SUPPLEMENTARY APPENDIX

Uncoupling of the Hippo and Rho pathways allows megakaryocytes to escape the tetraploid checkpoint

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Supplementary materials and methods

Cultures of MKs and erythroblasts derived from human CD34$^+$ cells in serum-free liquid medium

CD34$^+$ cells were isolated using immunomagnetic beads (AutoMacs; Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in serum-free medium in the presence of recombinant human thrombopoietin (TPO) (10 ng/mL; Kirin Brewery, Tokyo, Japan) and stem cell factor (SCF) (5ng/mL; Biovitrum, Stockholm, Sweden) to induce MK differentiation and in SCF (10ng/ml), erythropoietin (EPO, 3u/mL) and interleukin 3 (IL-3) (1ng/mL) for erythroid differentiation.

Cell transduction

CD34$^+$ cells were cultured with SCF and TPO and transduced at days 4-5 of culture with lentiviruses encoding GFP and three different short hairpin RNA (shRNA) targeting p53 (0, 2 and 4). Cells were also transduced with mCherry$^+$ lentivirus encoding shYAP or scrambled shRNA (Cnt). Lentiviral stocks were prepared and stored as previously described. (1) Details of the shRNA sequences are given in supplementary materials and methods.

List of antibodies

anti-p53, -BCL2A, –beta actin, γ-tubulin (Sigma-Aldrich), anti-BAX (Oncogene), anti-p21 (Beckton Dickenson), anti-BCL-2, LATS2, YAP, P-YAP(S127) and anti-BclxL (Cell Signaling), anti-MDM2 (BD Pharmingen), anti- PGC1alpha (Abcam), anti-GAPDH (Santa Cruz Biotechnology incorporation) and rat anti-Hsc70 (Stressgen).

List of qRT-PCR primers :

Gene Name	Sequence 5'-3'
p53	TTTGCGTGTGGAGTATTTGGAT
p53	TGTAGTGGATGGTGGTACAGTCAGA
DR5	CTCCTGAGATGTGCCGGAA
DR5	ATCACCGACCTTGACCATCC
BAK	CCCTGCCCTCTGCTTCTG
BAK	AAACGTAGCTGCGGAAAACC
Bax	GGTTGTCGCCCTTTTCTACTTTG
Bax	CAGTTCCGGCACCTTGGT
HPRT	GGCAGTATAATCCAAAGATGGTCAA
HPRT	TCAAATCCAACAAAGTCTGGCTTATAT

PPIA	GTCGACGGCGAGCCC
PPIA	TCTTTGGGACCTTGTCTGCAA
Noxa	CGGAGATGCCTGGGAAGAA
Noxa	CCAAATCTCCTGAGTTGAGTAGCA
MDM2	ACCACCTCACAGATTCCAGCTT
MDM2	GCACCAACAGACTTTAATAACTTCAAA
Bcl-2	GGCTGGGATGCCTTTGTG
Bcl-2	CAGCCAGGAGAAATCAAACAGA
MDM4	TCCAGAAAAAGAACTACAGAAGACGAT
MDM4	TCCAAGGTCCAGCCTAGATGTT
Bcl2L1	GGGCATTCAGTGACCTGACA
Bcl2L1	CCTGGGGTGATGTGGAGCTGGG
Bax	GGTTGTCGCCCTTTTCTACTTTG
Bax	CAGTTCCGGCACCTTGGT
p21	CCTTGTGGAGCCGGAGCT
p21	TTGCTGCCGCATGGG
Puma	CCCTGCCAGATTTGTGAGACA
Puma	CAGGAGTCCCATGATGAGATTGTA
LATS1	ACCATCCACGGCAAGATAGC
LATS1	GTGCAGCTCTCCGCTCTAAT
LATS2	CGCCCCTGGAGAGAGTGA
LATS2	CCTTTTGAAAATGTTCTTTCCTTCC
YAP1	TGACCCTCGTTTTGCCATGA
YAP1	GTTGCTGCTGGTTGGAGTTG
CTGF	CAAGGGCCTCTTCTGTGACTT
CTGF	GGTACACCGTACCACCGAAG
CYR61	CGCCTTGTGAAAGAAACCCG
CYR61	GGTTCGGGGGATTTCTTGGT
INHIBA	TTGCCAACCTCAAATCGTGCT
INHIBA	CCCACACTCCTCCACGATCAT
FSTL1	AGGGGACTCTGTGTTGATGC
FSTL1	GTTTCATCCTCCAGGGCACA
PGC1alpha	CCTGCATGAGTGTGTGCTCT
PGC1alpha	CAGCACACTCGATGTCACTCC
TAZ	TGGACCAAGTACATGAACCACC
TAZ	CTGGTGATTGGACACGGTGA
Cox4i	GGAGTCTTCCTCGATCCCGT
Cox4i	AGAGGTGGAAATTGCTCGCT
NRF1	AGGAACACGGAGTGACCCAA
NRF1	TGCATGTGCTTCTATGGTAGC
MOB1	TATGTTGCCTGAGGGAGAGGA
MOB1	GACATGACTGGACAGCTTGC
ATP synthase	TGCAAGGAACTTCCATGCCTC
ATP synthase	CGCCCAGTTTCTTCAAGATCAA
Cyt c	CTTTGGGCGGAAGACAGGTC
Cyt c	TTATTGGCGGCTGTGTAAGAG
PGC1beta	CGCTTTGAAGTGTTTGGTGAGATTG
PGC1beta	GCTGGAAGGAGGGCTCGTTG
TFAM	AGATTGGGGTCGGGTCACT
TFAM	CAAGACAGATGAAAACCACCTC
Cox5b	ATGGCTTCAAGGTTACTTCGC
Cox5B	CCCTTTGGGGCCAGTACATT

Sequences of shRNA

Three different short hairpin RNA (shRNA) targeting p53 (0, 2 and 4) shRNA-0: *gactccagtggtaatctac;* shRNA-2: *gagggatgtttgggagatg;* shRNA-4: *cggcgcacagaggaagaga;*

a short hairpin RNA (shRNA) targeting YAP1: *ccagttaaatgttcaccaa;* a shRNA control: *ttctccgaacgtgtcacgt* were used.

Transmission electron microscopy

To study the megakaryocyte morphology, samples were washed in 1× PBS, then fixed in 1.5% glutaraldehyde for 1 hour, and washed 3 times in 0.1 M phosphate buffer, pH 7.4. For morphologic examination, samples were post-fixed in 1% osmic acid, dehydrated in ethanol, and embedded in Epon by standard methods. Samples were counterstained and were observed on a Philips CM 10 electron microscope (Philips, Eindhoven, The Netherlands). To study the mitochondrial morphology by TEM, at least $2x10^6$ cells were pelleted. The medium was discarded and replaced by 2% glutaraldehyde (EMS, Hatfield, PA, USA) in 0.1 M Sörensen buffer pH=7.4, for 1 h at room temperature. Cells were post-fixed for 1 hour at room temperature with 1% osmium tetroxide and 1% potassium ferrocyanide (Sigma-Aldrich,France) (EMS,Hatfield, PA, USA) in cacodylate buffer. They were dehydrated by increasing concentrations of ethanol and finally embedded in Epon 812 epoxy resin (EMS, Hatfield, PA, USA). The polymerization was carried out by heating the sample during 48 hours at 56°C. Samples were then sectioned with a microtome (thickness 70 nm), and the sections were collected on collodion-carbon-coated copper grids. Sections were contrasted using aqueous uranyl acetate 2% (w/v) (Merck, France) and lead citrate solutions (Reynold's stain). The samples were observed with Zeiss 902 TEM in the filtered zero loss modes using a CCD array detector (Megaview III, Olympus). The sections were analysed for the number of mitochondria in a given section as well as the size of the mitochondria. The sizes of mitochondria were given as diameters of theoretical spheres using the SIS software (Olympus). To estimate the number and size of mitochondria in control and shYAP infected MKs, at least 18 sections corresponding to 18 individual MKs were analyzed per sample. The mean number of mitochondria per section and the mean size were plotted.

Micro-array analysis

Micro-array analysis data is part of a previously published study. (2) The raw data may be found at the Array Express data repository at the European Bioinformatics Institute under the accession numbers E-MTAB-1452. Analysis was performed according to the published protocol. (2)

ATP assay

The assay was performed according to the manufacturer's protocol (Abcam, Cambridge, UK). Briefly, MKs transduced with either shYAP or scrambled shRNA were stained with Hoechst and sorted on ploidy. The luminescence was read in a 96 well format in GloMax Multi+ luminometer (Promega, Wisconsin, USA).

