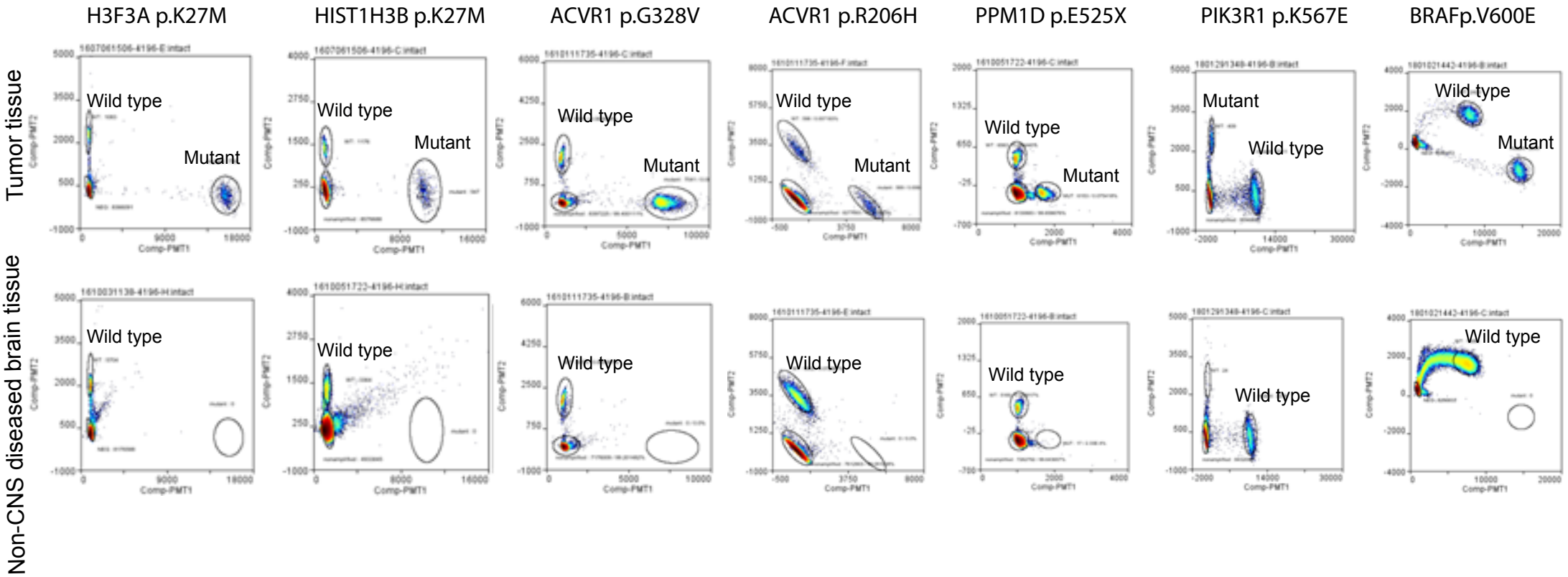
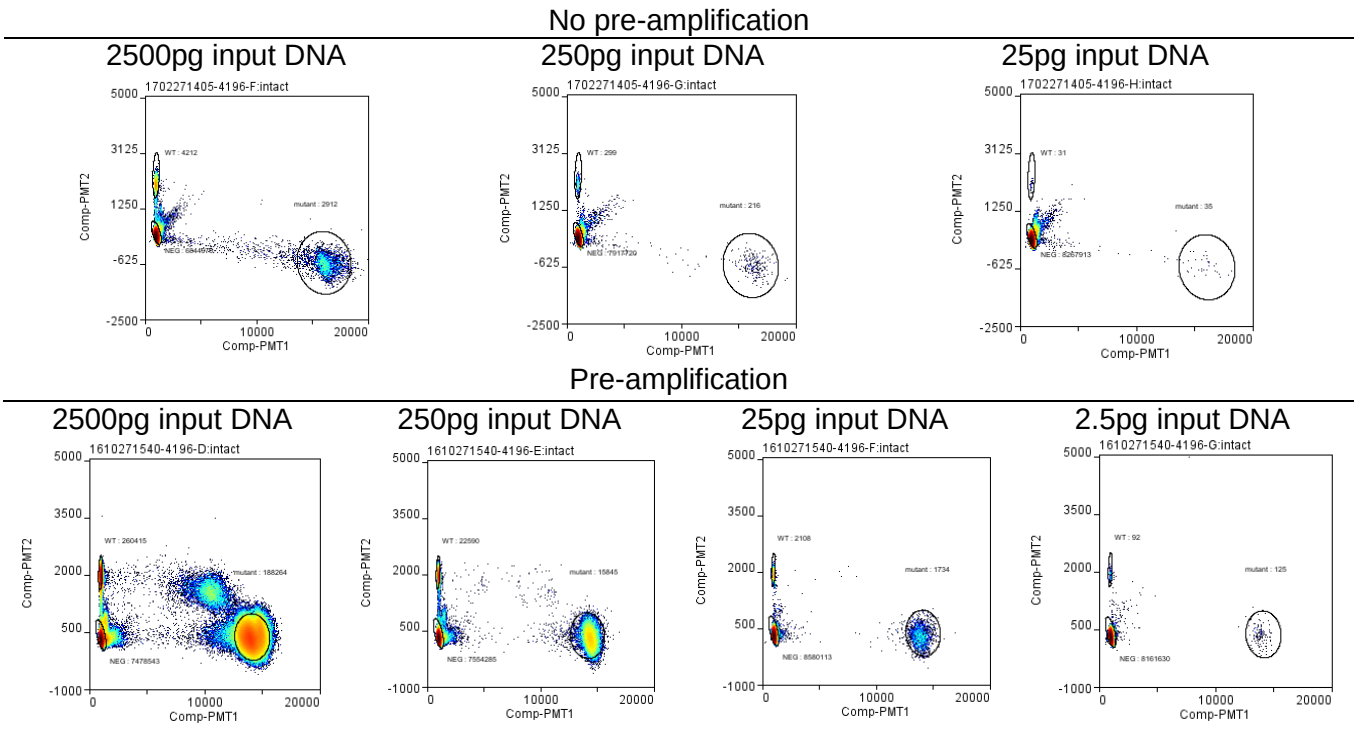
