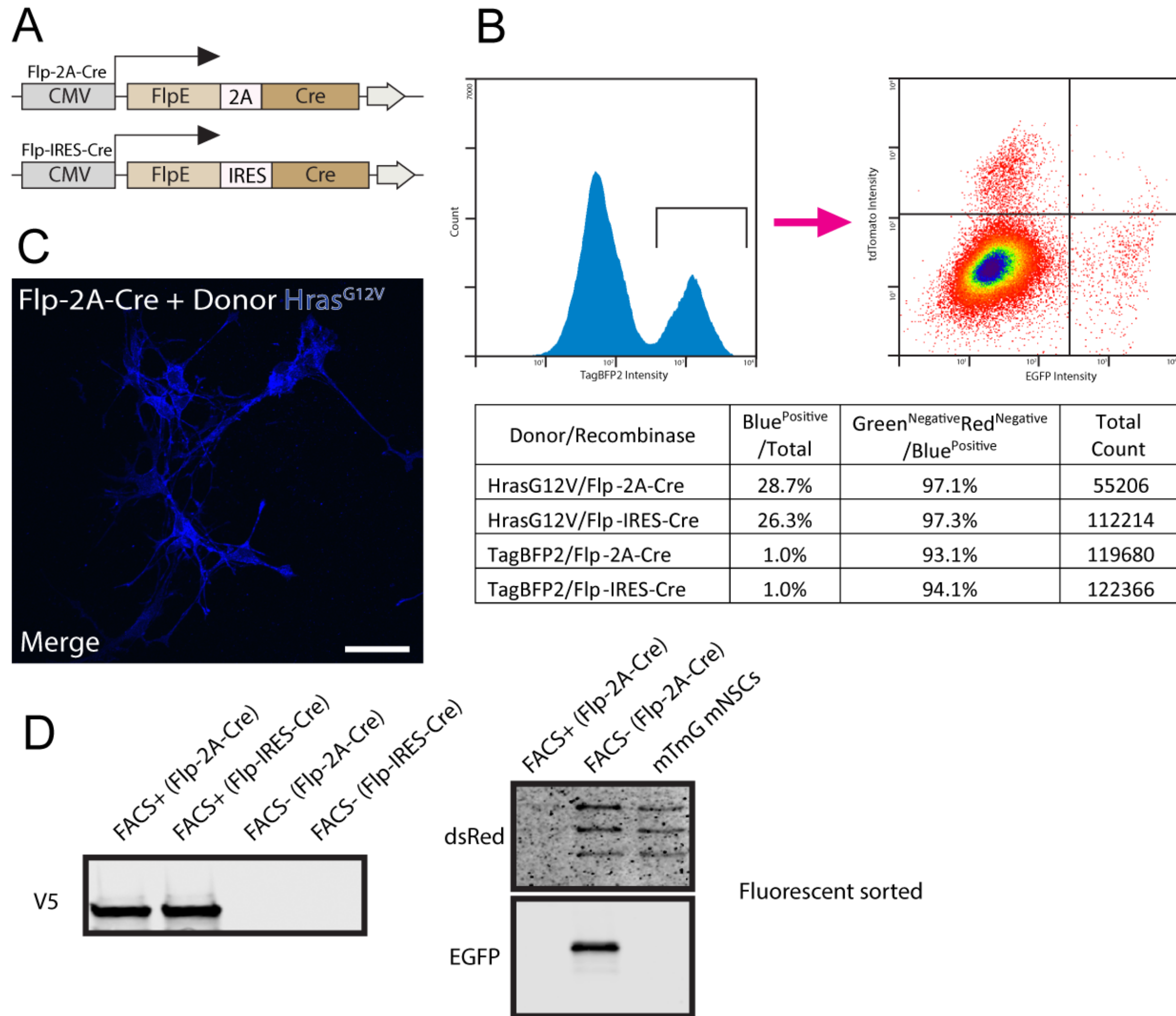


Donor/Recombinase	Blue^{Positive} /Total	Green^{Negative}Red^{Negative} /Blue^{Positive}	Total Count
HrasG12V/Flp-2A-Cre	28.7%	97.1%	55206
HrasG12V/Flp-IRES-Cre	26.3%	97.3%	112214
TagBFP2/Flp-2A-Cre	1.0%	93.1%	119680
TagBFP2/Flp-IRES-Cre	1.0%	94.1%	122366

