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Article

Far Upstream Element-Binding Protein 1 Regulates LSD1 Alternative Splicing to Promote Terminal Differentiation of Neural Progenitors

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SUMMARY

Loss of a cell's ability to terminally differentiate because of mutations is a selected genetic event in tumorigenesis. Genomic analyses of low-grade glioma have reported recurrent mutations of far upstream element-binding protein 1 (*FUBP1*). Here, we show that FUBP1 expression is dynamically regulated during neurogenesis and that its downregulation in neural progenitors impairs terminal differentiation and promotes tumorigenesis collaboratively with expression of IDH1^{R132H}. Mechanistically, collaborative action between SRRM4 and FUBP1 is necessary for mini-exon splicing of the neurospecific LSD1+8a isoform. LSD1+8a was downregulated upon loss of FUBP1 in neural progenitors, thereby impairing terminal neuronal differentiation and maturation. Reinforcing LSD1+8a expression in FUBP1-downregulated neural progenitors restored terminal differentiation and suppressed tumorigenesis; hence, LSD1+8a is an obligatory effector of FUBP1-dependent neuronal differentiation. These findings establish a direct role for FUBP1 in neuronal differentiation and also explain its tumor-suppressor function in the nervous system.

INTRODUCTION

Neural stem cells in the mammalian brain both self-renew and differentiate to produce neurons in a controlled manner (Kriegstein and Alvarez-Buylla, 2009). These cells follow the developmental hierarchy and differentiate into replication-arrested mature cells (Gage, 2000). Aberrant regulation of cellular differentiation may underlie various human developmental disorders, pathological conditions, and diseases including cancer. Glioma cells often exhibit anomalous developmental programs that disable terminal differentiation and sustain self-renewal (Jackson et al., 2006; Sanai et al., 2005). Restoration of differentiation capacities of glioma cells reduces tumorigenic potential, supporting the idea that impaired terminal differentiation contributes to glioma pathogenesis (Zheng et al., 2008, 2010).

Far upstream element-binding protein 1 (FUBP1) is a single-strand DNA- and RNA-binding protein. It regulates transcription, mRNA stability and translation, and splicing (Zhang and Chen, 2013). It is frequently overexpressed in several types of cancer, including hepatocellular carcinoma, non-small cell lung carcinoma, and gastric cancer; thus, it is considered a proto-oncogene (Baumgarten et al., 2014; Li et al., 2013; Malz et al., 2009; Rabenhorst et al., 2009; Singer et al., 2009; Zhang et al., 2013). Paradoxically, recent genomic studies on 1p19q co-deleted low-grade gliomas uncovered a frequent loss of FUBP1 functions in oligodendrogliomas (ODGs) by loss of heterozygosity of 1p and inactivating mutations, suggesting the potential of FUBP1 as a tumor-suppressor gene (Bettegowda et al., 2011; Sahm et al., 2012; Yip et al., 2012).

Existing genetic studies point to the role of FUBP1 in maintaining hematopoietic stem cells and supporting proliferation of neoplastic cells (Rabenhorst et al., 2009, 2015; Zhang et al., 2013). A *Fubp1* germline knockout (*Fubp1*$^{-/-}$) mouse was shown to be embryonic lethal starting at embryonic day 10.5. Phenotypically, these animals displayed a small body size associated with hypoplasia of multiple tissues with the exception of cerebral hyperplasia, suggesting a context-specific function of *Fubp1* (Zhou et al., 2016). *Fubp1*$^{-/-}$ embryos presented notable overall brain parenchymal hypercellularity without the normal lobation of the cerebellum and obvious organization of the diencephalic cortical layers (Zhou et al., 2016).

LSD1 (also known as KDM1A) is necessary for self-renewal of neural progenitor cells (NPCs) by repressing TLX target genes including CDKN1A and PTEN (Sun et al., 2010). It represses and activates transcription by mediating histone H3K4me1/2 and H3K9me1/2 or H4K20me3 demethylation, respectively (Laurent et al., 2015; Wang et al., 2007, 2015). Its expression is post-translationally downregulated by ubiquitin-mediated degradation during neurogenesis (Han et al., 2014). Sustaining its
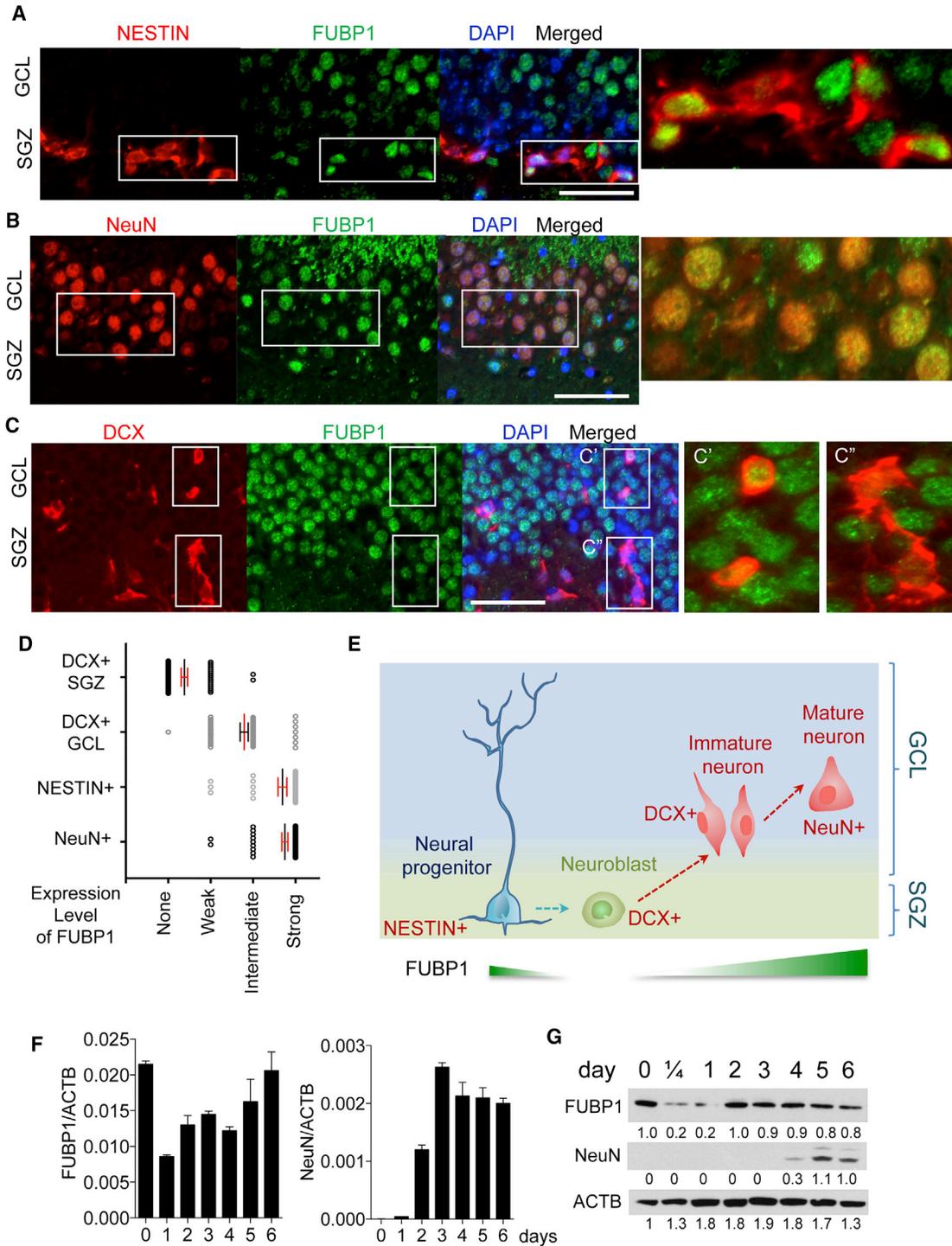
Figure 1. Expression of FUBP1 Is Dynamically Regulated during Adult Neurogenesis

(A–C) Immunofluorescence analysis of FUBP1 co-stained with NESTIN (A), NeuN (B), or DCX (C) in the adult mouse hippocampal dentate gyrus. White-boxed regions are presented in higher magnification on the right (C' and C'' for C). Scale bars, 50 μm.

