TRANSCRIPTIONAL REGULATORY MECHANISMS OF FREUD-1, A NOVEL MENTAL RETARDATION GENE

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ABSTRACT

The mechanisms that govern the repression of 5-HT1A receptor gene expression mediated by a novel mental retardation gene, Freud-1, were examined in HEK293 and SKNSH cells. In order to test the hypothesis that Freud-1 recruits co-repressors to its DNA element, affinity purification was used, which, combined with co-immunoprecipitation and pull-down assays, validated the potential Freud-1 interacting partners. The functional importance of these interactions was addressed by the use of siRNAs targeted towards the protein of interest and functional effects on 5-HT1A promoter recruitment and transcription verified by chromatin immunoprecipitation and quantitative RT-PCR assays, respectively. This study provides a possible mechanism of 5-HT1A receptor gene regulation by Freud-1, which, to mediate its action, recruits Swi/Snf and Sin3A/histone deacetylase (HDAC) complexes in non-neuronal HEK293 cells and Swi/Snf only in neuronal, 5-HT1A receptor-expressing SKNSH cells. Thus, Freud-1 has a dual mechanism of repression depending on cell type: HDAC dependent in HEK293 cells and HDAC independent in SKNSH cells. This dual type of repression is probably required for the different extents of repression in these cells. HDAC-dependent repression was associated with gene silencing, and is likely associated with a more compact chromatin structure that is inaccessible to other potential regulators. In contrast, HDAC-independent repression allows for 5-HT1A expression, and does not appear to induce compact chromatin structure. This may explain why a deletion in the Freud-1 gene leads only to non-syndromic mental retardation (NSMR) and not to additional phenotypes in non-neuronal tissues. In addition, I present evidence that Freud-1 is not

sumoylated at its consensus sumoylation sites and I present the lipid binding properties of Freud-1 and Freud-1 mutants.

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LIST OF ABBREVIATIONS

5-HIAA 5-hydroxyindoleacetic acid

5-HT 5-hydroxytryptamine (serotonin)

5-HT1A 5-hydroxytryptamine 1A receptor

5-HTP 5-hydroxytryptophan

5-HTR 5-hydroxytryptamine receptor

5-HTT 5-hydroxytryptamine transporter

AADC aromatic amine decarboxylase

AC adenylyl cyclase

ACh acetylcholine

ACTH adenocorticotropic hormone

Aki1 Akt kinase-interacting protein 1

ATP adenosine triphosphate

BAF Brg1/hBrm-associated factor

BDNF brain-derived neurotrophic factor

Brg1 Brahma-related gene-1

hBrm human brahma

[^{11}C] carbon 11

C2 protein kinase C conserved region 2

Ca^{++} calcium

CaM calmodulin

CaMK calcium calmodulin dependent protein kinase

CaMKII calcium calmodulin dependent protein kinase II

CARM1 coactivator-associated arginine methyltransferase-1

CC2D1A coiled-coil and C2 domain containing 1A

Cdk 1 cyclin B1-cyclin-dependent kinase 1

CNS central nervous system

CHIP chromatin immunoprecipitation assay

CHMP charged multivesicular body protein

CoREST co-repressor for repressor element-1-silencing transcription factor (also known as RCOR1)

CREB cAMP response element binding protein

CRE cAMP response elements

CRH corticotropin-releasing hormone

CSF cerebrospinal fluid

CtBP C-terminal binding protein

DA dopamine

Deaf-1 deformed epidermal autoregulatory factor 1

DNA deoxyribonucleic acid

DNMT DNA methyltransferase

DM14 *Drosophila* melanogaster 14

DRD2 dopamine-D2 receptor

DRE dual repressor element

DRN dorsal raphe nucleus

EDTA ethylene glycol tetraacetic acid

EE early endosomes

EGFR epidermal growth factor receptor

ELM2 EGL-27 and MTA1 homology 2 domain

EMSA electrophoretic mobility shift assay

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

G9a histone-lysine N-methyltransferase

GPCR G-protein coupled receptor

GR glucocortcoid receptor

H_3K_9 histone H3 lysine 9 methylase

HAT histone acetyltransferase

HBS HEPES buffered saline

HDAC histone deacetylase

Hes5 Hairy/Enhancer-of-split-5

HLH helix loop helix

HMT histone methyltransferase

HP1 heterochromatic protein 1

ICER inducible cAMP early repressor

KAP-1 Krab associated protein 1

Lgd lethal (2) giant disc protein

LSD1 lysine specific demethylase 1

MAO monoamine oxidase

MAOI monamine oxidase inhibitors

MD major depression

MDD major depressive disorder

MeCP2 methyl-CpG-binding protein

MR mental retardation

MRT3 mental retardation, nonsyndromic, autosomal recessive, 3

MVB multivesicular bodies

NA norepinephrine or noradrenaline

NADH nicotinamide adenine dinucleotide dehydrogenase

NcoR nuclear receptor corepressor-1

NFκB nuclear factor-kappa B

NICD Notch intracellular domain

NLS nuclear localization sequence

NRSE neuron restrictive silencer element

NRSF neural restrictive silencing factor (also known as REST)

NSMR non-syndromic mental retardation

NUDR nuclear deformed epidermal autoregulatory factor

NUMAC nucleosomal methylation activation complex

PAH paired amphipathic helix

PBAF polubromo-associated BAF complex

PBS phosphate buffered saline

PDK1 3'-phosphoinositide-dependent protein kinase 1

PET positron emission tomography

Pet-1 mouse transcription factor pheochromocytoma 12

PKA protein kinase A

PKC protein kinase C

PP2A protein phosphatase 2A

pRb retinoblastoma protein

RBBP4/7 retinoblastoma-associated proteins 4/7 also known as RbAP48/RbAP46

RE-1 repressor element 1

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

RNA ribonucleic acid

SAE SUMO activating enzyme

SANT SWI3/ADA2/NcoR/TFIIIB domain

SAP Sin3A-associated protein

SCP RNA polymerase II small CTD phosphatase

SENP sentrin/SUMO-specific protease

SERT serotonin reuptake transporter

SH3 Src homology 3 domain

SIM SUMO-interacting motif

SILAC Stable Isotope Labeling by Amino Acids in Cell Culture

Sin3 switch independent histone deacetylase component

SNP small nucleotide polymorphism

SRF serum responsive factor

SSRI selective serotonin reuptake inhibitor

SUMO small ubiquitin-related modifier

Swi/Snf mating-type switching and sucrose non-fermenting protein complex

TBP TATA-binding protein

TCA tricyclic antidepressants

TCA trichostatin A

TPD tryptophan depletion

TPH tryptophan hydroxylase

TPH1 tryptophan hydroxylase 1

TPH2 tryptophan hydroxylase 2

TSG tumor suppressor gene

WHO world health organisation

WINAC WSTF including nucleosome assembly complex

WSTF Williams syndrome transcription factor

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CHAPTER-I: Introduction

1-Serotonergic System

1.1 Overview

The serotonin system of the brain originates from neurons of the raphe nuclei that project widely through the brain. The serotonergic neurons of the raphe nuclei are the primary site of serotonin (5-HT) synthesis. These neurons project to various brain areas such as cortical, limbic and hypothalamic regions, brain regions known to be involved in the regulation of the mood, emotion, stress, etc (Jacobs & Azmitia, 1992; Tork, 1990). Reduction in serotonergic neurotransmission is implicated in the depressive illnesses and suicidal behaviors (Cools, Roberts, & Robbins, 2008; Doris, Ebmeier, & Shajahan, 1999; Mann, 1999).

Serotonergic neurons can be divided into 2 major groups on the basis of their distribution and main projections: the rostral and caudal groups. The rostral group is confined to the mesencephalon and rostral pons, with major projections to the forebrain. The caudal group extends from the caudal pons to the caudal portion of medulla oblongata with major projections to the caudal brainstem and to the spinal cord. The neurons in the caudal group are separated from the rostral group by a gap in the middle of the pons that is devoid of serotonin-containing neurons (Figure I-1) (Hornung, 2003).

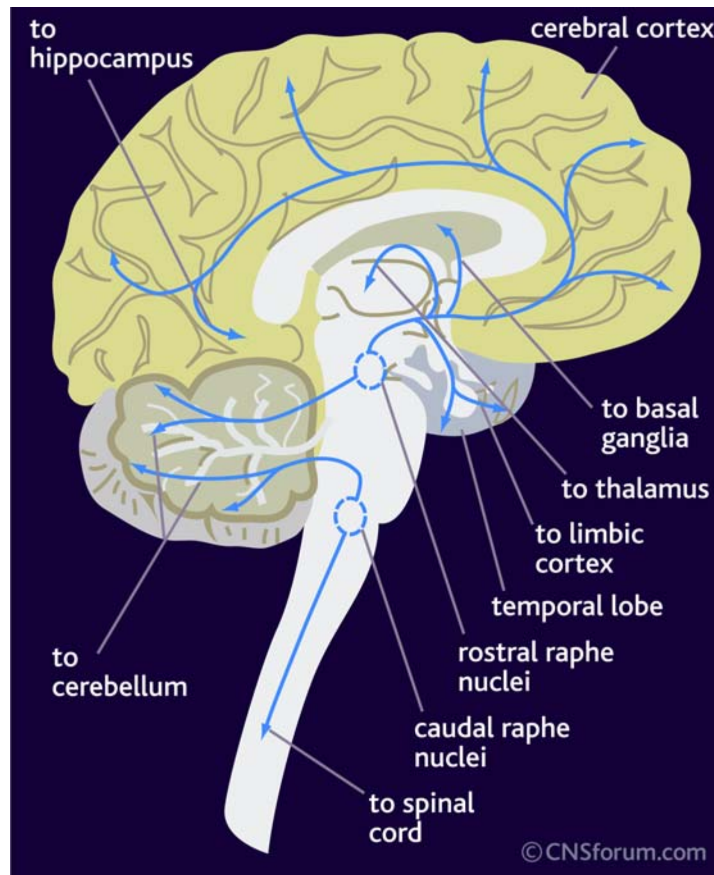

Figure I-1: The serotonergic projections. Serotonergic neurons projects broadly to different brain regions and can be organized in two groups: rostral and caudal. The neurons from the rostral raphe nuclei project to almost all of the regions of the brain such as the cortex, hippocampus, basal ganglia, thalamus, cerebellum and limbic regions which includes the cortical areas surrounding the brain stem. The neurons from caudal raphe nuclei project to cerebellum and to the spinal cord. Image obtained from http://www.cnsforum.com/imagebank/item/Neuro_path_SN_DPN/default.aspx

The raphe nuclei are collections of neurons, with poorly defined cytoarchitectonic limits. They contain heterogeneous populations of neurons, with distinct morphologies, projections and neurochemical characteristics in animals and humans (Meessen & Olszewsky, 1949; Olszewski & Baxter, 1954; Taber, Brodal, & Walberg, 1960). However, the serotonergic neurons are the best characterized constituents of the raphe nuclei. In 1964 the first localization of monoamines was done by histofluorescence techniques. These techniques revealed the preferential localization of serotonergic neurons near the midline by Dahlstrom and Fuxe (Dahlstrom & Fuxe, 1964). In 1975, immunohistochemical techniques further refined the visualization of serotonergic neurons. Finally, recent genetic studies have corroborated these results. The rostral raphe nuclei neurons are involved in the regulation of cerebral blood flow, sleep, circadian rhythms, cognition and mood. The caudal raphe nuclei are involved in cardiovascular function, nociception and movement. (Hornung, 2003; Filip & Bader, 2009).

1.2 Serotonin Synthesis and Synaptic Transmission

The monoamine neurotransmitter serotonin (also known as 5-hydroxytryptamine, 5-HT because of its chemical structure) is synthesized and used as a neurotransmitter by serotonergic neurons (Figure I-2). In 1930s, Erspamer and colleagues described serotonin as enteramine. They isolated it from the gut and it was shown to cause contraction of uterus (Erspamer & Asero, 1952). Then, serotonin was isolated as a vasoconstrictor substance thought to be contained in platelets (Janeway, Richardson & Park, 1918; Reid & Bick, 1942; Zucker, 1944). This substance was thought to increase vascular tone by virtue of its presence in serum. It was named after the Latin and Greek words serum and tonic respectively (Mohammad-Zadeh et al., 2008). Finally, serotonin was isolated from

vast amounts of blood, crystallized and named by Maurice M. Rapport in 1948 (Rapport, Green, & Page, 1948). It is one of the earliest neurotransmitters expressed during brain development and was identified in the cell bodies of brainstem raphe-nuclei (B1-9) by Dahlström and Fuxe in 1964 (Dahlstrom & Fuxe, 1964). Serotonin has been implicated in the regulation of several physiological functions such as mood, feeding, body temperature, sexual behavior, memory and cognition (Mann, 1999).

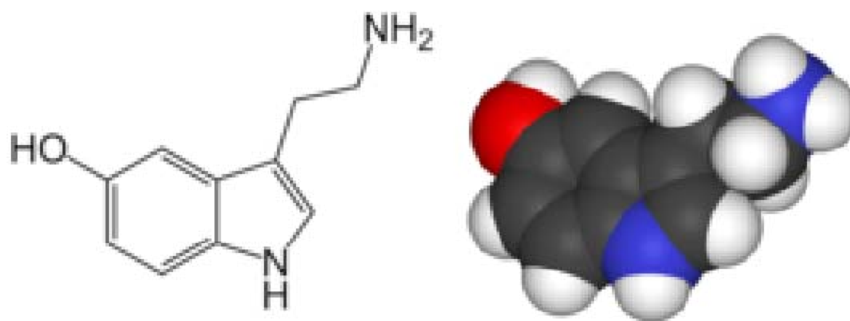

Figure I-2: The molecular structure of serotonin and its 3D view. The serotonin has relatively simple molecular structure with a primary amine and indole group and is 5-hydroxytryptamine. The serotonin chemical formula is $C_{10}H_{12}N_{20}$. Image was obtained from http://en.wikipedia.org/wiki/File:Serotonin-skeletal.png

Serotonin is synthesized from the essential amino acid L-tryptophan. The synthesis is initiated by the conversion of the amino acid tryptophan into intermediary 5-HTP (5-hydroxytryptophan) by the rate-limiting enzyme tryptophan hydroxylase (TPH). There are two isoforms of TPH: TPH1 is found in peripheral tissues that express serotonin (skin, gut, pineal gland) and in the central nervous system, while TPH2 is exclusively found in neuronal cells and is the predominant form in the brain (Walther et al., 2003; Walther & Bader, 2003). It has been shown that genetic polymorphisms in both these isoforms are associated with susceptibility to anxiety and depression (Zhang et al., 2006; Nash et al., 2005). The resulting 5-hydroxy-L-tryptophan (5-HTP) is then converted to 5-HT by 5-HTP decarboxylase (aromatic amine decarboxylase (AADC)). The availability of tryptophan in the extracellular fluids is a limiting step in serotonin synthesis (Figure I-3). The 5-HT is taken up into storage vesicles, the non-sequestered form of 5-HT is metabolized to 5-hydroxyindoleacetic acid by monoamine oxydase (MAO) (Boadle-Biber, 1993).

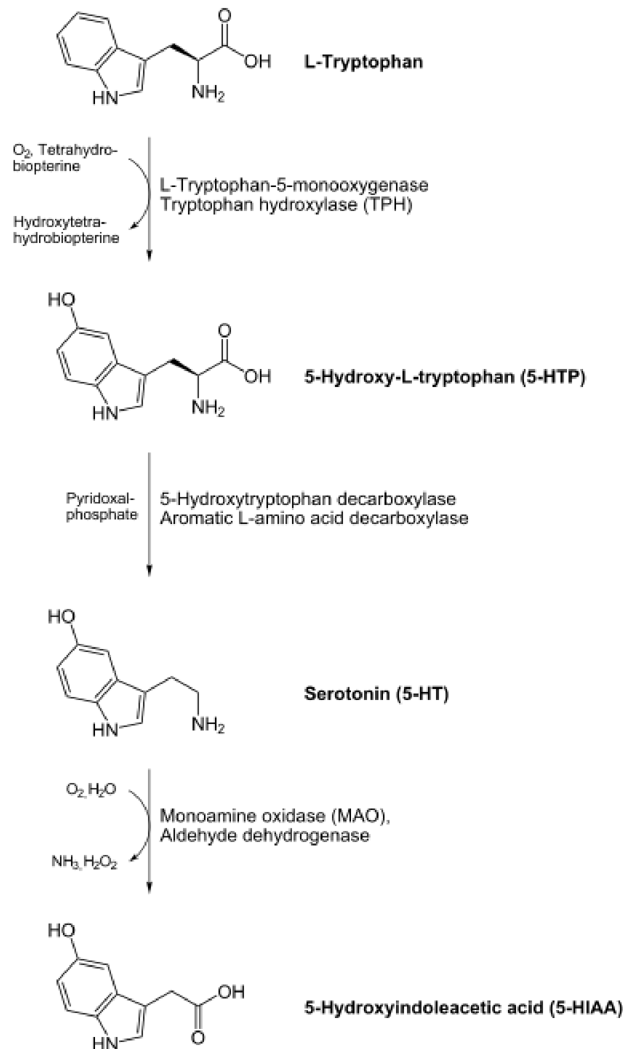

Figure I-3: Serotonin Biosynthesis. Serotonin is synthesised from L-tryptophan. L-tryptophan is converted to 5-hydroxy-L-tryptophan (5-HTP) by L-tryptophan-5-monooxygenase and Tryptophan hydroxylase (TPH) (rate-limiting step). The resulting 5-HTP is converted to serotonin (5-HT) by 5-Hydroxytryptophan decarboxylase (aromatic L-amino acid decaroxylase). Serotonin can be transformed to the inactive metabolite 5-Hydroxyindoleacetic acid (5-HIAA) by Monoamine oxidase (MAO), an aldheyde dehydrogenase. Image was taken from http://en.wikipedia.org/wiki/File:Serotonin_biosynthesis.svg

The release of the 5-HT into the synaptic cleft is the consequence of depolarization-induced calcium entry following the action potential. In the synaptic cleft 5-HT binds to and activates post-synaptic receptors, and through a negative feedback mechanism, also activates pre-synaptic terminal serotonin autoreceptors, the 5-HT1B receptor. The residual 5-HT is rapidly taken up by 5-HT transporter (SERT/5-HTT) back into the neuron in order to be recycled, packaged into new vesicles or metabolized by MAO, which is localized at the outer mitochondrial membrane (Figure I-4) (Sibille & Lewis, 2006).

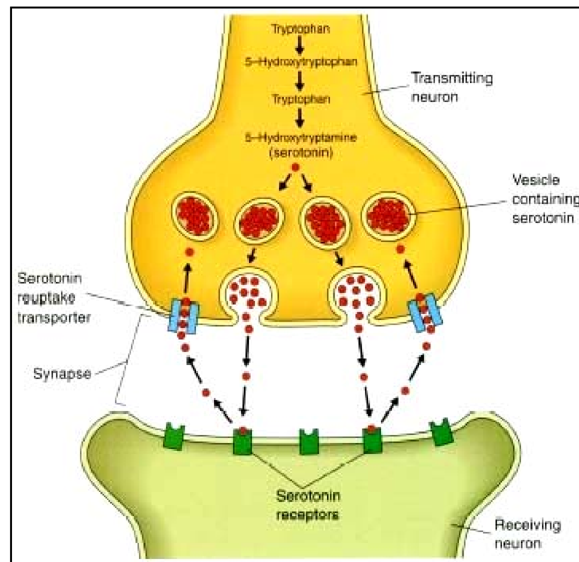

Figure I-4: A diagram of serotonin synaptic terminal. Upon activation of action potential, serotonin is released from vesicles into synaptic cleft by exocytosis where it binds to post-synaptic serotonergic receptors. The excess serotonin can be taken back up into the pre-synaptic neuron by the serotonin reuptake transporter (SERT) (a site of action of SSRIs) where it is degraded by MAO. Image is taken from http://www.humanillnesses.com/Behavioral-Health-Fe-Mu/Medications.html

1.3 Serotonin Receptors

The function of serotonin in the organism is complex. One reason for this is the diversity of serotonin receptors located directly on smooth muscle cells, on nerve endings and on neuron cell bodies and dendrites throughout the central nervous system. In general, 5-HT receptors are G-protein coupled receptors (GPCR), except 5-HT3, and are classified and grouped into 7 families on the basis of their sequence identity and on the nature of the second messenger systems to which they are coupled. There are 15 known mammalian receptors subtypes and 7 distinct families that include 5-HT1A/B/D/E/F, 5-HT2A/B/C, 5-HT3A/B/C/D/E, 5-HT4, 5-HT5A/B, 5-HT6 and 5-HT7. The 5-HT receptors are present pre- and post-synaptically, and are also present on the cell bodies and dendrites of neurons (Barnes & Sharp, 1999; Hannon & Hoyer, 2008; Hoyer, Hannon, & Martin, 2002; Pandey, Davis, & Pandey, 1995).

5-HT1 receptors are linked to $G_{i/o,}$ which are pertussis toxin-sensitive G proteins. They couple negatively to adenylyl cyclase. In addition, through the Gβγ subunit, these receptors couple to opening of G-protein inward rectifying potassium channels (GIRKs), and this leads to membrane hyperpolarization and inhibition of firing. There are five receptor subtypes in this family: 5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E and 5-HT1F. In humans, they share 40-63% overall nucleotide sequence identity (Hannon & Hoyer, 2008).

There are only three types of 5-HT2 receptors: 5-HT2A, 5-HT2B and 5-HT2C. They exhibit 46-50% of amino acid sequence identity and couple preferentially to $Gq/_{11}$ to increase phospholipase C activity, generating inositol phosphates and mobilizing

cytosolic [Ca^{2+}]. These receptors can also couple to $G_{12/13}$, known to mediate long term structural changes in the cell (Hannon & Hoyer, 2008).

5-HT3 receptor family has 5 members: 5-HT3A, 5-HT3B, 5-HT3C, 5-HT3D and 5-HT3E, although 5-HT3C, D and E have been reported as nucleotide sequences without any report confirming any direct roles of these subtypes. 5-HT3 receptors belong to the ligand-gated ion channel receptor superfamily, similar to the nicotinic acetylcholine, glycine or GABA-A receptors, and share electrophysiological properties and structural motifs with the Cys-loop transmitter-gated superfamily of ligand-gated ion channels (Hoyer, 1990). They are located on central and peripheral neurons, where they trigger rapid depolarization due to the opening of their non-selective cation channel (Na^+, Ca^{++} influx, K^+ efflux) (Hoyer et al., 1994; Humphrey, Hartig, & Hoyer, 1993).

$5\text{-}HT_4$, $5\text{-}HT_6$ and $5\text{-}HT_7$ receptors are considered as separate receptor classes because of their limited (<35%) overall sequence identities and 5-HT5 receptor is a class apart because its signaling remains unclear as does its function. $5\text{-}HT_4$, $5\text{-}HT_6$ and $5\text{-}HT_7$ receptors all couple preferentially to G_s and promote cAMP formation, by activation of various adenylyl cyclases. The intracellular messenger cAMP interacts with various targets, such as the phosphorylating enzyme protein kinase A (PKA), but also cyclic nucleotide-gated ion channels, leading to the modulation of calcium ion flux and membrane excitability, other cellular processes. PKA phosphorylates cAMP-responsive transcription factors, such as the cAMP response element binding protein (CREB), which leads to changes in gene expression, and thus may promote long term changes in cellular responses (Hannon & Hoyer, 2008).

1.4 Serotonin Receptor Signaling

In the classic paradigm of receptor activation and desensitization, GPCR function is mediated and modulated through two ubiquitous and conserved mechanisms: G-protein activation and β-arrestin function (DeWire, Ahn, Lefkowitz, & Shenoy, 2007; Gainetdinov, Premont, Bohn, Lefkowitz, & Caron, 2004). Agonist binding to the receptor stabilizes conformations that activate heterotrimeric G proteins and this leads to canonical second-messenger signaling. The GPCR is desensitized within minutes of agonist binding. This involves a general mechanism of agonist-induced phosphorylation of the intracellular domains of GPCRs followed by binding of arrestins to the intracellular loops and carboxy-terminal tails of agonist-activated GPCRs thereby targeting the receptor to intracellular compartments (DeWire et al., 2007; Ferguson, 2001). However, many recent studies suggest that this mechanism is probably more complex than initially thought. In the new emerging model, GPCR signaling is regulated by proteins that interact with the receptor within a cellular microenvironment (Urban et al., 2007; Violin & Lefkowitz, 2007) or by lipid rafts and adaptor proteins (Bjork, Sjogren, & Svenningsson, 2010). For example, 5-HT2A receptor seems to be regulated by scaffolding proteins and protein kinases (Allen, Yadav, & Roth, 2008).

1.5 Serotonin and Depression

Many studies have described the involvement of the serotonergic system in major depression. Although other systems seem also to be involved, such as noradrenargic, dopaminergic neurotransmitter systems, etc., their involvement seems to be indirect (Albert & Francois, 2010). Major depression appears to result from decreased

serotonergic neurotransmission (aan het Rot, Mathew, & Charney, 2009; Jans, Riedel, Markus, & Blokland, 2007; Millan, 2004; Tremblay & Blier, 2006; Wong, Perry, & Bymaster, 2005). The gravity of this disorder is demonstrated by the fact that depression results in suicide in 15% of patients suffering from severe depressive episodes (Mann, Brent, & Arango, 2001; Mann, 2005) and is twice as frequent in women as in men (Doris et al., 1999; Fava & Kendler, 2000). Currently, this disorder is difficult to treat because we do not have effective therapies and it is expected to increase from fourth to second (first in economically rich countries) highest global burden of diseases by 2030 (Albert & Francois, 2010; Mathers & Loncar, 2006; Ustun, Ayuso-Mateos, Chatterji, Mathers, & Murray, 2004). The serotonergic system plays a major role in many physiological and behavioral disorders. In addition to the major depression, the serotonergic system is also involved in anxiety, schizophrenia, mania, autism, obesity, drug addiction, migraine, hypertension, pulmonary hypertension, eating disorders, vomiting and irritable bowel syndrome (Hoyer et al., 2002; Filip & Bader, 2009). The serotonin system was linked to these disorders by the use of animal models and by evidence from living humans with these disorders. For example, knockout of the transcription factor pheochromocytoma 12 ETS (Pet-1), which is involved in the development of serotonergic system, results in aggression and anxiety phenotypes in mice (Hendricks et al., 2003). In humans, positron emission tomography (PET) studies in living subjects have provided a link between altered 5-HT1A receptor expression to panic disorders (Neumeister et al., 2004).

1.6 Antidepressant Treatments

The main strategy used for the treatment of depression is to increase the serotonin availability in the synaptic cleft. The first pharmacological therapies were introduced in the mid-1950s (Keller, 2003; Pacher & Kecskemeti, 2004). The way the monoamine oxidase inhibitors (MAOIs) increase serotonin is by blocking degradation of 5-HT by MAO. Examples of MAOIs include phenelzine (Nardil), isocarboxazid (Marplan) and tranylcypromine (Parnate). The major problem with this type of antidepressant is the associated side effects and toxicity such as dizziness, orthostatic hypothension, weight gain or sexual dysfunction. In addition, they are inconvenient because they require food restrictions (no high tyramine foods), multiple daily doses (Gumnick & Nemeroff, 2000) and are involved in serious drug interaction profiles (Keller, 2003).

The pharmacotherapy of depression advanced with the use of tricyclic antidepressants (TCA) (eg. Imipramine) in the mid-1950s (Pacher & Kecskemeti, 2004). The exact mechanism of action of TCAs is not well understood. They increase the monamine availability in the synaptic cleft by blocking serotonin, norepinephrine and more weakly dopamine reuptake. In contrast to MAOIs, TCAs are more convenient and less toxic, but they are not very selective and affect multiple neurotransmitter systems inside and outside the brain which have been associated with side effects such as drowsiness, dry mouth, urinary retention, constipation and they can even cause cardiac conduction delays and proarrythmic delays (Pacher, Kohegyi, Kecskemeti, & Furst, 2001; Peretti, Judge, & Hindmarch, 2000; Martinez & Marangell, 2004).

The problem of the unwanted side effects produced by TCAs was resolved by introduction of selective serotonine reuptake inhibitors (SSRIs) (eg. Citalopram,

Fluoxetine) in mid-1980s (Lieberman, Golden, Stroup, & McEvoy, 2000; Peretti et al., 2000). SSRIs act like TCA by blocking serotonin reuptake, except that they are specific to serotonin only and this is why they have reduced side effects. SSRIs block the SERT and consequently increase the availability of serotonin in the synaptic cleft to stimulate postsynaptic neurons (Lieberman, 2003). This is a simplistic view of the mechanism of action of SSRIs, since SSRIs become effective only after 2-3 weeks of treatment (Albert & Lemonde, 2004).

2- Serotonin 5-HT1A Receptors

2.1 Overview

The serotonin system plays an important role in psychoemotional, cognitive and motor functions in the CNS. Among all 5-HT receptor subtypes, the 5-HT1A receptor has been most strongly associated with pathogenesis and treatment of anxiety and depressive disorders. 5-HT1A agonists are well documented to be effective in the treatment of depressive disorders and anxiety. If used alone, 5-HT1A-specific ligands such as buspirone and ipsapirone are weak antidepressants. Nevertheless, when used in combination with SSRIs, pindolol can accelerate and potentiate their action by selectively targeting 5-HT1A autoreceptor (Artigas et al., 1996; Blier & Ward, 2003; Albert & Lemonde, 2004). Initially to determine 5-HT1A receptor expressing regions, tritium ([^{3}H]) labeled 5-HT1A ligands (8-OH-DPAT, WAY-100635, 5-HT) were used (Zifa et al., 1988; Laporte et al., 1994; Burnet et al., 1997) followed by the use of carbon-11 ([^{11}C]) labeled ligands in PET imaging which allows visualization of receptor distribution in living humans (Pike et al., 1995; Cselenyi et al., 2006). In recent studies, 5-HT1A

receptors have been also associated with various CNS disorders, including not only depressive disorders (e.g., delayed onset of action and refractory symptoms), but also schizophrenia (e.g., cognitive impairment and antipsychotic-induced extrapyramidal side effects) and Parkinson's disease (e.g., extrapyramidal motor symptoms and L-DOPA-induced dyskinesia) (Ohno, 2010).

2.2 Structure and Function

5-HT1A receptors are G-protein coupled receptors (GPCRs), they have seven transmembrane domains that couple to heterotrimeric guanine nucleotide binding regulatory proteins (G-proteins). 5-HT1A receptors signal via Gi/Go proteins to inhibit adenylyl cyclase (AC) and calcium channels, in addition to activating potassium channels to reduce neuronal firing and neurotransmitter secretion (Albert & Lemonde, 2004; Lanfumey & Hamon, 2004). The 5-HT1A receptor gene is located on human chromosome 5q11.1-q13 and encodes a protein for 422 amino acids in humans, mice and rat (Hannon & Hoyer, 2008; Lanfumey & Hamon, 2004). Since the identification of 5-HT1A as a distinct receptor in 1983 by ligand binding studies (Gozlan et al., 1983) and due to the anxiolytic and antidepressant properties of its ligands (Robinson et al., 1990), the 5-HT1A receptor has become one of the best characterized serotonin receptors (Barnes & Sharp, 1999).

In rodents, 5-HT1A receptors are involved in several physiological, behavioral, cognitive and developmental functions. Stimulation of 5-HT1A receptors facilitates the release of acetylcholine (ACh), noradrenaline (NA), corticotropin-releasing hormone (CRH), adenocorticotropic hormone (ACTH) and modulates cortisol blood levels (Fink

& Gothert, 2007; Jorgensen, 2007; Lanfumey & Hamon, 2004). In addition, the activation of 5-HT1A receptors can induce reduction in 5-HT and glutamate brain levels and decrease in growth hormone secretion (Fink & Gothert, 2007; Jorgensen, 2007; Roth, 2006) 5-HT1A receptors are also involved in motor behavior. For example, their activation evokes flat body posture, forepaw treading, tail flick, lower lip retraction and locomotor activation. They are also involved in pain perception and emotional behavior. 5-HT1A receptor activation leads to analgesia and induces anxiolysis (Kayser et al., 2007; Lanfumey & Hamon, 2004; Roth, 2006). Further evidence for the role of 5-HT1A receptors in regulation of emotions comes from studies of 5-HT1A knockout mice, in which the 5-HT1A knockout animals showed elevated anxiety-related behavior (Heisler et al., 1998; Parks, Robinson, Sibille, Shenk, & Toth, 1998; Ramboz et al., 1998) and an antidepressant-like phenotype (Heisler et al., 1998).

2.3 Distribution and Regulation

5-HT1A receptors are widely distributed in the CNS. They are principally located in the hippocampus, cingulate and enthorhinal cortices, lateral septum and mesencephalic raphe nucleus. 5-HT1A receptors function as autoreceptors in the raphe nuclei on the soma and dendrites of 5-HT neurons where they control cell firing. They are also a major postsynaptic receptor expressed in several limbic areas where they cause neuronal hyperpolarization due to the activation of G-protein coupled K+ channels. In the raphe nucleus, activated 5-HT1A autoreceptors activate potassium channels and inhibit voltage-dependent calcium currents (Lanfumey & Hamon, 2004). The neurotransmission of the neurons positive for 5-HT1A receptors is highly regulated by 5-HT1A receptor activity,

protein expression and localization. Prolonged activation of the receptors induces their desensitization and blocks the response to released neurotransmitter (Le Poul et al., 1995; Riad, Watkins, Doucet, Hamon, & Descarries, 2001). As a result, the receptors have to be either *de novo* transcribed and translated from the HTR1A gene (Albert & Lemonde, 2004; Albert & Francois, 2010; Czesak, Lemonde, Peterson, Rogaeva, & Albert, 2006; Meijer, Williamson, Dallman, & Pearce, 2000; Parks & Shenk, 1996; Wissink, Meijer, Pearce, van Der Burg, & van Der Saag, 2000; Wissink, van der Burg, Katzenellenbogen, & van der Saag, 2001) or resensitized by recycling back to the plasma membrane to continue signaling (Bhattacharyya, Puri, Miledi, & Panicker, 2002).

