The Cellular Degradation of Crouzon Syndrome Mutant FGFR2 Protein

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Abstract

Humans and mice with Crouzon Syndrome craniosynostosis have distinct craniofacial features including dome-shaped skulls, wide-set bulging eyes, premature fusion of coronal and facial sutures, and a severely retrusive midface. Surgical intervention is the only available treatment. Crouzon Syndrome is caused by mutations in the *FGFR2* gene, which have also shown correlation to severe neurodevelopmental disorders including Autism Spectrum Disorder. It remains unknown how the Crouzon mutations cause the distinct craniofacial phenotype. The goal of this study is to gain insight into the mechanism of Crouzon Syndrome by investigating the preferred method of mutant $FGFR2^{C342Y}$ degradation. We have previously shown that $FGFR2^{C342Y}$ exhibits dimerization and increased degradation. Based on these findings, we hypothesize that $FGFR2^{C342Y}$ is recognized as aggregated by the cell and thus degraded via autophagy. In order to influence autophagy in a laboratory setting, we treated cells with rapamycin, a known inducer of autophagy. To test the hypothesis, we studied protein amounts in cells following rapamycin treatment and transient transfection of wild type FGFR2 or $FGFR2^{C342Y}$ plasmids. If autophagy is a preferred degradation mechanism in Crouzon Syndrome, we anticipate less $FGFR2^{C342Y}$ protein inside the cells following treatment. This work will further our understanding of how $FGFR2^{C342Y}$ is intracellularly processed, potentially providing clarity on how the distinct Crouzon Syndrome phenotype arises. Without early intervention, the neurological and psychosocial consequences of the disease can be devastating to affected individuals. Information gained from this project can ultimately contribute to new treatments and quality of life improvements.

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Scientific Acknowledgements

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Introduction

General Overview

Cranial Development and the Coronal Suture

The formation of the skull is integral to fetal development. Within even the first eight weeks of pregnancy, precursors to the skull bones are established. The skull, also known as the cranium, is subdivided by location: the viscerocranium of the face and the neurocranium which protects the brain (Jin et al., 2016). The primary structure of the neurocranium is the cranial vault, which consists of the frontal, parietal, temporal, and occipital bones (Figure 1). During embryonic development, these bones form independently from condensed mesenchyme cells, and remain separated by cartilaginous sutures until after birth (Figure 2). In a healthy human, these sutures later ossify into bone and the cranial vault becomes whole. This mechanism exists to allow flexibility in the cranium, which is important when exiting the birth canal as well as postnatal brain growth.

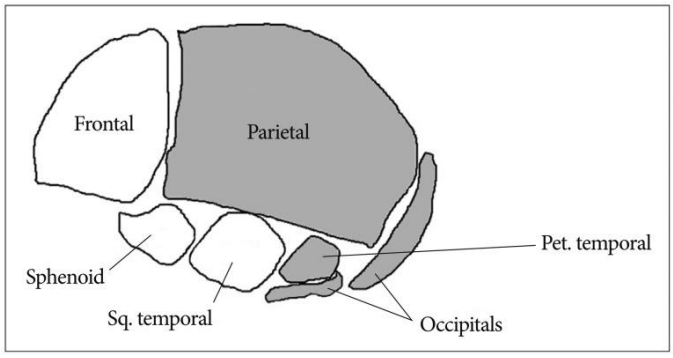

Figure 1. Skeletal structure of the skull, with derivation of mesodermal (gray) and neural crest (white) lineages (Jin et al., 2016).

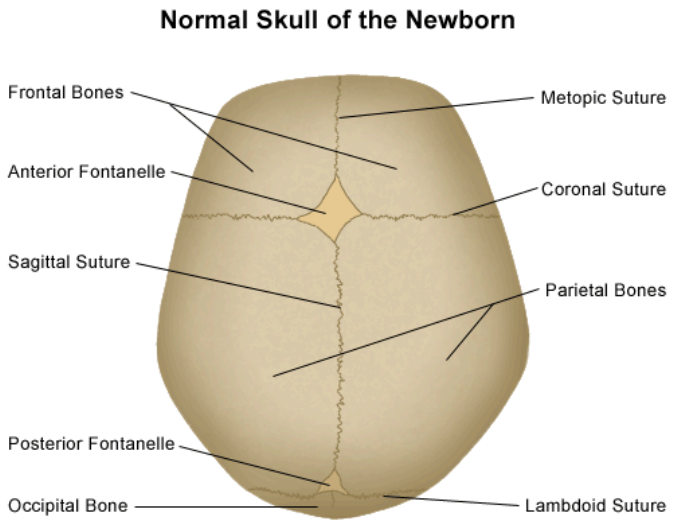

Figure 2. Map of cartilaginous sutures of a healthy newborn skull (stanfordchildrens.org).

An important feature of this mechanism is the development of the coronal suture, which lies between the frontal and parietal bones (Figure 2). These bones derive from different cell lines, making the coronal suture a unique biological distinction. The frontal bone is a descendant of the neural crest lineage, where the parietal bone is a descendant of the mesodermal (Figure 1). These cell lines differentiate independently and at different speeds, but can be influenced by each other (Trainor, 2005). A complex signaling system exists to regulate this differentiation and ensure proper growth of the cranial vault and specifically the coronal suture. When this system is disrupted, either by genetic or environmental factors, cranial defects arise.

Fibroblast Growth Factor Receptors

Fibroblast growth factors (FGFs) and their respective receptors (FGFRs) play a key role in a variety of fundamental growth processes, including the regulation of cranial vault development. FGFRs are a family of tyrosine kinases, meaning they catalyze a phosphorylation reaction of the amino acid tyrosine. The addition of a phosphoryl group to the tyrosine of a protein acts as a signal to initiate or terminate different cell processes (Paul and Mukhopadhyay, 2004). FGFRs lie embedded in the cell membrane, with three extracellular domains responsible for the affinity and binding of a specific ligands. Upon ligand binding, a variety of downstream intracellular effects can occur to mediate cell needs. These effects are due to the receptor's ability to dimerize, recruit molecules to the cell membrane, and autophosphorylation (Hatch, 2010). It is known that

mutations in the FGFR family result in craniofacial abnormalities and a condition known as craniosynostosis (Hatch, 2010).

Craniosynostosis and Crouzon Syndrome

Craniosynostosis is the premature fusion of cranial bones due to loss of cranial suture tissue. It can lead to abnormal skull and facial shapes and high intracranial pressure, with potential to negatively impact brain development. In rare cases it may lead to cognitive delays, learning disabilities, reduced quality of life, and death (Warren et al., 2012). Craniosynostosis can occur with an identified genetic mutation, or in isolation. As outlined previously, the mutations associated with this condition are often in FGFRs. A plethora of craniosynostosis syndromes resulting from FGFR mutations have been identified, including Apert, Crouzon, JacksonWeiss, Pfeiffer, and Muenke syndromes (Hatch, 2010).

The most common FGFR-related craniosynostosis syndrome is Crouzon Syndrome, which has been linked to several point mutations in the third extracellular domain of FGFR2 (Hatch et al., 2006) (Figure 3). Crouzon syndrome is characterized by distinct craniofacial features including premature fusion of the coronal suture, dome-shaped skulls, wide-set bulging eyes, and a severely retrusive midface (Figure 4) (Liu et al., 2013; Reardon et al., 1994). Surgical intervention is the only existing treatment.

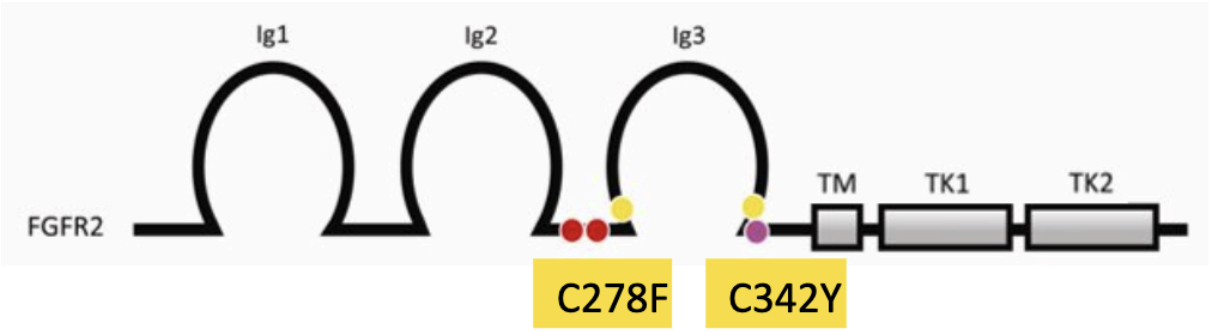

Figure 3. Schematic of FGFR2 and a map of the third domain mutations associated with craniosynostosis. Yellow = Crouzon Syndrome. (Adapted from Figure 3 of Hatch, 2010).