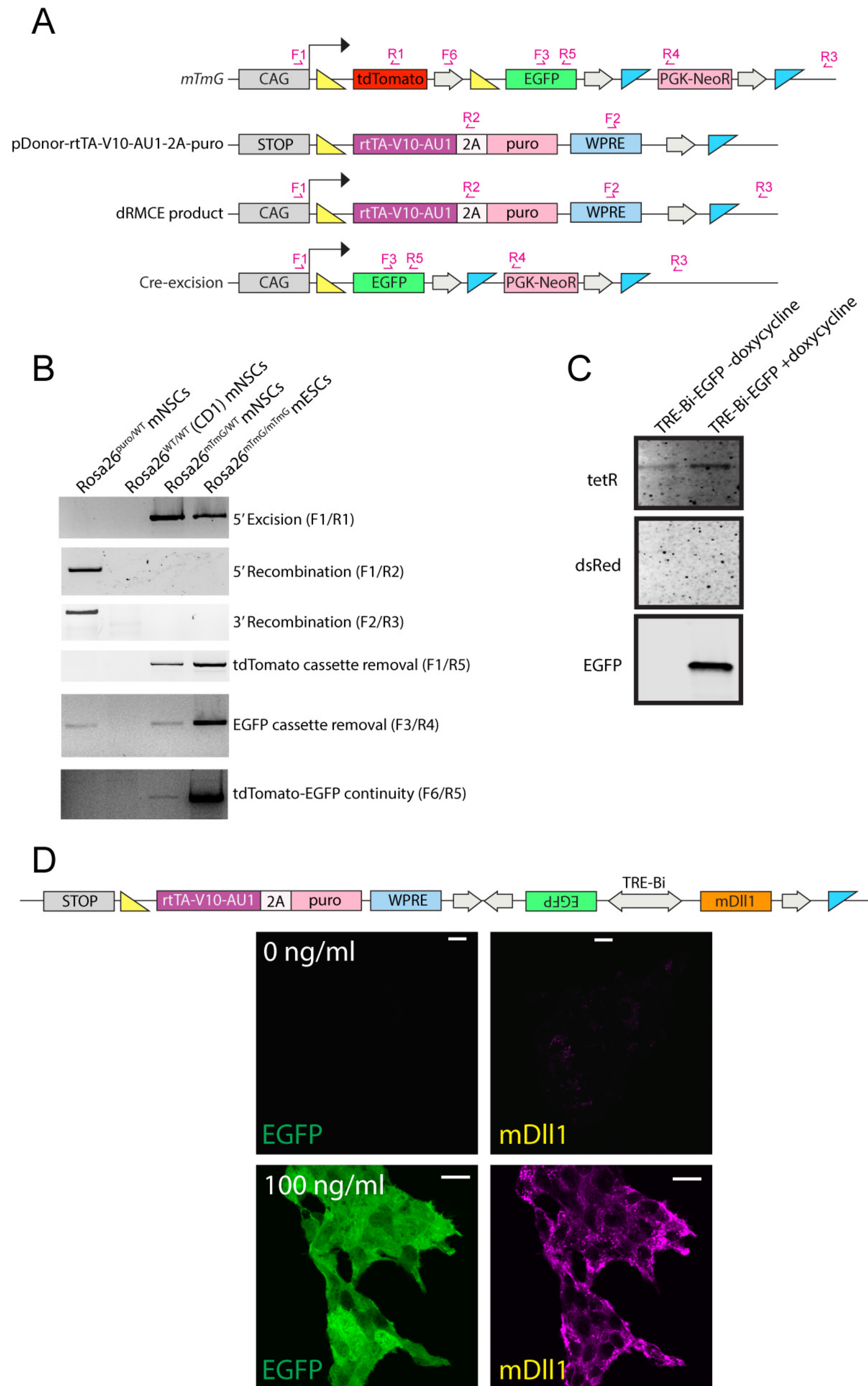
Supplementary Figure 1: Measurement of MADR efficiency in heterozygous *mTmG* mNSCs by FACS analysis, and confirmation of correct protein translation at non-clonal population level

A) Schematic of recombinase-expressing plasmids employed in this study[19].

B) FACS analysis indicates the approximate MADR efficiency in neural stem cells, and no obvious difference between Flp-2A-Cre and Flp-IRES-Cre in their catalytic efficiencies

C) Sorted cells express *Hras*^{G12V} but not tdTomato or EGFP. Scale bar: 50µm

D) Western blot indicating normal transgene production from non-clonal aggregate cells and lack thereof in FACS-negative population. Removal of tdTomato expression is also observed.