2.4 Serotonin Receptor 5-HT1A and Feedback Regulation

Many animal and clinical studies have convincingly associated the 5-HT1A receptor with depression and anxiety (Albert & Francois, 2010). For example, 5-HT1A-null mice have increased anxiety behavior (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998) and fail to respond to treatment with SSRIs (Santarelli et al., 2003). On the other hand, mice with a specific 30% increase in 5-HT1A autoreceptors in the raphe nuclei display reduced serotonergic activity but with no change in anxiety behavior (Richardson-Jones et al., 2010). These animal studies suggest that reduction in post-synaptic 5-HT1A receptors is involved in anxiety, while increase in pre-synaptic 5-HT1A autoreceptors is involved depression. Although the results of human studies of depression are conflicting, they do point towards the same mechanism in which increase in 5-HT1A autoreceptor levels are correlated with decreased serotonergic activity and with depression (Albert & Francois, 2010).

These findings are particularly interesting because they corroborate our early hypothesis in which we put forward evidence that an increase in 5-HT1A autoreceptors levels would be involved in depression and in attenuating the action of SSRIs (Albert, Lembo, Storring, Charest, & Saucier, 1996; Albert & Lemonde, 2004). The progression of changes in 5-HT1A autoreceptor levels induced by SSRI treatments is summarized in Figure I-5. Particularly interesting is that antidepressants act only after 2-3 weeks of delay, suggesting that adaptive changes in gene expression are necessary for the SSRI action. The delay in clinical response to SSRI can be attributed to feedback inhibition of serotonergic neurons by 5-HT1A autoreceptors. The feedback inhibition mechanism due to the 5-HT1A receptors present on serotonin neuron cell bodies and dendrites (autoreceptors), which can regulate serotonergic neuron firing by inhibiting raphe activity upon local release of 5-HT within the raphe nuclei (Albert, Lembo, Storring, Charest, & Saucier, 1996; Pineyro & Blier, 1999). Upon acute treatment with SSRIs, SERT is blocked which increases 5-HT levels in the synaptic cleft acting on post-synaptic neurons, the 5-HT excess acts on pre-synaptic 5-HT1A autoreceptors. They inhibit 5-HT neuron firing. However, after 2-3 weeks of delay, the clinical effect is observed, at a time when the activity and level of 5-HT1A autoreceptors is decreased. With the removal of inhibitory 5-HT1A feedback, neuronal firing is recovered and the excess 5-HT can act on post-synaptic neurons and leading to clinical improvement in depression.

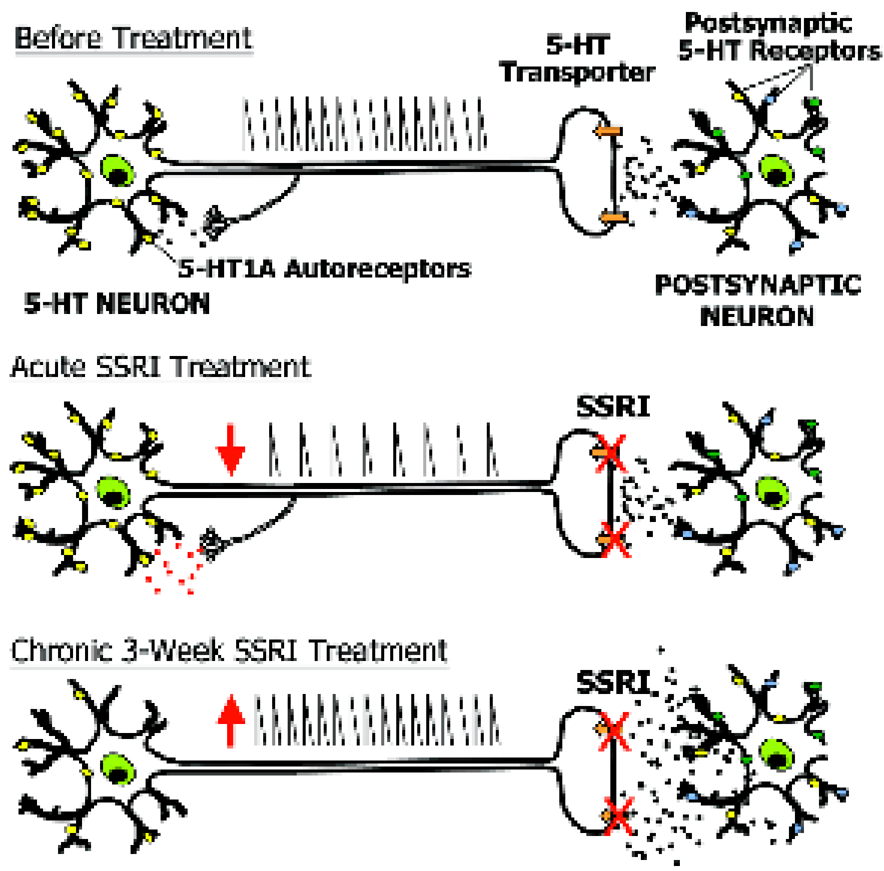

Figure I-5: Diagram representing the changes in serotonergic neurotransmission mediated by 5-HT1A autoreceptor after acute and chronic treatment with SSRIs. Before treatment, the activation of 5-HT1A autoreceptors by 5-HT released from axon collaterals or by diffusion from the synaptic cleft modulates serotonergic firing. Upon acute SSRI treatment, the SERT is blocked consequently increasing free 5-HT, as well as 5-HT1A autoreceptor inhibition. This results in decreased neuron firing. After chronic (3-week) SSRI treatment, the neuronal firing is increased presumably due to homologous desensitization of 5-HT1A receptors, which increases 5-HT release. As a result, the antidepressant effects are observed. Image is obtained with permission from Albert & Lemonde, 2004.

The adaptive changes induced by SSRIs are unlikely to only involve in 5-HT1A receptor desensitization because this mechanism is rapid and cannot account for a 2-3 week delay. A more plausible explanation is that the 5-HT1A receptors desensitization is insufficient and they are downregulated by the decrease in their expression (Albert & Lemonde, 2004). Since then, the search for transcriptional regulators of 5-HT1A autoreceptors has begun. Recent advances in our understanding in the genetic regulation of 5-HT1A receptors could make possible the selective targeting of 5-HT1A receptor gene expression in pre-synaptic versus post-synaptic neurons, which may lead to the improved antidepressant therapies (Albert & Francois, 2010).

3-Transcriptional Regulation of 5-HT1A Receptor Gene

3.1 Overview of Transcription

At the point of conception, each individual inherits parental DNA and this DNA is converted to proteins, which produce the corresponding characteristics of the individual. The central dogma of molecular biology is that DNA is transcribed to RNA, which is translated to proteins. Each step of this process is strictly regulated to ensure that a certain gene is transcribed only in the appropriate tissue. For example, neuronal genes are transcribed only in neuronal tissues and silenced in non-neuronal tissues (Latchman, 1998).

In eukaryotes, DNA is packaged into chromatin. The DNA is wound around a core complex of histones (H2A, H2B, H3 and H4), forming an octameric structure called the nucleosome (Figure I-6) (Luger, Mader, Richmond, Sargent & Richmond, 1997). The

DNA is locked in place by an additional histone (H1) around the nucleosome and this allows higher order structures to form. Inside the chromosome, the chromatin can be highly condensed into transcriptionally inactive (silenced) heterochromatin or decondensed, potentially transcriptionally active euchromatin (Elgin & Grewal, 2003; Venters & Pugh, 2009; Wegel & Shaw, 2005). Consequently, gene expression can be controlled by the chromatin structure. The nucleosomal arrangement can be altered by multi-protein complexes with enzymatic activities (Felsenfeld & Groudine, 2003; Kinyamu & Archer, 2004) At least two highly conserved chromosome-modifying enzymatic activities have been extensively studied and reported in the literature: the chromatin structure can be altered through disruption of histone-DNA contacts by ATP-dependent chromatin remodeling complexes or by posttranslational modifications of histone tails (Kouzarides, 2007; Trotter & Archer, 2007). Protruding from nucleosome of core and linker histone tails can be modified by covalent modifications such as acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, ADP-ribosylation, citrullination and N(epsilon)-lysine formylation (Fukuda, Sano, Muto, & Horikoshi, 2006; Houben et al., 2007; Ito, 2007; Sato, 2007; Selvi & Kundu, 2009; Shilatifard, 2006; Shukla, Chaurasia, & Bhaumik, 2009; Thompson & Fast, 2006; Weake & Workman, 2010; Wisniewski, Zougman, Kruger, & Mann, 2007; Wisniewski, Zougman, & Mann, 2008).

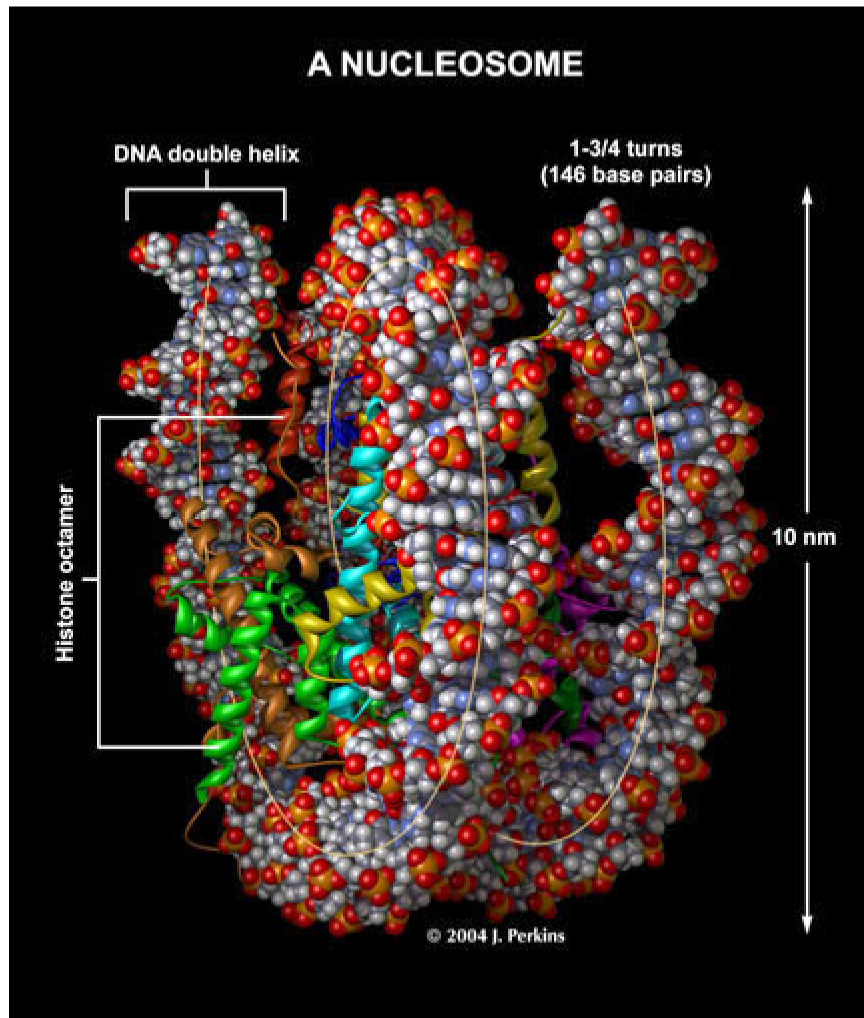

Figure I-6: Schematic representation of nucleosome. The nucleosome core particle contains two copies of each protein (H2A, H2B, H3 and H4) forming an octamer and 146 base pairs (bp) of superhelical DNA wrapped around the histone octamer. It is the principal structure that determines DNA accessibility. Image is obtained from http://bio.research.ucsc.edu/people/boeger/ResearchInterests.htm

In eukaryotes transcription is mediated by three classes of RNA polymerases such as RNA polymerase I (large rRNA), RNA polymerase II (mRNA) and RNA polymerase III (tRNA and other small RNAs) (Archambault & Friesen, 1993; Russell & Zomerdijk, 2006). All of the protein coding genes and some of the genes coding for small nuclear RNAs involved in splicing are transcribed by RNA polymerase II. RNA polymerase II is capable of unwinding the double stranded DNA template, synthesizing a RNA copy and rewind the DNA all by itself (Kornberg, 2007a; Kornberg, 2007b). However, RNA polymerase II is incapable of promoter recognition or transcription initiation and requires a number of other proteins. The initiation of transcription requires a multi-component complex containing RNA polymerase II and transcription factors (Brown, 1984). The general transcription factors such as TFIIA, B, D, E, F and H ensure accurate promoter targeting and initiation. They also assist with promoter melting and DNA unwinding (Naar, Lemon, & Tjian, 2001).

The TATA box is found in most eukaryotic genes, however it is absent in some genes. In a study characterizing the major promoter elements of over 1000 human genes, TATA boxes were identified in only 32% of promoters examined. In addition, the initiator motifs were identified in 85%, GC boxes in 97%, CAAT boxes in 64% and CpG islands in 48% of promoters examined (Suzuki et al., 2001; Suzuki, Tsunoda et al., 2001). The TATA box is an AT-rich sequence (consensus TATAA/TAA/T) found about 30 base pairs upstream of transcriptional start site (Latchman, 1998). It has been shown by mutagenesis or relocation of this sequence studies that it plays an essential role in accurately positioning the start site of transcription (Breathnach & Chambon, 1981).

In the absence of the TATA box, TATA box binding protein (TBP)-associated factor (TAF) complex is still required for promoter recognition. However, there are examples of TATA-less and initiator-less promoters. Since the refinement of our experimental techniques that enabled genome-wide discovery and characterization of core promoters, there is growing evidence that TATA-containing promoters with a static transcription initiation start are rather an exception than a rule. In fact, most genes have multiple promoters that have multiple start sites with many cis-regulatory elements (Sandelin et al., 2007). Gene transcription can be also influenced by sequences located distally from the core promoter that is essential for gene transcription. Enhancer or silencer elements can modulate the gene expression. Enhancers function by binding to activators and silencers function by binding to repressors (Latchman, 1998). This mechanism of gene modulation is particular for certain situations for example for tissue specific expression (Kadonaga, 2004; Naar et al., 2001). The transcriptional initiation is the first point in the control of gene expression, but every step between genomic DNA to protein can be regulated. For example transcriptional elongation (Sims, Belotserkovskaya, & Reinberg, 2004), the stages of RNA maturation and the transport of mature RNA to the cytoplasm (Latchman, 1998) and the other steps can be regulated.

3.2 Transcriptional Activation and Repression

Almost 50 years ago, Jacob and Monod suggested that only repressors regulate genes. In this paradigm, the genes are constitutively active unless repressed by a specific repressor (Jacob & Monod, 1961). However, it has been shown that in the absence of its repressor, a prokaryote gene is still expressed, but only at the basal level unless

stimulated by an activator (Beckwith, 1996). In the last two decades, many eukaryotic transcriptional activator proteins were described and they function following ‘recruitment’ model in which transcriptional activators, by virtue of protein-protein interaction (Mapp, Ansari, Ptashne, & Dervan, 2000; Ptashne & Gann, 1990; Ptashne & Gann, 1997; Ptashne, 2005; Venters & Pugh, 2009; Wang, Muratani, Tansey, & Ptashne, 2010; Zaman et al., 1998; Zaman, Ansari, Gaudreau, Nevado, & Ptashne, 1998) recruit the transcription machinery to DNA. In eukaryotes, an activator must recruit to the gene the large multi-protein complexes composed of at least 50 proteins. Some of these complexes modify histones and increase affinity for other complexes that can recruit to the gene the complexes that remove nucleosomes. The resulting multi-complex interacts with and attracts polymerases and promotes elongation of transcription resulting in the expression of a specific gene (Mapp, Ansari, Ptashne, & Dervan, 2000; Ptashne & Gann, 1990; Ptashne & Gann, 1997; Ptashne, 2005; Venters & Pugh, 2009; Wang, Muratani, Tansey, & Ptashne, 2010; Zaman et al., 1998; Zaman, Ansari, Gaudreau, Nevado, & Ptashne, 1998).

In eukaryotes, repression often involves recruitment, just as in activation, of the transcription machinery. Consequently, the specific repressor recruits repressing components to specific genes. These transcriptional machineries can recruit histone- and DNA-modifying enzymes that change chromatin structure and therefore repress the gene expression. There are two types of transcriptional repressors: passive or active (Cowell, 1994). The passive repressors lack intrinsic repressing activity and a repression domain. They function by competing with transcriptional activators for DNA binding, by forming inactive heterodimers with transcriptional activators making them incapable of

interaction with DNA, or by binding to coactivators required for the transcriptional activator proteins. For example, inducible cAMP early repressor (ICER) represses transcription mediated by the cAMP response element via blocking of the DNA-binding site using its basic region. ICER can also induce formation of nonfunctional heterodimers with wild-type basic region leucine zipper proteins. In the nervous system, ICER protects neurons against cytotoxic challenges by counteracting cAMP response element binding protein (CREB) (Jaworski et al., 2003). Moreover, the SP1-like zinc finger proteins compete for a common GC-rich DNA binding site. The decision between which discrete sets of genes are turned on or off is made by the balance between SP1, a transcriptional activator, and SP1-like transcriptional repressor (Kaczynski et al., 2001). Additional example of passive repressor is IkB which function by sequestering the NFkB transcription factor complex in the cytosol (Karin, 1999).

In contrast, active mammalian transcriptional repressor proteins have an intrinsic repression activity that targets the chromatin organization of the genome and is activator-independent. This type of repression can function over long distances of DNA sequence and two types of active transcriptional repression can be distinguished: transcriptional repression via chromatin remodeling and histone deacetylation and gene silencing via histone methylation and heterochromatin formation (Thiel, Lietz & Hohl, 2004).

3.3 Chromatin Remodeling

ATP-dependent enzymatic remodeling complexes can remodel chromatin architecture. They use energy derived from ATP hydrolysis to actively alter the nucleosomal structure (Johnson, Adkins, & Georgel, 2005). The open chromatin structure

is usually associated with transcriptional activation and the closed structure of chromatin is associated with transcriptional repression (Figure I-7). A number of chromatin ATP-dependent remodeling complexes have been identified in the literature that modulate the arrangement and stability of nucleosomes in a non-covalent manner. They can be divided into four major subfamilies mainly characterized by their central catalytic subuinit: Brg1 (or hBrm) from Swi/Snf complex, ISWI from ISWI complex, Mi-2 from NuRD complex and Ino80 from INO80 complex (Clapier & Cairns, 2009; Eberharter & Becker, 2004; Sif, 2004). However, the focus of this thesis will be on Swi/Snf complex that contains Brg1 as the central ATPase subunit.

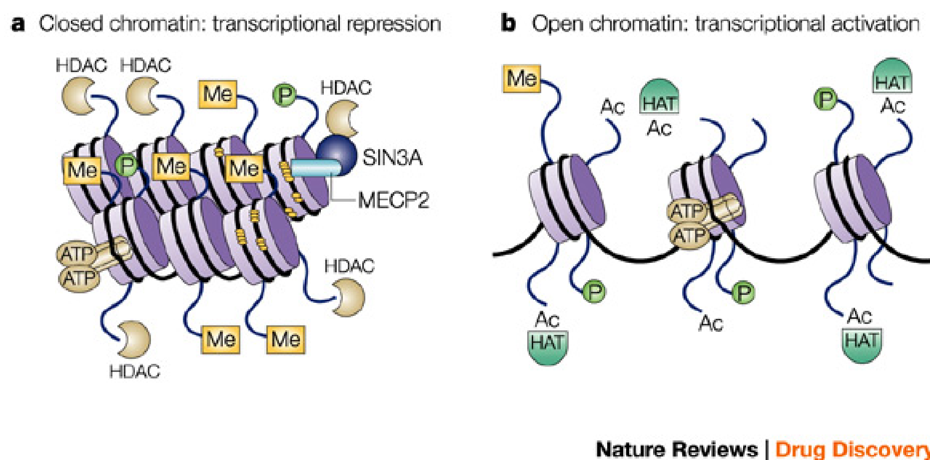

Figure I-7: Diagram of chromatin structure. The chromatin structure can be remodeled by chromatin remodeling complexes and coregulators. The chromatin structure modified by chromatin remodeling complexes and corepressors such as Sin3A/HDACs, MeCP2 leads to the closed chromatin structure and transcriptional repression. When the chromatin structure is modified by chromatin remodeling complexes and coactivators with HATs it results in opened chromatin structure and transcriptional activation. Image is obtained with permission from Johnstone, 2002.

Although Brg1 and hBRM (Brahma) subunits share a high degree of sequence identity (74%) and display similar biochemical activities in vitro, they can play different roles in various cellular processes including proliferation and differentiation (Khavari, Peterson, Tamkun, Mendel, & Crabtree, 1993; Phelan, Sif, Narlikar, & Kingston, 1999; Randazzo, Khavari, Crabtree, Tamkun, & Rossant, 1994). The Brg1 or hBrm protein is the central catalytic ATPase of the Swi/Snf chromatin remodeling complex. The Swi2 (Brg1 homologue) was first identified in yeast by genetic screens searching for proteins important for mating-type switching (Swi) and sucrose non-fermenting (Snf) (Neigeborn & Carlson, 1984; Stern, Jensen, & Herskowitz, 1984; Winston & Carlson, 1992). Later, the suppressors of these Swi and Snf mutations were identified which included genes encoding histones and other chromatin-associated proteins. This suggested that they might be involved in transcriptional regulation through chromatin remodeling (Sif, 2004; Winston & Carlson, 1992). Homologues of yeast Swi2/Snf2 have been identified in humans, *Drosophila* and mice (Dingwall et al., 1995; Khavari et al., 1993; Kwon, Imbalzano, Khavari, Kingston, & Green, 1994; Laurent, Treich, & Carlson, 1993; Muchardt & Yaniv, 1993; Randazzo et al., 1994; Tamkun et al., 1992; Wang et al., 1996; Wang, Xue et al., 1996).

The Swi/Snf enzymatic complexes are large multimeric complexes, which are thought to be recruited to specific gene targets through association with DNA-binding transcription factors and coregulators. They contain a multitude of distinct DNA-binding motifs, which have role in targeting Swi/Snf activity to gene-specific promoter. It has been proposed that these DNA-binding motifs do not direct the complex to specific gene

sequences, but rather work in concert with gene-specific activation domains within transcription factors or histone-binding factors.

Human Swi/Snf complexes contain one of two mutually exclusive ATPase-containing subunits (Brg1 or hBrm) and approximately 10-12 BAF (Brg1-associated factor) subunits or other proteins involved in gene regulation and nucleosome assembly, genomic stability and maintenance. The most purified complexes contain core subunits Brg1 (or hBrm), BAF170, BAF155, BAF47/Ini1, BAF60, BAF57, BAF53 and actin. Human Swi/Snf complex can be further subdivided into BAF and PBAF (Polybromo-associated BAF) complexes because they can be distinguished by the presence of specific subunits. For example, the BAF complex has BAF250 a/b and BAF180, BAF200 proteins, which can be found exclusively in PBAF complex. WINAC complex (WSTF including nucleosome assembly complex) consists of at least 13 components (BAF 250, 170, 155, 60a, 57, 53, 47 and WSTF (Williams syndrome transcription factor)). This complex also includes proteins associated with DNA replication such as TopoIIb and CAF-1p150 and transcription elongation factor FACTp140 that are exclusive to the WINAC complex. The histone-modifying enzyme, coactivator-associated arginine methyltransferase-1 (CARM1) is present only in the NUMAC complex with other BAFs. NcoR (Nuclear receptor corepressors-1) complex has only a few BAF members, such as BAF170, BAF155 and BAF47, in addition to its exclusive members HDAC3 and KAP-1 (Krab associated protein 1). The Sin3A/HDAC complex is composed of Sin3A, HDAC1, HDAC2 and PRMT5 in addition to BAFs. Each of these complexes, summarized in Figure I-8, has important cellular function by modifying chromatin structure.

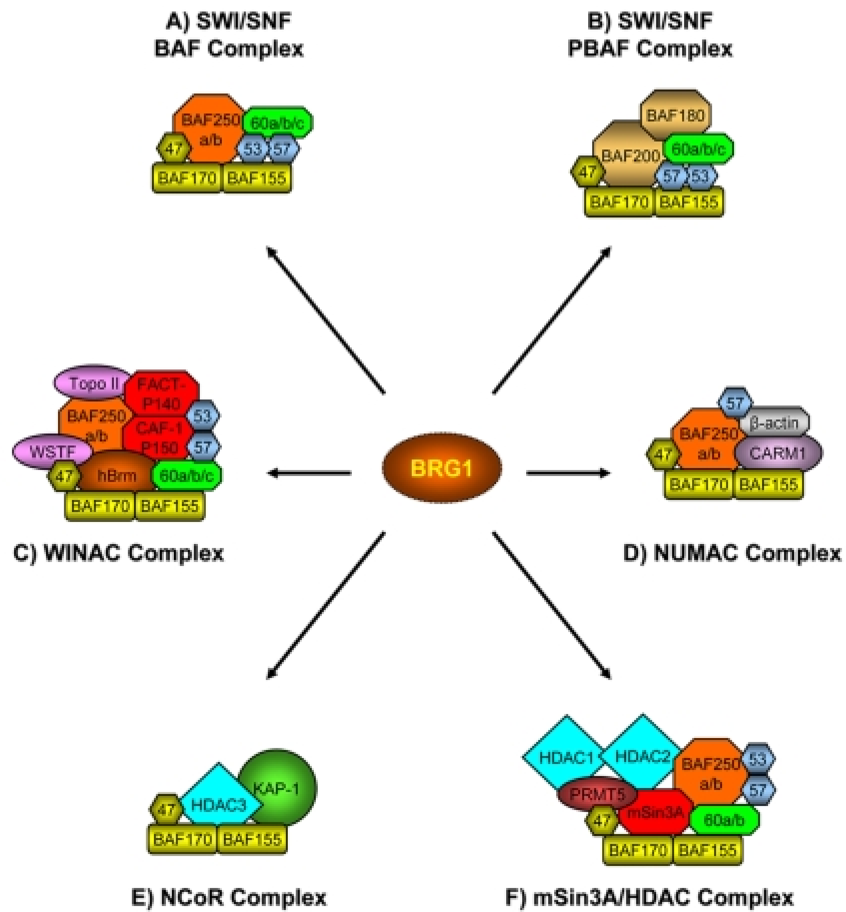

Figure I-8: Brg1 containing complexes. Depending on their composition, Swi/Snf complexes can be divided into 6 different complexes: A) BAF, B) PBAF, C) WINAC, D) NUMAC, E) NcoR and F) Sin3A/HDAC (Trotter & Archer, 2008).

If we look at individual components of the Swi/Snf complex, their roles in chromatin remodeling and gene regulation are not completely understood. However, increasing evidence points towards a mechanism in which combinatorial assembly causes sophistication and specificity of their functions. For example, in neural development, exchanges of subunits accompany the transitions from pluripotent to multipotent stem cells and post-mitotic neurons (Ho et al., 2009; Ho, Ronan et al., 2009; Yoo & Crabtree, 2009). In human, mouse or rat, BAF57 undergoes neuron-specific slicing of exons II, III and IV leads to at least 3 isoforms with truncated N-terminal mainly expressed in the nervous system (Kazantseva et al., 2009). BAF57 is a key subunit that has been documented to mediate the interaction between the remodeling complex and transcription factors. Its expression is limited by BAF155/BAF170 subunits (Chen & Archer, 2005). However, Hah and colleagues have shown that BAF57 has important role in maintaining the proper subunit composition of the complex, in addition to its roles in cell cycle progression through transcriptional regulation of a subset of cell cycle-related genes (Hah et al., 2010). BAF57 is not an essential subunit: for example, it is not required for CD4 chromatin remodeling (Wan et al., 2009). Moreover, the recruitment of BAF57 to the promoter doesn't guarantee alteration of gene transcription. In macrophages, BAF57 has been shown to be regulated by Ca2+/calmodulin, which regulates gene transcription without affecting complex recruitment (Lai et al., 2009). It is still unclear which subunits are required for activation or recruitment of the complex to which promoters. In vitro, it has been shown that only two subunits, Brg1 and BAF155 are sufficient for activation of the β-globin promoter (Kadam et al., 2000). BAF155 is likely important for the protection of the complex from proteasomal degradation and might affect nuclear

localization of the complex, similar to murine SRG3 (murine homolog of BAF155) (Sohn et al., 2007). Hence, the assembly of individual units and their isoforms of Swi/Snf complex is tailored for specific actions of the complex depending on the context it is in.

The most characterized BAF subunit is Brg1. It has been shown to be equally important for transcriptional activation and transcriptional repression (Trotter & Archer, 2008). Mainly, Brg1 has been described in transcriptional activation, however when Brg1 associates with Rb proteins (Zhang et al., 2000) or Sin3A/HDAC complex (Sif, Saurin, Imbalzano, & Kingston, 2001) or NcoR corepressor complex (Underhill, Qutob, Yee, & Torchia, 2000) or other proteins, Brg1 is involved in transcriptional repression (Trotter & Archer, 2008). Brg1 can inhibit promoters either in HDAC-dependent manner in the association with REST (Ooi & Wood, 2007) or HDAC–independent manner in the repression of nuclear hormone receptors such as GR (Inayoshi et al., 2005). In addition, Brg1 can be both coactivator and corepressor at the same promoter. The modes can be switched to coactivator or corepressor mode when the same estrogen receptor (ER)-responsive promoters recruit Brg1 by different cofactors in response to ligand or antagonist that will either activate or repress gene transcription respectively. For example, when Brg1 is recruited by HDAC1, p300, and prohibitin in response to antagonist, the promoter activation decreases, in contrast to the recruitment of Brg1 in response to agonist, where gene transcription is activated (Zhang, Chambers, Faller, & Wang, 2007). Thus, BRG1 mediates both transcriptional activation as well as repression, and can switch between co-activator and co-repressor function.

3.4 Histone Modifications: Acetylation, Deacetylation and Methylation

Histones can be acetylated by histone acetyltransferases (HATs) and deacetylated by histone deacetylases (HDACs) (Fukuda et al., 2006). Histone acetylation involves the transfer of an acetyl group from acetyl coenzyme A to the ε-amino group of lysine residue. This modification reduces the net positive charges of the core histones, leading to a decrease in their binding affinity to DNA. The subsequent displacement of the termini from the nucleosome unfolds it and this provides the access for transcription factors. Histone acetylation is associated with transcriptional activation (Wade, Pruss, & Wolffe, 1997). In contrast, histone deacetylation by HDACs removes the acetyl group from the ε-amino group of lysine residues of histones, allowing ionic interactions between negatively charged DNA phosphate backbone and the positively charged amino termini of the core histones and resulting in a more compact chromatin structure. The resulting chromatin structure is not easily accessible for the transcriptional machinery and associated with transcriptional repression.

An additional histone modification associated with transcriptional repression is DNA methylation, which occurs at the C-5 position of cytosine nucleotides within the dinucleotide $C^{m}pG$. It is a heritable modification that is catalyzed by DNA methyltransferases (DNMTs) (Shilatifard, 2006).

Thus, depending on the modification, histones are either associated with compact chromatin structure (eg. by acetylation, methylation), or released from it (deacteylation, demethylation).

3.5 Transcriptional Repressors: REST, pRb and MeCP2

3.5.1 REST

Recent studies have demonstrated that a single transcription factor can recruit multiple chromatin-modifying enzymatic complexes, uncovering interdependencies among individual enzymes that affect gene regulation. Repressor element-1 silencing transcription factor (REST), also known as neuron restrictive silencer element (NRSE), was identified in 1995 as a protein that binds to repressor element-1 (RE-1) sequences that are present in the rat *Scn2a2* (also known as *Nav1.2*) and *Stmn2* (also known as *SCG10*) genes (Chong et al., 1995; Schoenherr & Anderson, 1995). REST is a zinc finger protein that is mainly expressed in non-neuronal cells. It binds to the 21bp RE-1 site through eight C_2H_2 zinc fingers (Chong et al., 1995; Schoenherr & Anderson, 1995). Although mice lacking REST appear normal until embryonic day 9.5, they die by embryonic day 11.5 from widespread apoptotic death, which results in malformations in the developing nervous system and restricted growth, highlighting the importance of REST in development of nervous system. In addition, several neuronal genes were de-repressed in non-neuronal tissues and in neural progenitors suggesting that REST has important role in determining neuronal phenotype (Chen et al., 1998). Initially, REST was thought to be a master regulator of neuronal genes and is still known as a transcriptional repressor of neuronal genes in non-neuronal tissues, but growing evidence shows that the role of REST is complex and tissue dependent (Ooi & Wood, 2007; Ooi & Wood, 2008).

REST mediates gene repression through the recruitment of two separate corepressor complexes: Sin3A and CoREST (Andres et al., 1999; Grimes et al., 2000; Huang, Myers, & Dingledine, 1999; Naruse, Aoki, Kojima, & Mori, 1999; Roopra et al., 2000) in addition to other corepressors such as the histone H3 lysine 9 (H_3K_9) methylase, G9a (Tachibana, Sugimoto, Fukushima, & Shinkai, 2001) and the NADH-sensitive corepressor, CtBP (Zhang, Piston, & Goodman, 2002). The last ones can be recruited directly by REST (Garriga-Canut et al., 2006; Roopra et al., 2000). The G9a and CtBP also have been found associated with the CoREST complex (Lee, Wynder, Cooch, & Shiekhattar, 2005; Shi et al., 2003; Shi et al., 2005; Zhao, Subramanian, & Chinnadurai, 2006). The Sin3A complex contains two class I HDACs (HDAC1 and HDAC2), two class II HDACs (HDAC4 and HDAC5) and the retinoblastoma-associated proteins RbAP48 (also known as RBBP4) and RbAP46 (also known as RBBP7) that are thought to interact with histones in addition to several other proteins with unknown function (Alland et al., 2002; Fleischer, Yun, & Ayer, 2003; Zhang et al., 1998). The association between Sin3A and HDAC4 with HDAC5 is lost when the HDACs are phosphorylated by calcium/calmodulin-dependent protein kinase (CAMK) (Nakagawa et al., 2006). On the other hand, CoREST complex also contains HDAC1 and HDAC2 (Andres et al., 1999; You, Tong, Grozinger, & Schreiber, 2001) in addition to a histone H_3K_4 demethylase, LSD1 (Shi et al, 2004), the component of Swi/Snf complex Brg1 (also known as SMARCA4) (Battaglioli et al., 2002) as well as CtBP (Garriga-Canut et al., 2006; Zhang et al., 2002) and methyl-CpG-binding protein, MeCP2 (Ballas, Grunseich, Lu, Speh, & Mandel, 2005; Lunyak et al., 2002).

