Role of Deltex-1 as a Transcriptional Regulator Downstream of the Notch Receptor*

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Intercellular signaling through the cell-surface receptor Notch plays important roles in a variety of developmental processes as well as inpathogenesis of some human cancers and genetic disorders. However, the mechanisms by which Notch signals are transduced into cells still remain elusive. Here we investigated the signaling mechanisms for Notch in the cell fate control of neural progenitor cells. We show that Deltex-1 (DTX1), a mammalian homolog of Drosophila Deltex, mediates a Notch signal to block differentiation of neural progenitor cells. We found that a significant fraction of DTX1 proteins were localized in the nucleus and physically interacted with the transcriptional coactivator p300. Through its binding to p300, DTX1 inhibited transcriptional activation by the neural-specific helix-loop-helix-type transcription factor MASH1, and this mechanism is likely responsible for the differentiation inhibition of neural progenitor cells. Our results further suggest that DTX1 regulates transcription independently of the previously characterized Notch signaling pathway involving RBP-J and HES1/HES5. Thus, DTX1 serves as an important signaling component downstream of Notch that regulates transcription in the nucleus.

Notch is the evolutionarily conserved transmembrane receptor family in both vertebrates and invertebrates (1, 2). The Notch receptors receive signals from their ligands expressed on the surface of neighboring cells, thereby mediating signals elicited by cell-cell interactions (3). Accumulating evidence has established that the Notch signaling system plays vital and universal roles in development of a variety of tissues and organs (1, 2). Recent advances have further revealed that either loss-of-function or gain-of-function mutations of the genes encoding Notch receptors and their ligands are involved in pathogenesis of human diseases (1).

Based on the above findings, recent intensive efforts have been begun in studies on the intracellular signaling mechanisms downstream of Notch. Much of our knowledge about the molecules involved in Notch signaling has come from studies in fly and nematode. In Drosophila, Suppressor-of-Hairless (Su(H)) is an important signaling component (1, 2). Genes encoding proteins homologous to Su(H) have also been identified in both Caenorhabditis elegans and vertebrates and termed lag-1 and RBP-J (also termed CBF-1 and KFB2), respectively (4). All of the proteins belonging to the Su(H)/RBP-J/LAG-1 family physically associate with the intracellular domain of Notch receptors (5–12). Furthermore, Drosophila Su(H) acts in the nucleus as a transcriptional regulator of the Enhancer-of-Split gene complex (E(spl)-C), one of the major targets for Notch signals (13, 14). An analogous mechanism has been implicated in vertebrates, in which RBP-J activates the E(spl)-C homologs Hes1 and Hes5 (6, 7, 9, 10). Vital roles for RBP-J, HES1, and HES5 in normal development of vertebrates have been demonstrated in gene-targeting studies in mice (15–17).

Nevertheless, more recent studies on Drosophila have demonstrated the presence of Notch functions independent of Su(H) and E(spl)-C (13, 18–20). Signaling mechanisms independent of RBP-J and HES1/HES5 have also been implicated in mammalian cells (3, 17, 21–23). Supporting this idea, a novel signaling pathway involving deltex has recently been characterized in Drosophila (24, 25). Three closely related members of the Deltex family (DTX1, DTX2, and DTX3) have also been identified in mammals (26–28). All of these fly and mammalian Deltex homologs share many common features in structure, suggesting their important roles in Notch signaling. However, details of their biological functions remain largely unknown.

Neurons and glia in the vertebrate nervous system are generated from multipotential neural progenitor cells. During development, neural progenitor cells continue proliferation without overt differentiation, whereas at later stages, they stop dividing and differentiate at particular times and positions. Many lines of previous studies have demonstrated that Notch is involved in this cell fate control event (29). Recent studies have further demonstrated the important roles of the conventional Notch signaling components such as RBP-J, HES1, and HES5 in vertebrate neurogenesis. However, the signaling
mechanisms by which the Notch receptors control the differentiation of neural progenitor cells still remain poorly understood.

To better understand the functions of Notch signaling in neural development, we examined the intracellular signaling mechanisms in neural progenitor cells in vitro. We took advantage of the immortalized cell line MNS-70, which exhibits many of the properties of multipotential neural progenitor cells (30–32). Here we show that constitutive expression of a dominant-active form of Notch1 blocked differentiation of MNS-70 cells, and overexpression of the human Deltex homolog DTX1 mimicked this inhibition. Our results suggest that these inhibitory actions of Notch1 and DTX1 are attributable to their ability to block transcriptional activation by the neural-specific basic helix-loop-helix (bHLH) transcription factor MASH1. We found that significant fractions of endogenous and exogenous DTX1 proteins were localized in the nucleus and physically associated with the transcriptional coactivator p300. Several lines of evidence suggest that this action of DTX1 is independent of the signaling pathway involving RBP-J and HES1/HES5. Together, our results support the idea that DTX1 regulates nuclear transcription through targeting p300, thereby serving as an important signaling component downstream of Notch.

