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The Role of RNA Editing Within Calcium-dependent Activator for Secretion 1 in Large Dense Core Vesicle Fusion

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THE ROLE OF RNA EDITING WITHIN CALCIUM-DEPENDENT ACTIVATOR FOR SECRETION 1 IN LARGE DENSE CORE VESICLE FUSION

A Master's Thesis

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By

Kaylee M. Mathiason

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ABSTRACT

Calcium-dependent activator protein for secretion 1 (CAPS1) is involved in synaptic vesicle and large dense core vesicle fusion by bridging vesicles and the plasma membrane prior to vesicle fusion and cargo release. *CAPS1* is subject to a post-transcriptional modification called RNA editing. RNA editing recodes a single amino acid in the region of the protein that interacts with the vesicle. This recoding event within *CAPS1* has cellular consequences in neurons where edited *CAPS1* overexpression increases basal secretion from neural synaptic vesicles. Our goal was to investigate how RNA editing within CAPS1 affects large dense core vesicle secretion in an insulinoma cell type that secretes insulin. We modified *CAPS1* editing frequency within insulin-secreting cells, then measured non-stimulated and glucose stimulated secretion. We found cells with increased non-edited *CAPS1* had less basal secretion compared to normal insulin-secreting cells, but edited *CAPS1* did not affect secretion. Our study provides insight into how even slight modifications of *CAPS1* RNA editing frequency could have profound effects on cellular secretion, which may suggest that *CAPS1* editing is paramount to regulating cellular processes like neurotransmission and glucose homeostasis.

KEYWORDS: CAPS1, RNA editing, secretion, stimulated secretion, GSIS, INS-1 cells

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INTRODUCTION

Cells are complex machines, housing many subcellular processes that coordinate to make the cell function. The human body relies on collaboration of its many cells by cell-to-cell communication via the secretion of proteins, hormones, and other chemicals. Secretion is vital in regulation of glucose levels, immunity, and neurotransmission and is an intricate process controlled by a host of cellular factors. Disruption of secretion can cause disease, be a symptom of disease, or may occur during the treatment of disease. Regardless of the origin, disruption in this tightly controlled process can lead to drastic cellular and physiological consequences. For example, type II diabetes inflicts about 10% of the United States population. Type II diabetes develops when the body fails to release enough insulin or to respond to insulin signals that control circulating glucose levels (reviewed in Koeck et al., 2015). The failure to control glucose levels causes a wide array of complications, such as nerve damage, heart disease, increased risk for developing other conditions, and more. The fight to cure diabetes is ongoing, but understanding how insulin release occurs and the mechanisms of its regulation will help us better understand the disease and potential treatments.

Secretion

Secretion is the process by which cellular products are released into an extracellular space via fusion between a vesicle and plasma membrane. This event provides the means for cell to cell communication and is fundamental to cell survival. Though all cells release an array of contents that act as chemical signals on target cells, different cell types release different vesicle types with different cargos. There are two major types of vesicles, small-clear vesicles (SVs) and

large dense-core vesicles (LDCVs), which are utilized by a myriad of cell types. For example, SVs release neurotransmitters and LDCVs release signaling peptides from neurons. Endocrine cells typically utilize LDCVs to secrete hormones and other large peptides (Jahn and Südhof, 1999). The two types of vesicles also differ in origin, where SVs bud from early endosomes and LDCVs originate from the *trans*-Golgi network (Kelly, 1993). Though there are stark differences between the two, they follow the same basic fusion mechanics.

Fusion. Merging of vesicle and cell membrane lipid bilayers releases vesicle contents into the extracellular space. There is some evidence that suggests two major modes of fusion can occur: Full collapse fusion and "kiss-and-run" (Figure 1; reviewed in Rorsman and Renstrom, 2003). Full collapse fusion occurs when the vesicular membrane collapses completely and merges fully with the plasma membrane, releasing all cargo. "Kiss-and-run" is the process where a fusion pore forms, releasing a portion of cargo, then closes to re-form an independent vesicle (Rutter and Hill, 2006).

The two modes of fusion both follow the same basic process for fusion pore formation. Since each membrane is composed of acidic lipids, the membranes have an overall negative charge, which should cause repulsion of the vesicular and plasma membranes. However, electron microscope images reveal that medially the vesicular and cellular membranes are attracted to one another, while laterally, the two membrane oppose one another. This results in a vesicular and plasma membrane medial bilayer bending (Greengard, Valtorta, Czernik, and Benfenati, 1993). Some evidence suggests positively charged amino acids in vesicle membrane-spanning proteins may decrease the overall negative charge of the vesicle membrane, consequentially increasing the electrostatic attraction between vesicle and plasma membranes (Williams, Vicôgne, Zaitseva, McLaughlin, and Pessin, 2009). The medial bilayer bending eventually forms a stalk that allows

lipid-mixing between the vesicle and plasma membrane, and then the fusion pore opens. During full collapse fusion, the vesicle membrane lipids mix completely and the two bilayers merge. During "kiss-and-run", after a fusion pore forms, the membranes oppose one another medially, pushing the vesicle internally from the membrane. This is a transient process, where vesicles undergoing this type of fusion can repeatedly fuse with the membrane, release partial cargo, fully release, or retract from the membrane for future fusion (Ceccarelli, Hurlbut, and Mauro, 1973).

Some literature suggests LDCVs in certain cell types prefer the kiss-and-run mode of fusion. For example, cultured INS-1 (insulinoma) cells demonstrate "kiss-and-run" as the primary release mechanism, where vesicles release partial contents approximately 90% of the time and fully fuse with the membrane about 10% of the time (reviewed by Rorsman and Renstrom, 2003). However, direct imaging indicates insulin-containing LDCVs in pancreatic islet cells fuse completely with the membrane following stimulation (Ma et al., 2004). These vesicles lingered at the membrane following stimulation, but did not undergo partial release, only full fusion. SVs may also utilize both full fusion and "kiss-and-run", though the degree is still in question (Aravanis, Pyle, and Tsien, 2003).

Docking and priming. Vesicles undergo a series of events, termed "docking" and "priming", that prepare them for fusion. Typically, docking refers to anchoring of vesicles to the plasma membrane. Priming refers to subsequent events that ready the vesicle for fusion, such as protein-protein interactions or conformational changes in those proteins (Klenchin and Martin, 2000). Some evidence suggests these two events may be separate and distinct in neuroendocrine cells (Nofal, Becherer, Hof, Matti, and Rettig, 2007). Distinction between to two processes are supported by molecular manipulations that can alter the amount of readily releasable vesicles (primed) without altering the number of docked vesicles (Gulyás-Kovács et al., 2007). Other

sources characterize priming and docking by vesicle mobility at the membrane: Vesicles that fused with the membrane within eight seconds were most likely primed vesicles and were stationary at the fusion site prior to fusion. Vesicles that fused with the membrane after eight seconds of stimulation traveled a greater distance along the membrane prior to fusion and were most likely docked, but not primed (reviewed in Verhage and Sørensen, 2008).

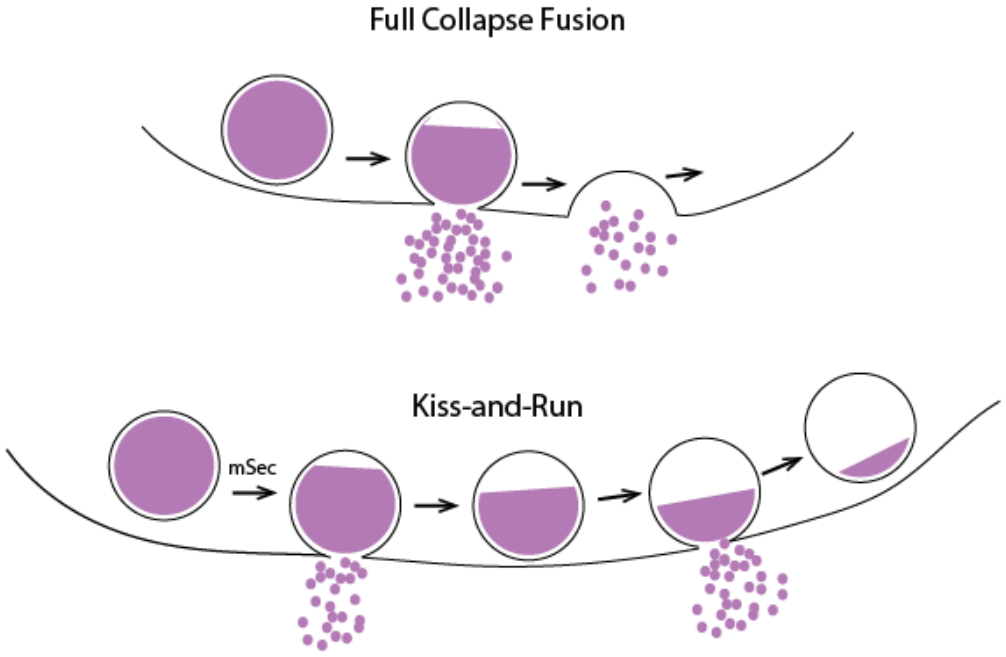

Figure 1. Full collapse and "Kiss-and-Run" vesicle fusion. Vesicles that undergo full collapse fusion will fully release cargo (purple) and the membrane will merge with the plasma membrane (Top). Vesicles that undergo "Kiss-and-Run" mode of fusion form a fusion pore, release partial contents, and retract from the membrane (Bottom). At times, this vesicle can transiently refuse multiple times prior to full fusion.

Docking and priming proteins. Proteins are central to docking and priming. Soluble N-ethylmaleimide-sensitive factor attachment receptors (SNAREs) are a family of proteins that direct vesicles to any target membranes within the cell and can also recruit proteins for further vesicular stabilization. Each SNARE protein contains a SNARE motif, a sequence approximately

65 amino acids long that forms a coiled-coil domain. SNAREs syntaxin, synaptobrevin (Vamp-2), and synaptosomal nerve-associated protein 25 (SNAP-25), form a quartet of helices that interact with calcium-sensor synaptotagmin to form the highly stable SNARE complex (Poirier et al., 1998). Two of the four helices are motifs from SNAP-25 and the others are from syntaxin and Vamp-2. Syntaxin, Vamp-2, and synaptotagmin each contain a C-terminal transmembrane region (TMR) that embeds the protein into their respective lipid membranes. Synaptotagmin and Vamp-2 are referred to as vesicular SNARES (v-SNARES) and are embedded in vesicular membranes via their single coiled TMR. The target SNARE (t-SNARE) SNAP-25 and calcium sensor synaptobrevin associate with the cellular membrane. Synaptobrevin contains a TMR while SNAP-25 is a peripheral protein and only associates with the cytosolic surface of the plasma membrane (reviewed in Goda, 1997).

Several members of the syntaxin family have been discovered, some which are involved in fusion mechanics at the plasma membrane. These isoforms may play a role in targeting specificity, as different isoforms are localized to different areas of the plasma membrane in polarized cells, such as kidney cells (Low et al., 2007). Some syntaxins, like Syntaxin-1, is further subdivided into isoforms 1A and 1B. The two isoforms are closely related and show some functional redundancy in regulation of SV fusion of vesicles in the RRP (Mishima et al., 2014). Both are heavily expressed in the nervous system (Bennett, Calakos, and Scheller, 1992) though Syntaxin-1A is crucial to the docking process for LDCVs in cultured adrenal cells (PC-12 cells) (S. Park et al., 2016). Syntaxin-3 has been found in specific vesicles involved in stimulated release, but much unknown regarding its localization (Band and Kuismanen, 2005). syntaxin contains a C-terminal SNARE domain which interacts directly with synapatobrevin and SNAP-25 to form the center of the SNARE complex. The N-terminal region of syntaxin contains

an Habc domain that is comprised of three alpha helices that binds to its own SNARE domain when inactive. This state is commonly referred to as “closed” syntaxin and is generally incapable of participating in the SNARE complex or vesicle docking. Conversion to “open” syntaxin is required for vesicle fusion and depends on the presence of calcium and other proteins (Borisovska, 2018). The open confirmation of syntaxin is characterized by its unbound N terminal SNARE motif which makes it available for interactions with other SNARE proteins, an essential function for vesicle fusion.

Prior to SNARE complex formation, the tSNARES and vSNARES are associated with the plasma and vesicular membranes as *cis*-SNARE complexes. To ready for fusion, tSNARE TMRs of proteins SNAP-25 and synaptotagmin homodimerize, causing the SNARE complex to extend perpendicular from the cell membrane. v-SNARE TMRs also homodimerize leaving the cytosolic N-termini free to eventually interact with the N-termini of the t-SNARE complexes (reviewed in Ramakrishnan, Drescher, and Drescher, 2012).

As the vesicle approaches the membrane, the *cis*-SNARE complexes interact to form a *trans*-SNARE complex, the conformation associated with vesicle docking. Docking is achieved by rigid SNARE linker regions within the alpha helical bundle “zippering” the two associated membranes together into proximity. This process requires precise timing and simultaneous bending of all SNAREs. Premature folding of one SNARE TMR into the alpha helical bundle could prevent the association of other TMRs (Lou and Shin, 2016). In the event where SNARE domains are not anchored to the plasma membrane, the un-associated TMRs then relieve the tension from their linker regions by kinking (Borisovska et al., 2012). This halts SNARE zippering and prevents vesicle fusion (Han, Pluhackova, Bruns, and Böckmann, 2016). Precise timing of TMR association is consistent with pore formation and full fusion. However, not all

vesicle types have the same hurdles for full fusion. For example, it may be more difficult for larger vesicles, like LDCVs, to fuse completely with the membrane, as the energetic barrier is higher compared to small vesicles, such as SVs. These fusion energy barriers may be overcome by increased numbers of SNARE complexes at fusion sites. SVs are associated with small and point-like fusion sites that contains clusters of SNARE complexes. The point-like shape of the fusion site allows rearrangement for SNARE complexes on either site of the fusion site and likely contributes to lower energy barriers (Risselada and Mayer, 2020).

Some cell types use calcium-mediated exocytosis, where a rise in intracellular calcium is required to overcome the energy barrier and trigger vesicle fusion. When cell calcium levels are low, synaptotagmins exist embedded in the vesicular membrane via the TMR. Synaptotagmin also contains two cytoplasmic C2 domains connected to the TMR by a flexible linker region. Following an influx of calcium, the C2A region of synaptotagmin can bind two calcium ions while the C2B domain can bind three. In this state, the C2A can bind to syntaxin while the basic amino acid residues in the C2B domain interact with acidic phosphatidylinositol bisphosphate (PIP_2) in the lipid bilayer. Therefore, the rise in intracellular calcium triggered by various cellular signaling pathways may trigger *trans*-SNARE complex or fusion pore formation (Chapman, 2008).

Munc18-1 is a protein in the Munc18/Sec1 family and is responsible for directing vesicles to appropriate target areas on the cell surface for secretion. Predominantly in neurons, Munc18-1 aids half-zippered SNARE bundles into full-zippered complexes (Shen et al., 2015). In chromaffin cells, Munc18-1 has a high binding for Syntaxin-1 (Arunachalam et al., 2008). Munc18-1 chaperones Syntaxin-1 delivery to the plasma membrane, which is consistent with the findings that deletion of Munc18-1 greatly reduces Syntaxin-1 presence in the plasma membrane

(Verhage et al., 2000). Munc18-1 also stabilizes Syntaxin-1 to the plasma membrane. It holds the SNARE protein in a closed conformation where the Syntaxin Habc domain interacts with the SNARE domain (Dulubova et al., 1999). Though Munc18-1 is primarily expressed in neural tissue, functions of Munc18-1 are important for LDCV fusion in other tissue types. In PC-12 cells, the depletion of Munc18-1 reduces LDCV docking and abolishes cell secretion completely (Voets et al., 2001). This evidence suggests that without Munc18-1, Syntaxin-1 is absent from the fusion sites or is held in an open conformation, decreasing vesicle fusion. However, in cultured kidney cells, absence of Munc18-1 halts secretion, but docked LDCV counts remained unchanged (Verhage et. al, 2005). This evidence indicates Munc18-1 may play a larger role in stabilization of Syntaxin-1, rather than SNARE complex assembly, and may help coordinate the timing of SNARE zippering to promote full zippering of the SNARE complex (Risselada and Mayer, 2020).

Munc18-1 contains a Munc homology domain (MHD), the region of the protein that interacts with Syntaxin-1. Many other proteins contain MHDs. For example, Munc-13 contains two MHDs near the C-terminus and alters the anchored, closed syntaxin into the open conformation in the brain. The MHD closest to the N-terminus contains the syntaxin interacting domain, though both MHDs are required to open syntaxin, freeing the SNARE protein for SNARE complex formation and LDCV docking (Wang et al., 2017).