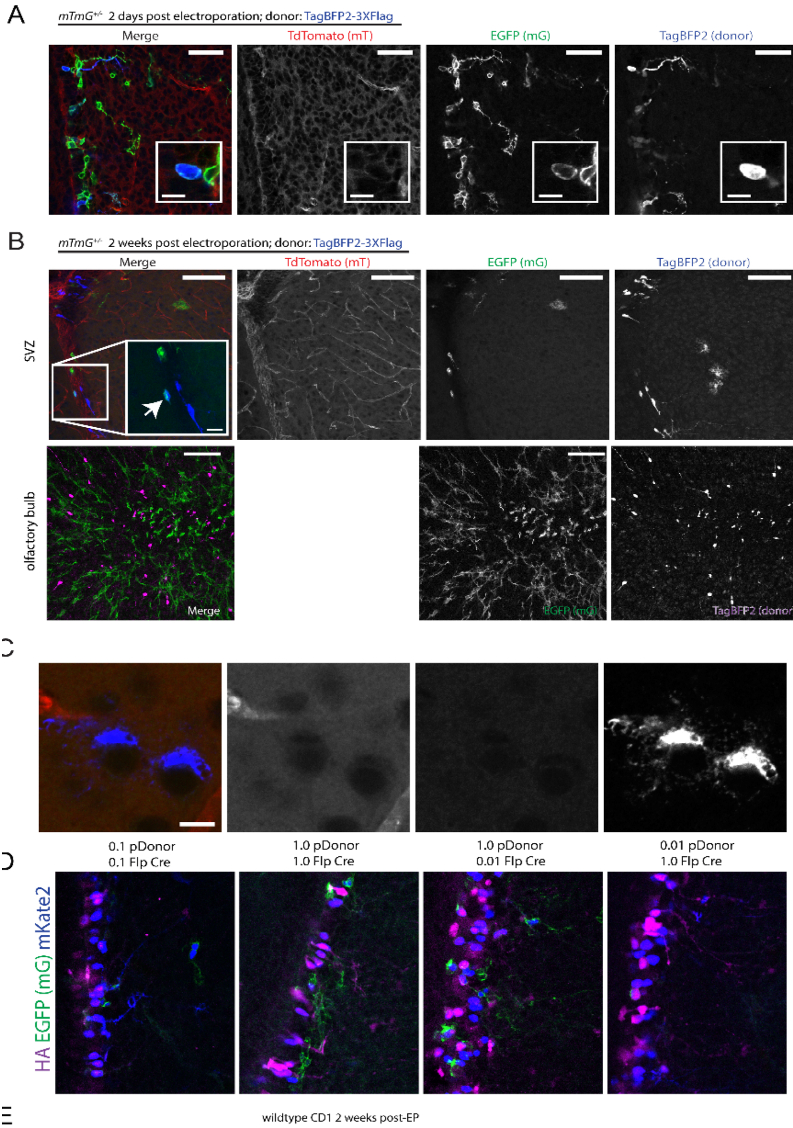
Supplementary Figure 2: PCR screening and western blot analysis confirms MADR-mediated excision of tdTomato cassette and integration of donor cassette at *Rosa26*^{mTmG} locus

A) Schematics of plasmids and alleles subject to PCR analysis at denoted sites. Primers used are listed in **Supplementary Table 1**

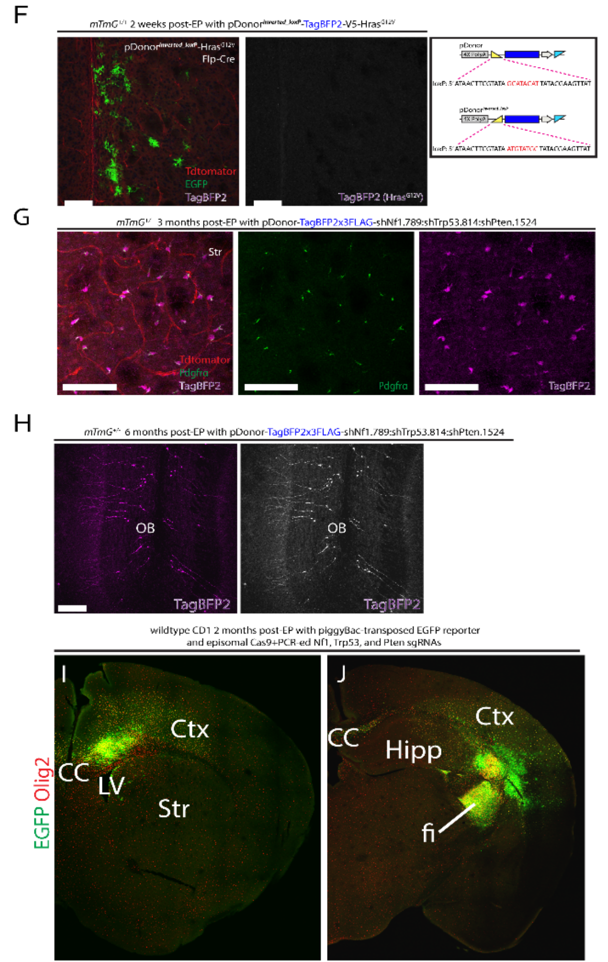
B) PCR screening analysis reveals that rtTA-V10-AU1 cassette is correctly integrated downstream of CAG-promoter in cells that are resistant to puromycin treatment

