Underlying mechanisms which regulate Equilibrative Nucleoside Transporter 1 (ENT1): From fundamental forms of regulation to unifying signalling pathways

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Abstract

Equilibrative nucleoside transporter 1 (ENT1) is a membrane protein critical for the movement of nucleosides across biological membranes. ENT1 plays a role in nucleoside salvage, cell signalling, cardioprotection, and the uptake of nucleoside analog drugs involved in the treatment of cancer, viruses, and parasites. Although ENT1 is clinically relevant as a drug transporter, relatively little is known about how it is regulated. I have identified two gaps in our knowledge of ENT1 – how calcium regulates ENT1-dependent nucleoside flux, and the role of *N*-linked glycosylation on ENT1.

I have defined a novel mode of ENT1 regulation, whereby direct binding of CaM to ENT1 modulates nucleoside flux in a calcium-dependent manner. Moreover, I have shown that in a physiologically relevant model (U-87 MG, human glioblastoma cell line) that activation of NMDA receptors led to increased intracellular calcium, which increased chloroadenosine flux that can be blocked with the NMDA receptor antagonist MK-801 (50 μM) or the CaM antagonist W7 (50 μM). Calcium-dependent regulation of ENT1 is relevant in many cell types, since both ENT1 and CaM are ubiquitously expressed.

I also examined *N*-linked glycosylation of ENT1. Previous work performed in yeast and oocytes have established that ENT1 is glycosylated at Asn48, but expression of a glycosylation deficient hENT1 mutant in a human cell line has not been published. I predicted that glycosylation was necessary for proper ENT1 function, and would alter protein abundance at the plasma membrane. Using chloroadenosine transport assays, I showed that N48Q mutant ENT1 protein is non-functional, and using NBTI binding analysis and immunofluorescence I identified that there was less glycosylation aberrant ENT1 at the plasma membrane compared to wild type protein.

Furthermore, we have previously established in the lab that ENT1 has a variety of protein-protein interactions with other ENTs. I established here that removing *N*-glycosylation from ENT1 blocked the ability for ENT1 to have protein interactions with wild type ENT1. This work provides an understanding of novel mechanisms of ENT1 regulation which can be used as the basis of future clinically relevant research to enhance nucleoside drug uptake.

This work is dedicated to my wife Amanda, my parents, my sister, my family, and friends. Without their unwavering support, and wholehearted encouragement, this would not have been possible.

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I would like to thank Dr. Imogen Coe for her guidance and advice throughout the project. I appreciate the time we spent discussing experimental methods, the results, and future plans. It was reassuring to know that if I ever had any doubts or concerns, I had someone to rely on for creativity, motivation, and support. I could not be more grateful for having such an inspiring mentor, who provided not just a framework to build scientific models effectively, but helped me develop skills pivotal for success as I progress onward in life.

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Lastly, I would like to thank my family and friends who believed in me. Your encouraging words comforted me when things seemed like they weren't going as planned. Laboratory research requires patience and determination, and when mine felt like it was wearing thin and things were going awry, I knew I always had people who I could count on to uplift me and put things in perspective. Your reassurance has helped me get where I am today.

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1.1 Nucleosides and their Biological Relevance

This body of work aims to elucidate fundamental regulatory mechanisms which govern the movement of nucleosides in and out of cells, and the roles of several proteins of the purinome, the network of purinergic signalling proteins, in this movement.

Nucleosides are biologically significant molecules, central in cell signalling, nucleic acid synthesis, and energy metabolism (King et al. 2006a, Young et al. 2013). Cells primarily attain nucleosides from the digestion of nucleic acids and absorption following dietary consumption (Ohyanagi et al. 1989). These hydrophilic glycosylamines have a structure containing a nucleobase paired with a ribose or deoxyribose sugar bound through an *N*-glycosidic linkage (Figure 1). These molecules fall under one of two categories: purines or pyrimidines. Adenosine, guanosine, and inosine constitute the purines (Rose 1923), while the pyrimidines consist of cytidine, uridine, and thymidine (Cerecedo 1927).

The drive to understand the underlying mechanisms which lead to heredity, following the work of Darwin and Mendelson, guided pioneers like Friedrich Miescher (who generated crude extract of nucleic acids and coined the term ‘nuclein’ in 1869, but was published posthumously) to isolate DNA and its substituent components (Dahm 2005). Since then, biologists have been keenly interested in nucleosides. Over a century ago, the metabolism of thymidine and uracil was examined by analysis of urine in test animals in the 1920’s (Deuel 1924). Eventually, Avery et al. (1944) demonstrated that the transforming principle seen in *S. pneumonia* (Griffith 1928) is due to DNA and not protein, suggesting that DNA is the chemical basis for heredity. The compilation of

work by Levene (constituents of DNA; Levene and London 1929), Chargaff (fixed ratios of nucleotides; Chargaff et al. 1950), Franklin and Wilkins (Crystal structure of DNA in 1953; Franklin and Gosling 1953), culminated with Watson and Crick (1953) determining the chemical structure of DNA. It has been well established that nucleosides are integral for the formation of nucleic acids. Nucleosides are rapidly phosphorylated upon entry into the cell, forming nucleotides (Kalckar 1950), in a process where first the kinase nucleoside phosphorylase produces a nucleotide monophosphate, which can subsequently be phosphorylated into nucleotide di- and tri-phosphates (Van Rompay et al. 2000).

Nucleosides also play an important role in energy metabolism, as adenosine and guanosine act as precursor molecules for generating ATP and GTP as described above (Griffith and Jarvis 1996, Baldwin et al. 1999). In all living cells, purines are fundamental components for cellular activity. For instance, ATP and GTP are used by cells to perform energy dependent enzymatic reactions. Salvage pathways from the extracellular environment is the preferred pathway for regenerating the nucleoside pool (Murray 1971). Moreover, the uptake of nucleosides from the extracellular milieu is necessary for cell types lacking enzymes for the *de novo* synthesis of nucleotides such as cell types in the CNS, bone marrow, and intestines (King et al. 2006a, Leung et al. 2001). Nucleoside pools in the extracellular milieu accumulate from the breakdown of trinucleotides by extracellular phosphatases, as well as by the release of nucleosides from the cell or neighbouring cells (Yegutkin 2008). Intracellularly, the tightly regulated degradation of nucleoside triphosphates by ecto-enzymes can lead to excess nucleosides, where there is an ongoing turnover of nucleosides between incorporation into RNA, DNA, and existing as free nucleosides (Feinendegen et al. 1961). The regulation of both nucleoside and nucleotide pools, inside and outside the cell, are critical for a variety of signalling pathways. Nucleosides (chiefly

adenosine), the network of proteins which control their movement, and their role in signalling pathways will be explored in the next section.

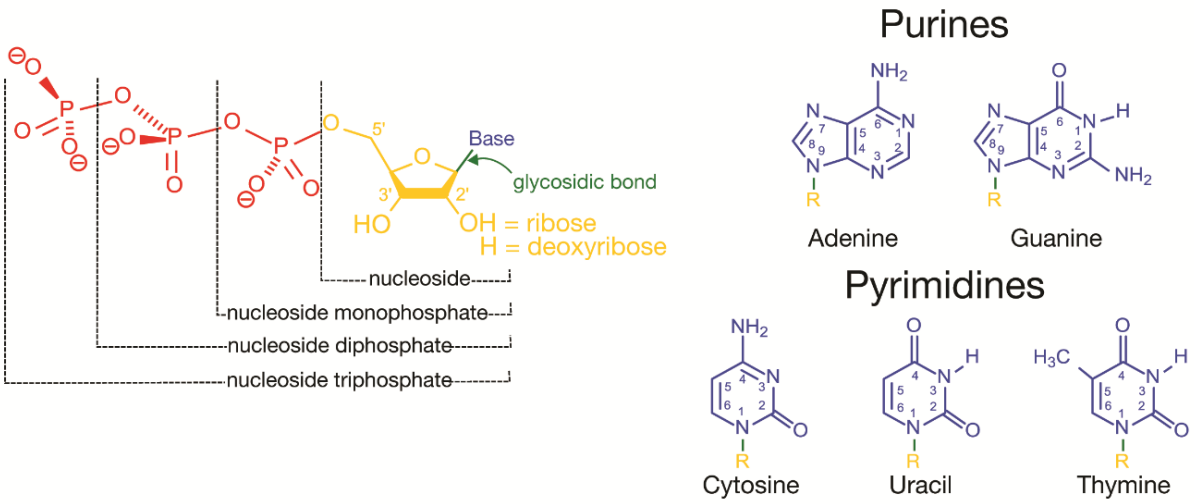

Figure 1: Structure of purine and pyrimidine nucleosides and nucleotides. Nucleosides consist of a nitrogenous base (right), and a ribose or deoxyribose sugar. When phosphorylated, these molecules are called nucleotides. Adapted from public domain image (https://en.wikipedia.org/wiki/Nucleoside#/media/File:Nucleotides_1.svg).

1.2.1 The Purinome [Adapted from dos Santos-Rodrigues et al. (2014) with permission from Elsevier Limited]

The purinergic signalling complex of a cell (the purinome) is a molecular network of purinergic ligands, receptors, enzymes, channels, and transporters (Volonté and D'Ambrosi 2009). Purinergic signalling is essential in the central nervous system (CNS) and cardiovascular system (CVS), where purinergic ligands, such as ATP and adenosine, act as autocrine and paracrine hormones. The role of ATP as an extracellular signalling molecule was first observed by Drury and Szent-Györgyi in 1929, when they demonstrated that extracellular ATP and adenosine had effects on heart rate and cardiovascular function (Drury and Szent-Györgyi 1929). Later, experiments by Holton (1959) demonstrated that ATP release followed antidromic stimulation of sensory nerves in rabbits. The important role of ATP and adenosine in extracellular signalling was corroborated by Ginsborg and Hirst (1972) when they showed that acetylcholine release could be modulated by adenosine. The concept that ATP could act as a neurotransmitter was later inferred from these and other findings by Burnstock in 1972, and this work defined purinergic signalling and regulation as a new and exciting field of study (Burnstock 1972). Further research has helped to elucidate the role of ATP and adenosine as players in purinergic signalling, but there is still much that remains unknown regarding the interactions between various proteins. There are thousands of distinct proteins that use purines as cofactors, implying that the purinome is diverse, ubiquitous, and essential to cellular function (Haystead 2006, Burnstock et al. 2011a, Coddou et al. 2011, Dale 2011, Zylka 2011, Schetinger et al. 2007, Samsel and Dzierzbicka 2011, Ferrero 2011, Lane et al. 2011). Our current understanding of adenosine function has come from many years of research, and some of the landmark studies will be described in the next section.

1.2.2 Adenosine

Extracellular adenosine levels play a critical role as a signalling molecule and examples can be seen by its ability to modulate vasodilation, insulin secretion, lipolysis, and inflammation (Ralavic and Burnstock 1998, Dunwiddie and Masino 2001). Adenosine also behaves as a neuromodulator within the central nervous system (CNS). However, adenosine is not stored and released from vesicles near synapses, so it is not considered a neurotransmitter. It instead modulates synaptic transmission by influencing transmitter release by interaction with adenosine receptors, which can both lead to changes in polarization or regulating the rate of neurotransmitter release (Cunha 2001). Adenosine has been shown to modulate a large variety of functions from memory, alertness, aggressiveness, anxiety, motor-activity, neuro- and cardioprotection, cognition, sleep, and arousal (Fredholm et al. 2005) and is clinically relevant, as adenosine effects have been correlated with diseases such as multiple sclerosis, Parkinson's disease, Huntington's disease, depression, schizophrenia, epilepsy, or stroke (Fredholm et al. 2005). The effects adenosine has on the body are profound, as it regulates energy use in cells and also acts as a signal which reduces cellular stress (Mubagwa and Flameng 2001). Basal levels of adenosine in the extracellular environment have been reported at concentrations ranging from about 30 to 150 nM (Conlay et al. 1997, Wall et al. 2007, Hui et al. 2012). Metabolic stress from stimuli such as a hypoxic challenge, ischemia, or inflammation leads to large and rapid increase in extracellular adenosine which can lead to protective effects in these tissues (Rose et al. 2010).

1.2.3 Adenosine Triphosphate (ATP)

ATP has dual function as a cellular source of energy and as an extracellular signalling molecule. ATP's effects on the heart and vasculature were first described by Drury and Szent-Györgyi in 1929. It is now known that ATP can be released as cotransmitter from nerves in both peripheral and central nervous systems (Burnstock 1976, Burnstock 2009). ATP is stored in vesicles and is released or co-released with other neurotransmitters, such as GABA (Pankratov et al. 2006). ATP can be hydrolyzed to adenosine by membrane bound ecto-enzymes which act on purinergic receptors (P2). The P_2 receptors are classified one of two families: the ionotropic, ligand-gated P_2X receptors, and the metabotropic G protein-coupled P_2Y receptors (Burnstock 2007).

1.2.4 P_2X receptors

The P_2X receptor subclass are ligand-gated ion channel receptors with seven receptor subtypes, $P_2X_{1\text{-}7}$. These receptors are permeable to Ca^{2+}, Na^{+}, and K^{+}, and have relative ion permeability depending on their oligomerization state (Burnstock 2007, Pankratov and Lalo 2014). P_2X receptors are composed of three subunits which form either homo- or heteromers. All homomers except P_2X_6 can allow ion flow, where P_2X_6 is only functional in a heteromer (Burnstock 2007). The other functional P_2X receptor heteromers found to date are $P_2X_{2,3}$, $P_2X_{1,2}$, $P_2X_{1,5}$, $P_2X_{2,6}$, $P_2X_{4,6}$, and $P_2X_{1,4}$ (Burnstock 2007).

1.2.5 P_2Y Receptors

The P_2Y receptor subclass are characterized by their status as G-protein coupled receptors. Originally five mammalian members, P_2Y_1, P_2Y_2, P_2Y_4, P_2Y_6, and P_2Y_{11}, were shown to activate the G_q and G_{11} which then activates the PLC, IP_3 and intracellular Ca^{2+} signalling (Ralevic and Burnstock 1998) but more recently another group of members were identified, P_2Y_{12}, P_2Y_{13}, and P_2Y_{14}, which linked to the G_i and G_o, which reduces adenylyl cyclase levels and modulates ion channels (Abbracchio et al. 2009). P_2Y receptors are not ubiquitous, and are expressed primarily in the CNS (Ralevic and Burnstock 1998). P_2Y receptor ligands include the adenine nucleotides ATP and ADP, and the uracil nucleotides UTP and UDP (Abbracchio et al. 2009). Similar to the P_2X subclass, oligomerization dictates P_2Y function as they are able to form both homo- and heterodimers with other members of the P_2Y family or A_1 receptors (Fischer and Krügel 2007). Intriguingly, purinoreceptors have been shown to localize intracellularly as well as at the cell surface for reasons that are yet to be determined (Burnstock 2015). This suggests that there may be more mechanisms in which purinoreceptors regulate the functions of the cell.

1.2.6 Adenosine Receptors

Adenosine's role in autocrine and paracrine signalling through interactions with adenosine receptors at the plasma membrane make it a widely studied molecule (Figure 2). Purinergic receptors at the cell surface mediate the cellular response to extracellular levels of adenosine. There are four types of G-protein coupled receptors, A_1, A_{2A}, A_{2B}, and A_3. These receptors can influence adenylyl cyclase activity which has numerous downstream effects for cellular signalling pathways (Figure 2). A_1 and A_3 (high and low affinity, respectively) have an inhibitory response and ligand binding leads to a decrease in adenylyl cyclase leading to a decrease in cAMP. Conversely, A_{2A} and A_{2B} (high and low affinity, respectively) are excitatory and lead to an increase in adenylyl cylcase, leading to an increase in cAMP (Burnstock 2007). Adenosine receptors, as with P_2 receptors, play an important role in the purinome and are widely studied (Figure 2). For example, A_3 receptors stimulated by elevated adenosine levels, resulting from prolonged high glucose levels, led to renal fibrosis (Kretschmar et al. 2016).

Purinergic signalling is critical for learning and memory, sleep, locomotive activity, mood, and behavior (Burnstock et al. 2011b). Moreover, dysfunction of this signalling plays a role in a wide array of neurodegenerative diseases, psychiatric disorders, migraines, epilepsy, cognitive impairment, and neuropathic pain (Burnstock et al. 2011b). These receptors are important in the regulation of many cellular processes, for example, the regulation of other membrane proteins. The next section will examine solute carriers, a family of membrane transporters, in more detail.

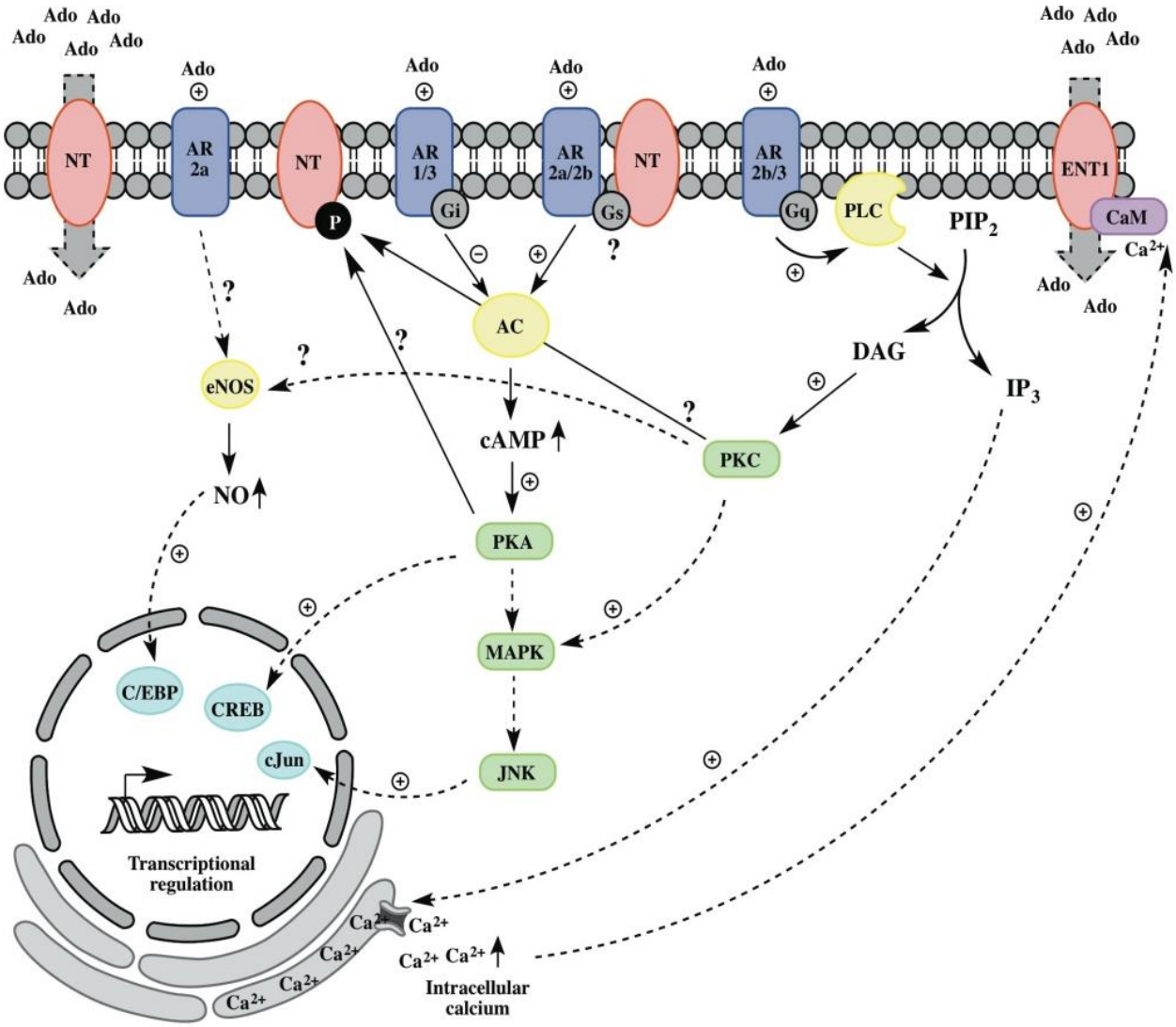

Figure 2: Cross-talk between members of the purinome. The interaction between receptors, transporters, kinases, etc. is a complex network which can regulate numerous downstream events as well as feeding back and regulating each other. Reprinted from Adenosine Signaling Mechanisms: Pharmacology, Functions and Therapeutic Aspects, volume 1, Chapter 2: Adenosine Membrane Transporters, pages 13-34, copyright (2015), Natalia Grañé-Boladeras, Alex Bicket, Maliha Zafar, Alexandre dos Santos-Rodrigues, and Imogen R. Coe, with permission from Nova Science Publishers, Inc.

1.3.1 Solute Carriers

Membrane proteins make up a significant proportion of the human proteome with approximately 27% of all proteins having a transmembrane domain (Almén et al. 2009). These proteins play a variety of important roles in cells: acting as enzymes, receptors, and transporters. The **Sol**ute **C**arriers (SLCs) are a group of integral membrane transport proteins which move a wide variety of solutes across biological membranes. The members are highly diverse, and most of the initial members of each family were identified through molecular cloning or protein purification. The SLC family nomenclature was initiated by the HUGO Gene Nomenclature committee to provide consistent naming for the gene family. There are currently, as of January 2016, 396 members of the SLCs, with 52 gene families (http://www.genenames.org/cgi-bin/genefamilies/set/752). Although these families all have over 20% sequence similarity, there is negligible homology between the members (Höglund et al. 2011). Almost all of the families consist of multispan membrane proteins and are commonly found at the plasma membrane, but there are instances of localization to the mitochondrial or nuclear membranes. Of these transporters, many have 8 to 14 transmembrane domains (hydrophobic regions of the protein), with most having a characteristic large extracellular loop and a cytosolic *C*- and *N*-terminal regions. Notable exceptions are SLC13, SLC24, SLC28, and SLC29 with an external *C*-terminus, SLC10 with an extracellular *N*-terminus, and SLC5, SLC38, and SLC39 with both external *C*- and *N*-termini (Pedersen et al. 2016).

1.3.2 Nucleoside transporters

In relation to the purinome, relatively little is known about the proteins which translocate nucleosides across biological membranes, the nucleoside transporters. Understanding the role of nucleoside transporters is valuable to the field since how nucleosides and nucleoside analog drugs (to be discussed in greater detail in section 1.5) cross biological membranes is a determinant of how effective signalling cascades will operate, and will often determine the efficacy of a drug. Understanding how nucleoside transporter regulation and function is modulated via the other members of the purinome is clinically relevant, especially with CNS and CVS therapies where adenosine signalling is abundant (Knapp et al. 2006, Murray and Bussiere 2009).

The nucleoside transporters consist of two gene families, SLC28 and SLC29. SLC28, the concentrative nucleoside transporters (CNTs), are cation-dependent symporters and have been shown in most cellular contexts to transport nucleosides unidirectionally. There are three CNT members expressed in humans: CNT1-3. The other nucleoside transporter family is SLC29, the equilibrative nucleoside transporters, which comprise four members (ENT1-4) in humans, are Na^+-independent, passive transporters (Griffith and Jarvis 1996). They have 11 putative transmembrane domains with a cytoplasmatic *N*-terminus and an extracellular *C*-terminus, a large extracellular loop between transmembrane domains 1 and 2, and a large intracellular loop between transmembrane domains 6 and 7 (Sundaram et al. 1998). They are characterized by their sensitivity NBTI (*S*-(4-Nitrobenzyl)-6-thioinosine), a specific, tight binding inhibitor of ENT1. ENT1 is sensitive to NBTI at nanomolar concentrations, while ENT2 is insensitive until micromolar concentrations (Parkinson et al. 2011). ENT and CNT substrate, distribution, and distinguishing characteristics are outlined in Table 1. ENTs and CNTs both transport nucleosides but do not

possess any significant sequence or structural similarities with each other or with any other protein family.

Table 1: Common features of nucleoside transporters. Reprinted from Adenosine Signaling Mechanisms: Pharmacology, Functions and Therapeutic Aspects, volume 1, Chapter 2: Adenosine Membrane Transporters, pages 13-34, copyright (2015), Natalia Grañé-Boladeras, Alex Bicket, Maliha Zafar, Alexandre dos Santos-Rodrigues, and Imogen R. Coe, with permission from Nova Science Publishers, Inc.

NT	Substrate	Tissue distribution	Distinguishing features
CNT1	Pyrimidine nucleosides	Kidney, liver, small intestine.	Substrate specificity
CNT2	Purine nucleosides and uridine	Heart, skeletal muscle, liver, kidney, intestine, pancreas, placenta and brain	Substrate specificity
CNT3	Pyrimidine and purine nucleosides	Widely expressed but most abundant in mammary gland, pancreas, bone marrow, trachea and intestine	Can use the Na^+ or H^+ gradient for nucleoside co-transport
ENT1	Pyrimidine and purine nucleosides	Ubiquitous	Very sensitive to inhibition by NBTI
ENT2	Pyrimidine, purine nucleosides, and nucleobases	Ubiquitous	Nucleobase transporter, role in nuclear membrane
ENT3	Pyrimidine, purine nucleosides, and adenine (at pH 5.5)	Ubiquitous	Intracellular localization, activity is pH dependent
ENT4 (PMAT)	Adenosine (at pH 5.5) and organic cations including serotonin	Ubiquitous	pH dependent adenosine transport, transports organic cations

1.3.3 Concentrative Nucleoside Transporters (SLC28)

The concentrative nucleoside transporters (CNTs) are transmembrane proteins of the SLC28 family and actively transport nucleosides unidirectionally across biological membranes (King et al. 2006a). CNTs are symporters, thus requiring a cation gradient in order to function, providing the energy to move nucleosides against their concentration gradient. CNTs have been identified in eukaryotes and prokaryotes with three isoforms (CNT1-3) found in mammals (King et al. 2006a). All CNTs transport only nucleosides, not nucleobases, but have different substrate specificity (Table 1). CNT1 has increased preference for pyrimidine nucleosides and is localized in the liver, kidney, intestine, and brain (Pennycooke et al. 2001). CNT2 has preference for purine transport, with the ability to transport uridine, and is more widely expressed in humans compared to CNT1 with it being expressed in the kidney, heart, liver, skeletal muscle, pancreas, placenta, brain, cervix, prostate, small intestine, rectum, colon, and lung (Pennycooke et al. 2001, Gray et al. 2004). CNT3 transports purines and pyrimidines, and is broadly expressed in mammary glands, pancreas, bone marrow, trachea, intestine, liver, lung, placenta, prostate, testis, brain, and heart tissues (Che et al. 1995, Gray et al. 2004). A crystal structure of *Vibrio cholerae* CNT (Figure 3), which possesses 39% amino acid identity to hCNT3, suggests that the functional unit of CNTs is a homotrimer (Johnson et al. 2012). Moreover, the three dimensional structure suggests that there are 8 transmembrane domains, which contradicts two dimensional membrane topology models which suggested that there were 13 transmembrane domains (Hamilton et al. 2001, Gray et al. 2004). Studies have suggested that mammalian CNTs have large intracellular *N*-terminal and extracellular *C*-terminal tails, which are not present in prokaryotic isoforms. This suggests that the terminal tails may be a more recent evolutionary addition and its importance in higher organisms

is highlighted by the region's importance for sorting and protein-protein interactions (Wang et al. 1997, Pinilla-Macua et al. 2012). Localization of CNT3 has also been shown to be regulated via Galectin-4 in HT-29 cells (Fernández Calotti et al. 2015). CNT2 and CNT3 have also been shown to be regulated by purinergic receptors in cholangiocytes (Godoy et al. 2014). Furthermore, mutational analyses have provided evidence to support the roles of Ser319, Ser353, Leu354, and Gln320 in CNT1 as well as Gly313, Met 314, Thr 347, and Val348 in CNT2 in substrate selectivity (Wang et al. 1997, Hamilton et al. 2001). Intriguingly, replacing transmembrane domains 8 and 9 of hCNT2 with hCNT1 domains, hCNT2 was converted from a pyrimidine specific transporter to a purine transporter (Wang et al. 1997). These studies highlight the importance of transmembrane domains 7, 8, and 9 in substrate selectivity.

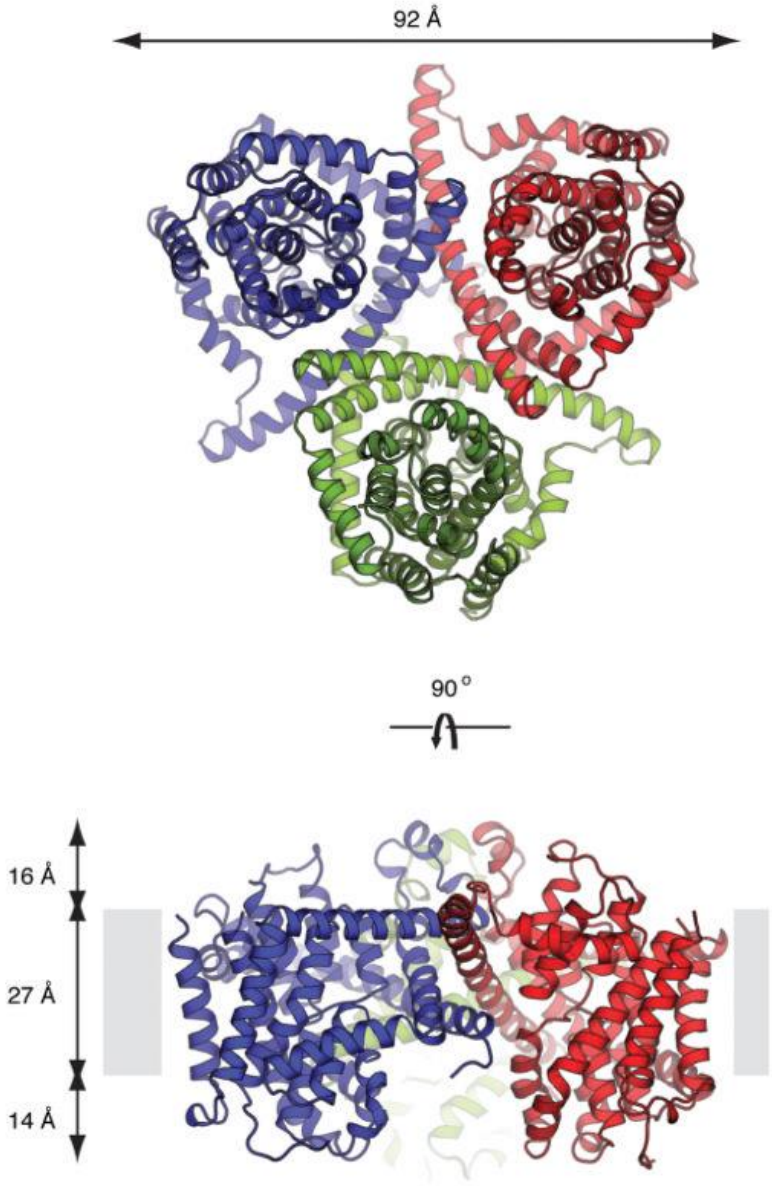

Figure 3: Three dimensional structure of VcCNT. The structure of VcCNT highlights the presence of transmembrane domains spatially oriented along the plasma membrane, which suggests that there are not 13 transmembrane domains but rather 8 per protein. This also suggests that these proteins form a functional trimer. Adapted from Johnson et al. (2012).

1.3.4 Equilibrative Nucleobase Transporter 1 (SLC43A3)

Until recently, only ENT1 and ENT2 (SLC29A1 and SLC29A2) were identified as able to transport nucleobases, which did not account for observations in red blood cells where adenine uptake halted in the presence of hypoxanthine but not nucleosides (Furukawa et al. 2015). The previously orphan transporter from the amino acid transporter family SLC43A3 has recently been identified as an equilibrative nucleobase specific transporter, designated equilibrative nucleobase transporter 1 (ENBT1). These transporters had previously been shown to be glycosylated, although the residue is unconfirmed, and are highly expressed in the liver and heart (Bodoy et al. 2013). Since the function of ENBT1 as a nucleobase transporter was only recently uncovered, the role ENBT1 plays in the purinome is still unknown.