Calcium-dependent activator protein for secretion 1 (Caps1), another important protein involved in exocytosis, also contains an MHD, which is required for LDCV fusion (W. Li et al., 2011). Caps1 MHD domain, in conjunction with DUF1041 (domain of unknown function 1041), make up the DAMH (DUF1041 and MHD1) domain (Figure 2) (Khodthong, 2011) which interacts directly with syntaxin. DAMH spans from Caps1 amino acid residues 859 to 1036 and

is comprised of four helical segments that run parallel and anti-parallel, connected by short, flexible loops. Hydrophobic amino acids are retained on the inside of the domain while charged residues face outward, promoting its ability for protein-interactions. (Zhou et al, 2019). Caps1 binding to syntaxin is accomplished by DAMH charged residues in outer helical regions interacting directly with the syntaxin linker region. Evidence suggests binding of DAMH to syntaxin is accomplished prior to vesicle docking (Khodthong, Kabachinski, James, and Martin, 2011). This could mean that Caps1 DAMH domain opens and stabilizes syntaxin prior to *trans*-SNARE formation (Zhou, 2019). This function is similar to Munc-13, the protein that binds to syntaxin and transitions it from the closed to open state (Betz, Okamoto, Benseler, and Brose, 1997). However, Caps1 and Munc-13 have distinct roles in LDCV fusion. LDCV fusion in CAPS1 knockout neurons and chromaffin cells could not be rescued by Munc-13 overexpression (Liu et al., 2010).

Caps1 also interacts with exocytosis machinery and vesicles by other functional domains that are required for exocytosis (van Keimpema, Kooistra, Toonen, and Verhage, 2017): the coiled-coil domain (C2), LDCV binding domain (LDCVBD), and pleckstrin homology domain (PHD) (Figure 2). The LDCVBD interacts directly with the vesicle (Figure 3). The PH domain interacts with PIP_2 in the plasma membrane and the C2 with phospholipids also in the target membrane. The culmination of these interactions in conjunction with the DAMH domain suggest that Caps1 tethers the vesicle at the plasma membrane and perhaps facilitates *trans*-SNARE complex formation during fusion (reviewed in James and Martin, 2013).

The Caps1 C2 domain is comprised of residues 397-516 and forms a coiled-coil domain. Coiled coils are the second most common domain, often present in proteins that self-associate or heterodimerize with other proteins. The domain can have anywhere from two to five alpha

helices, containing 7-11 amino acid repeats, which allow the helices to wind around one another to form a supercoil (Lupas and Gruber, 2005). Typically, C2 domains are calcium-dependent, however, the C2 domain in Caps1 does not appear to recognize calcium directly (Martin, 2014). Instead, Caps1 C2 domain plays a role in homodimerization. In the cytoplasm and *in vitro*, Caps1 appears to exist as a monomer. However, when at the plasma membrane and on vesicles, Caps1 dimerizes via the C2 domain. When this C2 domain is deleted, Caps1 can no longer facilitate LDCV fusion, indicating that dimerization is an important and required component for vesicle docking and/or priming (Petrie et al., 2016).

The PH domain of Caps1 spans amino acid residues 523-624 and makes two perpendicular anti-parallel beta sheets, connected by loops that can vary in length. The cytosolic facing amino acids in the Caps1 PH domain are basic, and therefore are electrostatically attracted to acidic elements. Though this domain can interact with acidic phospholipids, it appears to only bind to the plasma membrane phospholipids and not vesicular phospholipids. The PH domain in Caps1 is attracted to the PIP_2 in the plasma membrane (Grishanin et al., 2002), which is modeled to allow Caps1 to bring the LDCV close to the membrane for docking.

Caps1 bridges the plasma membrane and LDCV by interacting with PIP_2 in the membrane via PHD and with the outer surface of the vesicle membrane via the C-terminal LDCVBD (Figure 4). This interaction is important in bringing the vesicle close to the membrane for docking, but also plays a role in clustering dense core vesicles to the interior of the cells. Caps1 mutants lacking a functional C2 domain led to more dispersed LDCVs. However, Caps1 with functional C2 domains capable of dimerizing leads to dense core vesicles that are more tightly clustered together (Petrie et al., 2016).

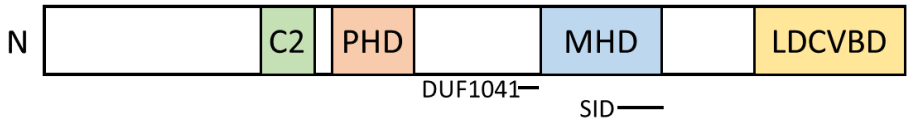

Figure 2. Secondary structure of calcium-dependent activator for secretion 1 and major functional domains. Caps1 secondary structure in reference to N-terminus (N) and contains calcium binding domain (C2, green), Plekstrin homology domain (PH, orange), Munc homology domain (MHD, blue) and LDCV binding domain (LDCVBD, yellow). The locations of the domain of unknown function (DUF1041) and syntaxin interacting domain (SID) are indicated underneath the Munc homology domain.

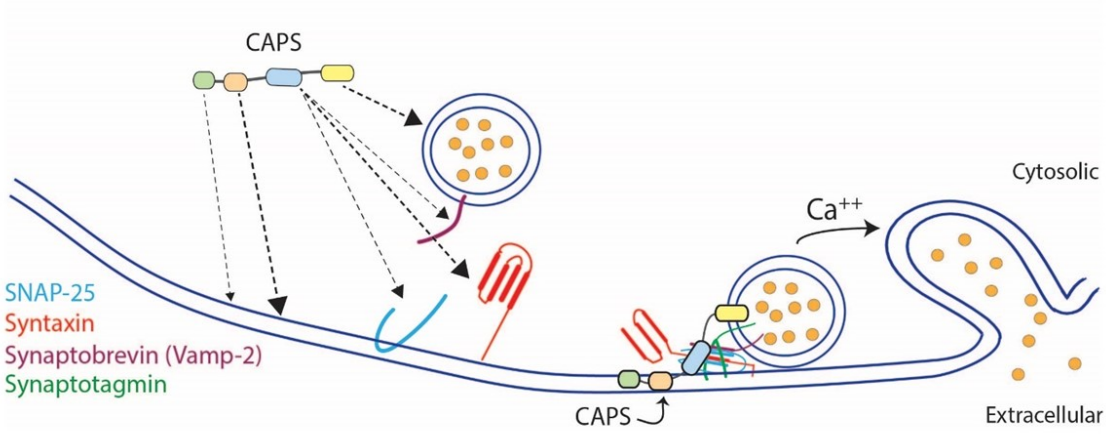

Figure 3. Caps1 interactions with exocytosis machinery and plasma membrane. Caps1 major functional domains and their interactions are illustrated. Strength of interactions are depicted by arrow weight. The C2 domain (green) has a weak interaction with the plasma membrane. Plekstrin homology domain (orange) strongly interacts with acidic proteins in the membrane, specificially PIP_2, further stabilizing Caps1 at the membrane. Munc homology domain (blue) interacts with synaptobrevin, SNAP-25, and strongly associates with syntaxin (open conformation). The LDCV binding domain (yellow) strongly interacts with vesicle membranes. Together, these interactions suggest that Caps1 helps tether the vesicle to the plasma membrane during docking and/or priming, which helps to facilitate calcium mediate fusion of the vesicle and release of vesicle contents (gold) into the extracellular space.

Regulated Secretion

The fusion machinery and docking and priming proteins, such as Caps1, enable vesicle fusion resulting from external stimulation. Outside stimuli (e.g. action potentials, ligand binding, etc.) generate signals that lead to an increase in intracellular calcium, which triggers vesicular fusion and release of cargo. The vesicle approaches the plasma membrane and docks independent of the calcium level. When cytosolic calcium levels are low, Munc18-1 is bound to syntaxin, preventing the transition to the open state and preventing the vesicle from fusing completely with the membrane. When calcium binds to synaptotagmin, synaptotagmin alters conformation, displacing Munc18-1, and inducing complete synaptotagmin binding with the SNARE complex, fully zippering the two membranes together and allowing the membranes to fuse (Tang et al., 2006). This calcium-mediated exocytosis is often referred to as stimulated or evoked secretion.

Even in the absence of outside stimuli to trigger secretion, vesicles may still fuse with the membrane, an event often described as "spontaneous release" (Kavalali, 2015). Spontaneous release of SVs may still be dependent upon calcium, as calcium binding affinity of synaptotagmin can vary to trigger calcium-dependent exocytotic events even with low levels of intra-cellular calcium (Xu, Pang, Shin, and Südhof, 2009). These events are asynchronous, unlike evoked release that occurs in a manner timed with an action potential or extracellular trigger. There may be a specific subpopulation of vesicles capable of spontaneous fusion marked by proteins similar to synaptotagmin, but have high-affinity calcium binding properties (Chung and Raingo, 2013).

LDCVs releasing neuropeptides from neuroendocrine cells have been reported to undergo slow spontaneous release. Vesicle fusion resembled "kiss-and-run", where cargo was released

slowly during spontaneous fusion due to narrow fusion pore formation and slow peptide diffusion rates. Upon stimulation, the same vesicles that were undergoing slow spontaneous fusion can be triggered to fuse completely after vesicle pores continued to open and narrow repeatedly before finally fusing completely (Vardjan, Stenovec, Jorgačevski, Kreft, and Zorec, 2007).

Vesicles are arranged into three functional pools; the readily releasable pool, recycling pool, and the reserve pool (Rizzoli and Betz, 2005). In mouse islet cells, approximately 1-5% of all vesicles are held in the readily releasable pool (RRP), the pool of vesicles docked and primed at the cell membrane, awaiting an outside stimulus that will trigger rapid release (Barg, Eliasson, Renström, and Rorsman, 2002) (Figure 4). Most of the remaining LDCVs belong to the reserve pool, which have yet to undergo docking and priming. In pancreatic beta cells, the RRP is 0.2-1% of total insulin granules (P. Rorsman and Renstrom, 2003). In neurons, approximately 10-20% of synaptic vesicles make up the recycling pool (RP), where vesicles are mobile and scattered in the synaptic terminals and participate in fusion and vesicle recycling with moderate stimulation. The remaining vesicles are fixed, or tethered, in a reserve pool away from the periphery of the cell and require strong stimulation to migrate to the cell surface and release (Rizzoli and Betz, 2005).

Vesicles are also organized temporally, and their release helps define phases of fast, slow, and sustained release (Braun, Ramracheya, Johnson, and Rorsman, 2009). During fast release, RRP vesicles fuse with the plasma membrane within milliseconds after an increase in cytosolic calcium (reviewed in Komatsu, Takei, Ishii, and Sato, 2013a). These vesicles are docked and primed at the plasma membrane surface at the time of stimulation, contributing to their timely release. A slower sustained release occurs when groups of unprimed vesicles that are released

slowly and consistently throughout several seconds of continued stimulation (reviewed in Stevens et al., 2011), likely because the RRP has been depleted. The vesicles released in this sustained phase are thought to be recruited from the reserve and recycling pools and require time to complete docking and become fusion competent (Rorsman and Renstrom, 2003).

While vesicle distance from the plasma membrane is often used to diagnose docking, this does not always correlate to release time. Only a small subset of vesicles at the membrane release immediately upon stimulation. Some estimates show that LDCVs release about 30% of morphologically docked insulin granules rapidly upon stimulation (P. Rorsman and Renstrom, 2003). This initial phase of release are vesicles that are primed, while the later phases correlated with unprimed insulin granules (Ohara-Imaizumi et al., 2007).

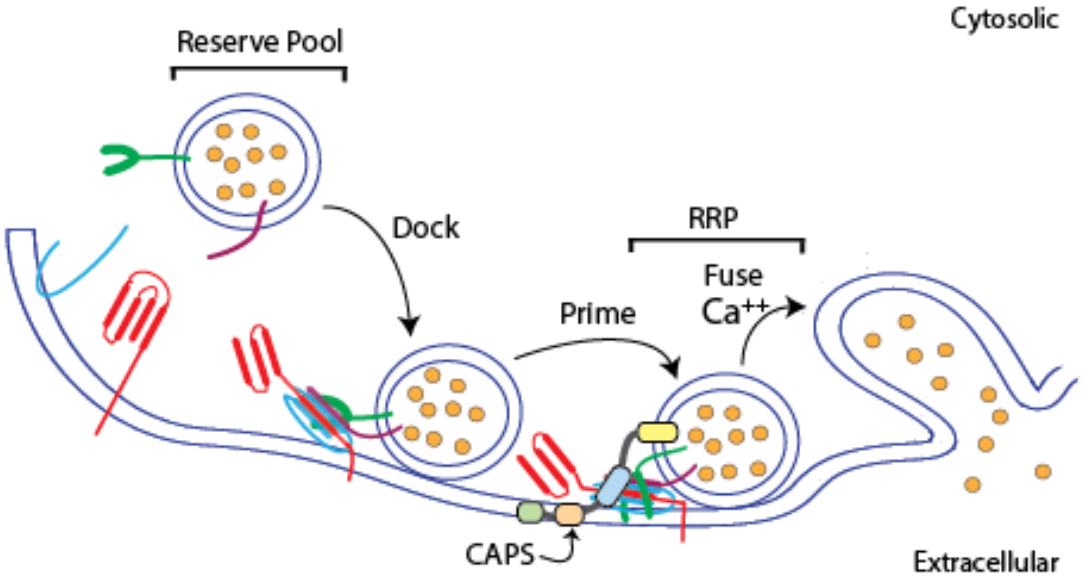

Figure 4. Conversion of reserve pool vesicles into docked, primed, and readily releasable vesicles. When a large stimulus occurs, reverse pools migrate to the plasma membrane to replace the readily releasable pool. Synaptotagmin (green) and synaptobrevin (purple) interact with syntaxin (red) and SNAP-25 (blue) during docking. Exocytosis machinery and additional proteins such as Caps1 interact, the SNARE complex fully zippers, and the vesicle is immobile at the membrane (primed). These primed vesicles are a part of the readily releasable pool (RRP). Upon a stimulus that raises the intracellular calcium level, the docked and primed vesicles of the RRP rapidly fuse and release cargo into the extracellular space.

Glucose-stimulated exocytosis. Pancreatic beta cells undergo regulated secretion in response to increased blood glucose levels. This release of insulin stimulates glucose uptake from the blood into the cell to use as energy. These pancreatic beta cells are one of three types of cells located in pancreatic islets and make up approximately 75% of islets. Alpha cells make up 20% of islets and secrete glucagon to release stored glucose when circulating glucose levels are low (reviewed in Chen, Cohrs, Stertmann, Bozsak, and Speier, 2017). The other 5% of pancreatic islet cells are delta cells, which counter the activity of neighboring alpha and beta cells. Delta cells secrete somatostatin to inhibit both glucagon and insulin secretion when glucose levels approach homeostasis (Patrik Rorsman and Huising, 2018).

Glucose stimulate insulin secretion (GSIS) from pancreatic beta cells is a highly regulated process is a prominent example of hormone release from LDCVs. Insulin release is biphasic, where the first phase of secretion releases vesicles held in the RRP while the release of vesicles from the RP pertain to the sustained second phase of release. Biphasic release occurs via two distinct pathways: the K_{ATP} channel-dependent and K_{ATP} channel-independent pathways (Komatsu, Takei, Ishii, and Sato, 2013). In the former, glucose traverses the beta cell plasma membrane via GLUT2 receptors in rodents and GLUT1 and 2 in humans and is processed through glycolysis and the citric acid cycle (TCA). By-products adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) are key metabolic coupling factors in the GSIS K_{ATP} channel-dependent signaling pathway. Through glycolysis, NADP+ accepts an electron, becomes NADH, and shuttles the electron into the mitochondrial electron transport chain. This creates a hydrogen gradient used to power ATP synthetase, which converts adenosine diphosphate (ADP) into ATP, increasing the ratio between cellular ATP and ADP (reviewed in Rorsman and Renstrom, 2003). Increasing ATP closes K_{ATP} channels and consequentially

depolarizes the cell (Cook and Hales, 1984). Depolarization opens calcium ion channels proximal to sites of exocytosis, which allows calcium binding to SNARE calcium sensor machinery, and triggers the fusion of insulin granules in the RRP (Wollheim and Sharp, 1981; Satin, 2000).

The K_{ATP} channel-independent pathway of insulin secretion is less understood, and is responsible for the second phase of insulin release (Taguchi, 1995). Though there are many hypotheses that aim to explain the second phase of release, it is heavily supported that replenishing TCA intermediates drives the second phase of insulin release forward. One specific intermediate, malonyl-CoA, has a directly proportional relationship with insulin secretion, such that an increase in malonyl-CoA increases insulin release under GSIS. Malonyl-CoA functions by preventing fatty acid (FA) binding to carnitine palmitoyl-transferase and inhibiting transport into the mitochondria where FA oxidation occurs. This oxidation occurs via LC-CoA, and without FA in the mitochondrion, LC-CoA accumulates in the cytosol where it can be esterified into diacylglycerol (DAG) and PIP_2 (reviewed in Komatsu et al., 2013). DAG is also produced following glucose-induced calcium influx. Calcium activates phospholipase C, which hydrolyzes PIP_2 into inositol triphosphate and DAG. DAG is also involved in the docking and priming process. DAG binds to Munc-13, inducing a conformational shift, and tightens the SNARE complex. This induces vesicular and plasma membrane fusion (Xu et al., 2017).

