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# The Role of DIAPH1 in the Megakaryopoiesis

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« Normal and Pathological Hematopoiesis »

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**THESE DE DOCTORAT**

**pour obtenir le grade de**

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**Le rôle de DIAPH1 dans la mégacaryopoïèse**

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## Abstract

Megakaryocytes (MKs) are the highly specialized precursor cells that produce platelets via cytoplasm extensions called proplatelets. Proplatelet formation (PPF) requires profound changes in microtubule and actin organization. Formins are a family of highly conserved eukaryotic proteins with multidomains that govern dynamic remodeling of the actin and microtubule cytoskeletons. Most formins are Rho-GTPase effector proteins. DIAPH1, a member of the formin family, is a mammalian homolog of *Drosophila diaphanous* gene that works as an effector of the small GTPase Rho and regulates the actomyosin cytoskeleton as well as microtubules. It contains the Rho-binding domain in the N-terminal and two distinct regions of formin homology, FH1 in the center and FH2 in the C-terminus. DIAPH1 coordinates microtubules and actin cytoskeleton through its FH2 and FH1 regions respectively, making DIAPH1 an ideal candidate in cell functions that depend closely on the cooperation between the actin and microtubule cytoskeletons.

The objective of the project was to decipher the role of DIAPH1 in megakaryopoiesis. At the end of the MK maturation, PPF and MK migration are associated with profound changes in cytoskeleton organization. Due to its dual function in actin polymerization and microtubule stabilization, DIAPH1 was an obvious candidate to play an essential role in PPF and MK migration.

Our results showed that DIAPH1 expression increased during MK differentiation, whereas DIAPH2 and DIAPH3 expression decreased suggesting that DIAPH1 may play a more important role than DIAPH2 and DIAPH3 in the late stages of MK differentiation. Immunostaining showed that DIAPH1 co-localized with F-actin, tubulin and myosin IIa along the plasma membrane and proplatelet. Using a knockdown strategy with shRNA and expression of an active form of DIAPH1, we showed that DIAPH1 is an important effector of Rho that negatively regulates PPF by remodeling actin and microtubule cytoskeletons. A previous work of our team has shown that Rho-ROCK also negatively regulates PPF by inhibiting myosin IIa activation. By the double inhibition of the DIAPH1 and the ROCK/Myosin pathway, we showed that DIAPH1 and ROCK played additive roles in the negative regulation of PPF.

These observations suggest that the cooperation between DIAPH1 and ROCK is required for the formation of cell structures dependent on actomyosin, such as the stress fibers and the contractile ring. Collectively, these results strongly suggest that cooperation of DIAPH1/microtubules and ROCK/Myosin may regulate PPF by modifying the balance between actomyosin and microtubules.

**Keywords:** Megakaryocyte, Proplatelet formation, DIAPH1, Myosin, Actin, Microtubule, Cytoskeleton, Rho

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## Résumé

Les mégacaryocytes sont les précurseurs cellulaires hautement spécialisés qui produisent des plaquettes via des extensions cytoplasmiques appelées proplaquettes. La formation des proplaquettes exige de profonds changements dans l'organisation du cytosquelette: microtubules et actine. Les formines sont une famille de protéines hautement conservées chez les eucaryotes composées de plusieurs domaines qui régulent le remodelage et la dynamique du cytosquelette d'actine et des microtubules. La plupart des formines sont des effecteurs protéiques des Rho-GTPase. DIAPH1, un membre de la famille des formines, est un homologue chez les mammifères du gène *diaphanous* de la drosophile qui fonctionne comme un effecteur de la petite GTPase Rho et régule le cytosquelette d'actomyosine ainsi que les microtubules. Il contient le domaine de liaison à Rho (Rho-binding domain) dans sa partie amino-terminale et deux régions distinctes d'homologie aux formines, FH1 localisé au centre de la protéine et FH2 dans la partie carboxy-terminale. DIAPH1 co-régule le cytosquelette des microtubules et d'actine à travers respectivement ses régions de FH2 et FH1. DIAPH1 est donc un gène candidat idéal dans toutes les fonctions cellulaires qui exigent une coopération étroite entre cytosquelettes d'actine et de microtubules.

L'objectif de ce projet de thèse était d'étudier le rôle de DIAPH1 dans la mégacaryopoïèse. A la fin de la maturation des mégacaryocytes, la formation des proplaquettes et la migration sont associées à des modifications importantes de la structure du cytosquelette. Nous avons émis l'hypothèse que grâce à sa double fonction dans la polymérisation de l'actine et la stabilisation des microtubules, DIAPH1 pourrait jouer un rôle essentiel dans les temps terminaux de la différenciation mégacaryocytaire.

Nos résultats ont montré qu'au cours de la différenciation mégacaryocytaire, l'expression de DIAPH1 augmente, alors que celles de DIAPH2 et DIAPH3 diminuent, ce qui suggère que DIAPH1 pourrait jouer un rôle plus important que DIAPH2 et DIAPH3 dans les stades tardifs de la différenciation mégacaryocytaire. Les études en immunomarquage montrent que DIAPH1 co-localise avec l'actine F, la tubuline et la myosine IIa en niveau de la membrane plasmique et des proplaquettes. Nous avons étudié la fonction de DIAPH1 par des stratégies d'inactivation (knockdown) et de surexpression d'une forme active de DIAPH1. Les résultats montrent que DIAPH1 est un effecteur important de Rho, pour réguler négativement la formation des proplaquettes en remodelant le cytosquelette d'actine et les microtubules. Le travail antérieur de notre équipe avait montré que Rho-ROCK régulait aussi négativement la formation des proplaquettes, en inhibant l'activation de la myosine IIa. En inhibant simultanément DIAPH1 et ROCK/myosine, nous avons montré que ces deux voies jouent un rôle additif dans l'inhibition de la formation des proplaquettes.

Ces résultats suggèrent que la coopération entre les voies DIAPH1 et ROCK/myosine est nécessaire pour la formation de structures cellulaires dépendant de l'actomyosine, telles les fibres de stress et l'anneau contractile en agissant à la fois sur le remodelage du cytosquelette et en assurant un équilibre entre l'actomyosine et microtubules.

**Mots-clés:** Mégacaryocytes, Formation des proplaquettes, DIAPH1, Myosine, Actine, Microtubules, Cytosquelette, Rho

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## Abbreviation

ADP, Adenosine diphosphate  
AML, Acute myeloid leukemia  
AMKL, Acute megakaryoblastic leukemia  
APC, Adenomatous polyposis coli  
Cdc42, Cell division control protein 42  
CLP, common lymphoid progenitor  
CMP, Common myeloid progenitor  
CXCR4, CXC chemokine receptor type 4  
DAD, Diaphanous auto-regulatory domain  
DID, Diaphanous-inhibitory domain  
DMS, Demarcation membrane system  
DRFs, Diaphanous-related formins  
EB3, End-binding protein three  
ECM, Extracellular matrix  
Erk1/2, Extracellular signal-related kinase 1/2  
ERG, ETS-related gene  
ETS, E26 transformation-specific or E-twenty six  
FH, Formin homology  
FGF-4, Fibroblast growth factor-4  
FLI-1, Friend leukemia integration 1  
FOG-1, Friend of GATA-1  
GABP $\alpha$ , GA-binding protein alpha  
GAPs, GTPase-activating proteins  
GBD, GTPase-binding domain  
GEFs, Guanine-nucleotide exchange factors  
GFI-1B, Growth factor independent 1B  
HSCs, Hematopoietic stem cells  
IF, Immunofluorescence  
IL, Interleukin  
IMS, Invaginated membrane system  
JAKs, Janus family of protein kinases  
KO, Knockout  
LIF, Leukemia inhibitory factor

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MAL, Megakaryocytic acute leukemia  
MAPK, Mitogen-activated protein kinase  
mDia, mammalian Diaphanous-related formins  
MDS, Myelodysplastic syndromes  
MEP, Megakaryocyte-erythroid progenitor  
MLC, Myosin light chain  
MK, Megakaryocyte  
MPL, Myeloproliferative leukemia virus oncogene  
MPPs, Multipotent progenitors  
MPD, myeloproliferative disorder  
MRLC, Myosin regulatory light chain  
MRTFs, Myocardin-related transcription factors  
MT, Microtubule  
mTOR, Mammalian target of rapamycin  
MYH, Myosin heavy chain  
MYB, Myeloblastosis  
NF-E2, Nuclear factor erythroid 2  
PAK1/2, p21-activated kinase  
PF4, Platelet factor 4  
PI3K, Phosphoinositide-3 kinase  
PKC, Protein kinase C  
PPF, Proplatelet formation  
SDF-1, Stromal cell-derived factor  
SOCS, Suppressor of cytokine signaling  
SRF, Serum response factor  
STATs, Signal transducers and activators of transcription  
ROCK, Rho-associated protein kinase  
RUNX, Runt-related transcription factor  
TPO, Thrombopoietin  
vWF, von Willebrand Factor  
WASP, Wiskott Aldrich syndrome protein  
WB, Western blot

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## Chapter I: Introduction

All blood cells are derived from hematopoietic stem cells (HSCs). HSCs reside in the bone marrow and are the only long life cells of the hematopoietic system, capable to self renew and to differentiate towards all hematopoietic cells. One of the lineages derived from HSCs is the megakaryocytic cell line; the entire process of differentiation of this cell line is called megakaryopoiesis or thrombopoiesis and gives rise to platelets. Megakaryocyte (MK) is the bone marrow cell that has the ability to produce circulating platelets (Kaushansky, 2008).

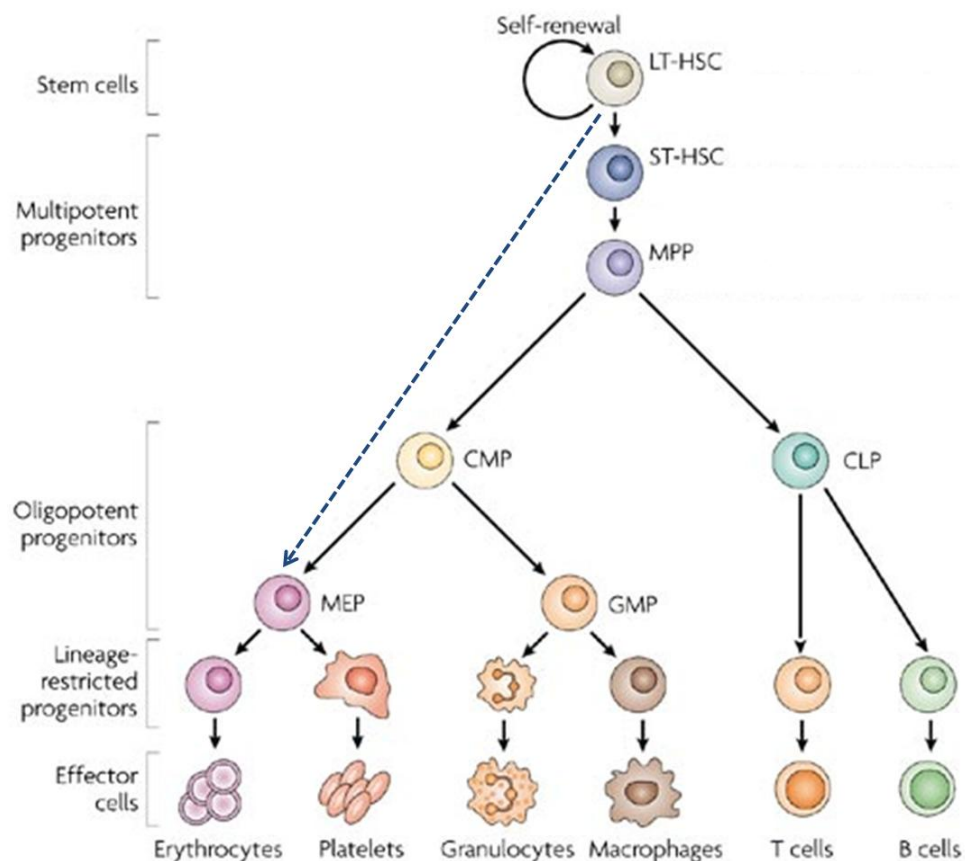
During hematopoiesis, HSCs give rise to multipotent progenitors (MPPs), a cell that has lost its self-renewal capacities, but has retained the capacity to differentiate towards all hematopoietic multi-lineages (Figure 1). Generally, it is believed that the multipotent progenitors will subsequently give rise to two types of progenitors, the common lymphoid progenitor (CLP) (Kondo et al., 1997) and the common myeloid progenitor (CMP) (Akashi et al., 2000). CLP then will give rise to all lymphoid cells including natural killer cells, T and B cells and dendritic cells, while CMP will be at the origin of all myeloid lineages including the granulocyte/macrophage progenitor and the MK-erythroid progenitor (MEP) (Debili et al., 1996; Szalai et al., 2006). MEPs are bipotential precursors that give rise to cells of both erythroid and megakaryocytic lineages. Nevertheless, recent studies have suggested that the MEP may directly derive from the HSCs to yield either the erythroid or megakaryocytic lineages without the CMP intermediate (Adolfsson et al., 2005; Forsberg et al., 2006). Furthermore, it has also been suggested the presence of MK biased HSCs expressing the CD41 or the vWF (von Willebrand Factor), which allow a rapid platelet recovery in case of bone marrow transplantation. However this classical model is presently challenged by a model in which committed progenitors play a central role in producing blood cells in homeostatic conditions.

### 1 Megakaryopoiesis

Megakaryopoiesis is a complex and integrated differentiation process, which is highly regulated at multiple stages. The MK differentiation from MK progenitors includes the switch from mitosis to endomitosis and proplatelet formation (PPF). After proliferation by mitosis at the level of progenitors, MKs enter into endomitosis and increase their ploidy, then they develop an extensive internal demarcation membrane system (DMS), now called invaginated membrane system (IMS), which serves as a membrane reservoir and then mature to release platelets through the formation of long pseudopods, which are called proplatelet (Italiano et al., 1999a). Platelets are shed into vascular

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sinusoids within the bone marrow by these processes. Platelets are essential in homeostasis and thrombosis and also in other processes such as the development of innate immunity, angiogenesis and metastasis. MK differentiation is regulated both positively and negatively by transcription factors and by the cytokine signaling.



**Figure 1:** The hematopoietic hierarchy

Overview of the differentiation of hematopoietic stem cells (HSCs) towards the different hematopoietic lineages.

HSCs are both multipotent and capable of self-renewal. LT-HSCs (long-term hematopoietic stem cell) represent the ‘true’ stem cells that have important self-renewal capacities and produce ST-HSCs (short-term hematopoietic stem cell) with limited self-renewal capacities and multipotent potential and subsequently the MPPs (multipotent progenitor) without self-renewal capacity. MPPs give rise to CLPs (common lymphoid progenitor) and CMPs (common myeloid progenitor). The CLPs then give rise to lymphocytes including T cells and B cells, while CMPs give rise to GMPs (granulocyte-macrophage progenitor) that produce granulocytes and macrophages and MEPs (megakaryocyte-erythrocyte progenitor) that produce erythrocytes and megakaryocytes/platelets. LT-HSCs can directly give rise to MEPs without the CMP intermediate (Figure adopted and modified from (Sharpless and DePinho, 2007) ).

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## **1.1 Transcriptional regulation of megakaryopoiesis**

A lot of transcription factors are involved in the regulation of megakaryopoiesis (Chang et al., 2007b; Goldfarb, 2007). During megakaryopoiesis, series of transcription factors coordinately regulate the MK-specific gene expression.

Through loss-of-function studies in mice and analysis of human diseases, various transcription factors have been identified, which are involved in MK differentiation, polyploidization, proplatelet formation (PPF) and platelet shedding. Multiple transcription factors, including RUNX1 (AML1), GATA-1/GATA-2, FOG-1 (Friend of GATA-1), GFI1b, NF-E2, FLI-1 (Friend leukemia integration1) and MYB, form large protein complexes that regulate the MK differentiation both positively and negatively (Figure 2) (Chang et al., 2007b). Some important examples are developed below.

### **1.1.1 GATA-1/FOG-1 related complex**

GATA-1 is a member of the GATA transcription factor family and is involved in cell growth and malignancy. GATA-1 protein regulates the expression of nearly all erythroid and MK specific genes. It also plays a role in erythroid development by regulating the switch from fetal to adult hemoglobin. GATA-1 plays also a crucial role in MK development acting as either activator or repressor depending of the protein complexes (Chang et al., 2007b). GATA-1 regulates the main MK-specific genes such as GPIIB, PF4 (platelet factor 4), GPIb $\alpha$ ,  $\beta$ -TG, GPIX or GPV.

FOG-1 (Friend of GATA-1) is one of the most important co-factors of GATA-1. The GATA-1/FOG-1 complex is critical in promoting MK/Erythroid lineage differentiation. The X chromosome-linked GATA-1 contributes to the differentiation of hematopoietic progenitors into erythroid and MK cells, and also to the development of eosinophil and mast cells. GATA-1 and FOG-1 physically interact and their complex is essential for GATA-1 functions in different cellular contexts. FOG-1 contributes to the regulation of cell type-specific gene expression in erythroid and MK differentiation (Tsang et al., 1997). Mice lacking FOG-1 have an absence of megakaryopoiesis and an arrest in erythropoiesis (Tsang et al., 1998). Loss of the GATA-1/FOG-1 interaction leads to an obvious decrease in membrane protein expression and to an increase in reactive oxygen species accumulation, which disrupts the function of GATA-1 in erythrocyte development (Hasegawa et al., 2012).

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GATA-1 mutations in the site of interaction with FOG-1 lead to an inherited human disorder characterized by an X-linked dyserythropoietic anemia associated with a macrothrombocytopenia: platelets are giant and immature with abnormal membrane complexes (Freson et al., 2001; Shivdasani et al., 1997). GATA-1 deficient MKs fail to undergo terminal differentiation and exhibit a significant hyperproliferation *in vitro*. *gata-1* knockout (KO) mice die at the embryonic stage because of a failure in erythroid maturation with a blockage at the proerythroblast stage (Fujiwara et al., 1996; Fujiwara et al., 2004). GATA-1 and GATA-2 have overlapping functions at the yolk sac stage required for normal hematopoiesis, loss of either leads to embryonic lethality in KO mice due to a failure in erythroid maturation and the expansion of progenitors, suggesting that either GATA-1 or GATA-2 is essential to initiate blood formation in the embryo (Fujiwara et al., 2004).

Mutations in exon 2 leading to a short form of GATA-1 (GATA-1s) are associated with all cases of Down syndrome-associated transient myeloproliferative disorder or transient leukemia (TL) (Greene et al., 2003), which may progress to acute megakaryoblastic leukemia (AMKL) (Rainis et al., 2003; Wechsler et al., 2002). GATA-1 mutations are present *in utero* demonstrating that it is an early event in leukemogenesis. GATA-1 mutation at birth may serve as a biomarker for an increased risk of Down syndrome-related AMKL (Pine et al., 2007; Shimada et al., 2004b).

GATA-1 can interact with the myeloid PU.1 transcription factor and can repress PU.1-dependent transcription and myeloid differentiation (Nerlov et al., 2000). The competition between GATA-1 and PU.1 play an important role in determining the hematopoietic cell fate in CMP (Chou et al., 2009). Huang and colleagues have demonstrated that GATA-1 can regulate not only MK maturation by activation of platelet-specific gene expression, but also MK polyploidization by modulating STAT1 (Huang et al., 2007).

GFI-1B (growth factor independent 1B), a zinc-finger proto-oncogene acting as transcription repressor, is essential for erythroid and MK lineage development by interacting with GATA-1 (Saleque et al., 2002). Taken together, all these findings emphasize the requirement of GATA-1 for MK development and platelet biogenesis.

### **1.1.2 MYB**

c-MYB proto-oncogene protein, known as a transcriptional activator, is a member of the MYB (myeloblastosis) family of transcription factors. MYB family proteins contain three domains: an N-terminal DNA-binding domain, a central transcriptional activation domain and a C-terminal regulatory



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domain involved in transcriptional repression. c-Myb is highly expressed in multipotent hematopoietic progenitors and plays an important role in the regulation of hematopoietic cell differentiation and in oncogenesis (Oh and Reddy, 1999).

Loss of c-Myb results in an embryonic lethality due to a failure of hematopoietic development. Suboptimal level of c-Myb expression induces the commitment of MPP toward the MK lineage and macrophage, while high level may control the erythropoiesis and lymphopoiesis (Emambokus et al., 2003). In addition, c-Myb mutant mice exhibit a myeloproliferative phenotype with an important MK expansion and a major increased platelet production without requiring TPO signaling (Carpinelli et al., 2004; Garcia et al., 2009).

Studies have shown that c-Myb activity may affect the balance between MK lineage differentiation and other hematopoietic lineages (Emambokus et al., 2003; Frampton et al., 1995). In another study, it has been shown that a transgenic insertion inducing a marked decrease of c-Myb expression in the MEP, leads to an increase in MK number and a decrease in erythroid progenitors (Mukai et al., 2006). Expression of the c-Myb in the MEP favors erythropoiesis, and is down-regulated during megakaryopoiesis (Metcalf et al., 2005). These findings indicate that c-Myb is a crucial regulator of the MK/Erythroid lineage fate.

Furthermore, studies have shown that MYB is a target of the microRNA-150 (miR-150) in human and is negatively regulated by miR-150. MYB and miR-150 interaction is essential for embryonic development and probably oncogenesis (Lin et al., 2008). By gain- and loss-of-function studies, Lu J and colleagues have shown that miR-150 is expressed in MK development and in a post-transcriptional manner decreases c-Myb expression and directs MEP differentiation toward the MK lineage (Lu et al., 2008). Another study suggests that TPO regulates the level of miR-150 and favors MK engagement of the MEP by down-regulating c-Myb (Barroga et al., 2008).

Collectively, c-MYB has a complex involvement in the regulation of proliferation and establishment in the hematopoietic hierarchy, but inhibits MK differentiation.

### **1.1.3 ETS family**

ETS (E26 transformation-specific or E-twenty six) family is one of the largest families of transcription factors. All ETS family members are identified by a highly conserved DNA binding domain, which is called ETS domain with a winged helix-turn-helix structure (Lee et al., 2005; Yordy and Muise-

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Helmericks, 2000). In addition to DNA binding function, ETS domain is also involved in protein-protein interaction. ETS family proteins are implicated in a wide range of functions including the regulation of cell differentiation, cell cycle control, cell migration, cell proliferation, apoptosis and angiogenesis. ETS-domain proteins function as either transcriptional repressors or transcriptional activators. 29 ETS genes in humans and 28 genes in the mouse have been described; among them, some are expressed in megakaryopoiesis and play a role in the development of erythroid and megakaryocytic lineages, such as *FLI-1*, *GABPA*, *ETS1*, *ETS2* and *ERG* (ETS-related gene).

FLI-1 is the most investigated ETS family member and is required for normal MK maturation by controlling MK-specific gene expression. It can cooperate with GATA-1 and FOG-1 to activate the expression of late MK genes such as *GPIX*, *GPIb $\alpha$* , and *PF4*. *fli-1* KO affects both vascular development and megakaryopoiesis, which leads to embryonic lethality at day 11.5 of embryogenesis with loss of blood vessel integrity and a partial block in MK differentiation (Hart et al., 2000). Another group has shown that *fli-1* mutant mice have a profound hematopoietic phenotype and hematopoiesis is severely impaired at mid-gestation, which provide *in vivo* evidence for the role of Fli-1 in the regulation of hematopoiesis and hemostasis (Spyropoulos et al., 2000). In addition, FLI-1 and GATA-1 work in cooperation to activate the expression of MK-specific genes (Eisbacher et al., 2003): FLI-1 enhances the GATA-1 activity at MK promoters and represses the activity of erythroid factors on erythroid promoters (Starck et al., 2003).

In human, the Paris Trousseau syndrome and Jacobsen syndrome have similar congenital anomalies with a thrombocytopenia and giant platelet  $\alpha$ -granules, which are the consequence of a FLI-1 haploinsufficiency due to a chromosome 11 deletion at 11q23.3 (Breton-Gorius et al., 1995; Raslova et al., 2004). However, *fli-1* deletion in adult mice induces a mild thrombocytopenia with a defect of bone marrow MK maturation and modifies several myeloid lineage commitment decisions and accelerates proliferation arrest and terminal erythroid differentiation (Starck et al., 2010).

ERG is an ETS family member extremely homologous with FLI-1. One study has shown that ERG is essential for definitive hematopoiesis in the embryo, adult HSC regulation and maintenance of the normal number of blood platelets (Loughran et al., 2008). Evidence from genetic approaches suggests that FLI-1 is required for megakaryopoiesis and ERG, more for normal adult HSC function. Double heterozygous KO mice for *fli-1* and *erg* display a more dramatic phenotype than those with a single deletion, concerning the thrombocytopenia, defect in MK numbers and MK maturation (Kruse et al., 2009). These results suggest that FLI-1 and ERG act in synergy and may regulate common target genes.

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In addition, some other members of the ETS family are also involved in the regulation of megakaryopoiesis, such as TLE-1, ETS1 and GABP $\alpha$  (GA-binding protein alpha). *GABP $\alpha$* -deficient mice show a decrease in immature MKs and an increase in mature MKs. In addition, the ratio between GABP $\alpha$ /FLI-1 decreases during MK maturation. In agreement with their pattern of expression, it has been shown that GABP $\alpha$  regulates early stages of MK differentiation, particularly the expression of early MK-specific genes such as *GPIIb* and *MPL* (Pang et al., 2006).

Furthermore, FLI-1 can interact with RUNX-1 by direct protein-protein interaction and results in a synergistic transcriptional activation of the *MPL* promoter. They are associated in a transcriptional protein complex that also includes GATA-1 and FOG-1. FLI-1 dephosphorylation affects RUNX-1 binding and inhibits *in vitro* fetal liver differentiation of MK. These data underscore the interaction of FLI-1 and RUNX-1 during MK development (Huang et al., 2009).

#### **1.1.4 RUNX1**

RUNX1, also known as AML1 (acute myeloid leukemia 1) or CBFA2 (core-binding factor subunit alpha-2), is a member of the RUNX (Runt-related transcription factor) family. RUNX-1 is a DNA-binding subunit of the core binding transcription factor complex, which is expressed in almost all hematopoietic cells and contributes to the commitment of hematopoietic stem and progenitor cells by regulating or interacting with other elements (Okuda et al., 2001).

RUNX1 may interact and cooperate with GATA-1 in MK differentiation and during hematopoietic development in mammals (Elagib et al., 2003; Waltzer et al., 2003). RUNX1 is one of the rare transcription factors that is involved in MK differentiation, but inhibits erythroid differentiation, suggesting that RUNX1 plays an essential role in the MK/Erythroid lineage commitment (Niitsu et al., 1997). An inducible *runx-1* KO leads to a marked defect in megakaryopoiesis with a thrombocytopenia, highlighting its important role in MK differentiation (Ichikawa et al., 2004; Sun and Downing, 2004).

It has been shown that *runx1* haploinsufficiency is associated with alterations in the early mesoderm development of ES cell differentiation (Lacaud et al., 2004), as well as it influences the temporal and spatial generation of HSC in mouse embryos (Cai et al., 2000). In the hematopoietic system, it induces an increase in myeloid progenitors and may lead to leukemogenesis (Yamashita et al., 2005).

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Several RUNX1 target genes have been identified during MK differentiation, such as *CBFβ*, *MPL*, *IEX-1*, *MYL9* (myosin light chain) (Hamelin et al., 2006; Heller et al., 2005; Jalagadugula et al., 2010; Song et al., 1999), as well as *p19<sup>INK4D</sup>*, which is involved in the endomitotic arrest and MK maturation (Gilles et al., 2008). Germ-line heterozygous mutations and somatically point mutations are found in *RUNX-1*, all resulting in loss of transcriptional activity. Germ line mutations induce familial platelet disorder with predisposition to develop acute myeloid leukemia (AML) (Harada et al., 2004; Heller et al., 2005; Song et al., 1999).

The role of RUNX1 on the control of HSC fate may be achieved by regulating the MPL promoter both positively and negatively, this dual activity being related to the binding partner according to the cell types (Sato et al., 2008). The *RUNX1* mutation related thrombocytopenia may result from a low MPL receptor expression in MK although low MPL expression is usually associated with a thrombocytosis in several murine models, but more obviously from altered expression of numerous genes encoding the actomyosin cytoskeleton (Heller et al., 2005).

### **1.1.5 SRF/MAL**

SRF (serum response factor), a member of the MADS (MCM1, Agamous, Deficiens and SRF) domain-containing transcription factor, binds to the serum response element in the promoter/enhancer region of many target genes and confers the recruitment of other transcriptional cofactors. SRF is widely expressed and plays an important role in regulating the activity of many genes including immediate early genes and genes involved in cytoskeleton and muscle development, as well as genes involved in cell cycle regulation, growth and differentiation (Chai and Tarnawski, 2002; Miano, 2010; Morita et al., 2007; Schratt et al., 2002).

MAL (megakaryocytic acute leukemia, also known as MKL1, BSAC and MRTF-A), a member of the MRTF (myocardin-related transcription factor) family, is one of the most important cofactors of SRF (Vartiainen et al., 2007). There are different extracellular stimuli that can activate SRF. One is the MAPK (mitogen-activated protein kinase) pathway, which acts through the ternary complex factors, the other is the small GTPase pathway acting through the MRTF family proteins (Cen et al., 2003; Hill et al., 1995). The MAL/SRF interaction is regulated by the Rho-actin pathway (Miralles et al., 2003; Morita et al., 2007). A recent study has shown that SRF and MAL regulate the myofibroblast differentiation by responding to both TGF-β1 and RhoA signaling (Small, 2012).

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SRF and MAL play a crucial role in MK terminal differentiation including PPF. *Mrft-A (Mal)* KO mice exhibit a decrease of platelet count in peripheral blood and a reduced ploidy in bone marrow MKs, suggesting the involvement of MAL in MK differentiation (Cheng et al., 2009). *MAL* knockdown resulted in dysmorphic MKs with disorganized DMS and  $\alpha$ -granules heterogeneously scattered in the cytoplasm, as well as a decreased expression of MYL9 and MMP9 (Gilles et al., 2009). *Srf* KO mice exhibit a similar phenotype with an abnormal DMS, a reduced platelet count and a down-regulation of actin cytoskeleton gene expression as well as an abnormal stress fiber formation and actin distribution (Halene et al., 2010). These indicate that MAL/SRF complex is involved in normal MK maturation and PPF by regulating MYL9 and MMP9.

MAL is shuttling from the cytoplasm to the nucleus. When localized in the nucleus, it activates SRF target genes transcription, suggesting that MAL subcellular localization is one important mechanism to control SRF activity (Vartiainen et al., 2007). A recent study has shown that MAL localization is disrupted by drugs inhibiting RhoA activity or actin polymerization, revealing that MAL subcellular localization and function is dependent on Rho GTPase family and actin organization (Smith et al., 2013). These data widen the study of the molecular mechanisms that regulate MK differentiation. MAL16 (also known as MKL2, MRTF-B), a homologue of MAL, is also expressed in MK and participates in MK differentiation. By a double KO strategy, it has been demonstrated that MAL and MAL16 play a redundant and crucial role in MK maturation and platelet formation (Smith et al., 2012). Together, these findings confirm that SRF/MAL is necessary for MK maturation and platelet production, partly by regulating cytoskeleton genes.

### 1.1.6 NF-E2

The transcription factor NF-E2 (nuclear factor erythroid 2), belongs to the basic-leucine zipper family. It is a heterodimer complex containing two basic subunits, a widely expressed 18-kDa (p18<sup>NF-E2</sup>) subunit (Andrews et al., 1993b) related to chicken Maf proteins and a tissue-restricted 45-kDa (p45<sup>NF-E2</sup>) subunit (Andrews et al., 1993a), whose expression is restricted to erythroid cells, MKs and mast cells.

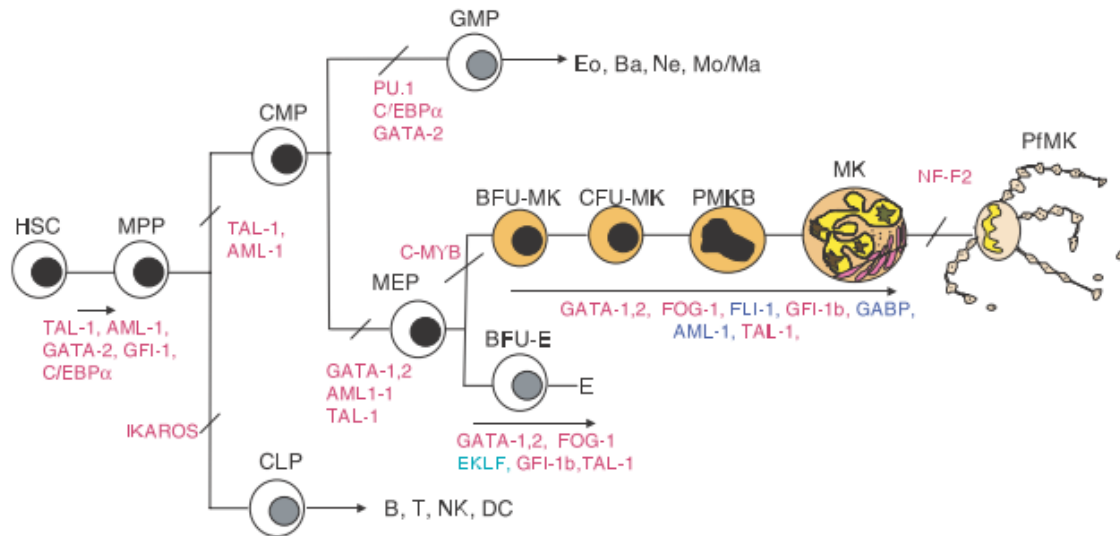
The NF-E2 complex is essential in erythroid and MK maturation and differentiation. *p45<sup>NF-E2</sup>* KO mice are lethal at birth as a consequence of a profound thrombocytopenia with an increased number of MKs, which present evident defect in both DMS development and  $\alpha$ -granules distribution and an absence of PPF (Shivdasani et al., 1995). Other genetic evidence has indicated a crucial role for p45<sup>NF-E2</sup> and small Maf in the terminal MK maturation and platelet production. The double KO mice for the two

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NF-E2 partners, MafG and MafK show a similar thrombocytopenia as p45<sup>NF-E2</sup> KO mice with an absence of proplatelets (Lecine et al., 1998; Motohashi et al., 2000; Onodera et al., 2000). Furthermore, these mutant mice develop a severe anemia accompanied by an abnormal erythrocyte morphology and modifications in the membrane protein composition. These findings provide evidences that NF-E2 plays an important regulatory role in erythropoiesis (Onodera et al., 2000), more particularly by regulating globin gene transcription.

Several genes have been established as regulated by p45<sup>NF-E2</sup> in MKs directly or indirectly, such as Thromboxane synthase,  $\beta$ 1-tubulin, Rab27b, 3 $\beta$ -HSD (3beta-hydroxysteroid dehydrogenase) and Lims1 (Chen et al., 2007a; Deveaux et al., 1997; Lecine et al., 2000; Nagata et al., 2003; Tiwari et al., 2003), all of which are involved in PPF and platelet biogenesis. Recently, *Selp* and *Myl9*, two other genes have been identified as direct p45<sup>NF-E2</sup> targets, which are also implicated in platelet production and function (Fujita et al., 2013). All these observations provide strong evidence that NF-E2 is essential for establishing normal platelet function as well as for generating the proper number of platelets.

Some other transcription factors also participate in hematopoiesis, MK differentiation and platelet production, such as zinc finger protein GFI-1b (growth factor independent 1B transcription repressor). GFI-1b is a crucial proto-oncogenic transcriptional regulator necessary for MK/Erythroid lineage development and differentiation. Dependent on the promoter and cell type context, GFI-1b functions as transcriptional repressor or transcriptional activator. It may also regulate cytokine signaling by repressing the activity of some promoters.



**Figure 2:** Overview of the transcription factors involved in megakaryopoiesis

Multiple transcription factors regulate megakaryopoiesis at different developmental stages. HSC, hematopoietic stem cell; MPP, multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte-monocyte progenitor; Eo, eosinophil; Ba, basophil; Ne, neutrophil; Mo/Ma, monocyte/macrophage; MEP, megakaryocyte/erythrocyte progenitor; BFU-MK, burst-forming unit megakaryocyte; CFU-MK, colony-forming unit megakaryocyte; PMKB, promegakaryoblast; MK, megakaryocyte; PfMK, proplatelet forming megakaryocyte; BFU-E, burst-forming unit erythrocyte; NK, natural killer cell; DC, dendritic cell (figure from (Chang et al., 2007b) ).

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## 1.2 TPO/MPL signaling in megakaryopoiesis

Cytokines and multiple compounds of bone marrow microenvironment are also involved in the regulation of MK differentiation. Numerous cytokines can stimulate MK progenitor proliferation, such as GM-CSF (Granulocyte-macrophage colony-stimulating factor), IL (interleukin)-3, IL-6, IL-11, IL-12 and erythropoietin. Nevertheless, other cytokines participate in the regulation of MK maturation and platelet release, such as IL-1 $\alpha$  and leukemia inhibitory factor (LIF) (Vainchenker et al., 1995). Thrombopoietin (TPO) is the most important cytokine for MK differentiation, more particularly in homeostatic conditions, as well as it plays a central role in HSC regulation (Kaushansky, 2006).

TPO is also known as megakaryocytic growth and development factor or MPL (Myeloproliferative leukemia virus oncogene) ligand. TPO is a glycoprotein which functions as a hormone and is produced by the liver and at a lesser extent by kidney and regulates platelet production in homeostasis. TPO acts throughout all stages of megakaryopoiesis, including MK progenitor proliferation and MK polyploidization. It also increases MK size by promoting protein synthesis. The TPO receptor, MPL has been identified as the proto-oncogene of the viral oncogene *v-mpl*, a member of the hematopoietic receptor super-family that was identified as the transformation factor of the murine myeloproliferative leukemia virus.

The TPO/MPL signaling pathway regulates platelet production and conversely the platelet number in a feedback manner negatively regulates TPO level. TPO is constitutively produced and the circulating TPO level is regulated both by platelets, its end products and MKs. TPO binds to MPL expressed by platelets and is endocytosed before being degraded. The blood platelet number thus regulates the TPO level. Low platelet level leads to higher level of plasmatic TPO that increases the number of MKs enhancing their further maturation. On the other hand, high platelet level decreases the availability of TPO for MKs. The discovery of TPO has contributed profoundly to platelet biology because it allows the development of cultures that generate MKs *in vitro*. This has greatly facilitated studies of MK differentiation and platelet related disorders. TPO and now synthetic MPL ligands are potential thrombopoietic and hematopoietic agents that are used as therapeutic drugs to stimulate expansion of HSC *ex vivo* and to treat some thrombocytopenia (Basser, 2002).

TPO/MPL has remarkable functions in MK differentiation and thrombopoiesis through the TPO-dependent signaling pathways (Figure 3) (Geddis, 2010; Santos and Verstovsek, 2011). These pathways include the Janus family of protein kinases (JAKs)/signal transducers and activators of



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transcription (STATs) pathway (Schulze et al., 2000), the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway (Rojnuckarin et al., 1999), the phosphoinositide-3 kinase (PI3K)/Akt/mTOR pathway (Nakao et al., 2008; Pasquet et al., 2000; Soda et al., 2008) and the nuclear factor kappa B (NF- $\kappa$ B) pathway (Zhang et al., 2002). The important role of TPO has been demonstrated by animal experiments. TPO and MPL are essential for MK growth and development in mouse models, KO of one of them induces reduces numbers of MKs and of circulating platelets to approximately 10% of the normal (Gurney et al., 1994; Murone et al., 1998).

MPL does not have intrinsic kinase activity, which is dependent on the cytoplasm tyrosine kinase JAK2. JAK2 kinase activates the downstream signaling pathways, such as the STATs, PI3K and the MAPK that activate and mediate the cellular response to TPO (Geddis et al., 2002). This cascade signaling allows cell proliferation and differentiation. MPL and JAK2 are involved in multiple inherited and malignant disorders leading to thrombocytosis, thrombocytopenia and aplastic anemia.

TPO binding changes MPL conformation leading to JAK2 phosphorylation. Activated JAK2 kinase phosphorylates MPL, which induces the binding of the different STAT family members and their subsequent phosphorylation (Schulze et al., 2000). This leads to the dimerization of the activated STAT proteins and then to their translocation to the nucleus where they act as transcription factors by binding to different STAT-response DNA sequences. Constitutive activation of JAK2/STAT pathway induces a cytokine-independent growth and transformation, such as the V617F mutant of JAK2 in myeloproliferative disorders, TEL-JAK2 in leukemia or a constitutively active STAT5 in different leukemia cell lines (Harir et al., 2007; Najfeld et al., 2007).

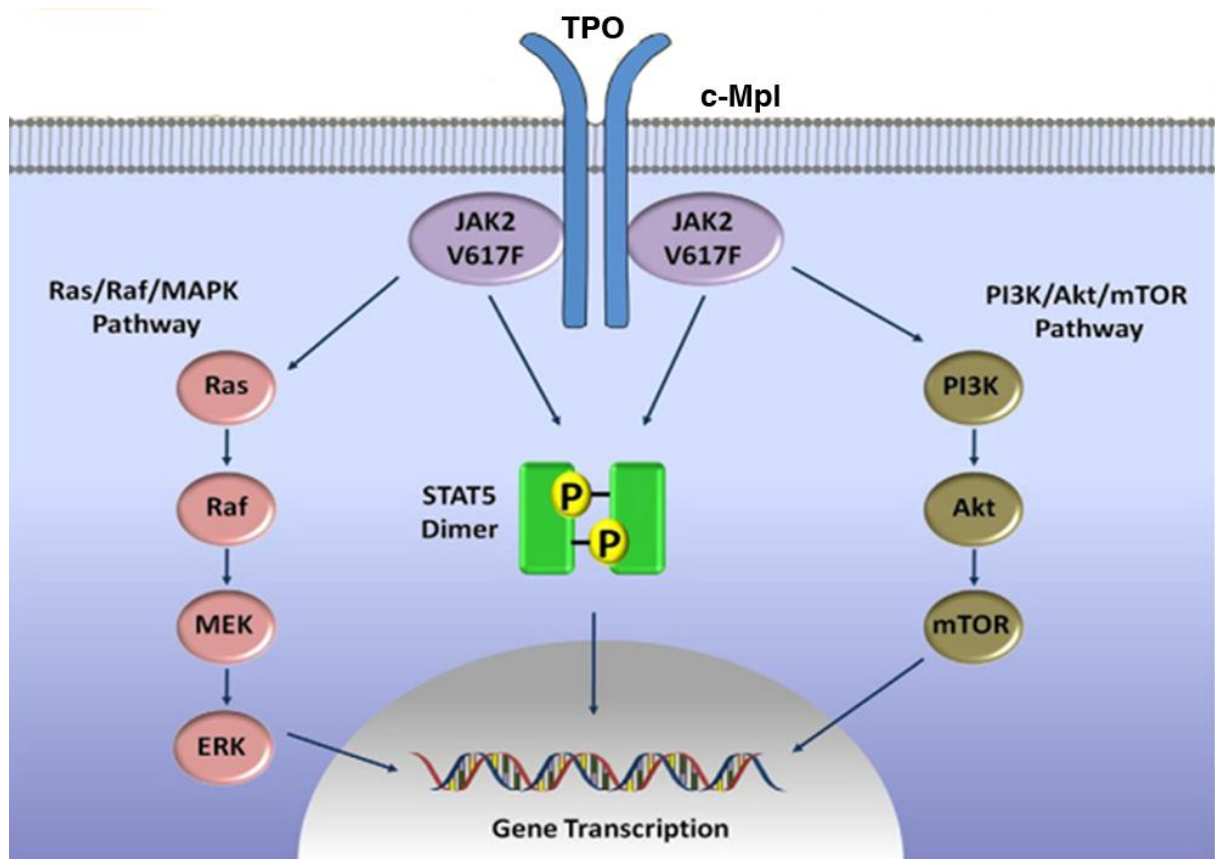
In addition, JAK2 can activate the small Ras-GTPase and the MAPK pathway to stimulate the activation of extracellular signal-related kinase 1/2 (ERK1/2). Studies have shown that TPO-induced MAPK signaling pathway has important role although controversial in normal MK differentiation (Rojnuckarin et al., 1999). TPO signaling activates Ras-GTPase based on the binding of the adaptor protein SHC to the phosphorylated MPL and the assembly of a complex consisting of the adaptor protein GRB2 (Growth factor receptor-bound protein 2) and the guanine nucleotide exchange factors (GEFs) like SOS (Son of sevenless), and then RAS activates RAF-1, ERK1/2 and MEK (mitogen-induced extracellular kinase) (Avruch et al., 2001).

