Recruitment by the Repressor Freud-1 of Histone Deacetylase-Brg1 Chromatin Remodeling Complexes to Strengthen HTR1A Gene Repression

Tatiana Souslova¹, Kim Mirédin¹, Anne M. Millar¹, and Paul R. Albert¹

¹Ottawa Hospital Research Institute (Neuroscience) and UOttawa Brain and Mind Research Institute, 451 Smyth Road, Ottawa, ON K1H 8M5, Canada

Abstract

Five-prime repressor element under dual repression binding protein-1 (Freud-1)/CC2D1A is genetically linked to intellectual disability and implicated in neuronal development. Freud-1 represses the serotonin-1A (5-HT1A) receptor gene HTR1A by histone deacetylase (HDAC)-dependent or HDAC-independent mechanisms in 5-HT1A-negative (e.g., HEK-293) or 5-HT1A-expressing cells (SK-N-SH), respectively. To identify the underlying mechanisms, Freud-1-associated proteins were affinity-purified from HEK-293 nuclear extracts and members of the Brg1/SMARCCA chromatin remodeling and Sin3A-HDAC corepressor complexes were identified. Pull-down assays using recombinant proteins showed that Freud-1 interacts directly with the Brg1 carboxyl-terminal domain; interaction with Brg1 required the carboxyl-terminal of Freud-1. Freud-1 complexes in HEK-293 and SK-N-SH cells differed, with low levels of BAF170/SMARCC2 and BAF57/SMARCE1 in HEK-293 cells and low-undetectable BAF155/SMARCC1, Sin3A, and HDAC1/2 in SK-N-SH cells. Similarly, by quantitative chromatin immunoprecipitation, Brg1-BAF170/57 and Sin3A-HDAC complexes were observed at the HTR1A promoter in HEK-293 cells, whereas in SK-N-SH cells, Sin3A-HDAC proteins were not detected. Quantifying 5-HT1A receptor mRNA levels in cells treated with siRNA to Freud-1, Brg1, or both RNAs addressed the functional role of the Freud-1-Brg1 complex. In HEK-293 cells, 5-HT1A receptor mRNA levels were increased only when both Freud-1 and Brg1 were depleted, but in SK-N-SH cells, depletion of either protein upregulated 5-HT1A receptor RNA. Thus, recruitment by Freud-1 of Brg1, BAF155, and Sin3A-HDAC complexes appears to strengthen repression of the HTR1A gene to prevent its expression inappropriate cell types, while recruitment of the Brg1-BAF170/57 complex is permissive to 5-HT1A receptor expression. Alterations in Freud-1-Brg1 interactions in mutants associated with intellectual disability could impair gene repression leading to altered neuronal development.

Correspondence to: Paul R. Albert.

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Compliance with Ethical Standards

Conflict of Interest Statement The authors declare that they have no conflict of interest.

Keywords

Transcription; Chromatin remodeling; Histone deacetylase; Gene repression; Intellectual disability

Introduction

Five-prime repressor element under dual repression binding protein-1 (Freud-1), also known as CC2D1A, MRT3, and Aki1, is a multifunctional protein that is expressed widely in the brain and throughout development [1–3]. It functions as a calcium-regulated transcriptional repressor of serotonin-1A (5-HT1A) and dopamine-D2 receptors [1, 4, 5]. In addition, Freud-1 has been implicated in regulating NFkappaB expression [6–8] and has cytosolic functions of signaling and vesicle trafficking [9]. C-terminal truncated mutants of Freud-1 have been genetically linked to nonsyndromal intellectual disability [2, 8, 10], suggesting a role for Freud-1 in brain development. Consistent with a role in neuronal development, global knockout of Freud-1 in mice results in post-natal death and impaired neuronal differentiation in vitro [8, 11]. Conditional knockout of Freud-1 in post-natal forebrain results in cognitive impairment and anxiety phenotypes [3], implicating Freud-1 in cognitive and emotional development.

Freud-1 represses through a dual repressor element (DRE) identified in 5-HT1A and dopamine-D2 receptor genes [1, 5, 12], with greater activity in cells that express the target gene. In 5-HT1A-expressing neuronal cells but not 5-HT1A-negative nonneuronal cells, RNA knockdown or calcium-mediated inhibition of Freud-1 increases 5-HT1A receptor expression, indicating reversibility of repression in 5-HT1A-expressing cells [1]. Similarly, dopamine-D2 receptor expression in D2 receptor-positive cells is induced upon RNA knockdown of Freud-1 [4]. Furthermore, Freud-1 recruits different epigenetic mechanisms for complete versus reversible repression of the HTR1A promoter: HDAC-dependent mechanisms in 5-HT1A-negative cells, but HDAC-independent repression in 5-HT1A-expressing cells [13]. These findings suggest that Freud-1 recruits different protein complexes to mediate complete versus reversible repression in different cell types.

Repressors like REST [14] mediate HDAC-dependent gene repression by recruiting corepressor (e.g., Sin3A, CoREST) and histone deacetylase (HDAC) complexes, and their associated proteins RbAP46/RbAP48, as well as Sin3A-associated proteins (SAPs) [15]. In addition, Brg1 or Brm chromatin remodeling complexes are recruited and have been shown to mediate both activation and repression of gene expression [16]. Brg1 is the catalytic ATPase subunit of Swi/Snf chromatin remodeling complex [17]. Chromatin remodeling complexes contain the core subunits (Brg1 or Brm) and Brg1/Brm-associated factors (BAFs): BAF170, BAF155, BAF47, BAF60, BAF57, BAF53, and actin [18], which together open chromatin structure and stabilize transcription factor binding [19].