DAG also plays indirect roles in regulating exocytosis via PKC activation. DAG moves through the plasma membrane and activates PKC. For example, in chromaffin cells, SNAP-25 phosphorylation by PKC causes consequential rapid exocytosis and vesicle recruitment to the membrane (Nagy et al., 2002). Caps1 is phosphorylated by PKC in neural cells (Nishizakis, Walent, Kowalchyk, and Martins, 1992) though this process does not seem to have clear effects

on Caps1 activity (Nojiri et al., 2009). Though PKC regulates proteins, it may play a bigger role in vesicle rearrangement and migration by interacting directly with the cellular cytoskeleton.

PKC, the Cytoskeleton, and Vesicle Mobility. The cytoskeleton plays an important role in vesicular mobility, both restraining vesicles and guiding them to their targeted sites for exocytosis. Electron micrographs of chromaffin cells show a large web of actin filaments interacting with outer membrane of vesicles (Nakata and Hirokawa, 1992). Filaments are arranged asymmetrically within the cell where inner regions of the cytoplasm lack filamentous bundles, while regions closer to the plasma membrane contain filamentous bundles between LDCVs. These bundles may explain the compartmentalization of vesicles to specific pools in the cell and offer a method for controlling release of secretagogues (Giner et al., 2011).

The cytoskeleton that divides vesicles into pools is not static as previously thought (Aunis and Bader, 1988), and is PKC-induced in many cells including pancreatic beta, chromaffin, and neural cells (Y. S. Park et al., 2006a; Vaughan, Walker, and Peers, 1998). Increased PKC activation is directly proportional to both first and second phases of insulin release (Vitale, Seward, and Trifaro, 1995). It is interpreted that sustained release of insulin occurs through the recruitment of vesicles from the reserve pool to the RRP by the PKC-directed release of myristoylated alanine-rich C-kinase substrate (MARCKS) from the plasma membrane. The translocation of PKC to the membrane releases MARCKS which correlates with filamentous actin disassembly (Y. S. Park et al., 2006b) and rearrangement (D. Giner et al., 2011). Actin disassembly allows vesicles in the RP to replenish the ones released from the RRP following the same calcium influx (Rodriguez Del Castillo et al., 1990; Giner et al., 2011; Parsons, Coorssen, Horstmann, and Almers, 1995).

Glucose-stimulated secretion and syntaxins. SNAREs are likely the key to the first, rapid phase of insulin release during GSIS. Pancreatic beta-cells rely predominantly on Syntaxin-1A and Syntaxin-3 for insulin release. In mice lacking Syntaxin-1A, pancreatic beta cells did not undergo first phase insulin release. However, the second sustained phase was preserved (Henquin, 2000). This suggests the SNARE activity and machinery are different for each insulin release phase. SNAP-25 and Syntaxin-1A expression is decreased in islets of type II diabetic patients (Ostenson et al., 2006). Furthermore, monitoring fluorescently tagged Syntaxin-1A and insulin in pancreatic cells during glucose stimulation assays shows that LDCVs and Syntaxin-1A interact during the first phase of release, but do not during the second release phase (Ohara-Imaizumi et al., 2007). Conversely, Syntaxin-3 is most frequently localized to vesicles associated with the second phase of insulin release. Syntaxin-3 ablation results in the disruption of the second phase. This suggests that different syntaxin isoforms are involved with different modes of release, where Syntaxin-1 frequently regulates release from the RRP and Syntaxin-3 regulates vesicle release from the RP (Zhu et al., 2013).

CAPS1 and RNA Editing

As discussed earlier, Caps1 is a key factor in regulated secretion via its major functional domains: C2, PH, DAMH, and LDCVBD. Caps1 participates in docking and/or priming by bridging the plasma membrane and vesicle, promoting fusion (Figures 3 and 4). Caps1 is a key component of secretion and its disruption has severe consequences. Caps1 deficiency results in decreased SV release from mouse neurons (Sadakata et al., 2013). More extremely, Caps1 is essential to life, as ablation in mice is embryonically lethal (Wang et al., 2000). Caps1 is essential due to its role in secretion, but it also plays a role in disease progression. Decreased

Caps1 expression in liver tumor cells results in tumor progression and poor patient prognosis while overexpression in liver tumor cells increases activation of cellular apoptotic pathways which decreases tumorigenesis (Xue et al., 2016). Similarly, overexpressing Caps1 negatively regulates tumorigenesis in cholangiocarcinomas (Weng et al., 2019). Thus, Caps1 may act as a tumor suppressor in certain cell lines, though the mechanisms behind this involvement are unclear.

The transcript encoding Caps1 is modified post-transcriptionally through a process termed Adenosine-to-Inosine (A-to-I) RNA editing (Li et al., 2009). A-to-I editing alters a genetically encoded glutamic acid (GAG) into a glycine (GGG) at amino acid 1250 in humans and 1252 in mice (Miyake et al., 2016).

RNA editing. RNA editing is a post-transcriptional modification where nucleotides within a transcript are inserted, deleted, modified, or substituted. Post-transcriptional base substitutions occur when an individual nucleotide is altered to generate a different nucleotide. Two specific types of base substitutions due to RNA editing are cytidine-to-uracil (C-to-U) and A-to-I editing. A-to-I editing modifies adenosine (A) into inosine (I) by deamination, which replaces the amine group on the adenosine base with a double-bonded oxygen. Inosine and guanosine are structurally similar, so if editing occurs within a coding region, ribosomal machinery recognizes I as G. If the editing event occurs with a codon, it has the potential to alter the amino acid sequence of the encoded protein. For example, modification of a genomically encoded GAG to GIG causes preferential pairing with tRNA anticodon CCC during translation (Figure 5), which results in the addition of glycine, instead of glutamate, within the LDCVBD of the growing Caps1 polypeptide (Li et al., 2009).

RNA-editing can provide a means for adaptability and diversity (Maas, Melcher, and Seeburg, 1997; Rueter and Emeson, 1998). Diversity not only pertains to the variability between organisms, but also the variability within a single organism. RNA-editing is rarely an all-or-nothing event, allowing multiple isoforms of a non-coding RNA or protein to exist simultaneously. A-to-I RNA editing within coding regions is relatively rare (Xu and Zhang, 2014), and only 3% of human transcripts contain recoding RNA editing sites where editing will alter the coding potential of the mRNA (Liscovitch-Brauer et al., 2017). These recoding events are highly conserved in mammals and each event seems to result is significant alteration of protein function (Pinto, Cohen, and Levanon, 2014).

The recoding of a protein by RNA editing can result in dramatic physiological consequences. For instance, in apolipoprotein B mRNA, a CAA codon that is deaminated to the UAA stop codon. This reaction results in truncated ApoB48 protein variant, while the unedited mRNA is translated into the full ApoB100 (Teng, Verp, Salomon, and Davidson, 1990). The truncated protein operates quite differently than the full ApoB100. The unedited ApoB transcript results in the major protein constituent of very low-density lipoproteins in the liver and aids in cholesterol transport, while ApoB48 is produced in the small intestine, aiding in fat absorption and secretion of chylomicrons (Teng et al., 1990). These distinct proteins arise from the same gene, further supporting the concept that not all proteins are encoded directly by the genome.

Another example how RNA editing can have physiological consequences lies with the GluA2 subunit within AMPA receptors. RNA editing occurs within the transcript, altering genomically encoded glutamine to arginine within the narrowest part of the ion channel (reviewed in Rosenthal, 2015). Glutamine at this point in the channel causes the receptor to be impermeable to calcium, which is detrimental to neural activity. However, the alteration to

arginine allows calcium permeability. This editing event from glutamine to arginine occurs in near 100% of the transcripts and is the most prevalent RNA editing event known. Furthermore, reduced RNA editing of GluA2 is lethal to neurons and potentially to the organism (reviewed in Rosenthal, 2015).

A majority of RNA editing occurs in noncoding regions of pre-mRNAs, such as 5' and 3' untranslated regions (UTRs) and introns, or within non-coding RNAs (Peng et al., 2012). Approximately 90% of the known editing sites within noncoding regions occur in Alu repeats. Alu repeats are SINEs (short interspersed elements) that are frequently located near or within gene-rich regions and can affect gene regulation (Krebs et al., 2018). If Alu repeats are located within transcribed regions, and occur back-to-back and inversely, the resulting RNA can form a hairpin or duplex. Since the enzymes that catalyze RNA editing target regions of double stranded RNA, the inverted Alu repeats are a target for A-to-I editing. Multiple A-to-I modifications can occur in this transcript, even though this region is not designed to be specifically targeted for A-to-I editing. This non-specific editing of long regions of dsRNA is known as hyper-editing. Hyper-editing occurs within *CAT2* 3'UTR due to an Alu repeats within the last exon. Inverted Alu repeats within the 3'UTR form a duplex and is hyper-edited, where up to 50% of A nucleotides are altered (Prasanth et al., 2005). *CAT2* is only one of the millions of examples how Alu repeats influence RNA editing. In fact, Alu repeats are widespread in the in the human transcriptome and are responsible for the majority of RNA editing within the human transcriptome. This hyper-editing differs from regulated site-selective editing, but both require a Adenosine Deaminases Acting on RNA (ADAR) for catalysis (reviewed in Rosenthal, 2015).

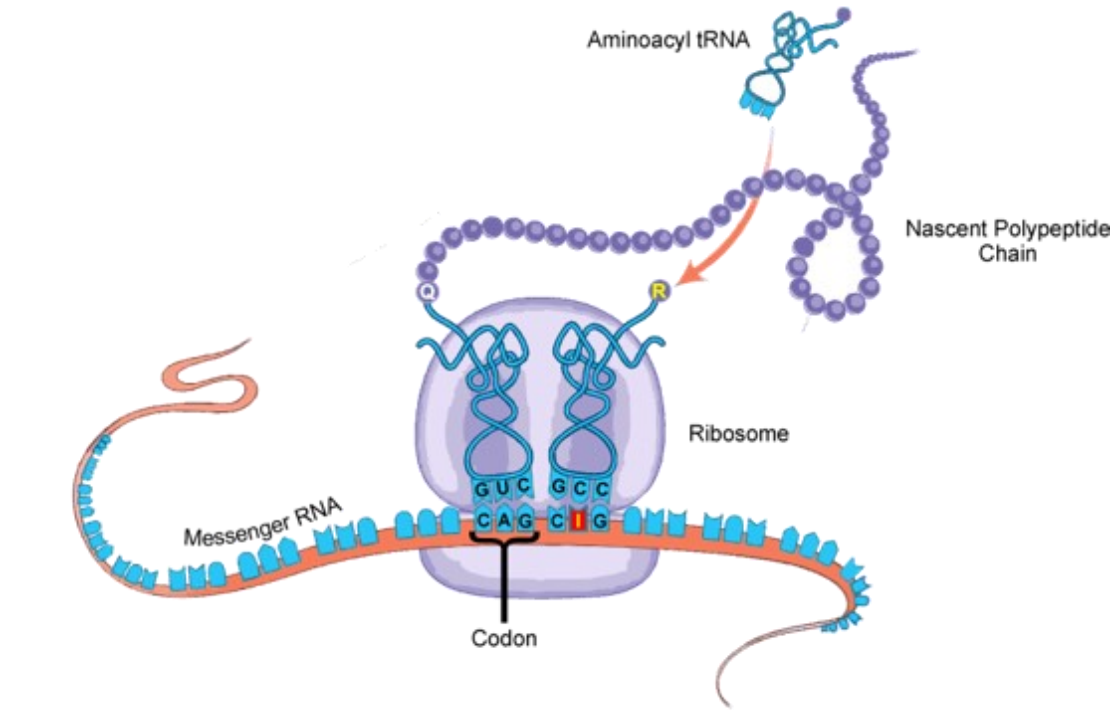

Figure 5. A-to-I RNA editing during translation. Following A-to-I editing events where adenosine is modified into inosine, ribosomes fail to recognize structural differences between guanosine and inosine. Consequentially, ribosomes pair inosine with a cytosine nucleotide during translation. While the genomically encoded CAG codon normally codes for a Glutamic acid (Q), the edited codon (CIG) would encode an arginine (R) in the nascent polypeptide chain.

Adenosine deaminases acting on RNA. ADARs are the enzymes that catalyze A-to-I RNA editing. ADAR proteins are not sequence specific, but instead are structure specific, recognizing double stranded RNA (dsRNA) through their dsRNA-binding domains (dsRBD). When ADARs hyper-edit, their dsRNA substrates are typically long without bulges or loops, whereas selective editing events typically occur within imperfect dsRNA substrates, containing loops and bulges (Lehmann and Bass, 1999). The differences in ADAR editing activity between

these two types of substrates could provide insight into how substrate structure regulates ADAR binding and editing.

Three different ADAR genes exist in vertebrates. ADAR1, ADAR2 and ADAR3 are similar in sequence and have dsRNA binding activity (Chen et al., 2000). Only ADAR1 and ADAR2 have catalytic capabilities and homodimerize (Gerber and Keller, 1999; Kim, Wang, Sanford, Zeng, and Nishikura, 1994; C. X. Chen et al., 2000; Cho et al., 2003). Though ADAR1 and ADAR2 have similar structures and capabilities, the roles are not redundant as ADAR1 does not rescue ADAR2 editing deficiency (Chalk, Taylor, Heraud-Farlow, and Walkley, 2019). ADAR1 and ADAR2 edit distinct targets, but also coordinately regulate a subset of RNA editing sites (Costa Cruz et al., 2020).

ADAR1 is derived from the *ADAR* gene in chromosome 1, spanning over 46,000 bp and encoding 17 different exons. Within the *ADAR* gene are four different promoter regions. Three of these regions are constitutively active while one promoter region is interferon inducible. The constitutive promoter will produce a 110 kilodalton protein (ADAR1p110) and interferon will induce production of a longer 150kD version (ADAR1p150) (reviewed in Nishikura, 2010).

ADAR1p150, ADAR1p110 and ADAR2 all contain a catalytic deaminase domain, double stranded RNA binding domains, and a nuclear localization signal (NLS). The NLS allows ADAR to reside in the nucleus and edit transcripts co-transcriptionally and/or immediately after transcription (Stefan Maas and Gommans, 2009; Rodriguez, Menet, and Rosbash, 2012). ADAR1p150 also contains a nuclear export signal that allows it to shuttle out of the nucleus to potentially edit transcripts in the cytoplasm. ADAR1p150 contains two Z DNA binding domains at its N-terminus, while p110 contains only one (dsRBMs; Figure 6).

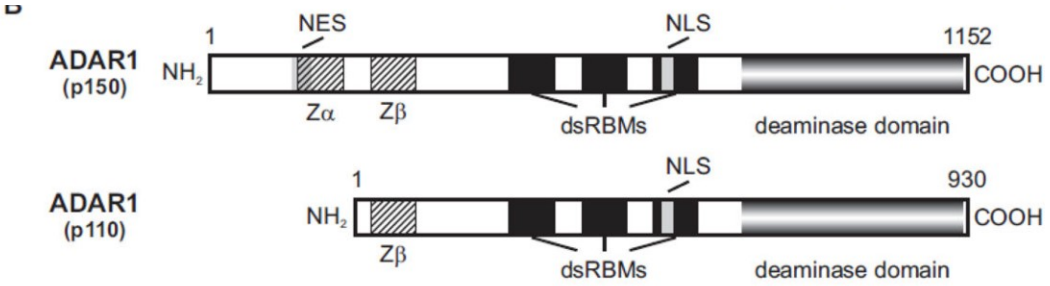

Figure 6. ADAR1 isoforms. ADAR1p150 contains and extended N-terminus with one more Z DNA binding domain (Zα) and a Nuclear Export Signal (NES), compared to ADAR1p110. Both contain dsRNA binding domains (dsRBMs), nuclear localization signal (NLS) and a deaminase domain (adapted from Hood et al., 2014).

Physiological consequences of ADAR and RNA editing. ADAR proteins are required for survival. Mice without ADAR2 experience seizures and die shortly after birth. This is primarily due to GluA2 editing, which is required to be maintained at near 100% for maintenance of calcium permeability. Mice that contain a synthetically edited GluA2 gene (genomic A to G substitution) but lack ADAR1, survive (Higuchi et al., 2000). Lack of ADAR1 leads to embryonic death due to widespread apoptosis (Herbert, Wagner, and Nickerson, 2002). This is likely because ADAR1 acts as an important interferon suppressor and protects organisms from inflammation, cancer, and autoimmune disorders (reviewed in Gallo and Locatelli, 2012).