1.3.5 Equilibrative Nucleoside Transporters

The equilibrative nucleoside transporters (ENTs) belong to the SLC29 gene family and mediate nucleosides down their endogenous concentration gradients (Griffith and Jarvis 1996). ENTs are evolutionarily ancient membrane proteins (Sankar et al. 2002), with four ENT members identified in mammals (ENT1-4), and with homologs existing in fungi, protozoans, nematodes, insects, and plants but not in bacteria (Cabrita et al. 2002, Acimovic and Coe 2002). The prototypical ENT, ENT1, is the best characterized due to its clinical implications on the uptake of nucleoside analog drugs (further discussion in section 1.5, see Tables 3 and 4). ENT1 is ubiquitously expressed in human tissues although the expression levels vary between tissues, with

high levels of expression observed in the heart, skeletal muscle, colon, bladder, uterus, stomach, pancreas, pituitary gland, adrenal gland, kidney, liver, small intestine, bone marrow, and placenta (Pennycooke et al. 2001). Most of the ENT1 life cycle takes place at the plasma membrane, yet some work has identified ENT1 at the mitochondria (Nivillac et al. 2011, Lee et al. 2006). ENT1 transports purines and pyrimidines with K_m of about 50 to 680 μM and does not transport nucleobases at physiologically relevant concentrations (Ward et al. 2000, Baldwin et al. 2004). Mammalian ENT1 is highly homologous to its isoform ENT2, with about 69% sequence similarity (Crawford et al. 1998). ENT2, originally referred to as NBTI-insensitive nucleoside transporter, and as mentioned previously, is much less sensitive to NBTI. ENT2 is broadly expressed in tissues and is found in skeletal muscles, heart tissue, the pancreas, placenta, prostate, brain, kidney, lung, and thymus (Pennycooke et al. 2001). ENT2 transports purines, pyrimidines, and nucleobases (Yao et al. 2002). In terms of structure and function, ENT3 is not as well characterized (Hyde et al. 2001). Mammalian ENT3 is broadly expressed and is rarely localized in the plasma membrane, but is primarily found in the lysosomes, endosomes, and mitochondria, which may function to free nucleosides from recently digested nucleic acids in these vesicles (Govindarajan et al. 2009, Baldwin et al. 2005). ENT3 is insensitive to NBTI with broad substrate selectivity, transporting purines, pyrimidines, and nucleobases (excluding hypoxanthine). ENT4 was identified based on its similar topology to ENT1-3, and has a broad distribution, found in the brain, skeletal muscle, kidney, heart, and liver, but has low sequence similarity (approximately 20%) to the other ENTs (Acimovic and Coe 2002, Engel et al. 2004). ENT4 is able to transport adenosine at an acidic pH, and is inhibited by dipyridamole and dilazep, supporting the inclusion as an ENT (Baldwin et al. 2005, Barnes et al. 2006, Zhou et al. 2010). ENT3 and ENT4 have also been shown to be pH-dependent (Baldwin et al. 2005, Barnes et al. 2006).

1.4.1 Equilibrative Nucleoside Transporter 1 structure, function, and life cycle

Putative membrane topology of ENTs has 11 transmembrane domains, with an intracellular *N*-terminus and extracellular *C*-terminus (Figure 4). There is a large extracellular loop between transmembrane domains 1 and 2 of ENT1, a large intracellular loop between transmembrane domains 6 and 7 predicted to be critical for regulation of the protein, with a variety of putative protein binding sites, and phosphorylation sites (Yap et al. 2000, Reyes et al. 2011b, Hughes et al. 2015). Putative pore forming transmembrane domains (2, 4, 5, 9, 10, and 11) are required for nucleoside transport (Endres and Unadkat 2005, SenGupta et al. 2002, SenGupta and Unadkat 2004, Park and Hammond 2012). ENT1 is ubiquitously expressed in humans, but the levels of expression change depending on individual, tissue type and cell type (Pennycooke et al. 2001). ENT1 was originally identified due to its sensitivity to inhibition by NBTI, a nucleoside analog that tightly binds ENT1 near the predicted binding pocket, since other ENT family members are not sensitive to NBTI in nanomolar concentrations. NBTI interacts with ENT1 on transmembrane domains 3 to 6 at a high affinity binding site with noncovalent interactions on the extracellular side of the protein (Sundaram et al. 2001).

A crystal structure for ENT1 has yet to be resolved, but the *Leishmania donovani* nucleoside transporter 1.1 (LdNT1.1) has been used as a model for determining ENT1 structure (Valdés et al. 2004). *Ab initio* structural models and computational modeling for extracellular and intracellular closed conformations have identified candidates for the transmembrane domain helices in ENT1 which are proposed to form an intracellular gate (Valdés et al. 2009, Valdés et al, 2012, Valdés et al, 2014, Figure 5). Additionally, the *C*-terminal transmembrane domains have been shown to be required for correct protein folding and trafficking of ENT1 when expressed in

COS-7 and MCF-7 cell lines (Nivillac et al. 2009) and in *X. laevis* oocytes (Aseervatham et al. 2015). It has been postulated that translocation of nucleosides by ENT1 may occur through alternating between outward and inward facing conformations, but to date, has not been directly shown (Bone et al. 2007, Ramadan et al. 2014, dos Santos-Rodrigues et al. 2014). After translation, ENT1 is trafficked to the plasma membrane. Work conducted on GFP-hENT1 in COS-7 cells suggests that the complete life cycle of the protein is ~14 hours, from translation, microtubule-dependent trafficking to the plasma membrane, and finally, clathrin-dependent endocytosis and lysis (Nivillac et al. 2011). The half-life of hENT1 pools at the plasma membrane are approximately 7 hours (Nivillac et al. 2011).

There is a clear relationship between structure, function, and expression of ENT1 in drug uptake which makes understanding ENT1 critically important for optimizing drug delivery and efficacy (Cano-Soldado and Pastor-Anglada 2012). Each individual has a unique profile of CNT and ENT expression (Pennycooke et al. 2001), which could lead to customized treatment for nucleoside drug treatments. This is important since ENT1 function is critical for the uptake of many nucleoside analog drugs (Errasti-Murugarren and Pastor-Anglada 2010). For example, ENT1 has been shown to play a critical role in absorption of ribavirin (Endres et al. 2009).

The regulatory mechanisms which control ENT1 expression, localization, and function will be explored in detail in the following sections (Figure 6).

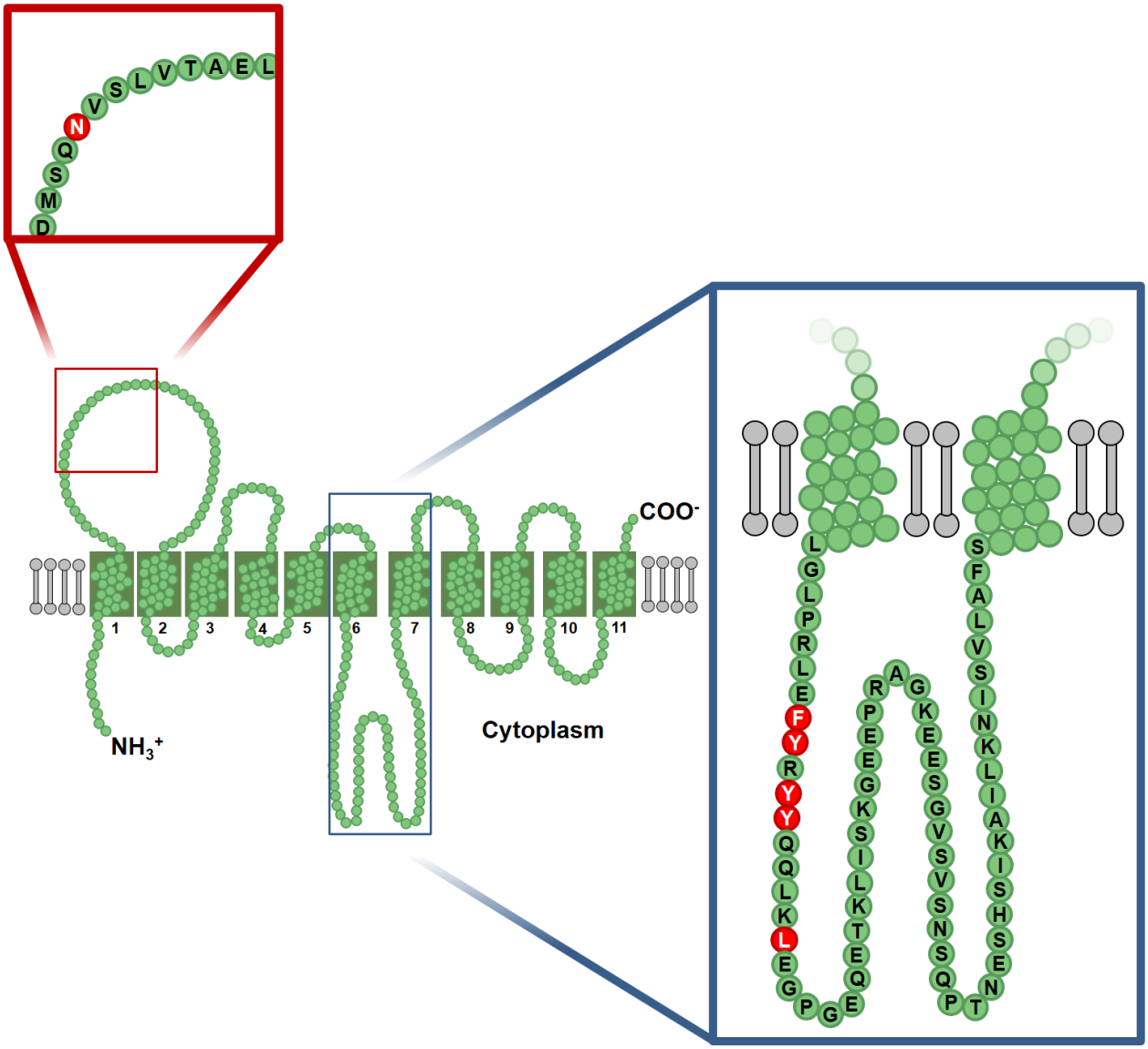

Figure 4: The predicted two-dimensional topology of ENT1. ENT1 has 11 putative transmembrane domains. The large intracellular loop between transmembrane domains 6 and 7 (blue box) is predicted to be critical for regulation of the protein and contains phosphorylation sites, as well as a calmodulin binding site (residues highlighted in red; discussed in Chapter 2). The extracellular loop between transmembrane domains 1 and 2 (red box) contains a glycosylation site at Asn48 (residue highlighted in red; discussed in Chapter 3).

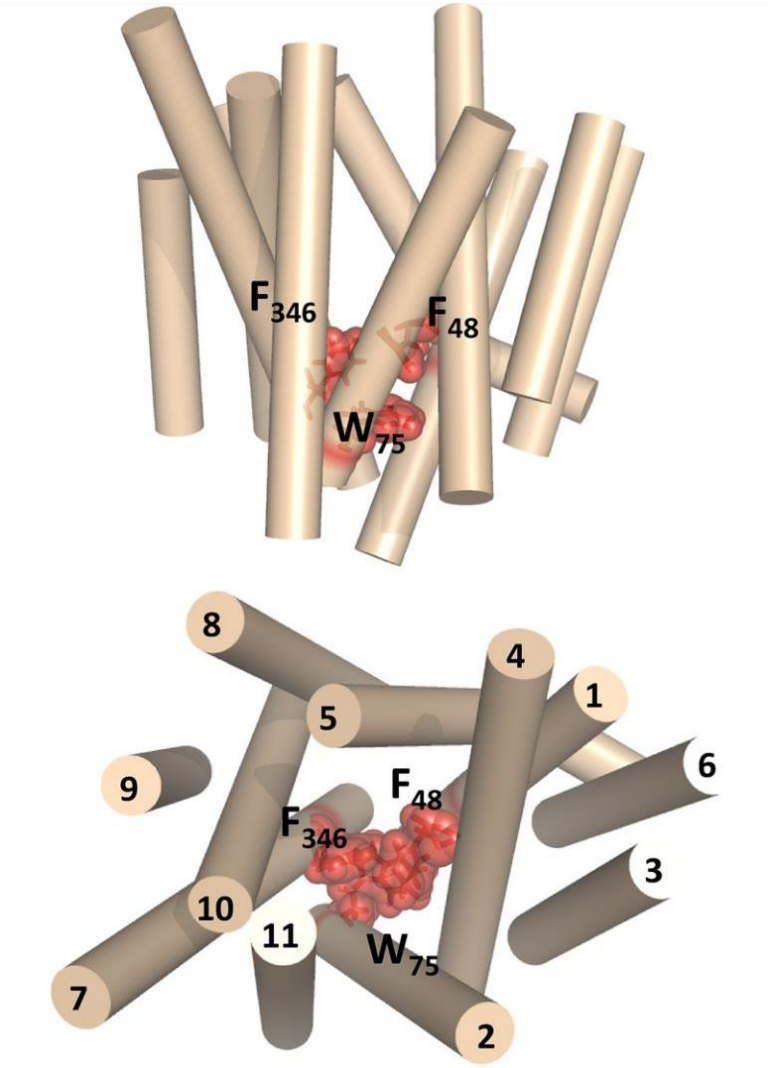

Figure 5: The three dimensional structure of LdNT1.1 *ab initio* model. This supports the critical role of aromatic amino acids in the proposed extracellular gate formed by transmembrane domains 1, 2, and 7. Figure adapted from Valdés et al. (2009).

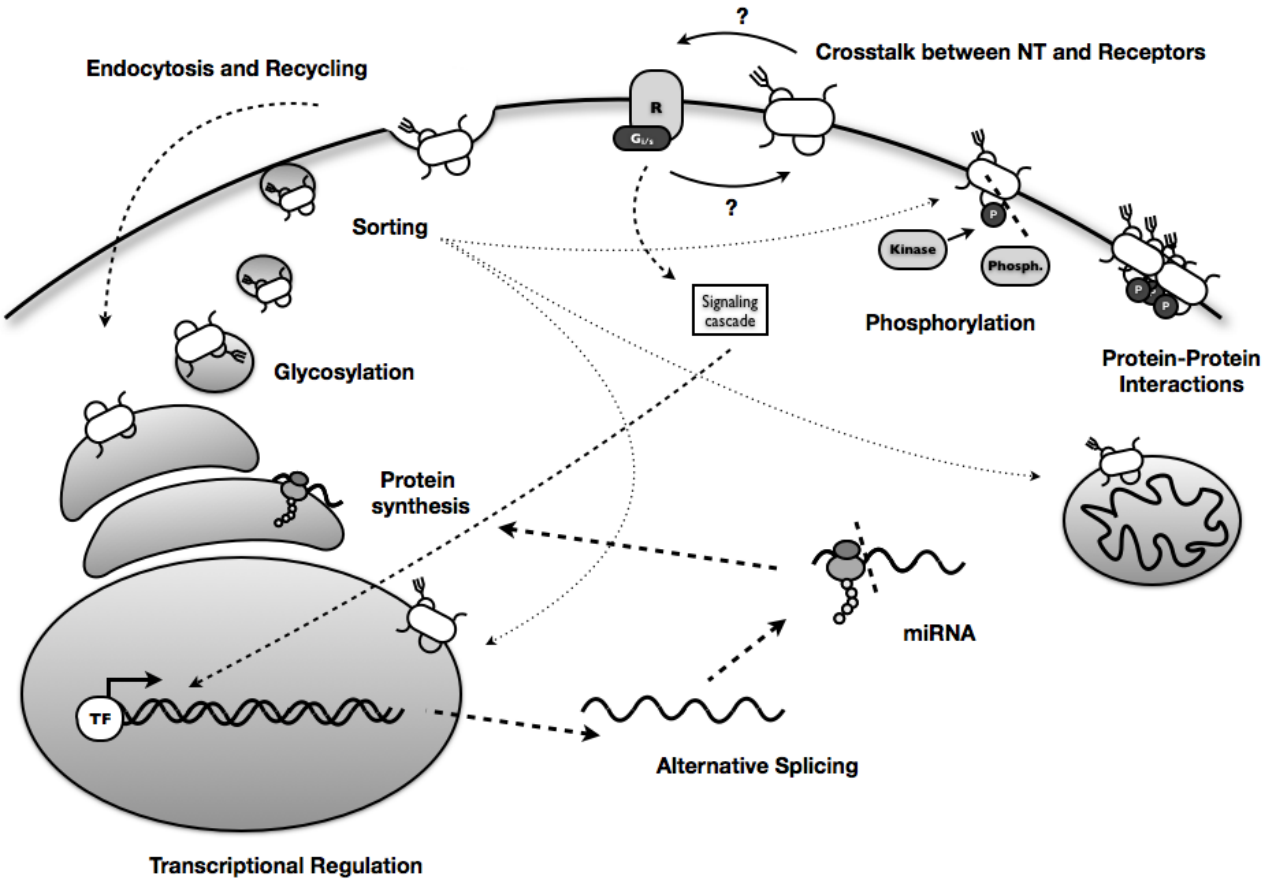

Figure 6: Overview of ENT regulation in the cell. This figure is an overview of how various mechanisms regulate ENT1 localization and function. Adapted from dos Santos-Rodrigues et al. (2014).

1.4.2 Transcriptional regulation of ENT1

ENT1 transcriptional regulation has been well characterized in mouse and human genes, with each isoform having a unique set of promoter consensus sequences. In mENT1, GATA-1, IRF-2, Pit-1, myogenin, CREB, Sp-1, Ap-2, MAZ, and GR consensus sequences have been identified in the 5′-flanking sequence (Choi et al. 2000). Other work has suggested that cJun binding the promoter region at an AP-1 site down regulates mENT1 in HEK293 cells, suggesting a role for JNK-cJun signalling pathway in the transcriptional regulation of mENT1. There are many possible transcription factors which may regulate hENT1, particularly in the -255 bp to +1 bp region (Abdulla and Coe 2007). Also, since hENT1 expression is highly variable between individuals, tissues, and cell types, a transcriptional mechanism of hENT1 regulation seems likely (Pennycooke et al. 2001). In mouse macrophages, macrophage-colony stimulating factor was shown to upregulate ENT1 mRNA as well as function which promoted proliferation (Soler et al. 2001). In CLL cells, hENT1 expression was enhanced by IL-4 and PMA (Fernández Calotti et al. 2008). It has been shown that MAZ and Sp-1 are critical positive regulators for hENT1 (Choi et al. 2000). Conversely, hENT1 is down regulated in the epithelium, heart, and lungs by HIF-1 and NF-κB–mediated transcriptional repression of the hENT1 promoter (Eltzschig et al. 2005, Morote-Garcia et al. 2009, Morote-Garcia et al. 2013). Interestingly, there have been three mENT1 splice variants identified but none for hENT1 (Choi et al. 2000, Robillard et al. 2008).

1.4.3 Phosphorylation

Protein phosphorylation is a reversible post-translational modification which occurs in both prokaryotes and eukaryotes, and is a critical means of post-translational regulation of proteins (Humphrey et al. 2015). Analysis of the human genome suggests that more than 30% of human proteins are phosphorylated at least once (Pinna and Ruzzene 1996, Cohen 2002). Prediction of phosphorylation sites is possible in many cases by identifying consensus sequences in the amino acid sequence (Table 2). Of the significant proportion of proteins which are phosphorylated, global-mass spectroscopy studies have predicted that the ratio of residues phosphorylated between serine, threonine, and tyrosine on human proteins are approximately 79.3% on serine, 16.9% on threonine, and on 3.8% tyrosines residues (Olsen et al. 2006). Phosphorylation of these residues occurs by the transfer of a phosphate mediated by a kinase and the removal of phosphates on these residues is performed by phosphatases, which occurs any time after the protein leaves the ER (Procino et al. 2003). Since phosphorylation is reversible, there is an important balance between the presence and absence of a phosphate on a site. The negatively charged phosphate addition in many cases causes conformational changes on the protein, and can also block protein binding sites. Phosphorylation has been shown to activate and deactivate proteins, modulate protein function, translocation, oligomerization, and the accessibility of protein binding domains (Machowska et al. 2015, Wagih et al. 2016).

There are putative phosphorylation sites in ENT1 and ENT2 which may be regulated via PKA, PKC, CKII, and CKQ (Kiss et al. 2000, Stolk et al. 2005, Robillard et al. 2008). When stimulated by PKC, there is an increase in ENT1-dependent nucleoside transport which suggested phosphorylation may be regulating ENT1 (Coe et al. 2002). PKA and PKC phosphorylation of

ENT1 was established at multiple sites of the large intracellular loop of ENT1 *in vitro*, suggesting that phosphorylation is indeed a key regulator of ENT1 (Reyes et al. 2011b). Disrupting phosphorylation of ENT1 has been shown to have functional consequences, for example, inhibiting CKII activity reduced ENT1 affinity for NBTI, and reduced ENT1 protein abundance at the plasma membrane (Bone et al. 2007). Furthermore, direct ENT1 phosphorylation has been identified at Ser281 in a PKC-dependent manner in response to adenosine A_1 receptor activation in pig kidney cells (Hughes et al. 2015).

Table 2: Common kinase consensus sites. Adapted from Ubersax and Ferrel (2007).

Kinase	Full Name	Consensus phosphorylation site
PKA	Protein kinase A/cAMPdependent protein kinase	R-R-X-S/T-Φ
CDK	Cyclin-dependent kinase	S/T-P-X-K/R
ERK2	Extracellular-regulated kinase-2	P-X-S/T-P
CK1	Casein kinase-1	pS-X-X-S/T
CK2	Casein kinase-2	S/T-D/E-X-E/D
CaMK2	Calmodulin-dependent protein kinase-2	R-X-X-S/T
EGFR	Epidermal growth factor receptor	E-E-E-Y-F
PKB/AKT	Protein kinase B	R-X-R-X-X-S/T

1.4.4 Glycosylation

With more than half of the mammalian proteome predicted to be glycosylated (Apweiler et al. 1999), and with an Mgat-1 (a glycotransferase) gene mutation leading to prenatal mortality in mice (Ioffe and Stanley 1994), it is clear that glycosylation is a post-translational modification that is critically important. There are two types of glycosylation, *N*- and *O*- linked. The addition of an *N*-linked glycan occurs in the ER, while *O*-linked is a transient event which occurs to cytosolic residues. To date, *O*-linked glycosylation (the transient addition of a glycan to an OH of a Ser or Thr residue on the intracellular side of proteins) has been identified in other members of the SLC family of proteins (Tse et al. 1994, Counillon et al. 1994), but the physiological relevance of this interaction with these proteins has yet to be determined so the focus of this section will be on *N*-linked glycosylation. This modification was thought to only occur in eukaryotes until the 1980's when *H. salinarum* was identified to modify proteins with a tetrasaccharide using the same common motif found in eukaryotic proteins (Wieland et al. 1983, Wieland et al. 1985). This suggests that *N*-linked glycosylation is an evolutionarily conserved modification and highlights its importance.

N-linked glycosylation is initiated in the ER during protein folding by the addition of a dolichol phosphate precursor saccharide to Asn residues with the appropriate consensus motif at the extracellular side (the lumen of the ER) of the membrane (Mookerjea et al. 1983). The large oligosaccharide chain, consisting of 3 Glucose, 9 Mannose, and 2 *N*-Acetylglucosamine molecules in higher eukaryotes, is transferred to an *N*-linked glycosylation site NX(S/T) where X is any residue but proline (Kornfeld and Kornfeld 1985). The oligosaccharyltransferase (OST), the enzyme responsible for transferring the oligosaccharide of the dolichol phosphate precursor to the

protein, requires 14 downstream (towards the *C*-terminus) residues and 12 upstream (towards the *N*-terminus) residues to add the glycan to multispan membrane proteins (van Geest and Lolkema 2000, Mohorko et al. 2011). The *N*-glycan is trimmed, and modified as it matures through the ER and Golgi.

Since such a large proportion of the proteome is *N*-glycosylated, it is unsurprising that this modification is often critical for the function of membrane transporters (Pedersen et al. 2016). Eukaryotic cells are coated with a layer of carbohydrate and membrane glycolipids known as the glycocalyx, which acts as support for membrane proteins, contributes to cell adhesion, and protecting the plasma membrane from chemical stress (van den Berg et al. 2006). The addition of an *N*-glycan to a membrane protein is critical for a number of reasons. For instance, the localization of membrane transporters is critical, particularly in polarized cells when altered expression and distribution of membrane proteins is required to regulate function of each membrane (Orlando and Guo 2009). Polarized cells exist in many cellular contexts, and any dysfunction can lead to serious maladies. Dependence on *N*-glycosylation for correct sorting, the mechanism in which proteins are differentially transported to targeted areas in or outside of the cell, is observed with the sulfate transporter NaSi-1 (Regeer et al. 2007) and with the glycine transporter GLYT2 in polarized kidney cells (Martinez-Maza et al. 2001). Sorting is not always affected though, for example, the sodium-dependent, purine-selective nucleoside transporter SPNT has the same distribution regardless of glycan addition in the renal epithelium (Mangravite and Giacomini 2004). There are also many described cases where glycosylation is required for proper folding of the protein. For example, glycosylation of the glucose transporter GLUT4 enhances protein folding and conformational stability resulting in increased cell surface expression (Tortorella and Pilch 2002). Although glycosylation is often critical for proper protein targeting and folding, it is also common

for proper localization (Asano et al. 1993, Olivares et al. 1995, Melikian et al. 1996, Martinez-Maza et al. 2001, Hoover et al. 2003, Chen et al. 2006, Paredes et al. 2006, Unal et al. 2008, Subramanian et al. 2008, Dorn et al. 2009, Hayashi and Yamashita 2012, Console et al. 2015) as well as function (Levy et al. 1998, Choi et al. 2003).

As with most SLC family members (Landolt-Marticorena and Reithmeier 1994), ENTs have a large extracellular loop between TMDs 1 and 2. For ENT1, this loop contains an NxS motif at Asn48 (Figure 4) with over 12 residues upstream, and 14 downstream between it and the closest transmembrane domain (adequate space for the recruitment of the oligosaccharyltransferase to add the backbone of the glycan chain onto the nascent protein), which made this site likely to be *N*-glycosylated (Kwong et al. 1986, Mohorko et al. 2011). ENT2 shares the glycosylation site an N48 as an NxT motif, as well as an NxT motif at N57, both are glycosylated. The first study on ENT1 glycosylation was undertaken by Vickers et al. (1999) and reported that *N*-glycosylation-defective mutant ENT1 was characterized by a higher ENT1 protein abundance at the plasma membrane, was functional, and had reduced affinity for NBTI. ENT1 glycosylation was later examined by expressing wild type, N48Q-, N277Q-, and N288Q-ENT1 in *X. laevis* oocytes, which confirmed that Asn48 was the only amino acid glycosylated in a non-mammalian model (Sundaram et al. 2001).

ENT1 is required for the uptake of many nucleoside analog drugs used in treating cancer, viruses, and parasites, and plays a key role in cardioprotection, purinergic signalling pathways, learning, and behaviour (Hillgren et al. 2013, Rose et al. 2010, dos Santos-Rodrigues et al. 2014, Knight et al. 2010). Understanding the fundamental mechanisms of regulation for ENT1 are necessary to guide research on optimizing patient outcomes using these drug therapies. Although glycosylation of ENT1 is important for drug design and enhancing drug uptake with currently used

therapies, relatively little is known about how glycosylation effects ENT1 function when expressed in mammalian cells. In the next section, I will further discuss the clinical relevance of ENTs.

1.5 Clinical significance of ENTs

The cloning of the first nucleoside transporters in 1997 (Griffiths et al. 1997) has led to the beginning of exciting research on how regulation of these proteins can improve the efficacy of nucleoside analog drugs, providing a significant clinical role for our research. Nucleoside transporters are an important route for many classes of drugs to enter the cell. Intervention using nucleoside analogs, synthetically generated compounds which are modified versions of native nucleosides transported via nucleoside transporters, have been beneficial for the treatment of various forms of cancers, cardiovascular disorders, neurological conditions as well as viral and parasitic infections (Hillgren et al. 2013).

Nucleoside analog drugs have similar structures to the naturally occurring nucleosides which results in them following a similar path into the cells, from uptake to metabolism. There are currently 34 Food and Drug Administration (FDA) and European Medicines Agency (EMA) approved nucleoside analog drugs listed within the Drugbank database (Wishart et al. 2008). The demand for new and more effective therapeutics is ever growing, driving the development of experimental nucleoside analogs (Jordheim et al. 2013), particularly to meet the demand for compounds or adjuvant therapies which can overcome drug resistance (Peng et al. 2008, Cai et al. 2008). Many of the nucleoside analog drugs available are used in the treatment of cancer. The first

nucleoside analog approved as a treatment for cancer was when the pyrimidine analog cytarabine (AraC) entered the market in 1969 with FDA approval under the trade name Cytosar-U®. It is widely used to treat hematological malignancies such as acute lymphoblastic leukemia, chronic myelocytic leukemia, erythroleukemia, and mantle-cell lymphoma (Lamba 2009). AraC is primarily transported into the cell via ENT1 (Damaraju et al. 2003, Hubeek et al. 2005, Zhang et al. 2007). It is then metabolized by phosphorylation to form the activated form of the drug, AraCTP. This compound is cytotoxic through its incorporation into nascent DNA and RNA, which leads to subsequent chain termination of the nucleic acids. Individuals or cell types with decreased expression of SLC29A1 is associated with reduced level of intracellular AraC uptake which leads to increased cancer resistance to AraC (Hubeek et al. 2005, Sarkar et al. 2005, Kanno et al. 2007). This supports our understanding that cells unable to transport nucleoside analogs will have reduced efficacy resulting in patients not responding to drug therapy. Similar patterns emerge with other nucleoside analog drugs. Another pyrimidine analog is gemcitabine (Figure 7), which is used as the primary or adjuvant therapy in the chemotherapeutic treatment of pancreatic carcinomas and adenocarcinomas, non-Hodgkin's lymphomas, non-small lung cancer, cervical cancer, bladder cancer, and breast cancer (Pérez-Torras et al. 2008, de Sousa Cavalcante and Monteiro 2014, Di Marco et al. 2016, Oguri et al. 2007, Massari et al. 2015, Moysan et al. 2013). With gemcitabine, ENTl is the primary route of entry into the cell and is therefore a predictor of survival with adjuvant gemcitabine monotherapy (Morinaga et al. 2012, Mackey et al. 1998), but ENT2, CNTl, and CNT3 also play a role in uptake of gemcitabine (Santini et al. 2011). The mode of action this drug takes is similar to AraC, and once phosphorylated, is incorporated into nucleic acids. Gemcitabine differs in its ability for the diphosphate metabolite to inhibit ribonucleotide reductase, which reduces the pool of available deoxynucleotide triphosphates for DNA synthesis. This leads to a

compounding effect, since less nucleosides are available in the nucleoside pools, then more gemcitabine will be incorporated into nucleic acids. Gemcitabine also acts in a third mechanism, whereby drug uptake leads to increased radiosensitivity (Pauwels et al. 2005). It is postulated that gemcitabine incorporation leads to higher propensity of DNA breakage by radiation therapy (Doyle et al. 2001). As with AraC, reduced ENT1 expression has detrimental effects on patient outcomes (Spratlin et al. 2004). Purines are also used in treatment of cancer through similar mechanisms as discussed above; for example, fludarabine is used for threatment of lymphoproliferative disease (King et al. 2006b). Among the 14 nucleoside analog drugs used for the treatment of cancer listed at this time (Table 3), ENT1 is identified for 13 of them as a route of entry for the drug. The only instance is floxuridine, which primary entry into the cell is through CNT1, CNT2 and CNT3 (Pastor-Anglada and Pérez-Torras 2015, Lang et al. 2001, Smith et al. 2004, Hu et al. 2006).

Parasites do not have anabolic enzymes required for *de novo* synthesis of nucleotides, therefore solely rely on their host for the uptake of nucleosides to meet their demand. This exposes them to treatment with nucleoside analog drugs. Some parasites which are treated with nucleoside analog drugs are *Plasmodium falciparum* (malaria), *Trypanosoma brucei* (African trypanosomiasis) and *Toxoplasma gondii* (toxoplasmosis) (Frame et al. 2015A, Landfear 2010, Frame et al. 2015B). Novel inhibitors of PfENT1 identified have also been proposed as a method to treat malaria, which is important due to drug resistance that can occur (Frame et al. 2015B).

Nucleoside analog drugs are commonly used to treat viral infections like the human immunodeficiency virus (HIV), Hepatitis B, and Hepatitis C viruses (Varatharajan and Thomas 2009, Liu et al. 2016, Iikura et al. 2012). Some examples of these drug are ribavirin, and zidovudine (Figure 7, Table 4). Antiviral drugs have a similar mechanism of action as cancer drugs,

as they are phosphorylated following uptake but instead of initiating strand termination, viral DNA incorporation into the host DNA is blocked due to competition with the drug metabolites, or by acting as reverse transcriptase inhibitors (Clercq and Field 2006).

Evidence to support a role of ENT1 on the development of diabetic nephropathy has recently been established where prolonged high glucose exposure in renal kidney cells led to decreased ENT1-dependent adenosine uptake and subsequent fibrosis of the tissue resulting from adenosine receptor A_3 activation (Kretschmar et al. 2016). There are also a variety of drugs that interact with nucleoside transporters such as dilazep, dipyridamole, and draflazine (Figure 7) which can alter adenosine levels leading to carioprotective effects or vasodialatory effects (Rose et al. 2010, Noji et al. 2004, Chakrabarti and Freedman 2008, Paproski et al. 2008). The widespread use of these drugs highlight the importance of understanding nucleoside transporters not only for the uptake of drugs used for interfering with DNA replication, cell division, and DNA integration, but treatment of blood pressure, blood clotting, and cardio- or neuroprotection following a stroke or heart attack.

