Genome-wide methylomic and transcriptomic analyses identify subtype-specific epigenetic signatures commonly dysregulated in GBM and GSCs

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Supplementary materials:

1. **Supplementary Figure Legends**
2. **Supplementary Figures 1 to 12**
3. **Supplementary Tables 1, 4 and 7**
4. **References**

Supplementary Figure Legends:

Supplementary Figure 1: The TCGA GBM patient samples with barcodes from four subtypes of GBM that are used for genome-wide methylation and expression profiling. 450K methylation and gene expression array data from a total of 93 GBM tumors were downloaded from the TCGA. These tumors include 22 PN, 29 MES, 29 CL, and 13 N subtype tumors. As illustrated in Figures 1 and 2, individual subtype-associated DNA methylation genes, *i.e.* one subtype versus others, such as PN versus others or comparison between PN versus MES subtypes in GBM, were generated, which correlate to their gene expression status. The DNA methylation genes in GBM were also compared to GSC methylation genes in specific subtypes to identify commonly shared epigenetic signature between GBM and GSCs (Figure 4A).

Supplementary Figure 2: Clustering of PN and MES GSCs and β-value distribution in all GSC and GBM subtypes. (A) PN and MES GSCs exhibit distinct methylome based on 5,062 most variable CpG loci in these samples. (B) Density plots of β-value distribution in all GSCs and GBM subtypes. The mean of the β-values for methylation level for each CpG site was calculated for PN, MES, and U1 in GSCs, as well as for PN, MES, CL, and N in GBM, respectively. The density plot was made using R package. The red and green arrow represents the variations in β-value distributions between GSCs and GBM bulk tumors in respective subtypes. This global methylation profiling was further analyzed to generate subtype-associated hypomethylation and hypermethylation signatures unique to either GSC or GBM, or common between them.

Supplementary Figure 3: Subtype-specific distribution of hypermethylated and hypomethylated CpGs with respect to gene features in GSCs and GBM bulk tumors. (A) With respect to gene features, hypermethylated CpGs (text in red above) are dominant in TS1500 and gene body regions in PN, MES and U1 GSCs. (B) In GBM bulk tumors, hypermethylated CpGs (text in green above) in PN and MES subtypes are also prominent in gene body region. (C) and (D) Distribution of hypomethylated CpG loci in GSCs (C) and GBM (D) in respective subtype. (E) and (F) Distribution of differentially methylated CpG loci when compared CpG loci of PN with MES subtypes in GSCs (E) and (F) GBM bulk tumors.

Supplementary Figure 4: Methylation and expression status of all the genes that are methylated in PN or MES subtypes common in GSCs and GBM. Five genes *i.e. OCIAD2, GCNT2, SP100, MT2A* and *CFLAR* are methylated in PN but unmethylated in MES subtypes in both GSCs (A) and GBM (B). These genes are also silenced in PN and have ≥1.5-fold higher levels of expressions in MES compared to PN in GSCs (C) and GBM (D). Similarly, 16 genes are methylated in MES but unmethylated in PN subtypes in both GSCs (E) and GBM (F). These genes are silenced in MES and have ≥1.5-fold higher levels of expression in PN compared to MES in GSC (G) and GBM (H). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Supplementary Figure 5: Methylation status of genes in PN and MES tumors that are randomly divided as test and validation sets in GBM. PN and MES GBM tumors are randomly divided into two groups as test and validation sets to validate the methylation status of genes commonly occurring in PN and MES subtypes. (A) Five genes are methylated in PN but unmethylated in MES both in randomly chosen test sets and validation set in GBM. These genes are also

methylated in PN GSCs and unmethylated in MES GSCs. (D) Sixteen other genes are methylated in MES but unmethylated in PN both in randomly chosen test, and validation sets in GBM. These genes are also methylated in MES GSCs and unmethylated in PN GSCs. Arrows with gene name in bold red, genes from PN-methylated/silenced or MES-methylated/silenced, did not show statistically significant methylation in GBM validation dataset *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Supplementary Figure 6: Global methylation profile of *MIDN* in GBM bulks tumors and GSCs. A) Global methylation profiling of *MIDN* (a MES methylated gene consisting of 23 CpG loci in total on 450K array) in GBM tumors and GSCs. B) An average β-value of all CpG loci in respective subtypes for *MIDN* showing its methylation status in specific regions in respective subtypes of GBM tumors and GSCs. **$p<0.01$; ***$p<0.001$.

Supplementary Figure 7: Methylation profiles of representative genes with differentially methylated multiple CpGs in representative individual samples in GSCs and GBM from 450K arrays. *CFLAR* (A) and *SP100* (B) are methylated in PN but unmethylated in MES and are commonly in GSCs and GBM in individual samples. *MIDN* (C) and *NOTCH1* (D) are methylated in MES but unmethylated in PN and are commonly in GSCs and GBM. These samples include 4 MES and 6 PN GSCs, and 29 MES and 22 PN TCGA GBM bulk tumors (Supplemental Figure 1). An average β-value ≥ 0.4 was considered as methylated and <0.3 was considered as unmethylated for genes that are statistically significant ($p<0.05$) between the two groups/subtypes. Average β-value between 0.3 and 0.4 was considered as intermediate.

Supplementary Figure 8: Experimental validation of methylation and expression status of individual genes by CoBRA and/or bisulphite sequencing and qRT-PCR. (A) Methylation analysis by CoBRA and direct/PCR bisulphite sequencing shows that *MIDN* is methylated in MES GSCs but unmethylated in PN GSCs. Bisulphite sequencing confirmed the methylation of individual CpG loci in *TMCC1* gene. (B) Direct bisulphite sequencing of *NOTCH1* and *ARHGEF7* also confirmed that individual CpG loci in the CoBRA amplified region of these genes are frequently methylated in MES GSCs but unmethylated in PN GSCs. Green and orange circles represent individual CpG in respective samples. Each arrow denotes the differentially methylated CpG site from 450K array data analyzed between PN and MES that were used as reference to design CoBRA primers around that region. (C) and (D) qRT-PCR analyses. *SP100* (C) and *CFLAR* (D) are expressed in MES but silenced in PN GSCs. Treatment of 5-Aza-dC led to re-expression of *SP100* (C) and *CFLAR* (D) in representative PN GSC lines (right graphs), demonstrating that these two genes are dysregulated by DNA methylation in the PN GSCs. (E) and (F) qRT-PCR analyses. *MIDN* (E) and *NOTCH1* (F) are silenced (methylated) in MES GSCs but expressed in PN GSCs. Treatment of 5-Aza-dC led to re-expression of *MIDN* and *NOTCH1* in representative MES GSCs, indicating that these genes are dysregulated by DNA methylation in the MES GSC subtype. (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

Supplementary Figure 9: Dysregulated expressions of MES- and PN-associated methylation of genes common in GBM and GSCs correlate to clinical prognosis of patients with GBM or all glioma in two independent datasets including TCGA. (A) to (H), Kaplan-Meier survival analyses of TCGA LGG + GBM, TCGA glioma, TCGA GBM, and several other glioma or GBM datasets. Low levels of expression of *SP100* that is methylated/silenced in PN but unmethylated/expressed

in MES (A), and high levels of expression of *ARHGEF7* (B), *NOTCH1* (C), *MIDN* (D), *KCNQ2* (E), *ATXN10* (F), *USP54* (G), and *TUB* (H) that are methylated/silenced in MES but unmethylated/expressed in PN predict better clinical prognosis of patients with GBM or glioma, respectively.

Supplementary Figure 10: Multivariate analyses of *CFLAR* and *TMCC1* genes on clinical prognosis of glioma patients including *TP53* and *IDH1* mutation status, age, and gender. Low expression of *CFLAR* (A) that is methylated/silenced in PN but unmethylated/expressed in MES and high expression of *TMCC1* (B) that is methylated/silenced in MES but unmethylated/expressed in PN correlates to better clinical outcomes of patients with gliomas. Multivariate clinical survival analyses using *CFLAR* and *TMCC1* expression in glioma samples correlate to *IDH1* and *TP53* mutation status, age, or gender show that the clinical prognosis of *CFLAR* and *TMCC1* methylation/expression status is independent of *TP53/IDH1* mutation status, age, and gender.