To identify Freud-1-associated protein complexes, coimmunoprecipitation studies and functional characterization were done. We find that in 5-HT1A-negative cells, Freud-1 recruits Brg1 and Sin3A/HDAC corepressor complexes to HTR1A gene promoter. In contrast, in 5-HT1A-expressing neuronal cells, Freud-1 recruits Brg1 without Sin3A/HDAC complex to mediate HDAC-independent repression. Full repression of the HTR1A gene in

5-HT1A-negative cells was reversed by depletion of both Freud-1 and Brg1, while reversible repression in 5-HT1A-expressing cells was reversed by depletion of either Freud-1 or Brg1, suggesting that recruitment of the Brg1-Sin3A-HDAC complex is critical for full repression.

Methods

Cell Culture, Transient Transfections, and siRNA Treatments

Human embryonic kidney (HEK) 293 cells and human neuroblastoma 5-HT1A-expressing cells (SK-N-SH, ATCC) 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% CO₂. Cell lines were grown to 50–60% confluence, and the medium was replaced 12 h prior to transfection. Cells were transfected using the calcium phosphate coprecipitation method [12] with 5 µg of human Freud-1 or human Brg1 expression plasmids. The cells were analyzed 72 h post-transfection.

Stealth siRNA targeting hFreud-1 (5'-GGCG CUCUAUCAGACAGCAAUUGAA-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 a final siRNA concentration of 20 and 25 nM, respectively. Transfection efficiency control was performed with BLOCK-ITTM 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 Proteins

The full-length human Brg1 plasmid (pcDNA3.1D-hBRG1-V5/His) was generously supplied by Dr. Trevor K. Archer, NIEHS [20]. Bacterial expression Brg1 constructs of N- and C-terminal regions on both sides of the central catalytic domain were generated by PCR with the following primers:

C-Brg1 (amino acids 1075 to 1647) forward, 5'-GGAT CCTACCGAGCCTCGGGTAAA-3' and reverse, 5'-CTCG AGTCAGTCTTCTTCGCTGCC-3'; N-Brg1 (amino acids 1 to 656) forward, 5'-CAGTACCCTTCACCAGATCT ATGTCC-3' and reverse, 5'-AGATCTATCAGACC TCGGAGCTACTTCAT-3'. PCR fragments were ligated into the pGEM-T-easy vector (Promega) and then sub-cloned for bacterial expression into the pGEX-4T-3 vector by enzymatic restriction. Full-length and deletion mutants of Freud-1 in N-terminal pFlag-CMV2 were kindly supplied by Dr. N. Fujita [21]. Freud-1 C2 and NSMR in pTriex4 vector were subcloned into the pFlag-CMV2 vector by enzymatic restriction. All constructs were verified by DNA sequencing.

Recombinant hFreud-1 in pTriEX-4 [5] or GST-Brg1 plasmids were transformed into BL21 (DE3) *E. coli* (Novagen), grown overnight in 2xYT broth with 100 µg/µl ampicillin and induced at OD₆₀₀ = 0.6 with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Wisent) at 37 °C for 3 h. Cells were harvested and proteins extracted. Protein purification was done on ice unless otherwise mentioned, and buffers were supplemented with protease inhibitors: 1.5 µg/µl aprotinin, 1 µg/µl leupeptin, 0.14 mM PMSF. For GST-tagged proteins, bacterial

pellets resuspended and lysed (2 h) in 2 ml lysis buffer (1% Triton X-100, 1 mM DTT, and 1 mg/ml lysozyme in PBS). Cell lysates were sonicated (6–10 × 15 s at 200–300 W), centrifuged (10,000g × 30 min, 4 °C), the supernatants filtered (0.45 µm, Millipore) and incubated with 250 µl 50% slurry of Glutathione Sepharose4G Fast Flow (GE Healthcare) for 1 h, 22 °C. Beads were pelleted (500g × 5 min), washed 3× in 10 ml PBS, resuspended in 500 µl PBS and stored at 4 °C. For S/His-tagged Freud-1 proteins, bacterial pellets were lysed 30 min in 5 ml/g HisPur Cobalt buffer (50 mM Na₃PO₄, 300 mM NaCl, 10 mM imidazole; pH 7.4 and 1 mg/ml lysozyme), sonicated, centrifuged and supernatants incubated in 1 ml 50% HisPur Cobalt beads (ThermoScientific) for 1 h 4 °C with shaking. The beads were loaded onto a column equilibrated with HisPur Cobalt buffer, washed 3× in buffer and eluted 4× with 1 ml elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole; pH 7.4). Protein extracts were dialyzed against PBS at 4 °C overnight. Protein content was then assessed with a Bradford assay; protein extracts were aliquoted and stored at –80 °C.