EXPERIMENTAL PROCEDURES

Plasmids—Mouse Notch1 cDNA fragments were engineered to encode the following amino acids (GenBank\textsuperscript{TM}/EBI Data Bank accession number Z18860): Notch1-A, 1704–2531; Notch1-B, 1704–2097; Notch1-C, 1704–1976; Notch1-D, 1848–2372; Notch1-E, 1848–2075; Notch1-F, 1704–1847; and Notch1-G, 1744–2372. The FLAG tag epitope sequence was attached in frame to the N-terminal ends of these constructs. The Myc tag was attached to the C-terminal end of human DTX1 to yield DTX1-Myc (25). Deletion mutants of human DTX1 (accession number AF053700) and p300 (accession number U01877) were obtained by polymerase chain reactions to encode the following amino acids (GenBank\textsuperscript{TM}/EBI Data Bank accession number D16464) was cloned into pGVB (TOYO-INKI) to yield pHes1-Luc. pE7a-Luc contains seven repeats of the E box motif (CAGGTG) upstream of the \( \beta \)-actin core promoter (33). The pHNF4-Luc and pGal4-UAS-Luc reporters were described previously (34). Luciferase reporter assays were performed using NIH3T3, HEK293, and MNS-70 cells. The cells were grown to a density of \( 8 \times 10^5 \) (NIH3T3 and HEK295) or \( 5 \times 10^5 \) (MNS-70) cells/well of six-well dishes. Transient transfection was performed by the standard calcium precipitation method or by the FuGene-6 lipofection method (Roche Molecular Biochemicals). Each well received an appropriate reporter plasmid (pHes1-Luc DNA, 0.5 \( \mu \)g; pE7a-Luc DNA, 2 \( \mu \)g; pGal4-UAS-Luc DNA, 0.5 \( \mu \)g; or pHNF4-Luc DNA, 1 \( \mu \)g) in combination with various amounts of expression plasmids. The \( \beta \)-galactosidase expression plasmid pEF-BOS-\( \beta \)-gal was included to normalize the results for transfection efficiency.

Western Blotting and Immunoprecipitation—Expression of MASH1 and nestin proteins in MNS-70 cells was examined as described previously (32). Physical interactions of various proteins were examined by co-immunoprecipitation assays. COS-7 cells transfected with various expression plasmids or rat embryos were lysed in buffer containing 50 mM Hepes-NaOH (pH 7.5), 50 mM KCl, 0.5\% (v/v) Nonidet P-40, 2.5 mM EGTA, 2.5 mM EDTA, 10\% (v/v) glycerol, 1 mM dithiothreitol, 2 \( \mu \)M phenylmethylsulfonyl fluoride, 2 \( \mu \)M leupeptin, and 2 \( \mu \)M aprotinin. Protein complex was recovered by immunoprecipitation and subjected to Western blot analysis with monoclonal anti-tag antibodies (anti-Flag M2 antibody (Sigma; anti-Myc 9E10 antibody; anti-HA 12CA5 antibodies, Roche Molecular Biochemicals) and goat anti-p300 antibody (Santa Cruz Biotechnology). Nuclear and cytoplasmic proteins were fractionated by the method described previously (35). The proteins fractionated from equal numbers of cells (3 \( \times 10^5 \) COS-7 cells and 1 \( \times 10^5 \) MNS-70 cells) or from 2\% of the total homogenate of an E14.5 rat embryo were subjected to Western blotting. The distribution of ERK (extracellular signal-regulated kinase) proteins was examined as a control. Immunoreactive bands were visualized and quantified with a Vistra ECF Western blotting kit (Amersham Pharmacia Biotech) by measuring fluorescence intensity using FluorImager SI.

Yeast Interaction Trap Assay—Plasmid constructs were designed to express various fragments of DTX1 and p300 as fusion proteins with Gal4-DDB and Gal4-TAD, respectively. The yeast strain FJ69-4A, in which expression of the HIS3 gene is under the control of the GAL1 promoter, was transformed with various combinations of the plasmids. The transcriptional activity of HIS3 was monitored to detect protein-protein interactions as described previously (36).

