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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 fopamine 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 capability 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

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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[2] 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 Cys_2/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 deacetylase, 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), 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 neuronally 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 10% 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*

[2] The abbreviations used are: KLF, Krüppel-like factor; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; DRG, dorsal root ganglia; qPCR, quantitative PCR; EV, empty vector; E13, E15, and E18, embryonic day 13, 15, and 18, respectively; HAT, histone acetyltransferase.

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™) 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, Frederick, MD). PCR products were examined on a 2% agarose gel and assessed relative to hypoxanthine-guanine phosphoribosyltransferase mRNA levels. ImageJ software version 1.43 (National Institutes of Health, Bethesda, MD) was used to measure the intensity of bands. For qPCR arrays (SA Biosciences), PC12 cells were transduced with adenovirus (multiplicity of infection = 200) containing the control empty vector (EV), KLF11, or KLF11ΔHP1 construct. Six hours later, cells were treated with 100 ng/ml NGF for 48 h, and cells were lysed. Total mRNA was extracted and reverse-transcribed into cDNA, which was used in the qPCR gene array (Rat Neurotransmitter Receptors and Regulators PCR Array, SA Biosciences). The mRNA levels in KLF11- or KLF11ΔHP1-transduced cells were compared with EV-infected PC12 cells. Changes in gene expression were validated with semiquantitative RT-PCR.

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; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), HP1α (Millipore, Billerica, MA), and p300 (Millipore) were used. Protein G beads (Millipore) were used to collect protein-DNA complexes. Elution buffer (1% SDS, 0.1 M $NaHCO_3$) and proteinase K were used to elute protein-DNA complexes from the beads and then to reverse cross-link the protein-DNA complexes to free DNA. DNA samples were purified, and protein enrichment on the promoter of each target gene was tested by RT-PCR using Platinum Taq (Invitrogen) according to the manufacturer's protocol. PCR products were examined on a 2% agarose gel.

Reporter Assays—*Drd2* promoter reporter (3 μg) and other protein constructs (5 μg) were introduced into 10^6 PC12 cells using the Cell Line Nucleofector Kit V (Basel, Switzerland) according to the manufacturer's protocol and then plated equally into three wells of rat tail collagen-coated 6-well plates (technical triplicate). Cells were lysed 48 h later, and the relative luciferase activity was measured using the Luciferase Assay System (Promega) and a Turner 20/20 luminometer. Each experiment was repeated at least three times, and S.E. values were calculated from all biological replicates. Total protein concentrations were measured and used for normalization in all experiments.

Western Blot and Immunoprecipitation—Standard Western blot techniques were used to determine the levels of KLF11 and DRD2 protein. 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™ 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-^{32}P]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 $MgCl_2$, 10 μM $ZnCl_2$, 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™ 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 adenovirus 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 adenovirus 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-