Evidence indicates ADAR RNA editing capabilities have a direct effect on cellular function. Deficits in ADAR RNA editing but not in dsRNA binding function lead to locomotion defects in *Drosophila* (Deng et al., 2020). Additionally, ADAR1 has been gaining attention for its involvement in innate immune functions. Gene expression assays in *Drosophila* with catalytically inactive ADAR demonstrate increased expression of proteins involved in innate immunity (Deng et al., 2020). ADAR2 editing capabilities play a pivotal role in regulated secretion. Disabling ADAR2 editing capabilities impairs GSIS within rat INS-1 cells and secretion is rescued when wild-type ADAR2 is reintroduced. Deficiency in secretion could be

explained by decreased expression of Munc18-1 and synaptotagmin when ADAR2 is not present. This indicates ADAR2 RNA editing function plays an important role in GSIS (L. Yang et al., 2010).

RNA editing, *CAPS1* and secretion. The *CAPS1* transcript is edited with an average of about 20% frequency in mouse and human tissue. However, adrenal glands experience an increased editing rate, near 80%, while 40% of *CAPS1* transcripts are edited in pancreatic tissue (Miyake et al., 2016). The wide-spread and conserved RNA editing of *CAPS1* suggests that its editing is important to physiology of the organism.

RNA editing of *CAPS1* transcript has significant effects on regulated secretion. Mice expressing only the edited transcripts demonstrated increased LDCV exocytosis in mice. In addition, these mice are leaner compared to the control mice due to increased metabolic rates. The increased metabolism in these mice is thought to be due primarily to the increased secretion of dopamine from LDCV (Miyake et al., 2016). In cultured neurons, increasing the ratio between Caps1 edited and Caps1 non-edited isoforms decreased spontaneous release from SVs (Ulbricht et al., 2017). Additionally, edited Caps1 reorganized the neural RP. Edited Caps1 decreased RP volume and increased the sphericity of the RP, suggesting edited Caps1 tightly clustered vesicles.

Caps1 interacts with different isoforms of exocytosis machinery. For example, Caps1 associates with Syntaxin-1A linker region peptides but does not bind to Syntaxin-3 linker region peptides (Daily, Boswell, James, and Martin, 2010). Additionally, evidence suggests edited Caps1 strongly interacts with Syntaxin-1 linker region while non-edited Caps1 binds less effectively (Miyake et al., 2016). These interactions between edited Caps1 and Syntaxin-1A may explain how edited Caps1 increases SV secretion from neurons. However, this is only a piece of

the bigger picture. Further information is needed to understand Caps1 mechanics and how editing of the transcript affects LDCV secretion from endocrine cells.

Investigating the Role of CAPS1 RNA Editing on Secretion

Modification to RNA-editing of *CAPS1* affects secretion from LDCV and SVs. A minor decrease in editing frequency of *CAPS1* mRNA (5 %) increases spontaneous release of SVs (Ulbricht et al., 2017). Expression of only edited Caps1 in mice have increased secretion from LDCVs in isolated chromaffin cells (Miyake et al., 2016) It is not clear if *CAPS1* editing affects on LDCV release are universal to all cell types, or are unique to chromaffin types of cells. It is also unclear if *CAPS1* editing effects on spontaneous release occur outside of neurons or on LDCVs, as non-stimulated events are largely un-reported. Our goal is to determine the role of *CAPS1* mRNA editing on regulation of LDCV secretion in insulinoma cells. To address this goal, we will alter RNA-editing frequency of *CAPS1* in INS-1 cells. Previous mechanisms for modulating the presence of edited *CAPS1* include overexpression of edited *CAPS1* cDNA (Ulbricht et al., 2017) and altering the genomic A to a G within the *CAPS1* E/G site (Miyake et al, 2016). We will use several methods, including CRISPR/Cas13 technologies, in attempts to manipulate the editing level of *CAPS1* RNA.

We will measure secretion from INS-1 cells by quantifying secretagogues within cell culture media. Basal secretion of secretagogues during normal, non-stimulating conditions will provide insight on how *CAPS1* editing affects spontaneous LDCV fusion. I expect increasing editing frequency of *CAPS1* within cultured INS-1cells will decrease spontaneous release of LDCVs, detected by fewer secretagogues in the INS-1 media under basal (low glucose) conditions. We will use high glucose conditions to induce GSIS and test how variations in

CAPS1 editing levels affect efficiency of LDCV fusion under stimulating conditions. GSIS is expected to increase in the presence of edited Caps1 if calcium-dependent secretion is modulated in INS-1 cells similarly to PC-12 and chromaffin cells.

Manipulating *CAPS1* editing. The INS-1 cell line is a pancreatic beta insulinoma derivative from *Rattus norvegicus*. The INS-1 cells express Caps1, which is likely important for robust GSIS from these cells. The *CAPS1* RNA in these cells is almost entirely nonedited. We will use three potential methods to increase editing of *CAPS1*. In the first, we will overexpress ADAR1. It is possible that increasing the enzyme responsible for RNA editing within *CAPS1* mRNA will increase editing frequency. However, ADAR1 and 2 expression levels do not directly correlate with the extent of editing frequency in mouse neurons (Jacobs, Fogg, Emeson, and Stanwood, 2009), therefore, it is possible that increasing ADAR expression in INS-1cells may not increase editing within *CAPS1* mRNA. Second, we will attempt to increase editing of *CAPS1* using a relatively new tool, RNA Editing for Programmable A to I Replacement, or REPAIR. REPAIR uses a catalytically inactive Cas13 fused with a hyperactive ADAR deaminase domain. A small RNA complementary to the *CAPS1* E/G editing site will guide the Cas13-ADAR fusion to the RNA, where the Cas13-ADAR will edit the RNA (Qu et al., 2019). The chances of this method being successful are limited by the targeting efficiency of the guide RNA (gRNA). Therefore, if the REPAIR is unsuccessful, we will attempt to knockdown the endogenous *CAPS1* RNA using wild-type Cas13b, and replace expression of *CAPS1* from plasmids expressing either edited (G) or nonedited (E) *CAPS1*. Finally, if our attempts to knockdown *CAPS1* are unsuccessful, we will increase the proportion of edited to non-edited Caps1 variants in INS-1 cultures by expressing edited *CAPS1* cDNA from a plasmid. While this final method has its limitations and may limit the resolution, it may be considered more similar

to disease or physiological conditions where we expect editing to more subtly change, rather than to entirely disrupt endogenous editing ratios.

Measuring LDCV secretion from INS-1 cells. After altering the levels of *CAPS1* editing within INS-1 cells, we will utilize this model to study secretion. We will administer cDNA encoding fluorescently tagged secretagogues and measure fluorescence in cell media during both non-stimulated and stimulated conditions. Secretagogues in the media represent LDCV exocytosis, where greater fluorescence correlates with more LDCV fusion events. Secretagogue fluorescence will first be measured following incubation under low glucose, non-stimulating conditions. Afterward, cells will be incubated in high glucose cell culture media to induce GSIS and secretagogue fluorescence will be measured again. I expect cells in which edited Caps1 is increased will show lower fluorescence in the media following basal conditions compared to cells with only non-edited Caps1. Less fluorescence would indicate fewer LDCV fusion events during basal conditions, meaning the presence of edited *CAPS1* decreases spontaneous LDCV fusion and editing is more controlled. I expect INS-1 cells to secrete more of the fluorescent marker in the presence of high glucose compared to baseline if GSIS is working properly. Additionally, I expect cells greater levels of edited Caps1 to have higher levels of GSIS than those with only non-edited Caps1. Greater secretion of the fluorescent marker would indicate more LDCV fusion events during stimulation, an observation consistent with previous findings in chromaffin cells (Miyake et al., 2016).

Impact of study. Successful modulation of edited Caps1 in INS-1cells and measurement of LDCV exocytosis will provide insight on how Caps1 affects LDCV secretion. If our hypothesis is correct and increased edited Caps1 increases LDCV exocytosis following stimulation and decreases spontaneous LDCV release in non-stimulating conditions, it is possible

Caps1 could be a therapeutic target to rescue secretion of other secretagogues, such as insulin. Since INS-1 cells release insulin form LDCVs in response to high glucose levels, edited Caps1 may also improve stimulated release of insulin similarly to the fluorescent secretagogues used in this experiment. Improving the release of insulin from pancreatic β-cells may have therapeutic potential for type II diabetes, a disease that has diminished insulin secretion in response to high blood glucose levels.

This study will also provide insight into the therapeutic potential for manipulation of RNA editing *in vivo*. If slight modulations *in CAPS1* editing, rather than complete obliteration or replacement, are capable of altering secretion, then perhaps slight perturbations of editing are responsible for phenotypes associated with disease. Furthermore, methods that can alter editing of *CAPS1*, even slightly, could potentially improve these phenotypes. Though our results may give insight on edited Caps1 and secretion, our study is confined within the scope of cultured INS-1cells. While we expect these results to be applicable across systems, utilization of these methods with other cell lines would broaden the scope and provide further insight on Caps1 and its role in cell secretion.

Finally, it is interesting that the biochemical alteration of a single amino acid in a large protein (~150 KDa) would affect secretion efficiency, even when only a fraction of the proteins contain the alteration. While RNA editing substitutes a non-polar glycine for the genomically encoded glutamic acid (ionic), the biochemical characteristics that facilitate changes in Caps1 function are not yet known. This work will provide insight into the qualities and conditions that RNA editing and Caps1 isoforms play in important cellular process.

MATERIALS AND METHODS

Plasmid DNA Isolation and Purification

To meet our goal and understand how RNA editing frequency within *CAPS1* affects LDCV secretion from INS-1 cells, we first needed to modify RNA editing frequency. We attempted to modify frequency by REPAIR, that uses CRISPR RNA complementary to *CAPS1* RNA, and catalytically inactive Cas13 attached to the RNA editing ADAR domain (Cas13-ddADAR). The crRNA brings Cas13 close to *CAPS1* and the targeted adenosine nucleotide for RNA editing. Since ddADAR is close to the editing site, theoretically, it should modify adenosine to inosine. To accomplish REPAIR, CRISPR-Cas13-ddADAR (Addgene 103849; pRU 86), non-targeting crRNA (Addgene 103868; pRU 88), crRNA backbone (Addgene 103854; pRU 87) and CRISPR-Cas13 (Addgene 103862; pRU 91) plasmids were obtained from Addgene (Cox et al., 2017), and streaked onto Luria Broth agar plates containing 100 µg/mL ampicillin for selective pressure, and incubated at 37℃ overnight. Single colonies were isolated and inoculated in 10 mL of Luria Broth and 100 µg/mL ampicillin and incubated at 37℃ overnight in Benchmark Incu-shaker. These inoculations were then transferred the following day to 150 mL of Luria Broth and ampicillin. Plasmids were isolated via the Wizard Plus SV Midiprep kit and supplied protocol (Promega A1460) and stored at -20 °C. Cas13-ddADAR functions in conjunction with a gRNA that is unique and complementary to the target. Therefore, the gene encoding gRNA needed to be made and inserted into the crRNA backbone plasmid.

Cloning Guide RNA into crRNA Backbone

The crRNA plasmid first needed to be digested into a linear strand for gRNA gene insertion. The crRNA backbone was subject to a 10 µL restriction digest comprised of 2 µg of

purified crRNA backbone plasmid, 1 µL of FastDigest *Bpi*I (ThermoFisher Scientific FD1014), and NEB buffer 3.1. The reaction was incubated in a water bath for 10 minutes at 37 ℃. To prevent the linear crRNA backbone from circularizing, the vector was dephosphorylated with 1 µL of Antarctic phosphatase (NEB M0289S) and incubated at 37 ℃ for 30 minutes. DNA was purified using the Promega Wizard SV Gel and PCR Clean-Up System and supplied protocol (A9280) and stored at -20 °C.

The crRNA in the backbone plasmid lacks the targeting RNA complementary to the *CAPS1* transcript. Thus, oligonucleotides (Table 1) were used to insert a crRNA gene cassette into the vector. To prepare for cloning, the oligonucleotides were first phosphorylated at the 5' end in 40 µL reaction mixtures comprised of oligonucleotides (1 µg), 1 X T4 ligase buffer (Promega M1801), and T4 polynucleotide kinase (NEB M0201S). The reactions were incubated at 37 ℃ for 1 hr in a water bath and heat inactivated at 65 ℃ for 20 minutes in a water bath. Twenty microliters of each phosphorylated oligonucleotide were then combined, and the mixture was incubated at 95 ℃ for 5 minutes in PCR machine, then ramped to 24 ℃ at 1.5 ℃/minute that resulted in the double-stranded gRNA cassette.

The double-stranded DNA containing the crRNA gene, was ligated into the vector. A 15:1 (insert:vector) molar ratio was calculated utilizing the NEBioCalculator. Two hundred ng of dephosphorylated crRNA backbone, annealed oligos, 1 X ligation buffer (Promega M1804), and 1 µL of rapid DNA ligase (ThermoFisher Scientific K1422) were filled to 10 µL total volume. Reactions were incubated at room temperature for 15 minutes. The resulting plasmids were transformed into Competent DH5α cells for amplification.

Transformation. For transformation, 5 µL of ligation reaction was mixed with 50 µL of Competent DH5α cells (Thermofisher Scientfic 18258012), incubated on ice for 30 minutes, and

heat shocked at 42 ℃ for 35 seconds via bead warmer. Five-hundred µL of Luria Broth was added, and mixtures were incubated in incu-shaker at 37 ℃ at 200-220 rpm for 1 hr. Full mixtures were plated on Luria Broth and 1 µg/mL ampicillin plates and incubated overnight in VWR 1540 incubator at 37 ℃ overnight.

Plasmid screening. To be ensure our gRNA genes were correctly inserted into crRNA backbone plasmids, the colonies from the transformation were screened for the presence of the crRNA gene by colony polymerase chain reaction (PCR) (Green and Sambrook, 2019). PCR reactions were made in PCR tubes were filled to 20 µL and comprised of 1 X DreamTaq Green Mastermix (Thermofisher Scientific K1082), M13 reverse primer, and 100 pg/µL *CAPS1* pre-mRNA-targeting antisense (oRU 64) or *CAPS1* mRNA-targeting antisense (oRU 63). Individual colonies were randomly picked and simultaneously resuspended in double-distilled water within individually marked PCR tubes and streaked onto labeled locations on an LB with 1µL/mL ampicillin plate. The remaining reagents for PCR were added to the bacteria suspension, and the PCR reactions were cycled in the conditions listed in Table 2. Five microliters of each PCR reaction were subject to Bluegel electrophoresis (according to Minipcr). Colonies corresponding to 500 bp PCR product compared to 100 bp GeneWiz ladder were isolated from the streaked plates and the plasmid DNA isolated via Wizard midiprep kit. Plasmids were stored at -20 °C prior to transfection within INS-1 cells.

Thawing INS-1 Cells

To use REPAIR to modify RNA editing frequency within *CAPS1*, the INS-1 cell line was obtained from liquid nitrogen storage and thawed to 37 ℃ via water baths, pipetted into 15 mL

Table 1: Oligonucleotides Used in This Study

Name	oRU	Sequence
CAPS1	oRU 48	AATGATCACACTTTTGGTGGCAAAGTTTG
	oRU 49	CTGTCCTTCATGCTGATACCTTGTAAG
2c minigene	oRU 50	ATTAGAATTCTATTTGTGCCCCGTCTGG
	oRU 51	CAACCGATCAAACGCAATGTT
CAPS1 pre-mRNA sense	oRU 61	CAACGATGTCCTTCGTGATAAGGTCAATGAGGAGAT GTATATAGAAAGGTTATTTGATGTC
CAPS1 pre-mRNA antisense	oRU 64	CAACGATGTCCTTCGTGATAAGGTCAATGAGGGGAT GTATATAGAAAGGTTATTTGATGTC
CAPS1 mRNA sense	oRU 62	CAACGATGTCCTTCGTGATAAGGTCAATGGGAGATGT ATATAGAAAGGTTATTTGATGTCAATC
CAPS1 mRNA antisense	oRU 63	CAACGATGTCCTTCGTGATAAGGTCAATGGGAGATGT ATATAGAAAGGTTATTTGATGTCAATC
M13 Reverse	M13R	AGCGGATAACAATTTCACACAGG

Table 2: Thermocycler Conditions for Colony PCR

Step	Temperature, ℃	Time	# of Cycles
Initial denaturation	95	3 min	1
Denaturation	95	30 s	
Annealing	50	30 s	34
Extension	72	1 min	
Final Extension	72	10 min	1

conical tubes filled with 10 mL 37 ℃ media (ThermoFisher Scientific 118750854; media recipes outlined in Table 3), and transferred drop-wise via 25 mL pipette to 10 cm Falcon plates containing 5 mL of warm media. Plates were incubated at 37 ℃ with 5 % CO_2 for 3 hours, then aspirated and replaced with new media. Three mL of media was added to INS-1 cells every few days for 2 weeks until cells were adhered to the plate.

