Supplemental Information:

Supplemental Methods:

Cell culture

To isolate single GNS 144 cell clones, cells were plated at a density of 1cell/well in 96 well Primaria plates in GNS media and incubated at 37°C in a humidified 5% CO_2 atmosphere. Once visible colonies formed in wells, the clones were dissociated with Accutase (Life Technologies) and expanded to obtain an adequate number of cells for FISH and immunofluorescence assays.

Construction of stable GNS 179 cell lines

Stable GNS 179 cells were constructed by nucleofection of plasmids expressing GFP, GFP-Kif2A, GFP-MCAK, or GFP-MCAK Hypir (kind gift L. Wordeman) as previously described with minor modifications (1). Kif2A, MCAK, and MCAK-Hypir were cloned into a modified pEGFP vector (Clontech) with AscI and PacI in the multiple cloning site (kind gift A. Straight). For nucleofection, Amaxa program T-020 was used and cells recovered in GNS serum-free media with 250µg/ml G418 selection. After nucleofection, cells were sorted based upon GFP expression to enrich for a stable pool of GFP+ cells. Cell sorting was performed by the Immunoassay and Flow Cytometry Shared Resource at the Geisel School of Medicine (Hanover, NH).

Immunofluorescence

Immunofluorescence was performed as described in the main method section with minor modifications. For NESTIN immunofluorescence, cells were fixed in 3.5% paraformaldehyde, pH 6.8 for 5 mins, washed 2 x 5 mins TBS-0.1% triton,

and blocked with Abdil. The primary antibody was used at 5µg/ml mouse NESTIN (R&D Systems). For experiments with the GFP expressing cell lines NESTIN or SOX2 antibodies were combined with 2µg/ml rabbit CENP-A (kind gift A. Straight) to mark centromeres. For Tuj1 immunofluorescence, cells were fixed in 3.5% paraformaldehyde, pH 6.8 for 5 mins, washed 2 x 5 mins TBS-0.1% triton, and blocked with Abdil. The primary rabbit Tuj1 antibody was used at 2µg/ml (Covance). For GFAP immunofluorescence of GNS 144 and GliNS2 TICs, cells were fixed with ice-cold methanol for 3 mins, washed 2 x 5 mins TBS-0.1% triton, and blocked with Abdil. The primary mouse GFAP antibody was used at 5µg/ml (Sigma).

All images were acquired with a cooled CCD camera (Andor Technology) mounted on a microscope (Eclipse Ti; Nikon). 0.2µm or 0.5µm optical sections in the z axis were collected with a planApo 60× 1.4 NA oil immersion objective at room temperature. For experiments comparing the frequency of expression of proteins of interest, images for each cell line were acquired with the same acquisition parameters and exposure times.

Quantification of Protein Expression

To determine the percent of cells expressing a protein of interest, maximum intensity projections were compiled from Z-stack images using Elements software (Nikon). Maximum intensity projections were subsequently scaled equivalently and single cells were then categorized as positive or negative for expression of a protein of interest. Specifically, for NESTIN and SOX2 experiments, non-tumor initiating osteosarcoma U2OS cells served as a negative

background control and CB660 cells as a positive control as indicated in the figure legends.

For quantification of SOX2 levels, four equivalent elliptical regions of interest (ROI) were randomly placed in the nucleus of a cell. The mean intensity of each ROI was then averaged to quantify SOX2 levels per cell. For quantification of NESTIN levels, four equivalent elliptical ROIs were randomly placed in the cytoplasm of a cell. The mean intensity of each ROI was then averaged to quantify NESTIN levels per cell.

Nocodazole Arrest Assay

Cells were plated on 12mm poly-D-lysine/laminin coated coverslips (Corning) and incubated overnight at 37°C in a humidified 5% CO_2 atmosphere. The next day, cells were treated with 100ng/ml nocodazole (Acros) for the indicated time. At the appropriate time point, cells were fixed with 3.5% paraformaldehyde, pH 6.8 for 5 mins, washed 2 x 5 mins TBS-0.1% triton, and blocked in Abdil. For H3 phosphoSer10 immunofluorescence, the primary rabbit antibody (Cell Signaling Technologies) was used at 1:500. The remaining steps were performed as described in the main methods section. Mitotic cells were scored as positive for H3 phosphoSer10 signal.

Differentiation Experiments

To differentiate GNS cells along the astrocyte lineage, cells were incubated in GNS media in the absence of growth factors EGF and FGF and the addition of 10ng/ml BMP-4 (R&D Systems) for 8 days. To differentiate GNS cells along the

neuronal lineage, cells were incubated in GNS media in the absence of the growth factors EGF and FGF for 8 days.

p53 and p21 Assays

Cells were plated at ~75% confluency in 6 well tissue culture plates and incubated overnight at 37°C in a humidified 5% CO_2 atmosphere. The next day wells were treated with DMSO or 1μM doxorubicin (Sigma) for 4hrs at 37°C. Subsequently, cells were dissociated with Accutase (Life Technologies) and washed twice with PBS. Cell pellets were stored at -20°C. Cell pellets were lysed with the following buffer: 20mM K^+Hepes, 0.5M NaCl, 1% igepal, 1mM DTT, 10mM EDTA, 10mM EGTA, 1mM PMSF, 10μg/ml pepstatin, and 10μg/ml leupeptin. Total cell protein was separated by SDS-PAGE and transferred to a poly-vinylidene fluoride membrane. For p53 detection, a membrane was incubated with the following primary antibodies 1:1000 mouse p53 (Santa Cruz) or 1:5000 mouse actin (Seven Hills Bioreagents) in 4% milk and TBS-0.2% tween. For p21 detection, a membrane was incubated with the following primary antibodies 1:1500 rabbit p21 (Santa Cruz) or 1:5000 mouse actin (Seven Hills Bioreagents) in 1% milk and TBS-0.05% tween. Primary antibodies were detected with HRP-conjugated secondary antibodies followed by chemiluminescence.

For sequencing p53 in GNS cells, RNA was purified from cell pellets following the Qiagen RNA mini-prep protocol. Subsequently, cDNA was amplified and purified following the Thermo Verso cDNA kit. p53 cDNA was PCR amplified with the following primers 5'-ATGGAGGAGCCGCAGTCAGA-3' and 5'-

TCAGTCTGAGTCAGGCCC-3'. PCR products were run on a 1% agarose gel and bands the size of p53 were gel extracted following the Qiagen gel extraction protocol. The PCR products were sequenced with the PCR primers and the additional primer 5'-CTGGAGTGAGCCCTGCTCC-3'.

Statistical Analysis

A Fisher's exact two-tailed test was performed for expression analysis of proteins of interest.

Supplemental Figure Legends:

Figure S1: GNS cells express the stem cell marker NESTIN. (A) Representative images of NESTIN expression in negative control U2OS cells that are non-tumor initiating osteosarcoma cells, positive control CB660 NSCs, and TICs GNS 179, GNS 144, and GliNS2. Late passage is 20 or more passages in culture relative to early passage cells. Shown in the images are DNA (blue) and NESTIN (green). Scale bar, 10μm. (B) Percentage of cells expressing NESTIN. N≥400 cells scored and *$p<0.05$, Fisher's exact test compared with the negative control U2OS cells.

Figure S2: GNS cells express the stem cell marker SOX2. (A) Representative images of SOX2 expression in negative control U2OS cells that are non-tumor initiating osteosarcoma cells, positive control CB660 NSCs, and TICs GNS 179, GNS 144, and GliNS2. Late passage is 20 or more passages in culture relative to early passage cells. Shown in the images are DNA (blue) and SOX2 (green). Scale bar, 10μm. (B) Percentage of cells expressing SOX2. N≥400 cells scored and *$p<0.05$, Fisher's exact test compared with the negative control U2OS cells.

Figure S3: GNS cells are multi-potent in vitro. (A) Representative images of TICs GNS 144 and GliNS2. Late passage is 20 or more passages in culture relative to early passage cells. Cells grown in the presence of the growth factors EGF and FGF maintain an undifferentiated stem-like phenotype and do not express the astrocyte marker GFAP. Cells grown in the absence of EGF and

FGF and in the presence of BMP4 differentiate and express the astrocyte marker GFAP. Shown in the images are DNA (blue) and GFAP (green). Scale bar, 10μm. (B) Percentage of cells expressing GFAP. N≥200 cells scored. (C) Cells grown in the presence of the growth factors EGF and FGF maintain an undifferentiated stem-like phenotype and do not express the neuronal marker Tuj1. Cells grown in the absence of EGF and FGF differentiate and express the neuronal marker Tuj1. Shown in the images are DNA (blue) and Tuj1 (green). Scale bar, 10μm. (B) Percentage of cells expressing Tuj1. N≥200 cells scored.

Figure S4: GNS cells have a functional spindle assembly checkpoint and TICs display extensive karyotype heterogeneity. (A) CB660 NSCs and TICs GNS 179, GNS 144, and GliNS2 were treated with 100ng/ml nocodazole for the indicated time. At each time point, cells with phosphorylated histone H3 at Ser10 were scored as mitotic. Three independent experiments were averaged with N>200 cells counted per replicate. The errors bars indicate standard deviation. (B) Representative images of CB660 NSCs and TICs GNS 179, GNS 144, and GliNS2 scored as mitotic as indicated by positive histone H3 Ser10 phosphorylation. Shown in the images are DNA (blue) and H3 phosphoSer10 (green). Scale bar, 10μm. (C) Representative images of FISH analysis for the TICs GNS 179, GNS 144, GNS 144 Clone E, and GNS 144 Clone G. Chromosome copy number variations were determined using centromeric DNA probes for chromosomes 2, 3, 7, and 10. Two independent experiments were performed for all cell lines. Scale bar, 10μm.

Figure S5: SKY karyotypes of GliNS2 cells. Twenty-three karyotypes of GliNS2 cells.

