The Effect of Freud-1/CC2D1A Knockout on EGF Receptor Activation

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Abstract

CC2D1A (coiled-coil and C2 domain containing protein 1A), also known as Freud-1, has been identified as a transcriptional repressor of the serotonin receptor 5-HT1A, a regulator of endosomal budding and an activator of NF-KB signaling. It also acts as a scaffold that promotes activity of the PI3K/Akt pathway upon stimulation by the epidermal growth factor (EGF). Moreover, several studies highlight naturally occurring mutations of CC2D1A in humans that produce varying degrees of intellectual disorder and autism.

Use of the Cre-LoxP system to conditionally knockout CC2D1A in mice has provided promising results regarding its effect on 5-HT1A expression and behaviour. This thesis aims to extend the use of this knockout model by studying cell signaling activity in mouse embryonic fibroblasts (MEFs), derived from the CC2D1A$^{flx/flx}$ transgenic line, that have been treated with a commercially available Cre recombinase to completely knock out CC2D1A. I hypothesize that CC2D1A directly regulates EGF receptor activity and that its Cre-mediated knock down *in vitro* will entirely block cell signaling pathways activated by the EGF receptor.

Western blot analysis demonstrated that, after Cre-mediated CC2D1A knockout, Akt and Erk1/2 phosphorylation were still maintained upon EGF treatment. In addition, overexpressing Freud-1 via transfection had no effect on cell signaling compared to the wild-type control. Analysis of recombinant Freud-1 constructs reveal that a C-terminal truncation enhances its ability to bind to PIP2 and PIP3 – phospholipids essential to the Akt pathway. In addition, immunocytochemistry analysis demonstrates a responsiveness of CC2D1A to EGF treatment. Altogether, these data highlight a unique and effective way in carrying out gene knockout *in vitro* while also emphasizing the need to further investigate CC2D1A's importance in regulating cell signaling pathways and functional compensation by other homologous proteins.

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List of Abbreviations

5-HIAA: 5-hydroxyindoleacetic acid

5-HT: 5-hydroxytryptamine

5-HTTP: 5-hydroxytryptophan

AAA-ATPase: ATPase associated with various cellular activities ATPase

AADC: aromatic L-amino acid decarboxylase

Aβ: Amyloid-β

Aki1: Akt kinase-interacting protein 1

ALS: amyotrophic lateral sclerosis

AMP: adenosine monophosphate

ANOVA: analysis of variance

αS: alpha synuclein

ATP: adenosine triphosphate

ASD: autistic spectrum disorder

bp: base pair

BSA: bovine serum albumin

CAMK: Ca^{2+}/calmodulin-dependent protein kinase II

cAMP: cyclic AMP

CC2D1A: coiled-coil and C2 domain-containing protein 1a

Cdk: cyclin-dependent kinase

cGMP: cyclic GMP

CHMP: charged multivesicular body protein

CNS: central nervous system

CNV: copy number variation

CRE: cAMP response element

CREB: cAMP response-element binding protein

DAPI: diamidino-2-phenylindole

DMEM: Dulbecco's Modified Eagles Medium

DNA: deoxyribonucleic acid

Doa4: degradation of alpha-4

DRE: dual repression element

DRN: dorsal raphe nucleus

dsRNA: double-stranded RNA

EGF: epidermal growth factor

EGFR: EGF receptor

EMSA: electrophoretic mobility shift assay

ES: embryonic stem cell

ESCRT: endosomal sorting complex required for transport

FBS: fetal bovine serum

Freud-1: five-prime repressor element under dual repression binding protein 1

FTD: frontotemporal dementia

GMP: guanine monophosphate

GSK-3: glycogen synthase kinase 3

HIV: human immunodeficiency virus

HLH: helix-loop-helix

HRP: horseradish peroxidase

ICC: immunocytochemistry

ID: intellectual disorder

IGF: insulin growth factor

IKK: IKB kinase

iNOS: inducible NO synthase

IRF3: interferon regulatory factor 3

***Lgd*:** lethal (2) giant disc

LTD: long-term depression

LTP: long-term potentiation

MAO: monoamine oxidase

MAPK: mitogen-activated protein kinase

MAVS: mitochondrial antiviral-signaling protein

MDA5: melanoma differentiation-associated gene 5

MDD: major depressive disorder

MEFs: mouse embryonic fibroblasts

mRNA: messenger RNA

mTOR: mammalian target of rapamycin

MVB: multivesicular body

NEMO: NF-KB essential modulator

NF-KB: nuclear factor kappa-light-chain enhancer of activated B cells

NIK: NF-KB-inducing kinase

NMD: nonsense-mediated mRNA decay

NSC: neural stem cells

NSID: nonsyndromic intellectual disorder

PA: phosphatidic acid

PAMP: pathogen-associated molecular patterns

PBS: phosphate-buffered saline

PBS-T: PBS and Triton X-100

PDE: phosphodiesterase

PDK1: 3-phosphoinositide-dependent protein kinase 1

PFA: paraformaldehyde

PI3K: phosphoinositide-3-kinase

PIP2: phosphatidylinositol-4,5-bisphosphate

PIP3: phosphatidylinositol-3,4,5-trisphosphate

PKA: protein kinase A

PKB: protein kinase B

PS: phosphatidylserine

PTB: phosphotyrosine binding

PtdIns3P: phosphatidylinositol-3-phosphate

PTEN: phosphatase and tensin homolog

PVDF: polyvinylidene fluoride

RHD: Rel homology domain

RLR: RIG-1-Like Receptor

RNA: ribonucleic acid

ROS: reactive oxygen species

RTK: receptor tyrosine kinase

RT-PCR: reverse-transcriptase polymerase chain reaction

SDS-PAGE: sodium dodecyl sulfate – polyacrylamide gel electrophoresis

SNP: single nucleotide polymorphism

SOP: sensory organ precursor

SSRI: selective serotonin reuptake inhibitor

SVZ: subventricular zone

TAD: transcriptional activation domain

TAK: TGFβ-activated kinase 1

TAPE: TBK1-associated protein in the endolysosomes

TANK: TRAF family member-associated NF-KB activator

TBK-1: TANK-binding kinase 1

TBS: Tris-buffered saline

TBST: TBS + Tween 20

TLR: Toll-Like Receptor

TNF: tumour necrosis factor

TPH2: tryptophan hydroxylase 2

TRAF: tumour necrosis factor receptor-associated factor 2

TSS: transcriptional start site

VEGF: vascular endothelial growth factor

VMAT: vesicular monoamine transporter

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Chapter 1: Introduction

1.1 – Freud-1/CC2D1A

1.1.1 – Discovery and Structural Characterization of Freud-1/CC2D1A

Freud-1, formally known as coiled-coil and C2 domain-containing protein 1a (CC2D1A), is a highly conserved protein involved in a wide array of processes that are crucial to gene expression, cellular growth and proliferation, and organismal survival. CC2D1A was initially identified as both a potential activator of the NF-KB signaling pathway and a transcriptional repressor of the serotonin receptor 5-HT1A. (Matsuda et al., 2003; Ou et al., 2003; Basel-Vanagaite et al., 2006). Further highlighting its widespread importance within the cell, this gene is also referred to as Akt kinase-interacting protein 1 (Aki1) and TBK1-associated protein in the endolysosomes (TAPE) (Nakamura et al., 2008; Chen et al., 2012)

The CC2D1A gene encodes a protein that spans 951 amino acids in humans (GenBank Accession Number NP_060191.3) and 943 amino acids in mice (GenBank Accession Number NP_666082.2). Computer analysis of their sequence homology indicates 82% identity between both species. Moreover, in all known homologs, the Freud-1/CC2D1A gene encodes a protein containing four 60-aa DM14 domains that are conserved within the Freud-1 family and of unconfirmed function, a helix-loop-helix domain which is likely involved in DNA-binding and a C2 domain that is considered a locus for calcium-mediated phospholipid-binding (Ou et al., 2003).

The CC2D1A gene is capable of generating two different protein isoforms. The long isoform (Freud-1_L) contains all the domains described above. Moreover, evidence points to it being the most expressed variant in human tissue and the one responsible for most of its DNA-binding

capability. By contrast, the short isoform (Freud-1_S) contains only the third and fourth DM14 domain, along with the HLH and C2 regions. Transcription of Freud-1_S is initiated by an upstream in-frame transcriptional start site, and is the most abundant isoform in rodent cells as confirmed by Western blot analysis (Rogaeva and Albert, 2007)

1.1.2 – Evolutionary Origin of Freud-1/CC2D1A

The evolutionary origin of CC2D1A can be traced back to a gene expressed in Drosophila melanogaster called lethal (2) giant disc (*Lgd*). This gene was originally characterized as a tumour suppressor given that, upon mutation, extensive hyperplasia could be observed in the larva's imaginal discs (Bryant and Schubiger, 1971). Mechanistically, the growth suppression enabled by lethal (2) giant disc functions by restricting the activity of the Notch pathway – a ligand-dependent signaling mechanism important for cell growth and organismal development (Verheyen et al., 1996; Klein, 2003). While, the protein encoded by the *Lgd* gene only shares 33% amino acid identity with Freud-1, they are structurally very similar in that they both contain four N-terminal DM14 domains and a C-terminal C2 domain (Gallagher and Knoblich, 2006).

1.2 – Freud-1/CC2D1A as a transcriptional repressor of 5-HT1A

Serotonin (5-HT, 5-hydroxytryptamine), a member of the monoamine family, contributes to a variety of physiological functions. Some of its many roles in the periphery include mediating pain recognition, blood pressure regulation and digestion (Tokunaga et al., 1998; Miwa et al., 2001; Morecroft et al., 2006). However, its involvement as a neurotransmitter in the central nervous system (CNS) has many clinical implications and is the subject of numerous research topics. In particular, an imbalance of serotonin levels in the brain is associated with the onset of major depressive disorder (MDD).

In the CNS, 5-HT metabolism mostly occurs in a component of the midbrain known as the dorsal raphe nucleus (Sheard and Aghajanian, 1968; Weiss and Aghajanian, 1971; Shields and Eccleston, 1972). Synthesis begins with the conversion of tryptophan to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase 2 (TPH2), which is then transformed into 5-HT by aromatic L-amino acid decarboxylase (AADC). Metabolism of 5-HT into 5-hydroxyindoleacetic acid (5-HIAA) is performed by monoamine oxidase (MAO) (Johnston, 1968; Zhang et al., 2004). Upon formation of the neurotransmitter, it is packaged into secretory vesicles by vesicular monoamine transporter (VMAT) and released by calcium-dependent exocytosis to various brain targets such as the hippocampus, prefrontal cortex and limbic system, which, altogether, allow for regulation of mood and behaviour. Serotonin is able to exert these numerous effects on the central nervous system by binding to a broad range of receptors. In total, there are fifteen different serotonergic receptors in mammals which are grouped into seven families. Most of them consist of seven transmembrane domains and are G-protein coupled, with the exception of the 5-HT3 receptor (Barnes and Sharp, 1999).

The 5-HT1A receptor, among others, bears significant relevance to the study and treatment of depression and anxiety. This serotonin receptor is coupled to a $G_{i/o}$ protein which, upon ligand binding, leads to an inhibition of adenylyl cyclase-mediated cyclic AMP (cAMP) synthesis. This decrease in intracellular cAMP, along with the activity of $G_{\beta\gamma}$ subunits, causes Ca^{2+} channels to close and K^+ channels to open, ultimately resulting in hyperpolarization of the neuron and suppression of neurotransmission (Albert and Lemonde, 2004).

Moreover, the 5-HT1A receptor assumes two major functional roles in the CNS. Firstly, it is highly expressed as a postsynaptic heteroreceptor in the hippocampus, limbic system and cortex where it mediates serotonergic actions (Gozlan et al., 1995). These heteroreceptors are found on

both pyramidal neurons and interneurons in these various serotonergic targets and are, thus, capable of mediating either excitatory or inhibitory neurotransmission upon agonist binding (Aznar et al., 2003). Secondly, 5-HT1A functions as a somatodendritic autoreceptor in the serotonin-producing neurons of the dorsal raphe nucleus (DRN), wherein it is responsible for establishing a negative-feedback loop that permits the monitoring of 5-HT production and release. Essentially, as serotonin is being released from the DRN, it is able to bind to 5-HT1A autoreceptors expressed on adjacent neurons to eventually inhibit further release of the neurotransmitter. This mechanism ultimately ensures a tight regulation of the total amount of serotonin that is distributed throughout the CNS at any given time (Albert and Lemonde, 2004).

There are instances, however, in which dysregulation of serotonin production and output occur that has a negative impact on one's mood and behaviour. The most noted case of this is major depressive disorder (MDD) and is linked to a reduction of total 5-HT in the brain (Krishnan and Nestler, 2008). Patients suffering from MDD experience states of low self-esteem, anhedonia, depressed mood and apathy (American Psychiatric Association, 2013). In addition, some cases of MDD are associated with a level of impulsivity and aggression which can sometimes lead to suicide (Dumais et al., 2005). The current treatment method for this disease is the administration of selective-serotonin reuptake inhibitors (SSRIs), which inhibit the reuptake of serotonin in the synaptic cleft (Gartside et al., 1995). This prolongs the monoamine's effects and compensates for its decreased CNS concentrations in MDD patients.

The specific causes of MDD are currently unclear. Nonetheless, observations of a significant increase in the expression of 5-HT1A autoreceptors in the brain tissue of MDD patients who commit suicide have been well-documented (Stockmeier et al., 1998). Moreover, it is known that the beneficial effects of SSRI treatment take up to 3 weeks to manifest, and that this is

preceded by desensitization and internalization of 5-HT1A autoreceptors (Riad et al., 2004). Altogether, these implications of 5-HT1A overexpression in the onset of mood disorder have sparked increased focus on the transcriptional mechanisms which regulate its production in the neuron.

The transcriptional regulatory regions of the 5-HT1A gene are located in an upstream region of its transcriptional start site (TSS) that spans approximately 1500 bases. Immediately ahead of the TSS lie promoter elements specific to the transcriptional enhancers Maz, Sp1 and NF-KB (Parks and Shenk, 1996). These are general enhancers which are associated with numerous other proteins and, consequently, do not contribute the 5-HT1A's tissue specificity. However, the receptor's expression is also controlled by several repression elements which attract proteins that are specifically found in neuronal tissue. These include a CpG island ~1000 bp upstream of the TSS which attract the transcriptional repressors Deaf-1, HES-1 and HES-5 (Jacobsen, et al., 2008; Czesak et al., 2012). In this instance, it contains a polymorphism which is split into two alleles: a normal C allele and a risk G allele. The risk allele is associated with resistance to SSRI treatment, as well as onset of schizophrenia (Huang et al., 2004; Lemonde et al., 2004; Wang et al., 2008; reviewed in Le François et al., 2008).

