THE TRANSCRIPTIONAL REPRESSOR CC2D1A/FREUD-1 INTERACTS WITH THE CHROMATIN REMODELING PROTEIN BRG1

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Abstract

The serotonin-1A (5-HT1A) receptor plays an important role in the regulation of the serotonin (5-HT) system as an autoreceptor on 5-HT neurons. The transcription factor CC2D1A/Freud-1 is a potent repressor of the 5-HT1A promoter in neuronal but not in non-neuronal cells. The clinical relevance of Freud-1 is evident in a naturally occurring mutation, resulting in a truncated form of Freud-1 lacking its C-terminal half, that is associated with non syndromic mental retardation in humans. Thus, it is of interest to clarify the structure and function of Freud-1. As Freud-1 was shown to interact with the transcriptional regulator Brg1 at the 5-HT1A promoter, identification of the structural domains mediating the Brg1/Freud-1 interaction is required to assess the role of Brg1 in Freud-1 repression. In this study, I used pull-down assays with recombinant proteins, co-immunoprecipitation studies and immunofluorescent staining with confocal microscopy to show that Freud-1 interacts directly with the C-terminus of Brg1 and that the C-terminal domain of Freud-1 is required for this interaction.

Résumé

Le récepteur de la sérotonine-1A (5-HT1A) est un important régulateur de l'ensemble du système de la sérotonine (5-HT) comme autorécepteur sur les neurones sérotoninergiques. Le facteur de transcription CC2D1A/Freud-1 réprime fortement le promoteur du 5-HT1A dans les cellules neuronales mais pas dans les cellules non-neuronales. Chez l'humain, une mutation spontanée qui mène à la synthèse d'une forme tronquée de Freud-1 (sans la moitié C-terminale) se traduit par un retard mental. Il est donc primordial de clarifier les déterminants structurels et la fonction de Freud-1. Il a été proposé que Freud-1 s'associe au régulateur de la transcription Brg1 sur le promoteur du 5-HT1A. Il est essentiel de connaître les domaines structurels grâce auxquels Freud-1 et Brg1 interagissent afin de comprendre le rôle de Brg1 dans la répression de Freud-1. Pour mener à bien ce projet, j'ai employé la précipitation de protéines recombinantes, les analyses par co-immunoprécipitation et l'immunofluorescence avec microscopie confocale. Je montre que Freud-1 se lie directement au C-terminus de Brg1 et pour ce faire utilise son domaine C-terminal.

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List of Abbreviations

5-HT 5-hydroxytryptamine (serotonin)

5-HT1A 5-hydroxytryptamine 1A receptor

5-HTT 5-hydroxytryptamine transporter

AC adenylyl cyclase

ACh acetylcholine

ADP adenosine diphosphate

ATP adenosine triphosphate

BAF Brg1/hBrm-associated factor

Brg1 Brahma-related gene-1

Brm Brahma

C2 protein kinase C conserved region 2

Ca^{2+} calcium

CaMK calcium calmodulin dependent protein kinase

cAMP cyclic adenosine monophosphate

CC2D1A coiled-coil and C2 domain containing 1A

CHIP chromatin immunoprecipitation assay

CHMP charged multivesicular body protein

DM14 *Drosophila* melanogaster 14

DNA deoxyribonucleic acid

DNMT DNA methyltransferase

DRD2 dopamine-D2 receptor

DRE dual repressor element

DRN dorsal raphe nucleus

E. coli *Escherischia coli*

EGFR epidermal growth factor receptor

ESCRT endosomal sorting complex required for transport

FRE five primed repressor element

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

Freud-2 five prime repressor under dual repression binding protein 2

GPCR G-protein coupled receptor

GR glucocortcoid receptor

HDAC histone deacetylase

HLH helix loop helix

HMT histone methyltransferase

HSA helicase/SANT-associated

IKK IκB kinase

ISWI imitation SWI

K+ potassium

Lgd lethal (2) giant disc protein

MBD methyl CpG-binding proteins

NFκB nuclear factor-kappa B

nNRE negative NFκB-response element

NR2B N-methyl D-aspartate receptor subtype 2B

NRE NFκB-response element

NSMR non-syndromic mental retardation

PBS phosphate buffered saline

PDK1 3'-phosphoinositide-dependent protein kinase 1

QLQ glucine-leucine-glucine

RE-1 repressor element 1

REST repressor element 1 silencer of transcription [also known as NRSF]

RNA ribonucleic acid

Sin3 switch independent histone deacetylase component

SSRI selective serotonin reuptake inhibitor

SWI/SNF mating-type switching and sucrose non-fermenting protein complex

Vm membrane potential

WT wild type

ZFP zinc finger protein

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1. Introduction

Rationale

5-HT1A receptors

The multiple serotonin (5-hydroxytryptamine; 5-HT) receptors in the serotonergic system mediate the diverse effects of serotonin. 5-HT, a biogenic monoamine, is an important neurotransmitter in the central and peripheral nervous system. It plays a role in a number of behavioral functions, including mood, sleep cycles, aggression, appetite and learning (Veenstra-VanderWeele et al., 2000; Mohammad-Zadeh et al., 2008) and is implicated in neuropsychiatric conditions, including depression, anxiety disorders, obsessive-compulsive disorders, psychosis, eating disorders, substance abuse and dependence (Lucki, 1998; Lesch, 2001).

5-HT1A receptors are among the most abundant and widely distributed 5-HT receptors in the brain. They exist pre-synaptically (as autoreceptors) on the soma and dendrites of 5-HT neurons that project from the raphe nuclei to the cortex, limbic system and hypothalamus. They also exist post-synaptically (as heteroreceptors) on target neurons of 5-HT projections. They are G-protein coupled receptors, coupled to Gi/Go and inhibit adenylyl cyclase, thus leading to decreased calcium entry, reduced neuronal firing and decreased neurotransmitter release (Figure 1; Albert and Lemonde, 2004; Lanfumey and Hamon, 2004). 5-HT1A autoreceptors exert a negative feedback on 5-HT release by controlling the firing rate of pre-synaptic 5-HT neurons. Therefore the activity of the 5-HT1A receptors allows fine control over the entire 5-HT system via pre- and post-synaptic regulation.

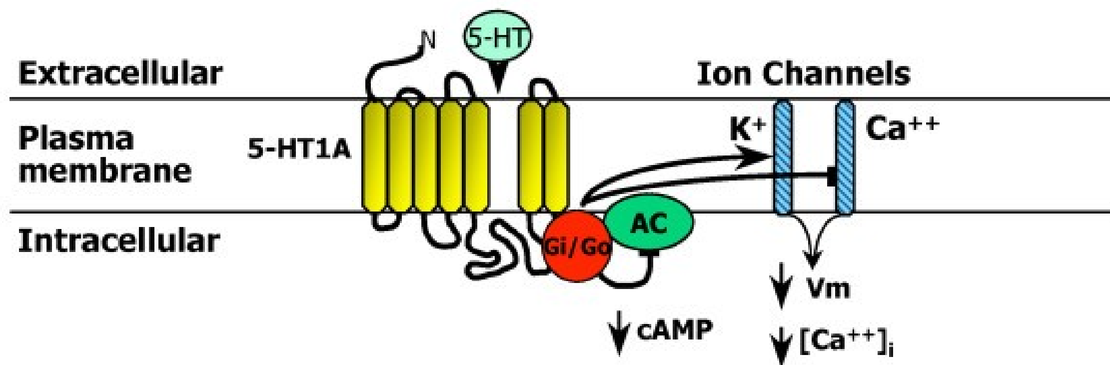


Figure 1. Neuronal signaling of the 5-HT1A receptor. The major signaling pathways of the 5-HT1A receptor in neurons are shown. The 5-HT1A receptor is shown figuratively as a heptahelical G-protein coupled receptor that couples via inhibitory G proteins (Gi/Go) to inhibit adenylyl cyclase (AC) and reduce cAMP levels; to open G-protein inward rectifying potassium channels (K^+) to reduce membrane potential (Vm); and to inhibit voltage-gated calcium channels (Ca^{2+}) and reduce intracellular free calcium concentration ([Ca^{2+}]i). Reused with permission from BioMed Central Ltd. (Molecular Brain Research) under the terms of the Creative Commons Attribution License. Copyright 2011 Albert et al.

5-HT1A over-expression is involved in decreased serotonergic neurotransmission and has been linked to major depression and suicide. Increased anxiety-like behaviors are observed in mice lacking the 5-HT1A gene (Kusserow et al., 2004) whereas rescue of 5-HT1A receptor expression in the early postnatal period reduces anxiety-like behaviors (Gross et al., 2002). Thus, proper 5-HT1A expression during development determines aspects of adult behavior and investigating the regulation of the expression of the 5-HT1A auto- and heteroreceptors is important both in development and in adulthood.

Anxiety disorders and major depression are often treated with selective serotonin reuptake inhibitors (SSRIs) that increase serotonergic transmission, implicating the serotonin system in these disorders. The delay of onset of SSRI action (4-6weeks) has been proposed to be due to the need for desensitization of the serotonin 1A autoreceptors on the soma and dendrites of serotonergic neurons in dorsal raphe nuclei (DRN) (Figure 2). Accordingly, patients with major depression have reduced 5HT-1A mRNA levels in the dorsolateral prefrontal cortex and the hippocampus (López-Figueroa et al., 2000) but increased autoreceptors levels in the DRN (Stockmeier et al., 1998). In addition, transgenic mice with a 30% repression of 5-HT1A autoreceptors show an increased and rapid response to SSRI treatment (Richardson-Jones et al., 2010), suggesting that the transcriptional repression of the 5-HT1A autoreceptors could be critical to an effective response to SSRIs.

These findings briefly illustrate the importance of clarifying the mechanisms that regulate the levels of expression of 5-HT1A autoreceptors.

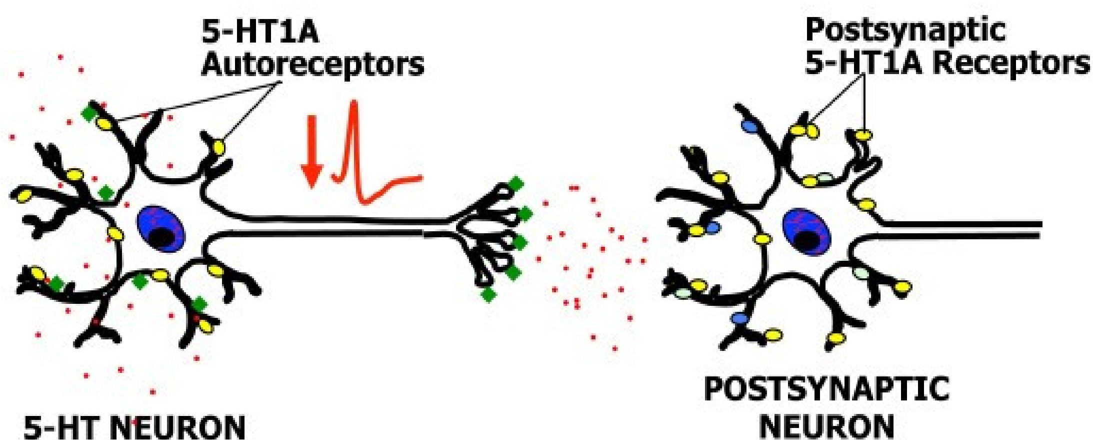


Figure 2. 5-HT1A autoreceptor-mediated negative feedback on 5-HT neurons. A model of a serotonergic neuron (5-HT neuron) projecting to a target neuron (postsynaptic neuron) is shown, with 5-HT1A receptors depicted as yellow ovals, and other 5-HT receptors as other colored ovals. Acutely, SSRI's rapidly enter the brain and block 5-HT transporters (green diamonds), located at the raphe serotonin nerve terminal, but also at the cell body and dendrites in the raphe nuclei. Inhibition of reuptake leads to accumulation of extracellular 5-HT (red dots) at both sites once released by depolarization. Activation of 5-HT1A autoreceptors located at the cell body and dendrites leads to inhibition of neuronal firing rate (red action potential), thus compensating for increase in 5-HT induced by SSRI treatment, resulting in little change in 5-HT neurotransmission initially. Reused with permission from BioMed Central Ltd. (Molecular Brain Research) under the terms of the Creative Commons Attribution License. Copyright 2011 Albert et al.

The 5-HT1A gene promoter has been dissected and studied to elucidate the role of its regulatory sequences and their binding partners. The distal promoter contains a series of activator elements and repressor elements used to repress the gene in rat, mouse and human (Figure 3; Ou et al., 2000). Major repression has been shown to require the presence of a 31 base pair (bp) conserved dual repressor element (DRE) (-1555/-1524) constituted of a 14 bp 5' repressor element (Five prime repressor element, FRE) and a 12 bp 3' repressor element (Three prime repressor element, TRE) (Figure 3; Ou et al., 2003; Parks and Shenk, 1996; Storring et al., 1999). Deletion or mutation of this region de-represses transcription of the 5-HT1A gene in all cell types tested (Rogaeva and Albert, 2007). Mutation of the FRE de-repressed 5-HT1A transcription by 10-fold in raphe RN46A cells but not in non-neuronal cells, supporting the role of the FRE as an important regulator of 5-HT1A neuronal expression (Ou et al., 2000). Transcriptional activity at the FRE is repressed by the Five prime REpressor Under Dual repression binding protein-1 (Freud-1) (Ou et al., 2003). Because Freud-1 is a potent repressor at the 5-HT1A promoter in neurons, changing its activity may be an effective way to modify the level of expression of 5-HT1A receptors. The ability to modulate 5-HT1A receptors expression could increase our knowledge of the regulation of the 5-HT system and in turn improve the treatment of several central nervous system disorders.