Figure S6: GNS cells have an aberrant p53 response. (A) Control RPE-1 and CB660 cells and the TICs GNS 144, GNS 179, and GliNS2 were treated with 1μM doxorubicin for 4hrs to induce DNA damage. Late passage refers to 20 or more passages in culture relative to early passage cells. Western blots of p53 protein levels (upper panel) and p21 protein levels (lower panel). Actin was used as a loading control. (B) The p53 DNA binding domain was sequenced in the TICs GliNS2, GNS 179, and GNS 144. DNA sequencing results identified base changes in the p53 sequence. (C) The protein coding sequence of p53 from amino acids 160-269 with the mutations in GNS 144, GliNS2, and GNS 179 cells identified from the DNA sequencing results.

Figure S7: PDX TICs and non-TICs display extensive karyotype heterogeneity. (A) Representative images of FISH analysis for the glioblastoma PDX lines T3691 and T3946. Chromosome copy number variations were determined using centromeric DNA probes for chromosomes 2, 3, 7, and 10. Scale bar, 10μm. (B) Summary table of the modal chromosome copy numbers and mode deviations for all three PDX lines derived from the data in Figure 4 panels B and C. (C) Three karyotypes of T3691 CD133+ TICs and five karyotypes of T3691 CD133- non-TICs.

Figure S8: Expression of NESTIN and SOX2 in GNS 179 GFP, GFP-Kif2A, GFP-MCAK, and GFP-MCAK Hypir cells. Representative images of NESTIN (A) or SOX2 (C) expression in GNS 179 GFP, GFP-Kif2A, GFP-MCAK, or GFP-MCAK Hypir expressing cells. Shown in the images are DNA (blue), the GFP expression construct (green), and NESTIN or SOX2 (red), and centromeres (grayscale). Scale bar, 10μm. (B) Percentage of GNS 179 GFP cell lines expressing NESTIN. U2OS cells are a negative control for NESTIN expression. Top panel bar graph shows the percent of cells expressing NESTIN with data pooled from duplicate coverslips. N≥162 GFP+ cells scored for the GNS 179 cell lines and *$p<0.05$, Fisher's exact test compared with negative control U2OS cells. Bottom panel bar graph is a quantification of NESTIN levels in the cytoplasm of N=50 GFP+ cells per cell line with the errors bars indicating standard deviation. (D) Percentage of GNS 179 GFP cell lines expressing SOX2. U2OS cells are a negative control for SOX2 expression. Top panel bar graph shows the percent of cells expressing SOX2 with data pooled from duplicate coverslips. N≥174 GFP+ cells scored for each GNS 179 cell line and *$p<0.05$, Fisher's exact test compared with negative control U2OS cells. Bottom panel bar graph is a quantification of SOX2 levels in the nucleus of N=50 GFP+ cells per cell line with the errors bars indicating standard deviation.

Figure S9: Estimated frequency of self-renewal in vitro for GNS 179 GFP, GFP-Kif2A, GFP-MCAK, and GFP-MCAK Hypir cells. In vitro limiting dilution

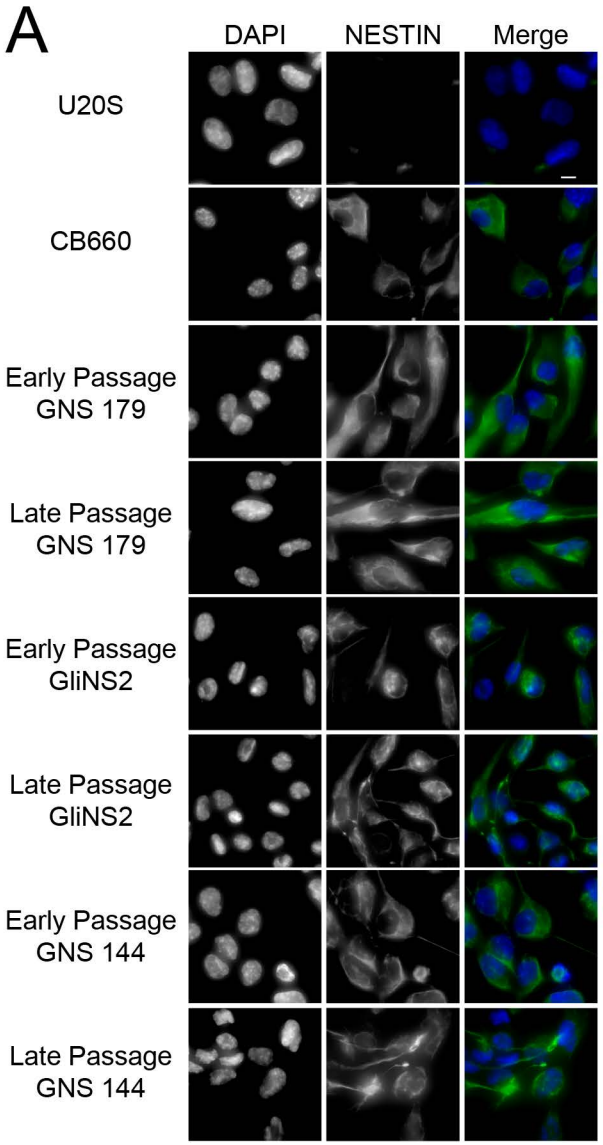
assays in GNS 179 GFP, GFP-Kif2A, GFP-MCAK, or GFP-MCAK Hypir expressing cells. N=3 trials.

Figure S10: Sorting of low and high expressing GNS 179 GFP, GFP-Kif2A, GFP-MCAK, and GFP-MCAK Hypir cells. FACS profiles of negative control parental GNS 179 cells and GNS 179 GFP, GFP-Kif2A, GFP-MCAK, and GFP-MCAK Hypir expressing cells. Cells within gate P1 were sorted as the low GFP expressing cells and cells within gate P2 were sorted as the high GFP expressing cells. For the negative control parental GNS 179 cells, cells within the region before gate P1 were sorted.

Figure S11: Astrocyte differentiation of GNS 179 GFP, GFP-Kif2A, GFP-MCAK, and GFP-MCAK Hypir cells. Representative images of GNS 179 GFP, GFP-Kif2A, GFP-MCAK, or GFP-MCAK Hypir expressing cells. Cells grown in the presence of the growth factors EGF and FGF maintain an undifferentiated stem-like phenotype and do not express the astrocyte marker GFAP. Cells grown in the absence of EGF and FGF and in the presence of BMP4 differentiate and express the astrocyte marker GFAP. Shown in the images are DNA (blue), the GFP expression construct (green), GFAP (red), and centromere (grayscale). Scale bar, 10μm.

Supplemental References:

1. Pollard SM, Yoshikawa K, Clarke ID, Danovi D, Stricker S, Russell R, et al. Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. Cell Stem Cell. 2009;4:568–80.


A
DAPI
NESTIN
Merge
U20S
CB660
Early Passage GNS 179
Late Passage GNS 179
Early Passage GliNS2
Late Passage GliNS2
Early Passage GNS 144
Late Passage GNS 144

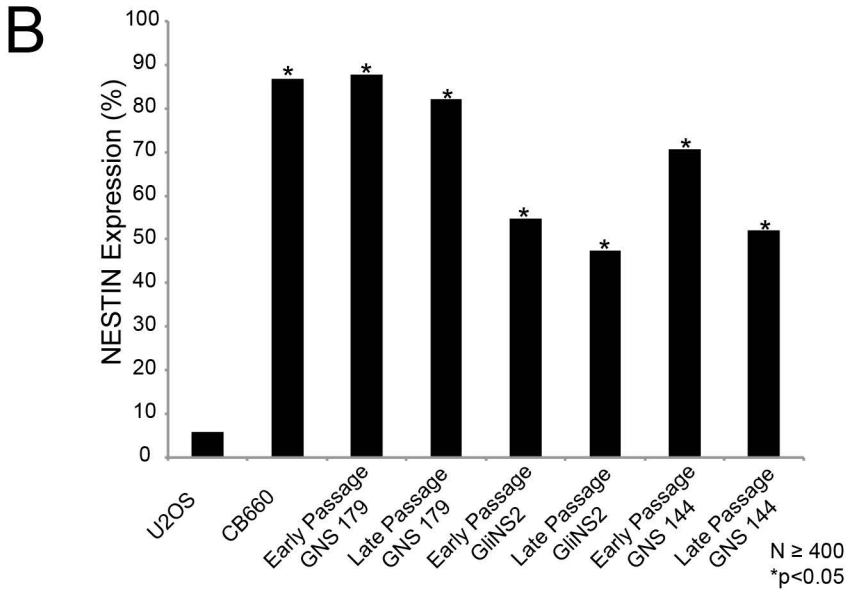
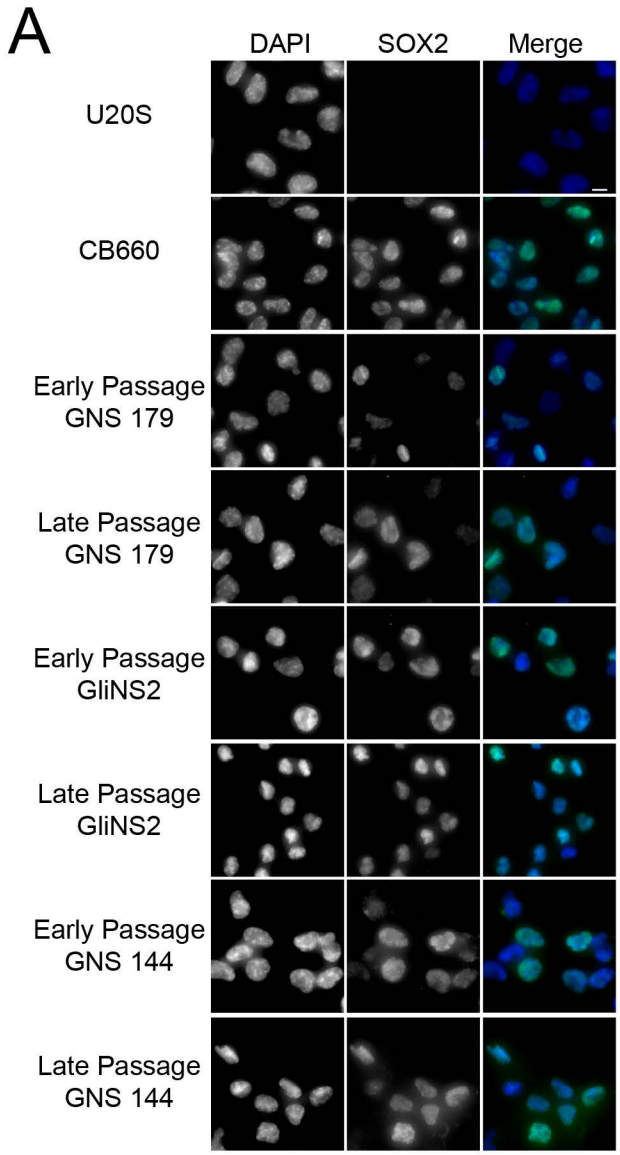