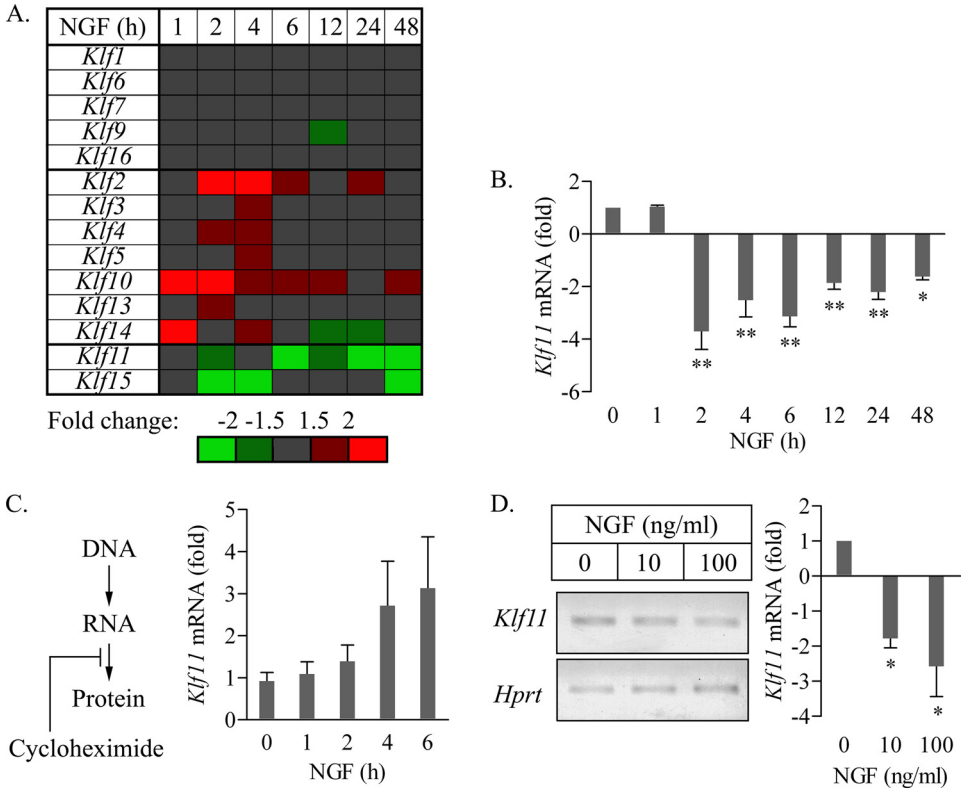

FIGURE 1. **Klf11 is down-regulated during NGF-induced neuronal cell differentiation.** *A*, in PC12 cells, the mRNA -fold changes of *Klf* family members upon 100 ng/ml NGF treatment were measured using semiquantitative PCR. -Fold changes of mRNA were calculated as the ratio of *Klf* levels in NGF-treated 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. 1*A*). 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. 1*B* shows that *Klf11* mRNA level decreased, -3.50 ± 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. 1*C* 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. 1*D*). This effect was dose-dependent because values of gene expression changed from -1.78 ± 0.12-fold in cells treated with 10 ng/ml