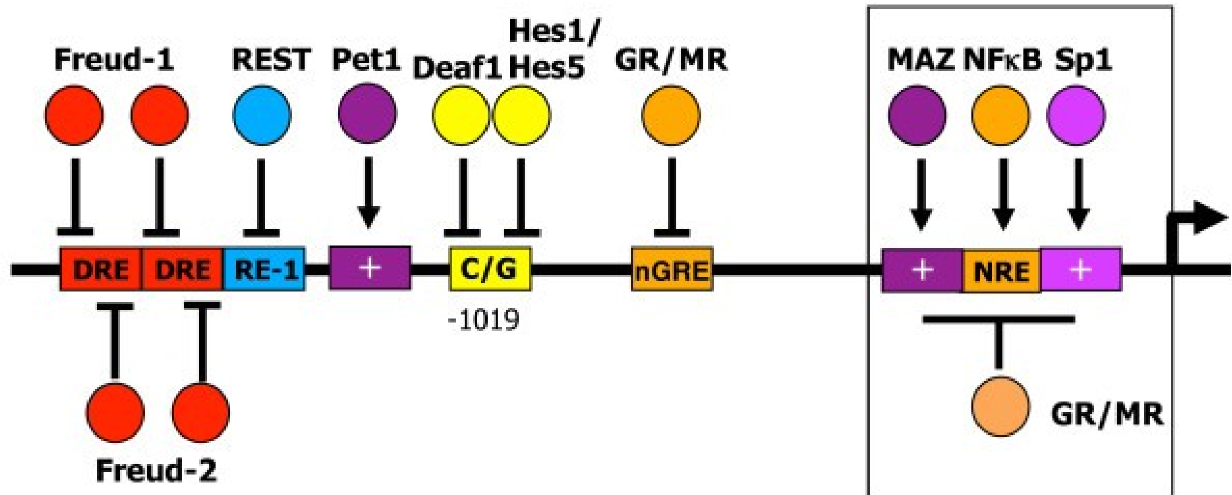


Figure 3. Identified promoter elements of the human 5-Ht1A receptor gene. The location of identified DNA elements on the 5-HT1A 5' regions flanking the start of translation (bold arrow) are shown figuratively. Identified activators (arrows) or repressors (bars) of transcription are also shown. Within the minimal promoter (box) there are GC-rich Sp1 and MAZ elements (+); NFkB response element (NRE); and glucocorticoid receptors (GR/MR) that inhibit transcription by blocking Sp1. Further upstream in a repressor/enhancer region, a negative glucocorticoid response element (nGRE) also mediates direct GR/MR-induced repression. Hes and Deaf1 proteins repress the 5-HT1A promoter at the C(-1019) allele, while in serotonin neurons Pet-1 exerts strong enhancer activity. A strong repressor region that silences expression in 5-HT1A negative non-neuronal cells, but also represses in 5-HT1A-positive neuronal cells is located upstream that includes elements for REST (RE-1), Freud-1 and Freud-2 (DRE). Reused with permission from BioMed Central Ltd. (Molecular Brain Research) under the terms of the Creative Commons Attribution License. Copyright 2011 Albert et al.

Where Freud-1 meets Brg1

Recent co-immunoprecipitation studies in our group suggested that Freud-1 associates with Brg1, a catalytic subunit of the chromatin remodeling SWI/SNF complex. Brg1 is implicated in transcriptional regulation and DNA replication, repair and recombination and is found in the nucleus as the central enzymatic subunit of multi-protein complexes, including SWI/SNF complexes (Trotter and Archer, 2004). Brg1 was found at the 5-HT1A gene promoter by ChIP experiments and knocking down both Brg1 and Freud-1 in HEK293 cells resulted in a 3.5 fold increase in 5-HT1A mRNA levels (Souslova and Albert, unpublished). These findings suggest that Brg1 plays a role in Freud-1-mediated repression of the 5-HT1A gene and provide the basis for the current studies regarding the interaction of Brg1 with Freud-1 and its potential as a tool to alter 5-HT1A receptors expression.

Before we can address the role of Brg1 and the SWI/SNF complex at the 5-HT1A gene promoter we need to answer the following questions:

- Is the interaction between Brg1 and Freud-1 direct or indirect?
- Which domains in Freud-1 and Brg1 mediate their interaction?

Once we understand more about how the two proteins interact, it will be possible to prevent their binding and study how this impacts the activity of the 5-HT1A promoter and in turn the level of expression of the 5-HT1A receptor. The following questions will then need to be addressed:

- Are the interacting domains of Freud-1 and Brg1 sufficient for the influence of Brg1 on the promoter activity and if not which other domains are required?

- Can we change Freud-1 activity in a predictable manner by preventing its interaction with Brg1?
- How will changes in Freud-1 activity alter 5-HT1A autoreceptors levels?

Literature Review

1. Freud-1

1.1. Freud protein family

Freud-1 is officially designated Coiled-coil and C2 Domain containing 1A (CC2D1A) and the gene encodes a 951 amino acid protein (predicted size ~104 kDa). The protein was identified by a yeast one-hybrid screening of mouse brain cDNA library using its target DNA sequence, the dual regulatory element in the 5-HT1A gene promoter (Ou et al., 2003). Freud-1 belongs to a protein family that includes Freud-1/CC2D1A and Freud-2/CC2D1B that have 40.8% amino acid identity in human (Rogaeva et al., 2007a). Freud-1 binds to both DREs but preferentially to the 5’ DRE (Lemonde et al., 2004) whereas Freud-2 binds to the 5’ or 3’ DRE and is weakly expressed in the raphe nucleus (Hadjighassem et al., 2009). Freud-1 and Freud-2 mediate together the dual repression of the 5-HT1A promoter in many cell types pre- and post-synaptically, restricting the expression of 5-HT1A receptors to appropriate neurons. Based on studies showing its role and localization in raphe cells (Ou et al., 2003; Lemonde et al., 2004; Albert and Lemonde, 2004; Rogaeva et al., 2007a), Freud-1 appears to be the dominant repressor of 5-HT1A autoreceptors expression (Albert et al., 2011).

1.2. Freud-1 structure

Human Freud-1/CC2D1A has conserved orthologues in the invertebrate *Caenorhabditis elegans* as well as in the rat and mouse (82.1% amino acid identity between rodent and human proteins) (Rogaeva et al., 2007a). Freud-1 sequence contains four *Drosophila melanogaster* (DM14) domains of unknown function, conserved in Freud-1 and Freud-2, a helix-loop-helix (HLH) domain that may be involved in DNA binding as well as cofactor recruitment (Rogaeva and Albert, 2007), a protein kinase C conserved region 2 (C2) domain present in proteins using calcium-dependent phospholipids binding and involved in protein-protein interactions (Basel-Vanagaite et al., 2006), a proline-rich domain implicated in protein binding (Williamson, 1994), and coiled-coil oligomerization motifs (Burkhard et al., 2001). It also contains putative sites for phosphorylation by protein kinase A and C and calcium/calmodulin-dependent protein kinase (CAMK) II/IV and Freud-1 repressor activity is inactivated by CAMK-mediated phosphorylation (Basel-Vanagaite et al., 2006; Ou et al., 2003) (Figure 4).

1.3. Known roles of other Freud-1 isoform

The Freud-1 isoform first identified in mouse is a short ~65 kDa isoform translated from a downstream ATG site (mFreud-1s DEL; Figure 4; Ou et al., 2003). It strongly represses the 5-HT1A gene despite lacking the two first DM14 domains and the proline-rich domain, suggesting that these regions are not required for its repressor activity at the 5-HT1A promoter (Rogaeva et al., 2007a). On the other hand, deletion of 8 residues in the C2 domain abolishes Freud-1 repressor activity and reduces its DNA-binding ability (mFreud-1s DEL, Figure 4; Ou et al., 2003).

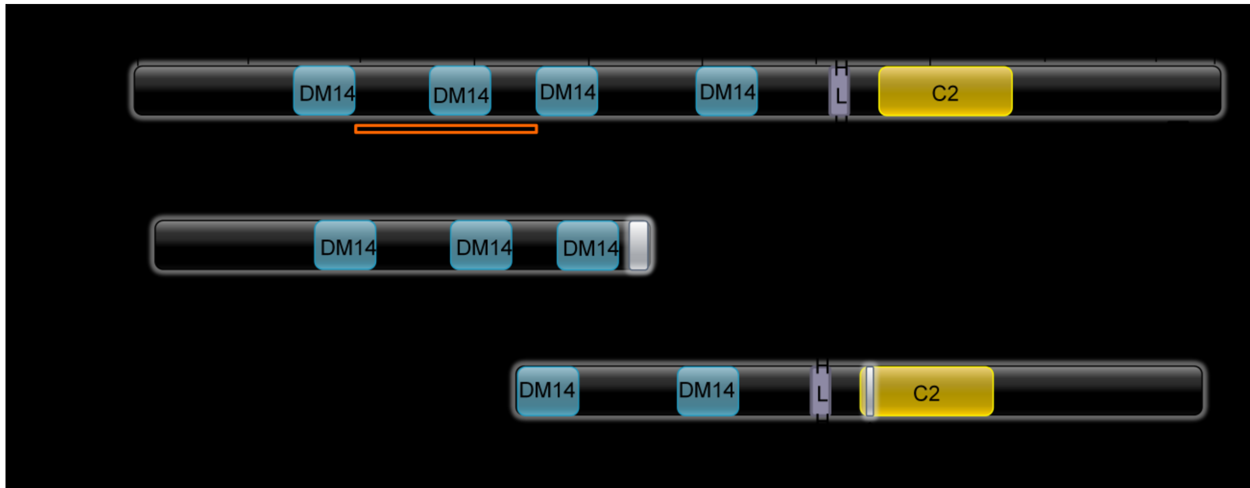


Figure 4. Schematic representation of Freud-1 isoforms. Long isoform of human Freud-1 with four DM14 domains, one helix-loop-helix (HLH), proline-rich region (diagonal line filled box), three coiled coil motifs (white boxes), and one C2 domain is depicted. hFreud-1 found in the patients with NSMR is also shown (hFreud-1_L NSMR), lacking fourth DM14, HLH, and C2 domains but containing an additional 30 nonsense amino acids (black box) preceding a termination codon. Finally, the short isoform of mouse Freud-1 is demonstrated without two N-terminal DM14 domains (mFreud-1_S). Furthermore, a location of the eight-amino-acid deletion that abolishes Freud-1 DNA binding abilities is shown. Amino acid scale is depicted at the top of hFreud-1_L counted from the most upstream methionine codon. Adapted from Rogaeva et al., 2007a with permission. Copyright 2007 John Wiley and Sons, Inc.

A truncated version of Freud-1 (437 a. a.) has been identified in a family, with affected individuals being diagnosed with Non Syndromic Mental Retardation (NSMR). NSMR is a form of mental retardation characterized by cognitive impairment in absence of physical and neurological deficits and is the most diagnosed form of mental retardation (Raymond, 2006; Ropers, 2006). Recently, Shi et al. (2012) linked two polymorphisms in CC2D1A/Freud-1 to mental retardation in a Han Chinese population with a family based association approach, supporting the implication of Freud-1 in cognitive function. In the NSMR mutant of Freud-1, exons 14-16 are deleted, generating a frameshift mutation in the mRNA leading to premature stop codon. The result is a truncated Freud-1 protein with an additional 30 residue long "nonsense" peptide at the C-terminal end (Figure 4; Basel-Vanagaite et al., 2006). This truncated mutant lacks the fourth DM14, the HLH and the C2 domains. The previously documented importance of the C2 domain in Freud-1 repressor activity (Ou et al., 2003) suggests that the NSMR mutant has lost its repressor activity (Rogaeva et al., 2007a). An important question is to define the role of the C-terminus of Freud-1 in its transcriptional regulation.

1.4. Tissue and subcellular localization of Freud-1

Freud-1 RNA is highly expressed in the central nervous system compared with many peripheral regions supporting its role in the regulation of neuronal genes (Ou et al., 2003). Freud-1 mRNA is expressed in pyramidal neurons of the cortex and the hippocampus and to a greater extent in the neurons of the raphe nuclei. In the latter, Freud-1 protein and RNA are strongly expressed in 5-HT neurons, supporting the role of Freud-1 in the regulation of 5-HT1A autoreceptors expression (Albert and Lemonde, 2004). Consistent with a regulatory role, increased calcium

levels cause a CAMK-dependent inhibition of Freud-1-activity that in turn induces 5-HT1A gene transcription in raphe RN46A cells (derived from E13 rat raphe nucleus) (Ou et al. 2003). Moreover, Freud-1 overexpression decreases 5-HT1A receptor levels in both raphe and non-neuronal myoblast cells but the 5-HT1A gene is de-repressed by antisense Freud-1 in raphe cells only (Albert and Lemonde, 2004).

The subcellular localization of Freud-1 supports its role as a transcription regulator in the raphe nucleus as well as its other cellular functions (see below). The short mouse isoform of Freud-1 –that has a strong repressor activity - displays a mainly nuclear and partially cytosolic localization in sections of dorsal raphe nucleus, hippocampus, cortex and substantia nigra of adult rat brain. It was strictly nuclear in raphe RN46A cells and primary cultures of embryonic cortical and hippocampal cells (Ou et al., 2003). Conversely, an antibody to the first 50 amino acids of the full length Freud-1 revealed mainly cytosolic localization in human U2OS osteosarcoma cells (Basel-Vanagaite et al., 2006). Moreover, Freud-1 staining is mostly nuclear in hippocampal neurons whereas it appears to be localized in both the cytoplasm and the nucleus in cortical neurons. These data suggest that the subcellular localization of Freud-1 is dependent on the cell-type (Ou et al., 2003). However, Freud-1 does not have a consensus nuclear localization or nuclear export sequence, therefore the mechanism of its nuclear-cytosolic shuttling remains unknown (Rogaeva et al., 2007a).

1.5. Other functions of Freud-1/CC2D1A

Several cellular functions of Freud-1 have been reported. It was shown to activate NFκB through the canonical IκB kinase (IKK) pathway which is involved in cell regulation and cell survival and this activation requires the C2 domain (Zhao et al., 2010, Matsuda et al., 2003). In

addition, Freud-1 promotes Akt activation via its role as an epidermal growth factor receptor (EGFR)-selective scaffold protein for the 3-phosphoinositide-dependent protein kinase 1 (PDK1)/Akt pathway that is critical to cell survival and metabolism (Nakamura et al., 2008). The *Drosophila* orthologue of Freud-1, identified as Lethal (2) giant disc (Lgd), was shown to regulate endosomal trafficking and activation of Notch, a protein that is involved in many developmental and pathological processes (Gallagher and Knoblich, 2006; Jaekel and Klein, 2006; Childress et al., 2006). In the innate immune system, Freud-1 is a regulator of the TLR3 and TLR4 pathways (Chang et al., 2011). Moreover, in the late stage of the endosomal sorting complex required for transport (ESCRT)-III pathway, budding of human immunodeficiency type 1 (HIV-1) virions relies on charged multivesicular body 4 (CHMP4) proteins (Usami et al., 2012). Meanwhile Freud-1 and Freud-2 bind to the core domain of CHMP4 to regulate CHMP4 polymerization and promote inhibition of HIV-1 budding. While in the cell cycle, Nakamura et al. (2009, 2010) showed that Freud-1 localizes to centrosomes where it interacts with cohesin and regulates centriole cohesion during mitosis. Finally, Freud-1 has been identified as a transcriptional repressor of not only the serotonin 1A receptor (5-HT1A) but also of the dopamine D2 receptor (DRD2) genes (Ou et al., 2003; Rogaeva et al., 2007b). Thus, Freud-1 is a multi-functional protein with roles in both receptor signaling and internalization as well as in transcriptional and cell cycle regulation. However, the question that remains is which functions of Freud-1 underlie the symptoms of non syndromic mental retardation.

