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Biochemical Characterization of the Endoribonuclease, EndoU, and its Role in the Negative Selection of Developing Thymocytes

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Cell, Molecular, and Developmental Biology

by

Kristen Dias

March 2021

Dissertation Committee:
Dr. Fedor Karginov, Chairperson
Dr. Weifeng Gu
Dr. David Lo

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The Dissertation of Kristen Dias is approved:

Committee Chairperson

University of California, Riverside

ACKNOWLEDGEMENTS

I would like to sincerely thank everyone at UCR who helped me during my PhD studies. This project would be middling without the expertise and input that I received.

I would like to thank my dissertation committee who suggested experiments and guided me on the EndoU project. For a long time, it felt like I would only have unsupported data that only contained minor and negative results. With my committee's guidance I was granted stronger conclusions and learned to be a better experimentally driven scientist.

Dr. Weifeng Gu's suggestion to include the mass spectrometry experiment gave excellent support to the RNA-seq data and it further gave insight into possible targets of EndoU. My second chapter would have been rather shallow without this experiment.

When I first joined our laboratory, we had little to know mouse work. Dr. David Lo trained me on how to perform thymic extractions in a proper manner. Furthermore, his suggestion to quantify the activity of EndoU was excellent. My first chapter would have been gels with smears rather than beautiful graphs, if it were not for these suggested experiments. I regret not observing calcium localization, as per his suggestion, because now it looks like this would have been a particularly good experiment that could have added to the second chapter conclusions, but I will strongly suggest this to the next graduate student. Dr. Lo's input made the first and third chapters come together nicely.

Lastly, I would like to thank Dr. Fedor (Ted) Karginov. I could not ask for a better mentor. When I first joined the lab, I could perform PCR and kit based minipreps. His constant support, morning talks, and experimental guidance allowed me to work tirelessly on this project, learn amazing techniques, and feel respected. I am constantly amazed and inspired by his level of intellect and ability to analyze. I am still a young scientist and so I hope to be at his level one day. There would be no dissertation if I had any other PI.

Dr. Fedor Karginov analyzed the RNA-sequencing results.

Dr. Majid Ghassemian (UC San Diego, BPMSF facility) analyzed the mass spectrometry data.

DEDICATION

I dedicate this thesis to Cam Vogel. You were a dear friend, confidant, and peer. I will always remember watching Breaking Bad with you and us calling out the periodic symbols in the titles; I still do this whenever I re-watch episodes. The best time I ever had, in undergrad, was when we became temporary roommates. While I am not the biggest fan of lizards, I came to appreciate yours. While you were always outgoing and lovable, I was reserved and quiet and it was simply amazing to watch you love others and be loved by all. You will forever be in my heart, my head, and my dreams.

ABSTRACT OF THE DISSERTATION

Biochemical Characterization of the Endoribonuclease, EndoU, and its Role in the Negative Selection of Developing Thymocytes

by

Kristen Dias

Doctor of Philosophy, Graduate Program in Cell, Molecular, and Developmental Biology
University of California, Riverside, March 2021
Dr. Fedor Karginov, Chairperson

RNA binding proteins (RBPs) are an important but a poorly characterized group of proteins that affect all stages of the lifecycle of RNA transcripts, also known as post-transcriptional regulation. Within this class of proteins are RNA endonucleases. These RNA endonucleases have been shown to, both, upregulate and downregulate the propensity of a cell to undergo programmed cell death, known as apoptosis. The endoribonuclease poly-U specific, EndoU, is one such RNA endonuclease that has been implicated in promoting apoptosis but has not been fully characterized to show how it elicits this cell death. EndoU shows a tissue expression pattern in highly transient cells that are primed for cell death. Here, EndoU was investigated using the immature thymocyte cell line, VL3-3M2, at the biochemical level to show that the mammalian EndoU used calcium, likely as an allosteric cofactor, for its activity. Further biochemical characterization revealed that it was regulated by a large-transiently interacting cofactor that broadened its activity. This cell line was also used to characterize the cellular role for

EndoU and was found that it positively regulated pro-apoptotic, calcium homeostasis, and cell motility related genes. Further support was given to this regulation by EndoU, when protein sequencing of the likely EndoU complex was shown that it included the pro-apoptotic proteases, calpain 1 and 2. The complex also likely included RBPs that bound ribosomal RNA and were involved in tRNA processing, an indication that EndoU may cleave these RNAs during a calcium burst. Finally, it was shown that, in mice, EndoU promoted the negative selection of self-reactive thymocytes but not death by neglect, a result not previously seen before. While EndoU could be characterized further, the results showed that this enzyme was a highly regulated endoribonuclease that, *in vitro*, did not prefer a specific substrate, but, *in vivo*, it was shown to enhance apoptosis likely due to the release of these regulating factors.

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INTRODUCTION

The mammalian endoribonuclease, EndoU, is a poorly characterized RNA binding protein expressed in high turnover cells

ABSTRACT

RNA binding proteins (RBPs) are a relatively large but poorly characterized class of proteins that are important for modulating the post-transcriptional regulation of genes. A portion of these RBPs are RNA endonucleases, some of which are involved in regulating programmed cell death. The mammalian endoribonuclease, EndoU, is poorly characterized both biochemically and at the cellular level. However, due to its unique expression pattern in tissues containing high turnover cells, it is likely involved in regulating apoptosis. In this chapter, the concentration will be on introducing the role of RNA binding proteins, with a short review on apoptosis-regulating endoribonucleases. The chapter will delve into what is known about the biochemical features and cellular role of EndoU. Finally, the chapter will explain the investigative measures taken to characterize EndoU at the biochemical, cellular, and organismal level and how this characterization relates to programmed cell death.

RNA binding proteins shape the transcriptome

1. RNA binding proteins affect post-transcriptional regulation

In eukaryotes, gene expression is generally well controlled by transcription factors. However, further regulation and fine tuning of gene expression occurs through post-transcriptional regulation. This regulation is orchestrated by RNA binding proteins (RBPs) that affect a wide array of molecular and cellular functions. RBPs are important for post transcriptional gene regulation by processing RNAs, proper localization, and translational repression as a few examples. These RBPs control gene expression at all points in the lifecycle of a transcript. As pre-mRNAs are transcribed, RBPs known as splicing factors will assemble to form the spliceosome to remove introns. The effects of proper splicing allow transcription to continue and alternative splicing can increase the complexity of the proteome by giving proteins novel domains (Reviewed in (H.-C. Chen & Cheng, 2012). After transcription is completed, the transcript is exported to the cytoplasm covered in RBPs that can control the fate of these transcripts. The human genome contains around 1500 RBPs, however only a handful have been characterized (Castello et al., 2012). In this way, RBPs play an integral role in shaping the transcriptome. Many, but not all RBPs work by identifying specific sequences on transcripts based on their binding affinity to those sites. In the case of Argonaute (Ago), binding preference is directed by microRNA (miRNA) complementarity to allow Ago to subsequently destabilize the transcript through recruitment of decapping or deadenylation machinery or through translational repression (Guo et al., 2010; R. C. Lee et al., 1993; Wightman et al., 1993). The binding sites of other RBPs, such as Elav like 1 (ELAVL1),

are based on the affinity of their residues for certain RNA sequences and can stabilize mRNAs by protecting them from RNA degradation (Brennan-Laun et al., 2014). . With these abilities in mind, RBPs have the capacity to augment the transcriptome in a relatively quick manner.

2. Ribonucleases affect apoptosis

A subclass of RBPs are RNA endonucleases (RNases). Generally, RNases have not been found to bind to RNAs in a sequence specific manner. They have a broad specificity through short binding sequences, of mono or dinucleotide motifs, or through specific structures of RNAs (Tomecki & Dziembowski, 2010). Target specificity of these RNases can also be through binding of other RBPS, as is the case for Drosha which cuts double stranded RNA (dsRNA) due to its binding partner, DGCR8, recognition of ssRNA in hairpins to produce pre-miRNAs (J. Han et al., 2006). RNases can have a variety of functions within a cell, but many play a role in apoptosis.

Endoribonuclease U-specific (EndoU) is an RBP that has been characterized to cleave ssRNA at U-rich regions creating 2'-3' cyclic phosphate and 5' OH ends (Laneve et al. 2003). An EndoU orthologue is classified as such, based on the presence of the XendoU domain. The family of EndoU proteins are poorly characterized as their biochemical characterization still needs to be fleshed out and their cellular function remains, yet, unclear.

2.1 RNase L is a pro-apoptotic antiviral RNase

RNase L is an RNA endonuclease that induces apoptosis in virally infected cells. RNase L is essential for the progression of the innate immune type I interferon response pathway and is the effector of the 2'-5' oligoadenylate synthetase (OAS) pathway. During viral infections of cells, double stranded RNA (dsRNA) is produced whether the viral genome is composed of RNA or DNA. OAS binds and is activated by this dsRNA to produce 2'-5' AMP oligomers from rATP monomers. RNase L binds these oligomers and becomes active to cleave ssRNAs. It has been shown that RNase L cleaves RNAs in a non-specific manner, preferring UU and UA dinucleotides (J.-Q. Han et al., 2004). It was found that RNase L activity led to an increase in interferon ß production due to pathogen recognition receptors identifying the 3' ends of cleavage products as non-self which leads to cell death (Malathi et al., 2007).

2.2 Onconase is a cytotoxic and cytostatic RNase from *Rana pipiens*

Onconase was originally identified as a cytotoxic and cytostatic anti-tumor protein from *R. pipiens* oocyte extracts where it was able to prevent cell cycle progression in small quantities and cause cell death in larger quantities on various tumor cell lines (Darzzynkiewicz, 1988). It was later characterized as a ribonuclease belonging to the RNase A superfamily based on sequence homology and RNase-like activity that was necessary for its cytostatic and cytotoxic effects on A-253 human squamous carcinoma cells (Ardelt et al., 1991). These anti-tumor effects were also seen *in vivo* when M109 Madison mouse cell carcinoma cells intraperitoneal (IP) transplanted mice were also IP injected with onconase and shown to survive longer than their vehicle control

counterparts (Mikulski et al., 1990). The mode of transport into the cell is quite interesting since it seemed that Onconase was able to specifically target malignant cells while avoiding toxicity to healthy cells. While a possible onconase receptor has been discussed, no receptor has been identified (Boix et al., 1996). A more likely explanation for entry and, indeed, its ability to distinguish between healthy and malignant cells is due to the anionic distribution at the outer plasma membrane surface of cancerous cells and the cationic nature of Onconase (Turcotte et al., 2009). Onconase is now in its third stage of clinical trials as an anti-cancer medication and it will be interesting if other RNases can elicit the cytotoxicity that onconase can.

2.3 IRE1 signals ER stress and augments the apoptotic response

In eukaryotic cells, the ER is comprised of two distinct regions: the smooth endoplasmic reticulum and he rough endoplasmic reticulum (RER). The RER is the site of co-translational translocation of proteins destined for the ER, golgi, lysosomes, plasma membrane ore secretion. Within the RER there is a high rate of protein folding, disulfide bond formation, and post translational modifications. This high rate of protein folding is necessary due to the extreme environments that these proteins are destined for. For instance, the high ionic environment of the extracellular matrix, the low pH of the lysosome, and even the μM concentration of calcium within the ER relative to the nM concentrations in the cytosol. Due to the high rate of protein folding, there is an increased risk of misfolding which indicates that the ER is under stress. If acute ER stress occurs, then the unfolded protein response (UPR) is activated. The three main effectors of the

UPR are the transmembrane ER proteins; inositol requiring enzyme 1 (IRE1), PERK, and ATF6 (Y. Chen & Brandizzi, 2013). IRE1 contains a stress sensing luminal domain that allows for dimerization upon ER stress and a cytosolic RNA endonuclease domain (Credle et al., 2005). The luminal domain binds to and is inactivated by the ER hsp70-like chaperone protein, immunoglobulin binding protein (BiP), under normal protein folding conditions, however under ER stress, BiP is recruited away from the luminal domain allowing for IRE1 homodimerization (Pincus et al., 2010; J. Zhou et al., 2006). This homodimerization then allows for trans-autophosphorylation of the cytoplasmic kinase domain which allows for the reveal of the nucleotide binding pocket of the kinase extension nuclease (KEN) domain (K. P. Lee et al., 2008). IRE1 is capable of cleavage through recognition of stem loop structures found on the X-box binding protein (Xbp1) unspliced transcript which are then cleaved to undergo unconventional splicing of said transcript to allow for translation (Calfon et al. (2002), Korennykh et al. (2011)). Xbp1 is a transcription factor that upregulates genes involved in increasing ER protein folding and therefore reduce ER stress (A. H. Lee et al., 2003). Interestingly, it seems as though IRE1 prevents apoptosis through caspase cleavage at its cytoplasmic linker region that can induce antiapoptotic events in the mitochondria (Shemorry et al., 2019). While IRE1 has been shown to be anti-apoptotic, ER stress does induce apoptosis eventually.

EndoU expression patterns suggest a role in cell death

3.1 EndoU is only expressed in high turnover cells, suggesting a role in cell death

In humans, the endoribonuclease poly-U specific enzyme (EndoU) has only been shown to be expressed in squamous epithelial layers of the epidermis, esophagus, and cervix, as well as in the placental trophoblasts as shown by immunohistochemical staining for the EndoU protein (Figure 1A,(Uhlen et al., 2015)). These are all cell types with high turnover rates that undergo a program of differentiation and cell death within days/weeks.

Head and neck cancer is mainly found to be oral squamous cell carcinoma (OSCC) and can form due to carcinogens increasing genetic dosage of Ha-ras or mutations in tumor suppressor genes, loss of pro-apoptotic transcription factor, P53, function, or loss of control over cell cycle transitions (reviewed in (Deshpande & Wong, 2008)). Based on human protein atlas results, EndoU expression is found at variable levels in head and neck cancer. A more favorable outcome occurs in individuals with high EndoU levels which could indicate that EndoU is able to combat cancer progression, likely through the induction of apoptosis (Figure 1B, (Uhlen et al., 2017)). This favorable outcome due to its expression is in line with EndoU being a pro-apoptotic enzyme.

3.2 EndoU expression patterns in developing thymocytes

Another class of cells, where EndoU is expressed, are thymocytes that can either survive or undergo apoptosis during their development with the vast majority undergoing the former. The function of a matured thymocyte (T cell) is to recognize foreign antigen, presented by major histocompatibility complexes (MHCs) on the surfaces of infected cells, through their T cell receptors (TCR). There are two main classes of T cells categorized by their TCR co-receptor: $CD4^+$ and $CD8^+$ T cells. They can either recruit the innate and adaptive immune system to infected cells presenting antigen, as is the case for $CD4^+$ T cells, or actively kill infected cells in the case of $CD8^+$ T cells (reviewed in (Gaudino & Kumar, 2019). Having a diverse repertoire of TCRs is paramount for a strong immune system, however the TCRs must not react to self-antigens. T cells partially accomplish the goal of differentiating between self and non-self through their initial development in the thymus at the double negative stages (DN; $CD4^-$, $CD8^-$). The TCR is composed of an alpha chain, located on chromosome 14 in humans and mice, and a beta chain, located on chromosomes 7 and 6 in humans and mice respectively, that are rearranged at their respective loci through a process called V(D)J recombination (reviewed in (Lieber, 1991)). Each locus is comprised of three regions containing multiple possible exons with one exon selected for each region during VDJ recombination. After this genomic rearrangement has occurred, the TCR can be expressed if the recombination has produced a series of in-frame mutations. After the

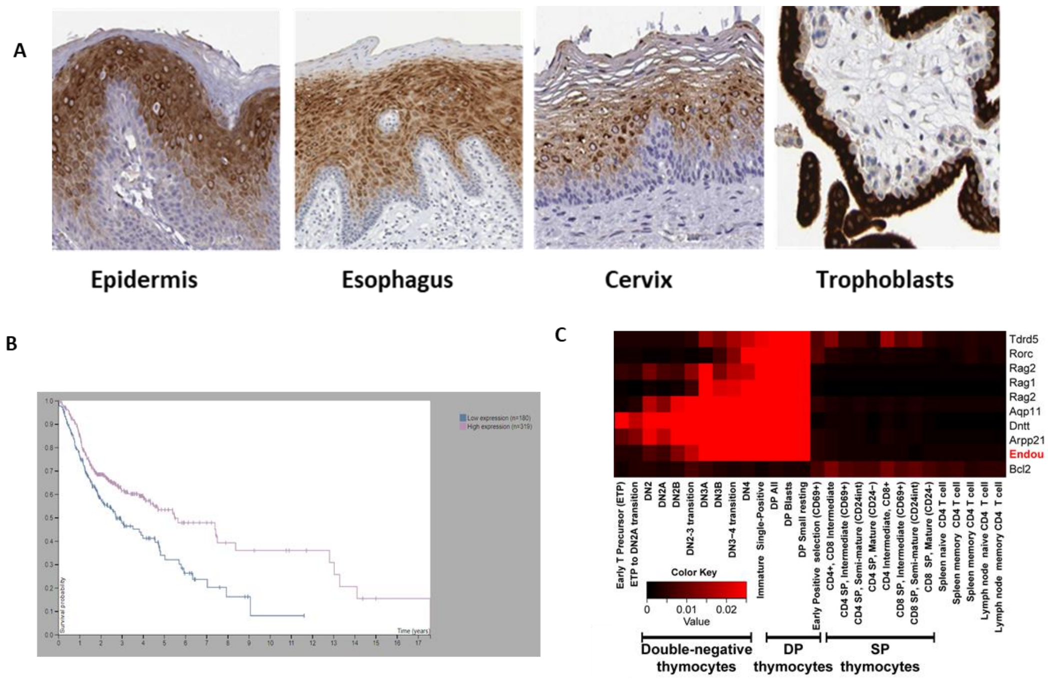

Figure 1: The EndoU expression pattern indicates a role in cell death.
(A) Human protein atlas results of EndoU protein expression based on IHC staining (brown) in human squamous epithelia of various tissues(Uhlen et al., 2015). (B) Human protein atlas results of survival rates correlated to EndoU expression. The purple line indicated high expression and the blue line indicated low expression (Uhlen et al., 2017). (C) Immgen microarray results of various genes in developing mouse thymocytes (Benoist et al., 2012).

TCR and its co-receptors, CD4 and CD8, are expressed, the thymocyte is now in the double positive (DP; $CD4^+$, $CD8^+$) stage where they undergo massive apoptosis at a rate of around 90%. This high rate of cell death is necessary to prevent self-antigen recognition and non-functional TCR thymocytes from escaping the thymus. The thymocyte is interrogated by cortical thymic epithelial cells (cTECs) for proper TCR formation and self-reactivity by MHC representation of a repertoire of self-antigens which will cause a response by the thymocyte depending on the TCR affinity for the antigen. If a thymocyte has a properly formed TCR then it will be able to recognize the MHC. If the TCR is functional but has affinity for the self-antigen presented by an cTEC, then the TCR will recognize the MHC and the self-antigen, inducing strong binding which causes apoptosis of the thymocyte; a process called negative selection. If the TCR is non-functional then there will be no interaction with the cTEC and the thymocyte will undergo apoptosis in a process called death by neglect. If the TCR is functional but has no affinity for the self-antigen, then there will be a weak interaction between the cTEC and the thymocyte allows the cell to survive; a process called positive selection (Brewer et al., 2002). In mice, microarray data of EndoU shows that EndoU levels increase as thymocytes develop and drop off just before mature thymocytes are formed (Figure 1C, (Benoist et al., 2012)). EndoU transcript levels gradually increase as thymocytes undergo TCR rearrangement in the DN stages with maximal expression at the double positive (DP; $CD4^+$, $CD8^+$) stage. Once the thymocytes undergo positive selection, these transcript levels immediately drop. This could mean that high-expressing EndoU thymocytes perform programmed cell death, while low expressing EndoU cells survive.

Alternatively, EndoU levels could be high during the DP stage in all thymocytes but its levels drop in thymocytes with proper TCRs and non-self-reactive TCRs. This means that EndoU levels likely remain high if the DP thymocytes undergo negative selection or death by neglect. Consistent with these observations, EndoU has been previously shown to promote apoptosis in self-reactive B cells, however its ability to promote cell death has not been directly tied with either its molecular function or regulation (Poe et al., 2014). Since EndoU is expressed only in cells that will undergo cell death and it has been shown to promote apoptosis in B-cells, it is likely that EndoU promotes cell death in self antigen reactive thymocytes in a similar manner to self-antigen exposed B cells, however, it may also be shown that EndoU could promote general apoptosis.

3.3 XendoU functions as a calcium dependent endoribonuclease in *Xenopus laevis*

The founding member of the EndoU family, XendoU, was characterized to cleave RNAs internally in *Xenopus laevis* oocyte nuclear extracts (Caffarelli et al., 1997). It was later indicated that in oocyte nuclear extracts, the preferred XendoU cofactor was calcium, wherein it cleaved RNAs in a non-specific manner but to a higher degree than if any other divalent metal was used as a cofactor *in vitro* and *ex vivo* (Schwarz & Blower, 2014). Originally, Mammalian EndoU, termed placental protein 11, was characterized as a serine protease (Grundmann et al., 1990) however, due to its sequence homology to XendoU and its ability to cleave RNAs, the protease characterization was later revised . It has not been shown that the mammalian EndoU is calcium dependent, however, it is

likely the case because the XendoU domain is well conserved between human and *X. laevis* with a sequence identity of about 40% and the secondary structure along with predicted 3-D models closely match the XendoU crystallographic structure (P. Laneve et al., 2008).

