Post-transcriptional regulation of neural stem cell fate by the RNaseIII Drosha

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Abstract

Stem cells are found in several organs where they are committed to differentiate into tissue specific somatic cells. In the developing and adult mammalian brain neural stem cells (NSCs) have the ability to differentiate into different cell types, the neurons and glia. NSCs differentiation is tightly regulated in order to ensure e.g. the correct formation of a six-layered isocortex during embryogenesis or in the adult to contribute to cognition. A major role in controlling NSC maintenance and differentiation plays post-transcriptional regulation. The RNaseIII Drosha, which is involved in miRNA biogenesis, was recently shown to directly inhibit specific mRNAs in a non-canonical, miRNA-independent manner, thereby controlling stem cell maintenance. It remained elusive if the non-canonical function of Drosha is also involved in cell fate decisions. During my PhD I investigated the role and requirement of Drosha in embryonic and adult NSC fate decision.

During embryogenesis, cortical development is a temporal tightly organized process. First, deep-layer neurons are generated followed by upper-layer neurons. To study the role of Drosha in cortical development, I performed NSC-specific conditional knock-out (cKO) experiments. Drosha cKO at defined developmental stages revealed that early during development Drosha is involved in controlling the timing of deep- and upper-layer neuronal differentiation and NSC maintenance. My biochemical results suggest that Drosha regulates deep-layer specification by inhibiting the deep-layer specific transcription factor Ctip2 in a miRNA-independent manner.

Under physiological conditions, adult hippocampal NSCs are bi-potent, giving rise to neurons and astrocytes but not to oligodendrocytes. However, when we deleted Drosha in hippocampal NSCs, they activated an oligodendrogenesis pathway. We demonstrated that Drosha inhibits oligodendrogenesis by directly repressing the expression of the transcription factor NFIB in a miRNA-independent manner by cleaving and destabilizing its mRNA. These results demonstrate that adult hippocampal NSCs intrinsically are multipotent but Drosha restricts their fate.

In summary, the results of my PhD work show that Drosha plays a crucial role not only in NSC maintenance but also in NSC fate decision in the

embryonic and adult brain. We find that during these processes, Drosha balances the expression of several differentiation factors thereby potentially fine-tuning the differentiation program. It will be of future interest to investigate how this specific miRNA-independent function of Drosha is targeted and if such a function is conserved in other stem cell populations.

Abbreviations

CLIP	Crosslinked immunoprecipitation
Ctip2	COUP-TF (chicken ovalbumin upstream promoter transcription factor) – interacting protein 1, also named Bcl11b
DG	Dentate gyrus
DL	Deep layer
GCL	Granule cell layer
IZ	Intermediate zone
miRNA	microRNA
mRNA	Messenger RNA
Ngn2	Neurogenin 2
NFIB	Nucleofactor 1B
NSC	Neural stem cell
OB	Olfactory Bulb
SGZ	Subgranular zone
SVZ	Subventricular zone
UL	Upper layer
VZ	Ventricular zone

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Content

1. Introduction

Stem cells are undifferentiated cells that are committed to generate tissue specific somatic cells and have the ability to self-renew. During embryonic development embryonic stem cells can differentiate into all germlines, the ectoderm, endoderm and mesoderm. Later they commit to more specialized stem cells to form the different organs. Stem cells also exist in the adult organism to maintain normal turnover in organs including the skin, blood or in some specific areas of the brain.

Embryonic and adult NSCs have the ability to give rise to different cell types such as neurons and glia. NSCs are fascinating stem cells, which build the whole brain during neurogenesis and in the adult brain contribute to some learning and adaptive changes induced by environmental changes (Kintner, 2002). While embryonic and adult neurogenesis share several transcriptional regulators, the molecular control for fate acquisition and maintenance can differ greatly (Gotz et al., 2016, Urbán and Guillemot, 2014). Therefore, I will focus on embryonic and adult neurogenesis separately.

1.1 Embryonic brain development

1.1.1 Neural induction and formation of the neural tube

The initial step in the development of the nervous system is the gastrulation followed by the neurulation. Gastrulation in mammals begins after the implantation of the blastocyst in the uterus as local invagination of a subset of cells, reorganizing the single cell-layer blastula into a trilaminar gastrula. The implanted blastocyst consists of the inner cell mass containing the distal epiblast, giving rise to the germ layers and the proximal hypoblast forming the extraembryonic structures and the extraembryonic ectoderm. The blastocyst is sourranded by the trophoblast that will develop into a large part

of the placenta (Gilbert, 2003). At this step during development the embryo consists of three germ layers, namely the outer ectoderm, middle mesoderm and inner endoderm (Solnica-Krezel and Sepich, 2012). Together with the position of the invagination of the mesoderm and the endoderm, the vertebrate embryo has a defined midline, anterior-posterior, and dorsal-ventral axis. The mesoderm will give rise mostly to muscles and blood, the endoderm mostly to inner organs and the ectoderm to skin and the nervous systems (Gilbert, 2003).

Subsequent gastrulation the middle part of the ectoderm gets specified to neural ectoderm. The neuroectoderm will generate the central and most of the peripheral nervous systems. This region is defined by inductive signals from the notochord that expresses sonic hedgehog (Shh) (Ybot-Gonzalez et al., 2002). The notochord is a mesoderm cylinder in the midline of the embryo that extends from the mid-anterior to the posterior region of the embryo and disappears later in development. The inductive signals from the notochord induce the differentiation of a subset of neuroectodermal cells into neural precursor cells, inducing neurulation. During neurulation the neural precursors thicken into a columnar epithelium to form the neural plate (Fig. 1.1 A). The lateral ends of the neural plate, the neural plate borders, fold inward, converting the neural plate into a tube. At this developmental stage the neural precursors are called neuroepithelial cells. At the same time, the neural crest cells at the neural plate border detach from the dorsal neural folds. The neural crest cells migrate along specific pathways that expose them to additional inductive signals, which influence their fate differentiation. Eventually the neural crest cells generate different progeny such as melanocytes of the skin, smooth muscles and in the head region to bone and cartilage but mostly they differentiate into the peripheral nervous system (Fig. 1.1 B, C) (Purves et al., 2004).

The induction of the neuroectoderm was long debated and many different models were suggested. The predominant and widely accepted model from Leivine and Brivanlou (Levine and Brivanlou, 2007) suggests that the early embryo is in a default neural state and that this state actively needs to be blocked to allow the formation of other tissues. Mesoderm and endoderm are

defined by the activation of BMP, Nodal, Wnt and FGF signals at the posterior side during gastrulation. Whereas the neural tissue is “induced” through an inhibition of these posterior signals by the organizer early during gastrulation allowing a local anterior region of the epiblast to remain neural tissue. These specified neural cells further move from the distal epiblast to the anterior epiblast (Levine and Brivanlou, 2007).

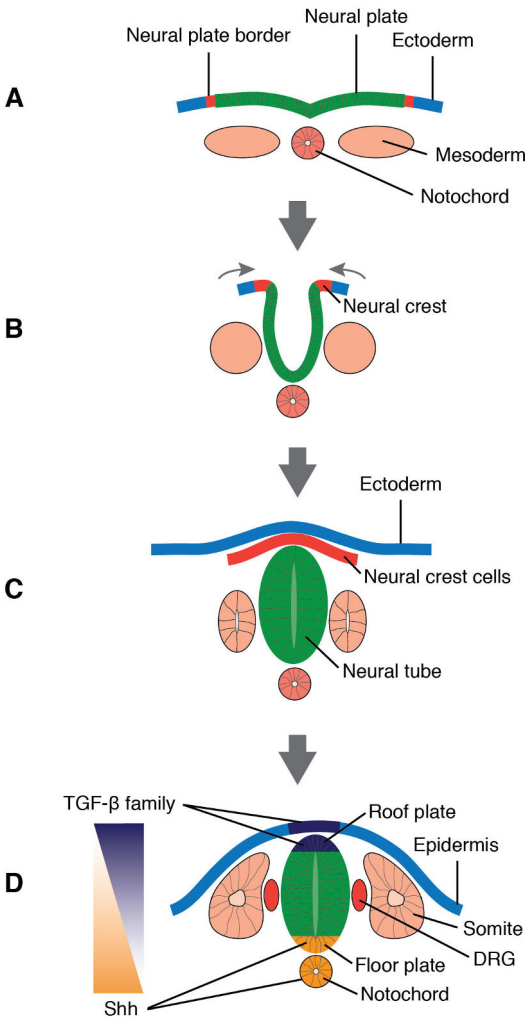

Figure 1.1 - Neural tube formation
(A-D) A coronal section through the developing embryo. (A) The neural plate originates from the ectoderm and thickens. The neural plate border separates the ectoderm from the neural plate. (B) The neural plate invaginates until the neural plate borders eventually meet, which are then called neural crest. (C) After the closure of the neural tube, the neural crest cells detach from the epidermis. (D) The notochord starts to degenerate and some mesoderm cells differentiate into the somites (precursors of skeleton muscle). Most of the neural crest cells differentiated into the peripheral nervous system and generate the dorsal root ganglia (DRG) after migrating along specific pathways. The floor plate and roof plate define the specific dorso-ventral pattern of the neural tube.
Adapted from (Purves et al., 2004)

1.1.2 Patterning and regional specifications

During development a subset of the neural precursors in the neural tube differentiate into neurons and form the nervous system in a precisely defined pattern along the dorsal-ventral and anterior-posterior axis. This is a temporally and spatially highly organized process. Cells at the ventral midline of the neural tube differentiate into the floorplate and the cells at the dorsal midline into the roofplate (Fig. 1.1 D). These structures together with the notochord define a dorso-ventral polarity of the neural tube during development. These are transient structures that disappear after the initial formation of the neural tube. The floorplate together with the notochord release the morphogen Sonic hedgehog (Shh) and the roofplate together with the ectoderm produce TGF-β. This results in a ventral high – dorsal low gradient of Shh signaling and an inverse TGF-β signaling gradient (Fig. 1.1 D). In the developing spinal cord the combination of these paracrine factors specify a mosaic of transcription factors and the generation of defined cell types along the dorso-ventral axis (Chamberlain et al., 2008). The exact expression of the transcription factors that are expressed at a specific time and place are determined by the concentration as well the duration of the exposure to the morphogens (Harfe et al., 2004). This allows for example the initial expression of Olig2 in the floor plate followed by the expression of Nkx2.2 and finally the expression of Shh (Ribes and Briscoe, 2009). The patterning of the dorsal neural tube however is not yet fully understood. The roofplate expresses and secrets BMPs, however they are not behaving as classical morphogenes, since they cannot act over long distances like Shh (Hogan, 1996, Hu et al., 2004). Additionally, there are several members of the TGF- β family and Wnt family required for proper patterning of the dorsal neural tube (Kiecker and Lumsden, 2012). Therefore the dorsal patterning of the spinal cord is more complex than the ventral patterning and it is likely that qualitative as well as quantitative mechanisms are involved.

Nevertheless, the patterning of the dorso-ventral axis is quite well understood compared to the patterning along the anterio-posterior axis. It is known that the formation of the spinal cord, the brainstem, midbrain and

forebrain with its basic anatomical structures starts soon after neurulation. During development the neural tube undergoes morphogenetic movements, which bend, fold and compress thereby forming the different brain regions (Fig. 1.2). At the moment a prominent view is that the anterior-posterior compartmentalization is established by the maintenance and refinement of morphogen patterns (Wurst and Bally-Cuif, 2001). To date several distinct signal patterns along the anteroposterior and dorsoventral axis are described to specify neuronal identities at the mid-hindbrain border such as Wnt-1, FGF-8 and Shh (Fig. 1.2) (Carlson, 2014). Once the neural tube is formed and the anteroior-posterior pattern is established, the neural progenitors start to differentiate and form the central nervous system.

1.1.3. Neurogenesis in the dorsal cortex

The adult brain develops from the neural tube, a single layer of pseudostratified neuroepithelial cells. Early during neurodevelopment, in mice at around embryonic day (E) 9, the neuroepithelial cells divide symmetrically to expand the progenitor pool. During division the nucleus of these cells undergo interkinetic nuclear migration along the apical-basal axis. During S-phase the cell body of the precursors is situated close to the basal surface

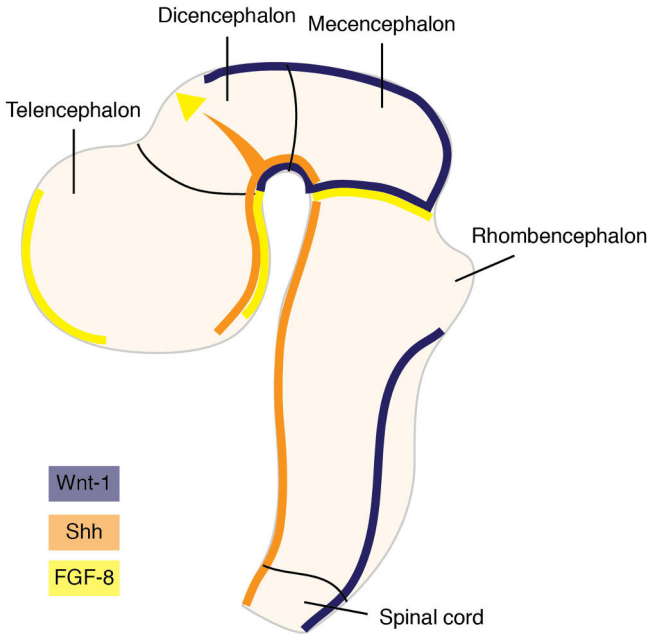

Figure 1.2 - Anterior-posterior patterning of the neural tube
Sagittal view on the developing mammalian brain. The interplay of the Wnt-1, Shh and FGF-8 signals specify neural identities.
Adapted from (Carlson, 2014).

and it migrates downwards apically during G_2-phase. At the apical surface the neuroepithelial cells divide symmetrically producing two identical neuroepithelial cells. This movement of the cell bodies gives the neuroepithelium a pseudostratified layered appearance (Takahashi et al., 1993, Chenn and McConnell, 1995). It is not yet fully understood how the positioning of the cell body interferes with the cell cycle progression. However, a recent study demonstrated that arresting the nuclear migration during G_2-phase inhibits mitotic entry, showing that correct interkinetic migration is necessary for neuroepithelial cells to progress from G_2- into M-phase (Fig. 1.3) (Hu et al., 2013). The precursors in the neural tube have typical epithelial features including adherence junctions and tight junctions at the apical side and express neuroepithelial markers such as intermediate filament Nestin (Kriegstein and Gotz, 2003).

At the onset of cortical neurogenesis in mice at around E10 the neuroepithelial cells transform into radial glial cells, the NSCs (Kriegstein and Alvarez-Buylla, 2009). NSCs maintain Nestin expression and also start to express astrocyte proteins including Glutamate Transporter (GLAST), Tenascin-C (TN-C) and Brain Lipid-Binding Protein (BLBP) (Kriegstein and Gotz, 2003). They lose the tight junctions whereas the adherens junctions and their apical-basal polarity remain conserved (Aaku-Saraste et al., 1996).

NSCs have a characteristic radial morphology with long processes that extend from the apical lumen of the neural tube to the basal pial surface and their soma form the ventricular zone (VZ). They still undergo interkinetic migration though their soma only migrates within the VZ (Fig. 1.3) (Haubensak et al., 2004). The radial scaffold of the NSCs is used for newborn neurons to migrate along into the growing cortex (Rakic, 1971). NSCs first generate neurons and later glia. NSCs can divide symmetrically to produce two identical daughter cells with equal distributed constituents to maintain the stem cell pool. Symmetric division takes mainly place in the neuroepithelium to amplify the progenitors but decreases as neurogenesis progresses. NSCs also have the ability to divide asymmetrically, giving rise to a NSC and a more differentiated daughter cell. During the neurogenic phase,

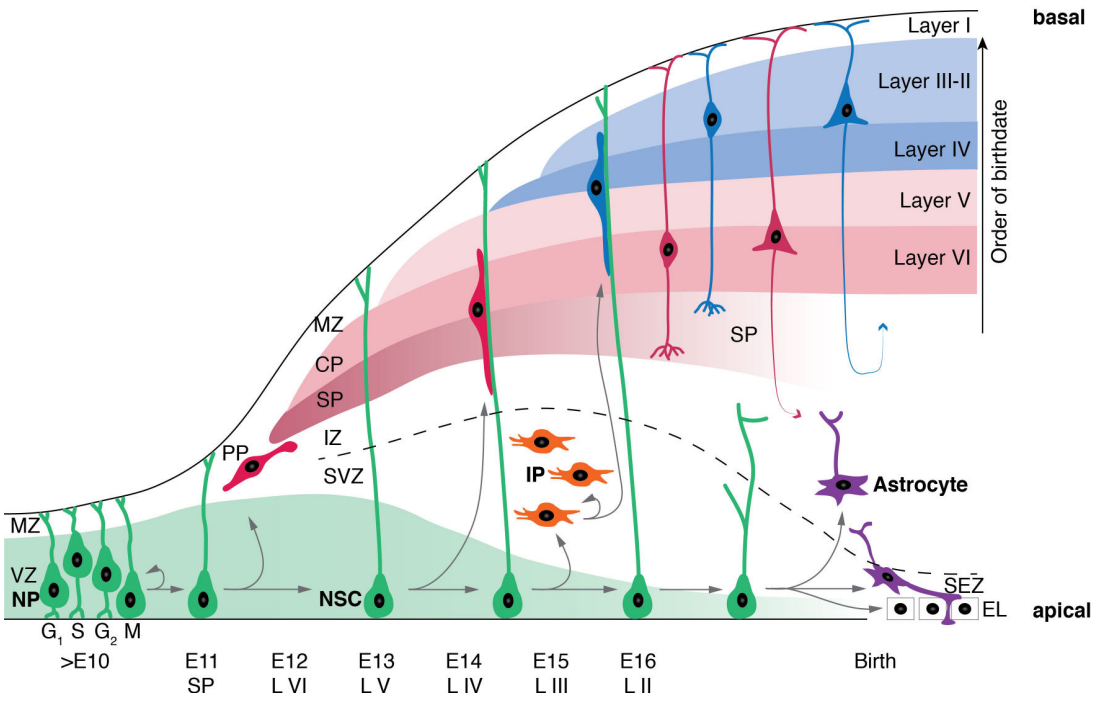

Figure 1.3 – Corticogenesis

Early during neural development, neural progenitors, the neuroepithelial cells (NP) located in the ventricular zone (VZ, green) divide symmetrically to expand the progenitor pool. At E10.5 neuroepithelial cells transit into NSCs. During neurogenesis NSCs generate intermediate basal progenitors (IP, orange), which at E13.5 migrate to the subventricular zone (SVZ). At E11 NSCs start to divide asymmetrically to generate postmitotic neurons that migrate along the NSCs through the intermediate zone (IZ) to reach the mantle layers. First projection neurons settle within the preplate (PP) which is later separated by the cortical plate (CP) into the marginal zone (MZ) that gives rise to Layer I and the subplate (SP) lying below Layer VI. The diverse layer-specific projection neurons are generated sequentially from E11.5 to E17.5. First the neurons destined for the SP are generated, followed by the neurons destined for the deep layers (Layer VI – V, red) and finally the neurons destined for the upper layers (Layer IV – II, blue). After completion of major neurogenesis at E17.5 the radial scaffold of the NSCs is dismantled and the progenitors start to generate cortical astrocytes (purple) and subependymal zone (SEZ) astrocytes (purple, adult NSCs) and give rise to the ependymal layer (EL). The tangential migration and positioning of interneurons is not illustrated. Adapted from (Kwan et al., 2012).

NSCs preferentially proliferate asymmetric to self-renew and give rise to an intermediate progenitor or a neuron (Fig. 1.3) (Noctor et al., 2001, Miyata et al., 2001). Intermediate progenitors are not anchored to either the apical or basal surface. They express Tbr2, Ngn2 and NeuroD1, function as amplifying cells, populating the subventricular zone (SVZ). In contrast to the NSCs the intermediate progenitors lose their apical-basal polarity and undergo mitosis in the SVZ without any obvious interkinetic migration. They are committed to the neuronal linage and undergo a number of proliferative divisions, dividing mostly symmetrically to produce two neurons (Haubensak et al., 2004, Noctor et al., 2004, Englund et al., 2005, Hevner et al., 2006). The asymmetric division of NSCs together with the amplification of the intermediate progenitors is crucial during corticogenesis, since it allows the generation of a large amount of neurons while maintaining the stem cell pool.

The mammalian neocortex consists of six cortical layers each composed of specific subsets of neurons with characteristic morphology, electrophysiology and markers, astrocytes, oligodendrocytes and microglia. Each cortical layer can be defined by a specific subset of excitatory, glutamatergic pyramidal neurons that have a stereotypic projection pattern

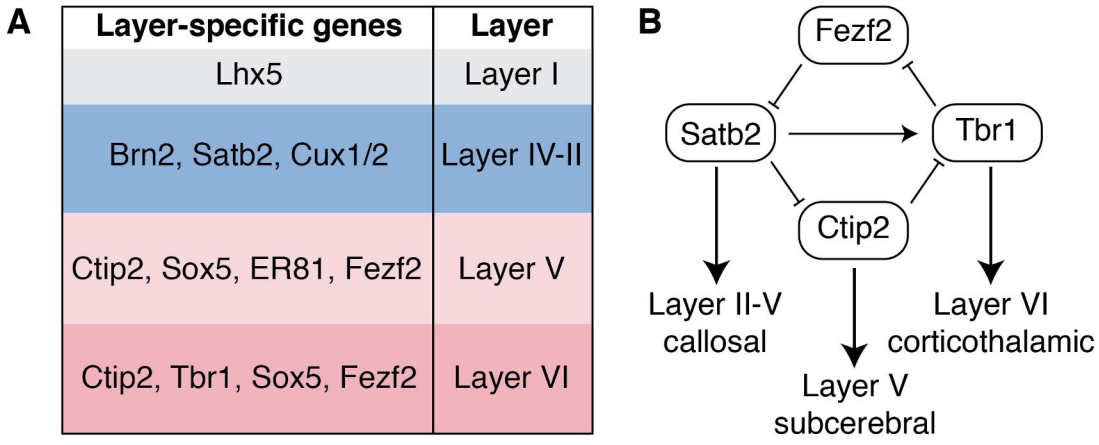

Figure 1.4 - Layer-specific gene expression in the mouse neocortex

(A) Each cortical layer consists of a specific subset of glutamatergic projection neurons. This figure presents some of the most used and known layer markers. (B) Schematic of genetic interplay between the layer markers Fezf2, Ctip2, Satb2 and Tbr1 to define the identity and projection of the pyramidal neurons. Adapted from (Srinivasan et al., 2012).

and inhibitory GABAergic interneurons. In contrast to the excitatory projection neurons that develop from progenitors in the dorsal telencephalon, interneurons are generated from progenitors in the ventral telecenphalon, more precisely the medial, lateral and caudal ganglionic eminence. They migrate tangentially to the neocortex where they locally connect as inhibitors (Wichterle et al., 2001, Cobos et al., 2001, Wonders and Anderson, 2005). Intriguingly, the ventrally derived interneurons integrate into the same cortical layer as the dorsal glutamatergic neurons that are born at the same time (Butt et al., 2005). While intermediate neurons make about 20% of the cortical neurons, the pyramidal projection neurons are in a majority making the other 80%. Glutamatergic projection neurons have a pyramidal shaped soma, contain several dendrites directing the basal surface and a single neurite towards the apical surface (Garcia-Lopez et al., 2006).

During corticogenesis the dorsal neural progenitors differentiate into the projection of the specific layers in a tightly controlled temporal structure. Newly born glutamatergic neurons migrate in an inside-out fashion, where early born neurons (mouse E10-E14) first populate deep layers and later born neurons (mouse E14-E17) migrate past the deep layers, populating progressively the superficial layers building the multilayered dorsal cortex (Fig. 1.3) (Greig et al., 2013). During their migration the newborn neurons undergo morphological and molecular differentiation and start to express layer specific genetic markers (Kwan et al., 2012). It is known that four genes, Fezf2, Satb2, Ctip2 and Tbr1, regulate the stereotypic projections in each cortical layer. These genes are in a complex interplay with each other by inhibiting or activating each other, thereby defining the development of subcortical or callosal projections (Fig. 1.4) (Srinivasan et al., 2012).

After the major neurogenesis period is completed at E17.5 in mice, NSCs start to differentiate into glia cells (Fig. 1.3). First NSCs differentiate into astrocytes and at around birth they differentiate into oligodendrocytes while the glia cells fully maturate postnatally (Kessaris et al., 2006, Rowitch and Kriegstein, 2010).

The complex formation of the six-layered neocortex needs to be tightly regulated. Hence, cortical development involves a precise coordination of

many aspects in cell biology including the timing of cell-cycle exit, differentiation, fate restriction, survival, and migration.

1.1.4 Expression of Transcription Factors during corticogenesis

A molecular control on the transcriptional level is provided by transcription factors. They are well known to coordinate the acquirement of the correct fate of differentiating cells (Iwafuchi-Doi and Zaret, 2016). This is also the case during embryonic neurogenesis. NSCs express Pax6 and Hes5, intermediate progenitors Tbr2 and Neurogenins (Ngns). Neural differentiation is initiated by NeuroDs and mature neurons finally express the layer- and projection-specific transcription factors including Ctip2 or Brn2 (Englund et al., 2005, Uittenbogaard et al., 2010, Imayoshi et al., 2010, Sun et al., 2001, Greig et al., 2013). NSCs express Notch receptors that induce the expression of Hes genes. Hes genes repress proneural genes in an oscillatory fashion, thereby inhibiting the transition of NSCs to intermediate progenitors (Shimojo et al., 2014, Shimojo et al., 2008). Furthermore, the paired box transcription factor Pax6 is known to be crucial to promote proliferation and differentiation of NSCs (Quinn et al., 2007). A loss of Pax6 function reduces cortical neurons.

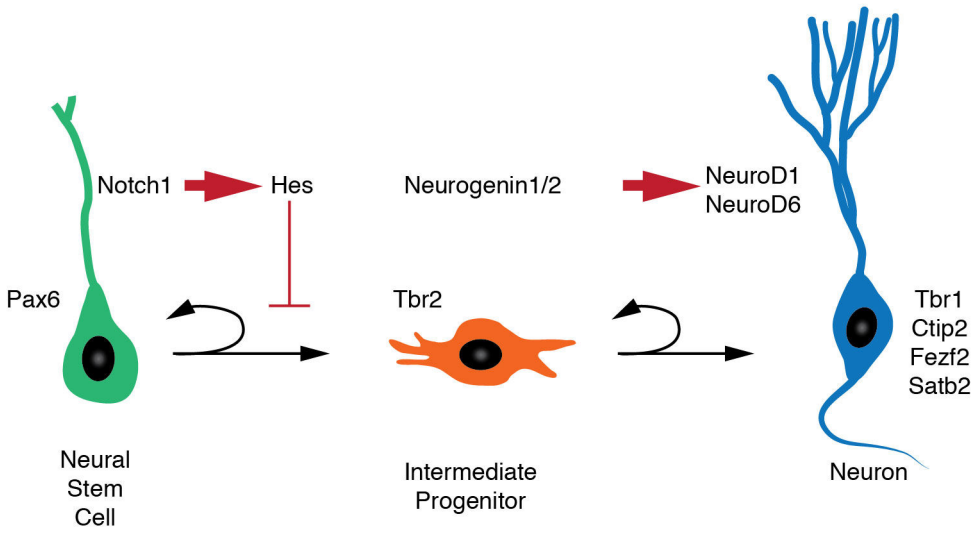

Figure 1.5 - Regulation of neurogenesis by transcription factors
During neural differentiation the progressive cell types are defined by a specific expression pattern of transcription factors.

Pax6 directly controls the transcription factor Ngn2 and Ngn2 in turn induces neural differentiation (Scardigli et al., 2001, Heins et al., 2002). Once the NSCs overcome the Notch-dependent maintenance signals, they start to become intermediate progenitors, migrate to the SVZ and express the proneural genes Ngn2 and T-box transcription factor Tbr2. The intermediate progenitors still proliferate and depend on Tbr2, since deletion of Tbr2 leads to severe microcephaly (Arnold et al., 2008). Further during differentiation NeuroD1/6 induce neuronal maturation, survival and migration of the intermediate progenitors, which is expressed in some mitotically active progenitors in the upper SVZ (Kim, 2013) (Fig. 1.5). Upon final differentiation the mature neurons start to express their layer-specific markers. Currently, no markers are known to distinguish the specific projection neuron subtypes among the progenitors. For this reason much less is known about what genes control the gradual commitment of progenitors to their distinct subtypes of pyramidal projection neurons (Molyneaux et al., 2007).

1.2 Adult neurogenesis

Traditionally neurogenesis was viewed to occur only during embryonic and perinatal development (Ming and Song, 2005). Only in 1965 Altmann and Das´s pioneer work showed the presence of newly generated cells in the dentate gyrus of the postnatal rat (Altman and Das, 1965). With the introduction of bromodeoxyuridin (BrdU), a nucleotide analogue used as a lineage tracer, the field made enormous progress (Kuhn and Gage, 1996). To date it has been demonstrated that life-long neurogenesis exists in several mammals, including humans (Eriksson et al., 1998).

Adult neurogenesis is defined as a process of generating functional neurons from adult NSCs that occurs throughout life in specific brain regions. Adult NSCs share structural and biological markers with astrocytes. They proliferate slowly and self-renew throughout life. NSCs generate actively dividing intermediate cells, called intermediate progenitors. Intermediate progenitors divide faster than NSCs, which allow them to amplify the stem cell pool prior to differentiation (Morshead et al., 1994). Similar to embryonic

neural progenitors it is unknown whether the intermediate progenitors are already biased to a specific fate commitment (Taylor, 2011). As soon as the progenitors are committed they are called neuroblasts, they rarely divide, eventually become postmitotic and develop into mature neurons, indistinguishable from the embryonically developed neurons (van Praag et al., 2005).

In mammals NSCs are found in two distinct regions, the SVZ of the lateral ventricular wall of the striatum and the hippocampus (Fig. 1.6). In the SVZ NSCs are located in the lateral wall (LW), where differentiated immature neuroblasts migrate along the rostral migratory stream to the olfactory bulb and differentiate into interneurons that integrate into local circuits (Fig. 1.6 blue) (Doetsch et al., 1999). In the hippocampus, NSCs are found in the subgranular zone (SGZ) of the dentate gyrus (DG) and immature neuroblasts generate DG glutamatergic granule nerons (Fig. 1.6 green) (Seri et al., 2004). The life-long process of NSC maintenance and differentiation is achieved by highly regulated control mechanisms including extrinsic signals as diffusible

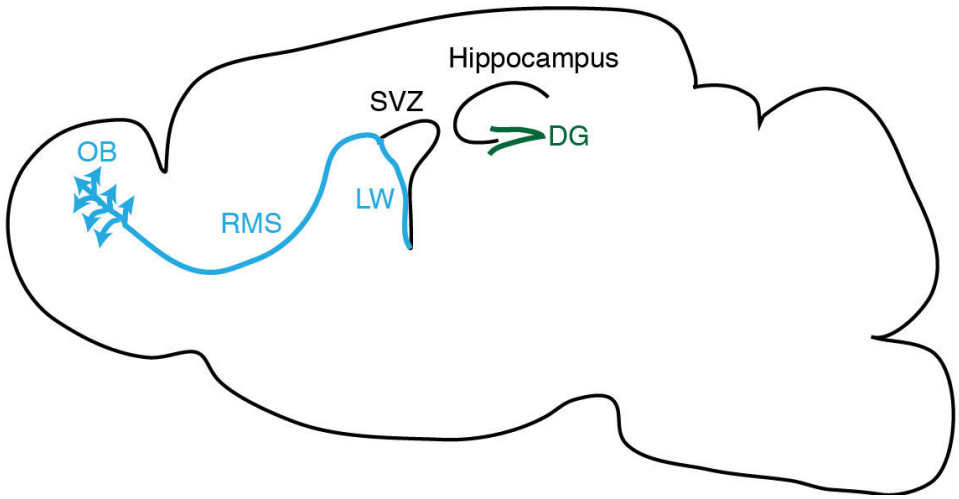

Figure 1.6 - Adult neurogenic niches of the murine brain

Sagittal scheme of a mouse brain representing the neurogenic niches. In the adult murine brain neurogenesis occurs in two distinct regions. In the lateral wall (LW) of the subventricular zone (SVZ), where newborn neurons migrate along the rostral migratory stream (RMS) into the olfactory bulb (OB) (blue) and in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus where neurogenesis is stationary (green).

and contact-mediated signals or intrinsic pathways including transcription factors or epigenetic regulators (Ihrie and Álvarez-Buylla, 2011, Sun et al., 2011).

In the following section I would like to focus in more detail on adult neurogenesis in the dentate gyrus, the region I studied during my PhD.

1.2.1 Adult hippocampal neurogenesis

Adult neurogenesis in the DG of the hippocampus has been found in rodents and primates, including humans (Spalding et al., 2013), where neurogenesis is believed to be crucial for some forms of learning and memory (Zhao et al., 2008). In the adult DG neuronal differentiation follows a strict hierarchy. DG NSCs are called type-1 cells and reside in the SGZ where they exist in radial and horizontal morphologies (Fig. 1.7, green). Radial NSCs project through the granule cell layer (GCL), divide very infrequently and are therefore referred to quiescent NSCs. Horizontal type-1 cells on the contrary divide more frequently and are defined as active NSCs (Lugert et al., 2010) and generate more committed progeny. The existence of quiescent adult NSCs is believed to be a mechanism to retain the stem cell pool by preventing its exhaustion through differentiation. Moreover, a quiescent stem cell pool is less prone to DNA mutations that can accumulate during cell division and DNA replication, therefore, a dormant pool of NSCs can reduce the formation and propagation of potentially dangerous chromosomal aberrations (Cheung and Rando, 2013, Llorens-Bobadilla et al., 2015, Shin et al., 2015). However, it still remains unclear what mechanisms regulate the activation of quiescent stem cells, though some extrinsic and intrinsic pathways have been identified to be involved in this process including the Notch-signalling (Rolando and Taylor, 2014, Lugert et al., 2010, Breunig et al., 2007, Ehm et al., 2010).