C) Western blot analysis of the cell line from **Fig. 2C** showing the expression of rtTA-V10-AU1 and also EGFP upon doxycycline induction

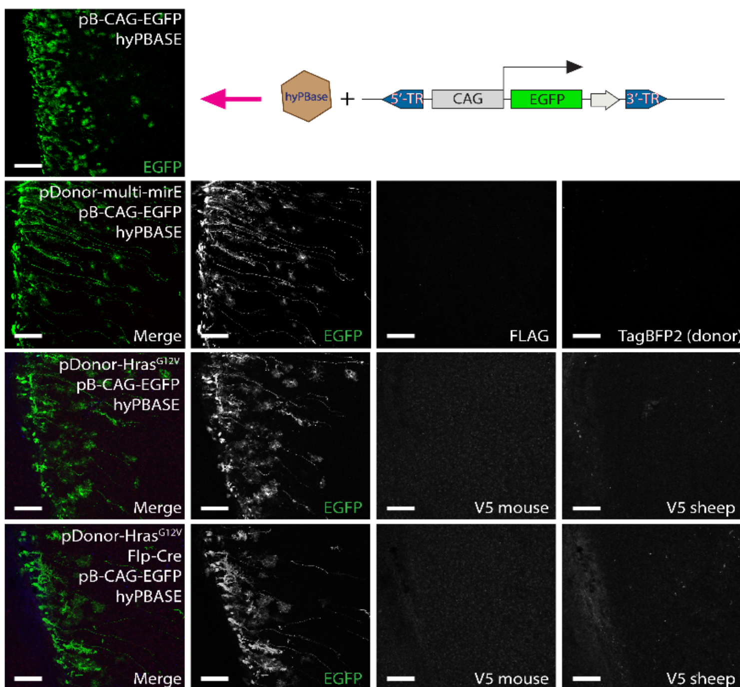
D) TRE cell line with a bidirectional tet-response element that expresses EGFP and Dll1 upon doxycycline treatment without leakiness or wide expression level distribution. Scale bars: 20µm



A
mTmG+/- 2 days post electroporation; donor: TagBFP2-3XFlag
Merge
TdTomato (mT)
EGFP (mG)
TagBFP2 (donor)
B
mTmG+/- 2 weeks post electroporation; donor: TagBFP2-3XFlag
Merge
TdTomato (mT)
EGFP (mG)
TagBFP2 (donor)
SVZ
olfactory bulb
Merge
EGFP (mG)
TagBFP2 (donor)
C
D
0.1 pDonor
0.1 Flp Cre
1.0 pDonor
1.0 Flp Cre
1.0 pDonor
0.01 Flp Cre
0.01 pDonor
1.0 Flp Cre
HA EGFP (mG) mKate2



