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The physiological roles of *Drosophila* ADAR and modifiers

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Abstract

ADAR (Adenosine Deaminases acting on RNA) family proteins are double-strand RNA binding proteins that deaminate specific adenosines into inosines. This A-to-I conversion is called A-to-I RNA editing and is well conserved in the animal kingdom from nematodes to humans. RNA editing is a pre-splicing event on nascent RNA that may affect alternative splicing when the editing occurs in the exon-intron junction or in the intron. Also, editing may change biological function of small RNAs by editing the pre-microRNAs or other noncoding RNAs. Editing also alters protein amino acid sequences because inosine in the mRNA base pairs with cytosine and is therefore read as guanosine.

In mammals, there are three ADAR family proteins, ADAR1, ADAR2, and ADAR3, encoded by three different genes. So far, no enzymatic activity of ADAR3 is detected. The most frequently edited targets of ADAR1 and ADAR2 are regions covering copies of *Alu* transposable elements in primates. In addition, loss of some specific editing events leads to profound phenotypes when the editing does not occur correctly. For example, some human neural disorders – such as epilepsy, forebrain ischemia, and Amyotrophic Lateral Sclerosis – are known to be associated with abnormally edited ion channel transcripts.

Drosophila has a single ADAR protein (encoded by the *Adar* gene) that is highly conserved with human ADAR2 (encoded by the *ADARB1* gene). To date, 972 editing sites have been identified in 597 transcripts in *Drosophila*, and approximately 20% of AGO2-associated esiRNAs are edited. Similar to mammals, many ion channel-encoding mRNA transcripts undergo ADAR-mediated A-to-I editing in *Drosophila*. While *Adar1* null mice die at the embryonic stage and *Adar2* null mice die shortly after birth due to seizures, *Adar* null flies are morphologically normal and have normal life span under ideal conditions. However, *Adar* null flies exhibit severe neurodegeneration and locomotion defects from eclosion, whilst *Adar* overexpression (OE) is lethal.

To better understand the physiological role of RNA editing and ADAR, and to shed light on ADAR-related human disease, I used *Drosophila Adar* mutant flies as a model organism to investigate phenotypes, and to find chromosomal deletions and specific mutations that rescue the neural-behavioural phenotype of the *Adar* null mutant flies.

Using the publicly available chromosomal deletions collectively covering more than 80% of the euchromatic genome of Chromsome III, I performed a genetic screen to find rescuers of the lethality caused by *Adar* overexpression. I confirmed that mutation in *Rdl (Resistant to dieldrin*, the gene encoding $GABA_A$ receptor main subunit) rescues. This rescue was not likely caused by effects on *Adar* expression level or activity. Driven by the hypothesis that the rescue may be due to reduction in GABAergic input to neurons, I recorded spontaneous firing activity of *Drosophila* larval aCC motor neurons using *in vivo* extracellular current recording technique. As expected, the neurons overexpressing *Adar* had much less activities compared with wild type neurons. Also, I found that *Adar* null fly neurons fired much more and showed epilepsy-like increased excitability. Although feeding PTX (Picrotoxin), a $GABA_A$ receptor antagonist, failed to rescue the lethality, reducing the expression of *GAD1* to reduce synthesis of GABA was able to rescue the ADAR overexpression lethality. These results suggest that ADAR may fine-tune neuron activity synergistically with the GABAergic inhibitory signal pathway.

I used MARCM (mosaic analysis using a repressible cell marker) to detect cell-autonomous phenotypes in *Adar* null cells in otherwise wild type flies. Although neurodegeneration, observed as enlarged vacuoles formation in neurophils, was detected both in histological staining and EM images, the *Adar* null neurons marked with GFP from early developmental stages were not lost with age. Nevertheless, swelling in the axons or fragmentation of the axon branches of *Adar* null neurons was sometimes observed in the midbrain.

By comparing the Poly-A RNA sequencing data from *Adar* null and wild type fly heads, we detected significant upregulation of innate immune genes. I confirmed this by qRT PCR and found that inactive ADAR reduces the innate immune gene transcript levels

almost as much as active ADAR does. Further, using the locomotion assay, I confirmed that reintroducing inactive ADAR into *Adar* null flies can improve the flies' climbing ability.

Based on the *Adar* null flies having comparatively low viability, I performed a second deficiency screen to find rescuers of *Adar* null low viability using the same set of deficiencies as in the lethality rescue screen described above. I found seven deletions removing 1 to 37 genes that significantly increased the relative viability of the *Adar* null flies. However, not all the rescuing deficiencies also improved the *Adar* null locomotion. One rescuing gene, *CG11357* was mapped from one of the rescuing deficiencies, and some mutant alleles of *cry, JIL-1* and *Gem3* also showed significant effects on the *Adar* null fly viability. The single gene viability rescuers were also not necessarily locomotion or neurodegeneration rescuers. Although the initial aim was to find neural-behavioural rescuing genes from the viability screen, the viability rescuers found in the screen are more likely to play a role in different aspects of stress response for survival.

Declaration

I declare that the work in this PhD is my own, unless otherwise stated. The work in this PhD has never been submitted for any other degrees.

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Thank you all so much!

Abbrevations

General Abbreviations

aa	amino acids
AED	anti-epilepsy drug
AGS	Aicardi–Goutières syndrome
ALS	amyotrophic lateral scleorsis
BDSC	bloomington Drosophila stock centre
BSC	bloomington stock centre
BWA	Burrow-wheeler aligner
CARD	caspase recruitment domains
Chip	chromatin immunoprecipitation
Chr.	chromosome
CLIP	UV cross-linking and immunoprecipitation
CNS	central nervous system
DCV	Drosophila C virus
DD	death domain
Df	deficiency
DSH	dyschromatosis symmetrical hereditaria
dsRBD	double-strand RNA binding domain
DXV	Drosophila X virus
EC50	half maximal effective concentration
EGG	electroencephalography
EM	electronic microscope
FADD	FAS-associated death domain
FDA	food and drug administration
FRET	fluorescent resonance energy transfer
GO	gene ontology
HEK293	human embryonic kidney 293
HIV	human immunodeficiency virus
hs	heat shock
KO	knockout
MARCM	mosaic analysis with repressible cell markers
MB	mushroom body
MEF	mouse embryonic fibroblast
ModENCODE	Model Organism ENCyclopedia Of DNA Elements

NES	nuclear export signal
NLS	nuclear localization signal
OE	overexpression
PCR	polymerase chain reaction
PRKM	read per kilobase per million
qRT PCR	quantitative real-time PCR
RISC	RNA induced silencing complex
RNAi	RNA interference
S.E.M	standard error of the mean
seq	sequencing
SIGMAV	Sigma virus
TISC	Toll-induced signaling complex
ts	temperature sensitive
UPR	unfolded protein response
UV	ultraviolet
α	alpha
β	beta
γ	gamma
κ	kappa

Unit Abbreviations

°C	degrees centigrade
µl	microlitre
bp	base pairs
cm	centimetre
g	gram
kb	kilo base
l	litre
m	metre
M	molar
ml	mililitre
mM	microlitre
mol	moles
ms	milisecond
n	nano

nm	nanometre
nmol	nanomolar
nt	nucleotide
pA	picoampere
s	second
V	volt

Cellular molecules Abbreviations

IP6	inositol hexaphosphate
A	Adenosine
C	Cytosine
cDNA	complementary DNA
DNA	deoxyribonucleic acid
dsRNA	double strand RNA
esiRNA	endogenous small interefering RNA
G	Guanosine
GABA	gamma-aminobutyric acid
I	Inosine
IFN	Interferon
miRNA	micro RNA
mRNA	messanger RNA
ncRNA	noncoding RNA
pre-mRNA	pre-messenger RNA
pri-miRNA	primary micro RNA
RNA	ribonucleic acid
shRNA	small hairpin RNA
siRNA	small interefering RNA
snoRNA	small nucleolar RNA
ssRNA	Single strand RNA
T	Thymine
U	Uracil

Cellular component Abbreviation

DAP	mesodiaminopimelic acid
ECS	editing site complemetary sequences
ER	endoplasmic reticulum
LINE	long interspersed nucleotides element
LPS	lipopolysaccharides
ORF	open reading frame
PGN	peptidoglycan
SINE	small interspersed nucleotides elements
UAS	upstream activation sequence
UTR	untranslated regions
κB-RE	NF-κB response element

Chemical Abbreviation

Amp	ampicilin
Ca^{2+}	calcium ion
CO_2	carbon dioxide
DTT	dithiothreitol
H_2O	water
HCl	hydrochloric acid
HE	haematoxylin and eosin
K^+	potassium ion
KCl	potassium chloride
LB	Luria broth
Na^+	sodium ion
NaCl	sodium chloride
PBS	phosphate saline buffer
PFA	paraformaldehyde
pH	potential of hydrogen
PTX	picrotoxin
ROS	reactive oxygen species
Tris	Aminotris(hydroxymethyl)methane

Protein and Gene Abbreviation

ADAR	adenosine deaminases acting on RNA
$5HT_2C$	Serotonin (5-hydroxytryptamine, 5-H) receptor subtype C
Act	actin
AGO 2	Argonaute 2
AMP	antimicrobial peptides
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
arm	armadillo
atg 5	autophagy related gene 5
AttD	attacin D
awd	abnormal wing discs
Bcl-2	B-cell lymphoma 2
BK channels	large conductance Ca^{2+} activated potassium channel
Cas	Castor
Cg	collagen
Chc	Clathrin heavy chain
Crc	Calreticulin
CREB	cAMP response element-binding protein
Cry	cryptochrome
Dcr	Dicer
Def	defensin
DIF	dorsal-related immunity factor
DREED	death-related ced-3/Nedd2-like protein
Drs	drosomycin
elav	embryonic lethal abnormal vision
Fas II	Fasciclin 2
FLP	Flipase
GAD1	Glutamate decarboxylase 1
Gem3	Gemin3
GFP	green fluorescent protein
GluR-B	Glutamate receptor β subunit
GNBP	Gram-negative binding proteins
HSP70	heat shock protein 70kDa
IKK	I-κB kinase
IL-6	Interleukin-6
IMD	immune deficiency
IRE-1	Inositol requiring enzyme 1
ISGs	interferon-stimulated genes

JNK	Jun-N-terminal kinase
Kv1.1	Potassium voltage-gated channel subfamily A member 1
LGP2	laboratory of genetics and physiology 2
MAPKKK	mitogen activated protein kinase kinase kinase
MAVS	mitochondrial antiviral signaling
MDA5	melanoma differentiation-associated gene 5
mef	Myocyte enhancer factor 2
MyD88	myeloid differerntiation primary-response gene 88
NAF-1	Nutrient-deprivation autophagy factor-1
neur	neuralised
NF-κB	nuclear factor -κB
nwk	nervous wreck
PGRP	peptidoglycan-recognition protein
Pka-C2	cAMP-dependent protein kinase 2
PKR	protein kinase R
PO	phenoloxidase
PRR	pattern-recognition receptors
PtdIns(3)-P	Phosphatidylinositol 3-phosphate
Rdl	resistant to dieldrin
RIG-I	retinoic acid-inducible gene 1
RLR	RIG-I like receptors
rtp	retinophilin
shab	Shaker cognate b
slimb	supernumerary limbs
slo	slowpoke
SMN	survival motor neuron
SPE	Spȁtzle processing enzyme
syt	synaptotagmin
TAB2	TAK1-binding protein 2
TAK1	TGF-β-activated kinase 1
TGF	transforming growth factor-β
TLR	Toll like receptors
TOR	Target of rapamycin
Tot	turandot
TRIF	TIR domain-containing adapter influencing IFN-β
Upd	unpaired
Xbp-1	X box-binding protein 1

Amino Acid code Abbreviations

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

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1 CHAPTER I: Introduction

From wonder into wonder, existence opens.

— Lao Tzu

1.1 ADAR family proteins and A-to-I RNA editing

Proteins of the ADAR family are enzymes responsible for converting adenosines to inosines in double-stranded RNAs (dsRNAs). The ADAR proteins bind to substrates that are either long and perfectly paired or short and imperfectly paired dsRNAs (A Gallo et al. 2003; Ring et al. 2004), and then convert certain adenosine (A) residues in RNA into inosines (I). This reaction involves a water molecule which is added to the 6-position to form a hydrated intermediate (Polson et al. 1991). The target substrates of ADAR include repetitive noncoding RNAs, virus RNAs, mRNAs, microRNAs, and endogenous small interfering RNAs (esiRNA) (Brenda L Bass 1997; A Gallo et al. 2003; Ring et al. 2004; Kawamura et al. 2008; Athanasiadis et al. 2004; Taylor et al. 2005; Kawahara, Zinshteyn, Sethupathy, et al. 2007).

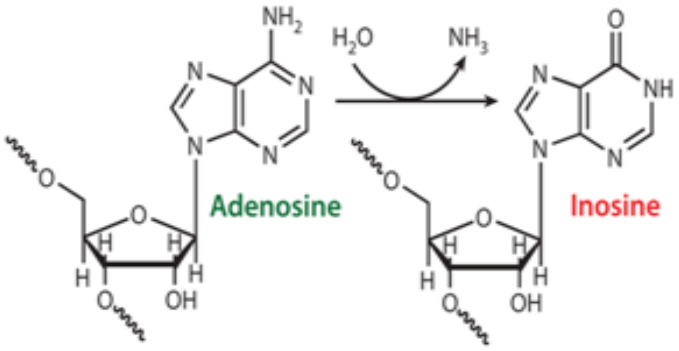
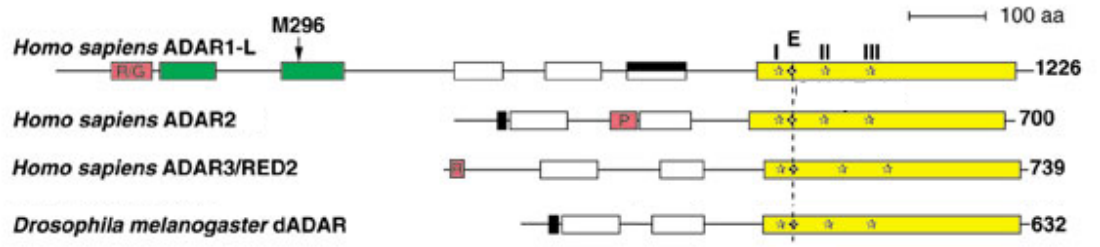


Figure 1.1 A-to-I conversion chemical reaction mediated by ADAR. Figure is taken from Keegan et al., 2004.

The ADAR proteins are evolutionarily conserved from worms to human beings. They have two to three double stranded RNA binding domains and one deaminase domain (Figure 1.2 A and B) (Keegan et al. 2001) .

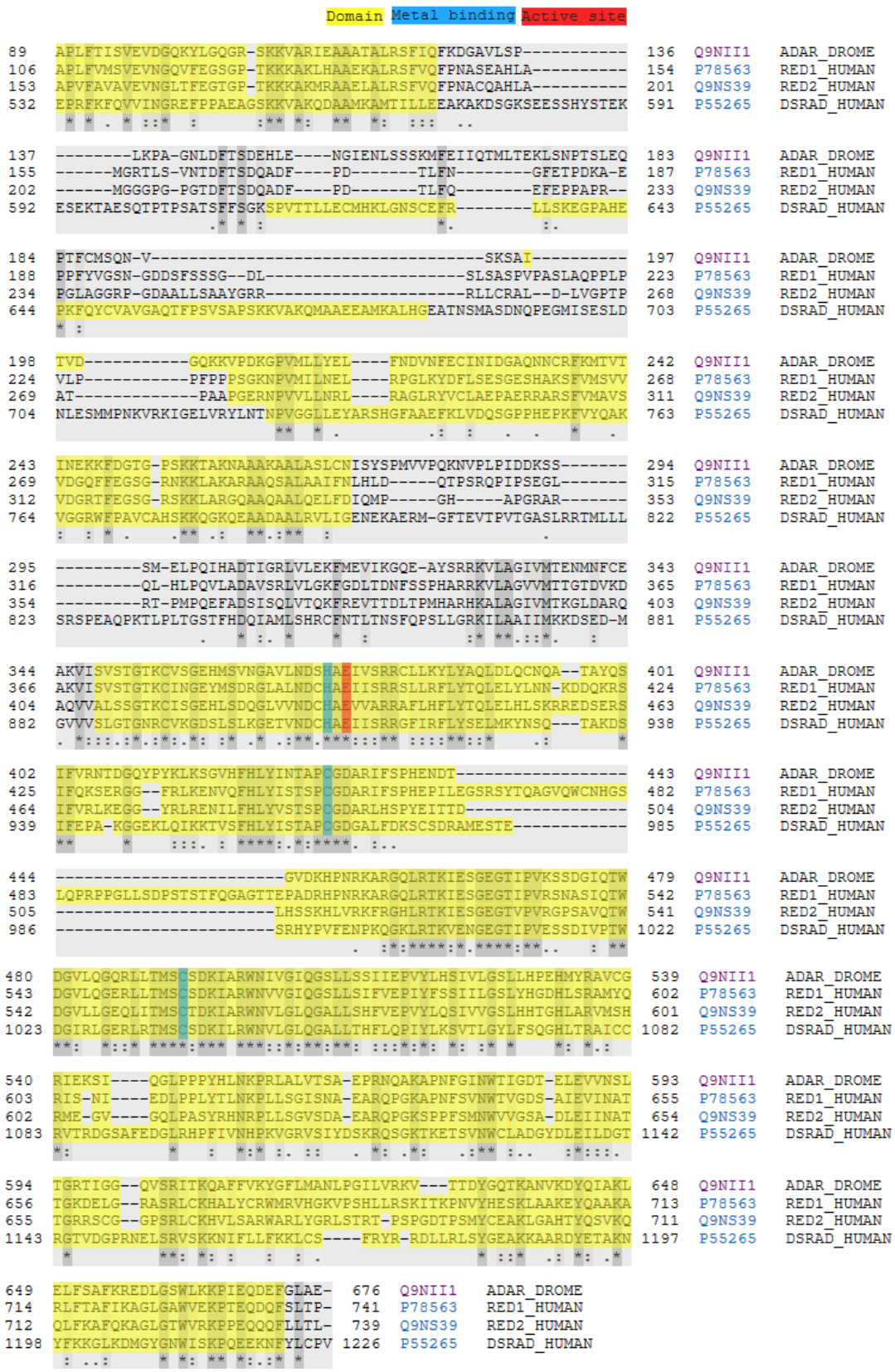
A

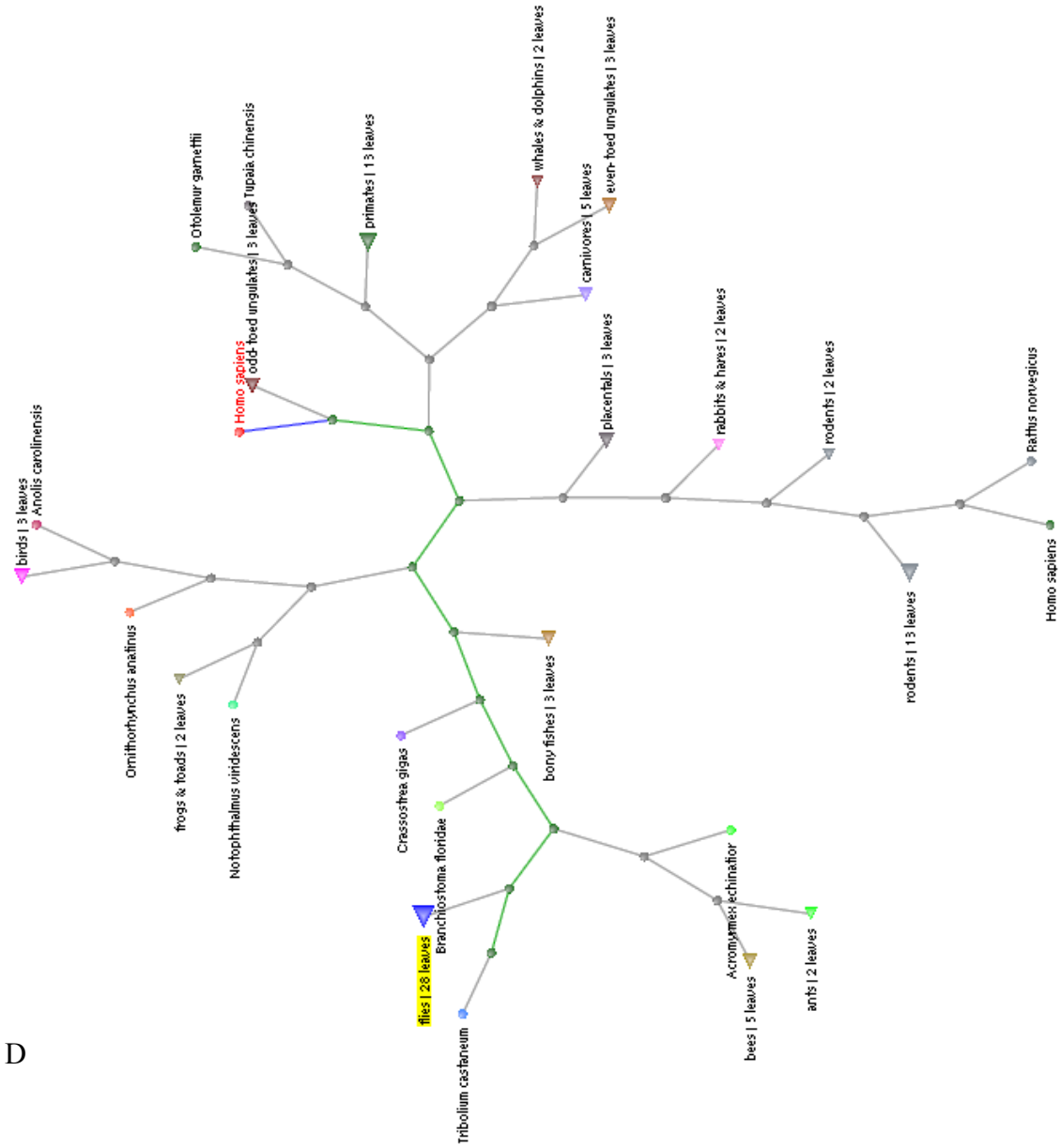


B

DNA binding Domain

```
1    ------------------------------------------------------------    0  Q9NII1   ADAR_DROME
1    ------------------------------------------------------------    0  P78563   RED1_HUMAN
1    ------------------------------------------------------------    0  Q9NS39   RED2_HUMAN
1    MNPRQGYSLSGYYTHPFQGYEHRQLRYQQPGPGSSPSSFLLKQIEFLKGQLPEAPVIGKQ   60  P55265   DSRAD_HUMAN

1    ------------------------------------------------------------    0  Q9NII1   ADAR_DROME
1    ------------------------------------------------------------    0  P78563   RED1_HUMAN
1    ------------------------------------------------------------    0  Q9NS39   RED2_HUMAN
61   TPSLPPSLPGLRPRFPVLLASSTRGRQVDIRGVPRGVHLRSQGLQRGFQHPSPRGRSLPQ  120  P55265   DSRAD_HUMAN

1    ------------------------------------------------------------    0  Q9NII1   ADAR_DROME
1    ------------------------------------------------------------    0  P78563   RED1_HUMAN
1    ------------------------------------------------------------    0  Q9NS39   RED2_HUMAN
121  RGVDCLSSHFQELSIYQDQEQRILKFLEELGEGKATTAHDLSGKLGTPKKEINRVLYSLA  180  P55265   DSRAD_HUMAN

1    ------------------------------------------------------------    0  Q9NII1   ADAR_DROME
1    ------------------------------------------------------------    0  P78563   RED1_HUMAN
1    ------------------------------------------------------------    0  Q9NS39   RED2_HUMAN
181  KKGKLQKEAGTPPLWKIAVSTQAWNQHSGVVRPDGHSQGAPNSDPSLEPEDRNSTSVSED  240  P55265   DSRAD_HUMAN

1    ------------------------------------------------------------    0  Q9NII1   ADAR_DROME
1    ------------------------------------------------------------    0  P78563   RED1_HUMAN
1    ------------------------------------------------------------    0  Q9NS39   RED2_HUMAN
241  LLEPFIAVSAQAWNQHSGVVRPDSHSQGSPNSDPGLEPEDSNSTSALEDPLEFLDMAEIK  300  P55265   DSRAD_HUMAN

1    ------------------------------------------------------------    0  Q9NII1   ADAR_DROME
1    ------------------------------------------------------------    0  P78563   RED1_HUMAN
1    ----------------------------MASVLGSGRGSG------GLSSQLKCKSKRR   25  Q9NS39   RED2_HUMAN
301  EKICDYLFNVSDSSALNLAKNIGLTKARDINAVLIDMERQGDVYRQGTTPPIWHLTDKKR  360  P55265   DSRAD_HUMAN

1    ---------------------------------------------MKFDSR---------V    7  Q9NII1   ADAR_DROME
1    ---------------------------------------------MDIEDEENMSSSSTDV   16  P78563   RED1_HUMAN
26   RRRRSKRKDKVSILST-------------FL---APFKHLSPGITNTEDDDTLSTSSAEV   69  Q9NS39   RED2_HUMAN
361  ERMQIKRN-TNSVPETAPAAIPETKRNAEFLTCNIPTSNASNNMVTTEKVEN---GQEPV  416  P55265   DSRAD_HUMAN
                                                        :.         *

8    --MLNSA-NNNS------PQH-PVSAPS-----------------DI----NMNGYNRKL   36  Q9NII1   ADAR_DROME
17   --KENRNLDNVS---PKDGST-PGPGEG-----------------SQLSNGGGGGPGRKR   53  P78563   RED1_HUMAN
70   --KENRNVGNLA---ARPP-------PS-----------------GDRARGGAPGAKRKR  100  Q9NS39   RED2_HUMAN
417  IKLENRQEARPEPARLKPPVHYNGPSKAGYVDFENGQWATDDIPDDLNSIRAAPGEFR--  474  P55265   DSRAD_HUMAN
       *    .                 .                                *  *

37   PQKRGYEMPKYSDPKKKMCKERIPQ----PKNTVAMLN----ELRHGLIYKLESQTGPVH   88  Q9NII1   ADAR_DROME
54   PLEEGSNGHSKYRLKKRRKTPGPVL----PKNALMQLN----EIKPGLQYTLLSQTGPVH  105  P78563   RED1_HUMAN
101  PLEEGNGGHLCKLQLVWKKLSWSVA----PKNALVQLH----ELRPGLQYRTVSQTGPVH  152  Q9NS39   RED2_HUMAN
475  ---AIMEMPSFYSHGLPRCSPYKKLTECQLKNPISGLLEYAQFASQTCEFNMIEQSGPPH  531  P55265   DSRAD_HUMAN
                                  ** :  *           :   .*:** *
```


Domain
Metal binding
Active site


Otolemur garnettii
Tupaia chinensis
primates | 13 leaves
whales & dolphins | 2 leaves
even-toed ungulates | 3 leaves
carnivores | 5 leaves
odd-toed ungulates | 3 leaves
Homo sapiens
placentals | 3 leaves
rabbits & hares | 2 leaves
rodents | 2 leaves
Rattus norvegicus
Homo sapiens
rodents | 13 leaves
birds | 3 leaves
Anolis carolinensis
Ornithorhynchus anatinus
frogs & toads | 2 leaves
Notophthalmus viridescens
bony fishes | 3 leaves
Crassostrea gigas
Branchiostoma floridae
flies | 28 leaves
Tribolium castaneum
Acromyrmex echinatior
bees | 5 leaves
ants | 2 leaves

D

C


DSRAD_HUMAN
ADAR_DROSOPHILA
RED1_HUMAN
RED2_HUMAN

Figure 1.2 ADAR phylogeny. (A) Conserved domains and residues of human ADARs and *Drosophila.* ADAR family proteins have two to three dsRBDs (white boxes) and one deaminase domain (yellow box). Human ADAR1 p150 also has two Z-DNA binding domains (green boxes) and an R-G rich domain. The shorter ADAR1 (ADAR p110) protein begins at M296 in the second Zβ Z DNA binding domain. In the second dsRBD of ADAR2, there is a proline (P) rich sequence, and ADAR3 has an ssRNA-binding domain at the N-terminus. Black boxes refer to nulcear localisation sequences (NLS). Three chelating residues involved in Zn^{2+} binding in each ADAR deaminase domain (marked as five-point stars) and glutamate (E) residues important in the active sites (aligned, four-point star) are marked. Numbers indicate total amino acids. Figure adapted from Keegan et al., 2004. (B) Alignment of *Drosophila* ADAR (ADAR_DROME), human ADAR2 (RED1_HUMAN), human ADAR3 (RED2_HUMAN), and human ADAR1 (DSRAD_HUMAN) full amino acid sequences. Green highlights DNA binding domain which exists only in ADAR1 but not in other ADAR proteins. Yellow highlights protein domains – dsRNA binding domains and deaminase domain. Blue highlights metal ion (Zn^{2+}) binding site and red highlights enzymatically active site. Dark grey aligns conserved sites. The alignment comparison is conducted at Uniprot website. http://www.uniprot.org/align/201305065027SU3DYU (C) Amino acid conservation guide tree of *Drosophila* ADAR (ADAR_DROME), human ADAR2 (RED1_HUMAN), human ADAR3 (RED2_HUMAN), and human ADAR1 (DSRAD_HUMAN). The tree is generated at http://www.uniprot.org/align/201305065027SU3DYU Uniprot website. (D) Unrooted tree view of *Drosophila* ADAR and its orthologous in different species. Subtrees (leaves) that contain sequences with common Blast Name are collapsed. Yellow highlights *Drosophila* ADAR and green line leads to human ADAR2 (Red font) from the evolutionarily predicted root ADAR. The tree is generated at NCBI blast webpage, using Fast Minimum Evolution Tree Method. Parameters set for the methods are: Max Seq difference: 0.85; Distance: Grishin (Protein). http://www.ncbi.nlm.nih.gov/blast/treeview/treeView.cgi

Based on the protein domain organization, *Drosophila* ADAR is most similar to ADAR2 and then ADAR3 (Figure 1.2 A-C) with two dsRNA binding domain and one deaminase domain. ADAR1, with its additional one dsRNA binding domain and DNA-binding domains, predicts quite different substrate specificity and physiological functions compared with the rest of ADAR proteins. It is shown that main role of ADAR1 is to edit long-repeat dsRNA promiscuously, and has important physiological role in hematopoietic stem cell differentiation and immune-control (Hartner et al. 2009; Laxminarayana et al. 2007; Feng et al. 2009). ADAR2 edits more site-specific sites in dsRNA, and its main physiological role is shown in controlling neuron physiology by

guarding ion channel activities (Kittler 2006; Higuchi et al. 2000a; Ohlson et al. 2007) Intriguingly, ADAR3 is exclusively expressed in the neural system but the physiological role of ADAR3 is unknown (Chen et al. 2000). Human ADAR2 rescues the *Drosophila* ADAR knockout neural-behavioural phenotypes almost as well as *Drosophila* ADAR does while human ADAR1 does not rescue locomotion defects of the ADAR knockout flies. These similarities between domain organizations of different ADAR proteins are correlated with physiological functions of the proteins. *Drosophila* ADAR is the evolutionary early ancestor of human ADAR2 (Figure 1.2 D). It is thought that ADAR1 is lost in the insect while conserved in the mammals (Keegan et al. 2011). However unexpectedly, human ADAR1 short isoform p110 could rescue age-dependent neurodegeneration of *Drosophila* (Keegan et al. 2011). Some other evidence, like identification of widely edited esiRNA for instance, also suggests that *Drosophila* ADAR may have some physiological roles of mammalian ADAR1 like a role in immunity or development of the animal.

The specificity and the mechanism of the deamination process hugely depend on the structure of ADAR proteins. Although the full-length ADAR protein structure has not been solved, the structure of each domain of ADAR is well-studied.

1.1.1 ADAR Substrates

The ADAR proteins bind to substrates that are either long and perfectly paired or short and imperfectly paired dsRNAs (Gallo et al. 2003; Ring et al. 2004).

Long and perfectly paired dsRNAs *in vivo* can be formed by the base-pairing of inverted *Alu* elements or LINEs (Long Interspersed Nucleotide Element) in primates, or SINEs (small Interspersed Nucleotide Elements) in mouse, or viral RNAs, or in untranslated regions (UTR) of mRNAs (Levanon et al. 2004; Athanasiadis et al. 2004; Osenberg et al. 2010; Zahn et al. 2007). The A-to-I conversion in these substrates is non-specific (Bass 1997). Non-specific editing has been estimated to occur at approximately 13,000

adenosines in about 1,700 human genes in a computational search for editing sites of whole human transcriptiome (Levanon et al. 2004).

In vitro and *in vivo* studies show that A-to-I editing of the dsRNAs leads to a reduction in RNAi efficiency (Yang et al. 2005; Scadden and Smith 2001; Wu et al. 2011). But recently, ADAR1 is shown to form duplex with Dicer to increase the efficiency of miRNA production(Ota et al. 2013). In addition, emerging evidence shows that dsRBD binding activity of ADAR, independent of the editing activity, interferes with miRNA processing (Heale, Keegan, McGurk, et al. 2009; Vesely et al. 2012).

Targeting of ADAR to short and imperfectly paired dsRNAs allows selection of adenosines at specific sites (Higuchi et al. 1993). This type of editing occurs mostly in exons of pre-mRNA that form imperfect double strands between the regions surrounding the editing sites and editing site complementary sequences (ECS) located mostly in nearby intronic regions (Figure 1.3) (Higuchi et al. 1993; Reenan 2005).

When A-to-I editing occurs in the open reading frame (ORF), inosine and base pairs with cytosine is read as guanosine (A-to-I-to-G) by the translational machinery. In that way, ADAR greatly increases the diversity of the proteins, especially neural proteins, and can affect alternative splicing or stability of the target dsRNAs. Also, A-to-I editing can interact with the RNAi pathway by targeting the precursors of siRNAs and miRNAs (Knight and Bass 2002; Kawahara, Zinshteyn, Chendrimada, et al. 2007).

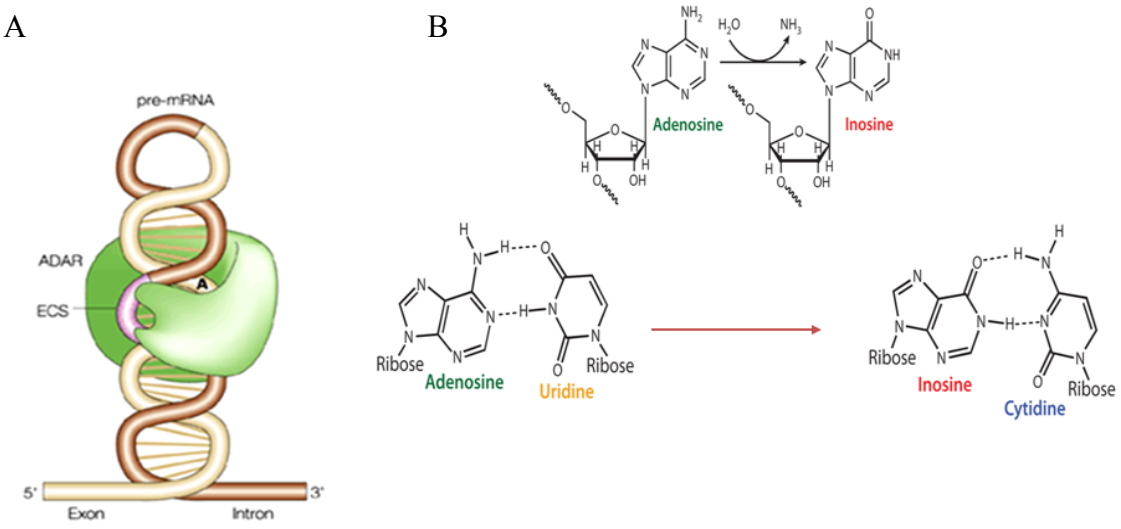


Figure 1.3 Editing of pre-mRNA requires ECS (Editing site complementary sequences) and changes base-pairing. (A) dsRNA formed between the edited site and downstream intron in the pre-mRNA. ECS base pairs with the sequence surrounding the edited A residue. ADAR binds and edits the site. (B) Adenosine base-pairs with Uridine, but Inosine base pairs with Cytidine, read as Guanosine. Thus, A-to-I conversion in the pre-mRNA changes the genetic information read by the translational machinery. Both (A) and (B) are taken from Keegan *et.al*, 2004.

1.1.2 Mutant phenotypes and human diseases

ADAR mutants and alterations in editing of many mRNAs and noncoding RNAs are associated with some human diseases, including dermatosis, mental diseases, motor neuron diseases, cancers and inflammations (Maas et al. 2006; Tariq and Jantsch 2012) .

Some neurological diseases are associated with abnormal editing levels in the ion channel transcripts (Table 1.1) (Niswender et al. 2001). For example, editing levels affect pharmacological properties of Kv1.1 channels and $5HT_{2c}$ receptors (Niswender et al. 1999; Berg et al. 2001; Decher et al. 2010; M. Singh et al. 2011). It is also shown that expression of unedited $GABA_A$ receptor is crucial for synapse formation in the

developing brain, and editing causes a delay in response to GABA (Ben-Ari et al. 2007; Rula et al. 2008).

One of the well-studied diseases related with A-to-I RNA editing alteration is sporadic ALS (Amyotrophic Lateral Sclerosis) disease. ALS is one of the most common motor neuron diseases, and altered editing of *GluR-B* Q/R site has been proposed (Kawahara et al. 2003). Kawahara et al. observed reduced editing at the *GluR-B* Q/R site in motor neurons of sporadic ALS patients in comparison to 100% editing in controls, indicating a crucial role of RNA editing in sporadic ALS (Kawahara et al. 2004). The *GluR-B* Q/R site in the key AMPA receptor (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) subunit is the principal target of ADAR2. In normal motor neurons, the *GluR-B* Q/R site is edited with 99.9% efficiency (O'Connell et al. 1997), which prevents high influx of Ca^{2+} ions through the AMPA receptors. But the unedited GluR-B permits the high influx of Ca^{2+} ions that may cause glutamate-excitotoxic neuron death (Shaw and Ince 1997). It is shown that mutating Q into R in the *GluR-B* Q/R site is sufficient to rescue seizures and early death of *Adar2* knockout mice (Higuchi et al. 2000).

ADAR1 mutations have been identified in more than 130 familial cases of dyschromatosis symmetrica hereditaria (DSH), an autosomal dominant disorder found mainly in China and Japan (Zhang et al. 2004; Liu et al. 2006; Kondo et al. 2008; Miyamura et al. 2003), and recently in an immune-mediated neural-developmental disorder Aicardi-Goutières syndrome (AGS) (Rice et al. 2012) (Aicardi and Goutières 1984). So far, most diseases reported to involve *ADAR1* mutations show inflammatory features and this part will be introduced in Section 1.3 in more details.

Table 1.1 Abnormal editing of ion channels and human diseases.

Edited channels	Edited sites	Effect of editing	Reported or predicted human diseases
Glutamate-gated ion channels	*GluR-2*, 3,4,5,6; Q/R site, R/G site, I/V site etc.	Editing at Q/R sites reduces calcium permeability.	ALS, epilepsy
$5HT_{2C}$	Up to five A-to-I events	Fully edited isoforms revealed a 40-fold decrease in serotonergic potency.	Forebrain ischemia, depression and suicide, Prader-wili Syndrome
$GABA_A$ receptor	Channel gating region, one I/M site in *Gabra-3*	EC_{50} is around 50% for the non-edited channel.	Epilepsy and neurodegeneration (?)
Kv1.1	Sixth transmembrane domain, I/M site.	Editing reduces inactivation rate, and reduces sensitivity to highly-unsaturated fatty acids.	Multiple sclerosis, epilepsy, and autoimmune diseases (?)

The question marks (?) refer to cases where there is no experimental evidence showing that editing level changes are involved in the diseases, but there are reports of disease mutations in the listed ion channels.

Homozygous *Adar1* null mice and *Adar2* null mice were both generated. *Adar1* null mice were generated in two different groups almost at the same time and both reported embryonic lethality occurring E11.5 and E12.5 (Wang et al. 2004; Hartner et al. 2004). *Adar1* null embryos show an anemia phenotype and are slightly retarded in growth

shortly before E11.5, with much reduced hematopoietic cells in the liver. *Adar1* null mouse embryos showed wide-spread apoptosis at near E10 which is likely the direct cause of the embryonic lethality (Wang et al. 2004; Hartner et al. 2004). Homozygous *Adar2* null mice die postnatal (P) between P0 and P20 and became progressively seizure-prone after P12. Surprisingly, these phenotypes are completely rescued by introducing homozygous edited $GluR^R$ alleles (Higuchi et al. 2000). This suggests that GluR-B is the main target of ADAR2.

The biological role of ADAR3 is not known. ADAR3 did not edit either endogenous or synthetic dsRNA *in vitro* although it shares 50% protein sequence identity with ADAR2 (Melcher et al. 1996). Also mysteriously, ADAR3 expression is restricted to brain and no phenotypes were found in *Adar3* knockout mice (Faul, Higuchi; Seeburg, unpublished).

1.1.3 Structure-based studies of ADAR

1.1.3.1 dsRBDs

ADAR proteins bind to target transcripts through the dsRBDs (~65 amino acids) with its conserved αββα topology that specifically binds to the A-form RNA helix (Masliah et al. 2012) or the stem-loop structure (Ramos et al. 2000).

The solution structure of the two ADAR2 dsRBDs bound to the dsRNA substrate of *GluR-2* R/G edited site (Figure 1.4A) revealed two important aspects of the interaction between ADAR2 dsRBDs and the transcript substrate (Stefl et al. 2010).

Firstly, the contacts the two dsRBDs make with the substrate are different. The first dsRBD of ADAR2 contacts the dsRNA apical loop that caps the RNA hairpin, whereas the second dsRBD of ADAR2 does not contact the apical loop, but binds dsRNA near the edited base (Stefl et al. 2010). The apical loop was shown to be essential for the substrate recognition in the case of the Rnt1p dsRBD (Wu et al. 2004). However, it is

not clear whether the interaction between Asn 87 and Glu 88 in rat ADAR2 dsRBD1 and the apical loop in the GluR-2 R/G site substrate is essential.

Secondly, ADAR2 dsRBDs recognize the sequence of RNA as well as the shape of the RNA (Stefl et al. 2010). Both the ADAR2 dsRBDs recognize the RNA helix via two sequence-specific contacts at two consecutive RNA minor grooves. One of the sequence-specific contacts is a hydrogen-bond formed between a single G of RNA to the amino-groups in the β1-β2 loop of each dsRBD. The other one is a hydrophobic contact to the adenine H2 via methinone in α1 helix (Stefl et al. 2010). Stefl and the co-workers further demonstrated that the sequence-specific contacts are important for editing (Stefl et al. 2006).

Substrate selectivity of ADARs may depend on the dsRBDs. Mammalian ADAR1 and ADAR2 share some substrates but also have specificity. The main structural differences between ADAR1 and ADAR2 are numbers of dsRBDs and the spacing between the dsRBDs (Stefl et al. 2006; Strehblow et al. 2002). In addtition, ADAR1 dsRBDs have longer α1 helices and do not have ADAR2 equivalent of Met 84 and Met 238 (Stefl et al. 2006). All these may explain the different substrate specificity of ADAR1 and ADAR2 (Bass 2002; Lehmann and Bass 2000).

The dsRBDs of ADARs also direct ADARs to compete with DICER for the same RNA substrates (Kawahara et al. 2007; Yang et al. 2006). Even inactive ADAR with functional dsRBDs can modulate pri-miRNA processing, suggesting an enzymatic activity-independent role of ADARs guided by dsRBDs (Heale, Keegan, McGurk, et al. 2009).

1.1.3.2 Deaminase domain

The C-terminus of each ADAR family protein contains the deaminase domain with an enzymatic activity to deaminate adenosine residues to inosine. Two parallel α helices (α2-α3) of the deaminase domain contain residues that are essential for the deaminating activity (Lai et al. 1995). These essential residues in the catalytic hub of the deaminase

domain coordinate zinc ion that bind water to form the nucleophil, and a conserved glutamate residue accepts a proton from the nucleophilic water (Figure 1.4B). When this glutamate residue (E396 in human ADAR2) is mutated to alanine, ADAR proteins lose their deaminase activity completely (Lai et al. 1995; Haudenschild et al. 2004).

The deaminase domain of ADAR2 has been determined by X-ray crystallography in 2005. The substrate binding surface forms a positive electrostatic field, and Macbeth and coworkers argued that this structure likely facilitates binding of dsRNA (Figure 1.4B) (Macbeth et al. 2005). A zinc ion buried in the active site is ligated by C451, C516 and H394 in the catalytic domain of ADAR2 with the water molecule (Macbeth et al. 2005). In the active catalytic hub, T375 and R455 residues are important for the catalytic activity of ADAR2 (Macbeth et al. 2005). T375 is suggested to prevent C to U deamination and to act as a hydrogen bond donor, and R455 may approach N7 of the editable adenosine that assist editing (Goodman et al. 2011).

The most striking finding was the presence of inositol hexakisphosphate (IP_6) in the core of the domain (Macbeth et al. 2005) (Figure 1.4B). IP_6 is known to be associated with surfaces of some proteins to affect interactions with other proteins (Hanakahi and West 2002; Reineke et al. 2007), but IP_6 has never been seen buried within a protein domain as in ADAR2. IP_6 is very tightly associated with the core of the ADAR2 structure (Macbeth et al. 2005). It is predicted that existence of IP_6 is crucial for the editing activity of ADAR (Macbeth et al. 2005).

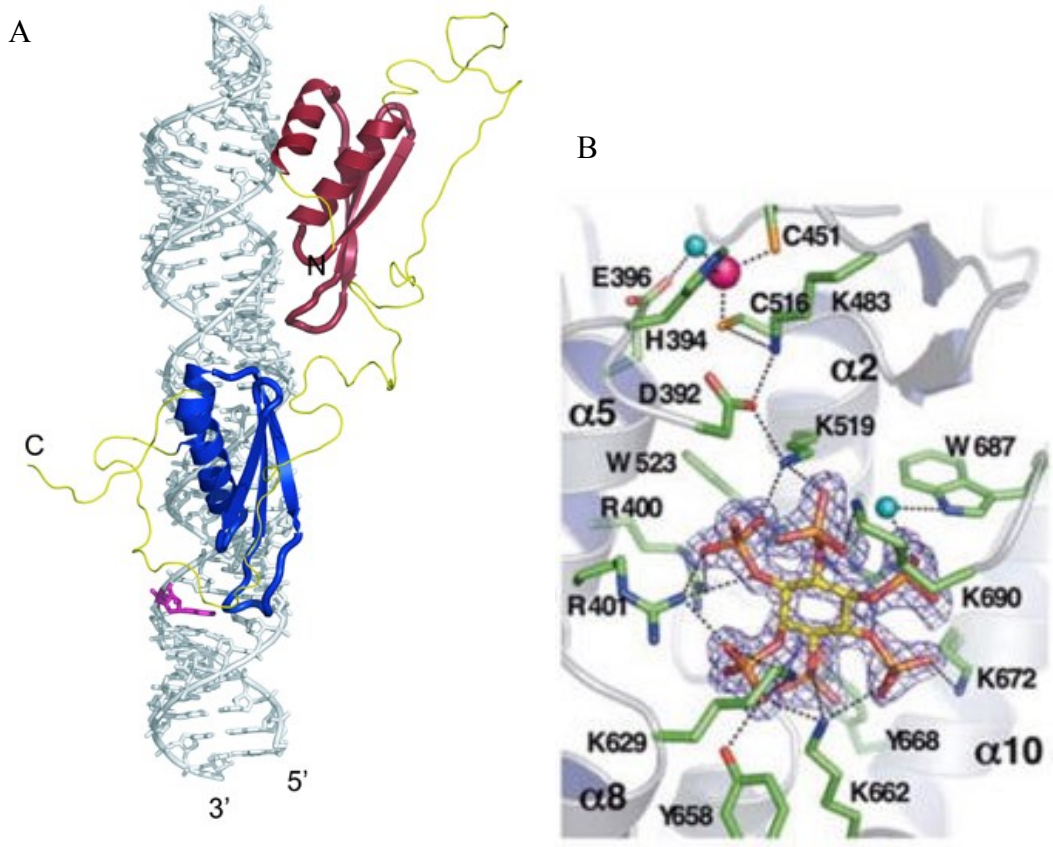


Figure 1.4 Reconstructed NMR structure of ADAR2 dsRBDs bound to the *GluR-2* R/G substrate and active site of the ADAR2 deaminase domain. (A) dsRBDs of ADAR2 bound to the *GluR-2* R/G site. Red: dsRBD1, Blue: dsRBD2. The grey helix represents *GluR-2* RNA and the edited adenosine is in pink. Figure is taken from Stefl *et al*., 2006. (B) The active site of the ADAR2 deaminase domain. Hydrogen bonds (blue dotted lines) connect Zn^{2+} to conserved amino acids in the hub of active site. IP_6 (yellow hexagon) is some distance away from Zn^{2+} (pink ball) in the hub of active site, but may communicate with the active site hub through a chain of hydrogen bonds (dash lines). The interaction of IP_6 with W532 and W687 is mediated by water (aqua sphere). The nucleophilic water (aqua sphere) is near Zn^{2+}. Figure is taken from Macbeth *et al.*, 2005.

1.1.3.3 Z DNA-binding domains

ADAR1, but not other ADAR family proteins, has two related Z-DNA binding domains in its N terminal, Zα and Zβ. Zβ has no binding capacity for Z-DNA (Herbert et al.

1997). Zα is present only in the interferon-inducible cytoplasmic form of ADAR1 (ADARp150) while Zβ is present in both ADAR1 isoforms (ADAR p150 and ADAR p110) (George and Samuel 1999; Schwartz et al. 1999). Zα not only binds Z-DNA, but can also binds Z-RNA (Brown et al. 2000). The inclusion of Zα may be related with the anti-viral function of ADAR1 by binding to negatively supercoiled viral RNAs (Wittig et al. 1991; Placido et al. 2007).

1.1.3.4 Dimerization is needed for editing

ADARs have not formed dimmers in any crystals so far, but biochemical data suggests that dimerization is needed for the editing activity of ADAR proteins. A study using FRET (Fluorescence resonance energy transfer) analysis showed that ADAR1 and ADAR2 both make dimers, including heterodimers *in vivo*, dependent on the dsRBDs (Chilibeck et al. 2006). In *Drosophila*, ADAR, which is highly conserved with ADAR2, is shown to dimerize, RNA substrate-dependently (Gallo et al. 2003). However, human ADAR2 was found as a monomer, when the analytical gel filtration analysis and equilibrium sedimentation were used (Macbeth et al. 2004). There is a hypothesis suggested by Poulsen *et al.* that dimerization of ADAR proteins may depend on the amount of RNA substrate present (Poulsen et al. 2006). With excess RNA substrate, only one ADAR binds per substrate molecule, instead of forming a dimer, which may explain the substrate inhibition phenomenon (Poulsen et al. 2006; Hough and Bass 1994). Nevertheless, it is also possible that some unknown factors may determine the dimerization of ADAR proteins that are constantly in equilibrium between monomer and dimer.

1.1.4 Cellular localization of ADARs

ADAR1 p150, the interferon-inducible isoform accumulates mostly in the cytoplasm (Patterson and Samuel 1995; Poulsen et al. 2001; Desterro et al. 2003) while ADAR1 p110 and ADAR2 are mostly located in the nucleoli (Sansam et al. 2003; Desterro et al.

2003). All these ADAR family proteins shuttle constantly either between nucleolus and nucleoplasm or between nucleus and cytoplasm (Desterro et al. 2003).

ADAR1 has a nuclear localization signal (NLS) sequence in the dsRBD3 (Eckmann et al. 2001; Strehblow et al. 2002) and a nuclear export signal (NES) sequence in the Zα domain (Poulsen et al. 2006). ADAR1 p150, with both NLS and NES, shuttles between cytoplasm and nucleus and ADAR1 p110 also shuttles between nucleus and cytoplasm though it does not have an NES (Eckmann et al. 2001; Poulsen et al. 2001; Fritz et al. 2009). ADAR1 shuttling is mediated by dsRBD3 with its interaction with Transportin-1 and exportin-5 (Fritz et al. 2009). It is not clear how ADAR2 shuttles between the nucleolus and nucleoplasm. It is also not clear why the ADAR1 and ADAR2 proteins are mostly in the nucleoli instead of in the nucleoplasm where their targets are. They relocate to nucleoplasm when extra substrate is produced from transfected plasmids (Desterro et al. 2003).

1.1.5 Physiological regulation of ADAR family proteins

ADAR RNA editing has been studied for more than two decades. Purified ADAR proteins can edit their substrates *in vitro* without any cofactors (O'Connell et al. 1998) and it was believed that the ADAR enzymes require no cofactors.However, there are still several challenges to fully understand the regulation of RNA editing *in vivo*.

In mice, *Adar1* expression is found to be controlled by multiple tissue-specific promoters, and *Adar1 p150* expression is triggered by the interferon-inducible promoter during virus infections (George et al. 2005). ADAR2 and its editing activity are shown to be metabolically regulated by nutritional status in pancreatic islets beta-cells (George and Samuel 1999; Gan et al. 2006). In the high-fat fed insulin-resistant mouse model, pancreatic *Adar2* expression increased nearly two-fold whereas in the diet restricted mice, the expression of *Adar2* was repressed (Gan et al. 2006).

Recently, the phosphorylation-dependent prolyl-isomerase Pin1 has been shown to interact with ADAR2 as a positive regulator required for nuclear localization. The E3 ubiquitin ligase WWP2 plays a negative role by binding to ADAR2 and catalyzing its ubiquitination and subsequent degradation (Marcucci et al. 2011). It is not known which phosphatases and kinases are involved or how they regulate ADAR2.

ModEncode study revealed that Drosophila Adar expression level is affected by some chemical treatment including ethanol, caffeine or paraquat (Graveley et al. 2011). However, it is not clear how environmental factors regulate *Drosophila Adar* which is highly conserved with ADAR2, and is the only ADAR family protein in *Drosophila*.

1.2 Regulation and the targets of *Drosophila* ADAR

1.2.1 *Drosophila Adar* gene and ADAR protein

Drosophila ADAR has two dsRBDs and one RNA editing domain, sharing high structural and functional homology with human ADAR2 (also known as RED1) and human ADAR3 (also known as RED2).

Drosophila melanogaster has a single *Adar* locus at cytogenic position 2B 6-7, near the tip of the X chromosome (Palladino et al. 2000a). Expression of *Adar* is developmentally controlled by two different promoters. The 4A promoter is active at early stages, and the stronger 4B promoter is active only after metamorphosis. *Adar* pre-mRNAs undergo alternative splicing and self-editing to produce different ADAR protein isoforms with different editing activities (Figure 1.5) (Palladino et al. 2000b).

There are two principal transcripts in the embryo and two further additional transcripts in the adult. The two adult-specific transcripts predominate in the adult stage (Palladino et al. 2000a). Exclusion of exon 3a, resulting in the 3/4 isoform is adult-specific and is predominant after metamorphosis (Palladino et al. 2000a). Inclusion of exon 3a adds 38 amino acids, increasing the distance between two dsRBDs of ADAR. Minor transcripts include an alternative -1 exon in both 3/4 and 3a isoforms that produces proteins with 12 more amino acids at the N-terminus (Figure 1.5) (Palladino et al. 2000b).

C In addition, exon 7 has an editing site, with editing occuring mainly after metamorphosis (Keegan et al. 2005). Exclusion of exon 7 was also observed in embryos (Ma et al. 2002). Self-editing of exon 7 is likely a mechanism to fine-tune the editing activity of ADAR, since the edited ADAR isoform shows lower editing activity on some well-known edited transcripts (Keegan et al. 2005).

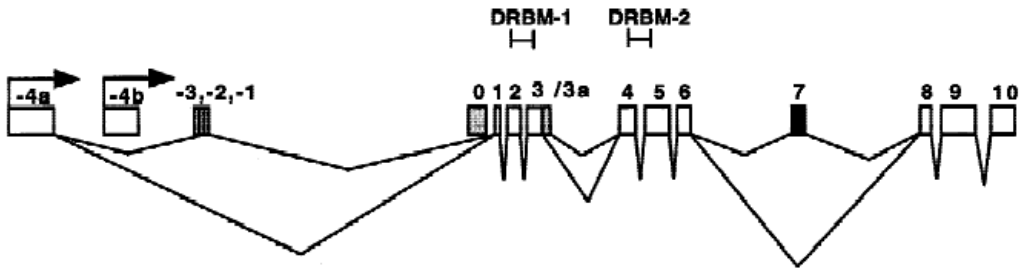


Figure 1.5 Gene structure of *Drosophila Adar.* Two promoter regions are suggested, -4a and -4b. Alternative splicing points are linked with lines. Exons -3,-2,-1 are alternatively included as is the extended exon 3a. Exon 7 is where self-editing occurs. DRBM-1 and DRBM-2 refer to the two dsRBD-encoding regions. Figure is taken from Palladino *et.al*, 2000 (b).

1.2.2 *Drosophila Adar* mutant phenotypes

In 2000, Palladino et al. generated a series of *Adar* mutations on the X chromosome by imprecise excision of a P element and examined their phenotypes. The *Adar* null and hypomorphic flies show strong adult neural-behavioural defects including uncoordinated locomotion, temperature sensitive paralysis, seizures, and progressive neural degeneration evidenced by vacuole formation in the brain mushroom bodies (MB) (Palladino et al. 2000a). The *Adar* null male flies lost courtship behaviour completely (Palladino et al. 2000a). Despite all these defects, the originally characterized $Adar^{1F4}$ mutant flies are morphologically normal and not short-lived (Palladino et al. 2000a).

Several phenotypes of the *Adar* mutant flies might be expected based on the known edited target genes, even though loss of RNA editing in channel transcripts may not affect function as severely as null mutations in the same channels. For instance, *cac* mutants affecting the voltage-gated calcium channel a1 subunit exhibit temperature sensitive convulsions, uncoordination, and defects in male courtship song (Smith, Peixoto, Kramer, et al. 1998; Smith et al. 1996; Smith, Peixoto and Hall 1998). *para*

mutants affecting the voltage-gated sodium channel gene also have behavioural phenotypes like temperature sensitive-paralysis and cold-sensitive lethality (Loughney et al. 1989; Hanrahan et al. 2000). Mutations in the recently identified edited transcripts *Atp alpha* and *CG31116* cause neurodegeneration (Ryder et al. 2007). The neural behavioural defects of the *Adar* mutant flies would be expected to derive from malfunctioning of many membrane channel proteins and trafficking proteins. A study using RNAi to knock down *Adar* in different cell types showed that reducing *Adar* activity in discrete subsets of neuronal cells cannot phenocopy the pan-neuronal *Adar* knockdown, suggesting that normal locomotion requires pan-neuronal expression of *Adar* (Jepson & R A Reenan 2009).

1.2.3 *Drosophila* ADAR substrates

The number of known edited transcripts in *Drosophila* increased dramatically from initial serendipitous identification of a few edited transcripts, to the currently known set comprising transcripts 4% of all fly genes discovered in the ModENCODE project. By studying the developmental transcriptome using extensive RNA sequencing analyses, Graveley et al. identified 972 edited positions within transcripts of 597 genes (Graveley et al. 2011). The majority (64.8%) of the edited sites alter amino acids, while 20.7% of the edited sites are silent and the remaining 14.5% occur in untranslated regions (Graveley et al. 2011). Recently, Rodriguez and colleagues also found there is extensive editing in the introns of nascent RNAs by Nascent-seq (Rodriguez et al. 2012). They also discovered that the editing occurs mostly (93%) cotranscriptionally (Rodriguez et al. 2012). However, loss of editing did not affect levels of edited transcripts (Rodriguez et al. 2012).

Several important common features of the edited sites in *Drosophila* are found, based on the observations on the 972 ModENCODE edited sites of *Drosophila* mRNA. Firstly, exons containing editing sites are more highly conserved than unedited exons (Graveley et al. 2011; Hoopengardner et al. 2003; Jepson and Reenan 2007). Secondly, the

frequency of editing generally increases with development (Graveley et al. 2011). Graveley and coworkers found that editing often begins in the late pupal stages and many edited events are only observed in the adult stage (Graveley et al. 2011). Thirdly, three classes of potential editing-associated sequence motifs, named Motif A, B, and C, are predicted by computational analysis (Figure 1.6). Motif C is less abundant than Motif A or B, but is most strongly associated with the edited sites. The adenosine residue in the 3'end is the edited residue (Graveley et al. 2011). Interestingly, these three motifs are mostly 5' of the edited adenosine residue, whereas the ADAR dsRBDs are known to bind mainly 3' of the edited adenosine residue (Stefl et al. 2010).

Before the ModENCODE project identified near 600 edited transcripts, 55 edited transcripts were found by serendipity and by computational and comparative genomic approaches (Hoopengardner et al. 2003; Sixsmith and Reenan 2007). In fact, the edited transcripts are expressed in every tissue of *Drosophila* though the brain still has the most abundant edited transcripts. In addition, the functional categories of the edited transcripts span a wide range, including transporter activity, enzymatic activity and binding activities, based on the classification of molecular functions of encoded proteins (AmiGO analyses).

There are so many edited transcripts in *Drosophila* and many even have multiple sites in one transcript changing the amino acid sequences that it is almost impossible now to examine the physiological consequences of the editing at each edited transcript. Still, some extensive studies have been carried on to study effects of RNA editing on several ion channels including the ligand-gated $GABA_A$ receptor subunit *Rdl* and the voltage-gated potassium channel *Shab*.

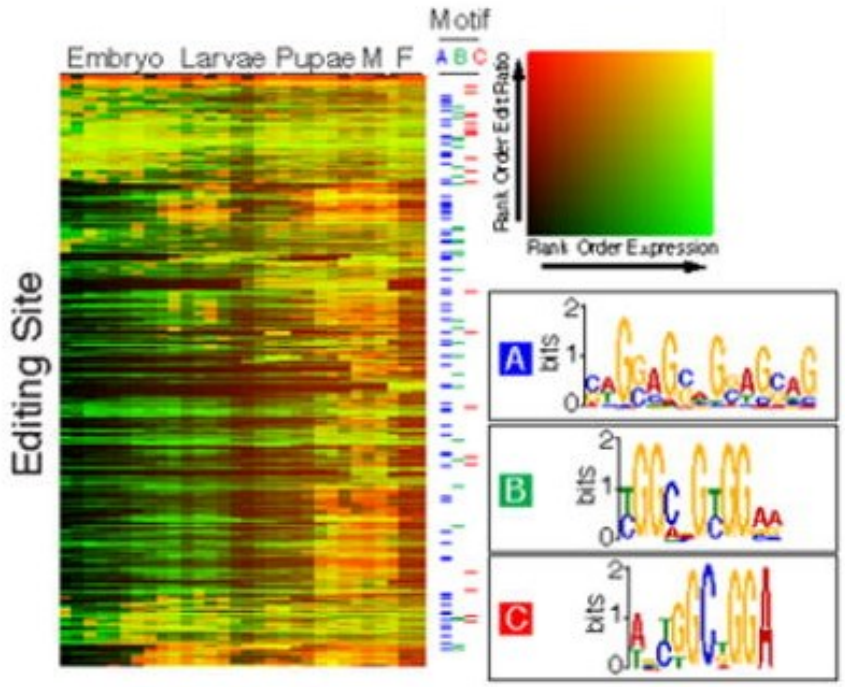


Figure 1.6 Potential common motifs of *Drosophila* ADAR editing sites. In the left box heat map, rows represent edited sites, ordered with ranks of expression levels (green) and editing ratios (red) at all developmental stages. Pictogram represents editing motifs A, B and C. Figure is taken from Graveley *et al.*, 2011.

RDL mediates fast synaptic inhibition through $GABA_A$ receptors and shares 30%-38% identity with vertebrate $GABA_A$ receptors (Hosie et al. 1997). RDL can form functional $GABA_A$ receptors as a homomer when expressed in *Xenopus larvis* eggs and also forms a heteromer with LCCh3. However, a study of pharmacological agonist and antagonist effects suggest that RDL likely forms a heteromer in flies (Lee et al. 2003). However, the physiological composition of $GABA_A$ receptor is not clear.

Jones and coworkers performed a detailed study to show that RNA editing, in combination with different splice variants, fine-tunes the GABA potency of RDL (Jones et al. 2009). *Rdl* transcripts are processed to produce four splicing isoforms by

alternative splicing events involving exon 3 (variants a and b) and exon 6 (variants c and d) (Buckingham et al. 2005). In the adult head, the bd variant is 26 times more abundant than the ad variant, and more than 300 times more abundant than the least abundant ac variant (Jones et al. 2009). Four editing sites in *Rdl* change amino acid residues; the RG site is in the N-terminal extracellular domain where ligand binds, the IV site is in the transmembrane domain, and the ND site and MV sites are in the intracellular domain (Figure 1.7) (Buckingham et al. 2005). The IV site in transmembrane helix I is consistently nearly 100% edited over all developmental stages in all the four different splice variants of *Rdl* (Jones et al. 2009). The editing levels at other sites are much lower, and increase generally with development (Jones et al. 2009; Graveley et al. 2011). Editing levels at these sites also differ between splice variants, especially in the bd variant in the adult where the RG and MV sites where have higher editing levels than in other splicing variants (Jones et al. 2009).

GABA potencies of the splicing isoforms and editing variants were measured using voltage-clamp analyses in *Xenopus* oocytes expressing the different variants (Table 1.2). Jones and colleagues examined the GABA EC_{50} (half maximal effective concentration) of different splicing isoforms with only the IV site fully edited and found that the ad variant is more sensitive to GABA than the bd variant (Jones et al. 2009). They also showed the effects of combinations of editing at different sites and splicing isoforms on the GABA EC_{50}. Among 16 different combinations tested, the editing of RG plus IV plus ND in the bd background showed the highest EC_{50} and the editing of IV plus ND in the ac background showed the lowest EC_{50} (Jones et al. 2009). Whereas, the fully edited variant had the highest $EC_{50,}$ approximately 7 times higher than the unedited variant in the ac background (Table 1.2) (Jones et al. 2009). The fully edited ac variant (the least sensitive ac variant) is still more sensitive than the most sensitive bd variant (Jones et al. 2009). All these observations lead to the conclusion that RNA editing in combination of alternative splicing has the potential to profoundly influence GABA-mediated inhibition.

