Krüppel-like Factor 11 Differentially Couples to Histone Acetyltransferase and Histone Methyltransferase Chromatin Remodeling Pathways to Transcriptionally Regulate Dopamine D2 Receptor in Neuronal Cells*

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Background: Chromatin-mediated events utilized by Krüppel-Like factors in neurons remain undefined. Results: Krüppel-Like factor 11 couples to antagonistic chromatin pathways (p300 versus heterochromatin protein 1) to regulate the dopamine D2 receptor gene. Conclusion: This is the first description of mechanisms underlying Krüppel-like factor-mediated functions in neurons. Significance: This knowledge expands our understanding of chromatin-mediated mechanisms that influence homeostasis in highly specialized cells.

The importance of Krüppel-like factor (KLF)-mediated transcriptional pathways in the biochemistry of neuronal differentiation has been recognized relatively recently. Elegant studies have revealed that KLF proteins are important regulators of two major molecular and cellular processes critical for neuronal cell differentiation: neurite formation and the expression of neurotransmitter-related genes. However, whether KLF proteins mediate these key processes in a separate or coordinated fashion remains unknown. Moreover, knowledge on the contribution of chromatin dynamics to the biochemical mechanisms utilized by these proteins to perform their function is absent. Here we report the characterization of two antagonistic, chromatin-mediated mechanisms by which KLF11, also known as TIEG2 (transforming growth factor-β-inducible early gene 2) and MODY VII (maturity onset diabetes of the young VII), regulates transcription of the dopamine D2 receptor (Drd2) gene. First, KLF11 activates transcription by binding to a distinct Sp-KLF site within the Drd2 promoter (−98 to −94) and recruiting the p300 histone acetyltransferase. Second, Drd2 transcriptional activation is partially antagonized by heterochromatin protein 1 (HP1), the code reader for histone H3 lysine 9 methylation. Interestingly, KLF11 regulates neurotransmitter receptor gene expression in differentiating neuronal cell populations without affecting neurite formation. Overall, these studies highlight histone methylation and acetylation as key biochemical mechanisms modulating KLF-mediated neurotransmitter gene transcription. These data extend our knowledge of chromatin-mediated biochemical events that maintain key phenotypic features of differentiated neuronal cells.

Studies performed during the last 3 decades have revealed that an unexpectedly large number of human gene promoters are regulated by the reversible binding of GC-binding transcription factors. Sp1, one of the first mammalian transcription factors to be characterized, has served as a well established paradigm for GC-binding transcriptional activators. In addition, because Sp1 GC-rich sites are most often located in close proximity to the transcriptional start site, studies on the regulation of these cis-regulatory sites have increased our understanding of the role that certain key subunits of the RNA polymerase II holoenzyme play in transcriptional initiation (1, 2). Recent discoveries have revealed the existence of a large repertoire of proteins, which share similar DNA binding domains with Sp1, can bind to the GC-rich sites previously attributed exclusively to Sp1, and differentially regulate gene transcription. Several of these proteins antagonize Sp1-mediated transcriptional activation, leading to gene silencing, endowing GC-rich sites with the capacity to function as “on” and “off” switches in gene regulation (3). Conceptually, these studies have definitively changed the paradigm for the regulation of promoters via proximal GC-rich sites from the initial notion of Sp1 as a single master regulator (activator) to a more dynamic model. The new paradigm informs us that a large variety of transcription factors target GC-rich sites to achieve different promoter states (silenced versus activated). Last, this information has excellent predictive power for designing experiments to investigate how proximal GC-rich sites serve to scaffold different transcription factors as...
KLF11 Regulation of Neurotransmitter Receptors

well as chromatin-activating and -silencing complexes on a large number of genes important for the regulation of most biochemical processes.

Fortunately, the discovery and investigations of the KLF family of Sp1-like transcription factors have extended our understanding of how GC-rich sites in proximal promoters are regulated. This KLF protein family is composed of 17 members that regulate gene expression programs underlying a substantial number of biological and pathobiological processes and are conserved from organisms ranging from Drosophila melanogaster to humans (3, 4). These proteins are structurally characterized by three Cys2/His2 zinc finger DNA binding domains at their C termini, which are remarkably similar to the corresponding region of Sp1, and variant transcriptional regulatory motifs at their N termini (5). Similar to Sp1, the conserved KLF zinc fingers recognize GC-rich sequences (4). The variant N-terminal domains recruit chromatin-remodeling co-regulators that dictate the function of KLF proteins as transcriptional activators, repressors, or both (3). Biochemical studies using both cell and animal models reveal that these domains can mediate sequence-specific regulation of promoters by recruiting distinct histone-modifying enzyme complexes, including p300, CREB-binding protein (CBP), p300/CBP-associated factor, C-terminal binding protein, SIN3-histone deacetyase, and histone methyltransferases, to GC-rich regions of promoters (6–8). However, the types of chromatin remodeling machines that are recruited to proximal cis-regulatory GC-rich promoter sequences by KLF proteins to regulate gene expression remain poorly understood. Moreover, whether distinct domains that mediate coupling to these chromatin pathways perform either synergistic or antagonistic functions remains to be firmly established. Thus, addressing these questions will expand our knowledge of how GC-rich regulatory sequences and distinct KLF-mediated recruitment of chromatin remodeling machines are coupled to transcriptional regulation, chromatin dynamics, and epigenetics.

