# Determining fundamental cis-regulatory elements directing selective, efficient, and novel RNA editing

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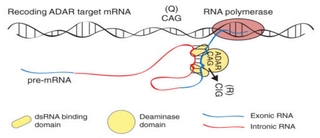
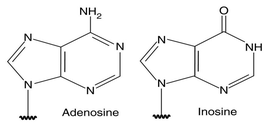
## A-to-I RNA Editing

- Adenosine deaminase acting on RNA (ADAR), mediates the conversion of adenosine to inosine on dsRNA duplexes through post-transcriptional hydrolytic deamination.[4]



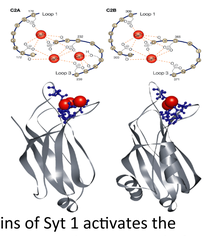

- When ADAR modifies the adenosine into inosine, the ribosomal machinery of the cell detects insoine as guanosine and therefore A-to-I RNA editing will result in a codon change.



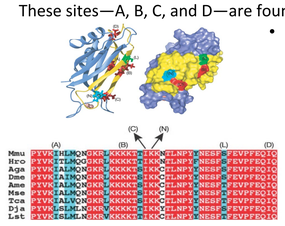

- ADAR substrates can fall into two categories: short, imperfect RNA duplexes and long, perfectly paired RNA duplexes. The former are subject to specific RNA editing. The latter are subject to promiscuous editing, through which 40-50% of adenosines can be exchanged with inosines.
- ADAR recognizes short, imperfect dsRNAs formed by complimentary exons and introns. These intronic sequences are known as editing site complementary sequences (ECSs).
- *Drosophila* without *adar* experience:
  - Extreme incoordination
  - Seizures
  - Temperature-sensitive paralysis
  - Lack of courtship in males
  - Neurodegeneration
- Although the severity of these symptoms increases with age, morphology and lifespan, in fact, appear normal.
- In humans improper editing has been linked to:
  - Suicidal depression
  - Schizophrenia
  - Prader-Willi syndrome
  - Amyotrophic Lateral Sclerosis
  - Epilepsy.
  - Glioblastoma
  - Aicardi-Goutieres Syndrome[5]

## Synaptotagmin 1

- Synaptotagmin 1 (Syt 1) acts as a calcium sensor, regulating calcium dependent fast and synchronous neurotransmitter release.
- Syt 1 consists of a short amino terminal region, a transmembrane domain spanning the membrane once, and a cytoplasmic carboxy-terminus.[1]




- Calcium binding to the $C_2$A and $C_2$B domains of Syt 1 activates the phospholipid binding ability of the $C_2$ domains, resulting in vesicle fusion and neurotransmitter release.
- Mutations of the $C_2$B domain, however, prevented Syt 1 phospholipid binding and vesicle fusion.
- Binding of $Ca^{2+}$ to $C_2$B domains triggers oligomerizaiton of Syt 1, an important step of neurotransmitter release.[2]
- There are four editing sites within the $C_2$B domain of *Drosophila* Syt 1. These sites—A, B, C, and D—are found on the ninth exon.




- Editing at site A is low (~5%), but converts Isoleucine to Valine. Editing at sites B and C is intermediate (~50%), resulting in a Lysine-to-Arginine and Isoleucine-to-Valine amino acid changes, respectively. Site D experiences the highest level of editing (~95%), converting Isoleucine to Methionine.[3]
- Two downstream, intronic ECSs, termed E1 and E2, have also been shown to direct specific editing of sites C and D.
- When eight base pair mutations were introduced into E1 and E2, editing at sites C and D was abolished.[3]