It is still not fully understood how REST is mediating gene repression, but recent studies are suggesting the emerging mechanism summarized in Figure I-9. The initial step in the transcriptional regulation of genes is the recruitment of REST to RE-1 sites. The zinc finger domain recognizes the RE-1 and this interaction between REST and DNA is stabilized by the ATP-dependent chromatin-remodelling enzyme, Brg1.Then, the N-terminus of REST interacts with the Sin3 complex and the C-terminus interacts with the CoREST complex. CtBP is recruited only in the presence of a low level of NADH and dissociates from the REST complex in the presence of high levels of NADH. Once the association between REST and DNA is stabilized, HDACs are recruited by Sin3 or CoREST and they remove acetyl groups from H3 and H4 lysine residues. The deacetylation of H_3K_9 stimulates LSD1 activity, which consequently removes di- and monomethylation marks from H_3K_4 in addition to providing substrate for G9a-mediated methylation (Ooi & Wood, 2007). This results in the formation of an altered chromatin landscape. Methylated H_3K_9 can recruit the heterochromatic protein HP1 through its chromodomain and this will result in complete and long-term silencing of genes (Figure I-10) (Ooi & Wood, 2007; Thiel, Lietz & Hohl, 2004).

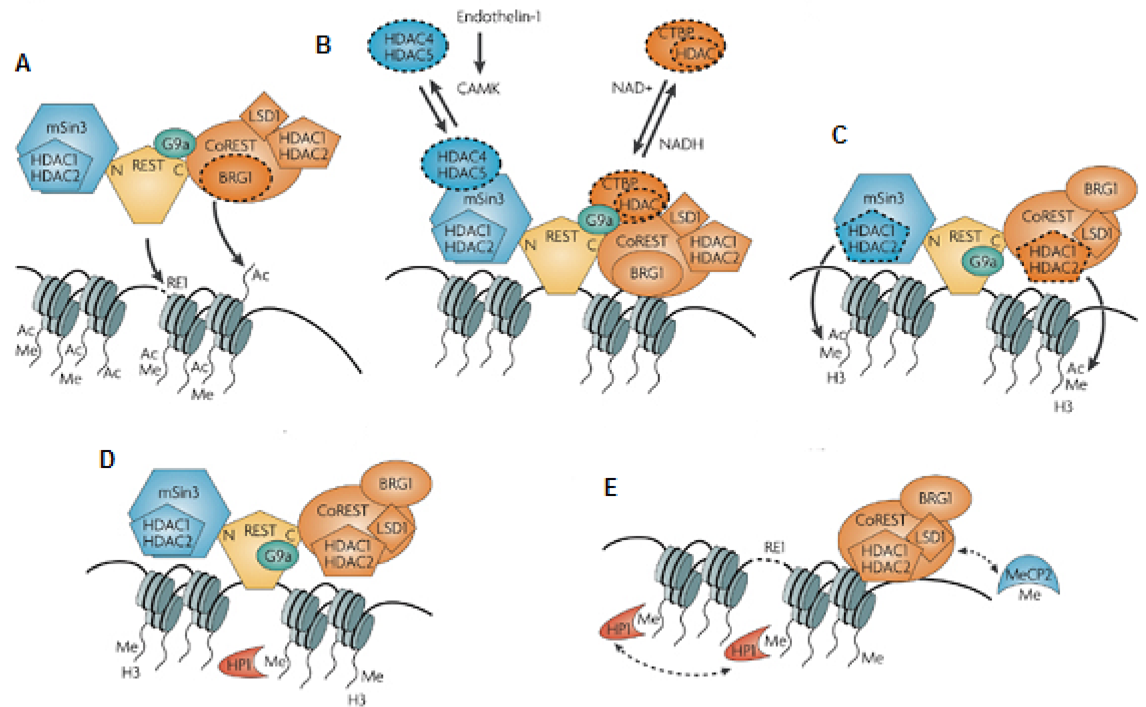

Figure I-9: Transcriptional Regulation of neuronal genes in non-neuronal tissues by REST and its corepressor complex. (**A**) REST recognizes RE-1 sites and its binding is stabilized by Brg1. The N-terminus interacts with Sin3A complex while the C-terminus interacts with CoREST complex. (**B**) These large complexes bring HDACs and other proteins to the region. (**C**) HDACs deacetylate the histones and demethylases demethylate them. (**D**) This brings HP1 and MeCP2 to the region (**E**) resulting in very compact chromatin structure and gene silencing. Thus, REST is capable to induce gene silencing to the adjacent regions by recruiting specific corepressor complexes. Image is adapted with permission from Ooi & Wood, 2007.

REST can mediate short-term transient repression and long-term gene silencing and modulate transcription rates. Although REST can recruit multiple complexes, the complexes recruited can be different in different cell types. For example, in Rat-1 fibroblasts and mouse neural stem cells, REST recruits Sin3 and CoREST to the *Snc2a2* promoter, however it recruits only Sin3 to the *Stmn2* promoter (Greenway, Street, Jeffries, & Buckley, 2007; Jepsen et al., 2000; Lunyak et al., 2002). This differential recruitment of one complex and not the other will produce a potential difference in the chromatin structure that might underlie the observation that REST binding can mediate both short-term repression and long-term gene silencing. Moreover, in the absence of REST, its corepressor complexes can maintain gene repression. For example, during neural stem-cell differentiation, REST binding is lost from RE-1 sites, but CoREST complex remains bound and continues to mediate the repression of associated genes facilitated by DNA methylation and MeCP2 recruitment (Ballas et al., 2005). This suggests that long-term gene repression can be maintained after only transient repressor occupancy by initiating stable corepressor-chromatin interactions. However, it is still not clear how long this type of repression can persist (Ooi & Wood, 2007). Finally, REST can also modulate transcription rates by binding to the TATA-binding protein (TBP) that results in the inhibition of the preinitiation complex (Murai, Naruse, Shaul, Agata, & Mori, 2004). Its interaction with RNA polymerase II small CTD phosphatases (SCPs) inhibits the activity of polymerase (Yeo et al., 2005). Consequently, REST can induce the gene silencing to the adjacent regions or transient repression depending on the transcriptional corepressors it recruits and thus REST can regulate transcription rates.

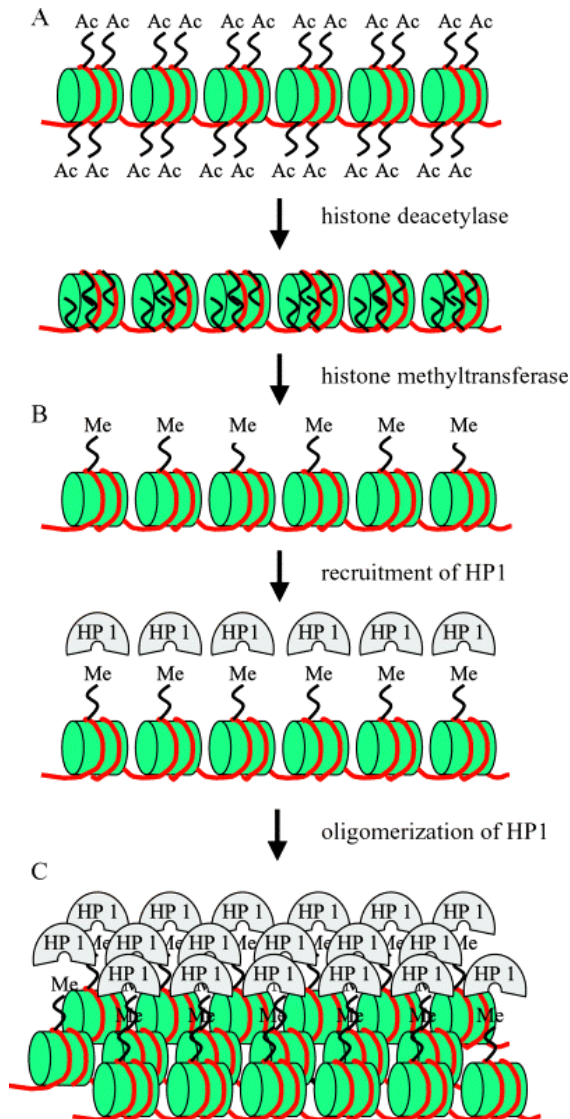

Figure I-10: Gene Silencing by sequential formation of facultative heterochromatin. (**A**) Deacetylation of histones is induced by the recruitment of HDACs by transcriptional repressors such as REST. Once histone tails are deacetylated they become substrates for histone methyltransferases (eg. SUV39H1), which methylates Lys9 of histone H3. (**B**) The methylated Lys9 of histone H3 becomes a binding site for HP1. (**C**) Recruitment of HP1 protein results in their oligomerization and dimerization. Consequently, chromatin structure forms a compact facultative heterochromatin. Image is taken with permission from Thiel, Lietz & Hohl, 2004.

3.5.2 pRb

The retinoblastoma protein (pRb) belongs to a family of proteins (pRb, p107 and p130) that repress transcription of E2F-regulated genes (Flemington, Speck, & Kaelin, 1993; Frolov et al., 2001; Frolov & Dyson, 2004; Helin & Ed, 1993). The E2F transcription factor mediates the temporal expression of a large set of genes that are needed for cell proliferation (Bracken, Ciro, Cocito, & Helin, 2004; DeGregori & Johnson, 2006; Farnham, Slansky, & Kollmar, 1993; van Ginkel, Hsiao, Schjerven, & Farnham, 1997). Consequently, if not stopped by pRb, E2F mediated transcription may lead to the formation of cancer. In fact, pRb is one of the most studied tumor suppressor genes, which is thought to be absent or misregulated in over 90% of human cancers (Hanahan & Weinberg, 2000; Sherr & McCormick, 2002).

The pRb family members have distinct binding sites for E2F proteins and chromatin modifying enzymes that enables them to recruit regulatory complexes to E2F-regulated promoters (Lee, Russo, & Pavletich, 1998; Rubin, Gall, Zheng, & Pavletich, 2005). They have been reported to interact with over 100 different proteins, but most of their interacting proteins are chromatin-associated proteins with roles in transcriptional regulation and chromatin modifications. Through these many different interactions, pRb family of proteins appear to coordinate extensive programs of gene expression that are important for cells to either proliferate or to differentiate. In addition, activation or inactivation of pRb can also have general consequences on chromatin condensation associated events such as mitosis, senescence and the formation and/or redistribution of heterochromatin. Consequently, pRb members might not simply act at the level of

individual promoter, but they may act more broadly to control local organization of chromatin structure (Longworth & Dyson, 2010).

The transcriptional mechanisms by which pRb represses E2F are not completely understood. However, it seems that pRb is able to mask the transactivation domain of E2F (passive repression) and it recruits the HDACs, Sin3A, Swi/Snf chromatin remodelers, histone methyltransferases (HMTases) and histone demethylases (active repression) (Brehm et al., 1998; Dunaief et al., 1994; Luo, Postigo, & Dean, 1998; Magnaghi-Jaulin et al., 1998; Nielsen et al., 2001; Singh, Coe, & Hong, 1995). Subsequently, the transcription is repressed via histone deacetylation by HDACs and chromatin remodeling by Swi/Snf. The HMTase Suv39H1 methylates histone H_3K_9 and creates binding site for the methyl lysine-binding protein HP1, providing a chromatin mark that is linked to transcriptional repression and that is able to potentially affect chromatin configuration over a much larger region (Bannister et al., 2001; Lachner, O'Carroll, Rea, Mechtler, & Jenuwein, 2001; Longworth & Dyson, 2010; Nielsen et al., 2001; Rea et al., 2000). Thus, pRb like REST can induce gene silencing to adjacent regions.

3.5.3 MeCP2

In vertebrates, DNA is subject to methylation of the C-5 position of cytosine nucleotide within the dinucleotide CpG. DNA methylation is commonly associated with transcriptional repression, because it affects gene expression by interfering with the binding of sequence-specific transcription factors. In addition, it may also influence chromatin structure directly by transforming the methylated DNA sequences into a

condensed state. Methylated DNA interacts with methylation-specific transcriptional repressor proteins. One of the most prominent proteins is methylcytosine-binding protein, MeCP2. It binds to single methyl-CpG base pair regardless of the sequence context via its methyl-CpG-binding domain (Nan, Meehan, & Bird, 1993). MeCP2 represses transcription by recruiting HDACs to methylated DNA regions (Jones et al., 1998; Nan et al., 1998). This results in denser packing of the chromatin. MeCP2 is thought to serve as a global transcriptional silencer (Jones et al., 1998; Nan, Cross, & Bird, 1998).

In addition to binding methylated DNA, MeCP2 associates with various co-repressor complexes such as Sin3A, NCoR, and c-Ski at the sites of its occupancy (Jones et al., 1998; Kokura et al., 2001). MeCP2 has been identified to be transcriptional repressor of the brain-derived neurotrophic factor (BDNF) promoter III. In mouse and rat, upon membrane depolarization and calcium influx in postmitotic neurons, MeCP2 is phosphorylated by CaMKII and dissociates from its binding site in the BDNF promoter (Chen et al., 2003; Martinowich et al., 2003; Zhou et al., 2006). However, treatment with HDAC inhibitor, trichostatin A (TCA) was able only to partially relieve MeCP2-mediated transcriptional repression, suggesting that MeCP2 may have a second mechanism to repress transcription aside from the recruitment of HDACs (Thiel, Lietz & Hohl, 2004).

3.6 Corepressors: Sin3A and CoREST

3.6.1 Sin3A

The Sin3A proteins are associated with HDACs and HDAC activity is required for their action (Alland et al, 1997; Hassig et al, 1997; Heinzel et al, 1997; Kadosh and Struhl, 1997; Laherty et al, 1997; Nagy et al, 1997; Zhang et al, 1997). They act as transcriptional corepressors in association with many transcriptional repressors such as MeCP2 (Nan et al, 1998), REST (Grimes et al, 1999), and others. Sin3 proteins have four imperfect repeats of a paired amphipathic helix (PAH) motif, which mediate protein-protein interactions (Wang et al, 1990). In vivo, Sin3A is associated with large multiprotein complex containing HDAC1, HDAC2, RbAP46/48, SAP180, SAP130, SAP45/mSDS3, SAP30, SAP18, SAP25 and other yet uncharacterized proteins (Alland et al, 2002; Fleischer et al, 2003; Hassig et al, 18; Laherty et al, 1998; Zhang et al, 1997; Zhang et al, 1998; Shiio et al, 2006).

Sin3A complexes contain different components in sub-stoichiometric quantities, however the exact mechanism regulating complex formation is still unclear (Kuzmichev et al, 2002; Sif et al, 2001; Xue et al, 1998). It has been shown that SDS3 and SAP30 function as 'bridge' between the Sin3A complex and other protein complexes. Recent genetic studies in mice have demonstrated that Sin3A is essential gene involved in cell fate determination (Cowley et al, 2005; Dannenberg et al, 2005). Nevertheless, the association of Sin3A with many transcriptional repressors required for diverse cellular processes suggests that Sin3A might also be important in regulating 5-HT1A receptor expression by repressors such as Freud-1 and REST.

3.6.2 CoREST

The transcriptional co-repressor for repressor element-1-silencing transcription factor (CoREST) has been identified in mammals and was shown to play an important role in regulating neuronal gene expression and neuronal stem cell fate. Initially, CoREST (also known as REST corepressor1 [RCOR1]) was identified as a cofactor of the REST transcriptional repressor. Although, the CoREST complex is best characterized for its role in regulating REST target gene expression, it is recruited by additional transcription factors and probably has additional roles (Lakowski, Roelens, & Jacob, 2006).

The CoREST protein has two distinct repressor domains that appear to act cooperatively: an N-terminal domain between amino acids 102 and 195 (the ELM2 and SANT1 domains), and a C-terminal domain spanning amino acids 321-442, including SANT2 domain (Ballas et al., 2001). The ELM2 (EGL-27 and MTA1 homology 2) domain is a small domain of unknown function that might be important for DNA binding or protein-protein interactions. The SANT (SWI3/ADA2/NcoR/TFIIIB) domain is a conserved motif similar to Myb DNA-binding domains have been implicated in binding to histone H3 tails and helping to present these tails to associated enzymes that can modify them (Boyer, Latek, & Peterson, 2004). The CoREST protein is part of a corepressor complex with six proteins: REST, HDAC1, HDAC2, LSD1 and BRAF35 (also known as HMG20B) and BHC80 (also known as PHF21A). BRAF35 and BHC80 have no predicted enzymatic activity and their exact role in the complex is presently unclear (Lakowski et al., 2006).

CoREST interacts with REST through its ELM2 domain (Andres et al., 1999; Ballas et al., 2001). The interaction between CoREST and HDAC1 is mediated through the ELM2 and SANT1 domains (You et al., 2001). In addition, LSD1 binds to CoREST through a domain that maps between the two SANT domains, but the presence of SANT2 domain is required for CoREST stimulation of LSD1 activity (Shi et al., 2005). The CoREST protein seems to function by recruiting HDACs at the RE1 sites of REST protein (Belyaev et al., 2004; Lunyak et al., 2002). Moreover, the presence of CoREST also has been associated with the recruitment of MeCP2 to the NaChI/Nav1.2 locus in the rat. This links histone modifications with DNA methylation and the spreading of repressed chromatin away from the sites of REST binding to repress adjacent genes (Lunyak et al., 2002). In addition, the CoREST complex has been shown to associate with components of the Swi/Snf complex (Battaglioli et al., 2002; Watanabe et al., 2006). Finally, CoREST has been shown to be present in a dynamic complex including both histone acetylases and deacetylases (Yamagoe et al., 2003). Currently, it is unclear when CoREST associates with additional factors and when it acts alone (Lakowski et al., 2006). In summary, REST is a strong repressor that recruits multiple proteins to induce a spreading of closed chromatin conformation and coordinately repress groups of adjacent genes.

3.7 Serotonin Receptor 5-HT1A Transcriptional Regulation

The 5-HT1A receptor gene is intronless and has a TATA-less promoter that contains several GC-rich sequences proximal to the start codon (Figure I-11) (Parks & Shenk, 1996). Its expression is driven by multiple strong Sp1/Maz enhancers (Albert et al., 1996; Parks & Shenk, 1996; Storring, Charest, Cheng, & Albert, 1999), well-known

zinc finger enhancer proteins that recognize GC-rich regions (Song et al., 2001; Song, Ugai, Kanazawa, Sun, & Yokoyama, 2001; Song et al., 2003). 5-HT1A promoter has several NFkB response elements and is positively regulated by NFkB homo- (p50/p50) and heterodimers (p50/p65). The transcriptional activation induced by NFkB can be repressed by glucocorticoid receptor (GR) (Zhong and Ciaranello, 1995; Meijer et al, 2000).

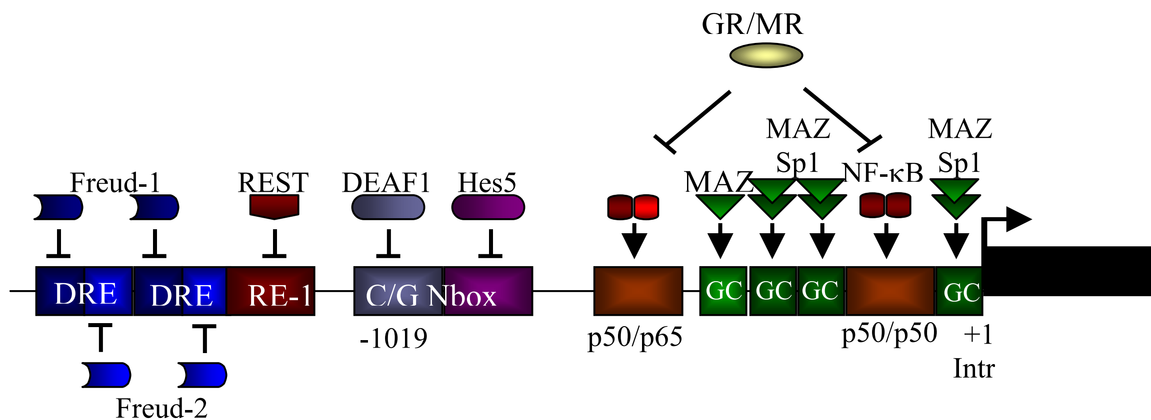

Figure I-11: Diagram representing transcriptional regulatory elements of the human 5-HT1A gene. The activators include Maz/SP1. Transcriptional activation can be induced by NFkB on NFkB response elements by NFkB homo- (p50/p50) and heterodimers (p50/p65). Repression can be induced by GR and MR, although the specific mechanism is still unclear. The major repressors are Freud-1 and Freud-2 on the DRE element and REST on the RE-1 element. The regulators Deaf1 and Hes1/Hes5 modulate 5-HT1A transcription through a polymorphic element C(-1019)G. Image modified with permission from Albert & Lemonde, 2004.

The upstream repressor region is the major region involved in repression of 5-HT1A receptor gene transcription. Interestingly, the 5-HT1A receptor gene can be repressed and also modulated by a different repressors depending on cell type (Albert & Francois, 2010). For example, Freud-1/CC2D1A (Five-prime Repressor Element Under Dual repression binding protein) can repress pre-synaptic neurons through the 5’ portion of the DRE where Freud-2/CC2D1B can repress post-synaptic neurons through 3’ part of the DRE (Hadjighassem et al., 2009; Lemonde, Du, Bakish, Hrdina, & Albert, 2004; Ou et al., 2003). The DRE (dual responsive element) is comprised of 5’ and 3’ repressor elements (Figure I-12) (Ou et al., 2000).

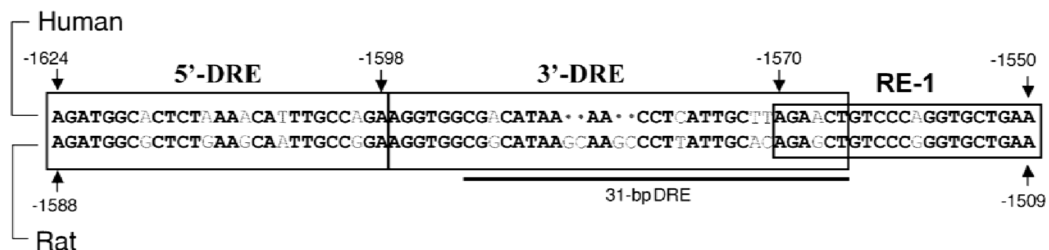

Figure I-12: Sequence alignment of the 5-HT1A receptor gene. Alignment shows that a repressor region between –1624 and –1550 of the human gene shares 81% nucleotide identity with the corresponding region of rat gene. Bold nucleotides represent sequence identity. Importantly, this schema shows the two consecutive repeats of DREs (5′-DRE and 3′-DRE), and overlapping RE-1 from human and rat 5-HT1A promoters. Image is taken with permission from Lemonde, Rogaeva & Albert, 2004.

Other important regulators of 5-HT1A transcription include Deaf1 and Hes1/Hes5 proteins. They regulate transcription through a palindromic element in human 5-HT1A promoter at the C-, but not the G-allele of a polymorphic element C(-1019)G (Lemonde et al., 2004). Intriguingly, Deaf1 can repress or enhance 5-HT1A receptor gene depending on the tissue and cell type. In raphe cells, Deaf1 represses 5-HT1A autoreceptor, but in certain non-serotonergic neurons, Deaf1 enhances 5-HT1A autoreceptor RNA and proteins (Czesak et al., 2006; Lemonde et al., 2003). The Hes proteins are exclusively expressed in neural precursors and immature neurons assuring the expression of 5-HT1A receptors in serotonergic neurons (Jacobsen, Vanderluit, Slack, & Albert, 2008). Finally, adjacent to the DRE, the RE-1 site was identified, responsible for silencing 5-HT1A receptor expression in non-neuronal cells (Figure I-12) (Lemonde, Rogaeva, & Albert, 2004; Parks & Shenk, 1996; Wissink et al., 2000).

5-HT1A receptor gene repression mediated by Freud-1 can be HDAC-independent in neuronal cells and HDAC-dependent in non-neuronal cells, whereas the transcriptional gene repression mediated by REST is only HDAC-dependent in both cell types (Lemonde, Rogaeva et al., 2004). This differential repression and modulation of 5-HT1A in pre- and post-synaptic neurons opens the door to the possibility of targeting the expression of 5-HT1A receptors by improving our antidepressant therapy (Albert & LeFrancois, 2010).

4-Freud-1

4.1 Structure, subcellular localization and function

In recent studies examining transcriptional regulation of 5-HT1A receptor gene, a protein called Five-prime Repressor under dual repression binding protein 1 (Freud-1)/CC2D1A was identified by our laboratory (Ou et al., 2003). Freud-1 is also known as CC2D1A, MRT3 and Aki1 protein belongs to a novel protein family composed of two proteins: Freud-1 and Freud-2 (Albert & Lemonde, 2004).

Human Freud-1 protein is highly conserved through evolution and contains (Figure I-13) four DM14 domains (*Drosophila melanogaster* 14 domain) of unknown function, one helix-loop-helix domain (HLH), a protein kinase C C2 domain, a proline-rich domain thought to be involved in protein binding (Williamson, 1994), coiled-coil oligomerization motifs (Burkhard, Stetefeld & Strelkov, 2001) and putative phosphorylation sites for protein kinases A and C and two strong consensus calcium calmodulin-dependent protein kinase (CaMK) II/IV sites (Basel-Vanagaite et al., 2006; Ou et al., 2003). Freud-1 is a transcriptional repressor and its activity can be both HDAC-dependent and –independent (Lemonde, Rogaeva & Albert, 2004) and CaMK-dependent (Ou et al., 2003; Rogaeva, Galaraga & Albert, 2007).

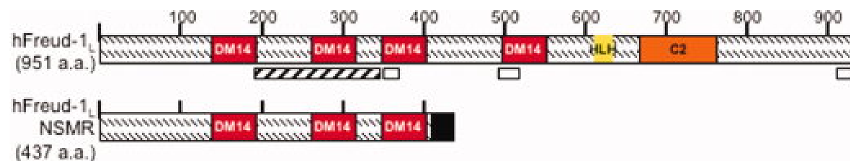

Figure I-13: Schema representing Freud-1 and its isoforms structure. Long isoform of human Freud-1 with four DM14 domains, one helix-loop-helix (HLH), proline-rich region (diagonal line filled box), three coiled coils motifs (white boxes) and one C2 domain is demonstrated. Amino acid scale is depicted at the top of hFreud-1L counted from the most upstream methionine start codon. The isoform linked to non-syndromic mental retardation (NSMR) has only three DM14 domains and lacks the rest of the sequence. The image is adapted with permission from Rogaeva & Albert, 2007.

Freud-1 is broadly expressed through the rat brain, with particularly high levels in cortical and hippocampal regions. Freud-1 is coexpressed with 5-HT1A receptors, suggesting an important function for Freud-1 to regulate 5-HT1A recepor expression in the central nervous system (Ou et al., 2003; Rogaeva & Albert, 2007). Freud-1 can repress the 5-HT1A receptor gene (Ou et al., 2003) and dopamine-D2 receptor gene (Rogaeva et al., 2007). Recently, a deletion in the Freud-1 gene was linked genetically to

non-syndromic mental retardation (NSMR) (Basel-Vanagaite et al., 2003; Basal-Vanagaite et al., 2006). Initially, analysis detected an autosomal recessive locus on 19p13.12-p13.2 (Basel-Vanagaite et al., 2003) which was subsequently mapped to a ~900kb region with a 3.6kb deletion in the CC2D1A. This deletion removes a region included within introns 13 to 16 and introduces a frame-shift mutation resulting in a premature stop codon after translation of 30 nonsense amino acids (Figure I-13). Finally, Freud-1 is a novel regulator of serotonin-1A and dopamine-D2 receptors that mediate the actions of serotonin and dopamine and regulate their levels and activity. Thus, Freud-1 could be a multisite integrator that acts at signaling, internalization and transcriptional levels to determine the activity of the serotonin and dopamine systems. An understanding of the mechanisms of Freud-1-mediated repression is important for better understanding of transcriptional regulation of serotonin and dopamine receptors which are important drug targets for mental diseases.

4.2 Other functions

In addition, recent studies on Notch trafficking in *Drosophila* by several groups has identified a lethal (2) giant disc (*lgd*) protein which is the ortholog of mammalian Freud-1, to be a regulator of endosomal trafficking of Notch and its activation (Jaekel and Klein, 2006; Gallagher and Knoblich, 2006; Childress et al, 2006), trafficking of Delta, Epidermal Growth Factor Receptor (EGFR) and possibly of other proteins (Gallagher & Knoblich, 2006). The role of Freud-1 in endosomal trafficking is further supported by the preliminary identification of Freud-1 interaction with charged multivesicular body proteins CHMP 4A, 4B and 4C, the proteins of human endosomal sorting complex

required for transport ESCRT-III (Tsang et al, 2006). Another study done by Nakamura et *al* (2008) has identified Freud-1 as a novel scaffold for the PDK1/Akt pathway. They have found that Freud-1 is required for EGF-mediated Akt phosphorylation and activation by forming EGFR/Freud-1/PDK1/Akt complex. Also, they describe the importance of Freud-1 for the regulation of cell survival and proliferation (Nakamura et al., 2008). Taken together, these studies suggest that Freud-1 may be implicated in the regulation of several receptors such as EGFR, Notch, Delta, 5-HT1A receptor and Dopamine D2 receptor through different mechanisms depending on its cellular localization. In the cytoplasmic compartment, Freud-1 may control the level of existing receptors or their intracellular targeting through endocytosis; in addition, Freud-1 may signal through Akt to prevent the synthesis of future receptors in the nucleus by transcriptional repression of these receptors genes.

In addition to its involvement in endocytic receptor trafficking and Akt signaling, Nakamura and colleagues have identified an additional role for Freud-1 (also known as Akt kinase-interacting protein 1 (Aki1)) independent of its nuclear or cytoplasmic functions. They have found that when Freud-1 localizes to centrosomes, it regulates centriole cohesion during mitosis and interacts with cohesin in centrosomes (Nakamura et al, 2009). Their group also showed that Freud-1 is phosphorylated on the Ser (208) by the cyclin B1-cyclin-dependent kinase 1 (cdk1) complex during mitosis (Nakamura et al, 2010).

Finally, in HEK293 cells, Freud-1 has been shown to be mildly upregulate NFkB-like activity (Matsuda et al, 2003). Furthermore, Zhao and colleagues have shown that the activation of NFkB by Freud-1 requires its C2 domain. This activation is dependent on

the ubiquitin-conjugating enzyme Ubc13 and their results suggest that Freud-1 activates NFkB through the canonical IKK pathway (Zhao et al, 2010).

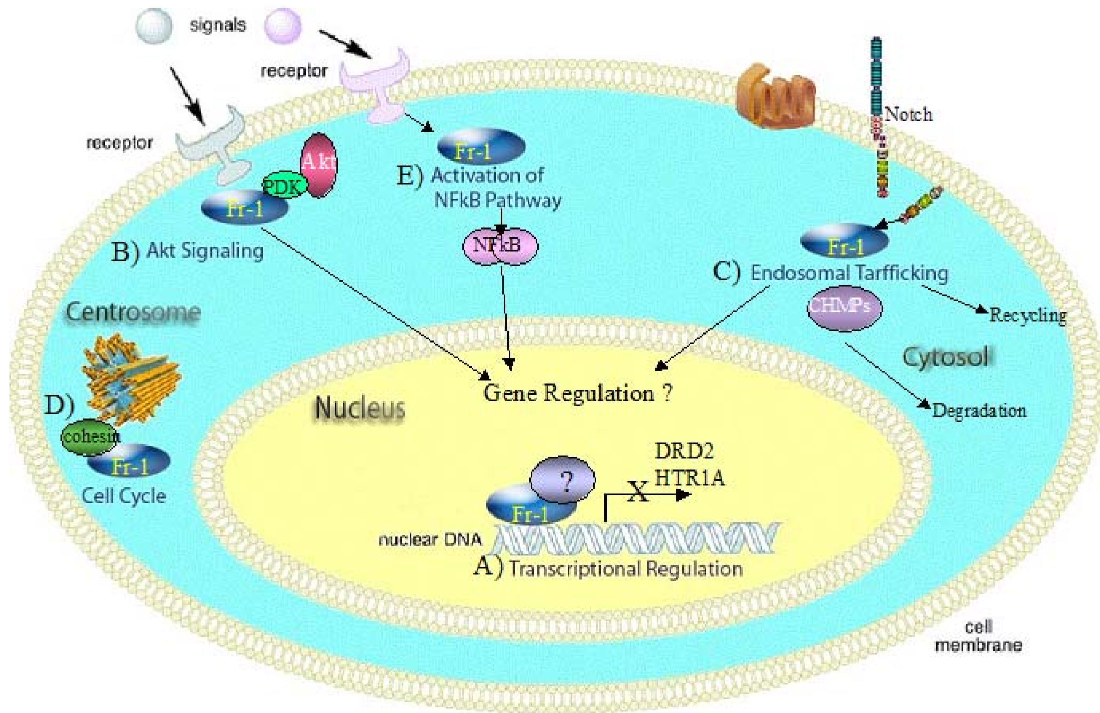

Figure I-14: Schematic Representation of Multiple Functions of Freud-1. (**A**) In the nucleus, Freud-1 has been identified to be a transcriptional repressor of 5-HT1A and dopamine D2 receptor genes (Ou et al., 2003; Rogaeva et al., 2007). (**B**) In the cytosol, Freud-1 has been shown to act as a scaffolding protein for Akt signaling (Nakamura et al., 2007). (**C**) Mammalian Freud-1 has been shown to be an interacting protein with CHMPs proteins (Tsang et al., 2006) and in *Drosophila,* the Freud-1 ortholog has been shown to be involved in the endosomal trafficking of several receptors suggesting a role for mammalian Freud-1 in endosomal trafficking of receptors (eg. Notch) (Jaekel & Klein, 2006; Gallagher & Knoblich, 2006; Childress et al., 2006). (**D**) In the centrosome, Freud-1 interacts with cohesin to regulate centriole cohesion (Nakamura et al., 2009; Nakamura et al., 2010). (**E**) Finally, in the cytosol, Freud-1 has been shown to have an

additional function in the activation of NFkB pathway through the canonical IKK pathway (Zhao et al., 2010). Taken together the cytosolic functions of Freud-1 might have an indirect role in gene regulation.