In the current study, we designed experiments to shed light on the biochemical mechanisms utilized by KLF proteins to regulate gene expression and to determine key phenotypic traits during cell differentiation. For this purpose, we selected well established neuronal models of cell differentiation, which, at the same time, help to fill a gap in knowledge, because only limited research has been performed on KLF proteins in neurons. Emerging elegant studies have shown the involvement of KLF proteins in neuronal cell differentiation and maturation (9–11). For instance, Moore et al. tested all KLF proteins to highlight their role(s) in axon growth (12), a key feature in neuronal cell differentiation. Additionally, recent reports have implicated KLF7 and KLF16 in the regulation of dopaminergic gene expression and KLF11 in the regulation of a neurotransmitter-related gene, monoamine oxidase, conspicuous events associated with neuronal cell differentiation (13–15). These studies, together with the fact that neuronal induced pluripotent stem cells can be derived from KLF-transfected cells (16), indicate that mechanistic knowledge on KLF proteins might be applicable to the field of regenerative medicine in the nervous system. In particular, there is a paucity of knowledge regarding the set of genes regulated by KLF proteins in neurons and the chromatin pathways that KLF proteins engage as biochemical switches during induction or maintenance of neuronal phenotypes. Thus, by extending this information, with a focus on chromatin remodeling, our study sought to further define how KLF transcription factors mechanistically regulate prominent phenotypic features in differentiating cells. The combined cellular, biochemical, and molecular analyses performed here reveal a novel KLF-mediated mechanism for regulating the transcription of Drd2, involving functional antagonism between two distinct chromatin pathways. Because this mechanism is dissociated from those that mediate neurite formation, this outcome has implications for understanding how neurite formation and neurotransmitter-related gene expression, two processes germane to neuronal differentiation, are independently regulated. Dysregulation of Drd2 receptor expression is a defining feature of neuronal dysfunctions that contribute to the pathobiology of common and debilitating human conditions, including addiction, schizophrenia, and Parkinson disease (17–21). Thus, this new biochemical information is likely to have significant biomedical relevance.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—PC12 cells were cultured in Dulbecco’s modified Eagle’s medium high glucose with l-glutamine medium, supplemented with heat-inactivated (57 °C, 1 h) 10% horse serum (Invitrogen) and 5% fetal bovine serum (Midsci, St. Louis, MO), and 0.5% penicillin-streptomycin (Sigma). PC12 cells were cultured on plates coated with rat tail collagen (BD Biosciences). Cells were allowed to adhere overnight prior to treatment with nerve growth factor (NGF; BD Biosciences). Cells were allowed to adhere overnight prior to treatment with nerve growth factor (NGF; BD Biosciences), adenoviral transduction, or cycloheximide (Sigma). The pancreatic epithelial cells, PANC1, which express the Drd2 receptor, were cultured by our laboratory as described previously (22). Dorsal root ganglia (DRG) neurons were obtained from mice pups at E13. All animal protocols were approved by the Mayo Clinic Animal Care and Use Committee. Dissociated and neurally enriched cultures were obtained by 20 μM 2,5-fluoro-2-deoxyuridine (Sigma) and 20 μM uridine (Sigma) treatment for 3 days using established methods (23). Stable cultures of neurons without Schwann cells or fibroblasts were maintained in Eagle’s minimal essential medium containing 15% calf bovine serum (Hyclone, Logan, UT), 7 mg/ml glucose (Sigma), and 1.2 mM l-glutamine (Invitrogen) and were treated with NGF at concentrations of 10 or 100 ng/ml. Whole DRG explants were cultured on rat tail collagen-coated 35-mm plastic dishes. Nine to twelve DRG were placed on each plate at least 10 mm apart. Cultures were maintained in Eagle’s minimal essential medium containing 15% calf bovine serum, 7 mg/ml glucose, 1.2 mM l-glutamine, and varying concentrations of NGF.

**Plasmids and Recombinant Adenovirus**—For all of our studies, we used human wild type and mutant KLF11 cloned into pcDNA3.1/His (Invitrogen) and rat wild type and mutant Drd2
promoters cloned into pGL3 (Promega, Madison, WI). The sequences of both the KLF11 protein and the Drd2 promoter are remarkably conserved both in structure and function across evolution. The potential GC-rich KLF11 binding sites are 95% conserved from rodents to humans. All mutants of the Drd2 promoter were made using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA) according to the manufacturer’s protocol. The p300 dominant negative (human p300DN) construct was from Upstate Biotechnology, Inc. (Lake Placid, NY). All constructs were confirmed by sequencing at the Mayo Clinic Molecular Biology Core Facility. Epitope-tagged (6XHis-Xpress^{TM}) KLF11, KLF11ΔHP1 (24), and empty vector (Ad5CMV) were generated as recombinant adenovirus in collaboration with the Gene Transfer Vector Core at the University of Iowa.

**GST Fusion Protein Purification**—GST and GST fusion protein purification was performed as described previously (25).

**RT-PCR and qPCR Gene Array**—Total mRNA was extracted using the RNeasy minikit (Qiagen, Valencia, CA) and was reverse transcribed into cDNA using SuperScriptIII (Invitrogen) according to the manufacturer’s protocol. Using the cDNA as template, mRNA for Klf family members or neurotransmitter receptors was amplified by semiquantitative RT-PCR or qPCR (SA Biosciences). PCR products were examined on a 2% agarose gel and assessed relative to hypoxanthine-guanine phosphoribosyltransferase mRNA levels.

**Chromatin Immunoprecipitation (ChIP) Assay**—Primer sets were designed (2–3 pairs/gene) in the promoter region of the possible target genes. The cells were lysed on ice in cell lysis buffer with protease inhibitor. After centrifugation, the cell pellet was resuspended in nuclear lysis buffer containing protease inhibitor. The resulting nuclear extract was sonicated on wet ice and then immunoprecipitated with appropriate antibodies. Antibodies against His tag (for recombinant KLF11 and KLF11ΔHP1, OMNI D8) and Drd2 (Millipore) were used at 1:1000 dilution. HRP-conjugated secondary antibody (1:1000 dilution) and chemiluminescence were used to detect the protein levels. Immunoprecipitation was done as described previously (25). EZview^TM^ Red anti-FLAG M2 affinity gel (Sigma) was utilized to immunoprecipitate recombinantly expressed KLF11 or KLF11ΔHP1. Subsequently, Western blot was performed to detect protein complexes with anti-FLAG (Sigma) and anti-HP1α (Millipore).