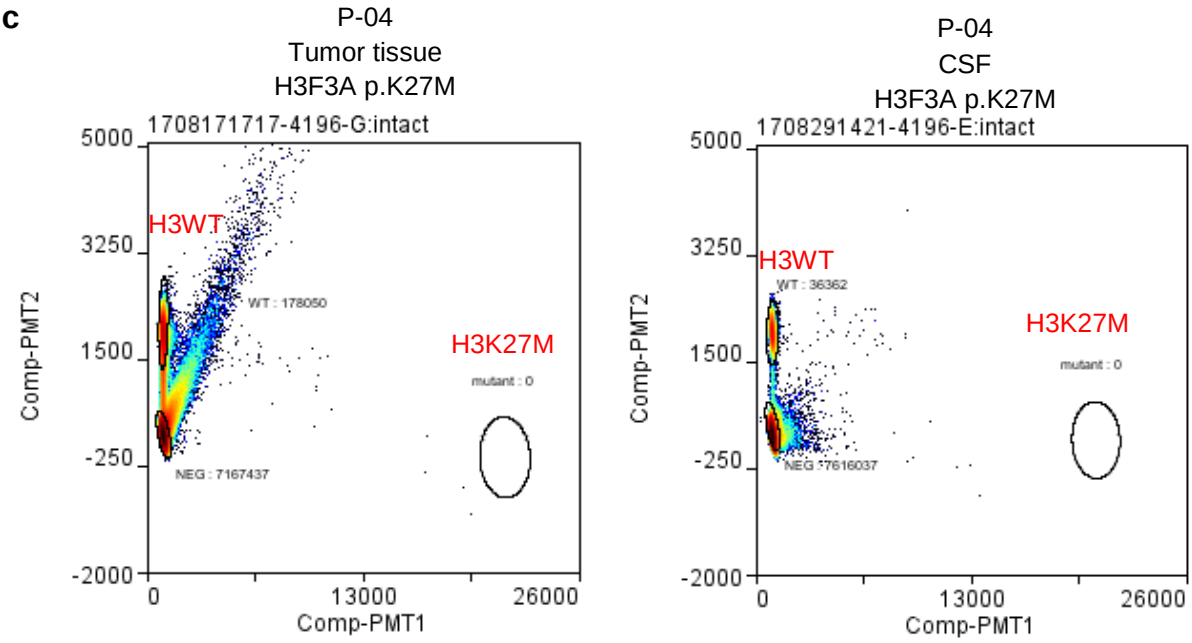
a


