransferase activity at H3K27 sites and other sites on histones. Most of the transcription regulators, particularly neural transcriptional regulators that are likely to regulate *Adar* specifically have not been mapped yet, and the *Adar* transcriptional

control sequences have not been defined. The cAMP response protein CREB is a possible regulator of *Adar*, based on mammalian data (Gan *et al.*, 2006; Peng *et al.*, 2006) and this could provide a link between *Adar* expression and neuronal activity.

The *Adar* transcripts have long 5'-UTRs with alternatively spliced exons. Based on the estimated relative abundances of different splice forms these are expected to generate predominantly two different protein isoforms starting specifically at the alternative exons -1 or +1. The inclusion of alternative exon -1 results in a protein being expressed with an additional 12 amino acids at the amino terminus. Two other starting methionines, in the more rarely included exon -2 and exon 0, produce two different protein isoforms that share high homology at the amino terminus (MKFDS and MKFEC) (Palladino *et al.*, 2000c).

Transcripts are spliced to include or exclude alternative exon 3a with exclusion of this exon occurring in the adult-specific splicing pattern. The ADAR 3/4 isoform predominates after metamorphosis (Palladino *et al.*, 2000a). Exon 3a has a rare non-consensus splice donor site (GCAAG vs. GTAAG) and it may be that a specific splicing enhancer contributes to the inclusion of exon 3a (Marcucci *et al.*, 2009). Interestingly, the inclusion of exon 3a introduces an additional 38 amino acids, modifying the distance between dsRBM1 and dsRBM2, a spacing that resembles that of vertebrate ADAR1 rather than ADAR2. There is a very strong correlation between the presence of adult exon 4b in the UTR and the adult splicing pattern deleting exon 3a.

The adult splicing pattern also correlates strongly with RNA editing at exon 7 in the transcript. Also, in embryos particularly, transcripts accumulate in which exon 7 is spliced out. This may serve to restrain ADAR activity in embryos as truncated ADAR proteins are predicted (Ma *et al.*, 2002a). Most of exon 7, though not the splice junctions, is predicted to form a large dsRNA structure involved in editing (Keegan *et al.*, 2005). This structure may affect the splicing of exon 7.

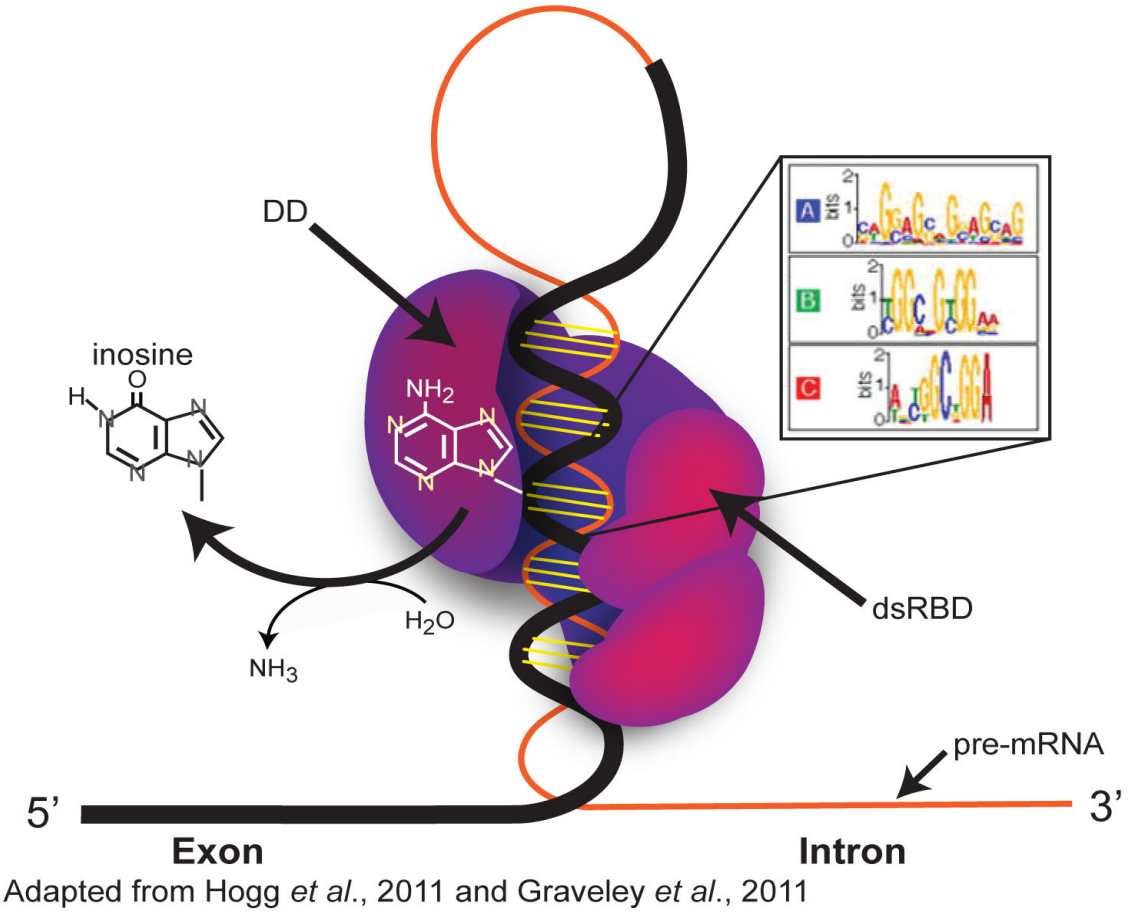
Drosophila ADAR contains two dsRBDs within the amino terminal half of the protein: dsRBM1 (53-133aa) and dsRBM2 (196-273aa) (Figure 1.2). dADAR protein with the alternative exon 3a inserted between the two dsRBMs, known as dADAR3a, rescues

Adar mutant phenotypes less efficiently than the adult-typical ADAR 3/4 isoform (Keegan *et al.*, 2005). Binding to RNA is necessary for formation of dADAR homo- or hetero-dimers and for editing activity. Sequences within the first 46 amino acids and the first dsRBM are required for dimerization (Gallo *et al.*, 2003). However, the main determinant of ADAR specificity lies in the deaminase domain at the carboxyl terminus and this is based on domain exchange experiments between mammalian ADAR1 and ADAR2. The deaminase domain contains three zinc-binding motifs (at positions 372, 430 and 493) that are essential to coordinate zinc near the active site glutamate at position 374. The self-editing event that takes place in the catalytic domain of the protein changes a serine residue (S) close to the zinc-chelating motif II to a glycine (G) residue. In adult flies, ADAR edits its own pre-mRNA with 40% efficiency to encode an ADAR 3/4G edited isoform that is eight fold less active by *in vitro* measurements and that rescues *Adar* mutant phenotypes less efficiently than the unedited isoform (Keegan *et al.*, 2005). It is not known what the physiological role of the self-editing event is. Understanding this presumably requires further study of factors regulating the activity of ADAR itself.

1.5 *Drosophila* transcripts that are edited by dADAR

ADARs bind a dsRNA helix of >20 base pairs (bps) and deaminate adenosines (i) non-selectively or (ii) selectively, implying that a few specific adenosines are chosen and edited (Nishikura *et al.*, 1991). Generally, completely base-paired dsRNA duplexes longer than 100 bps are edited promiscuously. However, in long (>100 bps) and imperfect dsRNAs containing bulges and single base-pair mismatches, adenosines are recognised and edited selectively (Lehmann and Bass, 1999). Most of the known site-specific editing sites lie within coding regions. In this case the region surrounding the editing site base pairs with a downstream intronic region called editing site complementary sequence (ECS) (Figure 1.4). This was originally identified by analysis of the RNA structure of vertebrate *GluR2* pre-mRNA (Higuchi *et al.*, 1993). Additionally, in *Drosophila* some ECS elements are not a single sequence unit as in the vertebrate glutamate receptor transcripts but consist of elements of six bases or more that may not be arranged sequentially in the genome but come together to pair with the edited region and form pseudo-knots (Reenan, 2005). Generally dsRNA duplexes as editing site/ECS duplexes tend to be short in *Drosophila* compared to those seen in vertebrate transcripts. Computational analysis of the *Drosophila* transcriptome identified three potential editing associated motifs (Figure 1.4).

In *Drosophila* 127 editing sites in 55 *Drosophila* transcripts mostly expressed within the CNS were identified and studied. Recently 972 new editing sites have been identified in 597 *Drosophila* transcripts by poly(A)$^+$ RNA-seq data. This indicates that 4% of the fly transcriptome is edited and a third of the editing events modify codons (Graveley *et al.*, 2011), the editing levels are usually lower than 100% with both edited and unedited isoforms generated. Interestingly, RNA editing increases immediately after metamorphosis and continues to increase throughout pupal and adult stages whereas some of the transcripts that are edited from the late embryonic stage.



Adapted from Hogg *et al*., 2011 and Graveley *et al*., 2011

Figure 1.4: *Drosophila* ADAR binds dsRNAs. The dsRBD, of *Drosophila* ADAR (in purple) recognizes and binds dsRNAs in which an exon (in black) basepairs with the downstream editing complementary sequence (ECS) within the intron (in orange). ADAR catalyses the deamination of a specific adenosine that is contained within the exonic sequence via its deaminase domain, DD. Therefore the adenosine (in white) is converted into inosine (in black). Recently Gravely and co-workers, identified 3 motifs (A, B and C) that are associated with RNA editing event in *Drosophila melanogaster* (Graveley *et al*., 2011); these three motifs are shown in the upper window on the right. 'A' and 'B' motifs are more common, whereas motif 'C' is more strongly associated with RNA editing site. The last A is the edited nucleotide in motif 'C' that is generally present on the sense strand of the transcript.

This reflects the increased enzymatic activity of the adult isoform, ADAR3/4. The overall expression patterns of the transcripts that are edited do not change as much as *Adar* between embryos and adult. Consistent with previous studies (Hoopengardner *et al.*, 2003; Jepson and Reenan, 2007), editing site-containing exons are more highly conserved than unedited exons as they have to form dsRNA. Most of edited proteins reside in ion channel complexes or in the membrane protein structure in plasma membranes, membrane bounded vesicles and mitochondrial membranes. Based on the classification of molecular functions of encoded proteins, most of them function as protein binding, nucleotide binding, lipid binding and ion binding. Only a few edited proteins have enzymatic activities such as kinases and phosphatases. Edited proteins can be classified in 7 groups based on their functional activity (Stapleton *et al.*, 2006); (1) Voltage-gated ion channels, (VGIC) such as *cacophony*, (Smith *et al.*, 1998a) and *paralytic*, (Hanrahan *et al.*, 2000); (2) Ligand-gated ion channels, (LGIC) such as *DrosGluCl*, (Semenov and Pak, 1999) and *Dalpha5,* (Grauso *et al.*, 2002a); (3) Proteins involved in synaptic release machinery (*Synaptotagmin1,* Ca^{2+} sensor; *Dunc-13*, SNARE binding; *Complexin*, SNARE protein, *EndoA*, promotes synaptic vesicle budding); (4) Vesicular trafficking proteins (*Rab26*, GTPase; *Rlip*, ral GTPase activator; *Rab3-GEF*, Rab guanyl-nucleotide exchange factor; *Alpha-Adaptin*, component of endocytosis subunit of AP-2; *Syd*, kinesin-dependent axonal transport; *Spin*, endosome to lysosome transporter); (5) Ion homeostasis proteins (*Cpn*, Ca^{2+} sequestration; *Nckx30C*, K^+-dependent Na^+,Ca^{2+} antiporter; *Atp-alpha*, Na^+, K^+ exchanging ATPase; *CG32699*, Ca^{2+} binding, acyltransferase activity); (6) Cytoskeletal components (*Spir*, actin nucleation factor; *Atx2*, regulator of actin filament formation); (7) Protein involved in signal transduction (*e1F-2beta,* transcription factor; *Fatp*, Long-chain fatty acid transporters; *CG12013*, glutathione peroxidase; *Cyp9b1*, Cytochrome P450).

1.6 *Adar* alleles

In 2000, two different *Adar* alleles have been reported and characterized. The first one is known as *Hypnos-2*^{P} and it is a loss of function allele in which the sequence of the second dsRNA binding motif and the catalytic domain of *dAdar* gene has been deleted (Figure 1.5). This mutant has been identified because its recovery from anoxic stupor is significantly prolonged (Ma *et al.*, 2001). Its sensitivity to perfusion followed by anoxic conditions seams to be dependent to the absence of editing of channels-encoding pre-mRNAs (Chen *et al.*, 2004; Ma *et al.*, 2001; Xia *et al.*, 2005). In fact *in vitro* electrophysiologic studies have shown that these membrane proteins, especially the voltage-gated Na^{+} and Ca^{2+} channels, play a pivotal role in nerve cell injury caused by O_2 deprivation (Choi and Rothman, 1990; Haddad and Jiang, 1997; Kimura *et al.*, 1998; Vornov, 1998). In this sense, *Hypnos-2*^{P} mutants are very resistant to oxidant injury (Ma *et al.*, 2001). Furthermore those flies suffer from a very mild age-dependent degeneration in the cortical neurons of the medulla and lobula complex as well as in the lamina (Ma *et al.*, 2001).

In the same year *Drosophila Adar* gene has been identified as unique gene expressing an adenosine deaminases acting on RNA enzyme. Then, different alleles were generated by *p*-Element mobilization and balanced using *FM7c* balancer gene as for *Adar* gene lies on X chromosome (Palladino *et al.*, 2000a). Furthermore the chromosome with the mutant allele for *Adar* has been marked with *y* and *w*: therefore mutant males are distinguishable not only by lack of *Bar* eye phenotype, but also by the colour of the body and the eyes that are, respectively, yellowish and white in comparison to wild-type flies. If males express a wild-type X chromosome due to female non-disjunction, then males would not bear the *y* mutation; once again they are distinguishable by body colour.

Adar^{1F4} is a hypomophic allele in which both 4A and 4B promoters upstream the coding region of *Adar* gene have been deleted (Palladino *et al.*, 2000a). Even though the promoter region has been deleted (Figure 1.5), *Adar* gene is still expressed; this might be due to other regulatory elements within the open chromatin region in which *Adar* gene is located. However the expression level is very low and only 1/10 of the protein that is

normally expressed can be detected within the central nervous system by immunostaining (McGurk, 2007). Furthermore the little protein present only edits its own *Adar*-encoding pre-mRNA while none of the known *Adar*-target transcripts are edited (Keegan *et al.*, 2005). The phenotypic characterization of the $Adar^{1F4}$ flies shows an age-dependent vacuolization affecting specifically the retina where the photoreceptors appear disorganized when compared to the more compact and organized retinal structure of wild-type flies (Palladino *et al.*, 2000b). Additionally $Adar^{1F4}$ flies undergo progressive vacuolization of the synaptic neuropil in mid-brain regions (Palladino *et al.*, 2000a). Beside the age-dependent neurodegeneration, $Adar^{1F4}$ flies show locomotion impairments immediately after eclosion (McGurk, 2007).

A third allele, referred as $Adar^{5G1}$, was previously generated (Palladino *et al.*, 2000a) and characterised in our laboratory by Leeanne McGurk (McGurk, 2007). It is a null-allele for *dAdar* expression. In fact, those flies carry a chromosomal deletion of 36.5 kb that covers the *Adar* gene and another gene downstream to *Adar*, *CG38206*. However this gene is not involved in the *Adar* phenotype as *Adar* transgene expression can rescue the $Adar^{5G1}$-associated phenotype (McGurk, 2007). Females, heterozygous for the $Adar^{5G1}$ allele, develop into morphologically normal adults: they are fertile and mate and lay eggs normally. $Adar^{5G1}$ males have a reduced viability: it is approximately 30% compared to wild-type flies or to heterozygous $Adar^{5G1}$ females or *FM7c* males from the same stock (McGurk, 2007). The lethal phase causing the reduced mutant fly viability has not been determined exactly because it seams to be spread throughout fly development. The complete deletion of *Adar* gene causes behavioural defects such as impairment of locomotion. The open-field locomotion assay was used to quantify locomotion defects. *Adar*-null flies almost completely lose the capability to walk normally and the assay revealed a reduction of over 80% compared to wild-type fly locomotion. The imperfect walking and the locomotion defects may explain the sterility affecting $Adar^{5G1}$ males that has been previously observed in our laboratory (personal communication Leeanne McGurk). In fact mutant males are unable to mate with mutant or wild-type females, but no gross morphological defects were detected in the testis of mutant adults. Before a successful mating can occur, *Drosophila* has to engage in

courtship which is a highly ritualistic and complex behaviour, consisting of a series of stereotyped interactions between the male and female (Hall, 1994; Greenspan and Ferveur, 2000). Therefore the defect in mating may be due to uncoordinated and defective movements of mutant flies rather than to a failure in their reproductive system. As well as in *Adar*^{1F4} flies, *Adar*^{5G1} mutants show an age-dependent neurodegeneration that is, however, much faster. This rapid vacuolisation specifically affects the mushroom body (MB) calyces and retina (McGurk, 2007). Mushroom body are involved in learning and memory as well as locomotion activation in response to external stimuli, in particular odours.

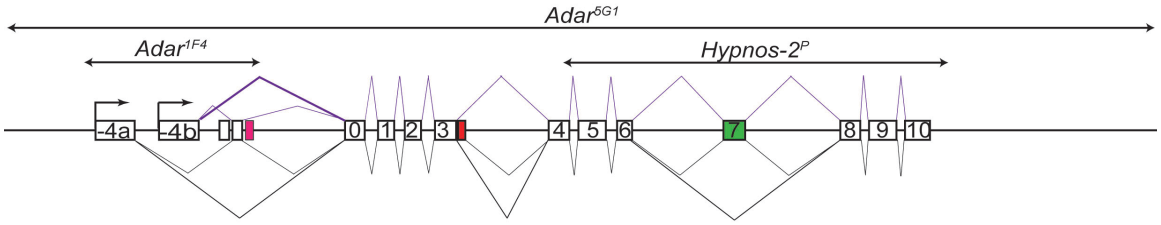


Figure 1.5: *Drosophila Adar* alleles. *Hypnos-2*^{P} allele is a loss of function allele in which the genomic region encoding the catalytic domain of dADAR has been deleted. *Adar*^{1F4} allele has been generated by removal of -4a, -4b and 5'UTR regions upstream the ADAR-encoding sequence. It is a hypomorphic allele. *Adar*^{5G1} is a null allele in which the deletion covers the whole gene.

In summary, *Adar*^{5G1} mutant flies show a reduced viability and locomotion defects that are succeeded by age-dependent neurodegeneration. The complete rescue of all *Adar*-null phenotypes is dependent on the expression of the active form of the enzyme, *UAS-Adar3/4* transgene, driven by *Cha-GAL4* driver (McGurk, 2007). The *Cha-GAL4* driver expresses GAL4 in the neuronal cell bodies and in neuronal projections on central and peripheral nervous system in embryos, larvae and adults. In fact, choline acetyltransferase immunoreactivity has been detected in the optical lobe including the lamina cartridges and in most of the neurophil of the brain and strong staining in the MB calyces has been observed. Thus, *Cha-GAL4* driver expresses *UAS-Adar3/4* transgene in most of the neurons in the *Adar*^{5G1} background, rescuing all the phenotypes associated to

the lack of ADAR. Interestingly, the expression of the inactive form of the protein (transgene: *UAS-Adar3/4 E374A*) using *Cha-GAL4* driver rescues only the neurodegeneration phenotype (personnel communication Leeanne McGurk). Hence, the impaired and uncoordinated movement is the most difficult phenotype to suppress and requires the catalytically active form of the enzyme. It is not clear why the inactive form of the enzyme, and therefore the only presence of the protein, is sufficient to suppress and/or prevent the neurodegeneration and only this phenotype. This might be due to it being able to bind specific mRNAs; in fact ADAR binds pre-mRNAs and this is can modulate RNA splicing (Grauso *et al.*, 2002b; Marcucci *et al.*, 2009) and processing of miRNAs (Heale *et al.*, 2009). Alternatively, the rescue by the inactive protein might related to the presence of inositol-1,2,3,4,5,6-hexakisphosphate (Ins P_6) molecule within the deaminase domain. Ins P_6 molecule was first identified within the catalytic domain of adenosine deaminases acting on tRNA, ADATs, or on RNAs, ADARs. *Drosophila* ADAR shares 62% of identity with human ADAR2. The amino acid sequence that is important for the coordination of Ins P_6 is also conserved (Figure 1.6). Those hypothesis might also explain why *Adar*^{5G1}-dependent neurodegeneration is suppressed not only by the expression of the human ADAR1 and ADAR2 (catalytically active enzyme) but also by expressing human ADAR3 that is catalytically inactive and expressed mostly and specifically in the CNS. Nevertheless it seams obvious that the locomotor phenotype depends on the loss of editing activity of the enzyme; in fact the rescue is possible only by expressing the active form of dADAR meaning that the presence of *Drosophila* ADAR protein itself is not sufficient to rescue this phenotype.

Figure 1.6: amino acid sequence coordinating the of Inositol-1,2,3,4,5,6-hexakisphosphate (Ins P6) molecule within the deaminase domain of ADAR enzymes. As shown, the sequence is highly conserved throughout the evolution. The red boxes show the identical amino acids conserved within the catalytic domain of both hADAR2 and dADAR. The asterisks show the amino acids involved in the coordination of Inositol-1,2,3,4,5,6-hexakisphosphate (Ins P_6) molecule.

1.7 Characterization of the neurodegeneration affecting *Adar*^{5G1}-null flies

Adar^{5G1} mutants suffer from age-dependent vacuole formation in the mushroom body; (McGurk, 2007) the neurodegeneration is faster null-mutants compared to the hypomorphic allele, *Adar*^{1F4}. This neurodegeneration phenotype is dependent on the lack of *Adar* gene because it can be rescued by expressing the *Drosophila Adar* transgene (encoding either the active or inactive form of the protein) in the null-background (McGurk, 2007). The vacuolization reflects neuronal loss that is independent to apoptosis activation. In fact, the expression of *UAS-p35* transgene driven to cholinergic neurons does not suppress the neurodegenerative phenotype within mushroom body and retina of aged-*Adar*^{5G1} flies. P35 is an anti-viral protein that inhibits the protein cascade activating apoptosis-mediated cell-death. These data suggest that the neuronal loss must be driven through another cellular pathway and not by apoptosis activation.

Also electron microscopy images of *Adar*^{5G1} optic lamina, frontal brain and retina confirm the progressing degeneration of the mutant fly's retina. In fact large vacuoles among ommatidia disrupt the retina structure (Figure 1.7). Nevertheless, photoreceptors show large double-layer multilamellar vesicles containing cytosolic material such as mitochondria. These structures are been found not only in photoreceptors but also within neurons of the frontal part of the mutant fly's brain. This structure resembles autophagosomes that is one of the hallmarks of autophagy. Literally, autophagy refers to the ability of the cell to eat itself where "auto" means "itself" and "phagy" means "to eat". This is a housekeeping mechanism where cellular components that otherwise might become toxic are auto-digested. Hence damaged organelles and protein aggregates are engulfed in double membrane vesicles, known as autophagosomes, which fuse to lysosomes to form autophagolysosomes; the contents are then degraded and lipids, sugars and amino acids are retrieved. Autophagy is also used under starvation condition when there is an increased demand of energy, amino acids or lipids leading to

breakdown of internal components to support cell metabolism. This process is tightly regulated through the TOR signaling pathway and it is a crucial event especially in quiescent cells in order to avoid the accumulation of protein aggregates and damaged organelles that are not diluted by cell division. Considering that neurons are quiescent cells it is not surprising that TOR and autophagy have been linked to several neurodegenerative diseases characterized by accumulation of insoluble protein aggregates. In fact rapamycin treatment clears accumulated misfolded protein by blocking TOR activity and inducing autophagy activation thereby reducing the pathology hallmark; this has been demonstrated in mouse models of Alzheimer's and Huntington's diseases (Ravikumar *et al.*, 2004; Spilman *et al.*, 2010). Also *Drosophila* has proven itself to be a good genetic model for these two diseases, in fact it was demonstrated in *Drosophila* that cell-autonomous activation of autophagy that is mediated by suppression of TOR activity clears toxic polyQ-containing protein aggregates and insoluble tau protein accumulation which can lead to neuronal degeneration associated with the aging processes (Berger *et al.*, 2006; Wang *et al.*, 2009). Unlikely *Drosophila* models of Alzheimer, Parkinson or Huntington's disease, $Adar^{5G1}$ mutant fly brain do not show any insoluble protein aggregates by EM images. Therefore the stress that causes the neurodegeneration observed in $Adar^{5G1}$ animals is different from what is observed in proteinopathies. Therefore, $Adar^{5G1}$ neuronal loss might be dependent to a soluble stress, such as the case of *Phospholipase C* (*norpA*)-mediated retinal degeneration (Wang *et al.*, 2009). In the case of *Phospholipase C* mutant flies, the degeneration does not involve the formation of protein aggregates but the formation of toxic rhodopsin-arrestin complexes after phototransduction signal (Alloway *et al.*, 2000; Kiselev *et al.*, 2000; Iakhine *et al.*, 2004). The toxic rhodopsin-arrestin complexes are completely soluble and therefore not visible by electron microscopy. However EM images of *Phospholipase C*-mutant fly brain show the progressing activation of autophagy and the accumulation of autophagosome structures (Wang *et al.*, 2009). This means that TOR signaling can increase autophagy, which removes toxic soluble protein complexes thereby reducing the cellular toxicity also of un-aggregated proteins.

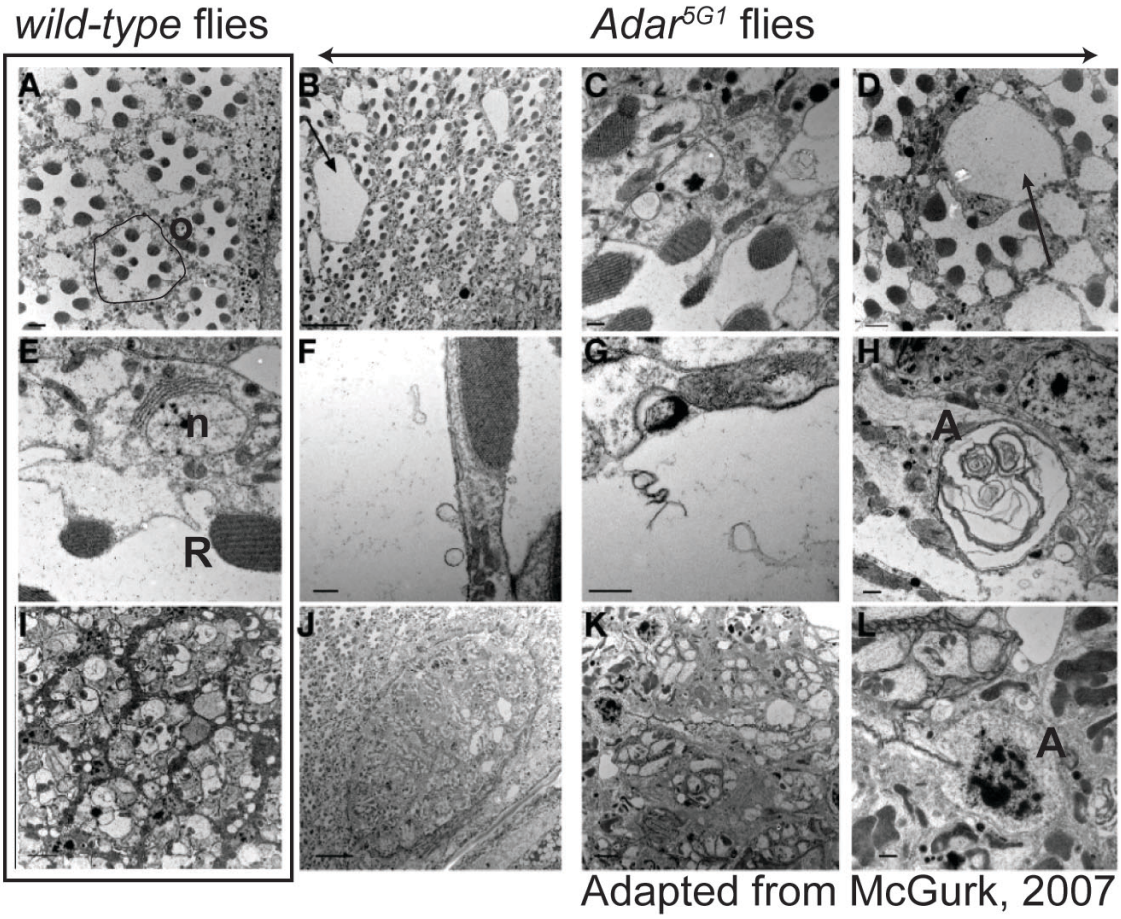


Figure 1.7: Electron microscopic analysis of photoreceptor neurons of *Adar*^{5G1}-null mutant flies. (A, E and I) Morphology of ommatidia (O) in wild-type flies at 30 days is normal. Sections of ommatidia were analyzed at a level at which seven of eight photoreceptor neurons are seen (A and E) and at the base of the photoreceptor neurons (I). n in E indicates nucleus: R, rhabdomere. B, C and D show ommatidia of *Adar*^{5G1}-null flies were large vacuole between photoreceptors are observed (black arrows). F, G and H show intracellular structures of ommatidia of *Adar*^{5G1}-null flies. Abnormal vesicles have been observed (F and G). A in H indicates autophagosome-like structure characterized by a double layer membrane surrounding cytosolic material. The same structure has been observed also in L that shows a section at the base of ommatidia of *Adar*^{5G1}-null flies together with J and K.

1.8 Autophagy and the *Drosophila* model of human neurodegenerative disease

Autophagy has also been linked with neuronal differentiation and homeostasis. For example, a decrease in autophagy causes a delay in neuronal differentiation, while a decrease in TOR activity through rapamycin administration causes neurite outgrowth (Zeng and Zhou, 2008). Impairments in autophagy activation or the misregulation of either the expression or the activity of proteins involved in this process have been linked with neurodegenerative disorders.

TOR is a negative regulator of autophagy activation and interestingly its expression is increased in the brain of patients affected by Alzheimer's disease, AD (Caccamo *et al.*, 2010; Spilman *et al.*, 2010). Indeed a down-regulation of TOR activity by drug administration ameliorates $A\beta_{1-42}$ aggregate levels and tau pathology that are the main hallmarks of AD. $A\beta_{1-40}$ and $A\beta_{1-42}$ are the cleavage products of amyloid precursor protein (APP) generated by β- and γ-secretases. Early $A\beta_{1-42}$ aggregates are degraded by autophagosomes (Gouras *et al.*, 2005). At late stages of AD, $A\beta_{1-40}$ and $A\beta_{1-42}$ are also secreted, forming extra-cellular aggregates. Alzheimer's disease has been modelled in *Drosophila melanogaster* where it has been shown that autophagy is activated in response to Aβ accumulation (Finelli *et al.*, 2004). However $A\beta_{1-42}$ expression increases, leading to an age-dependent impairment of autophagy activation, extensive neuronal damage and death (Ling *et al.*, 2009; Ling and Salvaterra, 2011). AD is classified as a taupathy as it has an abnormal accumulation of hyper-phosphorylated tau, a microtubule-associated protein (Lee *et al.*, 2001). Mutant tau induces neurodegeneration in the *Drosophila* model of AD that is mediated by an up-regulation of cell-cycle progression (Khurana *et al.*, 2006).

TOR activation enhances the degenerative phenotype in a cell-cycle dependent manner. Modifications in the TOR-signalling pathway have been linked to Huntington's disease, HD (Ravikumar *et al.*, 2004). HD is characterised by accumulation of an extended poly-

glutamine (Q)-tract contained in huntingtin protein, htt. Htt is also essential for fast axonal trafficking and the mutated isoform of the protein, mhtt, has been associated with impaired transport of vesicles and mitochondria along axon (Gunawardena *et al.*, 2003; Trushina *et al.*, 2004). Dynein is a minus end–directed microtubule motor that moves cargo from the distal ends of axons toward neuronal cell bodies. Interestingly it has also been shown that mhtt-containing aggregates include components of the trafficking machinery. Furthermore dynein mutations interfere with autophagy-mediated mhtt-clearance (Ravikumar *et al.*, 2005) as dynein is also involved in the transport of autophagosomes to lysosomes via microtubules. Mutations in the dynein gene are also found in one of familial cases of amytrophic lateral sclerosis (fALS) (Munch *et al.*, 2004).

ALS is a neurodegenerative disease affecting lower and upper motor neurons that leads to respiratory failure and fatality. Post-mortem spinal cords of ALS patients and spinal cords of a mouse genetic model of ALS disease have shown an increased number of cytosolic vesicles that are morphologically similar to autophagosomes (Morimoto *et al.*, 2007; Li *et al.*, 2008; Hetz *et al.*, 2009). Only 5-10% of ALS cases are familial; and are due to a mutation in a specific gene such as superoxide dismutase 1 (SOD1), Vesicle-associated membrane protein-associated protein B/C (VAMP-B/C), TAR DNA binding protein 43 (TDP 43), or Fused-In-Sarcoma (FUS) protein (Chai *et al.*, 2008; Watson *et al.*, 2008; Estes *et al.*, 2011; Lanson *et al.*, 2011). The majority of ALS cases are sporadic and they do not have a defined aetiology. Among all *Drosophila* models of ALS, the ALS-associated TDP-43 aggregation has been linked with autophagy by a genetic interaction between TDP-43 and Ubiquilin 1, a proteasome targeting factor that is also involved in AD etiology (Kim *et al.*, 2009; Hanson *et al.*, 2010).

1.9 Project outline

Adar-null flies show reduced viability and locomotion defects that are succeeded by age-dependent neurodegeneration (McGurk, 2007). By rescue experiments, the locomotor impairments and the neurodegeneration might be dependent on two different functions of the protein. In fact the walking phenotype rely on the enzymatic activity of the protein as for only the expression of the catalytically active form of the enzyme suppresses the aberrant phenotype. It is still unclear whether one unedited transcript or the accumulated effect of many unedited transcripts is responsible for the severe phenotypes observed in *Adar* mutants and also, which biological pathways are most severely affected. Different is the case of the neurodegeneration whereby the protein itself is able to rescue this phenotype without rescuing the editing events of the transcripts that are edited by ADAR. Little was known of the neurodegenerative phenotype affecting *Adar*-null flies when I have started my Ph.D. studies. As previously described, the neuronal loss affecting our mutant flies was not driven by the activation of apoptosis-programmed cell-death. Nevertheless the phenotype was not dependent on the loss of RNA editing function of the protein. Therefore the aim of my project was to (i) characterize the new role of the *Drosophila* ADAR protein, trying to explain why the inactive protein prevents or slow down the neurodegeneration affecting $Adar^{5G1}$ flies, and (ii) identify new modifiers that suppress the *Adar*-associated phenotypes. Therefore I performed a forward genetic screen crossing *Adar*-null flies with a set of well-characterized deletion lines called the *DrosDel* collection (Ryder *et al.*, 2007). The idea was to rescue the *Adar*-null dependent phenotypes without restoring the RNA editing events. Firstly I tried to rescue the viability that is the first obvious phenotype associated to the loss of *Adar* gene. This might give me an indication of whether the deletion that I was testing contained a gene or a combination of genes, which suppress the effect mediated by the lack of *Adar*. The effects of genetic interactors do not dependent on modifications catalyzed by ADAR activity or the regulation of *Adar* expression levels as for $Adar^{5G1}$ allele does not express any detectable level of *Adar* transcripts (Palladino, Keegan, *et al.,* 2000) or ADAR protein (McGurk, 2007). After the identification of new modifiers of

the reduced *Adar*^{5G1} viability phenotype, I investigated whether the same modifiers could also suppress the locomotor impairments and/or the neurodegenerative phenotype. The activity of mutant flies was analyzed with a two-minute open-field locomotion assay, whereas neurodegeneration was assayed by histology techniques.

The second aim of my PhD was to elucidate which is the cellular stress that arises from the lack of *Adar*. This would facilitate in the understanding the function of ADAR and its role in *Drosophila* physiology beyond its RNA editing activity. Therefore I tested whether there was an indication of three major cellular stresses: (i) modification of intracellular calcium concentration (ii) an increase in reactive oxygen species levels and (iii) unfolded protein response mediated by ER stress.

In summary the main aims of this PhD were:

1. Perform a forward genetic screen to identify new modifiers of *Adar*-null fly viability. The modifiers were then analyzed to determine if they could also rescue the locomotion defects and the neurodegeneration phenotypes of *Adar*^{5G1} flies.
2. Identify the cellular stress that arises from the lack of the *Adar* gene.

CHAPTER 2: Materials and Methods

2.1 *Drosophila* methods

2.1.1 Fly maintenance

All fly stocks were raised on standard corn meal-agar medium (made with water, yeast soy flour, yellow cornmeal, light malt extract, agar, light corn syrup and propionic acid) prepared by the technicians at the Michael Swann kitchen at Kings Building's, University of Edinburgh. Fly stocks were maintained at 18°C and crosses were performed at 25°C in tubes supplemented with yeast.

2.1.2 Fly strains

A complete list of *Drosophila* fly strains from the *DrosDel* collection used for the genetic screen is listed in Table 2.1. The whole *DrosDel* collection was obtained from Dr. Giuseppa Pennetta, at the University of Edinburgh. More information related to the collection is available at *DrosDel* website: www.drosdel.org.uk. *P*-Element-containing strains, listed in Table 2.2, were obtained from Bloomington Stock Centre; from there I also got the following strains: *UAS-S6K*^{KQ}, *UAS-Thor*, *UAS-Atg5-eGFP*. The *UAS-Atg1[GS10797]* strain was a kind gift from Prof. Thomas Neufeld, at the Masonic Cancer Center, University of Minnesota. *UAS-Xbp1-eGFP* and *UAS-NinaE*^{G69D} strains were a kind gift from Prof. Hermann Steller, The Rockefeller University, New York. The *UAS-Camgaroo-eGFP* strain was a kind gift of Prof. Ronald Davis, at the Baylor Collage of Medicine, Houston. The RNAi strains for complex I protein NaDH Dehydrogenase subunit 75, ND75, were obtained from VDRC Stock Center, Vienna.

Table 2.1: *DrosDel* strains used for the genome-wide forward genetic screen for suppressors of reduced *Adar*-null fly viability. Each of these strains carries a chromosomal deletion covering the left arm of the second chromosome (2L). The cytogenetic location of each deletion is annotated. Males coming from these strains were used in a genome-wide forward genetic screen for suppressors of reduced *Adar*^{5G1} viability.

Name	*Deletion genotype*	*Cytogenetic location of deletion*				*Bloomington*
		start	*band*	*end*	*band*	***numbers***
Df(2L)ED5878	w[1118]; Df(2L)ED5878, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5878/SM6a	67365	21B1	161120	21B3	9353
Df(2L)ED19	w[1118]; Df(2L)ED19, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED19/SM6a	159063	21B3	285763	21B7	8901
Df(2L)ED62	w[1118]; Df(2L)ED62, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED62/SM6a	480873	21D1	826788	21E2	8937
Df(2L)ED87	w[1118]; Df(2L)ED87, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED87/SM6a	568095	21E2	852827	21E2	8677
Df(2L)ED94	w[1118]; Df(2L)ED94, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED94/SM6a	568095	21E2	1036969	21E2	8908
Df(2L)ED108	w[1118]; Df(2L)ED108, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED108/SM6a	1119134	21F1	1420528	22A1	24629
Df(2L)ED123	w[1118]; Df(2L)ED123, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED123/SM6a	1985930	22B8	2222091	22D4	8943
Df(2L)ED136	w[1118]; Df(2L)ED136, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED136/SM6a	2492935	22F4	2753125	23A3	9176
Df(2L)ED501	w[1118]; Df(2L)ED501, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED501/SM6a	7423926	27F7	7800182	28C4	24128
Df(2L)ED508	w[1118]; Df(2L)ED508, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED508/SM6a	7576630	28B1	7800182	28C4	8944
Df(2L)ED647	w[1118]; Df(2L)ED647, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED647/SM6a	8543972	29E1	8959148	29F5	8678
Df(2L)ED690	w[1118]; Df(2L)ED690, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED690/SM6a	9437469	30B3	9918174	30E4	24133
Df(2L)ED692	Df(2L)ED692/SM6a	9571188	30B12	9918174	30E4	150393
Df(2L)ED697	w[1118]; Df(2L)ED697, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED697/SM6a	9616844	30C1	9918192	30E4	150093
Df(2L)ED695	w[1118]; Df(2L)ED695, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED695/SM6a	9699225	30C5	9918192	30E4	8041
Df(2L)ED701	Df(2L)ED701/SM6a	9699225	30C5	9948344	30F1	150094

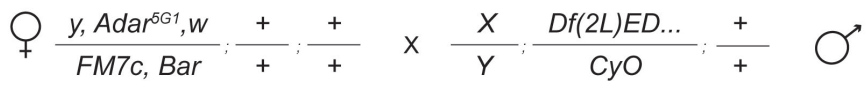
Name	*Deletion genotype*	*Cytogenetic location*				*Bloomington numbers*
		start	*band*	*end*	*band*	
Df(2L)ED700	w[1118]; Df(2L)ED700, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED700/SM6a	9897524	30E4	9918192	30E4	8042
Df(2L)ED737	Df(2L)ED737/SM6a	10220877	31B1	10321975	31D7	150520
Df(2L)ED761	w[1118]; Df(2L)ED761, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED761/SM6a	11808835	33A2	12436439	33E5	24109
Df(2L)ED778	w[1118]; Df(2L)ED778, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED778/SM6a	12545800	33E9	13165545	34A7	7420
Df(2L)ED784	w[1118]; Df(2L)ED784, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED784/SM6a	13004448	34A4	13332060	34B6	7421
Df(2L)ED3	w[1118]; Df(2L)ED3, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}CG2316[ED3]/SM6a	14490575	35B2	15333760	35D1	6963
Df(2L)ED800	w[1118]; Df(2L)ED800, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED800/SM6a	14490575	35B2	15332688	35D1	9192
Df(2L)ED1102	w[1118]; Df(2L)ED1102, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED1102/SM6a	16350236	35F12	16684883	36A10	24113
Df(2L)ED1158	Df(2L)ED1158/SM6a	16791519	36B1	17450371	36C9	150106
Df(2L)ED1165	Df(2L)ED1165/SM6a	17112846	36C1	17473293	36C9	150107
Df(2L)ED1315	w[1118]; Df(2L)ED1315, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED1315/SM6a	20085397	38B4	20917519	38F5	9269
Df(2L)ED1317	w[1118]; Df(2L)ED1317, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED1317/SM6a	20638580	38D1	20917519	38F5	9175
Df(2L)ED1384	Df(2L)ED1384/SM6a	20922881	38F5	21397328	39D2	150120
Df(2L)ED1455	Df(2L)ED1455/SM6a	21052126	39A1	21657677	39E6	150509
Df(2L)Exel7794	w[1118]; Df(2L)Exel7022/CyO	14490575	35B2	15068040	35C1	7794
Df(2R)ED4061	w[1118]; Df(2R)ED4061, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED4061/SM6a	20290110	60C8	20560724	60D13	9068
Df(2L)ED2809	w[1118]; Df(2L)ED2809, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED2809/SM6a	67365	21B1	72671	21B1	9180
Df(3L)ED4978	w[1118];Df(3L)ED4978,P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED4978/TM6C,cu[1]Sb[1]	15333599	35D1	1533766	35D1	8101

BLOOMINGTON NUMBER	GENOTYPE	GENE AFFECTED name	number
22878	y1 w67c23; Mi{ET1}CG5142MB00716	Ser-Thr specific kinase	CG5142
20794	y1 w67c23; P{EPgy2}A16EY14223	A16, chromatin binding protein	CG9933
23044	y1 w67c23; Mi{ET1}CG9932MB01281	metallo peptidase	CG9932
28402	y1 w*; P{EP}CG9934G13471	ubiquitin protein ligase	CG9934
10013	w1118; PBac{PB}CG9932c00144/CyO	metallo peptidase	CG9932
11218	y1 w67c23; P{lacW}Tork17004/CyO	TOR	CG5092
25363	w1118; Mi{ET1}TorMB07988	TOR	CG5092

Table 2.2: List of P-element-containing strains for those genes that are present within the overlapping region of *Df(2L)*ED778 and *Df(2L)*ED784 deleted regions. The expression of the gene was reduced by a *P*-element insertion. The strain numbered 23044 and 10013 show a reduced expression of *CG9932* gene.

2.1.3 Genetic screen and ageing experiment

y, Adar^{5G1}*, w / FM7, Bar ;* $^{+}/_{+}$*;* $^{+}/_{+}$ virgins were collected at 18°C. 10 females were crossed with 7 $^{X}/_{Y}$*; Df(2L)/CyO;* $^{+}/_{+}$ male flies. The genetic scheme is shown in Figure 2.1. The crosses were performed at 25°C in vials supplemented with yeast. Each single cross was tip daily for 10 days and all the vials kept. The F_1 generation was collected daily for 20 days. The genotypes were recognised phenotypically by eye and wing shape and annotated. In fact, F1 males have four possible genotypes (Figure 2.1 highlighted in red). For aging experiment one hundred *y,Adar*^{5G1}*,w /Y* male flies were collected and maintained individually in vials not supplemented with yeast at 18°C for 30 days; each vial was tipped on daily. Ten *y, Adar*^{5G1}*, w / FM7, Bar ;* $^{+}/_{+}$*;* $^{+}/_{+}$ virgins were crossed with seven *y[1]w[67c23];P{w[+mC]=lacW}Tor[k17004]/CyO* (Referred to as *Tor*^{k17004}) or seven with *w[1118]; Mi{ET1}Tor[MB07988]* (Referred to as *Tor*^{MB07988}) male flies. From those two crosses, one hundred male flies with the following genotype *y,Adar*^{5G1}*,w; Tor*^{k17004} and *y,Adar*^{5G1}*,w; Tor*^{MB07988} were also collected and used for aging experiments. As controls one hundred male flies with the following genotypes *FM7c, FM7c;Tor*^{k17004} or *FM7c; Tor*^{MB07988} were used.