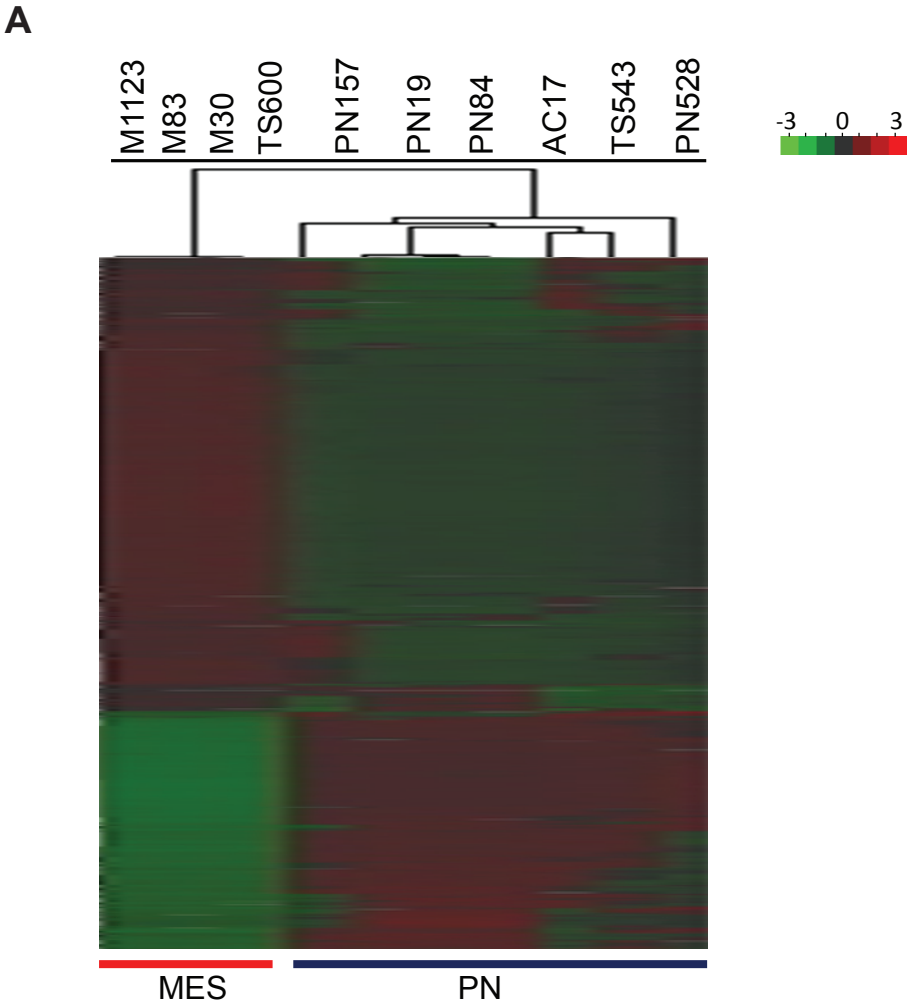
Supplementary Figure 11: Multivariate analyses of MES-methylated genes on clinical prognosis of glioma patients including *TP53* and/or *IDH1* mutation status. Multivariate clinical prognosis of (A) *ARHGEF7* and (B) *NOTCH1* that are methylated/silenced in MES but unmethylated/expressed in PN tumors and (C) *SP100* that is methylated/silenced in PN but unmethylated/expressed in MES tumors correlating to *TP53* and/or *IDH1* mutation status show that the clinical prognosis of these genes are independent of *TP53* mutation status of these genes in patients. However, the clinical prognosis of *NOTCH1* correlating to *IDH1* mutation was not significant (data not shown).

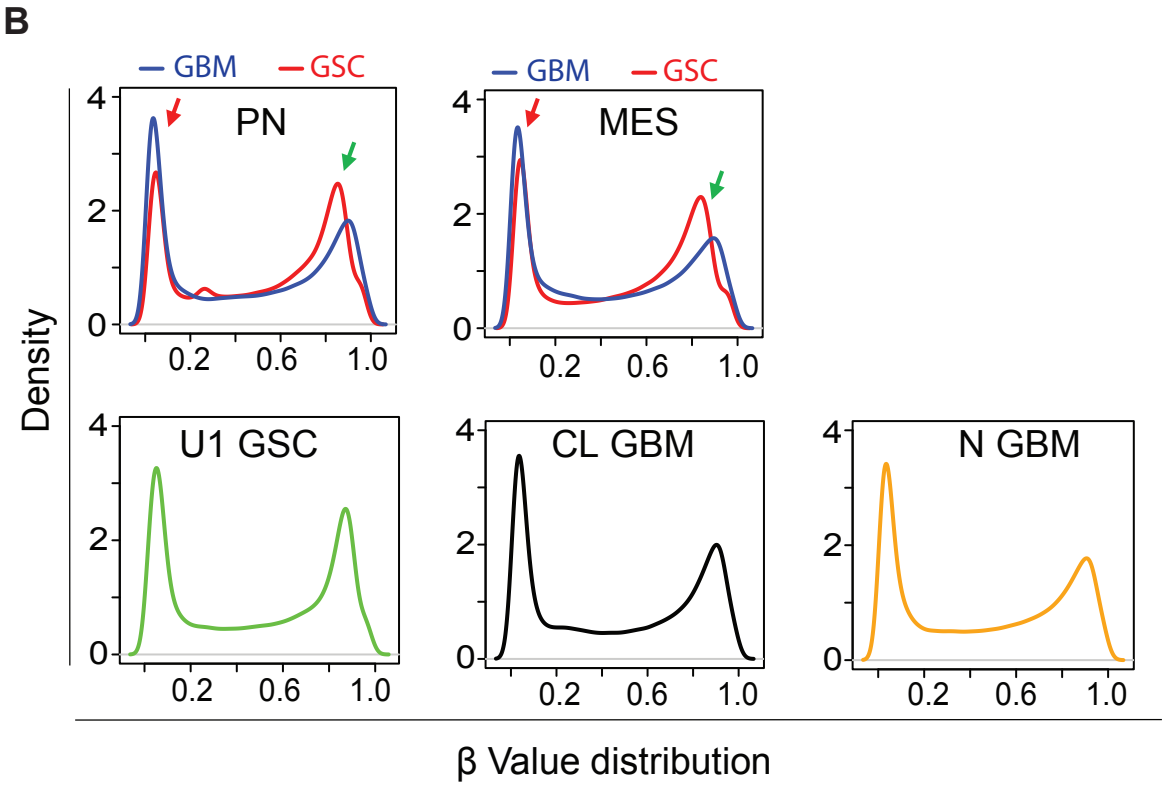
Supplementary Figure 12: Combined gene signature that are either methylated in PN or MES correlate to poorer clinical outcomes of patients with high-grade glioma (HGG). Clinical prognosis of combined gene signature is consistent with the prognosis of individual genes in respective category. In datasets of patients with HGG (GSE4271), (A) high levels of expression of the PN-methylated/silenced combined gene signature *CFLAR*, *SP100*, and *OCIAD2* correlate to poor clinical prognosis. (B) Low levels of expression of the MES-methylated/silenced combined gene signature, *TMCC1*, *USP54, TUB,* and *NOTCH1* correlate to poor clinical outcomes.

Supplementary Figure 1

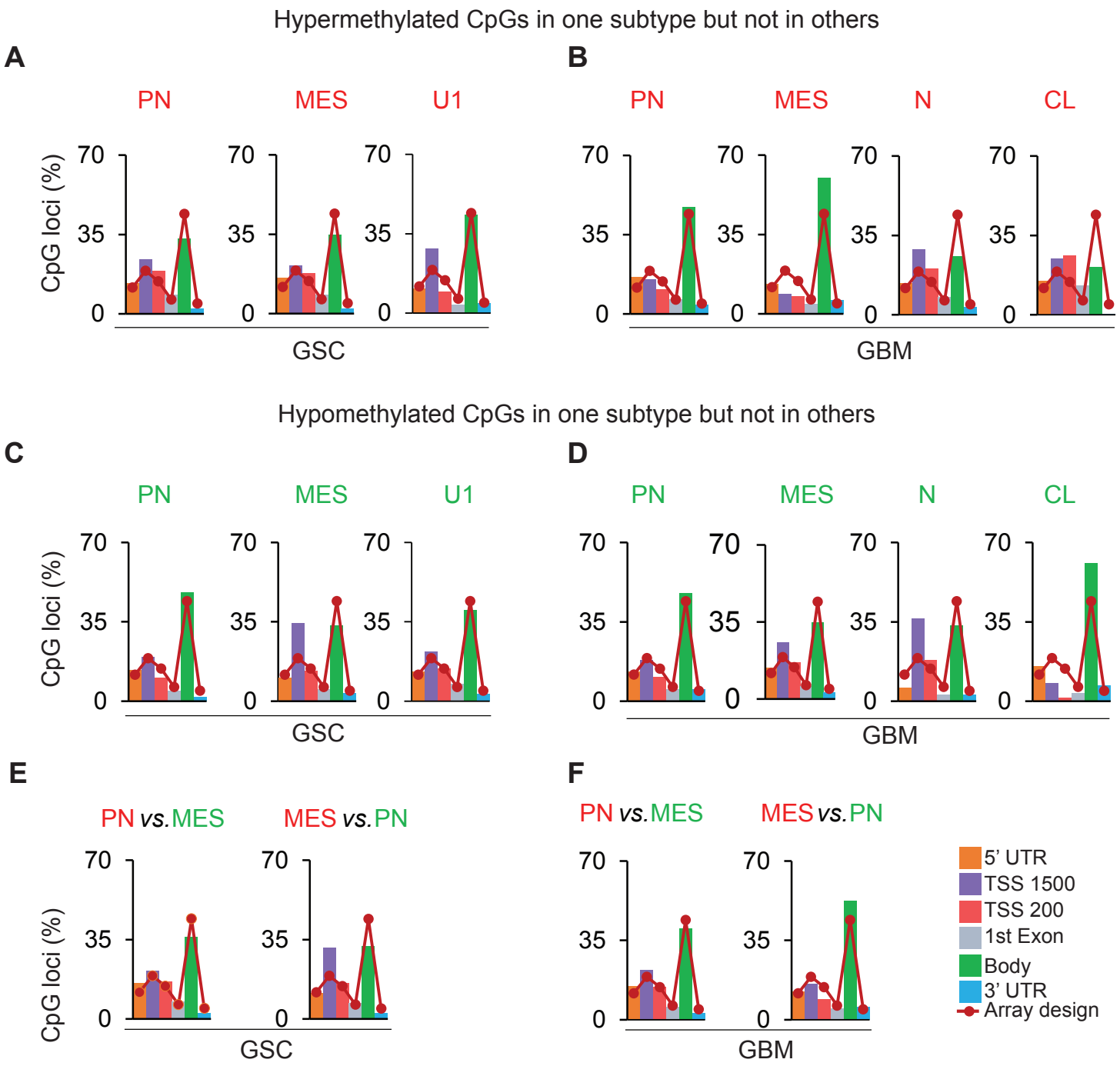
PN (n=22)	MES (n=29)	N (n=13)	CL (n=29)
TCGA-06-5416-01	TCGA-06-0152-01	TCGA-06-0171-01	TCGA-06-0125-01
TCGA-06-5417-01	TCGA-06-0152-02	TCGA-06-0171-02	TCGA-06-0125-02
TCGA-06-6389-01	TCGA-06-0190-01	TCGA-06-0221-01	TCGA-06-0211-01
TCGA-06-6391-01	TCGA-06-0190-02	TCGA-06-0221-02	TCGA-06-0211-02
TCGA-19-0957-01	TCGA-06-0210-01	TCGA-06-5411-01	TCGA-06-1804-01
TCGA-19-0957-02	TCGA-06-0210-02	TCGA-06-5413-01	TCGA-06-5408-01
TCGA-19-5956-01	TCGA-06-0650-01	TCGA-06-5859-01	TCGA-06-5414-01
TCGA-19-5960-01	TCGA-06-5412-01	TCGA-12-5295-01	TCGA-06-5415-01
TCGA-26-1442-01	TCGA-06-5418-01	TCGA-12-5301-01	TCGA-06-5856-01
TCGA-26-5133-01	TCGA-06-5858-01	TCGA-28-5204-01	TCGA-06-6390-01
TCGA-26-5134-01	TCGA-14-0736-01	TCGA-32-1980-01	TCGA-12-5299-01
TCGA-26-5135-01	TCGA-14-0736-02	TCGA-76-4927-01	TCGA-14-1402-01
TCGA-28-2510-01	TCGA-14-0781-01	TCGA-76-4929-01	TCGA-14-1402-02
TCGA-32-5222-01	TCGA-14-1034-02		TCGA-19-5950-01
TCGA-41-5651-01	TCGA-19-1389-01		TCGA-19-5951-01
TCGA-76-4925-01	TCGA-19-1389-02		TCGA-19-5952-01
TCGA-76-4932-01	TCGA-19-5947-01		TCGA-19-5954-01
TCGA-76-4934-01	TCGA-19-5955-01		TCGA-19-5958-01
TCGA-76-4935-01	TCGA-26-5136-01		TCGA-19-5959-01
TCGA-76-6191-01	TCGA-26-5139-01		TCGA-26-5132-01
TCGA-76-6192-01	TCGA-28-2501-01		TCGA-28-5219-01
TCGA-76-6285-01	TCGA-28-5207-01		TCGA-28-5220-01
	TCGA-28-5208-01		TCGA-28-6450-01
	TCGA-28-5209-01		TCGA-32-1979-01
	TCGA-28-5213-01		TCGA-76-4926-01
	TCGA-28-5214-01		TCGA-76-4928-01
	TCGA-28-5215-01		TCGA-76-4931-01
	TCGA-28-5216-01		TCGA-81-5910-01
	TCGA-28-5218-01		TCGA-87-5896-01

