

A noncoding RNA regulates the neurogenin1 gene locus during mouse neocortical development

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The proneural basic helix–loop–helix (bHLH) transcription factor neurogenin1 (*Neurog1*) plays a pivotal role in neuronal differentiation during mammalian development. The spatiotemporal control of the *Neurog1* gene expression is mediated by several specific enhancer elements, although how these elements regulate the *Neurog1* locus has remained largely unclear. Recently it has been shown that a large number of enhancer elements are transcribed, but the regulation and function of the resulting transcripts have been investigated for only several such elements. We now show that an enhancer element located 5.8–7.0 kb upstream of the mouse *Neurog1* locus is transcribed. The production of this transcript, designated *utNgn1*, is highly correlated with that of *Neurog1* mRNA during neuronal differentiation. Moreover, knockdown of *utNgn1* by a corresponding short interfering RNA inhibits the production of *Neurog1* mRNA in response to induction of neuronal differentiation. We also found that production of *utNgn1* is suppressed by polycomb group (PcG) proteins, which inhibit the expression of *Neurog1*. Our results thus suggest that a noncoding RNA transcribed from an enhancer element positively regulates transcription at the *Neurog1* locus.

The mammalian central nervous system (CNS) is composed of a great variety of neurons and glial cells, all of which must be generated from multipotential neural precursor cells (NPCs) in the correct number and at specific times and locations during embryogenesis (1, 2). Proneural basic helix–loop–helix (bHLH) proteins play central roles in the specification of neuronal fate and the subsequent differentiation processes (3). Among these proteins, neurogenin1 (*Neurog1*) is a key regulator of neuronal fate specification in various regions of the neural tube, including those that give rise to the neocortex, midbrain, hindbrain, and dorsal and ventral spinal cord of the CNS, as well as in portions of the peripheral nervous system (4–7). In the developing mouse neocortex, ablation of *Neurog1* along with the related protein neurogenin2 (*Neurog2*) results in the loss of deep-layer neurons (8), and conversely, overexpression of *Neurog1* results in premature neuronal differentiation of NPCs at the expense of glial fate (9, 10). These observations suggest that expression of the *Neurog1* and *Neurog2* genes must be tightly controlled in a spatiotemporal manner during development to ensure the generation of specific subsets of neurons and establishment of the fine architecture of the CNS.

Spatiotemporal control of gene expression is generally mediated by regulatory elements including enhancers. Previous studies have revealed several enhancer elements that control expression of the *Neurog1* gene, including the lateral stripe element (LSE), the anterior neural plate element (ANPE), and the LATE; which were originally identified as tissue-specific enhancers in zebrafish and found to be conserved among various vertebrate genomes including the mouse genome (11–13). Deletion of the 4-kb mouse genomic region including LATE and ANPE significantly reduced overall expression of *Neurog1* in a transgenic reporter assay (14). Therefore, although this 4-kb region has been implicated in tissue-specific regulation, it might also contain an essential general enhancer or locus control region. It has remained elusive, however, how these regions regulate *Neurog1* expression and how their activity is regulated.

Several molecules have been implicated in the regulation of *Neurog1* expression during neocortical development. For instance, Wnt signaling induces expression of *Neurog1* via β -catenin and T

cell specific transcription factor (TCF)/lymphoid enhancer binding factor (LEF) transcription factors in neocortical NPCs (15, 16). The homeodomain transcription factor Pax6, which contributes to neocortical regional identity, also positively regulates expression of *Neurog1* (12, 17). In contrast, Polycomb group (PcG) proteins suppress the *Neurog1* promoter during the gliogenic stage of neocortical development when the neurogenic potential of NPCs is restricted (18). Whether or how these molecules affect the enhancers of *Neurog1* has remained unclear.

Various mechanisms have been proposed for the regulation of a gene by a corresponding enhancer (19). Classically, enhancers are viewed as clusters of DNA elements that bind transcription factors, which in turn interact with the mediator complex or transcription factor IID to facilitate the recruitment of RNA polymerase II (as well as that of chromatin modifying enzymes) to the promoter region through “DNA looping” (20). However, recent studies have indicated that enhancer sequences are not simply binding sites for transcription factors and cofactors; rather, many of them are also transcribed to generate noncoding RNAs when the transcription of corresponding genes takes place (21–26). The functions of such enhancer-associated noncoding RNAs have only just begun to be elucidated, such as in the case of those derived from the *Snail1* and *HoxA* gene loci (23–25).

In this study, we found that an enhancer region spanning from between LATE and ANPE to LATE is transcribed in mouse neocortical NPCs and that expression of this transcript (designated *utNgn1*) correlates well with that of *Neurog1* mRNA. Knockdown experiments revealed that *utNgn1* is necessary for the effective transcription of *Neurog1* in neocortical NPCs, suggesting that one of the enhancers of the *Neurog1* locus functions via generation of its transcript. Furthermore, the amount of *utNgn1* was found to be increased by Wnt signaling and to be down-regulated by PcG proteins. We noticed that the *utNgn1* locus in neocortical NPCs harbors histone H3 lysine 27 trimethylation (H3K27me3), a histone mark catalyzed by PcG proteins, as well as histone H3 lysine 4 trimethylation (H3K4me3) and histone H3 lysine 9 or 14 acetylation (H3K9/K14ac), marks of active transcription (27). Our results thus suggest that the enhancer of *Neurog1* regulates the expression of *Neurog1* via its transcript, and PcG proteins suppress a target gene not only directly by occupying promoter regions but also indirectly by occupying their enhancers.

