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Haematologica 2016 [Epub ahead of print]

doi:10.3324/haematol.2016.149914

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

Anita Roy1,2,3*, Larissa Lordier1,2,3*, Catherine Pioche-Durieu2,3,4, Sylvie Souquere2,3,5, Lydia Roy1,6, Philippe Rameau3, Valérie Lapierre7, Eric Le Cam2,3,4, Isabelle Plo1,2,3, Najet Debili1,2,3, Hana Raslova1,2,3# and William Vainchenker1,2,3#

1Institut National de la Santé Et la Recherche Médicale (INSERM) UMR1170, Equipe labellisée par la Ligue Nationale contre le Cancer, Villejuif, France
2Université Paris-Saclay, Villejuif, France
3Gustave Roussy, Villejuif, France
4Centre Nationale de la Recherche Scientifique (CNRS), UMR 8126, Gustave Roussy, Villejuif, France
5CNRS UMR 8122, Gustave Roussy, Villejuif, France
6Assistance Publique des Hôpitaux de Paris (AP-HP), Service d’hématologie Clinique, Hôpital Henri Mondor, Créteil, France
7Gustave Roussy, Unité de Thérapie Cellulaire, Villejuif, France

* The authors contributed equally
# The authors contributed equally

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Corresponding authors:

Dr. William Vainchenker, INSERM UMR1170, Gustave Roussy, Université Paris-Saclay, 114 rue Edouard Vaillant, 94805 Villejuif, France; Phone: (+33)1 42 11 53 63; Fax: (+33) 1 42 11 52 40; Email: William.Vainchenker@gustaveroussy.fr

Key words: Megakaryocyte, Hippo, RhoA, p53, LATS2, YAP, mitochondria, polyploidization

Word counts: Main text: 3998; Abstract: 198

Figures: 7

Category: Research Article
ABSTRACT

Megakaryocytes are naturally polyploid cells that increase their ploidy by endomitosis. However, very little is known regarding the mechanism by which they escape the tetraploid checkpoint to become polyploid. Recently, it has been shown that the tetraploid checkpoint was regulated by the Hippo-p53 pathway in response to a down regulation of Rho activity. We therefore analyzed the role of Hippo-p53 pathway in the regulation of human megakaryocyte polyploidy. Our results revealed that Hippo-p53 signaling pathway proteins are present and are functional in megakaryocytes. Although this pathway responds to the genotoxic stress agent etoposide, it is not activated in tetraploid or polyploid megakaryocytes. Furthermore, Hippo pathway was observed to be un-coupled from Rho activity. Additionally, polyploid megakaryocytes showed increased expression of YAP target genes when compared to diploid and tetraploid megakaryocytes. Although p53 knockdown increased both modal ploidy and proplatelet formation in megakaryocytes, YAP knockdown caused no significant change in ploidy while moderately affecting proplatelet formation. Interestingly, YAP knockdown reduced the mitochondrial mass in polyploid megakaryocytes and decreased expression of PGC1α, an important mitochondrial biogenesis regulator. Thus, the Hippo pathway is functional in megakaryocytes, but is not induced by tetraploidy. Additionally, YAP regulates the mitochondrial mass in polyploid megakaryocytes.
INTRODUCTION

Megakaryopoiesis is a unique model of differentiation characterized by a physiological polyploidization and a maturation that leads to platelet production.\textsuperscript{1, 2} Polyploidy in megakaryocytes (MKs) is achieved by endomitosis, which corresponds to defective cytokinesis and karyokinesis.\textsuperscript{3-5} An increase in ploidy is believed to augment cell size, a crucial parameter for increasing platelet production.\textsuperscript{6, 7} While the modal ploidy of MKs in the bone marrow is 16N, the ploidy of individual MKs can reach 64N or more.\textsuperscript{7, 8} This indicates that MKs are able to escape the 4N control called the tetraploid checkpoint either because they are devoid of the tetraploid checkpoint machinery or can overcome this checkpoint that normally exists in most cell types except embryonic stem cells and undifferentiated embryos.\textsuperscript{9}

Key checkpoints exist in cells and include the tetraploid checkpoint that ensures cessation of proliferation and apoptosis of tetraploid cells. This is essential as tetraploidy not only promote genetic instability, but also contributes to tumorigenesis.\textsuperscript{10, 11} Reports have identified the Hippo-p53 pathway as an important component of the tetraploid checkpoint.\textsuperscript{12, 13} The conserved Hippo tumor suppressor pathway consists of the STE (yeast Sterile20 kinase) family protein kinases (MST1/2) which when activated can phosphorylate LATS1/2.\textsuperscript{14} This activates the kinase activity of LATS1/2 leading to direct interaction and phosphorylation of the transcription co-activators YAP/TAZ. Phosphorylated YAP/TAZ is sequestered in the cytoplasm resulting in the inhibition of target gene transcription.\textsuperscript{15} In contrast when upstream kinases are inactive, YAP/TAZ can translocate into the nucleus and activate transcription of their target genes. More recent studies have described the tumor suppressor LATS2 as a key link between p53 and tetraploid arrest. p53 has long been known to play a central role in this checkpoint since \textit{p53} knockout cells were found to be prone to accumulate tetraploid cells that divide subsequently.\textsuperscript{16, 17} The Hippo pathway is triggered in response to tetraploidy.\textsuperscript{12} This stabilizes p53 through direct interaction of LATS2 with MDM2 leading to p53 stabilization and proliferation arrest of tetraploid cells.\textsuperscript{13}
The role of p53 during MK differentiation has been previously studied. It has been shown that p53−/− mice showed increased numbers of MKs with higher ploidy, more particularly in stress conditions 18-20. Further, p53 stabilization by MDM2 inhibitors was found to impair all stages of megakaryopoiesis including polyploidy and proplatelet formation 20, 21. However, no information exists about the Hippo pathway in MKs. In this study we analyzed the role of the Hippo-p53 pathway in the regulation of human MK polyploidy. MKs were observed to harbor a functional Hippo-p53 pathway that responds to genotoxic stress, but not to polyploidy by decoupling the Hippo pathway from Rho activity.