Supplementary Figure 2

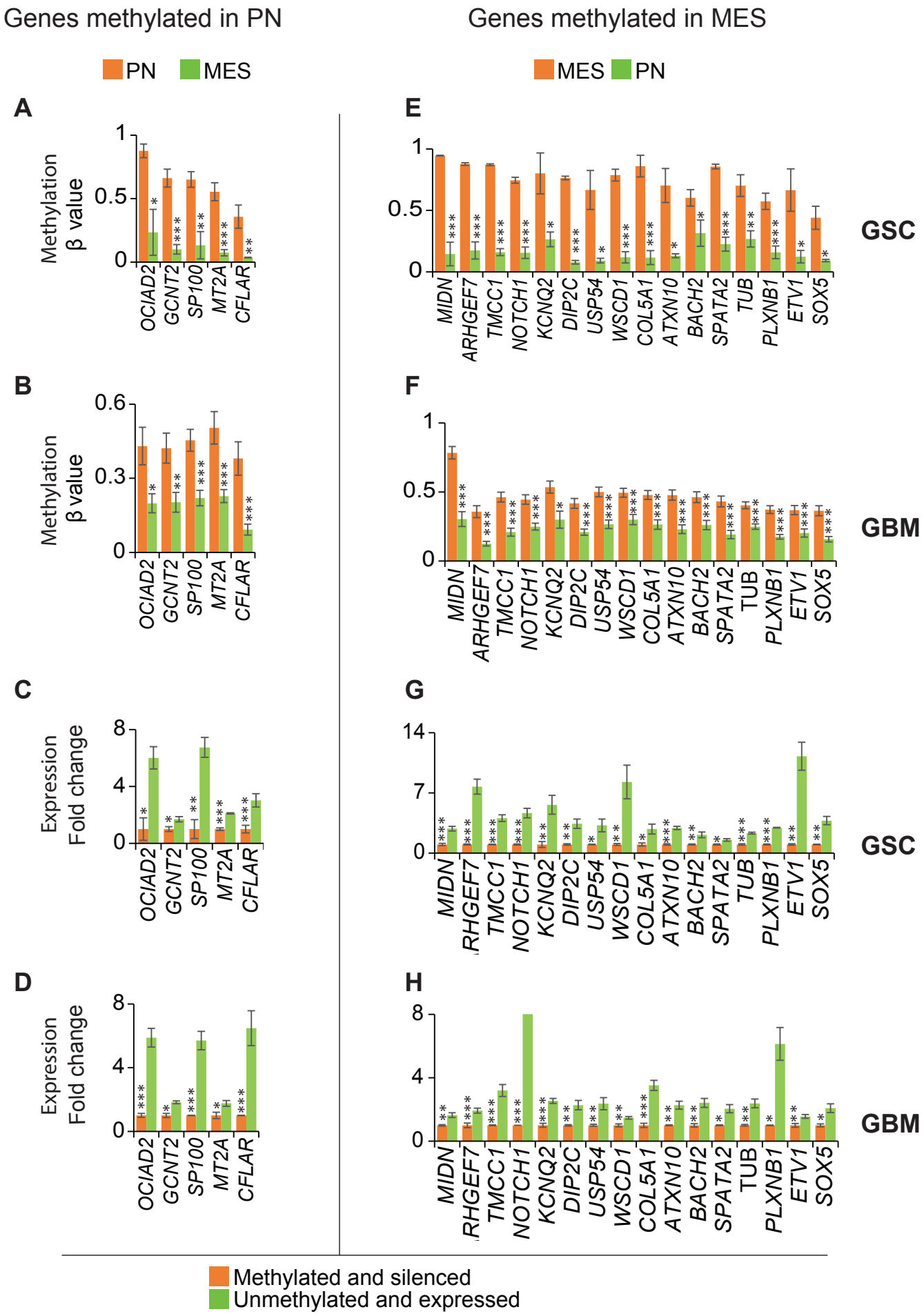




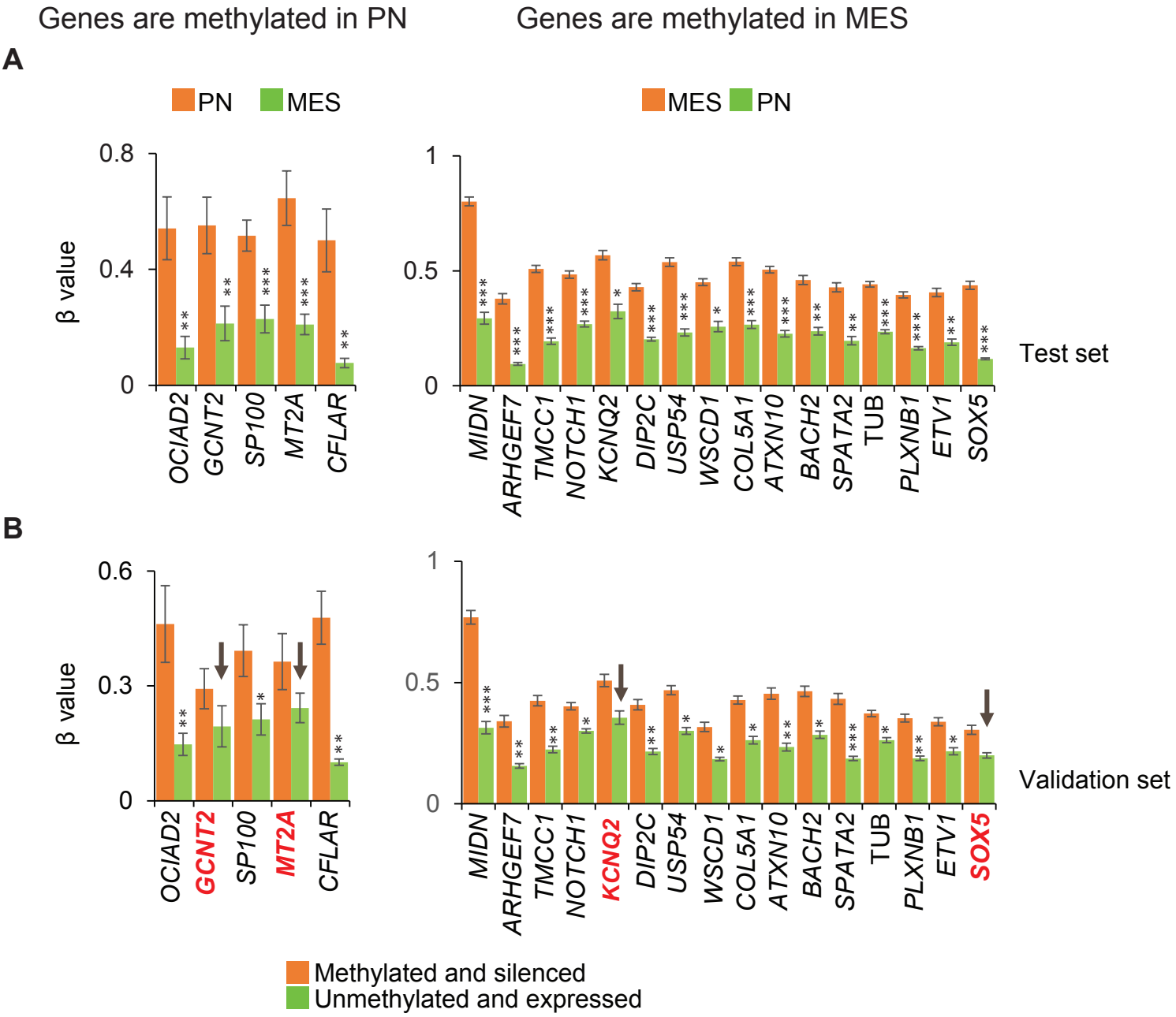
Supplementary Figure 3



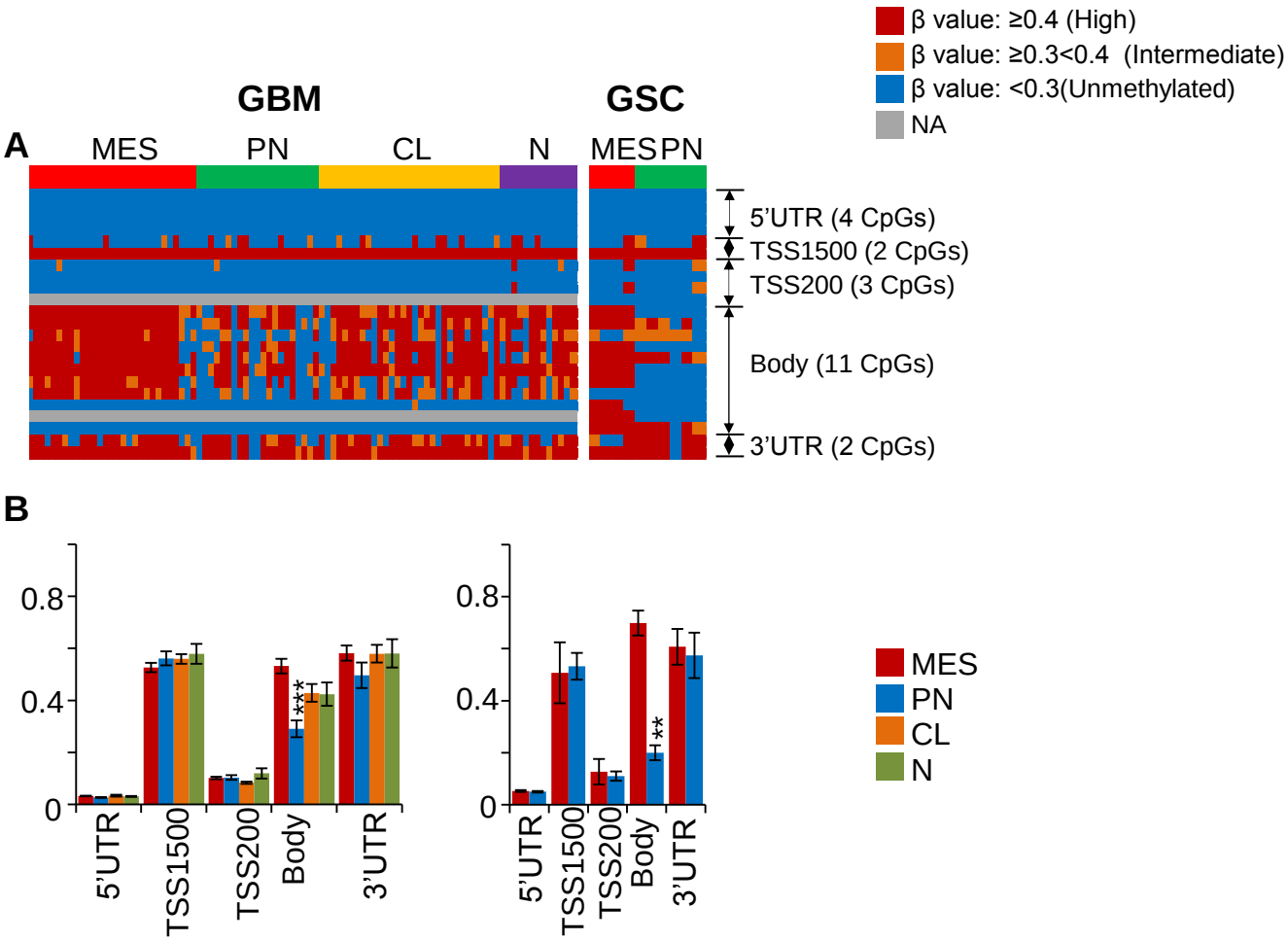
Supplementary Figure 4



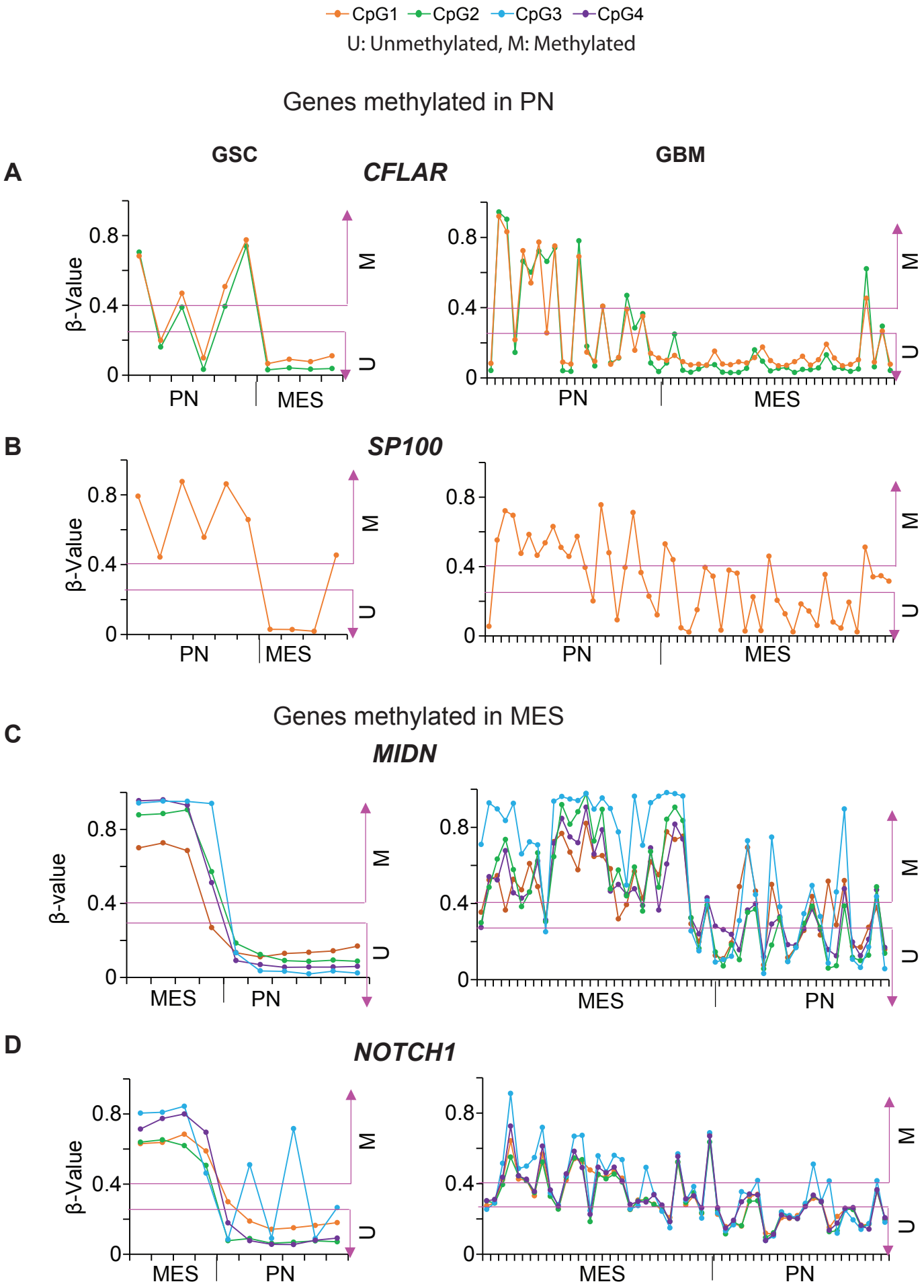
Supplementary Figure 5



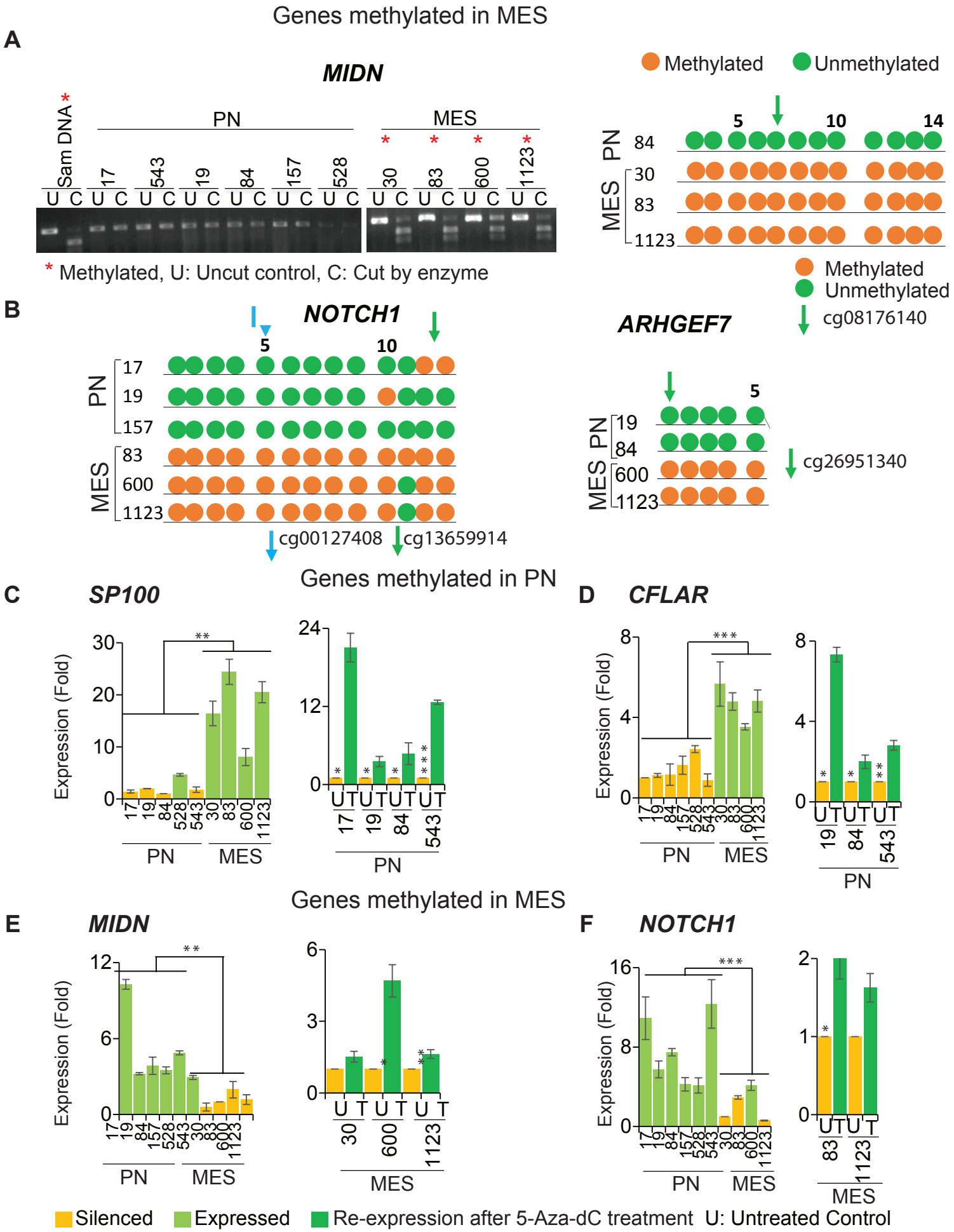
Supplementary Figure 6



Supplementary Figure 7

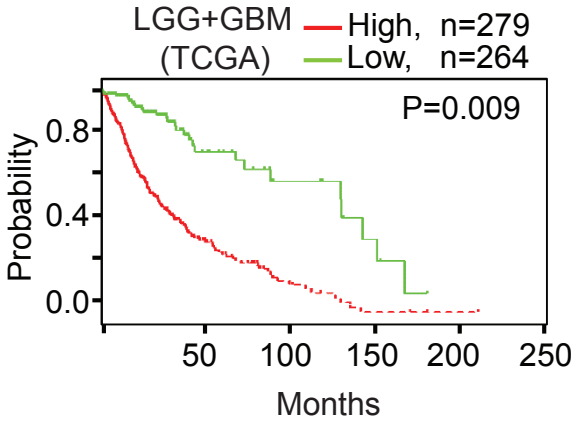