Subcellular Fractionation

Subcellular fractionation was performed as previously described [22]. Briefly, cells were washed 3× with PBS, harvested and allowed to pre-swell for 10 min at 4 °C in extraction buffer. Then the 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 MgCl₂, 0.1% Nonidet-P40, 0.5 mM dithiothreitol, 0.5 mM spermidine, 0.15 mM spermine, 1 mM phenylmethylsulphonyl fluoride, 1× protease inhibitor cocktail (Roche, Laval, Qc, Canada)). Lysates were centrifuged (2 min, 6000×g) and supernatant reserved as cytosolic fraction. The nuclear pellet was collected and washed 3× with wash buffer (50 mM NaCl, 20 mM Na-HEPES, pH 7.6, 25% glycerin, 0.2 mM EDTA, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM spermidine, 0.15 mM spermine, 1 mM phenylmethylsulphonyl fluoride, 1× 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 MgCl₂, 0.5 mM dithiothreitol, 0.5 mM spermidine, 0.15 mM spermine, 1 mM phenylmethylsulphonyl fluoride, 1× protease inhibitor cocktail) for 30 min on ice. Fractions were verified for lack of cross-contamination between nuclear and nonnuclear compartments by Western blot (data not shown).

Antibodies and Western Blots

Anti-hFreud-1L (1:10,000), generated against bacterially expressed and purified (Nitrilotriacetic beads, Qiagen) S/His-tagged human Freud-1L (Cedarlane, Hornby, ON, Canada), was used for affinity purifications, Western blots, and IPs [5]. Antibodies were used at the following dilutions: BAF170 (1:1000), BAF155 (1:1000), and BAF57 (1:2000) (Bethyl Laboratories); Brg1 (1:2000; Upstate) and Sin3A (1:1000; Abcam); and HDAC-1 (1:1000), HDAC-2 (1:1000), and β-actin (1:10,000) (Sigma). For in vitro pull-down assay, 1:20,000 goat anti-GST (GE Healthcare) and 1:5000 rabbit antiS-HRP conjugated (Novagen) were used. For Western blots of co-IP, anti-Brg/SNF2 (rabbit) at 1:2000 (Millipore) and anti-DYKDDDDK (rabbit) at 1:2000 (Cell Signaling) were used. Secondary antibodies used were anti-goat at 1:20,000 (Jackson ImmunoResearch), anti-rabbit at 1:2000 (Cell Signaling), and anti-mouse at 1:2000 (Jackson ImmunoResearch).

For Western blot analysis, polyvinylidenedifluoride membranes were blocked in TBST with 5% (w/v) milk for 1 h 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 HEK-293 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). As the corresponding bands were weak or not visualized in the control lane, it was not analyzed by mass spectrometry; instead, the interaction between Freud-1 and proteins with high peptide abundance was validated by IP studies (see "Results"). 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/assay was used. The lysate was not pre-cleared and was 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). For Flag IP, 20 µl of anti-Flag M2 affinity gel (Sigma) were added and incubated (16 h, 4 °C). The supernatant was then collected, and the beads were washed 3× 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 2× 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 µg of nuclear lysate from cells transfected with S-Freud-1 and incubated overnight at 4 °C. The

following day, the beads were washed 3× with 1 ml of NETIN buffer and boiled in 2× Loading buffer. The resulting elutions were examined by Western blot. For GST pull-down assays, equal amounts of Glutathione Sepharose beads bound to GST, GST-N-Brg1, or GST-C-Brg1 were incubated with 1–1.5 µg of purified S/His-tagged Freud-1 protein (30 min, 22 °C) in 1 ml GST pull-down buffer with end-over-end mixing. Beads were washed 3× in 1 ml PBS; centrifuged at 500g × 5 min at 4 °C, and eluted by boiling 5 min in 2× Loading buffer.

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 3× with PBS and cross-linked for 15 min at 22 °C in PBS supplemented with 1 mM MgCl₂ and 1% formaldehyde (v/v), rinsed 3× 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), 20× 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 analyzed using Q-PCR with primers designed to amplify the 151-bp region containing 5-HT1A-5' DRE (5'-CTGTCTTCTCTTTCTAAAAAGTTGTTG-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 Qtaq™ DNA polymerase mix (Clontech). The PCR products were verified on a 1% agarose gel. A negative control amplifying a 276-bp region within the 5-HT1A open reading frame was also examined with primers (5'-AGTGTGAATGGAGAGTCGGG-3') and (5'-CACTGTCTTCCTCTCTCGGG-3'). Cycling conditions were as follows: 94 °C for 1 min, then 40 cycles of 94 °C for 20 s, 58 °C for 30 s, 72 °C for 20 s, and a final extension of 72 °C for 10 min on a Biometra T gradient cycler, with product size and intensity confirmed on 1.2% agarose gel.

Quantitative Real-Time RT-PCR (RT-qPCR)

RNA was isolated from cells using the TRIZOL Reagent (Invitrogen), followed by DNase treatment using the TURBO DNAase-free™ 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 as follows: 95 °C for 10 min 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 5-HT1A to GAPDH were obtained as $10 \times (CtT - CtG)$, 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 mean $\pm$ SEM of the indicated number of replicates.