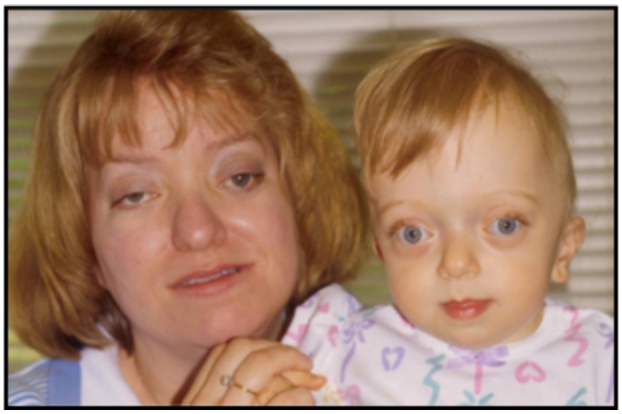

Figure 4. A mother and daughter showing the distinct Crouzon phenotype.

FGFR2 and Neurodevelopment

The Central Nervous System

Genes associated with craniosynostosis are known to play important roles in the development of the brain and nervous system. In fact, previous literature has established various relationships between FGFR2 specifically and the central nervous system (CNS). When normal FGFR2 production is disrupted in the telencephalon, rates of proliferation and cell death are altered (Ever et al., 2007). Similarly, in FGFR2 conditional knockout mice in radial glial cells, severe brain defects are observed. Such effects often include a lack of corpus callosum and hippocampal fissure, decreased cortical volume, and reduced surface area and thickness of the cortex, notably in the medial prefrontal cortex (Stevens et al., 2010). These findings confirm the integral role of FGFR2 in mediating cell proliferation and differentiation not only in bone, but also in the CNS.

Studies have also noted relationships between FGFR2 and cognitive development. In a study using conditional *Fgfr2* knockouts in embryonic and adult mouse hippocampal progenitor cells, significant memory and learning deficits were observed. These results were obtained through Morris Water Maze and object recognition trials, establishing both short- and long-term shortfall (Stevens et al., 2012). This strongly suggests that FGFR2 plays a role in the encoding of memories.

In addition to hippocampal cells, embryonic and neonatal loss of *Fgfr2* in mouse astroglia has shown to cause behavioral changes. Significant changes for both stages of knockouts include

locomotor hyperactivity, increased sociability, reduced anxiety-like behavior, and decreased working memory capabilities (Stevens et al., 2023). These results bring to mind the biological breadth FGFR2, beyond physical cell changes. Studies such as this one pose questions about whether *FGFR2* mutations might be involved in neurodevelopmental disorders such as Attention Deficit Hyperactivity Disorder (ADHD), as well as anxiety and depression.

Neurodevelopmental Disorders

Recent literature has tried to answer this question of a relationship between *FGFR2* mutations and neurodevelopmental disorders through forward genetics. In particular, an increasing number of individuals with severe Autism Spectrum Disorder (ASD) also exhibitting *FGFR2* mutations are being discovered. A genotyping study of children showing signs of ASD found an inherited $FGFR2^{A1295G}$ mutation with equivocal dysmorphology such as deep-set eyes and dolichocephaly (Tammimies et al., 2015). Additionally, a case study of an individual with a rare skin condition caused by a known $FGFR2^{C382R}$ mutation, was also found to have ASD (Gracia-Darder et al., 2023). Another study reported three siblings with neuropsychological impairments representative of ASD, caused by unbalanced translocation of the chromosome harboring the *FGFR2* gene (Coci et al., 2017). A comprehensive review correlated these findings with a 12-year old male patient showing severe ASD and ADHD, carrying a de novo *FGFR2* mutation (Nicotera et al., 2023). Although the literature is limited, these few reports suggest a potential relationship between neurodevelopment and ASD and the presence of an *FGFR2* gene mutation.

In addition to human studies, the link between FGFR2 and neurodevelopment has been investigated using a mouse model. Mice with downregulated *Fgfr2* expression in the somatosensory cortex showed changes in core behaviors related to ASD (Szczurkowska et al., 2018). This literature further suggests a causality between FGFR2 function and neurocognitive development disorders like that of ASD, although more investigation is required. The association could have vast implications for identifying and treating these disorders. Given that one of the most common results of an *FGFR2* somatic mutation is craniosynostosis, this literature raises the question of whether *FGFR2*-associated craniosynostosis syndromes are linked to defects in the CNS.

Neurological Consequences of Craniosynostosis

Craniosynostosis is known to be associated with neurological problems. Primarily, the premature fusion of a cranial suture often causes increased intracranial pressure (ICP), which can be related to cognitive defects. In a clinical study of 92 children, a higher ICP resulted in lower IQ (Renier et al., 1982**)**. These results were later built upon in a comprehensive review of single-suture sagittal craniosynostosis patients with more extensive IQ testing. A significant decrease in verbal IQ and performance IQ was observed, pointing to visual-motor impairment (Magge et al., 2002). Additionally, patients studied showed much higher rates of learning disabilities than the non-affected population (Magge et al., 2002). For individuals with craniosynostosis, higher ICP was associated with higher rates of learning disabilities (Magge et al., 2002).

Syndromic-specific craniosynostosis has also been studied with regards to learning disabilities. Behavioral testing of children with Apert and Muenke syndromes has revealed that craniosynostosis puts a child at increased risk for developing an intellectual disability, internalizing, and social and attentional issues (Maliepaard, 2014). Higher levels of problems surrounding behavior and emotion were also observed (Maliepaard, 2014). Among patients with general craniofacial abnormalities, not limited to craniosynostosis, one in ten children are later identified to have an intellectual disability (Junaid et al., 2022). This rate proved to be much higher than that of children with other birth defects such as gastrointestinal, urogenital, and musculoskeletal. Interestingly, higher rates of ASD were also observed in patients with craniofacial abnormalities (Junaid et al., 2022).

Individuals with *FGFR2*-associated craniosynostosis syndromes have also been studied in the neurological setting. Fetuses with Pfeiffer Syndrome (PS) were observed to have brain abnormalities such as megalencephaly, ventricular dilation, and enlarged temporal lobes (Khonsari et al., 2012). The paper suggests these anomalies are cause for cognitive screening in PS infants and could explain increased levels of cognitive impairment (Khonsari et al., 2012). Similarly in a study of Apert Syndrome patients, another *FGFR2*-associated craniosynostosis syndrome, psychometric research revealed that CNS malformations caused by the syndrome resulted in severe mental deficits (Cohen and Kreiborg, 1990). Although significantly more research is needed, literature suggests a relationship between craniosynostosis syndromes and

cognitive deficits. Evidence supports that this relationship also exists in individuals with Crouzon Syndrome specifically.

Neurological and Psychological Consequences of Crouzon Syndrome

It's clear that high ICP can have devastating neural consequences. Many individuals with Crouzon Syndrome have high ICP, with one study finding that 61.2% of individuals have experienced at least one instance of high ICP. (Abu-Sittah et al., 2016). In cases where high ICP is not relieved, severely distorted ocular globe morphology is possible. It has been observed that individuals with Crouzon Syndrome have markedly different eye shapes in all three dimensions, often resulting in vision impairment with at least one documented case of glaucoma (Bhattacharjee et al., 2022). A second case report also notes Crouzon Syndrome-induced optic nerve atrophy increasing vision impairment in one eye, as well as instances of cornea disease and crossed eyes (Pal et al., 2012). Given that many individuals with Crouzon Syndrome receive surgery to relieve high ICP, research of the optic consequences of Crouzon Syndrome-induced ICP is limited in scope.

In addition to ophthalmic issues, Crouzon Syndrome has been found to result in brain abnormalities such as ventriculomegaly and reduced thickness of the corpus callosum (Pal et al., 2012). There is also evidence to suggest individuals with Crouzon Syndrome may experience compromised learning and developmental issues, similar to other craniosynostosis syndromes. In two independent case studies where the individual did not undergo surgical intervention, Crouzon Syndrome-associated intellectual disabilities, previously known as mental retardation, was reported (Padmanabhan et al., 2011; Balyen et al., 2017).

Recent studies have revealed that the dysmorphic craniofacial features of Crouzon Syndrome can also result in negative psychosocial consequences for affected individuals. A study of 31 adults with Crouzon Syndrome revealed a rate of bullying three times higher than that of the control group without Crouzon Syndrome, with 33% of the patients reporting suicidal thoughts (Fischer et al., 2014). The study additionally found significantly lower education levels among individuals with Crouzon Syndrome (Fischer et al., 2014). A similar review found a distinct decline in quality of life in Crouzon Syndrome patients as compared to those without any

craniosynostosis, and notably poor participation in society compared to other syndromes (Sakamoto et al., 2021**)**. The article suggests that because Crouzon Syndrome most typically presents with only facial deformities, patients are not recognized as disabled and thus expected to integrate normally into society (Sakamoto et al., 2021**)**.

This literature highlights the importance of more research into Crouzon Syndrome in order to relieve affected individuals of these debilitating pathological and social consequences. Investigations into how *FGFR2* mutations result in the Crouzon Syndrome phenotype are critical to learning more about the mechanisms of the disease, which opens up the possibilities of finding potential therapeutics or adjunctive therapies to lessen the morbidity associated with craniofacial surgical correction.

FGFR2 Crouzon Mutations

Expression

Crouzon Syndrome is linked to several point mutations in the third immunoglobulin (Ig-3) domain of FGFR2. In wild type FGFR2, a disulfide bridge between C278 and C342 exists to stabilize Ig-3 (Figure 5A). The most common Crouzon Syndrome mutations are those which substitute either member of this bridge, typically C278F or C342Y, causing a remaining free cysteine (Figure 5B) (Kress et al., 2000).

