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Using Mouse Models to Unlock the Secrets of Non-Synonymous RNA Editing.

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Abstract

The deamination of adenosine to inosine by RNA editing is a widespread post-transcriptional process that expands genetic diversity. Selective substitution of inosine for adenosine in pre-mRNA transcripts can alter splicing, mRNA stability, and the amino acid sequence of the encoded protein. The functional consequences of RNA editing-dependent amino acid substitution are known for only a handful of RNA editing substrates. Many of these studies began in heterologous mammalian expression systems; however, the gold-standard for determining the functional significance of transcript-specific re-coding A-to-I editing events is the generation of a mouse model that expresses only one RNA editing-dependent isoform. The frequency of site-specific RNA editing varies spatially, temporally, and in some diseases, therefore, determining the profile of RNA editing frequency is also an important element of research. Here we review the strengths and weaknesses of existing mouse models for the study of RNA editing, as well as methods for quantifying RNA editing frequencies *in vivo*. Importantly, we highlight opportunities for future RNA editing studies in mice, projecting that improvements in genome editing and high-throughput sequencing technologies will allow the field to excel in coming years.

Keywords

RNA editing; post-transcriptional regulation; inosine; adenosine deaminase; ADAR; mouse models; protein diversity

1. INTRODUCTION

The selective conversion of adenosine to inosine (A-to-I) is a widespread post-transcriptional processing event that modifies the sequence of precursor and mature transcripts. Adenosine deaminases acting on RNA (ADAR) enzymes catalyze this process

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by recognizing and binding to regions of double-stranded RNA (dsRNA), deaminating adenosines within the dsRNA structure [1,2]. RNA editing that occurs within coding regions is typically highly regulated, affecting only select adenosines within short stretches of an imperfect dsRNA structure. Promiscuous hyper-editing of multiple adenosines within an extended RNA structure occurs at a low frequency within coding regions and is likely the consequence of near ubiquitous expression of ADARs and the transcription of genomic repeat elements [3].

Because inosine residues in RNA base pair with cytosines, A-to-I editing has the potential to affect several aspects of the RNA lifecycle, including processing, regulation, and coding potential. Splice sites may be introduced or disrupted by editing within pre-mRNA. For example, ADAR2 edits its own pre-mRNA to generate an alternative 3' splice site, resulting in the production of a truncated isoform that lacks the catalytic region of the full-length protein [4]. miRNAs are generated from dsRNA precursors and thus are likely targets of RNA editing. This editing can disrupt production of the miRNA, as well as affect miRNA target recognition by altering the seed sequence [5–8]. Editing within mRNA can also generate new miRNA binding sites [9]. Finally, A-to-I RNA editing events occurring within coding regions can potentially generate alternative protein isoforms by introducing RNA editing-dependent amino acid substitutions.

RNA editing is widespread in the human genome, largely due to the presence of primate-specific repetitive Alu sequences. More than 90% of the RNA editing events in human occur at low rates within regions that are transcribed from mammal-specific inverted Alu repeats [10–13]. Approximately 150 RNA editing sites overall are found within non-repetitive sequence, occur within the coding region of mRNA and alter the amino acid sequence of the resulting protein. Furthermore, around 30 of these re-coding sites are conserved across mammals species, including human and mouse [10,14]. This small conserved data set contains RNA editing events that occur at higher frequencies than other non-conserved editing events and have extreme contextual evolutionary constraints [14], suggesting that these events provide functionally distinct products that benefit organismal fitness. Indeed, non-synonymous RNA editing events on 14 different substrates have been verified to alter the functional properties of the encoded protein (Table 1). Several of those non-synonymous events re-code a single amino acid to a residue that is chemically similar to the genomically-encoded residue, yet still result in discrete physiological changes. For example, A-to-I editing of *KCNA1* converts an isoleucine to valine in the voltage-gated potassium channel Kv1.1, generating a channel with an inactivation rate ~20 times greater than in the non-edited channel [14,15].