H3F3A p.K27M
HIST1H3B p.K27M
ACVR1 p.G328V
ACVR1 p.R206H
PPM1D p.E525X
PIK3R1 p.K567E
BRAFp.V600E
Tumor tissue
Non-CNS diseased brain tissue
Wild type
Mutant
Comp-PMT1
Comp-PMT2

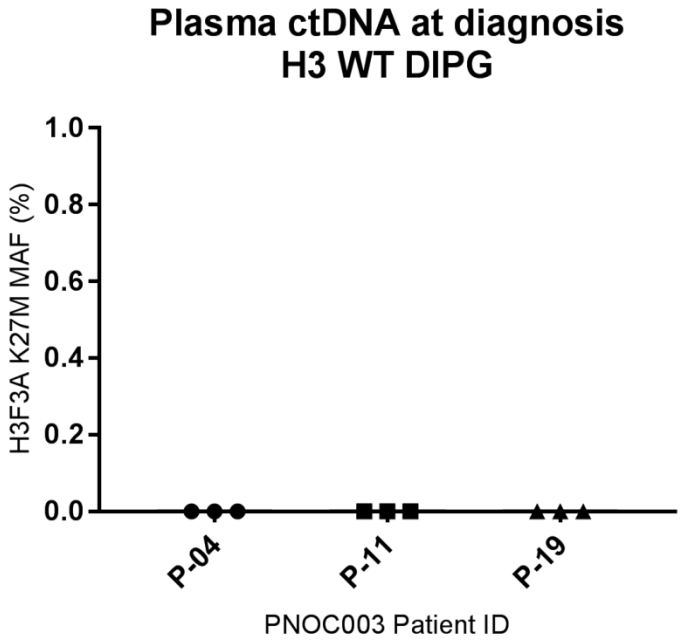
b


No pre-amplification
2500pg input DNA
250pg input DNA
25pg input DNA
1702271405-4196-F:intact
1702271405-4196-G:intact
1702271405-4196-H:intact
Comp-PMT2
Comp-PMT1
Pre-amplification
2500pg input DNA
250pg input DNA
25pg input DNA
2.5pg input DNA
1610271540-4196-D:intact
1610271540-4196-E:intact
1610271540-4196-F:intact
1610271540-4196-G:intact

