Investigating Potential Multi-Protein Complexes Involving Emx2

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INVESTIGATING POTENTIAL MULTI-PROTEIN COMPLEXES INVOLVING EMX2

A Thesis
Presented to
The Graduate Faculty
Central Washington University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Chemistry

by
Nicole Renee Enger
December 2019
We hereby approve the thesis of

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ABSTRACT

INVESTIGATING POTENTIAL MULTI-PROTEIN COMPLEXES INVOLVING EMX2

by

Nicole Renee Enger

December 2019

The neocortex of the mammalian brain is allocated into specialized areas during embryonic development. In mice, as well as humans, the neocortex develops four primary areas: somatosensory, auditory, visual, and motor. In the adult neocortex, the boundaries between these four areas are marked by differences in cellular architecture and gene expression patterns. However, these physical boundaries are not evident during embryonic development of this structure. Rather, the neocortex initially appears uniform across its expanse, with one exception: a handful of proteins that regulate neocortical arealization are generated in gradients across the expanse of the neocortex and control the size of the future neocortical areas. The most highly characterized protein involved in this process is Emx2. Previous researchers in our lab have identified two Emx2-interacting proteins, Cnot6l and QkI (which exists in three forms) that interact with each other. These interactions were initially identified by a yeast two-hybrid screen and then confirmed by pull-down assays. The goal of this project is to use GST pull-down assays determine whether or not various combinations of these five proteins can interact to form a larger complex. The results indicate that larger protein complexes can be formed between Emx2 with Cnot6l and any of three QkI isoforms, as well as larger complexes are formed with
Emx2 and any combination of two of the three QkI proteins. The QkI isoforms can also
form larger complexes with each other and with Emx2 and Cnot6l. However, it was
determined that QkI-6 and QkI-7 may or may not interact with QkI-5 and Cnot6l
simultaneously. These results are inconclusive due to the two proteins only differing by
six amino acids and overlapping of the two proteins on the resulting western blots.
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CHAPTER I
INTRODUCTION

Introduction to the Neocortex

The largest region of the mammalian forebrain is the neocortex, which comprises the outermost layer of the cerebral cortex. The neocortex is the major subdivision of the cerebral cortex and is located between two other regions, the archicortex (hippocampus) and the paleocortex (olfactory cortex) (Manuel et al., 2015). The neocortex has superficial layers comprised of grey matter containing cell bodies and unmyelinated fibers that surround the deeper white matter that is concentrated with myelinated fibers. The neocortex is responsible for higher-order thinking and processes conscious sensory perception, motor functions, and thoughts. The core responsibilities of the neocortex are performed within the four specialized areas of the neocortex; somatosensory area which processes input from touch sensors, visual area which processes input from the eyes, auditory area which processes input from ears, and motor area which is responsible for voluntary muscle movement. In all mammals, the four regions are termed as primary sensory areas and in adults are distinguishable by sharp boundaries marked by differences in cellular architecture and gene expression patterns (O’Leary and Sahara, 2008). The positions of these neocortical areas are conserved across mammalian species (Figure 1), suggesting that the processes involved in the formation of these areas evolved from a common ancestor (Krubitzer, 2007). One example of this is demonstrated by naturally blind animals, such as the blind mole rat (Tobler et al., 1998). The visual area within the neocortex remains present in these blind animals despite a lack of input of visual
information; rather the visual system is used for circadian functions (Tobler et al., 1998). Similarly, these observations are seen when visual loss is experimentally induced; the alteration of size, organization, and connections to the visual area are the same as in naturally blind animals (Krubitzer, 2007).

**Neocorticogenesis**

In the mouse model, the most commonly studied animal model for neocortical development, the production of neocortical neurons begins at embryonic (E) day 10 and ends around E17 which results in a six-layered structure (Figure 2) (O’Leary and Sahara, 2008). Most neocortical cells at the onset of neocorticogenesis are undifferentiated neuroepithelial cells (NE) which differentiate into radial glial cells (Pilaz and Silver, 2015). Radial glial cells extend a process to the surface of the neocortex forming a scaffold for newborn neurons to migrate. The remainder of early neocortical cells are Cajal-Retzius neurons, which are positioned superficial to the radial glial cells and originate from regions adjacent to the growing neocortex. Thus, the neocortex starts with two layers: radial glial cells make up the deep ventricular zone (VZ) while Cajal-Retzius neurons form the superficial marginal zone (MZ) (Molnár et al., 2014). The

---

**Figure 1. The mouse and human neocortex areas.** The primary sensory areas of the mouse (left) and human (right) neocortex are highlighted. The somatosensory (red) of both are rostral compared to the auditory (yellow) and the visual (blue) areas, which are positioned in increasingly caudal positions. Not shown is the motor area, which is rostral to the somatosensory area for both mouse and human brain. Sizes of the areas are not to scale between the mouse and human neocortex (Krubitzer, 2007). R: rostral (anterior); C: caudal (posterior).
Radial glial cells then begin to divide and these daughter cells go on to make up the vast majority of cells within the neocortex through a series of complicated events. The first wave of radial glial mitotic events gives rise to outer radial glial and intermediate progenitors that form the subventricular zone (SVZ) that lies just above the VZ (Custo Greig et al., 2013). At E10.5, excitatory projection neurons are produced and form the pre-plate (PP) which migrate superficially into expanding the cortical plate (CP) until they reach the Cajal-Retzius neurons in the MZ (O’Leary and Nakagawa, 2002). The migrating cortical plate neurons are instructed to stop at the boundary of the MZ and CP due to the secretion of the protein reelin, which acts as a stop signal, by the Cajal-Retzius neurons (Rice and Curran, 2001). The formation of the six layers is thus completed in an inside-out formation; each generation of daughter neurons arising from the radial glial
cells in the VZ migrate radially past previous cohorts and settle at the edge of the MZ (Manuel et al., 2015).

**Neocortical Arealization**

Neocortical neurons are pre-destined with identities belonging to one of the areas within the neocortex; motor, visual, somatosensory, etc. The process through which these primary areas are formed is termed neocortical arealization. A handful of transcription factor (TF) proteins mediate the process of neocortical arealization. TFs are a class of proteins responsible for turning genes on and off. The TFs that participate in neocortical arealization are expressed in the neocortical VZ in a graded fashion (O’Leary et al., 2007). The four proteins that have been implicated as regulators of neocortical arealization include: Emx2 (homeodomain TF), Pax6 (paired-box TF), Coup-TFI (orphan nuclear receptor), and Sp8 (zinc finger TF) (Figure 3). The biochemical mechanisms through which these TFs regulate the formation of the neocortex is not clearly

![Figure 3. Graded expression of neocortical arealization TFs. Emx2 is expressed in a high caudal-medial to low rostral-lateral pattern; Pax6 is expressed in the opposite pattern, high rostral-lateral to low caudal medial; Coup-TFI is expressed in a high caudal-lateral to low rostral-medial pattern; and Sp8 is expressed in a high rostral-medial to low caudal lateral pattern. R: rostral; C: caudal; M: medial; L: lateral (O’Leary et al., 2007).](image-url)
understood. However, the underexpression or overexpression of these proteins does result in changes in the sizes of the four primary areas in a predictable manner.

**Coup-TFI**

Coup-TFI is an orphan nuclear receptor (orphan refers to the fact that its ligand is unknown) that is heavily expressed in the developing central nervous system and throughout the brain, including the neocortex (Zhou et al., 1999). Coup-TFI is expressed in a high caudo-lateral to low rostro-medial gradient within the neocortex and stops abruptly at the boundary between the somatosensory and motor areas. To assess the role of Coup-TFI during neocortical area patterning, Coup-TFI was genetically inactivated in the mouse cortex by crossing mice carrying a floxed allele of Coup-TFI with Emx1-Cre (Emx1 is expressed in radial glial cells during neocorticogenesis) mice which resulted in the elimination of Coup-TFI expression in the neocortex beginning at E10 (Armentano et al., 2007). Inactivation of Coup-TFI function in the neocortex results in a radical reduction in size of the sensory areas (S1, V1, A1) and the majority of the neocortex taking on a motor area fate, suggesting that Coup-TFI suppresses the motor cortex fate (Armentano et al., 2007). Although the inactivation of Coup-TFI in the neocortex has a dramatic effect on the areas, the graded expression of Pax6 and Emx2 are maintained (Zhou et al., 2001). This data indicates that Pax6 and Emx2 may function independently of Coup-TFI during early neocortical arealization before Pax6 and Emx2 expression ceases.
Sp8

The zinc-finger TF Sp8 is expressed from E9.5 to E13.5 in a high rostro-medial to low caudo-lateral gradient within the neocortex (Sahara et al., 2007). In order to determine the effects on the primary areas due to loss of function of Sp8, Sp8 conditional knock-out (cKO) mice were generated by crossing Sp8 floxed mice with mice expressing Cre-recombinase controlled by the Foxg1 promoter (Zembrzycki et al., 2007). Using retrograde labeling of the thalamocortical projections, lipophilic dyes, Dil (red) and DiO (green) were used to monitor the visual and somatosensory areas, respectively. Compared to the wild type (wt) mice, Sp8 cKO mice exhibit a somatosensory fate, where the majority of the visual area becomes a part of the somatosensory area compared to the wt (Zembrzycki et al., 2007). The lack of Sp8 affects the expression levels of both Emx2 and Pax6 in the neocortex; at E.9.5 using whole mount in situ hybridization to measure the levels of the two TFs, Pax6 was down-regulated, while Emx2 was up-regulated in Sp8 cKO mice (Zembrzycki et al., 2007).

Pax6

As mentioned previously, Pax6 is a paired-box TF and is expressed in a high rostro-lateral to low caudo-medial gradient during neocorticogenesis. Homozygous Pax6 small-eye mutant mice (Sey/Sey) experience a rostral shift; the size of the motor and somatosensory areas decrease in size and the visual area increases in size (Bishop et al., 2000). This observation was confirmed using genetic markers such as the Cadherin6 and Cadherin8 mRNAs, which define the boundaries of the primary areas (Bishop et al., 2002). Based on these results with an absolute loss of Pax6, one would assume that
overexpression would induce the opposite effects. However, one study found that in fact, overexpression of Pax6 results in little to no effects on the size of the primary areas. This study used the developing cortex of Pax77 YAC transgenic mice, which carry extra copies of the human Pax6 (human and mouse Pax6 proteins are identical), the hemizygous PAX77+ and homozygous PAX77++ (Manuel et al., 2007). The steepness of the cortical Pax6 gradient was measured by using fluorescent Pax6 immunoreactivity between the rostral and caudal poles. The expression levels demonstrated that there was no significant difference in Pax6 expression levels between the wt and PAX77+ cortex (Manuel et al., 2007). This data demonstrates that although Pax6 mutant mice exhibit a rostral shift, overexpression has no effect on arealization, and thus Pax6 may not be as important in the regulation of the neocortex during this process.

**Emx2**

Emx2 is a homeodomain TF (253 amino acids, 28 kDa) that belongs to a class of proteins known as homeobox TFs and is homologous to the *empty spiracles* gene (*ems*) in *Drosophila melanogaster* (fruit fly) (Figure 4). Emx2 is expressed in a high caudo-medial to low rostro-lateral gradient within the neocortex during early embryogenesis. Loss of function studies using Emx2 mutant mice revealed an overall caudal shift in neocortical arealization, where the size of the motor and somatosensory areas increases in size while the visual area decreases in size (Figure 5) (Bishop et al., 2007).
Gain of function studies using nestin-Emx2 (ne-Emx2) resulted in overexpression of Emx2 compared to wild type (wt) and confirmed Emx2’s involvement in arealization. In ne-Emx2 mice, a rostral shift is exhibited, increasing the size of the visual area and decreasing the size of both the motor and somatosensory areas (Figure 5) (Hamasaki et al., 2004). The effects of overexpression and underexpression of Emx2 demonstrate that the levels of Emx2 directly controls the sizes and positions of the primary neocortical areas.

**Figure 5. Changes in Emx2 expression and its effects on the size of neocortical areas.** (A) Graded expression of Emx2 in normal (wild type) mouse neocortex (top) and normal size and location of the four neocortical areas (bottom); (B) In ne-Emx2, mice overexpress Emx2 (top) and the neocortical area patterning exhibits a shift in the rostral direction (demonstrated by the arrows) resulting in an increased size of V1 (visual) area and a decrease in M1 (motor) and S1 (somatosensory) areas (bottom); (C) In Emx2 +/- (heterozygous) mice, Emx2 expression is decreased (top) and the neocortical areas are shifted in the caudal direction, resulting in an increased size of M1 and S1 areas and a decrease in size of the V1 area (bottom). R: rostral; C: caudal; L: lateral. (O’Leary and Kroll, 2009).
Ems, the fruit fly homolog of Emx2, is expressed in anterior regions of *Drosophila* embryos and plays a major role in development of head structures (Simeone et al., 1992). *Drosophila ems* mutants have disrupted growth, or no growth at all of its major head structures, including the antennal sense organs, dorsal arms, and the vertical plates (Dalton et al., 1989). Similarly, Emx2 overexpression and underexpression affects its involvement in many developmental processes throughout the body, including the urogenital system, hair cell development, scapula formation, and the neocortex (Miyamoto et al., 1997; Holley et al., 2010; Pellegrini et al., 2001; Bishop et al., 2000).