## Abstract

Adenosine Deaminase Acting on RNA (ADAR), modifies double-stranded RNA molecules through post-transcriptional hydrolytic deamination of specific adenosines, turning them into inosines (A-to-I RNA editing), which the ribosomal machinery detects as guanosine. These modifications often result in an amino acid change with profound consequences in protein function. *Synaptotagmin 1* is a calcium sensor protein of the nervous system that has a pivotal role in the synapses for calcium dependent fast and synchronous neurotransmitter release. Intronic elements directing RNA secondary structures identified in the *Syt 1* locus have been shown to direct editing *in vitro*. Here, through precise genetic engineering of *syt 1* locus, we show a complex system of RNA editing *in vivo*. Each editing site consists of an adenosine with a specific level of editing across stages of development and tissues, controlled by an autonomous element. Simple modifications of these elements could have a big effect on editing level of a specific site. Here, we have shown that by modifying an element, editing could be completely abolished in a site; and by mimicking an element of a mosquito, editing levels would mimic that of mosquito's respective editing site as well; and also a completely new editing site can appear through modification of an element. We further show that mutations that cause modifications in the dsRNA secondary structure of *syt 1*, can not only modulate the editing efficiency of a specific site, but can also abolish editing at another site. This work offers evidence into the evolutionary development of how RNA editing sites appeared over the course of adaptive evolution. Further works could focus on implications of these changes in editing sites through behavioral analyses and electrophysiology and also further manipulation of editing in order to develop a desired phenotype.

## Methods

**Genetic Engineering and RNA Extraction**

Animals were genetically engineered through ends-out homologous recombination. Male drosophila were collected in 3 to 5 different biological replicates, and RNA was extracted from both heads and thoraxes of 20 male adults in each biological replicate.

**RT-PCR and Sequencing**

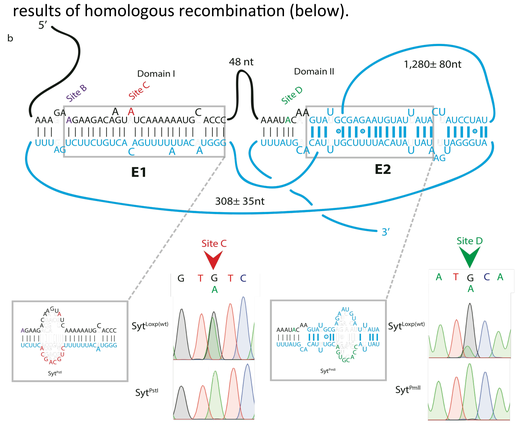
Reverse transcription and PCR were performed using synaptotagmin-specific primers. Amplified DNA was further isolated through electrophoresis with 1.5% agarose gels. Next-Gen illumina dye sequencing was used to sequenced the cleaned DNA.

**RNA Editing Analysis**

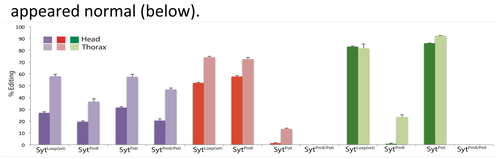
At each editing sites, we measured the relative G and A peak height in the sequencing results, and by G peak height over A+G peak height (G/(G+A)) we measured the editing level. This number falls between 0 and 1, with 0 indicating no editing and 2 indicating full editing.

## Autonomous Regulation of Sites C and D


- We introduced 8 base pair mutations in E1 and E2 by homologous recombination. E1 and E2 were no longer complimentary to exon 9 (above).
- A *PstI* restriction site was inserted into element 1 and a *PmlI* restriction site was inserted in order to confirm the results of homologous recombination (below).

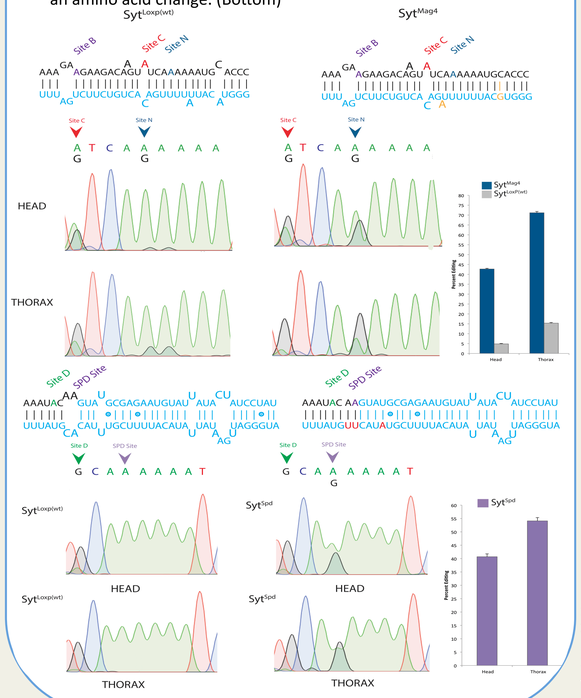



- In *PstI* mutants, site C editing was abolished, while editing at sites B and D remained normal. In *PmlI* mutants, editing was abolished at site D, while editing at sites B and C remained normal. For double mutants, neither site C nor site D was edited, but editing at site B appeared normal (below).