The PI3K signaling pathway also participates downstream of JAK2 in megakaryopoiesis. It has been shown that TPO up-regulates platelet  $\alpha$ -granule secretion and aggregation induced by thrombin through PI3K signaling (Kojima et al., 2001). TPO stimulates PI3K phosphorylation and activation of

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the threonine/serine kinase AKT (also known as protein kinase B, PKB) that enhances the TPO-induced survival and proliferation of MK (Nakao et al., 2008; Soda et al., 2008). mTOR (mammalian target of rapamycin), a downstream target of the PI3K/AKT pathway, is involved in the growth and the ploidy of mature MKs. Inhibition of mTOR by rapamycin reduces MK size and ploidy, through an inhibition of the G1/S transition, via a decrease of p21 and cyclin D3, and a delay in MK maturation preventing PPF. These results suggest that mTOR plays an important role in MK proliferation and maturation (Raslova et al., 2006).

Furthermore, TPO has a major function in the regulation of HSC and progenitors *in vivo* and *in vitro* (Bruno et al., 2003; Kaushansky, 2006; Pick et al., 2002). Studies of *c-mpl* KO mice have shown that TPO/MPL is associated with early hematopoietic progenitor development, while HSCs also express MPL and depend on TPO signaling for their maintenance and expansion (Fox et al., 2002). Studies have shown that TPO plays an important role in maintaining HSC quiescence in the bone marrow niche and in DNA gene repair after double strand DNA breaks by stimulating NHEJ (de Laval et al., 2014). *tpho* and *c-mpl* KO mice show not only a thrombocytopenia, but also a decrease in HSCs and in early progenitors of all hematopoietic lineages (Murone et al., 1998). Thus, the TPO/MPL axis plays a crucial role for hematopoiesis and megakaryopoiesis. Interestingly, in human, hereditary homozygous or composite heterozygous loss of function mutations of MPL lead to a thrombocytopenia with an absence of MK, which subsequently progresses to aplastic anemia, demonstrating that the TPO/MPL axis is also crucial for the maintenance of human HSCs.



**Figure 3:** TPO-induced signaling pathways in megakaryopoiesis

TPO binds to its receptor Mpl and the conformational changes on the homodimer induced by TPO binding result in activation of JAK2 by transphosphorylation, which initiates and activates downstream signaling pathways including the STATs, PI3K/Akt/mTOR and Ras/Raf/MAPKs pathways. TPO, thrombopoietin; MPL, Myeloproliferative leukemia virus oncogene; JAK, Janus family of protein kinase; PI3K, Phosphoinositide-3 kinase; mTOR, mammalian target of rapamycin; MAPK, mitogen-activated protein kinase (Figure adopted from (Santos and Verstovsek, 2011) ).

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Recent data suggest that the roles of TPO and MPL/JAK2 on MK differentiation are more complex than previously thought. First, it has been shown that a strong signaling through MPL/JAK2 induces an arrest in proliferation and a 'senescence' like phenotype. Second, *jak2* and *c-mpl* KO during MK differentiation induce a thrombocytosis associated with high TPO level. This suggests that the role of TPO is indispensable in early stages of hematopoiesis and MK differentiation, but is not required for terminal differentiation. MPL plays an important role in late MK differentiation stages, not as a signaling receptor, but more as a receptor involved in TPO clearance.

As the main cytokine regulating MK development and platelet production, TPO is critical to maintain the homeostatic balance and prevent a hematopoietic deficiency, such as thrombocytopenia and aplastic anemia. TPO/MPL signaling is also negatively regulated in order to maintain this homeostatic balance. For instance, the JAK/STAT pathway activation induces the transcription of members of the suppressor of cytokine signaling (SOCS) family, which can inhibit JAK signaling in different ways according to the member (Alexander and Hilton, 2004). TPO signaling and JAK2 activity can be inhibited by TPO induced SOCS1 and SOCS3, respectively (Hookham et al., 2007; Wang et al., 2000). LNK is an adaptor protein that binds JAK2 and regulates its activity. LNK negatively regulates growth of HSCs, erythroid and megakaryocytic cells by inhibiting TPO/MPL signaling pathway (Seita et al., 2007; Tong and Lodish, 2004).

Overall, TPO is the most important hematopoietic cytokine for MK differentiation and platelet production; both a positive and a negative regulation of TPO signaling are necessary for homeostasis and hematopoietic development.

Chemokines and cellular interactions are also involved in megakaryopoiesis and thrombocytopoiesis. The two chemokines that have crucial roles on megakaryopoiesis and platelet production are SDF-1 (Stromal cell-derived factor1, also known as CXCL12) and PF4 (CXCL4). SDF-1 belongs to the CXC family, which is involved in MK differentiation and in homing of HSCs to the bone marrow. SDF-1 and fibroblast growth factor-4 (FGF-4) can restore platelet production in *thpo*<sup>-/-</sup> and *c-mpl*<sup>-/-</sup> mice by mediating interaction of MK progenitors with the bone marrow vascular niche (Avecilla et al., 2004). CXCR4 (CXC chemokine receptor type 4) is the SDF-1 receptor, which is expressed during the entire MK differentiation from early progenitor to platelet production. SDF-1-induced migration of mature MK through endothelial cell layers leads to an increase in platelet production (Lane et al., 2000). A study has shown that molecular upregulation of CTAPIII in MKs, a CXC cytokine, is associated with maturation and is involved in cellular interactions with extracellular matrix (ECM) and platelet production (Deutsch et al., 2000).

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## **2 Cytoskeleton and MK differentiation**

MK differentiation is associated with multiple cell changes at the level of the cytoplasmic membrane and of secretory granules and in the cytoskeleton. Reorganization of cytoskeleton including MT (microtubule), actin filaments and myosin, plays an essential role in MK differentiation and migration, particularly in PPF and platelet release. Because of the dramatic morphological changes that occur during proplatelet production, the cytoskeleton mechanics that drive these processes have become a central issue in studies of megakaryopoiesis.

### **2.1 MK maturation**

The late stages of MK differentiation include the switch from mitosis to endomitosis and PPF. After proliferation by mitosis, the MK performs endomitosis to increase its ploidy and then matures to release platelets through the formation of long pseudopods, which are called proplatelets (Deutsch and Tomer, 2006; Italiano et al., 1999a). The former process by inducing MK polyploidization increases the cell volume, while the latter is essential for platelet production. At last, the mature MK migrates to the vascular niche where platelets are directly released into the blood stream.

#### **2.1.1 Endomitosis/polyploidization**

When MKs progressively differentiate from HSCs, they lose their ability of proliferation and become polyploid through a process called endomitosis (Bluteau et al., 2009). The MK endomitotic cell cycle appears to be nearly identical to a normal proliferative cell cycle, but lacking cytokinesis (Figure 4). During differentiation, diploid pre-megakaryoblasts give rise to tetraploid megakaryoblasts and then larger and more polyploid pro-MKs and MKs by rounds of DNA replication.

During cell cycle, MKs firstly undergo a proliferative 2N stage which is the same as for other hematopoietic cells and subsequently begin endomitosis and accumulate successively a DNA content of 4N, 8N, 16N, 32N, 64N and eventually 128N in a single polylobulated nucleus before proceeding with their final maturation and subsequent PPF (Figure 4) (Chang et al., 2007b; Zimmet and Ravid, 2000). The hallmark of a mature MK is to be a large cell containing a single, multilobulated and polyploid nucleus (Geddis, 2010). Mature MKs may have a diameter of up to 80  $\mu$ M and are the largest hematopoietic cells in the bone marrow. This massive size allows each MK to produce several thousands of platelets.

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Though not completely known, the mechanisms regulating MK endomitosis begin to be better understood. Endomitosis is a form of “endoreduplication” process, in which the cell cycle is regulated as in the mitotic process. During polyploidization, MKs undergo normal cell cycle progression i.e., G1, S, G2 and M phases, but the M phase is incomplete (Ravid et al., 2002). After M phase, MKs re-enter into a G1 phase to initiate a subsequent cell cycle in order to increase their DNA content. Initial studies have suggested that the endomitotic cell cycle continues till anaphase A and then skips anaphase B and cytokinesis, because the subsequent late stages of mitosis were not observed (Nagata et al., 1997; Vitrat et al., 1998). Later using time-lapse microscopy, it was observed the presence of a telophase and the onset of a cytokinesis, but endomitotic MKs were unable to complete the late cytokinesis (Geddis and Kaushansky, 2006; Lordier et al., 2008).

In order to explain the defects in late stages of the mitosis in endomitosis, studies have been first focused on the central spindle. Studies have shown that two components of the central spindle, Aurora B and survivin, are absent in endomitotic MKs (Kawasaki et al., 2001; Zhang et al., 2001; Zhang et al., 2004). Nevertheless, in human MKs, Aurora B and survivin are present and are normally localized including in the midbody (Bluteau et al., 2009; Geddis and Kaushansky, 2004). Aurora B is functional both at the metaphase/anaphase transition and during the late stages of endomitosis (Lordier et al., 2010). However, in contrast to normal mitosis, survivin and Aurora B are dispensable for polyploidization. Several studies have demonstrated that endomitosis is associated with a defect cleavage furrow, which cannot confer the abscission forces required for separation of two daughter cells. Following G1 and S phases, endomitotic MKs enter M phase and at anaphase, they separate the two pairs of chromosomes and begin to form a cleavage furrow (Geddis et al., 2007; Vitrat et al., 1998). However, nuclei fail to completely divide and cleavage furrows regress before cytokinesis completion, leading to the generation of a single cell with a multilobulated nucleus (Geddis et al., 2007). In higher ploidy MKs, cleavage furrow formation and ingression are also observed, but at much lower level than in the 2N/4N stages.

Endomitosis is a failure in cytokinesis and appears to be related to a defect in contractile forces related to the actin/myosin II complex. RhoA is one important regulator of cytokinesis and of the contractile ring assembly. RhoA is present in the cleavage furrow of MKs and is partially functional because inhibition of RhoA or ROCK increases MK ploidy (Lordier et al., 2008). The late failure of cytokinesis in endomitotic MKs seems to be related to a defect in RhoA activation/deactivation and/or to a defect in myosin II present at the cleavage furrow. Both mechanisms have been demonstrated. On the one hand, it has been shown that there is no accumulation of MYH9 in the contractile furrow of MKs and that MYH10, which can accumulate in the cleavage furrow of MKs, is transcriptionally silenced by RUNX1 during endomitosis (Lordier et al., 2012a). On the other hand, a defect of RhoA

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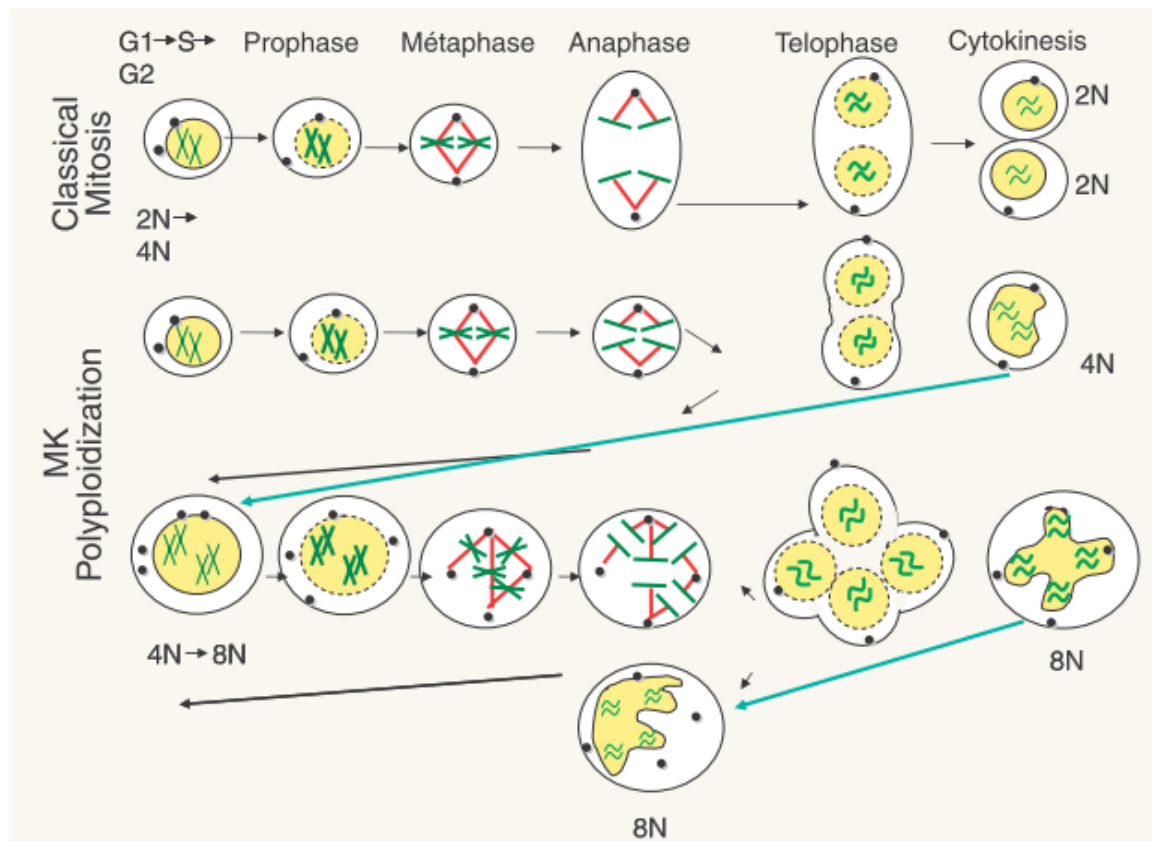
activation in the cleavage furrow has been directly illustrated due to a downregulation of two RhoA-GEF (GEF-H1 and ECT2) preventing the localization and activation of RhoA in the contractile ring (Gao et al., 2012).

A defect in karyokinesis is also observed in MK in addition to a cytokinesis failure (Lordier et al., 2012b). Recent study has identified two protein-tyrosine phosphatases Shp1 and Shp2 as important regulators of MK differentiation, showing that Shp1 and Shp2 function in endomitosis, and emphasize the importance of Shp1 and Shp 2 in MK maturation and ultimately in platelet production (Mazharian et al., 2013).

Several other regulators have been demonstrated to be involved in MK endomitosis process, such as cyclin D1/D3 and cyclin E. MKs express very high level of cyclin D3 in endomitosis, while overexpression of cyclin D1 leads to an increase in MK ploidy level, suggesting that the D-type cyclins function in MK endomitosis and polyploidization (Sun et al., 2001; Wang et al., 1995). In addition, cyclin E KO mice have a significant defect in MK polyploidization without altering proliferation of the other hematopoietic cells (Geng et al., 2003). MK endomitosis process can stop DNA duplication at any ploidy level between 2N and 128N, thus one possibility to explain platelet heterogeneity may be that platelets originating from different classes of polyploid MKs have different functions. However, there is some evidence that polyploidization may not affect *per se* gene expression, but is mostly involved in the regulation of gene expression in a differentiation-related manner (Raslova et al., 2007). This implies that polyploidization may be directly integrated into the MK differentiation program.

The majority of neonatal MKs derived from cord blood have a low ploidy, suggesting they have a reduced capacity to produce platelets (Mattia et al., 2002). Recent studies have suggested that neonate platelets have a prolonged survival that counteracts this relative defect in production (Liu et al., 2014).

In contrast to other cells that become polyploid in response to stress, MK polyploidization occurs during normal homeostatic differentiation. Polyploidy is a manner of increasing platelet production, as polyploidy is associated with an increase in DNA and protein synthesis leading to an augmentation in the MK cytoplasm volume. In addition, polyploidization may be a way to significantly increase metabolic pathways and to modify the level of gene expression, as MK polyploidization results in a functional gene amplification, all alleles being transcriptionally active except for those localized on the X chromosome (Raslova et al., 2003). Polyploidization is also a way to partially counterbalance a haploinsufficiency in case of mutations in key MK genes and thus to better keep a genomic integrity. This is particularly important as MK terminal differentiation is associated with high ROS levels, which may promote double DNA strand breaks.



**Figure 4:** Scheme of the megakaryocyte endomitosis

The overview of the endomitotic process in megakaryocyte, which results in polyploidization (Figure adopted from (Chang et al., 2007b) ).



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## 2.2 Proplatelet formation and platelet release

Each day,  $2 \times 10^{11}$  platelets are produced in humans by a highly regulated mechanism. The biology of platelet formation is unique, as platelets arise from cytoplasm fragmentation of their marrow precursors, the MKs. Circulating blood platelets are specialized cells that function to prevent bleeding and minimize blood vessel injury. Platelets circulate in a non-activated form, when stimulated, they change their form and spread on the affected tissue to generate a physical barrier that prevents blood loss. Platelets are essential for homeostasis. Thrombocytopenia is a main clinical disorder across several pathological conditions, including immune thrombocytopenic purpura, hematological malignancies, particularly myelodysplastic syndrome, leukemia and other malignant bone marrow infiltration, chemotherapy or drug toxicity, intravascular coagulation, aplastic anemia and viral infection including HIV and Ebola. A better understanding of the platelet formation mechanisms will give the chance to improve therapy of thrombocytopenia.

The mechanism of platelet biogenesis has been studied for many years; up to now, there are two models proposed to explain platelet production from mature MKs, which are not mutually exclusive. In the fragmentation model, within the MK cytoplasm, the prepackaged platelets are released by fragmentation within DMS. This hypothesis is supported by electron microscopy analysis of platelet producing from MKs explosive-fragmentation within internal membranes (Mori et al., 1993). When cultured on subendothelial extracellular matrix, mature MKs are stimulated to release platelets by a highly efficient explosive fragmentation of the entire cytoplasm (Caine et al., 1986; Eldor et al., 1986). Alternatively, in the PPF model, mature MKs in the bone marrow develop numerous and long branching cytoplasm processes that extend into the marrow sinusoidal blood vessels, where they fragment and release individual platelets into the circulation (Figure 5) (Italiano et al., 1999a; Junt et al., 2007; Patel et al., 2005a). Using live cell microscopy, Italiano and colleagues have observed the development of a network of branching proplatelets in cultured MK, then platelet assembly and their bud off at the end of each proplatelet (Italiano et al., 1999a). Another group has extended these results *in vivo* by dynamic imaging of the bone marrow compartment and show that fluorescently-labeled MKs form proplatelets into marrow vascular sinusoids and release large fragments making a link between PPF and their rupture by shear forces (Junt et al., 2007). PPF is thus an essential intermediate in platelet biogenesis, but is not yet sure that it is the only manner for MKs to produce platelets.

The platelet production process is a dynamic and organized process beginning after the end of the endomitosis and the increase of cell size by organelles and granules synthesis. The platelet release in contrast to all the other steps of megakaryopoiesis takes place in the blood stream and is regulated by

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the shear stress. The MT cytoskeleton undergoes a profound remodeling during PPF. In immature MKs, MTs translocate from the center to the cell cortex. Cortical MTs organize into thick bundles situated beneath the plasma membrane. These tubules further grow and form repeated branching structures, which develop a beaded appearance at short intervals along their length. The whole MK cytoplasm is consumed in proplatelets, except a nuclear mass surrounded by a thin rim of cytoplasm that consequently is degraded by apoptosis and phagocytosed by macrophages. During proplatelet evagination, the MT bundles turn and bring opposing bundles in contact and then interlink together in the proplatelet shaft. MTs sliding in the shaft extends the proplatelet and then MTs detach from the shaft to further fragment and release platelets (Hartwig and Italiano, 2006). After the whole MK cell body has been converted into proplatelet, the nucleus is eventually extruded and degraded, while individual platelets are released from the proplatelet ends.

Apoptosis is the process of programmed cell death that may occur in multicellular organisms and confer advantages for organism life cycle. The role of apoptosis is a controversial issue concerning MK development and functions, especially in platelet production (Kile, 2014). There are two distinct apoptotic pathways in mammals: the intrinsic pathway and extrinsic pathway, they seem to be convergent and both might be implicated in megakaryopoiesis and thrombopoiesis (Youle and Strasser, 2008). These two apoptotic pathways can be triggered by chemotherapy or infection. The intrinsic apoptotic pathway is controlled by the BCL-2 family proteins, which can be either pro-apoptotic (including Bax, BAD, and Bak) or anti-apoptotic (including Bcl-2, Bcl-xL, Bcl-w). The extrinsic apoptotic pathway can be triggered by ligands binding to death receptors belonging to the tumor necrosis factor receptor family, such as Fas or tumor necrosis factor receptor-1 (Youle and Strasser, 2008).

Concerning apoptosis and PPF there are presently opposite results. On the one hand, it has been demonstrated by several genetic approaches in the mouse that PPF absolutely requires a state of resistance to apoptosis with an excess of anti-apoptotic genes of the BH3 family, Mcl1 and Bcl-xL over the apoptotic members (Kodama et al., 2012). Studies have shown that at the end of MK differentiation, the activation of the intrinsic anti-apoptotic pathway is involved in platelet production (Kile, 2009), and also in platelet survival (Mason et al., 2007; Zhang et al., 2007). Platelet survival is regulated by an apoptotic process, which is dependent of the equilibrium between anti-apoptotic and apoptotic BH3 family members. Indeed a recent study has demonstrated that MKs and platelets possess functional BAK/BAX-mediated intrinsic apoptotic pathway and FasL-inducible extrinsic apoptotic pathway (Josefsson et al., 2014). Both pathways must be restrained during MK growth and development to allow normal platelet production. MKs possess a functional extrinsic apoptotic pathway, which can be activated by FasL, but the activation of apoptotic pathway by FasL does not

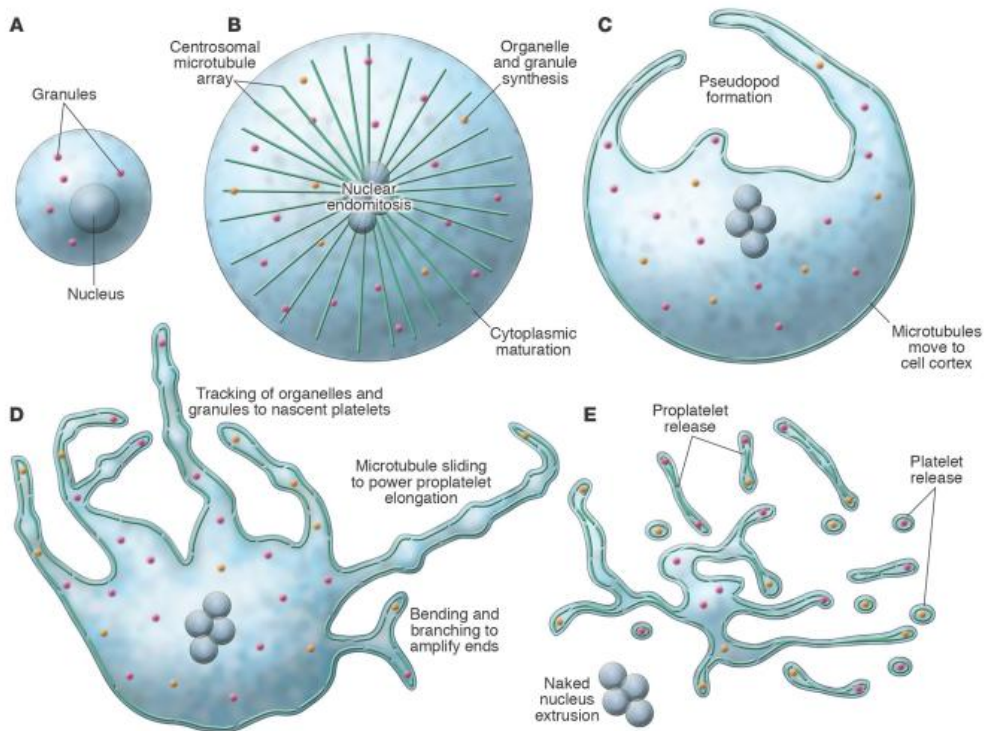
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stimulate platelet production and leads to failure of PPF (Josefsson et al., 2014). Livin is an intracellular anti-apoptotic protein, which belongs to the inhibitor of apoptosis protein family and acts by binding and inhibiting caspases. Recent study has shown that Livin has a role in thrombopoiesis by regulating the apoptotic and anti-apoptotic balance in MK endomitosis and platelet production (Abd-Elrahman et al., 2013).

On the other hand, Bcl-xL over expression in MKs induces an abnormally developed platelet DMS and cell margin extensions, as well as impaired PPF (Kaluzhny et al., 2002). Furthermore, two studies have suggested that members of the apoptotic machinery also contribute to the PPF (Clarke et al., 2003; De Botton et al., 2002). Clarke and colleagues have shown that distinct apoptotic factors accumulate in mature MKs including caspase activation. De Botton et al have shown that this activation of caspase is compartmentalized in the cytoplasm and recent studies suggest that it occurs in the mitochondria. Caspase inhibitors are able to inhibit PPF (Clarke et al., 2003; De Botton et al., 2002). Presently, the caspase substrates are not yet known and may be components of the cytoskeleton. However it remains possible, as none of the caspase inhibitors used is totally specific that other proteases close to caspases are required for PPF. Indeed in the mouse, caspase 9 KO does not inhibit PPF.

It remains that this controversy is not completely solved, but it is not excluded that platelet release may occur by different mechanisms: *in vivo* during the homeostatic production platelets may be formed by the standard PPF which requires a strong anti-apoptotic machinery; *in vitro*, in the shear or in case of acute thrombocytopenia when platelet release is accelerated, it may require additional mechanisms involving a caspase activation that may be initially independent of apoptosis. However, after platelet release, apoptosis is involved in the destruction of the MK nucleus that remains surrounded by a rim of cytoplasm. Further studies are required to elucidate the function of apoptosis in MK differentiation and to further investigate the role of the apoptotic machinery in platelet release with the hypothesis that the apoptotic machinery is used for platelet production in a different way than apoptosis.

Platelet shedding must occur directly in the circulation. During maturation, MKs originally located in the marrow close to the osteoblasts migrates to the sub-endothelium region near sinusoids. Proplatelets must cross the endothelial barrier to enter into the marrow sinusoids. *In vivo* imaging has shown that platelets can be shed from proplatelet extension directly in marrow sinusoids (Junt et al., 2007). There are three main determinants that are associated with platelet production, including DMS or IMS, MT and actin filaments. The process and regulation of PPF is based on both MT and actin cytoskeleton (Patel-Hett et al., 2008; Patel-Hett et al., 2011; Patel et al., 2005b; Thon et al., 2012; Thon et al., 2010).



**Figure 5:** Overview of platelets formation from mature megakaryocytes

Successive events occur during MK transition from immature cells (A) to individual platelet shedding (E). (B) MKs undergo rounds of endomitosis, organelle synthesis, and cytoplasm maturation and expansion, as well as centrosomal MT array. (C) Before the onset of proplatelet formation, centrosomes disassemble and microtubules translocate to the cell cortex. PPF begins with the thick pseudopodia formation. (D) Sliding of overlapping microtubules drives proplatelet elongation; while organelles are tracked into proplatelet ends to give rise to nascent platelets. Proplatelet elongation continues expansion, bending and branching to amplify proplatelet ends. (E) The entire megakaryocytic cytoplasm is converted into proplatelets, which are released from the cell. The nucleus is eventually extruded from proplatelets, and individual platelets are released from proplatelet ends (Figure is from (Patel et al., 2005a) ).

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### 2.2.1 DMS or IMS

Proplatelet elongation requires an increase in plasma membrane, which is performed by the DMS or IMS. The network of DMS contains the membrane cisternae and tubules originally thought to fragment the cytoplasm into channelized areas, from where platelets are shed. The process of MK maturation is associated with formation of secretory granules, increased cytoskeleton proteins and development of a complex membrane system. DMS or IMS arises from the invagination of the MK plasma membrane and forms a network of membrane channels that serves as a membrane reservoir for platelet formation (Radley and Haller, 1982; Schulze et al., 2006). At all development stages, the DMS remains continuous on the cell surface. A recent study has demonstrated that the DMS biogenesis from the plasma membrane occurs by four successive steps, but a part of the DMS directly originates from the Golgi system (Eckly et al., 2014). The DMS is also associated with both the MT and the actin filaments during PPF, and is evaginated to form pseudopodial processes during PPF (Patel et al., 2005b; Schulze et al., 2006). A recent study has shown that CIP4 (Cdc42-interacting protein 4) is involved in the formation of plasma membrane and platelet production in MKs by impairing membrane-cytoskeleton remodeling (Chen et al., 2013).

During development, numerous different types of granules including lysosomes,  $\alpha$ - and dense granules are formed. These granules as well as other MK organelles such as mitochondria and ribosomal RNAs are transferred into the nascent platelets.  $\alpha$ -granules are formed in the Golgi and contain both endogenously produced proteins and proteins derived from the extracellular environment through receptor-mediated endocytosis or pinocytosis. The DMS directly participates in platelet production, whereas special platelet organelles such as  $\alpha$ - and dense granules through their content play important roles in platelet function, but also in more general phenomenon such as bone marrow remodeling and formation of the hematopoietic niche, innate immunity and endothelium activation. PF4 and vWF are component of  $\alpha$ -granules that are synthesized by MKs and follow the normal route for packaged proteins. They are detected early in MK differentiation in the endoplasmic reticulum and Golgi, then their expression increases during differentiation and they localize into  $\alpha$ -granules (Schmitt et al., 2001). Some platelet proteins, such as the GPIIb/IIIa, are synthesized and directed to the MK surface membrane and then to  $\alpha$ -granules, may be through an endocytic pathway. Other proteins such as fibrinogen or immunoglobulins are not synthesized by MKs, but are endocytosed to the  $\alpha$ -granules by their respective receptors GPIIb/IIIa or the Fc receptor. This may imply that a part of the GPIIb/IIIa is activated in MKs to bind fibrinogen. Individual organelles including granules migrate from the cell body to the proplatelets, with approximately 30% of them in motion at any given time (Richardson et al., 2005).

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### 2.2.2 Microtubules

MTs are the main component of mitotic spindle participating in cell division and play roles in intracellular transport by interaction with dynein and kinesin, as well as in several other cellular processes. MTs play an important role in transport of mitochondria, granules and other vesicular organelles into proplatelets (Richardson et al., 2005). MTs may also convey spliceosomes, a phenomenon that may be important to permit protein synthesis in platelets (Denis et al., 2005). PPF depends on the MT function because treatment of MKs with drug that disassembled MT, such as colchicine or nocodazole, can block PPF (Tablin et al., 1990). Dynamic MT reorganization is essential for PPF and defects in their assembly lead to an absence of PPF and thrombocytopenia. MTs are the main structural component of proplatelet and a continuous MT polymerization is required for proplatelet enlargement. However, it is the sliding of overlapping MTs driven by dynein within cortical bundles that acts as the primary motor for proplatelet elongation. Dynein, a minus-end MT motor protein, localizes along the length of the proplatelet MT array and appears to provide directly the forces for MT sliding, because inhibition of dynein blocks PPF by disassembly of the dynactin complex (Patel et al., 2005b). Thus, the contribution of cytoplasmic dynein and dynactin to MT sliding appears to be key events for driving proplatelet elongation.

Visualization of MT dynamics in living MKs has provided insights into the role of MTs in PPF. EB3 (End-binding protein three), a MT plus end-binding protein associated with polymerizing MTs, is fused to GFP and has been expressed in mouse MKs and used as a marker to follow the dynamics of MT plus ends (Patel-Hett et al., 2008; Patel et al., 2005b). Time-lapse fluorescence microscopy of proplatelet-producing MKs expressing EB3-GFP has shown that, as proplatelets extend, MT polymerization occurs continuously throughout the entire proplatelet, including the swellings, the shaft and the tips (Patel et al., 2005b). The EB3-GFP has also shown that MTs polymerize in both directions in proplatelets, toward the cell body and the tips, revealing that the MTs composing the bundles have a mixed polarity.

In order to elucidate the mechanism of MT functions in late stages of MK maturation, Thon et al. have identified a large, intermediate discoid stage in platelet production, which they call 'preplatelet' (Thon et al., 2010). The preplatelets are large discoid anucleate platelet precursors that have the capacity to convert reversibly into elongated proplatelet by twisting MT-based forces that can be visualized in proplatelets expressing GFP- $\beta$ 1-tubulin. Larger MT coils may undergo twisting to continue MT polymerization and form barbell shaped proplatelet. During barbell PPF, dynamic and bidirectional assembly of MT coils mediate platelet cytoskeleton arrangement as  $\alpha$ - and dense granules track to distal proplatelet tips.

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The platelets release from the ends of proplatelets occurs at an increasing rate in time during culture, as large proplatelets undergo successive fission along the midbody (Thon et al., 2010). When pseudopodia begin to elongate, MT bundles align in the proplatelet shafts to form a loop at the free end of proplatelet extension. It is believed that mature platelets are only formed and released from the tips of proplatelet extension, but not from the swelling along the extension (Italiano et al., 1999a; Italiano et al., 2007). MT bundles form discrete loops at the ends of proplatelets, then the MT marginal band will form the outline of the discoid platelets (Geddis, 2009).

$\beta$ 1-tubulin isoform is unique to the MK lineage, particularly for platelets. The discoid form of platelet is maintained by a marginal band of tightly coiled MT. A functional polymorphism in  $\beta$ 1-tubulin has been found in humans, which modifies platelet morphology and functions (Freson et al., 2005). Mice lacking  $\beta$ 1-tubulin have shown a decreased PPF with thrombocytopenia and spherical platelets due to abnormal marginal MT band structure (Italiano et al., 2003).  $\beta$ 1-tubulin mutations lead to macrothrombocytopenia in CKCS (Cavalier King Charles Spaniels) dogs. These mutations induce an instability of the  $\alpha/\beta$ -tubulin dimers leading to impaired PPF (Davis et al., 2008). A mutation of  $\beta$ 1-tubulin has been found in a patient with a congenital macrothrombocytopenia (Kunishima et al., 2009). This  $\beta$ 1-tubulin mutant may not transport organelles from the MKs into proplatelets. In addition,  $\beta$ 1-tubulin is not expressed in NF-E2-deficient MKs (Lecine et al., 2000), which have an absence of PPF, this defect being partially rescued by reintroduction of  $\beta$ 1-tubulin. These results link the NF-E2 and  $\beta$ 1-tubulin with PPF, and provide further molecular insights into the role of MTs in platelet production.

### **2.2.3 Actin**

The actin cytoskeleton plays an important role at both early and late stages of platelet biogenesis. At early stages, cytoplasmic polymerized actin is associated with DMS and actin is highly aggregated in cultured MKs during PPF, while inhibition of actin polymerization blocks PPF before pseudopodia elongation. This may be the consequence of an association of actin cytoskeleton with DMS and of actin function in PPF (Rojnuckarin and Kaushansky, 2001; Schulze et al., 2006). Actin assembly does not play an indispensable role in proplatelet extension, but actin filaments are enriched at the sites of proplatelet bifurcation suggesting that actin is involved in proplatelet branching and amplification of proplatelet tips, a phenomenon which may be important for the regulation of platelet production (Italiano et al., 1999a; Italiano et al., 1999b). In addition, actin cytoskeleton may participate in platelet functions, as actin regulates platelet shape both in unstimulated and activated platelets (Fox, 2001).

Actin filaments are the thin filaments of the cytoskeleton and act as tracks for the movement of myosin molecules that attach to the microfilament and walk along them. Myosin motoring along F-actin filaments generates contractile forces, which are called actomyosin fibers, both in muscle as well

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as in most non-muscle cell types. Actomyosin plays an important role in the regulation of PPF and platelet production, though the mechanisms are not completely understood. It has been shown that myosin II and its upstream regulator RhoA control the onset of PPF because altered myosin II activity leads to the premature initiation of proplatelets within the marrow space. Inhibition of myosin light chain phosphorylation or the absence of the main myosin heavy chain MYH9 increases PPF *in vitro* (Chen et al., 2007b; Eckly et al., 2009), while activation of Rho through ROCK and phosphorylation of MLC2 decrease PPF *in vitro* (Chang et al., 2007a). Even more, activation of Cdc42 and Rac increases PPF.

It has also been demonstrated that actin fibers assembly near the MK internal membranes, suggesting that the actin/myosin complex may play a role both in PPF and in generation of forces that promote platelet release from proplatelets (Schulze et al., 2006). In Wiskott Aldrich syndrome patients, loss of function mutations in WASP (Wiskott Aldrich syndrome protein), an actin-regulating protein, are also associated with a thrombocytopenia, which combines a peripheral platelet destruction and a defect in platelet production related to an absence of MK egress from the bone marrow (Sabri et al., 2006).

Furthermore, myosin II may interact with MTs to generate the forces that impair the protrusion required for PPF. This interaction between the actin and MT cytoskeleton may be extremely important in the late stages of MK differentiation, but also in platelets. A recent study has focused on the functional crosstalk between F-actin and MTs systems by using confocal microscopy and has shown an interdependence between MTs and F-actin dynamics in the morphological platelet changes induced by thrombin (Severin et al., 2013). Overall, the role of actomyosin in PPF is complex, because it is required at three steps: MK motility, PPF and generation of the contractile forces required for platelet abscission. Further studies should be performed to elucidate the functional and molecular interaction among actin, myosin and MT in platelets.

In addition, recent studies indicate that ECM regulates PPF. Integrin receptors link the cytoskeleton changes in the MK with the ECM environment. For example, collagen I interaction with its receptors  $\alpha$ II $\beta$ 1 and GPVI inhibit PPF within the marrow (Balduini et al., 2008; Sabri et al., 2004), while constitutive activation of integrin  $\alpha$ IIb $\beta$ 3 leads to an abnormal PPF and inhibits thrombopoiesis (Ghevaert et al., 2008). When they begin to migrate towards the endothelium barrier, MKs respond to other components of ECM such as vWF, fibrinogen or collagen type IV, which may promote PPF and platelet release by interacting with different receptors (Larson and Watson, 2006b). A differential response to extracellular factors provides a mechanism to prevent premature platelet release until MKs reach the vascular niche, suggesting the existence of a spatial and temporal regulation during platelet production (Larson and Watson, 2006a).



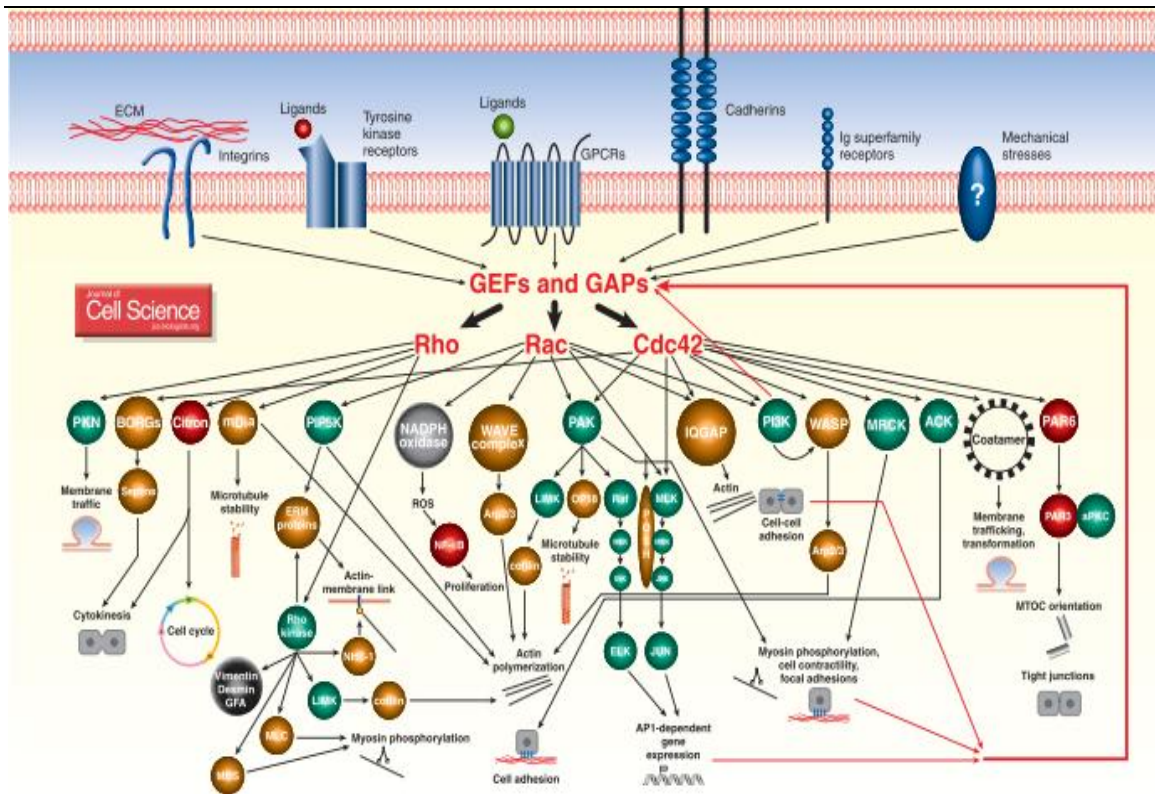
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There is evidence that FGF4 and SDF-1 also play an important role in MK marrow localization and migration as well as in PPF (Avecilla et al., 2004). At the end of maturation, there are controversial results on the role of SDF-1. On one hand, SDF-1 induces MKs migration and PPF, on the other hand mature MKs present a decrease in response to SDF-1 resulting in a reduction in the marrow retention force, which may facilitate their exit from the marrow (Riviere et al., 1999). This decreased function of the SDF-1 receptor CXCR4 is due to the over-expression of RGS16, a negative regulator of G protein-coupled receptor signaling (Berthebaud et al., 2005).

### **2.3 Rho-GTPase during megakaryopoiesis**

The Rho-GTPase is a family of small (~21 kDa) signaling G proteins that, belongs to the Ras superfamily. The Rho-GTPase proteins work as molecular switches that control intracellular signaling networks, such as organelle development, cytoskeleton dynamics, cell proliferation, cell differentiation, cell adhesion, apoptosis, cell migration and other common cellular functions (Boureux et al., 2007; Bustelo et al., 2007; David et al., 2012; Etienne-Manneville and Hall, 2002; Raftopoulou and Hall, 2004). The Rho-GTPase proteins mediate these signal transduction pathways and cellular processes by multiple effectors and are regulated by GEFs (Guanine-nucleotide exchange factors) and GAPs (GTPase-activating proteins), which permit their switch between inactive GDP and active GTP forms (Aslan and McCarty, 2013; Bos et al., 2007; Etienne-Manneville and Hall, 2002; Schwartz, 2004).

There are about 20 members of Rho-GTPase family in human, among which Cdc42, Rac1 and RhoA are the most common members that have been studied. These three members have different functions on the dynamics of the actin filaments: in fibroblasts Cdc42 affects filopodia extension, Rac1 lamellipodia formation and RhoA stress fiber formation. In MK differentiation, the Rho-GTPase-binding proteins Cdc42, Rac1 and RhoA regulate platelet production and function by mediating cytoskeleton reorganization (Bodie et al., 2001; McCarty et al., 2005; Pleines et al., 2010). Each Rho-GTPase protein has numerous downstream effectors proteins. When activated, Rho-GTPase proteins bind to their effectors and participate in various cell processes (Figure 6). Rho-GTPases are the most important regulators during MK differentiation, more particularly on PPF (Aslan and McCarty, 2013).