	X ; Df(2L)ED...	Y ; CyO
y, Adar^{5G1}, w ; +	X / y, Adar^{5G1}, w ; Df(2L)ED... / +	Y / y, Adar^{5G1}, w ; CyO / +
FM7c, Bar ; +	X / FM7c, Bar ; Df(2L)ED... / +	Y / FM7c, Bar ; CyO / +

	X ; CyO	Y ; Df(2L)ED...
y, Adar^{5G1}, w ; +	X / y, Adar^{5G1}, w ; CyO / +	Y / y, Adar^{5G1}, w ; Df(2L)ED.. / +
FM7c, Bar ; +	X / FM7c, Bar ; CyO / +	Y / FM7c, Bar ; Df(2L)ED... / +

Figure 2.1: Scheme of the genetic crosses used for the genome-wide forward genetic screen for suppressor of reduced *Adar*^{5G1} fly viability. *y, Adar*^{5G1}*, w / FM7c* females were crossed with males from different strains of the *DrosDel* collection. Those males carry a chromosomal deletion within the second chromosome (*X / Y ; Df(2L)ED.. /* +). Each F1 male fly was collected (red boxes) and its genotype was recognised phenotypically and counted.

2.1.4 Open field locomotor assay

The locomotion activity of 7-days-old male flies was measured by 2-minutes open-field locomotor assay. A 60-mm plate was divided into 9 different areas with approximately the same surface area. The number of times a fly moved to different areas within 2 minutes was counted. The flies tested were the following: w^{1118}, *y,Adar*^{5G1}*,w,* *y,Adar*^{5G1}*,w;Tor*^{k17004} , *y,Adar*^{5G1}*,w;Tor*^{MB07988} , *y,Adar*^{5G1}*,w;Cha>UAS-Atg1[GS10797]* and *y,Adar*^{5G1}*,w;Cha>UAS-Atg5*. The genotypes of flies were checked by CO_2 anaesthesia and the test performed the day after during morning hours. The test was repeated three times for each mutant and then averaged. More than 10 flies for each genotype of interest were used. The average locomotion for each genotype was plotted in Microsoft Excel and the standard error was calculated.

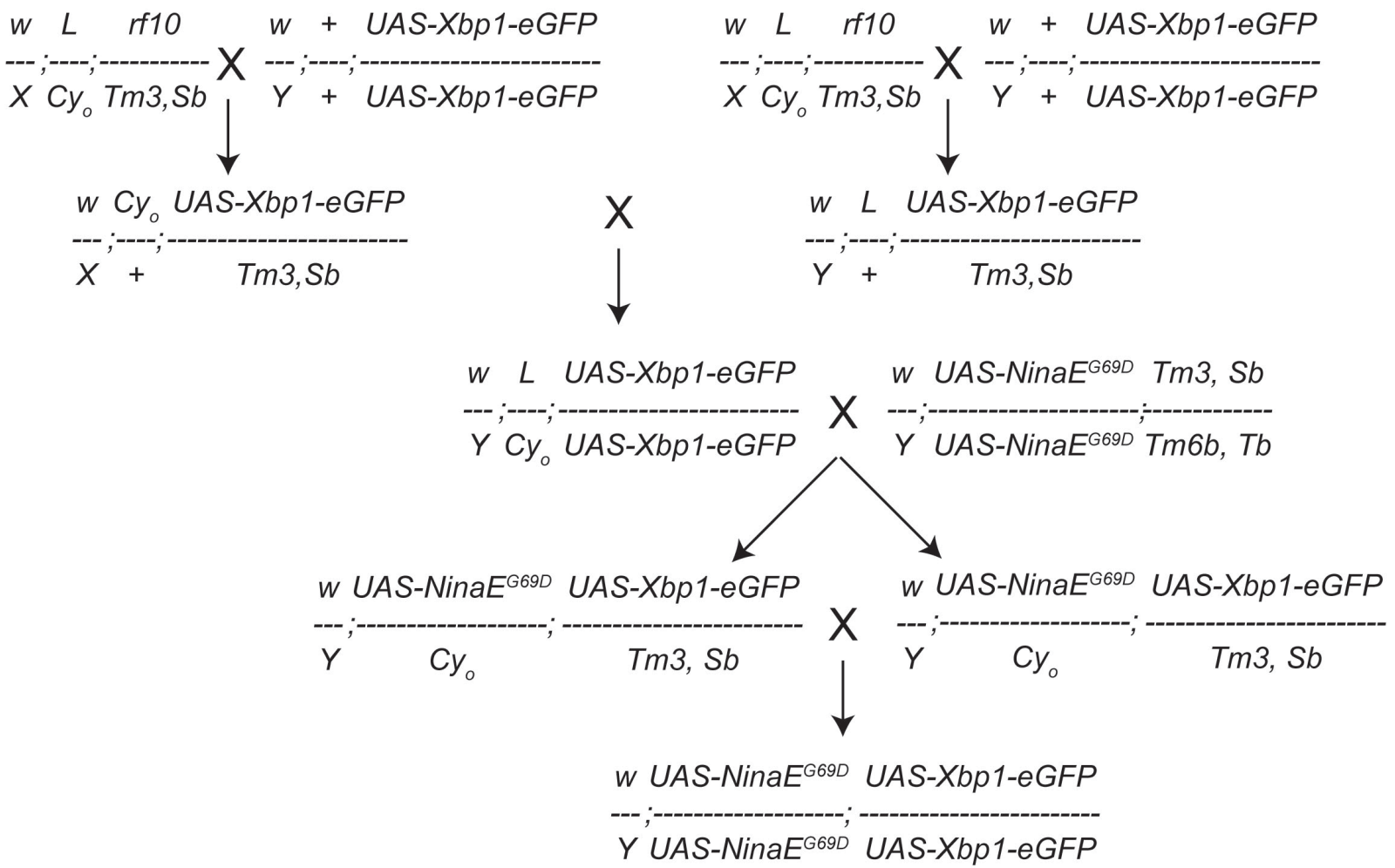
2.1.5 Fly movie

To confirm the locomotion data of the open-field assay, *Adar*^{5G1} mutant flies and the rescued flies, *Adar*^{5G1}*; Tor* ^{MB07988} were recorded while walking. Videos were taken with the NIKON AZ100 macroscope and QImaging-RETIGA EXi camera. The sequence of images was analyzed with IPLab software and exported to QuickTime with a speed of 15 Frames/Second.

2.1.6 Creation of the transgenic line w; UAS-NinaE^{G69D}; UAS-Xbp1-eGFP

The genetic crosses to create *w; UAS-NinaE*^{G69D}*; UAS-Xbp1-eGFP* transgenic fly strain are shown in Figure 2.2. *y,w; UAS-ninaE*^{G69D}*/CyO; Sb/Tm6b* and *w; +/+; UAS-Xbp1-eGFP/UAS-Xbp1-eGFP* were gift from Prof. Hermann Steller.

Figure 2.2: The crossing scheme to generate *UAS-NinaE*^{G69D}*; UAS-XBP1-eGFP* transgenic line. The balancer of the first chromosome is *w*, *white minus* that gives white eyes phenotype. On the second chromosome the balancers are: (i) *L*: gives smaller eyes (ii) *CyO*, *Curly* that gives curly wings. The balancers for the third chromosome are: (i) *Tm3, Sb, Stubble* that results in short and thick thoracic bristles. (ii) *Tm6b, Tb: Humeral* and *Tubby* have shorter and fatter larvae, pupae and adults with additional macrochaetas on humeri and (iii) *Rf (10), Ebony* that gives darker pigmentation in the thorax.


w L rf10 / ---;----;---------- / X Cy_o Tm3,Sb
X
w + UAS-Xbp1-eGFP / ---;----;---------- / Y + UAS-Xbp1-eGFP
w L rf10 / ---;----;---------- / X Cy_o Tm3,Sb
X
w + UAS-Xbp1-eGFP / ---;----;---------- / Y + UAS-Xbp1-eGFP
w Cy_o UAS-Xbp1-eGFP / ---;----;---------- / X + Tm3,Sb
X
w L UAS-Xbp1-eGFP / ---;----;---------- / Y + Tm3,Sb
w L UAS-Xbp1-eGFP / ---;----;---------- / Y Cy_o UAS-Xbp1-eGFP
X
w UAS-NinaE^G69D Tm3, Sb / ---;----------;---------- / Y UAS-NinaE^G69D Tm6b, Tb
w UAS-NinaE^G69D UAS-Xbp1-eGFP / ---;----------; ---------- / Y Cy_o Tm3, Sb
X
w UAS-NinaE^G69D UAS-Xbp1-eGFP / ---;----------; ---------- / Y Cy_o Tm3, Sb
w UAS-NinaE^G69D UAS-Xbp1-eGFP / ---;----------; ---------- / Y UAS-NinaE^G69D UAS-Xbp1-eGFP

2.2 Histology techniques

2.2.1 Wax embedding

The neurodegeneration of 30-days-old w^{1118}, $y,Adar^{5G1},w,$ $y,Adar^{5G1},w;Tor^{k17004}$, $y,Adar^{5G1},w;Tor^{MB07988}$ and *y,Adar*^{5G1}*,w;Cha>UAS-Atg5* male flies was analysed by haematoxylin and eosin (H&E) staining. Flies' heads were dissected and fixed with Carnoy's fixative (6:3:1, ethanol:chloroform:acetic acid) for 8 hours by rotating at room temperature. The samples were re-hydrated by sequentially washes of 30 minutes with 60%, 70%, 80%, 90% and 100% ethanol at room temperature. The next steps were performed in a Sakura Tissue Tek VIP tissue processor (Sakura). The ethanol was cleared with two washes of xylene at 35°C followed by three more washes at 60°C. Before embedding the samples, they were washed 3 times with paraffin-wax at 50°C. Thus, the tissues were embedded in a paraffin-wax block for cutting. 5 micron sections of fly heads were cut with a Leica microtome with the ACCU-edge low profile blades (Sakura). Sections were floated out in a 42°C water bath and attached to Superfrost plus slides (VWI international) and baked at 50°C overnight.

2.2.2 Haematoxylin and Eosin (H&E) staining

The paraffin-wax was removed from the sections by washing the slides with xylene, 3 times for 5 minutes. They were re-hydrated by 3 changes of 100% ethanol (5 minutes each) followed by one 2 minutes change in each 90%, 70%, 50%, and 30% ethanol and finally washed for a few minutes in water. After that, the slides were stained with haematoxylin for 3 minute and differentiated in acid/alcohol (1% chloridric acid in 70% ethanol) for few seconds. Before stained them in eosin (3:1, aqueous Eosin:alcohol, and 0.05% acetic acid) for 2 minutes, they were well washed in running tap water for at least 5 minutes. After a quick rinse in water and in 100% ethanol, the slides were placed in absolute alcohol for 1 minute. Another three changes of 100% ethanol of 5 minutes each were then performed followed by another three washes with xylene. The slides were subsequently mounted in Depex mounting medium (EMS).

2.2.3 Microscopy

Images were captured with Zeiss Axioplan 2 - compound microscope, which comprised of a Coolsnap HQ CCD camera (Photometrics Ltd, Tucson, AZ) with Plan-neofluar objectives (Carl Zeiss, Welwyn Garden City, UK). Images were captured with a neofluar objectives at 40X (with a numerical aperture of 1.3) and at 63X (with a numerical aperture of 1.25). Image capture and analysis were performed using an in-house scripts written for IPLab Spectrum (Scanalytics Corp, Fairfax, VA). The brightness and contrast were altered with the advanced histogram section in IP Lab Spectrum. This was done by manually setting the minimum and maximum pixel intensities on the histogram.

2.3 Immunohystochemistry

2.3.1 Lysotracker staining

The lysotracker probe (LysoTracker® Red DND-99, Molecular Probes, Invitrogen) was used to assess autophagy in live cells. It is a fluorescent acidotropic dye for labelling and tracking acidic organelles. The autophagy level was detected in *Drosophila* larval brains and fat body. These tissues were dissected in cold PBS and then incubated with LysoTracker® Red DND-99 (1 µl of dye in 10ml of cold PBS) for 5 minutes in ice. After 5 washes (2 minutes each) in PBS, the tissue was mounted in Vectashield DAPI and viewed with a fluorescent microscope, Zeiss Axioplan 2. Lysotracker dye was used to stain 10 larval brains of w^{1118}, *y,*$Adar^{5G1}$*,w* and *y,*$Adar^{5G1}$*,w;*Tor^{k17004} and larval fat body of w^{1118}, $Adar^{5G1}$*;Cg-GAL4>,* $Adar^{5G1}$*;Cg-GAL4>UAS-Adar3/4* and $Adar^{5G1}$*;Cg-GAL4>UAS-Adar3/4E374A.*

2.3.2 2'7'-dichlorofluorescein (H2DCF) and Dihydroethidium (DHE) staining

One of the techniques used for detecting intracellular hydrogen peroxide depends on oxidation of the non-fluorescent substrate 2',7'-dichlorofluorescein (H2DCF) to a green fluorescent product. Dihydroethidium (DHE), by virtue of its ability to freely permeate cell membranes is used extensively to monitor superoxide production (Owusu-Ansah, 2008). Therefore *y,Adar^{5G1},w/Y;Collagen-GAL4* and control (*X/Y;Collagen-GAL4>UAS-ND75i/+*) larval fat body was dissected and stained with 10μM of H2DCF in cold PBS for 10 minutes in a dark chamber at room temperature followed by three 5-minute washes in PBS. In the case of DHE dye, the tissue was dissected in Schneider's insect medium. After staining with DHE dye at the final concentration of 30μM for 5 minutes in a dark chamber, the tissue was washed 3 times with PBS. The tissue was then mounted in Vectashield DAPI and viewed with a fluorescent microscope, Zeiss Axioplan 2**.**

2.3.3 Fluo-4 AM staining

Fluo-4AM, Molecular Probes, (Invitrogen) is one of many fluorescent calcium indicators now available because of the crucial role of calcium within cells. The most important properties of calcium indicators are an absorption spectrum compatible with excitation at 488 nm by argon-ion laser sources and a very large fluorescence intensity that increases in response to Ca^{2+} binding, typically more than 100-fold. Fluo-4, AM is a cell-permeable acetoxymethyl (AM) esters (Figure 2.3B). Fluo-4 is an analog of fluo-3 with the two chlorine substituents replaced by fluorines, resulting in increased fluorescence excitation at 488 nm and consequently higher signal levels for confocal microscopy, flow cytometry and microplate screening applications. The intracellular calcium concentration was detected in live fat cells dissected from *y,Adar^{5G1},w^{-} /Y ;Collagen-GAL4 Drosophila* larvae in cold PBS and stained with 0.4 μM) and viewed directly under a fluorescent microscope.

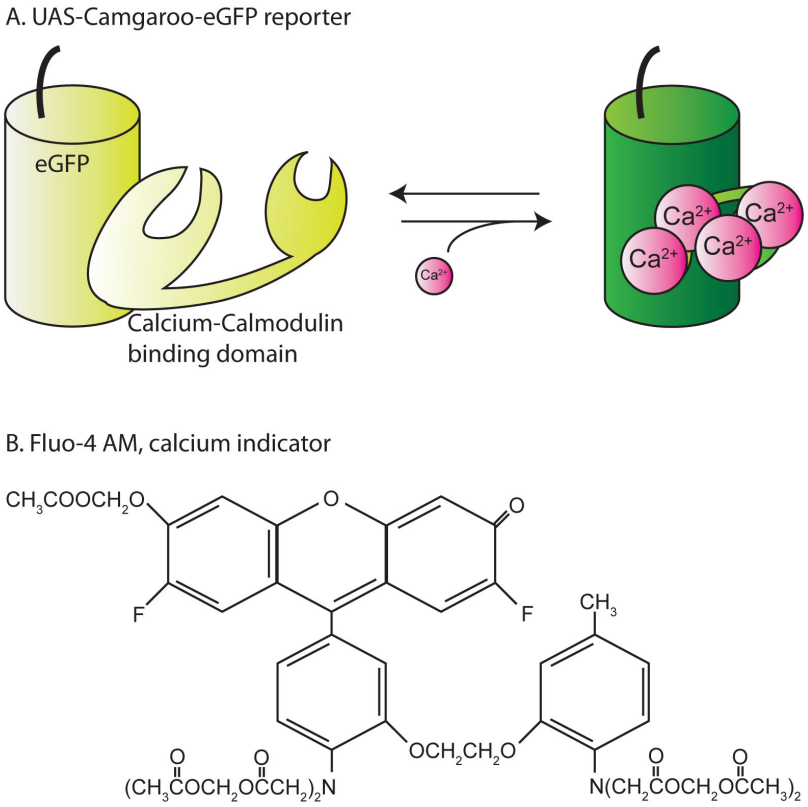


Figure 2.3: Detection methods to investigate variation in intracellular calcium ions concentration. Two methods were used to investigate whether *Adar*-null fat body have a variation in the level of free-calcium ions. The first method took advantage of transgenic flies with the *UAS-Camgaroo-eGFP* reporter (A). The eGFP sequence has been fused with Ca-Calmodulin binding domain sequence. Therefore upon binding of four free calcium ions the ca-calmodulin domain changes its conformation thereby enhancing the eGFP signal. Fluo-4 AM (Invitrogen) is the second method used to detect variation in the level of free calcium and its chemical structure is shown (B).

2.3.4 GFP-antibody staining

The GFP-antibody immunostaining was used to increase the probability of detecting any signals of GFP-fused transgene expressed in *Adar*^{5G1} fat cells using *Collagen-GAL4* driver. Shorthly fat body was dissected in cold PBS and fixed with Bouin's fixative (SIGMA) for 10 minutes at room temperature. The tissue was then blocked for 2 hours at room temperature with a 1:10 dilution of normal goat serum, NGS, in PBT (PBS + 0.1% Triton X100), incubated overnight at 4°C with 1 μg/mL mouse anti-GFP primary antibody (Roche Applied Sciences), washed and detected with Alexa-coupled goat anti-mouse IgG secondary antibody.

In one case, the GFP-immunostaining was used to enhace the signal of *UAS-Camgaroo-eGFP* reporter that detects increased level of intracellular free calcium ions (Figure 2.3A). The transgene contains a *UAS* sequence followed by the sequence of calcium-binding domain of Calmodulin fused to enhanced-GFP; the calcium binding leads to increased and enhanced GFP fluorescence. Therefore, *y,Adar*^{5G1}*,w*$^{-}$ */ Fm7,P{GAL4-Kr.C}DC3,P{UAS-GFP.S65T}DC7;Collagen-GAL4/Collagen-GAL4;+/+* virgins were crossed with males carrying the *UAS-Camgaroo* report inserted on the second chromosome, *X/Y;UAS-Camgaroo-2/CyO;+/+*. The GFP expression associated to the balancer, *Fm7,P{GAL4-Kr.C}DC3,P{UAS-GFP.S65T}DC7*, and the GFP associated with the reporter were distinguished by analyzing the different expression pattern. In fact the *Krupple* promoter drives *UAS-GFP* expression uniquely within the imaginal disc of first instar larvae; in contrast the expression of the reporter is driven within the fat cells.

Also the signal of the *UAS-Xbp1-EGFP* reporter was enhanced by immunostaining. *UAS-Xbp1-eGFP* reporter detects a short-term response of the endoplamic reticulum (ER) reacting to unfolded protein stress (Ryoo *et al.*, 2007). The sequence of enhanced GFP was inserted at the 3' end of the putative *Ire-1* splice site of *Xbp1*, deleting the C-terminus region if XBP1 protein (Figure 2.4). IRE-1 is an ER stress-responsive element that mediates the splicing of *Xbp1* triggering the unfolded protein response

(Plongthongkum *et al.*, 2007). EGFP is out of frame without IRE-mediated splicing, but is in frame after splicing. For that reason, in the presence of ER stress, EGFP signal can be detected by fluorescent microscopy and the signal detection can be enhanced by immuno-staining of the tissue of interest with GFP antibody. Hence, *y,Adar*^{5G1}*,w*$^{-}$ */ Fm7,P{GAL4-Kr.C}DC3,P{UAS-GFP.S65T}DC7;Collagen-GAL4/Collagen-GAL4;+/+* virgins were crossed with males that had the *UAS-Xbp1-eGFP* construct inserted on the third chromosome. *UAS-ninaE*^{G69D}*;UAS-Xbp1-eGFP* transgene was expressed within fat cells taking advantage of *Collagen-GAL4* driver and this was used as a positive control.

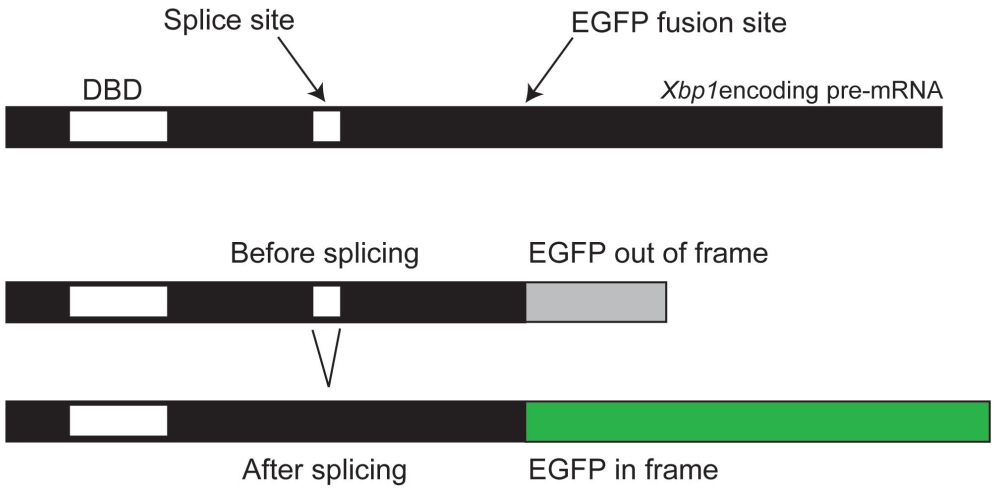


Figure 2.4: *UAS-Xbp1-eGFP* reporter. The *Xbp1*-encoding pre-mRNA contains the DNA binding domain, DBD, and intron that is removed in response to an increased misfolded-protein level by the nuclease activity of IRE-1. The region where eGFP was inserted is also shown. DBD: DNA binding domain.

CHAPTER 3: Genetic screen for suppressors of reduced *Adar*^{5G1} fly viability

3.1 Introduction

Drosophila melanogaster has only one *Adar* gene that encodes for two different ADAR isoforms, ADAR3/4 and ADAR3a. It lies on the X chromosome. The $Adar^{5G1}$ allele was generated by the mobilization of a promoter *P*-element and it is balanced using the *FM7c* balancer gene, which gives a dominant *Bar* eye phenotype in hemizygotes (Palladino *et al.*, 2000a). It is a null allele. In fact neither transcript nor protein levels have been detected in those mutant animals. The chromosomal deletion of 36.5 kb covers the *Adar* gene and another gene downstream to *Adar*, *CG38206* that is not involved in the *Adar* phenotype as *Adar* transgene expression can rescue the $Adar^{5G1}$ phenotype (McGurk, 2007). Those mutant flies have a reduced viability; it is not clear when the lethal phase occurs: preliminary data suggest this happens after the larval stage, during metamorphosis. In fact, no lethality in animals that carry $Adar^{5G1}$ allele has been observed until the end of the third-instar larval stage. This observation was possible by taking advantage of *y,* $Adar^{5G1}$*,w / FM7c , P{GAL4-Kr.C}DC3,P{UAS-GFP.S65T}DC7;* $^{+}/_{+}$*;* $^{+}/_{+}$ strain. In this case, the *FM7c* balancer chromosome is marked with green fluorescent protein (GFP) and can be used to balance the $Adar^{5G1}$ allele; GFP expression is driven indirectly by a *Kruppel* (*Kr*) promoter, via the yeast GAL4-UAS regulatory system. GFP fluorescence can be seen in embryos as early as the germ band extension stage, and can also be seen in larvae, pupae, and adults. Therefore the balancer can be used to identify hemizygous male $Adar^{5G1}$ progeny. It is still unclear whether mutant animals die during metamorphosis or whether the lethality occurs all through larval development with an increase at pupation.

The initial aim of the following study was to identify new modifiers that suppress the reduced viability associated with the $Adar^{5G1}$ allele. One hypothesis is that the lack of ADAR causes a constitutive stress with cumulative effects that induces progressive degeneration and locomotion phenotypes; this might reflect also the reduced viability, albeit the stress has not been identified yet. However there might be two different stressed that lead to the locomotive impairments rather than the neurodegeneration. This

idea comes from the fact that the inactive protein is able to rescue only the neurodegenerative phenotype. Therefore the presence of the protein itself might alleviate the stress caused by the lack of *Adar*. Different is the case of the walking impairments: In fact it is the lack of the RNA editing events on the pre-mRNA encoding edited proteins to generate this aberrant phenotype.

In order to elucidate the stress affecting $Adar^{5G1}$-null flies and leading to the aberrant phenotypes, I performed a forward genetic screen using a set of well-characterized deletion lines called the *DrosDel* collection (Ryder *et al.*, 2007). The *DrosDel* collection has been generated to perform genome-wide genetic interaction screens; the core deficiency kit is composed of 270 genetically heterogeneous deletions covering approximately 92% of the *Drosophila* genome. Deficiencies covering the left arm of the 2nd chromosome (2L) were used to screen for suppressors of reduced $Adar^{5G1}$ viability. Initially, 35 deficiency strains from the *DrosDel* collection (Ryder *et al.*, 2007) which cover approximately 70% of the left arm of the second chromosome (2L) (Figure 3.1)

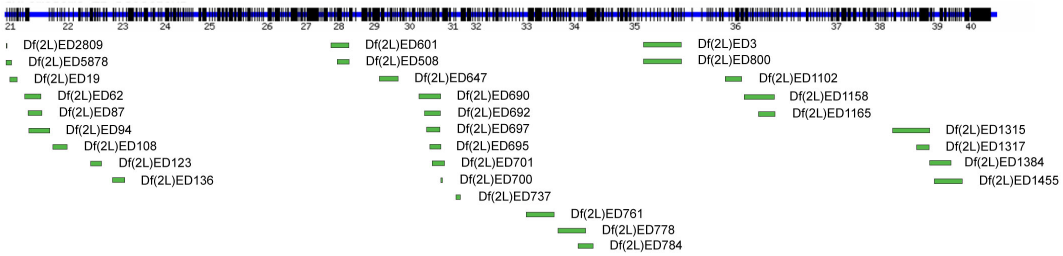


Figure 3.1: Chromosome 2L coverage map using the *DrosDel* collection strains available. The blue bar represents the left arm of the second chromosome. The black lines represent the chromosome band. Each deletion is illustrated by the green bar that represents the size and region deleted. All the available strains are included in the charter with their reference names.

Crosses were transferred to a new culture vial every day to prevent competition between *Adar*^{5G1} mutant larvae and their non-mutant siblings. Locomotion defects related to the lack of the RNA editing enzyme, ADAR, may start during early development. The reduction of *Adar*^{5G1} fly viability may be dependent on problems with locomotion and as a consequence a reduced number of larvae are able to come out from the food and complete their development. Thus, the expected ratio of adult flies emerging can be quantified, and assessed for a suppressive effect on the reduced viability of the *Adar* mutant phenotype.

In the case of positive results, the cross was repeated at least three times. Also additional fly strains carrying overlapping deletions were used to narrow down the region of interest.

3.2 Results

3.2.1 Genetic screen for suppressors of Adar5G1 reduced viability

The first visible phenotype that affects *Adar*^{5G1} mutant flies is the reduced viability at eclosion. It has been previously reported that just 20-30% of mutant flies eclose normally when compared to wild type flies (personal communication, Leeanne McGurk).

Once the crosses were performed, male progeny were collected daily, and numbers for each genotype were counted, annotated and plotted (Figure 3.2). Most of the strains tested did not significantly affect the viability of the mutant phenotype, which remained between 20-40% compared to wild-type viability. Interestingly, two deletions were able to increase the number of eclosed *Adar*^{5G1} males. The first deletion, *Df(2L)*ED1455 increased the viability to 60%. Information from the *DrosDel* website (http://www.drosdel.org.uk/), revealed that this deletion covers a region of approximately 600 Kbp, removing 193 genes. However the most promising result was the *Df(2L)*ED778 deletion as in this case only 44 genes are deleted from the cytogenetic region 33E9-34A7. The collection was subsequently searched to find deletions surrounding the deleted *Df(2L)*ED778 region; *Df(2L)*ED784 partially overlaps with *Df(2L)*ED778 at its centromeric end (Figure 3.3). The *Df(2L)*ED784 mutant strain increased viability to 55%. Therefore the region of interest was narrowed down to a 161 kbp deletion containing eleven genes, 6 of them have known or bioinformatically inferred function (Figure 3.3).

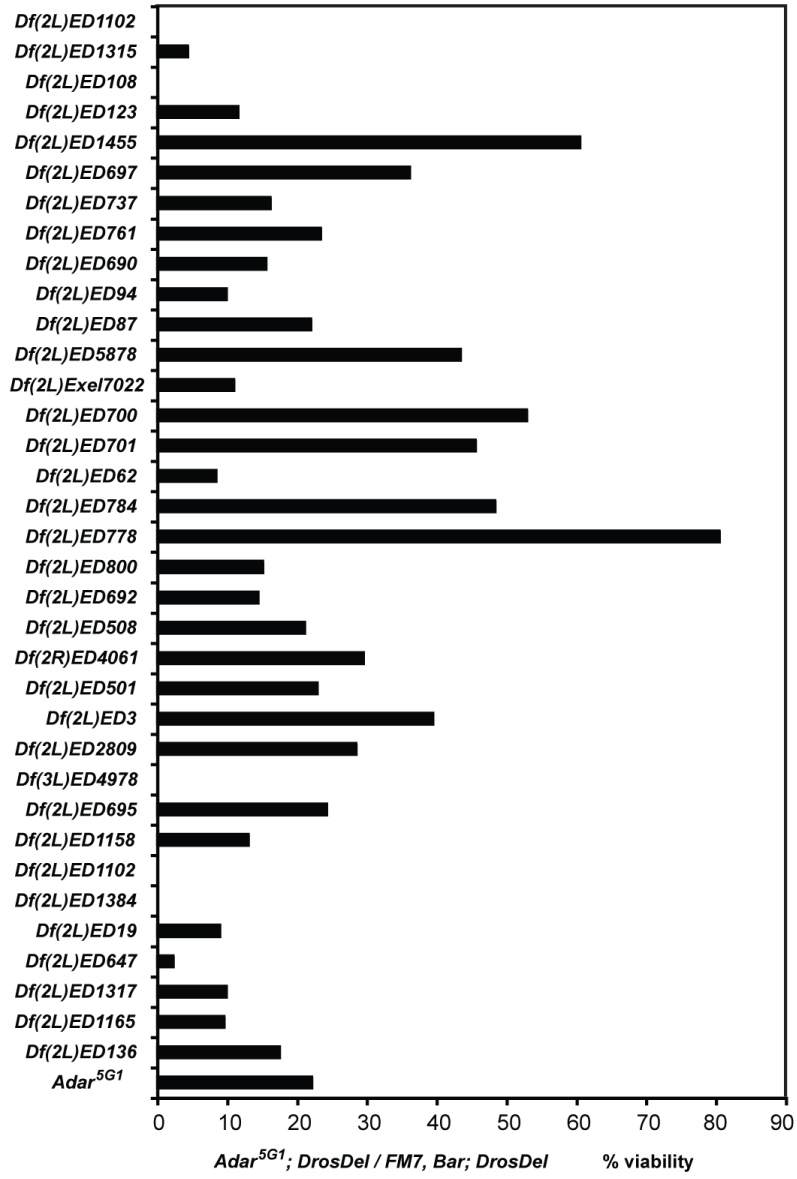


Figure 3.2: Genome-wide forward genetic screen for suppressors of reduced *Adar*^{5G1} viability. F_1 male flies derived from the cross between *Adar*^{5G1} virgins and males carrying a specific chromosomal deletion were collected and counted. The number of *Adar*^{5G1} males carrying a specific chromosomal deletion (y, *Adar*^{5G1}*, w / Y; Df(2L)ED.. /* +) was compared with the number of flies having the same deletion and the *FM7c* balancer (*FM7c / Y; Df(2L)ED.. /* +). The ratio calculated is referred as percentage of viability in the presence of the deletion. Independently of the presence of most of the deletions, the mutant fly viability stays around 20-40%. Only in one case the viability rises up till 80% (Df(2L)ED778).

3.2.2 Candidate modifiers of $Adar^{5G1}$ viability within the 2L chromosome region at cytogenetic position 34A4-34A7

After analyzing the results of the independent crosses, two overlapping deletions that increased $Adar^{5G1}$ viability were identified. Investigating the deleted genes, 6 out of 11 genes; Target of rapamycin, CG9934, A16, CG9932, CG5182, Pk34A were annotated in FlyBase (www.flybase.org) as follows:

- *Target of rapamycin*, (Cytogenetic position 34A4), is referred to by the symbol *Dmel\Tor* (CG5092, FBgn0021796) in FlyBase. This gene encodes a Ser-Thr specific kinase that regulates fly organ development as well as cellular growth and proliferation by positive regulation of ribosome biogenesis. It also is a sensor of stress as under starvation conditions or upon increase of reactive oxygen species, TOR activity is reduced, which increases autophagy activation that is critical for cellular survival (Yen and Klionsky, 2008).
- The gene *CG9934* encodes an ubiquitin-ligase protein which is inferred by sequence similarity. It contains a U-box domain which is typical of the ubiquitin conjugation factor E4, and a zinc finger domain (Wojcik *et al.*, 2004; Avery *et al.*, 2009).
- A16 has been suggested to be involved in chromatin assembly and/or disassembly.
- The protein encoded by the gene *CG9932* has a zinc finger domain; it is involved in bristle and wing disc development (see www.flybase.org).
- CG5182 is predicted to have carnitine O-acetyltransferase activity based on sequence similarity (see www.flybase.org).
- The gene *Pk34A* encodes a Ser-Thr protein kinase (see www.flybase.org).

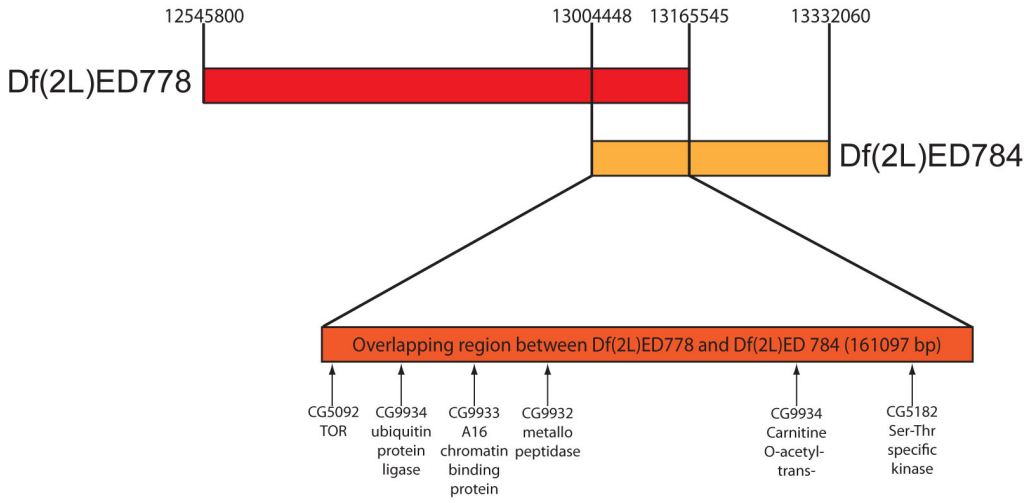


Figure 3.3: Overlapping region between *Df(2L)*ED778 and *Df(2L)*ED784. The red bar represents the chromosomal region deleted in *Df(2L)*ED778 strain. The yellow bar represents the deletion *Df(2L)*ED784. In orange, the overlapping deleted region and all annotated genes present in this deletion are shown.

The functions of the other five genes, CG31856, CG5122, CG9928, CG16978 and CG5204, are still unknown.

The next step was to identify which of these 11 genes was responsible for the increased viability of *Adar* mutant flies. Strains from the Exelixis *P*-element insertion collection were used to specifically reduce the expression of each candidate gene from the deletion analysis. There were 7 different strains available to reduce the expression of 5 out of the 11 annotated genes (Table 2.2).

A short description of each p-Element insertion line used is as follow:

- Bloomington Stock number 22878: this line contains the *Mi{ET1}* transgene at the transposable element insertion site within the third exon of *CG5142* gene, thereby disrupting its expression. This Ser-Thr specific kinase seems to be involved in cilia assembly.

- Bloomington Stock number 20794: the *EPgy2* transgene is inserted within the 3'-untranslated coding region (UTR) of *A16* gene thereby modifying the stability of the mRNA
- Bloomington Stock number 23044 and 10013: *CG9932* gene expression is disrupted by the insertion of two transgenes: the first; *Mi{ET1}*, is inserted within the regulatory region downstream of the open-reading-frame of the gene. The other, *Pbac{PB}*, is located within the first intron of the transcript.
- Bloomington Stock number 28402: this line contains *P{EP}* transgene at the transposable element insertion site in the second untranslated exon within the 3'UTR of *CG9934* gene which encodes a ubiquitin ligase.
- Bloomington Stock number 11218 and 25363: *Target of rapamycin*, (*Tor*) gene expression is reduced by *P{lacW}* or *Mi{ET1}* trangenes (respectively Tor^{K17004} and $Tor^{MB07988}$ stock lines) within the coding region .

The *y, Adar*^{5G1}*, w / FM7c, Bar;* $^{+}/_{+}$*;* $^{+}/_{+}$ mutant virgins were crossed with males from these new stocks. I found that a *P*-element insertion in the *Tor* gene resembled the rescue observed with the deletion *Df(2L)*ED778 (Figure 3.4). This data suggests that reduced $Adar^{5G1}$ mutant fly viability can be suppressed by the deletion *Df(2L)*ED778 by reducing the expression level of *Tor*.

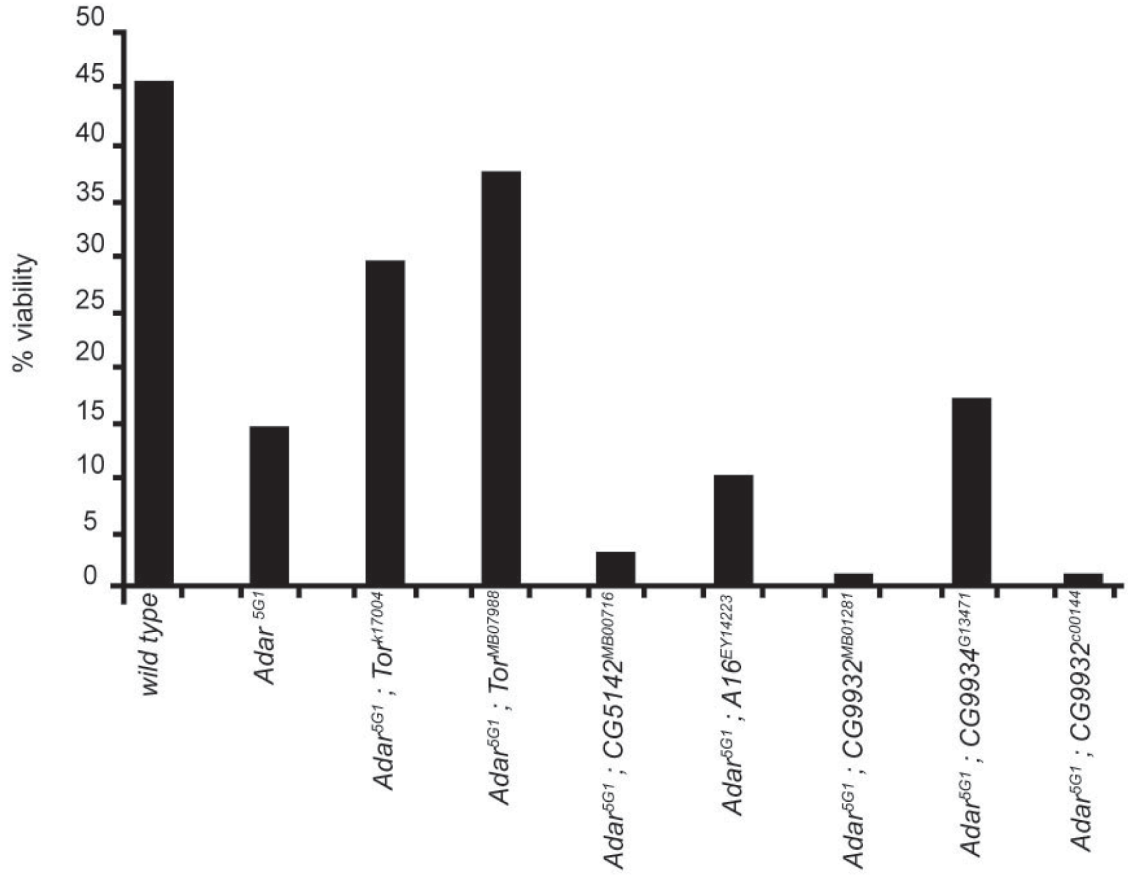


Figure 3.4: Effect on *Adar*^{5G1} viability mediated by knocking down the expression of specific genes. All the annotated genes within the overlapping region between *Df(2L)*ED778 and *Df(2L)*ED784 have been listed in Table 2.2. All the *P*-element containing strains available for these genes were crossed with *Adar*^{5G1} to determine which gene was responsible for the suppression of the reduced viability observed with *Df(2L)*ED778 and *Df(2L)*ED784 strains. Interestingly only lowering the expression of *Tor* gene increases the mutant fly viability.

3.3 Discussion

It has been proposed that ADAR is required for adult brain function (Keegan *et al.*, 2005). In particular, it may play a key role during central nervous system (CNS) formation as well as being important for its function (Keegan *et al.*, 2005). Most of the transcripts that ADAR edits are involved in different aspects of neuronal survival, for example neuron transmission such as voltage and ligand gated channels, in neuronal maintenance such as pumps that can maintain and restore intracellular ion homeostasis perturbed by synaptic transmission, as well as in axonal growth and transport (Stapleton *et al.*, 2006).

The biological function of the ADAR enzymes appears to be conserved throughout evolution. For example, the role of ADAR in neuronal function has also been investigated using squid as an animal model. Rosenthal and colleagues found that RNA editing is a critical event for the regulation of Na^+/K^+ ATPase transport velocity (Colina *et al.*, 2010). Furthermore, in mammals the glutamatergic neuron excitability is dependent upon the editing level of the pre-mRNA encoding the GluR-2 subunit of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor. In fact homozygous mice mutant for the *ADAR2* gene have apparently normal embryonic development; however they die during or shortly after weaning and are prone to seizures (Higuchi *et al.*, 2000b). This phenotype is dependent on the lack of editing at the Q/R site in the *GluR-2* transcript; and the $ADAR2^{-/-}$ phenotype can be rescued by expressing a transgene encoding the edited form of the transcript $GluR2^{R}$ in an $ADAR2^{-/-}$ background (Higuchi *et al.*, 2000b). Neurodegeneration has also been observed in *Drosophila* mutants for the *Adar* gene (Palladino *et al.*, 2000c). Also, locomotion defects and reduced viability are also observed in $Adar^{5G1}$ mutants.

It is still unclear whether one unedited transcript or the accumulated effects of many unedited transcripts is responsible for the severe phenotypes observed in *Adar* mutants and also which biological pathways are most severely affected. Also, it is not known what gene or combination of genes could rescue the phenotype. These questions were

addressed by performing a forward genome-wide genetic interaction screen to identify genes that could suppress the reduced viability as this is an obvious experimental approach in characterizing the *Adar*-null flies.

It is important to remember that the environmental condition can also influence fly development, for example alteration in external temperature can influence the timing of different fly developmental stages (Economos and Lints, 1986). Therefore all experiments were performed in a controlled environment. Also, even changes in ribonucleotide concentration within the fly media can influence larval growth and developmental timing (Sang and Burnet, 1963). For that reason maintaining the same experimental conditions was crucial. Furthermore a genetic screen to rescue the viability is an unusual approach, and not routinely performed and to date has not been reported in the literature.

Surprisingly few forward genetic screens which have used the *DrosDel* collection have been reported, even though it is a powerful tool for fly genetics. The reason might be related to the hemizygosity of the flies that are generated. In fact the mutants which have the genotype; *y, Adar^{5G1}, w / FM7c, Bar; Df(2L) / +;* $^{+}/_{+}$ are hemizygous for a specific region of the second chromosome; hence they contain a single allele of a gene without the corresponding allele. Flies hemizygous for the deletion can suppress and/or enhance specific phenotypes. Therefore in *Drosophila melanogaster* homozygous flies are preferred to address experimental questions as they enhance the observed phenotype. In the screen I performed, the rescue of viability in males was the readout, so halving the dosage of a gene indicated that there is a strong genetic interaction. Therefore this genetic approach is valid as it is reveals that even with half the dosage of a gene is sufficient to suppress the reduction in viability.

A deletion of the chromosomal region 34A4-34A7 significantly increases the viability of *Adar^{5G1}* males. This effect seems to be dependent on the deletion of the *Tor* gene. Thus, a reduction to half the dosage of the *Tor* gene is sufficient to suppress the reduced *Adar^{5G1}* viability. This result supports the hypothesis that first, reduction in gene dosage can be a strong indicator of a genetic interaction and second, hemizygosity in *Drosophila* is sufficient to show enhancing or suppressive effects on a phenotype.

The same rescuing result was obtained using two different *Drosophila* lines that have reduced *Tor* gene expression. The first strain, referred as Tor^{k17004} (Bloomington stock number: 11218) has the following genotype: $y^{1}, w^{67c23}; P\{lacW\}Tor^{k17004} / CyO$. The transgene $P\{lacW\}Tor^{k17004}$ is inserted in the cytological map location 34A4 within the 3'-untranslated region (3'UTR) of the *Tor* gene and reduces its expression by 75% (Zhang *et al.*, 2000). The second strain used, referred as $Tor^{MB07988}$ (Bloomington stock number: 25363) has a transposable element insertion site within the fourth intron of the *Tor* gene (w[1118]; Mi{ET1}Tor[MB07988]). While flies null for *Tor* do not survive to adulthood, certain trans-heterozygous allelic combinations are viable. TOR is the *Drosophila* homolog of the mammalian protein of the same name.