Results

utNgn1 Is Transcribed from an Enhancer Region of the *Neurog1* Locus.

A 4-kb region located 3.8–7.8 kb upstream of the transcription start site (TSS) of mouse *Neurog1* has been implicated in regulation of *Neurog1* expression in most expression domains of the mouse embryo (14), suggesting the existence of a general enhancer within this region. Given that this region contains a CpG island (CGI) between ANPE and LATE (Fig. 1A), we hypothesized that it might be transcriptionally active (28). We therefore first examined

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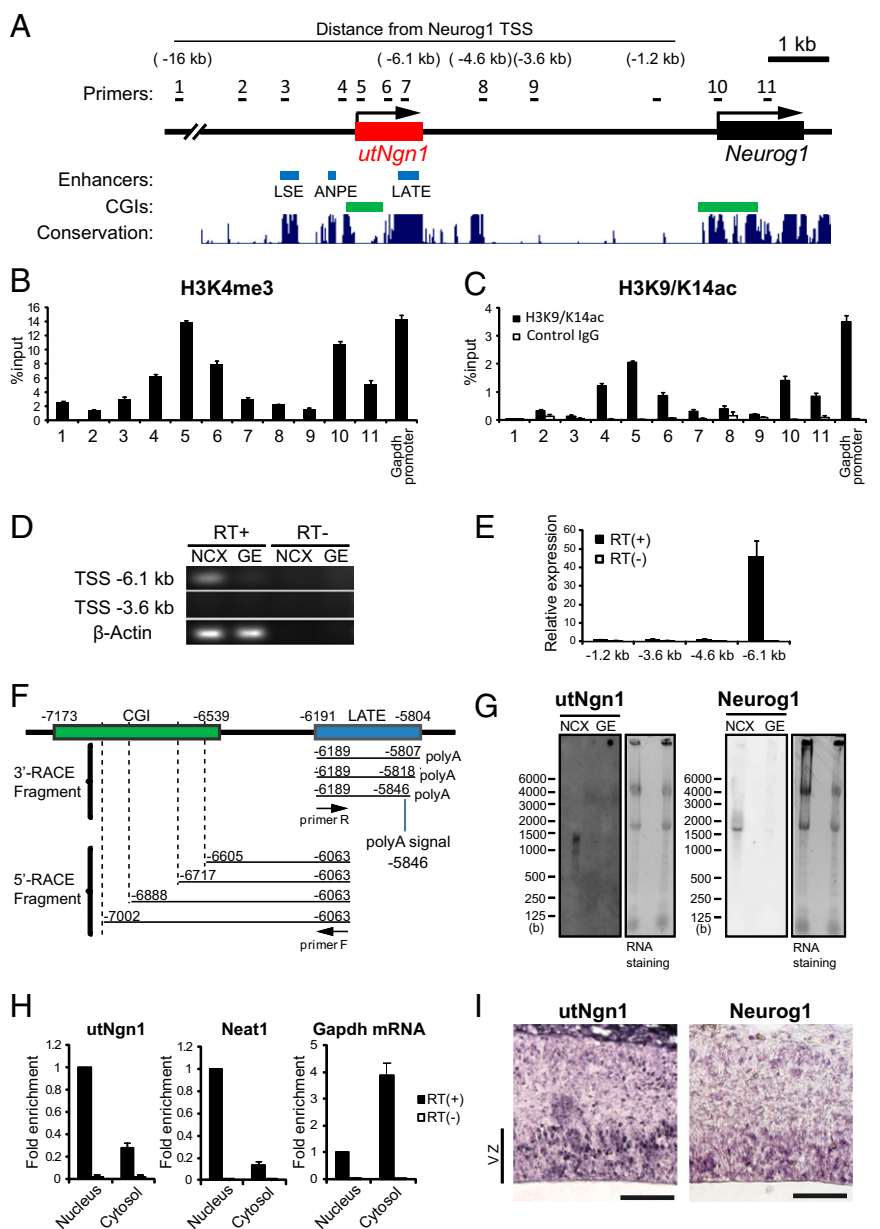
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whether the region surrounding this CGI contains the H3K4me3 mark, which is associated with the promoters of actively transcribed genes (or genes in a state permissive for transcription). We cultured NPCs prepared from the mouse neocortex at embryonic day (E) 11.5 for 3 d in vitro (DIV) as neurospheres (E11.5 + 3DIV culture). Chromatin immunoprecipitation (ChIP) assay revealed the presence of this modification in the region ~ 7 kb upstream of the TSS of *Neurog1* (Fig. 1A, primer 5), with a peak in H3K4me3 at the CGI (Fig. 1B). The amount of H3K4me3 at the CGI was similar to that at the promoter of *Neurog1* as well as at the promoter of the actively transcribed gene *Gapdh*, suggesting that the region surrounding the CGI might be transcribed. This notion was further supported by the observation that H3K9/K14ac, another histone modification associated with actively transcribed genes, was also present in the region surrounding the CGI (Fig. 1C).