B
NESTIN Expression (%)
100
90
80
70
60
50
40
30
20
10
0
U2OS
CB660
Early Passage GNS 179
Late Passage GNS 179
Early Passage GliNS2
Late Passage GliNS2
Early Passage GNS 144
Late Passage GNS 144
N ≥ 400
*p<0.05

Figure S1


A
DAPI
SOX2
Merge
U20S
CB660
Early Passage GNS 179
Late Passage GNS 179
Early Passage GliNS2
Late Passage GliNS2
Early Passage GNS 144
Late Passage GNS 144

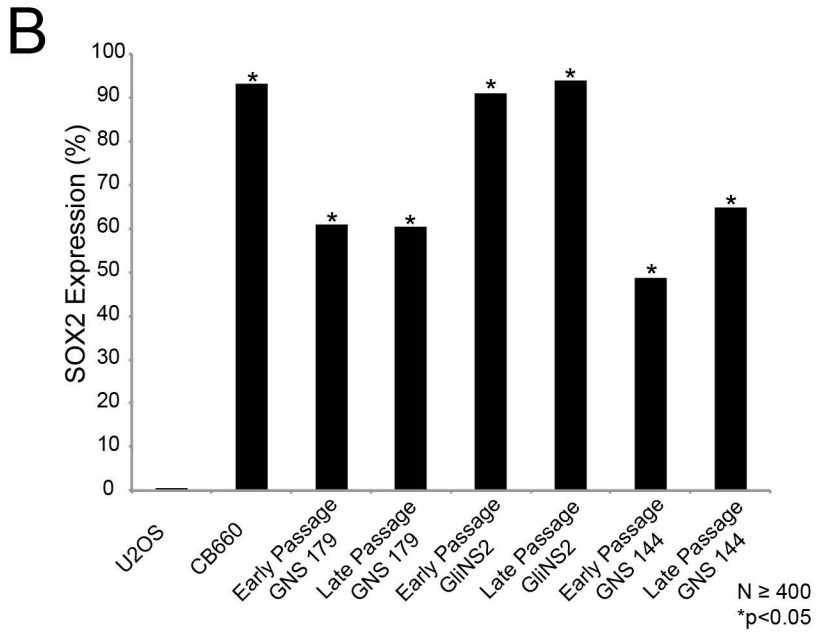
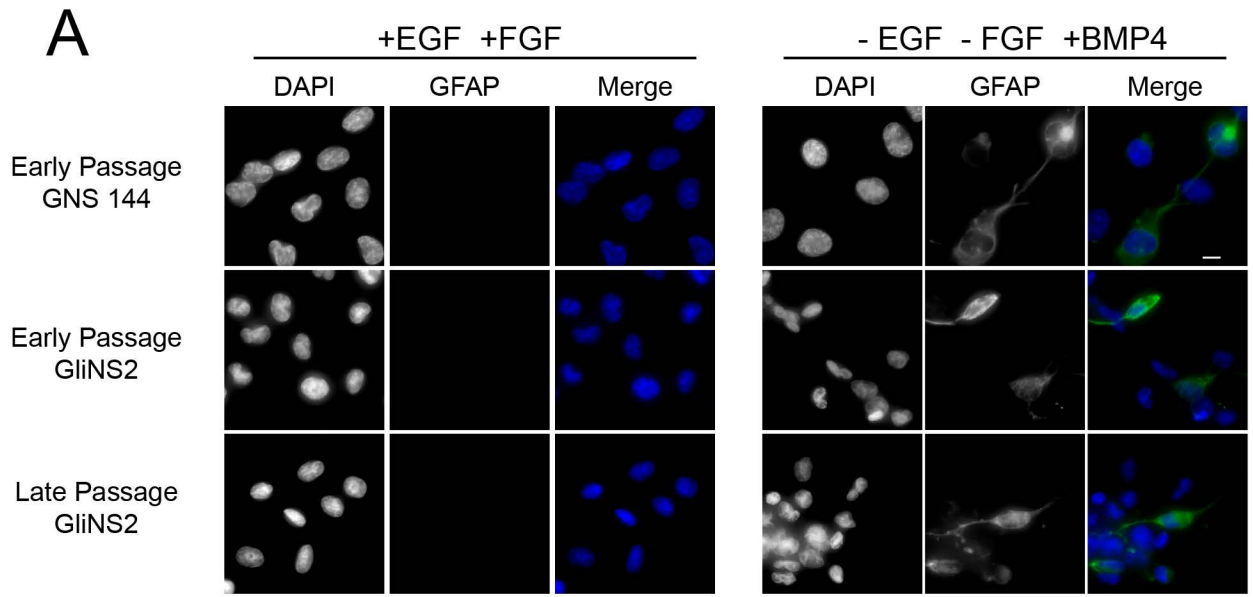
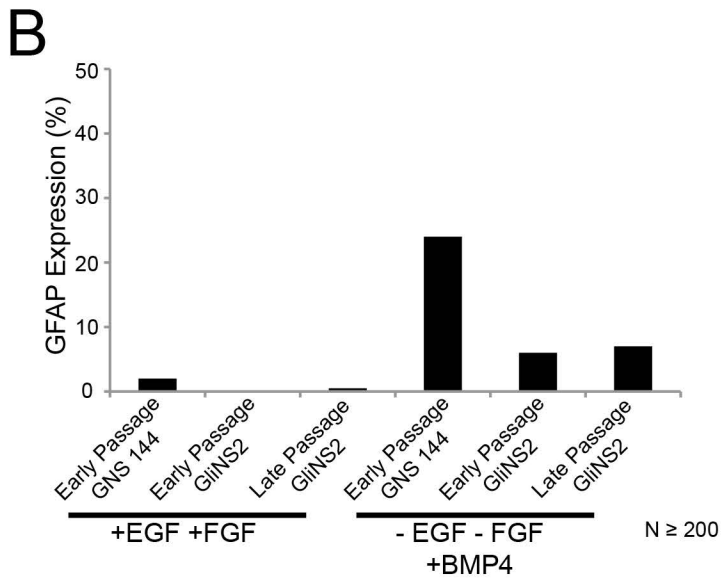
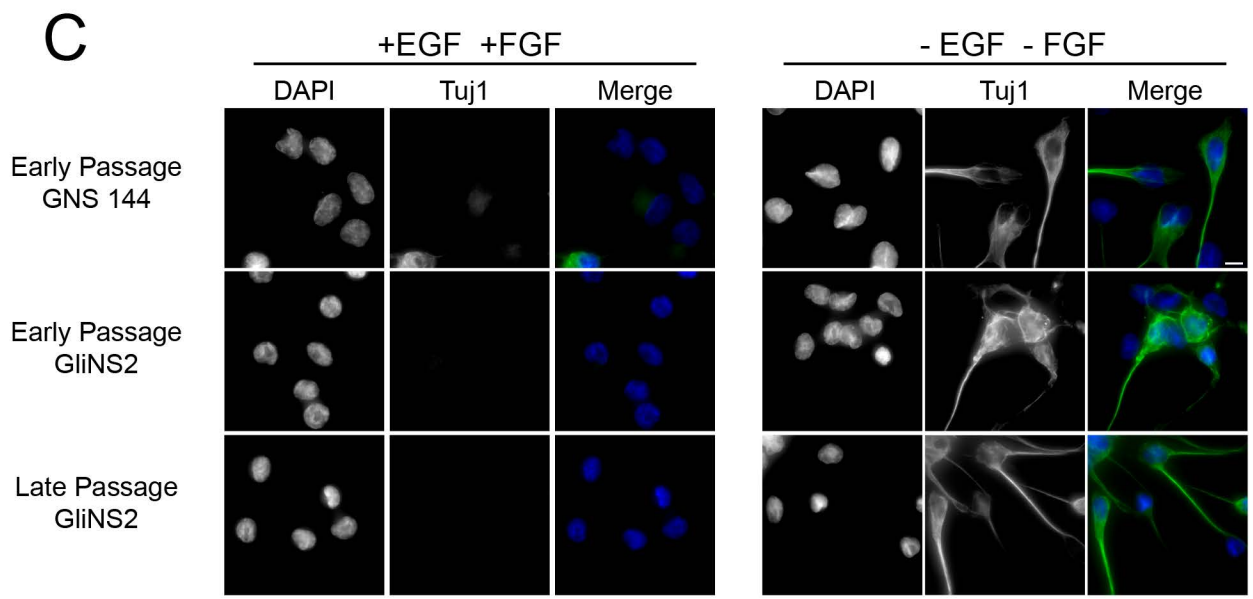