Table 3: INS-1 cell media

Ingredients mixed with RPMI 1640	Concentration
10% Heat inactivated Fetal Bovine Serum	
1% Penicillin/Streptomycin	
Na Pyruvate	1 mM
HEPES	10 mM
2-mercaptoethanol	50 μM

INS-1 Cell Transfection

When INS-1 cells become overcrowded, cell growth stunts. To aid cell division, cells require passaging into new plates with room to grow. INS-1 cell growth slows at 80-85 % confluence and therefore needs passage. Full media volume (~10 mL per 10 cm^2 dish) was transferred to a 15 mL conical tube and centrifuged (Sorvall Legend X1R) at 50 x g for 5 minutes. While centrifuging the media, cells were trypsinized and incubated in the CO_2 incubator at 37 ℃ for 10 minutes. The cells were disrupted by pipetting up and down and the suspension distributed equally to 6 wells in a 6-well plate to prepare for transfection. These wells received 1:1 ratio of old, centrifuged (conditioned) media to fresh media.

Cell transfections are most successful during active cell division. To make sure cells were in division and to give them time to recover from passaging, cells were incubated for ~24 hrs. Cell media was aspirated, replaced with warm 37 °C RPMI only, and placed in the CO_2 incubator while transfection mixtures were made. These transfection mixtures were made in microcentrifuge tubes by mixing 250 µL of RPMI only, 3 µg of DNA (or increasing amounts of 3, 5, and 7 µL for transfections in Table 4), and 17 µL of polyethylenimine (PEI) (Longo, Kavran, Kim, and Leahy, 2013) or 7.5 µL *Trans*IT-2020 (TI; Mirus MIR 5400). After incubation at room temperature for 30 minutes, the transfection mixture was added to the 6-well plates (according to Table 4-8 schemas) and incubated for 24-72 hours. Cells that were transfected with YFP and either PEI or TI were incubated for varying hours to determine optimal incubation time for peak YFP fluorescence. Statistical analyses of transfections were performed by *One-Way ANOVA* and multiple comparisons. Statistical error of the mean was calculated by calculating cohort standard deviation and dividing by square root of the sample size (n).

REPAIR transfection. To investigate whether CAPS1 editing frequencies could be manipulated by REPAIR, INS-1 cells were transfected with cDNA encoding for Cas13-ddADAR (pRU 86, Addgene 103849) and one of three possible gRNA-expressing vectors (Table 5): Targeting *CAPS1* pre-mRNA (pRU 89; cloned from Addgene 103854), targeting *CAPS1* mRNA (pRU 90; cloned from Addgene 103854), or a non-targeting (negative control) gRNA (pRU 88, Addgene 103868).

ADAR1p150 transfection. To investigate whether increasing amounts of ADAR1 cDNA endogenously altered RNA-editing of *CAPS1* transcript, cells were given varying amounts of plasmids containing ADAR1p150 (pRU 20; generously gifted from Ronald B. Emeson, Vanderbilt University, made by Jennifer Hood; scheme listed in Table 6). A plasmid expressing

the serotonin receptor type 2c ($5HT_{2C}$) minigene (pRU 58; generously gifted from Ronald B. Emeson) was used as a control.

Overexpression of CAPS1 isoforms. Alternative to increased expression of ADAR and REPAIR, cells were given cDNA (Table 7) encoding fluorescently-tagged proteins including phogrin-mCherry (pRU 51, generous gift form David Piston), AcGFP (pRU1, Clonetech), NPY-YFP (pRU 54, generous gift from Ronald Emeson), and/or CAPS1-GFP (pRU 48 and pRU 49). Plasmids encoding edited Caps1 (pRU 28) and non-edited Caps1 (pRU 29; Table 8) isoforms were transfected to manipulate editing ratios. Cells were incubated for 60 hrs post-transfection.

Table 4: PEI and TransIT-2020 Transfection

Wells	Transfection Contents
1 and 2	Non-transfected
3	PEI only
4	PEI and YFP
5	TI
6	TI and YFP

Table 5: CAPS1 REPAIR Transfection

Wells	Transfection Contents
1 and 4	Cas13-ddADAR and non-targeting gRNA
2 and 5	Cas13-ddADAR and gRNA 1
3 and 6	Cas13-ddADAR and gRNA 2

Table 6: ADAR1p150 Transfection

Wells	Transfection Contents
1 and 4	3 µg 2c minigene
2 and 5	5 µg ADAR1p150 and 3 µg 2c minigene
3 and 6	7 µg ADAR1p150 and 3 µg 2c minigene

Table 7: Fluorescently-tagged Secretagogue Transfection

Wells	Transfection Contents
1 and 2	Non-transfected
3	YFP
4	Phogrin-mCherry
5	NPY-GFP
6	Phogrin-mCherry and NPY-GFP

Table 8: CAPS1 Isoforms and NPY-GFP Transfection

Wells	Transfection Contents
1 and 2	Blank cells
3	Non-transfected (TI only)
4	NE-*CAPS1* and NPY-GFP
5	E-*CAPS1* and NPY-GFP
6	NPY-GFP

RNA Isolation from INS-1 cells

To see if REPAIR or increased ADAR expression modified editing frequency within CAPS1, we needed to convert *CAPS1* RNA into cDNA and Sanger sequence. RNA from all transfected cells (Tables 5 and 6) was isolated by adding 200 µL of Trizol (Tri reagent; Ambion Life Technologies 15596026) to each of the wells in the 6-well plate. The mixture was transferred to labeled 1.5 mL microcentrifuge tubes containing 40 µL of Chloroform. Following vortexing and centrifugation according to Sigma Aldrich Trizol RNA isolation protocol, the aqueous solution was transferred to a microcentrifuge fresh tube. To further purify the RNA, an additional 200 µL of Tri reagent and 40 µL of Chloroform were added to the RNA solution in a new tube. Samples were vortexed and centrifuged for 5 minutes, the aqueous solution transferred to a new tube. Precipitation of RNA was accomplished by adding equal volume 95 % room temperature ethanol. The pellet was washed with 70% ethanol. The dried pellet was resuspended in 50 µL house-made RNase-free water.

RT-PCR

RNA was converted into cDNA and the area surrounding the CAPS1 editing site was amplified in order to determine editing of the transcript. Utilizing the High-Capacity cDNA Reverse Transcription (RT) kit (Applied Biosystemsms 4368814), 0.5 – 1 µg of RNA, 1 X RT buffer, 1 X RT random primers, 0.5 X dNTPs, 1 µL RT, and 0.5 µL RNasin (Promega A7973) were filled to 20 µL and placed in the thermocycler at 25 °C for 10 minutes, 37 °C for 1 hr, and 85 °C for 5 minutes. A control RT reaction was also performed for each sample that lacked RT. Five µL of each RT-PCR reaction, 1 X DreamTaq Master Mix (Thermo Scientific K1071) and 400 ng of oRU 48 and oRU 49 (seqeunces listed in Table 1), were used in each PCR reaction. A

negative control lacking any RT-PCR was used as a no-template control. Volumes were filled to 50 µL and cycled under the conditions listed in Table 9.

Table 9: CAPS1 Thermocycler Conditions

Step	Temperature, °C	Time	# of Cycles
Initial denaturation	95	5 min	1
Denaturation	95	30 s	
Annealing	57	30 s	32
Extension	72	35 s	
Final Extension	72	10 min	1

Analyzing *CAPS1* RNA Editing Frequency

RT-PCR samples were sent to GeneWiz (South Plainfield, NJ) for purification and Sanger sequencing. Editing frequency for the E/G site was determined from the electropherogram traces. The sum of the area under both the A peak and G peak (inosine) were calculated using ImageJ (https://imagej.nih.gov/ij/download.html). The inosine peak was then divided by the sum of the areas under the curve to determine percent of edited (black peak) RT-PCR products.

Stimulating INS-1 Cells

To understand how LDCV secretion is affected by *CAPS1* edited and non-edited isoforms, INS-1 cells needed to be stimulated for LDCV secretion and then measured. Since a fluorescence reader (SpectraMax) was used to measure secretion, the normal RPMI containing phenol-red was replaced with clear DMEM. Additionally, since we were measuring both basal and stimulated secretion, we used glucose-free media so glucose concentrations could be manipulated. Phenol red-free and glucose-free DMEM (Gibco A1443001) and glucose were mixed to 2 mM and 16.7

Table 10: Plasmid reference list

Experiment	Construct	pRU	Purpose
REPAIR	Cas13-ddADAR	86	Manipulate RNA editing frequency of *CAPS1*
	crRNA-backbone	87	For cloning of gRNA
	gRNA1	89	Target REPAIR to CAPS1 pre-mRNA
	gRNA 2	90	Target REPAIR CAPS1 mRNA
	Non-targeting gRNA	88	Negative control for REPAIR
	Cas13	91	CAPS1 knockdown
Overexpression of ADAR	ADAR1p150	20	Increase Editing Frequency of *CAPS1*
	2c minigene	58	Transfection control
Overexpression of CAPS1	Edited CAPS1	28	Increase edited *CAPS1* within INS-1 cells
	Non-edited CAPS1	29	Increase non-edited *CAPS1* in INS-1 cells
Secretion	YFP	52	View transfection
	NPY-GFP	53	Secretagogue
	Phogrin-mCherry	51	Secretagogue

mM glucose solutions to make basal and secretion media, respectively. NPY-YFP (pRU 54) and non-tagged CAPS1 (pRU 28 and pRU 29; Table 8) transfected cells were incubated with 2 mM glucose basal media for 2 hrs. The media was removed and transferred to chilled microcentrifuge tubes, centrifuged, then transferred to a new 6-well plate, and the fluorescence analyzed with SpectraMax plate reader (wavelengths listed in Table 11). Fluorescence was read by the

SpectraMax “fluorescence” measurement tool. Full area of each well of the 6-well plate was read with a point density of 7 nm. Following measurement, media was then replaced with 16.7 mM glucose secretion media. After 2 hours, secretions (media) were then transferred to a new 6 well plate and fluorescence was read on the SpectraMax. The SpectraMax fluorescence options and set-up was tailored to a 6-well clear bottom plate with reading density ~7. Following readings, the same cells were harvested in 200 µl of Trizol and stored at -80 °C. Basal secretion was subtracted from stimulated secretion to find total glucose-stimulated secretion fluorescence. Fluorescence between edited and non-edited *CAPS1* and NPY-GFP were analyzed for significance via *One-way ANOVA* followed by multiple comparisons.

Table 11: Excitation and Emission of Fluorescently-tagged Proteins and Secretagogues

pRUs	**Fluorescent Tag**	**Excitation (wv)**	**Emission (wv)**
pRU 1	YFP	485 nm	535 nm
pRU 51	mCherry	554 nm	620 nm
pRU 48, 49, 54	GFP	395 nm	509 nm

RESULTS

Our goal was to manipulate RNA editing frequency and analyze how altering the editing frequency of *CAPS1* mRNA, and ultimately the ratio between edited and non-edited Caps1 protein, affects INS-1 secretion. INS-1 cells predominantly express unedited Caps1, so increasing *CAPS1* editing was attempted with CRISPR-Cas13-ddADAR, a system incorporated with a catalytically inactive Cas13 protein fused with a hyperactive ADAR deaminase domain (Cas13-ddADAR). This system relies on tracrRNA and unique crRNA to target Cas13-ddADAR to the *CAPS1* E/G editing site (Cox et al., 2018; Figure 7). The tracrRNA contains a long stretch of nucleotides that form a loop bound by the inactive Cas13 nuclease. The tracrRNA is fused to the crRNA and delivered by the Cas13b-crRNA backbone plasmid while the deactivated Cas13 is expressed via a separate vector (Cox et al., 2018; Figure 8).

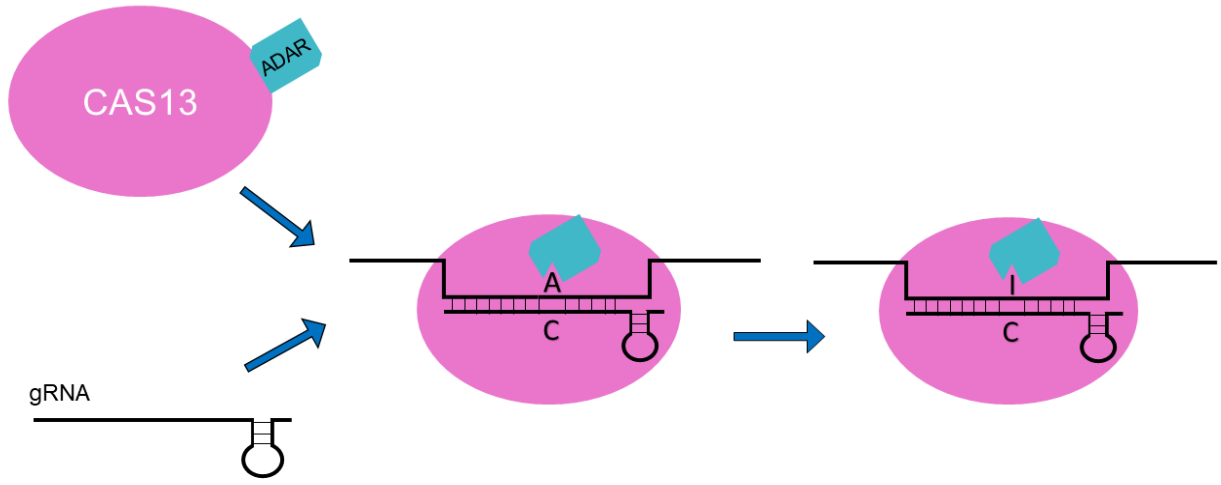

Figure 7. Using CRISPR/Cas13-ddADAR to manipulate RNA editing of CAPS1 transcripts. Catalytically inactive Cas13 is fused with ADAR2 hyperactive deaminase domain (ADAR). The gRNA specifies the target adenosine nucleotide by hybridizing with the editing region of the transcript. This brings Cas13-ADAR close to the adenosine (A) targeted for editing. The cytosine (C) directly across from the A promotes adenosine deamination into inosine (I) of the mismatched adenosine.

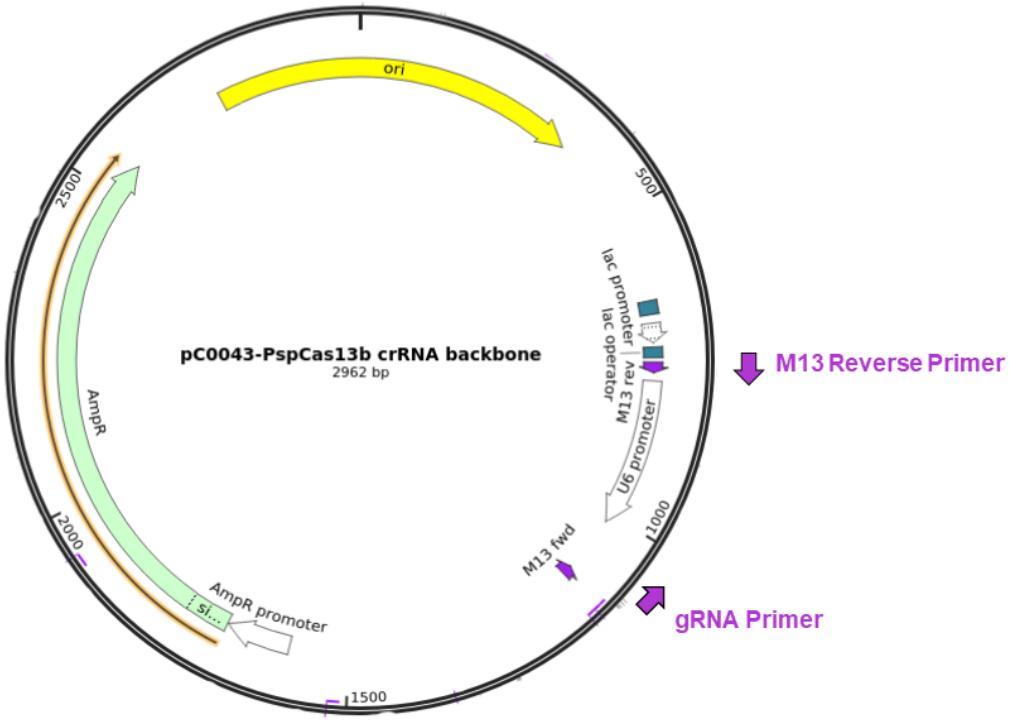

Figure 8. The vector utilized to clone *CAPS1* oligonucleotides and CRISPR RNA to form vectors containing *CAPS1* gRNA1 and *CAPS1* gRNA2. Plasmid characteristics include the origin of replication used for DH5α cells (Ori, yellow arrow), a gene encoding ampicillin resistance (green arrow) for selective pressure, a U6 promoter (white arrow) for transcription of the gRNA in INS-1 cells. The locations of primers utilized for PCR-based clone screening are identified by purple arrows (Addgene 103854).

To make the crRNA unique to the *CAPS1* E/G site, two crRNAs were designed targeting *CAPS1* pre-mRNA (gRNA 1) and *CAPS1* mRNA (gRNA 2). Each gene for the crRNA was made by annealing two strands of complementary oligonucleotides and cloning this 30 nucleotide-long cassette into the Cas13b-crRNA backbone. The resulting full gRNA gene will be expressed from the U6 promoter, a RNA Polymerase III promoter (Kunkel, Maser, Calvet, and Pederson, 1986). Following transcription, the gRNA will bind to the targeted *CAPS1* region. The nucleotide directly across from the E/G site will not be complementary to the A targeted for

deamination. Instead, the nucleotide directly across from A is C, as crRNA with this specific structure increases editing frequency by Cas13-ddADAR (Qu et al., 2019).

