Supplementary Materials

Extended Materials and Methods

1. ChIP-qPCR.

ChIP experiments were performed through procedure as described in (Shelby A. Blythe 2009) with minor modifications. Briefly, 50 embryos at the desired stages were fixed in 1% formaldehyde for 10 minutes at room temperature followed by washing in 0.125 M glycine for 10 minutes. After washing 3 times in pre-cold PBS, embryos can be stored at -80 degree. RIPA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 140 mM NaCl, 1% Triton X-100, 0.2% SDS, 0.1% Sodium Deoxycholate) was used to homogenize the fixed embryos followed by incubating embryos on ice for at least 10 min. After centrifugation, supernatant was discarded and the pellet was resuspended in RIPA buffer. Sonication was performed by 24 cycles with 2s on and 3s off. After centrifugation, the supernatant was pre-cleared using 10 µl blocked Dynabeads protein A (Life Tech, #10001D, Massachusetts, USA) or Dynabeads protein G (Life Tech, #10003D). Blocking buffer was 0.5% BSA, 0.5% Tween20 in PBS. After blocking, the supernatant was diluted 3 times using SDS-free RIPA buffer. 1% diluted supernatant was stored as input. 1 µg antibody (αMyc, αKDM3A, αH3K9me2, αH3K4me3, αH3K27ac, see detailed information for antibodies in the supplementary Table S2) or normal IgG was used for immunoprecipitation at 4 degree overnight. 20 µl blocked Dynabeads were used to pull down antibodies for at least 3 hours. Dynabeads were then washed by Buffer I

(20 mM Tris-HCl pH8.0, 0.1% SDS, 1% Triton X-100, 2mM EDTA, 150 mM NaCl), Buffer II (20 mM Tris-HCl pH8.0, 0.1% SDS, 1% Triton X-100, 2mM EDTA, 500 mM NaCl), Buffer III (10 mM Tris pH8.0, 0.25 M LiCl, 1% NP-40, 1% Na-Deoxycholate, 1 mM EDTA), TE Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Use 2 washes with each and every buffer. TES Buffer (50 mM Tris-HCl pH8.0, 10 mM EDTA, 1% SDS) was used to incubate Dynabeads for 20 min at 65 degree. Then Proteinase K was added to reverse crosslinks and digest proteins at 65 degree overnight. ChIP DNA was purified using phenol-chloroform and resuspended in TE buffer. Then the qPCR programs were performed using an FQD-96A^{TM} machine (BIOER, Hangzhou China).

2. ***Neuron induction and Co-immunoprecipitation (CoIP)***

Mouse neuronal cells were obtained by treating NE-4C cells with 1 □M all-trans retinoic acid (Schlett and Madarasz, 1997). Human neuronal cells were generated from primitive neural stem cells derived from H9 human stem cell line, following established protocol as described (Yan et al., 2013).

For immunoprecipitation analysis, cells from one well of 6-well plate were lysed with TNE buffer (10 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA) containing proteinase inhibitor cocktail (Roche), and 40% of the total lysate was used for immunoprecipitation with 0.5 □g of anti-KDM3A (Abcam) and anti-Ngn2 (Santa Cruz Biotechnology) antibodies, with 1.5 mg of Dynableads A (Novex, Life Technology) as instructed. A quarter of the eluted antigen product was subjected to

western blotting with anti-KDM3A and anti-Neurog2 antibodies. As input control, 12.5 μg of protein lysate was also loaded.

3. Dual-luciferase reporter assay

Dual-luciferase reporter assay was performed using Promega luciferase system (E1960, Promega, Wisconsin, USA) following procedures provided in the manufacture's manual. Briefly, 20 pg wild type or mutant tubb2b promoter plasmid (subcloned into pGL3-basic at MluI/XhoI sites) and 10 pg Renilla plasmid were injected into animal pole of 4-cell stage embryos with/without 100 pg *neurog2* or β-*gal* mRNA. Embryos were collected at stage 14/15. Each treatment group was sampled as triplicates. 30 counts of embryos were collected for each sample. Embryo samples were then lysed using 450 □l Passive Lysis Buffer. After centrifugation, supernatant was collected for dual-luciferase test using a Synergy H1 Hybrid Reader (BioTek, Winooski, USA). Data presented in the supplementary figure S6B represent mean +/- SEM (standard error of mean) from three independent experiments.

References:

Schlett, K. and Madarasz, E. 1997. Retinoic acid induced neural differentiation in a neuroectodermal cell line immortalized by p53 deficiency. J Neurosci Res 47(4): 405-15.

Yan, Y., Shin, S., Jha, B. S., Liu, Q., Sheng, J., Li, F., Zhan, M., Davis, J., Bharti, K., Zeng, X. et al. 2013. Efficient and rapid derivation of primitive neural stem cells and generation of brain subtype neurons from human pluripotent stem cells. Stem Cells Transl Med 2(11): 862-70.

Supplementary Tables

Supplementary Table S1: in situ probe constructs used in this study.