B
SOX2 Expression (%)
100
90
80
70
60
50
40
30
20
10
0
U2OS
CB660
Early Passage GNS 179
Late Passage GNS 179
Early Passage GliNS2
Late Passage GliNS2
Early Passage GNS 144
Late Passage GNS 144
N ≥ 400
*p<0.05

Figure S2


A
+EGF +FGF
- EGF - FGF +BMP4
DAPI
GFAP
Merge
Early Passage GNS 144
Early Passage GliNS2
Late Passage GliNS2


B
GFAP Expression (%)
50
40
30
20
10
0
Early Passage GNS 144
Early Passage GliNS2
Late Passage GliNS2
+EGF +FGF
- EGF - FGF +BMP4
N ≥ 200


C
+EGF +FGF
- EGF - FGF
DAPI
Tuj1
Merge
Early Passage GNS 144
Early Passage GliNS2
Late Passage GliNS2

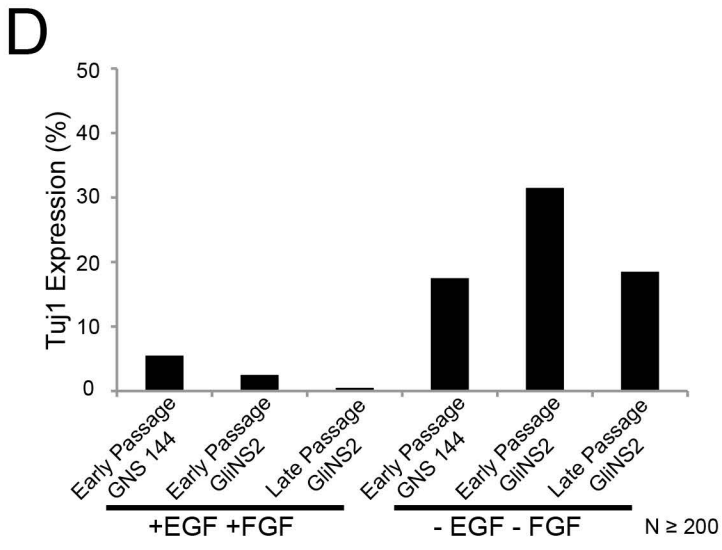

D
Tuj1 Expression (%)
50
40
30
20
10
0
Early Passage GNS 144
Early Passage GliNS2
Late Passage GliNS2
+EGF +FGF
- EGF - FGF
N ≥ 200

Figure S3

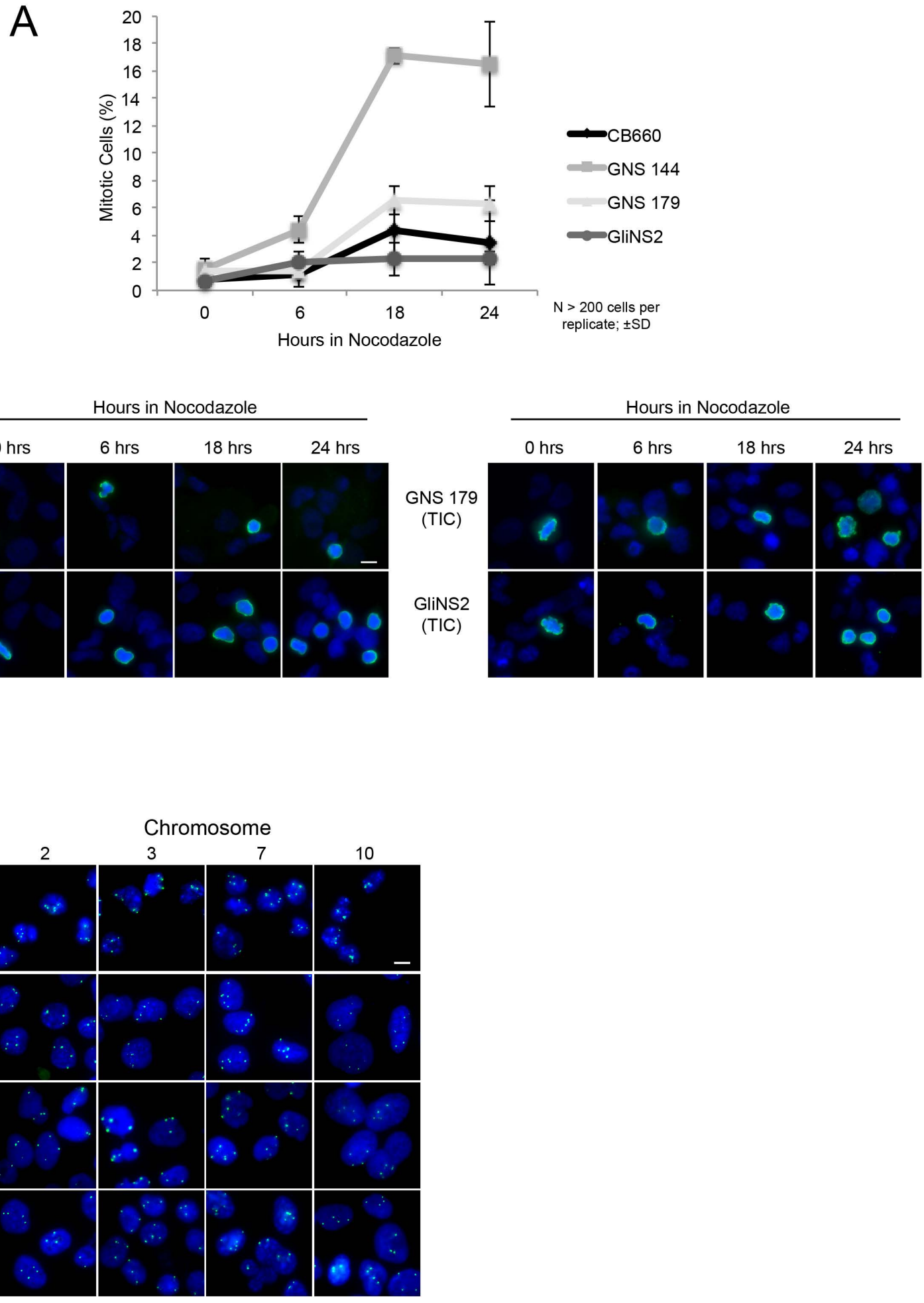

A
Mitotic Cells (%)
20
18
16
14
12
10
8
6
4
2
0
0
6
18
24
Hours in Nocodazole
CB660
GNS 144
GNS 179
GliNS2
N > 200 cells per replicate; ±SD
B
Hours in Nocodazole
0 hrs
6 hrs
18 hrs
24 hrs
CB660 (NSC)
GNS 144 (TIC)
GNS 179 (TIC)
GliNS2 (TIC)
C
Chromosome
2
3
7
10
GNS 179 (TIC)
GNS 144 (TIC)
GNS 144 (TIC) Clone E
GNS 144 (TIC) Clone G