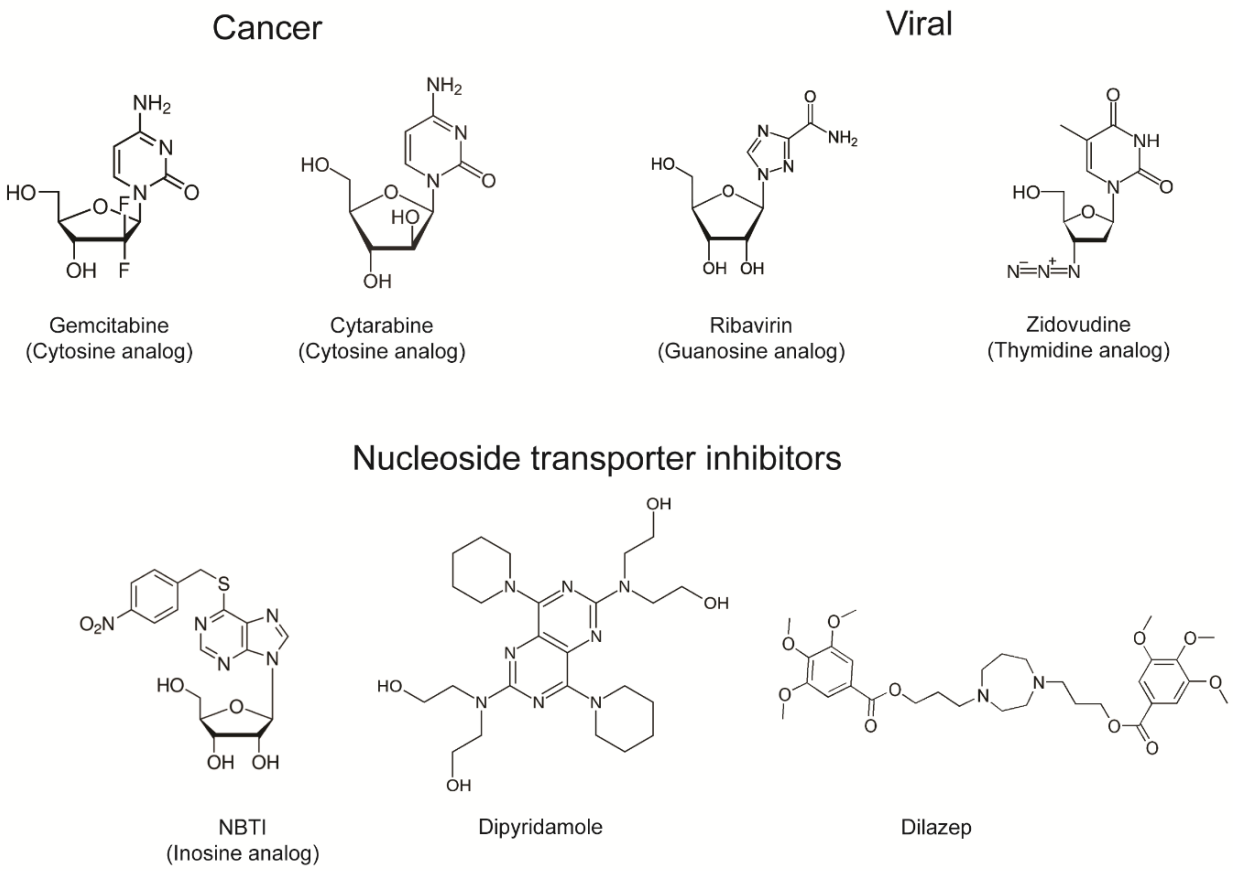

Figure 7: Examples of nucleoside analog drugs, and nucleoside inhibitors which are transported, bind, or inhibit ENT1.

Table 3: FDA approved nucleoside analog drugs used for the treatment of cancer. Adapted from Pastor-Anglada and Pérez-Torras (2015).

Drug	Therapeutic use	Analogous structure	FDA approval	Identified uptake transporters
Mercaptopurine	Lymphoproliferative diseases	Purine	1953	hCNT3, hENT1, hENT2
Cytarabine	Lymphoproliferative diseases	Pyrimidine	1969	hCNT1, hENT1, hENT2
Fludarabine	Lymphoproliferative diseases	Purine	1991	hCNT2, hCNT3, hENT1, hENT2
Pentostatin	Lymphoproliferative diseases	Purine	1991	hENT1, hENT2
Cladribine	Lymphoproliferative diseases	Purine	1993	hCNT2, hCNT3, hENT1, hENT2
Azacitidine	Lymphoproliferative diseases	Pyrimidine	2004	hCNT1, hCNT2, hCNT3, hENT1,hENT2,
Clofarabine	Lymphoproliferative diseases	Purine	2004	hCNT2, hCNT3, hENT1, hENT2
Nelarabine (AraG)	Lymphoproliferative diseases	Purine	2005	*hENT1 and hENT2*
Decitabine	Lymphoproliferative diseases	Pyrimidine	2006	hENT1, hENT2
Floxuridine	Solid tumors	Pyrimidine	1970	hCNT1, hCNT2, hCNT3
Gemcitabine	Solid tumors	Pyrimidine	1996	hCNT1, hCNT3, hENT1, hENT2, hENT3
Capecitabine (5-DFUR)	Solid tumors	Pyrimidine	1998	*hCNT1, hCNT2, hCNT3, hENT1 and hENT2*
5-Fluorouracil	Solid tumors	Pyrimidine	1998	hENT1, hENT2, hOAT2

Table 4: FDA approved drugs used for the treatment of viruses. Adapted from Pastor-Anglada and Pérez-Torras (2015).

Drug	Therapeutic use	Analagous structure	FDA approval	Identified uptake transporters
Ribavirin	HCV	Purine	1998	hCNT2, hCNT3, hENT1, hENT2
Sofosbuvir	HCV	Purine	2013	hOATP1B1
Adefovir	HBV	Purine	2003	hOAT1, hOAT3
Entecavir	HBV	Purine	2004	hOAT1, hOAT3, hPEPT2
Telbivudine	HBV	Pyrimidine	2006	
Lamivudine	HIV, HBV	Pyrimidine	1995	hENT3, hOCT1, hOCT2, hOCT3
Tenofovir	HIV, HBV	Purine	2001	hOAT1, hOAT3
Emtricitabine	HIV, HBV	Pyrimidine	2003	hOCT1, hOCT2, hOCT3
Zidovudine	HIV	Pyrimidine	1987	hCNT1, hCNT3, hENT2, hENT3, hOAT1, hOAT2, hOAT3, hOAT4
Didanosine	HIV	Purine	1991	hCNT2, hCNT3, hENT1, hENT2, hENT3
Zalcitabine	HIV	Pyrimidine	1992	hCNT3, hENT1, hENT2, hOCT1, hOCT2
Stavudine	HIV	Pyrimidine	1994	hCNT1, hCNT3, hENT3
Abacavir	HIV	Purine	1998	hOCT1, hOCT2, hOCT3
Acyclovir	Herpes	Purine	1982	hOCT1, hOAT1, hOAT2
Penciclovir	Herpes	Purine	2002	hOAT1, hOAT2, hOAT3
Famciclovir (Penciclovir)	Herpes	Purine	2007	
Ganciclovir	Herpes, CMV	Purine	1989	hOCT1, hOAT1, hOAT2
Valaciclovir (Acyclovir valyl ester)	Herpes, CMV, EBV	Purine	1996	hPEPT1, hPEPT2, ATB(0,+)
Cidofovir	CMV	Pyrimidine	1996	hOAT1, hOAT3
Valganciclovir (Ganciclovir valyl	CMV	Purine	2001	hPEPT1, hPEPT2, ATB(0,+)

1.6.1 Calcium

The divalent ion calcium (Ca^{2+}) is the most abundant mineral in the body, and has many critical roles in the body. Over 99% of calcium is stored in teeth and bones where it plays a structural role, but it is also an ubiquitous secondary messenger. Calcium has the unique ability to precipitate both inorganic and organic anions in approximately millimolar concentrations, which makes it essential for calcium to be removed, sequestered, or compartmentalized in cells (Williams 2006). Calcium signalling is involved in numerous events such as synaptic transmission in neurons, apoptosis, muscle contraction, CaM kinase dependent phosphorylation, exocytosis, cell motility, or TCF- SRF- and CRE-linked gene expression (Bootman 2012, Johnson et al. 1997). The paramount importance of calcium signalling is highlighted by its conserved evolutionary role. Calcium pumps, channels, cation exchangers, and calcium binding proteins are found in prokaryotes and aid in maintaining calcium homeostasis (Plattner and Verkhratsky 2015).

Towards the end of the 19th century, Sydney Ringer discerned that trace levels of calcium were required to support a heartbeat in *X. Laevis* cardiomyocytes (Ringer 1883). The mechanism in which muscle contraction is dependent on calcium was discovered in 1951 when it was shown that calcium release from the sarcoplasmic reticulum binds to troponin C and triggers muscle contraction (Bozler 1951, Deluca and Engstrom 1961, Ebashi and Kodama 1965). Furthermore, propagation of calcium action potentials were identified (Fatt and Katz 1953, Fatt and Ginsborg 1958). This work led to the paradigm shifting idea that calcium channels are ubiquitously expressed in all excitable cells (Hagiwara and Byerly 1981). Clinically relevant interventions were identified when nifedipine was characterized, and is still widely used today for the treatment of cardiovascular disease (Fleckenstein 1983). The identification of calcium channel blockers led to

pharmacological identification of the voltage-gated calcium channels (L-type, P-type, Q-type, N-type, R-type, and T-type calcium channels) which led to calcium influx in response to changes in membrane potential (Miller 1987). Ligand-gated calcium channels such as P_2X receptors (Burnstock and Kennedy 1985), as previously discussed, was identified as the source of an increase in intracellular calcium levels (Iredale and Hill 1993). It was also established that NMDA receptor activation led to increased intracellular calcium (MacDermott et al. 1986). These rapid changes in calcium concentration that led to downstream calcium signalling events are made possible through tightly regulated calcium homeostasis. Calcium homeostasis evolved to allow mammalian cells to produce a vast calcium gradient (10^{-3} M in the extracellular fluid, and 10^{-7} M free intercellular calcium) across the plasma membrane (Stini 1998). Extracellular calcium levels are maintained at a range of 1.1 to 1.4 mM via calcium-sensing receptors and regulated by parathyroid hormone, calcitonin and calcitriol (D'Souza-Li 2006). Inside the cell, calcium levels are tightly modulated and actively sequestered to maintain the low calcium concentration (~100 nM) by actively pumping calcium into organelles like the ER, Golgi, and endocytic vacuoles which have about 100-fold higher calcium concentrations (Figure 8), pumping calcium out of the cell, or by sequestering calcium binding proteins (Clapham 2007).

Calcium signalling is ubiquitous across eukaryotic cells, which affects hundreds of proteins. Calcium signal transducers, proteins which undergo a conformational shift in the presence of calcium, generally bind to calcium in the low micromolar concentrations since $[Ca^{2+}]_i$ (intracellular calcium concentration) generally rests at about ~100 nM (Chin and Means 2000). Following a calcium signalling event (i.e. calcium spark, calcium wave, etc.), restoring the calcium gradient is critical for maintaining the specificity of the signal. Any lag in restoring intracellular calcium concentrations can lead to transducers becoming desensitized to the calcium signals.

Calcium signals are also often localized to a very small area due to the rapid sequestering and chelation of the free ions, which allows another level of coordination for signal transducers whereby the origin of the calcium signal can have a large impact on the downstream effect. An example of this is prolonged raised local calcium concentration in the cytosol leads to calcineurin activation that dephosphorylates NFAT (Pinto et al. 2015). NFAT is then transported to the nucleus where it can activate immunity related genes (Figure 9). Calcium binds to a variety of proteins containing EF-hand motifs, which trigger a conformational change that allows calcium signal transduction to occur (i.e. binding different subset of proteins in low calcium concentration vs. high concentration). For instance, calmodulin (CaM), a small calcium signal transducer which modulates many proteins in the body through protein-protein interactions, or calcineurin, a phosphatase found to activate T cells (Chin and Means 2000, Yamashita et al. 2010). An example is the regulation of the TORC2 (also known as CREB regulated transcription coactivator 2) pathway by calcium (Clapham 2007). Increased cytosolic calcium activates calcineurin which dephosphorylates TORC2, and leads to inhibition of SNF1-like kinase 2 (Screaton et al. 2004). This enables TORC2 to move to the nucleus and activates genes associated with cell viability.

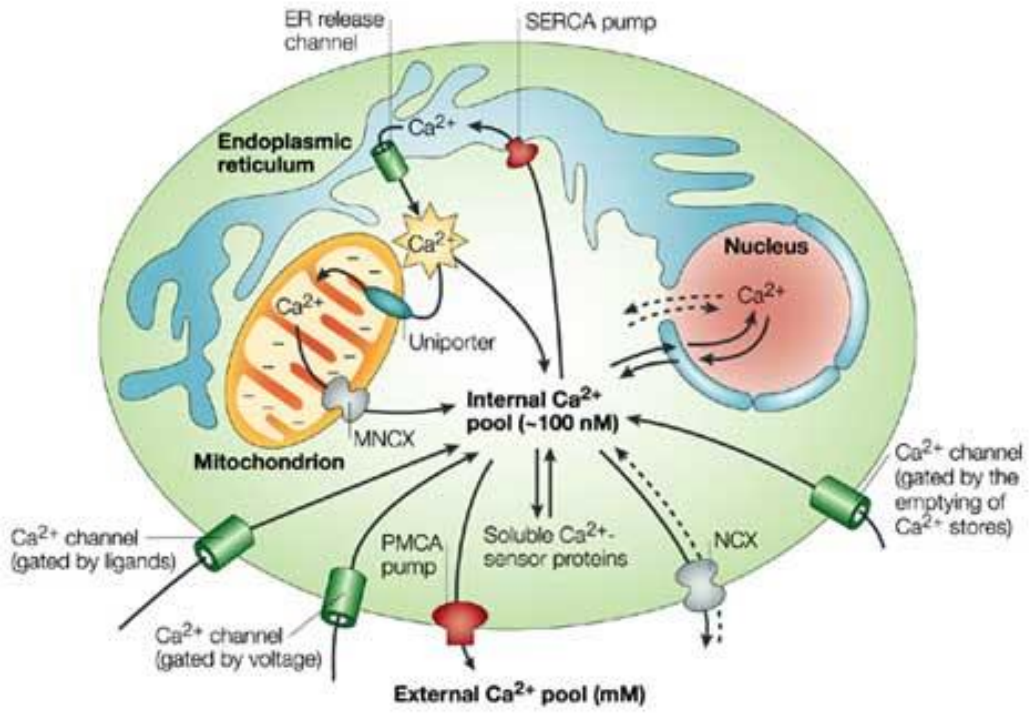

Figure 8: Typical calcium signalling in the cell. Calcium channels, pumps, and calcium binding proteins maintain the low concentration of intracellular calcium, which allows calcium to be a potent signal to downstream signalling events. Figure adapted from Carafoli (2003).

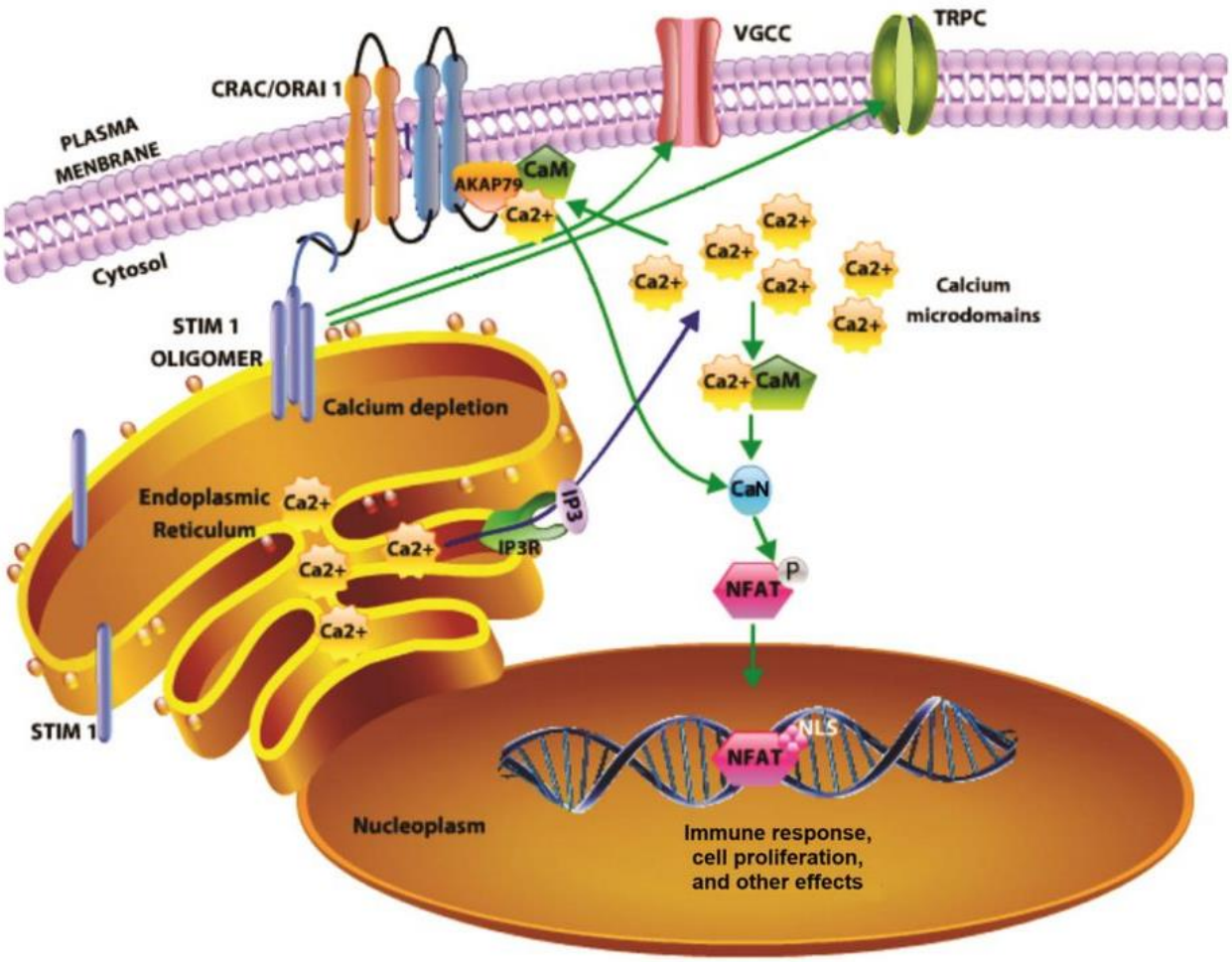

Figure 9: Ca^{2+}/CaM dependent regulation of NFAT, a transcriptional regulator of immunity. Microdomains of increased calcium concentration arising from STIM1 activating ORAI1 (calcium release-activated calcium channel protein 1), VGCC (voltage-gated calcium channel), and TRPC (transient receptor potential cation channels), leads to CaM activation. CaM binds to calcineurin (CaN) which dephosphorylates NFAT. Dephosphorylated NFAT is transported to the nucleus where it binds DNA and regulates gene expression. Figure adapted from Pinto et al. (2015).

1.6.2 Roles of Ca^{2+}/Calmodulin in regulating intracellular signalling

Calmodulin (CaM) activity was first discovered in brain and heart tissue by Cheung (1970) as well as Kakiuchi and Yamazaki (1970) independently as an activator of cyclic nucleotide phosphodiesterase (PDE). Shortly after, Teo and Wang (1973) showed that CaM, at the time known as the "PDE-activating factor", activity was calcium-dependent. CaM was aptly named, **cal**cium-**modul**ated prote**in,** since it was a calcium signal transducer. CaM is the prototypic member of the calcium binding EF-hand proteins, and is ubiquitously expressed in all eukaryotic cells. CaM acts as an intermediate messenger which transduces calcium ion signals by calcium binding one of four calcium binding sites (on one of the four EF-hand domains) that leads to a conformational change in the protein (Chin and Means 2000).

CaM is a relatively small protein, with a molecular weight of 16.7 kDa, yet it is critical for cellular regulation in almost every cellular process, including: apoptosis, immune response, inflammation, metabolism, muscle contraction, and synaptic plasticity (Cheung 1980, Chin and Means 2000). The protein structure is quite symmetrical with two roughly similar large globular domains each containing two EF-hand domains, deemed the *N*- and *C*-terminal globular domains, which give the CaM its dumbbell like shape (Figure 10). These two domains are connected via a flexible linker region, each with two calcium binding sites. These calcium sites bind in a positively cooperative manner with the calcium binding sites at the *C*-terminal domain binding free calcium ions with a K_d of ~1 μM and the calcium binding sites at the *N*-terminal domain binding with a K_d of ~10 μM (Linse et al. 1991). Each domain of the protein also contains a large hydrophobic cleft, which are the primary interaction domains (Babu et al. 1988).

Since the intracellular calcium concentration can rapidly change from 100 nM to 10 µM following cell signalling, the previously mentioned calcium binding affinities allows CaM to bind a different subset of proteins following a cell signalling stimuli that results in an increase of intracellular calcium. Hundreds of CaM binding partners have been identified, with many new protein-partners being uncovered with new large screens (Yap et al. 2000, Shen et al. 2005, Kaleka et al. 2012, Sengprasert et al. 2015). Of these binding partners, there are currently five binding motifs for CaM: 1-10, 1-14, 1-16 and IQ (Yap et al. 2000). Many of CaM protein partners exhibit a calcium-dependent interaction, but calcium-free CaM (apo-CaM) is also able to interact with many of these proteins, and certain proteins exclusively bind to apo-CaM through the IQ-domain such as neuromodulin or neurogranin (Alexander et al. 1988, Huang et al. 1993). Whether mediating calcium signalling events in response to stimuli, or interacting with protein partners in the absence of calcium, CaM is pivotal in almost every known cell signalling pathway, so will have numerous direct and indirect downstream effects in any context. One research objective I set out to answer was what the physiological relevance of the CaM-ENT1 interaction.

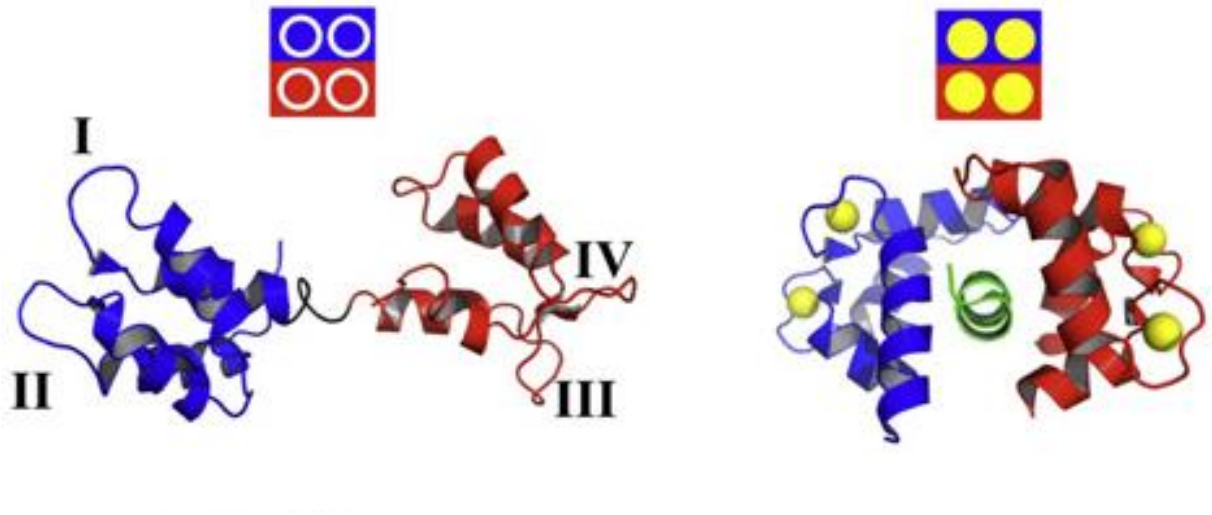

Figure 10: The three dimensional structure of calmodulin. The structure of Apo-CaM (left), and Ca^{2+}/CaM (right) binding to CaMKII highlights the major structural change between low and high calcium concentrations. Adapted from Feldkamp et al. (2011).

1.7 Research Aims

Most drugs require transporters to pass through the cell membrane, thus the efficacy of drugs relies heavily on the presence, and activity of transporters. This has led to a growing incentive for performing membrane transporter research. Understanding the mechanisms which regulate ENT1 is clinically relevant. Research has previously linked calcium, as well as glycosylation to regulate nucleoside flux. The mechanisms in which they operate are not well established, and this body of work aims to elucidate these pathways and how they regulate ENT1. I hypothesized that given the previous work in the literature had shown a relationship between calcium and nucleoside flux, as well as the identification of glycosylation on ENT1 when expressed in yeast, that these pathways would be critical for proper ENT1 function. My specific research objectives were to confirm that there is a calcium-dependent, physical protein-protein interaction between ENT1 and CaM, to determine the role of Ca^{2+}/CaM on the regulation of ENT1 in a physiologically relevant context, and to show that ENT1 glycosylation is critical for proper localization, function, and oligomerization of the protein.

Chapter 2: Novel regulation of Equlibrative Nucleoside Transporter 1 (ENT1) by receptor-stimulated Ca^{2+}-dependent calmodulin binding.

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Author contributions

Alex Bicket performed co-immunoprecipitation, fluorescence anisotropy, transport assays, live cell calcium imaging, and Western blotting with the assistance of Zlatina Naydenova. Pedram Mehrabi performed the MYTH screen, with the assistance of Igor Stagljar and Victoria Wong, and the NMR, with the assistance of Logan Donaldson. Alex Bicket assisted with the writing of the manuscript. Imogen R. Coe, the corresponding author, led and designed the project, and wrote the final version of the manuscript.

Abstract

Equilibrative nucleoside transporters (ENTs) facilitate the flux of nucleosides, such as adenosine, and nucleoside analog (NA) drugs across cell membranes. A correlation between adenosine flux and calcium-dependent signalling has been previously reported, however, the mechanistic basis of these observations is not known. Here we report the identification of the calcium signalling transducer, calmodulin (CaM) as an ENT1 interacting protein, via a conserved classic 1-5-10 motif in ENT1. Calcium-dependent human ENT1-CaM protein interactions were confirmed in human cell lines (HEK293, RT4, U-87 MG) using biochemical assays (HEK293) and the functional assays (HEK293, RT4) which confirmed modified nucleoside uptake which occurred in the presence of pharmacological manipulations of calcium levels and CaM function. Nucleoside and NA drug uptake was significantly decreased (~12% and ~39% respectively) by chelating calcium (EGTA, 50 μM; BAPTA-AM, 25 μM) while increasing intracellular calcium (thapsigargin, 1.5 μM) led to increased nucleoside uptake (~26%). Activation of NMDA receptors (in U-87 MG) by glutamate (1 mM) and glycine (100 μM) significantly increased nucleoside uptake (~38%) except in the presence of the NMDA receptor antagonist, MK-801 (50 μM), or CaM antagonist, W7 (50 μM). These data support the existence of a previously unidentified novel receptor-dependent regulatory mechanism whereby intracellular calcium modulates nucleoside and NA drug uptake via CaM-dependent interaction of ENT1. These findings suggest that ENT1 is regulated via receptor-dependent calcium-linked pathways resulting in an alteration of purine flux, which may modulate purinergic signalling and influence NA drug efficacy.

Keywords: ENT1; interactome; calcium; calmodulin (CaM); regulation

Introduction

Equilibrative nucleoside transporter 1 (ENT1), the prototypic member of the SLC29 family of transporters, is an integral membrane protein responsible for transporting nucleosides, and nucleoside analog drugs across cellular membranes (King et al. 2006a, Young et al. 2013). There are four members of the SLC29 family and two isoforms, ENT1 and ENT2. These proteins are clinically significant and are essential for the efficacy of many cytotoxic nucleoside analog drugs used to treat cancer, viral, and parasitic infections (Hillgren et al. 2013). ENT1 is also a key player in the purinome where it modulates adenosine flux in many tissues affecting a number of purinergic signalling pathways (Rose et al. 2010, Grenz et al. 2012)

While there is an increasing understanding of the structural and functional aspects of ENT family members (Valdés et al. 2014, Cano-Soldado and Pastor-Anglada 2012) and their role and relevance to clinical outcomes (Endres et al. 2009, Gusella et al. 2011), our understanding of the mechanisms of regulation of ENTs is still limited. Previous work has identified a number of potential regulatory mechanisms such as protein phosphorylation (Coe et al. 2002, Bone et al. 2007, Reyes et al. 2011b), possibly as a consequence of receptor activation (Hughes et al. 2015), and these mechanisms have been implicated in functional aspects of ENT-dependent nucleoside uptake (Bone et al. 2007, Ramadan et al. 2014, Santos-Rodrigues et al. 2014) although a direct relationship between phosphorylation and function remains to be demonstrated.

ENT1 has been shown to be clinically significant. For example, ENT1 expression is required for the efficacy of many cytotoxic nucleoside analog drugs and is responsible for adenosine flux of in many tissues. This highlights how ENT1 is a critical member within the purinome, yet relatively little is known about ENT1 regulation. Early work in guinea pig

myocardial cells demonstrated that exposure to elevated calcium led to a phasic release of adenosine (DeWitt et al. 1983). Similar findings have also shown a correlation between calcium flux and nucleoside flux. For example, calcium-dependent release of radiolabelled adenosine in cerebellar granule cells (Schousboe et al. 1989), and the involvement of calcium channels in the release of adenosine (Latini et al. 1997) showed a direct relationship between calcium signalling and adenosine release. These findings, along with observations that calcium-dependent excitation secretion coupling of adenosine was reduced in the presence of NBTI (Fredholm et al. 1994, Cunha et al. 1996) and dipyridamole (Meghji et al. 1985) provided evidence to support a role of ENT1 in calcium evoked adenosine flux. This information has been widely known for over 30 years yet evidence to demonstrate a mechanism has remained elusive.

Progress towards understanding this phenomenon was made when calcium-dependent release of adenosine in chick retinal cells was evoked with NMDA (Paes-de-Carvalho et al. 2005). Moreover, this adenosine release was shown to be occurring through an NBTI-sensitive manner, and was CAMKII dependent. This supported previous work in the hippocampus, where NMDA receptors have been shown to mediate electrically evoked adenosine release whereby injection of glutamate, NMDA, or quisqualate directly induced an increase in adenosine concentrations (Pedata et al. 1991). This had also been observed with NMDA, glutamate, or kainate induced adenosine release in cortical slices (Hoehn and White 1990) or with glutamate playing a role in calcium-dependent adenosine release in cerebellar granule cells (Schousboe et al. 1989). Other examples of studies identifying NMDA and glutamate evoked calcium-dependent adenosine release are well explained in a review by Latini and Pedata (2001). There is also evidence that activation of $P2X_7$ receptors led to an influx of calcium which resulted in a twelve-fold increase purine flux in astrocytes (Ballerini et al. 1996). Furthermore, in rat cortical neurons, calcium, NMDA, and

CaMKII were implicated in adenosine release (Zamzow et al. 2009). And more recently, in mouse hippocampal slices, about 40% of stimulated-adenosine release was shown to occur by ENTs, with the remaining adenosine pool resulting from the metabolism of ATP by ecto-nucleotidases (Wall and Dale 2013).

The overwhelming evidence of crosstalk between calcium and purinergic signalling pathways was the foundation of my model. For this project, I aimed to illustrate that calcium-dependent adenosine flux is mediated in a calmodulin (CaM) dependent manner. I used a variety of functional assays to provide strong evidence that calcium modulates nucleoside flux, and that this interaction is CaM-dependent. I also used co-immunoprecipitation, and fluorescence anisotropy to determine if there is a physical interaction between CaM and ENT1, and if this interaction was calcium dependent. I also aimed to show the mechanism in which this occurs, and that in a physiologically relevant model, I could demonstrate that glutamate activation of NMDA receptors could lead to increased intracellular calcium as well as increased Ca^{2+}/CaM-dependent nucleoside flux in human cells. I have defined a mechanistically novel mode of regulation of ENT1 which explain previous observations linking receptor-dependent calcium pathways to purinergic signalling and provide a novel route for potential enhancement of nucleoside analog drug efficacy (Paes-de-Carvalho et al. 2005, Zamzow et al. 2009, Wall and Dale 2013). Furthermore, we used a proteomic approach to characterize the interactome and identified calmodulin (CaM) as a novel calcium-dependent interacting protein partner of ENT1. We confirmed that a functionally relevant, calcium-dependent interaction exists between ENT1 and CaM in human cell lines and defined a mechanistically novel mode of regulation of ENT1. These data also suggest that nucleoside analog drug uptake may be affected by variations in calcium signalling depending on the context

suggesting both opportunities for enhancement of nucleoside analog drug efficacy as well contraindications with calcium influencing drugs.

Materials and Methods

Materials

[^{3}H]-2-chloroadenosine and [^{3}H]-gemcitabine were purchased from Moravek Biochemicals, (Brea, CA, USA), BAPTA-AM from EMD Millipore Corporation (Billerica, MA, USA), cOmplete Mini protease inhibitor cocktail from Roche (Basel, Switzerland), and CALP2, gemcitabine hydrochloride, and W7 hydrochloride from R&D Systems (Minneapolis, MN, USA).

Bait Construction for MYTH

Despite repeated attempts, we were unable to successfully clone hENT1 in the membrane yeast two-hybrid (MYTH) vector and therefore used mENT1 for the MYTH screen. Cloning of mENT1 into a MYTH vector, pTLB-1 (Dualsystems Biotech AG, Schlieren, Switzerland) allows for the bait protein to be fused to the *N*-terminal region of the Cub transcription factors. To achieve this, mENT1 cDNA was amplified from its host plasmid by standard PCR using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). The mENT1 cDNA was then ligated into both MYTH vectors via homologous recombination via a standard yeast transformation. The yeast strain THY.AP4 (MATa leu2, ura3, trp1 :: (lexAop- lacZ) (lexAop)-*HIS3* (lexAop)- ADE2) was employed. To check for self-activation of the MYTH vectors with

mENT1 cDNA, NubG/NubI tests were performed whereby yeast carrying the mENT1 MYTH vector was transformed with control plasmids Fur4, NubI, Ost1 NubI (positive control), Ost1, NubG, and Fur4 NubG (negative control) by standard yeast transformation. Following growth on SD-WL plates, a range of serial dilutions from 1:100 - 1:10000 was performed and samples spotted onto SD-WLAH plates with varying amounts of 3-amino-1,2,4 triazole (3-AT), up to 100 mM and allowed to incubate at 30˚C for 2-5 days.