In Situ Probe		
Items	**Linearization Enzymes**	**Vectors**
Kdm3a	BglII/T7	pCS107
Sox2	HindIII/T7	pCS107
Otx2	NotI/T7	pCS107
Rx	ClaI/T7	pCS107
Pax6	BamHI/T7	pCS107
Six3	ClaI/T7	pCS107
Sox3	BamHI/T7	pCS107
Xk81b2	HindIII/T7	pCS107
Msx1	EcoRI/T7	pCS107
Zic1	EcoRI/T7	pCS107
Sox9	ClaI/T7	pCS107
Snail1	ClaI/T7	pCS107
En2	XbaI/T3	pBSKS
Krox20	BamHI/T7	pCS107
Hoxb9	XbaI/T3	pBSKS
Ath5	ClaI/T7	pCS107
Neurog2	BamHI/T7	pCS107
Ascl1	BamHI/T7	pCS107
Neurod1	ClaI/T7	pCS107
Tubb2b	BamHI/T7	pCS107
Dll1	EcoRV/T7	pCS2
Runx1	ClaI/T7	pCS107
HB9	EcoRI/T7	pCS107
xHox11L2	EcoRI/T7	pCS107

Supplementary Table S2: antibodies used in this study

Antibody		
Antibody	**Supplier**	**Cat.#**
Anti-H3	Cell signaling	9715
Anti-H3K9me1	Millipore	07-450
Anti-H3K9me2	Abcam	ab1220
Anti-H3K9me3	Millipore	07-523
Anti-H3K9ac	millipore	06-942
Anti-H3K27ac	millipore	07-360
Anti-H3K27me3	millipore	ABE44
Anti-H3K4me3	millipore	07-473
Anti-Kdm3A	Abcam	ab80598
Anti-Kdm4A	Abcam	ab70786
Anti-Myc	Santa Cruz	sc-40
Anti-Flag	Sigma	F1804
Anti-HA	Roche	11867423001
Anti-Neurog2	Santa Cruz	sc-19233
Anti-phospho-Erk1/2(Thr-202/Tyr204)	Cell signaling	9101
Anti-Erk1/2(p44/42 MAPK)	Cell signaling	9102

Supplementary Table S3: oligos and primers used in this study

Click here to Download Table S3

Supplementary Table S4: KDM3A MS qPCR raw data

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Supplementary Figure

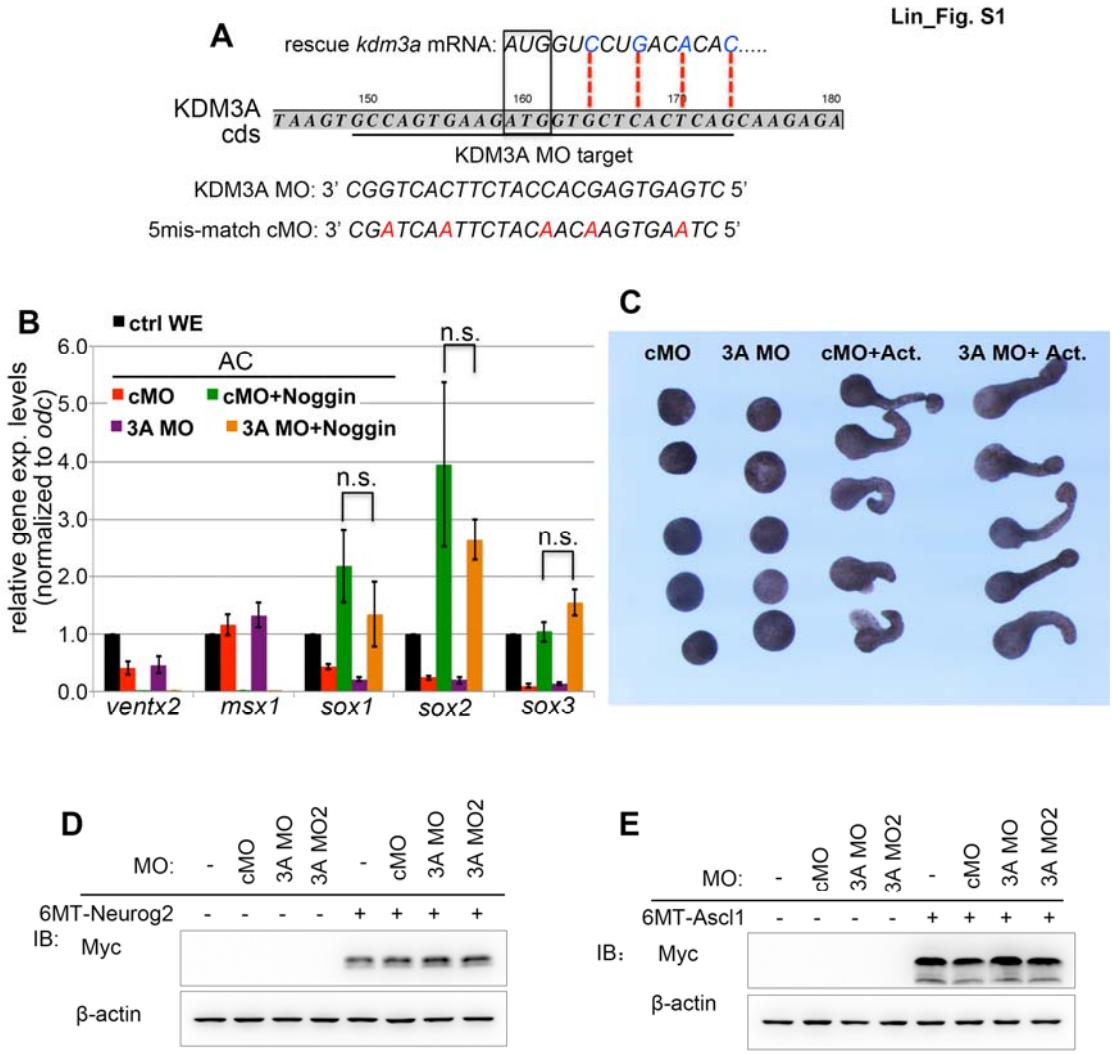

Fig. S1 (related to Fig. 1). MO-mediated depletion of KDM3A and assessment of the function of KDM3A in the developmental potential of naïve ectoderm.

