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Departamento de Biología Molecular

# Unconventional cell cycles in polyploidization
# The megakaryocyte paradigm

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CERTIFICA

Que la Tesis Doctoral titulada "Unconventional cell cycles in polyploidization: The megakaryocyte paradigm" ha sido realizada en el Centro Nacional de Investigaciones Oncológicas y tutelada en el Departamento de Biología Molecular de la Universidad Autónoma de Madrid.

El trabajo realizado por Marianna Trakala reúne todas las condiciones requeridas por la legislación vigente y la originalidad y calidad científica para poder ser presentada y defendida con el fin de optar al grado de Doctor.

Y para que conste donde proceda, firmo el presente certificado.

Madrid a 5 de Mayo de 2014

Dr. Marcos Malumbres
Tutor y Director de la Tesis

**A**

ACBD5: Acyl-CoA Binding Domain containing 5

Ad: Adenovirus

AMKL: Acute MegaKaryoblastic Leukemia

ANKRD26: Ankyrin Repeat Domain 26

APC/C: Anaphase-Promoting Complex/Cyclosome

ATM: Ataxia Telangiectasia Mutated

ATR: ATM and Rad3-related

ATP: Adenosine TriPhosphate

**B**

BM: Bone marrow

BSA: Bovine Serum Albumin

β-TrCP: β-Transducin repeat-Containing Protein

**C**

C: sets of chromosomes

Cdc20: cell division cycle 20

Cdc25: Cell division cycle 25

Cdk: Cyclin-dependent kinases

CFP: Cyan Fluorescence Protein

CLP: Common Lymphoid Progenitor

CMP: Common Myeloid Progenitor

CMV: Cytomegalovirus

CPC: Chromosome Passenger Complex

**D**

DAPI: 4',6-diamidino-2-phenylindole

DMEM: Dulbecco's modified Eagle's medium

DMS: Demarcation Membrane system

DTT; Dithiothreitol

**E**

EDTA: Ethylenediaminetetraacetic acid

ERK: Extracellular signal-Regulated Kinases

**F**

FACS: Fluorescence Activated Cell Sorting

FBS: Fetal Bovine Serum

FITC: Fluorescein IsoThioCyanate

FL: Fetal Liver

**G**

G1: Gap phase 1

G2: Gap phase 2

GATA-1: Globin Transcription factor 1

GFP: Green Fluorescence Protein

GMP: Granulocyte-Macrophage Progenitor

GTPase: Guanine nucleotide triphosphatase

γH2AX: phosphorylated histone H2AX

**H**

HBSS: Hanks' Balanced Salt solution

HEK293: Human Embryonic Kidney

HEL: Human Erytroblastic Leukemia

HEPES: 4-(2-HydroxyEthyl)Piperazine-1-EthaneSulfonic acid

HSC: Hematopoiestic Stem Cells

**I**

IMDM: Iscove's Modified Dulbecco's Medium

**K**

KFP: Katushka Fluorescence Protein

**L**

LSK: $Lin^-$-$Sca^+$-$kit^+$

**M**

MACS: MAgnetic Cell Sorting

MAPK: Mitogen Activated Protein Kinases

Mastl: Microtubule-associated serine/threonine-protein kinase-like

MBP: Myelin Basic Protein

MCM: Mini-Chromosome Maintenance

MEFs: Mouse Embryonic Fibroblasts

MEP: Megakaryocyte-Erythrocyte Progenitor

MOI: Multiplicity Of Infection

Mpl: Myeloproliferative, leukemia protein

**N**

NHS: N-HydroxySuccinimide

NOC: Nocodazole

**O**

ORC: Origin Recognition Complex

**P**

PBS: Phosphate-Buffered Saline

PE: phycoerythrin

PGK: Phospho Glycerate Kinase

pH3: phospho-Histone H3

Plk1: Polo-like kinase 1

PMSF: PhenylMethylSulfonyl Fluoride

PP2A: Protein Phosphatase 2

PreRCs: Pre-Replication Complexes

PrP: Platelet rich Plasma

**R**

Rb: Retinoblastoma

RhoA: Ras homolog gene family, member A

RPMI: Roswell Park Memorial Institute medium

RT: Room Temperature

**S**

SCF: Skp1–Cul1–F-box protein complex

SDS-PAGE: Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis

Skip1: S-phase kinase-associated protein 1

Skip2: S-phase kinase-associated protein 2

**T**

THC2: Thrombocytoepenia 2

TGC: Trophoblast Giant Cells

TPO: Thrombopoietin

**U**

U2OS: Human bone OsteoSarcoma

UTR: UnTranslated Region

**V**

VSVG: Vesicular Stomatitis Virus Glycoprotein

VWF: Von Wilebrand Factor

**W**

WT: Wild Type

# Presentación

# Presentación

La poli-ploidización es un proceso natural mediante el cual diversos tipos celulares aumentan la ploidía mediante la replicación del ADN en ausencia de segregación cromosómica. Este proceso forma parte del desarrollo normal de varios tejidos en plantas y animales, aunque las alteraciones en ploidía son también un hecho característico de la transformación maligna en cáncer. Los megacariocitos normalmente sufren varias rondas de poli-ploidización para convertirse en células gigantes capaces de generar plaquetas, aunque la base molecular de este processo se desconoce en detalle. En el presente estudio hemos analizado la relevancia de varios reguladores del ciclo celular en el proceso de megacariopoiesis y en los eventos de poli-ploidización que tienen lugar durante la maduración de megacarioticos. Mastl es una quinasa que inhibe fosfatasas durante mitosis y cuyo gen se encontró inicialmente mutado en pacientes con trombocitopenia. Mediante la generación de ratones *knockin* que llevan la mutación correspondiente, hemos encontrado que Mastl se requiere para atenuar la actividad de los complejos PP2A-B55 y modular la señalización por MAPK durante la maduración de estas células. Para profundizar en el control de la maduración de megacariocitos y la formación de plaquetas, hemos analizado el efecto de la eliminación genética en estas células de varios reguladores del ciclo celular que participan en la entrada, progresión y salida de mitosis. El complejo APC/C (Anaphase-promoting complex)-Cdc20 es necesario para la salida de endomitosis ya que los megacariocitos deficientes en Cdc20 se detienen permanentemente en mitosis dando lugar a trombocitopenia severa en los animales. El APC/C es sin embargo prescindible para la producción de plaquetas cuando se activa por el cofactor Cdh1, una proteína requerida para endoreplicación en otros sistemas. Sorprendentemente, la eliminación de Cdk1, una quinasa esencial para la mitosis en todas las células analizadas, no produce trombocitopenia. Esto se debe a la capacidad de los megacariocitos deficientes en Cdk1 de endoreplicar como un ciclo celular alternativo. Los megacariocitos deficientes en Cdk1 generan plaquetas de manera normal cuando se estimulan con TPO. La eliminación concomitante de la quinasa Cdk2 en megacariocitos deficientes en Cdk1 resulta en eventos de re-replicación acompañados por estrés replicativo. Inesperadamente, estos megacariocitos son capaces de madurar apropiadamente y formar plaquetas utilizando re-replicación en lugar de endomitosis o endoreplication. La ablación genética de Cdk1 o Cdk1 y Cdk2 es capaz de rescatar los defectos en el número de plaquetas observados en los ratones deficientes en Cdc20; indicando que la endoreplicación y la re-replicación es capaz de reemplazar funcionalmente a la endomitosis in vivo. En conjunto, este análisis sugiere que la poli-ploidización funcional puede ser obtenida a través de múltiples endociclos alternativos in vivo. Estos datos no sólo arrojan luz sobre cuestiones básicas de la biología celular, sino que también pueden tener interés en posibles nuevos enfoques terapéuticos contra los ciclos celulares mitóticas en células cancerígenas.