RESULTS

DTX1 Inhibits Differentiation of Neuroepithelial Progenitor Cells—To examine the functions of Notch1 and DTX1 in the neural progenitor cell line MNS-70, we established its sublines that constitutively express either a dominant-active (DA) mutant of Notch1 (herein termed Notch1-A) (Fig. 1A, B) or full-length human DTX1 (Fig. 1B). Previous studies have demonstrated that truncated mutant forms of Notch receptors in which the large extracellular domains are deleted acquire ligand-independent signaling activities (4). Thus, we expected to see that forced expression of Notch1-A would transduce constitutive Notch signals into cells. Likewise, in Drosophila,
overexpression of deltex recapitulates some of the phenotypes of DA alleles of Notch (25). As described previously (30–32), these sublines remained undifferentiated in monolayer culture, and the majority (>98%) of the cells expressed the intermediate filament protein nestin, a marker for undifferentiated neural progenitor cells (data not shown). Their differentiation could be conditionally induced through the formation of cell aggregates in floating culture and subsequent reseeding on poly-d-lysine-coated dishes (see “Experimental Procedures” for details). Upon differentiation induction, ~20% of the cells became microtubule-associated protein-2-positive neurons in cultures of both the parental MNS-70 cells and the H1-1 control subline cells (Table I). Another 60% of the cells expressed glial fibrillary acidic protein, a specific marker for astrocytes; and consequently, the percentage of nestin-positive cells was decreased to ~10%. The remaining cells probably corresponded to intermediate progenitor subtypes that transiently emerge during differentiation of MNS-70 cells (30, 32). In contrast, three independent sublines expressing Notch1-A generated much lower percentages of neurons and glia under the same conditions, and the majority of these cells expressed nestin (32). Forced expression of DTX1 also blocked generation of neurons and glia in three independent sublines (Table I).

We examined at which step(s) Notch1-A and DTX1 blocked differentiation of MNS-70 cells. We have previously shown that the neural-specific bHLH transcription factor MASH1 plays a critical role in an initial differentiation step of neural progenitor cells (32). Consistently, MASH1 was transiently induced during differentiation of MNS-70 cells (Fig. 1C). Similar transient expression of MASH1 was observed in the sublines NA-25 and DX-2, which constitutively express Notch1-A and DTX1, respectively. Thus, induction of MASH1 per se was not affected by overexpression of Notch1-A or DTX1. One of the functions of MASH1 in MNS-70 cells is the down-regulation of nestin (32); and coincident with the induction of MASH1, the level of nestin was markedly decreased in the parental cells (Fig. 1D). In contrast, the expression levels of nestin remained high in the Notch1-A- and DTX1-expressing sublines. The above characteristic phenotypes were observed in all of the sublines listed in Table I. These results suggest that Notch1-A and DTX1 inhibit the step at which expression of nestin is down-regulated and that the activity (but not the expression) of MASH1 is blocked by Notch1-A and DTX1 in MNS-70 cells.

**Physical Interactions between DTX1 and Notch1 in Mammalian Cells**—Next we examined how DTX1 is involved in Notch signaling. Although a physical interaction between Notch1 and DTX1 has been detected in yeast cells (27, 28), such an inter-

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**TABLE I**

*Generation of neurons and glia upon induction of differentiation of MNS 70 and its sublines*

A differentiation culture of each cell line was subjected to immunostaining with specific antibodies for microtubule-associated protein-2 (MAP2), glial fibrillary acidic protein (GFAP), and nestin. The percentages of cells positive for each marker among the total cells are shown.

| Cell line | MAP2 ≤ cells | GFAP ≤ cells | Nestin ≤ cells |
|-----------|--------------|--------------|----------------|
| Parent, MNS-70 | 18.9 ± 3.4 | 58.6 ± 5.4 | 8.9 ± 2.3 |
| Control, H1-1 | 21.6 ± 2.1 | 56.3 ± 4.7 | 9.7 ± 3.4 |
| Notch1-A | | | |
| NA-19 | 0.3 ± 0.2 | 0.6 ± 0.2 | 98.2 ± 1.2 |
| NA-25 | 0.5 ± 0.3 | 1.5 ± 0.8 | 95.7 ± 2.6 |
| NA-26 | 0.9 ± 0.5 | 3.5 ± 1.4 | 94.1 ± 4.9 |
| DTX1 | | | |
| DX-2 | 1.7 ± 0.8 | 4.2 ± 3.7 | 89.6 ± 4.9 |
| DX-12 | 3.1 ± 2.6 | 6.4 ± 3.5 | 86.2 ± 6.5 |
| DX-13 | 4.1 ± 2.5 | 5.9 ± 3.8 | 85.1 ± 5.7 |

* p < 0.05 compared with the control value.
action has not been directly demonstrated in mammalian cells. We first addressed this issue by immunoprecipitation assays in COS-7 cells. DTX1 is composed of three conserved domains termed domains I–III (Fig. 2A). Full-length DTX1 and its deletion mutants containing the N-terminal domain I, which is composed of two tandem repeats of ~70 amino acids; Domain II is rich in proline, serine, and threonine residues; and Domain III contains a ring-H2-type zinc finger motif. Interaction between Notch1-A and deletion mutants of DTX1. Flag-tagged Notch1-A and HA-tagged DTX1 mutants (HA-DTX1-A–F) were expressed in COS-7 cells, and their interactions were examined by immunoprecipitation assays. The combinations of transfected plasmids (7.5 μg each) and antibodies used for immunoprecipitation (IP) and immunoblotting (IB) are shown in each panel. The lower blot shows the expression of DTX1 mutants in WCLs (indicated by arrowheads). C, interaction of full-length DTX1 with various Notch1 intracellular fragments. Flag-tagged Notch1 mutant constructs (FLAG-Notch1-A–F) were coexpressed with DTX1-Myc in COS-7 cells. The lower two panels show the expression of tagged proteins in WCLs (indicated by arrowheads), whereas the upper panel shows Notch1-bound DTX1-Myc in immunoprecipitated samples (arrow). D, co-immunoprecipitation of Myc-tagged full-length DTX1 with HA-tagged mutants of DTX1. In each panel, – and + indicate transfection with the control and corresponding expression plasmids, respectively. The small brackets on the right sides of B–D indicate the positions of the heavy (IgH) and light (IgL) chains of immunoglobulin proteins used for immunoprecipitation.