EndoU remains to be fully characterized at the biochemical and cellular level

4.1 The investigation into EndoU activity

To explore how an enzyme is active within a cell, it can be necessary to remove it from the cellular context and explore its substrate specificity and, if it is not known, the regulation put forth on such an enzyme. Through immunoprecipitation experiments, the first chapter showed that mammalian EndoU utilizes calcium, and it is highly regulated. In the second chapter, this high regulation was seen in the cell line used as they did not display a cellular phenotype, likely due to their ability to overcome EndoU's apoptotic effects, and EndoU dependent RNA production was very slight and specific. However, RNA-sequencing showed that EndoU was regulating pro-apoptotic genes and it was binding to pro-apoptotic proteins. Finally, to use a better model of apoptosis, mouse thymocytes were used to characterize negative selection and it was found that EndoU was promoting apoptosis.

REFERENCES

Ardelt, W., Mikulski, S. M., & Shogen, K. (1991). Amino acid sequence of an anti-tumor protein from Rana pipiens oocytes and early embryos. Homology to pancreatic ribonucleases. *Journal of Biological Chemistry, 266*(1), 245-251. doi:10.1016/s0021-9258(18)52427-3

Benoist, C., Lanier, L., Merad, M., Mathis, D., & Immunological Genome, P. (2012). Consortium biology in immunology: the perspective from the Immunological Genome Project. *Nat Rev Immunol, 12*(10), 734-740. doi:10.1038/nri3300

Boix, E., Wu, Y., Vasandani, V. M., Saxena, S. K., Ardelt, W., Ladner, J., & Youle, R. J. (1996). Role of the N terminus in RNase A homologues: differences in catalytic activity, ribonuclease inhibitor interaction and cytotoxicity. *J Mol Biol, 257*(5), 992-1007. doi:10.1006/jmbi.1996.0218

Brennan-Laun, S. E., Ezelle, H. J., Li, X. L., & Hassel, B. A. (2014). RNase-L control of cellular mRNAs: roles in biologic functions and mechanisms of substrate targeting. *J Interferon Cytokine Res, 34*(4), 275-288. doi:10.1089/jir.2013.0147

Brewer, J. A., Kanagawa, O., Sleckman, B. P., & Muglia, L. J. (2002). Thymocyte apoptosis induced by T cell activation is mediated by glucocorticoids in vivo. *J Immunol, 169*(4), 1837-1843. doi:10.4049/jimmunol.169.4.1837

Caffarelli, E., Maggi, L., Fatica, A., Jiricny, J., & Bozzoni, I. (1997). A Novel Mn++-Dependent Ribonuclease That Functions in U16 SnoRNA Processing inX.Laevis. *Biochemical and Biophysical Research Communications, 233*(2), 514-517. doi:https://doi.org/10.1006/bbrc.1997.6487

Calfon, M., Zeng, H., Urano, F., Till, J. H., Hubbard, S. R., Harding, H. P., Clark, S. G., & Ron, D. (2002). IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature, 415*(6867), 92-96. doi:10.1038/415092a

Castello, A., Fischer, B., Eichelbaum, K., Horos, R., Beckmann, B. M., Strein, C., Davey, N. E., Humphreys, D. T., Preiss, T., Steinmetz, L. M., Krijgsveld, J., & Hentze, M. W. (2012). Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. *Cell, 149*(6), 1393-1406. doi:10.1016/j.cell.2012.04.031

Chen, H.-C., & Cheng, S.-C. (2012). Functional roles of protein splicing factors. *Bioscience reports, 32*(4), 345-359. doi:10.1042/BSR20120007

Chen, Y., & Brandizzi, F. (2013). IRE1: ER stress sensor and cell fate executor. *Trends Cell Biol, 23*(11), 547-555. doi:10.1016/j.tcb.2013.06.005

Credle, J. J., Finer-Moore, J. S., Papa, F. R., Stroud, R. M., & Walter, P. (2005). On the mechanism of sensing unfolded protein in the endoplasmic reticulum. *Proc Natl Acad Sci U S A, 102*(52), 18773-18784. doi:10.1073/pnas.0509487102

Darzzynkiewicz, Z. C., S.P. Mikulski, S. M. Ardelt, W. J. Shogen, K. (1988). Cytostatic and cytotoxic effects of Pannon (P-30 Protein), a novel anticancer agent. *Cell Tissue Kinetics*(21), 169-182.

Deshpande, A. M., & Wong, D. T. (2008). Molecular mechanisms of head and neck cancer. *Expert Rev Anticancer Ther, 8*(5), 799-809. doi:10.1586/14737140.8.5.799

Gaudino, S. J., & Kumar, P. (2019). Cross-Talk Between Antigen Presenting Cells and T Cells Impacts Intestinal Homeostasis, Bacterial Infections, and Tumorigenesis. *Front Immunol, 10*, 360. doi:10.3389/fimmu.2019.00360

Grundmann, U., Romisch, J., Siebold, B., Bohn, H., & Amann, E. (1990). Cloning and Expression of a cDNA Encoding Human Placental Protein 11, a Putative Serine Protease with Diagnostic Significance as a Tumor Marker. *DNA and Cell Biology, 9*(4), 243-250. doi:10.1089/dna.1990.9.243

Guo, H., Ingolia, N. T., Weissman, J. S., & Bartel, D. P. (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature, 466*(7308), 835-840. doi:10.1038/nature09267

Han, J.-Q., Wroblewski, G., Xu, Z., Silverman, R. H., & Barton, D. J. (2004). Sensitivity of Hepatitis C Virus RNA to the Antiviral Enzyme Ribonuclease L Is Determined by a Subset of Efficient Cleavage Sites. *Journal of Interferon & Cytokine Research, 24*(11), 664-676. doi:10.1089/jir.2004.24.664

Han, J., Lee, Y., Yeom, K.-H., Nam, J.-W., Heo, I., Rhee, J.-K., Sohn, S. Y., Cho, Y., Zhang, B.-T., & Kim, V. N. (2006). Molecular Basis for the Recognition of

Primary microRNAs by the Drosha-DGCR8 Complex. *Cell, 125*(5), 887-901. doi:https://doi.org/10.1016/j.cell.2006.03.043

Korennykh, A. V., Korostelev, A. A., Egea, P. F., Finer-Moore, J., Stroud, R. M., Zhang, C., Shokat, K. M., & Walter, P. (2011). Structural and functional basis for RNA cleavage by Ire1. *BMC Biology, 9*(1), 47. doi:10.1186/1741-7007-9-47

Laneve, P., Gioia, U., Ragno, R., Altieri, F., Di Franco, C., Santini, T., Arceci, M., Bozzoni, I., & Caffarelli, E. (2008). The tumor marker human placental protein 11 is an endoribonuclease. *J Biol Chem, 283*(50), 34712-34719. doi:10.1074/jbc.M805759200

Lee, A. H., Iwakoshi, N. N., & Glimcher, L. H. (2003). XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol Cell Biol, 23*(21), 7448-7459. doi:10.1128/mcb.23.21.7448-7459.2003

Lee, K. P., Dey, M., Neculai, D., Cao, C., Dever, T. E., & Sicheri, F. (2008). Structure of the dual enzyme Ire1 reveals the basis for catalysis and regulation in nonconventional RNA splicing. *Cell, 132*(1), 89-100. doi:10.1016/j.cell.2007.10.057

Lee, R. C., Feinbaum, R. L., & Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell, 75*(5), 843-854. doi:https://doi.org/10.1016/0092-8674(93)90529-Y

Lieber, M. R. (1991). Site-specific recombination in the immune system1. *The FASEB Journal, 5*(14), 2934-2944. doi:https://doi.org/10.1096/fasebj.5.14.1752360

Malathi, K., Dong, B., Gale, M., Jr., & Silverman, R. H. (2007). Small self-RNA generated by RNase L amplifies antiviral innate immunity. *Nature, 448*(7155), 816-819. doi:10.1038/nature06042

Mikulski, S. M., Ardelt, W., Shogen, K., Bernstein, E. H., & Menduke, H. (1990). Striking increase of survival of mice bearing M109 Madison carcinoma treated with a novel protein from amphibian embryos. *Journal of the National Cancer Institute, 82*(2), 151-153. doi:10.1093/jnci/82.2.151-a

Pincus, D., Chevalier, M. W., Aragon, T., van Anken, E., Vidal, S. E., El-Samad, H., & Walter, P. (2010). BiP binding to the ER-stress sensor Ire1 tunes the homeostatic

behavior of the unfolded protein response. *PLoS Biol, 8*(7), e1000415. doi:10.1371/journal.pbio.1000415

Poe, J. C., Kountikov, E. I., Lykken, J. M., Natarajan, A., Marchuk, D. A., & Tedder, T. F. (2014). EndoU is a novel regulator of AICD during peripheral B cell selection. *J Exp Med, 211*(1), 57-69. doi:10.1084/jem.20130648

Schwarz, D. S., & Blower, M. D. (2014). The calcium-dependent ribonuclease XendoU promotes ER network formation through local RNA degradation. *J Cell Biol, 207*(1), 41-57. doi:10.1083/jcb.201406037

Shemorry, A., Harnoss, J. M., Guttman, O., Marsters, S. A., Komuves, L. G., Lawrence, D. A., & Ashkenazi, A. (2019). Caspase-mediated cleavage of IRE1 controls apoptotic cell commitment during endoplasmic reticulum stress. *Elife, 8*. doi:10.7554/eLife.47084

Tomecki, R., & Dziembowski, A. (2010). Novel endoribonucleases as central players in various pathways of eukaryotic RNA metabolism. *RNA, 16*(9), 1692-1724. doi:10.1261/rna.2237610

Turcotte, R. F., Lavis, L. D., & Raines, R. T. (2009). Onconase cytotoxicity relies on the distribution of its positive charge. *The FEBS Journal, 276*(14), 3846-3857. doi:https://doi.org/10.1111/j.1742-4658.2009.07098.x

Uhlen, M., Fagerberg, L., Hallstrom, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, A., Kampf, C., Sjostedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C. A., Odeberg, J., Djureinovic, D., Takanen, J. O., Hober, S., Alm, T., Edqvist, P. H., Berling, H., Tegel, H., Mulder, J., Rockberg, J., Nilsson, P., Schwenk, J. M., Hamsten, M., von Feilitzen, K., Forsberg, M., Persson, L., Johansson, F., Zwahlen, M., von Heijne, G., Nielsen, J., & Ponten, F. (2015). Proteomics. Tissue-based map of the human proteome. *Science, 347*(6220), 1260419. doi:10.1126/science.1260419

Uhlen, M., Zhang, C., Lee, S., Sjostedt, E., Fagerberg, L., Bidkhori, G., Benfeitas, R., Arif, M., Liu, Z., Edfors, F., Sanli, K., von Feilitzen, K., Oksvold, P., Lundberg, E., Hober, S., Nilsson, P., Mattsson, J., Schwenk, J. M., Brunnstrom, H., Glimelius, B., Sjoblom, T., Edqvist, P. H., Djureinovic, D., Micke, P., Lindskog, C., Mardinoglu, A., & Ponten, F. (2017). A pathology atlas of the human cancer transcriptome. *Science, 357*(6352). doi:10.1126/science.aan2507

Wightman, B., Ha, I., & Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell, 75*(5), 855-862. doi:https://doi.org/10.1016/0092-8674(93)90530-4

Zhou, J., Liu, C. Y., Back, S. H., Clark, R. L., Peisach, D., Xu, Z., & Kaufman, R. J. (2006). The crystal structure of human IRE1 luminal domain reveals a conserved dimerization interface required for activation of the unfolded protein response. *Proc Natl Acad Sci U S A, 103*(39), 14343-14348. doi:10.1073/pnas.0606480103

CHAPTER 1

Biochemical characterization of mammalian EndoU reveals a highly regulated enzyme that uses calcium signaling

ABSTRACT

EndoU is a poorly characterized RNA endonuclease that has been shown to be expressed in immature double positive (DP; $CD4^+$, $CD8^+$) thymocytes and may have a role in apoptosis. Using the DP thymocyte line, VL3-3M2, EndoU was biochemically characterized through immunoprecipitation followed by RNase assays. The results indicated that mammalian EndoU used calcium, likely in an allosteric manner, as a cofactor. Because calcium usage is often used as a regulator, further characterization of EndoU regulation was determined through its spatial organization. EndoU localized to the nucleus but was not highly active and even though much less localized to the cytoplasm, it held higher cytoplasmic activity. This high cytoplasmic activity was due to a large cofactor that augmented the specificity of EndoU. The high regulation on and calcium dependency of EndoU within this cell line indicated that EndoU was likely involved in the apoptotic pathway.

INTRODUCTION

Calcium is the most prominent secondary messenger and can activate a broad range of functions within a cell (reviewed in (Clapham, 2007). ER stress can induce intrinsic apoptosis by creating a calcium flux. When calcium levels within the cell are

dysregulated due to ER stress, calcium is released to activate the calcium dependent cysteine protease, calpain, which in turn cleaves pro-caspase 12 to form the mature form (Martinez et al., 2010). Because XendoU has been shown to be calcium activated (Schwarz & Blower, 2014), it was likely that mammalian EndoU was also calcium activated.

To interrogate EndoU's biochemical activity, the immature double positive ($CD4^+$, $CD8^+$; DP) thymocyte cell line, VL3-3M2 was used. This cell line was derived from a mouse thymic lymphoma that displays characteristics of DP thymocytes. Upon stimulation with antibodies against the TCR co-receptors, CD3 and CD28, or PMA/ionomycin stimulation these cells display early aspects of maturation (Rag1 downregulation and CD5 upregulation); however, this cell line does not fully differentiate into single positive ($CD4^+$ or $CD8^+$; SP) thymocytes (Groves et al., 1995). Because EndoU has been shown to promote cell death in autoreactive B cells and because of its pattern of expression in high turnover cells, the functional role of EndoU is likely to promote cell death in any tissue where it is expressed. In this chapter, biochemical characterization of EndoU will be employed to show that it is highly regulated, and due to this regulation, how it had the potential to induce apoptosis.

RESULTS

EndoU was knocked out of the VL3-3M2 cell line to investigate its function

EndoU expression in VL3-3M2s resembled that in thymocytes, as seen by antibody stimulation against CD3 and CD28 TCR co-receptors and was likely performing the same or similar function in both VL3-3M2s and primary mouse thymocytes (Figure 2A). Therefore, EndoU was knocked out in this cell line to investigate its function. The CRISPR system, a highly efficient tool to create genetically null mutants was used in conjunction with templated replacement of the EndoU locus by homology directed repair (HDR) to replace most of the locus with a neomycin resistance cassette (neo^r). By using the VL3-3M2 cell line, knockout cells were created to identify how EndoU could affect developmental cell death through the biochemical characterization and regulated potential of EndoU.

CRISPR/Cas9 utilizes a single guide RNA (sgRNA) to specifically target a single locus but off-target loci can still be cleaved. To overcome phenotypic variances due to off-target cleavage or clonal differences, multiple sgRNAs along with multiple clones were designed and produced, respectively, to create EndoU null VL3-3M2s. Based on the predictive strengths of sgRNAs, two sgRNAs were designed to cut the first intron and two sgRNAs were designed to cut the last exon of the EndoU locus. To aid in the selection of clonal EndoU knockouts, the EndoU locus was replaced with neo^r using flanking homology arms to the respective cut sites (Figure 2B). Clonal selection was then carried out, followed by PCR screening for successful, bi-allelic, integrants of neo^r (Figure 2C).

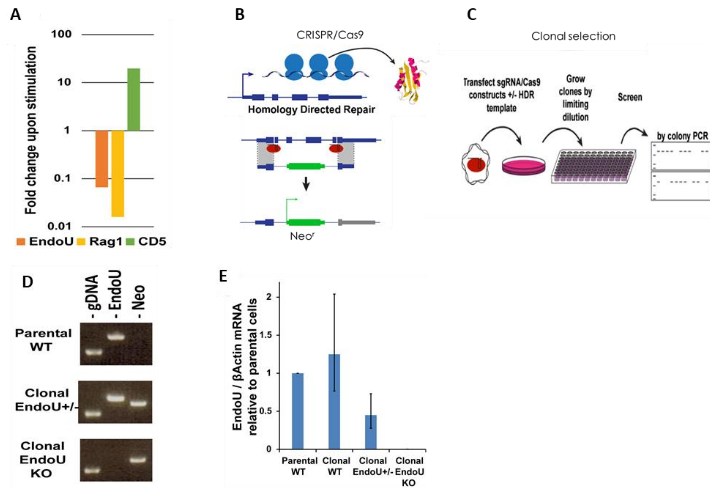

Figure 2: EndoU was knocked out in the VL3-3M2 cell line
(A) RT-qPCR results of stimulated VL3-3M2s by antibodies against the TCR co-receptors CD3 and CD28. (B) Outline of the strategy used to replace EndoU with the Neo^r cassette using HDR. (C) Outline of clonal selection and screening of Neo^r cassette integrants. (D) representative results of PCR screening on clones selected with neomycin (E) RT-qPCR validation of successful knockouts of EndoU.

Screening was carried out through PCR amplification of the EndoU locus with sets of primers designed to amplify neo^r in the EndoU locus, undisturbed wildtype (WT) locus, and outside the locus as a control (Figure 2D). Since there was no clean EndoU antibody,

RT-qPCR was used to validate 6 candidate knockouts that were then used to identify an EndoU dependent molecular phenotype (Figure 2E).

Knocking out EndoU in VL3-3M2 cells reveals an EndoU dependent phenotype induced by calcium

Since EndoU has been characterized as an endoribonuclease and calcium dependent, an assay was developed to determine if there was a molecular phenotype. Cell lysates from WT, KO or EndoU-rescued (FLAG-HA tagged or untagged) VL3-3M2 cells were treated with 5 mM calcium, followed by running extracted RNA on a denaturing PAGE to determine the RNA integrity as a result of EndoU activity with KO lysate serving as a control. No difference between RNA integrity was observed in untreated cell lysates as compared to the KO control, however, when calcium was added to these cytoplasmic lysates, differences could be observed. The loss of the most abundant RNA species such as tRNAs, 5S rRNA, and 5.8S RNA was clearly visible if either WT EndoU or rescued EndoU was present in the cytoplasmic lysates treated with 5 mM calcium and therefore showed that there was an EndoU dependent phenotype (Figure 3). Because EndoU functions as an endoribonuclease, the RNA degradation, seen in this experiment, was likely due directly to EndoU activity. After discovering that WT EndoU and EU-HA were functionally

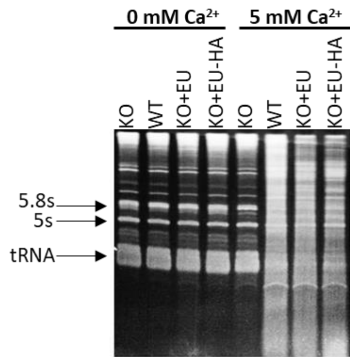

Figure 3: Calcium induces the EndoU molecular phenotype. Total RNA from KO, WT, or EndoU rescued VL3-3M2 lysates treated with or without calcium.

similar and had a dependency on calcium, characterization of the enzyme could be determined through immunoprecipitations.