# *Abstract*

**Abstract**

Polyploidization is a natural process by which specific cell types increase ploidy by replicating DNA in the absence of chromosome segregation. This process is part of the normal development of specific tissues in plants and animals, and altered ploidy is also observed as an accompanying feature of malignant transformation in cancer. Megakaryocytes normally poplyploidize to become mature giant cells able to generate platelets However, the molecular basis of polyploidization in these cells is mostly unknown. In the present study we have analyzed the relevance of well-established cell cycle regulators in the process of megakaryopoiesis and more specific in the particular polyploidization events that take place during the development and maturation of these platelet-producing cells. Mastl, a kinase regulating phosphatase complexes during mitosis, was initially found in humans as a gene mutated in human thrombocytopenia. By generating knockin mice carrying the corresponding mutation, we describe here that this protein is required to attenuate PP2A-B55 activity and to modulate MAPK signaling and proplatelet formation during the development of megakaryocytes. To further understand the control of megakaryocyte maturation and platelet formation by cell cycle regulators, we specifically ablated several cell cycle regulators involved in mitotic entry, progression and exit, using a megakaryocyte-specific Cre model in the mouse. Using these models, we demonstrate here that unperturbed megakaryocytes undergo endomitosis *in vivo,* a process in which cells replicate the DNA but chromosomes are not segregated due to incomplete mitosis. The Anaphase-promoting Complex (APC/C)-Cdc20 is necessary for endomitotic exit since Cdc20-null megakaryocytes were arrested in mitosis leading to severe thrombocytopenia. However, APC/C is dispensable for platelet production when activated by the Cdc20-related cofactor Cdh1, a protein required for endoreplication in other systems. Strikingly, ablation of Cdk1, a cell cycle kinase essential for mitosis in all cells analyzed, does not lead to thrombocytopenia development. The reason behind this observation is the ability of Cdk1-null megakaryocytes to endoreplicate as an alternative cell cycle. Mature megakaryocytes arising from endoreplicative cell cycles were able to shed platelets upon TPO stimulation *in vitro,* even though this procedure was carried out less efficiently. Further ablation of Cdk2 kinase in Cdk1-null megakaryocytes resulted in re-replication events. Unexpectedly, megakaryocytes were able to undergo functional polyploidization using re-replication and Cdk1; Cdk2 double mutant mice displayed normal differentiation of megakaryocytes in the absence of thrombocytopenia. Interestingly, genetic ablation of Cdk1 and Cdk2 rescued platelet defects in Cdc20-deficient mice indicating that endoreplication and re-replication can functional replace endomitosis in vivo. Altogether, this analysis suggests the presence of multiple endocycles that can be alternatively used during functional polyploidization. These data not only shed light on basic cellular biology questions but may also be of relevance for possible therapeutic approaches against cancer mitotic cell cycles.

# 1. Introduction

## 1.1. Genome ploidy in eukaryotes

### 1.1.1. Polyploidy

Multicellular organisms have a wide variety of cells in order to accomplish their diverse and complex necessities. One interest aspect of this cellular diversity is that many cells in diploid organisms are polyploid. Polyploid cells that contain multiple sets of chromosomes were first observed in plants in 1910 (Strassburger 1910) since then, polyploidy has been studied in a variety of organisms from bacteria to higher eukaryotes as well as in humans. Polyploidy can take place as part of a well-coordinated developmental program but also as a response to certain stresses, either physiological or not, which in turn can lead to pathological conditions and disease (Figure 1.1). For this reason is important not only to understand the requirements for polyploidy in several tissues in the mammalian organisms but also the molecular mechanisms that underlay this process and how they can be modulated.

Polyploidy mainly arises from cell cycles known as endocycles which are divergent from the typical mitotic process, although there are also other mechanisms that can contribute to polyploidy such as cellular fusion (Edgar et al., 2014). A conventional cell cycle can be divided in two periods: Mitosis and interphase (Figure 1.2). The name "mitosis" comes from the Greek word "*mitos*" meaning thread to describe the threadlike chromosomes that appear just before cell division and then are separated longitudinally into two new cells resulting from the original cell. The interphase which actually prepares cells for division during mitosis can be subdivided further into distinct parts: the S phase (where S is for DNA "Synthesis") during which the cells will replicate its entire genomic material, and two Gap phases, G1 and G2, in which cells prepare to proceed to S phase or to mitosis respectively (Figure 1.2).
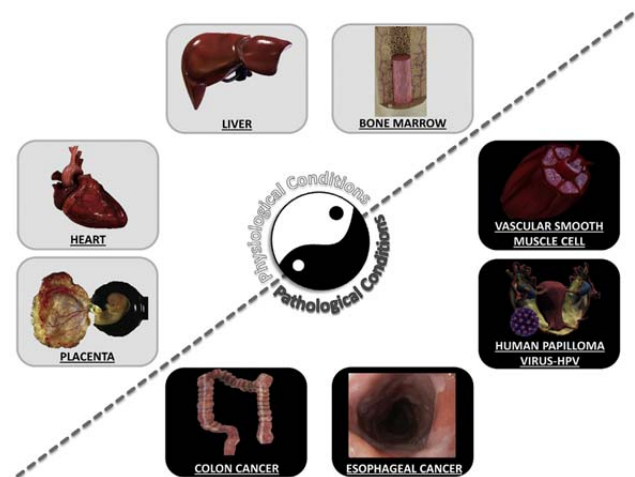


Figure 1.1. **Polyploidy during physiological and pathological processes**. Polyploid cells are generated during physiological processes, such as embryogenesis (placenta: trophoblast giant cells), postnatal development (heart: cardiomyocytes or liver: hepatocytes), and terminal differentiation (bone marrow megakaryocytes); and also during pathological processes, such as hypertension (vascular smooth muscle cells), virus infection (human papilloma virus), and tumorigenesis (esophageal and colon cancers).

### 1.1.2. Endocycles

Endocycling refers to unconventional cell cycling with variations from the canonical G1-S-G2-M cell cycle during which the cell executes S phase replicating its genome, either entirely or partially, but without segregation of the replicated genome into

two or more daughter cells. Endocycles use much of the same cell machinery that regulates the transition from G1 to S phase as mitotic cell cycles and mainly differ from mitotic cells in their capability to segregate chromosomes and divide (Edgar et al. 2014). Some examples of polyploid cells that result from endocycling in mammals are the trophoblast giant cells (TGCs) in the placenta, hepatocytes in the liver, and megakaryocytes in the bone marrow. Each of these cell types undergoes a different kind of endocycling such as endoreplication, endomitosis or acytokinetic mitosis. Although many variations of the nomenclature exit, we will define the different processes as shown in Figure 1.2.
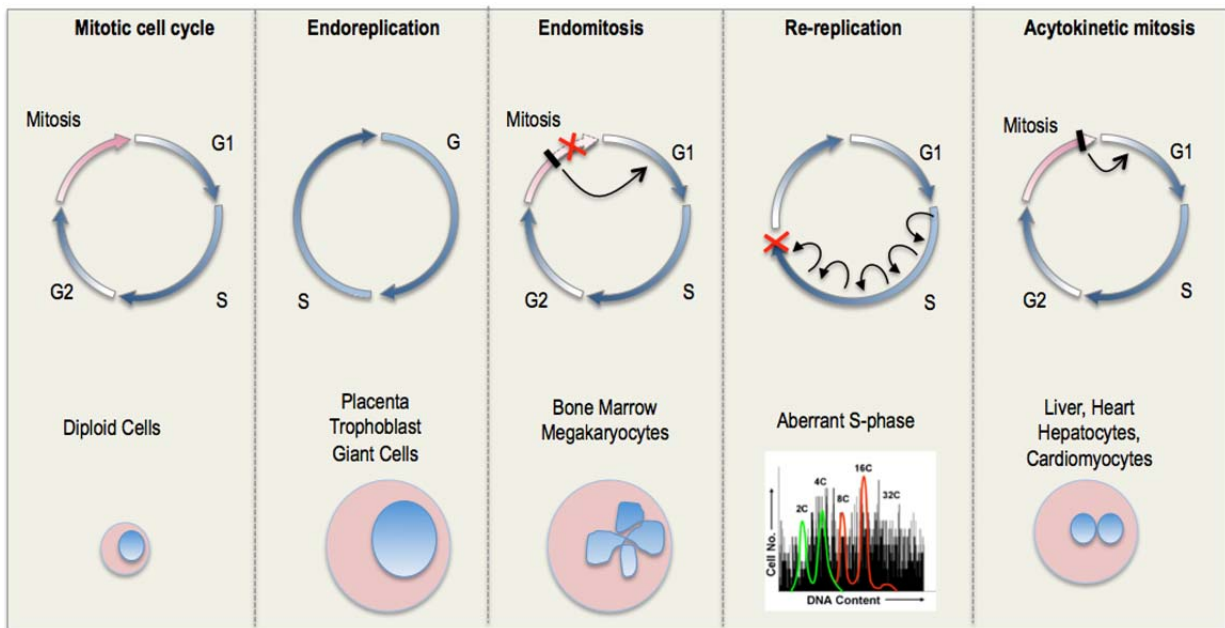