The *Tor* gene encodes a Serine-/Threonine-dependent kinase. It is involved in ribosome biogenesis. It induces cellular growth and proliferation by phosphorylating eukaryotic initiation factor 4E (eIF-4E)-binding protein (4E BP) and p70 S6 kinase (also known as S6K), to increase the translation rate (Miron *et al.*, 2003). However TOR also acts as sensor for three pathways that can sense three different sources of cellular stress (Yen and Klionsky, 2008). TOR is a sensor for:

1. Nutrient level, through the Akt/PKB pathway
2. Hypoxia
3. Impairment in energy, through AMPK pathway.

Under stress conditions TOR activity is reduced with a subsequent increase in autophagy to rescue the cell. Previously, Partridge and colleagues correlated TOR activity with *Drosophila* lifespan (Bjedov *et al.*, 2010). They provide the first evidence that reduced TOR activity through administration of rapamycin increases the average lifespan of flies. It is important to determine if the complete lack of *Adar* also influences fruit fly lifespan; in fact it was previously shown that the hypomorphic allele of *Adar*, called $Adar^{1F4}$, does not affect mutant fly lifespan (Palladino *et al.*, 2000c). However reduced viability of $Adar^{5G1}$ is just one of the effects of the *Adar* deletion. It would be of interest to examine other phenotypes of $Adar^{5G1}$ and elucidate whether reduced *Tor* expression has any effect on them.

CHAPTER 4: Loss of function mutations in the *Tor* gene suppress *Adar*-null phenotypes

4.1 Introduction

TOR (Target of Rapamycin) is a Serine-/Threonine-dependent-kinase that is a member of the phosphatidylinositol kinase-related kinase family (Jacinto and Hall, 2003). It was first identified in *S. cerevisiae* where there are two proteins with the same activity; TOR1 and TOR2, which form two functionally different complexes, TORC1 and TORC2, respectively (Kunz *et al.*, 1993; Helliwell *et al.*, 1994). Subsequently, proteins homologous to TORs have been found in all eukaryotes that have been examined. In mammals (Brown *et al.*, 1994; Chiu *et al.*, 1994; Sabatini *et al.*, 1994), flies (Oldham *et al.*, 2000), and worms (Long *et al.*, 2002), there is only one gene encoding one protein with TOR activity that exists in two distinct complexes, with different sets of protein cofactors.

TOR is a key regulatory protein because it responds to and integrates a variety of upstream signals from different pathways. According to the type of input signal it receives, TOR can react by regulating different pathways, including protein translation, autophagy, and metabolism. Additionally, TOR activity is down-regulated in response to intracellular and extracellular stress and signals such as starvation and growth factor deprivation. In the case of accumulation of damaged organelles or protein aggregates, autophagy is induced by decreasing TOR activity which rescues the cell from the source of stress. Therefore, TOR is one of the main players that control cellular homeostasis. Moreover it is able to arrest the cell cycle or induces cellular growth and proliferation depending on the level of stress on the cell. For these reasons, the regulation and biological role of TOR is actively studied by many groups.

Drosophila melanogaster is an ideal model to dissect the diverse biological roles of TOR because of the low redundancy of the fly genome. Also, *Drosophila* TOR shares approximately 56% identity to mammalian TOR, with higher conservation within the kinase and FRB (FKBP-rapamycin-binding) domains (Zhang *et al.*, 2000). Thus, the role of TOR in different biological pathways is highly conserved in *Drosophila*. As

previously mentioned, TOR regulates cellular growth and proliferation, and alleles of *dTor* show an extended larval period with little or no growth and increased sensitivity to rapamycin (Zhang *et al.*, 2000). Additionally, cells lacking *dTor* exhibit reduced size, reduced proliferation in multiple tissues, reduced nucleolar size, aggregation of lipid vesicles and growth arrest that is cell type specific (Zhang *et al.*, 2000). Consequently, flies null for *Tor* expression do not survive to adulthood.

Studies by the Partridge group have focused on dTOR and specifically, they are interested in the question of how diet correlates with the ageing processes. Indeed, it has been hypothesized that some dietary regimes, like caloric or methionine restriction, cause lifespan extension by decreasing TOR activity (Kaeberlein *et al.*, 2005; Powers *et al.*, 2006). Even the administration of relatively low doses of rapamycin decreases the activity of TOR kinase causing an increase in life span (Bjedov *et al.*, 2010).

It has been shown in the previous chapter that a decrease in *Tor* gene expression rescues the viability of the *Adar*-null mutant. This has been confirmed both with a partial deletion of the second chromosome (*Df(2L)*ED778 and *Df(2L)*ED784 strains) and with two different *p*-Element insertion lines, referred to as Tor^{k17004} and $Tor^{MB07988}$ (Zhang *et al.*, 2000).

In this chapter, the effect of a reduction in *Tor* expression will be further investigated to determine whether the beneficial effects extend beyond increasing *Adar* mutant life span, thereby resembling the effect of rapamycin. Reduced viability and reduced life span are not the only phenotypes associated with the lack of the ADAR RNA editing enzyme; mutant flies also show locomotion defects, which are succeeded by age-dependent neurodegeneration. Therefore a systematic approach was used to investigate whether reducing *Tor* expression also affects these phenotypes.

4.2 Results

4.2.1 Lifespan experiment

Previously, it has been observed that *Adar*^{5G1} have a reduced life-span (McGurk, 2007). However the actual decrease in life-span was not recorded. Therefore I had to first determine the actual reduction in *Adar*^{5G1} before studying the effects of TOR on the mutant.

Mutant flies were collected and kept for 30 days at 25°C. During this period, the number of dead flies was daily annotated. Finally the number of surviving flies was compared with the number of the flies at the beginning of the experiment, thereby calculating the percentage of survivors. Because *Adar*-null flies have locomotion defects, mutants were transferred to a new culture vial every day in order to prevent them from being stuck on the food and dying.

Interestingly, after 15 days, the mortality of *Adar*^{5G1} male flies increases to 50%. Nevertheless the number of survivors continued to decrease until the mortality reaches approximately 98% at day 30 (Figure 4.1, black line). *Adar*^{5G1}*; Tor*^{k17004} and *Adar*^{5G1}*; Tor* ^{MB07988} male flies were collected and kept individually in vials. As controls, male flies with the following genotypes: *FM7c,Bar*, *FM7c,Bar; Tor*^{k17004} and *FM7c,Bar; Tor* ^{MB07988} were used. The advantage of using these genotypes as controls was that firstly, they came from the same crosses therefore they share the same genetic background. Secondly, it was possible to ascertain how the decrease in *Tor* expression interferes with the genetic background independently of the *Adar* deletion.

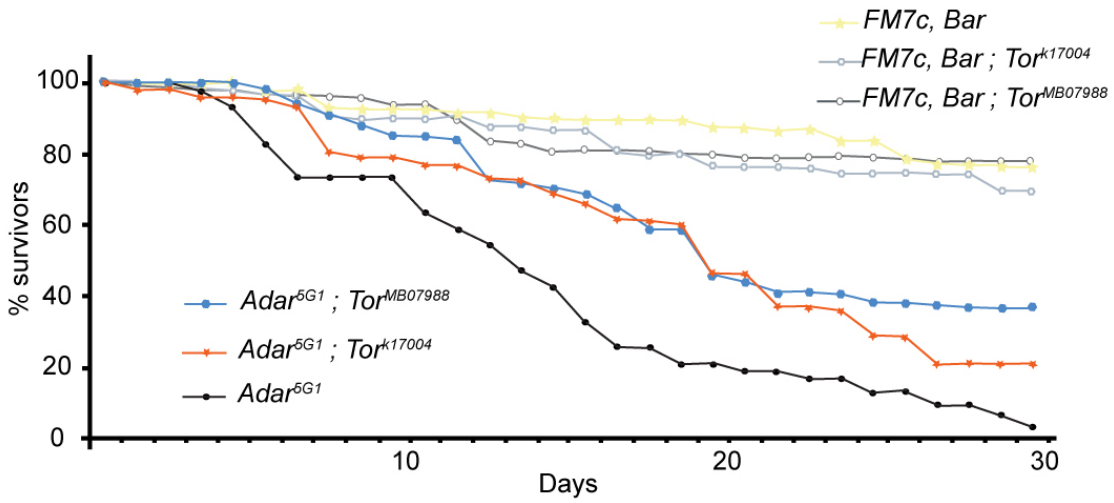


Figure 4.1: Loss of function mutations in *Tor* increase life span of *Adar*^{5G1} mutant flies. A hundred flies for each genotype were collected and kept individually in vials. Flies were flipped daily and the number of dead flies counted. Thus, the percentage of survivors was calculated for a period of 30 days. Interestingly, the mortality associated to the lack of *Adar* reaches 50% within 15 days and goes up till 98%. The reduced *Tor* gene expression suppresses the *Adar*-null phenotype, especially immediately after eclosion (between day 1 and 15), increasing mutant fly viability by 2 fold.

I found that the p-Element insertion in the *Tor* gene not only suppresses the viability of *Adar*^{5G1} males, but also reduced the mortality associated with the lack of *Adar*. Males carrying the balancer *FM7c*, mostly survive for more than 30 days (and the percentage of the survivors was approximately 80%). However the mortality of *Adar*^{5G1}*; Tor*^{k17004} and *Adar*^{5G1}*; Tor* ^{MB07988} male flies varied from 60 to 70% after 15 days, and decreased to 20% at 30 days (Figure 4.1, orange and blue line, respectively). Thus, reducing *Tor* expression partially suppresses the mortality associated with the *Adar* deletion. This effect is *Adar*-specific as the genetic background does not affect the life span of the control flies.

It is not clear how reducing *Tor* expression can positively influence both the viability and lifespan of ADAR mutants. To address this it was necessary to determine if TOR also affected the other *Adar*-null phenotypes.

4.2.2 Locomotion defects

The deletion of *Adar* causes behavioural defects such as impairment of locomotion. The open-field locomotion assay is used to quantify locomotion defects. *Adar*-null flies almost completely lose the capability to walk normally and the assay revealed a reduction of over 80% compared to wild-type locomotion (Figure 4.2).

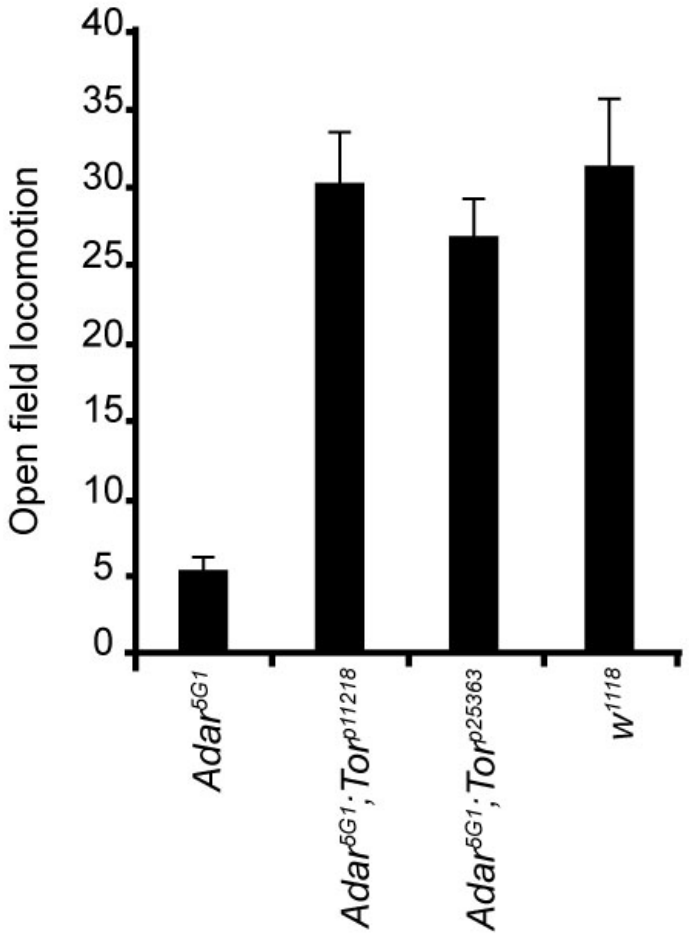


Figure 4.2: Loss of function mutations in *Tor* increase the locomotion in *Adar*^{5G1} mutant flies in an open-field locomotion assay. *Adar*-null mutant flies show locomotion defects; two minute open field locomotion assay reveals that the ability of mutant flies to walk in an open field arena is dramatically reduced compared to wild-type animals. Lowering *Tor* expression rescues the locomotion ability of mutant animals. Standard error bars are shown.

A short movie was recorded to gain further insight into the locomotion defects (Movie 1, see CD-Rom). First, flies show a defective posture as their muscles appear not have enough strength to support their body weight and their legs are spread wide apart. When induced to move by an external stimulation, they show additional symptoms such as uncontrollable tremors, imbalance and uncoordinated movements. Furthermore, anterior legs drag the posterior ones that are completely motionless. The imperfect walking and the locomotion defects may explain the sterility affecting $Adar^{5G1}$ males that has been previously observed in our laboratory (personal communication Leeanne McGurk). In fact mutant males are unable to mate with mutant or wild-type females, but no gross morphological defects were detected in the testis of mutant adults. Before a successful mating can occur, *Drosophila* has to engage in courtship which is a highly ritualistic and complex behaviour, consisting of a series of stereotyped interactions between the male and female (Hall, 1994; Greenspan and Ferveur, 2000). Therefore the defect in mating may be due to uncoordinated and defective movements of mutant flies rather than to a failure in their reproductive system.

As previously shown, a decrease in *Tor* expression can have a positive effect on $Adar^{5G1}$ flies as it increases their viability. I wanted to elucidate whether the effect mediated by TOR was specific for viability, or whether it could also affect other phenotypes, such as the impairment in locomotion. To address this question, the locomotion activity of 7-day-old male flies of the genotype; $Adar^{5G1}$; Tor^{k17004} and $Adar^{5G1}$; $Tor^{MB07988}$ was assayed (Figure 4.2). The open field assay revealed a general increase in locomotion in the mutant flies.

A short movie was recorded following the movements of an $Adar^{5G1}$; $Tor^{MB07988}$ mutant fly revealing that the main locomotion impairments were rescued. In particular, neither leg tremors nor posture problems that were previously observed were detected. I also observed that when a mutant fly fell down, it was able to right itself back to its original position. Therefore the movements are generally more coordinated and synchronized.

Thus, it is possible to conclude that decreasing *Tor* expression can also suppress the locomotion phenotype that is caused by the lack of the RNA editing enzyme ADAR.

4.2.3 Neurodegeneration

Most of the transcripts that ADAR edits encode ligand and voltage-gated ion channels and are important for the function and maintenance of the CNS. Therefore a deletion of *Adar* more than likely will affect the CNS. For example, in *hypnos-2*^{P} which is a deletion of *Adar*, the flies suffer from a very mild degeneration in the cortical neurons of the lamina (Ma *et al.*, 2002b). Additionally Palladino *et al*. reported that in *Adar*^{1F4} which is a hypomorphic mutant of *Adar*, the flies undergo progressive vacuolization of the synaptic neuropil (Palladino *et al.*, 2000c).

Adar^{5G1} flies show a rapid vacuolisation that specifically affects the mushroom body (MB) calyces and retina (McGurk, 2007). Mushroom body is involved in learning and memory as well as locomotion activation in response to external stimuli, in particular odours. Thus, they are an important region in the *Drosophila* adult brain. *Drosophila* photoreceptor neurons are another neuronal subtype extensively used to study the biological events and the molecular mechanisms that lead to neurodegeneration and are used to model human disease such as Alzheimer's (AD), Parkinson's (PD) and Huntington's disease (HD) in flies (Lu and Vogel, 2009). It has also been shown that TOR has a neuroprotective role in these *Drosophila* neurodegenerative diseases models. In fact reducing *Tor* expression thereby increasing the level of autophagy can result in stress-causing aggregates being completely removed such as the TAU aggregates in *Drosophila* model of Alzheimer's disease (Khurana *et al.*, 2006). Furthermore TOR-regulated autophagy regulates photoreceptor degeneration in *Drosophila* (Wang *et al.*, 2009). The cause of the neurodegenerative phenotype in *Adar*-null flies is unknown. Since it was shown that TOR prevents degeneration in flies, I hypothesised that it might suppress *Adar*^{5G1} neurodegeneration. To investigate this, *Adar*^{5G1}, *Adar*^{5G1}*; Tor*^{k17004} and *Adar*^{5G1}*; Tor* ^{MB07988} flies were collected and aged for 30 days. Heads of aged flies were then sectioned and stained with haematoxylin and eosin.

As previously observed, *Adar*-null fly brains undergo rapid degeneration that affects not only the retina, but also the mushroom body calyces (Figure 4.3 C-D). As expected, reduced *Tor* expression in an *Adar*^{5G1} background suppressed the neurodegenerative

phenotype. The mushroom bodies did not show vacuolated structures (Figure 4.3 E and G). Also, the organization of the retina was almost completely restored (Figure 4.3 F and H).

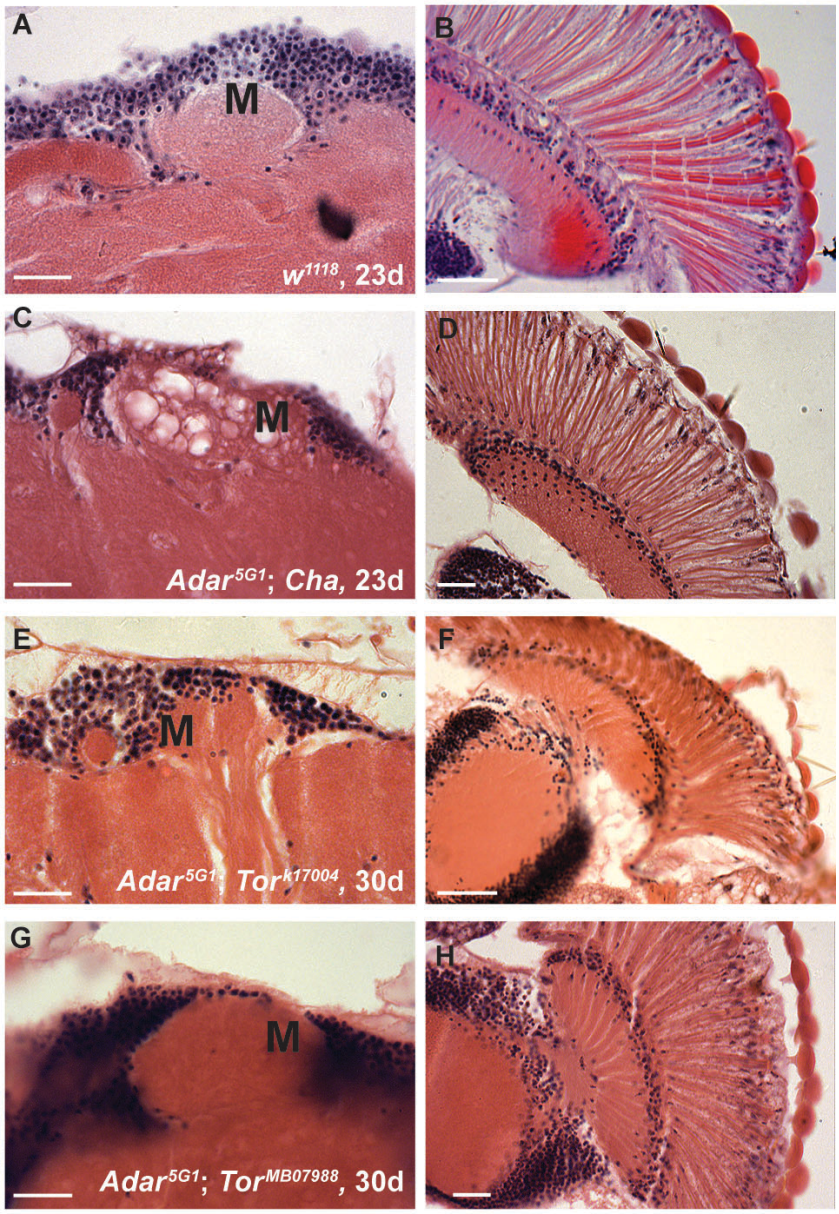


Figure 4.3: Loss of function mutations in *Tor* suppress the neurodegenerative phenotype of the *Adar*^{5G1} mutant. Section of mushroom body, M, calyx (63X) and retina (40X) of 23-days-old wild-type flies is shown in figure A and B, respectively. C and D show 23-day-old *Adar*^{5G1} flies show neurodegeneration affecting specifically MB calyx (C) and fly retina (D). E-F and G-H show sections of fly heads of *Adar*^{5G1}*; Tor*^{k17004}and *Adar*^{5G1}*; Tor*^{MB07988} genotypes, respectively. In E, the olfactory projection tract through the brain to MB is seen. Scale bars: 20μm.

4.3 Discussion

TOR is a key sensor in development and responds to different cellular and systemic signals thereby controlling cellular growth, proliferation and cell cycle. In *Drosophila*, this Ser/Thr protein kinase can increase the animal's life span as it responds to hormone-sensitive pathways such as insulin and growth hormone signalling. Nevertheless, dietary regimes cause lifespan extension or reduction by modulating TOR activity (Chapman and Partridge, 1996; Mair and Dillin, 2008).

Adar^{5G1} flies have a severe reduction of their life span though it is not clear what causes this reduction. However reduced *Tor* expression can significantly suppress the reduced lifespan phenotype in *Adar* mutants. This result supports previous data that show how reducing TOR activity increases life span. However in the case of *Adar*, there is no evidence of a modification in the hormone-responsive pathways. Also there is not a modification in the dietary regime in which flies are held.

Moreover not only lifespan of *Adar* mutant flies can be increased but also the locomotion impairments are suppressed by reducing *Tor* expression. It is possible that the life span is increased because mutant flies do not longer die becoming stuck in the food. In fact, reducing *Tor* expression corrects the mutant fly posture as well as their walking movements, which are comparable to normal flies. This has been demonstrated by using an open-field locomotion assay that measures locomotion activity and a short recorded movie that showed an improvement in the coordination and the organization of movement in the rescued mutant.

The cause underlying the locomotion defects due to the lack of RNA editing is still unknown. It would be interesting to study this aspect of the phenotype more. The abnormalities might be dependent on the progressive loss of the structure or function of the central and/or peripheral (mostly motor-) neurons. The evidence of progressive degeneration characterized by vacuoles within fly retina and within the mushroom bodies of *Adar* mutants appears to support the first hypothesis. Consistent with this,

neurons forming mushroom bodies are involved in locomotion activation in response to external stimuli. Thus, the defective mobility in *Adar*-null flies may be due to a problem in organizing movements. Furthermore, the neurodegenerative phenotype is suppressed by decreasing *Tor* expression. This result is supported by previous reports, which demonstrate that TOR activity has a central role in preventing tauopathy-related degenerative phenotypes in *Drosophila* (Khurana *et al.*, 2006). Therefore, the rescue of locomotion activity in *Adar* mutants may be dependent on the suppression of the loss of central neurons, which organize movement.

Alternatively, the defects might be caused by a faulty function or structure in motor neurons. It would therefore be interesting to investigate motor neuron morphology in *Adar* mutants. This would elucidate whether the lack of editing affects only central neurons, as has been previously demonstrated, or whether it has widespread effects throughout the whole nervous system. In this case, the rescue might occur at the synaptic level as it has been shown that TOR participates in controlling synaptic plasticity (Hu *et al.*, 2010). Additionally, many of the transcripts edited by ADAR are expressed at the neuromuscular junction (Stapleton *et al.*, 2006). For example, it is clear that transcripts encoding voltage and ligand-gated ion channels must be edited at a specific position to constitute functional channels (Stapleton *et al.*, 2006). Actually, no work has yet been performed to demonstrate that in the absence of *Adar*, the synapses are affected.

CHAPTER 5: Autophagy suppresses *Adar*-null associated phenotypes

5.1 Introduction

In the previous chapter I described how the reduction of the expression of *Tor* gene could suppress all the *Adar* null phenotypes. As previously mentioned, TOR controls metabolic activity in response to intracellular cues and extracellular stimuli. TOR is present within two distinct physical and functional complexes, TOR containing complex 1 (TORC1) and TORC2, firstly characterized in budding yeast, *Saccharomyces cerevisiae* (Helliwell *et al.*, 1994). These two complexes differ in their protein composition; TORC1 contains a regulatory associated protein of TOR (Raptor) rather than the rapamycin-insensitive companion of TOR (Rictor)/AVO3, present within TORC2. Both complexes contain GβL (G-protein β-subunit like protein), which stimulate TOR activity (Jacinto *et al.*, 2004; Sarbassov *et al.*, 2004).

The function of TORC2 is still not well defined. It has been reported to regulate the actin cytoskeleton by modulating protein kinase C and Rho-family small GTPases (Schmidt *et al.*, 1997; Helliwell *et al.*, 1998; Jacinto *et al.*, 2004; Sarbassov *et al.*, 2004). In contrast TORC1 mainly regulates cell growth and protein synthesis by phosphorylating two key translational regulators; eukaryote initiation factor 4E-binding protein (4E-BP) and p70 S6 kinase (S6K) (Nakashima *et al.*, 2010). Upon TOR phosporylation, there is a decrease in the 4E-BP affinity for eIF4E (Haghighat *et al.*, 1995) and S6K phosphorylates ribosomal protein S6 that is a component of 40S ribosomal subunit (Hara *et al.*, 1997; Hara *et al.*, 1998; Roux *et al.*, 2007). In both cases, this allows cap-dependent translation to occur.

It is therefore important to elucidate whether the reduction in *Tor* gene expression in *Adar* mutant flies is dependent on a general suppression of the initiation of cap-dependent protein translation.

TOR not only stimulates anabolic processes by increasing protein synthesis, but it also inhibits autophagy, which is a major catabolic process (Noda and Ohsumi, 1998). In fact, *autophagy specific gene 1* protein (ATG1) and ATG13 interaction which is essential for the nucleation step of autophagy initiation, is prevented by their TOR-mediated phosphorylation (Chang and Neufeld, 2009). When TOR activity is decreased, as occurs when under an increased demand of catabolic processes, autophagy takes place because of the stable interaction between ATG1 and ATG13 (Chang and Neufeld, 2009).

Therefore the possibility arises that the previously described rescue of *Adar*-null phenotypes is dependent on an increased autophagy activation rate. Earlier studies support this idea as it has been shown that enhanced autophagy prevents neurodegeneration, clearing protein aggregates in a *Drosophila* model of Huntington's disease (Ravikumar *et al.*, 2004; Berger *et al.*, 2006).

The major aim of the following chapter is to investigate whether decreasing *Tor* expression rescues the *Adar* mutant phenotype through a general decrease in protein translation, rather than through an increase in the autophagy activation rate.

5.2 Results

Fly genetics was used to elucidate which pathway was involved in rescuing the *Adar*^{5G1}-related phenotypes when *Tor* expression was reduced. The two pathways that depend on TOR activity-mediated regulation were tested. Both (i) cap-dependent translation and (ii) autophagy pathways were manipulated genetically to mimic the effect of a reduction in *Tor*.

5.2.1 Reducing general levels of translation does not affect Adar-null fly locomotion

TOR stimulates messenger RNAs (mRNAs) translation by inhibiting 4E-BPs and activating S6 kinase. It has been shown that reduced S6K activity extends life span in nematode (Hansen *et al.*, 2007), fruit fly (Kapahi *et al.*, 2004) and mice (Selman, 2009). The same effect has been observed in *C. elegans* by depleting eIF4E, which is inhibited by 4E-BP (Syntichaki *et al.*, 2007a, b). Longevity is reduced in *Drosophila* by loss of 4E-BP (Zid *et al.*, 2009). Indeed, 4E-BP (known as THOR in *Drosophila*) binds eIF4E and this impairs the recruitment of the 40S ribosomal subunit to the cap structure present at the 5'-end of eukaryotic mRNAs. Therefore TOR's influence on translation could explain the *Adar*-null phenotype. Reducing *Tor* expression causes a loss of function of S6 kinase and 4E-BP and accordingly, a reduced translation rate. Consequently the rescue of the *Adar*-null life span, mediated by suppression of *Tor* expression, might be due to reduced activation of translation.

To test this hypothesis, a dominant-negative isoform of S6K was used. The dominant-negative protein was generated by replacing a conserved lysine in the ATP binding site by glutamine (*UAS-dS6K*^{KQ}) (Bjedov *et al.*, 2010). This mutant was previously used in

lifespan experiments to prove that reducing mRNA translation initiation and ribosomal biogenesis increases the average life of *Drosophila* demonstrating a new role for S6K and mRNA translation in ageing (Bjedov *et al.*, 2010).

The *UAS-S6K*^{KQ} transgene were expressed using the GAL4-UAS regulatory binary system. For specific expression in cholinergic neurons, transgene expression was driven using the following strain*, Adar*^{5G1}*, w; Cha-GAL4 (19B), UAS-GFP S65T (Cha-GAL4 driver)*. Thus, *Cha-GAL4* driver expresses *UAS-S6K*^{KQ} in most of the neurons in the *Adar*^{5G1} background. The role of S6K was previously investigated in ageing using *Drosophila* as animal model however this phenotype is too variable and could be dependent on the locomotion impairment in the case of *Adar*-null flies. For this reason, I decided to use the reduced locomotion activity of *Adar*^{5G1} mutants as the phenotype to be studied to determine if it was suppressed. It was analysed using a two-minute open-field locomotion assay.

I found that the overexpression of the dominant negative isoform of S6 kinase does not rescue the locomotion activity of *Adar*-null flies (Figure 5.1). The overexpression of *Thor* was also tested. Dietary restriction mediates life span extension in *Drosophila* by enhancing 4E-BP activity (Syntichaki *et al.*, 2007a, b). Overexpression of *Thor* in transgenic flies encoding the *UAS-Thor* construct was not sufficient to suppress the defective walking phenotype of *Adar*^{5G1} mutants.

In summary, using either a dominant-negative isoform of S6 kinase or overexpressing *Thor* does not suppress the *Adar*^{5G1} locomotion impairments. These data suggest that the rescue mediated by decreasing *Tor* expression, does not act through translation.

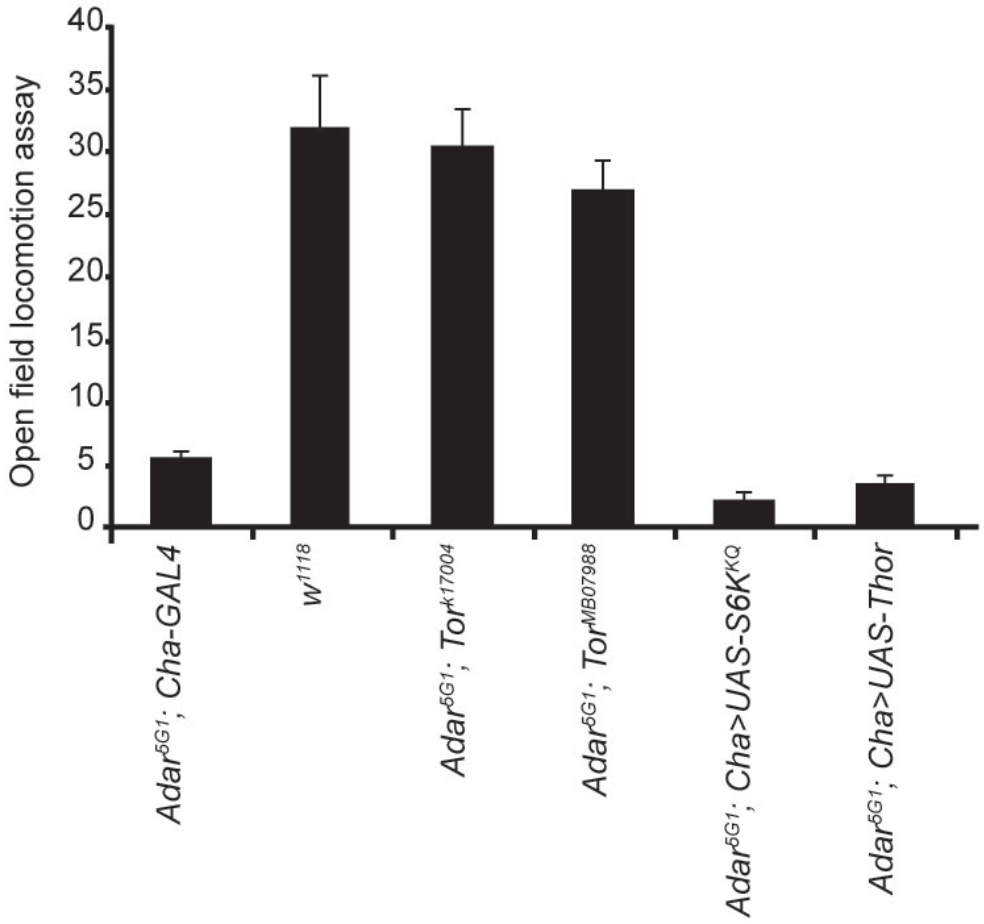


Figure 5.1: Reduction of cap-mediated protein translation does not suppress the Adar5G1 locomotion phenotype in an open-field locomotion assay. *Adar*-null mutant flies show locomotion defects in two-minute open-field locomotion assay as previously described (Figure 4.2). Their locomotion activity is dramatically reduced compared to wild-type animals. Lowering *Tor* expression (*Adar*^{5G1};*Tor*^{p11218} and *Adar*^{5G1};*Tor*^{p25363}) rescues the locomotion deficits of mutant animals. Reduced *Tor* expression influences protein translation impairing the ribosomal assembly and the first initiation step. The same effect is achieved over-expressing *UAS-S6K*^{KQ}, a dominant negative isoform of S6K, and *UAS-Thor* that inhibits translation initiation interacting with eIF4E. The figure shows that in both cases the locomotion impairments affecting *Adar*-null flies are not rescued reducing the translation activation suggesting that the *Tor*-mediated effect in *Adar*^{5G1} flies is not mediated throughout this pathway. Error deviation bars are shown.

5.2.2 Autophagy

Autophagy is a catabolic process involving the degradation of cellular components through the lysosomal machinery. It is a tightly-regulated process that is involved in cell growth, development, and homeostasis. It helps to maintain a balance between the synthesis, degradation, and subsequent recycling of cellular products. At the cellular level, cytosolic material is sequestrated within a unique double-membrane cytosolic vesicle called an autophagosome. The sequestration of cellular material can be selective as cargo proteins deliver proteins, damaged organelles or invasive microbes to the autophagosome. It can also be nonspecific, involving the engulfment of bulk cytoplasm (see chapter 1.6). Autophagosome formation requires two steps, first a nucleation step followed by an extension. The first step commences when the ATG1/ATG13 complex is maintained and not longer disassembled by TOR-mediated phosphorylation (Chang and Neufeld, 2009). Their interaction is crucial for the recruitment of essential proteins where the membrane (focus) begins to extend. During the extension step, the membrane is stretched out until it completely engulfs cytosolic material and organelles within a double layered membrane.

Thus, reduced *Tor* expression might rescue *Adar*-null phenotypes by enhancing autophagy activation. To test this hypothesis, transgenic flies expressing a *UAS-Atg1[GS10797]* construct were crossing with *y, Adar^{5G1}, w; Cha-GAL4 (19B), UAS-GFP S65T* virgins. Both ATG1 and ATG13 proteins are involved in autophagy activation and are also targeted by TOR (Scott *et al.*, 2004; Scott *et al.*, 2007; Chang and Neufeld, 2009). However most of the published work is focused on ATG1. ATG1 is also involved in axon cargo transport, regulation of synaptic transmission and vesicle density at the active zone, synaptic growth and organization (Toda *et al.*, 2008). Whereas, reducing *Tor* expression might increase autophagy activation, lack of ATG1 phosphorylation can influence other mechanisms. However this could explain why the locomotion impairment in *Adar^{5G1}* is rescued by *Tor*. To test this hypothesis *y, Adar^{5G1}, w; Cha-GAL4> UAS-Atg1[GS10797]* male flies (Scott *et al.*, 2007) were collected and a 2 minute-open field locomotion assay was performed.

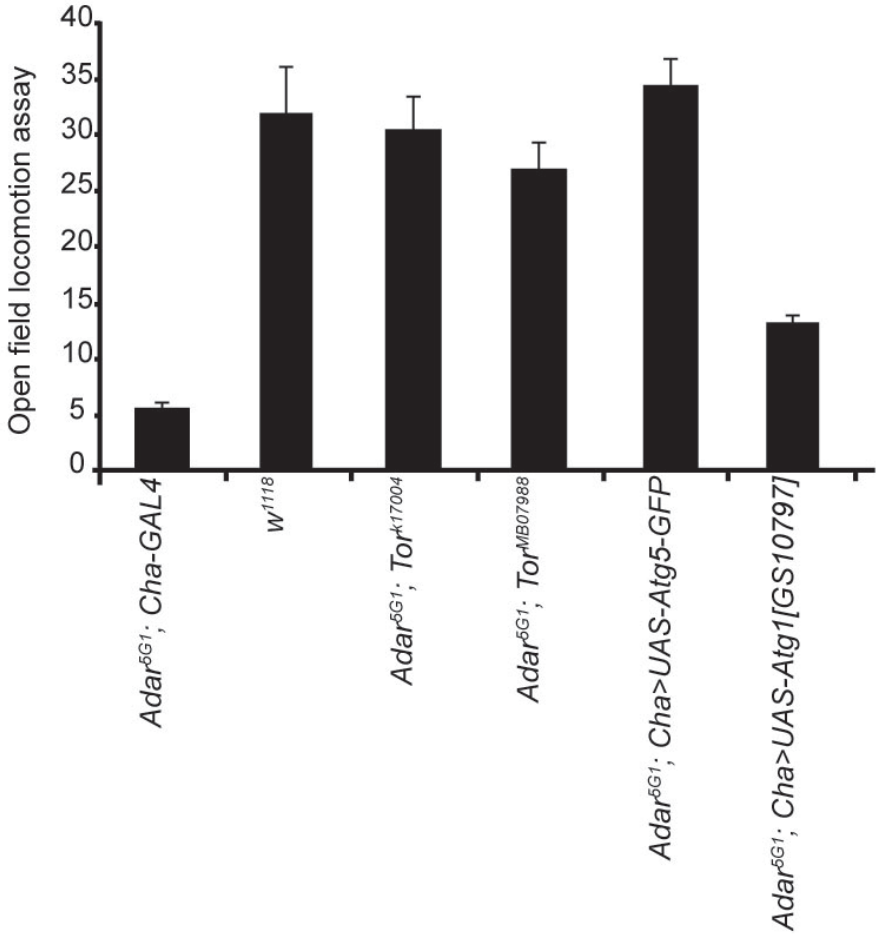


Figure 5.2: UAS-Atg5-GFP over-expression suppresses the *Adar*^{5G1} locomotion phenotype in an open-field locomotion assay. Lowering *Tor* expressing induces autophagy; therefore its positive effect on *Adar*^{5G1} mutants' locomotion activity might be mediated by increasing autophagy activation rate. To test this hypothesis *UAS-Atg1[GS10797]* and *UAS-Atg5-eGFP* were expressed in *Adar*-null animals using *Cha-GAL4* driver. Albeit, *UAS-Atg1[GS10797]* expression only partially suppresses *Adar*-null locomotion impairments, *UAS-Atg5-eGFP* reduces the defects bringing the locomotion activity of mutant flies comparable to wild-type, *Adar*^{5G1}*;Tor*^{p11218} and *Adar*^{5G1}*;Tor*^{p25363}, animals. These results suggest that *Tor*-mediated rescue acts through increased autophagy activation rate. Error deviation bars are shown.

Compared to control animals (*y, Adar*^{5G1}*, w; Cha-GAL4*), the locomotion activity of *Adar*^{5G1}*; Cha>Atg1[GS10797]* transgenic flies was increased by two fold (Figure 5.2); therefore *Atg1* expression partially suppresses the defective locomotion of *Adar*-null flies. However, as shown in figure 5.2, its rescue is less when compared to the effect of the loss-of-function mutations in the *Tor* gene.

Atg5 encodes an autophagy factor required during the extension step (Mizushima *et al.*, 2010). It is an essential component of autophagosome formation and it able to induce autophagy activation. As it has been shown that the presence of *Atg5* is sufficient to boost autophagy activation, I used *UAS-Atg5-eGFP* transgenic flies to confirm that the effect of reduced *Tor* expression was mediated by increased activation rate of autophagy pathway. *UAS-Atg5-eGFP* expression was driven in cholinergic neurons of *Adar*^{5G1} male flies using with *y, Adar*^{5G1}*, w; Cha-GAL4 (19B), UAS-GFP S65T* strain. Upon collection of *Adar*^{5G1}*; Cha>Atg5-eGFP* male flies, the locomotion activity of mutant animals was tested. I found that their locomotion activity was comparable to control flies (figure 5.2) and to *Adar*^{5G1}*; Tor*^{p11218} and *Adar*^{5G1}*; Tor*^{p25363} mutants, as well. In summary, the same effect previously observed when reducing *Tor* gene expression is found by increasing autophagy activation by overexpressing *Atg* autophagy genes.

5.2.3 Autophagy activation suppresses Adar^{5G1}-related neurodegenerative phenotype

ATG1 and ATG13 are able to active the cascade of events leading to autophagosome formation (Chang and Neufeld, 2009). Even though ATG13 is essential within the ATG1-ATG13 complex for autophagy initiation, most studies have focused on ATG1. As mentioned previously ATG1 has additional roles however, its overexpression does not rescue the locomotion impairment to the same extent as reducing *Tor* gene expression.

A complete rescue is obtained expressing *UAS-Atg5-eGFP* transgene in cholinergic neurons. Hara *et al.* (2009) have reported that mice deficient for *Atg5* in neuronal cells develop progressive deficits in motor function that are accompanied by the accumulation of cytoplasmic inclusion bodies in neuron (Hara *et al.*, 2006). They showed a typical axon swelling phenotype, characteristic of neurodegeneration, affecting cerebellar nucleus, cerebral cortex and nucleus gracile of *Atg5*$^{-/-}$ mice. Moreover, these results proposed a new role for ATG5 as a neuroprotective factor, as well as an autophagy protein (Kuma *et al.*, 2004; Hara *et al.*, 2006; Komatsu *et al.*, 2006). However its neuroprotective role might be dependent upon and linked to its function in autophagy activation.

Interestingly, *Atg5* null mice have similar phenotypes to *Adar*-null flies. It would be interesting to understand whether overexpression of the *UAS-Atg5* transgene in cholinergic neurons suppresses the neurodegeneration as well as the aberrant locomotion. Thus, *y, Adar*^{5G1}*, w; Cha-GAL4> UAS-Atg5-eGFP* flies (and its own controls: *y, Adar*^{5G1}*, w; Cha-GAL4*) were collected and aged for 30 days, sectioned and stained with H&E. Because of the expression of *UAS-Atg5-eGFP* transgene, the region of mushroom body did not show the typical vacuolization due to the lack of *Adar* gene. Also, the architecture of the retina of the *Adar*-null flies was restored and was morphologically similar to control flies.

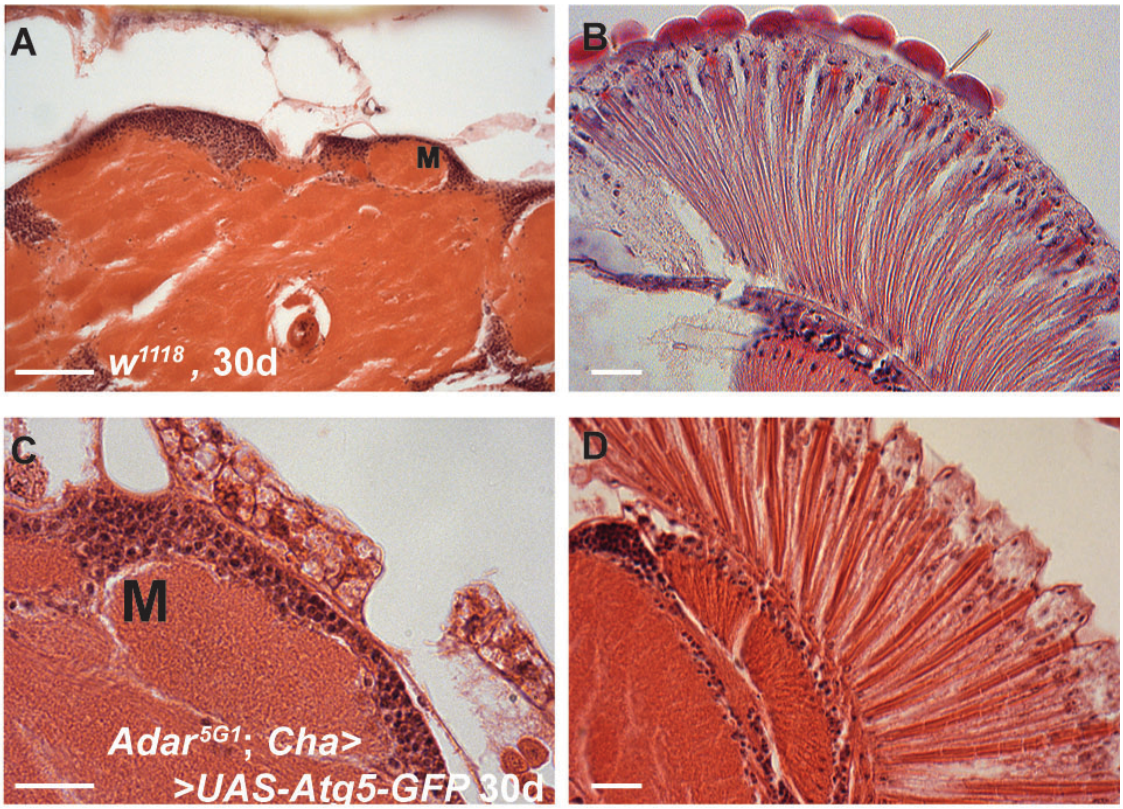


Figure 5.3: Increased *Atg5* expression suppressed the neurodegenerative phenotype affecting *Adar*^{5G1} mutant fly brain. Section of mushroom, M, calyx and retina of 30-days-old wild-type flies is shown in figure A and B, respectively. The expression of *UAS-Atg5-eGFP* transgene in the cholinergic neurons of 30-days-old *Adar*-null flies (*Adar*^{5G1}*;Cha>Atg5*) rescues the neurodegeneration affecting specifically MB calyx (C) and fly retina (D) of mutant animals. Scale bars in B, C and D: 20μm. Scale bar in A: 50μm

5.2.4 Validation of autophagy in 2nd instar larval brain.

The reduced viability, the locomotion impairment and the neurodegeneration, characteristic of *Adar*^{5G1} mutants are due to the lack of *Adar* gene. All of these phenotypes are suppressed by expression of the *UAS-Adar* transgene in the *Adar*-null background. These phenotypes are also rescued by increasing autophagy level. This is achieved by either reducing *Tor* gene expression, or increasing the expression level of autophagy factors such as *Atg1* and *Atg5* which induce autophagy. As previously mentioned autophagy controls steady-state of the cell. Either an excessive or an insufficient activation of autophagy can lead to cell death through self-digestion or own degeneration mediated by accumulation of damaged organelles, respectively. Consequently its activation is fine tuned especially in the case of stress, when the cell induces autophagy to overcome it but not excessively so as to prevent damage its cellular compartments.