METHODS

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

Leukapheresis and cord blood samples were obtained after approval from the Assistance Publique des Hopitaux de Paris. All participants to this study gave their informed written consent in accordance to the Declaration of Helsinki. The study was approved by the Local Research Ethics Committee of Hospital Saint Louis, Paris for the cord blood samples and the Local Research Ethics Committee of Institut Gustave Roussy, Villejuif. Details of culture conditions are given in the supplementary materials and methods

Cell sorting and flow cytometry

Cell sorting and analysis of ploidy level were previously described.4

Real Time quantitative PCR

Primers for qRT-PCR were designed using Primer Express Software (Perkin-Elmer Applied Biosystems, Foster City, CA) and were synthesized by Eurogentec (Angers, France). qRT-PCR was carried out in the ASI Prism GeneAmp 5700 (Perkin-Elmer Applied Biosystems) using the Power SYBR-Green PCR Master Mix (ABI) containing the specific primers (1.2mM). The expression levels of all genes were
calculated relatively to \textit{HPRT} and \textit{PPIA1}. Details of primer sequences are provided in the supplementary materials and methods.

\textbf{Immunofluorescence}

The cells were plated on poly-L-lysine-coated slides (O. Kindler GmbH&Co, Freiburg, Germany) for 1h at 37°C. Immunofluorescence staining was performed using mouse anti-p53, anti-β1-tubulin (Sigma-Aldrich) or anti-YAP antibodies and appropriate secondary antibodies conjugated with Alexa-488 or Alexa-546 ((Molecular probes Life Technology). TOTO-3 iodide or DAPI (Molecular probes, Life Technology) was applied for nuclear staining. Cells were examined under a Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Le Pecq, France) or Leica TCS SP8 MP (Leica Microsystems, Wetzlar, Germany) with a 63X oil immersion objective.

\textbf{Western blot analysis}

Western blots were performed as described previously.\textsuperscript{22} Details of the primary antibodies are given in supplementary materials and methods.

\textbf{Proplatelet formation assay}

CD41\textsuperscript{*}GFP\textsuperscript{*} or CD41\textsuperscript{*}mCherry\textsuperscript{*} MKs were sorted at day 8 of culture and proplatelet formation was evaluated as previously described.\textsuperscript{22} A total of 200 cells per well were counted during 4 days. Images were obtained using an inverted microscope (Carl Zeiss, Göttingen, Germany) at a magnification of 40X using the Axio Vision 4.6 software.

\textbf{Transmission Electron microscopy}

Detailed description may be found in the supplementary material and methods.

\textbf{Statistical Analysis}
Student’s t test and one-way Anova test were employed as applicable to the data set to test the significance of the data.

RESULTS

_Hippo-p53 pathway proteins are expressed in MKs_

The Hippo-p53 pathway constitutes the tetraploid checkpoint. The Hippo-p53 pathway constitutes the tetraploid checkpoint. 12 We investigated the status of this pathway in human MKs at different ontogenic stages. Analysis of previously reported global micro array expression data revealed that key genes of the pathway were expressed in MKs derived from human cord blood and adult cytapheresis (Supplementary Figure S1). This was confirmed by real time analysis of mRNA expression in _in vitro_ cultured mature MKs (defined as CD41*CD42+ cells) derived from cord blood and adult cytapheresis (Supplementary Figure S1). Furthermore, adult MKs were sorted on day 6 of culture on the expression of CD41 and further cultured to the end of MK maturation. A representative data of the ploidy distribution across the days of culture is shown in Supplementary Figure S2. We observed an initial increase followed by a marked decrease in the _p53_ transcript level at the end of MK maturation with a corresponding increase in the expression of _p21_. No statistically significant change was observed in the mRNA expression of _BCL2L1, BAX_ and _MDM2_ (Figure 1A). In agreement with the mRNA expression profile, p53 protein expression remained constant and decreased at the end (day 13) of _in-vitro_ MK differentiation (Figure 1B). Moreover, two negative regulators of p53, MDM2 and MDMX, were also present in MKs (Figure 1B and C). Treatment of sorted CD41+ MKs with the proteasome inhibitors ALLN and MG132 dramatically increased the expression of p53, demonstrating that p53 was mainly regulated by proteosomal degradation during MK differentiation (Figure 1C). The mRNA expression of Hippo pathway genes LATS1, LATS2 and TAZ remained invariant during the course of MK maturation. However, a consistent and significant increase in the expression of the transcriptional targets of YAP (CTGF, CYR61, FSTL1 and INHBA) was observed indicating that YAP activity increased
in mature MKs (Figure 1D). This was associated with an unchanged YAP protein level (Figure 1E), but with an increased nuclear localization of the protein (Figure 1F). Reminiscent of p53, the LATS2 protein expression remained fairly constant and decreased only at the very end of MK maturation (Figure 1E). Interestingly and in contrast to the mRNA levels, protein expression of LATS2 and p21 decreased at day 13 of in vitro culture. This may be due to the fact that day 13 MKs are at the very end of their maturation with a heterogeneous population of MKs in terms of ploidy and proplatelet production. Together, our results reveal that genes of the Hippo-p53 pathway are expressed throughout the various stages of MK maturation.