**Figure 6:** The main effector pathways of Rho-GTPases family

Regulation of the numerous effectors stimulated downstream Rho-GTPases family. ROCK, mDia, citron kinase and PKN are effectors acting downstream Rho A. ROCK including ROCK1 and ROCK2 are the major effectors, which can regulate myosin light chain phosphorylation and myosin regulatory chain that induce contractility. LIMK works downstream of RhoA to phosphorylate cofilin and release actin monomers to promote actin polymerization. Another RhoA effector, mDia mediates both actin polymerization and MT stabilization. Rac has multiple effectors that mediate direct and indirect effects on cytoskeleton and gene expression, respectively. An important role of Rac is the formation of lamellipodia by the Arp2/3 complex activation. Both Rac and Cdc42 bind and activate PAK1, PAK2 and PAK3. WASP is main effector of Cdc42 that mediates filopodia formation. (Figure is adapted from (Schwartz, 2004)).

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### 2.3.1 RhoA during megakaryopoiesis

During MK development, RhoA plays multiple roles in platelet biology, such as regulation of endomitosis, PPF, platelet release, platelet activation and function (Chang et al., 2007a; Lordier et al., 2008; Suzuki et al., 2013). In addition, RhoA mediates actin assembly, actomyosin contractility and MT dynamics, which are important for MK differentiation and platelet generation (Piekny et al., 2005). After activation, RhoA binds to two main downstream effectors including ROCK (Rho-associated protein kinase) and mDia (mammalian diaphanous homologue) to regulate the cytoskeleton dynamics. mDia is a member of formin family protein involved in *de novo* actin nucleation and extension of non-branched actin filaments and alignment of MTs (Schirenbeck et al., 2005), while the Rho/ROCK pathway plays a key role in the activation of myosin IIa providing the contractile forces required for platelet formation and allowing full spreading of platelets (Calaminus et al., 2007; Calaminus et al., 2008; Ono et al., 2008). ROCK is a kinase that activates myosin regulatory light chain (MRLC), either directly or through the inactivation of myosin phosphatases and functions as a key regulator of actomyosin contractility (Ueda et al., 2002).

RhoA regulates platelet production, shape change and secretion by controlling actin cytoskeleton forces. Expression of a constitutively active RhoA leads to a decrease in PPF, probably by preventing the folding of pseudopodia extensions from DMS (Chang et al., 2007a). RhoA deficient mice have a pronounced macrothrombocytopenia with platelet count about half the number of WT mice and changes in platelet shape. This leads to a prolonged tail bleeding time (Pleines et al., 2012). These results suggest that RhoA contributes to platelet production and platelet function in homeostasis and thrombosis. Using a similar mouse model, another group has observed the same platelet phenotype. In addition, the RhoA deficient MKs have a larger size with a higher ploidy and abnormal stiff membranes (Suzuki et al., 2013). These findings suggest that RhoA is necessary for normal MK development and platelet production.

RhoA contributes to the assembly of the contractile ring by polymerizing actin filaments and by activating myosin through ROCK. Two RhoA effectors, ROCK and citron kinase regulate the actin contractile ring, which generates the contractile forces (Yamashiro et al., 2003). During MK differentiation, the GEF regulating RhoA must be down-regulated in endomitosis, which allows a low RhoA activation leading to contractile ring disassembly, cleavage furrow regression and subsequently cytoskeleton failure (Gao et al., 2012; Geddis et al., 2007). RhoA-mediated activation of ROCK and inhibition of myosin light chain (MLC) phosphatase induce MLC phosphorylation, leading to actomyosin modulation and conversion of platelet shape from discoid to spherical. Myosin IIa

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deficiency affects F-actin organization and organelle distribution in MKs, which leads to macroplatelets with abnormal organelle content, suggesting that myosin IIa is required for proplatelet fragmentation, platelet abscission and the distribution of organelles within platelets (Pertuy et al., 2014). Other RhoA effectors may also contribute to the assembly of the actin ring, such as mDia and phosphatidylinositol 5-kinase (PIP5K) (Glotzer, 2009). These findings support that RhoA is as a multifunctional regulator in MK development and platelet production.

RhoA-mediated platelet contraction may also have a role in thrombus stability under shear flow conditions, as RhoA and its downstream effectors can stabilize and maintain platelet-matrix and platelet-platelet interactions. It has been shown that RhoA may play a role in regulating the stability of  $\alpha$ IIb $\beta$ 3 and integrin adhesion contacts, while it also sustains stable platelet-matrix interactions under conditions of high shear stress (Schoenwaelder et al., 2002). In addition, stress fiber formation on collagen is repressed by inhibition of either ROCK by Y27632 or myosin II by blebbistatin. Inhibition of ROCK also results in a significant decrease in thrombus formation *in vivo* (Calaminus et al., 2007). These data support the contention that as an effector of RhoA, myosin IIa contractility is necessary for maintenance of platelet structure during spreading on collagen and essential for thrombus stability.

PKC (protein kinase C) is a family of serine/threonine kinases, which have been commonly studied in thrombopoiesis. Their expression varies during human MK development and controls MK maturation and platelet shedding. PKC $\epsilon$  is one member of the PKC family, its down-regulation in cultured murine MKs leads to a decrease in PPF and the production of larger platelets (Gobbi et al., 2013). Furthermore, RhoA inhibition rescues the proplatelet defects induced by PKC $\epsilon$  down-regulation. Thus, these data suggest that a decreased RhoA activity increases PPF. Interestingly, this seems contradictory to the RhoA deficient mice in which RhoA is necessary for normal MK development and PPF (Pleines et al., 2012; Suzuki et al., 2013), but in agreement with the results obtained in culture. This may due to differences in experimental procedures and the origin of the MKs used in the different studies. Alternatively, RhoA may act in opposite ways at different stages of MK differentiation, being required at early steps and inhibiting PPF at late stages. Furthermore regulation of RhoA may greatly differ in *in vitro* and in *in vivo* experiments.

### **2.3.2 Cdc42 during megakaryopoiesis**

Cdc42 is necessary for platelet filopodia formation, granule secretion and aggregation, as well as platelet activation and function, which are involved in platelet mediated thrombosis and hemostasis (Akbar et al., 2011; Pleines et al., 2010). Cdc42 deficient mice exhibit a thrombocytopenia and a slightly increase in platelet size, as well as a decreased life span *in vivo*. PAK1/2 (p21-activated kinase) are effectors downstream of Cdc42. Activation of PAK1/2 is significantly reduced in Cdc42 deficient

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platelets and leads to a failure in the formation of filopodia or lamellipodia on fibrinogen or CRP (collagen-related peptide) (Akbar et al., 2011). An acceleration of arterial occlusive thrombus formation with prolonged bleeding times is also observed. Cdc42 deficient platelets also show an increased granule secretion and an enhanced aggregation on collagen under flow, with a reduced filopodia extension after adhesion on immobilized vWF, suggesting a link with GPIb signaling. All these observations imply that Cdc42 plays an important role in various aspects of platelet production, such as regulation of platelet activation and granule organization.

Cdc42 activation is commonly associated with filopodia formation. Early studies support this contention in platelets because Cdc42 is found to be abundant. Stimulated by TRAP (thrombin receptor-activating peptide) or ADP (Adenosine diphosphate), Cdc42 translocates to the platelet cytoskeleton through the activation of the  $\alpha$ IIb $\beta$ 3 integrin followed by actin polymerization and tyrosine kinase activation (Dash et al., 1995). Additionally, treatment of platelets with secramine A, a Cdc42-specific inhibitor, blocks platelet adhesion on collagen and collagen-induced platelet aggregation (Dash et al., 1995). Another group has demonstrated that actin polymerization and Cdc42 activation are required for activation of integrin  $\alpha$ 2 $\beta$ 1, thus regulating platelet adhesion to collagen and their subsequent activation (Pula and Poole, 2008). Cdc42 can also be rapidly and extensively activated by PAR-1 stimulation in solution and associated with actin cytoskeleton in resting platelets (Azim et al., 2000), as well as after activation by PAK stimulation that leads to early platelet shape change (Vidal et al., 2002).

WASP is a main effector of Cdc42 that mediates filopodia formation. Active Cdc42 induces the activation of WASP by changing its conformation, which leads to Arp2/3 complex activation, resulting in an increase actin turnover and initiating the formation of parallel actin bundles. Its major role concerns the branching of the actin filament. Cdc42 can also bind to and activate IRSp53 to subsequently recruit the Ena/vasodilator-stimulated phosphoprotein (VASP) family protein Mena, thus promoting filopodia elongation (Krugmann et al., 2001).

A recent study has focused on the respective role of Rac and Cdc42 in PPF. Using conditional KO mice, Pleines and colleagues have shown that Cdc42 and Rac1 have redundant functions in PPF and platelet functions (Pleines et al., 2013). The double Cdc42/Rac1 KO leads to a macrothrombocytopenia with an abnormal platelet morphology as well as impaired platelet function and PPF *in vitro*. Double KO mice also show nearly normal actin structures and assembly, but surprisingly MK tubulin organization is severely disrupted. This disruption in MT organization and stability inhibits PPF and results in a decrease of platelet release (Pleines et al., 2013). This study introduces Rac1 and Cdc42, as novel, and functionally redundant regulators of MT stabilization and dynamics. Together, these studies suggest that the combined action of Rac1 and Cdc42 is crucial for

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platelet production, particularly by regulating MT dynamics.

### 2.3.3 Rac1 during megakaryopoiesis

Rac-GTPase proteins are involved in a wide variety of cellular functions such as cell polarity, vesicular trafficking, cell cycle and transcriptional dynamics (Bustelo et al., 2007). There are three isoforms of Rac-GTPase, Rac1, Rac2 and Rac3, among which only Rac1 is expressed in platelets and involved in actin cytoskeleton reorganization. Early studies of Rac in platelet function have shown that Rac is activated in platelets upon stimulations with different agonists, including collagen, thrombin and fibrinogen (Soulet et al., 2001; Suzuki-Inoue et al., 2001). Use of different inhibitors has shown that Rac and PAK are activated during platelet spreading on collagen, suggesting that they are downstream effectors of  $\alpha 2\beta 1$  integrin, as well as the Src family kinases and PI3-kinase.

Rac1 is involved in the regulation of platelet secretion and aggregation, and its effectors control the formation of platelet lamellipodia. Rac1 is required for lamellipodia formation when platelet spread on surfaces of fibrinogen, vWF, laminin and collagen (McCarty et al., 2006; McCarty et al., 2005). Using Rac-deficient platelets, it has been shown that the formation of lamellipodia on collagen and laminin is Rac1-dependent (McCarty et al., 2005). Furthermore, Rac1 has been shown to be crucial for GPVI-regulated platelet spreading as well as in sustaining shear-resistant platelet aggregates under dynamic flow conditions both *in vitro* and *in vivo*. Rac1 is also required to maintain the stability of the platelet aggregation under physiological shear conditions as well as for thrombus stability at sites of injury. Another study using both Rac1 KO mice and Rac1 inhibitor-treated mice has shown that loss or inhibition of Rac1 blocks platelet secretion and prolongs the bleeding times, suggesting the Rac1 is required for platelet secretion and aggregation as well as for platelet functions (Akbar et al., 2007).

Rac has a role in PI3K activation in platelets, while PI3K activation is also required to completely activate Rac, supporting a feedback model of platelet activation that control platelet lamellipodia formation, secretion and thrombus stability. Phospholipase C-gamma2 is activated downstream of the immunoreceptor tyrosine activation motif (ITAM) receptor GPVI, which is one of the platelet collagen receptors. Rac1 may play a role in PLC-gamma2 activation through a mechanism independent of tyrosine phosphorylation (Piechulek et al., 2005). In platelets, Rac1 is required for PLC-gamma2 activation downstream of GPVI/ITAM and is essential for thrombus formation *in vivo* (Pleines et al., 2009). Rac1 is also involved in GPVI-mediated platelet aggregation,  $\alpha I I b \beta 3$  activation, granule secretion and ADP secretion downstream of calcium mobilization. Rac1 also participates in vWF-induced activation of  $\alpha I I b \beta 3$  integrin upstream of PI3K/Akt pathway to drive platelet responses to GPIb-IX (Delaney et al., 2012).

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Similar to Cdc42, Rac translocates to the platelet cytoskeleton after activation. During PAR-1-mediated platelet activation, Rac translocates to the cell periphery, binds to the plasma membrane where it can interact with phosphoinositide kinases implicated in actin assembly (Azim et al., 2000). Rac is also found in the center of activated platelets, which is enriched in platelet granules and membranes. Rac1 activation is also required for platelet integrin-mediated p38 and ERK phosphorylation, suggesting that in addition to its role in cell spreading, Rac1 has also a role in clot retraction by MLC phosphorylation through p38 and ERK, independently of the Rho and ROCK pathway (Flevaris et al., 2009).

Rac1 has multiple effectors that mediate effects on the cytoskeleton and gene expression. One important role of Rac is the formation of lamellipodia by the activation of the Arp2/3 complex. Rac1 downstream of PLC-gamma2 and calcium signaling regulates platelet secretion, aggregation and spreading processes by activating Rac effectors, such as the Arp2/3 complex and the PAK family. Both Rac and Cdc42 bind and activate PAK1, PAK2 and PAK3. Platelets express different PAK isoforms, and like Rac, PAKs are activated as platelets spread on collagen in a Src- and PI3K-dependent manner (Azim et al., 2000; Vidal et al., 2002). In platelets, PAKs act downstream of Rho-GTPase to control the intracellular cortactin distribution. The interaction of cortactin with the Arp2/3 complex is associated with the activation of actin polymerization in lamellipodia structures (Urano et al., 2001). The adaptor protein SLP-76 has also been proposed to potentiate PAK activity downstream of Rac activation to mediate platelet lamellipodia formation (Oberfell et al., 2001).

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## 3 Formin family

### 3.1 Formin family

Formins (formin homology proteins) are a family of proteins highly conserved in eukaryotes, which possess several domains and govern the dynamic remodeling of both actin and MT cytoskeleton (Bartolini and Gundersen, 2010; Chesarone et al., 2010; Goode and Eck, 2007). Formins are involved in a wide range of cellular functions, such as cell polarity, cytokinesis, cell migration and SRF transcriptional activity. They also play a role in embryonic development (Faix and Grosse, 2006; Sagot et al., 2002a). Most formins are Rho-GTPase effector proteins (Kuhn and Geyer, 2014). Formins can also interact with diverse signaling molecules and cytoskeleton proteins, while certain formins have also been assigned with nucleus functions (Baarlink and Grosse, 2014; Young and Copeland, 2010).

#### 3.1.1 Molecular structure of formins

Formins are characterized by the presence of three FH (Formin homology) domains (FH1, FH2 and FH3), although some members of the formin family do not necessarily contain all these three domains (Kitayama and Uyeda, 2003; Wallar and Alberts, 2003). The most highly conserved domains of formins are FH1 and FH2, both of them are involved in the control of actin assembly (Evangelista et al., 2002; Goode and Eck, 2007; Sagot et al., 2002a; Watanabe and Higashida, 2004).

The proline-rich FH1 domain mediates interactions with a variety of proteins, including the actin-binding protein profilin, SH3 (Src homology 3) domain proteins, and WW domain proteins. Profilin is required to bind to actin monomers and the FH1 domain of the formins to induce actin polymerization. The FH2 domain remains continually associated with the fast-growing barbed end of the actin filament while the FH1 domain increases the rate of assembly by directing the association of profilin–actin monomers above the theoretical diffusion-limited rate (Kovar et al., 2006; Romero et al., 2004).

The FH2 domain is also required for the self-association of formin proteins through the ability of FH2 domain to associate (Shimada et al., 2004a; Takeya and Sumimoto, 2003). The FH2 dimer nucleates actin filament assembly by interacting directly with actin and stabilizing actin polymerization, including dimers and trimers. The FH2 domain binds directly to G- and F-actin and has been shown in many formins to nucleate actin molecules, elongate actin filaments and control actin nucleation



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(Goode and Eck, 2007; Higashida et al., 2013). FH2 domains act as the functional center of formins, which nucleates new actin filaments and associates with the fast-growing end (barbed end) of actin filaments (Bear, 2002; Higgs, 2005; Pollard, 2002; Zigmond, 2004). The FH2 domain differs quite a lot among individual formins, reflecting the different cellular functions and regulatory mechanisms of the actin polymerization factors. Thus, most of the FH2 domain may be dedicated to an actin assembly role.

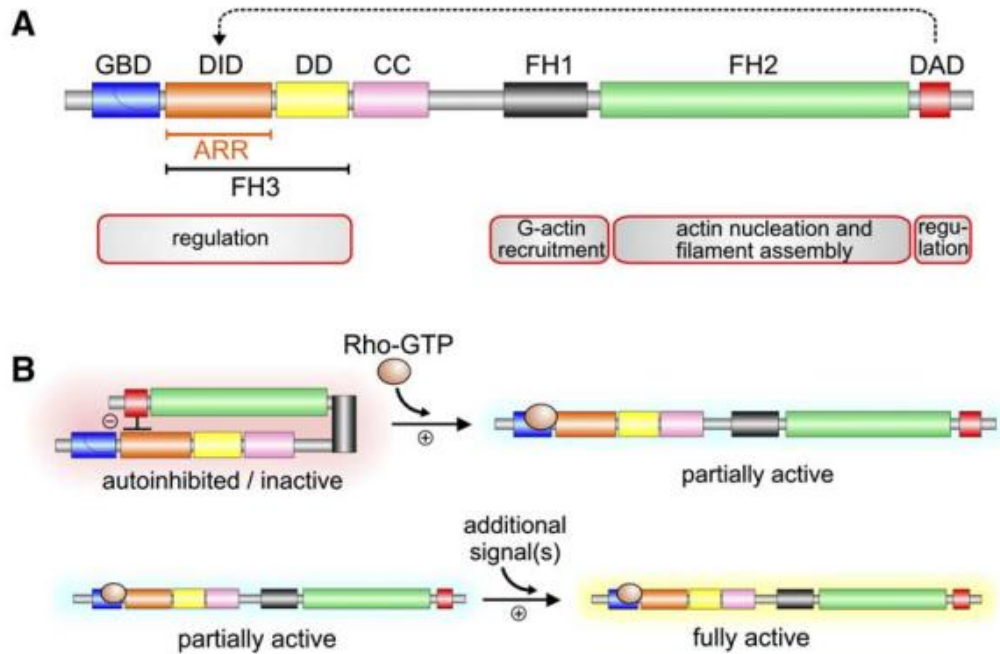
In addition, certain members of formins can be auto-inhibited, and are classified as Diaphanous-related formins (DRFs) (Alberts, 2001; Watanabe et al., 1999), because they are homolog to the *Drosophila* gene *diaphanous* (Castrillon and Wasserman, 1994). In addition to the conserved FH1 and FH2 domains, DRFs contain a conserved N-terminal regulatory domain and a C-terminal Diaphanous-autoinhibitory domain (DAD). The core regulatory domain at the N-terminal includes several adjoining domains: a GTPase-binding domain (GBD) required for binding to small GTPases, which is contiguous to the FH3, a Diaphanous-inhibitory domain (DID), which is a subdomain of the GBD/FH3 domain, and a dimerization domain (DD). A molecular scheme of the domain architecture of DRFs is shown in Figure 7. The GBD domain is a bifunctional auto-inhibitory domain that interacts with activated Rho family members. The DAD domain can mediate auto-inhibition through interactions with the DID, while the intra-molecular interaction between the C-terminal DAD and its N-terminal DID leads to the auto-inhibition of DRFs (Alberts, 2001; Lammers et al., 2005; Li and Higgs, 2003; Watanabe et al., 1999). However, the intra-molecular auto-inhibition can be relieved upon the interaction with an active Rho-GTPase, such as Rho, Rac or Cdc42, which binds to GBD and disrupts the DAD-DID interaction to release the DAD domain away from the DID domain, leading to a partial activation of the DRFs (Faix and Grosse, 2006; Li and Higgs, 2005; Nezami et al., 2006; Rose et al., 2005). Addition of the DAD to mammalian cells induces actin filament formation, stabilizes MTs and activates serum-response mediated transcription. However, this activation process requires high concentrations of Rho-GTP, so the release of the auto-inhibition requires membrane-associated factors that cooperate with Rho-GTP (Seth et al., 2006).

Some formins contain a FH3 domain between the GBD and the FH1 domain, which is the least conserved module among the different domains and is involved in the regulation of formin activity. The FH3 domain is required for sub-cellular localization and directs formins to the correct intracellular

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localization, such as the mitotic spindle or bud tips in yeast (Kato et al., 2001; Ozaki-Kuroda et al., 2001; Sharpless and Harris, 2002). Alternatively, some part of the FH3 domain may contribute to interactions with binding partners, such as the Rho small GTPases (Westendorf et al., 1999). In the inactive state of formins, the FH3 domain recognizes the C-terminal DAD to generate an intramolecular auto-inhibited complex. After the Rho-GTPase binding to GBD, the conformation changes from an auto-inhibited state to an active state.

The conservation of the formin homology domains suggests that all formins have similar molecular activities and underscores the need to characterize their structure and function.



**Figure 7:** Scheme of the structure and the molecular regulation of Diaphanous-related formins

(A) Among formin proteins, besides the conserved FH1 and FH2 domain, DRFs contain conserved regulatory domains and a C-terminal Diaphanous auto-inhibitory domain (DAD). The core region at the N-terminal includes several contiguous subdomains including a GTPase binding domain (GBD), a Diaphanous-inhibitory domain (DID), and a dimerization domain (DD). (B) Auto-inhibition of DRFs. It has been shown that the interaction between the DID and DAD domains inhibits the activity of the FH2 domain. The binding of specific Rho-GTPases to GBD can relieve this auto-inhibition by directly release the DAD domain away from the DID domain, leading to a partial activation of the DRFs (Li and Higgs, 2005; Rose et al., 2005). Alternatively, the Diaphanous-related formins that bind to activated Rho GTPases. (Figure from (Faix and Grosse, 2006) ).

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### 3.1.2 Formin functions in the regulation of actin cytoskeleton

Formins act as regulators of actin cytoskeleton organization that regulate the dynamics of actin filaments. In contrast to the Arp 2/3 complex, formins nucleate directly the formation of unbranched actin filaments. For example, Bni1p and Cdc12p have a direct function involving actin nucleation. Overexpression of the FH1FH2 domains of Bni1p and Cdc12p in yeast increases the formation of actin cables (Kovar et al., 2003; Pruyne et al., 2002; Sagot et al., 2002b), while deletion analysis reveals that the Bni1p FH2 domain is sufficient for actin filament nucleation (Pruyne et al., 2002). Cytochalasin B inhibits actin polymerization through binding to the fast-growing (barbed) end of F-actin filaments. Polymerization induced by Bni1p is inhibited by cytochalasin B, indicating that the nucleated filaments grow predominantly at the barbed end. *In vitro* studies of Bni1p (Pring et al., 2003; Sagot et al., 2002b) and Cdc12p (Kovar et al., 2003) have shown that they bind to the barbed end of actin filaments and increase the overall step of actin polymerization by promoting the actin nucleation rate, a process occurring in cells independently of the Arp2/3 complex (Pollard and Borisy, 2003). However, binding of Bni1p to the barbed end reduces the polymerization-depolymerization rate, without blocking the barbed-end actin dynamics (Pring et al., 2003). Alternatively, Cdc12p binds to the barbed end like capping protein and inhibits actin assembly-disassembly dynamics. This suggests that Bni1p and Cdc12p are actin-filament-capping proteins, but unlike traditional capping proteins that bind to the barbed end and prevent filament elongation, they bind to the barbed end of filaments and promote the growth rate.

Actin nucleation is regulated by profilin, a ubiquitous G-actin-binding protein, which induces the addition of monomers to the barbed end of actin filaments. High concentration of profilin decelerates the rate of FH1FH2-induced actin assembly, while low profilin concentration accelerates the actin polymerization rate (Pring et al., 2003; Sagot et al., 2002b). The presence of profilin greatly reduces formin nucleation activity, while interactions between profilin-actin with the FH1 domain accelerate their elongation activity at the FH2-capped barbed ends (Chesarone et al., 2010). Profilin can not only promote the polymerization rate of the filaments, but also stimulate actin filaments barbed-end elongation, suggesting that profilin may have two different roles in formin-dependent actin assembly: both nucleation and elongation of actin filaments.

Alternatively, there is evidence that some specific DRFs may promote nucleus actin assembly in a signal-dependent manner (Baarlink and Grosse, 2014). Under certain conditions, some DRFs can change of cellular compartments by shuttling between cytoplasm and nucleus. A study has shown that

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there is a functional nuclear localization signal in the N-terminus of mDia2 and at least one functional nuclear export signal in the C-terminus (Miki et al., 2009).

Formin-induced nucleation may be further regulated by formin post-translational modifications or by the binding partners. For example, formin Bni1p is regulated by phosphorylation *in vivo*. It has been shown that a particular sub-domain of FH2 and formin-interacting proteins participate in the regulation of actin assembly. The N-terminal region of Rho1p targets the Bni1p FH2 domain bound to the translation elongation factor eF1A and bundles actin filaments, implying a Rho1p-mediated reorganization of the actin cytoskeleton (Liu et al., 2002; Umikawa et al., 1998). Deletion of the eF1A-binding site within Bni1p induces a disruption in actin assembly (Umikawa et al., 1998), while overexpression of eF1A leads to reassembly of actin filaments that correlates with an activated Bni1p (Munshi et al., 2001).

The tumor suppressor protein adenomatous polyposis coli (APC) is one of the *in vivo* binding partner of the formin mDia1 during actin filament assembly. It has been shown that APC nucleates actin assembly and synergizes with tmDia1, while they are associated to overcome the inhibition of actin filament formation induced by capping protein and profilin, suggesting a collaboration between different actin assembly-promoting factors (Okada et al., 2010). Another group has shown that APC recruits actin monomers and binds to the tail region of mDia1 to form a complex, which initiates the actin filament formation and represses the inhibition of actin filament formation induced by capping protein and profilin. At the onset of filament polymerization, the complex deaggregates and mDia1 moves to the growing barbed ends, while APC remains at the site of nucleation (Breitsprecher et al., 2012). Thus, the two assembly factors directly interact to initiate filament assembly and then separate to retain independent associations with either ends of the growing filament. APC is an efficient nucleator that allows new filaments assembly, even at a high profilin level, where formins are less efficient. Alternatively, mDia1 has an independent role in protecting filament elongation from capping proteins and enhances the polymerization rate.

As actin nucleator, formin assembles unbranched actin filaments and typically associates with the growing barbed end of actin filaments (Pollard, 2007). Formins are also involved in the regulation of linear bundles of actin filaments, such as stress fiber formation, filopodia structures, lamellipodia formation and contractile ring during cytokinesis. In cultured mammalian fibroblasts, formin mDia1 plays an essential role in Rho-mediated stress fiber formation (Tominaga et al., 2000; Watanabe et al., 1997; Westendorf et al., 1999). Using live-cell microscopy analysis, recent studies have shown that mDia1 assembles stress fibers, which are associated with focal adhesions where actin polymerization occurs (Hotulainen and Lappalainen, 2006). mDia1 is involved in stress fiber assembly both in

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fibroblasts and in myofibroblasts (Anderson et al., 2004). Formin FMLN2 may promote the rate of filament elongation by protecting the growing end of the filament from actin-capping proteins and by attaching the filament ends to the plasma membrane (Blanchoin and Michelot, 2012).

In addition, formins play a role in many organisms by modulating actin activity, such as cytokinesis and transcriptional activation of the SRF. A general requirement of formins in cytokinesis implies that formins may regulate the formation of the actin contractile ring. Use of RNAi to decrease the expression level of the formin CYK-1 has shown that this formin regulates cortical microfilaments assembly and is essential for cytokinesis in the early embryo, while Arp2/3 is dispensable (Severson et al., 2002). Rho-mediated stimulation of formin protein and profilin is required for assembly of the actin contractile ring (Tolliday et al., 2002). The formin Cdc12 is a component of the cell division ring and is required for the primary F-actin ring formation cables that generates the actin contractile ring (Arai and Mabuchi, 2002). The contractile ring is the active site of actin assembly, this actin polymerization activity requires Cdc12 and Arp3, profilin and WASP, while Arp 2/3 complex and formins induced actin polymerization are crucial for cytokinesis (Pelham and Chang, 2002). Thus, formation of some actin structure may require coordination of formins and the Arp2/3 complex.

### **3.1.3 Formin functions in regulation of microtubule cytoskeleton**

In addition to their actin cytoskeleton activity, formins also play important roles in regulating the dynamics of MT cytoskeleton (Bartolini and Gundersen, 2010; Chesarone et al., 2010; Gundersen et al., 2004; Ishizaki et al., 2001; Kato et al., 2001). Formins participate in multiple cellular processes and signaling pathway by remodeling MT cytoskeleton, such as regulation of cell migration, cell division, and metastasis and embryo development.

In mammalian cells, mDia formins act as a scaffold regulator downstream of Rho and modulate MT function and organization as well as promote MT stabilization (Kato et al., 2001; Palazzo et al., 2001). In mouse embryonic fibroblasts, active Rho and mDia1 colocalize at the cell front. Loss of function analysis has indicated that mDia1 is required for MT stabilization and cell migration, as well as necessary for the formation of orientated and stable MTs during wound healing, suggesting that Rho-mDia1 signaling functions during cell movement, while Galha12/13 is dispensable in this process (Goulimari et al., 2005). mDia1 mediates formation of stable MTs by interaction with the MT tip proteins APC and EB1, thereby capturing MTs at the cell cortex to promote cell migration (Wen et al., 2004). Formins also directly bind to MTs by their FH2 domain. This interaction is important in promoting the capture and stabilization of MTs oriented towards the leading edge of migrating cells. mDia also promotes the capture of MTs by the kinetochore during mitosis and the alignment of MTs along actin filaments (Bartolini and Gundersen, 2010). mDia is sufficient to generate and orient stable

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MTs and DRFs are a part of the conserved pathway that regulates the dynamics of MT ends. Microinjection of serum-starved NIH 3T3 cells with DNA encoding a constitutively active form of mDia2 or DNA encoding the DAD auto-inhibitory domain, leads to activation of endogenous mDia1 and stimulates the formation of stable MTs oriented towards the wound edge (Palazzo et al., 2001).

In addition, mDia formins colocalize with a subset of MTs in some cells and bind directly to MTs *in vitro*. mDia1 localizes to the mitotic spindle in HeLa cells mitotic spindle and plays a role in the spindle-cleavage furrow interaction during cell division (Kato et al., 2001). mDia1 and mDia2 stabilize MTs by reducing the tubulin subunit exchange at the plus ends. The exact mechanism sustaining this effect is not yet fully understood. However, the affinity of formins for actin is much higher than for MTs (Chesarone et al., 2010). By expression of constitutively active forms of thirteen mammalian formins in HeLa and NIH3T3 cells, another group has shown that regulation of MT acetylation is likely a general formin feature and that FH1-FH2 domain of mammalian formins is sufficient to induce MT acetylation (Thurston et al., 2012). Therefore, these data support an important functional link between mDia formins and the regulation of MT cytoskeleton.

Furthermore, formins may regulate multiple cell processes by coordinating the MT and actin cytoskeleton. By catalyzing actin polymerization and stabilizing MTs, mDia1 plays also an important role in cell migration (Yamana et al., 2006). ACF7 (actin crosslinking family 7), the spectraplakins family member that has both actin and MT binding domain, acts as an integrator of MT-actin dynamics. ACF7 may bind along MT and is required for MT stabilization downstream of active mDia, as well as it regulates MT dynamics and enhances the link between MT and polarized F-actin (Kodama et al., 2003). Alternatively, as it is the case for actin polymerization and SRF activation, the mDia1 FH2 domain is essential for stable MT formation, suggesting that actin-dependent processes may nevertheless be involved (Wen et al., 2004). Thus, formins mediate crosstalk between the actin and MT cytoskeletons.

### **3.2 Formin associated with megakaryopoiesis**

In mammals, there are fifteen formins that cluster into eight different sub-families based on their sequences and domain architectures (Schonichen and Geyer, 2010), among which the DRFs are grouped into four subfamilies, including mDia (mammalian Diaphanous-related formin), Daam, FMNL and FHOD that commonly share a similar domain organization. mDia includes three members, mDia1, mDia2 and mDia3 and acts downstream of Rho-GTPases to assemble actin assembly. Alternatively, mDia may interact with microfilaments as well as regulation of MT dynamics to

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establish and maintain cell polarity during migration and to sustain asymmetric division and other cytoskeletal-related activities.

mDia1 is present in eukaryotic cells, localizes at the mitotic spindle and midbody and participates in multiple cytoskeleton-mediated cellular activities and organism formation, including stress fiber and filopodia formation, MT dynamics control, endocytosis, phagocytosis, activation of SRFs and formation of adherent junctions (Chesarone et al., 2010). DIAPH1 (Diaphanous-related formin 1, also known as mDia1, Drf1) is encoded by the gene *DRF1 (DIAPH1)*, which is located on chromosome 5q31.3, a chromosomal region that often undergoes a deletion in myelodysplastic syndromes (MDS, true 5q-syndrome or other MDS, but outside the minimum deletion). DIAPH1 is the homolog of the *Drosophila diaphanous* gene and belongs to the formin family. As a member of formin family, DIAPH1 works as an effector downstream of Rho-GTPase and regulates the actomyosin cytoskeleton as well as MT dynamics. In addition, inhibition of DIAPH1 may increase SDF1-mediated MK migration and our data show that it may be involved in PPF in the marrow.

Depending on the cell type and stage in the cell cycle, mDia1 localizes to the cell cortex, trafficking endosomes, cleavage furrow, midbodies, centrosomes and the cytoplasm MT-organizing center crucial for cell division (Tominaga et al., 2000). Recent findings from KO mouse models or non-neuronal tumor cell lines *in vitro* suggest that, as a Rho-effector, mDia1 plays an essential role in the maintenance of the adherent junction and polarity of neuroepithelial cells in multiple brain regions and in modulating actin dynamic that drives tangential migration of neuronal precursors (Shinohara et al., 2012; Thumkeo et al., 2011; Zaoui et al., 2008).

Despite their important functions in cell biology and development, few studies have directly investigated the role of formins in late stage MK differentiation, particularly in PPF and platelet release. Previous studies have shown that human platelets contain the formins mDia1 and Daam1, both are effectors of Rho and are platelet actin assembly factors directly regulated by Rho GTPases (Higashi et al., 2008). Daam1 and mDia1 may contribute to shape change and aggregation in activated platelets by their actin assembly activity. Because mDia1 is 10-fold more potent than Daam1 in actin assembly activity, these two DRFs may have distinct functions, but little is known about their precise functions as well those of other formin family members in platelets.

Thomas and colleagues have found that there are six formins present in mouse MKs (mDia1, mDia2, Daam1, Fmn11, Fmn13 and FHOD1), mDia3 being also found in human MKs (Thomas et al., 2011). There are four formins (mDia1, mDia2, Daam1 and FHOD1) present in human platelets while only three formins (mDia1, Daam1 and FHOD1) are detected in mouse platelets, confirming previous reports for mDia1 and Daam1 (Higashi et al., 2008), but the presence of mDia2 and FHOD1 was



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reported for the first time (Thomas et al., 2011). FHOD1 is an effector of Rho kinase in platelets and may be involved in the formation of platelet stress fibers. The most well characterized mammalian formin is mDia1, which is present both in mouse and in human platelets. However, mDia1 KO analysis has revealed the absence of alteration in platelet count, fibrinogen binding, tyrosine phosphorylation, P-selectin surface expression, platelet spreading or clot retraction in response to both G protein-coupled and tyrosine kinase-linked receptors (Thomas et al., 2011). Another group has shown that there is no evident difference in mDia1 expression level between quiescent and activated platelets, but PI3-kinase mediates the thrombin-induced platelet aggregation through the mDia1 pathway (Gao et al., 2010).

### 3.3 DRFs-related diseases

Formins are implicated in a growing number of cancers and other diseases, which may due to their important roles in cytoskeleton remodeling including nucleation and elongation of non-branched actin filaments and the control of MT dynamics, two processes, which are tightly associated with cell division and migration.

Upon the activation of GTP-bound Rho, mDia1 generates linear actin filaments implicated in the maintenance of polarity during adhesion, migration, division in immune cells and neuroepithelial cells and in driving tangential migration of cortical interneuron in rodents. Previous studies have shown that mDia1 (DFNA1) is associated with non-syndromic autosomal hearing loss (Lynch et al., 1997), as well as in neuronal migration of cortical interneuron and neuron development (Shinohara et al., 2012). A recent study has shown that patient with a homozygous nonsense DIAPH1 alteration has microcephaly, as well as reduced height and weight (Ercan-Sencicek et al., 2014). mDia1 protein is expressed in human neuronal progenitors during mitotic cell division and has a major impact in the regulation of spindle formation and cell division. These findings indicate that DIAPH1 has a crucial role in brain development and exhibits species differences in its function.

Human DIAPH1 (homolog to mouse mDia1) located at 5q31.3, has essential roles in actin and MT remodeling, cell division and in response to adhesive and migratory stimuli. It has been shown that mDia1 acts as a main effector in a tumor-suppressor network that is involved in several 5q minus-related hematopoietic disorders, such as MDS (Eisenmann et al., 2009). *mDia1* KO mice show a splenomegaly, a bone marrow and splenic fibrosis, an hypercellularity with extramedullary hematopoiesis in the spleen and liver and the presence of immature myeloid cells with a high nucleus/cytoplasm ratio. This phenotype is similar to a human mixed MPD (myeloproliferative disorder)/MDS, suggesting that defective mDia1 expression or mDia1 function may contribute to

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myeloid malignancies (Peng et al., 2007). In addition to the myeloproliferative defect, *mDia1* KO mice also develop a lymphopenia with reduced T cell populations in lymphoid tissues, suggesting *mDia1* is also associated with some formin-mediated immune diseases (Eisenmann et al., 2007). Mitf-induced downregulation of *DIAPH1* gene expression leads to an increased ROCK-dependent invasiveness in melanoma cells (Carreira et al., 2006).

In addition, members of the DRF family (DRF1-DRF3) are required for invadopodia formation, suggesting that DRFs are involved in aggressive breast cancer cells (Lizarraga et al., 2009), while inhibition of the activation of mouse *mDia1* (homolog to human *DIAPH1*) blocks the protrusion formation in carcinoma cells (Sarmiento et al., 2008). *mDia1* also directly binds to leukemia-associated Rho-GEF to mediate RhoA activity (Kitzing et al., 2007), which is associated with a wide range of cancers (Sahai and Marshall, 2002).

In human cells, high expression of *FMNL1* (formin-like protein 1) is associated with leukemia (Favaro et al., 2003) and non-Hodgkin lymphoma (Favaro et al., 2006), while overexpression of *FMNL2* is involved in metastasis of colorectal cancer cells (Zhu et al., 2008). *FMN1* and *mDia1* are also crucial for macrophage phagocytosis and immune function (Brandt et al., 2007; Colucci-Guyon et al., 2005; Seth et al., 2006). Therefore, additional studies are needed to elucidate the involvement of formins in inherited immune disorders.

Formin mutations also cause defects in fertility and development. Loss-of-function analysis show that *mDia1* and *FMN2* are crucial for normal gamete formation (Castrillon and Wasserman, 1994; Dumont et al., 2007; Leader et al., 2002) and embryonic development (Afshar et al., 2000; Grosshans et al., 2005; Magie et al., 1999), while *FMN1* is essential for normal limb development (Zhou et al., 2009). An early study links chromosomal truncation of human *Dia2* (homologous to mouse *mDia3*) and oogenesis, while *Dia2* defect leads to premature ovarian failure (Bione et al., 1998). Genome-wide studies have identified formins gene mutations in brain and pancreatic tumors (Jones et al., 2008; Parsons et al., 2008).

Formins are promising targets for cancer therapies, as T cells engineered to recognize a *FMN1* peptide exhibit potent anti-tumor activity (Schuster et al., 2007). Furthermore, *mDia1* KO mice exhibit a mixed MPD/MDS syndrome, particularly by the proliferation of hematopoietic progenitors, indicating that defective *mDia1* expression or function may contribute to myeloid malignancies. It suggests that *mDia1* may act as a tumor suppressor gene and may be as an attractive therapeutic target in MDS and MPD (Peng et al., 2007). Lash L.L and colleagues have identified two DID-DAD disruptor molecules that are called intra-mimics (IMM-01 and -02). *In vivo* analysis of these two disruptor molecules shows that they have the ability to slow tumor growth in a mouse xenograft model of colon cancer

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(Lash et al., 2013). Furthermore, these two disruptor molecules may trigger actin assembly and MT stabilization, cell-cycle arrest, serum response factor-mediated gene expression and apoptosis. Collectively, these data suggest that DID-DAD disruptor molecules and DRFs protein peptide can be used as a new general strategy for therapeutic targeting of the cytoskeleton remodeling machinery of cancer cells.

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## **Chapter II: Results and Conclusion**

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# 1 Results about non muscle Myosin II in MK polyploidization

## Introduction

MKs are the highly specialized precursors that have the ability to produce platelets. During differentiation, MKs undergo polyploidization (endomitosis), leading to an increase in cell size and protein synthesis that precedes platelet production. Polyploidy is a manner of increasing platelet production, as the increase in DNA or proteins synthesis and MK cytoplasm volume is parallel to the level of ploidy. MKs give rise to platelets by remodeling their cytoplasm into long pseudopodia extensions that are called proplatelets, which serve as assembly lines for platelet production (Chang et al., 2007b; Geddis, 2010; Machlus and Italiano, 2013; Ravid et al., 2002). Endomitosis is a unique MK differentiation process that is the consequence of a late cytokinesis failure associated with a contractile ring defect. This cytokinesis failure is related to a defect both in Rho activation and myosin II accumulation in the contractile ring (Gao et al., 2012; Geddis et al., 2007; Geddis and Kaushansky, 2006; Lordier et al., 2008).

Herein, our two studies show that MYH10 is the only non-muscle myosin II present in the contractile ring during MK mitosis and endomitosis, but its expression is silenced during MK differentiation. This induces the switch from mitosis to endomitosis and allows MK polyploidization. Contrary to MYH14, which is not detectable, MYH9 is well expressed during MK differentiation, but is not recruited in the contractile ring whether MK are in mitosis or endomitosis (Lordier et al., 2008). These results revealed that MYH9 and MYH10 must display distinct functions during MK polyploidization and cytokinesis.

In this study, we have also tried to elucidate the role of RUNX1 in regulating MYH10 expression and MK polyploidization.

## Results

First, we have studied the expression of the different heavy chains of non-muscular myosin (MYH) during megakaryopoiesis by qRT-PCR and WB (western blot) analysis. Results showed that the expression of MYH9 increased during MK differentiation whereas MYH10 expression decreased and became undetectable in mature MKs and blood platelets. MYH14 is not expressed during MK differentiation. Immunofluorescence analysis has shown that MYH10 accumulates at the contractile ring (midzone and midbody) and is implicated in the mitosis/endomitosis transition. MYH9 expression

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increases during MK differentiation, but is not recruited in the contractile ring irrespective of MK mitosis or endomitosis. Indeed, MYH9 is diffusively present in the cytoplasm of endomitotic MKs. These data suggest that MYH10 and MYH9 must have distinct functions during MK polyploidization and cytokinesis.

Study of MK-restricted *myh9* KO mice showed that there is no significant change in MK ploidy compared to control mice. Addition of 50  $\mu$ M blebbistatin in culture, a small molecule inhibitor of myosin II, leads to an increase in mean ploidy level of *myh9* KO MK. Using human cells we observe changes in ploidy level induced by MYH10 knockdown or its overexpression. MYH10 knockdown by shRNA induces a marked increase in MK ploidy, while its overexpression induces an increase in 2N MKs and a decrease in mean MK ploidy level. These results suggest that MYH10 is the main myosin II isoform implicated in MK mitosis and endomitosis and its silencing is associated in the switch from mitosis to endomitosis.

Next, we analyze whether accumulation of myosin in the contractile ring is associated with some myosin activity. Immunofluorescence showed that MLC2 is phosphorylated at the end of telophase in the midbody in both dipolar mitotic/endomitotic MKs and multipolar endomitotic MKs. This result suggests that some myosin II activity is present at the contractile ring at the end of MK telophase. Afterwards, we have studied the effects of MK treatment with the myosin inhibitor blebbistatin to explore the role of myosin II activity on furrow ingression. Cytometry and real-time video show that myosin II activity inhibition increases ploidy level, while the furrow ingression is almost completely inhibited. These data suggest that myosin II is implicated in MK furrow ingression and that its activation have a negative role in MK polyploidization.