During differentiation, type-1 cells give rise to fast proliferating type-2 cells (Fig. 1.7, orange). Type-2 cells are intermediate precursor cells (IPs), which are divided into two subtypes, the early progenitors type-2a expressing Mash1 and the early neuroblasts type-2b expressing Tbr2 and DCX. It has been shown that the early neuroblasts are the proliferating cells, amplifying the

progenitor pool (Lugert et al., 2012). Type-2 cells eventually give rise to type-3 cells, the fate-committed neuroblast (Fig. 1.7, yellow). Neuroblasts on the other hand proliferate rarely and eventually mature into granule neurons that can integrate into the local DG circuit (Fig. 1.7, blue) (Ehninger and Kempermann, 2008, Seri et al., 2004). In humans, up to 35% of the neural hippocampal circuits contain newborn neurons (Spalding et al., 2013), where in the murine brain this is estimated to be about 10% (Santos et al., 2007, Imayoshi et al., 2008). Therefore, adult neurogenesis essentially contributes to brain plasticity (van Praag et al., 2005).

Under physiological conditions, adult NSCs of the hippocampus are bi-potential, they can generate neurons and astrocytes, however, not oligodendrocytes (Bonaguidi et al., 2011, Rolando et al., 2016). Nonetheless, it has been shown that DG NSCs can differentiate into oligodendrocytes when

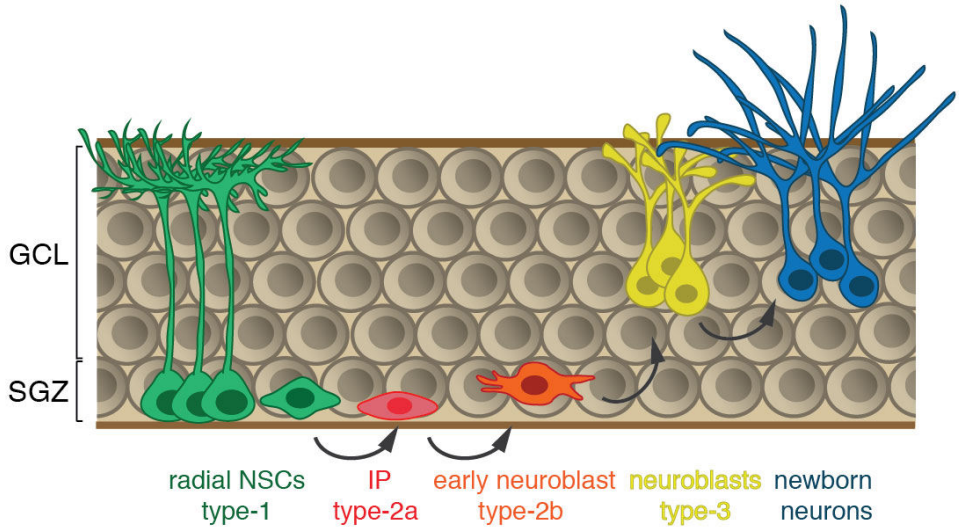

Figure 1.7 - Adult Hippocampal neurogenesis

NSCs populate the subgranular zone (SGZ) of the dentate gyrus (DG) and have a radial or horizontal morphology (type-1, green). Radial type-1 NSCs project through the granule cell layer (GCL) and divide less frequent than the horizontal type-1 NSCs. Therefore radial type-1 cells are defined as quiescent and horizontal type-1 cells as active NSCs. During differentiation type-1 cells generate type-2 intermediate progenitors (IP, red). IPs eventually generate neuroblasts (orange) that expand before differentiating into postmitotic neuroblasts (yellow), which become mature newborn neurons (blue). Adapted from (Rolando et al., 2016).

Mash1 is overexpressed or Nf1 is deleted *in vivo* (Braun et al., 2015, Jessberger et al., 2008, Sun et al., 2015) or when DG NSCs are co-cultured with neurons *in vitro* (Song et al., 2002, Suh et al., 2007). These results suggest, that DG NSCs intrinsically are multipotent. Generally, the maintenance, differentiation and fate commitment of adult NSCs is a complex interplay between several extrinsic and intrinsic factors within a defined local microenvironment. Over the last decades several key factors and signalling mechanisms have been revealed regulating the neurogenic process.

Notch signalling for example has been shown to be crucial to maintain NSCs. Notch signalling is inhibiting neurogenic differentiation by suppressing the expression of proneural factors and allowing astrocytic differentiation (Gaiano and Fishell, 2002, Ehm et al., 2010). Furthermore, Notch signalling modulates dendritic morphogenesis during neuronal maturation (Breunig et al., 2007, Dahlhaus et al., 2008). Furthermore, the maintenance of quiescent NSCs has been shown to be dependent on the morphogen BMP. In the hippocampus BMPs are secreted by granule neurons, NSCs and other cells of the niche. They promote quiescence and the exit of cells from the cell cycle (Mira et al., 2010). Moreover BMPs also control the maturation rate of newborn neurons in the hippocampus (Bond et al., 2014). It is believed that such a dual role of the BMPs is regulated by the expression of different BMP receptors along the neurogenic lineage (Mira et al., 2010). As essential regulators of gene expression, transcription factors play a crucial role during adult neurogenesis. Several transcription factors have been identified to be expressed at specific stages of adult neurogenesis controlling the transcriptional program during differentiation. The SRY-related high-mobility group (HMG) box (Sox) family member Sox2 for example is expressed in type 1 and 2a cells and controls the proliferative capacity and multipotency of NSCs (Favaro et al., 2009, Steiner et al., 2006). Sox2 itself can be regulated by diverse signalling pathways including Notch (Ehm et al., 2010). Furthermore transcription factors of the Hes family, the Forkhead O-box (FoxO), the transcriptional regulator Hmga2 or the nuclear factor 1 (NFI) are expressed in NSCs. All these transcription factors regulate the expression of cell-cycle inhibitors, differentiation inhibitors and signalling pathways involved

in regulating NSC behaviour (Goncalves et al., 2016). As a negative regulator of transcription the repressor element 1-silencing transcription/neuron-restrictive silencer factor (REST/NRSF) is required to maintain NSCs in an undifferentiated and quiescent state. REST is expressed in aNSCs in the DG and recruits corepressors CoREST and Sin3a to inhibit the neuronal differentiation program (Gao et al., 2011, Kim et al., 2015). Furthermore, PTEN is repressing the proliferation of aNSCs. It has been shown that PTEN deleted aNSCs undergo symmetric cell division at the expense of differentiation (Bonaguidi et al., 2011)

In addition, it has been shown that epigenetic regulators are involved in the differentiation of adult NSCs including DNA methylation or miRNAs (Yao et al., 2016). During my PhD we showed that the RNaseIII Drosha intrinsically blocks adult NSCs to differentiate into oligodendrocytes by inhibiting the transcript of NFIB (Rolando et al., 2016). These results revealed another mechanism of adult neurogenesis on the post-transcriptional level. Altogether this shows that precise differentiation and maintenance of adult NSCs is a highly complex process.

1.3 Book chapter – MiRNA-Dependent and Independent Functions of the Microprocessor in the Regulation of Neural Stem Cell biology

As discussed above, the maintenance and differentiation of NSCs is a highly complex process involving many aspects in cell biology as for example strict control of the transcriptome. Well-known regulators of mRNA stability and expression are miRNAs. miRNAs are short non-coding RNAs, first discovered in 1993 in *C.elegans* (Lee et al., 1993), which post-transcriptionally regulate gene expression by inhibiting or degrading complementary mRNAs. To date we know that about 60% of the human transcriptome is miRNA-regulated (Friedman et al., 2009) by more than two thousand known miRNAs (Kozomara and Griffiths-Jones, 2014, Griffiths-Jones et al., 2006). This variety of post-transcriptional regulation is implicated

in a diversity of biological functions, including neurogenesis.

Furthermore, our group and others have demonstrated that the microprocessor, a key complex involved in miRNA biogenesis, also has the ability to directly inhibit specific mRNAs (Fig. 1.8) in a non-canonical miRNA-independent manner. We recently showed that this direct destabilization of mRNAs by the microprocessor influences the fate of embryonic and adult NSCs (Knuckles et al., 2012, Rolando et al., 2016). The following book chapter summarizes the recent findings of miRNA-dependent and miRNA-independent regulations of the miRNA-machinery on neurogenesis (Erni et al., 2017).

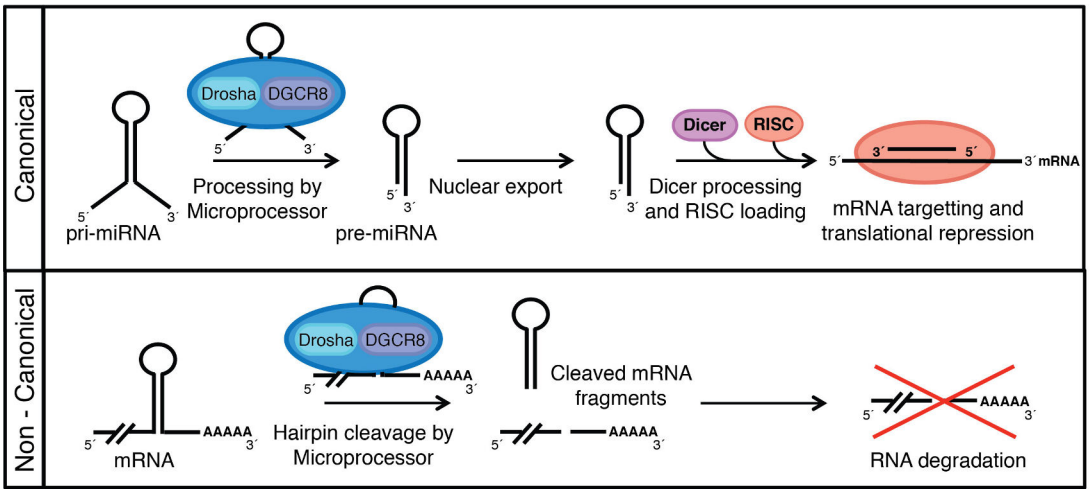

Figure 1.8 - Canonical and non-canonical function of the microprocessor

Canonical: Drosha together with DGCR8 build the central components of the microprocessor, catalyzing the production of pri-miRNAs to pre-miRNA in the nucleus. Following nuclear export the pre-miRNA is processed further by Dicer to a ~22 nucleotide long miRNA and loaded onto the RNA-induced silencing complex (RISC) to target mRNAs.

Non-canonical: Drosha binds hairpins on mRNAs and directly cleaves them, thereby destabilizing the transcripts.

1.3.1 Contribution

For the chapter “miRNA-Dependent and Independent Functions of the Microprocessor in the Regulation of Neural Stem Cell Biology“ (Erni et al. 2017) I wrote the text except from the part describing adult neurogenesis in 6.2 and I generated the figure 6.1.

The text is published in chapter 6 in “Essentials of Noncoding RNA in Neuroscience” and available under the following DOI link:
https://doi.org/10.1016/B978-0-12-804402-5.00006-6

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CHAPTER

6

MiRNA-DEPENDENT AND INDEPENDENT FUNCTIONS OF THE MICROPROCESSOR IN THE REGULATION OF NEURAL STEM CELL BIOLOGY

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INTRODUCTION

Stem cells exist in different organs of the body where they are committed to generate tissue specific somatic cells. In the developing and adult mammalian brain, neural stem cells (NSCs) generate neurons and glia. NSCs build the whole brain during embryonic development and enable the adult brain to adapt to environmental changes and to contribute to certain forms of memory by means of adult neurogenesis. During mammalian neural development, the complex structures of the brain are formed from a single layer of neuroepithelial cells that line the vesicular surface of the neural tube. In mice at around embryonic day 9, the neuroepithelial cells give rise to the NSCs, which in turn differentiate into the different neuronal subtypes and glia in a tightly controlled spatiotemporal manner (Molyneaux et al., 2007). NSCs persist in restricted regions of the postnatal brain where they support neurogenesis throughout life thus allowing brain plasticity and adaptation (Ming and Song, 2012). NSC regulation involves a precise coordination of several intrinsic and extrinsic factors (Song, 2008). A crucial intrinsic mechanism to finely regulate gene expression involves microRNAs (miRNAs) (Ha and Kim, 2014). miRNAs are short noncoding RNAs that posttranscriptionally regulate gene expression by targeting complementary mRNAs, thereby inducing cleavage and degradation or by inhibiting translation (Bartel, 2004). miRNAs are involved in all cellular processes and are expressed in all cell types including NSCs, where they affect maintenance and differentiation (Shi et al., 2010; Lang and Shi, 2012; Kawahara et al., 2012; Meza-Sosa et al., 2014). Interestingly, recent findings revealed that miRNA biogenesis components have more complex functions than expected and they are not restricted to classical miRNA-directed inhibition of mRNA expression. In this chapter, we will summarize the current knowledge about the canonical and noncanonical miRNA pathways and their influence on NSC maintenance and differentiation (Fig. 6.1).

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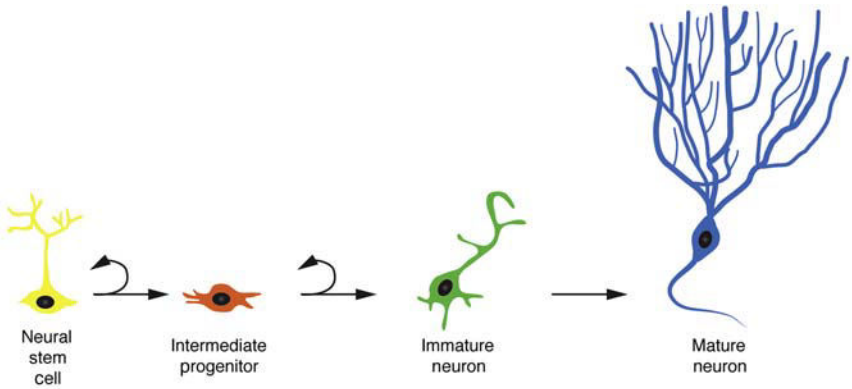
(A)



(B)

	Proliferation	Differentiation	Maturation	
miRNA	miR-9 Laneve *et al.* 2010 miR-92b Nowakowski *et al.* 2013 miR-106b~25 Brett *et al.* 2011 miR-137 Szulwach *et al.* 2010 miR-184 Liu *et al.* 2010 miR-379-410 Winter 2015	let-7 b Nishino *et al.* 2008 miR-9 Zhao *et al.* 2009 Lang and Shi 2012 miR-124 Neo *et al.* 2014 Visvanathan 2007 Cheng *et al.* 2009 Makeyev *et al.* 2007 miR-137 Sun *et al.* 2011 Silber *et al.* 2008 miR-128 Zhang *et al.* 2016 miR-184 Liu *et al.* 2010 miR-379-410 Winter 2015	miR-9 Dealoly *et al.* 2010 miR-200 Choi *et al.* 2008 miR-132 Luikart *et al.* 2011 miR-137 Smrt *et al.* 2010 miR-379-410 Winter 2015	Microprocessor-dependent
mRNA	Neurogenin 2 Knuckles *et al.* 2012	NeuroD1 Knuckles *et al.* 2012 NeuroD6 Knuckles *et al.* 2012 NFIB Rolando *et al.* 2016		
alternative splicing		REST Raj *et al.* 2011	Disabled-1 Yano *et al.* 2010 Neurexin Iijima *et al.* 2011 PSD-95 Zheng *et al.* 2012	Suggested to be microprocessor-dependent
snoRNA	SNORD 44, 47, 74-81 Schouten *et al.* 2012			

FIGURE 6.1 Influence of the microprocessor on neurogenesis

(A) Schematic of neurogenesis. NSCs self-renew and give rise to intermediate progenitors, which proliferate, and give rise to immature neurons (INs), which differentiate into mature neurons. (B) Table of RNA species processed by the microprocessor. The upper two layers represent the microprocessor-dependent miRNAs and mRNAs that affect neurogenesis. Several miRNAs are known to influence NSC proliferation, neuronal differentiation, and maturation. Recent studies discovered microprocessor-dependent regulation of mRNAs that are involved in NSCs proliferation and differentiation. The lower two lines highlighted in grey indicate potential microprocessor targets involved in the regulation of neurogenesis through alternative splicing or snoRNAs.

EMBRYONIC AND ADULT NEUROGENESIS

The mammalian neocortex is a complex six-layered structure, responsible for processing sensory information, coordinating motor output, and mediating cognitive functions (Greig et al., 2013). The neocortex is populated by neurons, inhibitory interneurons, and excitatory projection neurons and glial cells, astrocytes, oligodendrocytes, and microglia. The interneurons are generated from ventral

NSCs and migrate tangentially to the neocortex where they connect as inhibitors in local circuits (Wichterle et al., 2001; Cobos et al., 2001; Wonders and Anderson, 2005). In contrast, the excitatory projection neurons are generated from progenitors in the dorsal telencephalon and connect to local as well as distant brain regions (Gorski et al., 2002; Molyneaux et al., 2007). The cortical projection neurons originate from the telencephalic wall or ventricular zone (VZ), which is populated by undifferentiated neuroepithelial cells and then radial glial cells. These are the NSCs that establish the VZ (Haubensak et al., 2004). NSCs have a radial morphology and span the cortex from the apical luminal to the basal pial surface. This scaffold is used by newborn neuronal progeny that migrate along the radial processes into the growing cortical plate (Rakic, 1971). Early during neurogenesis, NSCs predominantly proliferate symmetrically to expand the stem cell pool, at later stages, they switch and preferentially proliferate asymmetrically to self-renew and give-rise to an intermediate progenitor or a neuron (Noctor et al., 2001; Miyata et al., 2001). Intermediate basal progenitors are not attached to the VZ and function as transient amplifying cells, populating the subventricular zone (SVZ). They undergo a limited number of proliferative divisions and mostly divide symmetrically to produce two immature neurons (Haubensak et al., 2004; Noctor et al., 2004) for more detail, see Chapter 5, The Cell Biology of Neural Stem and Progenitor Cells and Neocortex Expansion in Development and Evolution of this book by Huttner and colleagues. These newborn neurons migrate radially to generate the cortex in an inside-out fashion. Early-born neurons populate the deeper layers (Layers VI and V), whereas later-born neurons migrate through these deeper layers to progressively populate more superficial layers, until the 6 layers of the isocortex are formed by birth (Greig et al., 2013). After neurogenesis is complete, NSCs switch fate and start to differentiate into glia for more detail, see Chapter 10, Transcriptional and Epigenetic Control of Astrogliogenesis of this book by Berninger and colleagues. Astrogliogenesis during late embryonic and early postnatal periods is followed by a wave of oligodendrogenesis (Kessaris et al., 2006; Rowitch and Kriegstein, 2010) for more detail, see Chapter 11, microRNAs in Oligodendrocyte Myelination and Repair in the Central Nervous System of this book by Lu and colleagues. On the other hand, microglia have a nonneuronal origin and develop from haemotopoietic cells.

All of these complex NSC behaviors, maintenance, differentiation, fate switching and migration during cortical development need to be tightly coordinated in order to achieve proper brain formation and function. Among the key factors regulating these processes, Notch signaling is crucial in regulating neurogenesis (Gaiano and Fishell, 2002). Notch pathway activates the expression of the basic helix−loop−helix (bHLH) transcription factors Hes1 and Hes5, which are required for NSC maintenance by inhibiting the expression of the proneural factors including Neurogenin2 (Ngn2). In NSCs, the expression of the *Hes* and *Ngn2* genes oscillate out of phase. A sustained expression of Ngn2 initiates NSC differentiation into intermediate progenitors (Shimojo et al., 2008; Imayoshi et al., 2013). Intermediate progenitors subsequently differentiate into neurons upon expression of neural bHLH determination factors including NeuroD1 or NeuroD6 (Bond et al., 2012). The expression of the transcription factors in this cascade during neocortical development needs to be strictly modulated, which includes posttranscriptional regulation. The oscillatory behavior of Ngn2 for example can be explained by direct transcript degradation. Indeed, it has been shown that Ngn2 mRNA degradation plays a pivotal role in preventing aberrant accumulation of neurogenic factors that would otherwise result in abnormal and precocious neurogenesis (Knuckles et al., 2012).

NSCs self-renew in the embryo and produce neurons and glia until they transform into parenchymal astrocytes, ependymal cells, or remain as adult stem cells in the two adult niches, the SVZ of the wall of the lateral ventricles (Furutachi et al., 2015) and the subgranular zone of the hippocampal

dentate gyrus (DG) (Kriegstein and Alvarez-buylla, 2011). Under physiological conditions, adult NSCs exhibit structural and biological markers of astrocytes. Adult NSCs proliferate slowly, retain the ability to self-renew throughout life, and generate actively dividing intermediate cells that function as transit amplifying progenitors (TAPs). NSCs have distinct features in the lateral ventricular and hippocampal germinative areas (Kriegstein and Alvarez-buylla, 2011). NSCs in the lateral ventricle produce immature neuroblasts migrating in chains to the olfactory bulb where they differentiate into local interneurons (Lois and Alvarez-Buylla, 1994; Hack et al., 2005), while in the hippocampus, NSCs generate glutamatergic granule neurons (Seri et al., 2001). Moreover, SVZ but not DG NSCs also generates myelinating oligodendrocytes (Menn et al., 2006; Lugert et al., 2010; Bonaguidi et al., 2011).

In the adult neurogenic niches, fine regulation of the balance between stem cell preservation and production of differentiated progeny is achieved by interactions between extrinsic signals and intrinsic pathways based on the activity of intrinsic determinants including transcription factors (Ihrie and Álvarez-Buylla, 2011). In addition, recent work has highlighted the role of epigenetic regulators in the control of adult neurogenesis (Sun et al., 2011b). Ultimately, epigenetic regulation could represent the link between external environmental influence and internal transcriptional and posttranscriptional control of gene expression in neural progenitors of the adult brain for more detail, see Chapters 7 and 10 of this book.

ROLES OF CANONICAL miRNAs DURING NEUROGENESIS

MiRNA BIOGENESIS

Embryonic and adult neurogenesis requires fine regulation of signaling pathways and gene expression. miRNAs are abundantly expressed in the brains of embryos and adults where they influence NSC maintenance and differentiation as well as the integration of neurons into complex circuits (Bartel, 2004; Ji et al., 2013). miRNA biogenesis starts when a long primary transcript (pri-miRNA) containing the local stem-loop structure of the miRNA sequence is processed by the microprocessor, a large complex including the RNase III Drosha and the RNA binding protein (RBP) DGCR8 (Pasha in flies and worms). The microprocessor crops the pri-miRNA and produces a 60−70 nucleotide (nt) stem-loop pre-miRNA (Lee et al., 2003). This pre-miRNA is subsequently exported to the cytoplasm and further processed by the RNase III Dicer, generating a 22-nt double-stranded RNA duplex (Bohnsack et al., 2004; Lund et al., 2004; Ketting et al., 2001). The mature single-stranded lead-miRNA binds to the RNA-induced silencing complex (RISC) directing it to complementary mRNA targets and results in transcript repression either through mRNA cleavage and degradation or translational repression (Hammond et al., 2001; Ha and Kim, 2014). In mammals, it is estimated that more than 60% of all mRNAs are under miRNA control (Bartel, 2009). Single miRNAs can target several mRNAs, and one mRNA can be regulated by different miRNAs (Bartel, 2009). Therefore, it is believed that miRNAs function to fine-tune gene expression. miRNAs have been shown to influence neurogenesis by regulating the transcripts of key proteins involved in progenitor proliferation and differentiation (Lang and Shi, 2012; Kawahara et al., 2012).

MiRNAs IN NSCs

First evidences for miRNA influencing neurogenesis came from the genetic ablation of *Dicer* in the neurogenic regions of the mouse brain. Conditional *Dicer* deletion in Emx1 expressing dorsal

telencephalic NSCs causes an impaired neuronal differentiation and cell death of progenitors and neurons thus resulting in a smaller cortex (De Pietri Tonelli et al., 2008). Similar results were obtained by depleting Dicer in Foxg1 expressing NSCs, which also induced loss of NSCs and a failure of neuronal differentiation (Davis et al., 2008). Furthermore, Dicer deficiency results in abnormal development of the CNS including failure of proper morphogenesis of the cerebellum, midbrain and the cortex (De Pietri Tonelli et al., 2008; Kawase-Koga et al., 2009; Huang et al., 2010; Choi et al., 2008). Recently, different miRNAs were identified to be involved in NSC maintenance and differentiation. In the following paragraphs, we summarize a few of the miRNAs discovered to be involved in neurogenesis (Fig. 6.1).

The let-7 miRNA was one of the first miRNA discovered in *Caenorhabditis elegans* and is highly conserved throughout evolution. The let-7 family of miRNAs varies only in a few nucleotides whereupon let-7a, b, c, and e are expressed in the brain and are upregulated upon neuronal differentiation (Lang and Shi, 2012). let-7 controls neurogenesis through different mechanisms (Fig. 6.1). For example, let-7b induces neurogenesis by repressing the transcripts of the orphan nuclear receptor TLX and the cell cycle regulator cyclin D1 (Zhao et al., 2010). In addition, let-7b overexpression reduces NSC proliferation and induces neuronal differentiation by directly repressing high mobility group AT-hook 2 (Hmga2) expression (Nishino et al., 2008). let-7 miRNAs can be repressed by the RBP Lin28. During embryonic stem cell (ESC) commitment to the neural lineage, Lin28 inhibits let-7a expression through specific binding to pri-let-7a, thereby inhibiting processing of the pre-miRNA to pri-miRNA by the microprocessor (Rybak et al., 2008). Early during neuronal differentiation, the RBP Musashi1 potentiates the inhibitory effect of Lin28 on let-7 miRNA by enhancing the localization of Lin28 to the nucleus (Kawahara et al., 2011).

Another well-known miRNA involved in neurogenesis and highly expressed in the embryonic and adult mouse brain is miR-9 (Fig. 6.1). First evidence for miR-9 being involved in neurogenesis came from overexpression experiments, which led to decreased proliferation and induced differentiation of NSCs (Zhao et al., 2009). miR-9 targets multiple transcripts including those encoding TLX, FoxG1, Sirtuin, RE1-Silencing Transcription Factor (REST), Meis2, and Gsh2, thereby regulating differentiation and maintenance of NSCs in a cellular and context-specific manner (Shibata et al., 2011; Delaloy et al., 2010). Several mechanisms have been proposed to explain this contradictory function of miR-9 during regulation of NSCs. For example, TLX repression by miR-9 induces neurogenesis, but TLX itself represses miR-9 expression. Therefore, miR-9 inhibits NSC proliferation and induces differentiation via a feedback loop with TLX (Zhao et al., 2009). Another feedback regulation of miR-9 has been shown via REST. REST suppresses miR-9 during NSC proliferation by occupying the miR-9-2 promoter, but REST can be removed by cAMP response element-binding (CREB) during differentiation (Laneve et al., 2010). Thus, these are examples of mechanisms that allow the same miRNA to have different functions on neurogenesis depending on the cellular context.

miRNA-124 is an abundant neural miRNA that induces embryonic and adult neural differentiation through several mechanisms (Fig. 6.1). miR-124 directly targets the transcriptional repressor Ezh2 thereby promoting neuronal differentiation and inhibiting astrocytic differentiation of embryonic mouse NSCs (Neo et al., 2014). Another target of miR-124 is the Small CTD Phosphatase 1 (SCP1). SCP1 is expressed in non neuronal tissue and at low levels by NSCs. SCP1 repression by miR-124 induces neuronal differentiation in chick embryos (Visvanathan et al., 2007). Moreover, during adult neurogenesis, miR-124 is upregulated when TAPs differentiate into neuroblasts, and

its expression remains high in differentiated olfactory bulb neurons. Furthermore, miR-124 targets the SRY-box containing gene 9 (Sox9) mRNA in adult NSCs, thereby promoting neurogenesis (Cheng et al., 2009). miR-124 also influences neural-specific alternative splicing by repressing the polypyrimidine tract binding protein 1 (PTBP1), which is a repressor of neural-specific splicing. PTBP1 repression by miR-124 induces an upregulation of PTBP2, which in turn favors neural-specific splicing and induces neuronal differentiation in embryonic NSCs (Makeyev et al., 2007). Like miR-9, miR-124 can also be repressed by REST (Conaco et al., 2006).

Another miRNA involved in neurogenesis is miR-137 (Fig. 6.1). miR-137 promotes the differentiation of adult SVZ NSCs (Silber et al., 2008). It represses lysine-specific histone demethylase 1 (LSD1), thereby inhibiting NSC proliferation and promoting neuronal differentiation. miR-137 in turn is suppressed by TLX and LSD1. This regulatory loop provides a coordinated expression of LSD1 and miR-137 during the transition of NSC from proliferation to differentiation, providing a control mechanism during neurogenesis (Sun et al., 2011a). On the other hand, overexpression of miR-137 in adult DG NSCs favors their proliferation by repressing Ezh2 (Szulwach et al., 2010). Moreover, miR-137 has been shown to be involved in neuronal maturation by repressing Mib1 and inhibiting dendritic morphogenesis (Smrt et al., 2010). Thus, similar to miR-9, miR-137 has divergent functions at different stages of neurogenesis depending on its targets and regulation.

During brain development, miRNAs are expressed in a spatiotemporal manner suggesting a contribution to neurogenesis at different stages. Therefore, miRNA expression needs to be finely tuned by regulatory networks. Thus, when studying miRNAs, it is important to consider the miRNA targets, since feedback regulatory circuits are often found in miRNA function and regulation. Several miRNAs often function with a complex synergistic interplay. Hence, it will be important to have comprehensive analyses of miRNA biogenesis, targets, and regulation during neurogenesis. A recent study took advantage of the miRNA deep sequencing methods to examine the profile of NSCs (Zhao et al., 2014). Zhao et al. (2014) sequenced miRNAs from rosette NSCs (R-NSCs) derived from Rhesus monkey embryonic stem cells (rmESCs) and compared their miRNA expression profile with rmESC, early and late passage R-NSCs and neural progenitor cells. They discovered 451 of the 466 annotated rhesus miRNAs were expressed in R-NSCs, whereas the different cell types expressed specific sets of miRNAs. This approach revealed several miRNAs that are expressed by neural progenitors but not known to be involved in neurogenesis including miR-374, miR-758, and miR-889 (Zhao et al., 2014). By comparing mRNA with miRNA sequence data, Zhao et al. (2014) proposed that miRNAs negatively regulate the expression of specific signaling pathways. They correlated high expression of two Hedgehog regulatory genes Growth Arrest-Specific 1 and Patched 1 with low miRNA expression targeting their transcripts, suggesting that specific sets of miRNAs regulate Hedgehog signaling during neurogenesis (Zhao et al., 2014). It will be important to unravel the interplay between the expression of specific miRNA classes, their mRNA targets and their regulators to fully understand the impact of miRNA on neurogenesis.

MiRNA-INDEPENDENT FUNCTIONS OF THE MICROPROCESSOR

miRNAs play pivotal roles during neurogenesis. However, components of the miRNA biogenesis pathway have a direct influence on neurogenesis without acting through the 22 nt mature miRNAs

(Knuckles et al., 2012). Drosha and DGCR8 build the core components of the microprocessor, catalyzing the nuclear step of miRNA biogenesis. However, the microprocessor also regulates stability of other RNA classes including mRNAs and small nucleolar RNAs (snoRNA) (Chong et al., 2010; Knuckles et al., 2012; Macias et al., 2012; Heras et al., 2013). Evidence for noncanonical functions of the microprocessor came from comparisons of Drosha/DGCR8- and Dicer-deficient cells. Some cell-types from distinct tissues show overlapping phenotypes when Drosha/DGCR8 and Dicer knockouts are compared, thus suggesting a common pathway (Chong et al., 2008; Teta et al., 2012; Bezman et al., 2010; Berdnik et al., 2008). However, this is not always the case. Importantly, Drosha- but not Dicer-deficiency results in precocious differentiation of NSCs in vivo (Knuckles et al., 2012). On the other hand, Dicer but not Drosha depletion in the eye leads to macular degeneration (Kaneko et al., 2011; Tarallo et al., 2012). Together, these results suggest independent functions of both enzymes. The first evidence that the microprocessor can act independent of miRNAs came from genome-wide comparisons of Drosha- and Dicer-knockdown in *Drosophila* Schneider S2 cells (Kadener et al., 2009). As expected, Drosha-knockdown leads to accumulation of several miRNA precursors but surprisingly also to mRNAs that are under the control of Drosha but which were not altered in Dicer-knockdown cells. Interestingly, Evofold hairpin predictions (Pedersen et al., 2006) revealed that some of the Drosha mRNA targets have strongly conserved structural hairpins in their sequences. Therefore, it was proposed that Drosha processing could affect specific coding genes (Kadener et al., 2009).

The first microprocessor mRNA-target identified was that of DGCR8/Pasha (Han et al., 2009; Kadener et al., 2009). Drosha-depletion leads to DGCR8 mRNA accumulation indicating that Drosha inhibits DGCR8 expression in an autoregulatory mechanism to control microprocessor levels (Han et al., 2009; Kadener et al., 2009). It has been shown that DGCR8 mRNA contains hairpins in the coding sequence and the 5′-UTR, which are conserved amongst organisms and that are targeted and processed by the microprocessor (Han et al., 2009). Taken together, these data suggests that miRNA-biogenesis pathway is autoregulated by a negative feedback loop where DGCR8 levels are the limiting factor (Han et al., 2009).

Additional transcriptional analyses have underlined miRNA-independent functions of Drosha. In thymocyte progenitors many transcripts are upregulated in Drosha but not in Dicer-deficient cells (Chong et al., 2010). Furthermore, many of these regulated mRNAs contained pri-miRNA-like structures that are cleaved by the microprocessor in a miRNA-independent way (Chong et al., 2010). In line with this observation, comparison of Drosha and Dicer deletion in dendritic cell progenitors also revealed a miRNA-independent role of Drosha, where Drosha controls the development of dendritic cells by targeting the hairpin-containing mRNAs of Myl9 and Todr1 thereby repressing their expression (Johanson et al., 2015). Interestingly, transcriptome-wide mRNA cleavage patterns revealed additional Drosha-dependent mRNA substrates. Comparison of wild-type and Drosha-knockout ESCs identified a variety of mRNA targets including DGCR8 and Calcipressin-3 transcripts. These putative targets are upregulated in Drosha-knockout ESCs thus suggesting that Drosha-mediated cleavage directly affects their expression (Karginov et al., 2010).