Another repressor element, located 1500 base pairs upstream of the TSS, was discovered by Ou et al. (2000) and appears to have a very strong effect on 5-HT1A expression. This element contains two overlapping DNA-binding regions and is aptly named the dual repression element (DRE). It was later discovered through yeast one hybrid assays that Freud-1 (**F**ive-prime **R**epressor **E**lement **U**nder **D**ual repression binding protein-**1**) binds specifically to the 5' region of this element in order to induce a very strong repression of 5-HT1A expression in neuronal and non-neuronal cell types. A homolog of Freud-1, dubbed Freud-2 (CC2D1B), binds specifically

to the 3' region of the DRE and, in non-neuronal cells, works synergistically with the former to almost completely halt production of 5-HT1A. However, out of the two, only Freud-1 is expressed in serotonergic neurons, which has led to the conclusion that it is a key component in the dynamic regulation of 5-HT1A autoreceptor levels (Ou et al., 2003; Hadjighassem, et al., 2008; Hadjighassem et al., 2011).

The regulation of Freud-1's DNA-binding ability is primarily driven by Ca^{2+}/calmodulin-dependent protein kinase II (CAMK) activity. Since the C2 domain enables such functions as calcium-dependent lipid binding and protein phosphorylation, it was thought that addition of Ca^{2+} and ATP would consequently reduce Freud-1's affinity to the DRE. It was accordingly demonstrated through electrophoretic mobility shift assay (EMSA) and luciferase assays that induction of calcium influx significantly reduced Freud-1-mediated transcriptional repression, however application of a CAMK inhibitor rescued its function. Therefore, Ca^{2+}-mediated cell signaling via CAMK was deemed responsible for negatively-regulating Freud-1's DNA-binding activity (Ou et al., 2003).

It is important to note that Freud-1's involvement in transcriptional regulation is not exclusive to the 5-HT1A receptor. It was later discovered that a DRE is also present upstream of the dopamine-D2 receptor gene and elicits the same repressive effects. In the case of D2, however, the DRE is also accompanied by two single-nucleotide polymorphisms (SNPs), A/G and A/C. It was also observed, though luciferase reporter assays, that Freud-1-induced repression of the D2 receptor was significantly reduced in the A/G allele (Rogaeva et al., 2007). The consequences of this allelic variation is currently unknown.

1.3 – Freud-1 as a scaffold protein

Much of a cell's regulation of metabolic activity originates from ligand-receptor interactions which activate cell signaling pathways that ultimately induce the expression of proteins involved in cellular growth, division, survival and apoptosis. As previously mentioned, the structural nature of CC2D1A suggests that it evolved from the lethal (2) giant disc protein found in *D. melanogaster*. *Lgd's* main function in development is as a regulator of the Notch pathway. In *Drosophila*, Notch is especially important in determining the fate of sensory organ precursor (SOP) cells – a crucial step in the organism's morphological development. When *Lgd* function is compromised, Notch signaling is abolished since the Notch receptor, which is internalized upon activation, becomes trapped in early endosomes. This ultimately leads to lack of development of bristles which are necessary for the organism's sensory functions (Gallagher and Knoblich, 2006).

Similarly, several studies have shown that knockdown of Freud-1 compromises the integrity of several signaling pathways and endosomal trafficking mechanisms. Among the affected pathways are Akt signaling, NF-KB activation and the ESCRT pathway. Altogether, Freud-1/CC2D1A has been identified as a crucial regulator of cellular functions implicated in cell growth and survival, neuronal differentiation and endosomal trafficking.

1.3.1 – Freud-1/CC2D1A and the ESCRT pathway for endocytosis

Cells constantly undergo protein turnover in order to maintain optimal homeostasis. This involves highly regulated processes in which older proteins are targeted for degradation so that newly synthesized polypeptides take their place. The proteins that are destined for lysis are often ubiquitinated which subsequently permits their assortment into vesicles to be escorted to

lysosomes. Before they reach their ultimate fate, however, these vesicles are first collected into larger structures known as multivesicular bodies (MVBs), which carry them to their final destination (Palade, 1955; Haigler et al., 1979).

The formation of MVBs is a highly regulated process that heavily relies on the endosomal sorting complex required for transport (ESCRT) pathway. It is mainly responsible for the creation of the subvesicles needed to compartmentalize the material contained within the endosome. The cell encodes several different proteins which are sequentially recruited and coordinate the recognition, recruitment, budding and disassembly steps that make up the ESCRT pathway. In addition, this pathway is known to play a role in cytokinetic abscission, the final step in cell division in which two autonomous cells are formed. Moreover, retroviruses, such as HIV, are capable of exploiting this pathway in order to carry out infection (reviewed extensively in Henne et al., 2011). Furthermore, ESCRT dysregulation has been observed in various neurological ailments such as Alzheimer's, dementia, Parkinson's and Huntington's diseases.

The ESCRT pathway is a multi-step process. Prior to initiation, ubiquitination of the endosomal membrane must occur. This permits the first step in the sorting process, which involves recognition of the cargo and binding of the membrane residues by ESCRT-0, a heterodimeric protein consisting of the subunits Hrs (Vps27) and STAM1/2 (Hse1). Dimerization occurs via interaction between the coiled-coil domains of both of these subunits (Asao et al., 1997; Prag et al., 2007). ESCRT-0 binds specifically to the endosomal membrane through interactions with phosphatidylinositol-3-phosphate (PtdIns3P) (Raiborg et al., 2001). In addition, ESCRT-0 binds to ubiquitin, thus providing a pattern recognition step that ultimately leads to recruitment of ESCRT-I (Ren and Hurley, 2010).

ESCRT-I is a heteromeric protein complex consisting of the Tsg101 (mVPS23), Vps28, Vps37 and hMvb12 subunits (Katzmann et al., 2001; Bishop and Woodman, 2001; Bache et al., 2004; Morita et al., 2007). This complex functions as a bridge between ESCRT-0 and ESCRT-II, and as such, bears an elongated shape with a coiled-coil stalk on one end and a globular head on the other (Kostelansky et al., 2006; Kostelansky et al., 2007). Interactions between ESCRT-0 and -1 are mediated by the Hrs subunit of the former and the Vps23 domain of the latter (Katzmann et al., 2003). By contrast, the Vps28 group directly binds ESCRT-II via its C-terminus (Gill et al., 2007).

The ESCRT-II complex is a heterotetramer consisting of the subunits Vps22 and Vps36, and two subunits of Vps25 (Babst et al., 2002b). Crystal structure analysis reveals a Y-shaped protein wherein Vps22 and Vps36 form the base and Vps25 make up the branches (Hierro et al., 2004). The main functional domain for this complex is the GLUE domain contained in Vps36, which binds with high affinity to ESCRT-I (Teo et al., 2006). In addition, this domain interacts with PtdIns(3)P and, together with ESCRT-0, provides a high degree of endosomal localization (Slagsvold et al., 2005). Once ESCRT-II is localized at the MVB, it subsequently recruits ESCRT-III in order to proceed with the next step of the pathway (Teo et al., 2004).

ESCRT-III is a heteromeric complex consisting of the following four subunits known as charged multivesicular body proteins (CHMPs) in mammals: CHMP6 (Vps20); CHMP4 (Snf7); CHMP3 (Vps24); CHMP2 (Vps2) (Babst et al., 2002a). When this pathway is inactive, ESCRT-III's monomers remain dispersed throughout the cytoplasm and assume an autoinhibited resting state (Zamborlini et al., 2006; Shim et al., 2007). Its migration to the endosome occurs when the Vps25 subunit of ESCRT-II binds to CHMP6 (Teo et al., 2004). CHMP6 then recruits CHMP4, which undergoes a homo-oligomerization step that is halted by the binding of CHMP3 and

CHMP2 (Teis et al., 2008; Saksena et al., 2009). The CHMP4 polymer is required for the recruitment of the Bro1/Alix adaptor protein, which is responsible for bringing in the degradation of alpha-4 (Doa4) enzyme in order to deubiquinate the bound cargo vesicle (Odorizzi et al., 2003; Luhtala and Odorizzi, 2004).

The main role of ESCRT-III is to induce the membrane budding step characteristic of vesicle formation (Teis et al., 2008). In order to complete this step, ESCRT-III must bind to an additional complex known as Vps4-Vta1, whose role is to disassemble the entire ESCRT complex and release the vesicle from its parent membrane. The dissociation of ESCRT-III is a highly endergonic step and is therefore achieved by its binding to the oligomeric protein Vps4 which is classified as an ATPase associated with various cellular activities ATPase (AAA-ATPase) (Babst et al., 1998; Gonciarz et al., 2008). As well, the accessory factors Ist1, Did2, Vta1, and Vps60 are recruited in order to provide stability to the weak interaction between ESCRT-III and Vps4 (Henne et al.,2011). In addition, these secondary proteins assist Vps4 function by promoting its ATPase activity (Azmi et al., 2006). Finally, once Vps4 oligomerization is finalized, Vta1 creates a heteromeric complex with it. Altogether, the Vps4-Vta1 complex binds the multiple CHMP4 subunits in order to enhance its interaction with ESCRT-III and mediate the dissociation process (Yu et al., 2008).

Malfunction of the ESCRT pathway has been implicated in the manifestation of several neurodegenerative diseases in which neuronal autophagy is the primary cause of symptoms. For example, it had been reported that a cohort of patients suffering from frontotemporal dementia (FTD), in which widespread cortical atrophy is observed, carried a mutation in the CHMP2 isoform CHMP2B. This resulted in abnormal mRNA splicing which ultimately affected the integrity of the protein structure (Skibinski et al., 2005). Moreover, an unrelated mutation of

CHMP2B was also found to be associated with amyotrophic lateral sclerosis (ALS), a disease characterized by neurodegeneration (Parkinson et al., 2006).

Parkinson's disease is another neurodegenerative condition, wherein the protein α-synuclein (αS), highly expressed in neurons, accumulates at an abnormal level at brain lesions. These soluble proteins eventually aggregate to form plaques, known as Lewy bodies, which are toxic to the neuron, thus leading to cell death. In an unaffected individual, αS undergoes constant turnover, thus preventing plaque formation. The ESCRT pathway is responsible for regulating αS degradation (Spencer et al., 2010). Moreover, compromising the functionality of both Vps4 and CHMP2B have been shown to contribute to αS accumulation (Hasegawa et al., 2011; Kurashige et al., 2013)

In addition to its implications with neurological conditions, the ESCRT pathway is also used by HIV during the budding process. Specifically, interactions between viral protein $p6^{Gag}$ and the ESCRT-1 subunit TSG101 take place (Stuchell et al., 2004). In addition, the membrane fission step required for budding is reliant on ESCRT-III, with the knockdown of CHMP2 and CHMP4 yielding the greatest inhibitory effect on this process (Zamborlini et al., 2006; Morita et al., 2011). Much of HIV's ability to interact with ESCRT-III is driven by ALIX's prior engagement of the CHMP4 polymer. Moreover, transient recruitment of the ATPase Vps4 can be observed immediately prior to HIV particle release, thus providing additional confirmation that the virus hijacks the ESCRT pathway during its budding process (Baumgartel et al., 2011).

Interestingly, it was discovered that CC2D1A/Freud-1 binds with high affinity to the CHMP4 isoform CHMP4B (Martinelli et al., 2012). Mutational studies indicate that Freud-1 constructs lacking the DM14 domains are unable to form this interaction, thus providing a possible

functional role for the previously uncharacterized region. Moreover, overexpressing the protein leads to a dominant negative phenotype that ultimately suppresses HIV release (Usami et al., 2012). Such involvement with the ESCRT pathway is not surprising given that its homolog, *Lgd*, was also shown to regulate CHMP4 function in *Drosophila* via its DM14 domains (Troost et al., 2012).

1.3.2 – Freud-1/CC2D1A is a regulator of the NF-KB pathway

The NF-KB pathway is arguably one of the most important and well-studied cell signaling mechanisms. Known fully as nuclear factor kappa-light-chain-enhancer of activated B cells, it refers to a family of transcription factors which are activated by various growth factors and cytokines that bind specifically to the KB promoter region found upstream of many genes. It was originally implicated in immune response-mediated protein expression due to the rapid translocation of NF-KB proteins that was observed upon pathway stimulation – the full effect can be detected within minutes (Sen and Baltimore, 1986). It was later demonstrated that this pathway has a major involvement in cell growth and division (Kaltschmidt et al., 1999). In addition, constitutive activation leads to the rapid cell proliferation observed during oncogenesis (Oya et al., 2001).

The NF-KB family of transcription factors is also referred to as the Rel family, due to the characteristic Rel homology domain (RHD). This RHD region is responsible for the protein dimerization and DNA-binding necessary in maintaining the integrity of the overall pathway (Ghosh et al., 1990; Kieran et al., 1990). The Rel/NF-KB proteins can be further classified into two groups whose main distinguishing factor is the additional functional domains contained within their respective structures. The first consists of the p105 and p100 constructs, which are ultimately cleaved into p50 and p52, respectively (Coope et al., 2002; Fan et al., 1991; Héron et

al., 1995; Moorthy et al., 2006). Along with the RHD domain, they also bear repeats of the inhibitory domain ankyrin in the C-terminus (Hatada et al., 1992). The second group consists of the Rel proteins, c-Rel, v-Rel, p65 (RelA) and RelB. They primarily differ from p100 and p105 due to the presence of a large C-terminal transcriptional activation domain (TAD) (Rice et al., 1986; Simek et al., 1988; Bull et al., 1990; Ruben et al., 1992; Ryseck et al., 1992; reviewed in Gilmore, 2006). It is important to note that these two groups of proteins are capable of both homo- and heterodimerization. Homodimers of p50 or p52 are known to be transcriptionally repressive, while heterodimers between NF-KB and rel proteins enhance gene expression (Brown et al., 1994; Plaksin et al., 1993).

There are two main routes to NF-KB activation: the canonical and non-canonical pathways. The former involves tight regulation imposed by IKB, which binds to a p50/RelA heterodimer in order to render it inactive (Brown et al., 1993). Stimulation involves receptors which, upon ligand binding, interacts with adaptor proteins such as tumour necrosis factor receptor-associated factor 2 (TRAF2) and TGFβ-activated kinase 1 (TAK1). This allows for recruitment and phosphorylation of the IKB kinase (IKK) complex which contains a regulatory subunit, NF-KB essential modulator (NEMO), and two serine-threonine kinase subunits, IKKα and IKKβ. IKK subsequently phosphorylates IKB which leads to its ubiquitination (Zandi et al., 1997; Sakurai et al., 2003). This process induces IKB proteolysis, which permits release of the p50/RelA complex and its translocation to the nucleus in order to activate gene expression (Traenckner et al., 1995).