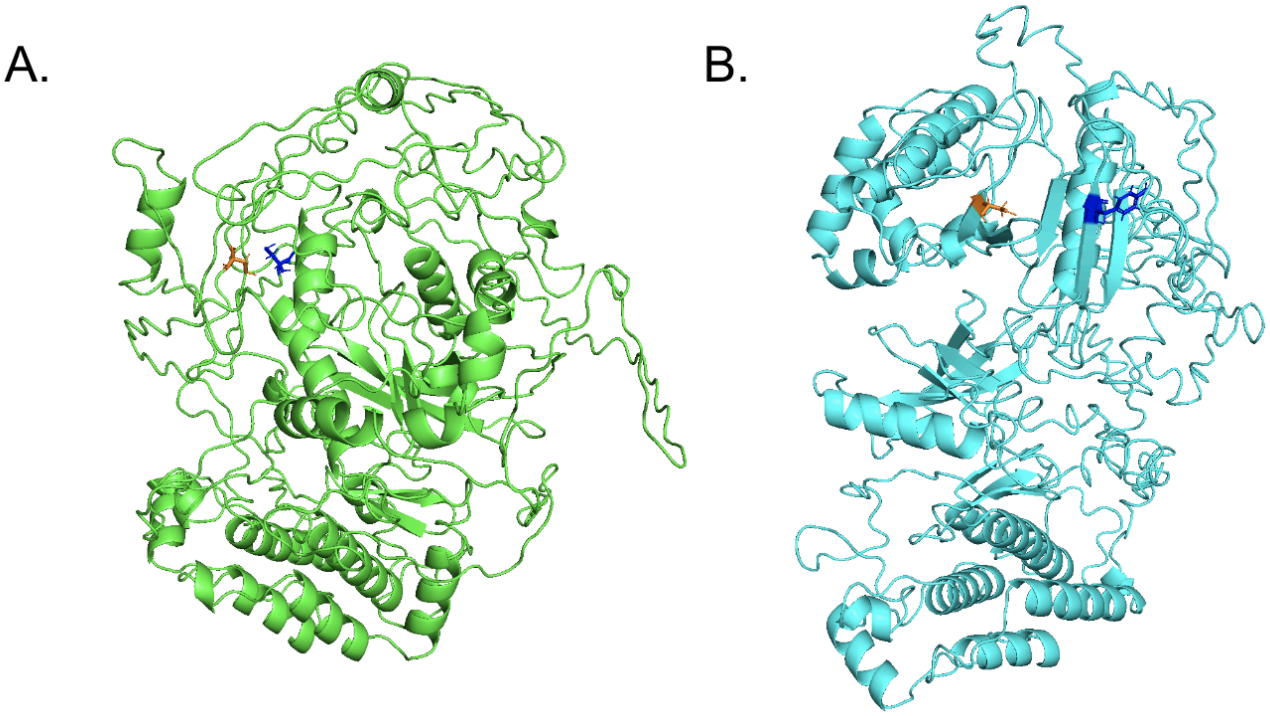

Figure 5. A. Wild type FGFR2 protein structure representation depicting proximity between C278 (orange) and C342 (blue) residues **B.** FGFR2^{C342Y} protein structure representation demonstrating how the C342Y mutation causes loss of the disulfide bond, leaving the C278 residue exposed. (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

When the native cysteine bond is intact, FGFR2 can become phosphorylated to produce downstream signals in the presence of the FGF ligand. However, in both C278F and C342Y Crouzon mutations, a cysteine residue is lost, causing its binding partner to "inappropriately" bind to another residue. In a *Xenopus* in vitro model, this has been shown to result in a mutant dimer (Robertson et al., 1998). It has been established in a fibroblast cell line, NIH3T3, that a significant proportion of mutant receptors exist as dimers (Mangasarian et al., 1997). Both the dimer and mutant monomer also show evidence of phosphorylation in the absence of its FGF signal, pointing to ligand-independent phosphorylation**,** although this finding was obtained through non-mammalian model organisms (Mangasarian et al., 1997). This evidence suggests that the Crouzon mutations may result in an FGFR2 receptor that is always active (Robertson et al., 1998; Mangasarian et al., 1997).

Similar studies have come to the same conclusion specifically with the C342Y mutation. Based on the appearance of a phosphorylated dimer under non-reducing conditions, authors suggested that C342Y in humans is “activating” or “gain of function” (Neilson and Friesel, 1995; Galvin et al., 1996). However, these experiments were performed using *Xenopus* frog eggs as the model organism, which are less analogous to humans than mouse cells and specifically cranial bone cells.

Published *in vivo* data may contradict this conclusion that the C342Y mutation is “gain of function”. While the similarities have not been explicitly linked in the literature, phenotypic similarities have been observed between the *Fgfr2*$^{C342Y/+}$ mouse model of Crouzon syndrome with that of the *Fgfr2IIIc-/-* mouse. Both mouse models exhibit coronal suture fusion, midface hypoplasia, and small body size (Eswarakumar et al., 2004; Liu et al., 2013). When bred together, the resulting *Fgfr2*$^{C342Y/–}$ mouse exhibits more severe craniofacial defects (Pfaff et al., 2016), suggesting that the Crouzon syndrome-associated mutation is not “activating” or cannot rescue the loss of the WT *Fgfr2* allele (Figure 6). This contradiction suggests there might be more to the C342Y mutation than how it functions in the membrane. This prompted the Hatch lab to investigate how FGFR2 and FGFR2^{C342Y} are trafficked and potentially degraded in the cell.

Figure 6. Alizarin-red stained mouse skulls depicting craniofacial skeletal growth (Pfaff et al., 2016).

Trafficking and Degradation

FGFRs are known to be N-glycosylated, with expression in fully glycosylated, partially glycosylated, or unglycosylated forms (Feige and Baird, 1988). Fully glycosylated FGFR2 is preferentially expressed on the cell membrane, which suggests that glycosylation plays an important role in cell membrane trafficking (Zhang et al., 2001; Feige and Baird, 1988). In fact,

Crouzon mutant FGFR2 in fibroblast cells shows higher levels of partially glycosylated protein, which suggests fewer receptors are successfully trafficked to the cell membrane (Mangasarian et al., 1997). It has previously been established that the alternate Crouzon mutant protein $FGFR2^{C278F}$ shows diminished glycosylation and increased degradation compared to wild type FGFR2 (Hatch et al., 2006). While the exact degradation methods of this isoform as well as $FGFR2^{C342Y}$ remain unknown, our lab has recently found this conclusion to be consistent in Crouzon *Fgfr2*$^{C342Y/+}$ mouse primary calvarial cells (Figure 7).

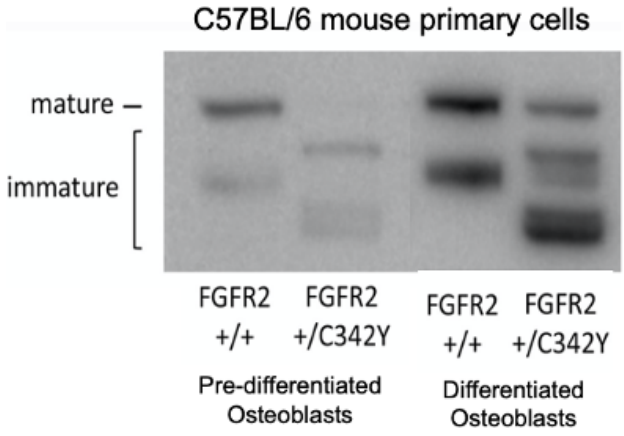

Figure 7. Primary calvarial cells from Crouzon *Fgfr2*$^{C342Y/+}$ mice exhibit incomplete glycosylation compared to cells obtained from wild type mice. Results obtained by Hwa Kyung Nam of the Hatch Lab.

Many intracellular protein degradation mechanisms exist, with two main pathways (Cooper and Hausman, 2000). The first is known as the ubiquitin-proteasome pathway, where faulty proteins are tagged with ubiquitin. Ubiquitin is then recognized by the proteasome and the protein is degraded using ATP. This pathway plays a major role in regulating proteins involved in the cell cycle (Cooper and Hausman, 2000). The second major pathway is lysosome-mediated degradation, a mainly non-selective process where proteins are taken in by the lysosome and digested by enzymes housed there (Cooper and Hausman, 2000). Many different methods exist to shuttle proteins to either become ubiquitinated or digested in a lysosome. One such method is endoplasmic reticulum-associated degradation (ERAD), where misfolded, unassembled, and mislocalized proteins are targeted and sent to a specific degradation destination (Krshnan, et al., 2022). A second method is autophagy, which might be related to Crouzon Syndrome.

Specific Goals

The goal of this project is to discover more information regarding how the Crouzon mutant FGFR2 protein functions within the cell, specifically its degradation mechanism and how that possibly leads to the craniofacial phenotype. While previous work in the field suggests a constitutively active mutant receptor and thus "gain of function" mutation, phenotypic observations of the mutant and knockout mouse contradict this conclusion (Pfaff et al., 2016). This work aims to shed light on the cause of the discrepancy. As preliminary studies show $FGFR2^{C342Y}$ is exhibiting increased degradation, insight about the mechanism for this degradation is integral to understanding the mutation (Hatch et al., 2006).