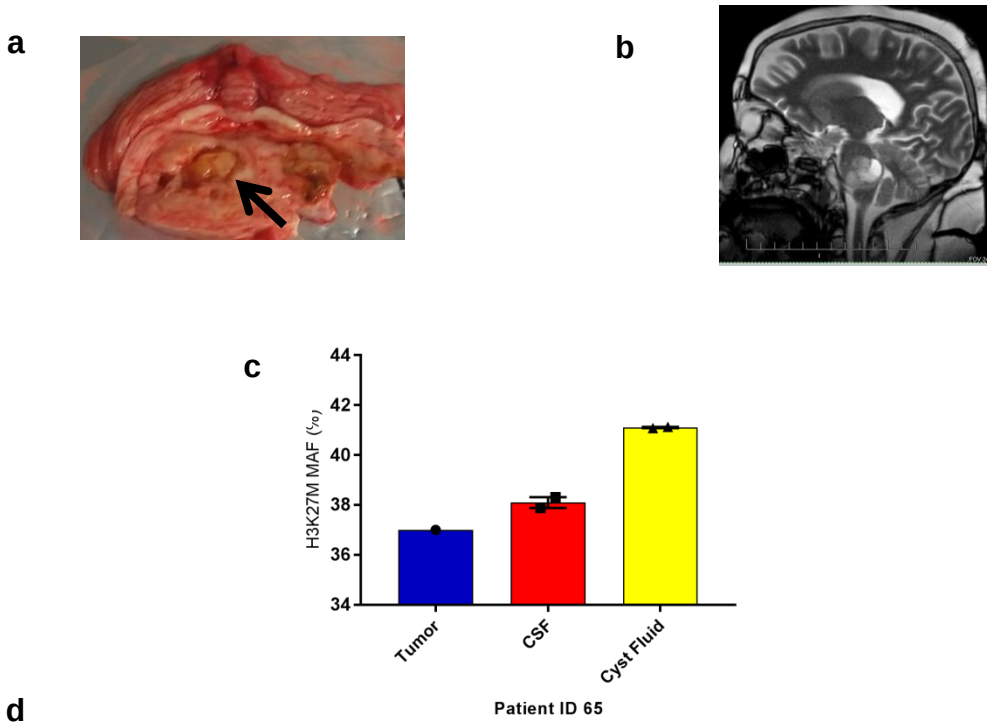
c


P-04
Tumor tissue
H3F3A p.K27M
1708171717-4196-G:intact
H3WT
H3K27M
WT : 178050
mutant : 0
NEG : 7167437
P-04
CSF
H3F3A p.K27M
1708291421-4196-E:intact
WT : 36362
NEG : 7616037
Comp-PMT2
Comp-PMT1

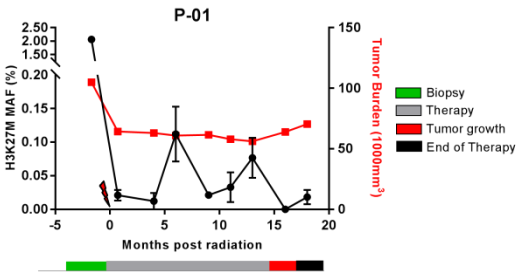
d

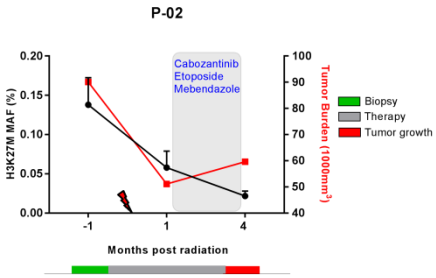


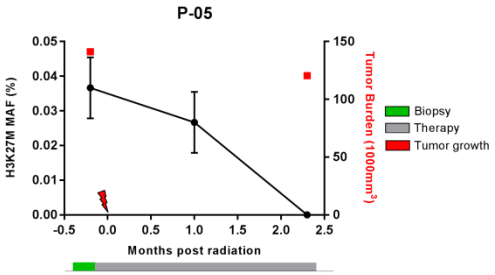
Supplementary figure 1. Genomic DNA validation of probes, assessing sensitivity, and specificity of ddPCR platform. (a) Genomic DNA validation for specific detection of mutant and wild type alleles of genes encoding driver mutations in pedatric diffuse midline gliomas. Representative ddPCR plots for mutation detection using tumor genomic DNA from seven MLG patients and two non-CNS diseased brain tissue controls. **(b)** Genomic DNA from frozen tumor tissue obtained at post-mortem, from a H3.3 K27M mutant DIPG patient was diluted and used to assess for sensitivity. **(c)** Matched tumor tissue and CSF collected at postmortem from the same Histone 3 wild type (H3 WT) DIPG patient was negative for H3F3A p.K27M. **(d)** Plasma collected at initial diagnosis was negative for *H3F3A* p.K27M in three H3 wild type DIPG patients.