In addition, the novel high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS-CLIP) identified several novel putative RNA species that the microprocessor complex binds. HITS-CLIP of DGCR8 from HEK 293T cells identified miRNAs, several long noncoding RNAs, snoRNAs, and mRNAs. Interestingly, several of the mRNA targets of DGCR8 contain predicted RNA secondary structures that resemble pri-miRNA. Some of these mRNAs have

been shown to be cleaved by the microprocessor, thereby repressing their expression (Macias et al., 2012). HITS-CLIP of DGCR8 and Drosha from human ESCs identified mostly miRNAs bound to the microprocessor and only a few mRNAs and snoRNAs. However, several of these mRNAs were shown to be directly processed by the microprocessor, destabilizing these transcripts (Jung et al., 2014; Seong et al., 2014). Thus, the microprocessor has a complex role in regulating several classes of RNAs and can have different functions depending on the cell type and context.

The noncanonical functions of the microprocessor represent a rapid and efficient way to influence gene expression. During neurogenesis, a fast regulation of the transcriptome and proteome is essential for the maintenance and differentiation of NSCs. The noncanonical functions of the microprocessor are predominant in the early regulation of embryonic neurogenesis (Knuckles et al., 2012). Loss of Drosha or DGCR8 in NSCs of the forebrain results in a loss of NSCs and precocious neuronal differentiation, whereas Dicer-deficiency does not. Drosha binds to and negatively regulates the stability of the proneural gene Ngn2 and the neural determination factor NeuroD6, thereby maintaining NSCs in concert with Notch signaling. Ngn2 and NeuroD6 contain evolutionarily conserved hairpins resembling pri-miRNA structures, which can be bound by Drosha. 3′RACE revealed Drosha-dependent cleavage of Ngn2 mRNA (Knuckles et al., 2012). Interestingly, comparison of Drosha- and Dicer-deficient NSCs did not reveal significant changes in miRNA profile, suggesting that the miRNAs are relatively stable and that microprocessor-induced phenotypes are miRNA-independent. These data indicate that the microprocessor facilitates embryonic NSC maintenance by directly blocking the accumulation of mRNAs encoding for critical differentiation factors (Fig. 6.1). Interestingly, the microprocessor is not only crucial for embryonic neurogenesis but also affects maintenance and fate restriction of adult NSCs (Rolando et al., 2016). Here, Drosha acts independently of miRNAs to regulate neuronal versus glial cell fate acquisition from adult NSCs by directly targeting mRNAs essential for gliogenic differentiation (Fig. 6.1).

Pri-miRNA transcripts are often located within the introns of genes. Moreover, the microprocessor and the spliceosome, which are responsible for pre-mRNA splicing, could interact with the same sequences as the microprocessor physically associates with the spliceosome (Gregory et al., 2004). Drosha has been shown to enhance exon splicing in vitro and in vivo. For example, the alternatively spliced eIF4H exon 5 is predicted to form a hairpin loop that resembles a Drosha substrate. The microprocessor can indeed bind and cleave exon 5 of eIF4H thus precluding its inclusion in the mRNA (Kataoka et al., 2009; Havens et al., 2014). This indicates that the microprocessor has a role in splicing that is distinct from its role in miRNA biogenesis. In addition, microprocessor-dependent alternative splicing can produce mirtrons from the spliced-out introns that mature into functional miRNAs (Okamura et al., 2007; Ladewig et al., 2012; Wen et al., 2015). Moreover, the microprocessor regulates retrotransposable elements, which are mobile DNA elements. The microprocessor can bind and cleave the retrotransposable elements LINE-1 and Alu-containing pre-miRNA like stem-loop structures, thereby acting as a defense against human genome integrity (Heras et al., 2013).

In addition to the miRNA-independent effects of the microprocessor on transcript regulation, the microprocessor could directly influence transcription by either promoting or delaying the transcriptional initiation (Gromak et al., 2013; Wagschal et al., 2012). It has been shown that binding of the microprocessor to promoter-proximal regions of human genes leads to an upregulation of transcription through Drosha binding to the RNA Polymerase II (Gromak et al., 2013). Furthermore, the microprocessor is involved in RNA Polymerase II pausing and premature

transcriptional termination by opening the transcript for exonucleolytic degradation by Xrn2 and Rrp6 (Wagschal et al., 2012). However, the mechanisms underlying microprocessor-dependent activation or inhibition of mRNA transcription are still relatively unclear.

Taken together, findings over the last few years uncovered different and unexpected noncanonical roles of the microprocessor. These versatile functions are involved in a broad range of biological processes including direct transcriptional regulation and splicing. However, our understanding of the mechanisms underlying these alternate functions of the microprocessor is limited and it needs further investigation. It would be of major interest to understand how the multifaced microprocessor orchestrates brain development and homeostasis.

ALTERNATIVE DROSHA AND DGCR8 COMPLEXES

DGCR8 and Drosha-knockouts exhibit different phenotypes indicating that they may also function separately and possibly interact within other complexes (Macias et al., 2012; Luhur et al., 2014). HITS-CLIP experiments for DGCR8 revealed mRNAs, lncRNAs, snoRNAs, and retrotransposable elements as putative targets (Macias et al., 2012). The discovery that DGCR8 controls snoRNA stability in a Drosha-independent manner confirmed the existence of an alternative DGCR8 complex in association with other nucleases than Drosha. Recently, novel proteins have been found associated with DGCR8 and Drosha using mass spectrometry analysis of DGCR8 or Drosha coimmunoprecipitation assays. This study revealed that DGCR8 forms a complex with the nuclear exosome that targets and degrades mature snoRNAs (Macias et al., 2015). DGCR8 only interacts with the exonuclease when it is localized within the nucleolus, suggesting that, in the nucleoplasm, DGCR8 processes pri-miRNA in complex with Drosha whereas in the nucleolus DGCR8 induces degradation of snoRNAs by interacting with the exonuclease. Moreover, some snoRNAs can be further processed into functional miRNAs (Ender et al., 2008; Scott and Ono, 2011).

The binding between Drosha and DGCR8 can be modulated by other proteins, which can be expressed in a cell and time-specific manner. The transcriptional repressor MeCP2 is implicated in Rett syndrome and autism spectral disorders and MeCP2 binds methylated DNA and recruits histone deacetylase complex (HDAC) (Chahrour and Zoghbi, 2007; Ramocki et al., 2009; Guy et al., 2011). However, MeCP2 can regulate gene expression posttranscriptionally by suppressing miRNA processing (Cheng et al., 2014). MeCP2 competes with Drosha to bind DGCR8 resulting in a reduction of miRNA biogenesis. Deep sequencing of MeCP2-knockout hippocampal tissue revealed an upregulation of mature miRNAs (Cheng et al., 2014). In line with this, overexpression of MeCP2 in mouse cortical neurons represses miRNA maturation and inhibits dendritic and spine growth by suppressing miR-134 which targets CREB, LIMK1, and Pumilio2 that play critical roles during neurodevelopment (Cheng et al., 2014).

Drosha also interacts with different binding partners that could potentially orchestrate its cleavage substrates. One example for an alternative RBP partner for Drosha is the TAR DNA-binding protein 43 (TDP-43). It has been shown that TDP-43 can directly interact with Drosha and a TDP-43 loss of function reduces Drosha in human neuroblastoma cells in vitro. Interestingly, TDP-43 is also involved in Drosha substrate recognition (Di Carlo et al., 2013). Interaction between Drosha

and TDP-43 is required for Drosha-dependent cleavage of Ngn2 mRNA but not DCGR8 mRNA. However, this mechanism still needs to be evaluated in vivo (Di Carlo et al., 2013).

These data support the hypothesis that the microprocessor exists in different complexes, thereby operating on different RNA targets. It will be of further interest to elucidate the alternate Drosha and DGCR8 complexes in NSCs and their functions on neurogenesis.

CONCLUSIONS

Neurogenesis is controlled by a hypostable transcriptome (Hsieh, 2012). miRNAs represent an efficient way to induce translational repression by blocking translation or inducing cleavage of specific transcripts. miRNA-independent functions of the microprocessor through direct binding and cleavage of specific mRNAs add an additional layer of regulation to neurogenesis (Fig. 6.1). The microprocessor affects embryonic NSCs maintenance and prevents differentiation, thus allowing normal brain development (Knuckles et al., 2012). Moreover, our data indicate that Drosha targets several mRNAs and modulates NSC differentiation in the adult brain in regions with active neurogenesis (Rolando et al., 2016). Therefore, the microprocessor is crucial in controlling mRNA levels of key genes involved in NSC maintenance and differentiation by cleaving mRNAs harboring stem loops with characteristics of pri-miRNA. Whether the Drosha-processed hairpin can be further processed by Dicer and RISC to produce a silencing miRNA-like molecule as has been shown for some mirtrons (spliced introns) and snoRNAs remains unknown. Thus, it will be of interest to elucidate whether noncanonical microprocessor cleavage leads to the production of functional miRNAs from processed mRNAs.

Recent studies have identified different and unexpected functions of the microprocessor. Our knowledge is based on experiments performed on immortalized cell lines, and it is a priority to understand how the diverse microprocessor functions are tissue and cell-type specific. Interestingly, some of the alternative microprocessor target RNA species are known to be involved during neurogenesis including alternative splicing variants and snoRNAs (Fig. 6.1). However, it is unclear if the microprocessor's regulation of neurogenesis involves these mechanisms. Alternative splice variants are particularly prominent in the nervous systems and play important roles during neurogenesis (Norris and Calarco, 2012) (Fig. 6.1). Alternative splicing allows multiple mRNA isoforms to be generated through the use of different and alternate splice sites, which is an important mechanism of gene regulation that contributes to transcriptome and proteome diversity (Nilsen and Graveley, 2010; Wang et al., 2008). It will be of interest to address whether Drosha is involved in processing neural-specific splice variants.

One of the alternatively spliced transcripts crucial for neuronal differentiation and maturation is the transcriptional repressor REST. In NSCs, REST occurs in the active isoform and it promotes proliferation, whereas in neurons it exists in an inactive isoform (Raj et al., 2011). PSD-95, an important scaffolding protein essential for synaptic maturation and plasticity of excitatory neurons, is another example of neural-specific splicing. PTBP1/2 induce increased exon skipping of PSD-95 leading to the degradation of the protein. Interestingly, PTBP1 is highly expressed in NSCs where it inhibits PSD-95 accumulation, whereas it is absent in neurons to allow functional PSD-95

expression for synapse formation. Interestingly, PTBP1 binds to Drosha in human cell lines and, therefore, Drosha could be involved in PSD-95 splice regulation (Macias et al., 2015).

Another alternatively processed RNA family regulated by DGCR8 is that of the snoRNAs. snoRNAs posttranscriptionally process RNA by methylation and are involved in various biological processes including rRNA modifications, alternative splicing, transcriptional regulation, genomic imprinting, and cell-cycle regulation (Mehler and Mattick, 2007). snoRNAs are expressed tissue and context specific and are especially abundant in the brain where they are believed to be involved in neurogenesis (Schouten et al., 2012). Growth arrest-specific 5, for example, is expressed in adult DG NSCs and harbors several snoRNAs, SNORD 44,47,74−81, which are assumed to be involved in NSC maintenance and differentiation (Smith and Steitz, 2010; Schouten et al., 2012) (Fig. 6.1). These data suggest that DGCR8 could have influence on neurogenesis via snoRNAs.

The composition of different microprocessor complexes, target recognition during the noncanonical functions, and how RNAs containing pre-miRNA-like loop structures escape microprocessor-mediated cleavage, is still open questions. The microprocessor is almost ubiquitously expressed but it is able to process distinct RNAs in specific cell-types and compartments. For example, the noncanonical function of DGCR8-dependent snoRNA production is restricted to the nucleolus, whereas pre-miRNA processing is carried out in the nucleoplasm (Macias et al., 2015). Therefore, it is possible that Drosha and DGCR8 interact with specific partners that trigger compartmental and cell-type specific functions in RNA processing. Moreover, specific proteins can compete for hairpin containing microprocessor RNA recognition sites and protect the transcripts from processing. One example is Lin28, which inhibits let-7 miRNA maturation by protecting the pre- and pri-miRNA structure from RNAse III cleavage (Thornton and Gregory, 2012; Heo et al., 2009). Moreover, Lin28 can bind to mRNAs with a GGAGA sequence within loop structures that are enriched within exons and untranslated regions of mRNAs including its own and that of other RBPs (Wilbert et al., 2012). Interestingly, a recent study supports the hypothesis that Lin28 inhibits microprocessor targets by demonstrating that Drosha directly mediates the destabilization of Lin28 mRNA targets via their Lin28-responsive elements (Qiao et al., 2012). Therefore, it is possible that a similar mechanism involving Lin28 or some of the other >1500 annotated RBPs in the genome could protect microprocessor RNA targets from cleavage, thereby stabilizing their transcripts when needed.

The identification of noncanonical functions for the microprocessor complex opens new perspectives in the field of NSC biology. Further analysis will aim to provide new insights into the complex role of the microprocessor in controlling gene expression during neurogenesis.

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2. Results

2.1 Manuscript – Drosha regulates the timing of neural stem cell differentiation

During cortical development NSCs generate different neurons in a tightly controlled temporal order, thereby forming a six-layered neocortex. This complex process needs to be tightly controlled on different levels. One level includes the regulation of gene expression, for example through post-transcriptional regulation. Here we show that Drosha plays a significant role during neurogenesis. We discovered that NSCs are Drosha-dependent to time the differentiation of deep- and upper-layer neurons. We propose Drosha controls the timing of deep-layer neurogenesis by regulating the post-transcript of the deep-layer marker Ctip2. This study provides further evidence that RNA modification plays a crucial role during embryonic neurogenesis.

2.1.1 Contribution

The following manuscript is formatted to submit to Development. For this manuscript I performed all experiments, except the RNA Immunoprecipitation of Ctip2 hairpins (Fig. 6F), I prepared all the figures and wrote the text.

Drosha controls neural stem cell fate-decision during cortical differentiation

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Running title

Drosha regulates cortical development

Summary statement

Drosha post-transcriptionally regulates the fate of neuronal progenitors

Abstract

Cortical development is a highly complex process, during which neural stem cells (NSCs) give rise to distinct neuronal layers in a tightly controlled temporal order, forming a six-layered neocortex. This process involves the precise regulation of gene expression. Here, we show that the RNaseIII Drosha plays a crucial role during early corticogenesis. Using a conditional knock-out system, we deleted Drosha in NSCs at defined time points during cortical development. Our results show that early but not late NSCs depend on Drosha to maintain the stem cell pool and to regulate temporal differentiation. We find that Drosha deletion in NSCs early during development increases the potential to differentiate into deep-layer neurons, while they are less likely to commit to the upper-layer lineages. Furthermore, we show that Drosha directly regulates the mRNA stability of the deep-layer specific transcription factor Ctip2. Based on these findings we propose that Drosha controls the timing of deep-layer neurogenesis by directly down-regulating the transcripts of layer-specific factors as Ctip2. Our study highlights the crucial role of RNA modification for fate determination during embryonic cortical development.

Key Words

Drosha, neurogenesis, corticogenesis, Ctip2, post-transcriptional regulation, neural fate

Abbreviations

cKO	Conditional Knock-Out
CLIP	Crosslinked-Immunoprecipitation
CS	Coding sequence
E	Embryonic day
E/E	Tamoxifen administration at E/Analysis at E
IP	Intermediate progenitor
IZ	Intermediate zone
hp	Hairpin
Ngn2	Neurogenin 2
NSC	Neural stem cell
SVZ	Subventricular zone
TAM	Tamoxifen administration
VZ	Ventricular zone
wt	Wild type

Introduction

The mammalian neocortex is a highly complex organ containing a large diversity of neuronal subtypes and glia cells, enabling the organism to perceive, understand and react to its environment. During mammalian neurodevelopment the neuroepithelium of the neural tube gives rise to the morphological complex central nervous system. Along the luminal side of the neural tube the NSCs form the ventricular zone (VZ) and give rise to the different neuronal and glial cell types during development (Anthony et al., 2004). Cerebral development is a temporal tightly regulated process that results in the formation of a six-layered isocortex. Dorsal NSCs first differentiate into deep-layer glutamatergic neurons expressing Tbr1 and Ctip2, followed by upper-layer neurons expressing Brn2, Satb2 and Cux1 later during development (Angevine and Sidman, 1961, Greig et al., 2013).

It is unclear whether all NSCs are multipotent and sequentially differentiate into the neuronal subtypes of the different cortical layers or if there are different pools of NSCs with more restricted potential that give-rise to specific neuronal subtypes (Franco and Muller, 2013). Independent of NSC potency, however, the timing of the sequential differentiation of NSCs must be tightly regulated. Although the timing and sequence at which individual neuronal subtypes and cortical layers are formed is well understood, the molecular mechanism(s) controlling NSC fate decisions in this precise temporal manner remains elusive. It has been shown that cell-intrinsic programmes, in addition to extrinsic signals, play a major role in NSC fate decision (Song, 2008, Toma et al., 2014). The essential effectors of Notch the bHLH genes Hes1 and Hes5 act as transcriptional repressors of neuronal differentiating factors, maintaining NSCs in an undifferentiated state (Ohtsuka et al., 2001, Hatakeyama et al., 2004). However, the right combination of signalling cues can overcome this repression and induces neuronal differentiation. Hence, a reversible silencing mechanism enables neural progenitors to prevent premature differentiation while maintaining the capacity to rapidly differentiate upon receiving differentiation signals (Kageyama and Nakanishi, 1997, Hirabayashi and Gotoh, 2010, Yao et al., 2016, Lilja et al., 2013, Kageyama et al., 2010). Major players controlling the equilibrium state

of NSCs include epigenetic mechanisms and mRNA regulators (Yao et al., 2016, Pilaz and Silver, 2015). The RNaseIII Drosha is involved in miRNA biogenesis (Bartel, 2004) and has recently been shown to control stem cell differentiation by directly inhibiting specific mRNAs in a miRNA-independent manner (Chong et al., 2008, Knuckles et al., 2012). Particularly, Drosha was shown to maintain NSCs in an undifferentiated state by preventing the accumulation of the proneural gene Neurogenin 2 (Ngn2) (Knuckles et al., 2012). Furthermore, it was recently demonstrated that the miRNA-independent function of Drosha plays a crucial role in fate decision of adult NSC (Rolando et al., 2016). These findings suggest that Drosha is involved in controlling NSC fate during neurodevelopment and in the adult brain. During neurodevelopment, the expression of differentiation factors is tightly controlled, thereby timing the layer-specific differentiation (Hirabayashi and Gotoh, 2010).

Hence, Drosha is a potential post-transcriptional regulator of neuronal differentiation factors. To examine the temporal role of Drosha in cortical development, we conditionally knocked-out (cKO) Drosha from NSCs at defined time points during cortical development. Our results revealed that Drosha is required during early neurogenesis to maintain the pool of NSCs and to limit the differentiation of deep-layer neurons. Additionally, we find that early Drosha-deleted NSCs are less likely to commit to upper-layer neuronal fate. *In vitro* experiments show that deletion of Drosha results in an up-regulation of the deep-layer transcription factor Ctip2 and that Drosha binds to the evolutionarily conserved hairpins in the Ctip2 mRNA. Based on these findings we propose a potential mechanism where Drosha regulates NSC fate and differentiation by controlling levels of Ctip2 on the transcript level.

Results

Drosha is required for early NSC fate decision

During embryonic development NSCs express high level of the Notch target Hes5 (Basak and Taylor, 2007). Therefore, we used Hes5:CreER^{T2} mice carrying a floxed Drosha allele to generate Drosha cKO in NSCs and examined the effects on NSC fate decision and a Rosa26-CAG::EGFP reporter to lineage trace individual NSCs and their progeny where Cre had been active (Fig. 1A). Tamoxifen (TAM) induced recombination resulted in a significant reduction of Drosha expression in GFP$^+$ NSCs (Fig S1A,B). To study the temporal requirement of Drosha during cortical development, we induced recombination by TAM administration to the pregnant mothers at embryonic days (E) E10.5, E12.5 and at E14.5 (Fig. 1B). The effect of Drosha deletion was analysed at E18.5 when the cytoarchitecture of the cortex was fully developed in control mice (Fig. 1B). To focus on the role of Drosha in cell fate decision, we investigated the commitment of differentiating Drosha cKO NSCs to either the deep layer (Layer VI+V) or the upper layer (Layer IV-II) fates. When we administrated TAM at E10.5 and analysed the animals at E18.5 (E10.5/E18.5) we observed more GFP$^+$Ctip2$^+$ cells in the deep-layers of the cortex in Drosha cKO than in control embryos. This suggested, that Drosha cKO NSCs generate more Ctip2$^+$ deep-layer neurons than wt NSCs (Fig 1C,D). However, Drosha cKO at later time points (E12.5 and E14.5) resulted in no significant differences in the differentiation of GFP$^+$Ctip2$^+$ deep-layer neurons at E18.5 (Fig. 1D).

We then analysed the effect of Drosha cKO on Brn2$^+$ upper-layer (IV-II) neuron production. Induction of Drosha cKO at E10.5 and E12.5 resulted in a significant reduction of GFP$^+$Brn2$^+$ upper-layer neurons at E18.5 (Fig. 1E,F). Upon TAM treatment at E14.5 we did not observe significant changes in upper-layer neurons differentiation (Fig. 1F). These results suggest that Drosha plays a role in the fate decision of NSCs during early cortical development.

Drosha is required to limit the differentiation of NSCs into deep-layer neurons

To study the temporal requirement of Drosha in NSC fate decision, particularly in controlling deep-layer differentiation, we analysed Drosha cKO embryos two, four, six and eight days after TAM administration at E10.5 (Fig. 2A). In order to determine if Drosha cKO NSCs showed a higher tendency to commit to deep-layer fate, we compared the number of GFP^+Ctip2^+ cells in the deep layers of Drosha cKO and control embryos. We only observed a significant increase in GFP^+Ctip2^+ cells in E10.5/E18.5 embryos (Fig. 1D), while this increase was not observed at earlier developmental stages (E10.5/E12.5, E10.5/E14.5, E10.5/E16.5) (Fig. 2B, S2E). In addition induction of Drosha cKO at E12.5 did not result in significant differences in deep-layer neuron differentiation (E12.5/E14.5, E12.5/E16.5, Fig. 2C, S2E). Therefore we examined the expression of another deep layer neuron-marker Tbr1 and observed similar results (Fig. 2D-G). We detected an increase in GFP^+Tbr1^+ neurons in E10.5/E18.5 embryos but not before E18.5 and when we induced recombination at later stages. These findings suggest that deletion of Drosha results in the appearance of ectopic deep-layer $Ctip2^+$ as well as $Tbr1^+$ neurons between E16.5 and E18.5, at a developmental stage where deep-layer differentiation is normally already completed.

During most of the neurogenic period NSCs generate neurons via intermediate progenitors (IP) a committed cell type that can undergo a limited number of proliferative divisions before differentiating into neurons (Hevner et al., 2006). To investigate whether increased NSC differentiation into deep-layer neurons in Drosha cKO embryos is a result of increased production of IPs, we analysed the expression of Tbr2. We did not observe significant changes in the number of GFP^+Tbr2^+ cells at any stage of our analysed Drosha cKO time points (Fig. S2A-C). These results suggest that the increase in deep-layer neurons in Drosha cKO embryos is not a result of increased IP production. However, Drosha cKO did result in a significant reduction in the thickness of the subventricular zone (SVZ) and the VZ. This indicates a loss of stem and progenitor cells (Fig. S2A, D), suggesting that Drosha is required for the maintenance of the neuronal progenitor pool.

Drosha is required for the maintenance of the NSC reservoir

Since the progenitor pool was reduced in Drosha cKO (Fig. S2A, D) and that Drosha is known to be crucial for NSC maintenance (Knuckles et al., 2012), we investigated whether Drosha cKO affected the number of NSCs. Staining for the cortical NSC marker Pax6 revealed a decrease in the number of GFP^+Pax6^+ NSCs in the VZ of Drosha cKO E10.5/E18.5 and E12.5/E18.5 embryos (Fig. 3A-D). However, this phenotype only manifested at E18.5, earlier time points did not result in significant differences in NSC number (Fig. 3C,D). Later TAM induction at E14.5 did not result in changes in the number of GFP^+Pax6^+ NSCs (Fig. S3).

Furthermore, we investigated the mitotic activity in Drosha cKO NSCs staining for the G2/M phase marker phospho histone H3 (pH3). The number of mitotic G2/M phase NSCs localized at the apical side of the VZ showed a tendency to be reduced at E10.5/E14.5 and E10.5/E16.5 and was significantly reduced at E10.5/E18.5 in Drosha cKO embryos compared to control embryos (Fig. 3E,G). Induction at E12.5 did not result in significant changes in the number of GFP^+pH3^+ NSCs, however, we observed a slight tendency of reduced proliferation at E12.5/E16.5. These data suggest that during development Drosha is crucial to maintain NSCs until E12.5.

Drosha inhibits and binds the mRNA of the transcription factor and deep-layer marker Ctip2

To examine whether we can recapitulate the *in vivo* role of Drosha in NSC maintenance *in vitro*, we performed Drosha cKO in neurosphere forming cells. Drosha cKO inhibited both neurosphere growth as indicated by reduced neurosphere diameter (Fig. S4A-C) and sphere forming capacity, suggesting a loss of self-renewing NSCs (Fig S4D). This indicates that Drosha regulates NSC maintenance *in vitro*, as observed *in vivo*.

Deep-layer neuron specification has been shown to depend on a regulatory network containing the transcription factor Ctip2 (Shimizu et al., 2010, McKenna et al., 2011, Arlotta et al., 2005). Our data suggested that early NSCs show an increased and potentially prolonged differentiation into

Ctip2^{+} neurons upon deletion of Drosha. Therefore, we took advantage of the *in vitro* neurosphere system to address whether Drosha is involved in the regulation of Ctip2 expression. Drosha is known to directly inhibit mRNA expression through binding and cleavage of conserved hairpin loops in mRNAs (Han et al., 2009, Chong et al., 2010, Knuckles et al., 2012, Macias et al., 2012, Rolando et al., 2016). *In silico* analysis of evolutionarily conserved hairpins in mRNAs revealed that the Ctip2 mRNA contains several conserved hairpins (Fig. 4A), making Ctip2 a potential target for Drosha-mediated cleavage (Pedersen et al., 2006). Therefore, we examined whether Drosha inhibits Ctip2 expression by performing RT-qPCR analysis on Drosha cKO embryonic neurospheres (Fig. 4B). Drosha cKO neurospheres showed increased expression of Ctip2 and the known Drosha target DGCR8 (Han et al., 2009) (Fig. 4C). These results suggest that Drosha inhibits the expression of Ctip2 in NSCs.

To examine whether Drosha directly binds the Ctip2 transcripts in NSCs, we performed a cross-linked immunoprecipitation (CLIP) with endogenous Drosha (Fig S4E). Our results revealed that the Ctip2 transcript was bound by Drosha in NSCs (Fig 4D). To test whether the hairpins of Ctip2 convey Drosha association, we placed the Ctip2 hairpins in a synthetic mRNA encoded by the psiCheck reporter vector downstream of Renilla Luciferase (rLuc) (Fig. 4E). The five hairpins in the coding sequence (CS) of Ctip2 are very close to each other, therefore we clustered the first three and the last two hairpins into two vector constructs (Fig. 4A). To determine Ctip2 hairpin binding by Drosha, we expressed the hairpin containing rLuc mRNAs in N2a cells together with Drosha-FLAG and performed a CLIP. All Ctip2 hairpin-containing transcripts were bound more efficiently to Drosha than the control rLuc transcript (Fig. 4F). The Ctip2 CS hairpin 1, UTR hairpin 1 and UTR hairpin 3 were bound most efficiently by Drosha (Fig. 4F). These data indicate that Drosha can bind Ctip2 mRNA via multiple hairpins in its transcript.

In summary, our data suggest that early during development Drosha inhibits Ctip2 expression via interaction with the evolutionarily conserved hairpins of the Ctip2 transcript. Therefore, Drosha mediated repression of

layer-specific transcription factors might be a potential mechanism by which Drosha controls deep-layer differentiation.

Discussion

During mammalian neurodevelopment NSCs give rise to distinct neuronal subtypes in a tightly regulated manner. Precise coordination of neuron production is ensured by several regulatory mechanisms that include post-transcriptional modification. Here, we utilized a genetic approach to knock-out Drosha from NSCs at defined time points during cortical development. A floxed Drosha allele allowed us to genetically delete Drosha in a mosaic manner and to investigate the behaviour of Drosha cKO NSCs in an otherwise wild type environment. Using this approach, we have for the first time addressed the temporal requirement of Drosha during cortical development.

We find that Drosha is essential in early but not late NSCs to control proper sequential differentiation into deep- and upper-layer neurons. An early knock-out of Drosha (E10.5) results in increased differentiation of deep-layer neurons between E16.5 and E18.5, a time where deep-layer differentiation is completed in control animals. Since we did not observe changes in the number of IPs and NSCs before E16.5, we conclude that increased numbers of deep-layer neurons are a result of a prolonged differentiation phase. These findings suggest a prominent role of Drosha in terminating deep-layer differentiation in early NSCs. Furthermore, we observed a reduction in upper-layer $Brn2^+$ neurons and the neural progenitor pool when Drosha was deleted at E10.5 and E12.5. These results suggest that Drosha also plays a crucial role in maintaining the NSCs that give rise to upper-layer neurons.

Along this line we find that Drosha cKO at E14.5, the beginning of upper-layer differentiation, had no effect on neuronal differentiation or NSC maintenance, suggesting that Drosha function is critically before E14.5. These results suggest that upon initiation of upper-layer differentiation Drosha cKO has no effect on neuronal differentiation or NSC maintenance.

Early Drosha cKO at E10.5 and E12.5 had striking effects on the NSC pool at late developmental stages, resulting in a significant reduction of neuronal progenitor numbers at E18.5. Our lab previously showed that Drosha knock-down in E13.5 NSCs leads to precocious differentiation at E15.5 in a miRNA-independent way (Knuckles et al., 2012). Similar to the here presented results, this previous study did not observe an increase in IPs

but a reduction of proliferating NSCs and a slowing of their cell-cycle upon Drosha knock-down. The combined results of these two studies suggest that Drosha ablated NSCs undergo direct differentiation without generating Tbr2-expressing IPs (Noctor et al., 2004). It will be of future interest to investigate what role Drosha plays in this process.

Several studies highlight miRNA-independent functions of Drosha in regulating several cellular processes, including fate decision (Han et al., 2009, Chong et al., 2010, Karginov et al., 2010, Knuckles et al., 2012, Rolando et al., 2016). Here, we provide evidence that Drosha can directly modulate the mRNA level of the layer-specific transcription factor Ctip2, providing a potential mechanism by which Drosha controls temporal differentiation of NSCs. Ctip2 is an important transcription factor involved in deep-layer neuron specification (Chen et al., 2008, Kumamoto et al., 2013). Recently, it was suggested that a slight disturbance in the transcription factor composition can influence neuronal fate decision (Toma and Hanashima, 2015). Hence, Drosha potentially fine-tunes the neuronal fate decision by modulating the expression of transcription factors, such as Ctip2.

Being a main component of miRNA biogenesis pathway, Drosha ablation in NSCs results in a reduction of miRNA levels, however this reduction only manifests with a temporal delay (Knuckles et al., 2012). Therefore, it is of interest to distinguish between miRNA-dependent and miRNA-independent Drosha cKO phenotypes. For this reason, we can compare our data with published Dicer cKO data. As the cytoplasmic RNaseIII involved in miRNA maturation, Dicer KO serves as ideal model to analyse miRNA-dependent functions (Volvert et al., 2012). Similar to Drosha cKO, also Dicer cKO in NSCs was shown to effect several developmental processes, highlighting the versatile functions of Dicer depending on the developmental stage (Volvert et al., 2012). As Drosha, also Dicer is involved in the timing of deep-layer differentiation (Saurat et al., 2013, De Pietri Tonelli et al., 2008). However, early Dicer cKO resulted in prolonged differentiation of $Tbr1^+$ but not $Ctip2^+$ deep-layer neurons as we observed for Drosha (De Pietri Tonelli et al., 2008, Kawase-Koga et al., 2009, Saurat et al., 2013). These results suggest that Drosha potentially regulates Tbr1 differentiation in a miRNA-dependent and

Ctip2 differentiation in a miRNA-independent manner. Similar to Drosha cKO also Dicer cKO mice revealed a reduced neural progenitor pool (De Pietri Tonelli et al., 2008). However, *in vitro* results of neurospheres suggest, that Drosha maintains NSCs predominantly by inhibiting differentiation and decelerating of the cell-cycle (Knuckles et al., 2012), whereas Dicer maintains the intermediate progenitor pool by preventing apoptosis of differentiating cells (Kawase-Koga et al., 2010). Furthermore, Dicer cKO NSCs could be propagated indefinitely, whereas Drosha cKO NSCs where lost after few passages (data not shown) (Knuckles et al., 2012, Kawase-Koga et al., 2010, Andersson et al., 2010). These results suggest different mechanisms of the maintenance of the neural progenitor pool between the two RNases.

The field of RNA modification and processing is emerging over the last few years (Frye et al., 2016). In neurobiology, RNA modification is currently intensely studied in the field of brain plasticity (Nainar et al., 2016). However, during neurodevelopment little is known about the role and function of post-transcriptional regulation (Pilaz and Silver, 2015). The here provided insight into the role of Drosha during corticogenesis adds to the evolving field of RNA modification, especially, demonstrating how post-transcriptional regulation can influence fate choice and commitment.