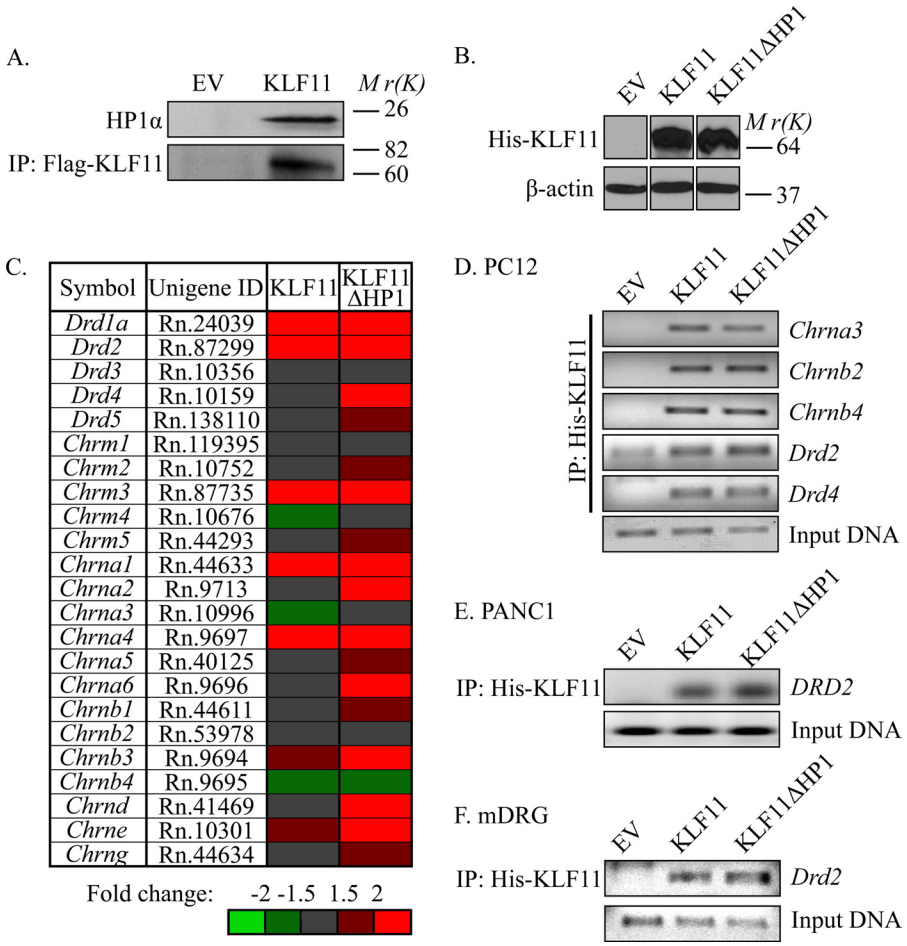

FIGURE 2. **Klf11 switches transcriptional patterns of neurotransmitter receptor expression.** *A*, KLF11 interacts with HP1α, allowing its coupling to the HP1-HMT repression system. Cells were transfected with EV control or FLAG-tagged KLF11. Upon immunoprecipitation (*IP*) with anti-FLAG affinity gel, Western blot was performed using an HP1α-specific antibody. *B*, PC12 cells virally transduced with EV control, KLF11, or KLF11ΔHP1 were treated with NGF (100 ng/ml) for 48 h. Levels of different neurotransmitter receptor mRNA were measured using a qPCR gene array. Western blot confirmed overexpression of His-tagged KLF11 and KLF11ΔHP1 by adenoviral transduction at the time of harvest. Loading control was β-actin. *C*, qPCR array data showing the mRNA level changes of dopaminergic and cholinergic receptors in KLF11- or KLF11ΔHP1-overexpressing PC12 cells. The -fold changes in mRNA levels are *color-coded* according to the *scale* presented. *D*, ChIP assay on PC12 cells transduced with EV, KLF11, or KLF11ΔHP1 showing KLF11 occupancy on multiple neurotransmitter receptor promoter regions, identifying *Chrna3*, *Chrnb2*, *Chrnb4*, *Drd2*, and *Drd4* as *bona fide* targets of KLF11. KLF11ΔHP1 binding to these same promoters indicates that the deletion mutation did not alter DNA recognition and binding ability. *E*, ChIP results from pancreatic epithelial cells confirm that both KLF11 and KLF11ΔHP1 occupy the human *DRD2* promoter. *F*, similar to PC12 and pancreatic epithelial cells, ChIP results from mouse DRG neurons show that both wild type KLF11 and KLF11ΔHP1 occupy the mouse *Drd2* promoter.

NGF to −2.58 ± 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 Switches Transcriptional Patterns of Neurotransmitter Receptor Expression during Cell Differentiation—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 P*XVX*L domain (amino acids 487–491), which couples this transcription factor to the HP1-HMT system (Fig. 2*A*), 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