NAD^+/NADH assay

The assay was performed using NAD/NADH Glo Assay according to the manufacturer's protocol (Promega,). Briefly, MKs transduced with either shYAP or scrambled shRNA were stained with Hoechst 33342 and sorted on ploidy. The luminescence was read in a 96 well format in GloMax Multi+ luminometer (Promega).

Apoptosis assay

Apoptosis was measured by Annexin V detection kit (BD Pharmingen, San Diego, CA). Mitoprobe $DiIC_1(5)$ vitality and apoptosis assay were performed on the sorted $CD41^+$ cells at day 12 of culture (Molecular probes Life Technology). Apoptosis and cell viability were measured on a LSRII (Becton Dickinson Mountain View, CA).

BrDU assay

Cells were incubated with 10 μM BrDU for 1 hr at 37°C, stained with the anti-CD41-APC (BD Pharmingen) for 30 min at 4°C and with Hoechst 33342 and analyzed on LSRII with the Cellquest software package (BD Biosciences).

Mitochondrial staining andanalysis of mitochondrial mass

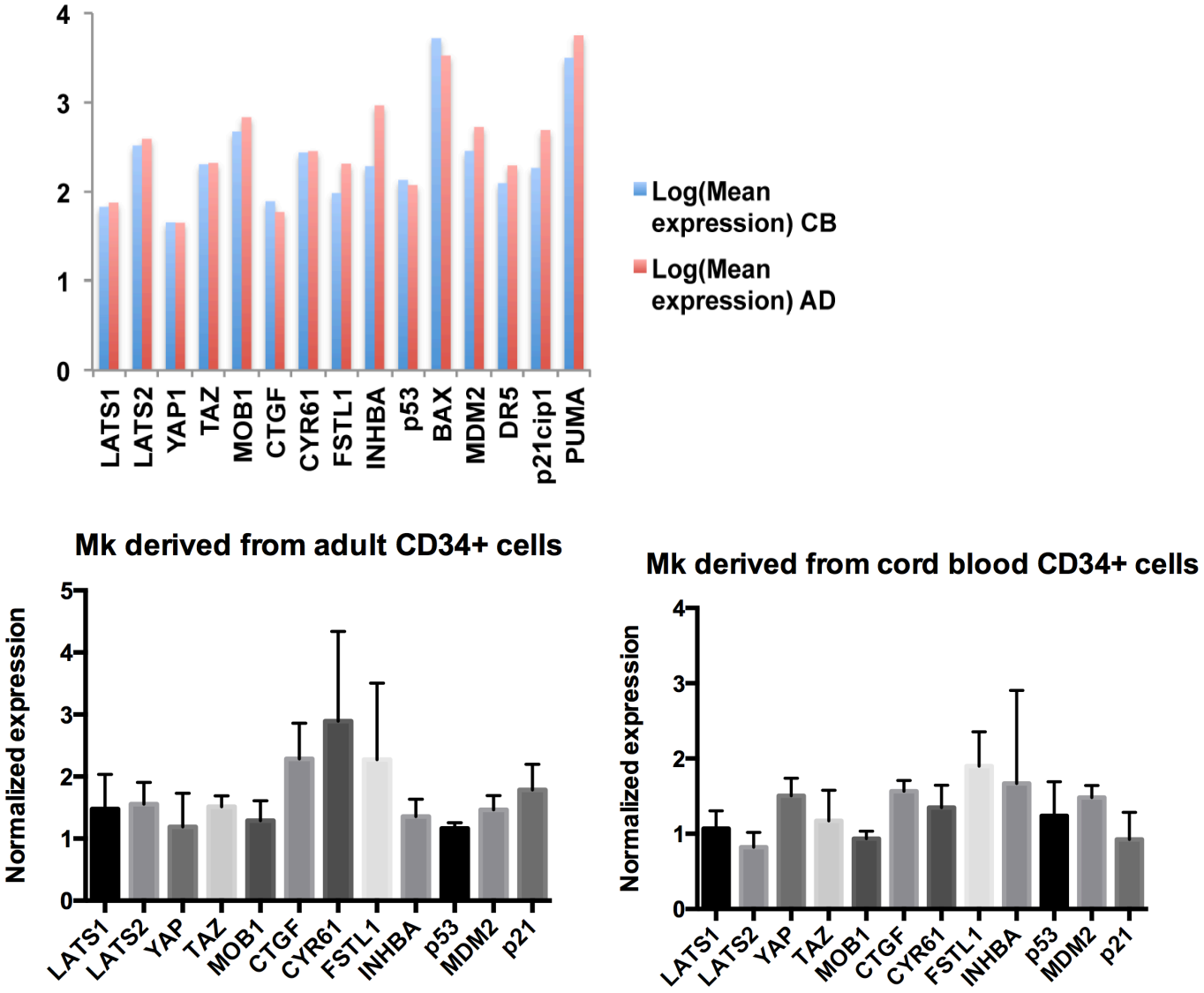
MK cells were grown in media containing mitotracker red (100 nM) or mitotracker green dyes (50 nM) (Molecular probes, Life Technologies) for 30 min. Cells were washed twice in 1X PBS to remove excess mitotracker dye. After washing, cells were observed directly by confocal microscopy. For flow cytometric analysis, cells were co-stained with Hoechst 33342 and anti-CD41 antibody. In MKs

infected with scrambled or shYAP, the $CD41^+$ $mcherry^+$ cell fraction was analyzed for mitotracker green and Hoechst 33342 intensity. The intensities of mitotracker dye (as represented by intensity of mitotracker green) at each ploidy fraction (as represented by the intensity of Hoechst 33342) was plotted.

REFERENCES

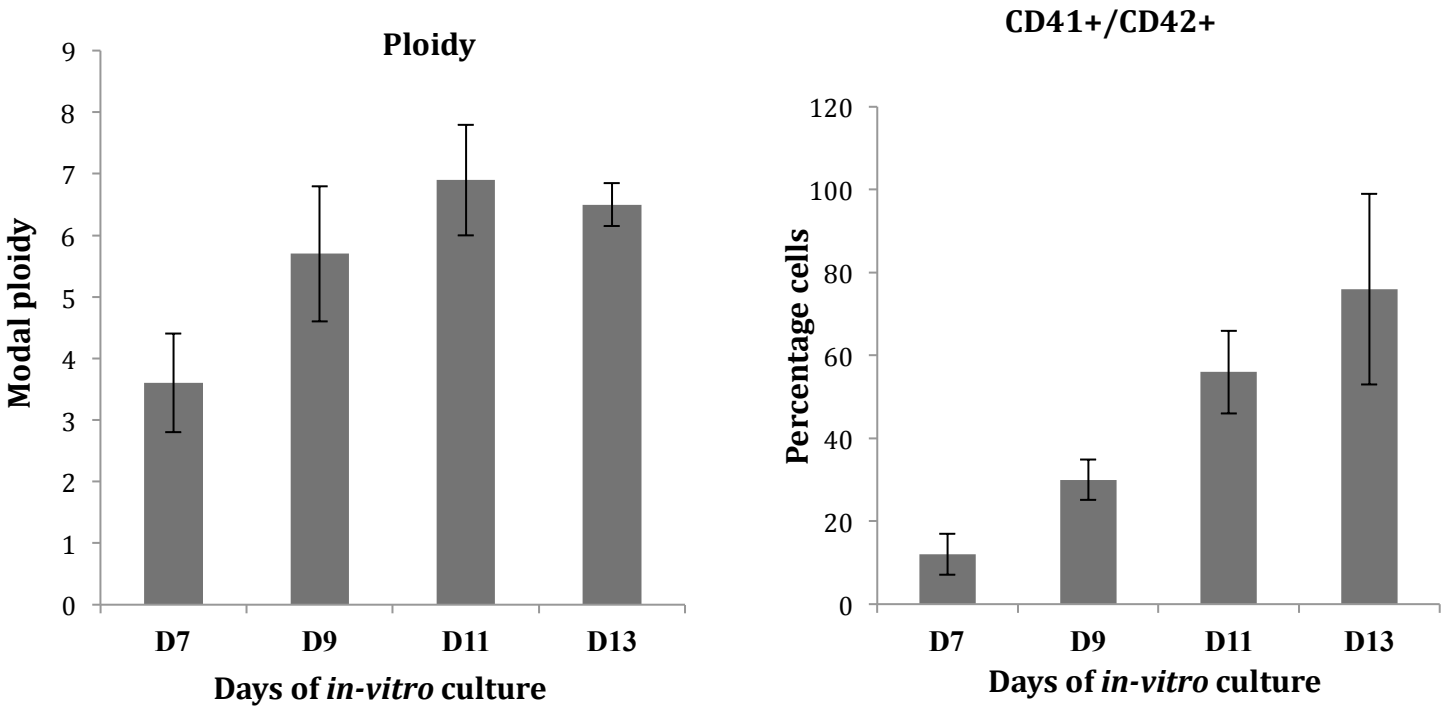
1. Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science. 1996 Apr 12;272(5259):263-7.
2. Bluteau O, Langlois T, Rivera-Munoz P, Favale F, Rameau P, Meurice G, et al. Developmental changes in human megakaryopoiesis. J Thromb Haemost. 2013 Sep;11(9):1730-41.