MYTH Assay

Membrane yeast two-hybrid (MYTH) assays were performed as previously described (Stagljar et al. 1998, Fetchko and Stagljar 2004, Snider et al. 2010, Snider et al. 2013, Lam et al. 2015). The yeast strain THY.AP4 (MATa leu2, ura3, trp1 :: (lexAop- lacZ) (lexAop)- HIS3 (lexAop)- ADE2) was transformed with the mENT1 pTLB-1 bait plasmid and the 11 day whole mouse embryo NubG-X prey plasmid using the lithium acetate method (Gietz and Woods 2002), and plated on SD-WL plates. Colonies were selected based on size and shape, diluted in 100 µL of ddH_2O and spotted onto SD-WL and SD-WLAH +/- 3-AT X-Gal plates. Colonies that grew and exhibited β-galactosidase activity were scored as positive for an interaction between the bait and prey.

Positive prey plasmids that were selected from the positive colonies obtained from the MYTH screens were retransformed into THY.AP4 yeast with mENT1 pTLB-1 bait plasmid, as well as control bait plasmid that consisted of an artificial bait protein which will not interact with any protein. The transformants are plated serially in triplicates on SD-WL and SD-WLAH X-GAL plates with 100 mM 3-AT. Colonies that showed β-galactosidase activity in both the mENT1 and

artificial bait transformants were determined to be self-activating and discarded. Those that did not show self-activation were selected for further screening.

Bioinformatic Prey Analysis

Calmodulin (CaM) was identified as a putative protein partner of ENT1. The human (GI:1845344) and mouse ENT1 (AF131212) protein sequences were analyzed and compared to known CaM interactors in the Calmodulin Target Database (Yap et al. 2000).

Cell Culture

RT4 (HTB-2) cells, a human bladder epithelial cancer cell line, were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were grown in McCoy's 5A medium supplemented with 10% FBS. For [^{3}H]-gemcitabine uptake assays, cells were left to attach for 24 h.

HEK293 cells, a transformed human embryonic kidney cell line, were grown in standard DMEM supplemented with 10% (v/v) FBS.

U-87 MG (HTB-14) cells, a human brain glioblastoma epithelial cancer cell line, were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were grown in EMEM supplemented with 10% FBS.

RT4, HEK293, and U-87 MG cell lines were incubated in 5% (v/v) CO_2 and 95% (v/v) air at 37°C. Cells were plated in 6-well plates for uptake assays, 10 cm plates for NBTI binding analysis, and 60 mm plates for Western blotting analysis. Cells for live cell imaging were seeded on #1.5 glass bottom dishes (MatTek corporation, Ashland, MA).

Rationale for cell line choice

HEK293 cells were chosen for biochemical and functional assays because they have well known nucleoside transporter characteristics, they are comparatively low maintenance, exhibit rapid growth, and are reliably adherent during transport assays.

To confirm our proposed mechanism of Ca^{2+}/CaM regulating nucleoside flux, I used U-87 MG cells, an immortalized *Homo sapiens* glioblastoma cell line which express mRNA for NR2A and NR2B subunits, show presence of NR2B protein, and for which electrophysiological data suggest Ca^{2+} influx results from ligand- or voltage-gated calcium channels (Stepulak et al. 2009, Jiang et al. 2010, Ducret et al. 2003). Recent studies on the role of NMDA receptor signaling have identified physiologically relevant NMDA receptors in glial cells linked to physiologically relevant roles (Dzamba et al. 2013, Palygin et al. 2010, Palygin et al. 2011). While U-87 MG cells are not a classic model for NMDA receptor signalling studies, they possess glutamate/glycine activated NMDA receptor dependent regulation of metalloproteinase activity and proliferation (Ramaswamy et al. 2013). These cells are also reliably adherent and thus suitable for functional transport assays. I confirmed the presence of the NR1 subunit by Western blotting analysis, verified the NMDA receptors were functional by performing fluo-4 live cell calcium imaging to confirm that glutamate and glycine treatment led to an increase in calcium transients (blocked in presence of the tight binding, non-competitive NMDAR antagonist MK-801), and used MK-801 treatment in my 2-chloroadenosine transport assays to corroborate previous observations (Paes-de-Carvalho et al. 2005) that NMDAR activation led to a sodium-independent nucleoside flux.

Construct design for HA-ENT1

An HA tag was cloned into the hENT1 coding sequence after the amino acid at position 64 (HA tag bolded). The HA tag is located at the beginning of the first, large extracellular loop. The sequence was submitted to DNA 2.0 (Menlo Park, CA) for generation of a mammalian expression vector to express HA-hENT1.

```
1   MTTSHQPQDR YKAVWLIFFM LGLGTLLPWN FFMTATQYFT NRLDMSQNVS LVTAELSKDA
61  QASA YPYDVP DYA APAAPLP ERNSLSAIFN NVMTLCAMLP LLLFTYLNSF LHQRIPQSVR
121 ILGSLVAILL VFLITAILVK VQLDALPFFV ITMIKIVLIN SFGAILQGSL FGLAGLLPAS
181 YTAPIMSGQG LAGFFASVAM ICAIASGSEL SESAFGYFIT ACAVIILTII CYLGLPRLEF
241 YRYYQQLKLE GPGEQETKLD LISKGEEPRA GKEESGVSVS NSQPTNESHS IKAILKNISV
301 LAFSVCFIFT ITIGMFPAVT VEVKSSIAGS STWERYFIPV SCFLTFNIFD WLGRSLTAVF
361 MWPGKDSRWL PSLVLARLVF VPLLLLCNIK PRRYLTVVFE HDAWFIFFMA AFAFSNGYLA
421 SLCMCFGPKK VKPAEAETAG AIMAFFLCLG LALGAVFSFL FRAIV
```

Co-immunoprecipitation of CaM using HA-hENT1 bait

HEK293 cells transfected with HA-ENT1 and after approximately 36 hours, were lysed with NP-40 buffer (50 mM Tris-HCl, 150 mM NaCl, 1% (v/v) NP-40, 1 mM Na_3VO_4, 50 mM NaF, and protease inhibitor cocktail) by homogenizing with a 1 mL syringe and 26 g needle and then centrifuged at 8000 rpm for 25 minutes in a bench top centrifuge to pellet cellular debris and organelle. Protein concentration was determined using a modified Lowry protein assay (BioRad, Hercules, CA) and 600 µg of protein lysate was loaded to a column with 20 µl of anti-HA beads (Thermo Scientific, Waltham, MA). The protein was agitated using a rotator for 2 hours at room temperature (approximately 23°C), and was then washed 6 times with TTBS containing 2 mM

$CaCl_2$, or without calcium in the presence and absence of EGTA. The immunoprecipitated protein was recovered by adding protein loading buffer, boiled at 95°C for 10 minutes (Thermo Scientific) and was supplemented with 2 µl of 1 M DDT. Elution and flow-through protein was analyzed by SDS-PAGE followed by immunoblotting against CaM (1^o antibody, Epitomics, Cambridge, MA, 1716-1, 1:1000 dilution; 2^o antibody, HRP-conjugated anti-rabbit 1:4000). Densitometry was performed using ImageJ.

Protein expression for NMR analysis and fluorescence anisotropy

A chimeric gene encoding the large intracellular loop (residues 228-290) of hENT1, in tandem with a 6xHis-ubiquitin tag, and an intervening thrombin site, was designed and commercially synthesized in the expression vector, pJexpress 401 by DNA 2.0 (Menlo Park, CA). A human calmodulin (CaM) clone was kindly provided by Dr. Mitsu Ikura (University of Toronto, Toronto, ON) and consisted of CaM cloned into a pET15b with 6xHis tagged and an interpolated thrombin site at the *N*-terminal. CaM fusion peptides were expressed in a conventional M9 minimal media (in 1 L batches) with ^{15}N-ammonium chloride as the only source of nitrogen, while conventional LB media was used to generate peptides for fluorescence anisotropy. The 6xHis-ubiquitin-hENT1 loop proteins were expressed in LB-Kan in a culture of *E. coli* BL21:DE3 cells. The cells were grown to an OD_{600} of 0.8 and then induced with IPTG for 3 hours. Cells were then centrifuged (10000 x g for 10 min) and lysed with a French press. The fusion peptides were purified from the cell lysate by a combination of standard nickel affinity and gel filtration chromatography.

NMR Measurements

Uniformly ^{15}N-labeled CaM at 0.12 mM in PBS, supplemented with 10% D_2O and 3 µM of $CaCl_2$ was titrated with 6xHis-ubiquitin-hENT1 at a 1:2 ratio in a ^{15}N-edited HSQC (heteronuclear single quantum coherence) spectra (768x80 pts) were acquired on a 600 MHz Varian NMR spectrometer (Jessard et al. 1991). HSQC spectra can provide evidence for protein-protein interactions if there are shifts in peaks when comparing the spectra of the free protein with the spectra of two interacting proteins.

Fluorescence Anisotropy

Fluorescence anisotropy is a technique that analyzes the ratio of polarized light to total light emitted from a fluorophore and enables the measurement of binding constants between a fluorophore-tagged molecule and an untagged molecule (Cano-Soldado and Pastor-Anglada 2012). A complex of a fluorescently tagged peptide with a binding partner will rotate more slowly than unbound peptide. Fluorescence anisotropy can therefore be used to determine the dissociation constant (K_d) of interacting proteins and has previously been used to measure the affinity between CaM and other membrane proteins (Grycova et al. 2015, Edrington et al. 2007).

Fluorescence anisotropy measurements were made with a Cary Eclipse Fluorescence Spectrophotometer at room temperature. Titrations were performed in Hepes buffer (25 mM Hepes, 200 mM NaCl, 2 mM $CaCl_2$, pH 7.4) with hENT1 peptide concentrations of 0, 2, 4, 8, 16, 32, 64, 75, 95 µM. The hENT1 intracellular loop peptides were fluorescently tagged and consisted of the putative 1-5-10 CaM binding site in the large intracellular loop of hENT1 (residues 224 –

245) with three C-terminal lysines added to enhance solubility, a PEG spacer, and an *N*-terminal fluorescein derivative (FAM) tag (CanPeptide Inc, Pointe-Claire, Québec) as follows:

Wild type: LGLPRLEFYRYYQQLKLEGPGKKK

ΔCaM-5:LGLPRLE**RR**R**HR**QQLK**R**EGPGKKK

ΔCaM-3: LGLPRLE**R**YRY**R**QQLK**R**EGPGKKK

The peptides were kept at a constant concentration of 2 μM during the titrations. Fluorescence anisotropy was measured at an excitation wavelength of 494.0 nm and an emission wavelength of 523.0 nm. Anisotropy, R, is measured using the equation:
$R = (I_{vv} - G * I_{vh})/(I_{vv} + 2 * G * I_{vh})$ where I_{vv} represents both polarizers in the vertical position, I_{vh} represents the perpendicular polarizer in the horizontal position, and G is the G-factor. Average relative anisotropy was calculated, after at least three experiments per peptide, by non-linear regression analysis using GraphPad Prism version 5.04 for Windows.

[^{3}H] Uptake Assays

[^{3}H]-2-chloroadenosine and [^{3}H]-gemcitabine uptake were measured as previously described (Coe et al. 2002). Cells were incubated for 10 sec in sodium-free transport buffer (pH 7.4) containing 20 mM Tris-HCl, 3 mM K_2HPO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose, 130 mM *N*-methyl *D*-glucamine, permeant (10 μM 2-chloroadenosine or gemcitabine) and radiolabeled nucleosides at room temperature, since uptake kinetics are the same as with 37°C transport assays (Boleti et al. 1997). The uptake was stopped by rapid aspiration of permeant solution and immediate washing of cells 3 times with ice-cold sodium-free transport buffer

containing 100 nM NBTI and 30 μM dipyridamole. Cells were lysed in 2 M NaOH for 48h at 4°C. Aliquots were taken to measure protein content (modified Lowry protein assay, Bio-Rad) and nucleoside uptake (standard liquid scintillation counting). Nucleoside uptake was expressed as picomoles per milligram of protein per unit time.

Calcium levels were manipulated by replacing media with calcium free buffer, or by treating cells with media containing BAPTA-AM (25 μM), EGTA (50 μM), or thapsigargin (1.5 μM) for one minute prior to the beginning of the uptake assay. Treatment with the CaM antagonist W7 (50 μM) was for 1 minute in media. Cells were FBS-starved for 20 hours prior to treatment.

For glutamate (1 mM) and glycine (100 μM) treatment, cells were washed twice with 37°C pre-heated HBSS (140 mM NaCl, 5 mM KCl, 20 mM Hepes, 4 mM glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$), incubated for 10 minutes in HBSS at 37°C, then cells were incubated for 20 minutes after the addition of 1 mM glutamate and 100 μM glycine at 37°C.

Live cell calcium imaging with Fluo-4

Glass bottom dishes of HEK293 cells or U-87 MG cells had media replaced with fresh DMEM +10% FBS or EMEM + 10% FBS respectively. Cells were incubated with 4 μM Fluo-4 (Molecular Probes) and 0.02% (w/v) Pluronic F-127 at 37°C in 5% CO_2 for 45 minutes.

After incubation, HEK293 cells were washed twice with pre-warmed 1xPBS, and were imaged in media + 10% FBS. HEK293 cells following 1.5 μM thapsigargin treatment were imaged using Zeiss AxioObserver spinning disc confocal microscope was used to capture a single plane every 5 seconds using a 40x oil immersion objective (N.A. = 1.40), exciting with the 488 nm laser. Basal fluorescence intensity was established by imaging HEK293 cells in media for 5 minutes, and then upon adding thapsigargin (t=0), cells were imaged for 10 minutes.

Calcium signalling of U-87 MG cells was assessed by quantifying calcium signalling events and was measured using the Zeiss LSM 700 inverted confocal microscope with image acquisition of one focal plane every 5 seconds using a 10x objective (N.A. = 0.45) following the imaging protocol previously described (Weber and Waldman 2014). Following incubation with fluo-4 for 45 minutes, cells were washed twice, pre-incubated with HBSS for 20 minutes, and were then imaged for 10 minutes in HBSS to establish the basal rate of calcium transients. This was determined by counting the number of cells experiencing a rapid, large, transient increase in fluorescence intensity over time in the set field of view (Weber and Waldman 2014). To determine the change in calcium signalling events, cells were imaged for 10 minutes following treatment with either 1 mM glutamate and 100 µM glycine, or 100 µM glycine. After 10 minutes of image acquisition, MK-801 was added to the plate (50 µM) and images were recorded for 10 minutes. Data analysis was performed using Zen Blue 2013 software for Windows, and histogram was generated using GraphPad Prism 5.04 for Windows.

Western blotting analysis

U-87 MG cells were homogenised in lysis buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM NaCl, 1 mM EDTA and protease inhibitors cocktail) through freezing (liquid nitrogen) - thawing (42°C water bath) in total of 4 cycles followed by 3 cycles of sonication. Lysate was centrifuged at 14000 rpm for 25 minutes at 4°C to pellet cellular debris and organelle. The supernatant was then centrifuged for 1.5 hours at 55,000 rpm at 4°C. The crude membranes pellet was resuspended in membranes solubilising buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% Triton X-100 and 0.5% SDS). The protein concentration was determined using a modified Lowry protein assay (BioRad, Hercules, CA). Positive control (crude synaptoneurosome preparation of mouse brain

tissue) protein was provided by the Ramsey lab, and was prepared by following the protocol from Li et al. (2010). 100 µg of U-87 MG crude membranes protein and 0.5 µg of positive control protein was each mixed with protein loading buffer, incubated for 10 minutes at 55°C and was analyzed following SDS-PAGE and Western blotting. The nitrocellulose membranes (Bio-Rad, Hercules, CA) were blocked for 45 minutes in 5% milk, incubated with rabbit polyclonal anti-ENT1 antibody (Abcam, Cambridge, MA; ab48607), and mouse monoclonal anti-NMDAR1 antibody (EMD Millipore, Billerica, MA; mab1586) in 1% (v/v) milk over night at 4°C using manufacturers recommended dilutions. After washing with TTBS, the nitrocellulose membranes were incubated with HRP conjugated secondary antibodies in 1% (v/v) milk for 1.5 hours at room temperature, followed with two washes of TTBS and one wash with TBS, each for 10 minutes. ECL was added, the membrane was exposed to film, and then the film was developed.

Statistical Analysis

For experiments with two conditions, one-tailed student t-tests were performed since our hypotheses were one directional in nature (i.e. we were interested in detecting the decrease in nucleoside uptake when treating with a drug, not interested in detecting the change, both an increase or decrease, after treating with a drug). With three or more conditions, to confirm there were one or more significant differences, we used one-way analysis of variance (ANOVA) since our hypotheses were also one directional. This test requires normality, homogeneity between the variances, and independence between each condition. Normality and homogeneity were confirmed in GraphPad prior to analysis, and independence was ensured through experimental design. The Newman-Keuls post-hoc test was performed where appropriate, since it is robust to violations of normality and relatively small sample sizes could lead to data approaching a skew

(not enough to violate the assumption of normality but could impair the degree of significance). Most graphs summarize three independent experiments (n is listed in caption for each figure), and all raw data from each independent experiment coincides with the same trend (i.e. for each independent experiment, the drug treatment led to a decrease, albeit, to varying extents).

Standard error (SE) was used instead of standard deviation (SD) where appropriate, since these experiments had outliers (objectively determined to be outside 2*SD, likely due to human or technical error) thus required normalization for differences in population. All statistical analyses were performed using GraphPad Prism 5.04 for Windows.

Results

Identifying the role of calcium in regulating sodium-independent nucleoside and nucleoside analog drug uptake

Previous work has shown a correlation between calcium levels and adenosine flux in neural cells (Zamzow et al. 2009, Wall and Dale 2013). To confirm that calcium regulates ENT-dependent nucleoside and nucleoside analog drug transport in other cell types, I measured chloroadenosine uptake in HEK293 cells and gemcitabine uptake in RT4 cells in the presence of intracellular (BAPTA-AM) and extracellular (EGTA) chelators of calcium. My data show that chelating either extracellular and/or intracellular calcium results in significantly reduced (~12% for EGTA and ~39% for BAPTA-AM) nucleoside transport in HEK293 cells (Fig. 1A) and chelation of intracellular calcium decreases gemcitabine transport (~24%) in RT4 cells (Fig. 1B) confirming a relationship between calcium levels and nucleoside transport in these cells.

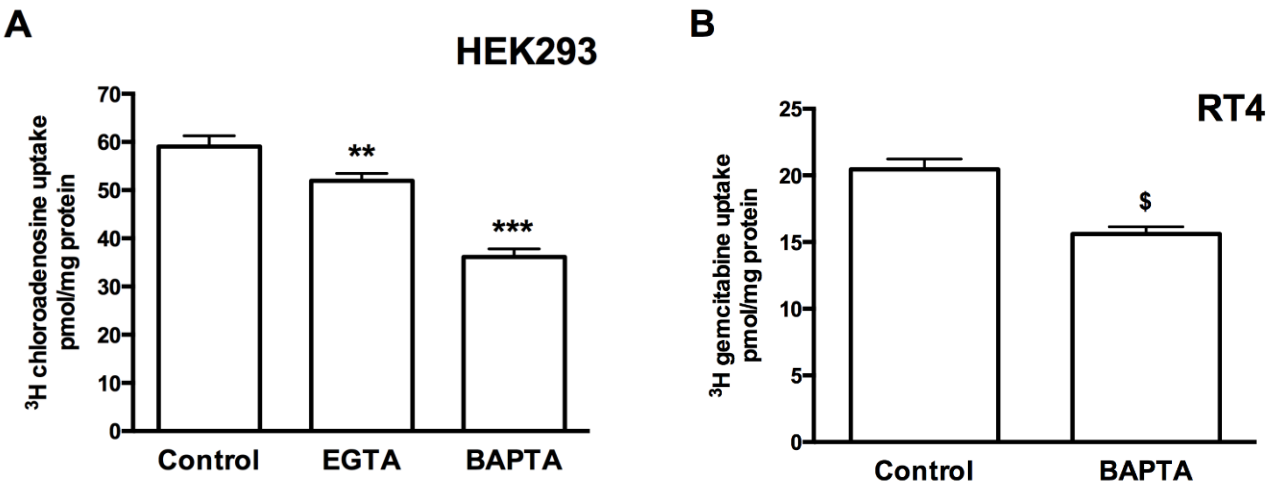

Figure 1: Reduced calcium levels lead to reduced nucleoside transport.

A) HEK293 cells were treated (1 min) in Ca^{2+}-containing, Ca^{2+}-free buffer + 50 μM EGTA, or Ca^{2+}-free buffer + 25 μM BAPTA-AM. EGTA and BAPTA-AM treatments significantly reduced [^{3}H]-chloroadenosine uptake. Pooled data from three individual experiments, with each condition conducted in sextuplicate, are presented as mean ± SEM (One-way ANOVA with Newman-Keuls multiple comparison post hoc test, **P<0.01, ***P<0.001, n=3).

B) RT4 cells maintained briefly (1 min) in calcium-free condition with the addition of 25 μM BAPTA-AM had significantly reduced [^{3}H]-gemcitabine uptake compared to control. Pooled data from three individual experiments (n=3), with each condition conducted in sextuplicate, are presented as mean ± SEM (t-test, $^{\$}$P<0.0001).

Identifying ENT1-binding protein candidates

Given previous observations that calcium signalling regulates adenosine flux in some cell types (Paes-de-Carvalho et al. 2005, Zamzow et al. 2009), we suspected that CaM could be the underlying mechanism of action through direct interaction with ENT1. We conducted an *in silico* analysis of the hENT1 sequence using the Calmodulin Target Database (Yap et al. 2000). A putative CaM binding site was found between residues 224 and 244 of hENT1. CaM binding sites are based on potential binding to bulky hydrophobic residues such as Phe, Val, Ile, Leu, or Trp, in a number of different motifs (Rhoads and Friedberg 1997). Comparisons to other CaM interacting proteins and threading of the putative CaM binding domain onto a *Drosophila melanogaster* myosin light chain kinase template, led to the identification of Phe231, Tyr235, and Leu240 as putative interacting amino acids of Ca^{2+} loaded CaM and confirmed a putative 1-5-10 interaction motif (Fig. 2A). This potential CaM binding site is within the long unstructured loop of ENT1 situated in the cytoplasm between transmembrane domains 6 and 7 (Fig. 2B) and modeling of this sequence suggested that the putative interacting amino acids could interact as typically predicted for calmodulin and its target proteins (Fig. 2C). Subsequently, a mENT1 bait was used to successfully screen approximately 2 x 10^6 transformants from a NubG-X 11 day whole mouse embryo library. Clones identified in the screen underwent bait validation (data not shown) and a total of 26 prey that interacted with the mENT1 bait were identified. Putative interactors consisted of proteins found in a variety of locations and involved in a diversity of functions, such as the plasma membrane located G-protein coupled receptor, Gpbar1 (also known as TGR5), the cytoplasmic metabolic proteins such as glucose phosphate isomerase (GPI), the metabolic signalling protein such as myotrophin and cytoplasmic cytoskeletal proteins such as tubulin. Intriguingly, a number of mitochondrial proteins were also identified as putative interactors

including the adenine nucleotide translocator, SLC25A4. Given our interest in calcium-dependent regulation of nucleoside transport, we focused on further analysis of the CaM-ENT1 interaction. Functionally and physiologically relevant interactions of any of the other identified putative interactors need to be confirmed by further studies.

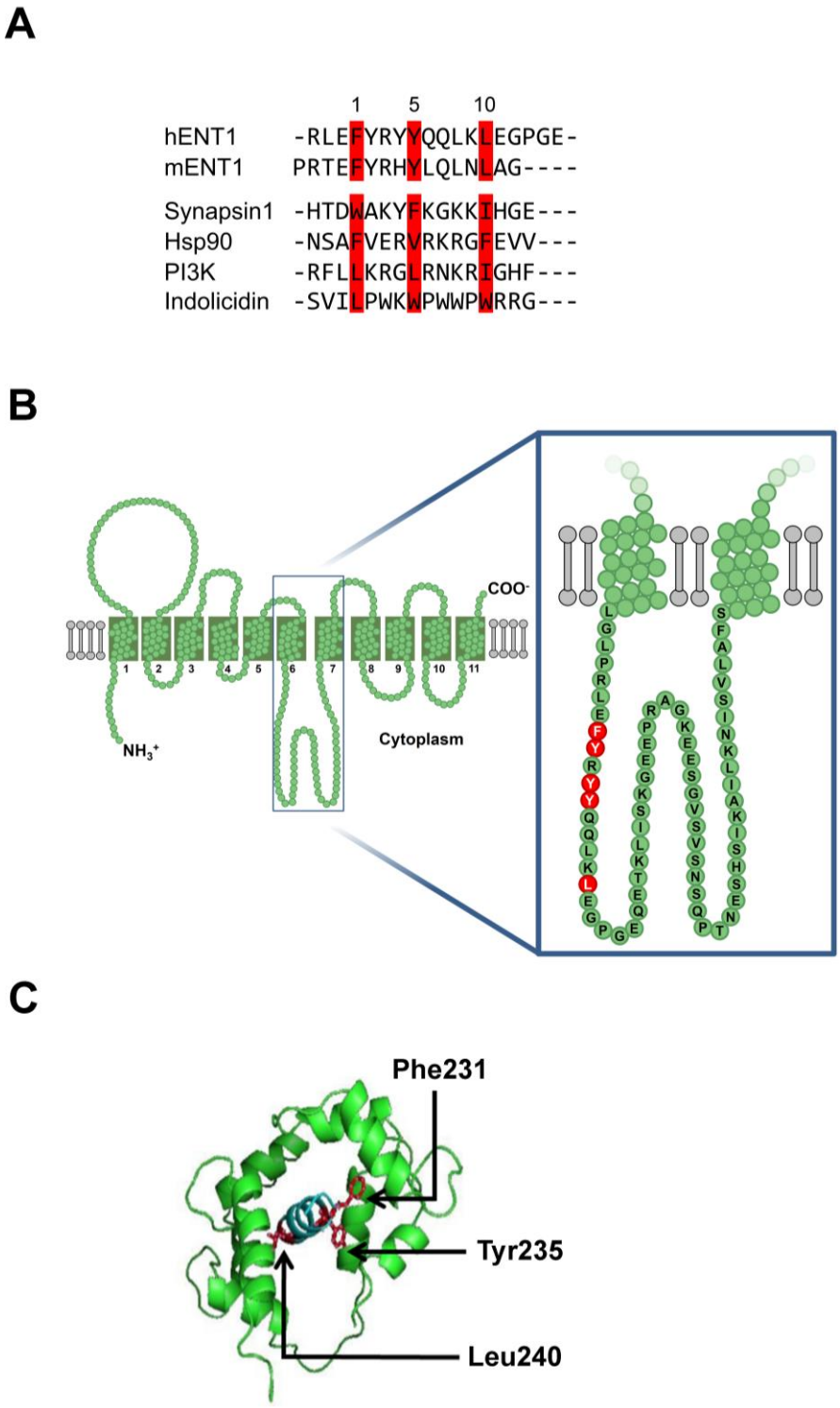

Figure 2: Calmodulin binding site in ENT1.

A) Sequence analysis of hENT1 and mENT1 identifies a 1-5-10 CaM binding motif (amino acids highlighted in red) along with other confirmed CaM binding sites.

B) Location of the 1-5-10 motif within the large intracellular loop of ENT1.

C) Modeling of putative CaM/ENT1 loop region showing putative interacting amino acids.

CaM binds to 1-5-10 interaction motif of ENT1-peptide

The putative 1-5-10 motif we identified in ENT1 is conserved in a number of other isoforms in other species (Fig. 3) where calcium has been implicated as a regulator of nucleoside transport (e.g. chicken, Paes-de-Carvalho et al. 2005; rat Zamzow et al. 2009; mouse, Wall and Dale 2013). We have previously confirmed that the intracellular loop between transmembrane domains 6 and 7 of ENT1 is generally unstructured and flexible (Reyes et al. 2011a) and we used NMR spectroscopy to confirm a biochemical interaction between CaM and hENT1. Since full-length hENT1 is highly hydrophobic and biochemically challenging to work with, we used a construct consisting of the intracellular loop for analyses. The large intracellular loop of hENT1 is predominantly unstructured but binding of CaM is predicted to force a conformational change in the unstructured loop to an alpha-helical conformation. ^{15}N-labeled CaM was titrated with the ubiquitin-hENT1 loop construct and the limited amount of line broadening confirmed that the hENT1 loop binds to CaM (Fig. 4A) in the presence of calcium, similar to other studies which have examined the interaction of a protein domain with CaM (Piazza et al. 2015).

To confirm that the predicted interacting amino acids (Phe, Tyr, Leu) of the 1-5-10 CaM-ENT1 interaction motif actually interact with CaM, I used fluorescence anisotropy to measure the interaction between the wild-type version of this region of ENT1 and mutants in which the 1-5-10 motif was disrupted. The affinity of wild type ENT1-loop and CaM was experimentally determined to be moderate (K_d = 4.54 ± 0.57 μM; n=4), while the affinity of the ΔCaM-3 mutant

(Phe, Tyr, Leu mutated to Ala) was 5-fold reduced (K_d = 22.9 ± 1.62 μM; n=3), confirming that mutation of the 1-5-10 motif severely comprises the interaction between CaM and CaM binding region of ENT1 (Fig. 4B). Mutation of 5 residues (ΔCaM-5) resulted in even lower affinity (K_d = 80.7 ± 6.13 μM; n=3), likely due to the loss of compensatory interactions as a consequence of the hydrophobic tyrosines situated next to the 1-5 motif residues (Phe, Tyr). Interaction did not occur in the absence of calcium (data not shown).

Figure 3: Multiple sequence alignment of mouse, rat and chicken ENT isoforms (Clustal 2.1) showing conservation of the putative 1-5-10 CaM binding site in species in which calcium regulation of nucleoside transport has been implicated.

A **B**

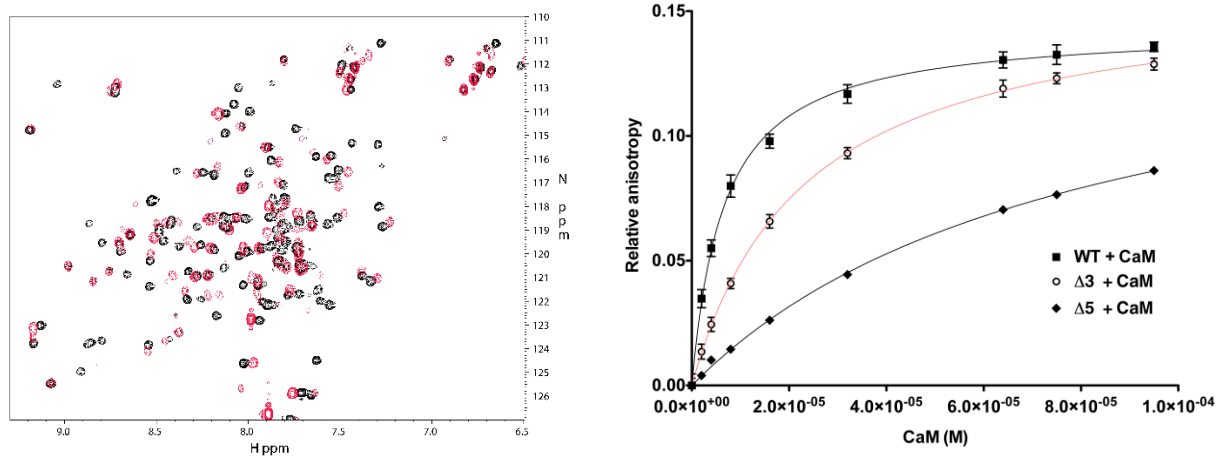

Figure 4: CaM and the ENT1-loop interact in the presence of calcium via the 1-5-10 motif.

A: HSQC spectrum of NMR analysis of interaction between CaM and hENT1-loop shows binding in the presence of calcium. Comparison of peaks representing CaM loaded with calcium (black) to CaM bound to hENT1-loop (red) indicates a protein-protein interaction is occurring.

B: Mutation of the residues comprising the 1-5-10 motif of the hENT1-CaM interaction domain reduces affinity of CaM for the hENT1-loop. Fluorescence anisotropy confirms that wild type hENT1-loop has a moderate affinity, K_d of 4.54 ± 0.57 μM (n=4) while the ΔCaM-3 mutant has 5-fold lower affinity, K_d of 22.9 ± 1.62 μM (n=3), confirming that mutation of the 1-5-10 motif abrogates the interaction between CaM and the CaM binding region of hENT1. Moreover, the ΔCaM-5 mutant with 5 altered residues resulted in an even lower affinity with a K_d of 80.7 ± 6.13 μM (n=3).