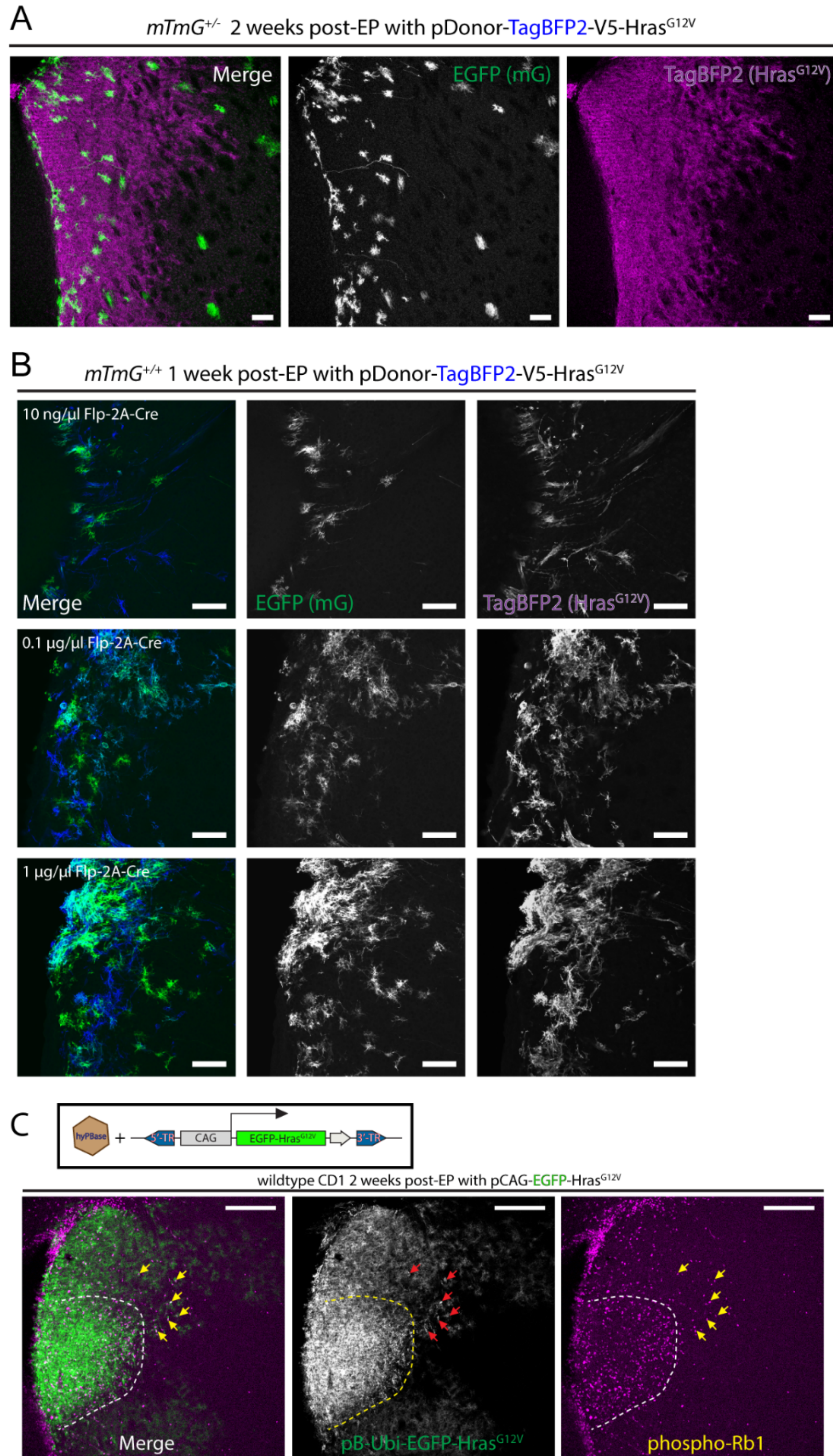
F
Tdtomato
EGFP
TagBFP2
G
mTmG+/- 3 months post-EP with pDonor-TagBFP2x3FLAG-shNf1.789:shTrp53.814:shPten.1524
Str
Tdtomato
Pdgfra
TagBFP2
Pdgfra
TagBFP2
H
mTmG+/- 6 months post-EP with pDonor-TagBFP2x3FLAG-shNf1.789:shTrp53.814:shPten.1524
OB
OB
TagBFP2
TagBFP2
wildtype CD1 2 months post-EP with piggyBac-transposed EGFP reporter
and episomal Cas9+PCR-ed Nf1, Trp53, and Pten sgRNAs
I
J
EGFP Olig2
Ctx
CC
LV
Str
Ctx
CC
Hipp
fi



E
wildtype CD1 2 weeks post-EP
pB-CAG-EGFP
hyPBASE
EGFP
hyPBase
5'-TR
CAG
EGFP
3'-TR
pDonor-multi-mirE
pB-CAG-EGFP
hyPBASE
Merge
EGFP
FLAG
TagBFP2 (donor)
pDonor-Hras^{G12V}
pB-CAG-EGFP
hyPBASE
Merge
EGFP
V5 mouse
V5 sheep
pDonor-Hras^{G12V}
Flp-Cre
pB-CAG-EGFP
hyPBASE
Merge
EGFP
V5 mouse
V5 sheep