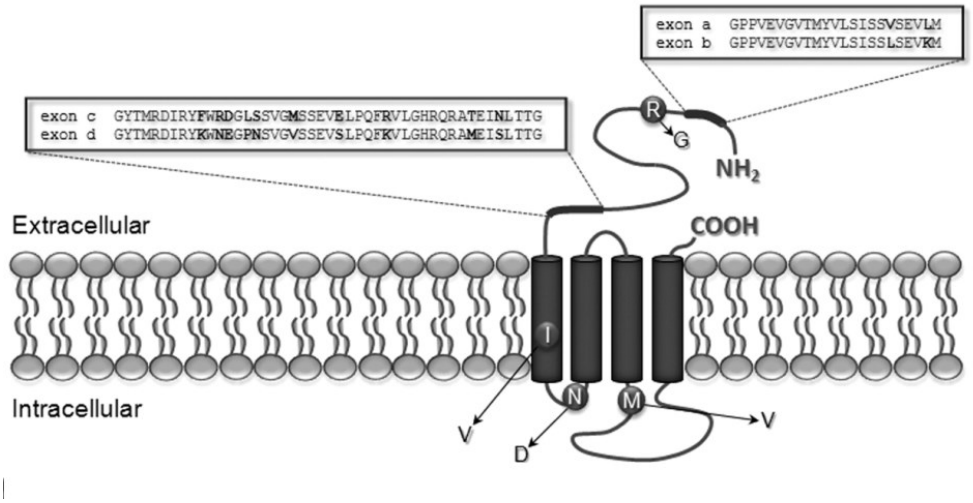


Figure 1.7 Schematic structure for RDL in the cell membrane. The alternatively spliced exons and four editing sites changing amino acid sequences and their locations are marked. Arrows indicate the amino acid changes caused by ADAR A-to-I changes. Figure is taken from Jones *et al.,* 2009.

Another detailed study was carried out on the SHAB voltage-gated K channel. Five highly edited sites in the *Shab* transcript were first discovered by comparing cDNA with genomic DNA sequences (Ryan et al. 2008) and the ModENCODE study later detected eight edited sites in Shab including two silent sites. Ryan and coworkers compared the electrophysiologies of singly unedited with the fully edited Shab isoforms in *Xenopus* oocytes. They found that the edited channel is less prone to open, thus enhancing the excitability of a neuron containing the edited channels (Ryan et al. 2008). However, the effects of loss of editing on the kinetics of channel gating seem to predict an opposite effect, because loss of editing slows both activation and deactivation of Shab. (Ryan et al. 2008).

Table 1.2 The GABA EC_{50} values for edited isoforms in combination with ac and bd splice forms of of RDL.

	ac			bd		
	EC_{50}, μM	SEM	n	EC_{50}, μM	SEM	n
R122G plus I283V plus N294D	4.2	1	4	192.9	57.3	4
R122G plus N294D plus M360V	8.5	0.8	4	182.8	19.2	4
R122G plus I283V plus M360V	24.9	12.3	5	143.5	13.4	4
Fully edited	15.4	3.3	5	118.6	32.2	7
M360V	20.6	4.2	5	95	36.1	3
N294D plus M360V	4.5	0.6	5	83.6	19.4	4
R122G plus M360V	11.3	2.5	5	81.8	8.4	5
I283V	4.7	1.9	5	72.6	36.5	4
N294D	11.1	4.3	5	66.9	14.7	4
R122G	18.5	4.5	3	65.6	7.4	4
Unedited	2.7	0.5	5	54	16.6	4
R122G plus I283V	5.3	1.6	5	53.2	13.8	5
R122G plus N294D	6.4	1.2	4	52.6	8.2	6
I283V plus M360V	4.9	1.7	4	52.3	12.2	3
I283V plus N294D plus M360V	3.7	0.4	5	42.3	14.4	5
I283V plus N294D	3.2	0.2	5	27.4	3.9	6

Overall, it is complicated to fully understand the functional consequence of A-to-I editing because the editing event is temporally and spatially regulated and generates complex combinations of isoforms (Graveley et al. 2011; Jones et al. 2009).

Recently, adenosine to guanosine conversions were also found in 18% of AGO-2 associated 21-mer small RNAs (Kawamura et al. 2008), suggesting that precursors of

this subset of endogenous small interfering RNAs (esiRNAs), which are primarily derived from transposons, are probably edited by *Drosophila* ADAR.

1.3 ADAR and innate immunity

1.3.1 Human ADAR1 and immune-mediated pathologies.

Recently, *ADAR1* mutations are documented in Aicardi-Goutières syndrome (AGS) (Rice et al. 2012). AGS is an immune-mediated neural-developmental disorder (Aicardi & Goutières 1984). Ten different missense mutations of ADAR1 were found in ten families with AGS, including a *Gly1007Arg* mutation that showed strong inhibition of the editing activity of ADAR1 (Rice et al. 2012). ADAR1 is known to prevent aberrant activation of interferon-stimulated genes (ISGs) (Hartner et al. 2009), suggesting that *ADAR1* mutations may contribute to the AGS disease pathology because the mutations are unable to turn off interferon induced immunity.

There are several hypotheses about how ADAR1 negatively regulate ISGs. One of the most compelling hypotheses is that multiple IU pair-dsRNAs derived from non-specific editing by ADAR1 inhibit ISG induction (Vitali and Scadden 2010). Solid evidence is shown by Vitali and Scadden that IU-dsRNAs specifically bind to MDA5 or RIG-1 and inhibit activation of IRF3 (IFN-regulatory factor) which is essential for induction of ISGs (Vitali & Scadden 2010). Their experiments were carried out by transfecting Hela cells with multiple IU-dsRNAs as well as Poly (I:C) that induces ISGs. MDA5 and RIG-1 are cytosolic dsRNA sensors for the immune system, which will be discussed in the following section 1.3.2.

However, there are many other possible mechanisms whereby ADAR1 inhibits the ISGs as the Vitali and Scadden experiments may not match normal physiology. For instance, ADAR1 deficient cells may produce immunoreactive dsRNA, or there are unidentified important transcripts in immune-regulation.

In both the *Adar1* knockout mouse cells and the *Adar* ^{5G1} null fly, our group also observed induction of innate immunity, indicating possible links between ADARs and

innate immunity. The possible mediator of this crosstalk between ADAR and innate immunity is dsRNA which can be a substrate of ADAR as well as a ligand for sensors that induce innate immunity. In this section, I will review the sensors for dsRNA in mammals, and the innate immunity in *Drosophila*.

1.3.2 dsRNA recognition by the innate immune system in mammals.

In mammals, it has been shown that TLR (Toll-like receptors) and RLR (RIG-1-like receptors) are the main sensors of the host defense against viral infections by recognizing dsRNAs. PKR (Protein kinase R) and the RNAi machinery may also serve as sensors for some viral RNA detections.

1.3.2.1 TLRs

The first TLR shown to in sensing viral nucleic acid was TLR3 (Alexopoulou et al. 2001). TLR3 binds dsRNA that is longer than 40 base pairs without a high degree of sequence specificity (Botos et al. 2009). The signal through TLR3 is mediated by TRIF (TIR domain-containing adapter including IFN-β) and ultimately induces expression of ISGs (Häcker et al. 2006; Oganesyan et al. 2006), whereas the other TLR proteins including the ssRNA-recognizing TLR7 and ssDNA-recognizing TLR9 signal via MyD88 (Kawai et al. 2004). Instead of localizing to the cell surface, TLR family proteins localize mostly to the ER (endoplasmic reticulumn) and the ER-resident protein Unc93b1 seems to control the exit of TLR from ER and multiple proteases are required for activation of TLR (Leifer et al. 2004; Barbalat et al. 2011).

1.3.2.2 RLR

RLR family proteins include RIG-1, MDA5 and LGP2. The full length RIG-1 and MDA5 both have CARD domains in their N terminus, a central DEAD box helicase/ATPase domain, and a C-terminal regulatory domain whereas LGP2 lacks CARD domains necessary for IRF3 activation (Yoneyama et al. 2005). IRF3 is the

transcription factor that activates the IFN-β promoter in response to viral dsRNA or Poly (I:C) infection (Yoneyama et al. 2004).

Little is known about how RLRs recognizes dsRNA, but there is evidence showing that the C-terminal domain of RIG-I, which is necessary to prevent constitutive activation of IRF3, binds to dsRNA (Cui et al. 2008; Takahasi et al. 2008). In recognizing synthetic dsRNA, it is shown that MDA5 preferentially recognizes long (>2 kb) Poly (I:C), whereas RIG-I recognizes smaller polymers (as short as 70 bp) (Kato et al. 2008). Whether LGP2 functions as a positive or negative regulator of the RIG-1/MDA5 pathway is still not clear with some contradictory experimental evidence. It is not known how RLR family proteins distinguish self and non-self RNAs. One hypotheses is that the host distinguishes its own dsRNA by Inosine residues introduced by ADAR (Yoneyama et al. 2005; Satoh et al. 2010).

Activation of IRF3 is mediated by MAVS (mitochondrial antiviral signaling, also known as CARDIF, IPS-1 or VISA) that is located in the mitochondrial outer membrane and this localization is necessary or MAVS to activate IRF3 (Seth et al. 2005). Interestingly, MAVS was also found to localize to peroxisomes, inducing anti-viral genes independent of type I IFN induction, which occurs more rapidly than the signals through IRF3 (Dixit et al. 2010).

1.3.2.3 PKR

PKR was shown to respond to multiple cellular stresses including viral infections. PKR has a dsRNA binding domain and it has been proposed that PKR binds viral dsRNA and activates itself to limit viral replication. Activated PKR phosphorylates the translation initiation factor eIF-2 to inhibit translation (Williams 1999). Study by Schulz *et al.* shows that PKR is required for production of type I interferon proteins in response to a subset of viral infections independent of its function of phosphorylating eIF-2 (Schulz et al. 2010). Therefore PKR is also a dsRNA sensor that activates ISGs but it is not clear how this PKR signaling action is activated by viral dsRNAs.

1.3.2.4 RNAi

Small RNAs associated with AGO proteins with viral siRNA features were cloned from mammalian cells infected with poliovirus and wNv (Parameswaran et al. 2010). Parameswaran et al. also found that the virus-derived small RNAs increased when type 1 interferon receptors are mutated, indicating possible crosstalk between the viral dsRNA induced RNAi and type I INF pathways (Parameswaran et al. 2010). Several mammalian viruses are shown to encode proteins with RNAi-suppressor activity, further indicating that RNAi may play a role in anti-viral defense in the mammals (Li and Ding 2006). However, it is still not clear whether virus derived small RNAs that show siRNA features mediate specific silencing of viral RNAs in mammals.

1.3.3 Defense against viruses in *Drosophila*

The main innate immunity pathway against virus in *Drosophila* is RNA interference pathway (Wang et al. 2006). The Toll and IMD pathways are also involved although it is still not clear whether they play a crucial role in anti-viral immunity (Dostert et al. 2005; Zambon et al. 2005). Also, the JAK-STAT signaling pathway is reported to participate in antiviral immunity (Dostert et al. 2005).

Several families of viruses are known to infect *Drosophila*. The study of viral infections started much later and is very limited in *Drosophila* compared with the identification of the systemic innate immune pathways against fungal and bacterial infections. Viruses (DCV as an example) have been shown to need clathrin-mediated endocytosis to enter the host cells both *in vitro* and *in vivo* (Cherry and Perrimon 2004). Mutations in the genes encoding components of clathrin-coated vesicles, incluing *αAdaptin*, *awd*, *chc* and *syt* showed either completely or significantly impaired resistance to the viral infections (Cherry and Perrimon 2004).

1.3.3.1 Two anti-viral activites of Dcr-2: RNAi and viruses sensing.

Viral dsRNAs trigger immune defenses against viral infections through RNA interference. Wang and coworkers demonstated that Dcr-2, Ago-2, and R2D2 are essential to silence a dsRNA virus (Wang et al. 2006). Based on the known siRNA pathway, it is clear that Dcr-2 cleaves the dsRNA into small interfering RNAs (siRNA) (Lee et al. 2004). Then R2D2 bridges the loading of Dcr-2-siRNA complex to RISC (RNA-induced silencing complex) by tightly biding to Dcr-2 (Liu et al. 2003). The core component of RISC is Ago-2 that is essential to guide siRNA to cleave or repress the translation of the target mRNA (Miyoshi et al. 2005).

Wang and coworkers demonstrated the essential roles of Dcr-2, Ago-2, and R2D2 by showing accumulation of the viral RNAs and reduced survival in each of these gene mutant flies (Wang et al. 2006). They also detected FHV (Flock house virus) siRNA accumulation after FHV injection in adult flies which is not observable when *dicer-2* or *r2d2* are mutated (Wang et al. 2006).

In the *dcr-2* or *r2d2* mutants, expression levels of AMP (anti-microbial peptide) genes were comparable with wild type flies after viral infections, indicating that induction of the Toll and IMD signal pathways are not compromised in the RNAi-deficient flies (Wang et al. 2006). Also, no alteration of the JAK/STAT responsive gene *vir-1* expression was detected in the *dcr-2* or *r2d2* mutants, suggesting that JAK/STAT pathway is independent of signaling from Dcr-2 anti-viral response (Wang et al. 2006).

A recent study showed that ATP-sensitive potassium channels (K_{ATP}) mediate resistance to FHV in the heart, in an RNAi-dependent manner (Eleftherianos et al. 2011), suggesting that ion channels also play a role in the anti-viral immunity. However, *Drosophila* cells infected persistently with FHV did not show siRNA-directed RNA silencing (Flynt et al. 2009). It is shown that most of the viral derived siRNAs did not bind to AGO2, which may explain the lack of RNAi in these viral infections and this effect may be signaling from Dcr-2 rather than through RNAi.

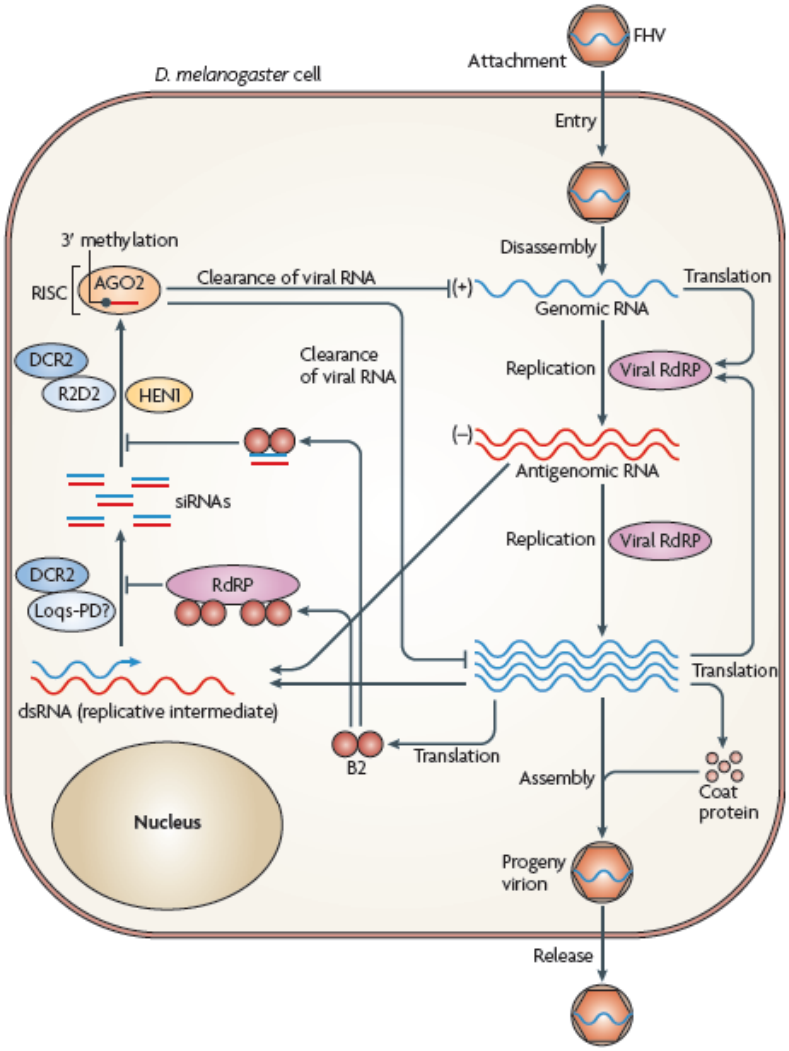


Figure 1.8 Key steps in the RNAi-mediated antiviral immunity by FHV infection in *Drosophila*. Following the entry of FHV virions, the genomic positive-strand (+) RNA is translated as well as being used for negative-strand (-) RNA synthesis by viral RNA-dependent RNA polymerase (RdRP). New (+)RNA is made from the (-)RNA and Dicer 2 (DCR2) recognizes the dsRNA formed between the 5' –terminal nascent progeny (+) RNA and the (-) RNA and triggers RNAi, guiding specific clearance of viral RNAs. The B2 protein, a vrial suppressor of RNAi encoded by FHV, inhibits the RNAi mediated antiviral immunity by inhibiting viral siRNA production and by sequestering viral siRNAs by binding to siRNAs. Loqs-PD, loquacious-isoform PD. Figure is taken from *Ding,* 2010.

1.3.3.2 The Toll pathway is important in immunity against viral infection

The involvement of the Toll and IMD pathways in virus immunity was found in the study of *Drosophila* X virus (DXV) infection. DXV is a member of the *Birnavirus* family and has an icosahedral nucleocapsid and bisegmented dsRNA genome (Zambon et al. 2005). Infection with DXV causes anoxia and eventually leads to death of the host (Teninges et al. 1979).

Infection with DXV induced expression of AMP genes to the similar levels as *Escherichia coli* infection which turns on both the Toll and IMD signalling pathways (Details of the pathways are reviewed in Section 1.3.4) (Zambon et al. 2005). Tsai *et al.* also detected upregulated expression of the peptidoglycan-recognition protein PGRP-SA and AMP genes including *Drosomycin-B*, *Metchnikowin* and *Defensin* in both DXV and DCV (*Drosophila* C virus) infection (Tsai et al. 2008). Sigma virus (SIGMAV), however, upregulates expression levels of different PGRP transcripts and AMP transcripts, including PGRPSB1, PGRP-SD*, Diptericin-A, Attacin-A, Attacin-B, Cecropin-A1*, and *Drosocin* (Tsai et al. 2008).

Intriguingly, ectopic expression of any single AMP genes does not enhance immunity against DXV in Toll or IMD deficient flies, suggesting that anti-viral defense occurs more at the cellular level than at the humoral (Zambon et al. 2005). Still, the Toll pathway deficient flies were much more susceptible to infection with DXV (Zambon et al. 2005). Zambon et al. hypothesized that the cellular debris released during apoptosis caused by the virus infection may turn on the Toll signal, similarly to cytokines released by hemocytes turning on the Toll signal in mammals (Zambon et al. 2005). In turn, the Toll signal may induce the proliferation of hemocytes which may attack the cells infected by the virus through sensing the aberrant apoptosis (Trudeau et al. 2001; Qiu et al. 1998; Basset et al. 2000).

1.3.3.3 The JAK/STAT pathway

Apart from inducing the expression of the Toll and IMD pathway genes, viral infections also triggered activation of the JAK/STAT pathway, inducing expression of *vir-1* and *TotM*, and *CG12780* (Details of the pathway are reviewed in Section 1.3.4) (Dostert et al. 2005). Genetic data suggest that the Jak kinase Hopscotch is required but not sufficient for controlling the viral load in the infected flies (Dostert et al. 2005). The effector of the JAK/STAT pathway, Vir-1, is specifically and substantially induced by viral infections, but not by fungal or bacterial infections nor by many general environmental stress (Dostert et al. 2005). In addition, *hop* (also known as JAK) mutant flies are more sensitive to DCV infection than wild-type flies, indicating that the JAK/STAT pathway is required for the anti-viral immunity.

1.3.4 Defense against bacteria and fungi: NF-κB pathway

1.3.4.1 Recognition of microbial infections

The main anti-bacterial and fungal defense is through the Toll and the IMD pathways that activate NF-κB (nuclear factor -κB) family proteins of *Drosophila*. The Toll signaling pathway activates two different NF-κB family tansactivators: DIF (dorsal-related immunity factor) and Relish, respectively, that lead to expression of different AMPs in the fat body which is equivalent to mammalian liver. In general, Toll receptors sense gram positive bacteria and fungi, whereas the IMD pathway is mainly activated by gram negative bacteria.

Recognition of microbial challenges mainly involves peptidoglycan-recognition proteins (PGRPs) which are pattern-recognition receptors (PRRs) that form complexes with bacterial cell wall components. The PGRP family members share the PGRP domain, and evolutionarily related to the bacteriophage type II amidases. Some of the PGRP family

proteins (known as recognition PGRPs) lost this catalytic activity and became microbial sensors (Kim et al. 2003).

Recognition sites of PGRPs are buried in the inner layer of the bacterial cell wall which is beneath the outer layer of Gram-positive bacteria or LPS (lipopolysaccharides) of Gram-negative bacteria (Gobert et al. 2003). This inner layer of bacteria is a layer of polymeric glycan chains formed by peptidoglycan (PGN) that is crossed linked by peptidic stems (Lugtenberg and Van Alphen 1983; Navarre and Schneewind 1999). Most gram-positive bacteria have a lysine residue in the third position of the PGN peptidic stem whereas Gram-negative and some Gram-positive bacilli have a mesodiaminopimelic acid (DAP) residue in the same position (Lugtenberg and Van Alphen 1983; Navarre and Schneewind 1999). Recognition PGRPs distinguish these differences and activate either Toll pathway or IMD pathway. PGRP-SA and PGRP-SD bind to Lys-type PGN, and activate the Toll pathway, and PGRP-LC and PGRP-LE bind to DAP-type PGN and activate the IMD pathway (Figure 1.8) (Royet et al. 2011).

Another important PRR that detects Gram-positive bacterial and fungal infections are Gram-negative binding proteins (GNBP, also known as β-glucan recognition proteins, βGRP) (Gobert et al. 2003; Lihui Wang et al. 2006). GNBP1 binds to a restricted range of Lys-type PGN, and cleaves polymeric Lys-type PGN chains *in vitro* (Wang et al. 2006). GNBP3 recombinant protein binds to β-(1,3)-glucans in the fungal cell wall, and is shown to be required for activation of the Toll pathway by alkali-treated preparations of fungal cell wall (Gottar et al. 2006).

Apart from the structural components of the microorganisms, certain pathogen virulence factors such as fungal proteases and some chitinases can also be detected by the *Drosophila* innate immune system. A fungal protease used by *Beauveria bassiana* to digest the host insect cuticle was shown to activate the Toll pathway (Ligoxygakis et al. 2002). The fungal protease cleaves the inactive *Drosophila* haemolymph zymogen Persephone at a defined position, into an active serine protease (Ligoxygakis et al. 2002).

Then, Persephone triggers the Toll pathway, as will be discussed in the Section on the Toll pathway signaling.

1.3.4.2 The Toll pathway

Both pathogen structural components recognized as as a non-self and virulence factors recognized as a signal of danger trigger the Toll pathway. PGRP-SA, PGRP-SD and GNBP1 sensing Lys-type PGN, GNBP3 sensing β-glucans, and CLIP-domain serine proteases (Persephone for instance) activated by fungal proteases leads to the activation of a cascade of proteases (Gottar et al. 2006). Recently, it has been found that Persephone, which was thought to be activated specifically in fungal infections is activated by virulence factors of Gram-positive bacteria as well (Chamy et al. 2008). Two more CLIP proteases, Grass and spirit, and two regulators Sphinx 1/2 and Spheroide, are found to play a role in the sequential activation of the cascade of proteases in the Toll pathway through a large-scale RNAi screen (Kambris et al. 2006). Grass was first shown to be only activated in Gram-positive bacterial infection when knocked down by approximately 60%. However, the study of an imprecise excision of *Grass* gene revealed that Grass is also activated in fungal infection, synergistically with Persephone (El Chamy et al. 2008). The cascade of proteases that is involved in the Toll pathway is not fully identified and the relationship between the identified proteases and the Toll pathway still need to be elucidated. The ultimate protease of the cascade is SPE (Spätzle processing enzyme). SPE cleaves the precursor of dimeric Spätzle, a cytokine that is structurally related to neurotorphins (Jang et al. 2006). The cleaved C-terminal of Spätzle (Spätzle C106) is released to bind to and activate Toll receptor through the conformational changes in the receptor (Hu et al. 2004; Weber et al. 2003).

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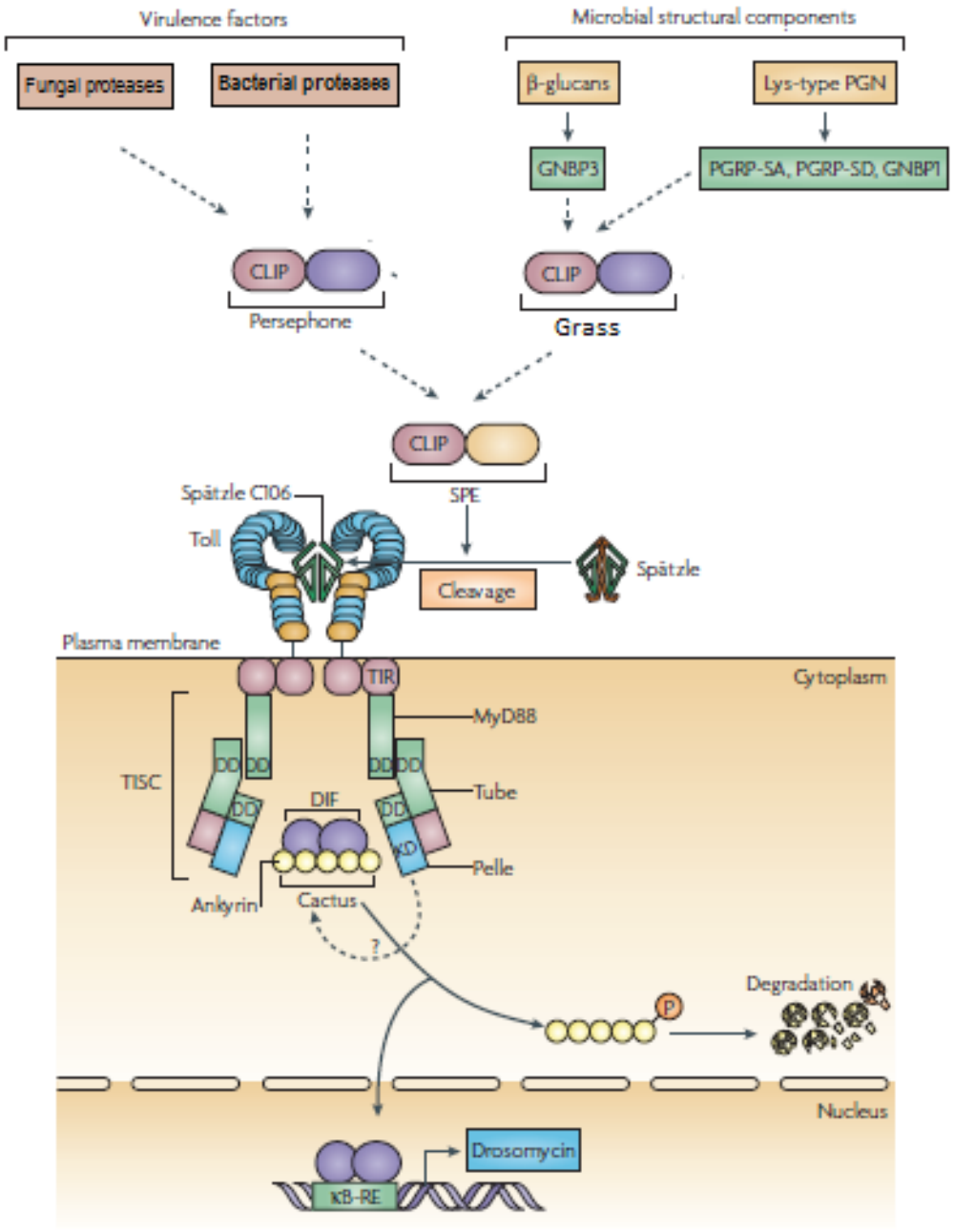


Figure 1.9 The Toll signaling pathway. Both virulence factors and cell wall components of most Gram-positive bacteria and fungi trigger the Toll signal pathway. The signal is transduced by pattern-recogntion proteins, a cascade of proteases, and eventually lead to cleavage of Spätzle by SPE, which in turn triggers assembly of TISC and releases DIF to translocate to nucleus. DIF turns on expressions of some AMP genes such as Drosomycin to kill the invaders. Figure adapted from *Ferrandon* et al., 2007.

Upon activation by Spätzle, the intracytoplasmic part of the Toll receptor (TIR domain) is assembled with the Toll-induced signaling complex (TISC) which is composed of the cytoplasmic adaptor MyD88 (myeloid differentiation primary-response gene 88), Tube and a serine-threonine kinase Pelle (Sun et al. 2004). All these three proteins have a death domain (DD) each (Sun et al. 2004). Tube, with its bivalent DD, mediates the assembly of the MyD88-Tube-Pelle complex. Activation of Pelle kinase activity following the assembly of the trimeric complex leads to phosphorylation and degradation of Cactus, a homologue of the mammalian inhibitor of NF-κB (Belvin et al. 1995). It is not clear how the signal leads to the phosphorylation of Cactus.

Removal of Cactus allows Dorsal (in the embryos, for development) or DIF (in the adult, for innate immunity) to be translocated to the nucleus and to bind to NF-κB response elements (κB-RE). In turn, genes encoding AMPs, such as *Drosomycin*, will be expressed. It is suggested that besides the degradation of Cactus, some post-translational modifications are likely needed for full activity of Dorsal or DIF (Ferrandon and Imler 2007)

1.3.4.3 The IMD pathway

To sense infection by Gram-negative bacteria, unexposed PGN of Gram-negative bacteria must become accessible to the PGRP. The current leading model for the Gram-negative bacteria detection has the following several phases. Firstly, short PGN fragments, such as TCT, are released during cell-wall remodeling of the Gram-negative bacteria when they grow or proliferate. These short PGN fragments are detected by PRGP-LCx—PGRP-LCa (Takehana et al. 2004; Kaneko et al. 2006; Mellroth et al. 2005), and this turns on the IMD pathway. As a consequence, AMPs and hemocytes attack the bacteria, leading to the release of large fragments of DAP-type PGN, which will be detected by membrane-bound PGRP-LCx receptors (Lim et al. 2006). PGRP-LF, which does not have a PGN-docking groove negatively-regulates the IMD signaling pathway through competitively binding to PGRP-LCx ectodomain (Basbous et al. 2011).

This model hypothesize that the immune system may sense proliferation but not the presence of bacteria (Ferrandon and Imler 2007).

PGRP-LC detects extracellular PGNs and PGRP-LE binds to intracellular PGNs. The N terminus of PGRP-LC and PGRP-LE both have motifs resembling the RIP homotypic interaction motif (RHIM) required to initiate IMD signaling (Kaneko et al. 2006), but PGRP and IMD interact through some unidentified adaptors (Kaneko et al. 2006).

IMD triggers the phosphorylation and cleavage of the NF-κB-like transcription factor Relish (Hedengren et al. 1999; Silverman et al. 2000; Stöven et al. 2000). The N-terminal DNA-binding REL domain translocates to the nucleus and bind to the promoters of *Cecropin A1* gene and other AMP genes (Stöven et al. 2000).

The phosphorylation of Relish initiated by IMD involves activation of the IKK (I-κB Kinase) signaling complex by the MAPKKK transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) and the TAK1-binding protein 2 (TAB2) (Vidal et al. 2001; Neal Silverman et al. 2003). The activation of TAK1 and the IKK complex is also suggested to involve K63-linked polyubiquitin conjugation. Genetic data suggest the involvement of the E2 ubiquitin enzyme. Bendless, the RING-finger containing protein DIAP2 as a potential E3 ligase, and *Drosophila* homologs of human ubiquitin-conjugating enzymes Ubc13 and UEV1a (Gesellchen et al. 2005; Kleino et al. 2005; Chen 2005; Zhou et al. 2005; Leulier et al. 2006).

Cleavage of Relish involves IMD to recruit FADD (FAS-associated death domain) and which in turn recruits the caspase-8 homologue DREDD (death-related ced-3/Nedd2-like protein) (Zhou et al. 2005). The cleavage of Relish by DREDD is independent of the proteosome (Wang et al. 2005). Although genetically separately regulated downstream of IMD activation, it is thought that the phosphorylation tags Relish for cleavage (Silverman et al. 2000). In addition, the ubiquitin-proteosome pathway and a *Drosophila* homologue of the FAS-associating factor 1, Caspar, negatively regulate the IMD pathway (Khush et al. 2002; Kim et al. 2006).

In addition, the IMD signal pathway activates the JNK (the JUN N-terminal kinase) pathway through the TAK1-TAB2 complex (Silverman et al. 2003). The physiological role of JNK signaling in the systemic host defense is still not clear.

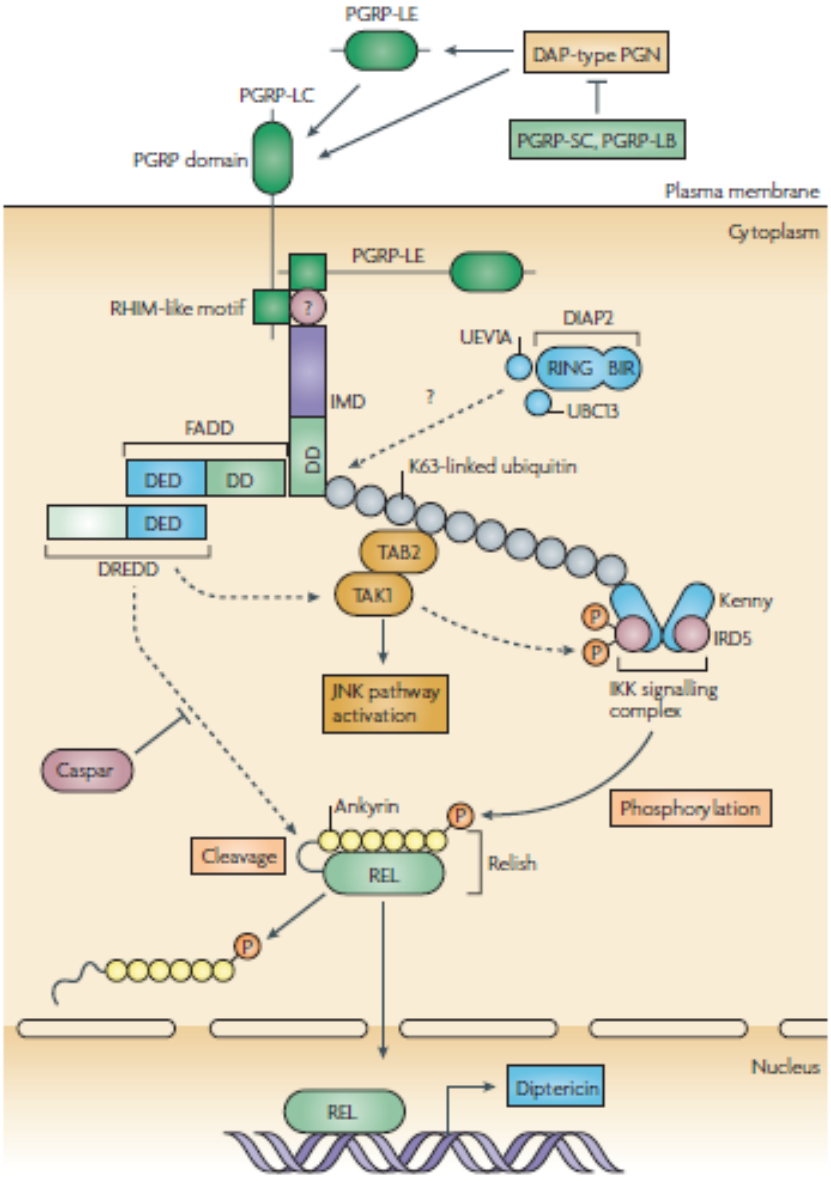


Figure 1.10 The IMD signaling pathway. DAP-type PGN from Gram-negative bacteria and some Gram-positive bacteria is recognized and triggers the IMD pathway.

Activation of IMD triggers downstream components including ubiquitin enzymes and negative regulators such as Caspar and eventually activates REL. REL turns on transcription of AMPs like *Diptericin*. In the process of the IMD signal activation, the JNK pathway is also activated. Figure is taken from *Ferrandon et al*., 2007.

1.3.4.4 Production of AMPs

AMPs play key roles in innate immunity against bacterial or fungal infections in both *Drosophila* and mammals (Zasloff 2002). They have a low molecular weight of below 5 kDa and a positive net charge at physiological pH, and most of them have conserved protein secondary structures (Bulet et al. 1999).

Activation of the Toll or IMD pathways both lead to expression of AMPs, and the expression level of the AMP genes reflect the degree of activation of the Toll or IMD pathways (Ferrandon and Imler 2007). Different AMPs have specialized activities against different types of microbial infections. For example, Defensin protects flies against Gram-positive bacteria but not Gram-negative bacteria or fungi, whereas Attacin or Drosomycin protect flies from Gram-negative bacteria or fungi (Lemaitre 1997). The Toll and IMD pathway seem to function synergistically as shown from the activation of both pathways in experimental challenges with various microbes (Tanji et al. 2007).

1.3.4.5 JAK/STAT pathway

During the early stages of the septic injury in mammals, IL-6, one of the cytokines released locally that induces systematic changes, activates the JAK/STAT signal pathway in the hepatocyte (Fattori et al. 1994; Kopf et al. 1994). This eventually leads to translocation of STAT dimers to the nucleus to turn on the transcription of genes that encode AMP proteins to attack bacteria (Alonzi et al. 2001; Li 2008).

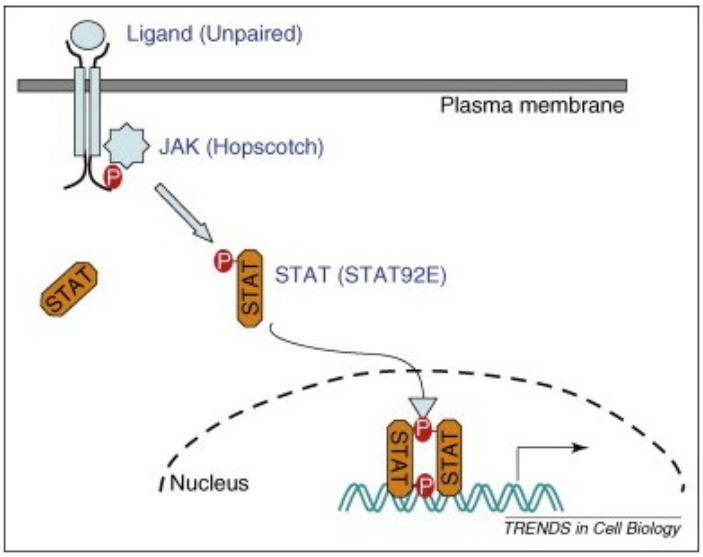


Figure 1.11 The canonical Drosophila JAK/STAT signaling pathway. Unpaired is the ligand for Dome, whose activation turns on JAK kinase Hopscotch and further activates STAT (STAT92E in Drosophila), and STAT turns on effector genes of JAK/STAT pathway. Figure is taken from *Li et al*., 2008.

Some evidence shows that the JAK/STAT pathway is also activated in septic injury in *Drosophila*. JAK/STAT plays an important role in many aspects of *Drosophila* development and stem cell maintenance (Arbouzova and Zeidler 2006). Upon infection by bacteria, expression of Upd3, a hemocyte-specific Upd family cytokine, is induced and binds to the Domeless receptor on fat body cells, which in turn activates the kinase Hopscotch. Hospscotch then induces the translocation of STAT to the nucleus where it turns on the expression of many proteins required for cell-survival. In mammals, the JAK/STAT pathway turns on the expression of anti-apoptotic B cell lymphoma -2 (Bcl-2) family proteins, and in *Drosophila*, it turns on the expression of Turandot family proteins like *TotA* and *TotM* (Agaisse et al. 2003; Li 2008). However, the biological role of this pathway in the immune response is not clear. Firstly, the gene expression induced by the JAK/STAT pathway is not infection-specific but induced by many other stresses. In addition, TotA, which is substantially induced, does not prevent the growth of the

bacteria (Agaisse et al. 2003). Secondly, the *hop* mutants that impairs the JAK/STAT pathway do not show any defects in immunity against bacteria (Agaisse et al. 2003).

1.3.4.6 Cellular immune responses independent of the Toll and IMD pathways.

GNBP3 was found to be required for survival of *Drosophila* after *C. albicans* fungal infection, independent of the Toll signal pathway, but by activating phenoloxidase (PO) enzymes (Matskevich et al. 2010). PO triggers several proteolytic cascades, one of which leads to melanization at the cuticular wound site of *Drosophila* after septic injury (Nappi and Vass 1993). During this catalytic cascade, reactive oxygen species (ROS) are produced, which is believed to attack the invaders (Nappi and Vass 1993). PO is also activated by the Toll pathway (Tang n.d.).

Some extracellular PGRPs also act as scavengers through enzymatic degradation of PGN (in the case of PGRP-SB) or as opsonins for phagocytosis (in the case of PGRP-SC). Also, PGRP-LEfl promotes autophagy to eliminate intracellular bacteria such as *Listeria monocytogenes* (Yano et al. 2008).

1.4 Thesis outline

There were two initial aims for my thesis project.

1. Identify genetic modifiers of *Drosophila Adar* overexpression phenotypes.
2. Find rescuers of neural-behavioural phenotypes of $Adar^{5G1}$ null flies, using the flies as a motor neuron disease model.

I started with heterozygous genetic deficiency screens to approach these aims. All the experimental and data analysis methods and materials are described in Chapter 2. Chapter 3 is the work to address the first aim, by conducting a genetic screen to find the rescuers of lethality caused by *Adar 3/4 S* overexpression. Chapter 4 is the study of $Adar^{5G1}$ null mutant flies. The main finding of the work described in this chapter is the induction of innate immune genes in the $Adar^{5G1}$ flies. Chapter 5 describes the experimental approaches to address the second aim and the findings from the heterozygous deficiency screen. At last, Chapter 6 summarizes the main findings of the thesis work and discusses potential physiological roles newly identified for *Drosophila* ADAR and future directions.

2 CHAPTER II: Methods

Two years' work wasted, I have been breeding those flies for all that time and I've got nothing out of it.

— Thomas Hunt Morgan

2.1 Fly methods

2.1.1 Fly maintenance and fly strains

Fly stocks were maintained at 18°C, on a 12 hour light/dark cycle. All the fly stocks were raised on standard corn meal agar media from the fly media kitchen in the Michael Swann Building at Kings Buildings, University of Edinburgh. For making general crosses, 12 to 16 virgin female flies were collected at 18°C and crossed with 5 to 8 male flies. The fly crosses were set up in standard food vials in the 25°C incubator. The parent flies were flipped to new food vials every two or three days, and their progeny were collected up to 15 days after the crossing date to avoid counting any second generation progeny.

Fly strains obtained from elsewhere or generated in our group prior to this thesis work are all listed in this Chapter. Table 2.1 lists the *Adar* mutant flies, wild type flies and different GAL4 driver lines. Descriptions of balancer chromosomes with their markers are listed in Table 2.2.

Table 2.1 *Adar* mutants, wild type controls and driver lines.

Fly strain symbols	Descriptions	Genotypes	References
Adar 3/4 dsRBD	N terminal dsRBD 1-234 AA.	*y, w^{1118}; UAS-3XFlag Adar 3/4 dsRBD/ TM3 Sb*	unpublished
Adar 3/4 EA	Inactive *Adar3/4*, E367A.	*w^{1118}; UAS-Flag Adar 3/4 E367A / TM3 Sb*	Keegan et al., 2005.
Adar 3/4	Unedited wild type *Adar 3/4* isoform.	*w^{1118}; UAS-Flag Adar 3/4 / TM3 Sb*	Keegan et al., 2005.

Adar3/4 S [ts]	Ineditable *Adar* with temperature-sensitive (ts) GAL80.	*w^{1118}; L/SM5 CyO; UAS-Adar 3/4S, UAS-GAL80$^{ts\ 10}$ /TM3 Sb*	Keegan et al., 2005.
Adar3/4 S OE[ts]	Ineditable *Adar* with *Act-GAL4*, and GAL80[ts]	*w^{1118};Actin 5c-GAL4/SM5 CyO; UAS-Adar 3/4S, UAS-GAL80$^{ts\ 10}$/ TM3 Sb*	Keegan et al., 2005.
Adar^{5G1}/ FM7	*Adar* null deletion	*y,Adar^{5G1},w^{1118}/FM7 B^1 g^4 sc ^{8} sn^{x2} v^{Of} w^a y^{31d}*	Palladino et al., 2000b
Adar^{5G1}/ FM7,GFP	*Adar* null with FM7,GFP balancer	*y,Adar^{5G1},w^{1118}/FM7c, P{GAL4-Kr.C}DC1, P{UAS-GFP.S65T}DC5*	/
Actin-5c-GAL4	Ubiqutious GAL4 driver	*P{Act5C-GAL4}25FO1*	Bloomington 4414
201Y-GAL4	Mushroom body gamma neuron GAL4 driver	*P{GawB}Tab2[201Y]*	Bloomington 4440
armadillo-GAL4	Ubiqutious GAL4 driver, in *arm*+ pattern	*P{GAL4-arm.S}11*	Bloomington 1560
Cg-GAL4	Hemocyte GAL4 driver, expression in larval fat body	*w^{1118}; P{Cg-GAL4.A}2*	Bloomington 7011
Cha-GAL4	Cholinergic neuron GAL4 driver	*P{Cha-GAL4.7.4}19B*	Bloomington 6793
Elav-GAL4	Pan-neuronal GAL4 driver	*P{GawB}elav[C155]*	Bloomington 5144
Mef2-GAL4	Muscle GAL4 driver	*P{GAL4-Mef2.R}3*	Bloomington 27390
OK6-GAL4	Motor neuron GAL4 driver	*P{GawB}OK6*	Kuppers-Munther et al., 2004
RRa-GAL4	Larval aCC motor neuron GAL4 driver	*P{eve-GAL4.RRa}*	Baines RA.
RRa-GFP	Larval aCC motor neuron GAL4 driver with GFP expression	*UAS-mCD8-GFP; RRA-GAL4*	Baines RA.

w^{1118}	Wild type	*w^{1118}*	/

Table 2.2 Chromosomal balancers and markers

Symbol	Linked balancer	Phenotype	Chromosome
FM6	*In(1)FM6, y[31d] sc[8] dm[1] B[1]*	White and Bar eye phenotype.	X
FM7a	*In(1)FM7, y[31d] sc[8] w[a] v[Of] B[1]*	Apricot and Bar eye phenotype.	X
CyO	*In(2LR)O, Cy[1] dp[lvI] pr[1] cn[2]*	Curly wings.	2
FM7a;CyO	*FM7a, l(1)TW24[1]/oc[1] ptg[3] l(1)TW1[cs]; CyO/l(2)DTS91[1]*	Apricot and Bar eye with Curly wings.	X, 2
TM3,Sb	*In(3LR)TM3, kni[ri-1] vvl[sep] p[p] l(3)89Aa[1] Ubx[bx-34e] e[1]*	Short and thick thoracic bristles.	3
TM6B, Tb	*In(3LR)TM6B, Antp[Hu] e[1]*	Small body size with short and crowded bristles on the shoulder.	3
FM7iGFP	*FM7i,p{ActGFP}JMR3/C(1)DX, y^1 f^1*	Bar eye, and green fluorescence in body.	X

2.1.2 Expressing transgenes using the *GAL4* driver system

Before the start of the project, the *$Adar^{5G1}$* mutant fly strains were combined with *Actin 5c-GAL4* or *Cha-GAL4*, each on Chromosome II. Also, our lab had already generated a series of *Drosophila UAS-Adar* and *UAS-Rdl* transgenic lines prior to the start of the project. These transgene constructs, designed by Dr. Liam Keegan, and microinjected

into *Drosophila* embryos are listed in Table 2.1. The scheme for combining *Adar*^{5G1} with new drivers, including *armadillo-GAL4* and *OK6-GAL4* is shown in Figure 2.1.

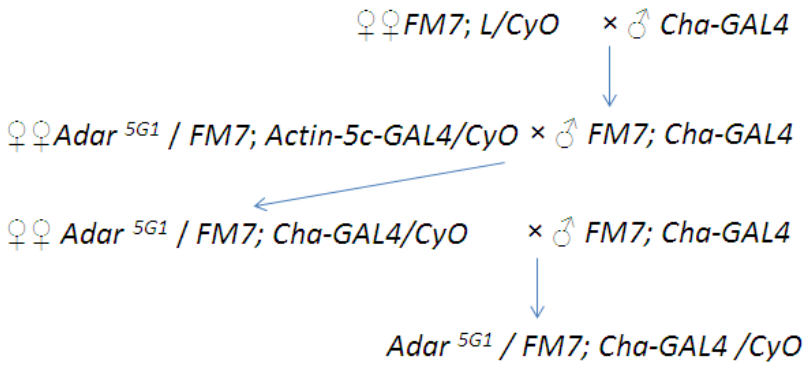


Figure 2.1 Scheme for combining *Adar*^{5G1} with *Cha-GAL4* or other drivers on Chromosome II. Firstly, male flies with the *Cha-GAL4* driver on Chromosome II were crossed with virgin female double balancer *FM7; L/CyO* flies, and male Bar eye flies were selected to cross with virgin *Adar*^{5G1}*/FM7; Actin-5c-GAL4/CyO* flies. Virgin *Adar*^{5G1}*/FM7; Cha-GAL4/CyO* flies were collected based on their eye and wing phenotypes and single-crossed to *FM7; Cha-GAL4* males to make stocks.

2.1.3 Generating flies for MARCM analysis

Adar^{5G1} was recombined with FRT19A using the y^{1},*w*^{1118},*P{neoFRT}19A* strain (Bloomington 1709), and the recombinant y^{1},*Adar*^{5G1},*w*^{1118},*P{neoFRT}19A* X chromosome was combined with specific GAL4 drivers. In this project, *Cha-GAL4, 201Y-GAL4, G01116-GAL4,* and *Collagen-GAL4* were used to generate cell clones with green fluorescence in cholinergic neurons, mushroom body neurons, projection neurons, and fat body cells in the larvae, respectively. Somatic clones were generated in progeny of crosses to the MARCM fly strain *P{neoFRT}19A, P{tubP-GAL80}LL1, P{hsFLP}1,*

w[*]; P{UAS-mCD8::GFP.L}LL5 (Bloomington 5134) to mark the *Adar*^{5G1} null single cells with GFP following loss of the GAL80 repressor in specific cell types. The scheme is shown in Figure 2.2, using *Cha-GAL4* as an example. The crossing scheme for combining *Adar*^{5G1}*, FRT19A* with the *GAL4* driver was the same as described in Figure 2.1.

The antibiotic G418 used was purchased as powder from Sigma (CAS#: 108321-42-2), and made up into a 5mg/ml solution in water. To select neomycin-resistant flies *(FRT19A* flies*)*, 0.2ml of the G418 solution was spread on top of approximately 10ml of fly food in each vial. Once the antibiotic was absorbed by the fly food, the parent flies were put into the vials to lay eggs for two days and then removed. Eggs laid by parent flies that do not have neomycin resistance were used as the negative control, to make sure no progeny was born from the neomycine containing vials. The fly lines determined to be *Adar*^{5G1}*, FRT 19A* were crossed with *FM7; L/CyO* males, and the F1 non-Bar eye virgin females with curly wings were crossed with *FM7; L/CyO* males again to make *Adar*^{5G1}*,FRT19A/FM7; L/CyO* fly line. This line was used to cross with different GAL4 driver lines of interest on Chromosome II.

After allowing *Adar*^{5G1}*, FRT19A/FM7; Driver-GAL4* females crossed with *FRT19A, tub-Gal80, hspFlp; UAS-mCD8:GFP* to lay eggs for twenty hours in fly food vials. The F1 embryos were immediately heat-shocked in a 37°C water bath for one hour. To detect neural degeneration and to examine neuron morphology, female round eye, non-curly wing adult flies were aged to 5 days, 30 days, or 60 days and their brains were dissected to be examined under the confocal microscope. To obtain MARCM clones in the fat body, fly eggs collected during six hours of egg-laying were heat-shocked for one hour, and female progeny flies with GFP positive cells were dissected at early third instar larvae stage.

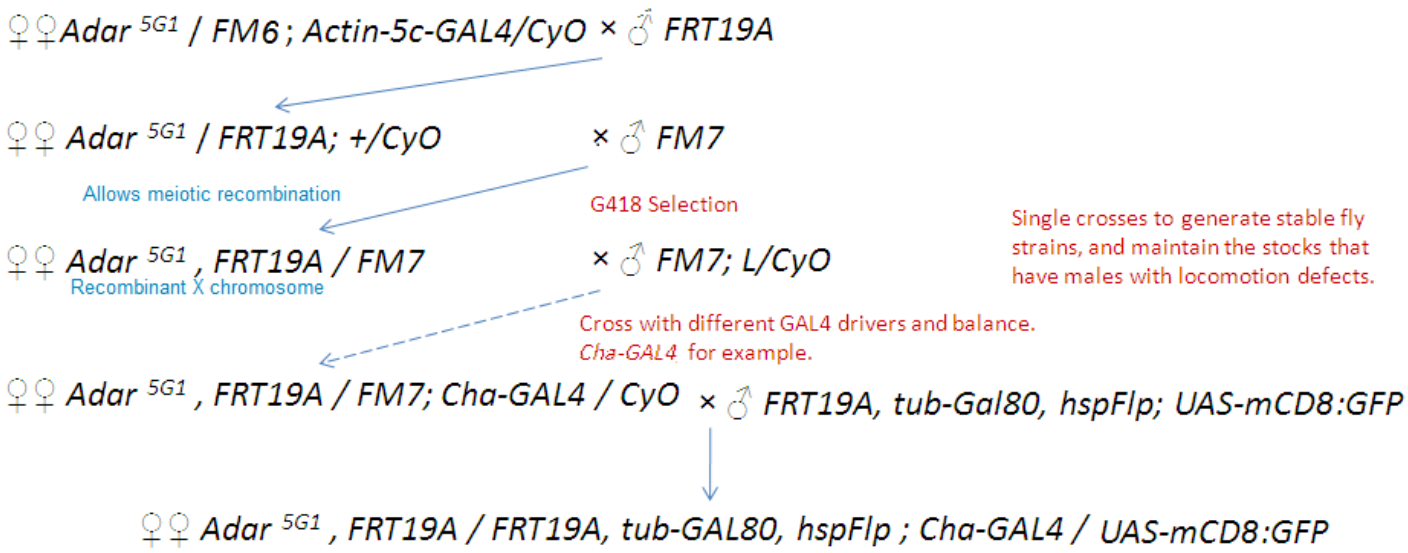

♀♀Adar 5G1 / FM6 ; Actin-5c-GAL4/CyO × ♂ FRT19A
♀♀ Adar 5G1 / FRT19A; +/CyO × ♂ FM7
Allows meiotic recombination
G418 Selection
♀♀ Adar 5G1 , FRT19A / FM7 × ♂ FM7; L/CyO
Recombinant X chromosome
Single crosses to generate stable fly strains, and maintain the stocks that have males with locomotion defects.
Cross with different GAL4 drivers and balance.
Cha-GAL4 for example.
♀♀ Adar 5G1 , FRT19A / FM7; Cha-GAL4 / CyO × ♂ FRT19A, tub-Gal80, hspFlp; UAS-mCD8:GFP
♀♀ Adar 5G1 , FRT19A / FRT19A, tub-GAL80, hspFlp ; Cha-GAL4 / UAS-mCD8:GFP
Heat shock at different developmental stages,
Observe the Adar 5G1 /Adar 5G1; Cha-GAL4/ UAS-mCD8:GFP clones in flies at different ages.

Figure 2.2 MARCM scheme for generating *Adar^{5G1}* clones. *Adar^{5G1}/FM6; Actin-5c-GAL4/CyO* virgin females were crossed with FRT19A male flies and the F1 virgin progeny that do not have the FM6 balancer but do have the CyO balancer were collected. These flies were then crossed with FM7 balancer flies in G418-containing vials. Female flies born from the crosses were then crossed with *FM7;L/CyO* double balancer male flies individually. In each cross, after one or two generations, the round eyed male flies from each line were examined for their locomotion to determine which line had recombinant X chromosomes. Only the fly lines having *Adar^{5G1}* recombined with FRT19A were retained and combined with the desired GAL4 drivers as shown in Figure 2.1. Virgin female *Adar^{5G1}*, *FRT19A* flies with GAL4 drivers on Chromosome II were crossed with *FRT19A* MARCM flies. The eggs or larvae from these crosses were heat-shocked to generate *Adar^{5G1}* null cell clones expressing membrane GFP within the tissue and cell populations determined by the choice of GAL4 drivers.

2.1.4 Screening scheme to identify deficiencies increasing *Adar^{5G1}* viability.

Viability of *Adar^{5G1}* relative to sibling *FM7* male flies carrying different deficiencies on Chr.III was counted by comparing the numbers of *Adar5G1;; Df* and *FM7;; Df* flies from the same vials (Figure 2.3). Most of the deficiency stocks were ordered from Bloomington Drosophila Stock Centre (BDSC) and some DrosDel strains were generous gifts from Dr. Guisy Pennetta's Group in the University of Edinburgh (The deficiency stocks used for the screen are listed in the Appendix, Supplementary table1). DrosDel collections are the newest deficiency collections, and are generated from an isogenic background with clearly defined break points (Ryder et al. 2007). Some Exelixis (Artavanis-Tsakonas 2004) and BSC (Bloomington Stock Centre) deficiencies (Cook et al. 2012) were also ordered from BDSC to either complete the coverage of particular regions or to narrow down locations of causative genes within large rescuing deficiencies of interest. In addition, single mutants in some specific genes within several rescuing deficiencies were tested for their effects on *Adar^{5G1}* null flies. Such mutants included shRNA stocks from the VDRC stock centre (Dietzl et al. 2007) or loss-of function mutants from BDSC. All these fly lines are listed in Table 2.3.

As shown in the Figure 2.3, the effect of each deficiency on *Adar*^{5G1} viability was calculated by comparing relative numbers of male flies born from the same crosses. This is necessary because many deficiencies affect viability and calculations of rescue effects must allow for reduced deletion viabilities.

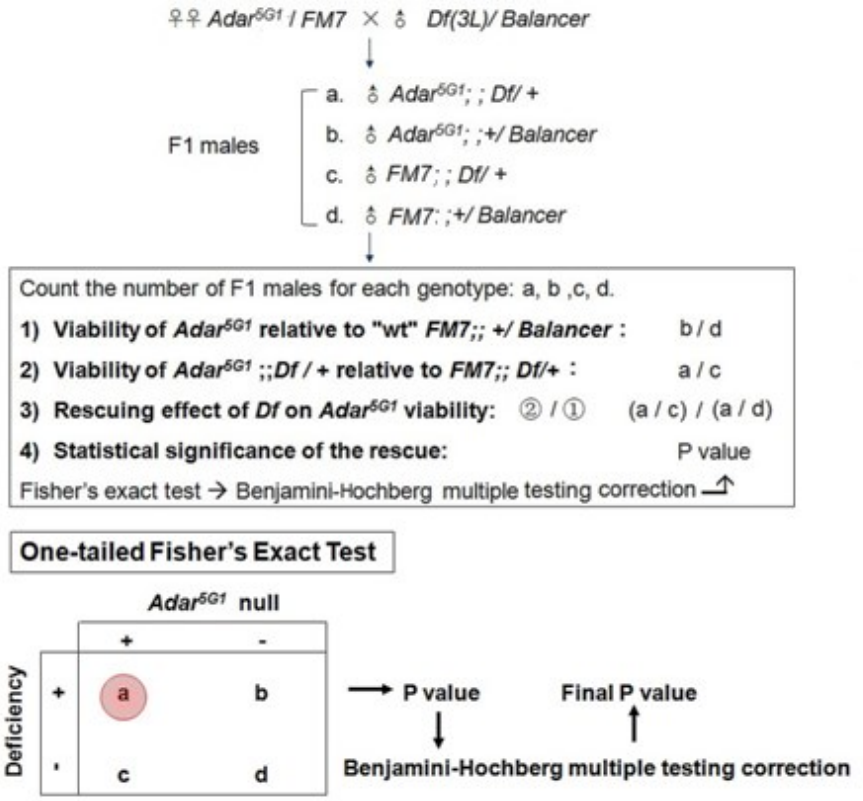


Figure 2.3 Calculating *Adar*^{5G1} viability in the heterozygous deficiency screen for rescue of *Adar*^{5G1} viability. *Adar*^{5G1}*/FM7* virgin females were crossed with the deficiency-bearing male flies, and the number of male progeny of each genotype was counted for viability calculation. The way of calculation is shown in the box. a,b,c,d in the box refer to the four progeny genotypes.

The p value for the null hypothesis that the Deficiency has no effect on *Adar*^{5G1} viability was calculated using Fisher's Exact Test followed by the Benjamini-Hochberg multiple testing corrections. In the 2X2 contingency table for the Fisher's Exact test [p= (a+b)! (c+d)! (a+c)! (b+d)! /a! b! c! d! n!], n is the total number of the progeny in each cross and the values of a,b,c,d were given to the numbers of *Adar*^{5G1}*;;Df/+* (Double mutant flies, *Adar*^{5G1} with heterozygous deficiency), *Adar*^{5G1}*;;Balancer* (*Adar* null) , *FM7;;Df/+* (heterozygous deficiency) , and *FM7;; Balancer/+* (wild type) each from the same crosses (Figure 2.3).

Table 2.3 Mutants and RNAi lines used in the screen

Mutants on ChrIII	BDSC Number	shRNA targets	VDRC number
akirin[EY08097]	20018	*Axin*	7748
Bre1[01640]	10066	*Bruno-3*	35525
capa[MB07374]	11565	*Capa*	41124
cas[j1C2]	11713	*Cas*	2928
CG11357[EY12484]	12070	*CG10089*	17991
CG31475[MB03509]	17736	*CG12091*	13985
CG5873[c00427]	20838	*CG14820*	15456
Cralbp[c05953]	27893	*CG32392*	34537
Cry[d10630]	9555	*CG7470*	38955
Cry[MB01493]	11541	*CG8564*	24127
dikar[KG00884]	26348	*Cnc*	51271
E(z)[731]	12116	*KO*	31266
Gem3[rL562]	24073	*Lqf*	35948
JIL-1[3]	19331	*Lsp-1 gamma*	50108
JIL-1[Scim]	26380	*mthl-8*	4071
neur[11]	13156	*Nwk*	21910
pum[13]	24470	*pak3*	39843
pum[Msc]	12079	*Rab11*	22198
Rab1[e01287]	14572	*Rab26*	43730
Rdl[1]	6374	*Rac2*	50349
Rdl[MD-RR]	2747	*Rdl*	100429
Rel[E20]	3254	*Smid*	35965
Rel[neo36]	2186	*Takr99D*	1372
S6k[07084]	17936	*Trio*	40137

scny[02331]	1687	*Trp*	1365
sec8[Delta1]	9457	*VPS16A*	23769
smid[C161]	10273	*VVL*	47182
smid[j6B8]	5692	*Adar*	7763
trp[1]	9046		
trp[9]	631		
Wrinkled[1]	1675		

2.1.5 Determining the lethal stage of *Adar 3/4S* OE.

To collect eggs, around 50 young females of *Adar 3/4S* OE and 30 males were put into an egg-laying chamber on a yeasted grape juice plate. After 6 hours, the parent flies were flipped out, and the number of embryos was counted. The second instar larvae number was counted after another 60 hours, and the number of pupae were counted on day 7 after the egg-laying. The number of eclosed adults was counted until day 13.

2.1.6 Determining effects of temperature on *Adar 3/4S* OE lethality.

The temperature of raising collected embryos was switched from 25°C to 29°C after day 1 or day 2 and so on up to day 8 and flies were shift to complete development at 29°C. Alternatively, the temperature was switched from 29°C to 25°C at different stages from day 1 till day 10.

2.1.7 Open filed locomotion assay

A 30mm petri dish, divided into nine equal areas with one central circle and 8 equal distance radiant lines, was used for the assay. Two-day old individual flies were put in the dish and the dish was tapped on the bench to make the fly start walking around. The number of lines crossed in a three minute period was recorded. For each fly line, six to ten individual flies were tested and each fly was tested three times in immediate

succession. The open field locomotion bar graph was plotted using the average number of line-crossings for each fly line. The assay was carried out at around 10-11 am.

2.1.8 Climbing assay

A column of 1.5 cm diameter and 20cm height cut from a 25ml plastic pipette was used for the assay. The height of the column was divided into 120 equally distanced lines, starting with 0 at the bottom. For each test, one 2-day old fly was put into the column. The highest line the fly climbed to in one minute was recorded. The scores were given from 0 to 120 depending on the highest point the flies reached in one minute and divided by 120 to calculate a climbing index for each score. For each genotype, six to ten individual flies were tested three times each to acquire an average score. A two-tailed Student t-test was carried out to calculate the p value compared with the *Adar* ^{5G1} null fly group. The climbing assay was carried out at room temperature, at around five o' clock in the afternoon.

2.1.9 Fly locomotion monitoring

The DAM2 *Drosophila* activity monitor (TriKinetics Inc, MA, US) was used at room temperature with a 12hr light/ dark cycle. With the data collection software set to bin collected data in 1 hour intervals, the monitor read how many times each fly broke the beam in the middle of the horizontally placed tubes. Four to eight flies for each genotype were monitored simultaneously for at least 24 hours. Data were acquired using DAMSystem software from the same company.