Figure S4

GliNS2 Karyotypes

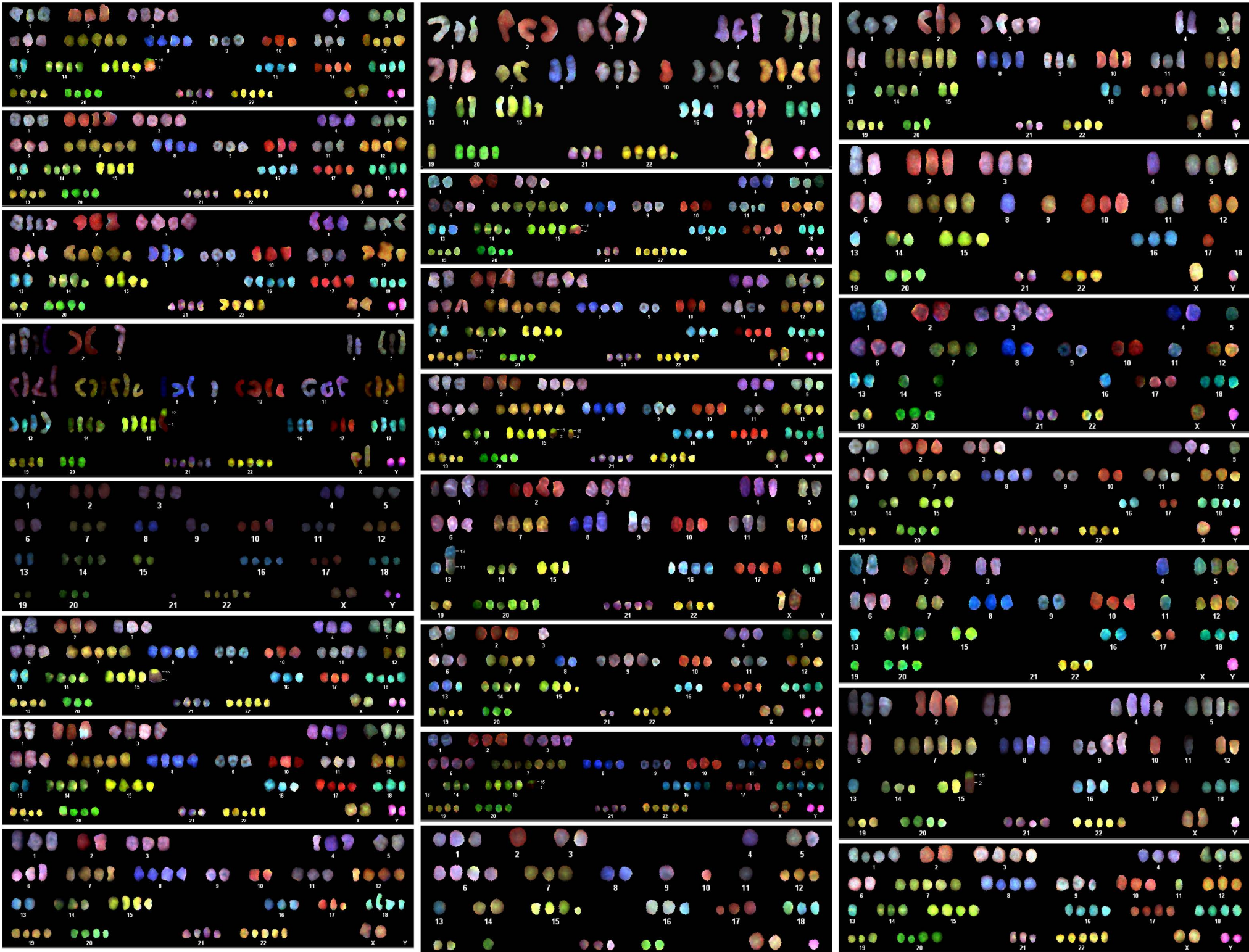
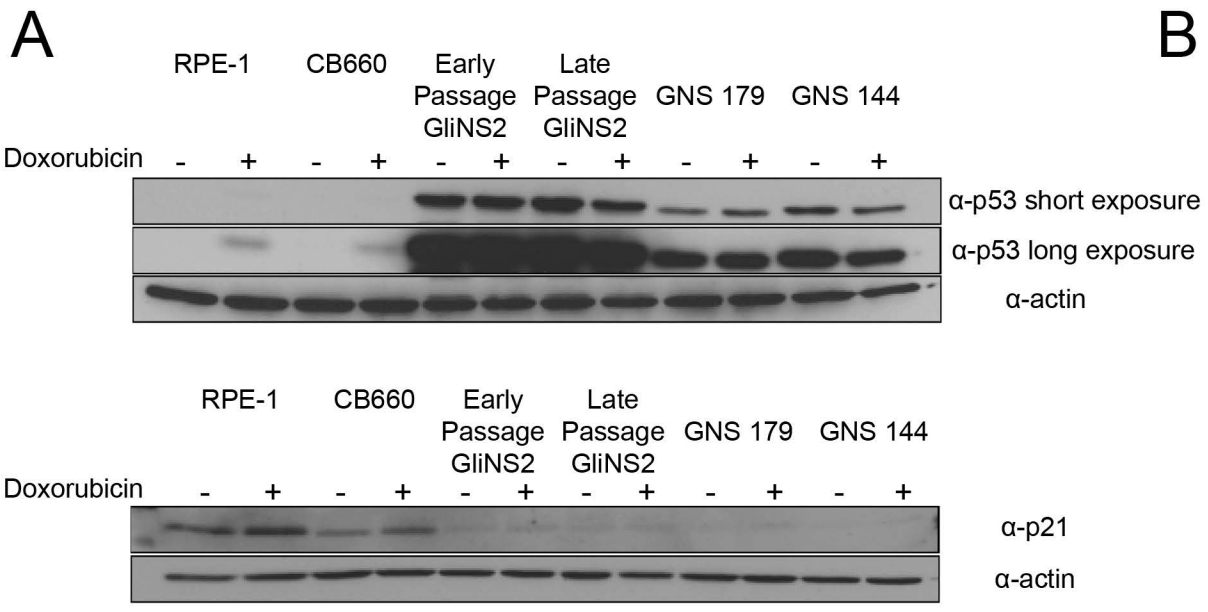
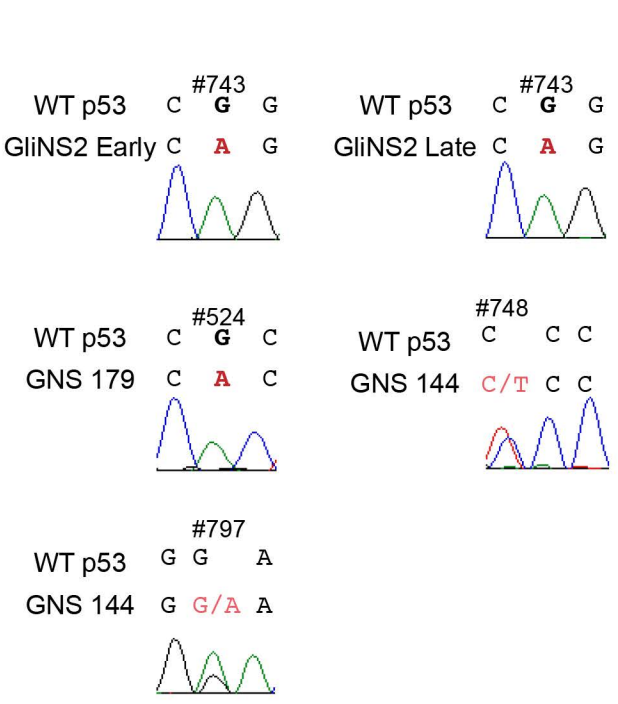


Figure S5


A
RPE-1
CB660
Early Passage GliNS2
Late Passage GliNS2
GNS 179
GNS 144
Doxorubicin
α-p53 short exposure
α-p53 long exposure
α-actin
α-p21
α-actin


B
WT p53
GliNS2 Early
GliNS2 Late
GNS 179
GNS 144
#743
#524
#748
#797

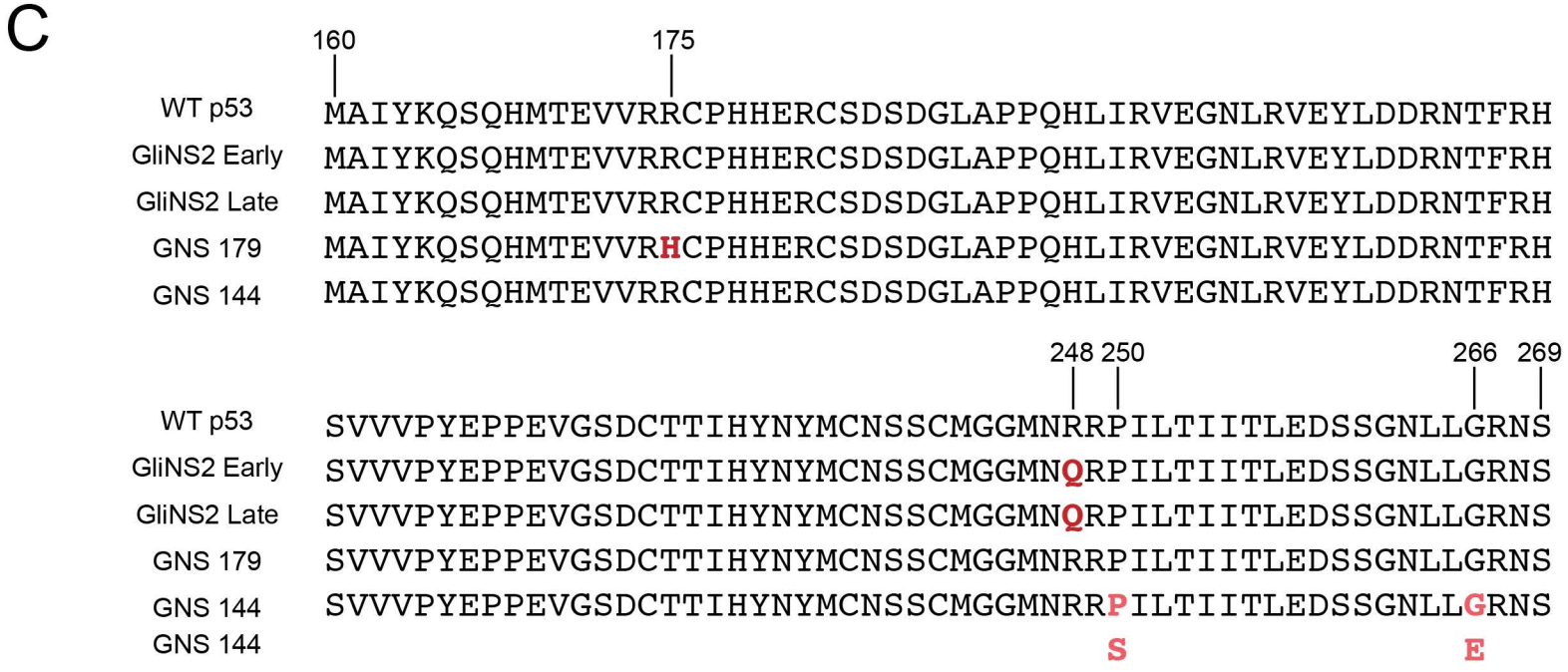
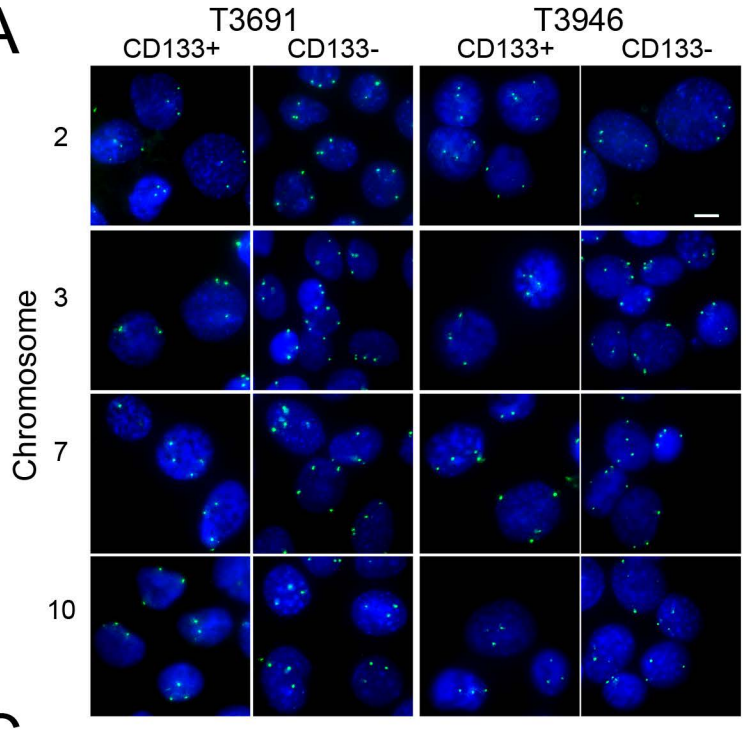