Results

Identification and Validation of Human Freud-1-Interacting Partners

In order to identify proteins involved in Freud-1 repressor activity, affinity purification of endogenous Freud-1 from HEK-293 nuclear extracts was done using an antibody raised against the human Freud-1 long form and that recognizes multiple Freud-1 isoforms [5]. As shown by Western blot for Freud-1, the affinity column efficiently purified endogenous Freud-1 compared to IgG control, with minimal background (Fig. 1a). From the affinity-purified fraction of HEK-293 cell nuclear extracts (Fig. 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 nonspecific interactions [23] (Supplementary Table I). Proteins identified with highest coverage and probability percentages are summarized in Table 1. The mass spectrometry analysis confirmed that the antibody recognized Freud-1, since it purified hFreud-1 (22 peptides with 28% coverage) and recognized multiple Freud-1 isoforms (Fig. 1b). 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 (Fig. 1b). The enrichment of BAF155 and BAF170 was further confirmed in two additional Freud-1 affinity purification experiments (Rogaeva and Albert, unpublished data). 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.

In order to verify the interaction of these proteins with endogenous Freud-1, coimmunoprecipitation studies using antibody to hFreud-1 were done in human HEK-293 kidney cells (Fig. 2, top left) or 5-HT1A-expressing SK-N-SH neuroblastoma cells (Fig. 2, bottom left). 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 from HEK-293 cells contained Freud-1 (control), Brg1, BAF170, BAF155, and Sin3A. The Freud-1 long form has two variants (130/100 kDa) that may reflect posttranslational modification, and the predominantly nuclear 130-kDa form was enriched in our nuclear extracts and immunoprecipitates [5]. These results were confirmed by pull-down using anti-S-tag-agarose beads in HEK-293 cells transiently transfected with S-Freud-1 (Fig. 2, top right). Similar results were obtained from IP and pull-down assays in neuronal SK-N-SH cells, which contained Freud-1 (control), Brg1, and BAF170, but were depleted of BAF155 and Sin3A (Fig. 2, bottom). Conversely, IP for each of the Freud-1-interacting proteins identified above brought down S-Freud-1 (Fig. 3a) and the immunoprecipitated protein (Fig. 3b). However, antibody to Sin3A more effectively immunoprecipitated S-Freud-1 in HEK-293 compared to SK-N-SH extracts, consistent with a cell-specific interaction. Similarly,

HDAC1 and HDAC2 were also enriched in S-Freud-1 IP from HEK-293 cells compared to SK-N-SH cells, while BAF155 appeared to be a nonspecific signal in both cell lines. Taken together, these results suggest that Freud-1 interacts with nuclear proteins in a cell-specific manner and fails to recruit Sin3A/HDAC in the 5-HT1A-expressing SK-N-SH cells compared to 5-HT1A-negative HEK-293 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 [13].

Direct Interaction Between Freud-1 and Brg1

Since Brg1 is a component of the Freud-1 nuclear protein complex, we used in vitro pull-down assay with recombinant bacterially purified Freud-1 and Brg1 proteins to address whether these proteins interact directly (Fig. 4). For Brg1, N- and C-terminal fragments flanking the catalytic ATPase domain were fused with GST, purified and combined with purified S/His-Freud-1 (wild-type and mutants). The multiple bands observed likely reflect instability of Freud-1 expressed in bacteria. Pull-down for GST revealed a strong signal for wild-type Freud-1 with GST-C-Brg1, but not GST or N-Brg1, indicating that Freud-1 interacts with the C-terminal portion of Brg1. Upon deletion of the C-terminal portion of Freud-1 in the C757 construct, the interaction was greatly attenuated, and abolished in the NSMR construct. These data indicate that the C-terminus of Freud-1 is the primary determinant of binding to the C-terminus of Brg1.

To further investigate the interaction of Freud-1 with Brg1, HEK-293 cells were transfected with a series of Flag-tagged human Freud-1 deletion mutants, total cell extracts were immunoprecipitated using anti-Flag column, and Flag-Freud-1 or Brg1 detected by Western blot (Fig. 5). In cells transfected with full-length Freud-1 but not vector-transfected cells, immunoprecipitation of Brg1 was observed and was stronger when Brg1 was transfected. For simplicity, only Flag-Freud-1 constructs were transfected. Progressive Freud-1 C-terminal deletion led to a loss of Brg1 binding, beginning with the 762 mutant, while deletion of individual N-terminal DM14 domains did not affect the interaction with Brg1. These findings further indicate that Freud-1 interacts with Brg1 primarily via the C-terminal region adjacent to the C2 domain.

Cell Type-Specific Recruitment of Brg1 and Sin3A/HDAC Complexes to the HTR1A Promoter

To determine whether the identified Freud-1-interacting proteins are present with Freud-1 at the HTR1A promoter, we performed quantitative ChIP experiments using Q-PCR to quantify Freud-1 recognition of its element, the 5-HT1A DRE (Fig. 6a). In HEK-293 cells, the -fold enrichment of 5-HT1A DRE compared to IgG control of the following protein was as follows: 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 SK-N-SH cells, Freud-1 (2.77), Brg1 (4.82), BAF170 (12.87), BAF155 (3.04), and BAF57 (8.19) were recruited to the HTR1A promoter region at significant levels, but not Sin3A (0.23) or HDAC1/2 (0.22 and 0.47, respectively) (Fig. 6b). Specific amplification of the 5-HT1A DRE was visualized by gel electrophoresis following

semi-quantitative PCR amplification (Fig. 6c). Together, these results indicate that different protein complexes are recruited to the Freud-1 binding site on the HTR1A promoter, depending on the cell type. In 5-HT1A-negative HEK-293 cells, Freud-1, Swi/Snf and Sin3A/HDAC complexes were recruited to the HTR1A promoter region. In 5-HT1A-expressing neuronal SK-N-SH cells, the transcriptional regulation of HTR1A gene was achieved through the recruitment of Freud-1 and the Brg1-Swi/Snf complex, but without the Sin3A/HDAC complex.