(A) Schematic depiction of the start codon spanning sequence of KDM3A CDS and the KDM3A MO target sequence. The sequence of a 5mis-match mutant version of KDM3A MO, designed cMO, shown in comparison with the KDM3A MO. (B) qPCR analyses of gene expression in animal cap explants. cMO: 80ng; 3A MO: 80ng.

Noggin mRNA: 200 pg. WE: whole embryo; AC: animal cap explants. ns: no significance according to two-tailed Student *t*-test. (C) Animal cap explants treated with or without Activin protein. 80ng cMO and 3A MO were injected into the animal pole at the 2-cellstage and animal caps were dissected at the stage 8.5 and treated with or without Activin (5 ng/ml) for four hours. All explants were then cultured in simple saline to the sibling stage 18. (D, E) Western blot data showing that KDM3A MO/MO2 injection did not affect the expression of microinjected 6MT-Neurog2 (D) or 6MT-Ascl1 (E). cMO: 80 ng; 3A MO: 80 ng; 3A MO2: 80 ng. 6MT-*neurog2* mRNA: 500 pg; MT-*ascl1*: 200 pg. MO and mRNA were sequentially injected into both cells at the 2-cell stage. Embryos were lysed at stage 11.

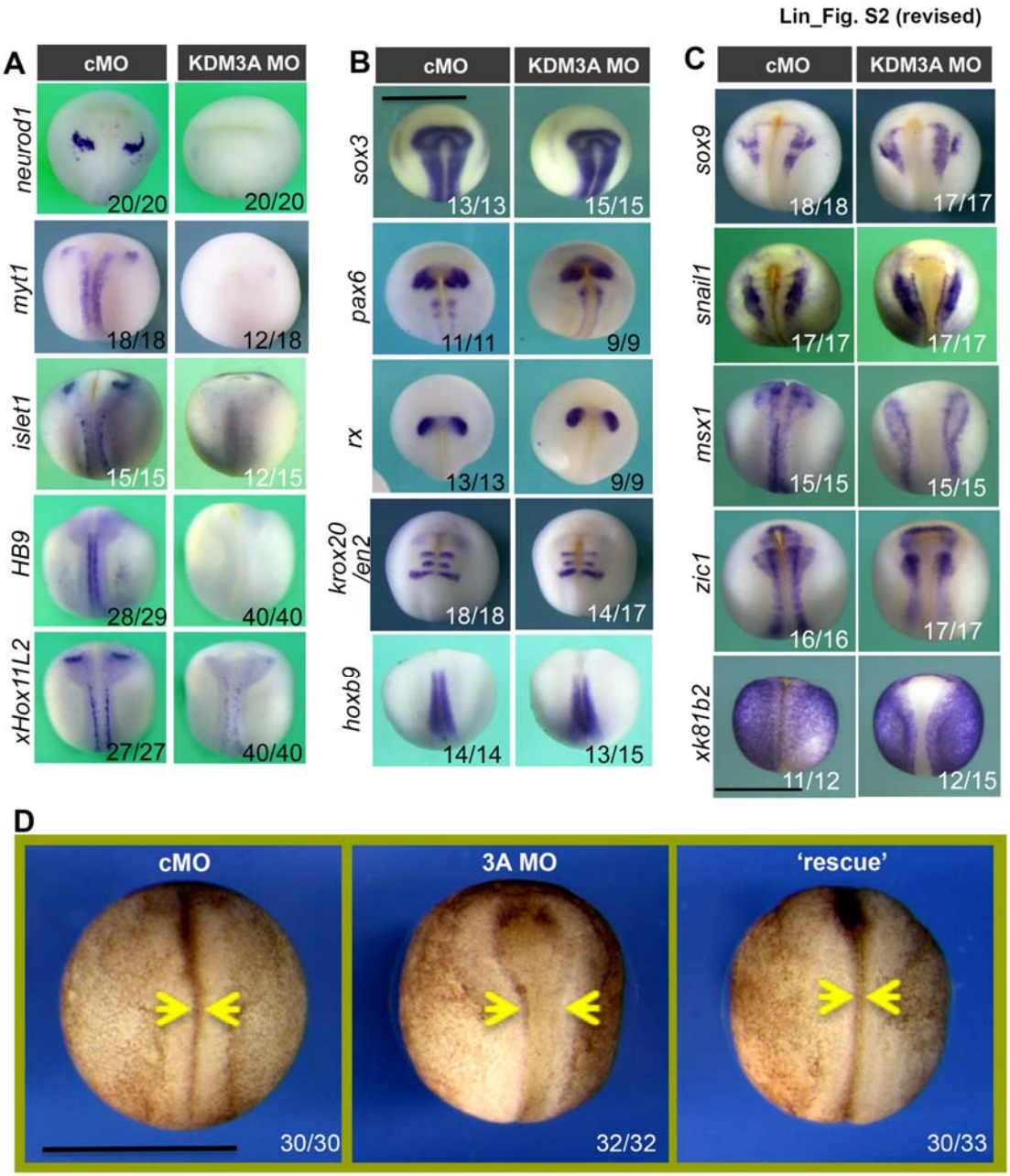

Fig. S2 (related to Fig. 2). Assessment of the effects of KDM3A depletion on neural development.