The non-canonical pathway's main distinction is that it does not involve IKB. It is, instead, tightly regulated via the conversion of p100 to p52. Specifically, p100 dimerizes with RelB and remains constitutively inactive (Solan et al., 2002). Upon ligand-binding, adaptor proteins are

recruited to the activated receptor, which then mediate phosphorylation of the NF-KB-inducing kinase (NIK). NIK then phosphorylates IKKα which proceeds to do the same for p100 (Ling et al., 1998). This process enables cleavage of p100 to p52 (Xiao et al., 2003), which permits translocation of the entire p52/RelB complex into the nucleus (Xiao et al., 2004).

The NF-KB pathway is widely implicated in the central nervous system. Firstly, its activation has been well-described in glial cells, wherein pro-inflammatory cytokines were shown to induce gene expression similar to previous descriptions of immune responses in the periphery. Such instances were associated with traumatic impact on the brain which induced inflammation. This sort of inflammation has also been shown to occur in Alzheimer's disease, where Amyloid-β (Aβ) plaques increase neurotoxicity by elevating levels of reactive oxygen species (ROS). It was observed via hippocampal culture studies that treatment with tumour necrosis factor (TNF), a major inducer of the NF-KB pathway, could significantly reduce the toxic impact of Aβ (Barger et al., 1995).

The NF-KB pathway is also important in learning and synaptic plasticity. Through the generation of a mouse line lacking the TNF receptor, Albensi and Mattson (2000) observed abolishment of long-term depression (LTD) while long-term potentiation (LTP) was maintained. This was further confirmed through *in vitro* studies in which treatment of KB decoy DNA conferred the same effect. In addition, Meffert and colleagues (2003) demonstrated using RelA deficient mice that abolishing this pathway led to severe learning deficits. In addition, they observed that nerve stimulation led to a migration of NF-KB, specifically p50/RelA, from the synapse to the nucleus, and that this phenomenon was dependent on Ca^{2+}/calmodulin-dependent kinase II (CAMKII) (Meffert et al., 2003).

Activation of the NF-KB pathway can also be observed during neuronal proliferation. It was previously demonstrated in early postnatal mice that NF-KB activation during early development is dependent on glutamate-mediated stimulation of NMDA receptors and that this plays a role in the differential gene expression needed to permit migration of cerebellar granule cells (Guerrini et al., 1995). In 2000, Bournat and colleagues used PC12 cells, a precursor line derived from the neural crest, to demonstrate that Wnt-1 overexpression exhibited an anti-apoptotic effect via elevation of NF-KB activity.

As mentioned earlier, Freud-1/CC2D1A was originally identified as an activator of NF-KB. Matsuda and colleagues (2003) made this inadvertent discovery during their broad analysis of several previously unidentified genes with potential associations with this pathway. In a study published in 2010, Zhao and colleagues realized this connection and further observed that CC2D1A was capable of interacting with IKK and that this enabled phosphorylation of IKB. Moreover, it was demonstrated in this study that deletion of the first two DM14 domains and creating a C-terminal truncation of CC2D1A significantly decreased overall NF-KB induction. In addition to this, siRNA-mediated knockdown of TRAF2 and TAK1 inhibited CC2D1A-mediated NF-KB activation, further indicating its association with the canonical pathway. In a follow-up study, Zhao and colleagues (2011) observed in CC2D1A-deficient mice a significant increase in the rate at which synaptic vesicle trafficking occurred, as well as elevated IPSC amplitude. While the authors did not demonstrate a link between these observations and NF-KB activation, they did attribute their data to a possible role for CC2D1A in endosomal trafficking in the synapse similar to the function of its homolog *Lgd*.

1.3.3 – Freud-1/CC2D1A and the innate immune system

The innate immune system is the first line of defense for any organism. It is nonspecific and involves the production of cytokines and complement in order to assist the function of the antigen-mediated, adaptive immune system. Innate immunity relies on the activation of two main signaling pathways: RIG-1-Like Receptor (RLR) signaling and Toll-Like Receptor (TLR) signaling.

TLRs detect pathogen-associated molecular patterns (PAMPs) found on the plasma membrane or contained within endosomes. There are two subgroups of TLRS: cell surface (TLR1, 2, 4, 5, 6), which detect lipids, proteins and glycoproteins, and endosomal (TLR3, 7, 8, 9), which recognize foreign nucleic acids that have been internalized by the cell via endosomes. There are two potential adaptor proteins which permit downstream signaling upon receptor activation: MyD88 and TRIF. MyD88 recruitment leads to phosphorylation of IKKβ, which then activates NF-KB (Adachi et al., 1998). TRIF, by contrast, stimulates the TRAF family member-associated NF-KB activator (TANK) which phosphorylates TANK-binding kinase 1 (TBK-1). This leads to both NF-KB and IRF3 induction (Sato et al., 2003). MyD88 acts as an adaptor for all receptors except TLR3, while TLR4 is capable of interacting with both MyD88 and TRIF. Altogether, TLR activation ultimately leads to interferon and pro-inflammatory cytokine production.

Freud-1/CC2D1A is also referred to as TBK1-associated Protein in Endolysomes (TAPE). This is due to the discovery of its ability to act as a scaffold in order to recruit TRIF to either activated TLR3 or TLR4. In doing so, CC2D1A/TAPE is able to facilitate activation of signaling pathways that strongly promote IFN-β production. In addition, it was shown that CC2D1A colocalizes with Rab5 and LAMP1 markers, indicating that this specific role occurs in endolysosomes (Chang et al., 2011).

RLR signaling further enhances the efficacy of the innate immune system by enabling the detection of viral RNA in the cytoplasm. The two main receptors that permit this surveillance are RIG-1 and melanoma differentiation-associated gene 5 (MDA5). Both receptors detect double stranded RNA (dsRNA), however, RIG-1 also has a high affinity to 5' triphosphate viral RNA (Pichlmair et al., 2006; Wu et al., 2013). Once activated, these receptors migrate to the mitochondria where they subsequently interact with the mitochondrial antiviral-signaling protein (MAVS). MAVS activation leads to phosphorylation TBK1 and IKK. This ultimately activates interferon regulatory factor 3 (IRF3) and NF-KB in order to induce transcription of cytokines and interferon (Fitzgerald et al., 2003; Seth et al., 2005). CC2D1A/TAPE is also a regulator of RLR signaling. Through co-immunoprecipitation studies, it was shown to physically interact with both the Rig-1 and MDA5 receptors. In addition, depletion of CC2D1A results in significant inhibition of RLR-mediated IFN-β stimulation (Chen et al., 2012).

1.3.4 – Freud-1/CC2D1A and the Akt pathway

The PI3K/Akt pathway is a major mechanism involved in cell cycle progression and survival, cell division, and gene expression. It is also implicated in neuronal development processes such as axon and dendrite formation, cell proliferation, cell size determination and excitability. Its dysfunction is widely associated with cancer formation and, thus, much of the published literature surrounds its role in oncogenetics.

Akt, also known as protein kinase B (PKB), refers to a family of three structurally-related serine/threonine kinases: Akt1, widely expressed across all cell types; Akt2, which is found in insulin-sensitive organs such as the liver and skeletal tissue; Akt3, which is particularly absent in insulin-sensitive tissue. These isoforms are characterized by an N-terminal pleckstrin homology domain, responsible for lipid-binding and a C-terminal kinase region with serine and threonine

specificity (Cheng et al., 1992; Masure et al., 1999). Akt kinase activity is stimulated by growth factors, such as IGF (insulin growth factor) and EGF (epidermal growth factor), which bind to receptor tyrosine kinases (RTKs). These RTKs are homodimers whose subunits contain an extracellular ligand-binding region, a transmembrane domain and a cytoplasmic kinase domain containing several tyrosine residues with the potential for autophosphorylation. Dimerization of these subunits is ligand-induced and activates the tyrosine kinase domains (Yarden and Ulrich, 1988). The activated receptor subsequently phosphorylates phosphoinositide-3-kinase (PI3K) which goes on to convert phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3). This action permits the migration of Akt and 3-phosphoinositide-dependent protein kinase 1 (PDK1) to the plasma membrane. PDK1 then phosphorylates Akt in its C-terminal kinase domain (Auger et al., 1989; Burgering and Coffer, 1995; Currie et al., 1999; Biondi et al., 2002).

Once Akt has been phosphorylated, it can then engage a number of downstream pathways. In particular, the Akt-mediated stimulation of the serine/threonine kinase known as mammalian target of rapamycin (mTOR) is a well-documented response elicited during stem cell proliferation. This Akt/mTOR pathway is able to integrate stimuli from sources such as growth factors, stress hormones and nutrients in order to permit the cell to determine the ideal conditions for growth and division. Hyperactivity, however, can also induce tumorigenesis, which is why the cell employs inhibitory proteins such as phosphatase and tensin homolog (PTEN) to revert PIP3 to PIP2, thus regulating the level of Akt activity in the cell. (Lee et al., 1999; Gary and Mattson, 2002; Altomare et al., 2004)

The PI3K/Akt pathway is a significant contributor to neurogenesis. Mainly, its activation by various growth factors inhibits apoptotic pathways in neural stem cells (NSC). Conversely, by

specifically inhibiting Akt, cell death is a direct result (Zhang et al., 2002). Moreover, it was shown that Akt-mediated NSC growth occurs by activating the downstream target glycogen synthase kinase 3 (GSK-3) and that this mechanism functions collaboratively with cyclic GMP (cGMP) signal transduction to induce neurogenesis in the subventricular zone (SVZ) of the hippocampus (Wang et al., 2005). In addition to this, NSCs maintain a high level of reactive oxygen species (ROS) in the cytoplasm which acts to mediate growth processes that are Akt-dependent (Le Belle et al., 2011).

Growth factor induced activation of the Akt pathway is also linked with neuroprotective effects in cases of neurodegenerative diseases. Huntington's disease is one such condition and is characterized by severe dementia and involuntary movement. The cause of this disease is genetic: a polymorphism in the Huntingtin gene creates an abnormally long poly-glutamine stretch in the protein which results in selective death of neurons in the striatum. The observed cell death is specifically caused by the formation of mutant Huntingtin inclusion bodies. *In vitro* administration of insulin growth factor (IGF) was shown to cause degradation of the protein aggregates due to phosphorylation of Huntingtin by Akt. Moreover, Akt activity appears to be compromised in patients suffering from Huntington's disease (Humbert et al., 2002).

The Akt pathway is also involved in mediating recovery following traumatic brain injury. For example, Kilic and colleagues (2006) demonstrated that vascular endothelial growth factor (VEGF) receptor expression increased in damaged neurons following focal cerebral ischemia. Activation of these receptors led to increased Akt activity which resulted in downregulation of inducible NO synthase (iNOS) – an enzyme responsible for free radical formation. This ultimately led to increased neuroprotection and a restoration of the blood brain barrier in the affected areas (Kilic et al., 2006).

In a 2008 study, Nakamura and colleagues discovered that CC2D1A serves as a scaffold during Akt activation – a role that led to an additional name for this protein, Akt-kinase-interacting protein 1 (Aki1). Through co-immunoprecipitation experiments, it was demonstrated that Freud-1 binds to both PDK1 and Akt. Through domain deletion experiments, it was further observed that the fourth DM14 domain is necessary for the formation of a PDK1-Akt-Aki1 complex. Additionally, as confirmed by siRNA CC2D1A knockdown, this scaffolding function only occurred in response to stimulation by epidermal growth factor (EGF) (Nakamura et al., 2008). The role of Aki1 appears to be tissue specific, with evidence that it elicits strong EGF-induced Akt activation in a select number of lung and pancreatic cancer cell lines (Yamada et al., 2013; Ohtsubo et al., 2014). However, in others, it was shown that the presence of Aki1 exhibited little effect on pathway activation (Ohtsubo et al., 2014). Nonetheless, CC2D1A/Freud-1/Aki1 appears to be a potential target for chemotherapy, as siRNA knockdown induces apoptosis in the cancer cell lines used to study its relationship with PI3K/Akt signaling (Nakamura et al., 2008; Yamada et al., 2013; Ohtsubo et al., 2014).

1.3.5 – Additional evidence of Freud-1/CC2D1A's role as a scaffold protein

Freud-1/CC2D1A contributes as a scaffold for other pathways similar to those already described. Firstly, there is recent evidence that it is required for normal function of the protein kinase A (PKA) pathway (Al-Tawashi et al., 2012). This is a cAMP-dependent pathway and is thus activated upon stimulation of G-protein coupled receptors due to the immediate production of the cyclic AMP. Upon activation, PKA migrates to the nucleus in order to phosphorylate transcription factors such as the cAMP response-element binding protein (CREB) which then binds to cAMP response elements (CRE) within the genome in order to modulate gene transcription. Al-Tawashi and colleagues (2012) demonstrated that Freud-1/CC2D1A migrates

towards the cell surface when the concentration of cAMP increases. Moreover, CREB-phosphorylation is abolished in transgenic mice containing truncated Freud-1. This ultimately results in reduced synapse number and dendrite length in neurons, thus severely affecting plasticity (Al-Tawashi et al., 2012).

In a follow-up study, Al-Tawashi and colleagues (2013) also discovered that CC2D1A serves as a regulator of phosphodiesterase (PDE) activity. The PDEs are particularly responsible for breaking down cAMP and is, thus, necessary in maintaining its steady-state concentration. Freud-1 appears to have the ability to specifically interact with the PDE isoform, PDE4D. Furthermore, mutational experiments indicate that the DM14 domains in CC2D1A are necessary in permitting this interaction. Altogether, the current understanding is that Freud-1 both enables cAMP's function as an activator of PKA and facilitates its degradation by PDE4D (Al-Tawashi et al., 2012; Al-Tawashi et al., 2013).

1.4 – Freud-1/CC2D1A's involvement in centrosome maintenance and cell division

The centrosome is an integral component in the maintenance of an animal cell's size and shape. It is an organelle consisting of two centrioles whose main function during the cell's growth phase is to maintain microtubule organization. The centrosome's function, however, is fairly dynamic. During division, it will duplicate, disengage and travel to opposite ends of the cell in order to serve as spindle poles which are needed to separate sister chromatids. Separase, a cysteine protease, is the enzyme responsible for centrosomal separation during cell division. Interestingly, CC2D1A translocates to the centrosome during mitosis, and siRNA-mediated knockdown leads to multipolar spindle formation (Nakamura et al., 2009). This is a severe anomaly which leads to abnormal cell division and uneven distribution of sister chromatids. The ultimate result is increased cell death and a reduction in cell viability. In addition to CC2D1A's

association with the centrosome, it also forms a complex with cohesin, a protein involved in separation of sister chromatids. Specifically, Freud-1 will bind to the Scc1 domain of cohesin (Nakamura et al., 2009)

Cell cycle progression is regulated primarily by Cyclin and cyclin-dependent kinases (Cdk). There are several different cyclin isoforms which serve specific roles during different stages of the cell cycle. In particular, cyclin B1 is responsible for regulating the transition from the G2 phase to the mitotic phase. Cyclin B1 and Cdk will dimerize and subsequently phosphorylate factors involved in cell division. In regards to CC2D1A, Cyclin B1-Cdk will phosphorylate its Ser^{208} residue – a necessary step in permitting the CC2D1A-cohesin complex to form (Nakamura et al., 2010).