HA-hENT1 and CaM co-immunoprecipitate in the presence of calcium

The loss of high affinity binding for CaM to the interaction domain on hENT1 when the 1-5-10 domain was altered suggested that a CaM-hENT1 complex would form in the presence of calcium. To test this, anti-HA conjugated agarose beads were loaded with HEK293 cell lysate expressing HA-hENT1. HA-ENT1 was immunoprecipitated with CaM in the presence of calcium, but this association was significantly reduced in the absence of supplementary calcium and by the addition of EGTA (Fig. 5A) to less than 20% of control levels based on densitometric analyses (Fig. 5B).

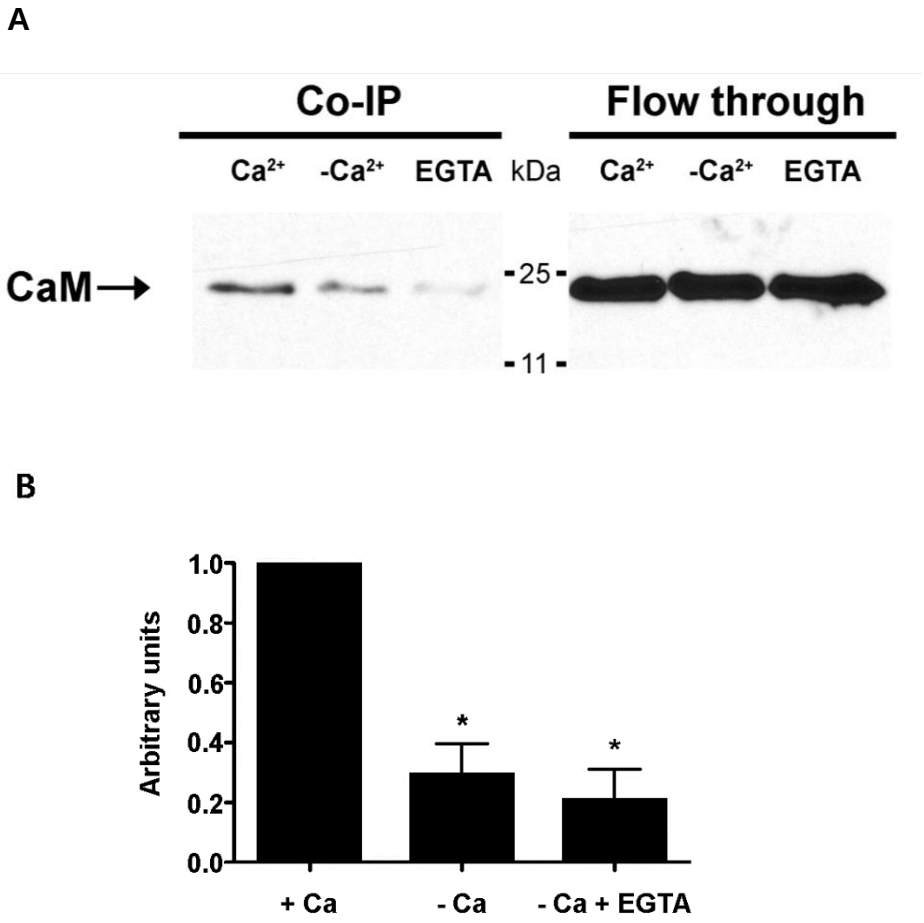

Figure 5: CaM and ENT1 interact in presence of calcium.

A: CaM is co-immunoprecipitated (Co-IP) with HA-ENT1 bait in the presence of 2 mM $CaCl_2$ (lane 1). Signal intensity is reduced with the absence of calcium (lane 2) and virtually abolished in the presence of the calcium chelator EGTA (lane 3). Right side of blot shows flow-through (FT) from same experiment. Representative blot, repeated 4 times with similar results (+Ca and -Ca n=4, EGTA n=2). Markers are 75, 25, and 11 kDa.

B: Densitometric analysis of immunoblots shows significant decrease in intensity of signal in the absence and/or chelation of calcium (pooled data, mean ± S.D, +Ca and -Ca n=4, EGTA n=2, *$P<0.05$).

Increased intracellular calcium levels leads to increased sodium-independent nucleoside uptake

Having confirmed that there was a biochemical interaction between CaM and hENT1, acting through a 1-5-10 motif within the large intracellular loop, we determined whether this was functionally significant. My data show that reducing calcium in cells leads to lower levels of nucleoside flux suggesting that varying calcium levels inside the cell results in modulation of nucleoside flux, so I hypothesized that increasing intracellular calcium would lead to an increased nucleoside uptake. To confirm this, I treated HEK293 cells with thapsigargin, a non-competitive SERCA inhibitor that leads to a gradual $[Ca^{2+}]_i$ increase in cells as they lose the ability to effectively sequester cytosolic calcium. My data show that a brief (1 min) exposure of HEK293 cells to thapsigargin (1.5 μM) led to a significant increase (~26%) in nucleoside transport (Fig. 6A). The increase in intracellular calcium levels stimulated by thapsigargin treatment was confirmed by calcium imaging and the calcium dye Fluo-4 (Fig. 6B). These data confirm that changes in intracellular calcium levels modulate ENT-dependent transport of nucleoside and nucleoside analog drugs in human cells.

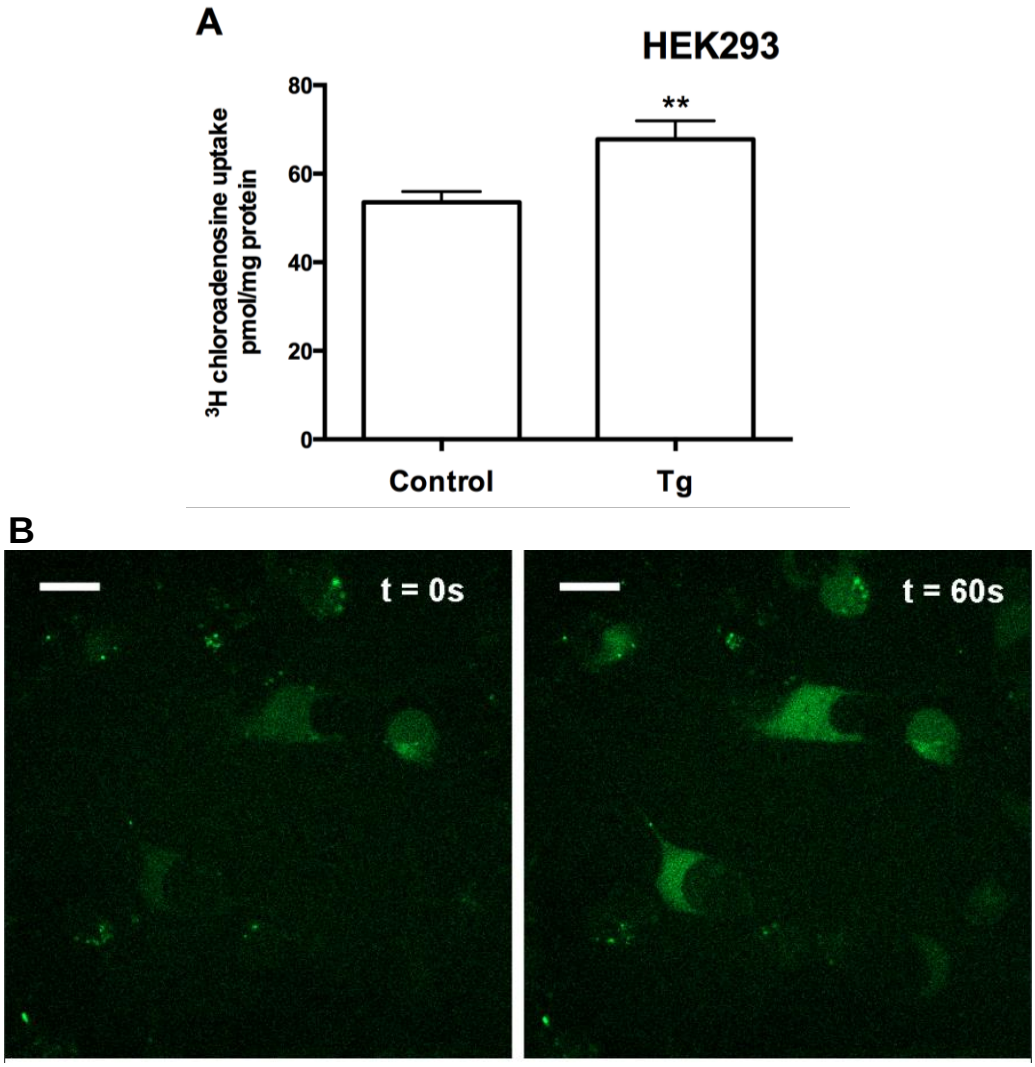

Figure 6: Increasing intracellular calcium results in increased nucleoside transport.

A) HEK293 cells were treated (1 min) in the presence or absence of 1.5 μM thapsigargin (Tg). Pooled data (n=3) with each condition conducted in sextuplicate, mean ± SEM (t-test, **P<0.01). B) Increased intracellular calcium level following 1.5 μM Tg treatment was confirmed with live cell imaging of HEK293 cells preloaded with Fluo-4 calcium indicator. HEK293 cells on glass bottom dishes at 5% CO_2 at 37 °C were treated with 1.5 μM Tg (initial condition t = 0s on left). Following treatment, fluorescent intensity (excited with 488 nm) gradually increased and 1 minute after treatment, there was a substantial increase in fluorescence intensity (t=60s shown on right). The scale bars represent 20 μm.

Blocking CaM-binding reduces uptake of nucleosides

After confirming there was a calcium dependent protein interaction between CaM and hENT1, and a calcium-dependent modulation of nucleoside flux, I hypothesized that the mechanism of regulation of ENT1 function was via protein-protein interactions between ENT1 and CaM. To confirm this, I treated HEK293 cells with W7, a cell permeable antagonist of CaM which would disrupt an endogenous CaM-ENT1 interaction (Osawa et al. 1998). Cells treated with W7 had significantly decreased sodium-independent nucleoside uptake compared to control (Fig. 7). These data corroborate my previous observations and suggest that calcium-dependent CaM-ENT1 interactions affect transporter function.

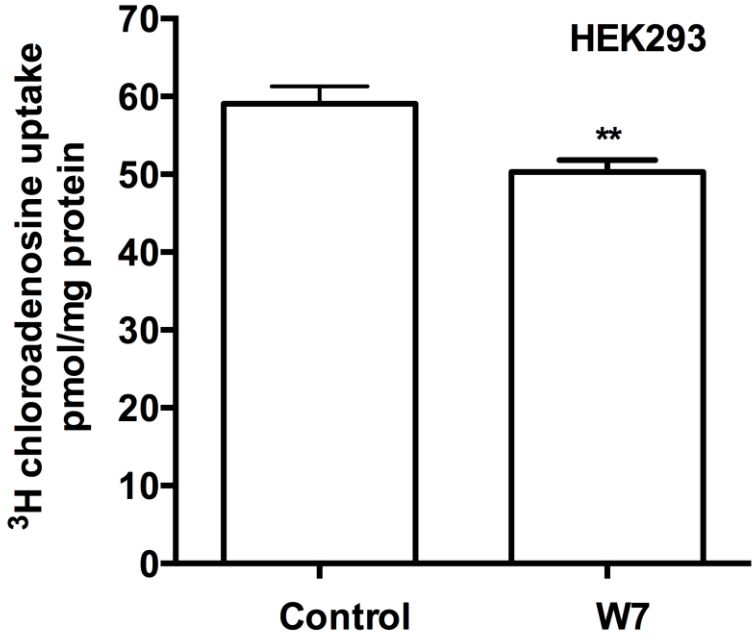

Figure 7: Blocking CaM interaction significantly reduces nucleoside transport.

HEK293 cell treated for 1 min with Ca^{2+}-containing buffer + 50 μM W7 had significantly less [^{3}H]-2-chloroadenosine uptake compared to 0.075% DMSO control. Pooled data from three

individual experiments (n=3) with each condition conducted in sextuplicate, are presented as mean ± SEM (t-test, **P<0.01).

Increasing $[Ca^{2+}]_i$ by NMDA receptors stimulation leads to increased ENT1-dependent transport, but is blocked with treatment with NMDA receptor or CaM antagonists

Manipulation of calcium levels and antagonizing CaM results in modulation of ENT-dependent nucleoside flux supporting my hypothesis that ENT1 function is regulated by a Ca^{2+}/CaM interaction. This mechanism explains previous observations made in cultured avian retinal cells (Paes-de-Carvalho et al. 2005), in which activation of glutamate receptors promotes a calcium-dependent and transporter-mediated release of purines, implying an important physiological link between glutamate signalling and ENT-dependent purine flux in the CNS. I therefore used the human cell line U-87 MG to determine if glutamate receptor activated calcium-dependent signalling leads to modulation of nucleoside uptake. I confirmed that these cells express NMDAR1 and ENT1 (Fig. 8A). I then observed that stimulation of NMDA receptors by glutamate and glycine leads to an increase in intracellular calcium (Fig. 8B) and a significant increase in nucleoside uptake, which can be blocked by the NMDA antagonist MK-801 (Fig. 8C) and the CaM antagonist W7 (Fig. 8D). Taken together, these data confirm that ENT1 is subject to receptor activated calcium-dependent calmodulin regulation, which can modulate the flux of both nucleosides and nucleoside analogs (Fig. 9).

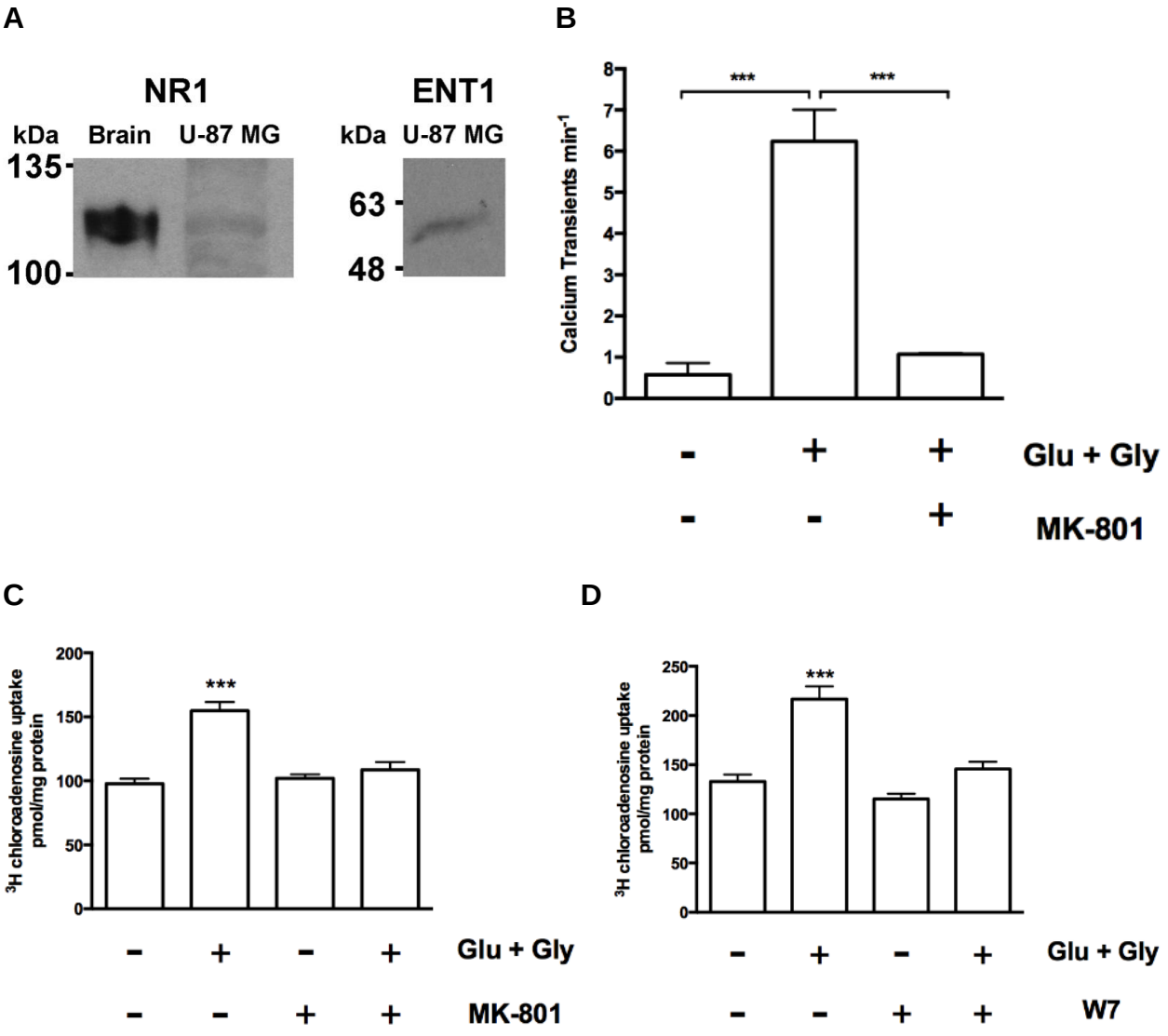

Figure 8: NMDA-receptor activation leads to CaM-dependent increased nucleoside transport.

A) Western blot analysis confirmed the presence of ENT1 and NMDAR1 protein in U-87 MG cells. A mouse synaptoneurosome preparation (0.5 µg protein loaded) was used as a control for NMDAR1. Crude membrane preparations (100 µg protein loaded) of U-87 MG cells were used. Representative image shown, experiment repeated 3 times.

B) Increased intracellular calcium levels in U-87 MG cells following glutamate (1 mM) and glycine (100 μM) treatment was confirmed by live cell imaging with cells preloaded with Fluo-4 calcium indicator (data not shown). Calcium transients, the rapid increase and subsequent gradual decrease of calcium in in the cell, in a field of view of a plate of U-87 MG cells on glass bottom dishes at 5% (v/v) CO_2 at 37 °C were quantified at basal levels (in HBSS following 20 minute incubation), then imaged in HBSS containing glutamate (1 mM) and glycine (100 μM) for 10 minutes, and then imaged following the addition of the NMDA receptor inhibitor MK-801 (50 μM). Bars represent mean ± standard deviation of pooled data from three independent experiments (one-way ANOVA with Newman-Keuls multiple comparison post hoc test, ***P<0.001). Glycine (100 μM) treatment alone had no effect in twice repeated experiment (data not shown).

C) U-87 MG cells were pre-treated for 10 minutes with HBSS, then treated for 20 minute with or without glutamate (1 mM) and glycine (100 μM) in the presence or absence of MK-801 (50 μM) in HBSS. Pooled data from three individual experiments (n=3), with each condition conducted in sextuplicate, are represented as mean ± SEM (one-way ANOVA with Newman-Keuls multiple comparison post hoc test, ***P<0.001).

D) U-87 MG cells pre-treated for 10 minutes with HBSS, then treated for 20 minute with or without 1 mM glutamate and 100 μM glycine in the presence or absence of W7 (50 μM) in HBSS. Pooled data from three individual experiments (n=3), with each condition conducted in sextuplicate, are represented as mean ± SEM (one-way ANOVA with Newman-Keuls multiple comparison post hoc test, ***P<0.001).

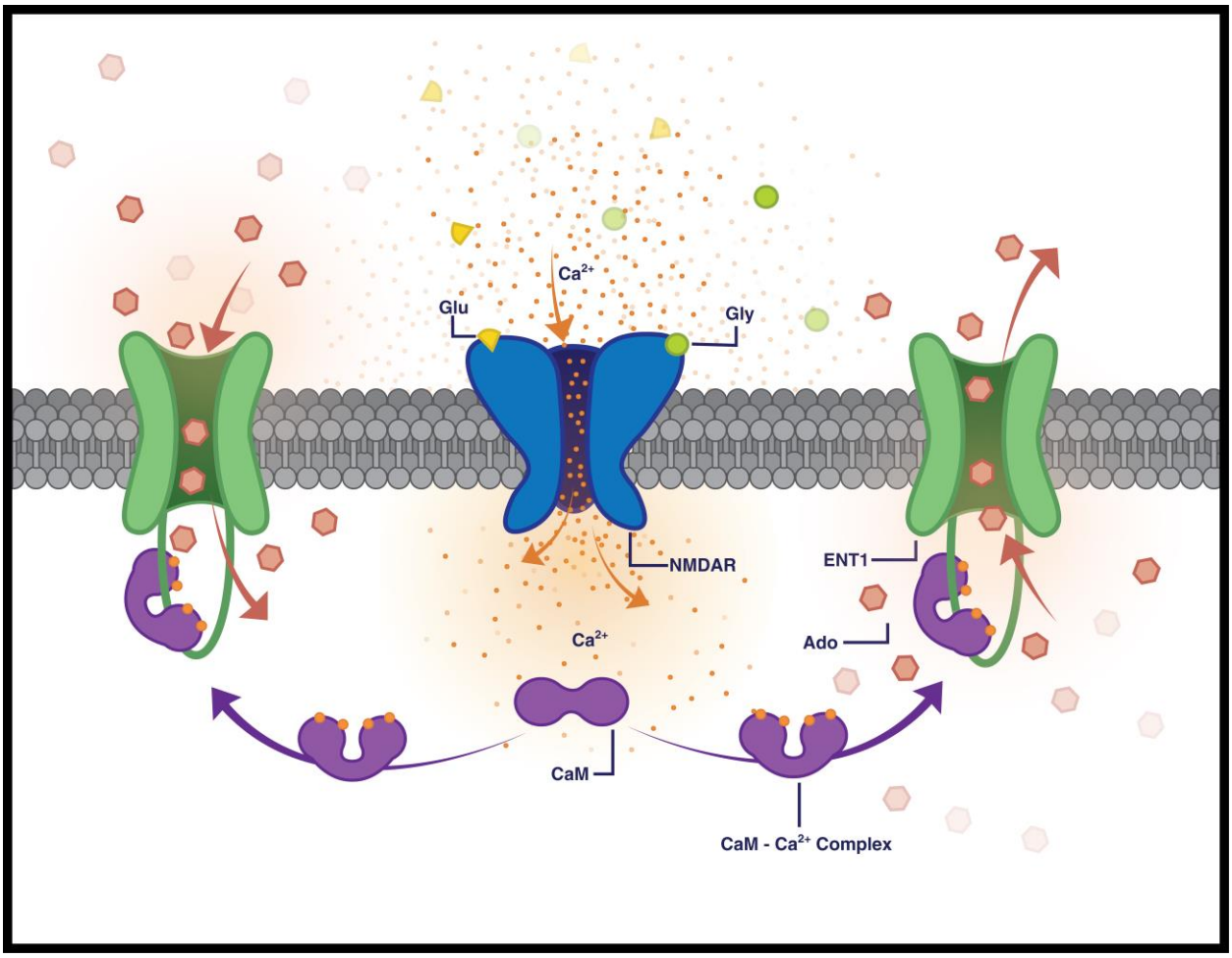

Figure 9: ENT1 is regulated by receptor-stimulated calcium signalling to modulate nucleoside flux.

This cartoon depicts a putative model for receptor dependent regulation of adenosine transport involving NMDA receptors, CaM and ENT1. Glutamate/glycine stimulation of NMDA receptors leads to calcium influx resulting in local increases in intracellular calcium and calcium binding to CaM which results in a shift from apo-CaM to a Ca^{2+}/CaM complex. CaM binds to ENT1 (in the presence of increased calcium), leading to an increase in nucleoside flux, which can result in either increased nucleoside uptake (left) or increased nucleoside release (right) depending on the endogenous adenosine concentration gradient.

Discussion

The cloning of the first equilibrative nucleoside transporter, hENT1 (Griffiths et al. 1997), led to interest in understanding underlying regulatory mechanisms and physiological importance of this prototypic isoform, in nucleoside analog drug delivery and purinergic signalling in the cardiovascular and central nervous systems (Rose et al. 2010, Grenz et al. 2012, Santos-Rodrigues et al. 2014, Rose and Coe 2008). As a component of the purinome, it is likely that ENT1 is subject to feedback regulation by several signalling pathways via a variety of mechanisms (Santos-Rodrigues et al. 2014). We hypothesized that protein-protein interactions are likely to play a role in regulation of ENT1 and speculated that calcium related proteins would be good candidates for interactors. Therefore we undertook a study to identify and characterize putative interactors using the MYTH approach. MYTH screening is specifically designed for membrane proteins such as transporters and identifies a range of putative interactors, representing a diversity of functions. While we cannot confirm at this point that all these putative interactors represent physiologically relevant partners for ENT1, our data suggest that ENT1 may interact with a variety of proteins, possibly in the form of a protein complex or a metabolon, whereby a transient complex between nucleoside related enzymes and proteins can function.

Here, I have identified a calcium-dependent interaction between CaM and ENT1. CaM binds hundreds of proteins in the cell varying widely in function (Ishida and Vogel 2006). Some examples are the EGF-receptor, IP_3 receptor, dopamine transporter, phosphatidylinositol 3-kinase, beta-2-glycoprotein, ATP-binding cassette transporter A1, and SLC9A7 (Martín-Nieto and Villalobo 1998, Sun et al. 2013, Padmanabhan et al. 2008, Joyal et al. 1997, Klaerke 1997, Iwamoto et al. 2010, Kagami et al. 2008). These proteins are functionally diverse, and the role

CaM plays when binding these proteins varies as well. In the case of EGF-receptor, CaM binding prevented kinase-dependent phosphorylation (Martín-Nieto and Villalobo 1998). With IP_3 receptors, CaM binding caused a conformational change in the protein which is required for activation. CaM can also enhance protein activity, as seen in the dopamine transporter where CaM regulated dopamine transporter function through CaMKII-dependent phosphorylation, or in phosphatidylinositol 3-kinase and SLC9A7 (a plasma membrane Na^+/H^+ exchanger) where CaM binding increased protein function (Padmanabhan et al. 2008, Joyal et al. 1997, Kagami et al. 2008). CaM binding can also protect against proteolysis as seen with the ATP-binding cassette transporter A1 (Iwamoto et al. 2010). These examples highlight the diversity of proteins which interact with CaM and the varied effect this interaction has, but in each case shown here, this interaction plays an important role in regulation of the protein.

The presence of CaM among the putative interactors suggested our prediction that protein-protein interactions might underlie previous observations (Paes-de-Carvalho et al. 2005, Wall and Dale 2013) of calcium-dependent regulation of nucleoside flux might be correct. I confirmed the site of interaction between CaM and ENT1 as a 1-5-10 motif which is located in the large intracellular loop between transmembrane domains 6 and 7. The large loop was identified as a potential regulatory target when ENT1 was first cloned (Griffiths et al. 1997) and many subsequent studies have suggested that this region is important functionally or in terms of regulation (Reyes et al. 2011b, Bone et al. 2007, Ramadan et al. 2014, Valdés et al. 2014, Cano-Soldado and Pastor-Anglada 2012). However, this is the first report to demonstrate a biochemical interaction with another protein and I confirmed that amino acids phenylalanine 231, tyrosine 235 and leucine 240 contribute to the transient interactions between the ENT1 loop and calmodulin, in the presence of calcium, to regulate ENT1. Moreover, previous studies

demonstrating the regulation of nucleoside flux by calcium have been done in models (rat, mouse, chicken) which also possess ENTs with the 1-5-10 motif in the large intracellular loop, shown here to be involved in calcium-dependent CaM regulation of ENT1 (Fig. 9). The presence of a putative CaM binding site in ENT1 isoforms in other vertebrates in this region suggests that Ca^{2+}/CaM regulation of equilibrative nucleoside transport is perhaps widely distributed phylogenetically and thus likely to be a fundamental mechanism of regulation of this protein family.

Calcium levels and adenosine levels have previously been shown to affect each other (Gerwins and Fredholm 1992, Peakman and Hill 1995, Spirli et al. 2012). To reduce potentially confounding effects of regulation of purine nucleoside metabolism by calcium, we routinely conduct transport analyses within the linear phase of transport (prior to permeant concentration reaching equilibrium) thereby ensuring we are focusing our attention on the regulation of transport rather than metabolism of the substrate.

A number of studies have implicated calcium as a potential regulatory component of a poorly understood feedback mechanism that regulates nucleoside flux. It is well established that NMDA-type receptor activation results in a rapid increase in intracellular calcium leading to a wide variety of effects and that NMDA-type glutamate receptor activated Ca^{2+}/CaM-dependent CaMKII are key regulators of synaptic plasticity underlying learning and memory (Hell 2014). Intriguingly, it is now clear that ENT1 plays a significant role in a variety of purinergic- and glutamatergic-dependent behavioural responses, since ENT1 knockout mice show altered goal-directed behaviours and altered addictive responses to ethanol (Nam et al. 2013, Chen et al. 2010, Chen et al. 2007). The findings presented in this paper provide a mechanistic basis for previous observations in cultured avian retinal cells and mouse hippocampal slices (Paes-de-

Carvalho et al. 2005, Wall and Dale 2013) where glutamate receptor activated calcium influx leads to enhanced efflux of nucleosides via ENT1 (in a process that involves CAMKII in avian cells). Moreover, our findings may provide an explanation for the glutamatergic and adenosinergic-dependent behavioural effects noted in ENT1 knockout mice.

The existence of calcium-regulated ENT1 supports a model that incorporates a feedback relationship between receptor coupled (NMDA-type glutamate or other) calcium signalling, CaM binding and altered ENT1 function, leading to modulation of extracellular adenosine levels and subsequent adenosine receptor signalling events. Since Ca^{2+}/CaM modulation of ENT1 exists in different cell types, this regulation may be widely distributed and perhaps universal for this isoform. Regulation of membrane transport activity by direct interaction of Ca^{2+}/CaM has been shown for other SLC families, such as SLC9A7, where identification and characterization of the interactome (Kagami et al. 2008) suggests Ca^{2+}/CaM regulation.

It remains to be seen whether Ca^{2+}/CaM binding to ENT1 is involved in shielding the large intracellular loop from phosphatases or ubiquitination, as seen in other membrane proteins (O'Donnell et al. 2013, Iwamoto et al. 2010). For instance, Ca^{2+}/CaM-dependent calcineurin activity in mouse and rat hippocampal synapses results in dephosphorylation of endocytic proteins that initiated both rapid and slow endocytosis (Sun et al. 2010). A number of consensus kinase target sites and a number of studies have inferred a role for phosphorylation or kinase-dependent processes in regulation of ENTs (Bone et al. 2007, Ramadan et al. 2014, Paes-de-Carvalho et al. 2005, Chaudary et al. 2004, Coe et al. 2002, Coe et al. 1996). CaM-dependent phosphorylation of a plasma membrane solute carrier has been previously described for aquaporin-0 (AQ0), a water and small solute channel exclusively expressed in eye lens cells and the underlying mechanism of regulation has been identified (Reichow et al. 2013). Our research

has shown that the large intracellular loop of ENT1 can be phosphorylated (*in vitro* and *ex vivo*) directly (Reyes et al. 2011b) by PKC and PKA suggesting that this is a potential regulatory mechanism, although no functional correlate has yet been found. Intriguingly, a role for CaMKII has been identified in regulation of adenosine flux via ENT1 in avian retinal cells (Paes-de-Carvalho et al. 2005). Consensus sites are not well conserved between species and a convincing CaMKII target site was not identified in the mammalian sequences. However CaMKII and phosphatases can mutually inhibit each other (Grey and Burrell 2010) and a role for PP1/2A in regulating the ethanol sensitivity (which is kinase dependent) of the adenosine transporter in neuronal cells (Coe et al. 1996) has been reported. Taken together, these data suggest that CaMKII could regulate ENT1 via phosphatase-dependent removal of phosphorylation sites, such as Ser279, 286 and Thr274 (Reyes et al. 2011b), which are located in the second half of the large intracellular loop, while the CaM binding domain resides in the proximal part of the loop. Thus, as intracellular calcium levels rise, CaM interacts with the ENT1 loop, altering the conformation of the previously unstructured loop and possibly changing accessibility of the phosphorylation sites. Functional consequences of this regulation are changes in overall rates of nucleoside flux. Further work should identify if kinases or phosphatases such as CaMKII or calcineurin bind to CaM during the CaM-ENT1 interaction and result in a change in the phosphorylation state of ENT1. Our data has shown that CaMKII did not appear as a putative interactor of mENT1 or hENT2 based on MYTH screens (Bicket et al. 2016, Grañé Boladeras et al. unpublished). It is possible that the involvement in nucleoside flux observed by Paes-de-Carvalho et al. (2005) was through another mechanism and not via direct ENT1 phosphorylation. It is also worthy of noting that the CaM binding site of mENT1 is shifted compared to hENT1, which may also play a factor in the MYTH screen since mENT1 was used for the screen. I do not yet know whether

Ca^{2+}/CaM regulates ENT1 via CaMKII-dependent phosphorylation at the intracellular loop, but there are sufficient data to propose this as a potential mechanism worth exploring for the future.