Figure 1.2. **Endocycles *versus* mitotic cell cycles.** Mitotic cell cycles are characterized by a gap phase (G1) prior to S-phase (S) where DNA synthesis occurs and then follows another gap phase (G2) prior to mitosis (M) during which the cell is divided in two daughter cells. Endocycling refers to cell cycles that result in polyploid cells and consists of variances of the conventional mitotic cell cycle. **Endoreplication** consists of G and S phases without entry into mitosis and trophoblast giant cells in the placenta arise from endoreplicative cell cycles resulting in a single polyploid nucleus. **Endomitosis** or abortive mitosis is the cell cycling during which cells do enter in mitosis but skip the late evens of anaphase, telophase, and cytokinesis. In late anaphase, the nuclear envelope reforms around the segregated chromosomes, leading to the formation of a single polylobulated nucleus. Megakaryocytes use endomitosis for polyploidization **Re-replication** results from aberrant regulation in which DNA synthesis is initiated multiple times at individual origins of replication within a single S phase. This results in an indistinct DNA content as depicted by black lines in this hypothetical FACS profile (Y-axis is cell number and X-axis is DNA content). Green represents the diploid mitotic cell cycle profile, with 2C and 4C peaks. Red represents endoreplication cycles that result in distinct populations of cells with more than a 4C DNA content. Although re-replication is not properly an endocycle, we will include it here for simplicity. During **Acytokinetic mitosis,** cells do enter mitosis and segregate chromosomes but fail to perform cytokinesis resulting in binucleated cells. Hepatocytes in liver and cardiomyocytes in heart during development polyploidize through acytokinetic mitosis.

Endoreplication refers to an endocycle in which successive rounds of S-phase followed by G-phase occur without intervening mitosis, resulting in cells with a single polyploid nucleus (Figure 1.2). This process of endoreplication has been extensively investigated in flies (*D. melanogaster*) and TGCs (Edgar and Orr-Weaver, 2001; Lee et al. 2009; Edgar et al., 2014; Sher et al. 2013). In contrast to *Drosophila* endocycling in which heterochromatin and specific euchromatin regions are underreplicated and reduced in copy number, in TGCs the genome is uniformly and integrally duplicated by clearly delineated genome doublings. This is an important distinction from the aberrant process of re-replication which is characterized by uncontrolled, continuous reinitiation of DNA synthesis within a given S phase, leading in increases in DNA content without clearly recognizable genome doublings (Arias & Walter 2007). Re-replication can lead as well to polyploidy even though it does not exist in nature and can result only from perturbations to the molecular mechanisms that control the ''once and only once'' firing of replication origins during a normal diploid S phase, and is thought to be a source of genome instability that contributes to cancer.

Another major form of endocycling is the acytokinetic mitosis during which cells that have entered and finished mitosis do not complete cytokinesis, giving rise to binucleated cells. This process occurs during liver and heart development to produce multinucleated hepatocytes (mainly binucleated) or cardiomyocytes. In contrast to cardiomyocytes, which generate a contractile cytokinetic ring that is not functional for separating daughter cells (Engel et al. 2006), hepatocytes never form an actinomyosin ring during the binucleation process (Margall-Ducos et al. 2007; Duncan 2013).

Endomitosis (also known as abortive mitosis) is another type of endocycle that has been described to take place in megakaryocytes, the cell precursors of platelets. Normal mitotic cells undergo nuclear envelope breakdown in prometaphase (P/M), alignment of chromosomes to the equator of the cells in metaphase (M) and chromatide segregation in anaphase (A). Then, nuclear envelope reformation occurs in telophase (T) and the cytoplasm is splitted into the two new nuclei to form two completely separated daughter cells during cytokinesis (Figure 1.3). To reach polyploidy through endomitosis, cells enter mitosis but skip the late evens of anaphase, telophase, and cytokinesis (Figure 1.3). In anaphase, the nuclear envelope reforms around partially segregated chromosomes, leading to the formation of a single polylobulated nucleus with a genome content up to 64-128 sets of chromosomes (64-128C) in humans, where each lobe presumably contains a diploid genome (Ravid et al. 2002). Eventually after several rounds of polyploidization megakaryocyte suffer a process of maturation during which cells are withdrawn from the cell cycle in a senescent-like state (Besancenot et al. 2010). Megakaryocyte maturation comes to completion with the release of platelets into the bloodstream.

**Figure 1.3. Mitotic and endomitotic cell cycles.** Mitotic cells undergo nuclear envelope breakdown in prometaphase (P/M), alignment of chromosomes to the equator of the cells in metaphase (M) and chromatid segregation in anaphase (A). These cells then proceed to the last part of mitosis (telophase, T) during which nuclear envelope reformation and furrow ingression occurs. The cytoplasm is then divided into two new nuclei to form two completely separated daughter cells during cytokinesis. In the endomitotic cell cycle, cells enter mitosis but skip the late evens of anaphase, telophase, and cytokinesis. In anaphase, the nuclear envelope reforms around the segregated chromosomes, leading to the formation of a single polylobulated nucleus with a genome content up to 64C in the mouse or 128C in humans, where each lobe presumably contains a diploid genome. Eventually after several rounds of polyploidization megakaryocyte suffer a process of maturation during which cells are withdrawn from the cell cycle in a senescent-like state. Upon maturation, megakaryocytes form pro-platelets that are ablt to release platelets in the blood stream.

The currently favored model of platelet production recognizes that differentiated megakaryocytes extend long cytoplasmic processes, designated proplatelets. Proplatelets function as the assembly lines of platelet production and comprise platelet-sized swellings in tandem arrays that are linked by thin cytoplasmic bridges (Figure 1.3).

## 1.2. Molecular basis of the cell cycle

To investigate the molecular basis that determines endocycling we need first to review the molecular basis that underlies the normal mitotic cell cycle since endocycles use much of the same cell cycle machinery.