Materials and Methods

Animal Husbandry

Mice (*mus musculus*) were maintained on a 12-hour day-night cycle with adequate food and water under specific pathogen-free conditions according to Swiss federal regulations. All procedures were approved by the Basel Cantonal Veterinary Office (license numbers ZH-TAY and 2537). Hes5::CreER^{T2}, Rosa26-CAG::EGFP, Hes5::GFP, Drosha$^{fl/fl}$ mice have been described elsewhere (Lugert et al., 2012, Tchorz et al., 2012, Chong et al., 2008). All mice were maintained on a C57BL6 background. Noon at the day of the vaginal plug was considered as embryonic day 0.5 (E0.5). Cre-recombinase activity from the Hes5CreER^{T2} locus was induced by TAM with a single administration by gavage (Sigma; 2 mg/gavage in corn oil).

Tissue preparation and immunohistochemistry

Pregnant females were sacrificed by CO_2 inhalation, embryos were isolated, put on ice and decapitated. Brains were dissected on ice, post-fixed overnight in 4% PFA in PBS and cryoprotected in 30% sucrose at 4°C overnight. Brains were embedded and frozen in OCT (TissueTEK) on dry ice. Tissue was cryo-sectioned (Leica) as 20 µm coronal sections, collected on Superfrost glass slides (Thermo Scientific) and stored at -20 °C until use.

For immunostaining, sections were incubated overnight at room temperature with primary antibody diluted in blocking solution of 3% normal donkey serum (Jackson ImmunoResearch) and 0.2% Triton X-100 in PBS. Sections were washed three times in PBS and incubated at room temperature for 1 hour with the corresponding secondary antibodies in blocking solution and counterstained with DAPI (1 µg/ml). For Ctip2, Pax6, pH3, Tbr1 and Tbr2 (Millipore), antigens were recovered at 80 °C for 20 min in sodium citrate solution (10 mM, pH 6.0) and for Tbr2 (Abcam) 30 min at 80 °C in 10mM Tris Base, 1mM EDTA Solution, pH9. Stained sections were embedded in mounting medium containing diazabicyclo-octane (DABCO; Sigma) as an anti-fading agent and visualized using a Zeiss Observer with Apotome. Antibodies used for immunolabeling were goat anti- Brn2 (1:250, sc-6029, Santa Cruz), rat anti-Ctip2 (1:400, Abcam), rabbit anti-Pax6 (1:400, PRB-

278P, Covance), rabbit anti-pH3 (1:200, Millipore), rabbit anti-Tbr1 (1:500, Abcam), chicken anti-Tbr2 (1:250, Millipore), rabbit anti-Tbr2 (1.:1000, Abcam). Secondary antibodies: Alexa488/Cy3/Cy5/Alexa647 conjugated anti-chicken, mouse, goat, rabbit, rat and sheep immunoglobulin (1:500, Jackson Immunoresearch).

Neurosphere cultures and Adeno-Cre adenovirus infection *in vitro*

Rosa26-CAG:EGFP$^{fl/+}$ and *Drosha*$^{fl/fl}$ *Rosa26-CAG::EGFP*$^{fl/+}$ brains of E14.5 embryos were transferred to L15 medium (Gibco). The dorsal forebrain was dissected, the meninges and olfactory bulbs were removed and mechanically dissociated in a papain based solution and cultured in the presence of FGF2 as described previously (Basak and Taylor, 2007, Giachino and Taylor, 2009). Cre recombinase–expressing adenovirus (adeno-Cre) infection and analysis of neurosphere number were performed as described previously (Nyfeler 2005).

RNA Isolation and RT-qPCR

Total RNA was isolated using the phenol-chloroform method (Trizol, Life Technologies) and resuspended in water. RNA was treated with RNase-free DNase I (Roche) to remove genomic DNA contamination. First-strand cDNA was generated using BioScript (Bioline) using random hexamer primers followed by quantitative PCR using SensiMix SYBR kit (Bioline). For expression analysis of genes of interest we used the comparative Ct method using Rpl29 and mRpl19 as normalizing genes (Zhou et al., 2010) performed on a Rotor-Gene Q (Qiagen). Used RT-qPCR:

Rpl29:
fwd ACAGAAATGGCATCAAGAAACCC
rev TCTTGTTGTGCTTCTTGGCAAA
Six3:
fwd TCAGCAGAGTCACCGTCCAC
rev TGGAGGTTACCGAGAGGATCG
Drosha:

fwd GACGACGACAGCACCTGTT
rev GATAAATGCTGTGGCGGATT
DGCR8:
fwd GGAGCTAGATGAAGAAGGAACAGG
rev GTAAAGCGTCCACATCATTGTCAA
Ctip2:
fwd CATGAGAGCGACCCATCTCT
rev CAGCAGCAGCTCCTCTTCTT)

Endogenous CLIP with Nerual Stem Cells

Experiment was carried out as described in (Rolando et al., 2016).

Crosslinking and immunoprecipitation

Experiment was carried out as reported recently (Rolando et al., 2016) with minor modifications. psiCheck2 vectors containing the Ctip2 hairpins were transfected together with p3X-FLAG-CMV or pCK-Drosha-WT-FLAG (Han et al., 2009, Knuckles et al., 2012). Immunoprecipitation of Drosha was performed using Dynabeads G (Invitrogen) coupled to anti-Drosha antibody (Cell Signaling, D28B1).

Quantification and Statistical Analysis

Stained sections were observed with a Zeiss Observer with Apotome. Distinct cortical layers were defined according to Molyneaux *et. al.* (Molyneaux et al., 2007). Absolute numbers of $GFP^{+}marker^{+}$ are corrected for recombination efficiency and layer thickness-difference between Drosha cKO and wt embryos. Statistical comparisons were conducted by two-tailed unpaired Student's t test. Significance was established at $p < 0.05$.

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Competing interests

The authors do not declare any competing or financial interests in this work.

Author contributions

A. Erni, C.R., A. Engler and N.I. designed and performed experiments and evaluated and interpreted the data. V.T. conceived and designed the project and evaluated the data. A. Erni and V.T. wrote the paper and prepared the figures. All authors edited and proofread the manuscript.

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Figure legends

Figure 1 – Drosha is required for NSC-differentiation during early corticogenesis

(**A**) Scheme of the Hes5::CreERT2, the Rosa26-CAG::EGFP reporter and the floxed Drosha allele and Cre-mediated rearrangements. (**B**) Scheme of recombination induction with TAM and chases after Drosha ablation. (**C**) GFP^+Ctip2^+ deep-layer projection neurons (arrowheads) in wt and Drosha cKO animals. TAM induction at E10.5, analysis at E18.5. (**D**) Quantifications of absolute GFP^+ $Ctip2^+$ cells at E18.5 when TAM was administrated at E10.5, E12.5 or E14.5. (**E**) GFP^+Brn2^+ deep-layer projection neurons (arrowheads) in wt and Drosha cKO animals. TAM induction at E10.5 or E12.5, analysis at E18.5. (**F**) Quantifications of absolute GFP^+ $Brn2^+$ cells at E18.5 when TAM was administrated at E10.5, E12.5 or E14.5.

Data are presented as mean ±SD, D: wt/cKO E10.5 and E12.5 n=3, E14.5 n=4, F: wt/cKO E10.5, E12.5, wt E14.5 n=3, cKO E14.5 n=4. Two-sided Student´s t-test: *p < 0.05. Scale bar = 50µm.

Figure 2 – Termination of deep-layer differentiation is Drosha-dependent

(**A**) Scheme of sequential neuronal differentiation during corticogenesis. (**B**) Quantifications of absolute GFP^+Ctip2^+ cells in layer VI-V 2, 4 and 6 days after TAM administration at E10.5. (**C**) Quantifications of absolute GFP^+Ctip2^+ cells in layer VI-V 2 and 4 days after TAM administration at E12.5. (**D,E**) GFP^+Tbr1^+ neurons in layer V in wt and Drosha cKO animals. TAM induction at E10.5 (D) and E12.5 (E), analysis at E18.5. (**F**) Quantifications of absolute GFP^+Tbr1^+ cells in layer V 2, 4 and 6 days after TAM administration at E10.5. (**G**) Quantifications of absolute GFP^+Tbr1^+ cells in layer VI 2 and 4 days after TAM administration at E12.5. Data are presented as mean ±SD, wt/cKO TAM E10.5; Analysis E12.5/E14.5/E16.5, TAM E12.5; Analysis E14.5 n=3. TAM E12.5; Analysis E16.5 n=4. Two-sided Student´s t-test: *p < 0.05. Scale bar = 50µm.

Figure 3 – Drosha is required for NSC maintenance

(**A,B**) GFP^+Pax6^+ NSCs in the VZ in wt and Drosha cKO animals with TAM induction at E10.5 (A) and E12.5 (B), analysis at E18.5. (**C**) Quantification of absolute GFP^+Pax6^+ cells 2, 4, 6 and 8 days after TAM administration at E10.5. (**D**) Quantifications of absolute GFP^+Pax6^+ cells 2, 4 and 6 days after TAM administration at E12.5. (**E,F**) GFP^+pH3^+ NSCs in the apical VZ in wt and Drosha cKO animals with TAM induction at E10.5 (E) and E12.5 (F), analysis at E18.5. (**G**) Quantifications of absolute apical GFP^+pH3^+ cells 2, 4, 6 and 8 days after TAM administration at E10.5. (**H**) Quantifications of absolute apical GFP^+pH3^+ cells 2, 4 and 6 days after TAM administration at E12.5. Data are presented as mean ±SD. C: TAM E10.5; Analysis E14.5, E16.5 wt/cKO n=3, TAM E10.5; Analysis E12.4, E18.5 n=4. D: n=3, G: TAM E10.5; Analysis E14.5 wt/cKO, E16.5 wt, E18.5 wt n=3, TAM E10.5; Analysis E12.5 wt/cKO, E16.5 cKO, E18.5 cKO n=4. H: TAM E12.5; Analysis E14.5 wt/cKO, E18.5 cKO n=3, TAM E12.5; Analysis E16.5, E18.5 n=4 . Two-sided Student´s t-test: *$p < 0.05$, **$p < 0.01$. Scale bar = 50µm.

Figure 4 – Drosha inhibits the transcript of Ctip2

(**A**) Scheme of the Ctip2 transcript, containing several evolutionarily conserved hairpins (hp) in the coding sequence (CS) and the 3'UTR. (**B**) RT-qPCR on Drosha ablated embryonic neurospheres. (**C**) CLIP of endogenous Drosha from NSCs pull-down Ctip2 mRNA. DGCR8 and Six3 mRNAs were used as positive and negative controls, respectively. (**D**) Scheme of psiCheck Renilla Luciferase constructs (rLuc) used for RNA Immunoprecipitation with Drosha. Ctip2 CS and UTR hps were inserted in the SV40 UTR of the rLuc. (**E**) RT-qPCR analysis of Drosha pulled-down rLuc constructs containing the Ctip2 hps transfected iton N2a cells, represented as relative pull-down to an empty rLuc vector. Data are presented as mean ±SD n B and C, n=3, E: n=1.

Figure S1 – Cre-expressing cells efficiently reduce Drosha expression

(**A**) Genotypes of wt and Drosha cKO animals and schematic representation of TAM administration and chase duration. (**B**) RT-qPCR of FACS sorted cells, TAM E10.5 6, FACS E16.5. Two-sided Student´s t-test: p***<0.001, data are presented as mean ±SD, n=3.

Figure S2 – Drosha depletion does not change IP generation but decreases the neural progenitor pool

(**A**) GFP^+Tbr2^+ cells in the VZ/SVZ in wt and Drosha cKO animals with TAM induction at E10.5, analysis at E18.5. (**B**) Quantifications of absolute GFP^+Tbr2^+ cells 2, 4, 6 and 8 days after TAM administration at E10.5. (**C**) Quantifications of absolute GFP^+Tbr2^+ cells 2, 4 and 6 days after TAM administration at E12.5. (**D**) Thickness of VZ/SVZ in Drosha cKO and wt embryos after TAM induction at E10.5 or E12.5, analysis at E18.5. (**E**) GFP^+Ctip2^+ neurons in layer VI+V in wt and Drosha cKO animals. TAM induction at E10.5 or E12.5, analysis at E18.5. Data are presented as mean ±SD. B and C: n=3, D: TAM E10.5; Analysis E18.5 n=4, TAM E12.5; Analysis E18.5. Two-sided Student´s t-test, **p < 0.01, ***p < 0.001. Scale bar = 50µm.

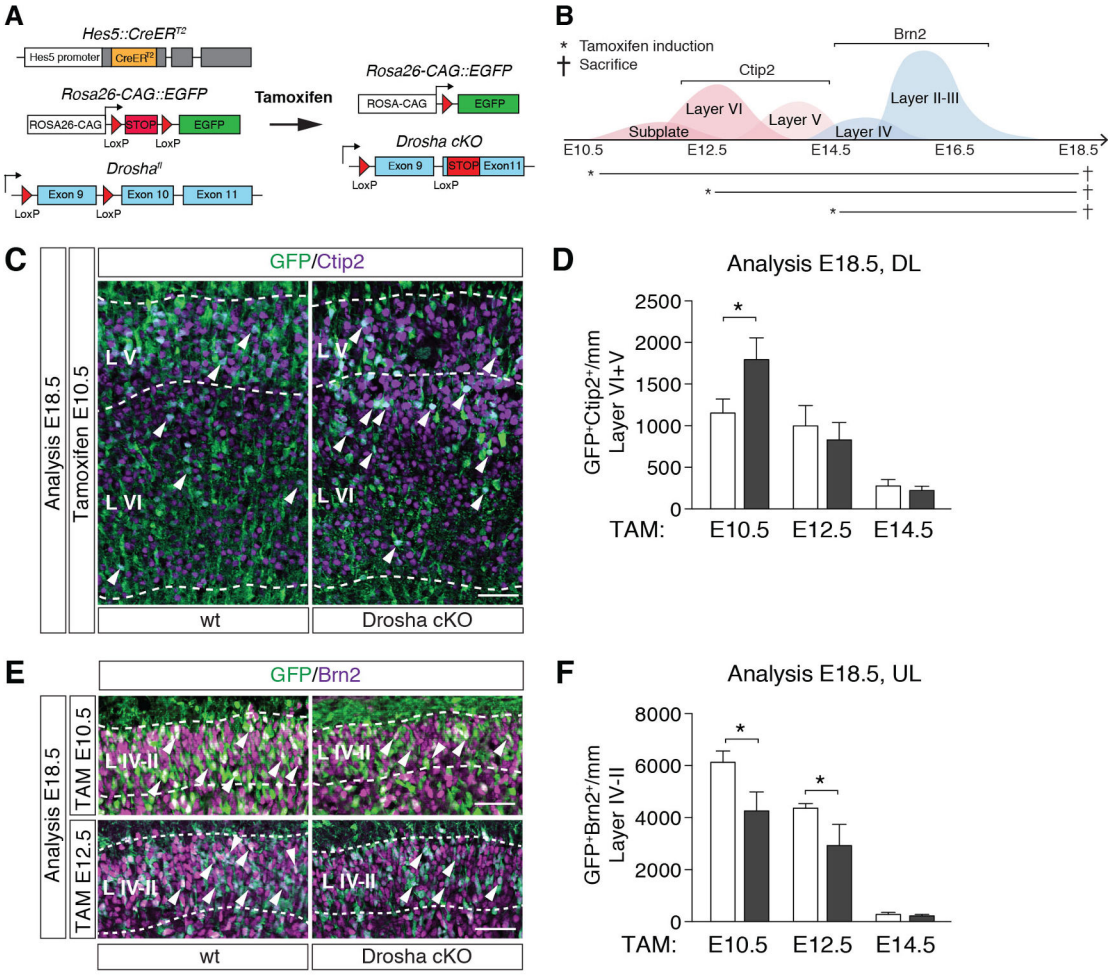
Figure S3 – Drosha is required for NSC maintenance of early NSCs

(**A**) Scheme of E14.5 recombination induction with TAM and a 4-day chase until E18.5. (**B**) GFP^+Pax6^+ NSCs in the VZ of wt and Drosha cKO animals with TAM induction at E14.5, analysis at E18.5. (**C**) Absolute numbers of GFP^+Pax6^+ NSCs in the VZ. Data are presented as mean ±SD, n=3. Two-sided Student´s t-test. Scale bar = 50µm.

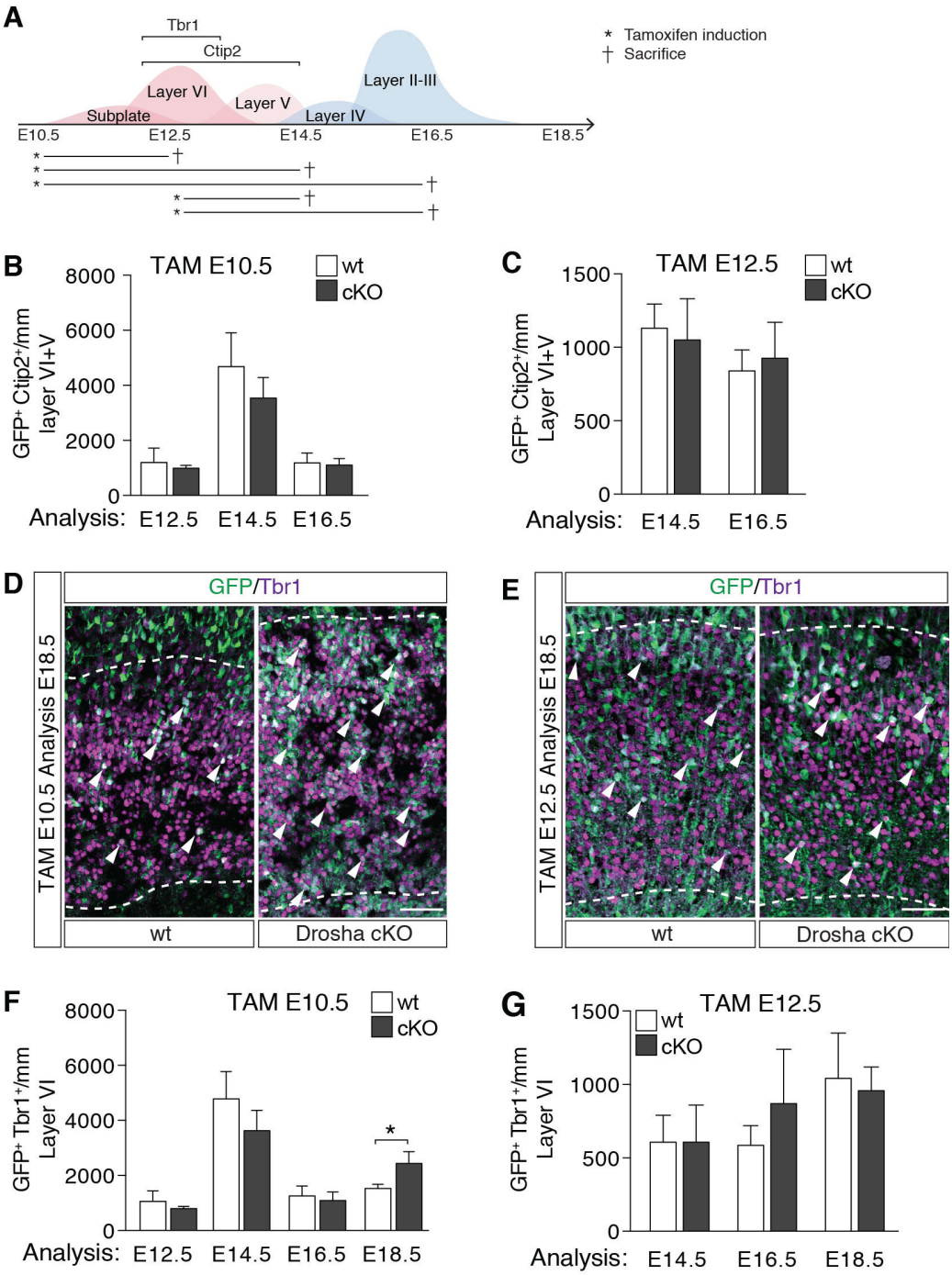
Figure S4 – Drosha depletion reduces neurosphere-growth

(**A**) Embryonic neurospheres after Adeno-Cre induced gene recombination. (**B-C**) Diameter of neurospheres 5 days after Adeno-Cre-infection (n≥49). (**D**) Sphere-forming capacity of Drosha cKO cells (n=12). (**E**) Scheme of Drosha ablation from embryonic neurospheres using Adeno-Cre virus, followed by FACS and RT-qPCR analysis of recombined GFP^+ cells. (**F**) Scheme of CLIP

procedure using NSCs followed by RT-qPCR analysis. Data are presented as mean ±SD, two-sided Student´s t-test: ***p < 0.0001. Scale bar = 100 µm.



Erni *et.al.* Figure 1



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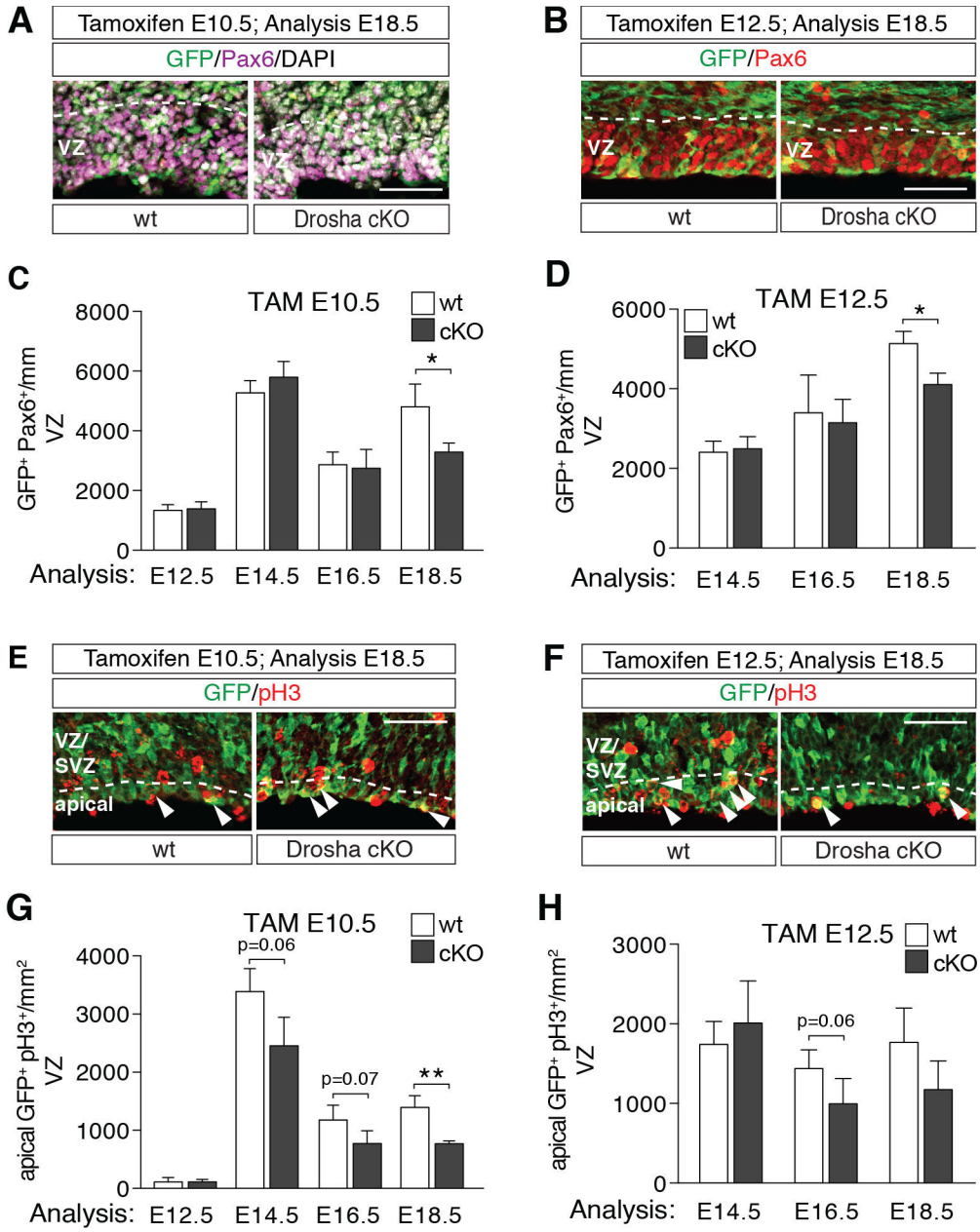

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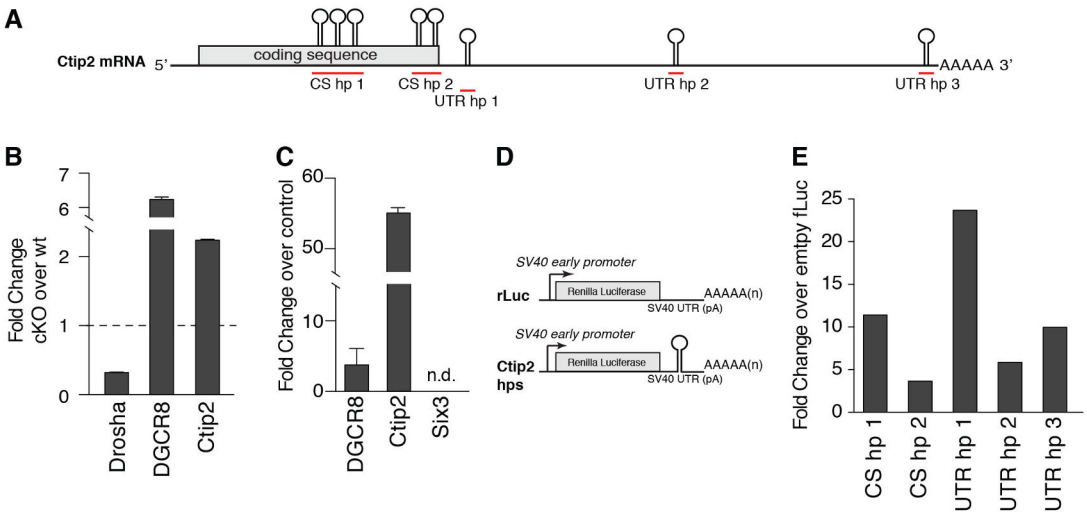

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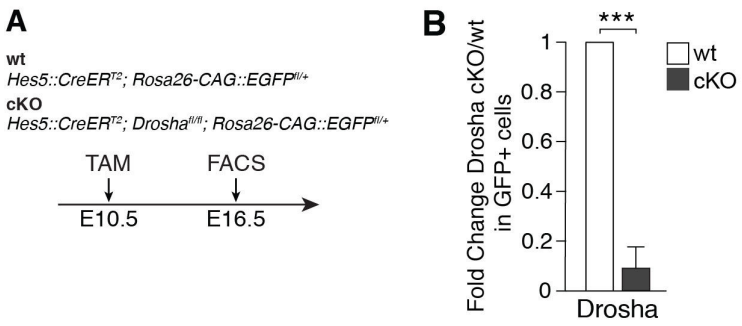

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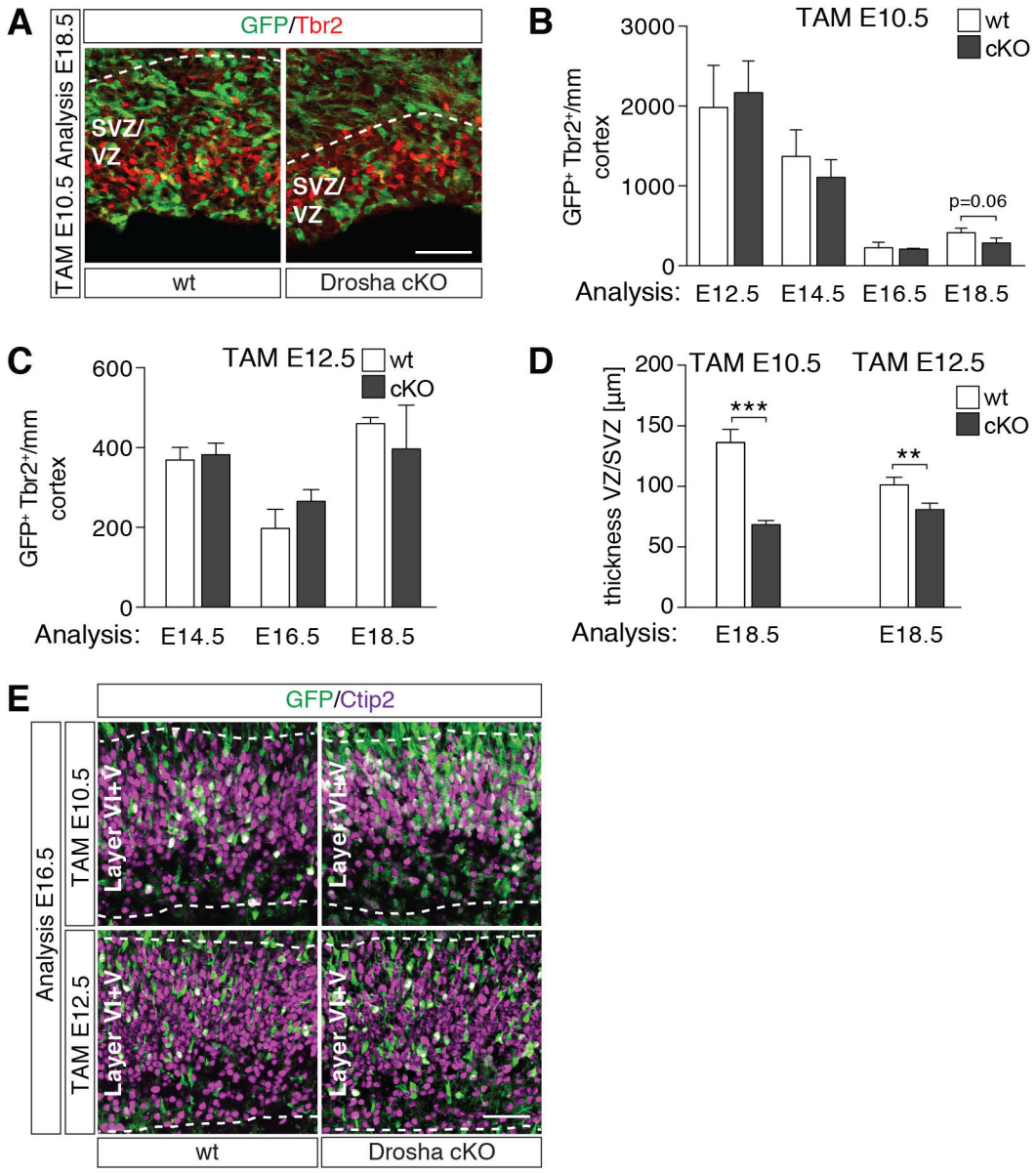

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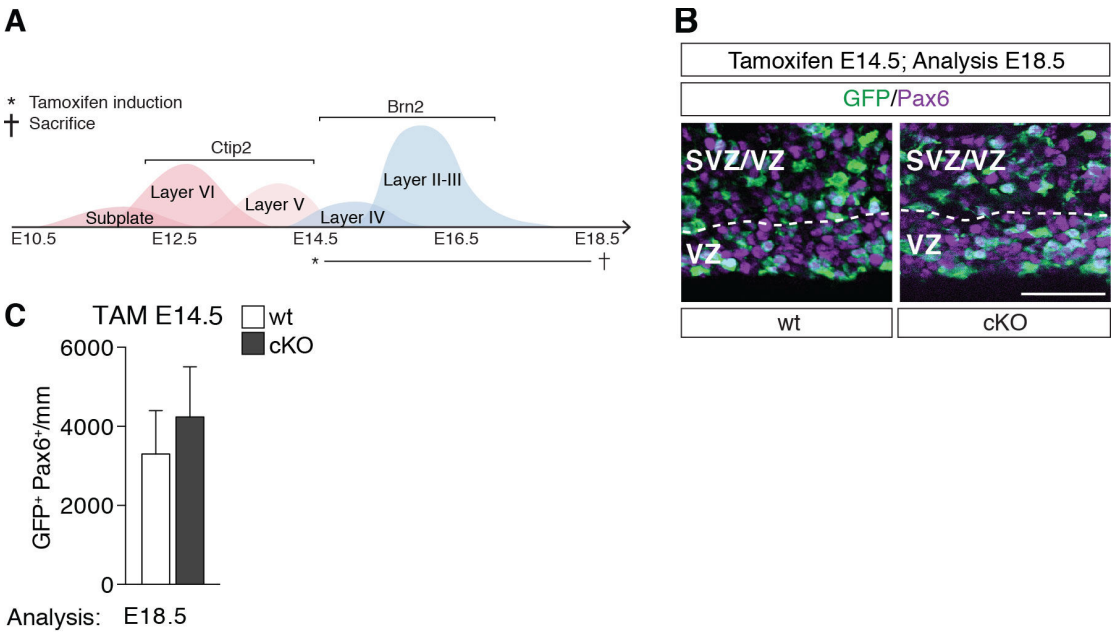

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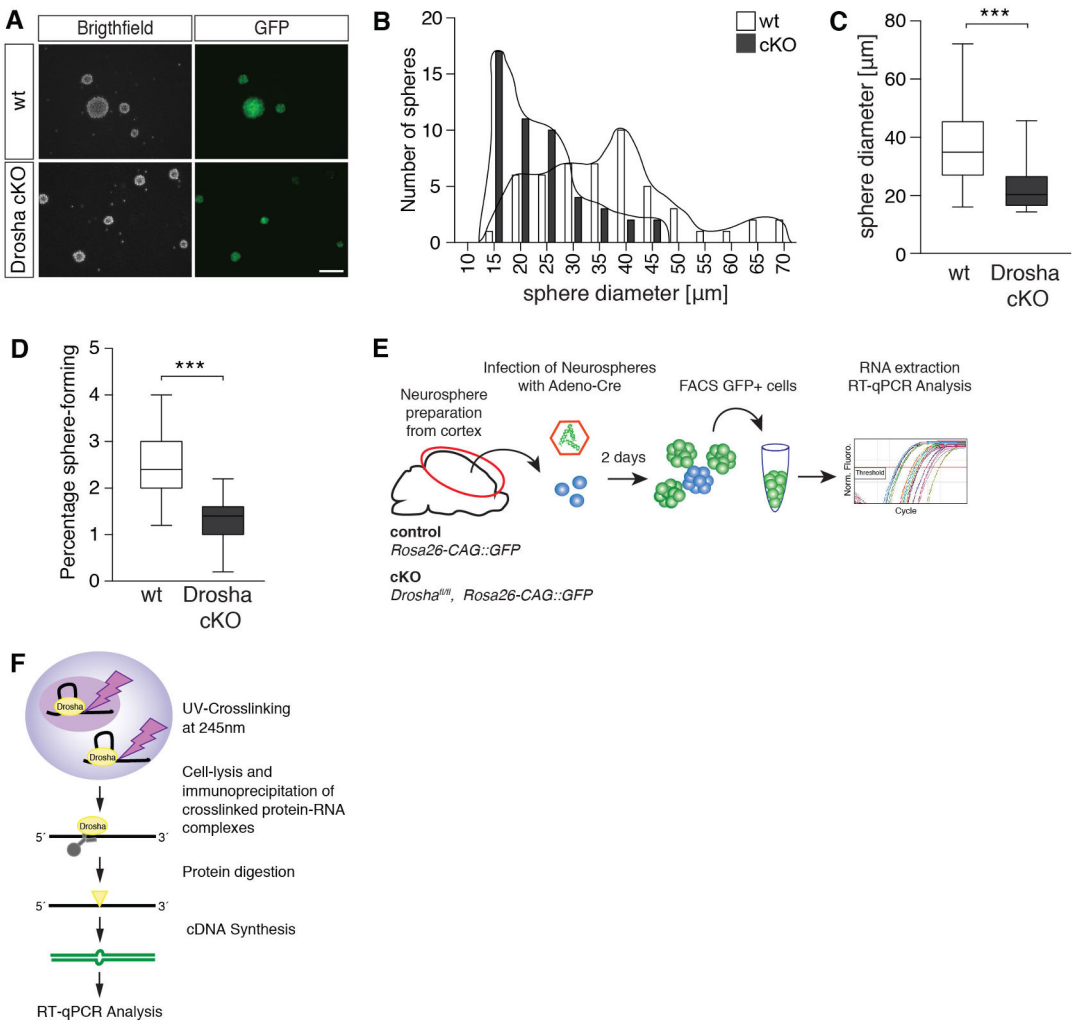


Erni *et.al.* Supplementary Figure 4

2.2 Additional results

In the following section I show and discuss additional data investigating the effect of Drosha deletion on NSCs during the development of the central nervous system.