Biochemical characterization of reaction conditions

Determining the optimal reaction condition of EndoU was important because it could be used to help characterize enzyme by discovering if there was a preferred substrate *in vitro* as well as compare the *in vitro* conditions with the environmental conditions within a cell. While it is not always the case that an enzyme's optimal conditions exactly match to cellular conditions, an approximation of conditions can be used to figure out where and how environmental factors may contribute to an enzyme's activity. To determine the optimal reaction conditions for EndoU, immunoprecipitations (IPs) of EU-HA were utilized. To first identify a preferred substrate, IP'd EU-HA was given 10 pmol of a variety of synthetic RNAs with varied length and sequence and the reaction proceeded for 20 minutes. The *in vitro* specificity of EndoU was indeterminate as it cleaved all RNA substrates well, however the highest amount of degradation was seen when the 50mer substrate was used (Figure 4A). The 50mer was then used in future IP'd EU-HA assays in various environments over the course of 50 minutes with an aliquot of the reaction taken at each time point to determine optimal reaction conditions (Figure 4B). Densitometric analysis was performed to quantify the amount of substrate cleaved over time with the initial reaction rates determined by calculating the regression line of the linear portion of the reaction (Figure 4C). The initial reaction rates were then used to determine if they

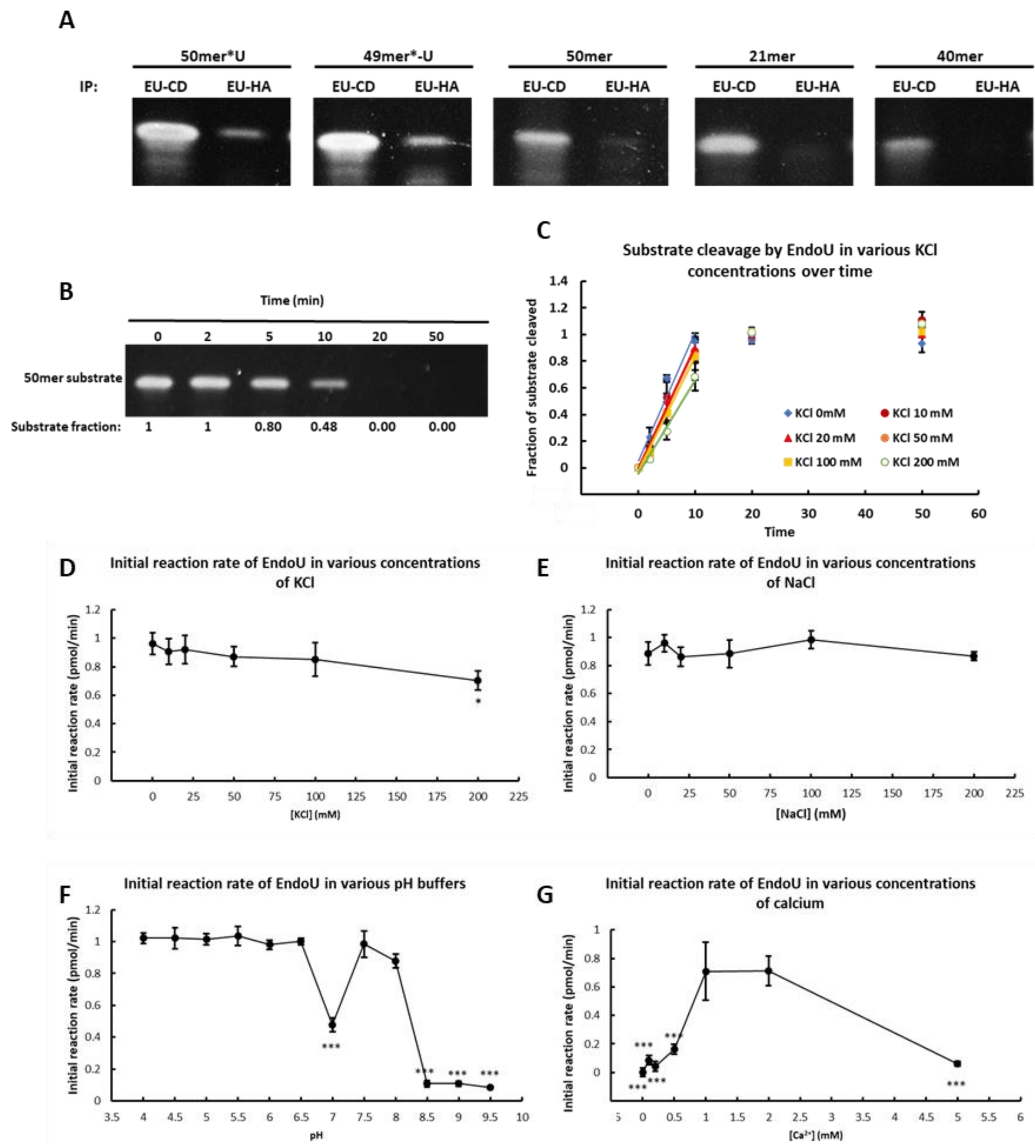

Figure 4: EndoU optimal reaction conditions indicate high activity in diverse environments. (A) IP'd EndoU activity on RNA substrates of varied length and sequence. Catalytically dead EndoU (EU-CD) was used as a negative control. (B) Representative image of the 50-mer substrate RNA run on a denaturing PAGE in optimum reaction conditions over time for IP'd EndoU. (C) Representative graph of EndoU activity over time as measured by the fraction of substrate cleavage. Initial reaction rate shown as the linear portion of the reaction. Error bars represent SEM (n=3). (D-G) Initial reaction rates in various titrated conditions. Error bars represent standard error of the coefficient (n=3).
* $p < 0.05$, *** $p < 0.001$

were significantly different from each other by using the standard error of the regression (Figures 4D-G).

To determine the monovalent ion influence on EndoU activity, various concentrations of NaCl or KCl were used in the reaction. The apparent monovalent salt dependency was absent from these experiments however initial reaction rates trended lower as KCl concentrations increased (Figure 4D). Because this trend was not seen with NaCl and 10 mM NaCl showed the highest initial reaction rate, 10 mM NaCl was determined to be an optimal reaction condition (Figure 4E). The monovalent salt did not give a clear representation of where EndoU was active within a cell.

The next environmental condition tested was the pH preference. Since pH within a cell, including organelles, can range from pH 4.5-8, a broad pH range was used for this experiment. Interestingly, EndoU initial reaction rates were high in acidic buffers and more neutral buffers while reaction rates were abolished in more basic buffers (Figure 4F). This showed that EndoU had high activity at a range of pHs ranging from acidic implying that EndoU could be active in both the lysosome and cytoplasm.

Since calcium was necessary to observe the EndoU phenotype and it has been shown that XendoU used it as a cofactor, it was likely that calcium directly interacted with EndoU. First, to determine if EndoU could be directly activated by calcium and, second, to determine at what concentration EndoU reaction rate was the highest, IP'd EU-HA was

treated with calcium and given the 50mer substrate. IP'd EU-HA was activated by calcium at relatively high concentrations where initial reaction rates were highest at 1 mM or 2 mM calcium (Figure 4G). While calcium levels inside of a cell will never reach 1 mM, a correlation of increasing amounts of calcium with increasing initial reaction rates was observed. Interestingly, EU-HA initial reaction rates were abolished at 5 mM calcium. This may have been due to an extreme in calcium concentration that negatively impacted EndoU activity.

The secondary messenger, calcium, is the EndoU cofactor

To validate that EndoU was dependent on calcium for RNA degradation, FLAG-HA tagged EndoU (EU-HA) was immunoprecipitated from rescued knockouts and given a 50mer RNA, as substrate, in various divalent metals, at a concentration of 2 mM, to determine the activating metal. The reaction rate was observed by running the substrate

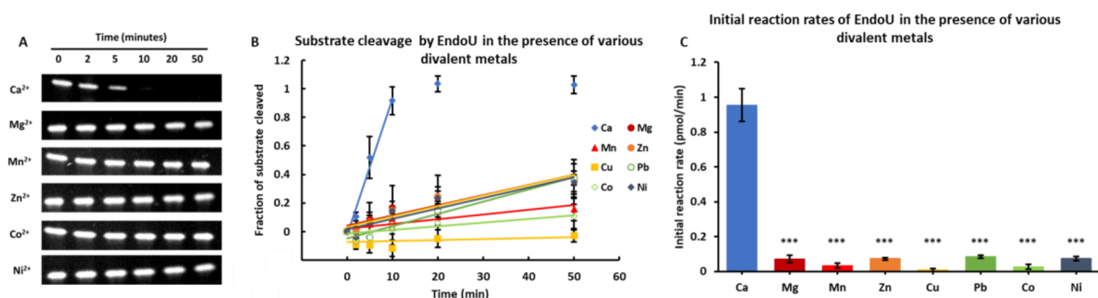

Figure 5: Calcium activates EndoU *in vitro*
(A) Representative image of 50-mer degradation over the course of an IP'd EndoU reaction in 2 mM of various divalent metals. (B) Quantification of 50-mer substrate cleavage over time. Initial reaction rates shown as the linear portion of the reaction. Error bars represent SEM (n=3). (C) Initial reaction rates of EndoU in various divalent metals. Error bars represent standard error of the regression coefficient (n=3).
*** $p < 0.001$

out on a denaturing PAGE gel at 0, 2, 5, 10, 20, and 50 minutes (Figure 5A). Quantification of the degraded products showed that EndoU was only able to completely degrade the 50mer in calcium (Figure 5B). Initial reactions rates showed that calcium significantly contributed to EndoU activity while the other tested divalent metals stimulated little to no activity (Figure 5C). These results have shown that EndoU activity was dependent only on the secondary messenger, calcium. This was the first time that mammalian EndoU has been shown to directly utilize calcium as a cofactor which implies that EndoU was active in a calcium regulated pathway.

Eukaryotic EndoU contains an extra domain that may be used to chelate calcium

Initially XendoU was predicted to bind a divalent metal and use it as part of the reaction (Cafarelli et al. 1997). However, the bacterial homolog to EndoU has not been shown to use a divalent metal, though it contains most of the XendoU domain (Michalska et al., 2018). Surprisingly, the human SARS-CoV EndoU, nsp15, does use manganese as a cofactor *in vitro* (Ivanov et al., 2004). Aligning the EndoU domains from eukaryotic species to viral and bacterial species, it was clear that the viral and bacterial species lacked an N-terminal domain present (Figure 6) in the eukaryotic species while the catalytic domain was conserved (Figure 6 asterisks). This eukaryotic sub-domain was intriguing because it could be responsible for the binding of calcium.

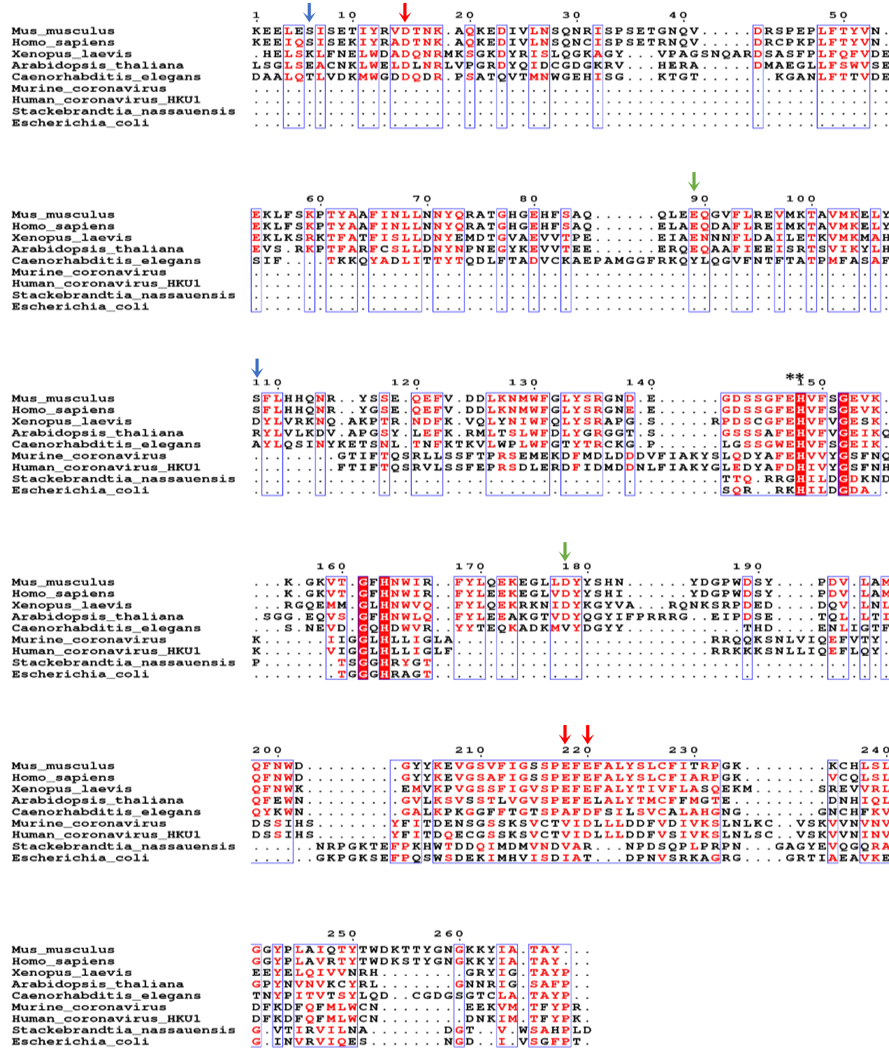

Figure 6: Alignments between eukaryotic, viral, and bacterial EndoU domains reveal a eukaryotic specific sub-domain

ClustalX alignments between eukaryotic, coronavirus, and bacterial EndoU domains. Red arrows indicate acidic residues predicted to bind calcium, green arrows indicate conserved acidic residues not predicted to bind calcium, and blue arrows indicate non-conserved residues not predicted to bind calcium. Asterisks represent alanine substitutions used to create catalytically dead EndoU

EndoU likely uses calcium as an allosteric activator

Since the sub-domain was present in eukaryotes, which utilize a divalent metal, and absent in viral, which utilize manganese, or prokaryotes, which do not utilize a cofactor, but all cleaved RNAs a hypothesis could be made that EndoU did not use calcium as part of the catalytic reaction but rather as an allosteric activator. By overlaying the crystal structure of the bacterial EndoU with XendoU, two separate domains were identifiable and when comparing showed the extra domain cradling the RNA binding pocket and catalytic core (Figure 7A). The eukaryotic domain could, therefore, be responsible for binding calcium to cause a conformational change in XendoU that was favorable for RNA cleavage. Additionally, software that predicts calcium binding sites within crystal structures was used (W. Zhou et al., 2015) (Figure7A white/green balls). While there were a few positions that were close to the catalytic core of both the XendoU and bacterial EndoU, most of the positions were predicted to be in the additional XendoU domain.

Conserved acidic residues near the predicted calcium binding sites were hypothesized to be functionally important for calcium binding. D153, E356, and E358 were then mutated to their basic analogues to determine the calcium dependence (Figure 6 red arrows). As a positive control, a catalytically dead mutant introduced into the knockout cells was used (Figure 6 asterisks). The catalytically dead version of EndoU contained residues E258 and H256 mutated to alanines to disrupt catalysis based on previous work in human EndoU (P. Laneve et al., 2008). To control for the possibility that conserved residues

were important for structural integrity, conserved acidic residues that were not predicted to bind calcium, D316 and E227, were mutated to their basic analogues, asparagine and glutamine, respectively (Figure 6 green arrows). Furthermore, to control for the possibility that any mutation could disrupt EndoU's structural integrity, not conserved and not predicted residues were mutated and examined (Figure 6 blue arrows). Mutants in the predicted and conserved residues, D153, E356, and E358, would be expected to have activity resembling the catalytically dead mutant. To overcome any differential expression levels, IP beads were saturated so that mutant and EU-HA could be compared to each other (Figure 7B). The results showed that the predicted D153N mutant had low activity, as it did not reach complete degradation until 50 minutes, and the E358Q mutant never reached complete degradation of the substrate however the predicted glutamate 356 did show a relatively fast reaction rate (Figure 7C). Quantification of substrate cleavage revealed that the E358Q mutant reaction closely resembled the EU-CD reaction (Figure 7D). Comparing the initial reaction rates to EU-CD revealed that E358Q was not significantly different than EU-CD and, while D153N did have lower activity than any of the negative controls, D153N and E356Q had significantly higher initial reaction rates (Figure 7E). Because other conserved and acidic residues, when mutated, did not abolish EndoU activity but mutating E358 did, it was likely that this residue was, at least in part, responsible for binding calcium.

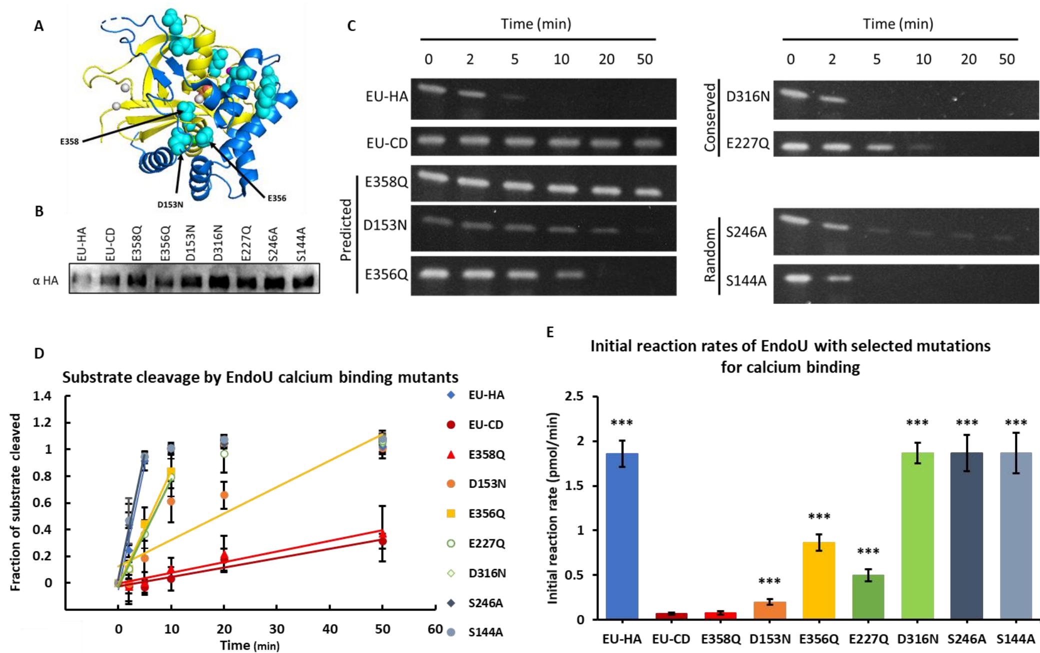

Figure 7: Calcium is likely an allosteric cofactor
(A) Crystallographic structure of XendoU from *X. laevis*. Cyan residues represent residues predicted to bind calcium that include the conserved E356 and E358 residues (arrows). Blue ribbons represent the animal domain and yellow ribbons represent the conserved domain between bacteria and animals. (B) Representative western blot of IP'd EU-HA with various mutations. (C) Representative nuclease assays of IP'd mutant EU-HA. (D) Quantification of substrate cleavage by mutant IP'd EU-HA. Error bars represent SEM (n=3). (E) Initial reaction rates of mutant IP'd EU-HA from D. Error bars represent standard error of the coefficient (n=3)
***= P<0.001 Student's T test on slopes comparing to EU-CD

Due to the utilization of calcium as a cofactor by mammalian EndoU and because no metal cofactor was needed for the bacterial EndoU, predicted and conserved acidic residues were expected to be important for binding calcium. The results showed that because these predicted and conserved residues, when mutated, caused a decrease in EndoU activity, this implies that these residues were important for binding calcium and because they were distant from the catalytic core, calcium caused a conformational change to EndoU rather than participating in catalysis.

EndoU mainly localizes to the nucleus but has higher activity in cytoplasmic lysates

To look further into the regulation of EndoU, it was necessary to determine if its cellular localization impacted its activity. Surprisingly, upon fractionation and immunoblotting for EU-HA, EndoU was found localized mainly to the nucleus even though, as previously seen in figure 3A, cytoplasmic EndoU activity was remarkably high (Figure 8A).

To clarify the local activity of EndoU, EU-HA and EU-CD were fractionated into cytosolic and nuclear fractions which were then treated with 5 mM calcium. Due to the differences in nuclear and cytosolic major RNA species, with nuclear RNAs being more varied in length, degradation patterns were obscured. Nonetheless, comparisons could be made between nuclear and cytoplasmic EndoU activity. The degradation of nuclear RNA

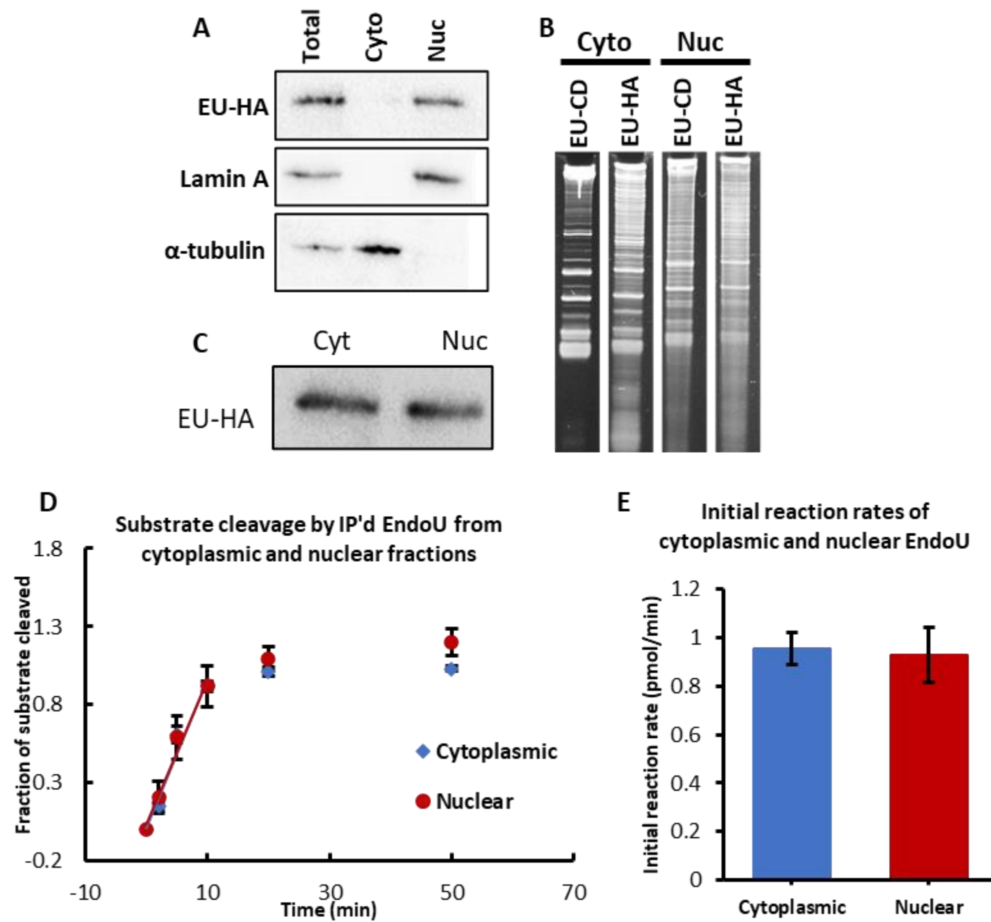

Figure 8: EndoU is localized mainly to the nucleus but cytoplasmic activity is higher
(A) Representative immunoblot showing EndoU, Lamin A, and α-tubulin from total, cytoplasmic and nuclear lysate fractions. (B) RNA from lysate fractions treated with 5 mM calcium. (C) Representative immunoblot of cytoplasmically derived and IP'd EndoU compared to nuclear derived IP'd EU-HA. (D) Quantifications substrate cleavage of cleavage assays from IP'd EU-HA derived from their respective fractions. Error bars represent SEM (n=3) (E) Initial reaction rates of IP'd EndoU derived from their respective fractions. Error bars represent standard error of the coefficient (n=3)

species by EU-HA, compared to EU-CD, was not as drastic as the degradation of cytoplasmic RNA species of EU-HA, when compared to EU-CD (Figure 8B). This showed that nuclear EndoU was somehow less efficient than cytoplasmic EndoU which implied that cytoplasmic EndoU was augmented to be more effective than nuclear EndoU, in cell lysates, or the nuclear EndoU was inhibited in these lysates. To determine if there was a difference in the cytoplasmic EndoU complex compared to the nuclear EndoU complex, EU-HA was immunoprecipitated, and given the 50-mer substrate, from either the nuclear or cytoplasmic fraction. Since EndoU localized mostly to the nucleus, it was confirmed that equivalent amounts of EU-HA were IP'd from each fraction (Figure 8C). EU-HA IP'd from each fraction showed that there was no difference between the extent of cleavage between each fraction (Figure 8D). Initial reaction rates also confirmed that there was no difference between the two fractions (Figure 8E). Taken together, these results indicated that EndoU had higher activity in the cytoplasm, but this higher activity was not due to the EndoU complex.