Freud-1 Recruits Brg1 to the HTR1A Promoter in Nonneuronal Cells

Brg1 is the central catalytic ATPase subunit of Swi/Snf complex that is required for its function [16]. We addressed whether the Swi/Snf complex is required to stabilize Freud-1 recruitment to the HTR1A DRE site or vice versa, by using siRNA to knockdown the expression of Freud-1 or Brg1. Western blots indicate that treatment of HEK-293 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 (Fig. 7a). The same observations were made using SK-N-SH cells (Fig. 7b). 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 (Fig. 8). When HEK-293 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 HEK-293 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 inhibited (Fig. 8a). The dependence of Brg1 binding to the DRE on Freud-1 suggests that Freud-1 is required for Brg1 recruitment to the HTR1A promoter region in HEK-293 cells, while Freud-1 binding appeared to be insensitive to Brg1. In neuronal SK-N-SH cells, treatment with siRNA to Brg1 or Freud-1 significantly abolished the recruitment of Freud-1 and Brg1 to the promoter region (Fig. 8b), 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 HTR1A promoter in HEK-293 cells in which the gene is silenced, while it requires association with Brg1 to bind the promoter in 5-HT1A-expressing SK-N-SH cells.

Depletion of Freud-1, Brg1, or Both Results in Increased 5-HT1A mRNA Levels

To determine the role of Brg1 in Freud-1-mediated HTR1A gene repression, we examined the effect of siRNA-mediated depletion of Brg1 or Freud-1 on 5-HT1A receptor expression using RT-qPCR analysis to quantify 5-HT1A mRNA levels. In 5-HT1A-deficient HEK-293 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 (Fig. 9a), indicating that a redundant action of both factors maintains silencing of the HTR1A gene. In 5-HT1A-expressing SK-N-SH cells, treatment with siRNA to either Freud-1 or Brg1 upregulated 5-HT1A receptor RNA (Fig. 9b), indicating that both factors are required for repression of the 5-HT1A receptor. The

combination of Freud-1 and Brg1 depletion resulted in no change in 5-HT1A RNA levels, 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. Together with the ChIP results, these data 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.

Discussion

Cell-Specific Freud-1/Brg1 Complexes for HTR1A Gene Repression

Freud-1 is a widely expressed protein that participates in silencing 5-HT1A and dopamine-D2 receptor gene expression in nonneuronal cells, together with REST/NRSF [13]. In neuronal cells that express these receptors, Freud-1 plays a major role in reversible repression of these genes [1, 4, 12, 13]. Here, we addressed whether different sets of proteins interact with nuclear Freud-1 depending on cell type (nonneuronal HEK-293 vs. neuronal SK-N-SH cells) and examined their role in Freud-1 repressor activity. In both cell types, Brg1 was coprecipitated with Freud-1 and recombinant Freud-1 directly interacted with the C-terminal domain of Brg1 in vitro, which required the Freud-1 C-terminal region. Previously, the calcium-responsive silencer CREST was shown to bind to the N-terminal domain of Brg1 in vitro and implicated in calcium-dependent activation of c-fos and NR2B promoters [24]. Because the Brg1 N-terminal was partially degraded, we cannot rule out the possibility that Freud-1 may also bind to the Brg1 N-terminal. The Brg1 C-terminal is targeted by the transcription factor EVI1 [25] and contains an Rb-binding domain, an AT-hook domain that participates in chromatin remodeling, and a bromodomain that interacts with histones and other proteins via acetyl-lysine side chains [16, 26]. The Freud-1 C-terminal region contains four lysine residues that are predicted to be acetylated (PAIL), although bacterially expressed proteins often lack acetyl-lysine [27], suggesting that other sites may mediate the Freud-1/Brg1 interaction. In addition, the Freud-1 C-terminal contains a glutamine-rich (aa 877–913, MotifScan) and coiled-coil motif (aa 906–927, Ensembl) that could mediate protein interactions. Interestingly, lysine to glutamine substitution of 5 residues mimics a constitutively lysine-acetylated phenotype in the transcription factor Aire [28], suggesting that the Freud-1 poly-glutamine motif could recruit Brg1 via its bromodomain, but this remains to be tested.

Although Freud-1/Brg1 complexes were present in both HEK-293 and SK-N-SH cells, Freud-1 bound different Brg1-associated complexes in these cells. BAF155, Sin3A, HDAC1, and HDAC2 were enriched in the Freud-1 co-IP from HEK-293 cells. In contrast, BAF170 and BAF57 were enriched in the Freud-1 complex in SK-N-SH cells, while Sin3A, HDAC1, and HDAC2 were absent or depleted. Consistent with our results, the expression of BAF170 and BAF57 are codependent and compete with the level of BAF155 in the Brg1 complex [29, 30]. The presence of BAF155 in the Freud-1/Brg1 complex in nonneuronal HEK-293 cells resembles the esBAF complex identified in embryonic stem cells, while the BAF170/BAF57-containing complex in neuronal SK-N-SH cells is similar to npBAF complex in neuronal precursor cells [31]. In neuronal cells, BAF57 exists as at least three different

isoforms with N-terminal truncations [32], and BAF57 can influence Swi/Snf subunit composition [33]. Differential recruitment of Brg1 complexes to Freud-1 could be driven by different isoforms BAF57 in SK-N-SH neuronal cells compared to HEK-293 cells. Differential recruitment of Sin3A by these distinct Freud-1/Brg1 complexes could account for the lack of Sin3A in neuronal Freud-1/Brg1 complexes.