2. Brg1

2.1. Brg1 is a chromatin remodeling protein

The packaging of DNA into nucleosomes creates a physical barrier to nuclear processes, such as transcription, that hinders the binding and association of DNA regulatory proteins at given promoter sites (Chen et al., 2006; Workman and Kingston, 1998; Wolffe, 2001). Mammalian chromatin occurs as “inducible” transcriptionally accessible euchromatin or as “silent” transcriptionally inaccessible heterochromatin (Horn and Peterson, 2006). The regulation of transcription thus often requires changes in chromatin structure that are frequently mediated by enzymatic multi-protein complexes (Felsenfeld and Groudine, 2003; Kinyamu and Archer, 2004). At least two forms of enzymatic activities have been described that alter chromatin structure by disrupting histone-DNA contacts. They rely on covalent modification of histone tails through acetylation, methylation, phosphorylation, ubiquitination, sumoylation and/or ADP ribosylation or ATP-dependent chromatin remodeling complexes (Kouzarides, 2007; Trotter and Archer, 2007). The latter require energy from ATP hydrolysis (Johnson et al., 2005). Numerous ATP-dependent chromatin remodeling complexes have been described and are usually separated in four groups: SWI/SNF, ISWI, NuRD and INO80 (Eberharter and Becker, 2004; Sif, 2004). The central catalytic unit of SWI/SNF complexes is either Brg1 or Brm (Brahma). They share 74% amino acid sequence identity and have similar biochemical activities *in vitro* (Khavari et al., 1993; Phelan et al., 1999; Randazzo et al, 1994); however they can assume different regulatory roles in various processes in the cell including proliferation and differentiation (Bultman et al., 2000; Reyes et al., 1998).

2.2. Brg1 was first identified in yeast

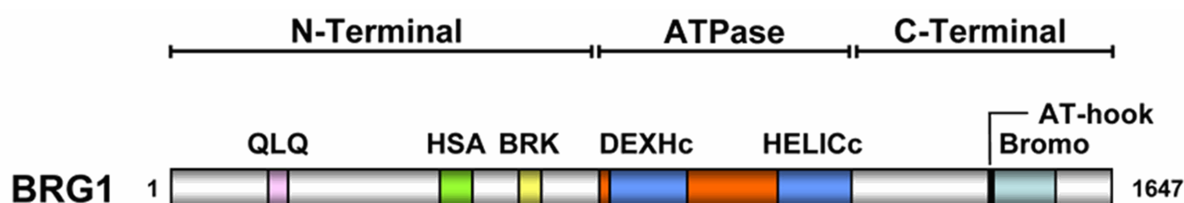
The Brg1 homologue, Swi2, was first identified in yeast through genetic screening for proteins involved in mating-type switching (SWI) and sucrose non-fermenting (SNF) (Neigeborn and Carlson, 1984; Stern et al., 1984; Winston and Carlson, 1992). In yeast, the catalytic subunit of SWI/SNF complexes is Swi2/Snf2 (Pazin and Kadonaga, 1997; Peterson and Tamkun, 1995). SWI2/SNF2 has known homologues in *Drosophila*, mice and humans (Dingwall et al., 1995; Khavari et al., 1993; Muchardt and Yaniv, 1993; Randazzo et al., 1994; Tamkun et al., 1992; Wang et al., 1996).

2.3. Brg1 structure

Brg1 contains a conserved catalytic ATPase domain and C-terminal bromodomain, an AT-hook motif and the QLQ, HSA and BRK N-terminal domains (Figure 5; Khavari et al.; Fan et al., 2005). The bromodomain is implicated in the recognition of acetylated lysine residues within H3 and H4 tails (Chandrasekaran and Thompson, 2007; Shen et al., 2007). Acetylated residues located within targets promoters may be used as a platform of interactions for the recruitment and/or assembly of bromodomain-containing coregulator complexes such as SWI/SNF. The AT-hook motif could be involved in DNA-binding or in recruiting the complex to acetylated lysine residues within histone tails (Singh et al., 2006). Although the function of Brg1 glutamine-leucine-glutamine (QLQ) domain is not clear, those motifs often mediate protein-protein interaction or aid forming the structural conformation of the protein. HSA domains are found in helicases and other eukaryotic DNA-binding proteins (Doerks et al., 2002) and BRK domains are associated with transcription (Allen et al., 2007; Doerks et al., 2002) but their function remains unknown.

2.4. SWI/SNF complexes

Mammalian SWI/SNF complexes contain the enzymatic central subunit Brm or Brg1 and 10-12 other proteins, including Brg1-associated factors (BAFs) that are for the most part orthologues of corresponding yeast proteins (Nie et al., 2000; Wang et al., 1996; Xue et al., 2000). Most human SWI/SNF purified complexes contain Brg1 (or Brm), BAF170, BAF155, BAF47, BAF60, BAF57, BAF53 and actin (Wang et al., 1996). Based on their subunit composition, they are further divided into polybromo-associated BAF (PBAF) and BAF complexes (Trotter and Archer, 2008). DNA-binding motifs within SWI/SNF complexes are thought to be important to recruit the SWI/SNF complexes to their DNA targets by cooperating with activation domains in transcriptional or histone binding factors to increase binding and chromatin remodeling efficiency (Peterson and Workman, 2000). The recruitment and stabilization of the SWI\SNF complex to gene-specific promoters is thought to be mediated by several direct and indirect interactions involving one or several BAF proteins. It might also involve zing finger protein (ZFP), a family of eukaryotic regulatory factors (Trotter and Archer, 2008).



Conserved Domain	Putative Function
QLQ	This conserved Gln, Leu, Gln motif is postulated to be involved in mediating protein interactions.
HSA	This domain is predicted to bind DNA and is often found associated with helicases.
BRK	The function of this domain is unknown. It is often found associated with helicases and transcription factors.
DEXHc	Domain associated with DEXH-box helicases. A diverse family of proteins involved in ATP-dependent DNA or RNA unwinding, needed in a variety of cellular processes. This domain contains the ATP-binding region.
HELICc	This domain is found in a wide variety of helicases and helicase related proteins. helicases share the ability to unwind nucleic acid duplexes with a distinct directional polarity; they utilize the free energy from nucleoside triphosphate hydrolysis to fuel their translocation along DNA, unwinding the duplex in the process.
AT-hook	a small DNA-binding protein motif. AT-hook motifs are frequently associated with known functional domains seen in chromatin proteins and in DNA-binding proteins . AT-hook motif is an auxiliary protein motif cooperating with other DNA-binding activities and facilitating changes in the structure of the DNA either as a polypeptide on its own or as part of a multidomain protein
Bromo	Bromodomains can interact specifically with acetylated lysine.

Figure 5. Domain architecture of Brg1. The BRG1 chromatin remodeling protein contains an evolutionarily conserved ATPase region, as well as domains found within the N- and C-terminus. Conserved domain identification and predictions were performed using the NCBI specialized BLAST conserved domains database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Reused with permission from The Nuclear Receptor Signaling Atlas (NURSA) under the terms of the Creative Commons Non-Commercial Attribution License. Copyright 2008 Trotter and Archer.

2.5. Overview of the functions of Brg1

Brg1-containing complexes can be recruited to their target promoters by associating with BAF subunits or transcription factors capable of binding DNA. Recruiting and stabilizing Brg1-containing SWI/SNF is likely to involve multiple interactions. One or more subunit may play a critical role in this process depending on the stage of transcription, nucleosomal composition and promoter architecture (Trotter and Archer, 2008). SWI/SNF subunits Brg1, BAF250, BAF60a and BAF57 have been shown to mediate critical interactions with nuclear receptors including glucocorticoid receptor, estrogen receptor, progesterone receptor, androgen receptor, retinoic acid receptor, vitamin D3 receptor and the peroxisome proliferator-activated receptor γ (Aoyagi et al., 2005; Chen et al., 2006; Debril et al., 2004; Dilworth et al., 2000; Ichinose et al., 1997; Lemon et al., 2001; Link et al., 2005; Trotter and Archer, 2004; Trotter and Archer, 2007). For example, BAF250 and/or BAF60a mediate the interaction between the glucocorticoid receptor (GR) and Brg1. SWI/SNF is recruited to GR-responsive promoters through both the DNA-binding and protein-protein interactions capabilities of BAF250 (Trotter and Archer, 2007). BAF57 mediates the interactions of SWI/SNF with several nuclear receptors including glucocorticoid receptor, estrogen receptor and androgen receptor (Garcia-Perdero et al., 2006; Hsiao et al., 2003; Link et al., 2005).

Brg1 function has often been associated with transcriptional activation, notably through nuclear receptors. However, Brg1 also plays a role in gene silencing through interactions with a variety of transcriptional co-repressors. (Gaston and Jayaraman, 2003; Underhill et al., 2000). Brg1 forms a repressor complex inhibiting cell cycle proteins such as cyclin A, D1 and E (Giacinti and Gordano, 2006; Zhang et al., 2000) through its interaction with retinoblastoma tumor

repressor (Zhang et al., 2000). Notably, Brg1 is part of the mSin3A/HDAC complex that participates in the transcriptional repression of a number of genes (Sif et al., 2001). Brg1 is also found in transcriptional repression complexes with histone deacetylases (HDACs), methyl CpG-binding proteins (MBDs) and histone methyltransferases (HMTs) where ATP-dependent chromatin remodeling is combined with a array of gene silencing mechanisms (Bilodeau et al., 2006; Dacwag et al., 2007; Datta et al., 2005; Pal et al., 2003; Xu et al., 2006). Recently, Brg1 has been shown to modulate activity-dependent regulation of c-fos and NR2B genes in the brain (Qiu and Ghosh, 2008), mediating both activation and repression of its target genes. Also, the repressor element-1-silencing transcription factor (REST) is a transcriptional regulator that binds the repressor element-1 (RE-1) sequence to repress the expression of neuronal genes (Battaglioli et al., 2002; Schoenerr and Anderson, 1995). To function, REST associates with HDAC1, HDAC2, Brg1, BAF170, BAF57, histone demethylase LSD1 and histone methyltransferase G9a, among others (Battaglioli et al., 2002; Gu et al., 2005; Roopra et al., 2004; Shi et al., 2004). Together, these studies show that Brg1 has the ability to associate with a large array of proteins. However, for many of these interactions, the mechanism at work *in vivo* has yet to be determined (Trotter and Archer, 2008).

2.6. Brg1 at the 5-HT1A promoter

Freud-1 and Brg1 are immunoprecipitated together both in 5-HT1A negative (HEK293) and positive (SK-N-SH) cells. Previous studies (Souslova and Albert, unpublished) showed that Freud-1 also immunoprecipitates with mSin3A, HDAC1, HDAC2, BAF155, BAF170, BAF47, BAF57 in HEK293 cells as well as with BAF57, BAF155 and BAF170 in SK-N-SH cells, suggesting that Freud-1 associates with a SWI/SNF or mSin3A/HDAC complex. Furthermore, it has been shown

by ChIP that Brg1 is present at the 5-HT1A promoter and simultaneous knock down of Brg1 and Freud-1 in HEK293 cells increased 5-HT1A mRNA levels (Souslova and Albert, unpublished). These findings suggest that Freud-1-mediated repression of HT1AR transcription may be affected by Brg1.

Hypothesis

I hypothesized that Brg1 would interact with Freud-1 directly or indirectly through specific domains on Brg1 and Freud-1. And therefore I also hypothesized that they are likely to co-localize in the nucleus of cells.

Objectives

Within the scope of this Masters project, I set out:

1. To determine if Brg1 binds directly or not with Freud-1
2. To find out which domains/regions in Freud-1 and Brg1 are required for their interaction in HEK293 cells
3. To show if Freud-1 and Brg1 co-localize in HEK 293 cells

Approaches

To assess if the binding was direct or not, I performed an in vitro pull-down assay with recombinant, bacterially expressed Freud1 and N-terminal and C-terminal regions of Brg1. I produced the two fragments of Brg1 by PCR of the N- and C-terminal regions on both sides of the central catalytic domain. I inserted the PCR products into the pGEM-T-easy vector and then subcloned the inserts into the pGEX-4T-3 vector that is suitable for bacterial expression with a GST tag. I designate those mutants as N-Brg1 (amino acids 1 to 656) and C-Brg1 (amino acids 1075 to 1647). I obtained a wild-type (WT) Freud1 construct in pTriex4 suitable for bacterial expression with His and S tags as well as extracts of purified recombinant ΔC2, ΔC-terminal and NSMR Freud1 from Anne Millar, a PhD student in our group.

I further probed for the domain of Freud1 that mediates its interaction with Brg1 by co-immunoprecipitating Brg1 in nuclear lysates of HEK293 cells transfected with Flag-tagged wild-type Freud1 and deletion mutants (Table 2). I then performed immunoblot analysis for Flag and endogenous Brg1.

To show the subcellular localization of Freud1 and Brg1, I used immunofluorescent staining of HEK293 cells transfected with Flag-Freud1 and V5-Brg1 or untransfected (to look at endogenous Freud1) followed by confocal microscopy.

2. Material and Methods

Plasmids

Brg1 fragments were generated by PCR with the following primers.

C-Brg1 forward primer: 5'-GGATCCTACCGAGCCTCGGGTAAA-3' and

C-Brg1 reverse primer: 5'-CTCGAGTCAGTCTTCTTCGCTGCC-3'.

N-Brg1 forward primer: 5'-CAGTACCCTTCACCAGATCTATGTCC-3' and

N-Brg1 reverse primer: 5'-AGATCTATCAGACCTCGGAGCTACTTCAT-3'.

PCR fragments were first ligated into the pGEM-T-easy vector (Promega) and then subcloned for bacterial expression into the pGEX-4T-3 vector by enzymatic restriction. Full length and deletion mutants of Freud-1 in N-terminal pFlag-CMV2 were given to us by Dr. N. Fujita (Nakamura et al., 2008).

I prepared other constructs that were only used for preliminary studies not presented in this thesis, however, will be used in future studies. Freud-1 C2 deletion and Freud-1 HLH deletion in pTriex4 vector were obtained from Anne Millar and subcloned into the pFlag-CMV2 vector by enzymatic restriction. Freud-1 C855 deletion in pFlag-CMV2 vector was generated by PCR of the full length pFlag-CMV2-Freud-1 with the following primers, forward: 5'-GGCGTTTGACCAATGAGAGCGTCTGGAGC-3'

and reverse: 5'-GCTCCAGACGCTCTCATTGGTCAAACGCC-3'.