(A, B, C) Control (cMO) and KDM3A morphant (3A MO) embryos at stage 18*in situ* hybridized with indicated marker genes. (A) Neuronal genes. (B) Markers for neural

progenitors (*sox3, pax6*) and the anteroposterior pattern (*rx*, *krox20*, *en2*, and *hoxb9*). (C) Markers for neural plate border, neural crest specification and epidermis. (D) Dorsal view of embryos at stage 18 showing that KDM3A depletion by injecting 60 ng 3A MO delays neural tube closure, which was partially rescued by injecting back of 500 pg the MO-resistant *kdm3a* mRNA.

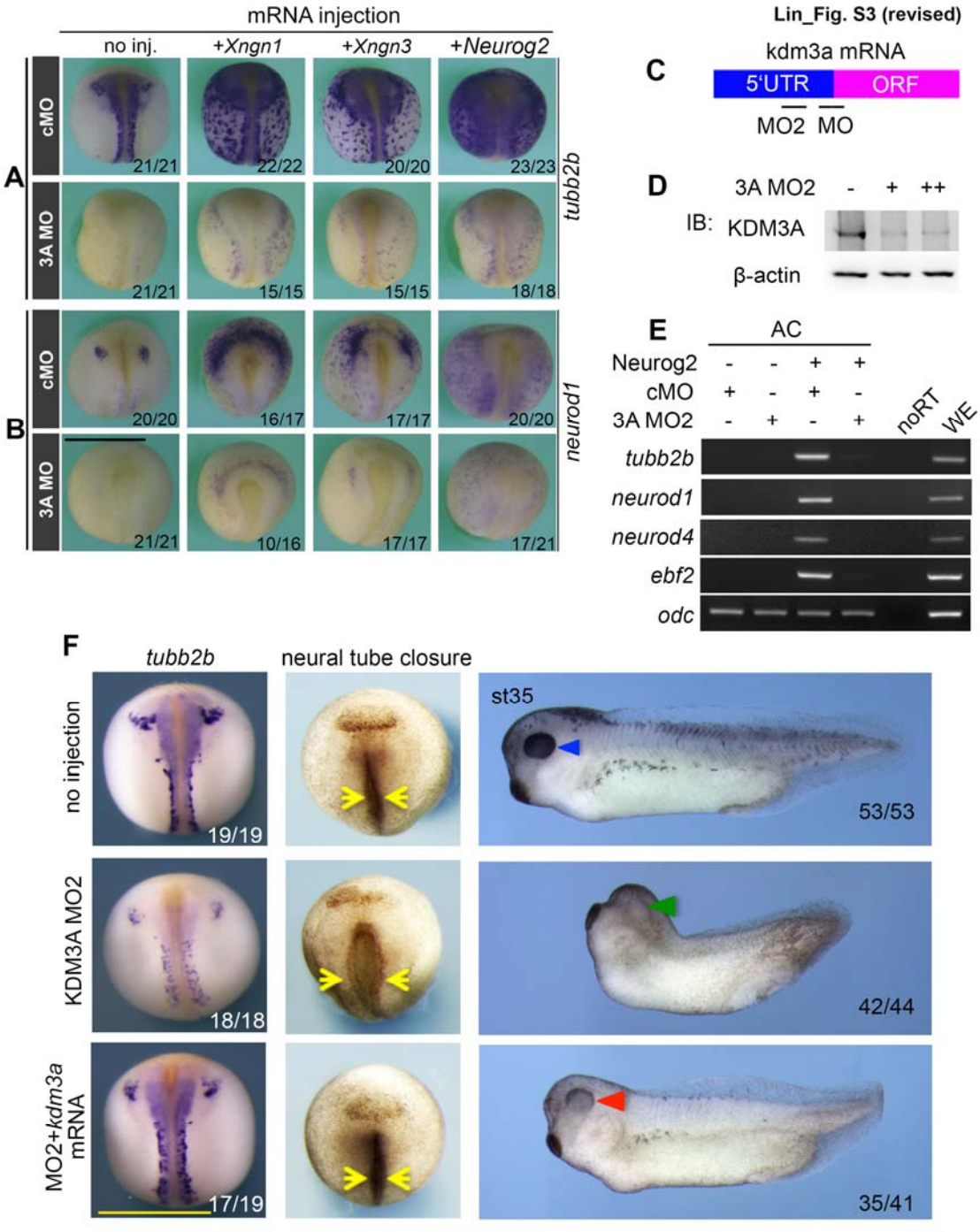

Fig. S3 (related to Fig. 2). KDM3A is required for primary neurogenesis in *Xenopus* and depletion of KDM3A using a second MO (MO2). (A, B) Embryos at stage 18 in situ hybridized with *tubb2b* (A, dorsal view) or *neurod1* (B, dorsal anterior view). 80 ng 3A MO or cMO was injected into both cells at the 2-cell stage. mRNA encoding Xneurog1 (100 pg), Xneurog3 (100 pg), or mouse Neurog2 (50 pg) was injected into two dorsal cells at the 4-cell stage. (C) A schematic depiction of KDM3A MO and MO2 targeting different locations that are critical for the translation control of *kdm3a* mRNA. (D) Western blot detection of KDM3A protein at stage 11 after KDM3A MO2 injection at the 2-cell stage (40 and 80 