Emx2 is expressed in epithelial cells of the developing urogenital system of mice, and homozygous Emx2 mutant mice die at birth due to the incomplete development of the urogenital system that includes a lack of kidneys, ureters, gonads, and genital tracts (Miyamoto et al., 1997). Emx2 expression is also necessary for scapula formation, but is not the sole gene responsible for the development of this structure. Other genes implicated in the process of scapula formation are the Pbx genes. Specifically, Pbx1 protein physically interacts with Emx2 protein during scapula development (Capellini et al., 2010). One study found that Emx2<sup>-/-</sup> mice have a complete absence of the scapula, coupled with a major part of the ilium is missing, suggesting that Emx2 plays a direct role in patterning of these structures (Pellegrini et al., 2001). Overexpression of Emx2 in the developing chick limb does not cause any malformations of the scapular blade; however, it does induce the formation of an additional posterior digit (Pröls et al., 2004).
Protein-Protein Interactions

Although the graded expression of these TFs clearly regulates neocortical arealization, it is still unclear how these graded expression patterns translate into areas separated by sharp borders. By investigating additional proteins that interact with the TFs that influence the formation of these boundaries, we hope to acquire information as to how these boundaries are formed. This experimental approach has already proved to be helpful in this regard: Glutathione S-transferase (GST) pull-down assays have demonstrated that Emx2 interacts with Sp8 (Zembrzycki et al., 2007). These assays showed that a deletion mutant of Emx2 that lacks the homeobox does not bind full length GST-Sp8 or GST-Sp8 lacking the zinc fingers, but full length Emx2 interacts with both full length GST-Sp8 and GST-Sp8 lacking zinc fingers (Zembrzycki et al., 2007). Although this interaction between these two neocortical TFs has been identified, little is understood about the mechanisms through which the other TFs regulate the formation of the neocortex and the protein-protein interactions mediating this process.

As previously mentioned, Emx2 interacts with Pbx1 during scapula development. Emx2 expression is significantly reduced in the forelimb of Pbx1/2 mutant mice, and somewhat reduced in Pbx1/3 mutant mice (Capellini et al., 2010). However, the Emx2⁻/⁻ mutation does not affect Pbx1/3 expression levels in the forelimbs (Capellini et al., 2010). Emx2 and Pbx1 form a DNA-bound heterodimer on a specific oligonucleotide sequence used during scapula formation. To investigate whether this heterodimer can induce transcription, a luciferase reporter construct was utilized. The results demonstrate that binding of the Emx2-Pbx1 heterodimer to the specific DNA sequence activated
transcription of the luciferase reporter gene, whereas Pbx1 without Emx2 does not induce transcription at this given DNA sequence (Capellini et al., 2010).

A yeast two-hybrid screen using Emx2 as bait conducted in the Kroll lab identified QkI-7 and Cnot6l as potential Emx2-binding partners (Groves et al., 2019). Additional yeast two-hybrid assays confirmed that Emx2 interacts with Cnot6l, its paralog Cnot6, and QkI-7, but not the other two QkI isoforms, QkI-5 and QkI-6 (Figure 6). GST pull-down assays were used to confirm the interactions between Emx2 and both Cnot6l and QkI-7 (Gillman, 2016). Surprisingly, these assays also revealed that Emx2 interacts with the other two QkI isoforms, QkI-5 and QkI-6 (Figure 7) (Gillman, 2016; Groves et al., 2019). This same set of experiments also demonstrated that each of the QkI isoforms interact with Cnot6l. It is worth noting here that each of the QkI isoforms can

![Figure 6. Yeast two-hybrid assays demonstrate Emx2 interacts with QkI-7, Cnot6l, and Cnot6.](image)

(A) Yeast two-hybrid assay shows Emx2 bait interacts with QkI-7 prey, but does not interact with QkI-6 or QkI-5 prey; (B) yeast two-hybrid assay shows Emx2 bait interacts with both Cnot6l and Cnot6 prey (Groves et al., 2019).
form both homodimers and heterodimers with each of the other isoforms (Chen and Richard, 1998).

**Figure 7. GST pull-down assay demonstrates 6xHis-Emx2 interacts with GST-Cnot6l, GST-Qki-5, GST-Qki-6, and GST-Qki-7.** Lane 1: input 6xHis-Emx2; lane 2: protein marker; lane 3: 6xHis-Emx2 does not interact with GST bait; lane 4: 6xHis-Emx2 interacts with GST-Cnot6l; lane 5: 6xHis-Emx2 interacts with GST-Qki-5; lane 6: 6xHis-Emx2 interacts with GST-Qki-6; lane 7: 6xHis-Emx2 interacts with GST-Qki-7 (Gillman, 2016; Groves et al., 2019).

**Qki Proteins**

The Qki gene produces three major alternatively spliced mRNA’s, Qki-5 (341 amino acids, 37 kDa), Qki-6 (319 amino acids, 35 kDa), and Qki-7 (325 amino acids, 36 kDa) that are identical aside from their C-terminal regions. The Qki proteins belong to the signal transduction and activation of RNA (STAR) family containing K-homology (KH) RNA binding domains (Hayakawa-Yano et al., 2017). Each Qki isoform contains the STAR/GSG domain, which consists of an extended KH domain flanked by the N-terminal QUA1 and C-terminal QUA2 sequences (Figure 8). The Qki isoforms form homo- and hetero-dimers through the GSG domain, which is mediated by the QUA1 region of their STAR/GSG domains. GST pull-down assays comparing Qki isoforms containing the *quaking* lethal point mutation, located at amino acid E48G, and wild type Qki demonstrated that this specific mutation prevents self-association but does not affect
RNA binding (Chen and Richard, 1998). However, deletion of any portion of the QkI STAR/GSG domain results in decreased or complete elimination of RNA binding ability.

The Quaking viable (qk') mutation is the result of a deletion within the promoter and enhancer region of the qk gene (Ebersole et al., 1996). Homozygous qk' mice develop a rapid tumor by postnatal day 10, primarily in the hind limbs and experience increasingly severe seizures as they mature (Sidman et al., 1964). This phenotype results from demyelination of the central nervous system (CNS) caused by a significant decrease in expression of QkI-6 and QkI-7 in myelinating cells, but not QkI-5 (Hardy et al., 1996). The deletion is hypothesized to have an effect on the parkin gene (PARKN), resulting in early onset Parkinson’s disease. Homozygous qk' mutation results in complete deletion of parkin co-regulated gene (PACRG) and the shared PACRG/PRKN promotor (Lockhart et al., 2004). Western blots were unable to detect PACRG or parkin protein in homozygous qk' animals, however, loss of function is not lethal.

**Figure 8. Amino acid sequence for the three QkI isoforms.** The QkI gene produces three major alternatively spliced mRNA’s that differ by only the C-terminal end (purple region); QkI-5 (30 unique amino acids), QkI-6 (8 unique amino acids), and QkI-7 (14 unique amino acids). The QUA1 (required for homo- and hetero-dimerization), QUA2, and KH domain are conserved regions of the mRNA sequence (STAR/GSG Domain) (Groves et al., 2019).
As previously stated, the three QkI isoforms are identical aside from their C-terminal amino acid sequence. The 30 unique amino acids in the C-terminus end of QkI-5 contains a novel nuclear localization sequence (NLS) composed of seven conserved amino acids; RVHPYQR in *Mus musculus* (also conserved in humans). A construct containing these seven amino acids from QkI-5 fused to GFP localizes to the nucleus, whereas QkI-5-GFP lacking the 30 C-terminal amino acids (without the NLS) localized primarily in the cytoplasm of HeLa cells (Wu et al., 1999). When the two arginine residues in the seven amino acid NLS sequence were mutated to alanines, the nuclear localization of QkI-5 was eliminated entirely, demonstrating that the arginine residues are essential for nuclear localization of the QkI-5 protein. This suggests that the conserved seven amino acids are responsible for localizing QkI-5 primarily in the nucleus, however, QkI-5 has also been shown to shuttle between the nucleus and the cytoplasm (Wu et al., 1999). An early study indicated that the other isoforms, QkI-6 and QkI-7, are localized only in the cytoplasm of oligodendrocytes and Schwann cells (Hardy et al., 1996). However, recent studies have demonstrated slightly different localization patterns for each of the three isoforms; immunolocalization assays using isoform-specific antibodies identified QkI-5 to be primarily nuclear with some cytoplasmic localization, whereas QkI-6 and QkI-7 are found throughout the cell, with QkI-6 localized in the cytoplasm more than QkI-7 in C2C12 myoblast cells (Fagg et al., 2017). It is worth noting, however, that different cell lines will have various expression levels of the QkI isoforms depending on their need or involvement in that given cell, which may affect the distributions of the QkI isoforms. The ability of QkI-5 to shuttle in and out of the nucleus is likely connected
to its ability to interact with QkI-6/7, suggesting that there could be more complex associations of the three isoforms including heterodimerization in both the nucleus and cytoplasm, as well as transportation of RNAs from the nucleus to the cytoplasm.

The QkI proteins were each tested to determine their ability to induce apoptosis. This analysis determined that QkI-7 has a greater ability to induce apoptosis compared to the other isoforms due to the 14 unique amino acid “killer sequence” in the C-terminus of QkI-7 (Pilotte et al., 2001). The ability for the QkI proteins to dimerize and affect QkI-7 induced apoptosis was tested by co-transfecting GFP-QkI-7 with GFP, GFP-QkI-5, or GFP-QkI-6, followed by assessment of cell death using the nuclear stain DAPI. Coexpression of either GFP-QkI-5 or GFP-QkI-6 with GFP-QkI-7 suppressed the apoptotic activity of QkI-7, whereas coexpression of GFP did not block apoptosis, demonstrating that heterodimerization of the proteins likely suppressed the ability of QkI-7 to induce apoptosis (Pilotte et al., 2001).

All three QkI isoforms are expressed in neural tube cells within the ventricular zone (VZ) at E10.5 (Hardy, 1998). Once neural progenitors begin to differentiate and migrate radially to form the neocortex, QkI protein expression is restricted to neural progenitors within the VZ and no other neocortex cells. QkI proteins are down-regulated in neural cells as they begin to differentiate and migrate away from the VZ. This observation is also consistent with QkI levels in neural progenitors of the spinal cord (Hardy et al., 1996; Hardy, 1998).
Cnot6l

Cnot6l (555 amino acids, 63 kDa) is a protein that contains two domains: an amino-terminal leucine-rich repeat domain (LRR) which allows for protein binding and a carboxyl-terminal endonuclease-exonuclease-phosphatase domain (EEP) that exhibits ribonuclease activity (Figure 9A). Cnot6l’s LRR contains five leucine-rich repeats where the fifth repeat is shielded by two α-helices (Figure 9B) (Basquin et al., 2012). The LRR domain allows Cnot6l to bind to Caf1 (Cnot7). The presence of specific mutations within the LRR inhibits the ability of Cnot6l to bind to Caf1. The crystal structure of a truncated form of human Cnot6l protein reveals that the EEP domain consists of a two-layered α-β sandwich fold and contains two Mg(II) ions that are required for hydrolysis of the target RNA phosphodiester backbone (Figure 9C) (Wang et al., 2010). Five highly conserved residues; Asn195, Glu240, Asp410, Asp489, and His529 are required for Mg(II) binding. Enzymatic activity is abolished when any one of three Cnot6l active site mutations (E240A, D489A, and H529A) are made (Wang et al., 2010).
Coimmunoprecipitation experiments compare the binding of wild type and mutated Cnot6l to Caf1. The most important mutation, the D357A/F358A LRR mutation, resulted in little to no detectable levels of Cnot6l-Caf1 interaction, demonstrating that Caf1 interacts with Cnot6l at this specific location (Clark et al., 2004). A deadenylase assay was used to demonstrate that the LRR is necessary for the nuclease activity of Cnot6l to function properly. Compared to wild type Cnot6l, Cnot6l lacking the LRR (nearly the entire LRR) does not exhibit deadenylase activity (Clark et al., 2004).
Cnot6l is a peripheral component of the CCR4-NOT protein complex (Figure 10), which is involved in regulating mRNA metabolism at both the transcription and post-transcriptional levels (Zhang et al., 2016; Kruk et al., 2011). Cnot6l’s paralog, Cnot6, is also a peripheral component of the CCR4-NOT complex. Cnot6 and Cnot6l are deadenylase enzymes associated with the CCR4-NOT complex and are responsible for the removal of the poly(A) tail of mRNA (Zhang et al., 2016). Removal of the poly(A) tail targets the mRNA for degradation. Although Cnot6 and Cnot6l have the same biochemical function, they have different roles. For example, Cnot6l is involved in cell proliferation by regulating mRNA levels of the cell cycle inhibitor p27KIP1, whereas Cnot6 is not involved in cell proliferation (Bartlam and Yamamoto, 2010).

The Cell Cycle Regulator, p27KIP1

The cell cycle is the process through which DNA is replicated and the cell divides via mitosis (Figure 11). The first phase, G1 (gap 1), is when the cell is active and growing. This is where the first checkpoint is located, where DNA damage is assessed.
and taken care of through repair mechanisms (Cooper and Hausman, 2015). The second phase, S (synthesis), is when DNA is actively being replicated. A second checkpoint is located in this phase to again check for DNA damage. During the next phase, G₂ (gap 2), the cell continues to grow while proteins are synthesized in preparation for the final phase, mitosis (Cooper and Hausman, 2015). Directly before mitosis, another DNA damage checkpoint occurs.

Before the cell divides, the final checkpoint allows for assessment of the spindle assembly - this is crucial as incorrect spindle assembly results in an unequal separation of the chromosomes. If the cell is unable to pass any of the checkpoints then the apoptotic pathway is initiated (Cooper and Hausman, 2015). The cyclin-dependent kinase (CDK) inhibitor p27KIP1 is a cell cycle regulating protein that inhibits the cell cycle at the G₀/G₁ phase by interacting with and regulating other CDKs. Interestingly, the mRNA encoding this protein interacts with the QKI proteins as well as

Figure 11. The cell cycle and checkpoints associated with each phase. The cell cycle is a process through which DNA is replicated and the cell divides via mitosis. The first phase is G₁, where the cell is active and growing. This phase contains a checkpoint to assess for any DNA damage. The second phase, S, is where DNA is actively being replicated. Again, a checkpoint is located here to check for DNA damage. The third phase, G₂, the cell is growing and proteins are synthesized in preparation for mitosis. DNA damage is assessed here too. The final phase, M, is directly before the cell goes through mitosis. The spindle assembly is checked, and if not properly aligned, the cell goes into cell cycle arrest (Cooper and Hausman, 2015).
Cnot6l (Laroque et al., 2005; Morita et al., 2007), the protein-protein binding partners of Emx2 (Groves et al., 2019).

