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Report

Transcriptional Elongation Factor Elongin A Regulates Retinoic Acid-Induced Gene Expression during Neuronal Differentiation

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SUMMARY

Elongin A increases the rate of RNA polymerase II (pol II) transcript elongation by suppressing transient pausing by the enzyme. Elongin A also acts as a component of a cullin-RING ligase that can target stalled pol II for ubiquitylation and proteasome-dependent degradation. It is not known whether these activities of Elongin A are functionally interdependent in vivo. Here, we demonstrate that Elongin A-deficient (Elongin $A^{-/-}$) embryos exhibit abnormalities in the formation of both cranial and spinal nerves and that Elongin $A^{-/-}$ embryonic stem cells (ESCs) show a markedly decreased capacity to differentiate into neurons. Moreover, we identify Elongin A mutations that selectively inactivate one or the other of the aforementioned activities and show that mutants that retain the elongation stimulatory, but not pol II ubiquitylation, activity of Elongin A rescue neuronal differentiation and support retinoic acid-induced upregulation of a subset of neurogenesis-related genes in Elongin $A^{-/-}$ ESCs.

INTRODUCTION

The synthesis of mRNA in eukaryotes is regulated by the concerted action of a set of transcription factors that control the activity of RNA polymerase II (pol II) during the initiation and elongation stages of transcription. Although the initiation stage of transcription by pol II was long thought to be the primary site for regulation, a growing body of evidence indicates that transcript elongation can also be rate limiting and is an important target for gene regulation by a diverse collection of elongation factors that promote efficient elongation of transcripts by pol II in vitro (Saunders et al., 2006). Elongin is a member of a family of elongation factors that can increase the overall rate of RNA chain elongation by decreasing the frequency and/or duration of transient pausing by pol II as it traverses the DNA template (Conaway et al., 2000; Shilatifard et al., 2003).

Elongin is a three-subunit complex composed of the transcriptionally active Elongin A subunit and two positive regulatory subunits, Elongins B and C, which share sequence similarity with ubiquitin and with the Skp1 subunit of SCF ubiquitin ligases, respectively (Aso et al., 1995). Elongin A binds Elongins B and C through a sequence motif referred to as the BC box (Aso et al., 1996). Elongins B and C are subunits not only of the Elongin complex but are also members of a large family of Cullin 2 (Cul2)- or Cullin 5 (Cul5)-based ubiquitin ligases, in which a BC box containing substrate recognition subunit is linked through the Elongin BC heterodimer to a subcomplex composed of Cul2 or Cul5 and either of the small RING finger proteins Rbx1 or Rbx2. We and others have shown recently that Elongin A can act as the BC box substrate recognition subunit of a Cul5- and Rbx2-containing ubiquitin ligase that ubiquitylates the largest subunit of pol II (Rpb1) (Yasukawa et al., 2008; Harreman et al., 2009).

Although mammalian Elongin A is not essential for cell viability in vitro, Elongin A is essential in vivo. Elongin A-deficient (Elongin $A^{-/-}$) mouse embryos exhibit developmental abnormalities including central nervous system (CNS) anomalies and die between days 10.5 and 12.5 of gestation (Miyata et al., 2007). In addition, the Elongin A ortholog in *Drosophila* has been shown to be required for optimal expression of the heat shock gene *HSP70* and several *Hox* genes, including *Ultrabithorax* and *Abdominal-B* (Gerber et al., 2005; Chopra et al., 2009). Because Rpb1 ubiquitylation following pausing or arrest of pol II is now believed to be one mechanism that helps to ensure the overall efficiency of

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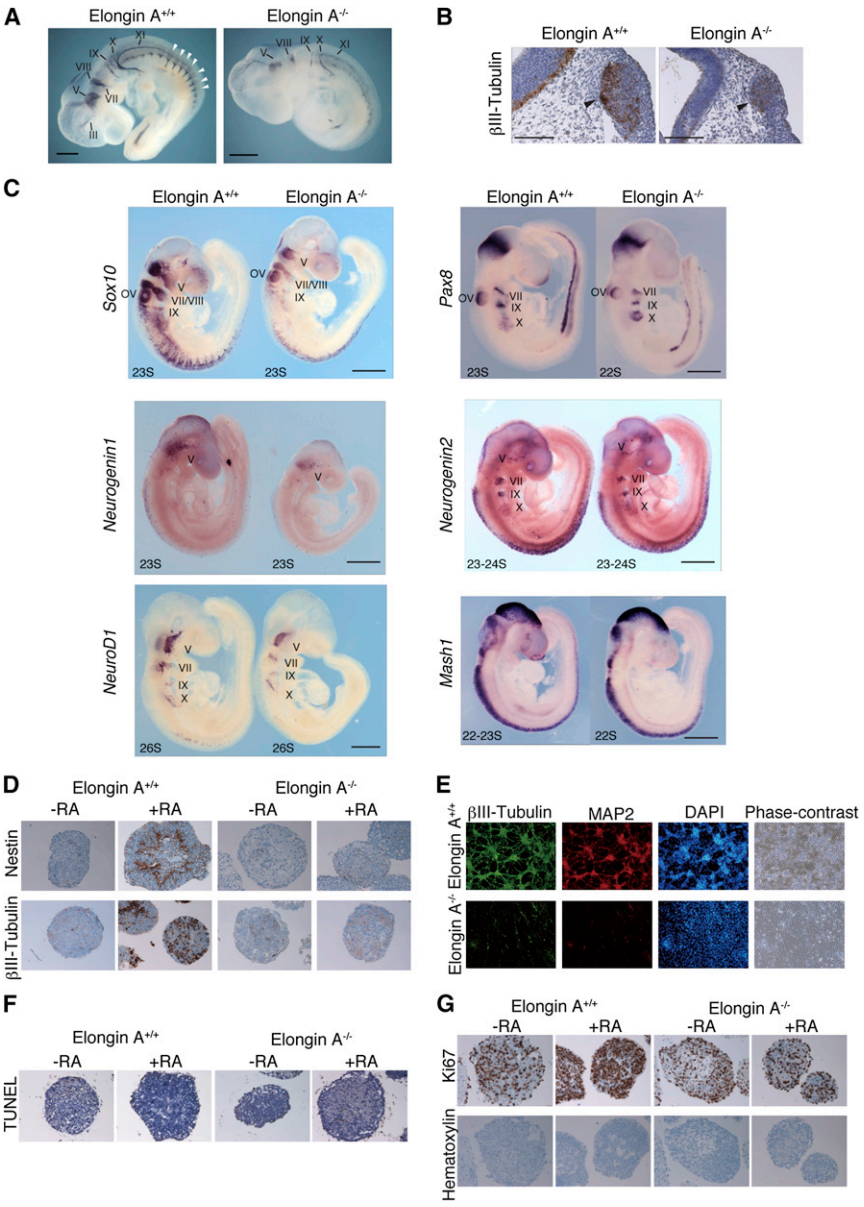

Figure 1. Elongin A$^{-/-}$ Embryos and ESCs Exhibit Impaired Neuronal Differentiation

(A) Neurons in E10.5 wild-type and Elongin A$^{-/-}$ embryos were visualized by whole-mount immunostaining using anti-neurofilament antibody 2H3. Arrowheads indicate DRG. III, oculomotor nerve; V, trigeminal ganglion; VII, facial ganglion; VIII, vestibulocochlear ganglion; IX, glossopharyngeal nerve; X, vagal nerve; XI, accessory nerve. Scale bars, 500 μm.

(B) Immunostaining of transverse sections through the trigeminal region of E10.5 wild-type and Elongin A$^{-/-}$ embryos using anti-βIII-tubulin antibody. Arrowheads indicate trigeminal ganglion. Scale bars, 100 μm.

(C) Whole-mount in situ hybridization of E9.5–E10.5 wild-type and Elongin A$^{-/-}$ embryos using indicated cRNA probes. S, somites; OV, otic vesicle; IX, glossopharyngeal ganglion; X, vagal ganglion. Scale bars, 500 μm.