We next performed RT-PCR analysis to search for transcripts derived from the region surrounding the CGI and detected PCR amplicons originating from a position ~ 6.1 kb upstream of the TSS

of *Neurog1* in the mouse neocortex at E13.5 (Fig. 1A and D). Furthermore, quantitative RT-PCR (qRT-PCR) analysis revealed that a significant level of transcription occurs in this position compared with other intergenic regions such as ~ 1.2 , 3.6, and 4.6 kb upstream of the TSS of *Neurog1* (Fig. 1A and E). Next, to determine the 5' and 3' ends of the transcripts, we performed rapid amplification of cDNA ends (RACE) analysis (Fig. 1F). 5' RACE revealed multiple 5' ends within the CGI, consistent with previous observations showing that CGI promoters are accompanied by multiple initiation points within the CGI (29). 3' RACE revealed a polyadenylation signal at position -5.8 kb relative to the TSS of *Neurog1*. Consistent with these observations, Northern blot analysis with an antisense probe corresponding to positions -6.0 to -6.4 kb revealed a transcript of ~ 1.4 kb in a total RNA fraction isolated from the mouse neocortex at E13.5 (Fig. 1G). We now refer to this transcript derived from an enhancer region of *Neurog1* as *utNgn1* (upstream transcript of the *Neurog1* locus). The longest ORF of *utNgn1* potentially encodes a 49-amino-acid protein with

Fig. 1. A noncoding RNA, *utNgn1*, is transcribed from an enhancer region of *Neurog1*. (A) Schematic representation of enhancers and CpG islands (CGIs) of the mouse *Neurog1* locus. Blue and green boxes indicate enhancers (LATE, ANPE, and LSE) identified in zebrafish and CGIs, respectively. Black arrows indicate the direction of transcription of *utNgn1* (red box) and *Neurog1* (black box). Conservation plot and CGIs are adapted from the University of California Santa Cruz Genome Browser. Black bars indicate the positions of PCR primers used for RT-PCR or ChIP analysis. (B and C) Primary NPCs isolated from the E11.5 mouse neocortex (NCX) were cultured in suspension for 3DIV in the presence of fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF). Cells were then subjected to ChIP analysis with antibodies to H3K4me3 (B) or H3K9/K14ac (or with control IgG) (C) and with the PCR primers indicated in A or those specific for the promoter of *Gapdh*. Data are expressed as percentage of the input and are means \pm SEM for three samples. (D) Total RNA extracted from the NCX or ganglionic eminences (GE) of E13.5 mouse embryos was subjected to RT (or not) with an oligo (dT) primer followed by PCR with primers targeted to regions located 6.1 kb or 3.6 kb upstream of the transcription start site (TSS) of *Neurog1* as well as with those specific for β -actin (internal control). (E) Relative expression levels of *utNgn1* locus (*Neurog1* TSS -6.1 kb) in the neocortex compared with other intergenic regions (*Neurog1* TSS -1.2 , -3.6 , and -4.6 kb) are determined by qRT-PCR. Data represent means \pm SEM from three embryos at E13.5. (F) 5' and 3' RACE analysis of *utNgn1* with cDNA prepared from poly(A)⁺ or total RNA of the mouse neocortex at E12.5. Black arrows indicate PCR primers F and R. Four 5' ends and three 3' ends were determined by DNA sequencing in 5' RACE and 3' RACE, respectively. Amplified fragments are mapped on the *Neurog1* upstream region, and a polyadenylation signal (AAATAA) is shown. (G) Northern blot analysis of total RNA prepared from the E13.5 NCX or GE were performed with DIG-labeled RNA probes specific for *utNgn1* or *Neurog1* mRNA. Blots were also stained with SYBR Gold (Right). (H) Relative concentration of *utNgn1* in the nuclear and cytosolic fractions. Cells were prepared from the E12.5 mouse NCX and cultured in suspension for 3DIV. The same amount of total RNA extracted from nuclear or cytosolic fractions of NPCs were reverse transcribed. Relative amounts of the cDNA in each fraction were determined by qRT-PCR. Data are expressed relative to the corresponding value for concentration in the nuclear fraction and are means \pm SEM from three independent experiments. (I) In situ hybridization analysis of *utNgn1* and *Neurog1* mRNA in coronal sections of the E13.5 mouse NCX. VZ, ventricular zone. (Scale bar, 50 μ m).



no sequence similarity to any expressed sequences currently deposited in protein databases. Moreover, it may not be translated because fractionation of neocortical NPCs (E12.5 + 3DIV culture) revealed that the concentration of *utNgn1* is higher in the nucleus compared with the cytosol, which is characteristic of noncoding RNA (for instance, *Neat1*, *Malat1*, or *Xist*) (30) in contrast to the cytosol-enriched localization of coding RNA (mRNA) (Fig. 1H). We cannot, however, exclude the possibility that *utNgn1* is translated. Although *utNgn1* and *Neurog1* mRNA are encoded on the same strand, they do not appear to be components of the same transcript, given that neither the probe for *utNgn1* nor one for *Neurog1* mRNA detected a transcript spanning both target RNAs (>3 kb) in Northern blot analysis (Fig. 1G). In addition, we could not detect a transcript, by qRT-PCR, or association with histone H3 lysine 36 trimethylation (H3K36me3) (27), by ChIP analysis, for the region between the coding sequences for *utNgn1* and *Neurog1* mRNA (Fig. 1E and Fig. S1).