## Physical interactions of DTX1 with Notch1 in mammalian cells

### A. Schematic representation of the deletion constructs of Notch1 and DTX1 used in this study. In the upper panel, the characterized structures of the intracellular domain of Notch1 are indicated (for details, see Ref. 2). TM, transmembrane domain; RAM, RAM domain; NLS, putative nuclear localization signal; ANK, six tandem ankyrin-like repeats; OPA, glutamine-rich domain; PEST, the domain rich in proline, glutamate, serine, and threonine residues. In the lower panel, the conserved domain structures of human Notch1 are shown (see Ref. 26). Domain I contains two tandem repeats of ~70 amino acids; Domain II is rich in proline, serine, and threonine residues; and Domain III contains a ring-H2-type zinc finger motif. B, interaction between Notch1-A and deletion mutants of DTX1. Flag-tagged Notch1-A and HA-tagged DTX1 mutants (HA-DTX1-A–F) were expressed in COS-7 cells, and their interactions were examined by immunoprecipitation assays. The combinations of transfected plasmids (7.5 μg each) and antibodies used for immunoprecipitation (IP) and immunoblotting (IB) are shown in each panel. The lower blot shows the expression of DTX1 mutants in WCLs (indicated by arrowheads). C, interaction of full-length DTX1 with various Notch1 intracellular fragments. Flag-tagged Notch1 mutant constructs (FLAG-Notch1-A–F) were coexpressed with DTX1-Myc in COS-7 cells. The lower two panels show the expression of tagged proteins in WCLs (indicated by arrowheads), whereas the upper panel shows Notch1-bound DTX1-Myc in immunoprecipitated samples (arrow). D, co-immunoprecipitation of Myc-tagged full-length DTX1 with HA-tagged mutants of DTX1. In each panel, – and + indicate transfection with the control and corresponding expression plasmids, respectively. The small brackets on the right sides of B–D indicate the positions of the heavy (IgH) and light (IgL) chains of immunoglobulin proteins used for immunoprecipitation.

### B. A, schematic representation of the deletion constructs of Notch1 and DTX1 used in this study. In the upper panel, the characterized structures of the intracellular domain of Notch1 are indicated (for details, see Ref. 2). TM, transmembrane domain; RAM, RAM domain; NLS, putative nuclear localization signal; ANK, six tandem ankyrin-like repeats; OPA, glutamine-rich domain; PEST, the domain rich in proline, glutamate, serine, and threonine residues. In the lower panel, the conserved domain structures of human Notch1 are shown (see Ref. 26). Domain I contains two tandem repeats of ~70 amino acids; Domain II is rich in proline, serine, and threonine residues; and Domain III contains a ring-H2-type zinc finger motif. B, interaction between Notch1-A and deletion mutants of DTX1. Flag-tagged Notch1-A and HA-tagged DTX1 mutants (HA-DTX1-A–F) were expressed in COS-7 cells, and their interactions were examined by immunoprecipitation assays. The combinations of transfected plasmids (7.5 μg each) and antibodies used for immunoprecipitation (IP) and immunoblotting (IB) are shown in each panel. The lower blot shows the expression of DTX1 mutants in WCLs (indicated by arrowheads). C, interaction of full-length DTX1 with various Notch1 intracellular fragments. Flag-tagged Notch1 mutant constructs (FLAG-Notch1-A–F) were coexpressed with DTX1-Myc in COS-7 cells. The lower two panels show the expression of tagged proteins in WCLs (indicated by arrowheads), whereas the upper panel shows Notch1-bound DTX1-Myc in immunoprecipitated samples (arrow). D, co-immunoprecipitation of Myc-tagged full-length DTX1 with HA-tagged mutants of DTX1. In each panel, – and + indicate transfection with the control and corresponding expression plasmids, respectively. The small brackets on the right sides of B–D indicate the positions of the heavy (IgH) and light (IgL) chains of immunoglobulin proteins used for immunoprecipitation.