The QkI proteins interact with a specific sequence found in the 3’-untranslated regions of mRNAs: the QkI response element (QRE; ACUAAY-N(1-20)-UAAY). Some mRNA targets of the QkI proteins include myelin basic protein (Li et al., 2000), Krox-20 (Nabel-Rosen et al., 2002), and the cyclin-dependent kinase inhibitor (CDK) inhibitor p27KIP1 (Laroque et al., 2005). One study identified two possible binding sequences located in p27KIP1 recognized by QkI-6 and QkI-7; electrophoretic mobility shift assays demonstrated that QkI-6 and QkI-7 bind to the QRE (nucleotides 618-647) sequence of mouse p27KIP1 mRNA (Laroque et al., 2005). The half-life of p27KIP1 mRNA followed by the addition of actinomycin D (inhibiting translation) was tested when oligodendrocytes were transduced with control (AdGFP), QkI-6 (AdQkI-6), and QkI-7 (AdQkI-7) for 48 hours. After 18 and 24 hours, Ad-GFP induced control cells retained 56% and 44% of p27KIP1 mRNA compared to cultures transduced with QkI-6 and QkI-7 which maintained the majority of p27KIP1 mRNA of 98% and 81% after 18 and 24 hours, respectively (Laroque et al., 2005). This maintenance of p27KIP1 mRNA suggests QkI-6 and QkI-7 stabilize or protect p27KIP1 in oligodendrocytes. Overexpression of QkI-6 and/or QkI-7 in oligodendrocytes increased expression of p27KIP1 protein five-fold compared to the control in oligodendrocyte progenitor cells (Laroque et al., 2005). Oligodendrocyte progenitor cells infected with Ad-GFP, Ad-QkI-6, and Ad-QkI-7 were used to examine cell proliferation. Flow cytometry demonstrated that approximately 90%
of cells co-infected with plasmids encoding QkI-6 and QkI-7 were in the G₀/G₁ cell arrest compared to 70% of cells infected with Ad-GFP.

Overexpression of QkI-6 and QkI-7 increases p27\(^{\text{KIP1}}\) activity, whereas depleted levels of Cnot6l stabilizes expression of p27\(^{\text{KIP1}}\), resulting in an increase of cells moving into the G₀/G₁ cell arrest (Morita et al., 2007). This is most likely due to Cnot6l acting as a deadenylase on the poly(A) tail of p27\(^{\text{KIP1}}\). A poly(A) tail (PAT) assay demonstrated that Cnot6l depletion inhibits the degradation of the p27\(^{\text{KIP1}}\) poly(A) tail. The PAT assay employed reverse transcription followed by PCR with a primer that targets a specific sequence of the cDNA of interest (p27\(^{\text{KIP1}}\)). The poly(A) tail of p27\(^{\text{KIP1}}\) was significantly longer in Cnot6l-depleted cells compared to the control group where Cnot6l functions as a deadenylase (Morita et al., 2007). Cnot6l decreases expression of p27\(^{\text{KIP1}}\) in the cell by initiating the destruction of p27\(^{\text{KIP1}}\) mRNA by its deadenylase activity, whereas the opposite effects are seen by QkI-6 and QkI-7, which up-regulate or protect p27\(^{\text{KIP1}}\). These three proteins (Cnot6l, QkI-6, and QkI-7) interact with each other, as well as with Emx2 (Groves et al., 2019). There are potential interactions between Cnot6l, QkI-6/QkI-7, and p27\(^{\text{KIP1}}\) mRNA within the cell where the QkI isoforms could protect p27\(^{\text{KIP1}}\) from Cnot6l’s deadenylase activity.

To study the role of Cnot6l in cell proliferation and survival in MCF7 breast cancer cells, one group used small interfering RNA (siRNA)-mediated knockdown. Cnot6l mediated knockout resulted in a decrease in cell proliferation of MCF7 cells. This decreased cell proliferation was a result of a decrease of cells in the S phase of the cell cycle, coupled to an increase in cells in the G₁ phase as well as a significant fraction of
cells with sub-G1 DNA content, indicating the cells were undergoing the apoptotic pathway (Mittal et al., 2011). This could be due to Cnot6l’s inability to perform its deadenylase activity on p27^{KIP1} when there is depleted levels of Cnot6l in the cell.

**Project Summary**

Given that Emx2 interacts with Cnot6l and the three isoforms of QkI, and that the QkI isoforms interact with Cnot6l, the next step is to determine if Emx2 can form multi-protein complexes with its confirmed protein-binding partners. Pull-down assays were performed to determine whether these proteins interact in a larger protein complex. To accomplish this task, two prey proteins were used in GST-pull down assays with a single GST-bait protein by simultaneous addition of the two preys to the bait. The prey proteins used were 6xHis-Emx2, 6xHis-QkI-5, 6xHis-QkI-6, and 6xHis-QkI-7.

GST pull-down assays using a combination of 6xHis-Emx2 and either 6xHis-QkI-5 or 6xHis-QkI-6 yielded results consistent with the formation of a multi-protein complex when GST-QkI-7 or GST-Cnot6l is used as bait. Similarly, pull-down assays using a combination of 6xHis-Emx2 and either 6xHis-QkI-5 or 6xHis-QkI-7 yielded results consistent with the formation of a multi-protein complex when GST-QkI-6 or GST-Cnot6l is used as bait. Also, pull-down assays using a combination of 6xHis-Emx2 and either 6xHis-QkI-6 or 6xHis-QkI-7 yielded results consistent with the formation of a multi-protein complex when GST-QkI-5 or GST-Cnot6l is used as bait. The identities of the bait and prey proteins were then altered to allow the use of Emx2 as bait and two of the three QkI isoforms as prey. Pull-down assays using 6xHis-QkI-5 and 6xHis-QkI-6, results were consistent with the formation of a multi-protein complex when GST-QkI-7,
GST-Emx2, or GST-Cnot6l are used as bait. Likewise, pull-down assays using 6xHis-QkI-5 and 6xHis-QkI-7, results were consistent with the formation of a multi-protein complex when GST-QkI-6, GST-Emx2, or GST-Cnot6l are used as bait. However, when 6xHis-QkI-6 and 6xHis-QkI-7 were used for pull-down assays, results consistent with the formation of a multi-protein complex was formed only with GST-Emx2. The results show that prey protein is present when using GST-QkI-5 and GST-Cnot6l bait, but the two prey proteins are too close in size (6 amino acid difference) to determine which prey protein is retained by the two baits. Therefore, these results are inconclusive at this time and further experiments would need to be performed to determine whether 6xHis-QkI-6 or 6xHis-QkI-7 (or both) is interacting with GST-QkI-5 and GST-Cnot6l during simultaneous addition.
CHAPTER II

METHODS

Buffer recipes are given at the end of the methods section.

Protein Expression

Proteins used in this experiment were expressed in, and purified from, Rosetta 2(DE3)pLysS *E. coli*. The DNA sequences encoding the open reading frame of the bait proteins were cloned into pGEX-4T-1 to produce proteins with an N-terminal glutathione S-transferase (GST) tag (26 kDa). DNA encoding the open reading frame of the prey proteins were cloned into pET24(a) to produce proteins with a C-terminal 6xHis tag (1 kDa). These DNA constructs were provided to me at the start of this project. These expression plasmids were transformed into chemically competent Rosetta 2(DE3)pLysS *E. coli* using standard heat-shock procedure.

Bait and prey proteins were expressed under different conditions. Bait proteins were expressed by inoculating a 100 mL LB culture in a 500 mL flask with 1 mL of overnight culture containing ampicillin (Amp) and chloramphenicol (Cm). All bait proteins except GST-Cnot6l were grown at 37 °C, 300 rpm until reading an optical-density at 600 nm (OD$_{600nm}$) of approximately 0.6, at which point protein expression was induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. Following the two-hour expression, the culture was divided into two 50 mL conical tubes and centrifuged at 4 °C, 4,000 g for 20 minutes. The supernatant was discarded and cell pellets were kept at -20 °C until ready for cell lysis. GST-Cnot6l cultures were grown until the OD$_{600nm}$ was approximately 0.9, at which point the flask
was placed on ice and shaken (on ice) for 30 minutes at 200 rpm. The culture was
induced with IPTG to a final concentration of 1 mM and protein expression continued for
12 hours at 18 °C, 200 rpm and then divided and centrifuged under the same conditions
for all other bait proteins.

Prey proteins were expressed by inoculating a 250 mL LB culture in a 1 L flask
with 2.5 mL of overnight culture containing kanamycin (Kan) and Cm. Prey proteins
were grown at 37 °C, 300 rpm until OD_{600nm} was approximately 0.6 at which point the
cultures were induced with IPTG to a final concentration of 1 mM and expressed for 2
hours. Following the two hours, the cultures were placed on ice for five minutes, then
transferred to a 250 mL centrifuge tube, and centrifuged for 10 minutes, 12,000 g at 4 °C.
The supernatant was removed and the cell pellet was kept at -20 °C until ready for cell
lysis/purification.

**Collection of Uninduced and Induced Cells:**

For bait and prey protein expression, uninduced and induced cells were collected
to determine if the each protein was expressed. For bait proteins, a 300 μL sample from
the culture was collected before induction with IPTG as well as after the two-hour
expression period. These samples were spun down, the supernatant was removed and the
cells were resuspended in 50 μL 2X SDS-loading dye. For the prey proteins, a 1 mL
sample was collected before induction with IPTG as well as after the two-hour expression
period. These samples were spun down, the supernatant was discarded, and the cells were
resuspended in 100 μL 2X SDS-loading dye.
Cell Lysis by Sonication

For each bait protein, one tube of frozen *E. coli* cells representing a 50 mL expression culture was thawed at room temperature. Thawed cells containing bait protein were resuspended in 3.6 mL TGEM 0.1 (-) and 400 μL of 7X stock solution of cOmplete Mini, EDTA-free protease inhibitors (Roche). Cells were resuspended by passage through a 20-gauge needle 3-5 times to ensure complete resuspension and then aliquoted into four 1 mL samples on ice. Each sample was sonicated on ice using the Fisherbrand Model 505 Sonic Dismembrator with 1/8” microtip for 10 seconds on, 59 seconds off at 20% amplitude until the solution turned relatively clear. Following sonication, dithiothreitol (DTT) and phenylmethylsulfonyl fluoride (PMSF) were added to bait cell lysates at final concentrations of 1 mM and 0.2 mM, respectively. Samples were then centrifuged for 20 minutes at 4 °C, 12,000 g to remove any insoluble material. The supernatant was then collected and saved for long term storage by adding glycerol to 20% (bait proteins were not purified).

For the prey proteins, one tube of frozen *E. coli* cells representing one 250 mL culture was thawed at room temperature. The thawed cells containing prey protein were resuspended in 8 mL ice-cold sodium phosphate lysis/binding buffer with two crushed tablets of protease inhibitors (cOmplete Mini, EDTA-free). Cells were resuspended using 3-5 passages through a 20-gauge needle to ensure complete resuspension and the samples were aliquoted into eight 1 mL samples on ice. All eight samples were sonicated under the same conditions as stated for bait proteins, however, only PMSF was added to each sample after lysis to a final concentration of 0.2 mM. The samples were clarified by
Protein Purification

The following protocol was performed at 4 °C or on ice. To purify the prey proteins (containing a 6xHis tag), Ni-NTA-agarose beads were used. The beads were prepared in a 15 mL conical tube by washing 2 mL of bead slurry (1 mL bed volume) twice with 10-bed volumes (10 mL) of dH2O, followed by two additional washes using 10-bed volumes (10 mL) of sodium phosphate lysis/binding buffer. The beads were centrifuged after each wash step at 700 g, for 2 minutes at 4 °C. After washing the beads, 8 mL of cell lysate was added to the beads and incubated on an end-over-end mixer for 1-2 hours at 4 °C. After the incubation, the beads/lysate was gently poured into a column and the beads were allowed to settle at the bottom of the column. Once the beads had settled, the unbound material was allowed to flow through the column. Wash buffer was then pumped through the column with a peristaltic pump at a flow rate of 1 mL/min (set to prime (purge) at a setting of 10 with the smallest tubing assembly on the pump). First, 30 mL of 20 mM imidazole wash buffer was pumped through the column, followed by 30 mL of 30 mM imidazole wash buffer, and finally 30 mL of 40 mM imidazole wash buffer. After the final wash, 2 mL of 250 mM imidazole elution buffer was pipetted onto the column and gently agitated for 5 minutes by repeated pipetting of the bead/elution buffer. The samples were eluted from the column and collected in 1 mL aliquots. This was repeated two more times for a total of six eluted aliquots of prey protein. The proteins were stored on ice until ready for long-term storage. Each aliquot (15 µL) was
run on a polyacrylamide gel and stained with Coomassie blue to confirm the purification of protein before moving onto long-term storage. The three samples (max of 3 mL) with the most concentrated prey protein were then dialyzed.

_Dialysis_

The dialysis cassette (Thermo Scientific, Slide-A-Lyzer Dialysis Cassette) was first hydrated in TEM 0.1 (-) buffer for at least 10 minutes. The ~3 mL sample of purified prey protein was then injected into the dialysis cassette using an 18-gauge needle. The dialysis cassette was incubated for 4 hours at 4 °C in 600 mL TEM 0.1 (-) with gentle spinning using a spin bar. After 4 hours, the buffer was removed and 600 mL of fresh TEM 0.1 (-) buffer was added and incubated overnight at 4 °C with gentle spinning. In the morning, the buffer was removed and 600 mL of TGEM 0.1 (-) was added and incubated at 4 °C for an additional 2 hours. The purified prey protein was pulled from the cassette using 18-gauge needle and DTT and PMSF were added to final concentrations of 1 mM and 0.2 mM, respectively. Glycerol was not added as it was present in the final incubation period of dialysis in TGEM 0.1 (-). The prey protein was then frozen for long-term storage using a dry ice/ethanol bath. Prey protein was aliquoted into 100 μL samples and stored at -70 °C.