All constructs were verified by DNA sequencing.

Antibodies

Antibodies were diluted in 5% skim milk for western blots and in antibody dilution buffer for immunofluorescence studies. For western blots of in vitro pull-down assay I used anti-GST (goat) at 1:20,000 (GE Healthcare) and anti-S-HRP conjugated (rabbit) at 1:5,000 (Novagen). For western blots of co-IP I used anti-Brg/SNF2 (rabbit) at 1:2,000 (Millipore) and anti-DYKDDDDK (rabbit) at 1:2,000 (Cell Signaling). Secondary antibodies used were anti-goat 1:20,000 (Jackson ImmunoResearch), anti-rabbit at 1:2,000 (Cell Signaling), and anti-mouse at 1:2,000 (Jackson ImmunoResearch). For immunofluorescent staining I used anti-DYKDDDDK tag at 1:400, anti-V5 (mouse) at 1:300 (Invitrogen) and anti-hFreud-1L (rabbit; 1:1,000), generated against bacterially expressed and purified (Ni-nitrilotriacetic beads, Qiagen) S/His-tagged human Freud-1L (Cedarlane). Secondary antibodies were Alexa Fluor 488 donkey anti-rabbit at 1:1,000 (Invitrogen) and Alexa Fluor 594 donkey anti-rabbit at 1:1,000 (Invitrogen).

Bacterial expression of recombinant proteins

Plasmids were transformed in *Escherichia coli* BL21 DE3 competent cells with 25µM β-mercaptoethanol. A starter culture was made by growing a colony into 10 mL 2X YT (2YT) media (1.6% m/v bacto tryptone, 1.0% m/v yeast extract, 0.5% m/v NaCl) with 100 µg/µl ampicilin overnight at 37°C with agitation. 500 mL 2xYT with 100 µg/µl ampicilin was inoculated with the starter culture and grown at 37°C with agitation until OD600 = 0.600 (2-3 hours). At this point protein expression was induced for 1 hour (GST fusion proteins) or 3 hours (S/His-Freud-1) with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG; Wisent). Cells were centrifuged at 4000 g for 20 minutes at 4°C. Pellets were washed with water, centrifuged at 4000 g for 10 minutes at 4°C,

frozen and stored -20°C until protein purification. The bacterial expression protocol was adapted from the QIAGEN QIAexpressionist^{TM} (fifth edition), Protocol 8. *E. Coli* culture growth for preparative purification in the fifth edition.

Affinity purification of recombinant GST fusion proteins

All subsequent steps were done on ice unless otherwise mentioned and buffers were supplemented with protease inhibitors: 1.5 µg/ul aprotinin, 1 µg/ul leupeptin, 0.14mM PMSF. Bacterial pellets were first thawed, then resuspended and lysed (2 hours) in 2 ml lysis buffer (1X Phosphate buffered saline (PBS; 50mM potassium phosphate, 150mM sodium chloride; pH 7.4), 1% triton, 1 mM DTT and 1mg/ml lysozyme). Cell lysates were then homogenized by sonication (6-10X 15 sec bursts at 200-300 W with 15 sec rest periods) and centrifuged at 10 000 g for 30 minutes at 4°C. Supernatants were filtered (0.45µm filters, Millipore) and incubated with 250 µl 50% slurry of 4G fast flow GST sepharose (GE Healthcare) for 1 hour with end-over-end rotation at room temperature. Beads were pelleted for 5 minutes at 500 g and washed 3X in 10mL PBS. Finally protein-bound beads were resuspended in 500 µl PBS and used immediately or stored at 4°C. The affinity purification protocol was adapted from the protocol for Batch purification of GST-tagged proteins using Glutathione Sepharose 4FF from GE Healthcare's Recombinant protein purification handbook.

Affinity purification of recombinant S/His- Freud-1 proteins

Bacterial pellets were first thawed then resuspended and lysed 30 minutes in 5 ml/gram HisPur Cobalt wash/equilibration buffer (50mM sodium phosphate, 300mM sodium chloride, 10mM imidazole; pH 7.4 and 1 mg/ml lysozyme). For lysis the buffer was supplemented with protease inhibitors: 1.5 µg/ul aprotinin, 1µg/ul leupeptin and 0.14mM PMSF. Cell lysates were then homogenized by sonication (6X 10 sec bursts at 200-300 W with 10 sec rest periods), centrifuged at 10 000 g for 30 minutes at 4°C. Supernatants were incubated with 1 ml 50% slurry of HisPur-Cobalt bead (ThermoScientific) for 1 hour at 4°c with mild shaking. The lysate-HisPur Cobalt beads mixture was loaded into a column previously equilibrated with wash/equilibration buffer. The column was washed 3 times with 10 ml buffer and Freud-1 was eluted 4X with 1 ml elution buffer (50mM sodium phosphate, 300mM sodium chloride, 150mM imidazole; pH 7.4). Protein extracts were dialyzed against 1X PBS at 4°C overnight. Protein content was then assessed with a Bradford assay; protein extracts were aliquoted and stored at -80°C. The affinity purification protocol was adapted from the QIAGEN QIAexpressionist™ (fifth edition) Protocol #12 for batch purification of 6xHis-tagged proteins from *E. Coli* under native conditions.

In vitro pull-down assay

Equal amounts across samples of beads bound to either GST, GST-N-Brg1 or GST-C-Brg1 were incubated with 1-1.5µg of wild-type or mutant Freud-1 purified protein for 30 minutes at room temperature with end-over-end mixing in a total volume of 1 ml pull down buffer (1X PBS,

0.1% Triton X-100, 1 mM DTT, 1.5 µg/ul aprotinin, 1µg/ul leupeptin and 0.14mM PMSF). Beads were washed 3 times in 1X PBS and centrifuged at 500 g for 5 minutes at 4°C each time. Bound proteins were eluted by boiling 5 minutes in 2X SDS-PAGE sample buffer. Immunoblots were performed as previously described (Ghahremani et al., 1999). Briefly, samples were run on SDS-polyacrylamide gels, transferred to a polyvinylidenedifluoride (PVDF) membrane (Perkin Elmer), blocked in 5% skim milk in TBS with 0.02% Tween-20 (Fisher) (TBST) and blotted with primary antibody at 4°C overnight. Membranes were washed 3 times 15 minutes in 1X TBST, incubated with secondary antibody for 1-2 hours at room temperature, washed again 3 times for 15 minutes in 1X TBST before application of immobilon western chemiluminescent HRP substrate (ECL) (Millipore). Immunoblot analysis probed for GST and S-tag and densitometry was performed using Image J analysis software (NIH).

Tissue Culture

Human embryonic kidney (HEK) 293 cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) with 8% fetal bovine serum (Wisent) at 37°C in 5% CO2. Cells were split by trypsinization at 80% confluence. No antibiotics were added to cultures. For conservation, cells were frozen in 10% DMSO and stored in liquid nitrogen.

Nuclear extract preparation & Co-immunoprecipitation (co-IP)

HEK293 cells were transfected at 50-60% confluence in 10 cm plates using the calcium phosphate co-precipitation method previously described (Ou et al., 2000) after changing the

medium. In order to obtain roughly equal levels of protein expression, constructs were transfected with the following amounts: 4µg of Freud-1, ΔC499 and ΔC520; 4-6µg of ΔC560 and ΔC657; 4-8µg of ΔC762; 2-4µg of ΔDM141 and ΔDM14-4; 3-4µg of ΔDM14-2; 2µg of ΔDM14-3; 1-4µg of Brg1. Cells were washed with 1X PBS 16 hours post-transfection and harvested 48 hours post-transfection. Cells were trypsinized, resuspended in medium and centrifuged 5 minutes at 750 rpm. Pellets were washed with 1X PBS and centrifuged for 5 minutes at 750 rpm. Subsequent steps were done on ice and buffers were supplemented with protease inhibitors: 1.5 µg/ul aprotinin, 1µg/ul leupeptin and 0.14mM PMSF. Washed pellets were resuspended and lysed for 10 minutes in 1 ml cytoplasmic extraction buffer (10mM KCl, 10mM Na-HEPES, pH 7.6, 1.5mM MgCl2, 0.1% Nonidet P40). Whole cell lysates were then centrifuged 2 minutes at 6000 rpm, nuclear pellets were washed in 0.5ml of wash buffer (50mM NaCl, 20mM Na-HEPES, pH 7.6, 25% glycerin, 0.2mM EDTA, 1.5 mM MgCl2) and centrifuged 2 minutes at 6000 rpm. Washed nuclei were lysed in 0.5ml lysis buffer (50mM Tris HCl, pH 7.4, 150mM NaCl, 1mM EDTA and 1% Triton X-100) and their protein content was assessed with a Bradford assay (Bradford MM, 1976).

Equal amounts of nuclear extracts across samples were added to 20 µl 50% slurry of anti-Flag M2 affinity gel (Sigma) in lysis buffer to a final volume of 0.8-1ml and incubated overnight at 4°C with end-over-end mixing. Beads were then washed 3 times in tris-buffered saline buffer (TBS) (50mM Tris, 150mM NaCl, pH 7.6) 3 times or until A280 = baseline and proteins bound to resin were eluted by boiling in 2X SDS-PAGE sample buffer. Samples were used for SDS-PAGE and immunoblot analysis probing for DYKDDDDK-tag and Brg1. Co-IP was performed according

to Sigma's M2 Flag affinity gel technical bulletin Densitometry was performed using Image J analysis software (NIH).

Immunofluorescence and Confocal Laser Microscopy

HEK293 cells were grown in DMEM with 8% FBS on sterilized glass slides in 6-well plates and transfected at 40-60% confluence with appropriate plasmids (4 µg WT Freud-1 or empty vector and 1 µg Brg1 or empty vector) according to the calcium-phosphate method previously described (Ou et al., 2000). 16 hours post-transfection cells were washed with 1X PBS and the media was changed. 48 hours post-transfection cells were fixed 30 minutes in 4% paraformaldehyde in PBS, washed 3 times with PBS, permeabilized and blocked 1-2 hours in blocking buffer (1X PBS, 5% normal donkey serum, 0.3% Triton-X-100). Primary antibody was applied for 1 hour at room temperature or overnight at 4°C in antibody dilution buffer (1X PBS, 1% bovine serum albumin, 0.3% Triton X-100). Cells were washed 3 times with 1X PBS and secondary antibodies were applied for 1-2 hours at room temperature in the dark in antibody dilution buffer. Cells were washed twice with PBS, 3 times with H2O, and incubated with DAPI staining (Invitrogen) in H2O for 10 minutes. Cells were then washed 3 times with H2O and mounted on coverslips with DAKKO mounting reagent (Invitrogen). The immunostaining protocol was adapted from Cell Signaling's general immunofluorescence protocol. Cells were observed and images were captured with a Carl Zeiss LSM 510 confocal laser microscope and Zen software.

3. Results

Freud-1 binds directly to Brg1

To assess if Brg1 and Freud-1 could interact directly, I did an in vitro pull down assay with bacterially expressed purified recombinant proteins. Because of the large size of Brg1 (~5 kbp, 185 kDa), I generated by PCR N-Brg1 (amino acids 1 to 656) and C-Brg1 (amino acids 1075 to 1647) fragments of the protein, leaving out the central catalytic domain. Indeed, subcloning large cDNA fragments is usually avoided since the size of a large 5 kbp insert is similar to the size of many plasmids (4-5 kbp). Expressing large proteins is also challenging as they tend to form insoluble aggregates or accumulate in the endoplasmic reticulum more easily than smaller proteins. I subcloned them into the pGEX-4T-3 vector that is suitable for bacterial expression. Brg1 fragments were thus expressed as proteins fused at their N-terminus to GST. I obtained a WT Freud1 construct with His and S tags from Anne Millar. Plasmids were bacterially expressed in *E. coli* BL21 DE3 and purified by affinity under native conditions. In the interest of time, I also used extracts of purified recombinant ΔC2, ΔC-terminal and NSMR Freud1 prepared by Anne Millar. Thus, the assay used GST-N-Brg1, GST-C-Brg1 and GST as a control as well as His and S tagged WT, ΔC2, ΔC-terminal and NSMR Freud-1.

Using N- and C-terminal fragments of Brg1 also allowed us to ask which part of Brg1 interacted with Freud-1. Purified Freud-1 bound to C-Brg1, suggesting that Freud-1 interacts directly with the C-terminal end of Brg1. Interestingly, the NSMR isoform of Freud-1 (predicted size 44 kDa, runs at ~70 kDa) did not bind to C-Brg1, suggesting that the missing C-terminal half is required for the interaction (Figure 6). Freud-1 lacking the C2 domain (ΔC2, predicted size 94 kDa, runs

at ~150 kDa) appeared to bind C-Brg1 more weakly than wild-type Freud-1 (predicted size 104 kDa, runs as a doublet at ~135 & 100 kDa), while binding of the C-terminus deleted Freud-1 (ΔC757, predicted size 81 kDa runs at ~100 kDa) was greatly reduced. These data indicate that the C-terminus of Freud-1 is the primary determinant of binding to the C-terminus of Brg1.

While Figure 6 shows that His-S-ΔC2 lacking the C2 domain runs higher on a WB than the tagged full length wild-type Freud-1, all constructs used in the experiment were sequenced and protein preparations were checked using Coomassie Blue staining. Although the band of ΔC2 that was pulled down with C-Brg1 was larger than predicted, it matched the highest MW input band size indicating that ΔC2 bound to C-Brg1. Future experiments should identify the origin of this size difference in the ΔC2 protein.

GST-N-Brg1 did not interact with WT Freud-1 or with the ΔC2, ΔC757 or NSMR. However, these results must be interpreted with some caution since Figure 6 shows that GST-N-Brg1 - whose predicted size is ~100 kDa- was partially cleaved into GST (26 kDa), a 30 kDa fragment and fragments of higher molecular weight. GST-C-Brg1 whose expected size is ~90 kDa - appears cleaved to a much lesser extent.

A.