1.5 – Freud-1/CC2D1A mutants cause intellectual disorder

Intellectual disorder is defined as a marked impairment of conceptual, social and practical function. Altogether, these areas of cognition are responsible for allowing an individual to perform essential daily tasks such as reading, communication and personal care (American Psychiatric Association, 2013). There are several different variants of intellectual disorder, each distinguished by its severity. The most common type is non-syndromic intellectual disorder (NSID), which is characterized as having severe cognitive impairments but no additional physical ailments. NSID is known to affect up to 3% of the World's population. In order to be diagnosed with this disease, one must display hindrances in cognitive functions from birth and possess an IQ below 70. As of now, the search for a genetic cause of NSID has posed a real challenge.

Mutations in several genes have thus far been implicated in the manifestation of NSID. These mutations can either by autosomal or X-linked and are identified through widespread genetic screening of affected individuals. Many of the affected genes encode enzymes involved in maintaining normal neuronal metabolism and kinases involved in important signaling pathways, which altogether direct synaptic formation and ensure cell viability during neurogenesis. For example, in a 1998 study, a point mutation in PAK3, a kinase involved in G-Protein signaling, was discovered in sufferers of X-linked NSID. While this protein is highly expressed in postmitotic cortical and hippocampal neurons, no severe physical and developmental defects were observed in individuals who bore the mutation, thus reinforcing the notion that NSID only affects cognition (Allen et al., 1998).

An autosomal recessive mutation affecting the serine protease neurotrypsin was also associated with NSID. This enzyme is highly expressed in brain regions involved in memory and learning, and is highly localized at the presynaptic nerve ending, specifically in the membrane forming the synaptic cleft. The discovered mutation causes a 4 base pair (bp) deletion which leads to a truncation of the protein's structure (Molinari et al., 2002).

Other genetic defects have been shown to affect synapse formation and integrity of dendritic spines. In a 2009 study, it was discovered that autosomal recessive point mutations in SYNGAP1 cause NSID. SYNGAP1 is a RAS GTP-ase activating protein that is specifically associated with synapse formation. The investigators involved in this study identified three different point mutations in this protein which led to varying degrees of truncation, were all autosomal recessive in nature, and contributed to the manifestation of NSID (Hamdan et al., 2009).

Some NSID-associated mutants produce effects which could have more severe effects on the affected patient. One such mutation occurs in the gene encoding UPF3B, a regulator of nonsense-mediated mRNA decay (NMD). NMD is an important pathway involved in the detection and degradation of faulty mRNA products. UPF3B is a surveillance protein involved in the detection of these nonsense transcripts. Several X-linked point mutations have been discovered in this gene, some of which produce NSID. However, among these mutations, there are a number which, in addition to intellectual disorder, produce facial defects such as an abnormally long face and pronounced facial asymmetry (Tarpey et al., 2007).

A 2006 study revealed CC2D1A as a candidate gene for the manifestation of NSID in nine families originating from Israel. The discovered mutation yields a deletion of aa 408 to 547 and a frameshift mutation immediately after this deletion. The result is an extreme truncation that removes the entire C-terminal half of Freud-1/CC2D1A including the fourth DM14 and C2 domains (Basel-Vanagaite et al., 2006). A similar study uncovered single nucleotide polymorphisms (SNPs) among several families in a Han Chinese population which contributed to NSID (Shi et al., 2012). In this case, the structural effect of the SNP remains unclear. Moreover, in both studies mentioned, the effect of CC2D1A mutation on the cell is unknown.

1.6 – CC2D1A mutations and Autistic Spectrum Disorder

Autism is a highly heritable neurodevelopmental disorder that affects up to 0.6% of the population. It is associated with abnormalities in social interaction, communication and behaviour. Autism only denotes one condition among several which form a group known as Autistic Spectrum Disorder (ASD), among which Asperger and Rett disorder are included. Despite its highly heritable nature, wherein twin studies demonstrate over 60% concordance, finding genetic causes for ASD remain a challenge. Genetic analysis has shown that copy

number variations (CNVs) – inheritance of an abnormal number of alleles for a given gene – in genes coding for glutamatergic synaptogenesis are strongly associated with instances of ASDs (The Autism Genome Project Consortium, 2007).

CC2D1A has also been implicated in a number of instances of ASD. In these cases, point mutations in the gene have resulted in comorbidity between ASD and intellectual disorder, as well as epilepsy. The effect on CC2D1A's protein structure is severe, leaving only a 20 kDa N-terminal fragment lacking all of the functional domains. The consequences of this mutation are profound, yielding excessive NF-KB signaling activity. This ultimately leads to a reduction in dendritic complexity, thus indicating that CC2D1A mutation has a profound effect on synaptogenesis (Manzini et al., 2014).

1.7 – CC2D1A$^{-/-}$ transgenic mouse models

Several mouse models have been generated to study the effects of global Freud-1 knockout. Currently, all have resulted in lethal phenotypes. However, the degree to which removing CC2D1A from the genome varies among the studies published to date.

Zhao and colleagues (2011) were the first to attempt this feat. By inserting the LacZ reporter gene in place of the first exon of CC2D1A and cloning this construct into a mouse embryonic stem (ES) cell line, they were able to achieve complete knockdown in C57BL/6 mice. The result of this deletion was death of homozygous mice mere minutes after birth. The cause of death was determined to be postnatal respiratory failure. By contrast, heterozygous mice were still able to retain wild-type phenotypes (Zhao et al., 2011). In a more recent study, Al-Tawashi and colleagues (2012) engineered global CC2D1A$^{-/-}$ mice by replacing exons 8 to 14 with a neomycin phosphotransferase gene cassette and cloning the construct in mouse ES cells. The

resulting mutant mouse expressed a truncation which deleted all functional domains except the first DM14 region. The group observed lethality in the mice within the first 12h of birth. Moreover, the pups had a significantly lower weight, hunched back and abnormalities in the brain's vasculature (Al-Tawashi et al., 2012).

To circumvent the lethality of global $CC2D1A^{-/-}$ mice, efforts have been made by the Paul Albert research group to create a conditional inducible knockout transgenic line using the Cre-LoxP system. This is achieved by crossing two different breeds of mice. The first expresses the Cre recombinase enzyme conjugated to an ER^{T2} estrogen receptor engineered to bind specifically to tamoxifen. This $CreER^{T2}$ protein is driven by the tryptophan hydroxylase 2 (TPH2) promoter, thus allowing it to be expressed specifically in serotonin-producing neurons located in the DRN. The second mouse line expresses CC2D1A with LoxP sites flanking exons 12 and 14. Once crossed, the generated $CreER^{T2}$/flx-C2D1A mice are born and allowed to reach adolescence. At this point, they undergo a five day treatment of daily tamoxifen injections, whose purpose is to activate the ER^{T2} receptor. The activated receptor releases the Cre recombinase, which then translocates to the nucleus where it recognizes the LoxP sites and excises the flanked exons (Vahid-Ansari et al., 2014). The result is a truncated CC2D1A mutant with similar architecture to the naturally occurring NSID-causing mutant described by Basel-Vanagaite and colleagues (2006).

The purpose of this conditional, inducible CC2D1A knockout mouse is to denote changes in 5-HT1A autoreceptor expression and observe its effects on the animal's behaviour. Thus far, obtained immunohistochemical data indicate increased expression of the 5-HT1A autoreceptor in the DRN. This has been accompanied by anxiety- and depressive-like behaviour during tests

such as the elevated plus maze, forced swim test and novelty suppressed feeding (Vahid-Ansari et al., 2014).

1.8 – Purpose and Hypothesis

The generation of the conditional, inducible Cre-ER^{T2}/flx-CC2D1A mouse presents substantial potential for *in vitro* study. Mouse embryonic fibroblasts (MEFs) originating from this transgenic line have been previously extracted and immortalized. These cells permit the study of the molecular biological effects of Freud-1 knockdown, including its impact on transcription, signaling pathways and cell health. Moreover, the way by which the Freud-1 mutation was engineered may provide insight into the molecular biology of NSID.

Taking this into consideration, the purpose of this study is to investigate the impact of using a recombinant Cre recombinase to produce an NSID mutation in CC2D1A in MEF cells. There will be a focus on its effects on EGF receptor (EGFR) signaling, since it was previously discovered that Freud-1 acts as a scaffold during EGF-mediated Akt activation (Nakamura et al., 2008). This pathway is particularly important due to the role EGF plays in neurogenesis during prenatal development (Ostenfeld and Svendsen, 2004). If the previously described, naturally occurring CC2D1A mutants lead to abolishment of the Akt activity, which is important for neural stem cell growth and proliferation, this may indeed provide an explanation as to why non-syndromic intellectual disability is the prevailing symptom.

I hypothesize that the *in vitro* use of Cre recombinase to knock out CC2D1A expression in MEF cells will completely suppress EGF receptor activity. To test this, Akt and ERK1/2 phosphorylation will be analyzed via Western Blot, as these are direct consequences of EGF receptor activation. In addition, reverse-transcriptase polymerase chain reaction (RT-PCR) and

immunocytochemistry (ICC) will be used to verify the integrity of the knockout. The results obtained in this study may shed light on the molecular biological effects of mutant CC2D1A and how this might contribute to cognitive dysfunction. As well, the generation of a stable CC2D1A$^{-/-}$ cell line can potentially become an effective expression system in which a mutational analysis of Freud-1 may be performed in order to truly dissect its functional domains and their contribution to the numerous attributed roles.

Chapter 2: Materials and Methods

2.1 - Antibodies, Plasmids and Primers

Wild-type and mutant CC2D1A constructs were previously generated and subcloned into the pTriex.4 vector. Rabbit polyclonal anti-Akt and monoclonal anti-pAkt(Thr308) antibodies were provided by Cell Signaling Technologies and diluted 1/1000 for Western blot analysis. HRP-conjugated Erkl1/2 and rabbit monoclonal phospho-ERK1/2 antibodies were provided by Cell Signaling Technologies (CST) and diluted 1/1000 for Western Blots. Rabbit polyclonal anti-CC2D1A antibody was previously generated and diluted 1/20000 for Western blot analysis and 1/1000 for immunocytochemistry. Mouse anti-β-Actin antibody was provided by Sigma and diluted 1/20000 for Western Blots. HRP-conjugated anti-Rabbit IgG antibody was provided by Cell Signaling Technologies and diluted 1/2000 for Western Blots.

Primers were synthesized by Integrated DNA Technologies as follows:

- Freud-1 N-Terminus (exons 2-6)
  - Forward: 5’-GCCCTAGAGAAACTGAAAGG-3’
  - Reverse: 5’-AGAAGCCAACTGGGAAGTAG-3’
- Freud-1 C-Terminus (exons 17-23)
  - Forward: 5’-CCTTCAGTGTCATCAAGGTC-3’
  - Reverse: 5’-CAGCCACCTCTCAGTTGTAG-3’
- β-Actin
  - Forward: 5’-GACAACGGCTCCGGCATGTG-3’
  - Reverse: 5’-TGGCTGGGGTGTTGAAGGTC-3’

2.2 - Cell Culture

Mouse embryonic fibroblasts (MEFs), previously harvested and immortalized using a protocol outlined by Xu (2005) from the flx-CC2D1A transgenic mouse line received from Dr. Chiara Manzini and Dr. Chris Walsh (Harvard University, Boston, MA), were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. Cells were passaged every 48h and grown in a 10cm culture dish (Corning). Cells were resuspended by incubation with Trypsin-EDTA solution. The cell suspension was diluted in 10mL DMEM with 10% FBS and seeded onto the desired plate. Cells were seeded in a 1:10 dilution with media.

2.3 - TAT-Cre Treatment

MEFs were seeded in 12-well culture plates at a density of $5x10^4$ cells/well and left to grow overnight at 37°C + 5% CO_2. Media was changed the following day with serum free DMEM and TAT-Cre was administered at a final concentration of 2μM. Cell health was assessed qualitatively every 30 minutes. After 4 hours, Cre treatment was halted by washing cells twice with 1X PBS and changing the media to DMEM + 10% FBS. Cells were left to reach full confluence and then passaged in 10 cm culture dishes (Corning). Protein levels were assessed through Western Blot. Procedure was repeated until complete knock down of the desired protein was achieved.

2.4 - EGF Treatment

MEFs were seeded in a 6-well culture plate (Falcon) at a density of $1x10^6$ cells/well and incubated with DMEM containing 10% FBS overnight at 37°C + 5% CO_2. The following day, medium was replaced with DMEM containing 0.1% FBS and incubated for an additional 24h.

The following morning, recombinant human epidermal growth factor (ThermoFisher) was added to the media at a final concentration of 50ng/mL at various time points. Cells were then lysed in 1mL 1x Laemmli Buffer (2% (w/v) SDS, 10% Glycerol, 60mM Tris-HCL (pH 6.8), 0.01% (w/v) bromophenol blue, 5% β-mercaptoethanol), vortexed, and boiled for 10 minutes. Protein expression and phosphorylation levels were then assessed using Western Blot.

2.5 - Transfection and Phenotype Rescue

MEFs were seeded in 6-well culture plates (Falcon) at a density of $1x10^5$ cells/well and incubated overnight. The following day, plasmids were mixed with FastFect transfection reagent (Feldan) at a 1:2 DNA-to-reagent ratio following manufacturer's protocols. Transfection mixtures were applied drop-wise to each well and cells were incubated overnight at 37°C + 5% CO_2. Media was changed the following day and cells were incubated in the same conditions for an additional night. Cells were lysed the following day after EGF Treatment.