ng). (E) Semi-quantitative PCR analyses of gene expression in animal caps with indicated treatment. cMO: 80 ng. 3A MO2: 80 ng. *neurog2* mRNA: 100 pg. (F) Effects of KDM3A MO2 injection on *tubb2b* expression analyzed through WISH (left column, dorsal view), neural tube closure (middle column, dorsal anterior view, yellow arrows demarcate the closing neural tubes), and later development (right column, lateral view, solid triangles indicate eyes). cMO: 60 ng. 3A MO2: 60 ng. 3A MO2-resistant *kdm3a* mRNA: 500 pg.

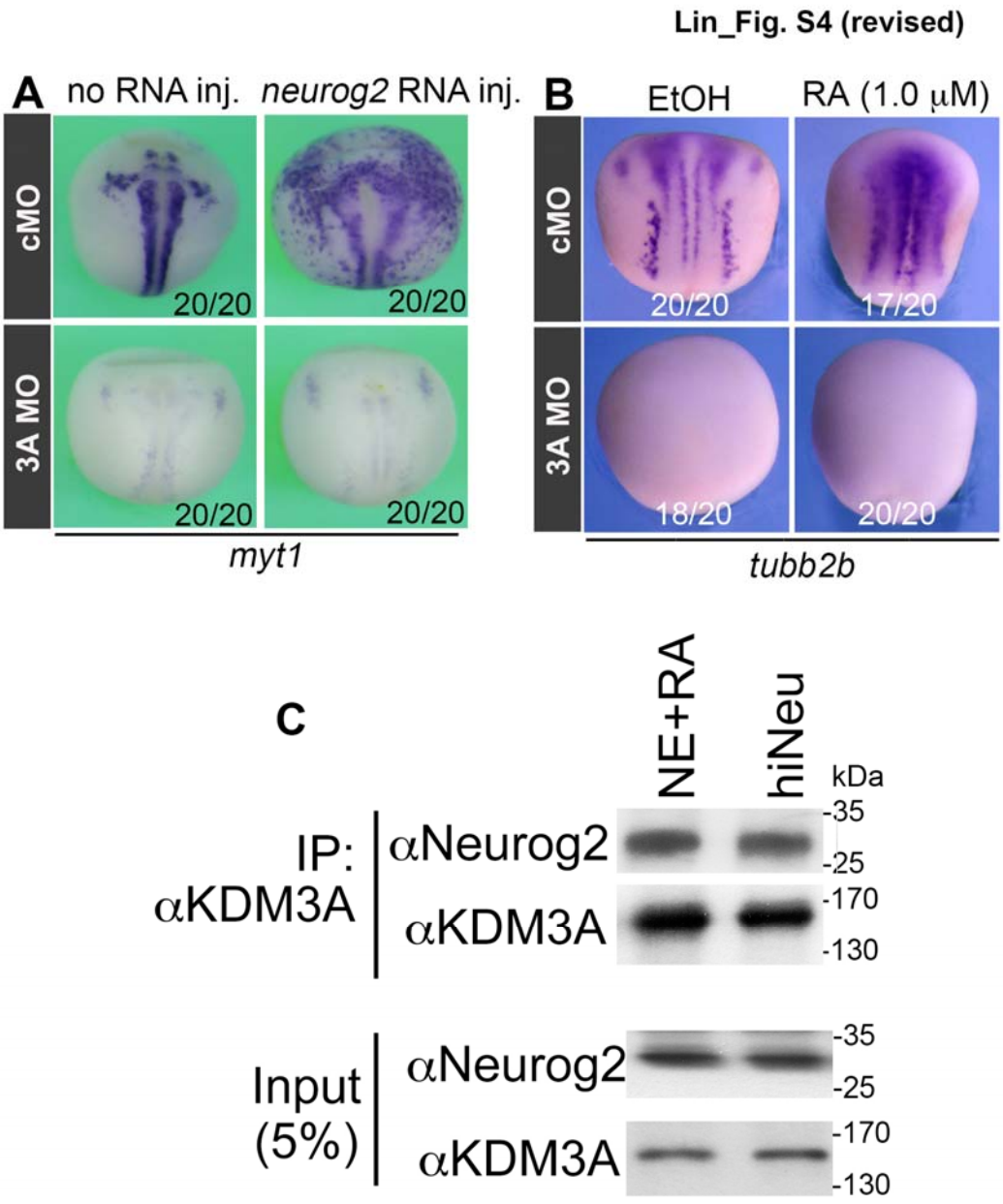

Fig. S4 (related to Figs. 3 and 4). Assessment of functional and physical interactions between KDM3A and Neurog2.

(A) WISH detection of *myt1* in embryos at stage 18 (dorsal view). cMO and 3A MO: 80 ng. *neurog2* mRNA:100 pg. (B) Embryos at stage 15/16 in situ hybridized with

tubb2b (dorsal view). 3A MO and cMO: 80 ng. RA (1.0 μM) was added to culture medium from stage 12 to stage 15/16. (C) Two different types of cells (RA-induced NE-4C cells and human induced-Neu neural stem cells) were lysed and subjected to CoIP for detecting interaction between the endogenous KDM3A and Neurog2. A KDM3A antibody was used to pull down endogenous KDM3A protein, and a Neurog2 antibody was used to detect the signal of endogenous Neurog2 protein.