Inputs

Wild-type ΔC757 ΔC2 NSMR

WB: S tag

230 150 100 80 60

WT

NSMR

Wild-type ΔC757 ΔC2 NSMR

C-Brg1 N-Brg1 GST C-Brg1 N-Brg1 GST C-Brg1 N-Brg1 GST C-Brg1 N-Brg1 GST

150 100 80 60

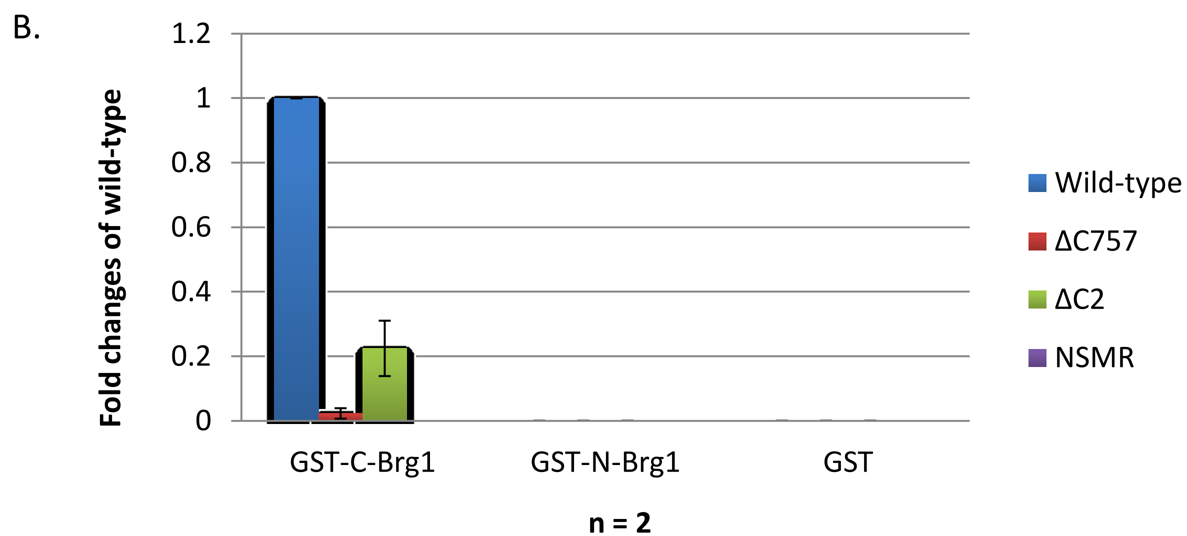
Pull down: GST

WB: S tag

100 80 60 50 40 30 25

Pull down: GST

WB: GST



C.

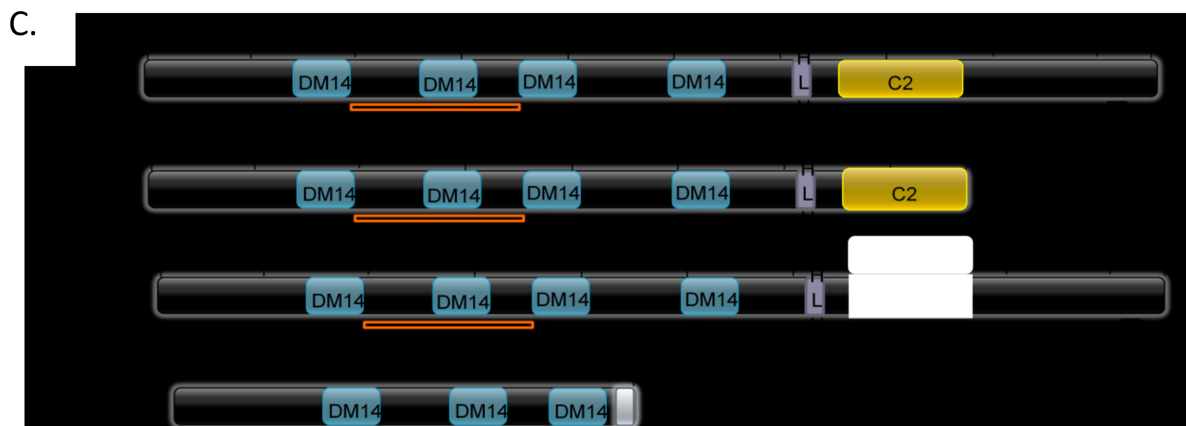


Figure 6. Wild-type and C-terminal mutants of Freud-1 bind to GST-C-Brg1.

(A) shows pull down of wild-type and mutants Freud-1 (right) and inputs of wild-type and mutants Freud-1. Lower panel shows loading controls for GST fusion proteins. Emphasized are full-length C-Brg1 (90 kDa; black arrowhead), higher band for N-Brg1 (white arrowhead, ~50 kDa), main fragment of N-Brg1 (green arrowhead, ~30 kDa) and GST (blue arrowhead, 26 kDa). As a negative control, wild-type and mutant forms of Freud-1 did not bind to recombinant GST attached to beads. S-tagged recombinant purified wild-type, ΔC757, ΔC2 and NSMR Freud-1 were incubated 30 minutes at room temperature with beads-bound GST-C-Brg1, GST-N-Brg1 or GST as indicated. After washes and protein elution, immunoblot analysis was performed for GST and S-tag. Representative blot of 2 independent experiments. (B) Densitometry analysis of in vitro pull down assays assessed binding between wild-type and mutants of Freud-1 and GST-C-Brg1 and GST-N-Brg1 fragments. Mean fold changes to wild-type Freud-1 are shown with standard error. n=2. (C) Structural alterations in the Freud-1 constructs assayed are depicted.

Which Freud-1 regions are required to interact with Brg1?

To determine which Freud1 domain(s) is required for its interaction with Brg1 *in vivo*, I performed co-immunoprecipitation studies with protein extracts from HEK293 cells expressing transfected Flag-tagged WT or mutants of human Freud-1 as well as His/V5-tagged human Brg1 or endogenous human Brg1. Samples were run on a SDS-PAGE and immunoblotted for analysis.

I first optimized the co-immunoprecipitation procedure with WT constructs of Flag-Freud1 and His/V5-Brg1 using nuclear extracts in order to target nuclear proteins.

I co-transfected WT Freud-1 with Brg1 as well as appropriate controls (Freud-1's empty vector (vector F: pFlag-CMV2) with Brg1's empty vector (vector B: pCDNA3.1D); Brg1 with vector F; Freud-1 with vector B). Figure 7 depicts a representative blot of those experiments. The top panel labeled "IP: Flag" shows that Flag-Freud-1 was detected only when transfected (Input lane) and was effectively pulled down by the Flag resin (IP lane). V5-His-Brg1 was only detected when transfected (Inp lane) and was pulled down with Flag-Freud-1 on the Flag resin (IP lane). Therefore, V5-His-Brg1 was specifically co-immunoprecipitated on the Flag resin. The lower panel labeled "IP: His" shows that V5-His-Brg1 was detected only when transfected and was effectively pulled down by the His-binding resin. Flag-Freud-1 was only detected when transfected and was pulled down with V5-His-Brg1 on the His resin. Therefore, Flag-Freud-1 was specifically co-precipitated on the HisPur resin.

This phase validated the co-immunoprecipitation of wild type constructs with nuclear extracts on both affinity resins.

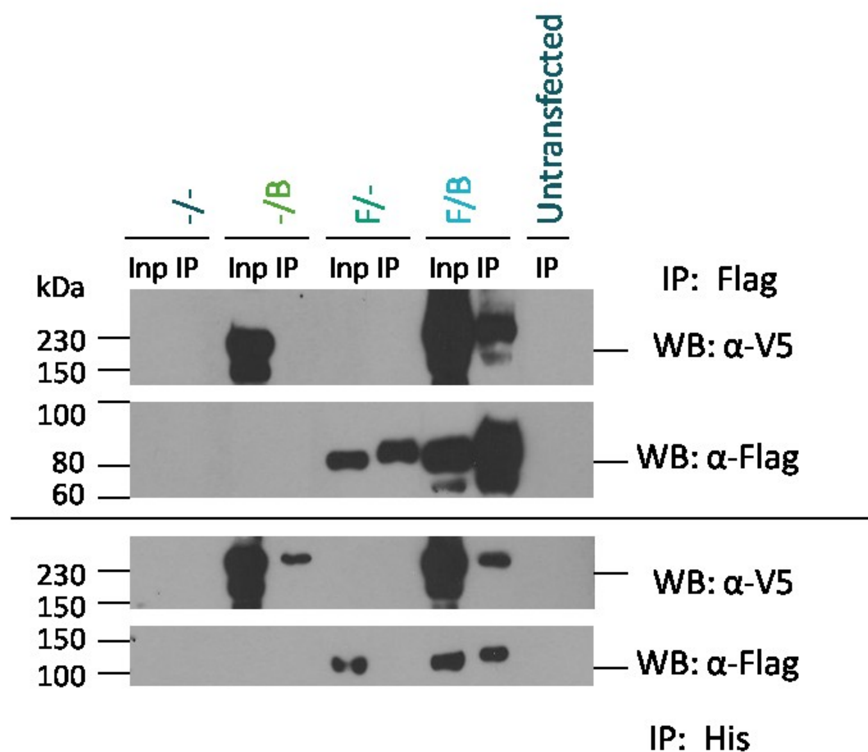


Figure 7. **Initial validation of the co-IP procedure with nuclear extracts.**

HEK293 cells were transfected as indicated and nuclear proteins were extracted. Immunoprecipitations followed by SDS-PAGE analysis and Western Blot were performed as labeled. IPs (IP) are shown beside the corresponding input (Inp) for each condition. -/-: pFlag-cMV2 with pcDNA3.1D-V5-His; -/B: pFlag-cMV2 with pcDNA3.1D-V5-His-hBrg1; F/-: pFlag-cMV2-hFreud-1 with pcDNA3.1D-V5-His; F/B: pFlag-cMV2-hFreud-1 with pcDNA3.1D-V5-His-hBrg1. Representative blots of 3 independent experiments.

Co-IP with Freud-1 deletion mutants

Having optimized the co-IP with wild type Freud-1, I repeated the procedure to include Freud-1 deletion mutants. Flag-tagged WT Freud1 and the nine mutants portrayed in Fig. 8 were co-transfected with His/V5-tagged Brg1 or its empty vector. Freud1 empty vector was also co-transfected with Brg1. Pull downs were performed with the His-binding resin. As shown in figure 9 with a representative blot, ΔC499 ΔDM14-1, ΔDM14-4 and WT Freud-1 bound as well without V5-His-Brg1 (lower panel) than with it (upper panel) indicating that those Flag-tagged proteins attach to the His-binding beads. Since it occurs with different Flag constructs it is likely to be due to the Flag tag binding to the HisPur cobalt resin, making the co-immunoprecipitation of Flag constructs independent of their interaction with Brg1. Other Flag-constructs also attach to beads in absence of Brg1 but to a lesser extent than when Brg1 is present. Increasing the amount of DNA transfected as indicated by the gradient sign leads to a stronger binding signal both specifically and non-specifically. ΔC762 was not pulled down. However, later experiments showed that ΔC762 is expressed when transfected in a greater amount. This experiment was performed with the Freud1 deletion mutants and repeated more than fifteen times however the results obtained lacked consistency. Globally, experiments gathered at this stage showed more reliable binding of WT Freud-1, ΔDM14-3 and ΔDM14-4 whereas the other constructs showed high levels of non-specific binding.

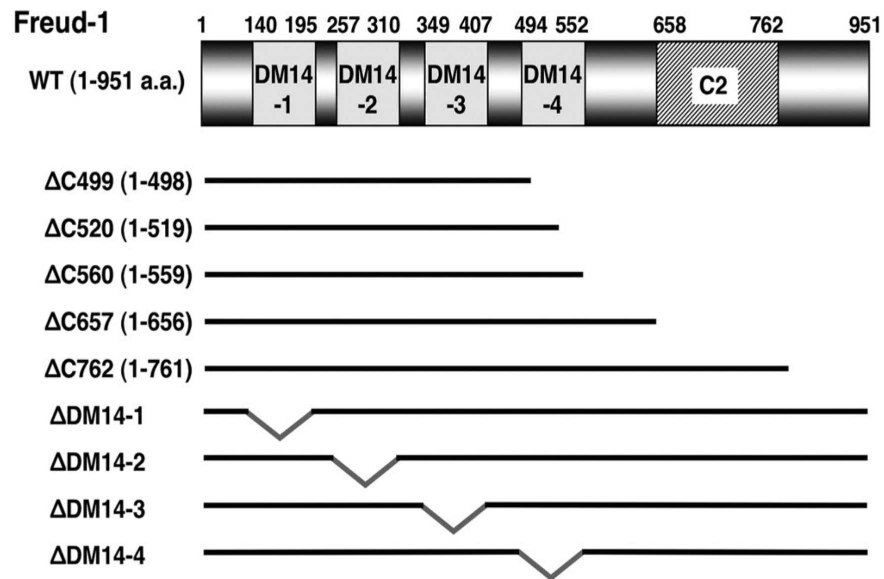


Figure 8. **Representation of Freud-1 deletion mutants and wild type used in the co-IP studies.** Freud-1 mutants. Adapted from Nakamura et al., 2008 (figure 2). Reproduced with permission. Copyright 2008 American Society for Microbiology.

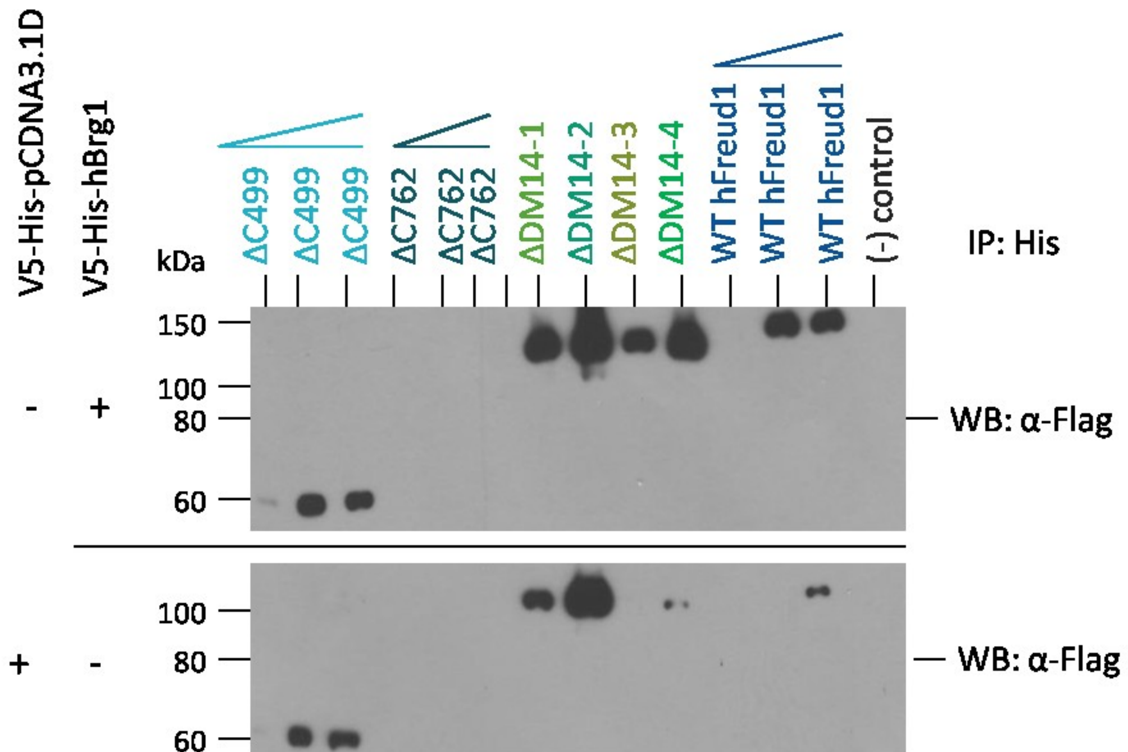


Figure 9. **Lack of specificity of the co-IP of certain Freud-1 mutants on a HisPur Cobalt resin.**

HEK293 cells were transfected as indicated and nuclear proteins were extracted. Immunoprecipitations followed by SDS-PAGE analysis and Western Blot were performed as labeled. Freud-1 constructs are indicated as transfected above each lane and co-transfected Brg1 constructs are indicated on the left. The gradient symbol indicates increasing amount of transfected DNA. Representative blots of 15 independent experiments.