SUPPLEMENTARY FIGURE S1



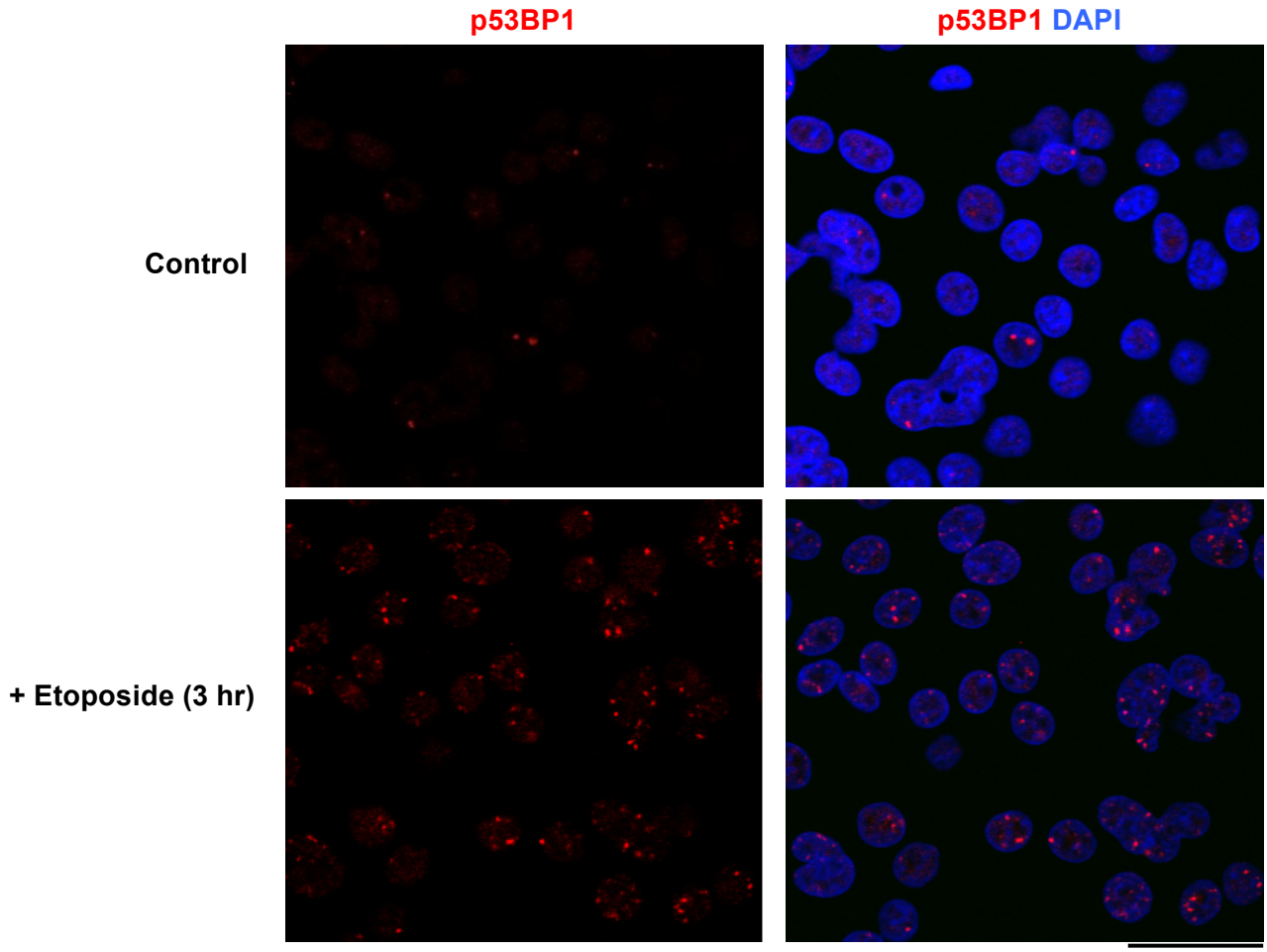
S1: Hippo-p53 pathway gene expression. Upper panel : The micro-array expression of Hippo-p53 circuit genes in MKs derived from cord blood (CB) and adult cytapheresis (AD). Plot indicates the Log of the normalized mean expression of the individual genes. The raw data may be found at the Array Express data repository at the European Bioinformatics Institute under the accession numbers E-MTAB-1452. **Lower panel** : Relative expression of Hippo-p53 pathway genes normalized against HPRT in MKs derived from adult or cord blood CD34+ cells. Data represents mean ± SEM of three independent experiments.

SUPPLEMENTARY FIGURE S2



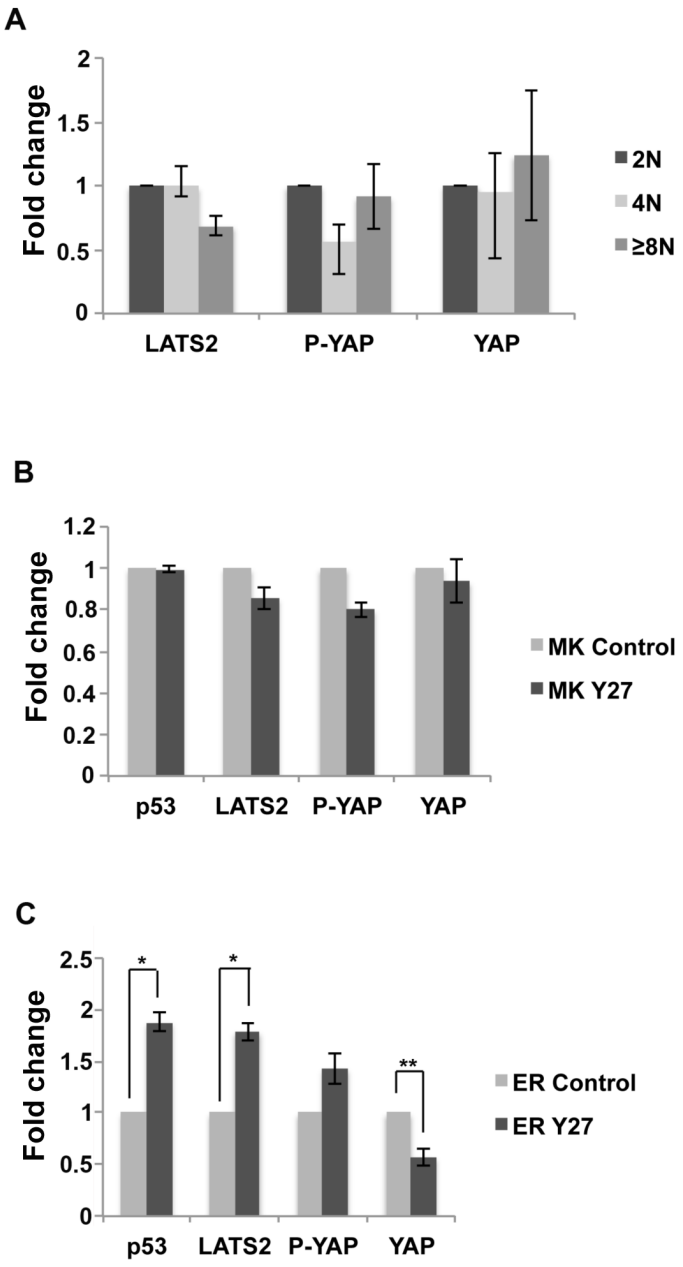
S2: Ploidy distribution in MKs. Left panel : The distribution of modal ploidy in *in vitro* cultured human adult MKs at different days of culture. Right panel : The percentage of CD41+CD42+ MKs in culture at the days analysed is shown. The data in this figure represents the mean ± SEM of two independent experiments.