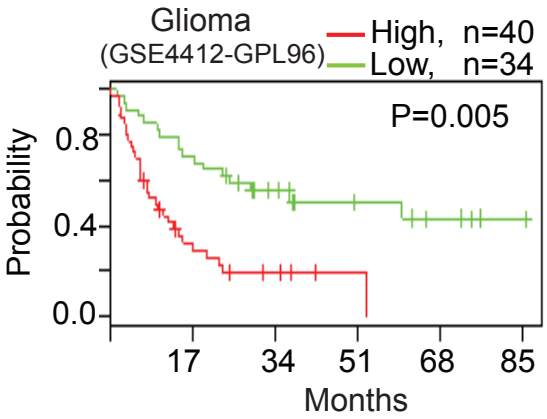
Supplementary Figure 8



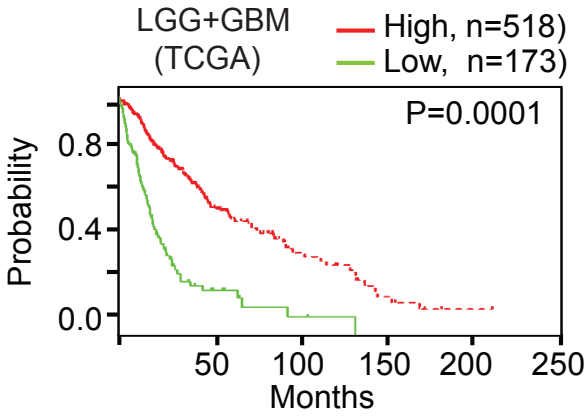
Supplementary Figure 9

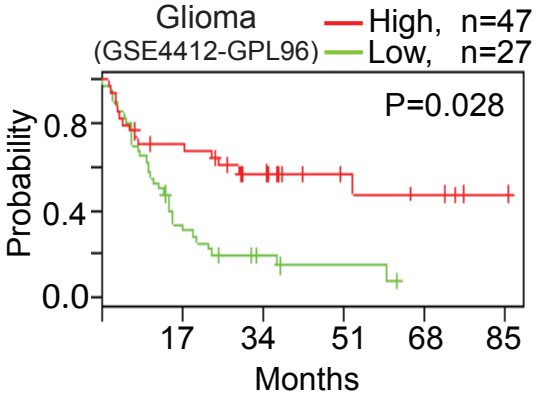
A ***SP100, methylated in PN***



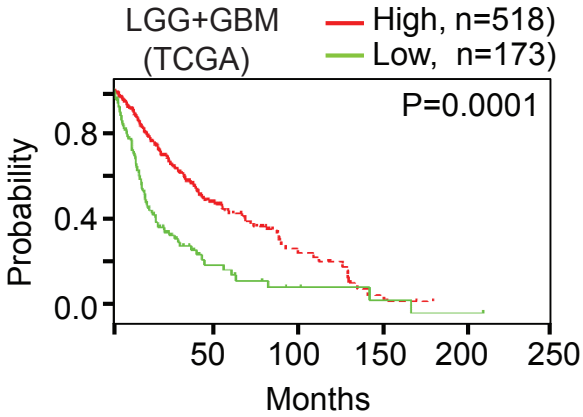


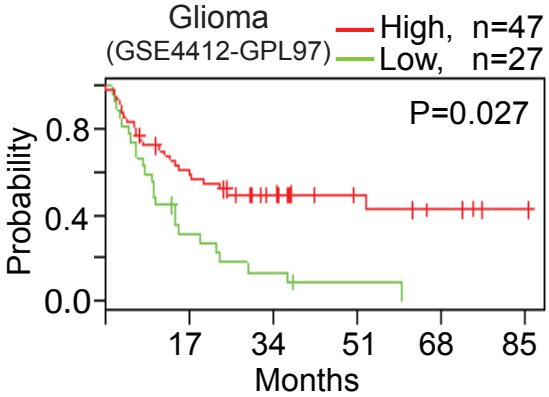
B ***ARHGEF7, methylated in MES***



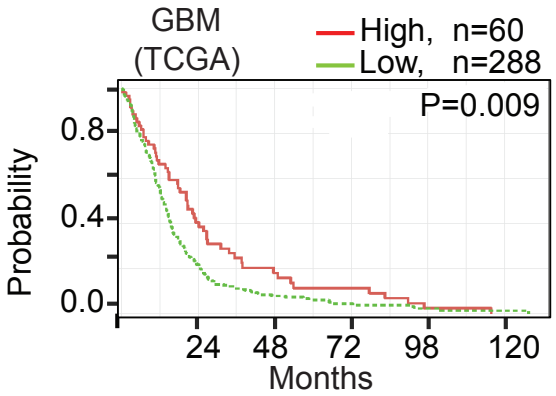