The transcription factor RUNX1 plays a crucial role in MK differentiation by regulating genes involved in MK differentiation and platelet functions, including MYL9/MLC2 and the CDK inhibitor p19<sup>INK4D</sup>. During polyploidization, MYH10 expression is repressed by RUNX1. Thus, the RUNX1/MYH10 pathway seems an important regulator of the switch from mitosis to endomitosis and polyploidization during MK differentiation.

## **Conclusion**

Previous studies have shown that polyploidization occurs by endomitosis, a process characterized by a cytokinesis failure related to a defect in Rho activation (Gao et al., 2012; Lordier et al., 2008). We show that this cytokinesis failure is also associated with a defect in myosin II accumulation in the contractile ring.

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Using WB and immunofluorescence analysis, we could show that MYH9 expression increases during MK differentiation, but does not accumulate in the MK contractile ring. In contrast, MYH10 accumulates in the MK contractile ring, but its expression is silenced by RUNX1 during differentiation. This result is identical with previous results showing that MYH10 is involved in cytokinesis and that its knockdown in cell lines leads to multinucleated polyploid cells. These results indicate that MYH10 is the main myosin II isoform whose expression regulates the switch from mitosis to endomitosis in MK.

It is interesting to observe that RUNX1 and the same transcription factor complex regulate both the endomitotic process and MK differentiation explaining that the endomitotic process is transcriptional regulated in a MK specific manner and explains the coupling between MK terminal differentiation and polyploidization. Thus, RUNX1 mediates MYH10 silencing is required for the switch from mitosis to endomitosis, linking polyploidization with MK differentiation. This explains why in the KO of RUNX1 and member of the RUNX1 complex such as FLI-1 and GATA-1 or in diseases involving in these transcription factors, MKs are hypoploid.

It remains a very important question to be solved why MYH9 does not accumulate in the contractile ring of MKs whereas it is able to do it in non-MK cells?

Studies in the second manuscript using transgenic mice with chimeric myosins II have shown that the localization of MYH10 in the contractile ring is related to its carboxy terminus. It remains now to understand why and answer to this question will be crucial to understand the mechanisms, which are responsible of MK polyploidization. Is there any link between the defect in Rho activation and this defect in myosin II accumulation or is it related to some scaffold protein necessary for MYH9 accumulation in the contractile ring that is not expressed during MK differentiation?

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## Article1

### ARTICLE

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# RUNX1-induced silencing of non-muscle myosin heavy chain IIB contributes to megakaryocyte polyploidization

Larissa Lordier<sup>1,2,3,\*</sup>, Dominique Bluteau<sup>1,2,3,\*</sup>, Abdelali Jalil<sup>2</sup>, Céline Legrand<sup>1,2,3</sup>, Jiajia Pan<sup>1,2,3</sup>, Philippe Rameau<sup>2</sup>, Dima Jouni<sup>1,2,3</sup>, Olivier Bluteau<sup>1,2,3</sup>, Thomas Mercher<sup>2,3,4</sup>, Catherine Leon<sup>5,6</sup>, Christian Gachet<sup>5,6</sup>, Najet Debili<sup>1,2,3</sup>, William Vainchenker<sup>1,2,3</sup>, Hana Raslova<sup>1,2,3</sup> & Yunhua Chang<sup>1,2,3</sup>



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Megakaryocytes are unique mammalian cells that undergo polyploidization (endomitosis) during differentiation, leading to an increase in cell size and protein production that precedes platelet production. Recent evidence demonstrates that endomitosis is a consequence of a late failure in cytokinesis associated with a contractile ring defect. Here we show that the non-muscle myosin IIB heavy chain (MYH10) is expressed in immature megakaryocytes and specifically localizes in the contractile ring. MYH10 downmodulation by short hairpin RNA increases polyploidization by inhibiting the return of 4N cells to 2N, but other regulators, such as of the G1/S transition, might regulate further polyploidization of the 4N cells. Conversely, re-expression of MYH10 in the megakaryocytes prevents polyploidization and the transition of 2N to 4N cells. During polyploidization, MYH10 expression is repressed by the major megakaryocyte transcription factor RUNX1. Thus, RUNX1-mediated silencing of MYH10 is required for the switch from mitosis to endomitosis, linking polyploidization with megakaryocyte differentiation.

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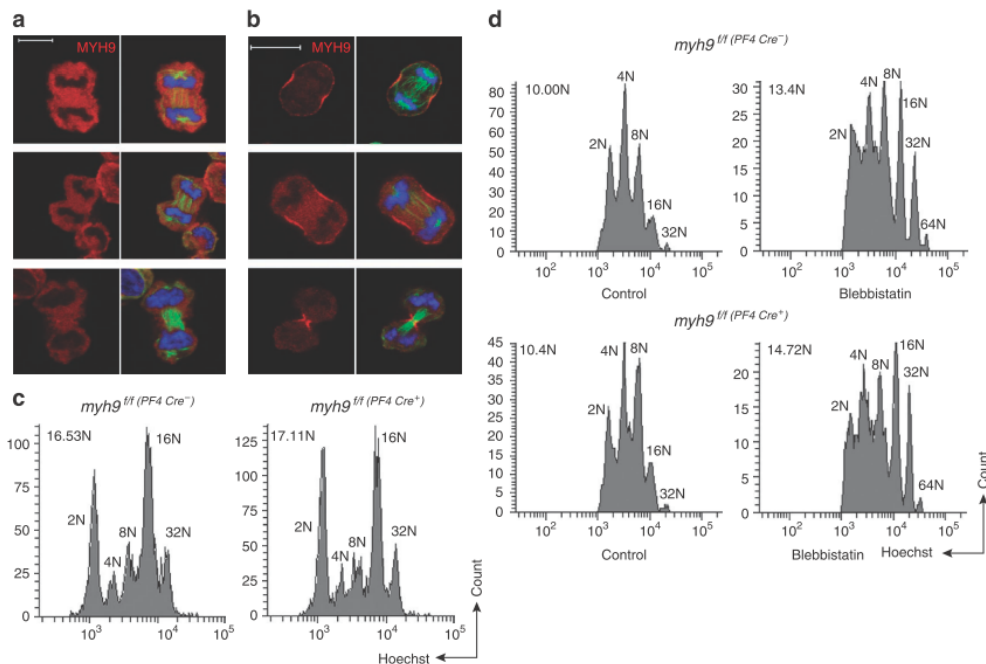
**P**latelets, the blood cells involved in haemostasis, arise in mammals from the fragmentation of the cytoplasm of a giant cell called megakaryocyte (MK)<sup>1,2</sup>. The large cell size is related to polyploidization, a unique process that is an integral part of MK differentiation<sup>3</sup>. The process of polyploidization, which is denoted as endomitosis<sup>4</sup>, corresponds to repeated incomplete cell cycles marked by abortive mitosis at the late phase of cytokinesis<sup>5,6</sup> and generates cells with ploidy of  $2N^x$  with a modal ploidy of  $16N^{3,4}$ . In contrast to other cells undergoing failure of cytokinesis, MK contain a single polylobulated nucleus. At the  $2N$  to  $4N$  transition, cleavage furrow ingression occurs, but subsequently regresses with a backward movement between the two daughter cells<sup>5,6</sup>. A similar phenomenon is observed at higher ploidy with a less profound furrow ingression<sup>7,8</sup>. The cleavage furrow is related to the development at the equator of a contractile ring consisting of myosin II and F-actin, an actomyosin complex generating the forces necessary for furrow ingression and cell separation<sup>9</sup>. Our previous results have shown that there was a very limited accumulation of non-muscle myosin IIA (MYH9) in the contractile ring of MK undergoing mitosis or endomitosis, but the existence of furrow ingression suggested that a myosin II activity still persisted in endomitosis<sup>6</sup>.

In this study, we investigated the presence of MYH10 and showed that it is expressed in immature MK where it accumulates in the contractile ring, and that its expression is silenced during differentiation. MYH10 downregulation increases the ploidy level, and inversely, MYH10 overexpression decreases the ploidy level. Moreover, we show that the Runt related transcription factor 1 (RUNX1) negatively regulates MYH10 expression and thus links the polyploidization process with terminal MK differentiation.

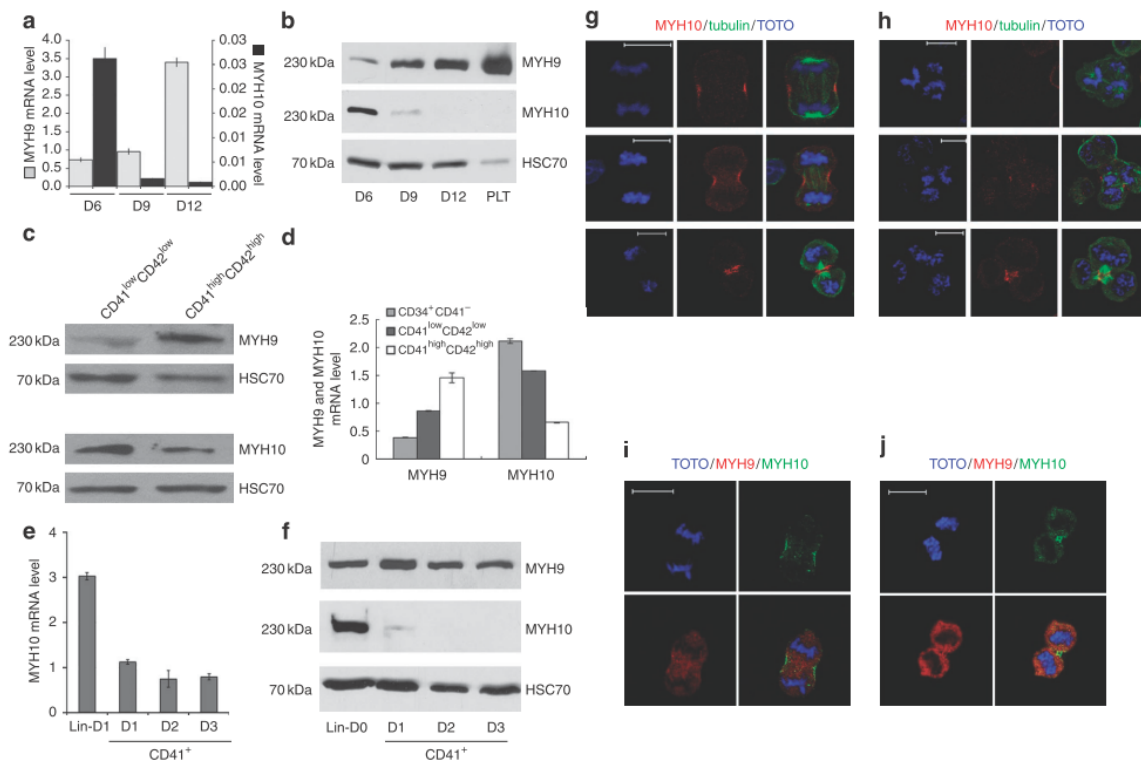
## Results

**MYH9 does not have an important role in MK endomitosis.** First, we confirmed an almost complete absence of MYH9 accumulation in the contractile ring during MK mitosis or endomitosis (Fig. 1a) opposite to primary erythroblasts undergoing mitosis (Fig. 1b). MYH9 has been considered as the only non-muscle myosin II heavy chain expressed in MK and platelets<sup>10</sup>. Therefore, we studied MK-restricted *myh9* knock-out (KO) mice (*myh9<sup>fl/fl</sup>(PF4<sup>Cre</sup>+/+)*) to examine the role of MYH9 in MK ploidization<sup>11,12</sup>, expecting an increase in MK ploidy. Surprisingly, no significant difference in the *in-vivo* MK ploidy level between *myh9<sup>fl/fl</sup>(PF4<sup>Cre</sup>-/-)* (17.11N,  $n=6$ ) and control mice (*myh9<sup>fl/fl</sup>(PF4<sup>Cre</sup>+/+)*; 16.53N,  $n=4$ ,  $P=0.22$ , *t*-test, ploidy measured in MK $\geq 8N$ ) was seen (Fig. 1c). Blebbistatin, a small molecule inhibitor of myosin<sup>13,14</sup>, has been recently shown to increase MK ploidy<sup>15</sup>. Addition of 50  $\mu M$  blebbistatin (an enantiomer mixture)<sup>16</sup> in culture led to an increase in MK mean ploidy level that was similar between *myh9<sup>fl/fl</sup>(PF4<sup>Cre</sup>+/+)* and control mice (from 10.40N to 14.72N,  $P=0.01$ , *t*-test, for *myh9<sup>fl/fl</sup>(PF4<sup>Cre</sup>+/+)* mice, and from 10.00N to 13.40N,  $P=0.01$ , *t*-test, for control mice,  $n=3$ , ploidy measured in MK $\geq 8N$ ; Fig. 1d).

**MYH10 is expressed in the contractile ring of MK.** As blebbistatin inhibits all myosin II isoforms<sup>14</sup>, we investigated the expression of the three non-muscle isoforms MYH9, MYH10 and MYH14 during human and mouse MK differentiation. We differentiated hematopoietic progenitors (CD34<sup>+</sup> cells for human and lineage-negative cells for mouse) in the presence of thrombopoietin (TPO), the growth factor regulating MK development<sup>2</sup>. The MKs were sorted by platelet-specific membrane glycoprotein CD41 (CD41<sup>+</sup>). MYH14 was



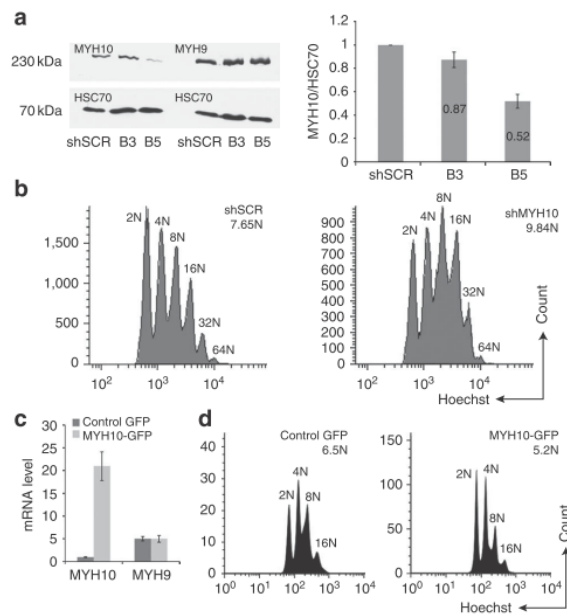
**Figure 1 | Blebbistatin increased ploidy of *myh9* KO MK.** (a, b) Human MKs were derived from CD34<sup>+</sup> cells and were studied at day 8 of culture. Cells were deposited on polylysine-coated slides, fixed and stained for tubulin ( $\alpha$  and  $\beta$ , green) and MYH9 (red). DNA was stained with TOTO (blue). The accumulation of MYH9 in the contractile ring was compared between MK undergoing mitosis or endomitosis (a) and primary mitotic erythroblasts (b). Scale bars represent 10  $\mu m$ . (c) The ploidy of murine MK was studied after double staining with an anti-CD41 antibody and Hoechst labelling. The histograms show the ploidy level ( $\geq 8N$ ) of bone marrow MK from *myh9<sup>fl/fl</sup>(PF4<sup>Cre</sup>-/-)* (17.11N,  $n=6$ ) and control *myh9<sup>fl/fl</sup>(PF4<sup>Cre</sup>+/+)* mice (16.53N,  $n=4$ ). (d) Ploidy levels of MK ( $\geq 8N$ ) derived from the fetal liver of *myh9<sup>fl/fl</sup>(PF4<sup>Cre</sup>+/+)* and control mice and treated with myosin II inhibitor blebbistatin (50  $\mu M$  in DMSO) or without (DMSO). The increase in mean ploidy induced by blebbistatin was similar for *myh9<sup>fl/fl</sup>(PF4<sup>Cre</sup>+/+)* mice (10.40N–14.72N;  $P=0.01$ , *t*-test;  $n=3$ ) and control mice (10.00N–13.40N;  $n=3$ ,  $P=0.01$ , *t*-test).



**Figure 2 | MYH10 is expressed during MK differentiation.** (a,b) CD41<sup>+</sup> MKs were derived from adult blood CD34<sup>+</sup> cells and studied at three different days (D6 to D12, corresponding to cells with increasing maturity). Blood platelets (PLT) were studied as a control for the more mature cells of the lineage. Increase of MYH9 and decrease of MYH10 at mRNA and protein levels were detected by real time RT-PCR (a) and western blotting (b). (a) Real time RT-PCR was normalized to PPIA. (c,d) MYH10 downregulation along the increase in CD41 and CD42 expression. CD41<sup>+</sup> MKs were derived from cord blood CD34<sup>+</sup> cells. Cells were sorted according to CD34, CD41 and CD42 expression at day 10 of culture. MYH9 and MYH10 expressions were analysed by western blotting (c) and real-time RT-PCR (d) in CD34<sup>+</sup>CD41<sup>-</sup> cells, and/or CD41<sup>low</sup>CD42<sup>low</sup> and CD41<sup>high</sup>CD42<sup>high</sup> MK. Real-time RT-PCR was normalized to HPRT. (e,f) Expression of MYH10 during murine MK differentiation was analysed by real-time RT-PCR (e) and western blotting (f). Lin<sup>-</sup> cells were purified from mouse fetal liver (Lin-D0, analysed by western blotting) and cultured in the presence of TPO and SCF for 3 days. After 24 h, CD41<sup>+</sup> cells were sorted from total population (D1, analysed by RT-PCR) and analysed at day 1, 2 and 3 of culture (D1-3 CD41<sup>+</sup>). (e) Expression of MYH10 transcripts was studied by real-time RT-PCR and normalized to HPRT. (f) Expression of MYH9 and MYH10 proteins by western blots. (g,h) MYH10 localization in human MK derived from CD34<sup>+</sup> cells from bone marrow. Cells were stained for tubulin ( $\alpha$  and  $\beta$ , green) and MYH10 (red). DNA was stained with TOTO (blue). Localization of MYH10 in dipolar 2N-4N mitotic/endomitotic MK (g) and in 4N-8N multipolar endomitosis (h) Scale bars represent 10  $\mu$ m. (i,j) Comparison of the localization of MYH10 (green) and MYH9 (red) in dipolar mitotic/endomitotic human MK (i) and (j) of dipolar mitotic/endomitotic human MK. DNA was stained with TOTO (blue). Scale bars represent 10  $\mu$ m. (a,d,e) Error bars represent s.d. of triplicate wells of one experiment. (b,c,f) Anti-HSC70 was used as the control of protein loading.

undetectable during MK differentiation, whereas MYH10 messenger RNA (mRNA) and protein were identified by quantitative reverse transcription (RT)-PCR and western blot analyses in undifferentiated MK. During human MK differentiation, MYH10 expression markedly decreased from day 6 to day 9, approximately at a time when MK have switched from mitosis to endomitosis, to become undetectable in mature MK (day 12) and blood platelets, whereas MYH9 expression increased (Fig. 2a and b). This inverse link between MYH10 expression and differentiation was confirmed by directly sorting cord blood CD34<sup>+</sup>-derived MK on the CD41 and CD42 expression at day 10 of culture. An important decrease in MYH10 expression, and inversely, an increase in MYH9 expression were detected at the protein and mRNA level in CD41<sup>high</sup>CD42<sup>high</sup> MK in comparison with CD34<sup>+</sup>CD41<sup>-</sup> cells and immature MK (CD41<sup>low</sup>CD42<sup>low</sup>; Fig. 2c and d). During murine MK differentiation, MYH9 expression remained stable, whereas MYH10 expression was hardly detectable as soon as 24 h after the beginning of the culture (Fig. 2e and f).

We used immunofluorescence labelling to investigate the recruitment of MYH10 to the contractile ring of mitotic and endomitotic human MK at day 6 of culture. At the 2N-4N transition, MYH10 accumulated at the midzone when the cleavage furrow started to form (Fig. 2g), and the percentage of positive cells increased with telophase progression. Only 24.5% of the cells ( $n=85$ , three independent experiments) with a cleavage furrow whose depth remained lower than 75% of cell transversal diameter accumulated MYH10. At later stage, telophase (depth of cleavage furrow >75%), 57% of the cells ( $n=141$ , three independent experiments) showed MYH10 accumulation. In multipolar endomitotic MK (4N to 8N or >8N), only 9% of the cells ( $n=42$ ; three independent experiments) with a cleavage furrow lower than 75% of the cell transversal diameter showed MYH10 accumulation and the protein was not located on the whole furrow (Fig. 2h). The percentage increased to 29% at the end of telophase (cleavage furrow >75% transversal diameter, 3 independent experiments and 17 late telophase cells counted). Moreover, MYH9 was diffusively present in the cytoplasm of



**Figure 3 | Changes in ploidy level induced by MYH10 knockdown or overexpression.** (a) Effect of shMYH10 on the level of MYH10 protein in human MK. Protein level was ascertained by western blotting. Anti-HSC70 was used as control of protein loading. (b) MYH10 knockdown increases human MK mean ploidy from 7.65N (shSCR control) to 9.84N (shMYH10 B5). GFP<sup>+</sup> MK analysed at day 9 of culture ( $n=3$ ,  $P=0.015$ ,  $t$ -test). (c) Transfection of a plasmid containing MYH10 in fusion with GFP induces overexpression of MYH10 mRNA in MK. Real-time RT-PCR was normalized to *HPRT* ( $n=4$ ,  $P=0.01$ ,  $t$ -test, error bars represent s.d. of triplicate wells from one experiment). (d) Overexpression of MYH10 in MK induces an increase in 2N ( $n=4$ ,  $P=0.009$ ,  $t$ -test) and a decrease in 8N cells ( $n=4$ ,  $P=0.01$ ,  $t$ -test).

endomitotic MK, whereas MYH10 was specifically localized in the contractile ring (midzone and midbody; Fig. 2i and j). Only a few human MK accumulated MYH9 at the cleavage furrow at the end of telophase<sup>6</sup>. Furthermore, we found that MYH9 did not accumulate in the contractile ring of dipolar murine MK in telophase (97 telophase cells were counted, 3 independent experiments). These results suggest that MYH10 is the main myosin II isoform present in the contractile ring of MK. Its downregulation during differentiation leads to a decreased myosin II accumulation in the contractile ring.

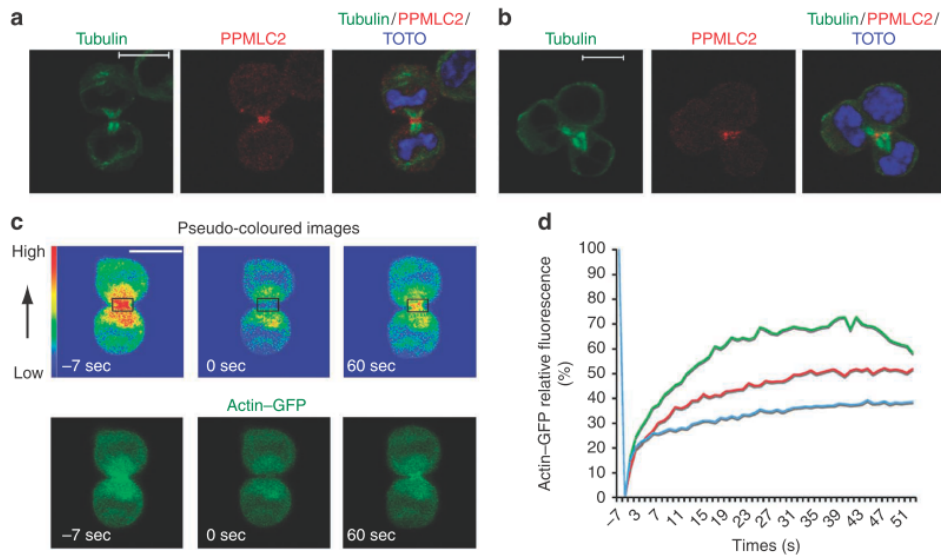
**MYH10 is involved in MK ploidy regulation.** To determine if MYH10 is really implicated in MK polyploidization, human progenitors (CD34<sup>+</sup> cells) were induced towards MK differentiation (stem cell factor (SCF) + TPO) and transduced at days 2 and 3 of culture with lentiviruses encoding green fluorescent protein (GFP) and two different short hairpin RNAs (shRNAs) targeting MYH10 (B3 and B5) or a scramble shRNA. Western blot analysis of the GFP-positive population sorted at 72 h after infection with the shMYH10-B5 showed an ~50% reduction in the MYH10 protein level, whereas the level of MYH9 protein remained unchanged (Fig. 3a). MYH10 knockdown resulted in a marked increase in MK ploidy, as attested by a shift in mean ploidy from 7.65N to 9.84N for shRNA-treated MK ( $n=3$ ,  $P=0.015$ ,  $t$ -test; Fig. 3b). The shMYH10-B3 was less effective in the downregulation of MYH10 (Fig. 3a), as well as in the increase of ploidy. By overexpressing a MYH10 mutated form, not identified by the shRNA, we could confirm in the COS7 cell line that

shMYH10 affects the cell cycle by a specific MYH10 knockdown and not by an off-target effect (Supplementary Fig. S1). Conversely, we re-expressed MYH10 in MK at day 5 of culture, using a vector containing MYH10 in fusion with GFP by nucleofection. Quantitative RT-PCR analysis of the GFP-positive populations (MYH10-GFP and control GFP) 48 h after nucleofection showed a 20-fold increase in MYH10 mRNA level, whereas MYH9 mRNA level remained unchanged (Fig. 3c). MYH10 overexpression increased the percentage of 2N MK from 30.5 to 41.9% ( $n=4$ ,  $P=0.009$ ,  $t$ -test) and reduced MK mean ploidy level (from 6.5N to 5.2N,  $n=4$ ,  $P=0.01$ ,  $t$ -test; Fig. 3d). Altogether, these results suggest that MYH10 is the main myosin II isoform implicated in MK polyploidization.

**Myosin II is activated during furrow ingression.** We then investigated if this low myosin accumulation in the contractile ring was capable to generate a myosin activity. Activation of myosin II was assessed by exploring the phosphorylation status of its regulatory light chain (MLC2) with an antibody recognizing PPMLC2-, Ser<sup>19</sup>- and Thr<sup>18</sup>-phosphorylated forms of MLC2. A positive signal was easily seen at late telophase in 68.6% ( $n=102$ , three independent experiments) of dipolar mitotic/endomitotic MK with a cleavage furrow  $\geq 75\%$  cell transversal diameter (Fig. 4a). PPMLC2 was also detected on the cleavage furrow of polyploid endomitotic MK examined at late telophase or during cytokinesis failure (Fig. 4b), but only for a minority of them (25%,  $n=24$ , three independent experiments). This experiment indicates that there still exists some myosin II activity at the contractile ring at the end of MK telophase, even with a reduced myosin heavy chain accumulation.

MLC2 phosphorylation regulates myosin II activity without affecting the recruitment of myosin II and F-actin in the contractile ring<sup>13,17,18</sup> and controls the actin dynamics (assembly and disassembly) necessary for completion of cytokinesis<sup>19</sup>. To study actin turnover, CD34<sup>+</sup> cells were transfected with a GFP- $\beta$ -actin construct and induced to differentiate into hematopoietic cells by a cocktail of growth factors. On the basis of the expression of the platelet-specific membrane glycoprotein CD41, GFP-positive cells were sorted into MK (CD41<sup>+</sup>GFP<sup>+</sup>) and non-MK (CD41<sup>-</sup>GFP<sup>+</sup>), and actin turnover was measured in the contractile ring at the end of cytokinesis by fluorescence recovery after photobleaching in dipolar mitosis or in endomitosis (Fig. 4c, Supplementary Movie 1). Fluorescence recovery in the contractile ring after bleaching was lower in MK than in non-MK cells ( $55.7 \pm 12.8\%$  with a  $t_{1/2}$  of  $6.3 \pm 2.5$  s ( $n=15$ ) versus  $77.6 \pm 10.9\%$  with a  $t_{1/2}$  of  $8.6 \pm 3.7$  s ( $n=16$ );  $P=0.0007$ ,  $t$ -test). This significant decrease in the actin-GFP fluorescence recovery in the contractile ring of MK as compared with control myeloid cells suggested that a high pool of non-dynamic F-actin existed at the end of cytokinesis of dipolar mitotic/endomitotic MK (Fig. 4d). Partial inhibition of myosin II activity by blebbistatin pretreatment at low concentration (12.5  $\mu$ M) increased the fraction of non-dynamic F-actin in the contractile ring of MK, as attested by a decreased fluorescence recovery ( $42.9 \pm 9.0\%$ , with  $t_{1/2}$  of  $2.94 \pm 1.35$  s,  $n=8$ ,  $P=0.01$   $t$ -test with blebbistatin; Fig. 4d). Although blebbistatin is inactivated by blue light, we think that myosin activity was not restored immediately and the time until recovery explains the greater stability of F-actin. Together, these results indicate that a myosin II activity still persists in the contractile ring of MK; however, its attenuation decreased the F-actin mobility, increasing the probability of a cytokinesis failure.

To investigate the role of residual myosin II activity on furrow ingression, blebbistatin was added at day 6 of culture when polyploidy begins in MK. Ploidy level was analysed by flow cytometry 72 h later in maturing MK identified by co-expression of two platelet-specific glycoproteins, CD41 and CD42. Blebbistatin significantly increased the mean ploidy from 6.47N to 11.27N at 25  $\mu$ M ( $n=3$ ,  $P=0.015$ ,  $t$ -test; Fig. 5a) and from 6.28N to 12.12N at 100  $\mu$ M ( $n=4$ ,  $P=0.008$ ,  $t$ -test; Fig. 5b). When examined by real-time video



**Figure 4 | Myosin II is in an active state in the contractile ring of MK. (a, b)** Human MKs were fixed and stained by an antibody recognizing Ser<sup>19</sup>- and Thr<sup>18</sup>-phosphorylated MLC2 (PPMLC2, red) to examine myosin II activation. In both dipolar mitotic/endomitotic MK (a) and multipolar endomitotic MK (b), PPMLC2 was seen at the end of telophase in the midbody. Scale bars represent 10  $\mu$ m. (c) Contractile ring in late telophase of a dipolar human MK was photobleached and images were acquired every second for 1 min after photobleaching. MKs were transfected with a plasmid encoding a fusion protein between actin and GFP (actin-GFP). The area outlined by the black square is the bleached region. Pseudo-coloured images with rainbow Lut according to GFP-signal intensity and GFP-actin of the corresponding images were presented. Times indicated are before (-7 s) or after (0 s and 60 s) bleaching. Scale bars represent 10  $\mu$ m. (d) Corresponding graphs of actin-GFP fluorescence recovery in the contractile ring of CD41<sup>+</sup> MK (red line), CD41<sup>-</sup> non-MK cells (green line) and MK treated with 12.5  $\mu$ M blebbistatin (blue line).

microscopy, H2B-GFP-expressing MK showed an apparently normal metaphase-to-anaphase transition in the presence of blebbistatin at 25  $\mu$ M (Supplementary Movie 2 and 3) as compared with controls (Supplementary Movie 4 and 5), but the furrow ingression was almost completely inhibited (Supplementary Movie 2 and 3 compared with Supplementary Movie 4 and 5; Fig. 5c and d). These data indicated that myosin II was implicated in MK furrow ingression and that its activation had a negative role for MK polyploidization.

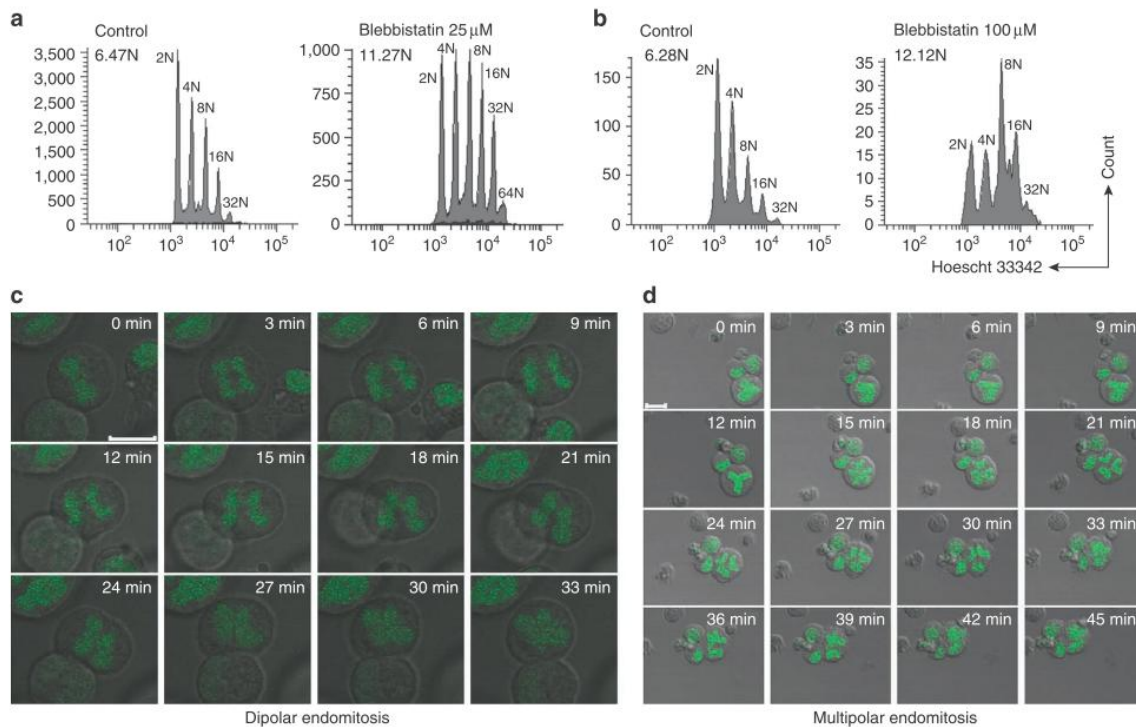
#### MYH10 is involved in the switch from mitosis to endomitosis.

There are two ways to increase MK ploidy: one is to augment the number of endomitotic cycles, the other is to promote the switch from mitosis to endomitosis by preventing 4N cycling cells to return to 2N. To determine the mechanism by which MYH10 knockdown modifies ploidy level, MK cultures were infected at day 0 and day 1 with the shMYH10-B5-encoding lentivirus. The cell cycle status of GFP-positive cells was analysed by bromodeoxyuridine (BrdU) incorporation 72h after infection (day 4 of culture; three independent experiments). MYH10 knockdown had no effect on BrdU incorporation in both 2N-4N (37% with shMYH10 and 36% with short hairpin scramble sequence (shSCR)) and >4N MK populations (6% with shMYH10 and 8% with shSCR; Fig. 6a). This result demonstrated that MYH10 knockdown had no impact in the G1/S transition for both diploid and polyploid MK. Simultaneous analysis of the ploidy level showed that MYH10 knockdown diminished the frequency of 2N MK, but increased that of  $\geq$ 4N MK, suggesting that MYH10 regulates the fate of 4N cells (Fig. 6b). To directly demonstrate this mechanism, 4N MKs were sorted after Hoechst 33342 labelling at day 5 of culture and cultured for additional 72h with and without blebbistatin (25  $\mu$ M) before ploidy analysis (Fig. 6c). After blebbistatin treatment, only a small fraction of 2N MKs were detected (3.4  $\pm$  2.3%), the majority of the cells remained 4N, and a part of MK increased their ploidy level (Fig. 6c, 4N

blebbistatin). Without blebbistatin treatment, a significant fraction of 4N cells returned to 2N, (30  $\pm$  6% 2N MK detected, three independent experiments,  $P=0.03$  *t*-test; Fig. 6c, 4N control). Collectively, these results demonstrated that MYH10 was involved in the switch from mitosis to endomitosis. This result is in agreement with previous results that have shown that MYH10 is involved in cytokinesis and that its knockdown in cell lines leads to multinucleated polyploid cells<sup>15,20,21</sup>.

#### RUNX1 mediates MYH10 silencing during MK differentiation.

The arrest of endomitosis is linked to MK maturation and RUNX1 has a role in this process by regulating genes involved in platelet functions, including myosin light chain 9 (*MYL9/MLC2*)<sup>22</sup> and the CDK inhibitor *p19<sup>INK4D</sup>* (ref. 23). Small diploid MKs are present in the bone marrow of *runx1* KO mice<sup>24,25</sup> suggesting that RUNX1 could also regulate the switch from mitosis to endomitosis by targeting MYH10. Using an *in silico* approach with the ChipMapper online software, we identified a putative conserved RUNX1 transcription factor-binding site in the *MYH10* promoter region (Fig. 7a, Supplementary Fig. S2). Chromatin immunoprecipitation (ChIP) assays demonstrated that RUNX1 could bind this site in MK (Fig. 7b, two independent experiments were performed). To test for its functional relevance, we cloned the promoter region (pMYH10\_luc) with or without mutation in the RUNX1-binding site (pMYH10mut\_luc) upstream of the *luciferase* gene (Fig. 7a). We performed gene reporter assays in the human erythroleukemia cell line. The mutation in the RUNX1-binding site led to a significant increase in luciferase activity ( $n=3$ ,  $P\leq 0.05$ , *t*-test; Fig. 7c). To better understand the regulatory role of RUNX1 in MK, we then transduced human CD34<sup>+</sup> cells induced for MK differentiation at day 1 and day 2 of culture with a lentivirus encoding an shRNA-targeting RUNX1 (shRUNX1)<sup>23</sup>. As shown in Fig. 7d, shRUNX1 treatment led to a significant decrease (30–50% depending on experiment) in



**Figure 5 | Effects of myosin II activity inhibition on MK ploidy and furrow ingression.** (a, b) Effects of myosin II inhibitors (blebbistatin) on human MK polyploidization. The ploidy level of CD41<sup>+</sup>/CD42<sup>+</sup> MK was analysed at day 9 of culture (72 h after addition of the inhibitor). MK mean ploidy was calculated from the number of cells in each ploidy class ( $n=3$ ). Blebbistatin was tested at two concentrations: (a) at 25  $\mu$ M, the mean ploidy level was increased significantly from 6.47N to 11.27N ( $n=3$ ,  $P=0.015$ ,  $t$ -test), and (b) at 100  $\mu$ M, from 6.28N to 12.13N ( $n=4$ ,  $P=0.008$ ,  $t$ -test). (c, d) Human MK transduced with H2B-GFP were treated with blebbistatin (25  $\mu$ M) and then filmed by real-time video microscopy. Almost no cleavage furrow was seen in diploid (c) and polyploid (d) endomitotic MK.

the RUNX1 transcripts. Real-time PCR showed a 1.5-fold increase in MYH10 expression (Fig. 7d), which was confirmed at the protein level (Fig. 8a), whereas flow cytometric analysis showed a decrease in the ploidy level of mature MK (CD41<sup>high</sup>CD42<sup>high</sup>;  $n=3$ ,  $P\leq 0.01$ ,  $t$ -test; Fig. 8b).

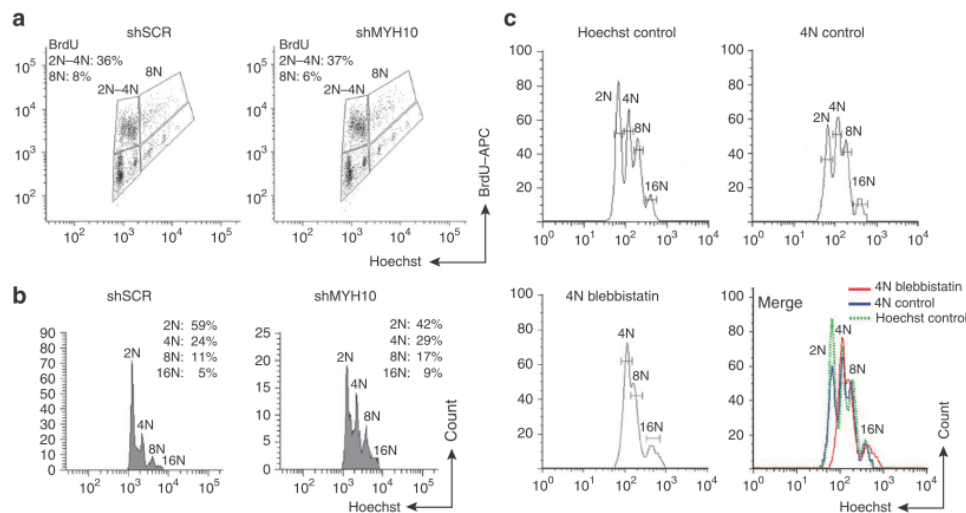
To confirm these results *in vivo*, we used a KO mouse model in which the conditional *runx1*<sup>*fl/fl*</sup> mouse strain<sup>24</sup> was crossed with Mx1-Cre mice. Efficient *Runx1* deletion was achieved in hematopoietic cells depleted in lineage markers (lineage-negative (Lin<sup>-</sup>)) 5 weeks after injection of polyinosinic-polycytidylic acid (pIpC) (Fig. 8c)<sup>24</sup>. *Runx1*-deficient bone marrow MK exhibited a decrease in ploidy level, as compared with control littermates (mean ploidy 12N versus 18N;  $n=3$ ,  $P\leq 0.01$ ,  $t$ -test; Fig. 8d). Bone marrow Lin<sup>-</sup> cells were cultured in the presence of TPO, and CD41<sup>+</sup> cells were sorted at day 2.5 of culture. Blebbistatin (50  $\mu$ M) induced a dramatic increase in ploidy in *runx1*<sup>*fl/fl*</sup>(Mx1 Cre<sup>+</sup>) MK (mean ploidy from 10.8N to 22.6N,  $n=3$ ,  $P=0.006$ ,  $t$ -test; Fig. 8e). A significant 1.7-fold increase of *Myh10* mRNA level was detected in *runx1*-deficient MK (Fig. 8f). Altogether, the RUNX1/MYH10 pathway appears as an important regulator of the switch from mitosis to endomitosis.

## Discussion

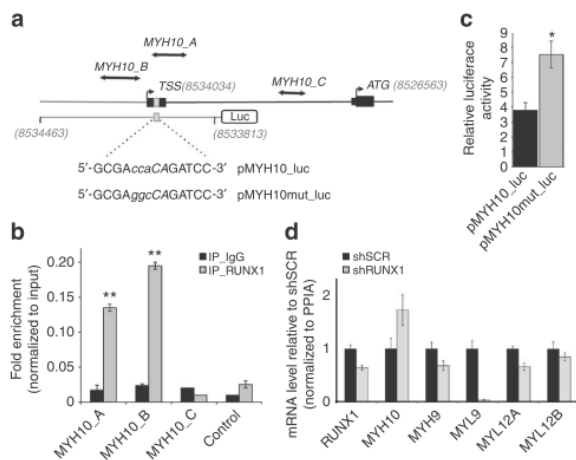
The mechanism of the switch from mitosis to endomitosis in MK is still a matter of controversy<sup>26–30</sup> with studies suggesting a cell cycle regulation defect, whereas others argue for a defect in the expression of the central spindle proteins. Our new findings demonstrate the key contribution of MYH10 downregulation in the process of the switch from mitosis to endomitosis. Evidence was provided that

endomitosis was a defect in late cytokinesis with an incomplete furrow ingression<sup>5–7</sup>. Here we demonstrate that immature MK express myosin IIB and that the *MYH10* gene is silenced during differentiation. We also show that the two non-muscle myosin II isoforms have distinct roles in MK. By accumulating in the contractile ring, MYH10 is involved in late phases of mitosis, whereas MYH9 appears to be mainly an organizer of the MK cytoplasm and is implicated in pro-platelet formation at late stages of differentiation<sup>10,12</sup>. Sustaining this hypothesis, a limited MYH9 accumulation was observed in the contractile ring of human MK<sup>6</sup>. Furthermore, MYH9 was not detected in the cleavage furrow of murine MK and an *myh9* KO did not modify the MK ploidy<sup>12</sup>. Therefore, it is likely that non-muscle myosin IIA and B have two distinct functions, both in human and murine megakaryopoiesis. These two myosin II isoforms have previously been shown to have distinct roles in cadherin junction of the epithelial cells<sup>31</sup> or in the erythroblast enucleation<sup>32</sup>. However, MYH10 silencing does not explain the entire polyploidization process. MYH10 silencing has a central role at the 2N–4N transition in MK by inducing failure of cytokinesis as also observed in MYH10-ablated cardiac myocytes<sup>21</sup> or COS cells<sup>15,20</sup>. However, MKs are not subsequently blocked in 4N. Thus, abnormalities in the G1/S transition check point might also be present in the 4N MK<sup>3,33,34</sup>. In addition, it remains to understand why MYH9 does not accumulate in the cleavage furrow of MK in contrast to other cell types; in fact, myosin II isoform localization may depend on distinct upstream signalling pathways as shown in epithelial cells<sup>31</sup>.