**Hippo-p53 pathway is functional in MKs**

To understand whether Hippo-p53 signaling pathway was functional in MKs, cells were treated with a genotoxic agent (etoposide). Staining for p53-BP1 confirmed the genotoxicity of a 3 hr treatment with 10μM Etoposide in MKs (Supplementary Figure S3). MKs were exposed to etoposide at various days of culture. Etoposide induced a drastic increase in the expression of both LATS2 and p53 (Figure 2A). Consistent with the canonical Hippo pathway, increased phosphorylation of YAP on ser127 was observed (Figure 2A). Enhanced LATS2 expression leading to increased p53 stability was reflected by increased p21 expression (Figure 2B). Under basal conditions, p53 protein was mostly cytoplasmic (>95%) in MKs. Upon etoposide exposure, p53 trafficked from the cytoplasm to the cell nucleus (Figure 2C). Moreover, consistent with its increased phosphorylation, YAP was completely sequestered in the cytoplasm of mature MKs (Figure 2D). Taken together, etoposide induced genotoxic stress was found to activate the Hippo-p53 axis in MKs.

**Hippo-p53 pathway is not activated in polyploid MKs**

We next checked whether polyploidy could induce the activation of Hippo and p53 pathway genes. MKs were sorted based on their ploidy level into diploid (2N), tetraploid (4N) and polyploid (≥8N) cell
populations. We did not detect any significant link between ploidization and the mRNA expression of p53, BAX, p21, MDM2 and MDMX genes. (Figure 3A). Similarly we did not detect any significant link between p53 protein expression and ploidy (Figure 3B). The expression of LATS2 also remained invariant at the different ploidy levels (Figure 3C and densitometric quantification in Supplementary Figure S4). YAP expression remained fairly constant in the three ploidy states with a modest, but not significant decrease in the phosphorylation of YAP in 4N ploidy stage (Figure 3C and Supplementary Figure S4). However, a pronounced increase in the expression of YAP target genes was observed in 4N and polyploid MKs indicating an increase in YAP transcriptional activity and therefore an inactivation of the Hippo pathway (Figure 3D). Thus, our data indicates that in MKs, the Hippo-p53 pathway failed to sense polyploidy as a genotoxic stress.

RhoA/ROCK pathway has been widely studied in relation to MK differentiation. RhoA and ROCK proteins are well expressed in MKs and have been shown to regulate ploidy and proplatelet formation. Because, reduced RhoA activity in tetraploid cells induced Hippo-p53 signaling and as MK differentiation and ploidization are associated with a decrease in RhoA activity, we checked if a further decrease in RhoA activity through ROCK inhibition (Y27632) could induce Hippo-p53 signaling in MKs. CD41^+CD42^+ MKs and CD71^+ erythroblasts sorted on day 5 of in-vitro culture were treated with Y27632. ROCK inhibition induced p53 expression in erythroblasts and reduced YAP expression increasing the ratio of phosphorylated YAP-S127 to total YAP (Figure 3E and densitometric quantification in Supplementary Figure S4). However, Y27632 treatment did not induce p53 expression in MKs. Additionally, there was no accompanying change in the expression of LATS2 or YAP and in the phosphorylation of YAP on ser127 (Figure 3E). Furthermore, increased MK ploidy was observed upon prolonged exposure to Y27632, a result that we have previously reported. Lastly, treatment of erythroblasts with ROCK inhibitor decreased the expression of YAP downstream target genes (Supplementary Figure S5) without effects on their expression in MKs. This indicated that in MKs in contrast to erythroblasts, RhoA activity is uncoupled from the Hippo pathway.
**p53 knockdown does not affect ploidy, but increases proplatelet formation in MKs**