Large-scale transcriptome information has advanced the RNA editing field by providing wide-angle knowledge regarding the identity of RNA editing substrates [11,13,16–19], tissue-and disease-specific RNA editing profiles [10,20–23], and the conservation of edited substrates [14,24–26]. Heterologous expression systems and animal models, particularly mouse, are useful for determining the function of RNA editing-dependent protein isoforms. The current review will discuss the strengths and limitations of these mouse models, as well as offer guidance for the future development of vertebrate models to study RNA editing. We will also discuss newer and more targeted sequencing approaches that often accompany

assessments of isoform-specific function, providing insight into the *cis*- and *trans*-factors required for recoding RNA editing events, as well as into the broader processes that regulate RNA editing.

2. MOUSE MODELS OF RNA EDITING

2.1. Evolution of Mouse Models to Study RNA Editing

There are two functional RNA editing enzymes in mammals, ADAR1 and ADAR2. Early studies of A-to-I editing employed ADAR1 and ADAR2 global knockouts to broadly assess the significance of RNA editing in establishing normal physiology. The ADAR2-null mouse revealed the critical ADAR2-dependent glutamine-to-arginine (Q607R) editing requirement within the GluA2 subunit of the ionotropic glutamate AMPA receptor [45]. ADAR1 is unique in that it encodes a constitutively expressed isoform (p110) in addition to an alternative isoform expressed from an interferon-responsive promoter (p150). Mice that lack expression of both ADAR1 isoforms [46,47], lack only the inducible p150 isoform [48], or express only editing-deficient ADAR1 (both p150 and p110) [49], all demonstrate lethality at embryonic day 11.5–13.5. The more overt phenotypes associated with ADAR1-deficiency are likely editing-independent and driven by ADAR1's unique interaction with the innate immune response. However, germline- and somatic-specific deletions have revealed that at least some developmental pathways are dependent on the A-to-I editing activity of ADAR1 [49,50]. More subtle editing-dependent effects due to perturbations at specific RNA editing sites may be masked in the global knockouts by functional overlap between ADAR1 and ADAR2 and/or compensatory measures at the cellular level. To better assess the functional consequences of A-to-I editing for *specific* ADAR substrates, investigators have developed more targeted vertebrate and invertebrate model systems, such as knock-in mice, that exclusively express either edited or non-edited substrate isoforms.

2.2. Always Edited/Non-Edited Mouse Models

The RNA encoding the 2C subtype of serotonin receptor can be edited at five sites to generate as many as 24 distinct protein isoforms that vary by one to three amino acids within the second intracellular loop of the receptor [51,52], a region that has an established role in G-protein coupling efficacy [53]. The genomically encoded INI isoform, contains isoleucine, asparagine, and isoleucine at amino acid positions 157, 159, and 161, respectively; whereas the fully-edited VGV isoform contains valine, glycine, and valine at the analogous positions [51,52]. Cell culture-based studies initially found that the VGV isoform of the receptor has dramatically reduced constitutive activity and G-protein coupling efficacy relative to the non-edited and partially edited 5HT_{2C} isoforms [51,52,54–56]. To determine the significance of this uniquely complex editing paradigm in vertebrate physiology, knock-in mice solely expressing the fully-edited VGV receptor isoform (5HT_{2C}-VGV) were generated [40,41]. The 5HT_{2C}-VGV knock-in mice displayed marked behavioral and physiological phenotypes, including a failure-to-thrive, decreased somatic growth, neonatal muscular hypotonia, and reduced food consumption followed by post-weaning hyperphagia [40,41]. By contrast, knock-in mice solely expressing the constitutively active non-edited INI receptor isoform (5HT_{2C}-INI) grew normally with no overt metabolic phenotypes [40]. When evaluated in a behavioral test battery, both 5HT_{2C}-INI and 5HT_{2C}-VGV mice exhibited

exaggerated anxiety-like behaviors in the elevated plus maze. However, in the forced-swim and tail suspension test paradigms, 5HT_{2C}-INI mice displayed increased depressive-like behavior, whereas 5HT_{2C}-VGV mice displayed increased antidepressant-like behavior [43]. These findings validated the idea that proper regulation of 5HT_{2C} editing patterns is essential for establishing normal physiology.