ENT1 plays a major role in the efficacy of uptake a large class of drugs used in a variety of clinical settings. It is also the target of drugs used to treat cardiac arrhythmias and other conditions. Consequently, a deeper understanding of the regulation of ENT1 may have positive implications for improved chemotherapeutics. Presence of ENT1 protein or mRNA has been reported as being a predictive indicator for sensitivity to nucleoside analog drugs (Achiwa et al. 2004, Klanova et al. 2014, Santini et al. 2011, Spratlin and Mackey 2010) but also as having no correlation to response (Bock et al. 2012). Relative levels (either protein or mRNA) of ENT1 may not be accurate correlates of drug response, especially if "low" levels of protein can activated to enhance update of drug and amplify effects. Indeed, proteins involved in calcium-dependent signalling have been reported to be significantly overexpressed/up-regulated in gemcitabine sensitive pancreatic (panc-1) cells and down-regulated in resistant cells in the absence of any observed change in levels of nucleoside transporters (Chen et al. 2011) and here, I demonstrated in a bladder cancer cell line that antagonism of CaM resulted in reduced uptake of the nucleoside analog drug, gemcitabine. Thus, modulation of Ca^{2+}/CaM-dependent signalling by manipulation of $[Ca^{2+}]_i$ may either enhance or compromise the efficacy of nucleoside analog drugs depending on the nature of the calcium effect. This may be particularly important in clinical situations where multiple drugs (e.g. nucleoside analogs, blood pressure medication) are involved.

The development of my model, centred on Ca^{2+}/CaM dependent regulation of ENT1, was based on previous work implicating calcium with nucleoside flux. As previously shown in rat cortical neurons, adenosine and inosine release were dependent on extracellular calcium (Zamzow

et al. 2009). Moreover, this nucleoside release was blocked by inhibiting NMDA receptors. I applied this model to a human glioblastoma cell line and showed that NMDA receptor activation led to an increase in intracellular calcium, which enhanced ENT1-dependent nucleoside flux in a CaM-dependent manner. This model can also be applied to numerous other cellular contexts, since both CaM and ENT1 are ubiquitously expressed. I would expect that the activation of other ionotropic receptors expressed in the cell could yield a similar effect. This highlights further potential interplay between other members of the purinome. Some examples could include P_2X activation by ATP which allows an influx of calcium ions into the cell, or P_2Y receptors which upon activation by the appropriate ligand (ATP, ADP, UTP, UDP, etc.) could lead to downstream signalling via the $G_{q/11}$ pathway, which results in stimulated release of intracellular calcium from the ER. Adenosine receptor A_{2B} is another example of a receptor where G_s subunit activation ultimately leads to an influx of intracellular calcium from intracellular stores. Although it is not yet clear if release of calcium from the ER is sufficient to initiate CaM binding to ENT1 at the plasma membrane since localization of the calcium signal is often critical due to rapid sequestering of the free ions, the most probable upstream regulator of ENT1 through Ca^{2+}/CaM would be ionotropic receptors and calcium channels due to their close proximity to ENT1 at the plasma membrane. Further work identifying potential candidates of upstream regulators which initiate the calcium signalling cascade would be beneficial, especially since expression of ionotropic receptors and calcium channels vary widely depending on environmental variables as well as cell type, yielding new responses depending on many different factors.

It is also widely known that calcium signalling plays a crucial role in cell differentiation and proliferation (Tennakoon et al. 2015, Pinto et al. 2015). These processes are coordinated through several known pathways, such as activation of kinases like CaMK, MAPK, PKC, and the

phosphatase calcineurin, which lead to transcription factor activity. A key example would be how increased calcium concentration led to a phosphorylation cascade that results in NF-κB activation and movement to the nucleus which activates genes associated with cell survival and proliferation (Moscat et al. 2008, Mellström et al. 2008, Pinto et al. 2015). Furthermore, CaM has been implicated in proliferation in its own right, as reducing CaM disrupts G_1 and M-phase progression, interfering with DNA replication (Rasmussen and Means 1989, Shapiro et al. 1998, Yu et al. 2005, Pinto et al. 2015). Another means of the cell regulating proliferation and differentiation in a calcium-dependent manner is through RasGF, whereby increased calcium concentration leads to phosphorylation of RasGP to make RasGFP, which along with PKC initiates the MAPK signalling cascade (Clapham 2007). Not all cells are proliferating or differentiating but these pathways are still relevant even to cells which are not. In contrast to the previous examples, cells found in the CVS like adult cardiomyocytes generally undergo hypertrophy or cell death following these types of cell signals (Olson 2006, Clapham 2007). It is clear that calcium and CaM are critical in modulating cell growth and fate, but this raises the larger question: why does calcium/CaM regulate ENT1?

As nucleic acids are polymers of nucleotides, it is clear that events like DNA and mitochondrial replication would lead to a high demand of nucleotides for the cell (Fasullo and Endres 2015, Taanman 1999). S-phase (synthesis phase) is when nucleotides are incorporated into nucleic acids in the nucleus, as the cell must replicate its entire genome (Shi et al. 2001, Brunskill et al. 2011, Romar et al. 2016). At the G_1/S checkpoint, the cell must assess whether it has sufficient resources to undergo DNA synthesis and begin expressing proteins necessary for DNA replication (Bell and Dutta 2002). The cell must respond appropriately to provide nucleosides adequate for its nucleic acid production needs.

The response of cells to meet increased nucleoside demand is met in part through regulation of nucleoside transporters and increased *de novo* synthesis (Warner et al. 2014). As seen in Lesch-Nyhan syndrome, where nucleotide pool imbalances stemming from a mutation in the hypoxanthine-guanine phosphoribosyltransferase gene led to increased uric acid production when these nucleobases cannot be made into nucleotides, *de novo* synthesis and salvage pathways must remain in balance or outcomes like excess uric acid accumulation and mutagenesis will occur (Rosenbloom 1968, Rampazzo et al. 2010, Fu et al. 2014, Fasullo and Endres 2015). There are serious consequences for the cell not meeting nucleotide demands, so during the S-phase nucleoside salvage is increased via nucleoside transporter regulation. As highlighted by Guillén-Gómez et al. (2012), nucleoside transporters are involved in inflammation, cell proliferation, oxidative stress, and are regulated via cytokines and cell-to-cell contact (Hirsh et al. 2007, Löffler et al. 2007, del Santo et al. 1998, Soler et al. 2001, García-Manteiga et al. 2003). I see this increasing demand for nucleosides in the cell cycle dependent expression of CNTs. CNT1 has been shown to be more abundant at the G_1-S transition (Valdés et al. 2002). Increased CNT1 is not only critical for the uptake of nucleosides but also as a transceptor (a transporter with receptor functionality) where it can also regulate cell cycle in a translocation-independent manner (Pérez-Torras et al. 2013). Similarly, ENT1 abundance at the plasma membrane more than doubles during the late G_1- and S-phases (Cass et al. 1979) and the supply of nucleotides regulated ENT1 plasma membrane abundance (Pressacco et al. 1995). Moreover, pro-fibrotic signals led to increased ENT1 expression and ENT1 was shown to be necessary for epithelial-to-mesenchymal transition (a pro-fibrotic event) in the kidney (Guillén-Gómez et al. 2012). These data support that nucleoside transporters are recruited to meet the increased demand for nucleotides during cell proliferation or differentiation.

It is clear that both calcium signalling and nucleoside transport are integral functions of cell cycle. My work has shown that ENT1 is regulated by CaM in a calcium-dependent manner and I have proposed a model whereby local increases in calcium levels can lead to increased ENT1-dependent nucleoside transport. One possible context in which this could be applied is that local increases in calcium from pro-proliferative pathways could lead to CaM binding ENT1, which leads to an increase in nucleoside transport across the plasma membrane. This, in concert with the uptake of nucleosides by CNTs, could meet the rising demand of the cell for nucleosides during the late G_1- and S-phases. Intriguingly, the Calmodulin Target Database detected a high probability, putative CaM binding domain for CNT1 (1-4-10 domain at Val519, Trp525, and Val528) which was previously thought to be extracellular based on the two-dimensional topology (Yap et al. 2000). Recent advances in the understanding of CNT structure have suggested that this region is intracellular, making it a possibility that CaM binds CNT1 at this region (Johnson et al. 2012). CNT2 and CNT3 also have high probability sites (between residues Leu118 and Val154, and between residues Phe273 and Leu289 respectively) but these may not be physiologically relevant due to proximity to transmembrane domain helices or existing extracellularly (Yap et al. 2000). It is plausible that both CNT1 and ENT1 are regulated by CaM in response to calcium, although there is no evidence to suggest a direct link between CNTs and calcium at this time. Both CNTs and ENTs are upstream regulators of calcium signalling through the transport of cyclic ADP-ribose. ENT2, CNT2, and CNT3 uptake of cyclic ADP-ribose in murine fibroblasts and human promyelocytic leukemia cell lines led to spikes in intracellular calcium concentrations following ryanodine receptor binding, which could lead to a calcium-mediated enhancement of proliferation (Guida et al. 2002, Guida et al. 2004, De Flora et al. 2004). This does not in itself

suggest that calcium regulates CNTs, but there are still many links between calcium signalling and nucleoside transporters as a whole.

The movement of nucleosides into the cell, which are promptly phosphorylated, leads to these accumulated molecules forming a nucleotide pool in the cell. Molecules like ATP and GTP are important for enzymatic reactions in the cytosol, but these pools must meet a higher required level in preparation of nucleotide polymerization during S-phase in the nucleus to ensure proper DNA replication (Anglana et al. 2003, Fasullo and Endres 2015). The source of the nucleoside/nucleotide pool required for replication is not well characterized, but previous work has identified that insufficient supply of nucleotides during DNA replication is devastating for the cell (Anglana et al. 2003, Fasullo and Endres 2015). The transport of molecules and proteins into the nucleus via the nuclear pore complex seems unlikely to supply the required nucleotides for replication (Weis et al. 1996, Warner et al. 2014, Fasullo and Endres 2015). Recently, work performed by a colleague in our lab has suggested that functional splice variants of ENT2 (HNP32 and HNP36) exist in the inner nuclear envelope to drive nucleosides into the nucleus and HNP32 and HNP36 knock down results in decreased proliferation and S-phase arrest (Grañe Boladeras et al. unpublished). This may also explain previous reports which identified ENTs at the nuclear envelope, which did not appear to have a clear function (Mani et al. 1998). This is supported by the existence of functional CNT3 at the outer nuclear membrane (Errasti-Murugarren et al. 2009). This work suggests that nucleosides are driven into the nuclear envelope via CNT3, and can passively move into the nucleus via HNT32 and HNT36 (Grañe Boladeras et al. unpublished). During a state of enhanced proliferation, the demand for nucleotides in the nucleus would be much higher. The recent development of fluorescent nucleoside analogs which are transportable by nucleoside transporters (Zhang et al. 2006, Claudio-Montero et al. 2015) could help explain where

these molecules accumulate (under basal conditions, they appear to be evenly distributed throughout the entire cell). Monitoring live cells synchronized in cell cycle with confocal microscopy or flow cytometry could reveal a shift in the pool towards the nucleus during the S-phase. The link between calcium stimulated proliferation and the signalling of nucleoside transporters to drive nucleosides into the cell (and potentially into the nucleus as well) is rational and merits further research.

Cell proliferation is of paramount importance, being implicated in many diseases (for instance, cancer, atherosclerosis, rheumatoid arthritis, psoriasis, idiopathic pulmonary fibrosis, scleroderma, birth defects, and cirrhosis of the liver), tissue repair, development, transplantation, fertilization, and immune function (Sporn and Harris 1981, Tennakoon et al. 2015, Gurtner et al. 2008, McArdle and Ashworth 1999, Franceschi 1989, Piltti et al. 2015, Ullman et al. 1990). There have been many links identified between purinergic signalling and cell proliferation. For instance, both adenosine nucleosides and nucleotides enhance tissue repair and activation of P_2Y receptors is critical for fibrosis (Ferrari et al. 2016). Purinergic signalling has also been recognized as a regulator of autocrine and paracrine stimulated proliferation, pro- or anti-apoptotic pathways, differentiation of stem cells, and immune system response, with P_1 and P_2 receptors playing key roles (Cavaliere et al. 2015). The mechanism uncovered in this chapter is a very important piece of the larger story, where cells tightly regulate their growth and differentiation. This work is applicable to innumerable clinical applications wherever dysfunction of proliferation, differentiation, or cell migration are associated with disease traits. Identifying a link between calcium signalling, a global regulator of proliferation and differentiation, and ENT1, an ubiquitously expressed nucleoside transporter critical for nucleoside salvage, provides a

mechanism in which the cell can regulate the nucleoside pool to ensure adequate nucleotides are available for DNA replication and ensure high fidelity transcription.

In summary, we have used a variety of novel techniques to identify the first set of putative interactors for the equilibrative nucleoside transporter, ENT1. These putative interactors span a variety of proteins raising the possibility of multiple interacting partners across a range of protein types. Moreover, I have confirmed that CaM binds to a defined region of the large intracellular loop of ENT1 in a calcium dependent manner suggesting that calcium signalling is a regulatory mechanism controlling some aspect of ENT1 behaviour. I have also described a novel receptor-dependent regulatory mechanism whereby intracellular calcium modulates nucleoside and nucleoside analog drug uptake via CaM-dependent interaction of ENT1. This report is the first to provide a mechanistic basis to explain calcium signalling-dependent regulation of nucleoside flux and provides novel insights into the importance of calcium in the varied roles of the *SLC29* family.

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Disclosures

The authors have no conflicts of interest, financial or otherwise, to declare.

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Supplementary Materials and Methods

Generating Mutant GFP-ΔCaM-ENT1 vector

A pEGFP-C1 vector containing the human ENT1 gene was already generated as described (Nivillac et al. 2009). Site directed mutagenesis was performed to introduce F231R, Y235R, and L240R mutations into the 1-5-10 motif of ENT1. These mutations as well as Y232R and Y234R mutations were introduced into another construct denoted GFP-Δ5-ENT1. The mutations were introduced by performing overlapping PCR (Ho et al. 1989). All mutations were verified using DNA sequencing.

GFP-tagged confocal microscopy

To discern any difference in localization between wild type and ΔCaM mutant hENT1, I used confocal microscopy. Wild type GFP-hENT1 and GFP-ΔCaM-hENT1 vectors were transfected into HEK293 cells for ~36 hours using the standard PolyJet protocol (SignaGen Laboratories). Following incubation, cells on coverslips were fixed with 4% (w/v) PFA for 15 minutes. Upon fixing, cells were permeabilized in 0.1% (v/v) Triton X-100, incubated with primary anti-FLAG (1:200) antibody solution in 1% (v/v) milk in TTBS for 1.5 hours and Alexa 594 fluorescent secondary antibody (1:500) for 45 minutes in 1% (v/v) milk in TTBS. Cells were then thoroughly washed, then stained with DAPI (Life technologies) using the provided protocol. Coverslips were mounted on glass slides using DAKO fluorescent mounting media.

Slides were viewed using a Zeiss LSM 700 Inverted Confocal microscope with a Plan Apochromat 63x oil immersion objective lens (N.A. = 1.40). Z-stacks between 8 and 12 at ~1 µm

intervals were collected. Zen Black for Windows (Zeiss) software was used for image acquisition and image processing.

Live cell calcium imaging

HTB-2 cells were imaged in DMEM + 10% FBS using 63x oil immersion objective (NA = 1.2) using a Zeiss AxioObserver spinning disc confocal microscope.

MTT assay

Cell viability is established by quantifying the amount of reduced tetrazolium salt, an event that occurs only in metabolically active cells following the treatment with MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide). MTT is a yellow product, and when reduced, a purple formazan product is formed.

HTB-2 cells were seeded onto 96-well plates, and incubated at 37°C and 5% (v/v) CO_2 for 24 hours. The media was then aspirated, and cells were treated with media in the presence or absence of 10 μM uridine for 2 hours in 37°C and 5% (v/v) CO_2. Following treatment, cells were thoroughly washed twice with pre-warmed PBS (37°C) and then media alone or media containing 100 nM gemcitabine. Cells were incubated at 37°C and 5% (v/v) CO_2 for 48 hours. Media was aspirated, and media containing MTT was added to cells as described in provided protocol (Sigma Aldrich). After 45 minute incubation, media was aspirated, DMSO was added to solubilize the purple salt products, and the absorbance was measured at 550 nm. Cell viability was determined by setting the control (media 2 hour treatment, media for 48 hours) to 100% and taking the ratio of absorbance for the remaining conditions. Bar graph presenting mean ± SEM was generated using GraphPad Prism version 5.04 for Windows.

Supplementary Results

Ca^{2+}/CaM regulates ENT1-dependent nucleoside flux

As I have shown, reducing calcium led to a decrease in sodium-independent adenosine flux. I predicted that increased intracellular calcium would also lead to an increase in transport. Caffeine is widely known to antagonize adenosine receptors at a concentration of about 50 μM, but at higher doses, caffeine is able to release intracellular calcium stores with an EC_{50} of 3 mM (Taniguchi 1969, Fredholm et al. 1999). To test my model that increased intracellular calcium would lead to greater chloroadenosine uptake, I treated HEK293 cells with 10 μM and 1 mM caffeine and measured chloroadenosine uptake to determine the role of caffeine on adenosine receptors alone, and the role of increasing intracellular calcium. There was a significant increase in transport with the 10 μM caffeine treatment but a significant decrease in transport with 1 mM caffeine compared to control (Figure 10; n=2). Since caffeine has many other effects on the cell and could inhibit nucleoside uptake through direct competition of the ENT1 substrate binding pocket, making the data more difficult to interpret, thapsigargin, a non-competative inhibitor of sarco/endoplasmic reticulum Ca^{2+} ATPases, was used to increase intracellular calcium levels for the transport assays.

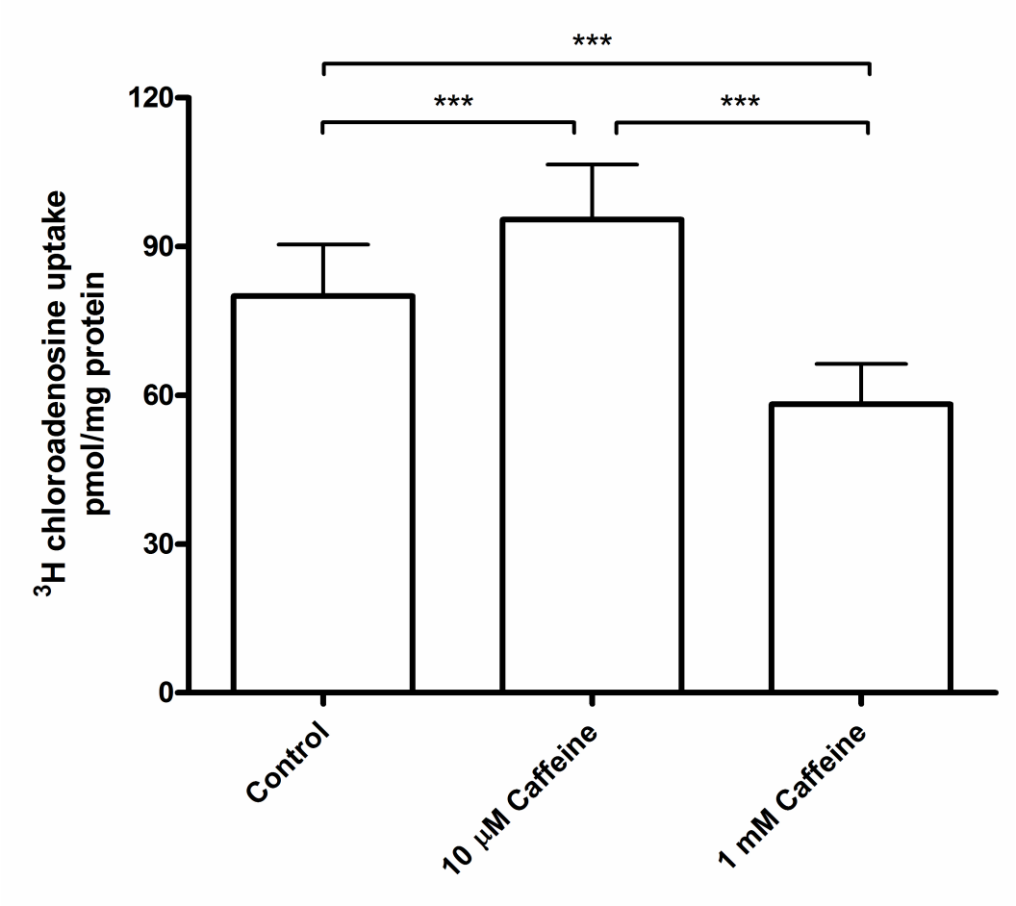

Figure 10: Effect of caffeine on chloroadenosine uptake was dependent on concentration. Caffeine antagonizes adenosine receptors at micromolar concentrations, and results in release of intracellular calcium at millimolar concentrations. There was a significant increase in transport with the 10 μM caffeine treatment but a significant decrease in transport with the 1 mM caffeine compared to control and is presented as mean ± SD (One-way ANOVA with Newman-Keuls multiple comparison post-test; n=2; ***P<0.001). Chloroadenosine transport assays were performed in sextuplicate using confluent cultured HEK293 cells.

As mentioned previously, thapsigargin led to a significant increase in nucleoside uptake in HEK293 cells (Figure 6A). Since gemcitabine is used for the treatment of bladder cancer, to provide a clinically relevant example of how increased calcium can lead to greater drug uptake, I acquired HTB-2 cells, an immortal human bladder cancer cell line, and performed radiolabelled gemcitabine transport assays. HTB-2 cells were treated with and without thapsigargin for 1 minute and there was not a significant difference (Figure 11A). When calcium imaging was conducted to confirm if thapsigargin treatment was effective at raising intracellular calcium concentrations on these cells, cells did not reach maximal fluorescent intensity until approximately 3 minutes (Figure 11B).

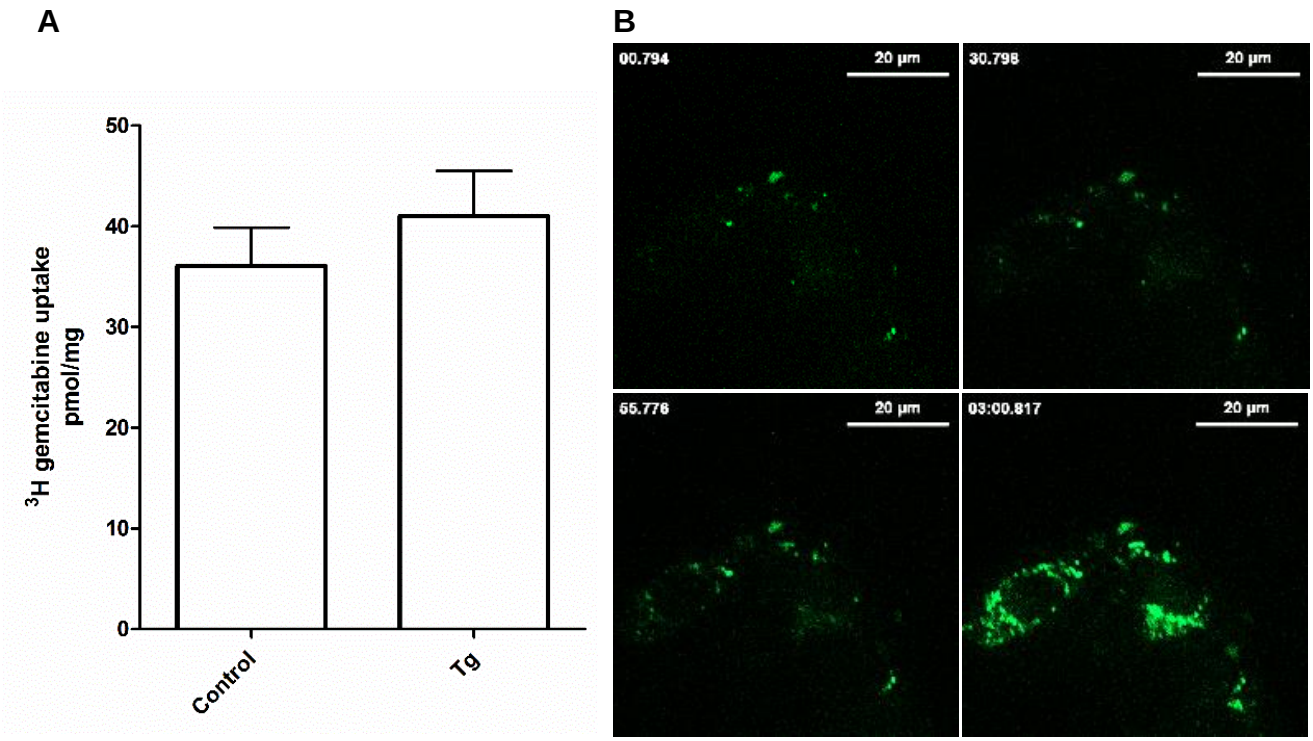

Figure 11: HTB-2 cells treated with thapsigargin did not have a significant difference in gemcitabine uptake after 1 minute due to minimal thapsigargin effect observed at that time point.

A. HTB-2 cells were predicted to have a significant increase in gemcitabine uptake after 1 minute treatment with thapsigargin, as seen with HEK293 cells. These results show there is no significant difference between treatment and control cells, and is presented as mean ± SEM (t-test; n=3). [^{3}H]-gemcitabine transport assays were performed in sextuplicate using confluent cultured HTB-2 cells and all treatments were for 1 minute.

B. Fluo-4 live cell calcium imaging with HTB-2 following thapsigargin treatment was used to determine the time at which a large increase in intracellular calcium levels were observed. The increase in calcium concentration observed within 1 minute of HEK293 cells with thapsigargin was not expected with HTB-2 since there was not a siginificant difference in uptake found between thapsigargin treatment and control. As seen in top left, top right, and bottom left panels (0 s, 30 s,

and 55 s respectively), there is relatively little difference in fluorescence intensity (indicative of low intracellular calcium levels). Only after 1 minute of treatment, as shown in the bottom right frame (3 minutes) is there a large increase in intracellular calcium. This lag explains the reduced gemcitabine increase. HTB-2 cells were imaged in DMEM + 10% FBS using 63x oil immersion objective (N.A. = 1.2) using a Zeiss AxioObserver spinning disc confocal microscope.

I have shown that calcium modulated nucleoside flux, and I had hypothesized based on reports in the literature and my preliminary data that CaM was regulating ENT1 function. To confirm this, I antagonized CaM with CALP2, a short cell-permeable inhibitory peptide which antagonizes the EF-hand of CaM (Villain et al. 2000), in HEK293 cells and measured nucleoside uptake. Cells incubated with 5 μM CALP2 for 1 minute had an approximate 20% reduction in nucleoside uptake compared to control (Figure 12). These results corroborated my previous results with W7 (Figure 7) as well as data from a colleague which demonstrated that CALP2 resulted in reduced gemcitabine efficacy (Stevanovic and Coe unpublished data).

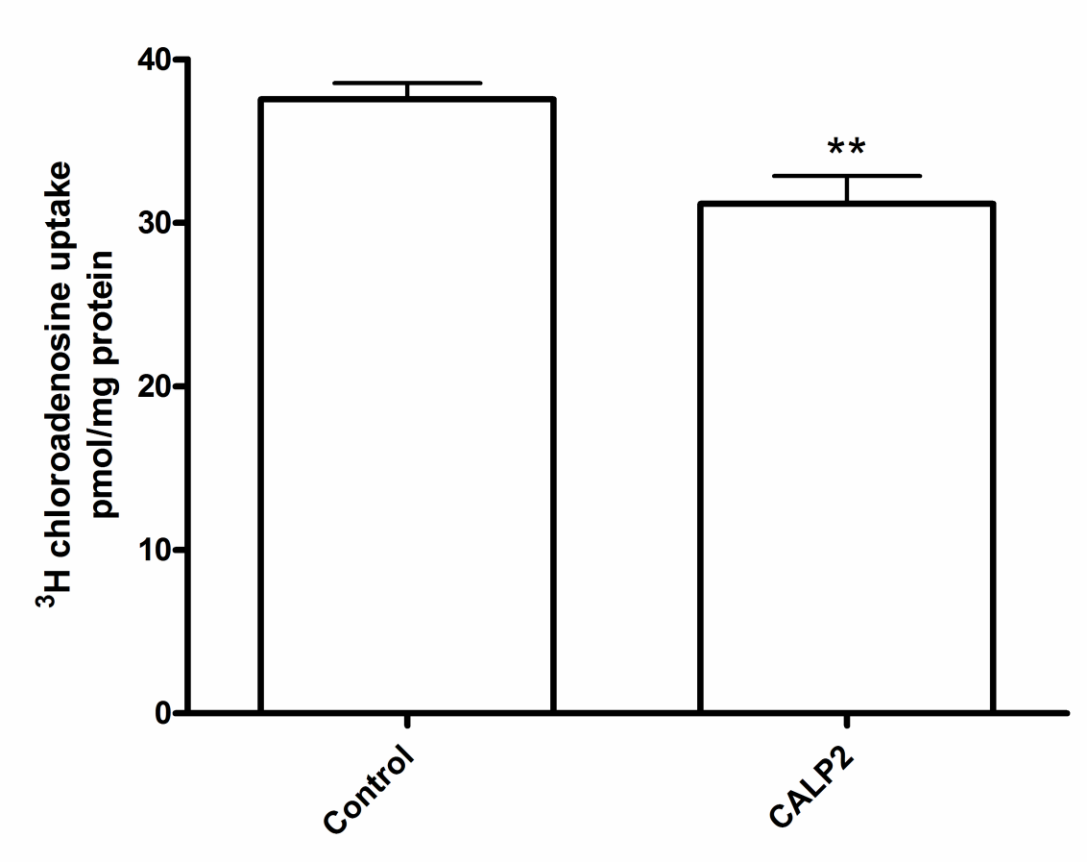

Figure 12: Antagonizing CaM with CALP2 reduced nucleoside uptake. CALP2 treatment (5 μM) resulted in a significant decrease in transport compared to control with pooled data from three independent experiments represented as mean ± SD (t-test; n=3; $^{**}P<0.01$). Chloroadenosine transport assays were performed in sextuplicate using confluent cultured HEK293 cells.

Abrogated CaM-ENT1 interaction leads to altered cellular distribution and reduced function

Functional assays have demonstrated that nucleoside flux is modulated via calcium, and can be reduced when antagonizing calmodulin with W7 or CALP2. Based on this and previous reports of calcium signals regulating movement of proteins to and from the membrane (Wu et al. 2009), I hypothesized that a change to residues in the 1-5-10 CaM binding motif on ENT1 would lead to a reduction in nucleoside uptake due to a reduction of ENT1 at the plasma membrane. Based on previous work in other SLC families, CaM has been shown to be important for trafficking these membrane transporters to and from the plasma membrane (Martinez-Maza et al. 2001). Based on this, I hypothesized that ΔCaM mutant ENT1 would be less abundant at the plasma membrane compared to wild type, and would have altered distribution when expressed in cells. To confirm that the ΔCaM mutant ENT1 was less abundant at the plasma membrane, I generated a ΔCaM mutant construct, overexpressed the mutant in HEK293 cells, and performed NBTI binding analysis. NBTI is an ENT1 specific, high affinity, non-transportable, nucleoside analog. Determining total NBTI binding sites on the cell surface indicates the presence of ENT1 proteins capable of binding nucleosides (Jarvis et al. 1982, Griffiths et al. 1997, Hyde et al. 2001). There was a significant increase in wild type hENT1 (B_{max} = 47.7±2.09 pmol/mg, n=3) over mutant ENT1 (B_{max} = 26.6±1.08 pmol/mg, n=3) and both were significantly higher than mock transfected cells (B_{max} = 1.39±0.10 pmol/mg, n=3) suggesting that there was significantly more wild type hENT1 protein at the plasma membrane than hENT1 mutant protein (Figure 13).

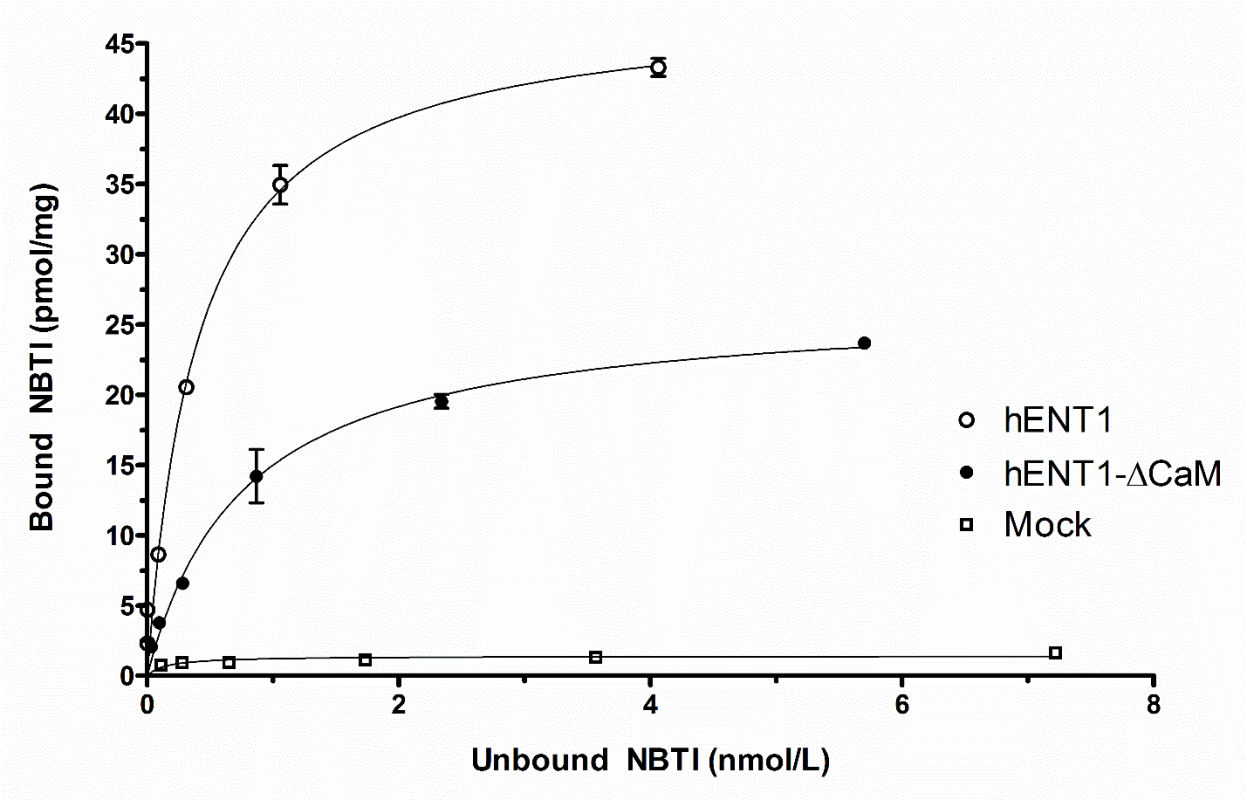

Figure 13: ENT1 mutations within the CaM binding site reduced the presence of over expressed GFP-hENT1 at the plasma membrane compared to over expressed wild type GFP-hENT1 protein. [^{3}H]-NBTI binding assays (n=3) performed with duplicate readings suggest that there is less over expressed ΔCaM mutant ENT1 at the plasma membrane compared to wild type in cultured HEK293 cells. Analyses of the NBTI binding curves yield a wild type GFP-ENT1 B_{max} of 47.7±2.09 pmol/mg of protein, a ΔCaM mutant ENT1 B_{max} of 25.6±1.07 pmol/mg of protein, and a mock transfected B_{max} of 1.39±0.10 pmol/mg of protein. Representative blot shown from three independent experiments with similar results.