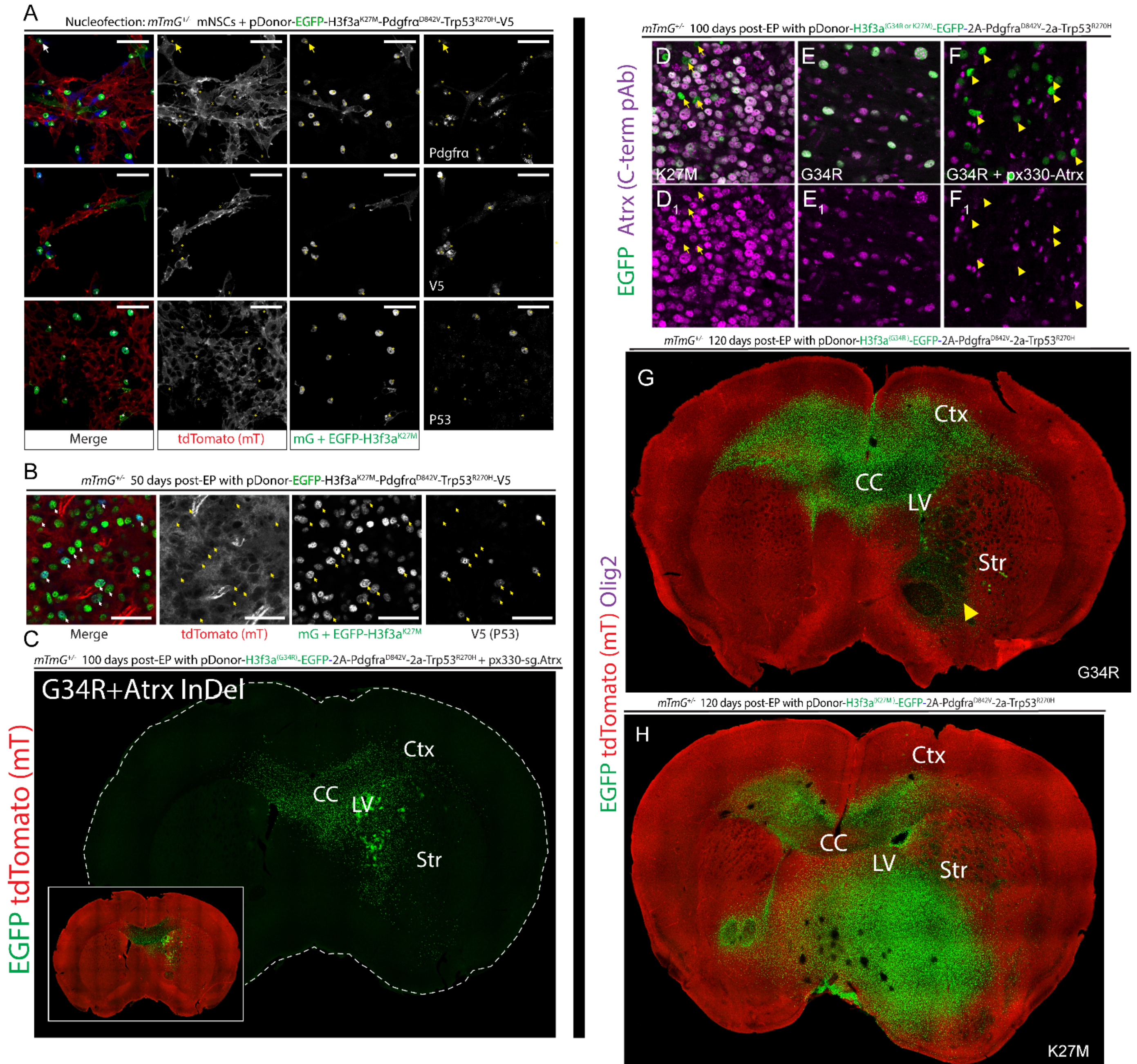
Supplementary Figure 3: Characterization of *in vivo* MADR and control experiments confirming specificity of integration

A) At 2 days post-EP, cells start expressing TagBFP2. Scale bars: 50µm; Insets: 10µm
B) Gliogenesis and radial glia 2 weeks post-EP. Arrow indicates rare green-and-blue double positive cells at the VZ. Neurons with both markers can be observed in the OB at this time point. Scale bars: 100µm; Inset: 20µm
C) High-magnification confocal image of a pair of TagBFP2+ satellite glia, which are negative for tdTomato and EGFP. Scale bars: 10µm
D) Representative images of SM_FP-HA (donor), EGFP (mG), and TagBFP2-nls (blue) from VZ of the plasmid titration quantitations depicted in **Fig. 3C**.
E) Lineage tracing of EP-ed cells in the VZ/SVZ with hyPBase-integrated EGFP reporter plus various donor vectors and recombinases do not show any integration by 2 weeks post-EP. Scale bars: 100µm
F) Donor vector with inverted loxP orientation fails to express $Hras^{G12V}$ and does not produce hyperplasia. (For comparison of integrated plasmid at same time point, see **Supplementary Fig. 4A**.) Scale bars: 100µm
G) At 3 months post-EP, cells expressing multi-miR-E tied to TagBFP2 reporter are predominantly Pdgfra+ OPCs. Scale bars: 100µm
H) TagBFP2+ neurons in the olfactory bulb of multi-miR-E MADR mice.
I-J) Episomal Cas9-mediated multiplex mutation of Nf1, Trp53, and Pten yield transformation of piggyBac-transposed EGFP+ cells into Olig2+ tumors localized near white matter tracts.



Supplementary Figure 4: Examination of *Hras^{G12V}*-recombined *mTmG* cells in the striatum

A) Two weeks post-EP shows clear lineage divergence between EGFP+ cells that underwent Cre-mediated excision of tdTomato cassette and Hras^{G12V}+ cells with successful MADR. Scale bars: 100µm
B) As low as 10 ng/µl recombinase-expression vector in EP mixture can catalyze MADR *in vivo*. Scale bars: 100µm
C) Brighter *EGPF-Hras^{G12V}* cells after pBase-mediated integration express phosphorylated Rb1. Scale bars: 200µm



Supplementary Figure 5: Characterization of multi-cistronic tumors.