To determine whether p53 knockdown facilitates human MK differentiation, CD34⁺ cells were induced into MK differentiation and transduced at day 3 or 4 of culture with GFP⁺ lentivirus encoding scrambled sequence or shp53 constructs (shp53-0, shp53-2, shp53-4). Transcript analysis of sorted GFP⁺CD41⁺ cells after 72hr of transduction demonstrated that shp53-0 alone almost completely depleted p53 whereas a combination of shp53-2 and shp53-4 (shp53-2/4) reduced the mRNA level by ~60% (Supplementary Figure S6i). A decrease in p53 expression was also seen at the protein level (Supplementary Figure S6ii). Reduced p53 expression was accompanied by a decrease in the expression of p53 targets such as p21, BAX, MDM2, DR5 and PUMA (Supplementary Figure S6i). We also checked that p53 expression was similarly down regulated at the different ploidy states (Supplementary Figure S7). In subsequent experiments, we used shp53-0 and confirmed our results with shp53-2/4. p53 knockdown had a modest effect on 2N and 4N ploidy MKs while significantly increasing the percentage of MKs with ploidy 8N and 16N (Figure 4A). It had no effect on MK differentiation as the percentage of mature CD41⁺CD42⁺ MKs was not significantly modified in culture (from 54% to 61% ; n=5) (Figure 4B). Furthermore, p53 down regulation had limited effects on MK cell cycle as attested by the incorporation of BrdU in the control and p53 knockdown samples at different ploidy states (Figure 4C). Lastly, as changes in p53 expression are frequently associated with genomic instability, we examined the separation of chromosomes and the number of centromeres during mitosis and endomitosis. We observed that centrosomes were paired and localized correctly and that the segregation and separation of these chromosomes was normal (Supplementary Figure S8). At the same time, p53 down regulation decreased MK response to apoptotic stimuli as observed in MK cells treated with 2 μM staurosporin, 4 μM etoposide and 1 μg/mL mitomycin C (Figure 4D). Next, we analyzed the proplatelet formation in p53 knockdown MKs. No effect was observed on proplatelet branching (Supplementary Figure S9i). However, p53 knockdown increased the number of proplatelet forming MKs. At day 14 of culture, a 4-fold increase was observed with shp53-0 and 3.5 fold increase with shp53-2/4 (Figure 4E, p=0.015). p53
Knockdown also caused a marked increase in cytoplasmic maturation as observed by an increased development of the demarcation membrane system (Supplementary Figure S9ii). At the same time, p53 knockdown did not affect the mRNA level of Hippo pathway genes (data not shown). Our results clearly indicate that p53 knockdown has minor effect on ploidy level furthermore demonstrating that in basal conditions p53 is not a major determinant of MK polyploidization, but markedly increases proplatelet formation.

**Knockdown of YAP moderately decreases proplatelet formation and does not affect MK ploidy**

To determine the effects of YAP on human MK differentiation, CD34+ cells were induced into MK differentiation and transduced at day 3 or 4 of culture with mCherry+ lentivirus encoding scrambled or shYAP. Western blot and real time PCR analysis on the sorted mCherry+CD41+ cells after 72hr of transduction demonstrated the efficacy of shYAP with >70% decrease in mRNA expression and a corresponding decrease in protein expression (Supplementary Figure S10i and ii). Reduction in YAP expression was also accompanied by a decrease in the expression of YAP target genes (Supplementary Figure S10ii). shRNA mediated knockdown of YAP did not significantly affect MK ploidy (Figure 5A), although YAP knock down by the shRNA was identical in all ploidy classes (Supplementary Figure S11). YAP knockdown did not affect MK differentiation, as the percentage of mature CD41+CD42+ MKs was not modified in culture in comparison to control cells (Figure 5B). At the same time, YAP knockdown had no significant effect on apoptosis as observed in MKs treated with etoposide (Figure 5C). In addition, we analyzed the proplatelet formation in YAP knockdown MKs. A moderate but significant decrease in the number of cells bearing proplatelets was consistently observed in YAP knockdown MKs (Figure 5D). Taken together, the results clearly indicate that YAP knockdown does not significantly affect ploidy, but decreases proplatelet formation. Given the increased expression of YAP target genes with the ploidy level and MK maturation, it was plausible that YAP had pleotropic functions in MK biology.

**YAP sustains the expression of PGC1α in MKs**
Therefore, we tried to understand the implications of increased YAP activity in polyploid MKs. We hypothesized that the increased cell size associated with polyploidy would require increased energy production. We therefore analyzed the mitochondria in MKs at different ploidy levels. Live cell imaging of mitochondria revealed that diploid and tetraploid (2N-4N) nuclei containing MKs and polyploid MKs have small, punctate mitochondria. Mitochondria were perinuclear in 2N-4N ploidy MKs while they were dispersed throughout the cytoplasm in polyploid MKs (Figure 6A). Electron micrographs also confirmed the perinuclear and dispersed localizations of mitochondria in the two ploidy classes. Furthermore mitochondria were observed to have clearly defined inner membrane with well-defined cristae (Figure 6B). Next, we analyzed the mitochondrial mass in the various ploidy states of MKs. Increase in MK ploidy was accompanied by an increase in mitochondrial mass (Figure 6C and D). An increase in the number of mitochondria was also evident in the electron micrographs of polyploid MKs. Furthermore, mitochondria in polyploid MKs were observed to be smaller in comparison to diploid-tetraploid MKs (Figure 6C and Supplementary Figure S12). We also analyzed the expression of key genes involved in mitochondrial biogenesis and mitochondrial fission-fusion kinetics in the three ploidy states. The mRNA expression of these genes remained unaltered with ploidy (Supplementary Figure S13i and ii). Together, this indicated that polyploidization was accompanied by an increase in mitochondrial mass.