(D) Quantitative analysis of FUBP1-positive cells from a marker-expressing cell (n = 50 cells from 3 different adult animals were scored). Error bars denote mean ± SEM.

(E) Summary of FUBP1 expression changes during adult hippocampal neurogenesis.

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transcription during neurogenic differentiation is necessary, as its neuron-specific isoform LSD1+8a is essential for terminal differentiation and maturation of neurons. Silencing of the neurospecific splice variant LSD1+8a delays neurite maturation in cortical neuronal cultures (Zibetti et al., 2010). Similarly, LSD1+8a promotes terminal neuronal differentiation by demethylating H3K9me1/2, which then derepresses neuronal gene expression (Laurent et al., 2015). In addition, it regulates neuronal activity-regulated transcription that is necessary for long-term memory formation by demethylating H4K20 (Wang et al., 2015).

FUBP1 is frequently overexpressed in many human cancers and strongly correlated with disease progression (Baumgarten et al., 2014; Li et al., 2013; Malz et al., 2009; Rabenhorst et al., 2009; Singer et al., 2009; Zhang et al., 2013). However, how it serves uniquely as a tumor suppressor in the brain is so far left unanswered. In this study, we hypothesized that FUBP1 plays an indispensable role in promoting terminal differentiation of neurons, and that lack of FUBP1 interferes with early-born neuronal cells exiting the cell cycle and predisposes these cells for transformation. Our study provides insights into the mechanism of how FUBP1 and isocitrate dehydrogenase (IDH) mutations together may converge on epigenetic aberrations and impair differentiation.

RESULTS

Expression of FUBP1 Is Regulated during Neurogenesis

FUBP1 is broadly expressed throughout the adult brain, primarily observed in the nucleus of neurons (Figure S1A). The glia-rich corpus callosum lacks FUBP1-positive cells, indicating neuronally enriched expression of FUBP1 in the adult brain. Consistently, double immunostaining of FUBP1 with a neuronal marker NeuN was strongly positive. In contrast, with an oligodendrocyte marker, Olig2$^+$, or an astrocyte marker, glial fibrillary acidic protein (GFAP)$^+$, cells showed a very low expression of FUBP1 (Figure S1B). We examined the expression of FUBP1 in neurogenic areas of the adult brain in order to understand its role during brain development. It is highly expressed in NESTIN-positive progenitors and NeuN-positive mature neurons (Figures 1A and 1B). Notably, doublecortin (DCX)-positive neuroblasts in the subgranular zone showed low to no signals while migrating ones in the granular cell layer increased

its expression to intermediate to strong levels (Figures 1C and 1D). These results suggest that the expression of FUBP1 is dynamically regulated during neurogenesis and may control key steps in committing to neuronal lineage and maturing into terminally differentiated neurons (Figure 1E).

Next, we tested whether this expression pattern is recapitulated in our neural progenitor cell cultures. Homozygous deletion or silencing of Ink/Arf locus is one of the most frequent genetic lesions of gliomas (Cancer Genome Atlas Research Network et al., 2015). We therefore used immortalized Ink/Arf$^{-/-}$ NPCs that maintain their multi-lineage differentiation capability (Bruggeman et al., 2005). Consistent with the tissue expression pattern, FUBP1 is highly expressed in NESTIN$^+$ NPC and terminally differentiated NeuN$^+$ cells (Figures S1C and S1D). In contrast, its expression is low to undetectable during early differentiation, as shown in DCX$^+$ or TUBB3$^+$ neuronal precursors. We generated a doxycycline-inducible NeuroD1-expressing NPC line (NPC^{ND1}), which terminally differentiates to NeuN-positive neurons in an expedited manner (Figure S2A). NPC^{ND1} cells effectively differentiate to DCX-expressing neuroblasts (~30%), within 2 days, in the presence of doxycycline (Figure S2B). In this system, almost all cells become quiescent within 5–7 days, as evidenced by a lack of 5-bromo-2'-deoxyuridine (BrdU)-positive cells following extended exposure (Figure S2C). During the early differentiation, the expression of FUBP1 declined followed by a progressive increase, while the neuronal marker NeuN continued to increase at mRNA and protein levels (Figures 1F and 1G). These results corroborate our findings that FUBP1 expression is regulated during neuronal differentiation.

Loss of FUBP1 Inhibits Terminal Neuronal Differentiation

Given that FUBP1 is precisely controlled in multiple steps during neuronal differentiation, we queried the role of FUBP1 in terminal neuronal differentiation and maturation. We knocked down the expression of FUBP1 in the NPC^{ND1} line (NPC^{ND1} FUBP1^{KD}). Two independent short hairpins for FUBP1 effectively decreased its expression (Figure S2D). Because both hairpins elicited indistinguishable biological outcomes (Figure S3A), we present detailed results from cultures generated from hairpin 1 for the current study. Clearly, the frequency of NeuN- or MAP2a/b-positive cells was significantly decreased in NPC^{ND1} FUBP1^{KD}

(F) qRT-PCR analysis of neuronal markers and FUBP1 expression by NPCs at indicated times after the onset of differentiation (mean ± SEM from 3 independent cultures).

(G) Representative western blot analysis of proliferating and differentiating NPC^{ND1} (+doxycycline) cells. Band intensities are indicated below each blot.