Correlation Between *utNgn1* and *Neurog1* mRNA Expression Patterns.

Long noncoding RNAs have previously been found to affect gene expression both positively and negatively (31). We therefore compared the expression pattern of *utNgn1* with that of *Neurog1* mRNA. We isolated various regions of the developing CNS, including the neocortex, ganglionic eminences, diencephalon, midbrain, hindbrain, and spinal cord, as well as nonneural tissues, including the heart, liver, and limb, from mouse embryos at E13.5. qRT-PCR analysis revealed that *utNgn1* was expressed in all regions of the CNS that expressed *Neurog1* mRNA, but neither RNA was detected in the nonneural tissues (Fig. 2A) (14). Moreover, the amount of *utNgn1* correlated well with that of *Neurog1* mRNA. The expression level of *utNgn1* is very low (1/30–1/300 of that of *Neurog1*) (Fig. 2A–D). Northern blot analysis also showed that both *utNgn1* and *Neurog1* mRNA were more abundant in the neocortex than in the ganglionic eminences (Fig. 1G). These results thus suggested that the expression of *utNgn1* is positively correlated with that of *Neurog1* mRNA.

We also examined the expression of *utNgn1* by in situ hybridization. An antisense probe for *utNgn1* yielded robust signals in a subpopulation of cells located within the ventricular zone (which contains NPCs) of the E13.5 mouse neocortex (Fig. 1I), the midbrain, and a dorsal part of the diencephalon (Fig. S2A and B). Importantly, the expression pattern of *utNgn1* largely overlapped with that of *Neurog1* mRNA, providing further support for a positive correlation between *utNgn1* and *Neurog1* mRNA expression in the developing CNS.

We next asked whether the expression level of *utNgn1* changes during neuronal differentiation of neocortical NPCs. NPCs freshly prepared from the neocortex at E11.5 were maintained in an undifferentiated state in the presence of fibroblast growth factor (FGF)2. Removal of FGF2 in such primary cultures results in neuronal differentiation and promotes the production of *Neurog1* mRNA and subsequently that of *βIII-tubulin* mRNA (32) (Fig. 2B). Here we found that, under the same conditions, growth factor deprivation also induced expression of *utNgn1* with a time course similar to that of *Neurog1* mRNA (Fig. 2B), indicating that the expression of *utNgn1* positively correlates with that of *Neurog1* mRNA and is induced during an early phase of neuronal differentiation of neocortical NPCs.

We next investigated how the production of *utNgn1* is regulated by extracellular signals, which regulate neuronal differentiation of NPCs. Notch signaling is essential for the maintenance of undifferentiated state of NPCs and suppress neuronal differentiation. We then induced neuronal differentiation of NPCs by treatment with *N*-[*N*-(3,5-difluoro-phenacetyl)-*L*-alanyl]-(*S*)-phenylglycine *t*-butyl ester (DAPT), a presenilin inhibitor that suppresses Notch signaling and thereby reduces the amount of *Hes1* and *Hes5* mRNA, a downstream effector of Notch signaling (33) (Fig. 2C). Exposure of the NPC cultures to DAPT induced the expression of *utNgn1* and that of *Neurog1* mRNA (Fig. 2C). Wnt signaling is another extracellular signaling that regulates neuronal differentiation and *Neurog1* expression in the mouse neocortex. We therefore examined whether Wnt signaling might also regulate *utNgn1* expression. Treatment of E11.5 neocortical NPCs with recombi-