2.6 - Western Blot

10uL of cell lysate were separated using SDS-PAGE for 1 hour at 60mA. Gels were transferred to a PVDF membrane overnight at 100mA. PVDF membranes were blocked for 1 hour at room temperature in 1x Tris-Buffered Saline and Tween 20 (TBST) (50mM Tris, 150mM NaCl, 0.1% Tween 20; pH 7.4) containing either 5% Powdered Skim Milk for total protein or 5% Bovine Serum Albumin (BSA) for phosphorylated protein. Membranes were incubated with the desired primary antibody diluted in TBST with 5% milk or BSA for 16h at 4°C. Blots were washed three times in 1x TBST for five minutes. Membranes were then incubated with the appropriate secondary antibody diluted in TBST with 5% milk or BSA for 1h at room temperature. The blots were then washed three times in 1X TBST for five minutes. Immobilon™ Western

Chemiluminescent HRP Substrate (EMD Millipore) was applied to the blots and protein levels were detected using autoradiography film (Denville). Digital images of the exposed film were acquired using an HP 400MFP LaserJet All-In-One printer. Band intensity was measured using ImageJ software and raw values for total protein were normalized to β-Actin levels. Measurement of protein phosphorylation was then normalized to the total protein being phosphorylated (e.g. total Erk1/2 and total Akt).

2.7 - Immunocytochemistry

Cells grown on coverslips (Fisher) were washed with 1X phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA). The cells were then blocked in PBS containing 0.03% Triton X-100 (PBS-T) supplemented with 0.5% Donkey serum for 1 hour at room temperature. The desired primary antibody, diluted in PBS-T, was applied to the samples and left to incubate for 16h at 4°C. Cells were then washed three times in PBS-T for five minutes each. Secondary antibody, diluted in PBS-T, was then applied to the samples and left to incubate for 2h at room temperature. Nuclear stain was achieved through a 5 minute incubation with a solution of 4',6-diamidino-2-phenylindole (DAPI) diluted in 1X PBS. Coverslips were washed twice in PBS and once with water. They were then mounted to slides using ImmuMount reagent (Thermo). Slides were then preserved at 4°C or analyzed under 630x magnification using the Zeiss LSM-510 confocal microscope.

Freud-1 fluorescence was quantified in whole cell and nucleus (nucleus = DAPI stained area) separately. Extranuclear localization was determined by taking the difference of the total and nuclear fluorescence. Three cells were quantified in three different slides; n=9. Values for integrated density, area of cell and mean fluorescence were acquired using ImageJ software and used to calculate total cellular fluorescence using the following formula:

Corrected total fluorescence = Integrated Density – (Area of selected cell X Mean fluorescence of background)

2.8 - mRNA Extraction and RT-PCR

Cells were seeded on a 6-well plate (Corning) and cultured for 48h. Samples were extracted via incubation with 1mL of Trizol reagent (Life Technologies) for 5 minutes at room temperature. 0.2mL of chloroform were added to each sample, shaken vigorously and centrifuged at 12000 x g for 15 minutes at 4°C. The aqueous phase (supernatant) was transferred to a clean Eppendorf tube and mixed with 0.5mL of anhydrous isopropanol. Samples were incubated for 10 minutes, after which they were centrifuged at 12000 x g for 10 minutes. Supernatant was discarded and the RNA pellet was resuspended in 1mL of 75% ethanol, vortexed and centrifuged at 7500 x g for 5 minutes. The resulting RNA pellet was air-dried for 5 minutes and resuspended in 20 µL nuclease-free H_2O. mRNA samples were quantified using the NanoDrop 2000 (Thermo Scientific) and the $A_{260/280}$ ratio was used as a screening measure for purity. Samples were then immediately used in downstream applications were stored at -80°C.

cDNA was synthesized from the extracted mRNA using the m-Mulv reverse transcriptase (Thermo Scientific). 1.5µg mRNA was used per reaction. Reaction mixture also included a final concentration of 4 µM random primers and 0.5 mM dNTPs. Nuclease-free H_2O was added to a final volume of 16µL. Samples were incubated at 65°C for 10 minutes to denature the mRNA. After this period, 1 µL m-Mulv reverse transcriptase, 1X reaction buffer and 1 µL RNAsin were added to the mixture. The reactions were then incubated at 42°C for 1 hour and then at 85°C for 10 minutes in order to inactivate the enzyme. Products were then either immediately used in downstream applications or stored at -20°C.

A Taq polymerase kit with Thermopol reaction buffer (New England Biolabs) was used to amplify the cDNA products. Each reaction mixture contained 1.5 µL of cDNA and nuclease-free H_2O was used as a negative control to screen for contamination. A final concentration 0.5mM dNTPs, 0.5µM forward and reverse primers and 1X ThermoPol Reaction Buffer were required for each reaction. Nuclease-free H_2O was used to bring the final volume up to 25 µL. 0.5 µL Taq DNA polymerase was added to each reaction mixture immediately before amplification.

The PCR parameters are as follows:

- Initial denaturation: 95°C for 30 sec
- Cycles:
    - Denaturation: 95°C for 30 sec
    - Primer Annealing: 50°C (Freud-1)/58°C (β-Actin) for 30 sec
    - Elongation: 68°C for 30 sec
- Final Elongation: 68°C for 5 min

The cycle numbers for β-Actin and Freud-1 are 25 and 30 respectively.

PCR samples were loaded onto 1.2% agarose gel containing ethidium bromide and electrophoresed at 120V for 45 minutes. The gel was analyzed under UV light and an image was acquired using AlphaQuant software. Band intensities were measured using ImageJ software.

2.9 - Purification of Recombinant Protein

All CC2D1A constructs were transformed into E. Coli BL21 DE3 competent cells. Colonies were inoculated in 2YT media and grown until an OD_{600} measurement of 0.6 was obtained. Protein expression was induced using IPTG at a final concentration of 1mg/L. The cultures were

centrifuged at 4500rpm for 15 minutes and the pellet was resuspended in equilibration buffer (50mM sodium phosphate, 300mM sodium chloride, 10mM imidazole, pH 7.4) containing protein inhibitors (0.1ug/ul leupeptin; 0.01mM PMSF; 1ug/mL aprotinin) and 1mg/mL lysozymes (Sigma-Aldrich). The cell suspension was incubated for 30 minutes at 4°C and then sonicated for six 10 second intervals. The suspension was then centrifuged at 8500 rpm for 30 minutes. The resulting supernatant was mixed with 2mL HisPur cobalt resin (ThermoScientific) and left to shake gently for 1 hour at 4°C. The lysate-resin mixture was then centrifuged for 2 minutes at 700 x g. The pellet was washed twice using equilibration buffer, centrifuging for 2 minutes at 700 x g after each time. Protein was then eluted using elution buffer (50mM sodium phosphate, 300mM sodium chloride, 150mM imidazole, pH 7.4) and centrifuged for 2 minutes at 700 x g. The supernatant was extracted and dialyzed in phosphate-buffered saline for 16h at 4°C. The resulting protein extract was quantified using Bradford assay and stored in 50ul aliquots at -80°C. Protein structure was verified using Western Blot.

2.10 - Lipid Binding Assay

PIP strips (Molecular Probes) were blocked using TBST + 0.1% ovalbumin for 1 hour. Strips were then incubated overnight at 4°C with 100ng of purified protein. This was followed by three fifteen minute washes in TBST + 0.1% ovalbumin. S-conjugate HRP antibody was then applied to each strip and left to incubate overnight at 4°C. This was followed by three washes. Strips were treated with Immobilon™ Western Chemiluminescent HRP Substrate (EMD Millipore) and exposed to autoradiography film. A digital image was obtained by scanning the film with an HP LaserJet 400MFP printer. Lipid binding intensity was quantified using ImageJ software.

2.11 - Statistical Analysis

All statistical analyses were performed using GraphPad Prism software. When comparing two different samples, Student's unpaired t-test was used. For experiments containing three or more different sample populations, analysis of variance (ANOVA) was performed followed by Tukey's post-hoc test for multiple comparisons. A confidence level of 95% was used as a benchmark for statistical significance.

Chapter 3: Results

3.1 – TAT-Cre mediated knockdown of Freud-1/CC2D1A

Knockdown of Freud-1 was executed with the purpose of assessing the integrity of EGF receptor signaling in the absence of this previously identified scaffold protein. This initial step in the study required 3 successive treatments of TAT-Cre (Excelgen) at a final concentration of 2 μM. Consistent with the manufacturer's guidelines, the first treatment reduced the presence of wild-type CC2D1A by 50% (Figure 1C). Further treatment led to undetectable Freud-1/CC2D1A protein levels in western blot and immunocytochemistry (Fig 1, B &D).

Treatment of TAT-Cre was expected to delete the C-terminal region of CC2D1A. The result was supposed to mimic the NSID mutation observed by Basel-Vanagaite (2006) in humans. This was achieved through the addition of LoxP sites flanking exons 12-14 of the mouse Freud-1 gene. The Cre recombinase would identify and excise these sites, thus removing the 3 exons (Figure 1a). In addition, a frameshift mutation would occur leading to a truncation approximately half the protein, removing the fourth DM14, helix-loop-helix and C2 domains.

The results of this study demonstrate that CC2D1A protein is entirely absent after TAT-Cre-induced knockdown. The NSID mutant was expected to be approximately 70 kDa in size – such a fragment could not be detected using western blot (Figure 1B). RT-PCR analysis, however, highlights a strongly significant reduction of Freud-1 mRNA levels in the knockout MEFs (Figure 2a). In addition, by amplifying the N- and C-terminus regions separately, it was possible to demonstrate that the C-terminal half of CC2D1A is significantly more reduced than the N-terminal, thus confirming the expected mechanism of action for the Cre recombinase in the flx-CC2D1A mouse fibroblasts (Figure 2b).

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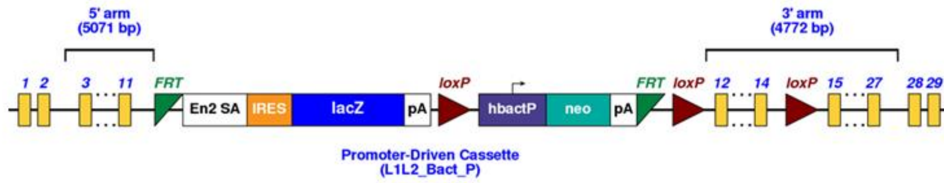

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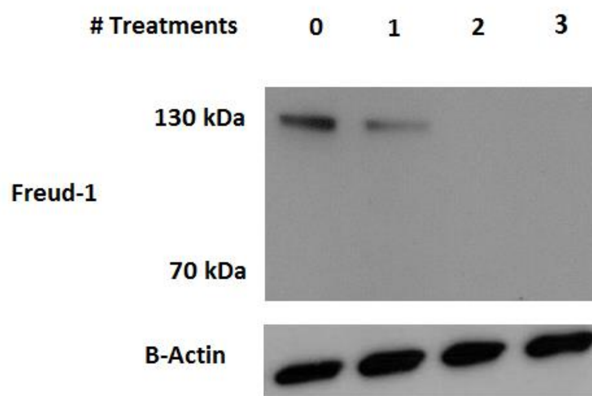

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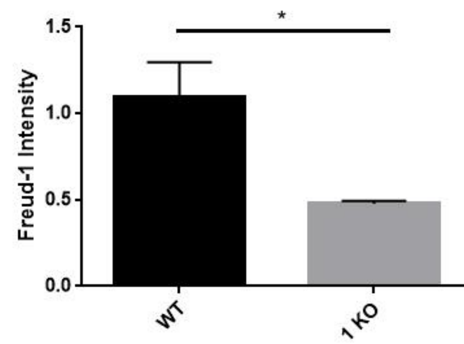

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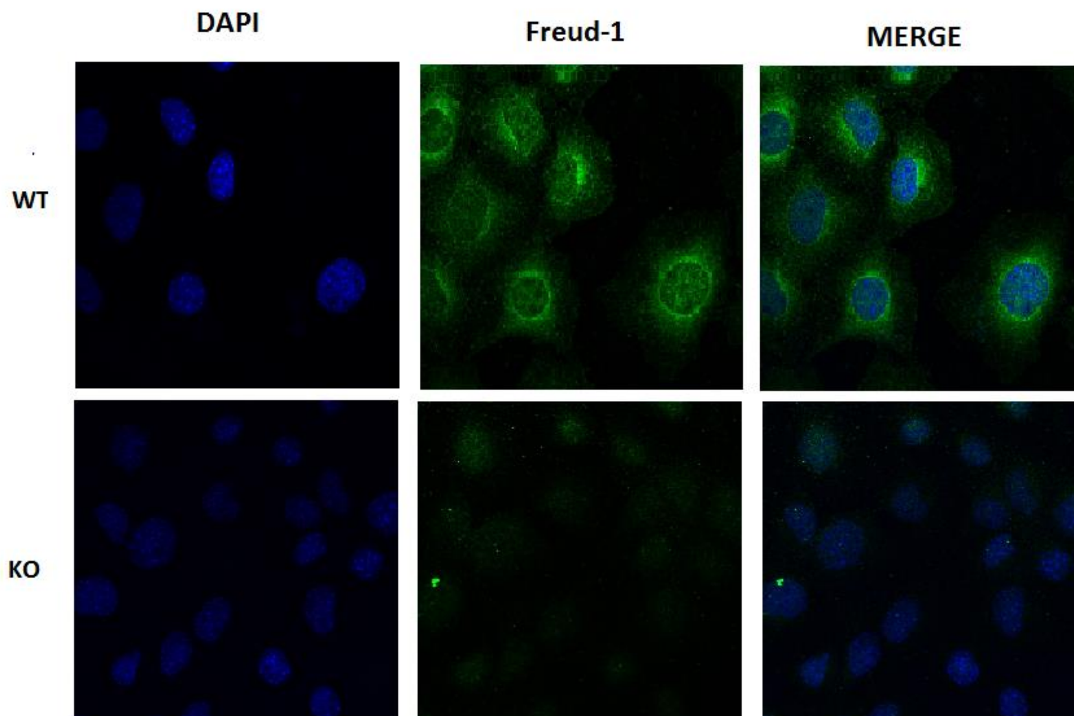
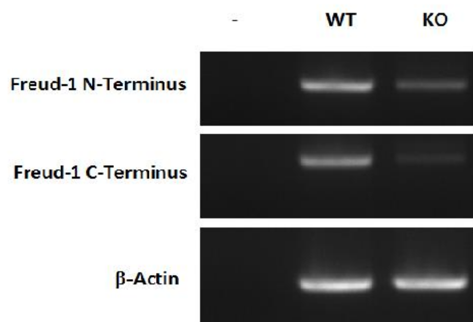


Figure 1. Effect of TAT-Cre treatment on Freud-1/CC2D1A protein levels. A) A schematic of CC2D1A containing LoxP regions flanking exons 12 and 14. Treatment with Cre-recombinase will excise this fragment and truncate the C-terminal region. B) Western blot representation of successive TAT-Cre treatments. Three treatments were sufficient for Freud-1 knockdown. C) Statistical analysis of protein levels in wild-type and single Cre treatment samples. Unpaired Student's t-test was performed on this data (*$p < 0.05$; N=3) D) Immunofluorescent validation of reduction of Freud-1 protein levels in MEFs.