EndoU interacts with a large-transient cofactor in the cytoplasm

Since EndoU appeared to be more active in cytoplasmic lysate when compared to immunoprecipitated EndoU this suggested that there was something in the lysate that was positively regulating EndoU but was not part of the EndoU complex. To identify if cytoplasmic EndoU had higher activity from a cofactor present in the cytoplasm, EU-HA was IP'd, treated with 5 mM calcium, and given various treatments of KO cytoplasmic lysate with cytoplasmic RNA used as substrate. First, to determine if a cytoplasmic

specific cofactor was present, and to what extent this cofactor was needed to activate EndoU, IP'd EU-HA was treated with cytoplasmic lysate at a low (5% lysate reaction volume) and high (90% reaction volume) concentration. Compared to the untreated control (no lysate added), the amount of RNA degradation seen in either amount of lysate addition, was higher due to more RNA entering the gel when any amount of cytoplasmic lysate was present (Figure 9A lanes 1-6). This cofactor was likely to be transiently interacting as it was not pulled down with EndoU and a low concentration was sufficient to increase EndoU activity. To further characterize the cofactor, cytoplasmic lysate was fractionated into low and high molecular weight, with a cutoff of 10 kDa, to determine if the relative size of the cofactor was a macromolecule or a small molecule. Interestingly, the macromolecule lysate instilled greater activity to EndoU than the small molecule lysate (Figure 9A lanes 8-10). To further validate this observation, cytoplasmic lysates were de-salted to show that buffering conditions did not alter the activity of EndoU (Figure 9A lanes 11-12). This showed that a large molecular weight cofactor was present in the cytoplasmic portion of the cell that was likely contributing to the high activity of EndoU seen in the cytoplasm.

Because 5 mM calcium was inhibitory when a specific substrate was used on IP'd EndoU (Figure 4G), it was necessary to determine if the calcium concentration was too high and the addition of, even a small amount, of cytoplasmic lysate was reducing the calcium concentration through calcium pumps of ER microsomes. If calcium levels were too high

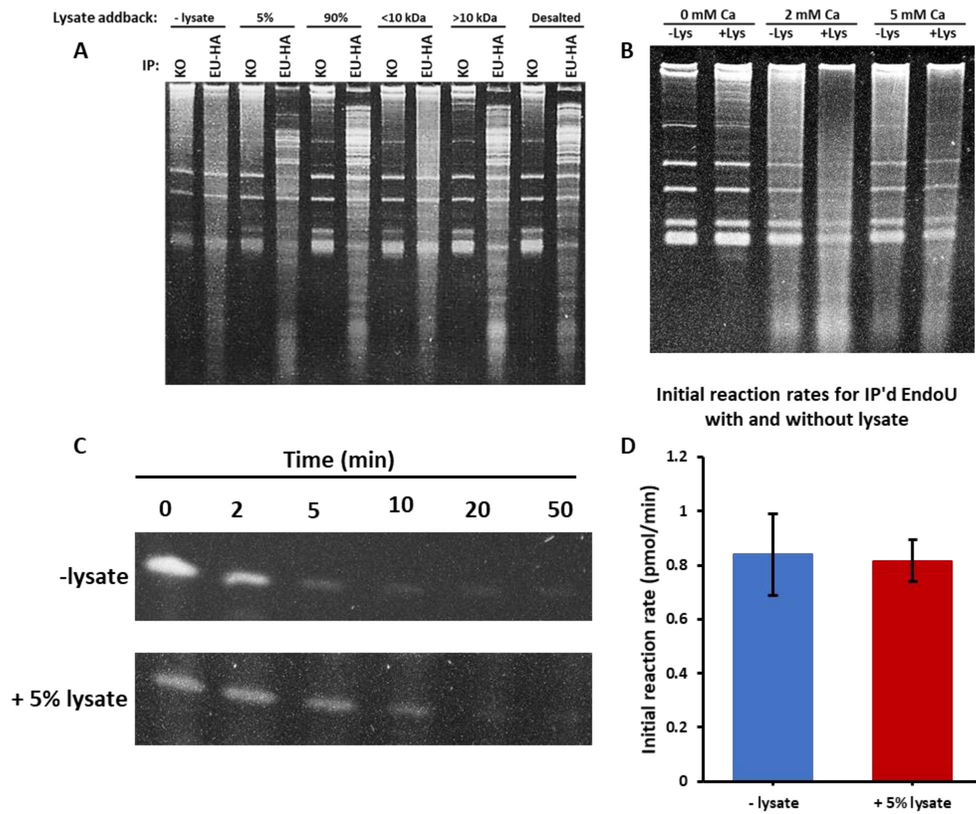

Figure 9: A large transiently interacting cofactor increases EndoU cytoplasmic activity
(A) Conditioned knockout cytoplasmic lysate added back to IP'd EU-HA in 5 mM calcium with cytoplasmic RNA as substrate. Percentages indicate the amount of lysate in the reaction volume. (B) 5% lysate addback in various calcium concentrations. (C) Representative images of nuclease assays on IP'd EU-HA with and without 5% KO lysate addback. (D) Quantification of initial reaction rates of IP'd EU-HA nuclease assays (n=3). Error bars represent standard error of the coefficient.

and being lowered by the lysate to a level that EndoU was highly active in, then at its optimal concentration, of 2 mM calcium, it would have lower activity at 2 mM than at 5 mM calcium. It was observed that when 5% lysate was added to IP'd EndoU and at either concentration of calcium, the activity was higher than no lysate added based on the amount of band degradation seen. Furthermore, the activity seen in lysate added back at 2 mM calcium was even higher than the degradation seen at 5 mM calcium with lysate added back based on the loss of the 5.8S and 5S RNAs (Figure 9B). This indicated that altered levels of calcium were not contributing to the enhanced activity endowed on EndoU by the lysate.

To quantify the activity enhancement by the cytoplasmic lysate, the specific 50mer was used in lieu of cytoplasmic RNA as a substrate (Figure 9C). Interestingly, the 50mer substrate was not cleaved at any rate higher in the presence or absence of cytoplasmic lysate (Figure 9D). This indicated that, because the only difference between the reactions in figures 9B and 9C was the substrate, somehow the cofactor was altering EndoU's preferred RNA substrates.

DISCUSSION

Knocking out EndoU in the VL3-3M2 cell line showed a molecular phenotype that was activated by calcium. Immunoprecipitations of EndoU followed by RNase assays provided framework to biochemically characterize the enzyme. Using these assays allowed for the characterization of the optimal environment for EndoU RNA RNase

activity, regulation through the subcellular local activity, and modulation by calcium. From these experiments, insight into how EndoU was regulated could be drawn.

To determine EndoU's preferred substrate, the optimum environment for EndoU activity was characterized. While no preference for a monovalent salt is found, it is likely that a minimal amount of NaCl or KCl is necessary for EndoU activity, this result may have been hidden since the IP wash buffer contains 100 mM NaCl and may contribute some amount of sodium to the overall monovalent salt concentration in the final reaction. In either case, a minimal requirement for monovalent salts is not observed however there is a slight preference for Na^+ at higher concentrations compared to K^+. The concentration of extracellular Na^+ ions is greater than intracellular Na^+ ions at around 150 mM to 5 mM, respectively, and the concentration of extracellular K^+ ions is lower than intracellular ions at around 5 mM to 150 mM, respectively. Taking these differential concentrations into account means that EndoU can likely function intracellularly or extracellularly.

EndoU's preference for a broad range of pHs suggests that it can be active within a few organelles of the cell. The high activity in low pH indicates that EndoU is capable of cleavage in the lysosome, however there would be no RNAs present within this organelle under normal circumstances. However, if a cell is virally infected, the virus can enter through the endocytic pathway. As endosomes mature, they become increasingly acidic until they fuse with the lysosome. The lysosome can have up to 500 μM calcium (Christensen et al., 2002; Lloyd-Evans et al., 2008). Viruses can escape the endocytic

pathway at any stage; however, some viruses will escape endocytosis at the lysosomal or even the autophagosomal stage (Berryman et al., 2012; Suikkanen et al., 2002). It is tempting to imagine that EndoU could destroy ssRNA viral genomes, if the virus enters the cell in this manner, however it is not clear how EndoU would have access to the genome since it would be surrounded by the viral envelope or nucleocapsid.

It has been previously shown that recombinant human EndoU and XendoU was able to cleave a radiolabeled snoRNA substrate in the presence of 5 mM manganese (P. Laneve et al., 2008), however, calcium was not included in these assays. Later, it was shown that recombinant XendoU had higher activity and produced more specific products when calcium was used as a cofactor, compared to manganese, if the substrate was total RNA from *X. laevis* egg extract (Schwarz & Blower, 2014). Based on the evidence presented here, mammalian EndoU uses calcium as a cofactor. While other divalent metals can slightly influence EndoU's activity, calcium is the preferred cofactor for mammalian EndoU which causes EndoU to degrade RNAs in a non-specific manner *in vitro*.

Through directed mutations in suspected calcium binding sites, these experiments suggest that calcium activates EndoU in an allosteric manner rather than taking part in the catalytic reaction. Calcium being likely used as an allosteric regulator is interesting since this could explain why other divalent metals or no divalent metals regulate EndoU outside the eukaryotic domain which can be seen with the severe acute respiratory syndrome (SARS) coronavirus EndoU which utilized manganese as a cofactor (Bhardwaj

et al., 2004; Ivanov et al., 2004) and the bacterial homolog to EndoU, which does not require a cofactor for activity (Michalska et al., 2018). The mutation in E358 causes complete loss of EndoU activity (Figure 7E) and in the alignment the acidic residue is conserved between eukaryotes and coronavirus (Figure 6 red arrow; position 220), this may indicate that this residue is able to generally bind divalent metals with another interacting sub-domain providing specificity of the divalent metal. While D153N does not completely abolish catalytic activity, the reduction, when compared to WT EndoU, is substantial. D153 could be part of the larger calcium binding pocket and a larger deletion of this region is necessary to determine important residues of the calcium binding pocket. To solidify this result, it would be possible to titrate the amount of calcium with the D153N mutant which, if shown that higher activity is seen in the higher concentrations of calcium, then this would indicate that mutation of this residue leads to decreased ability for EndoU to bind calcium.

These experiments show that EndoU is regulated through localization. EndoU localizes mainly to the nucleus but high RNase activity is seen in the cytoplasm, where EndoU levels are relatively low. The cytoplasmic activity is EndoU dependent, and, through immunoprecipitation experiments, this RNase activity can be attributed to EndoU. Interestingly, the predicted locale of EndoU is extracellular due to its N-terminal signal peptide and, further support for this is, the presence of two disulfide bond rich somatomedin B domains. The signal peptide would indicate that EndoU is co-translationally transported into the ER whereupon disulfide bridge formation would take

place. While it is still possible that EndoU can take the secretory pathway to some extent, it is shown from fractionation immunoblotting for EndoU showing nuclear localization, secretion is not preferred. This indicates that EndoU is spatially regulated within the cell, but it does not show how this regulation occurs. This leads to the options that EndoU is inhibited in the nucleus and/or activated in the cytoplasm. It is found that this high cytoplasmic activity is due to a large-transiently interacting cofactor, but it is not clear as to how this cofactor affects EndoU. While cytoplasmic RNA, used as a substrate, shows much more degradation in the presence of the cofactor, EndoU in the presence of a specific substrate and the cofactor, does not show this enhanced activity. The enhanced activity when cytoplasmic RNA is used as a substrate, but not when the 50mer is used, indicates that the targets of EndoU are broadened in a specific manner. In this scenario, EndoU can generally cleave all substrates at a certain rate but when the cofactor is present, EndoU is able to have a higher rate of cleavage on certain RNAs while maintaining its basal activity on others. Given the results, it is likely that the cofactor is also an RNA binding protein with an ability to guide EndoU to certain target RNAs. Regardless of how the cofactor is augmenting EndoU, this regulation also implies that EndoU is involved in a specific cytoplasmic pathway.

The most likely pathway that EndoU is involved in is the apoptotic pathway. EndoU has been shown to promote apoptosis in autoreactive B-cells (Poe et al., 2014). Autoreactive B cells likely undergo apoptosis through intrinsic apoptosis since mice that lack Fas expression but express autoantigen specific B-cell receptors were able to eliminate their

B-cells as well as WT mice (J. C. Rathmell & Goodnow, 1994). This indicates that apoptosis is occurring through the intrinsic pathway, and would thus utilize calcium as an effector and, because EndoU is calcium dependent, it is likely that EndoU promotes apoptosis the calcium signaling event of the intrinsic pathway.

MATERIALS AND METHODS

Cell culture

VL3-3M2 cells were cultured in RPMI 1640 (Corning) supplemented with 10 mM HEPES, 50 μM b-mercaptoethanol, 1X penicillin and streptomycin, and 10% FBS. Platinum-E (Plat-E) retroviral packaging cell line was cultured in DMEM (Corning) supplemented with 10% fetal bovine serum (Corning) and 10 units/ml of penicillin/streptomycin (Gibco). All cells were grown at 37 ^{o}C with 5% CO_2.

EndoU knockout cell generation

EndoU KO cells were generated as described (Sternburg et al., 2017) sgRNAs designed to target intron 1 and exon 11 of the EndoU locus were cloned into the pLx330 Cas9-Zhang sgRNA expression plasmid. A neomycin resistance cassette flanked by two 900 bp homology regions to intron 1 and exon 11 were assembled into the pUC-19 vector as previously described (Sternburg et al., 2017). The pLx330 Cas9-sgRNA expression plasmids and pUC-19 vector were electroporated into 1 X 10^7 VL3-3M2s at 340V for 47 milliseconds in Opti-MEM (Gibco). Neomycin selection was applied after two days. Clonal cells were generated and screened using primers flanking the intron 1 cut site.

EndoU tagged and mutant constructs

EndoU was PCR amplified from VL3-3M2 cDNA with primers containing XhoI and BglII cut sites and cloned into the pMSCV-PIG vector. The Q5 site-directed mutagenesis

kit (NEB) was used, according to the manufacturer's protocol, to add a C-terminal FLAG-HA tag, in pMSCV-PIG.

To mutate suspected calcium binding residues or control residues by subcloning EndoU-FLAG-HA into the pRL-TK. Mutations were made using the Q5 site-directed mutagenesis kit (NEB). Mutant EndoUs were then cloned back into pMSCV-PIG.

Viral production and stable integration of EndoU

VL3-3M2 clonal EndoU knockouts were rescued through viral integration of EndoU. Plat-E cells were calcium phosphate transfected with amphitropic, VSVG, pseudotyped retrovirus was produced in Plat-E cells through using calcium phosphate with 10 µg of pMSCV-PIG and 2.5 µg VSVG. Viral media was taken and used to infect 10^5 VL3-3M2 KOs using 30% viral media, 70% fresh media, and 20 µg/ml polybrene. Viral media and cells were immediately spun at 900 X g for 1 hour. Two days post transduction, cells were treated with 10 µg/ml puromycin for seven days.

RNA extractions

Total, cytoplasmic, or nuclear RNAs were extracted from whole cells, cytoplasmic, or nuclear fractions using 500 µl ribozol followed by two phenol chloroform (pH 5.2) extractions.

RT-qPCR

Superscript II reverse transcriptase was used for cDNA synthesis with 1 μg of total RNA as template. Taq-man with appropriate probes against EndoU (Cat. 4351372) were used in the RT-qPCR.

Immunoblotting

Whole cell, cytoplasmic fractions, or nuclear fractions were lysed or resuspended using SDS gel-loading buffer composed of 50 mM Tris-Cl (pH 6.8), 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, and 100 mM dithiothreitol. Proteins were separated by 10% SDS-PAGE, followed by transfer to 0.2 μm nitrocellulose membrane (Bio-Rad). The membrane was then probed with antibody; 1:1000 diluted anti-HA.11 (BioLegend), 1:500 diluted anti-lamin A (Sigma), or 1:1000 diluted anti-α-tubulin (Sigma). Membrane was washed using TBST composed of 25 mM Tris, 137 mM NaCl, 3 mM KCl and 0.1% (w/v) Tween-20, pH 7.4. The blot was then probed with 1:5000 diluted horseradish peroxidase-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology). The bound antibody was detected by radiance plus (azure biosystems) and imaged using a Bio-Rad ChemiDoc XRS+ imager.

Denaturing PAGE

2 μg of cytoplasmic/nuclear RNA or 10 pmol of 50mer RNA substrate was diluted in formamide-loading buffer composed of 80% (w/v) formamide, 10 mM EDTA (pH 8.0), 1 mg/ml xylene cyanol, 1 mg/ml bromophenol blue. RNAs were run on an 8% 7.5 M urea

polyacrylamide gel in 0.5X TBE. RNA bands were detected by SYBR green II (Sigma) and imaged on a UVP GelDoc-it Imaging System.

Cell lysis

Cell lysis was performed by washing cells once in 1X PBS and resuspended in hypotonic lysis buffer (10 mM KCl,). Cells were incubated on ice for 20 minutes. Isotonicity was achieved by bringing the KCl concentration to 100 mM. If the lysates were to be fractionated, then cells were centrifuged at 17000 X g for 20 minutes and the cytoplasmic fraction was separated from the nuclear fraction. The nuclear fraction was then treated with nuclear lysis buffer (400 mM NaCl, 5 mM Tris-Cl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) and incubated at room temperature for 20 minutes. Lysate was then spun at 17000 X g for 20 minutes to remove debris.

Immunoprecipitations:

Immunoprecipitations were carried out using Dynabeads protein-A. Beads were prepped with 16.7 μg/ml rabbit against mouse IgG (Fcγ) bridging antibody and 16.7 μg/ml (m α HA.11), sequentially. Lysate was exposed to prepped beads for 1 hour at room temperature.

RNase assays:

In a total volume of 10 μl reactions consisted of, unless indicated, 2 mM calcium, 100 mM Tris-Cl (pH 7.5), 10 mM NaCl, 1 μM 50mer or 2 μg cytoplasmic/nuclear RNA.

Beads were not calculated into the total reaction volume. Densitometry was used to quantify bands degradation using Quantity One (BioRad).

EndoU domain alignments

EndoU seed domains from bacteria, nidovirus (nsp15), and eukaryotes were taken from pFAM alignments (Mistry et al., 2021). Clustal X was used to align the bacterial domain to the C-terminal domain of the eukaryotic EndoU identified by Michalska et al. The N-terminal regions of the eukaryotic and viral EndoUs were aligned separately to determine if there was any conservation. As there was no conservation, the N-terminal domains of viral and eukaryotes were stitched back on the bacterial domain alignments, respectively, using BioEdit (Hall, 1999). Species were culled to only the ones shown and imported into ESPript 3.x to generate the figure (Robert & Gouet, 2014).

REFERENCES

Berryman, S., Brooks, E., Burman, A., Hawes, P., Roberts, R., Netherton, C., Monaghan, P., Whelband, M., Cottam, E., Elazar, Z., Jackson, T., & Wileman, T. (2012). Foot-and-mouth disease virus induces autophagosomes during cell entry via a class III phosphatidylinositol 3-kinase-independent pathway. *J Virol, 86*(23), 12940-12953. doi:10.1128/JVI.00846-12

Bhardwaj, K., Guarino, L., & Kao, C. C. (2004). The severe acute respiratory syndrome coronavirus Nsp15 protein is an endoribonuclease that prefers manganese as a cofactor. *J Virol, 78*(22), 12218-12224. doi:10.1128/JVI.78.22.12218-12224.2004

Christensen, K. A., Myers, J. T., & Swanson, J. A. (2002). pH-dependent regulation of lysosomal calcium in macrophages. *Journal of Cell Science, 115*(3), 599.

Clapham, D. E. (2007). Calcium Signaling. *Cell, 131*(6), 1047-1058. doi:https://doi.org/10.1016/j.cell.2007.11.028

Groves, T., Katis, P., Madden, Z., Manickam, K., Ramsden, D., Wu, G., & Guidos, C. (1995). In vitro maturation of clonal CD4+CD8+ cell lines in response to TCR engagement. *The Journal of Immunology, 154*, 5011-5022.

Hall, T. A. (1999). BioEdit : a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser., 41*, 95-98.

