The Hippo-p53 pathway in megakaryopoiesis

Praveen K Suraneni and John D. Crispino

Division of Hematology/Oncology, Northwestern University, Chicago, IL, USA

E-mail: j-crispino@northwestern.edu *doi:10.3324/haematol.2016.156125*

Megakaryocytes are among the largest and rarest cells in the body, accounting for approximately 0.01% of nucleated cells in the bone marrow. Their differentiation involves a progression from hematopoietic stem cell to the megakaryocyte progenitor and finally to platelets.[1] A key step in their maturation is the switch from a proliferating progenitor cell, which divides like any other cell, to a committed megakaryocyte that undergoes polyploidization through a modified form of the cell cycle termed endomitosis.[2] During endomitosis, megakaryocytes proceed through successive cell cycles without cell division to reach DNA contents of 32N, 64N, and even 128N. After completion of G1, S and G2 phases, committed megakaryocytes enter mitosis, transition through anaphase, separate their chromosomes, and initiate cleavage furrow formation.[3] However, the cleavage furrow regresses before cytokinesis is completed, resulting in formation of a single cell with a multi-lobulated, polyploid nucleus.[4]

Rho family small GTPases, including RhoA, Rac1, and Cdc42, are molecular switches that regulate various cellular processes including actin cytoskeleton reorganization, microtubule dynamics, cell cycle progression, cytokinesis, and platelet production.[5,6] The furrow regression seen in megakaryocytes appears to be due to a failure in either proper localization or activation of RhoA at the contractile ring.[7] Evidence in support of a critical role of the RhoA and its effector Rho kinase (ROCK) in the regulation of the switch to polyploidy includes the finding that its inhibition or knockdown leads to increased polyploidy of megakaryocytes.[7-10] Moreover, megakaryocyte-specific deletion of RhoA in mice resulted in macrothrombocytopenia due to premature release of platelets.[11] Interestingly, the megakaryocytes in the animals were larger and more highly polyploidy, consistent with the inhibitor data.

Among other processes, Rho A regulates the Hippo-p53 tumor suppressor pathway, which controls proliferation, differentiation and apoptosis of cells from *Drosophila* to mammals.[12] Decreased activity of Rho A contributes to the phosphorylation of the kinase LATS1/2 (Figure 1). Active LATS1/2 binds and inhibits MDM2, which releases p53 and allows for pathway activation.[13] In addition, LATS1 phosphorylation of YAP/TAZ leads to its cytoplasmic sequestration and degradation, and impairs expression of its pro-proliferative and anti-apoptotic target genes.[12] Recent studies by Ganem *et al.* demonstrated that the Hippo-p53 pathway controls the tetraploid checkpoint,[14]