SUPPLEMENTARY FIGURE S3



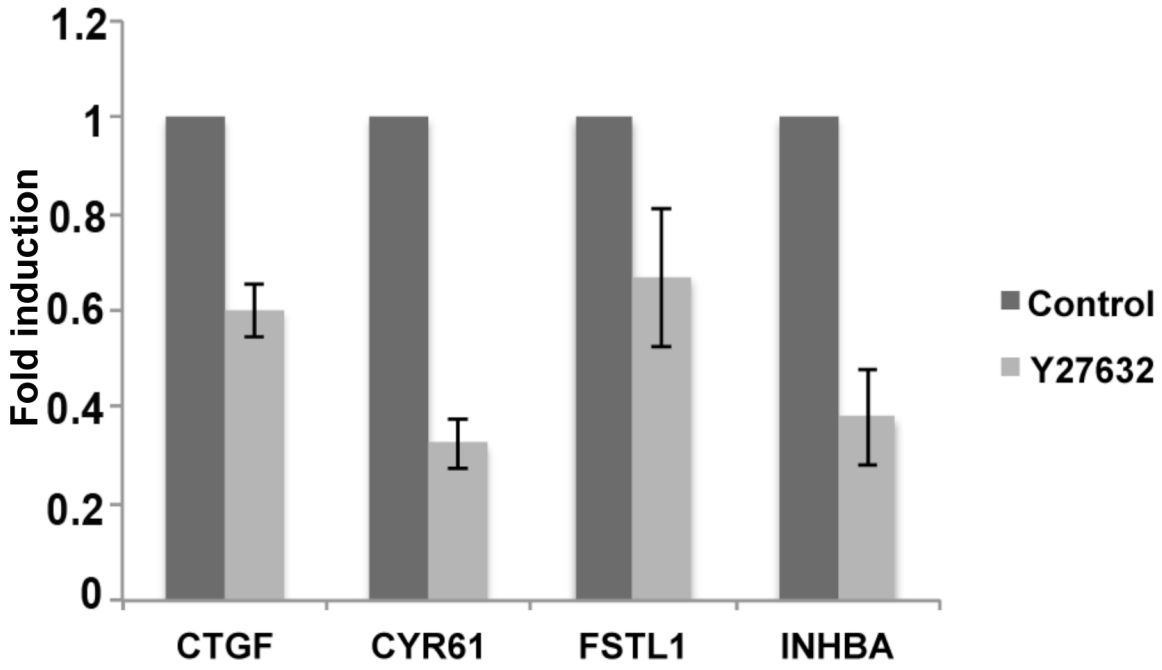
S3 : **Genotoxicity of Etoposide dose.** Control MKs and MKs treated with Etoposde were stained for p53-BP1 and DAPI. p53BP1 foci in the nucleus indicates DNA double strand breaks. A representative image of three independent experiments is shown. Scale bar = 30 μm.

SUPPLEMENTARY FIGURE S4



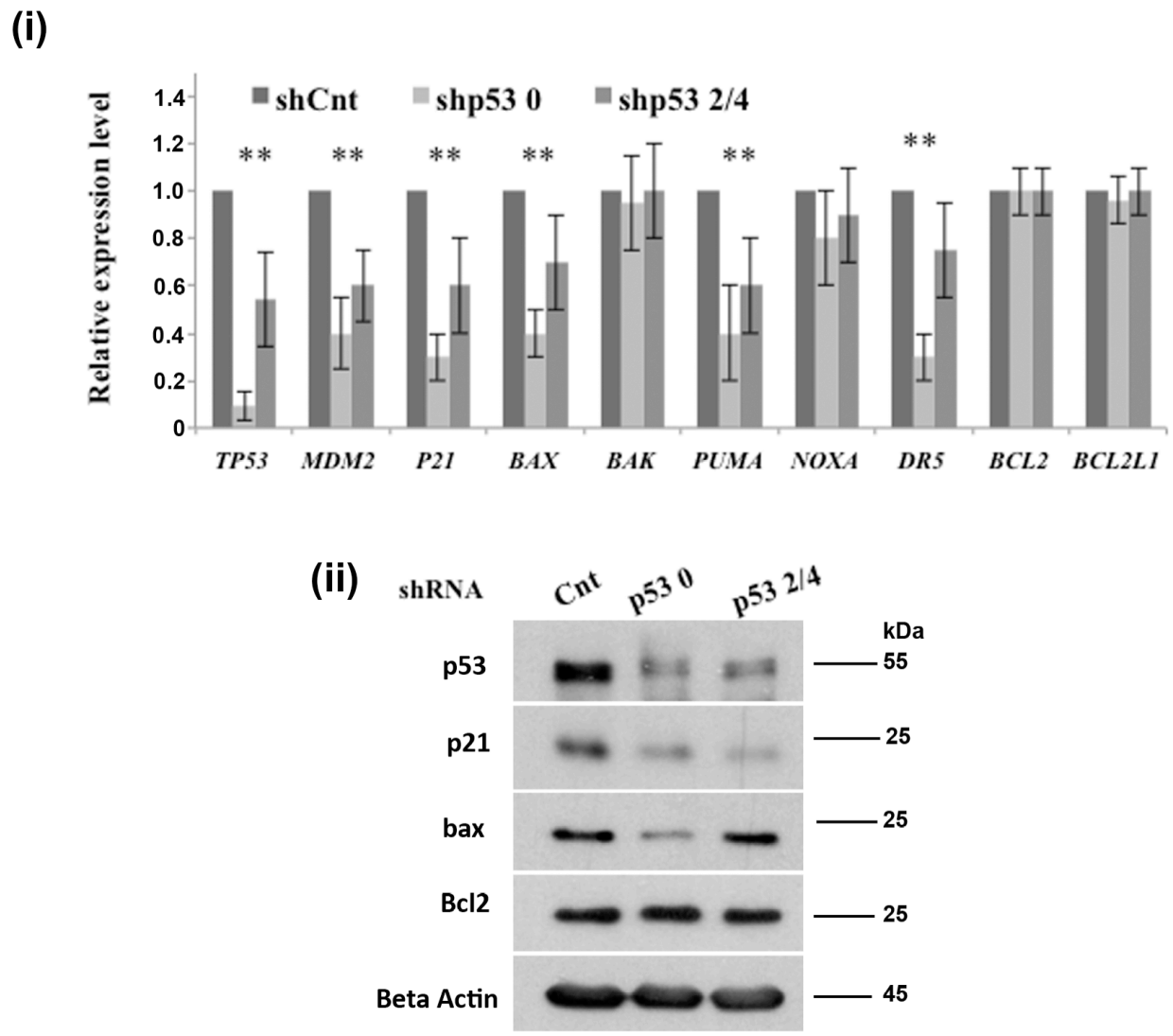
S4 : **Densitometric analysis of western blots corresponding to Figure 3.** (A) Densitometric analysis of western blot (Figure 3C) normalized against HSC70. Normalized intensity at 2N ploidy was considered as 1 and the corresponding intensities for 4N and ≥8N were plotted (n=3). (B-C) Densitometric analysis of western blot (Figure 3E) normalized against GAPDH. Normalized intensity of control MK and erythroblasts (ER) was considered as 1 and the corresponding intensities for Y27632 treated samples were calculated. Data represents mean ± SEM (n=3, **p<0,005, *p<0,02)

SUPPLEMENTARY FIGURE S5



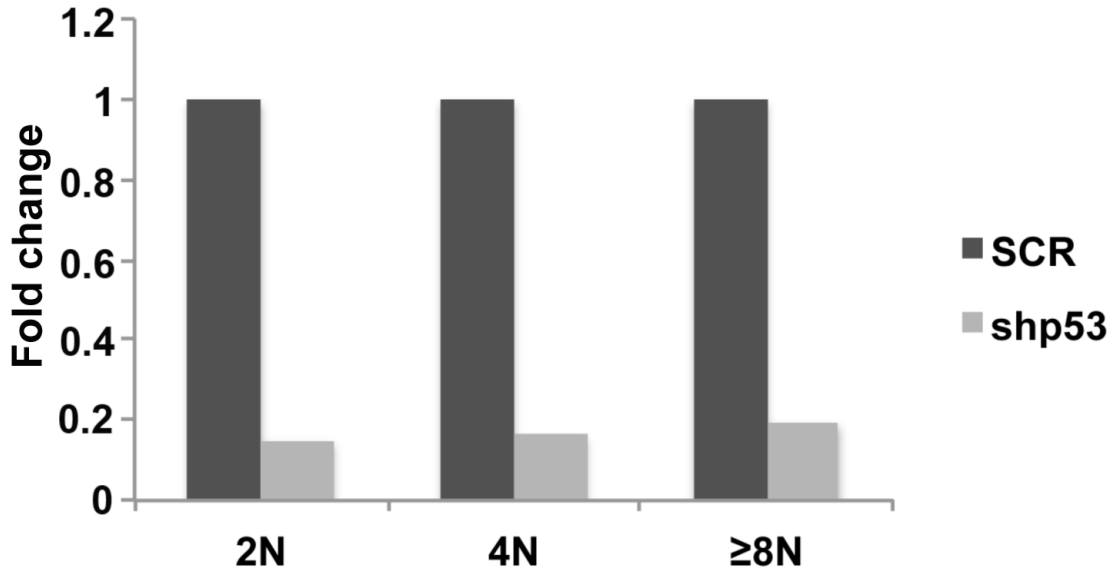
S5 : YAP target gene expression in erythroblasts treated with ROCK inhibitor. *In vitro* cultured CD71+ erythroblasts were treated with/without 10μM Y27632. The expression of YAP target genes was analysed by qRT-PCR. Data indicates relative expression in erythrobalsts treated with Y27632 with respect to control cells and normalized against *HPRT*. Data represents mean ± SEM (n=3, $p<0.02$).