A) In vitro assessment of transgene expression after MADR in heterozygous mTmG mNSCs shows the co-expression of nuclear EGFP with Pdgfra, V5 ($Trp53^{R270H}$), and P53. Note the presence of contaminating mG cells with membrane EGFP and no tdTomato or transgene expression. Scale bars: 50µm

B) Confirmation of Trp53 co-expression with nuclear EGFP (H3f3a). Scale bars: 50µm

C) Combined expression of MADR G34R/Pdgfra/Trp53 and a plasmid inducing CRISPR/Cas9-targeting of Atrx does not accelerate tumor formation

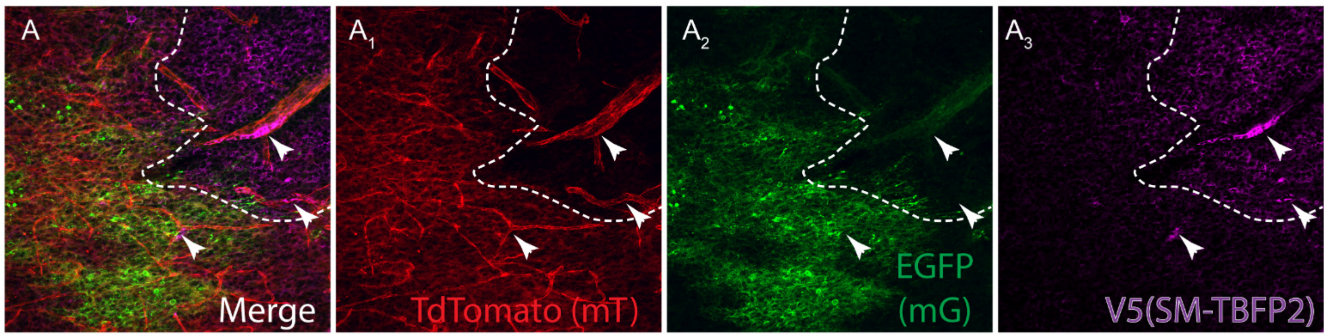
D) Atrx is expressed in the majority of EGFP+ cells in K27M tumors. A small subset of EGFP+ cells (yellow arrows) has lost Atrx antigenicity.

E) G34R cells at 100 days post-EP express Atrx.

F) CRISPR/Cas9 targeting leads to highly efficient loss of Atrx in EPed cells.

G) Cortically-infiltrating G34R tumor at 120 days post-EP.

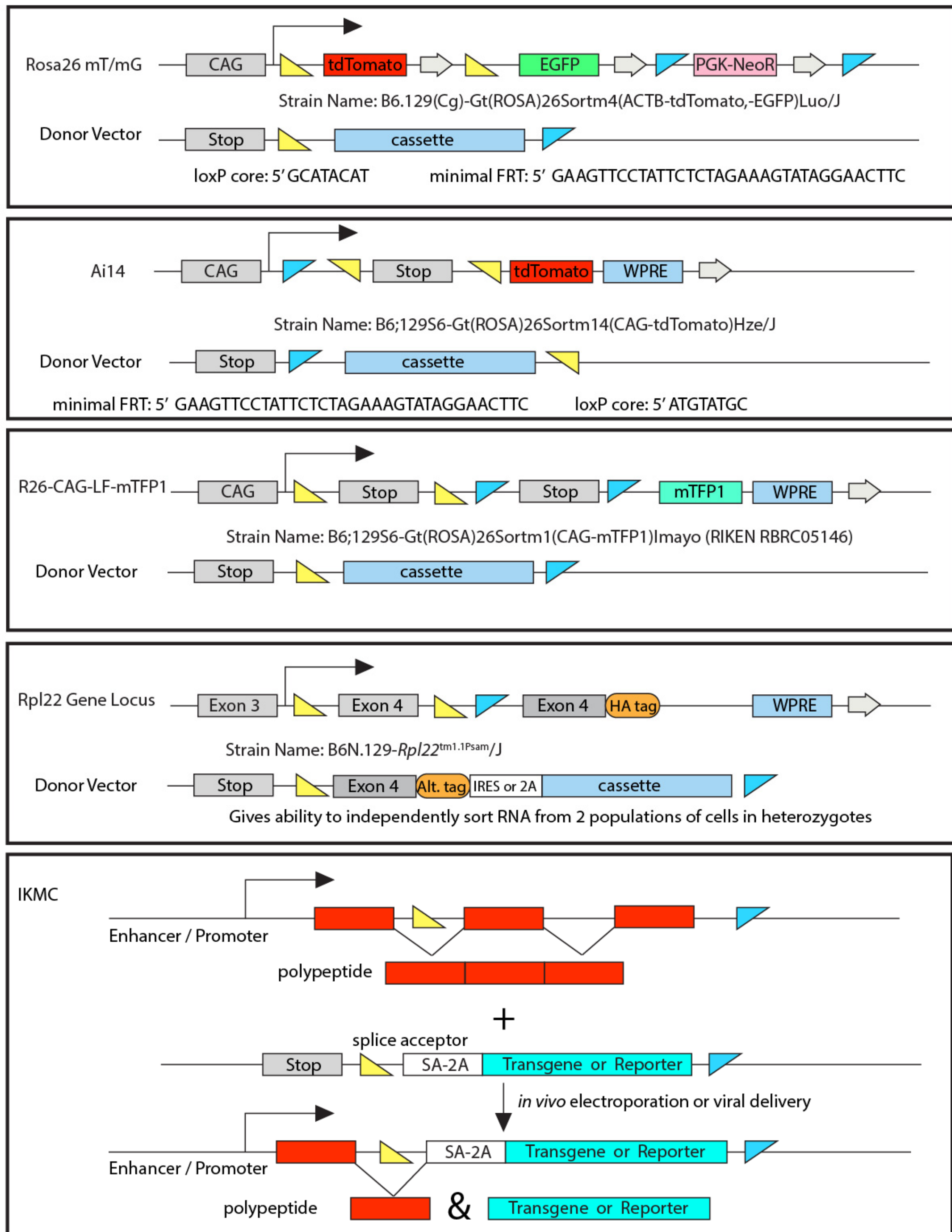
H) K27M tumor at 120 days post-EP is predominantly sub-cortical.