The level of autophagy activation can be detected with cellular markers such as ATG8 and LAMP-2A (also known as lysosome-associated membrane protein type-2A) which is a transmembrane protein present on lysosome surfaces. When autophagy occurs, the autophagosome is fused with lysosomes generating autolysosomes that degrade all vesicle contents. ATG8, (also known as LC3 or MAP-LC3, microtubule-associated protein 1 light chain 3), is a major constituent of autophagosomes and is a marker for them. During autophagy, the cytoplasmatic form of ATG8 (LC3 I) is recruited to this autophagosome where LC3 II is generated by site specific proteolysis and lipidation. Because they are transient structures, ATG8/LC3II signal represents the autophagic activity. Another method to detect the autophagic activity in living cell is to use LysoTracker probes that label and track acidic organelles, such as lysosomes.

Taking advantage of these tools, the level of autophagy was detected in 2nd instar larval brain of *Adar*^{5G1} mutants. Brains of 2nd instar larvae were analysed to avoid picking larvae at the 3rd instar stage because these larvae undergo massive autophagy activation prior to metamorphosis. Thus, upon dissection, brains were stained with Lysotracker dye and analysed with a fluorescent microscopy. Compared to control brains, *Adar*^{5G1} larval brains show increased lysosomal puncta, a characteristic of autophagy activation (Figure 5.4 A-B). Examination of *Adar*^{5G1}*; Tor*^{p11218} larval brains, reveal that the number of dots increased significantly (Figure 5.4, C). This result supports the hypothesis that in the *Adar*^{5G1} background, the rescue observed by reducing *Tor* expression is mediated by increasing autophagy level.

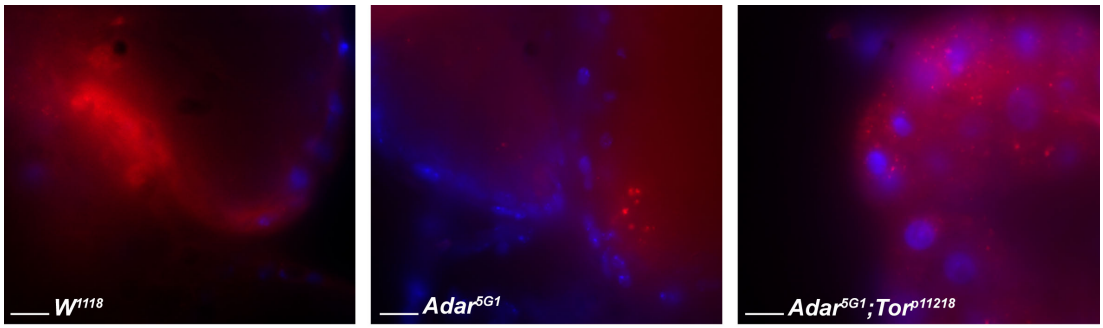


Figure 5.4: Lysotracker staining on larval brain. Ten second instar larval brains of wild-type, *Adar*^{5G1}, *Adar*^{5G1}*; Tor*^{p11218} animals were dissected and stained with LysoTracker® Red DND-99, Molecular Probes, Invitrogen (right, central and left panel, respectively). Dissected brains of wild-type animals do not show any specific lysotracker staining. Differently, *Adar*^{5G1} brains accumulate lysotracker dots that are increased in number in *Adar*^{5G1}*; Tor*^{p11218} animals. The images were captured using fluorescence microscope. Scale bars are shown in white: 20μm.

5.3 Discussion

In this chapter I have investigated which pathway downstram to *Tor* mediates the rescue of *Adar*-null phenotypes. In fact reducing *Tor* gene expression suppresses all phenotypes associated with the lack of ADAR. Moreover, the same effect is obtainable by increasing the autophagy activation rate by overespressing the *Atg* genes, *Atg1* and *Atg5*.

According to what has been previously reported the major known physiological functions of autophagy include: (1) degradation of misfolded proteins (Yorimitsu *et al.*, 2006), (2) removal of surplus or damaged organelles (Yen and Klionsky, 2008; Farre *et al.*, 2009), (3) generation of ATP in stressed or starved cells (Kahn *et al.*, 2005; Degenhardt *et al.*, 2006), (4) provision of nutrients during catabolism (Butterworth *et al.*, 1965; Butterworth *et al.*, 1988), (5) preservation of genomic stability (Mathew *et al.*, 2007) and (6) generation of signals for heterophagic removal of apoptotic corpses (Paludan *et al.*, 2005; Yano and Kurata, 2008; Yano *et al.*, 2008).

The ability of autophagy to defend cells against metabolic stress, through the generation of substrates to maintain macromolecular synthesis and ATP production, underlies its evolutionarily conserved role in promoting organism survival during a cellular stress.

The perturbation in *Adar*-null flies that arises because *Adar* gene is deleted is still unkown; however the complete rescue of all *Adar*-null phenotypes is dependent on (i) the reduction of *Tor* expression; (i) increased autophagy-activation rate; or (iii) on the expression of the active form of the enzyme *UAS-Adar3/4* (McGurk, 2007). Previous results suggest a new role for the ADAR protein in preventing neuro-degeneration which leads to the formation of vacuoles within the CNS of *Adar* mutant brain. This would suggest that when *Adar* is not longer present, the perturbation that arises can be suppressed by balancing the lack of a protein that prevents the degenerative phenotype. In other words, the perturbations are suppressed by increasing other pathways with the same protective effect such as autophagy. Intriguingly autophagy has a bypassing effect in rescuing *Adar*-null phenotypes; in fact the suppression is possible without restoring RNA editing transcripts.

As previously mentioned, the impaired and uncoordinated movement is the most difficult phenotype to suppress and requires the catalytically active form of the enzyme. Therefore locomotion was the phenotype I chosen to assay as this would allow me to assay for a complete rescue, which would require editing activity of the enzyme rather than the presence of *Drosophila* ADAR. However the inactive form of the protein, ADAR E/A, rescues the neurodegeneration, (personnel communication Leeanne McGurk).

It is not clear why the inactive form of the enzyme is sufficient to suppress and/or prevent the neurodegeneration. This might be due to it being able to bind specific mRNAs, in fact ADAR binds pre-mRNAs and this is can modulate RNA splicing (Grauso *et al.*, 2002b; Marcucci *et al.*, 2009) and processing of miRNAs (Heale *et al.*, 2009).

Alternatively, the rescue by the inactive protein might related to the presence of inositol-1,2,3,4,5,6-hexakisphosphate (Ins P_6) molecule within the deaminase domain. Ins P_6 molecule was first identified within the catalytic domain of adenosine deaminases acting on tRNA, ADATs, or on RNAs, ADARs. It is surrounded by 29 molecules of water and it is essential for the catalytic activity of the enzyme (Macbeth *et al.*, 2005). The amino acids that coordinate it, have been identified by (+) ion electrospray mass spectrometry and are thought to be critical for the folding of the ADAR and ADAT proteins (Macbeth *et al.*, 2005). *Drosophila* ADAR shares 62% of identity with human ADAR2. The amino acid sequence that is important for the coordination of Ins P_6 is also conserved. Interestingly, free Ins P_6 is important for a variety of cellular function such as RNA export, DNA repair, endocytosis and chromatin remodelling (York *et al.*, 1999; Hanakahi and West, 2002; Shen *et al.*, 2003; Steger *et al.*, 2003). Moreover it is known that Ins P_6 triggers calcium release from endomembrane stores (Lemtiri-Chlieh *et al.*, 2003). Therefore, is possible that a variation in the concentration of free Ins P_6 molecules might also serve as an intracellular messenger and ADAR expression and folding homeostatically regulates the intracellular Ins P_6 concentration.

CHAPTER 6: Identification of intracellular stress dependent on the lack of *Adar* gene

6.1 Introduction

Homeostasis is the ability of biological systems to adjust their internal physiological milieu in response to external or internal perturbation. In some cases when the perturbation persists, it becomes stress after a threshold has been reached. Autophagy is a housekeeping mechanism that can reflect a modified status of the cell. Its activation is tightly regulated because insufficient activation rather than excessive activation of autophagy leads to cell death. Autophagy has been extensively studied in the *Drosophila* fat body. The fat body is an important tissue because it can rescue its own stress cell-autonomously and it can provide sugars, lipids, amino acids and energy to support other tissues (non cell-autonomous response). It is a monolayer tissue, which has analogous functions to the liver and adipose tissue in mammals. This tissue responds when nutrients are in short supply through autophagy activation that sustains cell survival by maintaining ATP production. Furthermore it limits the accumulation of reactive oxygen species (ROS), degrading damage organelles such as mitochondria. During metamorphosis, larval fat cells undergo autophagy-mediated cell death highlighting the role of autophagy in development and cell differentiation. Recent studies emphasize the central role of the fat body in clearing infection through a complex inflammatory-like response involving both cellular and humoral defences (DiAngelo *et al.*, 2009). This tissue synthesizes and secretes antimicrobial peptides into hemolymph controlling the fruit fly's humoral response to antimicrobial infections (Hoffmann and Reichhart, 2002). Autophagy is a bulk degradation pathway that fat cells use to maintain their homoeostatic state. Fat cells are involved in both cell autonomous and non-cell autonomous stress-induced response in *Drosophila*. In this chapter I was keen to elucidate which stress affects $Adar^{5G1}$ mutant flies that can be suppressed by up-regulating autophagy activation. I tested whether there are increased reactive oxygen species (ROS), or a variation of intracellular calcium concentration and finally the ER-mediated unfolded protein response. Hence three brief paragraphs that show the correlation between those stresses and autophagy.

6.1.1 Reactive oxygen species (ROS)

Low ROS levels, specifically H_2O_2, can act as signaling molecules to oxidizing factors in a variety of pathways that lead to growth and survival. For example one oxidizing factor is the essential autophagy protein Atg4 (Scherz-Shouval *et al.*, 2007). Atg4 acts both as a conjugating and deconjugating enzyme during the autophagosome extension step and therefore its activity is thought to be tightly regulated. Following the initial cleavage of Atg8-like proteins, Atg4 must become inactive so as to ensure the conjugation of Atg8 to the autophagosomal membrane (Scherz-Shouval *et al.*, 2007). Later on, as the autophagosome fuses with the lysosome, Atg4 can be locally re-activated in order to delipidate and recycle Atg8. The delipidating activity of Atg4 is regulated through changes in redox potentials that take place under different conditions and at specific subcellular microenvironments.

High levels of ROS are also deleterious to cells resulting in programmed cell death (Jabs, 1999; Lee *et al.*, 2003; Macip *et al.*, 2003). For example malignant glioma cells treated with exogenous H_2O_2 activate autophagy as a survival pathway (Zhang *et al.*, 2009a). Even superoxide, O_2^-, which is generated in the mitochondria, has a role in the regulation of autophagy. In fact increased production of O_2^- leads to mitochondria damage and their selective degradation via autophagosomes (Kim *et al.*, 2007; Chen *et al.*, 2009). This process is called mitophagy and O_2^- is the predominant signaling molecule. O_2^- production is induced by prolonged starvation/glucose and amino acid deprivation which induces autophagy similar to H_2O_2 (Scherz-Shouval *et al.*, 2007). Superoxide is formed from oxygen, and is a by-product of respiration in the mitochondria. O_2^- is then converted by superoxide dismutase, SOD, into hydrogen peroxide, which is further detoxified into H_2O and O_2 by catalase or peroxidases (Oxidative Stress Relationship with Exercise and Training, 2006).

Several studies have shown that hypoxic exposure followed by perfusion induces free radical oxidative damage mediated by membrane lipid peroxidation (Wilhelm and Herget, 1999; Vesela and Wilhelm, 2002) and hydrogen peroxide seems to play a central role (Russell and Jackson, 1994; Kinnula *et al.*, 1995). To prevent this damage, cells

rapidly activate a variety of hypoxic-responsive adaptive mechanisms to reduce energy-intensive processes such as protein translation (Wouters *et al.*, 2005; Liu *et al.*, 2006). Furthermore in response to hypoxia, the *Redd1* gene and its *Drosophila* orthologs *Scylla* and *Charybdis* are overexpressed (Ellisen *et al.*, 2002; Sofer *et al.*, 2005; Reiling and Sabatini, 2006). REDD1 protein binds 14-3-3 inducing the dissociation of the inhibitory 14–3–3 from TSC2 and consequentially TORC1 inhibition. Therefore REDD1 specifically prevents PI3K/AKT-induced TSC2/14–3–3 association and TORC1 activation (DeYoung *et al.*, 2008). Hence in response to hypoxia, *Redd1* expression is increased and its binding to 14-3-3 drives the activation of TSC2 that inhibits TORC1 activity, blocking the activity of Rheb. This reduces the general level of cap-mediated protein translation and boosts autophagy activation (Figure 6.1).

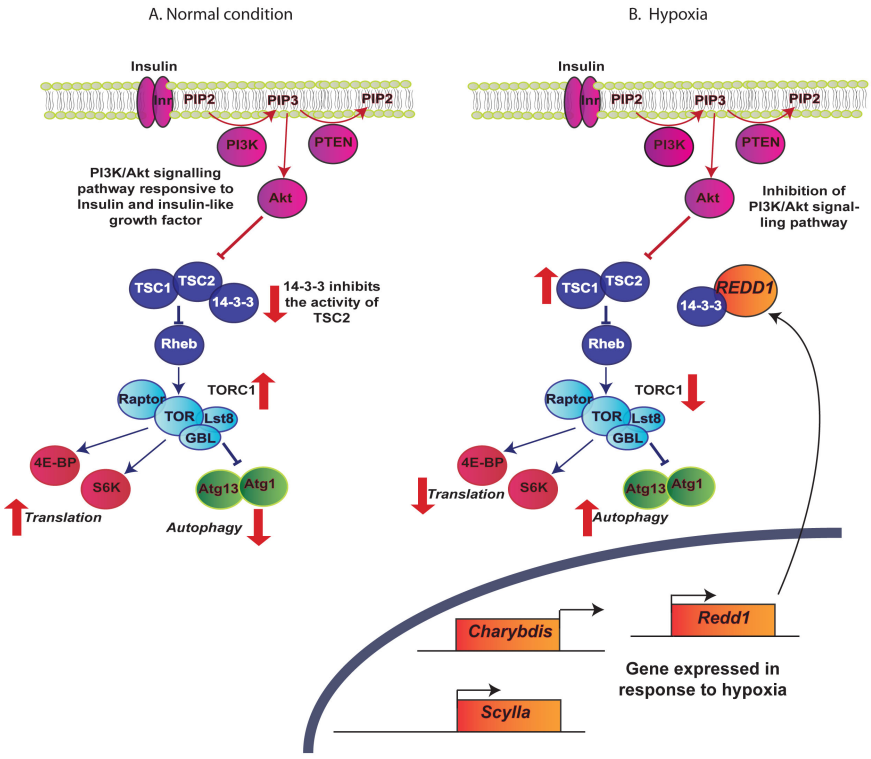


Figure 6.1: ROS responsive signaling pathway. Jnk pathway is activated by increased levels of reactive oxygen species (ROS). Under normal condition (A), the TSC1/TSC2 complex is decreased by the inhibitory effect of 14-3-3 on TSC2 activity. Therefore the activity of TORC1 complex is positively regulated and the cap-mediated translation takes place, while autophagy is inhibited. Under hypoxic conditions (B), Jnk pathway is activated. Therefore the hypoxia-responsive genes are expressed. Among them, *Redd1* gene expression is increased. REDD1 interacts with 14-3-3 and hence autophagy is positively regulated and can degrade intracellular organelles that have been damaged by increased ROS levels such as mitochondria and lipids.

6.1.2 Modification of intracellular free-Calcium concentration $[Ca]_i$

Calcium is an essential second messenger and its concentration has to be tightly regulated. It is stored within the ER where calcium mobilization induces autophagy through the calcium-dependent activation of AMP activated protein kinase, AMPK (Hoyer-Hansen *et al.*, 2007). This kinase is usually activated in response to ATP depletion by AMPK kinase (AMPKK)-mediated phosphorylation (Hawley *et al.*, 1995). In turn AMPKK activity is regulated by LKB1 (Hawley *et al.*, 2003) which is constitutively active (Lizcano *et al.*, 2004). AMPKK activation is also mediated by upstream calcium/calmodulin kinase kinase b, (CaMKK) in response to an increased in intracellular calcium concentration (Hawley *et al.*, 2005; Hurley *et al.*, 2005). AMPK signalling then inhibits the activity of TOR (Hoyer-Hansen and Jaattela, 2007) upon TSC2 phosphorylation (Figure 6.2A). This induces autophagy in TOR-dependent manner. Calcium also leads to an accumulation of Atg8-GFP foci suggesting that autophagosome formation is also caused by calcium. Further evidence supporting a direct role for calcium in the induction of autophagy was the finding that calcium phosphate precipitates induce autophagy in a Beclin-dependent manner (Gao *et al.*, 2008). Beclin forms a complex between the class III PI3 kinase Vps34 and p150, which facilitates assembly of the autophagosome (Sinha *et al.*, 2008).

However autophagy can be induced by lowering intracellular inositol or inositol 1,4,5-trisphosphate (IP_3) levels (Sarkar *et al.*, 2005) in a TOR-independent manner (Figure 6.2B). It is possible to modulate production of intracellular cAMP via adenylyl cyclase (AC) activity, cAMP acts on *Epac* which then activates a small G-protein Rap2B. Rap2B-GTP activates the phosphoinositide-specific phospholipase C, PLC-ε, converting phosphatidylinositol (4,5)-bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG). IP_3 induces the release of Ca^{2+} from the ER stores inhibiting autophagy activation. Therefore autophagy can be induced with the adenylyl cyclase inhibitor, 2'5'-dideoxyadenosine which decreases the levels of intracellular cAMP. Additionally the same effect can be achieved with carbamazepine (CBZ) and valproic acid (VPA) that

inhibit the inositol monophosphatase (IMPase). This enzyme converts inositol monophosphate (IP_1) into Ins, essential for PIP_2 synthesis (Maeda and Eisenberg, 1980). Consequently, IP_3 releases Ca^{2+} from the ER stores. Intracytosolic Ca^{2+} levels are also increased by L-type Ca^{2+} channel agonists. The L-type calcium channel is a type of voltage-dependent calcium channel, where "L" stands for long-lasting, referring to the length of activation. An increase in intracytosolic Ca^{2+} activates a family of Ca^{2+}-dependent cysteine proteases called calpains, which cleave and activate $G_{s\alpha}$. Activation of $G_{s\alpha}$, in turn, increases adenylyl cyclase activity to elevate cAMP levels, thereby forming a feed-forward loop (Sato-Kusubata *et al.*, 2000). For instance activation of TOR-independent pathway increases free calcium ions and also inhibits autophagy (Williams *et al.*, 2008).

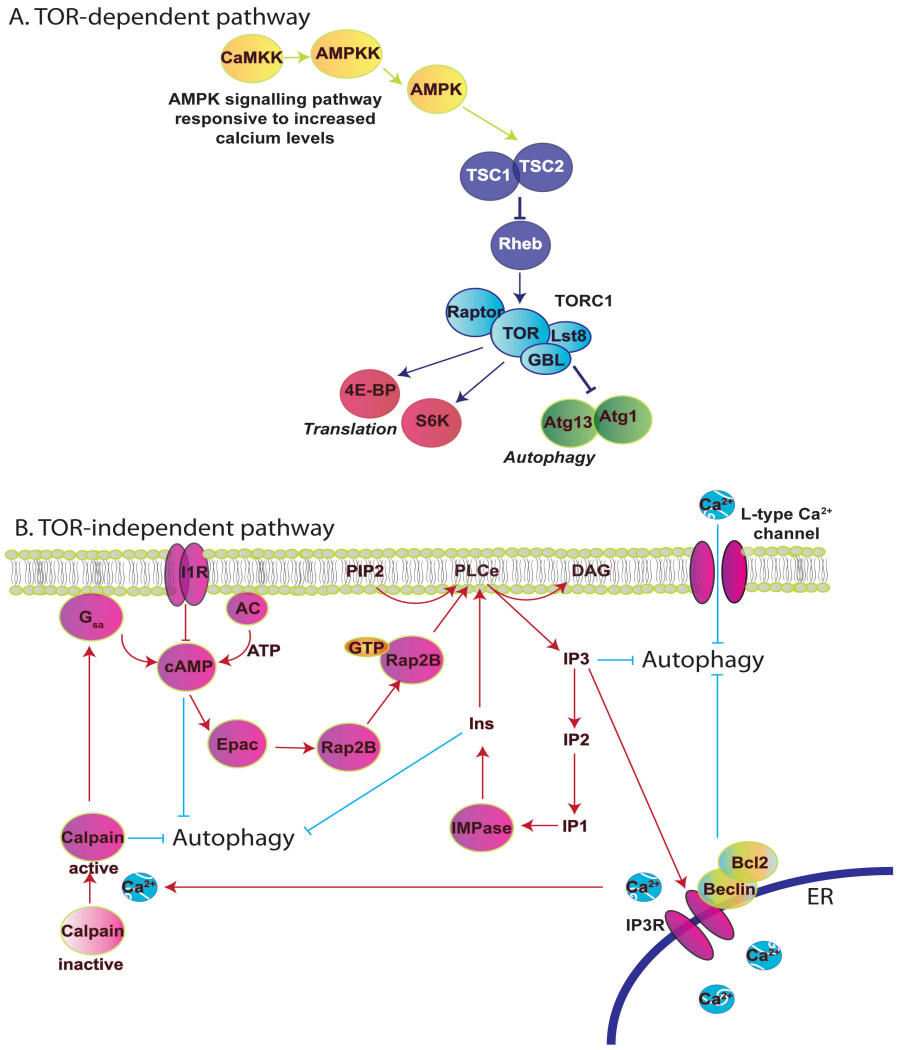


Figure 6.2: Calcium signaling pathways and autophagy. Variation in intracellular calcium concentration regulates autophagy activation through two distinct pathways, known as TOR-dependent (A) and TOR-independent (B) pathway. In the first case, increased free calcium activates calcium-calmodulin kinase kinase (CaMKK) that induces a decrease in TORC1 complex via AMPK activation. This results in enhanced autophagy activation in response to increase in intracellular calcium concentration via TOR kinase regulation. However autophagy and calcium signaling have been also linked through a different pathway that is independent of TOR kinase (B). In this case calcium inhibits autophagy; furthermore calcium activates calpain, a calcium-dependent, non-lysosomal cysteine proteases that also blocks autophagy. Nevertheless even Ins, and IP3, negatively regulate autophagy. In particular IP3 generates a feed forward loop to block autophagy. When it interacts with IP3 receptor (IP3R) releases calcium from the ER.

6.1.3 Detection of ER stress

The ER is an extensive network of cisternae held together by the cytoskeleton. The phospholipid membrane encloses the cisternal space (or lumen), which is connected to the perinuclear space. There are three types of ER known as the rough ER, smooth ER and sarcoplasmic reticulum (SR). The peculiar appearance of the rough ER (RER) is due to ribosomes however they are not a stable part of this organelle. Instead, a ribosome binds to the RER only when it begins to synthesize membrane proteins or proteins destined for the secretory pathway (Alberts, 2002). The RER and smooth ER (SER) are usually interconnected and the proteins and membranes made by the RER move into the SER to be transferred to other locations. The SER functions in several metabolic processes, including synthesis of lipids and steroids and their metabolism, metabolism of carbohydrates, regulation of calcium concentration and drug detoxification (Alberts, 2002). SR is a special type of smooth ER found in smooth and striated muscle. The fundamental difference between it and the other ERs is that the SR stores and pumps calcium ions. The SR contains large stores of calcium, which it sequesters and then releases when the muscle cell is stimulated thereby playing a major role in muscle excitation-contraction coupling (Alberts, 2002). In summary, the ER serves two major functions in the cell; it facilitates the proper folding of newly synthesized proteins destined for secretion, cell surface or intracellular organelles and it provides the cell with a Ca^{2+} reservoir. Persistent perturbations in the cellular environment normally result in an increase in protein synthesis, followed by an ER stress response which controls protein folding. The production of large amounts of misfolded proteins that exceed the functional capacity of the organelle triggers a physiological response in the cell, collectively known as the unfolded protein response (UPS). Inositol requiring 1 (IRE1) and dsRNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase, PERK, are two type I transmembrane protein kinase-endoribonucleases that act as sensors for the unfolded protein within the ER (Shamu and Walter, 1996; Liu *et al.*, 2002). In an inactive state the luminal domains of IRE1 and PERK are associated with BiP (Bertolotti *et al.*, 2000; Liu *et al.*, 2002; Liu and Kaufman, 2003). Upon ER stress, the huge excess

of unfolded proteins in the ER lumen dissociates BiP from the luminal domains of IRE1 and PERK. Subsequent IRE1/PERK oligomerizes which activates the endoribonuclease activity of IRE1 thereby catalyzing the unconventional splicing of *hac-1* pre-mRNA. The splicing of *hac-1* pre-mRNA allows its translation (Ruegsegger *et al.*, 2001). HAC-1 acts as a transcription factor, specifically binding UPR promoter element (UPRE) DNA motif to induce expression of proteins that help to alleviate ER stress (Mori *et al.*, 1992; Mori *et al.*, 1998). In *C. elegans*, *Drosophila melanogater* (Plongthongkum *et al.*, 2007) and mammals, IRE-1 plays a similar role in regulating the splicing of *Xbp1* which is the functional homolog of *Hac-1* in yeast (Figure 6.3).

ER stress affects autophagy-related genes which are evolutionarily conserved and are indispensable for autophagy. In yeast, the transcription factor Hac-1 transactivates *Atg8* during UPR (Bernales *et al.*, 2006). Furthermore mutations in ER stress-related proteins such as PERK can inhibit autophagy (Kouroku *et al.*, 2007). Also both thapsigargin and tunicamycin, which stimulate ER stress, also stimulate autophagy (Ogata *et al.*, 2006).

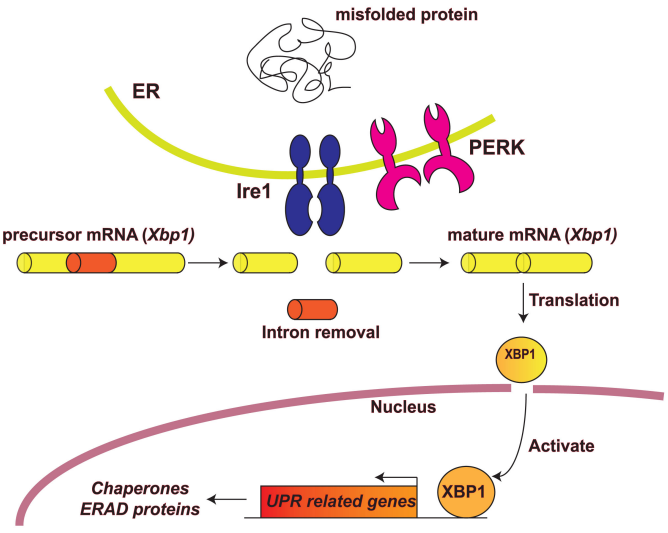


Figure 6.3: Activation of the signaling pathway in unfolded protein response. ER functions as storage for calcium it also facilitates folding of new synthesized proteins destined for secretion, cell surface or intracellular organelle. An increase in misfolded proteins induces the oligomerization of Ire1 receptor with the subsequent activation of its cytosolic endoribonucleases domain which catalyses the unconventional splicing event of *Xbp1* pre-mRNA. The mature mRNA of *Xbp1* is then translated and the protein is imported into the nucleus where promotes the expression of UPR-related genes, such as chaperones which assists the protein folding process and ER associated protein degradation (ERAD).

6.2 Results

6.2.1 Adar-null fat body and autophagy

Given the interaction between autophagy and the diverse functions which fat cells are responsible for, I hypothesised that this organ might help to elucidate which stress underlies the *Adar*-null phenotypes. Therefore, I first confirmed that the basal level of autophagy is increased in these cells. The fat body was dissected and stained with Lysotracker dye from ten GFP-negative *y,Adar^{5G1},w;Collagen-GAL4/Collagen-GAL4;+/+* male larvae. Wild-type larvae were used as controls. After staining, live tissues were immediately analyzed by fluorescent microscope.

Adar^{5G1} fat cells show a significant increase in Lysotracker staining (Figure 6.4 panel D) confirming what was previously observed in larval brains. However the level of autophagy enhancement in fat cells is much higher (comparison between figure 6.4 panel D and figure 5.4 panel B). This would suggest that fat body is more sensitive to the lack of *Adar* than the larval nervous system. This result might indicate that the fat body might respond to the stress that arises by the lack of the RNA editing enzyme by activating the main cellular pathway, which controls fluctuations in cell metabolism. However it is also possible that the fat cells are specifically affected by the lack of this enzyme. The first hypothesis would confirm the essential role of fat cells in mediating stress-induced metabolic adaptation and damage control through autophagy activation.

These data also suggest that ADAR it is not only important for the central nervous system, but also for other tissues. This is a far more intriguing possibility because most of the transcripts edited by ADAR are expressed in the central nervous system. In fact accordingly to ModENCODE database, only a few transcripts are edited within the fat body. Therefore, the lack of ADAR may affect fat cell homeostasis, suggesting that the enzyme has other unknown proprieties than its canonical function. Hence it would be

interesting to investigate whether the expression of *dAdar* in fat cells in the *Adar*-null background reduces autophagy activation.

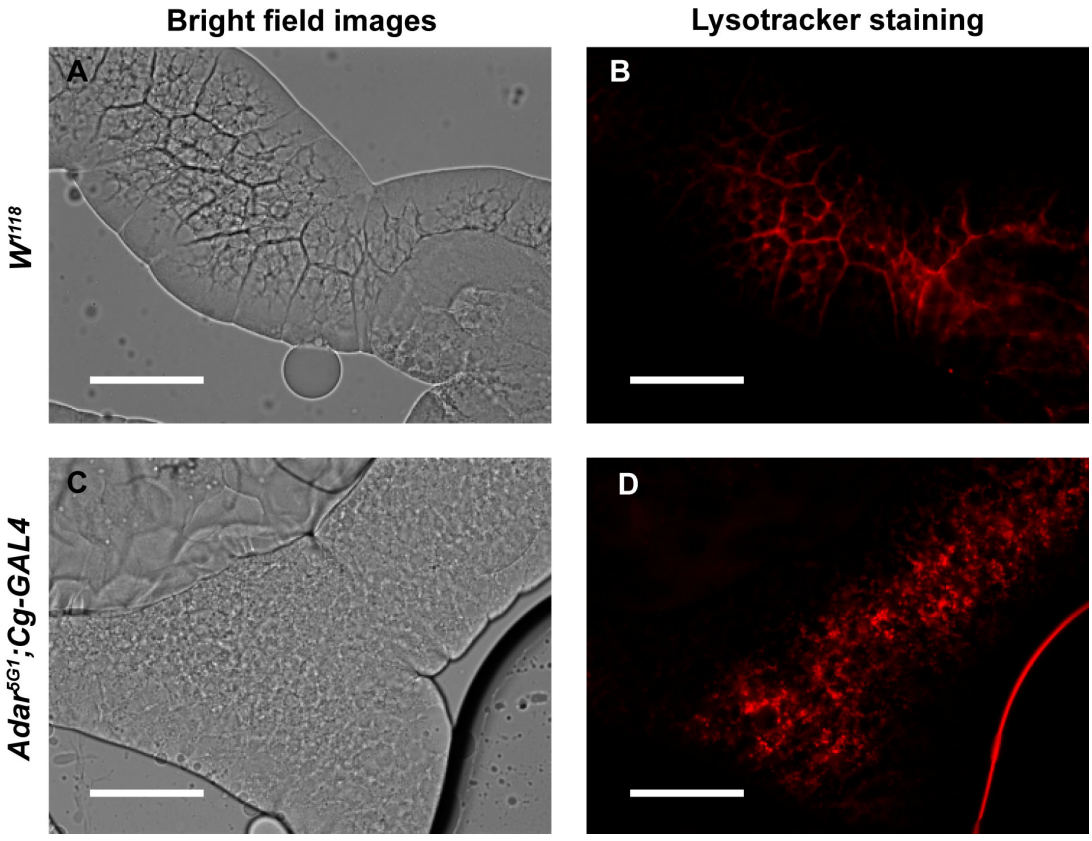


Figure 6.4: Lysotracker staining of *Adar*-null fat body. Lysotracker dye detects acidic vesicles such as lysosomes. Wild-type (A-B) and *Adar*-null (C-D) fat body were dissected and stained with Lysotracker dye, B and D respectively. Wild-type fat cells have a background staining that localizes with the cell membrane (B), $Adar^{5G1}$ fat body have an increased number of specific dots indicating enhanced autophagy activation. The bar scale is shown in white (50 μm).

The lack of *Adar* gene causes an increased activation rate of autophagy and it has been confirmed in mutant larval brain and fat body. This result was expected for the larval brain as most of the transcripts that ADAR edits are expressed in the central nervous system and are involved in its development, function and homeostasis (Stapleton *et al.*, 2006). However, *Adar*-null fat cells also have an autophagy-related phenotype, even though there are few edited transcripts present in this tissue. Physiologically, the fat

body controls lipid and sugar homeostasis and provides ATP molecules in the case of energy depletion. Therefore the effect observed in fat body might be dependent to a non-cell autonomous response of this tissue to a systemic and physiological stress. As previously mentioned, most of transcripts ADAR edits encodes voltage- and ligand-gated ion channel. Editing which results in re-coding of ion channels pre-mRNAs can influence the resting potential and the membrane repolarization of given neurons. *Shaker* is one example, it encodes a voltage-gated potassium channel and failure to edit *Shaker* transcripts changes the affinity of the pore resulting in channels that either fail to inactivate or inactivate incompletely (Hoopengardner *et al.*, 2003). Therefore more current passes through the pore of the channel in comparison to wild-type channels. This can result in variations in membrane potential of neurons and this might be a source of stress leading to neuronal cell death where it persists. The sodium-potassium exchange pumps make a major contribution to establishing and restoring the membrane potential (Kandel, Fourth edition). This is a complex of proteins embedded in the membrane that derive energy from ATP in order to maintain the correct concentration of ions between intra- and extra-cellular compartments. Hence neurons can adjust the internal concentration of ions in response to this perturbation by increasing the demand and uptake of sugars (Sokoloff *et al.*, 1977).

Drosophila adapts its energy requirement to its nutritional status by metabolic regulation, specifically through sugar and lipid homoeostasis. Sugar levels are maintained by neurosecretory cells located in the brain and ring gland that release adipokinetic hormone (AKH, the insect glucagons) and insulin into the haemolymph (Gade and Auerswald, 2003; Wu and Brown, 2006; Geminard *et al.*, 2009). AKH acts on fat cells that are the main storage reserve in which sugar and lipids accumulate. Here, the hormone activates glycogen phosphorylase enzyme, which converts glycogen in trehalose, an insect sugar, and is subsequently released in the haemolymph. It is also possible that a substantial demand for sugar leads to a massive mobilization of glycogen through the activation of glycogen autophagy. This is a special type of autophagy that is designated to the breakdown of cell glycogen within autophagic vacuoles. This process includes the sequestration of polysaccharide in the autophagosomes and its subsequent

degradation in the autolysosomes (Hers, 1964; De Duve and Wattiaux, 1966; Smith and Winkler, 1968). In order to elucidate whether the increased activation of autophagy in fat cells reflects a non-cell autonomous response of the fat body to a systemic stress, the expression of the *UAS-dAdar3/4* transgene was driven in *Adar*-null fat cells with the strain *y,Adar^{5G1},w^{-}/Fm7,P{GAL4-Kr.C}DC3,P{UAS-GFP.S65T}DC7;Collagen-GAL4/Collagen-GAL4;+/+*. The *UAS-dAdar3/4* construct encodes an isoform of dADAR which is abundantly expressed from the pupal stage and is catalytically active. If *Adar*-null fat cells respond in a non-cell-autonomous manner then the overexpression of ADAR protein would not rescue the autophagy phenotype because the stress is not suppressed. Therefore, *y,Adar^{5G1},w^{-};Collagen-GAL4>UAS-dADAR3/4* male larvae were selected, the fat body was dissected, stained with Lysotracker dye and analyzed by fluorescence microscopy. Surprisingly, the expression of *UAS-dAdar3/4* transgene was able to rescue the autophagy phenotype in the fat cells of *Adar*-null flies (figure 6.5 B' and C'). Therefore the fat body responds cell-autonomously to the lack of *Adar* and to the stress that arises from it, even though fat cells do not apparently express any essential transcripts that are edited by ADAR. Thus, ADAR protein might be sufficient to rescue the stress in this tissue, independently of its RNA editing activity. If this hypothesis was true it would a novel function for this protein within the fat body and the possibility to investigate it at a molecular and cellular level. To test this hypothesis, the expression of the inactive *UAS-dAdar3/4 E/A* transgene was driven within the *Adar*-null fat body. The fat body of *y,Adar^{5G1},w^{-};Collagen-GAL4>UAS-dAdar3/4 E/A* larvae were dissected and stained with Lysotracker dye and images of the tissue analyzed with fluorescence microscopy. The inactive form of dADAR was able to suppress the Lysotracker staining and hence the atypical autophagy activation (Figure 6.5 B' and D'). This result resembles what has been previously observed in aged adult brains (McGurk, 2007) confirming that the inactive form of dADAR is able to rescue the phenotypes due to the lack of *Adar,* such as an increased rate of autophagy activation in fat body. Moreover, this tissue is primarily affected by the absence of dADAR rather than being just a supportive tissue that responds to a systemic stress. Therefore the enhanced autophagy reflects the cell-autonomous response of the fat body due to the lack of the

enzyme. Additionally, the catalytic activity is not necessary and the expression of the catalytically inactive mutant is sufficient to prevent the stress.

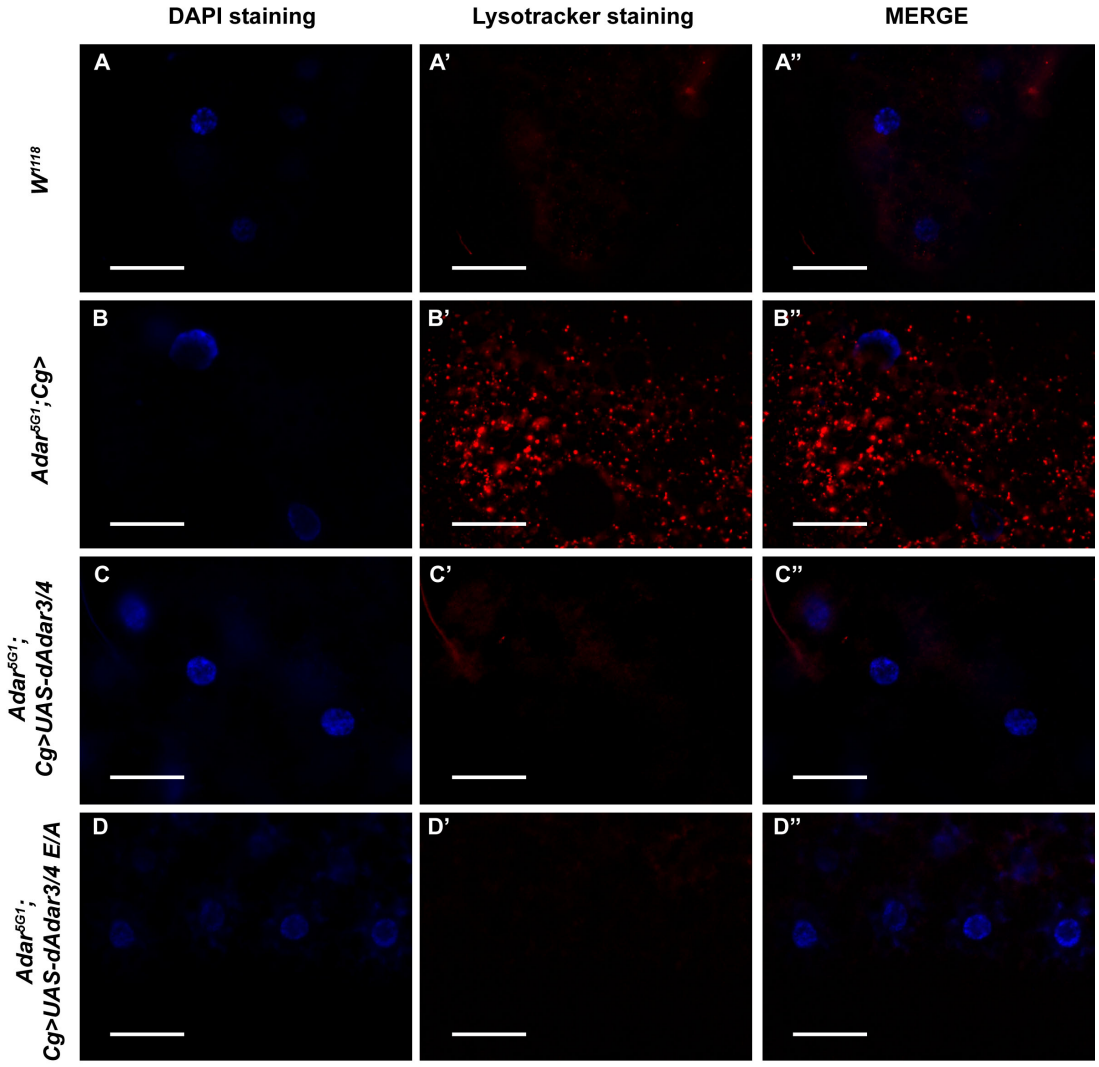


Figure 6.5: ADAR proteins rescue the autophagy-related phenotype in *Adar*-null fat cells. Wild-type (lane A), *Adar*^{5G1}*;Cg>* (lane B), *Adar*^{5G1}*;Cg>UAS-dAdar3/4* (lane C) and *Adar*^{5G1}*;Cg> UAS-dAdar 3/4 E/A* (lane D) fat body have been dissected and stained with DAPI (A, B, C and D) and lysotracker (A', B', C' and D') dyes. The merge of the two staining is shown (A'', B'', C'', and D''). Wild-type fat body does not have any lysotracker staining (A'and A''). *Adar*-null fat cells have an increased activation of autophagy (B' and B''). The expression of *dAdar* transgene, *UAS-dAdar3/4*, within the fat cells is sufficient to rescue the autophagy-related phenotype (C' and C''). The same result is obtained with the catalytically inactive isoform of dADAR protein, *UAS-dAdar3/4 E/A* (D' and D''). Scale bar is shown in white (50 μm).

6.2.3 Autophagy and the stress mediated by reactive oxygen species, ROS

Homeostasis is the ability of biological systems to adjust their internal physiological milieu in response to external or internal perturbation. In some cases when the perturbation persists, it becomes stress after a threshold has been reached. Autophagy is a housekeeping mechanism that can reflect a modified status of the cell. Its activation is tightly regulated because insufficient activation rather than excessive activation of autophagy leads to cell death. Autophagy has been extensively studied in the *Drosophila* fat body. I have demonstrated that the deletion of *Adar* enhances autophagy activation within fat body; furthermore this phenotype was suppressed by expressing either the active or the inactive form of *dAdar*. Therefore fat body responds cell-autonomously to a lack of *Adar* mediated stress. Unfortunately, the stress affecting *Adar*-null flies has not yet been identified. Therefore I investigated whether there was a correlation between an increase in different stresses and increased autophagy in *Adar* deficient flies.

The recovery from anoxic stupor is significantly prolonged when *Adar* gene is mutated. This is due to the absence of editing of channels encoding pre-mRNAs and leads to neuronal degeneration in aged flies (Ma *et al.*, 2001). In fact *in vitro* electrophysiologic studies have shown that these membrane proteins, especially the voltage-gated Na^+ and Ca^{2+} channels, play a pivotal role in nerve cell injury caused by O_2 deprivation (Choi and Rothman, 1990; Haddad and Jiang, 1997; Kimura *et al.*, 1998; Vornov, 1998). Furthermore *Adar* mutants are very resistant to oxidant injury and they recover their evoked potentials after anoxia much slower than wild-type flies (Ma *et al.*, 2001). Therefore longer exposure to hypoxic condition might lead to an increase in ROS, and therefore increase autophagy to prevent any dangerous accumulation of stressful agents. Thus the reduction of *Tor* expression removes the 'brake' to better rescue accumulated ROS.

The variations of intracellular ROS concentration were assessed with two redox-sensitive fluorescent probes: 2',7'-dichlorofluorescein (H2DCF) which is specific for H_2O_2 (Sundaresan *et al.*, 1995; Mills *et al.*, 1998) and dihydroethidium (DHE) which is

specific for O_2^- (Bindokas *et al.*, 1996; Li *et al.*, 2003; Rivera and Maxwell, 2005). These two fluorescent probes were used to investigate whether in a deletion of the *Adar* gene there is an accumulation of ROS.