Troubleshooting the co-IP to eliminate non-specific binding

At this stage, WT Freud1 and Freud1 deletion mutants of interest were co-transfected with Brg1 or its empty vector. Freud1 empty vector was also co-transfected with Brg1 and pull down was performed on the His-binding resin previously mentioned. I tested different experimental conditions in order to eliminate the binding of the Flag tag to the HisPur Cobalt resin; mainly I varied the incubation duration of the pull down and the composition of the buffer. Coating the beads with BSA 1% vs. 5% prior to IP didn't improve the specificity of the binding. The recommended composition of the HisPur Cobalt Equilibration/Wash buffer is 50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole; pH 7.4. To wash out more effectively non-specific interactions, I tested concentration gradients of Tween-20 (0.1-0.5%), Triton X-100 (0.0005-1%) and Imidazole (10-50 mM) as well as pH change (pH 6.0 vs. 7.4). Figures 10 and 11 provide detailed examples. Changing the pH and varying the Imidazole concentration of the buffer did not affect non-specific binding. Adding detergent to the buffer, either Tween-20 or Triton X-100, eliminated any detectable interaction until a concentration as low as 0.001% Triton X-100 was used (Fig. 10).

In order to find a Triton X-100 concentration that would eliminate only the non-specific binding for the different constructs of interest, I did the pull down with the three concentrations that were determined by the experiment showcased in figure 10. The difference between 0% and 0.001% Triton X-100 that was observed for WT Freud-1 in figure 9 was not repeated in figure 11 demonstrating variability between two identical experiments. Then, although the use of detergent decreased binding every time, each construct had a different

threshold concentration of detergent at which only the specific binding remained detectable. Thus, the constructs of interest could not all be tested adequately in the same conditions. This persistent variability undermined the validity of the experiment and mostly prevented conclusions to be drawn from it. It however reinforced the need to find a better experimental approach.

I explain further and discuss the steps taken to troubleshoot the co-IP and find a better approach in Appendices (p 82).

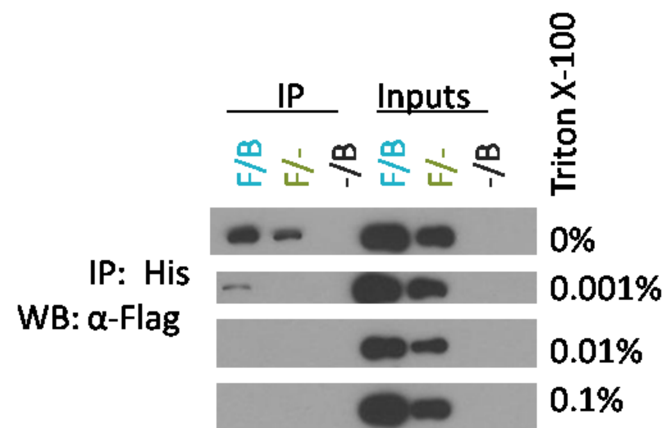


Figure 10. Titration of the concentration of Triton X-100 to eliminate non-specific binding of Freud-1.

HEK293 cells were transfected as indicated and nuclear proteins were extracted. Immunoprecipitations followed by SDS-PAGE analysis and Western Blot were performed as labeled. IP and input are shown for each condition. F/B: pFlag-cMV2-hFreud-1 with pcDNA3.1D-V5-His-hBrg1; F/-: pFlag-cMV2-hFreud-1 with pcDNA3.1D-V5-His; -/B: pFlag-cMV2 with pcDNA3.1D-V5-His-hBrg1.

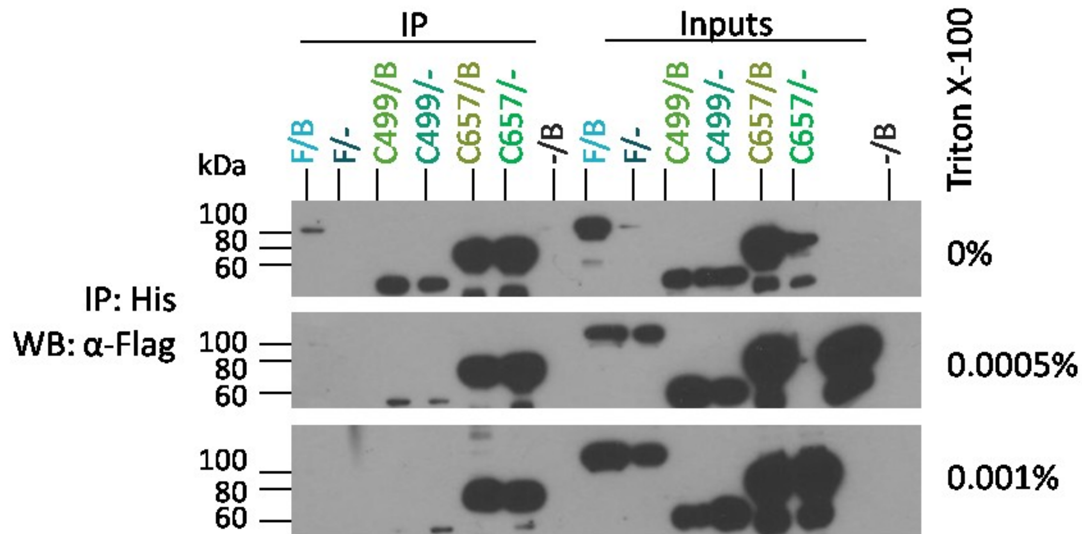


Figure 11. **Titration of the concentration of Triton X-100 to eliminate non-specific binding of Freud-1 and mutants of interest**.

HEK293 cells were transfected as indicated and nuclear proteins were extracted. Immunoprecipitations followed by SDS-PAGE analysis and Western Blot were performed as labeled. IP and input are shown for each condition. F/B: pFlag-cMV2-hFreud-1 with pcDNA3.1D-V5-His-hBrg1; F/-: pFlag-cMV2-hFreud-1 with pcDNA3.1D-V5-His; C499/B: pFlag-cMV2-ΔC499-hFreud-1 with pcDNA3.1D-V5-His-hBrg1; C499/-: pFlag-cMV2-ΔC499-hFreud-1 with pcDNA3.1D-V5-His; C657/B: pFlag-cMV2-ΔC657-hFreud-1 with pcDNA3.1D-V5-His-hBrg1; C657/-: pFlag-cMV2-ΔC657-hFreud-1 with pcDNA3.1D-V5-His; -/B: pFlag-cMV2 with pcDNA3.1D-V5-His-hBrg1.

One tag IP showed specific binding

I performed the co-immunoprecipitation with one tag instead of two in order to eliminate the non-specific binding of Flag to the His column. WT and mutant Flag-tagged hFreud-1 constructs were transfected with appropriate controls and immunoprecipitated on a Flag-affinity resin. Endogenous Brg1 was then detected by WB analysis (IB: anti-Flag and anti-Brg1). Immunoblotting for anti-Flag ensured that Flag-tagged proteins were pulled down while immunoblotting for anti-Brg1 showed the affinity of each Freud-1 mutant or wild-type protein for Brg1. Initially, I also co-transfected Brg1 with WT Freud-1 to compare the binding of transfected Brg1 with that of endogenous Brg1 to WT Flag-Freud-1. Binding was observed in both cases but was stronger when Brg1 was transfected. Since this experiment is based on the use of one tag, WT Freud-1 was transfected without Brg1 in subsequent experiments. Nuclear extract from untransfected cells was used to control for the immunoprecipitation of endogenous Brg1 with the Flag-affinity resin in absence of Flag-tagged proteins. Brg1 was transfected to control for the immunoprecipitation of overexpressed Brg1 with the Flag-affinity resin in absence of Flag-tagged proteins. Thus, using this approach endogenous Brg1 was effectively co-precipitated with Flag-Freud-1 as shown on Figure 12.

The C-terminal domain of Freud-1 is required to interact with Brg1

As depicted on Figure 12, mutants lacking one of the DM14 domains (Fig. 8) retained their ability to bind to Brg1 suggesting that none of the DM14 domains alone is required for the interaction with Brg1. This is not surprising since their homology suggests that they could

maybe compensate for one another if one DM14 was required for the interaction. The ΔC499, ΔC520, ΔC560 and ΔC657 mutants all lack the C2 domain and the C-terminal end of the protein (Fig. 8) and mostly lost their ability to bind to Brg1 suggesting that binding sites to Brg1 are located in the C-terminal domain of Freud-1. The ΔC762 truncation containing the C2 domain but lacking the remaining C-terminal end of the protein lost its binding ability as well. These findings suggest that the binding sites for Brg1 are located in the C-terminal domain of Freud-1 downstream of amino acid 762 and that the interaction does not involve the DM14 or C2 domains.

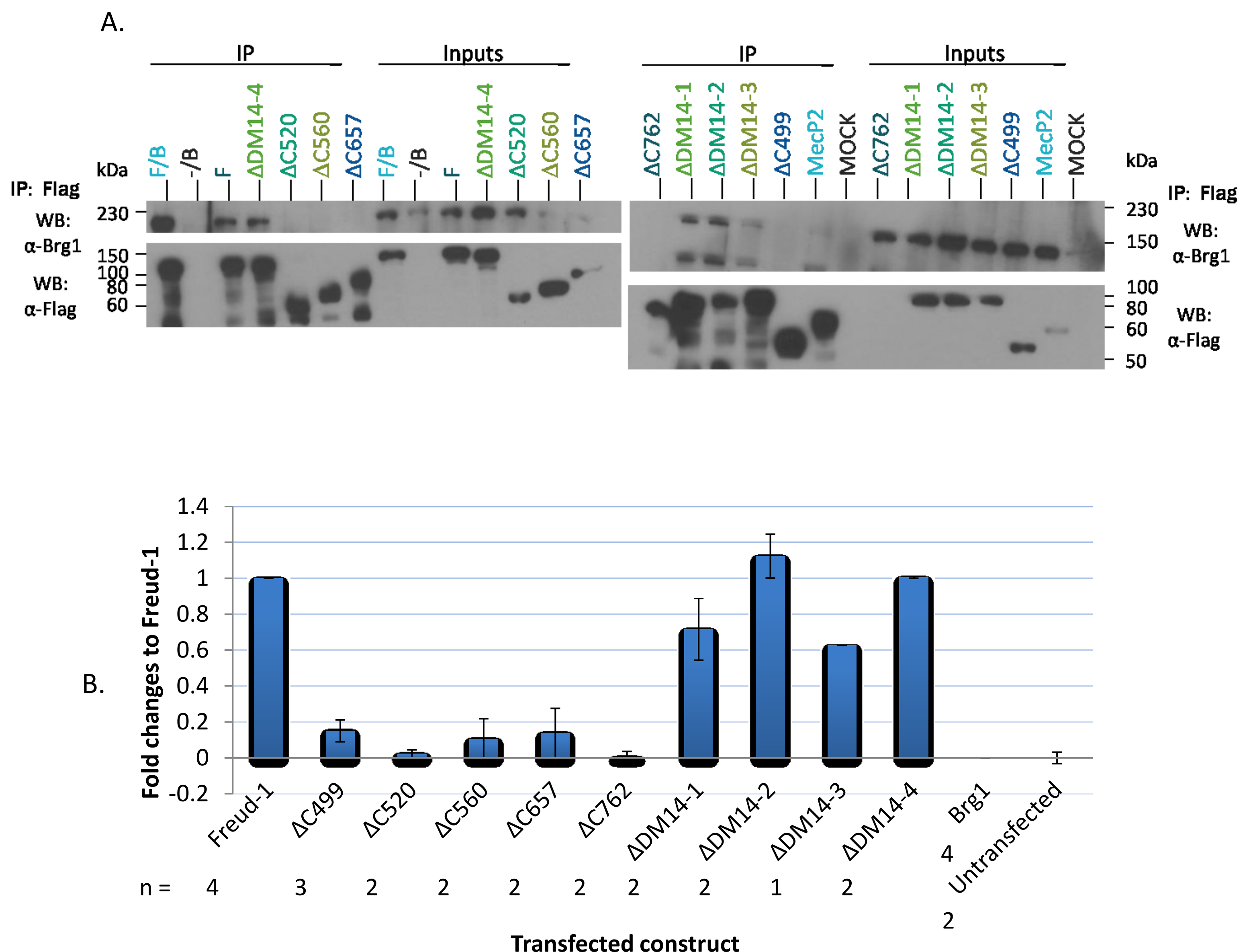


Figure 12. **Single tag co-IP of endogenous hBrg1 and Flag-Freud1 mutants.**

(A) HEK293 cells were transfected as indicated and nuclear proteins were extracted. Immunoprecipitations followed by SDS-PAGE analysis and Western Blot were performed as labeled. IP and input are shown for each condition. F/B: pFlag-cMV2-hFreud-1 with pcDNA3.1D-V5-His-hBrg1; -/B: pFlag-cMV2 with pcDNA3.1D-V5-His-hBrg1; F: pFlag-cMV2-hFreud-1; MOCK: untransfected; MeCP2: pFlag-cDNA3-MeCP2 was initially used as an additional negative control. As a negative control, Brg1 was transfected with the empty vector of Freud-1 constructs to verify that Brg1 did not bind to the resin. Flag-tagged wild type Freud-1 was used as a positive control. Representative blots of at least 2 independent experiments. (B) Densitometry analysis of co-IPs assessed binding of wild-type and mutant forms of Freud-1 to endogenous Brg1. Mean fold changes to wild-type Freud-1 are shown with standard error.