### Notch1 and DTX1 Inhibit Transcriptional Activation by MASH1—The above results indicate that overexpression of DTX1 can mimic the action of DA-Notch1, and its likely target is MASH1. Thus, we next examined the effect of DTX1 on MASH1-dependent transcription. MASH1 activated the reporter construct pE7-Luc containing the MASH1-binding E box motif (33). Notch1-A inhibited this activation in NIH3T3 cells. Importantly, DTX1 could mimic this inhibitory action of Notch1-A. DTX2, which is closely related to DTX1 in structure (28), also possessed the ability to inhibit MASH1 (data not shown). None of the deletion mutants of DTX1 (Fig. 2A) could inhibit the activity of MASH1 (data not shown). Instead, one truncated mutant (DTX1-B) that lacked domain III containing coprecipitation of RBP-J with Notch1-A (data not shown). Further studies will be needed, however, to define the relationships between the DTX1- and RBP-J-binding site(s) within the intracellular domain of Notch1.
a ring-H\(_2\)-type zinc finger motif acted as a dominant-negative form. DTX1-B could release MASH1 from inhibition not only by wild-type DTX1, but also by Notch1-A (Fig. 3B). This can be explained by a mechanism in which Notch-dependent inhibition is mediated by endogenous DTX1 and/or other members of the DTX family. Indeed, we detected the endogenous expression of DTX1 and DTX2 in NIH3T3 and MNS-70 cells (data not shown). Since DTX1-B can bind to wild-type DTX1 (Fig. 2D), the formation of hetero-oligomers with endogenous DTX1 is likely to be the basis of its blocking mechanism (see below). These results suggest that DTX1 plays an essential role in the inhibition of MASH1 by Notch signaling.

Downstream of Notch receptors, RBP-J induces the expression of HES1 and HES5, which can bind to and inhibit the activity of MASH1 (37). We next examined the relationship between DTX1 and this RBP-J/HES-dependent pathway. Ordentlich et al. (27) reported that DA-Notch1 (but not DTX1) activates an RBP-J-binding reporter construct. Here we tested whether DTX1 can modulate transcription of the Hes1 promoter, the well known natural target of Notch signaling. Hes1-Luc contains the genomic fragment of the mouse Hes1 gene upstream of the luciferase reporter gene. As described previously (6–10), Notch1-A strongly activated pHes1-Luc in NIH3T3 cells (Fig. 3C). A dominant-negative mutant of RBP-J, which carries a mutation abolishing effective high affinity binding to DNA (9), blocked the Notch1-A-dependent transactivation. Thus, the observed transactivation indeed recapitulates the activity of the RBP-J-dependent pathway. Under these conditions, DTX1 did not cause any transactivation at all (Fig. 3C). We confirmed that RBP-J (but not DTX1) activated the transcription of pHes1-Luc in other cell types, including MNS-70, HEK293, and COS-7 cells. Consistent with this, we did not detect a significant increase in the level of endogenous Hes1 or Hes5 mRNA in DTX1-expressing sublines of MNS-70 cells (data not shown).

In the above reporter assays, we observed distinct actions of DTX1 and RBP-J. Overexpression of RBP-J did not inhibit the transactivation of the E box reporter by MASH1, and dominant-negative RBP-J did not block the DTX1-dependent inhibition (Fig. 3A). Thus, DTX1 appears to inhibit MASH1 independently of the endogenous activity of RBP-J. Rather, overexpression of RBP-J abrogated the inhibition of MASH1 by Notch1-A. Conversely, overexpression of DTX1 inhibited the transactivation of the Hes1 promoter by Notch1-A, but not by RBP-J (Fig. 3C). These results could be explained by the idea that excess amounts of DTX1 and RBP-J prevent each other from binding to Notch1 and that they thereby act in a mutually antagonistic manner when overexpressed in cultured cells (38, 39).

We further examined how DTX1 regulates the MASH1-dependent transcription. MASH1 and other cell type-specific bHLH factors require heterodimerization with ubiquitous bHLH factors such as E47 (37). DTX1 inhibits the transcriptional activation by E47 (27, 28). In addition, RBP-J-induced HES1 and HES5 can inhibit MASH1 through heterodimerization with MASH1 or E47. Thus, it is possible that the inhibitory effect of DTX1 on MASH1 is not direct, but is instead mediated by the formation of hetero-oligomers with endogenous DTX1 and/or other members of the DTX family.

2 N. Yamamoto and M. Nakafuku, unpublished data.
indirectly through E47 and/or HES1/HES5. To distinguish between these possibilities, we constructed an expression plasmid coding for a fusion protein between the DBD of Gal4 and the TAD of MASH1 termed Gal4-MASH1-TAD. This fusion construct strongly activated the Gal4-binding reporter pGal4-UAS-Luc (Fig. 3D), which carries three tandem repeats of the Gal4-binding UAS. Both DA-Notch1 and DTX1 inhibited the activity of Gal4-MASH1-TAD, and the dominant-negative DTX1-B mutant canceled these inhibitory actions (Fig. 3D). The TAD of MASH1 used here lacked the bHLH domain responsible for heterodimerization with other bHLH factors, indicating that DTX1 has the ability to inhibit MASH1 independently of other bHLH factors. Altogether, the above results suggest that DTX1 can act in parallel to the RBP-J/HES pathway to inhibit the activity of MASH1 and that the C-terminal transactivation domain of MASH1 is a target for this inhibitory signal.