2.2.1 Drosha deletion in NSCs influences laminar layering

In the manuscript in section 2.1 we found that Drosha is required for proper NSC maintenance and neuronal differentiation. We therefore wanted to investigate the effect of Drosha cKO on cortical cytoarchitecture and laminar layering. For this reason, we analysed the thickness of the specific layers of the dorsal cortex during development using layer-specific markers including Pax6 (VZ), Tbr1 (Layer VI), Ctip2 (Layer VI+V) or Brn2 (Layer IV-II).

Analysing E10.5 TAM induced Drosha cKO embryos at E18.5 revealed that deep-layer VI and the IZ were significantly increased in thickness (Fig. 2.1 A). This is in line with our previous results that Drosha deleted NSCs differentiated into more Ctip2^{+} and Tbr1^{+} deep-layer neurons compared to wt as shown in section 2.1. Therefore, we hypothesise that the increase in deep-layer VI thickness in these embryos is a result of increased deep-layer differentiation. In contrast the thickness of the progenitor regions (VZ/SVZ) and the superficial layers (V-II) were significantly reduced (Fig. 2.1A). According to our previous results we propose that in E10.5 Drosha cKO NSCs reduce their proliferation and precociously differentiate into deep-layer neurons that populate the IZ and Layer VI. We suggest that this leads to a reduction in the progenitor pool and as a consequence subsequently formed layers are reduced in thickness.

To analyse the timing of layer reduction, we observed the effect of Drosha deletion in NSCs at E10.5 on layer thickness at defined time points after ablation at E14.5, E16.5 and E18.5. An initial increase was observed in the thickness of the VZ/SVZ at E14.5 followed by a decrease at E16.5 and E18.5. In addition, IZ thickness in Drosha cKO animals was initially (E14.5) reduced and at later time points (E18.5) significantly increased compared to control

embryos. We suggest that the initial enlargement of the neurogenic niche at E14.5 is a result of increased intermediate progenitor production from Drosha ablated NSCs consistent with our previous findings (Knuckles et al., 2012). We propose that increasing numbers of differentiating cells enter into the VZ/SVZ of Drosha cKO embryos, thereby expanding the domain. Furthermore, we suggest that the initial reduction of the IZ in Drosha cKO embryos could be a result of precociously differentiating NSCs, which potentially undergo direct neurogenesis without passing through the IP stage.

Comparing the cortex of E12.5 recombination-induced embryos, we observed that Drosha cKO had a smaller cortex at E18.5 compared to wt. In particular, the SVZ/VZ and the upper-layers IV-II showed a significant reduction in thickness (Fig. 2.1 B). Interestingly, at E14.5 and E16.5, the thickness of the cortical layers between Drosha cKO and wt embryos was indistinguishable (Fig. 2.1 B). Therefore, Drosha cKO reduces the neural progenitor pool between E16.5 and E18.5, at the time when upper-layer

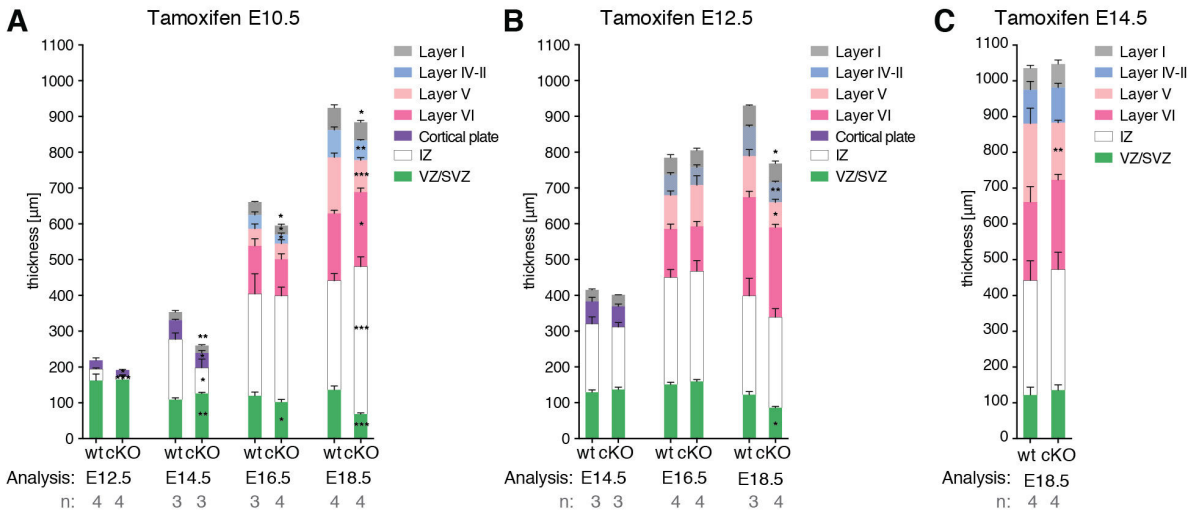


Figure 2.1 – Comparing cortical layering between wt and Drosha cKO embryos
(A-C) Drosha knock-out in NSCs was induced at E10.5 (A), E12.5 (B) or E14.5 (C) by a single TAM treatment. The cortical layers were measured 2, 4, 6 or 8 days after recombination, and analysed for expression of specific cortical markers (Pax6, Tbr1, Ctip2, Brn2). Data are presented as mean ±SD, n=3-4, two-sided Student´s t-test, *p < 0.05, **p < 0.005, ***p < 0.0005.

neurons are generated. In summary, these results suggest that Drosha cKO results in a reduced progenitor pool, an effect that manifests at the time when upper-layer neurogenesis occurs and hence leads to reduced upper-layer thickness, potentially due to precocious NSC exhaustion.

Drosha cKO at E14.5 did not affect cortical thickness, however, we observed a reduction in the deep-layer V at E18.5 (Fig. 2.1 C). In the manuscript in section 2.1 we showed that the proliferation and maintenance of NSCs and the differentiation of deep-layer glutamatergic neurons are unaffected in Drosha cKO embryos (manuscript Fig. 1, S3). Therefore, we suggest that a reduction in layer V could be a result from fewer glia cells populating Layer V. Furthermore the Hes5::CreER^{T2} approach also induces Drosha ablation in the ventral NSCs, the progenitors of the cortical interneurons. Hence, a reduction in Layer V could also result from fewer interneurons produced in Drosha cKO embryos. To conclude this finding, further investigations will be needed as immunohistochemical stainings for interneurons or glial cells markers.

2.2.2 Drosha deleted NSCs generally do not undergo apoptosis

Our previous results revealed a reduction in neural progenitors and a reduced thickness of the upper-layers when Drosha was deleted during early neurogenesis (manuscript Fig. 1 A-D, Fig. 2.1). This suggests that reduced proliferation and increased differentiation of Drosha cKO NSCs result in a smaller neural progenitor pool. Alternative Drosha cKO cells could undergo cell-death, thereby diminishing progenitor pool. To test this hypothesis we performed stainings for the apoptotic marker cleaved-Caspase 3. During our temporal analysis, we only observed a mild, but significant increase in apoptotic cells in the VZ of E14.5 Drosha cKO animals when recombination was induced at E10.5 (Fig. 2.2, 2.3). These results suggest that Drosha is required between E12.5 and E14.5 to prevent cell death in the progenitor pool.

Dicer was shown to be necessary for the survival of differentiating but not proliferative NSCs (De Pietri Tonelli et al., 2008). We therefore tested whether

apoptotic cells in the VZ of Drosha cKO embryos were NSCs or intermediate progenitors. Quantifications of GFP^+, $Sox2^+$ (a NSC marker) and cleaved Caspase 3^+ cells of E10.5 induced and E14.5 analysed embryos revealed no significant difference in recombined apoptotic NSCs between Drosha cKO and wt embryos (Fig. 2.3). Therefore, we hypothesize that the dying cells are intermediate progenitors or differentiating neurons. These findings suggest that cell-death at E14.5 may be miRNA-dependent. However, future experiments will be needed to test this hypothesis.

Concluding, these results suggest that apoptosis in Drosha cKO embryos is miRNA-dependent however not the main cause of the reduction in the neural progenitor pool and the reduced upper cortical layers.

2.2.3 Drosha deletion in NSCs results in an enlarged telencephalic vesicle

So far, we focused on the dorsal cortex of the developing brain. However, Hes5:$CreER^{T2}$ induces recombination in NSCs in the whole developing central nervous system (Basak and Taylor, 2007, Lugert et al., 2012). Therefore, we investigated if Drosha cKO results in phenotypes in other parts of the developing brain. We compared the cytoarchitecture of the brains on coronal

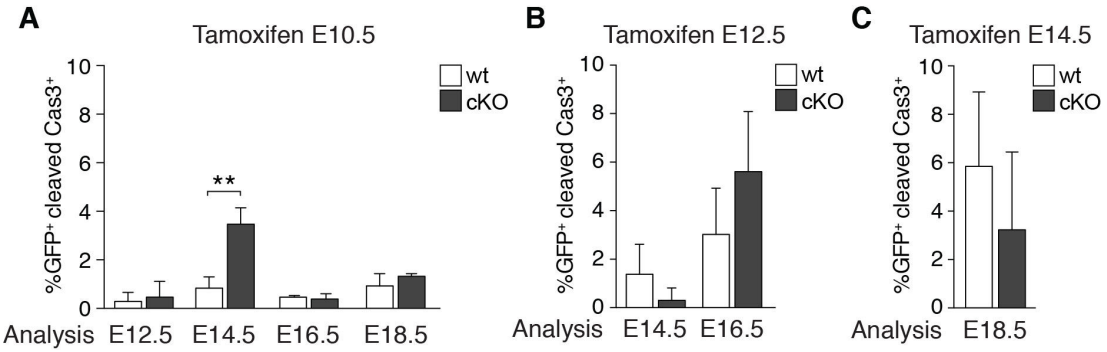


Figure 2.2 – Apoptosis in Drosha cKO embryos at E14.5

Quantification of apoptotic GFP^+ cleaved $Caspase3^+$ cells when TAM was administrated at E10.5 (A), E12.5 (B) or E14.5 (C). Data presented as mean values ±SD, wt. A: Analysis E12.5 n=4, Analysis E14.5/16.5/18.5 n=3, B, C: n=3. Two-sided Student´s t-test on arcsine: **$p < 0.005$

sections of E18.5 Drosha cKO and wt embryos stained for DAPI from different TAM induction time points (Fig. 2.4A+C). In addition to the disruption of the dorsal cortex, we observed an enlargement of the telencephalic vesicles (lateral ventricle) in Drosha cKO embryos (Fig. 2.4C). To obtain a more quantitative view of these phenotypes, we subdivided the ventricle into a dorsal, lateral and medial region of interest (Fig. 2.4B). Drosha cKO at E10.5 resulted in an elongation of all three ventricular walls at E18.5 (Fig. 2.4C-D). In contrast, Drosha cKO at E12.5 did not result in a significant enlargement of the ventricles, however, dorsal and medial ventricular walls tended to be more elongate (Fig 2.4 C-D). At E14.5 Drosha cKO resulted in a significantly elongation only of the dorsal side of the ventricle (Fig 2.4 C-D).

Enlargement of the ventricles has been described in different brain disorders with cognitive impairment (Garton and Piatt, 2004). It is known that an overproduction of cerebral spinal fluid (CSF) triggers hydrocephaly

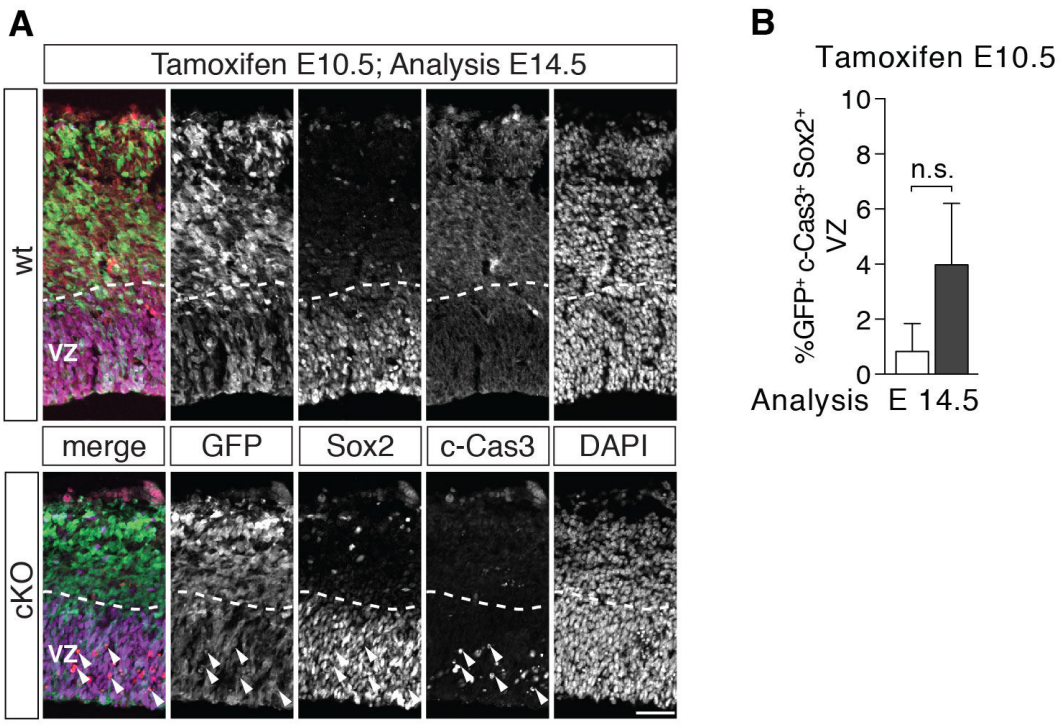
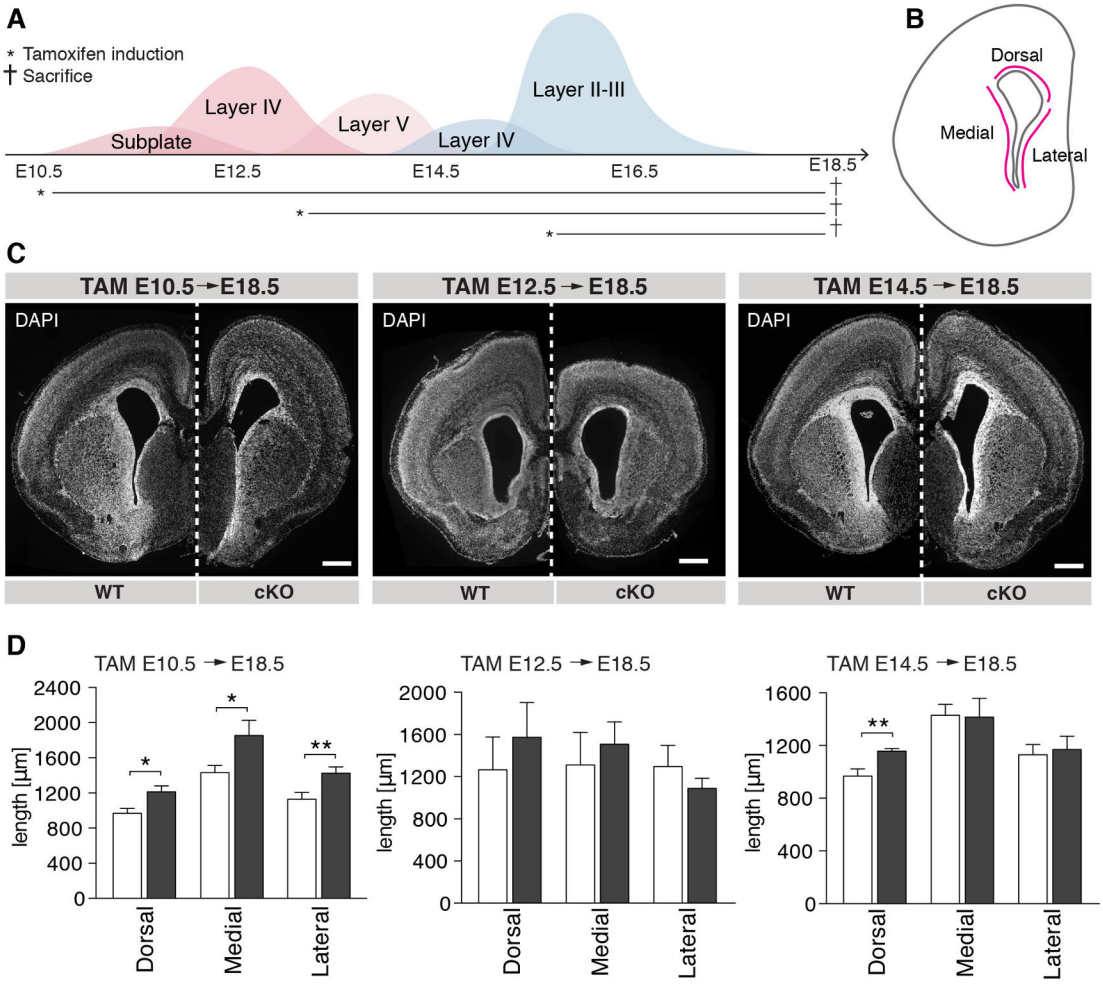


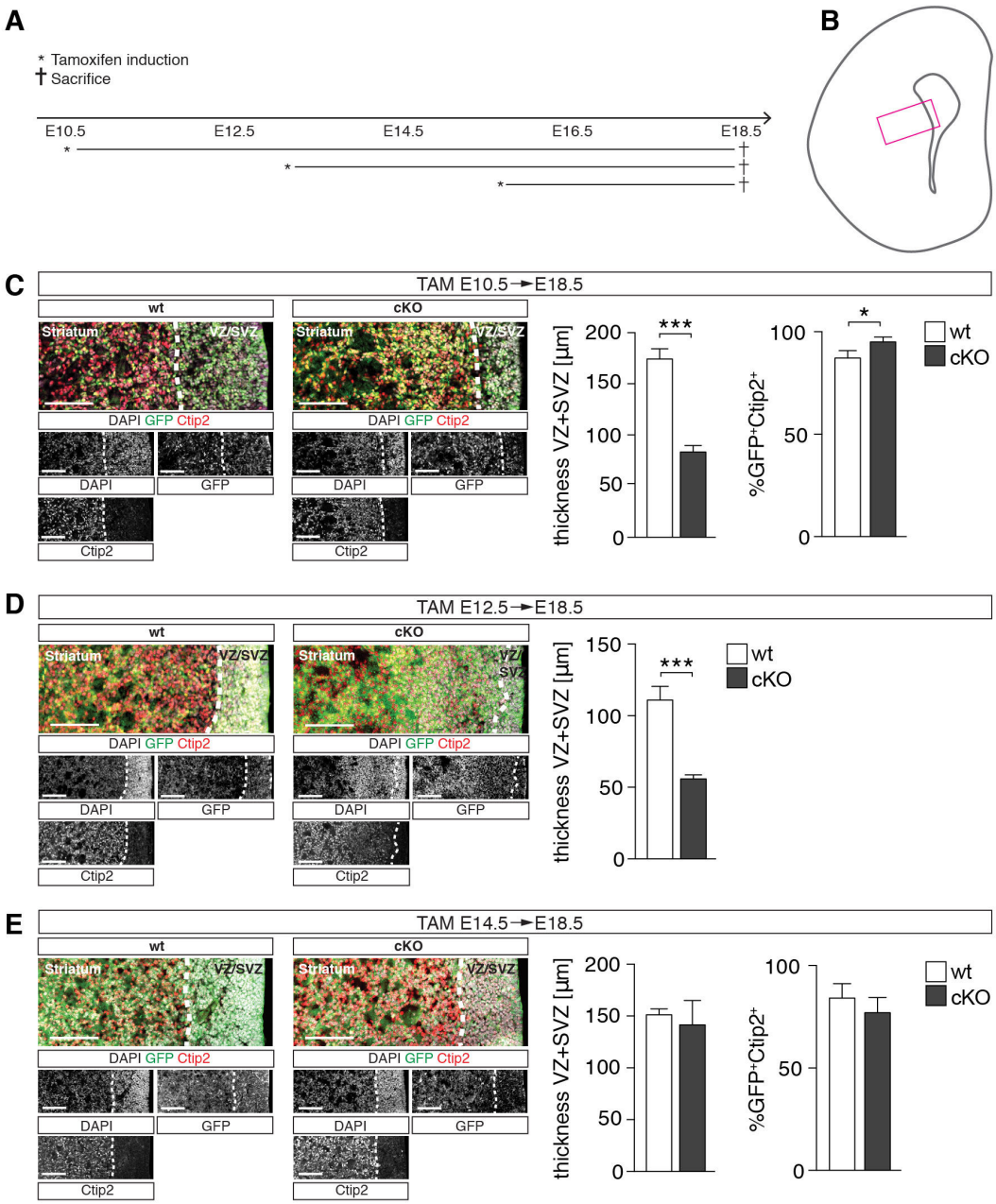
Figure 2.3 – E10.5 Drosha depletion leads to apoptosis at E14.5 in the VZ
(A) GFP^+ cleaved $Caspase3^+$ (c-Cas3) $Sox2^-$ cells (arrowhead) in the VZ of E10.5 recombination induced animals, analysed E14.5. (B) Quantification of $\%GFP^+$ c-$Cas3^+$ $Sox2^+$ cells in the VZ. Data are presented as mean ±SD n=3. Two-sided Student´s t test. Scale bar = 50µm.

(enlarged ventricles), which can be caused by a failure of absorption or an obstruction of the CSF passage (Ishihara et al., 2010). In the future it will be of interest to investigate what causes the ventricular enlargement in Drosha cKO embryos. Therefore, it will be interesting to examine if the morphology of the aqueduct and central canal, both involved in ventricular development are affected in Drosha cKO embryos.



2.4 - Drosha deletion leads to enlarged first ventricle

(A) Scheme of recombination induction with TAM and chases after Drosha depletion. (B) Definition of Dorsal, lateral and medial ventricle. (C) Coronal section of the forebrain, stained with dapi. Drosha depletion in NSCs was induced at E10.5, E12.5 or E14.5 and brains were collected at E18.5. On the left side are the wt and on the right side the Drosha cKO respectively. (D) Measurements of the ventricle on a specific coronal section (coronal section 9). Data are mean ±SD n=3-4 Two-sided Student´s t test, *p < 0.05, **p < 0.005, ***p < 0.0005. Scale bar = 1mm.



2.5 - Early Drosha depletion in ventral NSCs reduces the NSC-pool and induces differentiation into Ctip2$^+$ neurons

(A) Scheme of recombination induction with TAM and chases after Drosha depletion. (B) Scheme of a coronal section. The window indicates the regions unsed to characterize ventral NSCs. (C-E) Drosha depletion at E10.5, E12.5 or E14.5 and analysis at E18.5. Staining for medium spiny neurons with Ctip2. On the right measurements of VZ/SVZ thickness and %GFP$^+$Ctip2$^+$ cells in the striatum are shown. Data are mean ±SD n=3-4. VZ/SVZ length: two-sided Student´s t-test, %GFP$^+$Ctip2$^+$: two-sided Student´s t-test on arcsine, *p < 0.05, **p < 0.005, ***p < 0.0005.

2.2.4 Drosha deletion results in similar phenotypes in ventral and dorsal NSCs

Dorsal, as well as the ventral NSC express Hes5 (Basak and Taylor, 2007). However, these two populations give rise to different cell types and depend on distinct regulatory inputs (Greig et al., 2013, Chu and Anderson, 2014). Therefore, we investigated whether Drosha is as well involved in neurogenesis in the ventral telencephalon. We compared the ventral region of E18.5 wt embryos to Drosha cKO embryos that were treated with TAM at E10.5, E12.5 and E14.5 (Fig. 2.5A-B). E18.5 embryos that were administered with TAM at E10.5 showed a significant reduction in the progenitor zones as measured by the thickness of the VZ/SVZ, similar to the effect of Drosha cKO from the dorsal NSCs. Furthermore, ventral NSCs also showed increased differentiation into striatal GFP^+Ctip2^+ medium-sized spiny neurons (Fig 2.5C). Induction at E12.5 also resulted in a reduced thickness of VZ/SVZ compared to wt embryos (Fig 2.5D). Similar to the dorsal NSCs, Drosha deletion in ventral NSCs at E14.5 and analysis at E18.5 revealed no difference in VZ/SVZ thickness and striatal GFP^+Ctip2^+ medium-sized spiny neuron differentiation (Fig. 2.5E).

These results suggest that Drosha function is conserved in dorsal and ventral NSCs and supports that Drosha controls the formation of $Ctip2^+$ neurons.

3. Discussion

In this thesis I investigated the role of Drosha during neurogenesis. I showed that early NSCs are Drosha-dependent in respect to maintenance and timing of neuronal fate decision. Furthermore, my results suggest that Drosha directly controls Ctip2 expression, thereby regulating the timing of deep-layer neurogenesis.

The differentiation of specific cortical layers is defined by the expression of a defined set of different transcription factors (Srinivasan et al., 2012, Kumamoto et al., 2013, Toma and Hanashima, 2015). It has been shown that deep-layer neurons depend on the transcription factors Ctip2, Tbr1 and Fezf1/2 (Shimizu et al., 2010, Chen et al., 2008, McKenna et al., 2011). However, it remains unclear whether Drosha controls deep-layer fate solely by regulating Ctip2 and the previously identified proneural factor Ngn2 (Knuckles et al., 2012) or by a combination of additional transcription factors. Interestingly, all the transcription factors involved in deep-layer specification, Ctip2, Tbr1, Sox5 and Fezf1/2 contain evolutionarily conserved hairpins (Pedersen et al., 2006), suggesting that Drosha could process them. Recently it was suggested that DGCR8 together with Drosha regulates the transcriptional level of Tbr1 (Marinaro et al., 2017). Therefore we tested, whether Drosha is able to bind and eventually process Tbr1 mRNA. *In vitro* adult but not embryonic NSCs express Tbr1 and a CLIP assay of Drosha from adult NSCs did not pull-down Tbr1 (data not shown). These results suggest that Drosha may not regulate Tbr1 mRNA directly. Hence, it would be of interest to examine whether Drosha regulates the Sox5 or Fezf1/2 transcripts to reveal the overall effect of Drosha-dependent deep-layer specification of NSCs.

Drosha was initially identified as a core component of miRNA biogenesis. Therefore, Drosha deletion will affect miRNA abundance, and hence it will be important to discriminate between miRNA-dependent and miRNA-independent functions of Drosha. Embryonic NSCs showed significant differences in miRNA expression five days after Drosha depletion (Knuckles et al., 2012). However, the half-life of miRNAs is highly variable from several

hours to many days in the nervous system (Krol et al., 2010, Konopka et al., 2010). To investigate if Drosha cKO phenotypes are miRNA-dependent or miRNA-independent we can compare our data to published Dicer cKO studies. Dicer is also a central component involved in miRNA maturation. Therefore, phenotypes that are similar in Dicer cKO and Drosha cKO might be miRNA-dependent. Published Dicer cKO in NSCs revealed similar and different phenotypes compared to Drosha cKO during cortical development. Dicer cKO embryos for example were shown to have an enlarged first ventricle (Volvert et al., 2012), similar to Drosha cKO embryos. This suggests that ventricle-enlargement is potentially a miRNA-dependent phenotype. Dicer cKO embryos were also shown to have a reduced cortical thickness (De Pietri Tonelli et al., 2008, Kawase-Koga et al., 2009), similar to Drosha cKO embryos. However, the reduction in thickness was demonstrated to be the result of increased apoptosis in maturating neurons and not a reduction in proliferation of NSCs as we observe in Drosha cKO embryos. Therefore, the reduction in cortical thickness must have different origins in Drosha and Dicer cKO embryos, suggesting that both phenotypes are miRNA-independent.

Therefore, we hypothesize that Drosha has a combinatorial effect on NSC differentiation. We propose, that a potential miRNA-dependent role of Drosha is fine-tuning NSC fate while the non-canonical miRNA-independent role of Drosha is defining the fate of NSCs. Furthermore, we provide data that shows the role of Drosha in neurodevelopment in dorsal and ventral NSCs. In both areas maintenance of NSCs and differentiation into $Ctip2^{+}$ neurons is Drosha-dependent. Ventral NSCs generate striatal neurons and interneurons that migrate tangentially to their destination in the dorsal cortex (Arlotta et al., 2008, Wonders and Anderson, 2005). It will be of future interest to investigate whether Drosha is as well involved in the differentiation of interneurons. We have shown that Drosha maintains dorsal NSCs by regulating the Ngn2 transcript (Knuckles et al., 2012). However, Ngn2 is absent in ventral progenitors (Parras et al., 2002). Therefore, we propose that Drosha regulates ventral NSC maintenance through an Ngn2-independent mechanism. It will be of further interest to investigate the role of Drosha NSC maintenance ventrally and compare it to the dorsal mechanisms. Our studies suggest that post-

transcriptional regulation by Drosha has a significant impact on neural fate determination. This adds another piece to the evolving field of RNA modification, demonstrating how post-transcriptional regulation can influence fate commitment.

4. Materials

4.1 Transgenic animals

Dicer$^{fl/fl}$ mice have been described elsewhere (Tchorz et al., 2012).

4.2 Primers used for RT-qPCR

Dicer fwd	CAGTGCTGCAGTAAGCTGTG
Dicer rev	TCAATCATCCAGTGTTTCTTTC
NFIB fwd	CAGGAGCAAGATTCTGGAC;
NFIB rev	GGGTGTTCTGGATACTCTCAC
Olig2 fwd	TCCCCAGAACCCGATGATCTT
Olig2 rev	CGTGGACGAGGACACAGTC
Ngn2 fwd	ATGGCTGGCATCTGCTCTATTC
Ngn2 rev	CACATCAGAGAGGGAAAGTTTGGT
Sox10 fwd	AGCTCTGGAGGTTGCTGAAC
Sox10 rev	GCCGAGGTTGGTACTTGTAGTC
Tbr1 fwd	GCAGCAGCTACCCACATTC
Tbr1 rev	GTCCTTGGAGTCAGGAAAATTGT

4.3 Antibodies for immunohistochemistry

rabbit anti-cleaved-Caspase3 (1:200; 5A1E, Cell Signaling).

5. Publication - Multipotency of Adult Hippocampal NSCs In Vivo Is Restricted by Drosha/NFIB

Adult DG NSCs usually differentiate into neurons and astrocytes but not into oligodendrocytes. Intrinsically however they are suggested to have a three-linage potential, which has been demonstrated by *in vitro* oligodendrocyte differentiation by co-culture with neurons or *in vivo* by reprogramming with the transcription factor Ascl1 or the inactivation of Neurofibromin 1, which induces oligodendrogenesis (Braun et al., 2015; Jessberger et al., 2008; Song et al., 2002; Suh et al., 2007; Sun et al., 2015). This suggests that the fate restriction of DG NSCs is intrinsic and niche-independent. However it remained unclear how DG NSC potency and more specifically oligodendrocytic fate restriction is regulated. In our study we demonstrated that the RNaseIII Drosha, a component of the microprocessor, directly inhibits the expression of NFIB, thereby inhibiting the differentiation of NSCs into oligodendrocytes. These findings demonstrate that DG NSCs have a tri-lineage potential that is kept in check post-transcriptionally by Drosha.