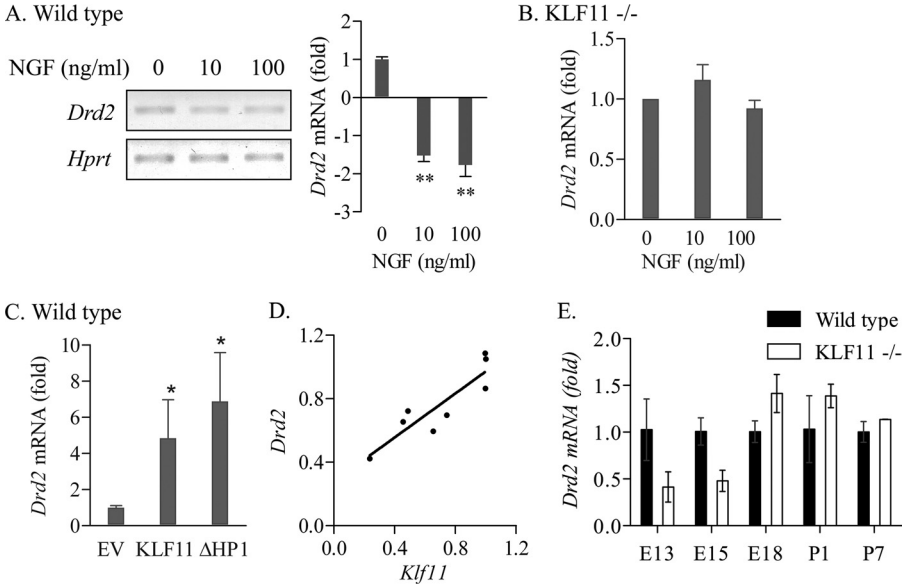

FIGURE 3. **In primary cultures of DRG neurons, NGF treatment induces down-regulation of *Drd2* mRNA.** *A*, semiquantitative RT-PCR was performed to measure mRNA level of *Drd2* in NGF-treated (48 h; 0, 10, or 100 ng/ml) DRG neurons from wild type mice. *Left*, representative image of the DNA gel showing *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 transduction was confirmed with Western blotting (Fig. 2*B*). A qPCR gene array was used to compare neurotransmitter receptor transcript levels across the treatment conditions (selected results shown in Fig. 2*C*). These experiments show that KLF11 overexpression increased transcript levels of *Drd1a* (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. 2*C*). 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. 2*D* 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. 1*D*; -1.8 ± 0.12-fold for 10 ng/ml NGF and -2.6 ± 0.25-fold for 100 ng/ml NGF) and *Drd2* (Fig. 3*A*; -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. 3*B*). Congruently, when KLF11 was overexpressed in DRG neurons of wild type mice, the mRNA level of *Drd2* was significantly increased (Fig. 3*C*). Congruently, overexpression of the KLF11ΔHP1 mutant induced even further increase in *Drd2* mRNA (Fig. 3*C*). The semiquantitative RT-PCR experiments shown in Figs. 1*D* and 3*A* demonstrate a strong positive corre-

lation between the mRNA levels of *Drd2* and *Klf11* (as percentages of change compared with no-NGF controls; $r = 0.88$; Fig. 3*D*). To gain insight at the whole organism level, we investigated whether selective ablation of the *Klf11* gene in mice affects the developmental pattern of *Drd2* expression using whole brain of developing *Klf11*$^{-/-}$ and wild type mice by qPCR (Fig. 3*E*). The mRNA levels of *Drd2* were lower in *Klf11* knock-out mice compared with the wild type at E13 and E15. Interestingly, this decrease in the levels of *Drd2* mRNA in *Klf11*$^{-/-}$ mice was compensated by E18. This result suggests that there may be other transcription factors, possibly other KLFs, that regulate transcription of *Drd2* and compensate for the loss of *Klf11* during later stages of embryonic development. Future studies using animals genetically engineered to have defects in these candidate compensatory KLF pathways might further extend these important observations. Collectively, these results demonstrate that KLF11 directly binds to and functions as a new transcriptional activator of the *Drd2* promoter.

KLF11 Recognizes and Binds to Specific Sequence on Drd2 Promoter to Regulate Transcription—Knowing that KLF11 functions in differentiating neuronal cells, to regulate expression of neurotransmitter receptors, we attempted to define this phenomenon mechanistically at a higher molecular resolution. We first located the precise KLF11 binding sites within the *Drd2* promoter through an extensive battery of transcriptional and chromatin-based assays. Previous studies have identified a critical 217-base pair promoter region upstream of the *Drd2* transcription start site to be important in transcriptional regulation (32). Based on the consensus sequence (CACCC, GGGCG) of KLF protein binding sites identified previously in our laboratory using both EMSA-based selection and SELEX assays (6, 33), we recognized five potential KLF11 *cis*-regulatory sites within the promoter region (−298 to −1). Fig. 4*A* depicts the location of these sites; nucleotides −122 to −114 (site 5), −98 to −94 (site 4), −85 to −81 (site 3), −69 to −65 (site 2), and −60 to −56 (site 1). To determine with which of these potential sites KLF11 actually interacts, we generated various deletion constructs of the *Drd2* promoter and tested their activity in a luciferase-based reporter assay. As shown in Fig. 4*B*, wild type promoter activity readily increased with KLF11 overexpression (7.77 ± 0.44-fold compared with EV control). This increased activation was conserved in the absence of site 5 (Δsite5, 7.0 ± 0.43-fold EV control). However, when sites 3, 4, and 5 were deleted, this effect was largely abolished (Δsite3–5, 1.69 ± 0.12-fold EV control). In fact, this result resembles that observed with the *Drd2* promoter lacking all possible KLF binding sites (Δsite1–5, 2.08 ± 0.46-fold EV control). Thus, these experiments demonstrate that site 3 and/or 4 are the key *cis*-regulatory sequences recognized by KLF11. This functional mapping was confirmed by EMSA studies. For this purpose, we utilized 39-bp-long probes (nucleotides −109 through −71) spanning both site 3 and site 4 which, in our hands, better reflect promoter context than the most commonly used 12-bp double-stranded oligonucleotides for Sp1-like proteins. KLF11 binding was observed with the wild type probe (Fig. 4*C*, *lane 3*) but was lost with mutation on site 4 ($^{-99}$GGGCGGG^{-93} to GGTTTGG; Fig. 4*C*, *lane 6*). Mutation on site 3 did not interfere with KLF11 binding to the probe ($^{-89}$CCCGGGCG^{-82} to CCTTTTTG; Fig. 4*C*, *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. 4*C*, *lane 12*). Specificity of KLF11 binding to the wild type probe was further confirmed with supershift experiments using control or KLF11 antibodies (Fig. 4*D*, *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. 4*D*, *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. 4*D*, *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. 4*B*, 1.70 ± 0.47-fold EV control). The data from both reporter assays and EMSAs 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. 2*B*). 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-PCR (Fig. 5*A*) and luciferase-based reporter assays (Fig. 5*B*). 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. 5*B*). 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. 5*C*). 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. 5*D*), 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-