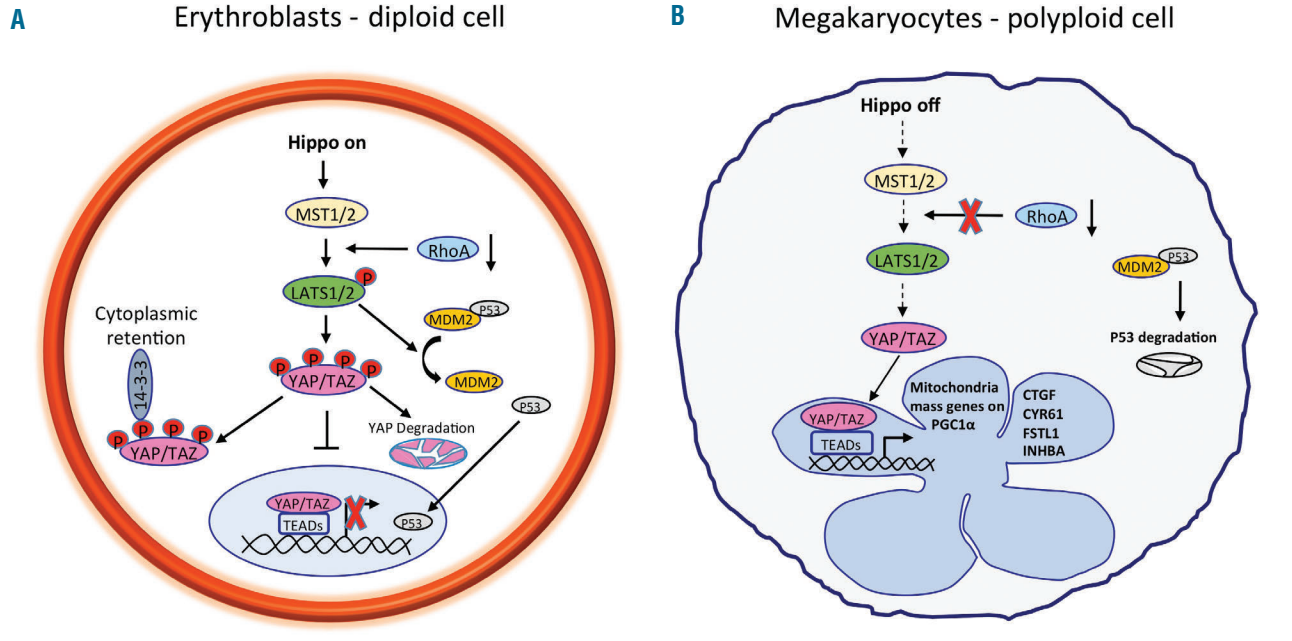

Figure 1. Comparison of the Hippo-p53 pathway in erythroblasts *versus* megakaryocytes. (A) In diploid cells, such as erythroblasts, a reduction in RhoA GTPase activity promotes the Hippo pathway by increasing the phosphorylation of LATS1/2. The LATS1/2 kinases in turn phosphorylate and inhibit the transcriptional co-activators YAP and TAZ by subsequent degradation and/or cytoplasmic retention. In parallel, LATS1/2 stabilizes p53 through its direct association with MDM2, which disrupts the MDM2-p53 interaction. (B) In polyploid megakaryocytes, the Hippo-p53 pathway remains off as the reduction in RhoA activity fails to activate LATS1/2, allowing YAP/TAZ to translocate into the nucleus and promote target gene expression.

which exists to prevent the continued growth of aneuploid cells. Moreover, the presence of extra centrosomes and actin filaments, a consequence of the increased number of chromosomes, was found to activate Hippo-p53 pathway by down-regulating RhoA activity.[14]

In proliferating cells, two guanine exchange factors, GEF-H1 and ECT2, play critical roles in cytokinesis by activating RhoA at the cleavage furrow.[15] By contrast, GEF-H1 and ECT2 are down-regulated during the 2N to 4N transition and during polyploidization beyond the 4N stage, respectively, resulting in suppression of RhoA signaling during endomitosis.[10] Given that RhoA activity is low in megakaryocytes undergoing polyploidization, one might predict that the Hippo-p53 pathway would be activated and prevent the process. However, a new study by Roy *et al.* published in this issue of *Haematologica* sheds new light on Hippo-p53 pathway function in megakaryocytes.[16] The critical observation is that, despite the presence of a functional Hippo-p53 pathway, low RhoA activation in megakaryocytes fails to activate the tetraploid checkpoint and instead allows for endomitosis. In addition, the sustained activation of YAP contributes to megakaryopoiesis by increasing expression of mitochondrial genes including PGC1α, which contributes to mitochondrial biogenesis.

To investigate how naturally polyploid cells such as megakaryocytes overcome the tetraploid checkpoint, the authors first validated the expression of Hippo-p53 pathway genes in human megakaryocytes at various developmental stages. Their results revealed that expression of LATS1, LATS2 and TAZ remain constant during MK maturation, but that there is a significant increase in the expression of YAP target genes, such as *CTGF*, *CYR61*, *FSTL1* and *INHBA*. This increase was associated with reduced levels of phosphorylated YAP but an overall high level of total YAP protein, indicative of an inactive Hippo-p53 pathway.

Next, the authors examined the functionality of the Hippo-p53 pathway by exposing megakaryocytes to genotoxic stress induced by etoposide. With this treatment, they observed a strong increase in both LATS2 and p53 along with a spike in the phosphorylation of YAP. Furthermore, exposure to etoposide was associated with translocation of p53 from the cytoplasm to the nucleus. These results reveal that there is an active Hippo-p53 surveillance pathway in human megakaryocytes.

To address whether polyploidy is interpreted as genotoxic stress in megakaryocytes, Roy *et al.* assessed the expression levels of p53 and Hippo pathway genes at different ploidy stages. However, there were no significant changes in the expression or the activity of p53 or level of YAP, and there was a steady rise in the expression of YAP target genes during polyploidization. This activation of YAP suggests that megakaryocytes fail to activate the Hippo pathway. To investigate this further, the Authors asked whether induced impairment of RhoA activity could force activation of the Hippo pathway in megakaryocytes. By treating cells with a ROCK inhibitor, they discovered that the Hippo pathway was, as expected, strongly induced in erythroid cells but that similar treatment did not drive Hippo signaling in megakaryocytes. This key result provides evidence that Hippo pathway activation is uncoupled from Rho kinase activity in endomitotic megakaryocytes.

Roy *et al.* also investigated the contributions of p53, which normally eliminates aberrant tetraploid cells and prevents tumor formation to megakaryopoiesis. Previous studies have shown that loss of p53 in mice was associated with increased megakaryocyte ploidy levels; this effect was exacerbated in stress conditions.[17] Furthermore, stabilization of p53 by MDM2 inhibition impaired polyploidization and proplatelet formation.[18,19] In this new report, knockdown of p53 was shown to result in a modest, but significant, increase in MK polyploidization as well as increased numbers of pro-platelet forming cells and cytoplasmic maturation.[16]