Screening Plasmids for Gene Insertion

The crRNA gene was ligated into the crRNA backbone and transformed into bacterial cells. Clones were screened for the presence of the gRNA gene via colony PCR where the primers bind to the backbone as well as the crRNA cassette itself. PCR products of 487 bp (gRNA1) or 490 bp piece of DNA (gRNA2) were amplified and indicated colonies contained a clone with the *CAPS1*-targeting crRNA gene. gRNA 1 gene was cloned in all five, or 100%, of screened colonies, as indicated by the presence of 487 bp bands (Figure 9A). Six colonies were screened for gRNA 2 gene and five, or 83%, of the clones were positive and therefore had the expected 490 bp band (Figure 9B). One positive clone containing each of the 487 or 490 bp PCR product was isolated. Purified plasmid DNA containing Cas13-ddADAR and plasmid containing gRNA1 or gRNA 2 genes were transfected into INS-1 cells to test the ability to manipulate RNA editing of *CAPS1*.

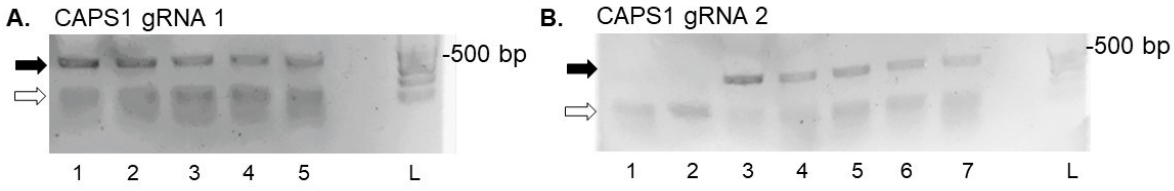

Figure 9. Screening for gRNA positive clones. Colony PCR of *CAPS1* gRNA 1 (A) and *CAPS1* gRNA 2 (B) cloned into the CRISPR crRNA backbone. The products were separated by agarose gel electrophoresis. The expected 487 bp (gRNA 1) and 490 bp (gRNA 2) products are indicated by a black arrow. (A) The lanes 1-5 contain PCR products from gRNA 1 screening. 100 bp ladder (L) is in the right lane. (B) PCR products screened for gRNA 2 in negative control, water only lane 1 and colonies lanes 2-6. 100 bp ladder (L) is in the right lane. Primer dimers are observed below PCR products and indicated by a white arrow in both gRNA 1 (A) and gRNA 2 (B) figures.

INS-1 Cell Transfection Results

INS-1 cells are difficult to transfect. In order to assure that the plasmids for REPAIR could be delivered to the cells, we tested for a reliable transfection method. The transfection efficiencies of two different transfection reagents, PEI and TransIT-2020, were compared against one another using cellular fluorescence after transfection of a YFP expressing vector. Relative fluorescence of cells transfected with a YFP-expressing vector using PEI was comparable to both PEI and TransIT-2020 negative controls (Figure 10). However, average fluorescence for TransIT-2020 was 13,000 RFU, approximately 10,000 more RFU than PEI transfection of the same YFP vector (Figure 10). TransIT-2020 was used for all assays, thereafter.

We also tested the optimal time for fluorescent detection after transfection. RFU from cells was measured 24, 36, 45, 60, and 72 hr after transfection of YFP with TransIT-2020. As expected, YFP RFU was comparable among PEI and YFP and negative controls. YFP RFU using TransIT-2020 peaked 60 hrs post-transfection (Figure 11). Therefore, cells were incubated 60 hrs before secretion experiments.

Comparing INS-1 Secretion Efficiency of Different Fluorescently-tagged Secretagogues

To accomplish our goal of understanding how edited *CAPS1* affects LDCV secretion, we needed to efficiently measure secretion of secretagogues from INS-1 cells. NPY is a neuroprotein secreted primarily by neurons, but is also highly expressed and secreted by INS-1 cells (Waeber et al., 1993) while phogrin is considered a transmembrane protein that associates with LDCVs in regulated secretion (Caromile, Oganesian, Coats, Seifer, and Bowen-Pope, 2010). We had fluorescently-tagged versions of each of these proteins available to us, therefore each were tested for their ability to be detected and secreted from INS-1 cells.

We first tested whether we could detect NPY-GFP and phogrin-mCherry in INS-1 cells post transfection. Sixty hours post-transfection with plasmids expressing each fluorescent protein, phogrin-mCherry and YFP RFU were barely detectible, while NPY-GFP RFU (~60,000 RFU) was much greater in INS-1 cells (Figure 12). This data suggests that NPY-GFP is more detectible in cells than phogrin-mCherry.

Fluorescence was expected to be detectable in the media if the secretagogues are secreted from the cells. YFP (control, not-secreted), Phogrin-mCherry, and NPY-GFP secretion were compared to determine which protein was secreted more efficiently from INS-1 cells. Following stimulation for 2 hrs with 16.7 mM glucose, YFP and phogrin-mCherry RFU in the media were comparable to the negative control, while NPY-YFP in media was significantly greater at ~30,000 RFU (Figure 13). These results suggested that NPY-YFP was more efficiently efficiently secreted by INS-1 cells. Therefore, NPY-GFP was used as the secretagogue in further experiments.

CAS13-ddADAR Directed Editing of CAPS1 Transcripts

Once optimal transfection reagents and secretagogues were determined, we aimed to modify editing frequency of *CAPS1*. Introducing Cas13-ddADAR with the *CAPS1* targeting gRNA was expected to increase editing of *CAPS1*, therefore increasing the detected ratio of edited to unedited *CAPS1*. INS-1 cells were transfected with plasmids containing Cas13-ddADAR cDNA and genes for gRNA 1, 2, or the non-targeting control gRNA (negative control). RNA was isolated from the transfected cells and the editing region of *CAPS1* was amplified by RT-PCR and sequenced with the Sanger method (Figure 14). The characteristic A/G double peak was seen at the E/G editing site in each sample. Editing levels were estimated based on the

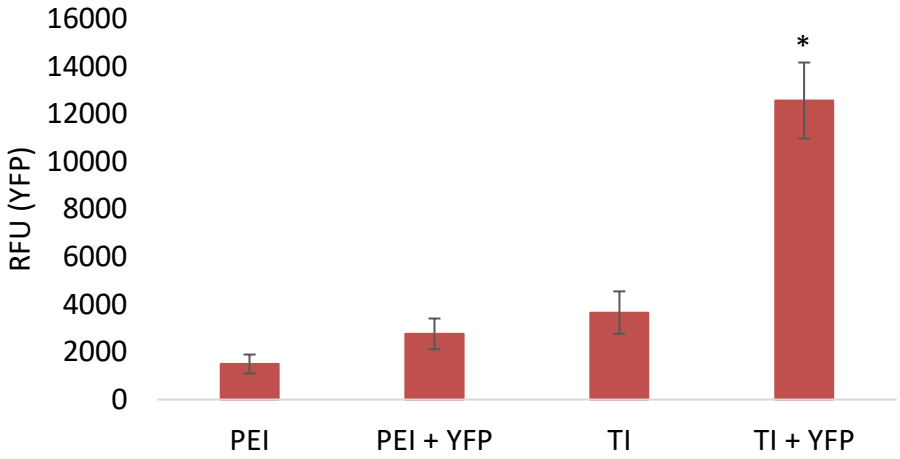

Figure 10. Relative fluorescence post-transfection with PEI and TransIT-2020. INS-1 cells were transfected with YFP using PEI and TransIT-2020 (N = 2, n = 4). YFP relative fluorescence units (RFU) were measured in cells transfected with YFP using PEI (YFP and PEI) or using Transit-2020 (YFP and TI). Negative controls were mock transfected (PEI and TI only). *ANOVA*, p = 0.002*. Error bars indicate standard error of the mean (SEM).

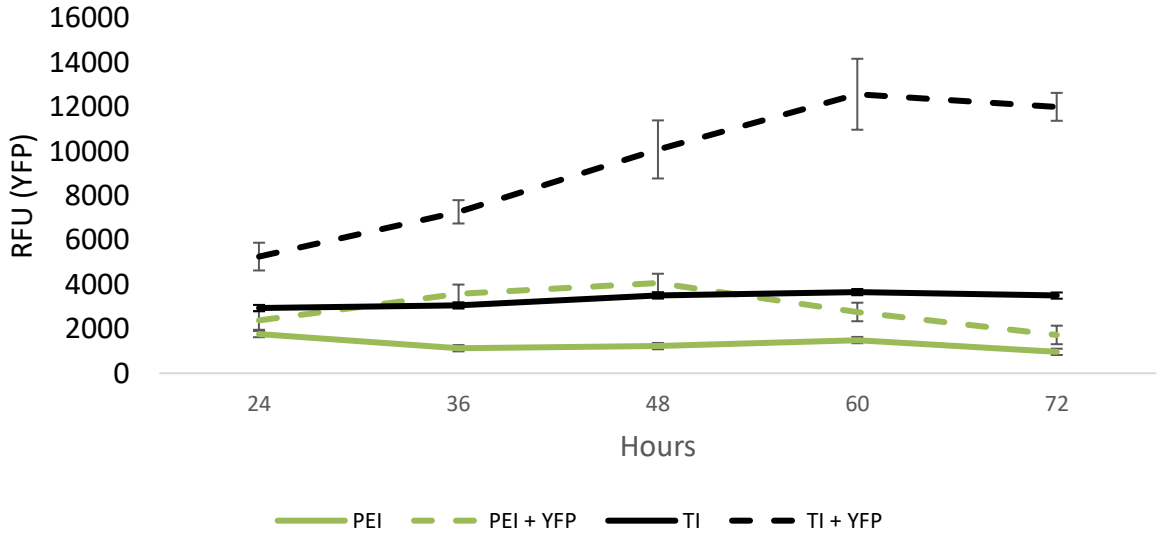

Figure 11. Relative cellular YFP fluorescence post-transfection. INS-1 cells were transfected with YFP (N = 3). Relative fluorescence (RFU) was measured from YFP and PEI, YFP and TransIT-2020 (TI), and negative controls at 24 -72 hours post-transfection. Error bars indicate SEM.

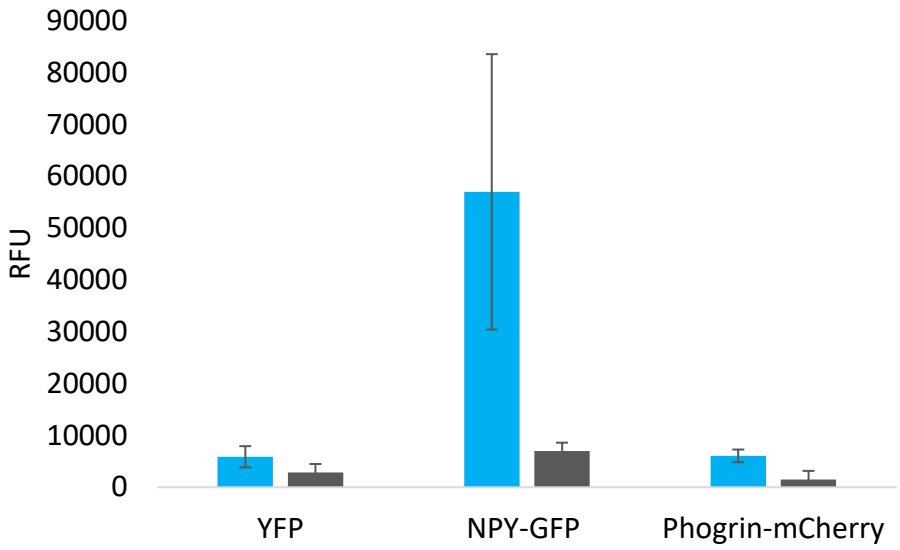

Figure 12. Relative fluorescence in INS-1 cells following transfection. INS-1 cells were transfected with GFP, NPY-YFP, or Phogrin-mCherry and the fluorescence of the cells was determined. Negative control cells (grey) were mock transfected and read at the same wavelengths as the experimental group (N = 3). *ANOVA*, $p > 0.05$. Error bars indicate SEM.

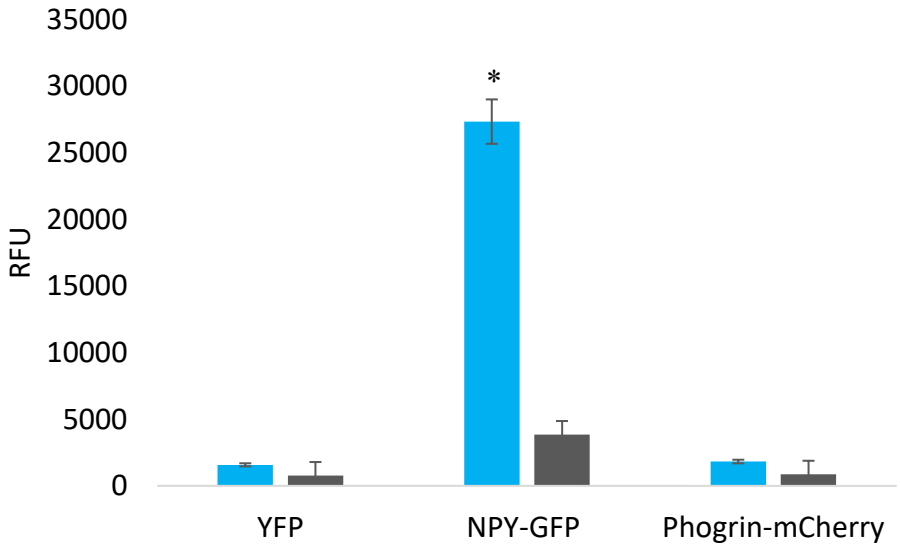

Figure 13. Relative fluorescence in media following glucose stimulation of INS-1 cells. INS-1 cells (N = 3) were treated with 16.7 mM glucose. Fluorescence in secreted media from NPY-GFP, YFP, and phogrin-mCherry transfected cells (blue) were read and compared against Mock transfected negative controls (gray). *ANOVA* followed by multiple comparisons, *$p = 0.002$. Error bars indicate SEM.

The area under the curves for nucleotides A (non-edited, genomically encoded) and G (edited). INS-1 cells transfected with Cas13-ddADAR plus gRNA1, gRNA2, or a control gRNA had an editing frequency of 15 %, 13 %, and 16 %, respectively (Figure 15). Since editing frequencies were similar in experimental and control groups, the ratio between edited and non-edited *CAPS1* were not manipulated via Cas13-ddADAR.

Since there were no observed differences in editing frequencies between experimental and control cells transfected with Cas13-ddADAR, an alternative method was used to manipulate RNA editing of *CAPS1* in INS-1 cells. Experimental cells were transfected with a plasmid that expresses ADAR1p150 cDNA and compared to non-transfected controls to determine if overexpression of the enzyme responsible for *CAPS1* RNA editing could increase the level of *CAPS1* RNA editing in INS-1 cells. RNA was isolated from transfected cells and the editing region of *CAPS1* RNA was amplified via RT-PCR and subjected to Sanger sequencing (Figure 14). INS-1 cells transfected with ADAR1-p150 had an editing frequency of 18% compared to the control which has 24% editing (Figure 16). There were no significant differences in editing between the control and experimental groups, thus *CAPS1* editing frequency was not influenced by ADAR1p150 overexpression.

Since neither method to manipulate editing of endogenous *CAPS1* mRNA was successful, we shifted to an alternative method to modify the ratios of edited to non-edited *CAPS1*. In this model, we overexpressed *CAPS1* edited and non-edited isoforms from cDNA containing constructs. Expressing edited *CAPS1* will increase the proportion of edited *CAPS1* within the cell. The change to editing was expected to be small considering *CAPS1* is robustly expressed from the INS-1 genome. We attempted to measure RNA editing from these cells to provide an exact estimate of *CAPS1* editing frequency, but the RT-PCR failed due to degradation

of the RNA in the samples. While the degree of RNA editing frequency within *CAPS1* is unknown, we proceeded with further experiments under the assumption that editing frequencies were altered, if only slightly.

