Supplementary Information

ASCL1 phosphorylation and *ID2* upregulation are roadblocks to glioblastoma stem cell differentiation

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Figure S1. Full length Western Blot gels. Full length gels for Fig. 1A (A), Fig. 1B (B), Fig. 1C (C), Fig. S2F (D), Fig. S3B (E), Fig. S4E (F), Fig. S4C (G).

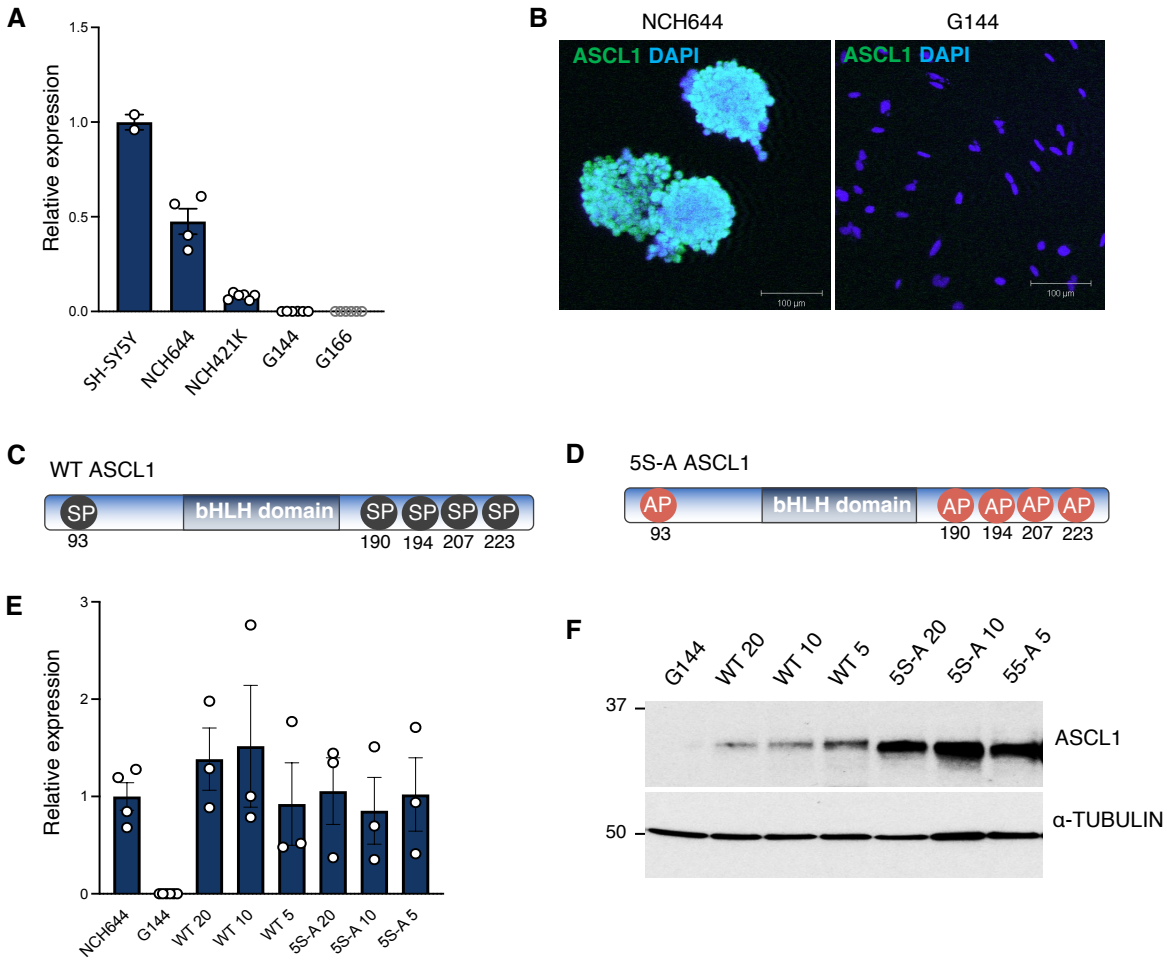

Figure S2. Expression of ASCL1 in GBM lines. (A) Expression of endogenous *ASCL1* mRNA in cell lines derived from primary human glioblastomas. The neuroblastoma line SH-SY5Y is used as a positive control. Data: mean ± s.e.m., normalized to *HPRT1*. (B) Immunofluorescence for ASCL1 in NCH644 and G144 cells. Scale bars: 100 µm. (C-D) Schematic representation of the 5 Serines, which are mutated to Alanine in phospho-mutant ASCL1 (5S-A ASCL1). (E-F) Expression of *ASCL1* mRNA (E) and protein (F) in G144 cells after 24 hours of dox induction. G144 cells have been transduced with dox inducible lentiviruses at different multiplicity of infection (MOI: 5, 10 or 20), as labelled. Data: mean ± s.e.m. normalized to *TBP*, n≥3