(D) EBs derived from Elongin A$^{+/+}$ and Elongin A$^{-/-}$ ESCs were grown in the absence or presence of RA. Paraffin sections were stained with anti-nestin or anti-βIII-tubulin antibody.

(E) Elongin A$^{+/+}$ ESCs and Elongin A$^{-/-}$ ESCs were subjected to 4−/4+ protocol for EB formation, and dissociated EBs were plated on a poly-L-lysine/gelatin-coated chamber slide for neuronal differentiation. Cells were fixed and double stained with anti-βIII-tubulin and anti-MAP2 antibodies. Total cells were viewed by staining of nuclei with DAPI. The corresponding phase-contrast images are also shown.

(F and G) TUNEL analysis (F) and staining with anti-Ki67 antibody (upper panels) and with hematoxylin (lower panels) (G) of sections of Elongin A$^{+/+}$ and Elongin A$^{-/-}$ EBs.

See also Figure S1.

RESULTS

Elongin A$^{-/-}$ Embryos Exhibit Severe Abnormalities in Both the Cranial and Spinal Nerves

We have previously shown that Elongin A$^{-/-}$ embryos die between days 10.5 and 12.5 of gestation. Elongin A$^{-/-}$ embryos exhibit poor development of the CNS accompanying significantly reduced thickness and cellularity of the cranial and spinal neuroepithelium (Miyata et al., 2007). In addition, some but not all Elongin A$^{-/-}$ embryos are smaller and developmentally delayed in comparison to their wild-type or Elongin A$^{+/-}$ littermates.

To characterize further the neural defects in Elongin A$^{-/-}$ embryos, we evaluated embryonic nervous system development in size- and stage-matched wild-type and Elongin A$^{-/-}$ embryos via whole-mount immunostaining of neurofilaments at E10.5. As shown in Figure 1A, the oculomotor nerve (III) was missing in the Elongin A$^{-/-}$ embryos. The formation of the trigeminal (V) and facial/vestibulocochlear (VII/VIII) ganglia, and the neurite outgrowth of glossopharyngeal (IX), vagal (X), and accessory (XI)

transcript elongation (Svejstrup, 2007), it remains unclear which activity of Elongin A, transcriptional elongation or Rpb1 ubiquitylation, in fact plays a more crucial role in gene expression in vivo. In this study, we demonstrate that Elongin A$^{-/-}$ mouse embryos and Elongin A$^{-/-}$ embryonic stem cells (ESCs) exhibit impaired neuronal development and differentiation, respectively. Furthermore, we show that Elongin A mutants that are defective in Rpb1 ubiquitylation but retain the ability to stimulate transcript elongation can rescue neuronal differentiation and retinoic acid (RA)-induced upregulation of a subset of neurogenesis-related and other genes in Elongin A$^{-/-}$ ESCs. Taken together, our results suggest that the elongation stimulatory activity of mammalian Elongin A plays a crucial role in the timely expression of a subset of genes required for neuronal development.

nerves were markedly impaired in Elongin A$^{-/-}$ embryos compared to wild-type embryos. In addition, outgrowth of spinal nerves and dorsal root ganglia (DRG) was severely disrupted in Elongin A$^{-/-}$ embryos. To clarify the mechanism underlying these defects, cell proliferation and apoptosis in embryos were examined by phospho-Histone H3 staining and cleaved caspase-3 and TUNEL staining, respectively. The trigeminal ganglia of Elongin A$^{-/-}$ embryos were much smaller compared with those of wild-type littermates, as indicated by the expression of βIII-tubulin, a representative marker of mature neurons (Figure 1B). No significant difference in phospho-Histone H3 staining was observed in the trigeminal ganglia between wild-type and Elongin A$^{-/-}$ embryos, whereas an increase in the number of both cleaved caspase-3^{+} and TUNEL^{+} cells was only observed in Elongin A$^{-/-}$ embryos (Figures S1A and S1B), indicating that these defects are not due to a decrease in proliferation but are at least in part due to an increase in cell death. Similar observations were made for facial/vestibulocochlear, glossopharyngeal, and vagal ganglia (data not shown).

Cranial ganglia are formed by contribution of two ectodermal-origin cell types, neural crest and ectodermal placodes, whereas DRG are formed exclusively from trunk neural crest cells. In an effort to determine whether the neurons of neural crest origin or of placodal origin are differentially affected in the cranial ganglia of Elongin A$^{-/-}$ embryos, we examined the expression of neural crest and placode lineage-specific marker genes at appropriate stages. We analyzed *Sox10* expression at E9.5 (23 somites), a time point when *Sox10* is specifically expressed in neurogenic neural crest cells, in wild-type and Elongin A$^{-/-}$ embryos. Expression of *Sox10* was markedly decreased in the cranial ganglia and DRG of Elongin A$^{-/-}$ embryos (Figures 1C and S1C), suggesting a role for Elongin A in migration and/or differentiation of neurogenic neural crest cells both in cranial and trunk regions.

In contrast to neural crest cells, ectodermal placodes start as a common thickened primordium of ectodermal origin that then segregates into various placodal territories with the help of surrounding signals (Schlosser, 2006). The time course of placode formation correlates with sequential expression of marker genes specifying each differentiation step. Descendants of *Pax8*-expressing cells in both the otic and epibranchial placode regions (Figure 1C) give rise to the vestibulocochlear and geniculate (distal facial) ganglia and their cranial nerves (Bouchard et al., 2004). We used *Pax8* expression as a marker for the forming otic and epibranchial placodes. Comparable expression patterns of *Pax8* were observed in the coalescing epibranchial placodes in wild-type and Elongin A$^{-/-}$ embryos (Figures 1C and S1C), suggesting that loss of Elongin A does not affect specification of otic and epibranchial placodes.

Neurogenin1, *Neurogenin2*, and *NeuroD1* encode proneural basic-helix-loop-helix (bHLH) transcription factors that are required for sensory neurogenesis (Bertrand et al., 2002). *Neurogenin1* is required for development of neurons of neural crest, trigeminal, and otic placodal origin, whereas *Neurogenin2* is required for that originating from epibranchial placodes (Fode et al., 1998; Ma et al., 1998). Expression of *Neurogenin1* in the trigeminal ganglia and trunk neural tube was significantly decreased; however, only a slight decrease of the *Neurogenin2* expression in the geniculate and petrosal (distal glossopharyngeal) ganglia was observed in Elongin A$^{-/-}$ embryos (Figures 1C and S1C). *NeuroD1*, a downstream transcriptional target of *Neurogenins* that is typically expressed in ganglionic cells (Fode et al., 1998; Ma et al., 1998), was obviously reduced in the trigeminal ganglia of Elongin A$^{-/-}$ embryos (Figures 1C and S1C), suggesting that the total number of neurons in these regions was decreased in Elongin A$^{-/-}$ embryos. Expression of *Mash1*, which encodes a proneural bHLH factor required for autonomic but not sensory neurogenesis (Bertrand et al., 2002), was, however, unchanged between wild-type and Elongin A$^{-/-}$ embryos (Figure 1C). Taken together, the observations that (1) in Elongin A$^{-/-}$ embryos, formation of cranial ganglia and DRG was severely impaired, and (2) expression of *Sox10* and *Neurogenin1* in those regions was obviously affected by the loss of Elongin A suggest the possibility that Elongin A plays a critical role in the development of the sensory nervous system at least in part by regulating the differentiation processes of neural crest cells.

