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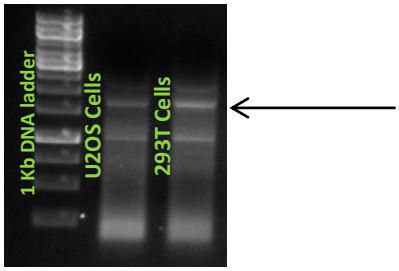

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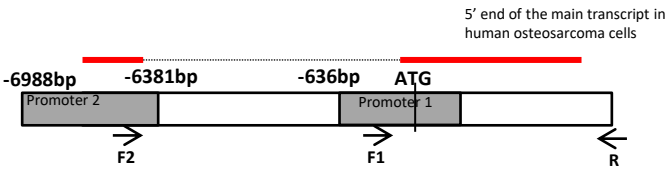

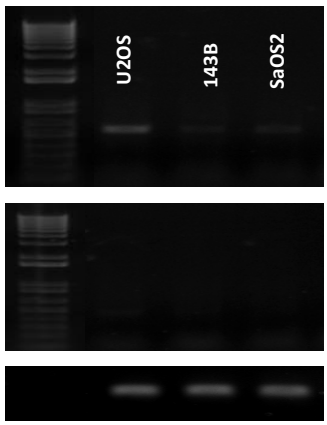

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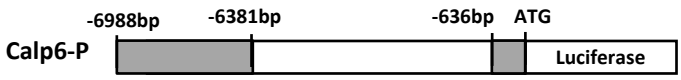

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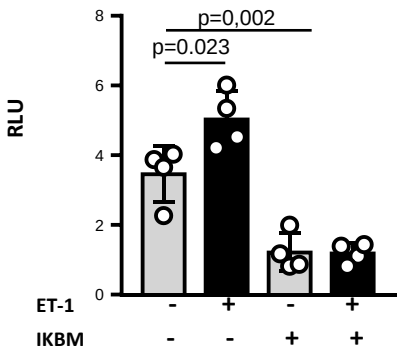
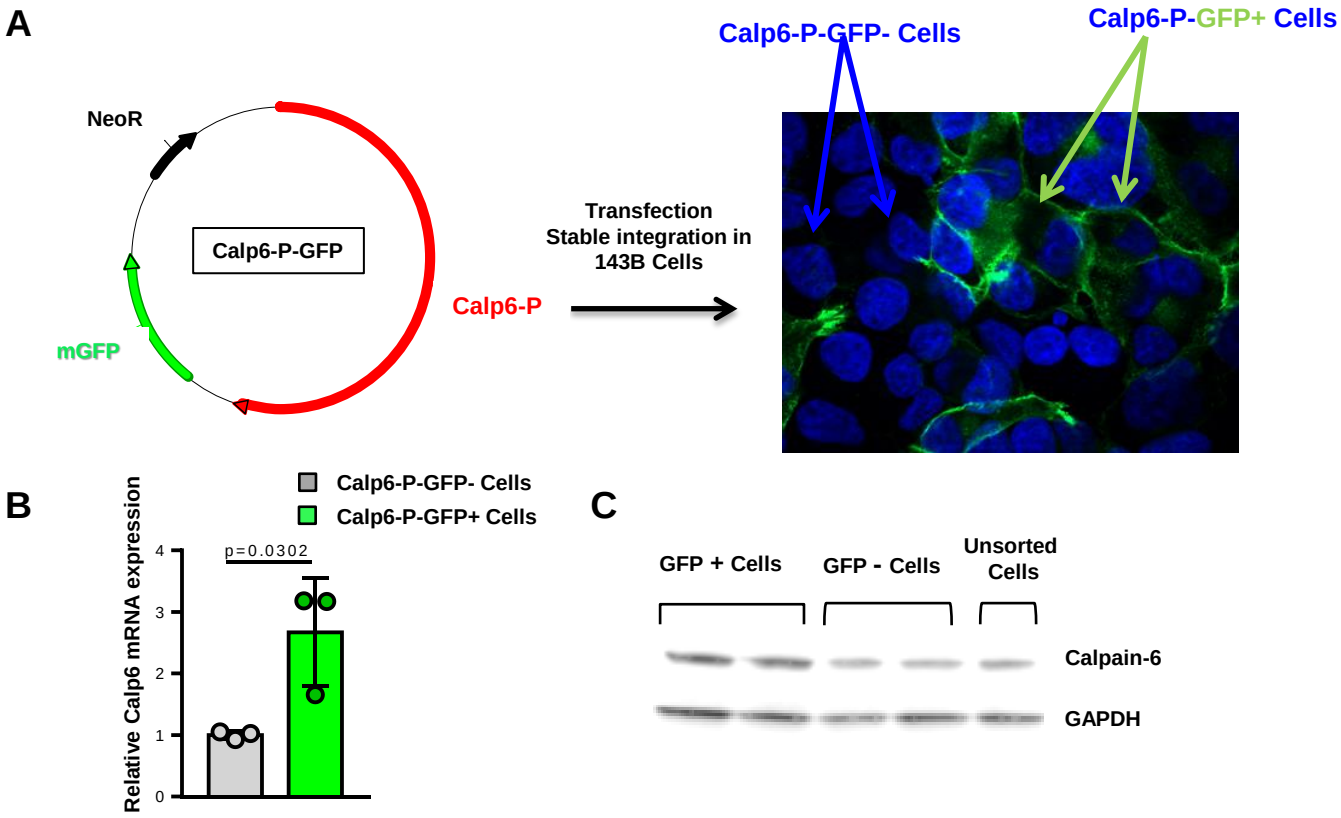
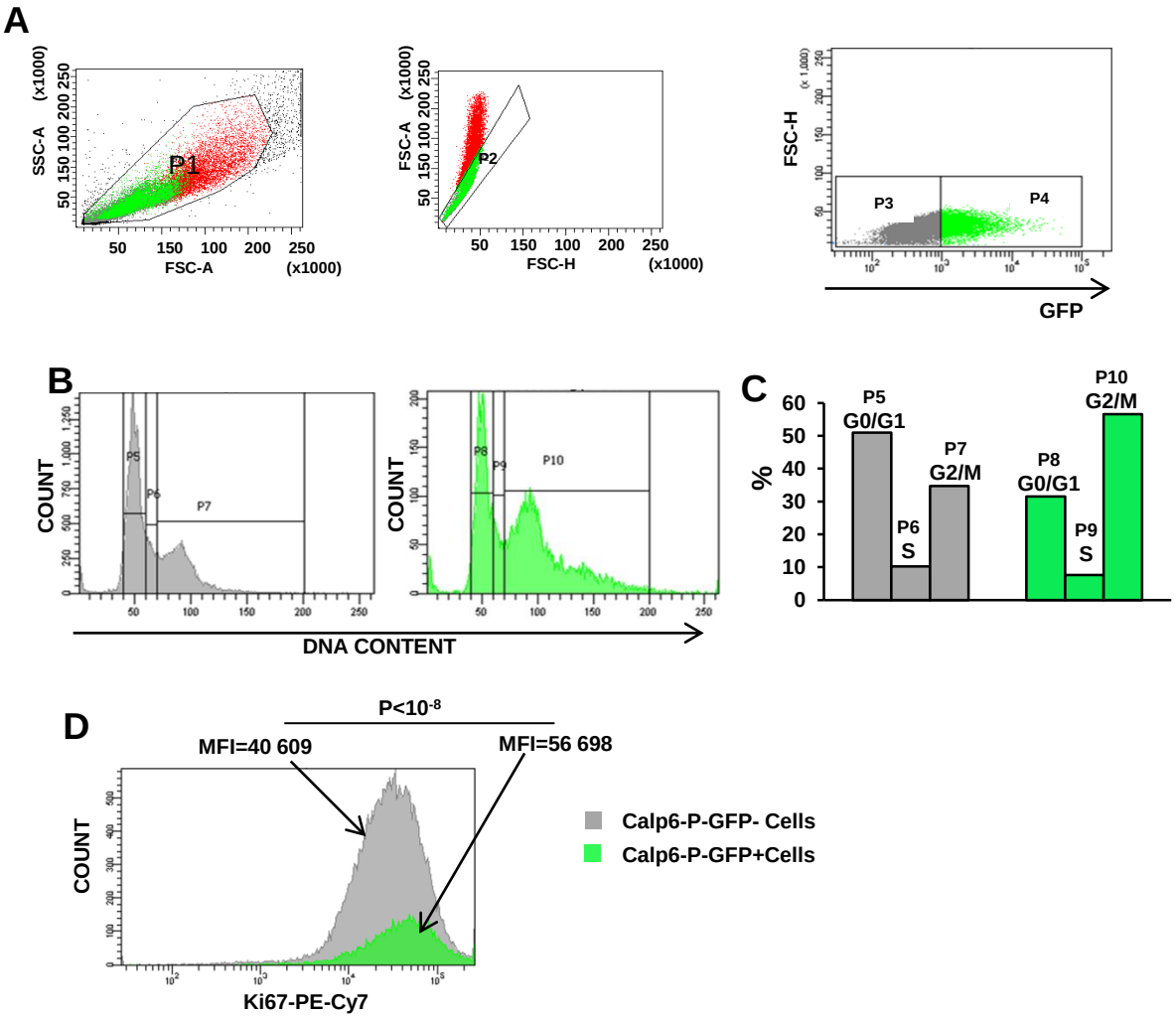