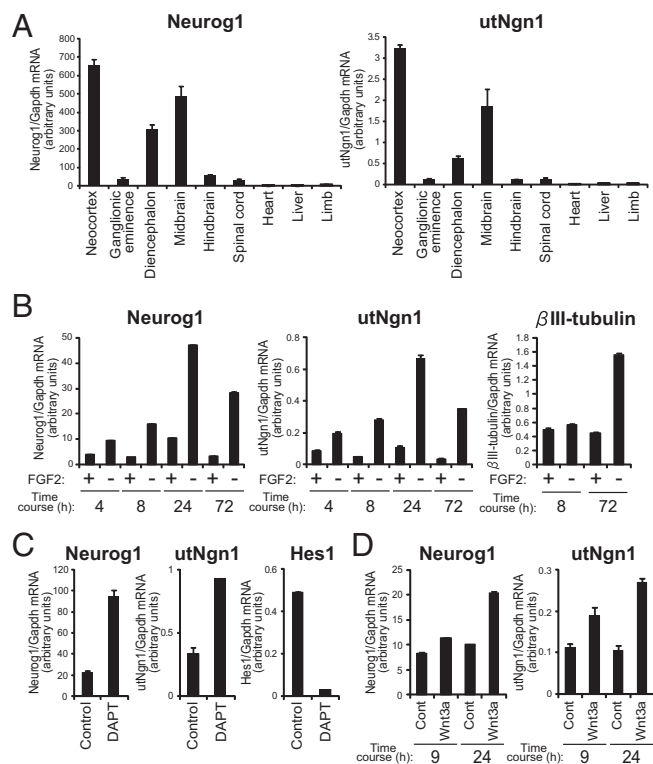


Fig. 2. Expression pattern of *utNgn1* is highly correlated with that of *Neurog1* mRNA. (A) Tissues were isolated from the neocortex, ganglionic eminences, diencephalon, midbrain, hindbrain, spinal cord, heart, liver, and limb of E13.5 mouse embryos. (B) Primary NPCs prepared from the E11.5 neocortex were cultured for 1 d and then incubated for the indicated times in medium with or without FGF2. (C) Primary NPCs prepared from the E11.5 neocortex were cultured for 2 d and then exposed to DAPT (5 μM) or dimethyl sulfoxide vehicle (control) for 7 h. (D) Primary NPCs prepared from the E11.5 neocortex were cultured without (control) or with Wnt3a for 9 or 24 h. (A–D) Total RNA was isolated from the cells or tissues and subjected to qRT-PCR analysis of each gene. Relative amounts of *Neurog1* and *utNgn1* were determined using a standard curve derived from a BAC clone, which contained both *Neurog1* and *utNgn1* loci, making the values of *Neurog1* and *utNgn1* transcripts comparable. Data are normalized by the amount of *Gapdh* mRNA and are means ± SEM ($n = 3$).

nant Wnt3a for 9 or 24 h increased the amounts of *utNgn1* and of *Neurog1* mRNA (Fig. 2D). These results further indicate that the expression of *utNgn1* positively correlates with that of *Neurog1* mRNA and is induced during neuronal differentiation of neocortical NPCs.

We also examined the expression of *utNgn1* in embryonic stem (ES) cells and ES-derived NPCs (Fig. S3) (34). Neither *utNgn1* nor *Neurog1* mRNA was detected in ES cells in the undifferentiated state (day 0), but both transcripts were found to be expressed after culturing the cells for 7 d under conditions that induce differentiation into NPCs (day 7). Again, these results supported a positive correlation between transcription of *utNgn1* and that of *Neurog1* mRNA during development.

***utNgn1* Positively Regulates *Neurog1* Expression.** Transcription of neighboring loci can be positively correlated as a result of the phenomenon known as transcriptional noise or “ripples” (35), which might be dependent on mechanisms including chromatin remodeling. We therefore investigated whether *utNgn1* functions in gene regulation or is simply a byproduct of *Neurog1* expression. To assess its possible role in *Neurog1* expression, we knocked down *utNgn1* in E11.5 NPCs using small interfering RNAs (siRNAs) targeted to three different sequences within *utNgn1* and three control siRNAs. Growth factor deprivation for 10 h induced *Neurog1* expression in cells without siRNA or with control siRNA, but this effect was markedly inhibited in cells harboring any of the

siRNAs specific for *utNgn1* (Fig. 3A and Fig. S4). We confirmed that the nuclear level of *utNgn1* was effectively decreased by siRNAs targeting *utNgn1* (Fig. 3B). We examined the expression of genes within 1 Mb of the *utNgn1* locus and found that knockdown of *utNgn1* did not reduce the expression of any of these genes except for *Neurog1* (Fig. 3C and Fig. S5A and B). We also found that depletion of *Neurog1* mRNA with a corresponding siRNA did not affect the amount of *utNgn1* (Fig. 3D). These results thus suggest that *utNgn1* is not simply a byproduct of transcription of a neighboring gene, but rather is a positive regulator of *Neurog1* expression, providing an example of gene regulation through the production of an enhancer-derived transcript.

Next we examined the biological function of *utNgn1*. Consistent with the proposed role of *Neurog1* in neuronal fate commitment in neocortical NPCs (8), we found that *utNgn1* knockdown partially inhibited the induction of *Tbr2* and *NeuroD1*, early markers of neocortical neuronal fate commitment, under a differentiation-inducing condition (Fig. S5C). Therefore, *utNgn1* might play a role in neuronal fate commitment of these cells.