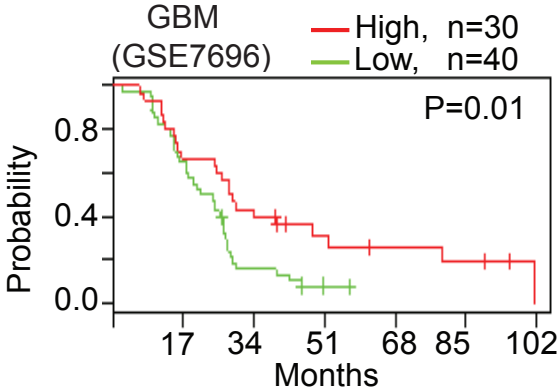
C ***NOTCH1, methylated in MES***





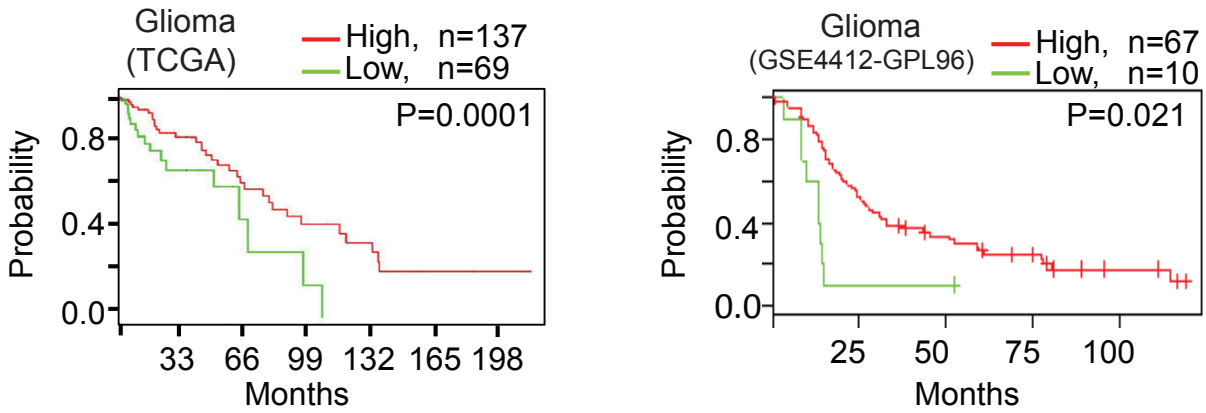
D ***MIDN, methylated in MES***



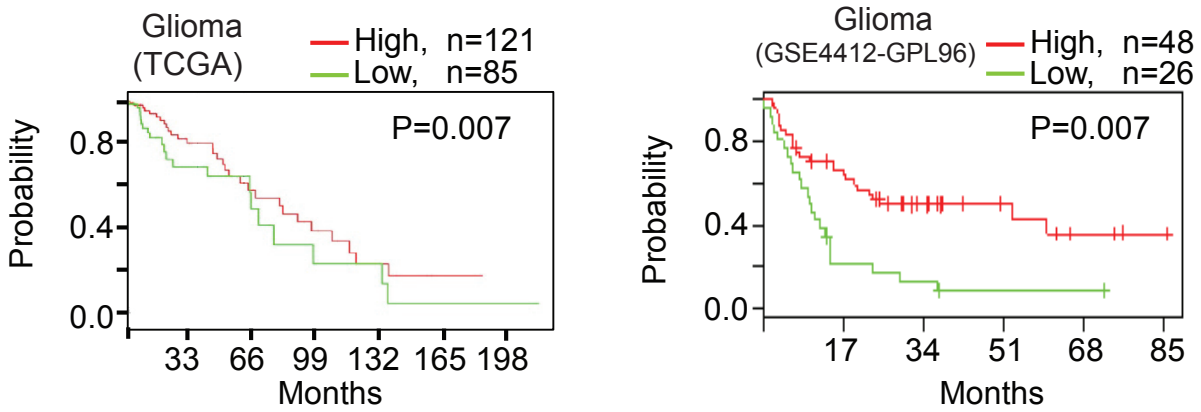


Supplementary Figure 9 continued

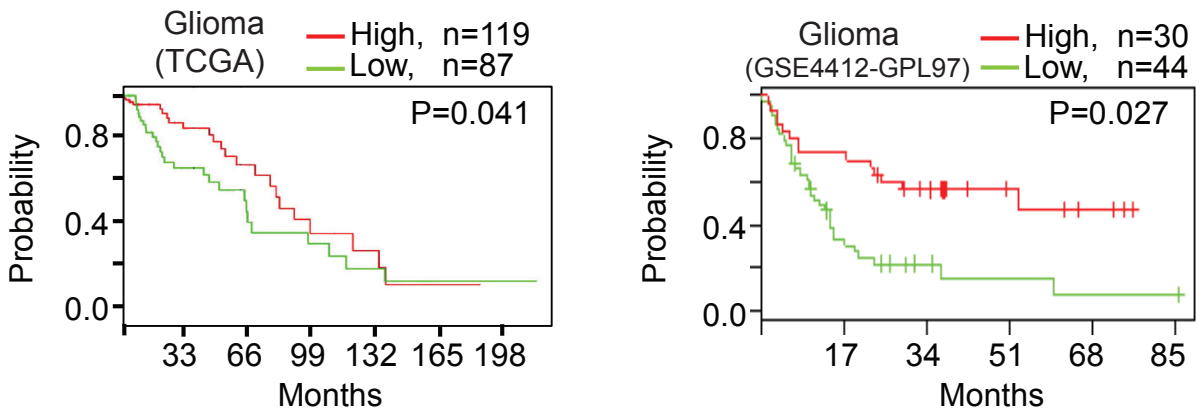
E ***KCNQ2, methylated in MES***



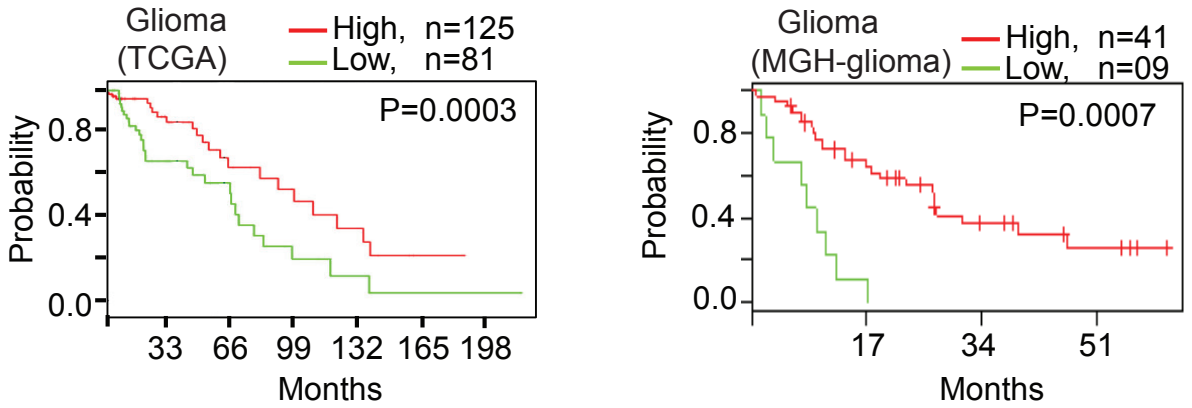
F ***ATXN10, methylated in MES***