A subsequent group also developed a dual knock-in mouse model aimed at maximizing 5HT_{2C} signaling activity by solely expressing the INI receptor and simultaneously ablating the expression of alternative splice variants. In comparison to 5HT_{2C}-VGV mice, these splice-null, 5HT_{2C}-INI mice did not produce an overt metabolic phenotype but did display mild alterations in fear conditioning and depressive-like behavior [42]. The basis for this model stemmed from a previous observation that a highly expressed truncated splice variant of the receptor (5HT_{2C}-tr) heterodimerizes with the full-length receptor in the endoplasmic reticulum to prevent trafficking of the heterodimer to the cell surface [57]. Therefore, researchers simultaneously eliminated this truncated product to maximize trafficking of the full-length receptor to the surface. Since the effects of the splice-null mutation were not measured separately from the editing mutation, the individual contribution of each mutation to the observed phenotypes is difficult to resolve.

A knock-in strategy was used to evaluate the physiological relevance of A-to-I editing in calcium-dependent activator for protein secretion 1 (CAPS1) [30]. A massively parallel target capture sequencing approach previously identified a single editing site within CAPS1 pre-mRNA that converts a glutamate to a glycine (E/G) within its C-terminal domain [16]. CAPS1 is a critical regulator of dense core vesicle (DCV) exocytosis [58–61]. To investigate the significance of RNA editing in CAPS1-mediated DCV exocytosis, investigators generated mice solely expressing the edited isoform. Electrophysiological and biochemical analyses revealed an upregulation of DCV exocytosis in both chromaffin cells and neurons in the edited mice. Behaviorally, these mice displayed marked hyperactivity that resulted in increased energy expenditure and a lean appearance. This work established clear functional significance for CAPS1 editing *in vivo* [30].

Editing of FLNA by ADAR2 results in a Filamin A protein with a glutamine to arginine substitution in a domain that is responsible for extensive protein interactions [25,32,62]. FLNA editing occurs at a high rate in vascular tissues, but that editing is reduced by nearly half in human populations with cardiovascular disease. A mouse model that expresses only non-edited FLNA has reduced cardiovascular fitness, with specific phenotypes including increased smooth muscle contraction and resting diastolic hypertension. Overall, this model demonstrates an important relationship between editing of FLNA and cardiovascular health [32].

Only a few additional mouse models have been generated in which either non-edited or edited ADAR substrates are exclusively expressed: GluA2 editing-deficient, GluA2 always-edited, GluK1 always-edited, GluK2 editing-deficient, and ADAR2 autoediting-deficient mice [45]. Each model has proved useful in providing initial *in vivo* validation for the functional significance of proper editing regulation. Nevertheless, these models are ultimately artificial and limited in scope in the sense that always edited or non-edited

conditions are scarcely observed in nature, with the noteworthy exception being the GluA2 subunit of AMPA receptors. Nevertheless, the phenotypes observed in these models can be used as guideposts to establish a physiologically-relevant range of effects for altered site-specific editing patterns. More suitable models that mimic the milder alterations to RNA editing commonly observed in disease models should also be considered. For example, the editing level of GluA2 transcripts in spinal motor neurons of amyotrophic lateral sclerosis patients is reduced by as much as 38% relative to normal controls [30,63–65].

Some disease models may provide insight on how to manipulate RNA editing frequency *in vivo*. For instance, single-nucleotide mutations occur within the double-stranded RNA structure required for A-to-I editing of *KCNA1* and are linked to a human disorder known as episodic ataxia type-1. These mutations destabilize the ADAR-recognized RNA structure and decrease A-to-I RNA editing of the transcript by up to 59% [44]. Based on this information, knock-in mouse models with silent substitutions that manipulate the structure of the RNA have the potential to tune RNA editing levels.