_Pull-down Assays_

Pull-down assays were performed by preparing glutathione agarose beads followed by the addition of bait and prey protein(s). Prey protein(s) were added simultaneously to determine how the addition of two prey proteins would affect the formation of multi-protein complex.
The volume of bait proteins used corresponded to approximately 10 μg of bait. This was determined by comparing two volumes of each bait protein, 3 μL and 10 μL, to a set of known concentrations of bovine serum albumin (BSA) ranging from 0.05 μg/μL to .16 μg/μL (Carter et al., 2013). Using ImageJ, the concentration of each bait protein was determined, thus the volume corresponding to 10 μg could be calculated (see results).

The volume of prey protein used was determined differently. Various volumes (see results) of each prey protein was run on a single gel, then transferred to nitrocellulose membrane for a western blot. The resulting western blots were analyzed by ImageJ. The volume of each prey protein used for the pull-down assays was determined by choosing volumes that give approximately equal amounts of area under the curve from the ImageJ analysis. The volume of bait protein was kept under 200 μL and the volume for prey proteins was kept under 25 μL.

Fifty microliters of glutathione agarose slurry (25 μL bed-volume of beads) were prepared by washing four times with 10-bed volumes (250 μL) of TGEM 0.1 (-), followed by centrifuging for 5 minutes at 500 g, room temperature. After the final wash, three-bed volumes of TGEM 0.1 (+) was added to the beads to create 100 μL suspension and were held on ice until the addition of bait proteins.

Bait proteins were thawed on ice and a volume corresponding to approximately 10 μg of bait was used (not to exceed 200 μL) and diluted to a total volume of 200 μL with TGEM 0.1 (+). The bait proteins were then centrifuged for 20 minutes, 12,000 g, at 4 °C to remove any insoluble materials. The bait proteins were then carefully transferred
to tubes containing prepared glutathione agarose beads and were incubated for one to two hours on an end-over-end mixer at room temperature.

After incubation, the bead/bait complex was washed three times with decreasing concentrations of NaCl: the bead/bait mix was washed once with 250 μL TGEM 1.0 (+), once with 250 μL TGEM 0.75 (+), and once with 250 μL TGEM 0.1 (+). After each wash step, the mixture was centrifuged for 5 minutes at 500 g, room temperature. After the final wash, 75 μL of TGEM 0.1 (+) was added to the bead/bait complex and held on ice until the addition of prey protein(s).

Prey proteins were thawed on ice and the volumes determined by ImageJ analysis were used for each reaction and diluted to a total volume of 25 μL with TGEM 0.1 (+). The prey proteins were then centrifuged for 20 minutes, 12,000 g, at 4 °C to remove any insoluble materials. Prey proteins were then added simultaneously.

*Simultaneous Addition of Prey:*

For experiments that used two prey proteins at the same time (simultaneous addition), the supernatant of two prey proteins (25 μL each prey) were added simultaneously to the appropriate bead/bait mixture on ice. This mixture was then incubated on ice on an end-over-end mixer at 4 °C for 6 hours or overnight.

After the incubation, the bait/prey complexes were washed six times with 750 μL ice-cold NEN (-), with centrifugation at 500 g for 5 minutes at room temperature. Proteins were eluted by adding 25 μL 2X SDS-loading dye, boiled for 5 minutes, cooled, and spun down.
Results were analyzed by running 20 μL of each sample on a SDS-PAGE gel and transferred to nitrocellulose membrane for a western blot. A 1:2000 dilution of primary antibody in blocking buffer (Anti-6xHis tag, ab137839) and a 1:10,000 dilution of secondary antibody in blocking buffer (Anti-Rabbit IgG (whole molecule)-Alkaline Phosphatase antibody produced in goat, A0418) were used for the western blot. Bands were resolved using complete AP developing buffer.

**Preliminary Pull-down Assays**

**Preliminary Assay 1:**

Glutathione agarose beads were prepared as described above (Pull-down Assays). Approximately 10 μg of each bait protein was diluted up to 200 μL with TGEM 0.1 (+) and incubated at 4 °C for 2 hours on an end-over-end mixer with 25 μL glutathione agarose beads in 75 μL TGEM 0.1 (+). The bead/bait complex was washed 3 times with HEPES/Tris (+) for 5 minutes, 500 g, at room temperature. Twenty microliters of purified 6xHis-Emx2 prey was diluted to a final volume of 25 μL with HEPES/Tris (+) and was added to each of the bead/bait complexes and incubated on an end-over-end mixer at 4 °C for 6 hours. The bait/prey complex was then washed 6 times with 250 μL ice cold NEN (-) with 5-minute centrifugation at 500 g, room temperature. The prey protein was eluted from the bead by adding 25 μL glutathione elution buffer (0.5 mL Tris-HCl, pH 10.23, 100 mg reduced glutathione, diluted to 10 mL with dH₂O) with gentle agitation for 15 minutes on an end-over-end mixer. The samples were centrifuged one final time for five minutes, 500 g, at room temperature. The supernatant was transferred to a clean tube containing 25 μL 2X SDS-loading dye. The samples were then analyzed by western blot.
**Preliminary Assay 2:**

Glutathione agarose beads and bait proteins were prepared as described above (Pull-down Assays). The bead/bait complex was washed three times with decreasing concentrations of NaCl: the bead/bait mix was washed once with 250 μL TGEM 1.0 (+), once with 250 μL TGEM 0.75 (+), and once with 250 μL TGEM 0.1 (+). For each experiment, 10 μL of 6xHis-Qkl-5 lysate was diluted to a final volume of 25 μL with TGEM 0.1 (+), added to each of the bead/bait complexes and incubated on an end-over-end mixer at 4 °C overnight. The following morning, the bait/prey complexes were centrifuged for 5 minutes, 500 g, at room temperature. The bait/prey complex was washed 6 times with ice cold NEN (-); twice with 250 μL, twice with 500 μL, and twice with 750 μL followed by 5-minute centrifugation at 500 g, room temperature. The prey proteins were eluted from the bead by adding 25 μL 2X SDS-loading dye. The samples were then analyzed by western blot.

**Preliminary Assay 3:**

Glutathione agarose beads and bait proteins were prepared as described above (Pull-down Assays). The bead/bait complex was washed three times with HEPES/Tris (+) for 5 minutes, 500 g, at room temperature. Purified 6xHis-Emx2 (20 μL) and lysate containing 6xHis-Cnot6l (20 μL) were diluted to a final volume of 25 μL with HEPES/Tris (+). The prey proteins were added to each of the bead/bait complexes and incubated on an end-over-end mixer at 4 °C overnight. The following morning, the bait/prey complexes were centrifuged for 5 minutes, 500 g, at room temperature. A 25 μL sample was collected and combined with 25 μL 2X SDS-loading dye and termed the
“unbound prey.” The bait/prey complex was washed a total of six times with ice cold NEN (-); twice with 250 μL, twice with 500 μL, and twice with 750 μL. Prey proteins were eluted as described above (Preliminary assay 1). The samples were then analyzed by western blot.

_Preliminary Assay 4:_

Duplicate sets of glutathione agarose beads and bait proteins were prepared as described above (Pull-down Assays). One set of bead/bait complexes were washed three times with decreasing NaCl in TGEM (+) buffers (Preliminary Assay 2) while the other set was washed three times with HEPES/Tris (+) buffer (Preliminary Assay 1). For each set, pure 6xHis-Emx2 prey (15 μL) and lysate 6xHis-QkI-5 prey (10 μL) were used and diluted to a final volume of 25 μL with either TGEM 0.1 (+) or HEPES/Tris (+) and incubated with bait proteins on an end-over-end mixer at 4 °C for 6 hours. After 6 hours, a 25 μL samples of unbound prey from all pull-down assays were collected and saved for later. All samples were washed a total of six times with ice cold NEN (-); twice with 250 μL, twice with 500 μL, twice with 750 μL. Proteins were eluted by addition of 25 μL 2X SDS-loading dye. Eluted prey proteins and unbound prey samples were analyzed by western blot.

_Preliminary Assay 5:_

Glutathione agarose beads and bait proteins were prepared as described above (Pull-down Assays). Bead/bait complexes were washes as described previously (Preliminary Assay 2). Fifteen microliters of purified 6xHis-Emx2 was diluted to 25 μL with TGEM 0.1 (+) then incubated with the bait proteins on an end-over-end mixer at 4 °C for 6 hours. After 6 hours, a 25 μL samples of unbound prey from all pull-down assays were collected and saved for later. All samples were washed a total of six times with ice cold NEN (-); twice with 250 μL, twice with 500 μL, twice with 750 μL. Proteins were eluted by addition of 25 μL 2X SDS-loading dye. Eluted prey proteins and unbound prey samples were analyzed by western blot.
°C overnight. Four duplicate experiments were run using GST-tag and 6xHis-Emx2, but were washed four different ways; (1) washed a total of six times with ice cold NEN (-); twice with 250 μL, twice with 500 μL, and twice with 750 μL; (2) washed a total of six times with ice cold TGEM 0.1 (-); twice with 250 μL, twice with 500 μL, and twice with 750 μL; (3) washed a total of six times; twice with 250 μL ice cold TGEM 0.1 (-) and four times with 250 μL ice cold TGEM 0.75 (-); (4) washed a total of six times; twice with 250 μL ice cold TGEM 0.1 (-), twice with 250 μL ice cold TGEM 0.75 (-), and twice with 250 μL ice cold TGEM 1.0 (-). Proteins were eluted by addition of 25 μL 2X SDS-loading dye. Eluted prey protein samples were analyzed by western blot.

Preliminary Assay 6:

Glutathione agarose beads and bait proteins were prepared as described above (Pull-down Assays). Bead/bait complexes were washes as described previously (Preliminary Assay 2). This assay used 6xHis-Emx2 with GST-tag and GST-QkI-5 (four experiments each). Prey proteins were prepared and incubated with bait proteins as described above (Preliminary Assay 5). The final wash steps used were; (1) six total washes with ice cold NEN (-); twice with 250 μL, twice with 500 μL, and twice with 750 μL; (2) six total washes with ice cold TGEM 0.1 (-); twice with 250 μL, twice with 500 μL, and twice with 750 μL; (3) six total washes; two washes with TGEM 0.1 (-) at 250/500 μL, twice with 250 μL TGEM 0.75 (-), and twice with 500 μL TGEM 0.75 (-); (4) six total washes; two washes with TGEM 0.1 (-) at 250/500 μL, two washes with TGEM 0.75 (-) at 250/500 μL, two washes with TGEM 1.0 (-) at 250/500 μL. Proteins
were eluted by addition of 25 μL 2X SDS-loading dye. Eluted prey proteins were analyzed by western blot.

**Preliminary Assay 7:**

Glutathione agarose beads were prepared as previously described (Pull-down Assays) except the beads were held on ice or at 4 °C, not at room temperature. Bait proteins used were prepared and incubated with beads as previously described (Preliminary Assay 2). The bead/bait complex was washed with decreasing NaCl concentration (Preliminary Assay 2) on ice or at 4 °C. The volume of prey protein used was determined by ImageJ and diluted to 25 μL with TGEM 0.1 (+). Prey proteins were then incubated with bait proteins as described (Simultaneous Addition of Prey). After the incubation period, bait/prey complexes were washed and prey proteins were eluted (Pull-down Assays). Eluted prey protein as well as unbound prey protein samples were analyzed by western blot.

**Preliminary Assay 8a:**

Preliminary Assay 7 was repeated with one change: the glutathione agarose beads were prepared/washed at room temperature, not on ice or at 4 °C. This includes the bead/bait complex wash steps, and the final wash steps of bait/prey. Unbound prey and eluted prey samples were analyzed by western blot.

**Preliminary Assay 8b:**

During preliminary assay 8a, 25 μL samples were collected directly after the bait proteins were incubated with the glutathione agarose beads. These samples were combined with 2X SDS-loading dye and analyzed by western blot using 1:4000 dilution
of anti-Glutathione S-Transferase (GST) antibody produced in rabbit (G7781) in blocking buffer.

*Preliminary Assay 9:*

Two sets of glutathione agarose beads were washed and prepared at room temperature (Pull-down Assays). To one of the two sets, 5 μL RNase A was added to the prepared glutathione agarose beads in 75 μL TGEM 0.1 (+). Approximately 10 μg of each bait protein diluted up to 200 μL in TGEM 0.1 (+) was then added to the beads and incubated for two hours on an end-over-end mixer at room temperature (RNase A is more active at room temperature than 4 °C). The bead/bait mix was washed with decreasing NaCl concentration at room temperature (Pull-down Assays). Prey proteins were diluted to 25 μL in TGEM 0.1 (+) then added to the bead/bait complex and incubated on an end-over-end mixer at 4 °C for 6 hours. The bait/prey complexes were washed six times with ice cold NEN (-). The prey proteins were eluted with 25 μL 2X SDS-loading dye and analyzed by western blot.

**Buffer Recipes**

Buffers were made without PMSF and DTT (-). Buffers containing PMSF and DTT (+) had final concentrations of 0.2 mM and 1 mM, respectively.