2.2 Molecular methods

2.2.1 Isolation of genomic DNA from *Drosophila*

Twenty to thirty anesthetized flies were homogenized in 200µl Solution A (Section 2.5) and an additional 200 µl Solution A was added and mixed. After 30 minutes' incubation at 65°C, 800 µl LiCl/KAc solution (Section 2.5) was added and tubes were left on ice for at least 10 minutes. The supernatant was then removed and the genomic DNA was precipitated using isopropanol followed by a 70% ethanol wash and finally suspended in 150 µl TE buffer (Section 2.5).

2.2.2 Isolation of RNA from *Drosophila*

RNA was extracted using the QIAGEN RNeasy kit based on the manufacturer's instructions. Ten to twenty flies were homogenized in 300µl RLT buffer with 30 µl Sigma concentrated stock of 2-Mercaptoethanol added. RNA was eluted in 30 µl of RNase free water with 1µl RNase inhibitor (RNasin® Ribonuclease Inhibitor, Promega, 20-40u/ µl) added and quantified using a Nanodrop. The quality of RNA was examined on a 2% agarose gel to check for intact rRNA bands to make sure that the RNA was not degraded. The RNA was stored in the -20 °C freezer for up to six months.

2.2.3 cDNA synthesis

500ng isolated RNA was used as the template to synthesize first strand cDNA in 20 µl reactions. Either oligo-dT primers or random primers were used with Superscript II Reverse Transcriptase (Promega). Reactions were performed according to the manufacturer's instructions.

2.2.4 Polymerase Chain Reaction (PCR)

2µl cDNA or 2µl plasmid DNA was added as the template to the reaction mixture. Fast start Taq polymerase from NEB was used as stated in the manufacturer's instructions.

2.2.5 Quantitative real-time PCR (qRT PCR)

cDNA made from 500ng RNA was used for quantification, with minus RT (reaction mix with no reverse transcriptase added when making cDNA) negative control and a water-only negative control. qRT PCR was performed using SYBER GREEN master mix, using either a BioRad (C1000^{TM} Thermal Cycler) instrument, or a Light Cycler® 480 (Roche). Prior to each comparison of gene expression, primers were tested for correlation factor and efficiency. All the qRT PCR results were normalized to *Gapdh* level and also to further standards. Error bars were added based on the standard error (Standard deviation divided by square roots of the number of repeats), and the p value was calculated using the unpaired student t-test. For each comparison, the cDNAs used were made at the same time using exactly the same protocol and starting with the same amount of RNA. The PCR primers used for Quantitative real-time PCR and for sequencing to determine editing levels at specific RNA editing sites are listed in Table 2.4.

Table 2.4 Primers for qRT PCR and for sequencing.

Gene name	Forward primer	Reverse primer
CG32243	GTGGAAACTGTGAGGGAGGA	GCCTCAAAATATCCGACGAA
CG11353	CATGAAACCCATTTGACACG	CCCAGCCAGTAGTTTTGACC
CG33777	ACTTCCTTGGATCCGGAGTT	TAATTCTCGACACGGGCTTT

CG42540	TGCGTACTCGCACATACGAT	CAGTAGCCTGGTCGAATGGT
mir4940	GCAACTTATCGATCGGGTGG	CGTGTCGTTGTATGTAAAATCGG
cg11357	GATCCCAATCTGATGCTCT	CAGTATTCCGGATAGAAACG
Adar	TGGACCTTCAGTGCAATCA	CCTCACCGGACTCGATTT
Def	GCTATCGCTTTTGCTCTGCT	GGTGTGGTTCCAGTTCCACT
AttD	AGTTTATGGAGCGGTCAACG	CGATCGGCTATGACTGTGAA
IM23	GCACGCAGATTGAGAATGAA	TAGGATTGGCCACCGACTAC
CecC	CATCAGTCGCTCAGTTTCCA	TTCCCAGTCCTTGAATGGTT
Drs	CTCCGTGAGAACCTTTTCCA	ACAGGTCTCGTTGTCCCAGA
AttC	TTGGGTGGATCACTCACATC	GCGTATGGGTTTTGGTCAGT
TotM	TTTATTTGAGCTGCCTTATGGT	TTTATTGGAATGGGTTGGAAAG
TotX	GCAGACAGGCAACAATTTGA	TATACCGGGTTCCGACTCTG
TotB	CACTTGCATTCCATTAAGTCC	TTGGAATAGGCCGAGCATAG
TotC	TACTATGCCTTGCCCTGCTC	CAGATTCCCTTTCCTCGTCA
TotA	TTCAGCGTTCCAAAAAGTCA	CGATACTCTCCCGTTCCTCA
Gapdh	ACGAGAGTAAAAGTGAAAAGACAGC	TCCGTTAATTCCGATCTTCG

Primers for site-specific editing	**Sequence**
sloND_F	GCGGGCATTATACATCTGCT
sloND_R	CGAGCAGAAAGAACACGAGA
sloSG_UTR_F1	GCCAATGTGCCCATGATAAC
sloSG_UTR_R1	TTGGGATGGACAAAATACACC
sloSG_R2	ATCAGCGTTAAGGCGTTTTG
sloUTR_F2	CGTACATTTGAACGATGGAGAA
cg33205F1	TGACCACTAACGACGCCATA

cg33205R1	CGCATCGTTTCCATTTCATT
cg33205R2	CGCATCGTTTCCATTTCAT
Rdl561F	TAAACATATCCGCTATTCTCGACTCC
Rdl960R	GGCGATCCATGGGGAAATATTGTAG
Rdl961F	AGCTGTGCCACATTGAAATCGAAAGC
Rdl1680R	TGTGGGCGTGGTGTCCATGCCCGTG
syt1381F	CGTTGAAGGAGAGGGCGGACAG
syt1860R	CCTTACTTCATGTTCTTCAGGATCTC
Ca alpha 1D	CGTTGATGGAGAGGGCGGACAG
Ca alpha 1D_2R	GCAATGTGAAACAGTGGCACCATGGC

2.2.6 Agarose gel electrophoresis

0.8% - 1% agarose gels were run in in 1× TBE buffer (Section 2.5) at 5v/cm. Ethidium Bromide was added to the melted gel (final concentration of 0.5µg/ml). 5× DNA loading buffer (QIAGEN) was added to samples. To quantify approximate yields of the samples, standards of known concentrations were run on the gel along with the samples and a DNA ladder (Invitrogen).

2.2.7 PCR product purification

When the PCR products were very clean, with a single strong band on each lane of the gel after electrophoresis, the remainder of the samples was purified using the QIAGEN PCR purification kit. Otherwise, PCR products were purified by the gel extraction method. After electrophoresis on the agarose gel, the PCR product bands were cut under

the UV light, and extracted from the gel using the QIAGEN gel extraction kit. All the steps followed the manufacturer's instruction.

2.2.8 pGEM-T® easy cloning

Purified PCR products were ligated with linearised pGEM-T easy vector following the Promega pGEM-T Easy Kit instructions. 5µl of the ligation mixture was transformed into competent *E.coli* (XL1 blue) cells (Chem Agilent Catalog #200249).

2.2.9 Bacterial transformations

100ng of plasmid or 5µl of the ligation mixture was incubated on ice for 30 minutes with 50µl XL1 blue cells. Then, the transformation mixture was heat-shocked at 42°C for 45 seconds, followed by a ten minute incubation on ice. 800 µl SOC medium (Section 2.5) was added to the mixture, and left shaking at 37°C for one hour. The transformed cells were plated on LB plates (Section 2.5) with appropriate antibiotics. The plates were then put in a 37°C incubator for 12-16 hours.

2.2.10 Plasmid DNA isolation

Single positive transformant colonies were picked from LB plates and shaken in 3ml LB medium (Section 2.5) for 12-16 hours to harvest enough cells for plasmid DNA isolation. The plasmids were extracted using the QIAGEN mini-prep kit following the manufacturer's instruction. The extracted plasmid DNA was quantified using the Nanodrop.

2.2.11 Sequencing

The Big Dye v3.1 (Applied Biosystems) sequencing kit was used following the manufacturer's instructions. The sequences were read on an AMI PRSM®3100 Genetic Analyser, and analysed using DNASTAR -Lasergene SeqMan software.

2.2.12 Editing level examination

cDNA made from total RNA was amplified using specific primers for the transcript regions of interest. Depending on the quantity and quality, the PCR product was either used directly for Sanger sequencing or cloned by ligation into pGEM-T easy vectors and transformation into competent cells for mini-preps. Editing levels of ADAR target sties were measured by comparing the heights of the Adenosine and Guanosine peaks ("Editing percentage"= "the height of Guanosine peak" / ("the height of Guanosine peak" + "the height of Adenosine peak") at the same position. For each comparison, three sequencing reactions were used to estimate an average editing level. In the second method of measuring editing level, 60-100 colonies for each genotype were picked from plated *E.coli* containing cloned RT PCR products. Each clone was sequenced individually using flanking T7 and SP6 primers and the number of clones containing Guanosine at the editing sites was divided by the total number of sequenced PCR clones to calculate the edited percentage.

2.3 Cell biology

2.3.1 Lysotracker Red staining of acidic lysosomes

The Lysotracker probe (LysoTracker® Red DND-99, Invitrogen, Cat. L-7528) was used to detect acidic organelles (lysosomes) in the cells. Lysotracker probes are widely used to assess autophagy in live cells. *Drosophila* early 3rd instar larval fat bodies and 2-day old adult midguts were dissected in cold PBS and then incubated with 100mM Lysotracker probe for 2 minutes. After three 2 minute washes with PBS, the tissues were fixed in 4% PFA (Section 2.5) for 2 minutes, followed by another three 2 minute washes with PBS. Fat body of at least ten early third instar fly larvae or ten adult male fly guts for each genotype were used for staining and quantification.

2.3.2 *Drosophila* adult CNS antibody staining

The anti-GFP antibody was used to enhance fluorescent signals from expression of GFP-fused transgene. Nc82 (DSHB, the University of Iowa) antibody was used to visualize neutrophil. Adult CNS was dissected in cold PBS and fixed with 4% formaldehyde (Section 2.5) at room temperature for 20 minutes. After three 20 minutes washes in 0.5% PBT, the tissue was blocked with 10% donkey serum in PBT for 1 hour at room temperature, and then incubated with primary antibody overnight at 4°C. GFP was detected using 1:250 rabbit anti-GFP (Invitrogen, Cat. A6455) and brain structure was detected using 1:40 mouse anti-nc82. The samples were washed with PBT three times for 20 minutes each after two quick washes with PBT. Secondary antibodies, Alexa-coupled donkey anti-mouse IgG and/or Alexa-coupled donkey anti-rabbit IgG, were added at 1:2000 dilution, and incubated at room temperature for 2 hours. The specimens were mounted in the VECTASHIELD® Mounting Medium with DAPI (Catalog No. H-1200) after another three 20 minute wash in PBT.

2.3.3 Imaging

The mounted specimens were viewed with a Nikon TiE-C1Si Confocal Microscope. NIS- Elements AR 4.0 software was used for acquiring images, which were modified using FIJI software. ×20, ×40, ×63 and ×100 objective lenses were used to take pictures.

2.4 Extracellular current recordings of *Drosophila* third instar larvae aCC motor neurons

2.4.1 Sample preparation

Intact brains from wandering third instar larvae, with imaginal discs and peripheral nerves attached were dissected in a Petri dish filled with external saline (Section 2.5). A brain was positioned in a drop of external saline on top of the pre-made sylgard (SYLGARD® 184 Silicone elastomer kit) slip. The specimen CNS was placed with the dorsal part facing up, immobilized by gluing the peripheral nerves on the sylgard and recorded immediately.

2.4.2 Clearing the neuron surroundings for recording

An enzyme pipette filled with 1mg/100µl type 41 protease (XIV Bacterial, from *Streptomyces grisens*) diluted in the external saline was used to make a hole on or near the midline of the CNS membrane. Then glia cells were removed carefully from one or two GFP positive neurons without damaging any synapses or cell bodies of the neurons with the enzyme pipette. Once the neurons were free from attached glia, the enzyme pipette was removed, and a patch pipette filled with external saline was connected to the electrode for current recording.

2.4.3 Recording the firing activity

The current changes on the membrane of a neuron were recorded for five minutes using the patch pipette, in a half sealed state. The current signal was recorded using the Integrating Patch Clamp (INTRA CEL. Axopatch 200B.) connected with the Patch

Axon Instrument (CV 203BU HEAPSTAGE US. Pat. 5,285, 012). The data were collected using Clampex® software.

2.4.4 Data analysis

Clampfit 9 software was used for the analysis. For each genotype, four or five neuronal extracellular current records were used. The analysis used three minute recording shortly after the second minute of recording data. In cases of baseline fluctuation, the baseline was manually adjusted before the software automatic threshold search. Counting the number of peaks as firing events was done automatically. A burst was defined at least four events in a row within a delimitation interval of 25ms. P values between different groups were calculated using a two- tailed student T-test. The Hazard graph shows the probability of having a firing event after the previous events. Hazard was calculated for each recording of a single neuron and the final graph was made using the mean of neurons of the same genotypes. The hazard graph was made on Excel using the data that have had basic statistics done in the Clampfit system. 5ms bin size was given for each recording and the number of the events in each bin was counted as BinCount. HazCount is the sum of the previous BinCounts ($HazCount_i = \sum BinCount_{i-1}$). Hazard= Bincount/ ($\sum$ BinCount – HazCount) . Error bar shows standard error.

2.5 Materials and preparations

Acidic alcohol 1% HCl 70% ethanol	**Carnoy's fixative** 900µl 100% ethanol 450µl chloroform 150µl acetic acid	**Eosin** Stock: 5g Eosin 100ml H_2O Working: 20ml eosin stock 80ml H_2O
PBT 1× PBS 0.5% Triton X100	**LiCl/KAc solution** 1 part 5M KAc 2.5 parts 6M LiCl	**Litium carbonate** 1g Lithium carbonate 100g Distilled water
Luria Broth (LB) 10g NaCl 10g Bacto-tryptone 5g Yeast extract Add ddH_2O to 1 litre	**LB agar** 10g NaCl 10g Bacto-tryptone 5g Yeast extract 15g Difco Agar Add ddH_2O to 1 litre	**4%Paraformaldehyde (PFA)** 1ml 37% Formaldeheyde 8.25ml ddH_2O
PBS 137mM NaCl 2.7mM KCl 10mM Na_2HPO_4 2mM KH_2PO_4	**1× External saline (Bath saline)** 7.9g 135mM NaCl 0.37g 5mM KCl 0.81g $MgCl_2.6H_2O$ 0.29g 2mM $CaCl_2.2H_2O$ 1.15g 5mM TES 12.32g 36mM sucrose Add water to 1 litre Add 5M NaOH to pH=7.15	**SOC medium** 20g Tryptone 5g Yeast Extract 2ml 5M NaCl 2.5ml 1M KCl 10ml 1M $MgCl_2$ 10ml 1M $MgSO_4$ 20ml 1M glucose Add to 1L ddH_2O
Solution A 100mM Tris-HCl, pH 7.5 100mM EDAT 100mM NaCl 0.5% SDS	**TBE (10X)** 108g Tris base 55g Boric acid 9.3g EDTA Add ddH_2O to 1 litre	**TE buffer** 10mM Tris-Cl, pH 7.5 1mM EDTA

3 CHAPTER III: Genetic screen for heterozygous deficiencies on Chromosome III that rescue lethality associated with *Adar3/4S* overexpression.

No amount of experimentation can ever prove me right; a single experiment can prove me wrong.

— Albert Einstein

3.1 Introduction

Prior to the commencement of this project, *Drosophila* transgenic lines that allow expression of different *Adar* cDNA constructs had already been made in our group. These cDNA constructs include expressing the naturally occurring ADAR unedited transcript that is capable of being edited by ADAR (*Adar 3/4*), the ADAR edited isoform (*Adar 3/4 G*), *Adar* that is unable to undergo self-editing (*Adar 3/4 S*), and inactive ADAR (*Adar 3/4 EA*). No noticeable morphological phenotypes were detected in the flies overexpressing the *Adar* constructs, except that the overexpression of *Adar3/4S* by the ubiquitously expressed *Actin 5c-GAL4* driver results in lethality in the adult fly (Keegan et al. 2005).

The ineditable *Adar 3/4 S* construct has a point mutation in the cDNA so that the self-editing (S/G) site in *Adar* exon 7 is mutated to another codon for serine 'TCT', that cannot be edited (Figure 3.1A). Whereas the wild type cDNA construct produces both serine and glycine isoforms, the cDNA construct (*Adar 3/4 S*) only produces the serine isoform. ADAR 3/4S protein has the highest editing efficiency for other transcripts (Keegan et al. 2005).

Adar 3/4 S was combined with *Actin 5c-GAL4*, and temperature sensitive *UAS-GAL80*, so that the flies express the *Adar 3/4 S* construct only at high temperature when GAL80 is inactivated (Figure3.1B). A 'Protein intron', known as an *intein*, was inserted into *GAL80* to make this temperature-sensitive GAL80. The *intein* autonomously splices itself out post-translationally at the permissive temperature (18°C), leaving the intact GAL80 protein. But at the restrictive high temperature, the *intein* stays in the *GAL80* transcript to make the protein nonfunctional or to promote degradation. At the permissive low temperature, $GAL80^{ts}$ inhibits transcription activation by binding to and inhibit *GAL4*, while at a higher temperature (29°C), $GAL80^{ts}$ loses its ability. The *Adar3/4S* OE [ts] is viable at 18°C which is partially permissive, and is lethal at 29°C.

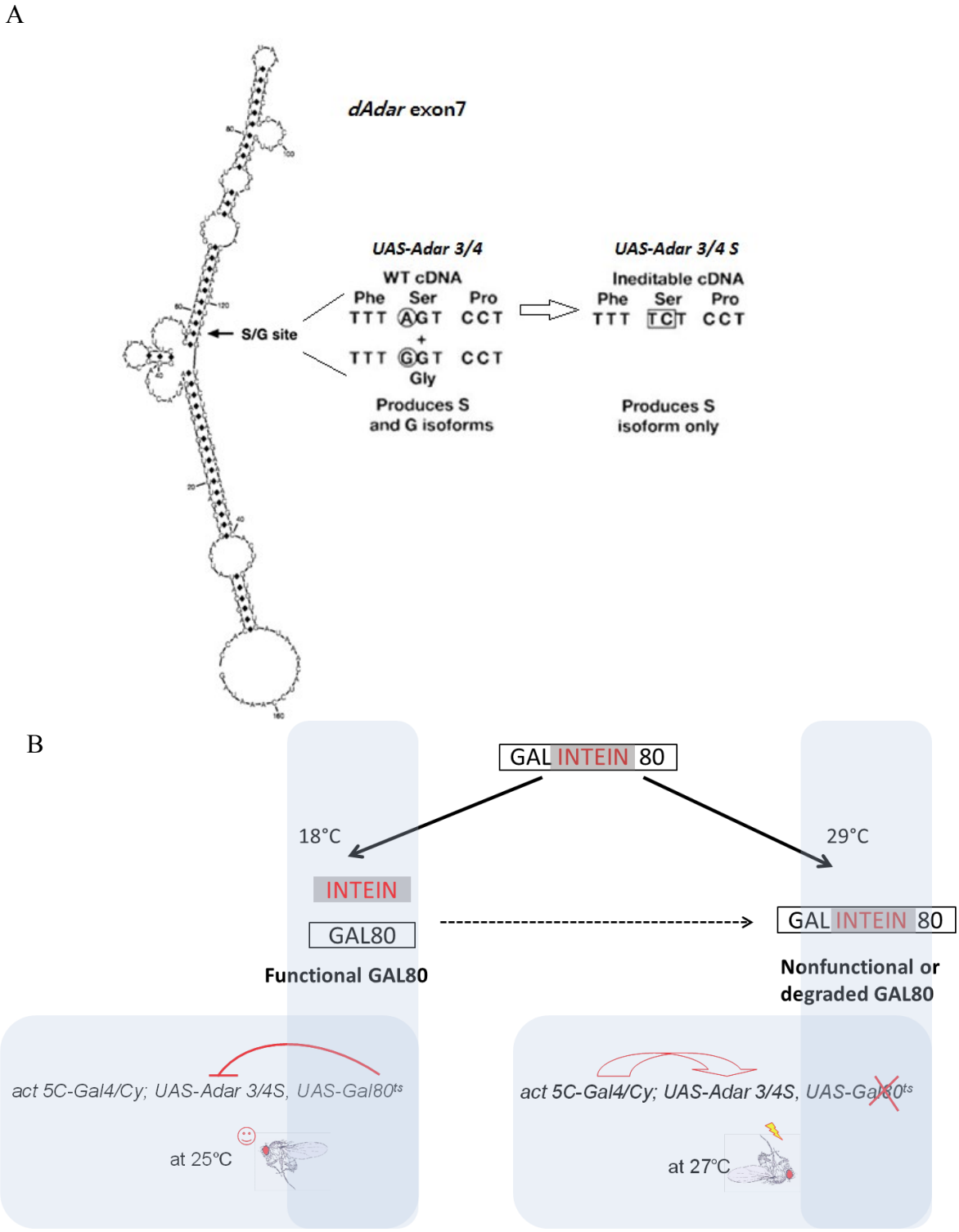


Figure 3.1 The ineditable *Adar 3/4 S* isoform and a diagram of the temperature-sensitive construct. (A) The arrow indicates the self-editing (S/G) site in the predicted

secondary structure of Exon 7 of the *Drosophila Adar*. Figure adapted from Keegan *et al.*, 2005. (B) Temperature-sensitive expression of the *Adar 3/4 S* isoform and the genetic screen utilizing the lethality scheme. 18°C and 29°C were the two extreme permissive and restrictive temperatures that were tested. *Act 5C-Gal4/Cy; UAS-Adar 3/4S, UAS-Gal80^{ts-10}* is the genotype of *Adar3/4S* OE [ts]. 25°C and 27°C conditions used in our experiments cover the most sensitive part of the range from permissive to restrictive temperatures.

The lethality caused by *Adar 3/4S* overexpression is likely due to the hyper-editing activity of this *Adar* isoform, since the flies overexpressing the inactive *Adar EA* driven by the same driver *Actin 5c-GAL4* was not lethal. The lethality caused by *Adar 3/4 S* is rescued by *Adar* RNAi construct. However, many aspects of the lethality caused by *Adar3/4S* OE were not clear, including the cause of the lethality and how the lethality occurs. Also not clear was whether there is a crucial developmental stage or tissue in which the lethality occurs. Is the lethality due to one or several abnormally edited transcripts or to some other unknown stress? This chapter describes experiments performed to address these questions, and to investigate the regulation of ADAR and RNA editing in *Drosophila*.

A deficiency genetic screen was designed to identify genes that rescue the lethality caused by *Adar 3/4S* ectopic overexpression. This screen aimed to find genetic modifiers that either affect the level or the activity of ADAR 3/4S or that play an important role downstream of ADAR protein in the normal physiological functions of the animal. The hypothesis is that deleted gene(s) that rescue the lethality will be either positive regulators of *Adar* or genes in a parallel pathway such that reduced copy number can re-establish homeostasis in the *Adar 3/4S* overexpressing flies. The less direct effect might be similar to the rescue of the *Adar^{5G1}* null mutant phenotypes by increased autophagy (Paro Thesis, University of Edinburgh, 2012).There is also a possibility that reducing

dosage of a gene whose transcript is hyper-edited and causing the lethality , would rescue the lethality due to *Adar 3/4S* overexpression.

3.2 Results

3.2.1 Overexpression of *Adar3/4S* is lethal.

The *Adar3/4S* OE [ts] *(w / w; Actin 5C-GAL4/Cy; UAS-Adar 3/4S, UAS-Gal80ts/ TM3,Sb*) flies were maintained at 18°C. To understand when and how they die, embryos and larvae at different developmental stages were counted and their percentage viability calculated at 25°C and 27°C. At 25°C, the flies were viable while at 27°C or at higher temperature, no flies survived to adulthood (Figure 3.2). w^{1118} wild type flies raised at 27°C were used as controls and raising the temperature to 27°C had little effect on the viability. At 25°C, temperature-sensitive GAL80 is not completely inactivated (Zeidler et al. 2004), therefore there is a low over-expression of *Adar* in the *Adar 3/4S* OE [ts] strain. At 27°C, the *Adar* OE [ts] flies were not viable, and the loss of viability was distributed over all developmental stages with dramatic reduction even in the number of hatched first instar larvae from the embryos (not shown).

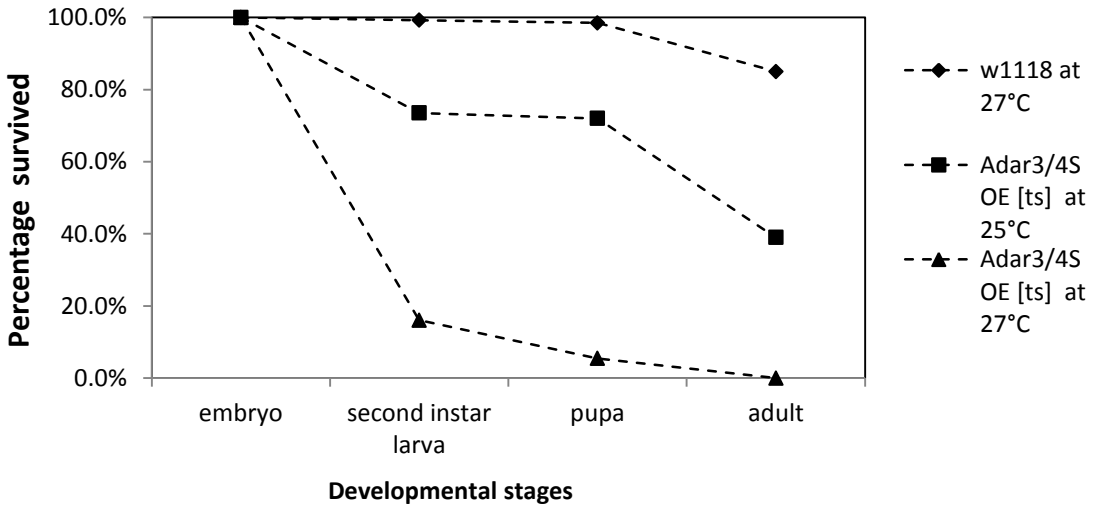


Figure 3.2 Survival of *Adar 3/4S* OE through developmental stages. Eggs were counted at the beginning and thereafter, the number of the live second instar larvae, pupae and adults were counted. w^{1118} wild type were flies raised at 27°C (n=400) and *Adar 3/4S* OE[ts] were raised at 25°C (n=200) and at 27°C (n=772).

In order to examine whether there is a crucial developmental stage when *Adar3/4 S* overexpression is lethal, the temperature was switched from 25°C to 27°C or from 27°C to 25°C at certain days, as described in Chapter 2. When the eggs/larvae were moved from 25°C to 27°C, no flies survived to adulthood regardless of the time of the switch in temperature. Intriguingly, the pupae, formed after seven days at 25°C, were still not able to develop further once placed at 29°C. This indicates that the expression level of *Adar* is crucial also in the pupal stage. When the eggs/larvae were raised at 27°C first and switched to 25°C, all died except when eggs and larvae were moved to 25°C after just 1 day at 27°C. This may be due to the fact that *Adar 3/4 S* is only approximately 1.5 X overexpressed on average at 27°C compared to 25°C during the embryonic stage while it is approximately 7 X overexpressed during the larval stage (Figure 3.3).

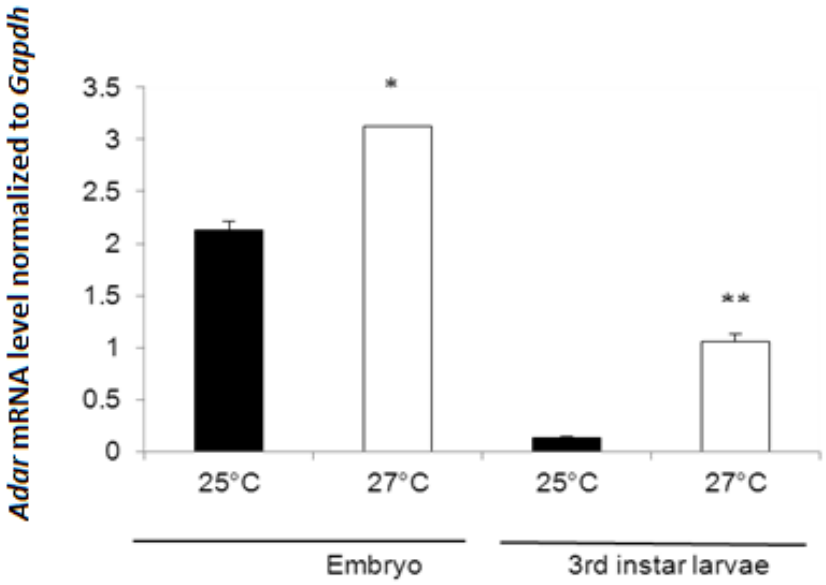


Figure 3.3 Relative *Adar* mRNA levels in *Adar 3/4 S* OE [ts] whole embryos and 3rd instar larvae at 25°C and at 27°C. Three independent replicates were performed for qRT PCR. The bar represents the mean value after normalizing to *Gapdh*. Error bars are standard error. Student t-test was used to calculate p value. * $p<0.5$, ** $p<0.05$. The black bar represents relative mRNA level from flies raised at 25°C and the white bar represents relative mRNA level from flies raised at 27°C. RT-PCR primers cover the *Adar* coding region and detect both endogenous *Adar* transcript and transcript expressed from the *Adar* cDNA construct.

Although *Adar 3/4 S* is overexpressed significantly in L3 larvae, some but not all sites that are edited by ADAR have increased editing levels. The *Ca alpha 1D* transcript encoding a well-studied muscle and CNS expressed voltage-gated calcium channel subunit did not show any increase in editing (Figure 3.4A). Small increases in the editing level are seen at one site out of the ten sites in *CG33205* (4.11% to 6.82%, no editing was detected in the other 9 sites in the L3 larvae), the S/G site in *slo* (45% to 60%), and the R/G site in *Rdl* (6.5% to 10.5%) (Figure 3.4B-D). The *CG33205* transcript was selected to investigate the editing levels because this newly identified edited

transcript has ten edited sites in a short (200bp) range of the 3'UTR that show a broad range of editing levels (Graveley et al. 2011). In the embryo, even though the *Adar 3/4 S* was not so significantly overexpressed, two edited sites in *Rdl,* the R/G site and the I/V site, had almost doubled the level of editing (Figure 3.4E). However, those editing levels are still very low. From the data here, it seems that the editing levels at sites in transcripts are not greatly increased by the lethal *Adar 3/4 S* overexpression.

Overexpressing *Adar* using the pan-neuronal *Elav-GAL4* driver, the muscle *Mef-GAL4* driver, or another weaker universal driver such as *arm-GAL4* did not lead to complete lethality.

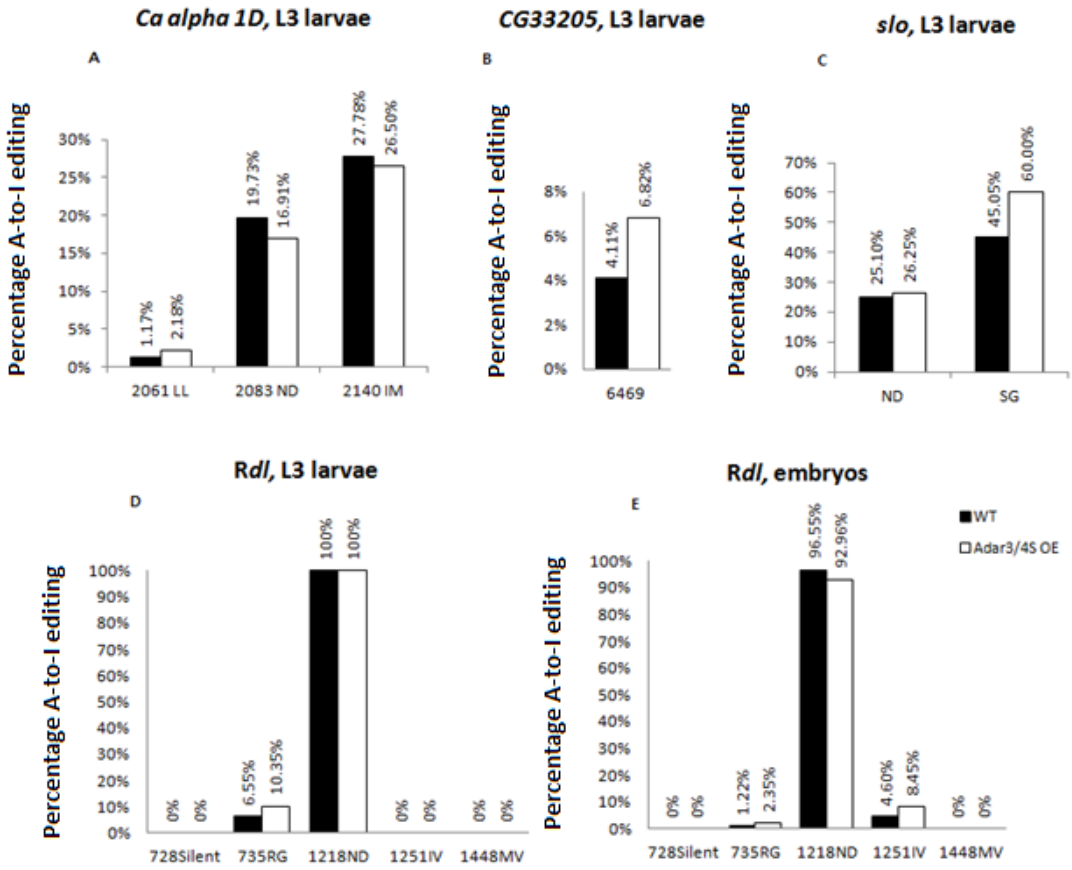


Figure 3.4 A comparison of RNA editing levels in transcripts in *Adar 3/4S* OE and wild type flies. Black columns represent the percentage editing at the indicated editing sites in the wild type control w^{1118} flies, at 25°C, and white columns represent the percentage editing at the *Adar 3/4 S* overexpressing flies, *Adar 3/4 S* OE [ts] at 27°C. The X axis shows the edited positions. The numbers indicate the amino acid sequence numbers, apart from in (B), where 6469 is the last four digits of the genomic DNA position in the 3'UTR of the mRNA. The single amino acid code is used to denote codon changes introduced by RNA editing events. The Y axis is the percentage RNA editing. (A-D) Editing of *Ca alpha-1D, CG332005, slo*, and *Rdl* transcripts from 3rd instar larvae respectively. (E) Editing in the *Rdl* transcript in late-stage embryos.

3.2.2 Deficiency screen for rescue of lethality in *Adar3/4S* OE flies

When the *Adar3/4S* OE [ts] flies were crossed to flies having deficiencies on Chromosome III at 29°C, no progeny overexpressing *Adar3/4*S were born. When the crosses were repeated at 27°C, seven heterozygous deficiencies rescued the lethality (Figure 3.5). The seven deficiencies were mapped to small regions (Table 3.1). However, as observed in another screen we carried out with deficiencies, there was little predictability when overlapping deficiencies were tested (Chapter 5).

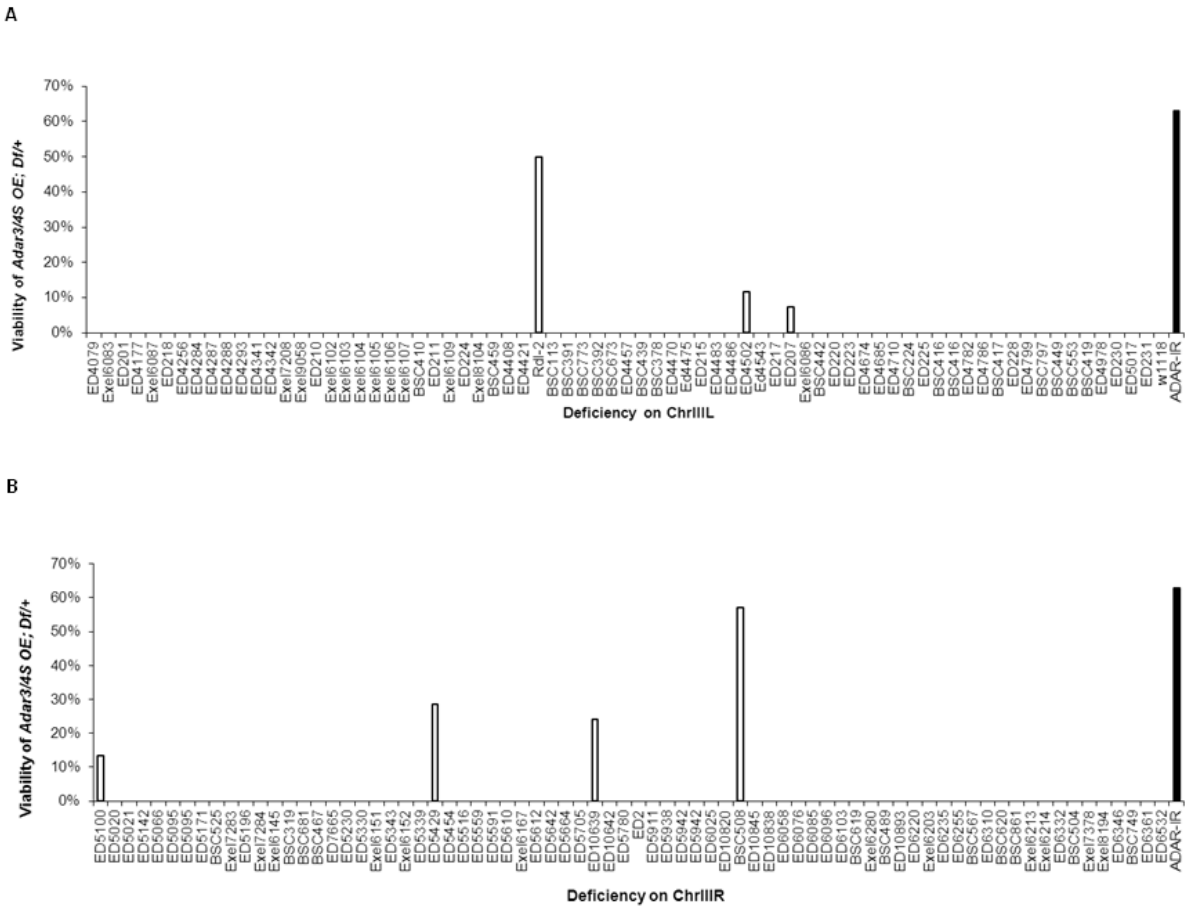


Figure 3.5 Deficiency screen for rescue of lethality caused by *Adar3/4S* overexpression. (A) Deficiency screen on Chromosome III left arm. (B) Deficiency screen on Chromosome III right arm. In both (A) and (B), the last column is the viability of *Adar3/4S OE, Adar RNAi* flies, as a positive control. The second last column on the X axis in A shows that no progeny survive from negative control crosses with w^{1118} wild type flies. The X axis lists the deficiencies crossed with *Adar3/4S* OE [ts] and the vertical axis shows the viability of *Adar3/4S OE, Df* flies.

Table 3.1 Mapping the genes present in the deficiencies that rescue *Adar 3/4 S* OE [ts] lethality.

Deficiencies	**Rdl-2**	**BSC508**	**ED5429**	**ED10639**	**ED5100**	**ED4502**	**ED207**
Including	nwk, Rdl S(CycEJP)3.4 Tequila		Exel6153 Exel6264	Exel7329 Exel7330	ED5020 ED5095 ED5066 BSC174 Exel6141	BSC614	BSC431 BSC250
Tested	-	-	-	-	-	-	-
Negative:	nwk IR, RdlIR	ED10820 ED10845 Rab11 IR	Exel6153 Exel6264 ED5454	Exel7329 ED10642 Pak3 IR	ED5020 ED5095 ED5066 BSC174	BSC614 ED4543	BSC289 ED4177
Positive:	+ Rdl^{1} Rdl^{CB-2L}	+ slmb			+ Exel6141		
Candidates	*Rdl*	*slmb*	*trabid* *dalmatian* *hyd*	*taranis* *Spineless* *Oxidation reductase etc.*	*Fip1* CG31523 CG14651 ED5021 *Aux etc.*	*capricious* *Acp70A* *Rgl* 4 unknown genes	*Aplip1, Mtacp1, LysB~S, etc.* BSC431 BSC250

Smaller deficiencies present in the rescuing large deficiencies or candidate genes affected by the deficiency are listed in the second row of the table. Overlapping or smaller deficiencies covered by the rescuing deficiencies, and candidate gene mutants were tested for viability rescue. Negative results and positive results are shown in the 'Tested' row. The last row lists candidate genes or smaller regions that may be responsible for the rescue.

RDL-2 and BSC508 deficiency effects were mapped down to single genes. *Rdl (Resistant to dieldrin)* null allele Rdl^{1} rescued the viability to 34% (n=94) and *slimb (Supernumerary limbs)* hypomorphic allele $slmib^{00295}$ rescued to 9.8% viability (n=82). Therefore, these two genes were the two strong candidates from the two deficiencies. In the *Adar3/4S* OE [ts] flies rescued by $slimb^{00295}$, the expression level of *Adar* mRNA was significantly reduced (Figure 3.6). In contrast, in the heterozygous RDL-2 deficiency or in the Rdl^{1} null mutant which is a large intergenic inversion covering multiple exons in *Rdl* (Ffrench-Constant et al. 1991), the surviving flies still had significantly high expression of *Adar* transcript (Figure 3.6). *Slimb* is an F-box/WD40 repeat protein, mediating proteolysis (J. Jiang and Struhl 1998). The possibility that *GAL4* is affected by a reduction in *slimb* expression has not been excluded. Therefore, this project focuses on understanding how the RDL-2 deficiency rescues the lethality of *Adar3/4S* overexpression without affecting the expression level of *Adar3/4S* much.

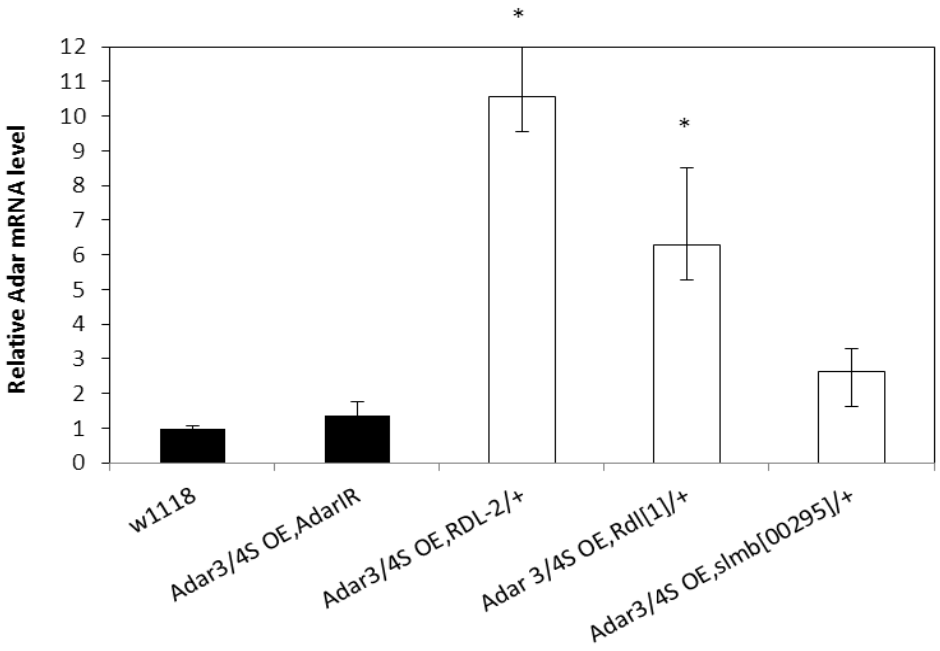


Figure 3.6 Relative *Adar* mRNA level in *Adar 3/4 S* OE adult flies rescued by *Rdl* and *Slmb* mutants. The expression level of *Adar* was normalized to *Gapdh*. The bar represents the relative expression level of *Adar* compared to that in wild type w^{1118}. Error bars are standard errors. Student's t-test was used to calculate the p value. * $p<0.5$

3.2.3 *Rdl (Resistant to Deldrin)* deficiency RDL-2 and *Rdl* mutants rescue the lethality caused by *Adar 3/4 S* overexpression.

Similarly to what was observed in the *Adar3/4S* OE larvae, in the RDL-2 strain that rescues the lethality, only a few edited sites had higher editing levels compared with wild type adult flies (Figure 3.7). Among the ten edited sites in the 3'UTR of *CG33205*, the last three 3' sites had significantly increased editing levels compared with *Adar3/4S OE, Adar IR* control at 27°C or the wild type control. However, none of the five specific editing sites in *Syt1 (Synaptotagmin1)* showed significant changes in editing levels compared to wild type flies.

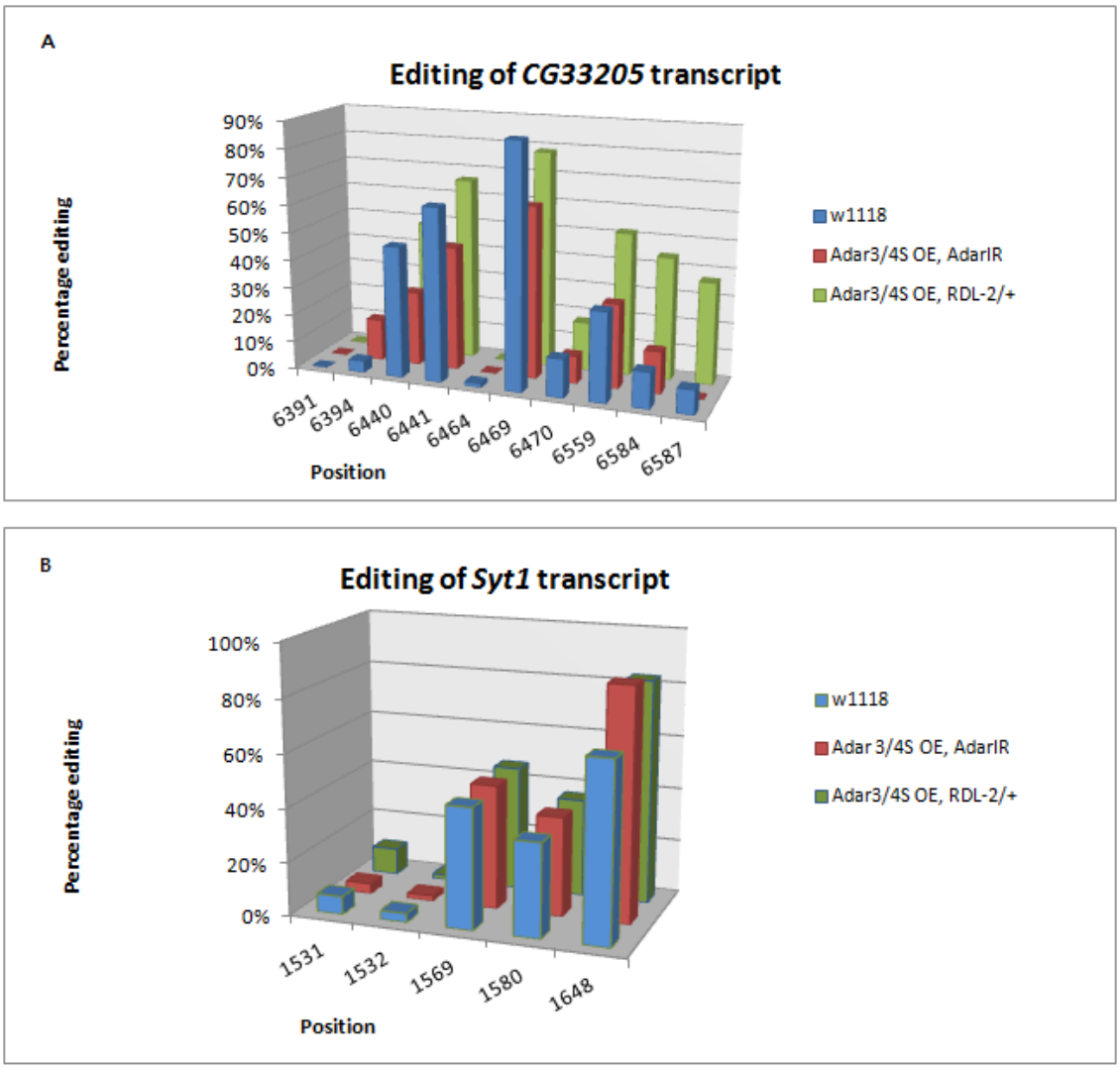


Figure 3.7 Comparison of site-specific editing in edited transcripts in wild type flies and *Adar 3/4 S* OE flies rescued with *AdarIR* or RDL-2. (A) Editing levels of the ten edited sites in the 3'UTR of *CG33200* in adult flies. (B) Editing levels of the four sites in the *Syt* transcript in adult flies. The X axis shows the edited positions in the *D.melanogaster* genome (Apr 2006, Dm3). The numbers are the last four digits of the genomic DNA position. w^{1118} is the wild type control, and *Adar3/4S* OE, *Adar* IR is the rescued control.

Since *Rdl* itself is edited by ADAR, I investigated whether changes in editing efficiencies at sites in *Rdl* contribute to the rescue of lethality by the RDL-2 deficiency. Editing efficiencies at *Rdl* sites in *RDL-2/+* flies and *Adar3/4S OE, RDL-2/+* flies were analysed. *RDL-2/+* flies bearing the RDL-2 deficiency in wild type *Adar* background had a significant increase in editing at the 735R/G site. A slight increase was also seen at the 728 silent site and the 1449 silent site but there was little change in editing at the other sites (Figure 3.8A). *Adar3/4S OE, RDL-2/+* flies showed a similar increase in editing at the R/G site as observed in the RDL-2/+ flies, and editing did not change or was slightly reduced at the other edited positions. *Rdl* has four different splicing isoforms and from the most abundant to the least abundant are bd, ad, bc and ac. There is a choice of 'a' or 'b' for exon 3, combined with a choice of 'b' or 'd' for exon 6 (Jones et al. 2009). Heterozygous *RDL-2* deficiency flies and *Adar3/4S OE, RDL-2/+* flies both have approximately 10% more of the bd isoform than the ad isoform (Figure 3.8B). No 'c' splicing isoform was detected.

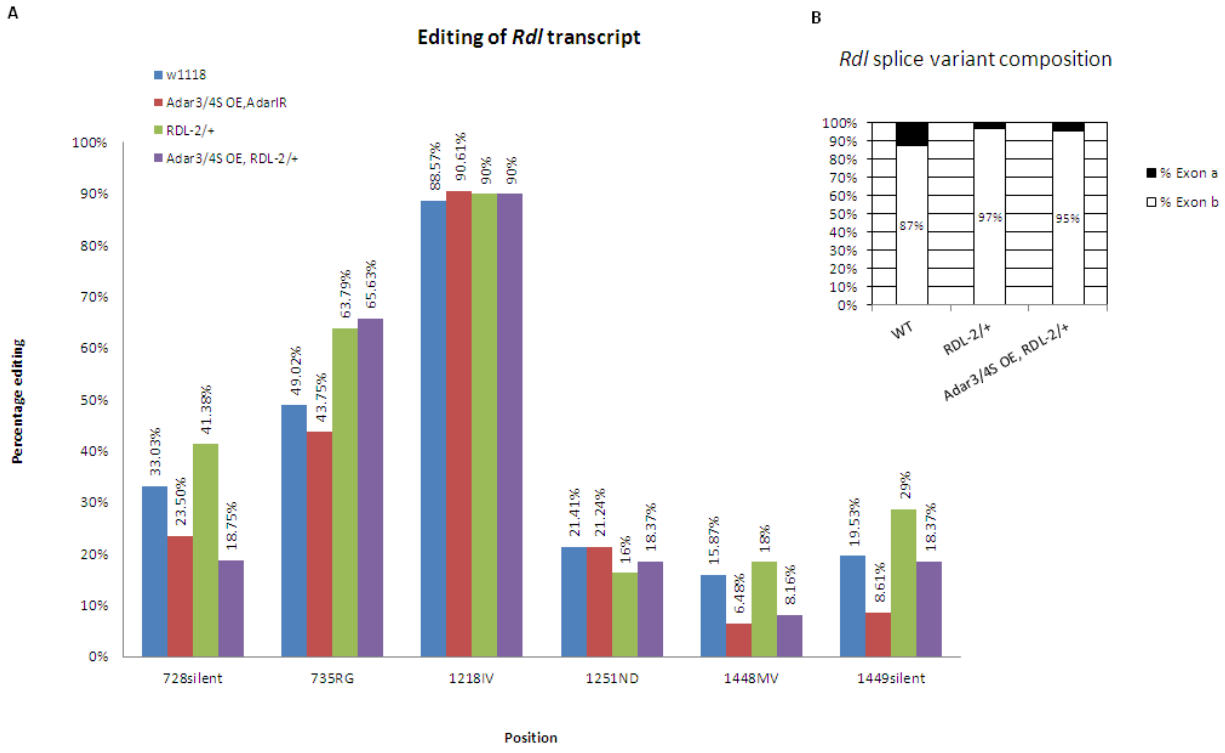


Figure 3.8 Changes in *Rdl* mRNA splicing and editing in the RDL-2 deficiency rescued flies. (A) Editing levels at sites in the *Rdl* mRNA in adult flies of different rescue genotypes. The horizontal axis shows the edited positions. The editing sites are named for the codon numbers and the resulting amino acid changes. The first letter is the genome-encoded amino acid and the second letter is the amino acid generated by RNA editing. (B) Exon a, exon b splicing isoform choices in the flies (n=60).

To confirm that the rescue was due to mutations in *Rdl*, the *Adar 3/4 S OE[ts]* flies were crossed with three additional different *Rdl* mutant lines ($Rdl^{MD\text{-}RR}$, $Rdl^{CB\text{-}2}$, $Rdl^{CB\text{-}2L}$) (Figure 3.9) and two *Rdl* RNAi lines at 27°C. *Adar 3/4 S OE;* $Rdl^{CB\text{-}2L}$/ + flies were viable but none of the other mutant alleles or the RNAi lines of *Rdl* that were tested rescued lethality. All the three *Rdl* mutant lines carry amino acid replacements that are caused by ethyl methanesulfonate (EMS) (Figure 3.9). Both $Rdl^{MD\text{-}RR}$ and $Rdl^{CB\text{-}2L}$ are documented to be dominant for picrotoxin (PTX, GABA antagonist) resistance (Ffrench-Constant et al. 1991). However, no phenotypes of $Rdl^{CB\text{-}2}$ were found.

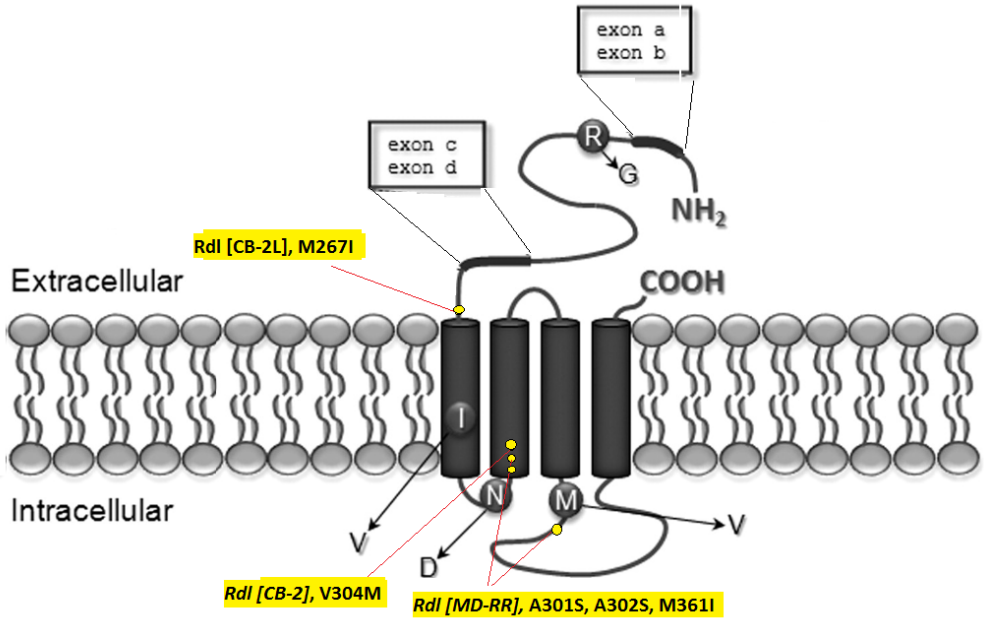


Figure 3.9 Schematic structure of RDL in the cell membrane and positions of alternative splicing sites, editing sites and point mutations. Two alternative splicing sites are in the extracellular region. Three *Rdl* mutants with their positions and amino acid changes are highlighted with yellow. Four editing sites are R122G, N294D, I283V and M360V. Figure adapted from *Jones et al*.,2009.

To test whether the rescue by *Rdl* mutants was due to a reduction in GABA signalling, I tested two different methods of reducing the GABAergic input—by feeding the *Adar 3/4 S* OE [ts] flies with GABA antagonist and by reducing synthesis of GABA. Picrotoxin (PTX) is a widely used $GABA_A$ receptor antagonist reported to effectively inhibit $GABA_A$ receptors that contain *Rdl*. Picrotoxin induces seizures in flies (Stilwell et al. 2006). Feeding different concentrations of PTX to the *Adar 3/4 S OE* [ts] larvae or to their parents did not rescue the lethality caused by *Adar*3/4S overexpression. However, when the *Adar 3/4 S OE* [ts] flies were crossed with $GAD1^{L352F}$, a strong hypomorphic mutant allele of *GAD1* (Glutamic acid decarboxylase 1, which encodes the enzyme that synthesizes GABA), *Adar 3/4 S* OE lethality was rescued to the same level as the

Adar3/4S OE flies having the *Rdl* heterozygous deficiency or a mutation in *Rdl* (Figure 3.10). The heterozygous $GAD1^{L325F}$ mutant shows approximately 50% reduced *GAD1* activity and is predicted to lead to reduced GABA inhibitory signal (Featherstone 2000).

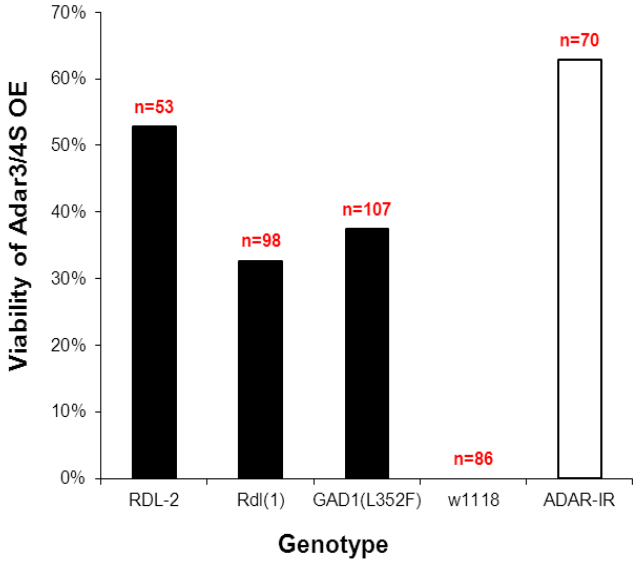
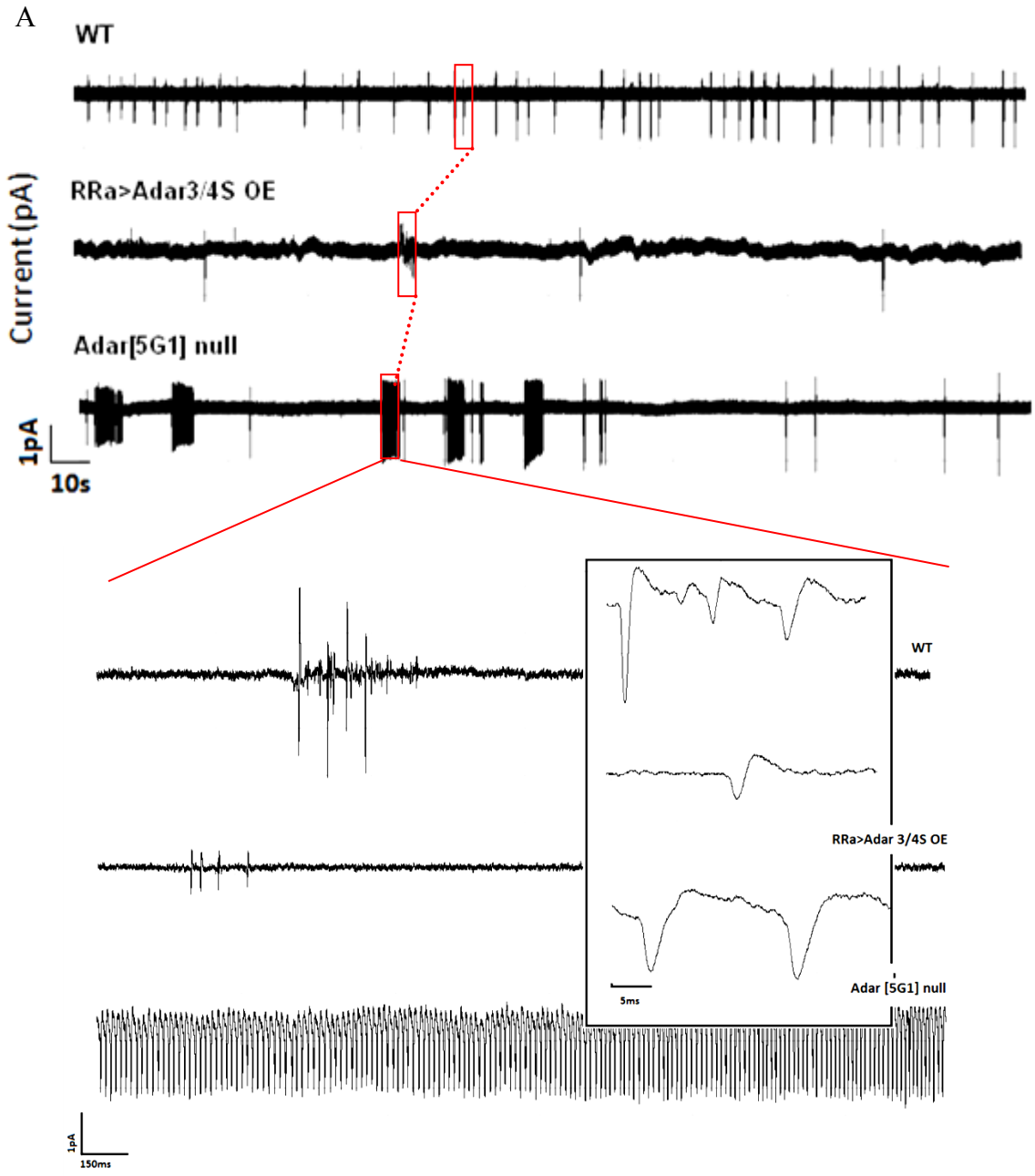


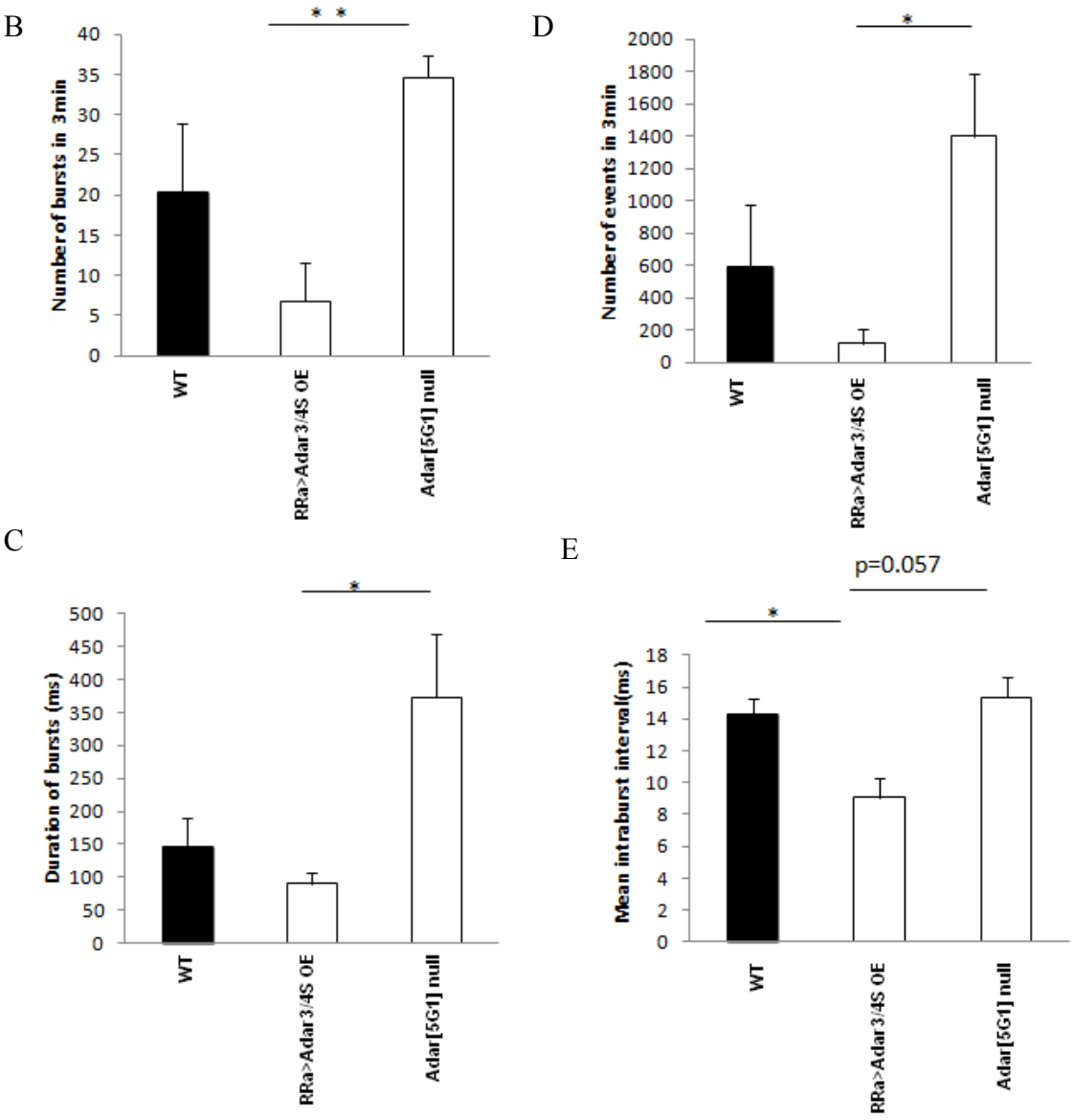
Figure 3.10 *Adar 3/4 S* OE viability rescue by mutants of *GAD1* and *Rdl.* The X axis show the genotypes of the flies crossed with *Adar3/4S OE* [ts] flies. The Y axis shows the viability of the F1 progeny that overexpresses *Adar3/4S,* compared with the expected Mendelian distribution. n is the total number of total progeny.