G ***USP54, methylated in MES***

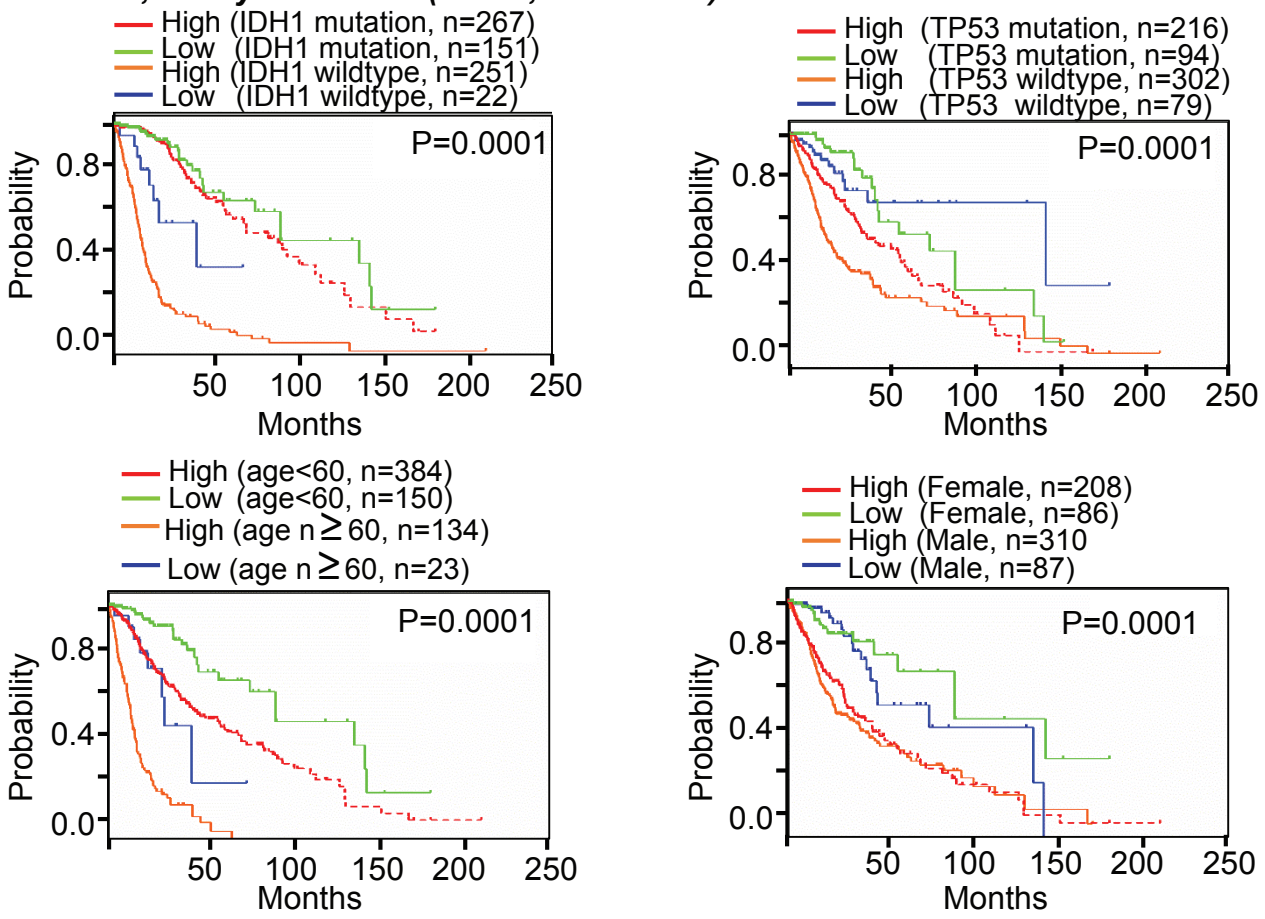


H ***TUB, methylated in MES***

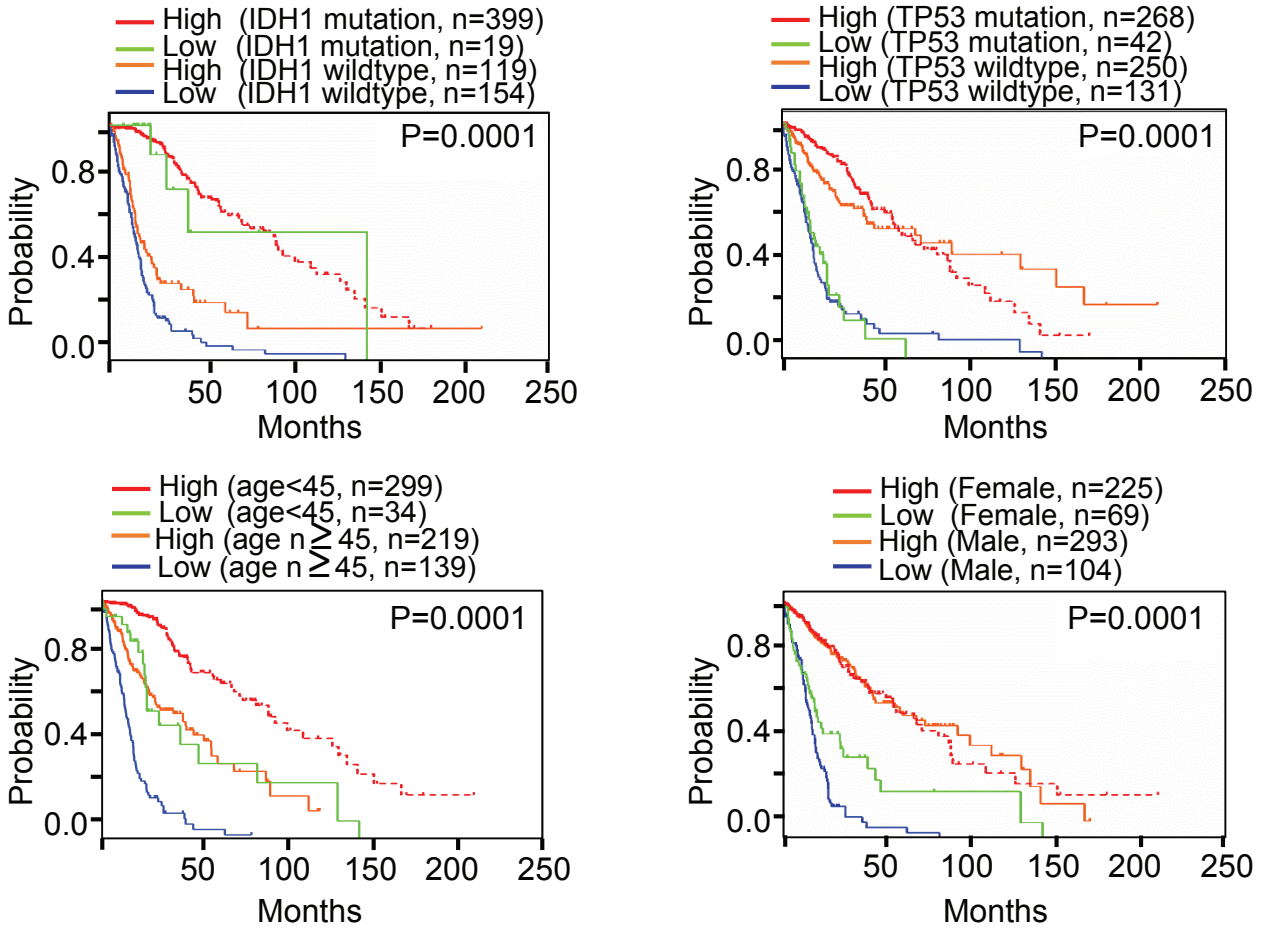


Supplementary Figure 10

A ***CFLAR, methylated in PN (TCGA, LGG+GBM)***

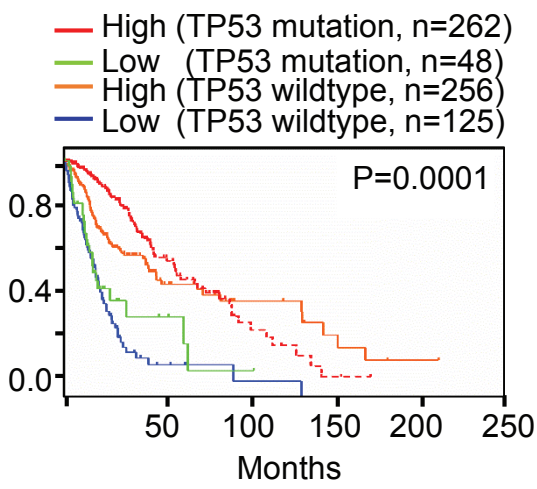


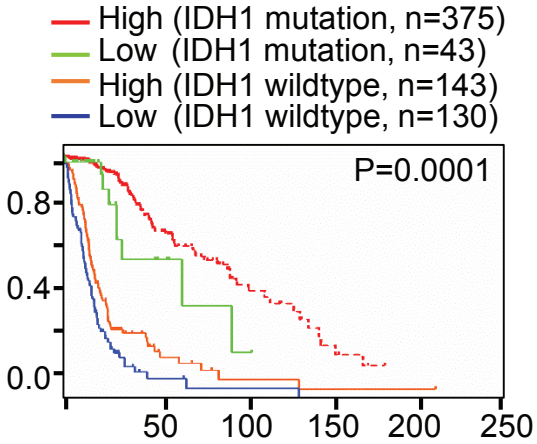
B ***TMCC1, methlyated in MES (TCGA, LGG+GBM)***



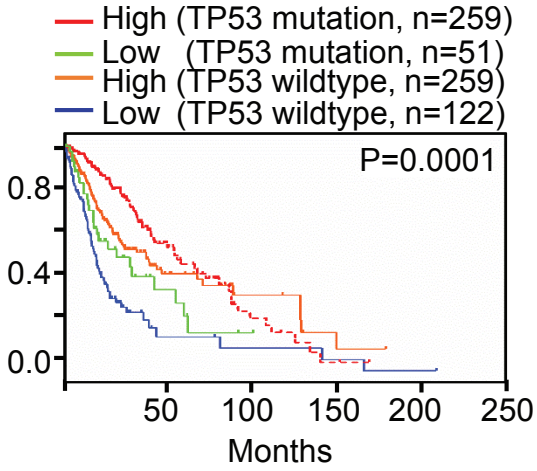