## 1.2.1. The mitotic cell cycle

The first requirement for cell cycle progression from G1 to S phase relies on the expression of cell cycle genes as a result of numerous mitogenic signals. These signals trigger the inactivation of the retinoblastoma protein (pRb) through phosphorylation by cyclin-dependent kinases (Cdks). pRb inactivation results in the activation of several transcription factors and in a massive transcription during G1 of genes required for the subsequent cell cycle phases (Malumbres & Barbacid, 2001). The Cdk-cyclin responsible for the first inactivating wave of pRb is the Cdk4/6-cyclin D complex. Partial inactivation of pRb and the other pocket family proteins p107 and p130 allows the activation of several transcription factors such as the E2F family responsible for the expression of genes required for the entry into the S-phase. One important target of E2F is cyclin E which can bind and activate Cdk1 and Cdk2. Cdk2-Cyclin E is thought to participate in pRb hyperphosphorylation and its complete inactivation and the subsequent entry into S-phase (Malumbres & Barbacid 2001), although this activity is dispensable in normal tissues (Ortega et al. 2003).

Once Cdk activity raises this allows the firing of the replication origins that have been "licensed" during the G1 phase. Licensing refers to the loading of the pre-replication complexes (PreRCs) onto the replication origins. Origin firing determines S-phase entry. Cdk1/2-cyclin A is then responsible to ensure the ''once and only once'' firing of replication origins by preventing the origin relicensing during late S-phase and G2 (Hook et al. 2007). During G2, the cell ensures that the genome doubling has been completed properly by the activation of checkpoints and prepares the cell for entry into mitosis.

An important event during G2 is centrosome organization during which the duplicated centrosomes move towards different poles of the cells. In addition to some Cdk family members, other kinases are specifically dedicated to drive centrosome duplication and maturation and to increase their ability to nucleate microtubules during these stages, including Aurora and Polo-like kinases or the never in mitosis a (NIMA)-related kinases (Neks; Malumbres, 2011). The critical molecular activity required for entry into mitosis is Cdk1 kinase. During the G2/M transition, a large number of proteins are phosphorylated in a Cdk1-dependent manner resulting in cytoplasmic changes, Golgi disassembly, nuclear envelope breakdown, and condensation of chromosomes. Some other kinase activities, mainly from Polo or Aurora families, participate in mitotic entry either by activating specific phosphatases such as the Cdc25c which relieves the phosphorylation-dependent Cdk1inhibition (Plk1), or by helping in the establishment of the proper chromosome structures and the mitotic spindle (Lens et al. 2010). In addition, a new kinase involved in the inactivation of phosphatases, known as Mastl, participates by favoring the balance between Cdk1-dependent phosphorylation and dephosphorylation by general phosphatases such as protein phosphatase PP2A (Malumbres 2011; Glover 2012). A particular group of kinases, represented by Bub1, BubR1 kinases and Mps1 (also known as TTK), are critical components of the spindle assembly checkpoint (SAC or mitotic checkpoint), a signaling pathway that monitors bipolar

attachment of chromosomes to the mitotic spindle (Nezi & Musacchio, 2009). This checkpoint ensures proper segregation of chromosomes to daughter cells. Finally, Aurora B and Plk1 as well as the GTPase (Guanine nucleotide triphosphatase) RhoA are involved in cytokinesis (Figure 1.4) (Malumbres 2011; Su et al. 2011).

Another important aspect of Cdk activity control is the ubiquitin-mediated proteolysis of key regulators such as cyclins and Cdk inhibitors. Two ubiquitin ligases, the SCF complex (Skip1-Cul1-F-box protein) and the APC/C (anaphase promoting complex/cyclosome), have a central role in cell cycle regulation. The SCF complex and the APC/C are both E3 ubiquitin ligases with similar structures (Nakayama & Nakayama, 2006; Skaar & Pagano 2009). The APC/C is active in mitosis and G1 while SCF exert it role mostly in interphase. Each is constituted of common subunits and a variable substrate recognition subunit that function as activator. Two activators of the APC/C, Cdc20 (cell division cycle 20) and Cdh1; and three F-box proteins in the SCF complex, Skip2 (S-phase kinase-associated protein 2), Fbw7 (F-box and WD-40 domain protein 7) and β-TrCP (β-transducin repeat-containing protein), are the most important in cell-cycle regulation (Nakayama and Nakayama, 2006; Skaar and Pagano 2009).
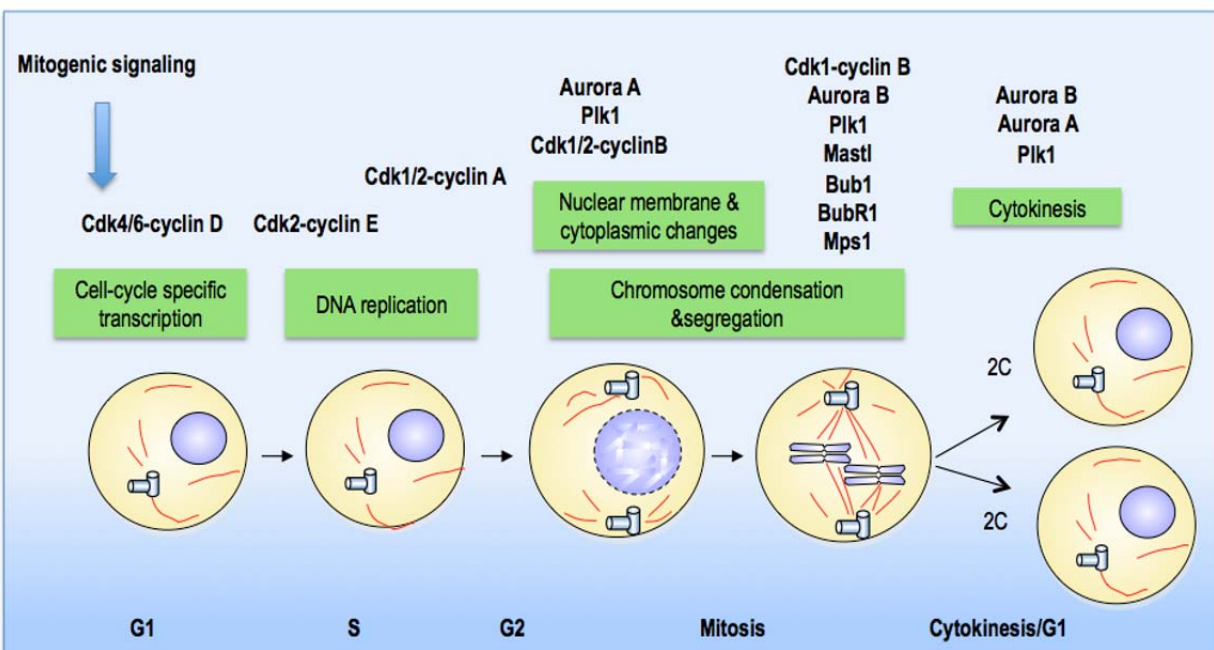


Figure 1.4 An overview of the mammalian cell cycle and its regulation by kinases. In response to mitogenic signaling, cells enter into the cell cycle through the Cdk4/6-cyclin D-dependent inactivation of pRb. Entry into S-phase may also depend on Cdk2-cyclin E complexes. During the S phase, the genome (blue) is duplicated. In G2, kinases such as Aurora A or Plk1 are important for centrosome separation and movement towards different poles of the cells. During the G2/M transition Cdk1 is activated by cyclin B and phosphorylates a variety of substrates involved in Golgi disassembly, nuclear envelop breakdown, and condensation of chromosomes. During mitotic exit, the cytoplasm is divided into two daughter cells through cytokinesis. Microtubules are represented as red lines. Adapted from Malumbres, 2011.