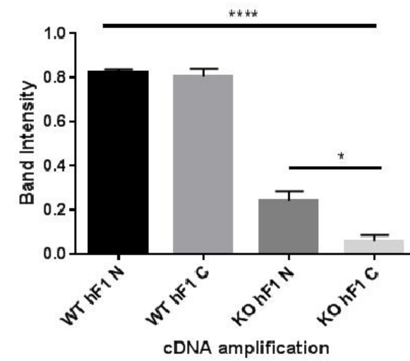
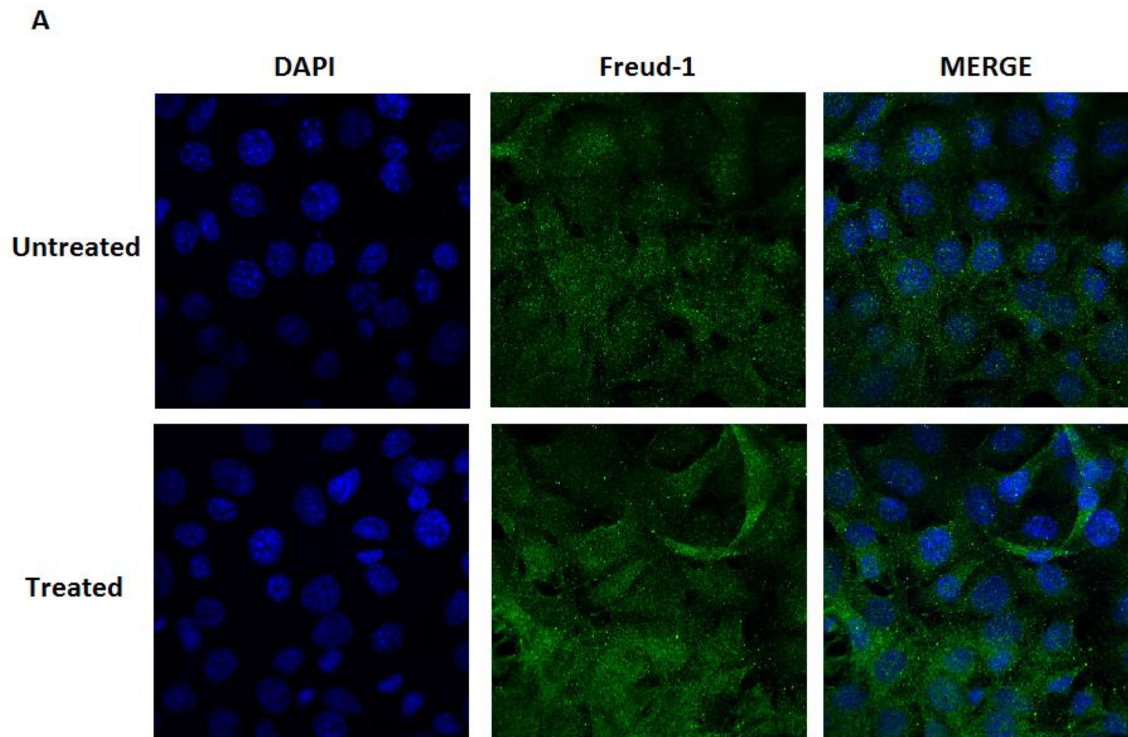
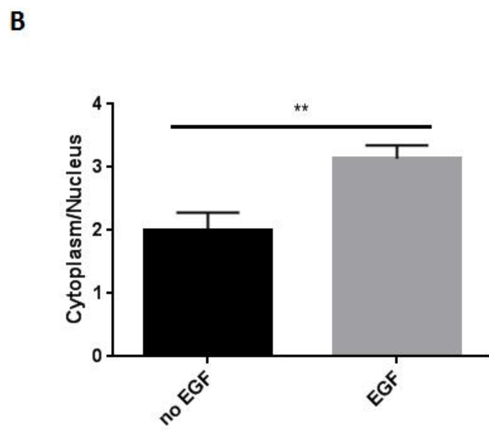


Figure 2. RT-PCR analysis of Freud-1 mRNA levels. A) cDNA fragments were generated from Freud-1 N- and C-terminal fragments and amplified by PCR. B) Statistical results of band intensity normalized to β-Actin expression. One-way ANOVA followed by Tukey's post-hoc test yielded ****$p < 0.0001$, N=3.

3.2 – CC2D1A translocates to the extranuclear space upon EGF treatment

Freud-1 has been shown to migrate to the plasma membrane in order to participate in signaling pathways (Al-Tawashi et al., 2012). Immunocytochemistry was used to verify that CC2D1A is indeed responsive to EGF treatment (Figure 3A). It was expected that Freud-1 would translocate from the nucleus into the cytoplasmic space upon growth factor treatment in order to engage in Akt signaling. Quantitative analysis of Freud-1 fluorescence indeed demonstrates a slight, yet significant, increase in its presence in the extranuclear compartment when cells are stimulated with EGF (Figure 3B). To control for human error in this analysis, a calculation of the ratio of total cell area to nuclear area was done in order to verify that the size of cells chosen for analysis were not larger in one sample group than the other. Statistical analysis of this calculation reveals no significant difference in cell size between treated and untreated samples (Figure 3C).





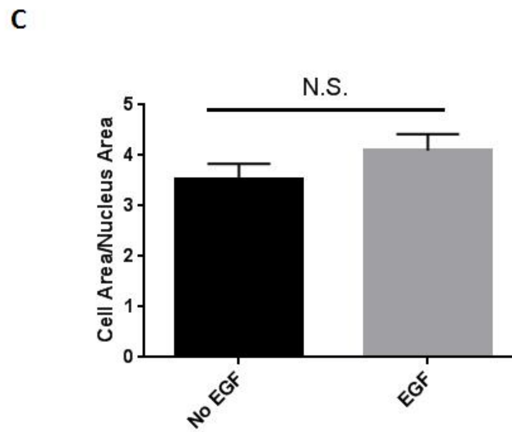


Figure 3. Immunofluorescent analysis of Freud-1 cellular localization. A) Immunocytochemistry performed on MEFs either untreated or stimulated by EGF. B) Statistical analysis of Freud-1's presence in cytoplasm. Unpaired Student's t-test was performed on this data (**p=0.0051; N=9). C) Statistical comparison of cell area between untreated and treated cells. Unpaired Student's t-test performed on this data yields p=0.2143 (N=9).

3.3 – EGFr signaling is unchanged in CC2D1A$^{-/-}$ MEFs

Gaining insight into the impact of Freud-1 knockdown on EGF receptor signaling required calibration of several parameters. Firstly, basal Akt activity needed to be significantly reduced in order to observe a measurable effect of EGF treatment. Titrating the concentration of fetal bovine serum in the DMEM from 10% to 0.1% was sufficient to reduce p-Akt(Thr308) phosphorylation. Akt(Thr308) was specifically analyzed in this study due to it being targeted for phosphorylation by the kinase PDK1, which was shown to have interactions with Freud-1. It is important to note that Akt has an additional phosphorylation site at Ser473 which is affected by an unknown kinase and there is no evidence of any contribution by Freud-1. In addition, the serum concentration of 0.1% was selected based on its use in previous publications, and the results of this study provide further validation for it (Figure 4B) (Vivanco and Sawyer, 2002; Nakamura et al., 2008). Thus, MEFs were maintained in media containing 0.1% serum for 24h before EGF treatment. Next, a time course was done to identify the optimal period of EGF incubation. The results from this assay indicate that 15 minute stimulation produced a maximal effect on Akt signaling (Figure 4C & D).

Activation of the EGF receptor stimulates both Akt and Erk signaling. Moreover, Freud-1's involvement in the PI3K/Akt pathway was specific to EGF induction (Nakamura et al., 2008). Therefore, it was important to test both pathways in order to determine whether Freud-1 regulated EGF receptor activity as a whole, or was just specific to Akt signaling. The data obtained in this study show that phosphorylation of Akt and Erk 1/2 significantly increased upon EGF treatment in both wild-type and knockout MEFs (Figure 5 B&C). In addition, the activation level of each pathway was not significantly affected in the absence of CC2D1A

(Figure 6 A&B). It is also important to note that no significant difference was observed in total Akt or Erk1/2 expression after Cre treatment or EGF stimulation (Figure 9; see Appendix).

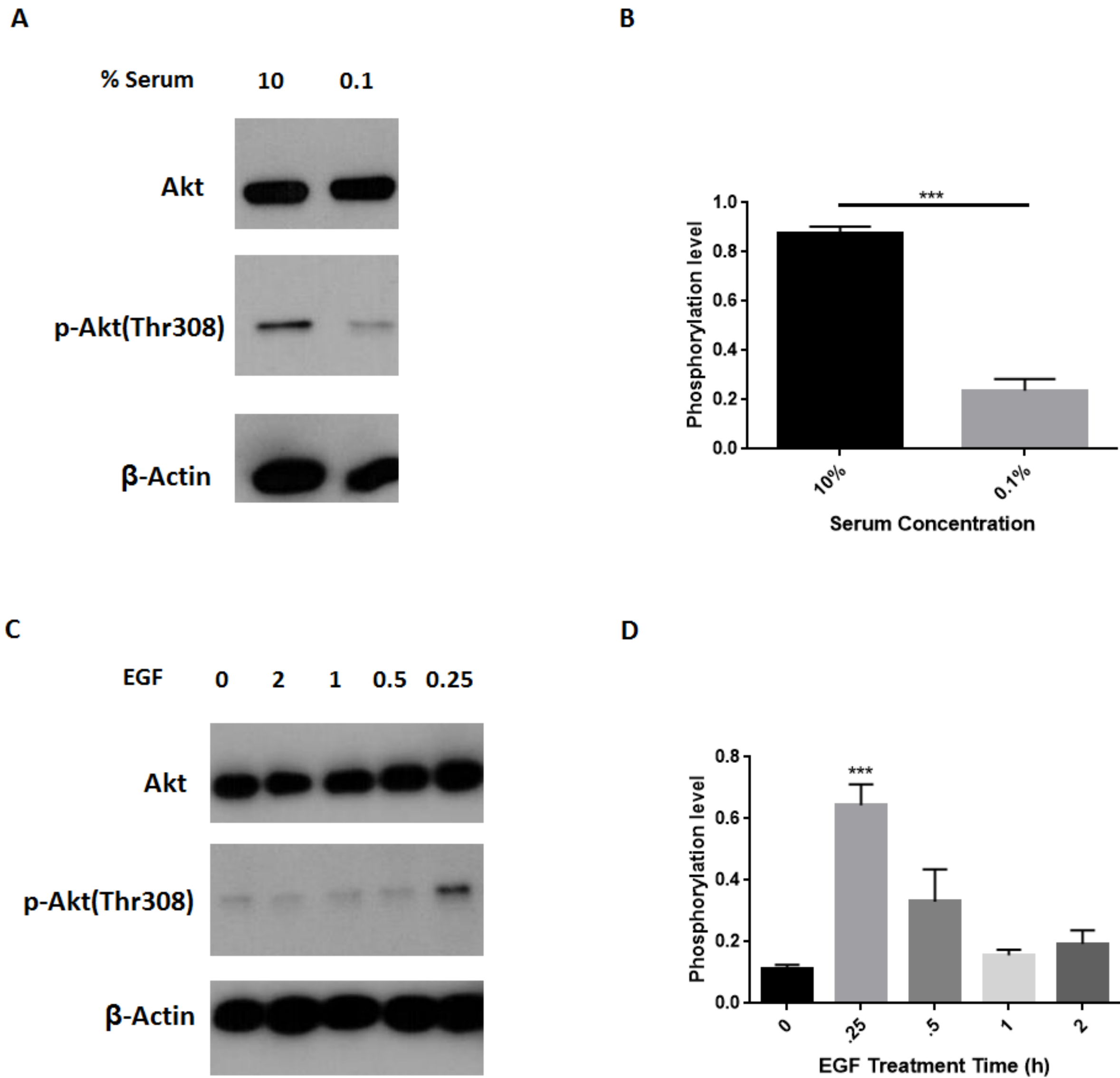


Figure 4. Optimization of EGF treatment. A) Western blot demonstrating the difference in Akt phosphorylation in the presence of either 10% or 0.1% FBS concentration. B) Quantification of the effect of reducing FBS concentration on Akt signaling. Unpaired student's t-test was performed on this data (***p=0.0003, N=3). C) Western blot analysis of EGFr signaling at varying time points. D) One-way Analysis of variance following by Tukey's post-hoc test of multiple comparisons was done on this data (***p=0.0006, N=3)