C
160
175
WT p53
GliNS2 Early
GliNS2 Late
GNS 179
GNS 144
MAIYKQSQHMTEVVRRCPHHERCSDSDGLAPPQHLIRVEGNLRVEYLDDRNTFRH
248 250
266 269
SVVVPYEPPEVGSDCTTIHYNYMCNSSCMGGMNRRPILTIITLEDSSGNLLGRNS
S
E

Figure S6

A



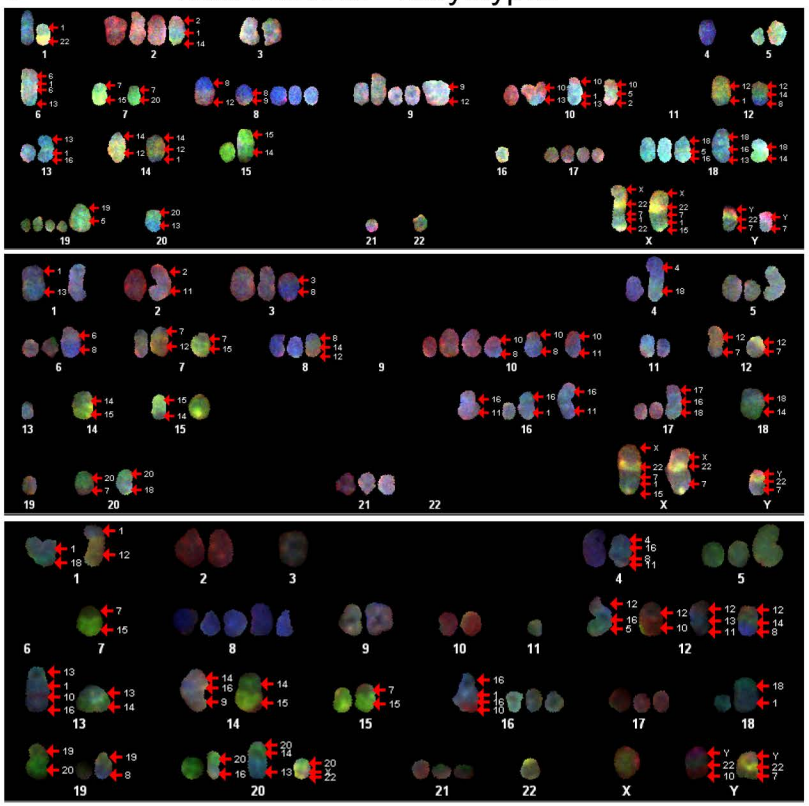
B

	Chromosome 2	Chromosome 3	Chromosome 7	Chromosome 10
T4121 CD133+	3 (15.8%)	3 (18.8%)	4 (19.0%)	3 (20.9%)
T4121 CD133-	3 (10.0%)	2 (12.9%)	4 (29.5%)	3 (15.4%)
T3691 CD133+	3 (24.0%)	3 (24.3%)	3 (63.5%)	2 (60.4%)
T3691 CD133-	3 (14.5%)	3 (19.7%)	2 (61.5%)	3 (60.9%)
T3946 CD133+	3 (21.5%)	3 (33.7%)	4 (42.7%)	3 (38.8%)
T3946 CD133-	3 (14.5%)	3 (20.9%)	4 (57.5%)	3 (55.7%)

Chromosome Mode (% Deviation)
N ≥ 96

C

T3691 CD133+ Karyotypes



T3691 CD133- Karyotypes

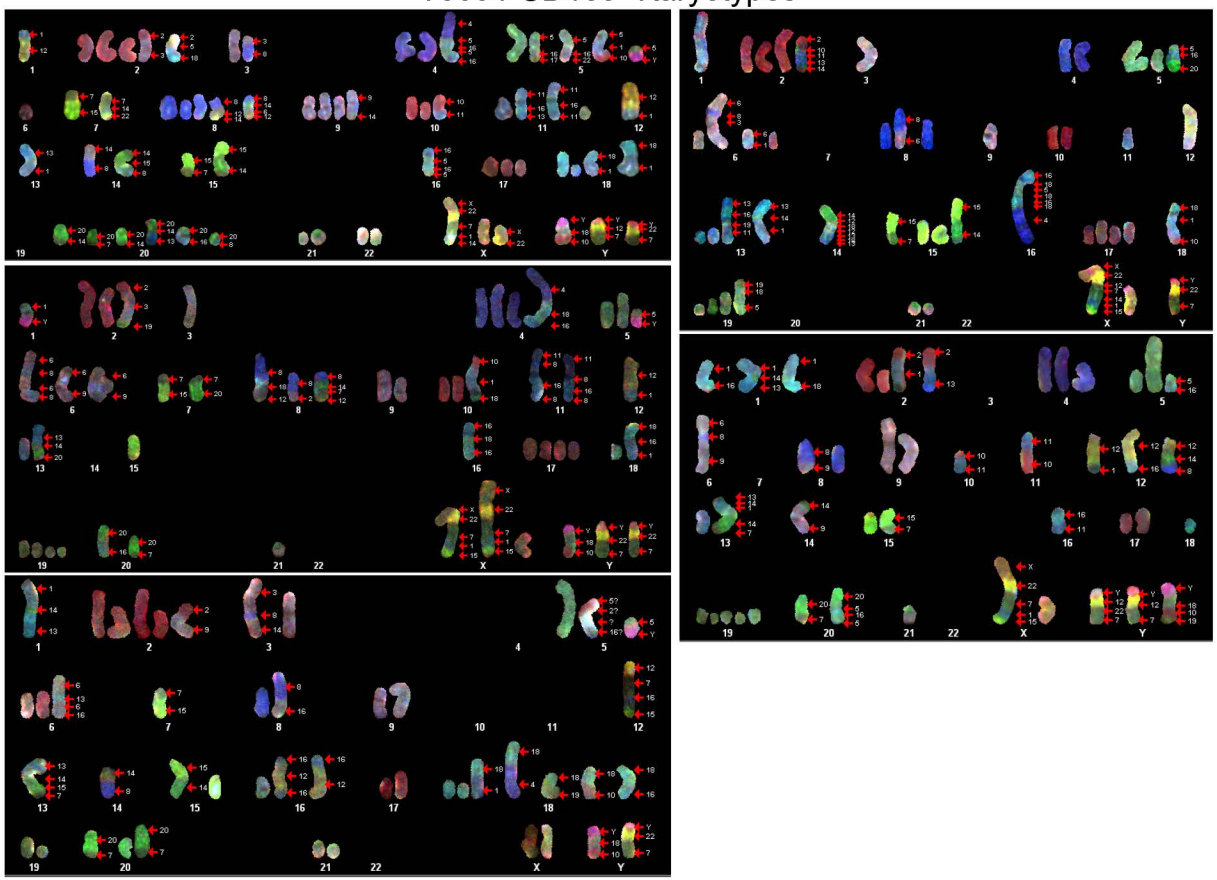
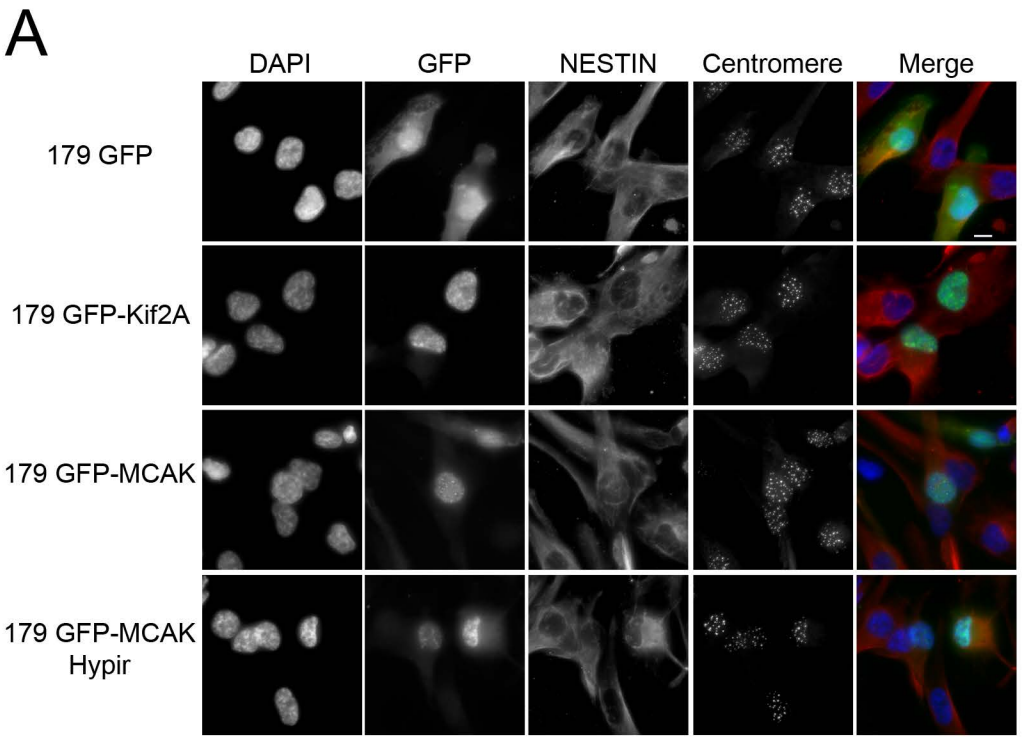
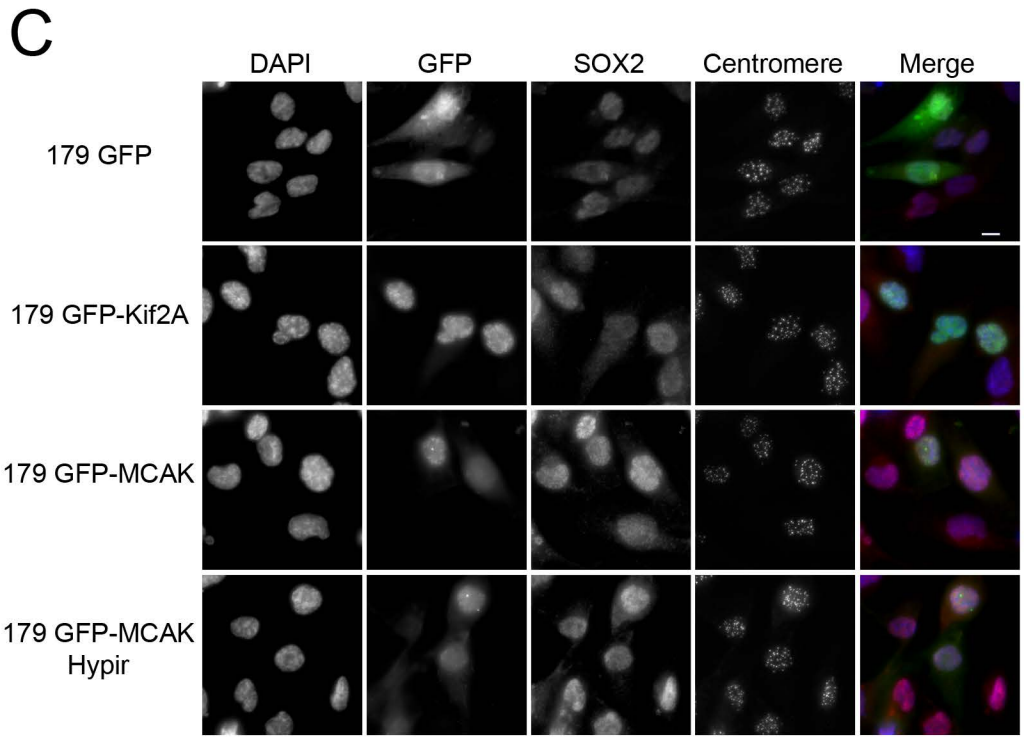