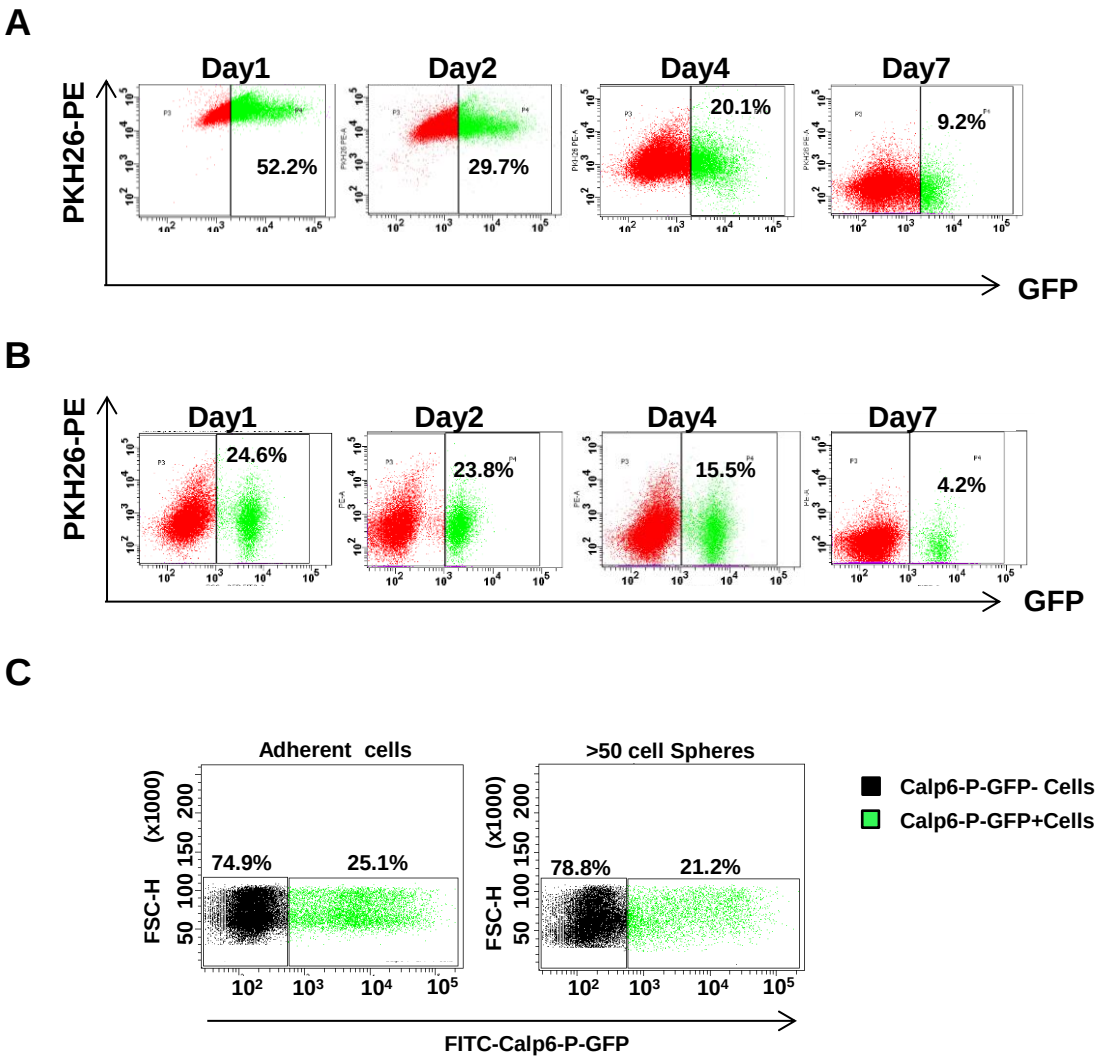
Supplementary Figure 1: **Identification of the active promoter and characterization of the regulatory sequence in the 5' sequence upstream coding region in *CAPN6***. (**A**) Rapid Amplification of cDNA Ends (RACE) was performed with mRNA extracts from U2OS and 293T cells. The internal primer was CTTTCGGGTAGCCACGAGCCAGGT to amplify the 5' end of the calpain-6 cDNA. (**B**) The longer PCR product (arrow) was cloned and sequenced. The sequence was aligned with the CAPN6 gene sequence showing that the mRNA originates from the distal promoter. Black letters are fragments of the CAPN6 sequence (Gene ID: 827; NC_000023.11) with putative distal and proximal promoters separated with the dotted line. Red letters are the sequence from the RACE experiment. (**C**) mRNA origin in the distal promoter was checked by RT-PCR with RNA extracts from U2OS, 143B and SaOS2 cell lines. F1 is the forward primer in the proximal promoter (promoter 1), F2 is the forward primer in the distal promoter (promoter 2), R is the reverse primer. Actin was a housekeeping gene. (**D**) The 6 988-bp sequence upstream ATG was cloned in the pGL4.10 vector (Promega). (**E**) Sarcoma cells were seeded in 24-well plates and transfected with the empty pGL4.10 plasmid or Calp6-P reporter construct with an empty vector or IKBM expressing vector (NF-KB inhibitor) and with phRL-SV40, a renilla expression plasmid that served as internal control. At 24 hr. after transfection, cells were cultured for 6 hr. in serum-free medium, then treated with 1 µg/mL endothelin 1 (ET-1) . Renilla and luciferase activity was measured by using chemoluminescent substrates. Data are mean ± SD from 4 wells evaluated by one way ANOVA. RLU, relative light units.