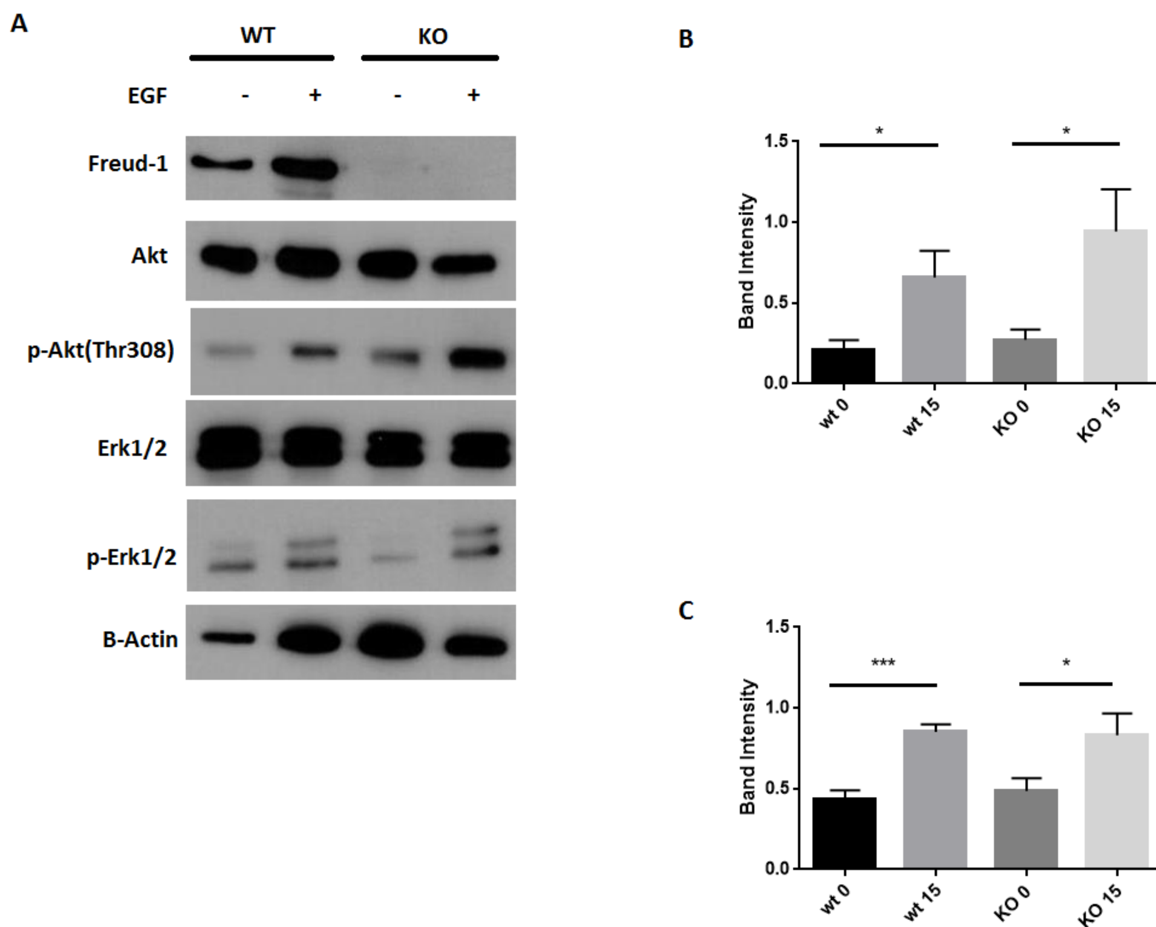


Figure 5. Comparison of EGF signaling in CC2D1A$^{+/+}$ and CC2D1A$^{-/-}$ MEFs. A) Western blot analysis of Akt and ERK phosphorylation upon EGF treatment. B) Quantitative analysis of Akt phosphorylation. Wild-type and knockout cells were calculated separately. Unpaired student's T-test was performed for each data set. (Wild-type: *p=0.0108; N=9/ Knockout: *p=0.0217; N=9). C) Quantitative analysis of ERK 1/2 phosphorylation. Wild-type and Knockout samples were grouped separately. Unpaired student's T-test was performed for each data set (Wild-type: ***p=0.0001; N=7/Knockout: *p=0.0488; N=7).

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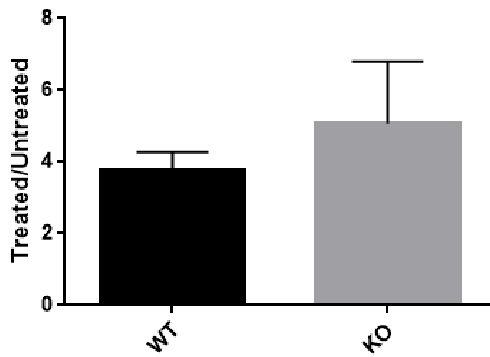

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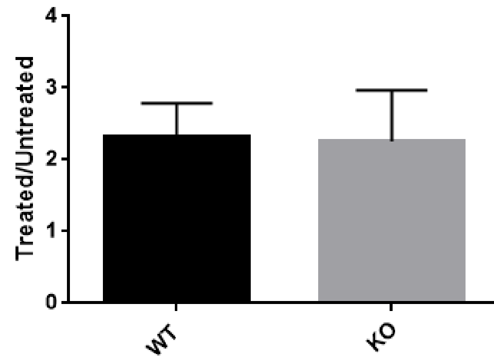

Figure 6. Analysis of Akt and Erk1/2 activation levels in wild-type and knockout MEFs. A) Comparison of Akt activation in wild-type and knockout cells. Unpaired Student's t-test was performed on the data (p=0.4762, N=9). B) Comparison of ERK1/2 activation in wild-type and knockout MEFs. Unpaired Student's t-test was performed on that data (p=0.9369; N=7)

3.4 – CC2D1A overexpression has no impact on EGFR signaling

It was previously demonstrated that Freud-1 overexpression has the potential of producing a dominant negative phenotype in regards to regulating NF-KB signaling (Manzini et al., 2012). To test this possibility in EGF signaling, CC2D1A$^{-/--}$ MEFs were transfected with 1 μg recombinant human Freud-1 subcloned in the pTriEx4 vector. Cells transfected with empty pTriEx4 vector and untransfected CC2D1A$^{-/-}$ MEFs were used as negative controls. Stimulated wild-type cells were also included as a positive control.

Transfection of 1 μg of Freud-1 was sufficient to induce overexpression of protein (Figure 7A). Interestingly, transfecting a lower amount of this plasmid led to no expression, thus it was not possible to attain similar Freud-1 protein levels to the wild-type cells. In addition, transfection efficiency was approximately 40% as determined by GFP plasmid. Analysis of Akt and Erk1/2 phosphorylation demonstrated a significant increase upon EGF treatment in all collected samples. However, there was no significant difference in activation of either pathway among the included sample groups (Figure 7, B&C).

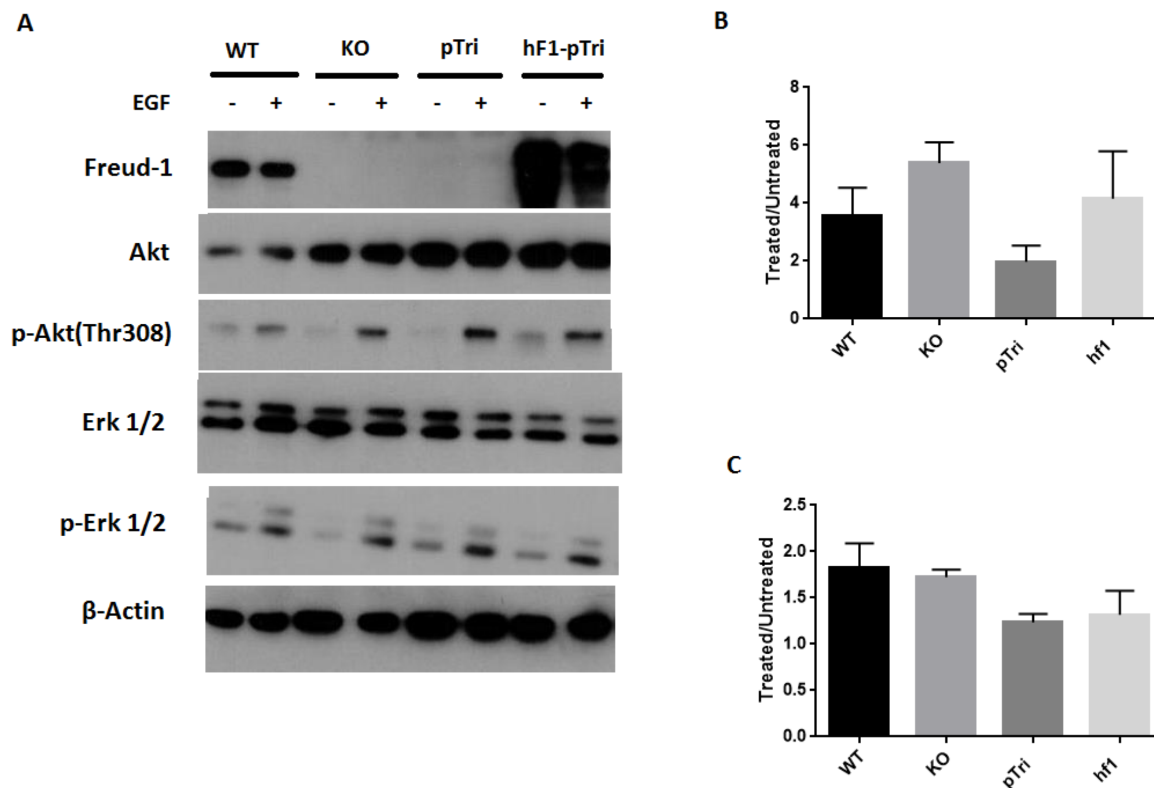

Figure 7. EGFR signaling in MEFs overexpressing CC2D1A. A) Western blot of Akt and ERK1/2 phosphorylation levels. B) Statistical analysis of Akt pathway activation in wild-type, knockout, empty vector and h-Freud-1 samples. One-way ANOVA followed by Tukey's post-hoc test was performed on this data set (p=0.2254; N=3). C) Statistical Analysis of Erk 1/2 pathway activation in wild-type, knockout, empty vector and h-Freud-1 samples. One-way ANOVA followed by Tukey's post-hoc test was performed on this data set (p=0.6313; N=3)

3.5 – CC2D1A NSID mutant binds strongly to membrane lipids

It was previously demonstrated that absence of phosphatidylinositol-3,4,5-trisphosphate (PIP3) was sufficient in abolishing CC2D1A's ability to form a heteromeric complex with PDK1 and Akt (Nakamura et al., 2008). Although this was impossible to verify in the CC2D1A$^{-/-}$ MEFs generated in this study due to undetectable Freud-1 protein levels, *in vitro* study of lipid binding using recombinant human wild-type and NSID CC2D1A constructs could gain insight into the gene's involvement with Akt signaling.

Recombinant constructs of wild-type and NSID Freud-1, subcloned in the pTriEx4 vector, were transformed in BL21 E.Coli bacteria and purified using HisPur Cobalt resin (ThermoScientific). The empty pTriEx4 vector was used as a negative control in order to screen for contamination by bacterial protein. The resulting purified proteins measured 130 kDa and 70 kDa for wild-type and NSID respectively (Figure 8B). This is consistent with previous analyses of the truncated NSID mutant which characterize a loss of the fourth DM14 domain and entire C-terminus (Figure 8A)

The lipid-binding strips used for this study contain a wide array of different phospholipids normally found in cellular and subcellular membranes. For quantitative purposes, only PIP3 and its precursor phosphatidylinositol-4,5-bisphosphate (PIP2) were analyzed due to their importance in Akt signaling (Auger et al., 1989). The results of this assay demonstrate that the binding affinity of NSID-CC2D1A to both PIP2 and PIP3 is strongly and significantly greater than the wild-type protein (Figure 8 D&E). The empty pTriEx4 vector was used as a negative control for this experiment to ensure no contamination by bacterial protein (Figure 8C).

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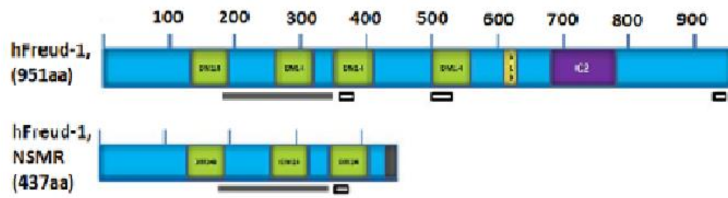

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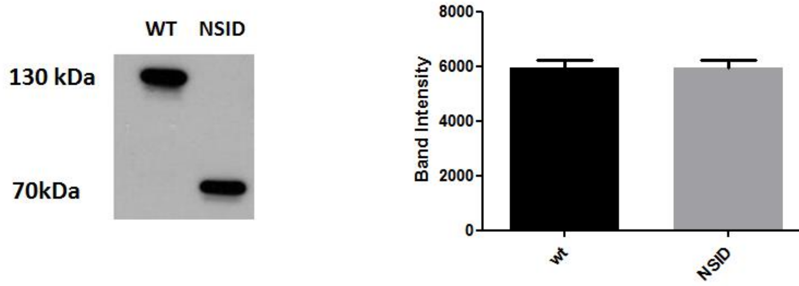

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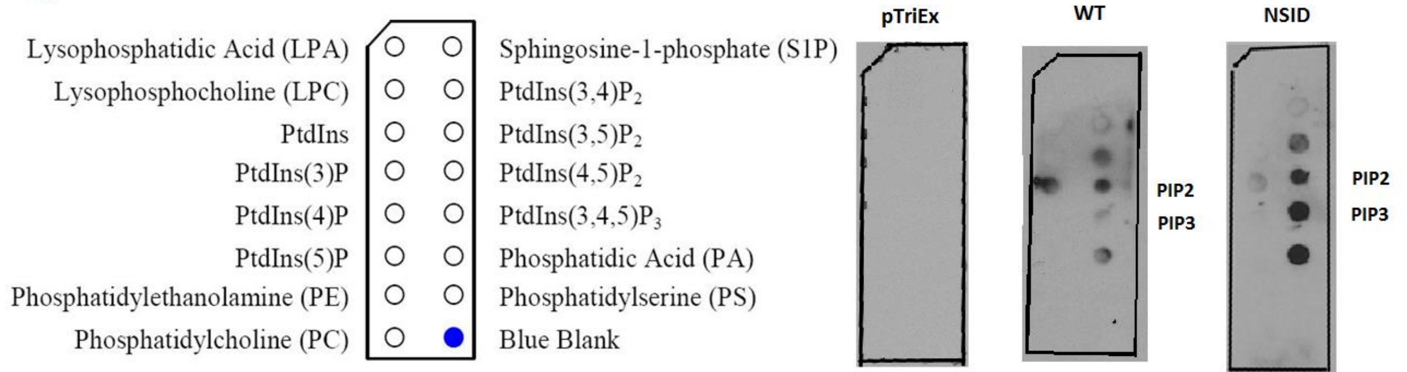

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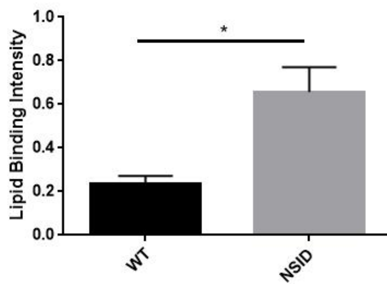

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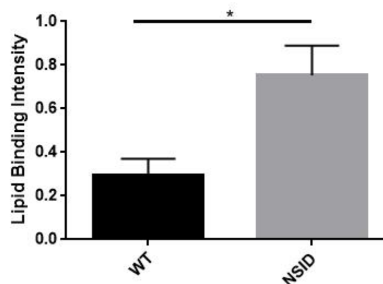

Figure 8. Phospholipid binding properties of WT-CC2D1A and NSID-CC2D1A. A) Structural diagram comparing wild-type and mutant Freud-1. The green areas represent the DM14 domains and the purple denotes the C2 region. B) Western blot of purified recombinant WT-CC2D1A (130 kDa) and NSID-CC2D1A (70 kDa). 10ng of protein were loaded into each well. Computer analysis and Student's t-test calculation of band intensity showed no difference in loading between WT- and NSID-CC2D1A ($p=0.9834$; $N=3$) C) PIP strips treated with 100ng of either wild-type or mutant protein. Lipids of interest are annotated. The empty pTriex vector was used as a negative control. A template displaying all lipids in the PIP strip has been included. D) Statistical analysis of PIP2 binding intensity. Unpaired Student's t-test was performed on this data (*$p=0.0240$; $N=3$). E) Statistical analysis of PIP3 binding intensity. Unpaired Student's t-test was performed on this data (*$p=0.0415$; $N=3$)

Chapter 4: Discussion

4.1 – TAT-Cre treatment produces an effective and stable Freud-1/CC2D1A knockdown

The recombinant Cre recombinase currently available on the market is engineered with a TAT peptide conjugated to its N-terminus. This is a cell-penetrating peptide derived from the HIV-1 virus and is used to ensure efficient endocytosis of the Cre enzyme by the cells of interest. The use of TAT-Cre was implemented in order to overcome the potential cytotoxicity associated with simply transfecting a Cre-expressing vector into cultured cells (Xu et al., 2008).