We next analyzed the effects of YAP knockdown on mitochondrial mass of MKs. Staining of mitochondria using mitotracker green followed by live cell imaging revealed that mitochondrial morphology remained unaltered in YAP knock down MKs (Supplementary Figure S14). YAP knockdown did not alter the mitochondrial mass in 2N-4N MKs. However, a significant decrease in mitochondrial mass was observed in cells with ploidy of greater than 8N (Figure 6D). Remarkably, YAP knockdown substantially decreased the expression of PGC1α, a key regulator of mitochondrial biogenesis (Figure 6E and 6F). PGC1α expression was also found to be constant between diploid, tetraploid and polyploid MKs (Supplementary Figure S13i). However, neither ploidy nor YAP knockdown had any significant effect on the expression of key genes of the oxidative phosphorylation system or other genes regulating
mitochondrial biogenesis (Figure 6E, S4). To check if the reduced mitochondrial mass affected cellular energetics, we assessed the ATP content along with cellular NAD⁺/NADH ratio (Supplementary Figure S15i and ii). No change in the ATP content was observed in diploid and tetraploid cells. However, a decrease was observed in the ATP content of polyploid cells demonstrating again that the regulation of YAP is important in polyploid MK cells. The NAD⁺/NADH ratio however remained unchanged in the three ploidy classes analyzed. Thus, YAP was found to regulate mitochondrial mass along with the expression of a key mitochondrial biogenesis regulator PGC1α.

DISCUSSION

MKs are endowed with unconventional properties that make them unique systems to study various biological processes, especially the switch from mitotic mode of cell division to endomitosis whereby the cells become polyploid. Thus MKs provide an ideal platform to study how naturally polyploid cells overcome the tetraploid checkpoint that normally arrests cell cycle. It was previously reported that the Hippo-p53 pathway maintains the tetraploid checkpoint and reduction of RhoA activity induced by extra centromeres was found to activate Hippo-p53 pathway.12 Here, we provide evidence for a functional Hippo-p53 axis in MKs. A schematic representation is shown that highlights the key players of the Hippo-p53 pathway (Figure 7). Previous reports have demonstrated the importance of the Hippo pathway in fly haematopoiesis.25, 26 It was also reported that key proteins of the Hippo pathway are expressed in Mantle cell lymphoma.27 Furthermore YAP, a key component of the Hippo pathway, is highly expressed in stem cells and decreases progressively during differentiation.28 Therefore, we first confirmed the expression of the Hippo and p53 pathway genes in MKs derived from cord blood or adult blood.

While various mechanisms induce the Hippo pathway activity, tetraploidy and induction of apoptosis activate both p53 and the Hippo pathway.12, 29 We employed a genotoxic agent etoposide to decipher the functionality of the Hippo-p53 pathway in MKs and found that it activates this pathway. Next, we
analyzed the expression of the components of this pathway in diploid, tetraploid and polyploid MKs. No appreciable change in their expression was detected between the various ploidy states. However, a consistent increase in the expression of YAP and its target genes was observed during polyploidization as well as during the course of MK differentiation. This indicates that the Hippo-p53 pathway does not sense polyploidy as a stress in MKs. This is in agreement with previous reports that indicated that tetraploid cells re-entering cell cycle harbored an inactive Hippo pathway.\textsuperscript{12} Although ploidization has minor effects on p53 expression and its activity, p21 expression markedly increased during late stages of megakaryopoiesis. This is related to regulation by other signaling pathways, such as the MAPK/ERK pathway.\textsuperscript{30, 31}

The activation of the Hippo-p53 pathway in tetraploid cells was reported to be acutely dependent upon RhoA activity. As RhoA activity was reported to decrease during the first endomitotic division of MKs, we assessed the effects of decreased RhoA activity on Hippo-p53 pathway.\textsuperscript{12, 24} Our data demonstrates that in contrast to erythroblasts, the Hippo pathway is decoupled from RhoA activity in MKs. Importantly, decreased RhoA activity has been previously linked to increased YAP phosphorylation in HEK293A cells.\textsuperscript{32} Our results show that decreased Rho/ROCK signaling did not induce YAP phosphorylation in MKs in contrast to erythroblasts. Moreover, in erythroblasts, inhibition of ROCK was also found to decrease the expression of total YAP protein while increasing the ratio of phosphorylated YAP to total YAP. This was consistent with previous reports that demonstrate that phosphorylation of YAP on S127 induced its degradation.\textsuperscript{33}

\textsuperscript{p53} knockdown induced a moderate, but significant increase in MK polyploidization, without significant increase in DNA replication. It is likely that the modest increase in polyploidization could be the consequence of a decreased basal apoptosis in \textsuperscript{p53} knockdown MKs. This is in agreement with previous results on \textsuperscript{p53}\textsuperscript{-/-} mice that show that \textsuperscript{p53} knockout only increased MK ploidy under stress conditions such as during induced thrombocytopenia, but not in basal conditions in the bone marrow.\textsuperscript{16, 17} However, \textsuperscript{p53} knockdown significantly increased proplatelet formation in accordance with our previous reports.\textsuperscript{21, 34}
Furthermore, *YAP* knockdown caused no significant change in ploidy, but a consistent and significant decrease in the percentage of proplatelet bearing MKs was observed.