3.2.4 *Adar* mutant larvae display changes in aCC motor neuron excitability

The rescue of the *Adar 3/4 S* OE lethality by *Rdl* and *GAD1* reduced function mutants led to the hypothesis that lethality of *Adar3/4S* overexpression may be due to highly suppressed neuronal excitability that could be rescued by a reduction in the fast

inhibitory GABA signal. If the hypothesis is true, the *Adar 3/4S* OE flies would have lower neuronal activity while *Adar* null flies would have higher neuronal activity.




B
Number of bursts in 3min
40
35
30
25
20
15
10
5
0
* *
WT
RRa>Adar3/4S OE
Adar[5G1] null
D
Number of events in 3min
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
WT
RRa>Adar3/4S OE
Adar[5G1] null
C
Duration of bursts (ms)
500
450
400
350
300
250
200
150
100
50
0
*
WT
RRa>Adar3/4S OE
Adar[5G1] null
E
Mean intraburst interval(ms)
18
16
14
12
10
8
6
4
2
0
p=0.057
*
WT
RRa>Adar3/4S OE
Adar[5G1] null

F

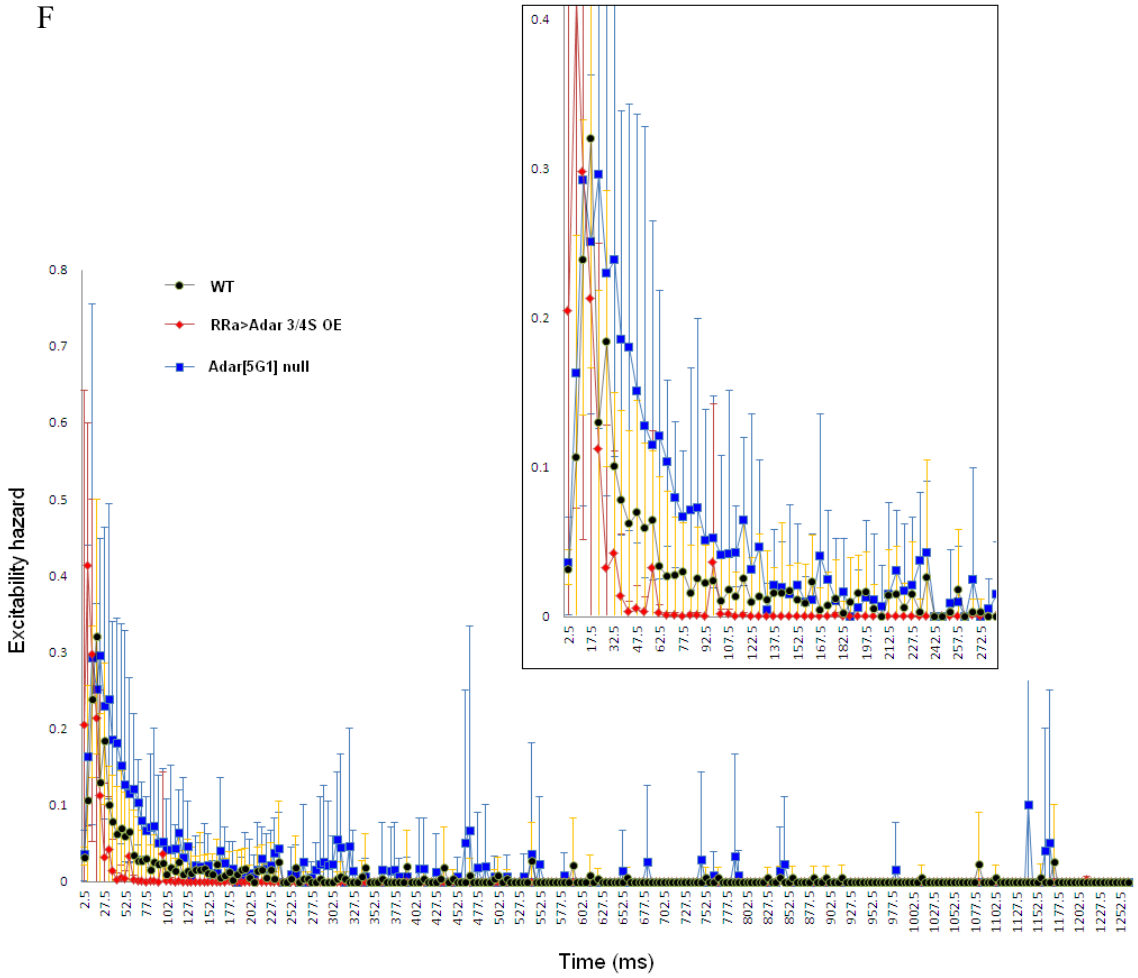


Figure 3.11 *In vivo* extracellular current recordings on *Adar*^{5G1} mutant and *Adar 3/4 S* OE aCC motor neurons. (A) Examples of single aCC neuron activity recordings from flies of three different genotypes, viewed at three different time scales. (B –E) Quantification of the firing activities. The bars are the averages of at least four recordings of different aCC cells. Error bars are standard errors. The p Value is calculated by Student's t-test. The black columns indicate the wild type control, and the white columns are the mutants. (F) Hazard: Hazard= BinCount(i) /(∑spike - ∑$_i$BinCount). The bin size is 2.5 milliseconds. The graph of the first 2772.5ms is enlarged in the box. Genotypes are WT: *UAS-GFP; RRa- GAL4*. *RRa>Adar3/4S OE*: *UAS-GFP / +; RRa-GAL4 / UAS-Adar3/4S*. *Adar[5G1]* null: *Adar5G1; UAS-GFP / +; RRa-GAL4 / +*.

To test this hypothesis, I recorded spontaneous neuronal activity using *in vivo* extra-cellular current recording on 3rd instar larval aCC motor neurons of *Adar* mutant flies. aCC motor neurons receive GABA input and are reported to have *Rdl* expression (Featherstone 2000). When the *Adar3/4S* isoform is specifically expressed in the aCC motor neurons using the *RRa-GAL4* driver, neurons showed significantly reduced firing activities. Reciprocally, $Adar^{5G1}$ null larval aCC motor neurons were hyper-active (Figure 3.11). The number of bursts (Figure 3.11B), durations of the bursts (Figure 3.11C), the number of firing events (Figure 5.11D), and the mean intraburst intervals (Figure 3.11E) were all decreased in *Adar 3/4 S* overexpressing aCC motor neurons, but increased in $Adar^{5G1}$ null aCC motor neurons. These quantifications showed a trend, however not all of these differences were statistically significant. A hazard graph, plotting the probability of having a firing event after previous firing events within a certain interval of time, showed that in $Adar^{5G1}$ flies, aCC motor neurons have higher excitability while *Adar 3/4 S* overexpressing neurons are much less active compared with wild type neurons (Figure 3.11F).

3.2.5 Overexpressing *UAS-Rdl* constructs in $Adar^{5G1}$ null flies

Since reduction in *Rdl* expression rescued the lethality caused by *Adar3/4S* overproduction, I wanted to elucidate whether overexpressing either edited or unedited *UAS-Rdl* constructs could rescue some of the phenotypes of the $Adar^{5G1}$ null flies. Prior to the start of the project, fly lines bearing *UAS-Rdl* cDNA constructs were generated to express the fully edited *Rdl* isoform (*Rdl_Ed*) and the fully unedited *Rdl* isoform (*Rdl_Un*) or the *Rdl* ac isoform that has only the IV site edited (the *Rdl ac* cDNA was a gift from Andrew Jones, Professor David Sattelle Group, University of Oxford). Overexpressing any of these three constructs with the *Actin 5c-GAL4* driver was lethal, but they were partially viable when a weaker *armadillo-GAL4* driver was used.

When $Adar^{5G1}$ was crossed with each of these three constructs in the absence of a *GAL4* driver, no changes in viability were observed in the progeny, with the exception being

the *Rdl_Un* flies which increased *Adar*^{5G1} viability slightly. *Adar*^{5G1}*; arm-GAL4/ Cy* flies were crossed with *UAS-Rdl_Un* homozygous flies at 25°C and 19°C separately to see the effects of different overexpression levels of *Rdl_Un*. The basic *GAL4/ UAS* system is sensitive to temperature as higher temperature leads to higher expression of UAS constructs (Fischer et al. 1988). Temperature indeed affected the populations of both *Adar*^{5G1} flies and *Rdl_Un OE* flies.

At 25°C, *Adar*^{5G1} has low viability of approximately 20-50% compared with *FM7* balancer flies in the *Adar*^{5G1}*/FM7* stock. Flies overexpressing *Rdl* constructs under *arm-GAL4* driver narrowly escape from lethality with a viability of only 5-8% compared with *UAS-Rdl_*Un flies without drivers. Among the progeny from crosses of *Adar*^{5G1}*; arm-GAL4/ Cy* flies and *UAS-Rdl_Un* homozygous flies at 25°C, *arm>Rdl_Un* flies showed the lowest viability, taking up 7% of the population. Surprisingly, loss of *Adar* increased the viability of *Rdl-Un* overexpressing flies by 2.7 folds (Figure 3.12A).

At 19°C, *Rdl_Un* OE has higher viability compared with 25°C. Greater viability for *UAS-Rdl_Un* construct is expected because *GAL4-driver* expression will be much lower at low temperature. At 19°C, the *Adar*^{5G1} viability is almost ten-fold less than at 25°C, but moderate overexpression of *Rdl-Un* increased *Adar*^{5G1} viability by five fold. In both 25°C and 19°C, combination of *Adar* null and *Rdl_Un* overexpression showed significant effects on each other's viability ($p<0.0005$ and $p<0.005$, respectively, by the Fisher's exact test).

However, neither overexpressing *Rdl_Un* construct using *armadillo-GAL4* driver nor *OK6* motor neuron driver improved *Adar*^{5G1}climbing performance. (Figure 3.12 B).

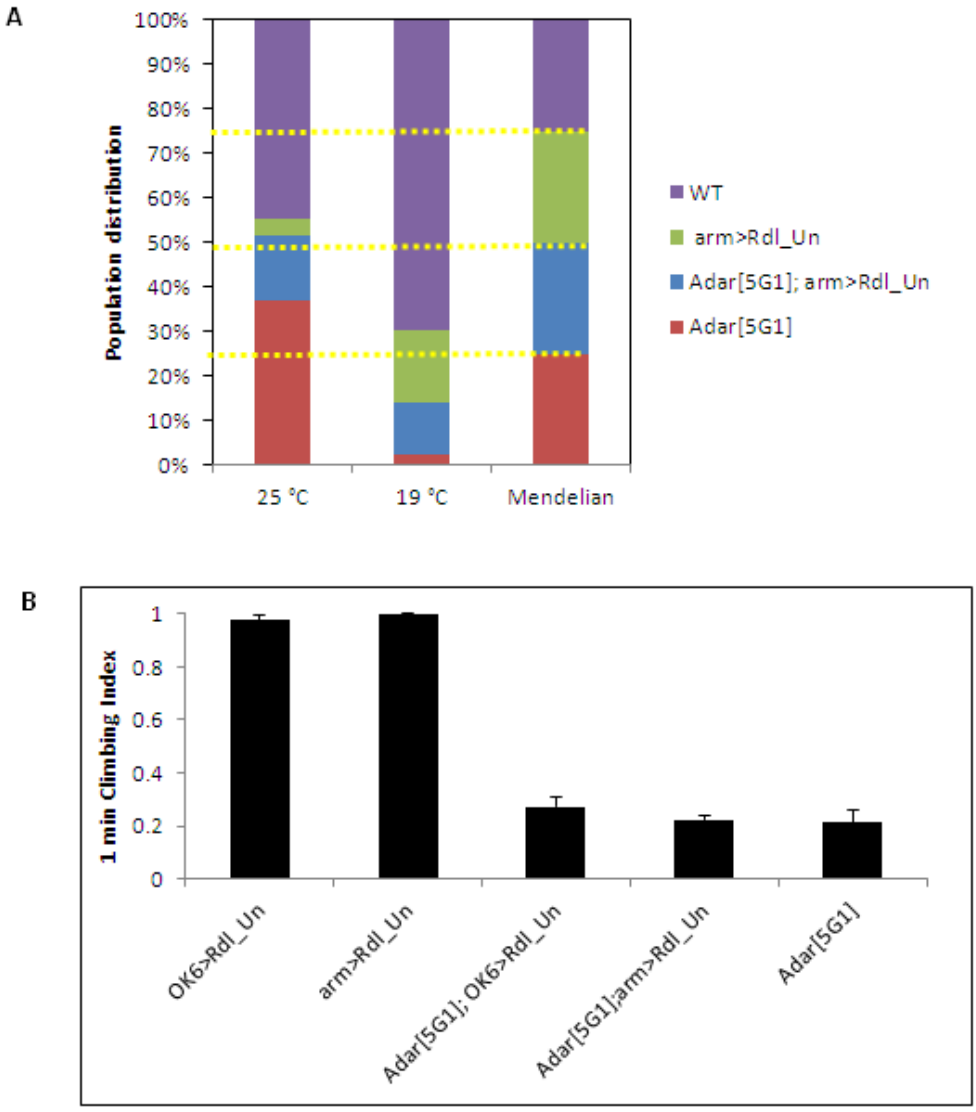


Figure 3.12 The effect of *Rdl_Un* overexpression on *Adar^{5G1}* flies. (A) Population distribution shows the listed four genotypes of the F1 generation from the cross *Adar^{5G1}; arm-GAL4* with *UAS-Rdl_Un/CyO*. Three stacked column bars indicate the distribution of F1 flies collected at 25°C (n=179), at 19°C (n=43), and the theoretical Mendelian distribution. Yellow dotted lines divide columns into four identical 25% areas. (B) Columns show average climbing index of the flies. Error bars are standard error. Genotypes are WT: *FM7; arm-GAL4/CyO. arm>Rdl_Un*: *FM7; arm-GAL4/ UAS-Rdl_Un. Adar[5G1];arm>Rdl_Un: Adar^{5G1}; arm-GAL4/ UAS-Rdl_Un. Adar[5G1]: Adar^{5G1}; arm-GAL4/Cy.*

3.3 Discussion

RNA editing mediated by ADAR is an essential biological process that has been proposed to diversify the genetic information to meet the physiological needs of the organism. Ectopically overexpressing the ineditable *Adar 3/4 S* isoform of the enzyme leads to lethality that occurs during all developmental stages. Lethality occurs continuously in embryos, larvae and pupae and lethality occurs if *Adar 3/4 S* overexpression is induced at any stage before the adult stage. The lethality is likely to be caused by a widespread physiological malfunction since the down-stream targets of ADAR are in every tissue in the fly. The *Adar3/4S* isoform has been shown to have the highest editing activity amongst the different *Adar* isoforms in *in vitro* editing assays with *Adar* exon 7 or *cac* editing site substrates (Keegan et al. 2005). Surprisingly, despite *Adar 3/4 S* transcript expression being increased more than six fold in *Adar 3/4 S* OE lethality-rescued flies, the editing level at sites in target transcripts did not change significantly. It suggests that there is some unknown factor(s) limiting the capacity of ADAR 3/4 to edit. It needs to be investigated what this limiting factor is. We have not totally excluded the possibility that *Rdl* mutants do affect *Adar 3/4 S* overexpression somewhat.

The deficiency genetic screen on Chromosome III to identify rescuers of *Adar3/4S* OE lethality was a tight screen that did not give many false positive results. Also, the rescuers identified from this screen were all partial rescuers; none completely reversed the lethality. *Slimb*, encoding a ubiquitin ligase, is an interesting candidate that may be a positive genetic regulator of ADAR since the heterozygous *slimb* hypomorphic mutant rescues the lethality associated with the *Adar3/4S* OE. This hypothesis can be tested only when the possibility that *slimb* affects the GAL4, GAL80^{ts}- UAS system is ruled out. Not included in this thesis is the deficiency screen data for Chromosome II and the X chromosome that was performed by two undergraduate students under my supervision. No rescuers were found among those chromosome deficiencies.

Rescue of the *Adar3/4S* OE lethality by *Rdl* deficiency RDL-2, heterozygous *Rdl* null allele Rdl^{1}, and by a mutation affecting the enzyme that produces GABA, *GAD1* strongly suggests that the GABA fast inhibitory signalling pathway and *Adar* mutants generate mutually compensating effects on neuronal physiology. The *in vivo* extra-cellular current recordings of aCC motor neurons from the *Adar* mutants support this hypothesis. The aCC motor neurons of $Adar^{5G1}$ null flies show higher neuronal excitability while *Adar3/4S* overexpressing aCC neurons have significantly reduced excitability. In the *Adar3/4S* OE flies, abnormally suppressed neuronal activity is probably one of the leading causes of the lethality, which can be corrected by a reduction in the fast inhibitory GABA signalling.

Xenopus oocyte electrophysiology studies on *RDL* isoforms revealed that editing at the edited R/G sites, the I/V and the N/D sites together caused a more than 7 fold increase in GABA EC_{50} compared with the editing of R/G site only, which is the most significant difference in EC_{50} among all the different combinations of edited site that were tested (Jones et al. 2009). Also, it was observed that the EC_{50} of the bd isoform is approximately 2.6 times higher than that of the ad isoform (Jones et al. 2009). If the mechanism is similar *in vivo*, then both the adjustments in editing and the particular spliced isoform more expressed in the heterozygous RDL-2 deficiency and *Adar3/4S OE, RDL-2/+*, render the GABA receptor less responsive to GABA signals. The reduction in *Rdl* expression also reduces the number of GABA receptors expressed (Hosie et al. 1997). In other words, the flies with the RDL-2 heterozygous deficiency have much reduced GABAergic inhibition of neuronal excitability compared with wild type flies.

On the other hand, moderate overproduction of the *Rdl_Un* construct in the $Adar^{5G1}$ null flies may increase the viability of the $Adar^{5G1}$ null flies by enhancing inhibitory signals to the hyperexcitable neurons. Besides the fully unedited *Rdl_Un* constructs, the other *Rdl* constructs tested for the rescue of $Adar^{5G1}$ viability were the fully edited *Rdl* construct and the construct that has only the I/V site edited. It is not ideal that all of these three constructs were ac spliced isoforms as that is the least abundant spliced isoform *in vivo* (Jones et al. 2009). Among these three constructs, *Rdl_Un* has the lowest GABA

EC_{50} when expressed in *Xenopus* oocytes (Jones et al. 2009). The fact that *Rdl_Un* has the highest sensitivity to GABA among the three constructs may explain why this construct but not the other two increased $Adar^{5G1}$ viability. Hence survival of *Adar* mutant flies seems to be highly related with GABA signalling.

Some of the negative results obtained appear to weaken the argument that reducing GABA inhibitory input rescues the lethality caused by *Adar3/4S* ectopic overexpression. These include the failure to rescue the lethality by PTX feeding, by *Rdl* RNAi or other *Rdl* mutants or by another large deficiency ED4421 that also deleted the *Rdl* gene. Firstly, the failure of the PTX feeding experiment was not unexpected. As discussed previously, overexpressing *Adar 3/4 S* is lethal at all developmental stages. In the case of genetic rescues, modifications commence at the start of the embryogenesis. However, PTX can be fed only during the feeding larval stage or to the parents so that the effect of PTX may extend to the early embryonic stage. PTX may not be obtained by feeding soon enough in first instar larvae or not be present in the wandering larvae or the pupae stages when the overproduction of *Adar 3/4 S* is very high. Secondly, *Rdl* RNAi may lower *Rdl* too much, which is the likely reason for its inability to rescue. GABA inhibitory signalling is crucial for the survival of the animals. Complete knockout of either *Rdl* or *GAD1* results in lethality. In the case of introducing RNAi against *Rdl* in the *Adar 3/4 S* OE[ts] flies, the flies were raised at a restrictive temperature 27°C. However, RNAi against *Rdl* in the wild type background is lethal with the *Actin 5c-GAL4* driver due to an efficient knockdown of *Rdl*. Compared with Rdl^{1} that is an inversion with the breakpoints inside the gene that disrupts the gene almost entirely, the other *Rdl* mutants $Rdl^{MD\text{-}RR}$, Rdl^{CB2}, and $Rdl^{CB\text{-}2L}$ are mutated at single amino acids (Figure 3.10) (Ffrench-Constant et al. 1991). It is not clear yet why $Rdl^{CB\text{-}2L}$ rescues the lethality of *Adar 3/4 S* OE while the other point mutants of *Rdl* cannot. The ED4421 deficiency deleted 642.8kb, 89 genes including *Rdl*, while the RDL-2 deficiency is predicted to have deleted approximately 23kb, 6 genes. Such a large deficiency as ED4421 is likely to introduce additional stress to the flies. To sum up, when carefully scrutinised these negative results do not really weaken the hypothesis.

Further investigation is necessary to confirm the hypothesis that altered RNA editing by ADAR affects neuron excitability and can be corrected by manipulating GABA signal. To elucidate the mechanism whereby ADAR controls neuronal excitability, the following experiments should be performed in the future:

1. Examination of the neuronal activity of the cell-autonomous *Adar* RNAi knockdown, by recording the firing activity of the single neurons that have *Adar* knocked down specifically in the aCC motor neurons (*UAS-GFP / +; RRa-GAL4 / UAS-Adar IR*). This experiment, together with the previous electrophysiological results will address the hypothesis that reduced ADAR activity makes neurons more excitable cell-autonomously.
2. Rescue neuronal activity by examining whether introducing *Rdl* or a *GAD1* mutation can correct the suppression of aCC single neuron activity by *Adar3/4S*-overexpression. The opposite experiment can also be performed where the activity of *Adar*-knockdown neurons can be analyzed when overexpressing *Rdl*.
3. Investigate the rescue of behaviour of $Adar^{5G1}$ null flies by feeding them GABA agonists, such as benzodiazepines.

It is important in the long term to elucidate the mechanism whereby *Adar* and RNA editing fine-tune neuronal activity. Many aspects of the living organism affect the excitability of the neurons. *Adar* may help control the neuronal activity by fine-tuning the properties of many ion channels and other cellular proteins by its editing activity and perhaps also by its double-strand RNA binding activity. This regulation by *Adar* is likely to be very complicated. Nevertheless, to unveil the mechanism by studying large Ca^{2+}-dependent Potassium (BK) channels and their control by *Adar* is a good starting point. Firstly, BK channels are the channels that directly control the firing patterns of neurons (Burdyga and Wray 2005). And secondly, several BK channels, including *slo*, and *shab* transcripts are edited in *Drosophila* (Ryan et al. 2008; Graveley et al. 2011). Therefore, studying the contributions of editing in these channels to control neuronal excitability is promising as well as experimentally feasible.

4 CHAPTER IV: Study of *Adar*^{5G1} null mutant phenotypes

Dare to be honest and fear no labor.

— Robert Burns

4.1 Introduction

Many phenotypes of *Adar* mutant flies have been described, including neurodegeneration and locomotion defects (Palladino et al. 2000a). More detailed aspects of $Adar^{5G1}$ null fly phenotypes have been described by previous PhD students in our group. McGurk described neurodegeneration in $Adar^{5G1}$ and showed that overexpressing the inactive *Adar* construct *Adar 3/4 EA* in cholinergic neurons rescues the neurodegeneration phenotype of $Adar^{5G1}$ but not the open field locomotion defects (McGurk Thesis, University of Edinburgh 2008). EM images showing multi-lamellar vacuole structures and standard 2µm autophagic vesicles in the $Adar^{5G1}$ null fly brain indicate that the neurodegeneration involves autophagy. Hogg performed a Microarray analysis and found that expression of many transcripts was altered in $Adar^{5G1}$ fly heads. *Adar 3/4 EA* expressed in cholinergic neurons of $Adar^{5G1}$ flies corrects some of these gene expression changes. More recently, Paro demonstrated that mutating *Tor* or overexpressing autophagy genes in cholinergic neurons were sufficient to prevent neurodegeneration and rescue locomotion defects in $Adar^{5G1}$ flies (Paro Thesis, University of Edinburgh 2012).

McGurk showed that $Adar^{5G1}$ null flies develop vacuoles from as early as 25 days, most prominently in the mushroom body (MB) calyces and the optic lobes. The abnormal membrane structures seen in the EM images included double membrane vesicles containing mitochondria which are recognizable as autophagic vesicles (McGurk Thesis, University of Edinburgh 2008). Cell death was detected using TUNEL staining in the brain fat cells but not in the neurons of the aged $Adar^{5G1}$ flies. In addition, overexpression of viral anti-apoptotic protein p35 (Hay et al. 1994; Lannan et al. 2007) in the cholinergic neurons did not rescue the neurodegenration (McGurk Thesis, University of Edinburgh 2008). The mechanisms of neuronal death in various *Drosophila* neurodegenerative models have been extensively investigated. It seems that the neurons do not die by typical apoptosis or necrosis in most fly models and this appears to be a feature particular to *Drosophila* rather than vertebrate neurodegenerations (McCall 2010). Recently, Trunova S. et.al found that in the *p35*

mutant *Drosophila* model of neurodegeneration, the neurons die through necrosis in the beginning of the neurodegeneration and later show an autophagic cell death phenotype (Trunova and Edward Giniger 2012). Neurodegeneration in $Adar^{5G1}$ may also involve autophagy or necrosis. $Adar^{5G1}$ flies provide an excellent model to study adult-onset neurodegeneration.

Expressing *Adar 3/4 EA* under *Cha-GAL4* driver control in the cholinergic neurons of $Adar^{5G1}$null flies was sufficient to prevent vacuole formation in the MB calyces of 30 day old flies (McGurk Thesis, University of Edinburgh 2008). Hogg repeated this result, and also found that *Adar 3/4 EA* corrected the expression levels of many transcripts altered in the $Adar^{5G1}$ heads by doing microarray analysis on the heads of $Adar^{5G1}$ null flies and two rescue lines, $Adar^{5G1}$*; Cha>Adar 3/4* and $Adar^{5G1}$*; Cha>Adar 3/4 EA*. In many cases, the rescue effect on expression of altered genes by cholinergic neuronal expression of *Adar 3/4 EA* was as complete as or more complete than with *Adar 3/4*. She argued that this may be due to the fact that *Adar 3/4 EA* construct is two to three fold more expressed than *Adar 3/4* (Hogg Thesis, University of Edinburgh 2010). Rescue of the $Adar^{5G1}$ phenotypes by inactive Adar is an interesting result and the mechanism is still not clear.

Continuing with McGurk's work, Paro found that *Tor* mutants or *Act-GAL4* driven overexpression of autophagy genes *Atg5* or *Atg1* rescue the $Adar^{5G1}$ null fly phenotypes, including rescue of low viability, locomotion defects, neurodegeneration and reduced longevity (Paro Thesis, University of Edinburgh 2012). *Atg5* overexpression showed better rescue than *Atg1* overexpression in her experiments (Paro Thesis, University of Edinburgh 2012). She also observed more LysoTracker Red staining in the fat bodies of $Adar^{5G1}$ null fly 3rd instar larvae compared with wild type, suggesting more autophagy in the $Adar^{5G1}$ null mutant flies (Paro Thesis, University of Edinburgh 2012). Up-regulating autophagy levels is well known to be beneficial in extending life span (Scott et al. 2004). Increased autophagy helps host-defence, and reverses neurodegenerative diseases presumably due to its role in removing toxic protein aggregates or reactive oxygen species (Lipinski et al. 2010; Ravikumar et al. 2004).

However, it is not obvious how increasing autophagy rescues the neurodegeneration phenotype and locomotion defects in $Adar^{5G1}$ null flies. In regard to the neurodegeneration phenotype of $Adar^{5G1}$, no obvious protein aggregates were found that may be a leading cause of the neurodegeneration. Intriguingly, overexpressing *p35* in cholinergic neurons did not rescue the massive vacuole phenotype in $Adar^{5G1}$ MB calyces but overexpressing *Atg5* in cholinergic neurons did (McGurk Thesis, University of Edinburgh 2008 and Paro Thesis, University of Edinburgh 2012). This suggests that the vacuoles observed in the HE stained sections of the fly brains were not part of an apoptotic cell death process. As supported by the EM images, the enlarged vacuoles may be accumulated pathological remnants of aberrant autophagy in the neurons. Increased autophagy is likely to be sufficient to turn these structures over at an early stage. The locomotion defects of $Adar^{5G1}$ flies may be a combined result of the defects in motor neurons, muscles and neuromuscular junctions, but may also reflect a general cellular fitness status. It is complicated to dissect the mechanism of the rescue by reducing *Tor* or increasing autophagy, without a clear picture of how the severe locomotion defects develop from loss of ADAR protein.

This chapter is mainly the continuation of work of previous students, attempting to address the mechanism of the severe neural-behavioural phenotype of $Adar^{5G1}$ flies and to further investigate the rescue of $Adar^{5G1}$ phenotypes by inactive *Adar 3/4 EA* and by *Tor* mutation. We sent poly-A tailed RNA samples from $Adar^{5G1}$ and w^{1118} wild type fly heads for Next Generation sequencing to determine the transcriptome changes caused by loss of *Adar.* Then, I performed qRT PCR to examine if inactive *Adar,* a *Tor* mutant, or overexpression of *Atg5* could rescue the expression changes of transcripts altered in $Adar^{5G1}$. In order to understand the cell-autonomous effect of $Adar^{5G1}$, I generated $Adar^{5G1}$ null cell clones in heterozygous mosaic flies using the MARCM technique.

4.2 Results

4.2.1 Innate immune response genes are up-regulated in poly A+ mRNA from *Adar*^{5G1} null fly heads.

Heads of 5-day old *Adar*^{5G1} null and w^{1118} wild type male flies were collected and poly A+ mRNA sequencing was carried out. The sequencing was performed in the Wellcome Trust Centre, Glasgow University sequencing facility. The sequencing was performed using the Ilumina Genome Analyser IIx. Single-end reads of 76bp were aligned and mapped to the fly genome by our collaborator Rui Zhang in Jin Billy Li's group at Stanford University (Figure 4.1A, Zhang R.). He further compared the transcriptome changes in the *Adar*^{5G1} and w^{1118} wild type fly heads (Figure 4.1A, Zhang R.).

Interestingly, Zhang found that the expression changes of edited genes between wild type and mutant were smaller than that of the whole transcriptome (Figure 4.1B, Zhang R.), indicating that ADAR does not control expression levels of edited transcripts.

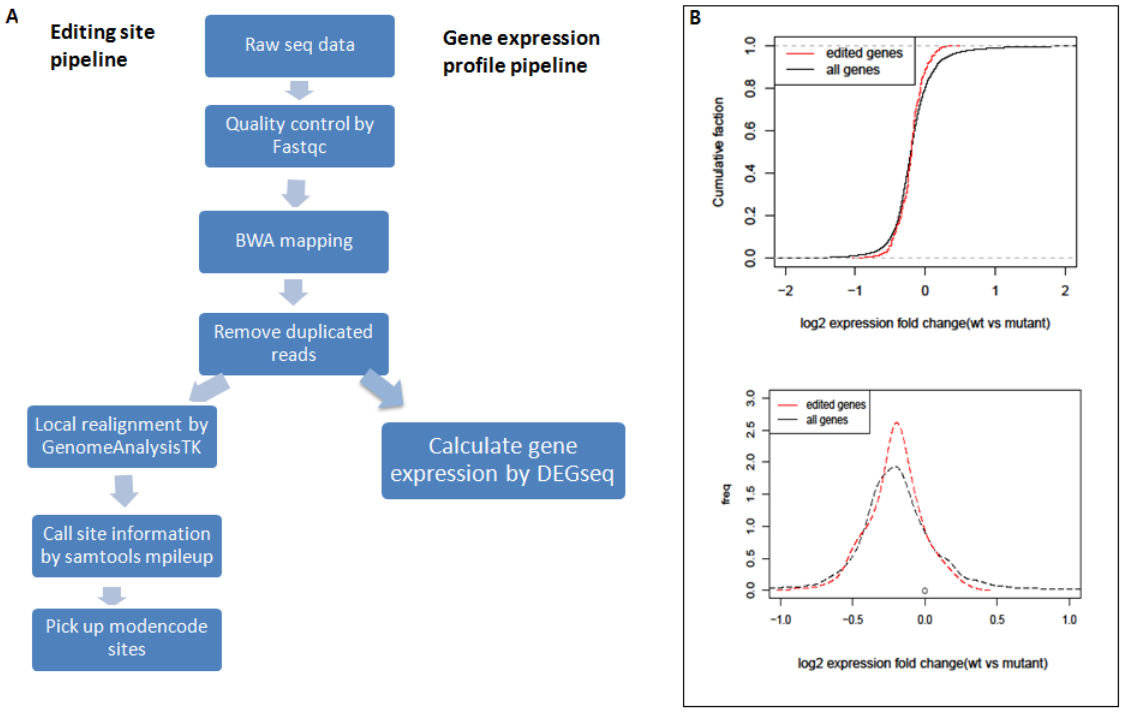


Figure 4.1 Poly-A+ RNA sequencing analysis of *Adar*^{5G1} and *w*^{1118} fly heads (Zhang, R.). (A) RNA sequencing analysis pipeline. Raw sequencing data underwent quality control by Fastqc, then BWA (Burrows-Wheeler Aligner) mapping (Li and Durbin 2009) and then removal of duplicated reads. The differences in gene expression levels were calculated by DEGseq (Wang et al. 2010), and the edited transcripts were analyzed by doing local realignment, calling site information, and picking up modEncode sites (Graveley et al. 2011) of RNA editing step by step after removing the duplicated reads. (B) The expression changes of edited genes between wt and mutant were smaller than that of the whole transcriptome. The X axis shows log2 expression fold changes (wt vs mutant). The Y axis in the upper chart shows the cumulative faction and in the lower chart shows the frequency of the reads.

Among the 14,624 mapped genes, 356 transcripts were down-regulated by more than two fold and 236 transcripts were up-regulated by more than two fold. I uploaded the lists of genes that changed more than two fold onto FlyMine (http://www.flymine.org), an integrated database for *Drosophila* and *Anopheles* genomics (Lyne et al. 2007). In terms of the chromosomal location distributions or tissue and developmental expression

patterns, there were no enrichments or biases in the expression-changed genes**.** However, there was a clear preference in the functional categories among the genes that were changed. The up-regulated genes were not significantly enriched in any pathways, but there were two overrepresented gene ontology terms—response to bacterium ($p<0.01$, 12 gene matches) and cellular response to heat ($p<0.01$, 6 gene matches) (Table 4.1). Also, there was one overrepresented protein domain; the stress-inducible humoral factor Turandot protein domain ($p<0.001$). These upregulated *Turandot* genes (five *Tot* genes out of six known *Tot* genes) are involved in responses to both heat and bacteria, as shown in Table 4.1. On the other hand, the down-regulated genes were overrepresented in the starch and sucrose metabolism pathway (P value<0.01), and Gene Ontology term analysis revealed that the gene classes were mainly involved in catabolism and reproduction (Table 4.2).

Table 4.1 Statistically significant categories of genes with transcripts increased more than two fold in *Adar*^{5G1} heads.

Gene name	WT: w^{1118}	*Adar*^{5G1}	Fold Change	Cellular response to heat	Response to bacterium
TotM	29.2232	7742.05	264.9285058	Y	Y
TotC	167.26	20216.2	120.8669072	Y	Y
TotA	904.345	45505.7	50.31886216	Y	Y
TotX	339.423	4459.89	13.13959249	Y	Y
IM23	263.698	2158.11	8.184073422	N	Y
Drs	397.826	2423.87	6.092813732	N	Y

Diedel	39.2918	178.992	4.555430328	N	Y
CecC	33.9743	89.8019	2.643233557	N	Y
AttC	384.485	651.826	1.695324215	N	Y
Hsp70Aa	186.169	296.359	1.591886833	Y	N
Edin	0	70.2873	10000	N	Y
TotB	0	31.1494	10000	Y	Y
CG6639	0	26.3876	10000	N	Y

'N' and 'Y' mean the gene does not or does belong to the gene ontology category. The numbers in the second and third columns are the number of reads in the RNA sequencing. Values for $Adar^{5G1}$ and w^{1118} are read per kilobase per million (RPKM) values from head poly A+ RNA sequencing.

Table 4.2 Statistically significant categories of genes down-regulated more than 2 fold in $Adar^{5G1}$ heads.

GO Term	p-Value	Matches
chitin metabolic process	7.32E-06	16
amino sugar metabolic process	1.12E-05	16
glucosamine-containing compound metabolic process	1.47E-05	16
carbohydrate metabolic process	1.86E-05	28
post-mating behavior	3.89E-05	8
aminoglycan metabolic process	4.24E-05	16

polysaccharide metabolic process	1.26E-04	16
amine metabolic process	1.26E-04	17
Proteolysis	7.61E-04	37
chitin catabolic process	8.07E-04	6
amino sugar catabolic process	8.07E-04	6
glucosamine-containing compound catabolic process	8.07E-04	6
multicellular organismal reproductive behavior	0.001823	11
sperm competition	0.003887	5
insemination	0.007622	5
reproductive behavior	0.008369	11
aminoglycan catabolic process	0.019393	6
oviposition	0.020907	5
polysaccharide catabolic process	0.020948	6
mating	0.023216	10
Copulation	0.029314	5

'Match' indicates the number of the down-regulated genes that match the gene ontology term.

4.2.2 Rescue of innate immune gene expression levels in *Adar*^{5G1} flies with different *Adar* rescue constructs.

In mammals, ADAR proteins were reported to be involved in immune responses (Samuel 2001; Hartwig et al. 2006; Rice et al. 2012). For example, transcription of the ADAR1 long isoform p150 is activated by interferon (George and Samuel 1999a). Also, in flies and vertebrates, *Adar* is reported to edit viral dsRNAs (Doria et al. 2009; Carpenter et al. 2009) . We decided to verify the expression changes in the innate immune genes from the RNA sequencing data. Could innate immune induction be the primary reasons for the pathological status of *Adar*^{5G1} flies? Do inactive *Adar 3/4 EA* or *Tor* mutation prevent immune induction and correct some or all of these expression changes?

To answer these questions, as well as to confirm the immune response transcript changes detected from RNA sequencing, I performed qRT PCR using cDNA made from total RNA of whole flies. *TotA, TotC, TotX*, and *Drs* from the up-regulated gene list and the two down-regulated immune responsive genes *AttD* (53 reads in wild type, and 0 read in *Adar*^{5G1} heads) and *Def* (1023 reads in wild type versus 108.67 reads in *Adar*^{5G1} heads) were selected for their expression profile changes in *Adar*^{5G1} whole flies, *Adar*^{5G1}; *Arm>Adar 3/4* flies, *Adar*^{5G1}*;Arm>Adar 3/4 EA* and wild type flies. In *Adar*^{5G1} null flies, the expression levels of *TotA, TotC, TotX* and *Drs* were much higher compared with wild type flies, which confirmed the RNA sequencing result (Figure 4.3). Unexpectedly, *AttD* and *Def* expression levels were also upregulated in *Adar*^{5G1} whole flies, and although upregulation was not as great as for the other four genes, the expression differences were still statistically significant (Figure 4.3). This may be due to higher expression of these genes in the digestive system than in the head.

Overexpressing the unedited wild type *Adar 3/4* or inactive *Adar 3/4 EA* in the *Adar*^{5G1} null fly background were both sufficient to correct the expression levels of *AttD, Def*, and *Drs* (Figure 4.3). Although the *Adar* constructs reduced *TotA, TotC*, and *TotX* gene expression levels in the *Adar*^{5G1} background, they did not correct the expression levels of

Turandot genes as well as the other three antimicrobial peptide (AMP) genes. It is very interesting that inactive *Adar 3/4 EA* also corrected these expression changes (Figure 4.3). This indicates that the double-strand RNA binding activity of *Adar* has an important physiological function in *Drosophila*.

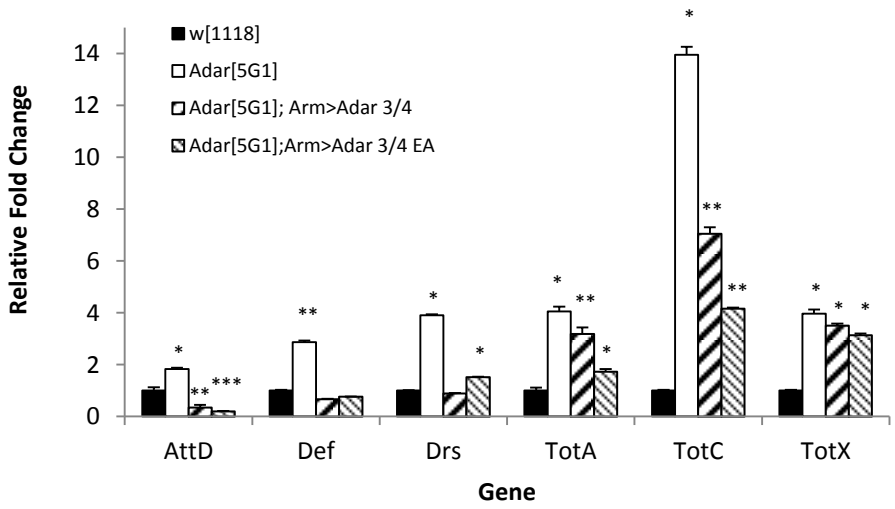


Figure 4.2 Relative immune transcript mRNA levels in *w*^{1118} wild type, *Adar*^{5G1} null or rescue flies expressing wild type *Adar 3/4* or inactive *Adar 3/4 EA* isoforms. Relative fold changes and p values were calculated compared with the expression level in *w*^{1118} wild type flies for each gene. * P<0.05, ** P<0.005, *** P<0.0005. For each gene, the expression level was normalized to the expression level in the wild type *w*^{1118} after normalizing to *Gapdh*. Error bars are standard error. Student t-test was used to calculate p value.

To examine whether *Adar 3/4 EA* also rescued the mobility of the *Adar*^{5G1} flies, I performed both open field locomotion assays and climbing assays. In both assays, overexpressing wild type *Adar 3/4* rescued the locomotion defects observed in *Adar*^{5G1}

flies as expected (Figure 4.4). However, the mobility of flies overexpressing *Adar 3/4 EA* or a construct expressing a truncated ADAR consisting only of double-stranded RNA binding domains of *Adar: Adar 3/4 dsRBD* gave different results in the two assays.

The open field locomotion assay shows the horizontal movement of the flies after being tapped to the bottom of a petri dish. No significant rescue of the $Adar^{5G1}$ by inactive *Adar 3/4 EA* or *Adar 3/4 dsRBD* was detected. This lack of rescue was seen when either motor neuron driver *OK6-GAL4* or ubiquitous driver *armadillo-GAL4* was used (Figure 4.4A). However, both the mutant constructs showed significant rescue in the climbing assay, although not as much as *Adar 3/4* (Figure 4.4B). A fly in the narrow column climbs up to the top naturally; a response known as negative geotaxis (Gargano and Martin 2005). Most wild type flies climb to the top in 20 seconds after being tapped to the bottom of the column but $Adar^{5G1}$ flies climb up to only approximately 20% of the height of the column in 1 minute. Interestingly, specifically overexpressing these constructs in motor neurons gave better rescue compared with using the *armadillo-GAL4* driver, suggesting that increased expression of *Adar* in motor neurons improves locomotion (Figure 4.4B).

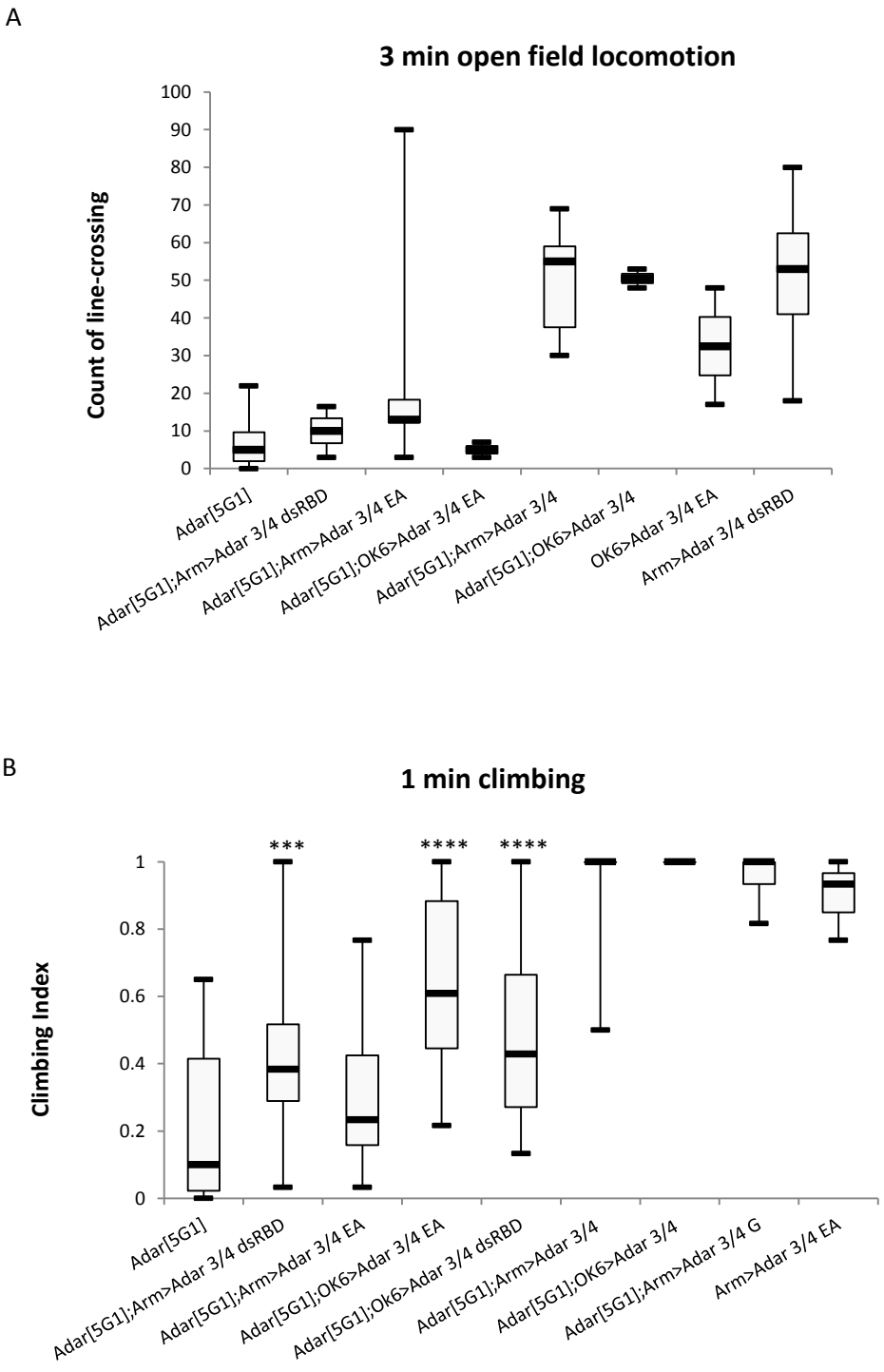


Figure 4.3 Locomotion of *Adar*^{5G1} flies and flies over-expressing catalytically active or inactive constructs of *Adar*. (A) 3 minute- open field locomotion. The Y axis shows the number of lines that the flies cross in 3 minutes. Positive controls are *Arm>Adar 3/4 dsRBD* and *FM7; OK6>Adar 3/4 EA* overexpressing *Adar* constructs in the wild type

background. (B) 1 minute climbing. The Y axis shows the climbing index of the flies. The positive control is *Arm>Adar 3/4 EA* expressed in the *Adar* wild type background. *** P<0.0005 **** P<0.00005 The P values are for comparisons with $Adar^{5G1}$ flies. Student t-test was used to calculate p value.

4.2.3 Heterozygous *Tor* mutations or *Atg5* overexpression rescue locomotion defects and gene expression changes in $Adar^{5G1}$ flies.

Paro found that reduction in *Tor* expression, or *Act-GAL4*-driven overexpression of an autophagy gene *Atg5* rescued reduced viability and open field locomotion defects in $Adar^{5G1}$ flies (Paro Thesis. University of Edinburgh 2012). I confirmed the rescue of the locomotion defect in $Adar^{5G1}$ by the hypomorphic P element insertion mutant Tor^{K17004} and by overexpression of *Atg5* using the *Act-5c-GAL4* driver in the climbing assay (Figure 4.5).

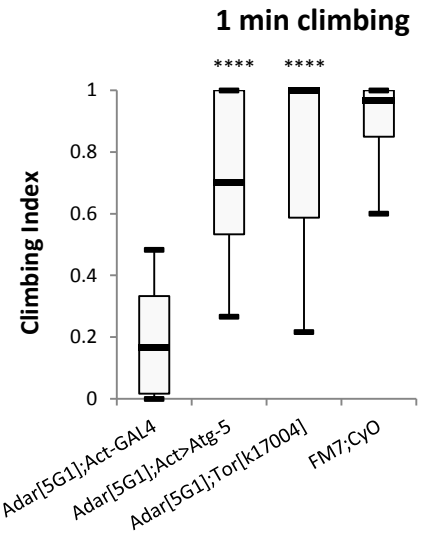


Figure 4.4 Rescue of *Adar*^{5G1} climbing defects rescue by *Tor*^{K17004} mutant and by *Atg-5* overexpression. *Adar [5G1];Act 5c-GAL4* is the negative control, and *FM7;CyO* is the positive control. **** P<0.0001, compared with *Adar [5G1];Act 5c-GAL4*. Student t-test was used to calculate p value. Box plot: Five lines from top to bottom are maximum, third quarter, median, first quarter, and minimal climbing indexes, respectively.

To examine whether upregulation of immune response genes is prevented by the *Tor*^{K17004} mutant or by overexpression of *Atg5*, I conducted qRT PCR analyses of stress and immune gene expression in the *Adar*^{5G1} flies. Either the *Tor*^{K17004} mutation or *Atg5* overexpression reduced the expression levels of *TotX, TotC,* and *TotA* significantly, but surprisingly increased *AttD* expression level (Figure 4.6). *Tor*^{K17004} decreased the expression levels of *Drs* and *Def* while overexpressing *Atg5* did not show any effect (Figure 4.6). This suggests that the rescue of the *Adar*^{5G1} null mutant phenotype by up-regulation of autophagy rescues the types of stress that drive *Tot* gene induction but the effects on immune gene expression are less predictable.

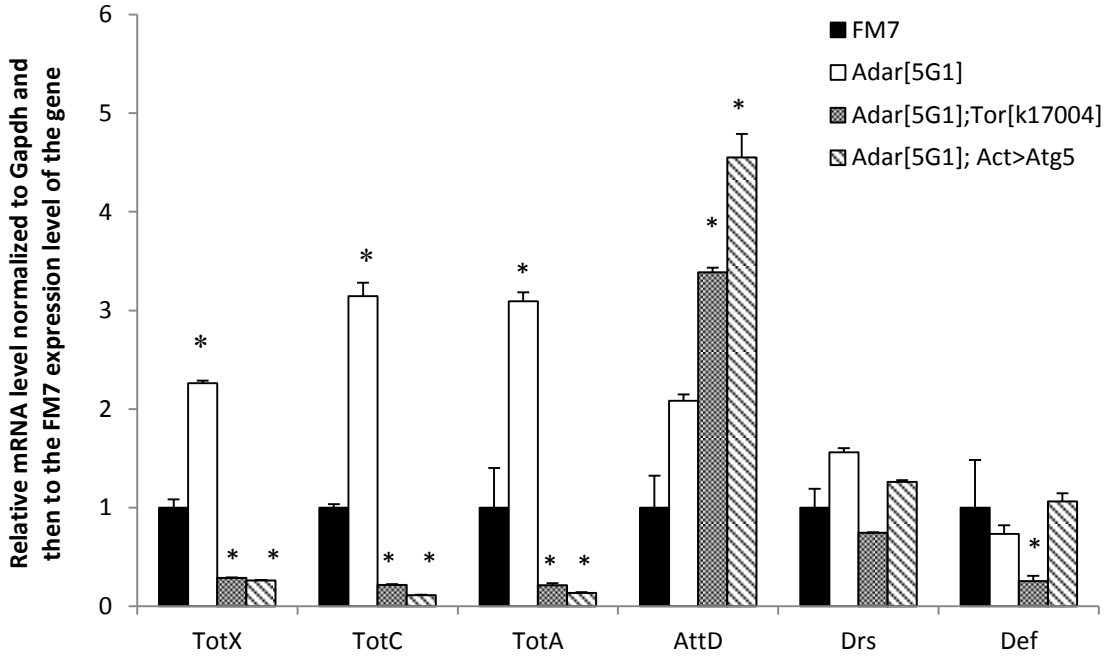


Figure 4.5 Relative mRNA levels of selected stress and immune genes in w^{1118} wild type, $Adar^{5G1}$ and in flies rescued by either Tor^{K17004} or ubiquitous overexpression of *Atg5* by *Actin 5c-GAL4* driver. Relative fold changes and P values were calculated compared with the expression level in w^{1118} wild type flies for each gene. * P<0.05. For each gene, the expression level was normalized to the expression level in the wild type w^{1118} after normalizing to *Gapdh*. Error bars are standard error. Student t-test was used to calculate p value.

Do the $Adar^{5G1}$ flies have activated autophagy caused by multiple stresses? Since most of the neural-behavioural phenotypes and abnormal transcriptome changes were detectable in the 5-day old adult flies, I examined autophagy level by Lysotracker Red staining the adult mid gut of $Adar^{5G1}$ flies, and quantified the staining intensity and the size of the stained dots. There was no significant change between $Adar^{5G1}$ adult fly gut and wild type flies (Figure 4.7).

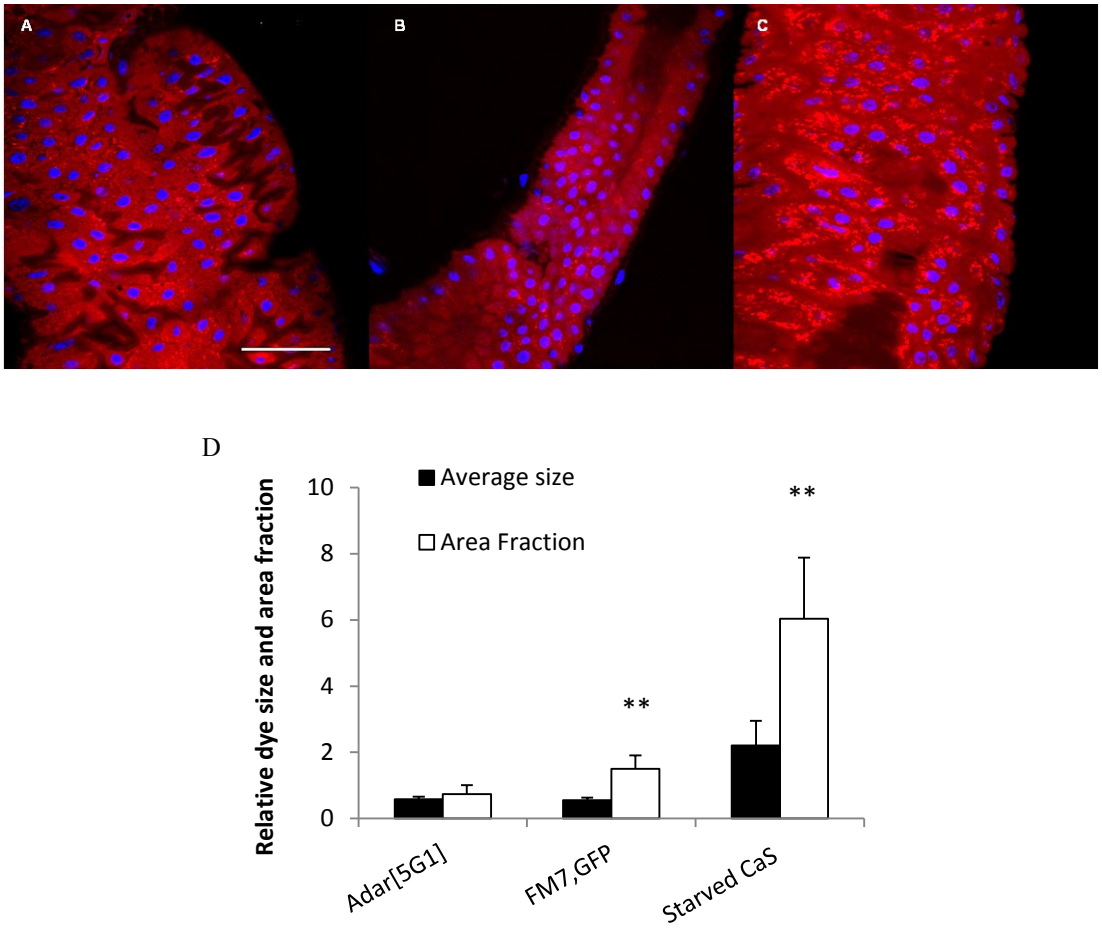


Figure 4.6 LysoTracker Red staining of *Adar*^{5G1} adult guts. A-C Red is LysoTracker Red staining, and blue is DAPI nuclei staining. Scale bar: 50µl. (A) *Adar*^{5G1}. (B) *FM7,* wild type. (C) Starved *CaS* as a positive control. (D) Quantification of the LysoTracker Red positive dots. The analysis was done in Image J. The average size and the total number of dots in an area of the same size was counted using the script in the software. Black columns show the average size of the dots and the white columns show the average area fraction. Three areas in each gut, and three different guts of each genotype was used for quantification. Error bars indicate SEM. ** P<0.005, comparison with *FM7,GFP* wild type flies. Student t-test was used to calculate p value.

4.2.4 MARCM analysis of *Adar*^{5G1} null cells in fat bodies and in the brain.

Fat bodies from early third instar *Adar*^{5G1}*, P{neoFRT}19A / P{neoFRT}19A, P{tubP-GAL80}LL1, P{hsFLP}1, w[*]; Collagen-GAL4/ P{UAS-mCD8::GFP.L}LL5* flies was dissected after clones were generated in embryos. There was variation in LysoTracker Red signal among different sheets of fat bodies (Figure 4.8; compare A to D, and C to F.). No differences in the LysoTracker Red intensity were detected in *Adar*^{5G1} homozygous clone cells compared with neighbouring wild type or heterozygote cells. This suggests that upregulation in the Lysotracker Red staining in the fat body sheets of individual larvae may be an indirect metabolic effect rather than being triggered directly by knocking down *Adar* cell autonomously (Figure 4.8).

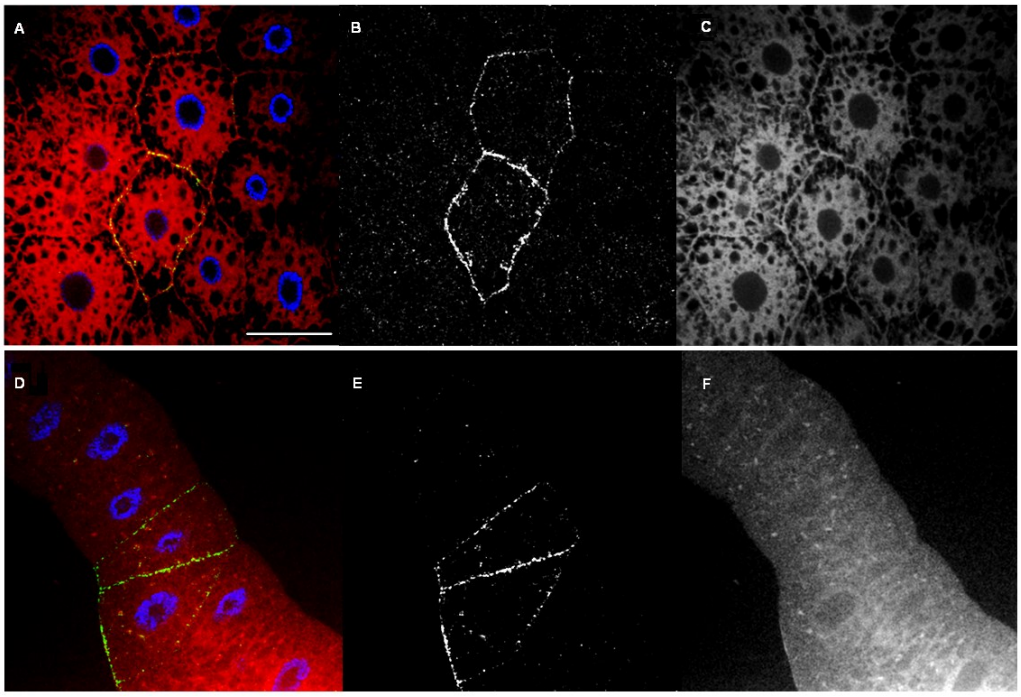


Figure 4.7 No increased LysoTracker Red staining in *Adar*^{5G1} cell clones in the fat body.Membrane GFP marks *Adar*^{5G1} null cell membranes. Blue is DAPI. A-C and D-F are two different sheets of fat body derived from two individual flies. Scale bar: 50µm.

To examine whether neuronal loss occurs in the *Adar*^{5G1} flies with the development of vacuoles, and to determine when and how the neuronal loss occurs, I used the MARCM system to mark and trace *Adar*^{5G1} mutant neurons in aging flies. Vacuoles were detected in the MB calyces in the *Adar*^{5G1} flies (McGurk Thesis, University of Edinburgh 2008). Therefore, I generated the *Adar*^{5G1} null cells in the MB neurons using the *201Y-GAL4* driver. The *Adar*^{5G1}*, P{neoFRT}19A / P{neoFRT}19A, P{tubP-GAL80}LL1, P{hsFLP}1, w[*]; 201Y-GAL4/ P{UAS-mCD8::GFP.L}LL5* animals were heat-shocked at embryo or first instar larval stages and the brain was dissected when flies were 30 days old, because the massive vacuole formation was detected by day 30 in the *Adar*^{5G1} flies. The GFP signals in the individually marked *Adar* null cholinergic neurons were still observed in 30-day old mosaic fly brains (Figure 4.9). The morphologies of the individual *Adar*^{5G1} cholinergic neurons were compared with wild type single neuron morphologies (Figure 4.9E) from an on-line data base (A.-S. Chiang et al. 2011). The database gives reconstructed single neuron confocal images from wild type MARCM clones driven by different neuronal drivers in the wild type. I also generated wild type cholinergic neuronal clones in *P{neoFRT}19A / P{neoFRT}19A, P{tubP-GAL80}LL1, P{hsFLP}1, w[*]; Cha-GAL4/ P{UAS-mCD8::GFP.L}LL5* flies to compare with *Adar*^{5G1} null neurons. In most of the cases, the *Adar*^{5G1} null neurons still maintained axon branches and looked normal morphologically (Figure 4.9 C-D'). Nevertheless, swelling in the axon or fragmentation of the axon branches was sometimes observed (Figure 4.9 B, B' and E, E') in the midbrain.

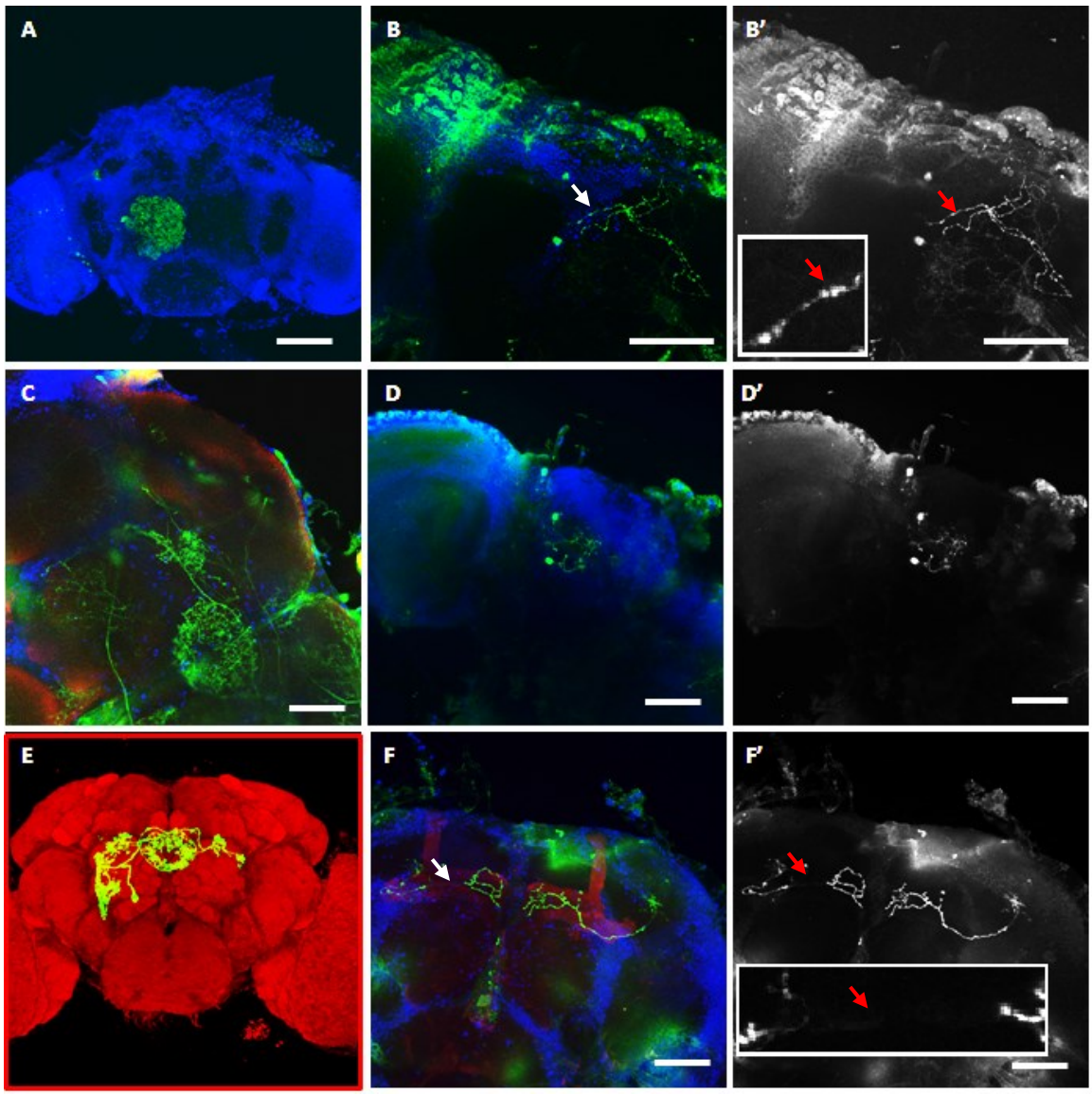


Figure 4.8 Example confocal images of *Adar*^{5G1} cholinergic neurons and mushroom body gamma neurons. The images are z-stack projections. (A and A') Wild type cholinergic neuron clones generated by MARCM using the *Cha-GAL4* driver. (B-D') *Adar*^{5G1} neurons generated using *Cha-GAL4*. The arrow points to an axonal branch where the swelling occurred and the inset box in B' shows an enlargement of this region. (E) Image from the online data base: http://flycircuit.tw/modules.php?name=clearpage& op=detail_table& idid=13456, ChaMARCM-F000194_seg001_lsm, shows the same neuron as the *Adar*^{5G1} clone shown in C. C and E marked the same neurons. (F and F') *Adar*^{5G1} neuron generated using *201Y-GAL4*. The arrow points to the axon where degradation occurred. (A-D', F and F') Green: mCD8-GFP marking *Adar*^{5G1} null neuron membrane. Red: FasII staining of MB gamma neurons in (F). Blue: DAPI staining. All

the images are the CNS from 30 day old flies while the clones were generated 16hours after 24 hour egg collection. Scale bar: 50μm.