Elongin A$^{-/-}$ ESCs Display Impaired Ability to Differentiate into Neurons

We next tested the ability of Elongin A$^{-/-}$ ESCs to form neuronal cell types. Elongin A$^{+/+}$ and Elongin A$^{-/-}$ ESCs were grown in suspension culture for 4 days without leukemia inhibitory factor (LIF) and for an additional 4 days with RA (4−/4+ protocol) to induce embryoid body (EB) formation (Figure S1D). As shown in Figures 1D and S1E, the expression of βIII-tubulin and of Nestin, a marker characteristic of neural progenitor cells, but not of glial fibrillary acidic protein (GFAP) or 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNPase), which are markers of astrocytes and oligodendrocytes, respectively, was significantly increased upon RA treatment in Elongin A$^{+/+}$ EBs. In contrast, expression of Nestin and βIII-tubulin was almost unaltered by RA in Elongin A$^{-/-}$ EBs. Dissociated EBs were also analyzed 3 days later for their morphology and for expression of βIII-tubulin and microtubule-associated protein 2 (MAP2), a marker of postmitotic neurons. The Elongin A$^{+/+}$ cultures displayed characteristic neuronal morphology: some cells were aggregated with each other, and their cell bodies and many dendrites were stained strongly with both βIII-tubulin and MAP2 (Figure 1E, upper panels), whereas almost none of the Elongin A$^{-/-}$ cells showed such characteristics (Figure 1E, lower panels). Cell proliferation and apoptosis in EBs were also examined by Ki67 staining and TUNEL assays, respectively. No significant difference in the number of TUNEL^{+} cells was observed between Elongin A$^{+/+}$ and Elongin A$^{-/-}$ EBs in the absence or presence of RA (Figure 1F), whereas an RA-induced increase in the number of Ki67^{+} cells was only observed in Elongin A$^{+/+}$ EBs (Figure 1G). Together, these results indicate that Elongin A makes a significant contribution to RA-induced neuronal differentiation of ESCs, most likely by promoting an early stage of the differentiation cascade.

Elongin A Is Involved in RA-Induced Expression of a Subset of Neurogenesis-Related Genes

To identify potential Elongin A targets during neuronal differentiation of ESCs, a cDNA microarray analysis comparing Elongin

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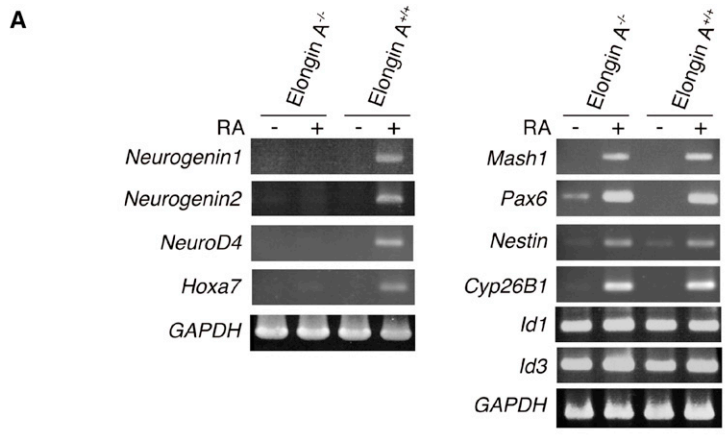

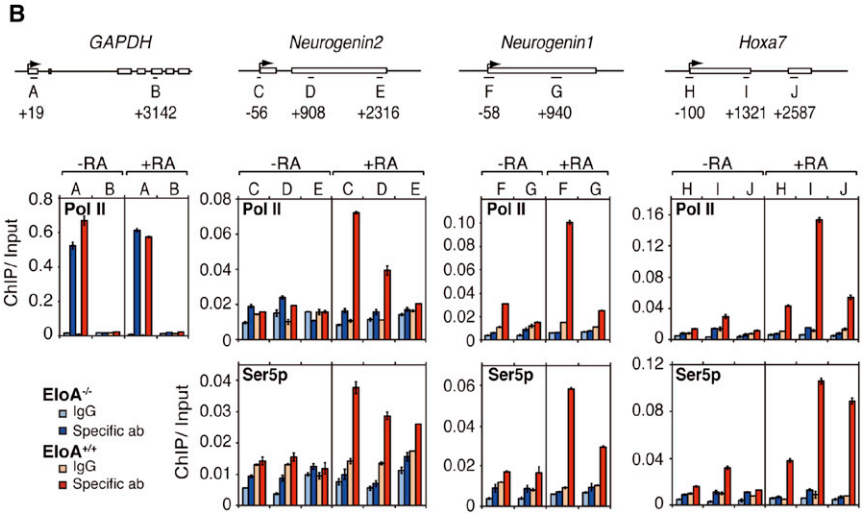

Figure 2. Elongin A Is Involved in RA-Induced Expression of a Subset of Neurogenesis-Related Genes

(A) Elongin $A^{-/-}$ ESCs and Elongin $A^{+/+}$ ESCs were subjected to 4−/4+ protocol for EB formation and were analyzed for the expression of various genes indicated in the figure by semiquantitative RT-PCR. See also Table S3.

(B) Effect of Elongin A on pol II occupancy on the *Neurogenin1*, *Neurogenin2*, *Hoxa7*, or *GAPDH* gene in the presence or absence of RA induction in EBs. Ig, IgG control, Specific ab, specific antibody. ChIP/Input is average from two biological replicates; error bars show data range. See also Table S4.

$A^{-/-}$ and Elongin $A^{+/+}$ EBs was performed. Of all the genes analyzed, 540 are known to be functionally related to neurogenesis, out of which 46 (8.5%) were significantly downregulated in Elongin $A^{-/-}$ EBs (Table S1). Moreover, a Gene Ontology (GO) analysis of the list of downregulated transcripts revealed a significant enrichment for genes whose products are involved in processes of nervous system development, such as neuron projection morphogenesis and development, axonogenesis, cell morphogenesis involved in neuronal differentiation, and neuronal development and differentiation (Table S2). These findings are consistent with the neuronal defects observed in vivo in Elongin $A^{-/-}$ embryos.

To verify the results of the microarray analysis and to confirm that the defect in RA-induced upregulation of these genes is truly dependent on the loss of Elongin A, Elongin $A^{-/-}$ and Elongin $A^{+/+}$ ESCs were subjected to 4−/4+ protocol for EB formation and analyzed for the expression of mRNA by semiquantitative RT-PCR. As shown in Figure 2A, RA-induced expression of *Neurogenin1*, *Neurogenin2*, *NeuroD4*, and *Hoxa7* was significantly impaired only in Elongin $A^{-/-}$ EBs. Whereas Neurogenins are involved in initiating neurogenesis, NeuroD4 is involved in mediating terminal differentiation (Bertrand et al., 2002). *Hoxa7* is a member of a paralogous group of homeobox-containing gene that controls region-specific differentiation during development and plays a regulatory role in CNS patterning and cell specification (Akin and Nazarali, 2005). In contrast, RA-induced expression of *Mash1*, *Pax6*, a paired homeodomain transcription factor gene required for neurogenesis (Simpson and Price, 2002), *Nestin*, and *Cyp26B1*, which encodes a cytochrome-P450 enzyme that catabolizes RA (Maden, 2002), was normal in both Elongin $A^{-/-}$ and Elongin $A^{+/+}$ EBs. Similarly, the expression of *Id1* and *Id3*, which code for negative HLH factors that inhibit the transcriptional activity of neurogenic bHLH transcription factors (Bertrand et al., 2002) and are not RA regulatory targets, was normal in Elongin $A^{-/-}$ EBs.

We then analyzed pol II occupancy on the *Neurogenin1*, *Neurogenin2*, and *Hoxa7* genes in EBs in the presence or absence of RA treatment using chromatin immunoprecipitation (ChIP). Very little pol II (either total or a form of pol II phosphorylated on Ser5 of the CTD) could be detected on the *Neurogenin2*, *Neurogenin1*, and *Hoxa7* genes in the absence of RA in Elongin $A^{+/+}$ and Elongin $A^{-/-}$ EBs. The amount of both total and Ser5-phosphorylated pol II detected in the promoters and the bodies of these genes was dramatically increased in the presence of RA in Elongin $A^{+/+}$ EBs, but not in Elongin $A^{-/-}$ EBs (Figure 2B). Taken together, these findings indicate that Elongin A contributes to RA-induced transcription of a subset of genes functionally related to neurogenesis.