Given the observed increase in YAP target gene expression both during the course of MK differentiation as well as polyploidization, we tried to identify its possible effects. Previous works had suggested links between mitochondria and the Hippo-p53 pathway. We hypothesized that polyploid MKs would have a large energy demand to sustain their size and functionality, which could plausibly be regulated by the Hippo-p53 pathways. YAP knockdown was found to decrease mitochondrial mass in polyploid MKs with ploidy greater than 8N while keeping the mitochondrial mass unchanged in diploid and tetraploid MKs. Although mitochondria in polyploid MKs were on average smaller when compared to mitochondria in diploid MKs, no significant differences could be observed in the expression of genes regulating mitochondrial fission-fusion dynamics. Instead, *YAP* knockdown substantially decreased the expression of PGC1α, a key mitochondrial biogenesis factor, which was associated with a decrease in ATP content in polyploid MKs. However, no significant change was observed in the NAD+/NADH ratio. Also no change was observed in the expression of factors regulating biogenesis like TFAM, which are themselves regulated by PGC1α. Thus, it appears that the expression level of PGC1α in *YAP* knockdown MKs is sufficient to drive biogenesis, albeit at a slower rate. Interestingly, activation of Yorkie (the fly homolog of YAP) did not alter the expression of PGC1α in *Drosophila*. Instead, activation of Yorkie increased mitochondrial fusion without altering the cellular ATP content. *YAP* mediated regulation of mitochondrial mass may explain its effects on proplatelet formation because it has recently been shown that mitochondria play a key role in platelet formation and function, particularly in stress conditions by controlling ROS production. Interestingly, mitochondria in diploid and tetraploid MKs were found clustered around the nucleus whereas they were distributed in the cytoplasm in polyploid MKs. The perinuclear localization of mitochondria has been reported previously to be associated with high nuclear ROS levels and increased expression of hypoxia associated genes. Therefore, mitochondrial biogenesis and its direct impact on the energetics of the cell could plausibly affect proplatelet formation. Further
research is required to fully understand the impact of cellular energetics on demarcation membrane formation, proplatelet formation and ultimately platelet function.

In summary, our results clearly show the existence of functional Hippo-p53 machinery in MKs that is not activated during MK polyploidization suggesting that the decrease in RhoA activity is not sensed in MKs. Finally, our study revealed the unexpected role of YAP in the regulation of mitochondrial biogenesis in polyploid MKs and proplatelet formation.

ACKNOWLEDGEMENTS

This work was supported by grants from Ligue Nationale Contre le Cancer (“Equipe labellisée HR 2013 and 2016”: A.R., I.P., N.D., W.V., H.R.) and Institut National de la Santé et de la Recherche Médicale (INSERM). A.R. was funded by a grant from FRM (SPF20140129106). I.P. and W.V are partners of the Laboratory of Excellence Globule Rouge-Excellence funded by the program “Investissements d’avenir”.

AUTHORSHIP CONTRIBUTIONS

A. R., L. L., H.R and W.V. conceived and designed the study, interpreted the data and wrote the paper. A. R. L. L., and L.R designed and performed experiments of cell and molecular biology. C.P.D, S.S. and E.L.C performed the ultrastructural studies. N.D and I.P. analyzed the data and provided essential reagents. P.R performed the cell sorting experiments. V.L. provided essential reagents.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

REFERENCES


FIGURE LEGENDS

Figure 1: Expression of Hippo-p53 pathway genes in MKs (A) qRT-PCR data indicating relative expression of p53 and related genes on days 10 and 13 with respect to day 8 of in-vitro cultured CD41⁺CD42⁺ MKs normalized against HPRT. Data represents mean ± SEM (n=3, **p<0.01). (B) Protein expression of p53, p21, BAX, MDM2 and BCL-XL (BCL2L1) in the CD41⁺ sorted cells during
MK differentiation and investigated by Western blot analysis. HSC70 indicates the loading in each lane. (C) The CD41\(^+\) sorted cells was treated with proteasome inhibitors (ALLN or MG132) for 3 hrs, then p53 level was investigated by Western blot. HSC70 indicates the loading in each lane.(D) qRT-PCR data indicating relative expression of Hippo pathway genes on days 10 and 13 with respect to day 8 of *in-vitro* cultured CD41\(^+\)CD42\(^+\) MKs normalized against *HPRT*. Data represents mean ± SEM (n=3, \(*p<0.05, \quad **p<0.01)\) Similar results were obtained using *PPIA*.  (E) Western blot analysis of Hippo pathway proteins and their expression in CD41\(^+\) MKs on different days of culture. HSC70 indicates the loading in each lane. (F) Confocal microscopic image of CD41\(^+\) MKs on different days of culture and stained for YAP and showing its localization in the cytosol and nucleus. DAPI was used to stain the nucleus. 150 cells were counted and categorized according to the distribution of YAP between nucleus (N) and cytosol (C) - Day 8: N>C(6%), N=C(21%), N<C(73); Day 13: N>C(74%), N=C(14%), N<C(12%). Scale bar = 100 \(\mu\)m.