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http://dx.doi.org/10.1016/j.stem.2016.07.003

5.1 Contribution

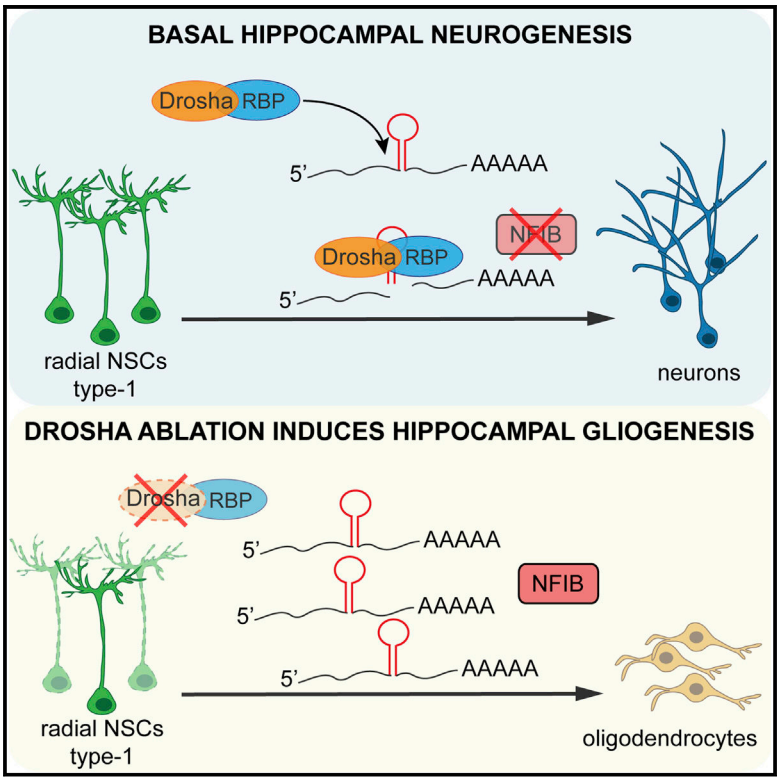
For this publication I performed the experiments summarized in figure 3, supplementary figure 3 A, B, C, E, F and I created the corresponding figures. Furthermore I generated the DG NSC cultures and was involved in editing the manuscript.

Short Article

Cell Stem Cell

Multipotency of Adult Hippocampal NSCs In Vivo Is Restricted by Drosha/NFIB

Graphical Abstract



Highlights

- Drosha regulates adult hippocampal stem cell maintenance
- Drosha inhibits oligodendrocytic differentiation of adult stem cells
- Drosha targets NFIB mRNA hairpin to inhibit expression and enable neurogenesis
- NFIB expression induces oligodendrocytic fate in adult hippocampal stem cells

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In Brief

Rolando et al. investigated the function of the RNaseIII Drosha in the regulation of adult hippocampal stem cell maintenance and differentiation. They found that Drosha directly inhibits the expression of the transcription factor NFIB through a miRNA-independent mechanism, thereby permitting neurogenesis and preventing oligodendrocyte fate commitment.

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November 3, 2016
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Cell Stem Cell
Short Article

Multipotency of Adult Hippocampal NSCs In Vivo Is Restricted by Drosha/NFIB

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SUMMARY

Adult neural stem cells (NSCs) are defined by their inherent capacity to self-renew and give rise to neurons, astrocytes, and oligodendrocytes. In vivo, however, hippocampal NSCs do not generate oligodendrocytes for reasons that have remained enigmatic. Here, we report that deletion of Drosha in adult dentate gyrus NSCs activates oligodendrogenesis and reduces neurogenesis at the expense of gliogenesis. We further find that Drosha directly targets NFIB to repress its expression independently of Dicer and microRNAs. Knockdown of NFIB in Drosha-deficient hippocampal NSCs restores neurogenesis, suggesting that the Drosha/NFIB mechanism robustly prevents oligodendrocyte fate acquisition in vivo. Taken together, our findings establish that adult hippocampal NSCs inherently possess multilineage potential but that Drosha functions as a molecular barrier preventing oligodendrogenesis.

INTRODUCTION

Somatic stem cells can generate progeny throughout life, but their fates are usually restricted, and they generate specific cell types in their respective tissue. Active adult neural stem cells (NSCs) are present in two regions of the brain: the subventricular zone (SVZ) of the lateral ventricles and the subgranule zone of the hippocampal dentate gyrus (DG) (Ihrie and Alvarez-Buylla, 2011; Kriegstein and Alvarez-Buylla, 2009). Although both SVZ and DG NSCs are multipotent, they generate specific neuron types. SVZ NSCs become fate restricted during embryonic development and generate multiple interneuron populations from topological locations in the lateral ventricle wall (Merkle et al., 2007). DG NSCs produce only granule neurons, which contribute to cognition, and loss or dormancy of stem cells during aging can result in psychological disorders and disease (Kronenberg et al., 2003; Petrus et al., 2009; Santarelli et al., 2003; Steiner et al., 2008). Whereas SVZ NSCs make a significant number of oligodendrocytes (Hack et al., 2004; Menn et al., 2006), new oligodendrocytes are normally not produced in the adult DG (Bonaguidi et al., 2011; Encinas et al., 2011; Lugert et al., 2010). In vitro, DG NSCs also rarely produce oligodendrocytes, although oligodendrocytic differentiation can be induced by their co-culture with neurons and in vivo by inactivation of the Neurofibromin 1 gene or reprogramming with the transcription factor Ascl1 (Braun et al., 2015; Jessberger et al., 2008; Song et al., 2002; Suh et al., 2007; Sun et al., 2015). This suggests an intrinsic and niche-independent fate restriction of DG NSCs that prevents oligodendrocyte formation. How DG NSC potency and particularly oligodendrocytic fate are restricted remains unclear.

Drosha is part of the microRNA (miRNA) microprocessor (Ha and Kim, 2014). However, Drosha can also cleave and directly destabilize mRNAs encoding proteins that regulate cell fate decisions (Chong et al., 2010; Han et al., 2009; Knuckles et al., 2012; Macias et al., 2012). During embryonic development, Drosha maintains embryonic NSCs in an undifferentiated, multipotent state by targeting and cleaving the mRNA of the proneural factor Ngn2 (Knuckles et al., 2012). This non-canonical function of Drosha does not require Dicer or miRNAs, and is a rapid mechanism for fate regulation.

Here, we examined how Drosha is involved in the regulation of DG NSC fate. We found that Drosha controls DG NSC maintenance and cell fate acquisition through a non-canonical regulation of the transcription factor nuclear factor IB (NFIB). We show that NFIB is required for the oligodendrocytic commitment by DG NSCs and propose that Drosha promotes neurogenesis and inhibits oligodendrocyte fate acquisition in the hippocampus by repressing NFIB.

RESULTS

Drosha Deletion from Adult DG NSCs Impairs Neurogenesis

NSCs in the DG of the adult mouse are Notch dependent and express the Notch target *Hes5* (Lugert et al., 2010, 2012). Drosha is expressed by most cells in the DG, including GFAP$^+$ and Hes5$^+$ radial NSCs (Figures S1A and S1B). To address the functions of Drosha in neurogenic DG NSCs, we treated *Hes5::CreER*^{T2} mice carrying floxed *Drosha* (Drosha cKO) or wild-type (wt) *Drosha* (ctrl) alleles with tamoxifen (TAM) and followed cell fate by lineage tracing (*Rosa26-CAG::EGFP*) (Figures 1A and S1A) (Lugert et al., 2012). Twenty-one days after TAM administration, Hes5$^+$

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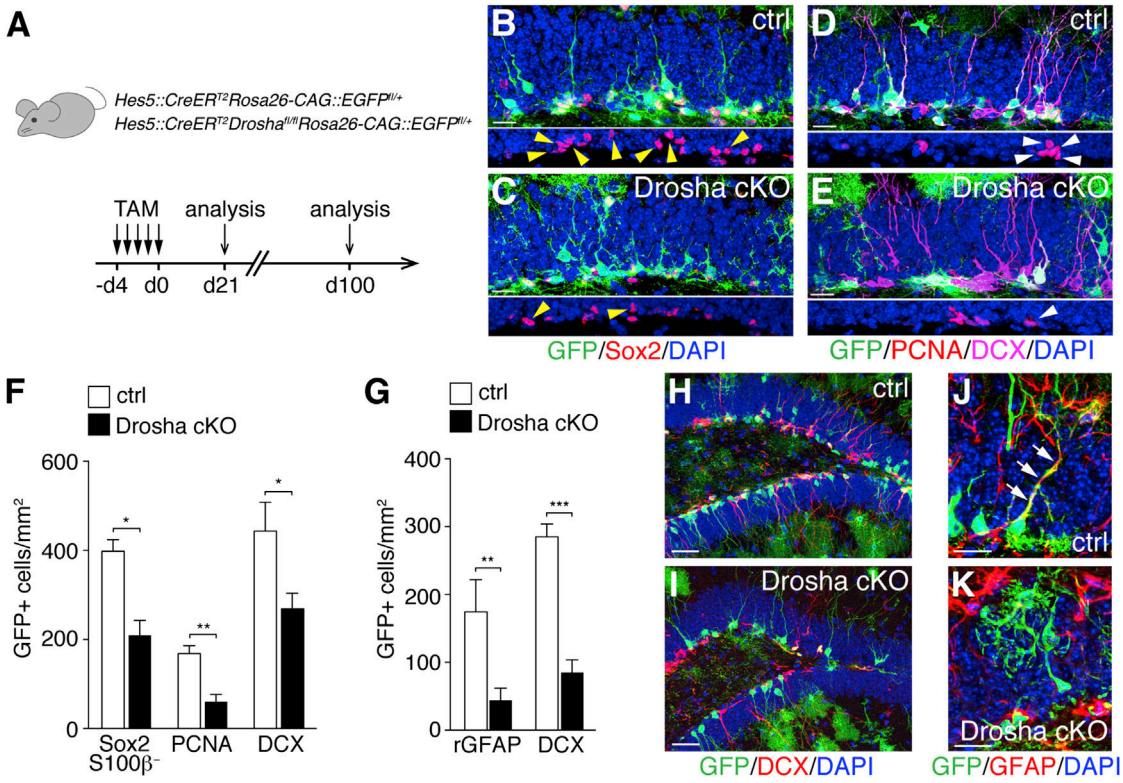

Figure 1. Drosha Deletion from Adult DG NSCs Impairs Neurogenesis In Vivo

(A) TAM induction regime and genotypes of *Hes5::CreER*^{T2} mice.

(B and C) GFP$^+$Sox2$^+$ NSCs (yellow arrowheads) in the DG of control (B) and Drosha cKO (C) animals at day 21.

(D and E) Proliferating cells (PCNA$^+$; white arrowheads) and DCX$^+$ neuroblasts in control (D) and Drosha cKO (E) animals at day 21.

(F) Quantification of GFP$^+$Sox2$^+$S100β$^-$ NSCs, proliferating GFP$^+$PCNA$^+$ progenitors and newly generated neuroblasts GFP$^+$DCX$^+$ in Drosha cKO and control animals at day 21 (control, n = 5; Drosha cKO, n = 5). Two-sided Student's t test: *p < 0.05, **p < 0.01.

(G) Quantification of radial GFP$^+$GFAP$^+$ NSCs and DCX$^+$ neuroblasts in Drosha cKO and control animals at day 100 (control, n = 5; Drosha cKO, n = 5). Two-sided Student's t test: **p < 0.01, ***p < 0.001.

(H and I) GFP$^+$DCX$^+$ neuroblasts in control (H) and Drosha cKO (I) animals at day 100.

(J and K) GFP$^+$GFAP$^+$ cells in control (J) and Drosha cKO (K) animals at day 100 (arrows in J; GFAP$^+$ radial process).

Data are mean ± SEM. The scale bars represent 20 μm in (B)–(E), (J), and (K) and 50 μm in (H) and (I). See also Figure S1 and Table S1.

NSCs and their progeny were Drosha deficient and generated fewer cells compared with controls (Figures S1B–S1D). Furthermore, the number of radial GFAP$^+$, Sox2$^+$, and mitotic (PCNA$^+$) NSC/progenitors and neuroblasts (DCX$^+$) was reduced in Drosha cKO animals (Figures 1B–1F and S1E). Decreased neurogenesis persisted in Drosha cKO animals at 100 days, and the reduction in newborn neurons (GFP$^+$NeuN$^+$) was accompanied by an increase in S100β$^+$ parenchymal astrocytes compared with controls (Figures 1G–1I and S1F–S1J). In addition, GFAP$^+$ putative radial NSCs were lost in Drosha cKO animals (Figures 1G, 1J, and 1K). Together these data suggest that Drosha is required for NSC maintenance and promotes neurogenesis in the DG at the expense of gliogenesis.

Quiescent DG NSCs activate, proliferate, and produce neuroblasts in response to seizures (Hüttmann et al., 2003; Sierra et al., 2015; Steiner et al., 2008). We addressed whether NSC-like progenitors remain in the Drosha cKO and can still respond to activating stimuli. We administered epileptogenic kainic acid (KA) to induce seizures in *Hes5::CreER*^{T2} Drosha cKO and control mice 21 days after TAM induction (Figure S1K). Whereas KA induced proliferation and an increase in neuroblasts in control animals (Figures S1L and S1M), neither proliferation (PCNA$^+$) nor neuroblast (DCX$^+$) production was increased following KA treatment of Drosha cKO mice (Figures S1L and S1N). This suggests that Drosha cKO diminishes the DG NSC pool and compromises progenitor reactivation.

Drosha cKO Induces Oligodendrocyte Commitment of NSCs

To examine whether Drosha controls neurogenesis by acting on quiescent NSCs, we ablated *Drosha* specifically in radial GFAP$^+$ NSCs by stereotactic infection with adenoviruses expressing Cre-recombinase under the control of the *gfap* promoter (adeno-*gfap*::Cre) (Figure S2A) (Merkle et al., 2007). Six days post-infection (dpi), most GFP-labeled, adeno-*gfap*::Cre-infected cells in the subgranular zone in control mice were GFAP$^+$ putative radial NSCs (Figures S2B–S2D). Twenty-one days post-infection, adeno-*gfap*::Cre-infected NSCs had generated mitotic (PCNA$^+$) progenitors and neuroblasts (DCX$^+$) in control animals, but Sox2$^+$ and PCNA$^+$ progenitors were almost absent, and

newly formed neuroblasts were reduced in Drosha cKO animals (Figures 2A–2E). Therefore, Drosha cKO DG NSCs lose stem cell potential, demonstrating that Drosha is essential for NSC maintenance and neurogenesis.

DG NSCs normally generate glutamatergic granule neurons and astrocytes but not oligodendrocytes (Bonaguidi et al., 2011). Following adeno-*gfap*::Cre-mediated Drosha cKO, a significant number of the newborn cells expressed Olig2 and Sox10, markers of oligodendrocyte progenitor cells (OPCs) (Figures 2D–2G). Similarly, we observed newly generated Sox10$^+$, Olig2$^+$, and NG2$^+$ OPCs in *Hes5::CreER*^{T2} Drosha cKO animals (Figures S2E–S2G). Thus, Drosha cKO induces a fate switch in DG NSCs to oligodendrocytes.

We performed clonal analysis of *Hes5::CreER*^{T2} Drosha cKO NSC fate. Two days after low-dose TAM induction, labeled NSCs were sparse in the DG (mean distance between clones = 184.3 ± 17.2 μm; Figures S2H and S2I). Twenty-one days post-TAM, 6 of the 41 clones examined in Drosha cKO animals contained OPCs but none in the controls (Figures 2H, 2I, S2J, and S2K). Interestingly, 1 clone contained neuroblasts, astrocytes, and oligodendrocytes, indicating tri-lineage potential of Drosha cKO NSC in vivo (Figure 2H).

We addressed whether Drosha controls oligodendrocyte production from mitotic GFAP$^-$ stem/progenitor cells. We infected dividing cells in the DG with a Cre-expressing retrovirus. We did not see oligodendrocyte formation in the Drosha cKO after retro-Cre virus infection, and active progenitors continued to generate neuroblasts (Figures S2L and S2M). These data suggest that Drosha deletion induces a fate shift in the quiescent NSC pool to oligodendrocyte production but not in active NSC/progenitors.

Dicer regulates miRNA maturation downstream of Drosha. To investigate whether Drosha regulates oligodendrocyte commitment of NSCs via miRNAs, we deleted Dicer (Dicer cKO) from radial DG NSCs with the adeno-*gfap*::Cre virus (Figure S2A). Dicer cKO did not affect the number of Sox2$^+$ progenitors (data not shown) and caused a minor decrease in neuroblasts, consistent with the role of Dicer in neuronal survival and maturation (Figures 2G, S2N, and S2O) (Davis et al., 2008). Unlike Drosha cKO, Dicer cKO did not induce oligodendrocytic differentiation of DG NSCs (ctrl versus Dicer cKO, $p = 0.56$; Figures 2F and 2G). Therefore, Drosha but not Dicer inhibits oligodendrocyte differentiation of adult DG NSCs in vivo, indicating that the mechanism of induced fate switching caused by the loss of Drosha does not primarily involve miRNAs.

Drosha cKO DG NSCs Produce Oligodendrocytes In Vitro

To investigate the mechanisms of Drosha-regulated NSC fate acquisition, we generated a self-renewing DG NSC culture system that recapitulates in vivo features of neurogenesis including expression of the progenitor markers Sox2 and BLBP (Figure S2P). Upon growth factor removal (−FGF2/−EGF), DG NSCs differentiated into neurons and astrocytes but not oligodendrocytes, indicating conserved intrinsic cell fate restriction (Figure S2Q; data not shown) (Bonaguidi et al., 2011; Lugert et al., 2010). We cultured DG NSCs from adult *Drosha*$^{fl/fl}$, *Dicer*$^{fl/fl}$, and *Drosha*$^{wt/wt}$*Dicer*$^{wt/wt}$ (control) animals that carried the *Rosa26-CAG::EGFP* Cre reporter. Following adeno-Cre viral transduction, we investigated the effects of Drosha and Dicer cKO (Figures S2R and S2S). Two days post-infection, BLBP$^+$ progenitors were reduced in the Drosha cKO compared with control and Dicer cKO cultures, similar to the reduction in progenitors after Drosha ablation in vivo (Figures 2J–2M). Both differentiated Drosha cKO and Dicer cKO NSCs generated fewer neurons in vitro (Figures 2M and S2T–S2V). However, we observed an increase in apoptotic cells (cleaved Caspase3$^+$) in the Dicer cKO cultures compared with Drosha cKO and control, confirming that Dicer is crucial for neuronal survival and providing an explanation for the reduction in neurons in its absence (Figure S2W). Drosha cKO induced an increase in NG2$^+$ OPCs in the cultures and this at the expense of neuron and astrocyte production (Figures 2K, 2M, and S2X). Dicer cKO induced a slight but not significant increase in NG2$^+$ OPCs in the cultures (ctrl versus Dicer cKO, $p = 0.27$; Figures 2L and 2M). Hence, DG NSCs retain a cell-intrinsic bias against oligodendrocyte differentiation in vitro, and Drosha controls this fate decision. We sorted Drosha cKO, Dicer cKO, and control DG NSCs 48 hr after adeno-Cre virus infection in vitro and determined the expression profiles of 381 miRNAs by microarray. Two hundred sixty miRNAs were detected in control DG NSCs (mean Ct values < 32), and their levels were not significantly changed 48 hr after Drosha cKO ($R^2 = 0.81$; Figure S2Y), even though the phenotypes were well established by this time. Dicer cKO resulted in moderate changes in miRNA levels after 48 hr ($R^2 = 0.66$; Figure S2Z), although Dicer cKO NSCs did not display an obvious phenotype at this time. Hence, Drosha cKO did not cause major global changes in miRNA levels, and any changes were less than in Dicer cKO DG NSCs. These data support that the mechanism of Drosha suppression of oligodendrocyte production by DG NSCs is independent of Dicer and miRNAs.

Drosha Binds and Cleaves the NFIB mRNA Regulating Expression

Drosha can bind and cleave hairpin loops in mRNAs (Chong et al., 2010; Han et al., 2009; Knuckles et al., 2012; Macias et al., 2012). In silico analysis (Evofold) (Pedersen et al., 2006) revealed two evolutionarily conserved hairpins in the mRNA of NFIB, a short 20-base hairpin in the 5′ UTR (5′ UTR HP) and a longer hairpin of 83 bases in the 3′UTR (3′ UTR HP) (Figure 3A). NFIB plays roles in the development of glial cells and myelin tracts (Barry et al., 2008; Deneen et al., 2006; Harris et al., 2015; Kang et al., 2012; Steele-Perkins et al., 2005). To examine whether Drosha binds directly to NFIB mRNA in DG NSCs, we performed cross-linked immunoprecipitation (CLIP) for endogenous Drosha protein and examined the bound RNAs (Figures S3A and S3B). NFIB mRNA cross-linked immunoprecipitated with Drosha from DG NSCs, as did the known target DGCR8 mRNA (Figures 3B and S3B) (Han et al., 2009; Knuckles et al., 2012).

In order to address whether either of the two NFIB mRNA hairpins convey Drosha association, we placed the 5′ UTR HP and 3′ UTR HP into the SV40 3′ UTR downstream of the *Renilla* Luciferase (rLuc) coding region of the psiCheck reporter vector (Figure 3C). We expressed 5′ UTR HP and 3′ UTR HP containing rLuc mRNAs in N2a cells and performed CLIP to address binding by Drosha. Both the 5′ UTR HP and 3′ UTR HP of NFIB bound to Drosha more efficiently than the SV40 3′ UTR sequence alone

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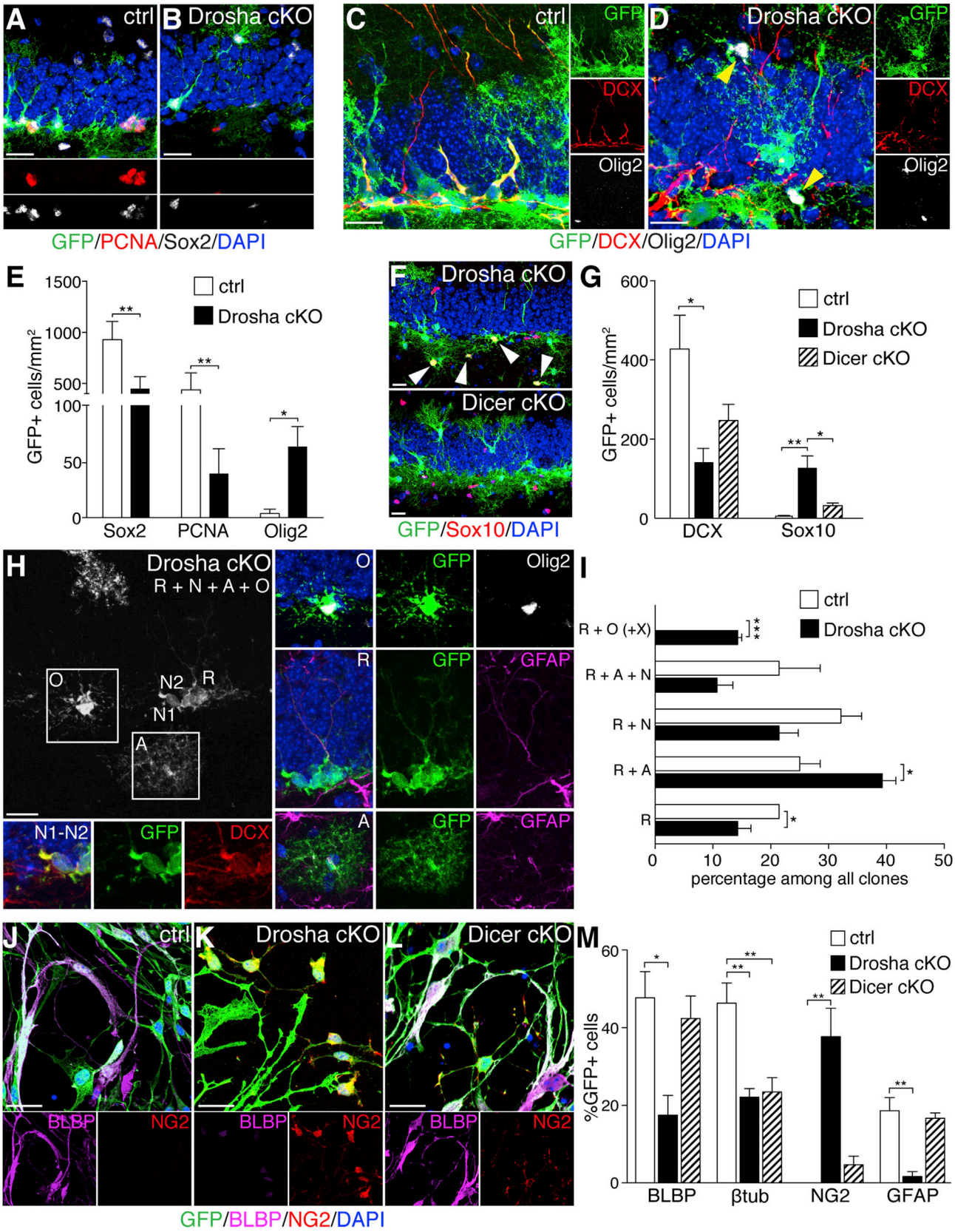

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(Figure 3D). These data suggest that both NFIB mRNA hairpins are bound by Drosha.

We evaluated whether Drosha cleaves the NFIB hairpins by in vitro processing assays (Figure 3E) (Lee and Kim, 2007). Incubation of in vitro transcribed NFIB 3′ UTR RNA with purified Flag-tagged Drosha resulted in cleavage and generation of RNA fragments (Figure 3F). NFIB 5′ UTR HP was not cleaved in vitro, suggesting that, although bound, it is not processed by Drosha (Figure S3C). We assessed whether fragmented NFIB mRNAs were present in DG NSCs in vivo by 5′ rapid amplification of cDNA ends (5′RACE). Multiple NFIB mRNAs fragmented in the vicinity of the 3′ UTR HP were detected in wt NSCs (Figure S3D). Fragmented NFIB transcripts were not detected in Drosha cKO NSCs, supporting that NFIB mRNA fragmentation at the 3′ UTR HP is dependent on Drosha (Figure S3D). Sequencing and mapping of 48 independent clones of the NFIB 5′RACE fragments supported the in vitro processing analysis (Figures 3F and S3D). The multiple fragmented RNA species suggest that either Drosha processing of the 3′ UTR HP is not as accurate as its processing of a pri-miRNA RNA or additional ribonucleases may be associated with the Drosha complex, and these cleave the RNAs further. We analyzed changes in NFIB RNA fragmentation in sorted NSCs following Drosha cKO compared with control by qRT-PCR over the 3′ UTR HP. Drosha cKO increased the relative levels of non-cleaved NFIB transcripts, confirming the Drosha-dependent destabilization of NFIB RNAs in vivo (Figure 3G).

To evaluate whether Drosha affects translation of NFIB 3′ UTR HP mRNAs, we performed Luciferase assays in cultured adult DG NSCs (Figure S3E). Drosha cKO increased Luciferase activity of an NFIB 3′ UTR HP containing synthetic mRNA (Figure S3F). Surprisingly, Dicer cKO also increased translation of the NFIB 3′ UTR HP containing Luciferase mRNA by an unknown mechanism, indicating that under these experimental conditions, Dicer can also regulate NFIB 3′ UTR HP containing mRNAs.

Drosha interaction with hairpins in mRNAs can result in destabilization of the transcripts (Han et al., 2009; Knuckles et al., 2012). We isolated *Hes5::CreER*^{T2} Drosha cKO and *Hes5::CreER*^{T2} control (Drosha$^{wt/wt}$) DG NSCs by fluorescence-activated cell sorting (FACS) based on GFP expression from the Cre-activated *Rosa26-CAG::EGFP* locus following acute induction with TAM (Figure S3G). Drosha mRNA levels were reduced in Drosha cKO cells compared with controls (Figure S3G). Interestingly, NFIB mRNA levels were increased in Drosha cKO NSCs, suggesting that Drosha suppresses NFIB mRNA expression in DG NSCs in vivo (Figure S3G). As cultured DG NSCs retain Drosha function and blockade of oligodendrocyte differentiation, we speculated that Drosha-dependent regulation of NFIB should also be present in vitro. We infected DG NSCs in vitro with adeno-Cre virus and isolated Drosha cKO and control NSCs by FACS 2 dpi (Figure S3H). NFIB and Sox10 mRNA levels were increased in cultured Drosha cKO but not in Dicer cKO NSCs (Figure S3H). Therefore, Drosha regulates NFIB mRNA levels in DG NSCs in vivo and in vitro.

Drosha cKO-Induced Oligodendrocytic Differentiation Depends on NFIB

We addressed whether NFIB is sufficient to drive oligodendrogenesis from adult DG NSCs. Overexpressed NFIB increased Sox10$^+$ and NG2$^+$ OPCs in DG NSC cultures and had a negative impact on neurogenesis (Figures 4A and S4A–S4E). Therefore, expression of NFIB is sufficient to induce programming of DG NSCs to oligodendrocytes. We addressed whether NFIB is required for the Drosha cKO-induced oligodendrocyte differentiation of NSCs. We ablated Drosha from DG NSCs in vitro with adeno-Cre viruses and simultaneously prevented NFIB mRNA accumulation by knockdown using specific endoribonuclease-prepared small interfering RNAs (esiRNAs) (Figure 4B). Twenty-four hours after esiRNA transfection, NFIB mRNAs were undetectable in DG NSCs compared with cells transfected with a control rLuc esiRNA (Figure S4F). Neither esiRNA rLuc nor esiRNA NFIB expression affected the differentiation of control DG NSCs (Figures 4C, 4D, S4G, and S4H). As expected, most Drosha cKO NSCs transfected with the esiRNA rLuc differentiated into NG2$^+$ OPCs (Figures 4C and 4E). In contrast, NFIB knockdown reduced NFIB expression and decreased oligodendrocytic differentiation of Drosha cKO cells (Figures 4C and 4F). Like their control counterparts, NFIB knockdown Drosha cKO NSCs adopted a neuronal fate or remained as progenitors (Figures 4G and 4H). Thus, Drosha negatively regulates DG NSC differentiation toward an oligodendrocytic fate by suppressing NFIB mRNA levels (Figure S4I). Upon Drosha cKO, inhibition of NFIB is released, and an oligodendrocytic differentiation program is activated (Figure S4J).

DISCUSSION

Adult NSC identity is orchestrated by complex regulatory gene networks and neurogenic niche microenvironments. Post-transcriptional modifications add an additional level of

Figure 2. Drosha Deletion from DG NSCs Induces Oligodendrocyte Fate Commitment

(A and B) GFP$^+$Sox2$^+$progenitors and GFP$^+$PCNA$^+$ mitotic cells in control (A) and Drosha cKO (B) animals at day 21 post-adeno-*gfap*::Cre virus infection.

(C and D) GFP$^+$DCX$^+$ neuroblasts and GFP$^+$Olig2$^+$ oligodendrocytes in control (C) and Drosha cKO (D) animals at day 21.

(E) Quantification of GFP$^+$Sox2$^+$, GFP$^+$PCNA$^+$ progenitors and GFP$^+$Olig2$^+$ oligodendrocytes in control and Drosha cKO day 21 after adeno-*gfap*::Cre virus infection (control, n = 3; Drosha cKO, n = 3) Two-sided Student's t test: *p < 0.05, **p < 0.01.

(F) GFP$^+$Sox10$^+$ oligodendrocytes in Drosha cKO and Dicer cKO animals.

(G) Quantification of GFP$^+$DCX$^+$ neuroblasts and GFP$^+$Sox10$^+$ oligodendrocytes upon Drosha cKO and Dicer cKO (control, n = 3; Drosha cKO, n = 3; Dicer cKO, n = 3). ANOVA with Bonferroni post hoc test: *p < 0.05, **p < 0.01.

(H) Tripotent clone derived from a single Drosha cKO NSC. A, astrocyte; N, neuron; O, oligodendrocyte; R, radial NSC.

(I) Quantification of clone composition in control and Drosha cKO (control clones, n = 28; Drosha cKO clones, n = 41). Two-sided Student's t test: *p < 0.05, ***p < 0.001.

(J–L) GFP$^+$BLBP$^+$ and GFP$^+$NG2$^+$ expression in cultured control (J), Drosha cKO (K), and Dicer cKO (L) NSCs 2 dpi with adeno-Cre virus.

(M) Quantification of neural lineage marker expression by adeno-Cre-infected (GFP$^+$) control, Drosha cKO, and Dicer cKO NSCs 2 dpi (n = 4). Kruskal-Wallis with Dunn post hoc test: *p < 0.05, **p < 0.01.

Data are mean ± SEM. The scale bars represent 20 μm. See also Figure S2 and Tables S2 and S3.