Ivanov, K. A., Hertzig, T., Rozanov, M., Bayer, S., Thiel, V., Gorbalenya, A. E., & Ziebuhr, J. (2004). Major genetic marker of nidoviruses encodes a replicative endoribonuclease. *Proc Natl Acad Sci U S A, 101*(34), 12694-12699. doi:10.1073/pnas.0403127101

Laneve, P., Gioia, U., Ragno, R., Altieri, F., Di Franco, C., Santini, T., Arceci, M., Bozzoni, I., & Caffarelli, E. (2008). The tumor marker human placental protein 11 is an endoribonuclease. *J Biol Chem, 283*(50), 34712-34719. doi:10.1074/jbc.M805759200

Lloyd-Evans, E., Morgan, A. J., He, X., Smith, D. A., Elliot-Smith, E., Sillence, D. J., Churchill, G. C., Schuchman, E. H., Galione, A., & Platt, F. M. (2008). Niemann-Pick disease type C1 is a sphingosine storage disease that causes deregulation of lysosomal calcium. *Nat Med, 14*(11), 1247-1255. doi:10.1038/nm.1876

Martinez, J. A., Zhang, Z., Svetlov, S. I., Hayes, R. L., Wang, K. K., & Larner, S. F. (2010). Calpain and caspase processing of caspase-12 contribute to the ER stress-induced cell death pathway in differentiated PC12 cells. *Apoptosis, 15*(12), 1480-1493. doi:10.1007/s10495-010-0526-4

Michalska, K., Quan Nhan, D., Willett, J. L. E., Stols, L. M., Eschenfeldt, W. H., Jones, A. M., Nguyen, J. Y., Koskiniemi, S., Low, D. A., Goulding, C. W., Joachimiak, A., & Hayes, C. S. (2018). Functional plasticity of antibacterial EndoU toxins. *Mol Microbiol, 109*(4), 509-527. doi:10.1111/mmi.14007

Mistry, J., Chuguransky, S., Williams, L., Qureshi, M., Salazar, G. A., Sonnhammer, E. L. L., Tosatto, S. C. E., Paladin, L., Raj, S., Richardson, L. J., Finn, R. D., & Bateman, A. (2021). Pfam: The protein families database in 2021. *Nucleic acids research, 49*(D1), D412-D419. doi:10.1093/nar/gkaa913

Poe, J. C., Kountikov, E. I., Lykken, J. M., Natarajan, A., Marchuk, D. A., & Tedder, T. F. (2014). EndoU is a novel regulator of AICD during peripheral B cell selection. *J Exp Med, 211*(1), 57-69. doi:10.1084/jem.20130648

Rathmell, J. C., & Goodnow, C. C. (1994). Effects of the lpr mutation on elimination and inactivation of self-reactive B cells. *The Journal of Immunology, 153*(6), 2831.

Robert, X., & Gouet, P. (2014). Deciphering key features in protein structures with the new ENDscript server. *Nucleic acids research, 42*(Web Server issue), W320-324. doi:10.1093/nar/gku316

Schwarz, D. S., & Blower, M. D. (2014). The calcium-dependent ribonuclease XendoU promotes ER network formation through local RNA degradation. *J Cell Biol, 207*(1), 41-57. doi:10.1083/jcb.201406037

Sternburg, E. L., Dias, K. C., & Karginov, F. V. (2017). Selection-dependent and Independent Generation of CRISPR/Cas9-mediated Gene Knockouts in Mammalian Cells. *J Vis Exp*(124). doi:10.3791/55903

Suikkanen, S., Sääjärvi, K., Hirsimäki, J., Välilehto, O., Reunanen, H., Vihinen-Ranta, M., & Vuento, M. (2002). Role of recycling endosomes and lysosomes in dynein-dependent entry of canine parvovirus. *Journal of virology, 76*(9), 4401-4411. doi:10.1128/jvi.76.9.4401-4411.2002

Zhou, W., Tang, G. W., & Altman, R. B. (2015). High Resolution Prediction of Calcium-Binding Sites in 3D Protein Structures Using FEATURE. *J Chem Inf Model, 55*(8), 1663-1672. doi:10.1021/acs.jcim.5b00367

CHAPTER 2

EndoU activity is highly attenuated when activated in VL3-3M2s but promotes the expression of pro-apoptotic genes

ABSTRACT

Because mammalian EndoU was found to be calcium activated, it was likely involved in apoptosis. Although EndoU did was not found to exhibit a phenotype in the VL3-3M2 cell line, it was shown to be active. Through RNA sequencing of resting and apoptotic VL3-3M2s, it was discovered that EndoU was upregulating genes involved in apoptosis and calcium homeostasis. Further characterization of EndoU's complex revealed that it likely bound to proteins involved in apoptosis, RNA binding, and cytoskeletal arrangement. While a phenotype was not observable in this cell line, a potential apoptotic role for EndoU was likely based on the characteristics of EndoU regulated pathways and the EndoU complex.

INTRODUCTION

In the previous chapter, EndoU was biochemically characterized to elucidate the possible cellular role. While the biochemistry showed that EndoU activity could function in many aspects of the cell, the high regulation and dependence on calcium indicated that its cellular role is through a calcium dependent pathway. The EndoU molecular phenotype showed that RNA degradation occurred when EndoU and calcium were present. While

the phenotype did not directly show that EndoU was activated by calcium, immunoprecipitation experiments made it clear that calcium directly activated EndoU, and no other divalent metal tested could increase catalytic activity. Furthermore, high activity was dependent on localization in the cytoplasm due to a relatively large cofactor.

The apoptotic pathway can use calcium as a secondary messenger because of the loss of homeostatic control of calcium in endoplasmic reticula or mitochondria (Jayaraman & Marks, 1997). Since EndoU is only expressed in the cells of tissue that will undergo cell death and the apoptotic pathway utilizes calcium, it is likely that EndoU promotes apoptosis in the cells where it is expressed.

EndoU cleaves RNAs to produce 2',3'-cyclic phosphate and 5' OH termini (Pietro Laneve et al., 2003). In yeast, 5'-OH mRNAs are recognized by the Trl1 kinase to phosphorylate this terminus. Following this phosphorylation event exoribonucleases, Xrn1 or Dxo1, can efficiently degrade the cleaved transcripts (Navickas et al., 2020). The targets of mammalian EndoU of have not yet been identified so it is unclear how its activity affects the transcriptome however it is thought that EndoU would downregulate genes based on the production of 5'-OH RNAs.

In this chapter, it was shown that the VL3-3M2 cell line did not exhibit an EndoU dependent phenotype. But the utilization of the 5'-OH allowed for detecting EndoU activity in whole cells by radiolabeling 5' ends. Even though EndoU activity was highly

attenuated, RNA-sequencing revealed that EndoU positively regulated pro-apoptotic genes. Through mass spectrometry it was further supported that EndoU could bind a variety of proteins that were involved with the cytoskeleton, RNA, and apoptosis.

RESULTS

EndoU slightly affects apoptosis in VL3-3M2 thymocytes

Since apoptosis is highly dependent on calcium and EndoU is capable of cleaving RNAs in the presence of calcium *in vitro*, it could affect the regulation of programmed cell death. To determine if EndoU influenced apoptosis in VL3-3M2s, wildtype (WT) and knockout (KO) clones were treated with either DMSO (vehicle) or 50 nM staurosporine, a general kinase inhibitor, for 6 hours and cell death was determined through the identification of cells double positive for 7AAD and annexin V (Figure 10A left). Under basal conditions, there was no difference between WT and KO cells (figure 10A right). Staurosporine significantly increased cell death in both the WT and KO clones (Figure 10B left). Under staurosporine treatment, there was a trend towards less cell death in Kos however this trend was not significant (figure 10B right). While the effect of EndoU on apoptosis was not significant, the trend was interesting and further investigated.

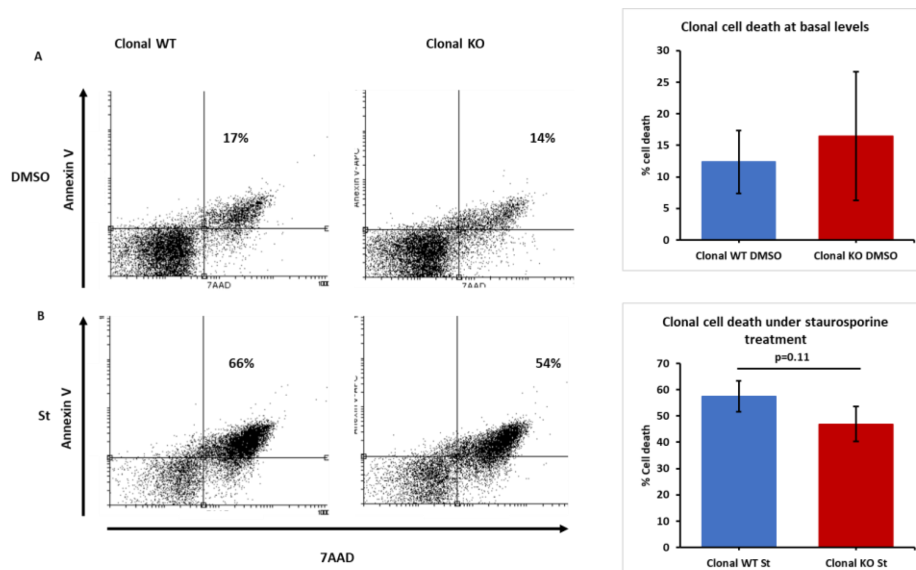

Figure 10: EndoU slightly lowers apoptosis in actively dying VL3-3M2 thymocytes
(A) Left: representative flow cytometry data of clonal VL3-3M2s treated with the vehicle control (DMSO). Percentages show annexin-V^+, $7AAD^+$ cells undergoing secondary necrosis. Right: Cell death of clonal WT or KO cells from 3 separate lines. (B) Left: representative flow cytometry from clonal VL3-3M@s treated with 0.05 µM staurosporine. Right: Cell death of clonal WT or KO cells from 3 separate lines treated with staurosporine. Error bars represent SEM. One tailed unpaired students T-test.

EndoU is active, but highly attenuated, in cells undergoing apoptosis

To support the hypothesis that EndoU could promote apoptosis in thymocytes, it was necessary to observe EndoU activity while cells were undergoing apoptosis. Apoptosis or calcium signaling was induced in VL3s using staurosporine or ionomycin, respectively. Ionomycin is a calcium ionophore that creates pores in plasma membranes that preferentially allow calcium through (Bennett et al., 1979). Since the calcium concentration within a cell's cytoplasm is much lower than the extracellular medium, calcium will enter the cell independent of apoptosis. In previous experiments done on cell lysate, the amount of EndoU dependent cleavage was extremely high in the presence of calcium (Figure 3A) however the amount of degradation seen in whole cells, due to EndoU activity was not observable, on a SYBR green II stained denaturing PAGE, when

ionomycin was used to allow calcium signaling to occur (Figure 11A). Since SYBR green II staining would only show major RNA species, it was possible that EndoU was active, however, the detection sensitivity was too low to observe EndoU dependent products. Because EndoU creates 5'-OH termini, aa more sensitive assay could be performed by phosphorylating the 5'-OH ends with a P^{32} containing phosphate through a polynucleotide kinase reaction. Total. Total RNA was extracted followed by radiolabeling of 5'-OH RNA species to observe any differences between WT and KO RNAs, which would indicate EndoU activity and likely direct targets (Figure 11A). Since observing radiolabeled RNAs was a sensitive technique that could show differences in clonal cells, three different clonal Kos were used as controls to compare banding patterns with WT radiolabeled RNAs. Under no treatment novel 5'-OH RNA bands were not produced. Although band intensities were not equivalent, this could have been due to clonal variability. Since no new bands were formed, this would indicate that EndoU was either not active under basal conditions or the sensitivity of this assay was not high enough. Under staurosporine or ionomycin treatment, however, EndoU dependent products could be seen since they were not present in any of the clonal Kos (Figure 11B boxes). This experiment showed that EndoU was active when calcium was released during apoptosis in cells as well as limited in its activity to a few specific substrates.

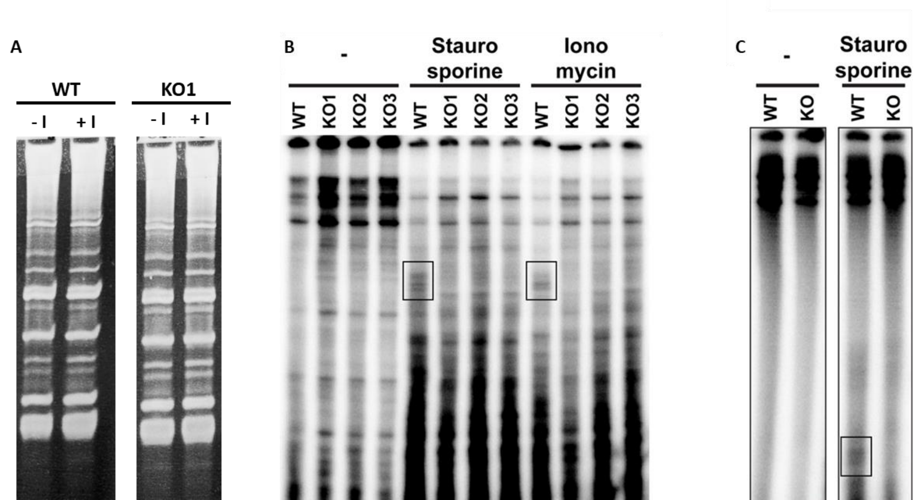

Figure 11: EndoU is cleaves RNAs during apoptosis and calcium signaling
(A) Total RNA from whole cells either untreated or treated with ionomycin (B) 5'OH radiolabeled total RNA from untreated, staurosporine, and ionomycin treated WT and three KO clonal cell lines. (B) 5'-OH radiolabeled poly-A selected RNAs from untreated and staurosporine treated WT and KO cells.

To further characterize which RNA species were affected by EndoU, WT and KO VL3s were treated with staurosporine, total RNA was extracted and then poly-A purified, followed by radiolabeling to determine if mRNAs could be affected by EndoU activity10B. Between WT and KO VL3s in the untreated condition, there were no differences in newly formed 5'-OH poly-A RNAs. During staurosporine treatment, however, EndoU dependent bands were formed (Figure 11C box). This experiment showed that EndoU affected poly-A RNAs, most of which are composed of mRNAs, during apoptosis and thus could be affecting protein coding genes through cleavage of mRNAs.

EndoU affects protein coding genes involved in apoptosis and calcium homeostasis

The likely outcome of mRNA cleavage is degradation of the transcript. If EndoU destabilized mRNAs through cleavage, then it would be shown in the transcriptome. To investigate if EndoU was altering potential pathways, RNA-seq was performed on untreated and staurosporine treated WT and KO VL3s for 3 hours (Figure 12A). To reduce clonal phenotypic variation, two KO clones were used in this experiment. Counts of differentially expressed genes (DEGs) showed that when untreated WT and KO cells were compared to their treated counterparts, ~1400 genes were differentially expressed and therefore indicating a change in cell state occurred (Figure 12B top rows). When comparing KO to WT in either treatment case, there were much fewer DEGs, at ~50 genes, and most of the DEGs were downregulated (Figure 12B bottom rows). The relatively high number of downregulated DEGs was surprising given that EndoU cleaves RNA, however the 2’3’ cyclic phosphate termini of EndoU cleavage products may allow them to persist after cleavage. The low number of DEGs may be due to the high attenuation of EndoU within whole cells as seen by the low amount of newly formed products in figure 11. Based on gene ontology (GO) enrichment, when compared to WT VL3s, Kos had decreased expression of genes involved in apoptosis regardless of treatment as seen by GO categories enriched for signaling in response to DNA damage by p53 genes and lymphocyte apoptotic process (Figures 12C, 12D and tables 1, 3). The

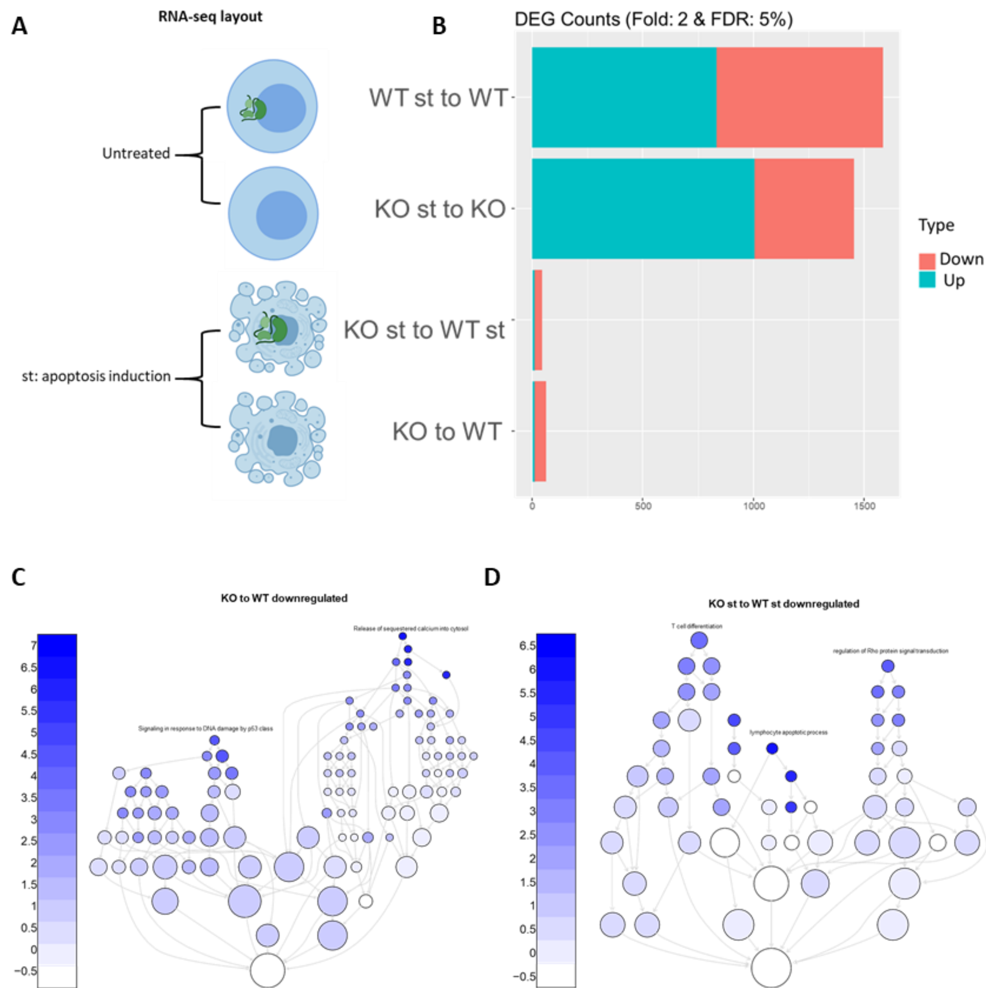

Figure 12: RNA-seq reveals EndoU upregulates genes involved in apoptosis
(A) Outline of the treatments performed on cells for RNA-seq. (B) DEGs in various comparisons of treated cells and genotype. (C) GO category enrichment in untreated cells. (D) GO category enrichment in staurosporine treated cells. St=staurosporine

downregulation of genes involved in signaling in response to DNA damage by p53 in the untreated case indicated that EndoU could be involved in priming cells for apoptosis. The down regulation of genes involved in lymphocyte apoptotic process in the staurosporine case likely meant that EndoU activity was increasing the abundance of these genes.