The proteins that coprecipitated with Freud-1 were also localized to the 5-HT1A DRE, indicating that Freud-1 actively recruits these protein complexes to the HTR1A promoter. Importantly, Freud-1/Brg1 recruited the Sin3A/HDAC to the HTR1A promoter in 5-HT1A-negative HEK-293 cells, but not in neuronal 5-HT1A-expressing SK-N-SH cells, suggesting that the Brg1 complex in the neuronal cells may be incapable of recruiting Sin3A to chromatin. Consistent with the lack of Sin3A/HDAC recruitment by Freud-1, we previously found that Freud-1-mediated repression is HDAC-independent in neuronal 5-HT1A-expressing cells [13]. However, in 5-HT1A-negative cells including HEK-293 cells, Freud-1-mediated repression is HDAC-dependent, consistent with recruitment of the Sin3A corepressor complex. Moreover, transcriptional repression of HTR1A promoter mediated by REST was HDAC-dependent in all cell types tested, consistent with HDAC recruitment by REST-associated corepressors CoRest and Sin3A [15, 34]. Like Freud-1, REST can also mediate reversible repression or long-term gene silencing through the action of different gene-specific corepressors, but their specific requirement repression vs. silencing is unclear [15, 34]. The Sin3A-HDAC complex stably interacts chromatin via Rb-binding proteins [35], and the recruitment of HDAC induces a compact chromatin structure, which would be expected to “shut-down” 5-HT1A receptor expression and prevent dynamic gene regulation. In contrast, in 5-HT1A-expressing cells, its promoter is dynamically regulated. For example, calcium-mediated activation of calmodulin-dependent protein kinase inhibits Freud-1 repression and induces the HTR1A gene in neuronal 5-HT1A receptor-expressing cells [1]. Taken together, our results suggest that Freud-1 recruits the Sin3A corepressor complex to mediate HDAC-dependent silencing of 5-HT1A receptor expression in nonneuronal cells, while recruitment of a non-Sin3A/HDAC Brg1 complex is involved in reversible HTR1A gene repression.

Depletion of Freud-1, Brg1, or both proteins by siRNA treatment revealed that different mechanisms are used to recruit the Brg1 complex to the HTR1A gene in HEK-293 and SK-N-SH cells. In HEK-293 cells, Brg1 recruitment was Freud-1-dependent, while Freud-1 recruitment was not affected by Brg1 depletion, suggesting that Freud-1 is firmly bound to the DRE site as part of the gene silencing complex, perhaps through recruitment of the Sin3A complex to stabilize its binding. In SK-N-SH cells, a decrease in either protein was sufficient to abolish their recruitment to 5-HT1A DRE, suggesting that Brg1 and Freud-1 interact to enhance Freud-1 binding to the DRE in the context of chromatin, as observed for REST-mediated gene repression [19]. This codependent recruitment of Freud-1 and Brg1 to the HTR1A promoter may involve Brg1 helicase activity to open the chromatin at the DRE site.

Differential Roles of Freud-1 Complexes in HTR1A Gene Regulation

The functional importance of Freud-1 and Brg1 to regulate 5-HT1A mRNA levels differed in HEK-293 and SK-N-SH cells. In HEK-293 cells, siRNA-mediated depletion of both Freud-1 and Brg1 was required to increase 5-HT1A mRNA levels, whereas in SK-N-SH cells, depletion of either was sufficient to upregulate 5-HT1A mRNA. Since in HEK-293 cells depletion of Freud-1 resulted in loss of Freud-1 and Brg1 at the 5-HT1A DRE but did not increase 5-HT1A mRNA levels, transcriptional repression of the HTR1A promoter is probably maintained through an alternative mechanism, such as by REST/Brg1 or Freud-2 mediated repression [13, 36]. Upon depletion of both Freud-1 and Brg1, 5-HT1A mRNA was increased in HEK-293 cells, suggesting the involvement of Brg1 in both Freud-1-dependent and Freud-1-independent regulation via REST, Freud-2, or other silencing mechanisms. These results indicate that in HEK-293 cells, HTR1A gene silencing is reprogrammable through abrogation of both Freud-1 repressor and Brg1 chromatin remodeling mechanisms. In SK-N-SH cells, treatment with either Freud-1 or Brg1 siRNA inhibited the recruitment of both Freud-1 and Brg1 to the 5-HT1A DRE and reversed Freud-1-mediated repression. Similarly, siRNA to Freud-1 also increased dopamine-D2 receptor RNA and protein levels in three different neuronal cells that endogenously express the receptor [4], indicating that in neuronal cells Freud-1 dynamically regulates the basal level of expression of its target genes. In contrast, in HEK-293 cells where Freud-1 target genes are not expressed, additional Brg1-mediated mechanisms appear to mediate silencing of these genes that can be reprogrammed by depletion of both Freud-1 and Brg1. Consistent with the role of Brg1 in reprogramming neural gene expression, nestin-mediated conditional knockout of Brg1 in neural precursor cells inducing neural gene expression and differentiation [37].