4.3 Discussion

Comparison of Poly A+ mRNA sequencing data of w^{1118} wild type and *Adar* ^{5G1} heads suggests that the $Adar^{5G1}$ mutant does not affect transcriptional levels of the edited transcripts more than other transcripts.

There are several possibilities to explain for the transcriptional changes in the *Adar* ^{5G1} flies. Loss of editing in some RNAs may lead to further transcriptional changes in other genes. Changes in the A-to-I edited level in the translated transcripts may further induce the changes in the transcription of other genes. For instance, loss of editing in the transcripts encoding membrane proteins like ion channels cause abnormal ion transmission in neurons (Jepson and Reenan 2009) that may cause stress to the cells. As a response, the cells activate expression of heat shock proteins and a range of emergency responses. These possibilities are not mutually exclusive.

Approximately 20% of esiRNAs (Kawamura et al. 2008), and some pre-miRNAs are edited in *Drosophila* (Heale et al. 2009). Differences in transcriptional levels of miRNA or siRNA target genes may be caused by changes in the amount of miRNA or siRNA production or by retargeting or other effects on mature miRNA or esiRNA due to A-to-I conversion. In *C. elegans*, it has been shown that loss of ADAR causes an accumulation of RNAi-dependent 23-24nt small RNAs from several loci throughout the genome that encode A-to-I edited dsRNAs in wild type (Wu et al. 2011). This supports a competitive role for ADARs acting against the RNAi pathway. It is possible that the fly genome shares a similar feature.

Alternatively, loss of dsRNA binding activity of ADAR may account for some of the transcriptional changes. Catalytically inactive ADAR with only two functional dsRNA binding domains corrects some of the down or up regulated genes when overexpressed in cholinergic neurons of *Adar* ^{5G1} flies (Hogg Thesis, University of Edinburgh 2011), which strongly indicates that the dsRNA binding activity of *Adar* may control transcriptional levels of some genes. If this is true, there must be a set of transcripts with

important physiological roles that ADAR binds to. This hypothesis can be supported by the existence of ADAR3 in mammals that does not have editing activity but retains dsRNA binding function. This hypothesis is further supported by the fact that inactive human ADAR1 p150 can inhibit the siRNA pathway in *Drosophila* (Heale, Keegan and O'Connell 2009). This provides the possibility that the unedited but bound targets of ADAR are some specific double-stranded small RNAs.

The over-represented gene categories among $Adar^{5G1}$-reduced transcripts are mainly involved in catabolic pathways and in reproduction while the upregulated transcripts respond to stress or infection. These gene enrichment results except the up-regulation of immune-related genes may be easily explained by the physiological adaptation to the stress caused by loss of *Adar*. However, the role of vertebrate ADAR1 clearly involve the immune system (Taylor et al. 2005; Toth et al. 2009). Significant upregulation of immune-related genes is intriguing. It may be the case that $Adar^{5G1}$ has significant upregulation of dsRNAs encoded by repetitive sequences and that accumulation of the dsRNAs from these sources are sufficient to induce anti-viral responses when ADAR is absent. Some individual transcripts of *Gypsy* and other retrotransposons show increased levels in the poly A+ RNA seq data but a more complete analysis requires total RNA Seq after ribosomal RNA depletion. Elevated dsRNA could induce immune genes through dsRNA sensor. Dicer2 has been proposed to act as such a sensor (Wang et al. 2006).

More interesting still is the rescue of $Adar^{5G1}$ gene expression changes by the inactive *Adar* construct. Catalytically inactive *Adar* rescued the neurodegeneration phenotype (McGurk Thesis, University of Edinburgh 2008 and Hogg Thesis, University of Edinburgh 2010.), and also improved the mobility of *Adar* null flies in the climbing assay. Undoubtedly, inactive ADAR protein has an important physiological role that requires binding to some double-stranded RNAs. ADARs may interact with other dsRNA-binding proteins such as the immune RNA sensors, to prevent them from signaling to induce immune responses. Using CLIP and other related techniques, we may be able to find a new set of the *Adar* target transcripts whose structured RNAs bind

to ADAR. Once these new target transcripts are found, we may be able to examine whether these dsRNAs trigger immune response in the animal.

Although we do not know how the innate immune response is induced, I confirmed that expression levels of *TotA*, *TotC*, and *TotX* are significantly upregulated in the $Adar^{5G1}$ fly. Expression levels of AMP genes *Drs*, *Def* and *AttD* are also moderately altered although the directions of changes are sometimes different between the head RNA Seq data and whole bodies overall. Induction of *Tot* genes can be triggered by a variety of severe environmental stresses including infection, heat stress, oxidation stress and other insults (Ekengren et al. 2001). Compared with the induction of heat-shock proteins or AMP, the upregulation of *Tot* genes is strong and consistent (Ekengren and Hultmark 2001).

Overexpressing *Adar 3/4 EA* or wild type *Adar 3/4* did not bring the expression of *Tot A, C* and *X* down to wild type levels, but decreased the expression of the AMP genes *AttD, Def*, and *Drs* to wild type levels. Overproduction of *Tot* genes is known to be protective and expression of one *Tot* gene does not induce more *Tot* gene expression or immune genes (Ekengren and Hultmark 2001). The high expression of *Tot* genes even when the *Adar* constructs were expressed in $Adar^{5G1}$ null flies suggest that flies overexpressing these *Adar* constructs are still under some stress, possibly caused by ectopic overproduction of ADAR protein per se. The down-regulation of AMP genes even by inactive ADAR may indicate that dsRNA binding activity of *Adar* can eliminate innate immune response caused by loss of ADAR. Is ADAR protein more specifically involved in the immune gene control whereas increased autophagy rescues a more general stress response? Might *Adar* knockout lead to an accumulation of immunogenic dsRNA whose accumulation or immunogenic property is normally prevented by binding to ADAR protein? To test this hypothesis more fully across the whole genome, we have sent RNA samples from $Adar^{5G1}$ null flies and wild type flies to be sequenced for total RNA and small RNA.

The effects of Tor^{K17004} mutant and of *Atg5* overproduction on the expression levels of *Tot* genes and AMP genes were quite different from the effects of the *Adar* constructs. The *Tot* gene expression levels were dramatically reduced while the AMP gene expression levels were not consistently affected. This suggests that increased autophagy removes the key cellular stress that underlies important phenotypes, but not the induced immune response caused by loss of *Adar.* Innate immune genes respond very selectively to different infections, so a more general examination of transcription may be needed to be clear about immune gene transcript effects.

Whether autophagy is activated in the $Adar^{5G1}$ null flies is unclear. In the 5-day old fly heads, no changes in transcripts of genes involved in autophagy were detected from RNA sequencing. In addition, I did not see differences in LysoTracker Red staining in guts of 5-day old $Adar^{5G1}$ flies compared to wild type flies. $Adar^{5G1}$ flies of 5 day old have not developed vacuoles in the brain yet, but show severe locomotion defects and an accumulation of stress gene transcripts most of which are likely to be cleared by upregulated autophagy. These results indicate that although causing severe stress, loss of *Adar* does not seem to induce a high level of autophagy in the young adult flies. Nevertheless, increased LysoTracker red dots were observed in the $Adar^{5G1}$ null larval fat body (Paro Thesis, University of Edinburgh, 2012). This did not seem to be a cell-autonomous effect, based on the MARCM clone analysis. The acidified lysosomes detected by LysoTracker represent the final step in autophagy (Levine and Klionsky 2004). If defects arise in the autophagy pathway itself, then increased acidified lysosomes may not be seen. Autophagy will need to be assessed using western blots to detect LC3 isoforms (Levine and Klionsky 2004). I did not detect differences in the LysoTracker Red dots between the homozygous $Adar^{5G1}$ fat body cell clones and the neighbouring wild type or $Adar^{5G1}$ heterozygous cells.

Mosaic analysis showed morphological defects in $Adar^{5G1}$ null neurons in the midbrain. Loss of *Adar* does not cause axonal degradation in every mutant neuron by 30 days. However, the incidence is much higher than in the wild type neurons. This again indicates that $Adar^{5G1}$ null neurons are much more prone to neurodegeneration. Innate

immune genes have been shown to play an important role in neurodegeneration (Nguyen et al. 2002; Greene & Whitworth 2005). So activation of immune genes in *Adar*^{5G1} may contribute to neurodegeneration. The cell-autonomous neural degeneration phenotype of *Adar*^{5G1} do not exclude the possibility that abnormal immune induction in *Adar* null glial cells also plays an important role in neurodegeneration in the *Adar*^{5G1} null flies. The *Adar*^{5G1} MARCM clone system can be used in future for a further study of cell-autonomous effects of *Adar*^{5G1} by staining with further cell markers, or to design a screen for rescue in a well-controlled *in vivo* system.

In summary, the experiments conducted in this chapter allow me to draw three main conclusions. First, innate immune genes and general stress response genes were up-regulated in the *Adar*^{5G1} null adult flies. Second, inactive *Adar* with dsRNA binding activity is capable of reducing the expression levels of AMP genes fully, and *Tot* genes partially, suggesting that ADAR protein affects primarily the innate immune response. In contrast, *Tor*^{K17004} mutation or *Atg-5* overproduction reduce the expression level of *Tot* genes but not the AMP genes fully, indicating that increased autophagy removes a more general stress caused by loss of *Adar*. Third, loss of *Adar* causes axon swelling and degradation cell-autonomously. Future experiments may focus on elucidating the role of dsRNA binding activity of *Adar* in innate immunity in *Drosophila*, and clarifying the stress caused by *Adar*^{5G1} at a single cell level.

5 CHAPTER V: Genetic screen for heterozygous deficiencies on Chromosome III that rescue the reduced viability of *Adar* ^{5G1}.

To kill an error is as good a service as, and sometimes even better than, the establishing of a new truth or fact.

- Charles Darwin

5.1 Introduction

The *Adar*^{5G1} null and *Adar*^{1F4} hypomorphic mutant flies show strong adult neural-behavioral defects (full description: Section 1.2.2, Chapter 1). Despite all these defects, the originally characterized *Adar*^{1F4} mutant which was subsequently shown to be strong loss of function rather than a null mutant is morphologically normal and not short-lived (Palladino et al. 2000a). The *Adar*^{5G1} null mutant fly strain bears a deletion that removes the entire *Adar* gene (Palladino et al. 2000a), resulting in the strongest phenotypes among the existing *Adar* mutant fly strains in terms of neurodegeneration, locomotion defects and low viability (McGurk Thesis, University of Edinburgh, 2008). For *Adar*^{5G1} though not for *Adar*^{1F4}, the reduction in viability at eclosion was sufficient to allow a viability rescue screen. The low viability may be an indication of the physiological competitiveness of *Adar*^{5G1} null larvae. Our starting hypothesis was that a deletion rescuing the low viability of *Adar*^{5G1} flies will bypass ADAR to remove general stress or other specific defects that lead to neural-behavioural phenotypes in *Adar*^{5G1}. If this is true, rescuers of the low viability will probably also rescue locomotion or neurodegeneration in *Adar*^{5G1} flies.

DrosDel, a very complete collection of well-defined genome deletions, became publically available in 2007, and this deficiency collection provided a genetic tool for the viability screen of *Adar*^{5G1} flies. The DrosDel deletion set made high-throughput genome-wide screens more interpretable because the DrosDel set is composed of molecularly mapped deletions on an isogenic background, covering ~77% of the Release 5.1 genome for *Drosophila melanogaster*. Each deletion covers an average of 44 genes or 368 kb. The Exelixis collection is also generated in an isogenic background, but with a much smaller average deletion size of 140kb (Parks et al. 2004). Both of these deficiency kits, in addition to the original and more recently made Bloomington Stock Centre (BSC) deficiencies, are now available to the public from Bloomington *Drosophila* Stock Centre (BDSC). All the DrosDel, Exelixis and new Bloominton deficiencies were generated by FRT-FLP recombination between P-element containing FRT sites which makes the deletion breakpoints precise and these have been confirmed

by sequencing (Roote and S Russell 2012). Compared with DrosDel deficiencies and Exelixis deficiencies, the size of BSC deficiencies vary from as small as one gene deletion to more than a hundred genes removed by a single deficiency. A combination of these three collections, assisted with old deficiencies of which the breakpoints are known with only cytogenetic accuracy, covers up to 98.9% of all the chromosome arms (Table 5.1, (Roote and Russell 2012)). This makes a genome wide screen for genetic modifiers of a mutation possible using the available deficiency kits.

Table 5.1 Percentage coverage of euchromatic genes by genetic deletions.

Chromosome arm	BSC deletions	Exelixis deletions[a]	DrosDel deletions[a]	BSC, Exelixis and DrosDel deletions	Unique to BSC deletions[b]	Other deletions[c]	All available deletions
X	82.7	18.0	57.6	92.3	26.9	5.9	98.1
2L	70.5	68.2	71.7	96.0	12.0	3.0	98.9
2R	88.5	45.7	52.4	95.9	26.8	2.3	98.2
3L	84.1	39.3	69.4	95.3	15.3	2.5	97.5
3R	83.0	59.6	73.0	95.7	11.4	3.2	98.9
4	0	0	54.1	54.1	0	42.4	96.5
Total	81.5	47.4	65.2	94.9	17.9	3.5	98.4

[a]Coverage by Exelixis or DrosDel deletion stocks maintained at the BDSC. Some deletions reported by Exelixis [5] were false positives; stocks for other deletions were lost. Stocks for some DrosDel deletions [9] were too weak to maintain. [b]Coverage provided by BSC deletions, but not Exelixis or DrosDel deletions. [c]Chemical- or irradiation-induced deletions plus *FRT*-derived deletions from individual investigators.

Cook R, Christensen S et al.*Genome Biology* 2012, 13:R21

Prior to the thesis work, Paro carried out a small scale deficiency screen on the left arm of Chromosome II for rescuers of $Adar^{5G1}$ low viability. She found that Df(2L)ED778 rescued the viability of $Adar^{5G1}$ significantly and mapped the effect to the *Tor* gene (Paro Thesis, University of Edinburgh, 2012). Further, she demonstrated that reduction in *Tor* or overproduction of *Atg5* also rescued neurodegeneration and locomotion defects in $Adar^{5G1}$ flies (Paro Thesis, University of Edinburgh, 2012). With this successful screen result on the Chromsome II L, we decided to do a large scale complete screen for deficiencies rescuing $Adar^{5G1}$ viability.

By doing a deficiency screen for *Adar*^{5G1} viability rescue, we expected to find positive results with deficiencies that deleted one of these genes:

1. Negative regulators of autophagy, such as *Drosophila* homologs of mammalian Bcl-2, NAF-1, PtdIns(3)-P phosphatase, Jumpy etc. (Liang 2010), that would further confirm the rescue observed by Paro.
2. Genes that contribute to a major stress that causes the pathological state of *Adar*^{5G1} flies. Although we predict that *Adar*^{5G1} null flies undergo a variety of stresses, including oxidation stress and probably ER stress, and stresses caused by upregulated immune responses, we do not know whether any of these stresses is a leading cause of the *Adar* mutant phenotype.
3. Genes encoding small RNAs that form double strand structures. This prediction is based on our hypothesis that the main stress in the flies may be caused by an accumulation of small RNAs that may induce stress or immune response when ADAR protein is not present.

With these hypotheses and predictions, I conducted a large scale forward genetic screen using the DrosDel deficiency set primarily, assisted by some Exel deficiencies and BSC deficiencies to increase the gene coverage. This chapter will describe the screen for *Adar*^{5G1} viability using the deficiencies on Chromosome III left and right arms.

The primary aim of the work presented in this chapter was to screen for deficiencies rescuing the low viability caused by the lack of ADAR RNA editing. Rescuing deficiencies may potentially also rescue the neural-degeneration and locomotion defects of the *Adar*^{5G1} null flies. The second aim is to better understand the physiological role of Adar on the basis of the genetic screen results.

5.2 Results

5.2.1 Six regions on Chrosomosome III rescue the low viability of *Adar*^{5G1} null.

DrosDel deficiencies (from BDSC, some were a kind gift from Dr. Penneta), Exelixis deficiencies (from BDSC) and BSC deficiencies (from BDSC) were used for the viability screen (Figure 5.1), covering approximately 80% of Chromosome III.

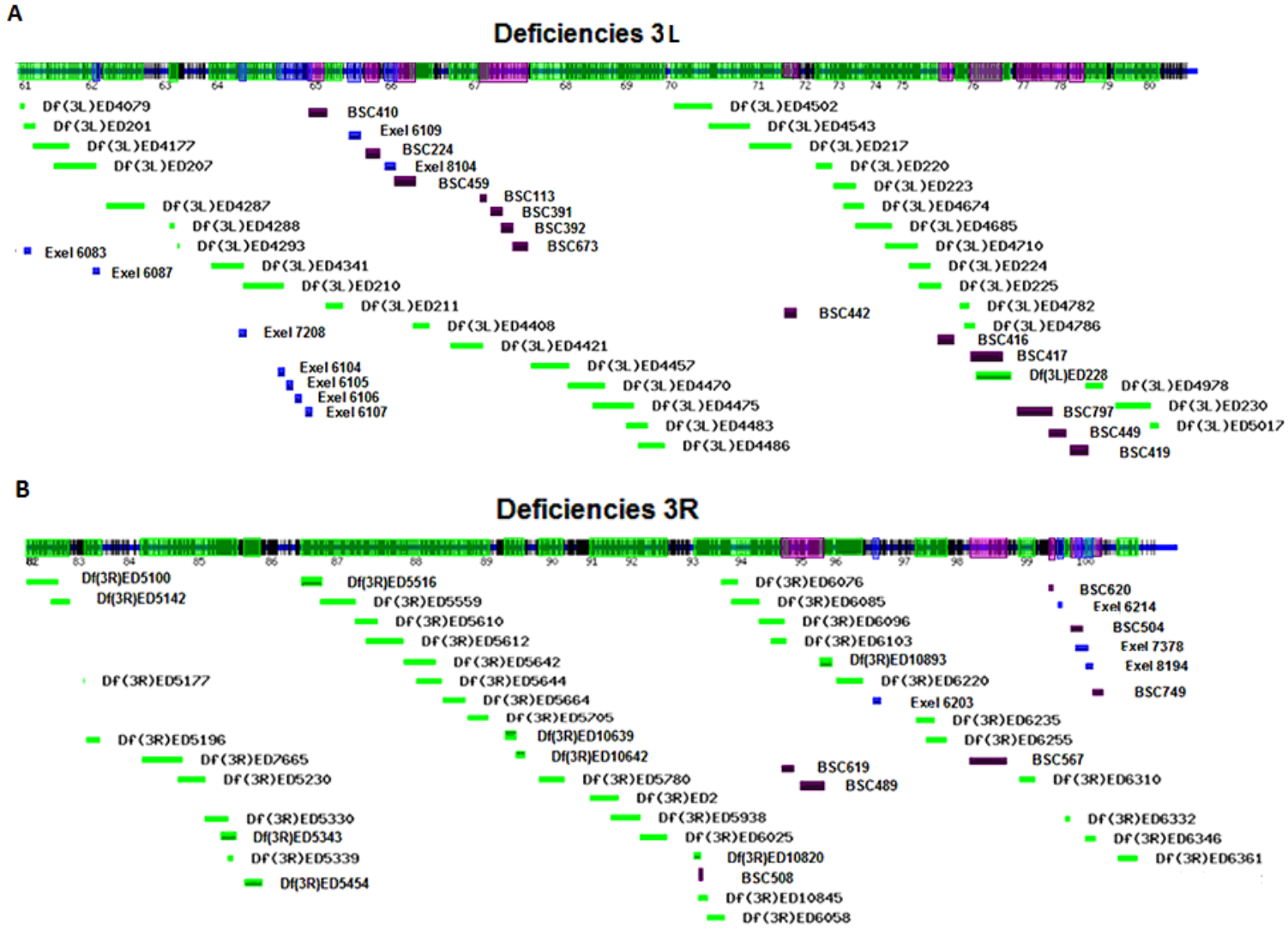

A
Deficiencies 3L
Df(3L)ED4079
Df(3L)ED201
Df(3L)ED4177
Df(3L)ED207
Df(3L)ED4287
Df(3L)ED4288
Df(3L)ED4293
Exel 6083
Exel 6087
Df(3L)ED4341
Df(3L)ED210
Df(3L)ED211
Exel 7208
Df(3L)ED4408
Df(3L)ED4421
Exel 6104
Exel 6105
Exel 6106
Exel 6107
Df(3L)ED4457
Df(3L)ED4470
Df(3L)ED4475
Df(3L)ED4483
Df(3L)ED4486
BSC410
Exel 6109
BSC224
Exel 8104
BSC459
BSC113
BSC391
BSC392
BSC673
Df(3L)ED4502
Df(3L)ED4543
Df(3L)ED217
Df(3L)ED220
Df(3L)ED223
Df(3L)ED4674
Df(3L)ED4685
Df(3L)ED4710
Df(3L)ED224
Df(3L)ED225
Df(3L)ED4782
Df(3L)ED4786
BSC442
BSC416
BSC417
Df(3L)ED228
Df(3L)ED4978
Df(3L)ED230
BSC797
Df(3L)ED5017
BSC449
BSC419
B
Deficiencies 3R
Df(3R)ED5100
Df(3R)ED5142
Df(3R)ED5177
Df(3R)ED5196
Df(3R)ED7665
Df(3R)ED5230
Df(3R)ED5330
Df(3R)ED5343
Df(3R)ED5339
Df(3R)ED5454
Df(3R)ED5516
Df(3R)ED5559
Df(3R)ED5610
Df(3R)ED5612
Df(3R)ED5642
Df(3R)ED5644
Df(3R)ED5664
Df(3R)ED5705
Df(3R)ED10639
Df(3R)ED10642
Df(3R)ED5780
Df(3R)ED2
Df(3R)ED5938
Df(3R)ED6025
Df(3R)ED10820
BSC508
Df(3R)ED10845
Df(3R)ED6058
Df(3R)ED6076
Df(3R)ED6085
Df(3R)ED6096
Df(3R)ED6103
Df(3R)ED10893
Df(3R)ED6220
Exel 6203
Df(3R)ED6235
Df(3R)ED6255
BSC567
Df(3R)ED6310
BSC619
BSC489
BSC620
Exel 6214
BSC504
Exel 7378
Exel 8194
BSC749
Df(3R)ED6332
Df(3R)ED6346
Df(3R)ED6361

Figure 5.1 Cytogenetic map positions of deficiencies tested for rescue of reduced viability *of Adar^{5G1}* (A) The deficiency map on Chromosome III L tested in the screen. (B) The deficiency map on Chromosome III R tested in the screen.The regions highlighted in green are covered by DrosDel deficiencies used; these are marked as ED followed with numbers. The violet color highlights regions deleted by BSC deletions used, and regions with blue highlights are covered by Exelixis deficiencies used. Exelixis deficiencies are marked as Exel followed by numbers. Regions without any highlights are the regions not covered in this deficiency screen, either because there were no available deficiencies for that region, or because the deficiency flies harboring heterozygous deletions were too weak and did not give enough progeny when crossed with *Adar^{5G1}* flies. The shown deficiencies are the majority of the deficiencies used in the screen. Approximately twenty more fly deficiency strains that were used for further narrowing down genetic effects are not shown in the map. The figure is modified from the DrosDel deletion official site.

The screen is based on the fact that *Adar^{5G1}* flies have a low viability, ranging from 20% to 50% compared with *FM7* balancer siblings. When heterozygous deficiencies on Chromosome III left arm (73 deficiencies) or right arm (72 deficiencies) were crossed to *Adar^{5G1}* flies, approximately one third of the *Adar^{5G1};;Df* flies showed at least a two-fold increase in their viability compared with sibling *Adar^{5G1} ;;Balancer* flies. However, the viabilities of *Adar^{5G1};;Df* flies in the different deficiency backgrounds were still lower than 80% in most of those cases. These viability comparisons (ie compared to *FM7;;Df* sibling flies) are all relative to their siblings and are affected by the population density in a single vial. Many variables in the environment like moisture and density of the food may also affect the relative viability. In order to minimize uncontrolled variables and reduce the false positive rate, a one-tailed Fisher's exact test and the Benjamini-Hochberg multiple hypothesis testing correction were used to calculate p values for the significance of the *Adar^{5G1}* viability rescue by each deficiency. Seven deficiencies from six regions of Chromosome III significantly increased *Adar^{5G1}* fly viability (Figure 5.2).

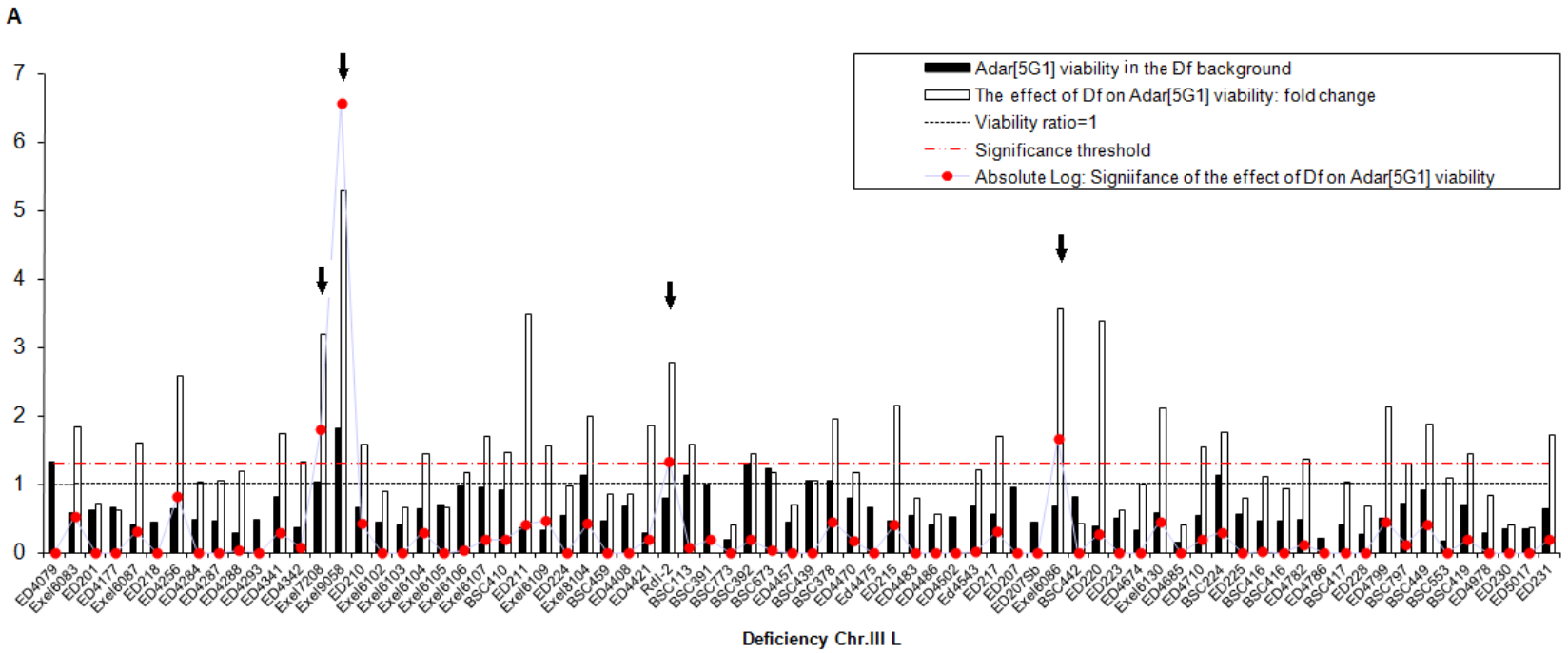
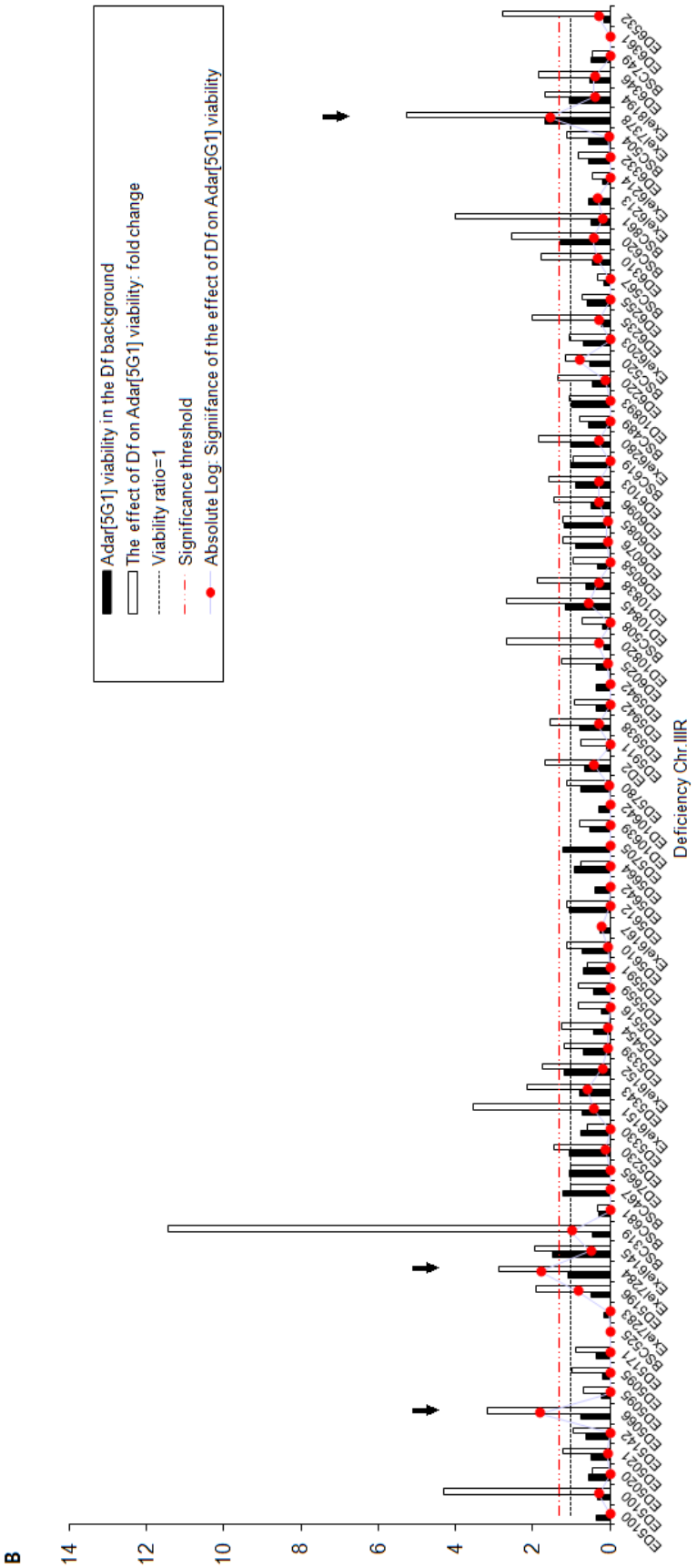

A
Adar[5G1] viability in the Df background
The effect of Df on Adar[5G1] viability: fold change
Viability ratio=1
Significance threshold
Absolute Log: Signiifance of the effect of Df on Adar[5G1] viability
0
1
2
3
4
5
6
7
ED4079
Exel6083
ED201
ED4177
Exel6087
ED218
ED4256
ED4284
ED4287
ED4288
ED4293
ED4341
ED4342
Exel7208
Exel8058
ED210
Exel6102
Exel6103
Exel6104
Exel6105
Exel6106
Exel6107
BSC410
ED211
Exel6109
ED224
Exel8104
BSC459
ED4408
ED4421
Rdl-2
BSC113
BSC391
BSC773
BSC392
BSC673
ED4457
BSC439
BSC378
ED4470
Ed4475
ED215
ED4483
ED4486
ED4502
Ed4543
ED217
ED207
ED207Sb
Exel6086
BSC442
ED220
ED223
ED4674
Exel6130
ED4685
ED4710
BSC224
ED225
BSC416
BSC416
ED4782
ED4786
BSC417
ED228
ED4799
BSC797
BSC449
BSC553
BSC419
ED4978
ED230
ED5017
ED231
Deficiency Chr.III L

Figure 5.2 Viability rescues in F1



***Adar^{5G1};;Df* males from ♀*Adar^{5G1}/FM7* X ♂*Deficiency/Balancer* crosses.** (A) Viability rescue by the deficiencies on Chromosome III left arm. (B) Viability rescue by the deficiencies on Chrosomosome III right arm. In both (A and B), on the X axis are the deficiency (Df) names in chromosome sequence order. Approximately 30% of the deficiencies overlap with each other. Black columns are *Adar^{5G1}* viability in each deficiency background, as a ratio of the numbers of *Adar^{5G1};;Df/+* and *FM7;;Df/+*. The black dashed line shows Y axis=1. White columns indicate the fold change of *Adar^{5G1}* viability that is due to the deficiency, corrected by taking account of the effect of the deficiency on viability among the *FM7* progeny (refer to Section 2.1.4, Chapter 2). Red dots are the absolute log of the p value. The p value tells the significance of the effect of deficiency on *Adar^{5G1}* viability. The red dashed line is the threshold of the significance, as the absolute log of p=0.05. Arrows indicate the cases of significant viability rescue. A one-tailed Fisher's exact test and the Benjamini-Hochberg multiple hypothesis testing correction were used to calculate p values for the significance of the Adar5G1 viability rescue by each deficiency.

The seven deficiencies that rescued *Adar^{5G1}* viability are Exel7208, Exel9058, RDL-2 and Exel6086 on Chr.IIIL and ED5066, Exel7284, and Exel7378 on Chr.IIIR (Figure 5.2). Exel9058 is entirely included in the deleted region of Exel7208. Therefore, these seven deficiencies point to six chromosomal regions that rescue *Adar^{5G1}* viability. Although their viability was increased, the rescued flies still showed noticeable age-dependent weaknesses, such as severe locomotion defects and early death, under normal handling conditions in the laboratory (most die by day 20, stuck on the food).

Nevertheless, four viability-rescuing deficiencies out of the six examined increased the locomotion of 2-day old adult flies significantly. Compared with *Adar^{5G1}* null flies, the viability rescued flies showed a wide range of climbing abilities. Apart from ED5066 and Exel6086, all the other viability-rescuing deficiencies improved the average climbing ability of the flies. The RDL-2 deficiency showed the best locomotion rescue, almost to the wild-type level and Exel9058, Exel7378, and Exel7284 showed similar moderate levels of rescue (Figure 5.3). However, only the RDL-2 rescue was comparable to the rescue by the *Tor* mutant (Figure 4.6, Chapter 4). ED5066 and Exel6086 did not improve the mobility of *Adar^{5G1}* flies at any stage.

We were interested in knowing whether the viability and locomotion rescuing deficiencies affect the general stress or immunity of the flies. I tested whether these deficiencies correct the expression levels of *Tot* genes and AMP genes in *Adar*^{5G1} flies, as seen in the rescues by *Tor* or *Adar* constructs. I examined mRNA levels of the six immune-related genes *TotA, TotC, TotX, AttD, Drs*, and *Def* using qRT-PCR in *Adar*^{5G1} flies bearing heterozygous deletions RDL-2, Exel7284, Exel9058, Exel6086 and Exel7378 respectively.

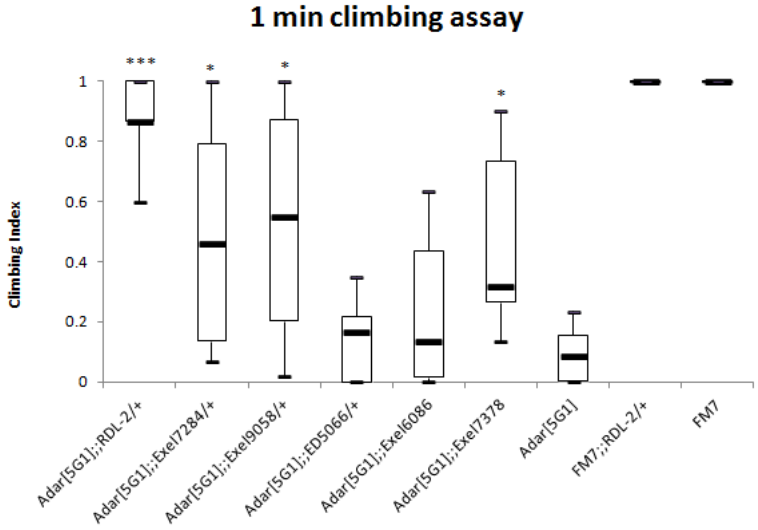


Figure 5.3 Climbing analyses of 2-day old *Adar*^{5G1} flies with deficiencies that rescue viability. Fly genotypes are shown on the X axis. The same number of flies was used for the analysis of each genotype. ***p<0.001 *p<0.05, compared with *Adar*^{5G1} flies. Student t-test is used to calculate p value. Box plot: Five lines from top to bottom are maximum, third quarter, median, first quarter, and minimal climbing indexes, respectively.

The *Def* expression level was not increased in the $Adar^{5G1}$ flies in this set of qRT comparisons, which used $Adar^{5G1}$ and *FM7* progeny from outcrosses to w^{1118} as the mutant and wild type controls. In previous qRT PCR experiments, gene expression levels were compared with w^{1118} wild type controls. This may be due to either the variability in the different batches of flies, or due to the fact that the gene expression levels were normalized to a different wild type. Although the *Def* expression level was not increased in $Adar^{5G1}$ flies compared to *FM7*, it was much reduced with heterozygous deficiencies Exel6086 and Exel9058. Exel7284, Exel7378 or RDL-2 deficiency did not affect the expression level of *Def* (Figure 5.4 and Table 5.2).

Expression of all *Tot* genes is reduced in all the rescued flies. This result indicates that the rescue of the $Adar^{5G1}$ mutant phenotype relieves stress that is induced by loss of ADAR. RDL-2 deficiency showed the least complete rescue of *Tot* gene expression levels. Surprisingly, the RDL-2 deficiency increased mRNA levels of *AttD, Drs* and *Def* in the viability-rescued flies, instead of reducing the expression as expected (Figure 5.4 and Table 5.2).

Exel6086 and Exel9058 also reduced the expression levels of *AttD*, *Drs* and even *Def*. Intriguingly, no *AttD* expression was detected at all in $Adar^{5G1}$*;;Exel9058/+* flies (Figure 5.4 and Table 5.2). If not considering *Def* expression level, Exel7284 consistently reduced expression of the other genes to the wild type level as well, while Exel7378 and RDL-2 increased these AMP gene expression level significantly (Figure 5.4 and Table 5.2).

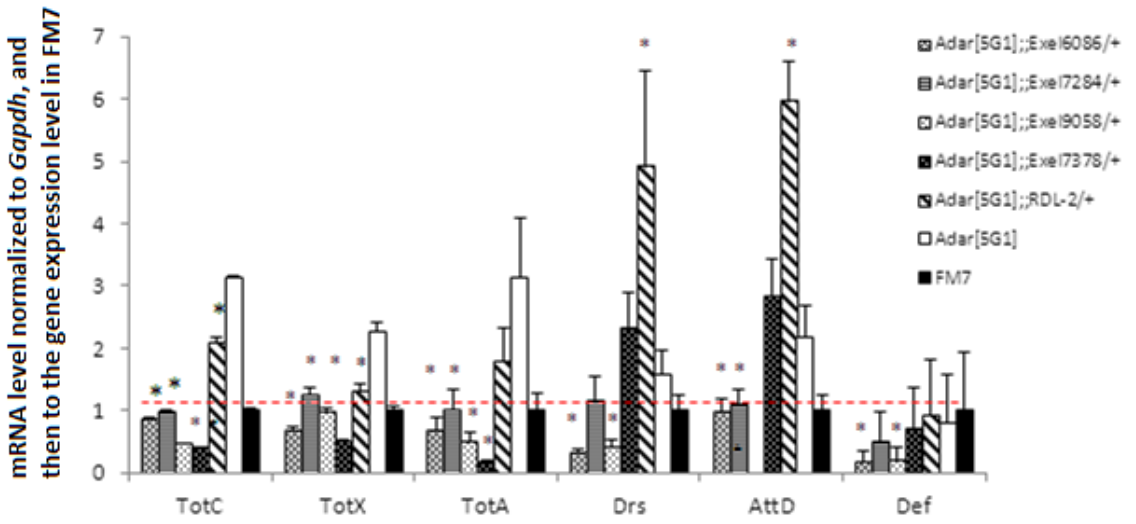


Figure 5.4 Relative expression levels of *TotC, TotX, TotA, Drs, AttD* and *Def* in *Adar*^{5G1} flies with rescuing deficiencies. Black bars represent expression levels of immune genes in *FM7* flies, used as wild type control in this assay. The expression level of each gene, as shown on the Y axis, is the relative expression level normalized twice, first to *Gapdh* expression level in the same flies, and then to the expression level of the same immune gene in the *FM7* flies. The red dashed line shows when the gene expression level is the same as in *FM7*. Error bars are S.E.M. The p values are for comparisons with the expression level of the same gene in *Adar*^{5G1}. Student t-test is used to calculate p value.

The *Tot* genes or AMP genes expression level rescues by these deficiencies were not tightly related with the level of the locomotion rescue (Table 5.2). Table 5.2 shows that overall, expression of *Tot* and AMP genes were corrected by rescuing deficiencies. The RDL-2 and Exel 7378 rescue different in detail but these also affect all the tested genes, even if the effect is most in the expected direction. In vertebrates, a set of approximately three hundred genes can be activated simultaneously by interferon. In *Drosophila*, the immune and stress genes are individually regulated (Ferrandon and Imler 2007).

Table 5.2 Summary of effects of viability-rescuing deficiencies on the expression levels of *Tot* genes and AMP genes in *Adar*^{5G1} and locomotion rescue.

Genes	Heterozygous deficiencies with *Adar[5G1]*:				
	Exel6086	**Exel7284**	**Exel9058**	**Exel7378**	**RDL-2**
TotC	↓	↓	↓↓	↓↓	↘
TotX	↓↓	↓	↓	↓↓	↓
TotA	↓	↓	↓↓	↓↓	↘
Drs	↓↓	↓	↓↓	↑	↑↑
AttD	↓	↓	↓↓↓	↑	↑↑
Def	↓	—	↓	—	—
Locomotion	—	+	+	+	+++

Downward arrows indicate that the expression levels of the genes in *Adar*^{5G1} combined deficiency is reduced compared with that in *Adar*^{5G1} flies. The number of arrows in each column indicates the degree of expression of change. One arrow indicates that the level of expression is back to near the wild type level; two arrows means that the expression level of the gene is much lower than in wild type; three arrows, only shown in the *AttD* expression level in *Adar*^{5G1}*; Exel9058/+*, means no detection of the expression from qRT PCR. '↘' indicates the case where the expression level of the gene is reduced but still higher than in the wild type. Arrows pointing up means the heterozygous deficiencies further increased the gene expression level above that seen in *Adar*^{5G1}. One or two arrows indicate the degree of increase. '—' symbols are placed in the columns where the gene expression levels were not changed in *Adar*^{5G1} compared with the wild type flies, and the deficiencies had no effect either. The significance of locomotion rescue is shown with "—" and one to three "+".

5.2.2 Mapping genes rescuing *Adar*^{5G1} within the rescuing deficiencies.

Mapping individual genes rescuing within these rescuing deficiencies was not very successful. Two unsuccessful attempts to map genes within the RDL-2 deficiency (Figure 5.5) and the Exel9058 deficiency (Figure 5.6A) are described below as examples.

5.2.2.1 Mapping genes within the RDL-2 deficiency.

The RDL-2 deficiency on Chr. 3L at 66F-67A was the only viability-rescuing deficiency that covers a gene encoding an edited transcript. Therefore, we were interested in knowing whether reduction in this edited transcript, *Rdl*, was responsible for the rescue of *Adar*^{5G1} viability. When *Adar*^{5G1} was crossed with *Rdl*^{1} or *Rdl*$^{MD\text{-}RR}$ mutant fly strains, the low viability was not rescued (Refer to Figure 3.10, Chapter 3 for information about the mutant alleles). Also, expressing shRNA against *Rdl* using the cholinergic neuron driver *Cha-GAL4* did not rescue the phenotype of *Adar*^{5G1} either (Table S4 and Figure S1 in the Appendix II). Therefore, it is not likely that reduction in *Rdl* expression accounts for the viability and locomotion rescue of the *Adar*^{5G1} flies. This was partially expected, because overexpressing *Rdl* unedited cDNA constructs in the *Adar*^{5G1} background increased the viability of the *Adar*^{5G1} flies (Figure 3.12 Chapter 3).

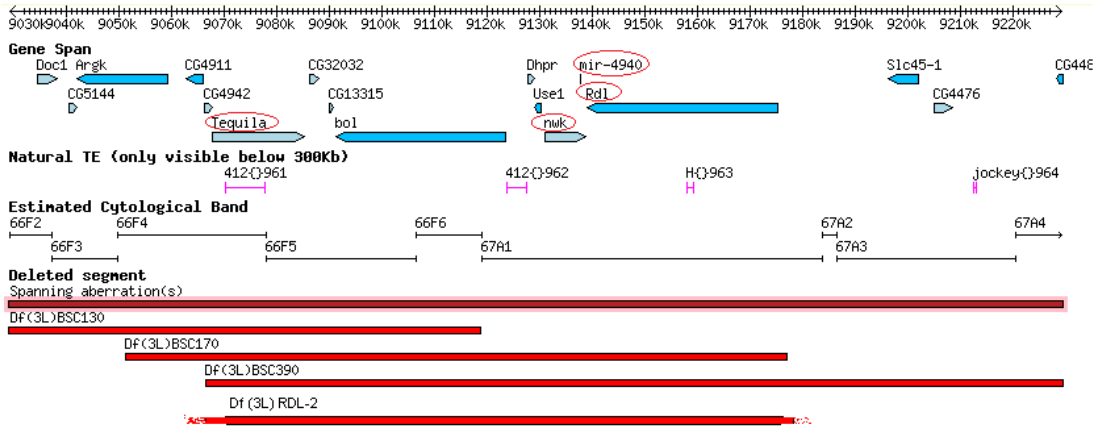


Figure 5.5 Genes and other deficiencies in the RDL-2 deficiency region. Red bars indicate the regions uncovered in the deficiencies. The break points of RDL-2 are not clear, but are reported to be in the 66F5 chromosomal region. The genes reported to be affected by the RDL-2 deletion are enclosed in the red circles.

RNAi against *nwk* did not rescue the viability or locomotion defects of $Adar^{5G1}$ either (Table S4 and Figure S1 in the Appendix II). We were interested in *mir4940* (Figure 5.6A), whose biological activities or downstream targets were not known. Very close to *Rdl*, the *mir4940* gene was deleted in the RDL-2 deficiency that rescues $Adar^{5G1}$ viability, but not affected in the Rdl^{1} mutant that did not rescue $Adar^{5G1}$ viability (Figure 5.6 B).

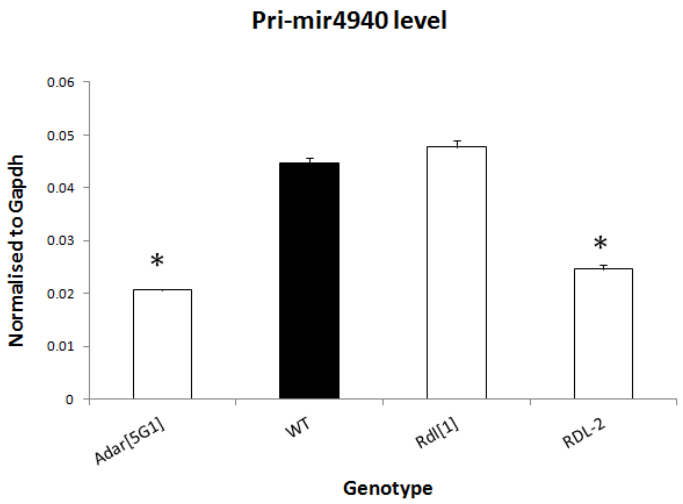


Figure 5.6 Expression levels of pri-mir4940 in adult *Adar*^{5G1} or deficiency male flies. Pri-mir4940 expression level, normalised to *Gapdh* in the adult male flies. *p<0.05, calculated in comparison with the expression level in wild type w^{1118} flies. Student t-test is used to calculate p value.

We argued that if mir4940 is responsible for the rescue, overproduction of this pri-micro RNA may worsen the phenotype of *Adar*^{5G1}. Unexpectedly, we did not get any surviving transformant flies that have UAS-mir4940 integrated in the genome.

To sum up, in the rescue of reduced *Adar*^{5G1} viability by the RDL-2 deficiency, we do not know whether there is a gene or some genes together that have a function on *Adar* viability and locomotion. The rescuing gene does not seem to *Rdl* or *nwk*. Also we could not rule out the possibility that the gene encoding mir4940 or some other proteins are responsible. It remains extremely surprising that a single deficiency such as RDL-2 rescues losses of viability associated with either ADAR loss or ADAR overexpression. No other rescuing deficiency identified in the *Adar 3/4 S* overexpression screen affected *Adar*^{5G1} viability.

5.2.2.2 Mapping genes within the Exel9058 deficiency.

The viability-rescue by Exel9058 on Chr. 3L (Figure 5.7A) was the most significant of all the rescuing deficiencies, and only one gene—*CG11357*, is indicated to be affected by Exel9058. *CG11357* is predicted to encode an N-linked glycosyl transferase (Schwientek et al. 2002). The expression level of *CG11357* in Exel9058 adult flies was 61% of that in wild type flies (Figure 5.7B). *Actin 5c-GAL4* driven RNAi knockdown of *CG11357* did not rescue the $Adar^{5G1}$ viability, although the RNAi efficiently knocked down the expression of the *CG11357* transcript (Figure 5.7B). Initially, we thought the RNAi against *CG11357* was not able to rescue because shRNA against *CG11357* knocked down expression of *CG11357* which is an essential gene, too much (Figure 5.7B). As we will show further here, mapping DrosDel rescue effects to deleted genes is unreliable. We tested whether deletions affect expression of flanking genes and found, unexpectedly, that the expression levels of the three genes (*CG32243, CG33777*, and *CG42540*) near the breakpoints of the deficiency Exel9058 increased significantly (Figure 5.7A and C). We do not know how far this effect extends but it does suggest that elevated expression of flanking genes may contribute to DrosDel rescue effects.

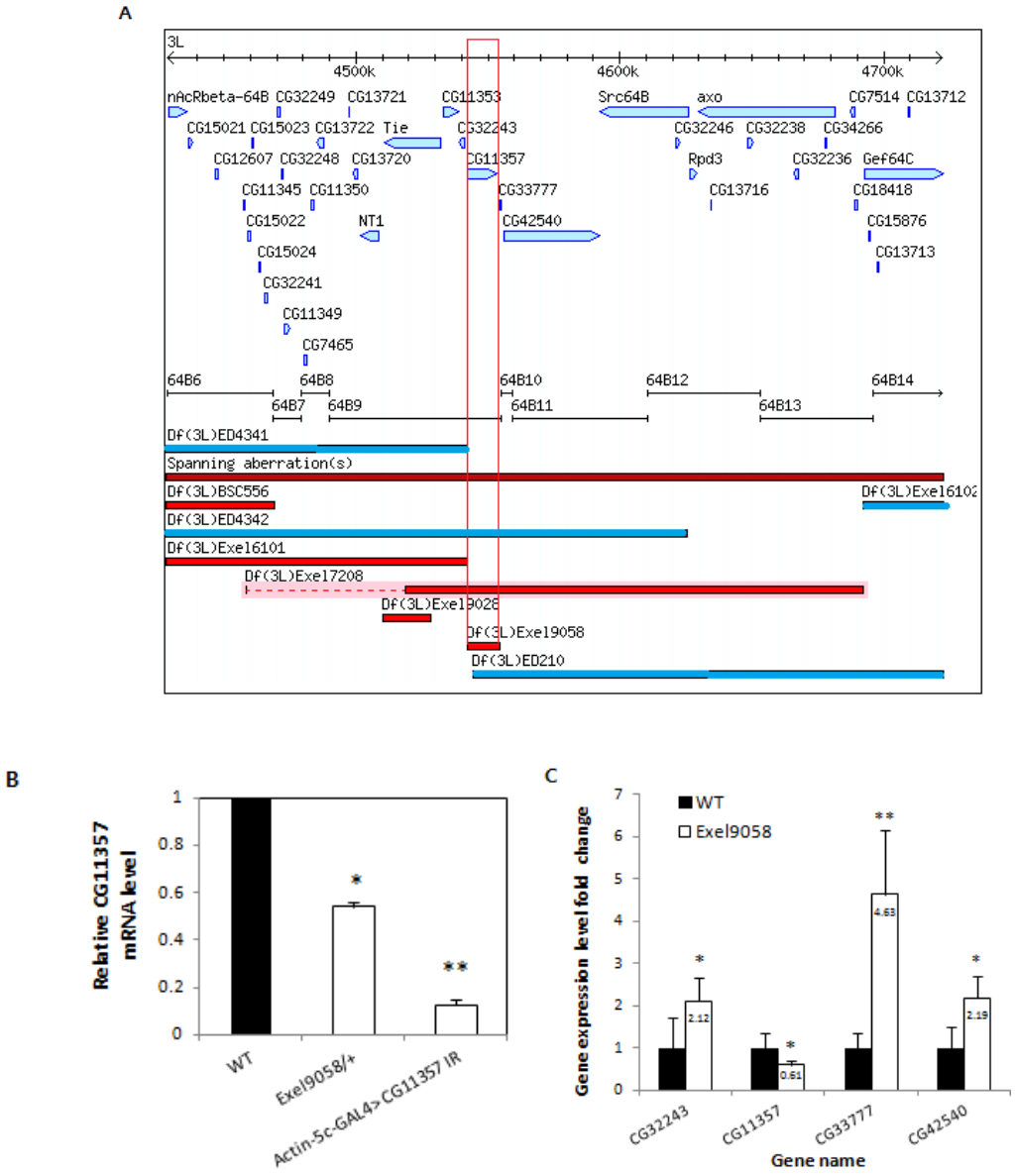


Figure 5.7 Exel9058 and the expression levels of *CG11357, CG33777* and *CG42540.* (A) The map of Exel9058 and its overlapping deficiencies. Red bars indicate the regions uncovered in the deficiencies indicated. The dashed line continuing from the left side of the Exel7208 red bar means the region is predicted to be deleted, while without solid evidence. The red box encloses the region deleted in Exel9058. The ED4341, ED4342, Exel7208, ED210 and Exel6102 were also tested in the primary viability screen. The deficiencies that did not show significant viability rescue of $Adar^{5G1}$ were highlighted with Blue. (B) Comparisons of *CG11357* mRNA expression levels in the wild type, Exel9058 and *Actin 5c> CG11357 RNAi* adult male flies, normalized to *Gapdh* level and then to *CG11357* expression level in w^{1118}. (C) Relative expression levels of *CG11357, CG33777* and *CG42540* in Exel9058 compared with w^{1118} adult male flies. For each

gene, the expression level was first normalized to *Gapdh,* and then to the gene expression level in w^{1118} wild type flies. Black columns indicate mRNA levels in the wild type flies. White columns indicate the relative mRNA level in Exel9058. * $p<0.05$ **$p<0.0005$ ***$p<0.0001$. Student t-test is used to calculate p value.

The big deficiency Exel7208 (Figure 5.7A), which deleted 237.5kb including *CG11357* and the three flanking genes mentioned above, rescued the viability of $Adar^{5G1}$, but ED4341 and ED210 that overlap with different parts of both Exel7208 and Exel9058 did not rescue the $Adar^{5G1}$ viability. Intriguingly, ED4342, a larger 354.1kb deficiency, which overlaps with Exel7208 and includes the Exel9058 region entirely, did not rescue the $Adar^{5G1}$ viability (Figure 5.1, Figure 5.4B). Considering all this information, it seems that the rescue of viability and locomotion of the $Adar^{5G1}$ flies by the deficiencies were not simply due to reduction of one single gene. In the case of the rescue by Exel9058, the viability rescue may have been caused by a combination of reduction in *CG11357* expression level and the overexpression of the neighbouring genes. The three genes close to *CG11357* are poorly annotated in the flybase. Their functions are not clear.

5.2.2.3 Mapping genes within the Exel7378 deficiency and other deficiencies.

The rescue effect of Exel7378 on Chr.3R at 100A (Figure 5.8) was narrowed down to smaller regions thanks to overlapping deficiencies that do not affect $Adar^{5G1}$ viability. Exel7378 significantly increased $Adar^{5G1}$ viability (P value=0.027), but the overlapping deficiencies BSC504 and Exel8194 that uncover parts of the same region as Exel7378 do not rescue $Adar^{5G1}$ viability (Figure 5.2B). This narrowed the genetic region to less than 70kb and ten potential rescuing genes (Figure 5.8, Table 5.3).

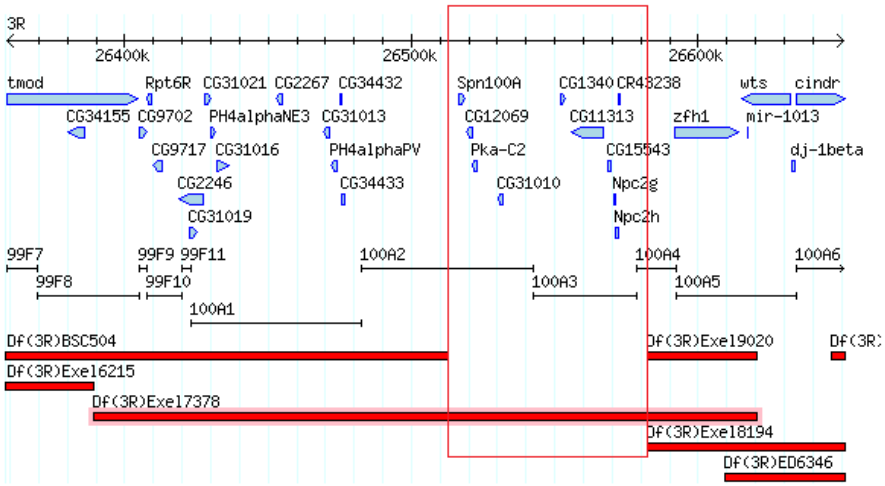
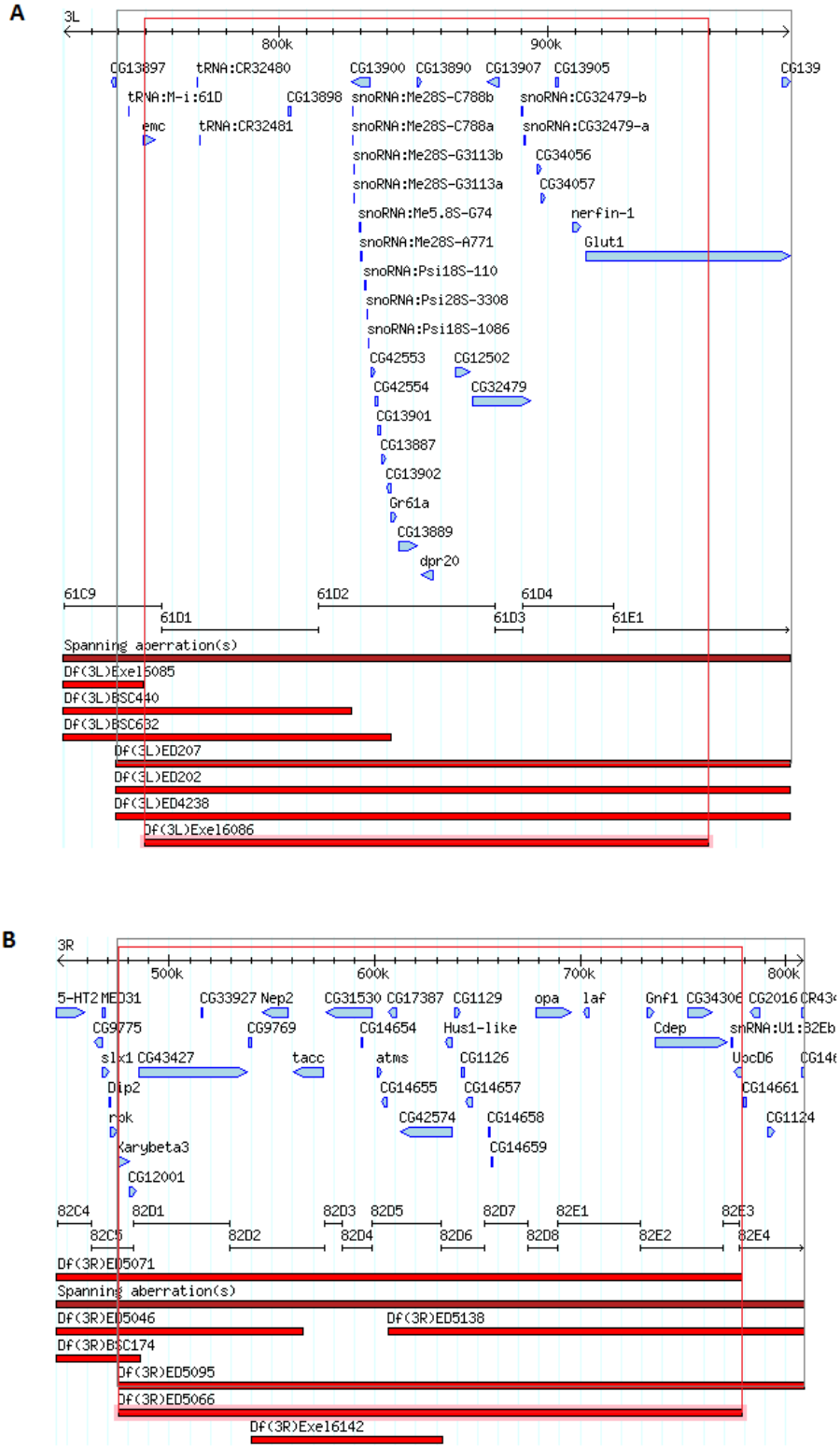


Figure 5.8 Genes and other deficiencies in the Exel7378 deficiency region. Red bars indicate the regions uncovered in the deficiencies marked above the red bars. The red box encloses the chromosomal region that is affected in Exel7378, but not in BSC504 or Exel8194 that did not rescue the viability of *Adar*^{5G1} flies.

Table 5.3Gene ontology descriptions of potential rescuing genes in the Exel7378 deficiency region.

ID	GO BIOLOGICAL PROCESS	GO MOLECULAR FUNCTION
Spn100A	-	serine-type endopeptidase inhibitor activity
CG12069	protein phosphorylation	protein serine/threonine kinase activity
	protein phosphorylation	ATP binding
		protein serine/threonine kinase activity
Pka-C2	protein phosphorylation	cAMP-dependent protein kinase activity
	protein phosphorylation	protein serine/threonine kinase activity
	neurogenesis	cAMP-dependent protein kinase activity
	protein phosphorylation	cAMP-dependent protein kinase activity
		ATP binding
CG31010	-	-
CG1340	translational initiation	mRNA binding
		translation initiation factor activity
		nucleic acid binding
		nucleotide binding
CG11313	proteolysis	serine-type endopeptidase activity
	proteolysis	serine-type endopeptidase activity
	hemolymph coagulation	
CG15543	nucleobase-containing compound metabolic process	nucleobase-containing compound kinase activity
		ATP binding
Npc2g	mesoderm development	sterol binding
	sterol transport	
	hemolymph coagulation	
Npc2h	sterol transport	sterol binding
	hemolymph coagulation	
CR43238	-	-

The other three deficiencies, Exel6086, ED5066, and Exel7284, were not narrowed down to smaller rescuing regions using the overlapping deficiencies. Instead, large deficiencies indicated as black boxes in Figure 5.9 that also covered the regions deleted in these deficiencies did not rescue the viability (Figure 5.2 and Figure 5.9). It appears that the simple interpretation that DrosDel effects are due to the genes removed by deletion cannot be consistently applied. It might be that many rescue effects are due to creation of aberrant or stronger enhancers across particular breakpoint junctions that increase expression of nearby genes as I have shown in Figure 5.7C.