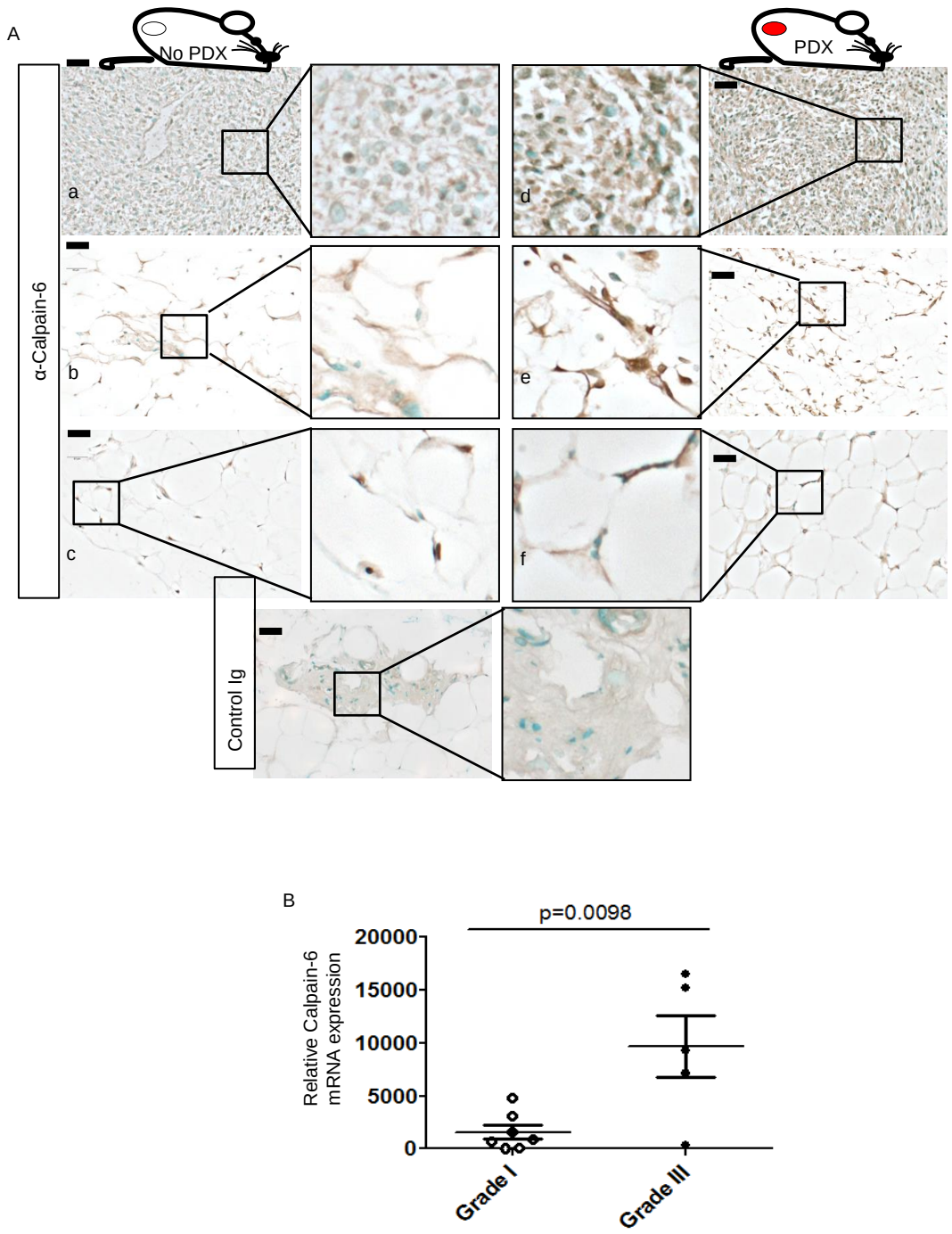
Supplementary Figure 2: **Calpain-6 and GFP expression are associated in human Calp6-P-GFP cells**. (**A**) Diagrammatic representation of the Calp6-P-GFP transgene. The reporter construct consisted in a sequence coding membrane GFP controlled by the calpain-6 regulatory sequence (Calp6-P). Sarcoma cells were transfected with this construction and selected with neomycin for stable integration. GFP expression was monitored by fluorescence microscopy. Calp6-P-GFP 143B cells were sorted according to GFP expression by flow cytometry in ImagoSeine platform (Paris Diderot University, Paris, France). (**B**) RT-PCR analysis of calpain-6 mRNA expression in sorted Calp6-P-GFP- and GFP+ 143B cells. Data are mean ± SD from 3 independent RNA extracts evaluated by one way ANOVA. (**C**) Western blot analysis of calpain-6 protein level with protein extracts from Calp6-P-GFP+, GFP- and unsorted cells. GAPDH was a loading control.