EndoU likely complexes with a variety of proteins involved in apoptosis, RNA binding, and cytoskeletal arrangement

To better understand a possible role for EndoU in apoptosis, it was important to determine the complex that EndoU was in. Rescued FLAG-HA tagged EndoU cells were used to immunoprecipitate EndoU from cytoplasmic and nuclear fractions with the parental KO cell line used as a control. Liquid chromatography followed by mass spectrometry (LC-MS) of the entire bead complex was performed. Log_2 fold enrichment of showed a variety of proteins enriched in the cytoplasm and/or the nucleus of EU-HA rescued cells (EU-HA cyt/nuc). EndoU was recovered in both the nuclear and cytoplasmic IP fractions to a relatively high level and was absent in the KO fractions (Figure 13C). In the cytoplasmic rescue IP fraction, many proteins that likely bound to EndoU were myosin and actin related (Figure 13A). Also found in EU-HA cyt, EndoU was likely binding to the thiol-proteases calpain 1 and 2 (CAN1, CAN2) which are positive regulators of ER stress induced apoptosis (Figure 13A, black arrows). In the

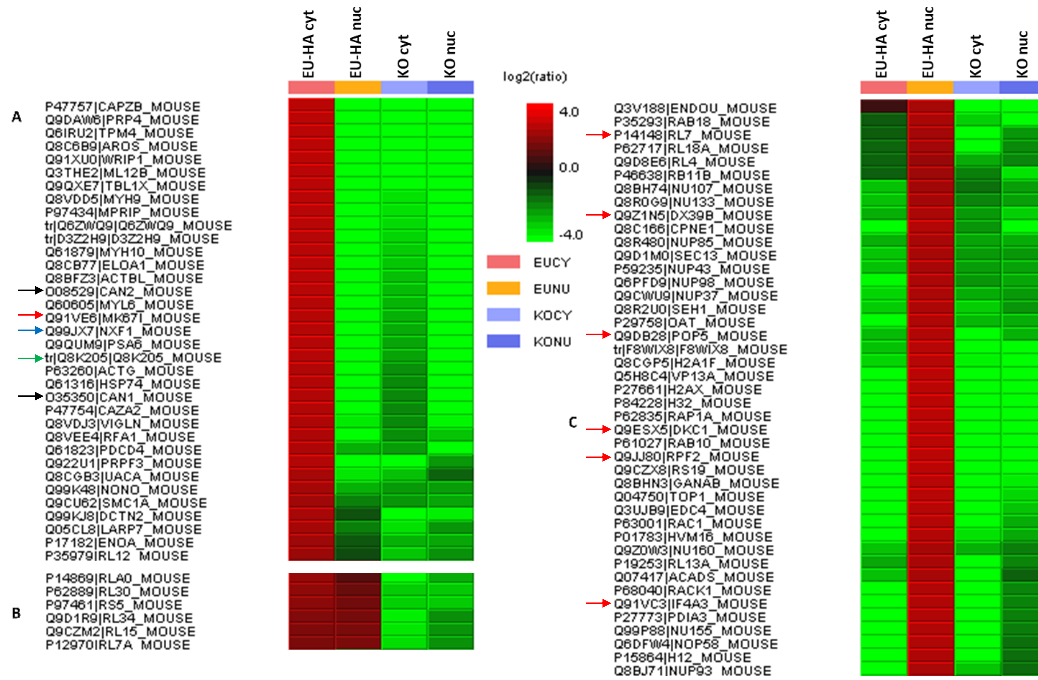

Figure 13: EndoU binds a variety of proteins in the cytoplasm and nucleus
Heat map showing log_2 ratio of proteins (>2 fold) likely binding to EndoU. (A) Proteins recovered which are exclusive to cytoplasmic EndoU. (B) Proteins recovered from both cytoplasmic and nuclear EndoU. (C) proteins recovered which are exclusive to nuclear EndoU (excluding EndoU).

cytoplasmic portion, EndoU was also likely binding RNA binding proteins (RBPs) mainly found in the nucleus that included MK67I, NXF1, and a subunit of the Pop1 RNP complex (Figure 13A red, blue, and green arrows, respectively). Furthermore, proteins that were found in both cytoplasmic and nuclear fractions that were likely binding EndoU were ribosomal proteins which could indicate that EndoU would be able to cleave ribosomal RNA (Figure 13B). The nuclear specific recovered proteins contained many nucleoporins or nucleoporin related proteins (Figure 13C). It was also observed that there were many more RBPs recovered from nuclear EndoU than cytoplasmic EndoU (Figure 13C red arrows). EndoU was therefore, likely binding to RBPs, perhaps to provide specificity to certain target RNAs.

DISCUSSION

In this chapter, EndoU was characterized at the cellular level. EndoU did not display a cellular phenotype in the VL3-3M2 cell line. At the basal level, EndoU KO cells did not undergo apoptosis at any appreciable rate less than the WT cells. Since this cell line is from a mouse thymoma, the reasoning behind why an EndoU dependent phenotype was not seen could be due to the nature of these cells. EndoU activity may be overcome by an undiscovered inhibitor within this cell line. The loss of EndoU, therefore, would not have a major impact on whether the cells would die at a slower rate or to a lesser extent. When treated with staurosporine, there was a trend towards apoptosis, but it was not significant. Therefore, there was a possibility that EndoU's pro-apoptotic function was highly diminished due to the nature of this cell line.

Since EndoU activity created unique products, 5'-OH radiolabeling was employed to determine a difference in banding patterns between WT and KO cells. While no differences were seen in the untreated case, under staurosporine treatment, distinct 5'-OH RNA species could be seen in an EndoU dependent manner. Since staurosporine caused apoptosis in these cells, as seen in figure 10, EndoU activity was likely attributable to the generation of these new 5'-OH RNA species. Interestingly, only a few new bands were observed. This is in line to what was observed in terms of the high amount of regulation put forth on EndoU. To further clarify if these newly formed RNA species were due to the calcium release during the apoptotic process or if this was due to an activating apoptotic factor, ionomycin was used to induce calcium signaling independent of apoptosis. The results showed that EndoU was active and the same sized RNA species were observed under ionomycin treatment that were seen under staurosporine treatment. This meant that EndoU activity was a direct result of the calcium signaling while these cells were undergoing apoptosis.

Calcium is the most prominent secondary messenger and signal transduction does occur outside the bounds of apoptosis. Many of these calcium regulated pathways regulate either cell growth/proliferation or cell death effected through calmodulin or calcinurin (reviewed in (Humeau et al., 2018)). Because there was a lack of, observable, EndoU activity in untreated cells this indicated that EndoU was either weakly active or not active within normal cycling VL3-3M2 cells (Figure 10). This cell line is highly proliferative,

so any calcium regulated pathways inducing cell growth or proliferation did not utilize EndoU activity. While it is possible that EndoU can be active during any calcium signaling event, the lack of activity seen in untreated cells, indicated that there weas high regulation put forth on EndoU that decreased or nullified EndoU activity in highly proliferative VL3-3M2s but during apoptosis, EndoU was active but still highly attenuated.

Regulation on EndoU activity, in whole cells, was relatively high when compared to lysate RNase assays (Figure 3). Surprisingly, the 5'-OH RNAs formed in an EndoU dependent manner were distinct and seen both when cells were undergoing apoptosis and when calcium signaling was high in these cells. The specific targets of EndoU cleavage are still unclear. While it has been shown that EndoU can process snoRNAs, *ex vivo*, in *X. laevis* oocytes, the activity was most likely attenuated due to the use of manganese as a cofactor and the use of calcium caused non-specific cleavage (Caffarelli et al., 1997). Other RNA endonucleases have been shown to have specific targets *in vivo*, such as IRE1 specificity for XBP1 and angiogenin on $tRNA^{glu}$ anticodon loop. Since, in this cell line, EndoU targets seemed to be very specific in the presence of calcium or during apoptosis, EndoU cleavage may rely on structure and U-specificity *in vivo*.

Because EndoU dependent bands were not observed in untreated cells, based on the 5'-OH radiolabeling of total RNA and poly-A RNA, changes in gene expression were not expected. Surprisingly, RNA-seq showed that EndoU affected gene expression in these

untreated cells. These effects in untreated cells were not likely due to EndoU's ribonuclease activity but if they were, then EndoU would likely have differential targets based on the concentration of calcium present. It is difficult to argue that EndoU can affect transcription, since it is not known if EndoU can bind to DNA. Gene ontology showed that genes involved in apoptosis and calcium homeostasis were downregulated in KO cells. This indicated that EndoU was important in the upregulation of these pro-apoptotic genes. This therefore indicates that EndoU may be multifunctional, and its non-catalytic activity may affect calcium homeostasis and the propensity for the cell to undergo apoptosis.

EndoU activity was seen on both poly-A and within total RNA in cells undergoing apoptosis. This implies that the transcripts changes seen in the RNA-seq could be affected by EndoU activity. These transcripts were mainly involved in apoptosis. When looking at the LC-MS results, the calpains were recovered. In neuronal cells, it has been shown that calpain 2 can cleave caspase 12 to induce apoptosis if cells are under ER stress (Martinez et al., 2010). Calpain 1 has been shown to promote caspase 3 activation by enhancing the release of cytochrome C through Bax cleavage in neutrophils and induce ER stress in the rat cardiomyocyte cell line, H9c2, to promote apoptosis (Altznauer et al., 2004; Zheng et al., 2015). The finding that EndoU upregulates genes involved in apoptosis and could bind both calpains, gives support to the role of EndoU in apoptosis. Since both EndoU and the calpains are calcium activated, they are likely activated by a calcium release at the same time. If EndoU binds calpain then it would be

interesting to determine how this interaction affects both enzymes. Since their substrates are quite different, it could be that one or both are inhibiting each other under basal calcium conditions while under calcium signaling, they bind calcium and are released from this inhibition.

While it was surprising that EndoU was highly attenuated in this cell line, the RNA-seq date and the likely EndoU complex show that EndoU is a good candidate for a pro-apoptotic protein.

MATERIALS AND METHODS

Cell culture

VL3-3M2 cells were cultured in RPMI 1640 (Corning) supplemented with 10 mM HEPES, 50 μM ß-mercaptoethanol, 10 U/ml penicillin/streptomycin, and 10% FBS.

Flow cytometry

Cells were stained with APC-annexin V at 1:200 (BioLegend). Annexin V staining was carried out in annexin V staining buffer composed of 10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$. 7AAD (bioLegend) was used at a concentration of 0.5 ng/ml.

Cell lysis

Cell lysis was performed by washing cells once in 1X PBS and resuspended in hypotonic lysis buffer (recipe). Cells were incubated on ice for 20 minutes. Isotonicity was achieved by bringing the KCl concentration to 100 mM. If the lysates were to be fractionated, then cells were centrifuged at 17000 X g for 20 minutes and the cytoplasmic fraction was separated from the nuclear fraction. The nuclear fraction was then treated with nuclear lysis buffer (400 mM NaCl, 5 mM Tris-Cl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) and incubated at room temperature for 20 minutes. Lysate was then spun at 17000 X g for 20 minutes to remove debris.

Poly-A RNA isolation:

The mRNA direct kit (Invitrogen) was used to isolate poly-A RNA from total RNA. Briefly, 100 µl of Dynabeads Oligo $(dT)_{25}$ were used to isolate mRNA from 75 µg of total RNA according to the manufacturer's protocol.

5'-OH Radiolabeling of RNAs:

Total RNA was extracted as previously described (see chapter 1 RNA extraction). 1 µg of RNA was used as a substrate for 1 U polynucleotide kinase in the presence of 0.167 µM ATP (γ-^{32}P) in a 1X buffer (70 mM Tris-HCl, 10 mM $MgCl_2$, and 5 mM DTT with a pH of 7.6). Radiolabeled RNAs were run on a 4% 7.5M urea polyacrylamide gel in 0.5X TBE.

RNA library preparation:

VL3-3M2 WT and two KO clones were either untreated or treated with 50 nM staurosporine for 3 hours. RNA was then extracted as previously described (see chapter 1, RNA extractions). The New England Biolabs NEBnext ultra RNA kit was used according to the manufacturers protocol using 1 µg of total RNA.

Immunoprecipitations

Immunoprecipitations were carried out using Dynabeads protein-A. Beads were prepped with 16.7 µg/ml rabbit anti-mouse Fcγ bridging antibody and 16.7 µg/ml mouse anti-

HA.11 (BioLegend), sequentially. Lysate was exposed to prepped beads for 1 hour at room temperature.

REFERENCES

Altznauer, F., Conus, S., Cavalli, A., Folkers, G., & Simon, H.-U. (2004). Calpain-1 Regulates Bax and Subsequent Smac-dependent Caspase-3 Activation in Neutrophil Apoptosis*. *Journal of Biological Chemistry, 279*(7), 5947-5957. doi:https://doi.org/10.1074/jbc.M308576200

Bennett, J. P., Cockcroft, S., & Gomperts, B. D. (1979). Ionomycin stimulates mast cell histamine secretion by forming a lipid-soluble calcium complex. *Nature, 282*(5741), 851-853. doi:10.1038/282851a0

Caffarelli, E., Maggi, L., Fatica, A., Jiricny, J., & Bozzoni, I. (1997). A Novel Mn++-Dependent Ribonuclease That Functions in U16 SnoRNA Processing inX.Laevis. *Biochemical and Biophysical Research Communications, 233*(2), 514-517. doi:https://doi.org/10.1006/bbrc.1997.6487

Humeau, J., Bravo-San Pedro, J. M., Vitale, I., Nunez, L., Villalobos, C., Kroemer, G., & Senovilla, L. (2018). Calcium signaling and cell cycle: Progression or death. *Cell Calcium, 70*, 3-15. doi:10.1016/j.ceca.2017.07.006

Jayaraman, T., & Marks, A. R. (1997). T cells deficient in inositol 1,4,5-trisphosphate receptor are resistant to apoptosis. *Molecular and Cellular Biology, 17*(6), 3005. doi:10.1128/MCB.17.6.3005

Laneve, P., Altieri, F., Fiori, M. E., Scaloni, A., Bozzoni, I., & Caffarelli, E. (2003). Purification, Cloning, and Characterization of XendoU, a Novel Endoribonuclease Involved in Processing of Intron-encoded Small Nucleolar RNAs in Xenopus laevis*. *Journal of Biological Chemistry, 278*(15), 13026-13032. doi:https://doi.org/10.1074/jbc.M211937200

Martinez, J. A., Zhang, Z., Svetlov, S. I., Hayes, R. L., Wang, K. K., & Larner, S. F. (2010). Calpain and caspase processing of caspase-12 contribute to the ER stress-induced cell death pathway in differentiated PC12 cells. *Apoptosis, 15*(12), 1480-1493. doi:10.1007/s10495-010-0526-4

Navickas, A., Chamois, S., Saint-Fort, R., Henri, J., Torchet, C., & Benard, L. (2020). No-Go Decay mRNA cleavage in the ribosome exit tunnel produces 5'-OH ends phosphorylated by Trl1. *Nat Commun, 11*(1), 122. doi:10.1038/s41467-019-13991-9

Zheng, D., Wang, G., Li, S., Fan, G. C., & Peng, T. (2015). Calpain-1 induces endoplasmic reticulum stress in promoting cardiomyocyte apoptosis following hypoxia/reoxygenation. *Biochim Biophys Acta, 1852*(5), 882-892. doi:10.1016/j.bbadis.2015.01.019

CHAPTER 3

EndoU does not affect apoptosis in death by neglect but plays a role in the negative selection of thymocytes

ABSTRACT

Previously, it was shown through biochemical characterization that EndoU is a calcium dependent and highly regulated endoribonuclease. In the thymocyte cell line, VL3-3M2, EndoU activity was highly attenuated but specific to a few targets in cells undergoing either apoptosis or when calcium signaling was present. Because most developing thymocytes die via either death by neglect, due to a nonfunctional T cell receptor, or negative selection, due to recognition of self-antigen, EndoU may play a role in these apoptotic processes. In this chapter, it will be shown that when comparing EndoU$^{+/+}$ mice to EndoU$^{-/-}$ mice, EndoU does not play a role in death by neglect nor does it influence the repertoire of mature T cells. But it will be shown that EndoU may promote negative selection by using EndoU WT and KO mice in an OT-II background. The purpose of which, would indicate that EndoU can prevent to a small extent, the escape of auto-reactive thymocytes from the thymus. The broader implication of EndoU promoting cell death during negative selection but not death by neglect is that EndoU is active in a specific apoptotic pathway that may be of importance in other tissues and even cancers.

INTRODUCTION

The thymus is a discrete bilobed organ, surrounded by a dense membranous capsule. Each lobe is further organized into lobules containing two microenvironments: the cortex, outer zone, and the medulla, inner zone. This organ is the site where the majority of thymocyte development occurs. The maturation of these thymocytes is paramount to the adaptive immune system by producing mature non-effector T cells and eliminating self-reactive thymocytes. Lymphoid progenitor cells (LPCs), derived from bone marrow, enter the thymus through postcapillary venules located between the cortex and medulla (Petrie et al. 2007, Foss et al. 2001). The LPCs migrate to the cortex where they undergo a series of differentiation processes with the main goal of developing a functional and non-self-reactive T cell receptor (TCR) with the vast majority of these TCRs consisting of an α- and ß-chain. The initial stages, termed double negative (DN) as they do not express the major TCR co-receptors, are most easily defined by the expression of CD44 and CD25 surface molecules followed by the double positive (DP) and single positive (SP) stages described as such based on the TCR co-receptors CD4 and CD8. As LPCs progress into the DN1 stage, they upregulate CD44 expression. In the DN2 stage, CD25 is also upregulated along with the recombinase activating genes, RAG1 and 2. It is at the DN2 stage where the ß-chain locus starts to undergo V(D)J recombination. This recombination continues into the DN3 stage where CD44 is downregulated and the invariant pre-TCRα (pTα) chain and CD3 TCR co-receptor genes are upregulated in order to complex with a correctly formed TCRß. Pairing of TCRß with the pTα and CD3 imparts survival, proliferation, and prevents further ß-chain rearrangement. Because most

DN3 thymocytes cannot make a functional ß-chain, there is a large amount of apoptosis but those thymocytes that have properly functioning TCRßs contribute to a high amount of proliferation within this stage. Entering the DN4 stage, replication ends and CD25 is downregulated. It is at this stage that, once again, RAG1 and 2 are upregulated to allow for V(D)J recombination of the α-chain locus. The thymocyte will then upregulate CD4 and CD8 to enter the double positive stage (DP). At the DP stage the α-chain locus will continue to rearrange until a functional α-chain is made. If the chain is not made correctly within a timely manner, the thymocyte will undergo death by neglect in around three days (Egerton et al., 1990). However, if the α-chain is made properly, pairing to the ß-chain will allow the thymocyte to undergo positive or negative selection to test the TCR for affinity to peptide loaded major-histocompatability complexes (pMHC). The amount of cell death within the thymus is massive at a rate of around 90% with

Glucocorticoid induced cell death is a calcium It was originally thought that glucocorticoid receptor (GR) stimulation is the regulating factor as they are highly sensitive to glucocorticoid treatment and do not display death by neglect when glucocorticoid synthesis is inhibited (King et al., 1995). However, when mice had reduced GR expression or were knockout for the GR, thymocyte apoptosis was not affected (Purton et al., 2000; Sacedón et al., 1999). Even though it still seems to be a topic of discussion, glucocorticoids increase death by neglect in the thymus. The glucocorticoid pathway likely causes a cytoplasmic influx of calcium since the use of the

calmodulin inhibitor, calmidazolium, prevented rat thymocyte apoptosis (McConkey et al., 1989) and the WEHI7.2 lymphocyte cell line (Dowd et al., 1991).

Apoptosis, or programmed cell death, can be initiated by extrinsic or intrinsic pathways. The extrinsic pathway is characterized by the initiation of ligand binding to death receptors. The intrinsic pathway is regulated by BCL-2 family members which include the initiating BH3-only proteins, pro-survival proteins, and pro-apoptotic effector proteins BAX and BAK (reviewed in (Czabotar et al., 2014). The intrinsic pathway is initiated by mitochondrial release of cytochrome C, Smac/Diablo, and HtrA2/Omi. Cytochrome C can bind to Apaf-1 which causes exposure of its caspase recruitment domain (CARD) which then allows for oligomerization and recruitment of procaspase-9 to form the mature apoptosome. Thymocytes undergoing apoptosis, either through negative selection or through positive selection, are likely to utilize the intrinsic pathway since mice deficient for BCL-2 family members, Bak and Bax, display decreased sensitivity to endogenous superantigen (Jeffrey C. Rathmell et al., 2002).

Mouse EndoU is a calcium regulated RNA endonuclease that is highly expressed in DP thymocytes. However, its role *in vivo* thymocytes has yet to be characterized. While, in this thesis, it is shown that EndoU has the propensity to induce apoptosis, a cellular phenotype has not been observed. This is likely due to the nature of studying EndoU in the thymocyte VL3-3M2 cell line, as this cell line is a cancer cell line that has likely overcome any EndoU dependent apoptosis. To overcome this problem, EndoU effects were observed in thymocytes null for EndoU (EndoU$^{-/-}$). The findings showed that

EndoU does not affect death by neglect or T cell maturity, EndoU did promote the negative selection of autoreactive $CD4^+$ single positive thymocytes using the OT-II transgenic background.

RESULTS

EndoU null mice are viable and do not show a dramatic phenotype

Because EndoU is expressed in placental trophoblast cells and keratinocytes, it was possible that EndoU could influence viability of offspring. To determine if there was a sex specific viability phenotype, the sex of pups was observed and chi^2 analysis showed no propensity for one sex over the other (Figure 14A). Furthermore, genotyping indicated that homozygous and heterozygous mice fell within Mendelian inheritance ratios of 1:2:1 based on 41 mice from 7 litters (Figure 14B). This indicated that EndoU null mice were viable regardless of sex.

Since it was expected that EndoU affected cell death of DP thymocytes and ~85-90% of thymocytes are DP, it would be expected that if these cells were undergoing apoptosis at a slower rate, then there would be an expected size difference in the thymus. Based on thymus to bodyweight ratios, there was no observable difference between WT, heterozygous, and KO thymi (Figure 14C). Looking deeper at the populations of thymocytes also revealed that there was no difference between WT or KO thymocytes at the DP or SP stages. This therefore indicated that development to the SP stage was unaffected by the loss of EndoU.