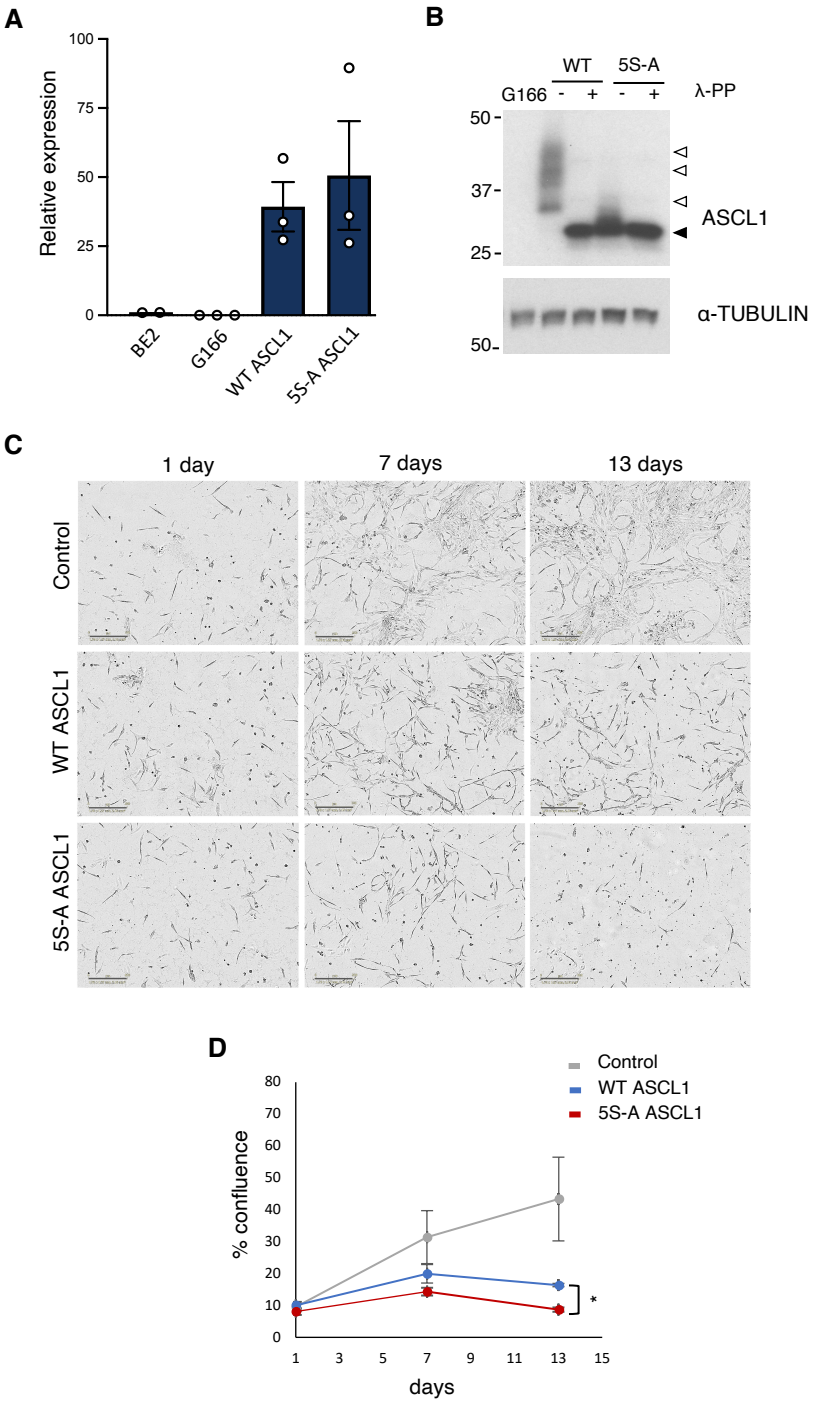

Figure S3. ASCL1 expression and phosphorylation in the G166 GBM cell line. (A-B) Expression of ASCL1 mRNA (A) and protein (B) in G166 cells 24 hours after WT and 5S-A ASCL1 induction, treated with and without phosphatase (λ-PP) as indicated. White and black arrowheads indicate phosphorylated and unphosphorylated ASCL1, respectively. (C) Representative images of G166 cells after growth factor withdrawal and dox induction of WT and 5S-A ASCL1 expression. Scale bars: 300 µm. (D) Quantification of cell confluence. Each data point is mean ± s.e.m. n=3 independent experiments; one-way ANOVA followed by the Bonferroni post-hoc test; *p≤0.05.