Since Freud-1 is involved in many different cellular processes (Figure I-14), the importance of this protein may be even greater than first hypothesized. It remains unclear whether the transcriptional, scaffolding, centrosomal or endosomal functions of Freud-1 are crucial in determining the mental retardation phenotype.

4.3 Mental retardation

Mental retardation (MR) affects up to 2% of the population. This mental disorder is attributed to environmental and genetic factors (Chechlacz & Gleeson, 2003; Raymond & Tarpey, 2006; Ropers, 2006). Diagnosis of MR is based on several criteria such as impairments in intellectual function, self-care and social/interpersonal skills (Branchi, Bichler, Berger-Sweeney & Ricceri, 2003). Mental Retardation lacking recognizable neurological or physical deficits is called Non-Syndromic Mental Retardation (NSMR). This type of MR is not well understood (Raymond & Tarpey, 2006; Ropers, 2006; Rogaeva et al., 2007). It has been documented that Freud-1 is a negative regulator of 5-HT1A receptor gene, suggesting that NSMR mutation which eliminates Freud-1 function as a repressor could result in an upregulation of 5-HT1A receptor gene (Rogaeva et al., 2007). Currently, there is no direct evidence of relationship between Freud-1 and depression, although 5-HT1A receptor dysfunction can contribute to depression (Albert & Lemonde, 2004; Berton & Nestler, 2006; Leonardo & Hen, 2006; Rogaeva et al.,

2007; Albert and LeFrancois, 2010). It is possible that an increase in the transcription of 5-HT1A receptor gene in NSMR patients contributes to cognitive impairment. Negative regulation of the dopamine D2 receptor gene is also important since dopamine D2 receptor is implicated in memory and cognitive performance (Cooper, Sagar, & Sullivan, 1993). As a result, the lack of functional Freud-1 in development can probably result in unbalanced serotonin 5-HT1A receptor gene and dopamine D2 receptor gene which could be responsible for memory deficits in NSMR patients.

For a long period of time in human history, mental illnesses such as depression were not considered physiological illnesses. Today, it is clear that mental illnesses involve disorders of brain function and medication forms an important part of treatment. The impact of mental illness on society is often underestimated. According to Health Canada and Statistics Canada, about 8% of Canadian adults will suffer from major depression at some point of their lives. Moreover, World Health Organization (WHO) reports that depressive disorders are the 4th leading cause, worldwide, of life years lost due to disability and are expected to rank 2nd by the year 2020 after heart diseases. In 1998, Health Canada estimated that in 1998, mental disorders were the 3rd highest source of direct health care costs at $4.7 billion and these numbers are underestimated according to Stephens and Joubert, 2001. In addition, it has been reported that the unemployment rate of persons with mental illness ranges from 70-90%, depending on the severity of disability. These are just few statistics that are published by Health Canada, Statistics Canada and WHO. But, behind these numbers there are people in society who need help. The numbers do not reflect the full scope of the burden, since mental illness affects not only those living with the problem but also society in general, whether through a family

member, a friend or simply the costs to health care system and support workers. Medications are available to treat these disorders such as antidepressants and antipsychotic drugs, but they are effective in less than 50% of subjects, and their molecular mechanisms of action are not well understood. Thus more research is needed to develop new approaches to treating mental illness.

5-Hypothesis and Objectives

Freud-1 may be an integrator of transcription and signaling involved in several mental illnesses, however the mechanism by which Freud-1 regulates gene expression is currently unknown. The objective of my research was to identify these mechanisms. I hypothesized that Freud-1, like other DNA-binding proteins, recruits co-activators or co-repressors to form complexes that mediate repression; and that Freud-1 activity is regulated by sumoylation.

My specific objectives were:

Objective 1: to identify Freud-1-associated partners and their roles in Freud-1 repression.

Objective 2: to determine if Freud-1 is sumoylated and its role in Freud-1 repression.

6-Approaches

In order to determine how transcriptional regulation of 5-HT1A receptor gene is mediated by Freud-1, we have purified Freud-1-associated proteins by affinity chromatography with a specific antibody against hFreud-1. The isolated proteins were identified by Mass Spectrometry and validated by co-IPs, reversed co-IPs and pull-down

assays. The presence of identified complexes on the 5-HT1A promoter was verified quantitatively by ChIP assays/ Q-PCR and qualitatively by PCR in non-neuronal HEK293 cells and neuronal 5-HT1A-expressing SKNSH cells. In addition, the importance of the interaction between isolated complexes and Freud-1 was assessed by depleting either Freud-1, Brg1 or both by using siRNAs towards indicated proteins and verifying their respective recruitment to the 5-HT1A promoter by ChIP assays. Finally, functional importance of the Freud-1/Brg1 interaction was assessed by measuring 5-HT1A mRNA expression in HEK293 and SKNSH cells after deletion by siRNA of Freud-1, Brg1 or both proteins in each cell type by Q-RT-PCR.

Moreover, in order to address whether Freud-1 is modified by SUMOylation, co-IPs were performed. Mutants in predicted Freud-1 sumoylation sites were constructed and tested by Western blot with specific antibodies towards S-Freud-1 and SUMO1. Reporter assays were also done to asses the activity of these mutants in transcriptional repression of 5-HT1A, DRE region.

Finally, Freud-1 lipid binding properties were assessed by using PIP lipid strips, where bacterially expressed S-Freud-1 was incubated with PIP lipid strips and Freud-1 binding assayed by Western blot.

CHAPTER II: Differential recruitment of the Sin3A co-repressor complex by Freud-1 for gene silencing versus reversible repression

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Abstract

Freud-1 is a repressor of the serotonin-1A (5-HT1A) receptor gene that recruits different complexes for gene silencing in 5-HT1A-negative (e.g., HEK293) cells or reversible repression in 5-HT1A-expressing neuronal cells (SKNSH). We identified Freud-1-associated proteins by mass-spectroscopy, using affinity purification of HEK293 nuclear extracts, which included members of the Swi/Snf BRG1 chromatin remodeling complex (BAF155, BAF57) and the Sin3A-HDAC co-repressor complex (SAP130, HDAC1, HDAC2, RBBP4/5). The composition and function of these complexes was compared in HEK293 cells or 5-HT1A-expressing SKNSH cells. Interactions with endogenous or transfected Freud-1 were validated by co-immunoprecipitation assays and the presence of these proteins at the 5-HT1A promoter was demonstrated by quantitative chromatin immunoprecipitation (ChIP) assay. In HEK293 cells the components of Swi/Snf-BRG1 and Sin3A-HDAC complexes were associated with Freud-1, whereas in SKNSH cells the Sin3A-HDAC proteins were not. The functional role of the BRG1 complex was addressed by measuring 5-HT1A receptor mRNA levels using Q-RT-PCR in cells treated with siRNA to Freud-1, Brg1 or both proteins. In HEK293 cells, 5-HT1A receptor mRNA levels were increased only when both Freud-1 and Brg1 were depleted, but in SKNSH depletion of either was sufficient to up-regulate 5-HT1A mRNA levels. These results are consistent with a model in which Freud-1 recruits the Swi/Snf-Brg-1 chromatin remodeling complex to mediate HDAC-dependent gene silencing through the Sin3A-HDAC complex in HEK293 cells, while in SKNSH cells only the Swi/Snf-BRG1 complex is required to mediate reversible HDAC-independent gene repression.

Introduction

Freud-1 (five prime repressor element under dual repression binding protein-1), also known as CC2D1A, MRT3 and Aki1, was initially identified as a repressor of 5-HT1A receptor gene (Ou et al, 2003) that recognizes the DRE element (dual repressor element) (Ou et al, 2000) and is colocalized with the 5-HT1A receptor in pre- and post-synaptic neurons of the serotonin system (Ou et al, 2003; Rogaeva & Albert, 2007; Szewczyk et al, 2010; Iyo et al, 2009; Kieran et al, 2010). Freud-1 also represses the dopamine-D2 receptor gene (Rogaeva et al., 2007) and is inhibited by CAMKII activation (Ou et al., 2003; Rogaeva et al, 2007). In addition to its transcriptional functions, mammalian Freud-1 acts as a scaffolding protein for Akt signaling (Nakamura et al, 2008), activates NFkB (Zhao et al, 2010), mediates endosomal trafficking (Tsang et al, 2006), and regulates centriole cohesion in the centrosome (Nakamura et al, 2009; Nakamura et al, 2010). Studies in *Drosophila* have identified lethal (2) giant disc (*lgd*) protein, the ortholog of mammalian Freud-1, as a regulator of endosomal trafficking of several receptor proteins (Jaekel & Klein, 2006; Gallagher & Knoblich, 2006; Childress et al, 2006). A C-terminal truncated mutant of Freud-1 has been linked to Non-Syndromic Mental Retardation (NSMR) (Basel-Vanagaite et al, 2006 Rogaeva et al., 2007), suggesting a role for Freud-1 in brain development. Thus Freud-1 is a multifunctional protein that is implicated in transcriptional regulation of 5-HT1A and dopamine-D2 receptors, which are crucial regulators of the serotonin and dopamine systems, respectively.

Freud-1-mediated repression is HDAC-dependent in 5-HT1A-negative cells, such as HEK293 or L6 myoblast cells. On the other hand, in cells that express 5-HT1A

receptors, such as rodent SN48 septal cells or rat RN46A raphe cells, Freud-1 mediates HDAC-independent repression of the 5-HT1A promoter (Lemonde et al, 2004). In addition, a consensus RE-1 element is located adjacent to the DRE of the 5-HT1A promoter that mediates repression by RE-1 silencing transcription factor (REST)/NRSF, which silences neuronal genes in non-neuronal tissues (Schoenherr & Anderson, 1995). REST mediates HDAC-dependent repression in both 5-HT1A-negative or 5-HT1A-positive cell types (Lemonde et al, 2004). REST has been shown to recruit two separate corepressor complexes: Sin3A-HDAC and CoREST-HDAC complexes (Andres et al., 1999; Grimes et al., 2000; Huang et al, 1999; Naruse et al, 1999; Roopra et al., 2000) and additional corepressor proteins (Tachibana et al, 2001; Zhang et al, 2002; Ooi et al, 2006; Ooi & Wood, 2007). Upon binding of REST to the RE-1 site, the interaction is stabilized by the chromatin remodeling protein Brg1 (Ooi et al, 2006), which recruits co-repressors Sin3A and CoREST, HDACs and other proteins to the gene silencing complex (Ooi & Wood, 2007). The mammalian Sin3A complex contains core enzymatic units HDAC1/HDAC2, retinoblastoma-associated proteins RbAP46/RbAP48, as well as Sin3A-associated proteins (SAPs) (Ayer, 1999; Kuzmichev et al, 2002; Lai et al, 2001; Skowyra et al, 2001; Fleischer et al, 2003; Meehan et al, 2004; Vannier et al, 1996; Alland et al, 2002)). Brg1 is the catalytic ATPase subunit of Swi/Snf chromatin remodeling complex (Muchardt & Yaniv, 1999). Purified Brg1 complexes contain core subunits Brg1 (or hBrm) and Brg1-associated factors (BAFs): BAF170, BAF155, BAF47/Ini1, BAF60, BAF57, BAF53 and actin (Wang et al, 1996). Brg1 has been shown to mediate both activation and repression of genes (Trotter & Archer, 2008). When Brg1

associates with the Sin3A/HDAC complex it mediates the transcriptional repression (Sif et al, 2001).

In this study, we addressed the protein complexes that associate with Freud-1 to mediate gene silencing or reversible repression in different cell types. We hypothesized that Freud-1 recruits different co-repressors to the 5-HT1A receptor gene depending on the cellular context to mediate HDAC-dependent silencing or HDAC-independent repression of the 5-HT1A receptor gene. We find that in non-neuronal cells, the transcriptional repression of 5-HT1A receptor occurs through the recruitment Swi/Snf and Sin3A/HDAC corepressor complexes to 5-HT1A receptor gene promoter. In contrast, in 5-HT1A-expressing neuronal cells, Freud-1 recruits Brg1 without Sin3A/HDAC complex, consistent with our previous findings (Lemonde et al, 2004). Furthermore, we show that gene silencing of the 5-HT1A is maintained by the presence of Sin3A-HDAC in Freud-1- or other Brg1-containing complexes, while repression in 5-HT1A-expressing cells was dependent on Freud-1-containing Brg1 complexes that lacked Sin3A-HDAC.

Methods

Cell Culture, Transient Transfections and siRNA Treatments

Human embryonic kidney (HEK) 293 cells and human neuroblastoma 5-HT1A expressing cells (SKNSHSKNSH; provided by Dr Lakshmi Devi, New York University, NY) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wisent, St-Bruno, QC) supplemented with 10% Fetal Calf Serum (Wisent). Cells were grown at 37°C in 5% CO_2. Cell lines were grown to 50-60% confluence and the medium was

replaced 12 h prior to transfection. Cells were transfected using calcium phosphate co-precipitation method with 5 μg of human Freud-1 expression plasmid. The cells were analyzed 72 h post-transfection.

Stealth siRNA targeting hFreud-1 (5'-GGCGCUCUAUCAGACAGCAAUUGAA-3'), (Invitrogen) and siRNA targeting Brg1/SMARCA4 (ON-TARGETplus SMARTpool; Dharmacon) as well as scrambled negative control (5'-GGCUCUCUAAGAGACAACUUGCGAA-3') (Invitrogen) were transfected using HiPerfect (Qiagen) transfection reagent at final siRNA concentration of 20 nM and 25 nM, respectively. Transfection efficiency control was performed with BLOCK-IT^{TM} Fluorescent Oligo (Invitrogen) demonstrating ~90% efficiency (data not shown). The cells were analyzed 72 or 104 h post transfection.

Plasmids, Production and Purification of Recombinant hFreud-1, Subcellular Fractionations

hFreud-1 cDNA in pTRiEX-4 (Rogaeva & Albert, 2007) was transformed into BL21 (DE3) E.coli (Novagen), grown overnight and induced at OD_{600}=0.6 with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Wisent) at 37°C for 3 h. Human Freud-1 was purified under native conditions using Ni-nitrilotriacetic beads (Qiagen, Mississauga, ON, Canada) and dialysed against DNA binding buffer (20mM HEPES, 0.2mM EDTA, 100mM KCl, 5% glycerol, pH 7.9).

Subcellular fractionation was performed as previously described by Czesak et al, 2006. Briefly, cells were washed 3x with PBS, harvested and allowed to pre-swell for 10 min at 4°C in extraction buffer. Following, pelleted cells were lysed for 10 min on ice

with complete extraction buffer (10 mM KCl, 10 mM Na-HEPES, pH 7.6, 1.5 mM $MgCl_2$, 0.1% Nonidet P40, 0.5 mM DTT, 0.5mM spermidine, 0.15 mM spermine, 1 mM phenylmethylsulphonyl fluoride, 1x protease inhibitor cocktail (Roche, Laval, Qc, Canada)). Lysates were centrifuged (2 min, 6000xg) and supernatant reserved as cytosolic fraction. The nuclear pellet was collected and washed 3x with wash buffer (50 mM NaCl, 20 mM Na-HEPES, pH 7.6, 25% glycerin, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 1 mM phenylmethylsulphonyl fluoride, 1x protease inhibitor cocktail (Roche)). The washed pellets were lysed in nuclear extraction buffer (500 mM NaCl, 20 mM Na-HEPES, pH 7.6, 25% glycerin, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 1 mM phenylmethylsulphonyl fluoride, 1x protease inhibitor cocktail) for 30 min on ice. Fractions were verified by western blot for cross-contamination between nuclear and non-nuclear compartments (data not shown).

Antibodies and Western blots

Anti-hFreud-1L (1:10000), generated against bacterially expressed and purified (Ni-nitrilotriacetic beads, Qiagen) S/His-tagged human Freud-1L (Cedarlane, Hornby, ON, Canada) was used for affinity purifications, western blots and IPs (Rogaeva and Albert, 2007). Antibodies were used at the following dilutions: BAF170 (1:1000), BAF155 (1:1000) and BAF57 (1:2000) (Bethyl Laboratories); Brg1 (1:2000; Upstate), Sin3A (1:1000; Abcam); HDAC-1 (1:1000), HDAC-2 (1:1000) and β-actin (1:10000) (Sigma).

For Western blot analysis, polyvinylidenedifluoride membranes were blocked in TBST with 5% (w/v) milk for 1hr and incubated with corresponding antibodies at 4°C overnight followed by 1 h incubation with horseradish peroxidase-linked anti-rabbit (1:5000; New England Biolabs, Pickering, ON, Canada) or anti-mouse (1:5000; Jackson Immunoresearch Laboratories, West Grove, PA, USA) secondary antibody, or in the case of anti-S antibody (1:5000) (Novagen), membranes were directly washed and developed with BM chemiluminescence blotting substrate (Roche).

Affinity Purification and Mass Spectrometry Identification.

Anti-Freud-1 affinity column was constructed according to manufacturer's instructions and the purification was done according to manufacturer's protocol (Pierce). Briefly, 0.9 mg of hFreud-1 antibody purified by Montage antibody purification kit (Millipore) was linked to the column for Freud-1 column and 0.9 mg purified rabbit IgG from pooled normal rabbit serum (Cedarlane) was linked to the control column. The total of 6 mg/column of HEK293 nuclear extract was purified by batch method as suggested by manufacturer's protocol. The resulting elutions were concentrated using Centricon 10K tubes (Millipore) and separated by large format 8% SDS-PAGE. The resulting gel was stained using Coomassie Blue, which is compatible with mass spectrometry, and 20 bands were cut from the gel and sent for protein identification at the Quebec Genomics Center, Proteomics Platform (Laval University Research Center, Quebec City, QC. Protein identification was performed using Applied Biosystems/SCIEX QSTAR XL LC/MS/MS system. The resulting files were analyzed using Mascot (Matrix Science) with fragment tolerance of 0.5 Da and parent tolerance of 2.0 Da while full tryptic

cleavage was required with up to two missed cleavages allowed. Modifications included carbamidomethylation of cysteines (fixed) and oxidation (variable). Scaffold (version Scaffold_2.1.03) was used to compile and assign probability scores. Peptide identifications were accepted if they could be established at greater than 95% and protein identification probability at 99% or higher.

Immunoprecipitation (IP), Pull-down and Chromatin Immunoprecipitation (ChIP)

For all experiments, 200 μg of nuclear lysate per assay were used. The lysates were not pre-cleared and were combined with corresponding antibodies (1:200) overnight at 4°C with rotation. The following day, 30 μl of protein-A agarose beads (GE HealthCare) were added and incubated (3 h, 4°C). The supernatant was then collected and the beads were washed 3x with 1 ml of NETIN buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.5% NP-40). The beads were then boiled for 5 min in 2x Loading buffer (200 mM Tris, pH 6.8, 0.8% SDS, 1.6% 2-mercaptoethanol, 0.04% Bromophenol blue, 4% glycerol) and subjected to Western blot analysis, 50% from the total elution volume was probed for the presence of Freud-1 (or control) and 50% was probed for the protein of interest.

For pull-down assays, 40 μl of S-protein agarose beads (Novagen) were added to 200 ug of nuclear lysates from cells transfected with S-Freud-1 and incubated overnight at 4°C. The following day, beads were washed 3x with 1 ml of NETIN buffer and boiled in 2x Loading buffer. The resulting elutions were examined by western blot.

ChIP assays were performed according to manufacturer's instructions (Upstate) with few modifications. One confluent 10-cm dish of healthy cells per sample was

washed 3x with PBS and cross-linked for 15 min at 22°C in PBS supplemented with 1 mM $MgCl_2$ and 1% formaldehyde (v/v), rinsed 3x with PBS and lysed. Shearing of genomic DNA to 100-400 bp fragments was done by sonication on ice with the addition of 212-300 µm glass beads (Sigma), 20x at the setting of 10 for 20 s each time (60 Sonic Dismembrator, Fisher, Ottawa, ON). The resulting lysates were incubated with corresponding antibodies overnight at 4°C. The next day, 30 µl of agarose beads were added, incubated for 2 h at 4°C, and the resulting complexes eluted according to manufacturer's instructions. De-cross-linking was done overnight at 65°C, followed by 1 h digestion with proteinase K (Sigma) and phenol/chloroform extraction, and ethanol precipitation. The results were analysed using Q-PCR with primers designed to amplify the 151-bp region containing 5-HT1A-5'DRE (5'-CTGTCTTCCTCTTTCTAAAAGTTGTTG-3') and 5-HT1A-3'DRE (5'-CCGGCTAGCCTCTCTTAATCTCAG-3'). The amplification cycles were 92°C for 10 min, 92°C for 30 s, 54°C for 30 s, 72°C for 30 s, 84°C for 20 s (25 cycles), terminated at 72°C for 10 min using a Rotor-Gene RG-3000 (Corbett Life Sciences, Sydney, Australia) and quantified using SYBR green (Molecular Probes, Eugene, OR, USA) incorporation with QtaqTM DNA polymerase mix (Clontech). The PCR products were verified on a 1% agarose gel.

Quantitative Real time RT-PCR (Q-RT-PCR)

RNA was isolated from cells using the TRIZOL Reagent (Invitrogen), followed by DNase treatment using the TURBO DNAase-free^{TM} kit (Ambion, Austin, TX) and cDNA was generated using M-MLV reverse transcriptase (Invitrogen). Parallel reactions

lacking M-MLV reverse transcriptase served as no reverse transcription (-RT) control. The resulting cDNA was analyzed for glyeraldehyde-3-phosphate dehydrogenase (GAPDH; control) (product number Hs02758991_g1) and serotonin-5HT1A (product number Hs00265014_s1) expression levels by Q-PCR analysis using TaqMan probe kits (Applied Biosystems, Foster City, CA, USA). Reactions were carried out in a Rotor Gene 3000 cycler (Corbett Research, Sydney Australia). The cycling program was: 95°C for 10min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. PCR products were verified by agarose gel electrophoresis (data not shown). Data was analyzed using RotorGene v6.0 software, which found the best fit for the standard curve. Relative amounts of 5HT1A to GAPDH were obtained as 10 X (CtT-CtG), where CtT is the threshold cycle for the target gene and CtG is the threshold cycle for the GAPDH. Duplicates were averaged and the resulting values were used to calculate means ± SEM.

Results

Identification and validation of hFreud-1-interacting partners

In order to identify proteins involved in Freud-1 repressor activity, affinity purification of endogenous Freud-1 from HEK293 nuclear extracts was done using a specific antibody to the more abundant human Freud-1 long form (130 kDa) (Rogaeva & Albert, 2007). As shown by Western blot for Freud-1, the affinity column efficiently purified endogenous Freud-1 compared to IgG control, with minimal background (Figure II-1A). From affinity purified fraction of HEK293 cell nuclear extracts (Figure II-1B), 20 bands were excised which showed enrichment of proteins relative to the IgG control. A total of 280 potential Freud-1-interacting proteins were identified, of which 84 proteins were eliminated as they belong to sepharose proteome and likely represent non-specific interactions (Trinkle-Mulcahy et al., 2008) (for complete list see Table 1 in Appendix). Proteins identified with highest coverage and probability percentages that are involved in transcriptional regulation are summarized in Table II-1. The mass spectrometry analysis confirmed that the antibody recognized Freud-1, since it purified hFreud-1 (22 peptides with 28% coverage). In addition, the eluted fraction contained Swi/Snf complex related proteins such as BAF155, BAF57, BAF47 and Sin3A complex related protein SPA130, Retinoblastoma Associated proteins RBBP4 and RBBP5 (Figure II-1B). This was an explorotary study that confirmed the presence of BAF155 to our earlier studies (data not shown) and therefore the control bands were not analyzed by Mass Spectrometry: instead, the interaction between Freud-1 and Swi/Snf proteins was validated by additional IPs (Figures II-2 and II-3). Together, these data suggest that Freud-1 interacts with

proteins involved in chromatin remodeling and gene repression, such as Swi/Snf-, Sin3A- and Rb-related proteins.

Table II-1: Freud-1 associated proteins identified by Mass Spectrometry. Total of 280 potential proteins were identified in 20 bands, 84 sepharose proteome related proteins were eliminated from analysis. Proteins with highest coverage and probability percentages are shown in the table. (Freud-1 22 peptides with 28% coverage).

PROTEIN	# PEPTIDES	% COVERAGE
Swi/Snf complex		
BAF155	**17**	**13**
BAF57	**15**	**34**
BAF47	**4**	**18**
Msin3A complex		
SP130	**2**	**2**
Retinoblastoma Binding Proteins		
RBBP5	**10**	**26**
RBBP4	**3**	**8**

Legend:

BAF155: SMARCC1, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1
BAF57: SMARCE1, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1
BAF47: SMARCB1, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1
SP130: SAP130, Sin3A associated protein, 130kDa
RBBP5/4: Retinoblastoma binding protein 5/4

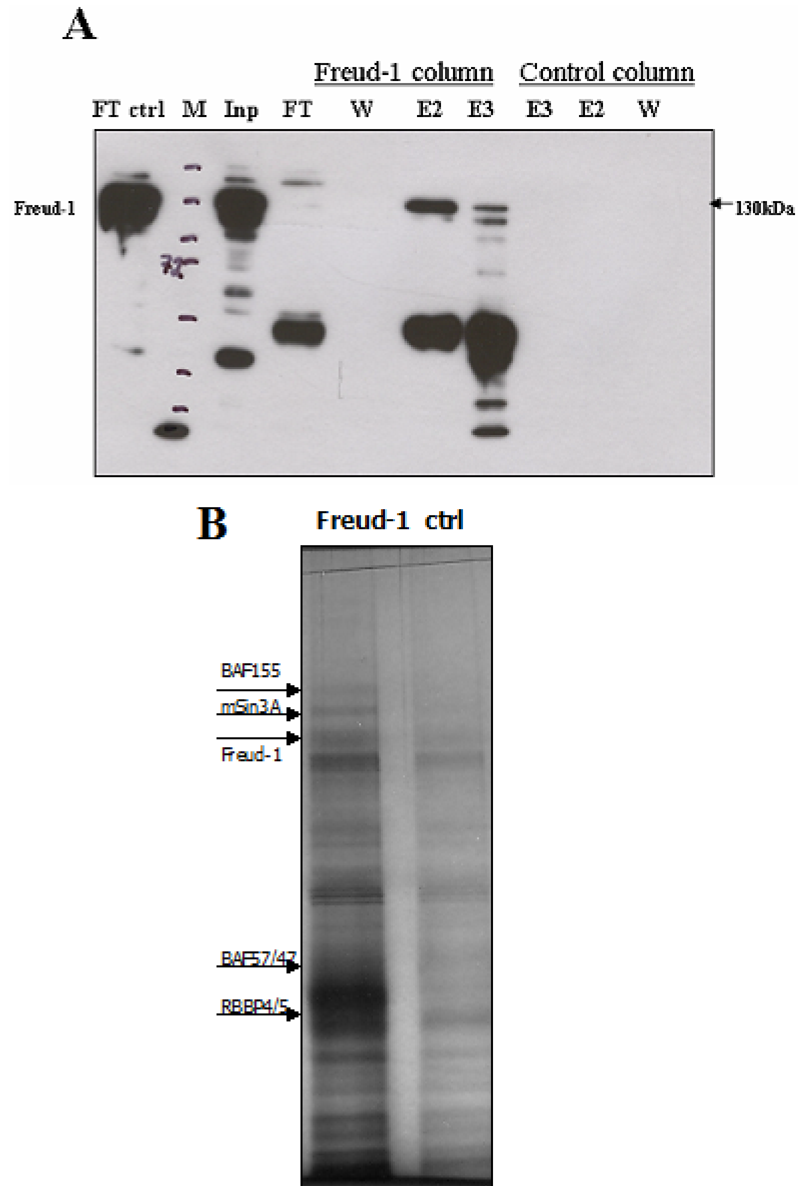


Figure II-1: Affinity purification of endogenous hFreud-1 and associated co-repressor complex. Nuclear extracts of HEK293 cells were immunoprecipitated using an antibody raised against bacterially-expressed full-length S-hFreud-1 and corresponding preimmune serum as a negative control (**ctrl**). (**A**) Western blot of affinity purified hFreud-1. hFreud-1 (arrow) was identified by Western blot using an antibody against full-length hFreud-1 raised in a different rabbit then the one used in column. Input (**INP**), flowthrough (**FT**), and elution (**E2** *and* **E3**) amounts loaded on the gel were equal in all

fractions. (**B**) Silver staining of hFreud-1 elution fractions from Freud-1 and control (preimmune) affinity column of HEK293 nuclear extracts. Arrows point to proteins identified by mass spectrometry analysis as hFreud-1, BAF155, BAF 47 or BAF57, RBBP4 or RBBP5, and Sin3A.

In order to validate the interaction of these proteins with endogenous Freud-1, co-immunoprecipitation studies using antibody to hFreud-1 were done in human HEK293 kidney cells (Figure II-2A) or SKNSH neuroblastoma cells (Figure II-2B). HEK293 cells do not express 5-HT1A receptors and exhibit strong HDAC-dependent silencing of the promoter by Freud-1, while SKNSH cells are neuronal cells that express 5-HT1A receptors in which Freud-1 repression is HDAC-independent (Lemonde et al., 2004). Pull-down assays were also performed in cells transiently transfected S-epitope-tagged Freud-1 (S-Freud-1) to definitively identify Freud-1. Upon immunoprecipitation with anti-Freud-1 but not IgG control, elution fractions were positive for the presence of Freud-1 (control), Brg1, BAF170, BAF155 and Sin3A in HEK293 cells (Figure II-2A). These results were confirmed by pull-down using anti-S-agarose beads in HEK293 cells transiently transfected with S-Freud-1 (Figure II-2C). Similar results were obtained from IP and pull-downs in neuronal SKNSH cells, which were positive for Freud-1 (control), Brg1, and BAF170, but negative for BAF155 and Sin3A (Figure II-2B, II-2D).

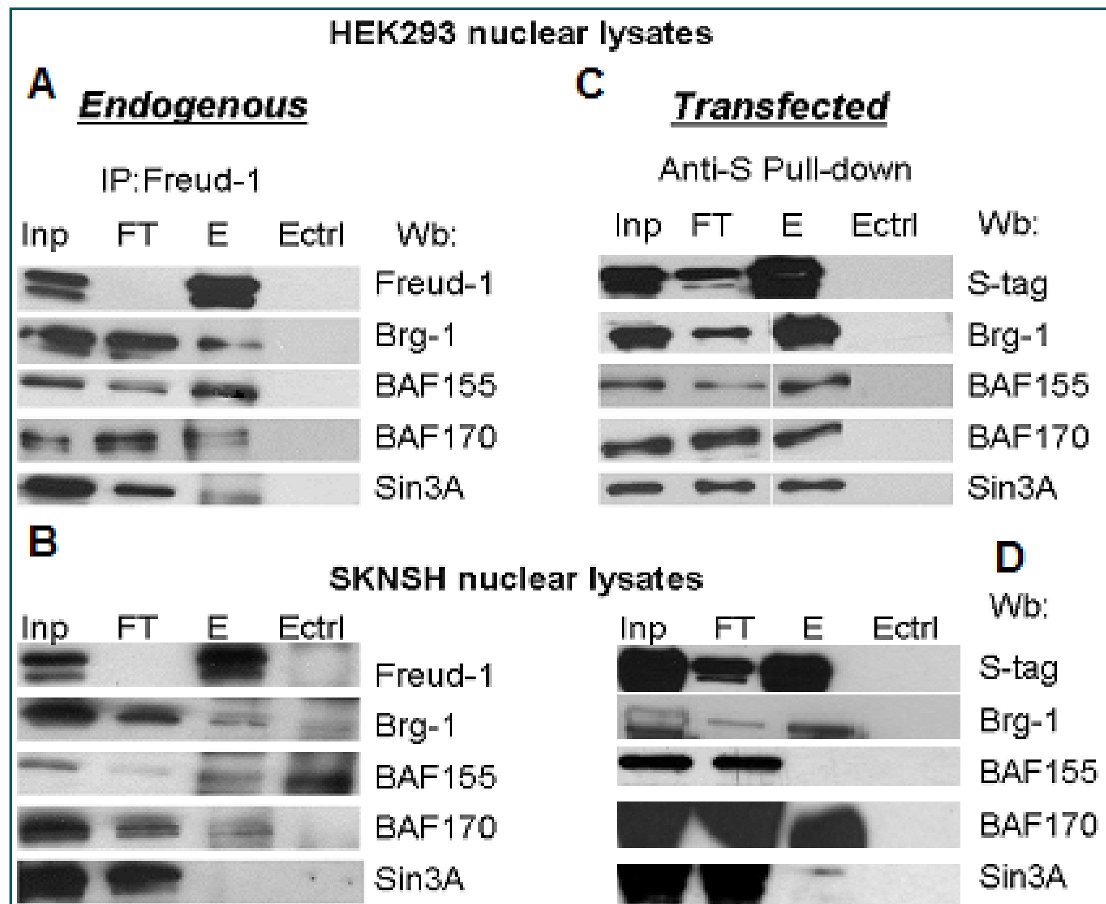


Figure II-2: Swi/Snf Brg-1-containing complex and Sin3A corepressor complex are hFreud-1-interacting partners. Analysis of proteins from nuclear lysates interacting with endogenous Freud-1. (**A**) HEK293 or SKNSH nuclear lysates (**B**) were examined by IP with full-length specific anti-hFreud-1L antibody. The presence of hFreud-1-interacting partners was detected by Western blot with corresponding specific antibodies in input (**Inp**), flow-through (**FT**) and elution fractions (**E**) and was absent in the elution from an identical column containing preimmune serum IgG (**Ectrl**). IP with hFreud-1 was positive for hFreud-1 indicating that IP is functional and for Brg-1, BAF155, BAF170 and Sin3A (Sin3A was absent in SKNSH cells). (**C**) Analysis of HEK293 nuclear lysates transiently transfected with S-tagged Freud-1 cDNA. Anti-S pull-down fractions were positive for S-Freud-1, Brg-1, BAF155, BAF170 and Sin3A. (**D**) Analysis

of SKNSH nuclear lysates transiently transfected with S-tagged Freud-1 cDNA. Anti-S pull-down fractions were positive for S-Freud-1, Brg-1, BAF170 but not Sin3A. For all Western blots, 20 μg of proteins per lane was loaded for **Inp** and **FT** fractions and 50% from total elution was loaded for **E** fraction.