The fat body of *y,Adar*^{5G1}*,w*$^-$*;Collagen-GAL4* male larvae were dissected and stained with both dyes. As a positive control the expression of the complex I protein NaDH Dehydrogenase subunit 75 (ND75) was reduced by driving the expression in the fat body of an RNAi construct made specifically against it with a *Collagen-GAL4* driver. It was previously observed that reduction of ND75 increases ROS in fat body, mimicking the effect of rotenone that inhibits mitochondria complex I (Owusu-Ansah, 2008). The images were capture with a fluorescent microscopy. As shown in figure 6.6 (panel B and C) *Adar*-null fat cells do not show any detectable staining using either H2DCF or DHE fluorescent dye. Therefore the absence of *Adar* gene does not increase the ROS production, which triggers an increased rate of autophagy activation.

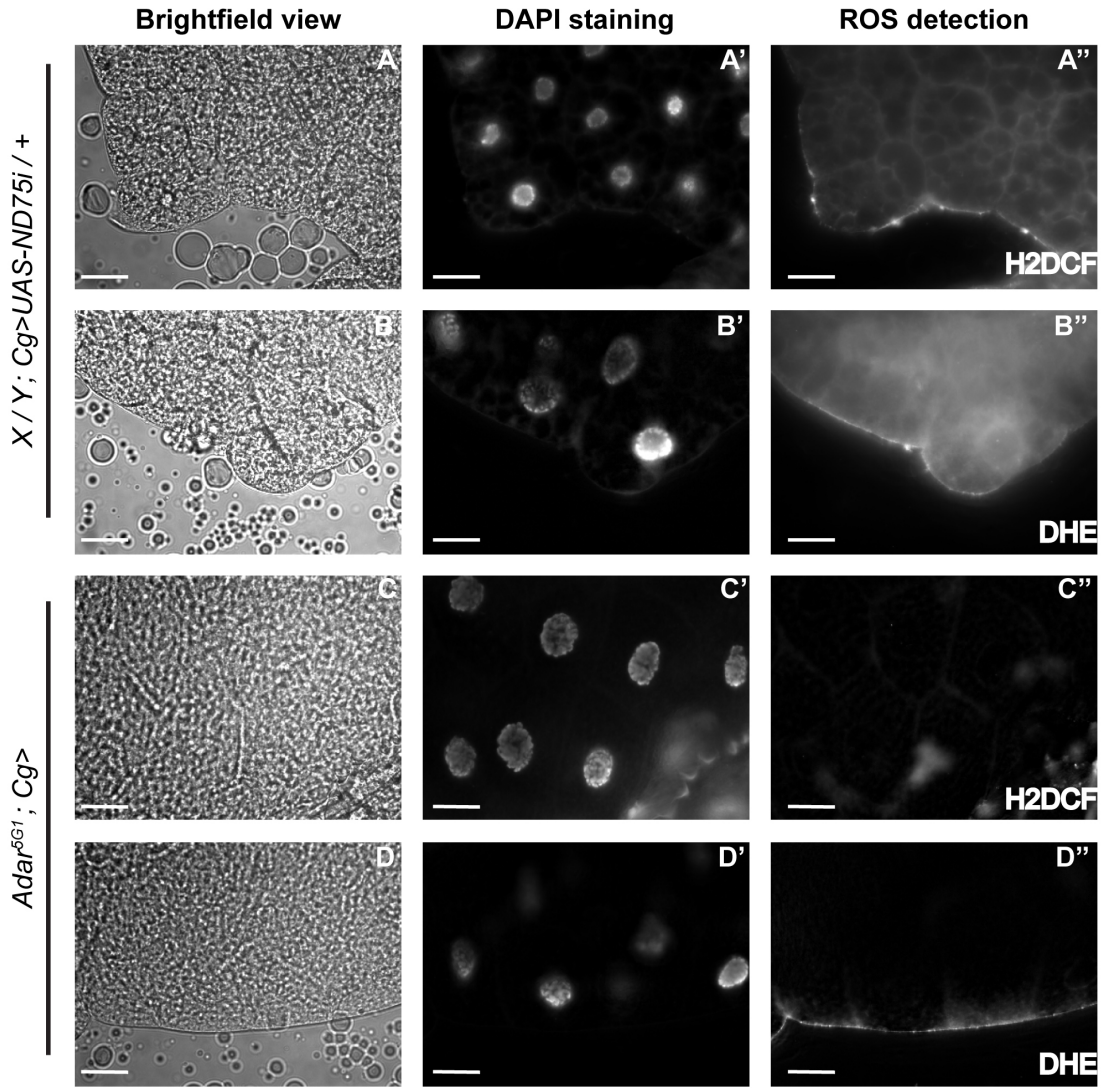


Figure 6.6: No detectable increased ROS levels in *Adar*^{5G1} fat body. Lanes A and B show fat body dissected from *Cg-GAL4>UAS-ND75i* larvae. The knock down of ND75 expression leads to an increased ROS levels that can be detected using H2DCF (A'') and DHE (B'') dyes. *Adar*^{5G1}*;Cg>* fat body (lane C and D) do not show increased ROS level using either H2DCF (C'') and DHE (D'') dyes. The first set of images (A, B, C and D) shows bright field of fat cells. A', B', C' AND D' show DAPI staining and A'', B'', C'' and D'' show fluorescent detection with ROS indicator dyes. Scale bar is shown in white (50um).

6.2.3 Autophagy and calcium

It has been previously described that likely Ca^{2+} can have an opposite effect on autophagy induction. This possibly depends on its concentration as low calcium levels can act as an internal messenger. A persistent accumulation of free calcium can be dangerous as it triggers a stress-mediated response (Zhang, 2010). In *Adar* mutant flies autophagy is enhanced when compared to wild-type flies. This could be due to an increased level of calcium and activation of the AMPK/TOR-dependent pathway. However, it is also possible that calcium concentrations rise because of the activation of a TOR-independent-pathway feed-forward loop. This pathway does not induce autophagy *per se* but constantly releases calcium. However a constant accumulation of calcium can be an important stress factor for the cell and may result in the activation of autophagy. In both of these cases reduced *Tor* gene expression boosts autophagy activation to reduce the stress.

To test whether variation in the concentration of calcium causes stress in *Adar*-null flies I took advantage of the transgenic fly strain that express *UAS-Camgaroo* reporter. This reporter is based on a GFP–calmodulin fusion (Baird *et al.*, 1999), where four free calcium ions bind the calmodulin domain inducing a conformational change in the fusion protein thereby activating and enhancing GFP fluorescence. *Camgaroo* reporters have previously been successfully used *in vivo* in flies to monitor neural activity (Yu *et al.*, 2003; Reiff *et al.*, 2005). The expression of *UAS-Camgaroo* transgene was driven in the *Adar*-null fat body with *y,Adar^{5G1},w^{-} / Fm7,P{GAL4-Kr.C}DC3,P{UAS-GFP.S65T}DC7;Collagen-GAL4/Collagen-GAL4;+/+* fly strain. The expression of the *UAS-Camgaroo* transgene does not show an increase of calcium concentration in *Adar^{5G1}* fat cells as for no difference has been observed with the control fat body. Fat cells of *y,Adar^{5G1},w^{-};Collagen-GAL4>UAS-Camgaroo2* male larvae were also dissected and immuno-stained with anti-GFP antibody to enhance the signal. In fact the signal depends on the conformational change of the transgene mediated by the binding of four calcium ions. Therefore upon calcium binding, the GFP antibody might specifically

recognize the GFP activated domain of the transgene. However no signal was still detected (Figure 6.7 B). This might mean that the transgene was not expressed or that there was no an increased intracellular calcium concentration. Therefore I tested the $[Ca]_i$ using the high-affinity calcium indicator; Fluo-4 AM. Fluo-4 AM is essentially nonfluorescent due to the intact acetoxymethyl (AM) ester derivative, unless it binds to Ca^{2+} (Figure 6.7 D).

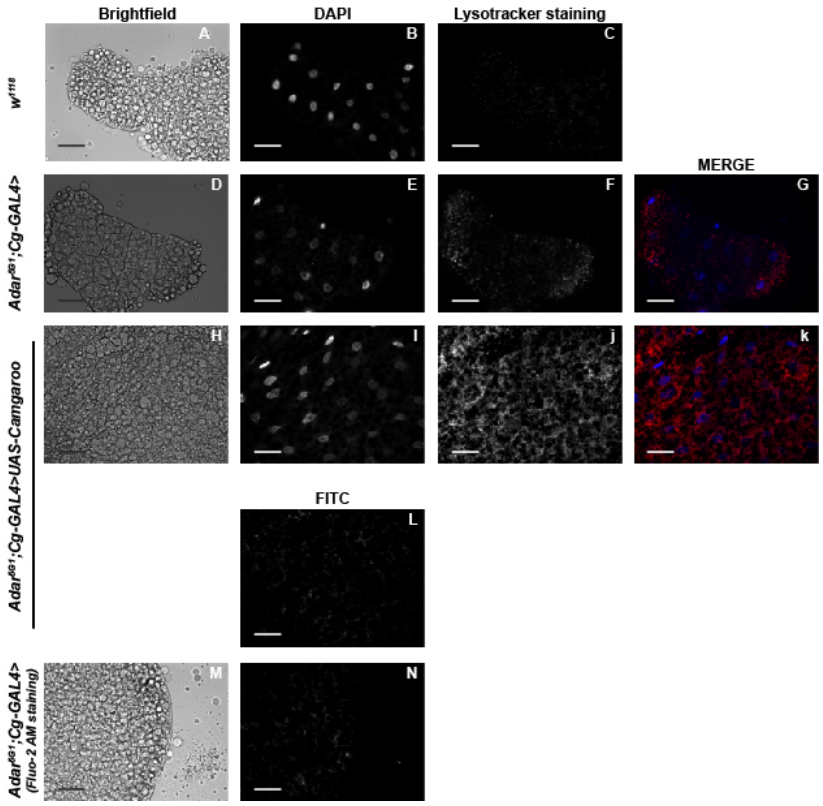


Figure 6.7: Variation of intracellular calcium concentration in *Adar*- null fat cells. *Adar*-null fat body (panel D-G) has increased autophagy activation (F and G). To test whether the stress that arises from the lack of ADAR enzyme is dependent to an alteration of intracellular calcium concentration, the *UAS-Camgaroo* reporter was driven in *Adar*-null larval fat cells (panels H-L). Therefore *Adar*^{5G1}*;Cg-GAL4/UAS-Camgaroo* fat body were dissected and stained not only with lysotracker dye (panel J) but also with anti-GFP antibody (L). *Adar*^{5G1}*;Cg-GAL4/UAS-Camgaroo* cells that are positive for lysotracker (J) do not show an increased of calcium concentration (L). The same result is showed staining *Adar*^{5G1} fat cells with Fluo-2 AM dye (N). Panel A, D, H and M show brightfield view of fat cells with the proper genotype. Panel B, A and I show DAPI staining. As negative, control *w*^{1118} larvae were used. The scale bars are shown in white (50µm).

The green-fluorescent emission (~525 nm) of Ca^{2+}-bound Fluo-4 AM is conventionally detected with optical filter that have been designed for fluorescein (FITC). Its absorption maximum is blue-shifted about 12 nm, as compared to other dyes such as Fluo-3, this results in increased fluorescence excitation at 488 nm and consequently a higher signal for the confocal laser-scanning microscopy. Fat body dissected from *y,Adar^{5G1},w^{-};Collagen-GAL4>* did not show any detectable staining confirming the previous result obtained with the *Camgaroo* reporter. In summary, I found no evidence for a modification in intracellular calcium concentration in *Adar*-null fat cells therefore the increased activation rate of autophagy is not due to aberrant calcium levels.

6.2.4 Autophagy and the unfolded protein response (UPR), mediated by the ER

Don Ryoo and colleagues have designed an enhanced *UAS-Xbp1-eGFP* reporter to detect a short-term response of the ER responding to unfolded protein stress (Ryoo *et al.*, 2007). They used this reporter to examine the UPR contribution to the progression of retinal degeneration in the class III autosomal dominant retinitis pigmentosa (ADRP) model in *Drosophila* (Ryoo *et al.*, 2007). The transgene expression was driven in the *Adar*-null fat cells with the *y,Adar^{5G1},w^{-}/Fm7,P{GAL4-Kr.C}DC3,P{UAS-GFP.S65T}DC7;Collagen-GAL4/Collagen-GAL4;+/+* fly strain. *y,Adar^{5G1},w^{-};Collagen-GAL4>UAS-Xbp1-eGFP* larvae were selected and the fat body dissected. The fat cells had increased GFP expression, reflecting an increased IRE-1 activity and consequently an increase in ER stress (Figure 6.8 G).

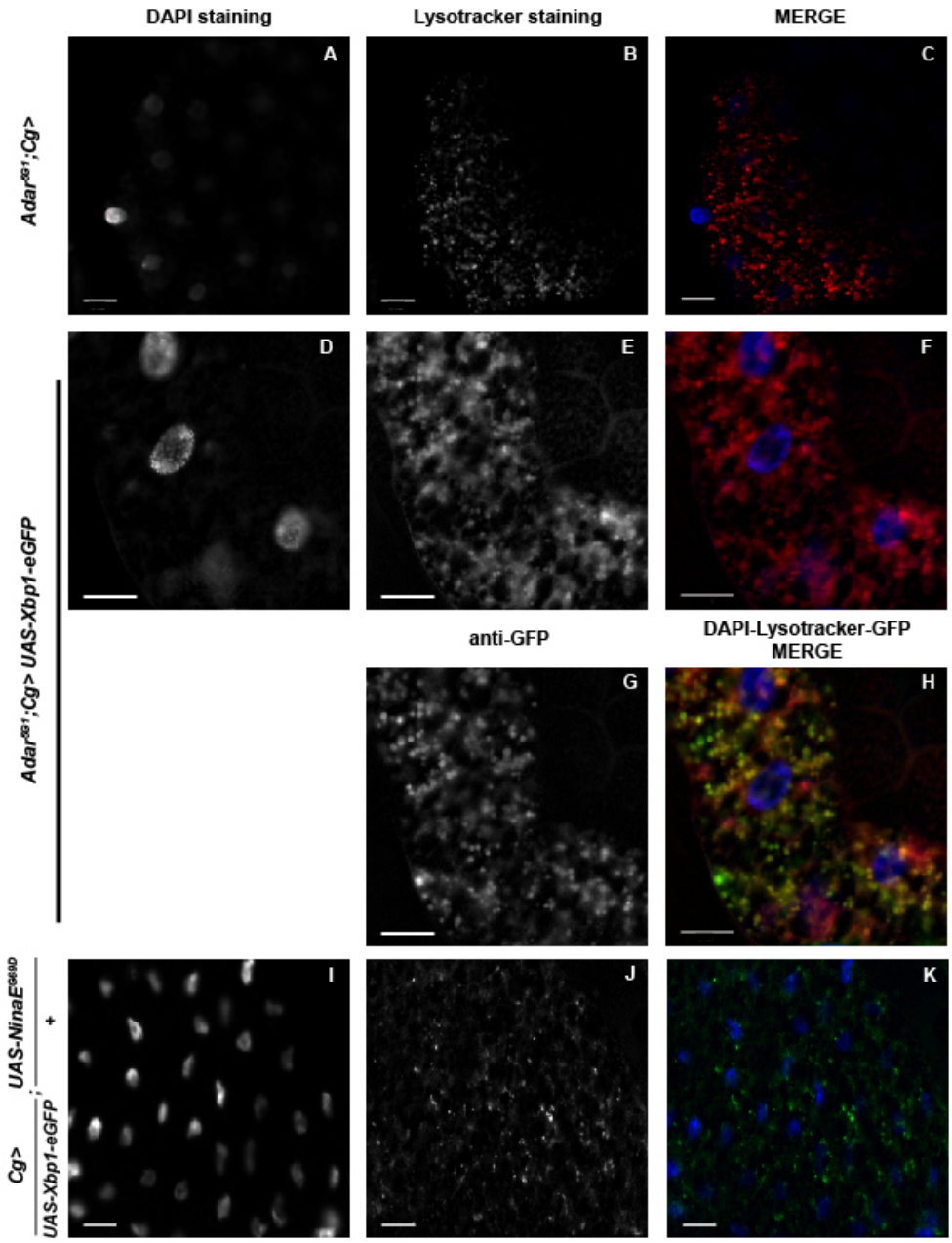


Figure 6.8: UPR in *Adar*-null fat body. *Adar*-null fat cells have increased autophagy activation (C and F). To test whether the stress that arises from the lack of ADAR enzyme is dependent to an enhanced UPR, the *UAS-Xbp1-eGFP* reporter expression was driven in *Adar*-null larval fat cells (panels D-H). The enhanced signal of lysotracker was confirmed (panels E and F). The *Xbp1-eGFP* signal was also detected with GFP antibody staining (G). The Xbp1-eGFP signal was not only detected within the nucleus as expected but also in the cytoplasm where it co-localizes generally with the lysotracker staining. The expression of *UAS-NinaE*^{G69D} transgene within fat body (I-K) has been used as positive control. In fact Ryoo *et al.* (2007) have been previously shown that its expression drives to ER-mediated UPR. The scale bars are shown in white: 50μm in A-C and I-K and 20μm in D-H.

6.3 Discussion

The fat body is the tissue in which autophagy studies have been primarily performed in *Drosophila melanogaster*. It is a monolayer cell tissue and is a valuable research tool in *Drosophila* because molecular and histology techniques can be easily combined with genetics by using specific genetic drivers such as *Collagen-GAL4*. Moreover it provides a good model to study developmental autophagy and the crosstalk between autophagy and apoptosis. Recently it has been shown that fat body releases antimicrobial peptides into the hemolymph thereby controlling an inflammatory-like response involving both cellular and humoral defences (Hoffmann and Reichhart, 2002). This is only one example in which fat cells respond non-cell autonomously to a systemic stress, in fact they provide lipids, sugars, ATP and antimicrobial peptides depending on the needs of the organism. These abilities reflect the properties of this tissue that has been under-appreciated especially in *Drosophila*.

Deletion of the *Adar* gene results in an up-regulation in autophagy activation. This was observed in larval brains and it was expected due to the lack of the RNA editing enzyme; therefore many transcripts can be no longer edited. However autophagy was also increased in the fat cells of null-*Adar* larvae and it reflects a cell autonomous response that specifically affects *Adar*-null fat cells. In fact the autophagy-phenotype can be suppressed by driving the expression of *UAS-dAdar3/4* in the fat body. Moreover, the phenotype is suppressed even by a catalytically inactive mutant of dADAR. This observation suggests that the presence of the protein *per se* is far more important than its catalytic activity, at least in this tissue. The same inactive protein, ADAR3/4 E/A, is also able to rescue the neurodegeneration affecting $Adar^{5G1}$ flies. It is intriguing how a catalytic inactive enzyme suppresses a stress-derived phenotype. This is still an unresolved question and addressing it may lead to the identification of a new ADAR function. Another way to better understand ADAR and its role in *Drosophila* physiology is to identify the source of the stress. For that reason three different stresses have been tested. The stresses were chosen because of their possible role in linking ADAR and

autophagy. None of the transcripts encoding autophagy genes are edited. Therefore I focused on studying the scientific literature to find possible connections between *Adar*, autophagy and cellular stress.

First, I investigated whether ROS had accumulated in the mutant fat cells. It was previously shown that *Adar* mutant flies recover slowly from anoxic stupor (Ma *et al.*, 2001). Also oxygen perfusion after a long period of anoxia increases the production of ROS. This suggests that mutant flies might be more sensitive to fluctuations in oxygen levels. Nevertheless variation in ROS enhances the activation of the autophagy pathway through up-regulation of JNK signalling which possibly boosts the expression of *Atg* genes via FOXO-transcription factor activity. Furthermore ROS can directly modify the redox state of ATG4 thereby modulating its activity (Scherz-Shouval *et al.*, 2007).

Second, I investigated whether the intracellular calcium concentration might also be another source of stress. The failure of RNA editing in neurons affect channels activity and permeability and this can change membrane resting potential thereby modulating the firing properties of neurons. Some of the transcripts encoding voltage-gated ion channels are highly edited and are channels permeable to Ca^{2+} such as *Cacophony* (Smith *et al.*, 1998b) and *Ca-α1D* (Grauso *et al.*, 2002b). Thus it is possible that variations in intracellular calcium levels can result in the neurodegeneration phenotype observed in *Adar*-null brains and to locomotor impairments. This stress can boost autophagy activation (Hoyer-Hansen *et al.*, 2007; Hoyer-Hansen and Jaattela, 2007; Gao *et al.*, 2008; Sinha *et al.*, 2008) and it may protect neurons from cell death. Additionally, calcium induces and controls vesicle-exocytosis in many cell types. For instance in neurons, it drives the fusion of neurotransmitter-containing vesicles to pre-synaptic membrane thereby controlling the activity of Synaptotagmin and SNARE proteins (Koh and Bellen, 2003; Tucker *et al.*, 2004; Chicka *et al.*, 2008). In fed larval brains, insulin-producing cells (IPCs) release insulin-like peptides (Dilp) through calcium-dependent granule exocytosis upon variation in the polarization state of the IPC membrane (Kaplan *et al.*, 2008). It is likely that fat cells use the calcium-dependent exocytosis to release metabolic signals and antimicrobial peptides into haemolymph, even though no study

has reported this yet. Fat cells also use calcium as an internal second messenger therefore its concentration must be tightly regulated.

Third, I hypothesized that the deletion of *Adar* would affect the function of the ER. The ER is an important organelle within the cell with many important roles, crucially it acts as a "quality-control" sensor for unfolded proteins triggering UPR. RNA editing *per se* is an important step during gene expression as it can generate protein diversity through recoding of genomic information. In fact during transcription, ADAR binds pre-mRNAs probably competing with other RNA-binding proteins such as splicing factors. RNA editing precedes RNA splicing. Therefore the presence of the protein can interfere with alternative splicing and this is independent of whether its catalytic activity can modify amino acid codons. Furthermore many of the transcripts edited by ADAR encode transmembrane proteins that are post-translationally modified within the ER. Hence the ER might be a sensor for the accumulation of misfolded proteins arising from aberrant RNA processing.

Adar-null fat cells have an up-regulation of ER stress mediated by UPR. This suggests that there is a modification in RNA metabolism. Moreover this hypothesis is also supported by the rescue with the catalytically inactive protein. In fact the enzymatic activity of the protein is not the only function of the protein, especially in the fat body where edited transcripts are few. What is more essential is the expression of the ADAR protein. This opens a new prospective concerning ADAR and its function in *Drosophila*.

CHAPTER 7: Discussion

7.1 Discussion

A-to-I RNA editing is a biochemical modification of specific adenosines within pre-mRNA. After binding to a specific adenosine, the amino group of the adenosine is hydrolytically deaminated by ADAR to generate inosine. The inosine base pairs with cytosine and it is read as guanosine by the ribosomal translational machinery. This type of RNA editing was firstly identified in *Xenopus laevis* and in mammalian cell culture (Bass and Weintraub, 1987; Rebagliati and Melton, 1987; Wagner and Nishikura, 1988). ADARs edit pre-mRNAs. Editing can occurs within the coding sequence changing the amino acid sequence and can alter the function of the protein as in the case of GluR-2, one of the four subunits of glutamate-gated AMPA receptors (Higuchi *et al.*, 2000a; Seeburg and Hartner, 2003; Peng *et al.*, 2006). Alterations in protein sequence with the generation of new protein isoforms can also be achieved by editing introns as it can generate alternatively spliced exons (Beghini *et al.*, 2000). RNA editing can also occur within the 5'- or 3'-untranslated region (UTR), which may influence mRNA stability and transport (Morse *et al.*, 2002).

In order to understand the biological relevance of these enzymes is important to investigate how their expression and enzymatic activity is regulated. Considering the number of editing events in *Drosophila* and the physiological importance of some of the edited transcripts in mammals, it has become crucial to understand how an organism can homeostatically response to variations in RNA editing In fact deficiency or misregulation of A-to-I RNA editing has been implicated in neurological diseases such as epilepsy (Brusa *et al.*, 1995; Higuchi *et al.*, 2000a), amyotrophic lateral sclerosis (ALS) (Kawahara *et al.*, 2004; Hideyama *et al.*, 2010) and depression (Niswender *et al.*, 2001; Gurevich *et al.*, 2002). However ADAR enzymes may have a more complex biological role in addition to generating protein diversity; hence animal model are critical to investigate the function of ADARs *in vivo*. A transgenic mouse lacking *ADAR2* is heterozygous viable and normal. Homozygous *Adar2* $^{-/-}$ mice have apparently normal embryonic development but die by 21 days after birth due to epileptic seizures

(Higuchi *et al.*, 2000a). The reason is due to the lack of editing at the Q/R site of *GluR-2* pre-mRNA (Higuchi *et al.*, 2000a). Furthermore conditional knockout *Adar2* $^{-/-}$ mice that is targeted to motor neurons shows a decline in motor function in the spinal cord and cranial motor nerve nuclei (Hideyama *et al.*, 2010). Different groups have generated *Adar1* $^{-/-}$ homozygous mice which is lethal by day E12.5 but the RNA that is edited by ADAR1 that causes this lethality is unknown (Hartner *et al.*, 2004; Wang *et al.*, 2004). The difference observed between *Adar1* $^{-/-}$ and *Adar2* $^{-/-}$ knock out mice is likely to reflect the different repertoires of transcripts edited by these two enzymes. However ADAR1 and ADAR2 can recognize and compete for the same RNA editing site as in the case of R/G site in *GluR-2* pre-mRNA (Wong *et al.*, 2001). Furthermore mammalians have other two *ADAR* genes, *ADAR3* and *TENR* that are catalytically inactive enzymes that are expressed in the CNS and testis, respectively; however their biological function is unknown (Schumacher *et al.*, 1995; Mittaz *et al.*, 1997b; Connolly *et al.*, 2005).

As mammals have four ADAR genes it is difficult to elucidate the biological role of each one individually as they may have compensatory effects on each other. Also the interaction between different pathways in which they are involved might hide the effect of their misregulation. For that reason, simpler model organisms such as *Drosophila* and *C. elegans* represent an important tool to study RNA processing, and particularly RNA editing *in vivo*. Recently our group have shown that *ADAR1* and *ADAR2* genes are conserved throughout evolution (Keegan *et al.*, 2011). In fact Cnidaria have two genes that encode two distinct RNA editing enzymes that appear to be true orthologs of human *ADAR1* and *ADAR2* genes (Keegan *et al.*, 2011). Among Arthropoda, Pancrustacea have lost one of them whereas Arachnida maintain both of them. *Drosophila melanogaster*, which belongs to the Insecta filum, have only one gene that encodes a protein which is a true ortholog of human *ADAR2* gene rather then a common ancestor of Chordata *Adar* genes (Keegan *et al.*, 2011). *Drosophila Adar* gene lies on the X chromosome at the cytogenetic position 2B6-7 (Palladino *et al.*, 2000c). Its gene expression is regulated by two upstream promoters; -4a and -4b, in a developmental dependent manner (Figure 1.3). The promoter -4a drives *dAdar* gene expression throughout all developmental stages, from embryos until adulthood, whereas -4b promoter becomes active

immediately after metamorphosis. Only transcripts expressed from the early promoter contain the alternatively spliced exon 3a; in fly gonads its inclusion within *dAdar*-encoding pre-mRNAs is enhanced by the binding of B52/SRp55 (Marcucci *et al.*, 2009). The alternative exon 3a encodes 38 amino acids and lies between the two dsRNA-binding domains, dsRBDs, of dADAR protein (Keegan *et al.*, 2005). The inclusion of this exon changes the distance between dsRBM1 and 2 of the protein, generating the dADAR3a; when this exon is not included the ADAR 3/4 isoform is generated (Keegan *et al.*, 2005). Albeit the *dAdar* gene is the true ortholog of human *ADAR2*, the space between the two dsRBD is similar to that of hADAR1 (Gallo *et al.*, 2003; Keegan *et al.*, 2005). Previous studies identified 127 sites in 55 *Drosophila* genes that undergo A-to-I RNA editing (Stapleton *et al.*, 2006) that are mostly expressed within the CNS. However *dAdar* is widely expressed. Recently Graveley and colleagues have used RNA-seq, tiling microarrays and cDNA sequencing to analyse the *Drosophila* transcriptome throughout its development, identifying 972 edited positions within transcripts of 597 genes (Graveley *et al.*, 2011). The majority of the edited positions modify codons therefore changing the amino acid. For example the pre-mRNA encoding *Quiver* is edited in 6 different positions. This gene encodes a potassium channel subunit that modulates the function of the voltage gated potassium channel SHAKER, whose pre-mRNA is also edited by ADAR (Hoopengardner *et al.*, 2003; Wang and Wu, 2010). The combination of the editing events in these two subunits of voltage-gated potassium channels may modulate their action potentials.

Albeit most of the known transcripts that are edited by ADAR are expressed within CNS, the work by Graveley and co-workers shows that RNA editing also occurs within other tissues raising the possibility that dADAR has a broader biological role than only CNS development and maintenance (Graveley *et al.*, 2011).

However the phenotypic characterization of the hypomorphic allele for *dAdar* expression, $Adar^{1F4}$, shows an age-dependent vacuolization affecting specifically the retina and mid-brain regions. The photoreceptors in $Adar^{1F4}$ animals appear disorganized when compared to the more compact and organized retinal structure of wild-type flies (Palladino *et al.*, 2000b). Also the *dAdar* loss of function allele, $Hypnos\text{-}2^{P}$ in which the

sequence of the second dsRNA binding motif and the catalytic domain has been deleted, has neuronal degeneration in the cortical neurons of the medulla and lobula complex as well as in the lamina (Ma *et al.*, 2001). This mutant is more sensitive to perfusion followed by anoxic conditions however we do not know the lack of editing of which specific transcript underlies this condition (Chen *et al.*, 2004; Xia *et al.*, 2005).

A third allele, referred as $Adar^{5G1}$, was previously characterised in our laboratory by Leeanne McGurk (McGurk, 2007). It is a null-allele for *dAdar* expression as the whole gene plus some of the surrounding region has been deleted by *P*-element mobilization (Palladino *et al.*, 2000c). The *Adar*-null animals are viable, even though the fly viability is reduced and is approximately 30% compared to wild-type animals. *Adar*-null flies show locomotion defects that are succeeded by age-dependent neurodegeneration that affects mainly mushroom bodies and retina structure (McGurk, 2007).

The initial aim of my work was to identify new modifiers that suppress the reduced fly viability associated with the $Adar^{5G1}$ allele. It is not obvious how the loss of *Adar* interferes with fly development; suppressor as well as enhancer screens are often used to find missing components of developmental pathway that cannot be found with traditional genetic screens. The genetic screen was performed with the *DrosDel* collection that consists of a set of fly strains each carrying a chromosomal deletion; therefore they are hemizygous for the deleted genes. The production of only 50% of wild-type level of the protein is still sufficient for normal development. However, when a mutation is present as the case of $Adar^{5G1}$ allele, a second mutation might be identified as a dominant suppressor in this "sensitized" background, if it is in the same pathway. For example the components in the signal-transduction pathway down-stream of *Sevenless*, a receptor tyrosine kinase, have been identified (Simon, 1994). The advantage of this screen is the ability to analyze the progeny coming from mutant flies. Nevertheless it is possible to use heterozygous flies therefore it is possible to use the whole genome without having to balance each mutation. However, since the collection became available, no genetic screen has been published using *DrosDel*. Additionally a phenotype such as viability is very sensitive to variations in external conditions such as amino acids composition and the pH of the media, etc; therefore the experimental

condition must be rigorously controlled otherwise they might hide the effect of the deletion.

When screening the left arm of the second chromosome, it was clear that the reduced viability phenotype of *Adar*^{5G1} mutant flies was suppressed by reducing the expression of the *Tor* gene. TOR is a key regulator of cellular homeostasis and one of its key functions is to control cap-mediated protein translation that is generally mediated by the activation of PI3K/Akt signalling pathway (Gao and Pan, 2001; Miron *et al.*, 2003; Hardie, 2004; Hu *et al.*, 2010). Nevertheless lowering *Tor* expression was able to rescue the reduced lifespan affecting *Adar*^{5G1} flies. This result is consistent with the link between TOR and aging in *Drosophila* (Kapahi *et al.*, 2004). In fact inhibition of TOR signalling together with dietary restriction increases *Drosophila* lifespan by decreasing cap-mediated translation (Kapahi *et al.*, 2004; Syntichaki *et al.*, 2007a; Zid *et al.*, 2009). The same result can also be achieved by up-regulating the activity of the negative regulators of TOR; TSC1/TSC2 and overexpressing 4E-BP the major inhibitor of translation (Kapahi and Zid, 2004; Kapahi *et al.*, 2004). In the screen I performed the dietary condition were not modified, but lowering *dTor* expression could result in a decrease in translation.

This hypothesis was then tested genetically; unexpectedly the rescue of *Adar*^{5G1} was clearly mediated by increasing autophagy activation (Figure 5.1 and figure 5.2). Autophagy is a self-degradation process whereby damaged organelles and protein aggregates are engulfed in double membrane vesicles which fused to lysosomes; the contents are then degraded and lipids and amino acids are retrieved. Autophagy is also used under starvation condition when there is an increased demand of energy, amino acids or lipids. This process is tightly regulated through the TOR signaling pathway. Therefore TOR controls and positively regulates protein synthesis and inhibits autophagy-mediated degradation, autophagy is a crucial event especially in quiescent cells in order to avoid the accumulation of protein aggregates and damaged organelles that are not diluted by cell division.

Considering that neurons are quiescent cells it is not surprising that TOR and autophagy have been linked to several neurodegenerative diseases characterized by accumulation of protein aggregates. In fact rapamycin treatment clears accumulated misfolded protein by blocking TOR activity thereby reducing the pathology hallmark and this has been demonstrated in mouse models of Alzheimer's and Huntington's diseases (Ravikumar *et al.*, 2004; Spilman *et al.*, 2010). Also *Drosophila* has proven itself to be a good genetic model for these two diseases, in fact it was demonstrated in *Drosophila* that cell-autonomous activation of autophagy that is mediated by suppression of TOR activity clears toxic polyQ-containing protein aggregates and insoluble tau protein accumulation which can lead to neuronal degeneration associated with the aging processes (Berger *et al.*, 2006; Wang *et al.*, 2009).

$Adar^{5G1}$ mutants suffer from age-dependent vacuole formation in the mushroom body (McGurk, 2007) this neurodegeneration is faster compared to the hypomorphic allele, $Adar^{1F4}$. This neurodegeneration phenotype is dependent on the lack of *Adar* gene because it can be rescued by expressing the *Drosophila Adar* transgene in the null-background (McGurk, 2007). I found that this neurodegeneration phenotype can be also rescued by lowering *Tor* expression or by inducing autophagy activation by increasing *Atg5* expression. However electron microscopy images of $Adar^{5G1}$ optic lamina, frontal brain and retina do not show any protein aggregates; therefore the stress that causes the neurodegeneration observed in $Adar^{5G1}$ animals is different from what is observed in proteinopathies such as Alzheimer's or Huntington's disease. A similar situation is observed in *Phospholipase C* (*norpA*) mediated retinal degeneration (Wang *et al.*, 2009) that has been also been modeled in *Drosophila*. Also in this case the degeneration does not involve the formation of protein aggregates but the formation of toxic rhodopsin-arrestin complexes after phototransduction signal (Alloway *et al.*, 2000; Kiselev *et al.*, 2000; Iakhine *et al.*, 2004). Moreover degeneration can be arrested by breaking rhodopsin-arrestin interactions or by increasing the cellular autophagy levels by lowering TOR activity (Wang *et al.*, 2009).

In summary these observations demonstrate that TOR signaling can increase autophagy, which removes toxic soluble proteins, and complexes thereby reducing the cellular toxicity of unaggregated proteins. This is reminiscent of Hutchinson–Gilford progeria syndrome (HPGS) which is caused by progerin, a mutant form of lamin A that alters nuclear structure and function however an increased activation rate of autophagy reverse all effects of progeria (Cao *et al.*, 2011).

In the second part of my PhD I wanted to elucidate the stress affecting *Adar*-null mutant flies that can be suppressed by increasing autophagy levels. Remarkably the effect of *Tor* on *Adar*-null phenotypes rescues without restoring the editing activity of ADAR. This has been also confirmed using the catalytically inactive isoform, ADAR3/4 E/A, that not only is able to rescue the neurodegeneration in adult brain (McGurk, 2007), but also suppresses the autophagy phenotype observed in larval *Adar*-null fat body (Figure 6.5). The stress affecting $Adar^{5G1}$ mutant flies was investigated in this tissue as autophagy has been extensively studied in this tissue in *Drosophila melanogaster*. It is the analogous to both mammalian liver and adipose tissue and, similarly, provides amino acids, lipids and glycogen. Fat cells also support other tissues in a non-cell autonomous manner. For example during metamorphosis they provide essential molecules to rebuild and re-organize body components upon autophagy activation (Rusten *et al.*, 2004). Fat cells respond cell-autonomously to the stress in the *Adar* null flies that is due to the lack of ADAR protein, rather than its enzymatic activity. Because RNA editing is crucial for proper development in *Drosophila,* we hypothesized and subsequentially demonstrated that the deletion of *Adar* affects the function of the ER which acts as "quality-control" sensor for unfolded proteins triggering the UPR and as well as a calcium storage. In fact *Adar*-null fat cells have increased of Ire1-mediated UPR activation.

For the first time, this work provides a link between RNA editing and ER stress via ADAR protein and this is independent of its catalytic activity. The stress causes an increased autophagy activation rate; this observation supports the major result because ER stress stimulates the assembly of pre-autophagosomal structures and subsequentially

autophagosomes are formed in a *Atg* protein dependent manner (Yorimitsu *et al.*, 2006). Additionally it has been shown that IRE1 signal transduction is linked to up-regulation of JNK signalling pathway that is furthermore involved in autophagosome formation in early phase of ER stress (Ogata *et al.*, 2006); nonetheless activated JNK signalling pathway can induce *Atg* genes expression (Wu *et al.*, 2009).

The autophagy-related phenotype and the rescue driven by the inactive protein have been observed not only in fat cells but also in $Adar^{5G1}$ mutant fly brain (McGurk, 2007). Therefore it is possible that the age-dependent vacuolization affecting *Adar*-null fly brain might be driven by ER stress and is similar to what is observed in Huntington and Parkinson's diseases (Qin *et al.*, 2003; Webb *et al.*, 2003; Ravikumar *et al.*, 2004).

Unlikely other *Drosophila* models of neurodegenerative disease, EM images of *Adar*-null flies do not show protein aggregates. This suggests that the stress that drives the neurodegeneration is soluble and cannot detect using electron microscopy technique; nevertheless it might involve *Adar* substrates independently of its enzymatic activity. Interestingly IRE-1 can dimerize with PERK (double-stranded RNA-activated protein kinase-like ER kinase (PERK)) (Pomar *et al*., 2003). PERK is an ER associated transmembrane protein that has been correlated with ER stress and autophagy (Avivar-Valderas *et al.*, 2011). Nevertheless its activation relies on an overload of RNA within the ER. Therefore I would be interesting to elucidate whether the absence of ADAR in Drosophila increases the total amount of 'free' (or not associated to ADAR) RNAs.

7.2 Future Perspectives

I would like to suggest experiments that would support the novel findings presented in this work:

1. Is the catalytically inactive rescue dependent to the catalytic domain rather than the RNA binding domain of dADAR?
2. How does the catalytic inactive protein rescue the autophagy-phenotype in *Adar*-null fat body?
3. Is the age-dependent vacuolization affecting *Adar*-null fly brain also dependent on enhanced ER stress?
4. Is there a significant increase of RNAs within $Adar^{5G1}$ mutants that can explain the ER stress?

7.2.1 Is the catalytically inactive rescue dependent to the catalytic domain rather than the RNA binding domain of dADAR?

I have shown that ADAR3/4 E/A, a catalytically inactive isoform, is able to suppress the autophagy phenotype of *Adar*-null fat body. Therefore the suppression of autophagy phenotype is not dependent on the enzymatic activity of the protein. The protein has two functional domains: the dsRNA binding domain and the catalytic deaminase domain. It would be interesting to investigate which domain mediates the observed rescue by making transgenic fly strains with the binary *UAS-GAL4 system* to express either the dsRBD or the deaminase domain. This result would demonstrate whether the suppression of the autophagy-phenotype is mediated by the ability of the protein to bind to dsRNA. This result would imply that ADAR not only catalyzes RNA editing events *per se*, but can also impact on other RNA processing events such as influencing RNA

splicing. If this is the case, would another protein with a similar RNA-binding specificity replace dADAR function? This question could be addressed by expressing the human ADAR proteins. In fact hADAR2 is the true ortholog of dADAR, whereas inactive hADAR3 could function as dADAR3/4 E/A.

However the editing independent rescue might be dependent to the inositol-1,2,3,4,5,6-hexa*kis*phosphate present within the deaminase domain of ADAR. This molecule is involved in signal transduction, regulation, energy transduction and ATP regeneration (Biswas *et al.*, 1978; Saiardi *et al.*, 2002). It is embedded within the catalytic deaminase domain of ADAR, surrounded by 29 molecules of water (Macbeth *et al.*, 2005) and it has been suggested that it is required for the correct folding of ADAR. However it is not clear whether inositol-1,2,3,4,5,6-hexa*kis*phosphate can diffuse in and out of the deaminse domain or whether it is stably embedded. In this case ADAR may have another unknown function beside its RNA editing activity.

7.2.2 How does the catalytic inactive protein rescue the autophagy-phenotype in Adar-null fat body?

The autophagy phenotype in *Adar*-null fat cells is caused by an increase in ER stress; and is generated by an increased in UPR. It would be interesting to further investigate ER stress to elucidate how it is linked with the lack of ADAR protein. For example, it would be interesting to explore whether this phenotype can also be suppressed by over-expressing other chaperone proteins. Alternatively, it may be possible to perform RNA-seq to identify transcripts that have undergone an incorrect post-transcriptional modification and therefore accumulated as unfolded proteins when translated in the ER.

7.2.3 Is the age-dependent vacuolization affecting Adar-null fly brain also dependent on enhanced ER stress?

ADAR3/4 E/A, which is the catalytically inactive isoform of ADAR, is able to rescue the autophagy phenotype in *Adar*-null fat body as well as the age-dependent neurodegeneration in $Adar^{5G1}$ fly brain. Albeit it is not clear which is the molecular stress underlies the neuronal loss in *Adar*-null fly brain, my work on larval fat body suggests that UPR of ER stress may be involved. It would be interesting therefore to explore whether there is an up-regulation of UPR within neurons using *UAS-Xbp1-eGFP* construct. Also it has been previously shown that ER stress can cause neuronal degeneration in model of Parkinson' and Huntigton's disease (Qin *et al.*, 2003; Webb *et al.*, 2003; Ravikumar *et al.*, 2004).

7.2.4 Is there a significant increase of RNAs in $Adar^{5G1}$ mutants that can explain the ER stress?

I wanted to elucidate whether the lack of ADAR protein in *Drosophila* affects RNA metabolism increasing the intracellular level of "free" (not associated to any RNA-binding protein) RNAs. This might be the cause of ER stress observed using *UAS-Xbp1-eGFP* reporter; nevertheless RNAs are absolutely soluble molecules and, therefore, impossible to notice through EM images. To prove this hypothesis, it would be interesting to extract proteins from *Adar*-null animals and w^{1118} control flies without losing RNAs and RNAs associated to proteins. The protein extract will be then immunoprecipitated using an antibody that recognises specifically dsRNAs (Wisskirchen et al., 2011; Pichlmair et al., 2009) and the RNAs subsequentially purified.

CHAPTER 8: References

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Appendix: poster and publication

Neurodegeneration in the brain of *Drosophila* lacking ADAR RNA editing is rescued by increasing autophagy

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Abstract

In humans ADAR RNA editing controls key properties of excitatory glutamate receptors. Loss of RNA editing is implicated in neurodegeneration and glutamate excitotoxic neuron death in stroke and ALS. *Drosophila* provides an excellent study model: in *Drosophila* ADAR RNA editing converts specific adenosines to inosines to change codons in fifty seven known transcripts. Inosine is read as guanosine during translation. Editing diversifies transcripts encoding mainly neuronal membrane proteins such as ion channel subunits, ion exchangers, membrane trafficking and cytoskeleton-associated proteins.

Drosophila mutant for the single *Adar* gene on the X chromosome are locomotion-defective and develop age-dependent vacuolization of mushroom body calyces and of the retina. We find that this neurodegeneration is not associated with extensive neuronal apoptosis. Instead intracellular membrane structures resembling those seen in autophagy mutants and in human lysosomal storage diseases appear and large fluid-filled vacuoles also develop.

A DrosDel screen for suppressors of the reduced viability at eclosion in an *Adar* null mutant showed that heterozygous *Tor* mutants rescue. Reduced *Tor* leads to increased autophagy. Overexpression of *Atg5* also rescues the reduced viability as well as the locomotion defects, neurodegeneration and the reduced longevity of the *Adar* null. Rescue of *Adar* mutant phenotypes by increased autophagy substantially bypasses the requirement for RNA editing.

Our findings are consistent with the idea that ADAR-related neurodegenerations in both flies and vertebrates involve autophagy even though vertebrate studies have focussed mainly on calcium-mediated effects. There has been debate on whether autophagy exacerbates or moderates neurodegenerative illnesses. Increased autophagy is clearly protective in the *Drosophila Adar* neurodegeneration and possibly also in human neurodegenerations associated with loss of ADAR function. RNA editing is essential for normal function in the fly but mechanisms allowing substantial bypass of RNA editing are identified by our genetic screen.