A
3L
800k
900k
CG13897
tRNA:CR32480
CG13900
CG13890
CG13907
CG13905
CG139
tRNA:M-i:61D
CG13898
snoRNA:Me28S-C788b
snoRNA:CG32479-b
emc
tRNA:CR32481
snoRNA:Me28S-C788a
snoRNA:CG32479-a
snoRNA:Me28S-G3113b
CG34056
snoRNA:Me28S-G3113a
CG34057
snoRNA:Me5.8S-G74
nerfin-1
snoRNA:Me28S-A771
Glut1
snoRNA:Psi18S-110
snoRNA:Psi28S-3308
snoRNA:Psi18S-1086
CG42553
CG12502
CG42554
CG32479
CG13901
CG13887
CG13902
Gr61a
CG13889
dpr20
61C9
61D2
61D4
61D1
61D3
61E1
Spanning aberration(s)
Df(3L)Exel5085
Df(3L)BSC440
Df(3L)BSC682
Df(3L)ED207
Df(3L)ED202
Df(3L)ED4238
Df(3L)Exel6086
B
3R
500k
600k
700k
800k
5-HT2
MED31
CG33927
Nep2
CG31530
CG17387
CG1129
opa
laf
Gnf1
CG34306
CG2016
CG9775
CG9769
CG14654
Hus1-like
Cdep
snRNA:U1:82Eb
slx1
CG43427
tacc
atms
CG1126
UbcD6
Dip2
CG14655
CG14657
CG14661
rok
CG42574
CG14658
CG1124
Xarybeta3
CG14659
CG12001
82C4
82D1
82D3
82D5
82D7
82E1
82E3
82C5
82D2
82D4
82D6
82D8
82E2
82E4
Df(3R)ED5071
Spanning aberration(s)
Df(3R)ED5046
Df(3R)ED5138
Df(3R)BSC174
Df(3R)ED5095
Df(3R)ED5066
Df(3R)Exel6142

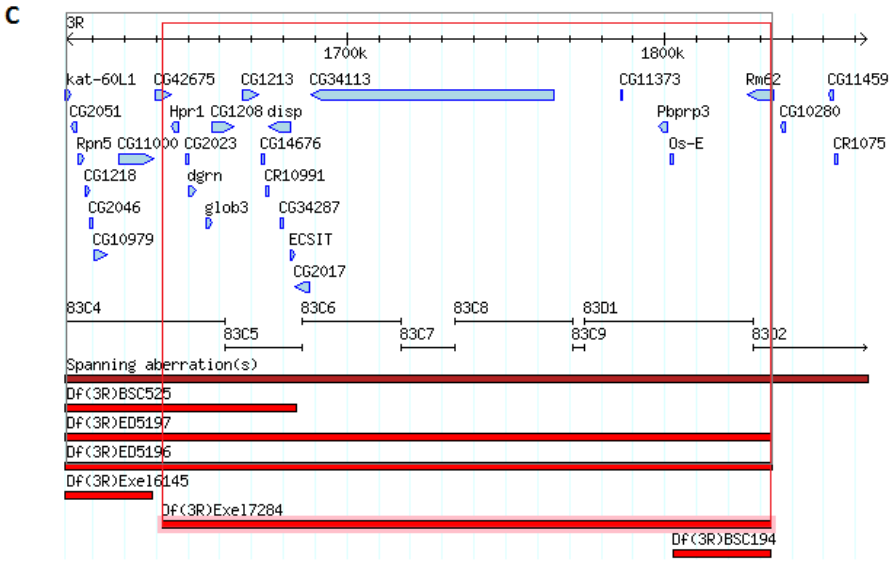


Figure 5.9 Schematic maps of deficiencies Exel6086, ED5066 and Exel7284. Red bars indicate the regions uncovered in the deficiencies marked above the red bars and red boxes enclose the chromosomal regions that are affected in *Adar*^{5G1} viability-rescuing deficiencies. Black boxes enclose the regions deleted in the deficiencies that did not rescue *Adar*^{5G1} viability.

5.2.3 Candidate approach identifies genes that ameliorate the *Adar*^{5G1} mutant phenotypes.

While the deficiency screen was being conducted for deficiencies rescuing *Adar*^{5G1} viability, some mutants and RNAi knockdowns of candidate genes were also tested. These genes (Table 2.2, Chapter 2) were selected because firstly, they were in regions deleted by deficiencies that rescued the low viability of *Adar*^{5G1} during the first-round genetic screen. Secondly, the annotations of the genes in the database suggest that they may play a role in neural development or diseases.

Amongst the 31 examined mutant alleles, four hypomorphic mutant alleles, *cry*^{d10630}, *JIL-1*^{scim}, *Gem3*^{rL562} and *neur*^{11} increased *Adar*^{5G1} viability when (Table S3, Appendix II).

cry^{MB08493} and *JIL-1*^{3} were also examined for the effect on *Adar*^{5G1} viability and neither of these mutants rescued *Adar*^{5G1} viability.

cry, or *cryptochrome* is a photoreceptor controlling circadian rhythms (Zheng et al. 2008). Both cry mutants are P-element inserted mutants, with the P-element inserted upstream of the transcription site for *cry*^{d10630} and in the third exon of *cry* for *cry*^{MB08493}. Phenotypic defects of neither *cry*^{d10630} nor *cry*^{MB08493} have been described.

JIL-1 encodes a histone kinase (H3-S10 specific), and functions as a negative regulator of chromatin silencing (Zhang et al. 2006). It is shown that reduction in *JIL-1* protein level leads to severely reduced euchromatic regions of polytene chromosomes and more condensed chromosome structures (Wang et al. 2001). The P-element inserted weak hypomorphic allele *JIL-1*^{scim} is a P-element inserted weak hypomorphic allele that reduces *JIL-1* protein level (Zhang et al. 2003). *JIL-1*^{3} (also known as *JIL-1*$^{Su(var)3-1[3]}$) is an EMS-induced gain-of-function allele (Bao et al. 2007), while *JIL-1*^{scim} is a hypomorphic allele. *JIL-1*^{3} significantly reduced the viability of *Adar*^{5G1} (Table S3, Appendix II).

Gem3 is a DEAD-box RNA helicase involved in larval motor neuron function that interacts with survival motor neuron SMN complex (Cauchi et al. 2010; Cauchi et al. 2008). *Gem3*^{rL562} is a P-element inserted amorphic allele (Shpargel et al. 2009).

Although *Adar*^{5G1} flies heterozygous for mutant alleles of *cry*^{d10630}, *JIL-1*^{scim}, *Gem3*^{rL562} still had locomotion defects at day 5, their mobility was much better at earlier days compared with *Adar*^{5G1} flies (Figure 5.10 A).

Adar^{5G1}*;;neur*^{11} flies were extremely sick from birth, so the viability-rescue by *neur*^{11} was not further considered for the locomotion rescue. *neur (neuralized)* is known to have ubiquitin-protein ligase activity and is involved in developmental process of many organs including neurogenesis and neuromuscular process (Yeh et al. 2000). The EMS induced mutant *neur*^{11} is a loss of function allele and the relatively normal viability of

Adar^{5G1} observed in the heterozygous of *neur*^{11} background may be due to the fact that *neur*^{11} heterozygous flies have similar viability with *Adar*^{5G1} compared to wild type flies.

None of 32 shRNA constructs against candidate genes expressed in *Adar*^{5G1} using *Cha-GAL4* driver significantly improved the *Adar*^{5G1} viability (Table S4 and Figure S1, Appendix II). The *UAS-shRNA* constructs were all viable in combination with the *Cha-GAL4* driver. It may be not easy to detect the effect however in a viability screen, since there are eight different progeny genotypes in each cross. Each standard cross produced only at most 200 flies in total and having eight genotype classes lowered numbers. Therefore, I conducted a mobility screen on *Adar*^{5G1} flies expressing shRNAs against candidate genes in the cholinergic neurons to find any potential rescuers that improve the *Adar*^{5G1} fly locomotion. The Trikinetics fly locomotion monitor was used for the mobility screen. Free horizontal movement of the 5 day old flies was recorded for 1 hour from 5pm-6 pm. Neither *JIL-1*^{scim} nor *cry*^{d10630} increased the flies' mobility, which was not unexpected based on rapid deterioration with age in the climbing assay. The *Adar*^{5G1} flies expressing shRNA against *rtp (retinophilin)* which reduced the *Adar*^{5G1} viability hardly walked around and the RNAi against *cas*, *mthl-8*, *crc*, or *CG10089* that improved the *Adar*^{5G1} viability slightly, did not improve *Adar*^{5G1} flies' mobility in this assay (Figure 5.10 B).

From these assays, it seems that there is a positive link between the viability and the early stage mobility and especially the climbing ability of young adult flies. However, none of the knockdowns effectively improved mobility of the *Adar*^{5G1} null flies.

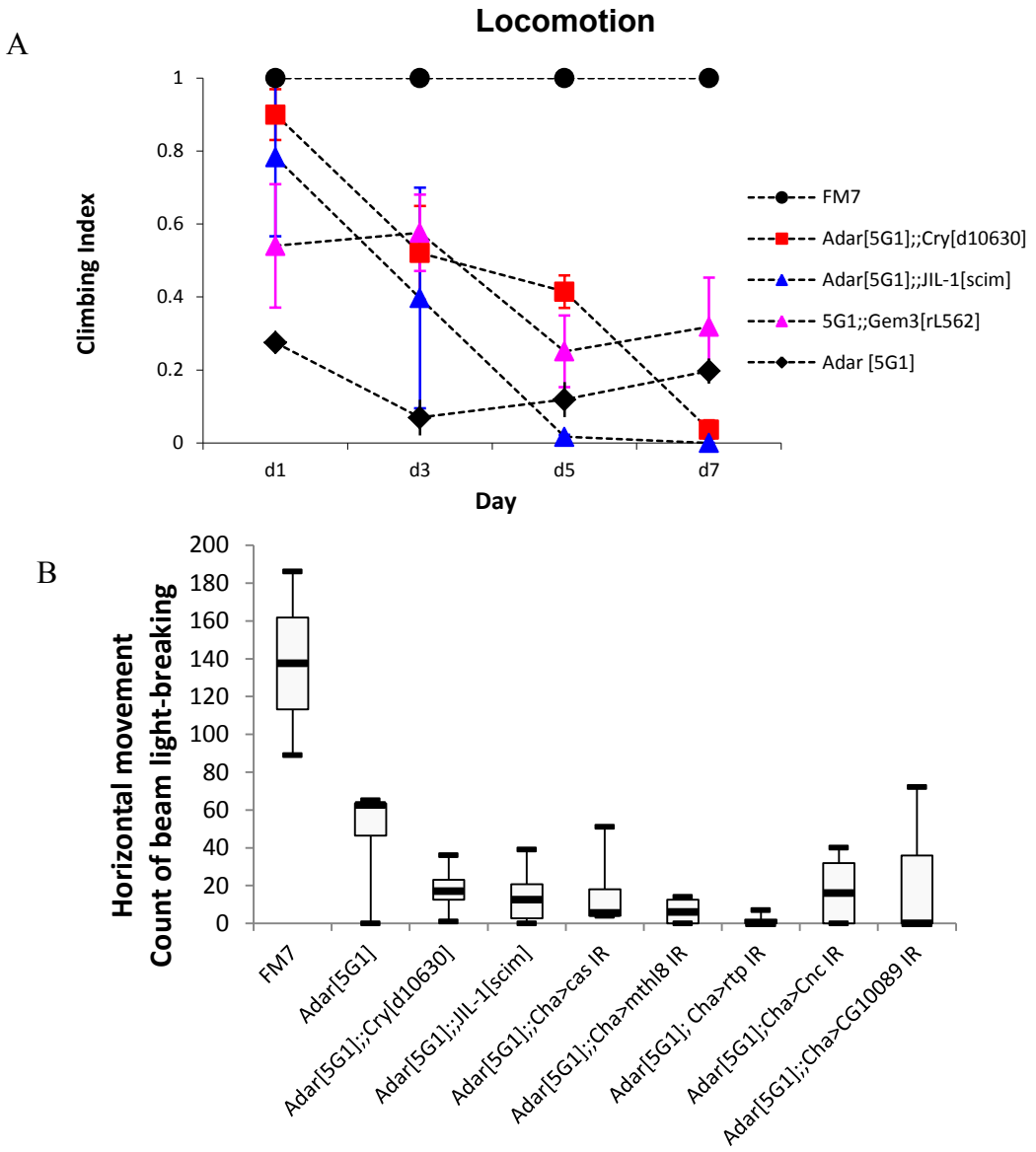


Figure 5.10 Locomotion tests on *Adar*^{5G1} flies bearing second mutations in rescue candidate genes. (A) Climbing ability was tested for each fly genotype on day 1, day 3, day 5 and day 7. The graph shows the average and the error bars are standard deviations. (B) Horizontal mobility of 5 day old flies. Count of beam light breaking in the horizontally placed Trikinetics locomotion monitor. The count is the sum of the 1 hour movement for each fly. Box plot: Five lines from top to bottom are maximum, third quarter, median, first quarter, and minimal climbing indexes, respectively.

5.3 Discussion

Our heterozygous deficiency screen on Chromosome III for the rescue of *Adar*^{5G1} mutant viability has identified six regions on Chromosome III that significantly improve the *Adar*^{5G1} viability. Not all these viability-rescuing deficiencies improve the locomotion defects of 2 day old flies, and none of them prevented the *Adar*^{5G1} null flies from developing more physical impairment with age like neurodegeneration and locomotion defects (data not shown). Very few viability-rescued flies lived to day 20. However, all these viability-rescuing deficiencies reduced expression levels of *Tot* genes, indicating that the viability rescue may act by removing the unknown stress caused by loss of ADAR in the flies. This common effect on *Tot* gene expression levels of the viability-rescuing deficiencies is the same as the effect of the *Tor*^{k17004} mutant or overexpression of *Atg5* though with less complete rescue effects in most cases.

Unexpectedly, the level of locomotion rescue was not comparable with the rescue of *Tot* gene expression levels. The Exel6086 deficiency that did not improve the mobility of the flies in the climbing assay consistently reduced the expression levels of all the *Tot* genes and AMP genes. In contrast, the RDL-2 deficiency that showed the best rescue of the *Adar*^{5G1} mobility showed the least complete rescue of *Tot* gene expression levels, and it even increased *Drs* an *AttD* expression levels significantly. This suggests that the causes of locomotion defects in the *Adar*^{5G1} flies involve more than general stress or immune response. The low viability of *Adar*^{5G1} flies is more likely primarily caused by general stress but full locomotion rescue is more difficult obtain. Motor neurons may be particularly important for locomotion rescue.

An effort to map the genes from the viability-rescuing deficiencies was not successful. Recently, we have found that the heterozygous deletions not only affect the genes deleted in the region, but also affect the expression levels of genes not included but close to the boundary of the deficiency. In addition, the expression levels of deleted genes are not always near 50% (personal communication, 52nd Drosophila Conference, San Diego,

2011). All these facts made the mapping of causative genes from the positive deficiencies difficult.

Exel9058 showed the most promising viability-rescuing phenotype and the deficiency also improved the climbing ability of *Adar*^{5G1} flies. In addition, the deficiency significantly reduced the expression levels of all the *Tot* genes and AMP genes examined. Although Exel9058 deleted only *CG11357*, it is still not confirmed yet whether the rescue was due to reduction in the expression level of *CG11357* or assisted by the increase in the expression levels of neighbouring genes *CG32243, CG32777*, or *CG42540*. InterPro domain predictions suggest that the protein encoded by *CG11357* is involved in protein glycosylation and has UDP-galactose:beta-N-acetylglucosamine beta-1,3-galactosyltransferase activity (Schwientek et al. 2002). There is not much experimental data available about this gene, but there is one report about the involvement of *CG11357* in stress responses: *CG11357* was in a list of two fold decreased mRNAs, induced by acute ER stress after the unfolded protein response (UPR) induced with the reducing agent dithiothreitol (DTT) in *Drosophila* S2 cells (Hollien and Weissman 2006). Hollien and Weissman demonstrated that these repressed mRNAs that are ER associated, including *CG11357* are destabilized by IRE before the expression changes mediated by XBP-1 takes effect, as the changes in expression of these genes are dependent on Inositol-requiring enzyme-1 (IRE1) and independent of X-box-binding protein 1 (XBP-1) (Hollien and Weissman 2006). They argued that such an effect relieves acute ER stress because it would relieve the burden on the ER more rapidly than the transcriptional turn-on of the protective mechanisms of the XBP-1–dependent pathway (Hollien and Weissman 2006). *CG11357* is an essential gene since homozygous deletion of *CG11357* is lethal. Too much knockdown of the expression levels of *CG11357* causes additional problems to the animal which may explain the failure to mimic the rescue effect by RNAi against *CG11357* although these knockdown flies were viable. No molecular function or biological role is known yet for any of the three genes whose expression levels are increased by Exel9058.

There are two strong candidates in the RDL-2 deficiency, the *mir4940* gene discussed in the Results is one, and the other one is *Tequila* which is predicted to have a serine-type endopeptidase activity (Ross et al. 2003). Like the *CG11357* transcript, the *Tequila* mRNA level was also reported to have reduced in response to DTT to relieve acute ER stress (Hollien and Weissman 2006).

The Exel6086 deficiency, although it did not improve the locomotion of $Adar^{5G1}$ greatly, did reduce both *Tot* gene expression levels and AMP gene expression levels. Exel6086 deletes 210 kb including eleven snoRNAs, two tRNAs and other protein coding genes. These snoRNAs interest us because through alternative processing, they might also act as small RNAs that could interact with ADAR and contribute to induce stress and immune response in the $Adar^{5G1}$ null flies. It would be interesting to see if the small RNAs in the Exel6086 deleted region accumulate in the $Adar^{5G1}$ fly or if the RNAs bind to ADAR in the normal physiological condition. If this is the case, then the rescue of $Adar^{5G1}$ phenotype by Exel6086 provides good evidence for our hypothesis that the noncoding RNAs play an important role in $Adar^{5G1}$ null fly phenotypes. It is also possible that *dpr20* removed in Exel6086 contributed to the reduction in AMP genes since *dpr20* encodes a protein of immunoglobulin subtype.

To sum up, from the $Adar^{5G1}$ viability-rescuing heterozygous deficiencies on Chromosome III, we could not define any genes that seem to play a role in autophagy or in any common stress-related genes. However, all these viability rescuing deficiencies reduced the expression levels of *Tot* genes, indicating that they somehow reduced certain stresses, presumably in different ways. Even without identifying individual rescuing genes, the general nature of rescuing effects does achieve some of the goals of the screen.

Finally, our results have implications for the successful use of DrosDel deficiencies in genetic screens. It appears from our data that knowing the deletions end points with base pair accuracy has not eliminated the difficulties that arise from using deficiencies. Our screen may have involved too subtle a phenotype but communication with other researchers suggests that this is not the main issue with DrosDel screens. It appears that

many of our most strongly rescuing deficiencies may rescue because of de novo genetic effects associated with the breakpoints. Flanking genes are upregulated in one case and genetic effects may depend on specific breakpoints and/or flanking genes in several other cases. In future work, it may be worthwhile to test UAS constructs overexpressing candidate genes adjacent to but not deleted by rescuing deficiencies as overexpression of these may contribute to rescues.

6 CHAPTER VI: Discussion

A fact is a simple statement that everyone believes. It is innocent, unless found guilty. A hypothesis is a novel suggestion that no one wants to believe. It is guilty, until found effective.

— Edward Teller

6.1 Summary of the results

The main findings of my thesis work are as follows:

1. In Chapter 3, I showed that reduction in GABA signalling can rescue the lethality caused by *Adar 3/4 S* OE. I also examined the effect of *Adar 3/4 S* OE and of the $Adar^{5G1}$ null on neuronal excitability using *in vivo* extracellular current recordings of aCC motor neurons. The observations that *Adar 3/4 S* OE motor neurons have significantly reduced excitability while the aCC motor neurons of $Adar^{5G1}$ null flies have higher neuronal excitability supports the hypothesis that one of the important physiological roles of ADAR and RNA editing is fine-tuning neuronal activity synergistically with GABA fast inhibitory signalling (Figure 6.1). I showed genetic and electrophysiological evidence to argue that manipulating *Rdl*-containing $GABA_A$ fast inhibitory receptor responses may rescue neuronal excitability of *Adar* mutants.
2. I confirmed that expression levels of *Tot* genes and AMP genes are significantly upregulated in the $Adar^{5G1}$ fly. The induction of systemic immunity is independent of editing activity of ADAR since the expression levels of AMP genes can be rescued by overexpressing catalytically inactive *Adar 3/4 EA* in the $Adar^{5G1}$ null background (Figure 6.1). Expression levels of *Tot* genes, an indicator of a variety of general stresses (Sophia Ekengren et al. 2001), however, are not rescued by the inactive *Adar* construct, indicating that loss of editing in ADAR substrates causes other stress in addition to induced immunity. Upregulation of autophagy by the Tor^{k17004} mutant or overexpression of *Atg5* eliminates the stress but not AMP gene expression level and rescues *Adar* mutant phenotypes without restoring the editing level in these substrates, seen from the reduction in the expression levels of *Tot* genes (Figure 6.1).
3. I generated $Adar^{5G1}$ MARCM clones, and showed cell-autonomous morphological defects in $Adar^{5G1}$ null neurons in the midbrain. Generation of $Adar^{5G1}$ MARCM clones can be a useful tool to further study cell-autonomous effects of $Adar^{5G1}$.

4. Seven deficiencies were identified in the heterozygous deficiency screen on Chr.III for rescuers of the *Adar*^{5G1} low viability. These viability-rescuing deficiencies also reduce expression levels of *Tot* genes upregulated in *Adar*^{5G1}. Although individual rescuing genes were not identified, the general nature of rescuing effects indicate that the low viability of the *Adar*^{5G1} flies can be rescued by removing stress caused by loss of *Adar*.
5. In addition, the deficiency genetic screen suggests that the genetic effects of the deficiencies may depend on specific breakpoints and/or flanking genes.
6. Individual gene mutant alleles *JIL-1*^{scim}, *Gem3*^{rL562}, and *cry*^{d10630} improve the low viability and locomotion defects of *Adar*^{5G1} flies. Experiments to understand mechanisms underlying these rescues may be continued.

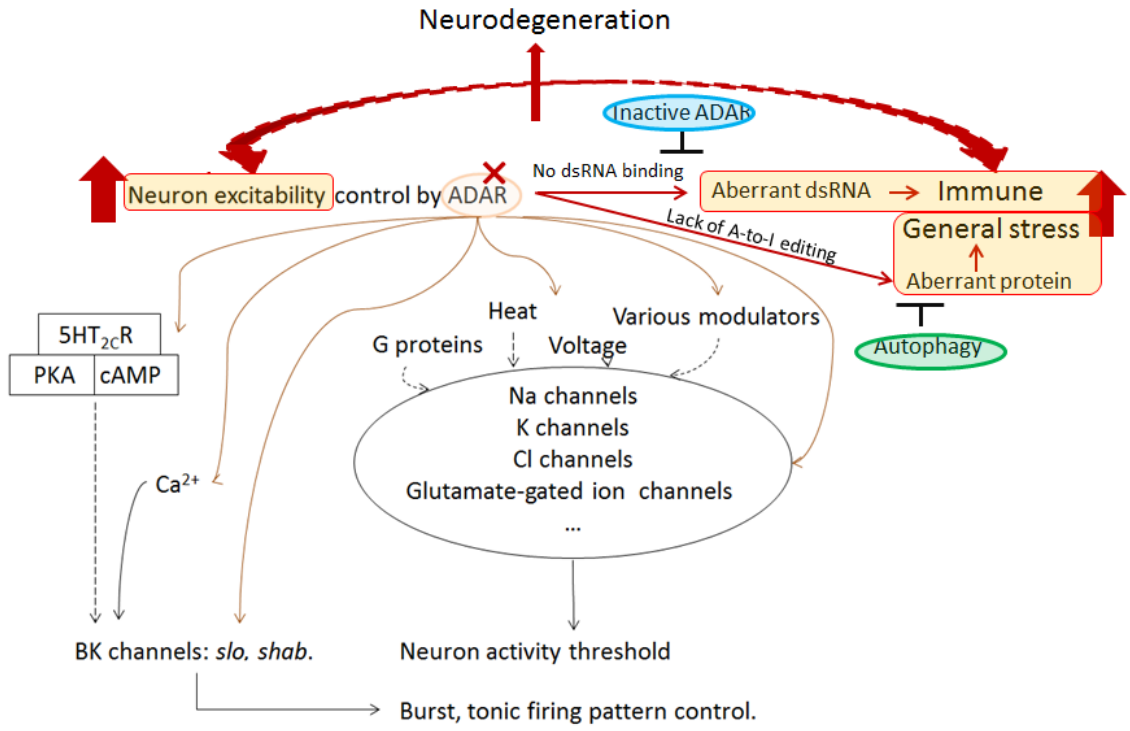


Figure 6.1 A schematic model of the physiological consequences of loss of ADAR and the rescue by inactive ADAR or enhanced autophagy. Loss of ADAR during early developmental stage leads to increased neuron excitability probably by controlling

many cellular components of neurons like serotonin receptor ($5HT_{2c}R$), Ca^{2+} transport, potassium channels and many ion channels that determine neuron activity threshold and firing pattern. Aberrant dsRNA accumulation may be the cause of immune activation which can be blocked by inactive ADAR expression, with its dsRNA binding activity. Upregulation of autophagy rescues general stress, probably by clearing aberrant protein accumulation which is caused by lack of ADAR editing. Abnormal neuron excitability in the early stage of the flies may also contribute to the defects in the adult flies by increasing general stress and probably also immune stress. All those physiological defects may contribute to age-dependent neurodegeneration.

6.2 A conserved yet distinct *Drosophila* ADAR.

Compared with vertebrates that have two functional ADAR family proteins ADAR1 and ADAR2, *Drosophila* has only one ADAR that is highly conserved with ADAR2. It is hypothesized that *Drosophila* ADAR is an orthologue of vertebrate ADAR2, and lost ADAR1 during eveolution (Figure 6.1) (Keegan et al. 2011). The hypothesis is supported by the observations that human *ADAR2* rescues age-dependent neurodegeneration and locomotion defects of $Adar^{5G1}$ flies whereas human ADAR1p150 does not (Keegan et al. 2011). But interestingly, ADAR1p110 does suppress the neurodegeneration in the $Adar^{5G1}$ flies (Keegan et al. 2011), suggesting that there are also functional overlaps between *Drosophila* ADAR and human ADAR1.

The abundance of site-specific editing events that change codons is much smaller in vertebrates compared with in *Drosophila*. A recent study identified 239 edited sites in 207 trancripts in human, and only 38 of them are predicted to change codons (Li et al. 2009). We know at least 972 sites within transcripts of 596 genes are edited and 630 of them are predicted to change codons in *Drosophila* (Graveley et al. 2011). Intriguingly, the *Drosophila Adar* mutant phenotype is not as severe as those caused by individual ADAR mutations in vertebrates. This suggests that the many codon changes caused by site-specific editing events have been highly evolutionarily selected.

Adar2 knockout mice die shortly after birth due to severe seizures and this can be completely rescued by expressing edited *GluR-B* receptor (Higuchi et al. 2000a). The rescued mice did not show any other profound defects, suggesting that the main physiological role of *Adar2* in mice is editing of the *GluR-B* Q/R site which prevents excess Ca^{2+} influx through AMPA receptors that is toxic to neurons (Higuchi et al. 2000b; Melcher et al. 1995). A microarray comparison between rescued *Adar2* KO by *GluR-B* R/R and *GluR-B* R/R mice revealed statistically significant upregulation of 80 genes overrepresented in nucleic acid metabolism, cellular growth, hematopoiesis, lipid, carbohydrate and amino acid metabolism, immune response, cancer, and cell-to-cell signaling functional annotations (Horsch et al. 2011). This indicates that *Adar2* knockout

mice are still physiologically challenged even though the major seizure phenotype is rescued by *GluR-B* R/R. This also hints overlaps between *Adar1* and *Adar2* phenotypes but this will require study of double mutant mice.

Adar1 null mice die by embryonic day E12.5 with defects in haematopoiesis and mutant MEFs (mouse embryonic fibroblast) are susceptible to stress-induced apoptosis (Wang et al. 2004; Hartner et al. 2004). This lethality caused by loss of *Adar1* has not been rescued nor is there any indications that a site specific editing event is involved. Recent work in our group showed that the lethality can be partially rescued (survival from E12.5 to birth) by modulating the NF κB signalling pathway (Keegan et al., unpublished data). An increased dsRNA accumulation and induction of interferon and other cytokines in the MEF cells from *Adar1* knockout mice are observed by anti-dsRNA antibody staining and cytokine expression examination (Greenwood and Mannion, unpublished data).

We observed significant induction of immune system genes in the $Adar^{5G1}$ flies as well, suggesting that ADAR possibly plays an important role in the *Drosophila* immune system which may be parallel to what happens in *Adar1* knockout mice. The temperature-sensitive paralysis in $Adar^{5G1}$ (Palladino et al. 2000a) and the recording of increased excitability in $Adar^{5G1}$ larval aCC motor neurons show similarities to *Adar2* knockout mice. Besides these phenotypes, *Adar* null flies develop locomotion defects, age-dependent neurodegeneration, resistance to paraquat, male sterility (Palladino et al. 2000a) and probably many other phenotypes that have not been identified yet. However, not like the *Adar2* knockout phenotype in mice, loss of *Adar* is not lethal in flies and no edited sites are found to be more important than the other edited transcripts.

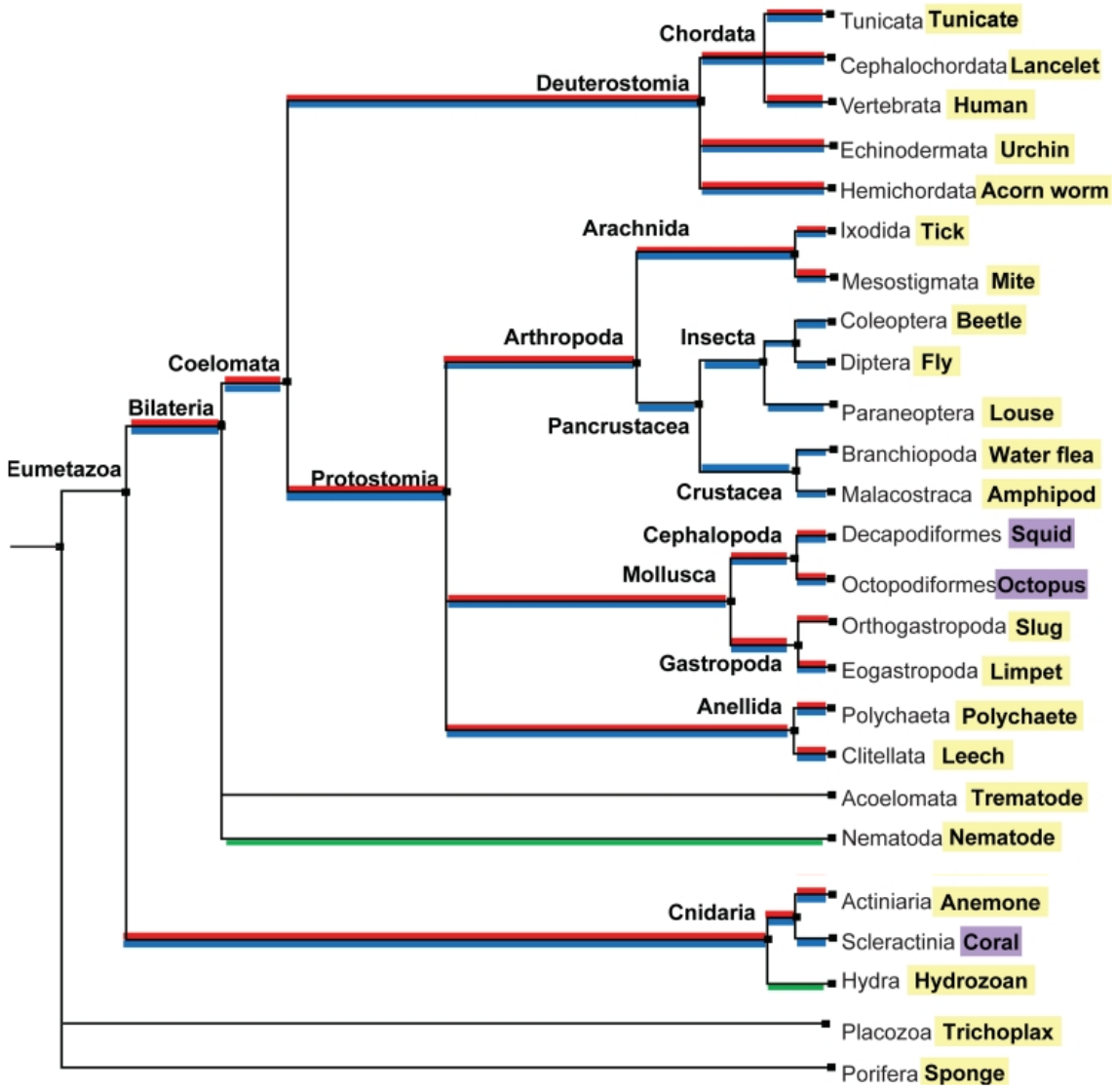


Figure 6.2 The phylogenetic tree of ADAR1 and ADAR2 genes in the Metazoa. Red lines and blue lines indicate positive identification of ADAR1 or ADAR2, respectively. Green line indicates ADARs that cannot be classified as either ADAR1 or ADAR2. Species names highlighted in yellow represent ADAR1 and ADAR2 orthologues identified in genome, available in on-line databases. Species names highlighted in purple represent the cases where ADARs were identified by cloning. Insecta and crustacean lack ADAR1. A similar loss of ADAR1 may have occurred in Corals or Hydrozoan but genome are not fully complete. Figure is taken from Keegan *et al.*, 2011.

Nevertheless, with so many severe defects, *Adar* null flies are morphologically normal (Palladino et al. 2000a). In an ideal situation without any stress, we may expect normal viability and uncompromised lifespan. Flies can live without ADAR and editing although they are very weak. In contrast, flies are unable to survive with overexpressed ADAR active since *Adar 3/4 S* overexpression causes lethality (Keegan et al. 2005). Editing, in general, increases with development and many edited events are only detectable at late pupal stages and adult stages (Graveley et al. 2011). Tight control of the level of editing may be needed. Ectopic overexpression of *Adar 3/4 S* from the embryonic stage, possibly by editing some substrates too much, may hinder normal development. The phenotypes of loss of ADAR are mostly detected at the adult stage and the phenotypes worsen with aging, which makes sense when we consider the temporal profile of editing in *Drosophila*.

6.3 A role of ADAR: fine-tuning neural activities?

Ectopic overexpression of *Adar 3/4 S* is lethal, indicating that inappropriate or excessive editing at some editable sites is deleterious to the development of the *Drosophila.* Reduction of the expression level of $GABA_A$ receptor subunit *Rdl* or reduction in the functional GAD1 protein level rescues the lethality.

One of the most important physiological roles of ADAR in *Drosophila* may be its role in guarding proper physiological functions of neurons and muscles and other organs. *Drosophila* ADAR may act like a conductor of an orchestra consisting of a variety of ion channels and other transcripts rather than affecting just one or two (Figure 6.2).

The fact that the *Rdl* transcript is edited may not be very relevant to rescue of the *Adar* overexpression phenotype. It may simply be more important that *Rdl* is the key inhibitory receptor in *Drosophila*. Neither *Rdl* nor other transcripts showed significant increases in editing levels in the *Adar 3/4 S* overexpressing larvae. Based on the *in vitro* elctrophysiological studies on *Drosophila* ion channels, editing affects the properties of the channels but not greatly (Ryan et al. 2008; Jones et al. 2009). It is likely that overexpression of *Adar 3/4 S* changed editing levels of only a subset of editing sites and the effects of these changes are quite mild for the functions of each edited transcripts. Nevertheless, the total effect of adding up these mild changes on the physiology of the fly may be very significant.

From my experiment results, it seems that *Adar 3/4 S* OE flies have much suppressed neuronal activities and this might be one of the most important reasons for the lethality. The fact that reducing GABA signalling during development of *Drosophila* rescues the lethality of *Adar 3/4 S* OE strongly supports the argument. In addition, I observed the hyper-excitable neuronal activities in the $Adar^{5G1}$ null larvae, in agreement with the hypothesis that editing fine-tunes neuronal activity synergistically with GABA fast inhibitory signalling pathway.

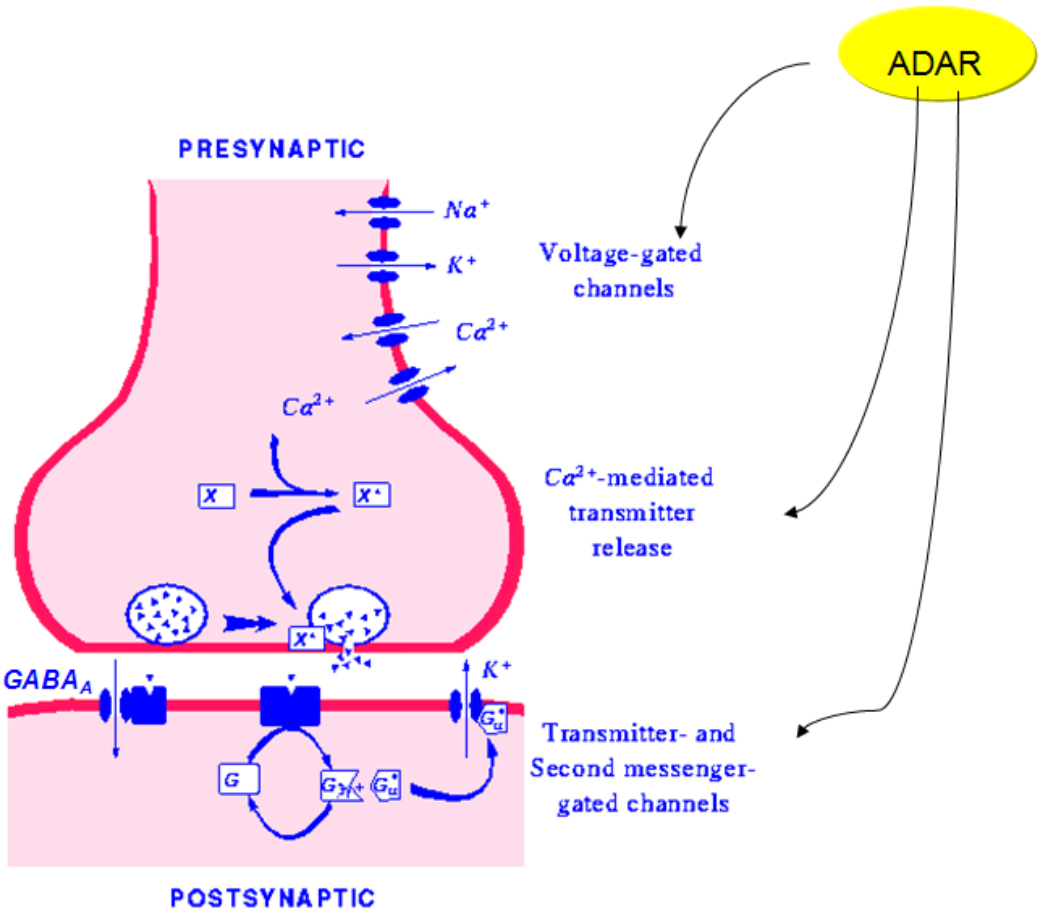


Figure 6.3 Schematic model of ADAR, ion channels and the synapse. ADAR edits ion channels and transcripts involved in synaptic transmission. ADAR plays an important role in regulating neuronal physiology by editing transcripts and by affecting the expression levels of many transcripts important for neuronal activities. By modulating the fast inhibitory signal through the $GABA_A$ receptor, the effect of ADAR mutation may be partially corrected. Figure is adapted from Destexhe et al., 1994.

To confirm the hypothesis, further experiments are needed. For instance, cell-autonomous effects of loss of ADAR, the dependence on editing activity rather than ADAR acting as an RNA-binding protein to control neuronal excitability, and the rescue of the neuronal excitability by manipulation of GABA signalling should be examined. These are easy experiments utilizing the *in vivo* extracellular current recording technique

as described in section 2.4 of Chapter II. It will be also interesting to determine the mechanism of the neuronal activity control by editing. The frequency of firing events of neurons is known to be directly controlled by large-conductance calcium and voltage-gated potassium channels (BK channels) and indirectly by calcium channels (Burdyga and Wray 2005). Therefore, to understand the mechanism of the neuronal excitability control by ADAR, we may need to study the effects of ADAR on these ion channels separately and sum up the effects. These can be done *in vitro*, but may not reflect the *in vivo* physiological situation, since the composition of the channels and the choice of isoforms for each ion channel subunit are not clear in a single cell model. One of the approachable ways to address the problem may be by building a computational model with published experimental data to predict the *in vivo* composition of ion channels and the proportional contributions of RNA editing on each edited channel transcripts.

The hypothesis that ADAR and editing fine-tune neuronal activities in concert with GABA fast inhibitory signalling is a novel idea, which is not yet reported in any animal models or human cases. After confirming the hypothesis in the flies, we may further test the hypothesis in vertebrates as a long-term goal. This physiological interaction of ADAR and GABA fast inhibitory signalling could operate similarly in adult mice. During development in mammals, GABA signalling is excitatory rather than inhibitory (Ben-Ari et al. 2007; Stein and Nicoll 2003).

Nevertheless, many editing of ion channels in mice or rat can be accommodated to the hypothesis that A-to-I RNA editing reduces neuronal excitability. For instance, in simplified scenarios, editing reduces calcium permeability of glutamate gated ion channel, decreases serotonergic potency of $5HT_{2C}$ receptors, makes $GABA_A$ receptors more sensitive to GABA, and reduces the inactivation rate of Kv1.1 channel (Table 1.1) (Gardiner and Du 2006; Decher et al. 2010; Ohlson et al. 2007). Summarizing these data seems to suggest that editing makes the excitatory ion channels less sensitive and the inhibitory ion channels more sensitive. In a sense, this is also true for *GluR-B* Q/R site. Another strong evidence is the observation that loss of ADAR2 induces seizures cell-autonomously in mice since the seizure can be rescued by introducing fully edited *GluR-*

B subunit (Brusa et al. 1995; Higuchi et al. 2000a). In other words, physiological roles of ADAR and RNA editing at the higher level of overall neuronal activities may be well conserved from insects to mammals.

One of the fastest and reliable ways to examine this hypothesis will be building a one-cell based computational model of neuronal activity of a mammalian pyramidal cell. Using the published data about the effect of editing on individual ion channels, we may be able to build a cell model of excitability thresholds and predict the hyper-excitable neurons in the complete loss of editing event. In addition, effects of stress from induced immunity on the neuronal physiology may be taken into consideration.

As discussed before, I propose that editing by ADAR functions to inhibit neuronal excitability in humans as well as in the insects and rodents. Based on this hypothesis, we may build a computational epilepsy model where excitatory ion channels are more excitable and the inhibitory channels are less active due to lack of RNA modification by ADAR protein. This will provide a comprehensive computational model in which epilepsy is not merely due to malfunction of one channel activity.

Around 50 million people worldwide have epilepsy and over 30% of these patients do not have seizure control even with the best available medication. My observation of the abnormal long burst of spontaneous activity of the $Adar^{5G1}$ null *Drosophila* larvae motor neuron is very similar to EEG (Electroencephalography, the hallmark of epilepsy in clinics) patterns recorded from pyramidal neurons of the catastrophic neonatal/infancy peilepsy disorders in humans. $Adar^{5G1}$ null flies are a good model for complicated age-independent epilepsy which so far does not have an animal model. In the PTX (Picrotoxin, $GABA_A$ receptor antagonist)-induced seizure model in flies, eight anti-epilepsy drug (AED) including Diazepam and Nifedipine were shown to reverse the effects (Stilwell et al. 2006). New combinations of FDA (Food and Drug Administration) proven drugs screened on the $Adar^{5G1}$ null fly model with these reported eight AED drugs as controls may be able to find new efficient AED drugs.

Yet to consider is the effect of non-specific editing on the neuronal excitability and the lethality. We do not know whether overexpressing *Adar 3/4 S* induces expression level changes in new categories of RNA such as non-edited transcripts or small RNAs. By doing the Next Generation RNA-seq on the *Adar 3/4 S* OE larvae compared with the wild type larvae of the same developmental stages, we may be able to detect additional problems in these flies.

6.4 The role of ADAR in the adult: a guardian against stress including immunity?

Adult flies have much higher editing levels in most of the substrate transcripts compared with the embryos or larvae. The direct consequence of loss of ADAR is the complete loss of editing and probably changes in the expression of many unedited transcripts. The changes in gene expression levels detected in both microarray and RNA sequencing analysis of $Adar^{5G1}$ null fly heads may be the systemic physiological response to the stress caused by loss of ADAR. Alternatively, the gene expression changes observed in $Adar^{5G1}$ null fly heads may be the effects mediated by RNA interference through changes in the production of miRNAs or retargeting caused by loss of ADAR.

The overall effects of loss of ADAR are making the flies very vulnerable in many aspects. In this sense, ADAR and RNA editing can be seen as the guardian for the flies from environmental and cellular stress.

One of the most exciting and interesting aspects is the relationship between ADAR and the immunity. Loss of ADAR significantly increased expression of AMP genes and also many proteases which may play important roles in the Toll and IMD signaling pathways. And more interestingly, catalytically inactive ADAR could rescue the expression changes of the AMP genes in the $Adar^{5G1}$ null flies. There are two possible explanations for the induction of AMP genes in the $Adar^{5G1}$ null flies. Loss of ADAR may make flies much more susceptible to the infections. Alternatively, loss of ADAR may mimic the infectious situation, which turns on the innate immunity without any infection, similar with the autoimmune diseases.

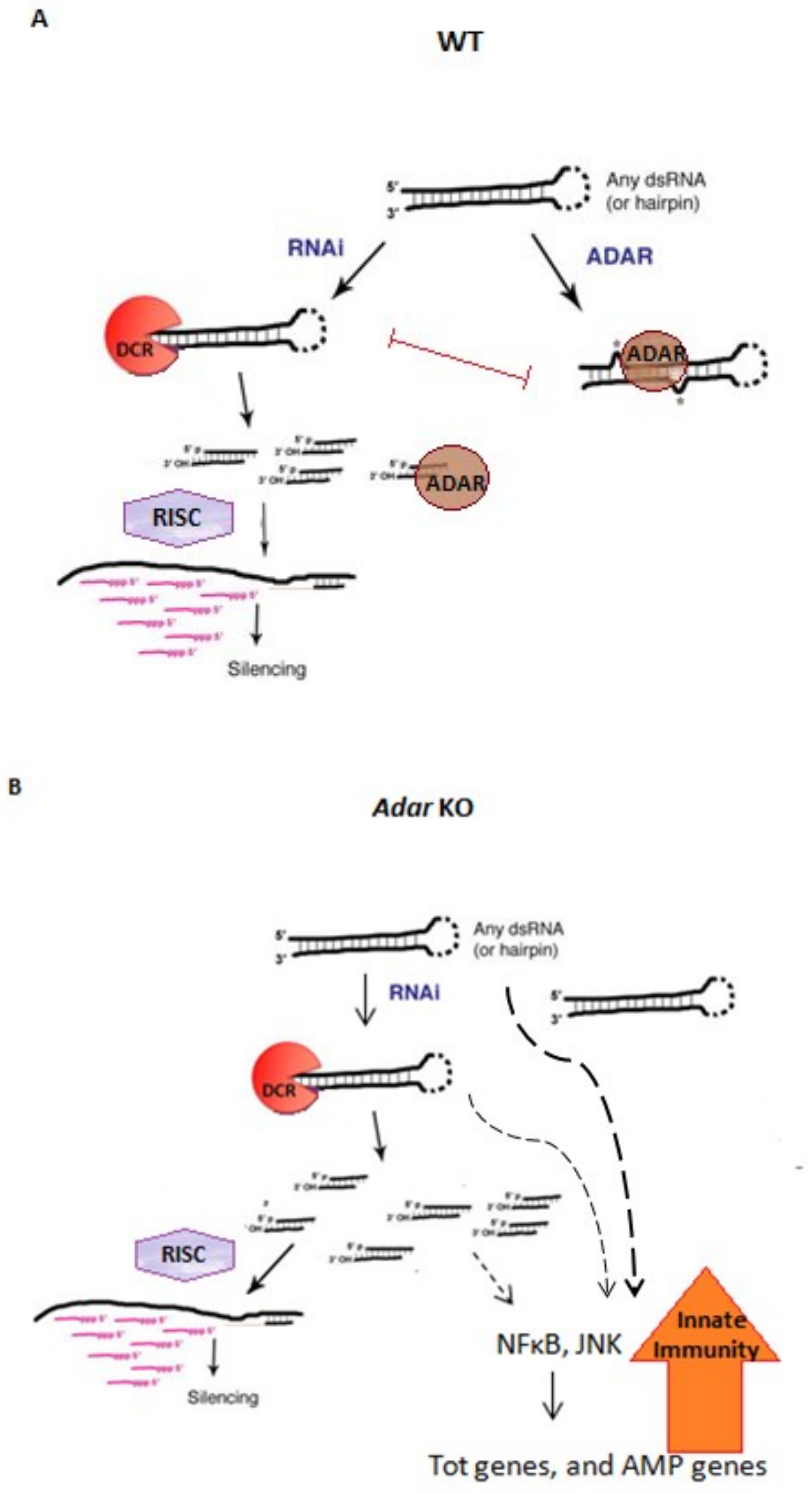
The first model involves the weakened physical barrier against the infection in the $Adar^{5G1}$ null flies compared to the wild type flies. The digestive tract of flies is the primary source of contact with microbes and many microbes cannot reach the intestinal epithelium because of the chitinous peritrophic matrix lining the midgut epithelium, secreted by the cardia (Nehme et al. 2007). Whether the induction of AMP genes are due

to the weakened physical barrier can be examined in many different ways. For example, we may examine the susceptibility of the *Adar*^{5G1} flies upon oral infections or observe the thickness of the peritrophic matrix using EM imaging. If *Adar*^{5G1} flies are more susceptible to the microbial infections and/or have thinner or much reduced peritrophic matrix, we may further predict that the loss of ADAR impaired the secretive functions of cardia.

In the other model, loss of ADAR induces immune responses through accumulation of some immunogenic materials which may be some non-coding double-stranded RNAs (Figure 6.3). There is experiment data in different organisms to suggest the possibility of this hypothesis. Firstly, knocking out ADAR family genes in *C elegans* leads to accumulation of 24nt small (Wu et al. 2011). The same may be the case in *Drosophila*, and it can be proven by comparing the small RNA pools between *Adar*^{5G1} and wild type flies by Next Generation sequencing analysis.

Immune activation may depend more on larger dsRNA precursors but also, short dsRNAs (siRNA and shRNA) have been shown to initiate immune response through TLR3 sequence-independently when transfected to HEK293 (human embryonic kidney 293) cells (Kariko et al. 2004). Once we identify classes of accumulated dsRNAs, we may prove the immunogenic nature of the RNAs. RNAs of distinct sizes or families that are enriched in the *Adar*^{5G1} null flies can either be purified or synthesized and transfected to S2 cells to determine the immune response by examining the expression levels of several AMP genes.

These two models are not mutually exclusive. We are interested in the editing-independent role of ADAR in the innate immunity, based on the result that catalytically inactive ADAR can correct the expression levels of the AMP genes induced in *Adar*^{5G1} null flies. RNA samples extracted from the *Adar*^{5G1} null flies and wild type flies will be sent for small RNA and ncRNA sequencing soon as the first step to test the working models. We expect to achieve clear answers by analyzing the sequencing data.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RISC
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NFκB, JNK
Innate
Immunity
Tot genes, and AMP genes

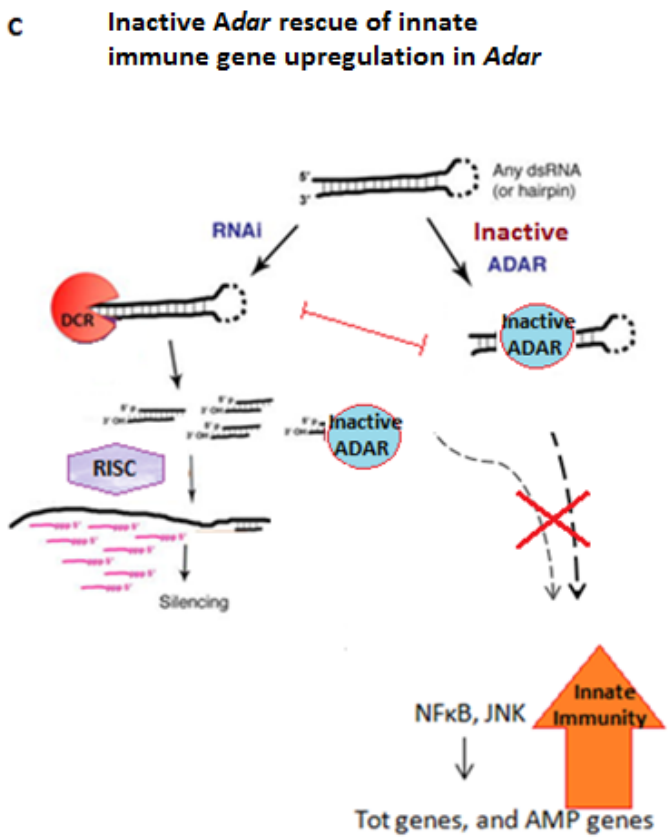


Figure 6.4 Schematic model of the competition between RNAi and ADAR for dsRNAs and the induction of innate immunity by the loss of ADAR. (A) In the wild type, ADAR binds to some long dsRNAs, which may inhibit access of Dicer to the dsRNA for further processing into the RNAi pathway. Alternatively, ADAR may bind to the small dsRNAs to interfere with the biological functions of small dsRNAs. (B) When ADAR is mutated or absent, an excessive amount of long dsRNAs and small dsRNAs may be produced. The accumulated dsRNAs are sensed by the immune system possibly because Dcr2 also acts as a signaling sensor. (C) Inactive ADAR, which does not have editing activity but have intact dsRNA binding domains, may bind to some long dsRNAs or small dsRNAs to prevent excessive amount of long dsRNAs and small dsRNAs from being detected by the immune system. Figure is adapted from *Wu et.al, 2011*.

Undoubtedly, ADAR is involved in immunity. The interferon inducibility of ADAR1 p150, the extensive editing of virus RNAs (including measles and HIV) by ADAR and

the ADAR1 mutant families with Aicardi-Goutières syndrome all links ADAR1 to the immune system (Sato et al. 2001; Doria et al. 2009; Rice et al. 2012; Liu et al. 1997). However, the relationship between ADAR1 and immune system is complicated and still needs to be elucidated. For the first time, we discovered that *Drosophila Adar*^{5G1} null flies also induced innate immunity. Elucidating the mechanism by which ADAR is involved in the immune system using *Drosophila* will shed light on studying the mechanism in the mammals and the treatment of immunological human diseases involving ADAR1 mutation.

7 CHAPTER VII: References

The great men of science are supreme artists.

— Martin H. Fischer

Agaisse, H. et al., 2003. Signaling Role of Hemocytes in Drosophila JAK/STAT-Dependent Response to Septic Injury. *Developmental Cell*, 5(3), pp.441–450. Available at: http://dx.doi.org/10.1016/S1534-5807(03)00244-2 [Accessed September 12, 2012].

Aicardi, J. & Goutières, F., 1984. A progressive familial encephalopathy in infancy with calcifications of the basal ganglia and chronic cerebrospinal fluid lymphocytosis. *Annals of neurology*, 15(1), pp.49–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6712192 [Accessed September 29, 2012].

Alexopoulou, L. et al., 2001. Recognition of double-stranded RNA and activation of NF-kB by Toll-like receptor 3. *Nature*. Available at: http://kendallasmith.com/pdf/Alexopolulou_Flavel_2001.pdf [Accessed October 15, 2012].

Alonzi, T. et al., 2001. Essential role of STAT3 in the control of the acute-phase response as revealed by inducible gene inactivation [correction of activation] in the liver. *Molecular and cellular biology*, 21(5), pp.1621–32. Available at: http://mcb.asm.org/content/21/5/1621 [Accessed September 27, 2012].

Arbouzova, N.I. & Zeidler, M.P., 2006. JAK/STAT signalling in Drosophila: insights into conserved regulatory and cellular functions. *Development (Cambridge, England)*, 133(14), pp.2605–16. Available at: http://dev.biologists.org/content/133/14/2605.short [Accessed October 3, 2012].

Artavanis-Tsakonas, S., 2004. Accessing the Exelixis collection. *Nature genetics*. Available at: http://www.nature.com/ng/journal/v36/n3/full/ng1316.html [Accessed October 10, 2012].

Athanasiadis, A., Rich, A. & Maas, S., 2004. Widespread A-to-I RNA editing of Alu-containing mRNAs in the human transcriptome. *PLoS Biol*, 2(12), p.e391. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15534692.

Bao Deng, H., Johansen, J., Girton, J., Johansen, K.M., X., 2007. Loss-of-function alleles of the JIL-1 histone H3S10 kinase enhance position-effect variegation at pericentric sites in Drosophila heterochromatin. *Genetics*, 176(2), pp.1355–1358.

Barbalat, R. et al., 2011. Nucleic acid recognition by the innate immune system. *Annual review of immunology*, 29, pp.185–214. Available at: http://www.annualreviews.org/doi/full/10.1146/annurev-immunol-031210-101340 [Accessed October 7, 2012].

Basbous, N. et al., 2011. The Drosophila peptidoglycan-recognition protein LF interacts with peptidoglycan-recognition protein LC to downregulate the Imd pathway. *EMBO reports*, 12(4), pp.327–33. Available at: /pmc/articles/PMC3077246/?report=abstract [Accessed August 1, 2012].

Bass, B L, 2002. RNA editing by adenosine deaminases that act on RNA. *Annu Rev Biochem*, 71, pp.817–846.

Bass, Brenda L, 1997. RNA editing and hypermutation by adenosine deaminase. *TIBS*.

Basset, A. et al., 2000. The phytopathogenic bacteria Erwinia carotovora infects Drosophila and activates an immune response. *Proceedings of the National Academy of Sciences of the United States of America*, 97(7), pp.3376–81. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=16247&tool=pmcentrez&rendertype=abstract [Accessed September 17, 2012].

Belvin, M.P., Jin, Y & Anderson, K. V, 1995. Cactus protein degradation mediates Drosophila dorsal-ventral signaling. *Genes & development*, 9(7), pp.783–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7705656 [Accessed October 2, 2012].

Ben-Ari, Y. et al., 2007. GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. *Physiological reviews*, 87(4), pp.1215–84. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17928584 [Accessed October 4, 2012].

Berg, K.A. et al., 2001. RNA-editing of the 5-HT(2C) receptor alters agonist-receptor-effector coupling specificity. *Br J Pharmacol*, 134(2), pp.386–92.

Botos, I. et al., The toll-like receptor 3:dsRNA signaling complex. *Biochimica et biophysica acta*, 1789(9-10), pp.667–74. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2784288&tool=pmcentrez&rendertype=abstract [Accessed October 16, 2012].

Brown, B.A. et al., 2000. The zalpha domain of the editing enzyme dsRNA adenosine deaminase binds left-handed Z-RNA as well as Z-DNA. *Proceedings of the National Academy of Sciences of the United States of America*, 97(25), pp.13532–6. Available at: http://www.pnas.org/content/97/25/13532.short [Accessed September 28, 2012].

Brusa, R. et al., 1995. Early-onset epilepsy and postnatal lethality associated with editing-deficient GluR-B allele in mice. *Science*, 270, pp.1677–1680.

Buckingham, Steven David et al., 2005. Insect GABA receptors: splicing, editing, and targeting by antiparasitics and insecticides. *Molecular pharmacology*, 68(4), pp.942–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16027231 [Accessed October 4, 2012].

Bulet, P. et al., 1999. Antimicrobial peptides in insects; structure and function. *Developmental & Comparative* .... Available at: http://www.sciencedirect.com/science/article/pii/S0145305X99000154 [Accessed October 7, 2012].

Burdyga, T. & Wray, S., 2005. Action potential refractory period in ureter smooth muscle is set by Ca sparks and BK channels. *Nature*. Available at: http://www.nature.com/nature/journal/v436/n7050/abs/nature03834.html [Accessed October 10, 2012].

Carpenter, J., Keegan, L. & Wilfert, L., 2009. Evidence for ADAR-induced hypermutation of the Drosophila sigma virus (Rhabdoviridae). *BMC* .... Available at: http://www.biomedcentral.com/1471-2156/10/75 [Accessed October 10, 2012].

Cauchi, R.J., Davies, K.E. & Liu, J.-L., 2008. A motor function for the DEAD-box RNA helicase, Gemin3, in Drosophila. G. A. Cox, ed. *PLoS genetics*, 4(11), p.e1000265. Available at: http://dx.plos.org/10.1371/journal.pgen.1000265 [Accessed October 10, 2012].

Cauchi, R.J., Sanchez-Pulido, L. & Liu, J.-L., 2010. Drosophila SMN complex proteins Gemin2, Gemin3, and Gemin5 are components of U bodies. *Experimental cell research*, 316(14), pp.2354–64. Available at: http://dx.doi.org/10.1016/j.yexcr.2010.05.001 [Accessed October 10, 2012].

El Chamy, L. et al., 2008. Sensing of "danger signals" and pathogen-associated molecular patterns defines binary signaling pathways "upstream" of Toll. *Nature immunology*, 9(10), pp.1165–70. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2768518&tool=pmcentrez&rendertype=abstract [Accessed August 8, 2012].

Chen, C.X. et al., 2000. A third member of the RNA-specific adenosine deaminase gene family, ADAR3, contains both single- and double-stranded RNA binding domains. *Rna*, 6(5), pp.755–67.

Chen, Z.J., 2005. Ubiquitin signalling in the NF-kappaB pathway. *Nature cell biology*, 7(8), pp.758–65. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1551980&tool=pmcentrez&rendertype=abstract [Accessed July 25, 2012].

Cherry, S. & Perrimon, Norbert, 2004. Entry is a rate-limiting step for viral infection in a Drosophila melanogaster model of pathogenesis. *Nature immunology*, 5(1), pp.81–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14691479 [Accessed August 13, 2012].

Chiang, A.-S. et al., 2011. Three-dimensional reconstruction of brain-wide wiring networks in Drosophila at single-cell resolution. *Current biology : CB*, 21(1), pp.1–11. Available at: http://www.cell.com/current-biology/fulltext/S0960-9822(10)01522-8 [Accessed October 4, 2012].

Chilibeck, K.A. et al., 2006. FRET analysis of in vivo dimerization by RNA-editing enzymes. *J Biol Chem*, 281(24), pp.16530–16535. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16618704.

Cook, R.K. et al., 2012. The generation of chromosomal deletions to provide extensive coverage and subdivision of the Drosophila melanogaster genome. *Genome biology*, 13(3), p.R21. Available at: http://genomebiology.com/2012/13/3/R21 [Accessed July 31, 2012].

Cui, S. et al., 2008. The C-terminal regulatory domain is the RNA 5'-triphosphate sensor of RIG-I. *Molecular cell*, 29(2), pp.169–79. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18243112 [Accessed October 24, 2012].

Decher, N., Streit, A. & Rapedius, M., 2010. RNA editing modulates the binding of drugs and highly unsaturated fatty acids to the open pore of Kv potassium channels. *The EMBO ....* Available at: http://www.nature.com/emboj/journal/vaop/ncurrent/full/emboj201088a.html [Accessed October 4, 2012].

Desterro, J.M. et al., 2003. Dynamic association of RNA-editing enzymes with the nucleolus. *J Cell Sci*, 116(Pt 9), pp.1805–1818. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12665561.

Dietzl, G. et al., 2007. A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature*, 448(7150), pp.151–156. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17625558.

Dixit, E. et al., 2010. Peroxisomes are signaling platforms for antiviral innate immunity. *Cell*, 141(4), pp.668–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20451243 [Accessed October 17, 2012].

Doria, M. et al., 2009. Editing of HIV-1 RNA by the double-stranded RNA deaminase ADAR1 stimulates viral infection. *Nucleic Acids Res*, 37(17), pp.5848–5858. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19651874.

Dostert, C. et al., 2005. The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. *Nat Immunol*, 6(9), pp.946–953. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16086017.

Eckmann, C.R. et al., 2001. The Human But Not the Xenopus RNA-editing Enzyme ADAR1 Has an Atypical Nuclear Localization Signal and Displays the Characteristics of a Shuttling Protein. *Mol Biol Cell*, 12(7), pp.1911–24.

Ekengren, S & Hultmark, D, 2001. A family of Turandot-related genes in the humoral stress response of Drosophila. *Biochemical and biophysical research communications*, 284(4), pp.998–1003. Available at: http://dx.doi.org/10.1006/bbrc.2001.5067 [Accessed August 22, 2012].

Ekengren, Sophia et al., 2001. A humoral stress response in Drosophila. *Current Biology*, 11(9), pp.714–718. Available at: http://dx.doi.org/10.1016/S0960-9822(01)00203-2 [Accessed September 11, 2012].

Eleftherianos, I. et al., 2011. ATP-sensitive potassium channel (K(ATP))-dependent regulation of cardiotropic viral infections. *Proceedings of the National Academy of Sciences of the United States of America*, 108(29), pp.12024–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3141999&tool=pmcentrez&rendertype=abstract [Accessed August 1, 2012].

Fattori, E. et al., 1994. Defective inflammatory response in interleukin 6-deficient mice. *The Journal of experimental medicine*, 180(4), pp.1243–50. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2191674&tool=pmcentrez&rendertype=abstract [Accessed October 3, 2012].