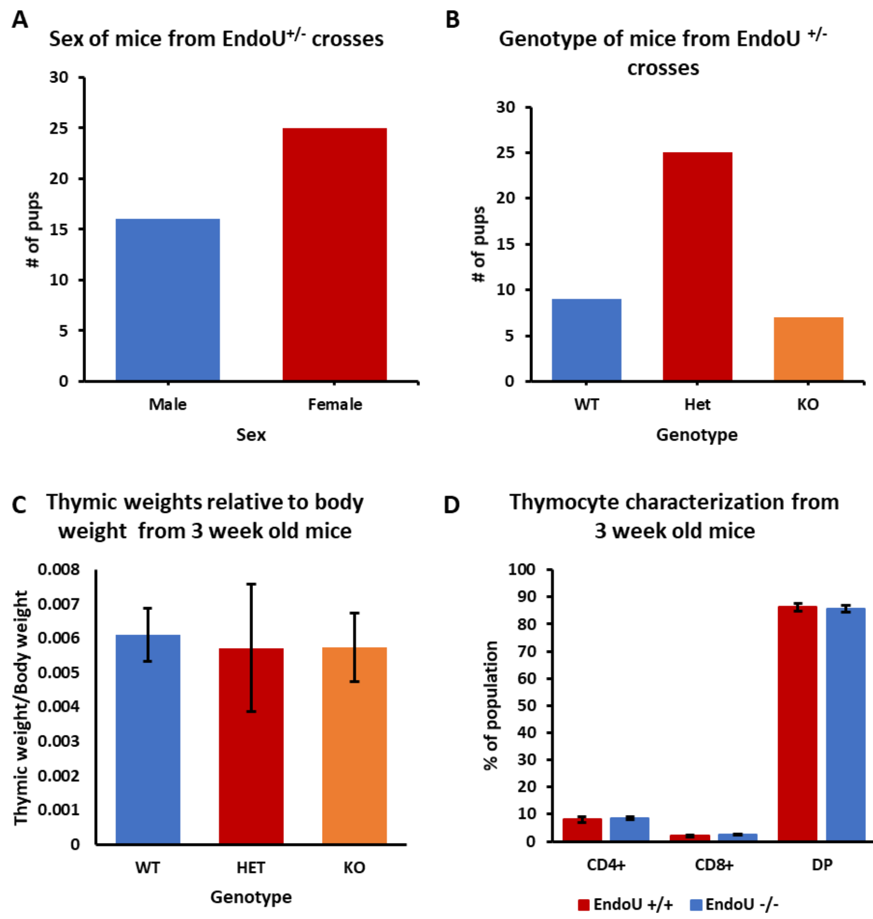

Figure 14: Broad phenotyping of pups from EndoU$^{+/-}$ crosses shows no observable phenotype.
(A) Sex of 35 pups from 6 litters. (B) Genotype of 41 pups from 7 litters. (C) Thymic weight relative to body weight from each genotype. 6 EndoU$^{+/+}$, 15 EndoU$^{+/-}$, and 4 EndoU$^{-/-}$ (D) Flow cytometry results of CD4 and CD8 staining of thymocytes from 3-week-old mice.

EndoU does not affect death by neglect

Because EndoU could be affecting apoptosis and 90% of DP thymocytes die through death by neglect, it was initially thought that EndoU was more likely to affect DP thymocytes dying in this manner. However, because DP thymocyte levels did not change between WT and KO mice (Figure 14B), EndoU was either not affecting the ability of DP thymocytes to die or the effects were minimal. To observe death by neglect directly,

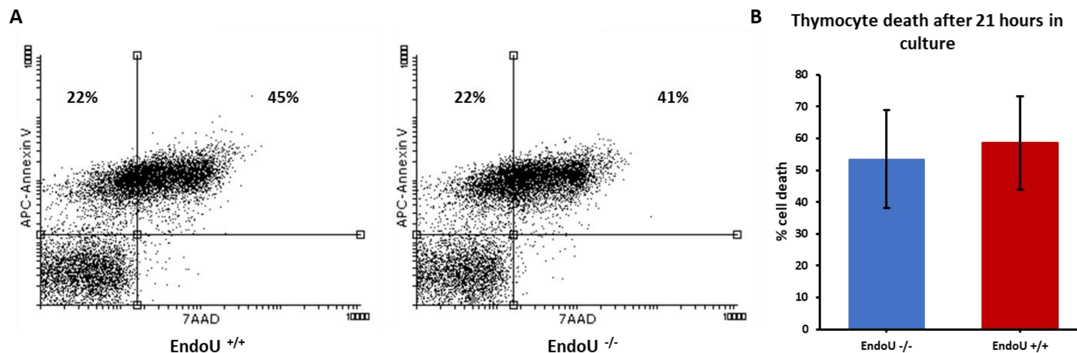

Figure 15: Death by neglect is not affected by the loss of EndoU
(A) Flow cytometry results of annexin V and 7AAD thymocytes cultured for 21 hours. (B) Quantification of thymocytes from three $EndoU^{+/+}$ and four $EndoU^{-/-}$ mice.

thymocytes were cultured for 21 hours and stained with 7AAD and annexin V to observe early (Annexin V^+, $7AAD^-$) and late apoptosis (Annexin V^+, $7AAD^+$) visualized in figure 15A. The results showed that EndoU was not affecting death by neglect in culture (Figure 15B).

EndoU does not affect differentiation of Th1 cells

To ensure that EndoU did not affect the maturation of thymocytes and T cells, up to the effector stage, $CD4^+$ T cells were purified through depleted magnetic activated cell

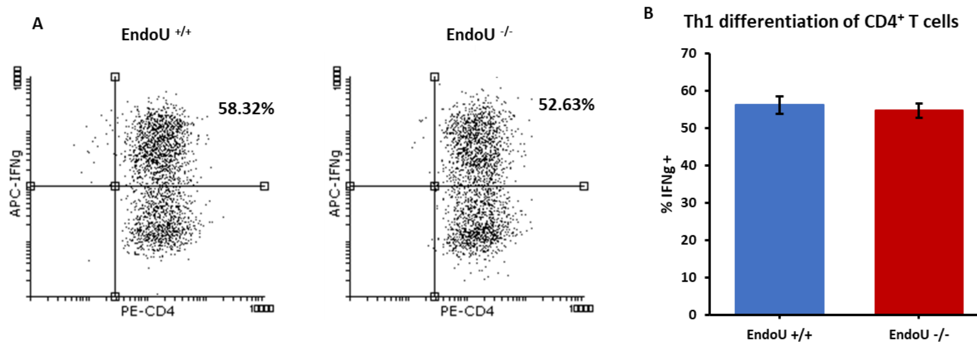

Figure 16: Th1 differentiation of CD4$^+$ T cells is unaffected by EndoU
(A) Representative flow cytometry of CD4$^+$ T cells from 6-week-old EndoU$^{+/+}$ and EndoU$^{-/-}$ mice stimulated with IL-2 and treated with brefeldin A. (B) Quantification of CD4$^+$ T cells positive for IFNγ

sorting, *in vitro* differentiated to Th1 T cells, and stimulated to produce IFNγ. It was observed that EndoU did not play a role in the developmental aspect of thymocyte maturation.

EndoU promotes the deletion of negatively selected thymocytes

Negative selection was another avenue for determining EndoU's cellular function. Because apoptosis through negative selection is a relatively rare event, differences due to EndoU, in the DP or SP populations of thymi, could have been missed. To observe negative selection directly, EndoU$^{-/-}$ mice were crossed into the OT-II background. OT-II mice have a transgenic MHC class II-restricted αßTCR that is specific to the chicken ovalbumin peptide containing residues 323-339 (OVAp)(Barnden et al., 1998). It has previously been reported that intraperitoneal injections (IP) of OVAp can elicit negative selection of thymocytes in OT-II mice (Bouillet et al., 2002; Mier-Aguilar et al., 2016; Zhan et al., 2003). 6-week-old EndoU$^{+/+}$ or EndoU$^{-/-}$ OT-II mice were IP injected with

either 1 mg of OVAp or PBS (vehicle) and euthanized 72 hours later to collect thymi. Total thymocyte cell counts were taken, and population ratios were characterized to determine the absolute quantities of DP and $CD4^+$ SP cells. Although the ratios of DP and SP thymocytes did not alter much due to these injections (Figure 17A), a significant difference of total thymocytes between PBS injected and OVAp injected mice could be observed (Figure 17B). This indicated that the IP injections of OVAp were causing thymocyte deletion. Although not significant, the OVAp injected $EndoU^{+/+}$ mice tended to have a lower abundance of thymocytes when compared to the $EndoU^{-/-}$ mice (Figure 17B). Measurements of total DP thymocytes revealed that there was no EndoU dependent phenotype within this population however the trend was observed that $EndoU^{+/+}$ mice had lower abundance of DP thymocytes when compared to $EndoU^{-/-}$ mice (Figure 17C). Interestingly, it was in the SP population, where a significantly lower abundance of $EndoU^{+/+}$ $CD4^+$ thymocytes than the $EndoU^{-/-}$ $CD4^+$ thymocytes, was observed (Figure 17D). These results indicated that IP injections using OVAp were reaching the thymus and causing negative selection to occur as seen by the loss of total thymocytes within the thymus. In the presence of OVAp, the loss of EndoU did not affect the DP to much extent, however, the $CD4^+$ SP population was almost two-fold higher. Thus, EndoU may be increasing the rate of SP thymocyte deletion through negative selection and maybe, to a small extent DP thymocytes.

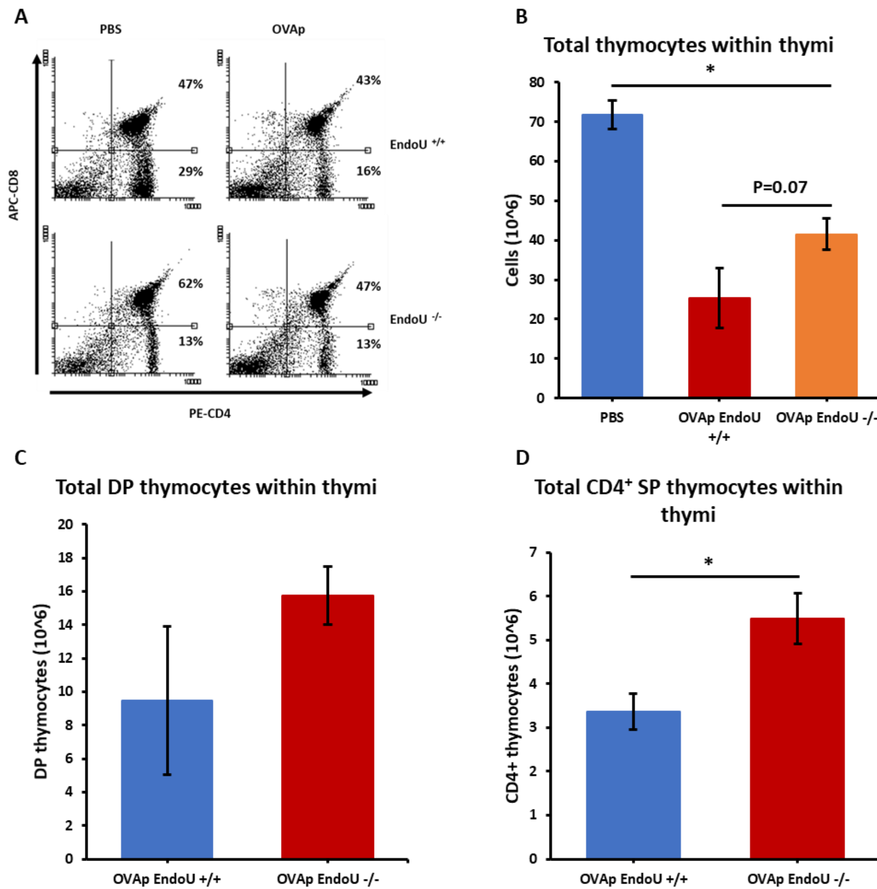

Figure 17: Negatively selected thymocytes are deleted at a lower rate in EndoU null OT-II mice

(A) Representative flow cytometry data of CD4 and CD8 stained thymocytes from IP injections of PBS or 1 mg OVAp in OT-II EndoU$^{+/+}$ or EndoU$^{-/-}$ 6-week-old mice. (B) Total thymocyte counts of mice injected with PBS or OVAp with respective genotypes. (C) Total DP thymocyte counts of mice injected with OVAp with respect to their genotypes in the OT-II background. (D) Total CD4^{+} thymocyte counts of mice injected with OVAp with respect to their genotype in the OT-II background. PBS injections were either EndoU$^{+/+}$ or EndoU$^{-/-}$ (n=2/genotype). Four mice per genotype were injected with 1 mg OVAp.

* $p < 0.05$

DISCUSSION

In this chapter, EndoU$^{+/+}$ and EndoU$^{-/-}$ thymocytes were characterized and their development processes were analyzed from the DP stage to the Th1 effector stage. The reason for analysis beyond the DP stage was because EndoU transcripts are known to be highest at the DP stage however, the protein may be stable enough to last through to the SP stage. To study EndoU's effects on death by neglect, a simple assay of observing cell death over a short time period in culture which showed that EndoU did not affect death by neglect. Characterizing the ability of CD4^{+} spleenocytes to differentiate into Th1 effector T cells was important to determine if the maturation process of thymocytes was unperturbed by the loss of EndoU. To determine this, equal amounts of CD4^{+} spleenocytes, from EndoU$^{+/+}$ and EndoU$^{-/-}$ mice, were differentiated *in vitro*. The results showed that, indeed, EndoU does not affect differentiation up to the effector stage of T cells. Finally, because thymocytes could undergo either apoptosis through death by neglect or negative selection, the latter of which is relatively rare compared to the former, it was important to determine if EndoU influenced negative selection. The OT-II mouse background was employed because the transgenic TCR against OVAp could be used to elicit a high amount of negative selection. Indeed, negative selection occurred if OVAp was IP injected. Interestingly, a lower amount of CD4^{+} SP thymocytes were lost if EndoU was absent which was indicative of a bypass in negative selection.

Negative selection of thymocytes requires a long interaction between the thymocyte's TCR and the antigen presenting cells which creates a high and sustained calcium

signaling event to occur. This has been observed in thymic selections that closely mimic *in vivo* negative selection (Melichar et al., 2013). The observation that EndoU promotes negative selection is in line with the cellular environment that EndoU prefers. While EndoU does promote negative selection, it does appear to be rather weak since it would be expected that a phenotype would be observed in non-transgenic mice, even with negative selection being rare.

Whether EndoU is active in the DP or SP stage remains unclear as there may be a recovery period in between the injection time and the thymus collection time. However, it is likely that there is some recovery and EndoU may be promoting the negative selection of both DP and SP thymocytes. This result means that EndoU could be playing a small but significant role in apoptosis. Since it is expressed in other high turnover tissues, it will be interesting to determine its apoptotic role in other cell types.

MATERIALS AND METHODS

Mice

EndoU$^{tm1(KOMP)Vlcg}$ (C57BL/6Ntac) embryos were ordered from the Jackson Laboratory and, through *in vitro* fertilization performed by UCI, recovered EndoU$^{+/-}$ mice. OT-II mice were ordered from The Jackson laboratory (Barnden et al., 1998).

EndoU$^{-/-}$ mice were crossed into the OT-II background containing the transgenic TCR (Tg) against OVAp (323-339)(Barnden et al., 1998). F1 littermates that Tg^{+}; EndoU$^{+/-}$ were crossed to each other. Experiments were performed on the F2 Tg^{+}; EndoU$^{+/+}$ homozygous mice.

All Mice were euthanized according to IACUC guidelines using CO_2 asphyxiation followed by cervical dislocation at 3-6 weeks of age.

Cell culture

Thymocytes were cultured in RPMI containing 10% FBS, 10 U/ml penicillin /streptomycin, 10 mM HEPES, 50 µM ß-mercaptoethanol and grown at 37 ºC with 5% CO_2.

***Ex vivo* differentiation of Th1 cells**

Spleenocytes were collected by crushing spleens over a 20 µm screen. CD4^{+} T cells were isolated using the MojoSort mouse CD4^{+} T cell isolation kit (BioLegend) according to

the manufacturer's protocol. Briefly, 10^7 spleenocytes were depleted of non-$CD4^+$ spleenocytes with an average recovery of 10^6 $CD4^+$ T cells. The $CD4^+$ T cells were then differentiated using the CellXVivo mouse Th1 cell differentiation kit according to the manufacturer's protocol (R & D Systems).

Flow cytometry

Thymi were crushed over a 20 µm screen. The screen was rinsed with 1X FACS buffer (5% BSA, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8mM KH_2PO_4, 2mM EDTA). Thymocytes were stained with the following concentrations PE-CD4 at 1:2000 (BioLegend), APC-CD8 at 1:200 (BioLegend), APC-IFNγ at 1:200, and APC-annexin V at 1:200 (BioLegend). Annexin V staining was carried out in annexin V staining buffer composed of 10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$. 7AAD (bioLegend) was used at a concentration of 0.5 ng/ml.

Thymocyte cell counting was performed using precision count beads (BioLegend). Beads were diluted with cells at a concentration of 100 beads/µl with thymocytes. Flow was stopped and data was taken after 1000 beads were counted.

OVA peptide injections

The ovalbumin peptide (OVA) (SIINFEKL) was custom purchased from BioMatik as a lyophilized powder. It was resuspended in 1X PBS at a concentration of 5 mg/ml. 200 µl

(1 mg) of OVA peptide was injected interperitoneally into six-week-old mice. 72 hours post injection, mice were euthanized and thymi were extracted.

REFERENCES

Barnden, M. J., Allison, J., Heath, W. R., & Carbone, F. R. (1998). Defective TCR expression in transgenic mice constructed using cDNA-based α- and β-chain genes under the control of heterologous regulatory elements. *Immunology & Cell Biology, 76*(1), 34-40. doi:https://doi.org/10.1046/j.1440-1711.1998.00709.x

Bouillet, P., Purton, J. F., Godfrey, D. I., Zhang, L.-C., Coultas, L., Puthalakath, H., Pellegrini, M., Cory, S., Adams, J. M., & Strasser, A. (2002). BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature, 415*(6874), 922-926. doi:10.1038/415922a

Czabotar, P. E., Lessene, G., Strasser, A., & Adams, J. M. (2014). Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat Rev Mol Cell Biol, 15*(1), 49-63. doi:10.1038/nrm3722

Dowd, D. R., MacDonald, P. N., Komm, B. S., Haussler, M. R., & Miesfeld, R. (1991). Evidence for early induction of calmodulin gene expression in lymphocytes undergoing glucocorticoid-mediated apoptosis. *Journal of Biological Chemistry, 266*(28), 18423-18426. doi:https://doi.org/10.1016/S0021-9258(18)55076-6

Egerton, M., Scollay, R., & Shortman, K. (1990). Kinetics of mature T-cell development in the thymus. *Proceedings of the National Academy of Sciences of the United States of America, 87*(7), 2579-2582. doi:10.1073/pnas.87.7.2579

King, L. B., Vacchio, M. S., Dixon, K., Hunziker, R., Margulies, D. H., & Ashwell, J. D. (1995). A targeted glucocorticoid receptor antisense transgene increases thymocyte apoptosis and alters thymocyte development. *Immunity, 3*(5), 647-656. doi:https://doi.org/10.1016/1074-7613(95)90135-3

McConkey, D. J., Nicotera, P., Hartzell, P., Bellomo, G., Wyllie, A. H., & Orrenius, S. (1989). Glucocorticoids activate a suicide process in thymocytes through an elevation of cytosolic Ca2+ concentration. *Archives of Biochemistry and Biophysics, 269*(1), 365-370. doi:https://doi.org/10.1016/0003-9861(89)90119-7

Melichar, H. J., Ross, J. O., Herzmark, P., Hogquist, K. A., & Robey, E. A. (2013). Distinct temporal patterns of T cell receptor signaling during positive versus negative selection in situ. *Sci Signal, 6*(297), ra92. doi:10.1126/scisignal.2004400

Mier-Aguilar, C. A., Cashman, K. S., Raman, C., & Soldevila, G. (2016). CD5-CK2 Signaling Modulates Erk Activation and Thymocyte Survival. *PLoS One, 11*(12), e0168155. doi:10.1371/journal.pone.0168155

Purton, J. F., Boyd, R. L., Cole, T. J., & Godfrey, D. I. (2000). Intrathymic T Cell Development and Selection Proceeds Normally in the Absence of Glucocorticoid Receptor Signaling. *Immunity, 13*(2), 179-186. doi:https://doi.org/10.1016/S1074-7613(00)00018-2

Rathmell, J. C., Lindsten, T., Zong, W.-X., Cinalli, R. M., & Thompson, C. B. (2002). Deficiency in Bak and Bax perturbs thymic selection and lymphoid homeostasis. *Nature Immunology, 3*(10), 932-939. doi:10.1038/ni834

Sacedón, R., Vicente, A., Varas, A., Morale, M. C., Barden, N., Marchetti, B., & Zapata, A. n. G. (1999). Partial blockade of T-cell differentiation during ontogeny and marked alterations of the thymic microenvironment in transgenic mice with impaired glucocorticoid receptor function. *Journal of Neuroimmunology, 98*(2), 157-167. doi:https://doi.org/10.1016/S0165-5728(99)00091-0

Zhan, Y., Purton, J. F., Godfrey, D. I., Cole, T. J., Heath, W. R., & Lew, A. M. (2003). Without peripheral interference, thymic deletion is mediated in a cohort of double-positive cells without classical activation. *Proc Natl Acad Sci U S A, 100*(3), 1197-1202. doi:10.1073/pnas.0237316100

Conclusions

Through these experiments it has been shown that EndoU is a pro-apoptotic RNA endonuclease. Chapter 1 shows that EndoU is calcium regulated through allosteric binding. Through immunoprecipitation of EndoU, reaction rates could be followed to identify the optimal reaction conditions of EndoU to show that it does not prefer any specific sequence. Further investigation shows that EndoU has a transient and large, likely proteinaceous, cofactor that broadens EndoU's specificity.

Chapter 2 shows that EndoU is highly attenuated in cells, yet it is still able to affect mRNA expression. It is shown that EndoU can promote the expression of pro-apoptotic genes. Furthermore, by looking at the EndoU complex, insights into how what EndoU could bind, implicates it in the apoptotic pathway. The calpains are important for calcium regulated apoptosis and because EndoU binds to many ribosomal proteins, indicates that when it is activated by calcium, it can cleave ribosomal RNAs in calcium activated apoptosis.

Chapter 3 shows that EndoU does not impact death by neglect however it does promote the apoptosis of negatively selected thymocytes. Because the effects are not strong, it could mean that EndoU is playing a small role in apoptosis. Through further investigations, it will be interesting to see to what extent, it plays a role apoptotic pathway.