SUPPLEMENTARY FIGURE S6



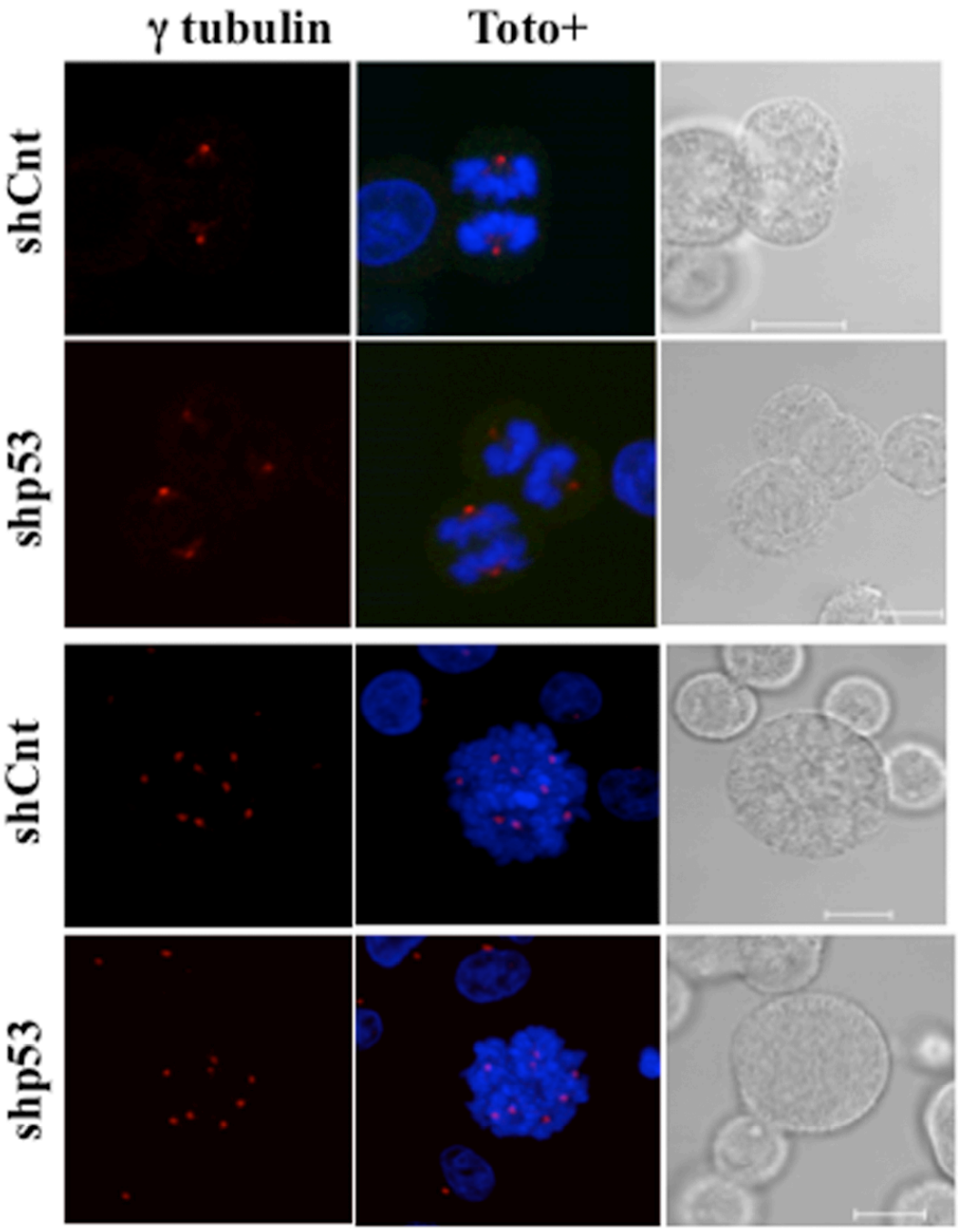
S6 : **Validation of p53 knockdown by shRNA.** MKs were transduced at day 4 of culture with a control lentivirus or a lentivirus encoding either shRNA p53-0 or shRNAs p53-2/4. (i) Real-time PCR was used to quantify p53-dependent genes in *p53* knockdown MKs at day 9 of culture. The relative expression was normalized against *HPRT*. Data represents mean ± SEM (n=5, **p<0.01). (ii) Western blot analysis of p53 protein level in $GFP^+/CD41^+$ sorted cells was analyzed at day 9 of culture. β-actin was used as internal loading control. A representative image of three independent experiments has been shown.

SUPPLEMENTARY FIGURE S7



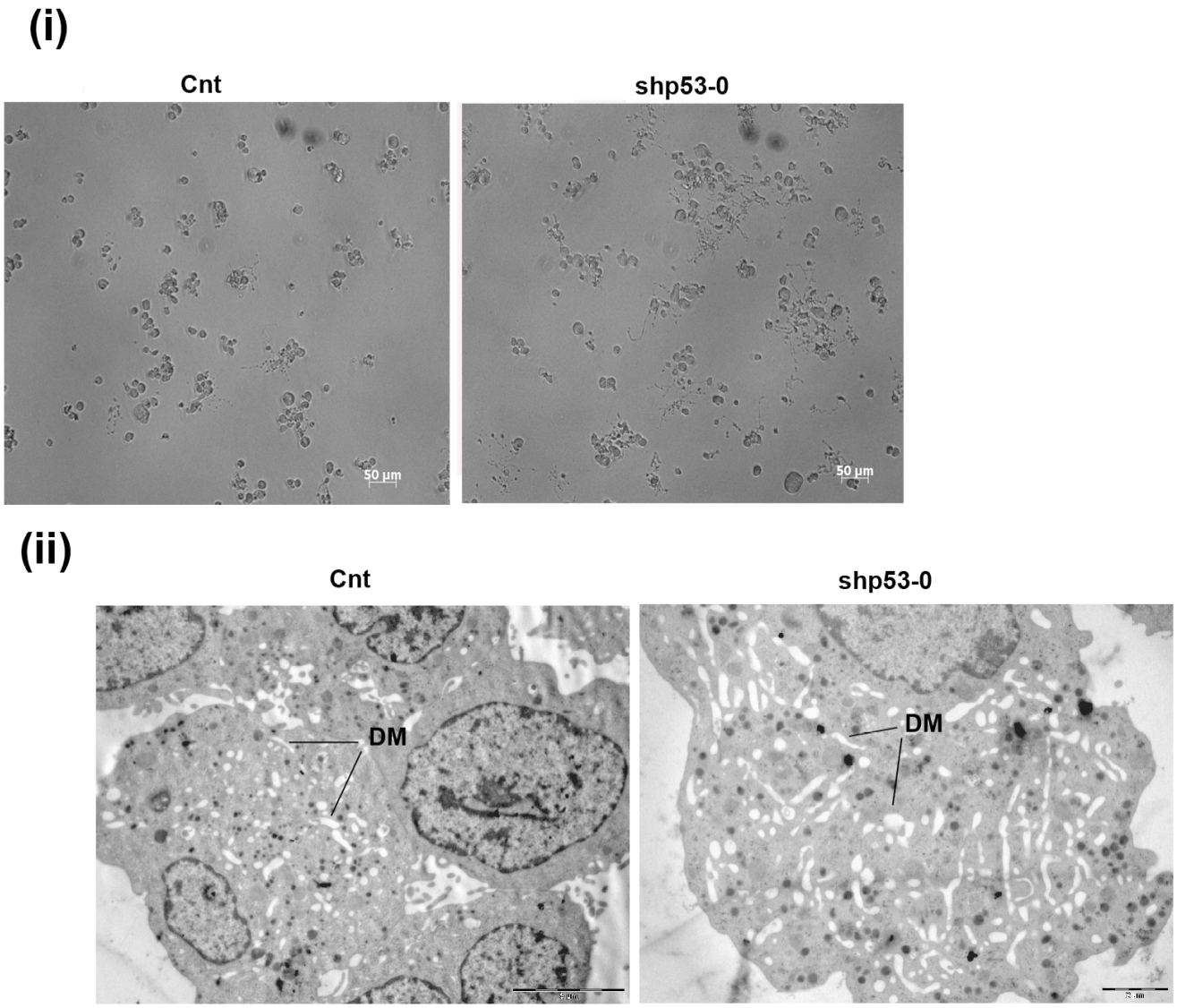
S7 : **Validation of p53 knock down across the various ploidy states.** Real-time PCR was used to quantify p53 expression in *p53* knockdown MKs sorted on their ploidy at day 10 of culture. The relative expression was normalized against *HPRT*. Normalized expression in scrambled MKs was considered as 1 and the corresponding relative expression in shp53 MKs was calculated for each ploidy state. Figure represents data obtained for one biological sampe with three technical replicates.