To confirm that there is reduced ΔCaM mutant ENT1 at the plasma membrane, I transfected HEK293 cells with the mutant and wild type constructs (containing a GFP tag) and used confocal microscopy to visualize the protein. Mutant ENT1 protein appeared to be distributed primarily within the cytosol with limited presence at the plasma membrane (Figure 14). Wild type ENT1 protein is present almost exclusively at the plasma membrane (Figure 14). These data supports further evidence that the CaM binding domain is important for correct ENT1 localization, and that this protein-protein interaction may play a key role in recruitment to the plasma membrane.

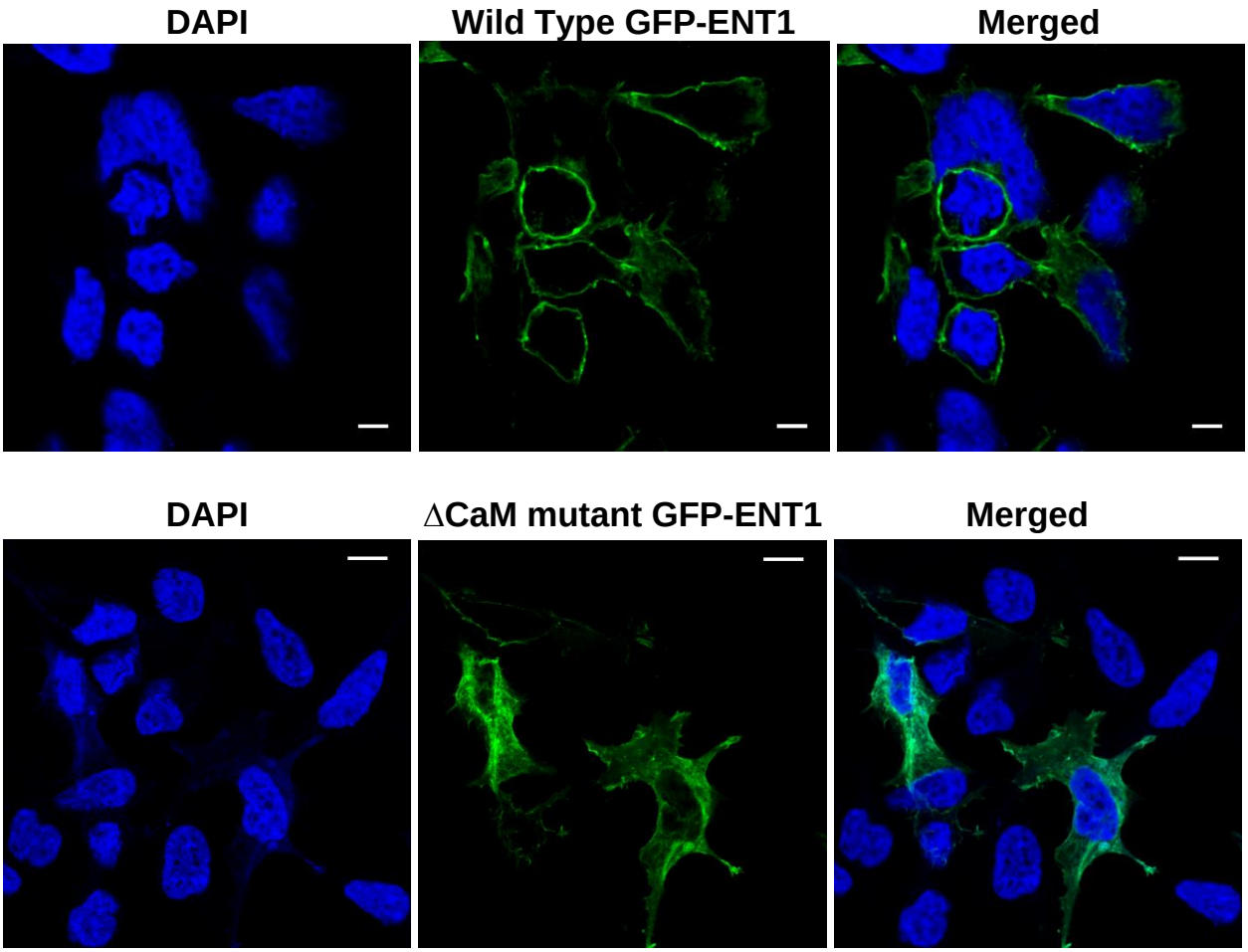

Figure 14: ΔCaM mutant GFP-hENT1 has an altered cellular distribution compared to wild type GFP-hENT1. GFP-ENT1 wild type (top) and ΔCaM mutant (bottom) proteins over expressed in live HEK293 cells are based on Z-stacks captured over time. Qualitative examination of these data suggests that mutant ENT1 is being held in the cell and does not adopt the uneven punctate distribution across the entire membrane as seen in wild type GFP-hENT1. Green fluorescence represents wild type GFP-ENT1, and blue fluorescence represents DNA. Green and blue fluorescence was achieved by excitation with 488nm and 405nm lasers respectively, with each signal acquired separately. Images represent a plane from a series of Z-stacks from one of three individual experiments. Scale bars represent 5μm.

As I have shown, modulating calcium levels as well as antagonizing CaM led to changes in nucleoside flux. To confirm that the change in nucleoside flux occurs due to the interaction between CaM and ENT1 at the 1-5-10 CaM binding site on ENT1, overexpressed the ΔCaM mutant in HEK293 cells, and performed chloroadenosine transport assays. I predicted that ΔCaM mutant protein would have reduced uptake compared to overexpressed wild type ENT1, but would be increased over endogenous levels of transport due to the increased ENT1 pool at the plasma membrane as shown by NBTI binding. There was a significant increase in both wild type and ΔCaM mutant ENT1 transport over endogenous, but wild type ENT1 transport was significantly greater than ΔCaM mutant (Figure 15). This demonstrates that ΔCaM mutant is still functional, and suggests that ΔCaM-ENT1 either has reduced activity, is less abundant at the plasma membrane, or has lower activity and reduced presence at the plasma membrane.

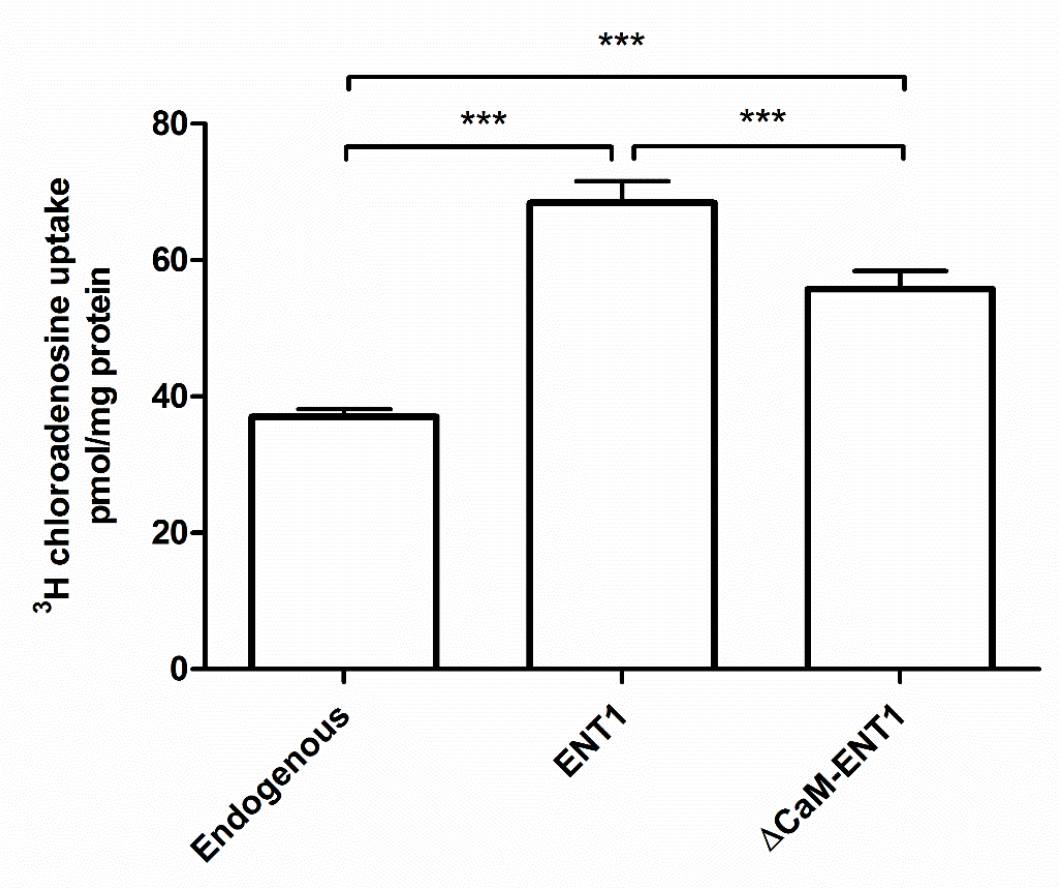

Figure 15: Overexpressing full length ENT1 containing mutations at the 1-5-10 CaM binding site in HEK293 cells leads to reduced nucleoside uptake compared to wild type ENT1. Endogenous transport, over expressed wild type ENT1 transport, and over expressed ΔCaM mutant ENT1 were significantly different in transport of chloroadenosine. Bars represent pooled data from three independent experiments represented as mean ± SEM (One-way ANOVA with Newman-Keuls post-test; n=3; $^{***}P<0.001$). Chloroadenosine transport assays were performed in sextuplicate using confluent cultured HEK293 cells

Substrate uptake leads to reduction of gemcitabine efficacy

As previously discussed, the efficacy of a variety of nucleoside analog drug therapies is dependent on ENT1, such as the anti-cancer drug gemcitabine (Achiwa et al. 2004). Previous work in our lab suggested that repeated translocation of substrate across the plasma membrane may lead to endocytosis of the protein (Zafar and Coe unpublished). Since I have shown that calcium regulates ENT1-dependent uptake of gemcitabine, and the CaM binding site is critical for localization of the protein, I hypothesized that treating cells with another substrate would lead to the internalization of ENT1, thus reducing the uptake of gemcitabine and reducing drug effectiveness. To test this, I treated HTB-2 cells with 10 μM uridine for 1 hour, washed the cells twice, then added media containing 100 nM gemcitabine for 48 hours and performed an MTT assay. I hypothesized that treatment with uridine, a transportable substrate by ENT1 which should have minimal receptor activity (unlike adenosine), would result in internalization of ENT1 and lead to increased viability. Cells treated with gemcitabine (1 hour media pre-treatment, 48 hour gemcitabine treatment) had significantly lower viability compared to control cells (1 hour media pre-treatment, 48 hour media treatment), while uridine and gemcitabine treatment (1 hour uridine pre-treatment, 48 hour gemcitabine treatment) was significantly different than both uridine gemcitabine treated and control cells (n=3; Figure 16). Uridine pre-treatment (1 hour uridine pre-treatment, 48 hour media treatment) was not significantly different than control (n=3). These data suggest that uridine treatment protects HTB-2 cells from 48 hour gemcitabine treatment.

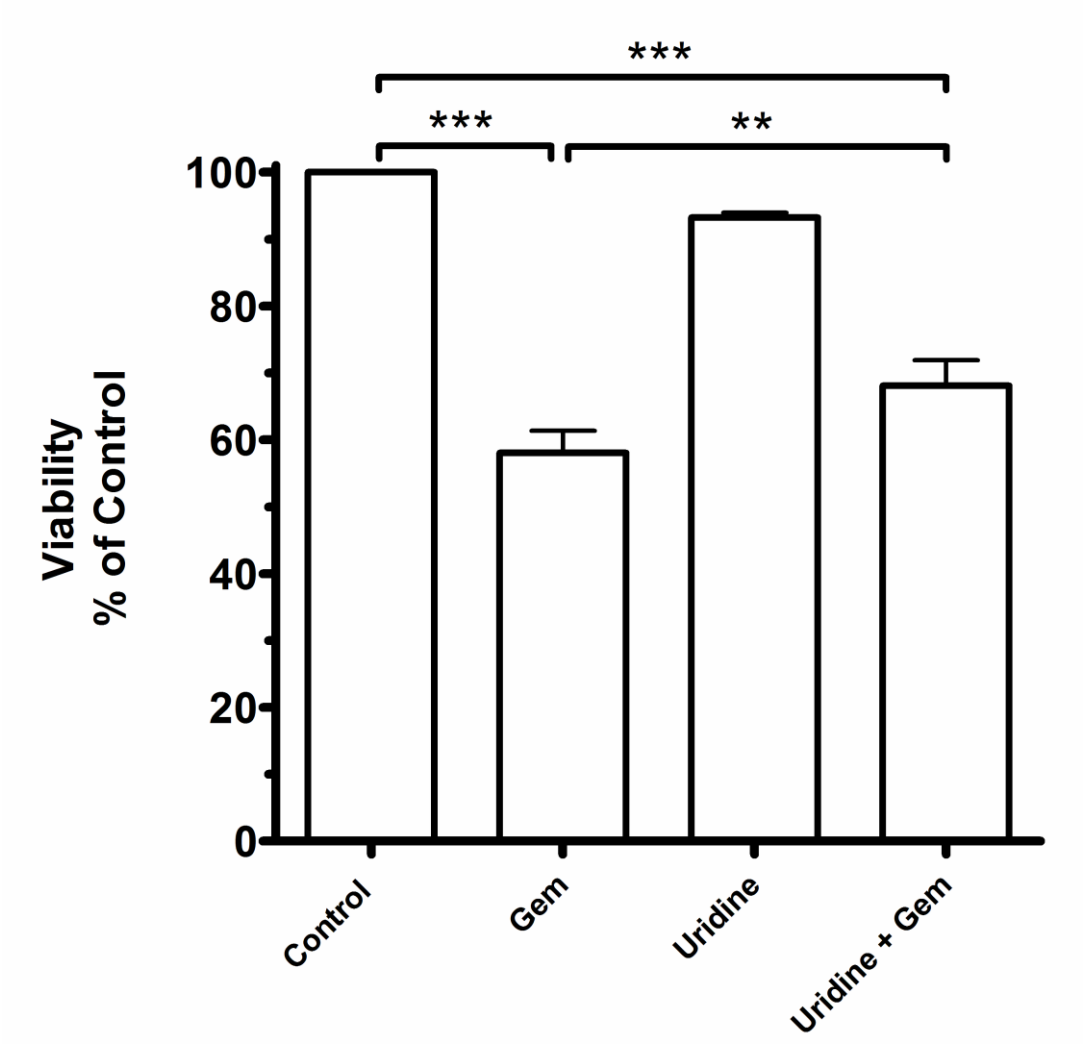

Figure 16: Uridine pre-treatment reduced gemcitabine cytotoxicity in HEK293 cells. Cells treated with uridine (10 μM, 2 hours) prior to gemcitabine treatment (100 nM, 48 hours) had reduced viability. Viability is quantified from absorbance of MTT at 550 nm. Figure presents pooled data from three independent experiments where each condition was measured in octaplicate as mean ± SEM (One-way ANOVA with Newman-Keuls multiple comparison post hoc test, $^{**}P<0.01$, $^{***}P<0.001$, n=3).

Chapter 3: *N*-linked glycosylation of N48 is required for Equilibrative Nucleoside Transporter 1 (ENT1) function

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This chapter is adapted from a manuscript which has been submitted to Bioscience Reports, and at the time of the submission of the dissertation, was under revision.

Author Contribution

Alex Bicket performed cloning, cell culture, Western blotting, microscopy, transport assays, NBTI binding analysis, co-immunoprecipitation, and assisted with the writing of the manuscript. Imogen R. Coe, the corresponding author, led and designed the project, and wrote the final version of the manuscript.

Abstract

Human equilibrative nucleoside transporter 1 (hENT1) transports nucleosides and nucleoside analog drugs across cellular membranes and is necessary for the uptake of many anti-cancer, anti-parasitic, and anti-viral drugs. Previous work, and *in silico* prediction, suggests that hENT1 is glycosylated at Asn48 in the first extracellular loop of the protein and that glycosylation plays a role in correct localization and function of hENT1. Site-directed mutagenesis of wild-type (wt) hENT1 removed potential glycosylation sites. Constructs (wt 3xFLAG-hENT1, N48Q-3xFLAG-hENT1 or N288Q-3xFLAG-hENT2) were transiently transfected into HEK293 cells and cell lysates were treated with or without PNGase-F, followed by immunoblotting analysis. Substitution of N48 prevents hENT1 glycosylation, confirming a single *N*-linked glycosylation site. N48Q-hENT1 protein is found at the plasma membrane in HEK293 cells but at lower levels compared to wt hENT1 based on NBTI binding analysis (wt 3xFLAG-ENT1 B_{max}, 41.5±2.9pmol/mg protein; N48Q-3xFLAG-ENT1 B_{max}, 13.5±0.45pmol/mg protein) and immunofluorescence microscopy. Although present at the membrane, chloroadenosine transport assays suggest that N48Q-hENT1 is non-functional (wt 3xFLAG-ENT1, 171±44.0pmol/mg protein; N48Q-3xFLAG-ENT1, 57.9±17.1pmol/mg protein; mock-transfected 74.3±19.6pmol/mg protein). Co-immunoprecipitation analyses suggest that N48Q ENT1 is unable to interact with self or with wt hENT1. Based on these data we propose that glycosylation at N48 is critical for the localization, function, and oligomerization of hENT1.

Summary

Our study confirmed that Asn48 of hENT1 is the only *N*-glycosylated residue when expressed in HEK293 cells, and loss of the *N*-glycan resulted in less hENT1 at the plasma membrane, as well as a loss of function and protein-protein self-interaction.

Short Title: hENT1 requires glycosylation of N48 for proper function

Keywords: Equilibrative Nucleoside Transporter 1; *N*-linked glycosylation; function; trafficking; immunofluorescence; oligomerization

Abbreviations:

Asn, asparagine; DAPI, 4',6-diamidino-2-phenylindole; DDT, dichlorodiphenyltrichloroethane; DMEM, Dulbecco's Modified Eagle Media; FBS, fetal bovine serum; HEK, human embryonic kidney; hENT1, Human Equilibrative Nucleoside transporter; HRP, horseradish peroxidase; NBTI, S-(4-Nitrobenzyl)-6-thioinosine; NP-40, Nonidet P-40 (octyl phenoxypolyethoxylethanol); PBS, phosphate buffered saline; PFA, paraformaldehyde; PNGase-F, peptide–*N*-glycosidase F; SDS, sodium dodecyl sulfate; SLC, solute carrier; TMD, transmembrane domain; TTBS, tris-buffered saline and tween 20; WT, wild type.

Introduction

Membrane transporter proteins enable movement of molecules across biological membranes. Nucleosides are hydrophilic molecules involved in cell signalling, DNA synthesis, and energy metabolism and require trans-membrane transport. The equilibrative nucleoside transporters (ENTs) comprise the solute carrier (SLC), SLC29 family (Hediger et al. 2004). ENTs passively facilitate movement of nucleosides down their concentration gradients (Cabrita et al. 2002) while CNTs (SLC28) are cation/nucleoside co-transporters which do not possess any sequence or known structural homology to ENTs (Young et al. 2013).

ENTs are critical for the uptake of many classes of nucleoside derivative drugs. ENT1 and ENT2 are clinically important drug transporters that are critical for drug delivery, and therefore efficacy, of many anti-cancer, anti-parasitic, and anti-viral agents (Hillgren et al. 2013). Moreover, ENTs modulate adenosine flux and thereby regulating purinergic responses (Dos Santos-Rodrigues et al. 2014, Rose et al. 2010).

Like most SLC proteins (Landolt-Marticorena and Reithmeier 1994), human ENT1, hENT1, is reported as being glycosylated at the large extracellular loop (Kwong et al. 1986). Glycosylation mutants of hENT2 expressed in mammalian cells show reduced transport and protein levels at the membrane (Ward et al. 2003). In contrast, glycosylation mutants of hENT1, expressed in *Saccharomyces cerevisiae*, show increased expression at the plasma membrane and functional transport (Vickers et al. 1999). Therefore, the role of glycosylation of hENT1 in human cells is unclear.

N-linked glycosylation of membrane transporters is important for function (Levy et al. 1998, Choi et al. 2003), trafficking (Martinez-Maza et al. 2001, Yao and Hersh 2007), stability (Asano et al. 1993, Melikian et al. 1996), and sorting (Vagin et al. 2009). Therefore I hypothesized

that non-glycosylated hENT1 would exhibit reduced recruitment to the plasma membrane resulting in lower hENT1-dependent transport. Reduced hENT1-dependent uptake of nucleoside analog drugs used in disease treatment has significant clinical implications since drug efficacy is correlated with hENT1 presence (Zhang et al. 2007). Since cancerous cells can exhibit global changes in cellular glycosylation (Christiansen et al. 2014), understanding the role of ENT1 glycosylation is clinically relevant. In this study, I show that *N*-linked glycosylation site of hENT1 is necessary for function.

Materials and Methods

In silico detection of putative glycosylation sites

I used NetNGlyc 1.0 to determine putative *N*-linked glycosylation sites in full length human ENT1 sequence (accession number NP_001071645).

Cell culture and transfection

HEK293 (human embryonic kidney cell line), commonly used in membrane protein glycosylation studies (Li et al. 2014, Filippo et al. 2011, Fan et al. 1997), were grown in DMEM supplemented with 10% (v/v) FBS in 10 cm^2 plates (NBTI binding and Western Blotting) or 6-well plates (transport assays) at 37°C with 5% (v/v) CO_2. Cells were transfected using the standard Polyjet protocol (SignaGen Laboratories) and incubated post transfection for ~36 hours. Equivalent transfection efficiency in wild type and mutant-transfected cells was confirmed by microscopy.

Generating N48Q-hENT1 and N288Q-hENT1 mutant constructs

Full-length hENT1 conjugated with a 3xFLAG tag in a pCDNA 3.1 vector was used as the template and point mutations were introduced using overlap extension PCR (Higuchi et al. 1988). To create the N48Q mutation, the AAT codon was substituted for a CAA, while the N288Q mutation used an AAT codon substituted for a CAG.

Immuno-blotting analysis

To determine which residues were *N*-glycosylated, I overexpressed wild type, N48Q, or N288Q mutant hENT1 protein in HEK293 cells, and treated lysates with and without PNGase-F, followed by immunoblotting analyses as previously described (Reyes et al. 2010).

NBTI binding assay

NBTI is a high affinity, tight-binding, non-transportable, ENT1-specific nucleoside analog used which can be used to analyze the presence of ENT1 (Jarvis et al. 1982, Griffiths et al. 1997, Hyde et al. 2001) as previously described (Chaudary et al. 2002). [^{3}H]-NBTI binding parameters (K_d and B_{max}) were determined from non-linear regression analysis using GraphPad Prism (v. 5.04).

[^{3}H]-2-chloroadenosine transport assay

To determine the functionality of N48Q-hENT1, I conducted [^{3}H]-2-chloroadenosine transport assays using HEK293 cells as previously described (Coe et al. 2002).

Immunofluorescence and point scanning confocal microscopy

I used immunofluorescence microscopy to investigate localization of wt 3xFLAG-hENT1 and N48Q-hENT1. Wt HA-hENT1 and N48Q-3xFLAG-hENT1 vectors were co-transfected into HEK293 cells as described above. To confirm differential localization of glycosylated ENT1 and non-glycosylated ENT1, both constructs were transfected into the cells with wt HA-ENT1 serving as both the positive control and the plasma membrane marker. Cells grown on coverslips were prepared as previously described (Nivillac et al. 2011) followed by incubation with anti-FLAG primary and Alexa488 or Alexa594 fluorescent secondary antibody (1:500, in 1% (v/v) milk in TTBS, 45 min). Slides were viewed using a Zeiss LSM 700 Inverted Confocal microscope with a Plan Apochromat 63x oil immersion objective lens (N.A.=1.40). Z-stacks (8 to 12 at ~1µm intervals) were collected. Zen Black (Zeiss) software was used for image acquisition and image processing.

Co-immunoprecipitation of wild type and glycosylation mutant FLAG-ENT1 using HA-ENT1 bait

HEK293 cells co-transfected (as described above) with HA-ENT1 and 3xFLAG-vector (either wild type ENT1, N48Q-ENT1, or hLa as a negative control) were lysed with NP-40 buffer ~36 hours post-transfection. Lysate was homogenized with 1mL syringe and 26g needle then centrifuged at max speed (15 min) on a bench top centrifuge to pellet cellular debris and organelles. Protein concentration was determined by modified Lowry protein assay (BioRad). To best equilibrate the strength of transfected protein bands between the constructs when immunoblotting, columns were loaded with transfected cell lysate as follows: wt 3xFLAG-ENT1 (100µg), N48Q-3xFLAG-ENT1 (1000µg), and 3xFLAG-hLa (600µg, cytosolic non-specific control), each with

20µl of anti-HA beads (Thermo Scientific). Protein was agitated overnight (approximately 18 hrs) at 4°C and washed 3 times with TTBS. Immuno-precipitated protein was recovered by boiling with 2x elution buffer (Thermo Scientific) and supplemented with 1 M DDT (2µl). Protein from elution and flow-through was resolved by SDS-PAGE and subjected to immuno-blotting as described above. The entire elution sample was added to the column for each condition, while wt ENT1 (1µg), N48Q-ENT1 (10µg), and hLa (1µg) was added for flow-throughs.

Statistical Analysis

With three conditions (mock, wild type, and N48Q mutant), to confirm there were one or more significant differences, we used one-way analysis of variance (ANOVA). Our hypothesis was one directional (i.e. we were interested in detecting the decrease in nucleoside uptake when treating with a drug, not interested in detecting the change, both an increase or decrease, after treating with a drug) thus making one-way ANOVA appropriate. For precision when using ANOVA, normality, homogeneity between the variances, and independence between each condition are required. Normality and homogeneity were confirmed in GraphPad prior to analysis, and independence was ensured through experimental design. The Newman-Keuls post-hoc test was performed, since it is robust to violations of normality and relatively small sample sizes could lead to data approaching a skew (not enough to violate the assumption of normality but could impair the degree of significance). All raw data from each independent experiment (n=3) coincides with the same trend (i.e. for each independent experiment, the wild type had higher activity, albeit, to varying extents for each experiment). All statistical analyses were performed using GraphPad Prism 5.04 for Windows.

Results

hENT1 possesses a single glycosylation site at Asparagine-48

In silico analyses suggested that N48 and N288 had the highest probability of glycosylation. N48 is predicted to be in the large extracellular loop and N288 is near a transmembrane domain (TMD), which may be exposed to the extracellular space depending on protein conformation (Fig. 1A). Both sites are physiologically plausible as targets, although N288 was less likely being close to a TMD (van Geest and Lolkema 2000). Previous work suggested ENT1 was glycosylated at N48 when expressed in *S. cerevisiae* (Vickers et al. 1999). My results suggest that wt hENT1, expressed in HEK293 cells, is a protein of 50 to 65 kDa and following PNGase-F treatment, the size of the protein is reduced to 50 to 55 kDa (Fig. 1B). In contrast, N48Q hENT1 mutant protein is 50 to 55 kDa in the presence and absence of PNGase-F confirming hENT1 is exclusively *N*-glycosylated at N48 in human cells with no evidence of glycosylation at N288.

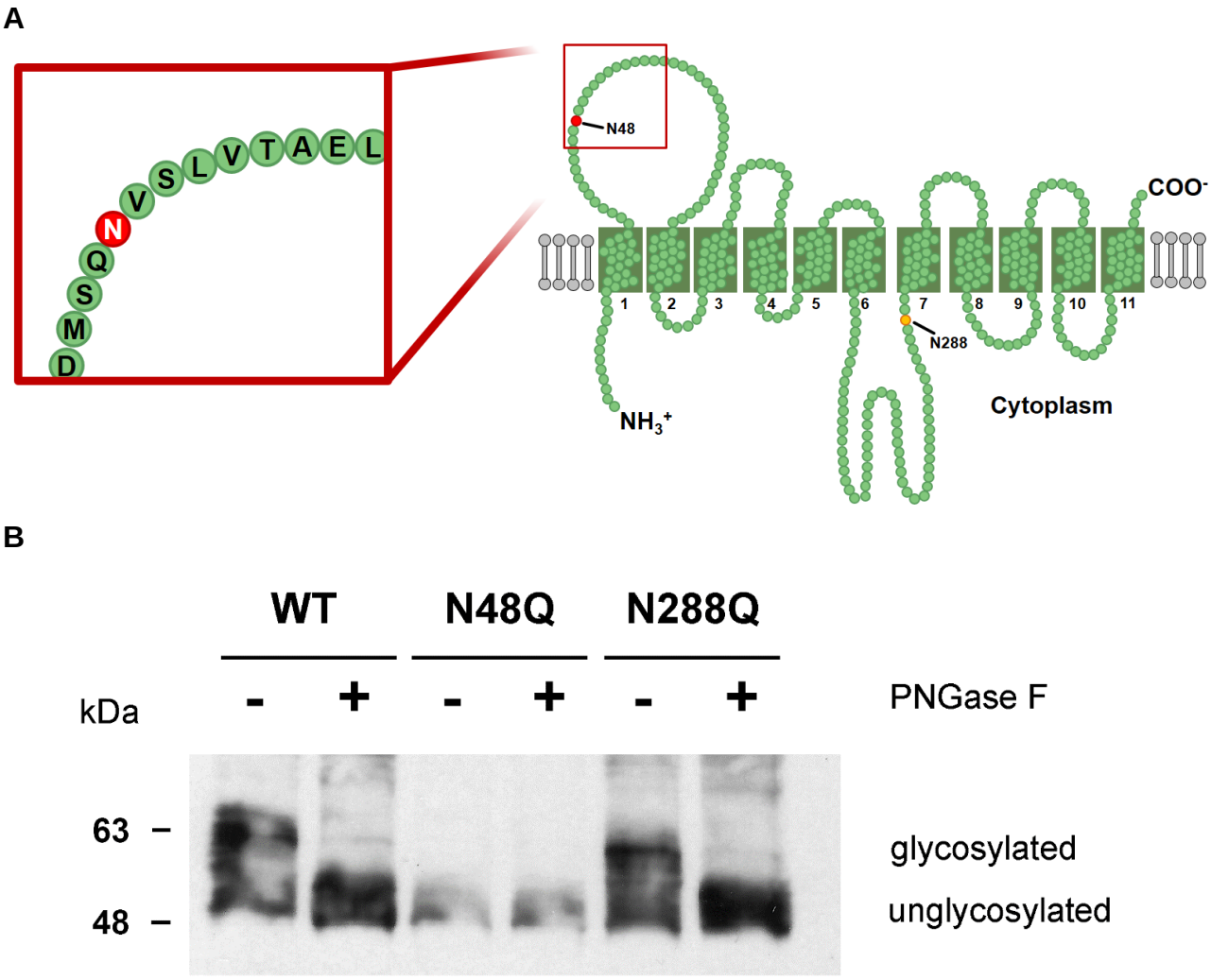

Figure 1: Predicted hENT1 topology and immunoblot identifying N48 as only *N*-linked glycan in human cells.

A. Putative two-dimensional membrane topology with *N*-glycosylation sites (NetNGlyc). Asn48, at large extracellular loop (red), has the highest probability. We did not rule out Asn288 (orange) near transmembrane domain 7 since the three-dimensional structure for ENT1 is not confirmed.