Identification of Elongin A Mutants that Differentially Affect E3 Ubiquitin Ligase or Transcription Elongation Activities

Immediately downstream of its BC box, Elongin A contains sequences similar to the canonical Cul5 box consensus ϕxxLPϕ Pxxϕxx[Y/F][L/I], which in other BC box proteins has been shown to specify assembly into Cul5-based ubiquitin ligases (Mahrour et al., 2008) (Figure 3A). We have previously shown

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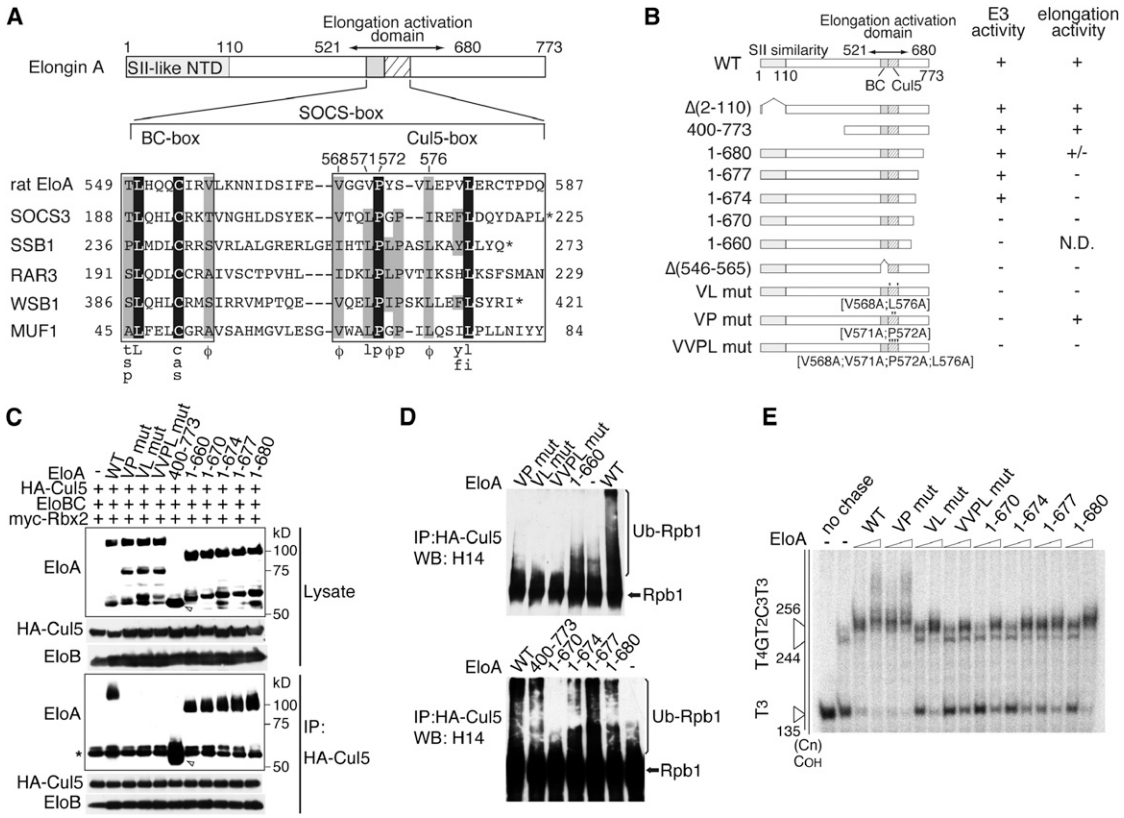

Figure 3. Identification of Elongin A Mutations that Selectively Inactivate Either Its Rpb1 Ubiquitylation or Transcriptional Elongation Activities

(A) Alignment of amino acid sequences of SOCS box proteins. The BC box and Cul5 box are indicated (boxed). Identical (black) and similar (gray) amino acids are shaded. Asterisks indicate C termini. Sequences are of the human proteins with the exception of Elongin A. Sequences of the SOCS box of Elongin A are perfectly conserved between rat and human and correspond to amino acids 550–588 in human Elongin A. The canonical BC box and Cul5 box sequences are indicated below each alignment. NTD, N-terminal domain.

(B) N-terminal, C-terminal, and Cul5 box point mutants of Elongin A analyzed in this study. On the right, the results of the assays shown in (D) and (E) are presented. Some of results were originated from previously published work (Aso et al., 1996; Yasukawa et al., 2008). N.D., not determined; WT, wild-type. VP mut, VL mut, and VVPL mut represent Elongin A[V571A;P572A], Elongin A[V568A;L576A], and Elongin A[V568A;V571A;P572A;L576A], respectively.

(C) Sf9 cells were coinfected with the baculoviruses indicated in the figure. Anti-HA immunoprecipitation was performed as described under Experimental Procedures. Total cell lysates and anti-HA immunoprecipitates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Arrowheads indicate Elongin A(400–773). The asterisk indicates the IgG heavy chain. IP, immunoprecipitation.

(D) Lysates from Sf9 cells infected with the indicated baculoviruses were immunoprecipitated with anti-HA antibody and were assayed for their ability to activate Rpb1 ubiquitylation as described under Experimental Procedures. Samples were analyzed on 6% SDS-PAGE and processed for western blotting (WB) with H14 antibody.

(E) The oligo (dC)-tailed template assays were performed as described under Experimental Procedures in the absence or presence of wild-type and indicated Elongin A mutants complexed with Elongin BC. The concentration of Elongin complexes was adjusted so that Elongin A was present in reaction mixtures at either 6 or 18 nM.

that amino acid sequences between residues 521 and 680 of Elongin A, which include both the BC box and Cul5 box consensus sequences, are required for both Rpb1 ubiquitylation and transcriptional elongation activities (Aso et al., 1996; Yasukawa et al., 2008). To investigate the possibility that these two activities are separable by further mutagenesis, we constructed three Cul5 box point mutants and four more C-terminal deletion mutants of Elongin A. Elongin A[V571A;P572A] (Elongin A-VP), Elongin A[V568A;L576A] (Elongin A-VL), and Elongin A[V568A;V571A;P572A;L576A] (Elongin A-VVPL) contain alanine substitutions of predicted key Cul5 box residues. Elongin A(1–677), Elongin A(1–674), Elongin A(1–670), or Elongin A(1–660) each contain additional small C-terminal deletions that we expected might affect both activities (Figure 3B).

Sf9 cells were coinfected with various combinations of baculoviruses encoding Elongins B and C, myc-Rbx2, HA-Cul5, and Flag-tagged wild-type or mutant Elongin A. Cul5-containing ubiquitin ligase complexes were immunoprecipitated from cell lysates with anti-HA antibody and analyzed for (i) the relative amount of Elongin A in each complex by immunoblotting and (ii) their abilities to activate Rpb1 ubiquitylation in vitro. As shown in Figures 3B and 3C, the Elongin A Cul5 box mutants Elongin A-VP, Elongin A-VL, and Elongin A-VVPL were severely impaired in their abilities to assemble into ubiquitin ligase complexes.

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Consistent with this observation, Cul5 complexes from cells expressing these Elongin A mutants failed to activate Rpb1 ubiquitylation above the low background level observed in the absence of Elongin A. Furthermore, the C-terminal deletions Elongin A(1–670) and Elongin A(1–660), which can assemble into Cul5 complexes, also failed to activate Rpb1 ubiquitylation above background. In contrast, the remaining C-terminal deletion mutants, Elongin A(1–677) and Elongin A(1–674), preserved levels of ubiquitylation activity similar to that of wild-type Elongin A (Figures 3B and 3D).

The relative transcriptional elongation activities of the wild-type and mutant Elongin As complexed with Elongin BC were then compared using the oligo(dC)-tailed template assay. In the experiment shown in Figure 3E, the ~135-nucleotide transcripts were chased for 4.5 min with a limiting concentration of UTP in the presence or absence of equivalent amounts of purified Elongin ABC complexes. Elongin ABC complexes containing Elongin A mutants Elongin A-VL, Elongin A-VVPL, Elongin A(1–677), Elongin A(1–674), and Elongin A(1–670) were profoundly impaired in their abilities to stimulate elongation by pol II. However, Elongin ABC complexes containing Elongin A-VP exhibited a level of activity comparable to that of the wild-type Elongin ABC complex.