Cdc20 is the crucial effector of the spindle assembly checkpoint and targets securin and mitotic cyclins for destruction, thereby promoting sister-chromatid separation and mitotic exit (Figure 1.5) (Peters, 2006; Manchado et al. 2010). Cdh1, on the other hand, facilitates exit from late mitosis and maintains G1 phase mediating the degradation of mitotic cyclins, non-Cdk mitotic kinases such as Aurora and Plk1 and some regulators of the formation of pre-replicative complexes (Figure 1.5). SCF-Skp2 targets inhibitors of the cell cycle such as p27Kip1, p21Cip1 and p57Kip2 for degradation and thereby promotes cell cycle progression. Fbw7 induces the degradation of positive regulators of the cell cycle, such as Myc, Jun, cyclin E and Notch. β-TrCP is a versatile F-box protein that recognizes several cell cycle regulators such as Emi1/2, Wee1A and Cdc25A/B (Nakayama & Nakayama 2006).
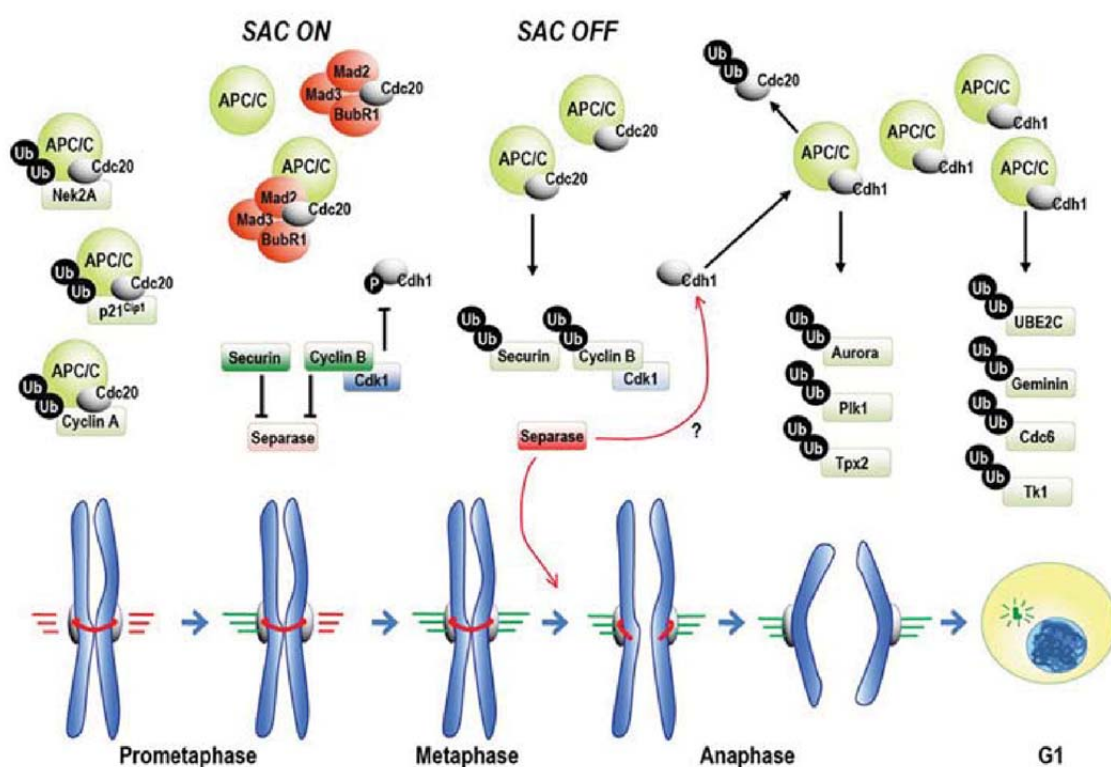


Figure 1.5. **Function of APC/C in the cell cycle.** During the early stages of mitosis, APC/C–Cdc20 ubiquitinates several substrates such as Nek2A, cyclin A and p21Cip1, promoting their degradation. Yet, in response to unattached kinetochores, the SAC is active and the APC/C is inhibited by sequestering of Cdc20 or non-functional APC/C–Cdc20 complexes also containing Mad2, Mad3 and BubR1. Under these conditions, securin and cyclin B–Cdk1 function as inhibitors of separase. Once all chromosomes are bi-orientated on a metaphase plate, the SAC is extinguished, resulting in APC/C–Cdc20 activation. APC/C–Cdc20 targets cyclin B1 and securin for degradation allowing separase activity, which cleaves the centromere cohesin complex leading to anaphase onset. The newly formed APC/C–Cdh1 complexes drive mitotic exit by targeting for destruction Plk1, Aurora A or Tpx2 among other substrates as well as Cdc20 itself. During G1 phase, APC/C–Cdh1 maintains low levels of Cdk activity by targeting A- and B-type cyclins and several regulators of DNA replication such as Geminin or Cdc6. Finally, the APC catalyses the auto-ubiquitination of its own E2 ubiquitin-conjugating enzyme UBE2C/UbcH10, leading to the stabilization of cyclin A, activation of Cdks and APC/C–Cdh1 inactivation. Source: (Manchado, et al. 2010).

## 1.2.2. Endoreplication

To convert the mitotic cell cycle into an endoreplicative one, the cell cycle must show two essential features. First, the processes of mitosis must be bypassed without blocking DNA replication and second the Cdk activity required for DNA replication has to be periodically inactivated to enable the transition into a G phase. The first requirement for bypassing mitosis is accomplished by inhibiting Cdk1 which is the Cdk that drives G2-to-M phase progression, while allowing unperturbed the S-phase Cdk activity that drives G1-to-S progression (Edgar and Orr-Weaver 2001; Lee et al. 2009; Edgar et al. 2014).

Several cell types can be experimentally forced into endoreplication by suppressing Cdk1 activity, which suggests that Cdk1 downregulation is sufficient to trigger endoreplication (Laronne & Rotkopf 2003). The second requirement for endoreplication which is accomplished by the SCF and the APC/C-Cdh1 ubiquitin ligases, has been clearly demonstrated by experiments in *D. melanogaster* showing that constitutive expression of Cyclin E, an S-phase Cdk activator, can block endocycling (Weiss et al. 1998). Also recently in murine TGCs has been demonstrated that APC/C activity oscillations take place during the endoreplicative cell cycles (Sakaue-Sawano et al. 2013). The underlying reason for this seems to be that the assembly of pre-replication complexes at origins of replication is inhibited by high levels of Cdk activity. Like mitotic cells, endocycling cells require a G1-like gap phase with low Cdk activity during which they can assemble PreRCs. Thus in this model that describes the progression of the endoreplicative cell cycles, G phases are characterized by low S-phase Cdk and Geminin activity and high APC/C-Cdh1 which allows PreRC formation. S phases have opposite characteristics: high S-phase Cdk and Geminin activity, which allows DNA replication but prevents PreRC formation.

During the switch from a mitotic cell cycle to endoreplication, Cdk1 inactivation is thought to occur through the action of APC/C-Cdh1 and the Cdk inhibitor p57Kip2 (Ullah et al. 2008; Edgar et al. 2014). APC/C-Cdh1 activity is involved in mitotic cyclin destruction therefore mediating Cdk1 inhibition. Cdh1 is essential for endoreplication both in flies (Sigrist & Lehner 1997) and mice (Garcia-Higuera et al. 2008). Cdh1 ablation in mouse embryos leads to embryonic lethality due to placental defects. Cdh1 null placentas present aberrant TGCs with small nuclei confirming the requirement for Cdh1 in endoreplicative cell cycles. Interestingly, overexpression of Cdh1 in U2OS cells is sufficient to induce endoreplication (Sorensen et al. 2000). On the other hand TGCs have been detected in p57Kip2 knockout mice, suggesting that p57Kip2 is not essential for endocycle entry, or at least has redundant functions (P. Zhang et al. 1998). Severe placental abnormalities occur in p57Kip2-deficient mice, but so far it is unclear whether these are due to a compromised mitotic-to-endocycle transition or to a more general failure of cell cycle exit in progenitor cells.