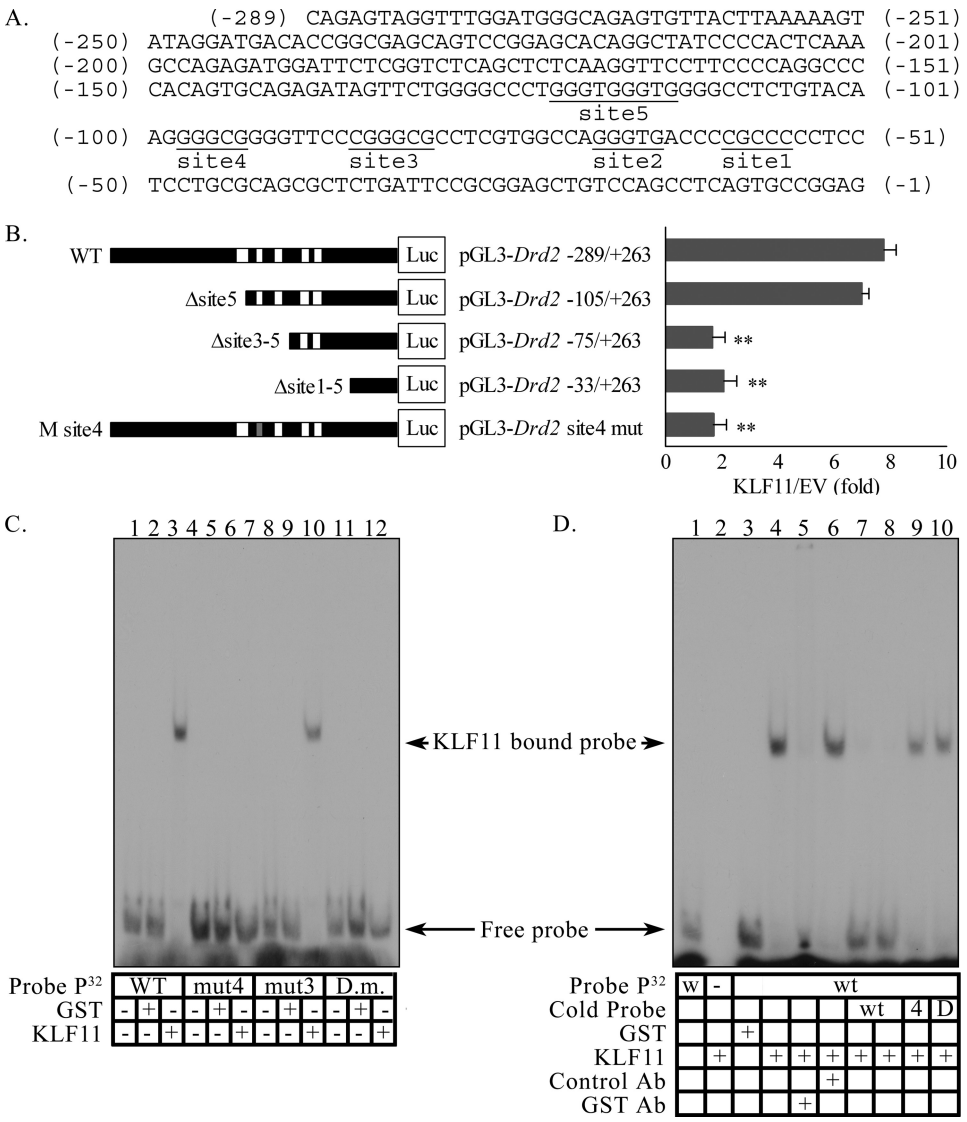

FIGURE 4. **KLF11 recognizes and binds to a specific sequence on the *Drd2* promoter to regulate transcription.** *A*, the sequence represents rat *Drd2* proximal promoter (−1 to −289 relative to TSS). Five possible KLF11 binding sites were identified based on the sequence (*underlined*, sites 1–5 relative 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 ($^{-99}$GGGCGGG^{-93} to GGTTTGG); *mut3*, mutation in site 3 ($^{-89}$CCCGGGCG^{-82} to CCTTTTTG); *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 3*) or recombinant KLF11 (*lanes 2* and *4–10*) 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. **, $p < 0.01$.

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 KLF11ΔHP1 (Fig. 5*D*). In complementary luciferase-based reporter assays, we expressed a dominant negative form of p300 (p300DN), lacking enzymatic activity, along with KLF11 or KLF11ΔHP1 (Fig. 5*E*). Expression of p300DN antagonized the increase observed with KLF11 (9.9 ± 0.17- to 6.1 ± 0.07-fold) and KLF11ΔHP1 (47 ± 0.02- to 2.1 ± 0.08-fold), without disrupting the binding of KLF11 to the *Drd2* promoter (Fig. 5*F*). 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.

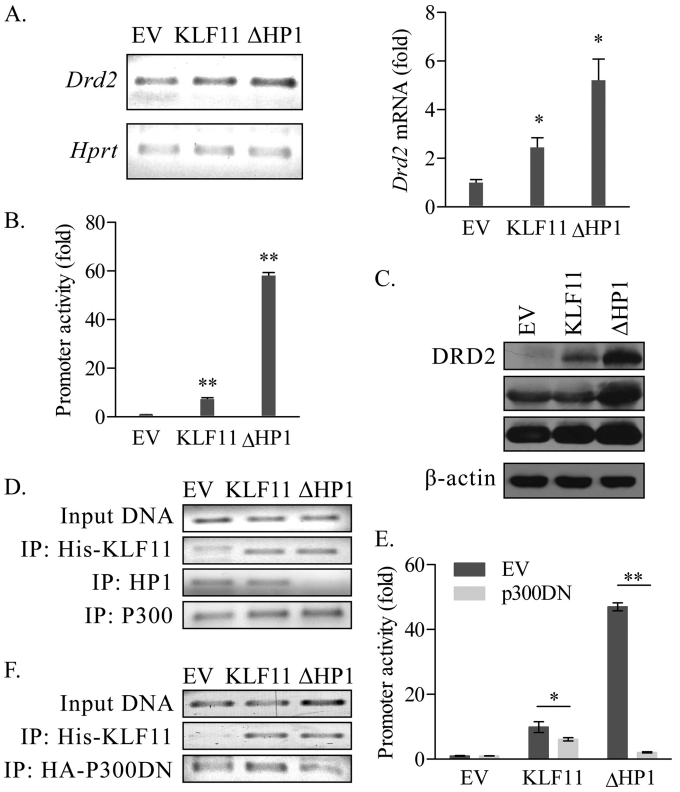