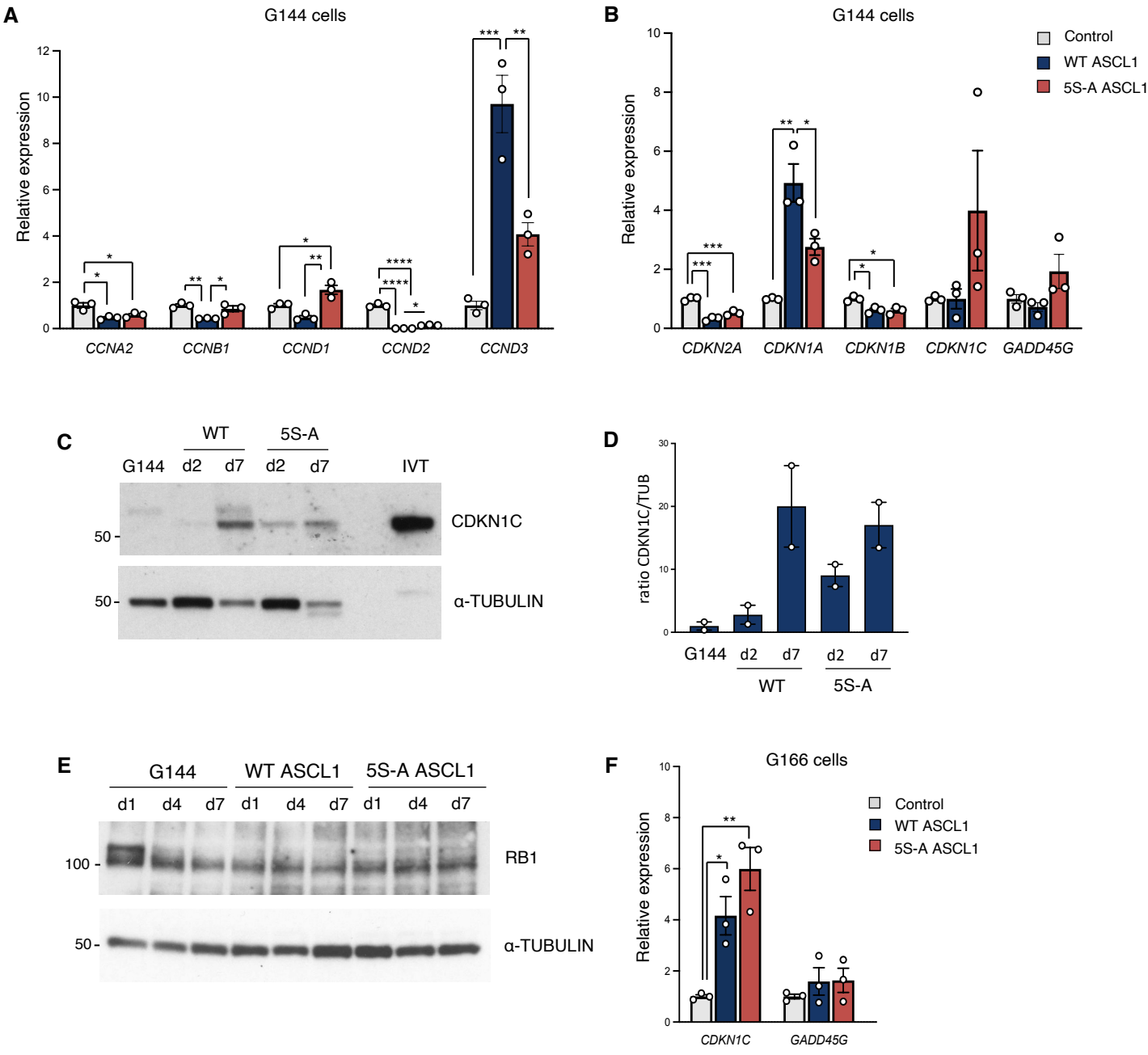

Figure S4. ASCL1-mediated regulation of cell cycle genes. (A-B) qPCR to measure expression of mRNAs for cyclins (A) and negative cell cycle regulators (B) in G144 cells, 24 hours post-WT or 5S-A ASCL1 induction. Data: mean ± s.e.m., normalized to *TBP;* n=3 independent experiments; one-way ANOVA followed by the Bonferroni post-hoc test; *p<0.05; ** p<0.01; ***p<0.001; ****p<0.0001. (C) Western blot of the cell cycle inhibitor CDKN1C in growing G144 cells and at day (d) 2 and 7 of growth factor withdrawal and dox induction of WT and 5S-A ASCL1 expression. (D) Quantification of the relative amount of CDKN1C, normalized to the level of TUBULIN expression. n=2 from different western blots from one experiment. *In vitro* translation (IVT) of CDKN1C has been used as a control. (E) Western blot showing expression and phosphorylation of the Retinoblastoma protein (RB1) in G144 cells at different days (d) of differentiation and upon induction of WT and 5S-A ASCL1 in differentiation conditions. (F) Expression of negative cell cycle regulators in G166 cells, 24 hours after WT or 5S-SA ASCL1 induction. Data: mean ± s.e.m., normalized to *TBP;* n=3 independent experiments; one-way ANOVA followed by the Bonferroni post-hoc test; *p<0.05; ** p<0.01.