**Electrophoretic Mobility Shift Assays (EMSAs)**—Wild type and mutant probes spanning KLF11 potential binding sites were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase according to the manufacturer’s protocol (Promega). Four micrograms of purified GST or GST fusion recombinant KLF11 were incubated in a buffer containing 20 mM HEPEs (pH 7.5), 50 mM KCl, 5 mM MgCl2, 10 μM ZnCl2, 6% glycerol, 200 μg/ml bovine serum albumin, and 50 μg/ml poly(dI-dC)-poly(dI-dC) for 7 min at room temperature. End-labeled probes, excess of cold probes, or antibodies were added as indicated to each reaction and incubated at room temperature for an additional 20 min. The samples were loaded onto a 4% non-denaturing polyacrylamide gel, run for 3 h at 200 V, vacuum-dried, and exposed to HyBlot CL^TM^ autoradiography film (Denville Scientific Inc., Metuchen, NJ).

**Neurite Outgrowth**—After appropriate NGF treatment, light microscopic images were acquired every 24 h. Random fields of view were taken from PC12 cells, and neurite outgrowth was assessed using stereology. From whole DRG explants, digital phase-contrast images were acquired every 24 h after appropriate NGF treatment. Radial neurite outgrowth was measured using ImageJ software. Neurite length was measured from the edge of the ganglion to the tip of the longest neuronal process.

**Neurite Collapse**—PC12 cells were treated with 100 ng/ml NGF for 7 days before being transduced with adenosivirus containing EV or KLF11. Once transduced, the cells were incubated in NGF-free media to inhibit further neurite outgrowth. Light microscopic images were taken every 24 h, and neurite lengths were measured using stereology. Whole DRG explants were treated with 10 ng/ml NGF for 48 h before being transduced with adenosivirus containing EV or KLF11. Once transduced, NGF concentration in the media was increased to 100 ng/ml to inhibit further neurite outgrowth, and digital phase-
KLF11 Regulation of Neurotransmitter Receptors

![Figure 1](image1.png)

**FIGURE 1. Klf11 is down-regulated during NGF-induced neuronal cell differentiation.** A, in PC12 cells, the mRNA fold changes of Klf family members upon 100 ng/ml NGF treatment were measured using semiquantitative PCR. -Fold changes of mRNA were calculated as the ratio of Klf levels in NGF-treated cells to those in cells without NGF treatment. Klf8 and Klf17 have not been identified in rats, and Klf12 was not amplified in our hands. The -fold changes in mRNA levels are color-coded according to the scale presented. B, qPCR was performed to measure the amount of Klf11 mRNA in PC12 cells treated with 100 ng/ml NGF. -Fold changes of Klf11 mRNA were calculated as the ratio of Klf11 mRNA levels in NGF-treated cells to those in cells without NGF treatment. C, in order to inhibit translation, PC12 cells were treated with cycloheximide (5 µg/ml) 2 h prior to NGF treatment. Klf11 mRNA levels were measured by qPCR at various time points after NGF treatment. D, semiquantitative PCR was performed to measure the Klf11 mRNA level in DRG neurons treated with 0, 10, or 100 ng/ml NGF for 48 h. -Fold changes of Klf11 mRNA were calculated as the ratio of Klf11 level in 10 or 100 ng/ml NGF-treated cells to that in cells without NGF treatment. Left, representative image of DNA gel. Right, averaged quantification of the DNA gels (n = 3). Error bars, S.E. *, p < 0.05; **, p < 0.01.

contrast images were taken every 24 h. Changes in radial neurite lengths were measured using ImageJ software.

**RESULTS**

**Family-wide KLF Screening Reveals Role for KLF11 in Neuronal Cell Differentiation—**To investigate which KLF transcription factors participate in NGF-induced neuronal cell differentiation, we initially performed an expression-based family-wide screening of all Klf family members in the neuron-like PC12 rat pheochromocytoma cell model as recently described (26, 27). For this purpose, we utilized RNA from PC12 cells treated with 100 ng/ml NGF for periods between 0 and 48 h (Fig. 1A). This screening step defined three different expression patterns for members of this transcription factor family: (i) mRNA levels of Klf1, -6, -7, -9, and -16 remained constant, regardless of NGF treatment (NGF-insensitive KLF genes); (ii) mRNA levels of Klf2, -3, -4, -5, -10, -13, and -14 increased within 6 h of treatment and returned to basal level by 48 h (NGF-inducible KLF genes); (iii) mRNA levels of Klf11 and -15 decreased with NGF treatment (NGF-down-regulated KLF genes) (Klf8 and -17 have not been identified in rats, and Klf12 was not amplified in our hands). Notably, among all observed effects, the decrease in Klf11 mRNA level was the most robust (p < 0.01, compared with t = 0 for all time points) and long lasting. Plotting of qPCR results of KLF11 in Fig. 1B shows that Klf11 mRNA level decreased, $-3.50 \pm 0.67$-fold at 2 h, and remained low even after 48 h of NGF treatment. To test the possibility that Klf11 is an NGF early response gene, translation was inhibited with cycloheximide (5 µg/ml) 2 h before NGF treatment, and Klf11 mRNA levels were measured over time. Fig. 1C shows that cycloheximide-induced inhibition of translation abolished the NGF-induced decrease in Klf11 mRNA. Hence, protein synthesis is required for the down-regulation of Klf11 transcription, which, together with the duration of its response, indicates that Klf11 is not an NGF early response gene but rather part of a delayed and long lasting response to NGF. Notably, genes that participate in defining the acquisition of distinct phenotypes in undifferentiated neuronal precursors often show the type of expression described above for Klf11 (28–30).