FIGURE 5. **KLF11 differentially couples to distinct chromatin pathways to regulate *Drd2*.** *A*, semiquantitative RT-PCR was performed to validate the qPCR gene array data showing an increase in *Drd2* transcription with KLF11 or KLF11ΔHP1 overexpression in PC12 cells. *Left*, representative image of the DNA gel; *right*, averaged quantification of the DNA gels ($n = 3$). *B*, luciferase assay showed changes in *Drd2* promoter activity with KLF11 or KLF11ΔHP1 overexpression in PC12 cells. *C*, Western blot showed increase in DRD2 protein levels in KLF11- and KLF11ΔHP1-overexpressing PC12 cells, consistent with the increase observed in transcript levels. Loading control is β-actin. *D*, ChIP assay was performed to show occupancy of His-KLF11, HP1, and p300 in the *Drd2* promoter region. When wild type KLF11 was present, both p300 and HP1 were also found on the promoter, whereas when KLF11ΔHP1 was present, only p300 was found on the promoter. *E*, luciferase assays were performed with a dominant negative p300 (p300DN) to confirm the involvement of this HAT in the regulation of *Drd2* promoter activity. PC12 cells were transduced with EV or p300DN, along with EV, KLF11, or KLF11ΔHP1 (Δ*HP*1). *Error bars*, S.E. *F*, ChIP assay showed that overexpression of p300DN did not affect the binding of KLF11 to *Drd2* promoter. *, $p < 0.05$; **, $p < 0.01$. *IP*, immunoprecipitation.