**Figure 2: Hippo-p53 axis is activated by genotoxic stress** (A) Western blot analysis of Hippo-p53 pathway proteins in CD41\(^+\) MKs treated with/without 10\(\mu\)M etoposide for 5hr. \(\beta\)-actin was used as loading control. (B) Western blot analysis of p53 and its target genes performed after 12 hr of etoposide-treatment at different days of culture (D5, D7 and D9). HSC70 was used as loading control. (C) p53 localization in untreated and 12 hr etoposide treated CD41\(^+\) MK cells. Cells were stained with anti-p53 antibody. TOTO-3 was used to stain the nucleus. Scale bar = 20 \(\mu\)m. (D) YAP localization in untreated CD41\(^+\) MK cells and CD41\(^+\) MK cells treated by 10 \(\mu\)M etoposide for 5 hr on day 10 of culture. Cells were stained with anti-YAP antibody. DAPI was used to stain the nucleus. Cells (150) were counted and categorized according to the distribution of YAP between nucleus (N) and cytosol (C). Whereas for control cells nearly 70% had YAP staining the nucleus, none of the cells in etoposide treated samples showed nuclear localization of YAP. (Scale bar = 30 \(\mu\)m)
Figure 3: Hippo-p53 axis is not activated by polyploidy (A) qRT-PCR data indicating relative expression of p53 and related genes in cultured MKs at different ploidy levels: 4N and ≥8N in comparison to 2N CD41⁺CD42⁺ MKs and normalized against HPRT. Data represents mean ± SEM of three independent experiments. No significant difference was observed. (B) p53 expression in the CD41⁺ MKs sorted on ploidy and investigated by Western blot analysis. HSC70 indicates the loading in each lane. The mean density of p53 normalized against HSC70 is indicated in the histogram plot below. (C) Protein expression of Hippo pathway genes in the CD41⁺ MKs sorted on ploidy and investigated by Western blot analysis. HSC70 indicates the loading in each lane. (D) qRT-PCR data indicating relative expression of YAP down stream target genes in MK cells of ploidy 4N and ≥8N with respect to 2N of in-vitro cultured CD41⁺CD42⁺ MKs and normalized against HPRT. Data represents mean ± SEM (n=4, *p<0.05, **p<0.01). (E) Western blot analysis of the expression of Hippo-p53 pathway proteins in CD41⁺ MKs and CD71⁺ erythroblasts (ER) treated for 5 hrs with 10 μM Y27632. GAPDH was used as a loading control. The corresponding DNA ploidy/cell cycle analysis analyzed after 72hr of culture with/without 10 μM Y27632 for each sample with Hoechst 33342 staining is provided. The data is a representative of 3 independent experiments. Densitometric analysis of the western blots are provided in Supplementary Figure S3. A significant increase in the percentage of polyploid MKs was observed with Y27632 (Control MKs = 3.50 ± 0.2%, MK + Y227632= 18.15 ± 2.5%, n=3, p<0.004).

Figure 4: p53 knockdown increases proplatelet formation. Cells were transduced at day 4 of culture with a control lentivirus encoding scrambled shRNA (Cnt) or lentiviruses encoding either shRNA p53-0 or shRNAs p53-2/4. (A) Representative image of the ploidy level of GFP⁺ CD41⁺CD42⁺ cell population as analyzed by Hoechst staining. The percentage of cells at each ploidy level was calculated. Data represents mean ± SEM of four independent experiments (*p<0.006, **p<0.002). (B) Flow cytometric analysis of mature MKs expressing CD41 and CD42 in the GFP⁺ cells at day 9 of culture. (n=5, p=0.03) (C) Flow cytometric analysis showing percentage of BrdU positive cells in control and p53 knockdown.
MKs (CD41\(^+\)GFP\(^+\) cells) at day 7 of culture. Data represents the mean ± SEM of three independent experiments. (D) Annexin V binding assay on \(p53\) knockdown or control cells at day 7 of culture exposed to 2 \(\mu\)M staurosporine (STS), 4 \(\mu\)M etoposide (Et), 1 \(\mu\)g/mL mitomycin C (Mit) and DMSO for 24 hrs was performed by flow cytometry. Data represents mean ± SEM (n=3, **p≤.01) (A) GFP\(^+\)CD41\(^+\) sorted cells were seeded at 2\times10^3 cells/well in 96-well plate. The percentage of proplatelet-forming MKs was estimated by counting MKs exhibiting one or more cytoplasmic processes with areas of constriction. A total of 200 cells per well were counted during 4 days. Error bars in histograms represent the SD of one representative experiment performed in triplicate wells. Similar results were obtained in 4 repeated experiments (n=4, **p=0.015).