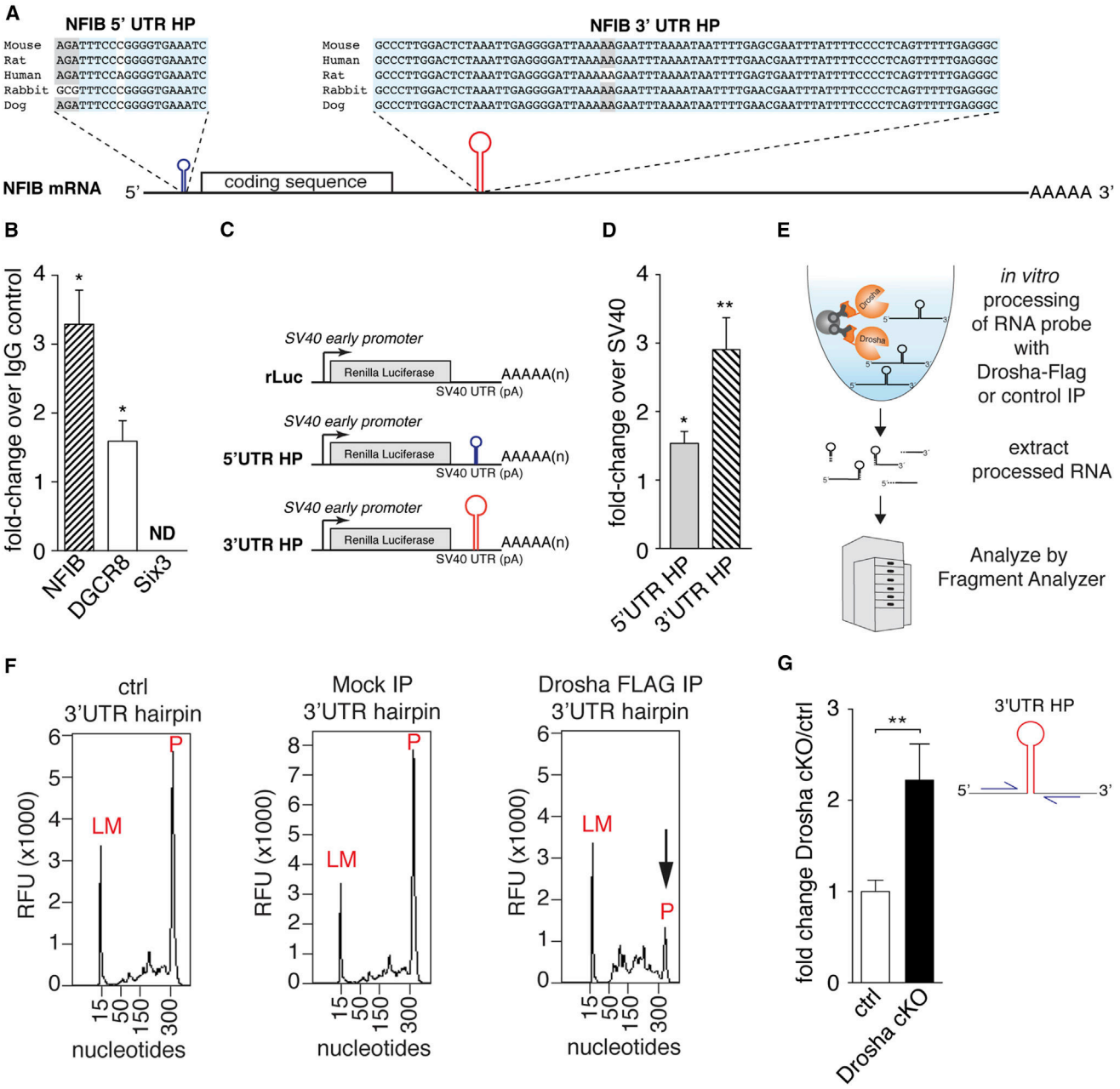

Figure 3. Drosha Binds and Cleaves NFIB mRNA in DG NSCs

(A) Evolutionary conserved hairpins 5′ UTR HP (blue) and 3′ UTR HP (red) in the NFIB mRNA sequence.

(B) Drosha CLIP-qRT-PCR of NFIB mRNA from DG NSCs. DGCR8 and Six3 mRNAs were used as positive and negative control CLIP targets, respectively (n = 3 replicates). Mann-Whitney test: *p < 0.05.

(C) Scheme of the psiCheck *Renilla* Luciferase constructs (rLuc) containing the NFIB 5′ UTR HP or 3′ UTR HP sequence in the SV40 UTR.

(D) qRT-PCR analysis of rLuc mRNA pulled down with Drosha from psiCheck-NFIB 5′ UTR HP and psiCheck-NFIB 3′ UTR HP transfected N2a cells relative to the pull-down from psiCheck-rLuc transfected cells (n = 3 replicates). Two-sided Student's t test: *p < 0.05, **p < 0.01.

(E) Scheme of the in vitro processing procedure.

(F) Capillary electrophoresis electropherograms of NFIB 3′ UTR HP RNA (probe) incubated with the beads alone (ctrl), incubated with mock IP sample, or flag-tagged Drosha IP (Drosha FLAG IP). Arrow points to degraded 3′ UTR HP probe. Loading marker (LM) and probe (P) are indicated.

(G) qRT-PCR analysis of the NFIB 3′ UTR HP in control and Drosha cKO NSCs 2 days after adeno-Cre infection.

Data are mean ± SEM.

regulation to NSC maintenance and differentiation. Growing evidence suggest that miRNA-independent functions of the microprocessor are conserved mechanisms that regulate several cellular processes in the nervous system and other tissues (Chong et al., 2010; Han et al., 2009; Karginov et al., 2010; Knuckles et al., 2012; Macias et al., 2012).

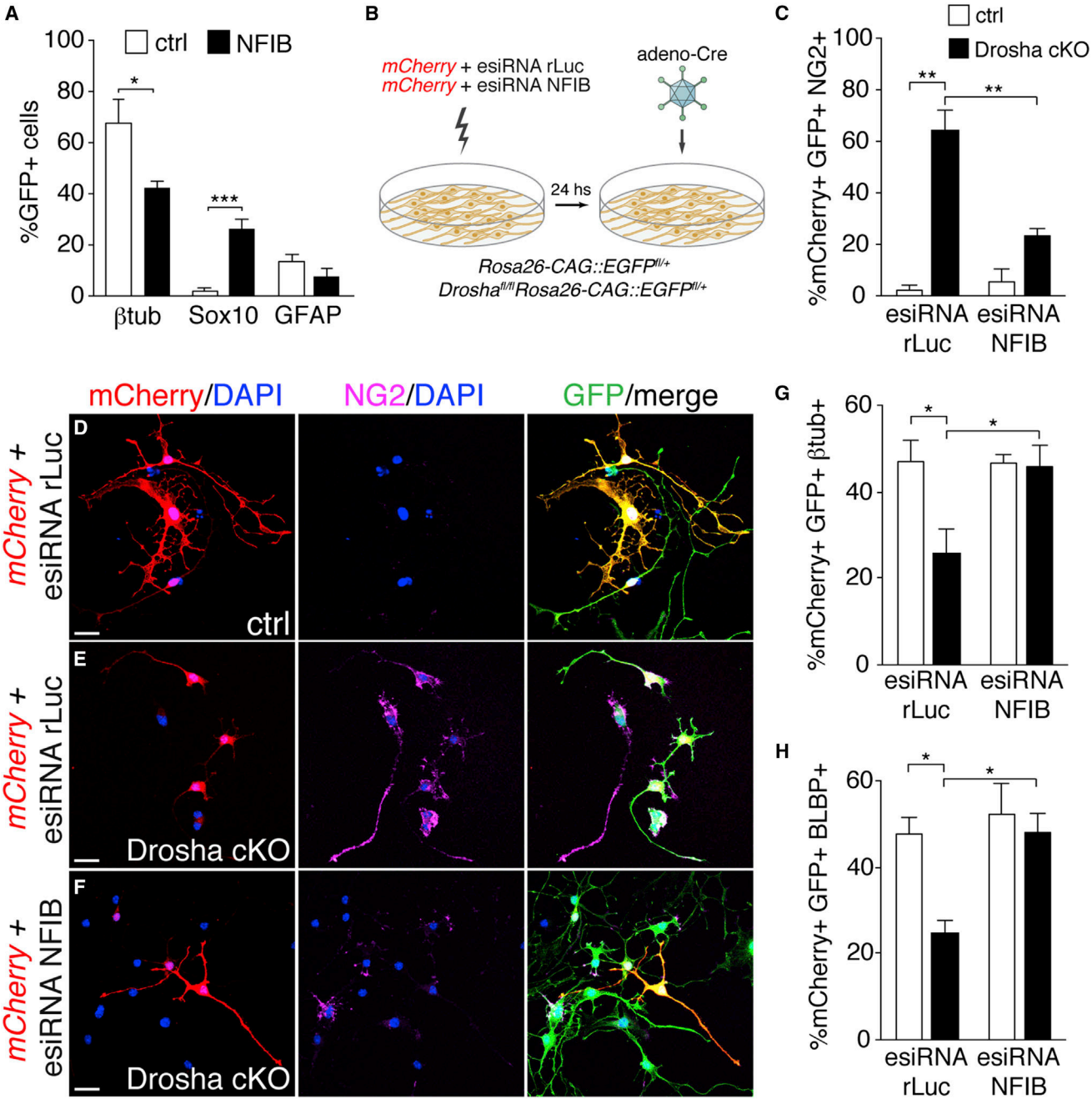

Figure 4. NFIB Knockdown Rescues Drosha cKO-Induced Oligodendrocyte Differentiation

(A) Quantification of lineage marker expression by NFIB overexpressing DG NSCs after 5-days of differentiation (n = 3 replicates). Mann-Whitney test: *$p < 0.05$, ***$p < 0.001$.

(B) Experimental paradigm of the nucleofection experiments.

(C) Quantification of adeno-Cre virus infected (GFP^+) $mCherry^+NG2^+$ OPCs in Drosha cKO and control NSCs nucleofected with control rLuc esiRNA or NFIB esiRNA.

(D–F) $mCherry^+$, GFP^+, and $NG2^+$ cells in adeno-Cre virus infected control NSC cultures nucleofected with the control esiRNA, Drosha cKO NSCs nucleofected with the control esiRNA (E), and Drosha cKO NSCs nucleofected with the NFIB esiRNA (F).

(G) Quantification of adeno-Cre virus infected (GFP^+) $mCherry^+βtub^+$ neurons from Drosha cKO and control NSCs nucleofected with rLuc esiRNA or NFIB esiRNA.

(H) Quantification of adeno-Cre virus infected (GFP^+) $mCherry^+BLBP^+$ progenitors from Drosha cKO and control NSCs nucleofected with control rLuc esiRNA or NFIB esiRNA.

Data are mean ± SEM. Biological replicates, n = 3. Kruskal-Wallis with Dunn post hoc test: *$p < 0.05$, **$p < 0.01$. The scale bars represent 20 μm.

Here we show that Drosha plays a central role in regulating progenitors of the adult DG by sustaining NSC potential. Upon *Drosha* ablation, DG NSCs are depleted, and gliogenesis increases at the expense of neurogenesis. By comparing Drosha cKO and Dicer cKO mice, we identified the transcription factor NFIB as a target of Drosha and showed that the blockade of NFIB expression is necessary for inhibiting oligodendrocyte formation and enabling neurogenesis in the adult DG. Therefore, Drosha regulates DG neurogenesis and gliogenesis at least partially through a miRNA and Dicer-independent, cell-intrinsic fate program.

CLIP experiments revealed that the microprocessor targets different RNA classes, including pri-miRNAs, small nucleolar RNA, long non-coding RNA, and mRNAs (Macias et al., 2012). The microprocessor interactome has been defined in human embryonic stem cells and indicates the importance of cell type and biological context (Seong et al., 2014). However, it is clear that several mRNAs are processed by the microprocessor, resulting in their destabilization (Chong et al., 2010; Johanson et al., 2015; Knuckles et al., 2012). The non-canonical functions of the microprocessor represent a rapid and efficient way to influence gene expression. Our understanding of the mechanisms underlying these alternative functions of Drosha and the microprocessor need further investigation. The Drosha-DGCR8 complex is required for miRNA biogenesis, but it is possible that other protein-protein interactions underlie the alternate functions of Drosha (Macias et al., 2015).

DG NSCs are fate committed to glutamatergic granule neuron and astrocytic fates in vivo (Bonaguidi et al., 2011; Lugert et al., 2010). How this intrinsic fate restriction is controlled remained unclear. In vitro studies showed that DG NSCs are able to generate oligodendrocytes only under specific conditions, including co-culture with neurons (Song et al., 2002; Suh et al., 2007). Furthermore, reprogramming of adult DG NSCs by *Ascl1* overexpression leads to a shift in fate from neuronal to oligodendrocyte differentiation (Braun et al., 2015; Jessberger et al., 2008). A potential link between Drosha and Ascl1 remains to be shown, but Ascl1 mRNA was not cross-linked immunoprecipitated with Drosha from DG NSCs (data not shown).

Clonal lineage tracing of DG NSCs in vivo showed symmetric and asymmetric neuron and astrocytic fates (Bonaguidi et al., 2011). Drosha cKO NSCs exited the stem cell pool and the cell cycle and generated few progeny. However, at the population and single-cell levels, DG NSCs retain the potential to generate all three cell lineages of the brain, but Drosha mediates the intrinsic restriction of oligodendrocyte differentiation potential.

NFI transcription factors can activate and repress gene transcription depending on the gene and cellular context (Chang et al., 2013; Gronostajski, 2000; Messina et al., 2010). NFIB influences stem cell maintenance and differentiation in several tissues, including in the SVZ, as part of a cross-regulatory network together with Pax6/Brg1 (Chang et al., 2013; Ninkovic et al., 2013). In addition, NFIB can repress Notch signaling in embryonic hippocampal NSCs by repressing *Hes1* promoter activity (Piper et al., 2010). Therefore, we speculate that induction of NFIB expression might lead to inhibition of stem cell genes and block of Notch signaling resulting in exhaustion of the DG NSC pool and differentiation. Moreover, we also show for the first time that NFIB has a central function in regulating oligodendrocyte fate commitment in the adult DG. It remains to be shown which genes are regulated downstream of NFIB. Although we cannot exclude that NFIB acts as a transcriptional repressor of genes required for neuronal differentiation and therefore indirectly promotes gliogenesis, NG2 is upregulated in response to Drosha cKO in an NFIB-dependent manner. Interestingly, *Cspg4* (the gene encoding NG2) has NFI binding motifs that are bound by NFIB, suggesting a direct regulation in DG NSCs (Chang et al., 2013). We believe this is the first demonstration of a non-canonical Drosha-mediated regulation of adult stem cell fate through a niche-independent intrinsic pathway. In the future, it will be important to understand the targets of this post-transcriptional pathway and whether stem cells are able to modulate Drosha activity to control cell fate in order to satisfy demand.

EXPERIMENTAL PROCEDURES

Animal Husbandry

The mice used have been described previously (Supplemental Experimental Procedures). Mice were maintained on a 12 hr day-night cycle with free access to food and water under specific pathogen-free conditions and according to Swiss federal regulations. All procedures were approved by the Basel Cantonal Veterinary Office (license numbers 2537 and 2538).

Hippocampal NSC Cultures, Adenoviral Infection, and Nucleofection

DG NSCs were isolated from 8-week-old mice as described previously (Lugert et al., 2010). DG NSCs were infected with an adeno-Cre adenovirus at a multiplicity of infection of 100 and fixed after 24 or 48 hr. DG NSC cultures were nucleofected using a mouse neural stem cell kit (Lonza) (Supplemental Experimental Procedures).

FACS

After TAM induction, NSCs were isolated from *Hes5CreER*^{T2}*Rosa26-CAG::EGFP*$^{fl/+}$ and *Hes5::CreER*^{T2}*Drosha*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$ using a FACSariaIII (BD Biosciences) (Supplemental Experimental Procedures).

RNA Isolation, qRT-PCR, and Analysis of miRNA Expression

Total RNA was isolated from cultured or sorted DG NSCs using Trizol reagent (Life Technologies). Analysis of gene expression was performed as described in Supplemental Experimental Procedures. miRNAs were isolated using mirVANA kit (ThermoFisher) following the miRNA enrichment procedure and quantified by TaqMan arrays (Life Technologies) (Supplemental Experimental Procedures).

In Vitro Processing of NFIB HP RNAs

In vitro processing was performed on 5′ and 3′ UTR NFIB HP RNAs as described previously with minor adaptations (Supplemental Experimental Procedures) (Lee and Kim, 2007).

5′ RACE

5′ RACE experiments were performed on 3 μg of total RNA of control and Drosha cKO NSCs following the manufacturer's instructions (Invitrogen) (Supplemental Experimental Procedures).

Luciferase Assay

DG NSCs were transduced with an adeno-Cre adenovirus at a multiplicity of infection of 100 with or without subsequent nucleofection 2 days later with the psiCheck2 containing the 3′ UTR HP or 5′ UTR HP or control psiCheck2 vectors (Supplemental Experimental Procedures).

Quantification and Statistical Analysis

Randomly selected, stained cells were analyzed with fixed photomultiplier settings on a Zeiss LSM510 confocal and Apotome2 microscope. For clonal

analysis, the entire hippocampus was sectioned and reconstructed as described previously (Bonaguidi et al., 2011) (Supplemental Experimental Procedures). Percentages were converted by arcsine transformation. Statistical comparisons were conducted by two-tailed unpaired Student's t test, Mann-Whitney test, one-way ANOVA, or Kruskal-Wallis with Dunn post hoc test as indicated. Statistical significance was assessed using GraphPad Prism software (GraphPad Software). Significance was established at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2016.07.003.

AUTHOR CONTRIBUTIONS

C.R., A. Erni, A.G., R.B., A. Engler, P.J.G., and M.M. designed and performed experiments and evaluated and interpreted the data. T.W. and S.J. contributed reagents. V.T. conceived and designed the project and evaluated the data. C.R., A. Erni, and V.T. wrote the paper and prepared the figures. All authors edited and proofread the manuscript.

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Supplemental Information

Multipotency of Adult Hippocampal NSCs In Vivo Is Restricted by Drosha/NFIB

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Figure S1, Related to Figure 1

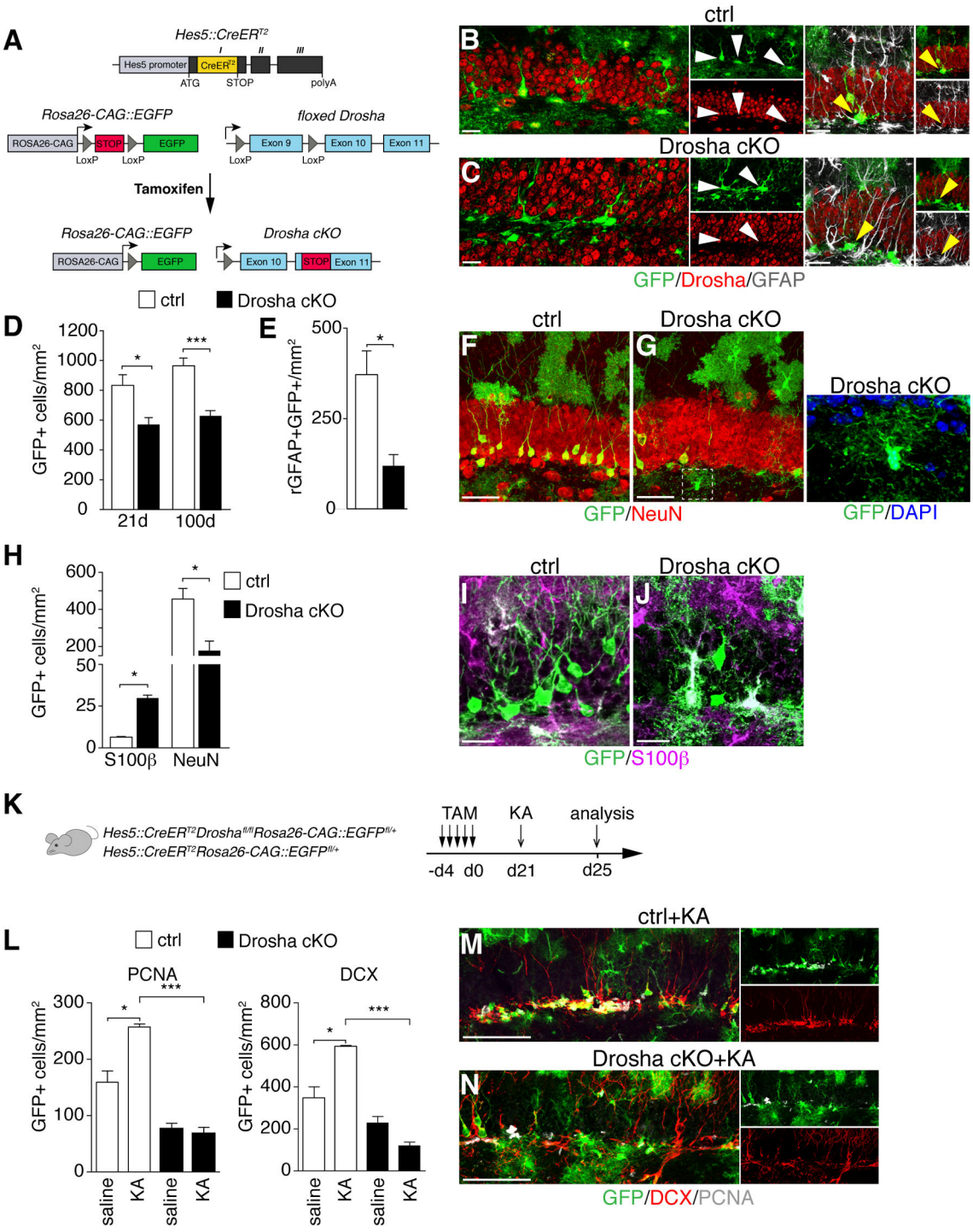

Figure S1. Drosha cKO from *Hes5::CreER^{T2}* expressing NSCs impairs neurogenesis in the DG. (A) Overview of the *Hes5::CreER^{T2}*, *Rosa26-CAG::EGFP* and floxed *Drosha* alleles and Cre-mediated gene rearrangements (Chong et al., 2008; Harfe et al., 2005; Lugert et al., 2012). TAM treatment induces Drosha cKO and constitutive expression of GFP from the *Rosa26-CAG::EGFP* reporter allele in *Hes5::CreER^{T2}*-expressing cells and their progeny. (B) Twenty-one days after TAM induction, GFP$^+$ *Hes5*-derived cells in control animals express Drosha (white arrowheads) and these include radial GFP$^+$GFAP$^+$ NSCs (yellow arrowheads). (C) Twenty-one days after TAM induction, GFP$^+$ *Hes5*-derived cells do not express Drosha in the Drosha cKO (white arrowheads) including *Hes5*-derived radial GFP$^+$GFAP$^+$ (yellow arrowheads). (D) Quantification of *Hes5*-derived (GFP$^+$) cells at d21 and d100 post-TAM induction in control and Drosha cKO animals (control n = 5, Drosha cKO n = 5. Two-sided

Student's t-test, *P<0.05, ***P<0.001). (E) Quantification of radial GFP^+GFAP^+ cells at d21 post-TAM induction in control and Drosha cKO animals (control n = 5, Drosha cKO n = 5. Two-sided Student's t-test, *P<0.05). (F and G) $NeuN^+$ mature neurons in control and Drosha cKO animals at d100 post-TAM induction. Inset and magnification on the right show an oligodendrocyte in Drosha cKO animals at d100 post-TAM induction. (H) Quantification of $GFP^+S100\beta^+$ astrocytes and GFP^+NeuN^+ newborn neurons at d100 post-TAM induction in control and Drosha cKO animals (control n = 5, Drosha cKO n = 5. Two-sided Student's t-test, *P<0.05). (I and J) $S100\beta^+$ mature astrocytes in the Drosha cKO compared to control animals at d100. (K) TAM induction and kainic acid (KA) treatment regime to study the activation of Drosha cKO progenitors after epileptic seizures. TAM was administered once per day for 5 consecutive days. KA was administered systemically 21 days after TAM induction and the mice analyzed 4 days later at d25. (L) Quantification of proliferative GFP^+PCNA^+ progenitors and GFP^+DCX^+ neuroblasts on d4 after KA treatment in control and Drosha cKO animals. (M and N) $PCNA^+$ and DCX^+ cells in control and Drosha cKO animals on d4 after KA treatment (control n = 3, Drosha cKO n = 4. One-way ANOVA with Bonferroni post-hoc test: *P<0.05, ***P<0.001). Data are mean ± SEM. Scale bars represent 20 μm in (B), (C), (F), (G), (I) and (J) and represent 100 μm in (M) and (N).

Figure S2, Related to Figure 2

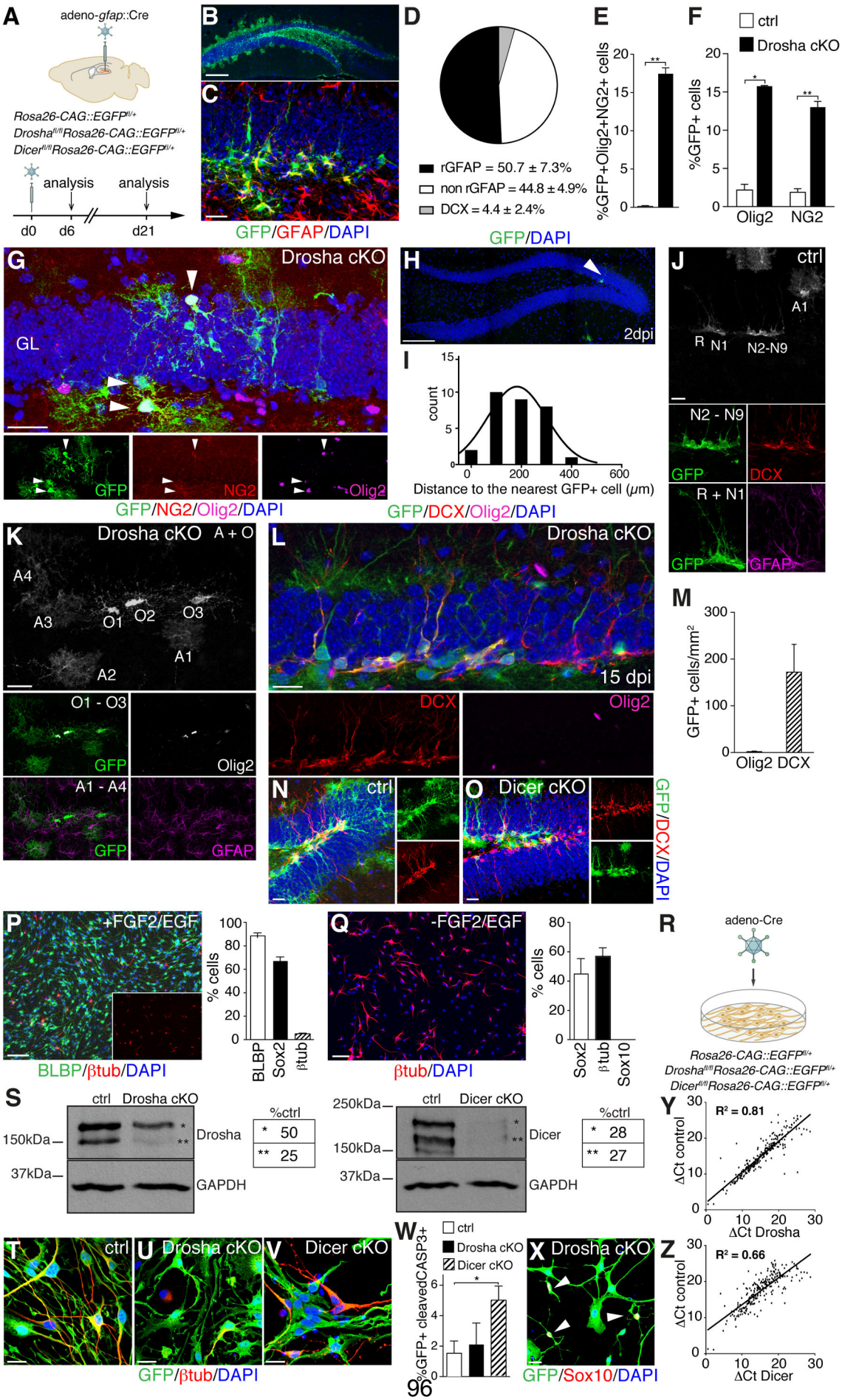

Figure S2. Adult hippocampal NSCs produce oligodendrocytes upon Drosha deletion *in vivo* and *in vitro*. (A) Experimental paradigm of adeno-*gfap::*Cre stereotactic intracranial injection and gene deletion from $GFAP^+$ radial NSCs and analysis at d6 and d21. (B) GFP expression from the recombined *Rosa26-CAG::EGFP* allele following adeno-*gfap::*Cre injection into the DG of *Rosa26-CAG::EGFP*$^{fl/+}$ mice. (C) GFP and GFAP expression at 6 dpi. (D) Quantification of GFP^+GFAP^+ and GFP^+DCX^+ at 6 dpi. (E) Quantification of $GFP^+Olig2^+NG2^+$ cells in the DG of Drosha cKO (*Hes5::CreER*^{T2}*Drosha*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$) and control (*Hes5::CreER*^{T2}*Rosa26-CAG::EGFP*$^{fl/+}$) animals (control n = 3, Drosha cKO n = 3. Two-sided Student's t-test: $^{**}P<0.01$). (F) Quantification of GFP^+Olig2^+ and GFP^+NG2^+ cells in Drosha cKO (*Hes5::CreER*^{T2}*Drosha*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$) DG NSCs and control (*Hes5::CreER*^{T2}*Rosa26-CAG::EGFP*$^{fl/+}$) animals (control n = 3, Drosha cKO n = 3. Two-sided Student's t-test: $^{*}P<0.05$, $^{**}P<0.01$). (G) $NG2^+$ and $Olig2^+$ oligodendrocytes in the DG of Drosha cKO (*Hes5::CreER*^{T2}*Drosha*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$) at d21 post-TAM induction (arrowheads). (H) Clonal analysis of GFP expression following low dose TAM administration of *Hes5::CreER*^{T2}*Rosa26-CAG::EGFP*$^{fl/+}$ mice after 2 days. (I) Quantification of the distance to the nearest GFP^+ cell 2 days after low dose TAM induction (n = 2 animals). (J) GFP, DCX and GFAP expression following low dose TAM administration of *Hes5::CreER*^{T2}*Rosa26-CAG::EGFP*$^{fl/+}$ animals after 21 days. A - astrocyte, N - neuron, R - radial glia. The cells of each cell-type in the clone are numbered in the image. (K) GFP, Olig2 and GFAP expression following low dose TAM induction of Drosha cKO at d21. A - astrocyte and O - oligodendrocyte. The cells of each cell-type in the clone are numbered in the image. (L) GFP, DCX and Olig2 expression d15 after retro-Cre virus infection of the DG of *Drosha*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$ animals. (M) Quantification of GFP^+DCX and GFP^+Olig2 cells d15 after retro-Cre virus infection of the DG of *Drosha*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$ animals. (N and O) GFP and DCX expression after adeno-*gfap::*Cre-mediated Dicer cKO (*Hes5::CreER*^{T2}*Dicer*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$) and infected control (*Hes5::CreER*^{T2}*Rosa26-CAG::EGFP*$^{fl/+}$) mice. (P) Expression and quantification of $BLBP^+$ and βtub^+ cells derived from NSCs grown in the presence of mitogens (FGF2 and EGF). (Q) βtub expression by cultured DG NSCs upon differentiation induced by mitogen removal and quantification of Sox2, βtub and Sox10 expressing cells (Biological replicates n = 2). (R) Experimental paradigm for gene ablation from cultured adult DG NSCs with adeno-Cre viruses. (S) Western-blot and quantification of Drosha and Dicer protein expression 72 hours after adeno-Cre virus mediated Drosha cKO and Dicer cKO, respectively. (T-V) βtub expression after adeno-Cre virus mediated Drosha cKO and Dicer cKO compared to control. (W) Quantification of $GFP^+cleavedCASP3^+$ cells in cultured control, Drosha cKO and Dicer cKO NSCs d4 post adeno-Cre virus infection (Biological replicates n = 4. Kruskal-Wallis with Dunn post-hoc test: $^{*}P<0.05$). (X) Cells expressing the oligodendrocyte marker Sox10 by Drosha cKO cells 2 days after adeno-Cre virus infection. (Y) ΔCT plots of relative miRNA expression profiles of control (y–axis) versus Drosha cKO (x–axis) DG NSC cultures 48 hours post adeno-Cre infection. Correlation coefficients R^2 = 0.81. (Z) ΔCT plots of relative miRNA expression profiles of control (y–axis) versus Dicer cKO (x–axis) DG NSC cultures 48 hours post adeno-Cre infection. Correlation coefficients R^2 = 0.66. Data are mean ± SEM. Scale bars represent 200 μm in (B), 100 μm in (H), (P) and (Q), 20 μm in (C), (G), (J), (K), (L), (N), (O), (T), (U), (V) and (X).