Potential Roles of Freud-1/Brg1 Complexes In Vivo

The presence of the Brg-1 complex with Freud-1 at the HTR1A promoter and its role in differential modes of gene regulation is interesting, given the convergent evidence that members of the Brg-1 complex are implicated in mental retardation, as is Freud-1 [9, 31, 38, 39]. In particular, the NSMR mutant that is linked to nonsyndromic intellectual disability [2, 8] truncates the protein, and abolished Freud-1 interaction with Brg1 and Freud-1-induced repression of the HTR1A gene (unpublished data). Similarly, nontruncating in-frame deletions or point mutations of Brg1 have been identified in Coffin Siris syndrome [40–42]. BAF170 is mutated in autism spectrum disorder [43] and was associated with Freud-1 in neuronal SK-N-SH cells implicating its role in Freud-1 regulation of neuronal genes. Thus, Freud-1-mediated recruitment of the Brg1 complex could contribute to regulation of genes involved in cortical development. Consistent with this, gene deletion of Freud-1 results in abnormal cortical development, reduced signaling to CREB, and impaired dendritic and synaptic maturation in vitro [8, 11, 44]. Further implicating Freud-1 in cognitive development, mice with conditional deletion of Freud-1 in the post-natal forebrain displayed cognitive impairment and anxiety behavior [3]. These results suggest that Freud-1 and Brg1-complex proteins are among a constellation of interacting transcriptional regulators implicated in cortical development [37].

However, the exact mechanism by which Freud-1 or Brg1 complexes result in altered cortical development remains unclear. Gene deletion of BAF170 in cortical progenitors reduces BAF57 recruitment to the Brg1 complex and results in increased cortical size, while its overexpression promotes BAF57 recruitment and reduces cortical size [30]. Furthermore, while Freud-1-mediated regulation of the HTR1A gene may play a role in adult behavior phenotypes such as depression or anxiety, it is likely that additional gene targets mediate Freud-1 actions in cortical development. Similarly, although specific Brg1 complexes have been implicated in neuronal differentiation, the gene targets of neuronal Brg1 complexes that are involved in cortical development remain unclear [31, 38]. Our data suggest that Freud-1 is at least one of the transcription factors that recruit the Brg1 complex to regulate specific gene expression.

Conclusion

We have shown that Freud-1 interacts with Brg1 to recruit different Brg1 complexes to the HTR1A promoter depending on the cell type. In HEK-293 cells, the Freud-1/Brg1 complex is esBAF-like and contains Sin3A/HDAC and BAF155 to mediate silencing of HTR1A gene that is dependent on both Freud-1 and Brg1. In neuronal 5-HT1A-expressing SK-N-SH cells, the Freud-1/Brg1 complex resembles npBAF and contains BAF170/BAF57. These results suggest that Freud-1 recruitment of Brg1 and Sin3A-HDAC complexes strengthens repression of the HTR1A gene to promote gene silencing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Affinity purification of endogenous hFreud-1 and associated corepressor complex. Nuclear extracts of HEK-293 cells were immunoprecipitated using an antibody raised against bacterially expressed full-length S-hFreud-1 (IP: Freud-1) and corresponding preimmune serum as a negative control (IP: control). **a** Western blot of affinity purified hFreud-1. Multiple hFreud-1 isoforms (*arrows*) were identified by Western blot using an antibody against full-length hFreud-1 long form raised in a different rabbit than the one used in column. Input (*Input*), flow-through (*FT*), wash (*W*), and elution (*E2* and *E3*) fractions are shown, and 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 HEK-293 nuclear extracts. *Arrows* point to bands containing proteins identified by mass spectrometry analysis as hFreud-1, BAF155, BAF 47, BAF57, RBBP4 or RBBP5, and Sin3A. Molecular weight markers (kDa) are indicated at the *left*

Fig. 2.

Swi/Snf Brg1-containing complex and Sin3A corepressor complex are hFreud-1-interacting partners. *Left panels:* analysis of proteins interacting with endogenous Freud-1 (*Endogenous*). HEK-293 (*top panels*) or SK-N-SH (*bottom panels*) nuclear lysates were examined by IP with antibody specific for full-length human Freud-1 [5]. 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 Brg1, BAF155, BAF170, and Sin3A (Sin3A was absent in SK-N-SH cells). *Right panels:* analysis of HEK-293 (*top*) nuclear lysates transiently transfected with S-tagged Freud-1 cDNA (*Transfected*). *Lines* indicate nonadjacent samples in the same blot. Anti-S pull-down fractions were positive for S-Freud-1, Brg1, BAF155, BAF170, and Sin3A. *Bottom:* analysis of SK-N-SH nuclear lysates transiently transfected with S-tagged Freud-1 cDNA. Anti-S pull-down fractions were positive for S-Freud-1, Brg1, BAF170, but not Sin3A. For all Western blots, 20 µg protein/lane was loaded for *Inp* and *FT* fractions and 50% from total elution was loaded for *E* or *Ectrl* fraction. Results were replicated in at least three independent experiments

Fig. 3.