The degradation mechanism specifically investigated by this project is autophagy. Autophagy is involved in the breakdown of misfolded and aggregated proteins (Glick et al., 2010). Because the Crouzon mutant has been observed to form a dimer, it is possible that $FGFR2^{C278F}$ is recognized as aggregated and may undergo autophagy. It is known that autophagy dysfunction can promote disease states, and that autophagy levels influence differentiation of osteoblasts (Klionsky et al., 2021). It has also been found that overactive autophagy in calvarial suture mesenchymal cells is an underlying mechanism of nonsyndromic suture fusion (Qiu et al., 2018). This evidence suggests that autophagy could be a mechanism involved with how the Crouzon mutation results in the known phenotype.

In order to influence autophagy in a laboratory setting, we treated cells with rapamycin, a known inducer of autophagy (Ren et al., 2022). Based on our previous findings of Crouzon mutant receptor dimerization, we hypothesize that autophagy is a preferred degradation mechanism of $FGFR2^{C342Y}$. To test this hypothesis, we studied protein amounts in cells following treatment with rapamycin and transient transfection of wild type FGFR2 or $FGFR2^{C342Y}$ plasmids. If autophagy is a preferred degradation mechanism in Crouzon Syndrome, we anticipate observing less $FGFR2^{C342Y}$ protein inside the cells following rapamycin treatment.

This work will ultimately further our understanding of how $FGFR2^{C342Y}$ is intracellularly processed and trafficked, potentially providing more clarity on how the distinct Crouzon

Syndrome phenotype arises. The neurological consequences of the disease can be devastating to affected individuals, and information gained from this project has the potential to inspire therapeutics for individuals with Crouzon Syndrome and ultimately improve their quality of life.

Materials and Methods

Cell Culture, Treatment, and Transfection

MC3T3-E1 mouse primary calvarial cells were thawed at passage 15 and cultured in alpha-MEM media fortified with 10% fetal bovine serum and 5% penicillin/streptomycin blend. Cells were incubated in a humid environment at 37°C with 5% CO2 and split when an estimated 80% confluency was reached. At passage 18, cells were counted via hemocytometer and then seeded in 9.6 cm^2 6-well plates.

In experiments where rapamycin treatment and transfection took place (experiments 1-2), cells were seeded at a density of $2.5x10^5$ cells/well in 1.5 mL media and treated either with 25nM rapamycin or equal volume DMSO as control. Twenty-four hours later, cells were transiently transfected with 1.2μg of either $FGFR2^{C342Y}$ or wild type FGFR2 V5-tagged plasmids and 4.5μL Attractene. Three hours after transfection, the complex-saturated media was removed and replaced with fresh media. The treatment and transfection resulted in six different groups of lysate: control MC3T3-E1 cells with and without rapamycin, FGFR2-V5 transfected cells with and without rapamycin, and $FGFR2^{C342Y}$-V5 transfected cells with and without rapamycin. Lysates were harvested 24 hours later.

In experiments where rapamycin optimization took place (experiments 3-4), cells were seeded at a density of $2.25x10^5$ cells/well in 2 mL media and non-control wells were treated with 25nM rapamycin (experiment 3) or 50nM rapamycin (experiment 4). Four time points were arbitrarily selected for treatment (24, 6, 3, and 1 hours prior to harvest) with a control group receiving no treatment, resulting in five groups of lysate.

Lysate Harvest and Protein Assay

Prior to collection, plates were placed on ice and each well was rinsed in a 4°C PBS/sodium orthovanadate solution. Lysates were collected using 60μL of a custom mix containing RIPA Lysis Buffer, protease inhibitor cocktail, phenylmethylsulfonyl fluoride, and sodium orthovanadate. Centrifugation was used to remove insoluble material, with the final product stored at -80°C.

Bicinchoninic Acid (BCA) Protein Assay was performed on the lysates using the Pierce™ BCA Protein Assay Kit and nine different albumin standards in the SpectraMax i3x (Molecular Devices). Microsoft Excel was used to plot the standard absorbance values and estimate the protein concentration of each sample.

Western Blot

Western blots were run using the protocol associated with Invitrogen Bolt Bis-Tris Plus gels, Bolt LDS sample buffer, and MOPS running buffer (ThermoFischer). Lysates were run under reducing conditions (disulfide bonds broken) through the addition of dithiothreitol. Non-reducing conditions (disulfide bonds maintained) did not contain dithiothreitol. Gel-membrane transfer took place under 100V in a standard Towbin transfer buffer for one hour, followed by a standard blocking step in 5% milk for 1 hour.

V5 primary antibody (Invitrogen R960-25) was used to visualize V5-tagged mutant and wild type FGFR2. LC3 (Novus Biologicals NB100-2220) was used to measure autophagy flux, and P62 (Cell Signaling #5114) as a marker of increased or decreased autophagy levels. GAPDH (Cell Signaling #2118) was used for the loading control. Membranes were exposed overnight to antibody-specific standard dilutions– 1:7500 for V5 and 1:1000 for all other primary antibodies. Anti-mouse was used as the secondary antibody for V5 and anti-rabbit was used for the remaining during a two hour incubation period the next morning. Standard rinses in 1x TBST took place after each antibody step. All membranes were visualized through five minute exposure to the SuperSignal™ West Pico PLUS Chemiluminescent Substrate and scanned via the ChemiDoc Imaging System (Bio-Rad).

Adobe Photoshop 2024

Adobe Photoshop 2024 was used in standard fashion to compile, crop, and label each western blot membrane image to create Figures 8-11.

Densitometry

ImageJ Software was used to perform densitometry measurements on each blot and quantify protein amount in ratio to the loading control. Microsoft Excel was used to compile the raw ImageJ data and visualize it into the graphs presented in Appendices 1-4.

PyMOL

Previously obtained lab pdb files for the mutant and wild type FGFR2 protein were imported into PyMOL. The residue identification and labeling tools allowed for the creation of Figure 5.

Results

Experiment 1

Cells were treated with rapamycin to induce autophagy and later transiently transfected with either FGFR2^{C342Y} or wild type plasmids. In both reducing and non-reducing blots, immature and mature wild type bands had a higher protein amount when rapamycin was added (Figure 8). This observation was verified by densitometry (Appendix 1A, 1B, 1D, 1E). Alternatively, FGFR2^{C342Y}-transfected cells showed lower protein amounts in the blots when treated with rapamycin. This was confirmed with densitometry for the reducing immature, non-reducing mature, and non-reducing immature bands (Figure 8, Appendix 1B, 1D, 1E). LC3 showed little change between treated and untreated cells (Figure 8). Densitometry of LC3 also showed no clear trend between treatment and autophagy flux (Appendix 1C, 1F).

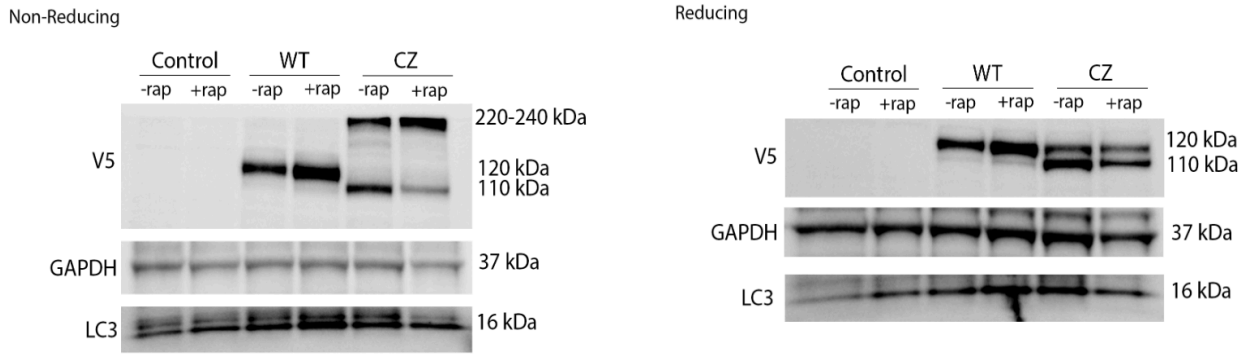

Figure 8. Experiment 1 Western blot results, displaying both reducing and non-reducing membranes. Higher protein amounts are visible in wild type groups treated with rapamycin (25nM, 51 hours prior to harvest). Lower protein amounts are visible in FGFR2^{C342Y} groups treated with rapamycin. LC3 shows no clear trend.

Experiment 2

Experiment 2 served as a repetition of experiment 1 in hopes of gaining more clarity on how rapamycin was influencing protein amount. Experiment 2 was consistent with experiment 1 in that wild type immature and mature bands showed a higher protein amount when rapamycin was added (Figure 9). This was quantified through densitometry calculations (Appendix 2A, 2B, 2D, 2E). An increase in protein amount following rapamycin treatment was also observed in the cells transfected with FGFR2^{C342Y}. (Figure 9, Appendix 2A, 2B, 2D). In contrast to experiment 1, both

reducing and non-reducing blots showed a notable increase in autophagic flux when rapamycin was added, indicating a change in autophagy activity (Figure 9, Appendix 2C, 2F).