SUPPLEMENTARY FIGURE S8



S8 : **Effect of p53 knockdown on mitosis.** Effect of *p53* knockdown on the mitotic or endomitotic process as observed by immunofluorescence labeling of shp53-0 and Cnt $CD41^+$ MKs. γ-tubulin (red) and TOTO-3 (blue) staining were visualized under a fluorescent light microscope at an original 60X magnification. (Bar = 10μm). Representative images shown here are obtained from three independent experiments.

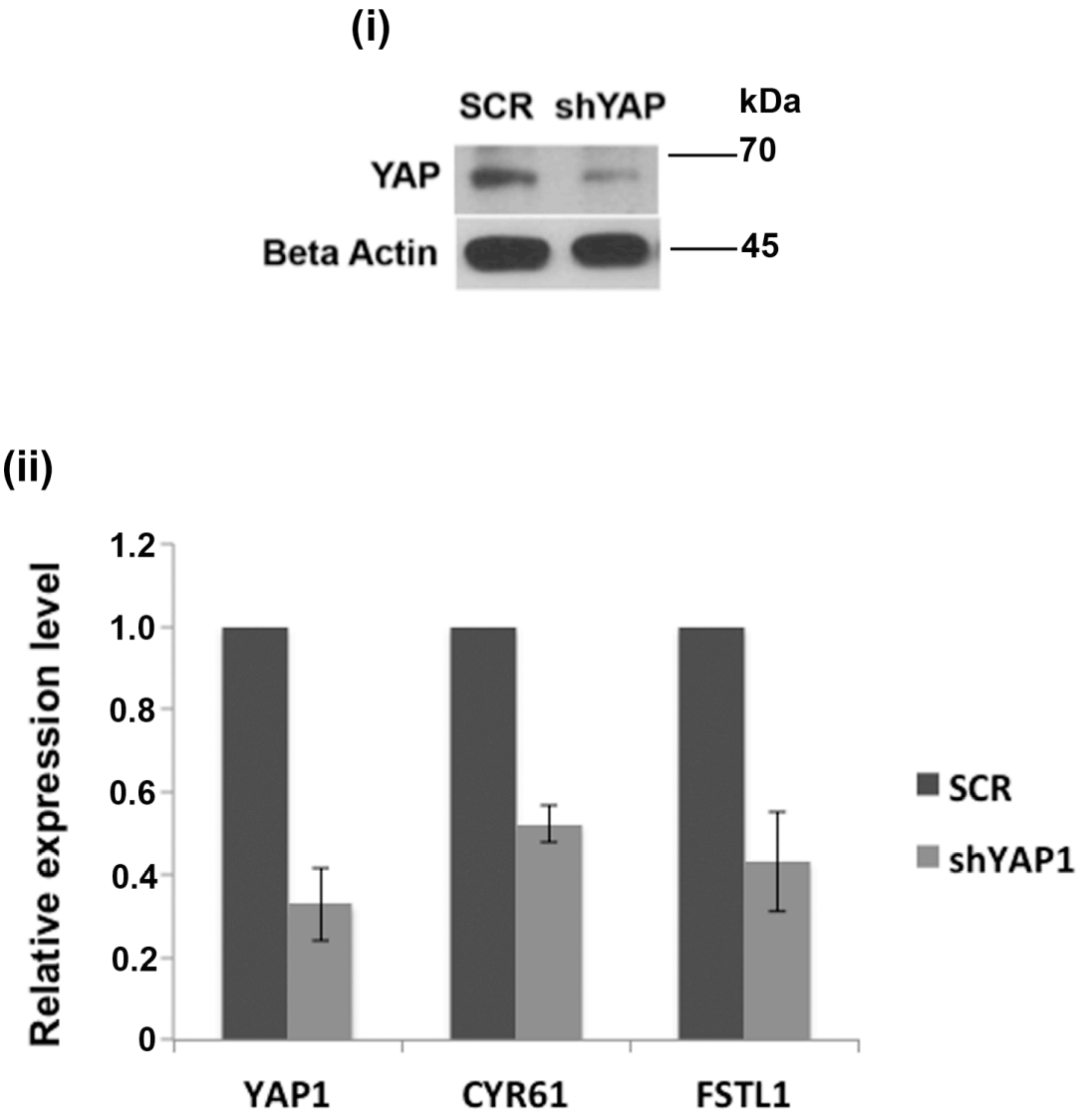
SUPPLEMENTARY FIGURE S9



S9: **Effect of p53 knockdown on proplatelet formation.** Cells were transduced at day 4 of culture with a control lentivirus and lentiviruses encoding either shRNA p53-0 or shRNAs p53-2/4. (i) $GFP^+/CD41^+$ sorted cells were seeded at $2x10^3$ cells/well in 96-well plate in triplicate. Three independent samples were analyzed for proplatelet formation. At least 150 MK cells were observed per well to determine changes in proplatelet branching. One representative control- and shp53-transduced proplatelet-forming MK is shown. (Bar = 50 µm). (ii) Ultrastructure of control and shp53 transduced MKs. MKs were sorted at day 10 of culture on the expression of GFP and CD41 and fixed.

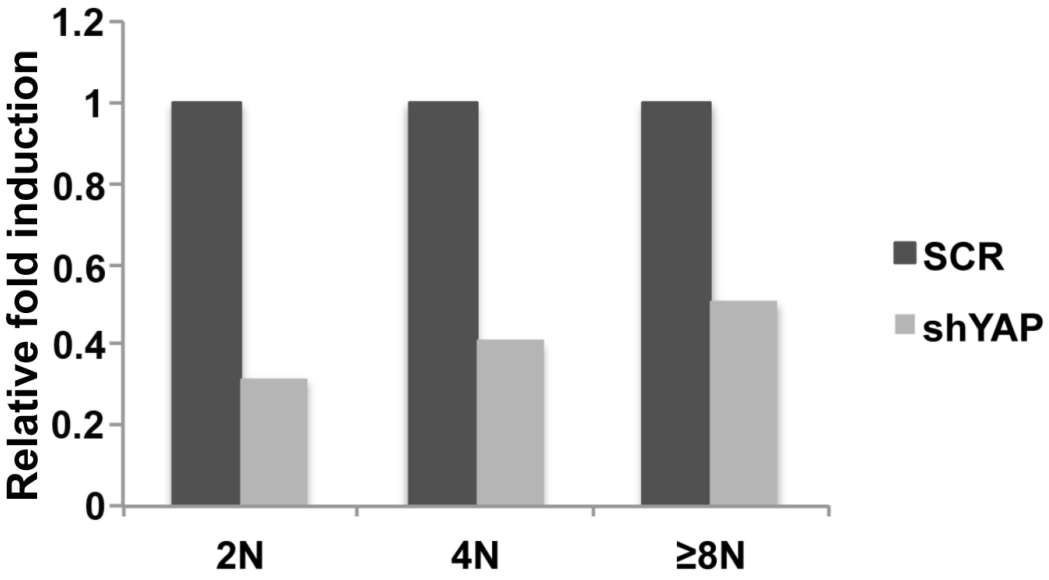
DM indicates demarcation membranes. At least 20 individual MK cells were observed for differences in ultrastructure. (Bar = 5μm)