SGZ, subgranular zone; GCL, granular cell layer. See also Figure S1.
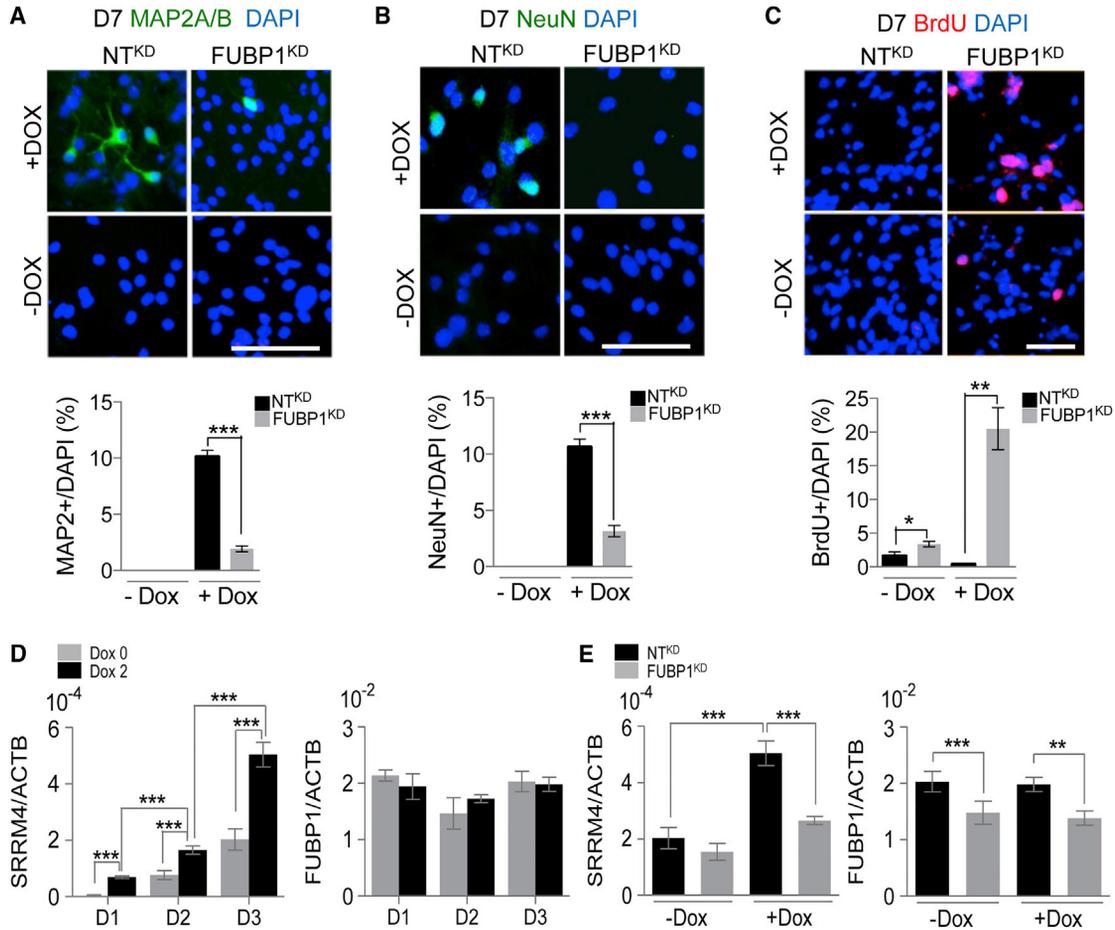
Figure 2. Loss of FUBP1 Expression Impairs Terminal Neuronal Differentiation

(A–C) Immunofluorescence analysis for (A) NeuN, (B) MAP2a/b, and (C) bromodeoxyuridine (BrdU) of differentiated NPC[ND1] NT[KD] or NPC[ND1] FUBP1[KD] in the presence (+DOX) or absence (−DOX) of doxycycline. Scale bars, 50 μm. Quantitation of representative experiments is plotted below (mean ± SEM from 3–4 independent cultures).

(D and E) Relative mRNA expression levels of SRRM4 and FUBP1 during NPC[ND1] differentiation (D) or NPC[ND1] NT[KD] versus NPC[ND1] FUBP1[KD] (E) were determined by qPCR (mean ± SEM from 3 independent cultures).

Statistical significance was determined by one-way ANOVA. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. See also Figures S2 and S3.

compared with non-targeted (NPC[ND1] NT[KD]) cells under doxycycline ($p < 0.001$) (Figures 2A and 2B). To determine whether impaired terminal differentiation resulted from a failure to exit the cell cycle, we performed a DNA synthesis-based cell proliferation assay. The number of BrdU-positive NPC[ND1] cells declined within 48 hr of differentiation initiation and became undetectable after prolonged (5–7 days) differentiation (Figure 2C). Surprisingly, a fraction of NPC[ND1] FUBP1[KD] continued to incorporate BrdU at 7 days after differentiation induction under doxycycline, indicating sustained proliferation. Even without enforced expression of NeuroD1, FUBP1[KD] cells showed higher BrdU incorporation compared with NT[KD] cells (Figures 2C and S3A). However, FUBP1 knockdown in the background of NeuroD1 induction elicited much greater

increase of BrdU incorporation. This is due to generally increased cell survival with NeuroD1 expression under differentiation induction (data not shown). These results suggest that FUBP1 is necessary for a complete cell-cycle exit for terminal neuronal differentiation.

To better define the molecular mechanism of the cell-cycle exit impaired by FUBP1 loss, we performed RNA sequencing (RNA-seq) analyses of differentiated NT[KD] and FUBP1[KD] cultures. Gene ontology analyses of differentially expressed genes under differentiation conditions indicated that cell-cycle genes (i.e., *CCNB1*, *CCNB2*, *CCND1*, *PLK1*, *BUB1*, *BUB1B*, *CDC20*, and *MCM5*) are the most represented category (Figure S3B). We further tested the role of cell-cycle deregulation in sustained mitotic phenotype of FUBP1[KD] cells. Treatment of PD0332991, a

CDK4/6 inhibitor, suppressed BrdU incorporation but failed to restore neuronal differentiation (Figure S3A and data not shown). These results together suggest that sustained proliferation is due to the impaired terminal differentiation rather than the direct role of FUBP1 in cell-cycle regulation.

FUBP1 Promotes the Expression of LSD1+8a during Neuronal Differentiation

Recent studies suggested a context-dependent splicing-regulatory function of FUBP1 (Jacob et al., 2014; Li et al., 2013; Miro et al., 2015). In agreement, many interacting proteins identified by mass spectrometry and confirmatory co-immunoprecipitation analysis belong to components of spliceosomes (e.g., PTBP2, NOVA1 and NOVA2, and SRRM4) (Table S1 and Figure S4), raising the possibility that FUBP1 could have a functional role in splicing control during neuronal differentiation. In particular, the interaction between FUBP1 and SRRM4 appears indirect and RNA dependent as it is abolished by RNase A treatment (Figure S4). Notably, our analysis of RNA-seq experiments for global alternative splicing (AS) events occurring in differentiating FUBP1^{KD} NPC showed increased exon skipping consistent with its reported function in exon inclusions (Jacob et al., 2014; Miro et al., 2015) (Figure S5). In addition, we calculated Z score of differential exon usage focusing on transcripts with little to no expression changes between NT^{KD} and FUBP1^{KD} cells. From the analysis, 838 differentially used exons with Z score of larger than 2 corresponding to 689 genes were identified, further supporting the role of FUBP1 in AS control (Table S2).

Among those neuron-specific splicing factors, we noted that the expression of SRRM4 is gradually increased in differentiating NPC^{ND1} cells (Figure 2D), consistent with its role in neuronal differentiation and maturation (Quesnel-Vallières et al., 2015). However, it failed to increase beginning from differentiation day 3 in NPC^{ND1} FUBP1^{KD} cells (Figure 2E). These results suggest that loss of FUBP1 affects SRRM4 expression, likely due to impaired neuronal differentiation.

Previously, several lines of evidence have suggested that LSD1 has a critical role in NPC differentiation and neuronal maturation. The LSD1 gene, which is highly conserved among vertebrates, consists of 19 exons. However, due to the existence of two additional exons (exon 2a and exon 8a) that can be included in mature LSD1 mRNA, four different LSD1 transcripts can be generated. Among these isoforms, LSD1+8a, which contains mini-exon 8a, is exclusively expressed in the nervous system (Zibetti et al., 2010). In addition, the expression of the LSD1+8a isoforms is upregulated during neuronal differentiation and throughout cortical development (Laurent

et al., 2015; Zibetti et al., 2010). Downregulation of LSD1+8a strongly inhibited neurite outgrowth and abrogated the establishment of the neurite network. A previous study reported that neuron-specific splicing factors such as SRRM4 and NOVA1 are necessary for LSD1+8a expression (Rusconi et al., 2015).