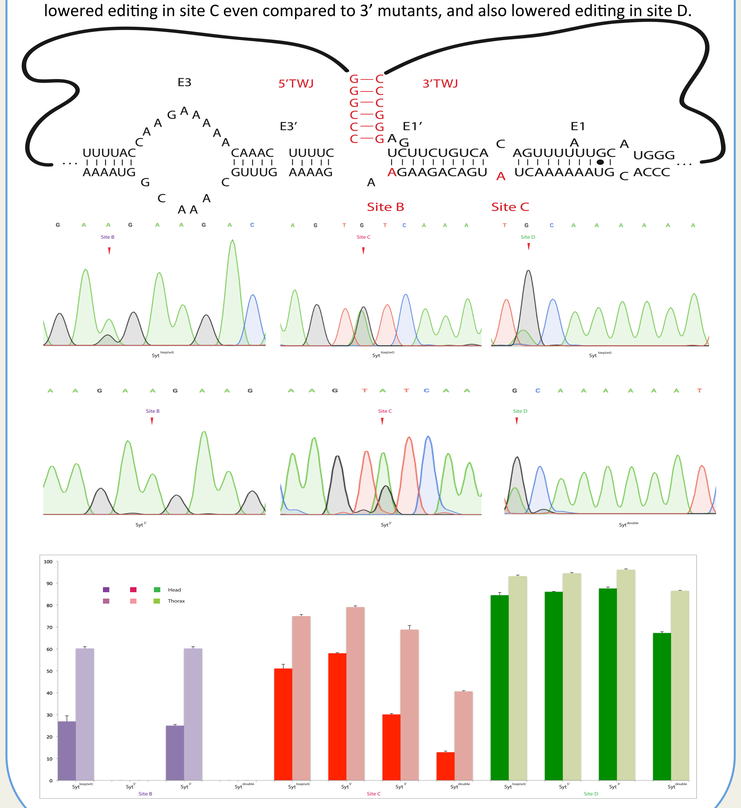

## The Birth of RNA Editing Sites

- We genetically engineered animals to mimic the E1 of *anopheles gambiae's* (mosquito) E1, termed MAG4, which differs in only 2 ribonucleotides from *Drosophila*'s E1. Editing at site E of drosophila was comparable to its equivalent in mosquito (site N). (Top)
- By homologous recombination, we altered the sequence of E2 to completely to compliment exon 9. This removed a bulge downstream of site D, which resulted in slightly higher editing at site D and a novel editing site, termed SPD, which does not lead to an amino acid change. (Bottom)



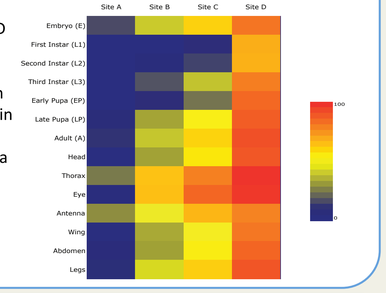

## The Three-Way Junction

- Three-way RNA junctions usually adopt a recurrent parallel-Y shape. This is formed when two of the helices form a coaxial stack, and a third helix establish one or more tertiary contacts, usually several base pairs apart from the junction.
- Mutants were generated through homologous recombination, and the success of the crosses were confirmed using maker gene in the recombination.
- Our results show that editing is abolished in site B in the 5' three-way junction mutants. Site C editing is lowered in 3' mutants. Double mutants demonstrate eliminated editing in site B, lowered editing in site C even compared to 3' mutants, and also lowered editing in site D.




## Future Direction and Next Steps

- Results from developmental and morphological analysis of sites A, B, C, and D (right).
- Although editing trend seems to be correlated with levels of ADAR expression in each developmental stage, differences within each developmental stage and within each morphological tissue imply the presence of a complex system of regulation that could be the subject of future research.
- The three-way junction research could help provide mechanistic information about this process at the RNA (structural) level.

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