FEATHERSTONE, D., 2000. Presynaptic Glutamic Acid Decarboxylase Is Required for Induction of the Postsynaptic Receptor Field at a Glutamatergic Synapse. *Neuron*, 27(1), pp.71–84. Available at: http://dx.doi.org/10.1016/S0896-6273(00)00010-6 [Accessed September 30, 2012].

Ferrandon, D & Imler, J., 2007. The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. *Nature Reviews* .... Available at:

http://www.nature.com/nri/journal/v7/n11/abs/nri2194.html [Accessed October 2, 2012].

Ffrench-Constant, R.H. et al., 1991. Molecular cloning and transformation of cyclodiene resistance in Drosophila: an invertebrate gamma-aminobutyric acid subtype A receptor locus. *Proceedings of the National Academy of Sciences of the United States of America*, 88(16), pp.7209–13. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=52263&tool=pmcentrez&rendertype=abstract [Accessed October 9, 2012].

Fischer, J.A. et al., 1988. GAL4 activates transcription in Drosophila. *Nature*, 332(6167), pp.853–856. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=3128741.

Flynt, A. et al., 2009. Dicing of viral replication intermediates during silencing of latent Drosophila viruses. *Proceedings of the National Academy of Sciences of the United States of America*, 106(13), pp.5270–5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2663985&tool=pmcentrez&rendertype=abstract [Accessed October 26, 2012].

Fritz, J. et al., 2009. RNA-regulated interaction of transportin-1 and exportin-5 with the double-stranded RNA-binding domain regulates nucleocytoplasmic shuttling of ADAR1. *Mol Cell Biol*, 29(6), pp.1487–1497. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19124606.

Gallo, A et al., 2003. An ADAR that edits transcripts encoding ion channel subunits functions as a dimer. *Embo J*, 22(13), pp.3421–3430. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12840004.

Gan, Z. et al., 2006. RNA editing by ADAR2 is metabolically regulated in pancreatic islets and beta-cells. *J Biol Chem*, 281(44), pp.33386–33394. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16956888.

Gardiner, K. & Du, Y., 2006. A-to-I editing of the 5HT2C receptor and behaviour. *Brief Funct Genomic Proteomic*, 5(1), pp.37–42. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16769676.

Gargano, J. & Martin, I., 2005. Rapid iterative negative geotaxis (RING): a new method for assessing age-related locomotor decline in< i> Drosophila</i>. *Experimental*

gerontology. Available at: http://www.sciencedirect.com/science/article/pii/S0531556505000343 [Accessed October 11, 2012].

George, C.X. & Samuel, C E, 1999a. Characterization of the 5'-flanking region of the human RNA-specific adenosine deaminase ADAR1 gene and identification of an interferon-inducible ADAR1 promoter. *Gene*, 229(1-2), pp.203–13. Available at: http://www.elsevier.com:80/cgi-bin/cas/tree/store/gene/cas_sub/browse/browse.cgi?year=1999&volume=229&issue=1-2&aid=11747.

George, C.X. & Samuel, C E, 1999b. Human RNA-specific adenosine deaminase ADAR1 transcripts possess alternative exon 1 structures that initiate from different promoters, one constitutively active and the other interferon inducible. *Proc Natl Acad Sci U S A*, 96(8), pp.4621–6. Available at: http://www.pnas.org/cgi/content/full/96/8/4621.

George, C.X., Wagner, M. V & Samuel, C E, 2005. Expression of interferon-inducible RNA adenosine deaminase ADAR1 during pathogen infection and mouse embryo development involves tissue-selective promoter utilization and alternative splicing. *J Biol Chem*, 280(15), pp.15020–15028. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15677478.

Gesellchen, V. et al., 2005. An RNA interference screen identifies Inhibitor of Apoptosis Protein 2 as a regulator of innate immune signalling in Drosophila. *EMBO reports*, 6(10), pp.979–84. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1369191&tool=pmcentrez&rendertype=abstract [Accessed September 25, 2012].

Gobert, V. et al., 2003. Dual activation of the Drosophila toll pathway by two pattern recognition receptors. *Science (New York, N.Y.)*, 302(5653), pp.2126–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14684822 [Accessed August 14, 2012].

Goodman, R.., Macbeth, M.R. & Beal, P.A., 2011. *Adenosine Deaminases Acting on RNA (ADARs) and A-to-I Editing (Google eBook)* Charles E Samuel, ed., Springer. Available at: http://books.google.com/books?hl=en&lr=&id=hx1DrRbFEGgC&pgis=1 [Accessed September 28, 2012].

Gottar, M. et al., 2006. Dual detection of fungal infections in Drosophila via recognition of glucans and sensing of virulence factors. *Cell*, 127(7), pp.1425–37. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1865096&tool=pmcentrez&rendertype=abstract [Accessed August 1, 2012].

Graveley, B.R. et al., 2011. The developmental transcriptome of Drosophila melanogaster. *Nature*, 471(7339), pp.473–479. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21179090.

Greene, J. & Whitworth, A., 2005. Genetic and genomic studies of Drosophila parkin mutants implicate oxidative stress and innate immune responses in pathogenesis. *Human molecular ....* Available at: http://hmg.oxfordjournals.org/content/14/6/799.short [Accessed October 10, 2012].

Häcker, H. et al., 2006. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature*, 439(7073), pp.204–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16306937 [Accessed October 24, 2012].

Hanakahi, L.A. & West, S.C., 2002. Specific interaction of IP6 with human Ku70/80, the DNA-binding subunit of DNA-PK. *The EMBO journal*, 21(8), pp.2038–44. Available at: http://dx.doi.org/10.1093/emboj/21.8.2038 [Accessed September 28, 2012].

Hanrahan, C.J. et al., 2000. RNA editing of the drosophila para Na(+) channel transcript. Evolutionary conservation and developmental regulation. *Genetics*, 155(3), pp.1149–1160.

Hao Zheng et al., 2008. Spatial and circadian regulation of cry in Drosophila. *Journal of biological rhythms*, 23(4), pp.283–95. Available at: http://jbr.sagepub.com/content/23/4/283.long [Accessed October 10, 2012].

Hartner, J.C. et al., 2009. ADAR1 is essential for the maintenance of hematopoiesis and suppression of interferon signaling. *Nat Immunol*, 10(1), pp.109–115. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19060901.

Hartner, J.C. et al., 2004. Liver Disintegration in the Mouse Embryo Caused by Deficiency in the RNA-editing Enzyme ADAR1. *J Biol Chem*, 279(6), pp.4894–4902. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14615479.

Hartwig, D. et al., 2006. The large form of ADAR 1 is responsible for enhanced hepatitis delta virus RNA editing in interferon-alpha-stimulated host cells. *J Viral Hepat*, 13(3), pp.150–157. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16475990.

Haudenschild, B.L. et al., 2004. A transition state analogue for an RNA-editing reaction. *J Am Chem Soc*, 126(36), pp.11213–11219. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15355102.

Hay, B., Wolff, T. & Rubin, G., 1994. Expression of baculovirus P35 prevents cell death in Drosophila. *Development*. Available at: http://dev.biologists.org/content/120/8/2121.short [Accessed October 10, 2012].

Heale, B.S., Keegan, L P, McGurk, L, et al., 2009. Editing independent effects of ADARs on the miRNA/siRNA pathways. *Embo J*, 28(20), pp.3145–3156. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19713932.

Heale, B.S., Keegan, L P & O'Connell, M A, 2009. ADARs have effects beyond RNA editing. *Cell Cycle*, 8(24), pp.4011–4012. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19949296.

Hedengren, M. et al., 1999. Relish, a central factor in the control of humoral but not cellular immunity in Drosophila. *Molecular cell*, 4(5), pp.827–37. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10619029 [Accessed October 2, 2012].

Herbert, A. et al., 1997. A Z-DNA binding domain present in the human editing enzyme, double-stranded RNA adenosine deaminase. *Proc Natl Acad Sci U S A*, 94(16), pp.8421–6.

Higuchi, M et al., 2000a. Point mutation in an AMPA receptor gene rescues lethality in mice deficient in the RNA-editing enzyme ADAR2. *Nature*, 406(6791), pp.78–81. Available at: http://dx.doi.org/10.1038/35017558 [Accessed July 13, 2012].

Higuchi, M et al., 2000b. Point mutation in an AMPA receptor gene rescues lethality in mice deficient in the RNA-editing enzyme ADAR2. *Nature*, 406(6791), pp.78–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10894545 [Accessed June 28, 2012].

Higuchi, Miyoko et al., 1993. RNA editing of AMPA receptor subunit GluR-B: A base-paired intron-exon structure determines position and efficiency. *Cell*, 75, pp.1361–1370.

Hollien, J. & Weissman, J.S., 2006. Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. *Science (New York, N.Y.)*, 313(5783),

pp.104–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16825573 [Accessed July 25, 2012].

Hoopengardner, B. et al., 2003. Nervous system targets of RNA editing identified by comparative genomics. *Science*, 301(5634), pp.832–836. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12907802.

Horsch, M. et al., 2011. Requirement of the RNA-editing enzyme ADAR2 for normal physiology in mice. *The Journal of biological chemistry*, 286(21), pp.18614–22. Available at: http://www.jbc.org/content/286/21/18614.long [Accessed October 23, 2012].

Hosie, A. et al., 1997. Molecular biology of insect neuronal GABA receptors. *Trends in Neurosciences*, 20(12), pp.578–583. Available at: http://dx.doi.org/10.1016/S0166-2236(97)01127-2 [Accessed September 30, 2012].

Hough, R.F. & Bass, B L, 1994. Purification of the Xenopus laevis dsRNA adenosine deaminase. *J. Biol. Chem.*, 269, pp.9933–9939.

Hu, X. et al., 2004. Multimerization and interaction of Toll and Spätzle in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, 101(25), pp.9369–74. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=438983&tool=pmcentrez&rendertype=abstract [Accessed October 2, 2012].

Hundley, H.A. & Bass, B L, 2010. ADAR editing in double-stranded UTRs and other noncoding RNA sequences. *Trends Biochem Sci*, 35(7), pp.377–383. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20382028.

Jang, I.-H. et al., 2006. A Spätzle-processing enzyme required for toll signaling activation in Drosophila innate immunity. *Developmental cell*, 10(1), pp.45–55. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16399077 [Accessed August 1, 2012].

Jepson, J.E. & Reenan, R A, 2009. Adenosine-to-inosine genetic recoding is required in the adult stage nervous system for coordinated behavior in Drosophila. *J Biol Chem*, 284(45), pp.31391–31400. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19759011.

Jepson, J.E. & Reenan, R A, 2007. RNA editing in regulating gene expression in the brain. *Biochim Biophys Acta*. Available at:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18086576.

Jiang, J. & Struhl, G., 1998. Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb. *Nature*, 391(6666), pp.493–6. Available at: http://dx.doi.org/10.1038/35154 [Accessed July 30, 2012].

Jones, A.K. et al., 2009. Splice-variant- and stage-specific RNA editing of the Drosophila GABA receptor modulates agonist potency. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 29(13), pp.4287–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19339622 [Accessed October 4, 2012].

Kambris, Z. et al., 2006. Drosophila immunity: a large-scale in vivo RNAi screen identifies five serine proteases required for Toll activation. *Current biology : CB*, 16(8), pp.808–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16631589 [Accessed July 16, 2012].

Kaneko, T. et al., 2006. PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan. *Nature immunology*, 7(7), pp.715–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16767093 [Accessed August 1, 2012].

Kariko, K. et al., 2004. Small Interfering RNAs Mediate Sequence-Independent Gene Suppression and Induce Immune Activation by Signaling through Toll-Like Receptor 3. *J. Immunol.*, 172(11), pp.6545–6549. Available at: http://www.jimmunol.org/content/172/11/6545.long [Accessed October 16, 2012].

Kato, H. et al., 2008. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *The Journal of experimental medicine*, 205(7), pp.1601–10. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2442638&tool=pmcentrez&rendertype=abstract [Accessed October 4, 2012].

Kawahara, Y. et al., 2004. Glutamate receptors: RNA editing and death of motor neurons. *Nature*, 427(6977), p.801. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14985749.

Kawahara, Y. et al., 2003. Human spinal motoneurons express low relative abundance of GluR2 mRNA: an implication for excitotoxicity in ALS. *J Neurochem*, 85(3), pp.680–689. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12694394.

Kawahara, Y., Zinshteyn, B., Sethupathy, P., et al., 2007. Redirection of silencing targets by adenosine-to-inosine editing of miRNAs. *Science*, 315(5815), pp.1137–1140. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17322061.

Kawahara, Y., Zinshteyn, B., Chendrimada, T.P., et al., 2007. RNA editing of the microRNA-151 precursor blocks cleavage by the Dicer-TRBP complex. *EMBO Rep*, 8(8), pp.763–769. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17599088.

Kawai, Taro et al., 2004. Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nature immunology*, 5(10), pp.1061–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15361868 [Accessed October 24, 2012].

Kawamura, Y. et al., 2008. Drosophila endogenous small RNAs bind to Argonaute 2 in somatic cells. *Nature*, 453(7196), pp.793–797. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18463636.

Keegan, L P et al., 2005. Tuning of RNA editing by ADAR is required in Drosophila. *Embo J*, 24(12), pp.2183–2193. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15920480.

Keegan, L P, Gallo, A & O'Connell, M A, 2001. The many roles of an RNA editor. *Nat Rev Genet*, 2(11), pp.869–78. Available at: http://www.nature.com/nrg/journal/v2/n11/full/nrg1101-869a.html.

Keegan, Liam P et al., 2011. Functional conservation in human and Drosophila of Metazoan ADAR2 involved in RNA editing: loss of ADAR1 in insects. *Nucleic Acids Research*, 39(16), pp.7249–7262. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21622951.

Keegan, Liam P et al., 2005. Tuning of RNA editing by ADAR is required in Drosophila. *The EMBO journal*, 24(12), pp.2183–93. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1150885&tool=pmcentrez&rendertype=abstract [Accessed April 10, 2012].

Khush, Ranjiv S et al., 2002. A ubiquitin-proteasome pathway represses the Drosophila immune deficiency signaling cascade. *Current biology : CB*, 12(20), pp.1728–37.

Available at: http://www.ncbi.nlm.nih.gov/pubmed/12401167 [Accessed October 2, 2012].

Kim, M. et al., 2006. Caspar, a suppressor of antibacterial immunity in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, 103(44), pp.16358–63. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1637587&tool=pmcentrez&rendertype=abstract [Accessed October 2, 2012].

Kim, M.-S., Byun, M. & Oh, B.-H., 2003. Crystal structure of peptidoglycan recognition protein LB from Drosophila melanogaster. *Nature immunology*, 4(8), pp.787–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12845326 [Accessed October 2, 2012].

Kittler, J.T., 2006. Censoring the editor in transient forebrain ischemia. *Neuron*, 49(5), pp.646–648. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16504939.

Kleino, A. et al., 2005. Inhibitor of apoptosis 2 and TAK1-binding protein are components of the Drosophila Imd pathway. *The EMBO journal*, 24(19), pp.3423–34. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1276168&tool=pmcentrez&rendertype=abstract [Accessed August 1, 2012].

Knight, S.W. & Bass, B L, 2002. The role of RNA editing by ADARs in RNAi. *Mol Cell*, 10(4), pp.809–17.

Kondo, T. et al., 2008. Dyschromatosis symmetrica hereditaria associated with neurological disorders. *J Dermatol*, 35(10), pp.662–666. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19017046.

Kopf, M. et al., 1994. Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature*, 368(6469), pp.339–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8127368 [Accessed September 1, 2012].

Lai, F., Drakas, R. & Nishikura, K, 1995. Mutagenic analysis of double-stranded RNA adenosine deaminase, a candidate enzyme for RNA editing of glutamate-gated ion channel transcripts. *J. Biol. Chem.*, 270(29), pp.17098–17105.

Lannan, E., Vandergaast, R. & Friesen, P., 2007. Baculovirus caspase inhibitors P49 and P35 block virus-induced apoptosis downstream of effector caspase DrICE

activation in Drosophila melanogaster cells. *Journal of virology*. Available at: http://jvi.asm.org/content/81/17/9319.short [Accessed October 10, 2012].

Laxminarayana, D. et al., 2007. Induction of 150-kDa adenosine deaminase that acts on RNA (ADAR)-1 gene expression in normal T lymphocytes by anti-CD3-epsilon and anti-CD28. *Immunology*, 122(4), pp.623–633. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17897325.

Lee, D., Su, H. & O'Dowd, D.K., 2003. GABA Receptors Containing Rdl Subunits Mediate Fast Inhibitory Synaptic Transmission in Drosophila Neurons. *J. Neurosci.*, 23(11), pp.4625–4634. Available at: http://www.jneurosci.org/content/23/11/4625.long [Accessed October 26, 2012].

Lee, Y. et al., 2004. Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell*. Available at: http://www.sds.vanderbilt.edu/singleton/BSCI275BmiRNA/12.pdf [Accessed October 3, 2012].

Lehmann, K.A. & Bass, B L, 2000. Double-stranded RNA adenosine deaminases ADAR1 and ADAR2 have overlapping specificities. *Biochemistry*, 39(42), pp.12875–84.

Leifer, C.A. et al., 2004. TLR9 is localized in the endoplasmic reticulum prior to stimulation. *Journal of immunology (Baltimore, Md. : 1950)*, 173(2), pp.1179–83. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2757936&tool=pmcentrez&rendertype=abstract [Accessed October 24, 2012].

Lemaitre, B, 1997. Drosophila host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proceedings of the* .... Available at: http://www.pnas.org/content/94/26/14614.short [Accessed October 7, 2012].

Leulier, François et al., 2006. The Drosophila inhibitor of apoptosis protein DIAP2 functions in innate immunity and is essential to resist gram-negative bacterial infection. *Molecular and cellular biology*, 26(21), pp.7821–31. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1636742&tool=pmcentrez&rendertype=abstract [Accessed August 7, 2012].

Levanon, E.Y. et al., 2004. Systematic identification of abundant A-to-I editing sites in the human transcriptome. *Nat Biotechnol*, 22(8), pp.1001–1005. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15258596.

Levine, B. & Klionsky, D.J., 2004. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell*, 6(4), pp.463–477. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15068787.

Li, F. & Ding, S.-W., 2006. Virus counterdefense: diverse strategies for evading the RNA-silencing immunity. *Annual review of microbiology*, 60, pp.503–31. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2693410&tool=pmcentrez&rendertype=abstract [Accessed October 24, 2012].

Li, H. & Durbin, R., 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics (Oxford, England)*, 25(14), pp.1754–60. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2705234&tool=pmcentrez&rendertype=abstract [Accessed July 13, 2012].

Li, J.B. et al., 2009. Genome-wide identification of human RNA editing sites by parallel DNA capturing and sequencing. *Science*, 324(5931), pp.1210–1213. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19478186.

Li, Willis X, 2008. Canonical and non-canonical JAK-STAT signaling. *Trends in cell biology*, 18(11), pp.545–51. Available at: http://dx.doi.org/10.1016/j.tcb.2008.08.008 [Accessed October 4, 2012].

Liang, C., 2010. Negative regulation of autophagy. *Cell Death & Differentiation*. Available at: http://www.nature.com/cdd/journal/vaop/ncurrent/full/cdd2010115a.html [Accessed October 10, 2012].

Ligoxygakis, P. et al., 2002. Activation of Drosophila Toll during fungal infection by a blood serine protease. *Science (New York, N.Y.)*, 297(5578), pp.114–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12098703 [Accessed August 14, 2012].

Lim, J.-H. et al., 2006. Structural basis for preferential recognition of diaminopimelic acid-type peptidoglycan by a subset of peptidoglycan recognition proteins. *The Journal of biological chemistry*, 281(12), pp.8286–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16428381 [Accessed August 8, 2012].

Lipinski, M.M. et al., 2010. Genome-wide analysis reveals mechanisms modulating autophagy in normal brain aging and in Alzheimer's disease. *Proc Natl Acad Sci U S A*, 107(32), pp.14164–14169. Available at:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20660724.

Liu, Q. et al., 2003. R2D2, a bridge between the initiation and effector steps of the Drosophila RNAi pathway. *Science*. Available at: http://www.sciencemag.org/content/301/5641/1921.short [Accessed October 3, 2012].

Liu, Q. et al., 2006. Two novel mutations and evidence for haploinsufficiency of the ADAR gene in dyschromatosis symmetrica hereditaria. *Br J Dermatol*, 154(4), pp.636–642. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16536805.

Liu, Yong et al., 1997. Functionally distinct double-stranded RNA-binding domains associated with alternative splice site variants of the interferon-inducible double-stranded RNA-specific adenosine deaminase. *J. Biol. Chem.*, 272(7), pp.4419–4428.

Loughney, K., Kreber, R. & Ganetzky, B., 1989. Molecular analysis of the para locus, a sodium channel gene in Drosophila. *Cell*, 58, pp.1143–1154.

Lugtenberg, B. & Van Alphen, L., 1983. Molecular architecture and functioning of the outer membrane of Escherichia coli and other gram-negative bacteria. *Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes*, 737(1), pp.51–115. Available at: http://dx.doi.org/10.1016/0304-4157(83)90014-X [Accessed October 3, 2012].

Lyne, R. et al., 2007. FlyMine: an integrated database for Drosophila and Anopheles genomics. *Genome biology*, 8(7), p.R129. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2323218&tool=pmcentrez&rendertype=abstract [Accessed August 14, 2012].

Ma, E. et al., 2002. Developmental expression and enzymatic activity of pre-mRNA deaminase in Drosophila melanogaster. *Brain Res Mol Brain Res*, 102(1-2), pp.100–4.

Maas, S. et al., 2006. A-to-I RNA Editing and Human Disease. *RNA Biol*, 3(1). Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17114938.

Macbeth, M R et al., 2005. Inositol hexakisphosphate is bound in the ADAR2 core and required for RNA editing. *Science*, 309(5740), pp.1534–1539. Available at:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16141067.

Macbeth, M R, Lingam, A.T. & Bass, B L, 2004. Evidence for auto-inhibition by the N terminus of hADAR2 and activation by dsRNA binding. *Rna*, 10(10), pp.1563–1571. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15383678.

Marcucci, R. et al., 2011. Pin1 and WWP2 regulate GluR2 Q/R site RNA editing by ADAR2 with opposing effects. *The EMBO journal*, 30(20), pp.4211–22. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3199391&tool=pmcentrez&rendertype=abstract [Accessed July 13, 2012].

Masliah, G., Barraud, P. & Allain, F.H.-T., 2012. RNA recognition by double-stranded RNA binding domains: a matter of shape and sequence. *Cellular and molecular life sciences : CMLS*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22918483 [Accessed September 13, 2012].

Matskevich, A.A., Quintin, J. & Ferrandon, Dominique, 2010. The Drosophila PRR GNBP3 assembles effector complexes involved in antifungal defenses independently of its Toll-pathway activation function. *European journal of immunology*, 40(5), pp.1244–54. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2978882&tool=pmcentrez&rendertype=abstract [Accessed July 28, 2012].

McCall, K., 2010. Genetic control of necrosis - another type of programmed cell death. *Current opinion in cell biology*, 22(6), pp.882–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2993806&tool=pmcentrez&rendertype=abstract [Accessed October 5, 2012].

Melcher, T. et al., 1996. A mammalian RNA editing enzyme. *Nature*, 379, pp.460–464.

Melcher, T. et al., 1995. Editing of AMPA receptor GluR-B pre-mRNA in vitro reveals site-selective adenosine to inosine conversion. *J. Biol. Chem.*, 270, pp.8566–8570.

Mellroth, P. et al., 2005. Ligand-induced dimerization of Drosophila peptidoglycan recognition proteins in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, 102(18), pp.6455–60. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1088352&tool=pmcentrez&rendertype=abstract [Accessed October 2, 2012].

Miyamura, Y. et al., 2003. Mutations of the RNA-specific adenosine deaminase gene (DSRAD) are involved in dyschromatosis symmetrica hereditaria. *Am J Hum Genet*, 73(3), pp.693–699. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12916015.

Miyoshi, K., Tsukumo, H. & Nagami, T., 2005. Slicer function of Drosophila Argonautes and its involvement in RISC formation. *Genes &* .... Available at: http://genesdev.cshlp.org/content/19/23/2837.short [Accessed October 3, 2012].

Nappi, A.J. & Vass, E., 1993. Melanogenesis and the generation of cytotoxic molecules during insect cellular immune reactions. *Pigment cell research / sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society*, 6(3), pp.117–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8234196 [Accessed October 3, 2012].

Navarre, W.W. & Schneewind, O., 1999. Surface Proteins of Gram-Positive Bacteria and Mechanisms of Their Targeting to the Cell Wall Envelope. *Microbiol. Mol. Biol. Rev.*, 63(1), pp.174–229. Available at: http://mmbr.asm.org/content/63/1/174.full [Accessed October 3, 2012].

Nehme, N.T. et al., 2007. A model of bacterial intestinal infections in Drosophila melanogaster. *PLoS pathogens*, 3(11), p.e173. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2094306&tool=pmcentrez&rendertype=abstract [Accessed October 15, 2012].

Nguyen, M., Julien, J. & Rivest, S., 2002. Innate immunity: the missing link in neuroprotection and neurodegeneration? *Nature Reviews Neuroscience*. Available at: http://lea.univ-lille1.fr/Menu_du_Site/Cours d'Immunologie/frame/immunologie_fonctionnelle/cerveau_fichiers/cerveau.pdf [Accessed September 12, 2012].

Niswender, C.M. et al., 2001. RNA editing of the human serotonin 5-HT2C receptor. alterations in suicide and implications for serotonergic pharmacotherapy. *Neuropsychopharmacology*, 24(5), pp.478–91.

Niswender, C.M. et al., 1999. RNA editing of the human serotonin 5-hydroxytryptamine 2C receptor silences constitutive activity. *J Biol Chem*, 274(14), pp.9472–8.

O'Connell, M A, Gerber, A. & Keegan, L P, 1998. Purification of native and recombinant double-stranded RNA-specific adenosine deaminases. *METHODS: A companion to Methods in Enzymology*, 15, pp.51–62.

O'Connell, M A, Gerber, A. & Keller, W., 1997. Purification of human double-stranded RNA-specific editase 1 (hRED1) involved in editing of brain glutamate receptor B pre-mRNA. *J Biol Chem*, 272(1), pp.473–478. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8995285.

Oganesyan, G. et al., 2006. Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. *Nature*, 439(7073), pp.208–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16306936 [Accessed October 24, 2012].

Ohlson, J. et al., 2007. Editing modifies the GABA(A) receptor subunit alpha3. *Rna*, 13(5), pp.698–703. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17369310.

Osenberg, S. et al., 2010. Alu sequences in undifferentiated human embryonic stem cells display high levels of A-to-I RNA editing. *PLoS ONE*, 5(6), p.e11173. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20574523.

Ota, H. et al., 2013. ADAR1 Forms a Complex with Dicer to Promote MicroRNA Processing and RNA-Induced Gene Silencing. *Cell*, 153(3), pp.575–589. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23622242 [Accessed April 26, 2013].

Palladino, M.J. et al., 2000a. A-to-I pre-mRNA editing in Drosophila is primarily involved in adult nervous system function and integrity. *Cell*, 102(4), pp.437–449.

Palladino, M.J. et al., 2000b. dADAR, a Drosophila double-stranded RNA-specific adenosine deaminase is highly developmentally regulated and is itself a target for RNA editing. *Rna*, 6, pp.1004–1018.

Parameswaran, P. et al., 2010. Six RNA viruses and forty-one hosts: viral small RNAs and modulation of small RNA repertoires in vertebrate and invertebrate systems. *PLoS pathogens*, 6(2), p.e1000764. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2820531&tool=pmcentrez&rendertype=abstract [Accessed October 5, 2012].

Parks, A.L. et al., 2004. Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. *Nature genetics*, 36(3), pp.288–92. Available at: http://dx.doi.org/10.1038/ng1312 [Accessed October 5, 2012].

Patterson, John B & Samuel, Charles E, 1995. Expression and regulation by interferon of a doubled-stranded-RNA-specific adenosine deaminase from human cells: Evidence for two forms of the deaminase. *Mol. Cell. Biol.*, 15(10), pp.5376–5388.

Placido, D. et al., 2007. A left-handed RNA double helix bound by the Z alpha domain of the RNA-editing enzyme ADAR1. *Structure*, 15(4), pp.395–404. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17437712.

Polson, A.G. et al., 1991. The mechanism of adenosine to inosine conversion by the double-stranded RNA unwinding/modifying activity: A high-performance liquid chromatography-mass spectrometry analysis. *Biochemistry*, 30, pp.11507–11514.

Poulsen, H. et al., 2001. CRM1 mediates the export of ADAR1 through a nuclear export signal within the Z-DNA binding domain. *Mol Cell Biol*, 21(22), pp.7862–71.

Poulsen, H. et al., 2006. Dimerization of ADAR2 is mediated by the double-stranded RNA binding domain. *Rna*, 12(7), pp.1350–1360. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16682559.

Qiu, P., Pan, P.C. & Govind, S., 1998. A role for the Drosophila Toll/Cactus pathway in larval hematopoiesis. *Development (Cambridge, England)*, 125(10), pp.1909–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9550723 [Accessed October 3, 2012].

Ramos, A. et al., 2000. RNA recognition by a Staufen double-stranded RNA-binding domain. *EMBO J.*, 19(5), pp.997–1009. Available at: http://www.emboj.org/cgi/content/full/19/5/997.

Ravikumar, B. et al., 2004. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat Genet*, 36(6), pp.585–595. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15146184.

Reenan, R A, 2005. Molecular determinants and guided evolution of species-specific RNA editing. *Nature*, 434(7031), pp.409–413. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15772668.

Reineke, J. et al., 2007. Autocatalytic cleavage of Clostridium difficile toxin B. *Nature*, 446(7134), pp.415–9. Available at: http://dx.doi.org/10.1038/nature05622 [Accessed July 27, 2012].

Rice, G.I. et al., 2012. Mutations in ADAR1 cause Aicardi-Goutières syndrome associated with a type I interferon signature. *Nature Genetics*, advance on. Available at: http://dx.doi.org/10.1038/ng.2414 [Accessed September 24, 2012].

Ring, G.M., O'Connell, M A & Keegan, L P, 2004. Purification and assay of recombinant ADAR proteins expressed in the yeast Pichia pastoris or in Escherichia coli. *Methods Mol Biol*, 265, pp.219–238. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15103076.

Rodriguez, J., Menet, J.S. & Rosbash, M., 2012a. Nascent-Seq Indicates Widespread Cotranscriptional RNA Editing in Drosophila. *Molecular cell*. Available at: http://dx.doi.org/10.1016/j.molcel.2012.05.002 [Accessed June 5, 2012].

Rodriguez, J., Menet, J.S. & Rosbash, M., 2012b. Nascent-Seq Indicates Widespread Cotranscriptional RNA Editing in Drosophila. *Molecular cell*, 47(1), pp.27–37. Available at: http://www.cell.com/molecular-cell/fulltext/S1097-2765(12)00354-1 [Accessed July 12, 2012].

Roote, J. & Russell, S, 2012. Toward a complete Drosophila deficiency kit. *Genome Biology*. Available at: http://genomebiology.com/2012/13/3/149/ [Accessed October 10, 2012].

Ross, J. et al., 2003. Serine proteases and their homologs in the Drosophila melanogaster genome: an initial analysis of sequence conservation and phylogenetic relationships. *Gene*, 304(null), pp.117–131. Available at: http://dx.doi.org/10.1016/S0378-1119(02)01187-3 [Accessed October 10, 2012].

Royet, J., Gupta, D. & Dziarski, R., 2011. Peptidoglycan recognition proteins: modulators of the microbiome and inflammation. *Nature reviews. Immunology*, 11(12), pp.837–51. Available at: http://dx.doi.org/10.1038/nri3089 [Accessed July 16, 2012].

Rula, E.Y. et al., 2008. Developmental modulation of GABA(A) receptor function by RNA editing. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 28(24), pp.6196–201. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2746000&tool=pmcentrez&rendertype=abstract [Accessed October 4, 2012].

Ryan, M.Y. et al., 2008. Characterization of five RNA editing sites in Shab potassium channels. *Channels (Austin)*, 2(3), pp.202–209. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18836299.

Ryder, E. et al., 2007. The DrosDel deletion collection: a Drosophila genomewide chromosomal deficiency resource. *Genetics*, 177(1), pp.615–629. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17720900.

Samuel, C E, 2001. Antiviral actions of interferons. *Clin Microbiol Rev*, 14(4), pp.778–809, table of contents. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11585785.

Sansam, C.L., Wells, K.S. & Emeson, R B, 2003. Modulation of RNA editing by functional nucleolar sequestration of ADAR2. *Proc Natl Acad Sci U S A*. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14612560.

Sato, S, Wong, S.K. & Lazinski, D.W., 2001. Hepatitis delta virus minimal substrates competent for editing by ADAR1 and ADAR2. *J Virol*, 75(18), pp.8547–55.

Satoh, T. et al., 2010. LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. *Proceedings of the National Academy of Sciences of the United States of America*, 107(4), pp.1512–7. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2824407&tool=pmcentrez&rendertype=abstract [Accessed October 24, 2012].

Scadden, A.D. & Smith, C.W., 2001. RNAi is antagonized by A-->I hyper-editing. *EMBO Rep*, 2(12), pp.1107–1111. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11743024.

Schulz, O. et al., 2010. Protein kinase R contributes to immunity against specific viruses by regulating interferon mRNA integrity. *Cell host & microbe*, 7(5), pp.354–61. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2919169&tool=pmcentrez&rendertype=abstract [Accessed October 16, 2012].

Schwartz, T. et al., 1999. Crystal structure of the Zalpha domain of the human editing enzyme ADAR1 bound to left-handed Z-DNA. *Science*, 284(5421), pp.1841–5.

Schwientek, T. et al., 2002. The Drosophila gene brainiac encodes a glycosyltransferase putatively involved in glycosphingolipid synthesis. *The Journal of biological chemistry*, 277(36), pp.32421–9. Available at: http://www.jbc.org/content/277/36/32421.long [Accessed October 10, 2012].

Scott, R., Schuldiner, O. & Neufeld, T., 2004. Role and Regulation of Starvation-Induced Autophagy in the< i> Drosophila</i> Fat Body. *Developmental cell*. Available at: http://www.sciencedirect.com/science/article/pii/S153458070400245X [Accessed October 10, 2012].

Seth, R.B. et al., 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell*, 122(5), pp.669–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16125763 [Accessed October 24, 2012].

Shaw, P.J. & Ince, P.G., 1997. Glutamate, excitotoxicity and amyotrophic lateral sclerosis. *J Neurol*, 244 Suppl , pp.S3–14. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9178165.

Shpargel, K.B. et al., 2009. Gemin3 is an essential gene required for larval motor function and pupation in Drosophila. *Molecular biology of the cell*, 20(1), pp.90–101. Available at: http://www.molbiolcell.org/content/20/1/90.long [Accessed October 20, 2012].

Silverman, N et al., 2000. A Drosophila IkappaB kinase complex required for Relish cleavage and antibacterial immunity. *Genes & development*, 14(19), pp.2461–71. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=316979&tool=pmcentrez&rendertype=abstract [Accessed October 2, 2012].

Silverman, Neal et al., 2003. Immune activation of NF-kappaB and JNK requires Drosophila TAK1. *The Journal of biological chemistry*, 278(49), pp.48928–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14519762 [Accessed September 22, 2012].

Singh, M. et al., 2011. Altered ADAR 2 equilibrium and 5HT(2C) R editing in the prefrontal cortex of ADAR 2 transgenic mice. *Genes, brain, and behavior*, 10(6), pp.637–47. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3150620&tool=pmcentrez&rendertype=abstract [Accessed October 4, 2012].

Sixsmith, J. & Reenan, R A, 2007. Comparative genomic and bioinformatic approaches for the identification of new adenosine-to-inosine substrates. *Methods Enzymol*, 424, pp.245–264. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17662844.

Smith, L.A. et al., 1996. A Drosophila calcium channel a1 subunit gene maps to a genetic locus associated with behavioural and visual defects. *J. Neuroscience*, 16(24), pp.7868–7879.

Smith, L.A., Peixoto, A.A., Kramer, E.M., et al., 1998. Courtship and visual defects of cacophony mutants reveal functional complexity of a calcium-channel a1 subunit in Drosophila. *Genetics*, 149, pp.1407–1426.

Smith, L.A., Peixoto, A.A. & Hall, J.C., 1998. RNA editing in the Drosophila DMCA1A calcium-channel alpha 1 subunit transcript. *J Neurogenet*, 12(4), pp.227–40.

Stefl, R. et al., 2006. Structure and specific RNA binding of ADAR2 double-stranded RNA binding motifs. *Structure*, 14(2), pp.345–355. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16472753.

Stefl, R. et al., 2010. The solution structure of the ADAR2 dsRBM-RNA complex reveals a sequence-specific readout of the minor groove. *Cell*, 143(2), pp.225–237. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20946981.

Stein, V. & Nicoll, R.A., 2003. GABA generates excitement. *Neuron*, 37(3), pp.375–378. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12575946.

Stilwell, G.E. et al., 2006. Development of a Drosophila seizure model for in vivo high-throughput drug screening. *European Journal of Neuroscience*, 24(8), pp.2211–2222. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17074045.

Stöven, S et al., 2000. Activation of the Drosophila NF-kappaB factor Relish by rapid endoproteolytic cleavage. *EMBO reports*, 1(4), pp.347–52. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1083746&tool=pmcentrez&rendertype=abstract [Accessed October 2, 2012].

Strehblow, A., Hallegger, M. & Jantsch, M F, 2002. Nucleocytoplasmic distribution of human RNA-editing enzyme ADAR1 is modulated by double-stranded RNA-binding domains, a leucine-rich export signal, and a putative dimerization domain. *Mol Biol Cell*, 13(11), pp.3822–3835. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12429827.

Sun, Huaiyu et al., 2004. Regulated assembly of the Toll signaling complex drives Drosophila dorsoventral patterning. *The EMBO journal*, 23(1), pp.100–10. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1271671&tool=pmcentrez&rendertype=abstract [Accessed October 2, 2012].

Takahasi, K. et al., 2008. Nonself RNA-sensing mechanism of RIG-I helicase and activation of antiviral immune responses. *Molecular cell*, 29(4), pp.428–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18242112 [Accessed October 4, 2012].

Takehana, A. et al., 2004. Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in Drosophila immunity. *The EMBO journal*, 23(23), pp.4690–700. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=533052&tool=pmcentrez&rendertype=abstract [Accessed August 1, 2012].

Tang, H., Regulation and function of the melanization reaction in Drosophila. *Fly*, 3(1), pp.105–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19164947 [Accessed October 3, 2012].

Tanji, T. et al., 2007. Toll and IMD pathways synergistically activate an innate immune response in Drosophila melanogaster. *Molecular and cellular biology*, 27(12), pp.4578–88. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1900069&tool=pmcentrez&rendertype=abstract [Accessed August 4, 2012].

Tariq, A. & Jantsch, Michael F, 2012. Transcript diversification in the nervous system: a to I RNA editing in CNS function and disease development. *Frontiers in neuroscience*, 6, p.99. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3391646&tool=pmcentrez&rendertype=abstract [Accessed October 4, 2012].

Taylor, D.R. et al., 2005. New antiviral pathway that mediates hepatitis C virus replicon interferon sensitivity through ADAR1. *J Virol*, 79(10), pp.6291–6298. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15858013.

Teninges, D. et al., 1979. Isolation and Biological Properties of Drosophila X Virus. *Journal of General Virology*, 42(2), pp.241–254. Available at: http://vir.sgmjournals.org/content/42/2/241.abstract?ijkey=116bc3c75fc2e0a22b9db5dbc9e3ba7649ecf82f&keytype2=tf_ipsecsha [Accessed October 3, 2012].

Toth, A.M. et al., 2009. RNA-specific adenosine deaminase ADAR1 suppresses measles virus-induced apoptosis and activation of protein kinase PKR. *J Biol Chem*, 284(43), pp.29350–29356. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19710021.

Trudeau, D., Washburn, J.O. & Volkman, L.E., 2001. Central role of hemocytes in Autographa californica M nucleopolyhedrovirus pathogenesis in Heliothis virescens and Helicoverpa zea. *Journal of virology*, 75(2), pp.996–1003. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=113996&tool=pmcentrez&rendertype=abstract [Accessed October 3, 2012].

Trunova, S. & Giniger, Edward, 2012. Absence of the Cdk5 activator p35 causes adult-onset neurodegeneration in the central brain of Drosophila. *Disease models & mechanisms*, 5(2), pp.210–9. Available at: http://dmm.biologists.org/cgi/content/abstract/5/2/210 [Accessed September 16, 2012].

Tsai, C.W. et al., 2008. Drosophila melanogaster mounts a unique immune response to the Rhabdovirus sigma virus. *Applied and environmental microbiology*, 74(10), pp.3251–6. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2394955&tool=pmcentrez&rendertype=abstract [Accessed August 14, 2012].

Vesely, C. et al., 2012. Adenosine deaminases that act on RNA induce reproducible changes in abundance and sequence of embryonic miRNAs. *Genome research*, 22(8), pp.1468–1476. Available at: http://genome.cshlp.org/content/22/8/1468.short [Accessed July 13, 2012].

Vidal, S. et al., 2001. Mutations in the Drosophila dTAK1 gene reveal a conserved function for MAPKKKs in the control of rel/NF-kappaB-dependent innate immune responses. *Genes & development*, 15(15), pp.1900–12. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=524699&tool=pmcentrez&rendertype=abstract [Accessed October 2, 2012].

Vitali, P. & Scadden, A.D., 2010. Double-stranded RNAs containing multiple IU pairs are sufficient to suppress interferon induction and apoptosis. *Nat Struct Mol Biol*, 17(9), pp.1043–1050. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20694008.

Wang, Lihui et al., 2006. Sensing of Gram-positive bacteria in Drosophila: GNBP1 is needed to process and present peptidoglycan to PGRP-SA. *The EMBO journal*, 25(20), pp.5005–14. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1618108&tool=pmcentrez&rendertype=abstract [Accessed August 1, 2012].

Wang, Likun et al., 2010. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics (Oxford, England)*, 26(1), pp.136–8.

Available at: http://bioinformatics.oxfordjournals.org/content/26/1/136.abstract [Accessed July 16, 2012].

Wang, Q. et al., 2004. Stress-induced apoptosis associated with null mutation of ADAR1 RNA editing deaminase gene. *J Biol Chem*, 279(6), pp.4952–4961. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14613934.

Wang, Q. et al., 2005. Vigilins bind to promiscuously A-to-I-edited RNAs and are involved in the formation of heterochromatin. *Curr Biol*, 15(4), pp.384–391. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15723802.

Wang, X.H. et al., 2006. RNA interference directs innate immunity against viruses in adult Drosophila. *Science*, 312(5772), pp.452–454. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16556799.

Wang, Y et al., 2001. The JIL-1 tandem kinase mediates histone H3 phosphorylation and is required for maintenance of chromatin structure in Drosophila. *Cell*, 105(4), pp.433–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11371341 [Accessed October 20, 2012].

Weber, A.N.R. et al., 2003. Binding of the Drosophila cytokine Spätzle to Toll is direct and establishes signaling. *Nature immunology*, 4(8), pp.794–800. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12872120 [Accessed October 2, 2012].

Williams, B.R., 1999. PKR; a sentinel kinase for cellular stress. *Oncogene*, 18(45), pp.6112–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10557102 [Accessed October 16, 2012].

Wittig, B., Dorbic, T. & Rich, A., 1991. Transcription is associated with Z-DNA formation in metabolically active permeabilized mammalian cell nuclei. *Proceedings of the National Academy of Sciences*, 88(6), pp.2259–2263. Available at: http://www.pnas.org/content/88/6/2259.short [Accessed September 28, 2012].

Wu, D., Lamm, A.T. & Fire, A Z, 2011. Competition between ADAR and RNAi pathways for an extensive class of RNA targets. *Nat Struct Mol Biol*. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21909095.

Wu, H. et al., 2004. Structural basis for recognition of the AGNN tetraloop RNA fold by the double-stranded RNA-binding domain of Rnt1p RNase III. *Proceedings of the*

National Academy of Sciences of the United States of America, 101(22), pp.8307–12. Available at: http://www.pnas.org/content/101/22/8307.short [Accessed September 28, 2012].

XuFeng, R. et al., 2009. ADAR1 is required for hematopoietic progenitor cell survival via RNA editing. *Proc Natl Acad Sci U S A*, 106(42), pp.17763–17768. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19805087.

Yang, W. et al., 2005. ADAR1 RNA deaminase limits short interfering RNA efficacy in mammalian cells. *J Biol Chem*, 280(5), pp.3946–3953. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15556947.

Yang, W. et al., 2006. Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. *Nat Struct Mol Biol*, 13(1), pp.13–21. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16369484.

Yano, T. et al., 2008. Autophagic control of listeria through intracellular innate immune recognition in drosophila. *Nature immunology*, 9(8), pp.908–16. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2562576&tool=pmcentrez&rendertype=abstract [Accessed September 11, 2012].

Yeh, E. et al., 2000. Neuralized functions cell autonomously to regulate Drosophila sense organ development. *The EMBO journal*, 19(17), pp.4827–37. Available at: http://dx.doi.org/10.1093/emboj/19.17.4827 [Accessed October 20, 2012].

Yoneyama, M. et al., 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *Journal of immunology (Baltimore, Md. : 1950)*, 175(5), pp.2851–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16116171 [Accessed October 24, 2012].

Yoneyama, M. et al., 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nature immunology*, 5(7), pp.730–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15208624 [Accessed October 24, 2012].

Zahn, R.C. et al., 2007. A-to-G hypermutation in the genome of lymphocytic choriomeningitis virus. *J Virol*, 81(2), pp.457–464. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17020943.

Zambon, R.A. et al., 2005. The Toll pathway is important for an antiviral response in Drosophila. *Proc Natl Acad Sci U S A*, 102(20), pp.7257–7262. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15878994.

Zasloff, M., 2002. Antimicrobial peptides of multicellular organisms. *Nature*. Available at: http://uregina.ca/suhdaey/courses/BIOC 430/reading/10R29 AMPs Nature.pdf [Accessed October 7, 2012].

Zeidler, M.P. et al., 2004. Temperature-sensitive control of protein activity by conditionally splicing inteins. *Nature biotechnology*, 22(7), pp.871–6. Available at: http://dx.doi.org/10.1038/nbt979 [Accessed July 16, 2012].

Zhang, Weiguo et al., 2003. Genetic and Phenotypic Analysis of Alleles of the Drosophila Chromosomal JIL-1 Kinase Reveals a Functional Requirement at Multiple Developmental Stages. *Genetics*, 165(3), pp.1341–1354. Available at: http://www.genetics.org/content/165/3/1341.long [Accessed October 20, 2012].

Zhang, Weiguo et al., 2006. The JIL-1 histone H3S10 kinase regulates dimethyl H3K9 modifications and heterochromatic spreading in Drosophila. *Development (Cambridge, England)*, 133(2), pp.229–35. Available at: http://dev.biologists.org/content/133/2/229.long [Accessed October 10, 2012].

Zhang, X.J. et al., 2004. Seven novel mutations of the ADAR gene in Chinese families and sporadic patients with dyschromatosis symmetrica hereditaria (DSH). *Hum Mutat*, 23(6), pp.629–630. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15146470.

Zhou, Rui et al., 2005. The role of ubiquitination in Drosophila innate immunity. *The Journal of biological chemistry*, 280(40), pp.34048–55. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16081424 [Accessed August 11, 2012].

Appendix: Supplementary materials, figures and publications

Appendix I Supplementary materials

Table1. Deficiencies used for the genetic screens

Symbol	Chromosome	Deletion Start	Deletion End	BDSC Number
ED4079	3L	40319	131780	8046
Exel6083	3L	104350	180193	7562
ED201	3L	123924	347941	8047
ED4177	3L	319846	1035182	8048
ED207	3L	738739	1568108	8053
Exel6086	3L	749809	959651	7565
Exel6087	3L	1478674	1586881	7566
ED4256	3L	1546104	1586663	8054
ED4287	3L	1795442	2551761	8096
ED4284	3L	1795442	1963552	8056
ED4288	3L	3070827	3149091	8057
ED4293	3L	3226338	3250564	8058
ED4341	3L	3905091	4542236	8060
ED4342	3L	4277987	4625372	8062
Exel7208	3L	4458589	4692071	7926
Exel9058	3L	4542105	4554415	7923
ED210	3L	4544234	5348442	8061
Exel6102	3L	4692405	4976311	7581
Exel6103	3L	4976403	5177896	7582
Exel6104	3L	5177896	5359162	7583
Exel6105	3L	5359162	5601375	7584
Exel6106	3L	5601375	5684102	7585
Exel6107	3L	5746110	5895644	7586
BSC410	3L	5763773	6483285	24914
ED211	3L	6211235	6545859	8063
Exel6109	3L	6736213	6936639	7588
BSC224	3L	6957557	7150109	9642
Exel8104	3L	7353086	7522363	7929
BSC459	3L	7427327	7999689	249663
ED4408	3L	7972207	8292674	8065
ED4421	3L	8738426	9377175	8066
RDL-2/Sb	3L	66F5	66F5	1688
BSC113	3L	9342609	9416591	8970
BSC391	3L	9439870	9690291	24415
BSC392	3L	9671803	9892355	24416

BSC673	3L	9756714	10174058	26525
ED4457	3L	10357051	11118909	9355
BSC439	3L	10500147	10957206	24943
BSC378	3L	10735370	11091445	24402
ED4470	3L	11090089	11826330	8068
ED4475	3L	11580140	12401701	8069
ED4483	3L	12270320	12686314	8070
ED4486	3L	12507519	13025585	8072
ED4502	3L	13220865	13986651	8097
ED4543	3L	13928325	14751140	8073
ED217	3L	14751170	15582196	8074
ED218	3L	15007168	15582196	8075
BSC442	3L	15467849	15613088	24946
ED220	3L	16080584	16404777	8077
ED223	3L	16444925	16883977	8079
ED4674	3L	16654384	17042518	8098
ED4685	3L	16884176	17605270	8099
ED4710	3L	17480563	18132399	8100
ED224	3L	17962303	18391619	8080
ED225	3L	18179245	18614437	8081
BSC416	3L	18572608	18884362	24920
ED4782	3L	18988994	19163802	8082
ED4786	3L	19094051	19288762	8083
BSC417	3L	19163798	19597367	24921
ED228	3L	19163806	19864908	8086
ED4799	3L	19163806	19288762	8085
BSC797	3L	20445923	20942833	27369
BSC449	3L	20850015	21196030	24953
BSC419	3L	21218032	21597878	24923
ED4978	3L	21526907	21873785	8101
ED230	3L	22127751	22827471	8089
ED5017	3L	22828597	22991401	8102
ED231	3L	22864916	22938620	8090
ED5100	3R	22995	912807	9226
ED5021	3R	22995	216113	9196
ED5020	3R	107408	216113	9075
ED5142	3R	279018	1090605	9198
ED5066	3R	475607	778404	8092
ED5095	3R	475607	912807	8093

ED5177	3R	1426351	1449817	8103
ED5196	3R	1510301	1833866	8681
Exel6145	3R	1542490	1638975	7624
Exel7284	3R	1641744	1833511	7953
BSC681	3R	2111067	2206257	26533
BSC467	3R	2365827	2824771	24971
ED7665	3R	2916249	3919805	8685
ED5230	3R	3803496	4478856	8682
ED5330	3R	4495308	5055517	9077
Exel6151	3R	4878552	4983798	7630
ED5343	3R	4859916	5178097	150524
Exel6152	3R	4983798	5073203	7631
ED5339	3R	5052798	5178097	9204
ED5454	3R	5552399	5937180	9080
ED5516	3R	7059892	7445622	8968
ED5559	3R	7394904	8269738	8920
ED5591	3R	8176253	8545732	9086
ED5610	3R	8269738	8821397	9087
ED5612	3R	8545707	9470856	9089
ED5642	3R	9509544	10307496	9279
ED5664	3R	10523031	11054571	24137
ED5705	3R	11117380	11619518	9152
ED10639	3R	12038635	12306942	9481
ED10642	3R	12279479	12450993	9482
ED5780	3R	12882199	13507523	8104
ED2	3R	14224953	14922493	6962
ED5911	3R	14568649	14991505	8683
ED5938	3R	14732356	15467758	24139
ED5942	3R	15052016	15660809	8922
ED6025	3R	15468450	16135241	8964
ED10820	3R	16774462	16937182	150268
BSC508	3R	16886325	16966208	25012
ED10845	3R	16890893	17122221	9487
ED10838	3R	16960036	17122221	9485
ED6058	3R	17122217	17545322	8923
ED6076	3R	17459227	17868550	8962
ED6085	3R	17706717	18413461	8923
ED6096	3R	18413403	19047691	8684
ED6103	3R	18724275	19084137	8963

BSC619	3R	18887281	19172138	25694
Exel6280	3R	19017039	19121235	7686
BSC489	3R	19273602	19768726	24993
ED10893	3R	19713027	19930781	28827
ED6220	3R	20369520	21009495	9211
Exel6203	3R	21341620	21463598	7682
ED6235	3R	22360956	22806229	7709
ED6255	3R	22624758	23107623	7723
BSC567	3R	23763552	24627253	25390
ED6310	3R	24964617	25337875	8961
BSC620	3R	25702740	25860612	25695
Exel6214	3R	25925104	26028690	7692
ED6332	3R	26103647	26215013	24141
BSC504	3R	26253789	26512985	25008
Exel7378	3R	26388946	26620677	7997
Exel8194	3R	26582117	26713967	7918
ED6346	3R	26609284	26874606	24142
BSC749	3R	26837657	27136770	26847
ED6361	3R	27434853	27904166	24143

Appendix II Supplementary figures

Table S1 Numbers of male progeny from the crosses of *Adar*^{5G1} females with heterozygous deficiencies on Chr3L and relative viability calculations.

	Number of male progeny				*Viability calculation*		
♀ ***Adar*^{5G1}** ×♂	*Adar*^{5G1}*;;Df* **a**	*Adar*^{5G1} **b**	*FM7;;Df* **c**	*FM7;;Bal* **d**	**a/c**	**(a/c) / (b/d)**	**P Value**
ED4079	73	0	55	0	1.33	-	1.00
Exel6083	57	21	98	66	0.58	1.83	0.30
ED201	38	44	61	50	0.62	0.71	1.00
ED4177	17	24	26	23	0.65	0.63	1.00
Exel6087	35	18	85	70	0.41	1.60	0.49
ED218	35	0	80	0	0.44	-	1.00
ED4256	31	12	49	49	0.63	2.58	0.15
ED4284	21	20	44	43	0.48	1.03	1.00
ED4287	15	10	33	23	0.45	1.05	1.00
ED4288	13	9	46	38	0.28	1.19	0.93
ED4293	27	0	57	0	0.47	-	1.00
ED4341	23	18	28	38	0.82	1.73	0.50
ED4342	15	11	42	41	0.36	1.33	0.84
Exel7208	55	16	53	49	1.04	3.18	0.02
Exel9058	85	27	47	79	1.81	5.29	0.00
ED210	58	36	89	87	0.65	1.57	0.37
Exel6102	29	19	66	39	0.44	0.90	1.00
Exel6103	39	41	95	66	0.41	0.66	1.00
Exel6104	53	28	83	63	0.64	1.44	0.50
Exel6105	27	18	39	17	0.69	0.65	1.00
Exel6106	36	15	37	18	0.97	1.17	0.92
Exel6107	22	9	23	16	0.96	1.70	0.65
BSC410	28	18	31	29	0.90	1.46	0.65
ED211	9	3	25	29	0.36	3.48	0.39
Exel6109	25	8	23	20	1.09	2.72	0.34
ED224	44	46	82	84	0.54	0.98	1.00
Exel8104	42	16	37	28	1.14	1.99	0.37

BSC459	29	29	64	54	0.45	0.84	1.00
ED4408	48	42	70	52	0.69	0.85	1.00
ED4421	18	4	63	26	0.29	1.86	0.65
Rdl-2	44	15	56	53	0.79	2.78	0.05
BSC113	37	5	33	7	1.12	1.57	0.84
BSC391	2	0	2	4	1.00	-	0.65
BSC773	2	4	11	9	0.18	0.41	1.00
BSC392	34	28	26	31	1.31	1.45	0.65
BSC673	28	22	23	21	1.22	1.16	0.92
ED4457	13	24	30	39	0.43	0.70	1.00
BSC439	22	22	21	22	1.05	1.05	1.00
BSC378	41	20	39	37	1.05	1.94	0.35
ED4470	105	78	132	115	0.80	1.17	0.66
Ed4475	36	0	55	0	0.65	-	1.00
ED215	16	11	34	50	0.47	2.14	0.39
ED4483	15	19	28	28	0.54	0.79	1.00
ED4486	17	31	43	44	0.40	0.56	1.00
ED4502	52	0	101	0	0.51	-!	1.00
Ed4543	13	8	19	14	0.68	1.20	0.96
ED217	26	19	47	58	0.55	1.69	0.49
ED207	61	0	64	0	0.95	-	1.00
Exel6086	33	11	49	58	0.67	3.55	0.02
BSC442	18	21	22	11	0.82	0.43	1.00
ED220	6	2	16	18	0.38	3.38	0.53
ED223	13	18	26	22	0.50	0.61	1.00
ED4674	15	16	46	49	0.33	1.00	1.00
ED4685	6	16	43	45	0.14	0.39	1.00
ED4710	15	14	28	40	0.54	1.53	0.65
BSC224	25	22	22	34	1.14	1.76	0.50
ED225	21	28	38	40	0.55	0.79	1.00
BSC416	15	16	33	39	0.45	1.11	0.96
BSC416	6	10	13	20	0.46	0.92	1.00
ED4782	19	18	39	50	0.49	1.35	0.75
ED4786	10	0	50	0	0.20	-	1.00
BSC417	12	12	30	31	0.40	1.03	1.00

ED228	8	14	30	36	0.27	0.69	1.00
ED4799	26	12	52	51	0.50	2.13	0.35
BSC797	26	26	36	47	0.72	1.31	0.75
BSC449	33	19	36	39	0.92	1.88	0.39
BSC553	1	2	6	13	0.17	1.08	1.00
BSC419	21	22	30	45	0.70	1.43	0.65
ED4978	5	7	17	20	0.29	0.84	1.00
ED230	12	13	35	15	0.34	0.40	1.00
ED5017	13	26	38	28	0.34	0.37	1.00
ED231	16	9	25	24	0.64	1.71	0.65

Table S2 Numbers of male progeny from the crosses of *Adar*^{5G1} females with heterozygous deficiencies on Chr3R and relative viability calculations.

	Number of male progeny				*Viability calculation*		
♀ **Adar^{5G1}** ×♂	*Adar^{5G1};;Df* **a**	*Adar^{5G1}* **b**	*FM7;;Df* **c**	*FM7;;Bal* **d**	**a/c**	**(a/c) (b/d)**	**P Value**
ED5100	49	0	140	0	0.35	-	1.00
ED5100	12	1	39	14	0.31	4.31	0.50
ED5020	28	5	50	4	0.56	0.45	1.00
ED5021	19	25	39	63	0.49	1.23	0.84
ED5142	17	17	27	26	0.63	0.96	1.00
ED5066	39	19	52	80	0.75	3.16	0.02
ED5095	19	19	81	56	0.23	0.69	1.00
ED5095	12	15	68	84	0.18	0.99	1.00
ED5171	27	22	80	58	0.34	0.89	1.00
BSC525	0	3	14	8	0.00	0.00	1.00
Exel7283	2	0	12	6	0.17	-	0.96
ED5196	37	26	41	63	0.90	2.19	0.15
Exel7284	65	20	61	54	1.07	2.88	0.02
Exel6145	49	29	33	38	1.48	1.95	0.32
BSC319	11	1	25	26	0.44	11.44	0.10
BSC681	10	30	37	37	0.27	0.33	1.00
BSC467	11	11	9	9	1.22	1.00	1.00
ED7665	34	29	32	28	1.06	1.03	1.00
ED5230	18	16	17	22	1.06	1.46	0.75
ED5330	22	25	29	19	0.76	0.58	1.00
Exel6151	17	3	24	15	0.71	3.54	0.37

ED5343	43	16	56	45	0.77	2.16	0.25
Exel6152	27	10	23	15	1.17	1.76	0.61
ED5339	25	21	36	36	0.69	1.19	0.88
ED5454	31	10	74	30	0.42	1.26	0.86
ED5516	11	12	50	45	0.22	0.83	1.00
ED5559	38	38	90	73	0.42	0.81	1.00
ED5591	35	38	50	31	0.70	0.57	1.00
ED5610	49	47	67	72	0.73	1.12	0.87
Exel6167	3	0	12	11	0.25	-	0.59
ED5612	21	16	20	17	1.05	1.12	0.97
ED5642	39	0	104	0	0.38	-	1.00
ED5664	33	34	36	28	0.92	0.75	1.00
ED5705	30	0	25	0	1.20	-	1.00
ED10639	17	20	32	29	0.53	0.77	1.00
ED10642	32	0	114	0	0.28	-	1.00
ED5780	22	21	30	32	0.73	1.12	0.95
ED2	43	26	67	68	0.64	1.68	0.37
ED5911	5	6	60	53	0.08	0.74	1.00
ED5938	45	24	57	47	0.79	1.55	0.50
ED5942	11	19	30	48	0.37	0.93	1.00
ED5942	38	0	105	0	0.36	-	1.00
ED6025	17	16	50	59	0.34	1.25	0.84
ED10820	10	3	65	52	0.15	2.67	0.50
BSC508	3	4	17	16	0.18	0.71	1.00
ED10845	25	11	22	26	1.14	2.69	0.28

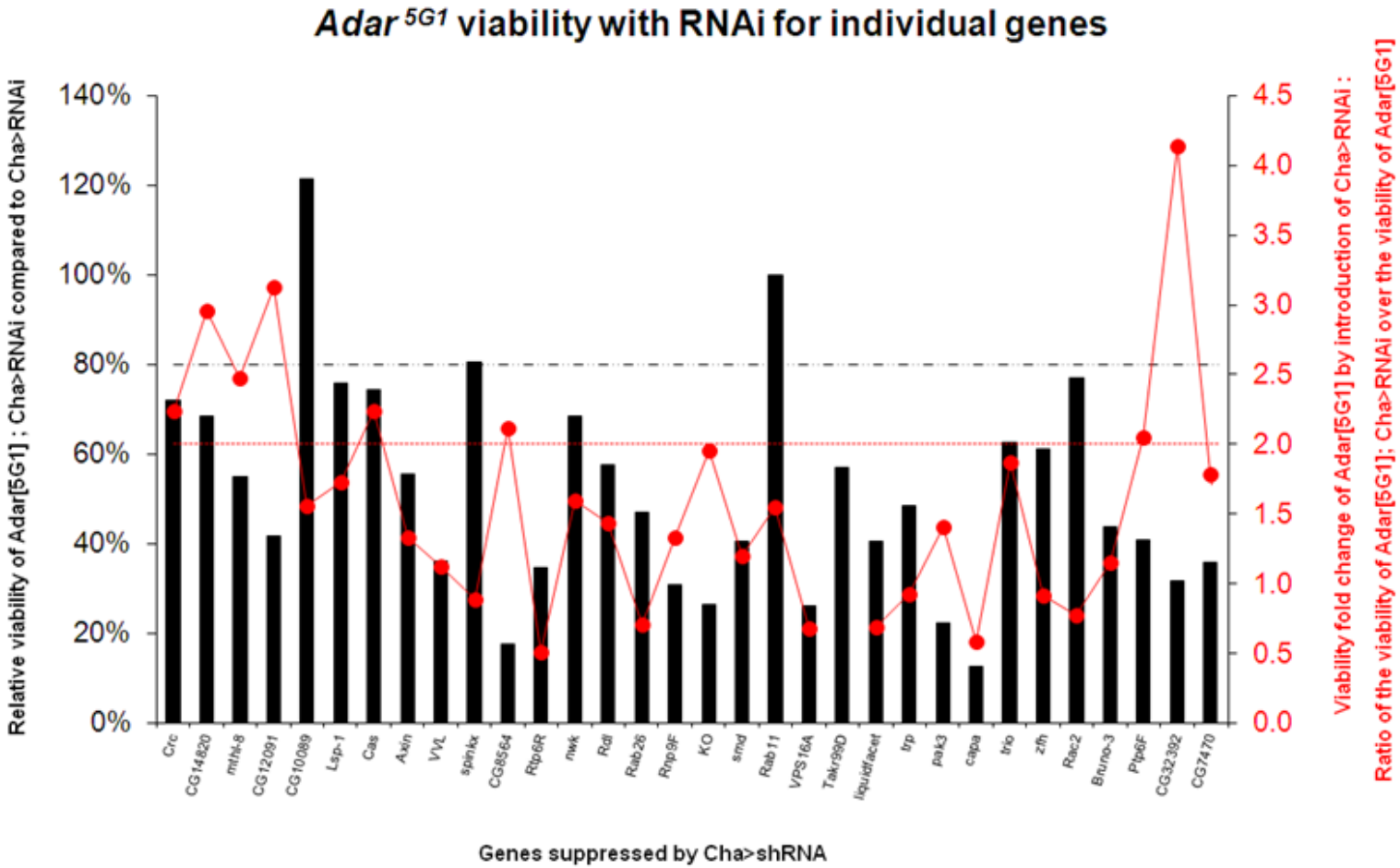
ED10838	15	14	24	42	0.63	1.88	0.50
ED6058	9	12	29	37	0.31	0.96	1.00
ED6076	33	22	38	31	0.87	1.22	0.84
ED6085	32	31	27	32	1.19	1.22	0.84
ED6096	38	26	79	79	0.48	1.46	0.50
ED6103	30	23	34	41	0.88	1.57	0.50
BSC619	18	36	18	34	1.00	0.94	1.00
Exel6280	37	14	36	25	1.03	1.84	0.49
BSC489	40	31	72	43	0.56	0.77	1.00
ED10893	44	36	45	38	0.98	1.03	1.00
ED6220	18	16	40	48	0.45	1.35	0.75
Exel6203	24	15	35	23	0.69	1.05	1.00
ED6235	13	6	60	56	0.22	2.02	0.50
ED6255	9	11	15	13	0.60	0.71	1.00
BSC567	5	17	33	34	0.15	0.30	1.00
ED6310	21	17	46	66	0.46	1.77	0.48
BSC620	23	10	18	20	1.28	2.56	0.36
BSC861	6	1	12	8	0.50	4.00	0.65
Exel6213	7	0	13	7	0.54	-	0.47
Exel6214	2	4	10	9	0.20	0.45	1.00
ED6332	20	13	36	19	0.56	0.81	1.00
BSC504	39	25	73	52	0.53	1.11	0.92
Exel7378	35	6	21	19	1.67	5.28	0.03
Exel8194	52	28	49	44	1.06	1.67	0.39
ED6346	25	16	49	58	0.51	1.85	0.39

BSC749	16	27	32	25	0.50	0.46	1.00
ED6361	0	10	9	15	0.00	0.00	1.00
ED6532	9	3	55	51	0.16	2.78	0.50

Table S4. Numbers of male progeny and relative viabilities from the crosses of *Adar*^{ex2} females to *UAS-shRNA* males.