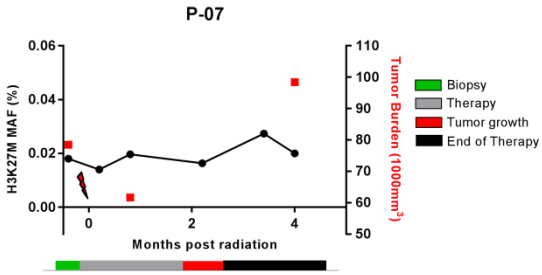


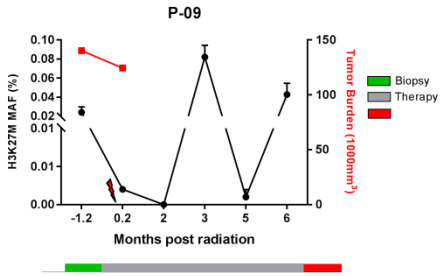
Supplementary figure 2. Novel detection of histone 3 mutation in fluid present in a brainstem tumor cyst found in a DIPG patient at postmortem. (a) Image of cyst (indicated by an arrow) in fresh tissue at postmortem. **(b)** MRI captured a month before postmortem indicating cyst in pontine tumor. **(c-d)** Higher MAF levels were found in cyst fluid (41%) compared to tumor tissue (37%), and CSF (38%) for *H3F3A* p.K27M.

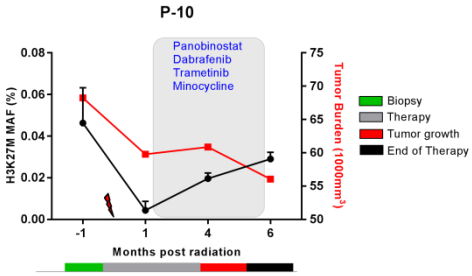

P-01
H3K27M MAF (%)
Tumor Burden (1000mm3)
Months post radiation
Biopsy
Therapy
Tumor growth
End of Therapy

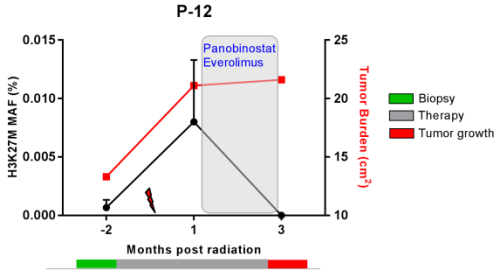

P-02
Cabozantinib
Etoposide
Mebendazole
H3K27M MAF (%)
Tumor Burden (1000mm3)
Months post radiation
Biopsy
Therapy
Tumor growth

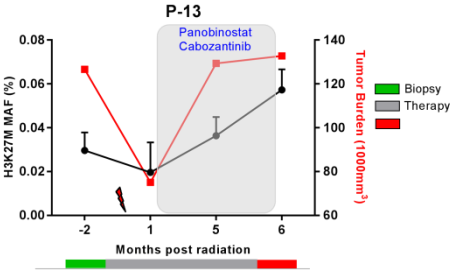

P-05
H3K27M MAF (%)
Tumor Burden (1000mm3)
Months post radiation
Biopsy
Therapy
Tumor growth

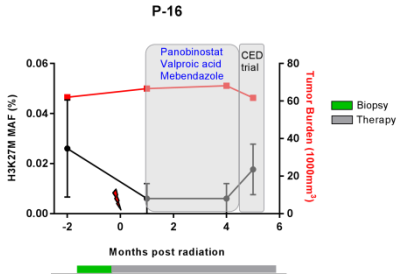

P-07
H3K27M MAF (%)
Tumor Burden (1000mm3)
Months post radiation
Biopsy
Therapy
Tumor growth
End of Therapy