Subsequently, we utilized a primary neuronal cell culture to confirm the expression pattern of this transcription factor during NGF-induced neuronal cell differentiation. Dissociated DRG neurons from E13 mouse embryos were treated with NGF (10 or 100 ng/ml) for 48 h. For control purposes, we harvested whole DRG without NGF treatment. Similar to the effect observed in PC12 cells, NGF treatment of DRG cultures induced decrease in Klf11 mRNA levels (Fig. 1D). This effect was dose-dependent because values of gene expression changed from $-1.78 \pm 0.12$-fold in cells treated with 10 ng/ml
NGF to $\sim 2.58 \pm 0.25$-fold with 100 ng/ml NGF. Thus, together, the results of this screening step show that KLF family members are actively regulated by differentiating growth factors (NGF), probably to regulate the expression of important neuronal genes. This idea led us to perform subsequent mechanistic experiments using KLF11 as a model for understanding how members of this family of transcription factors mediate neuronal gene expression.

**KLF11 Regulation of Neurotransmitter Receptors**

**Klf11 Switches Transcriptional Patterns of Neurotransmitter Receptor Expression**—We next tested the hypothesis that KLF11 functions as a pivotal switch for coupling chromatin-mediated pathways in neurons. Initially, we investigated whether this transcription factor influences two of the major processes that characterize neuronal differentiation: (i) a defined neurotransmitter receptor profile or (ii) neurite formation. Thus, the first series of experiments sought to determine the function of KLF11 in regulating the expression of neurotransmitter receptors. Notably, KLF11 can complex to multiple chromatin pathways, including histone acetyltransferases (HATs) (CBP or p300) (6) and Sin3-histone deacetylases (31). More importantly, in the context of this study, KLF11 contains a PXXV domain (amino acids 487–491), which couples this transcription factor to the HP1-HMT repression system (Fig. 2A), a recently identified and characterized corepressor system for KLF11 (24). Therefore, we analyzed the influence of these complexes on neurotransmitter receptor transcriptional regulation. Moreover, we determined whether these pathways either synergize or antagonize each other to provide the ultimate functional outcome in gene expression and differentiation. To address these conceptual queries, we virally transduced PC12 cells with either the wild type KLF11 or mutants that selectively disrupt coupling of KLF11 to either of the repressive chromatin pathways, Sin3-histone deacetylase or HP1. Because HAT binding occurs within the first zinc finger of KLF (6), which is responsible for its DNA binding, selective disruption of KLF11-mediated coupling to this chromatin...
KLF11 Regulation of Neurotransmitter Receptors

A. Wild type

NGF (ng/ml) 0 10 100

Drd2

Hprt

Drd2 mRNA (fold)

B. KLF11 −/−

NGF (ng/ml) 0 10 100

Drd2 mRNA (fold)

C. Wild type

EV KLF11 ΔHP1

D. Klf11

Drd2

Klf11

E. Wild type

KLF11 −/−

FIGURE 3. In primary cultures of DRG neurons, NGF treatment induces down-regulation of Drd2 mRNA. A, semiquantitative RT-PCR was performed to measure mRNA level of Drd2 in NGF-treated (48 h; 0, 10, or 100 ng/ml) DRG neurons from wild type mice. Left, representative image of the DNA gel showing Drd2 mRNA level. Right, averaged quantification of the DNA gels (n = 3). B, qPCR was performed to measure the mRNA level of Drd2 in NGF-treated (48 h; 0, 10, or 100 ng/ml) DRG neurons from Klf11 knock-out mice. Unlike DRG from wild type mice, Klf11 −/− DRG cells did not show a decrease in Drd2 mRNA with NGF treatment. C, qPCR was performed to measure the Drd2 mRNA levels in Klf11−/− or Klf11ΔHP1-overexpressing DRG neurons from wild type mice. -Fold changes of Drd2 mRNA were calculated as the ratio of Drd2 mRNA levels in control (EV)-infected cells to cells infected with KLF11 or KLF11ΔHP1 (ΔHP1). D, based on the semiquantitative RT-PCR results from wild type DRG neurons, correlation between KLF11 and Drd2 mRNA levels was plotted and calculated (y = 0.79x + 0.21, r = 0.88). E, qPCR was performed to measure the Drd2 mRNA levels in brains from Klf11 knock-out and wild type mice at different developmental stages (E13, E15, E18, and postnatal days 1 and 7 (P1 and P7)). The -fold changes represent Drd2 levels in knock-out mice compared with the wild type mice (n = 3–4). Error bars, S.E. *, p < 0.05; **, p < 0.01.

pathway was not possible. These cells were subsequently treated with NGF (100 ng/ml) for 48 h. Expression of KLF11 after transfection was confirmed with Western blotting (Fig. 2B). A qPCR gene array was used to compare neurotransmitter receptor transcript levels across the treatment conditions (selected results shown in Fig. 2C). These experiments show that KLF11 overexpression increased transcript levels of Drd2a (5.11-fold, p < 0.05), and Drd2 (2.88-fold, p < 0.01) compared with EV control. Interestingly, the Drd2 mRNA level in cells expressing KLF11 mutant defective in Sin3-histone deacetylase coupling did not differ from that in cells expressing KLF11 wild type (2.49-fold, p = 0.07, compared with EV control). In contrast, disruption of KLF11 coupling to HP1 (KLF11ΔHP1) resulted in a greater activation of Drd2 (5.12-fold, p < 0.001), revealing that loss of a basal repressing function mediated by this heterochromatin protein behaves as a rheostat mechanism that limits the activation of this gene. Concomitantly, transcript levels of several acetylcholine receptors, both muscarinic and nicotinic, were decreased with KLF11 overexpression (Fig. 2C). These changes in receptor expression pattern suggest that KLF11 is involved in establishing distinct phenotype changes in differentiating sensory neurons.