Modulation of p300-dependent Transcription by DTX1—The coactivator p300 plays an essential role in transcriptional regulation by bHLH factors (40–45). We next investigated whether p300 is involved in the DTX1-dependent signaling pathway. In HEK293 cells, the coactivator function of endogenous p300 is attenuated by the adenoviral oncoprotein E1A; and hence, p300-dependent transcription is sensitive to exogenous p300 (46). Exogenous expression of p300 indeed augmented the transactivation of the E box reporter by MASH1 (Fig. 4A). When expressed in combination with DTX1, p300 repressed the DTX1-dependent inhibition of MASH1. p300 also blocked the Notch1-A-dependent inhibition of MASH1. Conversely, overexpression of Notch1-A and DTX1 significantly attenuated the p300-dependent activation. Thus, p300 and DTX1 act in a mutually antagonistic manner in regulating the activity of MASH1.

The above results raised the possibility that p300 is a direct target for DTX1. To test this idea, we constructed a fusion protein between Gal4-DBD and an N-terminal fragment of p300 (p300-A) termed Gal4-p300-A (Fig. 4B). As described previously (40), Gal4-p300-A strongly activated the Gal4-UAS-Luc reporter in HEK293 cells (Fig. 4C). Coexpression of DA-Notch1 and DTX1 could inhibit this transactivation. These results support the idea that DTX1 regulates transcription by modulating the activity of p300. To further extend this notion, we examined the effects of DTX1 on another p300-dependent transcriptional regulatory system. HNF4 belongs to the nuclear hormone receptor superfamily. Cotransfection of an expression plasmid for HNF4 transactivated the HNF4-binding reporter pHNF4-Luc (34), and exogenous p300 further augmented this transactivation in HEK293 cells (Fig. 4D). DTX1 and p300 again exhibited a mutual antagonism in regulating the transcription by HNF4. Thus, DTX1 modulates the coactivator function of p300 under various contexts.

Nuclear Localization of DTX1 and Its Physical Interactions with p300—p300 is localized in the nucleus and physically associates with many classes of transcriptional regulators (46). However, a previous immunostaining study detected DTX1 proteins in the cytoplasm when exogenously expressed in cultured cells (27). Considering its regulatory role for transcription, we reexamined the subcellular distribution of DTX1 by an alternative method. When nuclear and cytoplasmic/membrane proteins were fractionated, FLAG-tagged MASH1 was almost exclusively (>97%) found in the nuclear fraction of COS-7 cells (Fig. 5A). In contrast, ERM proteins, which were used as a control here, were predominantly (85–90%) recovered in the cytoplasmic/membrane fraction (47). Under these conditions, >70% of the DTX1-Myc proteins were detected in the nuclear...
fraction. The distribution of DTX1 did not significantly change irrespective of the presence or absence of the nuclear form of Notch1 (Notch1-G) (11).

We also examined the localization of endogenous DTX1 and DTX2 proteins. Both anti-DTX1 and anti-DTX2 antibodies detected bands of 70–72-kDa proteins and some other bands with smaller sizes in the fractions of rat embryos at E14.5 (Fig. 5B, arrowheads and asterisks). These bands matched the full-length and degradation products of DTX1 and DTX2 expressed in COS-7 cells (data not shown). Approximately 20% of the endogenous DTX1 and DTX2 proteins were detected in the nuclear fraction, although they were much more abundant in the cytoplasm/membrane. DTX2 proteins were abundantly expressed in MNS-70 cells, and the majority were also present in the nucleus (Fig. 5C). Stronger immunoreactive bands were detected by transfection of a DTX2 expression plasmid, again being recovered in the nuclear fraction.

We further examined the distribution of endogenous DTX1 proteins in vivo. The expression of Notch1 was detected in actively proliferating neural progenitor cells (Fig. 5D, panel a) (38, 39). We also detected specific staining with anti-DTX1 antibody in these cells (Fig. 5D, panel b). Consistent with the above in vitro results, strong staining signals of DTX1 were found in cell nuclei (arrowheads), although DTX1 was also detected in the cytoplasm in some cells (arrows). This was in clear contrast with the distribution of immunoreactivity of the intracellular domain of Notch1 in the cytoplasm/membrane (Fig. 5D, panel a, arrowheads). Although nuclear Notch1 was barely detectable at this stage, signaling can still occur in these cells (11). In contrast, both Notch1 and DTX1 were localized in the nucleus in mature neurons (Fig. 5D, panels c and d, arrowheads) (38, 39).