Lin_Fig. S5 (revised)

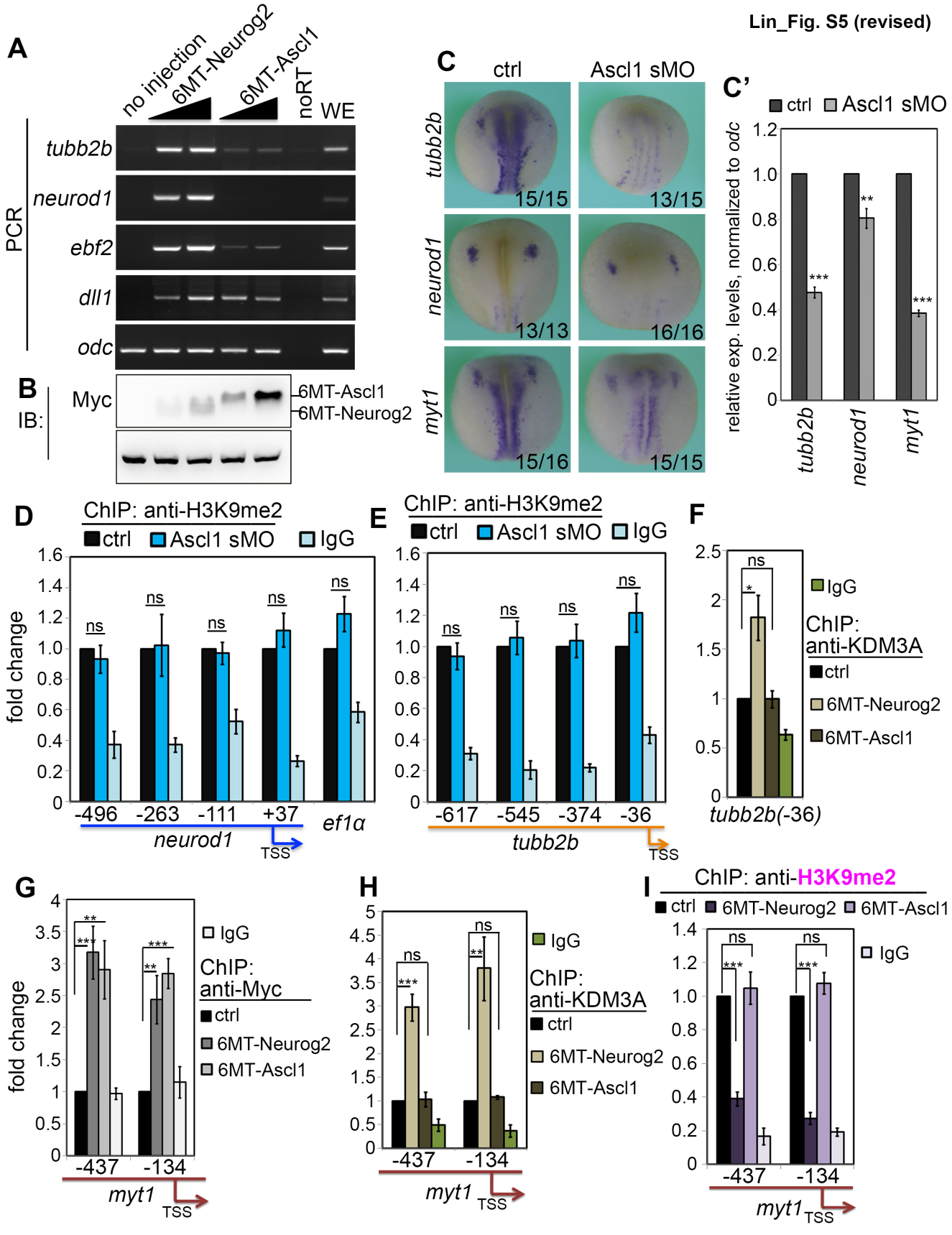

Fig. S5 (related to Fig. 5). Assessment of the activities of Ascl1 on neuronal gene expression and H3K9me2.

(A) Semi-quantitative PCR analyses of gene expression in animal cap explants treated with Neurog2 or Ascl1. (B) Western blot detection of overexpressed 6MT-Neurog2 and 6MT-Ascl1 in the animal cap explants prepared through the same procedure as done in (A). (C, C') WISH (C) and RT-qPCR (C') detection of gene expression in control and Ascl1 splice blocking MO (Ascl1 sMO, 80 ng)-injected embryos at the stage 18. * P<0.05; ** P<0.01; *** P<0.005, according to two-tailed Student t-test.. (D, E) Anti-H3K9me2 ChIP-qPCR analyses showing that 80 ng Ascl1 sMO did not alter the H3K9me2 marks on the promoter regions of *neurod1* (D) or *tubb2b* (E). (F) ChIP-qPCR detection of KDM3A on the -36 bp position of *tubb2b* promoter. 6MT-*neurog2* mRNA (500 pg) and 6MT-*ascl1* mRNA (200 pg) were individually injected at the 2-cell stage and embryos were then harvested at the stage 15 followed by ChIP-qPCR procedures. (G-I) ChIP-qPCR data showing the effects of ectopic Neurog2 and Ascl1 on the promoter region of *myt1*. Both ectopic Neurog2 and Ascl1 were able to bind *myt1* promoter (G). Only overexpressed Neurog2 increased the level of KDM3A (H), and decreased the H3K9me2 marks (I) on the promoter of *myt1*. * P<0.05; ** P<0.01; *** P<0.005; ns: no significance, according to two-tailed Student's t-test.