## 1.2.3. Re-replication

What enables endocycling cells to replicate their DNA many times within a single interphase is not entirely clear yet, but work in diverse mitotic systems does suggest what sort of mechanisms to expect. In eukaryotic cells, pre-RCs which consist at least of four factors, ORC, Cdc6, Cdt1 and MCM2-7, are assembled on chromatin in the G1 phase, rendering origins of DNA replication competent to initiate DNA synthesis (Arias & Walter 2007). When DNA replication commences in S phase, pre-RCs are disassembled, and multiple initiations from the same origin do not occur because new rounds of pre-RC assembly are inhibited. In most experimental organisms, multiple mechanisms that prevent pre-RC assembly have now been identified, and re-replication within the same cell cycle can be induced through defined perturbations of these mechanisms. In mammals, the main mechanisms to avoid re-replication are the Cdt1 proteolytic destruction, the sequestration of Cdt1 by Geminin and the Cdk1-mediated inhibition of ORC loading (Arias & Walter 2007; Hook et al. 2007) (Figure 1.6).

Cdt1 is ubiquitinated in a PCNA-dependent manner by the E3 ubiquitin ligase cullin based complex Cul4-Ddb1$^{Cdt2}$ during S-phase while during S and G2 phases is dependent on the SCF$^{Skip2}$ ubiquitin ligase (Li & Blow 2004). Although all these mechanisms show redundant and overlapping properties there are many evidences showing that the overall Cdk activity is the one that controls many of those mechanisms and hence the cell ensures a proper replication of its genome (Li & Blow 2004).
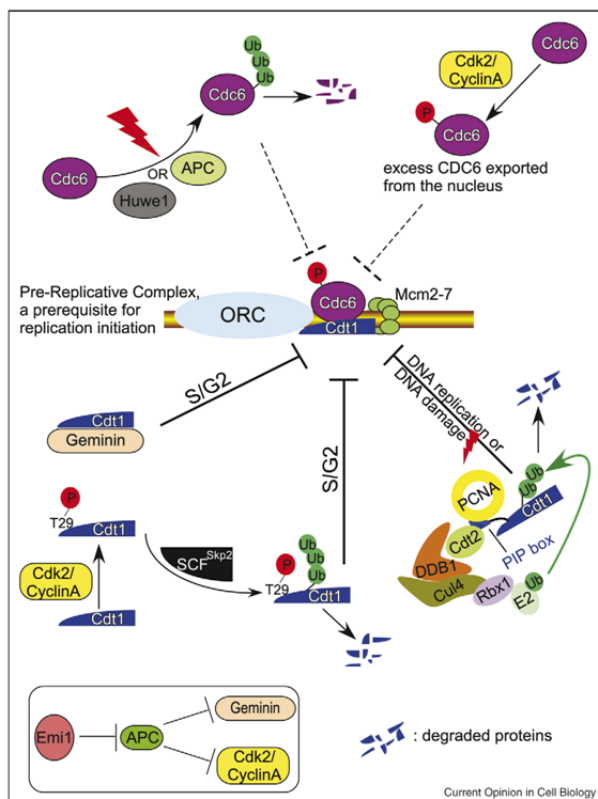


**Figure 1.6. Mechanisms to prevent re-replication in mammalian cells.** In the center is the pre-RC which is a pre-requisite for replication initiation. The pathways highlighted with black lines are known to inhibit pre-RC formation and by doing so have been demonstrated to prevent re-replication. The pathways denoted with dashed lines also inhibit pre-RC formation but have not yet been shown to prevent re-replication. The red lightening bolt denotes pathways activated by DNA damage. The boxed section shows Emi1 which has been shown to prevent rereplication by stabilizing geminin and cyclin A. Source: (Hook et al. 2007)

### 1.2.4. Endomitosis

Since the first description of megakaryocytes as the origin of blood platelets in 1906 by James H. Wright, only during the last years the molecular basis for endomitosis has been described (Geddis & Kaushansky 2006; Lordier et al. 2008; Gao et al. 2012). Endomitotic cycles as mentioned above are characterized by entry into mitosis, nuclear breakdown and formation of complex mitotic spindles with multiple asters. At the end of each endomitosis, however, megakaryocytes abort anaphase, reverse or fail to initiate cytokinesis, and gather all the chromosomes in a single nucleus (Ravid et al. 2002; Machlus & Italiano 2013). While key cytoskeletal and regulatory components, including RhoA, Aurora B, and the chromosome passenger complex (CPC), localize correctly in the spindle midzone in early anaphase of the first endomitotic cycle it has been showed that a cleavage furrow slowly begins to ingress and later regresses (Geddis & Kaushansky 2006). In subsequent cycles, furrow ingression is barely perceptible, and the contractile ring lacks nonmuscle myosin IIA. These observations highlight differences between the first and subsequent endomitotic cycles, with both types showing defective cleavage furrow ingression, a process that requires RhoA-mediated actomyosin contraction. Till recently, single components of the spindle midzone such as Aurora B (see Section 1.4.1) and cleavage furrow such RhoA were not conclusive offering satisfying reasons for abortive cytokinesis. The first complete and satisfying explanation for the abortive mitosis in megakaryocytes was given in 2012 by Gao et. al. They showed that RhoA localizes correctly in the spindle midzone and latter in furrow formation but fails to be activated, attributing this failure to reduced levels of two RhoA-activating guanine exchange factors (GEFs), GEF-H1 and Ect2 (Gao et al. 2012).

## 1.3. Significance of polyploidization in mammalian cells

To define the physiological role of polyploidization it is crucial to determine the consequences of increasing or reducing polyploidization in vivo. Recently, multiple mouse studies have provided surprising new insights into the physiological function of polyploidization. To gain insight into the possible reasons for polyploidy *in vivo* is necessary to analyze where and when polyploidization occurs during embryonic and postnatal development.

### 1.3.1. Trophoblast giant cells

TGCs are the first polyploid cell type during development and their formation occurs during implantation of mouse blastocysts at embryonic (E) day E4.5. TGCs differentiate from the trophectoderm layer in the blastocyst, are endocrine in nature, and are composed of an extremely large cytoplasm and polyploid nuclei. TGCs undergo multiple rounds of endoreplication and thereby accumulate DNA up to 1000C (Lee et al. 2009; Edgar et al.

2014). The physiological significance of polyploidization for TGC function is a matter of speculation (Lee et al. 2009; Pandit et al., 2013). Because TGC have a relatively short lifespan, it may be that polyploidization and the associated hypertrophy allow tissue growth with less time and energy expenditure. Another hypothesis is that polyploidization is important for the terminal differentiation of TGC. Polyploidy may increase their capacity for protein synthesis because TGC secrete a wide array of hormones and paracrine factors, including steroid hormones and prolactin related cytokines (Hu & Cross 2010). Furthermore, polyploidization through increased gene expression may supply the energy necessary for aggressive invasion of TGC into maternal tissue.

As mentioned above, Cyclin E1/E2, Cdh1, or Dp1 deficiency in mice results in embryonic lethality at E10.5–11.5 with marked reduction of polyploidization in TGCs (Geng et al. 2003; Parisi et al. 2003; Garcia-Higuera et al. 2008; Kohn 2003). Mutant Cyclin E1/E2 TGCs show equal expression of differentiation markers compared to wild type TGCs, indicating that polyploidization and TGC differentiation are not causally linked. Interestingly, embryonic lethality is rescued by providing cyclin E or Cdh1 mutant embryos with a wild type placenta, suggesting that the polyploidization defect in these mutant TGCs is the main cause of embryonic lethality. However, TGC-specific deletion of these molecules would be required to appropriately address this hypothesis.