It remains possible, and even enticing, that RNA editing could function in some instances as a dynamic mechanism to provide homeostatic regulation in response to changing stimuli. Investigating the potential dynamic aspects of editing regulation for a given substrate will likely require the development of more sophisticated conditional models in which the expression levels of specific isoforms can be altered in more refined way, perhaps spatio-temporally and/or similar to that modeled by disease states.

3. ANALYZING RNA EDITING FREQUENCY

3.1. A Case for Measuring Editing Profiles *in vivo*

High-throughput sequencing technology has provided an extensive library of inosine-containing transcripts that are available through the online databases DARNED [45,66] and RADAR [67]. Investigations into the physiological consequences of an individual RNA editing event, or a set of events, are often accompanied by more comprehensive, model-specific analysis of transcript-specific editing levels. Isolation of high-quality RNA from the tissue of interest is a critical first step in investigating the transcript sequence, for which several comprehensive technical guides are available [68].

For *in vivo* studies, it is important to recognize that the editing frequency of a given transcript can vary widely, perhaps providing clues as to the developmental times and/or anatomical locations where editing is more critical to fitness. Human CAPS1 transcripts, for example, are edited at ~15–20% across brain tissues but increase to 42% and 70% in pancreas and adrenal glands, respectively [30], indicative of the crucial role CAPS1 editing plays in secretion of hormones and peptides. More widespread analyses in human and mouse confirm that RNA editing patterns, and specifically those sites transcribed from non-repetitive regions of the genome, have distinct tissue-specific editing patterns [20,69,70]. Therefore, it is advisable as a general practice to obtain transcript editing frequencies from a broad sampling of tissues when conducting *in vivo* studies. Editing frequencies at re-coding sites do not appear to vary across mouse strains [26,71], and are consistent with those in human tissue [69]. Age, however, does affect RNA editing levels [72,73], suggesting that

while a study design may select any available laboratory mouse strain, careful considerations should be made with regard to developmental time points.

3.2. Techniques for Quantifying RNA Editing Frequency

Before quantification of apparent RNA editing by cDNA sequencing, it is imperative to cross-reference genomic sequence with cDNA sequence to rule out mis-identification of RNA editing sites due to potential SNPs or somatic mutations. Moreover, comparison of the genomic and cDNA sequences between individuals and samples may give some indication about the variables (i.e. conserved structure, age, conditions, disease state, tissue, etc.) that influence RNA editing. It should also be noted that even within a well-defined discrete tissue sample, transcript editing frequencies can vary in a cell type-specific manner. To illustrate, 5HT_{2C} mRNA is expressed in multiple cell populations within the arcuate nucleus of the hypothalamus, including POMC and NPY neurons [74]. RNA isolated from dissected hypothalamus or microdissected arcuate nucleus could, at best, provide an averaged editing profile for the various 5HT_{2C}-expressing cell types contained in this hypothalamic structure, but it cannot segregate the potentially unique editing levels within POMC or NPY neurons. A thioracil (TU)-tagging technique has been described for labeling and purifying RNA from specific cell types within intact complex tissues and may prove useful for obtaining editing profiles at this level of resolution [75,76].