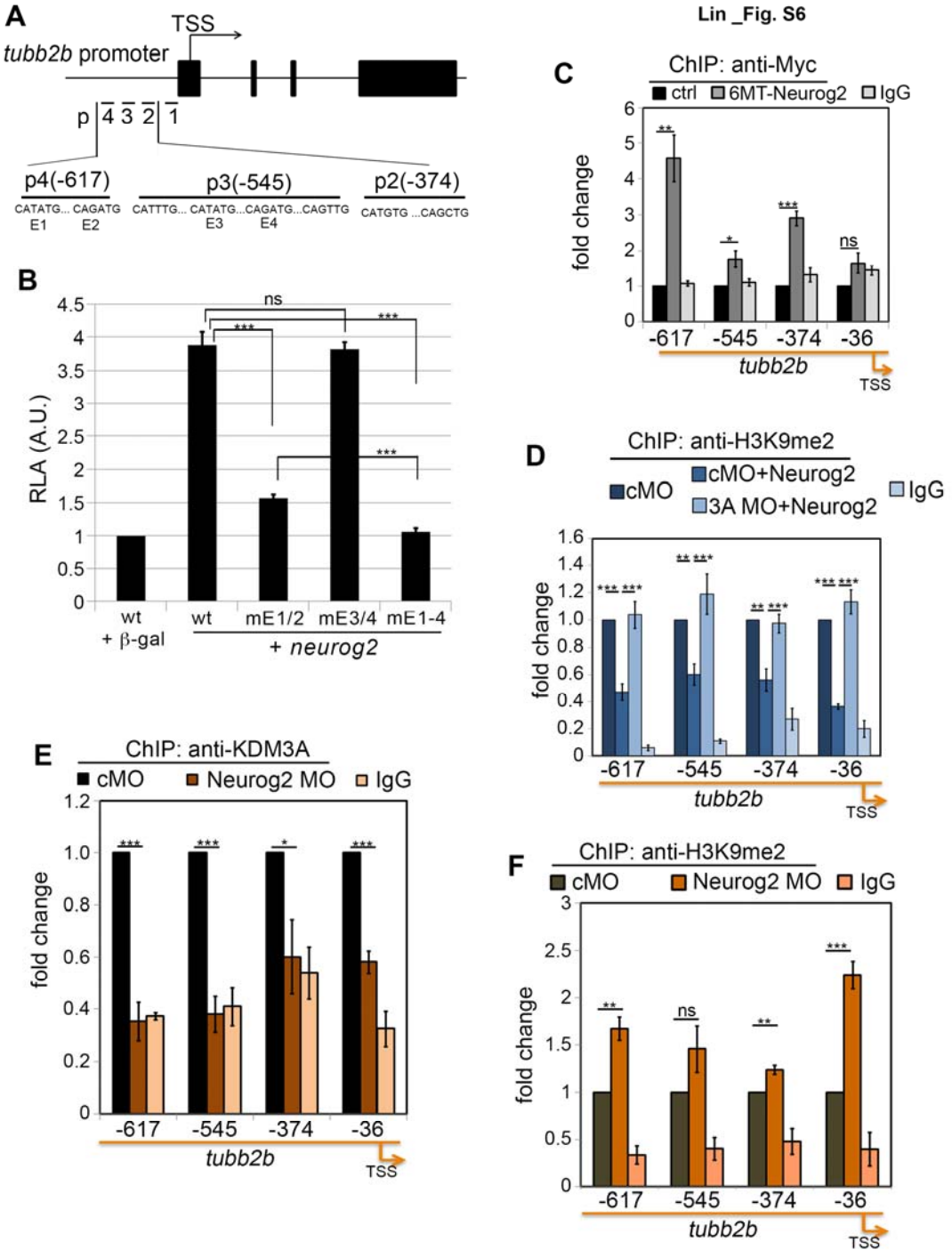

Fig. S6 (related to Fig. 5). Neurog2 transactivates *tubb2b* in a KDM3A-dependent manner.

(A) Schematics of the *tubb2b* promoter and upstream regulatory sequences that contain several degenerate E-box motifs. Locations of ChIP-qPCR primers are also shown. (B) Dual-luciferase reporter assays showing that the *tubb2b* promoter-driven luciferase reporter is responsive to overexpressed 100 pg *neurog2* depending the presence of intact E-box motifs (E1 and E2). (C) ChIP-qPCR analysis of the binding of overexpressed 6MT-Neurog2 on the promoter region of *tubb2b*. (D) Anti-H3K9me2 ChIP-qPCR analyses showing that 80 ng 3A MO abolished the ability of ectopic Neurog2 (100 pg) to decrease theH3K9me2 marks on the *tubb2b* promoter. (E) Anti-KDM3A ChIP-qPCR analyses at stage 15 indicating that Neurog2 MO (80 ng) but not standard MO reduced KDM3A bound on the *tubb2b* promoter. (F) Anti-H3K9me2 ChIP-qPCR at stage 18 showing that Neurog2 (80 ng) but not control MO increased the H3K9me2 marks on the *tubb2b* promoter. * $P<0.05$; ** $P<0.01$; ***$P<0.005$, ns: no significance, according to two-tailed Student's *t*-test.