Brg1 expression is nuclear whereas Freud-1 expression is widespread but mainly perinuclear

I observed the subcellular localization of Freud-1 and Brg1 in HEK293 cells. In untransfected HEK293 cells, endogenous Freud-1 staining is diffuse in the whole cell and can appear throughout the cell or concentrated in the perinuclear region (Figure 13 a). In the same cells transfected with Flag-Freud-1 and V5-Brg1, Brg1 is localized to the nucleus whereas Freud-1 has a more punctate staining present throughout the cell, although it seems to be more prominent around the nucleus and less inside of it (Figure 13 b, c). Thus, Freud-1 is diffusely expressed in the whole cell and more markedly in the perinuclear region on the contour of the nucleus whereas Brg1 is expressed only in the nucleus. Transfected Freud-1 and Brg1 weakly colocalize in the nucleus of HEK293. This finding is consistent with previous studies showing that Freud-1 was weakly expressed in the nucleus of HEK293 cells and that its subcellular localization varies between cell lines (Rogaeva et al., 2007).

As a control, Figure 14 (in appendix) shows the background staining associated with Flag-Freud-1 and V5-Brg1.

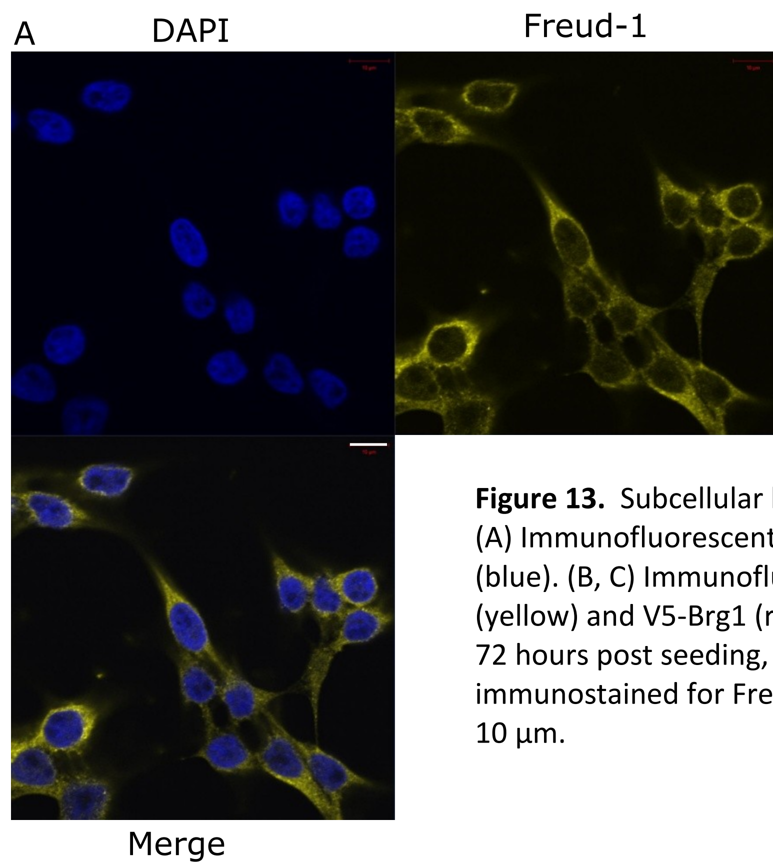


Figure 13. Subcellular localization of Freud-1 and Brg1.
(A) Immunofluorescent staining of endogenous Freud-1 (yellow) and DAPI (blue). (B, C) Immunofluorescent staining of transfected Flag-Freud-1 (yellow) and V5-Brg1 (red) and DAPI (blue). 48 hours post transfection or 72 hours post seeding, HEK293 cells were fixed, permeabilized and immunostained for Freud-1 (A) or Flag and V5 (B, C). Scale bar represents 10 µm.

B DAPI Flag-Freud-1

C DAPI Flag-Freud-1

V5-Brg1 Merge

V5-Brg1 Merge

4. Discussion

The C-terminus of Freud-1mediates its interaction with Brg1

Our results using purified bacterially expressed proteins show that Freud-1 can bind directly to the C-terminus of Brg1 and indicate that their interaction in cells is direct. Co-IP studies showed that deletion of both the C2 and the C-terminal domains of Freud-1 prevented its interaction with Brg1. Interestingly, deleting the C-terminal end reduced direct binding to the C-terminus of Brg1 as assessed by in vitro pull down assay with recombinant purified proteins. Furthermore, the NSMR isoform of Freud-1 – which lacks the C-terminal half of the wild type protein (both C2 and C-terminal domains deleted) - did not bind to Brg1. These findings indicate that the C-terminal end of Freud-1, perhaps including part of the C2 domain, binds to the C-terminus of Brg1. This is consistent with data from IP studies in cells, since the C-terminal truncations (ΔC762 and shorter mutants) lost their ability to bind to Brg1. Future IP studies will verify if the ΔC2 maintains its ability to bind to Brg1 in the cell. Furthermore, because Brg1 is part of a complex of at least ten subunits that tightly regulate its activity at numerous promoters, there could be additional interactions with the complex that occurs in the cell than what is observed between the two proteins within the in vitro pull down assay. Other subunits may serve to promote or prevent Freud-1/Brg1 interaction in spite of their ability to bind *in vitro*. In addition, the C2 and C-terminal domains of Freud-1 could both contain binding sites and may differentially mediate the interaction with Brg1.

Freud-1 and its mutants bound directly to the C-terminal domain of Brg1 that contains a DNA-binding AT-hook motif and a bromodomain that recognizes N-acetyl-lysines in histones and

other proteins (Trotter and Archer, 2008). The C2 and C-terminal domains of Freud-1 contain 8 and 5 lysines, respectively but it is unknown whether they are acetylated. We can speculate that the bromodomain of Brg1 may bind to acetylated lysines in the C2 and C-terminal end of Freud-1. Interestingly, ΔC762 contains a lysine (K757) that is not present in ΔC757 which ends at L756. If this lysine is one of several residues important for binding to Brg1, it could explain why ΔC762 was weakly interacting with Brg1 in co-IP studies whereas ΔC757 hardly bound in *in vitro* pull downs. Although lysine acetylation is not frequent in bacteria (Soppa, 2010) its occurrence has been reported (Liang et al., 2011). Furthermore, using purified bacterially expressed proteins Qiu and Ghosh (2008) showed that the N-terminal domain of Brg1 binds to the calcium-responsive transactivator (CREST). The resulting CREST-Brg1 complex regulated the activation of the c-fos and NR2B promoters in a calcium-dependent manner. To determine whether K757 is required for binding to Brg1 *in vivo*, future studies should compare the binding ability of ΔC757 and ΔC762 in co-IP studies. Post-translational acetylation of Freud-1 C-terminus may play a role in recruitment or assembly of the SWI/SNF complex at the 5-HT1A gene DRE. Future studies should test this hypothesis by assessing and mapping the acetylation of the C-terminus of Freud-1 with the appropriate acetylation assay. And they should elucidate the role of acetylation of the C-terminus by using co-IP studies to analyze the binding ability of C-terminus lysine-deficient mutants of Freud-1 generated by point-mutations. Because Figure 6 shows that N-Brg1 was more degraded and couldn't be detected at its full length compared to C-Brg1 we cannot rule out the possibility that Freud-1 may also bind to N-Brg1. Future studies should examine this aspect, optimizing the expression and purification of N-Brg1 to prevent its degradation. Moreover, when Brg1 interacts with a transcription factor, it can use distinct

domains to mediate the interaction and exert its activity on the given promoter region (Szewczyk et al., 2010). Thus it will be crucial next to determine which structural changes between Freud-1 and Brg1 can affect the transcriptional activity of the 5-HT1A promoter. This could be assessed by luciferase reporter assay based on the findings of this study.

This study increases our knowledge of the structure of the Freud-1 protein. Future studies will probe the role of its interaction with Brg1 and will expand on previous work in our group that showed the importance of the C-terminal domain for the DNA binding ability and the repression by Freud-1 (Ou et al., 2003).

Subcellular localization of Freud-1 and Brg1

Although we anticipated the cytosolic localization of Freud-1 based on its cytosolic functions (Nakamura et al., 2009; Chang et al., 2011; Gallagher and Knoblich, 2006; Nakamura et al., 2009; Matsuda et al., 2003), we hypothesized that it would also co-localize with Brg1 in the nucleus based on our co-IP results. We showed here that in HEK293 cells, Freud-1 is diffusely expressed in the whole cell and more markedly in the perinuclear region whereas Brg1 is expressed in the nucleus. Freud-1 expression appeared low compared to that of Brg1 or DAPI particularly when it was transfected. Thus Freud-1 signal in the nucleus was very weak in our experimental conditions. In addition, it was shown that Freud-1 distribution varies between cell lines and that its expression is lower in the nucleus than in the cytoplasm of HEK293 cells (Rogaeva and Albert., 2007) and Freud-1 staining is more nuclear in neurons than in other cells (Ou et al., 2003). We performed immunofluorescence in HEK293 in comparison to our co-IP

studies for which this cell type was a good model. However, future immunofluorescence studies should look at the co-localization of endogenous Brg1 and Freud-1 in neurons.

The variable localization of Freud-1 in different cell types could be explained by its multiple cellular functions. Indeed, Freud-1 has been reported to function as, among other things, a transcription factor (Ou et al., 2003), nucleosomes associated factor (Nakamura et al., 2009), regulator of Notch signaling on endolysosomes (Gallagher and Knoblich, 2006), upstream NF-kB activator (Matsuda et al., 2003), an innate immune regulator of the TLR3 and TLR4 pathways (Chang et al., 2011) and scaffold of a complex containing the EGF Receptor (Nakamura et al., 2008). A possible explanation is that since most of Freud-1 functions are cytoplasmic, the majority of the protein may reside outside of the nucleus to perform its non-nuclear functions and that only a very small fraction is necessary inside the nucleus. Moreover, HEK293 cells don't express the 5-HT1A gene and could need even less Freud-1 for its regulation given the presence of a REST complex at its promoter, repressing the expression of a neuronal gene in this non-neuronal cell type. Thus, Freud-1 localization is cell-type dependent and is unlikely to resemble that of other transcription factors given its varied functions performed mostly in the cytoplasm. Future studies will confirm its localization in neuronal cells. Besides, the expression of Freud-1 in both the cytoplasm and the nucleus in neuronal cells (Ou et al., 2003) suggests that it may exert its transcriptional repression in an activity-dependent manner. This could be tested in 5-HT1AR positive neurons by stimulating 5-HT1AR and assessing Freud-1 activity at the 5-HT1A gene promoter as well as its subcellular distribution.

In summary, my studies indicate that Freud-1 C-terminal domain directly interacts with the C-terminus of Brg1. These results suggest that Freud-1 mediated repression may involve a direct interaction with Brg1. Further studies attempting to specifically block this interaction are needed to confirm this hypothesis.

Future perspectives

In this study I used HEK293 cells - that don't express 5-HT1A receptors - to elucidate the structural determinants of the interaction between Freud-1 and Brg1 because of their ease of use. In 5-HT1A negative cells, the 5-HT1A promoter contains an upstream repressor element-1 (RE-1) sequence that overlaps the DREs bound by Freud-1 and Freud-2 (Figure 3). RE-1 is the target sequence of a REST complex that prevents the expression of neuronal genes in non-neuronal cells. Given their proximity, when the Brg1-containing SWI/SNF complex binds to the DREs, it could interact with a REST complex in non-neuronal cells to silence the promoter activity. Moreover, Freud-1-mediated repression is histone deacetylase (HDAC)-independent in neuronal cells expressing 5-HT1A but HDAC-dependent in non-neuronal cell lines pointing to differences in the mechanism of 5-HT1A gene repression between those cell types (Lemonde et al., 2004). Therefore, blocking Freud-1 repression in HEK293 cells may not increase the expression level of 5-HT1A receptors (Ou et al., 2003). Thus after successfully blocking the Freud-1/Brg1 interaction, future studies should assess its impact on the promoter activity in 5-HT1A positive cells and neurons.

I showed here that the C-terminal domain of Freud-1 mediates its binding to Brg1. Freud-2, the other known member of Freud-1's family, binds the 5-HT1A promoter at a 3' dual

regulatory element adjacent to the 5' dual regulatory element bound by Freud-1. Their C-terminal regions are highly conserved although Freud-2 lacks the latter 135 amino acid region of Freud-1 (815-950). This C-terminal extension contains kinase sites (GSK3, CK1, PKCu), four lysines (K823, K825, K860, K932), a CBF-B site involved in the DNA binding of CBF (875-895) and a polymorphic CAMKII/PKA site (A/T905). Because Freud-2 also represses the 5-HT1A promoter, its shorter C-terminal domain of Freud-2 might also contain binding sites for Brg1. If it is the case, the binding might take place in the C-terminus of Freud-2 and between amino acids 657 and 815 of Freud-1. Future studies should assess this hypothesis by testing whether Brg1 is co-precipitated with Freud-2. This finding will further delineate the domain of interaction with Brg1 in Freud-1 and Freud-2 and would expand the picture of Brg1-regulated repression at the 5-HT1A promoter.

This study shows that Freud-1 interacts directly with the C-terminus of Brg1 and that the C-terminal end and possibly the C2 domain of Freud-1 mediate this interaction. These findings expand our knowledge of how the structure of Freud-1 mediates its various functions in the cell and help us to gain insight into the possible impacts of Freud-1 dysfunctions. In the long-term, we aim to develop molecular tools that could alter, in a specific and predictable manner, the levels of 5-HT1A autoreceptors in the brain. To determine the potential of our strategy in this regard, we need to know whether Brg1 associates with Freud-1 on other promoters. So far, it has been shown that Freud-1 also regulates the transcription of the dopamine D2 receptor gene (Rogaeva and Albert, 2006) but the presence of Brg1 at this site has not been assessed. Also, future studies should determine if it is possible to alter the interaction of Freud-1 with Brg1 without disrupting their other cellular functions. This could represent a major roadblock

because Brg1 is a widespread transcriptional co-regulator and Freud-1 has multiple functions in the cell. Yet even in this case, elucidating Freud-1 repression could teach us more about the transcriptional regulation of the 5-HT1A receptor and direct us to better targets. More broadly it could increase our knowledge of the transcriptional response to changes in a cell's environment.