### 1.3.2. Megakaryocytes

Megakaryocytes are a second example of developmentally regulated polyploidization in mammals (Ravid et al. 2002; Thon & Italiano 2012; Machlus & Italiano 2013). The first polyploid megakaryocyte in mice appears around embryonic day E7.5 in the blood islands of the yolk sac (Xu et al. 2001). Megakaryocytes differentiate from hematopoietic stem cells in response to the cytokine thrombopoietin (TPO) (Kaushansky 2009) and at later stages of embryonic development megakaryocytes can be found in the liver, bone marrow, and spleen. Megakaryocytes give rise to platelets (thrombocytes; Figure 1.7), which are essential for blood clotting and angiogenesis.

Proplatelets can been observed extending from megakaryocytes in the bone marrow through junctions in the lining of blood sinuses where they have been hypothesized to be released into the circulation and undergo additional fragmentation into platelets (Patel et al. 2005). For proplatelet formation is necessary a complete transformation of the cytoplasm. The transformation unfolds over 3 to 10 hours and begins in a polarized fashion with the erosion of one side of the megakaryocyte cytoplasm (Figure 1.7). Thick pseudopodia-like processes initially form and then extend into thin tubes of uniform diameter of 2 to 4 μm. The thin tubules subsequently undergo a dynamic bending and branching process and develop periodic platelet-sized swellings that span their length (Figure 1.7). Proplatelet formation and elongation is dependent on microtubule function. Ultimately, the megakaryocyte is converted into a residual naked nucleus surrounded by an elaborate network of proplatelets.
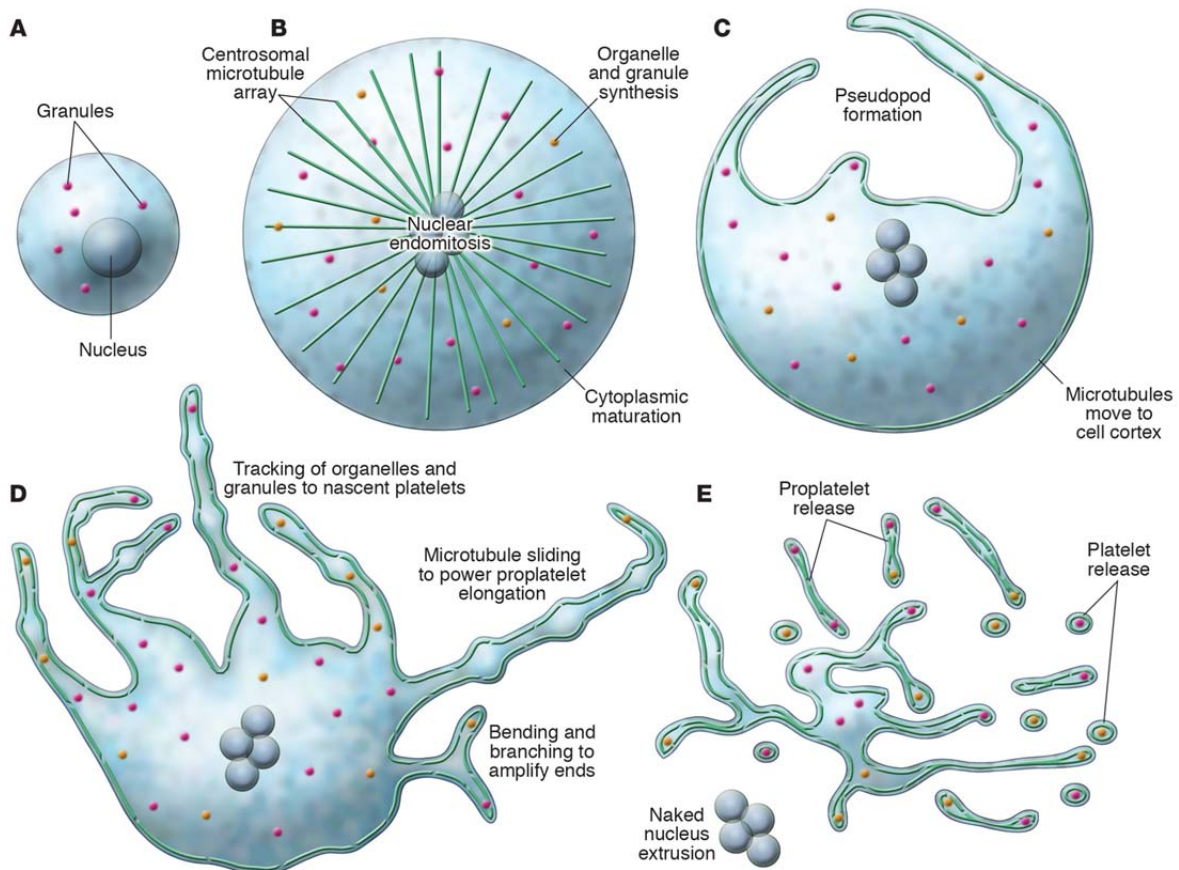
**Figure 1.7. Production of platelets by megakaryocytes.** As megakaryocytes transition from immature cells **(A)** to mature structures able to release platelets **(E)**, a systematic series of events occurs. **(B)** The cells first undergo nuclear endomitosis, organelle synthesis, and dramatic cytoplasmic maturation and expansion, while a microtubule array, emanating from centrosomes, is established. **(C)** After polyploidization of megakaryocytes and prior to the onset of proplatelet formation, centrosomes disassemble and microtubules translocate to the cell cortex. Proplatelet formation commences with the development of thick pseudopods. **(D)** Sliding of overlapping microtubules drives proplatelet elongation as organelles are tracked into proplatelet ends, where nascent platelets assemble. Proplatelet formation continues to expand throughout the cell while bending and branching amplify existing proplatelet ends. **(E)** The entire megakaryocyte cytoplasm is converted into a mass of proplatelets, which are released from the cell. The nucleus is eventually extruded from the mass of proplatelets, and individual platelets are later released from proplatelet ends. Source: (Patel et al. 2005).

The subsequent rupture of the cytoplasmic bridges between platelet-size segments is thought to release individual platelets into the circulation (Figure 1.7). It has been suggested that the large size of megakaryocytes and their high ploidy are essential for the release of thousands of enucleated platelets per cell (Kaushansky 2009). Accordingly mice deficient for TPO (de Sauvage et al. 1996) or for its receptor (Alexander et al. 1996) presented megakaryocytic hypopolyploidization that perfectly correlated with severe thrombocytopenia (low blood platelets levels). Similarly deficient mice for the stem cell leukemia factor

(SCL/TAL1), specifically in megakaryocytes, were profoundly thrombocytopenic due to aberrant polyploidization and maturation of megakaryocytes (Chagraoui et al. 2011).

## 1.4. Requirements for cell cycle regulators in endomitosis, megakaryocyte maturation and platelet formation

### 1.4.1. Cell cycle regulators in endomitotic process

The relevance of known cell cycle regulators during endomitosis has been a matter of speculation, and only few analysis of loss- or gain-of-function have been performed. Examination of the cyclin expression profile in both megakaryocytic cell lines and primary megakaryocytes has shown that Cyclin E, D3, and to a less extent cyclin D1, are expressed and upregulated upon treatment to ploidy-promoting factors such as TPO (Zimmet et al. 1997; Eliades et al. 2010). Importantly Cyclin D3 overexpression in megakaryocytes resulted in higher ploidy while overexpression of cylin D1 only provoked moderate increase in ploidy (Zimmet et al. 1997). Interestingly overexpression of Cyclin D1 was sufficient to rescue the polyploidization defects in megakaryocytes deficient for GATA-1, an essential transcription factor during megakaryopoiesis (Muntean et al. 2007). However, no significant differences were detected when cyclin D1 deficient mice were analyzed for mekagaryocytic polyploidization. This lack of phenotype was speculated to be due to a possible compensatory role of cyclin D3 since it was found highly upregulated in megakaryocytes deficient for cyclin D1 (Muntean et al. 2007).