KLF11 Directly Regulates Transcription of Some Neurotransmitter Receptors—Due to the importance of these observations for outlining novel biochemical mechanisms that regulate neurotransmitter receptors, we performed ChIP assays to test if any of these receptor genes were direct targets of KLF11. Fig. 2D shows that KLF11 occupies the promoter regions of Chrna3, Chrnb2, Chrnb4, Drd2, and Drd4. Our results, therefore, identified these neurotransmitter receptor genes as bona fide targets of KLF11 in neuronal cell populations. To maximize the chances of gaining valuable mechanistic knowledge, we focused more on the role of KLF11-mediated Drd2 regulation because this receptor exerts a pivotal role in normal neurobiology, drug addiction, schizophrenia, and other neuropsychiatric diseases.

ChIP-based experiments demonstrate that KLF11 binds to the DRD2 promoter in both mouse DRG neurons and a human pancreatic epithelial cell line that readily expresses DRD2 (Fig. 2, E and F). Thus, the interaction of KLF11 with this DNA regulatory region appears to be conserved in genomes ranging from rodents to humans. In addition, similar to our observations in rat PC12 cells, microarray experiments performed in the same human pancreatic cell used above for ChIP assays revealed that KLF11ΔHP1 expression increased the level of the human DRD2 mRNA by 2.27-fold (p < 0.02). Subsequently, we tested whether KLF11 exerts similar effects on Drd2 transcription in the primary cultures of DRG neurons. In this model, NGF treatment led to a concordant decrease in mRNA levels of both Klf11 (Fig. 1D; −1.8 ± 0.12-fold for 10 ng/ml NGF and −2.6 ± 0.25-fold for 100 ng/ml NGF) and Drd2 (Fig. 3A; −1.5 ± 0.09-fold for 10 ng/ml NGF and −1.7 ± 0.15-fold for 100 ng/ml NGF). This decrease of Drd2 mRNA upon NGF treatment was abolished in Klf11 knock-out mice, indicating that Klf11 plays a key role in transcription of Drd2 (Fig. 3B). Congruently, when KLF11 was overexpressed in DRG neurons of wild type mice, the mRNA level of Drd2 was significantly increased (Fig. 3C). Congruently, overexpression of the KLF11ΔHP1 mutant induced even further increase in Drd2 mRNA (Fig. 3C). The semiquantitative RT-PCR experiments shown in Figs. 1D and 3A demonstrate a strong positive corre-
KLF11 Regulation of Neurotransmitter Receptors

binding was observed with the wild type probe (Fig. 4, lane 9), suggesting that site 4 is the key site for KLF11 binding. Concordantly, a probe containing both mutations on sites 3 and 4 was not recognized by KLF11 (Fig. 4C, lane 12). Specificity of KLF11 binding to the wild type probe was further confirmed with supershift experiments using control or KLF11 antibodies (Fig. 4D, lanes 5 and 6). Competition assays were performed to demonstrate that KLF11 binding to the labeled wild type probe was abolished by the addition of a 50- or 100-fold amount of its unlabeled counterpart (Fig. 4D, lanes 7 and 8). In contrast, a 100-fold amount of unlabeled probe with site 4 mutation or unlabeled probe with a double (sites 3 and 4) mutation did not compete with the radioactive wild type probe from KLF11 (Fig. 4D, lanes 9 and 10). To validate site 4 as the KLF11 binding site, a luciferase plasmid containing the Drd2 promoter with a mutation in this site was created (M site4). Reporter assays showed that, similar to Δsite3–5 and Δsite1–5, the activity of M site4 Drd2 promoter did not significantly increase with KLF11 overexpression (Fig. 4B, 1.70 ± 0.47-fold EV control). The data from both reporter assays and EMSA assays combined with the knowledge inferred from mutation analyses constitute a rigorous demonstration that KLF11 activates Drd2 transcription by directly binding to a distinct GC-rich site within the Drd2 promoter (site 4 at nucleotides 98 to 94). Thus, KLF11 binding to this distinct site should be critical for linking this regulatory gene region to the chromatin pathways that are relevant to its regulation. Consequently, we next studied how this new KLF activator of Drd2 transcription couples to chromatin to mediate its function.

KLF11 Differentially Couples to Distinct Chromatin Pathways to Regulate Drd2—The qPCR gene array experiment described above showed that KLF11 expression increases Drd2 mRNA levels (Fig. 2B). In addition, disruption of KLF11 interaction with HP1 via expression of KLF11ΔHP1, induced a greater increase in Drd2 mRNA. This result was validated using both RT-qPCR (Fig. 5A) and luciferase-based reporter assays (Fig. 5B). Similar to the results observed at the mRNA level, KLF11 increased Drd2 promoter reporter activity (7.3 ± 0.86-fold), which was also further augmented by KLF11ΔHP1 expression (58.2 ± 2.13-fold) (Fig. 5B). Results at the protein level, obtained through Western blotting from PC12 cells overexpressing KLF11 or KLF11ΔHP1, were congruent with the data from mRNA and reporter studies, showing an increased level of the typical highly glycosylated membrane-bound DRD2 protein (>80 kDa), the intermediate form (~50 kDa), and the non-glycosylated (~25 kDa) cytoplasmic form (Fig. 5C). ChIP assay confirmed that both the KLF11 and KLF11ΔHP1 mutant proteins occupied the Drd2 promoter. In addition, HP1 occupancy of the Drd2 promoter was confirmed in control and KLF11-transduced cells, but was absent in cells transduced with KLF11ΔHP1 (Fig. 5D), confirming that this KLF11 mutant abolishes HP1 recruitment. The further activation of Drd2 transcription with KLF11ΔHP1 suggests that the HP1 chromatin silencing system operates to prevent an excessive activation of this receptor, which could result in neuronal dysfunction (20, 34).