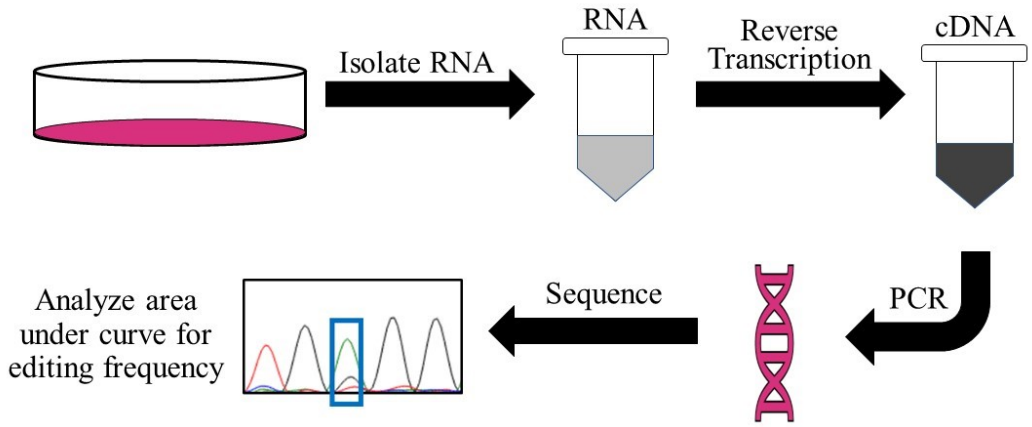

Figure 14. Determining *CAPS1* RNA editing levels. To determine RNA editing levels of *CAPS1* mRNA, total RNA was isolated from cultured cells then subjected to reverse transcription. The resulting cDNA was amplified through PCR and sequenced with Sanger sequencing. To calculate the editing frequency, the area under the G peak (resulting from edited transcripts) was divided by the total area of both A and G peaks at the editing site (boxed).

Stimulated and Basal Secretion

Once we decided on a model to manipulate RNA editing frequency of *CAPS1*, we proceeded to address our second aim to measure basal and stimulated secretion. After overexpressing edited and non-edited *CAPS1* cDNA in INS-1 cells, cells were treated with low glucose to provide conditions for basal secretion of NPY-GFP. After correcting for transfection and expression of NPY-GFP in the cells, the basal NPY-GFP secretion from cells also containing excess non-edited *CAPS1* was significantly decreased ($p = 1.9 \times 10^{-6}$) compared to those transfected with edited *CAPS1* and NPY-GFP only (Figure 17). The same cells were then treated

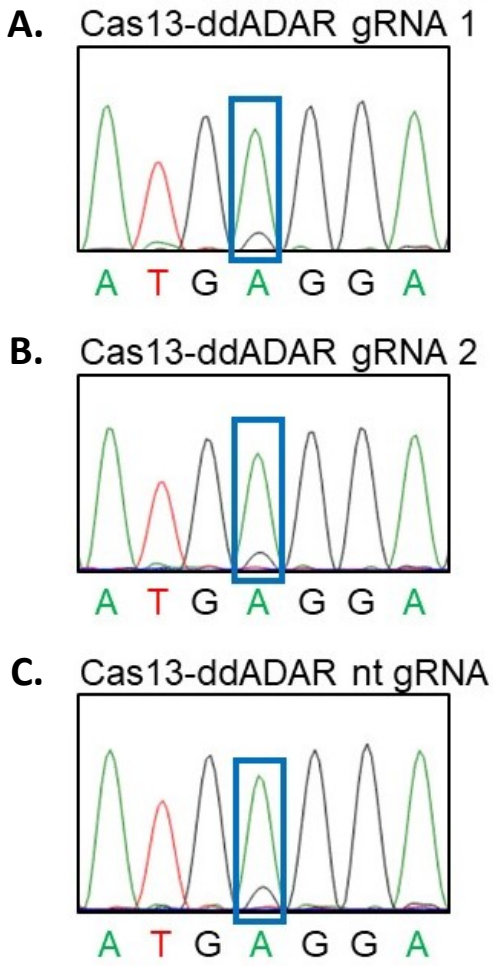

Figure 15. RNA editing of *CAPS1* by Cas13-ddADAR. INS-1 cells were transfected with Cas13-ddADAR and gRNAs. Total RNA was isolated and *CAPS1* mRNA was amplified via RT-PCR. Samples containing Cas13-ddADAR gRNA 1 (A) and Cas13-ddADAR gRNA 2 (B) were subjected to Sanger sequencing and compared to a non-targeting (nt) gRNA (C). Sanger sequencing electropherogram traces of the RT-PCR products are shown. *CAPS1* E/G editing site (blue box) contains both adenosine (green peak) from non-edited transcripts and guanosine (black peak) from edited transcripts. The genomically encoded sequence is shown below each sequence trace.

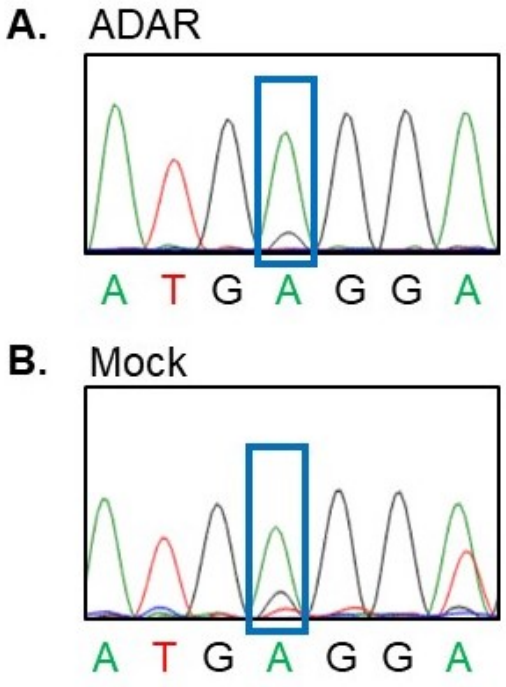

Figure 16. RNA editing of *CAPS1* in INS-1 cells by ADAR1p150. INS-1 cells were transfected with a plasmid expressing ADAR1p150 cDNA (A) and compared against mock transfection (B). Electropherogram traces from Sanger sequencing of the RT-PCR products are shown. The *CAPS1* E/G editing site (blue box) depicts adenosine (green peak) from non-edited transcripts and guanosine (black peak) from edited transcripts. The genomically encoded sequence is indicated below each sequence trace.

with high glucose (16 mM) to induce GSIS. Glucose stimulated secretion was measured by determining the increase in NPY-GFP secretion under stimulated conditions over the basal conditions. The results indicate inconsistent levels of secretion in all groups. At times, the level of secretion in stimulated cells was less than the basal secretion (resulting in negative glucose stimulated secretion levels, Figure 18). There were no statistical differences in stimulated secretion between cells expressing non-edited or edited *CAPS1*.

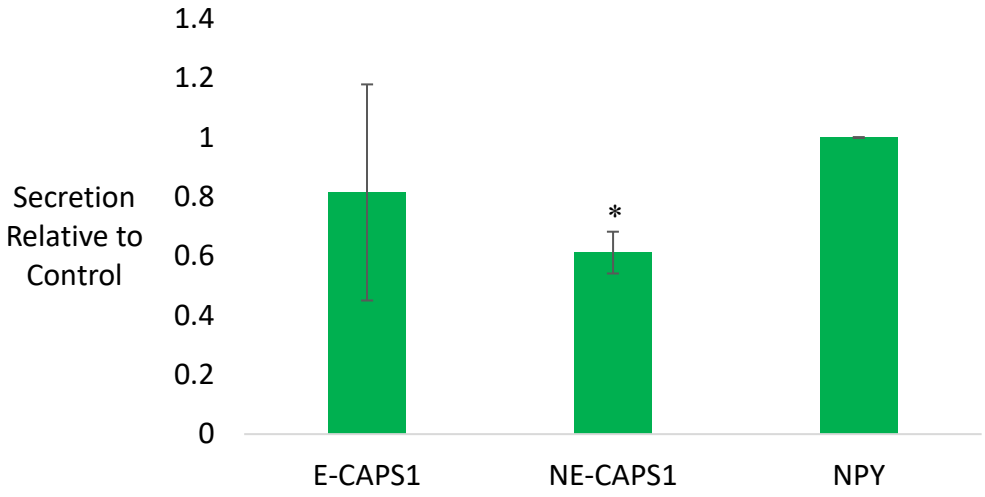

Figure 17. NPY-GFP Fluorescence Following Basal Secretion from INS-1 cells. INS-1 cells (N = 3, n = 6) were transfected with NPY-GFP and edited (E) or non-edited (NE) *CAPS1*. Secretion was compared to the cells containing only NPY (tagged with GFP and with no CAPS1 transfected). Error bars indicate SEM. *ANOVA*, *p = 1.9 x 10^{-6}.

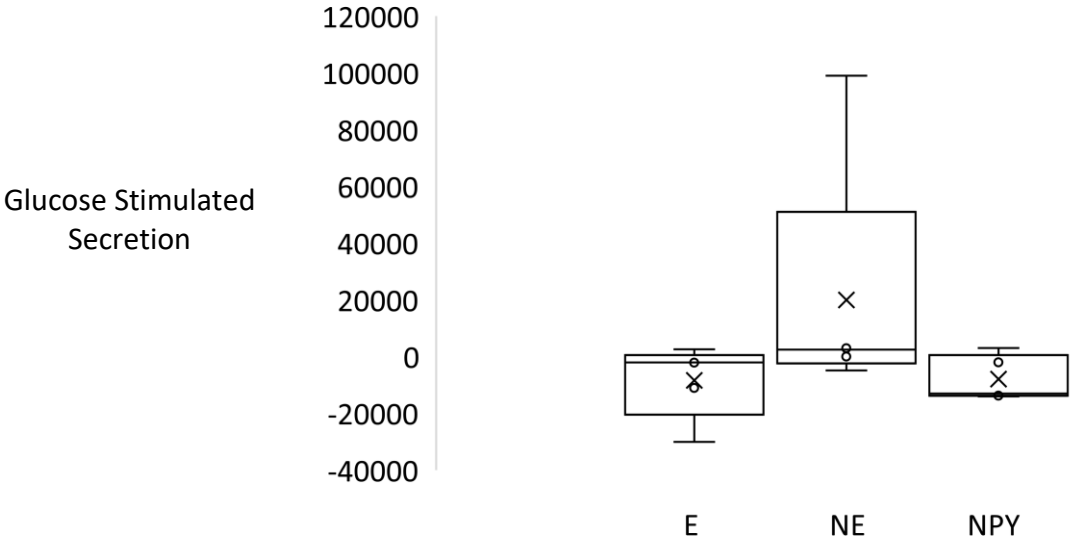

Figure 18. Stimulated secretion of NPY-GFP from INS-1 cells. INS-1 cells (N = 3, n = 6) were transfected with NPY-GFP and edited (E) or non-edited (NE) *CAPS1*. Secretion was measured after 2 hrs in 16mM glucose. Basal secretion from the same cells was subtracted from the stimulated secretion to determine glucose stimulated secretion. *ANOVA*, p > 0.05.

DISCUSSION

CAPS1 facilitates stimulated cell secretion by bridging LDCVs and the plasma membrane. RNA editing occurs within the *CAPS1* pre-mRNA, an event highly conserved across mammals (Miyake et al., 2016). This event recodes the transcript, substituting a glycine for a glutamic acid residue within the region of the protein that interacts directly with the vesicle. PC-12 cells expressing only edited *CAPS1* demonstrate increased LDCV secretion (Miyake et al., 2016), suggesting that the role of *CAPS1* RNA editing is to upregulate secretion.

RNA editing only occurs in about 20 % of *CAPS1* transcripts. Studies in cultured neurons showed that even slight increases in editing frequency significantly modify secretion efficiency of SVs (Ulbricht et al., 2017). We used a similar method of increasing levels of edited or non-edited *CAPS1* in insulinoma cells. We found that basal, or non-stimulated, secretion levels from LDCVs were significantly affected by changes in *CAPS1* editing while the effect of *CAPS1* editing on stimulated secretion was inconclusive.

Manipulating RNA Editing Frequency Within the *CAPS1* Transcript

To study the effects of *CAPS1* RNA editing on LDCV secretion from the insulinoma (INS-1) cells, we first sought to alter the level of *CAPS1* editing within INS-1 cells. Since INS-1 cells express primarily non-edited *CAPS1*, we attempted to increase editing of the endogenous *CAPS1* by simply administering exogenous ADAR1. If *CAPS1* RNA editing is limited by the amount of ADAR1 available within the cell, then transfecting cells with excess ADAR1 should upregulate editing. However, increasing ADAR1 levels within INS-1 cells did not affect editing frequency within the *CAPS1* transcript (Figure 16). This indicates that *CAPS1* RNA editing is not wholly dependent upon ADAR1 levels. It is relatively well known that ADAR expression

levels weakly correlate with RNA editing events**.** Regardless of ADAR expression levels RNA editing targets, like *GluR-2* and $5HT_{2c}$, are edited consistently (Jacobs et al., 2009). Additionally, interferon-induced upregulation of ADAR1 levels throughout the body, do not have a significant effect on editing within ADAR targets in the brain (Hood et al., 2014).

We also attempted to increase the level of editing within the endogenous *CAPS1* transcript by targeting Cas13-ddADAR to the E/G site (Figure 7). This REPAIR system requires a gRNA that will target Cas13-ddADAR to the desired editing location. We made two different guide RNAs: gRNA1 targeted *CAPS1* pre-mRNA by recognizing parts of both exon 28 and a downstream intron. gRNA2 complements a region from exons 28-29 within the mRNA. Following successful cloning of the gRNA gene (Figure 9), we transfected the plasmids expressing the gRNA with Cas13-ddADAR into INS-1 cells. The results show that REPAIR did not increase RNA editing frequency within *CAPS1* pre-mRNA and mRNA (Figure 15). This failure may be attributed to the double-stranded nature of *CAPS1* pre-mRNA and mRNA. The pre-mRNA is known to form a dsRNA duplex via complementary regions surrounding the editing site and in the downstream intron. This duplex is the structure recognized by ADAR1 for RNA editing (Miyake et al., 2016). Similarly, a region of the *CAPS1* mRNA containing the editing site is predicted to form an imperfect dsRNA structure by binding to a region within the 3'UTR (Figure 19). Since Cas13 requires single-stranded substrates (Cox et al., 2016.), it is possible the double-stranded *CAPS1* structures prohibited Cas13 interaction with the *CAPS1* mRNA.

We were unable to manipulate editing of endogenous *CAPS1* transcripts, so we shifted our attention to altering editing by expressing *CAPS1* edited and non-edited variants with cDNA constructs. Overexpressing *CAPS1* isoforms creates an artificial situation where we are both

adding *CAPS1* and altering ratios of edited to non-edited *CAPS1*. Ideally, we would not increase (or decrease) the amount of *CAPS1* in the cells, but instead, replace the mostly non-edited *CAPS1* with desired ratios of edited to non-edited *CAPS1*, allowing us to see how editing alone affects secretion. One way to do this would be to first knock down *CAPS1* mRNA, then express *CAPS1* isoforms using the cDNA clones. *CAPS1* knockdown could be accomplished by using catalytically active Cas13 and a gRNA complementary to *CAPS1*. Students in BMS 525 and BMS 625 (Fall 2020) showed that Cas13 can knockdown *CAPS1* using gRNAs targeting various exons. In these experiments, gRNAs targeting exons 3, 4, and 31 were successful in the knockdown of *CAPS1* mRNA (data not shown). This experiment will need slight modifications to be useful in manipulating *CAPS1* editing. First, the gRNA must target the 3'UTR of endogenous *CAPS1*. The cDNA clones expressing *CAPS1* variants do not contain the endogenous 3'UTR, so gRNA complementary to this location would allow us to target only the endogenous *CAPS1*, while simultaneously expressing the desired *CAPS1* variant from the cDNA clone. From there, editing frequencies and editing-dependent secretion from INS-1 cells could be analyzed by the methods outlined in this study.

Our model to overexpress *CAPS1* isoforms to modify RNA editing frequency also leads to increased total expression of Caps1. It is possible the overexpression of *CAPS1* could confound results and possibly occlude conclusions regarding RNA-editing dependent effects. One study replaced *CAPS1* with solely the edited variant and showed significant differences between secretion from LDCVs (Miyake et al., 2016). However, this model also has limitations in assessing how natural variations of *CAPS1* editing affect its role in secretion. Since only about 20 % of *CAPS1* transcripts are edited, it is unrealistic to expect *CAPS1* editing to increase to 100 % within an organism or tissue of an organism. More reasonably, natural conditions, such as

disease, may only slightly alter editing. Inflammation, a common symptom of disease, increases A-to-I editing occurrences up to 5 % in lymphocytes (J. H. Yang et al., 2003). We expect our model to be more physiologically relevant and resemble this slight modification to RNA editing frequency.

Similar overexpression of edited *CAPS1* in neurons, where *CAPS1* editing was only increased by 5 %, showed measurable effects on secretion from SVs (Ulbricht et al., 2017). We were unable to determine the exact ratio of editing in our transfected cells. We isolated RNA from the INS-1 cells used in our study and attempted to analyze the change in overall *CAPS1* editing levels but the RT-PCR was unsuccessful due to unstable RNA. This information may be crucial to determine the role of editing in our experiments: If differences in *CAPS1* editing frequency are not detectible between the transfected and non-transfected groups, then perhaps effects on secretion are limited. Thus, the lack of phenotype may be due to the lack of our influence on editing ratios, rather than a general lack of RNA editing-dependent effects on Caps1 function in LDCV secretion form INS-1 cells.