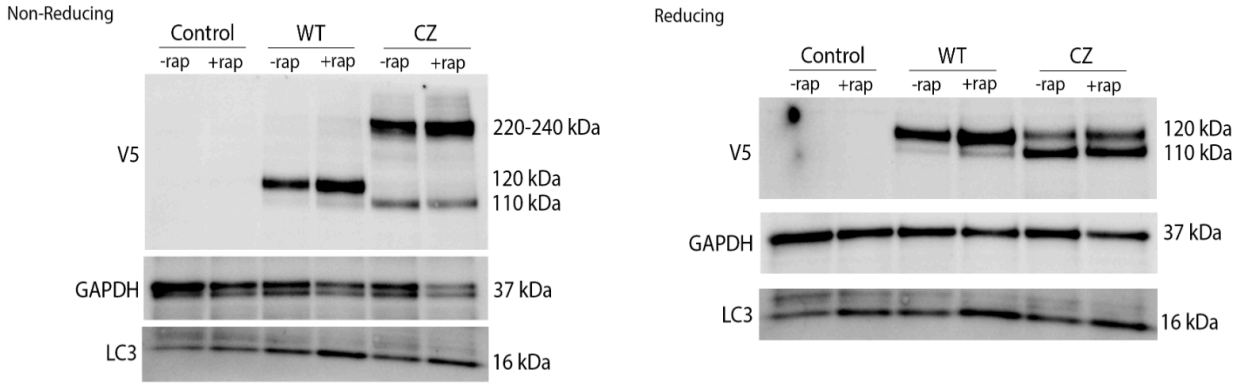

Figure 9. Experiment 2 Western blot results, displaying both reducing and non-reducing membranes. Higher protein amounts are visible in wild type groups treated with rapamycin (25nM, 51 hours prior to harvest). Higher protein amounts are also visible in $FGFR2^{C342Y}$ groups treated with rapamycin. LC3 shows an increase in protein amount when rapamycin was added.

Both trials fail to present consistent evidence of a correlation between rapamycin treatment, autophagic flux, and protein amount. This prompted us to perform a series of rapamycin optimization experiments in order to determine the most effective time and concentration of rapamycin to induce autophagy in MC3T3-E1 cells.

Experiment 3

Cells were treated with rapamycin at different time points and autophagy activity was visualized through both P62 and LC3 antibodies to determine the ideal collection time point that results in the most potent induction of autophagy. The P62 antibody indicates high levels of autophagy with a lower protein amount. The P62 bands at 1 and 6 hour treatment times showed higher autophagy levels than the other times (Figure 10). Densitometry analysis suggests that the 1 hour time point has the greatest change in autophagy compared to the control sample (Appendix 3A). Qualitatively, there is little difference in the LC3 bands (Figure 10). Densitometry analysis may suggest the 6 hour time point has the greatest change in autophagy, however, with only one data point we cannot statistically state that there is a difference between treatment groups (Appendix 3B).

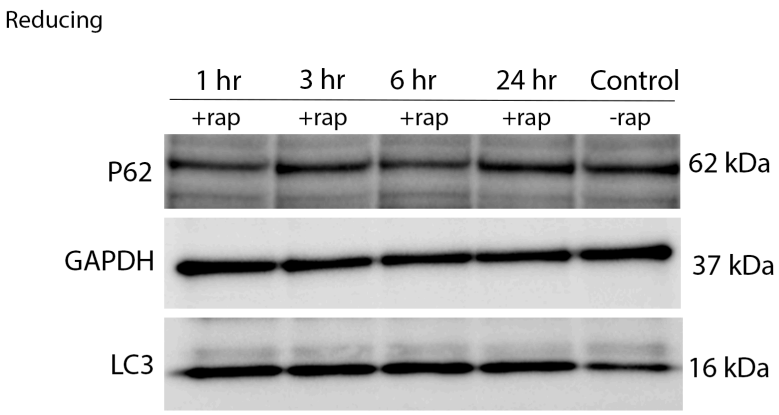

Figure 10. Experiment 3 Western blot results, displaying the rapamycin (25 nM) treatment times prior to harvest. Higher autophagy levels are visible after 1 and 6 hour treatment time points. LC3 shows no clear trend.

Experiment 4

Because we did not observe an obvious change in autophagy following 25nM rapamycin treatment in the previous experiments, we decided to treat the cells with 50nM rapamycin using the same time intervals to see if the previous dose was too weak. P62 revealed a small band at the 6 hour treatment, indicating high levels of autophagy (Figure 11). Densitometry confirmed a difference between the 6 hour treatment group and other time points (Appendix 4A). As with the previous experiment, we only have one replicate of this data, so we cannot make solid conclusions regarding the data due to lack of statistical replicates. The 3- and 24-hour treatment time points showed similar P62 results, with much thicker and darker bands than the 6 hour band (Figure 11). Based on the light LC3 band at the 6 hour time point, there was a change in autophagic flux between the 3 and 6 hour time points as well as the 6 and 24 hour time points (Figure 11). This change was quantified by densitometry (Appendix 4B).

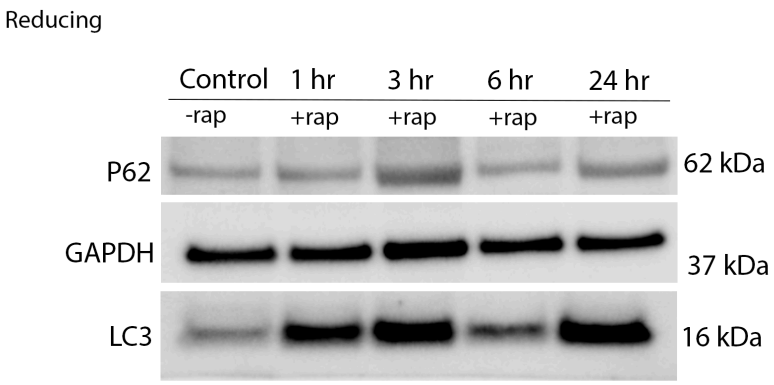

Figure 11. Experiment 4 Western blot results, displaying the rapamycin (50nM) treatment times prior to harvest. High autophagy activity clearly visibly at the 6 hour time point based on P62 band. LC3 indicates autophagic flux across all measured time points.

When comparing the results of experiments 3 and 4, a higher concentration of rapamycin suggests greater differences in protein amount for both P62 and LC3 (Figure 10, 11). While experiment 3 showed a similar protein amount for each time point, experiment 4 revealed varied blot darkness and sizing between groups, which indicates a more profound response to treatment (Figure 10, 11).

Discussion

Rapamycin Rebound

The results of experiments 1 and 2 do not provide us with evidence that autophagy is the preferred degradation mechanism of $FGFR2^{C342Y}$. The observation of greater protein amounts in samples that were treated with rapamycin was unexpected, given that rapamycin is an inducer of autophagy. A possible explanation for this phenomenon is that prolonged exposure to rapamycin could actually cause a rebound effect, where protein translation is actually increased. Rapamycin is known to inhibit mTORC1, a complex that controls ribosome recruitment for translation (Rabanal-Ruiz et al., 2017; Wang and Zhang, 2019). mTORC1 operates through the phosphorylation of two downstream substrates: 4E-BPs and S6Ks (Rabanal-Ruiz et al., 2017; Wang and Zhang, 2019). Rapamycin was found to inhibit S6K activity for the duration of treatment, however, 4E-BP was only disrupted within the first 6 hours of rapamycin treatment (Choo et al., 2008). After 6 hours of rapamycin treatment, 4E-BP was re-phosphorylated and protein translation was able to proceed (Choo et al., 2008). This unexpected finding from this one study could serve as a possible explanation of how translation was able to continue after we treated cells with rapamycin. However, we would have to conduct more experiments in order to prove that this is actually occurring in our current system.

It is important to note that the experiments performed in this study utilized the MC3T3-E1 cell type, which were not used by Choo et al. In order to support the idea that a rapamycin rebound effect was responsible for the results of our experiments 1 and 2, evidence is needed surrounding whether 4E-BP rapamycin resistance exists in MC3T3-E1 cells. However, because it is possible that rapamycin is losing efficacy after several hours, we hoped performing experiments 3 and 4 may reveal the optimal time to harvest lysates after treatment.

Rapamycin Efficacy

The data in experiment 4 suggest a greater change in autophagy with a higher concentration of rapamycin. The lower concentration in experiment 3 could explain why the results were inconclusive and why we were unable to identify a clear effect on autophagy activity.

Experiment 4 provides some insight into the efficacy of rapamycin on MC3T3-E1 cells. It appears that autophagy becomes active after about 1 hour of rapamycin treatment, and as suggested above, the effects of rapamycin may expire overtime. With this information, it makes sense that the 24 hour treatment group would have low autophagy activity if the effects of rapamycin wore off. Similarly, we'd expect the 1 and 3 hour treatment groups to have low activity, because autophagy would not have been active for very long at the time of lysate collection.

This interpretation aligns well with what was observed in the 6 hour treatment group where P62 revealed high autophagic activity and LC3 showed a stark difference in flux between the 3 and 24 hour time points (Figure 11, Appendix 4A, 4B). Analysis of experiment 4 suggests that 50nM rapamycin treatment 6 hours prior to collecting lysates will result in the most potent induction of autophagy.

Future Directions

We should first repeat the rapamycin optimization experiments, adding varying concentrations and time points, to either support or refute the interpretation provided above. This will allow us to draw statistically significant conclusions regarding the effect of rapamycin on autophagy prior to treating MC3T3-E1 cells that have been transfected with plasmids.