B. Immunoblot with wild type (3xFLAG-hENT1) and mutant (N48Q-3xFLAG-hENT1 and N288Q-3xFLAG-hENT1) cell lysates from transfected HEK293 cells, with and without PNGase-F treatment. Whole cell lysates were fractioned with 12% (v/v) SDS-PAGE and immunoblotted with anti-FLAG antibody.

N-linked glycosylation of N48 is required for hENT1 movement to the plasma membrane

Transporters often require glycosylation for effective recruitment to the plasma membrane and thus function (Martinez-Maza et al. 2001, Asano et al. 1993, Melikian et al. 1996, Olivares et al. 1995, Hoover et al. 2003, Chen et al. 2006, Paredes et al. 2006, Unal et al. 2008, Subramanian et al. 2008, Dorn et al. 2009, Hayashi and Yamashita 2012, Console et al. 2015). I therefore predicted that lack of glycosylation would interfere with trafficking of hENT1 at the plasma membrane and tested this using NBTI binding site saturation assays. These assays determine the number of total NBTI binding sites (where one NBTI binding site is equivalent to one hENT1 protein) present in a cell population. HEK293 cells transiently transfected with wild type 3xFLAG-hENT1 showed a higher maximal NBTI binding (B_{max}=41.5±2.9, n=3) compared to mock transfected cells (B_{max}=0.44±0.03, n=3) (Fig. 2A). N48Q mutant hENT1 transfected cells showed an NBTI binding (B_{max}=13.5±0.45, n=3) which is greater than that seen in the mock transfection, but much lower (~70%) than wild type 3xFLAG-ENT1 suggesting that non-glycosylated hENT1 is greatly reduced in presence at the plasma membrane.

To confirm that glycosylation abberant ENT1 is less abundant at the plasma membrane, I assessed the localization of wt hENT1 and N48Q hENT1 in HEK293 cells using confocal microscopy. To determine the effect of glycosylation on ENT1 distribution, I co-transfected HEK293 cells with FLAG-N48Q-ENT1 (glycosylation deficient mutant ENT1 protein) and HA-ENT1 (glycosylated ENT1 which localizes to plasma membrane). N48Q hENT1 has a primarily cytosolic distribution with some punctate regions at the plasma membrane (Fig. 2B). In contrast, wt hENT1 protein is clearly present almost exclusively at the plasma membrane (Fig. 2B).

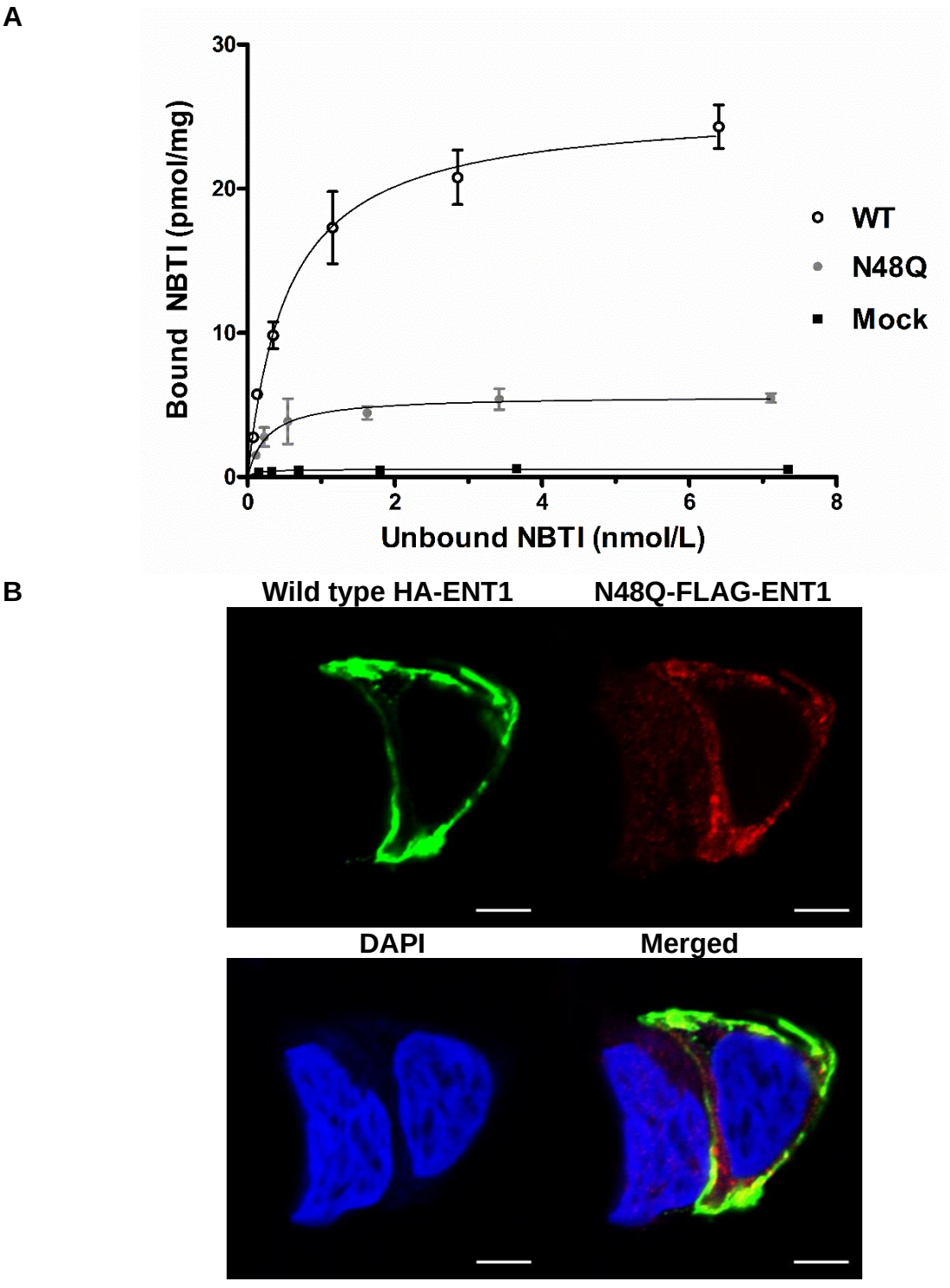

Figure 2: Loss of *N*-glycosylation reduces hENT1 presence at the plasma membrane.

A. HEK293 cells transfected with N48Q-3xFLAG-hENT1 exhibited a 3-fold decrease in hENT1 NBTI binding sites compared to transfected 3xFLAG-hENT1. Both N48Q hENT1 and wild type

hENT1 transfected cells showed an increase in NBTI binding sites compared to mock transfected. Error bars represent the mean±SD. Representative graph from three experiments, with each point conducted in duplicate.

B. Confocal microscopy of fixed HEK293 cells co-transfected with HA-hENT1 and *N*-linked glycosylation mutant N48Q-3xFLAG-hENT1. Cells were fixed and probed with anti-FLAG primary antibody then Alexa594 secondary and anti-HA primary antibody then Alexa488 secondary antibody, nuclei were stained with DAPI. Red fluorescence represents N48Q mutant hENT1, green fluorescence represents wild type hENT1, and blue fluorescence represents DNA. Red, green, and blue fluorescence were achieved by excitation with 555nm, 488nm, and 405nm respectively, with each signal acquired separately. Altered distribution is observed between HA-hENT1 (green) and 3xFLAG-N48Q-hENT1 (red). Images represent a plane from a series of Z-stacks from one of three individual experiments. Scale bars represent 5µm.

N48Q mutant ENT1 is non-functional in HEK293 cells

N48Q hENT1, while less abundant, is still present at the membrane and therefore I predicted that I would see reduced, but not absent, transport. However, data show an almost complete lack of functional transport compared to wt hENT1. Since N48Q-ENT1 does not have an increase in uptake compared to mock transfected cells, this suggests that N48Q hENT1, even if present at the plasma membrane, is non-functional (Fig. 3).

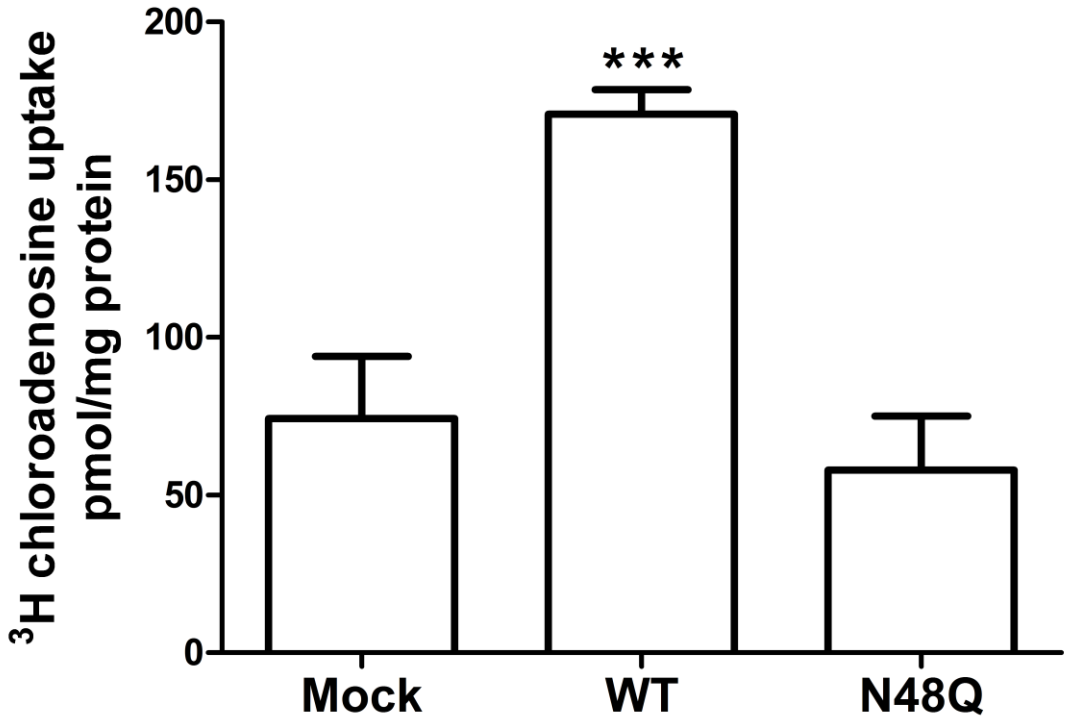

Figure 3: Chloroadenosine uptake by endogenous, wild type ENT1, and N48Q-ENT1 overexpressed protein suggests that glycosylation is required for sodium-independent nucleoside uptake in HEK293 cells. HEK293 cells were either mock transfected, transiently transfected with wild type 3xFLAG-hENT1, or with glycosylation mutant 3xFLAG-N48Q-hENT1. [^{3}H]-chloroadenosine uptake was the same between mock transfected and N48Q-hENT1 transfected cells, but both were significantly less than wild type hENT1 transfected cells. Graph represents pooled data from three individual experiments (n=3), with each condition conducted in sextuplicate. Error bars represent the mean±SD (One-way ANOVA with Newman-Keuls multiple comparison post hoc test, $^{***}P<0.0001$).

ENT1-ENT1 co-immunoprecipitation is disrupted with mutation of glycosylation site

These data suggest that glycosylation of hENT1 may have a functional role in addition to assisting trafficking of the protein to the membrane. Oligomerization plays an important role for the proper function of other SLC members (Ozaslan et al. 2003, Brast et al. 2012, Johnson et al. 2012). Our data show that ENT isoforms co-immunoprecipitate (ENT1-ENT1, ENT1-ENT2, and ENT2-ENT1) suggesting that ENTs form complexes with each other (Grañé Boladeras et al. unpublished). ENT-ENT interactions or oligomerization may have important functional roles, which are yet to be identified (Grañé Boladeras et al. unpublished). However, since we suspect that ENTs form dimers and we know that glycosylation of other transporters has been correlated with the formation of oligomers (Li et al. 2014, Filippo et al. 2011), I investigated the role of glycosylation of ENT1 in the formation of ENT dimers. As predicted, I observed that wt ENT1 co-immunoprecipitated with itself (HA-hENT1 with 3xFLAG-ENT1) but did not co-immunoprecipitate with 3xFLAG-N48Q-ENT1, or the cytosolic negative control protein 3xFLAG-hLa (Fig. 4). Although FLAG-N48Q-ENT1 expression is lower than wild type FLAG-ENT1, these data still indicate that N48Q mutant ENT1 is unable to form a complex with wild type ENT1.

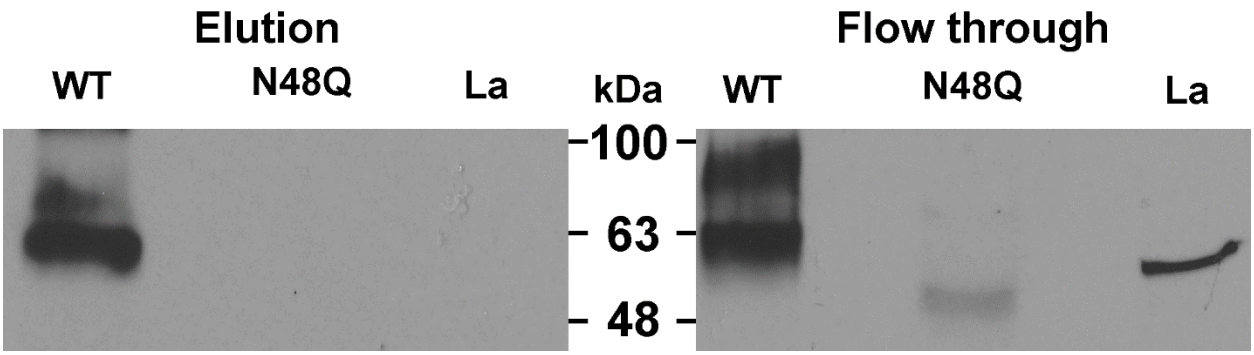

Figure 4: Co-immunoprecipitation analyses suggest glycosylation is required for hENT1-hENT1 interaction. HEK293 cells were transiently transfected with HA-ENT1 as well as with the indicated construct (WT, wild type 3xFLAG-hENT1; N48Q, 3xFLAG-N48Q-hENT1; La, FLAG-hLa), lysed, and co-immunoprecipitated using anti-HA beads (Thermo Scientific) as described in the Materials and Methods. Co-immunoprecipitation only occurred with wild type 3xFLAG-ENT1 (100µg lysate loaded to column), and not with *N*-glycosylation mutant 3xFLAG-N48Q-ENT1 (1000µg lysate loaded to column) and the cytosolic negative control protein 3xFLAG-hLa (600µg lysate loaded to column). Elution (bound protein) and flow through (unbound protein) were fractioned with 12% (v/v) SDS-PAGE and immunoblotted with anti-FLAG antibody.

Discussion

The post-translational enzymatic addition of glycans at aspargine residues (*N*-linked glycosylation) to proteins is responsible for modulating a wide range of functions and has a pivotal impact on cell growth, protein folding, oligomerization, and signal transduction (Imperiali and O'Connor 1999, Helenius and Aebi 2001, Vagin et al. 2009). Moreover, increasing incidences of aberrant glycosylation are being detected in neoplasmic tissues, suggesting a link between glycosylation state and tumourogenic phenotype (Meany and Chan 2011, Hakomori 1996, Hakomori 2002, Mechref et al. 2012, Tuccillo 2014). For these reasons, the study of glycosylation on drug transporters like ENT1 is clinically relevant.

Here, we provide evidence in support of the role of *N*-linked glycosylation in the function and localization of hENT1. We have confirmed previous reports (Kwong et al. 1986, Ward et al. 2003, Vickers et al. 1999) that the *N*-linked glycosylation site, N48, is unique for hENT1 expressed in a human cell line. The removal of this site significantly impacts functionality of hENT1 which contrasts with previous data that suggested N48Q-ENT1 expressed in *S. cerevisiae* is functional (Vickers et al. 1999). This difference may be due to promiscuous glycosylation, which is known to occur in the yeast model (Gemmill and Trimble 1999, Breidenbach et al. 2012, Cohen-Rosenzweig et al. 2014) resulting in *N*-glycosylation at non-canonical sequences (Chi et al. 2010) which could play a compensatory role and restore function of N48Q-hENT1. Our study also suggests that glycosylation contributes to, but is not solely responsible for, correct ENT1 localization, since non-glycosylated ENT1 is present at the plasma membrane and that glycosylation is necessary for hENT1 function. This corroborates previous work which suggested that hENT1 mutant protein lacking the extracellular loop expressed in *X. laevis* had reduced hENT1 protein abundance at the plasma membrane (Aseervatham et al. 2015). Several members

of the SLC family experience only a small or no functional effect when the *N*-linked glycosylation site is abrogated (Levy et al. 1998, Choi et al. 2003, Wong et al. 1998, Balamurugan and Said 2002). Typically, *N*-glycosylation leads to reduced transport activity as a consequence of reduced presence at the plasma membrane (Martinez-Maza et al. 2001, Asano et al. 1993, Melikian et al. 1996, Olivares et al. 1995, Hoover et al. 2003, Chen et al. 2006, Paredes et al. 2006, Unal et al. 2008, Subramanian et al. 2008, Dorn et al. 2009, Hayashi and Yamashita 2012, Console et al. 2015). Glycosylation is also often critical for protein sorting, where membrane proteins will be differentially expressed on apical and basolateral membranes. I used HEK293 as the cellular model for this study, a non-polarized cell line commonly used for the studying the glycosylation of other SLC members (Li et al. 2014, Filippo et al. 2011, Fan et al. 1997). Based on other reports in the literature, I would hypothesize that targeting of ENT1 would be disrupted in cell lines like in the canine renal epithelial cell line MDCK (where ENT1 is most abundant on the basolateral membrane) if there is disruption of glycosylation since it is often critical in protein sorting in polarized cells (Lai et al. 2002, Mangravite et al. 2003).

However, *N*-glycosylation may affect function in ways that are not related to trafficking or sorting. For instance, *N*-glycan deficient human erythrocyte anion transporter SLC4A1 (AE1) expressed in oocytes had reduced chloride transport yet had similar levels of surface protein abundance which authors attributed to non-ideal folding that effected function but not trafficking (Groves and Tanner 1994). Aberrant glycosylation can also reduce protein half-life, which is a common characteristic of other over expressed *N*-glycosylation mutants from SLC family members (Melikian et al. 1996, Chen et al. 2006, Subramanian et al. 2008, Muthusamy et al. 2015). Increased degradation of protein can result from changes in protein folding, as seen with OAT4 (SLC22A11) following *N*-glycan removal (Zhou et al. 2005).

Our data suggest that glycosylation of hENT1 contributes to correct localization of the protein as well as functionality of the protein at the membrane and we propose that this may be correlated with glycosylation-dependent protein-interactions between hENT1 proteins at the membrane as proposed for other SLC members. Glycosylation of hOCT2 (SLC22A2) in HEK293 cells is required for the formation of hOCT2 dimers (Brast et al. 2012). Similarly, serotonin transporter (SLC6A4) monomer, when expressed in CHO hamster ovary cells, require *N*-glycan addition to associate into functional homo-oligomers (Ozaslan et al. 2003). Our work (Grañé Boladeras et al. unpublished) has revealed that ENT isoforms interact with each other (ENT1-ENT1, ENT1-ENT2, ENT2-ENT2, etc.) although the functional significance of this observation remains unclear. In this study, I have shown that the loss of glycosylation abrogates interaction of ENT1 monomers and I therefore predict that oligomerization may be a fundamental form of regulation for the ENTs. This mechanism could explain previous data (Boleti et al. 1997) where a large increase in hENT1 protein at the membrane yields a relatively small increase in the translocation of substrate, as well explaining the presence of distinct ENT1 populations (possibly differentially glycosylated variants) at the plasma membrane (Boumah et al. 1992).

Interestingly, ENT1 only has one *N*-linked glycan, where most characterized SLC members have two or more (Pedersen et al. 2016). *N*-glycosylation is multi-purposed, and plays a role in protein stability, function, trafficking, and cell signalling, so the loss of the glycan on a single glycosylated protein is often detrimental. Some examples of SLC members which are only *N*-glycosylated once, at the large extracellular loop, are the glucose transporter GLUT1, the apical sodium-dependent bile ASBT, and the reduced folate carrier RFC (Asano et al. 1993, Muthusamy et al. 2015, Wong et al. 1998). Of these examples, *N*-glycosylation was essential for GLUT1 function, abundance at the plasma membrane, and stability (Asano et al. 1991, Asano et al. 1993)

whereas with ASBT, only function and protein half-life was affected by the *N*-glycan (Muthusamy et al. 2015). These reports show losses in function and plasma membrane protein abundance on par with the results I have described here. Interestingly, RFC showed no change in transport or membrane abundance between wild type and *N*-glycosylation mutant over expressed in K562 leucocytes (Wong et al. 1998). Although there is variability in the influence that glycosylation has on the protein, in the case of ENT1, it is clear that the *N*-glycan plays an important role.

The existence of a single glycosylation site makes these proteins, which heavily rely on proper glycan addition, vulnerable to single point mutations that would drastically alter the function of the protein. Given that SNPs are not found at the glycosylation site for ENT1, GLUT1, ASBT, or RFC proteins, and there are detrimental effects for non-SLC members containing a SNP at glycosylated Asn residues (Vogt et al. 2007, Pedersen et al. 2016), it appears that these sites are important for viability. In the case of ENT1, I have highlighted that glycosylation is required for function which is critical for nucleoside salvage, particularly in highly proliferative cells. It is clear that the glycosylation site of ENT1 is important for function of the protein and reliance on glycosylation for proper function is also seen with the glycine transporter GLYT2, where loss of all *N*-glycans results in non-functional protein (Martinez-Maza et al. 2001). Both GLYT2 and ENT1 are present at the plasma membrane – albeit, at a lower protein abundance – so I would expect that these *N*-glycan deficient proteins are in a non-functional conformation, potentially due to problems with protein folding.

Issues in protein folding may arise if the addition of the *N*-glycan is not sufficiently early on following protein translation, and could be necessary for proper conformation. I have provided evidence to support that ENT1 lacking *N*-glycosylation does not engage in ENT1-ENT1 protein interactions. Whether this is because of issues with protein folding and protein conformation, or if

the glycan is an essential part of forming this complex remains to be seen. This observation provides a potential mechanism to explain previous data which demonstrated that there is not a linear increase in ENT1-dependent activity when overexpressing ENT1 protein (Boleti et al. 1997). I often see an approximate 20- to 30- fold increase in ENT1 plasma membrane abundance when overexpressing GFP-ENT1 (as shown in Chapter 2), but this corresponds to a 2- to 3-fold increase in nucleoside transport. I predicted that this may have had to do with improper folding of a proportion of wild type overexpressed ENT1 protein, which has no transport activity but the NBTI binding site is still available, but as I have demonstrated in this chapter, availability of a protein partner may play a role. Although the functional significance of ENT-ENT interactions has not yet been well established, the binding of ENT1 to protein partners when overexpressed may be disrupted, as the ENT1 population may represent too much of the plasma membrane proteome reducing the availabilities of protein partners. Additionally, aberrantly glycosylated ENT1 could inhibit normally glycosylated ENT1 through a direct protein-protein interaction, similar to how ENT2 binding to ENT1 reduces ENT1-dependent activity (Grañé-Boladeras et al. unpublished data).

Overall, these findings show that the glycosylation of Asn48 is critical for hENT1 function and proper localization, and provides insight on a fundamental form of hENT1 regulation. These results can be used to guide further research on hENT1 glycan structure, and have potential to encourage further research in translating the role of hENT1 glycosylation state to clinical applications.

Conclusion

We confirm that N48 is the single site of glycosylation of hENT1 in human cells. NBTI binding, immunofluorescence microscopy, chloroadenosine uptake assays, and co-immunoprecipitation immunoblotting data suggest that hENT1 glycosylation at N48 is critical for the proper localization and function of the protein.

Declarations of interest

The authors have no conflicts of interest to declare.

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Chapter 4: Discussion and Potential Implications

Equilibrative nucleoside transporter 1 (ENT1) plays a critical role in the cell. The passive transport of nucleosides across cellular membranes is important for nucleoside salvage, purinergic signalling, and for the uptake of nucleoside analog drugs used in the treatment of cancer, viral infection, or parasitic infection (dos Santos-Rodrigues et al. 2014, Knight et al. 2010, Hillgren et al. 2013, Zhang et al. 2007). However, relatively little is known about the mechanisms that regulate ENT1. My thesis research provides insight into two gaps in our knowledge – how Ca^{2+}/CaM regulates ENT1 function, and the role *N*-glycosylation has on ENT1 – and provides context for future clinically relevant work.

In my analysis of the literature relating to the regulation of nucleoside flux from over the last 30 years, I identified a number of studies which implicated calcium signalling in the regulation of purine flux, yet the underlying mechanism of regulation was not determined (Paes-de-Carvalho et al. 2005, Zamzow et al. 2009, Wall and Dale 2013). Here, I have demonstrated that ENT1 plays a role in the cross-talk between calcium and purinergic signalling pathways with Ca^{2+}/CaM regulating ENT1 (Bicket et al. 2016). There are promising potential implications for optimizing nucleoside analog drug uptake synergistically with drugs like digoxin, a drug used in the treatment of atrial fibrillation, atrial flutter, or heart failure, or NMDA agonists like D-cycloserine, used for treating psychiatric diseases (Palatnick and Jelic 2014, Schade and Paulus 2015). These drugs result in an increase of intracellular calcium, and based on my research findings, should lead to enhanced nucleoside drug uptake (Bicket et al. 2016). This drug synergism could be particularly beneficial when drug resistance is an issue. It is also important to consider the potential for contraindications with other drugs such as calcium channel blockers, commonly used as an

antihypertensive drug, and ketamine, an NMDA receptor antagonist used for pain relief or treatment of depression of cancer patients (Chenniappan 2015, Hirota and Lambert 1996). Based on my model, drugs which reduce intracellular calcium may lead to a reduction of nucleoside flux, reducing the amount of nucleoside analog drug uptake. ENT1 regulation by calcium is a novel finding and should be carefully considered when moving forward with conducting trials where off target drug interactions occur.

Furthermore, my work suggests that CaM-ENT1 binding regulates ENT1 protein abundance at the plasma membrane. Since uptake of many nucleoside analog drugs is dependent on presence and action of ENT1, increasing protein abundance of ENT1 at the plasma membrane should enhance drug uptake. Here, I provide preliminary data showing that treatment with a high concentration of uridine, an endogenous ENT1 substrate, leads to protection from gemcitabine, a commonly used cancer drug. These data confirmed our hypothesis that repeated translocation of substrate would protect the cells. I predicted that increased translocation of substrate would lead to internalization of ENT1 thus lowering levels of drug uptake. Other work from our lab has shown that treatment with cytidine, another endogenous ENT1 substrate, resulted in lowered ENT1 abundance at the plasma membrane (Zafar et al. submitted). A lag period of reduced uptake could be observed following administration of the drug, or increased concentration of another ENT1 substrate, which highlights the importance of timing for the effective efficacy of the drug. Further work in identifying the potential link between drug delivery timing, concentration of the drug, and drug efficacy could prove to have a clinically relevant impact for nucleoside analog drug therapy.

There is also a growing interest in the scientific community in understanding the role of glycosylation in the development of disease and as a mechanism of targeted intervention to improve treatments of disease. Membrane proteins have substantial variation in the constitution of

the *N*-glycan chain that is found on the extracellular facing loops of the protein. For instance, many membrane proteins such as SLC1A2 and SLC44A1 have relatively unprocessed glycans constituted mostly of mannose, while others have elongated mannose or *N*-acetylglucosamine branches (Parker et al. 2013, Pedersen et al. 2016). I currently do not know what constitutes the glycan chain of ENT1, only that it is present at Asn48. Identifying the size of the ENT1 glycan chain, the degree of branching, the degree of mannose richness, as well as the presence of sialic acid or galactose in normal and cancer cells could provide a framework to differentiate between normal or cancerous cells since these factors commonly change under disease states like cancer (Tan et al. 2014, Christiansen et al. 2014, Pedersen et al. 2016). Differential ENT1 glycosylation in normal and abnormal cells has yet to be explored, but mass spectrometry is a technique commonly used to identify the constituents of the glycosyl moiety, and would be appropriate for determining the glycan constitution for ENT1 (Harvey 1993, Tsarbopoulos et al. 1994). Patients who have become non-responsive to nucleoside drug therapy may exhibit aberrant glycosylation of ENT1 leading to improper function of the protein since glycosylation appears to be linked to function (Bicket and Coe submitted). Interest in using antibodies to bind glycans on cancer cells to activate an immune system response to the cancer cells is growing (Dalziel et al. 2014, Vasconcelos-Dos-Santos et al. 2015). Further work to understand ENT1 glycosylation could prove valuable for the targeted treatment of cancer.

Increasing incidences of aberrant glycosylation are being detected in neoplasmic tissues, suggesting a link between glycosylation state and tumourogenic phenotype (Meany and Chan 2011, Hakomori 1996, Hakomori 2002, Mechref et al. 2012, Tuccillo 2014, Vasconcelos-Dos-Santos et al. 2015). Reduced ENT1-dependent uptake due to aberrant glycosylation could lead to ENT1-dependent drug resistance in cells. Since aberrant glycosylation is prevalent in cancer cells,

improper glycosylation of ENT1 in these cells could lead to reduced drug efficacy (Meany and Chan 2011, Tuccillo et al. 2014). A potential correlation between cell lines with low gemcitabine sensitivity and aberrant glycosylation was observed in the literature. For example, panc-1, a human pancreatic carcinoma cell line, and AsPC-1, a human pancreatic adenocarcinoma cell line, were resistant to gemcitabine and were glycosylation aberrant due to decreased expression of STT3A and STT3B (Hu et al. 2012, Pan et al. 2014). Work to either restore normal glycosylation in these cells or target aberrantly glycosylated ENT1 with antibodies to promote an immune system response are avenues which can be explored in the future (Taylor-Papadimitriou and Epenetos 1994, Dube and Bertozzi 2005, Pinho and Reis 2015).

We also believe that ENT1 oligomerization is an important form of regulation for the protein. ENT1 homo- or hetero-oligomers have been shown to exist (Grañé Boladeras and Coe unpublished data), and we have shown that this oligomerization requires ENT1 to be *N*-glycosylated. Future work to determine the role of glycosylation on ENT structure and function, could help to explain the existence of nucleoside analog drug resistant cell lines. If glycosylation contributes to the formation of a complex between ENT1 and other ENTs, then it could play a significant role in the function of the overall protein complex. For example, preliminary research has shown that protein-protein binding between ENT1 and ENT2 leads to reduced ENT1-dependent nucleoside flux, for reasons that are not yet clear (Grañé Boladeras and Coe unpublished data). Since *N*-linked glycosylation can modulate a wide range of protein functions leading to impacts on cell growth, protein folding, oligomerization, and signal transduction, it is important to understand the role that glycosylation plays in regulating a drug transporter like ENT1 (Imperiali and O'Connor 1999, Helenius and Aebi 2001, Vagin et al. 2009).

I have also addressed the importance of cell proliferation and the relationship to many diseases. Calcium signalling is critical for cell differentiation and proliferation with activation of proteins like CaMK, MAPK, PKC, and calcineurin regulating pro-proliferation genes (Tennakoon et al. 2015, Pinto et al. 2015). In proliferating cells, the salvage of nucleosides is valuable since *de novo* synthesis of nucleotides is energetically costly and failure to meet nucleotide demand during DNA replication will result in dire consequences for the cell (Anglana et al. 2003, Fasullo and Endres 2015). To ensure high fidelity DNA replication, nucleoside transporters are upregulated, increasing transporter abundance at the plasma membrane, which enhances nucleoside salvage (Cass et al. 1979, Pressacco et al. 1995, Valdés et al. 2002, Guillén-Gómez et al. 2012, Pérez-Torras et al. 2013). Overall, calcium signals lead to increased proliferation, which places a higher demand on the cell for nucleosides, so calcium upregulation of ENT1 would increase ENT1-dependent activity and aid in the salvage of nucleosides. Furthermore, there are many clinical applications associated with my work on calcium regulation of ENT1 since proliferation, differentiation, and cell migration are associated with many disease traits like cancer, atherosclerosis, rheumatoid arthritis, psoriasis, idiopathic pulmonary fibrosis, scleroderma, birth defects, and cirrhosis of the liver (Sporn and Harris 1981, Tennakoon et al. 2015). Moreover, proliferation is critical for tissue repair, development, transplantation, fertilization, and immune function (Gurtner et al. 2008, McArdle and Ashworth 1999, Franceschi 1989, Piltti et al. 2015, Ullman et al. 1990). I have identified a critical link between calcium signalling and ENT1, which describes a novel mechanism where the cell can regulate the intracellular nucleoside pool.

In conclusion, I have completed research which provides a greater understanding of two mechanisms of ENT1 regulation. My newly defined models provide a greater understanding of how ENT1 functions in the cell, and how both glycosylation and Ca^{2+}/CaM play a critical role in

integrating ENT1 with other signalling pathways. Since ENT1 expression and function varies depending on the individual, the tissue, and cell type, as well as the glycosylation state of the protein and the prevalence of calcium signalling in the cell, there are many opportunities for tailoring the best conditions for nucleoside analog drug delivery (Pennycooke et al. 2001, Bicket and Coe submitted). Future work in identifying potential drug interactions and optimizing nucleoside analog drug treatment for patients could greatly improve the efficacy of these drugs and improve patient outcomes.

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