We believe this model will provide insight into how RNA editing alterations that result from disease, or treatment of disease, affect secretion from LDCVs. For example, a common treatment for patients with Hepatitis C includes interferon treatments (Rong and Perelson, 2010). The interferon induces ADAR1p150 transcription (Hood et al., 2014). If this ADAR1p150 induction increases editing of *CAPS1* by as little as 5 %, it can potentially affect secretion Previous work investigating the connection between ADAR1p150 and RNA editing have focused on the brain and found no significant alterations to RNA editing (Hood et al., 2014). Preliminary data within Dr. Ulbricht's lab suggests that inflammation and ADAR1p150 induction does have a slight effect on *CAPS1* editing in peripheral tissues (data not shown). Our

work aims to investigate if small changes in *CAPS1* editing frequency affect secretion from LDCV vesicles.

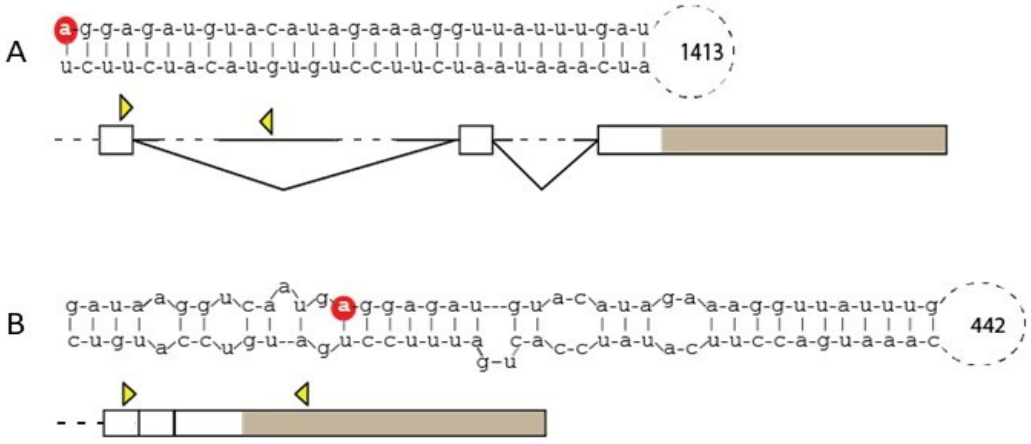

Figure 19. Double-stranded structures of *CAPS1* pre-mRNA and mRNA. *CAPS1* pre-mRNA forms a dsRNA structure via the E/G and ECS regions (A). It is predicted *CAPS1* mRNA can also form a dsRNA structure by complementary regions with exon 28 and section of the 3' UTR (B). The dsRNA structure is above each graphical representation of the transcript. Yellow arrows depict the relative location of half of the duplex within the transcript and the red region represents the E/G site. Shaded area represents the 3' untranslated region.

CAPS1 Editing in Glucose Stimulated Secretion

With our model in place, next, we sought to determine how altered *CAPS1* editing levels affect secretion from LDCVs. NPY-GFP was detected in the cells after transfection and was secreted into the media under high glucose conditions (Figure 13), suggesting that this secretagogue was detectible and efficiently secreted. The NPY used in this study was tagged with GFP. Moreover, instead of the full-length NPY, we used a peptide that only contained NPY signal sequence, a segment (28 aa) of the full NPY (36 aa) that is packaged in LDCV and secreted (El Meskini et al., 2001). NPY is known to be highly expressed and secreted by INS-1 cells (Waeber et al., 1993) and our work shows NPY-GFP we used is also loaded into and

secreted from INS-1 cells (Figure 13). The other secretagogue tested, phogrin tagged with mCherry, was barely detected in the cells or in the media (Figure 13). We believe the reason Phogrin-mCherry emitted low levels of fluorescence was due to the close excitation and emission wavelengths of mCherry (Table 11). It is possible that the SpectraMax plate reader used to detect fluorescence from cells and media is more sensitive to GFP, a fluorescent peptide with a larger range between excitation and emission wavelengths (Table 11).

After choosing an effective secretagogue, we attempted to stimulate secretion from INS-1 cells. We expected increased edited *CAPS1* to increase glucose stimulated secretion of NPY-GFP from LDCVs, however, the data on stimulated secretion was inconclusive. In most of the replicates, in all three groups (NPY only, NPY with non-edited *CAPS1*, and NPY with edited *CAPS1*) NPY-GFP secreted during high glucose conditions was not more than during low (non-stimulating) glucose conditions, suggesting that INS-1 cells were not strongly stimulated by glucose and glucose stimulated secretion did not occur (Figure 18). Future steps could focus on using a more robust mechanism to stimulate secretion. Potassium generates a larger stimulus than glucose and would likely result in total and efficient vesicle release. Using potassium as a stimulus would shift the focus from glucose stimulated insulin secretion to LDCV stimulated secretion, in general. This shift in focus would not significantly alter our goal to understand how RNA editing within *CAPS1* affects LDCV secretion and would be consistent with how experiments in other LDCV secreting cells (namely, PC-12 cells) are done.

If future steps continue to assess glucose-stimulated secretion, it may be worth to consider a different secretagogue. It is possible that NPY-GFP secretion is regulated differently than endogenous insulin release. In fact, NPY negatively modulates insulin secretion by interfering with adenylyl cyclase and decreasing ATP, which is required to trigger fusion of

insulin granules in the RRP (Morgan et al., 1998; Wollheim and Sharp, 1981). The NPY fragment used in our experiments encodes only the signal sequence, and it is unclear whether it has similar effects on secretion compared to the full NPY peptide (Morgan et al., 1998). It may be beneficial to bypass NPY-related potential complications by direct measurement of insulin secretion. Commercially available ELISA kits (ThermoFisher # ERINS) can be used to measure the amount of insulin in the media following glucose stimulation.

CAPS1 Editing and Basal Secretion

Glucose stimulated secretion is measured by subtracting secretion under low glucose conditions (2 mM glucose) from secretion induced by high glucose conditions (16 mM glucose). The levels of secretagogue in the media under low glucose (2 mM) conditions are a baseline level of secretion that is not normally measured on its own, nor compared between experimental conditions. Instead, this level of non-stimulated secretion is typically used as a constant, assumed to be equal among all cells or conditions. We found basal stimulation to be significantly decreased with non-edited *CAPS1,* even after accounting for the number of transfected cells (Figure 17). This is interesting considering Ulbricht et al. (2017) also showed that altering editing levels could affect spontaneous secretion from synaptic vesicles, but that stimulated secretion was not affected. Miyake et al did not report non-stimulated secretion levels from PC-12 cells with solely edited or non-edited *CAPS1* but did find small differences between stimulated secretion from these cells. Since the basal secretion is used in the calculations for stimulated secretion, unaccounted for changes in basal levels could skew the results and lead to mis-reporting alterations to stimulated secretion due to differences solely in basal secretion. This leaves the possibility that, in Miyake et al. (2016) studies and our studies, the *CAPS1* variants do

not affect stimulated secretion from cells but edited and non-edited *CAPS1* primarily regulates basal secretion.

Increasing edited *CAPS1* (to alter total editing by only 5 %) decreases synaptic vesicle pool volume and increases sphericity of vesicle pool, suggesting that *CAPS1* editing contributes to a tighter, more compact pool of SVs in the synapse (Ulbricht et al., 2017). This study also observed that edited *CAPS1* decreases basal secretion from synaptic vesicles and non-edited *CAPS1* increases the same basal secretion (Ulbricht et al., 2017). In our experiments, edited *CAPS1* had no effect on basal LDCV secretion, but increasing non-edited *CAPS1* levels resulted in decreased basal secretion of LDCVs in INS-1. It is possible that the contrasting effects of increasing non-edited *CAPS1* between these studies are due to differences between mechanisms of LDCV organization in INS-1 cells and SV in synapses. It is possible that *CAPS1* does not play the same role in vesicle organization for both vesicle types. To study INS-1 vesicle organization and its dependency on *CAPS1* editing, future studies could use a transmission electron microscopy or 3-dimensional structure illumination microscopy (3D-SIM) to see how edited *CAPS1* affects the distribution of LDCVs. For example, 3D-SIM experiments would use immunohistochemistry to visualize the location of vesicles within the cells and their dispersion relative to each other and the plasma membrane. If this study shows non-edited *CAPS1* in INS-1 cells results in more tightly clustered vesicles away from the membrane (similar to edited *CAPS1* in synapses), *CAPS1* organization capabilities may restrict secretion under non-stimulating conditions by physically retaining vesicles in the RP. This would also indicate that *CAPS1* editing-dependent effects on organization are dependent on the vesicle type and/or the cell type.

The E/G Site: A Means of Modifying *CAPS1* Function and Cellular Processes

One way that *CAPS1* isoforms may differentially affect vesicle organization is through *CAPS1* interaction with vesicle and with itself. *CAPS1* editing affects an amino acid within the domain of the protein that directly interacts with vesicles. *CAPS1* association with vesicles is also required for homodimerization through its C2 domain (Petrie et al., 2016). This creates an interesting model where *CAPS1* may organize vesicles by linking them together through its homodimerization (Figure 20). We do not understand how these two physical properties are linked with RNA editing, but it is hard to ignore the possibility that RNA editing may affect interaction with the vesicle and/or interaction with itself, thereby possibly affecting the overall vesicle organization.

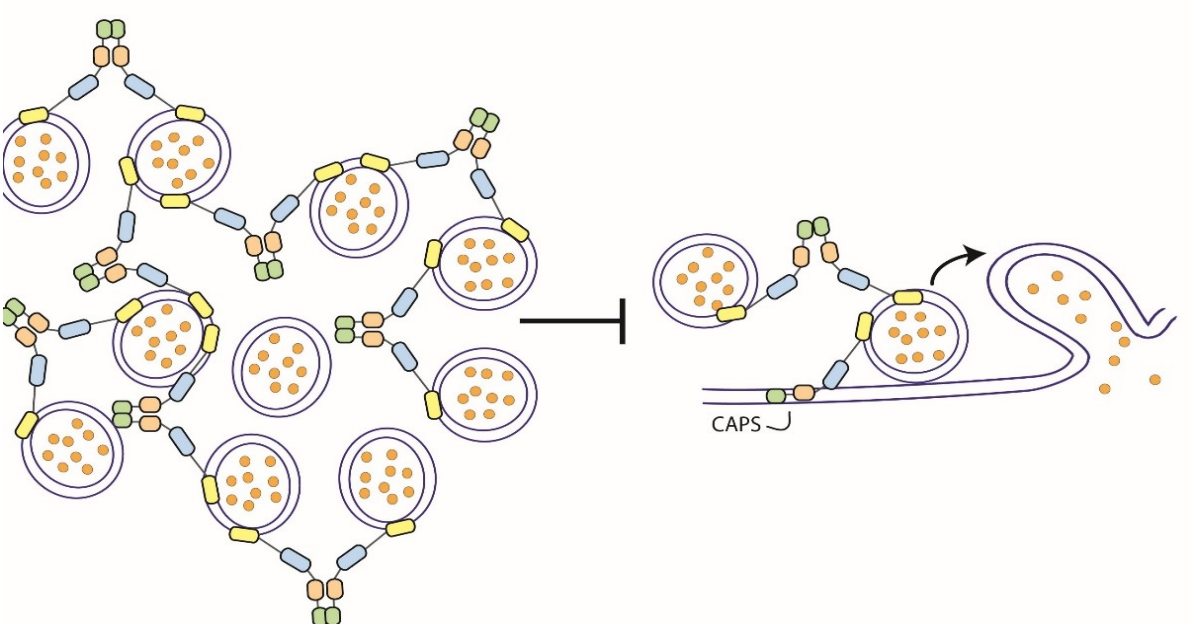

Figure 20. Model for Caps1-dependent vesicle organization. Caps1 homodimerizes via its C2 domain (green) and interacts with the vesicle through its LDCVBD (yellow). In this model, Caps1 links vesicles together and organizes them in the interior of the cell. This may also restrict vesicles in the reserve pool form moving toward the plasma membrane, where docking, priming and fusion occur.

It is possible that *CAPS1* editing may affect secretion for some cell types differently than others. In this case, perhaps *CAPS1* editing influences secretion from PC-12s and neurons differently than INS-1 cells due to unique properties of those cells. It would be interesting to expand these studies, identifying secretion phenotypes of other cell types and lines in response to alterations to *CAPS1* editing. Knowledge of how/if *CAPS1* editing affects stimulated and non-stimulated secretion in a variety of cell types may provide insight into how RNA editing dependent isoforms of *CAPS1* interact with subcellular machinery to affect secretion. It is possible editing-dependent phenotypes require a specific component unique to certain cell types to differentially affect non-stimulated or stimulated secretion. Identifying the factors common to cell types that have similar *CAPS1*-dependent secretion phenotypes could help identify the components or factors involved.

Recoding of *CAPS1* by RNA editing is known to alter protein-protein interactions. For example, Syntaxin-1A preferentially binds to edited-Caps1 compared to non-edited Caps1. While both closed and open forms of Syntaxin-1A bind to edited Caps1, the interaction between edited Caps1 and the open form is stronger (Parsaud et al., 2013). Syntaxin-1A is abundant in both PC-12 cells (Miyake et al., 2016) and neural cells (Vardar et al., 2016). However, Syntaxin-1A is mainly found at the membrane and involved with the first phase of insulin release within pancreatic beta cells, while Syntaxin-3 is associated with the second phase of insulin release (Zhu et al., 2013). Since Syntaxin-1A preferentially binds to edited Caps1, it is possible this relationship is responsible for secretion effects and for potential differences in *CAPS1* editing-dependent isoforms in different cell types. Perhaps, edited *CAPS1* had no effects on calcium-mediated secretion in INS-1 cells due to the limited availability of Syntaxin-1A. In this case, overexpressing Syntaxin-1A, or replacing Syntaxin-3 with Syntaxin-1A, while increasing levels

of *CAPS1* editing would have secretion effects similar to neurons or PC-12 cells. We might expect these editing-dependent phenotypes to deplete in a Syntaxin-1A knockout model, cementing the idea that editing-dependent *CAPS1* affects are dependent upon the presence of Syntaxin-1A. This experiment would also suggest editing of *CAPS1* preferentially affects the first phase of insulin release, and has a minimal to no effect on sustained, or second phase of insulin release.

Much remains unknown about the mechanism or why modification of a single residue within *CAPS1* affects secretion. Future studies focusing on the residue re-coded by RNA editing may be beneficial to understanding the mechanisms by which *CAPS1* and RNA editing are involved in secretion. To see the properties of glutamate and/or glycine that are responsible for editing-dependent effects on secretion, endogenous *CAPS1* could be replaced with variants that encode different amino acids at the E/G site and then test how this modifies secretion. One could also test the interaction of these variants with Syntaxin-1A or with the vesicle itself to help us understand the biochemistry of the interaction. It is possible that glycine and amino acids with similar charge and polarity have similar effects on secretion, similarly interact with Syntaxin-1A and with the vesicle, and similarly affect vesicle organization. Perhaps these qualities contrast the behavior of Caps1 isoforms containing amino acids charged similarly to glutamate. This information would provide insight into the impact glutamate, and ultimately RNA editing, has on Caps1 structure and its function in vesicle fusion.

Pursuit to Understand RNA Editing Within *CAPS1* and Its Effect on Secretion

To summarize, the results in our study bring clear future directions for how we can continue to uncover the mysteries of how RNA editing within *CAPS1* affects LDCV fusion. The

immediate next steps to continue this work include identifying and/or deploying alternate strategies to alter the editing frequency of *CAPS1* within the INS-1 cells. Stimulating secretion with high potassium is also an important step. Along the way, it may be prudent to identify an alternative secretagogue (e.g. insulin or Phogrin-YFP) and compare its secretion to that of NPY-GFP. More involved future projects could focus on how the biochemistry of the E/G site is involved in secretion, giving insights to how this one amino acid change can affect a highly regulated cellular process through protein-protein interactions, association with the vesicle, and/or organization of the vesicles themselves. Perhaps as the work moves forward, even more questions will be compelled, and this work will pave the way to a deeper understanding how LDCV fusion is regulated.

Information on how alterations to *CAPS1* editing frequency or increasing levels of *CAPS1* isoforms are important when considering potential therapies or consequences of biological conditions that might alter editing or *CAPS1* levels. For example, CAPS family has been shown to be involved in patients with intellectual disabilities. Dysregulation of *CAPS1* and *CAPS2* causes phenotypes similar to autism while deletion of *CAPS1* causes learning difficulties, delayed social development, and language impairments (reviewed in de la Hoz et al., 2015). Dysregulation of *CAPS1* and 2 in mouse pancreatic beta cells leads to loss of glucose sensitivity, a characteristic of diabetes (Speidel et al., 2008). Thus, it is clear that proper maintenance of neurotransmission and glucose tolerance rely on Caps1. *CAPS1* and its role in secretion are vital in maintaining body homeostasis. Therefore, regulating *CAPS1* is paramount to homeostasis and health, and RNA editing is just one of many steps involved in that regulation.

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