To further define the chromatin pathways utilized for KLF11-mediated transcriptional activation of Drd2, we exam-
ined coupling of this transcription factor to p300, a HAT. ChIP assays confirmed p300 occupancy on the Drd2 promoter in PC12 cells transduced with EV, KLF11, and KLF11/HP1 (Fig. 5D). In complementary luciferase-based reporter assays, we expressed a dominant negative form of p300 (p300DN), lacking enzymatic activity, along with KLF11 or KLF11/HP1 (Fig. 5E). Expression of p300DN antagonized the increase observed with KLF11 (9.9 ± 0.17- to 6.1 ± 0.07-fold) and KLF11/HP1 (47 ± 0.02- to 2.1 ± 0.08-fold), without disrupting the binding of KLF11 to the Drd2 promoter (Fig. 5F). Thus, p300 is necessary for KLF11-mediated Drd2 transcriptional activation, and HP1 appears to limit the level of transcriptional activation achieved by this HAT (antagonistic pathways). Thus, by balancing these two processes, KLF11 helps to achieve appropriate levels of Drd2 transcription. These data raise the possibility that selective inactivation of these pathways, as is known to occur under several physiological and pathophysiological situations (e.g. Rubinstein-Taybi syndrome), may tilt the transcriptional outcome to produce more or less Drd2 mRNA and protein. Thus, testing of this hypothesis in human tissues from affected patients may shed light as to whether they are more sensitive to drugs and processes that are dependent on KLF11-p300-mediated Drd2 expression.

KLF11 Regulation of Neurotransmitter Receptors

A. (-289) CAGAGTGGTTGGAATGCGACAGTTTTAAAGCT (-251)
   (-250) ATAGGATGACCCGCGAGAGTCGGAACACGTATCCCACCTCAAA (-201)
   (-200) GCCGAGATGAGATTCTGGTAAGCCTCAGAGGCTCCTTCCCAGGCC (-151)
   (-150) CAGAGTGGTTGGAATGCGACAGTTTTAAAGCT (-101)

B. site5
   (-100) ACGGCGGCTCCGCCGCTGAGCTGAGGCCGCGCCCTC (-51)

C. probe P
   WT
   Δsite5
   Δsite3-5
   M site4

D. Probe P
   wt
   Cold Probe
   GST
   KLF11
   Control Ab
   GST Ab

FIGURE 4. KLF11 recognizes and binds to a specific sequence on the Drd2 promoter to regulate transcription. A, the sequence represents rat Drd2 proximal promoter (−1 to −289 relative to TSS). Five possible KLF11 binding sites were identified based on the sequence (underlined, sites 1–5 relative to TSS). B, various Drd2 promoter deletion constructs were cloned upstream of the luciferase gene and co-transduced into PC12 cells with EV or KLF11 constructs. Relative luciferase activity with KLF11 expression is plotted (compared with EV values). C, in order to identify the binding site of KLF11 on the Drd2 promoter, EMSA was performed using recombinant KLF11 (lanes 3, 6, 9, and 12) or control GST protein (lanes 2, 5, 8, and 11) with radiolabeled double-stranded probe containing various mutations of the Drd2 promoter or probe alone (lanes 1, 4, 7, and 10). WT, wild type spanning residues between −109 and −71; mut4, mutation in site 4 (−94GGGCGGG−93 to GGTTTGG); mut3, mutation in site 3 (−89CCCGGGCG−82 to CCTTTTG); Dm, double mutation (sites 4 and 3). Specific complexes between KLF11 and probe and the free probe are indicated by arrows on the right. D, EMSA was performed with radiolabeled wild type rat Drd2 promoter probe spanning residues between −109 and −71 (lanes 1 and 3–10) with control GST protein (lane 2) or recombinant KLF11 (lanes 4 and 10) or probe alone (lane 1). Specific complexes between KLF11 and probe and the free probe are indicated by arrows on the left. A GST antibody (Ab) shifted recombinant KLF11-Drd2 probe complex (lane 5), whereas the same amount of anti-IgG did not (lane 6), indicating specificity. Excess unlabeled WT probe robustly competed for binding (50-fold (lane 7) and 100-fold (lane 8)), whereas unlabeled mutant probes did not (lane 9, site 4 mutant probe, 50-fold; lane 10, double mutant probe, 50-fold). Error bars, S.E. **, p < 0.01.
KLF11 Regulation of Neurotransmitter Receptors