Full reference list

Altznauer, F., Conus, S., Cavalli, A., Folkers, G., & Simon, H.-U. (2004). Calpain-1 Regulates Bax and Subsequent Smac-dependent Caspase-3 Activation in Neutrophil Apoptosis*. *Journal of Biological Chemistry, 279*(7), 5947-5957. doi:https://doi.org/10.1074/jbc.M308576200

Ardelt, W., Mikulski, S. M., & Shogen, K. (1991). Amino acid sequence of an anti-tumor protein from Rana pipiens oocytes and early embryos. Homology to pancreatic ribonucleases. *Journal of Biological Chemistry, 266*(1), 245-251. doi:10.1016/s0021-9258(18)52427-3

Barnden, M. J., Allison, J., Heath, W. R., & Carbone, F. R. (1998). Defective TCR expression in transgenic mice constructed using cDNA-based α- and β-chain genes under the control of heterologous regulatory elements. *Immunology & Cell Biology, 76*(1), 34-40. doi:https://doi.org/10.1046/j.1440-1711.1998.00709.x

Bennett, J. P., Cockcroft, S., & Gomperts, B. D. (1979). Ionomycin stimulates mast cell histamine secretion by forming a lipid-soluble calcium complex. *Nature, 282*(5741), 851-853. doi:10.1038/282851a0

Benoist, C., Lanier, L., Merad, M., Mathis, D., & Immunological Genome, P. (2012). Consortium biology in immunology: the perspective from the Immunological Genome Project. *Nat Rev Immunol, 12*(10), 734-740. doi:10.1038/nri3300

Berryman, S., Brooks, E., Burman, A., Hawes, P., Roberts, R., Netherton, C., Monaghan, P., Whelband, M., Cottam, E., Elazar, Z., Jackson, T., & Wileman, T. (2012). Foot-and-mouth disease virus induces autophagosomes during cell entry via a class III phosphatidylinositol 3-kinase-independent pathway. *J Virol, 86*(23), 12940-12953. doi:10.1128/JVI.00846-12

Bhardwaj, K., Guarino, L., & Kao, C. C. (2004). The severe acute respiratory syndrome coronavirus Nsp15 protein is an endoribonuclease that prefers manganese as a cofactor. *J Virol, 78*(22), 12218-12224. doi:10.1128/JVI.78.22.12218-12224.2004

Boix, E., Wu, Y., Vasandani, V. M., Saxena, S. K., Ardelt, W., Ladner, J., & Youle, R. J. (1996). Role of the N terminus in RNase A homologues: differences in catalytic activity, ribonuclease inhibitor interaction and cytotoxicity. *J Mol Biol, 257*(5), 992-1007. doi:10.1006/jmbi.1996.0218

Bouillet, P., Purton, J. F., Godfrey, D. I., Zhang, L.-C., Coultas, L., Puthalakath, H., Pellegrini, M., Cory, S., Adams, J. M., & Strasser, A. (2002). BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature, 415*(6874), 922-926. doi:10.1038/415922a

Brennan-Laun, S. E., Ezelle, H. J., Li, X. L., & Hassel, B. A. (2014). RNase-L control of cellular mRNAs: roles in biologic functions and mechanisms of substrate targeting. *J Interferon Cytokine Res, 34*(4), 275-288. doi:10.1089/jir.2013.0147

Brewer, J. A., Kanagawa, O., Sleckman, B. P., & Muglia, L. J. (2002). Thymocyte apoptosis induced by T cell activation is mediated by glucocorticoids in vivo. *J Immunol, 169*(4), 1837-1843. doi:10.4049/jimmunol.169.4.1837

Caffarelli, E., Maggi, L., Fatica, A., Jiricny, J., & Bozzoni, I. (1997). A Novel Mn++-Dependent Ribonuclease That Functions in U16 SnoRNA Processing inX.Laevis. *Biochemical and Biophysical Research Communications, 233*(2), 514-517. doi:https://doi.org/10.1006/bbrc.1997.6487

Calfon, M., Zeng, H., Urano, F., Till, J. H., Hubbard, S. R., Harding, H. P., Clark, S. G., & Ron, D. (2002). IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature, 415*(6867), 92-96. doi:10.1038/415092a

Castello, A., Fischer, B., Eichelbaum, K., Horos, R., Beckmann, B. M., Strein, C., Davey, N. E., Humphreys, D. T., Preiss, T., Steinmetz, L. M., Krijgsveld, J., & Hentze, M. W. (2012). Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. *Cell, 149*(6), 1393-1406. doi:10.1016/j.cell.2012.04.031

Chen, H.-C., & Cheng, S.-C. (2012). Functional roles of protein splicing factors. *Bioscience reports, 32*(4), 345-359. doi:10.1042/BSR20120007

Chen, Y., & Brandizzi, F. (2013). IRE1: ER stress sensor and cell fate executor. *Trends Cell Biol, 23*(11), 547-555. doi:10.1016/j.tcb.2013.06.005

Christensen, K. A., Myers, J. T., & Swanson, J. A. (2002). pH-dependent regulation of lysosomal calcium in macrophages. *Journal of Cell Science, 115*(3), 599.

Clapham, D. E. (2007). Calcium Signaling. *Cell, 131*(6), 1047-1058. doi:https://doi.org/10.1016/j.cell.2007.11.028

Credle, J. J., Finer-Moore, J. S., Papa, F. R., Stroud, R. M., & Walter, P. (2005). On the mechanism of sensing unfolded protein in the endoplasmic reticulum. *Proc Natl Acad Sci U S A, 102*(52), 18773-18784. doi:10.1073/pnas.0509487102

Czabotar, P. E., Lessene, G., Strasser, A., & Adams, J. M. (2014). Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat Rev Mol Cell Biol, 15*(1), 49-63. doi:10.1038/nrm3722

Darzzynkiewicz, Z. C., S.P. Mikulski, S. M. Ardelt, W. J. Shogen, K. (1988). Cytostatic and cytotoxic effects of Pannon (P-30 Protein), a novel anticancer agent. *Cell Tissue Kinetics*(21), 169-182.

Deshpande, A. M., & Wong, D. T. (2008). Molecular mechanisms of head and neck cancer. *Expert Rev Anticancer Ther, 8*(5), 799-809. doi:10.1586/14737140.8.5.799

Dowd, D. R., MacDonald, P. N., Komm, B. S., Haussler, M. R., & Miesfeld, R. (1991). Evidence for early induction of calmodulin gene expression in lymphocytes undergoing glucocorticoid-mediated apoptosis. *Journal of Biological Chemistry, 266*(28), 18423-18426. doi:https://doi.org/10.1016/S0021-9258(18)55076-6

Egerton, M., Scollay, R., & Shortman, K. (1990). Kinetics of mature T-cell development in the thymus. *Proceedings of the National Academy of Sciences of the United States of America, 87*(7), 2579-2582. doi:10.1073/pnas.87.7.2579

Gaudino, S. J., & Kumar, P. (2019). Cross-Talk Between Antigen Presenting Cells and T Cells Impacts Intestinal Homeostasis, Bacterial Infections, and Tumorigenesis. *Front Immunol, 10*, 360. doi:10.3389/fimmu.2019.00360

Groves, T., Katis, P., Madden, Z., Manickam, K., Ramsden, D., Wu, G., & Guidos, C. (1995). In vitro maturation of clonal CD4+CD8+ cell lines in response to TCR engagement. *The Journal of Immunology, 154*, 5011-5022.

Grundmann, U., Romisch, J., Siebold, B., Bohn, H., & Amann, E. (1990). Cloning and Expression of a cDNA Encoding Human Placental Protein 11, a Putative Serine Protease with Diagnostic Significance as a Tumor Marker. *DNA and Cell Biology, 9*(4), 243-250. doi:10.1089/dna.1990.9.243

Guo, H., Ingolia, N. T., Weissman, J. S., & Bartel, D. P. (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature, 466*(7308), 835-840. doi:10.1038/nature09267

Hall, T. A. (1999). BioEdit : a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser., 41*, 95-98.

Han, J.-Q., Wroblewski, G., Xu, Z., Silverman, R. H., & Barton, D. J. (2004). Sensitivity of Hepatitis C Virus RNA to the Antiviral Enzyme Ribonuclease L Is Determined by a Subset of Efficient Cleavage Sites. *Journal of Interferon & Cytokine Research, 24*(11), 664-676. doi:10.1089/jir.2004.24.664

Han, J., Lee, Y., Yeom, K.-H., Nam, J.-W., Heo, I., Rhee, J.-K., Sohn, S. Y., Cho, Y., Zhang, B.-T., & Kim, V. N. (2006). Molecular Basis for the Recognition of Primary microRNAs by the Drosha-DGCR8 Complex. *Cell, 125*(5), 887-901. doi:https://doi.org/10.1016/j.cell.2006.03.043

Humeau, J., Bravo-San Pedro, J. M., Vitale, I., Nunez, L., Villalobos, C., Kroemer, G., & Senovilla, L. (2018). Calcium signaling and cell cycle: Progression or death. *Cell Calcium, 70*, 3-15. doi:10.1016/j.ceca.2017.07.006

Ivanov, K. A., Hertzig, T., Rozanov, M., Bayer, S., Thiel, V., Gorbalenya, A. E., & Ziebuhr, J. (2004). Major genetic marker of nidoviruses encodes a replicative endoribonuclease. *Proc Natl Acad Sci U S A, 101*(34), 12694-12699. doi:10.1073/pnas.0403127101

Jayaraman, T., & Marks, A. R. (1997). T cells deficient in inositol 1,4,5-trisphosphate receptor are resistant to apoptosis. *Molecular and Cellular Biology, 17*(6), 3005. doi:10.1128/MCB.17.6.3005

King, L. B., Vacchio, M. S., Dixon, K., Hunziker, R., Margulies, D. H., & Ashwell, J. D. (1995). A targeted glucocorticoid receptor antisense transgene increases thymocyte apoptosis and alters thymocyte development. *Immunity, 3*(5), 647-656. doi:https://doi.org/10.1016/1074-7613(95)90135-3

Korennykh, A. V., Korostelev, A. A., Egea, P. F., Finer-Moore, J., Stroud, R. M., Zhang, C., Shokat, K. M., & Walter, P. (2011). Structural and functional basis for RNA cleavage by Ire1. *BMC Biology, 9*(1), 47. doi:10.1186/1741-7007-9-47

Laneve, P., Altieri, F., Fiori, M. E., Scaloni, A., Bozzoni, I., & Caffarelli, E. (2003). Purification, Cloning, and Characterization of XendoU, a Novel Endoribonuclease Involved in Processing of Intron-encoded Small Nucleolar RNAs in Xenopus laevis*. *Journal of Biological Chemistry, 278*(15), 13026-13032. doi:https://doi.org/10.1074/jbc.M211937200

Laneve, P., Gioia, U., Ragno, R., Altieri, F., Di Franco, C., Santini, T., Arceci, M., Bozzoni, I., & Caffarelli, E. (2008). The tumor marker human placental protein 11 is an endoribonuclease. *J Biol Chem, 283*(50), 34712-34719. doi:10.1074/jbc.M805759200

Lee, A. H., Iwakoshi, N. N., & Glimcher, L. H. (2003). XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol Cell Biol, 23*(21), 7448-7459. doi:10.1128/mcb.23.21.7448-7459.2003

Lee, K. P., Dey, M., Neculai, D., Cao, C., Dever, T. E., & Sicheri, F. (2008). Structure of the dual enzyme Ire1 reveals the basis for catalysis and regulation in nonconventional RNA splicing. *Cell, 132*(1), 89-100. doi:10.1016/j.cell.2007.10.057

Lee, R. C., Feinbaum, R. L., & Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell, 75*(5), 843-854. doi:https://doi.org/10.1016/0092-8674(93)90529-Y

Lieber, M. R. (1991). Site-specific recombination in the immune system1. *The FASEB Journal, 5*(14), 2934-2944. doi:https://doi.org/10.1096/fasebj.5.14.1752360

Lloyd-Evans, E., Morgan, A. J., He, X., Smith, D. A., Elliot-Smith, E., Sillence, D. J., Churchill, G. C., Schuchman, E. H., Galione, A., & Platt, F. M. (2008). Niemann-Pick disease type C1 is a sphingosine storage disease that causes deregulation of lysosomal calcium. *Nat Med, 14*(11), 1247-1255. doi:10.1038/nm.1876

Malathi, K., Dong, B., Gale, M., Jr., & Silverman, R. H. (2007). Small self-RNA generated by RNase L amplifies antiviral innate immunity. *Nature, 448*(7155), 816-819. doi:10.1038/nature06042

Martinez, J. A., Zhang, Z., Svetlov, S. I., Hayes, R. L., Wang, K. K., & Larner, S. F. (2010). Calpain and caspase processing of caspase-12 contribute to the ER stress-

induced cell death pathway in differentiated PC12 cells. *Apoptosis, 15*(12), 1480-1493. doi:10.1007/s10495-010-0526-4

McConkey, D. J., Nicotera, P., Hartzell, P., Bellomo, G., Wyllie, A. H., & Orrenius, S. (1989). Glucocorticoids activate a suicide process in thymocytes through an elevation of cytosolic Ca2+ concentration. *Archives of Biochemistry and Biophysics, 269*(1), 365-370. doi:https://doi.org/10.1016/0003-9861(89)90119-7

Melichar, H. J., Ross, J. O., Herzmark, P., Hogquist, K. A., & Robey, E. A. (2013). Distinct temporal patterns of T cell receptor signaling during positive versus negative selection in situ. *Sci Signal, 6*(297), ra92. doi:10.1126/scisignal.2004400

Michalska, K., Quan Nhan, D., Willett, J. L. E., Stols, L. M., Eschenfeldt, W. H., Jones, A. M., Nguyen, J. Y., Koskiniemi, S., Low, D. A., Goulding, C. W., Joachimiak, A., & Hayes, C. S. (2018). Functional plasticity of antibacterial EndoU toxins. *Mol Microbiol, 109*(4), 509-527. doi:10.1111/mmi.14007

Mier-Aguilar, C. A., Cashman, K. S., Raman, C., & Soldevila, G. (2016). CD5-CK2 Signaling Modulates Erk Activation and Thymocyte Survival. *PLoS One, 11*(12), e0168155. doi:10.1371/journal.pone.0168155

Mikulski, S. M., Ardelt, W., Shogen, K., Bernstein, E. H., & Menduke, H. (1990). Striking increase of survival of mice bearing M109 Madison carcinoma treated with a novel protein from amphibian embryos. *Journal of the National Cancer Institute, 82*(2), 151-153. doi:10.1093/jnci/82.2.151-a

Mistry, J., Chuguransky, S., Williams, L., Qureshi, M., Salazar, G. A., Sonnhammer, E. L. L., Tosatto, S. C. E., Paladin, L., Raj, S., Richardson, L. J., Finn, R. D., & Bateman, A. (2021). Pfam: The protein families database in 2021. *Nucleic acids research, 49*(D1), D412-D419. doi:10.1093/nar/gkaa913

Navickas, A., Chamois, S., Saint-Fort, R., Henri, J., Torchet, C., & Benard, L. (2020). No-Go Decay mRNA cleavage in the ribosome exit tunnel produces 5'-OH ends phosphorylated by Trl1. *Nat Commun, 11*(1), 122. doi:10.1038/s41467-019-13991-9

Pincus, D., Chevalier, M. W., Aragon, T., van Anken, E., Vidal, S. E., El-Samad, H., & Walter, P. (2010). BiP binding to the ER-stress sensor Ire1 tunes the homeostatic

behavior of the unfolded protein response. *PLoS Biol, 8*(7), e1000415. doi:10.1371/journal.pbio.1000415

Poe, J. C., Kountikov, E. I., Lykken, J. M., Natarajan, A., Marchuk, D. A., & Tedder, T. F. (2014). EndoU is a novel regulator of AICD during peripheral B cell selection. *J Exp Med, 211*(1), 57-69. doi:10.1084/jem.20130648

Purton, J. F., Boyd, R. L., Cole, T. J., & Godfrey, D. I. (2000). Intrathymic T Cell Development and Selection Proceeds Normally in the Absence of Glucocorticoid Receptor Signaling. *Immunity, 13*(2), 179-186. doi:https://doi.org/10.1016/S1074-7613(00)00018-2

Rathmell, J. C., & Goodnow, C. C. (1994). Effects of the lpr mutation on elimination and inactivation of self-reactive B cells. *The Journal of Immunology, 153*(6), 2831.

Rathmell, J. C., Lindsten, T., Zong, W.-X., Cinalli, R. M., & Thompson, C. B. (2002). Deficiency in Bak and Bax perturbs thymic selection and lymphoid homeostasis. *Nature Immunology, 3*(10), 932-939. doi:10.1038/ni834

Robert, X., & Gouet, P. (2014). Deciphering key features in protein structures with the new ENDscript server. *Nucleic acids research, 42*(Web Server issue), W320-324. doi:10.1093/nar/gku316

Sacedón, R., Vicente, A., Varas, A., Morale, M. C., Barden, N., Marchetti, B., & Zapata, A. n. G. (1999). Partial blockade of T-cell differentiation during ontogeny and marked alterations of the thymic microenvironment in transgenic mice with impaired glucocorticoid receptor function. *Journal of Neuroimmunology, 98*(2), 157-167. doi:https://doi.org/10.1016/S0165-5728(99)00091-0

Schwarz, D. S., & Blower, M. D. (2014). The calcium-dependent ribonuclease XendoU promotes ER network formation through local RNA degradation. *J Cell Biol, 207*(1), 41-57. doi:10.1083/jcb.201406037

Shemorry, A., Harnoss, J. M., Guttman, O., Marsters, S. A., Komuves, L. G., Lawrence, D. A., & Ashkenazi, A. (2019). Caspase-mediated cleavage of IRE1 controls apoptotic cell commitment during endoplasmic reticulum stress. *Elife, 8*. doi:10.7554/eLife.47084

Sternburg, E. L., Dias, K. C., & Karginov, F. V. (2017). Selection-dependent and Independent Generation of CRISPR/Cas9-mediated Gene Knockouts in Mammalian Cells. *J Vis Exp*(124). doi:10.3791/55903

Suikkanen, S., Sääjärvi, K., Hirsimäki, J., Välilehto, O., Reunanen, H., Vihinen-Ranta, M., & Vuento, M. (2002). Role of recycling endosomes and lysosomes in dynein-dependent entry of canine parvovirus. *Journal of virology, 76*(9), 4401-4411. doi:10.1128/jvi.76.9.4401-4411.2002

Tomecki, R., & Dziembowski, A. (2010). Novel endoribonucleases as central players in various pathways of eukaryotic RNA metabolism. *RNA, 16*(9), 1692-1724. doi:10.1261/rna.2237610

Turcotte, R. F., Lavis, L. D., & Raines, R. T. (2009). Onconase cytotoxicity relies on the distribution of its positive charge. *The FEBS Journal, 276*(14), 3846-3857. doi:https://doi.org/10.1111/j.1742-4658.2009.07098.x

Uhlen, M., Fagerberg, L., Hallstrom, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, A., Kampf, C., Sjostedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C. A., Odeberg, J., Djureinovic, D., Takanen, J. O., Hober, S., Alm, T., Edqvist, P. H., Berling, H., Tegel, H., Mulder, J., Rockberg, J., Nilsson, P., Schwenk, J. M., Hamsten, M., von Feilitzen, K., Forsberg, M., Persson, L., Johansson, F., Zwahlen, M., von Heijne, G., Nielsen, J., & Ponten, F. (2015). Proteomics. Tissue-based map of the human proteome. *Science, 347*(6220), 1260419. doi:10.1126/science.1260419

Uhlen, M., Zhang, C., Lee, S., Sjostedt, E., Fagerberg, L., Bidkhori, G., Benfeitas, R., Arif, M., Liu, Z., Edfors, F., Sanli, K., von Feilitzen, K., Oksvold, P., Lundberg, E., Hober, S., Nilsson, P., Mattsson, J., Schwenk, J. M., Brunnstrom, H., Glimelius, B., Sjoblom, T., Edqvist, P. H., Djureinovic, D., Micke, P., Lindskog, C., Mardinoglu, A., & Ponten, F. (2017). A pathology atlas of the human cancer transcriptome. *Science, 357*(6352). doi:10.1126/science.aan2507

Wightman, B., Ha, I., & Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell, 75*(5), 855-862. doi:https://doi.org/10.1016/0092-8674(93)90530-4

Zhan, Y., Purton, J. F., Godfrey, D. I., Cole, T. J., Heath, W. R., & Lew, A. M. (2003). Without peripheral interference, thymic deletion is mediated in a cohort of

double-positive cells without classical activation. *Proc Natl Acad Sci U S A, 100*(3), 1197-1202. doi:10.1073/pnas.0237316100

Zheng, D., Wang, G., Li, S., Fan, G. C., & Peng, T. (2015). Calpain-1 induces endoplasmic reticulum stress in promoting cardiomyocyte apoptosis following hypoxia/reoxygenation. *Biochim Biophys Acta, 1852*(5), 882-892. doi:10.1016/j.bbadis.2015.01.019

Zhou, J., Liu, C. Y., Back, S. H., Clark, R. L., Peisach, D., Xu, Z., & Kaufman, R. J. (2006). The crystal structure of human IRE1 luminal domain reveals a conserved dimerization interface required for activation of the unfolded protein response. *Proc Natl Acad Sci U S A, 103*(39), 14343-14348. doi:10.1073/pnas.0606480103

Zhou, W., Tang, G. W., & Altman, R. B. (2015). High Resolution Prediction of Calcium-Binding Sites in 3D Protein Structures Using FEATURE. *J Chem Inf Model, 55*(8), 1663-1672. doi:10.1021/acs.jcim.5b00367a