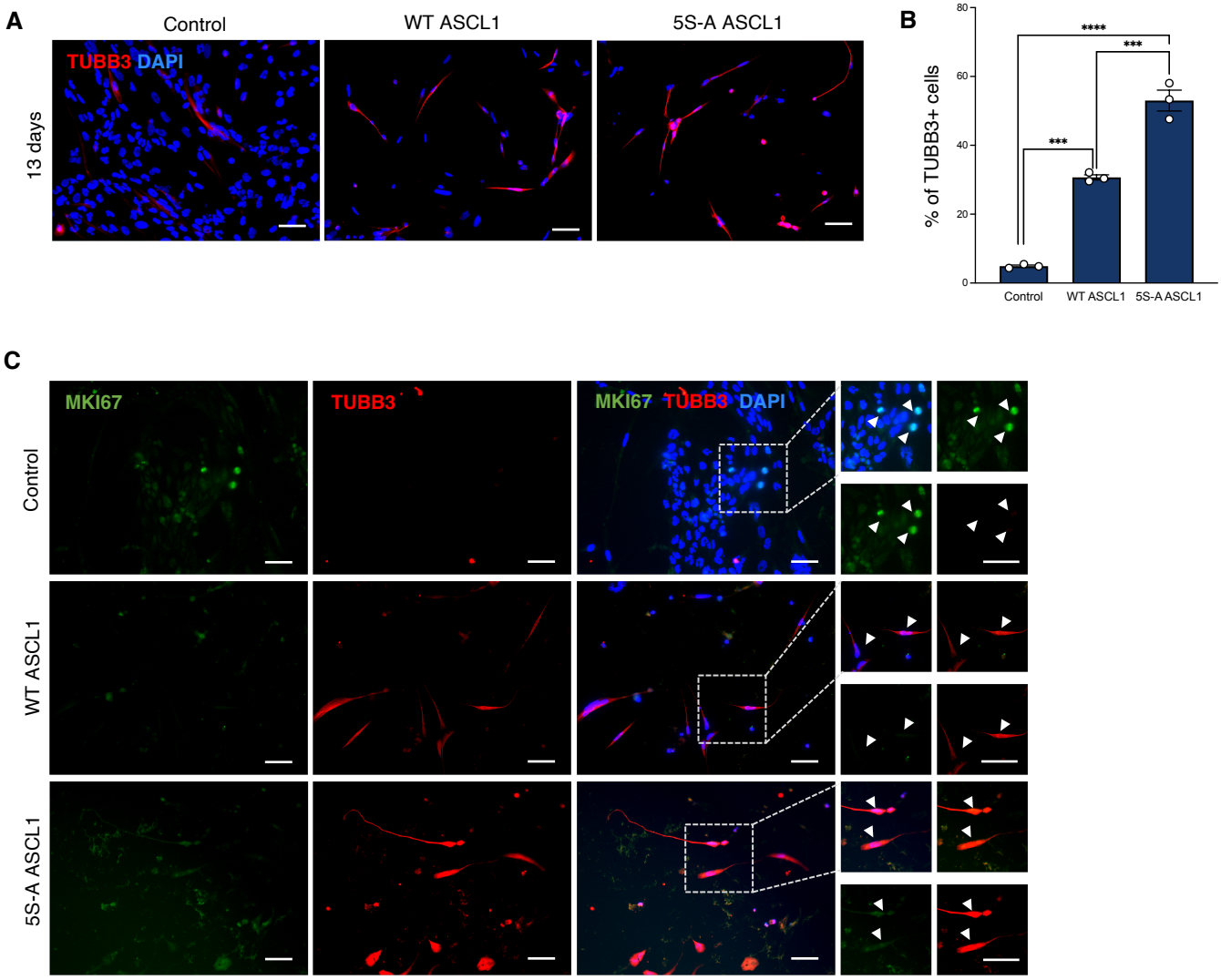

Figure S5. Effect of ASCL1 phosphorylation on G166 cell differentiation. (A) Immunostaining for the neuronal marker TUBB3 (red) in G166 cells cultured for 13 days without growth factors and in the presence of dox-induced WT or 5S-A ASCL1. Scale bars: 50 µm. (B) Quantification of the percentage of TUBB3^{+} cells over the total number of DAPI+ cells. Data: mean ± s.e.m. n=3 independent experiments; one-way ANOVA followed by the Bonferroni post-hoc test; ***p<0.001; ****p<0.0001. (C) Co-staining of TUBB3 and MKI67 at 13 days of differentiation upon WT and 5S-A ASCL1 induction. Scale bars: 50 µm.