Conversely, IP for each of the Freud-1-interacting proteins identified above brought down S-Freud-1 (Figure II-3A) and the immunoprecipitated protein (Figure II-3B). However, antibody to Sin3A immunoprecipitated S-Freud-1 only in HEK293, but not SKNSH extracts, consistent with a cell-specific interaction. Similarly, HDAC1 was also enriched in S-Freud-1 IP from HEK293 cells over SKNSH cells, while BAF155 appeared to be a non-specific signal in both cells. Taken together, these results suggest that Freud-1 interacts with proteins in a cell-specific manner, and fails to recruit Sin3A/HDAC in the 5-HT1A-expressing SKNSH cells compared to 5-HT1A-negative HEK293 cells, consistent with our previous studies showing that Freud-1-mediated repression is HDAC-dependent in 5-HT1A-negative cells and HDAC-independent in neuronal cells that express 5-HT1A receptors (Lemonde et al, 2004).

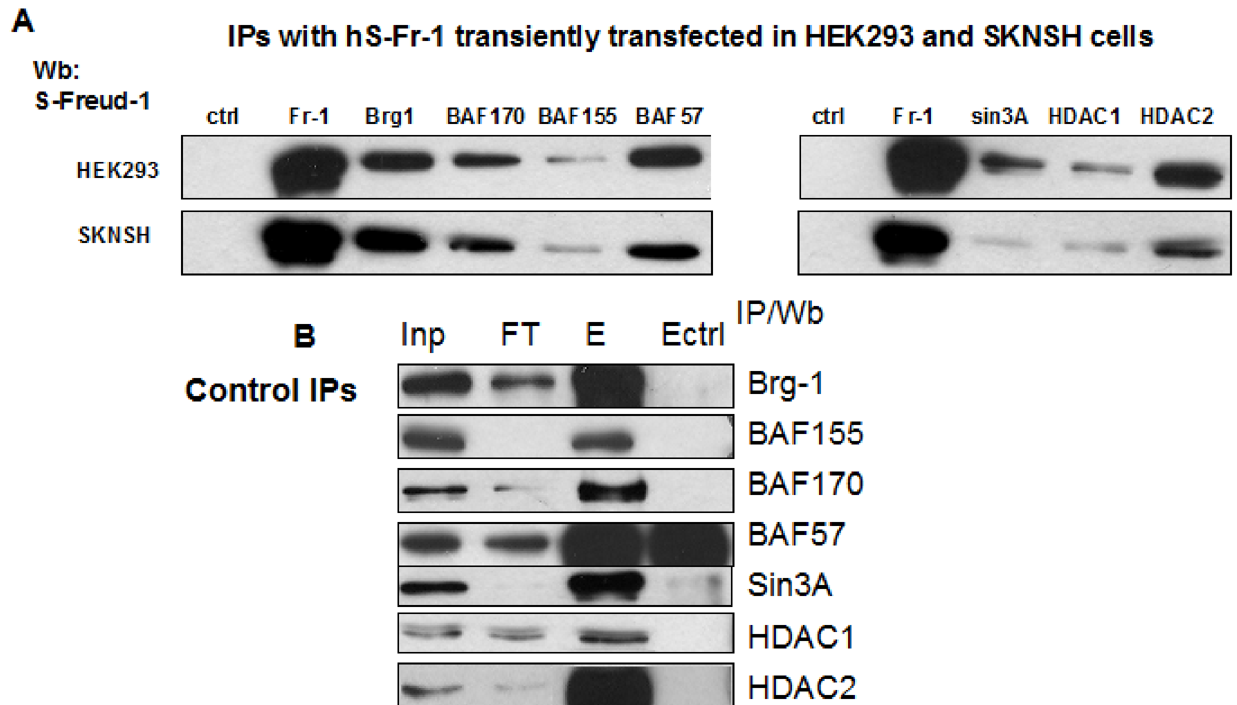


Figure II-3: Co-IP of Freud-1 with Brg1 and Sin3A complex proteins. (**A**) Co-IP studies. HEK293 and SKNSH cells were transiently transfected with S-tagged Freud-1 or not transfected (ctrl) and nuclear extracts harvested after 48 h. IP was done using specific antibodies against indicated proteins and was probed by Western blot for S-Freud-1 using specific antibody against S-tag. (**B**) Control IPs were performed in nuclear extracts from S-Freud-1-transfected HEK293 cells, and input (**Inp**), Flowthrough (**FT**) and elution fractions from 50% of the IP in **A**, were probed with antibodies each of the indicated proteins; control elution was from cells not transfected with S-Freud-1 (**Ectrl**).

Cell type-specific recruitment of Brg1 and Sin3A/HDAC complexes to the 5-HT1A promoter

To determine whether the identified Freud-1-interacting proteins are present with Freud-1 at the 5-HT1A receptor promoter, we performed quantitative ChIP experiments using Q-PCR to quantify Freud-1 recognition of its element, the 5-HT1A DRE (Figure II-4A). In HEK293 cells, the -fold enrichment of 5-HT1A DRE compared to IgG control of the following protein was: Freud-1 (1.79), Brg1 (3.82), BAF170 (2.32), BAF155 (4.81), BAF57 (1.63), Sin3A (6.06) and HDAC1 (7.82), HDAC2 (4.17) were enriched. In multiple experiments, these changes were statistically significant, except for BAF170 and BAF57. Consistent with the co-IP results, in neuronal SKNSH cells Freud-1 (2.77), Brg1 (4.82), BAF170 (12.87), BAF155 (3.04), BAF57 (8.19) were recruited to the 5-HT1A promoter region at significant levels, but not Sin3A (0.23) or HDAC1/2 (0.22 and 0.47 respectively) (Figure II-4B). Specific amplification of the 5-HT1A DRE was visualized by gel electrophoresis following semi-quantitative PCR amplification (Figure II-4C). Together these results indicate that different protein complexes are recruited to the Freud-1 binding site on the 5-HT1A promoter, depending on the cell line. In 5-HT1A-negative HEK293 cells, Freud-1, Swi/Snf and Sin3A/HDAC complexes are recruited to the 5-HT1A promoter region. In 5-HT1A-expressing neuronal SKNSH cells, the transcriptional regulation of 5-HT1A receptor gene is achieved through the recruitment of Freud-1, Swi/Snf complex without the Sin3A/HDAC complex.

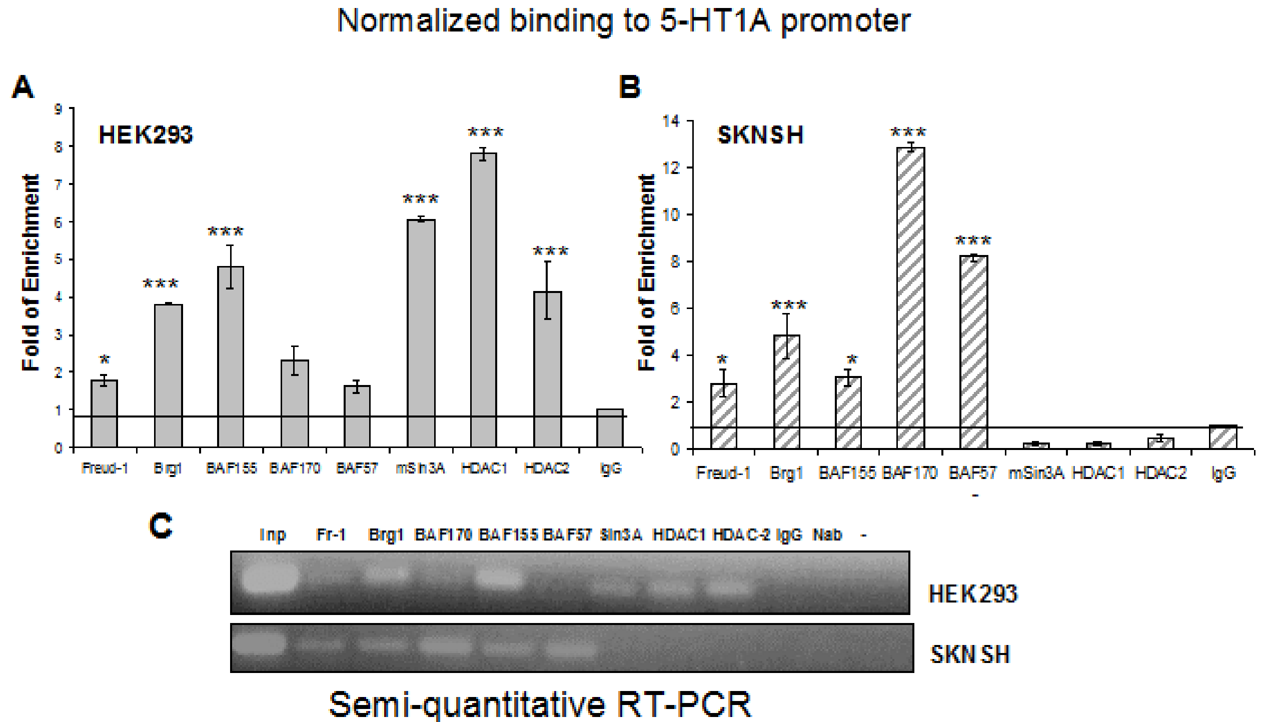


Figure II-4: Different complexes are present with Freud-1 on 5-HT1A promoter depending on the cell type. Chromatin Immunoprecipitation (ChIP) assay using indicated antibodies in HEK293 cells (**A**) and SKNSH cells (**B**) in the Freud-1-binding DRE region of 5-HT1A receptor gene was quantified by real time Q-PCR. Results are expressed as -fold of enrichment normalized to precipitation using IgG. Black line indicates level of background precipitation. Means and SEM shown are from at least three separate experiments; *p < 0.05; **p < 0.01, ***p<0.001 by one-way ANOVA with Dunnett's post-test compared to control IgG. (**C**) Semi-quantitative analysis of ChIP by RT-PCR. Samples were loaded in 2% agarose gel, non-immune rabbit IgG was used as negative control and non antibody control (Nab) was used to determine the level of background precipitation.

Freud-1 recruits Brg1 to 5-HT1A promoter region in non-neuronal cells

Brg1 is the central catalytic ATPase subunit of Swi/Snf complex that is required for its function (Trotter & Archer, 2008). We addressed whether the Swi/Snf complex is required to stabilize Freud-1 recruitment to 5-HT1A DRE site or vice-versa, by using siRNA to knock-down the expression of Freud-1 or Brg1. Western blots indicate that treatment of HEK293 cells with siRNA to Freud-1 reduced Freud-1, but not Brg1 protein expression. Conversely, when these cells were treated with siRNA to Brg1, Freud-1 protein expression was not affected (Figure II-5A, C). The same observations were made using SKNSH cells (Figure II-5B, D). Thus the siRNA treatment was effective and specific for the targeted protein.

The siRNA treatment also reduced the amount of the targeted protein (Freud-1 or Brg1) that was bound to the 5-HT1A DRE detected by ChIP in both cell lines (Figure II-6). When HEK293 cells were treated with siRNA to Freud-1, the relative -fold recruitment of the Freud-1-DRE complex was significantly reduced compared to untreated cells. Similarly, siRNA to Brg1 reduced the amount of Brg1 at the 5-HT1A DRE detected by ChIP with Brg1 antibody. When HEK293 cells were treated with siRNA to Freud-1, recruitment of both Freud-1 and Brg1 to the 5-HT1A DRE was significantly decreased, but with siRNA to Brg1 only Brg1 recruitment was reduced (Figure II-6A). The dependence of Brg1 binding to the DRE on Freud-1 suggests that Freud-1 is required for Brg1 recruitment to 5-HT1A promoter region in HEK293 cells, while Freud-1 binding appeared to be insensitive to Brg1. In neuronal SKNSH cells, treatment with siRNA to Brg1 or Freud-1 significantly reduced the recruitment of Freud-

1 and Brg1 to the promoter region (Figure II-6B), indicating that both proteins are necessary for efficient Freud-1 binding to the DRE and recruitment of Brg1 in these cells. Thus, Freud-1 is stably associated with the 5-HT1A promoter in HEK293 cells in which the gene is silenced, while it requires association with Brg1 to bind the promoter in 5-HT1A-expressing SKNSH cells.

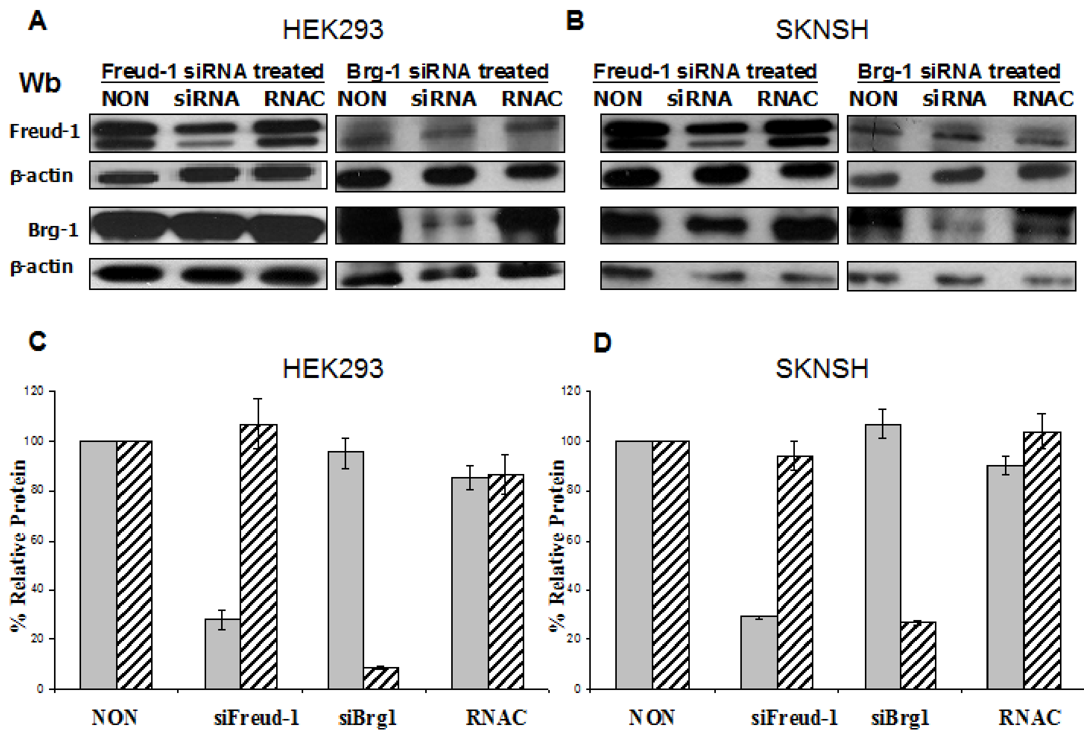


Figure II-5: Freud-1 or Brg-1 protein levels after siRNA treatment. Depletion of Freud-1 or Brg1 in HEK293 (A) or SKNSH (B) cells. Cells were treated for 72 h with 20 nM Freud-1 siRNA or 25 nM Brg1 siRNA (siRNA) or with siRNA control (RNAC) or not treated (NON). Freud-1 or Brg1 protein levels were measured by Western blot and quantified (C & D) using ImageJ software (NIH), with β-actin as loading control.

Freud-1 or Brg1 Recruitment to 5-HT1A promoter

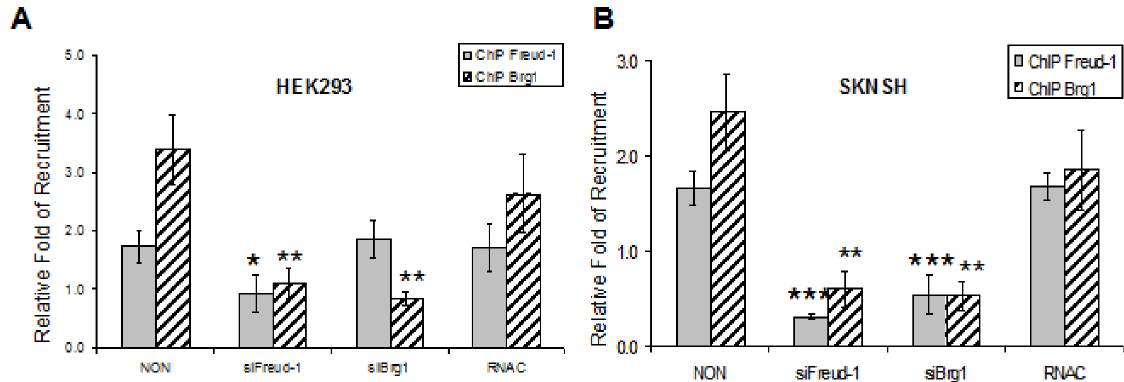


Figure II-6: Freud-1 or Brg-1 depletion by siRNA treatment decreases their recruitment to 5-HT1A promoter. ChIP assay in HEK-293 cells (**A**) or SKNSH cells (**B**). Cells were treated with Freud-1 or Brg1 siRNA and after 72 h, nuclei were isolated and quantitative ChIP assay for the 5-HT1A DRE region was done using the indicated antibodies; the level of 5-HT1A-DRE was quantified by real time Q-PCR and is expressed as -fold enrichment compared to IgG. Cells treated with Freud-1 or Brg1 siRNA show decreased recruitment of Freud-1 and Brg1, but with Brg1 siRNA in HEK293 cells no change in Freud-1 recruitment was seen. Mean ± SEM, $n \geq 3$. *$p < 0.05$; **$p < 0.01$, ***$p<0.001$. one-way ANOVA with Dunnett's post-test to compare to not-treated control (NON).

Depletion of Freud-1, Brg1 or both results in increased 5-HT1A mRNA levels

To determine the role of Brg1 in Freud-1-mediated 5-HT1A receptor gene repression we examined the effect of siRNA-mediated depletion of Brg-1 or Freud-1 on 5-HT1A receptor expression using Q-RT-PCR analysis to quantify 5-HT1A mRNA levels. In 5-HT1A-deficient HEK293 cells, 5-HT1A mRNA was not significantly altered from its background level when cells were treated with siRNA to either Freud-1 or Brg1 alone, but was dramatically increased when cells were treated with both siRNAs (Figure II-7A), indicating a redundant action of both factors maintains silencing of the 5-HT1A gene. In 5-HT1A-expressing SKNSH cells, treatment with siRNA to either Freud-1 or Brg1 up-regulated 5-HT1A receptor RNA (Figure II-7B), indicating that both factors are required for repression of the 5-HT1A receptor. The combination of Freud-1/Brg1 depletion resulted in no upregulation, suggesting that both factors are required for maximal repression, since reduction of either one increased expression of the receptor RNA as much as depleting both. This is consistent with the ChIP results, and together indicate that an interaction between Freud-1 and Brg1 is required to maintain Freud-1 binding to its element for reversible repression of the 5-HT1A receptor in neuronal cells. In HEK-293 cells, in which Freud-1 binding is independent of Brg1, the gene is silenced and requires removal of both Freud-1 and Brg1 to induce transcription.

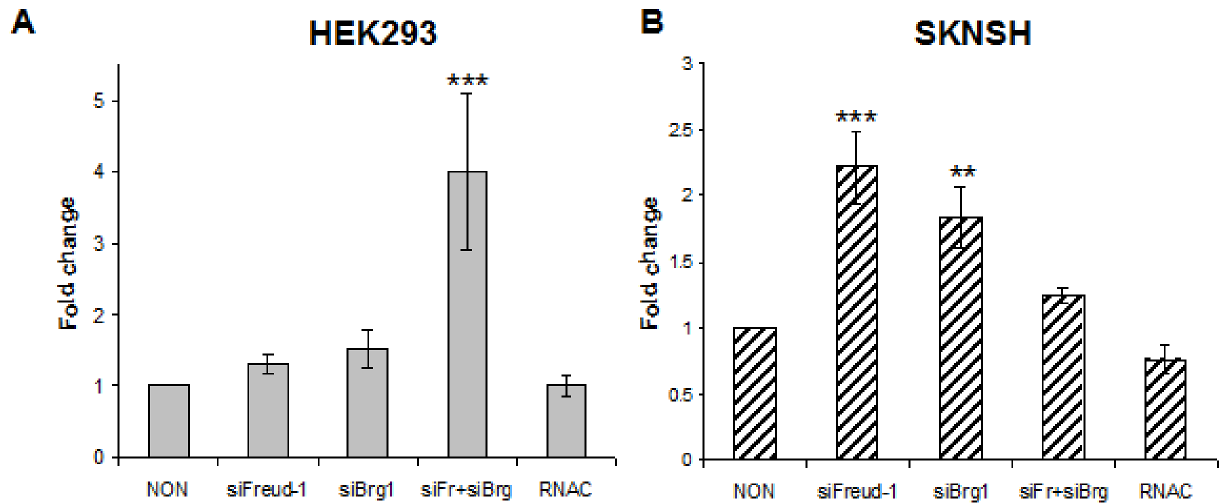


Figure II-7: Freud-1/Brg-1 complex represses 5-HT1A receptor expression. HEK293 (**A**) or SKNSH (**B**) cells were treated with the indicated siRNA (Freud-1, 20 nM; Brg1, 25 nM; control RNAC, 25 nM) or untreated (**NON**), harvested 72 h later, and relative 5-HT1A receptor mRNA levels measured using Q-RT-PCR, and presented as –fold change compared on untreated cells. The level of 5-HT1A RNA measured was 100-fold greater in SKNSH cells than in HEK293 cells. In 5-HT1A-negative HEK293 cells suppression of both Freud-1 and Brg1 induced 5-HT1A RNA, while in SKNSH cells, suppression of either was sufficient. Mean and SEM are shown from at least three independent experiments. Data were analyzed by one-way ANOVA with Dunnett's post-test compared to untreated control (NON), **p<0.01, ***p<0.001.

Discussion

Cell-specific Freud-1 complexes for 5-HT1A gene repression.

The focus of this study was to understand the transcriptional regulation of 5-HT1A receptor gene by the repressor Freud-1. Our data point towards two different mechanisms of Freud-1-induced repression, depending on the cell type. Initially, affinity purification of Freud-1-associated complexes from HEK293 nuclear extracts demonstrated the presence of proteins belonging to the Swi/Snf and Sin3A complexes. Further investigation has demonstrated that Freud-1 recruits Swi/Snf-Brg1 containing complex and Sin3A/HDAC complex to 5-HT1A promoter in HEK293 cells. In contrast, in neuronal 5-HT1A-expressing SKNSH cells, only the Swi/Snf complex, and not the Sin3A complex, was detected as a Freud-1-interacting complex. This suggests that transcriptional repression mediated by Freud-1 is HDAC-dependent in non-neuronal cells and HDAC-independent in neuronal cells. This is consistent with our previous finding that transcriptional repression mediated by Freud-1 is HDAC-dependent in non-neuronal cells and HDAC-independent in neuronal 5-HT1A-expressing cells (Lemonde et al., 2004). However, transcriptional repression of 5-HT1A promoter mediated by REST was HDAC-dependent in all cell types tested, consistent with HDAC recruitment by REST co-repressors CoRest and Sin3A.

In addition to different protein complexes, we observed differential recruitment of proteins to the Freud-1 site at the 5-HT1A promoter depending on cell type. ChIP assays (Figure II-4) showed that the levels of Freud-1 and Brg1 at the 5-HT1A DRE were similar in both HEK293 and SKNSH cells. However, the enrichment for BAF155 was

greater in HEK293 cells compared to SKNSH. Similarly, IP studies showed that BAF155 appeared to be absent from the Freud-1 complex in SKNSH compared to HEK293 cells (Figure II-2). In addition, striking differences in the enrichment of BAF170 and BAF57 in Freud-1 IPs were found, with increased levels of these proteins observed in SKNSH cells. In contrast, Sin3A, HDAC1 and HDAC2 were absent from the Freud-1 complex in SKNSH cells. Taken together, we find that transcriptional repression mediated by Freud-1 involves Swi/Snf-Brg1 and Sin3A/HDAC complexes in HEK293 cells, while in SKNSH cells, the Swi/Snf complex is involved but not the Sin3A complex. This differential recruitment could be due to different isoforms of the same protein in SKNSH neuronal cells, as shown for BAF57. The expression of BAF57 is limited by the level of BAF155/BAF170 subunits (Chen & Archer, 2005), and BAF57 has been also shown to play a role in determining Swi/Snf subunit composition (Hah et al., 2010), although it is not an essential subunit, as shown for chromatin remodeling of the CD4 gene (Wan et al., 2009). Thus, differential expression of BAF57 in HEK293 or SKNSH cells may result in different Freud-1/BRG1 complexes. In neuronal cells, BAF57 exists as at least 3 different isoforms with N-terminal truncated (Kazantseva et al., 2009), thus differential recruitment of Brg1 or Sin3A complex by neuronal BAF57 isoforms could account for the lack of Sin3A in neuronal Freud-1/Brg1 complexes.

The presence of HDAC-containing Freud-1 complexes in HEK293 cells fits with the HDAC-dependent repression at the 5-HT1A DRE by Freud-1 in these and other non-neuronal cells that do not express 5-HT1A receptors; while the lack of HDAC in the complex fits with the HDAC-independent repression observed in a variety of neuronal 5-HT1A-expressing cells (Lemonde et al., 2004). This suggests that HDAC-dependent

mechanisms are recruited by Freud-1 for long-term silencing of the 5-HT1A receptor gene. Together with our results, these studies suggest that depending on the complex recruited, Freud-1 will induce a reversible repression or long-term silencing of 5-HT1A DRE. In cells that lack 5-HT1A receptor expression, the 5-HT1A receptor gene is completely silenced. The recruitment of HDAC for transcriptional repression is known to induce an extremely compact chromatin structure, denying access to DNA binding proteins. Thus, the access to 5-HT1A DRE is strongly restricted by Freud-1 in complex with Swi/Snf and Sin3A/HDAC proteins, possibly in cooperation with REST and its multiple co-repressors, to ensure that the closed chromatin structure remains locked, although this silencing could be partly reversed by depletion of both Freud-1 and Brg1. In contrast, in 5-HT1A receptor-expressing cells, 5-HT1A receptor gene expression is present and dynamically regulated. For example, activation of calcium/CaMKII to inhibit Freud-1 induces the expression of the 5-HT1A receptor gene in neuronal, 5-HT1A receptor-expressing cells (Ou et al., 2003). Since in 5-HT1A-expressing SKNSH cells, Freud-1 recruits only Swi/Snf complex without the Sin3A/HDAC co-repressor, this may allow for dynamic regulation of receptor expression in these cells. Since the Sin3A complex forms a stable interactions with chromatin (Vermeulen et al., 2006), this complex would be expected to "shut-down" 5-HT1A receptor expression through recruitment of additional co-repressors to adopt a closed chromatin structure, and prevent dynamic gene regulation.

Freud-1 is not the only transcriptional repressor who will recruit different co-repressors depending on the cell type to induce a different chromatin complex. The classic example is REST, which can also mediate a transient repression and long-term

silencing (Ballas et al., 2005) at specific genes in different tissues through the action of different co-repressors (Ooi & Wood, 2007). In Rat-1 fibroblasts and mouse neural stem cells, REST recruits Sin3A and CoREST to the *Scn2a2* promoter, but only Sin3A is recruited at the *Stmn2* promoter (Lunyak et al., 2002; Greenway et al., 2006; Jepsen et al., 2000). The transcriptional factor NFkB is another example, where the sequence of its binding site will determine which cofactor to recruit (Leung et al., 2004).

Depletion of Freud-1, Brg1 or both proteins by siRNA treatment in HEK293 and SKNSH cells revealed that depending on cell type, different mechanisms are used to repress the 5-HT1A receptor gene (Figure II-6). In HEK293 cells, Freud-1 siRNA reduced Freud-1 protein levels and decreased both Freud-1 and Brg1 recruitment to 5-HT1A DRE promoter region. In contrast, decrease in Brg1 protein levels reduced Brg1 recruitment without affecting Freud-1 recruitment to 5-HT1A DRE, suggesting that Freud-1 is obligatory for Brg1 recruitment to the 5-HT1A-DRE in HEK293 cells. In HEK293 cells, Brg1 is probably recruited by Freud-1 to stabilize the DNA-Freud-1 interaction as it was seen for transcriptional gene repression mediated by REST (Ooi et al., 2006). This situation is different in SKNSH cells, in which a decrease in either protein was sufficient to abolish the recruitment of Freud-1 and Brg1 to 5-HT1A DRE promoter region. This result suggests that Brg1 is recruited to the 5-HT1A-DRE by Freud-1, but in turn stabilizes the binding of Freud-1 to the DRE in the chromatin context. The presence of Brg1 may enhance Freud-1 binding through its helicase activity by opening the chromatin at the DRE site. In contrast, Freud-1 appears to be strongly bound to the site in a Brg1-independent manner in HEK293 cells, perhaps through recruitment of Sin3A complex to stabilize its binding.

Differential roles of Freud-1 complexes in 5-HT1A receptor regulation

The functional aspect of Freud-1/Brg1 interaction was examined in HEK293 and SKNSH cells, in which different levels of 5-HT1A mRNA induction were observed. In HEK293 cells, inhibition of Brg1 function has been shown to induce neuronal genes expression presumably by abolishing gene repression mediated by REST (Ooi et al., 2006). No increase in 5-HT1A mRNA was observed in HEK293 cells treated with siRNA to Freud-1 or Brg1 alone, but a substantial increase was seen with the combination of both Freud-1 and Brg1 siRNA's. Depletion of Brg1 did not affect recruitment of Freud-1 to the 5-HT1A promoter (Figure II-6), and consistent with this repression of 5-HT1A gene was maintained. Since depletion of Freud-1 resulted in the absence of Freud-1 and Brg1 on 5-HT1A DRE but did not induce 5-HT1A mRNA, transcriptional repression in HEK293 cells is probably maintained through an alternative mechanism, such as by Freud-2 repression at the DRE (Hadjighassem et al., 2009), or by REST/Brg1-mediated repression via an RE-1 site adjacent to DRE site in 5-HT1A promoter (Lemonde et al., 2004; Parks & Shenk, 1996; Wissink et al., 2000). When cells are treated with siRNA to deplete both Freud-1 and Brg1, then repression by both Freud-1 and these alternate mechanisms (Freud-2 or REST) would be attenuated due to loss of Freud-1 and Brg1, leading to the increase in 5-HT1A mRNA levels. These results indicate that "silencing" is potentially reversible, but that it requires inhibition of multiple repressor mechanisms.

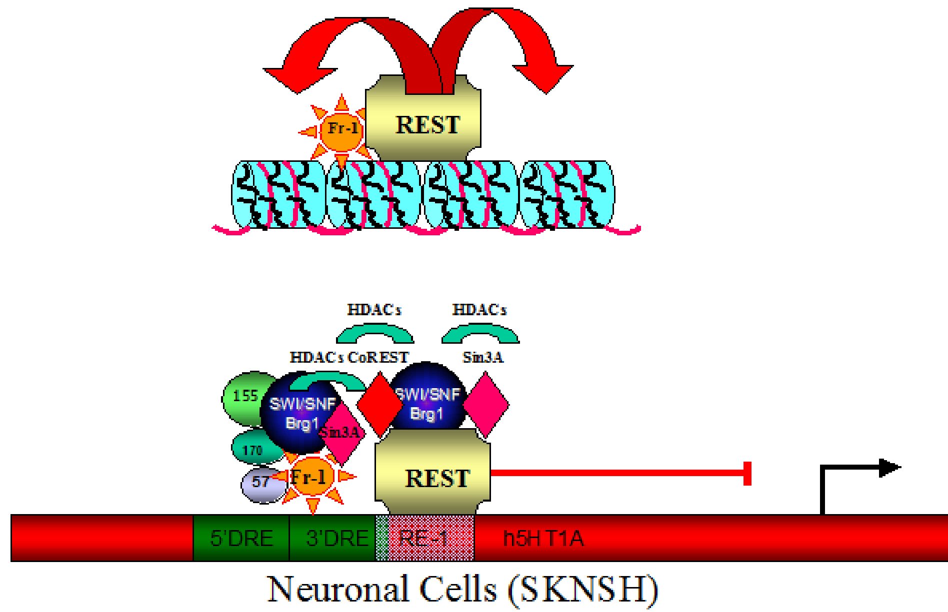
In SKNSH cells, treatment with either siRNA resulted in an increase in 5-HT1A mRNA levels since siRNA treatment with either protein abolished the recruitment of Freud-1 and Brg1 protein to the 5-HT1A DRE region of the promoter. In neuronal cells,

REST is present in small quantities and as a truncated form presumably not active (Ooi & Wood, 2007). Consequently, REST appears not to compensate for the absence of Freud-1. Surprisingly, no significant increase observed when cells were treated with siRNA to both proteins: since Brg-1 has multiple target genes, it is possible that altered regulation of another target upon Brg1 depletion might enhance Freud-1 repression or reduce promoter activity of the 5-HT1A gene. For example, in the absence of REST during neural stem cell differentiation, CoREST was able to maintain repression (Ballas et al., 2005). Similarly, upon depletion of Freud-1 and Swi/Snf proteins, other co-repressors may be able to compensate and maintain 5-HT1A receptor regulation.