- 7X stock protease inhibitors: 1 tablet of cOmplete Mini, EDTA-free protease inhibitors dissolved in 1.5 mL dH2O
- Sodium phosphate lysis/binding buffer: 50 mM NaH2PO4, 0.5 M NaCl, pH 7.9
- 20 mM, 30 mM, and 40 mM imidazole wash buffer: 50 mM NaH$_2$PO$_4$, 0.5 M NaCl, with 20 mM, 30 mM, or 40 mM imidazole from the 3 M imidazole stock solution
- 250 mM imidazole elution buffer: 50 mM NaH$_2$PO$_4$, 0.5 M NaCl, pH 7.9, 250 mM imidazole from the 3 M imidazole stock solution
- TGEM 0.1 (-): 0.1 M NaCl, 20 mM Tris-HCl pH 7.9, 1 mM EDTA, 5 mM MgCl$_2$, 20% glycerol, 0.1% IGEPAL-CA630.
- TGEM 0.75 (-): 0.75 M NaCl, 20 mM Tris-HCl pH 7.9, 1 mM EDTA, 5 mM MgCl$_2$, 20% glycerol, 0.1% IGEPAL-CA630.
- TGEM 1.0 (-): 1.0 M NaCl, 20 mM Tris-HCl pH 7.9, 1 mM EDTA, 5 mM MgCl$_2$, 20% glycerol, 0.1% IGEPAL-CA630.
- HEPES/Tris (-): 12 mM HEPES, 4 mM Tris-HCl, 50 mM NaCl, 10 mM KCl, 1 mM EDTA, pH 7.9.
- NEN (-): 150 mM NaCl, 1 mM EDTA, 0.5% IGEPAL-CA630, pH 6.5.
- TEM 0.1 (-): 0.1 M NaCl, 20 mM Tris-HCl pH 7.9, 1 mM EDTA, 5 mM MgCl$_2$, 0.1% IGEPAL-CA630.
- Blocking Buffer: PBST (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$ + 0.1% Tween-20), 5% nonfat powdered milk.
- Complete AP developing buffer (10 mL): 10 mL incomplete developing buffer (100 mM Tris-HCl, pH 9.0, 1 mM MgCl$_2$·H$_2$O, 150 mM NaCl), 100 μL NBT, 50 μL BCIP.
CHAPTER III

RESULTS

Determining Concentration of Bait Proteins

GST-tagged bait and 6xHis-tagged prey proteins were expressed in Rosetta 2(DE3)pLysS E. coli cells. Before conducting pull-down assays, the concentration of each bait protein needed to be determined so that approximately 10 μg of each bait protein would be used. A standard curve containing increasing amounts of bovine serum albumin (BSA) along with 3.5 μL and 10 μL of each bait protein lysate were resolved by SDS-PAGE and stained with Coomassie blue. The resulting acrylamide gels were photographed using the High Performance UV Transilluminator and analyzed using ImageJ (available from the NIH) and Microsoft Excel. The BSA standard curve was used to determine the concentration of the 3.5 μL and 10 μL lysate volumes for each bait protein. The concentration of GST-Emx2 was determined to be approximately 0.18 μg/μL (Figure 12).
Determining Approximately Equal Amounts of Purified Prey Proteins

Since the experiments described here are intended to assess the ability of multiple prey proteins to bind to a bait, it was important to include similar molar amounts of each purified prey protein so that the experiments were not biased for any of the prey proteins. Various volumes of each prey protein were resolved by SDS-PAGE and transferred to nitrocellulose membrane for western blot analysis (Figure 13). A picture of each developed western blot was taken and the results were analyzed using ImageJ and Excel (Tables 1 and 2).
Based on the results in Table 1 and Table 2, two new calculated volumes of each of the four prey proteins were chosen and analyzed by western blot (Figure 14). The two new volumes of each of the prey proteins were chosen based on a ratio calculation. Using Table 1, the two new volumes of each prey protein were chosen by approximating the volume of each prey protein that would result in a pixel count of approximately 5000 and
7000. The lower volumes, shown in table 1, were chosen because using higher volumes of each prey protein (specifically using the QkI proteins) resulted in the amount of prey proteins being too much (as demonstrated by the increased intensity of the bands in Figure 13b). The developed western blot was analyzed using ImageJ software (Table 3).

![Figure 14](image)

**Figure 14. Determining approximately equal concentrations of purified prey proteins.** Volumes used here were adapted from the first two western blots with pure prey protein to determine more accurate volumes for pull-down assays. Lane 1-2: 4.4 μL, 6 μL 6xHis-QkI-7; lane 3-4: 4 μL, 5.4 μL 6xHis-QkI-6; lane 5-6: 7.2 μL, 9 μL 6xHis-QkI-5; lane 7-8: 8 μL, 9.6 μL 6xHis-Emx2; lane 9, protein marker.

<table>
<thead>
<tr>
<th>Prey</th>
<th>Volume (μL)</th>
<th>Area</th>
<th>For about 16,000 Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>QkI-7</td>
<td>6.00</td>
<td>1.59x10^4</td>
<td>6.00 μL QkI-7</td>
</tr>
<tr>
<td></td>
<td>4.40</td>
<td>9.84x10^3</td>
<td></td>
</tr>
<tr>
<td>QkI-6</td>
<td>5.40</td>
<td>2.15x10^4</td>
<td>4.00 μL QkI-6</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>1.60x10^4</td>
<td></td>
</tr>
<tr>
<td>QkI-5</td>
<td>9.00</td>
<td>1.91x10^4</td>
<td>6.50 μL QkI-5</td>
</tr>
<tr>
<td></td>
<td>7.20</td>
<td>1.71x10^4</td>
<td></td>
</tr>
<tr>
<td>Emx2</td>
<td>9.60</td>
<td>1.62x10^4</td>
<td>9.50 μL Emx2</td>
</tr>
<tr>
<td></td>
<td>8.00</td>
<td>1.44x10^4</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3. Determination of final volumes of prey proteins to give approximately equal amounts of each prey protein for pull-down assays. ImageJ was used to analyze the data in Figure 14.**
The volumes resulting in approximately equal amounts of the four prey proteins was determined to be 6.00 μL 6xHis-QkI-7, 4.00 μL 6xHis-QkI-6, 6.50 μL 6xHis-QkI-5, and 9.50 μL 6xHis-Emx2.

**Preliminary Pull-down Assays**

*Preliminary Assay 1: Confirming previous results.* Before performing pull-down experiments to determine if three of the proteins are able to interact simultaneously to form a larger complex, I repeated pull-down assays previously performed by another student in Dr. Kroll’s lab (Gillman, 2016; Groves et al., 2019). I confirmed that pure 6xHis-Emx2 interacts with GST-QkI-5, GST-QkI-6, GST-QkI-7, and GST-Cnot6l. This assay also revealed that 6xHis-Emx2 interacts with GST-Emx2 (Figure 15).

![Figure 15](image1.png)

**Figure 15. Initial pull-down assays demonstrating 6xHis-Emx2 interacts with bait proteins GST-QkI-5, GST-QkI-6, GST-QkI-7, GST-Cnot6l, and GST-Emx2.** Purified 6xHis-Emx2 was incubated with each of the individual bait proteins. Prey protein was eluted by addition of 25 μL glutathione elution buffer. Lane 1, 10 μL pure Emx2 input; lane 2, protein marker; lane 3, GST/6xHis-Emx2; lane 4, GST-QkI-5/6xHis-Emx2; lane 5, GST-QkI-6/6xHis-Emx2; lane 6, GST-QkI-7/6xHis-Emx2; lane 7, GST-Cnot6l/6xHis-Emx2; lane 8, GST-Emx2/6xHis-Emx2.

*Preliminary Assay 2: Confirming 6xHis-QkI-5 interacts with each of the three QkI isoforms.* Next, pull-down assays using 6xHis-QkI-5 and 6xHis-QkI-7 prey proteins
with GST-QkI-5, -6, and -7 bait proteins were performed. These assays did not detect an interaction between 6xHis-QkI-7 and any of the three QkI bait proteins (data not shown), but this was due to issues with protein expression that were later resolved. However, 6xHis-QkI-5 did interact with the three QkI bait proteins (Figure 16).

![Figure 16. 6xHis-QkI-5 interacts with the QkI bait proteins GST-QkI-5, GST-QkI-6, and GST-QkI-7. Prey protein eluted by adding 25 μL 2X SDS-loading dye to beads. Lane 1, 3 μL 6xHis-QkI-5 input; lane 2, protein marker; lane 3, GST/6xHis-QkI-5; lane 4, GST-QkI-5/6xHis-QkI-5; lane 5, GST-QkI-6/6xHis-QkI-5; lane 6, GST-QkI-7/6xHis-QkI-5.](image)

**Preliminary Assay 3: Confirming the presence of prey protein not retained on the bait.** A previous pull-down assay with just the eluted prey proteins demonstrated that 6xHis-Emx2 and 6xHis-Cnot6l did not interact with GST-QkI-7 (data not shown). This same assay was repeated to determine if the prey proteins that were not retained by the bait were still present within the assay. Purified 6xHis-Emx2 along with lysate containing 6xHis-Cnot6l did not interact with GST-QkI-7 (Figure 17). However, both prey proteins were present in the supernatant rather than retained by the bait.
Preliminary Assay 4: Optimizing prey protein wash buffer for pull-down assays.

Pull-down assays in this project are based on assays performed previously in the Kroll lab (Gillman, 2016). These assays utilized two sets of wash buffers for different prey proteins: HEPES/Tris buffer for 6xHis-Emx2 and TGEM buffers for 6xHis-QkI-7. In order to perform the assays intended for this project (using multiple prey proteins), one buffer needed to be chosen for situations using the combination of 6xHis-Emx2 and 6xHis-QkI-7 (or the other QkI isoforms) in a single pull-down assay. I determined that prey proteins prepared in TGEM buffers produced cleaner results compared to when prey proteins were prepared in HEPES/Tris buffer (Figure 18).

Figure 17. Analysis of unbound prey proteins 6xHis-Emx2 and 6xHis-Cnot6l demonstrate a lack of interaction with the QkI-7 bait protein. Samples to the left of the protein marker are eluted prey samples collected by the addition of glutathione elution buffer. Samples to the right of the protein marker are the unbound prey protein. Unbound prey samples were collected directly after the bait and prey proteins were incubated together. Lane 1, 10 μL 6xHis-Cnot6l lysate input; lane 2, 3 μL pure 6xHis-Emx2 input; lane 3, GST-QkI-7/6xHis-Emx2; lane 4, GST-QkI-7/6xHis-Cnot6l; lane 5, GST-QkI-7/6xHis-Emx2+6xHis-Cnot6l; lane 6, GST/6xHis-Emx2; lane 7, GST/6xHis-Cnot6l; lane 8, GST/6xHis-Emx2+6xHis-Cnot6l; lane 9, protein marker; lane 10, GST-QkI-7/6xHis-Emx2; lane 11, GST-QkI-7/6xHis-Cnot6l; lane 12, GST-QkI-7/6xHis-Emx2+6xHis-Cnot6l; lane 13, GST/6xHis-Emx2; lane 14, GST/6xHis-Cnot6l; lane 15, GST/6xHis-Emx2+6xHis-Cnot6l.
Preliminary Assay 5: Optimizing final wash step to remove 6xHis-Emx2 from GST. Several previously performed pull-down assays showed 6xHis-Emx2 does not completely wash off when GST is used as the negative control (Figures 15, 17 and 18). I tested different final wash conditions in order to reduce the amount of background

Figure 18. TGEM wash buffers produced cleaner results than HEPES/Tris wash buffers for pull-down assays. These pull-down assay experiments are the same except for the buffer used, using pure 6xHis-Emx2 prey and lysate 6xHis-Qkl-5 prey. (A) Bead/bait complex was washed three times with decreasing NaCl concentration in TGEM (+) buffers; (B) Bead/bait complex was washed three times with HEPES/Tris (+). Lane 1, pure 6xHis-Emx2 prey; lane 2, lysate 6xHis-Qkl-5 prey; lane 3, protein marker; lane 4, GST/6xHis-Emx2; lane 5, GST/6xHis-Qkl-5; lane 6, GST/6xHis-Emx2+6xHis-Qkl-5; lane 7, GST-Qkl-6/6xHis-Emx2; lane 8, GST-Qkl-6/6xHis-Qkl-5; lane 9, GST-Qkl-6/6xHis-Emx2+6xHis-Qkl-5; lane 10, GST-Qkl-5/6xHis-Emx2; lane 11, GST-Qkl-5/6xHis-Qkl-5; lane 12, GST-Qkl-5/6xHis-Emx2+6xHis-Qkl-5.
binding of 6xHis-Emx2 to the GST bait. Four pull-down assays using GST bait and
6xHis-Emx2 prey, followed by four different washing conditions, were performed
(Figure 19). This assay determined that the best wash conditions for reducing the binding
of 6xHis-Emx2 prey to GST used six total washes with ice cold TGEM, twice with 250
μL TGEM 0.1 (-), four times with 250 μL TGEM 0.75 (-)

![Figure 19. Determining optimal wash conditions for GST bait and 6xHis-Emx2 prey.](image)

Each lane contains 6xHis-Emx2 prey incubated with GST bait. Lane contents describe how
the bait/prey complexes were washed. Lane 1, 3 μL pure 6xHis-Emx2; lane 2, protein marker;
lane 3, six total washes with ice cold NEN (-), twice with 250 μL, twice with 500 μL, and
twice with 750 μL; lane 4, six total washes with ice cold TGEM 0.1 (-), twice with 250 μL,
twice with 500 μL, and twice with 750 μL; lane 5, six total washes with ice cold TGEM, twice
with 250 μL TGEM 0.1 (-), four times with 250 μL TGEM 0.75 (-); lane 6, six total washes
with ice cold TGEM, twice with 250 μL TGEM 0.1 (-), twice with 250 μL TGEM 0.75 (-),
and twice with 250 μL TGEM 1.0 (-). Prey proteins eluted with 25 μL 2X SDS-loading dye.