The above results raised the possibility that DTX1 physically interacts with p300 in the nucleus. DTX1-Myc proteins were indeed recovered in immunoprecipitates with full-length p300 when expressed in COS-7 cells (Fig. 6A). DTX1 also interacted with its truncated fragments p300-A and p300-D (Fig. 6B). The physical interaction of DTX1 with p300 was further examined in yeast interaction trap assays. In yeast cells, significant interactions of DTX1 could be detected not only with p300-A and p300-D, but also with p300-B and p300-C (Fig. 6C). Thus, both the N- and C-terminal portions of p300-D can interact with DTX1, although both regions are probably required for an interaction that is detectable in immunoprecipitation assays. Among the three domains of DTX1, domain I specifically interacted with p300-A, whereas domain III interacted with p300-D.

Various bHLH factors interact with domain D of p300, which includes the third cysteine/histidine-rich region conserved among the p300 family of proteins (40–45). MASH1 was specifically co-immunoprecipitated with FLAG-p300-D (Fig. 6D). DTX1 also interacted with p300-D, raising the possibility that DTX1 inhibits the binding between MASH1 and p300. When increasing amounts of DTX1-Myc were coexpressed with MASH1, the levels of MASH1 coprecipitated with p300-D were significantly decreased; and conversely, the amounts of p300-D-associated DTX1 increased in a dose-dependent manner. Thus, DTX1 can competitively inhibit the interaction between MASH1 and p300.

**DISCUSSION**

Recent studies have implied the presence of Notch signaling pathways independent of RBP-J and HES1/HES5 (13, 17–21). Deltex is a strong candidate involved in such alternative signaling mechanisms in *Drosophila* (19, 24, 25). Here we have presented evidence supporting the idea that mammalian DTX1 is also involved in these pathways. DTX1 physically interacted with the intracellular domain of Notch1 and mimicked the action of DA-Notch1 to inhibit the transcriptional activity of the bHLH factor MASH1. Overexpression of a truncated form of DTX1 that is thought to act as a dominant-negative form blocked Notch-dependent inhibition of MASH1. Thus, DTX1 plays an important role in this aspect of Notch signaling. Inhi-
bition of neurogenic bHLH factors is one of the critical functions of Notch signaling during neural development (37). Consistent with this, like DA-Notch1, overexpression of DTX1 blocked differentiation of the neural progenitor cell line MNS-70. We detected a significant fraction of DTX1 proteins in the nucleus both in vitro and in vivo. Consistently, there are several clusters of basic residues in the predicted amino acid sequence of human DTX1 (e.g., RTQRRRRRLRRR, residues 161–172 in domain I; and RKTKKKHLKKSK, residues 379–390 in domain II) that potentially serve as nuclear localization signals (25). The related factor DTX2 also contains such putative nuclear localization signals (28). However, a previous study detected exogenous DTX1 proteins in the cytoplasm (27). This apparent discrepancy may be due to different methods used to detect protein distributions or different cell types used to express exogenous DTX1. Alternatively, the conclusions obtained by both studies may not be mutually exclusive. In fact, endogenous DTX1 and DTX2 proteins were abundant in the cytoplasmic/membrane fraction in developing embryos. Thus, such cytoplasmic/membrane DTX1 proteins may be retained by Notch receptors on the cell surface. Such a trapping mechanism has also been proposed for Drosophila Su(H) (5). However, a low expression level of the full-length Notch1 proteins in cultured cells currently precludes direct assessment of this prediction. It remains to be determined whether the subcellular localization of DTX proteins is regulated by Notch signaling and/or other mechanisms.

In line with this idea, recent studies have demonstrated that cleavage and nuclear translocation of the intracellular domain of the Notch receptor are responsible for transcriptional regulation (12, 48, 49). We can hypothesize that the cleaved Notch fragment binds to and activates DTX1 upon entering the nucleus. This scenario is analogous to a proposed mechanism for the action of RBP-J/Su(H) (6–11, 50). Alternatively, the intact Notch receptor may sequester DTX1 in the cytoplasm and translocate bound DTX1 into the nucleus upon activation by ligands. In fact, endogenous DTX1 and DTX2 proteins were abundant in the cytoplasmic/membrane fraction in developing embryos. Thus, such cytoplasmic/membrane DTX1 proteins may be retained by Notch receptors on the cell surface. Such a trapping mechanism has also been proposed for Drosophila Su(H) (5). However, a low expression level of the full-length Notch1 proteins in cultured cells currently precludes direct assessment of this prediction. It remains to be determined whether the subcellular localization of DTX proteins is regulated by Notch signaling and/or other mechanisms.