The Elongin A Transcriptional Elongation, but Not Its Rpb1 Ubiquitylation, Activity Is Required for Elongin A-Dependent Neuronal Differentiation and Gene Regulation

To examine whether the reintroduction of Elongin A into Elongin $A^{-/-}$ ESCs is able to rescue their neuronal differentiation, and if so, which domain of Elongin A is required for this activity, we transfected cDNAs encoding wild-type and various mutant Elongin As into Elongin $A^{-/-}$ ESCs (Figure 4A). These ESC clones were subjected to 4−/4+ protocol for EB formation and tested for RA-inducible expression of the neuronal marker βIII-tubulin. As shown in the western blots of Figure 4B, neither Elongin A [Δ(546–565)], which lacks its BC box and is defective in both transcriptional elongation and E3 ubiquitin ligase activities (Aso et al., 1996; Yasukawa et al., 2008), nor Elongin A(1–674) was able to rescue RA-induced expression of the neuronal marker βIII-tubulin. In contrast, RA-induced βIII-tubulin expression was restored in cells expressing the ubiquitylation-defective mutant Elongin A-VP.

In further experiments, we tested the ability of wild-type and mutant Elongin As to rescue RA-induced expression of *Neurogenin1*, *Neurogenin2*, *NeuroD4*, and *Hoxa7* mRNAs in EBs derived from Elongin $A^{-/-}$ ESCs. As shown in Figure 4C, a comparable level of RA-induced expression of these four genes was observed in EBs derived from ESCs transfected with wild-type Elongin A and Elongin A-VP, but not in EBs derived from ESCs transfected with Elongin A[Δ(546–565)] and Elongin A(1–674). In contrast, the expression of *Cyp26B1* was induced by RA regardless of the transfected Elongin A, and that of *Id1* was almost unaltered regardless of either the transfected Elongin A or RA treatment.

Taken together, these results argue that (1) mammalian Elongin A contributes to neuronal differentiation in ESCs, at least in part by regulating RA-induced expression of genes such as *Neurogenin1*, *Neurogenin2*, *NeuroD4*, and *Hoxa7*, and (2) Elongin A transcriptional elongation activity, but not its E3 ubiquitin ligase activity, is critical for these functions.

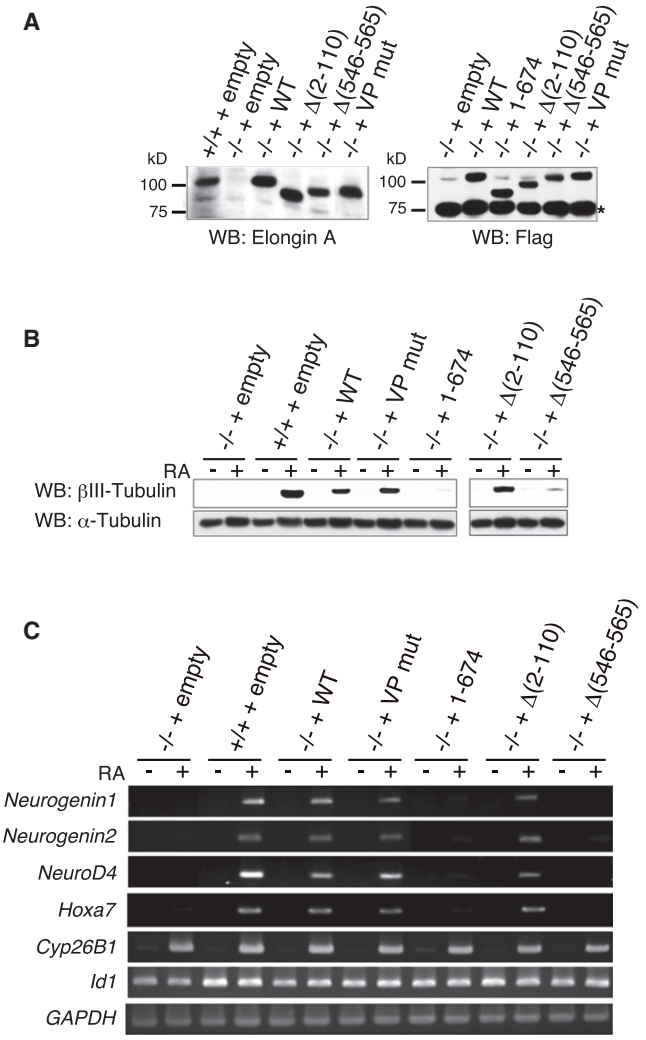

Figure 4. The Elongin A Transcriptional Elongation, but Not Its Rpb1 Ubiquitylation, Activity Is Required for Elongin A-Dependent Neuronal Differentiation and Gene Regulation

(A) Expression levels of Elongin A in Elongin $A^{+/+}$ ESCs or Elongin $A^{-/-}$ ESCs transfected with expression vectors encoding wild-type or various mutant Elongin A were analyzed by western blotting with anti-Elongin A (left panel) or anti-Flag antibody (right panel). VP mut represents Elongin A[V571A;P572A]. The asterisk indicates nonspecific bands.

(B and C) Various ESC clones in (A) were subjected to 4−/4+ protocol for EB formation and were analyzed for the expression of βIII-tubulin or α-tubulin by western blotting (B) and for the expression of genes indicated in the figure by semiquantitative RT-PCR (C).

DISCUSSION

RA signaling plays a key role in vertebrate embryogenesis. The embryonic distribution of RA correlates with neuronal differentiation and positional specification in the developing CNS (Maden, 2002). RA is also an important extrinsic inductive signal that can be used to induce neuronal differentiation of mouse ESCs in vitro

(Bain et al., 1995). Hundreds of genes have been shown to be regulated by RA during the processes of neuronal differentiation and neurite outgrowth, including those encoding transcription factors, extracellular proteins, neurotransmitters, growth factors, and cell surface receptors (Maden, 2002). Here, we have shown that (1) Elongin $A^{-/-}$ embryos exhibit reduced number in subpopulations of neural progenitors and differentiated neurons throughout the peripheral nervous system, (2) RA-induced neuronal differentiation is severely impaired in Elongin $A^{-/-}$ ESCs, and (3) Elongin A is required for transcription of a subset of RA-induced genes with roles in neuronal development and differentiation.

How might Elongin A participate in RA-induced pol II transcription? Our rescue experiments demonstrate that a functional Elongin A elongation activation domain is needed to rescue the neuronal differentiation and gene expression defects of Elongin $A^{-/-}$ ESCs; however, an Elongin A mutant that does not assemble into Cul5-containing ubiquitin ligase complexes and hence does not support Rpb1 ubiquitylation activity can still rescue these defects. Although ubiquitylation of pol II was originally detected as a response to DNA damage (Bregman et al., 1996), more recent results suggest that pol II ubiquitylation during transcript elongation is a frequent event that can occur in the absence of DNA damage; indeed, pol II ubiquitylation is now thought to be a general response to paused or arrested elongation complexes (Svejstrup, 2007). Therefore, Rpb1 ubiquitylation evoked by Elongin A and/or other ubiquitin ligases has been proposed to be a mechanism that ensures the overall efficiency of transcript elongation either by (1) reactivating the elongation complexes by recruiting 19S subparticle of the proteasome, which in turn stimulates transcription elongation (Ferdous et al., 2001), or (2) removing paused or arrested pol II from the DNA by proteasome-mediated degradation, thereby enabling the next pol II to transcribe the gene (Svejstrup, 2007).