Because (1) loss of FUBP1, SRRM4, or LSD1+8a impaired neuronal differentiation (Laurent et al., 2015; Raj et al., 2011) (Figures 2A and 2B), (2) FUBP1 and SRRM4 are functionally associated (Figure 2E), and (3) AS of LSD1 is critical during neuronal maturation, we hypothesized that FUBP1 along with SRRM4 may be required for the splicing of LSD1 to include mini-exon 8a. First, we analyzed the expression of LSD1+8a and LSD1−8a by using specific primers that detect each isoform (Figure 3A). Neuronal differentiation by NeuroD1 induction time-dependently increased the mRNA expression level of LSD1+8a in NPC^{ND1} while the levels of total LSD1 or LSD1−8a were only modestly increased (Figure 3B). It is noteworthy that LSD1−8a is much more abundant than LSD1+8a and accounts for the majority of LSD1 transcripts. Next, we tested whether loss of FUBP1 can influence the production of alternatively spliced isoforms of LSD1. Interestingly, the expression of LSD1+8a was significantly decreased in NPC^{ND1} FUBP1^{KD} NPCs, compared with NPC^{ND1} NT^{KD} cells (p = 0.0005, Figures 3C and 3D). On the contrary, the expression of LSD1−8a was increased and that of total LSD1 remained constant. These results suggest that splicing of LSD1+8a might be regulated by FUBP1 in NPC during differentiation.

To gauge the contribution of SRRM4 to LSD1+8a expression, we expressed human SRRM4 in NPC^{ND1} NT^{KD} and NPC^{ND1} FUBP1^{KD} cells. Endogenous SRRM4 expression was low on day 1 of differentiation (Figure 2D) and further decreased in NPC^{ND1} FUBP1^{KD} cells. However, enforced SRRM4 at this stage was sufficient to elevate LSD1+8a expression to detectable levels in both NPC^{ND1} NT^{KD} and NPC^{ND1} FUBP1^{KD} cells (Figure 3E). In contrast, it failed to increase LSD1+8a expression and NeuN expression on day 3 of differentiation in NPC^{ND1} FUBP1^{KD} cells (Figure 3F). These results together suggest that SRRM4 expression at early stages and FUBP1 levels in later stages of differentiation play a role as rate-limiting factors of LSD1+8a expression.

To test whether FUBP1 or SRRM4 physically associate with LSD1+8a pre-mRNA and regulate the splicing of mini-exon 8a during neuronal differentiation, we performed an RNA-immunoprecipitation (RNA-IP) experiment with differentiating NPC^{ND1}. We conducted the experiment 2.5 days after the onset of differentiation, when the expression of FUBP1, SRRM4, and LSD1+8a increases as the majority of NPC^{ND1} cells transit through late DCX stages (Figures 2D, 3B, 4A, and S2B). We searched
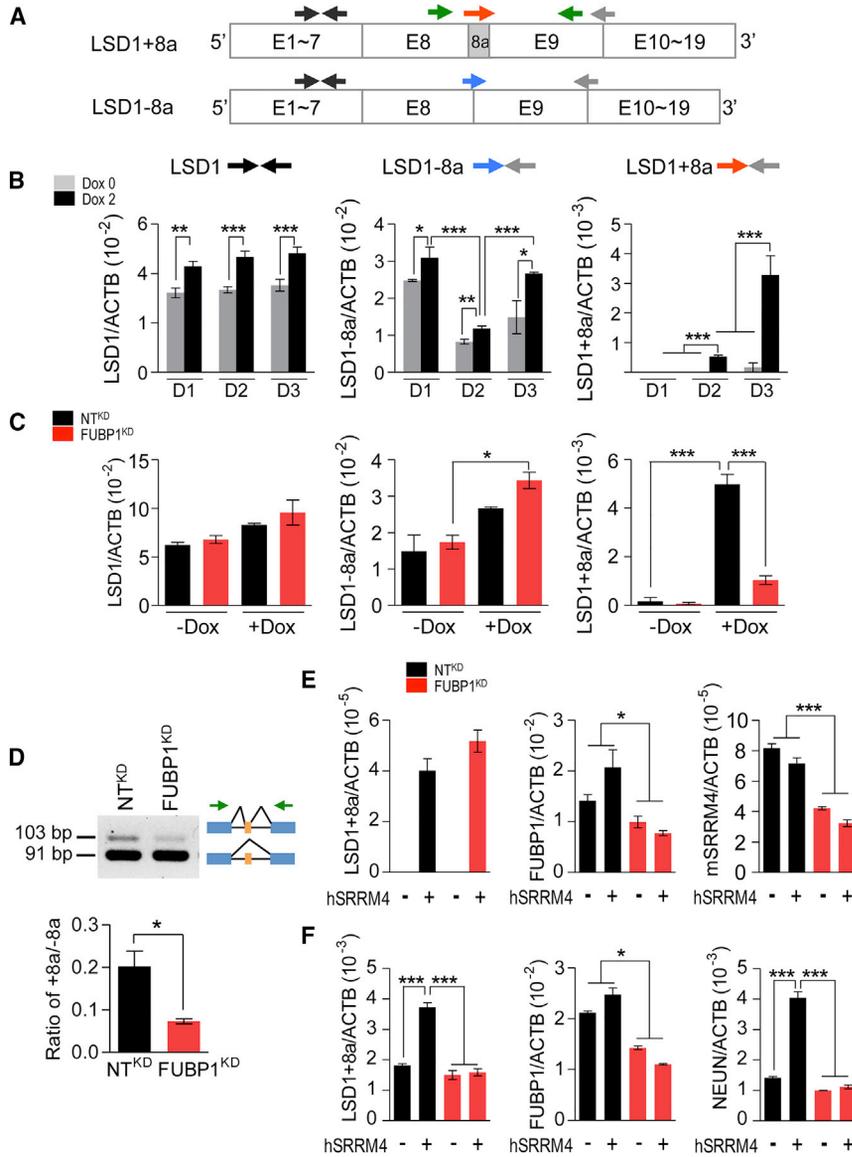
Figure 3. FUBP1 Promotes Inclusion of Neuron-Specific Exon 8a

(A) Schematic representation of primer sets that specifically detect total LSD1, LSD1+8a, and LSD1−8a.

(B and C) Relative expression levels of specific LSD1 isoforms during NPC[ND1] differentiation by doxycycline (Dox) induction (B) or NPC[ND1] NT[KD] versus NPC[ND1] FUBP1[KD] (C) were determined by qPCR (mean ± SEM from 3 independent cultures).

(D–F) RT-PCR detection of alternatively spliced LSD1+8a and LSD1−8a in Dox-induced NPC[ND1] NT[KD] versus NPC[ND1] FUBP1[KD]. A set of PCR primers with their location depicted as green arrows in (A) were used. Band intensity was plotted (mean ± SEM from 3 independent cultures). Statistical significance was determined by unpaired t tests. Relative mRNA levels of indicated genes were determined from NPC[ND1] NT[KD] versus NPC[ND1] FUBP1[KD] with human SRRM4 expression under proliferation (E) or on day 3 of differentiation (F) (mean ± SEM from 3 independent cultures). *p < 0.05; **p < 0.01; ***p < 0.001. Statistical significance was determined by one-way ANOVA. See also Figure S4.

for the TG-rich FUBP1-binding motif (Miro et al., 2015) in intron 8 and 8a of LSD1, and designed multiple pairs of primers that span the putative FUBP1-binding sites (Figures 4B and S6). We identified two strong binding sites (R1 and R5) among five candidates (Figures 4C, 4D, and S4). Similarly, SRRM4 showed strong binding near the 3′ end of intron 8, where the polypyrimidine tract preceding the putative UGC motif is present (Figures 4D and S6). The set of primers designed for a gene desert region failed to amplify RNA pulled down by immunoglobulin G (IgG), FUBP1, or SRRM4 antibodies, demonstrating the specificity of RNA-IP (Figures 4C and 4D). Primers amplifying the exonic region of GAPDH, which is not subject to FUBP1- or SRRM4-mediated splicing, failed to be enriched by the pull-down (Figure 4D).