Probing the transcriptional role of Freud-1 in the NSMR phenotype

Studies of neuronal activity-regulated gene transcription suggest that neuronal activity may modulate ATP-dependent chromatin remodeling complexes thus directing nucleosomes sliding and revealing or masking transcription factors binding sites (West and Greenberg, 2011). Indeed, Qiu and Ghosh (2008) demonstrated the contribution of Brg1 to activity-dependent gene transcription through its interaction with the calcium regulated transcriptional activator CREST at a Sp1 binding site. Furthermore, several SWI/SNF subunits can be represented by either member of their gene family: Brg1/Brm, BAF250a/b, BAF155/BAF170, BAF60a/b/c, BAF45a/b/c, BAF57, BAF53a/b. Genetic studies demonstrate that subunit exchange helps pluripotent embryonic stem cells to transition to multipotency and to the committed post-mitotic neuron (Hargreaves and Crabtree, 2011). Moreover the last step leading to post-mitotic neurons is accompanied by an essential switch in subunits forming an nBAF complex that appears to be unique to the nervous system (Lessard et al., 2007). Thus, highly defined selective assembly of SWI/SNF complexes takes place during the development of the nervous system and the relevance of those mechanisms to the developmental role of Freud-1 needs to be investigated. At the moment, it is unclear whether a similar developmental switch takes

place when Brg1 associates with Freud-1 at the 5-HT1A (and possibly DRD2) promoter, but the developmental role of Freud-1 is demonstrated in mice by the lethal phenotype of Freud-1 knock-out mice (Zhao et al., 2011). Given that Freud-1 mRNA is present in the murine brain developmentally (Basel-Vanagaite et al., 2006), our studies support the importance of the Freud-1 C-terminus in transcriptional repression and suggests that loss of the C-terminus may alter the development of the dopaminergic and serotonergic systems. Notably, the NSMR isoform of Freud-1 (437 out of 951 a. a.) lacks its C-terminal half and we show here that it does not bind to Brg1. Furthermore, mice lacking 5-HT1A receptors show cognitive deficits (Sarnnyai et al., 2000) while 5-HT1A antagonists increase cognitive performance in adult rodents (Sumiyoshi and Meltzer, 2004; Schechter et al., 2005). Thus, dysregulation of the expression of the 5-HT1A receptor alters cognitive functions and these effects may be timing and location-dependent. At present, it is unclear which domains and functions of Freud-1 could lead to the NSMR phenotype but our studies probe the relevance of investigating the transcriptional role of Freud-1. In the future, a NSMR knock-in mouse or a brain conditional knock-out mouse should be used to model behavioral and neurophysiological alterations in NSMR and clarify the contribution of Freud-1 transcriptional function.

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6. *Appendices*

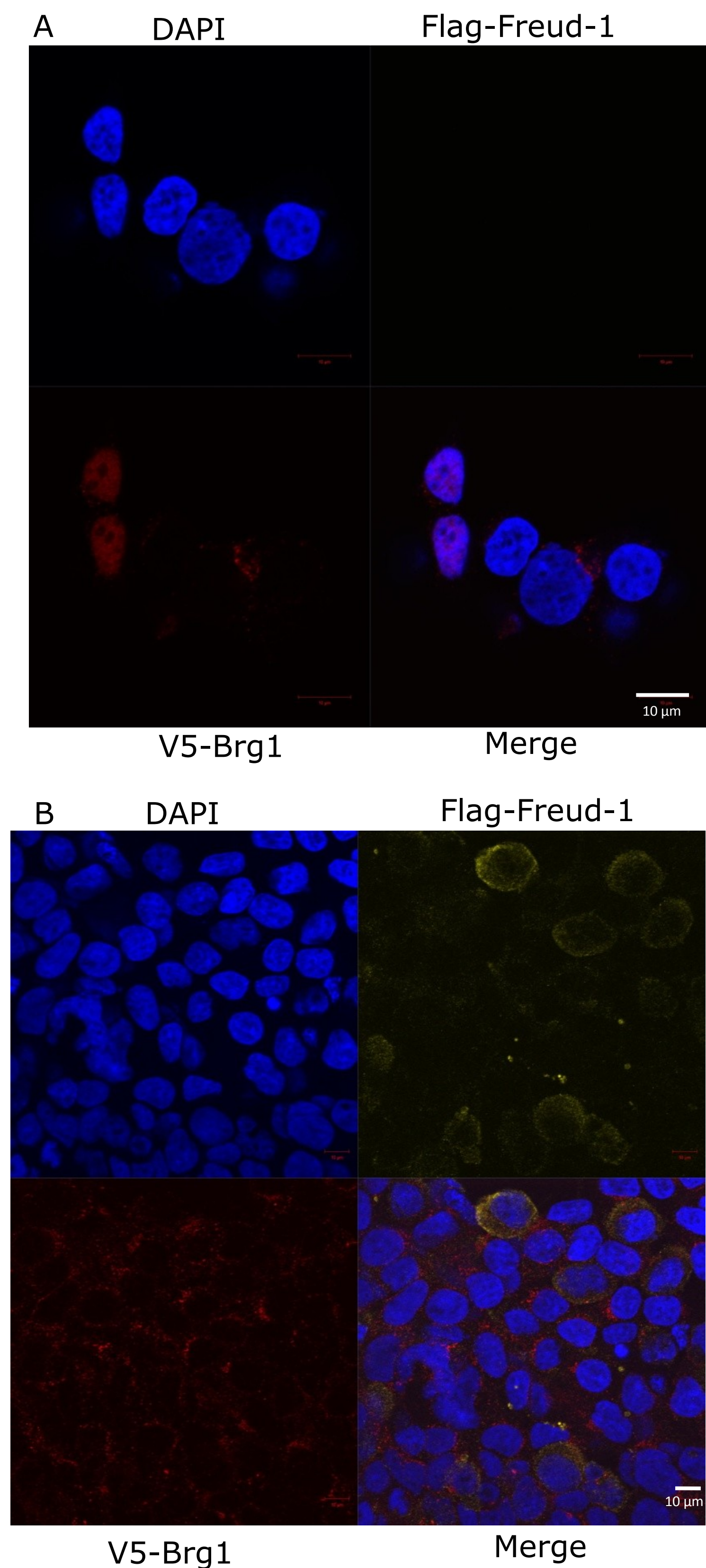

Figure 14. Controls ensured the specificity of the immunofluorescence staining. Background signal when staining for (A) V5-Brg1 and Flag-Freud-1 is shown. Cells were transfected with V5-Brg1 and stained for V5-Brg1 and Flag-Freud-1. (B) Cells were transfected with Flag-Freud-1 and stained for V5-Brg1 and Flag-Freud-1. DAPI staining (blue), Flag-Freud-1 (yellow) and V5-Brg1 (red) staining are shown in both cases. Scale bar represents 10 µm.

A single-tag co-IP removed experimental obstacles

Although I first successfully optimized the co-IP procedure with WT Freud-1 and Brg1 constructs, later experiments displayed a high amount of background, coincidently with the addition of Freud-1 deletion mutants to the assay. Because each protein has a different structure and can behave differently, I used a control for each mutant, i.e., co-transfecting Flag-tagged mutant and Brg1 empty vector. This showed that Flag-tagged Freud-1 and mutants were detected on the His-affinity resin when no His-tagged protein was transfected as well as when His-tagged Brg1 was transfected. This happened while following the same protocol previously used for WT constructs and their controls. The experiment was repeated many times while making sure to accurately follow the protocol that had yielded specific results earlier and using the same reagents to the extent to which it was possible. Results were inconsistent, sometimes specific and sometimes not, and the relative amount of signal and background varied between constructs but in spite of careful observation I couldn’t link a discrete cause to the variations in the outcome.

I then applied several strategies to prevent non-specific binding of Flag-tagged protein to the His-affinity resin. I increased the stringency of the washes by adding detergent to the buffer, a potassium phosphate (50 mM) and sodium chloride (300 mM) solution with a low Imidazole concentration (10mM) at neutral pH (7.4). Adding a mild detergent such as Triton X-100 or Tween-20 at low concentration to the buffer while washing an IP disrupts weak bonds and is a common strategy to decrease non-specific interactions. In our case, when 0.5% Tween-20 or Triton X-100 was added to the buffer no interaction at all could be detected. Thus, a

variety of low and very low concentration on a decreasing gradient was tested to look for the threshold at which the specific binding would remain while the non-specific binding wouldn't. At every concentration of detergent binding was completely lost until a threshold was reached between the concentrations of 0.01% and 0.001% Triton X-100. Repeating the exact same experiment with 0.001% Triton X-100 yielded slightly different results and after trying an intermediate concentration of 0.0005% without consistent satisfactory results, I explored other avenues. Moreover, as illustrated in figure 11, optimized conditions varied from one construct to another in spite of efforts made to have a similar level of expression between the constructs.

I asked whether changing the binding properties of the His-affinity resin would affect this non-specific interaction. In the wash and elution buffers (50 mM NaH2PO4, 300 mM NaCl, imidazole - 10 mM for washes and 150 mM for elution -, pH 7.4) the affinity of the His groups for the resin decreases as the imidazole concentration increases. Changing the pH of the buffer to 6.0 was expected to protonate a larger proportion of imidazole groups, increasing their affinity for their target His motifs. Testing in parallel imidazole concentrations of 10, 20 and 50 mM at pHs of 6.0 and 7.4 led to no visible difference on the specificity of the binding. Also, coating the resin with 1% or 5% BSA prior to the immunoprecipitation led to no improvements. These results suggested that further investigation was necessary to uncover the source of the non-specific binding and circumvent it.

So far, I had two hypothetical explanations for the persistent non-specific binding. The first was that because the immunoprecipitated proteins were overexpressed they could be present in such an important quantity that a proportion high enough to be detected would

always be bound to the resin. The second was that because proteins bound the resin in absence of transfected His-Brg1 and all carry a Flag-tag, it was likely that either the Flag tag or else shared amino acid sequence between the Freud-1 proteins bound to the His-affinity resin. Since the Flag tag is negatively charged it may interact with the Co^{2+} cations of the His-affinity resin.

To address the first hypothesis the next strategy consisted of reducing the total amount of overexpressed proteins to lower the concentration of Flag-tagged proteins in the nuclear extract thus reducing the chances that it would non-specifically attach to the beads. This change in conditions is also desirable because it resembles more physiological conditions. The amount of transfected DNA was thus re-assessed for each construct (WT and mutants of Freud-1 and WT Brg1) to transfect as little DNA as possible while maintaining perceived levels of expression above the level of detection by WB and as similar as possible across samples. This strategy didn’t solve the problem either. A possible explanation is that even in those conditions the amount of overexpressed Flag-tagged proteins might have been high enough to attach to the resin to the extent that the specific interaction was obscured by the background. Thus leading to no consistent difference between the signals detected for sample and control conditions.

I also sought to compete out the binding of Flag-tagged proteins to the resin with that of His-Brg1 proteins by decreasing the amount of Flag constructs transfected relative to the Brg1 construct. Since Flag-tagged Freud-1 proteins and His/V5-Brg1 were not detected with the same antibodies and that for an equal amount of protein loaded on two gels the signal

detected depends on many factors inherent to the WB procedure, it was not feasible to ensure a given ratio between His-tagged and Flag-tagged proteins.

Also, in standard conditions the lysates were incubated with the His-affinity resin for only 30 minutes and further reducing this duration didn't affect the non-specific binding.

I then opted for pre-clearing the lysates from the excess of Flag-tagged proteins, by incubating them with agarose beads G-25 (Sigma) prior to incubation with the affinity resin. This approach wiped out all signal in sample and control.

These results illustrate the challenge of finding a solution i) that could increase the ratio of signal to background in the co-IP experiments rather than eliminating all signal and ii) that could reach this goal consistently. Together, they led to the conclusion that the ratio of signal to background associated with the technique itself was too high for the experiment attempted and that it was necessary to change the technique to be able to address the question of interest, which is to determine which Freud-1 mutants have the region required for binding to Brg1.

There are other immunoprecipitation systems using agarose or non agarose beads pre-incubated with a highly specific and very low background antibody however they were not explored by lack of a suitable antibody.

Since all Freud-1 mutants carry the Flag-tag it wasn't possible to avoid the use of Flag-tag in an alternative approach. Subcloning the mutants in a vector carrying a different tag wouldn't warrant better results so it wasn't done at this point. To avoid the non-specific binding

of the Flag tag to the His-affinity resin the co-IP was performed with one tag instead of two. I did it by co-precipitating endogenously expressed Brg1 proteins with Flag-tagged WT and mutant Freud-1 in a Flag-affinity resin. This approach yielded specific results. Indeed, as shown in figure 12, transfected (-/B) and endogenous Brg1 (MOCK) were not detected in absence of flag-tagged proteins. This approach also had the advantage of probing for the Brg1 endogenously expressed rather than overexpressed.

For a given experimental approach, the extent to which it is possible to control the conditions should be sufficient to ensure repeatable results. This is usually achieved by repeating accurately an optimized protocol and using identical reagents. Using identical reagents from one experiment to the next is feasible to a certain extent but not absolutely. In a co-immunoprecipitation approach, this applies for example to the DNA preparations and the protein lysates.

Variability in the transfected DNA constructs is due to the fact that DNA preparations need to be replaced once they are used up and even if in theory it doesn’t make any difference experimentally it sometimes affects the level of expression of the construct by the cells. A way to avoid varying DNA preparation would be to prepare at the beginning of the project a number of DNA maxi-preparations for each construct and pool them together before measuring the concentration and aliquoting this pooled preparation. This would ensure that I use the same DNA preparation in all experiments throughout the project and eliminate one source of variability.

To compare results obtained by immunoblotting it is necessary to load the same amount of elution across samples on each blot and then to analyze the signal of those samples relative to the positive control (wild-type) and the negative control. My objective was more to qualitatively assess the presence or absence of an interaction than to quantitatively measure this interaction. If I wanted to compare quantitatively results obtained with two different antibodies it would be an asset to use an additional reference to the loading of equal amounts of proteins. At the beginning of the project, I would need to prepare and pool many nuclear extracts from cells transfected with WT Freud-1 and Brg1 before aliquoting them. Then loading an identical volume of this pooled extract on each blot throughout the project would provide a reference for each antibody and allow comparing samples and blots to one another. I didn't add this step to my approach because I had no knowledge of it at the time and didn't anticipate at the beginning of the project the problems that would later emerge.

The WB has experimental challenges inherent to itself. The specificity of the signal needs to be well verified and the conditions worked out to maintain a proper signal level. Since the signal varied sometimes from one experiment to the next executed in the same conditions, I didn't always maintain an identical signal at WB. Experimental variations also make it virtually impossible to always have the same signal; this is why I loaded a quantified amount of proteins that was equal between conditions.

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Title: Freud-1/Aki1, a Novel PDK1-Interacting Protein, Functions as a Scaffold To Activate the PDK1/Akt Pathway in Epidermal Growth Factor Signaling

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