	P		PP	P	N		NN	N	Viability	Rescue
♀ *Adar*^{ex2} × ♂	a *Cha-Gal4*	b *Cha>shRNA*	c *Triple balancers*	d *UAS-shRNA*	e *Adar*^{ex2}/*Y*; *Cha-Gal4*	f *Adar*^{ex2}/*Y*; *Cha>shRNA*	g *Adar*^{ex2}	h *Adar*^{ex2}/*Y*; *UAS-shRNA*	f/b	(f/b)/(g/c)
Crc	0	32	0	28	0	23	0	9	72%	2.2
CG14820	0	35	0	56	0	24	0	13	69%	3.0
mthl-8	0	40	0	54	0	22	0	12	55%	2.5
CG12091	0	36	0	15	0	15	0	2	42%	3.1
CG10089	0	28	0	9	0	34	0	7	121%	1.6
Lsp-1	10	29	0	25	8	22	0	11	76%	1.7
Cas	0	51	0	33	0	38	0	11	75%	2.2
Axin	24	27	2	12	15	15	0	5	56%	1.3
VVL	0	61	0	25	0	22	0	8	36%	1.1
Sphinkx1	0	46	0	31	0	37	0	28	80%	0.9
CG8564	13	17	0	12	3	3	0	1	18%	2.1
Rtp6R	0	69	0	29	0	24	0	20	35%	0.5
nwk	0	19	0	21	0	13	0	9	68%	1.6
Rdl	0	66	0	25	0	38	0	10	58%	1.4

Rab26	0	115	0	18	0	54	0	12	47%	0.7
Rnp9F	0	13	0	13	0	4	0	3	31%	1.3
KO	0	72	0	37	0	19	0	5	26%	2.0
smid	0	69	0	53	0	28	0	18	41%	1.2
Rab11	0	1	0	17	0	1	0	11	100%	1.5
VPS16A	0	46	0	68	0	12	0	26	26%	0.7
Takr99D	0	14	0	9	0	8	0	0	57%	–
Lqf	0	91	0	51	0	37	0	30	41%	0.7
trp	1	35	0	21	0	17	0	11	49%	0.9
pak3	0	58	0	69	0	13	0	11	22%	1.4
capa	0	16	0	14	0	2	0	3	13%	0.6
trio	0	8	0	9	0	5	0	3	63%	1.9
zfh	0	95	0	21	0	58	0	14	61%	0.9
Rac2	0	13	0	8	0	10	0	8	77%	0.8
Bruno-3	0	16	0	42	0	7	0	16	44%	1.1
Ptp6F	0	22	0	25	0	9	0	5	41%	2.0
CG32392	0	44	0	39	0	14	0	3	32%	4.1
CG7470	17	28	0	15	3	10	1	3	36%	1.8



Figure·S1·Relative·viabilities· of·progeny·*Adar5G1;·Cha>shRNA*·flies·expressing·shRNAs·against·candidate·genes.

Appendix III Publications

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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*^{1E4} 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 Y/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
Cac1D								
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								
1865 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
Nac 3'E								
1872 L/L	**100**	6	**76**	4	**85**	4	**100**	4
1873 I/V	**100**	6	**78**	4	**85**	4	**100**	4
2020 I/A	**100**	7	**97**	6	**100**	5	**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 $Adar^{1E4}$ 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 $Adar^{1E4}$ deletion removes promoters of *Adar* but leaves the coding sequence intact and its expression is at

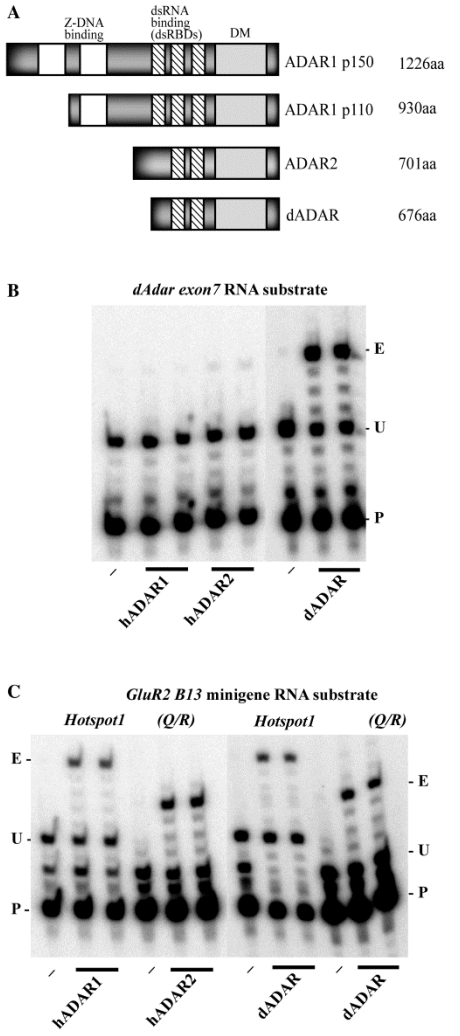

Figure 1. Comparison of human and *Drosophila* ADAR structures and activities on RNA substrates *in vitro*. (**A**) Domain structures of human and *Drosophila* ADARs. (**B**) *In vitro* RNA editing of a single site in the *Drosophila Adar exon* 7 substrate by duplicate samples of *Drosophila* and human 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

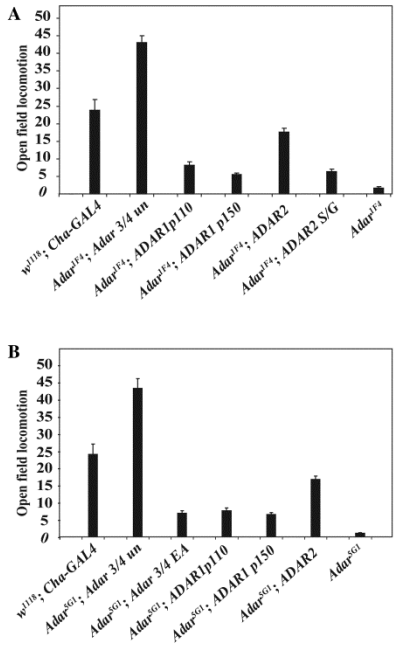

Figure 2. Human *ADAR2* rescues *Drosophila Adar* mutant locomotion defects. (**A**) Rescue by human ADAR2 of hypomorphic $Adar^{1F4}$ mutant open field locomotion defects with the strong neuron-specific *Cha-GAL4* driver. Neither the long nucleocytoplasmic shuttling human ADAR1p150 isoform nor the shorter 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 *Cα1D* 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
Cra1D												
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	**23**	5	**36**	9	**0**	7	**0**	13
2139 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]	2	[a]	1	**31**	6	**0**	2	[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	[a]	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 $Adar^{1E4}$ 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 $Adar^{1E4}$ 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 $Adar^{1E4}$ strain. Also RNA editing by ADAR1 is observed at more sites in ion channel transcripts in the $Adar^{1E4}$ 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**	1
2083 N/D	**97**	2	**0**	3	**31**	5	**2**	4	**0**	2	**0**	1
2097 L/L	**96**	2	**0**	3	**24**	5	**0**	2	**0**	1	**0**	1
2098 R/G	**96**	2	[a]	3	**29**	5	**32**	2	**0**	1	**0**	1
2140 I/M	**100**	2	**0**	4	**26**	5	**20**	4	**0**	1	**0**	1
Eag												
1864 K/R	**58**	3	**0**	2	**50**	2	**58**	2	**0**	7	**0**	4
2107 Y/C	**89**	5	**0**	2	**55**	3	**56**	5	**10**	7	**0**	4
2159 V/V	**16**	5	**0**	2	**10**	5	**24**	6	**0**	7	**0**	4
2163 N/D	**88**	5	**0**	2	**62**	5	**46**	6	**40**	7	**12**	4
2177 A/A	**0**	5	**0**	2	[a]	5	**0**	6	**0**	7	**0**	4
2560 K/R	**78**	3	**0**	1	**37**	2	**56**	2	**0**	3	**0**	2
Nic 34E												
1872 L/L	**100**	4	**0**	5	[b]		**76**	2	**0**	1	**0**	2
1873 I/V	**100**	4	**0**	5	[b]		**74**	2	**0**	1	**0**	2
2020 T/A	**100**	3	**0**	5	**82**	3	**80**	3	**26**	3	**0**	2
2023 I/V	**38**	1	**0**	5	**35**	3	[a]	3	**0**	3	**0**	2
2028 L/L	**35**	1	**0**	5	**29**	3	**15**	3	**0**	3	**0**	2
2037 I/M	**67**	1	**0**	4	**63**	3	**75**	3	**84**	3	**37**	1
2052 S/S	**71**	1	**0**	4	**60**	3	**8**	3	**27**	3	**0**	2
2062 I/V	**100**	3	**0**	5	**84**	3	**38**	2	**32**	3	**10**	1
2065 I/V	**53**	1	**0**	5	**42**	3	**13**	2	**54**	2	**17**	1
Rdl												
728 L/L	**23**	2	**0**	2	**29**	2	**34**	4	[a]	2	**0**	2
735 R/G	**65**	2	**0**	2	**52**	2	**64**	4	**15**	2	**0**	2
1218 I/V	**100**	3	[a]	2	**88**	2	**81**	4	**31**	2	**16**	2
1251 N/D	**22**	3	**0**	3	**0**	2	**0**	5	**0**	2	**0**	2
1448 Q/Q	**8**	3	**0**	3	**10**	2	**0**	3	**0**	2	**0**	2
1449 M/V	**22**	3	**0**	3	**12**	2	[a]	3	**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.
[b]Sites that we were unable to obtain sequence for.

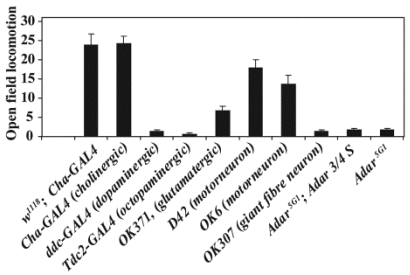

Figure 3. *Adar* expression in cholinergic or motor neurons is sufficient to rescue $Adar^{5G1}$ mutant locomotion defects. The chart shows open field locomotion in $Adar^{5G1}$ flies, $Adar^{5G1}$*; UAS-Adar 3/4 S* flies having this UAS construct in the absence of any GAL4 driver to induce expression 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 $Adar^{5G1}$

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 *ADARs*. 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 *ADARs* 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

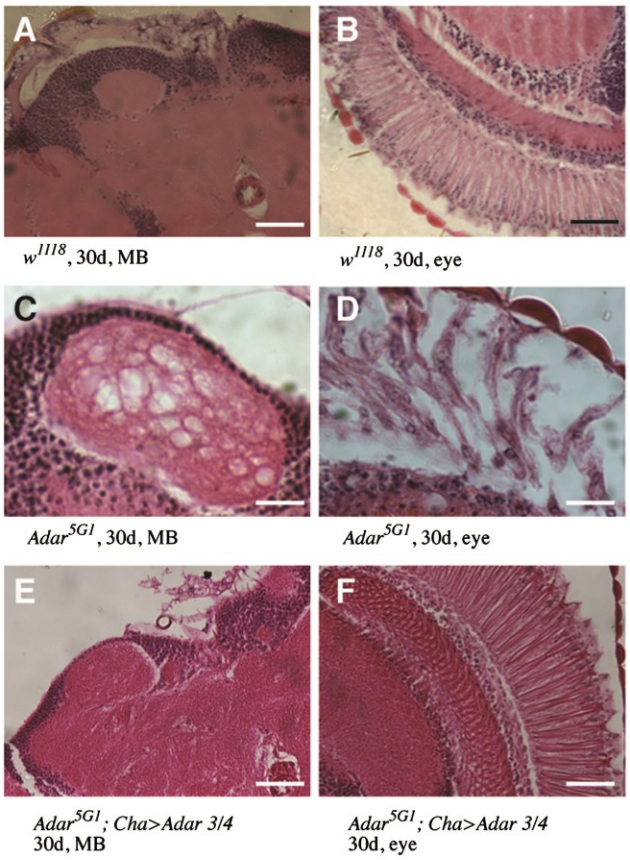

Figure 4. Suppression of neurodegeneration in $Adar^{5G1}$ mutant flies by *Drosophila* ADAR. (**A** and **B**). Haematoxylin and eosin stained frontal sections of 30-day-old wild-type (w^{1118}) heads show no neurodegeneration in the mushroom body calyces or in the eye. Scale bars: 20 μM. (**C** and **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

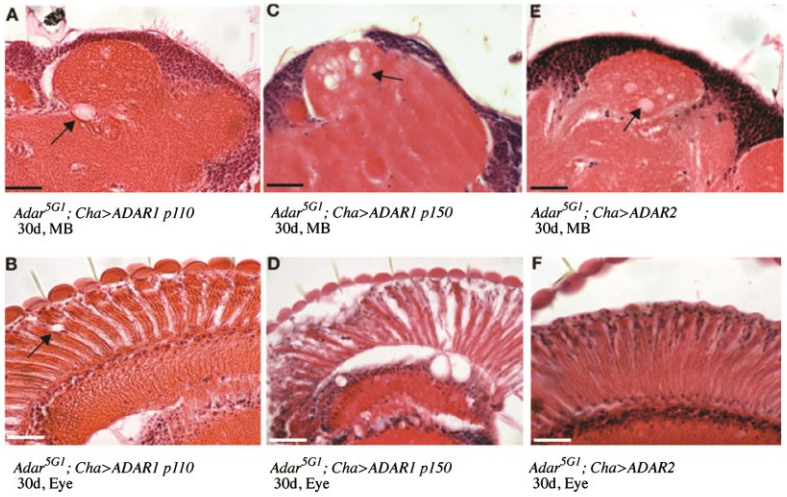

Figure 5. Suppression of neurodegeneration at 30 days in *Adar*^{5G1} mutant flies by human ADAR2. (**A** and **B**): Haematoxylin and eosin stained frontal sections of 30-day-old *Adar*^{5G1}; *Cha-GAL4, UAS-ADAR1p110* heads show rescue of neurodegeneration in the mushroom body (MB) calyces of the *Adar*^{5G1} 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

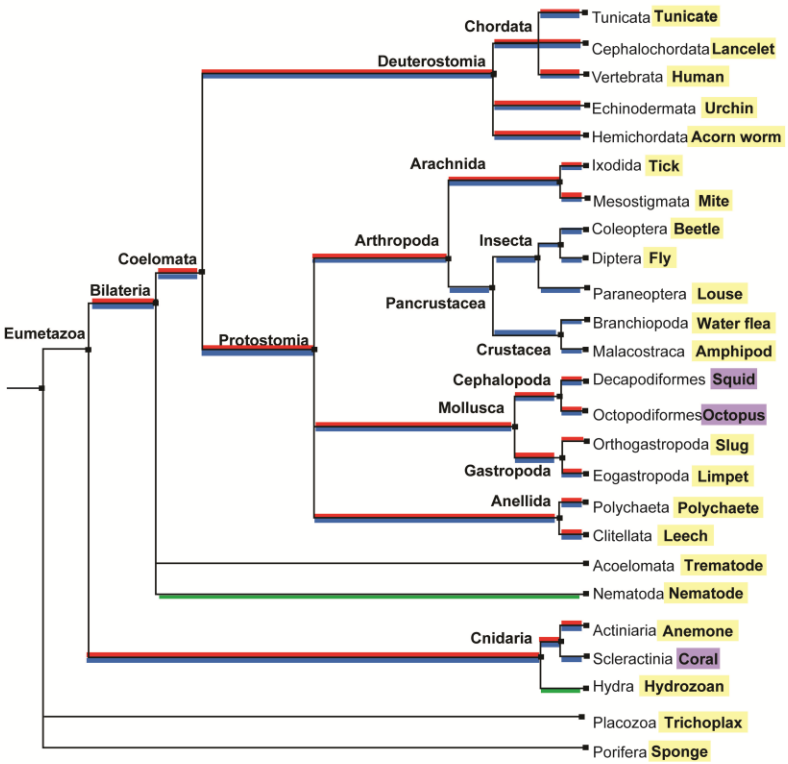

Figure 6. Occurrence of *ADAR1* and *ADAR2* genes in the Metazoa. The phylogenetic tree of species was obtained from Taxonomy Common Tree NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi). Species names at the ends of branches highlighted in yellow represent available genomes that were 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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REFERENCES

1. Heale,B.S.E. and O'Connell,M.A. (2009) Biological roles of ADARs. In Grosjean,H. (ed.), *DNA and RNA Modification Enzymes: Structure, Mechanism, Function and Evolution*. Landes Bioscience, Austin, pp. 233–258.
2. Nishikura,K. (2009) Functions and regulation of RNA editing by ADAR deaminases. *Annu. Rev. Biochem.*, **79**, 321–349.
3. Stefl,R., Oberstrass,F.C., Hood,J.L., Jourdan,M., Zimmermann,M., Skrisovska,L., Maris,C., Peng,L., Hofr,C., Emeson,R.B. *et al.* (2010) The solution structure of the ADAR2 dsRBM-RNA complex reveals a sequence-specific readout of the minor groove. *Cell*, **143**, 225–237.
4. Graveley,B.R., Brooks,A.N., Carlson,J.W., Duff,M.O., Landolin,J.M., Yang,L., Artieri,C.G., van Baren,M.J., Boley,N., Booth,B.W. *et al.* (2011) The developmental transcriptome of *Drosophila melanogaster*. *Nature*, **471**, 473–479.
5. Hoopengardner,B., Bhalla,T., Staber,C. and Reenan,R. (2003) Nervous system targets of RNA editing identified by comparative genomics. *Science*, **301**, 832–836.
6. Stapleton,M., Carlson,J.W. and Celniker,S.E. (2006) RNA editing in *Drosophila melanogaster*: new targets and functional consequences. *RNA*, **12**, 1922–1932.
7. Palavicini,J.P., O'Connell,M.A. and Rosenthal,J.J. (2009) An extra double-stranded RNA binding domain confers high activity to a squid RNA editing enzyme. *RNA*, **15**, 1208–1218.
8. Patton,D.E., Silva,T. and Bezanilla,F. (1997) RNA editing generates a diverse array of transcripts encoding squid Kv2

K+ channels with altered functional properties. *Neuron*, **19**, 711–722.
9. Rosenthal,J.J. and Bezanilla,F. (2002) Extensive editing of mRNAs for the squid delayed rectifier K(+) channel regulates subunit tetramerization. *Neuron*, **34**, 743–757.
10. Colina,C., Palavicini,J.P., Srikumar,D., Holmgren,M. and Rosenthal,J.J. (2010) Regulation of Na+/K+ ATPase transport velocity by RNA editing. *PLoS Biol.*, **8**, e1000540.
11. Higuchi,M., Maas,S., Single,F.N., Hartner,J., Rozov,A., Burnashev,N., Feldmeyer,D., Sprengel,R. and Seeburg,P.H. (2000) Point mutation in an AMPA receptor gene rescues lethality in mice deficient in the RNA-editing enzyme ADAR2. *Nature*, **406**, 78–81.
12. Li,J.B., Levanon,E.Y., Yoon,J.K., Aach,J., Xie,B., Leproust,E., Zhang,K., Gao,Y. and Church,G.M. (2009) Genome-wide identification of human RNA editing sites by parallel DNA capturing and sequencing. *Science*, **324**, 1210–1213.
13. Palladino,M.J., Keegan,L.P., O'Connell,M.A. and Reenan,R.A. (2000) *dADAR*, a *Drosophila* double-stranded RNA-specific adenosine deaminase is highly developmentally regulated and is itself a target for RNA editing. *RNA*, **6**, 1004–1018.
14. Palladino,M.J., Keegan,L.P., O'Connell,M.A. and Reenan,R.A. (2000) A-to-I pre-mRNA editing in *Drosophila* is primarily involved in adult nervous system function and integrity. *Cell*, **102**, 437–449.
15. Greger,I.H., Khatri,L. and Ziff,E.B. (2002) RNA editing at arg607 controls AMPA receptor exit from the endoplasmic reticulum. *Neuron*, **34**, 759–772.
16. Greger,I.H., Khatri,L., Kong,X. and Ziff,E.B. (2003) AMPA receptor tetramerization is mediated by Q/R editing. *Neuron*, **40**, 763–774.
17. Kawahara,Y., Ito,K., Sun,H., Aizawa,H., Kanazawa,I. and Kwak,S. (2004) Glutamate receptors: RNA editing and death of motor neurons. *Nature*, **427**, 801.
18. Peng,P.L., Zhong,X., Tu,W., Soundarapandian,M.M., Molner,P., Zhu,D., Lau,L., Liu,S., Liu,F. and Lu,Y. (2006) ADAR2-dependent RNA editing of AMPA receptor subunit GluR2 determines vulnerability of neurons in forebrain ischemia. *Neuron*, **49**, 719–733.
19. Hartner,J.C., Schmittwolf,C., Kispert,A., Muller,A.M., Higuchi,M. and Seeburg,P.H. (2004) Liver disintegration in the mouse embryo caused by deficiency in the RNA-editing enzyme ADAR1. *J. Biol. Chem.*, **279**, 4894–4902.
20. Wang,Q., Miyakoda,M., Yang,W., Khillan,J., Stachura,D.L., Weiss,M.J. and Nishikura,K. (2004) Stress-induced apoptosis associated with null mutation of ADAR1 RNA editing deaminase gene. *J. Biol. Chem.*, **279**, 4952–4961.
21. Hartner,J.C., Walkley,C.R., Lu,J. and Orkin,S.H. (2009) ADAR1 is essential for the maintenance of hematopoiesis and suppression of interferon signaling. *Nat. Immunol.*, **10**, 109–115.
22. Steitz,T.A. (1993) Similarities and differences between RNA and DNA recognition by proteins. In Gesteland,R.F. and Atkins,J.F. (eds), *The RNA World*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 219–237.
23. Lehmann,K.A. and Bass,B.L. (2000) Double-stranded RNA adenosine deaminases ADAR1 and ADAR2 have overlapping specificities. *Biochemistry*, **39**, 12875–12884.
24. Scadden,A.D. and O'Connell,M.A. (2005) Cleavage of dsRNAs hyper-edited by ADARs occurs at preferred editing sites. *Nucleic Acids Res.*, **33**, 5954–5964.
25. Putnam,N.H., Srivastava,M., Hellsten,U., Dirks,B., Chapman,J., Salamov,A., Terry,A., Shapiro,H., Lindquist,E., Kapitonov,V.V. *et al.* (2007) Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. *Science*, **317**, 86–94.
26. Ring,G.M., O'Connell,M.A. and Keegan,L.P. (2004) Purification and assay of recombinant ADAR proteins expressed in the yeast Pichia pastoris or in *Escherichia coli*. *Methods Mol. Biol.*, **265**, 219–238.
27. O'Connell,M.A., Gerber,A. and Keller,W. (1997) Purification of human double-stranded RNA-specific editase 1 (hRED1) involved in editing of brain glutamate receptor B pre-mRNA. *J. Biol. Chem.*, **272**, 473–478.
28. Ito,K., Awano,W., Suzuki,K., Hiromi,Y. and Yamamoto,D. (1997) The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development*, **124**, 761–771.
29. Salvaterra,P.M. and Kitamoto,T. (2001) *Drosophila* cholinergic neurons and processes visualized with Gal4/UAS-GFP. *Gene Expr. Patterns*, **1**, 73–82.
30. Keegan,L.P., Brindle,J., Gallo,A., Leroy,A., Reenan,R.A. and O'Connell,M.A. (2005) Tuning of RNA editing by ADAR is required in *Drosophila*. *EMBO J.*, **24**, 2183–2193.
31. Sanyal,S. (2009) Genomic mapping and expression patterns of C380, OK6 and D42 enhancer trap lines in the larval nervous system of *Drosophila*. *Gene Expr. Patterns*, **9**, 371–380.
32. Mahr,A. and Aberle,H. (2006) The expression pattern of the *Drosophila* vesicular glutamate transporter: a marker protein for motoneurons and glutamatergic centers in the brain. *Gene Expr. Patterns*, **6**, 299–309.
33. Melcher,T., Maas,S., Herb,A., Sprengel,R., Seeburg,P.H. and Higuchi,M. (1996) A mammalian RNA editing enzyme. *Nature*, **379**, 460–464.
34. Desterro,J.M., Keegan,L.P., Lafarga,M., Berciano,M.T., O'Connell,M. and Carmo-Fonseca,M. (2003) Dynamic association of RNA-editing enzymes with the nucleolus. *J. Cell. Sci.*, **116**, 1805–1818.
35. Sommer,B., Kohler,M., Sprengel,R. and Seeburg,P.H. (1991) RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. *Cell*, **67**, 11–19.
36. Heale,B.S., Keegan,L.P., McGurk,L., Michlewski,G., Brindle,J., Stanton,C.M., Caceres,J.F. and O'Connell,M.A. (2009) Editing independent effects of ADARs on the miRNA/siRNA pathways. *EMBO J.*, **28**, 3145–3156.
37. Peters,N.T., Rohrbach,J.A., Zalewski,B.A., Byrkett,C.M. and Vaughn,J.C. (2003) RNA editing and regulation of *Drosophila 4f-rnp* expression by *sas-10* antisense readthrough mRNA transcripts. *RNA*, **9**, 698–710.
38. Jin,Y., Zhang,W. and Li,Q. (2009) Origins and evolution of ADAR-mediated RNA editing. *IUBMB Life*, **61**, 572–578.
39. Tonkin,L.A., Saccomanno,L., Morse,D.P., Brodigan,T., Krause,M. and Bass,B.L. (2002) RNA editing by ADARs is important for normal behavior in *Caenorhabditis elegans*. *EMBO J.*, **21**, 6025–6035.
40. Reenan,R.A. (2005) Molecular determinants and guided evolution of species-specific RNA editing. *Nature*, **434**, 409–413.
41. Bhalla,T., Rosenthal,J.J., Holmgren,M. and Reenan,R. (2004) Control of human potassium channel inactivation by editing of a small mRNA hairpin. *Nat. Struct. Mol. Biol.*, **11**, 950–956.

Regulation and Functions of ADAR in *Drosophila*

Simona Paro, Xianghua Li, Mary A. O'Connell and Liam P. Keegan

Abstract *Drosophila melanogaster* has a single *Adar* gene encoding a protein related to mammalian ADAR2 that edits transcripts encoding glutamate receptor subunits. We describe the structure of the *Drosophila Adar* locus and use ModENCODE information to supplement published data on *Adar* gene transcription, and splicing. We discuss the roles of ADAR in *Drosophila* in terms of the two main types of RNA molecules edited and roles of ADARs as RNA-binding proteins. Site-specific RNA editing events in transcripts encoding ion channel subunits were initially found serendipitously and subsequent directed searches for editing sites and transcriptome sequencing have now led to 972 edited sites being identified in 597 transcripts. Four percent of *D. melanogaster* transcripts are site-specifically edited and these encode a wide range of largely membrane-associated proteins expressed particularly in CNS. Electrophysiological studies on the effects of specific RNA editing events on ion channel subunits do not suggest that loss of RNA editing events in ion channels consistently produce a particular outcome such as making *Adar* mutant neurons more excitable. This possibility would have been consistent with neurodegeneration seen in *Adar* mutant fly brains. A further set of ADAR targets are dsRNA intermediates in siRNA generation, derived from transposons and from structured RNA loci. Transcripts with convergent overlapping 3′ ends are also edited and the first discovered instance of RNA editing in *Drosophila*, in the *Rnp4F* transcript, is an example. There is no evidence yet to show that *Adar* antagonizes RNA interference in *Drosophila*. Evidence has been obtained that catalytically inactive ADAR proteins exert effects on microRNA generation and RNA interference. Whether all effects of inactive ADARs are due to RNA-binding or to even further roles of these proteins remains to be determined.

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Contents

1 Introduction

Drosophila has a single *Adar* gene encoding a protein closely related to vertebrate ADAR2. This makes *Drosophila* an excellent model to study conserved roles of ADAR2–type proteins in site-specific editing of CNS transcripts. This role of ADARs appears to have developed strongly in the evolution of *Drosophila* with many edited transcripts identified. Other roles of ADARs in non-specific RNA editing related to microRNA processing and RNA interference or as RNA-binding proteins are likely to be conserved also.

2 *Drosophila Adar* Gene Transcription, Splicing and RNA Editing

2.1 Adar *Gene Transcription*

The single *Adar* gene in *Drosophila melanogaster (D. melanogaster)* lies at cytogenetic position 2B6-7, near the tip of the X chromosome (Palladino et al. 2000a). Expression is highest in the CNS but also widespread outside the CNS at lower levels. Expression of *Adar* increases at metamorphosis. It was proposed that two different promoters, 4A and 4B, control the transcription of the *Adar* gene (Fig. 1). The constitutive 4A promoter is active all through fly development and 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

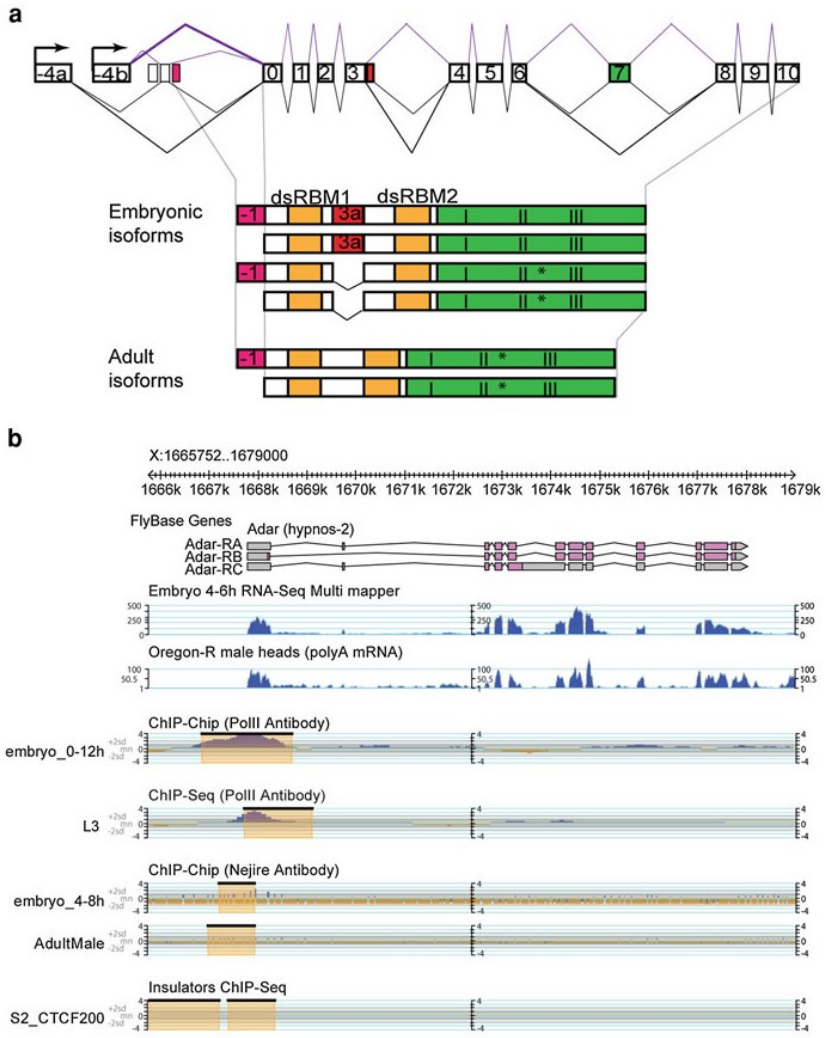

Fig. 1 **a**. *Adar* gene structure, embryonic splicing pattern (*below the gene*) and adult splicing pattern (*above the gene*), and ADAR protein isoforms expressed in embryos and adults. **b**. Selected *Adar* gene region tracks from ModENCODE browser showing embryonic and adult transcription, and binding patterns of RNA polymerase II, the enhancer-locating transcriptional coactivator P300/CBP and the insulator protein CTCF

promoter, based on finding cDNAs with an alternative 5′-exon derived from this region and 5′-RACE analysis (Palladino et al. 2000a).

For *Drosophila* genes and chromosomes a great deal of new information has been 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 proposed first exon−4B of the adult-specific transcript in adult flies, (see Fig. 1 and *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). Some exons may be underrepresented in RNA-Seq data for various reasons.

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 at the promoter in both embryos and adults, strong CTCF with some extension of Polymerase II more 3′ in the adult data (Fig. 1). Upstream of the constitutive promoter there is a very strong prediction of 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 180 kb downstream and 110 kb upstream of the *Adar* promoter. The promoter region also binds Origin Recognition Complex (ORC) proteins in embryo and at metamorphosis and this and other evidence suggests that the promoter region contains an origin of replication active at these times.

A possible enhancer immediately upstream of the constitutive promoter is suggested by binding of the *Drosophila* homolog of the transcriptional coactivator P300/CBP, which is encoded by the *Nejire* gene in *Drosophila* (Akimaru et al. 1997). This protein has been extremely valuable in locating enhancers in human and vertebrate genomes (Visel et al. 2009). CBP is CREB-binding protein, a transcriptional coactivator that binds to the DNA-binding cAMP response element binding protein CREB 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 transcription 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.

2.2 *Embryonic and Adult* Adar *Splice forms and ADAR Protein Isoforms*

The *Adar* transcripts have long 5′ UTRs with alternatively spliced exons. Based on the estimated relative abundances of different splice forms these transcripts 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. 2000b).

A constitutive splicing pattern is seen clearly in embryos that persists in the background also in adults but an adult-specific splicing pattern in a subset of transcripts is superimposed on this. 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 nonconsensus 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 the two double strand RNA binding motifs (dsRBM1 and dsRBM2), to 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 5′-UTR and the adult splicing pattern deleting exon 3a. The adult splicing pattern also correlates strongly with RNA editing at exon 7 in the *Adar* 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. 2002). Most of exon 7, though not the splice junctions, are predicted to form a large dsRNA structure involved in editing here (Keegan et al. 2005). This structure may affect the splicing of exon 7.

2.3 Adar *Mutant Phenotypes and Outstanding Questions in* Adar *Regulation*

The $Adar^{5G1}$ deletion removes the entire *Adar* gene. Under ideal conditions, $Adar^{5G1}$ mutants develop into morphologically normal adults and they perform functions necessary to sustain life (eating, respiration and metabolism) (Palladino et al. 2000b). However they display severe neuro-behavioural deficits such as slow uncoordinated locomotion, tremors and alteration of normal posture; furthermore they obsessively and frequently clean their wings and they are able to jump and fly but only when repeatedly provoked. The earlier characterized $Adar^{1F4}$ deletion mutant is intriguing; it deletes only the promoters and not the coding sequence and has some residual transcript expressed at a low level. It is phenotypically indistinguishable from $Adar^{5G1}$ but it edits the *Adar* transcript only and not any other target transcript that has been examined.

The main outstanding questions about *Adar* gene expression relate to how expression is controlled. Is transcription regulated by CREB or by neuronal factors needed for ubiquitous neural expression? Is *Adar* expression or self-editing regulated by neuronal activity?

3 The *Drosophila* ADAR Protein Isoforms

Drosophila ADAR contains two double strand RNA binding domains within the amino terminal half of the protein: dsRBM1 (53-133aa) and dsRBM2 (196-273aa). dADAR protein with the alternative exon 3a inserted between the two dsRBMs 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 vertebrate ADAR homo- or hetero-dimers and for editing activity. Sequences within the first 46 amino acids and the first dsRBM are required for dimerization of dADAR (Gallo et al. 2003).

However, based on domain exchange experiments between mammalian ADAR1 and ADAR2, the main determinant of ADAR specificity lie in the deaminase domain at the carboxyl terminus. The dADAR 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). In adult flies, ADAR edits its own mRNA with 40% efficiency to encode an ADAR 3/4 G edited isoform that is eightfold 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 will require further study of factors regulating the activity of ADAR itself.

4 Roles of *Drosophila* ADAR

There are three general categories of effects that we can distinguish for ADARs: site-specific RNA editing in transcripts, non-specific RNA editing in long dsRNA precursors in RNA interference pathways and potential RNA editing-independent roles, probably as RNA-binding proteins.

4.1 Site-Specific RNA Editing in Drosophila Transcripts and Consequences

4.1.1 Serendipitously Discovered Editing Sites Led to Searches for Further Sites

Site-specific RNA editing events were first detected serendipitously in *Drosophila* transcripts encoding ion channel subunits such as *cacophony (cac)* encoding the large, pore-forming subunit of the voltage-gated CNS calcium channel (Smith et al. 1996) and *paralytic (para)* encoding the large, pore-forming subunit of the voltage-gated sodium channel (Hanrahan et al. 2000). Other individually identified

edited transcripts included *DrosGluCl* encoding a glutamate-gated chloride channel subunit gene (Semenov and Pak 1999), the *Adar* transcript itself (Palladino et al. 2000a) and the *Dalpha5* transcript encoding the pore-forming subunit of a nicotinic acetylcholine receptor (Grauso et al. 2002).

Although no definite signature sequence motif was found for an ADAR editing site, editing site complementary sequences (ECSs) usually located in an adjacent intron form imperfect duplex RNA by base-pairing with the exon that contains the adenosine to be edited. This is as expected from studies of vertebrate glutamate receptor transcript editing (Higuchi et al. 1993). Based on the hypothesis that cis-elements required for editing site/ECS duplex formation will be conserved where RNA editing of particular sites is conserved between species. Hoopengardner et al. (2003) identified 16 new edited targets in *Drosophila* by comparing genome sequences of *D. melanogaster* and *D. pseudoobscura* to identify highly conserved exons. They examined 914 genes annotated as ion channels ($n = 135$), G protein-coupled receptors ($n = 178$), proteins involved in synaptic transmission ($n = 102$), and transcription factors ($n = 499$). All the edited transcripts they discovered by this method encode proteins functioning in rapid electrical and chemical neurotransmission, among which were seven voltage-gated ion channels (VGIC), five components of the synaptic release machinery, and four ligand-gated ion channels (LGIC). The number of edited sites differed from one to seven in each transcript. Nevertheless, due to the limited size of the screen pool and the possibility that there are some rapidly evolving ADAR editing events, this approach was not able to detect all the ADAR targets. It was found that in *Drosophila* some ECS elements are not a single sequence unit as in the vertebrate glutamate receptor transcripts but consist of fragments that are not arranged sequentially in the genome but come together in the transcript to pair with the edited region and stack along it (Reenan 2005).

Another systematic approach to identify ADAR targets was carried out using sequence data from the *Drosophila* Gene Collection project which set out to provide a sequence of one individual adult head cDNA with a complete protein-coding sequence for each gene in the genome (DGC; http://www.fruitfly.org/DGC). Stapleton et al. (2006) compared the cDNA clone sequences with genomic DNA and further experimentally verified 27 new targets of ADAR, expanding the categories of edited transcripts to seven. They identified three more classes of ADAR target transcripts: encoding vesicular trafficking proteins, ion homeostasis proteins and cytoskeletal components. However, it remained likely that not all edited transcripts were yet detected, partly because sites edited less than 100% might not be detected in individual cDNA sequences.

4.1.2 Four Percent of all *Drosophila* Transcripts have Site-Specific RNA Editing

The list of known site-specifically edited transcripts in *Drosophila* has recently been very dramatically increased by the publication of the ModENCODE study of the developmental transcriptome based on extensive RNA Seq analyses of

RNA from 72 samples and 30 distinct developmental stages. By analyzing the poly(A)+ RNA Seq data, Graveley et al. (2011) identified 972 edited positions within transcripts of 597 genes, which is around 4% of the *Drosophila* genes.

Graveley and colleagues observed several important common features of the edited sites in their sequencing data. Firstly, consistent with the earlier studies (Hoopengardner et al. 2003; Jepson and Reenan 2007), exons containing editing sites are more highly conserved than unedited exons. Secondly the frequency of editing increases throughout development; editing often begins in late pupal stages and many of the newly discovered sites are edited only in adult flies. Thirdly, editing levels do not correlate with the expression levels of the genes. Lastly, the majority of the edited sites (630) alter amino acid coding, 201 sites are silent, and 141 are within untranslated regions.

In addition, Graveley and colleagues identified by computational analysis three length classes of a potential editing-associated sequence motif having the edited A near the 3′end. Although motifs A and B are more common, Motif C, the shortest one, is observed to be most strongly associated with the editing sites and over-represented in early developmental editing events (Graveley et al. 2011). The other two motifs are longer than Motif C but rather similar and tend to have a G immediately 3′of the edited A and further Gs running 5′at −2, −5, −8 and −11 from the edited position i.e. G residues at every third base. Oddly, these conserved motifs are mostly 5′of the edited A, whereas the ADAR dsRBDs bind mainly 3′of the edited A (Stefl et al. 2010). It is not clear that these motifs will necessarily contribute to dsRNA duplex stretches as editing site/ECS duplexes tend to be short in *Drosophila* compared to those seen in vertebrate transcripts. Possibly the motifs reflect further interactions of substrate RNAs with ADARs or with other proteins.

Functional categories highly represented among the edited transcripts based on the classification of molecular functions of encoded proteins include transporter activity ($n = 66$), enzyme regulator activity ($n = 31$, mainly GTPase regulator activity), binding activity, catalytic activity and structural molecule activity ($n = 5$, all are genes encoding structural constituents of muscle). The most widely studied edited transcripts encode proteins with transporter activities. However, binding activity is the biggest category of molecular function among the edited transcripts, consisting of protein binding ($n = 132$), nucleotide binding ($n = 76$), lipid binding ($n = 14$) and ion binding ($n = 22$) classes. Edited transcripts included in catalytic activity categories include 31 genes with kinase activity and 17 genes with phosphatase activity. Analyzed from the cellular component aspect, most edited transcripts reside in membrane structures including ion channel complexes, plasma membranes, membrane bounded vesicles and mitochondrial membranes. Also, there are edited transcripts encoding components involved in cell projections, synapses, and cytoskeleton. (AmiGO analyses, and statistical analyses were carried out using the FlyMine website http://www.flymine.org)

4.1.3 Effects of Individual RNA Editing Events on Ion Channel Subunits and Other Proteins

Intriguing suggestions for the overall function of site-specific RNA editing have been made that now need to be re-examined with larger numbers of sites. One proposal is that editing events tend to change less conserved residues in highly conserved functional regions of proteins (Reenan 2005; Yang et al. 2008). A somewhat related suggestion is that editing events tend to alter evolutionarily conserved amino acid sequences in such a way as to introduce an evolutionarily novel residue at a conserved position in the genomic sequence (Tian et al. 2008). RNA editing is then evolutionarily restorative—as though a new, unedited, functional protein isoform is provided from the unedited transcripts while the isoform with the evolutionary consensus residue is provided by RNA editing.

It is not always obvious how significant the functional consequences of editing events in individual proteins are. Nevertheless, to our knowledge, where effects of RNA editing changes on protein functions have been sought they have been found. This suggests that editing events have been selected for effects on protein function even though the effects are sometimes subtle. Several extensively studied editing events include the one in ADAR itself which undergoes self-editing to reduce enzymatic activity, possibly as a fine-tuning mechanism for RNA editing regulation (Keegan et al. 2005).

If there are hints of patterns in the evolutionary selection of editing sites in protein domains then are there also conserved patterns in the effects of RNA editing on protein or neuron function? There are so many editing events in *Drosophila* transcripts that for most the biophysical or physiological consequences are, at best, merely predicted depending on the domains where the edited sites reside. However, recent studies of effects of RNA editing on several *Drosophila* ion channels do now allow these questions to be considered. The GABA receptor, for instance, is generally inhibitory with regard to neuronal excitability. Loss of RNA editing at sites in Rdl leads to increased responsiveness to GABA so that a lower concentration of GABA is sufficient for a half-maximal channel opening response i.e. loss of *Rdl* RNA editing is expected to make neurons less excitable (Jones et al. 2009). Does loss of RNA editing have parallel effects on other channels?

A very detailed study of the biophysical consequences of editing was conducted on *Shab*. *Shab* belongs to the voltage-gated potassium channel family, one of which contains the only specific adenosine position known to be edited by ADARs in chordates, mollusks and arthropods. The RNA structure that directs editing in that case is not conserved between chordates and arthropods so this may be an example of convergent evolution (Bhalla et al. 2004). The original discovery of editing sites in *Shab* by comparing their cDNA with genomic DNA revealed five highly edited sites but the ModENCODE data detects eight edited sites in *Shab* including two silent sites.

Four of the sites are fully edited so, using a two-microelectrode voltage clamp in *Xenopus* oocytes, Ryan et al. (2008) compared the effects of single unediting at

each of the edited sites to the genomic construct with no editing and to the fully edited version. The original five sites were the I583V site in the S4 voltage sensor, the T643A site in the pore helix, Y660C in the extracellular turret and T671A and I681V in the S6 segment. One functional consequence of RNA editing in *Shab* is to change the voltage dependence so that the edited channel is less prone to open, which would enhance the excitability of a neuron containing the edited channels. From this the predicted effect of loss of RNA editing is decreasing neuronal excitability.

The effects of loss of editing on the kinetics of channel gating seem to predict an opposite effect on neuronal excitability however. Loss of RNA editing in *Shab* slows both activation and deactivation. The authors suggest that slower activation resulting from loss of editing would tend to make neurons more excitable. Therefore it is unclear whether loss of *Shab* editing would tend to make a neuron more or less excitable overall.

Fully understanding the functional consequences of A-to-I conversion in each transcript is still challenging, especially for the transcripts that have multiple editing sites. The editing events are not only temporally but also spatially tightly regulated to give combinations of isoforms with different sites edited at different levels. For instance, a predominantly expressed edited isoform (68%) of Shaker in male wing tissue is found to have very low (1%) expression in the male head, and the most abundant isoform (27%) in the male head is not detected in the male wing tissue (Ingleby et al. 2009). Homologous recombination in *Drosophila* may be useful to distinguish roles of edited and unedited forms of *Adar* and other edited transcripts (Jepson et al. 2011).

4.2 RNA Editing and RNA Interference

4.2.1 Types of RNA Interference and Production of Different Small RNAs in *Drosophila*

RNA interference is a process of silencing gene expression at the transcriptional or posttranscriptional levels. Small RNAs (21–29 nucleotides) are involved in this process of gene silencing and several classes have been well described in *Drosophila* (Czech and Hannon 2011). These include short interfering RNAs (siRNAs), micro RNAs (miRNAs), repeat associated RNAs (rasiRNAs) and piwi-interacting RNAs (piRNAs).

Small interfering RNAs (siRNAs) are generated by the activity of Dicer2 enzyme which binds longer dsRNA precursors and releases RNA duplexes 21 nucleotides long on each strand with 2 base 3′ overhangs on each end. One strand is discarded and a single stranded siRNA remains in a RNA induced silencing complex (RISC) containing Argonaute2 (AGO2) protein, which then cleaves target RNA molecules complementary to the siRNA strand. This process can act on exogenously supplied dsRNA but when it acts on internally generated dsRNA

the products are referred to as endogenous siRNAs (esiRNAs) or repeat associated siRNAs (rasiRNAs).

The processing of pre-miRNAs is similar but these are first cleaved from an endogenously expressed transcript by Drosha enzyme and transported to the cytoplasm for cleavage by Dicer1 enzyme and maturation into mature miRNAs in a miRISC complex containing AGO1 protein. Mature miRNAs inhibit the translation of the complementary mRNA (most often binding to the 3′UTR).

piRNAs are generated particularly from transposon-associated RNAs by Dicer-independent processes and in *Drosophila* these are processed into complexes containing Piwi, Aubergine (Aub) or AGO3 proteins. One amplification process for piRNAs inolves Aub and AGO3 proteins binding opposite strands of triggering RNAs and engaging in ping-pong cleavage reactions that load further RNA copies into the silencing complexes. In mammals this class of RNAs are expressed only in the germline but in *Drosophila* piRNAs are found in germline and also to a lesser extent in somatic tissues (Li et al. 2009; Malone et al. 2009).

4.2.2 RNA Editing in esiRNAs Derived from Transposons, Structured RNA Loci and Convergently Transcribed Genes

The Siomis and their colleagues in Japan have shown that, among *Drosophila* endogenous siRNAs (esiRNAs) recovered from RISC complexes immunoprecipitated with an antibody to AGO2, 18% of all the 21 mer sequences showed A–G changes reflecting probable RNA editing of dsRNA precursors (Kawamura et al. 2008). This corresponds to an adenosine to inosine conversion once every 130 base pairs in precursor dsRNA and this level is similar to estimates of editing rates in mammalian microRNAs (Kawahara et al. 2008): editing of microRNAs has not been studied in *Drosophila*.

ADAR interactions with RNA interference pathways are expected since ADARs and Dicers both act on dsRNA and potentially compete for this substrate (Yang et al. 2005). In addition to a potential for competitive binding of the proteins it has been demonstrated that hyper editing of dsRNA in vitro inhibits cleavage by Dicer (Scadden and Smith 2001). Another experiment had shown that the Tudor-SN component of RISC binds and promotes degradation of hyper edited dsRNAs (Scadden 2005). While some proportion of dsRNA precursors that get edited still go on to contribute to RNA interference pathways with potential to alter the targeting of RISC complexes some portion of edited dsRNAs may be degraded.

The full range of sequences able to contribute to esiRNAs all seem to be equally editable (Kawamura et al. 2008). esiRNAs are derived primarily from transposons and from structured transcripts with potential to form long dsRNA. A very intriguing category of esiRNAs that are more abundant in *Drosophila* than in mice is derived from convergent transcripts with overlapping 3′ ends (Czech et al. 2008; Petschek et al. 1996). The *D. melanogaster* genome has 998 convergently transcribed gene pairs with annotated overlapping transcripts and different but partly overlapping subsets of these produce esiRNAs in ovaries and in Schneider S2 cell

cultures. Probably not all convergent overlapping transcript pairs are expressed in the same cells or pairing of UTRs may not be efficient because the numbers of esiRNAs produced are not as high as from structured RNA loci.

The very first A-to-I edited transcripts identified in *Drosophila* were discovered serendipitously as A to G discrepancies between cloned cDNAs and the corresponding genomic sequences of *RNA-binding protein 4F (Rnp4F)*, at 4F5 on the X chromosome (Petschek et al. 1996). *Rnp4F* encodes a protein homologous to human P110/Sart3 protein and to the U4/U6 snRNP recycling factor. Editing in this transcript arises because of convergent transcription of *Rnp4F* and another gene *Something about silencing 10 (Sas10)*, which encodes a nuclear, positively charged, protein. The *S. cerevisiae* ortholog of *Sas10* inhibits chromosomal silencing at the mating-type loci when overexpressed. *Sas10* shares a conserved domain with RNA-binding components of the exosome and U3.

The *Rnp4F* transcript is expressed early in embryogenesis but later in embryogenesis a longer *Sas10* transcript is produced that overlaps with the 3′ end of *Rnp 4F*, leading to a drop in *Rnp4F* transcript levels (Peters et al. 2003). The *Rnp4F* transcript now has adenosines converted to guanosine when cDNA and genomic DNA sequences are compared. In embryonic and larval stages *Sas10* is the much more strongly expressed of the two transcripts but in adults the level of both transcripts is low. The overlapping transcripts appear to trigger RNA interference. modENCODE data now shows that small RNAs are expressed that correspond to the region of transcript overlap in adult tissues, particularly in mutants of *Ago 2* or *r2d2*. Small RNAs from this region are present in AGO 1 complexes immunoprecipitated from adult cells such as ovarian somatic cell (OSC) cultures and from ovaries and these are particulary prominent in AGO 1 complexes immunoprecipitated from cells in which Ago 2 or *r2d2* are mutant or knocked down. Whether loss of RNA editing would help or hinder silencing at *Rnp4F* has not been determined.

The presence of RNA editing events in siRNAs is part of the evidence that these small RNAs are generated from dsRNA. There have not been any reports of editing events in piRNAs. In the case of piRNAs there may be no very extensive dsRNA involved in their formation or any dsRNA that is formed during their biogenesis may be bound within protein complexes and inaccessible to ADARs.

4.2.3 Consequences of RNA Editing for RNA Interference Phenomena

There is no clear published evidence that loss of *Adar* in *Drosophila* influences the potency of RNA interference effects. In the simplest case of pure antagonism loss of *Adar* function should make RNA interference more active, as occurs in *C. elegans* (Knight and Bass 2002). In *Drosophila*, mutations in genes encoding RNAi components did not rescue locomotion defects of *Adar* mutant flies but RNA interference has not been shown to have any relevance for locomotion defects in flies so this is not surprising (Jepson and Reenan 2009). The finding does not rule out the possibility of antagonistic effects of *Adar* on aspects of RNA interference. Such an

antagonism has been shown in the case of *white* hairpin-directed RNA interference in *Drosophila* when the cytoplasmically localized human ADAR1 p150 protein is overexpressed but neither *Drosophila Adar* mutations nor overexpression of the nuclear localized *Drosophila* ADAR or human ADAR2 have an effect in this assay. This is presumably because the *white* hairpin is cytoplasmic in this case and ADAR p150 is the only protein with a matched localization.

4.3 RNA Editing-Independent Roles of ADARs

ADARs edit microRNA precursors and thereby redirect RISC complexes containing edited microRNAs to new targets (Kawahara et al. 2007). A follow up study found however that the effect of ADAR binding to inhibit the processing of microRNAs from their precursors is stronger than the effect of retargeting (Heale et al. 2009a). This antagonism is independent of adenosine deamination activity. Stable, catalytically inactive ADAR proteins can be generated by mutating a glutamate residue in the deaminase active site to alanine. Such a catalytically inactive human ADAR1 protein was shown to inhibit processing of micro RNA precursors in vitro and in cultured human cells. This mutant ADAR1 was also shown to retain a substantial portion of the antagonistic effect against RNA interference in *Drosophila* that is exhibited by the wildtype ADAR1 protein. This data joins a range of other evidence that ADARs have important roles independent of deamination that probably arise mainly from their roles as RNA-binding proteins.

A different catalytically inactive ADAR1 mutation in two Japanese families was proposed to have more severe effects than other ADAR1 loss of function mutations because of a dominant negative effect on residual active ADAR1 in those patients (Heale et al. 2009b; Kondo et al. 2008). In *Drosophila* the inactive dADAR protein has been shown to be insufficient to rescue locomotion defects, consistent with the need to edit CNS transcripts. Further study of the relationship between ADAR and RNA interference requires a naturally-occurring, ideally nuclear-based, RNA interference phenomenon to act as a reporter. Whether there are aspects of ADAR function other than antagonizing RNA interference that the inactive protein can provide remains to be determined.

5 Conclusion

In conclusion, study of ADAR RNA editing in *Drosophila* developed from serendipitous findings to systematic discovery of the amazing 596 edited transcripts with 972 sites edited to date. Significant progress has been made in studying the effects of editing on some transcripts, most of which encode ion channels or other membrane proteins. However, there is still quite a long way to go to completely understand the physiological effects of editing on affected proteins in *Drosophila*. Whether editing events are evolutionarily selected to some common purposes and

how the editing profiles are fine-tuned remain to be determined. In addition, the effect of ADAR on small RNAs is still not clear. Sequencing of endogenous siRNAs detected vastly over-represented adenosine-to-guanosine mismatches reflecting ADAR editing of dsRNA precursors. Further investigations are needed to test whether cross regulation between A-to-I editing and other post transcriptional modification mechanisms like RNA interference exist.

References

Akimaru H, Hou DX, Ishii S (1997) *Drosophila* CBP is required for dorsal-dependent twist gene expression. Nat Genet 17:211–214

Bhalla T, Rosenthal JJ, Holmgren M, Reenan R (2004) Control of human potassium channel inactivation by editing of a small mRNA hairpin. Nat Struct Mol Biol 11:950–956

Czech B, Hannon GJ (2011) Small RNA sorting: matchmaking for Argonautes. Nat Rev Genet 12:19–31

Czech B, Malone CD, Zhou R, Stark A, Schlingeheyde C, Dus M, Perrimon N, Kellis M, Wohlschlegel JA, Sachidanandam R et al (2008) An endogenous small interfering RNA pathway in *Drosophila*. Nature 453:798–802

Gallo A, Keegan LP, Ring GM, O'Connell MA (2003) An ADAR that edits transcripts encoding ion channel subunits functions as a dimer. Embo J 22:3421–3430

Gan Z, Zhao L, Yang L, Huang P, Zhao F, Li W, Liu Y (2006) RNA editing by ADAR2 is metabolically regulated in pancreatic islets and beta-cells. J Biol Chem 281:33386–33394

Grauso M, Reenan RA, Culetto E, Sattelle DB (2002) Novel putative nicotinic acetylcholine receptor subunit genes, Dalpha5, Dalpha6 and Dalpha7, in *D. melanogaster* identify a new and highly conserved target of adenosine deaminase acting on RNA-Mediated A-to-I Pre-mRNA Editing. Genetics 160:1519–1533

Graveley BR, Brooks AN, Carlson JW, Duff MO, Landolin JM, Yang L, Artieri CG, van Baren MJ, Boley N, Booth BW et al (2011) The developmental transcriptome of *Drosophila melanogaster*. Nature 471:473–479

Hanrahan CJ, Palladino MJ, Ganetzky B, Reenan RA (2000) RNA editing of the *Drosophila* para Na(+) channel transcript. Evolutionary conservation and developmental regulation. Genetics 155:1149–1160

Heale BS, Keegan LP, McGurk L, Michlewski G, Brindle J, Stanton CM, Caceres JF, O'Connell MA (2009a) Editing independent effects of ADARs on the miRNA/siRNA pathways. Embo J 28:3145–3156

Heale BS, Keegan LP, O'Connell MA (2009b) ADARs have effects beyond RNA editing. Cell Cycle 8:4011–4012

Higuchi M, Single FN, Kohler M, Sommer B, Sprengel R, Seeburg PH (1993) RNA editing of AMPA receptor subunit GluR-B: a base-paired intron-exon structure determines position and efficiency. Cell 75:1361–1370

Hoopengardner B, Bhalla T, Staber C, Reenan R (2003) Nervous system targets of RNA editing identified by comparative genomics. Science 301:832–836

Ingleby L, Maloney R, Jepson J, Horn R, Reenan R (2009) Regulated RNA editing and functional epistasis in Shaker potassium channels. J Gen Physiol 133:17–27

Jepson JE, Reenan RA (2007) Genetic approaches to studying adenosine-to-inosine RNA editing. Methods Enzymol 424:265–287

Jepson JE, Reenan RA (2009) Adenosine-to-inosine genetic recoding is required in the adult stage nervous system for coordinated behavior in *Drosophila*. J Biol Chem 284:31391–31400

Jepson JE, Savva YA, Yokose C, Sugden AU, Sahin A, Reenan RA (2011) Engineered alterations in RNA editing modulate complex behavior in *Drosophila*: regulatory diversity of adenosine deaminase acting on RNA (ADAR) Targets. J Biol Chem 286:8325–8337

Jones AK, Buckingham SD, Papadaki M, Yokota M, Sattelle BM, Matsuda K, Sattelle DB (2009) Splice-variant- and stage-specific RNA editing of the *Drosophila* GABA receptor modulates agonist potency. J Neurosci 29:4287–4292

Kawahara Y, Zinshteyn B, Sethupathy P, Iizasa H, Hatzigeorgiou AG, Nishikura K (2007) Redirection of silencing targets by adenosine-to-inosine editing of miRNAs. Science 315: 1137–1140

Kawahara Y, Megraw M, Kreider E, Iizasa H, Valente L, Hatzigeorgiou AG, Nishikura K (2008) Frequency and fate of microRNA editing in human brain. Nucleic Acids Res 36:5270–5280

Kawamura Y, Saito K, Kin T, Ono Y, Asai K, Sunohara T, Okada TN, Siomi MC, Siomi H (2008) *Drosophila* endogenous small RNAs bind to Argonaute 2 in somatic cells. Nature 453:793–797

Keegan LP, Brindle J, Gallo A, Leroy A, Reenan RA, O'Connell MA (2005) Tuning of RNA editing by ADAR is required in *Drosophila*. Embo J 24:2183–2193

Knight SW, Bass BL (2002) The role of RNA editing by ADARs in RNAi. Mol Cell 10:809–817

Kondo T, Suzuki T, Ito S, Kono M, Negoro T, Tomita Y (2008) Dyschromatosis symmetrica hereditaria associated with neurological disorders. J Dermatol 35:662–666

Li C, Vagin VV, Lee S, Xu J, Ma S, Xi H, Seitz H, Horwich MD, Syrzycka M, Honda BM et al (2009) Collapse of germline piRNAs in the absence of Argonaute3 reveals somatic piRNAs in flies. Cell 137:509–521

Ma E, Tucker MC, Chen Q, Haddad GG (2002) Developmental expression and enzymatic activity of pre-mRNA deaminase in *Drosophila melanogaster*. Brain Res Mol Brain Res 102:100–104

Malone CD, Brennecke J, Dus M, Stark A, McCombie WR, Sachidanandam R, Hannon GJ (2009) Specialized piRNA pathways act in germline and somatic tissues of the *Drosophila* ovary. Cell 137:522–535

Marcucci R, Romano M, Feiguin F, O'Connell MA, Baralle FE (2009) Dissecting the splicing mechanism of the *Drosophila* editing enzyme; dADAR. Nucleic Acids Res 37:1663–1671

Palladino MJ, Keegan LP, O'Connell MA, Reenan RA (2000a) dADAR, a *Drosophila* double-stranded RNA-specific adenosine deaminase is highly developmentally regulated and is itself a target for RNA editing. RNA 6:1004–1018

Palladino MJ, Keegan LP, O'Connell MA, Reenan RA (2000b) A-to-I pre-mRNA editing in *Drosophila* is primarily involved in adult nervous system function and integrity. Cell 102:437–449

Peng PL, Zhong X, Tu W, Soundarapandian MM, Molner P, Zhu D, Lau L, Liu S, Liu F, Lu Y (2006) ADAR2-dependent RNA editing of AMPA receptor subunit GluR2 determines vulnerability of neurons in forebrain ischemia. Neuron 49:719–733

Peters NT, Rohrbach JA, Zalewski BA, Byrkett CM, Vaughn JC (2003) RNA editing and regulation of *Drosophila 4f-rnp* expression by sas-10 antisense readthrough mRNA transcripts. RNA 9:698–710

Petschek JP, Mermer MJ, Scheckelhoff MR, Simone AA, Vaughn JC (1996) RNA editing in *Drosophila 4f-rnp* gene nuclear transcripts by multiple A-to-G conversions. J Mol Biol 259: 885–890

Reenan RA (2005) Molecular determinants and guided evolution of species-specific RNA editing. Nature 434:409–413

Roy S, Ernst J, Kharchenko PV, Kheradpour P, Negre N, Eaton ML, Landolin JM, Bristow CA, Ma L, Lin MF et al (2010) Identification of functional elements and regulatory circuits by *Drosophila* modENCODE. Science 330:1787–1797

Ryan MY, Maloney R, Reenan R, Horn R (2008) Characterization of five RNA editing sites in *Shab* potassium channels. Channels (Austin) 2:202–209

Scadden AD (2005) The RISC subunit Tudor-SN binds to hyper-edited double-stranded RNA and promotes its cleavage. Nat Struct Mol Biol 12:489–496

Scadden AD, Smith CW (2001) RNAi is antagonized by A → I hyper-editing. EMBO Rep 2:1107–1111

Semenov EP, Pak WL (1999) Diversification of *Drosophila* chloride channel gene by multiple posttranscriptional mRNA modifications. J Neurochem 72:66–72

Smith LA, Wang XJ, Peixoto AA, Neumann EK, Hall LM, Hall JC (1996) A *Drosophila* calcium channel α1 subunit gene maps to a genetic locus associated with behavioural and visual defects. J Neurosci 16:7868–7879

Stapleton M, Carlson JW, Celniker SE (2006) RNA editing in *Drosophila melanogaster*: new targets and functional consequences. RNA 12:1922–1932

Stefl R, Oberstrass FC, Hood JL, Jourdan M, Zimmermann M, Skrisovska L, Maris C, Peng L, Hofr C, Emeson RB, Allain FH (2010) The solution structure of the ADAR2 dsRBM-RNA complex reveals a sequence-specific readout of the minor groove. Cell 143:225–237

Tian N, Wu X, Zhang Y, Jin Y (2008) A-to-I editing sites are a genomically encoded G: implications for the evolutionary significance and identification of novel editing sites. RNA 14:211–216

Visel A, Blow MJ, Li Z, Zhang T, Akiyama JA, Holt A, Plajzer-Frick I, Shoukry M, Wright C, Chen F et al (2009) ChIP-seq accurately predicts tissue-specific activity of enhancers. Nature 457:854–858

Vo N, Goodman RH (2001) CREB-binding protein and p300 in transcriptional regulation. J Biol Chem 276:13505–13508

Yang W, Wang Q, Howell KL, Lee JT, Cho DS, Murray JM, Nishikura K (2005) ADAR1 RNA deaminase limits short interfering RNA efficacy in mammalian cells. J Biol Chem 280: 3946–3953

Yang Y, Lv J, Gui B, Yin H, Wu X, Zhang Y, Jin Y (2008) A-to-I RNA editing alters less-conserved residues of highly conserved coding regions: implications for dual functions in evolution. RNA 14:1516–1525