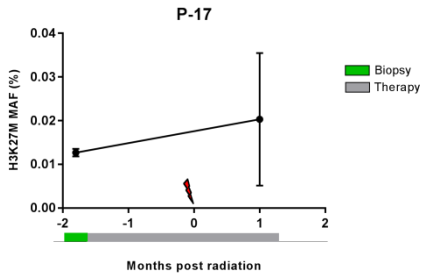

P-09
H3K27M MAF (%)
Tumor Burden (1000mm3)
Months post radiation
Biopsy
Therapy

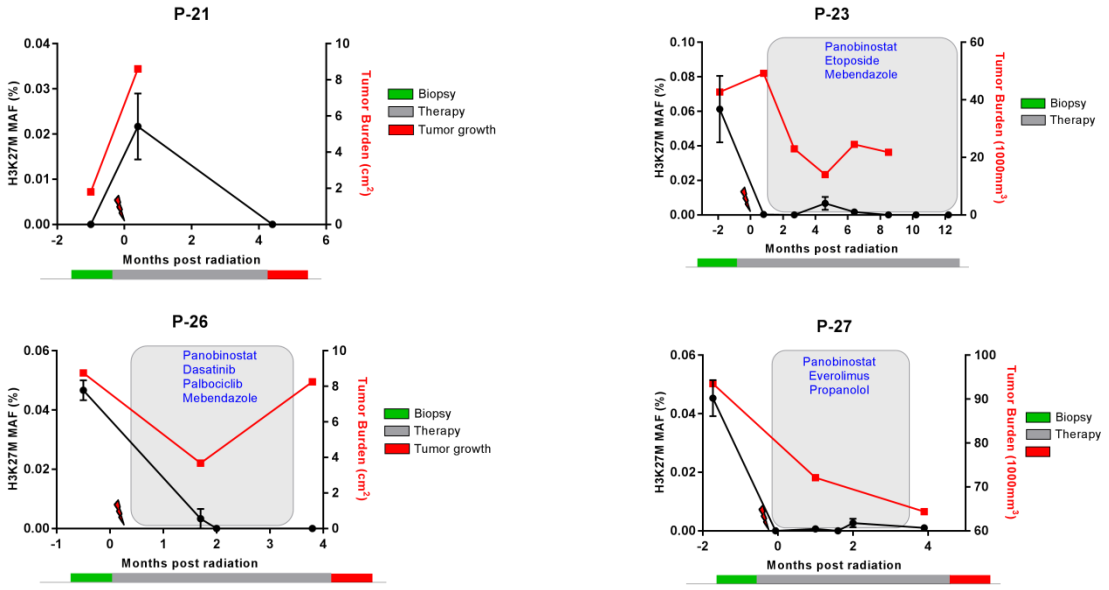

P-10
Panobinostat
Dabrafenib
Trametinib
Minocycline
H3K27M MAF (%)
Tumor Burden (1000mm3)
Months post radiation
Biopsy
Therapy
Tumor growth
End of Therapy


P-12
Panobinostat
Everolimus
H3K27M MAF (%)
Tumor Burden (cm2)
Months post radiation
Biopsy
Therapy
Tumor growth