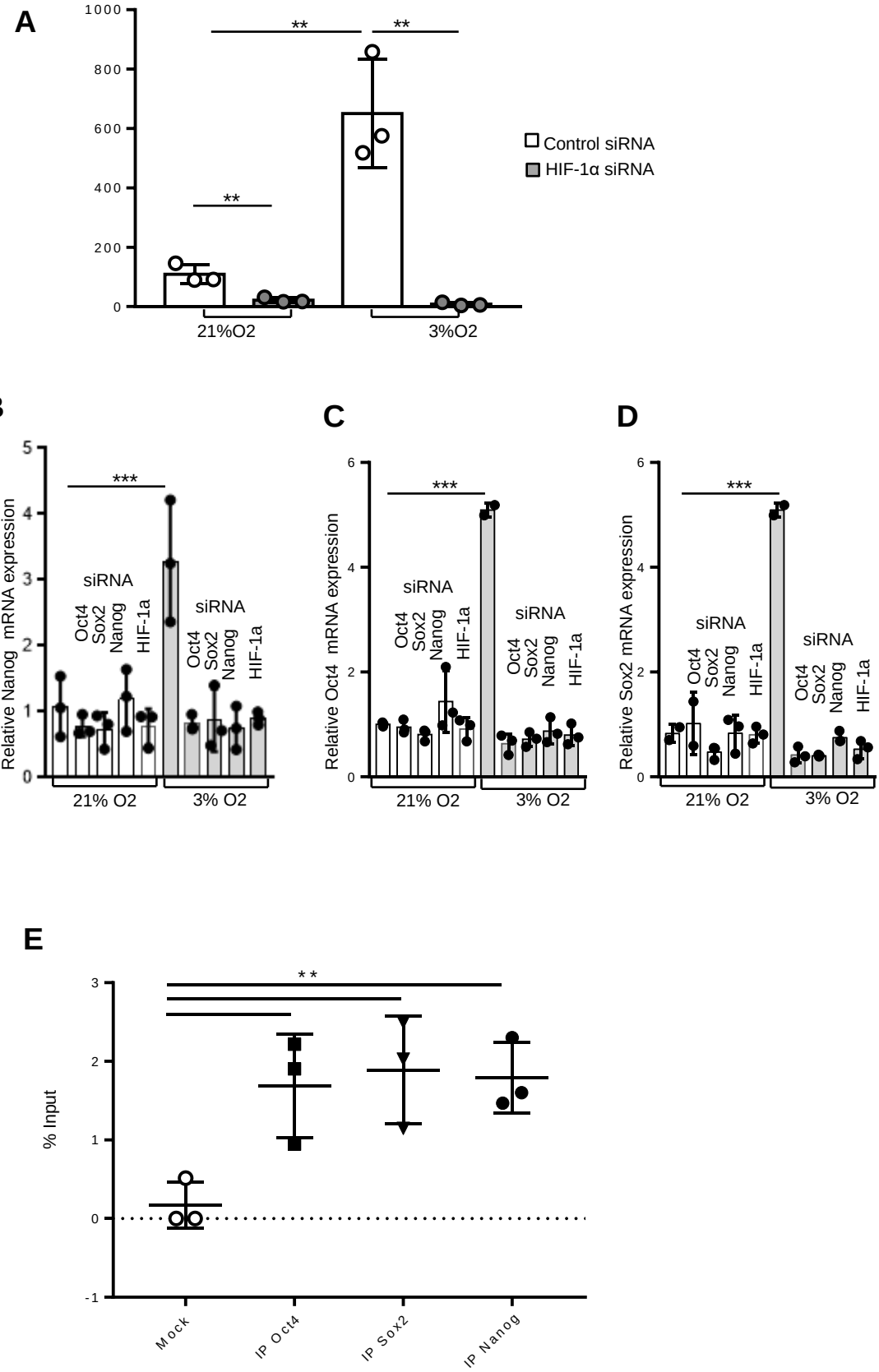
Supplementary Figure 3: **Switch in G2/M at the expense of G0/G1 and increased proliferation for calpain-6–expressing human TC71 Ewing cells.** (**A**-**C**) Flow cytometry of DNA content in Calp6-P-GFP- and GFP+ cells. (**A**) Gating strategy to select Calp6-P-GFP- and GFP+ cells. Starting number of cells was 50 000. The selected cells in P2 were analyzed for GFP expression. P3 and P4 contained 32 278 GFP- and 7684 GFP+ cells, respectively. (**B**) Dye Cycle Violet fluorescence was measured for GFP- and GFP+ cells. Cell cycle phases were determined according to DNA content. (**C**) The proportion of cells in each phase. (**D**) Calp6-P-GFP TC71 cells were labeled with a PeCy7-conjugated anti-Ki67 antibody. Mean fluorescence intensity (MFI) of Ki67 labeling for GFP- or GFP+ TC71 cells. N=50 000 cells. Means were compared by two-sided Student *t* test.