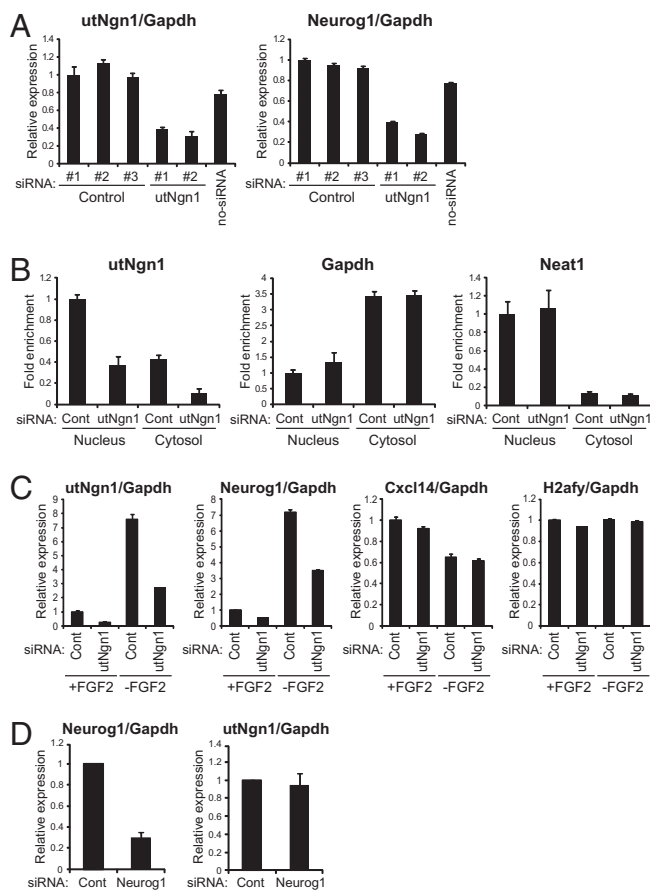


Fig. 3. *utNgn1* is required for the expression of *Neurog1* during neuronal differentiation. (A, C, and D) Primary NPCs prepared from the E11.5 neocortex were transfected with three independent control (siRNAs 1, 2, and 3), two independent siRNAs against *utNgn1* (*utNgn1* siRNA 1 and 2), or without siRNAs (no-siRNA) (A). Similarly, cells were transfected with control (siRNA 1), *utNgn1* (siRNA 1), or siRNA against *Neurog1* (C and D). Cells were cultured for 2 d with FGF2 and then for 10 h with or without FGF2. Total RNA was then extracted from the cells and subjected to qRT-PCR analysis of each gene. Data are normalized with the amount of *Gapdh* mRNA, are expressed relative to the corresponding value for cells transfected with the control siRNA 1, and are means \pm SEM ($n = 3$). (B) Primary NPCs prepared from the E11.5 neocortex were transfected with a control (siRNA 1) or with a combination of two independent siRNAs against *utNgn1* (*utNgn1* siRNAs 1 and 2). Cells were cultured for 3 d in suspension with FGF2 and EGF, harvested and fractionated, as described in Fig. 1H, and the amount of RNA in each fraction was determined.

***utNgn1*, but Not Its Truncated Form, Positively Regulates Expression of Its Downstream Gene.** To investigate whether *utNgn1* is capable of *cis*-regulating the transcription of its downstream gene, we performed a reporter gene assay. We designed Firefly luciferase (*luc2*) reporter constructs as schematized in Fig. 4A. In these constructs, the *luc2* gene is under the control of the *Neurog1* promoter either alone (a), with an intact *utNgn1* (b), or with a truncated *utNgn1* harboring early termination sequences (c). In vector c, we inserted early termination sequences in *utNgn1* between the CGI and the LATE enhancer to inhibit the expression of full-length *utNgn1* without affecting the LATE enhancer sequence itself. A synthetic poly(A) signal transcriptional pause site was inserted between *utNgn1* and the *Neurog1* promoter to prevent the direct effect of upstream transcription on reporter transcription. We found that the insertion of *utNgn1* into an upstream region of the *Neurog1* promoter significantly enhanced reporter gene transcription (Fig. 4B). Importantly, insertion of early termination sequences in *utNgn1* resulted in a loss of the increased reporter gene expression, suggesting the importance of an intact *utNgn1* transcript for this action (Fig. 4B). This result provides additional evidence indicating the importance of *utNgn1* for *Neurog1* gene transcription.

PcG Proteins Suppress *utNgn1* Expression. During the late stage of neocortical development, which is associated with the restriction of neuronal fate and activation of the gliogenic phase of NPCs, PcG proteins suppress the *Neurog1* promoter (18). We therefore asked whether PcG proteins also regulate *utNgn1* expression. ChIP analysis with neocortical NPCs (E11.5 + 3DIV or 9DIV) revealed that the *utNgn1* locus was broadly occupied by H3K27me3, a histone mark catalyzed by PcG proteins, at levels comparable to those at the promoter of *Neurog1* (Fig. 5A). Therefore, like the *Neurog1* locus, the *utNgn1* locus is also “bivalent,” harboring both H3K4me3 (active) and H3K27me3 (repressive) histone marks (36). Neocortical E11.5 + 3DIV and E11.5 + 9DIV NPC cultures are characterized by preferential differentiation into neurons and astrocytes, respectively, under the conditions adopted in the present study (18). The level of H3K27me3 at the *utNgn1* locus was substantially higher in E11.5 + 9DIV cultures than in E11.5 + 3DIV cultures (Fig. 5A), suggesting that this change is associated with the late (gliogenic) stage of neocortical NPC development. In contrast, the level of H3K4me3 at both *utNgn1* and *Neurog1* loci remained largely unchanged between E11.5 + 3DIV and E11.5 + 9DIV (Fig. 5B).

The high levels of H3K27me3 at the *utNgn1* locus suggest that PcG proteins may suppress the production of the enhancer-encoded transcript during the late developmental stage of neocortical NPCs. To test this hypothesis, we first examined whether the expression of *utNgn1* is indeed repressed in the late stage of neocortical NPC development. The level of *utNgn1*, as well as that of *Neurog1* mRNA, was greatly reduced in E11.5 + 9DIV cultures compared with that in E11.5 + 3DIV cultures (Fig. 5C). We observed similar reductions of both *utNgn1* and *Neurog1* in E17.5 + 3DIV cultures relative to E11.5 + 3DIV cultures (Fig. 5C), suggesting that the suppression of *utNgn1* expression is the result of developmental progression rather than culture duration. Consistent with this, the expression levels of both *Neurog1* and *utNgn1* in the neocortex were dramatically reduced during development (Figs. S2C and S6). We then examined whether the suppression of *utNgn1* production in the late stage of development is mediated by PcG proteins. To this end, we induced the conditional ablation of Ring1B, an essential component of Polycomb repressive complex 1 (37), by injecting tamoxifen intraperitoneally into *Ring1b*^{fllox/fllox}; NestinERT2-Cre mice (18, 38) at E12.5. The amount of *utNgn1* was markedly greater in the neocortex isolated from the Ring1B-deficient embryos at E18.5 compared with control embryos (Fig. 5D). Together, these results indicate that PcG proteins suppress *utNgn1* expression during the late stage of neocortical NPC development. PcG proteins thus appear to regulate *Neurog1* expression not simply by direct suppression of the gene itself but also by that of its enhancer.