SUPPLEMENTARY FIGURE S10



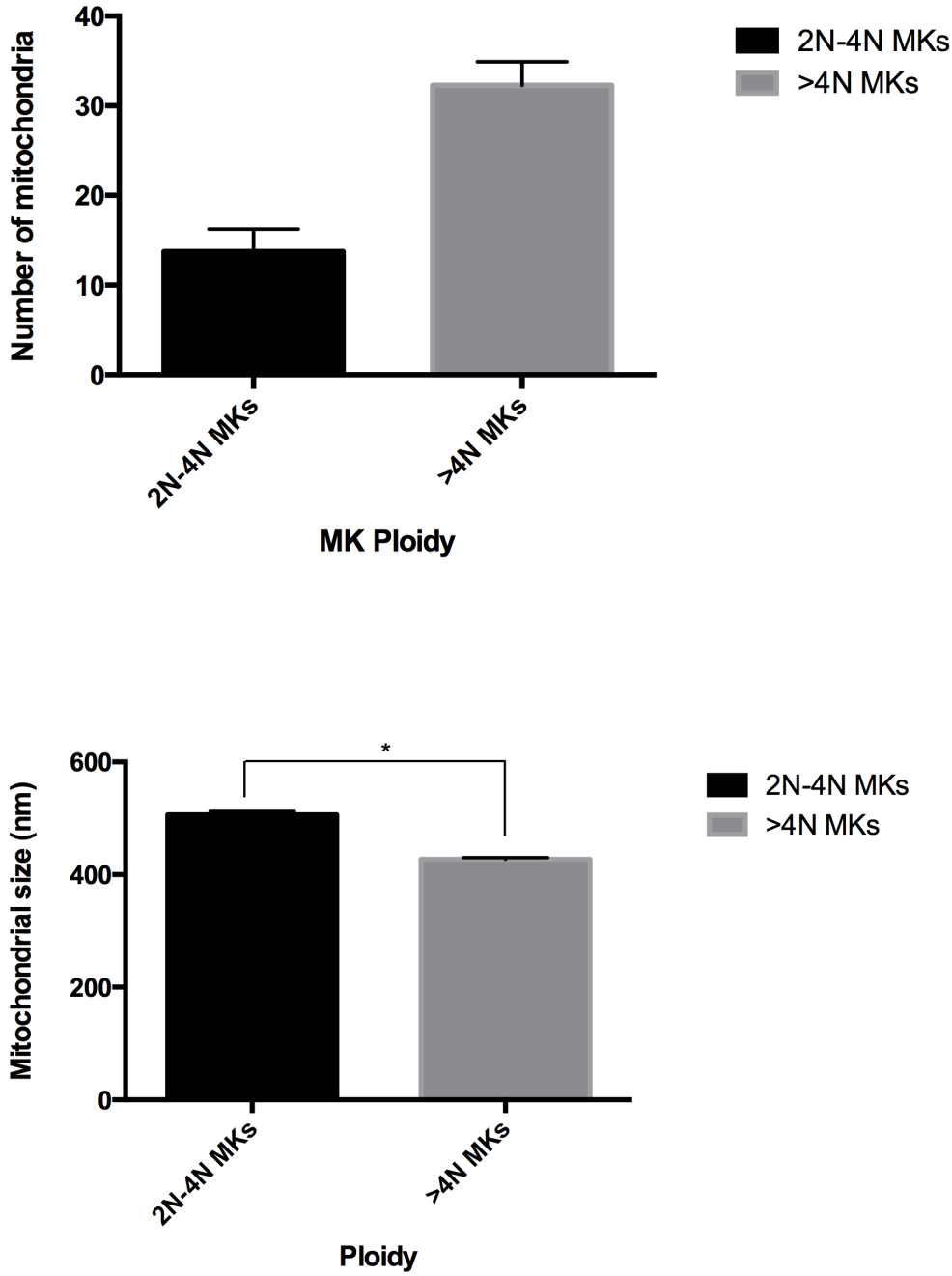
S10: **Validation of YAP knockdown**. (i) Western blot analysis of YAP expression in $CD41^+$ MKs transduced with lentivirus expressing either $mCherry^+$ scrambled (Cnt) or $mCherry^+$ shYAP. (ii) Real-time PCR was used to quantify YAP and its target gene expression in control and YAP knockdown MKs. Data represents mean ± SEM of three independent experiments normalized against *HPRT*. (n=3, $p<0.002$)

SUPPLEMENTARY FIGURE S11



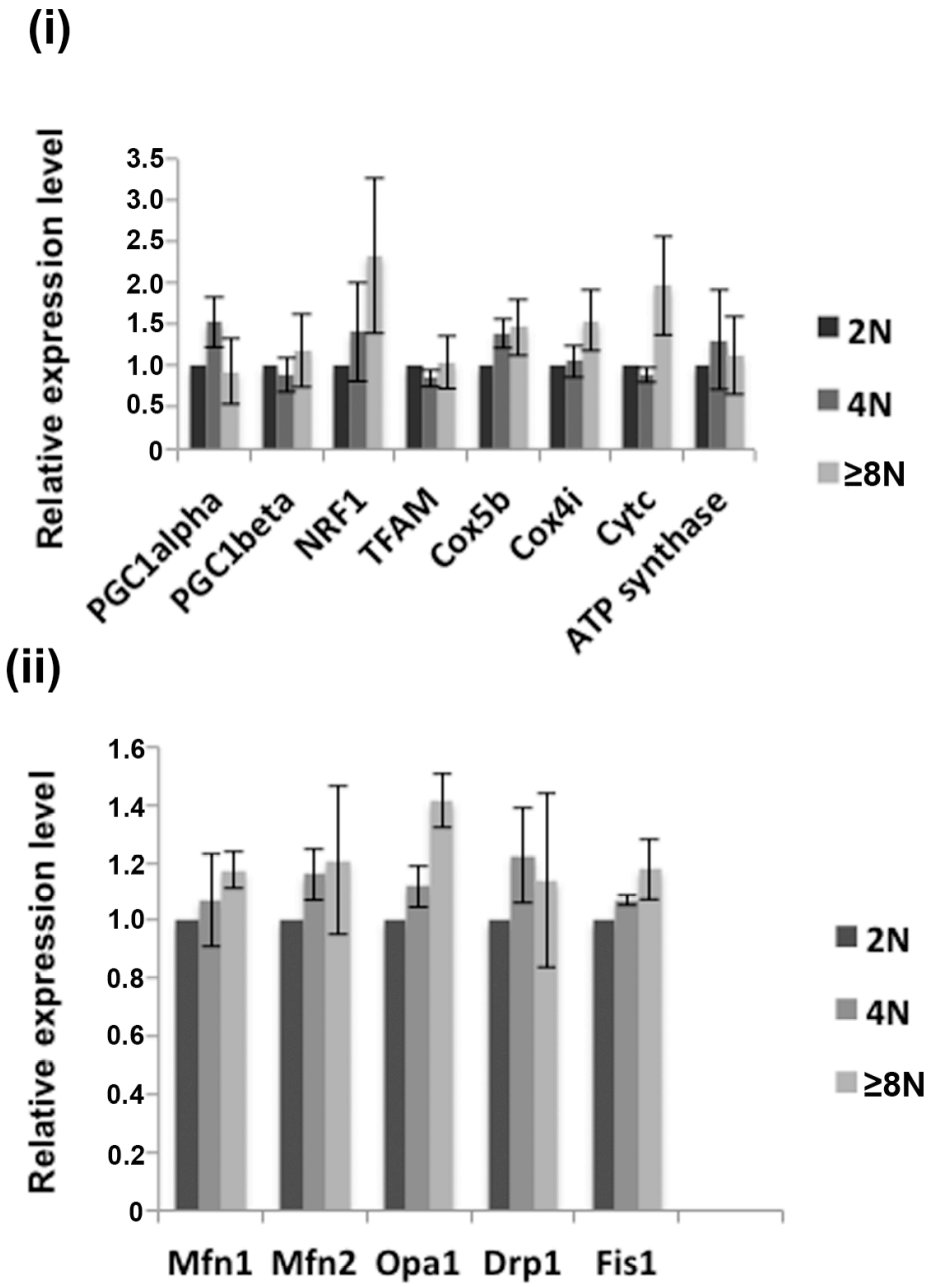
S11 : **Validation of *YAP* knock down across the various ploidy states.** Real-time PCR was used to quantify YAP expression in *YAP* knockdown MKs sorted on their ploidy at day 10 of culture. The relative expression was normalized against *HPRT*. Normalized expression in scrambled MKs was considered as1 and the corresponding relative expression in shp53 MKs was calculated for each ploidy state. Figure represents data obtained for one biological sampe with three technical replicates.