RUNX1, one of the main transcription factor involved in MK differentiation, directly regulates MYH10 expression and is



**Figure 6 | Effect of MYH10 knockdown on mitosis to endomitosis transition.** (a) BrdU incorporation in the control (shSCR) or after MYH10 knockdown (shMYH10). The BrdU-positive and -negative populations (day 4 of culture) were compared in 2N-4N and >4N MK transduced by shMYH10 (B5) or shSCR control lentivirus. BrdU incorporation in 2N-4N MK was 37% with shMYH10 and 36% with shSCR, and in >4N, MK was 6% with shMYH10 and 8% with shSCR (n = 3). (b) Analysis of ploidy levels of GFP<sup>+</sup>/CD41<sup>+</sup> MK in the BrdU experiment (day 4 of culture), (c) Effect of blebbistatin on cell cycle of sorted 4N MK. Hoechst control: MKs were incubated with Hoechst 33342 at day 5 of culture for 1 h and cultured for additional 72 h; 4N control: 4N MKs were sorted after Hoechst 33342 labelling at day 5 of culture and cultured for additional 72 h without blebbistatin; 4N blebbistatin: 4N MKs were sorted after Hoechst 33342 labelling at day 5 of culture and cultured for additional 72 h with blebbistatin (25 μM). Merge: Hoechst control, 4N control and 4N blebbistatin.



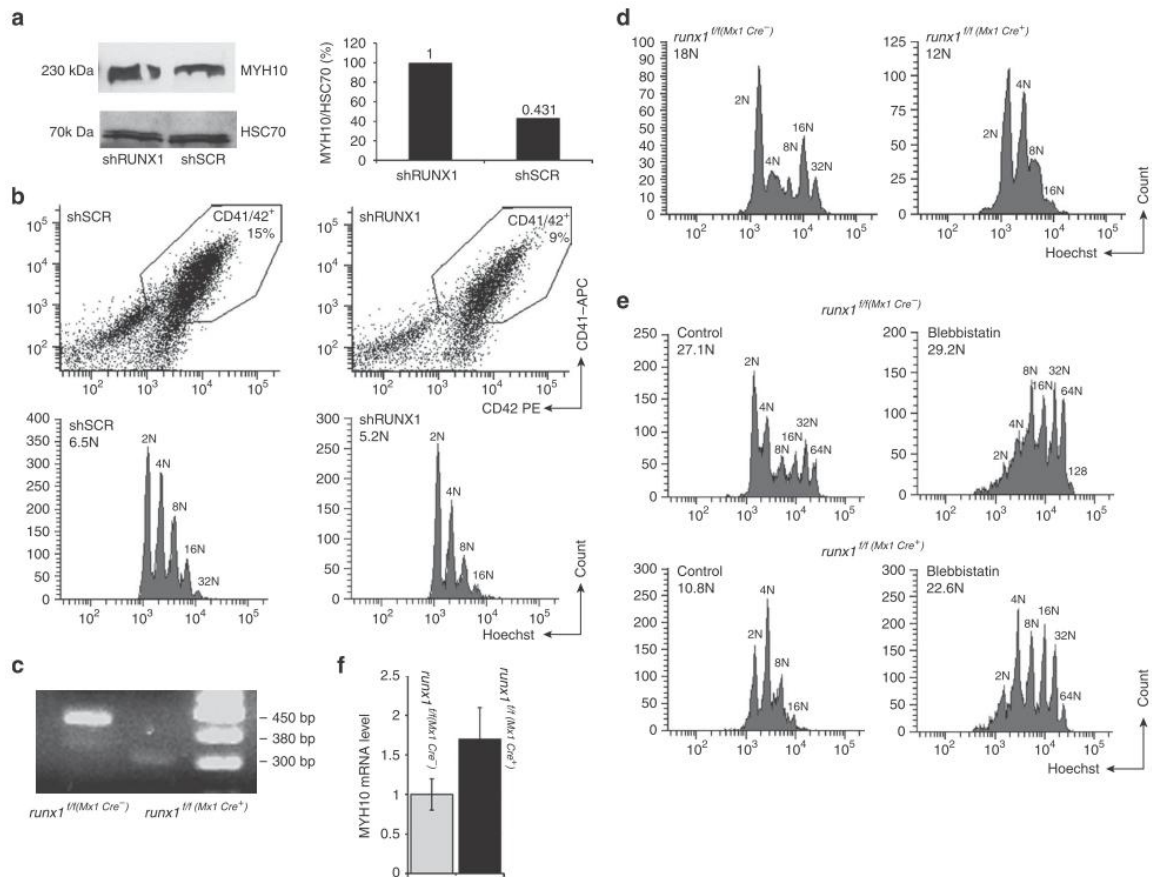
**Figure 7 | RUNX1 regulates negatively MYH10 transcription during MK differentiation.**

(a) Schematic representation of MYH10 promoter. Gray numbers indicate genomic location in the UCSC database (GRCh37/hg19). Gray box indicates RUNX1 putative binding site and black lines show MYH10\_A, \_B, and \_C amplicon fragments studied in ChIP experiment, respectively, localized in position: chr17:8533904-8534036, chr17:8534067-8534201, chr17:8530973-8531054. TSS indicates transcription start site. (b) Quantitative ChIP analysis was performed in MK. Primers encompassing (MYH10\_A and \_B) or not (MYH10\_C) the RUNX1 site were used to amplify input genomic DNA, and the DNA precipitated by antibodies against either normal immunoglobulin G (IgG) or RUNX1. A genomic region without RUNX1-binding site was used as negative control sequence (control). Values are normalized to input genomic DNA. Values obtained for fold enrichment using MYH10\_A and B were statistically significant as indicated. All the data are representative of three independent experiments performed in duplicate (n = 3, \*\*P ≤ 0.01, t-test, error bars represent s.d. of duplicate). (c) MYH10-promoter luciferase assay with (pMYH10mut\_luc) or without mutated RUNX1-binding site (pMYH10\_luc) in human erythroleukemia (HEL) cells. Two independent experiments were performed. (c) Illustrates one representative experiment (n = 3, \*P ≤ 0.05 error bars represent s.d.). (d) CD41<sup>+</sup>CD42<sup>+</sup>GFP<sup>+</sup> cells were sorted at day 6 of culture in presence of TPO. RUNX1, MYH10, MYH9, MYL9, MYL12A and MYL12B expression levels were analysed by real-time RT-PCR, expression level was normalized to PPIA and expressed relatively to shSCR (control). Error bars represent s.d. of triplicate wells from one experiment.

responsible for its repression during MK differentiation, which may explain the presence of low ploidy MK in *runx1*<sup>fl/fl</sup>(*Mx1* Cre<sup>+</sup>) mice<sup>24,25</sup>, as well as in hereditary or acquired haematological disorders associated with *RUNX1* loss-of-function mutations<sup>35,36</sup>. Low-ploidy MK has been observed in KO of other transcription factors, such as GATA1 or FLI-1 (refs 37,38). As these two transcription factors may be associated with RUNX1 in large transcription complexes<sup>39,40</sup>, they may similarly act on MYH10 expression. The observation that two non-muscle myosin II isoforms are present in MK and have distinct roles opens new avenues of research; for example, the MYH9/MYH10 ratio could be used as a marker of MK differentiation and dysfunction. Complete understanding of how MYH10 expression is regulated in numerous physiological and pathological conditions remains a question for further investigations.

**Methods**

**Mouse and in vitro cultures of murine MK.** The conditional *runx1* KO mouse strain was developed by Dr J. D. Gowney *et al.*<sup>24</sup> and was a gift from Dr. D. G. Gilliland (Boston, USA) and Dr. N. A. Speck (Philadelphia, USA). *Runx1*<sup>fl/fl</sup> mice were crossed with *Mx1*-Cre mice; deletion of *runx1* locus flanked by *LoxP* sites



**Figure 8 | Effects of RUNX1 knockdown or KO on MYH10 expression and MK ploidy level in human and mouse. (a)** shRNA-mediated RUNX1 knockdown increases MYH10 protein level (western blots) in sorted GFP<sup>+</sup> human MK at day 9 of culture. **(b)** At day 9 of culture, ploidy levels were analysed by flow cytometry in CD41<sup>+</sup>CD42<sup>+</sup>GFP<sup>+</sup> human cells ( $n=3$ ,  $P<0.01$ ,  $t$ -test). shRNA-mediated RUNX1 knockdown decreases MK ploidy level. **(c)** Five weeks after induction of *runx1* KO by injection of plpC, the excision by Cre-recombinase of *runx1* locus flanked by LoxP sites was verified by PCR on DNA isolated from bone marrow progenitors (Lin<sup>-</sup> cells). **(d)** The effect of *runx1* KO on ploidy level was analysed directly on bone marrow murine CD41<sup>+</sup> MK. **(e)** At day 2.5 in culture, ploidy level in MK derived from *runx1*<sup>fl/(Mx1 Cre<sup>+</sup>)</sup> mouse bone marrow was analysed by flow cytometry in CD41<sup>+</sup> cells cultured with (50  $\mu$ M) or without blebbistatin (for *runx1*<sup>fl/(Mx1 Cre<sup>+</sup>)</sup> mice,  $n=3$ ,  $P=0.006$ ,  $t$ -test; for *runx1*<sup>fl/(Mx1 Cre<sup>-</sup>)</sup> mice,  $n=4$ ,  $P=0.02$ ,  $t$ -test). **(f)** At day 2.5 in culture, CD41<sup>+</sup> cells derived from *runx1*<sup>fl/(Mx1 Cre<sup>+</sup>)</sup> mouse bone marrow were sorted and MYH10 expression level was analysed. Real-time RT-PCR was normalized to HPRT (a pool of CD41<sup>+</sup> cells from three *runx1*<sup>fl/(Mx1 Cre<sup>+</sup>)</sup> mice and from four *runx1*<sup>fl/(Mx1 Cre<sup>-</sup>)</sup> mice was analysed). Error bars represent s.d. of triplicate.

was induced by injection of the interferon-inducing agent plpC. Five weeks after *runx1* KO induction, platelet counts were performed. The *runx1* KO mice exhibited thrombopenia ( $2-3 \times 10^5$  platelets per ml), contrasting with mice negative for Cre-recombinase ( $\sim 1 \times 10^6$  platelets per ml).

The floxed *myh9* strain was crossed with PF4-Cre mice to obtain mice with deletion of the *MYH9* exon 1 in the MK lineage, as described previously<sup>11</sup>. Lin<sup>-</sup> cells were purified from the fetal liver (E14.5) after incubation with a mixture of rat monoclonal antibodies against lineage antigens (Gr1, TER119, CD11b, B220 and anti-CD3, 15  $\mu$ g ml<sup>-1</sup> each, Pharmingen, CA, USA) and depleted with Dynabeads coupled to a mouse antibody against rat immunoglobulin (Dyna, Norway). Lin<sup>-</sup> cells were cultured in the presence of murine (SCF, 10 ng ml<sup>-1</sup>, Peprotech, USA) and TPO (50 ng ml<sup>-1</sup>, Kirin Brewery, Tokyo, Japan) to obtain murine MK. Blebbistatin (50  $\mu$ M) was added at the onset of Lin<sup>-</sup> cell culture and MK ploidy was analysed after 48 h treatment by blebbistatin. Mice were housed and analysed at the Institut Gustave Roussy Animal Facilities (Villejuif, France) according to the national and institutional guidelines.

**In vitro MK culture derived from human CD34<sup>+</sup> cells.** CD34<sup>+</sup> cells were obtained in agreement with our Institute Ethic Committee (Assistance Publique des Hôpitaux de Paris) from the bone marrow of healthy patients, leukapheresis samples and cord blood. CD34<sup>+</sup> cells were isolated by an immunomagnetic cell sorting technique (AutoMacs; Miltenyi Biotec, Germany) and cultured in serum-free medium in the presence of recombinant human TPO (10 ng ml<sup>-1</sup>)<sup>41</sup>.

Depending on the experiments, MKs were sorted according to CD41, or CD41 and CD42 expression using Influx flow cytometer (BD, Mountain View, CA)<sup>42</sup>.

**Fluorescence recovery after photobleaching analysis.** CD34<sup>+</sup> cells were cultured 4 days in serum-free medium with recombinant human cytokines: either a combination of TPO (10 ng ml<sup>-1</sup>) and SCF (25 ng ml<sup>-1</sup>; Biovitrum AB, Stockholm, Sweden) or a combination of TPO, interleukin-3 (100 U ml<sup>-1</sup>, Novartis, Basle, Switzerland), SCF and fetal liver tyrosine kinase ligand (10 ng ml<sup>-1</sup>, Celldex Therapeutics Inc., Needham, USA). Cells were then transfected with the GFP- $\beta$ -actin construct by the Amaxa nucleofection system (Amaxa, France). Twenty-four hours after transfection, CD41<sup>+</sup>GFP<sup>+</sup> MKs were sorted. The CD41<sup>+</sup>GFP<sup>+</sup> cells (mainly myeloid precursors) were obtained from cultures performed with four growth factors and were used as control. Hoechst 33342 (5  $\mu$ g ml<sup>-1</sup>, Sigma, France) was added in the medium 5 min before observation under a laser-scanning microscope (LSM). In some experiments, blebbistatin, an enantiomer mixture of blebbistatin ( $\pm$ ), (Merck, Germany) dissolved in dimethyl sulfoxide (DMSO; 12.5  $\mu$ M) or the same volume of diluents (DMSO) was added in the medium 30 min before observation. Photobleaching experiments were performed with a Zeiss LSM (510, Carl Zeiss, Germany) using a  $\times 63/1.4$  numerical aperture oil objective at 37°C with 5% CO<sub>2</sub> in air. Bleaching of the marked region was carried out at 100% laser power with 100 iterations. After photobleaching, images were acquired every second for 1 min. Fluorescence recovery was analysed with the Zeiss Image Examiner and Excel softwares.



**Lentiviral constructs.** The EF1 $\alpha$ -H2BGFP fragment from the BOS-H2BGFP vector (BD Pharmingen) was excised and subcloned into an HIV-derived lentiviral vector (pRRLsin-PGK-eGFP-WPRE; Genethon, France) in place of the phosphoglycerate kinase (PGK)-enhanced GFP sequence.

**shRNA cloning and lentiviral construction.** shRUNX1-containing lentivirus was previously described<sup>23</sup>. Two shMYH10, and control SCR containing lentiviruses were constructed as previously described<sup>23</sup>.

**Lentivirus production.** Lentivirus particles were prepared by transient co-transfection of human HEK293T cells with lentiviral vector plasmids (H2B-GFP<sup>6</sup>, shRNA MYH10, shRNA RUNX1, shRNA SCR), the packaging plasmid pCMV $\Delta$ R8.74 and the VSV-G protein envelope plasmid. JetPrime (Ozyme, France) was used for transfection according to the manufacturer's instructions. Lentivirus stocks containing about 10<sup>9</sup> infectious particles per ml were kept frozen at -70°C. Viruses stocks were thawed and immediately used.

**Cell transduction.** CD34<sup>+</sup> cells were cultured with TPO and SCF. Lentiviral particles were added at a concentration of 10<sup>7</sup> infectious particles per 1 $\times$ 10<sup>5</sup> cells for 12 h followed by a second transduction, then cells were cultured in presence of TPO<sup>23</sup>.

**Electroporation.** pEGFP-C3-MHCB plasmid encoding for wild-type MYH10 cDNA was kindly provided by Professor Shoshana Ravid (The Hebrew University, Israel). To test for specificity of the shMYH10 in COS7 cell line, four nucleotides localized in the region recognized by the shMYH10 were mutated in MYH10 cDNA by directed mutagenesis (3928nt gcagc<sup>T</sup>/<sub>A</sub>ag<sup>T</sup>/<sub>C</sub>cttgagtc<sup>T</sup>/<sub>C</sub>ca<sup>A</sup>/<sub>G</sub> 3949nt, NM\_005964.1). To study MYH10 overexpression in primary MK, the cytomegalovirus promoter was replaced by the EF1 $\alpha$  promoter. pEGFP-EF1 $\alpha$ -MHCB and the control (pEGFP-EF1 $\alpha$ ) were delivered into MK by nucleofection (Amaxa Inc., MD).

**BrdU analysis.** CD34<sup>+</sup> cells were cultured with TPO and SCF, and transduced by the shMYH10 or shSCR at day 0 and day 1 culture. GFP<sup>+</sup> cells sorted at day 4 of culture were incubated with 10  $\mu$ M BrdU for 1 h at 37°C. Staining was performed with the BrdU flow kit according to manufacturer's instruction (BD Pharmingen). BrdU incorporation was analysed in CD41<sup>+</sup> cells stained with propidium iodide on a LSRII (Becton Dickinson).

**Ploidy analysis.** Hoechst 33342 (10  $\mu$ g ml<sup>-1</sup>) was added in the medium of cultured MK for 1 h at 37°C. Cells were stained with directly coupled monoclonal antibodies: anti-CD41 APC and anti-CD42 PE (BD Biosciences) for 30 min at 4°C<sup>26</sup>. Ploidy was measured in the CD41<sup>+</sup>/CD42<sup>+</sup> cell population (for chemical inhibitors test) and in the CD41<sup>+</sup>/CD42<sup>+</sup>/GFP<sup>+</sup> population (after lentiviral transduction) by means of a LSRII flow cytometer equipped with three lasers (360, 480 and 560 nm excitation; Becton Dickinson). The mean ploidy of human MK was calculated by the following formula: (2N  $\times$  the number of cells at 2N ploidy level + ... + 64N  $\times$  the number of cells at 64N ploidy level/the total number of cells). The mean ploidy of murine MK was calculated in  $\geq$ 8N MK.

**Mitosis and endomitosis transition analysis.** Hoechst 33342 (4  $\mu$ g ml<sup>-1</sup>) was added in the medium for 1 h at 37°C before cell sorting at day 5 of culture. The 4N sorted cells were cultured for additional 72 h with and without blebbistatin (25  $\mu$ M) before ploidy analysis in the CD41<sup>+</sup> population described above.

**Western blot analysis.** Cells were lysed in Laemmli 2 $\times$  buffer, and the lysates were then gently sonicated on ice. Samples were boiled 5 min in loading buffer and subjected to SDS-polyacrylamide gel electrophoresis gel. After transfer, nitrocellulose membranes were blotted with the following antibodies: rabbit anti-MYH10, anti-MYH9 (Cell Signaling, Cat. Number 3404 and Number 3403, both diluted at 1:1000) and anti-HSC70 antibodies (Stressgen, used at 0.2  $\mu$ g ml<sup>-1</sup>) followed by horseradish peroxidase-linked secondary antibodies and Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation, USA).

**Immunofluorescence.** Immunofluorescence was performed on CD41<sup>+</sup> MK sorted at day 6 of human culture<sup>42</sup> or day 2 of murine culture derived from Lin<sup>-</sup> liver fetal cells. Cells were examined under the Zeiss LSM. The following antibodies were used: rabbit anti-MYH10 and anti-MYH9 antibodies (as above, both diluted at 1:100), mouse anti-MYH9 (Sigma, used at 10  $\mu$ g ml<sup>-1</sup>), rabbit anti-phospho-MLC2 Thr<sup>18</sup>Ser<sup>19</sup> (PPMLC2; gift from Dr J. M. Staddon)<sup>43</sup>, and mouse anti- $\alpha$  tubulin and anti- $\beta$  tubulin antibodies (Sigma, both used at 20  $\mu$ g ml<sup>-1</sup>). Appropriate secondary antibodies were conjugated with Alexa 488 or Alexa 546 (Molecular Probes). TOTO-3 iodide (Molecular Probes) was applied for nucleus staining.

**Live-cell imaging by confocal video microscopy.** CD34<sup>+</sup> cells were cultured 3 days in the presence of TPO and then transduced by H2B-GFP lentivirus as previously described<sup>6</sup>. After transduction, cells were cultured in the presence of TPO for 48–72 h. Cells were then seeded in glass bottom culture dish (MatTek) and were imaged under the Zeiss LSM using a  $\times$ 63/1.4 numerical aperture oil objective at 37°C with 5% CO<sub>2</sub> in air. Serial images were obtained at 3- or 5-min interval. Image analyses were performed with the Zeiss Image Examiner software.

**Quantitative real-time PCR.** The mRNA isolation, reverse-transcription, and RT-PCR analyses were performed as described<sup>23</sup>. The expression levels of all genes studied were expressed relatively to housekeeping genes, *PPIA* for human cells and *HPRT* for murine cells, where the *PPIA* level was not comparable to that of MYH10. Primer sequences are listed in Supplementary Table S1.

**ChIP and promoter activity assays.** ChIP assays were performed with a ChIP assay kit (Millipore Upstate Biotechnology) using the anti-RUNX1 antibody (8  $\mu$ g per sample, sc-65, Santa Cruz Biotechnology). Assays were performed using chromatin prepared from human MK as previously described<sup>23</sup>. Immunoprecipitated DNA was analysed on a PRISM<sup>7</sup> 7700 sequence detection system using SYBR<sup>®</sup> green (Applied biosystems) in duplicate. Primer sequences are listed in Supplementary Table S1.

**Luciferase reporter assay.** The human erythroleukemia cells were transfected with the reporter plasmids, pMYH10<sub>luc</sub> and pMYH10mut<sub>luc</sub>, and co-transfected with TK-Renilla reporter (Promega, France) for normalization of transfection efficiency. Transfected cells were harvested at 48 h post-transfection. A dual luciferase assay was performed according to the manufacturer's instructions (Promega, France). The luciferase activity was measured with an AutoLumat LB953 luminometer (Berthold).

**Statistics.** Statistical significance was determined by Student's *t*-test. A *P*-value <0.05 was considered as statistically significant. Error bars represent s.d. of duplicate or triplicate.

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### Author contributions

W. V., H. R. and Y. C. co-directed the studies. H. R., Y. C., L. L., D. B. designed the experiments. L. L., Y. C., A. J., P. R., N. D., H. R. and J. J. P. performed the cell biology experiments. D. B., H. R. and O. B. performed the molecular biology experiments. T. M. provided transgenic mice. L. L., Y. C., C. Legrand, D. J., C. Léon, C. G. and J. P. performed mouse experiments. W. V., H. R. and Y. C. wrote the manuscript with contribution of all other authors. L. L. and D. B. contributed equally to this work. H. R. and Y. C. have contributed equally to this work.

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**Carboxyl-terminal-dependent recruitment of nonmuscle myosin II to megakaryocyte contractile ring during polyploidization**

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# Carboxyl-terminal-dependent recruitment of nonmuscle myosin II to megakaryocyte contractile ring during polyploidization

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## Key Points

- C-terminal domain determines myosin II localization to the MK contractile ring and the specific role of NMII-B in MK polyploidization.

Endomitosis is a unique megakaryocyte (MK) differentiation process that is the consequence of a late cytokinesis failure associated with a contractile ring defect. Evidence from *in vitro* studies has revealed the distinct roles of 2 nonmuscle myosin IIs (NMIIs) on MK endomitosis: only NMII-B (MYH10), but not NMII-A (MYH9), is localized in the MK contractile ring and implicated in mitosis/endomitosis transition. Here, we studied 2 transgenic mouse models in which nonmuscle myosin heavy chain (NMHC) II-A was genetically replaced either by II-B or by a chimeric NMHCII that combined the head domain of II-A with the rod and tail domains of II-B. This study provides *in vivo* evidence on the specific role of NMII-B on MK polyploidization. It demonstrates that the carboxyl-terminal domain of the heavy

chains determines myosin II localization to the MK contractile ring and is responsible for the specific role of NMII-B in MK polyploidization. (*Blood*. 2014;124(16):2564-2568)

## Introduction

Megakaryocytes (MKs) are the naturally polyploid hematopoietic cells that give rise to platelets.<sup>1-3</sup> Polyploidization occurs by endomitosis, a process characterized by a cytokinesis failure related to a defect both in Rho activation and myosin II accumulation in the contractile ring.<sup>4-8</sup> Our previous studies<sup>8</sup> have revealed that nonmuscle myosin II (NMII) B (MYH10) is the only NMII present in the contractile ring during MK mitosis and endomitosis, but its expression is silenced during MK maturation both in human and mice megakaryopoiesis. This induces the switch from mitosis to endomitosis and allows MK polyploidization. Contrary to NMII-C, which is undetectable, NMII-A is well expressed during MK differentiation but is not recruited in the contractile ring whether MKs are in mitosis or endomitosis.<sup>6,8</sup> These results revealed that NMII-B and NMII-A must display distinct functions during MK polyploidization and cytokinesis. However, these results were obtained with *in vitro* studies and need to be confirmed in *in vivo* models. Furthermore, some myosin II functions are determined by their distinct cellular localizations, which are controlled by the carboxyl (C)-terminal domain, whereas others are reliant on the motor activity of their amino (N)-terminal domain.<sup>9-13</sup> Here, we have studied 2 mouse lines, in which the murine NMII-A (*Myh9*) heavy chain first coding exon was disrupted either by a complementary DNA (cDNA) encoding a green fluorescent protein (GFP) human NMII-B ( $A^{b^*}/A^{b^*}$  mice) or by a cDNA encoding a chimeric GFP human nonmuscle myosin heavy chain (hNMHC) II-AB

(the N-terminal domain of NMII-A fused to the C-terminal II-B domain,  $A^{ab}/A^{ab}$  mice).<sup>10</sup> Results obtained with these 2 models provide *in vivo* evidence for the specific role of NMII-B on MK polyploidization. They reveal also the important role of the C-terminal domain of NMII, which determines the specific NMII-B localization in the MK contractile ring.

## Study design

### Mice

$A^{b^*}/A^{b^*}$  and  $A^{ab}/A^{ab}$  mouse lines were generated in the Adelstein's laboratory.<sup>10</sup> The detail of mice and genotyping are presented in supplemental Figure 1 (available on the *Blood* Web site).

### In vitro cultures and sorting murine MK

$Lin^-$  cells were isolated by using the Lineage Cell Depletion Kit (Miltenyi Biotec, CA) and cultured in serum-free medium as previously reported<sup>8</sup> and as described in supplemental Methods.

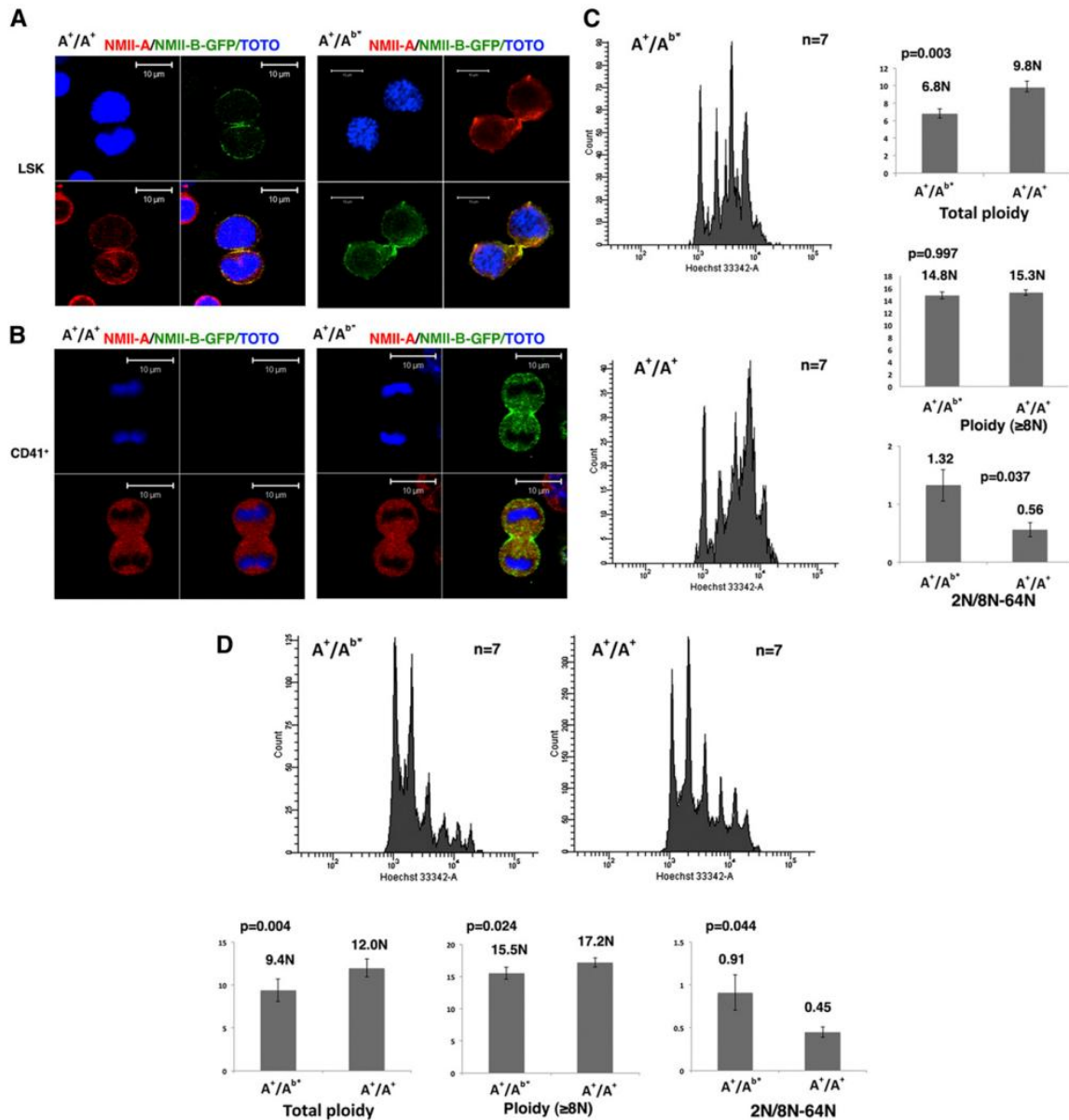
### $Lin^-Sca-1^+cKit^+$ (LSK) purification

$Lin^-$  bone marrow cells were isolated and labeled with PE-anti-Sca-1 (BD Pharmingen) and APC-anti-cKit (eBioscience) antibodies. LSK cells were sorted by an Influx flow cytometer.

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**Figure 1. The persistent NMII-B expression during MK differentiation induces a decreased MK polypldization.** (A) NMII-B (green) as well as NMII-A (red) are present in the contractile ring of LSK cells obtained from A<sup>+</sup>/A<sup>+</sup> WT mice (left) and A<sup>+</sup>/A<sup>b+</sup> heterozygote mice (right). Bars indicate 10 μm. At least 30 dividing MKs were observed with 6 heterozygous and WT mice in 3 independent experiments. (B) No specific accumulation of NMII-A and NMII-B is observed in the contractile ring of CD41<sup>+</sup> MKs from A<sup>+</sup>/A<sup>+</sup> WT mice (left). On the contrary, CD41<sup>+</sup> MKs derived from A<sup>+</sup>/A<sup>b+</sup> heterozygous mice (right) exhibited a specific localization of NMII-B-GFP (green) in the contractile ring, but without accumulation of NMII-A (red). Bars indicate 10 μm. At least 30 dividing MKs were observed with 6 heterozygotes and 6 WT mice in 3 independent experiments. (C) Persistence of NMII-B expression during MK differentiation decreases the bone marrow MK ploidy level (in vivo) (left). Histograms show the total ploidy, the ploidy level of MKs ≥8N, and the ratio between 2N and 8N to 64N of bone marrow MKs from A<sup>+</sup>/A<sup>b+</sup> heterozygous mice and control WT mice (A<sup>+</sup>/A<sup>+</sup>) (right). (D) Persistence of NMII-B expression during MK differentiation decreases the ploidy level of day-3 cultured MKs (in vitro) (top). Histograms show the total ploidy, the ploidy level of MKs ≥8N, and the ratio between 2N and 8N to 64N of cultured MKs derived from bone marrow of A<sup>+</sup>/A<sup>b+</sup> heterozygous mice and control WT mice (A<sup>+</sup>/A<sup>+</sup>) (bottom).

**In vivo and in vitro MK ploidy analysis**

Ploidy of MKs from mouse bone marrow or from culture (day 3 or day 4) was measured as previously reported.<sup>8</sup>

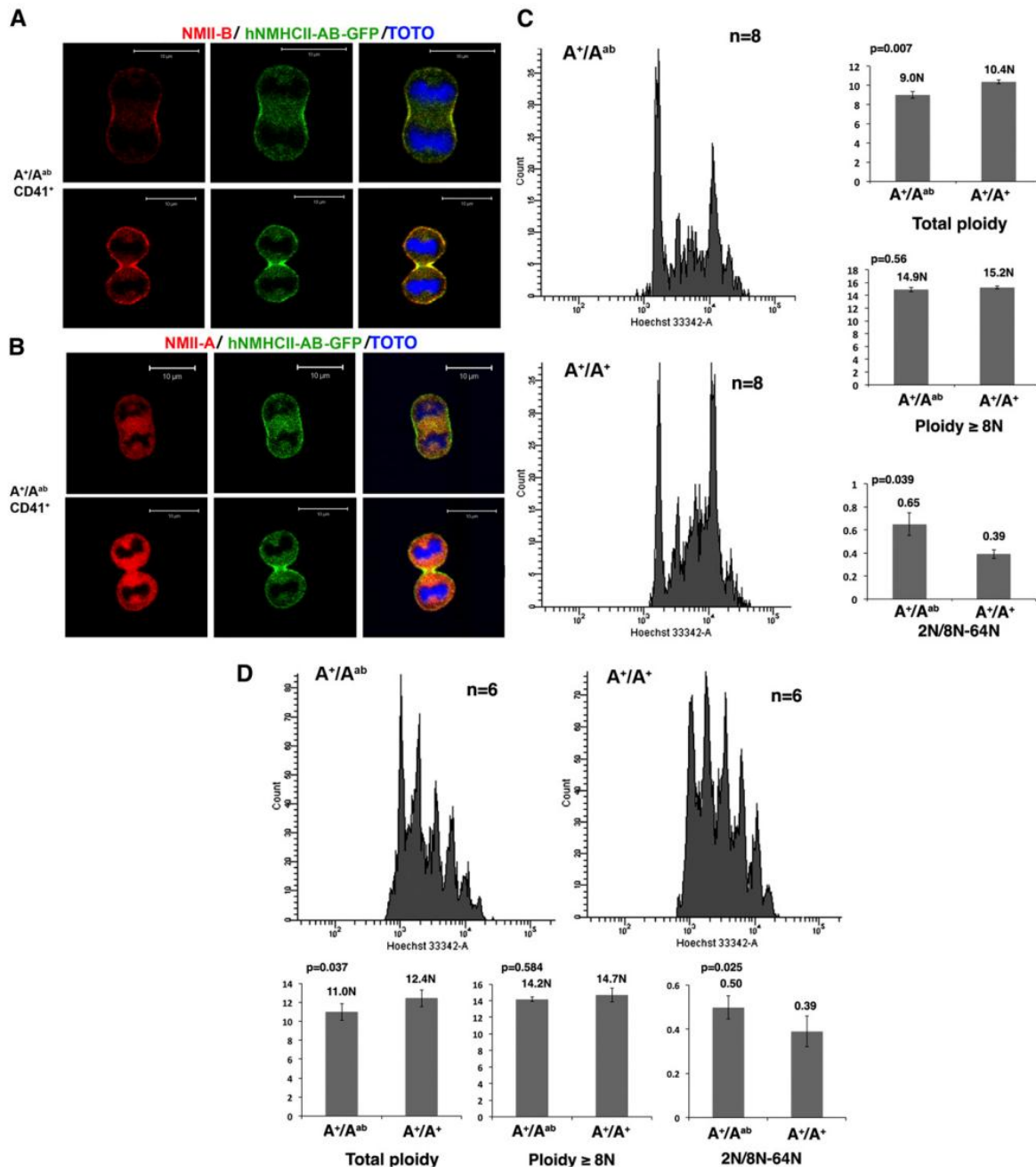
**Immunofluorescence**

Sorted LSK cells and CD41<sup>+</sup> MKs were grown 4 to 5 hours in serum-free medium containing recombinant murine thrombopoietin (TPO) and stem cell factor before performing immunolabeling as previously described.<sup>8,14</sup>

**Results and discussion**

**Persistence of NMII-B expression during MK differentiation induces a decreased MK polypldization**

The mouse line, in which the *NMII-A* first coding exon has been disrupted by cDNA encoding GFP-tagged *IIB*, was used to check the



**Figure 2. The C-terminal domain determines the myosin II-specific localization in the contractile ring and its role in polyploidization.** (A) CD41<sup>+</sup> MKs from A<sup>+</sup>/A<sup>ab</sup> heterozygous mice exhibit a specific localization of chimeric hNMHCII-AB protein in the contractile ring as revealed by an antibody against the C terminus of NMII-B (red) or by the GFP expression (green). Bars indicate 10  $\mu$ m. At least 30 dividing MKs were observed with 6 heterozygous and WT mice in 3 independent experiments. (B) An antibody against the C terminus of NMII-A revealed no specific localization of NMII-A tail (red) in the contractile ring of CD41<sup>+</sup> MKs from A<sup>+</sup>/A<sup>ab</sup> heterozygous mice. Bars indicate 10  $\mu$ m. At least 30 dividing MKs were observed with 6 heterozygous and WT mice in 3 independent experiments. (C) Expression of the chimeric hNMHCII-AB protein induces a decreased ploidy level of bone marrow MKs (left). Histograms show the total ploidy, the  $\geq$ 8N ploidy level, and the ratio between 2N and 8N to 64N of bone marrow MKs from A<sup>+</sup>/A<sup>ab</sup> heterozygous mice and control WT mice (right). (D) Expression of the chimeric hNMHCII-AB protein induces a decreased ploidy level of day-4 cultured MKs from A<sup>+</sup>/A<sup>ab</sup> heterozygous mice compared with WT control mice (A<sup>+</sup>/A<sup>+</sup>) (top). Histograms show the total ploidy, the  $\geq$ 8N ploidy level, and the ratio between 2N and 8N to 64N cultured MKs (bottom).

specific role of NMII-B on polyploidization. The complete substitution of NMII-A by II-B (homozygous mice) was lethal at embryonic day 9.5 to 10.5.<sup>10</sup> However, heterozygous A<sup>+</sup>/A<sup>b\*</sup> mice, which have persistent MYH10 expression associated with haploinsufficiency in MYH9

during MK differentiation, could provide an interesting model to study the specific role of NMII-B in MK polyploidization because mice with an MK-restricted *myh9* deficiency have normal polyploidization.<sup>15</sup> Therefore, if heterozygous (A<sup>+</sup>/A<sup>b\*</sup>) mice present with alterations in

MK ploidy level, this will be attributable to the abnormal MYH10 expression, but not the MYH9 haploinsufficiency.

First, in heterozygous ( $A^+/A^{b*}$ ) or WT (wild type,  $A^+/A^+$ ) LSK cells, both NMII-B and NMII-A were localized in the contractile ring (Figure 1A). Contrarily, in WT CD41<sup>+</sup> MKs, both NMII-A and NMII-B were absent from the contractile ring (Figure 1B, left panel, and supplemental Figure 2A). However, for heterozygous MKs, only NMII-B was still accumulated in the contractile ring (Figure 1B, right panel). Therefore, in mice as in humans, only NMII-B, but not NMII-A, has the property to accumulate in the MK contractile ring. However, because *MYH10* is silenced much faster during MK differentiation in mice compared with humans (supplemental Figure 2B),<sup>8</sup> no myosin II accumulates in the contractile ring of WT murine MKs.

We thus wondered if NMII-B persistence in the  $A^+/A^{b*}$  MKs could impair the switch from mitosis to endomitosis and inhibit MK polyploidization. In vivo analysis revealed a significantly lower MK ploidy level of heterozygous mice ( $A^+/A^{b*}$ ) (6.8N) than WT mice ( $A^+/A^+$ ) (9.8N,  $n = 7$ ,  $P = .003$ ). Interestingly, the heterozygous mice have more 2N MKs compared with WT control. The ratio between 2N MKs and 8N to 64N MKs was much higher in  $A^+/A^{b*}$  (1.32) vs WT (0.56,  $P = .037$ ) (Figure 1C). This implies that NMII-B persistence favors mitosis (4N returns to 2N) in the switch between mitosis/endomitosis. However, the ploidy level measured in MKs  $\geq 8N$  from  $A^+/A^{b*}$  mice (14.8N) was not lower than in WT (15.3N). Similar results were found in MKs obtained by in vitro culture (Figure 1D). Altogether these results suggest that once MKs enter endomitosis, MYH10 persistence does not alter the subsequent polyploidization. These results are in agreement with those obtained by Gao et al, which revealed a different regulation for the first cycle of polyploidization (2N to 4N) from the subsequent cycles.<sup>7</sup> MYH10 downregulation is thus only important for the first endomitosis cycle (ie, the switch from mitosis to endomitosis), as previously suggested by human MK culture.<sup>8</sup>

#### The C-terminal domain of MYH10 is responsible for the specific localization of myosin II in MK contractile ring

We then tried to understand why only NMII-B, but not NMII-A, is recruited to the contractile ring by studying another transgenic mouse line in which the N-terminal domain of GFP-tagged NMHCII-A is fused to the C-terminal II-B domain ( $A^{ab}/A^{ab}$  mice, chimeric hNMHCII-AB protein). The homozygous genotype is also lethal between embryonic day 11.5 and 12.5, and thus only heterozygous mice could be studied.<sup>11</sup>

We first checked if the chimeric protein accumulates in the contractile ring. Contrary to WT MKs, which presented neither NMII-A nor NMII-B in the midzone (supplemental Figures 2 and 3), a specific localization of hNMHCII-AB in the contractile ring was revealed either by GFP expression or by an antibody recognizing NMII-B C terminus (Figure 2A-B). This provides strong evidence that localization of myosin II-B in the MK contractile ring depends on the C terminus of the heavy chain. Indeed, NMII-B and NMII-A share 79% sequence amino acid identity, and the majority difference is within the C-terminal tail. As demonstrated by in vitro studies, the small portion of the extreme C terminus, which clusters significantly the differences in primary structure of NMII-A and NMII-B, is particularly important for NMII isoform-specific distribution and assembly (solubility).<sup>12,13</sup> Here, we provide the in vivo evidence

supporting the idea that the C-terminal region of NMII regulates the isoform-distinct distribution.

Moreover, in vivo and in vitro MK ploidy analyses showed that hNMHCII-AB accumulation in the contractile ring decreased total MK ploidy level without modifying the ploidy level of MKs  $\geq 8N$ . The 2N MK group in the heterozygous  $A^+/A^{ab}$  mice was also increased compared with WT control, which is confirmed by the ratio between 2N MKs and 8N to 64N MKs (Figure 2C, D). So, when the NMII-A N-terminal domain is properly localized in the MK contractile ring, it is capable of impairing the switch from mitosis to endomitosis, but in a less efficient manner than the NMII-B N-terminal domain, though both of them catalyze adenosine triphosphate hydrolysis. In fact, modifications in total ploidy level and the 2N MK/8N to 64N MK ratio observed between  $A^+/A^{ab}$  mice and their respective controls were less significant than those observed between  $A^+/A^{b*}$  mice and their controls. This may be related to the different kinetic properties of NMII-A and NMII-B, which increase the fitness of NMII-B for cytokinesis.<sup>10,11,16-18</sup>

Together, the work revealed the important role of myosin II during MK mitosis/endomitosis transition and the critical role of NMII-B silencing for polyploidization. A greater understanding of the recruiting mechanisms of NMII-A and NMII-B in the MK contractile ring will help us to better comprehend MK polyploidization and its regulation, as well as the normal cytokinesis process. This work also underscores the importance of the C terminus in NMII function. Mutations in both the C terminus and N terminus of the *NMII-A* gene can be observed in the inherited human macrothrombocytopenia, MYH9-related diseases. A better understanding of the different NMII domain functions will be helpful to better understand the molecular mechanism of MYH9-related diseases.