Retinal degeneration with autophagy in the *Adar*^{5G1} mutant

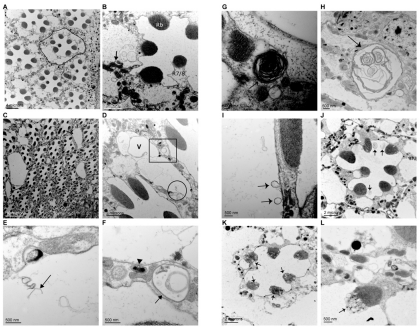
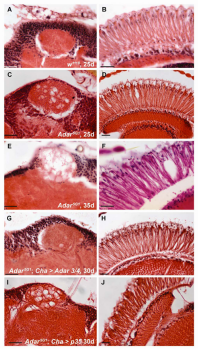


Fig. 2: A: The ommatidia of *w*^{1118} at 25 days. The black line outlines a single ommatidium comprising seven photoreceptor cells surrounded by and separated from neighbouring ommatidia by thin pigment cells containing red pigment granules. Lengths of scale bars are indicated in the individual panels. B: An ommatidium of 25 day old *w*^{1118} at higher resolution. The photoreceptor cells with light-detecting rhabdomeres (Rb) appear normal. The R7/R8 photoreceptor is indicated. Organelles such as mitochondria are identifiable (arrow). C: Retina of the *Adar*^{5G1} mutant at 25 days showing pigment cells with large vacuoles (arrows) between ommatidia. D: Higher resolution image of a single ommatidium in 25 day old *Adar*^{5G1} with vacuole (V) between photoreceptor cells of two ommatidia. E: Magnification of area within the circle in (D). Interrupted membrane (arrow) was observed inside the vacuole. F: Magnification of area within the square in (D). Membrane-bound vesicles (arrows) in the photoreceptors contain cellular components in an autophagosome-like structure surrounded by two or more membrane layers G and H: Multilamellar membrane structures (arrows) in a photoreceptor cell and within a glial cell close to the basement membrane between the retina and the lamina. I: Single membrane-bound vesicles pinching off from the photoreceptor (arrows) in early stages of photoreceptor degeneration. J: Larger multilamellar membrane structures budding off from the extracellular membrane of photoreceptor cells into the ommatidial cavity (arrows) at more advanced stages of degeneration. K: Extensive loss of pigment cells separating ommatidia in advanced stages of neurodegeneration. Photoreceptor cell cytoplasm and extracellular membrane are abnormal and vesicles bud from the rhabdomeres (arrows). L: Abnormal exocytosis from the rhabdomere in late stages. The extracellular membrane of the photoreceptor is not well defined.

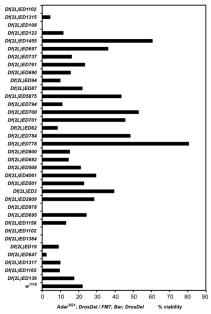
Neurodegeneration in *Adar*^{5G1}

Fig. 1: A and B: Mushroom body calyx and retina in the 25-day-old *w*^{1118} wild-type control strain. Black haematoxylin-stained nuclei of Kenyon cells overly the oval eosin red -stained mushroom body calyx on the posterior dorsal side of the brain. Frontal sections through the retina run parallel to the ommatidia. C and D: Vacuoles within the mushroom body calyx and thinning and separation of ommatidia in the retina of the 25-day-old *Adar*^{5G1} mutant. E and F: Heavily degenerated mushroom body calyx and retina in the 35 day old *Adar*^{5G1} mutant. G and H: Rescue of vacuolization in mushroom body calyx and retina in 30 day old *Adar*^{5G1}*; Cha-GAL4; UAS-Adar 3/4 S*. I and J: No rescue of vacuolization in mushroom body calyx and retina in 30 day old *Adar*^{5G1}*; Cha-GAL4; UAS-p35*. Scale bars: 20mm.



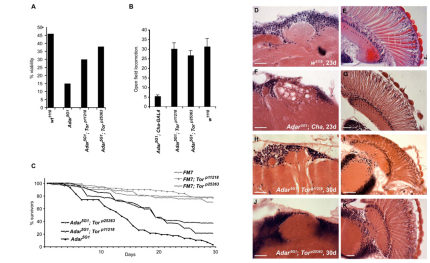
Screen for *Adar*^{5G1} viability modifiers

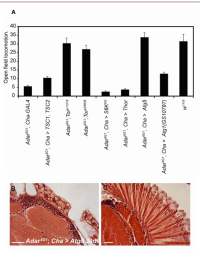
Fig. 3. Progeny are obtained by crossing Adar^{5G1} / FM7, Bar virgin females to w^{1118} males or males of Chr. 2L DrosDel / SM5, Cy deficiency stocks. The chart shows the ratio of Adar^{5G1} to FM7, Bar flies among male progeny in the presence of DrosDel deficiencies, or in their absence (w^{1118} cross at the bottom). Df(2L)ED778 and DF(2L)ED784 both increase *Adar*^{5G1} viability. The overlap of these deletions covers thirteen genes, one of which is the *Tor* Gene. P element insertion mutants and RNAi strains that lower expression of these genes were tested for Adar^{5G1} viability rescue.



Rescue of *Adar*^{5G1} phenotypes by reduced *Tor* activity

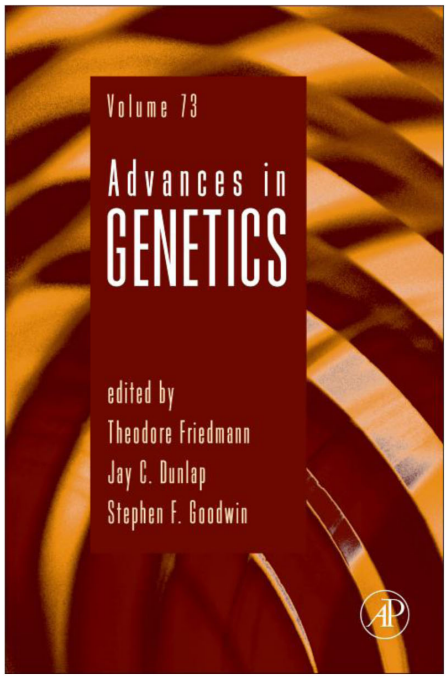
Fig. 4. A. Loss of function mutations in *Tor* increase the viability of the *Adar*^{5G1} mutation. B. Loss of function mutations in *Tor* substantially increase the locomotion of *Adar*^{5G1} mutant flies in a two-minute open-field locomotion test. C. Loss of function mutations in *Tor* increase the lifespan of the *Adar*^{5G1} mutant flies. D and E: Mushroom body calyx (63X) and retina (40X) of the 23-day-old *w*^{1118}. F and G: Mushroom body calyx and retina in the 23-day-old *Adar*^{5G1}*; Cha-GAL4* mutant. H and I: Mushroom body calyx and retina in the 25 day old *Adar*^{5G1}*; Tor*^{e11218}. The olfactory projection tract through the brain to the mushroom body is seen in this section. J and K: Mushroom body calyx and retina of 23 day old *Adar*^{5G1}*; Tor*^{e2536J} Scale bars: 20mm.





Rescue of *Adar*^{5G1} phenotypes by expression of autophagy (*Atg1, Atg5*) genes

Fig. 5. A. Rescue of open field locomotion defects by increased expression of autophagy genes but not by inhibition of autophagy or apoptosis nor by decreasing cap-dependent translation in *Adar*^{5G1}. B, C: Mushroom body calyx (63X) and retina (40X) in 30 day old *Adar*^{5G1}*; Cha-GAL4; UAS-Atg5*. Scale bars: 20mm.



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Functional conservation in human and *Drosophila* of Metazoan ADAR2 involved in RNA editing: loss of ADAR1 in insects

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ABSTRACT

Flies with mutations in the single *Drosophila Adar* gene encoding an RNA editing enzyme involved in editing 4% of all transcripts have severe locomotion defects and develop age-dependent neurodegeneration. Vertebrates have two ADAR-editing enzymes that are catalytically active; ADAR1 and ADAR2. We show that human *ADAR2* rescues *Drosophila Adar* mutant phenotypes. Neither the short nuclear ADAR1p110 isoform nor the longer interferon-inducible cytoplasmic ADAR1p150 isoform rescue walking defects efficiently, nor do they correctly edit specific sites in *Drosophila* transcripts. Surprisingly, human ADAR1p110 does suppress age-dependent neurodegeneration in *Drosophila Adar* mutants whereas ADAR1p150 does not. The single *Drosophila Adar* gene was previously assumed to represent an evolutionary ancestor of the multiple vertebrate ADARs. The strong functional similarity of human ADAR2 and *Drosophila Adar* suggests rather that these are true orthologs. By a combination of direct cloning and searching new invertebrate genome sequences we show that distinct *ADAR1* and *ADAR2* genes were present very early in the Metazoan lineage, both occurring before the split between the Bilateria and Cnidarians. The *ADAR1* gene has been lost several times, including during the evolution of insects and crustacea. These data complement our rescue results, supporting the idea that ADAR1 and ADAR2 have evolved highly conserved, distinct functions.

INTRODUCTION

The conversion of adenosine (A) to inosine (I) by RNA editing occurs in CNS transcripts in both *Drosophila* and humans, diversifying ion channels and many other proteins [for reviews see (1,2)]. The ADAR RNA editing enzymes recognize specific adenosines within RNA duplexes that form, typically by base pairing between edited exons and sequences in adjacent introns, in edited transcripts. ADARs have two or more double-stranded (ds) RNA binding domains that bind dsRNA (3), and a catalytic deaminase domain that also contributes to recognition of bases adjacent to the edited site (Figure 1A). Although the ADAR RNA editing enzymes are conserved, the editing events in particular transcripts are not; edited transcripts differ substantially between fly and human and no clear example of a conserved editing site has been found. In *Drosophila* editing is extensive. A recent study identified 972 edited positions within transcripts of 597 genes, 630 of which are predicted to alter protein-coding sequences (4) It is not known which editing events are responsible for the *Adar* phenotype (5,6). Other invertebrates such as the squid, a member of the Phylum Mollusca, also show extensive RNA editing of CNS transcripts (7–10). Vertebrates have far fewer editing events that result in recoding of transcripts and only one editing event is essential (11). One recent study identified 239 edited sites in 207 human transcripts, but only 38 are predicted to change codons (12).

Mutations to both *Drosophila* and vertebrate *ADAR* genes have catastrophic effects on the CNS. *Drosophila* has a single *Adar* gene and mutations cause a loss of locomotion in adult flies from birth and drastic age-dependent neurodegeneration (13,14). Vertebrates have two catalytically active *ADAR* genes and mutations in one of

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them, the CNS-expressed *Adar2* gene, leads to seizures and early postnatal death with localized hippocampal neurodegeneration in mice (11). The mouse *Adar2* mutant is rescued by genomically encoding a single residue change in a key AMPA class glutamate receptor subunit transcript that is normally introduced by editing. By replacing a glutamine (Q) codon with an arginine (R) codon within the region of *GluR2* transcripts that encodes the ion channel pore, *Adar2* mutant mice survive to adulthood. Editing at this site has the key functions of both restraining the assembly of AMPA receptors to synapses and blocking calcium entry through the resulting channels (15,16). Reductions in RNA editing efficiency at this site leads to production of calcium-permeable AMPA receptors and may be involved in disease symptoms such as motor neuron death through glutamate excitotoxicity in ALS (17), and selective neuron death following ischaemia in stroke (18).

Vertebrates have two other *ADAR* genes; *ADAR1* is widely expressed within the CNS as well as in mesoderm and haematopoietic lineages. Mutations in *Adar1* result in death of mouse embryos by embryonic day 12.5 with failure of haematopoiesis in the liver and overproduction of interferon (19–21), preventing the role of *Adar1* in the CNS from being assessed. ADAR1 has an intrinsic RNA editing site specificity that is distinct from that of ADAR2, however to date no site-specific editing event catalysed by ADAR1 has been found to be essential. This enzymatic substrate specificity is surprising considering the overall homology between the two proteins and also that the major groove in the A structure of dsRNA is inaccessible, rendering it difficult for proteins to read the actual base sequence of dsRNA substrates (22). Selection of particular adenosines for editing at different RNA editing sites is likely to be determined by the location of the edited base within the duplex and by its proximity to imperfect pairings between base pairs in each duplex structure (3). In addition both ADAR1 and ADAR2 have distinct yet overlapping preferences for particular nucleotides 5′ and 3′ of the editing sites when editing long dsRNA (23,24). There is some evidence of competition between ADAR1 and ADAR2 in editing: in neurons cultured from *Adar1*−/− ES cells loss of ADAR1 leads to increases in RNA editing by ADAR2 at some sites in transcripts encoding 5-HT_{2C} receptor (19,20).

Until recently the single *Drosophila Adar* gene appeared to be an invertebrate ancestor of both human ADARs and we wondered if it had similar or distinct substrate specificity to the human ADARs. As the edited sites in target transcripts are not conserved, the ADARs may also have diverged in their substrate specificities. We investigated this with RNA editing assays *in vitro* and by expressing the human ADARs in *Drosophila,* to determine if they can edit *Drosophila* transcripts, rescue locomotion defects and suppress neurodegeneration. It is advantageous to perform this analysis in *Drosophila* as there are a large number of editing sites in the fly to compare the editing site specificities of the different ADARs.

Surprisingly, we find that the editing specificity of an ADAR2-type protein is conserved from fly to human, allowing effective rescue of site-specific RNA editing events, locomotion defects and suppression of neurodegenerative phenotypes in *Adar* mutant flies by human ADAR2. ADAR1 does not efficiently edit most sites in *Drosophila* transcripts nor does it rescue the locomotion phenotype. However the different ADAR1 isoforms behave differently with regard to the neurodegeneration phenotype; ADARp110 suppress neurodegeneration whereas ADAR1p150 does not.

We conclude that *Drosophila Adar* is an orthologue of vertebrate *ADAR2*. By cloning *ADAR* genes from invertebrates and by examining data from genome sequencing projects, particularly that of the starlet sea anemone *Nematostella vectensis* (25), we show that *ADAR1* and *ADAR2* have evolved independently since early in Metazoan evolution. Both *ADAR1* and *ADAR2* genes are present in molluscs, annelids, echinoderms and even cnidarians. *ADAR1* appears to have been lost in some Arthropods, including insects, as well as in some other taxa.

MATERIALS AND METHODS

Comparison of RNA editing site specificities of *Drosophila* and vertebrate ADARs *in vitro*

All recombinant ADAR proteins were expressed and purified from *Pichia pastoris* as previously described (26). Poisoned primer extension assays in the presence of dideoxythymidine were performed with equivalent concentrations of ADAR proteins as described in (27).

Rescue of *Adar* mutant phenotypes in *Drosophila* by human *ADAR1* and *ADAR2*

cDNAs encoding full length human ADARs were cloned into the vector *pUAST* and multiple balanced transgenic *Drosophila* lines were generated with constructs inserted randomly at different locations on Chromosomes II or III. These construct lines were crossed to lines expressing GAL4 ubiquitously and strongly in all cells [*actin 5C-GAL4 25FO1* driver (28)], or strongly in cholinergic neurons [*Cha-GAL4 19B, UAS-GFP S65T* driver (29) also expressing an enhanced GFP from Chr. II]. To express ADARs in an $Adar^{5G1}$ mutant background under the control of the *Cha-GAL4* driver, for example, we crossed the *UAS-ADAR* lines to females of a strain that had the first and second chromosome genotypes *y, $Adar^{5G1}$, w/w, FM6 Bar; Cha-GAL4 / SM5 Cy* and picked male *y, $Adar^{5G1}$, w; Cha-GAL4, UAS-ADAR* progeny to measure rescue of mutant phenotypes.

We also constructed a strain that had the first and second chromosome genotypes *y, $Adar^{5G1}$, w /w, FM6 Bar; UAS-dADAR S / SM5 Cy*. This strain has no GAL4 driver but it allows the rescue effectiveness of drivers expressing GAL4 in different cell types to be tested. Crossing males of some GAL4 driver lines to females of this strain gives male *y, $Adar^{5G1}$, w; GAL4 driver; UAS-Adar S* progeny in which phenotypes are rescued by expression of the *UAS-dAdar S* construct in particular cell types.

Open field locomotion assay

We measured phenotypic rescue of *Adar*^{1F4} and *Adar*^{5G1} locomotion defects with an open field locomotion assay on flies expressing the human *UAS-ADAR* constructs 2–4 days after eclosion (30). Flies were collected using CO_2 and left for 1 day to recover before performing this assay. They were placed in a 30-mm petri dish divided into seven equal areas. The dishes were tapped and the number of times a fly walked over a line separating the zones was recorded for a 2-min period. This was then repeated a further two times for each individual fly. For each *UAS-ADAR* construct multiple different transgenic lines with random insertions were generated to control for variations in expression levels due to insertion sites. Locomotion rescue was measured for 10 or more flies from each of three different transgenic lines for each construct. RNA editing *in vivo* and protein expression levels were determined for the line of each construct that rescued locomotion best or that showed the darkest red eye colour, another correlate of expression levels at different sites of chromosomal insertion.

Other *Drosophila* GAL4 driver lines used in this study

w^{1118}*; Ddc-Gal4 L 4.3D* on Chr. II expresses GAL4 in the pattern of dopa decarboxylase which is involved in synthesis of the excitatory neurotransmitter dopamine in dopaminergic neurons. *Tdc2-GAL4 C 2* on Chr. III expresses GAL4 in the pattern of tyrosine decarboxylase which is involved in synthesis of the excitatory neurotransmitter octopamine in octopaminergic neurons. Expression of two of the three motor neurone driver lines have been examined in detail elsewhere (31). The OK6 line has a GAL4 enhancer trap insertion in the *Rapgap1* gene on Chr. II and is the driver line most highly specific for motor neurones. The D42 line is a GAL4 enhancer trap insertion in the *toll6* gene on Chr. III (31). It is expressed in a very small number of brain cells and in peripheral nervous system in addition to motor neurones. *w*^{1118}*; VGlut*^{OK371} has a GAL4 enhancer trap insertion on Chr. II in the gene encoding the vesicular glutamate vesicular uptake receptor (32), broadly expressed in all glutamatergic neurons including motor neurons. *w*^{1118}*; OK307* is a GAL4 enhancer trap insertion on Chr. II that is expressed specifically in the giant fibre descending jump escape neuron.

Haematoxylin and eosin staining

To characterize neurodegeneration 6-μm sections of paraffin wax-embedded *Adar*^{5G1} mutant heads were cut and stained with haematoxylin and eosin. To remove the wax the slides were taken through three 5-min incubations in Xylene. To re-hydrate, the slides were incubated twice in 100% ethanol for 2 min, 90% ethanol for 2 min, 80% ethanol for 2 min, 50% ethanol for 2 min, 30% ethanol for 2 min and finally in H_2O for 2 min. The slides were incubated in freshly filtered haematoxylin for 4 min and then in running tap water. Once the haematoxylin had washed out the slides were dipped twice into acid alcohol and again washed in running tap water. The slides were incubated in lithium carbonate for 3 min and then in water for 3 min. The slides were incubated in 1% eosin for 4 min and quickly washed in running tap water. The slides were dipped in 100% ethanol and then incubated three times in 100% ethanol each for 2 min. Before mounting the slides were incubated in Xylene three times, each for 5 min. The slides were mounted with D.P.X. and eyes were photographed at 40× and mushroom bodies at 63× with Zeiss Plan Neofluor objectives on a Zeiss Axiophot compound microscope with Coolsnap HQ CCD camera (Photometrics Ltd. Tuscon, AZ, USA) and images processed using IPLab Spectrum (Scanalytics Corp, Fairfax VA, USA) with all alterations of brightness and contrast covering the entire image.

Oligos, RT–PCR and sequencing

The oligos used in this study to perform RT–PCR and for sequencing the edited positions are listed in Supplementary Table S1.

Quantitating RNA editing activity *in vivo*

RNA was extracted from rescue and control male flies with Trizol reagent (Invitrogen) as described by the manufacturer and sequential RT–PCR was performed on the isolated RNA. To ensure that each RT–PCR product sequenced represents a distinct initial first strand cDNA, two separate RT reactions were performed. The majority of the editing sites were analysed by sequencing the RT–PCR reaction product pools and not by sequencing individual clones. We measured the relative heights of A and G peaks in electropherograms of RT–PCR product pools covering edited sites. Editing at each site was determined using multiple sequence chromatograms in each direction. To indicate the variability in this data: for percentage editing in adult male flies at *Eag* 2107 Y/C in Table 1 the standard error is ±2% flies and for editing at *Eag* 2159 V/V the standard error is ±2.9%. If editing appeared to be zero at a position but there was a low background in the electropherogram then we inserted an asterisk in the tables to represent this.

Phylogenetic analysis of invertebrate ADAR1 and ADAR2

Putative ADAR sequences were identified using blast searches (tblastn or blastp) against invertebrate genome sequences available at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=euk) and the Joint Genome Institute (JGI- (http://genome.jgi-psf.org/). Initially human ADAR1 and ADAR2 were used as query sequences. As we identified invertebrate homologues, they were used as queries as well. Cephalopod ADAR deaminase domains were cloned directly using cDNA samples and PCR primers based on other invertebrate ADAR sequences. Putative ADAR hits were defined as ADAR1 or ADAR2 using several criteria. First, the core deaminase domains were aligned with vertebrate

Table 1. Percentage RNA editing at specific sites in transcripts isolated from whole wildtype Canton S male or female flies, embryos and third instar larvae

	Male	*n*	Female	*n*	Embryo	*n*	Larva	*n*
Caα1D								
2061 L/L	**36**	4	**38**	4	**0**	5	0	2
2083 N/D	**97**	4	**95**	4	**22**	6	**20**	3
2097 L/L	**96**	4	**89**	4	[a]	4	**0**	3
2098 R/G	**96**	4	**92**	4	[a]	4	**0**	3
2140 I/M	**100**	2	**100**	4	**14**	6	**18**	3
Eag								
1864 K/R	**58**	11	**66**	4	**76**	2	**89**	3
2107 Y/C	**89**	11	**92**	5	**46**	3	**70**	5
2159 V/V	**16**	7	[a]	5	**0**	3	[a]	4
2163 N/D	**88**	7	**86**	5	**52**	3	**66**	4
2560 K/R	**78**	3	**60**	2	[a]	2	**0**	3
Nic 34E								
1872 L/L	**100**	6	**76**	4	**85**	4	**100**	4
1873 I/V	**100**	6	**78**	4	**85**	4	**100**	4
2020 T/A	**100**	7	**97**	6	**100**	4	**100**	3
2023 I/V	**38**	5	**30**	5	**16**	4	**17**	3
2028 L/L	**35**	5	**28**	3	**15**	2	**15**	3
2037 I/M	**67**	5	**60**	3	**41**	2	**48**	3
2049 L/L	**16**	4	**17**	3	**0**	2	[a]	3
2052 S/S	**71**	4	**63**	1	**40**	1	**40**	3
2062 I/V	**100**	4	**100**	2	**100**	2	**100**	3
2065 I/V	**53**	4	**41**	1	**15**	2	**11**	3
Rdl								
728 L/L	**23**	8	**23**	4	**0**	2	**0**	2
735 R/G	**65**	8	**68**	4	**0**	2	[a]	2
1218 I/V	**100**	8	**87**	8	**78**	2	**100**	2
1251 N/D	**22**	4	**14**	8	**0**	1	**0**	2
1448 Q/Q	**8**	4	**12**	7	**0**	2	**0**	2
1449 M/V	**22**	4	**20**	7	**0**	2	**0**	2

The left column lists the specific editing sites in target transcripts and the bold numbers indicate the percentage editing at that site in the different samples. The total number of RT–PCR reactions sequenced is represented by *n*.

[a]Editing is probably 0 however due to background in sequencing electropherogram 0 cannot be assigned to this position.

ADAR1 and ADAR2 using T-COFFEE (http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee_cgi/index.cgi) to assess general homology with residues previously defined as ADAR1 or ADAR2 consensus. Second, phylogenetic trees were generated using the entire deaminase domain. Alignments for ADAR1 and ADAR2 were generated using M-COFFEE (http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee_cgi/index.cgi). Both alignment files were joined by ClustalX2 (profile mode). Gap-rich columns were removed from each alignment. The tree was generated using Phylip Package (Protdist, Neighbor, Consense) (http://bioweb.pasteur.fr/phylogeny/intro-en.html). In the following cases only partial sequences were available; *Varroa destructor* (ADAR1 and ADAR2), *Helobdella robusta* (ADAR1), *Acropora millepora* (ADAR1) See Supplementary Table S2 for the names of species in different evolutionary groups and for sequence accession numbers. For these, separate phylogenetic trees were generated using the homologous regions from both human ADAR1 and ADAR2. Based on these trees the partial sequences were classified as either ADAR1 or ADAR2. All the accession numbers for ADAR1 and ADAR2 that were used in the alignment are in Supplementary Table S2.

RESULTS

Human ADAR1 and ADAR2 proteins show greater selectivity than *Drosophila* ADAR for specific sites *in vitro*

Human ADAR1 and ADAR2 proteins (Figure 1A), have been shown to have distinct editing site specificities for vertebrate transcripts. Using an *in vitro* poisoned primer extension assay in the presence of dideoxythymidine we compared the specific RNA editing activities of dADAR 3/4, human ADAR1p110 and human ADAR2 proteins on the *Adar exon 7* substrate from *Drosophila* which dADAR edits very efficiently *in vitro* (30) (Figure 1B) and on the *GluR2 B13* minigene substrate (Figure 1C). Fly and human ADAR proteins expressed in the yeast *Pichia pastoris* were purified and cross-species editing was tested using equivalent amounts of the different proteins sufficient for maximal editing of their specific substrates.

The vertebrate proteins are much less active on the *Drosophila Adar exon 7* substrate than dADAR 3/4 is. Human ADAR2 edits the *Adar exon7* site slightly more efficiently than human ADAR1p110, but the activity is significantly lower than that of *Drosophila* ADAR (Figure 1B). This data is in agreement with what was previously observed when all three enzymes were assayed on long dsRNA for promiscuous RNA editing and dADAR edited more sites than the two human proteins (24).

The dADAR 3/4 protein edits sites in the vertebrate substrate efficiently (Figure 1C). The *GluR2 B13* minigene substrate contains an exonic Q/R editing site that is preferentially edited by human ADAR2 and an intronic hotspot site that is preferentially edited by human ADAR1 (27,33). *Drosophila* ADAR is less selective than the human ADARs on the *GluR2 B13* minigene substrate, efficiently editing both the Q/R (ADAR2-preferred) site and the hotspot (ADAR1-preferred) site.

Because relatively few of the dsRNA structures that are required for editing have been fully defined in *Drosophila*, only a limited number of site-specific RNA editing events can be assayed *in vitro*. Since *Drosophila* has so many edited transcripts, a much larger number of edited sites can be studied *in vivo* in transgenic flies. By expressing human ADAR proteins we can elucidate if some *Drosophila* editing sites respond to human ADARs differently than the *dAdar exon7* site.

Human ADAR2 rescues locomotion defects in *Adar* mutant *Drosophila*

Constructs designed to express human *ADAR* cDNAs under UAS/GAL4 control were injected into *Drosophila* and transgenic lines were generated and balanced. To measure phenotypic rescues, human and *Drosophila* ADAR proteins were expressed in two different deletion strains of *Adar* in a range of tissue-specific expression patterns by means of the *GAL4-UAS* binary system. Both $Adar^{1F4}$ and $Adar^{5G1}$ mutants are equally grossly defective in open-field locomotion and totally lack RNA editing in all ion channel transcripts tested (Figure 2) (14). The $Adar^{1F4}$ deletion removes promoters of *Adar* but leaves the coding sequence intact and its expression is at

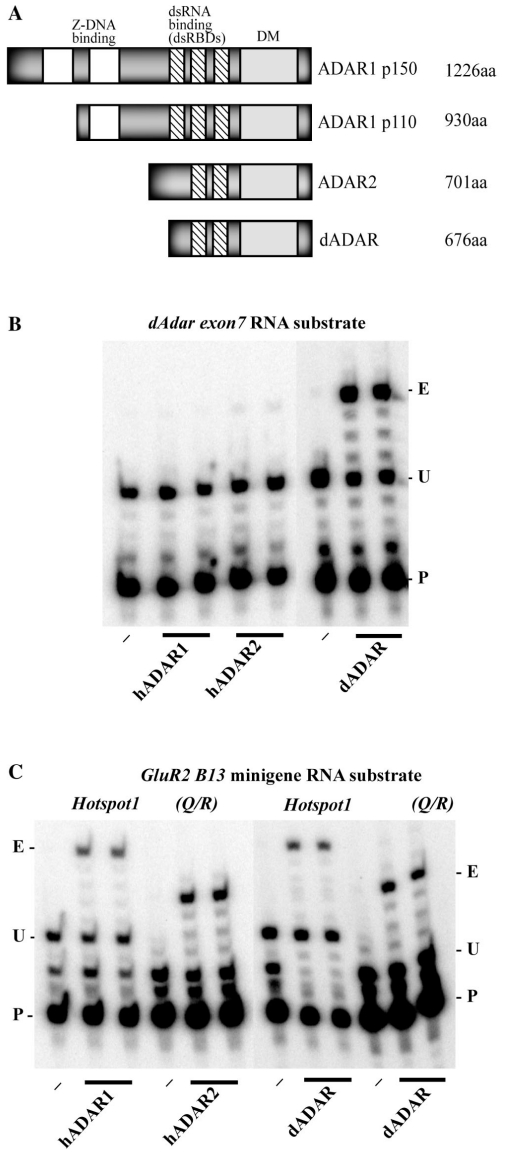

Figure 1. Comparison of human and *Drosophila* ADAR structures and activities on RNA substrates *in vitro*. (**A**) Domain structures of human and *Drosophila* ADARs. (**B**) *In vitro* RNA editing of a single site in the *Drosophila Adar exon 7* substrate by duplicate samples of *Drosophila* and human ADARs analysed by poisoned primer extension with dideoxythymidine. Dash indicates substrate RNA incubated without ADAR. For each primer extension reaction P (primer) indicates the end-labelled primer, U, (unedited) indicates the position of the next A after the primer in the template. On unedited templates primer extension terminates at the first A but if this is edited then primer extension continues to the next A, which is indicated with E, (edited). (**C**) *In vitro* RNA editing of two sites in the mammalian *GluR-2 miniB 13* substrate by *Drosophila* and human ADARs analysed with poisoned primer extension with dideoxythymidine. The *GluR-2 miniB 13* transcript contains an exonic Q/R editing site (unextended primer and unedited and edited extension product sizes indicated on the right) that is preferentially edited by human ADAR2 and an intronic hotspot site (primer and extension product sizes on the left) that is preferentially edited by human ADAR1.

least 10- to 20-fold lower (14). This strain shows residual RNA editing at only one identified site—the *Adar exon7* site. In later stages of this study we concentrate on the $Adar^{5G1}$ null mutant, as it completely removes the coding sequence and expresses no ADAR protein. In addition we found age-dependent neurodegeneration proceeds more rapidly in the $Adar^{5G1}$ null mutant.

Strong and widespread expression of ADAR proteins in both the $Adar^{5G1}$ and $Adar^{1F4}$ mutant brains was obtained using the *Cha-GAL4* driver: choline acetyl transferase encoded by the *Cha* gene is involved in the biosynthesis of acetylcholine, the major excitatory neurotransmitter in insect neurons. Because the *Drosophila Adar* gene is on the X chromosome, rescue phenotypes were measured in male flies that had the *Adar* mutation and that also had the *Cha-GAL4* driver construct and *UAS-ADAR* constructs.

Each of the two vertebrate ADARs yield viable flies when expressed under the control of the *Cha-GAL4* driver. Figure 2 shows a comparison of open field locomotion tests on $Adar^{1F4}$ (Figure 2A) or $Adar^{5G1}$ (Figure 2B) mutant flies that have *Drosophila* ADAR protein or different vertebrate ADARs expressed under the control of the *Cha-GAL4* driver. The *Adar* mutants are both grossly defective in locomotion and this defect is efficiently rescued by either the *Drosophila* ADAR 3/4 protein or human ADAR2 in either $Adar^{1F4}$ or $Adar^{5G1}$ mutant flies (Figure 2A and B) whereas the rescue with human ADAR1p110 or ADAR1p150 is barely above background and movement is not well coordinated. For each ADAR expressed the locomotion data represents an average of results obtained with three independent insertions of the relevant *UAS-ADAR* transgene and the results obtained with different insertion lines for each ADAR are consistent with each other. The wild-type control strain is w^{1118}*; Cha-GAL4*. This is an appropriate control because strong expression of GAL4 in neurons negatively affects locomotion in flies, (w^{1118} flies cross 57 lines in 2 min in this test.) Expression of ADAR 3/4 restores locomotion above the level seen in w^{1118}*; Cha-GAL4* but not quite to the level seen in w^{1118}. Locomotion rescue by *ADAR2* is not as strong as expected since it edits most *Drosophila* sites more efficiently than dADAR 3/4.

ADAR1 is expressed as either a cytoplasmic 150-kDa protein that shuttles in and out of the nucleus but accumulates in cytoplasm or as a shorter 110-kDa protein that is primarily localized to the nucleus (34). Neither isoform efficiently rescues the locomotion defects in either $Adar^{1F4}$ or $Adar^{5G1}$ mutant *Drosophila* (Figure 2A and B). There is a small effect of ADAR1 in improving the locomotion but a similar slight effect is seen with a catalytically inactive mutant form of *Drosophila* ADAR in which an essential

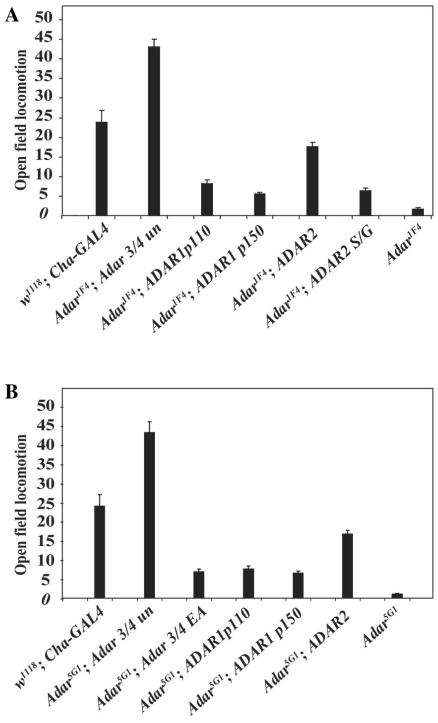

Figure 2. Human *ADAR2* rescues *Drosophila Adar* mutant locomotion defects. (**A**) Rescue by human ADAR2 of hypomorphic $Adar^{1F4}$ mutant open field locomotion defects with the strong neuron-specific *Cha-GAL4* driver. Neither the long nucleocytoplasmic shuttling human ADAR1p150 isoform nor the shorter human ADAR1p110 nuclear isoform rescue locomotion defects. (**B**) Rescue of locomotion in the $Adar^{5G1}$ null mutant.

glutamate residue at the catalytic site has been mutated to alanine (dADAR 3/4 EA, Figure 2B). Catalytic RNA editing activity at appropriate target sites is necessary for full locomotion rescue.

The equivalence of function between human ADAR2 and *Drosophila Adar* is further supported by the fact that ubiquitous expression of *UAS-ADAR2* with the *actin 5C-GAL4* driver is lethal to *Drosophila*; similar lethality was previously observed with the very active genome-encoded isoform of dADAR that has a serine residue as found in ADAR2 at the S/G RNA editing site in the deaminase domain (30). The lethality was attributed to premature editing of target transcripts during embryonic development, particularly in muscle tissue or heart which normally have lower ADAR expression than CNS. There is a very much weaker rescue of locomotion when the serine corresponding to the *Drosophila* self-editing site is mutated to glycine in ADAR2 (Figure 2A). Editing of the *GluR2 B13* minigene substrate at the Q/R site is reduced 8-fold by the serine to glycine mutation in poisoned primer extension assays (Supplementary Figure S1). Widespread ADAR1 expression under *actin 5C-GAL4* driver control is not fully lethal in *Drosophila* though viability is low and only small numbers of flies are obtained.

Human ADAR2 edits many *Drosophila* editing sites similarly to dADAR but ADAR1 edits only a subset of these sites

We do not know which individual RNA editing events or which combination of editing events in the known edited transcripts in *Drosophila* are the most essential. Therefore we chose to measure RNA editing levels in a subset of the known *Drosophila* transcripts that contain sites that are highly edited at functionally important amino acids (5). These sites were originally identified by comparative genomics due to strong evolutionary conservation among fly species of exonic sequences flanking some of the highly edited positions due to conservation of RNA duplex formation. We analysed 26 RNA editing sites in four transcripts in embryos, larvae and adult male and female flies to examine developmental RNA editing levels in these transcripts and to determine if there were sex-specific effects (Table 1). Editing levels were calculated using peak height measurements of A and G peaks in sequencing electropherograms of RT–PCR products covering each the edited sites. The analysis shows that amongst this set of transcripts some sites are fully edited such as the 1218 I/V site in the *Rdl (Resistance to Dieldrin)* transcript which encodes a pore-forming alpha subunit of a member of the inhibitory GABA-gated chloride channel family. Another transcript with fully edited sites, *Nic34E*, encodes a pore-forming subunit of acetylcholine receptors. Acetylcholine has widespread significance as an excitatory neurotransmitter in insect brain similar to that of glutamate in vertebrate brain.

As previously observed, editing at most sites is low in embryos and increases during development (13,30). There was a dramatic increase in editing of the *Caα1D* transcript encoding a muscle-type voltage-gated calcium channel that is expressed in both muscle and CNS at metamorphosis. The *Nic34E* transcript encoding a pore-forming subunit of a nicotinic acetylcholine receptor is always highly edited with two sites being edited to 100% even in early developmental stages. We decided that these sites would be informative to analyse rescue of RNA editing by human ADARs since they include sites constitutively edited by dADAR as well as sites with editing levels ranging from 0 to 100%. The constitutive editing of some of these sites throughout development (Table 1), is reminiscent of the human *GluR2 Q/R* site (35) and also suggests that these editing sites might be physiologically important. Editing of these transcripts was slightly higher in males than females.

We measured RNA editing levels in these transcripts in flies expressing either human ADAR proteins or *Drosophila* ADAR and compared these to editing levels seen in wild-type *Canton S* and *Adar* mutant flies (Tables 2 and 3). Expressing *Drosophila* ADAR 3/4 under the control of the *Cha-GAL4* driver in the $Adar^{5G1}$ background rescues RNA editing in these sites, substantially

Table 2. Percentage RNA editing at specific sites in transcripts from rescued $Adar^{5G1}$ flies expressing either dADAR, hADAR1p110, hADARp150 or hADAR2 under the control of the *Cha-GAL4* driver

	WT	*n*	*5G1*	*n*	*dAdar*	*n*	*ADAR2*	*n*	*ADAR1 P110*	*n*	*ADAR1 P150*	*n*
Caα1D												
2061 L/L	**36**	1	**0**	4	**0**	4	**18**	5	**0**	4	**0**	3
2083 N/D	**97**	2	**0**	4	**20**	4	**56**	4	**0**	4	**0**	3
2097 L/L	**96**	2	**0**	4	**0**	4	**20**	4	**0**	4	**0**	3
2098 R/G	**96**	2	**0**	4	**11**	4	**24**	4	**0**	4	[a]	2
2140 I/M	**100**	2	[a]	2	**0**	4	**25**	3	**0**	2	**0**	2
Eag												
1864 K/R	**58**	3	**0**	11	**14**	5	**10**	2	[a]	4	**0**	6
2107 Y/C	**89**	5	**0**	11	**21**	5	**36**	9	**0**	7	**0**	13
2159 V/V	**16**	5	**0**	7	**0**	3	**23**	7	**0**	7	**0**	13
2163 N/D	**88**	5	**0**	7	**52**	3	**30**	7	**0**	7	**10**	13
2560 K/R	**78**	2	[a]	3	[a]	1	**31**	6	**0**	3	[a]	11
Nic 34E												
1872 L/L	**100**	4	**0**	6	**16**	2	**54**	6	**0**	4	**0**	3
1873 I/V	**100**	4	**0**	6	**14**	2	**56**	6	**0**	4	**0**	3
2020 T/A	**100**	3	**0**	7	**55**	2	**79**	5	**0**	3	[a]	3
2023 I/V	**38**	1	**0**	5	**0**	2	**10**	5	**0**	3	**0**	3
2028 L/L	**35**	1	[a]	5	**0**	2	**19**	5	**0**	3	**0**	2
2037 I/M	**67**	1	[a]	5	**6**	2	**49**	5	**0**	2	**21**	7
2049 L/L	**16**	1	**0**	4	**0**	2	**0**	4	**0**	3	**0**	6
2052 S/S	**71**	1	**0**	4	**18**	2	**10**	3	**0**	3	**0**	6
2062 I/V	**100**	3	**0**	4	**46**	2	**31**	3	**0**	2	*	6
2065 I/V	**53**	1	**0**	4	**14**	2	**11**	3	**0**	2	**11**	4
Rdl												
728 L/L	**23**	2	[a]	8	[a]	6	**12**	4	**10**	11	[a]	8
735 R/G	**65**	2	[a]	8	**12**	6	**39**	4	**16**	11	[a]	8
1218 I/V	**100**	3	[a]	8	**43**	3	**81**	5	**0**	4	**0**	4
1251 N/D	**22**	3	**0**	4	**0**	3	**0**	12	**0**	4	**0**	4
1448 Q/Q	**8**	3	**0**	4	**0**	4	**0**	7	**0**	4	**0**	5
1449 M/V	**22**	3	**0**	4	**0**	4	**0**	7	**0**	4	**0**	4

The left column lists the specific editing sites in target transcripts and the bold numbers indicate the percentage editing at that site in the different samples. The total number of RT–PCR reactions sequenced is represented by *n*.
[a]Editing is probably 0 however due to background in sequencing electropherogram 0 cannot be assigned to this position.

though not completely (Table 2). Editing is completely dependent on dADAR as it is eliminated in the $Adar^{5G1}$ mutant and not restored by expression of a catalytically inactive dADAR 3/4 EA protein (data not shown). Human ADAR2 edits 22/26 sites analysed in *Drosophila* when expressed using the *Cha-GAL4* driver in $Adar^{5G1}$ (Table 2). The levels of editing at specific sites are generally similar to, and generally higher than, levels obtained for rescue by dADAR expressed under the control of the *Cha-GAL4* driver. We have repeated this with different drivers and the pattern of editing with ADAR2 is always similar to that with dADAR. Human ADAR1p110 and p150 display low levels of editing activity, 2/26 and 3/26 sites respectively were edited.

When the $Adar^{1F4}$ hypomorphic mutant background is used in rescue experiments with the *Cha-GAL4* driver the pattern of locomotion rescue is unchanged from that obtained in the $Adar^{5G1}$ null background, i.e. ADAR2 rescues and ADAR1 isoforms do not (Figure 2). Levels of RNA editing at most sites are higher in $Adar^{1F4}$ rescues with *UAS-dAdar* and *UAS-hADAR2* than in the $Adar^{5G1}$rescues with the same UAS-ADAR transgenic lines (Table 3), presumably due to some assistance from the low level of residual dADAR in the $Adar^{1F4}$ strain. Also RNA editing by ADAR1 is observed at more sites in ion channel transcripts in the $Adar^{1F4}$ rescue but the pattern of sites with high and low levels of editing is very different from that seen in wild-type flies or in rescues by *Drosophila* ADAR protein or human ADAR2 (Table 3). This is exemplified by editing of the *Nic 34E* transcript where sites that are normally edited to 100% are edited slightly or not all by ADAR1 yet other sites within in the same transcript are highly edited by ADAR1p110 (*Nic 34E* I/M site, 84%) Editing activity is due to ADAR1 itself and not to endogenous *Drosophila* ADAR protein because no editing is observed at any site in transgenic flies expressing catalytically inactive ADAR1 EA (not shown). We conclude that human ADAR1, even when it succeeds in editing ion channel transcripts in *Drosophila*, does not restore the wild-type pattern of editing.

The ADAR proteins are expressed at low levels and cannot be detected on immunoblots of total protein extracts from embryos, whole flies or fly heads. In the case of ADAR2 low level expression in mammalian cells is due to the activity of a specific E3 ubiquitin ligase (R. Marcucci, manuscript in preparation). To express ADARs strongly in embryos male flies of *UAS-ADAR* lines were crossed to *actin 5C-GAL4 / SM5 Cy* and soluble protein extracts were made from 48-h embryo collections. The FLAG-tagged ADAR proteins were immunoprecipitated from extracts with anti-FLAG antibodies and the proteins were detected on immunoblots with anti-FLAG or

Table 3. Percentage RNA editing at specific sites in transcripts from rescued $Adar^{1F4}$ flies expressing either dADAR, hADAR1p110, hADARp150 or hADAR2 under the control of the *Cha-GAL4* driver

	WT	*n*	*1F4*	*n*	*dAdar*	*n*	*ADAR2*	*n*	*ADAR1 P110*	*n*	*ADAR1 P150*	*n*
Cac1D												
2061 L/L	**36**	1	[a]	4	**13**	5	**24**	4	**25**	2	**29**	
2083 N/D	**97**	2	**0**	3	**31**	5	**2**	4	**0**	2	**0**	
2097 L/L	**96**	2	**0**	3	**24**	5	**0**	2	**0**	1	**0**	
2098 R/G	**96**	2	[a]	3	**29**	5	**32**	2	**0**	1	**0**	
2140 I/M	**100**	2	**0**	4	**26**	5	**20**	4	**0**	1	**0**	
Eag												
1864 K/R	**58**	3	**0**	2	**50**	2	**58**	2	**0**	7	**0**	
2107 Y/C	**89**	5	**0**	2	**55**	3	**56**	5	**10**	7	**0**	
2159 V/V	**16**	5	**0**	2	**10**	5	**24**	6	**0**	7	**0**	
2163 N/D	**88**	5	**0**	2	**62**	5	**46**	6	**40**	7	**12**	
2177 A/A	**0**	5	**0**	2	[a]	5	**0**	6	**0**	7	**0**	
2560 K/R	**78**	3	**0**	1	**37**	2	**56**	2	**0**	3	**0**	
Nic 34E												
1872 L/L	**100**	4	**0**	5	[b]		**76**	2	**0**	1	**0**	
1873 I/V	**100**	4	**0**	5	[b]		**74**	2	**0**	1	**0**	
2020 T/A	**100**	3	**0**	5	**82**	3	**80**	3	**26**	3	**0**	
2023 I/V	**38**	1	**0**	5	**35**	3	[a]	3	**0**	3	**0**	
2028 L/L	**35**	1	**0**	5	**29**	3	**15**	3	**0**	3	**0**	
2037 I/M	**67**	1	**0**	4	**63**	3	**75**	3	**84**	3	**37**	
2052 S/S	**71**	1	**0**	4	**60**	3	**8**	3	**27**	3	**0**	
2062 I/V	**100**	3	**0**	5	**84**	3	**38**	2	**32**	3	**10**	
2065 I/V	**53**	1	**0**	5	**42**	3	**13**	2	**54**	2	**17**	
Rdl												
728 L/L	**23**	2	**0**	2	**29**	2	**34**	4	[a]	2	**0**	
735 R/G	**65**	2	**0**	2	**52**	2	**64**	4	**15**	2	**0**	
1218 I/V	**100**	3	[a]	2	**88**	2	**81**	4	**31**	2	**16**	
1251 N/D	**22**	3	**0**	3	**0**	2	**0**	5	**0**	2	**0**	
1448 Q/Q	**8**	3	**0**	3	**10**	2	**0**	3	**0**	2	**0**	
1449 M/V	**22**	3	**0**	3	**12**	2	[a]	3	**0**	2	**0**	

The left column lists the specific editing sites in target transcripts and the bold numbers indicate the percentage editing at that site in the differen samples. The total number of RT–PCR reactions sequenced is represented by *n*.
[a]Editing is probably 0 however due to background in sequencing electropherogram 0 cannot be assigned to this position.
[b]Sites that we were unable to obtain sequence for.