The most common technique for measuring editing frequencies from purified RNA relies upon classical Sanger sequencing of RT-PCR products. Editing sites can be identified on chromatograms by the characteristic overlapping A/G peaks, although very low levels of editing (<10%) may prove difficult to resolve depending on the level of background noise [77]. Quantification of the relative A/G peak height can provide a reliable estimate of editing frequency; however, bulk sequencing from pooled RNA isoforms cannot discriminate the identity or frequency of individual RNA isoforms when multiple editing sites are present in a transcript (e.g. GluA2, 5HT_{2C}R; Table 1). In these cases, the combinatorial editing profile can be determined by sequencing individual sub-cloned RT-PCR products, a process that is often performed with limited statistical power. Arguably better methods of analysis use targeted high-throughput second-generation sequencing to differentiate and quantify the relative distribution of all possible transcript configurations within narrow ranges and with limited error [71,78]. The read-depth of either the Illumina MiSeq or HiSeq allows for extensive multiplexing using barcoded RT-PCR primers to enable simultaneous analysis of multiple tissue samples or replicates, thereby maximizing the data generated while minimizing time and cost per sample [71,78]. For groups of transcripts expressed at very low levels and/or with low-level editing, microfluidics-based multiplexed PCR amplification coupled with second-generation sequencing (mmPCR-seq) is a powerful way to increase sensitivity and robustly increase multiplexing capabilities, while maintaining accuracy and throughput [78]. Third-generation sequencing technologies developed by Pacific Biosciences and Oxford Nanopore provide significantly improved read-length, thus expanding the ability to sequence not only across multiple editing sites but also across entire >10 kilobase transcripts. The accuracy and depth of the sequencing information does decline, which can be compensated by additional trials, although this will significantly affect

the cost-efficiency of experiments to reliably determine isoform-specific editing frequency [79].

One additional consideration that remains relatively unexplored within the RNA editing field is the commonly-held assumption that transcript isoform editing frequencies accurately reflect the corresponding distribution of protein isoforms. However, an intriguing RNA-protein disparity in 5HT_{2C}-VGV mice challenges this assumption. 5HT_{2C}-VGV mutant mice expressed a region-specific increase in receptor protein (4000–7000%) relative to wild-type littermates using receptor autoradiography, saturation binding and Western blotting, with no concurrent change in the steady-state levels of total 5HT_{2C} transcripts [41]. This unprecedented disparity between 5HT_{2C} mRNA and protein levels suggests novel post-transcriptional mechanism(s) for regulating protein expression in an editing-dependent manner.

Protein expression from edited transcripts was recently verified by comparison of RNA-sequencing data to available mass spectrometry (MS) data. This post-hoc analysis was followed up by immunoprecipitation from cell lines overexpressing the edited or non-edited protein isoforms of two editing targets, then liquid chromatography with tandem mass spectrometry (LCMS/MS) to corroborate the presence of peptides corresponding to edited and non-edited COPA and Cog3 [31]. While this analysis was somewhat artificial (overexpression of synthetically edited isoforms), and not-performed in a quantitative way, it represents a forward step towards quantitative analysis of endogenous RNA editing-dependent protein expression.

To extend this proof-of-concept, a customized MS strategy may validate whether individual transcript editing profiles correspond to the protein isoform distribution; however, there are important technical considerations involved in this analysis. First, the availability of commercial antibodies capable of achieving sufficient homogeneity and yield from native samples by immunoprecipitation is often limiting. This potential limitation may be overcome by introducing epitope tag(s) into the target coding sequence, a prospect that is increasingly possible due to advances in specific and efficient CRISPR gene editing technology (Miura et al., 2018). Secondly, a suitable protease enzyme (e.g. trypsin) must be selected to cleave an efficiently ionizable peptide containing the RNA editing-dependent variable amino acid position. The method of fragmentation must avoid isoform-dependent bias, yet generate unique and identifiable isoform-specific profiles in mass spectrometry. The success of these methods is dependent on the overall protein sequence, but also on user expertise and trial and error.

4. SUMMARY

A-to-I RNA editing events are widespread in the human transcriptome, however, conserved site-specific editing events that affect the sequence of the encoded protein are relatively rare. RNA editing by ADARs is often incomplete, resulting in parallel expression of protein isoforms translated from edited or non-edited transcripts. In a handful of cases, the molecular and physiological consequences of RNA editing has been determined through studies in heterologous expression systems and/or mouse models that express only one of

the RNA editing-dependent protein isoforms. Remarkably, even subtle amino acid substitutions have consequences on the function of the encoded protein. We argue that more sophisticated models capable of fine-tuning RNA editing are required to provide information relevant to disease states.