Figure S7


A
DAPI
GFP
NESTIN
Centromere
Merge
179 GFP
179 GFP-Kif2A
179 GFP-MCAK
179 GFP-MCAK Hypir


C
DAPI
GFP
SOX2
Centromere
Merge
179 GFP
179 GFP-Kif2A
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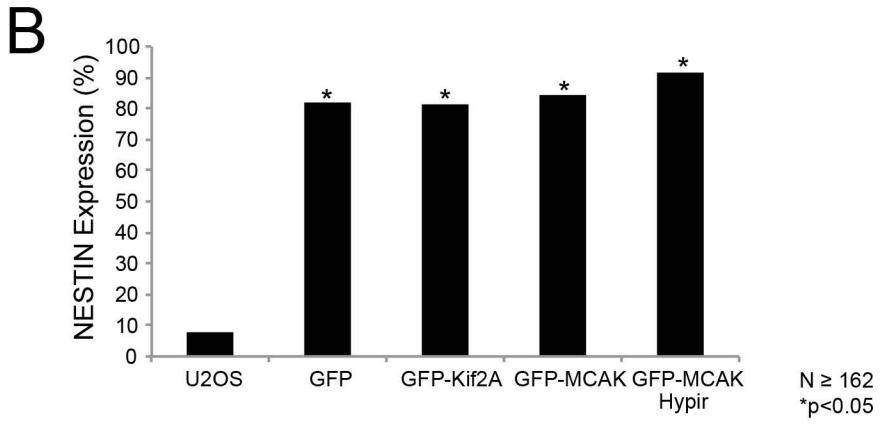
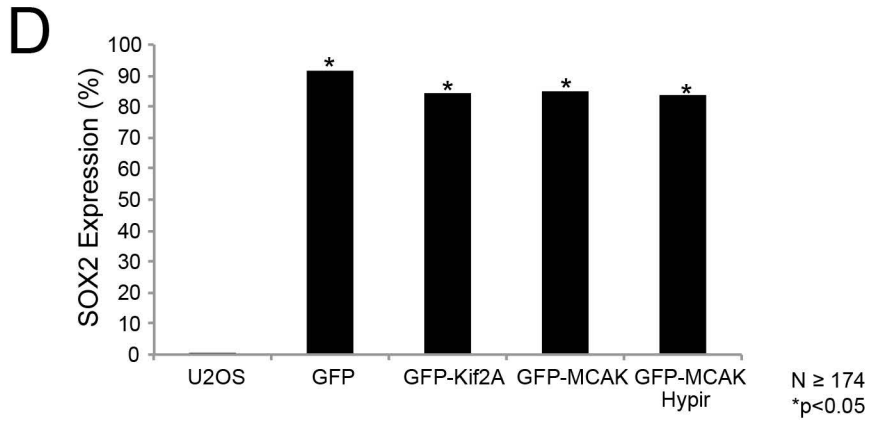
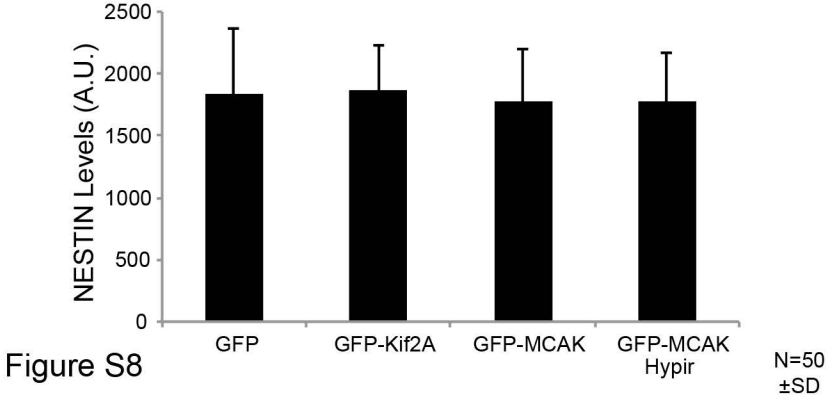
179 GFP-MCAK Hypir


B
NESTIN Expression (%)
100
90
80
70
60
50
40
30
20
10
0
*
U2OS
GFP
GFP-Kif2A
GFP-MCAK
GFP-MCAK Hypir
N ≥ 162
*p<0.05


D
SOX2 Expression (%)
100
90
80
70
60
50
40
30
20
10
0
*
U2OS
GFP
GFP-Kif2A
GFP-MCAK
GFP-MCAK Hypir
N ≥ 174
*p<0.05


NESTIN Levels (A.U.)
2500
2000
1500
1000
500
0
GFP
GFP-Kif2A
GFP-MCAK
GFP-MCAK Hypir
N=50
±SD

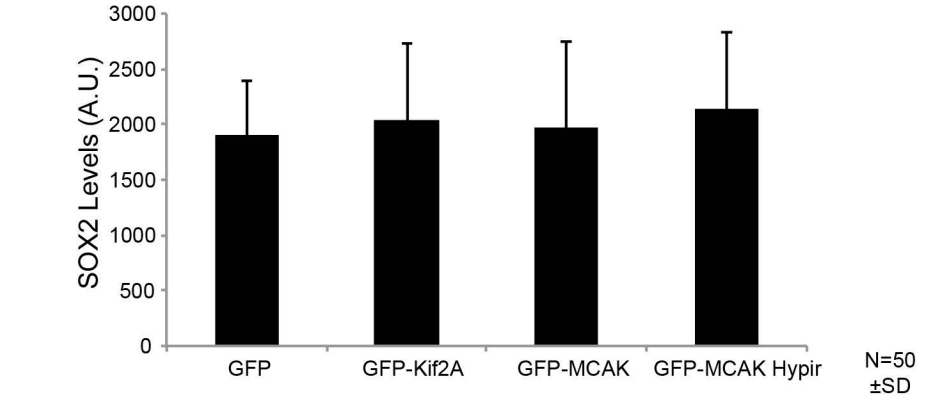

SOX2 Levels (A.U.)
3000
2500
2000
1500
1000
500
0
GFP
GFP-Kif2A
GFP-MCAK
GFP-MCAK Hypir
N=50
±SD