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#### Authorship

Contribution: I.B., J.P., C.L., S.B., A.R., and L.L. performed research; A.W. generated the 2 mouse lines; Y.C. designed and performed research; W.V. designed the research; and Y.C. wrote the paper with the help of W.V. and I.B.

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## 2 Results about karyokinesis in endomitosis

### Introduction

Endomitosis is a unique MK differentiation process where cells undergo repeated rounds of DNA replication, but fail to execute cytokinesis. This mitotic process ends prematurely due to aborted cytokinesis that leads to mature MKs that contain a DNA content of up to 128N, in contrast to normal diploid (2N) cells (Bluteau et al., 2009; Chang et al., 2007b; Zimmet and Ravid, 2000). The development of polyploid MKs results from the failure of cytokinesis associated with a contractile ring defect and absence of myosin II accumulation in the contractile ring (Gao et al., 2012; Geddis et al., 2007; Geddis and Kaushansky, 2006; Lordier et al., 2012a; Lordier et al., 2008). However, usually a defect in cytokinesis leads to multinucleated cells whereas polyploid MKs exhibit a single multinucleate nucleus. Herein, we have observed that a defect in karyokinesis is also present during endomitosis, which may explain the presence of a single nucleus.

### Results

Using MK derived from human CD34<sup>+</sup> cells, we studied the chromosome kinetics by DNA staining and nuclear membrane dynamic by laminA/C immunostaining. LaminA/C is an important component of the nuclear envelope, which is disassembled during the first stage of mitosis and reassembled around the chromosomes at the last stages of mitosis (Margalit et al., 2005). Using confocal microscopy, we observe that the nuclear membrane of endomitotic MKs remains disassembled until anaphase. At late telophase, the nuclear membrane starts to reassemble, but the nuclear membrane is not complete at the end of endomitosis leading to a single polylobulated nucleus after endomitosis. Next, by staining with TOTO and antibodies against laminin, we observe the presence of nucleoplasmic bridges (NPM) between segregated chromosomes during endomitosis. The histone 3 phosphorylation is spatially and temporally coordinated with mitotic chromosome condensation (Hans and Dimitrov, 2001). Therefore, we confirm the presence of NPM in endomitosis through the observation of a phospho-histone 3 signal expressed in the DNA bridges. Furthermore, by double staining, we have found that Phospho-H3 and laminA/C colocalize around segregated chromosomes in telophase MKs.

Defects in Fanconi proteins and BLM are one of the main causes of NPM (Naim and Rosselli, 2009a, b). WB data show that BLM is expressed in MKs, while immunofluorescence data show that both

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FANCD2 and BLM are present in NPM during MK endomitosis, suggesting these proteins are expressed and that they may be partially functional in MKs.

## **Conclusion**

Polyploidization is limited to specific cell types, such as MKs (Ravid et al., 2002). Polyploidy and aneuploidy are also common phenomena present in cancer cells (Storchova and Pellman, 2004). It is believed that aneuploidy always follows the re-entry in mitosis of tetraploid cells. By immunostaining with LaminA/C, TOTO and phospho-histone 3, we have shown the NPMs are present in dipolar and multipolar endomitosis. Taken together, we have shown the presence of a defect in karyokinesis during MK endomitosis characterized by the presence of NPMs, which are not completely resolved at the end of telophase. This defect increases with ploidy and may be involved in the formation of a single multilobulated nucleus in polyploid MKs. Therefore, it is important to study the precise mechanisms responsible for the presence of these NPM in MK endomitosis. Interestingly, we have shown that FANCD2 and BLM are present in these NPM and are expressed in MKs. It remains to understand at which level this pathway is altered during endomitosis because it is one of the most important pathway regulating NPM as well as Aurora B. It will be interesting to study if there are increased defects in nuclear bridges in the Fanconi disorders, which are associated with thrombocytopenia and if they may leads to a blockage in DNA replication followed by apoptosis, which may explain the thrombocytopenia.

## Presence of a defect in karyokinesis during megakaryocyte endomitosis

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<sup>†</sup>These authors contributed equally to this work.

**Keywords:** megakaryocyte, endomitosis, polyploidy, karyokinesis, cytokinesis

**Abbreviations:** MK, megakaryocyte; NPM, nucleoplasmic bridges

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**Keywords:** megakaryocyte, endomitosis, polyploidy, karyokinesis, cytokinesis

**Abbreviations:** MK, megakaryocyte; NPM, nucleoplasmic bridges

Megakaryocyte is the naturally polyploid cell that gives rise to platelets. Polyploidization occurs by endomitosis, a process corresponding to a late failure of cytokinesis with a backward movement of the daughter cells. Generally, a pure defect in cytokinesis produces a multinucleated cell, but megakaryocytes are characterized by a single polylobulated nucleus with a  $2^N$  ploidy. Here, we show the existence of a defect in karyokinesis during the endomitotic process. From late telophase until the reversal of cytokinesis, some dipolar mitosis/endomitosis and most multipolar endomitosis present a thin DNA link between the segregated chromosomes surrounded by an incomplete nuclear membrane formation, which implies that sister chromatid separation is not complete. This observation may explain why polyploid megakaryocytes display a single polylobulated nucleus along with an increase in ploidy.

## Introduction

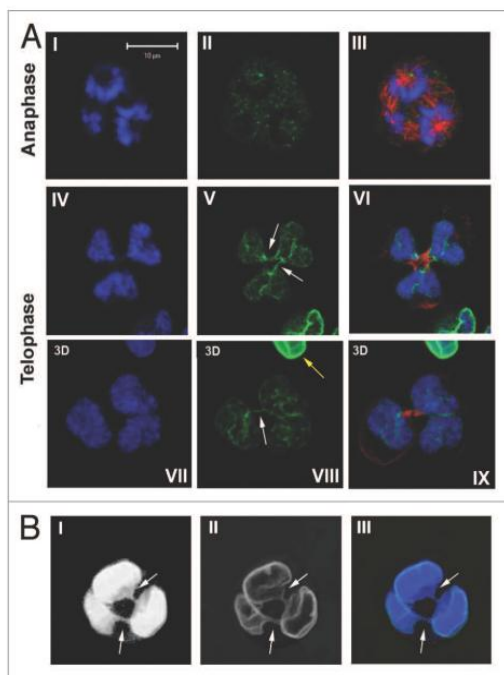
Platelets are continuously produced from megakaryocytes (MK) localized in the bone marrow. During differentiation, instead of dividing one cell to two cells, MKs replicate their DNA content without cell division and become polyploid by a process called endomitosis. Previous works showed that endomitosis corresponded to a failure of late cytokinesis.<sup>1,2</sup> A simple defect in cytokinesis generally induces multinucleated cells, whereas MK possess a single polylobulated nucleus with a  $2^N$  DNA content, which suggests there exists also a karyokinesis defect. By analyzing the nuclear kinetics during endomitosis, we observed the presence of nucleoplasmic bridges (NPM) in most multipolar endomitosis from telophase until the cytokinesis failure. These results suggest MK endomitosis presents a karyokinesis defect that probably favors a single polylobulated nucleus formation.

## Results

Recent studies have shown that MK endomitosis is an incomplete mitosis characterized by a cytokinesis failure<sup>1,2</sup> related to both a defect in Rho activation<sup>2,3</sup> and the absence of Myosin IIB accumulation in the contractile ring.<sup>4</sup> In this work, we tried to understand if a defect in karyokinesis was also present. We studied the chromosome kinetics by DNA staining and nuclear

membrane dynamics by laminA/C immunostaining. LaminA/C is one important component of the nuclear envelope, which is disassembled during the first stage of mitosis and reassembled onto the chromosomes at the last stages of mitosis.<sup>5</sup> During MK endomitosis, the nuclear kinetics is identical to what is observed during a classical mitosis until telophase; nuclear membrane breakdown began in prometaphase of endomitosis, as previously described.<sup>6,7</sup> The nuclear membrane remained disassembled until anaphase and then started to reassemble around the segregated chromosomes at late telophase (Fig. 1A, I–VI). However, the lamin signal remained very weak until the end of endomitosis when compared with the control cells in interphase (Fig. 1A, VII–IX). The nuclear membrane assembly was incomplete in agreement with the presence of condensed chromosomes, which explains why the nuclear membrane could still fuse to form a single polylobulated nucleus despite the late failure of cytokinesis (Fig. 1B). Some (15%) dipolar mitosis/endomitosis and most multipolar endomitosis (73%) exhibited a thin extension of lamin between segregated chromosomes from the beginning of nuclear membrane formation until the end of endomitosis (149 dipolar and 20 multipolar cells examined in three independent experiments) (Figs. 1A and B and 2B; see also Fig. S1). The percentage of MK presenting lamin extension in multipolar endomitosis was much higher than in the erythro-megakaryocytic cell line UT-7 (6%), which does not polyploidize in presence of GM-CSF.

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**Figure 1.** Presence of a defect in karyokinesis during endomitosis. (A) I–III: Until anaphase, lamin immunostaining shows that the nuclear membrane of endomitotic MK remains disassembled. IV–VI: At late telophase, the nuclear membrane starts to reassemble. IV–IX: The nuclear membrane is not complete at the end of endomitosis. White arrows, Lamin between segregated chromosomes; yellow arrow, one control cell not in mitosis. (B) A typical polylobulated MK, which possesses a single polylobulated nucleus after endomitosis. I, TOTO; II, lamin A/C; III, lamin A/C (green) and TOTO (blue); white arrows, DNA and lamin link between segregated chromosomes.

This abnormality corresponds to nucleoplasmic bridges (NPM), as staining with TOTO revealed the presence of DNA. Sometimes, the connection was so thin that TOTO staining was not obvious but could be observed after overexposing the images (Fig. 2A). This presence of DNA was confirmed by the presence of phospho-histone 3 (phospho-H3) in the bridges. The histone 3 phosphorylation is spatially and temporally coordinated with mitotic chromosome condensation.<sup>8</sup> The presence of phospho-H3 in the bridges confirmed the existence of DNA linking among the separating chromosomes (Fig. 2C and D). Significant 4N MKs in interphase presented two nuclei (19.5–46.6% in six experiments) suggesting in this case a pure cytokinesis defect. In contrast, the great majority of 8N MKs presented a single polylobulated nucleus (> 85% in six experiments). These results parallel the differences observed in the percentage of NPM in dipolar and multipolar endomitosis (Fig. 2B).

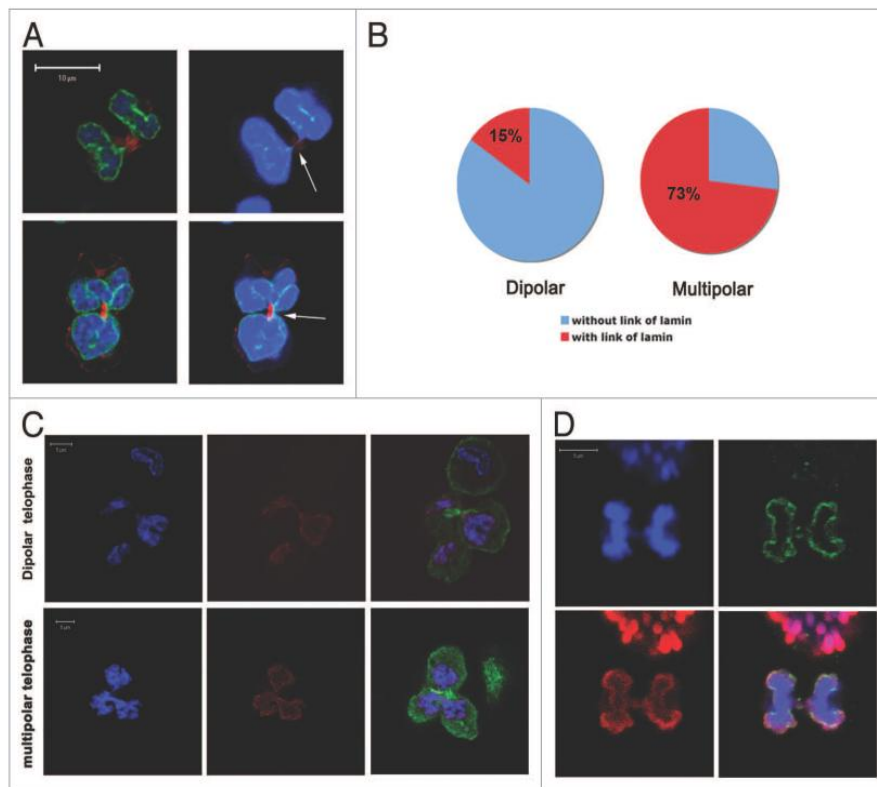
As Fanconi proteins and BLM play an important role in resolving these DNA links,<sup>9,10</sup> we therefore studied whether they were expressed in MKs. The western blot revealed BLM expression in MKs (Fig. 3A). Furthermore, FANCD2 and BLM were

detected in NPM during MKs endomitosis as in classic mitosis (Fig. 3B and C). This pathway hence seems to be at least partially functional in MKs.

Altogether, we have demonstrated a karyokinesis defect during MK endomitosis characterized by the presence of NPM, which are not completely resolved at the end of telophase. This defect increases with ploidy and may be involved in a single multilobulated nucleus formation in polyploid megakaryocytes. Finally, it will be important to determine the precise mechanisms responsible for the presence of these NPM in MK endomitosis.

## Discussion

This work revealed the persistence of NPM at telophase in most multipolar MKs endomitosis, but only in a few dipolar mitosis/endomitosis. In addition, a large percentage of 4N MKs was truly binucleated, whereas the majority of higher ploidy MKs was mononucleated. This is in agreement with previous observations showing the presence of small binucleated MKs in cord blood cultures.<sup>11</sup> After 4N, an increased defect in karyokinesis was seen with the presence of NPM in the great majority of endomitotic MKs. This may be related to the absence of a true anaphase B with a limited endomitotic spindle elongation<sup>6,7</sup> and the asymmetrical chromosome segregation in multipolar endomitosis.<sup>12</sup> The presence of unresolved NPM may also favor the cytokinesis defect, because abscission cannot occur when DNA linkage persists, which could explain why polyploid MKs may rarely return to mitotic cycles.<sup>13,14</sup> In addition, it could also explain why polyploid MKs have a single nucleus, because if the NPM did not resolve, the nuclear membrane is reassembled around unseparated DNA masses to form the link between segregated chromosomes. In human epithelial cells, it has been observed that cells showing chromatin bridges exhibited different fate: in most cases, bridge breakage occurred and cells eventually completed division; in contrast, most cells with unbroken bridges did not complete abscission, but resulted in two daughter cells connected with an ultra-fine DNA string, and the remaining cell became tetraploid with two nuclei (binucleated) coupled with an unbroken chromatin bridge.<sup>15</sup> The latter possibility is similar to what is observed in MKs. Several pathways are implicated in resolution of DNA bridges at the end of mitosis. The pathway best described concerns Aurora B activation. This kinase remains activated when chromatin is trapped between dividing cells. This activation promotes proper chromosome segregation by delaying abscission and prevents tetraploidization. If Aurora B activation is inhibited before the bridge resolved, furrow regression is observed, leading to cells with two nuclei connected by DNA bridges.<sup>16</sup> In MKs Aurora B activity could be observed at the end of MK endomitosis, but we cannot exclude that its activation is not sufficiently sustained to resolve the DNA bridges during most MK endomitosis or that another pathway is indispensable.<sup>17</sup> In favor of this last hypothesis, it has been recently suggested that sister chromatid disjunction at the NPM origin is frequently incomplete in human cells, but Fanconi proteins and BLM play a cooperative role in resolving these DNA links.<sup>9,10</sup> However, this pathway seems to be at least partially functional in MKs, as both



**Figure 2.** Presence of nucleoplasmic bridges (NPM) during endomitosis. **(A)** Presence of a DNA link between segregated chromosomes. **(A and B)** Tubulin  $\alpha$ , red; lamin A/C, green; TOTO, blue. Bar, 10  $\mu$ M. **(B)** Percentage of mitosis/endomitosis exhibits NPM. **(C)** Phospho-H3 is present in the NPM. Phospho-H3, red; Tubulin, green; TOTO, blue. Bar, 5  $\mu$ M. **(D)** Co-localization of Phospho-H3 and lamin around segregated chromosomes during MK telophase. Phospho-H3, red; lamin A/C, green; TOTO, blue. Bar, 5  $\mu$ M.

proteins were present in NPM. As NPM are induced by replicative stress, it is possible that polyploidization is associated to a replicative stress, such as *c-myc* activation,<sup>18</sup> leading to increased DNA linkage, which cannot be completely resolved by Aurora B or the FANCD1 and BLM pathways. However, we cannot exclude that in some endomitotic MK, the DNA bridge could be finally resolved because it was reported that polyploid MK could occasionally complete cytokinesis and divided into separated polyploid daughter cells.<sup>13,14</sup>

Altogether, we have demonstrated a defect in karyokinesis during MK endomitosis characterized by the presence of NPM, which are not completely resolved at the end of telophase. This defect increases with ploidy and may be involved in the formation of a single multilobulated nucleus in polyploid megakaryocytes. Finally, it will be important to determine the precise mechanisms responsible for the presence of these NPM in MK endomitosis.

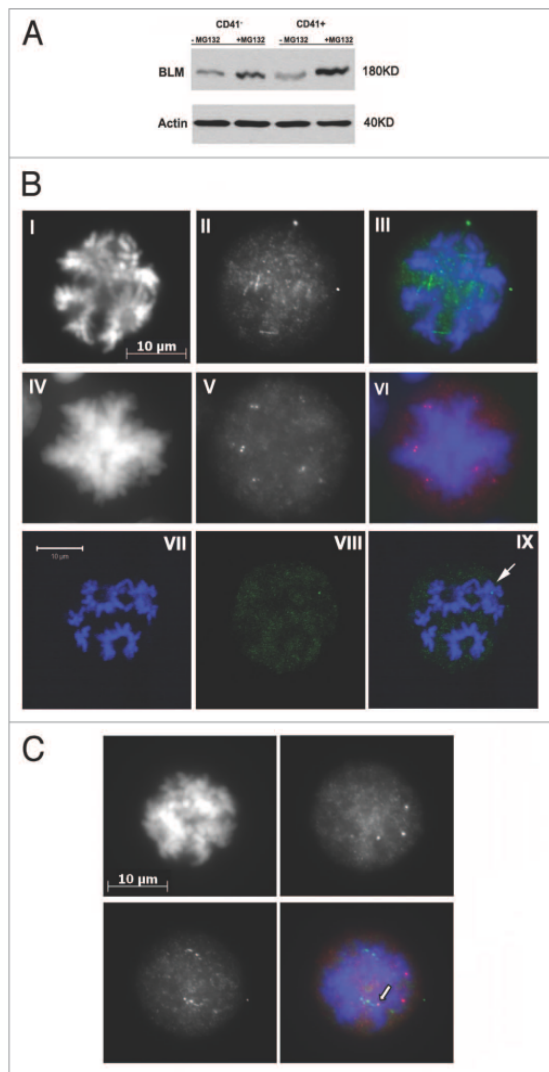
### Materials and Methods

**In vitro culture of megakaryocytes derived from human CD34<sup>+</sup> cells.** CD34<sup>+</sup> cells were isolated by an immunomagnetic

technique (Miltenyi Biotec) from the bone marrow of healthy subjects and cultured as described.<sup>2</sup>

**Separation of 4N and 8N MKs.** Hoechst 33342 (10  $\mu$ g/mL; Sigma) was added in MKs culture for 1h at 37°C. Cells were stained with the anti-CD41APC monoclonal antibody (MoAb) (PharMingen) for 30 min at 4°C. 4N and 8N CD41<sup>+</sup> cells were individually sorted as described.<sup>2</sup>

**Immunofluorescence.** Fixation and immunofluorescence were performed on CD41<sup>+</sup> MKs sorted at day 6 and cultured overnight before the experiments, as described previously.<sup>2</sup> Cells were examined under a LSM 510 (Carl Zeiss) confocal microscopy with a X63/1.4NA oil objective or an Olympus Atimes X70 fluorescence microscope with a X100/1.3 oil objective. The following antibodies were used: rabbit anti- $\alpha$  tubulin (1:100, ABR, CO), mouse anti-lamin A/C (1:100), mouse anti-phospho histone H3 (Ser10) (1:100, Millipore), mouse anti- $\alpha$  tubulin and mouse anti- $\beta$  tubulin MoAbs (Sigma, Saint Quentin Fallavier), rabbit anti-FANCD2 (1:1,000, Abcam) and goat anti-Bloom protein (BLM) (1:150, Santa Cruz). Appropriate secondary antibodies were conjugated with Alexa 488, Alexa 546 or Alexa 592 (Molecular Probes, Invitrogen). TOTO-3 iodide or DAPI (Molecular Probes) was



**Figure 3.** FANCD2 and BLM are present normally during MK endomitosis. (A) Western blot reveals BLM expression increased by proteasome inhibition. (B) I–III: BLM (green) localized to ultrafine anaphase bridges. IV–VI: FANCD2 (red) localized as spots on mitotic chromosomes. VII–IX: The arrow indicates one FANCD2 (green) spot on separating chromosomes during anaphase of endomitosis. (C) The arrow indicates a subset of BLM-stained bridges (green) connected to FANCD2 (red) spots on separating chromosome sets at anaphase. Bars, 10  $\mu$ M.

applied for nucleus staining. Three-dimensional images analyses were performed with Zeiss Image Examiner software.

**Cell line.** UT-7 cells were cultured in DMEM medium (Invitrogen) supplemented with 10% FBS and 2.5 ng/mL of hGM-CSF (Leucamax).

**Western blot.** Western blot was performed on CD41<sup>+</sup> and CD41<sup>-</sup> cells sorted at day 6 of MK culture as described.<sup>3</sup> MG132 (10  $\mu$ M) was added to some samples before cell collection.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Acknowledgments

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#### Supplemental Materials

Supplemental materials may be downloaded here:  
[www.landesbioscience.com/journals/cc/article/22712/](http://www.landesbioscience.com/journals/cc/article/22712/)

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### 3 DIAPH1 and proplatelet formation by megakaryocytes

#### Introduction

There is now strong evidence that actomyosin and MT cytoskeleton play an important role in MK differentiation, particularly in polyploidization, PPF and platelets release. Formins are a large family of proteins that are highly conserved. They are multidomain proteins that are involved in regulation of the dynamics and organization of both actin and MT cytoskeletons (Chesarone et al., 2010; Wallar and Alberts, 2003). Most formins are Rho-GTPase effector proteins (Kuhn and Geyer, 2014). DIAPH1 is one of RhoA effector that is encoded by the gene *DIAPH1* (also known as *Drf1*) and is located on chromosome 5q31.3, a chromosomal region that may often undergo a deletion in MDS. The phenotype of *drf1* KO mice, particularly the remarkable proliferation of hematopoietic progenitors, suggests that DIAPH1 may act as a tumor suppressor gene (Eisenmann et al., 2009; Peng et al., 2007).

As one member of formin family, DIAPH1 is a mammalian homolog of *Drosophila diaphanous* gene that works as an effector of the small Rho GTPase and regulates the actomyosin cytoskeleton as well as MTs. It contains the Rho-binding domain in the amino terminal and two distinct regions of formin homology, FH1 in the center of the molecule and FH2 in the carboxy-terminus. DIAPH1 coordinates MTs and the actin cytoskeleton through its FH2 and FH1 domains respectively, which induce and regulate the MT and actin polymerization. Thus DIAPH1 appears as a good candidate for playing a major function in MK differentiation and PPF.

In this work, we focus on the study of the role of DIAPH1 in MK proplatelet formation.

#### Results

First, we analyzed the expression of DIAPH during MK differentiation (see the article Figure 1). Relative to housekeeping gene HPRT, qRT-PCR analysis show that the expression of *DIAPH1* increases during MK differentiation, while *DIAPH2* and *DIAPH3* expressions decrease. Using the heat shock protein (HSC70) as protein loading control, WB analysis show the same pattern of expression for the protein as demonstrated for the mRNA. By immunofluorescence analysis, we find that DIAPH1 co-localizes with both actin and tubulin along the cytoplasm membrane in mature MKs, as well as along proplatelets, particularly in the swelling and the tips (Figure 8). The expression and localization analysis suggest that DIAPH1 may play a more important role than DIAPH2 and DIAPH3 in late stages of MK differentiation, particularly in the process of platelet formation.

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In order to study the role of DIAPH1, two shRNA (sh7, sh8) were constructed and vectorized in lentiviruses for functional studies (see the article Figure 2A-C). WB and qRT-PCR analysis show that these shRNA elicit a 40-60% decrease in DIAPH1 expression level compared to the control SCR, without changes in expression level of DIAPH2 and DIAPH3. The percentage of MKs bearing PPF was daily quantified during three days. The presence of at least one pseudopodial extension was considered as a PPF. DIAPH1 knockdown leads to a significant increase in PPF compared to the control SCR at day 13 and day 14 of culture (see the article Figure 2D-E). However, knockdown of DIAPH1 by shRNA decreases formation of actin stress fibers on collagen and the size of the stress fibers, which are thinner compared to the controls (see the article Figure 3A-B). This indicates that DIAPH1 is involved in the Rho-mediated assembly of actin stress fibers.

Furthermore, tubulin polymerization assays were performed and analyzed by immunofluorescence and WB. It is known that stable MTs accumulate a post-translational modification of tubulin called detyrosinated tubulin (or Glu-Tubulin), whereas dynamic MTs contain predominantly tyrosinated tubulin (Tyr-Tubulin) (Gundersen et al., 1984; Westermann and Weber, 2003). The ratio between Glu-Tubulin and Tyr-Tubulin is increased in MKs infected by DIAPH1 shRNA compared to control (see the article Figure 3F), indicating that loss of DIAPH1 increases MT stability. In addition, DIAPH1 knockdown MKs show more obvious MT elongation after nocodazole treatment compared to the SCR control treated MK (see the article Figure 3D-E and Figure 9).

To confirm the role of DIAPH1 induced by the shRNA strategy, a constitutive active form of mouse DIAPH1 (mDia1 $\Delta$ N3) was expressed in human MKs (see the article Figure 4A-B). In contrast to DIAPH1 knockdown, PPF is decreased in MKs expressing mDia1 $\Delta$ N3 compared to the control (empty vector infected MKs) (see the article Figure 4C). Remarkably, proplatelets of MKs expressing mDia1 $\Delta$ N3 often present a more simple structure with less branching and shorter extensions, which lead to a clear decrease in proplatelet area (see the article Figure 4D). Stress fiber formation is increased in MK expressing mDia1 $\Delta$ N3 compared to the control (see the article Figure 5A), while MT polymerization is decreased after nocodazole treatment (see the article Figure 5B). Immunofluorescent and WB analysis reveal that mDia1 $\Delta$ N3 expression decreases the ratio between Glu-Tubulin and Tyr-Tubulin, which indicates a loss in MT stability (see the article Figure 5C).

To demonstrate that the increase in PPF observed in DIAPH1 knock down MKs is specific, but not an off-target effect of the shRNAs, we perform rescue experiments by expressing mDia1 $\Delta$ N3 (see the article Figure 6A-B). For these experiments, we use the sh8-shRNA because this shRNA targets the endogenous DIAPH1, but not mDia1 $\Delta$ N3. The sh8-shRNA was cloned in a lentiviral vector containing as reporter gene, the cherry and a non-relevant shRNA SCR was also cloned in the same

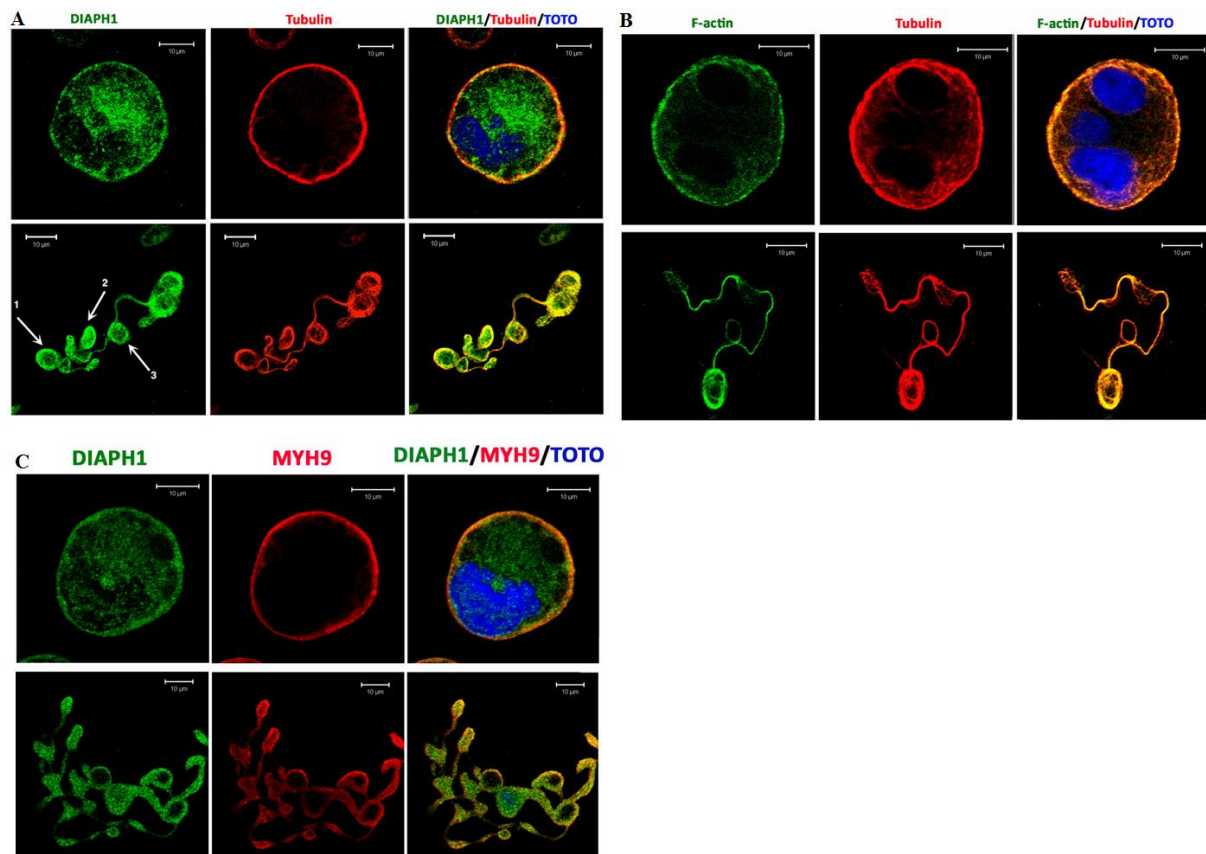
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vector to be used as a control. Double-infected MKs show a rescue in PPF, meaning in this case a decrease in PPF in comparison to DIAPH1 knockdown (see the article Figure 6C).

The RhoA/ROCK pathway plays a negative role in PPF by inducing myosin II activation (Chang et al., 2007a). Herein, our results imply that DIAPH1 may also play a negative role in PPF by remodeling the actin and MT cytoskeleton. As effectors of RhoA, DIAPH1 shows a similar localization as MYH9 in mature MK and in PPF suggesting that it can cooperate with myosin IIA to regulate actomyosin and MT dynamics during PPF. Inhibition of ROCK by Y27632 (10 $\mu$ M) or myosin II by blebbistatin (25 $\mu$ M) disturbs MK stress fiber formation, but increases tubulin polymerization by tubulin polymerization assays (see the article Figure 6D-E). WB results indicate that inhibition of ROCK and myosin II by Y27632 and Blebbistatin increases the ratio between Glu-tubulin/Tyr-tubulin (see the article Figure 7A), which means that ROCK and myosin II inhibit PPF by simultaneously increasing contractile force and reducing the stability of MTs.

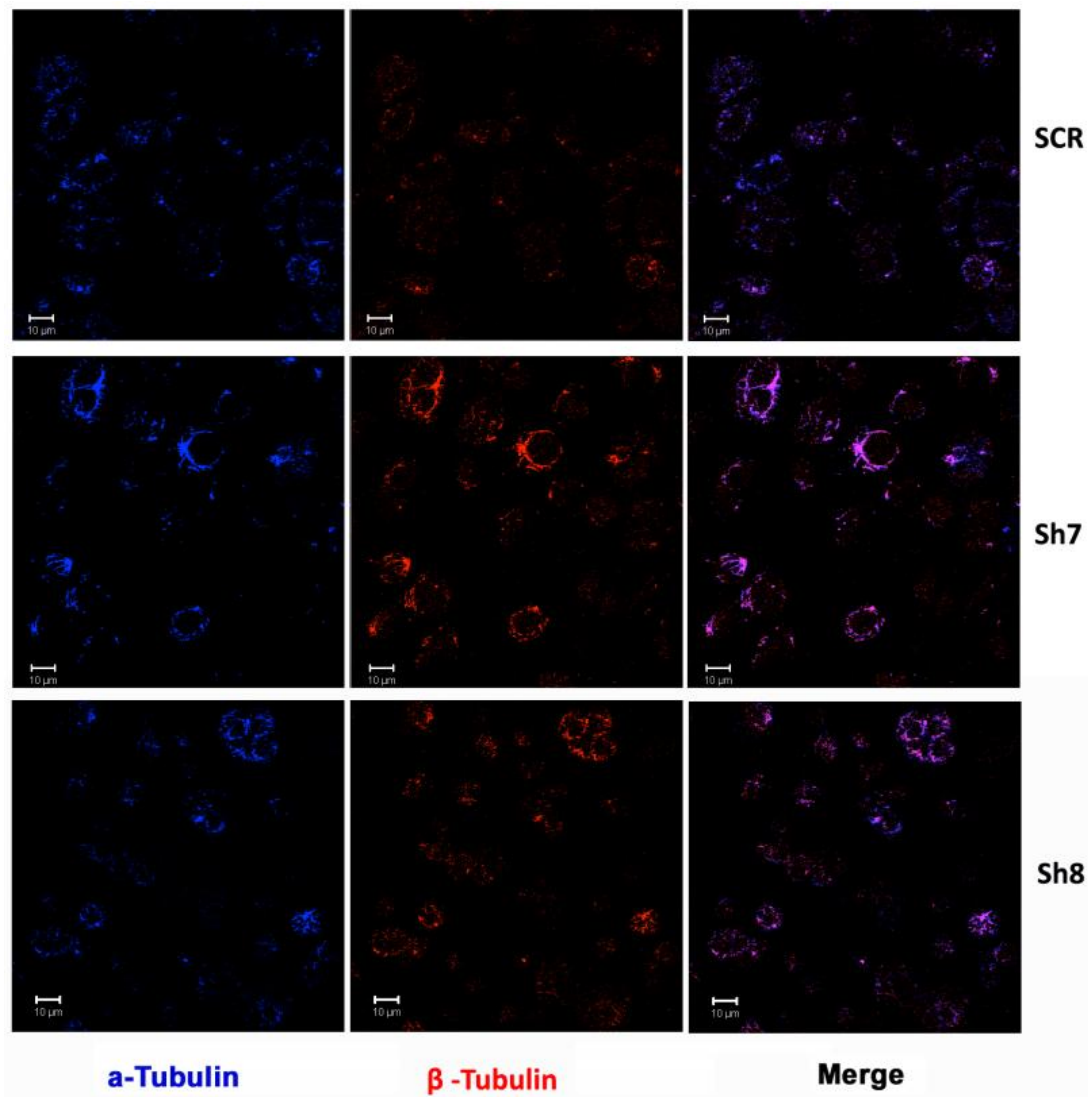
We then studied the relationship between Rho/ROCK/myosin and Rho/DIAPH1 pathways by treating MKs with ROCK and myosin II inhibitors (Y27632 and blebbistatin) after infection with the shRNA directed against DIAPH1. The increase in PPF observed in sh7- or sh8-transduced MK is further augmented when ROCK or myosin II inhibitors are added alone or the two in association (see the article Figure 7B). This double inhibition of both ROCK/myosin and DIAPH1 pathways further increases MT stability compared to a single DIAPH1 inhibition by analyzing the ratio between Glu-T and Tyr-T (see the article Figure 7E). Moreover, MKs double transduced by a shRNA against DIAPH1 cloned in a lentiviral vector containing cherry as reporter (sh8) and a shRNA against MYH9 cloned in a lentiviral vector containing GFP as reporter (shMYH9) show an increased PPF compared to a single knockdown (see the article Figure 7C). These data suggest that Rho/ROCK/myosin and Rho/DIAPH1 pathways have an additive role in PPF.

Finally, we performed additional experiments to test if DIAPH1 is implicated in platelet release by testing the number of platelets produced in culture (Figure 10). The number of platelet released into the culture supernatant from MK infected either by shRNA against DIAPH1 or by mDia1 $\Delta$ N3 was measured by flow cytometry. Three independent experiments were performed, and results reported to the different controls assigned as 1.0. We observe that *DIAPH1* knockdown just slightly increases platelet production *in vitro* (Figure 10A), while overexpression of the active form mDia1 $\Delta$ N3 results in a significant decrease in platelet production (Figure 10B). These results suggest that DIAPH1 probably contributes to the platelet release process.



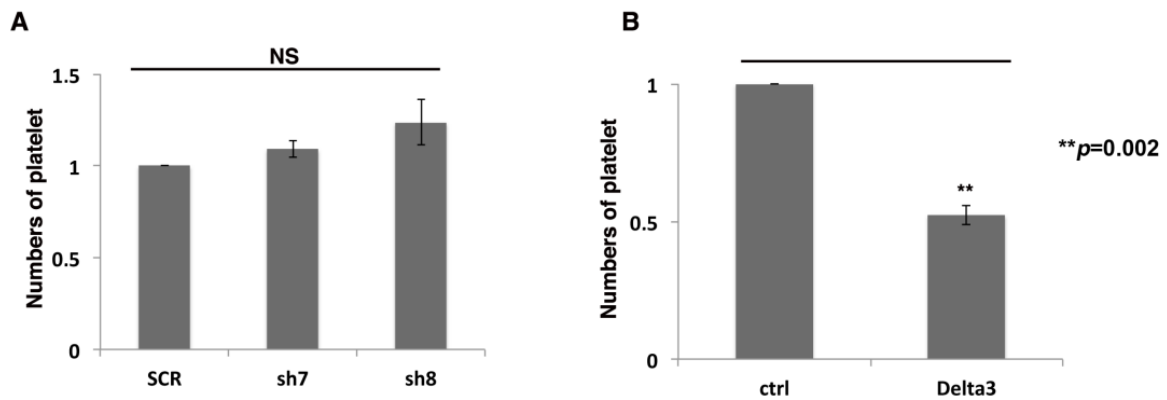
**Figure 8:** Localization of DIAPH1 during MK differentiation.

IF revealed the presence and co-localization of DIAPH1 with F-actin, tubulin and MYH9 in mature MK (8A. 8B. 8C. upper images) and proplatelet (8A. 8B. 8C. lower images). Cells were stained for DIAPH1 (green), F-actin (green), tubulin (red), MYH9 (red) and DNA was stained with TOTO (blue).



**Figure 9:** DIAPH1 knockdown increases tubulin polymerization and stability

CD41+ MK infected by SCR, sh7 or sh8 were plated on collagen-coated slides for 1h and then treated for another hour with nocodazole. Tubulin polymerization assay was performed 10 min after washing. MK infected with sh7 and sh8 show increased tubulin polymerization compared to the SCR control. Confocal microscopy image is taken for one zone showing the general tubulin polymerization state of cells.  $\alpha$ -tubulin, blue;  $\beta$ -tubulin, red.



**Figure 10:** Effect of DIAPH1 in platelet release *in vitro*

Number of platelet released into the culture supernatant from the MK infected either by shRNA-DIAPH1 (A) or by mDia1 $\Delta$ N3 (B) was measured by flow cytometry. The graph showed the relative number compared to the different controls assigned as 1.0 (3 independent experiments). *DIAPH1* knockdown only slightly increased platelet production *in vitro* but overexpression mDia1 $\Delta$ N3 induced a significant decrease in platelet production.

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## Conclusion

PPF is associated with morphological changes that require profound actin and MT cytoskeleton reorganization. There is strong evidence that PPF is mainly regulated by MTs (Italiano et al., 1999a), and that actin is involved in the branching structures that serve to increase the number of proplatelet ends and thus the platelet number. Although MTs are constantly assembling in proplatelets, the continuous MTs polymerization is required for proplatelet enlargement, but not for providing the forces for extension. Nevertheless, the sliding of overlapping MTs is necessary for proplatelet elongation, which may be impaired by inhibiting the function of MT-associated motor protein dynein.

In mammalian cells, mDia formins modulate MT and actin function and organization (Ishizaki et al., 2001; Kato et al., 2001; Li and Higgs, 2003; Palazzo et al., 2001). As a member of formin and an effector of RhoA, DIAPH1 has the ability to regulate both the actin and the MT cytoskeleton, this feature making it a good candidate for regulating PPF. Our results suggest that DIAPH1, in addition to ROCK, is another important effector of Rho during megakaryopoiesis that may also negatively regulate PPF. More surprisingly, our results show that DIAPH1 and ROCK are involved not only in Rho-mediated stress fiber assembly, but also in MT stability and dynamics regulation during PPF making a link between these two types of cytoskeleton.

Our results also show that the expression of two other DIAPH (DIAPH2 and DIAPH3) decrease during MK differentiation. It is particularly important to elucidate if this silencing is really required for polyploidization and MK maturation.

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## Article 4



blood

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### **The formin DIAPH1 (mDia1) regulates megakaryocyte proplatelet formation by remodeling the actin and microtubule cytoskeletons**

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**The formin DIAPH1 (mDia1) regulates megakaryocyte proplatelet formation by remodeling the actin and microtubule cytoskeletons**

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Short title: DIAPH1 (mDia1) and proplatelet formation

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Summary: 127 words

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**Key points:**

DIAPH1 (mDia1) is involved in both Rho-mediated actin polymerization and microtubule assembly and stability during proplatelet formation.

**Abstract**

Megakaryocytes (MKs) are the highly specialized precursor cells that produce platelets via cytoplasmic extensions called proplatelets. Proplatelet formation (PPF) requires profound changes in microtubule and actin organization. In this work, we demonstrated that DIAPH1 (mDia1), a mammalian homolog of *Drosophila* diaphanous that works as an effector of the small GTPase Rho, negatively regulates PPF by controlling the dynamics of the actin and microtubule cytoskeletons. Moreover, we showed that inhibition of both DIAPH1 and the Rock/myosin pathway increased PPF via coordination of both cytoskeletons. We provide evidence that two major effectors of the Rho GTPase pathway (DIAPH1 and Rock/myosin II) are involved not only in Rho-mediated stress fibers assembly but also in the regulation of microtubule stability and dynamics during PPF.

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## Introduction

Formins are highly conserved, eukaryotic, multidomain proteins that assemble the actin microfilament and microtubule cytoskeletons into architectures which physically support cell adhesion, migration during development, and responses to injury and infection.<sup>1-3</sup> The mammalian Diaphanous-related formins are encoded by the *DIAPH* genes. Three *DIAPH* isoforms have been described in mammals: *DIAPH1* (mDia1), *DIAPH2* (mDia3), and *DIAPH3* (mDia2). *DIAPH1* (mDia1)<sup>4</sup> is encoded by the *DIAPH1* gene localized on human chromosome 5q31.3. *Diap1*-deficient mice develop age-dependent myeloproliferative /myelodysplastic phenotypes, suggesting that *DIAPH1* may act as a tumor suppressor.<sup>5</sup> *DIAPH1* may be involved in the myeloid malignancies associated with the 5q deletion, because interstitial loss of a variable part of the long arm of chromosome 5 (5q-) is a frequent clonal chromosomal abnormality in human myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML).<sup>6-7</sup>

*DIAPH1* serves as an effector of Rho small GTP-binding proteins.<sup>4</sup> GTP-bound RhoA activates *DIAPH1* by interaction with the GTPase-binding domain (GBD) in its N-terminus.<sup>8-</sup><sup>9</sup> RhoA binding sterically disrupts autoinhibitory interactions between the diaphanous inhibitory domain (DID) within the N-terminal armadillo repeat region and the C-terminal Dia-autoregulatory domain (DAD).<sup>10-11</sup> Released from autoinhibition, the free formin homology-2 (FH2) domain collaborates with the formin homology-1 (FH1) domain bound to profilin-actin complexes to add monomers to the growing (plus [+]) ends of actin filaments.<sup>12</sup>

RhoA-activated *DIAPH1* also induces the selective stabilization of polarized microtubules in serum-deprived NIH 3T3 fibroblasts by binding microtubules directly to its FH2 domain.<sup>13</sup> This affects not only actin and microtubule dynamics but also other cellular processes.<sup>14</sup> Thus, *DIAPH1* is an ideal candidate for coordinating actin and microtubules during proplatelet formation.