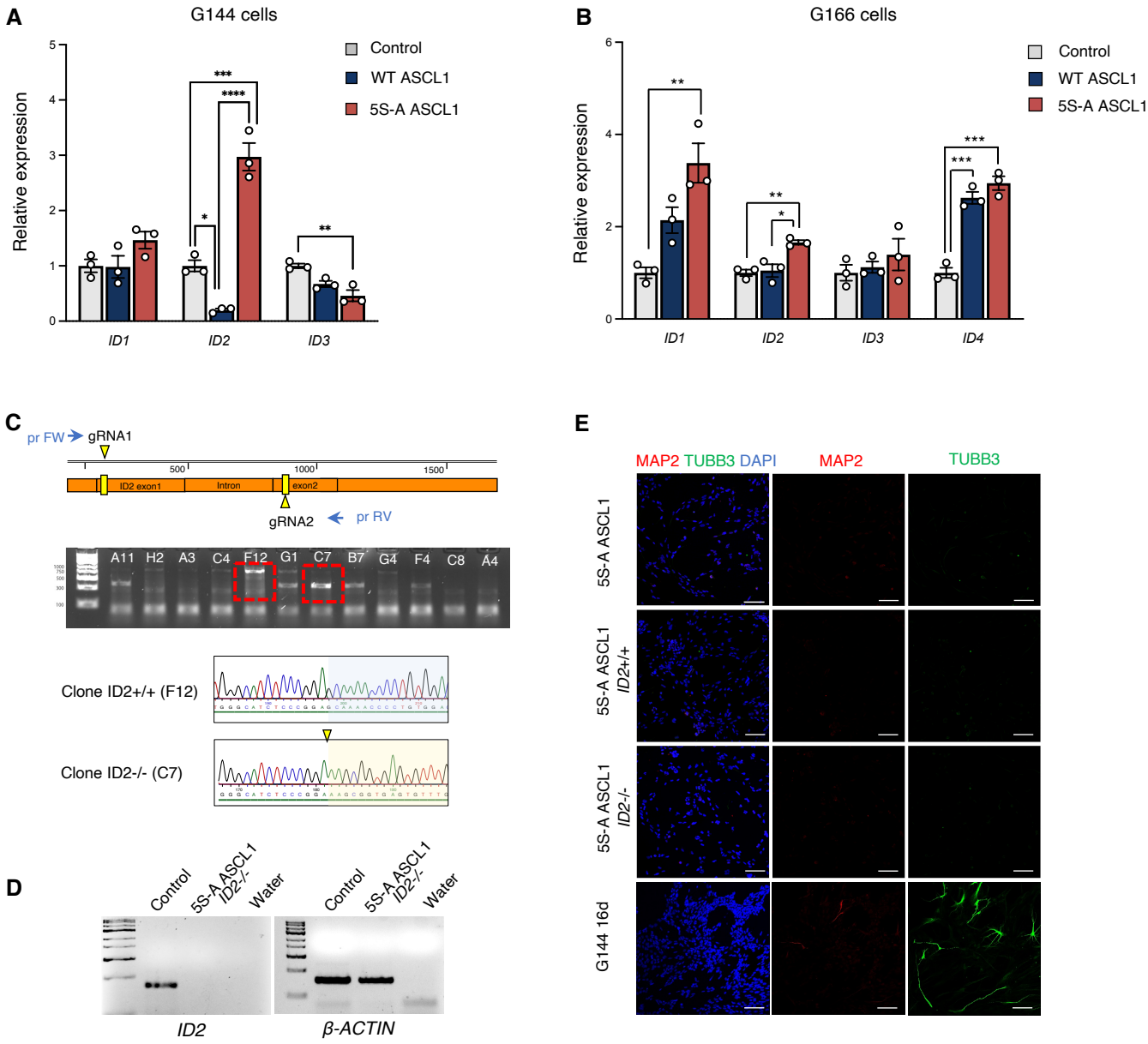

Figure S6. *ID* expression and generation of *ID2* knockout cells. (A-B) Relative mRNA expression of different members of the *ID* family, 24 hours after WT and 5S-A ASCL1 expression in G144 cells (A) and G166 cells (B).Data: mean ± s.e.m., normalized to *TBP*. n=3 independent experiments; one-way ANOVA followed by the Bonferroni post-hoc test; *p<0.05; ** p<0.01; ***p<0.001; ****p<0.0001. (C) Top panel: schematic diagram showing the position of the guide RNAs for *ID2* deletion; middle panel: PCR screening of different *ID2* mutated clones; bottom panels: snapshot of the sequencing data for $ID2^{+/+}$ F12 clone and $ID2^{-/-}$ C7 clone (yellow arrowhead indicates Cas9 cutting site). (D) PCR for *ID2* in Control and *ID2* CRISPR knockout cells. *β-ACTIN* is used as loading control. (E) Immunostaining for the neuronal markers TUBB3 (green) and MAP2 (red) in proliferative conditions. Positive control for the immunostaining: G144 cells after 16 days in differentiation conditions. Scale bar: 100 µm

	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
ASCL1	CATCTCCCCCAACTACTCCA	AACGCCACTGACAAGAAAGC
ASCL1 OE	CTCAACTTCAGCGGCTTTGG	CTCATCTTCTTGTTGGCCGC
PDGF	GATACCTCGCCCATGTTCTG	CAGGCTGGTGTCCAAAGAAT