To define the functional interaction between FUBP1 and SRRM4 in exon 8a inclusion, we performed a mini-gene reporter analysis (Cooper, 2005) (Figure 4E). Enforcing the expression of either FUBP1 or SRRM4 synergistically enhanced exon 8a inclusion (Figure 4F). Consistent with the RNA-IP result, mutations of either SRRM4 binding site or R5 (Figure 4E) abolished exon 8a inclusion (Figure 4G). Together, our results suggest that FUBP1 and SRRM4 bind to different regions within the introns 8 and 8a of LSD1 pre-mRNA and promote the inclusion of mini-exon 8a by AS in differentiating neurons. The LSD1+8a isoform may promote neuronal gene expression and generation of mature neurons as previously demonstrated (Laurent et al., 2015; Wang et al., 2015; Zibetti et al., 2010) (Figure 4H).
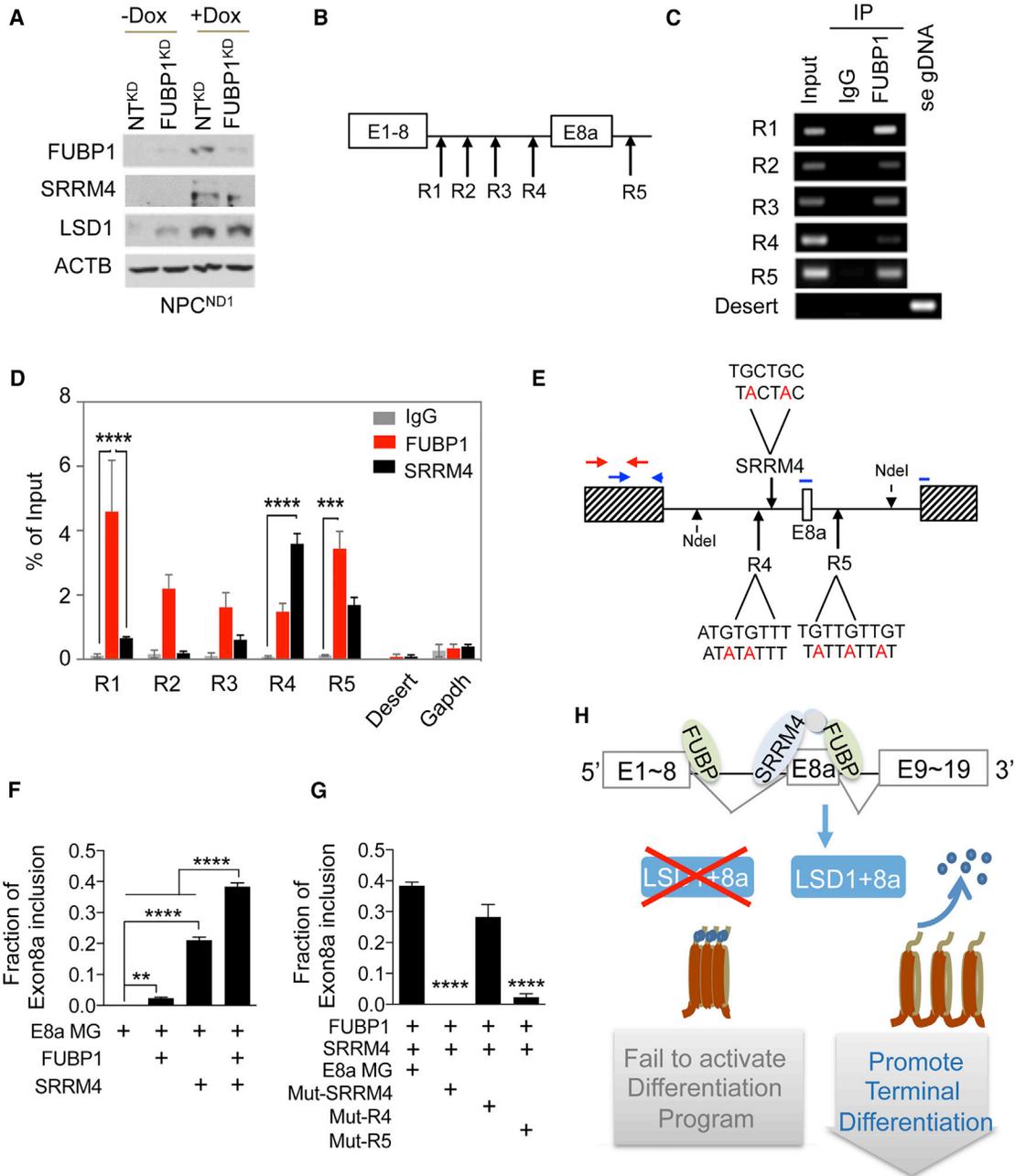
Figure 4. FUBP1 and SRRM4 Functionally Interact to Include Exon 8a

(A) Representative western blot analysis of NPC[ND1] cells differentiated for 3 days.

(B) Schematic presentation of relative location of candidate FUBP1-binding sites tested by RNA-IP.

(C) A representative PCR amplification of RNA-IP. Primers for gene desert region were used to determine background genomic DNA contamination.

(D) qPCR analysis of IgG versus anti-FUBP1 or anti-FLAG (SRRM4) antibody RNA-IP for R1 through R5 (mean ± SEM from 5 independent experiments). Statistical significance was determined by two-way ANOVA. ***p < 0.001; ****p < 0.0001.

(E) Schematic representation of the hybrid LSD1 exon 8a mini-gene used in transient transfection splicing assay. α-Globin and fibronectin EDB exons are indicated in shaded boxes. The primer pairs used in RT-PCR assay are shown as blue (8a included only) and red (total transcript) arrows. Specific mutations introduced in R4, SRRM4 binding site, and R5 are indicated in red.

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Restoration of LSD1+8a Expression Overcomes Impaired Terminal Neuronal Differentiation of FUBP1[KD] Cells

To validate LSD1+8a as an obligatory downstream effector of FUBP1, we tested whether reinforcing its expression rescues terminal neuronal differentiation in NPC[ND1] FUBP1[KD] cells. First, we determined the expression of FUBP1 upon induction of LSD1 (+/− 8a) on differentiation day 3 when the expression of FUBP1 begins to increase in NPC[ND1] cells with NeuroD1 induction (Figure 5A). Unexpectedly, FUBP1 expression was further increased when LSD1−8a or LSD1+8a was induced. This suggests that both LSD1 isoforms enhanced early neurogenic differentiation as evidenced by high levels of DCX expression. Next, aberrantly elevated BrdU incorporation was suppressed in response to LSD1+8a induction in NPC[ND1] FUBP1[KD] cells (Figure 5B). Notably, the expression of NeuN and the frequency of MAP2a/b-positive terminally differentiated neurons were restored in LSD1+8a-, but not in LSD1−8a-expressed NPC[ND1] FUBP1[KD] to the level of NPC[ND1] NT[KD] (Figures 5C and 5D). These observations suggest that expression of LSD1+8a is necessary for timely coordination of differentiation and cell-cycle exit to complete neurogenesis. Collectively, our results support the hypothesis that FUBP1 is a rate-limiting factor for the expression of necessary levels of LSD1+8a isoform and therefore essential for terminal neuronal differentiation.