Our results, however, are most consistent with the model that the elongation stimulatory activity of Elongin A, but not Elongin A-dependent pol II ubiquitylation, is required for production of full-length transcripts essential to rescue the neuronal differentiation defect of Elongin $A^{-/-}$ ESCs. In this scenario, the requirement for the Rpb1 ubiquitylation activity of Elongin A might be gene specific and would depend on the nucleotide sequences of the genes and, perhaps, on the structure and number of sites causing pausing or arrest of pol II. Importantly, our observations do not rule out the possibility that Rpb1 ubiquitylation might have a more general role because pol II is likely to be targeted for ubiquitylation by more than one E3 ubiquitin ligase in metazoan cells (Svejstrup, 2007; Yasukawa et al., 2008; Harreman et al., 2009).

Recent genome-wide studies have demonstrated that a large number of developmentally regulated genes contain paused pol II at their promoters and that a significant fraction of these encode transcription factors involved in neurogenesis and ectodermal development or components of cell signaling pathways (Zeitlinger et al., 2007). Thus, it is now believed that the transition of pol II from the promoter-proximal early to the productive elongation stage of transcription is a key regulatory step that facilitates rapid temporal and spatial changes in gene expression during differentiation and development in metazoans.

Although we have not yet succeeded in generating antibodies suitable for ChIP of Elongin A in mouse ESCs, Gerber et al. have reported that the *Drosophila* ortholog of Elongin A colocalizes with pol II at sites of active transcription on polytene chromosomes and that it is required, not only for proper metamorphosis, but also for heat shock gene expression (Gerber et al., 2004, 2005). More recently, Chopra et al. (2009) have presented evidence that in *Drosophila* embryos, the elongation factors P-TEFb, which relieves DSIF- and NELF-dependent pol II pausing (reviewed in Conaway et al., 2000; Shilatifard et al., 2003), and Elongin A are essential for optimal expression of at least a subset of *Drosophila Hox* genes and that mutations in the CDK9 subunit of P-TEFb and Elongin A exhibited additive effects particularly on the reduction in *Ultrabithorax* activity. Taken together with the findings reported here, these observations raise the possibilities that (1) Elongin A is a limiting factor for optimal expression of a subset of genes that undergo transient high-level expression in response to external signals and/or stimuli, and (2) Elongin A might function together with other elongation factors such as P-TEFb in a nonredundant, gene-specific manner during transcription.

Although our data implicate Elongin A's transcription elongation activity in RA-induced activation of transcription of a subset of neurogenic genes, the precise biochemical mechanism by which Elongin A participates in RA-induced transcriptional activation remains to be determined. Our ChIP data indicate that loss of Elongin A dramatically decreases pol II occupancy at both the transcribed regions and the promoters of several genes. We cannot rule out the possibility that these changes are secondary to Elongin A-dependent control of other transcriptional regulatory factors; however, an alternative possibility is that Elongin A not only contributes to elongation control but also affects transcription by enhancing pol II recruitment and/or stability of very early transcription complexes. Interestingly, results of a recent study support just such a role for another transcription elongation factor, eleven-nineteen lysine-rich in leukemia (ELL) (Byun et al., 2012).

In summary, our results argue that the transcription elongation activity of mammalian Elongin A is required for neuronal differentiation and development and has a crucial role in RA-induced transcription by pol II of a subset of genes critical for neurogenesis. In the future, it will be of considerable interest to explore in more detail how Elongin A's elongation activity contributes to gene regulation and to determine why some, but not all, RA-inducible genes are particularly dependent on Elongin A for their activation.

EXPERIMENTAL PROCEDURES

ESC Culture, Cell Lines, and Differentiation of ESCs

ESC culture and generation of Elongin $A^{+/+}$ and Elongin $A^{-/-}$ ESC clones expressing wild-type or mutant Elongin A were as described (Yasukawa et al., 2008). EB formation was carried out essentially as described by Bain et al. (1995).

Immunocytochemistry, Histological Analyses, RNA In Situ Hybridization, and ChIP

Immunocytochemistry, histological analyses, RNA in situ hybridization, and ChIP were performed using standard methods. See Extended Experimental Procedures for details. All animal care and experiments conformed to the Guidelines for Animal Experiments of Kochi University and were approved by the Animal Research Committee of Kochi University.

Production of Recombinant Proteins, Immunoprecipitation, Western Blotting, and Assays of Ubiquitylation and Transcription In Vitro

Expression of recombinant proteins in *E. coli* and Sf9 cells, immunoprecipitation, immunoblotting, and in vitro ubiquitylation and transcription were performed essentially as described (Aso et al., 1995; Yasukawa et al., 2008) and are detailed in the Extended Experimental Procedures.

RT-PCR, Microarray, and GO Analyses

RT-PCR and microarray analyses were performed essentially as described by Miyata et al. (2007) and are detailed in the Extended Experimental Procedures. Primer sets used are listed in Table S3. The GO analysis of biological processes was performed using the DAVID Bioinformatics Database (http://david.abcc.ncifcrf.gov).

ACCESSION NUMBERS

The GEO accession number for the microarray data reported in this paper is GSE30911.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, one figure, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.09.031.

LICENSING INFORMATION

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EXTENDED EXPERIMENTAL PROCEDURES

ESC Culture and Cell Lines

Elongin $A^{-/-}$ ESCs were generated from CCE ESCs as described earlier (Yamazaki et al., 2003). Elongin $A^{+/+}$ and Elongin $A^{-/-}$ ESC clones expressing wild-type or mutant Elongin A were generated as described by transfections of pCAG-IPG empty vector into CCE ESCs and Flag-tagged wild-type or mutant Elongin A in pCAG-IPG vector into Elongin $A^{-/-}$ ESCs, respectively (Yasukawa et al., 2008). ESCs were cultured on gelatin-coated dishes in the absence of feeder cells in ES medium; DMEM (Wako) supplemented with 20% fetal bovine serum (FBS), glutamine, non-essential amino acids, antibiotics, 100 mM β-mercaptoethanol, and recombinant LIF (Wako), as described (Yamazaki et al., 2003). Cells were collected and lysed in ice-cold buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM DTT, 0.5% Triton X-100, 10% glycerol, and a Complete Protease Inhibitor Cocktail (Roche Applied Science), and centrifuged at 10,000 g for 20 min at 4°C.

Differentiation of ESCs

For EB formation, ESCs were subjected to an 8-day induction procedure which consisted of 4 days of culture as aggregates without All-*trans*-retinoic acid (RA) (Wako) followed by 4 days of culture in the presence of RA (4-/4+ protocol) as described by Bain et al. (1995). In brief, ESCs were detached and dissociated into single cells with 0.25% trypsin-EDTA and then plated onto a bacteriological dish in ES medium without LIF at a density of 5×10^4 cells/ml. RA was added at 2 μM at day 4. EBs were collected at day 8 of culture and allowed to settle to the bottom of the tube for a few minutes. The cells were then washed once with phosphate-buffered saline (PBS) and incubated with 0.25% trypsin-EDTA for 5 min at 37°C. Trypsin was inactivated by addition of an equal volume of ES medium without LIF, and the dissociated cells were resuspended in DMEM/F12 (1:1) (Wako) containing 15% FBS and N2 (Invitrogen). The cells were plated on poly-L-lysine/gelatin-coated 4-well chamber slides at a density of 8.5×10^5 cells/1.7 cm^2.

Immunocytochemistry and Histological Analyses

For the whole-mount immunostaining, E10.5 embryos were fixed overnight in 4% paraformaldehyde at 4°C, and incubated in methanol containing 6% hydrogen peroxide to inactivate endogenous peroxidase. The samples were subsequently treated with DMSO/methanol and 2.5% Triton X-100, and then washed with TST (Tris-HCl buffered saline: 20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% Triton X-100). Next they were blocked with 5% non-fat dry milk in TST (TSTM), and afterward incubated with 2H3 anti-neurofilament monoclonal antibody (Developmental Hybridoma Bank) in TSTM for 2 days at 4°C. After washing with TST, the samples were incubated overnight at 4°C with peroxidase-conjugated goat anti-mouse IgG (GE Healthcare). Finally, they were washed with TST, pre-incubated with TST containing DAB (250 μg/ml) for 1 hr, and then reacted with the same concentration of DAB/TST containing hydrogen peroxide (0.003%) at 0°C for 15 min. The reaction was stopped by rinsing the samples with TST.