Figure S8

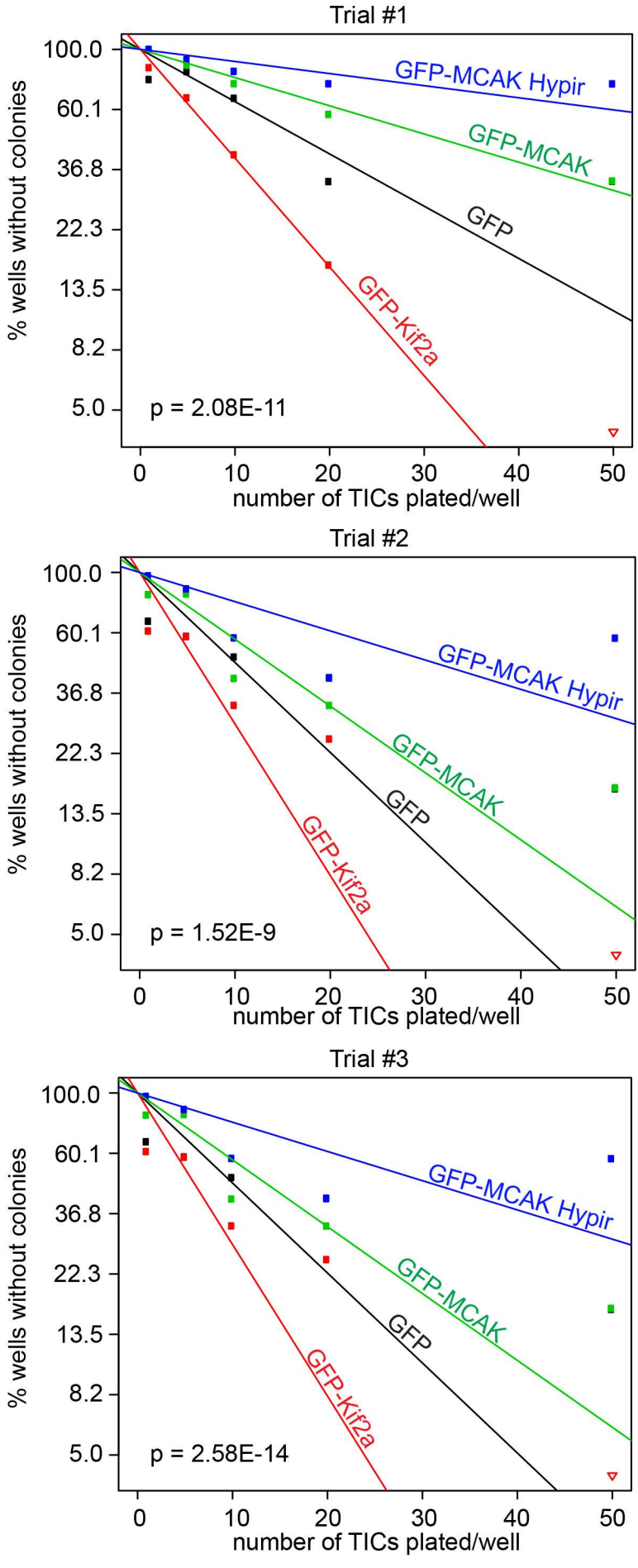
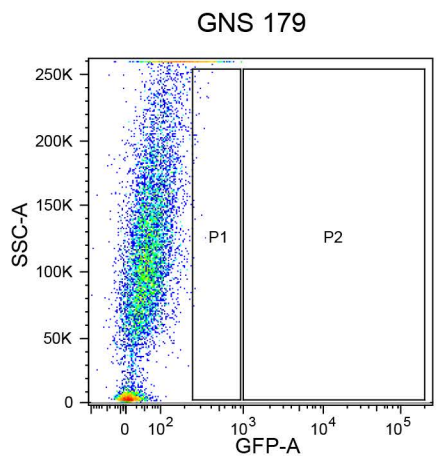
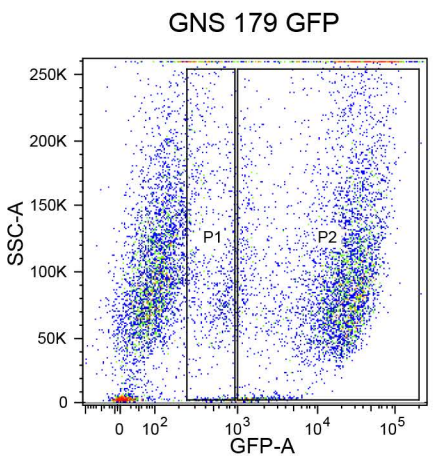
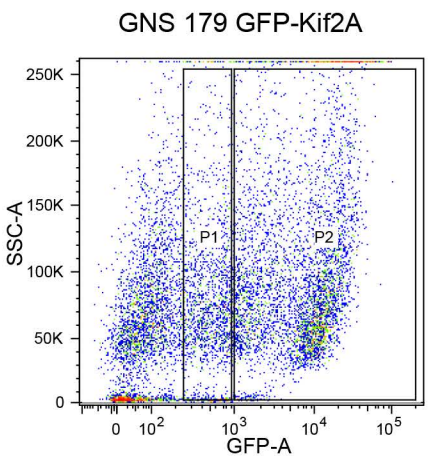
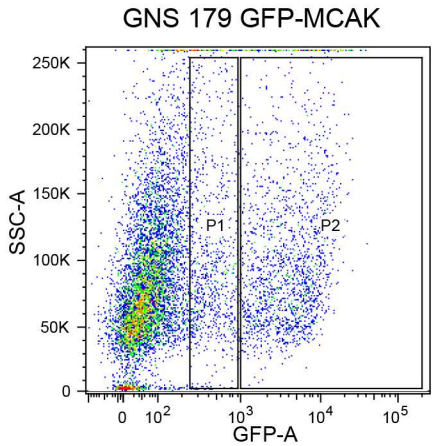

Trial #1
% wells without colonies
100.0
60.1
36.8
22.3
13.5
8.2
5.0
GFP-MCAK Hypir
GFP-MCAK
GFP
GFP-Kif2a
p = 2.08E-11
0
10
20
30
40
50
number of TICs plated/well
Trial #2
% wells without colonies
GFP-MCAK Hypir
GFP-MCAK
GFP
GFP-Kif2a
p = 1.52E-9
number of TICs plated/well
Trial #3
% wells without colonies
GFP-MCAK Hypir
GFP-MCAK
GFP
GFP-Kif2a
p = 2.58E-14
number of TICs plated/well

Figure S9


GNS 179
SSC-A
GFP-A
P1
P2
250K
200K
150K
100K
50K
0
0
10^2
10^3
10^4
10^5


GNS 179 GFP
SSC-A
GFP-A
P1
P2
250K
200K
150K
100K
50K
0
0
10^2
10^3
10^4
10^5


GNS 179 GFP-Kif2A
SSC-A
GFP-A
P1
P2
250K
200K
150K
100K
50K
0
0
10^2
10^3
10^4
10^5


GNS 179 GFP-MCAK
SSC-A
GFP-A
P1
P2
250K
200K
150K
100K
50K
0
0
10^2
10^3
10^4
10^5

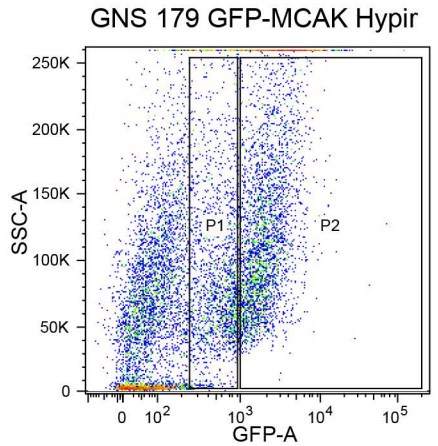

GNS 179 GFP-MCAK Hypir
SSC-A
GFP-A
P1
P2
250K
200K
150K
100K
50K
0
0
10^2
10^3
10^4
10^5

Figure S10

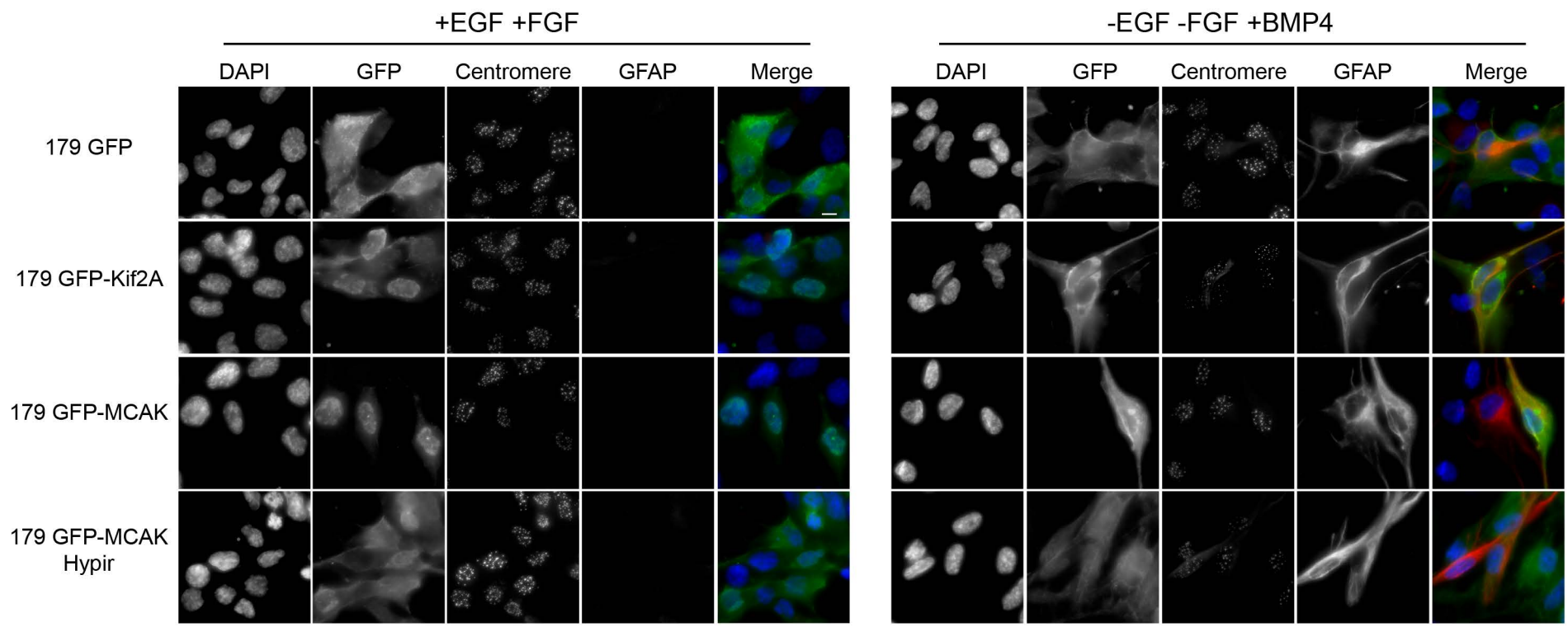

+EGF +FGF
-EGF -FGF +BMP4
DAPI
GFP
Centromere
GFAP
Merge
179 GFP
179 GFP-Kif2A
179 GFP-MCAK
179 GFP-MCAK Hypir

Figure S11