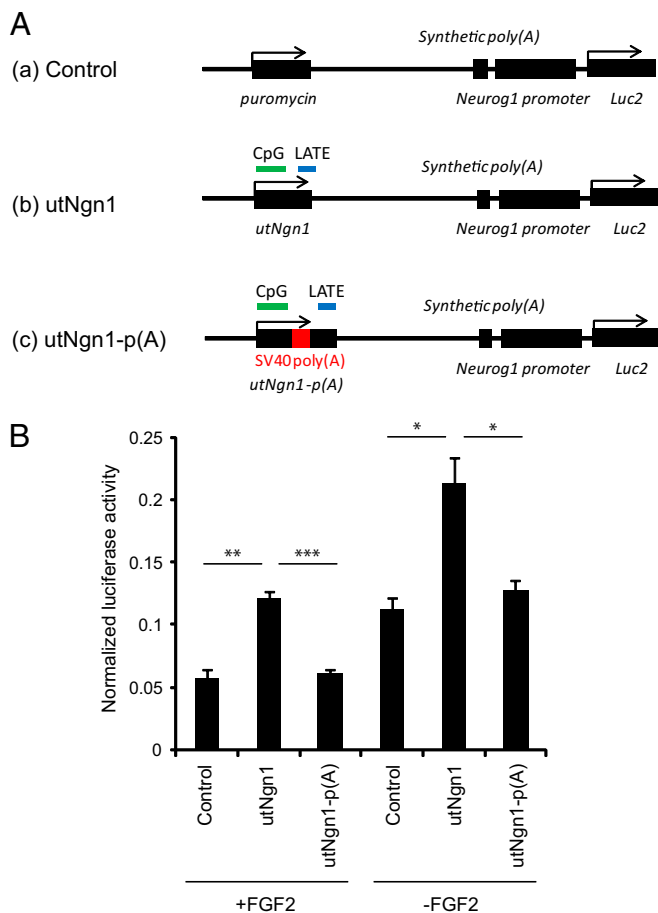


Fig. 4. Full-length *utNgn1*, but not truncated *utNgn1*, promotes expression of reporter gene. (A) Schematic representation of the reporter constructs. A 2.7-kb region of the *Neurog1* promoter was cloned into the promoter multicloning site of the pGL4.20 vector and used as a control (a). Full-length *utNgn1* was cloned and inserted into the upstream region of vector a (b). Two SV40 poly(A) sequences were tandemly inserted into the *utNgn1* region between the CGI and the LATE enhancer (c). (B) Luciferase reporter assay. Firefly vectors shown in A and the control Renilla vector were transfected into NPCs prepared from E11.5 neocortices. All cells were cultured with FGF2 for 12 h and then half were cultured with and half without FGF2 for 20 h. Data are normalized by the Renilla luciferase activity and are means \pm SEM ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by two-tailed Student t test.

Discussion

A significant portion of the mammalian genome generates non-coding RNAs (ncRNAs) including long intervening noncoding RNAs (lincRNAs) (39–41), some of which have been implicated in gene silencing associated with processes such as imprinting and X chromosome inactivation (42). However, recent studies have also begun to reveal roles of lincRNAs in gene activation (23–25). For instance, a set of ncRNAs (ncRNA-a) was shown to positively regulate their neighboring protein-coding genes, including those for *SCL*, *Snail1*, and *Snail2* (24). Similarly, *HOTTIP*, a lincRNA transcribed from the 5' end of the *HoxA* locus, was found to positively regulate the *HoxA* gene cluster (23). Our results now indicate that *utNgn1* is another lincRNA that positively regulates gene expression.

Recent genome-wide studies have revealed that gene promoters are marked by H3K4me3, whereas enhancers are often associated with H3K4me1 but are devoid of H3K4me3 (43). The enhancer region now shown to encode *utNgn1* therefore appears to be atypical in that it is associated with H3K4me3. Because this is also the case for the ncRNA-a and *HOTTIP* loci, the presence of H3K4me3 in the enhancer regions might be a specific charac-

teristic of regions encoding this “gene activator” class of lincRNAs. H3K4me1-enriched enhancers have also been shown to express RNA (eRNA), although the functions of these RNA molecules are unknown (21, 26). The transcripts from H3K4me1-enriched enhancers are devoid of polyadenylation, whereas long non-coding RNAs transcribed from H3K4me3-enriched enhancers including *utNgn1* harbor polyadenylation, possibly reflecting a difference in mode of regulation and/or function (21, 23–26). *utNgn1* is more similar to lincRNAs in this context, although the *utNgn1* locus does not harbor the H3K36me3 mark, which is a characteristic of lincRNAs.