The use of TAT-Cre in this study proved to be highly effective in completely suppressing expression of Freud-1/CC2D1A. This simple experiment entailed 3 successive four hour treatments of the recombinase and was associated with minimal cell death. Furthermore, this method of knockdown, while highly limited due to the requirement of LoxP sites in the genome of the cells used, is much more effective and stable than current methods of RNA interference since it completely removes the gene from the cell rather than target the mRNA for degradation. The resulting knockout cell line was maintained for over six months with no trace during Western blot analysis of emerging wild-type protein after frequent passaging. Moreover, the CC2D1A$^{-/-}$ MEFs could be effectively transfected using the FastFect reagent (Feldan), thus providing a stable expression system in which recombinant mutant Freud-1 could be studied.

The complete absence of Freud-1 protein in the Cre-treated MEFs was both interesting and unexpected. The CC2D1A$^{flx/flx}$ transgenic line was generated with the specific purpose of creating a truncated mutant protein similar to a naturally occurring phenotype in humans which causes NSID. Individuals bearing this mutation express a Freud-1 fragment measuring approximately 70 kDa (Basel-Vanagaite et al., 2006). No such truncations were observed in the

MEFs via Western blot analysis. RT-PCR of mRNA samples extracted from these cells, however, did indicate that levels of the N-terminal portion of Freud-1 are significantly greater than the C-terminus, confirming that the knockdown occurred as expected. However, altogether, mRNA production was significantly suppressed compared to the non-Cre-treated MEFs. This suggests that, in the mice on which this study is based, CC2D1A transcription is reduced and the resulting protein is degraded through an undetermined mechanism. This mechanism would be similar to that proposed by Manzini and colleagues (2014) who observed several different mutant Freud-1 genotypes which all contributed to varying degrees of intellectual disorder, but produced no detectable CC2D1A protein. However, the question as to how the particular NSID mutant outlined by Basel-Vanagaite (2006), upon which our transgenic mouse model is based, affects normal function remains unanswered.

4.2 – EGFR signaling is maintained in CC2D1A$^{-/-}$ MEFs

The results of this study demonstrate that EGF-induced Akt and Erk signaling are maintained after knocking out Freud-1 expression in the CC2D1A$^{flx/flx}$ MEFs. These results are not consistent with the study by Nakamura and colleagues (2008) which identified CC2D1A as an essential component to EGF-induced Akt activation. A number of potential explanations could be derived from these conflicting data.

Firstly, Freud-1's scaffolding action in regards to Akt signaling may be cell-specific. The data showing involvement of CC2D1A in EGF-mediated Akt activation were performed in HT1080 cells which are derived from human fibroblastic sarcoma and were chosen due to their low basal Akt(Thr308) phosphorylation (Nakamura et al., 2008). In follow-up studies, however, Freud-1 was shown to contribute to EGF signaling in certain types of cells derived from pancreatic

tumours, but had little impact in lung cancer lines and other pancreatic cancer lines (Yamada et al., 2013; Ohtsubo et al., 2014).

The possible explanation for why Akt signaling is maintained after Freud-1 knockout in some cell types including the CC2D1A$^{-/-}$ MEFs described in this thesis is compensation by other scaffold proteins which interact with the EGF receptor. EGFR itself is a receptor tyrosine kinase (RTK) that stimulates both the mitogen-activated protein kinase (MAPK) cascade and the PI3K/Akt pathway (Hubbard and Till, 2000; Vivanco and Sawyers, 2002; McKay and Morrison, 2007). One of the downstream targets of MAPK is the phosphorylation of Erk1/2 which then goes on to stimulate the expression of genes involved in cell growth and differentiation. Therefore, blotting for both phosphorylated Erk1/2 and Akt was important to test for complete EGF receptor activation in this study.

EGFR activation recruits several scaffold proteins which mediate signaling cascades that ultimately lead to the activation of MAPK and Akt signaling. Specifically, the scaffold protein Shc1, via its phosphotyrosine-binding (PTB) domain, immediately binds to the activated receptor. This then leads to the recruitment of the adaptor protein Grb2 which subsequently serves to gather the components involved in the aforementioned cell signaling pathways (Zheng et al., 2013). Despite the current evidence indicating Freud-1's crucial role in EGF-mediated Akt phosphorylation, it is unclear at which point it makes a contribution to this already vast network of scaffold and adaptor proteins that are regulating the receptor's function aside from the fact that it physically interacts with PDK1 through its DM14 domains (Nakamura et al., 2008).

In addition to the mentioned scaffolds which could potentially mediate EGFR activity in the absence of Freud-1, it is also important to consider the possibility that Freud-2/CC2D1B plays a

compensatory role in $CC2D1A^{-/-}$ MEFs. Computer analysis of the two homologs show that they share 46% amino acid identity. Despite this significant difference, they are quite topologically similar – Freud-2 also contains four DM14 domains and a C2 region. Functionally, CC2D1B targets CHMP4B to participate in the ESCRT pathway in a similar fashion to Freud-1 (Martinelli et al., 2012; Usami et al., 2012). As well, it is capable of binding to the dual repression element in order to regulate 5-HT1A expression – particularly in postsynaptic neurons (Hadjighassem et al., 2009; Hadjighassem et al., 2011). However, it was also observed that Freud-2 does not share Freud-1's ability to activate the NF-KB pathway (Manzini et al., 2014). Given this information, it would be worthwhile to study Freud-2/CC2D1B in regards to EGF receptor signaling to see whether it is capable of compensating in the event of a loss of Freud-1 expression.

When interpreting the results of this thesis, one must also take note of the possibility that a trace amount of mutant Freud-1 is present and capable of maintaining normal EGF signaling. Western blot and immunocytochemistry showed that, after three successive treatments with TAT-Cre, CC2D1A protein, wild-type or mutant, was completely absent in the knockout cell (Figure 1 B&D). However, analysis of mRNA levels showed that trace amounts of Freud-1 was detected in the Cre-treated cells and that the N-terminal portion was significantly more abundant than the C-terminus, thus confirming the expected nature of the knock down which generates a truncated form of the protein. This conclusion could be further reinforced by the data obtained using recombinant NSID-CC2D1A protein, which revealed greater binding affinity to PIP2 and PIP3, which are crucial lipids involved in Akt signaling (Currie et al., 1999). This result in itself was unexpected as it was previously thought that, since the NSID-causing mutation led to impairments in learning and cognition, it must be non-functional to a certain degree (Basel-Vanagaite et al., 2006). Moreover, it was demonstrated that deletion of the C2 domain

completely abolished phospholipid binding activity in a recombinant GST-tagged Freud-1 construct (Al-Tawashi et al., 2012). Since the NSID mutant also lacks the C2 domain, it was believed that it would also not have the ability to bind to phospholipids. However, one must take into consideration that the authors responsible for this discovery only observed binding of phosphatidic acid (PA) and phosphatidylserine (PS), not PIP2 or PIP3, and only provided qualitative evidence to support their conclusions. Moreover, the GST-tag used by Al-Tawashi and colleagues (2012) is relatively large and could potentially change Freud-1's secondary and tertiary protein structures, which would ultimately interfere with normal function. By contrast, the Freud-1 construct used in this thesis is His- and S-tagged, both of which are much smaller than a GST-tag and unlikely to have any influence in protein structure and function. Thus, any discrepancy between the lipid-binding results of this thesis and those previously published may likely be attributed to differences in experimental design.

Ultimately, if there is a trace amount of truncated Freud-1 in the Cre-treated MEFs, and the mutant is capable of enhanced binding to Akt-associated phospholipids, this could be a potential reason for why the hypothesis for this study could not be confirmed. If this is indeed the case, then it would explain why humans bearing a homozygous mutant CC2D1A genotype which causes protein truncation are capable of leading healthy lives, albeit with marked cognitive dysfunction (Basel-Vanagaite et al., 2006; Manzini et al., 2012). This possibility would also support the findings regarding the function of the DM14 domains, which up to this point have been shown to be crucial mediators of Freud-1's interactions with components of various cell signaling cascades such as PDK1 (Nakamura et al., 2008). Ultimately, it would be beneficial to perform the lipid binding assay on recombinant Freud-1 deletion mutants to confirm that the

DM14 domains are indeed responsible for binding PIP2 and PIP3 and whether this correlates with previously described protein-protein interactions.

4.3 – Future Directions

The results of this study open doors to a wide array of possible avenues through which one can study Freud-1's physiological importance as a scaffold protein. Firstly, the TAT-Cre mediated gene knockdown method could be applied to primary neuronal cultures in order to study the molecular biological outcomes on brain tissue. Given the low toxicity of this method, along with its stability and ease of use, it should prove to be an effective means of analyzing the integrity of signaling pathways and their impact on synaptogenesis and neuroplasticity. It could also be possible to measure cell health by performing experiments such as proliferation and apoptosis assays which would be indicative of any neurodegeneration that can occur upon mutation of CC2D1A. Such an approach would be useful and informative given that every study published so far has highlighted cognitive deficits as a result of Freud-1 mutation in humans (Basel-Vanagaite et al., 2006; Shi et al., 2012; Manzini et al., 2014).

Moreover, the generation of CC2D1A$^{-/-}$ MEFs has proven to be an effective expression system into which recombinant mutant plasmids can be transfected in order to attempt rescue of the wild-type phenotype. The results of this thesis demonstrate that these knockout MEFs are capable of expressing a high degree of recombinant human Freud-1 with little effect on cell health using the Fastfect reagent (Feldan), despite the fact that EGF signaling remained unaffected. Nonetheless, it remains possible, for example, to test the performance of potential mutants in luciferase assays as well as in pathways such as NF-KB, PKA and CREB to determine the functional characteristics of each of Freud-1's domains.

Additionally, due to the multifunctional nature of Freud-1, it is important to generate deletion mutants with which to conduct *in vitro* cell signaling, protein interaction and DNA-binding assays. The lipid binding assay performed in this thesis revealed much about how truncated Freud-1 may function inside the cell, however, it provided little information in regards to which domain is responsible for the observations made. Therefore, by deleting each of CC2D1A's domains individually, one could reliably identify which one is responsible for allowing this protein to function as a scaffold during cell signaling events.

This approach would also work for CC2D1A's other functions. In particular, by doing a domain deletion study using luciferase and electrophoretic mobility shift assay, it would be possible to determine whether the NSID mutant is capable of regulating 5-HT1A expression and which domain is responsible for exerting the DNA-binding functionality. One could then package these recombinant Freud-1 constructs into a lentivirus and attempt *in vivo* rescue of wild-type function in the conditional knockout mouse line.

4.4 – Concluding Remarks

Based on previous findings that siRNA-mediated knockdown of Freud-1 suppressed EGF-mediated Akt signaling in human cells, we hypothesized that CC2D1A regulated EGF receptor signaling entirely. A unique approach to gene knockdown was made in this study, whereby recombinant Cre recombinase was used to abolish Freud-1 expression in mouse embryonic fibroblasts derived from a conditional, inducible mouse knockout line. PI3K/Akt and MAPK pathways were stimulated via EGF receptor activation, and phosphorylation of Akt(Thr308) and Erk1/2 were measured by western blot using samples gathered from wild-type and knockout cells in order to verify the integrity of both signaling cascades.

The results obtained from the EGF-treated CC2D1A$^{-/-}$ MEFs indicate no change in cell signaling. Immunofluorescent study of Freud-1 cellular localization confirmed, however, that it is indeed responding to EGF receptor activation by migrating into the extracellular space. Moreover, lipid-binding assays highlight a potential hyperactive role for mutant Freud-1 in regards to Akt signaling. Altogether, this data reinforces the robust nature of a cell's signaling pathways and their importance in survival and proliferation. The results should also inspire new interest in the roles Freud-1 homologs play in compensating for lost function. In addition, the methods used in this study could be applied to different cell types in order to discern the differential roles for Freud-1 in the body and how loss-of-function contributes to intellectual disorder.

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Appendix

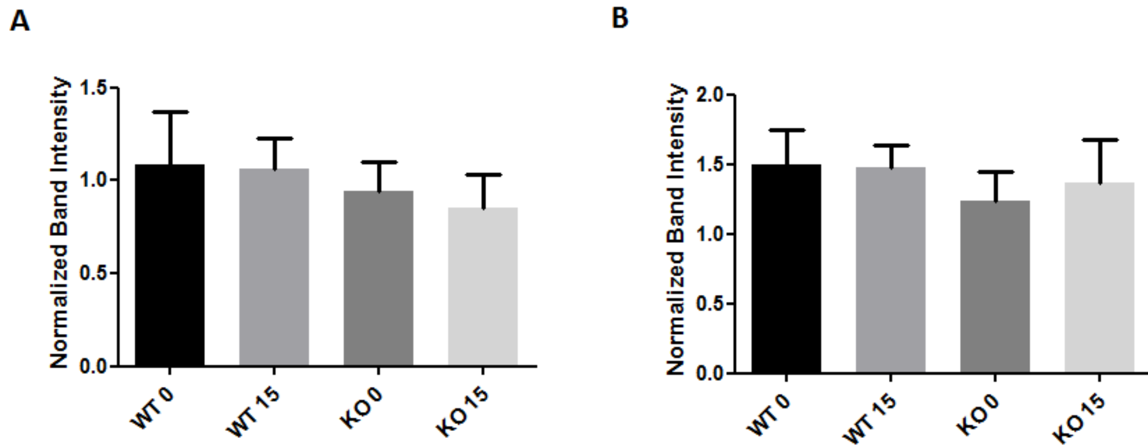

Figure 9. Quantification of total Akt and Erk1/2 expression. A) Statistical analysis of total Akt expression in wild-type and knockout MEFs before and after EGF treatment. One-way ANOVA was performed on this data (p=0.8388; N=10). B) Statistical analysis of total Erk1/2 expression in wild-type and knockout MEFs before and after EGF treatment. One-way ANOVA was performed on this data (p=0.8682; N=9)