Next, we queried whether LSD1+8a expression depends on FUBP1 under pathological conditions. Frequent loss of heterozygosity (LOH) of FUBP1 in 1p19q co-deleted ODG has been reported (Bettegowda et al., 2011; Cancer Genome Atlas Research Network et al., 2015). We have identified 8 tumors with FUBP1 LOH out of 12 ODGs (Figure 6A). Notably, these tumors showed significantly reduced expression of LSD1+8a but not LSD1−8a, corroborating our mechanistic findings (p = 0.0238, Figure 6B).

Loss of FUBP1 in the Background of IDH1[R132H]-Expressing NPCs Promotes Tumorigenesis *In Vivo*

FUBP1 mutations are associated with neomorphic IDH1 or 2 mutations (R132H or R172K, respectively) and inactivating mutations in capicua (CIC) on 19q13 (Bettegowda et al., 2011; Hartmann et al., 2010; Ichimura et al., 2009; Parsons et al., 2008; Reitman and Yan, 2010; Sahm et al., 2012; Yan et al., 2009; Yip et al., 2012) in ODGs. Despite abundant genetic evidence, the mechanism of how loss-of-function mutations of FUBP1 contribute to gliomagenesis remains unclear. On this basis, we first tested the combined effect of FUBP1 knockdown and expression of IDH1[R132H] in NPC culture. NT[KD] IDH1[R132H] NPCs lost BrdU incorporation, while FUBP1[KD] IDH1[R132H] NPCs continued to incorporate BrdU under differentiation conditions, suggesting that FUBP1 is necessary for a complete cell-cycle exit of NPCs (Figure 6C). Next, to determine the oncogenic potential caused by loss of FUBP1, we performed a tumorigenesis analysis of control or FUBP1-downregulated IDH1[R132H] PIK3CA[H1047R] (IP) NPC (Figure 6D). PIK3CA[H1047R] has one of the activating mutations of PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α) and accelerated tumorigenesis in our experiments. However, its expression alone or with IDH1[R132H] was not sufficient to drive gliomagenesis (not shown). Notably, intracranial injection of FUBP1[KD] IP NPCs resulted in tumor growth and reduced overall survival of tumor-bearing animals. NT[KD] IP NPCs failed to grow tumors (Figures 6E and 6F), suggesting that loss of FUBP1 is necessary to initiate the tumorigenesis. Lastly, we enforced LSD1+8a expression in FUBP1[KD] IP NPCs to test whether restoring terminal differentiation suppresses tumorigenesis. Consistent with our mechanistic model, LSD1+8a expression prevented the outgrowth of FUBP1[KD] IP NPCs *in vivo* (Figure 6G). Taken together, we find that loss of FUBP1 critically contributes to the initiation of tumorigenesis by sustaining the proliferation of IP NPCs *in vivo*.

DISCUSSION

The importance and non-redundant role of FUBP1 in the development of the mammalian brain has been demonstrated (Zhou et al., 2016). Nevertheless, the role of FUBP1 in lineage commitment and terminal differentiation of NPCs is uncharted. Our study demonstrated that FUBP1 expression is dynamically regulated during NPC differentiation and is indispensable for terminal neuronal maturation. Similarly, a recent study reported that loss of FUBP1 expression in embryonic stem cells delayed differentiation into the mesoderm germ layer, or diminished differentiation into the erythroid lineage, further corroborating its context-specific function in cell-fate regulation (Wesely et al., 2017). Similar to several other neuronal differentiation splicing factors such as PTBP2 and RBFOX1, its loss critically contributes to gliomagenesis through trapping NPCs in a slowly proliferating progenitor-like state

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(F and G) Mini-gene reporter assay following the transient transfection of indicated reporters and exogenous SRRM4 and FUBP1 (mean ± SEM from 3 independent experiments). Statistical significance was determined by one-way ANOVA. **p < 0.01; ****p < 0.0001.
(H) A schema for FUBP1 regulation of LSD1+8a expression and differentiation.
See also Figures S4–S6.
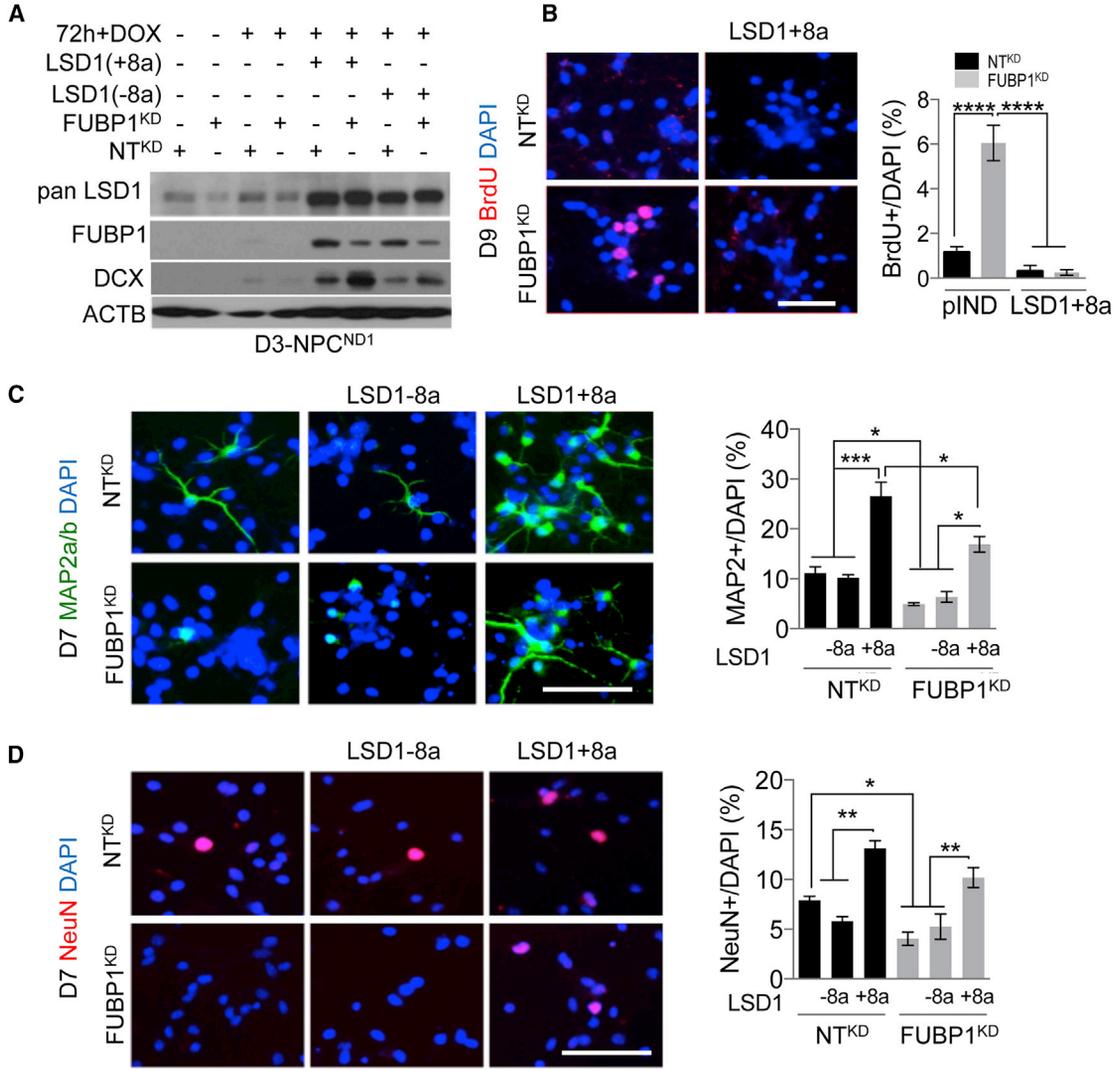
Figure 5. Expression of LSD1+8a Restores Terminal Neuronal Differentiation of FUBP1[KD] NPCs

(A) Representative western blot analysis of induced LSD1−8a and LSD+8a, FUBP1, and DCX expressions in NPC[ND1] cells differentiated for 3 days (∼50,000 cells/lane).