Taken together, these results suggest a mechanism of 5-HT1A receptor gene repression is different in HEK293 and SKNSH cells. The possible mechanisms are summarized in Figure II-8, although other possible corepressors could be involved, such as Rb-associated proteins that were identified in the original co-IP with Freud-1. In the future, it will be interesting to investigate if they are involved and how calcium/calmodulin affects Freud-1/Brg1 complex in neuronal cells.

Non-Neuronal Cells (HEK293)

A Gene Silencing



Neuronal Cells (SKNSH)

B Transient Repression

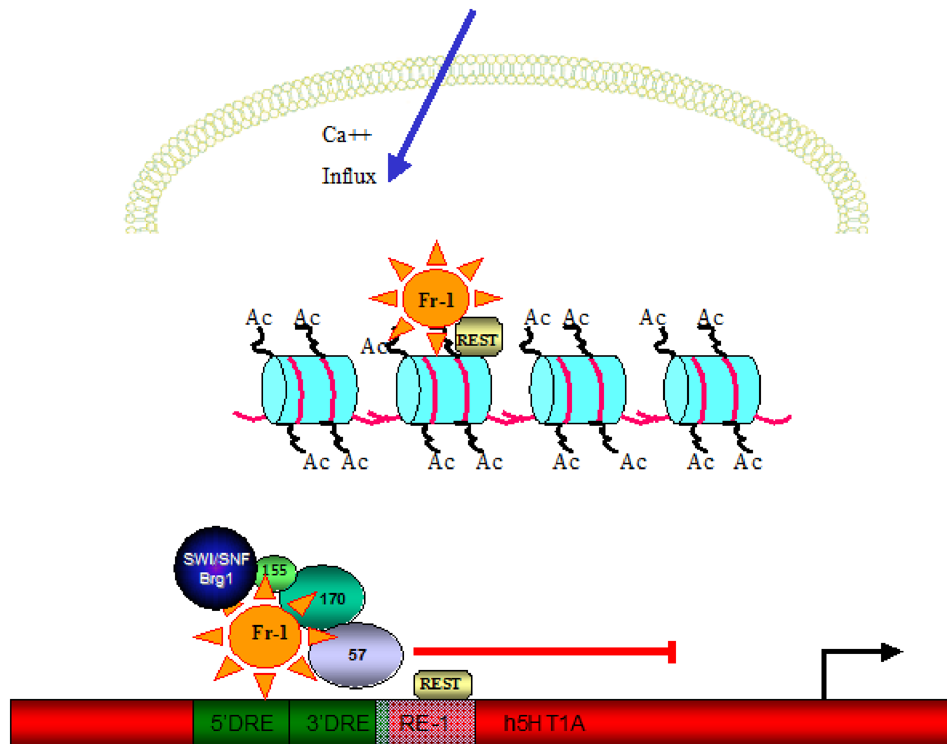


Figure II-8

Figure II-8: Transcriptional Mechanisms of Freud-1-Mediated Repression of 5-HT1A Receptor Gene. (**A**) In non-neuronal HEK293 cells, 5-HT1A receptor gene is silenced by Freud-1 and REST, the histone tails are probably deacetylated and the chromatin structure is very compact restricting all access to any possible activators. REST probably spreads gene silencing to adjacent regions (Ooi & Wood, 2007). Specifically, REST recruits HDACs through CoREST and Sin3A complexes (Ooi & Wood, 2007). Similarly, Freud-1 interaction with DRE is stabilized by Swi/Snf-Brg1 containing complex and BAF155, which allows the recruitment of Sin3A/HDAC complex leading to the deacetylation of histones. Together, Freud-1 and REST assure a complete silencing of 5-HT1A receptor gene. (**B**) In neuronal, 5-HT1A expressing SKNSH cells, REST, if any is present, is probably inactive and the repression of 5-HT1A receptor gene can be attributed to Freud-1. The chromatin structure is in a more open conformation with protruding acetylated histone tails giving the access to regulators. Specifically, Freud-1 interaction with DRE is probably stabilized through Swi/Snf-Brg1-containing complex and is possibly regulated by calcium/calmodulin through Freud-1/BAF57/BAF170 subunits. The transcriptional regulation of 5-HT1A receptor gene is HDAC-independent in this cell type.

In vivo significance:

The deletion in Freud-1 gene which introduces a frame shift mutation resulting in expression of a truncated protein was linked to NSMR. This isoform has the first three DM14 domains and lacks the rest of the sequence. In lymphoblasts of individuals affected with NSMR, this truncated form of Freud-1 is expressed. Because of the lack of the C2 domain that is implicated in Freud-1 DNA binding and repression (Ou et al., 2003), this truncated Freud-1-NSMR presumably lacks DNA binding and repressor activities (Basel-Vanagaite et al., 2006; Rogaeva et al., 2007). NSMR is a mental retardation condition without any other visible symptoms or phenotypes (Raymond & Tarpey, 2006; Ropers, 2006). This suggests that the predominant role of Freud-1 transcriptional repressor activity is necessary for proper development of cognitive function *in vivo*. Since in NSMR patients, Freud-1 is probably not repressing 5-HT1A and D2 receptor genes in neuronal tissues (Rogaeva et al., 2007), serotonergic and dopaminergic systems may be dys-regulated. However, other systems or organs do not appear to be affected in NSMR, possibly because transcriptional repression in non-neuronal cells is still maintained in NSMR patients possibly through Freud-2, REST, or co-repressors. The loss of other Freud-1 functions, such as scaffolding the Akt pathway (Nakamura et al., 2008) or Notch endocytosis (Childress et al., 2006; Jaekel et al., 2006; Gallagher et al., 2006) may also play a role in the NSMR phenotype.

Conclusion

We have shown that Freud-1 can recruit different complexes depending on the type of repression needed in a specific cell type. Freud-1 recruits Swi/Snf-Brg-1 containing and Sin3A/HDAC co-repressor complexes in non-neuronal HEK293 cells to induce long-term silencing of 5-HT1A receptor gene. For the induction of transient repression, Freud-1 recruits only Swi/Snf-Brg-1 containing complex in neuronal 5-HT1A-expressing SKNSH cells. These results suggest that different Freud-1 complexes are required for reversible repression vs. gene silencing.

CHAPTER III: Sumoylation and Lipid Binding Properties of Freud-1

1-Summary

The purpose of this chapter is to report my additional experimental approaches to address the mechanisms of Freud-1 function. Previously, it has been observed that Freud-1 appears as a doublet on the SDS-PAGE gels, differing in about 10-20kDa in size. The upper species is predominant in the nuclear fraction while the lower band is cytoplasmic (Rogaeva & Albert, 2007). These data and the fact that Freud-1 has predicted sumoylation sites in its C2 domain suggested that Freud-1 might be modified by sumoylation, which is known to be involved in negative regulation of transcription (Hay, 2005; Gill, 2005). I therefore hypothesized that Freud-1 is sumoylated and that this modification is required for its actions on transcription: either for the interaction with other proteins either for its translocation through C2 domain from cytoplasm to nucleus. I tested this hypothesis using IP and mutational approaches that indicate that Freud-1 is not modified by sumoylation. A second set of experiments was based on the finding that the C2 domain of the *Drosophila* homolog of Freud-1 (lethal giant discs, *lgd)* was reported to have lipid-binding properties important for its endosomal trafficking (Gallagher & Knoblich, 2006). Consequently, I hypothesized that the C2 domain of mammalian Freud-1 will have similar lipid binding properties as its *Drosophila* homolog. These studies present evidence that Freud-1 has similar lipid binding properties as its *Drosophila* homolog *lgd*, and report the lipid-binding properties of Freud-1 are affected by mutations of the C2 domain and CaMKII sites.

2-Introduction

2.1 Sumoylation

2.1.1 Overview

SUMOylation is a posttranslational modification of proteins involved in almost all aspects of cell development, function and senescence. This modification is critical for many neuronal processes such as neurogenesis, differentiation and development, presynaptic release, vesicle recycling and synaptic plasticity (Wilkinson, Nakamura & Henley, 2010). In mammals, SUMOylation is an essential modification as knockdown studies have shown that a deletion of Ubc9, the enzyme required for conjugation of all SUMO (small ubiquitin-related modifier) paralogues, abolishes all SUMOylation and results in abnormalities in chromosome segregation, nuclear organization and apoptosis in chicken DT40 lymophocyte cell line (Hayashi et al., 2002). In addition, Ubc9-knockout mice die at an early embryonic stage from defects in chromosomal segregation at mitosis and aberrant nuclear organization (Nacerddine et al., 2005).

2.1.2 SUMO paralogues

SUMOylation involves covalent modification of lysine residues in target proteins. SUMO or small ubiquitin-related modifier is a 97 amino acid protein (11kDa) that is covalently conjugated to lysine residues on target proteins. The enzymes involved in this enzymatic cascade are: E1 (SUMO-activating), E2 (SUMO-specific conjugating, Ubc9) and sometimes E3 (SUMO ligase, e.g. PIAS3) (Geiss-Friedlander & Melchior, 2007; Hay, 2005; Hilgarth et al., 2004; Johnson, 2004; Muller et al., 2001; Watts, 2004). The same enzymatic machinery is required for the conjugation of different SUMO paralogues

(Tatham et al., 2001). In mammals, there are four SUMO paralogues (SUMO1, SUMO-2, SUMO-3 and SUMO-4) (Bohren et al., 2004) that can modify distinct targets (Saitoh & Hinchey, 2000) or sometimes either of them (SUMO1 or SUMO2/3) can modify the same protein (Hardeland et al., 2002; Hofmann et al., 2000). When they are in their conjugable form, SUMO-2 and SUMO-3 differ only in three N-terminal residues. Theire amino acids sequences are approximately 95% identical to each other and sometimes they cannot be distinguished, consequently, they are known as SUMO2/3 (Wang & Dasso, 2009; Wilkinson & Henley, 2010). Interestingly, SUMO-4 has a restricted tissue distribution and possibly may be unable to SUMOylate target proteins. This is probably due to a proline residue, which would prevent SUMO-4 to maturate to its conjugable form (Owerbach et al., 2005). Furthermore, the SUMO-4 protein has never been detected endogenously (Wilkinson & Henley, 2010).

2.1.3 SUMOylation sites

Target proteins are SUMOylated at a consensus sequence designated ψKxD/E, where ψ is a large hydrophobic residue, K is the target lysine and D/E are acidic residues (Rodriguez et al., 2001; Sampson et al., 2001). However, it has been reported that not all proteins containing a putative SUMOylation consensus sequence are SUMOylated. In addition, many proteins have been reported to be modified at lysine residues other than those of the SUMOylation consensus site (Wilkinson, Nakamura & Henley, 2010). SUMO can be also conjugated to proteins in a non-covalent manner through the SUMO interacting motifs (SIMs) (Kerscher, 2007). SUMOylation is a rapidly growing field of study and so far, identified SIMs possess a hydrophobic core surrounded by acidic

flanking residues or serine residues that can be phosphorylated (Minty, Dumont, Kaghad & Caput, 2000; Song et al., 2004; Hannich et al., 2005; Hecker et al., 2006). Hence, SUMO can be conjugated to target proteins covalently at the SUMOylation consensus lysine sites and/or to other lysine residues. In addition, SUMO can be attached in a non-covalent manner to SIMs. Finally, SUMO can be conjugated to the same target protein via SUMO consensus site lysines or other lysines, and via SIMs.

2.1.4 Conjugation and deconjugation of SUMO

SUMO conjugation can begin after SUMO has been activated from its inactive, precursor form by a C-terminal cleavage mediated by SENP (sentrin/SUMO-specific protease) enzymes. This cleavage exposes a di-glycine motif important for SUMO conjugation to target proteins (Wilkinson & Henley, 2010). Each SUMO conjugation cycle requires SUMO protein ATP-dependent activation mediated by E1 enzyme, a heterodimer of SAE1 (SUMO activating enzyme 1) and SAE2 in mammals (Gong et al., 1999; Wilkinson & Henley, 2010). Following activation, SUMO proteins are transferred to the active site cysteine of the SUMO-specific conjugating enzyme Ubc9, which either alone or with SUMO E3 enzymes, catalyzes the conjugation of SUMO to the lysine residue in the target protein (Figure III-1) (Wilkinson & Henley, 2010).

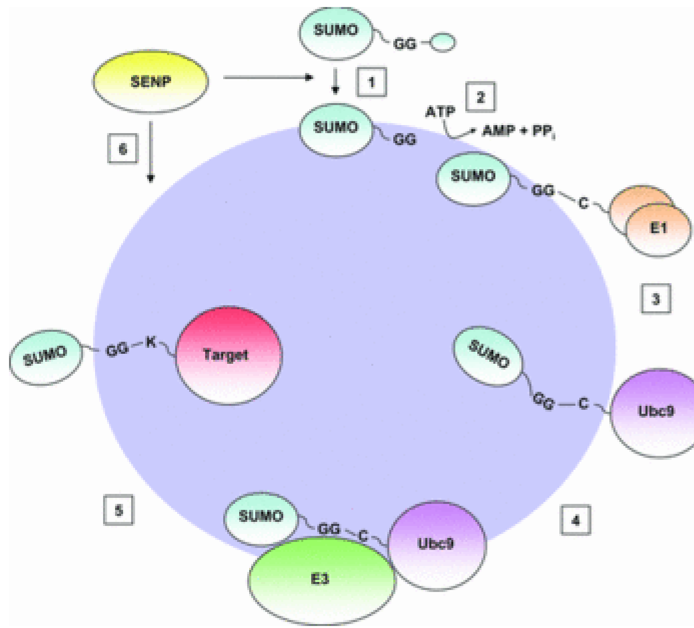


Figure III-1: Schematic representation of SUMOylation cycle. 1) SUMO precursor's C-terminal is cleaved by SENP. 2) Maturated SUMO is activated in ATP-dependent manner by heterodimer SAE1 & SAE2 in mammals. 3) SUMO proteins are passed to Ubc9. 4) Ubc9 can combine with E3 enzyme. 5) SUMO is conjugated to lysines on target proteins. 6) SUMO can be deconjugated from target proteins by the same SENP as the one that was required for maturation. Image is taken with permission from Wilkinson & Henley, 2010.

SUMOylation is a reversible protein modification. Desumoylation is mediated by the same SENP enzymes that are involved in the maturation of SUMO precursor. In mammals, there are six SENPs (SENP1, SENP2, SENP3, SENP5, SENP6, SENP7) that are organized into three groups (Yeh, 2009; Mukhopadhyay & Dasso, 2007). They differ in their cellular distribution, SUMO paralogue specificity and selectivity for SUMO maturation compared with deconjugation activities. SENP1 and SENP2 function both in the processing and deconjugation of SUMO1 and SUMO2/3 (Gong et al., 2000; Hang &

Dasso, 2002; Zhang, Saitoh & Matunis, 2002). SENP3 & SENP5 and SENP6 & SENP7 have higher specificity for SUMO2/3 (Nishida, Tanaka & Yasuda, 2000; Di Bacco et al., 2006; Gong & Yeh, 2006; Mukhopadhyay et al., 2006; Shen et al., 2009). However, SENP6 & SENP7 are not involved in maturation and deconjugation of monomeric SUMO-2/3 from target proteins, but they efficiently edit and/or deconjugate poly-SUMO-2/3 chains (Mukhopadhyay et al., 2006; Shen et al., 2009).

2.1.5 Functions of SUMOylation

The first SUMOylated protein discovered was the nuclear pore-associated GTPase activating protein RanGAP1 (Mahajan et al., 1997; Matunis, Coutavas & Blobel, 1996). Consequently, SUMOylation has been shown to be involved in many functions associated with nuclear pore complex and nucleocytoplasmic transport of proteins (Palanacade & Doye, 2008). Further studies have associated SUMOylation with protein stability, chromosome segregation and genome integrity, enzyme regulation, cellular structure and transcriptional regulation, specifically with transcriptional repression (Gill, 2004; Hay, 2005; Johnson, 2005; Seeler & Dejean, 2003; Gill, 2005; Lyst & Stancheva, 2007). Interestingly, before SUMOylation was well established, the consensus sumoylation site was thought to be a negative regulatory sequence in transcriptional factors (Iniguez-Lluhi & Pearce, 2000). The relationship of SUMO with HDACs, histones, the heterochromatin, Polycomb proteins and chromatin remodeling complexes leads to the recruitment of repressor complexes and transcriptional repression of genes (Garcia-Dominguez & Reyes, 2009). Thus, transcriptional repression of genes is the most prominent function of SUMOylation.

2.2 Lethal (2) Giant Disk (*lgd*) protein

2.2.1 Overview

Several recent studies on Notch trafficking in *Drosophila* have identified a lethal (2) giant disc (*lgd*) protein which is the ortholog of mammalian Freud-1, to be a regulator of endosomal trafficking of Notch and its activation (Jaekel & Klein, 2006; Gallagher & Knoblich, 2006; Childress et al, 2006), as well as trafficking of Delta, the Epidermal Growth Factor Receptor (EGFR) and possibly other proteins (Gallagher & Knoblich, 2006). The contention that Freud-1 has a role in endosomal trafficking of proteins is further supported by identification of Freud-1 as interacting protein with charged multivesicular body proteins CHMP 4A, 4B and 4C, proteins of the human endosomal sorting complex that are required for transport ESCRT-III (Tsang et al, 2006). Interestingly, depletion of CHMP4 (Vps32) or EAP20 (Vps25) also increases Notch signaling (Jekely & Rorth, 2003). Moreover, the murine ortholog of *lgd* functionally was able to replace *lgd* in *Drosophila* suggesting that the function is conserved among members of the *lgd* gene family (Jaekel & Klein, 2006).

2.2.2 *Lgd* genes

In *Drosophila*, imaginal disc development, in addition to other factors, depends on the *Drosophila* tumor suppressor genes (TSG). There are fifty TSG identified and they can be organized into two groups based on their mutant phenotype: tumorous and hyperplasic groups (Bryant et al., 1993; Watson et al., 1994). Deletion of tumorous TSGs

results in cells overproliferation that causes the invasion of new regions and the loss of epithelial and compartmental organization of the discs. In contrast, deletion of hyperplasic group causes over-proliferation without disturbing epithelial and compartmental organization of the discs. The lethal (2) giant disc (*lgd)* genes belong to the hyperplastic group of tumor suppressor genes (Bryant et al, 1993; Watson et al, 1994). Their loss results in massive over-proliferation of imaginal disc cells and extended larval life (Bryant & Schubiger, 1971; Bryant & Schmidt, 1990). In the case of ectopic activation of Notch, similar phenotypes to *lgd* deficiency were observed in wing development (Couso, Knust & Martinez Arias, 1995; de Celis & Bray, 2000; Diaz-Benjumea & Cohen, 1995; Klein & Martinez-Arias, 1998; Speicher et al., 1994), linking *lgd* with Notch regulation. This observation was proven to be correct and further studies have demonstrated that *lgd* act as a general repressor of Notch activity affecting also vein, eye and bristle development (Klein, 2003). Thus, *lgd* gene is involved in Notch regulation.

2.2.3 *Lgd* protein and its functions

The *lgd* gene encodes a novel 816 amino acids protein known as a regulator of endosomal trafficking (Jaekel & Klein, 2006), a protein with conserved C2 domain (Gallagher & Knoblich, 2006; Childress et al, 2006). The C2 domain is also known as calcium- and lipid-binding domain that has been linked to many functions such as protein-protein interactions, membrane recruitment, protein localization and trafficking (Nalefski & Falke, 1996; Ponting & Parker, 1996). The deletion studies of C2 domain have indicated that this domain is responsible for the binding of *lgd* to the phospholipids

present on early endosomes (Gallagher & Knoblich, 2006). In *Drosophila*, *lgd* over-expression or loss of function leads to the accumulation of Notch on endosomes and results in enlarged endosomal compartments (Gallagher & Knoblich, 2006; Childress et al, 2006; Jaekel & Klein, 2006) that contain Notch full-length receptor (Jaekel & Klein, 2006). Notch accumulates on early or late endosomes in *lgd* mutant cells in a ligand-independent manner, leading to disrupted protein sorting. However, it is not clear where exactly the Notch-*lgd* interaction occurs. In *Drosophila*, the action of *lgd* is reported to be between the actions of Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) and the late endosomal component Vps25 (EAP20) (Childress et al, 2006) which was confirmed by Gallagher and Knoblich (2006) who reported that *lgd* acts after Hrs. In conclusion, it is now well accepted by scientific community that *lgd* is a regulator of endosomal trafficking of Notch and possibly of other proteins.

2.2.4 Notch Signaling pathway

The Notch signaling pathway is an evolutionarily conserved intercellular communication process. Functions of Notch signaling include regulation of gene expression, embryonic development, cellular differentiation, cell fate determination, stem cell self-renewal, proliferation, apoptosis and angiogenesis. Aberrant Notch signaling is associated with a host of disorders (CADASIL, schizophrenia, cortical dysplasia, cancers and stroke) (Artavanis-Tsakonas et al., 1999). In animals, Notch plays a key role in both embryonic neural development and later brain plasticity. Notch pathway is essential to the proper formation and maintenance of multiple tissues and organs such as vasculoendothelial system and nervous system. Studies of the Notch pathway in mice

using gene targeting have been restricted to embryogenesis because of the resulting embryonic lethality phenotypes (Duarte et al, 2004; Hamada et al, 1999; Krebs et al, 2000, 2003; Swiatek et al, 1994; Xue et al, 1999). Notch 1 mouse mutant embryos die before E11.5 (Swiatek et al, 1994). These mutant embryos have hypoplastic brains and neural tube defects secondary to a loss of neuroblasts and premature neuronal differentiation. Over the past decade, many groups have examined mouse mutants in component of the Notch pathway, and have suggested that the Notch pathway is generally responsible for maintaining the progenitor state (Yoon & Gaiano, 2005).

The Notch gene was first characterized in *Drosophila*. The Notch gene encodes a receptor with a single transmembrane domain, which is activated by ligands such as Serrate (Ser) and Delta (Dl) (Kadesch, 2004). The interaction between receptors and ligands triggers two proteolytic events culminating in the release of Notch intracellular domain (NICD) (Kadesch, 2004). After ligand binding, Notch receptors are cleaved by ADAM10/ADAM17 and Presenillin (gamma-secretase complex) proteases, resulting in the release and translocation to the nucleus of the NICD. In the nucleus, NICD activates specific genes (ex: Hes, Hey, CHF, HRT, HERS) by interacting with the transcription factor RBPJ (recombination signal-binding protein for immunoglobulin kappa J region). The genes activated by Notch act as transcriptional regulators of other genes. For example, activation of Notch leads to the activation of the negative basic helix-loop-helix (bHLH) Hes genes, including Hes1 (mammalian hairy and Enhancer-of-Split homologue 1) (Kageyama & Nakanishi, 1997). Subsequently, Hes1 inhibits neuronal and muscle differentiation by negatively regulating other bHLH transcription factors (Sasai et al., 1992; Ishibashi et al., 1994). In spite of the apparent simplicity of this mechanism, it is

becoming clear that several mechanisms such as ubiquitination and endosomal trafficking regulate the activities of Notch and its ligands.

Notch and its ligands are transmembrane proteins whose signaling activity is influenced by vesicular trafficking. Endocytotic internalization and recycling of DSL (Delta/Serrate/Lag2) ligands in the signal-sending cell are required for successful Notch signaling (Nichols et al., 2007). Notch is usually degraded by trafficking to lysosomes. First, Notch is ubiquitinated by E3 ligase (AIP4 in human and Su[dx] in *Drosophila*) including Itch (the ubiquitin-protein isopeptide ligase) and WWP1 (WW domain containing E3 ubiquitin protein ligase 1), and internalized (Qiu et al, 2000; Jennings, 2007). The proteins of human endosomal sorting complex (ESCRT) are required for Notch processing: ESCRT-0 captures the ubiquitinated Notch and sequentially, Notch interacts with ESCRT-I, ESCRT-II and ESCRT-III. Notch is constitutively internalized into early endosomes and sorted to other endocytic compartments, such as recycling endosomes, multivesicular bodies (MVB), late endosomes and lysosomes. Each of these steps has complex effects on Notch signaling. They can promote signaling from ligand-induced Notch while preventing inappropriate signaling from the pool of inactivated Notch.

The knowledge about Notch signaling and its attenuation in endocytic compartments come from the systematic analysis of mutations inactivating endocytic regulators in *Drosophila*. Factors promoting cargo internalization from the cell surface and entry into early endosomes such as Dynamin and Rab5 (Rab-protein 5) are necessary for correct ligand-dependent Notch signaling (Lu & Bilder, 2005; Vaccari et al, 2008). In contrast, mutations in ESCRT components prevent Notch degradation and exhibit

important, ligand-independent signaling (Vaccari et al., 2008; Moberg et al, 2005; Thompson et al, 2005; Vaccari & Bilder, 2005; Herz et al., 2006). For example, inactivation of *lgd* protein results in endosomal accumulation of Notch and ectopic ligand-independent signaling activity (Childress et al, 2006; Gallagher & Knoblich, 2006; Jaekel & Klien, 2006) (Figure III-2).

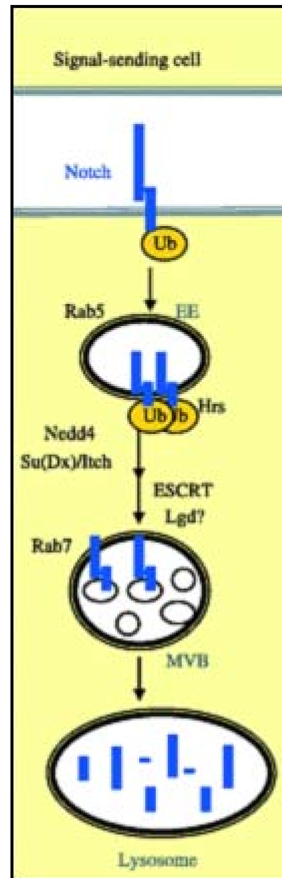


Figure III-2: Schematic representation of full-length Notch targeted to the degradative pathway independently from ligands. Once Notch has been ubiquitinated it is targeted to the degradation pathway. Full-length ligand-independent Notch is first found in early endosomes (EE) containing Hrs and Rab5. Following sequential ubiquitination by Nedd4 and Su(Dx)/Itch ubiquitin ligases, Notch is then targeted to multivesicular bodies (MVB) containing Rab7 by ESCRT proteins and *lgd*.

Consequently, Notch is recycled in lysosomes. Image is modified from Fiuza & Arias, 2007.

3-Methods

Cell Culture and Transient Transfections

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wisent, St-Bruno, Qc) supplemented with 10% Fetal Calf Serum (Wisent). Cells were grown at 37°C in 5% CO_2. Cell line was grown to 50-60% confluency and the medium was replaced 12 hours prior the transfection. Cells were transfected using calcium phosphate co-precipitation method with 5 μg of human Freud-1 expression plasmid or mutants. The cells were analyzed 72 hrs post-transfection.

Plasmids, Production and Purification of Recombinant hFreud-1 and mutants, Subcellular Fractionations

Recombinant hFreud-1 in pTRiEX-4 (Rogaeva & Albert, 2007), hFreud-1 without C2 domain and hFreud-1 CaMKII mutants were transformed into BL21 (DE3) E.coli (Novagen), grown overnight and induced at OD_{600}=0.6 with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Wisent) at 37°C for 3 hrs. Human Freud-1 and mutants were purified under native conditions using Ni-nitrilotriacetic beads (Qiagen, Mississauga, ON, Canada) and dialysed against DNA binding buffer (20mM HEPES, 0.2mM EDTA, 100mM KCl, 5% glycerol, pH 7.9).

Sumoylation mutants were made by multi-site-directed mutagenesis (Qiagen) according to manufacturer's instructions. Predicted sumoylation sites at K735, K746 and

K757 (Figure III-3A) in hFreud-1 in pTriEx4 were simultaneously mutated to R following the Qiagen protocol. hFreud-1 without C2 domain and CAMKII mutants (Figure III-4A, 5A) were generated using the QuickChange® II XL Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA))

Subcellular fractionation was performed as previously described by Czesak et al, 2006. Briefly, cells were washed 3x with PBS, harvested and allowed to pre-swell for 10min at 4°C in the extraction buffer without detergent. The cell were pelleted and lysed for 10 min on ice with complete extraction buffer (10mM KCl, 10mM Na-HEPES, pH 7.6, 1.5mM $MgCl_2$, 0.1% Nonidet P40, 0.5 mM DTT, 0.5mM spermidine, 0.15mM spermine, 1mM phenylmethylsulphonyl fluoride, 1x protease inhibitor cocktail (Roche, Laval, QC, Canada)). Lysates were centrifuged (2min, 6000xg) and supernatant reserved as the cytosolic fraction. The nuclear pellet was collected and washed 3x with wash buffer (50mM NaCl, 20mM Na-HEPES, pH 7.6, 25% glycerin, 0.2mM EDTA, 1.5 mM MgCl2, 0.5 mM DTT, 0.5mM spermidine, 0.15 mM spermine, 1mM phenylmethylsulphonyl fluoride, 1x protease inhibitor cocktail). The washed pellets were lysed in nuclear extraction buffer (500mM NaCl, 20mM Na-HEPES, pH 7.6, 25% glycerin, 0.2mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5mM spermidine, 0.15 mM spermine, 1mM phenylmethylsulphonyl fluoride, 1x protease inhibitor cocktail) for 30 min on ice. Fractions were verified by western blot for cross-contamination between nuclear and non-nuclear compartments (data not shown).

Antibodies and Western blots

Anti-hFreud-1L (1:10000), generated against bacterially expressed and purified (Ni-nitrilotriacetic beads, Qiagen) S/His-tagged human Freud-1L (Cedarlane, Hornby, ON, Canada) was used for affinity purifications, western blots and IPs (Rogaeva and Albert, 2007). For SUMO1 IPs and western blots (1:1000) was purchased from Cell Signaling, β-actin (1:10000) (Sigma).

For Western blot analysis, polyvinylidenedifluoride membranes were blocked in TBST with 5% (w/v) milk for 1hr and incubated with corresponding antibodies at 4°C overnight followed by 1hr incubation with horseradish peroxidase-linked anti-rabbit (1:5000; New England Biolabs, Pickering, ON, Canada) or anti-mouse (1:5000; Jackson Immunoresearch Laboratories, West Grove, PA, USA) secondary antibody or in the case of anti-S antibody (1:5000) (Novagen), membranes were directly washed and developed with BM chemiluminescence blotting substrate (Roche).

Pull-down Assays

For pull-down assays 40 μl of anti-S-protein agarose beads (Novagen) were added to nuclear lysates from cells transfected with SUMO1 and S-Freud-1 or S-Freud-1 mutants and incubated overnight at 4°C. The following day, beads were washed 3x with 1ml of NETIN buffer and boiled in 2x Loading buffer. The resulting elutions were tested for the presence of different proteins by Western blot analysis.

Reporter Assays

HEK293 cells were transiently transfected with 5μg of reporter construct, human 5-HT1A DRE (5'-DRE and 3'-DRE or both) placed 5' to SV40 promoter-luciferase, Freud-1 in pTriEx4, Freud-1 SUMO mutants in pTriEx4 and 5μg pCMV-βGal (Clontech, Mountain View, CA) using calcium phosphate precipitation. All plasmids were purified by maxiprep kit (Sigma, St-Louis, MO), quantified spectrophotometrically and verified by ethidium bromide staining. For reporter assays, triplicate samples were extracted using 200 μl of Reporter Lysis Buffer (Promega). The resulting supernatants were collected and assayed for luciferase activity using Promega Luciferase Assay system by VictorV5 luminometer (Perkin Elmer). All the samples were normalized to β-galactosidase activity using 4-methyl-umbelliferyl-β-D galactoside (Sigma) conversion to methylumbelliferone (λex=350nm, λem=450nm). The activity was normalized to pTriEx4 vector.