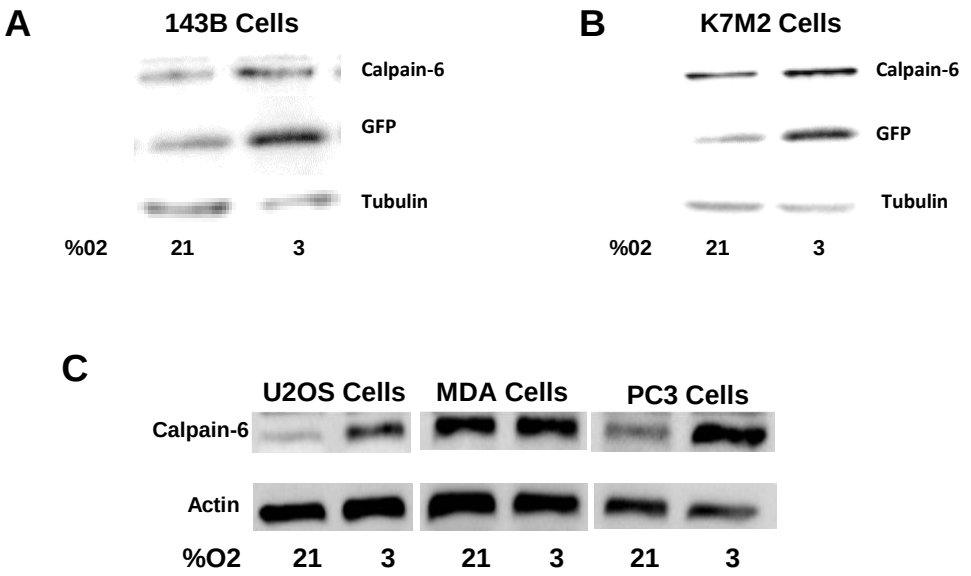
Supplementary Figure 4: Calp6-P-GFP+ cell population decreases during cell growth. (**A**,**B)** Flow cytometry of cell divisions. 143B cells (A) or K7M2 cells (B) were seeded at low density and labeled with PE-conjugated PKH26. Fluorescence of this membrane marker and GFP expression are reported at day 1, 2, 4 and 7. (**C**) Flow cytometry of the proportion of Calp6-P-GFP+ cells in adherent confluent culture and in large spheres comprising more than 50 cells. 50 000 cells were used as a starting cell population.