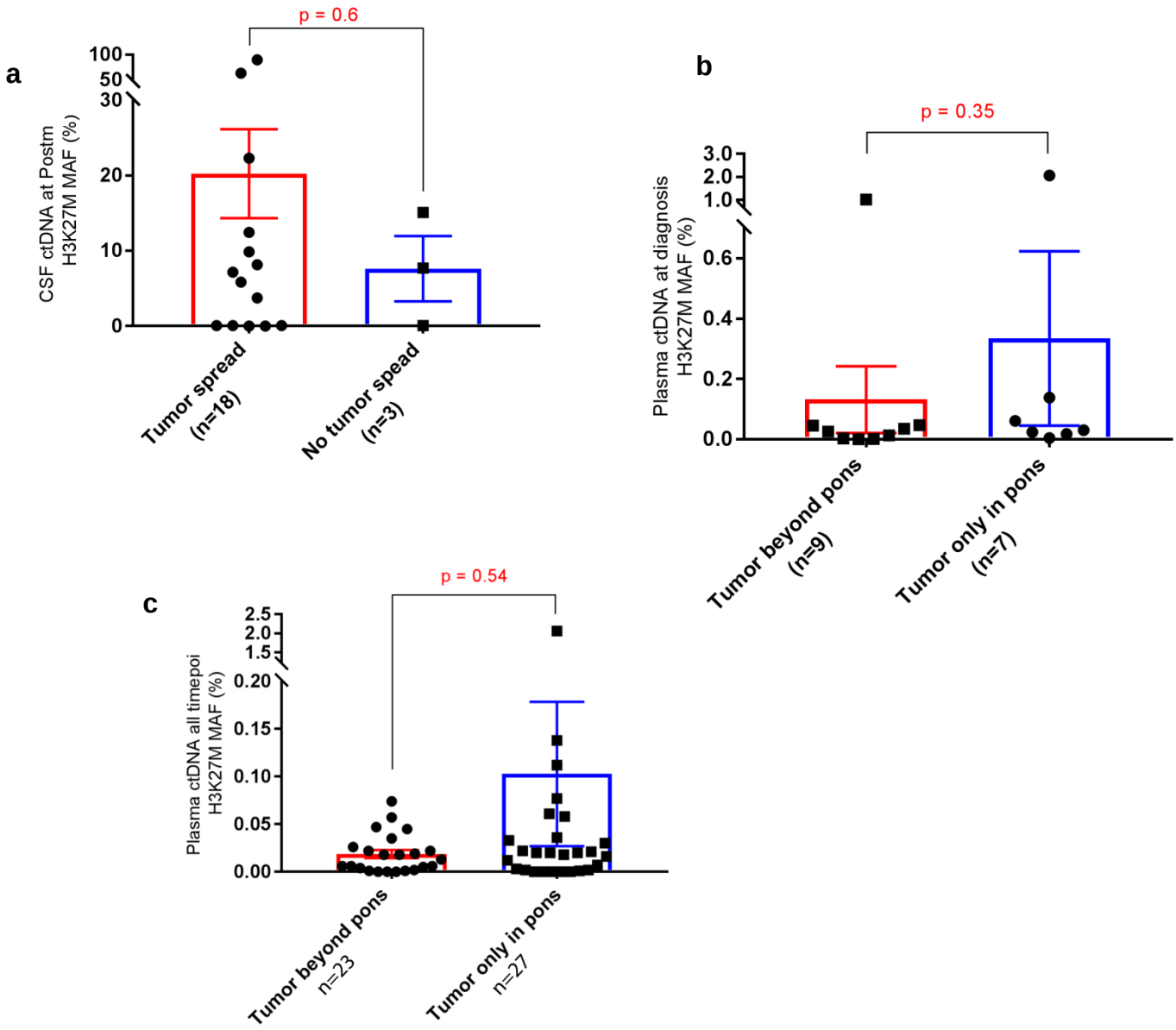

P-13
Panobinostat
Cabozantinib
H3K27M MAF (%)
Tumor Burden (1000mm3)
Months post radiation
Biopsy
Therapy


P-16
Panobinostat
Valproic acid
Mebendazole
CED
trial
H3K27M MAF (%)
Tumor Burden (1000mm3)
Months post radiation
Biopsy
Therapy


P-17
H3K27M MAF (%)
Months post radiation
Biopsy
Therapy



Supplementary figure 3. Longitudinal changes in plasma ctDNA in association with MR imaging findings and clinical assessments. Each line graph represents plasma ctDNA and MRI changes in an individual patient diagnosed with DIPG. Red line depicts MRI tumor measurements and black line depicts changes in temporal plasma ctDNA. Error bars are standard error of mean for technical triplicates of plasma ctDNA assessed for MAF of H3K27M. Colored legend at the bottom of each figure indicates the time point of plasma ctDNA and MRI assessment during course of disease: green for initial diagnosis/biopsy, grey for during therapy, red for tumor growth, and black for end of therapy following tumor growth/progression.