KLF11 Regulates Neurotransmitter Receptors Independently of Neurite Formation—We next investigated whether KLF11 chromatin-mediated pathways are involved in the second event of neuronal cell differentiation, namely neurite extension. Similar to results from the studies on neurotransmitter gene expression, our initial experiments revealed a correlation between increased neurite outgrowth after NGF treatment with reduced Klf11 expression (Fig. 1B). Thus, it is possible that high levels of KLF11 hinder neurite outgrowth or cause existing neurites to collapse. We tested these potential scenarios by examining the effects of KLF11 in preventing or reversing neurite outgrowth in DRG neurons. Primary cultures of whole DRG explants from wild type mouse embryos (E13) were plated and transduced with adenovirus carrying control (EV) or KLF11 constructs and subsequently treated with NGF to induce neurite extension. Neurite length was measured 24 and 48 h later under two different conditions: low NGF (10 ng/ml), which promotes maximum neurite outgrowth, and high NGF (100 ng/ml), which inhibits neurite outgrowth in this system (35). Neurite lengths in EV-treated and KLF11-overexpressing DRG did not differ at any time (Fig. 6, A and B), demonstrating that an increased level of KLF11 is not sufficient to suppress neurite outgrowth. Similarly, neurite outgrowth experiments in primary cultures of whole DRG from E13 Klf11−/− mouse embryos also revealed no differences in neurite outgrowth between Klf11−/− and wild type neurons (Fig. 6, A and B). KLF11 overexpression was confirmed by Western blotting at 48 h (Fig. 6C). Identical results were also recapitulated in PC12 cells treated with 100 ng/ml NGF (Fig. 7, A and B). Therefore, all of the experimental evidence presented in this study supports the view that KLF11 does not induce neuritogenesis. Consequently, we also determined whether KLF11 causes existing neurites to collapse. E13 wild type or Klf11−/− whole DRG were pretreated with 10 ng/ml NGF for 48 h to allow neurite formation. Cells were then transduced with either KLF11 or control adenovirus and incubated in medium with 100 ng/ml NGF to inhibit further neurite outgrowth. Neurite lengths were measured 24 and 48 h after infection. These conditions generated three different levels of KLF11: low (Klf11−/−, cells), normal (EV-transduced wild type cells), and high (KLF11-transduced wild type cells). However, the length of pre-existing neurites did not shorten over time in any of these groups (Fig. 6D). This result in primary neuronal cultures (DRG) was confirmed in experiments that compared neurite lengths between control and KLF11-transduced PC12 cells (Fig. 7C). In conclusion, during neuronal cell differentiation, KLF11 neither retards neurite outgrowth nor causes existing neurites to collapse, demonstrating that the function of this transcription factor is restricted to the regulation of neurotransmitter receptors with no obvious function in neuritogenesis.

DISCUSSION

The current study constitutes the first characterization of fundamental biochemical mechanisms utilized by KLF proteins to regulate neurotransmitter receptors, independent of their function in neurite formation. Among these mechanisms, we defined how KLF11 recognizes, binds, and regulates the Drd2 gene via a distinct GC-rich cis-regulatory motif (nucleotides −94 through −98). This information expands the knowledge on the repertoire and function of chromatin-mediated pathways, which are recruited by Sp–KLF sequence to transcriptionally regulate a target gene in neurons. The antagonism between the HP1 and p300 chromatin pathways works in a manner analogous to a rheostat to maintain appropriate levels of Drd2.

Although we chose Drd2 as a model for detailed mechanistic studies on KLF11, our experiments reveal that KLF11 directly binds to and up-regulates other dopaminergic receptor genes while down-regulating some cholinergic receptors. Thus, several neurotransmitter receptor genes are amenable to KLF11-mediated regulation. Similarly, we showed that KLF11-induced Drd2 regulation can also be observed in human pancreatic cells (Fig. 2E), which indicates that the mechanism we describe here is not limited to neurons but might be, at least in part, applicable to other types of cells in contexts yet to be defined.
Mechanistically, KLF11 activates transcription of Drd2 by directly binding to a specific promoter sequence and recruits both HP1 and p300. Transcriptional activation of Drd2 is accomplished by KLF11 interaction with the co-activator, p300, whereas HP1 interaction with KLF11 limits the level of activation. These results were confirmed and cross-validated using extensive assays to characterize this phenomenon by studying both the isolated Drd2 promoter (reporter assays) and endogenous promoter (ChIP assays) and by looking at both the mRNA level (gene array and PCR) and protein level (Western blot analyses). Key studies utilized recently generated Klf11−/− mice (26), to demonstrate that the results are reliably reproduced in primary neurons. Collectively, our results provide a basis for understanding the regulation of key GC-rich promoters involved in functions associated with phenotypic differentiation (patterns of neurotransmitter-related gene expression) in well established neuronal cell models.

The fact that NGF functions as a survival factor for DRG neurons creates a confounding variable for the experiments. DRG neurons consist of three populations that express specific receptors for different neurotrophins. The presence of NGF will tend to favor the survival of the NGF-responsive population that expresses the TrkA receptor but not others. Thus, both the entire repertoire of neurons in which KLF11 plays an

**FIGURE 6.** KLF11 regulates neurotransmitter receptors in DRG neurons independently of neurite formation. A, representative images of control, KLF11-overexpressing DRG from wild type or KLF11 knock-out animals. DRG were treated with various concentrations of NGF for 48 h. Collapsing images show DRG neurons at 24 h after adenovirus infection. B, quantification of neurite outgrowth. All measurements were normalized to 100 ng/ml NGF, EV conditions. C, Western blot was used to show overexpression of KLF11 from cell lysates harvested at 48 h after transduction. D, quantification of neurite lengths in collapsing experiment. Neurite lengths were measured at 0, 24, and 48 h after infection. All measurements were normalized to 0 h with the EV condition. Error bars, S.E.
important biological role and the cellular context that influences its functions (e.g., different neurotrophins) remain to be defined. We are optimistic that the current mechanistic work will serve to better inform the design of this type of investigation.