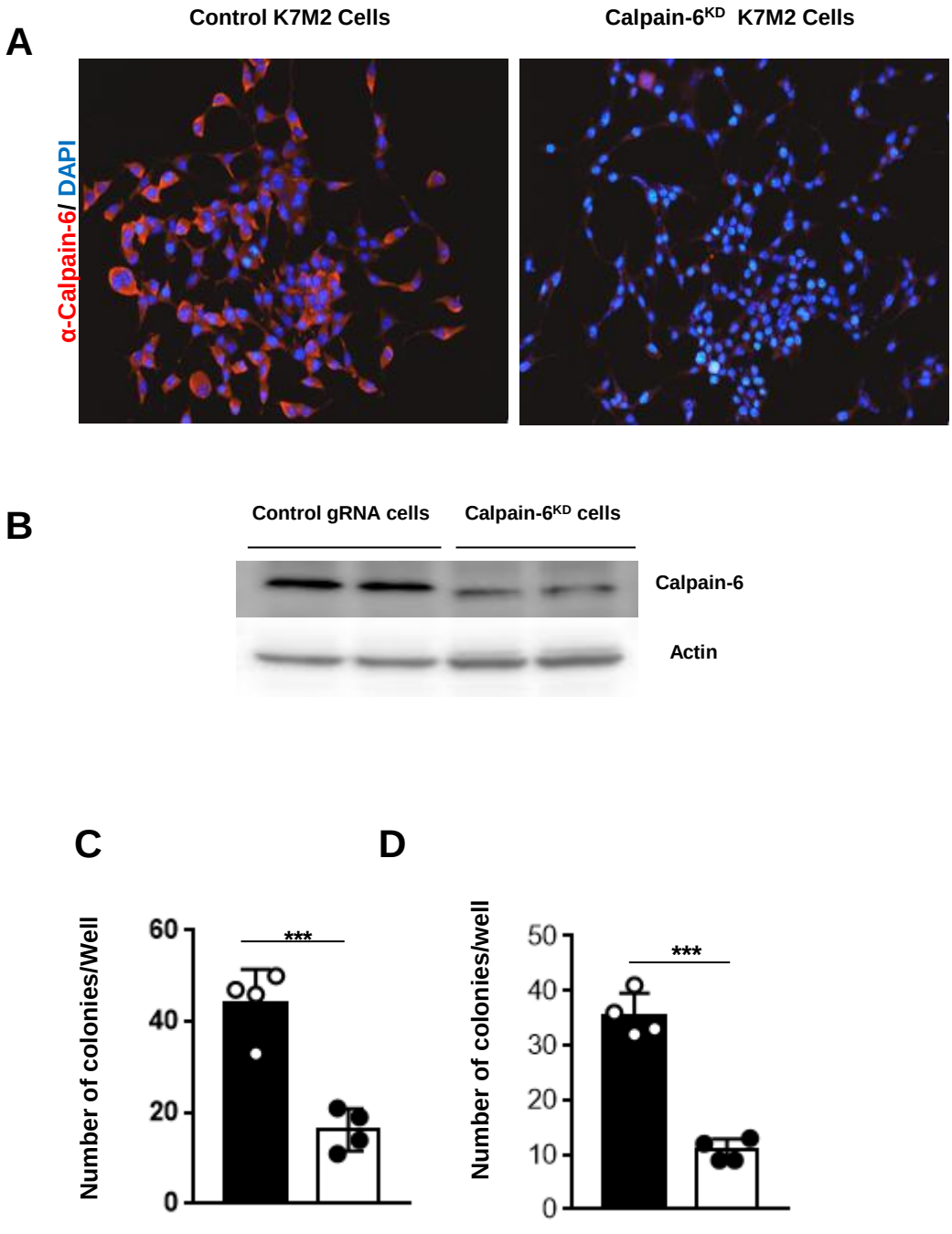
Supplementary Figure 5: Calpain-6 expression is associated with liposarcomas engraftment in mice and the highest grade. (A) Immunohistochemistry of calpain-6 in patient liposarcomas not engrafted (a, b, c) or successfully engrafted (d, e, f) in mice. Controls were rabbit Ig. (**B**) RT-PCR analysis of calpain-6 mRNA expression in patient samples of liposarcomas from grade I and III. Horizontal lines are mean; whiskers are SD..