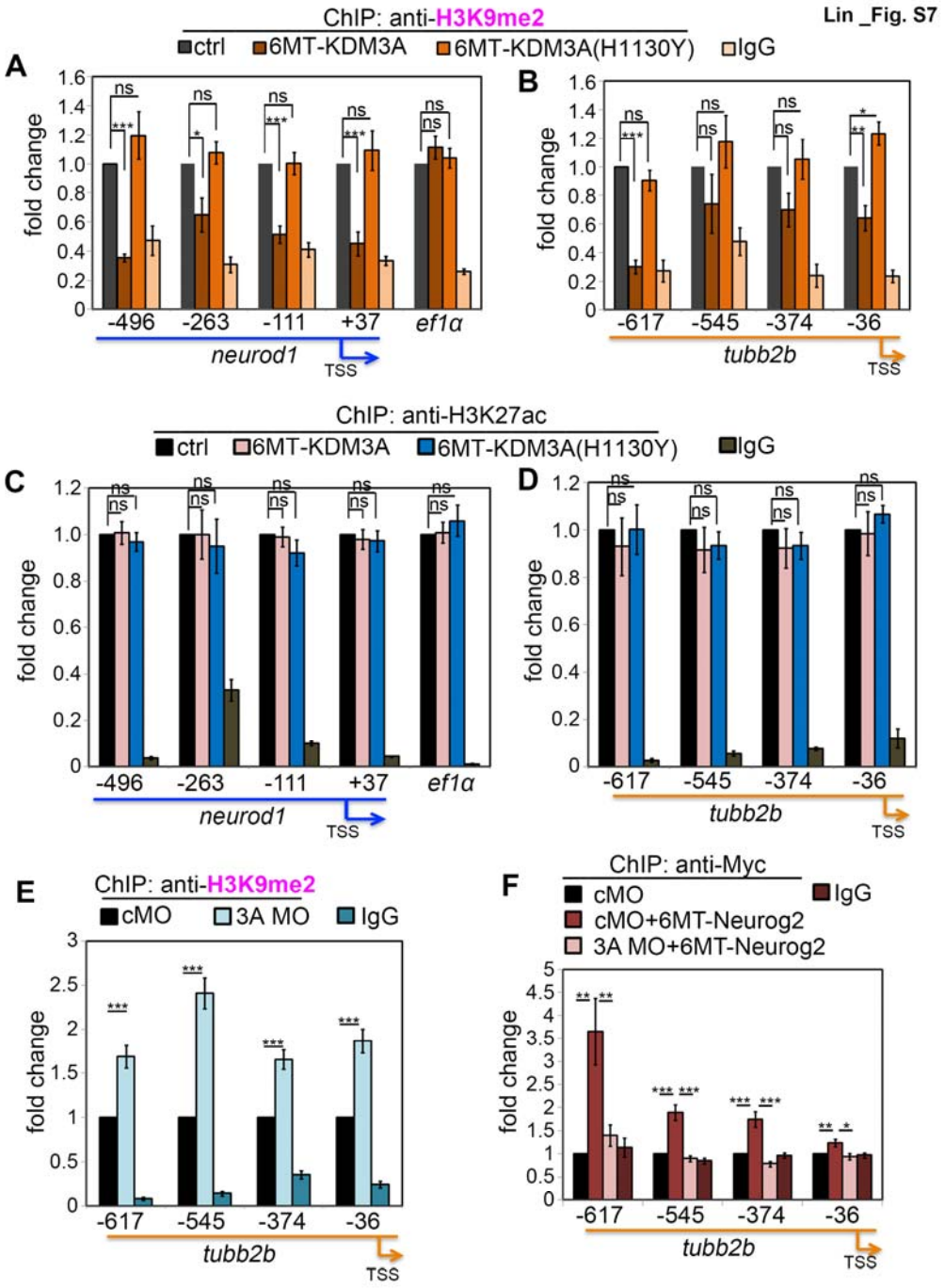

Fig.S7 (related to Figs. 5 and 6). KDM3A facilitates the chromatin binding of Neurog2.

(A, B) Anti-H3K9me2 ChIP-qPCR analyses indicating that overexpression of wild type 6MT-KDM3A (1 ng) but not a catalytic mutant 6MT-KDM3A (H1130Y, 1 ng) reduced the expression levels of H3K9me2 on the promoter regions of *neurod1* (A) and *tubb2b* (B). (C, D) Anti-H3K27ac ChIP-qPCR analyses indicating that ectopic 6MT-KDM3A or a catalytic mutant 6MT-KDM3A (H1130Y) did not alter the H3K27ac marks on the promoter regions of *neurod1* (C) or *tubb2b* (D). (E) Anti-H3K9me2ChIP-qPCR analyses showing 3A MO (80 ng) increased the H3K9me2 marks on the promoter region of *tubb2b*. (F) Anti-Myc ChIP-qPCR analyses indicating that 80 ng 3A MO but not cMO blocked the overexpressed 6MT-Neurog2 from accessing the promoter of *tubb2b*.