RNA editing is thought to be a dynamic process that contributes to the genetic diversity within an organism. Editing frequencies vary based on the tissue of expression and stage of development, which is potentially due to alternative regulatory strategies. Editing frequency is determined by sequencing cDNA and quantifying the relative proportion of edited to non-edited transcripts. The sequencing strategy selected to quantify RNA editing levels should consider the extent of editing or change in editing expected, as well as the number of editing sites present on the targeted transcript. Therefore, the most appropriate strategy for sequencing and analysis will depend on the target of interest and experimental goals. Sanger sequencing, as well as high-throughput next-generation sequencing methods can be used for quantitative analysis of RNA editing levels, but resolution of RNA editing-dependent protein isoforms may provide insight into RNA editing-mediated proteome content that may not always be reflected by the transcript editing percentage.

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ABBREVIATIONS

ADAR	adenosine deaminase acting on RNA
RNA	Ribonucleic acid
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats

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HIGHLIGHTS

- Functional consequences of re-coding RNA editing is commonly studied in mouse models.
- Existing mouse models include ADAR null and all-or-none editing of individual RNAs.
- *In vivo* studies should consider variables known to influence editing frequency.
- Sanger and next generation sequencing may be used to quantify RNA editing.
- Mass spectrometry may be useful to quantify RNA editing-dependent protein levels.

Table 1.**Functional Consequences of Site-Specific RNA Editing Events**

Gene	Protein	AA change	Editing-Dependent Functional Consequence	Validation*	Ref
<i>ADAR2</i>	Adenosine deaminase, RNA-specific 2	Premature stop	Loss of enzyme auto-editing	mouse model	[27]
<i>AZIN1</i>	Antizyme inhibitor 1	S>G	Cytoplasmic-to-nuclear translocation, promotes tumor growth	cell culture	[28,29]
<i>CADPS1</i>	Calcium-dependent secretion activator 1 (CAPS1)	E>G	Elevated dense core vesicle exocytosis	mouse model	[30]
<i>COG3</i>	component of oligomeric golgi complex 3	I>V	Promotes tumor growth and migration	cell culture	[29]
<i>COPA</i>	coatamer protein complex subunit alpha	I>V	Promotes tumor growth and migration	cell culture	[31]
<i>FLNA</i>	Filamin A	Q>R	Regulates smooth muscle contraction, hypertension, and aortic relaxation.	mouse model	[32]
<i>GABRA3</i>	γ -aminobutyric acid (GABA) A receptor, subunit $\alpha 3$	I>M	Reduction of cell surface expression and subunit composition	cell culture	[33,34]
<i>GRIA2</i>	GluA2 subunit of AMPA subtype of ionotropic glutamate receptor	Q>R	Attenuation of Ca^{2+} permeability Faster recovery from desensitization	mouse model	[35,36]
<i>GRIA3</i>	GluA3 subunit of AMPA subtype of ionotropic glutamate receptor	R>G	Faster recovery from desensitization	cell culture	[37]
<i>GRIA4</i>	GluA4 subunit of AMPA subtype of ionotropic glutamate receptor	R>G	Faster recovery from desensitization	cell culture	[37]
<i>GRIK1</i>	GluK1 subunit of kainate subtype of ionotropic glutamate receptor	Q>R	Attenuation of Ca^{2+} permeability	mouse model	[38]
<i>GRIK2</i>	GluK2 subunit of kainate subtype of ionotropic glutamate receptor	Q>R	Attenuation of Ca^{2+} permeability	mouse model	[39]
<i>HTR2C</i>	5-hydroxytryptamine (serotonin) receptor 2C (5HT _{2C})	I>V,M N>S,G,D I>V	Attenuation of receptor constitutive activity	mouse models	[40–43]
<i>KCNA1</i>	Voltage-gated K ⁺ channel, shaker-related subfamily (K _v 1.1)	I>V	Faster recovery from channel inactivation	oocytes	[15,44]

* Most advanced model available in the literature