Perhaps the most surprising finding of Roy *et al.* is the link between YAP and expression of the mitochondrial biogenesis regulator PGC1α during megakaryocyte differentiation and polyploidization. Although YAP regulated genes are generally thought to induce proliferation and survival of cells in the absence of Hippo-p53 activation, knockdown of YAP had no effect on polyploidization or apoptosis of megakaryocytes but rather the reduction of YAP did lead to decreased proplatelet formation and reduced mitochondrial mass, which the authors demonstrate is a notable feature of polyploid megakaryocytes. These findings are consistent with previous studies that suggested that YAP signaling also has a role in mitochondrial regulation in *Drosophila* and human cell lines.[20]

In summary, the study by Roy *et al.* provides critical mechanistic insights into how naturally polyploid megakaryocytes overcome the tetraploid checkpoint that normally functions as a tumor suppressor pathway. Their findings clearly show that although the Hippo-p53 pathway is intact in megakaryocytes, it is not activated during polyploidization. Furthermore, they provide novel insights into the contributions of YAP to mitochondrial biogenesis, which is a notable feature of the larger, polyploid cells. However, a few important questions remain unanswered. What is the nature of the disconnect between low RhoA activity and Hippo-p53 activation, and how do megakaryocytes escape Hippo-p53 activation during polyploidization? Also, what is the link between Rac1 and RhoA in megakaryocytes? A previous study observed a 2-fold increase in Rac1 activity in tetraploid compared to diploid cells; this increased Rac1 activity was further found to suppress RhoA activity, which, in turn, typically activates the Hippo pathway.[14] Comparing the level of Rac1 activity in megakaryocytes at different stages of polyploidization would be an interesting next step. Finally, a more comprehensive analysis of YAP target genes in endomitotic megakaryocytes would provide further insights into the way that this pathway contributes to increased mitochondrial mass and platelet production. In summary, the report by Roy *et al.* provides exciting and novel insights that improve our understanding of megakaryopoiesis and may lead to improved strategies to increase platelet production.

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Ibrutinib in the real world patient: many lights and some shades

Paolo Ghia[1] and Antonio Cuneo[2]

[1]Strategic Research Program on CLL and B Cell Neoplasia Unit, Division of Experimental Oncology, Vita-Salute San Raffaele University and IRCCS San Raffaele Scientific Institute, Milan; and [2]Department of Medical Sciences, Hematology Unit. University of Ferrara, Italy.

E-mail: ghia.paolo@hsr.it *doi:10.3324/haematol.2016.155986*

With an estimated incidence of about 4.92 cases per 100,000/year in Europe[1] and 14,620 new cases in 2015 in the USA,[2] chronic lymphocytic leukemia (CLL) is the most frequent leukemia in Western countries. While a minority of patients may attain long-lasting responses with chemoimmunotherapy,[3,4,5] relapse and treatment-resistant diseases develop in the majority of cases; infections, progressive disease and second primary tumors being the most frequent causes of death.[5] The first-in-class inhibitor of Bruton's tyrosine kinase (BTK), ibrutinib, was welcomed in 2013 as a new paradigm for the treatment of relapsed or refractory CLL, as it produced responses in 71% of the cases in a heavily pre-treated patient population who had few, if any, alternative treatment options.[6,7] After a median observation of 3 years,[8] exceptional overall survival (OS) and progression-free survival (PFS) rates were reported (79% and 69%, respectively), along with a low (12%) discontinuation rate due to adverse events. Following the publication of excellent efficacy data in patients with 17p deletion (del(17p)) or *TP53* mutations,[9,10] high expectations were generated in the belief that this drug was able to produce durable responses in the majority of patients, irrespective of the presence of unfavorable prognostic factors.[11]

However, the median age of the patients in the trials was 64 years[8] and only 32% of them had a Cumulative Illness Rating Scale (CIRS) score of >6.[12] This reflects the inclusion criteria in the clinical trials, which required that the patients had an Eastern Cooperative Oncology Group (ECOG) performance status of less than 2, with adequate liver and kidney function, no significant neutropenia or thrombocytopenia and who did not require warfarin or strong CYP3A4/5 inhibitors. Because CLL is diagnosed at a median age of 70-72 years and the majority of patients carry several comorbidities,[13] the efficacy and safety data published in the literature were obtained in a patient population which did not reflect the typical patient found in everyday practice.

Two papers in this issue of Haematologica[14,15] describe the efficacy and toxicity of ibrutinib in 315 and 95 real-world patients treated in the UK and in Sweden, respectively, within a named patient scheme or a compassionate use program. Both studies adopted rigorous methods, minimizing biases inherent in retrospective studies. The baseline characteristics of the enrolled patients are summarized in Table 1, along with the salient outcome measures.

Overall, these two studies are reassuring with regards to the excellent efficacy of this new class of inhibitors, even when utilized in routine clinical practice without the many constraints and controls typical of clinical trials. Though the follow up is still short (around 1-1.5 years), objective outcome measures, i.e., median discontinuation-free survival and PFS, remain in the comfort zone, with PFS values of 77% after 10 months among the Swedish patients, and not yet reached in the UK series. These PFS values also include *TP53* disrupted patients, and, in particular, those patients with del(17p), who make up one third