p300 directly binds to many classes of transcription factors, thereby activating transcription in the nucleus. Although the involvement of p300 in Notch signaling was not detected in a previous study (27), our results strongly suggest that DTX1 regulates transcription through the modulation of the coactivator function of p300. DTX1 counteracted with p300 to attenuate the MASH1- and HNF4-dependent transcription. Moreover, DTX1 and p300 could form a complex in both mammalian and yeast cells. Thus, the inhibition of the transcriptional activity of MASH1 and HNF4 likely involves the direct physical interaction between DTX1 and p300. Consistent with this idea,
DTX1 competitively inhibited the formation of the complex between MASH1 and the C-terminal domain D of p300. Conversely, DTX1-B, which lacks the C-terminal domain III and hence does not interact with p300-D, lost the inhibitory activity and instead acted as a dominant-negative form. Thus, the ability of DTX1 to interact with p300 is critical for its inhibitory action on MASH1. The N-terminal domain of p300 (p300-A) directly binds to the TATA-binding protein-containing basal transcription complex (40, 46), thereby acting as a TAD when tethered to the Gal4 DBD. DTX1 could also bind to this p300-A domain through its N-terminal domain I and inhibited the transactivation activity of the Gal4-p300-A fusion construct.

Thus, DTX1 presumably interacts with multiple domains of p300, thereby modulating its coactivator function at multiple steps.

In support of our findings, recent studies have revealed the important functions of p300 in differentiation of mammalian cells. For instance, p300 plays an essential role in MyoD-directed transcription and muscle differentiation of C2C12 cells (40–42, 45). Notch signals have been shown to inhibit both responses (3, 9). The bHLH factor Twist and the viral oncoprotein E1A also inhibit p300, thereby repressing myogenic differentiation (45, 51–53). Thus, it appears that two distinct classes of cellular proteins, i.e. DTX1 and Twist, regulate differentiation of mammalian cells by targeting the transcriptional coactivator p300, and the viral oncoprotein E1A mimics their actions. p300 is the major component of the histone acetylase coactivator complex and regulates diverse classes of transcription factors. p300 also interacts with cell cycle regulators and oncogenes and hence integrates a variety of signals to control cellular transcription, proliferation, and differentiation (46). Recent studies have also suggested that Notch signaling regulates transcription by modulating the histone acetyltransferase complex containing SMRT and HDAC1 (54, 55). Thus, functional and physical interactions between DTX1 and p300 may provide an important clue to the understanding of complex transcriptional regulation by Notch signaling and its diverse biological functions.

This study raises the important issue of how the DTX1- and RBP-J/HES-dependent signaling pathways operate downstream of Notch receptors. Several lines of evidence we presented here suggest that DTX1 acts independently of RBP-J and HES1/HES5. DA-Notch1 and RBP-J (but not DTX1) activated the Hes1 promoter. Conversely, DTX1 could inhibit the transcription by MASH1 even under conditions in which endogenous RBP-J was blocked by dominant-negative RBP-J. DA-Notch1 and DTX1 also inhibited the transactivation by Gal4-MASH1-TAD, which lacks the bHLH domain and hence is refractory to the inhibition by HES1 and HES5. These results collectively suggest that DTX1 can act in parallel to the conventional RBP-J/HES-dependent pathway to inhibit the activity of MASH1.

In Drosophila, Su(H) is involved in only a subset of the multiple functions of Notch (18–20). Vertebrate Notch is also implicated in a variety of biological processes, and at least some of the functions of Notch signaling are independent of RBP-J and HES1/HES5 (3, 16, 17, 22, 23). Thus, the DTX- and RBP-J-dependent pathways may be responsible for regulating different cellular responses. It is also possible that both pathways interact with each other under certain circumstances. For instance, overexpression of Drosophila Deltex induces an accumulation of Su(H) in the nucleus through interference of the association between Notch and Su(H) (25, 50). Our results, together with those in recent studies (38, 39), also suggest that the binding sites of mammalian RBP-J and DTX1 overlap within the intracellular domain of Notch1. Consistently, when overexpressed in cultured cells, DTX1 inhibited the RBP-J-mediated activation of the Hes1 promoter by DA-Notch1; and conversely, RBP-J blocked the DTX1-mediated inhibition of MASH1 by Notch1. Thus, DTX1 and RBP-J appear to act in either a synergistic or an antagonistic manner under distinct conditions. A recent study has also suggested that the expression of DTX1 is under the control of Notch signaling (56). In addition to DTX and RBP-J, we need to consider many other regulatory molecules that associate with the Notch receptor (1, 2). Notch appears to serve as a scaffold protein assembling multiple intracellular regulatory molecules; and hence, physical and functional interactions among these signaling proteins may play important roles in the coordinated regulation of multiple intracellular events. Further studies on Deltex in both flies and mammals should facilitate the understanding of divergent signaling pathways downstream of Notch, which will ultimately contribute to greater insight into the molecular mechanisms of vertebrate development and pathogenesis of human diseases.

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Role of Deltex-1 as a Transcriptional Regulator Downstream of the Notch Receptor
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