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. 1*B*). 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. 6*C*). 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 of 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. 6*D*). This result in primary neuronal cultures (DRG) was confirmed in experiments that compared neurite lengths between control and KLF11-transduced PC12 cells (Fig. 7*C*). 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. 2*E*), 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.

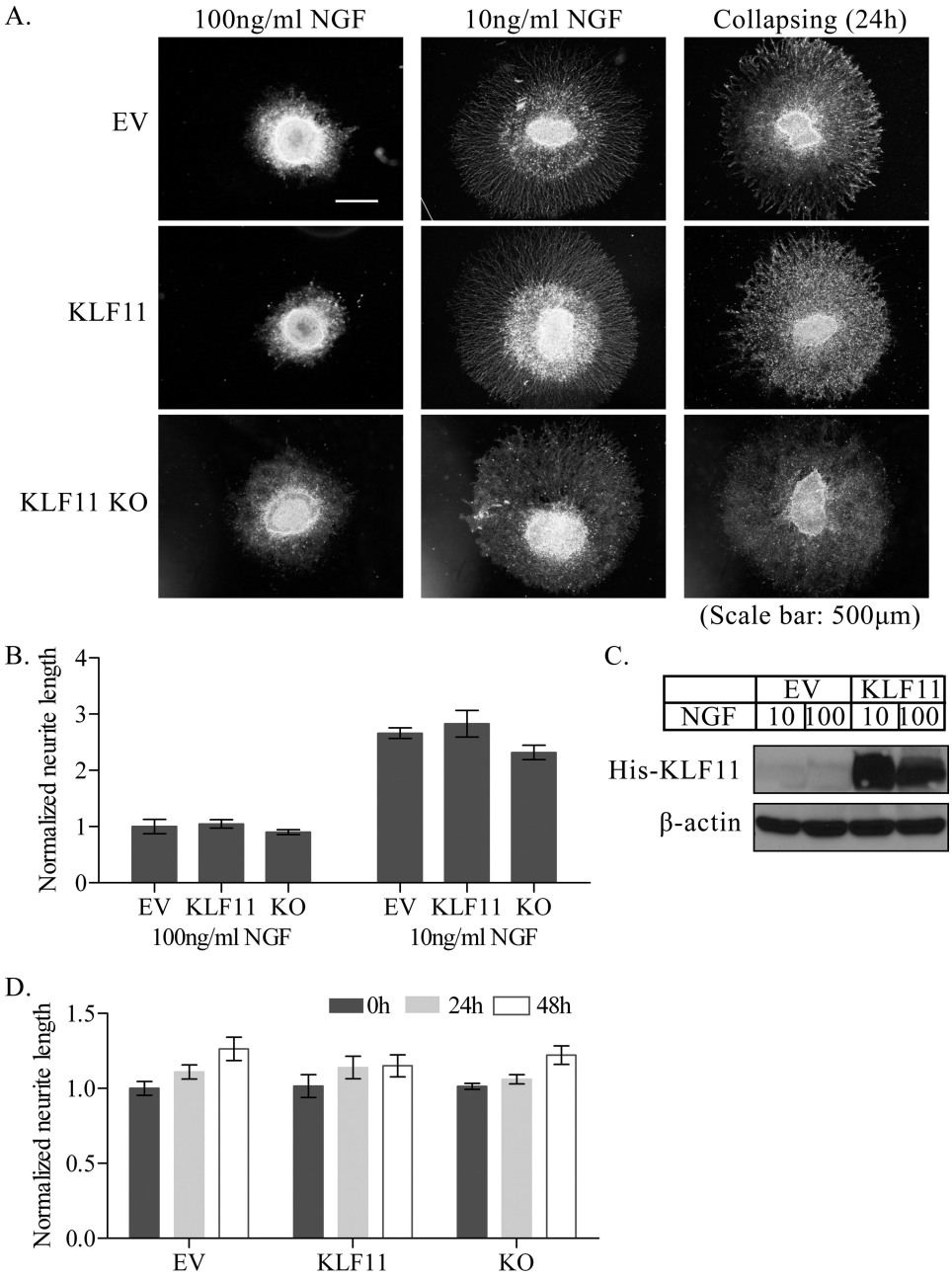

FIGURE 6. **KLF11 regulates neurotransmitter receptors in DRG neurons independently of neurite formation.** *A*, representative images of control, KLF11-overexpressing DRG from wild type or KLF11 knock-out animals. DRG were treated with various concentrations of NGF for 48 h. *Collapsing images* show 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.

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