(B–D) Immunofluorescence analysis for MAP2a/b (B), NeuN (C), or BrdU (D) incorporation of differentiated NPC[ND1] cells with indicated knockdown or overexpression. Scale bars, 50 μm. Quantitation of representative experiments is plotted on the right (mean ± SEM from 3 independent cultures). Statistical significance was determined by one-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

in the background of IDH1[R132H] expression. Prior to our study, the majority of biological functions of FUBP1 was paradoxically attributed to promoting proliferation. In agreement with this assumption, most human cancers were shown to express high levels of FUBP1, which predict poor prognosis (Baumgarten et al., 2014; Li et al., 2013; Malz et al., 2009; Rabenhorst et al., 2009; Singer et al., 2009; Zhang et al., 2013).

Our identification of LSD1+8a as a downstream effector of FUBP1 action in neuronal differentiation may explain the molecular mechanism of how FUBP1 and IDH1 muta-tions collaborate during gliomagenesis. LSD1+8a acts as demethylase of H3K9me2 that is required for derepression of gene expression for terminal neuronal differentiation (Laurent et al., 2015). Previous studies showed that IDH1[R132H]-expressing NPCs failed to differentiate to the astrocytic marker GFAP or neuronal marker MAP2- and Synapsin 1-positive cells (Lu et al., 2012; Rosiak et al., 2016). Impaired lineage differentiation was in part ex-plained by 2-hydroxyglutarate (2-HG) produced by IDH1[R132H], which inhibits histone demethylases including H3K9me2/3-demethylating KDM4C. This leads to the
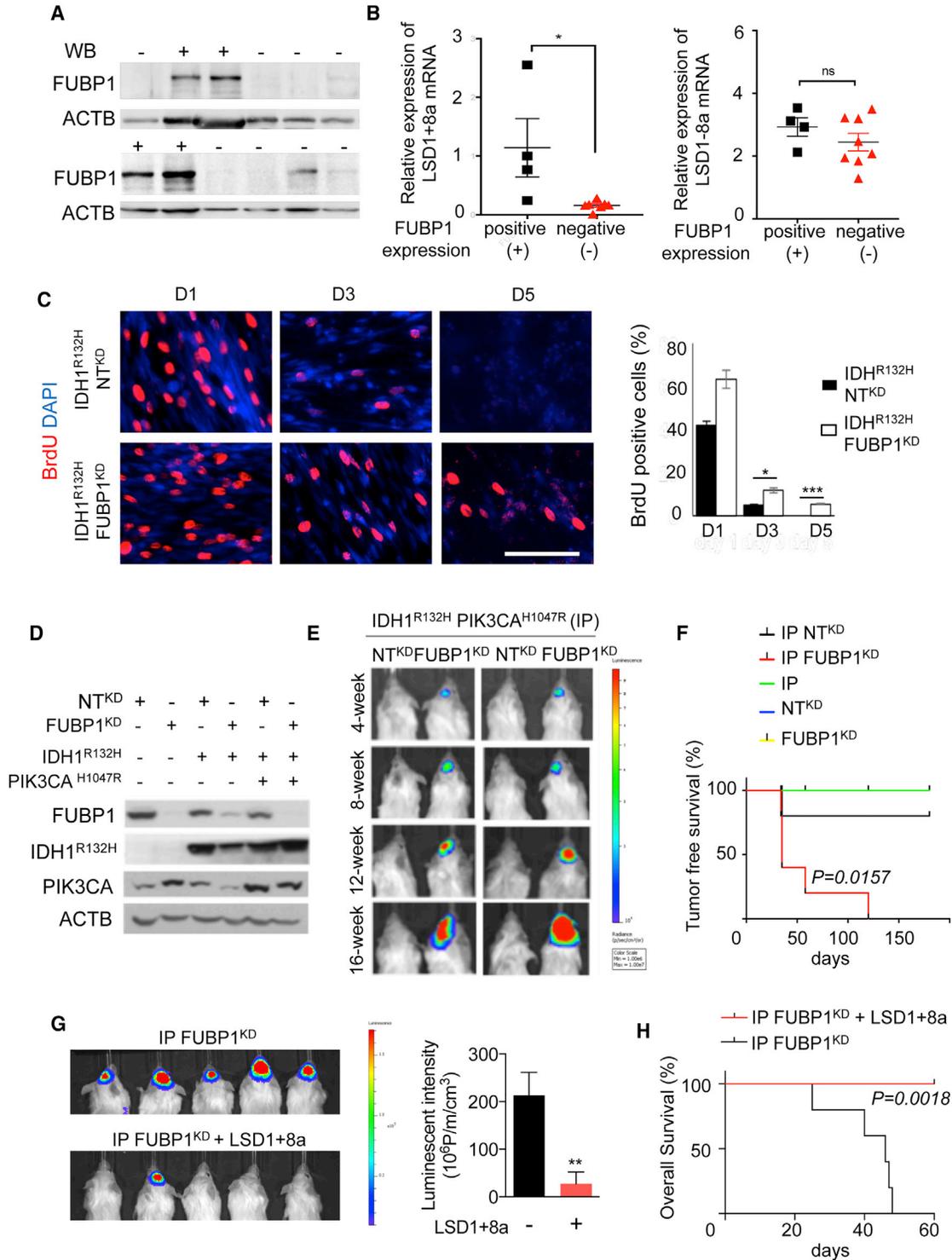
Figure 6. Loss of FUBP1 Collaboratively with IDH1^{R132H} and PIK3CA^{H1047R} Expression in NPCs Promotes Orthotopic Tumor Growth

(A) Representative western blot (WB) analysis of FUBP1 in ODG patient samples (n = 12) with 1p19q co-deletion.

(B) qPCR analysis of LSD1+8a and LSD1−8a mRNA levels from tumors from (A). *p < 0.05; ns, not significant.

(C) Immunofluorescence analysis of BrdU incorporation in differentiated IDH1^{R132H} NT^{KD} versus IDH1^{R132H} FUBP1^{KD} NPCs (day 1–5). Scale bar, 50 μm. Quantitation of BrdU-positive cells from a representative experiment is plotted in the graph on the right (mean ± SEM from 5 independent experiments). Statistical significance was determined by unpaired t tests. *p < 0.05; ***p < 0.001.

(legend continued on next page)

deregulation of gene expression subject to H3K9me2/3-mediated repression. Decreased expression of H3K9 demethylase LSD1+8a and accumulation of its inhibitory 2-HG in FUBP1^{KD} IDH1^{R132H} NPC may together synergistically block terminal differentiation.