Supplementary figure 4. Biofluid ctDNA and tumor spread as assessed by MRI, genomic, and/or histological studies. (a) Tumor extension beyond site of primary tumor (pons or thalamus) was determined based on MRI obtained prior to postmortem, or molecular and/or histopathology of autopsied whole brain specimens. CSF ctDNA was higher in 18 MLG patients with tumor extension as compared to three MLG patients without tumor spread. **(b-c)** Tumor involvement beyond pons was assessed by MRI review of patients enrolled in PNOC003. Plasma ctDNA and MRI collected from DIPG patients were analyzed **(b)** at initial diagnosis and **(c)** at time points during course of disease (initial diagnosis, during therapy, and tumor growth). Sample sizes in **(c)** indicate plasma samples representing 13 patients. A Mann Whitney-U test was performed for all statistical analyses. Unlike in CSF, ctDNA levels in plasma were not higher in DIPG patients with tumor spread.

Gene	cDNA change	Mutation (protein)	Wild type probe	Mutant probe	Forward Primer	Reverse Primer
H3F3A	c.83A>T	K27M	/5HEX/CA+C+T+C+T+T+GC/3IABkFQ/	/56-FAM/CA+CT+C+A+T+GCG/3IABkFQ/	5'-GTACAAAGCAGACTGCCCGCAAAT-3'	5'-GTGGATACATACAAGAGAGACTTTGTCCC-3'
HIST1H3B	c.83A>T	K27M	/5HEX/T+CGC+A+A+GAG+CG/3IABkFQ/	/56-FAM/TCGC+A+T+G+AGCG/3IABkFQ/	5'-ACAGACGTCTCTGCAGGCAAGC-3'	5'-GGCGGTAACGGTGAGGCTTT-3'
ACVR1	c.983G>T	G328V	/5YakYel/CC+CAA+G+G+GAAA/3IABkFQ/	/56-FAM/AC+C+CAA+G+T+GAA/3IABkFQ/	5'-CTTTAAATCTCGATGGGCAATGG-3'	5'-ATAGCTAGTGGTCTTGCACATT-3'
ACVR1	c.617G>A	R206H	/5YakYel/CTG+G+C+GA+GCC/3IABkFQ/	/56-FAM/TC+TG+G+T+G+AGC/3IABkFQ/	5'-AATTACCGACACACTCCAACA-3'	5'-GTGGCTCTGGTCTTCCTTT-3'
PPM1D	c.1573G>T	E525X	/5YakYel/AGC+C+CA+A+G+AAA/3IABkFQ/	/56-FAM/AG+C+C+CA+A+T+AAA/3IABkFQ/	5'-AATTTGAAGATGTCAACTCCTGG-3'	5'-AATTGGACTCTTCTAATGTCCTTT-3'
PIK3R1	c.1699A>G	K567E	/5FAM/CA+T+T+G+AA+C+C/ZEN/AG/3IABkFQ/	/56-YakYellow/AGCAT+T+A+AA/ZEN/+C+C+AG/3IABkFQ/	5'-GGCAGCTGAGTATCGAGAAAT-3'	5'-TCAAGTATTGGTCTCTCGTCTTTC-3'
BRAF	c.1799T>A	V600E	/5TET/TCGAGAT+TTC+ACT+GTAGCT/3IABkFQ/	/56-FAM/TCGAGAT+TTC+TCT+GTAGCT/3IABkFQ/	5'-ACCTCAGATATATTTCTTCATG-3'	5'-CCAGACAACTGTTCAAAC-3'

Supplementary table 3. Mutations analyzed in the study by ddPCR with corresponding design of primers and probes.