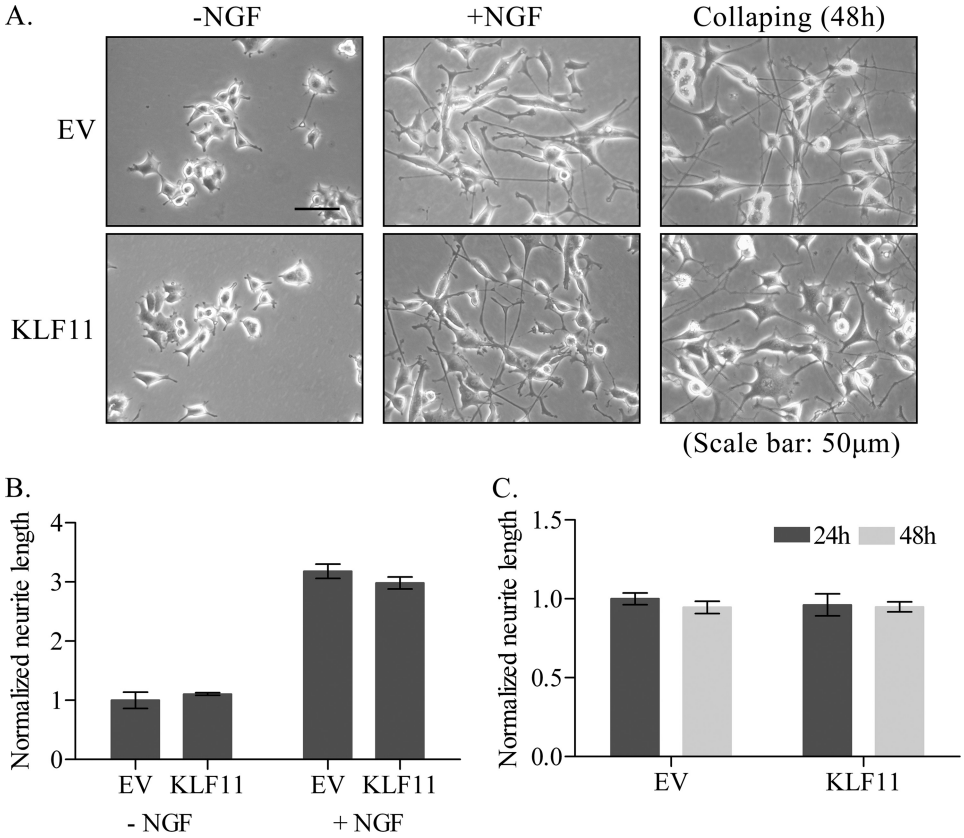

FIGURE 7. **KLF11 regulates neurotransmitter receptors in PC12 cells independently of neurite formation.** *A*, representative images of control or KLF11-overexpressing PC12 cells. *+NGF*, 100 ng/ml NGF treatment for 48 h. *Collapsing images* were taken 48 h after adenovirus infection and NGF retraction. *B*, quantification of neurite outgrowth in PC12 cells with or without NGF treatment, using stereology. All measurements were normalized to NGF, EV conditions. *C*, quantification of neurite lengths in a collapsing experiment. Neurite lengths were measured 24 and 48 h after transduction. All measurements were normalized to 24 h of 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-

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 Caiazzo *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 involved 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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