Preliminary Assay 6: Determining final wash buffer in pull-down assays that will
not interfere with interaction between 6xHis-Emx2 and GST-QkI-5. Preliminary Assay 5
was repeated using GST bait along with GST-QkI-5 bait with 6xHis-Emx2 prey, along
with an additional control; 6xHis-Emx2 was added directly to the glutathione agarose
beads (without GST bait) and the amount of bound and unbound 6xHis-Emx2 was
analyzed. This control demonstrates that 6xHis-Emx2 does not bind directly to the glutathione agarose beads without a bait protein present (Figure 20). This assay also demonstrated that TGEM buffers interfere with 6xHis-Emx2’s interaction with GST-QkI-5. Thus, the conditions for the final set of wash steps was chosen to involve six washes of 750 μL with ice cold NEN (-). These wash conditions were used for the remainder of the experiments to follow.

**Figure 20.** Determining optimal wash conditions for GST-QkI-5 bait with 6xHis-Emx2 prey so that Emx2 does not wash off of GST-QkI-5. Lane contents will describe how the bait/prey complex was washed. Lane 1, glutathione beads incubated with 6xHis-Emx2 prey (wash 1); lane 2, glutathione beads incubated with 6xHis-Emx2 prey (unbound material); lane 3, protein marker; lane 4, 3 μL pure 6xHis-Emx2; lane 5, GST/6xHis-Emx2 (wash 1); lane 5, GST-QkI-5/6xHis-Emx2 (wash 1); lane 6, GST/6xHis-Emx2 (wash 2); lane 7, GST/6xHis-Emx2 (wash 2); lane 8, GST-QkI-5/6xHis-Emx2 (wash 2); lane 9, GST/6xHis-Emx2 (wash 3); lane 10, GST-QkI-5/6xHis-Emx2 (wash 3); lane 11, GST/6xHis-Emx2 (wash 4); lane 12, GST-QkI-5/6xHis-Emx2 (wash 4). Prey proteins eluted with 25 μL 2X SDS-loading dye.

- **Wash 1:** six total washes with ice cold NEN (-); twice with 250 μL, twice with 500 μL, and twice with 750 μL.
- **Wash 2:** six total washes with ice cold TGEM 0.1 (-); twice with 250 μL, twice with 500 μL, and twice with 750 μL.
- **Wash 3:** six total washes; two washes with TGEM 0.1 (-) at 250/500 μL, twice with 250 μL TGEM 0.75 (-), and twice with 500 μL TGEM 0.75 (-).
- **Wash 4:** six total washes; two washes with TGEM 0.1 (-) at 250/500 μL, two washes with TGEM 0.75 (-) at 250/500 μL, and two washes with TGEM 1.0 (-) at 250/500 μL.

**Preliminary Assay 7: Pull-down assay using two purified prey proteins with washes at 4 °C.** After determining the optimal conditions for washing the bead/bait and bait/prey complexes, a pull-down assay using two prey proteins was attempted. This
assay suggested that 6xHis-Emx2 and 6xHis-QkI-6 do not bind to any of the bait proteins (GST-QkI-5 and GST-Cnot6l), when added separately or simultaneously (Figure 21). These results were a little surprising, since each of these preys do individually bind the bait proteins. Thus, additional preliminary assays, using different wash temperatures, were conducted.

**Figure 21. Initial pull-down assay utilizing purified 6xHis-Emx2 and 6xHis-QkI-6 to determine if prey proteins will bind bait when added simultaneously.** Glutathione agarose beads, bait proteins, and prey proteins were prepared, washed, and centrifuged on ice or at 4 °C. All incubation periods took place at 4 °C. (A) Prey protein eluted with 2X SDS-loading dye; lane 1, 2 μL purified 6xHis-Emx2 prey; lane 2, 1 μL purified 6xHis-QkI-6 prey; lane 3, protein marker; lane 4, GST/6xHis-Emx2; lane 5, GST/6xHis-QkI-6; lane 6, GST/6xHis-Emx2+6xHis-QkI-6; lane 7, GST-QkI-5/6xHis-Emx2; lane 8, GST-QkI-5/6xHis-QkI-6; lane 9, GST-QkI-5/6xHis-Emx2+6xHis-QkI-6; lane 10, GST-Cnot6l/6xHis-Emx2; lane 11, GST-Cnot6l/6xHis-QkI-6; lane 12, GST-Cnot6l/6xHis-Emx2+6xHis-QkI-6. (B) Unbound prey protein samples. Lane contents are identical to western blot (A).
**Preliminary Assay 8a: Pull-down assay using two purified prey proteins with washes at room temperature.** Preliminary assay 7 was repeated except for one small change: the glutathione agarose beads were completely prepared and washed (with bait and prey) at room temperature rather than at 4 °C (Figure 22). There is a significant increase in 6xHis-Emx2 prey protein retained by the GST-QkI-5 and GST-Cnot6l bait proteins, although there is still a lack of 6xHis-QkI-6 binding.

The 6xHis-Emx2 and 6xHis-QkI-6 prey proteins were purified and washed at room temperature rather than at 4 °C. Glutathione agarose beads were held on ice between washes (directly before addition of bait or prey), but centrifugation steps were done at room temperature, rather than at 4 °C. All incubation periods took place at 4 °C. (A) Prey protein eluted with 2X SDS-loading dye; lane 1, 2 µL purified 6xHis-Emx2 prey; lane 2, 1 µL purified 6xHis-QkI-6 prey; lane 3, protein marker; lane 4, GST/6xHis-Emx2; lane 5, GST/6xHis-QkI-6; lane 6, GST/6xHis-Emx2+6xHis-QkI-6; lane 7, GST-QkI-5/6xHis-Emx2; lane 8, GST-QkI-5/6xHis-QkI-6; lane 9, GST-QkI-5/6xHis-Emx2+6xHis-QkI-6; lane 10, GST-Cnot6l/6xHis-Emx2; lane 11, GST-Cnot6l/6xHis-QkI-6; lane 12, GST-Cnot6l/6xHis-Emx2+6xHis-QkI-6. (B) Unbound prey protein samples. Lane contents are identical to western blot (A).
**Preliminary Assay 8b: Analyzing the efficiency of GST-bait proteins to bind to glutathione agarose beads.** During the previous assay (8a), samples of the unbound GST-bait proteins (following incubation with the beads, but before washing) were collected. Since it is feasible that the lack of detected bait-prey interactions was due to an absence of GST-bait proteins binding to the beads, these samples were analyzed in order to determine whether the GST-bait proteins were binding efficiently to the beads (Figure 23). The results show that some of the bait protein does not bind to the glutathione agarose beads, but this is most likely a small fraction of all of the bait.

![Western blot image](image)

**Figure 23. Analysis of unbound GST-bait proteins following incubation with glutathione agarose beads.** Following the 2 hour incubation between the glutathione agarose beads and bait proteins, a 25 μL sample was collected to determine if the bait bound the beads. Lane 1, protein marker; lane 2, 3 μL GST input (26 kDa, indicated by the arrow); lane 3-5, bead/GST; lane 6, 3 μL GST-Qki-5 input (63 kDa, indicated by the arrow); lane 7-9, bead/GST-Qki-5; lane 10, 3 μL GST-Cnot6l input (89 kDa, indicated by the arrow); lane 11-13 bead/GST-Cnot6l. Western blot used Anti-GST primary antibody.

**Preliminary Assay 9: Pull-down assays are not RNA dependent.** Many of the proteins used in this experiment are RNA-binding proteins, thus the next thing I tested was whether these pull-down assays are RNA dependent. I ran duplicate experiments
using 6xHis-Emx2 and 6xHis-QkI-7 prey with GST-QkI-5, GST-QkI-6, GST-QkI-7, GST-Emx2, and GST-Cnot6l (Figure 24). These combinations of bait and preys were chosen since these interactions were previously confirmed. The addition of PureLink RNase A (Invitrogen, Cat. no. 12091039) did not increase or decrease the ability of the prey proteins to bind to the bait proteins. Incubating the bait proteins with the glutathione agarose beads at room temperature rather than at 4 °C increased the binding of bait to bead. This then allowed for more prey protein to be retained by the GST-bait proteins. These conditions, without the inclusion of RNase A, were used for the remainder of the experiments described.

**Figure 24. Pull-down assay utilizing bait and prey proteins are not RNA dependent.** (A) RNase A was not added to beads before addition of bait and prey proteins. Lane 1, 1 μL of purified 6xHis-Emx2; lane 2, GST/6xHis-Emx2; lane 3, GST-QkI-5/6xHis-Emx2; lane 4, GST-QkI-6/6xHis-Emx2; lane 5, GST-QkI-7/6xHis-Emx2; lane 6, GST-Cnot6l/6xHis-Emx2; lane 7, protein ladder; lane 8, 1 μL of purified 6xHis-QkI-7; lane 9, GST/6xHis-QkI-7; lane 10, GST-Emx2/6xHis-QkI-7; lane 11, GST-6xHis-Cnot6l/QkI-7; lane 12, GST-QkI-5/6xHis-QkI-7; lane 13, GST-QkI-6/6xHis-QkI-7. (B) 5 μL RNase A was added to the washed glutathione agarose beads before addition of bait proteins. Lane contents are identical to western blot (A).
**Pull-down Assays by Simultaneous Addition of Two Prey Proteins**

To determine if the four prey proteins, 6xHis-Emx2, 6xHis-QkI-5, 6xHis-QkI-6, and 6xHis-QkI-7, can interact in a multi-protein complex with any of the five bait proteins, pull-down assays were performed using the optimal conditions previously determined. The first pull-down assay using 6xHis-Emx2 and 6xHis-QkI-6 demonstrates that the two prey proteins interact simultaneously with GST-QkI-5, GST-QkI-7, or GST-Cnot6l bait proteins (Figure 25). The western blot shows that 6xHis-Emx2 has a stronger interaction with GST-QkI-5 than 6xHis-QkI-6 when the two preys are added separately to the bait protein, as well as when they are added simultaneously to GST-QkI-5 (Figure 25, lanes 7-9). A stronger interaction is determined by the intensity of the band; in this case 6xHis-Emx2 prey protein is represented by a darker, more intense band than the band representing 6xHis-QkI-6. In the next experiment, using GST-QkI-7 bait, both 6xHis-Emx2 and 6xHis-QkI-6 prey exhibit strong interactions (relatively similar intensity of both bands) with the bait separately and by simultaneous addition. Then, when GST-Cnot6l is used as bait, again, 6xHis-Emx2 exhibits a stronger interaction (more intensity of the band) with the bait compared to 6xHis-QkI-6 (weaker band intensity).
The ability for 6xHis-Emx2 and 6xHis-QkI-5 to interact simultaneously in a multi-protein complex with either GST-QkI-6, GST-QkI-7, or GST-Cnot6l was demonstrated (Figure 26). When 6xHis-Emx2 and 6xHis-QkI-5 are added to GST-QkI-6, 6xHis-Emx2 exhibits a stronger interaction compared to 6xHis-QkI-5. However, both interactions of the two prey proteins decrease when added together compared to when each prey is added to GST-QkI-6 bait individually. These same patterns are observed.
when GST-Cnot6l bait is used. When GST-QkI-7 bait is used, both 6xHis-Emx2 and 6xHis-QkI-6 demonstrate similar strengths in interaction with the bait protein.

<table>
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<th></th>
<th>No bait</th>
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<th>QkI-6</th>
<th>QkI-7</th>
<th>Cnot6l</th>
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<td>6xHis-QkI-5</td>
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</table>

Figure 26. 6xHis-Emx2 and 6xHis-QkI-5 interact simultaneously with GST-QkI-6, GST-QkI-7, and GST-Cnot6l. Glutathione agarose beads were initially washed at room temperature followed by incubation with GST-bait proteins for 1-2 hours at room temperature. Prey proteins were incubated with bait at 4 °C for 6 hours. (A) contains 20 μL of the eluted prey proteins; (B) 20 μL of unbound prey proteins. Unbound prey samples correspond to the eluted prey samples in (A). Lane contents described are identical for (A) and (B). Lane 1, 1 μL purified 6xHis-Emx2; lane 2, 1 μL purified 6xHis-QkI-5; lane 3, protein ladder; lane 4, GST/6xHis-Emx2; lane 5, GST/6xHis-QkI-5; lane 6, GST/6xHis-Emx2+6xHis-QkI-5; lane 7, GST-QkI-6/6xHis-Emx2; lane 8, GST-QkI-6/6xHis-QkI-5; lane 9, GST-QkI-6/6xHis-Emx2+6xHis-QkI-5; lane 10, GST-QkI-7/6xHis-Emx2; lane 11, GST-QkI-7/6xHis-QkI-5; lane 12, GST-QkI-7/6xHis-Emx2+6xHis-QkI-5; lane 13, GST-Cnot6l/6xHis-Emx2; lane 14, GST-Cnot6l/6xHis-QkI-5; lane 15, GST-Cnot6l/6xHis-Emx2+6xHis-QkI-5. 6xHis-Emx2 is approximately 35 kDa (lower bands) and 6xHis-QkI-5 is approximately 44 kDa (upper bands).

Pull-down assays using 6xHis-Emx2 and 6xHis-QkI-7 added simultaneously to GST-QkI-5, GST-QkI-6, or GST-Cnot6l was also demonstrated (Figure 27). Both 6xHis-Emx2 and 6xHis-QkI-7 interact equally (due to their similar intensities of bands for each prey) with GST-QkI-5 and GST-QkI-6. However, 6xHis-Emx2 has a stronger interaction with GST-Cnot6l than with 6xHis-QkI-7.
The ability for 6xHis-QKI-5 and 6xHis-QKI-6 to interact simultaneously in a multi-protein complex with either GST-QKI-7, GST-Emx2, or GST-Cnot6l was also demonstrated (Figure 28). As shown in the figure, 6xHis-QKI-5 has a stronger interaction with both GST-QKI-7 and GST-Cnot6l, whereas 6xHis-QKI-6 has a slightly weaker interaction with GST-QKI-7 and a much weaker interaction with GST-Cnot6l compared to 6xHis-QKI-5. When using GST-Emx2 as bait, both proteins interact with equal strength by simultaneous addition.
His-QkI-5 and His-QkI-6 were then used to determine if the two prey proteins can interact with either GST-QkI-6, GST-Emx2, or GST-Cnot6l by simultaneous addition (Figure 29). The two preys, His-QkI-5 and His-QkI-7 interact equally with GST-QkI-6 during simultaneous addition. When using GST-Emx2 bait, His-QkI-5 weakly interacts with the bait by itself as well as with the other prey, His-QkI-7. However, His-QkI-5 has a stronger interaction with GST-Cnot6l than His-QkI-7 when used by itself, as well as with both prey proteins present.