Supplementary Figure 6: Investigation of MADR glioma.

A) V5^{+} tumor-derived cell populations can be found juxtaposed to the Tdtomato+ vasculature in focal regions of the tumor.

Supplementary Figure 7:



Supplementary Figure 7: Alternative reporter mice amenable to *in vivo* MADR or MADR MAX, and extension of the method to Ribotrap and IKMC repository mouse lines with gene-trap alleles
There are existing CAG-based reporter mice that are similar to mTmG mice in construction and compatible with *in vivo* MADR to achieve mutant lineage tracing studies or orthogonal RNA isolation using Ribotrap heterozygotes. Additionally, this method can extend to thousands of gene-trap mice that, as an example, flank loxP and FRT around important exons. *in vivo* MADR at such loci would enable 1) lineage tracing of heterozygous/homozygous null cells at the locus, as well as 2) swapping the locus with a transgene.

Supplementary Table 1

F1	GCAACGTGCTGGTTATTGTGC	mtmg-cagF
F2	CTCAATCCAGCGGACCTTCC	mtmg-wpreF
F3	AGCAAAGACCCCAACGAGAAG	EGFP-F
F4	TGTCTGGATCCCCATCAAGC	mtmg-sv40F
F5	ATGCCCTGGCTCACAAATAC	rb glob pA F
F6	ACACAGGCATAGAGTGTC	SV40pA-F
R1	GATGACGGCCATGTTGTTGTCC	mtmg-tdtomatoR2
R2	TTTAACAGAGAGAAGTTCGTGGC	pTV-R
R3	GGAGCGGGAGAAATGGATATG	Rosa26-wildtype-R
R4	CGAAAGGCCCGGAGATGAGGAAG	PGKpromR
R5	TGATCGCGCTTCTCGTTGGG	EGFP653CSseq

Supplementary Table 2

Abcam 13970	Chicken anti-EGFP	1:5000
Abcam 95038	Goat anti-V5	1:1000
Aves	Chicken anti-Myc	1:500
BD Pharmingen	Rat anti-PDGFRα	1:500
Calbiochem	Sheep anti-p53	1:1000
Cell Signaling	rb anti-H3K27Me	1:1000
Cell Signaling	rb anti-Pdgfrb	1:500
Cell Signaling 3724	Rabbit anti-HA	1:2000
Cell Signaling 9308	Rabbit anti-pRb1	1:500
Clontech 9G9	Mouse anti-tetR	1:1000
Clontech 9G9	rb anti-dsred	1:1000
Dawen Cai (Univ of Mich)	Rabbit anti-dsRed	1:1000
Dawen Cai (Univ of Mich)	Guinea pig anti-mKate2	1:500
Invitrogen 46-0705	Mouse anti-V5	1:1000
Kerafast	rat anti-tdtomato	1:2000
R&D Systems	Sheep anti-Dll1	1:500
R&D Systems	Gt anti-Olig2	1:500
Sigma	Mouse anti-Flag	1:2500

Supplementary Table 1: Details on the primers used for PCR screening

Supplementary Table 2: Antibodies used in the study