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At the end of their maturation, MKs extend their demarcation membranes to form proplatelets.<sup>15</sup> Changes in cytoskeletal structures cause proplatelets to extend further and to re-localize organelles such as mitochondria in the proplatelet. Cytoskeletal changes are also involved in the final abscission and cleavage in platelet formation in the blood stream of marrow sinusoids. Actin undergoes continuous remodeling during proplatelet formation (PPF) and serves as the motor machinery for megakaryocyte (MK) migration in the bone marrow. Previous data from *in vitro* and *in vivo* models have underscored the key role of the Rho/Rock/myosin II pathway in PPF.<sup>16-17</sup> *MYH9* mutations in humans are known to lead to macrothrombocytopenia.<sup>18</sup> Microtubules also play a crucial role in PPF by facilitating the motor forces that elongate the shaft and the tips of proplatelets.<sup>19-23</sup> Because of its dual function on actin and microtubule cytoskeletons, we hypothesized that *DIAPH1* (mDia1) is an essential Rho effector in governing actin and microtubule dynamics in PPF in cooperation with Rho kinase.

To pursue this hypothesis, we studied the precise role of *DIAPH1* and its cooperation with other RhoA effectors such as Rock and myosin II in MK maturation and PPF. Our results show a crucial role for *DIAPH1* in generating F-actin structures and assisting the microtubule stabilization underlying PPF and platelet production.

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## Methods

### ***In vitro* culture of MKs derived from human CD34<sup>+</sup> cells**

In agreement with our Institute Ethic Committee (Assistance Publique des Hôpitaux de Paris), CD34<sup>+</sup> cells were obtained from leukapheresis samples after mobilization performed on patients, from the bone marrow of healthy patients undergoing hip surgery, or from umbilical cord blood. CD34<sup>+</sup> cells were isolated and cultured in serum-free liquid medium with recombinant human TPO (10 ng/mL, Kirin Brewery) and SCF (25 ng/mL, Biovitrum AB) for inducing MK differentiation, as previously described.<sup>16,24</sup>

### **shRNA cloning in lentiviral vector**

Two shRNAs against human *DIAPH1*, one shRNA against human *MYH9*, and a control scrambled sequence (SCR) were cloned separately into a lentiviral vector (pRRL-PGK-GFP or pRRL-PGK-Cherry) as previously described.<sup>25</sup>

### **mDia1ΔN3 cloning in lentiviral vector**

The mDia1ΔN3 sequence lacking autoregulatory components was provided by Shuh Narumiya.<sup>4,14</sup> It was verified by DNA sequencing and amplified by PCR using oligonucleotide adaptors containing Kazak and Stop sequences. The PCR product was digested at *MluI* sites in the adaptor sequence and then introduced into a HIV-derived lentiviral vector pRRL-EF1α-PGK-GFP.

### **Lentivirus production and cell transduction**

Lentivirus particles were prepared and stoked as previously described.<sup>25</sup> Purified CD34<sup>+</sup> cells were cultured for 4-5 days with TPO and SCF. Lentivirus particles were added at a concentration of 10<sup>7</sup> infectious particles/10<sup>5</sup> cells for 12 h, followed by a second transduction exactly done as the first. CD41<sup>+</sup>GFP<sup>+</sup>, CD41<sup>+</sup>Cherry<sup>+</sup>, or CD41<sup>+</sup>GFP<sup>+</sup>Cherry<sup>+</sup> cells were purified by cell sorting 48 h after infection (Influx flow cytometer, Becton Dickinson, CA).

### **Immunofluorescence**

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The cells were plated on poly-L-lysine-coated slides (O. Kindler GmbH & Co, Freiburg, Germany) or Horm type I collagen-coated slides (Nycomed, Austria) for 1 h (37 °C, 5% CO<sub>2</sub>, and 100% humidity). Immunofluorescence was performed as described previously.<sup>16</sup> The detailed information about first antibodies and fixation are presented in supplemental data. Cells were examined under a Zeiss laser scanning microscope (LSM 510, Carl Zeiss, Germany) with a 63X1.4NA oil objective.

#### **Western blot**

Western blot analysis was performed with 8% or 10% SDS-PAGE gel as described previously with the primary antibodies described in supplemental data.<sup>16</sup> The cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100) with a cocktail of proteinase and phosphatase inhibitors (Sigma, France).

#### **Stress fiber formation assay**

MKs derived from CD34<sup>+</sup> cells were infected with lentivirus and sorted by flow cytometry. The CD41<sup>+</sup>GFP<sup>+</sup> cells were then recultured for one additional day before layered on collagen-coated Super Frost slides (Menzel-Glaser, Germany) for 1 h (37 °C, 5% CO<sub>2</sub>, and 100% humidity) to induce stress fiber formation. Three hundred MKs were counted for each independent experiment and the percentage of MKs showing stress fibers was averaged for three independent experiments.

#### **Tubulin polymerization assay**

As described for the stress fiber assay, CD41<sup>+</sup>GFP<sup>+</sup> MKs were plated on collagen-coated slides for 1 h, then incubated with serum-free medium containing 5 μM nocodazole (Sigma, France) for 1 h. Slides were carefully washed 3 times with pre-warmed serum-free medium and put back in the incubator (37 °C, 5% CO<sub>2</sub>, and 100% humidity) to induce tubulin polymerization. After 10 min of incubation, pre-warmed extract solution (100 mM PIPES pH 6.9, 1 mM MgCl<sub>2</sub>, 5 mM EGTA, 0.5% Triton X-100) was added to the slides for 30 s to stop

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the reaction. Cells were fixed with 4% paraformaldehyde for 10 min at 37 °C, washed once by TBS (50 mM Tris, 150 mM NaCl, pH 7.6), and blocked with antibody diluting solution (TBS, 0.1% Triton, 2% BSA) for 10 min before starting IF staining as described previously.<sup>16</sup> The ratio of the  $\alpha$ -tubulin or  $\beta$ -tubulin fluorescence area compared to total cell area of 30-36 cells was quantified with Image J in three independent experiments. Error bars represent SEM of all the analyzed cells.

### **Quantitative real time PCR**

RT-PCR was performed with CD41<sup>+</sup>GFP<sup>+</sup>, CD41<sup>+</sup>Cherry<sup>+</sup>, or CD41<sup>+</sup>GFP<sup>+</sup>Cherry<sup>+</sup> MKs as previously described.<sup>26</sup> All gene-expression levels were normalized to that of the housekeeping *HPRT* gene.

### **Statistics**

Statistical significance was determined by a Student's *t*-test. A *P* value < 0.05 was considered statistically significant. Data are presented as means ( $\pm$  SD) except those noted as means ( $\pm$  SEM).

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## Results

### **DIAPH1 expression increases during MK differentiation**

To measure DIAPH1 expression during MK differentiation, western blots and quantitative RT-PCR analyses were performed on cultured CD41<sup>+</sup> MKs from day 6 to day 12. Compared to the protein loading control (HSC70) and relative to *HPRT* transcripts, DIAPH1 protein and RNA level increased during MK differentiation, particularly during the late days of culture; on the other hand, DIAPH2 and DIAPH3 protein and RNA expression levels, particularly those of DIAPH2, decreased (**Figure 1, A - C**).

### ***DIAPH1* knockdown by shRNA increases MK proplatelet formation**

To determine if DIAPH1 is implicated in MK PPF, human CD34<sup>+</sup> cells were induced *in vitro* towards MK differentiation and transduced at days 4 and 5 of culture with lentiviruses carrying GFP and either a control shRNA (SCR) or two different shRNAs targeting *DIAPH1* (sh7 and sh8). The CD41<sup>+</sup>GFP<sup>+</sup> cell population was sorted 48 h after infection, and RT-PCR and western blot analyses were performed on day 10 or 11 of culture. Quantitative RT-PCR showed a 50%–60% reduction in *DIAPH1* mRNA without significant changes in *DIAPH2* and *DIAPH3* mRNA (**Figure 2C**). DIAPH1 protein level was reduced 50%–60% by sh7 or sh8 treatment, while DIAPH2 (mDia3) and DIAPH3 (mDia2) protein levels remained unchanged (**Figure 2A and 2B**).

The percentage of MKs bearing proplatelets was quantified daily from day 12 to day 14 of culture. The presence of at least one pseudopodial extension was considered a PPF. Five independent experiments were performed (Figure 2D, E). *DIAPH1* knockdown resulted in a marked increase in the number of MKs bearing PPF relative to the control (SCR) at day 13 and 14 of culture.

### ***DIAPH1* knockdown decreases F-actin content, but increases tubulin polymerization**



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**and stability**

The adhesion of primary human MKs onto type I collagen substrate induces stress fiber formation, which depends on actin polymerization and myosin activation.<sup>27</sup> To evaluate the role of DIAPH1 on actin cytoskeleton reorganization, MKs were infected with sh7 or sh8 and made to adhere onto type I collagen-coated slides for a stress fiber assay on day 8 of culture (**Figure 3A**). In each independent experiment, we counted the number of MKs forming stress fibers in at least 300 MKs. A lower percentage of MKs transduced with sh7 and sh8 showed stress fiber formation: 26.3% of MKs infected with the control (SCR) versus 16.3% for sh7 and 16.1% for sh8 (**Figure 3B**). In addition, sh7- or sh8-infected MKs showed thinner stress fibers relative to the control (**Figure 3A**). *DIAPH1* knockdown did not change the pMLC2 level, suggesting that the reduction of stress-fiber formation results from a reduction of actin polymerization, not from inhibition of myosin II activation (**Figure 3C**). This result suggests *DIAPH1* knockdown could reduce contractile forces during MK differentiation.

The microtubule cytoskeleton plays an essential role in PPF, and DIAPH1 is known to stabilize and orientate microtubules.<sup>28</sup> To determine whether DIAPH1 could also affect microtubule dynamics during MK differentiation, tubulin polymerization assays were performed on sorted CD41<sup>+</sup>GFP<sup>+</sup> MKs infected with various shRNAs. Quite surprisingly, sh7- and sh8-infected MKs showed more obvious microtubule elongation after nocodazole inhibition compared to the SCR control (**Figure 3D, E; Supplemental Figure 1**). It is known that stable microtubules accumulate a posttranslationally modified form of tubulin recognized as detyrosinated tubulin (Glu-tubulin), whereas dynamic microtubules contain predominantly tyrosinated tubulin (Tyr-tubulin).<sup>29-30</sup> We found that the ratio between Glu-tubulin and Tyr-tubulin was increased in MKs infected by *DIAPH1* shRNA (**Figure 3F**), indicating that *DIAPH1* knockdown increases microtubule stability.

**Expression of a constitutively active DIAPH1 inhibits proplatelet formation**

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To confirm the role of DIAPH1 revealed by the shRNA strategy, a constitutively active form of murine DIAPH1 (mDia1ΔN3, an active mDia1 containing the FH1 and FH2 regions without the Rho-binding domain) was expressed in human MKs (**Figure 4A**). Two sets of RT-PCR primers were designed, one set specific for the constitutively active form of mDia1ΔN3 (*DIAPH1* Exo) and the other for endogenous *DIAPH1* (*DIAPH1* Endo). Expression of the endogenous and exogenous forms was analyzed in sorted CD41<sup>+</sup>GFP<sup>+</sup> MKs by quantitative RT-PCR (**Figure 4B**). PPF was counted from days 12 to 14 of culture. As shown in **Figure 4B**, total *DIAPH1* mRNA (*DIAPH1* Endo + *DIAPH1* Exo) increased about 4- to 6-fold in mDia1ΔN3-infected MKs relative to the empty vector infected MK controls. In contrast, PPF was decreased in MKs expressing mDia1ΔN3 (**Figure 4C**). Remarkably, proplatelets formed by MKs expressing mDia1ΔN3 often presented a simpler structure with less branching and shorter extensions, which lead to a clear decrease in proplatelet area (**Figure 4D, 4E and 4F**). Furthermore, *DIAPH1* knockdown only slightly increased platelet production *in vitro* in spite of the marked increase in PPF, but overexpression of mDia1ΔN3 induced a decrease in platelet production parallel to the decrease in proplatelet formation, suggesting that DIAPH1 contributes to the platelet release process (**Figure supplemental 2A and 2B**). Together, these data suggest that DIAPH1 activation inhibits platelet production.

#### **Expression of mDia1ΔN3 increases stress fiber formation but decreases tubulin polymerization and stability**

We performed assays to evaluate the effects of mDia1ΔN3 on actin and tubulin cytoskeleton, stress fiber formation, and tubulin polymerization on day 8 of MK culture. Stress fiber formation increased in MKs expressing mDia1ΔN3 (50.7%) relative to empty vector controls (38.5%) (**Figure 5A**), but tubulin polymerization after nocodazole treatment was lower in MKs expressing mDia1ΔN3 relative to the control (**Figure 5B**). In agreement with the immunofluorescence results, western blots showed that mDia1ΔN3 expression

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decreased the ratio between Glu- and Tyr-tubulin, indicating a loss in microtubule stability (**Figure 5C**).

### **Expression of mDia1ΔN3 rescues the effects induced by *DIAPH1* shRNA on proplatelet formation**

To demonstrate that the increase in PPF observed after *DIAPH1* knockdown was not an off-target effect, we performed rescue experiments using mDia1ΔN3. For these experiments, we used sh8 because it targets the endogenous *DIAPH1* but not mDia1ΔN3 (data not shown). To perform a double infection, sh8 was cloned in a lentiviral vector containing Cherry (a nonrelevant shRNA SCR was used as a control), and mDia1ΔN3 was cloned in a lentivirus vector expressing GFP (an empty vector was the control). Expression of these constructs was analyzed both by RT-PCR (with primer sets allowing distinction between *DIAPH1* Endo and mDia1ΔN3 mRNA) or by flow cytometry. sh8-cloned in the Cherry vector was as efficient as the previous vector in decreasing endogenous *DIAPH1* in CD41<sup>+</sup> MKs (**Supplemental Figure 3A**). Similarly, mDia1ΔN3 was well expressed as revealed by both mRNA and GFP levels (**Supplemental Figure 3B**). Double infections were performed in different combinations (shSCR+control, sh8+control, mDia1ΔN3+shSCR, and mDia1ΔN3+sh8), and the double-infected cells (Cherry<sup>+</sup>GFP<sup>+</sup>CD41<sup>+</sup>) were then sorted (**Figure 6A**). As shown in **Figure 6B**, sh8 decreased the level of endogenous *DIAPH1* without an effect on mDia1ΔN3. The increase in PPF induced by sh8 was lessened by mDia1ΔN3 expression (**Figure 6C**). Together, the rescue of *DIAPH1* expression abolished the increase in PPF induced by *DIAPH1* knockdown, demonstrating the sh8 specificity for *DIAPH1* and underscoring the role of *DIAPH1* in PPF.

### **Effects of double inhibition of *DIAPH1* and Rock/myosin II on proplatelet formation**

The RhoA pathway could also play a negative role in proplatelet formation by inducing myosin II activation.<sup>16,31</sup> We thus checked double inhibition of myosin IIA and *DIAPH1* in

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cytoskeleton dynamics regulation during PPF.

First, tubulin polymerization assays showed that inhibition of Rock by Y27632 (10  $\mu$ M) or myosin II by blebbistatin (25  $\mu$ M) decreased CD41<sup>+</sup> MK stress fiber formation but increased tubulin polymerization (**Figure 6D and Figure 6E**). Western blots revealed that Rock or myosin II inhibition increased Glu-/Tyr- tubulin ratio and slightly increased acetylated-tubulin (**Figure 7A**).

To verify the effect of a double inhibition of DIAPH1 and the Rock/myosin II pathway on PPF, MKs transduced with sh7, sh8, or an SCR control were incubated overnight just before PPF (about day 10 of culture) with Y27632 (10  $\mu$ M) or blebbistatin (25  $\mu$ M) alone or in combination. The increase in proplatelet formation observed in sh7- or sh8-transduced MKs was augmented when Rock or myosin II inhibitors were added alone or together (**Figure 7B**). Moreover, MKs double-transduced with an shRNA against *DIAPH1* cloned in a lentiviral vector containing Cherry (sh8) plus an shRNA against *MYH9* cloned in a lentiviral vector containing GFP (shMYH9) showed an increased proplatelet formation relative to a single knockdown (**Figure 7C and 7D**).

Finally, sh7-, sh8- or SCR-transduced MKs were incubated overnight with Rock or myosin II inhibitors at day 8 of culture and analyzed by western blots. In SCR-transduced MKs, Rock/myosin II inhibition increased Glu-/Tyr-tubulin ratio, which was further augmented in sh7- or sh8-transduced MKs. This suggests that the inhibition of both DIAPH1 and the Rock/myosin II pathway increases microtubule stability more than DIAPH1 inhibition alone (**Figure 7E**).

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## Discussion

Proplatelet formation is associated with morphological changes that require profound cytoskeleton reorganization. The mammalian Diaphanous-related formins directly promote actin filament growth. They could also stabilize microtubules or coordinate the dynamics of the microtubules and actin cytoskeleton.<sup>13-14,32</sup> These properties make DIAPH (mDia) molecules ideal candidates for the cytoskeletal assembly machines that build the structures supporting PPF. Further, DIAPH1(mDia1) is one of the major effectors of the Rho signaling pathway, which is a key regulator of PPF as outlined by *in vitro* and *in vivo* models.<sup>16-17</sup> Moreover, two genes encoding Rho GTPase guanine nucleotide exchange factors, *DOCK8* (dedicator of cytokinesis 8) and *ARHGEF3* (Rho guanine nucleotide exchange factor 3), were identified among 68 genomic loci reliably associated with platelet count and volume.<sup>33</sup> Mutations in *MYH9*, another important effector of Rho pathway, can lead to macrothrombocytopenia.<sup>18</sup> Until now, there has been no direct evidence that *DIAPH1* mutations are associated with disorders in platelet biogenesis. However, DIAPH1 as a main RhoA effector is implicated in PPF, either directly or in cooperation with other RhoA effectors, such as Myosin II or Rock.

During MK differentiation, DIAPH1 expression increases and DIAPH2 and DIAPH3 expression diminish. The different expression profiles suggest that these family members play specific roles in the differentiation process. MKs first become polyploid by endomitosis before proplatelet formation, with a corresponding increase in cell size and a concomitant elevation of actin content.<sup>16,34</sup> DIAPH1 expression also increases to allow normal actin assembly, a process required for normal cell function at the end of MK differentiation. DIAPH1 is a potent activator of serum response factor (SRF), which senses changes in actin monomer concentrations to induce the expression of cell structure genes.<sup>35</sup> Our data suggest a specific role of DIAPH1 in PPF and platelet shedding. In contrast, DIAPH2 (mDia3) and

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DIAPH3 (mDia2) are down-regulated, possibly to facilitate MK polyploidization and PPF.

mDia1 and mDia2 are required for cytokinesis depending upon the cell type,<sup>36-38</sup> and mDia3 is involved in stabilization of the kinetochores onto spindle microtubules.<sup>39-40</sup> Moreover, mDia2 contributes to the generation of an actin structure that maintains the cortical actin cytoskeleton,<sup>41</sup> and inhibition of mDia2 leads to non-apoptotic membrane blebbing, a process that resembles PPF in MKs. Other studies have shown that formin activation impairs the ability of oncogenic Ras to induce membrane extrusions that bud off from cells to form microvesicles (Davis-Turner and A.S.A., submitted for publication). A persistent MAPK activation also inhibits proplatelet formation in humans.<sup>42</sup> Therefore, hyperactivation of actin assembly may impair the ability of MKs to alter their membrane plasticity and to generate platelets. A selective decrease in the expression of some DIAPH could facilitate PPF. Another notable point is that when extracts of MKs from different culture days were analyzed by western blotting in 8% SDS-PAGE gels, the three DIAPHs each appeared as two distinct bands. These may correspond to different splice variants, as described for mDia3,<sup>43</sup> or to posttranslational changes. mDia1 and mDia2 can be both ubiquitinated (unpublished observation of Deward and Arthur) and phosphorylated.<sup>44</sup>

DIAPH1 expression increases during MK differentiation but *DIAPH1* knockdown increases PPF, which seems contradictory. It is likely that the increase during differentiation is related to the MKs becoming polyploid, which produces a large increase in cell size and is accompanied by an elevation in actin content.<sup>1,34</sup> An increase in DIAPH1 expression is required in order to regulate actin polymerization and maintaining a normal cell shape. At the end of MK differentiation, MKs promote membrane protrusion in order to form proplatelets. The contractile forces generated by Myosin II and F-actin restrain cytoplasmic extensions and maintain cell rigidity must be inactivated to trigger PPF and platelet shedding. That's why knockdown or inhibition of DIAPH1 or MYH9 increases the proplatelet formation.

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As we were unable to detect modification in MLC2 phosphorylation level after *DIAPH1* knockdown, we infer that mDia1 may contribute to actomyosin assembly only by promoting actin polymerization, but not by activating myosin II. It is possible that Rho activation regulates PPF by two independent pathways, the RhoA/Rock/Myosin IIA and the RhoA/DIAPH1 pathways. This is in line with our observation that the simultaneous inhibition of Rock and DIAPH1 induced a marked increase in PPF. Our results revealed that the two important effectors of RhoA pathway, DIAPH1 and ROCK cooperate to create the contractile forces that restrain cytoplasmic extensions and to inhibit PPF during MK differentiation. Our data also revealed that the Rho pathway must be shut down to allow appropriate PPF.

We examined whether DIAPH1 can regulate microtubule reorganization during PPF. Surprisingly, our results suggest that DIAPH1 plays a negative role in microtubule polymerization and stability. Indeed, it has been clearly shown that proplatelet extension is a microtubule-driven process with a continuous polymerization of microtubules at the free (plus) end. This contributes to PPF and to the sliding of overlapping microtubules, which likely drives proplatelet elongation.<sup>17-20</sup> Activated DIAPH1 could restrict proplatelet formation by two mechanisms, first by inducing stress fiber formation (contractile forces) and second by destabilizing microtubules.

The mechanism by which DIAPH1 (mDia1) contributes to microtubule stabilization is still a matter of debate. Early studies have suggested that actin and microtubules compete for mDia because the mDia FH2 domain functions in both actin assembly and microtubule stabilization.<sup>13,45</sup> There is also evidence that mDia regulates microtubule stability independently of actin nucleation.<sup>14,32</sup> DIAPH1 can also complex with other microtubule-binding proteins, including EB1 and the product of the familial colon cancer gene *APC1* (APC, adenomatous polyposis coli), or can regulate GSK3beta through a novel class of protein kinase Cs (PKCs) to promote microtubule stabilization.<sup>46-49</sup> Simultaneous loss of both mDia1

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and APC function profoundly impacts the myelodysplastic syndrome phenotype in mice (A.S.A, and S.K.G., unpublished observation), suggesting a collaboration in tumor suppression as well as in actin assembly.<sup>49</sup>

Moreover, inhibition of either Rock or myosin II could also increase microtubule elongation and stability. More importantly, their dual inhibition increased microtubule stability even more, as indicated by the Glu-tubulin/Tyr-tubulin ratio. The combined inhibition of Rock/myosin II and DIAPH1 also has a more significant effect on PPF inhibition than either single inhibition. However, there is no clear evidence for direct interactions between Rock and microtubules. It has been reported that inhibition of Rock or myosin II can increase acetyl-MT levels, a posttranslational modification associated with microtubule stabilization, via the phosphorylation of tubulin polymerization which promotes protein 1 and histone deacetylase 6 (HDAC6) activity.<sup>50-52</sup> In MKs, Rock/myosin II inhibition increased Glu-/Tyr- tubulin ratio and increase tubulin polymerization. However, we did not find an obvious change in the acetyl-tubulin level when Y27632 or blebbistatin is added. The molecular mechanism by which Rock regulates microtubules needs to be further studied.

Another interesting question is raised by our work because DIAPH usually increases microtubule stability in interphase cells, but we observed the opposite in MKs. A similar result was also reported in osteoclasts, in which Rho inhibition is correlated with an increased microtubule stabilization that is mediated by mDia2 through tubulin acetylation.<sup>53</sup> However, we did not observe any significant changes in microtubule acetylation level when we knocked down *DIAPH1* or expressed the active form of DIAPH1 in human MK, suggesting that regulation of tubulin acetylation may be more linked to mDia2 (DIAPH3) or to formins other than mDia1 (DIAPH1). All these observations suggest the complexity of the DIAPH pathway, whose effects on microtubule may depend on the cellular context and thus on cell type.



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Finally, *DIAPH* expression profiles are different between human and mouse MKs with a much higher *DIAPH2* and *DIAPH3* expression level in mouse than in human MK (unpublished results). The functional redundancy among different DIAPH molecules or other formin family members may explain why there is no evident abnormality in PPF (unpublished results), platelet number, and function in *DIAPH1* knock-out mice.<sup>5,54</sup>

Together, our work shows that the two main effectors of the Rho-GTPase pathway (DIAPH1 and Rock/myosin II) are involved in Rho-mediated stress fiber assembly as well as in the regulation of microtubule stability and dynamics during PPF.

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### **Author Contribution**

YC directed the studies. YC and WV designed the experiments. PJJ, YC, LL, and DM performed research. PR, YL, SMK-G, IB, and HM contributed to the performance of certain experiments. SN provided mDia1ΔN3. YC, ASA, and WV wrote and revised the manuscript with contribution of all other authors.

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**Competing financial interests**

The authors declare no competing financial interests.

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## Legends of figures

### Figure 1: DIAPH1, DIAPH2 and DIAPH3 expression during MK differentiation

**A:** CD41<sup>+</sup> MKs were sorted at day 6 of culture and recultured for various periods according to the experiments. In the western blots, HSC70 was used as the protein loading control; an 8% SDS-PAGE gel was used for protein separation.

**B:** The ratio between DIAPH (1, 2 or 3) and HSC70 protein was analyzed by Image J in

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three independent experiments. *DIAPH1* expression was increased, and *DIAPH2* and *DIAPH3* were decreased during MK differentiation.

**C:** Real-time RT-PCR (normalized to *HPRT*) was performed on  $CD41^+$  MKs collected at days 7, 9, and 12 of culture (three independent experiments). *DIAPH1* mRNA expression was increased during MK differentiation.

**Figure 2: *DIAPH1* knockdown by shRNA increases MK proplatelet formation**

**A:** Western blots revealed that shRNA (sh7 and sh8) targeting of *DIAPH1* decreased *DIAPH1* protein level (but not *DIAPH2* and *DIAPH3* protein expression) relative to control shRNA (scr). A 10% SDS-PAGE gel was used for protein separation.

**B:** *DIAPH1*(left), *DIAPH2* (middle), or *DIAPH3* (right) protein levels (relative to HSC70) were analyzed by Image J in three independent experiments. Only *DIAPH1* expression was decreased by sh7 and sh8.

**C:** RT-PCR (normalized to *HPRT*) results showed that only the *DIAPH1* mRNA level was decreased by sh7 and sh8 (four independent experiments).

**D:** *DIAPH1* knockdown resulted in a marked increase in MK proplatelet formation relative to the control (scr) (five independent experiments).

**E:** Phase contrast microscopy images (lens 20x) of day 13 culture of control (SCR), sh7, or sh8-infected MKs. There was more proplatelet formation in sh7- or sh8-infected cells.

**Figure 3: *DIAPH1* knockdown decreases stress fiber formation but increases tubulin polymerization and stability**

**A:** MKs transduced with different shRNAs (SCR, sh7, or sh8) after 1 h of adhesion onto a fibrillar collagen I substrate for a stress fiber formation assay. MKs infected with sh7 (middle) and sh8 (right) showed fewer stress fibers than did control (SCR) (left).

**B:** MKs transduced with sh7 and sh8 showed 16.3% and 16.1% stress fiber formation,

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respectively, relative to 26.3% for the control (SCR) in three independent experiments.

**C:** *DIAPH1* knockdown did not modify the ratio of pMLC2/MLC2 or of acetylated-tubulin/total tubulin. A 10% SDS-PAGE gels was used for protein separation (left). Three independent experiments were quantified with Image J (right).

**D:** CD41<sup>+</sup> MKs infected with SCR, sh7, or sh8 were plated on collagen-coated slides for 1 h and then treated for another hour with nocodazole. Tubulin polymerization assays were performed 10 min after washing. MKs infected with sh7 (middle) or sh8 (bottom) show increased tubulin polymerization relative to the control (top).  $\alpha$ -Tubulin is labeled in blue and  $\beta$ -tubulin in red. 3D: three dimensions.

**E:** MKs infected with sh7 or sh8 showed a greater  $\alpha$ -tubulin or  $\beta$ -tubulin fluorescence area relative to total cell area (Area%). We quantified 30-40 cells for each condition in three independent experiments using Image J. The data are presented as means  $\pm$  SEM. **Top:**  $\alpha$ -tubulin (SCR:  $8.04 \pm 1.02$ ; sh7:  $12.96 \pm 1.83$ ; sh8:  $12.37 \pm 1.69$ ). **Bottom:**  $\beta$ -Tubulin (SCR:  $6.60 \pm 0.96$ ; sh7:  $14.32 \pm 1.61$ ; sh8:  $12.15 \pm 1.58$ ).

**F:** Western blots revealed that MKs infected with sh7 or sh8 presented a higher proportion of Glu-tubulin and a lower proportion of Tyr-tubulin relative to SCR controls. A 10% SDS-PAGE gel was used for protein separation (top). The expression ratios between *DIAPH1* and HSC70 and between Glu-tubulin and Tyr-tubulin were quantified in three independent experiments using Image J (bottom).

#### **Figure 4: Expression of a constitutively active *DIAPH1* (mDia1 $\Delta$ N3) inhibits proplatelet formation**

**A:** CD41<sup>+</sup>GFP<sup>+</sup> MKs were sorted by flow cytometry after infection with mDia1 $\Delta$ N3 (right) or with the empty lentiviral vector (left) (pRRL-EF1 $\alpha$ -PGKGFP, control) at day 7 of MK culture.

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**B:** Quantitative real-time PCR for endogenous and exogenous mRNA expression in sorted CD41<sup>+</sup>GFP<sup>+</sup> MKs. Total *DIAPH1* mRNA expression was 3 to 4 folds higher in MKs transduced with mDia1ΔN3 relative to controls (cells transduced with pRRL-EF1α-PGKGFP) in three independent experiments.

**C:** PPF measured from day 12 to day 14 in MK culture was lower in MKs expressing mDia1ΔN3 than in controls (cells transduced with pRRL-EF1α-PGKGFP) in four independent experiments.

**D:** mDia1ΔN3-expressing MKs (bottom) often presented proplatelets with shorter extensions and less branching than controls (top); phase contrast images with a 20x lens.

**E:** Proplatelet area was measured by Image J in three independent experiments. The data are presented as mean ± SEM. mDia1ΔN3-infected MKs ( $0.0018 \pm 0.0002 \text{ mm}^2$ ,  $n = 32$ ) had a lower proplatelet area than controls ( $0.0008 \pm 0.0001 \text{ mm}^2$ ;  $n = 30$ ).

**F:** Proplatelet branch numbers were counted in three independent experiments. mDia1ΔN3-infected MK ( $6.57 \pm 0.94$ ,  $n = 32$ ) showed less branching than the controls (cells transduced with pRRL-EF1α-PGKGFP) ( $2.81 \pm 0.42$ ,  $n = 35$ ).

**Figure 5: Expression of a constitutively active DIAPH1 (mDia1ΔN3) increases stress fiber formation and decreases tubulin polymerization and stability**

**A: Left:** A stress fiber formation assay was performed in sorted CD41<sup>+</sup>GFP<sup>+</sup> MKs transduced with mDia1ΔN3 or with an empty vector as described in Materials and Methods. **Right:** A higher percentage (50.7%) of mDia1ΔN3 MKs showed stress fiber formation than did controls (38.5%) in three independent experiments.

**B: Left:** CD41<sup>+</sup> MKs infected by mDia1ΔN3 or the control vector were plated on collagen-coated slides and treated for 1 h with nocodazole. Tubulin polymerization assays were performed 10 min after recovery. mDia1ΔN3-infected MKs showed lower tubulin



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polymerization relative to controls. 3D, three dimensions. **Right:** MKs infected by mDia1ΔN3 showed lower  $\alpha$ -tubulin (top) or  $\beta$ -tubulin fluorescence area (bottom) relative to total cell area (Area %). We quantified 37 cells in each condition in three independent experiments using Image J. The data are presented as mean  $\pm$  SEM.  $\alpha$ -Tubulin: control:  $10.10 \pm 1.14$ ; mDia1ΔN3:  $6.64 \pm 0.73$ .  $\beta$ -Tubulin: control:  $6.14 \pm 0.69$ ; mDia1ΔN3:  $3.82 \pm 0.48$ .

**C:** Western-blot analysis and quantification were performed in sorted CD41<sup>+</sup>GFP<sup>+</sup> MKs transduced with mDia1ΔN3 or with an empty vector in three independent experiments, showing that mDia1ΔN3 expression decreased the ratio between Glu-tubulin and Tyr-tubulin but did not change the ratio between PMLC2 and MLC2. A 10% SDS-PAGE gel was used for protein separation.

**Figure 6: The constitutively active DIAPH1 (mDia1ΔN3) rescues the effects of shRNA-induced knockdown of DIAPH1**

**A:** CD41<sup>+</sup>GFP<sup>+</sup>Cherry<sup>+</sup> MK populations double-infected with two lentiviral vectors containing GFP or Cherry were purified by flow cytometry. In each combination (Top left: SCR+control; Bottom left: sh8+control; Top right: SCR+mDia1ΔN3; Bottom right: sh8+mDia1ΔN3), the CD41<sup>+</sup> population was first gated and then the double-labeled population was obtained by gating the GFP<sup>+</sup> Cherry<sup>+</sup> population in the previously gated CD41<sup>+</sup> population.

**B:** Quantitative RT-PCR showing the levels of endogenous and exogenous *DIAPH1* mRNA in the double-infected populations purified by flow cytometry (three independent experiments).

**C:** Proplatelet formation was counted in the double-transduced MK population CD41<sup>+</sup>GFP<sup>+</sup>Cherry<sup>+</sup>. The increase of proplatelet formation caused by the shRNA targeting DIAPH1 was abolished by mDia1ΔN3 expression (four independent experiments; proplatelet

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formation in SCR+control MKs was used as a normalized baseline).

**D:** Tubulin polymerization assays in CD41<sup>+</sup> MKs show that Rock or myosin II inhibition increased tubulin polymerization, but disturbed stress fiber formation. Top: Control; Middle: Blebbistatin; Bottom: Y27632. MKs were stained for tubulin ( $\alpha$  and  $\beta$ , green) and phalloidin-TRITC (red); DNA was stained with TOTO (blue).

**E:** Blebbistatin or Y27632 incubation increased  $\alpha$ -tubulin or  $\beta$ -tubulin fluorescence area relative to total cell area (Area%). Thirty to forty cells in each condition were quantified in three independent experiments using Image J. The data are presented as mean  $\pm$  SEM: control ( $5.77 \pm 0.85$ ); blebbistatin ( $13.63 \pm 1.99$ ); Y27632 ( $13.07 \pm 1.75$ ).

### **Figure 7: Effects of the double inhibition of DIAPH1 and Rock/myosin II on proplatelet formation**

**A: Left:** Western blot performed on CD41<sup>+</sup> MKs at day 8 of culture revealed Glu-tubulin, Tyr-tubulin and acetylated-tubulin expression after ROCK (Y27632, 10  $\mu$ M) or myosin II inhibition (blebbistatin 25  $\mu$ M) with overnight incubation. **Right:** The ratio of Glu-tubulin to Tyr-tubulin and quantification of acetylated-tubulin expression relative to HSC70 showed a clear increase of stable Glu-tubulin, but only a slight increase of acetylated-tubulin.

**B:** sh7-, sh8- or SCR-transduced MKs were incubated overnight just before PPF (at day 10 of culture) with inhibitors of Rock (Y27632, 10  $\mu$ M) or myosin II (blebbistatin 25  $\mu$ M). Inhibition of Rock and of myosin II combined with *DIAPH1* knockdown by shRNA increased proplatelet formation even more in three independent experiments.

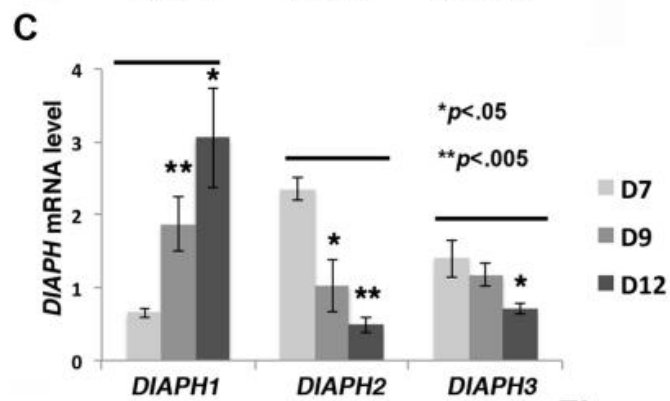
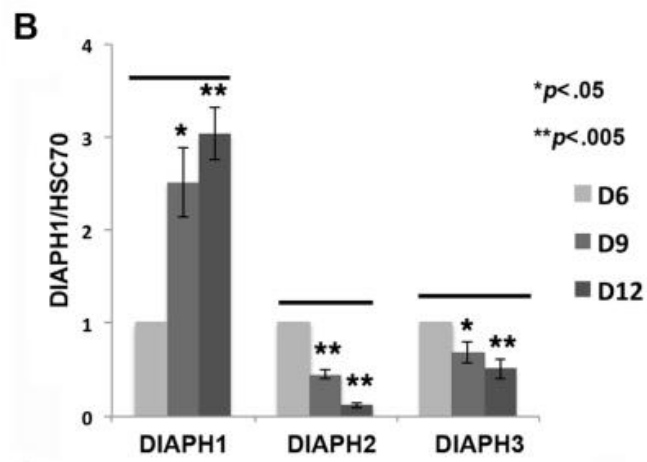
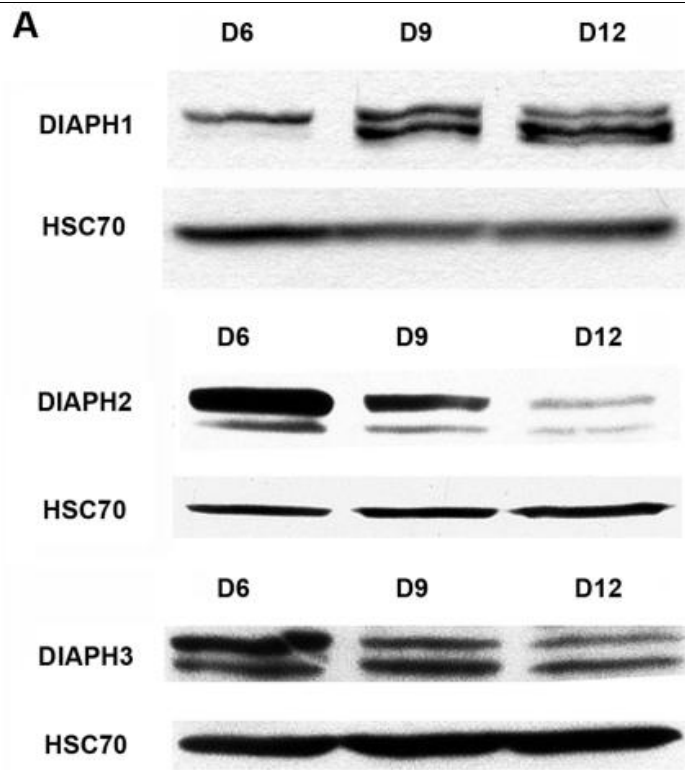
**C:** Knockdown of both *MYH9* and *DIAPH1* by shRNA increased proplatelet formation more the knockdown of either alone in three independent experiments. MKs were double-infected with two lentiviral vectors containing GFP (shMYH9 or SCR control) or Cherry (sh8 or SCR control). CD41<sup>+</sup>GFP<sup>+</sup>Cherry<sup>+</sup> populations (SCR+SCR, SCR+sh8, SCR+shMYH9, or

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sh8+shMYH9) were purified by flow cytometry.

**D:** Quantitative RT-PCR showing *DIAPH1* and *MYH9* mRNA levels in double-infected MK populations (SCR+SCR, SCR+sh8, SCR+shMYH9, or sh8+shMYH9). Cells were isolated by flow cytometry in three independent experiments.

**E: Top:** Western blots of sh7-, sh8-, or SCR-transduced MKs incubated overnight with inhibitors of ROCK (Y27632, 10  $\mu$ M) or myosin II (blebbistatin, 25  $\mu$ M) at day 8 of culture. Glu-tubulin levels increased even more after *DIAPH1* knockdown combined with Rock/myosin inhibition. A 10% SDS-PAGE gel was used for protein separation. **Bottom:** The ratios of DIAPH1/HSC70 and Glu-tubulin/Tyr-tubulin showed a clear decrease of DIAPH1 expression and an increase of stable Glu-tubulin after DIAPH1 knockdown combined with inhibitor.