Figure 5: YAP knockdown does not affect ploidy. Cells were transduced at day 4 or day 5 of culture with a control mCherry\(^+\) lentivirus encoding either scrambled (Cnt) or shYAP. (A) Representative image of ploidy distribution in mCherry\(^+\)CD41\(^+\)CD42\(^+\) cells analyzed by Hoechst staining. The percentage of cells at each ploidy level was calculated. Corresponding histogram plot represents the mean ± SEM of mean ploidy of five independent experiments. (p>0.05, indicates that differences between the two samples are not significant). (C) Flow cytometric analysis of mature MKs expressing CD41 and CD42 in the mCherry\(^+\) cells at day 9 of culture. Data represents mean ± SEM of four independent experiments. (n=4, p>0.05 indicates that the difference between the samples is not significant) (D) Annexin V binding assay in scrambled and YAP knockdown MK cells exposed to 10 \(\mu\)M etoposide (Et) for 5 hr was performed by flow cytometry. Data represents mean ± SEM of four independent experiments. (n=4, p>0.05 indicates that the difference between the samples is not significant) (D) mCherry\(^+\)CD41\(^+\) sorted cells were seeded at 2\times10^3 cells/well in 96-well plate. The percentage of PPT-forming MKs was estimated by counting MKs on day 11 or day12 exhibiting one or more cytoplasmic processes with areas of constriction. shYAP slightly but significantly decreased proplatelet formation. Data represents mean ± SEM of four independent experiments (n=4, *p<0.014).
Figure 6: YAP knockdown decreases mitochondrial mass (A) Fluorescence microscopic image of CD41+ MKs stained with mitotracker red. DAPI was used to stain the nucleus. Scale bar = 20 μm. (B) Electron micrographs of sections of MKs of 2N-4N ploidy (a,b) and ≥8N (c,d) ploidy states. Representative image shown at 4400X magnification (a,c) and mitochondria indicated at higher magnification of 21600X (b,d) of a field within marked by a box. Scale bars indicate 1 μm (a,c) and 200 nm (b,d). (C) The corresponding graph indicates the number of mitochondria versus the median mitochondrial diameter in 2N-4N and ≥8N ploidy MKs. The parameters are measured on ultrathin sections by TEM using a photos series taken at the same magnification. Further statistical analysis in provided in Supplementary Figure S12. (D) MK cells were transduced at day 4 or day5 of culture with a control mCherry+ lentivirus encoding either scrambled (Cnt) or shYAP. Cells were stained with mitotracker green, Hoechst and anti-CD41 antibody. The fluorescence intensity of mitotracker green in each individual ploidy state (2N, 4N etc.) was plotted against ploidy number and fitted against a straight line passing through origin by least square fit method (n=4, *p<0.07). (E) qRT-PCR data indicating relative expression of YAP and genes involved in mitochondrial biogenesis and oxidative phosphorylation in MK cells transduced with the indicated vectors. Relative expression of shYAP samples with respect to control (Cnt) was calculated. Data was normalized against HPRT. Data represents mean ± SEM of three independent experiments. (n=3, p<0.003) (F) Protein expression of PGC1α and YAP in the CD41+ MKs sorted on mCherry+ Cnt or mCherry+ shYAP and investigated by Western blot analysis. HSC70 indicates the loading in each lane. Densitometric analysis showing the normalized expression of PGC1alpha (mean ± SEM) of 3 independent experiments has been provided. (p<0.006)

Figure 7: Illustration of Hippo-p53 pathway in MKs. Low RhoA activity leads to increased Hippo pathway activity by increasing the expression and phosphorylation of LATS2 leading to increased interaction with MDM2. The interaction between LATS2 and MDM2 inhibits MDM2-p53 interaction thereby releasing p53, which can now enter the nucleus and act as a transcription factor blocking cell
cycle progression. At the same time, increased expression and phosphorylation of LATS2 increases phosphorylation of YAP. YAP is thus sequestered away from the nucleus thereby blocking its transcriptional activity. In MKs, the cells fail to respond to decreased RhoA activity and do not increase the expression of LATS2 or phosphorylation of YAP.
FIGURE 2
FIGURE 4
FIGURE 5
FIGURE 6
**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 :**

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**Sequences of shRNA**

Three different short hairpin RNA (shRNA) targeting p53 (0, 2 and 4) shRNA-0: *gactccagtgtaatctac*; shRNA-2: *gagggatgtttgggagatg*; shRNA-4: *cggcgcacagaggaagaga*; a short hairpin RNA (shRNA) targeting YAP1: *ccagttaaatgttceacca*; a shRNA control: *ttctccgaacgttgtcaagt* 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 2×10⁶ 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. 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.

**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 DiIC₅(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 and analysis 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

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 Etoposide 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.
**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 erythroblasts treated with Y27632 with respect to control cells and normalized against *HPRT*. Data represents mean ± SEM (n=3, p<0.02).
**SUPPLEMENTARY FIGURE S6**

(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.

**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.
**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 sample with three technical replicates.
**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.
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 2x10³ 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 as 1 and the corresponding relative expression in shp53 MKs was calculated for each ploidy state. Figure represents data obtained for one biological sample with three technical replicates.
**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).
**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).
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**

(i) **NAD⁺/NADH**

![Graph showing NAD⁺/NADH ratio](image)

(ii) **ATP content**

![Graph showing ATP content](image)

**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).