Dissociated EBs were cultured for 72 hr and fixed for 20 min in 2% paraformaldehyde/0.2% glutaraldehyde at room temperature (RT). The cells were rinsed with PBS twice and permeabilized with PBS containing 0.5% Triton X-100 for 20 min at RT and blocked in PBS containing 3% BSA for 1 hr at RT. Cells were then incubated for 2 hr at RT with anti-βIII-tubulin (TUJ1) (Covance) and anti-MAP2 Ab-2 (Lab Vision Corporation) antibodies. After washing with PBS three times, the cells were incubated for 2 hr at RT with secondary antibodies conjugated with Alexa 488 or Alexa 594 (Molecular Probes). For staining DNA, cells were treated with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) from Sigma. Slides were mounted in SlowFade (Molecular Probes) and examined with a universal fluorescence microscope (TE2000-S; Nikon).

E9.5-E10.5 embryos or EBs were fixed overnight in 4% paraformaldehyde at 4°C, dehydrated by using a graded series of ethanol washes, and embedded in paraffin. Sectioning of embryos stained in whole-mount was performed following a 1 hr postfixation in 4% paraformaldehyde at 4°C, impregnation of embryos with 20% sucrose/PBS, and mounting in OCT (Tissue-Tek). Immunostaining of sections was performed using following primary antibodies: anti-neurofilament (2H3), anti-Nestin (Chemicon); anti-βIII-tubulin (TUJ1), anti-GFAP (DakoCytomation), anti-CNPase (Sigma), anti-cleaved caspase-3 (Cell Signaling), anti-phospho-Histone H3 (Millipore) and anti-Ki67 nuclear antigen (YLEM). Peroxidase-conjugated anti-mouse or anti-rabbit antibody (GE Healthcare) or Alexa 488- or Alexa 594-conjugated anti-rabbit antibody were used as secondary antibodies. TUNEL assays were performed on cryosections of embryos or paraffin sections of EBs using the In situ Cell Death Detection kit (Roche Applied Science) according to the manufacturer's instructions. For staining DNA, sections were also treated with DAPI.

RNA In Situ Hybridization

E9.5-E10.5 embryos were fixed overnight in 4% paraformaldehyde at 4°C, dehydrated in methanol, and stored at −20°C in methanol until used in the staining protocol. Anti-sense digoxigenin-labeled mRNA riboprobes were synthesized for *Sox10, Neurogenin1, Neurogenin2, NeuroD1, Mash1,* and *Pax8* (gift from Dr. Bouchard). In situ hybridization for *Sox10, Neurogenin1, Neurogenin2* and *NeuroD1* were performed following a previously described standard protocol (Nagy et al., 2003) and for *Mash1* and *Pax8* were performed following a protocol described in Gammill et al., 2006.

ChIP

ChIP assays were performed essentially as described (Takahashi et al., 2011). EBs were cross-linked with 1% formaldehyde in PBS for 10 min at RT. EBs were resuspended and lysed in lysis buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA,

0.1% sodium deoxycholate, 0.5% N-lauroylsarcosine) containing a Complete Protease Inhibitor Cocktail and a PhosSTOP (Roche Applied Science), and were sonicated with a Bioruptor Sonicator (Cosmo Bio) for 50 × 30 s at the maximum power setting to generate DNA fragments of ~150-400 bps. Sonicated chromatin was incubated at 4°C overnight with 8-15 μg of normal IgG or specific antibodies. Pol II total Rpb1 (N-20, Santa Cruz) and Pol II Rpb1 CTD phospho Ser5 (Abcam) antibodies were used. Then, protein G Dynabeads (Invitrogen) was added and incubated for 2 hr at 4°C. Beads were washed 2 times with IP buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100), 2 times with high-salt buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 2 mM EDTA, 1% Triton X-100), 1 time with LiCl buffer (250 mM LiCl, 20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Triton X-100, 0.1% NP40, 0.5% sodium deoxycholate), and 2 times with TE buffer. Bound complexes were eluted from the beads with 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM DTT and 1% SDS by incubating at 50°C for 30 min with occasional vortexing. Crosslinking was reversed by overnight incubation at 65°C. Immunoprecipitated DNA and input DNA were treated with RNase A and Proteinase K by incubation at 45°C. DNA was purified using the QIAquick PCR purification kit (QIAGEN) or MinElute PCR purification kit (QIAGEN). Immunoprecipitated and input material was analyzed by quantitative PCR using SYBR Premix Ex Taq (Takara) on a StepOne PLUS (Applied Biosystems). ChIP signal was normalized to total input. Primer sets used are listed in Table S4.

Expression of Recombinant Proteins in Sf9 Insect Cells

A series of C-terminal deletion and Cul5-box point mutants of rat Elongin A, containing 3 × Flag tags at their C-termini, were subcloned into pBacPAK-His1. Recombinant baculoviruses were generated with the BacPAK baculovirus expression system (Clontech). Baculoviruses encoding wild-type rat Elongin A, rat Elongin B, rat Elongin C, human Cul5 containing an N-terminal HA tag, and mouse Rbx2 containing an N-terminal 3 × myc tag were described previously (Yasukawa et al., 2008). Baculovirus encoding Arapidopsis thaliana E1 with an N-terminal 6-histidine tag was provided by Dr. W. Krek.

Sf9 cells were cultured at 27°C in Grace's insect medium (GIBCO) with 10% FBS and kanamycin (100 μg/ml) and infected with the recombinant baculoviruses indicated in the figures. Seventy-two hours after infection, cells were collected and lysed in ice-cold buffer containing 40 mM HEPES-NaOH (pH 7.9), 150 mM NaCl, 1 mM DTT, 0.5% Triton X-100, 10% glycerol, and the Complete Protease Inhibitor Cocktail, and centrifuged at 10,000 g for 20 min at 4°C.

Expression of Recombinant Proteins in *Escherichia coli*

Human Ubc5a (hUbc5a) with an N-terminal 6-histidine tag and a C-terminal Flag tag was expressed in *E. coli* strain BL21 (DE3) and purified by Ni^{2+}–agarose chromatography as described previously (Yasukawa et al., 2008). After dialysis against 40 mM HEPES-NaOH (pH 7.9), 60 mM potassium acetate, 2 mM DTT, 5 mM $MgCl_2$, 0.5 mM EDTA, and 10% glycerol, proteins were stored at −80°C.

Immunoprecipitation and Western Blotting

Anti-Flag (M2) monoclonal antibody (Sigma), anti-HA (12CA5) monoclonal antibody (Roche Applied Science), anti-pol II monoclonal antibody (H14; Covance), anti-Elongin A and anti-Elongin B antibodies (Yasukawa et al., 2008) were used in immunoprecipitation and immunoblotting. Lysates of baculovirus-infected Sf9 cells were incubated with 3 μg of anti-HA (12CA5) antibody for 1 hr and then with protein A-Sepharose (GE Healthcare) for 1 hr at 4°C. Beads were washed five times in buffer containing 40 mM HEPES-NaOH (pH 7.9), 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.5% Triton X-100, 10% glycerol. Immunoprecipitated proteins or cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Immobilon-P membranes (Millipore), and analyzed by immunoblotting with the antibodies indicated in the figures. Immunoblots were visualized with either Western Lightning *Plus*-ECL (Perkin Elmer) or Immobilon Western (Millipore) according to the manufacturer's instructions.