Co-IP of Freud-1 with Brg1 and Sin3A complex proteins. **a** Co-IP studies. HEK-293 and SK-N-SH cells were transiently transfected with Stagged 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 HEK-293 cells, and input (*Inp*), flow-through (*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*). Results were replicated in at least three independent experiments

Fig. 4.

Freud-1 binds to the Brg1 carboxyl-terminal domain. Results from in vitro GST pull-down assay using recombinant GST-tagged Brg1 N-terminal (N-Brg1), C-terminal (C-Brg1) or GST, and probing by Western blot (WB) for GST or His/S-tagged human Freud-1L, wild-type (WT), ΔC757 or NSMR mutants. Above is the predicted Freud-1 domain structure including DM14 (*gray*), HLH (*white*), and C2 (*gray/white outline*) domains, and below are low complexity (*gray bar*) and coiled-coil domains (*black bar*). Freud-1 input is shown at left, and pull-down at right showing strong pull-down only for C-Brg1 of WT, weak for ΔC757, and not detectable for NSMR. The *lower right panel* shows loading controls for GST fusion proteins, C-Brg1 (90 kDa; *black arrowhead*), N-Brg1 (*white arrowhead*, ~50 kDa), N-Brg1 fragment (*gray arrowhead*, ~30 kDa), and GST (*gray arrowhead*, 26 kDa). *N* = 2. *Inset, lower left*: densitometric analysis of in vitro pull-down assays shown as fold-change to wild-type Freud-1, mean ± SEM

Fig. 5.

Coimmunoprecipitation of Flag-hFreud-1 mutants and hBrg1. HEK-293 cells were transfected as indicated in Methods and nuclear proteins were extracted.

Immunoprecipitations followed by SDS-PAGE analysis and Western blot. *Above*: two representative blots are shown and IPs were probed for Brg1 (*above*) and Flag (*below*). F/B: Flag-Freud-1 and hBrg1; -/B: Flag vector with hBrg1 (negative control); F: pFlag-cMV2-hFreud-1; MOCK: untransfected; MeCP2: Flag-MeCP2 (negative control). The hFreud-1 mutants are deleted in individual DM14 domains (DM14-1, 2, 3, 4), or C-terminal mutants truncated at the indicated amino acid. *Below*: densitometric analysis of co-IPs shown as fold change compared to wild-type Freud-1, mean $\pm$ SEM

Fig. 6.

Different complexes are present with Freud-1 at the HTR1A promoter depending on the cell type. Chromatin immunoprecipitation (ChIP) assay using the indicated antibodies for IP of nuclear extracts from HEK-293 cells or SK-N-SH cells. **a** Semi-quantitative analysis of ChIP by RT-PCR: DNA from each IP was amplified in the Freud-1-binding DRE region of HTR1A gene. Samples were loaded on 2% agarose gel; nonimmune rabbit IgG was used as negative control, and no antibody control (Nab) was used to determine the level of background precipitation. As negative control, the coding region of the HTR1A gene was amplified and showed no difference from IgG. **b, c** Quantitative ChIP: IP of HEK-293 (**b**) or SK-N-SH (**c**) nuclear extracts was done using the indicated antibodies and the amount of Freud-1-binding DRE region was quantified by real time Q-PCR. Results are expressed as %input DNA. *Black line* indicates level of background precipitation using preimmune serum

(IgG). 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

Fig. 7.

Freud-1 or Brg1 protein levels after siRNA treatment. Depletion of Freud-1 or Brg1 in HEK-293 (a) or SK-N-SH (b) cells. Cells were treated for 72 h with 20 nM Freud-1 siRNA or 25 nM Brg1 siRNA (siRNA) or with 25 nM siRNA control (RNAC) or not treated (NON). Freud-1 or Brg1 protein levels were measured by Western blot, with β -actin as loading control. c Data from at least three independent experiments were quantified using ImageJ software (NIH) and are presented as mean $\pm$ SD

Fig. 8.

Freud-1 or Brg1 depletion by siRNA treatment decreases their recruitment to the HTR1A promoter. ChIP assay in HEK-293 cells (a) or SK-N-SH 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 HEK-293 cells no change in Freud-1 recruitment was seen. Mean $\pm$ SEM, $n = 3$. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$. one-way ANOVA with Dunnett's post-test to compare to untreated control (NON)

Fig. 9.

Freud-1/Brg1 complex represses 5-HT1A receptor expression. HEK-293 (a) or SK-N-SH (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 RT-qPCR, and presented as -fold change compared on untreated cells. In 5-HT1A-negative HEK-293 cells, suppression of both Freud-1 and Brg1 induced 5-HT1A RNA, while in SK-N-SH 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), ** $p < 0.01$, *** $p < 0.001$

Table 1

Freud-1 associated proteins identified by mass spectrometry

Protein	No. of peptides	% Coverage
Swi/Snf complex		
BAF155	17	13
BAF57	15	34
BAF47	4	18
Sin3A complex		
SP130	2	2
Retinoblastoma binding proteins		
RBBP5	10	26
RBBP4	3	8

A total of 280 potential Freud-1-interacting proteins were identified in 20 bands, and 84 sepharose proteome-related proteins were eliminated from analysis. Proteins with highest coverage and probability percentages are shown in the table. For Freud-1, we obtained 22 peptides with 28% coverage *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, 130 kDa; *RBBP5/4* Retinoblastoma binding protein 5/4