<table>
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Figure 28. His-QkI-5 and His-QkI-6 interact simultaneously with GST-QkI-7, GST-Emx2, and GST-Cnot6l. Glutathione agarose beads were initially washed at room temperature followed by incubation with GST-bait proteins for 1-2 hours at room temperature. Prey proteins were incubated with bait at 4 °C for 6 hours. (A) contains 20 μL of the eluted prey proteins; (B) 20 μL of unbound prey proteins. Unbound prey samples correspond to the eluted prey samples in (A). Lane contents described are identical for (A) and (B). Lane 1, 1 μL purified His-QkI-5; lane 2, 1 μL purified His-QkI-6; lane 3, protein ladder; lane 4, GST/His-QkI-5; lane 5, GST/His-QkI-6; lane 6, GST/His-QkI-5+His-QkI-6; lane 7, GST-QkI-7/His-QkI-5; lane 8, GST-QkI-7/His-QkI-6; lane 9, GST-QkI-7/His-QkI-5+His-QkI-6; lane 10, GST-Emx2/His-QkI-5; lane 11, GST-Emx2/His-QkI-6; lane 12, GST-Emx2/His-QkI-5+His-QkI-6; lane 13, GST-Cnot6l/His-QkI-5; lane 14, GST-Cnot6l/His-QkI-6; lane 15, GST-Cnot6l/His-QkI-5+His-QkI-6. His-QkI-5 is approximately 44 kDa (upper bands) and His-QkI-6 is approximately 42 kDa (lower bands).
The final pull-down assay performed used 6xHis-Qkl-6 and 6xHis-Qkl-7 with either GST-Qkl-5, GST-Emx2, or GST-Cnot6l to determine if the proteins can interact simultaneously (Figure 30). When GST-Emx2 is bait, both 6xHis-Qkl-6 and 6xHis-Qkl-7 are seen bound to the bait. There is a slight overlap of the two bands representing each prey protein, so determining which has a stronger interaction with GST-Emx2 is inconclusive. When using the other two bait proteins, GST-Qkl-5 and GST-Cnot6l, the western blot shows that most likely only one of the two prey proteins is present but due to

<table>
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Figure 29. 6xHis-Qkl-5 and 6xHis-Qkl-7 interact simultaneously with GST-Qkl-6, GST-Emx2, and GST-Cnot6l. Glutathione agarose beads were initially washed at room temperature followed by incubation with GST-bait proteins for 1-2 hours at room temperature. Prey proteins were incubated with bait at 4 °C for 6 hours. (A) contains 20 μL of the eluted prey proteins; (B) 20 μL of unbound prey proteins. Unbound prey samples correspond to the eluted prey samples in (A). Lane contents described are identical for (A) and (B). Lane 1, 1 μL purified 6xHis-Qkl-5; lane 2, 1 μL purified 6xHis-Qkl-7; lane 3, protein ladder; lane 4, GST/6xHis-Qkl-5; lane 5, GST/6xHis-Qkl-7; lane 6, GST/6xHis-Qkl-5+6xHis-Qkl-7; lane 7, GST-Qkl-6/6xHis-Qkl-5; lane 8, GST-Qkl-6/6xHis-Qkl-7; lane 9, GST-Qkl-6/6xHis-Qkl-5+6xHis-Qkl-7; lane 10, GST-Emx2/6xHis-Qkl-5; lane 11, GST-Emx2/6xHis-Qkl-7; lane 12, GST-Emx2/6xHis-Qkl-5+6xHis-Qkl-7; lane 13, GST-Cnot6l/6xHis-Qkl-5; lane 14, GST-Cnot6l/6xHis-Qkl-7; lane 15, GST-Cnot6l/6xHis-Qkl-5+6xHis-Qkl-7. 6xHis-Qkl-5 is approximately 44 kDa (upper bands) and 6xHis-Qkl-7 is approximately 43 kDa (lower bands).
their very similar size (6 amino acid difference) it is impossible to determine which prey protein (or if both) are interacting with these two bait proteins.

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<th>Qki-5</th>
<th>Emx2</th>
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</table>

**Figure 30.** 6xHis-Qki-6 and 6xHis-Qki-7 interact simultaneously with GST-Emx2 but not with GST-Qki-5 or GST-Cnot6l. Glutathione agarose beads were initially washed at room temperature followed by incubation with GST-bait proteins for 1-2 hours at room temperature. Prey proteins were incubated with bait at 4 °C for 6 hours. (A) contains 20 μL of the eluted prey proteins; (B) 20 μL of unbound prey proteins. Unbound prey samples correspond to the eluted prey samples in (A). Lane contents described are identical for (A) and (B). Lane 1, 1 μL purified 6xHis-Qki-6; lane 2, 1 μL purified 6xHis-Qki-7; lane 3, protein ladder; lane 4, GST/6xHis-Qki-6; lane 5, GST/6xHis-Qki-7; lane 6, GST/6xHis-Qki-6+6xHis-Qki-7; lane 7, GST-Qki-5/6xHis-Qki-6; lane 8, GST-Qki-5/6xHis-Qki-7; lane 9, GST-Qki-5/6xHis-Qki-6+6xHis-Qki-7; lane 10, GST-Emx2/6xHis-Qki-5; lane 11, GST-Emx2/6xHis-Qki-7; lane 12, GST-Emx2/6xHis-Qki-6+6xHis-Qki-7; lane 13, GST-Cnot6l/6xHis-Qki-6; lane 14, GST-Cnot6l/6xHis-Qki-7; lane 15, GST-Cnot6l/6xHis-Qki-6+6xHis-Qki-7. 6xHis-Qki-6 is approximately 42 kDa (lower bands) and 6xHis-Qki-7 is approximately 43 kDa (upper bands).
CHAPTER IV
DISCUSSION

The largest region of the mammalian forebrain, the neocortex, is responsible for higher-order thinking, motor functions, thoughts, and processing conscious sensory perception. In less advanced mammals such as mice, these responsibilities are almost entirely performed within four specialized areas; somatosensory, visual, auditory, and motor. In humans and other “advanced” mammals, such as dolphins and apes, additional neocortical areas mediate complicated and/or abstract thoughts. However, the same four primary neocortical areas that control the basic processing of visual, auditory, somatosensory, and motor output are shared amongst all mammals (O’Leary and Kroll, 2009). The neocortex is composed of six stratified layers which arise from a population of progenitor cells, the radial glial cells, located in the ventricular zone (VZ) which expresses transcription factors (TFs) in gradients during embryogenesis. These graded expression patterns seen in the embryonic neocortex disappear by adulthood, when the four primary areas of the neocortex become distinguishable by sharp borders (O’Leary and Nakagawa, 2002). One of the most important TFs that helps mediate the process of neocortical arealization, and the main focus of this research, is Emx2. Emx2 is expressed in a high caudo-medial to low rostro-lateral gradient during neocortical arealization. Although the mechanism(s) through which Emx2 mediates the process of neocortical arealization is not fully understood, studies have demonstrated that underexpression or overexpression of Emx2 dramatically alters the sizes and positions of the four primary
areas. However, examining previously published protein-protein interactions of Emx2 may give some insight into how this TF is involved in neocortical arealization.

A few proteins have already been identified as Emx2 protein binding partners, namely Sp8 and eIF4e (Zembrzycki et al., 2007; Nédélec et al., 2004). Sp8 is a zinc finger TF that is expressed in a gradient opposing that of Emx2 along the rostral-caudal axis of the neocortical VZ. GST pull-down assays using the full-length Emx2 and Sp8 proteins demonstrated that these two protein physically interact. Deletion mutant analysis indicates that this interaction is mediated by the homeodomain portion of Emx2; deletion of the homeodomain of Emx2 eliminates binding with GST-Sp8 (Zembrzycki et al., 2007). These proteins are both TFs, and thus spend the majority of their time in the nucleus of the cell. However, the presence of Emx2 outside the nucleus has been reported in olfactory sensory neurons, where it is partially localized in axon terminals (Nédélec et al., 2004). This suggests that Emx2 is shuttled outside of the nucleus by proteins present in specific cell types. The eukaryotic translation initiation factor 4E (eIF4e) is an mRNA cap-binding protein that is part of a larger multi-protein structure that guides ribosomes to the initiation (start) codon (Marcotrigiano et al., 1999). GST-pull-down assays showed eIF4e directly interacts with Emx2 in the axon terminals of olfactory sensory neurons (Nédélec et al., 2004). This interaction suggests that Emx2 may have functions outside of the nucleus that are required to regulate gene expression post-transcriptionally. Again, this suggests that there may be other proteins responsible for shuttling Emx2 in and out of the nucleus when needed for post-transcriptional work.
Given the importance of protein-protein interactions to the function of proteins in general, coupled with the fact that interactions between Emx2 and other proteins have been shown to be of biological importance, a yeast two-hybrid screen using Emx2 as bait was conducted in the Kroll lab in order to identify additional Emx2-interacting proteins. This screen identified QkI-7 and Cnot6l as potential Emx2-binding partners (Groves et al., 2019). Yeast two-hybrid assays confirmed that Emx2 interacts with Cnot6l, its paralog Cnot6, and QkI-7, but not the other two Qki isoforms, Qki-5 and Qki-6. Additional yeast two-hybrid assays confirmed that the three Qki isoforms in both the bait and prey position form homo- and hetero-dimers (Groves et al., 2019; Chen and Richard, 1998). To further determine if these interactions demonstrated by yeast two-hybrid assays are accurate, GST-pull-down assays were conducted and confirmed the interactions between Emx2 and Cnot6l as well as, Emx2 and Qki-7 (Gillman, 2016). Surprisingly, these assays also revealed that Emx2 interacts with the other two Qki isoforms, Qki-5 and Qki-6 (Gillman, 2016; Groves et al., 2019). Complicating matters more, yeast two-hybrid assays also demonstrated that each of the Qki isoforms interact with Cnot6l.

The fact that Emx2 interacts with Cnot6l and the three isoforms of Qki, coupled with our evidence that Cnot6l interacts with each of these Qki proteins, suggests that a larger complex containing more than two proteins at a time may be able to form. This possibility was tested by a series of GST pull-down assays, using a single GST-tagged bait protein combined with two 6xHis-tagged prey proteins. For example, separate pull-down assays using 6xHis-Emx2 and 6xHis-Qki-5 prey proteins with GST-Qki-6, GST-Qki-7, or GST-Cnot6l were performed. The overall results of the experiments are
summarized in Table 4. The majority of the combinations using pairs of prey proteins with a single bait protein resulted in the apparent formation of larger protein complexes.

Table 4. Combinations of pull-down assays performed and the overall results of simultaneous addition of two prey proteins

<table>
<thead>
<tr>
<th>Bait</th>
<th>Emx2/Qkl-5</th>
<th>Emx2/Qkl-6</th>
<th>Emx2/Qkl-7</th>
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</tr>
<tr>
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<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* + indicate the prey is interacting with the bait; ++ indicates more of one prey is present than the second (due to greater intensity of the bands); - indicates neither prey proteins interact with bait; N/A indicates that the combination was not performed; inconclusive results show one or both of the prey are present but it is difficult to determine due to the relatively similar size of the given proteins

In some cases, one of the two prey proteins displays a stronger interaction with one of the bait proteins: for example, 6xHis-Emx2 bound more strongly than 6xHis-Qkl-5 to GST-Qkl-6. The only inconclusive results came from the pull-down assays using 6xHis-Qkl-6 and 6xHis-Qkl-7, which have similar molecular weights (they differ by only six amino acid residues). Based on the western blot, it was not possible to determine which of the two prey proteins, Qkl-6 or Qkl-7 are interacting with the GST-Cnot6l and GST-Qkl-5 bait proteins. However, it is possible to determine if either or both of these two prey proteins, Qkl-6 and Qkl-7, are interacting with the bait, GST-Cnot6l and GST-Qkl-5. The experiment could be repeated by using epitope specific antibodies to Qkl-6/Qkl-7 instead
of using anti-6xHis tag antibody, which would allow us to decipher which of the prey proteins are interacting (or both).

As noted in the table above, several combinations of these protein-protein interactions were not investigated (any of the prey proteins with itself in the bait form). However, we would predict that similar observations would be seen; that a larger protein complex would be formed. For example, an experiment utilizing 6xHis-Emx2 and 6xHis-QkI-5 prey with GST-Emx2 or GST-QkI-5 would most likely result in the interaction of all three proteins. We may assume this interaction would occur because 6xHis-Emx2 and 6xHis-QkI-5 interact with the two bait proteins, GST-Emx2 and GST-QkI-5 individually. The three isoforms of QkI also interact with each other in homo- and hetero-dimers as well as they interact in a trimeric complex (Table 4).

**CCR4-NOT Complex**

Cnot6l and its paralog Cnot6 are peripheral components of the CCR4-NOT complex. The CCR4-NOT complex is composed of nine subunits that interact with the Cnot1 core protein, which acts as a scaffold for the other Cnot proteins to bind (Kruk et al., 2011). The CCR4-NOT complex regulates many aspects of metabolism and the cell cycle, including regulating transcription, mRNA deadenylation, cell cycle arrest, and ubiquitination of target proteins (Collart, 2003; Zhang et al., 2016; Kruk et al., 2011). The CCR4-NOT complex is arguably most notably known for its deadenylation of the poly(A) tail of various mRNAs, which leads to the ultimate destruction of the target mRNAs. The importance of this role for the CCR4-NOT complex is evidenced by the fact that four proteins, Cnot6, Cnot6l, Cnot7, and Cnot8 each have deadenylase activity.
The complex is also associated with the ubiquitin-proteasome pathway that marks proteins for degradation. Specifically, the Cnot4 protein exhibits E3 ligase activity, which interacts with ubiquitin to target specific proteins for the degradation pathway (Lau et al., 2009).