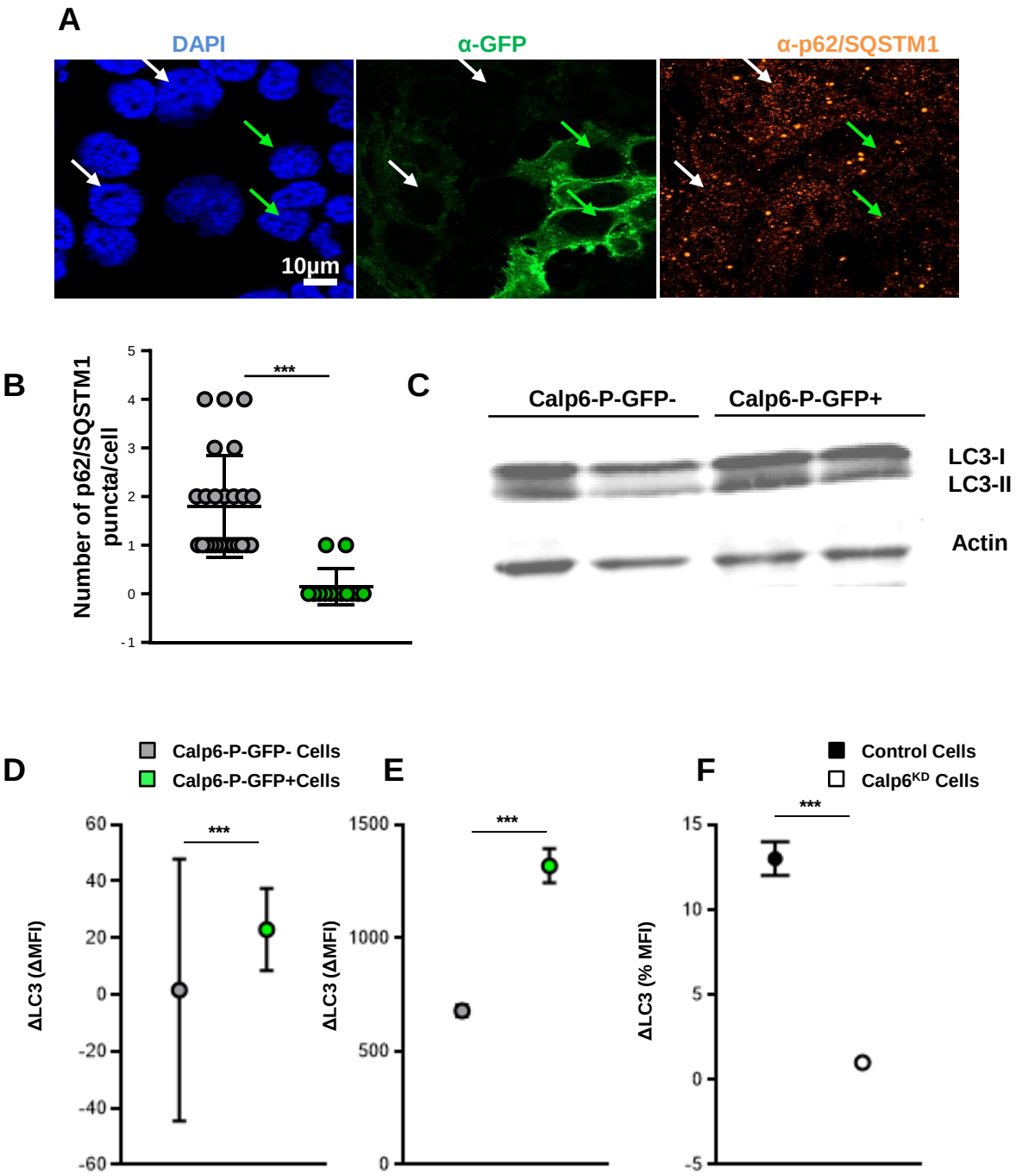
Supplementary Figure 6: Increased Oct4 and Nanog expression in hypoxia depends on the stem-cell pathway involving Oct4, Nanog and Sox2. (**A)** Sarcoma cells were seeded in 24-well plates and transfected with a control or HIF responsive elements containing plasmid (HRE) with control or HIF-1α siRNA. Luciferase served as reporter gene. The cells were cultured for 24H in 21% or 3% O2. phRL-SV40, a renilla expression plasmid that served as internal control. Renilla and luciferase activity was measured by using chemoluminescent substrates. Data are mean ± SD from 4 wells evaluated by one way ANOVA, RLU= relative light units. **(B-D**) RT-PCR analysis of siRNA inhibition of Oct4, Sox2, Nanog or HIF-1α on the hypoxia-dependent induction of Nanog (B) and Oct4 (C) and Sox2 (D). Data are mean ± SD in 3 independent wells evaluated by one way ANOVA. *** indicate p <0,001. (**E**) ChIP of genomic DNA from 143B cells with control Ig (Mock), anti-Oct-4 (IP Oct4), anti-Sox2 (IP Sox2) or anti-Nanog (IP Nanog) antibodies. Cells were cultured in 3% O_2. Immunoprecipitated chromatin was analysed by qPCR. Data are mean ± SD from 3 different replications. ** indicate p<0,01



Supplementary Figure 7: Hypoxia increases Calp6-P transactivation activity and calpain-6 expression. (**A-B**) Western blot analysis of calpain-6 and GFP protein levels from Calp6-P-GFP 143B (A) and K7M2 (B) cells. Cells were cultured for 24 hr with 21% or 3% O_2. Tubulin was a loading control. (**C**) Western blot analysis of calpain-6 protein level in cell lines cultured with 21% and 3% O_2. Actin was a loading control.



Supplementary Figure 8: Calpain-6 knockdown reduces stem cell colony formation. (**A**) Immunofluorescence of calpain-6 (red) in control and calpain-6 knockdown (calpain-6^{KD}) by RNA-guided CRISPR-Cas9 in K7M2 cells. (**B**) Western blot analysis of calpain-6 protein level in control and calpain-6^{KD} 143B cells. Actin was a loading control. (**C-D**) Control or calpain-6^{KD} human 143B (C) or TC71 (D) cells were seeded on non-adherent plastic coated with a growth factor-free matrix for cell attachment and cultured in serum-free stem cell medium. Data are mean ± SD of 4 independent wells evaluated by two-sided Student *t* test.



Supplementary Fig. 9: High autophagy in calpain-6 expressing cells. (**A**)) Representative immunofluorescence of GFP and p62/SQSTM1 in Calp6-P-GFP 143B cells. White and green arrows indicate GFP- and GFP+ cells. (**B**) Quantification of p62/SQSTM1 puncta in 143B cells cultured in 3% O_2. Data are mean ± SD in Calp6-P-GFP- and GFP+ cells by two-sided Student *t* test. (**C**) Western blot analysis of autophagosome-associated form of LC3, LC3-II, in 2 different protein extracts from sorted 143B Calp6-GFP- and GFP+ cells. (**D**) Flow cytometry quantification of ΔLC3 in mouse Calp6-P-GFP- and GFP+ K7M2 cells. n=89 000 GFP- cells n=3 300 GFP+ cells. (**E**) Flow cytometry quantification of ΔLC3 in human Ewing Calp6-P-GFP- and GFP+ TC71 cells. n=40 000 GFP- cells n=6 500 GFP+ cells. (**F**) Flow cytometry quantification of ΔLC3 in control and calpain-6^{KD} TC71 cells. n=50 000 control or calpain-6 KD cells. Cells were cultured in 3% O_2. Data are mean ± SD; *** indicate p values < 0.001 determined by two-tailed Student *t* test.