After the rapamycin protocol is optimized, experiment 1 should be repeated using the optimal rapamycin concentration and treatment time. Based on experiment 4, this would likely mean treating the cells with 50 nM rapamycin and harvesting the lysates 6 hours later. This is different from the original protocol, which used 25 nM rapamycin and harvested lysates a total of 51 hours after treatment. Based on those results, more data replicates are needed to draw any specific conclusions to test the hypothesis that autophagy is the preferred degradation mechanism of $FGFR2^{C342Y}$.

If results continue to be inconclusive, it would be important to investigate other possible mechanisms of degradation for the Crouzon mutant FGFR2 protein, such as the ubiquitin-proteasome pathway. Another aspect of this project that would be interesting to explore

is the intracellular interaction between wild type FGFR2 and FGFR2^{C342Y}. An understanding of whether these proteins interact *in vitro*, and in what cellular compartments, could reveal if the mutant protein has a dominant negative effect on wild type FGFR2. Results could shed light on the discrepancy between *Xenopus* experiments that have suggested a constitutively active receptor and published *in vivo* data.

Concluding Remarks

The work presented here can help provide insight on whether or not autophagy is the preferred degradation mechanism for the Crouzon mutant FGFR2 protein. Information about how FGFR2^{C342Y} is degraded is integral to understanding more about how the mutation causes the distinct craniofacial phenotype. It also has potential to shed light on the correlation between FGFR2 mutations, Crouzon Syndrome specifically, and the neurodevelopmental defects that have been observed. Gathering more information on the neurodevelopmental aspects of Crouzon Syndrome is also critical to improving quality of life for individuals who live with this condition.

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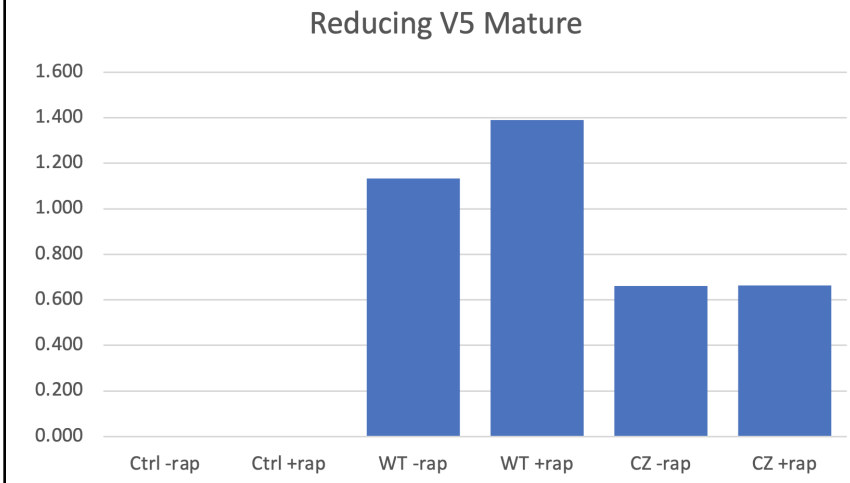
Appendices

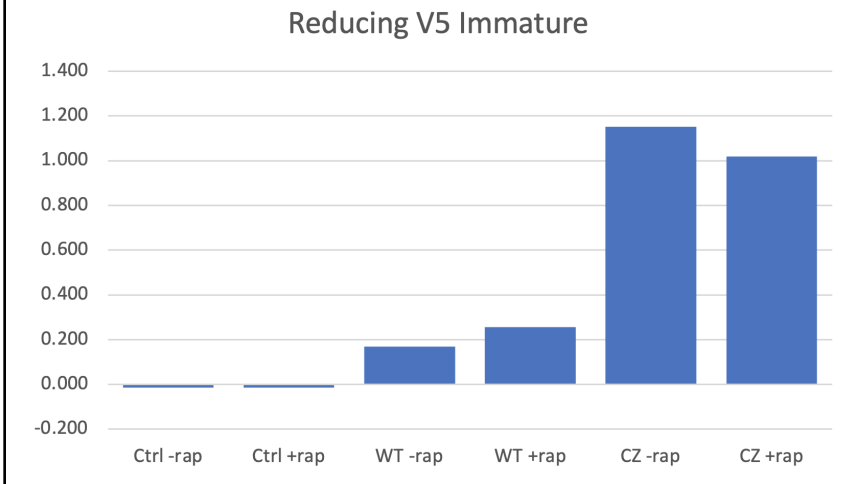
Appendix 1

Densitometry data associated with Figure 8.

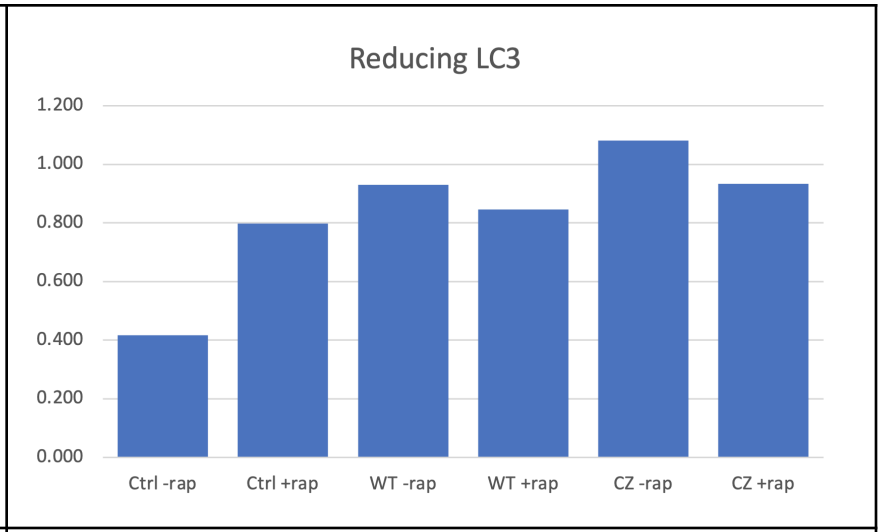
A.



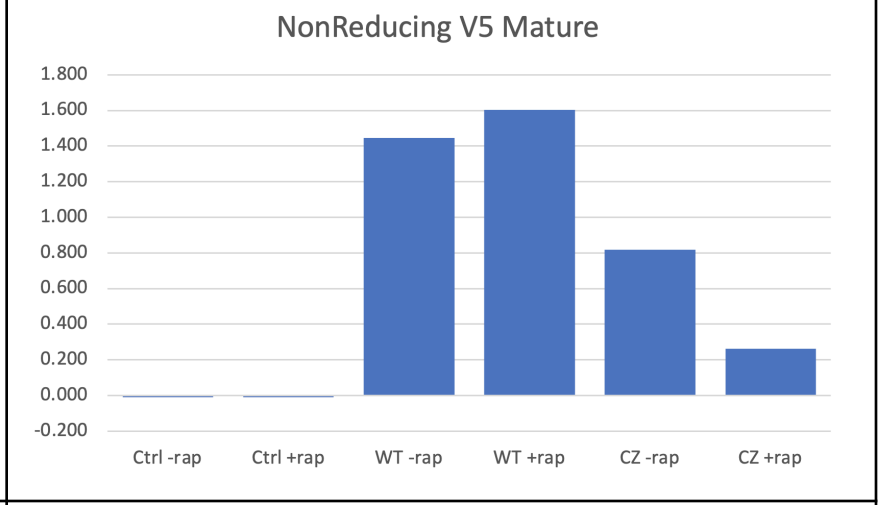
B.



C.


Reducing LC3
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Ctrl -rap
Ctrl +rap
WT -rap
WT +rap
CZ -rap
CZ +rap

D.


NonReducing V5 Mature
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
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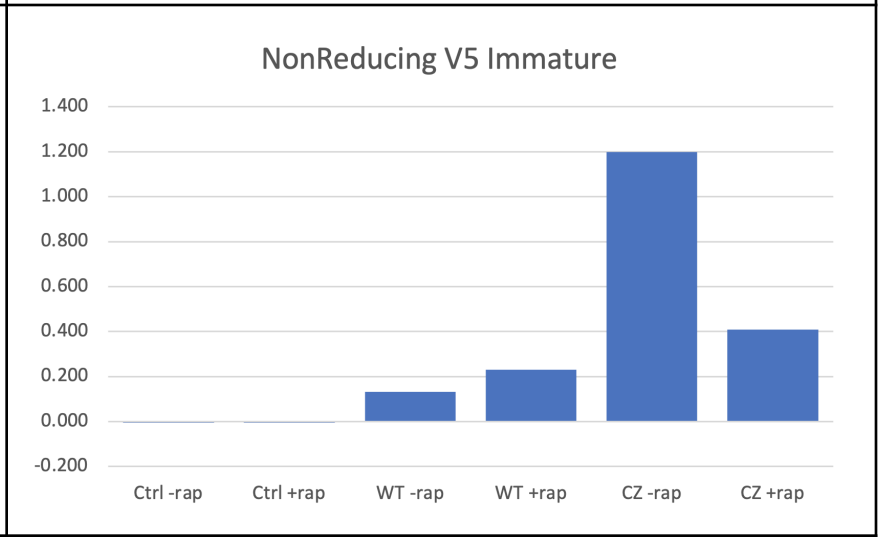
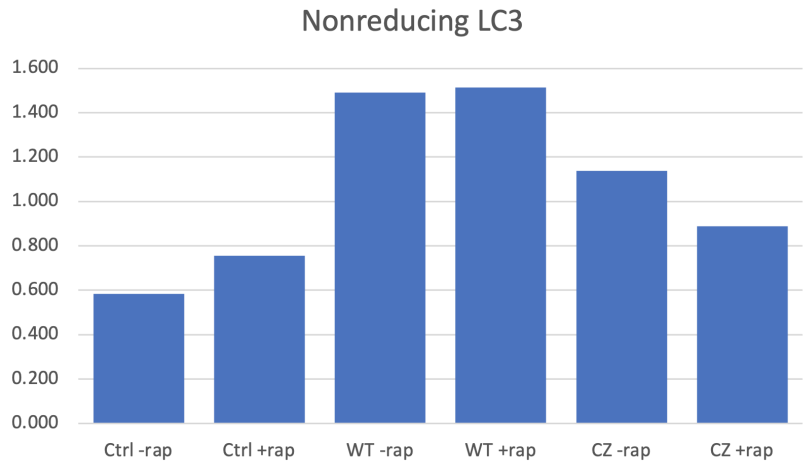
0.000
-0.200
Ctrl -rap
Ctrl +rap
WT -rap
WT +rap
CZ -rap
CZ +rap