As peripheral components, Cnot6l and Cnot6 can function outside of the CCR4-NOT complex. Cnot6l and Cnot6 contain two functional domains, a leucine-rich repeat domain (LRR) and an endonuclease-exonuclease-phosphatase domain (EEP) (Figure 9). The LRR domain allows for protein binding and the EEP exhibits ribonuclease activity (Wang et al., 2010). Although Cnot6 and Cnot6l have almost identical structures (78% identity, 88% similar) and the same biochemical function, they have different roles (Winkler and Balacco, 2013). For example, Cnot6l is involved in cell proliferation by regulating mRNA levels of the cell cycle inhibitor p27KIP1, whereas Cnot6 is not involved in cell proliferation and seems to have little to no effect on mRNA levels when knocked-out of cells (Bartlam and Yamamoto, 2010; Mittal et al., 2011). Cnot6l is localized in both the nucleus and the cytoplasm, depending on cell type. In NIH 3T3 cells, Cnot6l is primarily localized in the cytoplasm, but is distributed in both the nucleus and the cytoplasm in MCF7 cells (Morita et al., 2007; Mittal et al., 2011). Nuclear localization of Cnot6l is mediated by the LRR domain; deletion mutants lacking the LRR domain result in Cnot6l becoming localized exclusively in the cytoplasm (Mittal et al., 2011).

QkI Proteins

The QkI gene encodes an hnRNA that is alternatively spliced to make three predominate QkI mRNAs, which encodes three proteins, QkI-5, QkI-6, and QkI-7 that
are identical aside from their C-terminal amino acids (Figure 8). The three QkI isoforms belong to the signal transduction and activation of RNA (STAR) family and have K-homology (KH) RNA binding domains (Hayakawa-Yano et al., 2017). The STAR/GSG domain consists of the KH domain which is flanked by the QUA1 (which mediate homo- and hetero-dimerization of QkI proteins) and QUA2 domains (Chen and Richard, 1998).

Recent studies have demonstrated that in C2C12 myoblast cells, QkI-5 is primarily nuclear with some cytoplasmic localization, while QkI-6 and QkI-7 can be found throughout the cell. However, QkI-6 was shown to be localized more in the cytoplasm than QkI-7 (Fagg et al., 2017). These distributions are specific to C2C12 myoblast cells, whereas QkI-7 is almost exclusively localized in the cytoplasm of neocortical cells of newborn mice brains (Hardy, 1998). QkI-5 has demonstrated shuttling ability between the nucleus and the cytoplasm, and this ability may be shared by QkI-6 (Wu et al., 1999).

This leads to the possibility that the QkI isoforms bind to various RNA’s and shuttle them between the nucleus and the cytoplasm. This includes the p27\textsuperscript{KIP1} mRNA, which is responsible for arresting the cell cycle at the $G_0/G_1$ phase.

\textbf{p27\textsuperscript{KIP1}}

One of the major cell cycle regulating proteins is the cyclin dependent kinase (CDK) inhibitor p27\textsuperscript{KIP1} that arrests the cell cycle at the $G_0/G_1$ phase. The mRNA encoding this protein interacts with the QkI proteins and Cnot6l (Larocque et al., 2005; Morita et al., 2007), each which interact with Emx2 (Groves et al., 2019), as well as with each other in a large complex (Figures 25, 27, and 30). As a deadenylase, Cnot6l is responsible for degrading the poly(A) tail of various mRNAs, including p27\textsuperscript{KIP1}. In
Cnot6l-depleted cells, the poly(A) tail of p27\textsuperscript{KIP1} is significantly longer than cells containing Cnot6l (Morita et al., 2007). If Cnot6l is functioning properly in the cell, then less cells enter arrest at the G\textsubscript{0}/G\textsubscript{1} phase compared to cells containing depleted levels of Cnot6l.

The protein-protein binding partners of Cnot6l, QkI-6 and QkI-7 act to increase stability of, and/or protect, p27\textsuperscript{KIP1}. QkI-6 and QkI-7 interact with p27\textsuperscript{KIP1} mRNA via the QkI response element located between nucleotides 618-647 in the 3’-untranslated region of the mRNA (Larocque et al., 2005). Half-life assays demonstrated that cells containing both QkI-6 and QkI-7 retained 98% and 81% of p27\textsuperscript{KIP1} mRNA after 18 and 24 hours following actinomycin D treatment, which interferes with mRNA synthesis, compared to the control which retained only 56% and 48% of p27\textsuperscript{KIP1} mRNA after the same incubation times (Larocque et al., 2005). QkI-5 was not used in these experiments because it maintains oligodendrocytes in the undifferentiated form while expression of QkI-6 and QkI-7 promotes oligodendrocyte differentiation which is also regulated by p27\textsuperscript{KIP1} protein. Overexpression of QkI-6 and QkI-7 in oligodendrocytes increased expression of p27\textsuperscript{KIP1} protein five-fold compared to control cells (Larocque et al., 2005). QkI-7 can induce apoptosis due to its “killer sequence,” the last 14 amino acids in its C-terminal end, which is not shared by the other two QkI isoforms. This was demonstrated by comparing various truncated portions of QkI-7, which showed that when the last 14 amino acids of QkI-7 are removed, the percentage of apoptotic cells decreases by about 40% compared to QkI-7 containing the killer sequence (Pilotte et al., 2001). However, homo- and hetero-dimerization of the QkI proteins with QkI-7 inhibits the killer sequence.
from inducing apoptosis (Pilotte et al., 2001). Although the facts that QkI-7 induces apoptosis and p27KIP1 induces cell cycle arrest, compounded by the fact that QkI-7 interacts with p27KIP1 mRNA, suggests a possible connection that may not actually exist between QkI-7’s ability to induce apoptosis and its direct interaction with p27KIP1 mRNA (literature searches failed to indicate any connection).

Based on the results of this project, we have determined that some sort of larger multi-protein complexes involving Emx2 and any two of its binding partners, Cnot6l and the QkI isoforms. However, we are unsure of how these proteins are interacting with each other to form these larger complexes, thus more experiments would need to be conducted to better understand the specific interactions between these proteins.

**Future Studies**

Now that the results of this project suggest the formation of a larger complex, the next experiment that should be conducted is to determine which specific regions are required for these interactions by making deletion mutants of each of the proteins (Emx2, Cnot6l, and the QkI isoforms). By determining which regions of each of the proteins are important for interactions with the other proteins, we may be able to determine how the proteins are interacting. We already know that the QkI isoforms can interact with each other through the QUA1 domain, which allows them to form homo- and hetero-dimers (Chen and Richard, 1998). By making deletion mutants of this specific region, we could determine if the QkI’s interact with both Emx2 and Cnot6l through this same domain. In addition, we would want to determine which of the domains that Cnot6l has, the LRR (protein interactions) or the EEP (ribonuclease activity) domain is required for these...
interactions (Wang et al., 2010). If we find that one of these specific domains is required for direct interaction with any of the other proteins, we could continue to increase or decrease the size of the mutant to see how this affects the interaction of Cnot6l with the other proteins. The same experiment would be conducted using Emx2, as it contains the homeodomain (DNA binding) and another domain that could be directly involved in protein-protein interactions.

As demonstrated in this work, Emx2 interacts with QkI-6, QkI-7, and Cnot6l and each of the three proteins interact with each other to form a larger complex that may regulate p27\textsuperscript{KIP1} mRNA levels. The QkI isoforms provide stability and/or protection for p27\textsuperscript{KIP1} (Larocque et al., 2005) while Cnot6l degrades the poly(A) tail of p27\textsuperscript{KIP1} mRNA (Morita et al., 2007), thus the two protein activities oppose each other. Each of these proteins also bind Emx2, and it is feasible that the presence of Emx2 may regulate the stabilizing/degrading affects that QkI and Cnot6l have on p27\textsuperscript{KIP1} mRNA, respectively (Figure 31). In the most simple of scenarios, the interaction of Emx2 with Cnot6l or QkI-6/7 could inhibit their ability to either degrade or stabilize p27\textsuperscript{KIP1} mRNA. It is also possible, given the opposing affects that QkI-6/7 and Cnot6l have on the stability of p27\textsuperscript{KIP1} mRNA, that the interaction between Cnot6l and QkI-6 and/or QkI-7 demonstrated in this work may inhibit Cnot6l’s deadenylase activity and/or QkI-6/7’s stability/protection of p27\textsuperscript{KIP1}. Given the facts provided here, it will also be interesting to investigate whether having three of these proteins interacting in a larger complex (Emx2 with Cnot6l and QkI-6 or QkI-7) alters the ratio of p27\textsuperscript{KIP1} mRNA degradation/stabilization. However, we do not know how these proteins are interacting,
thus additional studies to investigate how the binding of Emx2 to either Cnot6l or the QKI
isoforms affects their ability to either degrade or protect p27<sup>KIP1</sup> would be necessary to
determine if Emx2 has any effect on these activities.
Figure 31. Potential outcomes of Emx2 interacting with Qki, Cnot6l, and p27KIP1 mRNA.
(A) Qki binds to p27KIP1 mRNA via the Qki response element (QRE) located between nucleotides 618-647 (GenBank accession number AK047669) and protects and/or stabilizes p27KIP1 mRNA when Qki is bound (Larocque et al., 2005); (B) Cnot6l is a deadenylase that removes the poly(A) tail of mRNA, including p27KIP1, which marks the mRNA for degradation; (C) If both Qki and Cnot6l are present and interacting with p27KIP1 mRNA, the outcome is unknown. The binding of Qki to the QRE may protect the mRNA from Cnot6l. If the mRNA is folded, it is possible that the two proteins could interact; the protein-protein interaction may inhibit Cnot6l’s deadenylase activity, protecting the mRNA, or the interaction may not have any affect at all; (D) Emx2 may interact with Qki when bound to p27KIP1 mRNA in which the mRNA may or may not be protected; (E) If Emx2 binds to Cnot6l when it is performing its deadenylase activity, the protein-protein interaction may inhibit Cnot6l from degrading the poly(A) tail, or Emx2’s presence may not have any affect; (F) If both Qki and Cnot6l are interacting with p27KIP1 mRNA and Emx2 comes into play, it is unknown if Emx2 can interact with either or both proteins (depending on mRNA folding). The overall outcome is unknown, p27KIP1 mRNA could be protected by Qki, or could still be degraded by Cnot6l.
To study how the addition of Emx2 may affect p27^KIP1 stability, a deadenylase/protection assay could be used (Morita et al., 2007). This type of assay involves transforming/transfecting (into either *E. coli* or cultured cells, respectively) plasmids encoding GST-tagged Cnot6l, QkI, and Emx2 proteins. Different combinations of the tagged proteins will then be purified using glutathione beads and then incubated with the p27^KIP1 mRNA substrate labeled with fluorescein isothiocyanate at the 5' end. Reaction mixtures would then be subjected to polyacrylamide denaturing gel to determine how the protein(s) affect the poly(A) tail of the RNA (Morita et al., 2007).

A similar assay could also be performed *in vivo* using cultured cells. First, plasmids encoding different combinations of Cnot6l, Emx2, and QkI would be transfected into an appropriate cell line (naturally expressing p27^KIP1 mRNA). Forty-eight hours after transfection with the expression plasmids, total mRNA will be isolated from the cells and the presence of p27^KIP1 mRNA will be analyzed by quantitative RT-PCR. Each of these protection assays could be used to see how Cnot6l and each of the QkI isoforms (QkI-6 and QkI-7) affects p27^KIP1 mRNA. Since Cnot6l and the two QkI isoforms interact, combinations of Cnot6l with each of the QkI’s as well as with all three proteins present, this would allow us to determine if the QkI’s can protect p27^KIP1 mRNA from Cnot6l. The same assay would be performed with Cnot6l and Emx2 as well as Emx2 with the QkI isoforms to see if p27^KIP1 mRNA is protected when Emx2 is present. Three of the proteins, Emx2, Cnot6l, and one of the QkI isoforms could then be used to determine if Emx2 helps to protect p27^KIP1 mRNA from Cnot6l or if degradation from Cnot6l will occur.
An electrophoretic mobility shift-assay (EMSA) could be employed to investigate if Emx2 is directly binding to p27\textsuperscript{KIP1} mRNA. This assay requires radioactively labeled mRNA, that when bound to protein, exhibits a shift on nondenaturing polyacrylamide gels. By increasing the concentration of Emx2 (the QkI isoforms and Cnot6l could be used as positive controls), we can determine if the protein binds to the mRNA. This experiment could be employed with each of these proteins individually with the mRNA and with two or three of the proteins present to see if all three proteins bind to p27\textsuperscript{KIP1} mRNA, resulting in a super shift in the mobility of the p27\textsuperscript{KIP1} mRNA.

As mentioned earlier on, Emx2 is a TF that is localized primarily in the nucleus. However, it is localized outside of the nucleus in the axon terminals of olfactory sensory neurons, where it binds to eukaryotic translation initiation factor eIF4E (Nédélec et al., 2004). Thus, it would be potentially interesting to study the nucleocytoplasmic distribution of Emx2 within cultured cells when the QkI isoforms and/or Cnot6l are overexpressed. Using GFP fused to Emx2 (or Emx2-specific antibody) would allow us to visualize where Emx2 is localized within the cell. It would be interesting to see if overexpression of any of these proteins shifts (or shuttles) Emx2 outside of the nucleus into the cytoplasm for other jobs unrelated to transcription (potentially RNA binding).
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