Interestingly, in different cell types, KLF11 can couple to different HAT pathways, namely CBP, p300, and p300/CBP-associated factor (6, 8). Notably, however, in our biochemical studies, p300 mediates the activating function of KLF11 on Drd2, thus defining this protein as a key HAT pathway that couples to KLF11 in neuronal cell populations. This is important in light of the fact that mutation and dysregulation of this HAT are found in human diseases characterized by abnormal neuronal function (36). The involvement of HP1, a co-repressor, in limiting transcriptional activation led us to discover an antagonism between activating and silencing pathways, which has a significant impact on the regulation of neurotransmitter receptors (e.g., Drd2). Interestingly, humans express three distinct HP1 isoforms that modulate gene silencing in a highly regulated manner (37). In this regard, although the present study identified HP1α as the isoform that couples to KLF11, we cannot rule out the possibility that HP1β and HP1γ can accomplish this function under different circumstances. A KLF11 mutant, which lacks the HP1 binding site (KLF11ΔHP1) without disrupting KLF11 DNA binding or its coupling to other chromatin co-regulators (24), loses the ability to antagonize HAT-mediated activation, thereby increasing its activation of the Drd2 promoter. This increase in Drd2 activation, due to impaired HP1 coupling, was almost abolished by expression of the dominant negative form of the p300 HAT, confirming this HAT as a molecule responsible for the transcriptional activation. Our results, taken together, demonstrate that KLF11-mediated transcriptional regulation of neurotransmitter receptors is not a simple “on” and “off” phenomenon but rather is regulated by different chromatin pathways that functionally interact to fine-tune the intensity of transcriptional activation or repression in a cell context-dependent manner.

The relevance of these results to the regulation of gene expression, cell differentiation, and phenotypic typing of neuronal cells deserves further discussion. For instance, because alterations in Drd2 expression are associated with several neurological diseases, knowledge of the KLF-p300-HP1 pathway combined with a new type of chromatin-centric/epigenetic pharmacology adds biomedical relevance. The dopaminergic system plays a major role in pleasure and reward. Abnormalities in this system have been implicated in diseases such as Parkinson disease, schizophrenia, drug addiction, and obesity (17–21). Specifically, reduced levels of DRD2, along with compulsive eating behavior, are associated with human obesity and drug addiction (18, 19). One hypothesis is that these compulsive eating or drug-taking behaviors reflect an attempt to re-establish the same intensity of DRD2 activation with fewer avail-
KLF11 Regulation of Neurotransmitter Receptors

able receptors. Therefore, understanding the mechanism of Drd2 transcriptional activation may offer a new perspective on drug development for obesity or drug addiction. Last, the fact that small molecule inhibitors for the pathways described here are currently available for clinical trials should fuel interest in studies to determine whether these drugs have either desired (therapeutic) or undesired (toxic) effects based on their potential to target Drd2 levels.

The present findings, combined with the limited data from previous studies on KLF proteins in neurons, are a foundation for better understanding the role of these proteins in neuronal biochemistry. KLF11 is the first KLF protein whose role in neurite formation and neurotransmitter receptor expression has been determined, revealing that neuronal cells are equipped with a means to regulate these phenomena independently. This role is different from that of other well known neuronal transcription factors that co-regulate both phenomena (e.g. NRSF/REST). Interestingly, at the onset of this study, the findings of altered KLF11 expression during neuronal differentiation suggested a causal role for this protein in promoting or preventing neurite extension. However, the lack of effect on neurite formation by KLF11 knockout or overexpression makes such a role less likely, highlighting the limitations of interpreting expression data in the absence of functional results. Thus, the fact that KLF11 functions in this manner is an indication that transcriptional regulation during neuronal differentiation displays different levels of complexity. Our study, however, does not rule out the possibility that some KLF proteins may mediate both phenomena in a highly coordinated manner.

Last, while this work was in progress, a few reports on KLFs in neuronal cell differentiation were published. These studies found that KLF4 and KLF10 can also be induced by NGF in the PC12 model, although the functional meaning of these observations remains to be further clarified (38, 39). On the other hand, Moore et al. (12) screened for different transcription factors in differentiating retinal ganglion cells and found nine KLF family members that changed axon growth significantly. KLF11 did not show this property, which we also observed in our experiments. Solid results reported by Caiazza et al. (14, 40) demonstrated that KLF7, expressed in the nervous system only during development, is required for differentiation of neuroectodermal and mesodermal cells as well as dopaminergic neurons in the olfactory bulb. Although highly valuable and informative, these previous studies did not provide clear insight into chromatin-mediated mechanisms by which KLF proteins induce or repress transcription of specific genes that lead to differentiation or axon growth. However, none of these studies investigated biochemical mechanistic investigations. Thus, the present report is the first to describe specific mechanisms of KLF-induced transcriptional regulation (chromatin pathways) in neuronal cell differentiation.

In conclusion, our data implicate KLF proteins in neuronal cell differentiation through the regulation of neurotransmitter receptors. More specifically, we show that KLF11 activates transcription of several of these molecules and, more in depth, the Drd2 gene, by binding directly to the promoter region. Subsequently, KLF11 recruits both the co-activator, p300, and the co-repressor, HP1. These data, showing that transcriptional regulation of neurotransmitter receptors in neuronal cells can occur by antagonism between two co-regulators, p300 and HP1, represent an advance in the fields of chromatin dynamics and neuroscience. In our view, it should be worthwhile to carry out future studies aimed at defining important membrane-to-nucleus signaling pathways that utilize such mechanisms. If we can specify the individual recruitment of these complexes, we might come to a better understanding of how chromatin can dynamically change the states of crucial promoters between activation and repression. Such insight would allow us to decipher how different KLF proteins utilize distinct chromatin remodeling machines to codify the execution of functions that have wide biological implications, including neurotransmitter gene expression and neuronal cell differentiation.

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