SUPPLEMENTARY FIGURE S12



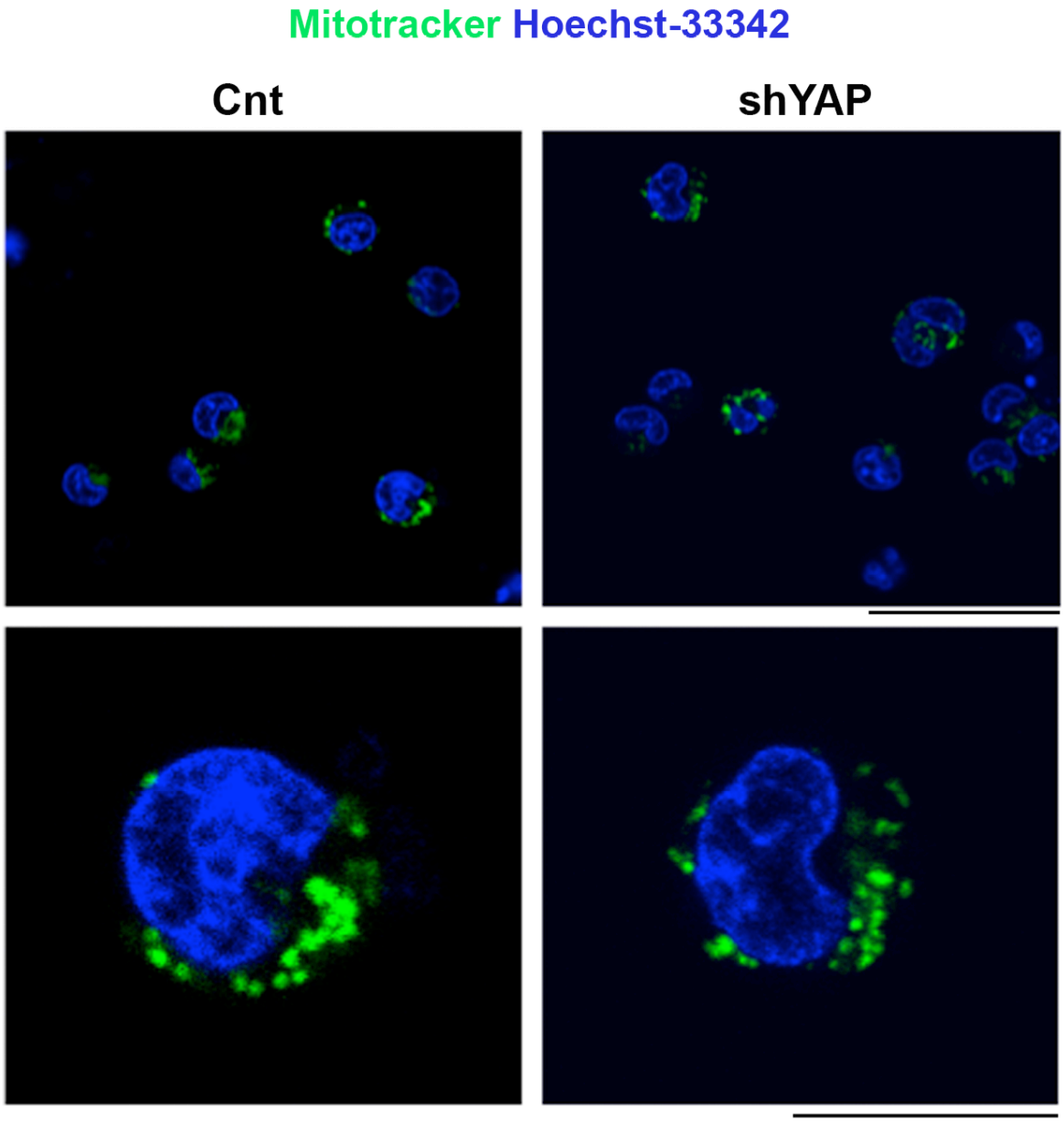
S12 : **Statistical analysis of mitochondrial size and number.** The number and size of mitochondria in 2N-4N MKs and >4N MKs was analyzed (Figure 6C). Data represents mean ± SEM. (*$p<0.01$) A negative correlation between the number of mitochondria and size was observed in each sample set ($r=-0.362$ for 2N-4N MKs and $r=-0.454$ for >4N MKs).

SUPPLEMENTARY FIGURE S13



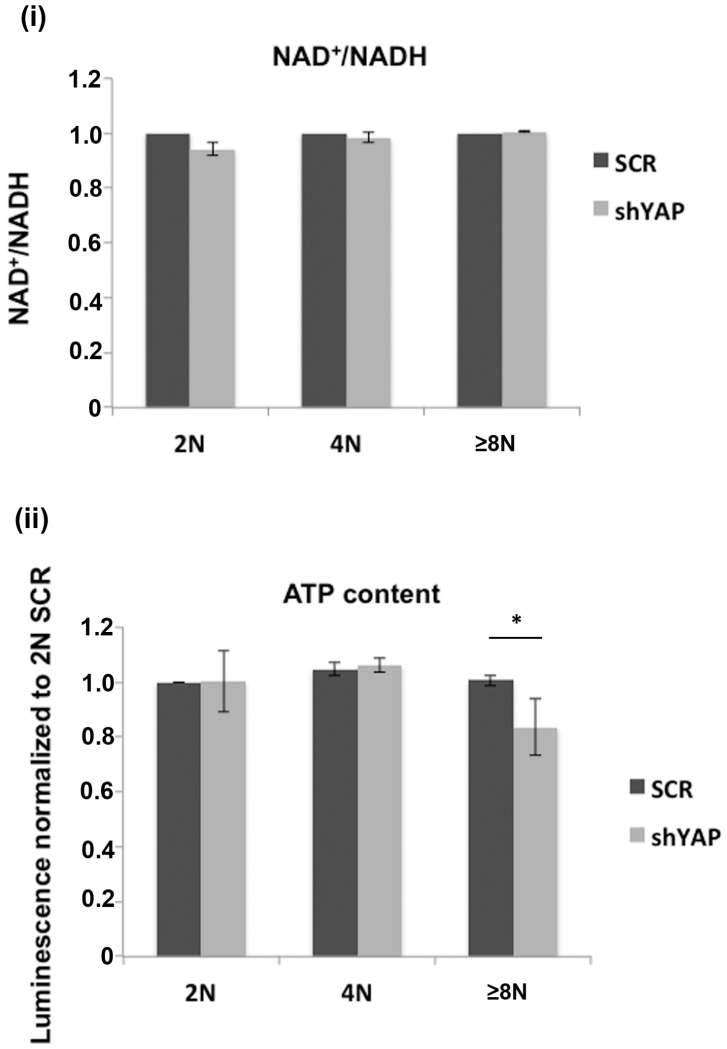
S13: Mitochondrial regulatory genes with ploidy. (i) Real-time PCR was used to quantify genes regulating mitochondrial biogenesis and oxidative phosphorylation in MKs sorted on their ploidy level. Data represents mean ± SEM of three independent experiments normalized against *HPRT* (n=3). (ii) Real-time PCR was used to quantify genes regulating mitochondrial fission-fusion kinetics in MKs sorted on their ploidy. Data represents mean ± s.e.m of two independent experiments normalized against *HPRT* (n=2).

SUPPLEMENTARY FIGURE S14



S14: Mitochondria in YAP knockdown MKs. (i) MKs were transduced on day 4 or day 5 of culture with lentivirus expressing scrambled (Cnt) or shYAP and were sorted on day 7 or day 8 on the expression of mCherry and CD41. MK cells were subsequently cultured with mitotracker green (30min) and Hoechst 33342 (1 hr). Images were acquired under a fluorescence microscope under a 63X oil immersion objective lens. (Scale bar for top panel : 30μm ; bottom panel : 15μm). Representative images shown here are obtained from two independent experiments.

SUPPLEMENTARY FIGURE S15



S15: Mitochondrial energetics with knockdown of YAP. (i) MK cells were transduced at day 4 of culture with a control lentivirus and lentiviruses encoding shYAP. $CD41^{+}CD42^{+}$ cells were sorted on ploidy. 8000-10000 cells were used in triplicate for each sample to measure NAD^{+}/NADH ratio. The ratio of NAD^{+}/NADH in 2N scrambled sample was considered as 1 and the relative ratio for the corresponding shYAP sample was calculated. Data represents mean ± SEM for two independent experiments (n=2). (ii) 3000 sorted 2N, 4N, ≥8N MKs were used in triplicate per sample to measure ATP content. The ATP content (in arbitrary luminescence unit) in 2N scrambled sample was considered as 1 and the relative ATP content for the corresponding shYAP samples was calculated. Data represents mean ± SEM for three independent experiments (*$p<0.05$).