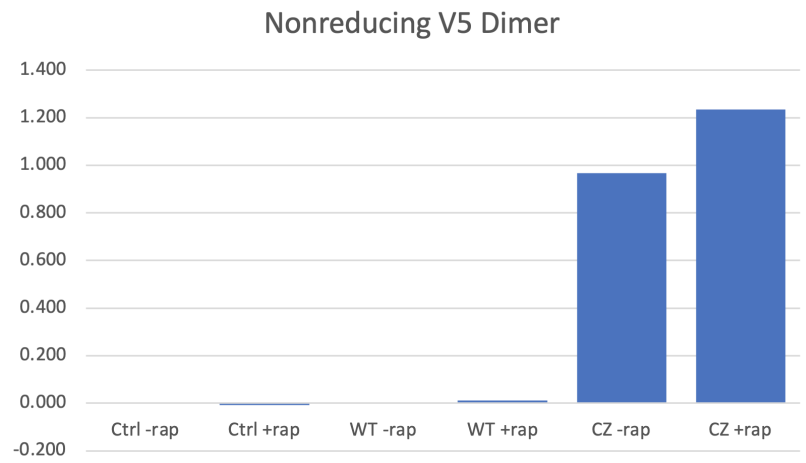
E.


NonReducing V5 Immature
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
-0.200
Ctrl -rap
Ctrl +rap
WT -rap
WT +rap
CZ -rap
CZ +rap

F.


Nonreducing LC3
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Ctrl -rap
Ctrl +rap
WT -rap
WT +rap
CZ -rap
CZ +rap

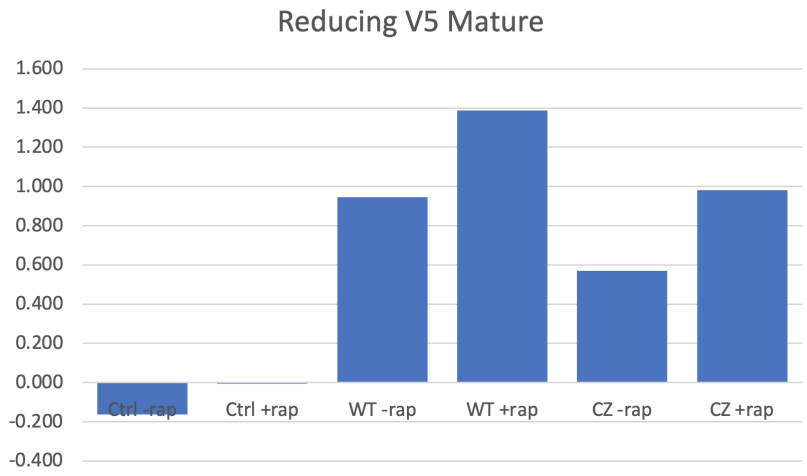
G.


Nonreducing V5 Dimer
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
-0.200
Ctrl -rap
Ctrl +rap
WT -rap
WT +rap
CZ -rap
CZ +rap

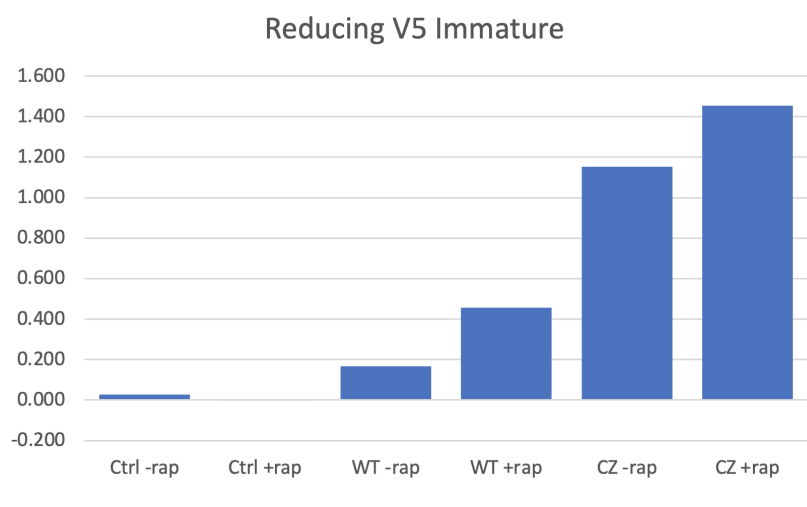
Appendix 2

Densitometry data associated with Figure 9.

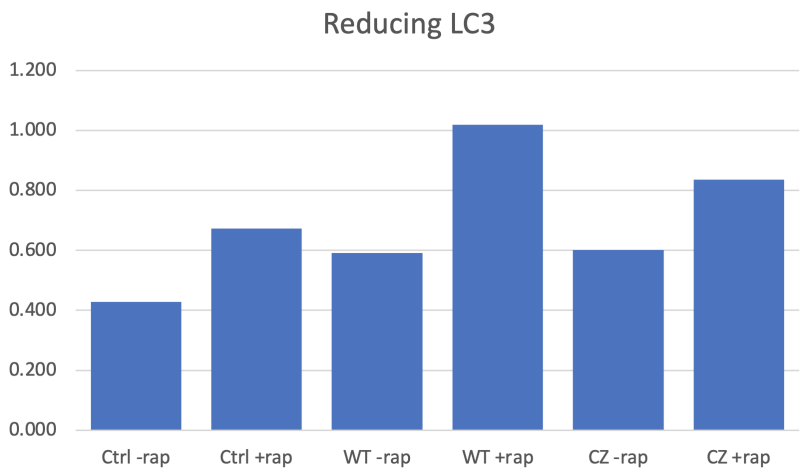
A.



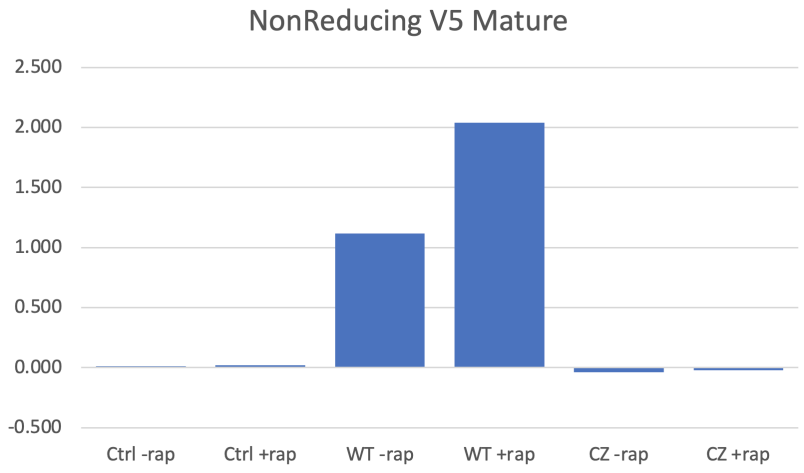
B.



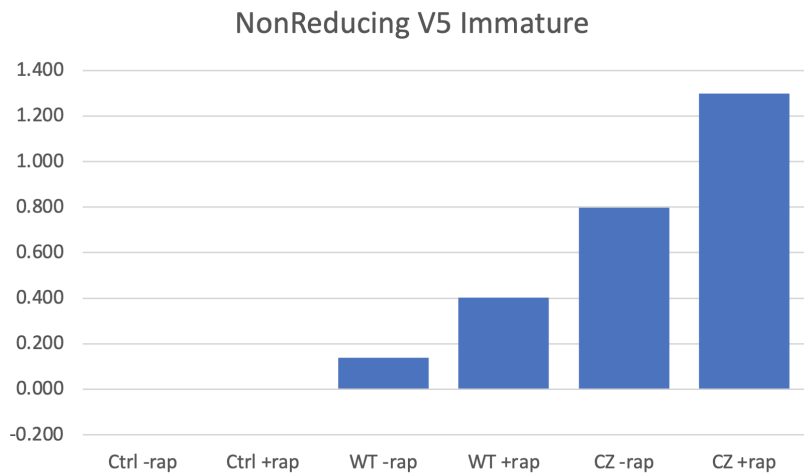
C.