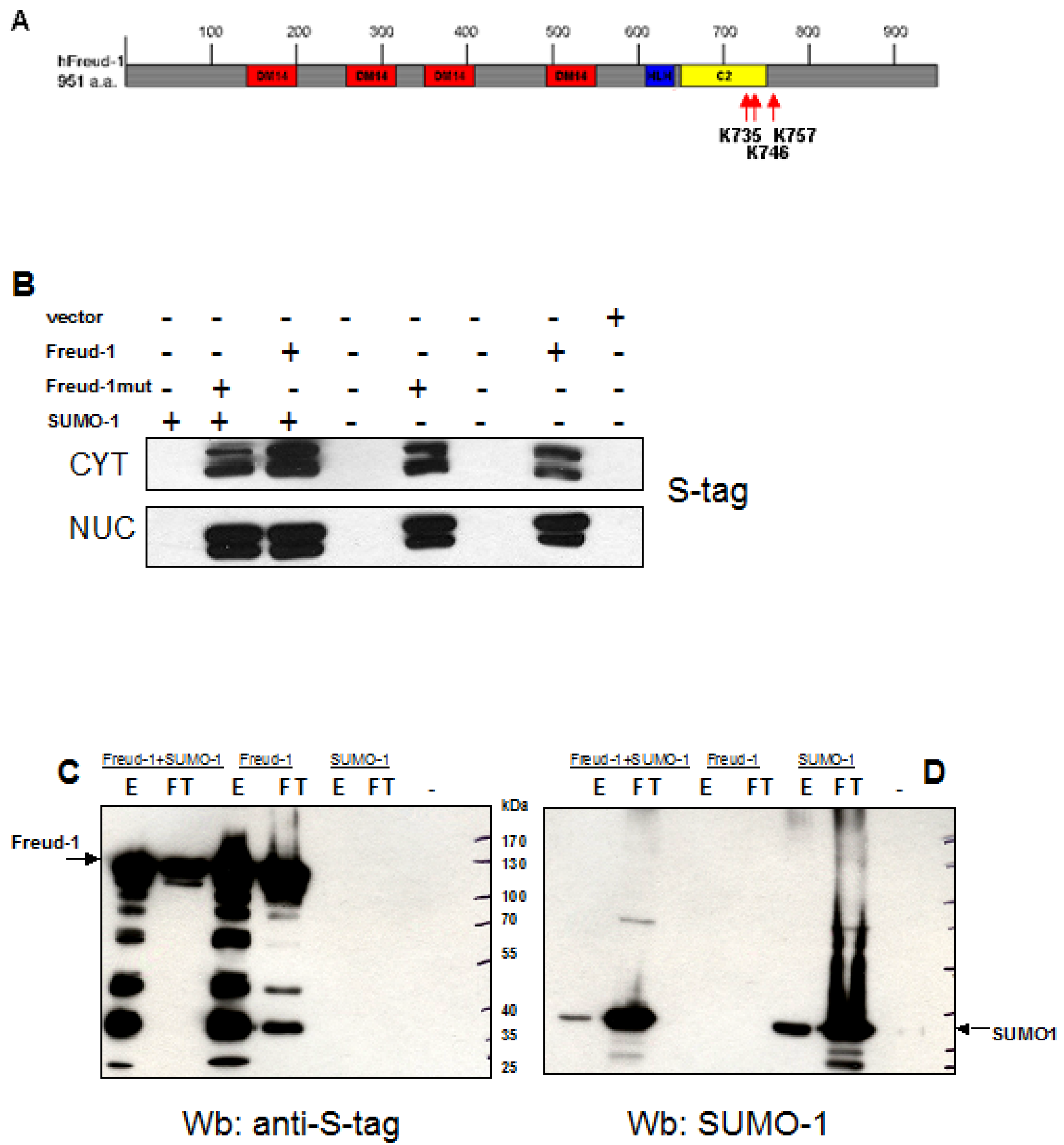
Lipid binding Assay

PIP Strips (Figure III-4B) (membranes containing 15 different phospholipid samples of 100pmol; Molecular Probes) were blocked in TBST with 0.1% ovalbumin for 1hr at room temperature and incubated with 50ng/ml of bacterially expressed and purified S-tagged hFreud-1 or S-tagged hFreud-1 mutants without C2 or CaMKII at 4°C overnight. The membranes were washed and incubated with anti-S-HRP (1:5000) antibody for 1hr. Finally, the membranes were washed and developed using BM chemiluminescence blotting substrate (Roche). Recombinant purified bacterially-expressed RASA3 protein was used as positive control for lipid binding.

4-Results

In order to test whether Freud-1 is modified by sumoylation resulting in a doublet band, Freud-1 predicted sumoylation consensus sites were mutated (lysine to arginine) to prevent their sumoylation and compared to wild-type Freud-1 for the presence of a doublet band for Freud-1 on Western blot. To address whether Freud-1 is sumoylated pull-down for SUMO1 was done; and to address functional roles of the putative sumoylation sites, the repressor activity of mutant vs. wild-type Freud-1 was assessed. Freud-1 has three predicted sumoylation sites at K735, K746 and K757 (Figure III-3A). The S-tagged Freud-1 sumoylation mutants or wild-type were transfected in HEK293 cells without or with SUMO1 and examined by Western blot using anti-S-Tag antibody (Figure III-3B). No difference between the mutants and Freud-1, with or without SUMO1, was observed for the presence of the doublet species in cytoplasmic or nuclear fractions. In pull-down assays done using anti-S-Tag and probing for S-Tag or SUMO1, no differences in the amount of SUMO1 pulled down without or with Freud-1 were observed (Figure III-3C, D), although equal amounts of Freud-1 were pulled down. The presence of SUMO1 in elutions of extracts not transfected with S-tag Freud-1 probably represents background binding of SUMO1 to the column. Finally, in HEK293 cells cotransfected with Freud-1 or mutants and 5-HT1A promoter-luciferase constructs, Freud-1 and mutants repressed 5-HT1A transcriptional activity to the same extent (Figure III-3E), indicating that the mutations did not alter the repressor activity of Freud-1. Taken together, these results indicate that Freud-1 does not appear to be modified by sumoylation, at least not at the predicted sumoylation consensus sites.

Figure III-3



E

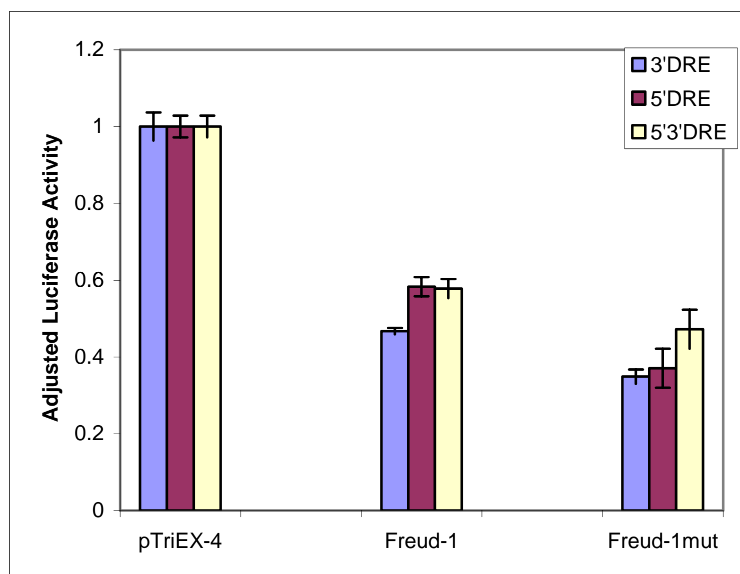


Figure III-3: *In vitro* sumoylation studies of human S-Freud-1L. (**A**) Predicted sumoylation sites at K735, K746 and K757 in human Freud-1L in pTriEx4 were all mutated by using multi-site directed mutagenesis to arginine residues (R). (**B**) The resulting mutant (Freud-1 mut) K735R, K746R, K757R was transiently transfected into HEK293 cells, without or with SUMO1. Cytosolic (**CYT**) and nuclear (**NUC**) fractions were examined by Western blot using anti-S-tag-HRP antibody and no difference was observed between Freud-1 and Freud-1mut, and both showed the presence of double band. (**C, D**) The sumoylation of Freud-1 was analyzed by anti-S pull-down assay of HEK293 cells transfected with constructs for S-tagged Freud-1, SUMO1 or both. Western blot with anti-S-tag showed the successful purification of S-Freud-1 (**C**), but when the same elution (**E**) and flow through (**FT**) fractions were analyzed by Western blot with anti-SUMO1 antibody (**D**), no difference was observed between the elution fractions of S-Freud-1/SUMO1 and SUMO1 alone, indicating a lack of sumoylation. (**E**) HEK293 cells were transiently transfected with human 5-HT1A DRE (5'-DRE and 3'-

DRE or both) placed 5' to SV40 promoter-luciferase, pCMV-βGal, and either Freud-1, Freud-1mut or vector (pTriEx4) and analyzed for luciferase activity adjusted to β-galactosidase activity and normalized to vector. The experiments were performed at least 3 times and shown as mean ± SEM. No significant difference was observed between mutants and Freud-1 analysed by one-way ANOVA with Dunnett's post-test.

The vast majority of known Notch pathway members and genetic interactions were first identified using fly genetics. In addition, Notch signaling pathway, its regulatory mechanisms and its role in neurogenesis are conserved from fly to vertebrates (de la Pompa et al, 1997). Since the C2 domain of *lgd* has been shown to have important lipid binding properties in Notch regulation in *Drosophila*, I decided to test the lipid binding properties of human Freud-1. To address the role of the C2 domain and flanking CAMK phosphorylation sites, I have compared the lipid binding properties of hFreud-1, hFreud-1 without C2 domain and hFreud-1 mutants in the CaMKII sites (Figure III-4; Figure III-5). hFreud-1 bound strongly to mono-phosphorylated inositides, and deletion of C2 domain abolished this binding, as observed for *lgd* mutants (Gallagher & Knoblich, 2006). A point mutation (P552S) shown to disrupt *lgd* function in *Drosophila* (Gallagher & Knoblich, 2006), also prevented lipid binding of hFreud-1 (Figure III-4 C). RASA3 is a good positive control since it is known to bind selectively to PI-3-P lipids (Figure III-4 D). Under lower stringency, hFreud-1 was able to also bind to bis-phosphorylated inositides, and mutation of the CaMKII sites did not alter Freud-1 lipid binding properties except for the phospho-mimic DD mutant, which lost the binding to bis-phosphorylated

inositides, but retained binding to mono-phosphorylated inositides (Figure III-5). While this lipid binding approach did not allow quantitative determination of affinity and specificity, these results indicate that mammalian Freud-1 interacts with membrane inositide lipids with a similar specificity and structural requirements as observed for *lgd*, and thus may play a role in the Notch endocytotic pathway. The observation that the Freud-1 DD mutant has an altered specificity for lipids suggests that phosphorylation of these sites could selectively affect the membrane targeting and sub-cellular localization of Freud-1.

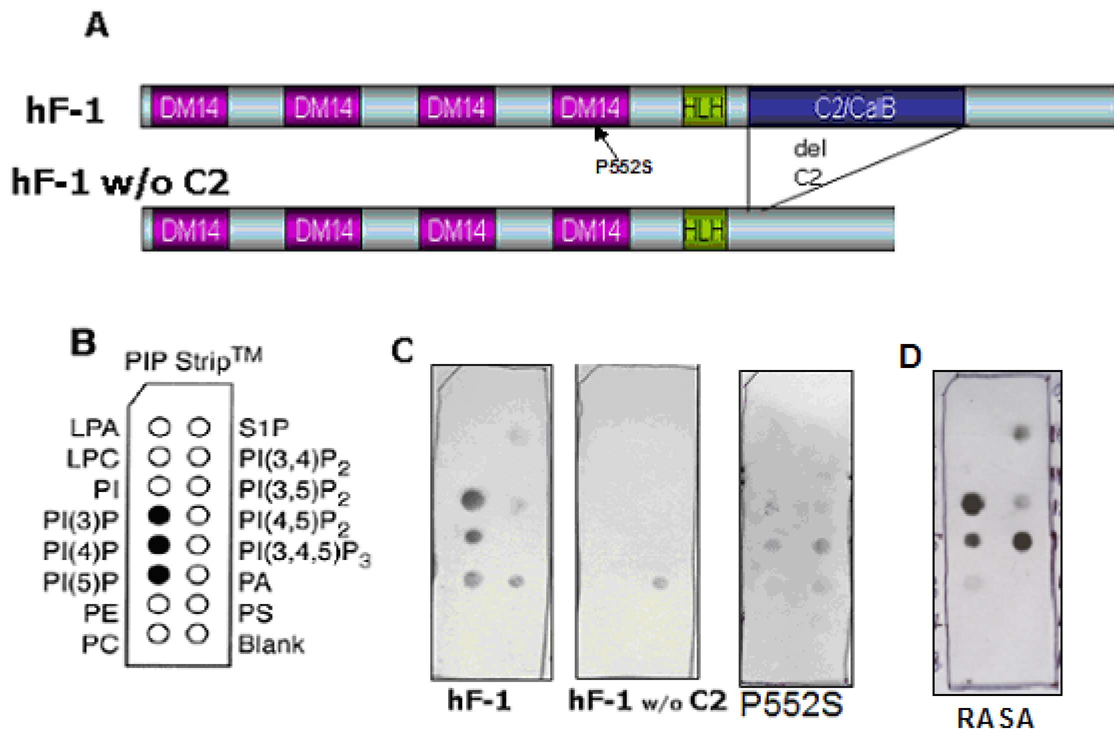


Figure III-4: Lipid binding properties of hFreud-1 (hF-1) and hFreud-1 C2 domain mutants. (**A**) Model of Freud-1, and the C2 deletion construct lacking only the C2 domain. (**B**) Schematic diagram of a PIP strip showing the lipid composition of each dot. (**C**) Lipid Binding Study. Recombinant purified bacterially-expressed hF-1 or mutant proteins were incubated with filter for 30 min, the filters washed and Freud-1 detected by blot with anti-Freud-1 antibody. Freud-1 bound strongly to mono-phosphorylated phosphatidyl inositides; deletion of the C2 domain dramatically reduced binding, the P552A point mutation also abolished binding. (**D**) Recombinant purified bacterially expressed RASA3 protein was used as positive control for lipid binding.

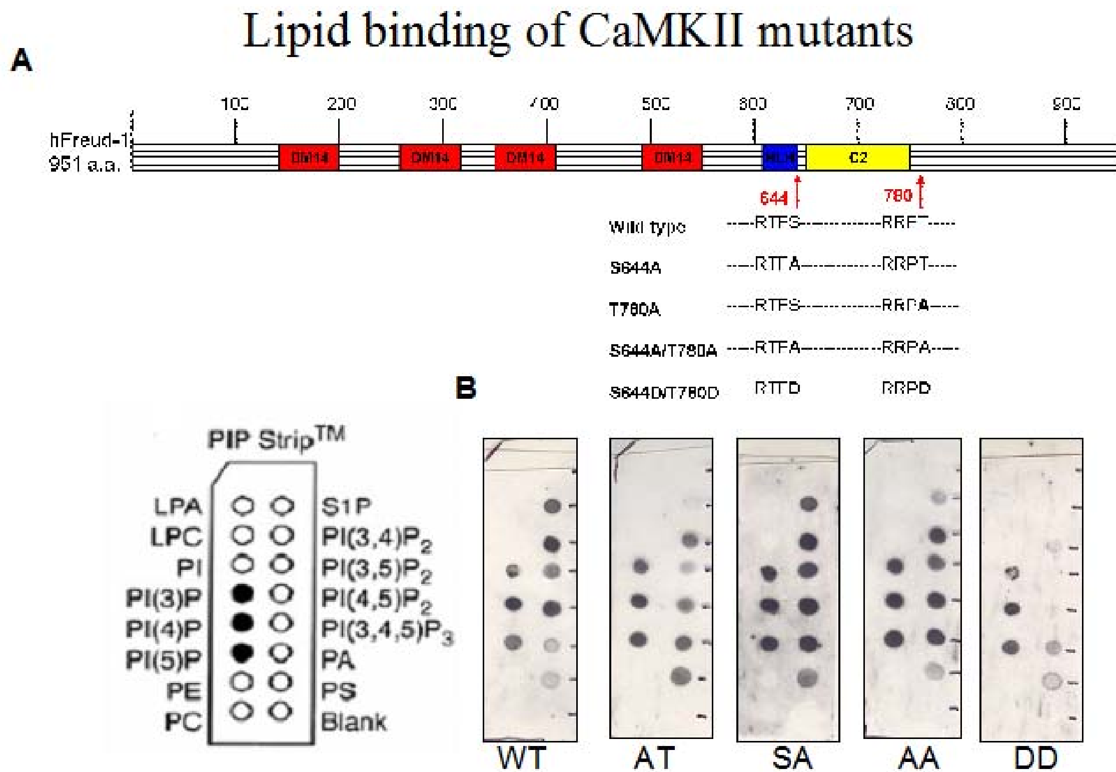


Figure III-5: Lipid binding properties of hFreud-1 (hF-1) and hFreud-1 CaMKII mutants. (A) Model of Freud-1 CaMK site mutants at sites S644 and T780. **(B)** Lipid Binding Study of bacterially-expressed hFreud-1 (**WT**) bound to both mono- and bis-phosphorylated inositides; Freud-1 mutants displayed same lipid binding properties except for the DD mutant, which displayed reduced binding to bis-phosphorylated phosphatidyl inositides.

6-Discussion

Freud-1 modification by sumoylation

Taken together, these data indicate that predicted sumoylation sites at K735, K746 and K757 are not involved in Freud-1 sumoylation. In the pull-down studies I did not detect SUMO1 conjugation to Freud-1 and it is probably not sumoylated, although we did not examine SUMO2/3. However, upon mutation of the consensus sumoylation sites the double band for Freud-1 remained, and there was no alteration in molecular weight detected by Western blot. It is possible that the slower migration of the doublet is due to some other modification such as phosphorylation. For example, a recent publication has shown that Freud-1 (also known as Aki) is a substrate for cyclinB1-cdk1, which slightly altered its migration (Nakamura, Naito, Arai & Fujita, 2010). In addition, Freud-1 is phosphorylated by CAMKII, however this did not appear to affect its migration (Galaraga et al., unpublished data). However, we cannot completely rule out a role for sumoylation of Freud-1, since proteins can be also sumoylated at the other lysines in the sequence than sumoylation consensus sites and SIMs (Wilkinson, Nakamura & Henley, 2010; Kerscher, 2007). One way to address this hypothesis would be to examine whether Freud-1 interacts with SIMs, which would detect sumoylation at any site.

The possibility that Freud-1 may be sumoylated remains interesting, since some chromatin-modifying complexes require SIMs and sumoylation sites for their architecture and function, such as NuRD and CoREST. However when these complexes are biochemically purified, the subunits that have remained assembled during the purification are not significantly sumoylated (Hay, 2005). Consequently, it is possible that at the steady state, a low proportion of the whole pool of a factor is sumoylated, leading to the

possibility that sumoylation can be a transient modification that is difficult to study biochemically. In certain cases SUMOylation has been reported to be a transient modification, the effect of which persists over a long time. For example, in neurons, only a small proportion of sumoylated GluR6 (glutamate receptor subunit 6) can be detected, but a large portion of GluR6 that undergoes dynamic sumoylation-mediated endocytosis (Martin, Nishimune, Mellor & Henley, 2007). The low detectable levels of sumoylated GluR6 can be attributed to the action of SENPs that rapidly remove SUMO from GluR6. Consequently, transient sumoylation of GluR6 mediates its endocytosis from the neuronal surface leading to a different cellular localization of GluR6. So there is GluR6 that has never been sumoylated at the surface and endocytosed GluR6 that was sumoylated and has had the SUMO removed (Wilkinson & Henley, 2010).

It will be very interesting to further explore the possibility of Freud-1 modification by sumoylation because Freud-1 is a transcriptional repressor that is present in the nucleus and cytoplasm. Sumoylation of transcriptional repressors such as Sp3 (stimulating protein 3) and LEF-1 (lymphoid enhancer-binding factor 1) causes their relocation to nuclear subdomains (condensed chromatin) that are associated with silencing of transcriptional activity (Sachdev et al., 2001; Ross, Best, Zon & Gill, 2002). In addition, sumoylation of transcription factors can lead to the recruitment of chromatin-modifying proteins, such as HDACs (Yang & Sharrocks, 2004) that will induce a repressive transcriptional environment that persists after removal of SUMO from the substrate (Wilkinson & Henley, 2010).

In conclusion, it is reasonable to think that although Freud-1 was found to be not sumoylated at the predicted sumoylation consensus sites in the present studies, Freud-1

might be sumoylated at the other lysine sites or at SIMs if there are any present. Moreover, sumoylation is a transient modification difficult to study because of the high activities of SENPs. It is possible that only small portion of Freud-1 is sumoylated and we are not able to detect it. The challenge in studying sumoylation of proteins by affinity-based approaches, as used in the present study, is due to the low steady-state levels of sumoylation *in vivo*. In order to test if Freud-1 is sumoylated we can use different approaches. For example, we can use the *in vitro* expression cloning (IVEC) method for SUMO target identification that circumvents the current challenges and complements the affinity-based approaches. This method allows for immediate validation and analysis of substrates through *in vitro* reconstitution (Gocke & Yu, 2009). In summary, more sensitive approaches may be useful to determine whether Freud-1 is sumoylated or not.

Lipid binding properties of Freud-1 C2 domain

Previous studies had shown that truncation of *lgd* at residue 664 (*lgd*ΔC2) lacking the C2 domain and C-terminal that fails to bind phospholipids. Here we found that specific deletion of the C2 domain of Freud-1 was sufficient to block phospholipid binding, clearly implicating the C2 domain in this function. The C2 domain is known as a calcium-phospholipid binding domain, and the C2 domain of Freud-1, seems to have similar lipid-binding properties as *lgd*. Another mutant (lgd^{24}) had a P557S point mutation, but was not further characterized for lipid binding or Notch endocytosis. Here we show that the analogous mutation (P552S) in Freud-1 completely blocked lipid binding, consistent with a role for lipid binding in the Notch signaling phenotype in *Drosophila*. These data suggest that mammalian Freud-1 might be also involved in

endosomal trafficking of proteins or down-regulation of receptors (e.g., the EGF receptor, see Gallagher & Knoblich, 2006) that might be important in development (e.g., Notch). It would be interesting to investigate further this hypothesis. One interesting difference was that Freud-1 did bind to bis-phosphoinositides, while *lgd* did not appear to. Bis-phosphoinositides are localized at the plasma membrane, consistent with additional functions of Freud-1 in receptor signaling. Recently Freud-1 has been shown to mediate Akt signaling through scaffolding with PDK1 (Nakamura, Naito, Tsuruo & Fujita, 2008), which is activated by the 3'-phosphorylated bis- and tris-phosphoinositides. Thus, lipid binding to bis-phosphoinositides such as PI(3, 4)P2 may recruit Freud-1 to the plasma membrane for its signaling function. Interestingly, the P552 residue is located within the DM14-4 domain, which was essential for Freud-1 mediated Akt activation, consistent with a role for lipid binding in the Akt scaffolding function of Freud-1. It is not known whether phosphorylation of CAMKII sites alters the Akt scaffolding function of Freud-1 as might be predicted from the lack of bis-phosphoinositide binding of the DD-Freud-1 phospho-mimic mutant.

Although initially, mammalian Freud-1 was identified to be a transcriptional repressor of serotonin 5-HT1A (Ou et al., 2003) and dopamine D2 receptor (Rogaeva et al., 2007) genes, it is not the only endosomal protein that would have other roles than endosomal functions. For example, similar to ESCRT-II subunits, mammalian ESCRT-III subunits were originally identified to have functions unrelated to endosomal trafficking functions (Slagsvold et al., 2006). Initially, chromatin modifying protein 1 (CHMP1) was isolated as a nuclear matrix protein controlling chromatin structure and cell cycle progression (Stauffer et al., 2001) and in later studies CHMP1 was found to be required

for correct endocytic membrane traffic (Howard et al., 2001). Consequently, CHMP was renamed charged multivesicular body protein. The function of CHMP1 in gene silencing is still remaining unchallenged, suggesting that the canonical mammalian subunits have a role in this process as it is seen for Freud-1. Taken together these reports strongly suggest that mammalian Freud-1 might be also involved in endosomal trafficking of Notch and maybe other proteins possibly by interacting with them as seen for EGFR receptor in mammalian cells (Nakamura, Naito, Tsuruo & Fujita, 2008).

In conclusion, the present studies suggest that the C2 domain of Freud-1 has important lipid binding properties similar to those identified in *Drosophila*. This provides a rationale for future studies possibly of the involvement of Freud-1 in Notch pathways or endocytic trafficking of receptors. Finally, I report lipid binding properties of Freud-1 mutants in CaMKII sites that can be used in the characterization of Freud-1 mutants studies.

CHAPTER IV: Conclusions & Future Perspectives

Conclusions

The current studies propose a dual mechanism of transcriptional repression of 5-HT1A receptor gene mediated by Freud-1. Depending on the cell type, Freud-1 recruits different co-repressors to mediate its action on 5-HT1A receptor gene. In non-neuronal HEK293 cells, Freud-1 recruits Swi/Snf and Sin3A/HDAC complexes to induce a very compact chromatin structure in the 5-HT1A DRE region leading to gene silencing. In contrast, in neuronal 5-HT1A receptor expressing SKNSH cells, Freud-1 mediates the transient repression through the recruitment of the Swi/Snf complex but not the Sin3A/HDAC components, providing access to potential gene regulation. These results suggest that the HDAC-dependent mechanism recruited by Freud-1 will be required for long-term silencing of the 5-HT1A receptor gene in non-neuronal cells, while HDAC-independent transient repression will allow dynamic regulation of 5-HT1A receptor gene in neuronal cells, for example by calcium/CaMKII regulated Freud-1. Additional studies of Freud-1 repression and repressor complexes in other cell types would be required to address how general this HDAC-dependent mechanism of cell type specific regulation is.

Moreover, Freud-1 is not the only transcriptional repressor known to recruit different co-repressors depending on the cell type in order to induce different chromatin complex. The classic example is REST, which can also mediate a transient repression and long-term silencing depending on the cell needs. Thus, the transcriptional repression of 5-HT1A receptor gene mediated by Freud-1 could be HDAC-dependent leading to gene

silencing and HDAC-independent involving transient repression depending on the cell needs.

The additional studies on Freud-1 posttranslational modification by SUMOylation indicate that Freud-1 is not SUMOylated. In addition, the importance of the C2 domain for lipid binding was demonstrated by analyzing the lipid binding properties of Freud-1 mutants where the lipid binding was decreased or abolished.

Future Perspectives

The current studies present the evidence that Freud-1 recruits different co-repressors to mediate the 5-HT1A receptor gene repression. Only two cell types were examined, the non-neuronal HEK293 cells and neuronal 5-HT1A expressing SKNSH cells. It would be interesting to test some additional cell types for example neuronal, but not 5-HT1A expressing cell type. In addition, it will be important to address the mechanism of interaction between Freud-1 and Brg1 proteins. In order to verify if the interaction is direct we can use *in-vitro* transcription studies, GST-pull-down assays and FRET. To dissect which domains are responsible for Freud-1/Brg1 interaction, the mutational approach can be used. We would expect that Freud-1 might be interacting with Brg1 through its HLH domain since this domain has been shown to be important for protein-protein interactions (Latchman, 1998). The bromodomain of Brg1 has been shown to interact with REST (Trotter & Archer, 2008) and this is why we would expect that this domain may also interact with Freud-1. The interaction between Freud-1 and

Brg1 is possibly necessary for stabilizing the Freud-1/DRE interaction as it was shown for REST/RE-1 (Ooi & Wood, 2007).

Moreover, it will be important to address which additional cofactors interact with Freud-1. It has been shown that REST recruits multiple factors to mediate its action (Ooi & Wood, 2007). This is why it is reasonable to expect that Freud-1 may also recruit multiple cofactors. To address with which other proteins Freud-1 interacts we could employ affinity approach combined to Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) method. This quantitative mass spectrometry method is useful in reliably distinguishing between specific and non-specific interactions from the beginning of the identification process (Trinkle-Mulcahy et al., 2008).

In addition, the present study has identified Rb-associated proteins to be Freud-1 interacting proteins. It will be important to validate this additional interaction by co-IPs and pull-down assays. Since Rb-associated proteins are the part of Sin3A complex, we could expect that this interaction will be validated.

In the present study, no evidence that Freud-1 is sumoylated at its predicted sumoylation consensus sites was found. But, it will be interesting to examine Freud-1 for interaction with SIMs. In addition, the lipid binding properties of C2 domain could be further explored. For example, we could examine their importance for nuclear shuttling of Freud-1 or receptor endocytosis.

In conclusion, the present thesis proposes the mechanisms of transcriptional regulation of 5-HT1A receptor gene by Freud-1 involves the recruitment of Swi/Snf and Sin3A/HDAC complexes in HEK293 cells to induce gene silencing. In contrast, in SKNSH cells, Freud-1 recruits only Swi/Snf complex to mediate 5-HT1A receptor gene

transient repression. Additional results present the evidence that Freud-1 is not sumoylated at its predicted consensus sumoylation sites. Freud-1 and Freud-1 mutants lipid binding properties are also described in this thesis. The involvement of Freud-1 in mental illnesses such as depression seems to be indirect through dysregulation of 5-HT1A receptor genes. Currently, it is unknown if Freud-1 is involved in any other diseases than NSMR. These studies provide a background for better understanding of molecular mechanisms of depression, NSMR and possibly other diseases involving Notch. The major aim of these studies was to provide a background for the development of better strategies in the treatment of these diseases in order to improve people's lives.

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APPENDICES

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Figures I-5 & I-11

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Figure I-7

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Table 1: Potential Freud-1-interacting proteins identified by Mass Spectrometry. The total of 280 proteins were identified, 84 proteins were eliminated from the study because they are known to belong to sepharose bead proteome.

Identified Proteins	Accession #	# Unique peptides
SFPQ_HUMAN Splicing factor, proline- and glutamine-rich [Homo sapiens (Human)]	P23246 (+1)	33
DDB1_HUMAN DNA damage-binding protein 1 [Homo sapiens (Human)]	Q16531	54
A8K008_HUMAN cDNA FLJ78387 [Homo sapiens (Human)]	A8K008 (+10)	3
Q05DF2_HUMAN SF3A2 protein (Fragment) [Homo sapiens (Human)]	Q05DF2 (+1)	17
SERPH_HUMAN Serpin H1 [Homo sapiens (Human)]	P50454	21
KU70_HUMAN ATP-dependent DNA helicase 2 subunit 1 [Homo sapiens (Human)]	P12956	25
A8K9W7_HUMAN cDNA FLJ77440, highly similar to Homo sapiens general transcription factor II, i (GTF2I), transcript variant 2, mRNA [Homo sapiens (Human)]	A8K9W7 (+4)	31
SMCE1_HUMAN SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1 [Homo sapiens (Human)]	Q969G3 (+1)	15
GRP78_HUMAN 78 kDa glucose-regulated protein [Homo sapiens (Human)]	P11021	25
IF2B1_HUMAN Insulin-like growth factor 2 mRNA-binding protein 1 [Homo sapiens (Human)]	Q9NZI8	25
A6NEM2_HUMAN Uncharacterized protein HCFC1 [Homo sapiens (Human)]	A6NEM2 (+1)	25
ELAV1_HUMAN ELAV-like protein 1 [Homo sapiens (Human)]	Q15717	21
C2D1A_HUMAN Coiled-coil and C2 domain-containing protein 1A [Homo sapiens (Human)]	Q6P1N0	23
KRR1_HUMAN KRR1 small subunit processome component homolog [Homo sapiens (Human)]	Q13601	23
ALDOA_HUMAN Fructose-bisphosphate aldolase A [Homo sapiens (Human)]	P04075	16
ROA2_HUMAN Heterogeneous nuclear ribonucleoproteins A2/B1 [Homo sapiens (Human)]	P22626	15
Q17RS0_HUMAN SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1 [Homo sapiens (Human)]	Q17RS0 (+2)	17
LSD1_HUMAN Lysine-specific histone demethylase 1 [Homo sapiens (Human)]	O60341	23
RFC4_HUMAN Replication factor C subunit 4 [Homo sapiens (Human)]	P35249	25
Q53HV2_HUMAN Chaperonin containing TCP1, subunit 7 (Eta) variant (Fragment) [Homo sapiens (Human)]	Q53HV2 (+1)	26

Identified Proteins	Accession #	# Unique peptides
TCPD_HUMAN T-complex protein 1 subunit delta [Homo sapiens (Human)]	P50991 (+1)	19
DNJA1_HUMAN DnaJ homolog subfamily A member 1 [Homo sapiens (Human)]	P31689	16
A8K984_HUMAN cDNA FLJ78259 [Homo sapiens (Human)]	A8K984 (+3)	24
YBOX1_HUMAN Nuclease-sensitive element-binding protein 1 [Homo sapiens (Human)]	P67809 (+2)	9
MO4L2_HUMAN Mortality factor 4-like protein 2 [Homo sapiens (Human)]	Q15014 (+1)	14
DDX21_HUMAN Nucleolar RNA helicase 2 [Homo sapiens (Human)]	Q9NR30	9
B2R5R9_HUMAN cDNA, FLJ92591, highly similar to Homo sapiens regulatory factor X, 5 (influences HLA class IIexpression) (RFX5), mRNA [Homo sapiens (Human)]	B2R5R9 (+2)	12
STRAP_HUMAN Serine-threonine kinase receptor-associated protein [Homo sapiens (Human)]	Q9Y3F4	11
SMC1A_HUMAN Structural maintenance of chromosomes protein 1A [Homo sapiens (Human)]	Q14683	15
B2RD27_HUMAN cDNA, FLJ96428, highly similar to Homo sapiens proteasome (prosome, macropain) 26S subunit, non-ATPase, 7 (Mov34 homolog) (PSMD7), mRNA [Homo sapiens (Human)]	B2RD27 (+1)	16
A6NL74_HUMAN Uncharacterized protein FUBP1 [Homo sapiens (Human)]	A6NL74 (+5)	16
PSIP1_HUMAN PC4 and SFRS1-interacting protein [Homo sapiens (Human)]	O75475	12
SRP72_HUMAN Signal recognition particle 72 kDa protein [Homo sapiens (Human)]	O76094	18
WDR82_HUMAN WD repeat-containing protein 82 [Homo sapiens (Human)]	Q6UXN9	11
RAI14_HUMAN Ankycorbin [Homo sapiens (Human)]	Q9P0K7 (+3)	12
RUVB2_HUMAN RuvB-like 2 [Homo sapiens (Human)]	Q9Y230	14
RUVB1_HUMAN RuvB-like 1 [Homo sapiens (Human)]	Q9Y265	14
RBBP5_HUMAN Retinoblastoma-binding protein 5 [Homo sapiens (Human)]	Q15291 (+1)	10
LA_HUMAN Lupus La protein [Homo sapiens (Human)]	P05455	12
Q96HL7_HUMAN EPB41L3 protein (Fragment) [Homo sapiens (Human)]	Q96HL7 (+3)	2
A8K4H1_HUMAN cDNA FLJ78268, highly similar to Homo sapiens fusion (involved in t(12;16) in malignant liposarcoma), transcript variant 1, mRNA [Homo sapiens (Human)]	A8K4H1 (+5)	8
SRFB1_HUMAN Serum response factor-binding protein 1 [Homo sapiens (Human)]	Q8NEF9	13
TOP1_HUMAN DNA topoisomerase 1 [Homo sapiens (Human)]	P11387	9
SNW1_HUMAN SNW domain-containing protein 1 [Homo sapiens (Human)]	Q13573 (+1)	13
LYAR_HUMAN Cell growth-regulating nucleolar protein [Homo sapiens (Human)]	Q9NX58	13