Supplementary Figure 11

A ***ARHGEF7, methylated in MES***

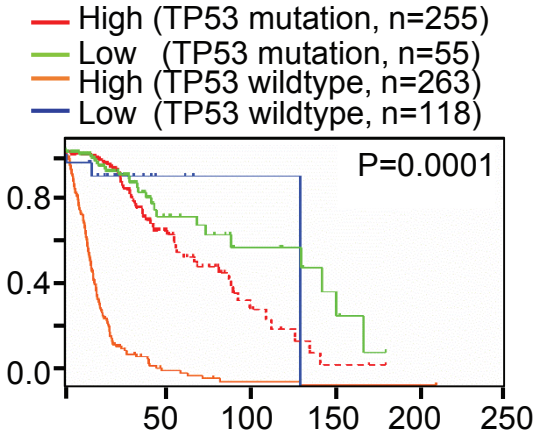


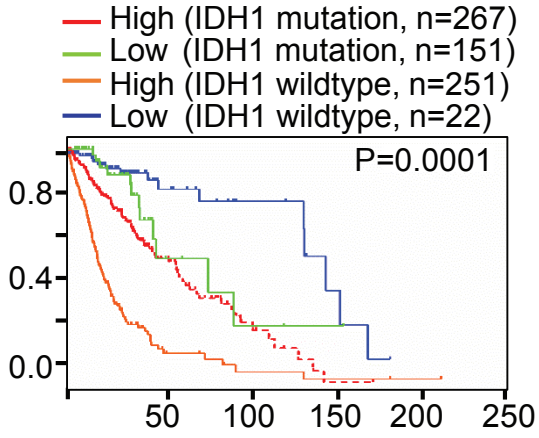


B ***NOTCH1, methylated in MES***



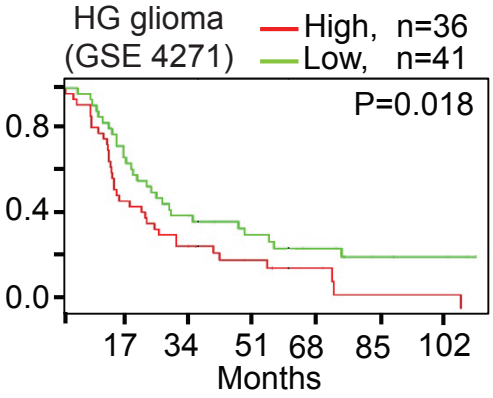
C ***SP100, methylated in PN***





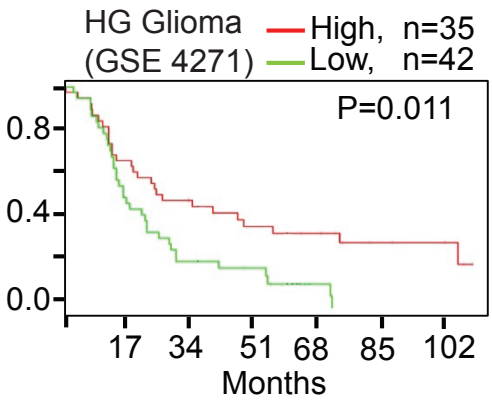
Supplementary Figure 12

A



CFLAR, SP100 and OCIAD2, methylated in PN
(Combined prognosis)