Sequential actions of splicing factors in neuronal differentiation could be deduced based on their expression patterns and phenotypic consequences of their loss. Among those we surveyed, expression of NOVA2 and SRRM4 was increased by NeuroD1 and as differentiation progresses in NPC^{ND1} cells (data not shown). NOVA1 and NOVA2 double-knockout mice showed delayed neuronal migration without neuronal differentiation defects in the developing brain (Leggere et al., 2016). SRRM4 mutant mice showed increased early-born immature TBR1$^+$ neurons and a decrease in NeuN$^+$ mature neurons and PAX6$^+$ neural progenitors. In addition, neuritogenesis defects were noted (Quesnel-Vallières et al., 2015). This phenotype is likely to be the most similar to the FUBP1 null defect, given the decline in NeuN$^+$ populations *in vitro* and the increase in early neurons *in vivo*. These may serve as limiting factors for neuronal maturation and their expression is also regulated by the action of differentiation stage-specific factors including FUBP1. We suspect that decreased SRRM4 expression in FUBP1^{KD} NPC is largely due to impaired neuronal differentiation and in part direct transcriptional regulation, based on our RNA-seq data showing a decrease even before the onset of differentiation. Our molecular model supports the differentiation cascade controlled by these factors (Figure 4H).

What tethers FUBP1 to the transcriptional regulator complex in proliferating cells as reported by others, and to spliceosomes responsible for the inclusion of exon 8a to the LSD1 transcript during terminal differentiation of neurons, remains to be further defined. Interestingly, our mass spectrometry analysis of FUBP1-interacting proteins identified SWI/SNF chromatin remodeling factors and histone subunits (Table S1). This is in agreement with the previously demonstrated role of FUBP1 as a transcriptional regulator. In particular, FUBP1 was shown to bind to the far upstream sequence element, only in proliferating cells, and upregulate c-Myc expression. It is plausible that specific epigenetic modifications or transcription machineries are required for FUBP1-mediated transcriptional regulation.

We also noted that mRNA levels and protein expression of FUBP1 are not always collinear. For example, FUBP1 protein levels are deeply reduced by a 20%–30% knockdown of its mRNA levels (Figure S2D). A previous study also reported such a discrepancy, suggesting possible regulation at post-translational levels (Wesely et al., 2017). Indeed, FUBP1 was identified as an authentic PARKIN E3 ligase substrate (Ko et al., 2006). How protein stability plays a role in FUBP1-dependent biological functions including neurogenic differentiation warrants further study.

Our mechanistic findings of FUBP1 action in glioma suppression warrant future investigations. Currently ODG is incurable despite the favorable prognosis. Our study may strengthen the rationale for novel mechanism-driven therapeutic strategies. Re-expression of key effector genes necessary for terminal differentiation may reduce undifferentiated progenitor state cells. One such approach would be normalization of aberrantly marked repressive H3K9me2/3 by inhibitors of H3K9 methyltransferases G9a or GLP. In a recent study, a dual G9a/GLP inhibitor UNC0642 was effective in restoring ATM expression and its function in IDH1-mutant hematopoietic cells where H3K9 methylation at the ATM promoter was elevated and transcriptionally repressive (Inoue et al., 2016). Similarly, an inhibitor of mutant IDH was shown to reverse blocked differentiation in gliomas and reduced tumor burdens in patient-derived xenografts (Rohle et al., 2013). Strategic combination of these inhibitors may prove effective in releasing cells trapped in early differentiation stages. In conclusion, our results reveal that these molecular mechanisms outlining how FUBP1 regulates NPC fate can help our understanding of ODG pathogenesis and facilitate the development of novel therapeutic strategies.

EXPERIMENTAL PROCEDURES

Animal experiments

This study was conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Institutional Animal Care and Use Committee.

NPC Cultures and Induction into Terminally Differentiated Neurons

Ink/Arf$^{-/-}$ NPCs isolated from the subventricular zone of P1 neonatal mouse brain were transduced with viral particles of pLenti-CMV-rtTA3-hygro (Addgene #26730) and Tet-O-Neurod1-puro (Addgene #52052) (NPC^{ND1}). After selecting the culture with 50 μg/mL hygromycin and 1 μg/mL puromycin for 3 days, the cells were divided and transduced with virus encoding short

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(D) Western blot analysis to confirm the genotype of experimental cultures.

(E and G) BLI of representative animals orthotopically injected with indicated genotypes of NPCs. **p < 0.01.

(F and H) Kaplan-Meier survival curves (n = 5–6 for each group). Note that curves for NT^{KD}, FUBP1^{KD}, and IP overlap in (F). p Values were calculated by log-rank (Mantel-Cox) test.

hairpin RNA (shRNA) for FUBP1 (shFUBP1-1, TRCN0000013293 or shFUBP1-7, TRCN0000230197) or non-targeting shRNA for control (NT[KD], SH016, pLKO.1-puro). LSD1−8a and LSD1+8a vectors were provided by Dr. Yang Shi. LSD1−8a and LSD1+8a were gateway cloned to pINDUCER vector (Addgene #44012). For the induction of terminally differentiated neurons, NPC[ND1] were plated on fibronectin- and poly-L-ornithine-coated surface. On day 0, culture medium was replaced with N2 containing BDNF (10 ng/mL, PeproTech), NT-3 (10 ng/mL, PeproTech), B27 (Invitrogen #17504044), and/or doxycycline (2 μg/mL). On day 2, 1% fetal bovine serum was added to the medium to support the fitness of the culture.

Mini-Gene Reporter Analysis

A mouse genomic fragment (780 bp) encompassing LSD1 exon 8a was PCR amplified and cloned into pTBNde(min) (Addgene #15125) using the NdeI site. The reporter was further mutagenized on R4, R5, and SRRM4 binding sites by a site-directed mutagenesis kit (Agilent QuikChange Lightning Site-Directed Mutagenesis Kit, #210518). NPC[ND1] cells on differentiation day 1 were transfected with 1.2 μg of reporter constructs by Lipofectamine 3000 (Thermo Fisher #L3000008) as previously described (Kim et al., 2015) and harvested for qRT-PCR analysis after 24 hr. Transcript including exon 8a was measured and normalized by the level of total α-globin and fibronectin EDB transcripts. Sequences of oligos used are listed in Supplemental Experimental Procedures.

Statistical Analysis

We determined experimental sample sizes on the basis of preliminary data. All results are expressed as mean ± SEM. GraphPad Prism software (version 7; GraphPad, San Diego, CA) was used for all statistical analysis. Normal distribution of the sample sets was determined before applying unpaired Student's two-tailed t test for two-group comparisons. ANOVA was used to assess the differences between multiple groups. The mean values of each group were compared by the Bonferroni's post hoc procedure. Differences were considered significant when $p < 0.05$.

ACCESSION NUMBERS

The accession number for the RNA-seq data reported in this paper is GEO: GSE108537.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.02.013.

AUTHOR CONTRIBUTIONS

Conception and Design, I.H. and J.P.; Development of Methodology, I.H. and T.Z.; Acquisition of Data, I.H., D.C., Y.N., D.-Y.K., H.O., and J.P.; Analysis and Interpretation of Data (e.g., statistical analysis, biostatistics, computational analysis), I.H., T.Z., H.O., J.Y., H.Z., Y.Y., and J.P.; Writing, Review, and/or Revision of the Manuscript, I.H., D.-Y.K., J.H., J.Y., Y.Y., and J.P.; Administrative, Technical, or Material Support (e.g., reporting or organizing data, constructing databases), I.H., H.Z., Y.Y., and J.P.; Study Supervision: Y.Y. and J.P.

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