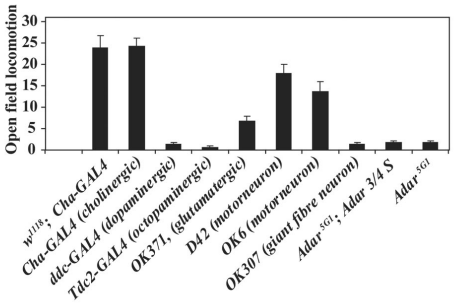

Figure 3. *Adar* expression in cholinergic or motor neurons is sufficient to rescue $Adar^{5G1}$ mutant locomotion defects. The chart shows open field locomotion in $Adar^{5G1}$ flies, $Adar^{5G1}$*; UAS-Adar 3/4 S* flies having this UAS construct in the absence of any GAL4 driver to induce expression or lines in which the *UAS-Adar 3/4 S* construct is expressed in the $Adar^{5G1}$ background under the control of different GAL4 drivers. The wild-type control is w^{1118}, *Adar* wild-type having a *Cha-GAL4* driver to control for locomotion effects of widespread and strong GAL4 expression. Drivers expressing GAL4 in motor neurons, giant fibre escape neurons and different chemical classes of neurons are indicated. Drivers expressing GAL4 specifically in motor neurons (OK6, D42 and OK371) and *Cha-GAL4* which expresses GAL4 in cholinergic neurons and some motor neurons direct efficient rescue.

anti-His antibodies. This allowed confirmation that proteins of the expected sizes are expressed at similar though not identical levels. The ADAR1p150 protein was not detected in this way but other evidence indicates that this protein is expressed and that it behaves differently than ADAR1p110 (36).

To ascertain if the fly and human proteins have similar levels of RNA editing activity in transgenic flies and therefore similar protein expression, we analysed non-specific RNA editing of the *Rnp-4F* transcript. This transcript is overlapped at the 3′-end by a convergently transcribed antisense transcript generated by read-through at the transcription terminator of the convergently transcribed gene (37). The resulting dsRNA is promiscuously edited by ADARs. Non-specific editing in the *Rnp-4F* transcript is rescued to the same level as in wild-type (approximately 14%) in *Adar* mutant flies rescued by expression of dADAR 3/4, ADAR1 p110 and p150 and human ADAR2 under *engrailed-GAL4* control.

Locomotion defects in *Adar* mutant flies are rescued by expression of ADAR specifically in motor neurons

We have tested rescue of the locomotion defect by ADARs using a wide range of GAL4 drivers in addition to *Cha-GAL4*. We constructed a strain that had $Adar^{5G}$

on the X chromosome and a *UAS-dAdar 3/4 S* construct on the second chromosome and crossed a number of different GAL4 drivers to this strain (Figure 3). Surprisingly the enhancer trap GAL4 driver lines D42 and OK6 that drive GAL4 and UAS construct expression specifically in motor neurons, give efficient rescue of the *Adar* locomotion defect (Figure 3). In *Drosophila* neuromuscular junctions are primarily glutamatergic. The GAL4 enhancer trap line OK371 has a GAL4 insert in the promoter region of the gene encoding the vesicular glutamate transporter and this line directs expression in motor neurons as well as widely in a range of other glutamatergic neurons in the brain. None of the driver lines tested has expression that is absolutely restricted to motor neurons although OK6 has very little expression elsewhere in the CNS (31). Also the locomotion rescue by all three GAL4 driver lines is consistent with motor neurons being the main focus of the locomotion defect. Among all GAL4 drivers we have tested those whose expression patterns are known to include motor neurons consistently give efficient locomotion rescue.

Drivers expressing in neurons of other pharmacological types implicated in the central control of movement such as *ddc-GAL4* (dopamine decarboxylase in dopaminergic neurons) or *Tdc2-GAL4*, (tyrosine decarboylase 2 in octopaminergic neurons) are not sufficient to direct locomotion rescue. Expression of ADARs in muscles, (*How(Held-out wings)-GAL4*) or in glia, (*nrv(nervana)-GAL4*) do not give rescue of walking defects (data not shown).

Human ADAR2 suppresses age-dependent neurodegeneration in *Adar* mutant *Drosophila*

$Adar^{1F4}$ flies undergo progressive vacuolization of the synaptic neuropile from 30 to 50 days (14). As the $Adar^{5G1}$ deletion mutant is less viable than the $Adar^{1F4}$ mutant strain it was hypothesized that the neurodegeneration in $Adar^{5G1}$ would be more aggressive. To characterize the neurodegeneration pattern of the $Adar^{5G1}$ mutant strain, $Adar^{5G1}$ mutant males were aged, and heads were sectioned at 30 days and stained with haematoxylin and eosin (Figure 4). This revealed that vacuolization occurred in the $Adar^{5G1}$ mutant as it did in the $Adar^{1F4}$ mutant. However the neurodegeneration was more aggressive in the $Adar^{5G1}$ mutant, not only affecting the retina (Figure 4D, compare to wild-type in B), but also the paired mushroom body (MB) calyces on the dorsal brain (Figure 4C, compare to wild-type in A). The mushroom body calyces are neuropil which is comprised of the dendrites of mushroom body Kenyon cells whose haematoxylin-stained nuclei lie above the calyces, and the axonal collaterals of projection neurons extending to them from the paired olfactory glomeruli on the ventral brain above the antennae.

To confirm that the neurodegeneration that had been observed in aged $Adar^{5G1}$ is due to the *Adar* deletion, the *UAS-Adar 3/4* transgenic line was crossed into $Adar^{5G1}$; *Cha-GAL4*. The $Adar^{5G1}$mutant male rescued by expression of *dAdar 3/4* in the cholinergic nervous system was aged to 30 days and the MB calyces and retina were analysed by haematoxylin and eosin staining of head sections. The vacuolization of the neuropil of the MB calyces and retina of the $Adar^{5G1}$; *Cha-GAL4* male rescued with *Adar 3/4* is significantly reduced compared to the $Adar^{5G1}$mutant strain at 30 days (Figure 4).

As neurodegeneration in the $Adar^{5G1}$ mutant strain is successfully suppressed by *Cha-GAL4*-driven expression of *dAdar*, it was therefore possible to compare suppression of this phenotype by human ADARs. We aged the transgenic flies to 30 days to visualize neurodegeneration (Figure 5). Human ADAR2 suppresses neurodegeneration of both the calyces of the mushroom body (Figure 5E) and in the retina (Figure 5F) as effectively as *Drosophila* ADAR in the $Adar^{5G1}$ mutant background in flies aged to thirty days. The suppression of neurodegeneration at thirty days is weaker with the nuclear p110 form of human ADAR1 (Figure 5A, ADAR1p110 calyx, Figure 5B, ADAR1p110 retina) but is lacking entirely with the cytoplasmically accumulating p150 isoform of ADAR1 (Figure 5C, ADAR1p150 calyx, Figure 5D, ADAR1p150 retina), suggesting that suppression of neurodegeneration is associated with nuclear localization of the ADAR proteins. It appears that suppression of neurodegeneration by ADAR proteins is easier to obtain than rescue of the locomotion defect.

Insects have lost the *ADAR1* gene

Human ADAR2 expressed in *Drosophila* matches the target site specificity of dADAR and rescues mutant phenotypes surprisingly well while human ADAR1 does not. These data suggest that *Drosophila Adar* may be a true orthologue of human *ADAR2* rather than an invertebrate gene ancestral to both vertebrate *ADAR*s. Because the *Drosophila* genome harbours a single *Adar* gene, this idea would imply that flies have lost an *ADAR1* orthologue. Sequence data from recent invertebrate genome projects supports this idea. Many genes that were previously assumed to have first appeared only at the separation of Chordates from invertebrates have now been found in some of the simplest invertebrates like cnidarians (25). Both the *ADAR1* and *ADAR2* genes are in this category.

Figure 6 shows results of our searches for invertebrate *ADAR*s mapped onto the phylogeny of all Metazoans that extend a previous report (38) (Supplementary Table S2). For all putative *ADAR* sequences, the deaminase domain was aligned with those from human *ADAR1* and *ADAR2*. In most cases each *ADAR* could be classified as an orthologue of *ADAR1* or *ADAR2* with a high degree of confidence (Supplementary Figures S2 and S3). Surprisingly, having discrete *ADAR1* and *ADAR2* genes is an ancient characteristic, present throughout the Eumetazoa lineage, including its oldest phylum, the Cnidaria. In a few cases, however, *ADAR1* appears to have been lost. For example, an *ADAR1* orthologue was not found in multiple insect and crustacean genomes. It was found in some arachnids, indicating that it was not lost in all arthropods. Among the cnidarians, hydrozoans also seem to have lost ADAR1, although it was present in anemones (its presence or absence in corals cannot be

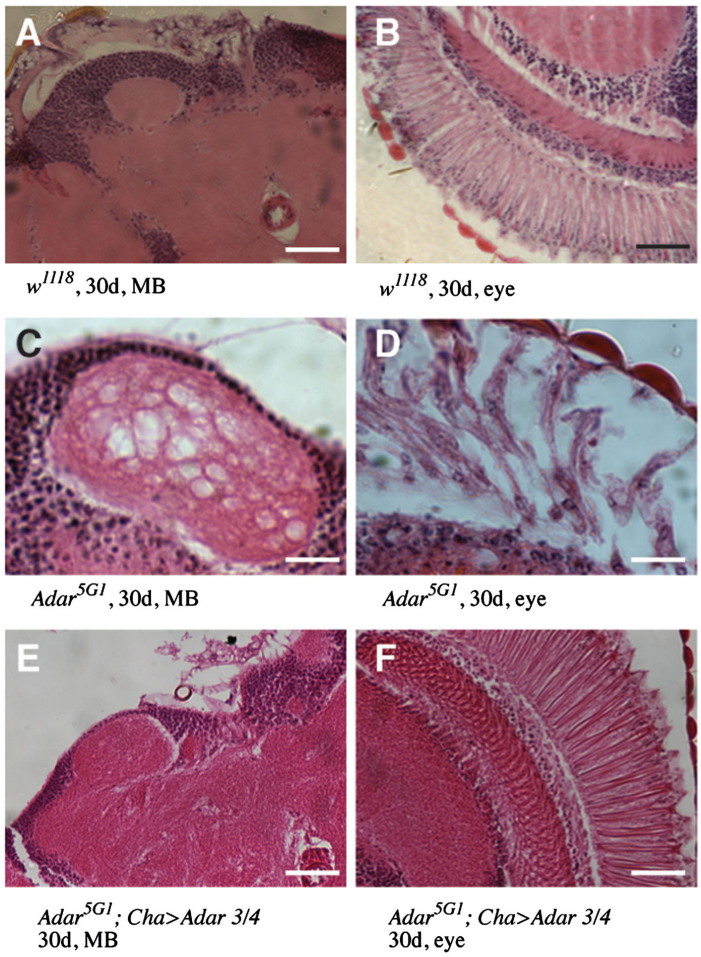

Figure 4. Suppression of neurodegeneration in $Adar^{5G1}$ mutant flies by *Drosophila* ADAR. (**A** and **B**). Haematoxylin and eosin stained frontal sections of 30-day-old wild-type (w^{1118}) heads show no neurodegeneration in the mushroom body calyces or in the eye. Scale bars: 20 μM. (**C** and **D**) Frontal sections of 30 day-old $Adar^{5G1}$ heads show vacuolization and loss of Mushroom Body calyx neuropil (C) and large vacuoles in the retina of the eye (D) of Scale bars: 5 μM. (**E** and **F**) Frontal sections of 30-day-old $Adar^{5G1}$; *Cha GAL4, UAS-Adar 3/4* heads show rescue of vacuolization in the MB calyx and in the eye. Scale bars: 20 μM.

clearly inferred because no genome is available, only a partial EST library). *ADAR2* appears to be more ubiquitous. In fact, the only genome that possibly lacks an *ADAR2* orthologue, but contains one for *ADAR1*, is *Aplysia*. However, the apparent absence of an *Aplysia ADAR2* could be due to incomplete coverage of the *Aplysia* genome. Interestingly, nematodes and flatworms have neither a true *ADAR1* nor *ADAR2* orthologue. The two *Adr* genes from *Caenorhabditis elegans* cannot be classified into either group (39).

Together the findings of an ancient Metazoan *ADAR2* conserved between fly and human, and loss of an ancient Metazoan *ADAR1* in insects explain the results of the rescue tests with human *ADAR*s in fly and account for the surprising similarity in target site preferences between human ADAR2 and *Drosophila* ADAR.

DISCUSSION

We find that the target specificity of an ADAR2-type protein is conserved from fly to human allowing effective rescue of *in vivo* RNA editing, locomotion and neurodegenerative phenotypes in flies by human ADAR2. Neither ADAR1p110 nor ADAR1p150 efficiently edit critical sites in *Drosophila* transcripts nor rescue the *Adar* mutant locomotion phenotype. This data was

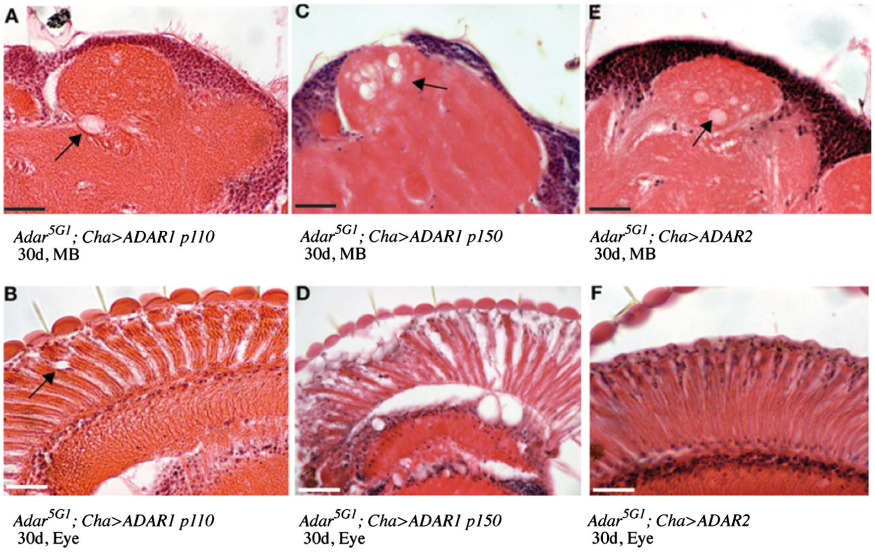

Figure 5. Suppression of neurodegeneration at 30 days in *Adar*^{5G1} mutant flies by human ADAR2. (**A** and **B**): Haematoxylin and eosin stained frontal sections of 30-day-old *Adar*^{5G1}; *Cha-GAL4, UAS-ADAR1p110* heads show rescue of neurodegeneration in the mushroom body (MB) calyces of the *Adar*^{5G1} mutant. (A). Some small vacuoles remain in the retina (B). Arrows indicate vacuolization. (**C** and **D**): Frontal sections of 30-day-old *Adar*^{5G1}; *Cha-GAL4, UAS-ADAR1p150* heads show lack of neurodegeneration rescue in the MB calyces of *Adar*^{5G1} (C) The retina degenerated rapidly (D). (**E** and **F**): Frontal sections of 30-day-old *Adar*^{5G1}; *Cha-GAL4, UAS-ADAR2* heads show rescue of vacuolization of the MB calyces (E) and the eye (F). Scale bars: 20 μM.

obtained before the recent increase in vertebrate genome sequences and is well explained by the identification of ancient Metazoan *ADAR1* and *ADAR2* genes in invertebrate genomes. Previously these *ADAR* genes had been identified only in Chordate genomes and not in *Drosophila* and other insects. We find that *ADAR2* is conserved in *Drosophila* and that *ADAR1* has been lost from insects and crustaceans but is present in Arachnid genomes. The data also show that the *Drosophila Adar* mutant represents a very useful genetic model for *ADAR2* loss of function effects in human disease even though different transcripts are edited in vertebrates and flies. Restoration of ADAR activity in motor neurons, a fundamental neuron type present in even the simplest metazoans, is sufficient to rescue locomotion defects in *Adar* mutant flies.

The lack of RNA substrates from *Drosophila* with defined ECS elements made it impossible to analyse the activities of ADAR1 and ADAR2 at many *Drosophila* editing sites *in vitro*. We find that RNA structures at specific editing sites in *Drosophila* are often difficult to predict from the genome sequence. Although vertebrate editing sites show easily recognized pairings between edited exons and editing site complementary sequence (ECS) elements that exist as contiguous stretches of sequence in nearby introns, some fly sites may have shorter fragmented ECSs, as shown for the *Drosophila synaptotagmin1 (Syt1)* transcript (40). To analyse rescue at more editing sites we expressed the human ADAR proteins in *Drosophila* and measured editing by these proteins in *Adar* mutant flies. We focused on 26 edited positions in four transcripts that were either constitutively highly edited at all developmental stages or edited only or predominantly in adult flies. We have also analysed other edited positions in many other transcripts, though not in such depth, and the overall pattern of editing at these other positions with different ADARs did not vary from our core set. Our data showed that the set of edited sites in *Drosophila* match the specificity of an ADAR2 enzyme but not an ADAR1 enzyme to a surprising extent, i.e. the fly ADAR does not appear to represent an evolutionary precursor that might combine features of two descendant vertebrate ADARs. This is consistent with greater sequence conservation between *Drosophila* ADAR and vertebrate ADAR2.

Human *ADAR2* expressed in *Drosophila* mirrors the function of the fly gene in many respects. We found that *actin 5C-GAL4* and other drivers that direct ubiquitous high level expression of *ADAR2* in embryos and larvae or *Mef 2-GAL4* that directs similarly premature high level expression in muscles and heart cause embryonic and larval lethality. We have previously observed similar lethality with the edited *dAdar S* isoform that is the most active *Drosophila* ADAR isoform (30). This is presumably due to some transcripts being edited inappropriately early in development. Expressing either an edited-equivalent *Drosophila UAS-ADAR 3|4 G* isoform or *UAS-ADAR2 G* do not cause this lethality. Human ADAR2 also

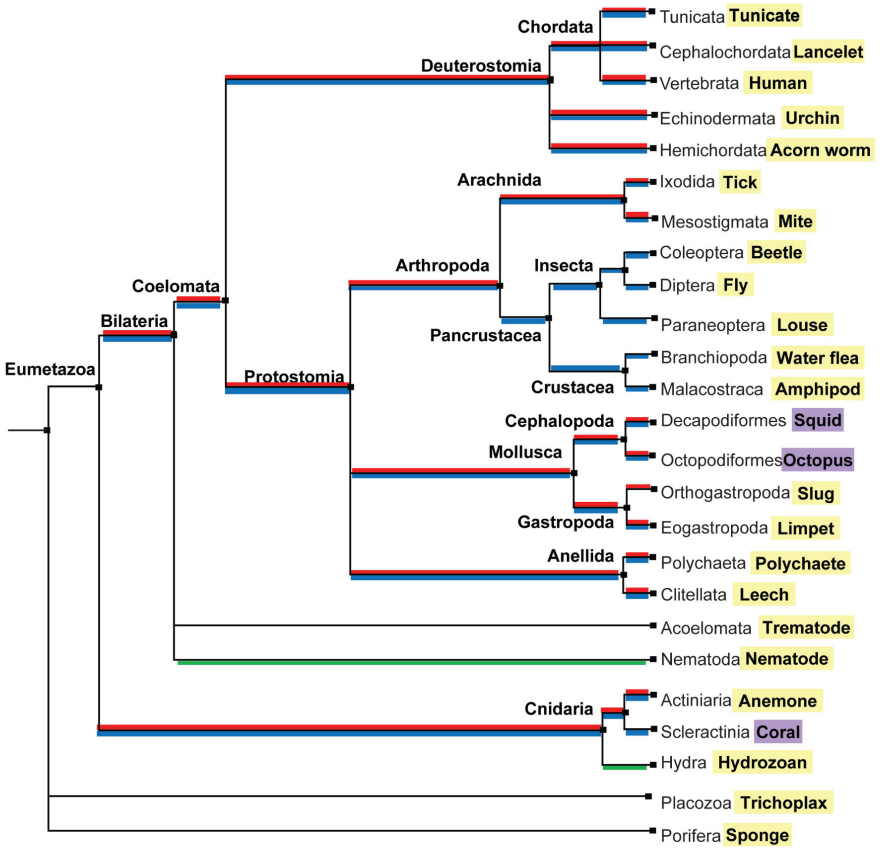

Figure 6. Occurrence of *ADAR1* and *ADAR2* genes in the Metazoa. The phylogenetic tree of species was obtained from Taxonomy Common Tree NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi). Species names at the ends of branches highlighted in yellow represent available genomes that were searched for *ADAR1* or *ADAR2* orthologues. Species names highlighted in purple were cases where *ADAR*s were identified by direct cloning (cephalopods) or searching EST resources (coral). Positive identification of *ADAR1* or *ADAR2* is coloured in red and blue, respectively. *ADAR*s that cannot be classified as either *ADAR1* or *ADAR2* are coloured in green.

rescues neurodegeneration in *Adar* mutant flies as does dADAR 3/4.

Human ADAR2 does not rescue locomotion defects in the *Adar* mutants as well as expected since in the best-rescuing *UAS-ADAR2* line sites in *Drosophila* transcripts are edited more effectively than in the best-rescuing dADAR 3/4 line (Tables 2 and 3). We do not know why this is. Since ADAR2 is less active than dADAR 3/4 in editing the *dAdar exon 7* site *in vitro* (Figure 1B) it might be expected that for ADAR2 to edit sites *in vivo* in *Drosophila* more efficiently than dADAR 3/4 would require a higher level of ADAR2 expression. We cannot rule out that ADAR2 is more highly expressed than dADAR 3/4 and has also some deleterious effect due to a higher expression level that interferes with locomotion rescue.

We do not understand why ADAR1p110 also rescues neurodegeneration but the finding suggests that rescue of neurodegeneration may not be dependent on rescue of site-specific RNA editing. ADAR proteins may have dosage-sensitive effects independent of their RNA editing specificities since ADAR1p110 is able to rescue neurodegeneration even though it does not edit correctly. The ability to rescue neurodegeneration correlates with predominant localization to the nucleus. It does not appear likely that rescued RNA editing of a subset of the *Drosophila* sites is the reason that ADAR1p110 rescues neurodegeneration, since ADAR1p150 edits most of the same sites to some extent, but we cannot rule out this possibility. Editing independent effects of ADARs expressed in motor neurons might also account for the small improvements in locomotion seen when ADAR1 isoforms

or inactive dADAR 3/4 EA are expressed in *Adar* mutant flies and might also contribute to the toxicity of high level ADAR1 isoform expression.

Ironically, even though the target specificity of ADAR2-like proteins is well conserved and *Drosophila* has many edited transcripts, there is no evidence that any editing sites are conserved between *Drosophila* and vertebrates. There is no evidence for editing of transcripts encoding ionotropic glutamate receptor subunits in *Drosophila* even though this family of genes is conserved with vertebrates; vertebrate glutamate receptor editing appears to have first evolved in fish. None of the many editing sites in *Drosophila* transcripts can be related to known editing sites in vertebrate homologues and the one known case where a fly and vertebrate transcript are edited at the equivalent codon appears to have arisen by convergent evolution rather than by conservation of the underlying dsRNA target structure (41). This makes more impressive the finding that human ADAR2 has retained specificity and rescues the *Drosophila Adar* mutant.

As *Drosophila* has lost ADAR1, the possibility existed that certain sites would remain ADAR1-preferred sites since dADAR may have a higher specific activity or a slightly broader specificity than the vertebrate ADARs (Figure 1B and C). However this has not occurred and the tested editing sites in *Drosophila* are all preferentially edited by ADAR2. RNA editing sites at sites once edited by ADAR1 may have adjusted to conform better with the ADAR2-like target specificity after ADAR1 was lost in insects and crustaceans. Now that so many RNA editing events have been detected in *Drosophila* (4), evolutionary comparisons across invertebrates may be able to establish whether some RNA editing events are conserved since the insects diverged from crustaceans or arachnids or more distant groups and perhaps also determine which ADARs edited these sites in more primitive invertebrates. We cannot exclude the possibility that human ADAR1 edits some completely unknown sites in RNA duplexes in *Drosophila* transcripts that might represent relics of ancient ADAR1 editing events. This could provide one explanation for the reduced viability associated with highly expressing ADAR1 isoforms but we did not see any evidence for new human ADAR1 RNA editing events close to the *Drosophila* editing sites examined in rescue lines *in vivo*. ADAR1-type sites retained in *Drosophila* might not be edited by *Drosophila* ADAR and it would require a genome-wide search by RNA Sequencing in ADAR1-expressing flies to detect them, if they are still present. It is not clear however that ADAR1 editing sites would be conserved since the beginning of modern insects. Whole genome sequences are available for only a limited number of insect and crustacean species so there could be some insects and crustaceans that do still have *ADAR1*. With the full extent of editing in humans still to be determined, 4% of *Drosophila* transcripts affected and indications that RNA editing may be even more widespread in squid studies on the evolutionary origins of RNA editing sites and the selective forces maintaining them will expand our understanding of the role of RNA in gene expression.

What is most surprising is that *ADAR1*, an essential gene in mammals, has been lost in some invertebrates. Is there a biological role of ADAR1 other than site-specific editing that became dispensable?

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Pin1 and WWP2 regulate *GluR2* Q/R site RNA editing by ADAR2 with opposing effects

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ADAR2 catalyses the deamination of adenosine to inosine at the *GluR2* Q/R site in the pre-mRNA encoding the critical subunit of AMPA receptors. Among ADAR2 substrates this is the vital one as editing at this position is indispensable for normal brain function. However, the regulation of ADAR2 post-translationally remains to be elucidated. We demonstrate that the phosphorylation-dependent prolyl-isomerase Pin1 interacts with ADAR2 and is a positive regulator required for the nuclear localization and stability of ADAR2. *Pin1*$^{-/-}$ mouse embryonic fibroblasts show mislocalization of ADAR2 in the cytoplasm and reduced editing at the *GluR2* Q/R and R/G sites. The E3 ubiquitin ligase WWP2 plays a negative role by binding to ADAR2 and catalysing its ubiquitination and subsequent degradation. Therefore, ADAR2 protein levels and catalytic activity are coordinately regulated in a positive manner by Pin1 and negatively by WWP2 and this may have downstream effects on the function of GluR2. Pin1 and WWP2 also regulate the large subunit of RNA Pol II, so these proteins may also coordinately regulate other key cellular proteins.

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Introduction

The AMPA class of glutamate-gated ion channel receptors (GluR) are impermeable to calcium if a GluR2 subunit is present in the tetrameric receptor (Hollmann *et al*, 1991; Verdoorn *et al*, 1991). This impermeability to calcium results from RNA editing of the *GluR2* transcript. The enzyme that catalyses this RNA editing event is a member of the family of adenosine deaminases that act on RNA (ADARs). ADAR2 specifically deaminates an adenosine residue in a glutamine (Q) codon to an inosine that is read as guanosine by reverse transcriptase and the translational machinery. ADAR2 converts the glutamine (Q) codon to an arginine (R) codon with 100% efficiency at the *GluR2* Q/R site changing a key residue in the ion channel pore and rendering AMPA receptors assembled with this subunit impermeable to calcium (Sommer *et al*, 1991). The editing event also regulates AMPA receptor assembly, slowing the passage of the GluR2 subunit through the ER thus ensuring correct receptor assembly (Greger *et al*, 2003). Failure of RNA editing at this site can lead to neuronal cell death due to the influx of calcium (Higuchi *et al*, 2000). A decrease in editing at this site has been reported in sporadic ALS motor neurons (Kawahara *et al*, 2004) and in hippocampal neurons following transient forebrain ischaemia in a rat model of stroke (Peng *et al*, 2006).

Mice that are null mutants for ADAR2 are seizure-prone and die within 3 weeks after birth (Higuchi *et al*, 2000). Lethality in these *Adar2*$^{-/-}$ mice can be rescued by knocking-in the edited isoform of GluR2 (*GluR2*^{R}). This experiment suggests that despite ADAR2 having other transcripts that it edits, the critical site is the Q/R site in *GluR2* transcripts. These rescued mice have a normal phenotype, suggesting that the unedited GluR2 isoform does not have an essential biological function.

For this deamination event to occur, ADAR2 must recognize and bind to double-stranded (ds)RNA that is formed at the editing site between the edited exon and the downstream intron (Higuchi *et al*, 1993). Identified transcripts edited specifically by ADAR2 are mostly expressed in the CNS even though the protein is also expressed in other tissues. RNA editing occurs before splicing and ADAR2 localizes to the nucleus. In some cells, ADAR2 accumulates within the nucleolus (Desterro *et al*, 2003; Sansam *et al*, 2003); however, this localization is dynamic. When transcripts that can be edited are overexpressed in these cells, ADAR2 relocalizes to the nucleoplasm (Desterro *et al*, 2003).

Until now the only regulator found to influence ADAR2 expression is CREB, which can induce ADAR2 expression in hippocampal CA1 neurons in rat brain (Peng *et al*, 2006). In this study, we demonstrate that ADAR2 is dynamically regulated post-translationally by the phosphorylation-dependent peptidyl-prolyl *cis/trans* isomerase Pin1 (peptidyl-prolyl isomerase NIMA interacting protein 1). Pin1 binds to a phosphorylated serine or threonine residue preceding a proline residue and catalyses the *cis/trans* isomerization of the peptide bond (Lu *et al*, 1999). This conformational change can have a range of consequences on the function of target proteins, altering catalytic activity, stability or subcellular localization (for review see Lu and Zhou, 2007). Pin1 binds to the amino-terminus of ADAR2 in a phosphorylation-dependent manner. In the absence of Pin1, ADAR2 protein is

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more labile and is mislocalized to the cytoplasm, where it is unable to edit pre-mRNAs and there is a decrease in editing of the Q/R and R/G sites in endogenous *GluR2* transcripts. Pin1 is therefore a positive regulator of ADAR2 editing activity.

We also identify a negative regulator of ADAR2 activity, which is WWP2; a HECT (homologous to the E6-AP C terminus) E3 ubiquitin ligase (Pirozzi *et al*, 1997). WWP2 binds to a conserved PPxY motif in ADAR2 and this interaction results in ubiquitination and subsequent degradation of ADAR2. An increase in the expression of WWP2 results in a decrease in ADAR2 protein level. This report of the post-translational regulation of ADAR2 demonstrates how RNA editing activity is controlled by coordinate action of two regulators.

Results

Phosphorylation sites near the N-terminus of ADAR2

When human ADAR2 was purified to homogeneity from HeLa cells, enzymatic activity was very labile (O'Connell *et al*, 1997). However, recombinant human ADAR2 protein purified after overexpression in the yeast *Pichia pastoris* is active and stable. To determine if the protein is regulated by post-translational modification, we performed mass spectrometry on recombinant ADAR2 purified from *P. pastoris* and identified two phosphorylated serines near the amino-terminus, serine (S) 26 and S31 (Supplementary Figure S1). Phosphorylation at S26 has been independently verified (Dephoure *et al*, 2008). The amino-terminal region of ADAR2 is of interest since it has been shown to be important for dimerization of the protein and autoinhibition of catalytic activity (Gallo *et al*, 2003; Macbeth *et al*, 2004).

ADAR2 interacts with Pin1

The phosphorylated residues near the N-terminus of ADAR2 are within potential recognition motifs (Ser/Thr-Pro) for the phosphorylation-dependent peptidyl-prolyl *cis*/*trans* isomerase Pin1, a well-conserved and extremely efficient enzyme for transducing post-translational modifications into conformational changes in key cellular proteins (Lu and Zhou, 2007). To determine whether Pin1 interacts with ADAR2, HEK293T cells were transiently transfected with a construct expressing ADAR2 bearing a FLAG epitope tag at the N-terminus and tetra-His tag at the C-terminus. After 24 h, the cells were harvested, whole cell protein extracts were immunoprecipitated with anti-FLAG monoclonal antibody and analysed by immunoblot detection of the immunoprecipitate with mouse anti-mitotic phosphoprotein monoclonal-2 (MPM-2) antibody (Davis *et al*, 1983), that recognizes the phosphorylated Pin1 motif (Ser/Thr-Pro) in proteins. As shown in Figure 1A, α-MPM-2 recognizes the FLAG-tagged ADAR2 protein. We mutated T32, as this is the residue that precedes the proline so it may be important for Pin1 binding. When the immunoprecipitation was repeated with alanine (A) substitutions for S26, S26/31 or T32 at the amino-terminus, the antibody recognized ADAR2 less efficiently and loss of binding of the MPM-2 antibody was particularly evident with the triple mutant ADAR2$^{S26A/S31A/T32A}$ (Figure 1A), suggesting that the amino-terminus of ADAR2 harbours phosphorylated S/T-P sites at the amino-terminus that are likely to bind Pin1.

The ability of ADAR2 to bind to Pin1 was next evaluated by *in vitro* binding assays with GST–Pin1 and recombinant ADAR2 purified from *P. pastoris*. As shown in Figure 1E (left panel), ADAR2 binds strongly to GST–Pin1 whereas ADAR2 did not interact with the GST beads alone. To map the interaction between Pin1 and ADAR2, ADAR2^{S26A} ADAR2$^{S26A/S31A/T32A}$ or an N-terminal deletion of ADAR2 from amino acid to 4–72 (Wong *et al*, 2003) were purified from *P. pastoris* (Figure 1B, right panel) and similarly tested for interaction with GST–Pin1. The interaction of GST–Pin1 with ADAR^{S26A} was slightly weaker than with wild-type ADAR2 and interaction was drastically decreased with the triple mutant ADAR2$^{S26A/S31A/T32A}$, and totally absent with ADAR2$^{\Delta4-72}$ (Figure 1B, left panel). To determine if the interaction with Pin1 depends on ADAR2 phosphorylation a transient transfection of ADAR2 into HEK293T cells was performed and the lysate was treated with λ phosphatase followed by a pull-down assay with GST–Pin1 beads. The interaction between ADAR2 and Pin1 was observed and this was abolished with a longer λ phosphatase treatment (Figure 1C).

As these experiments were performed *in vitro*, we then analysed the Pin1 ADAR2 interaction in HEK293T cells by transiently cotransfecting with constructs expressing FLAG tagged ADAR2 and HA-tagged Pin1. The cells were harvested after 24 h and an immunoprecipitation of the lysate was performed with anti-FLAG monoclonal antibody and the precipitate was detected on an immunoblot with an anti-HA antibody (Figure 1D). Only the wild-type ADAR2 interacted with HA-tagged Pin1. Neither the triple alanine mutant nor ADAR2$^{\Delta4-72}$ interacted with Pin1. In addition, ADAR2 that has mutations in both RNA-binding domains and cannot bind to dsRNA (ADAR2^{RRM1-2}) (Valente and Nishikura, 2007) does not interact with Pin1. Therefore, ADAR2 has to bind to RNA before it can interact with Pin1. In the *in vitro* binding assays with GST–Pin1 and recombinant ADAR2 purified from *P. pastoris* (Figure 1B), ADAR2 appears to interact with GST–Pin1 in the absence of dsRNA. However in our experience, it is difficult to eliminate all the dsRNA present in the purified protein fraction from yeast (Gallo *et al*, 2003) so therefore we presume that this *in vitro* reaction is also mediated by dsRNA. Similar results were obtained when HEK293T cells were transiently transfected with FLAG-ADAR2 followed by coimmunoprecipitation with endogenous Pin1 (Figure 1E). These results demonstrate that Pin1 binds to ADAR2 in a phosphorylation-dependent manner and that this interaction occurs at the amino-terminal of ADAR2 after it has bound to RNA.

Pin1 expression is required for optimal editing at the GluR2 Q/R site

Since ADAR2 converts a glutamine (Q) codon to an arginine (R) codon with 100% efficiency at the *GluR2* Q/R site in neurons, the important question is whether the interaction between Pin1 and ADAR2 affects editing activity at the critical *GluR2* Q/R site. To address this point, we analysed editing of the *GluR2* Q/R site in HeLa cells. To increase the level of editing at the Q/R site by ADAR2 in HeLa cells, we transiently cotransfected a plasmid encoding ADAR2 with the *GluR2 B13* minigene. The level of editing rose to 100%. We then co-transfected an siRNA specific for *Pin1* and editing fell to 53% (Figure 2A).

We also analysed editing at the Q/R site in the *GluR2 B13* minigene transcript (Higuchi *et al*, 1993) by endogenous

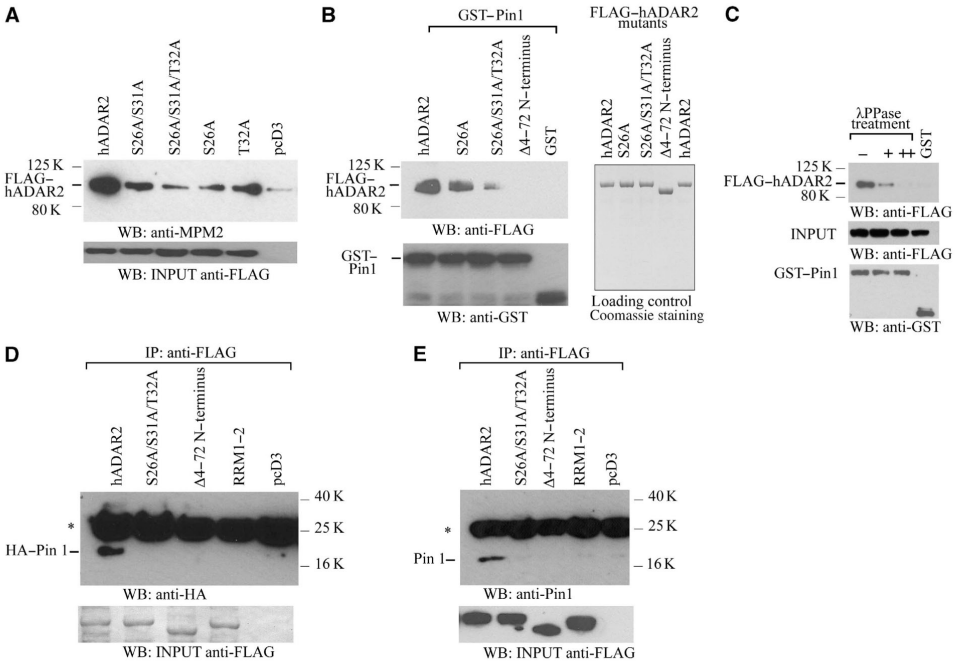

Figure 1 The amino-terminus of ADAR2 harbours a Pin1-binding site. (**A**) The anti-MPM-2 antibody recognizes potential Pin1 sites in ADAR2 purified after overexpression in *P. pastoris*. Immunoblot analysis with anti-MPM-2 antibody of anti-FLAG immunoprecipitates from lysates of HEK293T cells transfected with FLAG-tagged hADAR2, ADAR2$^{S26A/S31A}$, ADAR2$^{S26A/S31A/T32A}$, ADAR2^{S26A}, ADAR2^{T32A} or pcD3. The minor band in the lane with pcD3 is contamination from the neighbouring lane. ADAR input visualized with anti-FLAG antibody, lower panel. (**B**) Purified ADAR2 binds *in vitro* to Pin1 immobilized on beads. (Upper left panel) Immunoblot analysis with anti-FLAG antibody of the binding of FLAG-tagged ADAR2, ADAR2^{S26A}, ADAR2$^{S26A/S31A/T32A}$, ADAR2$^{\Delta4-72}$ bound to GST–Pin1 or GST on glutathione beads. (Lower panel) GST input visualized with anti-GST antibody. (Right panel) Purified ADAR proteins stained with Coomassie. (**C**) Binding of purified ADAR2 to Pin1 depends on phosphorylation of Pin1 sites on ADAR2. λ phosphatase treatment of lysate from HEK293T cells transfected with ADAR2 for 0 (−), 2 h (+), 3 h (++) prior to incubation with GST–Pin1. Immunoblot analysis of ADAR2 with anti-FLAG antibody. Middle and lower panels are input loading controls. (**D**) Pin1 binds to ADAR2 in HEK293T cells. Coimmunoprecipitation of ADAR2 and Pin1 performed with anti-FLAG antibody on HEK293T cell lysate cotransfected with HA–Pin1 and either FLAG-tagged ADAR2, ADAR2$^{\Delta4-72}$, ADAR2^{RRM1-2}, ADAR2$^{S26A/S31A/T32A}$ or pcD3. HA–Pin1 was detected with anti-HA antibody. Asterisks represent IgG light chain. (Lower panel) Immunoblot of input proteins with anti-FLAG antibody. (**E**) Endogenous Pin1 detected with anti-Pin1 antibody after immunoprecipitation with anti-FLAG antibody from cell lysates of HEK293T cells transfected with FLAG-tagged ADAR2, ADAR2$^{S26A/S31A/T32A}$, ADAR2$^{\Delta4-72}$, ADAR2^{RRM1-2} or pcD3. (Lower panel) Immunoblot of input proteins detected with anti-FLAG antibody. Asterisks represent IgG light chain.

ADAR2 and observed it was 60% efficient in this cell line; however, when siRNA specific for *Pin1* was cotransfected, the level of editing fell to 46% (Figure 2B). Editing was restored to 74% when *Pin1* was overexpressed in HeLa cells.

We also examined the effect of reducing Pin1 expression on editing of endogenously expressed *GluR2* transcript in a neuroblastoma cell line, SH-SY5Y (Figure 2C). In this cell line, ADAR2 was cotransfected with either a *Pin1*-specific siRNA or a control siRNA and editing of the endogenous *GluR2* transcript was analysed. Again the level of editing dropped from 100% to ~60% at the Q/R site when there was a reduction in Pin1 expression. We also analysed editing at the R/G site in the *GluR2* transcript and found it was 69% but dropped to 45% when siRNA specific for *Pin1* was cotransfected whereas editing was 73% when a control siRNA was cotransfected. The reduction in Pin1 expression for this experiment is shown in Supplementary Figure S2.

To examine the effect of complete Pin1 elimination, we cotransfected constructs expressing the *GluR2 B13* minigene and ADAR2 into an immortalized mouse fibroblast cell line derived from *Pin1*$^{-/-}$ mice (Figure 2D) (Fujimori *et al*, 1999). The editing activity at the Q/R site was ~50% and increased to 100% when a construct expressing Pin1 was reintroduced in these cells. All these experiments strongly suggest that ADAR2 requires Pin1 for maximal editing of the critical Q/Rand R/G sites in *GluR2* transcripts.