B



TMCC1, USP54,TUB, NOTCH1 methylated in MES
(Combined prognosis)

Table 1: Glioma Stem Cells (GSCs) used in 450K methylation array and expression array

GSCs	Subtypes	450K methylation array	Expression array
SYC11	NHA	YES	YES
NSC16	NSC		YES
M1123	MES	YES	YES
MD30	MES	YES	YES
TS600	MES	YES	
M83	MES	YES	YES
MD13	MES		YES
AC17	PN	YES	YES
PN528	PN	YES	YES
PN157	PN	YES	YES
PN19	PN	YES	
PN84	PN	YES	YES
TS543	PN	YES	
AC20	PN		YES
PN816	PN		YES
JK67	UNKNOWN	YES	
JK92	UNKNOWN	YES	
TS608	UNKNOWN	YES	
JK42	UNKNOWN*	YES	
JK44	UNKNOWN*	YES	
JK46	UNKNOWN*	YES	
JK59	UNKNOWN	YES	
JK83	UNKNOWN	YES	
TS576	UNKNOWN	YES	
TS586	UNKNOWN	YES	
TS603	UNKNOWN	YES	
JK34	UNKNOWN	YES	
JK16	UNKNOWN	YES	

NHA: Normal Human Astrocyles, NSC: Neural Stem Cell, PN: Proneural, MES: Mesenchymal, * :U1 group in 450K methylation clustering,

Table 4: Methylation status of the genes that are commonly dysregulated between GBM and GSCs in PN and MES subtypes.

Gene symbol	Gene name	Functions
Genes hypermethylated in PN compared to the MES Subtype		
CFLAR	CASP8 and FADD like apoptosis regulator	Regulates of cell death by inhibiting apoptosis [1]
GCNT2	Glucosaminyl (N-Acetyl) Transferase 2	Regulates the formation of blood group I antigen [2]
MT2A	Metallothionein 2A	Maintains homeostasis of metal ions in cells [3]
OCIAD2	Ovarian Carcinoma Immunoreactive Antigen-Like Protein	A modulator of gamma secretase that stimulates amyloid beta production [4]
SP100	SP100 Nuclear Antigen	Modulate replication process of DNA viruses [5]
Genes hypermethylated in MES compared to the PN Subtype		
ARHGEF7	Rho Guanine Nucleotide Exchange Factor 7	Regulates cell migration [6]
ATXN10	Ataxin 10	Inhibits apoptosis and promoters cytokinesis [7]
BACH2	BTB Domain And CNC Homolog 2	Plays roles in B cell development and apoptosis induction [8]
COL5A1	Collagen Type V alpha 1	Involved in cellular adhesion and extracellular matrix remodeling [9]
DIP2C	Disco Interacting Protein 2 Homolog C	Transcriptional factor binding [10]
ETV1	ETS Variant 1	Involved in Epithelial-Mesenchymal Transition (EMT) in pancreatic development [11]
KCNQ2	Potassium-Voltage Gated Channel Subfamily Q Member 2	Subunit of voltage-gated potassium channel in neuron [12]
MIDN	Midnolin	May be involved in regulation of neurogenesis in the nucleus [10]

NOTCH1	Notch1	Involved in Notch signaling regulating stem cells, cell proliferation, apoptosis and other processes [13]
PLXNB1	Plexin B1	A cell surface receptor regulates, angiogenesis, immune response and other cellular processes [14]
SOX5	SRY-box 5	Essential for BMP signaling and embryonic development [15]
SPATA2	Spermatogenesis Associated 2	Regulates spermatogenesis and mediates necroptosis [16]
TMCC1	Transmembrane and coiled coil domain 1	Plays roles in Endoplasmic Reticulum (ER) organization [17]
TUB	Tubby Bipartite Transcription Factor	Regulates microglial phagocytosis to maintain CNS homeostasis [18]
USP54	Ubiquitin Specific Peptidase 54	Associated with thiol-dependent ubiquitinyl hydrolase activity [10]
WSCD1	WSC Domain Containing 1	Involved in sulfotransferase activity and glucose metabolism [19]
MES specific hypermethylated gene (not methylated in other subtypes)		
AGPAT5	1-Acylglycerol-3-Phosphate O-Acyltransferase 5	Play roles in phosphatidic acid biosynthesis [20]

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Table 7: Primers used for experimental validation of methylation and expression of candidate genes

S.No.	Gene	Primer	Primer sequence
CoBRA Primers			
1	*CFLAR*	F	GGG TGT TTG GAT TTG GAT AGA AGG TT
		IF	GTG GTA YGT AGT AGA ATA AAG GTT ATT GAA ATT T
		R	CCA TTA CAT TCC AAC CTA AAC AAC AAA AAT A
2	*SP100*	F	GGT TTT GTA GGT TTT GTT GTT TGT TAG GTT
		IF	GAG AAT TTT TTG GAG TGA AAA AGG AGG AGA AAT
		R	CTT TCA TTT CAT TAT ATA ACA TCR CAT ACC TAT A
3	*WSCD1*	F	GGG GGT TTG GAA TGT TAG TTT AAA TAT TGT TT
		IF	GTA TAG AGT AGT TAT TGA GTG GTT GTA TAG GTT
		R	CTT ATA TAA CCT TCC CAA ACC TCC TA
4	*ARHGEF7*	F	GAT TGG TAG TGG AAG TTG TAA TTT ATT TGT AAA ATT
		IR	CTA AAA TAC CCA CTC CCT CTA CCA AA
		R	CAA ACA CAC AAC CAA ACC TAA CTC CCT A
5	*AGPAT5*	F	GGA AAT AGT TAT GTG TTT TAT TGA TTT TAT TGA
		IF	GTA GTT AGT TAG TTT ATT TAT TAG ATA TGG TAA GA
		R	CTA TCT CTC CCC AAT CTT TAA TTA CAC AA
6	*DIP2C*	F	GYG GGT GGT TGY GAG TTT TTA GGT T
		IF	GAA GTA GTT ATG TAG TGT TTG GTG ATT TGA TAA T
		R	CAC RCC AAC CAA AAA CCA CCA ACT A
7	*MIDN*	F	GGY GTT TGT ATA TTG GGG AYG TGT TT
		IR	CCA AAT CRA AAA CCR ATT AAA CCR AAA AAC ACT A
		R	CTA ACT CTA ACC CTA CAA CAA AAA AAC TA
8	*NOTCH1*	F	GAG TTG TGG TTA ATT TTY GTT TGA TAA TGG GGT
		IF	GTG GTT ATA GTT TAG TTT AGT TTA GTT TGT GTA T
		R	CCA AAA ACC AAA AAT CCC AAA CCA ACT AA
9	*SPATA2*	F	GTA YGG TTT GGY GTT TTA ATT TTT GGG TTG T
		IF	GGA GAA ATT AGT AGT TTT TGT YGT TGG GT
		R	CTT ACA AAA CCA TCC TAC TAC ATC TAC TA
10	*TMCC1*	F	GAT GTT TAT AGT TGG AGA AGA GAG GTA GAT AT
		IR	CCT AAA CTT CTC ACC TTC ACA ATC TCA A
		R	CCA CCA AAA AAC CRC AAT AAA ACT TCT AAT A
11	*MT2A*	F	GTA GTT AYG GTT ATG GGG GTT AGG AT
		IF	GGA ATT TAT AGT AAG GGT TGT AAG GAT AGT T
		R	CTT CCC CTA TAA AAA CTA AAA AAA AAA ACC CAA AT
Quantitative Reverse Transcription (qRT) primers			
1	*CFLAR*	F	GGA CTA TAG AGT GCT GAT GGC
		R	CAG TTG ATC TGG GGC AAC CAG
2	*SP100*	F	GCA AAG GAT GTT CAC GGA AGA C
		R	GTA CAG GGA CCA GGT TTC TAC
3	*NOTCH1*	F	GAC GTC ACC CAC GAG TGT G
		R	CAG TAC TGA CCT GTC CAC TCT G
4	*MIDN*	F	GGC TCT TCT CCA CAA AGA CAC
		R	CAG GAA GTC ACT GAC CTG CG
5	*TMCC1*	F	GCG GAT CGA ACG GTT GGA AG
		R	CGG GCT GTT TGT GCA ATC TTG