PDGFRA	CCACCGTCAAAGGAAAGAAG	CCAATTTGATGGATGGGACT
MBP	AAGAACTGCTCACTACGGCT	TGAATCCCTTGTGAGCCGAT
SOX10	GCTGAGTTGGACCAGTACCT	TCTGTCTTCACCTGGGCTTT
ADRA2C	GAGTACAACCTGAAGCGCACAC	GGAGGACAGGATGTACCAGGTC
DBN1	GAGGAAACTGAGGCAAAGAGGA	TCGGAGCCATCTTCATATGTGT
NTRK1	TTGCCTGCCTCTTCCTTTCTAC	ATTGTGGGTTCTCGATGATGTG
OLIG2	CAGAAGCGCTGATGG	TCGGCAGTTTTGGGT
ID1	ATCAGGGACCTTCAGTTGGAGC	GGAGACCCACAGAGCACGTAAT
ID2	CGACCCGATGAGCCTGCTAT	TCCGTGTTGAGGGTGGTCAG
ID3	GGTCACTGTAGCGGGACTTCTT	GTGGTTCATGTCGTCCAGCAAG
ID4	GCTCACTGCGCTCAACACCG	CTGGCTCGGGCTCAGGCGGC
CCNA2	GCACTCTACACAGTCACGGG	GTGTCTCTGGTGGGTTGAGG
CCNB1	TGTTGGTTTCTGCTGGGTGT	TGCCATGTTGATCTTCGCCT
CCND1	TTTGTTGTGTGTGCAGGGAG	TTTCTTCTTGACTGGCACGC
CCND2	CCGCAGTGCTCCTACTTCAA	GCCAAGAAACGGTCCAGGTA
CCND3	TTTGGGGCAGCACTGGTTTA	AGGCCAGGAAATCATGTGCA
CDKN2A	AGGGGTGCCACATTCGCTAA	GCCAGCCCCTCCTCTTTCTT
CDKN1A	GCACTTTGATTAGCAGCGGA	AGCCGAGAGAAAACAGTCCA
CDKN1B	AGAGACATGGAAGAGGCGAG	CCAAATGCGTGTCCTCAGAG
CDKN1C	GAGCCAATTTAGAGCCCAAAGA	AAGCTTTACACCTTGGGACCAG
GADD45G	GACACAGTTCCGGAAAGCACAG	AGCGTAAAATGGATCTGCAGCG
TBP1	AGCAGCAGCAACAGGCAGTG	TGGGGGAGGGATACAGTGGA
β-ACTIN	GCCCATCTACGAGGGGTATG	GTGGCCATCTCTTGCTCGAAG
ID2_GF1	CCGCCGAGTGCGGATAAAAG	
ID2_GR1	GTACGGGTAACACCGCGAAG	
ID2_SeqF1	AGCCGCCCCGCCGGGCTCGG	
ID2_SeqR1	GTAAGCATGCATTTACCAAA	

Table S1. List of primers for qPCR and ID2 sequencing