Pin1 has a role in the nuclear localization of ADAR2

Pin1 has many diverse activities within the cell and it can alter the cellular localization of its substrate, as occurs with β-catenin (Ryo *et al*, 2001). Although ADAR2 has been documented as nuclear, recent evidence demonstrated that in human motor neurons in spinal cord sections, ADAR2 is both nuclear and cytoplasmic (Aizawa *et al*, 2010). Interestingly, a deletion of the amino-terminal residues 4–72 renders ADAR2 cytoplasmic (Wong *et al*, 2003) and it has also been demonstrated that this region is required for nuclear localization as it contains a non-canonical NLS within the

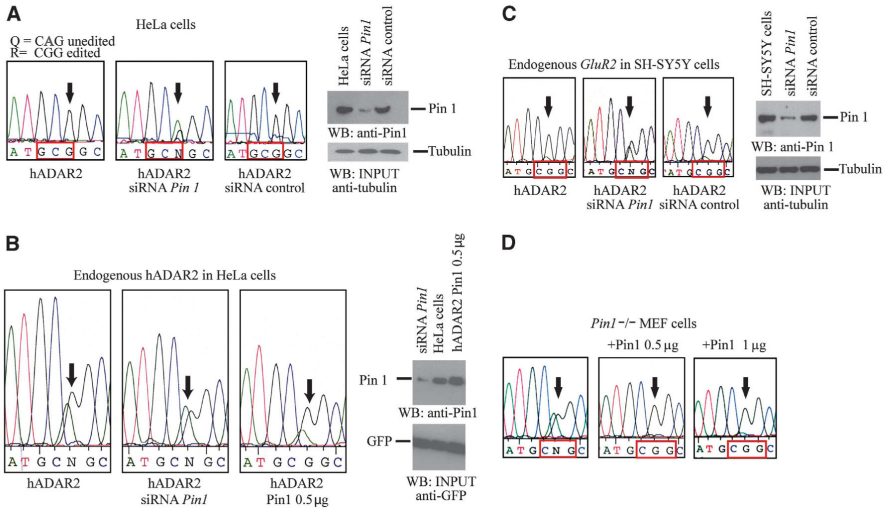

Figure 2 Pin1 is required for efficient editing at the *GluR2* Q/R site. (**A**) DNA sequence chromatograph of the RT–PCR product of the regior encompassing the Q/R site (arrow) encoded by the *GluR2 B13* minigene transiently cotransfected with ADAR2 (2 µg) in HeLa cells, editing i 100% (left chromatograph). Editing of the Q/R site drops to 53% when an siRNA specific for *Pin1* is cotransfected together with plasmid encoding both ADAR2 and the *GluR2 B13* minigene (middle chromatograph). Editing is 100% at the *GluR2* Q/R site when a control siRNA i cotransfected (right chromatograph). Immunoblot analysis of cell lysate from HeLa cells with either anti-Pin1 or anti-tubulin antibodies (righ panel). (**B**) (Left panel) Sequencing chromatogram of editing by endogenous ADAR2 at the Q/R site of RT–PCR product pools from the *GluR B13* minigene transcript that has been transiently transfected into HeLa cells. Arrows indicate Q/R editing site in all panels. Immunoblo analysis with anti-Pin1 antibody of HeLa cell extracts that have been cotransfected with GFP in the presence of either *Pin1*-specific siRNA, nc siRNA or HA–Pin1 construct (0.5 µg). Proteins are detected with anti-Pin1 antibody and anti-GFP antibody as a loading control (right panel) (**C**) Chromatograph of editing of endogenous *GluR2* transcript at the Q/R site in neuroblastoma SH-SY5Y cells transfected with ADAR2 (2 µg) Editing is 100% at the Q/R site (left chromatograph). A decrease in editing is observed when an siRNA specific for *Pin1* was cotransfected (middle chromatograph). A control siRNA does not affect editing when transfected (right chromatograph). Arrows indicate the Q/R site. Cel lysates of SH-SY5Y cells were analysed by immunoblot with either anti-Pin1 or anti-tubulin (right panel). (**D**) Chromatograph of editing at the Q/R site of *GluR2 B13* minigene transcript in *Pin1*$^{-/-}$ MEF cells transfected with ADAR2 (2 µg). Editing increased to 100% when *Pin1*$^{-/-}$ MEI cells were cotransfected with either 0.5 or 1 µg of a construct expressing Pin1. An arrow marks the Q/R editing site in all the chromatographs

first 64 amino acids (Desterro *et al*, 2003). As this deletion removes the Pin1-binding site, we wondered if preventing Pin1 binding also leads to mislocalization of ADAR2. To elucidate this we transiently transfected GFP-tagged ADAR2 into *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ MEF cells and performed immunofluorescence detection of ADAR2 (Figure 3A). In the absence of Pin1, wild-type ADAR2 is mislocalized in the cytoplasm (Figure 3A, lower panel). Mislocalization of ADAR2 is confirmed when nuclear and cytoplasmic fractionation is performed on *Pin1*$^{-/-}$ MEF cells transiently transfected with FLAG-tagged ADAR2 (Figure 3D). When Pin1 was reintroduced into these cells, the level of ADAR2 in the cytoplasm was significantly reduced (Figure 3B and D). This effect of Pin1 on the localization of ADAR2 requires Pin1 prolyl-isomerase enzymatic activity as a Pin1^{S67E} mutant that is catalytically inactive was unable to restore ADAR2 localization to the nucleus (Figure 3C). GFP–ADAR2 is localized to the nucleus when Pin1 is present; however, cytoplasmic localization of GFP–ADAR2 increases following cotransfection with catalytically inactive Pin1. Increased FLAG–ADAR2 is also observed in the cytoplasmic fraction of *Pin1*$^{-/-}$ MEF cells transiently transfected with FLAG-tagged ADAR2 (Figure 3D).

As Pin1 recognizes a phosphorylated serine or threonine preceding a proline, we replaced the phosphorylated amino acids as well as the prolines with alanine to determine if al were required for nuclear localization. As expected, the triple mutant FLAG–ADAR2$^{S26/S31A/T32A}$ was present in the cyto plasm (Supplementary Figure S3) and this appears slightly different to ADAR2$^{\Delta 4-72}$ that is more localized around the nuclear periphery (Supplementary Figure S4). When the proline mutants were generated; FLAG–ADAR2^{P27A} and FLAG–ADAR2^{P33A}, were transiently transfected into HeLa cells together with HA–Pin1 (Figure 4), FLAG–ADAR2^{P33A} was present in the cytoplasm as detected by immunofluores cence as well as by nuclear and cytoplasmic fractionation (Figure 4B and C) whereas FLAG–ADAR2^{P27A} is nuclear. This implies that the second proline is the critical one, thus the phosphorylation of T32 may be the critical site for Pin1 binding and P33 for isomerization. Notably, this is also the most conserved Pin1 site in vertebrate ADAR2 sequences (Supplementary Figure S1).

Pin1 stabilizes ADAR2

We wanted to elucidate if Pin1 had other effects on ADAR2 As Pin1 has been shown to influence the stability of proteins such as β-catenin (Ryo *et al*, 2001), NF-κB (Ryo *et al*, 2003) and p53 (Zacchi *et al*, 2002; Zheng *et al*, 2002), we wondered if Pin1 also influences the stability of ADAR2. The level o Pin1 was reduced in HeLa cells by transfecting either pSuper

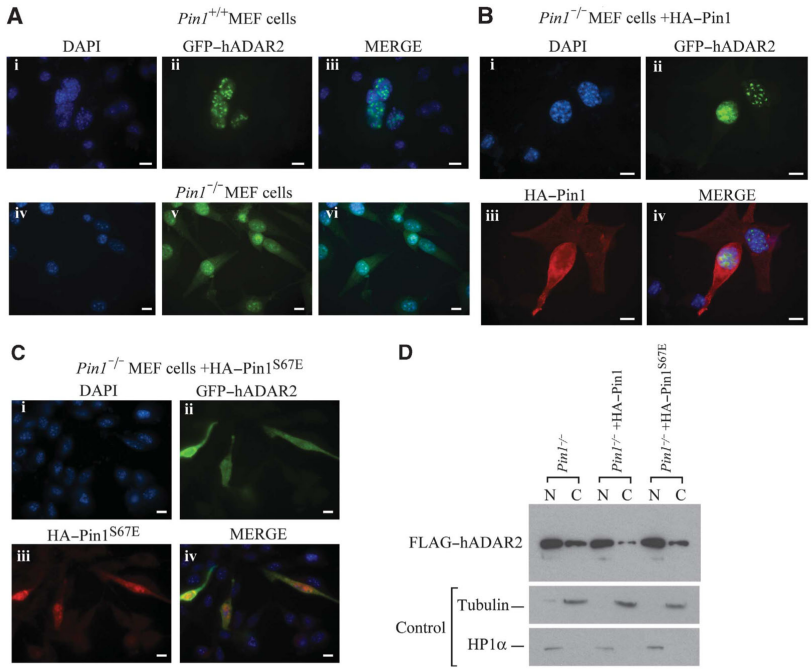

Figure 3 Pin1 is required for nuclear localization of ADAR2. (**A**) ADAR2 is mislocalized from the nucleus to the cytoplasm in *Pin1*$^{-/-}$ MEF cells. GFP–ADAR2 immunofluorescence in *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ MEF cells cotransfected with *GluR2 B13* minigene and GFP–ADAR2. DAPI staining of nuclei (i, iv), GFP fluorescence of cell (ii, v) and merged (iii, vi). (**B**) Nuclear localization of ADAR2 is restored in *Pin1*$^{-/-}$ MEF cells by transfection of HA–Pin1. GFP–ADAR2 (green) direct and HA–Pin1 (red) indirect immunofluorescence detection in *Pin1*$^{-/-}$ MEF cells cotransfected with *GluR2 B13* minigene and (ii) GFP–ADAR2 (green) and (iii) HA–Pin (red). (i) DAPI staining of nuclei. (iv) Merge of all three images. (**C**) Nuclear localization of ADAR2 depends on catalytic activity of Pin1. GFP–ADAR2 (green) and HA–Pin1^{S67E} (red) in *Pin1*$^{-/-}$ MEF cells cotransfected with *GluR2 B13* minigene and (ii) GFP–ADAR2 (green) and (iii) HA–Pin1^{S67E} (red). (i) DAPI staining of nuclei. (iv) Merge of all three images. All photographs were taken at the same exposure. Scale bar, 10 μm. (**D**) Nucleo-cytoplasmic fractionation. Immunoblot analysis with anti-FLAG antibody of nuclear and cytoplasmic fractions of *Pin1*$^{-/-}$ MEF cells transfected with FLAG–ADAR2 (lanes 1 and 2). Pin1 was cotransfected with FLAG–ADAR2 in *Pin1*$^{-/-}$ MEF cells (lanes 3 and 4). HA–Pin1^{S67E} was cotransfected in *Pin1*$^{-/-}$ MEF cells (lanes 5 and 6). (Lower panel) Immunoblot of fractionated *Pin1*$^{-/-}$ MEF cells with tubulin as a marker for cytoplasmic fraction and HP1α for nuclear fraction.

Pin1 siRNA (Rustighi *et al*, 2009) or a control pSuper *LacZ* siRNA so that the level of endogenous ADAR2 could be analysed (Figure 5A). A similar experiment was performed in the neuroblastoma cell line SH-SY5Y (Figure 5C). Twenty-four hours after transfection, cycloheximide was added to prevent further protein synthesis, a time course from 0 to 8 h was performed to chase the decay of ADAR2 protein and the samples were analysed by immunoblot analysis to determine ADAR2 levels. In both cell lines, the protein level of ADAR2 decreased when cycloheximide was added; however, the decrease was more dramatic when *Pin1* expression was reduced (Figure 5A–C). The stability of the triple mutant ADAR2$^{S26A/S31A/T32A}$ was also analysed after cycloheximide treatment (Figure 5D). As predicted this mutant protein was unstable as it could no longer interact with Pin1. Therefore, Pin1 affects the stability of ADAR2.

The E3 ubiquitin ligase WWP2 interacts with ADAR2

Mass spectrometry was performed on the original samples of ADAR2 purified from large quantities of HeLa cell nuclear fractions (O'Connell *et al*, 1997) and one of the proteins that copurified with ADAR2 was WWP2, an E3 ubiquitin ligase containing four WW domains as well as a HECT domain. As ADAR2 was unstable in the absence of Pin1, we wondered if this E3 ligase was involved. WWP2 can bind directly to a PPxY motif within its substrate. Analysis of the ADAR2 amino-acid sequence revealed that this motif was present twice, at the amino-terminus and carboxyl-terminus of ADAR2 and was highly conserved (Supplementary Figure S5).

To demonstrate that ADAR2 and WWP2 interact, HEK293T cells were transiently cotransfected with constructs expressing FLAG–ADAR2 and WWP2 with a c-myc epitope tag at its amino-terminus. An immunoprecipitation was performed with anti-FLAG monoclonal antibody to precipitate FLAG–ADAR2 and c-myc–WWP2 was present in this precipitate (Figure 6A). To determine which motif in ADAR2 WWP2 binds to, transient transfections were performed in HEK293T cells with constructs expressing full-length and truncated forms of ADAR2 all with FLAG epitope at their amino-terminus. Immunoprecipitation with anti-FLAG monoclonal antibody followed by immunoblot detection with an

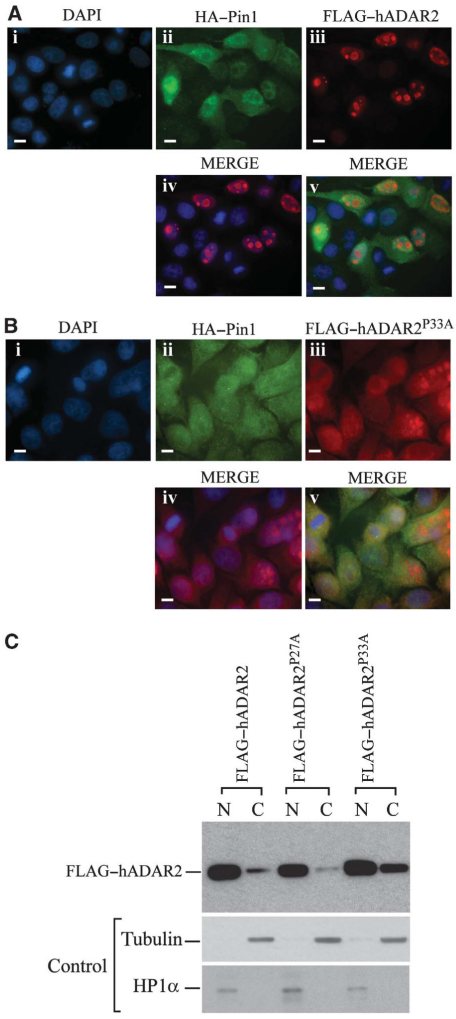

Figure 4 Proline 33 of ADAR2 is required for the Pin1 effect on ADAR2 nuclear localization. (**A**) Normal localization of ADAR2 and Pin1. Immunofluorescence of HeLa cells cotransfected with *GluR2 B13* minigene, FLAG–ADAR2 and HA–Pin1 stained with (i) DAPI, (ii) anti-HA–Pin1 (green), (iii) anti-FLAG–ADAR2 (red), (iv) Merge of DAP1 and FLAG and (v) merge of all three images. (**B**) Nuclear localization of ADAR2 depends on Proline 33. Immunofluorescence of HeLa cells cotransfected with *GluR2 B13* minigene, FLAG–ADAR2^{P33A} and HA–Pin1 stained with (i) DAPI, (ii) anti-HA–Pin1 (green), (iii) anti-FLAG–ADAR2^{P33A} (red), (iv) Merge of DAP1 and FLAG and (v) merge of all three images. All photographs were taken at the same exposure. Scale bar, 10 μm. (**C**) Nucleo-cytoplasmic fractionation of wild-type and ADAR^{P27A} and ADAR2^{P33A} mutants. Immunoblot analysis with anti-FLAG antibody of nuclear and cytoplasmic fractions of HeLa cells transfected with FLAG–ADAR2 (lanes 1 and 2), ADAR^{P27A} (lanes 3 and 4), ADAR2^{P33A} (lanes 5 and 6). (Lower panels) Immunoblot of fractionated HeLa cells with tubulin as a cytoplasmic marker and HP1α as a nuclear marker.

anti-WWP2 antibody showed that endogenous WWP2 interacts best with full-length ADAR2 and with a truncated protein containing the amino-terminus of ADAR2 (Figure 6B). The interaction of WWP2 with ADAR2 appears to be weaker with the site in the deaminase domain or when the amino-terminus of ADAR2 was deleted. We repeated this immunoprecipitation with anti-FLAG with mutants of ADAR2 in which the binding site for WWP2 in the amino-terminus, carboxyl-terminus or a combination of both binding sites were mutated (Figure 6C). The single mutants decrease the interaction between ADAR2 and WWP2 whereas the interaction was completely abolished with the double mutant.

When a cotransfection was performed in HeLa cells with a constant amount of a plasmid encoding ADAR2 and an increase in the plasmid expressing WWP2, a drastic decrease in ADAR2 protein level was observed (Figure 6D). However, when this experiment was repeated with the double ADAR2^{-PPxY} mutant then the level of the mutant protein did not change as it is no longer a substrate for WWP2. These experiments demonstrated that WWP2 can interact with ADAR2 via the PPxY motif present in ADAR2. An increased expression of WWP2 in HeLa cells resulted in a reciprocal decrease in ADAR2 levels; however, the protein level of the ADAR2^{-PPxY} mutant unable to bind WWP2 remained stable, demonstrating that WWP2 can cause a decrease in ADAR2 protein levels.

WWP2 poly-ubiquitinates ADAR2

To verify that ADAR2 is indeed poly-ubiquinated by WWP2, we performed an ubiquitin assay with extract from HEK293T cells. The cells were transiently cotransfected with ADAR2 or a mutant where either single or both PPxY motifs in ADAR2 were mutated and a further construct expressing WWP2. Poly-ubiquitination of ADAR2 was detected in extracts from cells cotransfected with constructs expressing wild-type ADAR2 and WWP2 proteins but there was a decrease in poly-ubiquitination with both the ADAR2$^{NH_2-PPxY}$ and ADAR2$^{COOH-PPxY}$ single mutants. Only in the presence of ADAR2^{-PPxY} was there a complete loss of poly-ubiquitination (Figure 7A). Poly-ubiquitination was also observed in the absence of V5-UBQ as there was sufficient endogenous ubiquitin in the cell extract (Figure 7A, lane 1).

To demonstrate that the proteosome affected the stability of ADAR2, a time course was performed in the presence of the proteosome inhibitor MG132 (Figure 7B). An increase in the level of ADAR2 was observed; however, there was not a reciprocal increase in the levels of the ADAR2^{-PPxY} mutant. This mutant could no longer bind WWP2 so the protein level could no longer be regulated by the proteasome, therefore, the proteosome inhibitor had no effect on its stability.

To determine if an increase in stability of ADAR2 would affect its localization, immunofluorescence was performed in *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ MEF cells and were transiently transfected with ADAR2^{-PPxY}. A cytoplasmic accumulation of ADAR2^{-PPxY} was evident (Figure 7C). Previously it had been difficult to observe an accumulation of ADAR2 within the cytoplasm as the protein was being degraded by WWP2 (Figure 3A). However, as the binding of WWP2 is impaired in the ADAR2^{-PPxY} mutant, the cytoplasmic accumulation is obvious. Nuclear and cytoplasmic fractionation was performed with the ADAR2^{-PPxY} mutant in MEF wild-type and

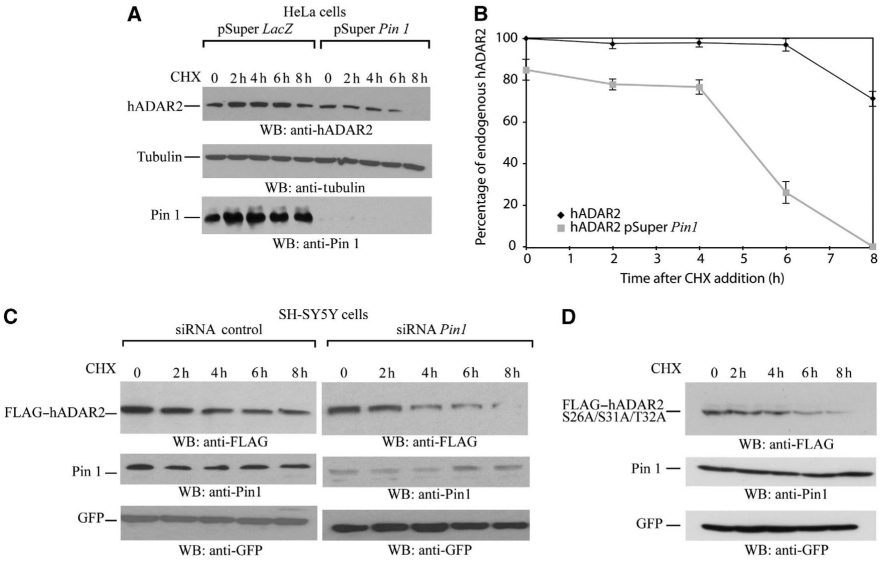

Figure 5 Pin1 contributes to stability of ADAR2 protein. (**A**) Knockdown of *Pin1* in HeLa cells destabilizes ADAR2 in a cycloheximide time course. HeLa cells were transfected with either pSuper *LacZ* or pSuper *Pin1*. Cycloheximide (50 μg/μl) was added to both and a time course from 0 to 8 h was performed. Cell lysates were analysed by immunoblot and the antibodies used were anti-ADAR2 (top panel), anti-tubulin as a loading control (middle panel) and Pin1 (bottom panel). (**B**) Quantification of (**A**). (**C**) *Pin1* knockdown destabilization of FLAG–ADAR2 in SH-SY5Y neuroblastoma cells. SH-SY5Y cells were cotransfected with FLAG-tagged ADAR2 and a control siRNA or *Pin1*-specific siRNA. Cycloheximide (50 μg/μl) was added to both and a time course from 0 to 8 h was performed. Cell lysates were analysed by immunoblot and the antibodies used were anti-FLAG (top panel), anti-Pin1 (middle panel) and GFP as loading control (bottom panel). (**D**) ADAR2 mutant in the Pin1-binding site is less stable. SH-SY5Y neuroblastoma cells were transfected with FLAG-tagged ADAR2$^{S26A/S31A/T32A}$ and cycloheximide (50 μg/μl) was added and a time course was performed from 0 to 8 h. Cell lysates were analysed by immunoblot and the antibodies used were anti-FLAG (top panel), anti-Pin1 (middle panel) and GFP as loading control (bottom panel).

Pin1$^{-/-}$ cells and the results were quantified (Figure 7D and E). It is clear that in the absence of Pin1 and if WWP2 cannot bind to ADAR2, there is a cytoplasmic accumulation of ADAR2 that is not observed under normal conditions.

Discussion

These data illustrate the complex regulation of ADAR2 by two proteins with opposing effects; Pin1 is a positive regulator of ADAR2 whereas WWP2 can bind and cause degradation. Pin1 and WWP2 regulate other protein such as the large subunit of RNA pol II and this regulation is conserved from yeast to mammals (Wu *et al*, 2001).

When ADAR2 is phosphorylated at the amino-terminus, it becomes a substrate for the phosphorylation-dependent prolyl-isomerase Pin1. The enzymatic activity of Pin1 is required for the localization and stability of ADAR2 in the nucleus. In the absence of Pin1, ADAR2 is unstable and is present in the cytoplasm. It can then interact with WWP2, an E3 ligase that results in its poly-ubiquitination and subsequent degradation by the proteasome (Figure 8). One direct consequence of this is a reduction in editing of the Q/R site and R/G sites in *GluR2* transcripts. The presence of unedited GluR2^{Q} subunit can have dramatic downstream effects as it can increase the trafficking of GluR2 subunit to the synapse as well as increasing the permeability of AMPA receptors to calcium ions.

The function of Pin1 is to isomerize a specific proline from the *cis* to *trans* conformation or *vice versa* (Ranganathan *et al*, 1997; Yaffe *et al*, 1997). Most biological processes require proline to be in the *trans* conformation; however, when the protein is translated the choice in conformation is dependent on the surrounding amino acids. If there is a pool of ADAR2 that is not phosphorylated and therefore not a Pin1 substrate, then this protein may not be fully active. This probably does occur as ADAR2 is expressed in various mammalian cell lines such as HeLa and SH-SY5Y but is not very active and for efficient editing of transcripts, additional ADAR2 must be transfected. There may be sufficient Pin1 present but the kinase required for the phosphorylation of the Pin1-binding site may be limited. This may explain the pool of inactive ADAR2 that has been observed as it may require phosphorylation and subsequent Pin1 activity. For example in the undifferentiated NT2 cell line, ADAR2 is well expressed; however, it requires differentiation of the NT2 to neuronal cells for efficient editing of *GluR2* Q/R; this occurs without any major change in ADAR2 expression (Lai *et al*, 1997). The authors of that study proposed that a post-translational regulatory mechanism is involved.

In the absence of Pin1, ADAR2 mislocalizes to the cytoplasm. It is difficult to detect ADAR2 as it is poly-ubiquitinated by WWP2 in the cytoplasm and degraded. Only when WWP2 is unable to bind to ADAR2 is high level of cytoplasmic accumulation of ADAR2 observed (Figure 7). There are two binding sites for WWP2 on the ADAR2 protein. The site

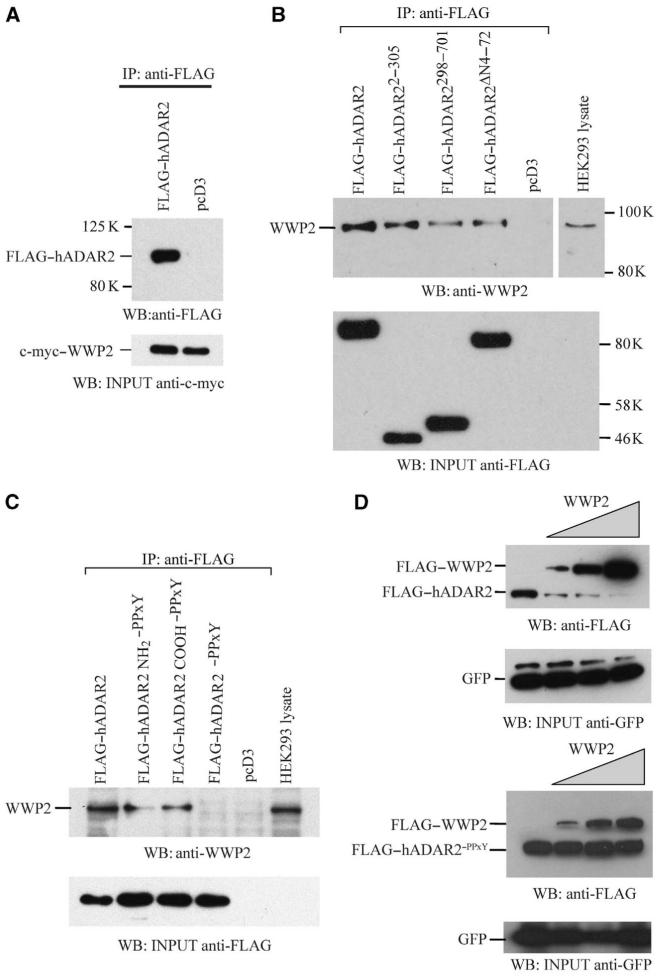

Figure 6 WWP2 interacts with ADAR2. (**A**) Coimmunoprecipitation of ADAR2 and WWP2 performed with anti-FLAG antibody in HEK293T cell lysate cotransfected with FLAG–ADAR2 and c-myc–WWP2 or pcD3 empty vector. FLAG–ADAR2 was detected with FLAG antibody. WWP2 input visualized with anti-c-myc antibody (bottom panel). (**B**) Endogenous WWP2 detected with anti-WWP2 antibody after immunoprecipitation with anti-FLAG antibody from lysates of HEK293T cells transfected with FLAG-tagged ADAR2, ADAR2$^{2–305}$, ADAR2$^{298–701}$, ADAR2$^{\Delta 4–72}$ or pcD3. Immunoblot of input proteins detected with anti-FLAG antibody (bottom panel). (**C**) Immunoprecipitation of FLAG–ADAR2 and mutants with mutations in the amino, carboxyl binding site for WWP2 or a combination of both was performed with anti-FLAG antibody in HEK293 cells. Endogenous WWP2 detected with anti-WWP2 antibody. (Lower panel) Immunoblot of input proteins detected with anti-FLAG antibody. (**D**) Immunoblot analysis with anti-FLAG antibody of lysates from HeLa cells cotransfected with FLAG–ADAR2 and increasing amount of FLAG–WWP2 (0.5, 1, 2.5 μg) (upper panel) and this experiment was repeated with the mutant ADAR2^{PPxY} that is unable to bind to WWP2 (lower panel). The efficiency of transfection was normalized to GFP expression. GFP input visualized with anti-GFP antibody is shown below both panels.

in the amino-terminus of ADAR2 appears to be more important for WWP2 interaction than the site in the deaminase domain despite the deaminase site being more conserved. The crystal structure for the deaminase domain of ADAR2 has been solved (Macbeth *et al*, 2005) and the PPLY amino acids are on the outside of the protein opposite to the region that has been proposed to interact with the RNA (Supplementary Figure S6). Therefore, these amino acids are easily accessible to WWP2.

The finding that ADAR2 is regulated by Pin1 and WWP2 opens up new avenues of research. Under normal conditions, ADAR2 is present within the nucleolus (Desterro *et al*, 2003; Sansam *et al*, 2003). However, once a substrate is transfected into the cell, it relocates to the nucleus and editing can occur. One key factor that is missing is the kinase that phosphorylates ADAR2 and instigates this complex regulation. Also, we would predict from our results both with the ADAR2 triple mutant (Figure 1) and with ADAR2^{P33A} (Figure 4) that Thr32

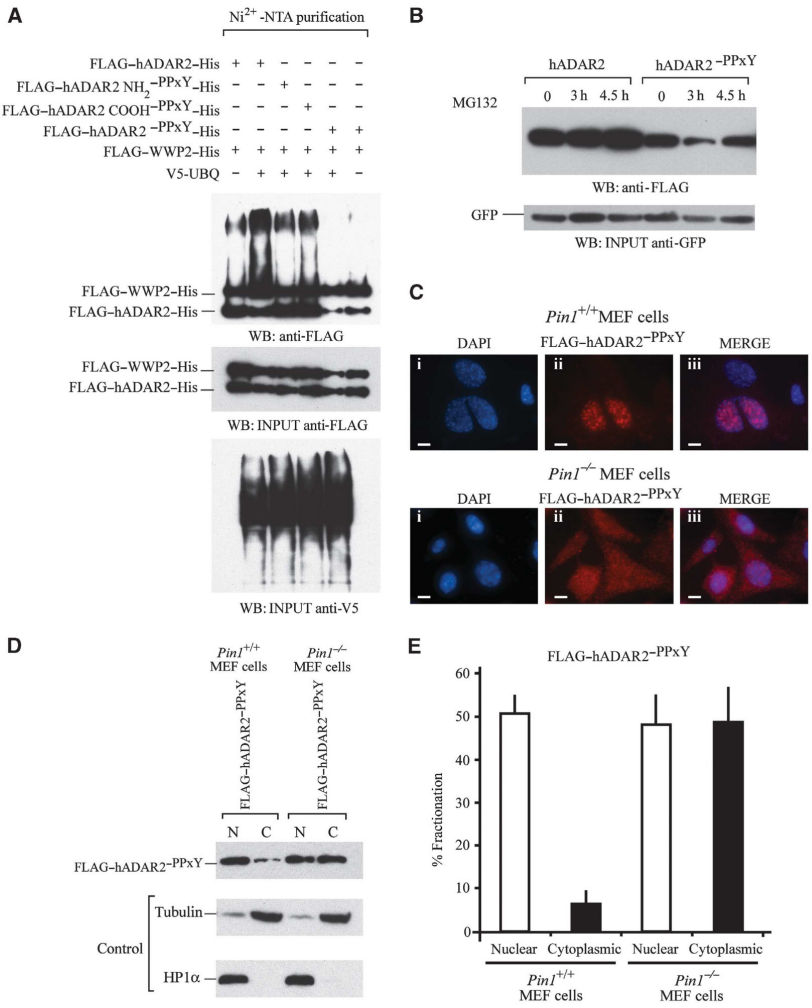

Figure 7 WWP2 is required for ADAR2 ubiquitination and subsequent degradation in the cytoplasm. (**A**) *In vivo* ubiquitination assays. FLAG–ADAR2–His, FLAG–WWP2–His and V5-UBQ were transfected in HEK293T cells followed by purification of ubiquitination complexes from lysates with Ni^{2+}-NTA. In lane 1, cotransfection was with FLAG–ADAR2–His, FLAG–WWP2–His. Lane 2, cotransfection was with FLAG–ADAR2–His, FLAG–WWP2–His and V5-UBQ. Lane 3, cotransfection of FLAG–ADAR2 NH_2^{-PPxY}–His, FLAG–WWP2–His and V5-UBQ. Lane 4, cotransfection of FLAG–ADAR2 $COOH^{-PPxY}$–His, FLAG–WWP2–His and V5-UBQ. Lane 5, cotransfection of FLAG–$ADAR2^{-PPxY}$–His (double mutant), FLAG–WWP2–His and V5-UBQ. Lane 6 is the same as lane 5 without the addition of V5-UBQ and is the negative control. (Middle panel) Immunoblot of input proteins detected with anti-FLAG antibody. (Lower panel) Immunoblot of V5-UBQ present in the purified complex detected with anti-V5 antibody. (**B**) Immunoblot at 24 h following transfection of FLAG–ADAR2 and FLAG–$ADAR2^{-PPxY}$ with 20 μM MG132 to inhibit protein degradation. The proteasomal inhibitor MG132 was added to both and a time course from 0 to 4.5 h was performed in HeLa cells. Cell lysates were normalized to GFP levels (lower panel). (**C**) Immunofluorescence of $Pin1^{+/+}$ and $Pin1^{-/-}$ MEF cells cotransfected with *GluR2* and FLAG–$ADAR2^{-PPxY}$ (double mutant). (i) DAPI staining of nuclei. (ii) Anti-FLAG–$ADAR2^{-PPxY}$ (red). (iii) Merge of DAP1 and FLAG. Scale bar, 10 μm. All photographs were taken at the same exposure. (**D**) Nuclear and cytoplasmic fractionation of FLAG–$ADAR2^{-PPxY}$ in $Pin1^{+/+}$ and $Pin1^{-/-}$ MEF cells. (Lower panels) Immunoblot of fractionated MEF cells with tubulin as a cytoplasmic marker and HP1α as a nuclear marker. (**E**) Quantification of (**D**).

is phosphorylated and that this is a key phosphorylation event. Pin1 interaction requires the binding of ADAR2 to dsRNA; however, we do not know whether the kinase phosphorylates ADAR2 in the bound or unbound state. Also, we do not know what happens after RNA editing has occurred. Is ADAR2 then a substrate for a phosphatase that results in its relocation to the nucleolus or is it exported and degraded? In the absence of Pin1, ADAR2 mislocalizes to the cytoplasm where it is a substrate for WWP2; however, the molecular mechanism underlying this mislocalization is

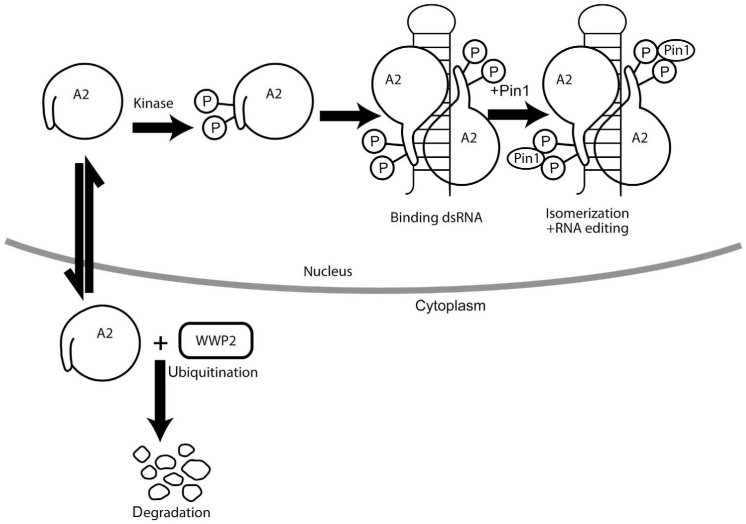

Figure 8 Schematic representation of the regulation of ADAR2 by Pin1 and WWP2. ADAR2 can exist as a monomer in the nucleus and has sequences at its amino-terminus that inhibit enzymatic activity (Macbeth *et al*, 2004). However, ADAR2 can be phosphorylated by an unknown kinase either when it is free or bound to dsRNA. ADAR2 is a substrate for Pin1 once it is bound to dsRNA and the active form of ADAR2 is a dimer (Gallo *et al*, 2003; Poulsen *et al*, 2006; Valente and Nishikura, 2007). The mechanism of dimer formation is still unclear. After RNA editing has occurred, we do not know the fate of ADAR2. However, in the absence of Pin1, ADAR2 mislocalizes to the cytoplasm where it is poly-ubiquitinated by WWP2 and is degraded by the proteasome.

unknown. It is important to elucidate how these various factors regulate the activity of ADAR2 as this will subsequently impinge on the properties of the AMPA receptor.

This report of post-translational regulation of ADAR2 reveals how ADAR2 is highly coordinated and regulated within the cell as this ultimately controls the calcium permeability and assembly of AMPA receptors. This opposing regulation by Pin1 and WWP2 is very analogous to that of the large subunit of yeast RNA polymerase (pol) II where the yeast orthologue of Pin1; *ESS1* binds to the C-terminal domain and positively regulates RNA pol II transcription whereas *RSP5*; a HECT-type E3 ligase similar to WWP2 mediates its ubiquitination and degradation (Wu *et al*, 2001). This regulation of RNA pol II large subunit by Pin1 and WWP2 is also conserved in mammals (Li *et al*, 2007; Xu and Manley, 2007). Therefore, we propose that these two proteins with opposing effects can act coordinately in the regulation and stability of other key cellular proteins.

The interaction of ADAR2 with Pin1 may explain why the Q/R site is edited to 100% in neurons. Pin1 is a key regulator of many proteins and processes within the cell; however, it is itself regulated by phosphorylation, which inhibits its activity (Lu *et al*, 2002; Lee *et al*, 2011). We hypothesize that as Pin1 is the hub of a regulatory network and that transient ischaemia or other insults lead to reduction in Pin1 activity. A decrease in ADAR2 activity would then ensue with a subsequent reduction in editing at the Q/R site in *GluR2* transcripts. This would result in increased calcium permeability of AMPA receptors that could have major effects depending on the region of the brain and the presence of calcium-binding proteins or calcium pumps within the particular neuron. If calcium-binding proteins are low as in the CA1 pyramidal neurons, then this could lead to neuronal cell death (Liu and Zukin, 2007). Therefore, we propose 100% editing of Q/R in the *GluR2* transcript is a 'quality control' measure that reflects a healthy neuron. Data from mice support this hypothesis as when the edited GluR2^{R} isoform has been knocked-in, the mice have no apparent phenotype despite a lack of the unedited isoform (Kask *et al*, 1998). An explanation why rats expressing GluR2^{R} are resistant to forebrain ischaemia in the vulnerable CA1 pyramidal neurons could be that the regulatory network from Pin1 to ADAR2 editing the *GluR2* Q/R site has been disrupted (Liu *et al*, 2004). Other experiments are required to rigorously test this hypothesis; however, if it is correct it will facilitate devising treatments to limit the neuronal damage associated with forebrain ischaemia.

Materials and methods

A more detailed Materials and methods section is provided in Supplementary Data.

Mass spectrometry of ADAR2

A measure of 1 μg of ADAR2 with FLAG and tetra-histidine epitope tags was purified after overexpression in *P. pastoris* as previously described (Ring *et al*, 2004). The purified protein was denatured in Novex LDS sample buffer plus 10 mM DTT at 65°C for 30 min and alkylated with 50 mM 4-vinylpyridine for 15 min at room temperature. The protein was separated by SDS–PAGE on a 4–12% MOPS NUPAGE gel, stained with colloidal Coomasie and digested with trypsin (5 μg/ml) in 50 mM ammonium bicarbonate. The phosphopeptides were enriched with PHOS-select resin (Sigma) and analysed on a 4700 TOF–TOF mass spectrometer as described previously (Beullens *et al*, 2005).

ADAR2 mutagenesis

The pcD3 construct expressing FLAG–ADAR2 has been previously described (Heale *et al*, 2009). All mutations were generated with the QuickChange mutagenesis strategy (Stratagene, La Jolla, CA) and were sequenced to verify the intended mutations.

S^{26} to A: 5′-ctggacaacgtggcccccaaggatggc-3′
T^{32} to A: 5′-tcccccaaggatggcagcgcacctgggcctgg-3′
$S^{26/31}$ to A: 5′-gtcccccaaggatggcgccacacctgggcctggcga-3′
$S^{26/31}/T^{32}$ to A: 5′-ggacaacgtggctcccaaggatggcgccgcacctgggcctg-3′
P^{27} to A: 5′-ctggacaacgtgtccgccaaggatggcagcaca-3′
P^{33} to A: 5′-cccaaggatggcagcacagctgggcctggcgagggctct-3′

ADAR2 was subcloned into pEGFP-C3 with the oligonucleotides: *Eco*RI-ADAR2 5′-ccggaattctgatggatatagaagatgaagaaaacatgagt-3′

ADAR2-*Sal*I (antisense) 5′-ccggtcgacctcagggcgtgagtgagaactggtc ctgctc-3′.

WWP2 cloning and mutagenesis

WWP2 was cloned by PCR amplification of cDNA clone IMAGE 100008816 (Gene Service) in the expression vector pENTR221 with the oligonucleotides 5′-*GGGGACAAGTTTGTACAAAAAAGCAGGCT CA*ATG**GACTACAAGGACGACGATGACAAA**GCATCTGCCAGCTCTAG CCGGGCA-3′ and antisense 5′-*GGGGACCACTTTGTACAAGAAAGCT GGGTC*CTA**ATGGTGATGGTGATGGTG**CTCCTGTCCAAAGCCTTCGG TCTC-3′ for subsequent gateway cloning (Invitrogen). Similarly, N- and C-terminal truncations of hADAR2 were constructed by PCR amplification of hADAR2 cDNA into the pGEM T-Easy vector with the oligonucleotides. 5′-*GGGGACAAGTTTGTACAAAAAAGCAGGC TA*TG**GACTACAAGGACGACGATGACAAA**GATATAGAAGATGAAGAA AAC-3′ and antisense 5′-*GGGGACCACTTTGTACAAGAAAGCTG GGTC*TA**ATGGTGATGGTGATGGTG**TGGCGTCTGATCCAAGTCCAA-3′ primers were used to produce a construct encoding only the N-terminal portion of hADAR2.

To produce a construct encoding the C-terminal portion of hADAR2 with the oligonucleotides 5′-*GGGGACAAGTTTGTA CAAAAAAGCAGGCTA*TG**GACTACAAGGACGACGATGACAAA**TTGC ACTTGGATCAGACGCCA-3′ and antisense 5′-*GGGGACCACTTTGTAC AAGAAAGCTGGGTC*TA**ATGGTGATGGTGATGGTG**GGGCGTGAGTGA GAACTGGTC-3′ were used. All primers were designed to incorporate 5′ FLAG (bold) and 3′ HIS epitope tags (underlined) at either end of the respective ORF, as well as the *att* recombination sites required for gateway cloning (italics). The purified PCR products were cloned by site-specific recombination into the donor vector pDONR221 to generate an entry clone. The entry clone was used in a second site-specific recombination reaction with the modified destination vector pcD3 (origin pcDNA3) to generate the expression clone (following the standard protocol, as described by Invitrogen).

To generate FLAG–ADAR2 NH_2^{-PPxY} (PPFY to AAFA) and FLAG–ADAR2 $COOH_2^{-PPxY}$ (PPLY to AALA) the following oligonucleotides were used:

FLAG–$ADAR2^{NH_2-PPxY}$ 5′-gacaaggcggcagcatttgccgtgggctcc-3′ and for FLAG–$ADAR2^{COOH-PPxY}$ 5′-gaggacctggcagctctcgccaccctcaac-3′

Immunoblot analysis, immunoprecipitation and GST pull down

Expression and purification of the GST-tagged proteins were performed as described (Buratti and Baralle, 2001). Immunoprecipitation was performed as described (Rustighi *et al*, 2009). Immunoblot analysis was performed with primary antibodies: mouse α-FLAG 1:3000 (Sigma), mouse α-HA 1:1000 (Sigma), mouse α-MPM-2 1:1000 (Upstate Cell Signaling) (Davis *et al*, 1983) mouse α-Pin1 1:500 (G-8) (Santa Cruz Biotechnology, Santa Cruz, CA) rabbit α-ADAR2 1:1000 (Sigma), mouse α-GST 1:5000 dilution (Amersham Pharmacia), overnight at 4°C, followed by an 1-h incubation with the appropriate secondary antibody (Dako).

Ubiquitination assay and proteasome-mediated degradation analysis

Cells were cotransfected with the indicated constructs at the following ratios: FLAG–ADAR2 and FLAG–$ADAR2^{-PPxY}$ 1 μg, FLAG–WWP2 and FLAG–WWP2 (C/A) 4 μg, V5-UBA 1 μg. After 24 h, cells were harvested and lysed under denaturing conditions, and ubiquitinated proteins were purified with Ni^{2+}-NTA agarose beads (QIAGEN) as described previously (Rodriguez *et al*, 1999). The cells in Figure 7A were incubated for 4.5 h with 20 μM MG132 (Calbiochem), 20 μM MG5 (Sigma) prior to lysis.

Cell lines, transfection conditions and RNA extraction

MEF cells were cultured in a Hypoxic incubator, 10% CO_2, 3% O_2 (Thermo Scientific HeraCell 150i) (Parrinello *et al*, 2003). Total RNA was extracted from cells with Trizol reagent (Invitrogen) and treated with Turbo DNA-free DNAseI beads (Ambion). cDNA synthesis was performed with random-hexamer primers. PCR of the *GluR2 B13* minigene was performed with primer, 5′-atggaagagaaacacaaagt-3′ that anneals to exon 11 and antisense primer 5′-gaatgataggaacct tctgc-3′ that anneals to intron 11 (Higuchi *et al*, 1993). PCRs conditions were 94°C for 3 min, followed by 28 cycles of: 94°C for 30 s, 54°C for 30 s, 72°C for 45 s and 72°C for 7 min. For endogenous *GluR2* transcript, 1 μg of DNAse-treated total RNA was used for cDNA synthesis and RT–PCR was performed with Superscript™III One step RT–PCR System (Invitrogen), was performed with primer, 5′-atggaagagaaacacaaagt-3′ and the antisense primer 5′-ttccctttggac ttccgcac-3′ that anneals to exon 13.

RNAi knockdown

siRNA or pSUPER transfections were performed in HeLa and SH-SY5S cells with Lipofectamine 2000 reagent (Invitrogen). Both siRNA against *GAPDH* and smart Pool of siRNAs against *Pin1* (LPIN1, Dharmacon Thermo Scientific) were added to a final concentration of 100 nM. The pSUPER*Pin1* and pSUPER*LacZ* were used as siRNA controls as previously described (Rustighi *et al*, 2009).

Indirect immunofluorescence

Cells were plated on sterile cover-slips in six-well plates at 2.5×10^5 cells/well and grown overnight before transient transfection of expression constructs with Fugene 6 transfection reagent (Roche). Indirect immunofluorescence was performed as previously (Ayala *et al*, 2008).

Preparation of cytoplasmic and nuclear extracts

Cytoplasmic and nuclear fractionation was performed with ProteoExtract Subcellular Proteome Extraction Kit (Merck) according to the manufacturer's instructions. Quantification of cytoplasmic and nuclear fractionation was performed with the IMAGEQUANT/TL (GE Heathcare Life Science).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: RM conceived, designed and performed the majority of experiments. JB generated many of the ADAR2 reagents. SP performed some experiments with Pin1. AC performed some experiments with WWP2. SH performed some experiments with WWP2. NM performed mass spectrometry. AB generated some of the Pin1 reagents. LPK was involved in experimental design and contributed to writing the manuscript. GDS was involved in experimental design, provided reagents and contributed to writing the manuscript. MAO'C was involved in experimental design and wrote the manuscript

Conflict of interest

The authors declare that they have no conflict of interest.

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