Identified Proteins	Accession #	# Unique peptides
A6NJ42_HUMAN Uncharacterized protein BUB3 (BUB3 budding uninhibited by benzimidazoles 3 homolog (Yeast), isoform CRA_a) [Homo sapiens (Human)]	A6NJ42 (+1)	9
SEPT9_HUMAN Septin-9 [Homo sapiens (Human)]	Q9UHD8 (+2)	7
A8MTP9_HUMAN Uncharacterized protein DDX52 [Homo sapiens (Human)]	A8MTP9 (+1)	10
PUR6_HUMAN Phosphoribosylaminoimidazole carboxylase [Homo sapiens (Human)]	P22234	8
SGF29_HUMAN SAGA-associated factor 29 homolog [Homo sapiens (Human)]	Q96ES7	9
PLRG1_HUMAN Pleiotropic regulator 1 [Homo sapiens (Human)]	O43660 (+1)	11
UFD1_HUMAN Ubiquitin fusion degradation protein 1 homolog [Homo sapiens (Human)]	Q92890 (+2)	8
DDX41_HUMAN Probable ATP-dependent RNA helicase DDX41 [Homo sapiens (Human)]	Q9UJV9	10
MRT4_HUMAN mRNA turnover protein 4 homolog [Homo sapiens (Human)]	Q9UKD2	11
B1ANR0_HUMAN Poly(A) binding protein, cytoplasmic 4 (Inducible form) (Poly(A) binding protein, cytoplasmic 4 (Inducible form), isoform CRA_e) [Homo sapiens (Human)]	B1ANR0 (+4)	7
FUBP2_HUMAN Far upstream element-binding protein 2 [Homo sapiens (Human)]	Q92945 (+2)	4
A8K622_HUMAN cDNA FLJ75871, highly similar to Homo sapiens staufen, RNA binding protein (STAU), transcript variant T3, mRNA [Homo sapiens (Human)]	A8K622 (+3)	8
TCPQ_HUMAN T-complex protein 1 subunit theta [Homo sapiens (Human)]	P50990 (+2)	9
A8K7D9_HUMAN cDNA FLJ78270, highly similar to Homo sapiens karyopherin alpha 2 (RAG cohort 1, importin alpha 1)(KPNA2), mRNA [Homo sapiens (Human)]	A8K7D9 (+4)	9
A8K4K2_HUMAN cDNA FLJ78025 [Homo sapiens (Human)]	A8K4K2 (+1)	9
P66B_HUMAN Transcriptional repressor p66-beta [Homo sapiens (Human)]	Q8WXI9	6
RFA1_HUMAN Replication protein A 70 kDa DNA-binding subunit [Homo sapiens (Human)]	P27694	7
Q53GY0_HUMAN Plastin 3 variant (Fragment) [Homo sapiens (Human)]	Q53GY0 (+1)	8
NCALD_HUMAN Neurocalcin-delta [Homo sapiens (Human)]	P61601	3
TAF4_HUMAN Transcription initiation factor TFIID subunit 4 [Homo sapiens (Human)]	O00268 (+2)	7
A6NCR2_HUMAN Uncharacterized protein ENSP00000343748 (HCG33299, isoform CRA_a) [Homo sapiens (Human)]	A6NCR2 (+3)	8
ECHA_HUMAN Long chain 3-hydroxyacyl-CoA dehydrogenase [Homo sapiens (Human)]	P40939	9
PSME3_HUMAN Proteasome activator complex subunit 3 [Homo sapiens (Human)]	P61289	7
PINX1_HUMAN Pin2-interacting protein X1 [Homo sapiens (Human)]	Q96BK5	7

Identified Proteins	Accession #	# Unique peptides
Q5VZJ7_HUMAN General transcription factor IIIC, polypeptide 4, 90kDa (General transcription factor IIIC, polypeptide 4, 90kDa, isoform CRA_b) [Homo sapiens (Human)]	Q5VZJ7 (+1)	6
RAD50_HUMAN DNA repair protein RAD50 [Homo sapiens (Human)]	Q92878 (+1)	6
A1A508_HUMAN PRSS3 protein [Homo sapiens (Human)]	A1A508 (+10)	2
KI67_HUMAN Antigen KI-67 [Homo sapiens (Human)]	P46013 (+1)	2
A6NHB7_HUMAN Uncharacterized protein RPL3 [Homo sapiens (Human)]	A6NHB7 (+2)	7
A6NFL0_HUMAN Uncharacterized protein ENSP00000306791 [Homo sapiens (Human)]	A6NFL0 (+1)	4
RFC2_HUMAN Replication factor C subunit 2 [Homo sapiens (Human)]	P35250 (+1)	5
NOMO3_HUMAN Nodal modulator 3 [Homo sapiens (Human)]	P69849 (+4)	7
DNJA2_HUMAN DnaJ homolog subfamily A member 2 [Homo sapiens (Human)]	O60884	7
MTA2_HUMAN Metastasis-associated protein MTA2 [Homo sapiens (Human)]	O94776	7
Q53EM5_HUMAN Transketolase variant (Fragment) [Homo sapiens (Human)]	Q53EM5 (+1)	7
B2R791_HUMAN cDNA, FLJ93335, highly similar to Homo sapiens PRP3 pre-mRNA processing factor 3 homolog (yeast) (PRPF3), mRNA [Homo sapiens (Human)]	B2R791 (+3)	7
RBM14_HUMAN RNA-binding protein 14 [Homo sapiens (Human)]	Q96PK6	8
MYST1_HUMAN Probable histone acetyltransferase MYST1 [Homo sapiens (Human)]	Q9H7Z6	5
Q9Y520_HUMAN Putative uncharacterized protein (HBxAg transactivated protein 2) [Homo sapiens (Human)]	Q9Y520 (+3)	2
A8K818_HUMAN cDNA FLJ75784, highly similar to Homo sapiens CD3E antigen, epsilon polypeptide associated protein (CD3EAP), mRNA [Homo sapiens (Human)]	A8K818	4
B2RBR9_HUMAN cDNA, FLJ95650, highly similar to Homo sapiens karyopherin (importin) beta 1 (KPNB1), mRNA [Homo sapiens (Human)]	B2RBR9 (+1)	4
T2EB_HUMAN Transcription initiation factor IIE subunit beta [Homo sapiens (Human)]	P29084	6
EXOS2_HUMAN Exosome complex exonuclease RRP4 [Homo sapiens (Human)]	Q13868	6
CNOT7_HUMAN CCR4-NOT transcription complex subunit 7 [Homo sapiens (Human)]	Q9UIV1 (+1)	6
A8K9K8_HUMAN cDNA FLJ75823, highly similar to Homo sapiens dimethyladenosine transferase (HSA9761), mRNA [Homo sapiens (Human)]	A8K9K8 (+1)	5
A6NE29_HUMAN Uncharacterized protein COPE (Coatomer protein complex, subunit epsilon, isoform CRA_e) [Homo sapiens (Human)]	A6NE29 (+3)	5
PP1G_HUMAN Serine/threonine-protein phosphatase PP1-gamma catalytic subunit [Homo sapiens (Human)]	P36873 (+1)	5
Q5U8W9_HUMAN Protein arginine methyltransferase 1 isoform 4 [Homo sapiens (Human)]	Q5U8W9 (+3)	4
HPBP1_HUMAN Hsp70-binding protein 1 [Homo sapiens (Human)]	Q9NZL4	3

Identified Proteins	Accession #	# Unique peptides
A8K4B4_HUMAN cDNA FLJ78441, highly similar to Homo sapiens nucleolar and spindle associated protein 1 (NUSAP1),mRNA [Homo sapiens (Human)]	A8K4B4 (+5)	4
A2A2Q9_HUMAN Chromosome 20 open reading frame 4 [Homo sapiens (Human)]	A2A2Q9 (+1)	6
RCOR1_HUMAN REST corepressor 1 [Homo sapiens (Human)]	Q9UKL0	6
C1QBP_HUMAN Complement component 1 Q subcomponent-binding protein, mitochondrial [Homo sapiens (Human)]	Q07021	5
B2R9I9_HUMAN cDNA, FLJ94417, highly similar to Homo sapiens WD repeat domain 57 (U5 snRNP specific) (WDR57), mRNA [Homo sapiens (Human)]	B2R9I9 (+1)	5
GBLP_HUMAN Guanine nucleotide-binding protein subunit beta-2-like 1 [Homo sapiens (Human)]	P63244 (+1)	4
TERF2_HUMAN Telomeric repeat-binding factor 2 [Homo sapiens (Human)]	Q15554	5
DNJC8_HUMAN DnaJ homolog subfamily C member 8 [Homo sapiens (Human)]	O75937	4
A8KAM9_HUMAN Peptidyl-prolyl cis-trans isomerase [Homo sapiens (Human)]	A8KAM9 (+2)	3
Q96F88_HUMAN Processing of 1, ribonuclease P/MRP subunit (S. cerevisiae) [Homo sapiens (Human)]	Q96F88 (+1)	4
TPP1_HUMAN Tripeptidyl-peptidase 1 [Homo sapiens (Human)]	O14773 (+3)	3
EF1G_HUMAN Elongation factor 1-gamma [Homo sapiens (Human)]	P26641 (+1)	4
B2R7X3_HUMAN cDNA, FLJ93645, highly similar to Homo sapiens chromatin assembly factor 1, subunit B (p60) (CHAF1B),mRNA [Homo sapiens (Human)]	B2R7X3 (+1)	4
A8MVJ7_HUMAN Uncharacterized protein SCFD1 [Homo sapiens (Human)]	A8MVJ7 (+2)	4
B2R713_HUMAN cDNA, FLJ93224 [Homo sapiens (Human)]	B2R713 (+1)	4
CDC5L_HUMAN Cell division cycle 5-like protein [Homo sapiens (Human)]	Q99459	5
B2RAX6_HUMAN cDNA, FLJ95176, Homo sapiens CGI-48 protein (CGI-48), mRNA [Homo sapiens (Human)]	B2RAX6 (+1)	5
VP26A_HUMAN Vacuolar protein sorting-associated protein 26A [Homo sapiens (Human)]	O75436	5
A8MUT5_HUMAN Uncharacterized protein DKC1 [Homo sapiens (Human)]	A8MUT5 (+2)	5
SNUT2_HUMAN U4/U6.U5 tri-snRNP-associated protein 2 [Homo sapiens (Human)]	Q53GS9	5
NXF1_HUMAN Nuclear RNA export factor 1 [Homo sapiens (Human)]	Q9UBU9	4
ECHB_HUMAN 3-ketoacyl-CoA thiolase [Homo sapiens (Human)]	P55084	4
A8K8J9_HUMAN cDNA FLJ77785 (Dynactin 2 (P50), isoform CRA_b) [Homo sapiens (Human)]	A8K8J9 (+4)	4
Q05D08_HUMAN PA2G4 protein (Fragment) [Homo sapiens (Human)]	Q05D08 (+2)	4
SNF5_HUMAN SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1 [Homo sapiens (Human)]	Q12824 (+3)	4

Identified Proteins	Accession #	# Unique peptides
A8MTG3_HUMAN Uncharacterized protein ZNF207 [Homo sapiens (Human)]	A8MTG3 (+4)	4
A6NCZ7_HUMAN Uncharacterized protein MAP7D3 [Homo sapiens (Human)]	A6NCZ7 (+3)	4
VAT1_HUMAN Synaptic vesicle membrane protein VAT-1 homolog [Homo sapiens (Human)]	Q99536	4
CDK7_HUMAN Cell division protein kinase 7 [Homo sapiens (Human)]	P50613	2
TR150_HUMAN Thyroid hormone receptor-associated protein 3 [Homo sapiens (Human)]	Q9Y2W1	3
A8JZY9_HUMAN cDNA FLJ78587 [Homo sapiens (Human)]	A8JZY9 (+4)	3
RBBP4_HUMAN Histone-binding protein RBBP4 [Homo sapiens (Human)]	Q09028	3
IMDH2_HUMAN Inosine-5'-monophosphate dehydrogenase 2 [Homo sapiens (Human)]	P12268	2
B0QYK0_HUMAN Ewing sarcoma breakpoint region 1 [Homo sapiens (Human)]	B0QYK0 (+5)	3
SR140_HUMAN U2-associated protein SR140 [Homo sapiens (Human)]	O15042 (+2)	2
B2R9U2_HUMAN cDNA, FLJ94557, highly similar to Homo sapiens FK506 binding protein 4, 59kDa (FKBP4), mRNA [Homo sapiens (Human)]	B2R9U2 (+1)	3
Q86T56_HUMAN Putative uncharacterized protein DKFZp451A123 [Homo sapiens (Human)]	Q86T56 (+1)	2
SAMD1_HUMAN Atherin [Homo sapiens (Human)]	Q6SPF0 (+1)	4
DDX5_HUMAN Probable ATP-dependent RNA helicase DDX5 [Homo sapiens (Human)]	P17844	4
WDR5_HUMAN WD repeat-containing protein 5 [Homo sapiens (Human)]	P61964	4
P15927-3 Isoform 3 of P15927 [Homo sapiens (Human)]	P15927-3 (+3)	4
A8K489_HUMAN cDNA FLJ76151, highly similar to Homo sapiens Wilms tumor 1 associated protein (WTAP), transcript variant 1, mRNA [Homo sapiens (Human)]	A8K489 (+1)	4
DHX15_HUMAN Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15 [Homo sapiens (Human)]	O43143	4
WDR68_HUMAN WD repeat-containing protein 68 [Homo sapiens (Human)]	P61962	3
NAT10_HUMAN N-acetyltransferase 10 [Homo sapiens (Human)]	Q9H0A0 (+1)	3
SERA_HUMAN D-3-phosphoglycerate dehydrogenase [Homo sapiens (Human)]	O43175 (+1)	3
B2R7M3_HUMAN cDNA, FLJ93510, highly similar to Homo sapiens JTV1 gene (JTV1), mRNA [Homo sapiens (Human)]	B2R7M3 (+1)	3
A8K670_HUMAN cDNA FLJ75703, highly similar to Homo sapiens nitric oxide synthase interacting protein (NOSIP), mRNA [Homo sapiens (Human)]	A8K670 (+1)	3
DHX9_HUMAN ATP-dependent RNA helicase A [Homo sapiens (Human)]	Q08211	2
A5D8W6_HUMAN Mortality factor 4 like 1 [Homo sapiens (Human)]	A5D8W6 (+2)	3
Q5IRN2_HUMAN DAZAP1/MEF2D fusion protein [Homo sapiens (Human)]	Q5IRN2 (+2)	3

Identified Proteins	Accession #	# Unique peptides
CPSF7_HUMAN Cleavage and polyadenylation specificity factor subunit 7 [Homo sapiens (Human)]	Q8N684 (+1)	3
B2R7V4_HUMAN cDNA, FLJ93619, highly similar to Homo sapiens PRP4 pre-mRNA processing factor 4 homolog (yeast) (PRPF4), mRNA [Homo sapiens (Human)]	B2R7V4 (+3)	3
K1967_HUMAN Protein KIAA1967 [Homo sapiens (Human)]	Q8N163 (+1)	2
O75100_HUMAN D-HSCDK2 [Homo sapiens (Human)]	O75100 (+3)	3
A8MSK1_HUMAN Lysyl-tRNA synthetase [Homo sapiens (Human)]	A8MSK1 (+3)	2
RPAC1_HUMAN DNA-directed RNA polymerases I and III subunit RPAC1 [Homo sapiens (Human)]	O15160 (+2)	2
ACLY_HUMAN ATP-citrate synthase [Homo sapiens (Human)]	P53396 (+1)	3
HCC1_HUMAN Nuclear protein Hcc-1 [Homo sapiens (Human)]	P82979 (+1)	3
A6NCD2_HUMAN Uncharacterized protein CCT6A [Homo sapiens (Human)]	A6NCD2 (+3)	3
A6NG42_HUMAN Uncharacterized protein TPD52L2 (Fragment) [Homo sapiens (Human)]	A6NG42 (+7)	3
ODP2_HUMAN Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial [Homo sapiens (Human)]	P10515 (+2)	3
OFC2B_HUMAN Oligonucleotide/oligosaccharide-binding fold-containing protein 2B [Homo sapiens (Human)]	Q9BQ15	3
LG3BP_HUMAN Galectin-3-binding protein [Homo sapiens (Human)]	Q08380 (+1)	3
RING2_HUMAN E3 ubiquitin-protein ligase RING2 [Homo sapiens (Human)]	Q99496	3
A8K414_HUMAN Malate dehydrogenase [Homo sapiens (Human)]	A8K414 (+2)	3
B2RDC1_HUMAN cDNA, FLJ96547, highly similar to Homo sapiens nucleostemin (NS), mRNA [Homo sapiens (Human)]	B2RDC1 (+2)	3
TALDO_HUMAN Transaldolase [Homo sapiens (Human)]	P37837	3
TOE1_HUMAN Target of EGR1 protein 1 [Homo sapiens (Human)]	Q96GM8	3
FA50A_HUMAN Protein FAM50A [Homo sapiens (Human)]	Q14320	2
Q5VU20_HUMAN SERPINE1 mRNA binding protein 1 [Homo sapiens (Human)]	Q5VU20 (+4)	2
IPYR_HUMAN Inorganic pyrophosphatase [Homo sapiens (Human)]	Q15181	2
DBPA_HUMAN DNA-binding protein A [Homo sapiens (Human)]	P16989	2
Q9BZZ5-2 Isoform 2 of Q9BZZ5 [Homo sapiens (Human)]	Q9BZZ5-2 (+1)	2
XRCC1_HUMAN DNA-repair protein XRCC1 [Homo sapiens (Human)]	P18887 (+2)	2
BCCIP_HUMAN BRCA2 and CDKN1A-interacting protein [Homo sapiens (Human)]	Q9P287 (+2)	2
CPSF6_HUMAN Cleavage and polyadenylation specificity factor subunit 6 [Homo sapiens (Human)]	Q16630 (+2)	2
M6PBP_HUMAN Mannose-6-phosphate receptor-binding protein 1 [Homo sapiens (Human)]	O60664 (+1)	2
P53_HUMAN Cellular tumor antigen p53 [Homo sapiens (Human)]	P04637 (+8)	2
SP130_HUMAN Histone deacetylase complex subunit SAP130 [Homo sapiens (Human)]	Q9H0E3 (+1)	2

Identified Proteins	Accession #	# Unique peptides
IF2B3_HUMAN Insulin-like growth factor 2 mRNA-binding protein 3 [Homo sapiens (Human)]	O00425	2
CCD22_HUMAN Coiled-coil domain-containing protein 22 [Homo sapiens (Human)]	O60826	2
TCRG1_HUMAN Transcription elongation regulator 1 [Homo sapiens (Human)]	O14776 (+1)	2
SRP54_HUMAN Signal recognition particle 54 kDa protein [Homo sapiens (Human)]	P61011	2
IGBP1_HUMAN Immunoglobulin-binding protein 1 [Homo sapiens (Human)]	P78318	2
STCH_HUMAN Stress 70 protein chaperone microsome-associated 60 kDa protein [Homo sapiens (Human)]	P48723 (+1)	2
A6NIG8_HUMAN Uncharacterized protein EIF4E2 [Homo sapiens (Human)]	A6NIG8 (+2)	2
IPO5_HUMAN Importin-5 [Homo sapiens (Human)]	O00410 (+1)	2
Q5JXM0_HUMAN Putative uncharacterized protein DKFZp564C0482 [Homo sapiens (Human)]	Q5JXM0 (+2)	2
B2RDH6_HUMAN cDNA, FLJ96613, highly similar to Homo sapiens vacuolar protein sorting 33A (yeast) (VPS33A), mRNA [Homo sapiens (Human)]	B2RDH6 (+2)	2
Q5T7G5_HUMAN Phosphoserine aminotransferase 1 (Phosphoserine aminotransferase 1, isoform CRA_a) [Homo sapiens (Human)]	Q5T7G5 (+1)	2
A4GYY8_HUMAN Septin (Fragment) [Homo sapiens (Human)]	A4GYY8 (+6)	2
CV028_HUMAN UPF0027 protein C22orf28 [Homo sapiens (Human)]	Q9Y3I0	2
B2RC06_HUMAN cDNA, FLJ95791, highly similar to Homo sapiens aurora kinase B (AURKB), mRNA [Homo sapiens (Human)]	B2RC06 (+1)	2
A6NE09_HUMAN Uncharacterized protein ENSP00000346598 [Homo sapiens (Human)]	A6NE09 (+3)	2
A8K4X1_HUMAN cDNA FLJ77520, highly similar to Homo sapiens brain and reproductive organ-expressed (TNFRSF1A modulator) (BRE), transcript variant 2, mRNA [Homo sapiens (Human)]	A8K4X1 (+6)	2
AMOT_HUMAN Angiomotin [Homo sapiens (Human)]	Q4VCS5 (+2)	2
CTBP1_HUMAN C-terminal-binding protein 1 [Homo sapiens (Human)]	Q13363 (+2)	2
VPS45_HUMAN Vacuolar protein sorting-associated protein 45 [Homo sapiens (Human)]	Q9NRW7	2
ORC2_HUMAN Origin recognition complex subunit 2 [Homo sapiens (Human)]	Q13416	2
Q53ES5_HUMAN MUS81 endonuclease homolog (Fragment) [Homo sapiens (Human)]	Q53ES5 (+2)	2
A8K7Q2_HUMAN cDNA FLJ77848 [Homo sapiens (Human)]	A8K7Q2	2

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HSP7C_HUMAN Heat shock cognate 71 kDa protein [Homo sapiens (Human)]	P11142	59
HSP71_HUMAN Heat shock 70 kDa protein 1 [Homo sapiens (Human)]	P08107	45
NUCL_HUMAN Nucleolin [Homo sapiens (Human)]	P19338	47
PARP1_HUMAN Poly [ADP-ribose] polymerase 1 [Homo sapiens (Human)]	P09874	66
PABP1_HUMAN Polyadenylate-binding protein 1 [Homo sapiens (Human)]	P11940	39
ILF3_HUMAN Interleukin enhancer-binding factor 3 [Homo sapiens (Human)]	Q12906	48
ROA1_HUMAN Heterogeneous nuclear ribonucleoprotein A1 [Homo sapiens (Human)]	P09651 (+1)	28
NPM_HUMAN Nucleophosmin [Homo sapiens (Human)]	P06748	24
ILF2_HUMAN Interleukin enhancer-binding factor 2 [Homo sapiens (Human)]	Q12905 (+1)	29
A8K525_HUMAN cDNA FLJ76817, highly similar to Homo sapiens non-POU domain containing, octamer-binding (NONO), mRNA [Homo sapiens (Human)]	A8K525 (+1)	29
RS3_HUMAN 40S ribosomal protein S3 [Homo sapiens (Human)]	P23396	29
SF3A1_HUMAN Splicing factor 3 subunit 1 [Homo sapiens (Human)]	Q15459	30
HNRPR_HUMAN Heterogeneous nuclear ribonucleoprotein R [Homo sapiens (Human)]	O43390	25
keratin 1 [Homo sapiens]	UPI000013CD4D	26
K1C10_HUMAN Keratin, type I cytoskeletal 10 [Homo sapiens (Human)]	P13645 (+1)	20
EF1A1_HUMAN Elongation factor 1-alpha 1 [Homo sapiens (Human)]	P68104 (+4)	18
HNRPD_HUMAN Heterogeneous nuclear ribonucleoprotein D0 [Homo sapiens (Human)]	Q14103 (+1)	23
THOC4_HUMAN THO complex subunit 4 [Homo sapiens (Human)]	Q86V81	11
ACTB_HUMAN Actin, cytoplasmic 1 [Homo sapiens (Human)]	P60709 (+4)	19
Q53F64_HUMAN Heterogeneous nuclear ribonucleoprotein AB isoform a variant (Fragment) [Homo sapiens (Human)]	Q53F64 (+3)	18
PTBP1_HUMAN Polypyrimidine tract-binding protein 1 [Homo sapiens (Human)]	P26599 (+2)	14
HNRPQ_HUMAN Heterogeneous nuclear ribonucleoprotein Q [Homo sapiens (Human)]	O60506 (+1)	13
HNRPG_HUMAN Processed heterogeneous nuclear ribonucleoprotein G [Homo sapiens (Human)]	P38159	15
B2R984_HUMAN cDNA, FLJ94268, highly similar to Homo sapiens histone 1, H1e (HIST1H1E), mRNA [Homo sapiens (Human)]	B2R984 (+4)	8
K22E_HUMAN Keratin, type II cytoskeletal 2 epidermal [Homo sapiens (Human)]	P35908	11
B2RCM1_HUMAN cDNA, FLJ96154, highly similar to Homo sapiens heat shock 70kDa protein 9B (mortalin-2) (HSPA9B),nuclear gene encoding mitochondrial protein, mRNA [Homo sapiens (Human)]	B2RCM1 (+2)	19
B0AZQ4_HUMAN cDNA, FLJ79494, highly similar to Structural maintenance of chromosome 3 [Homo sapiens (Human)]	B0AZQ4 (+2)	21

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K1C9_HUMAN Keratin, type I cytoskeletal 9 [Homo sapiens (Human)]	P35527	10
HNRPU_HUMAN Heterogeneous nuclear ribonucleoprotein U [Homo sapiens (Human)]	Q00839 (+1)	12
A8K6U7_HUMAN cDNA FLJ78252, highly similar to Homo sapiens heterogeneous nuclear ribonucleoprotein U-like 1 (HNRPUL1), transcript variant 1, mRNA [Homo sapiens (Human)]	A8K6U7 (+2)	9
A4D208_HUMAN Eukaryotic translation initiation factor 3, subunit 9 eta, 116kDa (Eukaryotic translation initiation factor 3, subunit 9 eta, 116kDa, isoform CRA_a) [Homo sapiens (Human)]	A4D208 (+4)	16
RL6_HUMAN 60S ribosomal protein L6 [Homo sapiens (Human)]	Q02878 (+3)	10
RS2_HUMAN 40S ribosomal protein S2 [Homo sapiens (Human)]	P15880 (+2)	15
PABP2_HUMAN Polyadenylate-binding protein 2 [Homo sapiens (Human)]	Q86U42	7
RL1D1_HUMAN Ribosomal L1 domain-containing protein 1 [Homo sapiens (Human)]	O76021 (+2)	13
A8MXP9_HUMAN Uncharacterized protein MATR3 [Homo sapiens (Human)]	A8MXP9 (+1)	10
ROA3_HUMAN Heterogeneous nuclear ribonucleoprotein A3 [Homo sapiens (Human)]	P51991 (+1)	7
ROA0_HUMAN Heterogeneous nuclear ribonucleoprotein A0 [Homo sapiens (Human)]	Q13151	10
B1AHM0_HUMAN DEAD (Asp-Glu-Ala-Asp) box polypeptide 17 (DEAD (Asp-Glu-Ala-Asp) box polypeptide 17, isoform CRA_c) [Homo sapiens (Human)]	B1AHM0 (+5)	10
HS90B_HUMAN Heat shock protein HSP 90-beta [Homo sapiens (Human)]	P08238	12
A8KAP3_HUMAN cDNA FLJ78483, highly similar to Homo sapiens elongation factor Tu GTP binding domain containing 2 (EFTUD2), mRNA [Homo sapiens (Human)]	A8KAP3 (+3)	11
MYH4_HUMAN Myosin-4 [Homo sapiens (Human)]	Q9Y623	11
HNRPM_HUMAN Heterogeneous nuclear ribonucleoprotein M [Homo sapiens (Human)]	P52272 (+1)	8
RCC1_HUMAN Regulator of chromosome condensation [Homo sapiens (Human)]	P18754 (+2)	9
G3BP2_HUMAN Ras GTPase-activating protein-binding protein 2 [Homo sapiens (Human)]	Q9UN86	7
RS6_HUMAN 40S ribosomal protein S6 [Homo sapiens (Human)]	P62753 (+1)	9
PCBP1_HUMAN Poly(rC)-binding protein 1 [Homo sapiens (Human)]	Q15365	7
ENOA_HUMAN Alpha-enolase [Homo sapiens (Human)]	P06733 (+2)	9
MAP4_HUMAN Microtubule-associated protein 4 [Homo sapiens (Human)]	P27816 (+9)	7
RL8_HUMAN 60S ribosomal protein L8 [Homo sapiens (Human)]	P62917	6
HS71L_HUMAN Heat shock 70 kDa protein 1L [Homo sapiens (Human)]	P34931 (+1)	3
PDIA1_HUMAN Protein disulfide-isomerase [Homo sapiens (Human)]	P07237	6
EIF3I_HUMAN Eukaryotic translation initiation factor 3 subunit I [Homo sapiens (Human)]	Q13347	8

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A8K2Y2_HUMAN cDNA FLJ78120, highly similar to Homo sapiens eukaryotic translation initiation factor 2, subunit 3 gamma, 52kDa (EIF2S3), mRNA [Homo sapiens (Human)]	A8K2Y2 (+1)	7
A8K485_HUMAN cDNA FLJ76788, highly similar to Homo sapiens splicing factor 3b, subunit 2, 145kDa (SF3B2), mRNA [Homo sapiens (Human)]	A8K485 (+5)	6
HSP72_HUMAN Heat shock-related 70 kDa protein 2 [Homo sapiens (Human)]	P54652	3
PDIA3_HUMAN Protein disulfide-isomerase A3 [Homo sapiens (Human)]	P30101	6
B0YJC4_HUMAN Vimentin variant 3 [Homo sapiens (Human)]	B0YJC4 (+2)	3
B2R7B5_HUMAN cDNA, FLJ93365, highly similar to Homo sapiens KH domain containing, RNA binding, signal transduction associated 1 (KHDRBS1), mRNA [Homo sapiens (Human)]	B2R7B5 (+3)	6
ACL6A_HUMAN Actin-like protein 6A [Homo sapiens (Human)]	O96019 (+2)	4
EIF3M_HUMAN Eukaryotic translation initiation factor 3 subunit M [Homo sapiens (Human)]	Q7L2H7	6
IF4A3_HUMAN Eukaryotic initiation factor 4A-III [Homo sapiens (Human)]	P38919	3
A2RTX1_HUMAN Myosin, heavy chain 6, cardiac muscle, alpha (Cardiomyopathy, hypertrophic 1) (Myosin, heavy polypeptide 6, cardiac muscle, alpha (Cardiomyopathy, hypertrophic 1), isoform CRA_a) [Homo sapiens (Human)]	A2RTX1 (+1)	4
A5PLK7_HUMAN RCC2 protein (Fragment) [Homo sapiens (Human)]	A5PLK7 (+1)	3
TBB5_HUMAN Tubulin beta chain [Homo sapiens (Human)]	P07437 (+2)	4
SF3B4_HUMAN Splicing factor 3B subunit 4 [Homo sapiens (Human)]	Q15427 (+1)	4
MYH1_HUMAN Myosin-1 [Homo sapiens (Human)]	P12882 (+1)	3
H1X_HUMAN Histone H1x [Homo sapiens (Human)]	Q92522	2
ACTN1_HUMAN Alpha-actinin-1 [Homo sapiens (Human)]	P12814 (+3)	4
A2ICT2_HUMAN Heat shock 70 kDa protein 4-like protein (Heat shock 70kDa protein 4-like, isoform CRA_b) [Homo sapiens (Human)]	A2ICT2 (+2)	4
RL4_HUMAN 60S ribosomal protein L4 [Homo sapiens (Human)]	P36578 (+1)	2
A8K7X6_HUMAN cDNA FLJ77432, highly similar to Homo sapiens poly(rC) binding protein 2 (PCBP2), transcript variant 2, mRNA (Poly(RC) binding protein 2, isoform CRA_b) [Homo sapiens (Human)]	A8K7X6 (+6)	2
EF1B_HUMAN Elongation factor 1-beta [Homo sapiens (Human)]	P24534 (+1)	2
ACTC_HUMAN Actin, alpha cardiac muscle 1 [Homo sapiens (Human)]	P68032 (+1)	3
A8K088_HUMAN cDNA FLJ78614, highly similar to Homo sapiens eukaryotic translation initiation factor 4A, isoform 1 (EIF4A1), mRNA [Homo sapiens (Human)]	A8K088 (+5)	3
HS90A_HUMAN Heat shock protein HSP 90-alpha [Homo sapiens (Human)]	P07900 (+1)	2
Q53GJ1_HUMAN DEAD (Asp-Glu-Ala-Asp) box polypeptide 47 isoform 1 variant (Fragment) [Homo sapiens (Human)]	Q53GJ1 (+2)	2

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MLE1_HUMAN Myosin light chain 1, skeletal muscle isoform [Homo sapiens (Human)]	P05976 (+2)	2
EIF3F_HUMAN Eukaryotic translation initiation factor 3 subunit F [Homo sapiens (Human)]	O00303	2
PHB2_HUMAN Prohibitin-2 [Homo sapiens (Human)]	Q99623 (+1)	2
RRP1B_HUMAN Ribosomal RNA processing protein 1 homolog B [Homo sapiens (Human)]	Q14684 (+3)	2
LDHA_HUMAN L-lactate dehydrogenase A chain [Homo sapiens (Human)]	P00338	2
B2R6X5_HUMAN cDNA, FLJ93166, highly similar to Homo sapiens heat shock 70kDa protein 6 (HSP70B') (HSPA6), mRNA [Homo sapiens (Human)]	B2R6X5	2
A8KA56_HUMAN cDNA FLJ77678, highly similar to Homo sapiens DEAD (Asp-Glu-Ala-Asp) box polypeptide 23, mRNA [Homo sapiens (Human)]	A8KA56 (+1)	2