Figure S3, Related to Figure 3

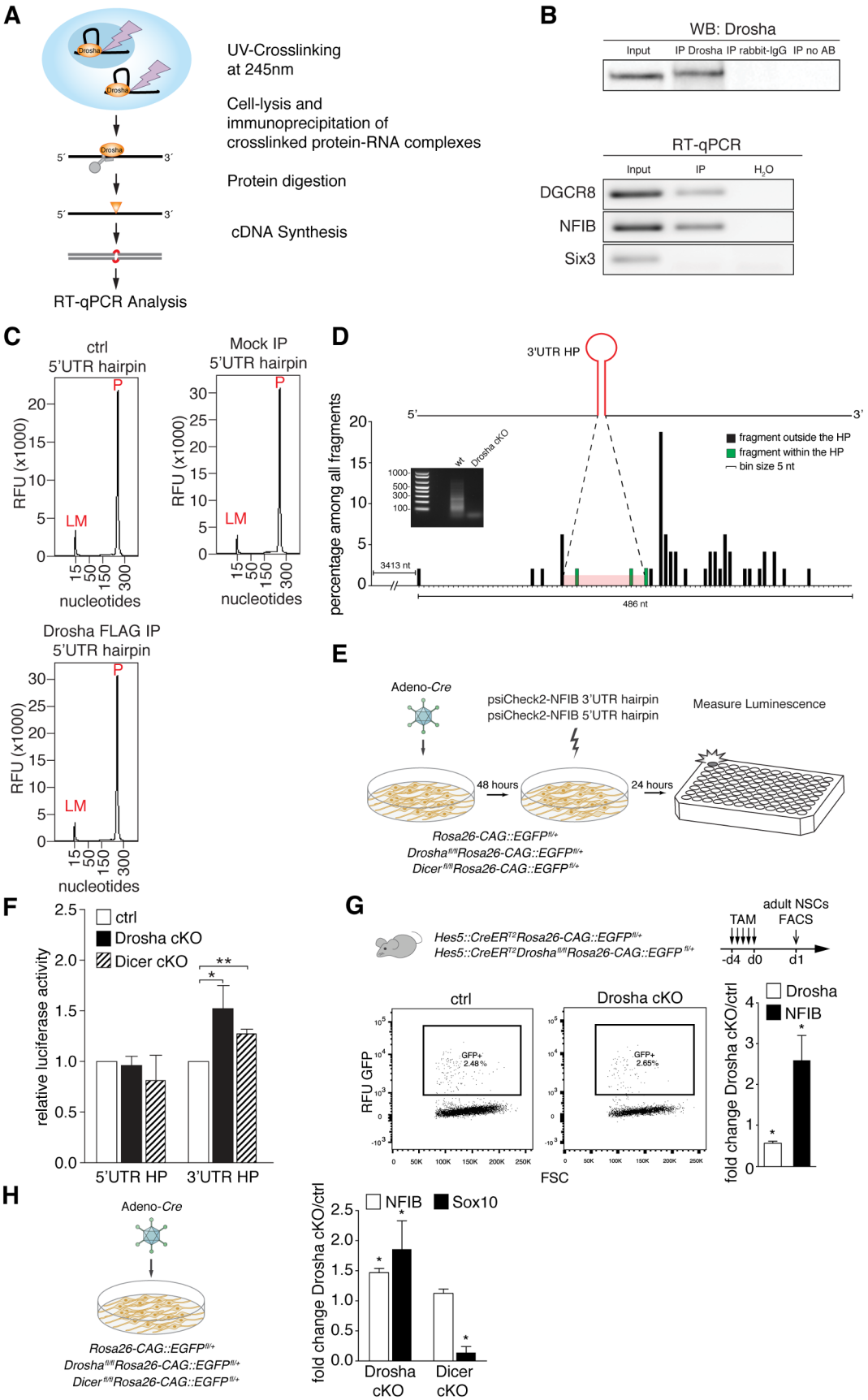

Figure S3. Drosha binds and regulates NFIB mRNA. (A) Scheme of the crosslinked immunoprecipitation (CLIP) procedure. (B) Western-blot for Drosha protein after immunoprecipitation. Rabbit IgG and bead-only (no AB) IPs were performed as negative controls. Drosha CLIP-quantitative RT-PCR for NFIB and DGCR8 (positive control) mRNAs. Six3 mRNA was used as a negative control mRNA in the CLIP experiments. (C) Fragment analyzer electropherograms of NFIB 5'UTR HP RNA probe, control incubated with the beads alone (ctrl) as degradation control, with mock IP, or with flag-tagged Drosha IP (Drosha FLAG IP). Loading marker – LM, full-length probe - P. (D) 5'RACE of NFIB 3'UTR mRNA in wild-type NSCs. Agarose gel of 5'RACE products of control and Drosha cKO NSCs. The diagram represents cleaved fragments identified by Sanger sequencing. Green and black bars identify respectively fragments within and distal to the hairpin sequence. Bin size corresponds to 5 nucleotides. (E) Scheme of luciferase assay. *Rosa26-CAG::EGFP*$^{fl/+}$ (control), *Drosha*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$ and *Dicer*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$ DG NSCs were infected with adeno-Cre viruses and subsequently transfected with psiCheck-NFIB 5'UTR HP or psiCheck-NFIB 3'UTR HP vectors before quantifying luciferase activity. (F) Relative luciferase activity of the psiCheck2 NFIB 5'UTR HP and 3'UTR HP vectors in control, Drosha cKO and Dicer cKO DG NSCs (Biological replicates n = 3. One-sided Student's t-test: *P<0.05, **P<0.01). (G) TAM induction regime for fluorescence activated cell sorting (FACS) of *Hes5::CreER*^{T2}-derived cells. TAM was administered to mice once per day for 5 consecutive days before FACS for GFP$^+$ cells at 1 day (d1) after induction. The GFP$^+$ population was gated on the basis of the GFP-negative population. Quantitative RT-PCR analysis of Drosha and NFIB mRNA levels in the FACSorted GFP$^+$ cells from the Drosha cKO (control n = 12, Drosha cKO n = 19. Two-sided Student's t-test: *P<0.05). (H) Scheme of the *in vitro* deletion assay. Quantitative RT-PCR analysis of NFIB and Sox10 expression by Drosha cKO and Dicer cKO NSCs 48 hours after adeno-Cre infection (Biological replicates n = 3. Two-sided Student's t-test: *P<0.05). Data are mean ± SEM.

Figure S4, Related to Figure 4

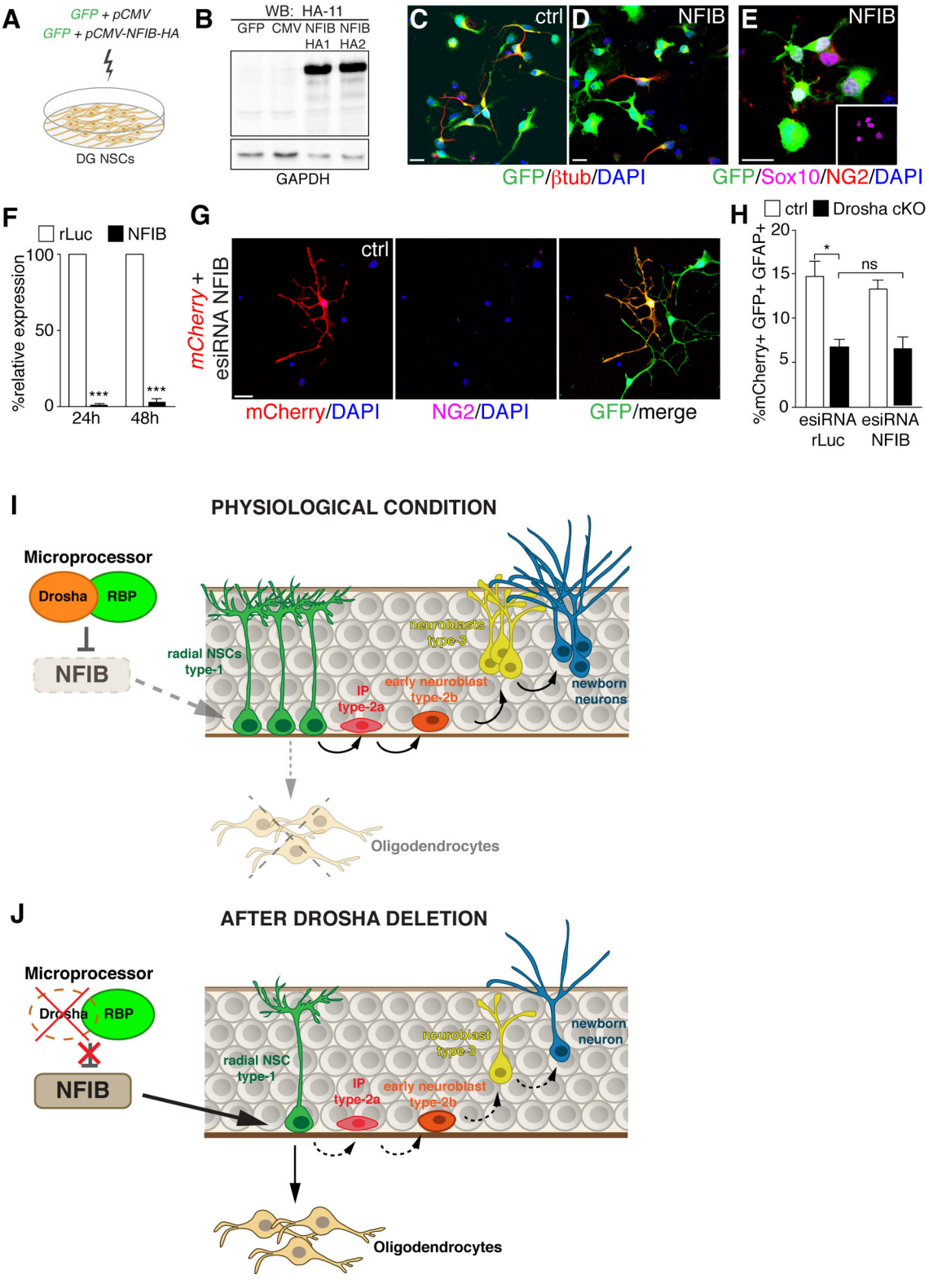

Figure S4. Drosha inhibits oligodendrocyte generation from DG NSCs through NFIB knockdown. (A) Gain of NFIB function experiments in cultured DG NSCs. pCMV-NFIB or empty pCMV expression vectors were nucleofected into cultured adult DG NSCs. (B) Western-blot analysis of transfected DG NSCs blotted for the HA-tagged NFIB (HA1, HA2 are experimental duplicates) compared to empty pCMV vector (CMV) and pCMV-GFP vector (GFP) only transfected cells. (C-D) βtub expression by pCMV (ctrl: C) and pCMV-NFIB (NFIB: D) transfected DG NSCs after 5 days of differentiation. (E)

Sox10 and NG2 expression by NFIB overexpressing DG NSCs after 5 days of differentiation. (F) Quantitative RT-PCR analysis of N2a cells transfected with the control esiRNA (rLuc) and esiRNA targeting NFIB. NFIB mRNA is not detectable 24 and 48 hours after esiRNA NFIB transfection (Biological replicates $n = 3$. Mann-Whitney test: $***P<0.001$). (G) Expression of the oligodendrocyte marker NG2 by control NSCs nucleofected with NFIB esiRNA. (H) Quantification of adeno-Cre infected (GFP^+), nucleofected $mCherry^+$, $GFAP^+$ astrocytes in Drosha cKO and control NSCs nucleofected with control esiRNA (rLuc) or NFIB esiRNAs (Biological replicates $n = 3$. Kruskal-Wallis with Dunn post-hoc test: $*P<0.05$). (I) Under physiological conditions, adult DG NSCs express the RNAseIII Drosha that targets NFIB mRNA and inhibits NFIB protein expression. DG *Hes5*$^+$ NSCs (type-1) produce DCX^+ neuroblasts via intermediate progenitors (IP) that mature into $NeuN^+$ granule neurons, but do not generate oligodendrocytes. (J) After Drosha deletion from adult DG NSCs, NFIB mRNA is up regulated. NFIB expression drives NSCs into oligodendrocyte differentiation at the expense of neuron production. RBP, RNA binding protein. Scale bars represent 20 μm in (C), (D), (E) and (G). Data are mean ± SEM.

Table S1, Related to Figure 1 and S1

	Mean ± SEM (GFP+ cells/mm^2) 5d Tamoxifen + 21d chase				
	GFP+	**Sox2+S100β-**	**PCNA+**	**DCX+**	**radial GFAP+**
Control	838.7 ± 65.3	398.0 ± 26.1	168.2 ± 17.8	444.3 ± 64.6	371.7 ± 65.5
Drosha cKO	577.5 ± 46.4	209.0 ± 34.1	60.5 ± 16.1	269.5 ± 34.3	119.4 ± 30.9
P-values (two-sided t-test)	0.03 (*)	0.01 (*)	0.002 (**)	0.04 (*)	0.02 (*)

	Mean ± SEM (GFP+ cells/mm^2) 5d Tamoxifen + 100d chase				
	GFP+	**Sox2+S100β+**	**NeuN+**	**DCX+**	**radial GFAP+**
Control	969.0 ± 52.4	6.4 ± 0.4	455.7 ± 57.5	284.9 ± 19.1	174.2 ± 47.7
Drosha cKO	625.3 ± 23.9	29.8 ± 1.8	177.3 ± 51.2	84.9 ± 19.4	43.2 ± 17.8
P-values (two-sided t-test)	0.003 (**)	0.002 (***)	0.02 (*)	0.0004 (***)	0.004 (**)

	Mean ± SEM (GFP+ cells/mm^2) 5d Tamoxifen + 21d chase + KA		
	GFP+	**DCX+**	**PCNA+**
Control	955.6 ± 53.8	590.3 ± 7.7	257.4 ± 5.4
Drosha cKO	530.0 ± 40.4	121.4 ± 15.8	68.2 ± 10.9
P-values (one-way ANOVA)	0.003 (**)	0.00004 (***)	0.0003 (***)

Table S1: Density of GFP$^+$ marker expressing cells in the adult DG *in vivo*. Table showing the density of GFP$^+$ cells expressing specific markers at d21 and d100 post-TAM induction and the density of GFP$^+$ cells expressing DCX and PCNA d21 after kainic acid (KA) administration in control and Drosha cKO animals. Values are mean ± SEM.

Table S2, Related to Figure 2

	Mean ± SEM (GFP+ cells/mm^2) adeno-*gfap::*Cre + 21dpi	
	DCX+	**Sox10+**
Control	427.8 ± 85.1	5.3 ± 2.1
Drosha cKO	141.8 ± 34.5	127.8 ± 39.7
Dicer cKO	247.9 ± 40.7	32.1 ± 6.4
P-values (one-way ANOVA + Bonferroni Post-Hoc) ctrl vs. Drosha cKO	0.03 (*)	0.0062 (**)
P-values (one-way ANOVA + Bonferroni Post-Hoc) ctrl vs. Dicer cKO	0.2 (ns)	0.5 (ns)
	Sox2+	**PCNA+**
Control	991.3 ± 80.4	259.2 ± 26.0
Drosha cKO	450.3 ± 116.7	39.3 ± 22.1
P-values (two-sided t-test)	0.01 (**)	0.003 (**)

Table S2: Density of GFP$^+$ marker expressing cells in the adult DG *in vivo* following adeno-*gfap*::Cre adenoviral infection. Table showing the density of GFP$^+$ cells expressing specific markers d21 after adeno-*gfap*::Cre adenovirus infection in control and Drosha cKO animals. Values are mean ± SEM, ns – not significant.

Table S3, Related to Figure 2

	Mean ± SEM (% GFP+ cells) adeno-Cre + 2 dpi				
	BLBP+	**NG2+**	**βtub+**	**GFAP+**	**aCASP3+**
Control	47.7 ± 6.7	0 ± 0	46.3 ± 5.1	18.6 ± 3.4	1.6 ± 0.8
Drosha cKO	17.4 ± 5.1	37.7 ± 7.2	22.1 ± 2.2	1.6 ± 1.2	2.4 ± 1.4
Dicer cKO	42.4 ± 5.7	4.7 ± 2.2	23.4 ± 3.6	16.6 ±1.3	4.7 ± 0.9
P-values (Kruskal-Wallis test) Ctrl vs. Drosha	0.03 (*)	0.001 (**)	0.0029 (**)	0.0011 (**)	0.99 (ns)
P-values (Kruskal-Wallis test) Ctrl vs. Dicer cKO	0.99 (ns)	0.27 (ns)	0.0037 (**)	0.99 (ns)	0.04 (*)

Table S3: Distribution of GFP$^+$ marker expressing cells in adult DG NSCs *in vitro* following adeno-Cre-mediated recombination. Table showing the distribution of GFP$^+$ cells expressing specific markers 2 days after adeno-Cre adenoviral infection of control, Drosha cKO and Dicer cKO DG NSCs *in vitro*. Values are mean ± SEM, ns – not significant.

Table S4, Related to Figure 4 and Figure S4

	Mean ± SEM (% mCherry+GFP+ cells) adeno-Cre + 2 dpi			
	NG2+	**BLBP+**	**βtub+**	**GFAP+**
Control + esiRNA rLuc	2.2 ± 1.8	47.8 ± 3.7	46.9 ± 4.4	14.7 ± 1.5
Drosha cKO + esiRNA rLuc	64.4 ± 10	24.6 ± 2.8	25.6 ± 2.9	6.7 ± 0.8
Control + esiRNA NFIB	4.7 ± 4.7	52.4 ± 7	46.5 ± 1.9	13.3 ± 0.8
Drosha cKO + esiRNA NFIB	23.1 ± 2.6	48.1 ± 4.5	45.7 ± 4.9	6.3 ± 1.3
P-values (Kruskal-Wallis test)	0.001 (**)	0.006 (**)	0.005 (**)	0.003 (**)

Table S4: Distribution of GFP and mCherry expressing cells in adult DG NSCs following NFIB knockdown *in vitro*. Table showing the distribution of GFP^+ marker expressing cells after NFIB knockdown and 2 days after adeno-Cre adenoviral infection of control and Drosha cKO DG NSCs *in vitro*. Values are mean ± SEM.

Supplemental Experimental Procedures

Transgenic animals

Hes5::CreER^{T2}, *Rosa26-CAG::EGFP*, *Drosha$^{fl/fl}$*, *Dicer$^{fl/fl}$* mice have been described elsewhere (Chong et al., 2008; Harfe et al., 2005; Lugert et al., 2012; Tchorz et al., 2012). All mice were maintained on a C57BL6 background and were 8-10 weeks old at the onset of the experiments. CreER^{T2}-recombinase activity from the *Hes5CreER^{T2}* locus was induced by Tamoxifen administration (Sigma; 2 mg/injection in corn oil) injected as a single dose intraperitoneal daily for five consecutive days. For *in vivo* clonal analysis animals received one single injection of Tamoxifen (48 mg/kg in corn oil).

Tissue preparation and immunohistochemistry

Mice were deeply anesthetized by injection of a ketamine/xylazine/acepromazine solution (150 mg, 7.5 and 0.6 mg per kg body weight, respectively). Animals were perfused with ice-cold 0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate buffer. Brains were isolated and post-fixed overnight in 4% paraformaldehyde in 0.1M phosphate buffer, and then cryoprotected with 30% sucrose in phosphate buffer at 4°C overnight. Brains were embedded and frozen in OCT (TissueTEK) and sectioned as 30 µm floating sections by cryostat (Leica). Free-floating coronal sections were stored at -20°C in antifreeze solution until use. For clonal analysis, coronal brain sections (45 µm) through the entire dentate gyrus were maintained in series.

Sections were incubated overnight at room temperature, with the primary antibody diluted in blocking solution of 1.5% normal donkey serum (Jackson ImmunoResearch), 0.5% Triton X-100 in phosphate-buffered saline. For clonal analysis, sections where incubated for 48 hours at 4°C, with primary antibody in blocking solution of 1.5% normal donkey serum (Jackson ImmunoResearch), 2% Triton X-100 in phosphate-buffered saline. Antibodies used: AN2 (1:5, gift of Prof. M. Trotter), activated cleavedCASP3 (Cell Signalling, rabbit, 1:500), BLBP (Chemicon, rabbit, 1:500), βtubulinIII (Sigma, mouse, 1:500), DCX (Santa Cruz, goat, 1:500), Drosha (Abcam, rabbit, 1:100), dsRed (Clonetech, rabbit, 1:500), GFAP (Sigma, mouse, 1:1000; Santa Cruz, goat, 1:500), GFP (AbD Serotec, sheep, 1:250; Invitrogen, rabbit, 1:700; AvesLabs, chicken, 1:500), NeuN (Millipore, mouse, 1:1000), NG2 (Chemicon, rabbit, 1:500), Olig2 (Millipore, rabbit, 1:500), PCNA (DAKO, mouse, 1:1000), S100β (Sigma, mouse, 1:200), Sox2 (Santa Cruz, goat, 1:500), Sox10 (Santa Cruz, goat, 1:500).

Sections were washed in phosphate-buffered saline and incubated at room temperature for 2 hours with the corresponding secondary antibodies in blocking solution. For clonal analysis sections where incubated for 24 hours at 4°C with the corresponding secondary antibody in blocking solution. Secondary antibodies and detection: Alexa488/Cy3/Alexa555/Alexa594/Alexa647/Alexa649 conjugated anti-chicken, mouse, goat, rabbit, rat and sheep immunoglobulin (1:500, Jackson Immunoresearch). Sections were then washed and counter-stained with DAPI (1 µg/ml). For PCNA and Drosha detection, antigens were recovered at 80 °C for 20 minutes in sodium citrate solution (10 mM, pH7.4). Stained sections were mounted on Superfrost glass slides (Thermo Scientific), embedded in mounting medium containing diazabicyclo-octane (DABCO; Sigma) as an anti-fading agent and visualized using a Zeiss LSM510 confocal microscope, Leica SP5 confocal microscope or Zeiss Apotome2 microscope.

Adeno-*gfap*::Cre adenoviral and retro-Cre retrovirus infections in the adult DG

Adult (8-10 week old) mice (*Rosa26-CAG::EGFP$^{fl/+}$*, *Drosha$^{fl/fl}$Rosa26-CAG::EGFP$^{fl/+}$*, *Dicer$^{fl/fl}$Rosa26-CAG::EGFP$^{fl/+}$*) were anesthetized in a constant flow of Isofluorane (3%) in oxygen and positioned in a stereotaxic apparatus (David Kopf instruments). Mice were injected with Temgesic subcutaneous (0.05 mg/kg body weight). The skull was exposed by an incision in the scalp and a small hole (1 mm) drilled through the skull. One µl of adeno-*gfap*::Cre adenovirus (titer 1x10^{12} infectious particles per ml) or retrovirus-Cre (titer 2.7x10^{7}, Braun et al., 2015) was injected in the DG using a sharpened borosilicate glass capillaries at the stereotaxic coordinates -2 mm anteroposterior, 1.5 mm lateral to Bregma and -2.0 mm below the surface of the skull. Mice were killed 6, 15 or 21 days after virus infection. Brain tissue was processed and analyzed by immunohistochemistry as described above.

Induction of epileptic seizures

Seizures were induced as described previously (Lugert et al., 2010), kainic acid (KA, Tocris Bioscience) was administered intraperitoneal at 30 mg/kg body weight. Seizures developed within 45 minutes after injection and spontaneously stopped within 2-3 hours. The mice were sacrificed 4 days after KA injection and the brains processed for immunohistochemical analysis as described above.

Hippocampal neural stem cell cultures

Brains of 8-week old *Rosa26-CAG::EGFP$^{fl/+}$*, *Drosha$^{fl/fl}$Rosa26-CAG::EGFP$^{fl/+}$*, *Dicer$^{fl/fl}$Rosa26-CAG::EGFP$^{fl/+}$* mice were isolated in L15 Medium (GIBCO) and sectioned live at 300 µm using a

McIllwains tissue chopper. The DG was micro-dissected from the rest of the hippocampus under a dissection binocular microscope avoiding contamination with tissue from the molecular layer, cerebral cortex and subventricular zone, digested in a Papain based solution and mechanically dissociated as described previously (Lugert et al., 2010). Cells were plated in 48-well dishes (Costar) coated with 100 μg/ml Poly-L-Lysine (Sigma) and 1 μg/ml Laminin (Sigma) in neural progenitor culture medium: DMEM:F12 (Gibco, Invitrogen), 2% B27 (Gibco, Invitrogen), FGF2 20 ng/ml (R&D Systems), EGF 20 ng/ml (R&D Systems). DG NSCs were differentiated by growth factor removal and continued culture. Cells were fixed for 10 minutes in 4% paraformaldehyde in 0.1M phosphate buffer and processed as described above.

Adeno-Cre adenovirus infection and AMAXA nucleofection *in vitro*

Rosa26-CAG::EGFP$^{fl/+}$, *Drosha*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$, *Dicer*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$ DG NSCs were transduced with an adeno-Cre adenovirus (titer 1x10^{11} infectious particles per ml) in growth factor free medium and plated at a density of 5x10^4 cells/cm^2 on poly-L-Lysine/Laminin coated coverslips. 48 hours later, the cells were fixed in 4% paraformaldehyde in 0.1M phosphate buffer and process as described above. For western-blot experiments, *Rosa26-CAG::EGFP*$^{fl/+}$, *Drosha*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$, *Dicer*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$ DG NSCs were transduced with an adeno-Cre adenovirus (titer 1x10^{11} infectious particles per ml) and collected in lysis buffer after 72 hours and processed for western-blot (see below)

Rosa26-CAG::EGFP$^{fl/+}$ and *Drosha*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$ adult DG NSC cultures were nucleofected according to the mouse neural stem cell kit instructions (Lonza). Briefly, DG NSCs were dissociated with trypsin and resuspended in the nucleofector solution to a final concentration of 10^6 cells/100μl. Cell suspensions were combined with either 100 pmol endoribonuclease-prepared siRNAs (esiNA) against NFIB or *Renilla* luciferase (Sigma). *pCAG::mCherry* was added at a ratio 1:3 to identify transfected NSCs. For overexpression, DG NSCs were combined with either pCMV (empty) or pCMV-HA-NFIB (kindly provided by Prof. Heiner Schrewe) vectors and pmaxGFP. NSCs were nucleofected with a Nucleofector 2b device (program A-033). NSCs were immediately transfer to neural progenitor culture medium and plated at the density of 5x10^4 cells/cm^2 on poly-L-Lysine/Laminin coated coverslips. 24 hours later, DG NSCs were transduced with an adeno-Cre adenovirus (titer 1x10^{11} infectious particles per ml) in growth factor free medium and fixed 2 dpi. For overexpression, DG NSCs were fixed 2 days post-nucleofection.

Fluorescence activated cell sorting

Hes5::CreER^{T2}*Rosa26-CAG::EGFP*$^{fl/+}$ and *Hes5::CreER*^{T2}*Drosha*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$ animals were induced with TAM for five consecutive days and brains collected 1 day after the last injection. NSCs were isolated as described above. Cells were washed with L15 medium (Gibco, Invitrogen), filtered through a 40 μm cell sieve (Miltenyi Biotec) and sorted by forward and side-scatter for live cells (control) and gated for GFP-negative (wild type levels) or GFP$^+$ populations with a FACSaria III (BD Biosciences). DAPI (5 mg/ml) was added to discriminate living NSCs. GFP$^+$ cells were used for RNA isolation and gene expression analysis (see below).

RNA Isolation and quantitative RT-PCR

Total RNA was isolated using the Trizol method (Life Technologies) and resuspended in water. RNA was treated with RNase-free DNase I (Roche) to remove genomic DNA contamination. First-strand cDNA was generated using BioScript (Bioline) and random hexamer primers followed by quantitative PCR using SensiMix SYBR kit (Bioline). Expression analysis of genes of interest was performed on a Rotor-Gene Q (Qiagen). Primers for quantitative RT-PCR were:

NFIB (Forward: CAGGAGCAAGATTCTGGAC; Reverse: GGGTGTTCTGGATACTCTCAC);
NFIB 3'UTR HP (Forward: TAAGTCCTTCAGCCCTTGGA ; Reverse: CTGAGGAGGCTGCAGCTAAG)
Sox10 (Forward: AGCTCTGGAGGTTGCTGAAC; Reverse: GCCGAGGTTGGTACTTGTAGTC);
Drosha Exon9-10 (Forward: GACGACGACAGCACCTGTT; Reverse: GATAAATGCTGTGGCGGATT);
DGCR8 (Forward: GGAGCTAGATGAAGAAGGAACAGG; Reverse: GTAAAGCGTCCACATCATTGTCAA);
Six3 (Forward: TCAGCAGAGTCACCGTCCAC; Reverse: TGGAGGTTACCGAGAGGATCG)
βactin (Forward: AGGTGACAGCATTGCTTCTG; Reverse: GGGAGACCAAAGCCTTCATA)

Analysis of miRNA expression

Total RNA was isolated from adeno-Cre adenovirus infected *Rosa26-CAG::EGFP*$^{fl/+}$, *Drosha*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$, *Dicer*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$ DG NSCs at 2 dpi using the mirVANA isolation kit following the miRNA enrichment procedure. miRNA profiling was performed on TaqMan arrays (Life

Technologies) with 500 ng of purified RNA according to manufacturer's instructions. Expression analysis was performed using the comparative cycle threshold (Ct) values.

Crosslinking and immunoprecipitation

N2a cells (ATCC) were transfected using Transfectin Lipid Reagent (BioRad) according to manufacturer's instructions with p3X-FLAG-CMV (Sigma) or pCK-Drosha-WT-FLAG (Han et al., 2009; Knuckles et al., 2012) together with psiCheck2 vectors containing the NFIB hairpins. The transfected cells were trypsinized and collected after 48 hours. The mouse NFIB 5' and 3' untranslated regions of 200bp fragments containing the hairpins were amplified by PCR and cloned into the NotI site of psiCheck2 vector (Promega). The cells were cross-linked with 0.5% paraformaldehyde in PBS for 10 minutes, the reaction was quenched by adding Glycine to a final concentration of 140 mM and the cells were lysed by sonication (10 pulses for 10 seconds). Immunoprecipitation was performed for 2 hours at 4°C using anti-Flag M2 Affinity Gel (Sigma-Aldrich). After washing with lysis buffer, the complexes were reverse cross-linked at 70°C for 1 hour. RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer´s instructions and processed as described above.
Primers: psiCheck2 (Forward: TGATCGGAATGGGTAAGTCC; Reverse: GGCCTTGATCTTGTCTTGGT).

Luciferase Assay

Rosa26-CAG::EGFP$^{fl/+}$, *Drosha*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$ and *Dicer*$^{fl/fl}$*Rosa26 CAG::EGFP*$^{fl/+}$ DG NSCs were transduced with adeno-Cre or adeno-GFP adenoviruses (see Adeno-Cre infection). 48 hours later, the NSCs were nucleofected with the psiCheck2 containing the 3'UTR or 5'UTR NFIB hairpins (see Crosslinking and Immunoprecipitation) using the AD1 Primary Cell 4D-Nucleofector Y Kit (Lonza) and program EH158. 24 hours post-nucleofection, luciferase activity was measured in a Centro LB 960 Microplate Luminometer (Berthold) using the Dual-Luciferase Reporter Assay System (Promega).

Endogenous CLIP in DG NSCs

A confluent 10 cm dish of DG NSCs was cross-linked at 254 nm at 300 mJ/cm^2 in a BioLink UV-Crosslinker. Cells were lysed with RIPA buffer (0.1M sodium phosphate pH 7.2, 150 mM sodium chloride, 0.1% SDS, 1% sodium deoxycholate, 1% NP-40) containing complete protease inhibitor cocktail (Roche) and afterwards treated with RNase-free DNase I (Roche). Immunoprecipitation was performed with Protein G Sepharose 4 Fast Flow (GE Healthcare Life Science). Rabbit anti-Drosha Antibody (1:200; D28B1; Cell Signaling) was coupled to the beads for 1 hour at RT, beads were washed three times with RIPA and immunoprecipitation was performed for 2 hours at 4°C. After washing the beads with RIPA buffer, the proteins were digested with 4 mg/ml recombinant PCR grade Proteinase K (Roche) for 1 hour at 37°C with shaking at 1000 rpm. First-strand cDNA synthesis and quantitative RT-PCR was performed as above.

Immunoprecipitation and Western-blot

Beads from the endogenous Drosha immunoprecipitation were resuspended in Lämmli-Buffer containing 2-mercaptoethanol, boiled for 5 minutes and collected at 12000 x g for 20 seconds. Protein samples were separated on 10% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). Primary antibody rabbit anti-Drosha (1:1000; D28B1, Cell Signaling), as secondary antibody HRP-conjugated anti-rabbit IgG (1:10000; Jackson ImmunoResearch). Detection was by chemiluminescence (ECL, GE Healthcare). To determine Drosha and Dicer protein expression, *Rosa26-CAG::EGFP*$^{fl/+}$, *Drosha*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$, *Dicer*$^{fl/fl}$*Rosa26-CAG::EGFP*$^{fl/+}$ DG NSCs were transduced with adeno-Cre adenovirus. 24 or 72 hours after infection, the cells were lysed in RIPA Buffer. The lysates were incubated 30 minutes on ice and clarified by centrifugation at 13,000 rpm for 20 minutes. Cell pellets were resuspended in Lämmli-Buffer 3X. Equal amount of protein were separated by 8% SDS-polyacrylamide gel and transferred to Immobilon-P membranes (Millipore). Primary antibodies: anti-HA tag (1:1000; mouse, Covance), anti-Dicer (1:300; rabbit, Sigma), anti-Drosha (1:1000; rabbit, Cell Signaling) and anti-GAPDH (6C5) (1:10000; mouse, Calbiochem). Secondary antibodies HRP-conjugated anti-rabbit IgG (1:10000; Jackson ImmunoResearch) and HRP-conjugated anti-mouse IgG (1:10000; Jackson ImmunoResearch). Detection was by chemiluminescence (ECL, GE Healthcare) and quantification by densitometry using ImageJ software (National Institutes of Health, USA).

***In vitro* processing**

In vitro processing experiments were performed as described previously with some adaptations (Lee and Kim, 2007). Briefly, N2a cells were transfected with pCMV Drosha-Flag or pCMV (empty) vectors. One day after transfection, total cell extracts were prepared in lysis buffer (20mM Tris-HCl, pH 7.8, 100mM KCl, 0.2mM EDTA, 20% (v/v) glycerol, 1mM PMSF) by sonication followed by RNaseA (Sigma) and DNaseI (Roche) treatment and centrifugation at 13400 g for 15 minutes. Total extracts were used for

immunoprecipitation in lysis buffer using Dynabead protein G (Life Technologies) coupled to mouse anti-Flag antibody (1:100, Sigma). 30 µl of the processing reaction were prepared and contained: 15 µl of beads from Drosha-Flag immunoprecipitated or uncoupled bead fraction, 6.4 mM $MgCl_2$, 0.75 µl RNase Inhibitor (Invitrogen) and 0.5-1 µg RNA probe containing the 5' UTR or 3'UTR NFIB hairpins transcribed with T7 RNA polymerase (NEB). The reaction was carried out at 25°C for 30 minutes. RNA was extracted using phenol/chloroform and subsequently analyzed on a fragment analyzer using the DNF-472 kit (AATI) and the Low Range ssRNA ladder (NEB).

5' RACE

5' RACE experiments were performed on control and Drosha cKO embryonic NSCs according to 5'RACE System for rapid amplification of cDNA ends version 2.0 kit instructions (Invitrogen). 3 µg of total RNA of control and Drosha cKO NSCs were used. Nested PCR products were cloned into pGEM-T easy vector (Promega) and sequenced by Sanger sequencing (Microsynth). Fragments were aligned to NFIB sequence using DNASTAR Lasergene.
NFIB RT Primer: AGATCTGTCAATACGAGAA
NFIB 1 Primer: GTTTTCCTAGCCTACCTGGCATT
NFIB nested Primer: TGCCTCTTTGTCTCTACGATGC

***In vivo* clonal analysis**

Confocal images were used to confirm GFP^+ cell identity according to immunohistological and morphological properties. Whole hippocampi were serially imaged. For 3D reconstruction, optical stacks from the entire DG were serially aligned using Reconstruct 1.1.0 software (Fiala, 2005). Reconstructed hippocampi were analyzed with Imaris Software (Bitplane) with the spot detection tool and manually refined to mark single NSC in the DG. Single cell coordinates were obtained and analyzed using an in-house MATLAB script (The MathWorks, Inc.) in order to get the distance to the nearest GFP^+ cell neighbor (mean: 184.3 ± 17.2 µm, at 2 days after Tamoxifen injection).

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