**Figure 1**

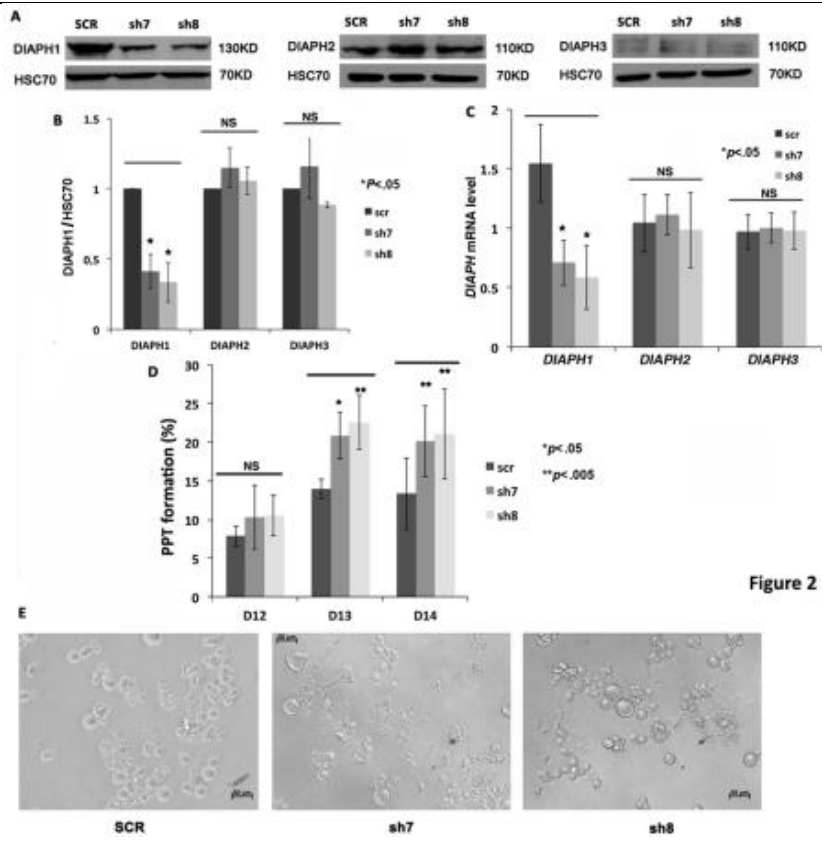


Figure 2

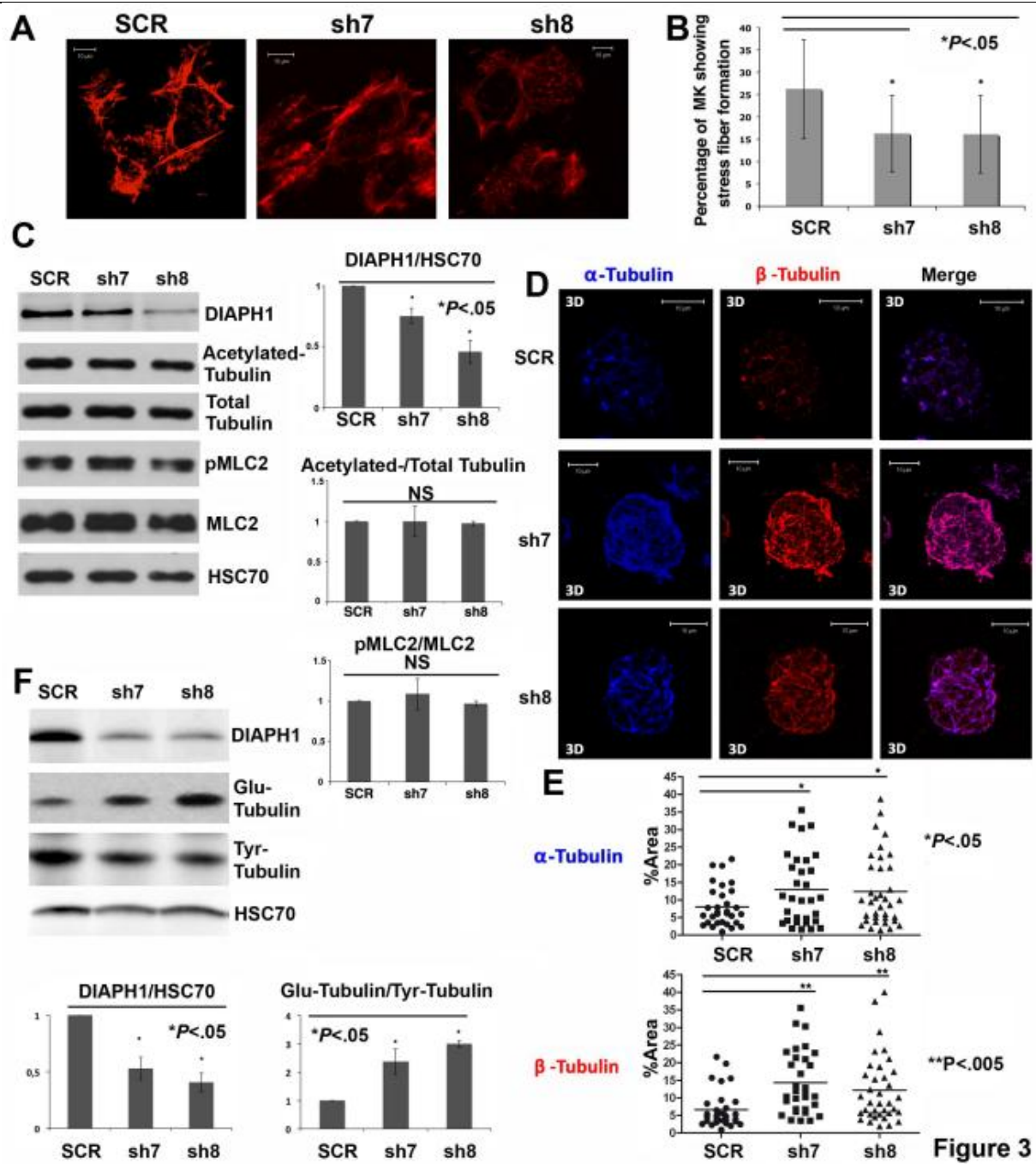
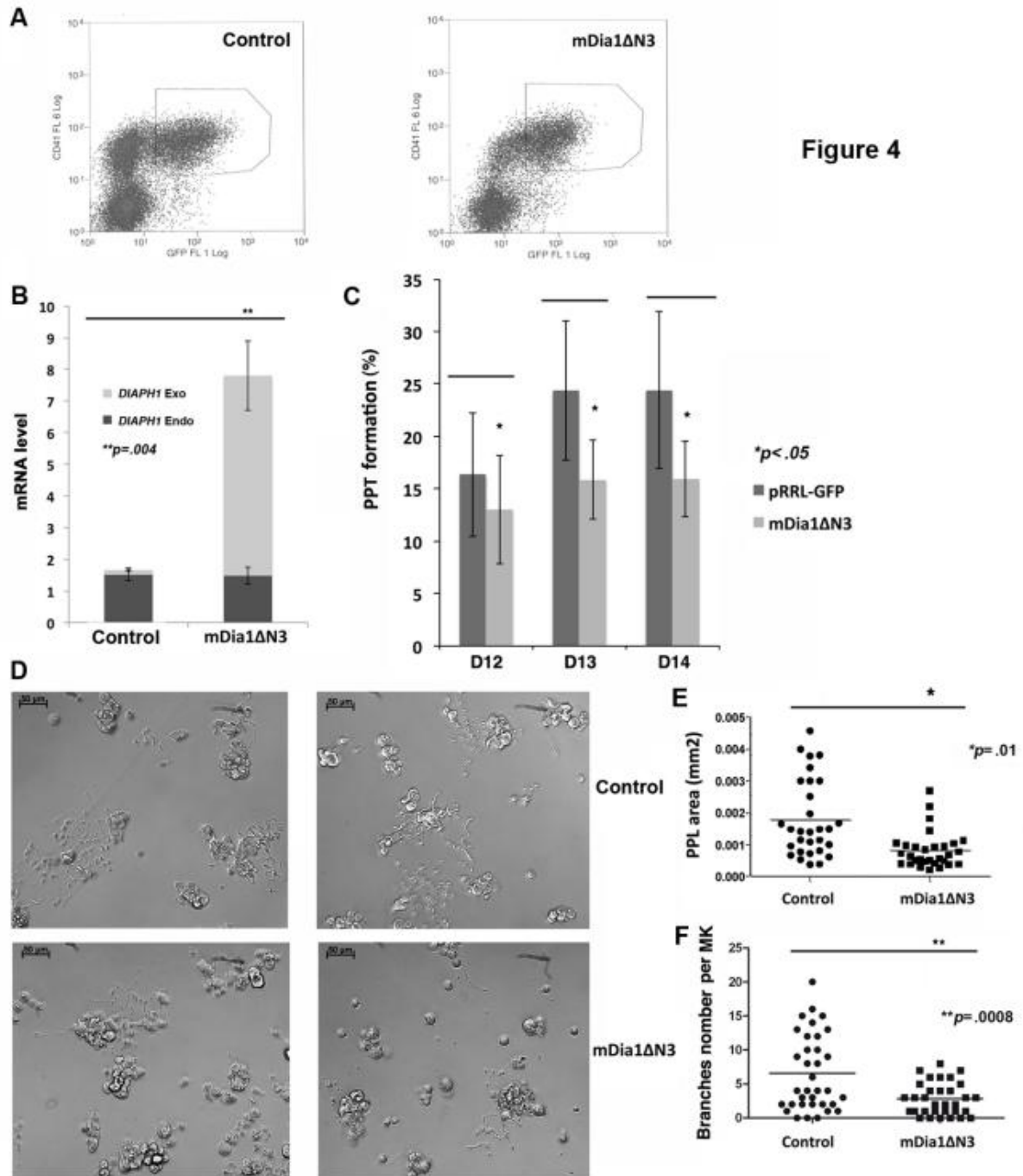
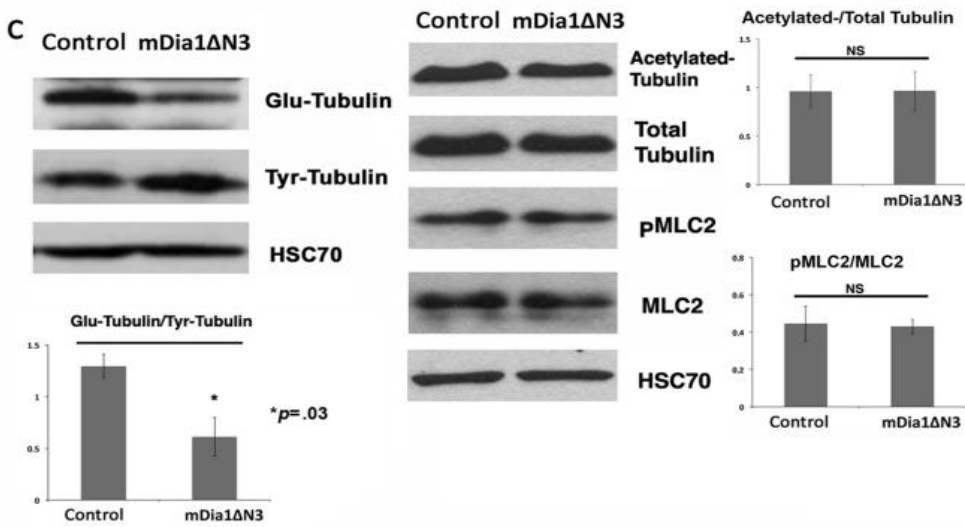
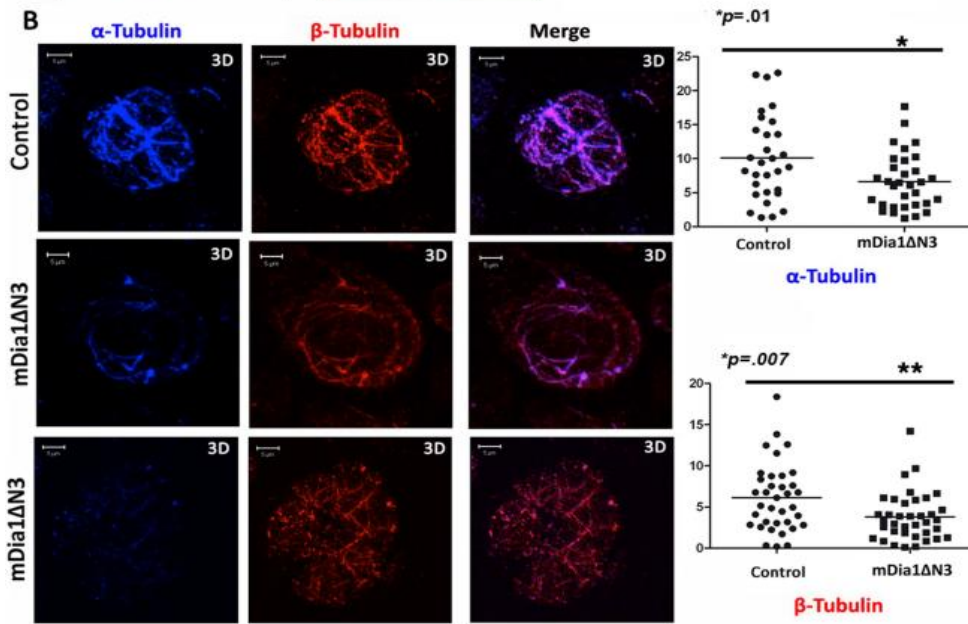
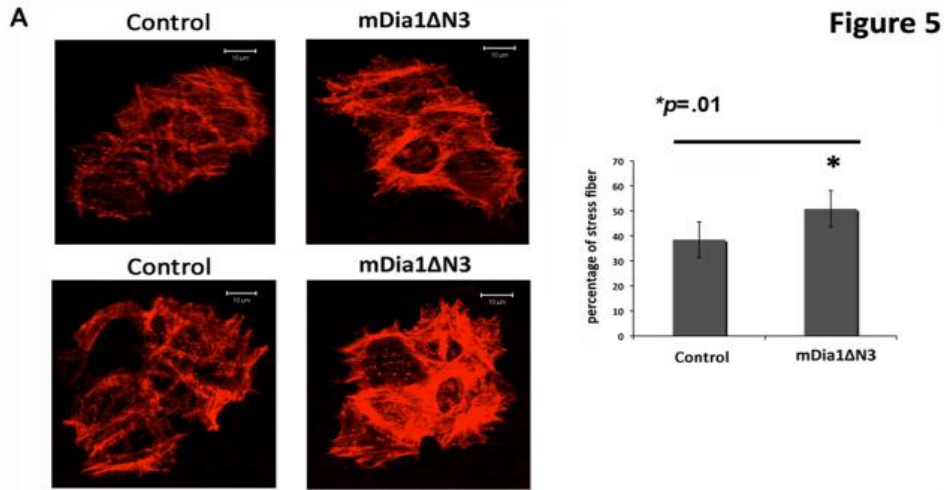


Figure 3

Figure 4







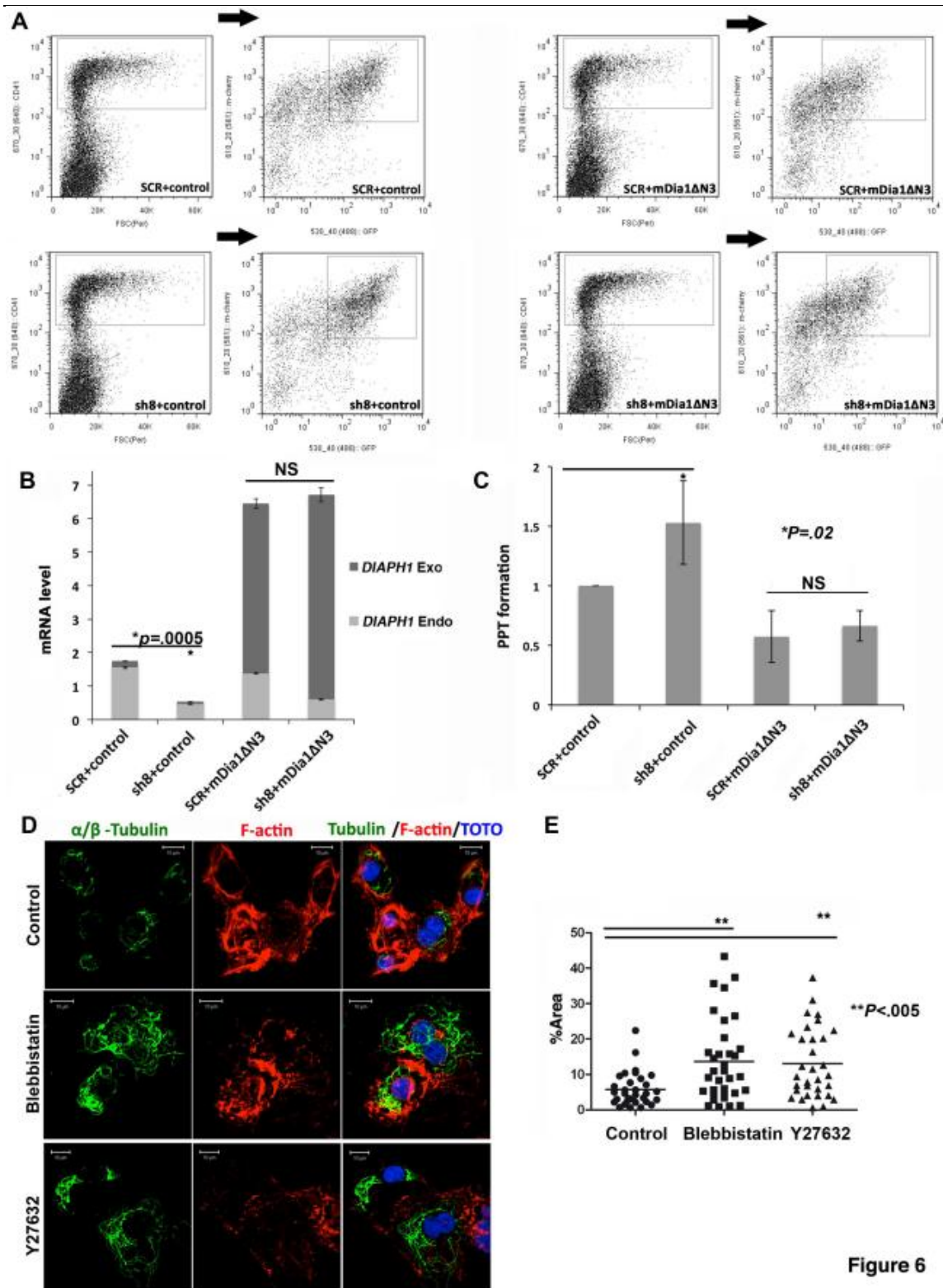


Figure 6

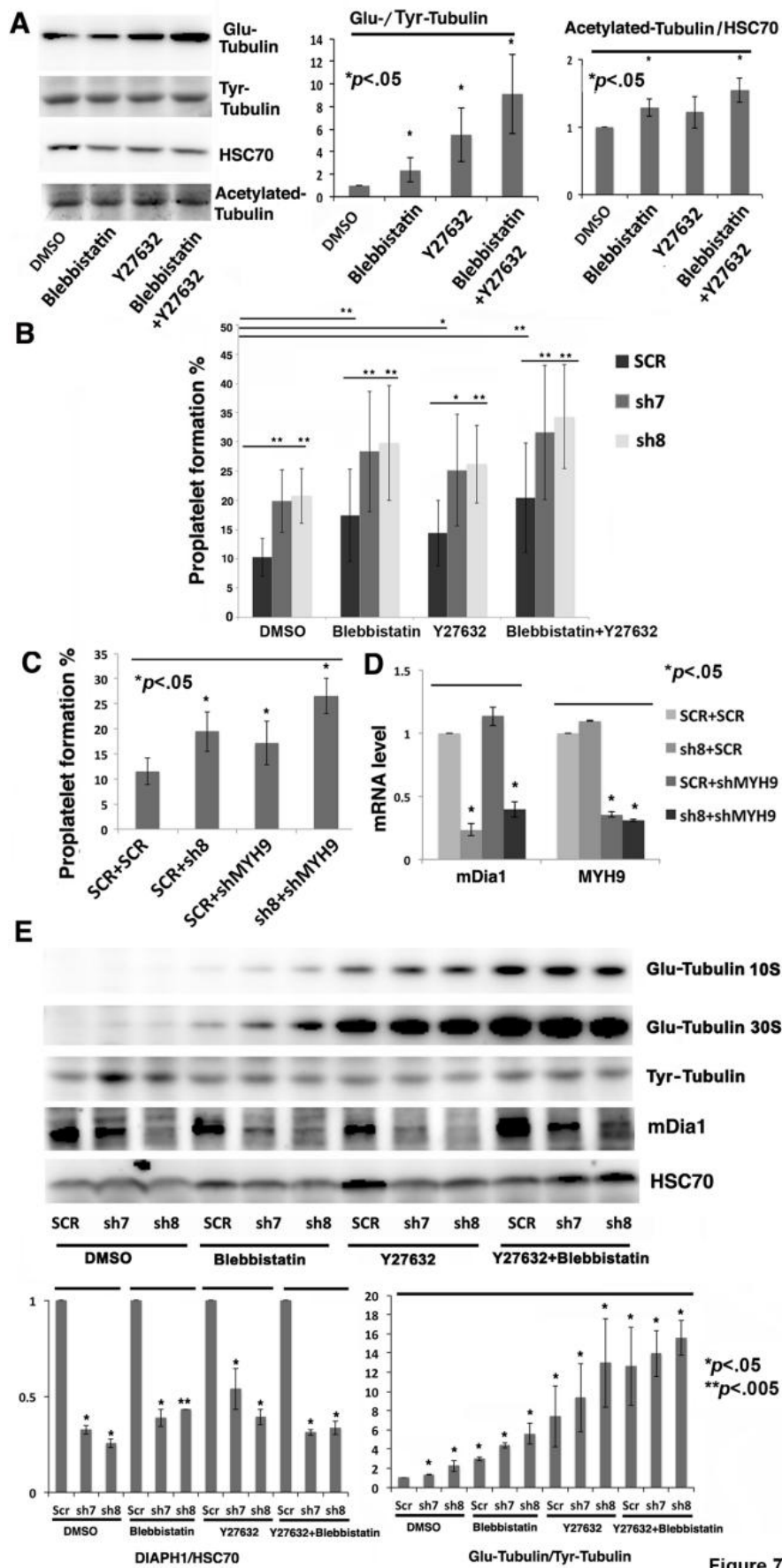


Figure 7

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# Chapter III: Discussion and Perspectives

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## **Rho/ROCK/Myosin pathway during megakaryopoiesis**

Recent studies have shown that MK endomitosis is an incomplete multipolar mitosis characterized by failure of both cytokinesis and karyokinesis producing a cell that contains a unique multilobulated nucleus. Polyploidy is a manner of increasing platelet production, as it induces a parallel increase in DNA and proteins synthesis and MK cytoplasm volume. The defect in cytokinesis is a late failure, consequence of a contractile ring defect related to alteration both in Rho activation and myosin II accumulation in the contractile ring (Gao et al., 2012; Geddis et al., 2007; Geddis and Kaushansky, 2006; Lordier et al., 2012a; Lordier et al., 2008). After activation, RhoA binds to numerous downstream effectors including ROCK and mDia, to regulate the cytoskeleton dynamics. As one of the main Rho effector, ROCK regulates MLC2 phosphorylation, which is necessary for actomyosin activation to provide essential contractile forces required for a diversity of cellular processes, such as cell contraction, cytokinesis, cell migration, and membrane blebbing (Kawano et al., 1999; Sebbagh et al., 2001). ROCK stimulates MLC2 phosphorylation at Ser19 and inactivates myosin phosphatase, producing actomyosin contractility (Amano et al., 1996; Narumiya et al., 1997; Ueda et al., 2002).

Our study has shown that MYH10 is the only non-muscle myosin II that accumulates in the contractile ring during MK mitosis and endomitosis, but its expression is silenced during MK maturation. This induces the switch from mitosis to endomitosis and allows MK polyploidization. MYH9 is well expressed during MK differentiation, but is poorly or not recruited in the contractile ring whether MKs were in mitosis or endomitosis (Lordier et al., 2008). These results reveal that MYH9 and MYH10 may display distinct functions during MK polyploidization and cytokinesis.

We have also demonstrated that RUNX1 mediates MYH10 silencing, which is required for the switch from mitosis to endomitosis, linking polyploidization with MK differentiation (Lordier et al., 2012a). The mechanism responsible for the specific accumulation of MYH10 in the MK contractile ring is not yet understood. As one characteristic of MK endomitosis is the low level of RhoA activation in the contractile ring, an attractive hypothesis would be that MYH10 accumulation in the contractile ring is more sensitive to Rho activation than MYH9. Alternatively, MYH9 and MYH10 may not have the same scaffold proteins to localize in the contractile ring and those specific for MYH9 may be lacking in MKs. Further experiments will be required to solve this question, which is one of the key determinants for understanding MK polyploidization and its regulation.

Furthermore, it has been shown that in FPD-AML patient (germ-line mutation of RUNX1), MYH10 is not silenced in MKs. It will be important to determine if both MYH9 and MYH10 have similar properties in PPF and platelet abscission because these patients have a thrombocytopenia. This type of experiments will require the use of knock in mice in which MYH10 is placed in the MYH9 locus

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(Figure 11). These experiments are ongoing in our laboratory and suggest that MYH10 is capable to induce the contractile forces necessary for platelet release, but in a less efficient manner than MYH9.

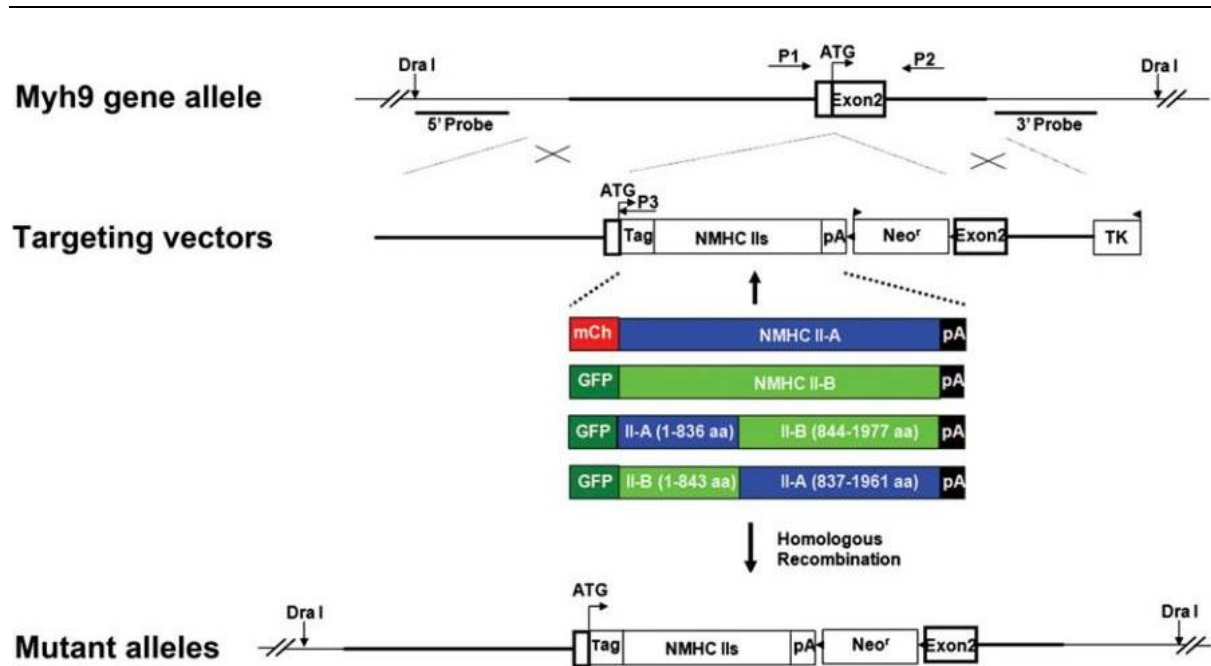
It has been shown that Rho/ROCK stimulates the actin/myosin contractile activity that may accelerate membrane retraction (Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005; Raftopoulou and Hall, 2004). This may inhibit the pseudopodia processes. In contrast, studies in fibroblasts have revealed that the two other major members of the Rho-GTPase family, Cdc42 and Rac, promote membrane protrusion. The fibroblast model has been extended to study the effects of Rho-GTPase in neurite outgrowth and differentiation. Rac and Cdc42 promote neurite formation, while Rho activation antagonizes this effect and causes neurite retraction (Etienne-Manneville and Hall, 2002; Govek et al., 2005; Van Aelst and Cline, 2004). On the basis of the neurite model, we have hypothesized that in MKs, Rac or Cdc42 may facilitate the protrusion forces required for PPF, whereas Rho/ROCK may generate opposite forces repressing cytoplasm extensions by MLC2 phosphorylation. At the early stage of MK differentiation, Rho activation predominates and restrains PPF. At the end of MK differentiation, the decrease of Rho activation coupled to an increase in Cdc42 activation confers an advantage to protrusion forces leading to PPF. Thus, when the Rho/ROCK pathway is inhibited at the onset of PPF in cultured MKs, the equilibrium in contractile forces in MKs is disrupted and leads to an increase in PPF. The fact that a dominant-negative expression of N-WASp prevents PPF by blocking actin fiber assembly along the demarcation membranes (Schulze et al., 2006) supports this hypothesis.

The molecular mechanisms leading to platelet release in the blood flow is not completely understood. Platelet biogenesis depends on the PPF, which is under the control of cytoskeleton organization. Cytoskeleton plays an important role in proplatelet elongation and platelet release. The ploidy level of MK may influence the number of produced platelets. During maturation, MKs increase cell size by proteins synthesis and develop the DMS for PPF and platelet release. In parallel, actin/myosin activity increases to get more contractility for keeping the normal MK shape. The regulation of Rho/ROCK through MLC2 phosphorylation may be associated with proplatelet constrictions and platelet shedding. Phosphorylated-MLC2 localizes along the cytoplasm extension, particularly in the swellings and tips of proplatelet. The increased expression of actin and phospho-MLC2 suggests that actin/myosin contractility might be necessary to keep a normal cell shape during polyploidization (Chang et al., 2007a). When MLC2 phosphorylation is inhibited, platelet size is increased in the absence of enough contractile forces, suggesting that actin/myosin may provide forces for both constrictions and retraction of proplatelet and regulation of platelet release and size. Previous data from *in vitro* and *in vivo* models have revealed the key role of the Rho/ROCK/myosin II pathway in PPF (Chang et al., 2007a; Suzuki et al., 2013). The Rho/ROCK pathway is implicated in platelet shape changes during activation by regulating MLC2 phosphorylation (Suzuki et al., 1999; Watanabe et al., 2001). MKs

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may interact with extracellular matrix to regulate platelet production. MKs adhesion on collagen type I leads to an inhibition of PPF likely through the activation of the Rho/ROCK pathway (Sabri et al., 2004). In addition, there is evidence showing that proplatelet elongation is regulated by MTs, while actin can play a negative role in PPF through Rho/ROCK effectors such as LIM kinase, which can inactivate the actin depolymerizing protein cofilin (Maekawa et al., 1999; Ohashi et al., 2000). Through inhibition of MLC2 phosphorylation by either Tat-C3 or Y27632 or P18, previous results of our group have revealed that the Rho/ROCK pathway negatively regulates PPF through MLC2 phosphorylation (Chang et al., 2007a). However, inhibition of ROCK by Y27632 or myosin II by blebbistatin disturbs MK stress fiber formation, but also modifies tubulin polymerization by tubulin polymerization assays. Furthermore, inhibition of ROCK and myosin II increases the ratio between Glu-tubulin/Tyr-tubulin, suggesting that ROCK and myosin II inhibit PPF by simultaneously increasing contractile force from actomyosin and reducing the stability of MTs.

Some human platelet disorders are associated with an increase in platelet size, which are due to defects in actin/myosin activation (Balduini et al., 2002). The Bernard-Soulier syndrome is associated with an absence or a decreased expression of the GPIb-GPIX-GPV complex that is linked to the membrane actin cytoskeleton and may participate in cytoskeleton transduction signaling leading to normal platelet formation. MYH9 is the only myosin isoform present in platelets, while the May-Hegglin syndrome is a rare inherited human macrothrombocytopenia caused by mutations in the MYH9; the precise effects of these mutations on MYH9 functions remain controversial. However, the fact that MYH9 mutations in human lead to a macrothrombocytopenia underscore the role of actomyosin in platelet production (Kunishima and Saito, 2010).



**Figure 11:** Generation of the Knock-in Mouse Lines.

Knock-in mice lines were generated for further studying the functions of MYH9 and MYH10 in PPF and platelet production. MYH10 is placed in the MYH9 locus: MYH9 first coding exon is disrupted either by a cDNA encoding GFP target human NMII-B ( $Ab^*/Ab^*$  mice) or by a cDNA encoding chimeric GFP-hNMHCII-AB (the N-terminal domain of NMII-A is fused to the C-terminal II-B domain,  $A^{ab}/A^{ab}$  mice) (Figure from Wang (Wang et al., 2010)).

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## **Rho/DIAPH1 pathway during megakaryopoiesis**

Formins are a conserved family of proteins that play key roles in cytoskeleton remodeling, including nucleate and elongate non-branched actin filaments and also modulate MT cytoskeleton dynamics (Bartolini and Gundersen, 2010; Schirenbeck et al., 2005). The PPF is associated with morphological changes that require profound actin and MT cytoskeleton reorganization. mDia is a member of formin family that acts as an effector downstream Rho-GTPase. Once activated RhoA binds to mDia and regulates the cytoskeleton dynamics. DIAPH1 (mDia1 or Drf1) has critical roles in actin remodeling in cell division and in response to adhesive and migratory stimuli. DIAPH1 coordinates MTs and the actin cytoskeleton through its FH2 and FH1 domains, respectively and thus appears as a good candidate for regulating MK differentiation and PPF.

DIAPH1 colocalizes with tubulin, F-actin or MYH9 in mature MK along the plasma membrane and along PPF in the swelling and in the tips. This co-localization may be important to understand the role of DIAPH1 in actin or tubulin cytoskeleton regulation and cooperation with myosin II in cortical contractile forces around MK.

Actin assembly is not required for MKs to extend proplatelets, but actin filaments are enriched at the sites of proplatelet bifurcation, suggesting that actin is essential for proplatelet branching structures leading to amplification of the number of proplatelet tips and thus of the produced platelets (Italiano et al., 1999a). DIAPH1 knockdown increases the number of PPF, while expression of a DIAPH1 active form decreases PPF. These results suggest that DIAPH1, in addition to ROCK, is the other important effectors of Rho during megakaryopoiesis, particularly in PPF. Therefore, DIAPH1 could be an important Rho effector involved in PPF in cooperation with ROCK by its dual function on actin and MT cytoskeleton.

On the other hand, the elongation of proplatelet required the forces derived from tubulin polymerization, MTs sliding, or may be a combination of both processes, while actin filament assembly may transmit contractile forces to the MT bundle to help its compression during loop formation (Italiano et al., 2007; Schwer et al., 2001; Thon et al., 2012; Thon et al., 2010). By knockdown or expression of an active form, our results have shown that DIAPH1 negatively regulates the PPF, with a direct effect on MT stability during MK differentiation, suggesting that DIAPH1 plays a negative role in MT polymerization and stability. Together, these data show that DIAPH1 could negatively regulate PPF by regulating MT reorganization. Thus, activated DIAPH1 could restrict PPF by two mechanisms, one by inducing stress fiber formation that generating contractile force, and the other by destabilizing MTs.



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Interestingly, it has been reported that activated formins have essentially a stabilizing effect on MTs by increasing both MT deetyrosination and acetylation (Ishizaki et al., 2001; Palazzo et al., 2001; Thurston et al., 2012). In contrast, our results show that DIAPH1 destabilizes MTs during PPF. Similar results have been reported in osteoclasts, where inhibition of Rho correlates with an increased MT stabilization; however, this is mediated by mDia2, but not mDia1 through tubulin acetylation (Destaing et al., 2005). We did not observe any significant change in MT acetylation level when we knockdown or express the active form of DIAPH1 in human MKs, suggesting that regulation of tubulin acetylation is mediated more by mDia2 or others formins than by mDia1. Thus, these different observations underscore the complexity of the DIAPH pathway on MT stability or destabilization, which may depend on the cellular context.

Furthermore, mDia may mediate important crosstalk between the actin and tubulin cytoskeleton. In mammalian cells, mDia modulates MT and actin reorganization (Ishizaki et al., 2001; Kato et al., 2001; Li and Higgs, 2003; Palazzo et al., 2001). Expression of an active form of mDia1 induces bipolar elongation of HeLa cells and alignment of MTs and F-actin bundles with the long axis of the cell (Ishizaki et al., 2001). However, mutation of conserved residues within the mDia1 FH2 domain abolishes this phenotype. As a member of formin and effector of RhoA, DIAPH1 affects actin stress fiber formation and the MT stability during PPF, suggesting that it regulates both actin and MT cytoskeleton. The precise mechanism by which DIAPH1 mediates stable MTs and coordinates MT and actin cytoskeletons will require further studies. It is not known whether this dual activity on actin and tubulin is restricted to certain formins such as DIAPH1 or represents a general property of this protein family.

PPF and platelet release are two independent processes. DIAPH1 knockdown increases PPF and normally it is expected that it would lead to a higher platelet production if the platelet release process is normal. However, DIAPH1 knockdown only induces a slight increase in platelet number, implying that DIAPH1 is implicated in the platelet release process, but in a positive way. Otherwise, there should be a parallel increase in the number of platelets produced and in PPF. These results suggest the DIAPH1 contributes to the platelet release process. Thus DIAPH1 may have two opposite effects in platelet production: decreasing PPF and increasing platelet abscission. Further studies will be required to precisely demonstrate that DIAPH1 is implicated in the platelet abscission and how it coordinates the forces required. Whatever, this implies that DIAPH1 must be tightly regulated during platelet production.

### **Linkage between ROCK/Myosin and DIAPH1 pathway during megakaryopoiesis**

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Most formins are Rho-GTPase effectors that nucleate, elongate and bundle actin filaments to support polarized cell division, adhesion and migration (Goode and Eck, 2007; Wallar and Alberts, 2003). The Rho-GTPases may induce specific types of actin cytoskeleton and regulate MT dynamics. GTP-bound RhoA binds to and directly activates formins by disrupting the DID-DAD intramolecular auto-inhibition that induces the binding through the FH1-FH2 domain to actin filaments and MTs and mediates their assembly (Schonichen and Geyer, 2010). Furthermore, cooperation between the RhoA effectors ROCK and the formins is required for the formation of stress fibers as required both F-actin polymerization and activation of myosin contractility for proper function (Narumiya et al., 2009).

At the end of maturation, MKs extend their demarcation membranes to form proplatelets. Changes in cytoskeleton structures cause proplatelets to further extend, to re-localize organelles, to fragment and then to release platelets into the blood stream of marrow sinusoids. The RhoA/ROCK pathway clearly plays a negative role in PPF *in vitro* by inducing myosin II activation. Alternatively, DIAPH1 also negatively regulates PPF. DIAPH1 colocalizes with F-actin and MYH9 along the plasma membrane in mature MKs and along the PPF, especially in the swelling and the tips of proplatelets, suggesting that it could cooperate with myosin IIA to regulate cytoskeleton dynamics during PPF and to generate contractile forces restraining cytoplasmic extensions during PPF and keeping a normal platelet shape. Changes in MLC2 phosphorylation level could not be detected after DIAPH1 knockdown, implying that mDia1 may contribute to actomyosin assembly, only by promoting actin polymerization, but not by activating myosin II. The double inhibition of both DIAPH1 and ROCK/myosin pathway has an additive effect on PPF and increases even more the MT stability. These two main effectors of the Rho-GTPase pathway, DIAPH1 and ROCK/myosin II, are involved not only in Rho-mediated stress fiber assembly but also in MT stability and dynamics regulation during PPF. These results suggest that a cooperation between DIAPH1 and ROCK is required for the formation of cell structures dependent on actin-myosin to generate the contractile forces that restrain cytoplasmic extensions and finally to inhibit PPF during MK differentiation.

Interestingly, DIAPH1 expression increases during MK differentiation, but DIAPH1 knockdown increases PPF. How to explain these two results which seems controversial? In fact, MKs become polyploid during differentiation, with a great increase of cell size and a parallel elevation of actin content (Chang et al., 2007a; Raslova et al., 2007). This increased actin content may require more DIAPH1 expression to regulate its polymerization, which is required for normal cell functions and cell shape. This may explain why DIAPH1 expression is increased during MK differentiation, particularly between day 6 and day 9 of culture when polyploidization occurs. However, at the end of differentiation, MK needs to promote membrane protrusion to form proplatelet. Actin and myosin could generate contractile forces restricting cytoplasm extensions and keeping a normal cell shape.

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Hyperactivation of actin assembly may impair the ability of MKs to alter membrane plasticity. Thus, DIAPH1 knockdown, as well as inhibition of ROCK/myosin II activity, could disrupt the force equilibrium and facilitate PPF. In addition, it is possible that DIAPH1 plays an important role in very late stages of MK differentiation by regulating platelet release. Thus, it appears that the regulation of DIAPH1 is certainly as important as its level of expression during MK differentiation.

In the present work, our results have shown the important role of DIAPH1 in the reorganization of the cytoskeleton during PPF in human MKs. However, the mechanism by which DIAPH1 acts on actin and tubulin during megakaryopoiesis should be studied in more details. Moreover, it should be important to investigate the effects of DIAPH1 mutated forms, such as those carrying mutations of the DID and/or the DAD domain to evaluate the role of DIAPH1 auto-activation in the regulation of MK differentiation and PPF. As mentioned before, both DIAPH1 and ROCK/myosin could negatively regulate PPF. However, the results of our group have shown that the ROCK/myosin pathway participates in the regulation of MK polyploidization. Our preliminary results show that knockdown of DIAPH1 slightly increases the polyploidization in *in vitro* cultured MKs, so it will be important to investigate the contribution of DIAPH1 to MK polyploidization. It will be also important to understand by which precise mechanisms DIAPH1 is capable to destabilize microtubules in MKs whereas it increases their stability in other cell types and if the FH2 domain is implicated in both processes.

Our work has revealed that DIAPH1 knockdown does not affect the expression of DIAPH2 and DIAPH3 in human MKs. Various formin family members are present in MKs and platelets, such as FHOD1, INF2 or DAAM1, thus there might be redundancy among this large protein family, at least for some functions. Therefore, it will be very interesting to elucidate whether other formins are involved in PPF and if they have redundancy or synergy effects on cytoskeleton organization during PPF and MK migration.

It has been shown that mDia2 is crucial for mammalian cell cytokinesis by providing an actin scaffold for contractile ring assembly and maintaining its position in the middle of a dividing cell (Watanabe et al., 2008). The expression of the two other DRFs, DIAPH2 (mDia3) and DIAPH3 (mDia2), decreases during MK differentiation. Thus, it will be very interesting to study if this silencing is required for MK polyploidization and PPF, particularly the function of DIAPH3 (mDia2) in cytokinesis and cell migration.

In addition, inhibition of DIAPH1 may increase SDF1-mediated MK migration, which is important for proper MK localization in the marrow. Both actin and MT cytoskeleton undergo continuous remodeling during PPF and serve as motor machineries for MK migration. Previous studies have

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shown that MAL/SRF complex was involved in the MK migration by regulating MYL9 and MMP9 (Gilles et al., 2009). Other evidence also reveals that Rho, mDia or Rho/ROCK are implicated in cell migration (O'Connor and Chen, 2013; Schofield et al., 2012; Shinohara et al., 2012). Thus, future studies should be focused on the precise functions and mechanisms by which Rho/DIAPH1 or Rho/ROCK affect MK migration, as well as the cooperation between DIAPH1 and ROCK/Myosin occurs during MK migration towards SDF-1 and during interaction with extracellular matrix, such as collagen I, fibronectin, vWF and fibrinogen.

Formins as potential activators of actin assembly in the cytoplasm and in turn of cytoplasmic actin polymerization may promote actin release from MAL protein for SRF transcriptional activity, while serum stimulates actin assembly inside the nucleus in a formin-dependent manner. Thus, it is interesting to study if DIAPH-mediated actin polymerization within the nucleus could control the serum-dependent MAL/SRF activity and related genes expression, which also may affect the PPF and MK migration.

Altogether, understanding of the molecular mechanism responsible for PPF and platelet release are important for basic research and understanding the mechanisms of some thrombocytopenia and thrombocytosis, but it may also important for improving the techniques to produce platelets *in vitro*, which may have consequences in the future for transfusion of *ex vivo* produced platelets.

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## Scientific contributions

### Publications:

1. **Pan J**, Lordier L, Meyran D, Rameau P, Lecluse Y, Kitchen-Goosen S, Badirou I, Mokrani H, Narumiya S, Alberts AS, Vainchenker W and Chang Y. The formin DIAPH1 (mDia1) regulates megakaryocyte proplatelet formation by remodeling the actin and microtubule cytoskeletons. *Blood*. 2014. Oct 8. pii: blood-2013-12-544924.
2. Badirou I, **Pan J**, Legrand C, Wang A, Lordier L, Boukour S, Roy A, Vainchenker W, Chang Y. Transgenic mouse models demonstrate the Carboxyl-terminal depended recruitment of non-muscle myosin II to megakaryocyte contractile ring during polyploidization. *Blood*. 2014. Sep 2. pii: blood-2014-06-584995.
3. Lordier L, **Pan J**, Naim V, Jalil A, Badirou I, Rameau P, Larghero J, Debili N, Rosselli F, Vainchenker W, Chang Y. *Cell Cycle*. Presence of a defect in karyokinesis during megakaryocyte endomitosis. *Cell Cycle*. 2012, 11(23):4385-9. **Lordier L and Pan J contributed equally to this work.**
4. Lordier L, Bluteau D, Jalil A, Legrand C, **Pan J**, Rameau P, Jouni D, Bluteau O, Mercher T, Leon C, Gachet C, Debili N, Vainchenker W, Raslova H, Chang Y. RUNX1-induced silencing of non-muscle myosin heavy chain IIB contributes to megakaryocyte polyploidization. *Nat Commun*. 2012. 6(3):717.

### Manuscripts under review:

- Idinath Badirou, **Jiajia PAN**, Sylvie Souquere, Celine Legrand, Gérard Pierron, Aibing Wang, Larissa Lordier, Anita Roy, Christian Gachet, William Vainchenker, Catherine Leon and Yunhua Chang. Distinct localizations and roles of nonmuscle myosin II during proplatelet formation and platelet release. (*Journal of Thrombosis and Haemostasis*)

### Conferences:

05/2012, Les 8<sup>èmes</sup> journées scientifiques de Roscoff. Poster presentation.

10/2012, Les 19<sup>èmes</sup> congrès 2012 du CHO (Club Hématopoïèse et Oncogénèse). Poster presentation.

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