Assay of Ubiquitylation In Vitro

Pol II was purified as described from rat liver nuclear extracts (Conaway and Conaway, 1990). To assay immunoprecipitated Elongin A complexes for their ability to activate Rpb1 ubiquitylation, Sf9 cells infected with the baculoviruses indicated in the figures were lysed as described above. After centrifugation at 10,000 g for 20 min at 4°C, the supernatants were immunoprecipitated with 3 μg of anti-HA (12CA5) antibody and 10 μl of protein A-Sepharose. The beads were mixed with ~100 ng of E1, ~100 ng of hUbc5a, 5 μg of bovine ubiquitin (Sigma), and an aliquot of purified pol II in a 20-μl reaction containing 20 mM Tris-HCl (pH 7.9), 2 mM DTT, 5 mM $MgCl_2$, 1.5 mM ATP, 10 mM creatine phosphate, 10 μg of creatine phosphokinase, and 5 mM NaF. Reaction mixtures were incubated for 30 min at 30°C. Reaction products were subjected to 6% SDS-PAGE and analyzed by Western blotting with H14 antibody.

Oligo(dC)-Tailed Template Assay of Elongation by RNA Polymerase II

Pol II was purified as described from rat liver nuclear extracts (Conaway and Conaway, 1990) and pulse-chase assays were carried out essentially as reported previously (Aso et al., 1995). Pol II (0.01 units) and 100 ng of pCpGR220S/P/X were incubated at 28°C in the presence of 20 mM HEPES-NaOH (pH 7.9), 20 mM Tris-HCl (pH 7.9), 2% polyvinyl alcohol, 0.5 mg/ml of bovine serum albumin, 60 mM KCl, 50 μM $ZnSO_4$, 7 mM $MgCl_2$, 0.2 mM DTT, 3% glycerol, 3 units of recombinant ribonuclease inhibitor, 50 μM ATP, 50 μM GTP, 2 μM CTP and 10 μCi [α-^{32}P]CTP. After 25 min labeling, 100 μM nonradioactive CTP, 2 μM UTP and the specified amounts of Elongin preparations were added, and the reactions were chased for 4.5 min. Transcripts were analyzed by electrophoresis through 6% polyacrylamide-7.0 M urea gels.

RT-PCR Analysis

Total RNA was isolated from EBs using a GenElute Mammalian Total RNA Miniprep kit (Sigma) according to the manufacturer's instructions. First strand cDNA was synthesized from 1 μg of total RNA, with oligo-dT primer and MuLV reverse transcriptase (Applied Biosystems). One-twentieth of each RT reaction product was used for PCR amplification. Primer sets are listed in Table S3.

Microarray Analysis

Total RNA was isolated from EBs using a RNeasy mini kit (QIAGEN) according to the manufacturer's instructions, and the quality was determined using an Agilent Bioanalyzer 2100 (Agilent Technologies). RNA (1 μg) was transcribed to DIG-labeled cRNA using an Applied Biosystems Chemiluminescent NanoAmp™ RT-IVT Labeling Kit (Applied Biosystems) according to the manufacturer's instructions. Microarray hybridization, processing, chemiluminescence detection, imaging, auto-gridding, and image analysis were performed according to Applied Biosystems protocols using the 1700 Chemiluminescent Microarray Analyzer software (Version 1.0.3). The Applied Biosystems Mouse Genome Survey Microarray, which contains approximately 34,000 features including a set of about 1000 controls, was used. Each microarray uses 32,996 probes targeted to 32,381 curated genes representing 44,498 transcripts.

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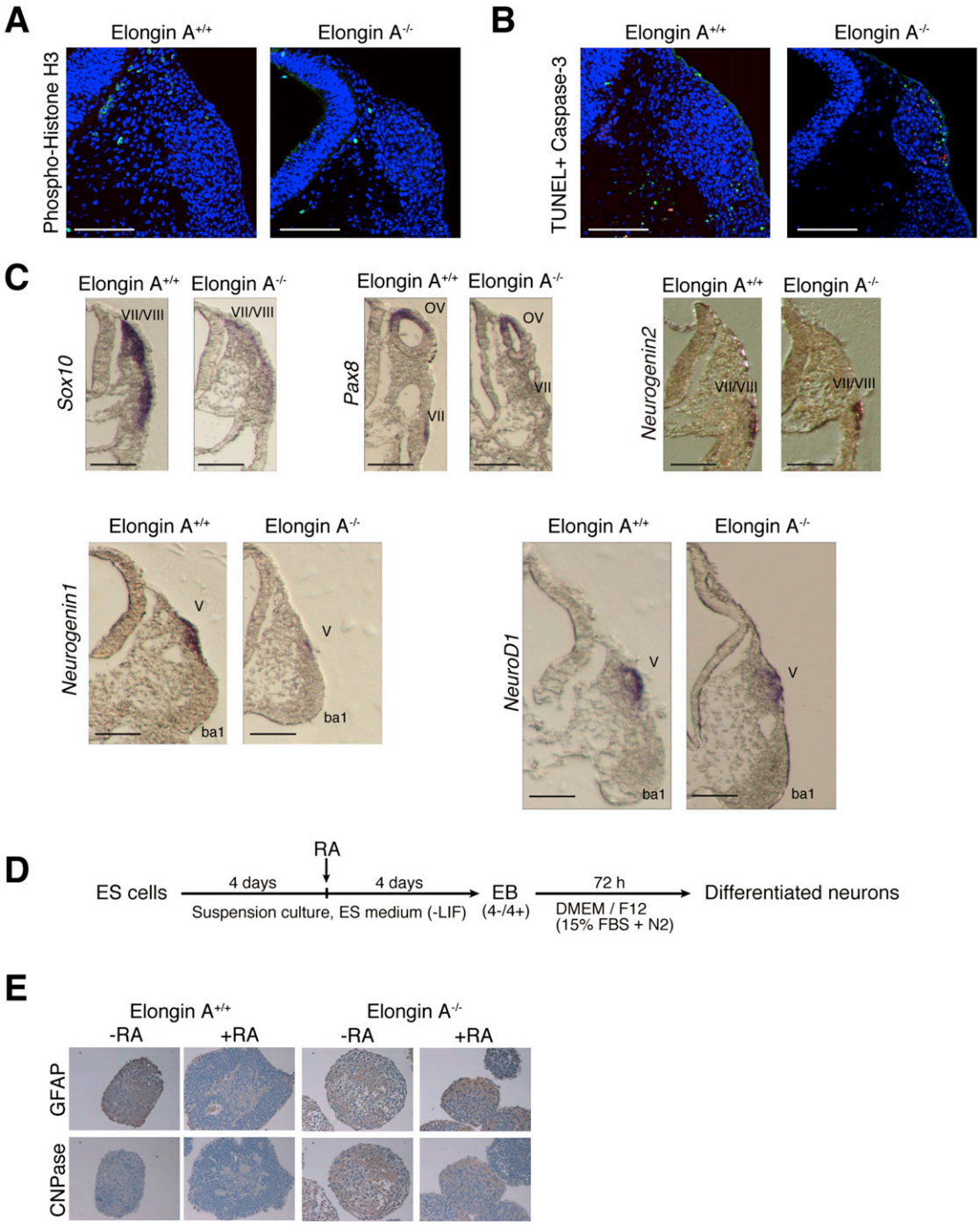

Figure S1. Phenotypic Analyses of Elongin A$^{-/-}$ Embryos and ESCs, Related to Figure 1

(A and B) Immunostaining with both anti-phospho-Histone H3 antibody (green) and DAPI (blue) (A) and staining with anti-cleaved caspase-3 antibody (red), TUNEL (green) and DAPI (blue) (B) of adjacent serial transverse sections through the trigeminal region of E10.5 wild-type and Elongin A$^{-/-}$ embryos. Bars = 100 μm.

(C) Transverse sections through the indicated regions of whole-mount in situ hybridization of E9.5-E10.5 wild-type and Elongin A$^{-/-}$ embryos using indicated cRNA probes. ba, branchial arch; OV, otic vesicle; V, trigeminal ganglion; VII, facial ganglion; VIII, vestibulocochlear ganglion. Bars = 100 μm.

(D) Experimental protocol for differentiation of ESCs with RA.

(E) EBs derived from Elongin A$^{+/+}$ and Elongin A$^{-/-}$ ESCs were grown in the absence or presence of RA. Paraffin sections were stained with anti-GFAP or anti-CNPase antibody.