Supplementary data. Table I : The list of primers

			Forward	Reverse
PCR	RACE	CAPN6		5’-CTTTCGGGTAGCCACGAGCCAGGT-3’
	TSS Checking	F1	5’-CCTCATTCTAGGCATAGTTCAACAC-3’	5’-TGACTTGTTGCCCACCATTA-3’
		F2	5’-TGGGGTTACCTGGCTAACAG-3’	
Cloning	Calp6-P		5’-CGGGGTACCACTGTGATGCA GTATAGTATTTATAGGGA-3’	5’-TCCCCCGGGAGTGTTGAACTATGCC TAGAATGAGGCCTCATTCTAGGC-3’
	mGFP		5’-AGCACAGTGGCGGCCGCATTA AGGGTTCCGGATCATC-3’	5’-TCTAGAGTCGCGGCCGCTTACTTGT ACAGCTCGTCC-3’
qPCR	Calpain-6	Human	5'-ATGATCCCCTGATGAACCGC-3'	5'-GTAAGTGCGCAGGTCCTTCT-3'
		Mouse	5'-TGCTGTTTCCACCTGCCTAA-3'	5'-TGGGATGTCAGGCTAGACCA-3'
	Oct4	Human	5'-AGCCTGGGGTACCAAAATG-3'	5'-AGCGATCAAGCAGCGACTAT-3'
	Sox2	Human	5'-TTGCTGCCTCTTTAAGACTAGGA-3'	5'-CTGGGGCTCAAACTTCTCTC-3'
	Nanog	Human	5'-TTCCTTCCTCCATGGATCTG-3'	5'-CTTTGGGACTGGTGGAAGAA-3'
	IL-1	Human	5'-TTCGAGGCACAAGGCACAA-3'	5'-TGGCTGCTTCAGACACTTGAG-3'
	IL-8	Human	5'-GAGCCAGGAAGAAACCACCG-3'	5'-TGGCAAAACTGCACCTTCACA-3'
	IL-6	Human	5'-GGCACTGGCAGAAAACAACC-3'	5'-GCAAGTCTCCTCATTGAATCC-3'
	p16	Mouse	5'-CGGGGACATCAAGACATCGT-3'	5'-TGAGGCCGGATTTAGCTCTG-3'
	p21	Mouse	5'-GACCAGCCTGACAGATTTCTATC-3'	5'-TAAGGCCGAAGATGGGGAAG-3'
	Actin	Human	5'-GAGAAGAGCTACGAGCTGCCTG-3'	5'-GGTAGTTTCGTGGATGCCACA-3'
	Actin	Mouse	5'-GGCTGTATTCCCCTCCATCG-3'	5'-CCAGTTGGTAACAATGCCATGT-3'
	HPRT	Mouse	5'-TGAAGCGCTGCCAGTATGT-3'	5'-GGTCGCTCAGAGCCTTGTA-3'
	PPia	Human	5'-GTCAACCCCACCGTGTTCTT-3'	5'-CTGCTGTCTTTGGGACCTT-3'
	GAPDH	Human	5'-CGAGATCCCTCCAAAATCAA-3'	5'-TGTGGTCATGAGTCCTTCCA-3'
ChIP	Cap6-P	Human	5’-AGAGCTTGGTACAGCCCAAA-3’	5’-TCCAGGGTAAGTTTGCGAAT-3’

Supplementary data. Table II : The list of antibodies. IF: Immunofluorescence; FC: Flow Cytometry; WB: Western Blot; IHC: Immunohistochemistry; ChIP: Chromatine ImmunoPrecipitation.

Protein	Host	Supplier	Dilution
Luciferase	Rabbit	Santa-Cruz	1:200 (IF)
Ki67	Rat	eBiosciences	1:200 (FC)
Calpain-6	Rabbit	Abbexa	1:10000 (WB)
	Goat	Santa-Cruz	1:100 (IF)
Pimonidazole Adducts	Mouse	Hypoxyprobe	1:100 (IF)
HIF-1α	Rabbit	Santa-Cruz	1:100 (IF)
GFP	Goat	Abcam	1:10000 (WB)
			1:500 (IHC)
GATA4	Mouse	Santa-Cruz	1:500 (WB)
			1:100 (IF)
Actin	Rabbit	Sigma-Aldrich	1:1000 (WB)
α Tubulin	Mouse	Santa-Cruz	1:1000 (WB)
GAPDH	Mouse	Santa-Cruz	1:1000 (WB)
LC3	Rabbit	Sigma-Aldrich	1:1000 (WB)
			1:200 (IF)
LC3-Biotin	Rabbit	Novus Biological	1:200 (FC)
Lamp2	Rat	Abcam	1:100 (IF)
Oct4	Rabbit	Cell signaling	1:50 (ChIP)
Sox2	Rabbit	Cell signaling	1:50 (ChIP)
Nanog	Rabbit	Cell signaling	1:50 (ChIP)
p62	Rabbit	Santa-Cruz	1:100 (IF)
Biotin	Mouse	Miltenyi	1:200 (FC)