Development is associated with strict regulation of the enhancers of developmental genes. It has been proposed that H3K4me1-enriched enhancers can be classified as either “poised” and “active” enhancers by the additional presence of the histone marks H3K27me3 and H3K27ac, respectively (44, 45). It remains unclear, however, whether these histone marks are functionally important for enhancer activity. Our results now suggest that the

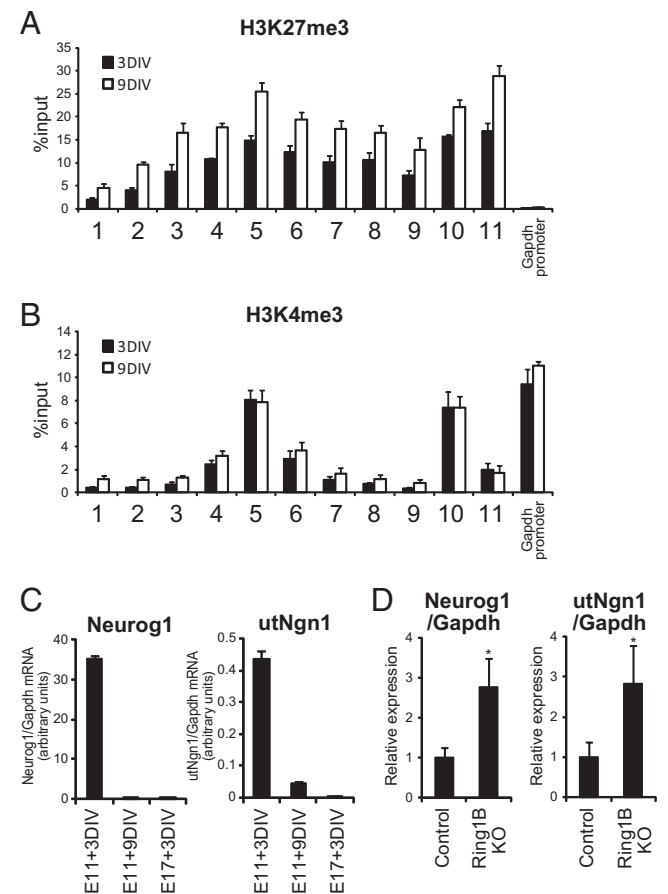


Fig. 5. PcG proteins repress the expression of *utNgn1* in the late stage of NPC development. (A and B) Primary NPCs isolated from the E11.5 neocortex were cultured for 3 or 9 DIV in suspension in the presence of FGF2 and EGF. Cells were then subjected to ChIP assays with antibodies to H3K27me3 (A) or to H3K4me3 (B) and with the PCR primers indicated in Fig. 1A. Data are means \pm SEM from three independent experiments. (C) Primary NPCs isolated from the E11.5 or E17.5 neocortex were cultured for 3 or 9 DIV as in A, after which total RNA was isolated and subjected to qRT-PCR analysis of *utNgn1* and *Neurog1* mRNA. Data are normalized by the amount of *Gapdh* mRNA and are means \pm SEM ($n = 3$). (D) Total RNA prepared from the neocortex of E18.5 *Ring1b*^{fllox/fllox}; NestinERT2-Cre (*Ring1B* KO) and *Ring1b*^{fllox/fllox} (control) embryos that were exposed to tamoxifen in utero at E12.5 were subjected to qRT-PCR analysis of *Neurog1* mRNA and *utNgn1*. Data are normalized by the amount of *Gapdh* mRNA, are expressed relative to the corresponding value for control embryos, and are means \pm SD from three embryos of each genotype. * $P < 0.05$ (Student t test).

utNgn1 locus is maintained in a poised (or inactive) state in the late stage of neocortical NPC development by a PcG protein-mediated mechanism, given that the locus is enriched with both H3K27me3 and H3K4me3 and that *utNgn1* expression was derepressed by ablation of Ring1B. These findings thus suggest that PcG proteins may directly suppress enhancers in addition to promoters for a class of developmental genes in a developmental context-dependent manner.

The mechanism of enhancers' regulation of gene expression and that of the regulation of enhancers themselves are fundamental to an understanding of gene control mechanisms. Given our finding that *utNgn1* is necessary for the efficient activation of *Neurog1* expression, a next key question will be how this transcript promotes gene activation, which is likely by recruitment of effector proteins, including histone modifiers or transcription factors, to the *Neurog1* promoter. Although recruitment or maintenance of mixed lineage leukemia (MLL) complex is implicated in the action of lincRNAs such as *HOTTIP* and *Mistral* (23, 25), other mechanisms likely account for the action of *utNgn1*, because the level of H3K4me3 (mediated by MLL complex) of the *Neurog1* promoter was not significantly reduced, despite a marked reduction of *utNgn1* expression, in the late developmental stages (Fig. 5). It will also be important to elucidate how PcG protein-mediated regulation of the *utNgn1* locus relates to that of the *Neurog1* promoter. It is possible that PcG proteins associated with the

enhancer might physically and functionally interact with those associated with the promoter. Further elucidation of *utNgn1*'s regulation and function should bring new insights into the role of enhancers and the regulation of transcription.

Materials and Methods

Full details of procedures are provided in *SI Materials and Methods*. Briefly, primary NPCs were isolated and cultured as described previously (18). The siRNA were transfected using Neon transfection system (Invitrogen). Antibodies used in ChIP assay were anti-H3K9K14Ac (Upstate; 06–599), anti-H3K27me3 (Upstate; 07–449), anti-H3K4me3 (Active Motif; 39916), anti-H3K36me3 (Abcam; 9050), and control rabbit IgG (Santa Cruz; sc-2027).

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