Reducing LC3
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Ctrl -rap
Ctrl +rap
WT -rap
WT +rap
CZ -rap
CZ +rap

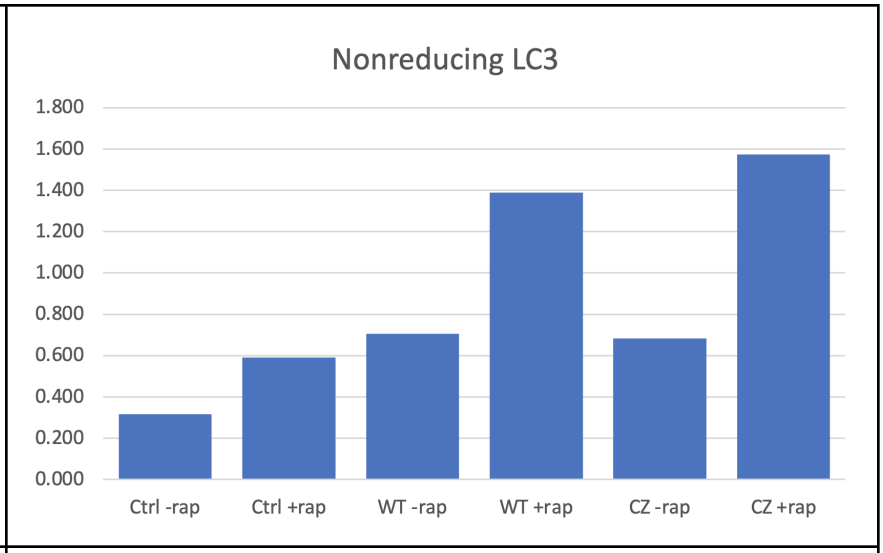
D.


NonReducing V5 Mature
2.500
2.000
1.500
1.000
0.500
0.000
-0.500
Ctrl -rap
Ctrl +rap
WT -rap
WT +rap
CZ -rap
CZ +rap

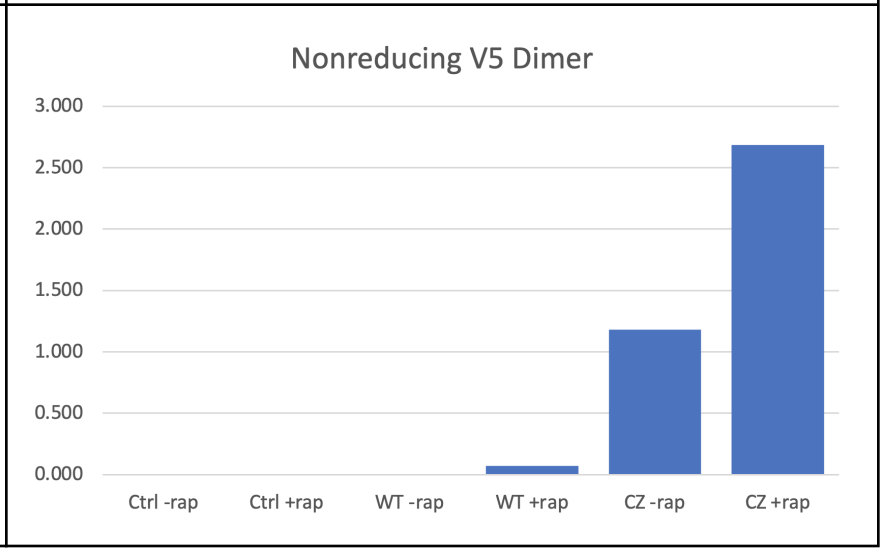
E.


NonReducing V5 Immature
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
-0.200
Ctrl -rap
Ctrl +rap
WT -rap
WT +rap
CZ -rap
CZ +rap

F.


Nonreducing LC3
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Ctrl -rap
Ctrl +rap
WT -rap
WT +rap
CZ -rap
CZ +rap

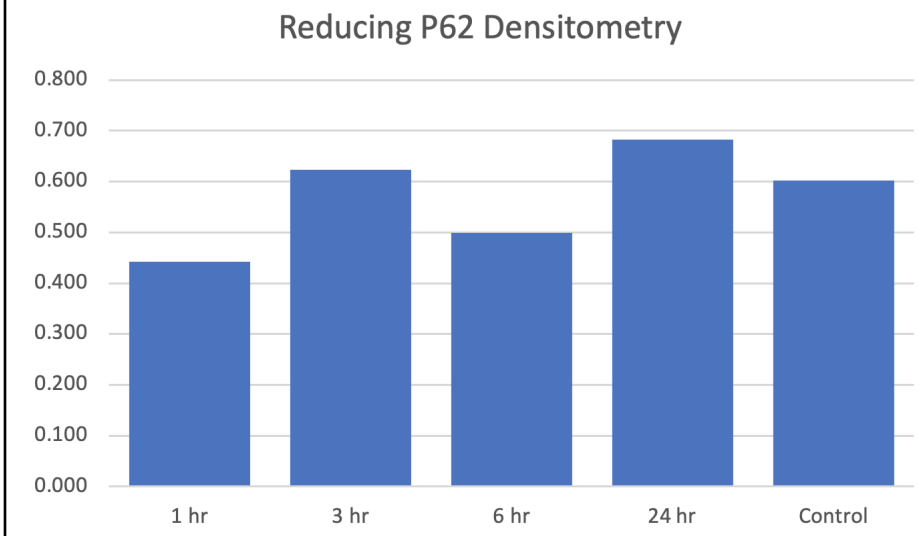
G.


Nonreducing V5 Dimer
3.000
2.500
2.000
1.500
1.000
0.500
0.000
Ctrl -rap
Ctrl +rap
WT -rap
WT +rap
CZ -rap
CZ +rap

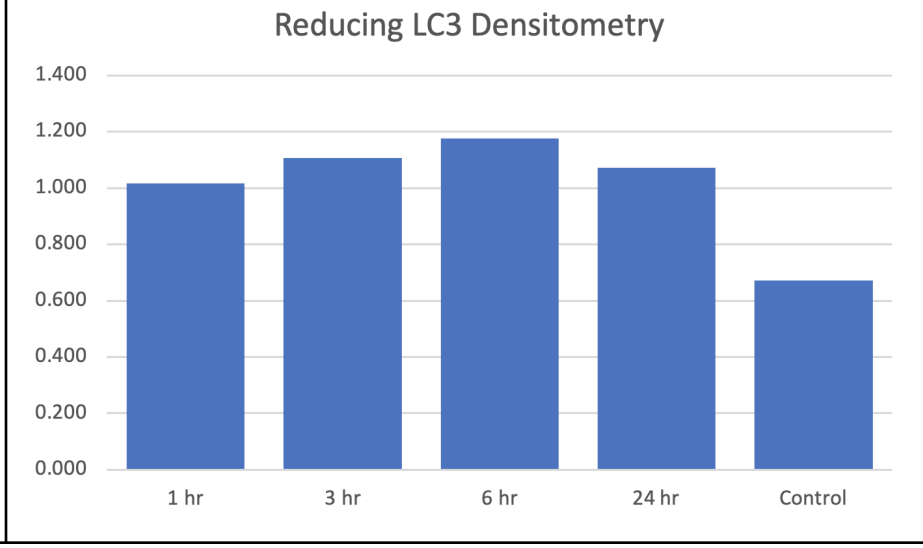
Appendix 3

Densitometry data associated with Figure 10.

A.



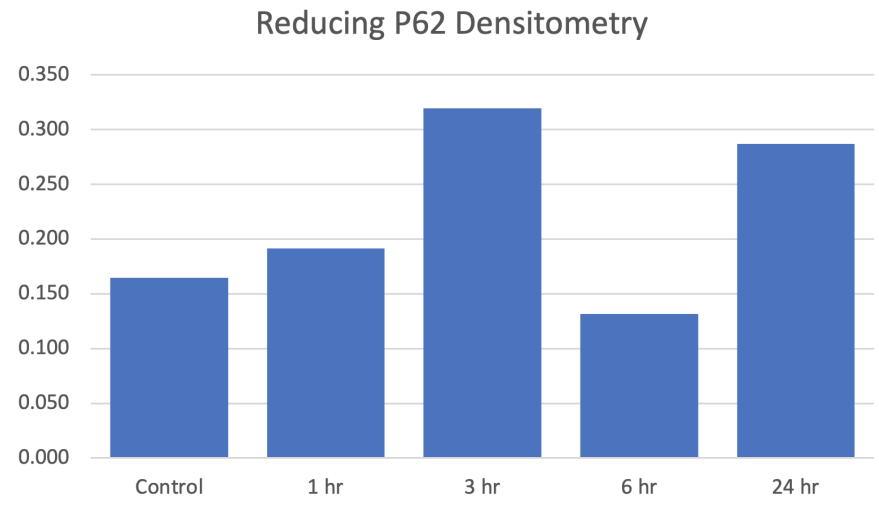
B.



Appendix 4

Densitometry data associated with Figure 11.

A.



B.