E-type cyclins have also been found to play an important role during polyploidization. Cyclin E1 and E2 double knock-out mice showed impairment of polyploidization in megakaryocytes (Geng et al. 2003). Accordingly overexpression of cyclin E specificaly in megakaryocytes led to enhanced ploidy both *in vivo* and *in vitro* comparable to levels achieved by TPO administration (Eliades et al. 2010).

While both p53 and p21Cip1 have been reported to be expressed in endomitotic megakaryocytes, deficiency for these proteins did not affect either megakaryocyte polyploidization or platelet production *in vivo* (Fuhrken et al. 2008; Apostolidis et al. 2012; Baccini 2001). Interestingly *in vitro* ablation of p53 in hematopoietic progenitors during its differentiation towards megakaryocytes resulted in higher ploidy megakaryocytes because of greater entry into S-phase and less induction of apoptosis (Apostolidis et al. 2012). Accordingly while p21Cip1 is not essential for the determination of the ploidy profile in normal megakaryocytes *in vivo,* overexpression of p21Cip1 caused the arrest of the endomitotic cycle (Baccini 2001).

The implication of mitotic regulators and, specifically, Aurora B in the endomitotic cycle has been a matter of debate. Aurora B kinase is a member of the CPC that changes its subcellular localization during mitosis (Carmena et al. 2012). Aurora B is associated with chromosomes during prophase, with inner centromeres during metaphase and early anaphase,

and with the midzone and midbody during late anaphase and telophase. Aurora B activity depends on its association with other chromosome passenger proteins (INCENP, Borealin, and survivin) as well as its autophosphorylation at threonine 232 (Thr232) located in the activating loop (Carmena et al. 2012). Aurora B is involved in chromosome congression at metaphase and chromosome separation at anaphase. In addition is essential for mitosis and cytokinesis since its ablation provokes premature mitotic exit and cytokinesis failure (Fernández-Miranda et al. 2011; Trakala et al. 2013). Accordingly it has been shown that Aurora B kinase exerts its role in cytokinesis by regulating mitotic kinesin-like protein 1 phosphorylation, which controls the abscission checkpoint to prevent tetraploid cell occurrence (Guse et al. 2005; Steigemann et al. 2009).

In endomitotic cell cycles it was initially shown that Aurora B was not expressed or only at a low level in murine megakaryocytes, and it was postulated that this deregulated expression was responsible for polyploidy (Kawasaki et al. 2001; Zhang et al. 2004). Subsequently, it was shown that Aurora B is not only expressed and functional during the early endomitosis phases of human megakaryocytes but also all along the endomitosis and whatever the ploidy (Geddis & Kaushansky 2004). Interestingly while chemical inhibition of *in vitro* megakaryocytic cultures did not alter polyploidization it did induced growth arrest and apoptosis in 2C and 4C megakaryocytic precursors during mitosis (Lordier et al. 2010). Importantly when Aurora B overexpression was induced in megakaryocytes no dramatic changes in polyploidization were observed (Nguyen et al. 2005). Similar results were obtained during the specific ablation of Survivin, another CPC component (McCrann et al. 2008; Wen et al. 2009; Ravid 2009).

### 1.4.2. Mastl and human thrombocytopenia

Mastl (Microtubule-Associated Serine/Threonine protein kinase-Like) is an additional cell cycle kinase recently characterized kinase because of its critical functions in the maintenance of mitosis (Figure 1.8) (Malumbres 2011; Glover 2012). Mastl, also known as Scant or Greatwall, was originally discovered in *Drosophila* screenings for cell cycle mutants (Archambault et al. 2007; Yu et al. 2004). Deficiency in this kinase leads to defective chromosome condensation and segregation in mitosis and this phenotype is due to hyperactivation of PP2A-B55 complexes (Vigneron et al. 2009; Yu et al. 2004; Álvarez-Fernández et al. 2013). In fact, Mastl is known to exert its role through phosphorylation of endosulfine and Arpp19, two small proteins that function as PP2A-B55 inhibitors (Figure 1.8)(Gharbi-Ayachi et al. 2010; Mochida et al. 2010).

PP2A is a heterotrimeric serine-threonine phosphatase composed of a catalytic subunit C, a structural subunit A and a regulatory subunit B, which confers substrate specificity. In mammals, there are 4 subfamilies of B regulatory subunits (B55, B56, PR72 and PR93), each one with multiple isoforms. In particular, depletion of PP2A-B55 complexes delays mitotic exit and postmitotic reassembly of various interphase structures (Schmitz et al. 2010). In

agreement with that, Cdc20-null cells arrest in metaphase and require both Cdk inactivation and the activity of PP2A-B55 phosphatase complexes to exit from mitosis (Manchado, et al. 2010). Little is known about the mammalian Mastl ortholog although its inhibition also results in defects in mitotic progression and mitotic exit in a PP2A-B55-dependent manner (Burgess & Vigneron 2010; Manchado et al. 2010; Voets & Wolthuis 2010; Álvarez-Fernández et al. 2013)

Independent of these studies a missense mutation was found in 2003 in a region of human chromosome 10p11-12 in patients with nonsyndromic autosomal dominant thrombocytopenia, also known as thrombocytopenia-2 (THC2) (Gandhi et al. 2003). These patients were characterized by incomplete differentiation of megakaryocytes and mild thrombocytopenia with an average platelet count of 60,000 platelets per microliter of blood. The mutation, a substitution of cytosine for guanidine (G to C) at nucleotide position 565, was present in all thrombocytopenic family members, causing a predicted substitution of aspartic acid for glutamic acid (E167D) in exon four of the FLJ14813 sequence (Gandhi et al. 2003). Using morpholino knockdown assays in zebrafish, it was shown that knockdown of Mastl in the embryo resulted in deficiency in circulating thrombocytes (Johnson et al. 2010). In addition to the E167D substitution in *MASTL*, additional mutations in two genes in the THC2 region of chromosome 10p11-12 have been recently linked to congenital thrombocytopenia. These include specific mutations in *ACBD5* and the 5' UTR of *ANKRD26*, the ankyrin repeat domain 26 gene (Pippucci et al. 2011). These three genes are very close in human chromosome 10p12.1 (*ACBD5-MASTL-YME1L1-ANKRD26*; centromeric to telomeric) and the relative contribution to thrombocytopenia is still under debate (Di Paola and Johnson, 2011; Pippucci et al., 2011). However, the role of Mastl in this process and its possible links to cell cycle regulation are currently unknown.



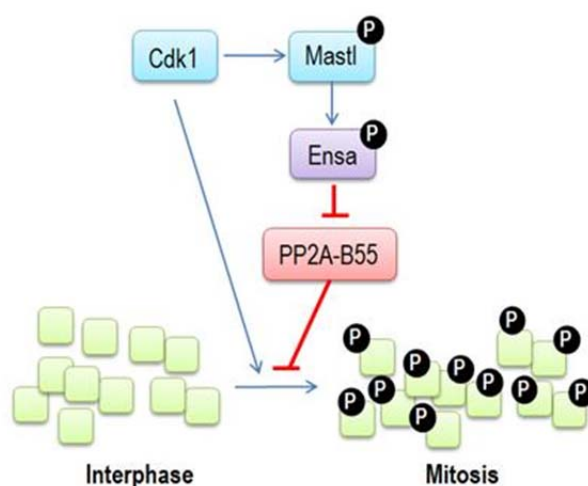**Figure 1.8. Role of Mastl as an inhibitor of PP2A phosphatases.** Mastl is activated in a Cdk-dependent manner and phosphorylates members of the Ensa family. These small molecules, when phosphorylated, bind and inhibit PP2A-B55 complexes. Since PP2A-B55 counteracts Cdk-dependent phosphorylations during mitosis, Mastl function as a critical enzyme to support Cdk-dependent phosphorylations during mitosis.

### 1.4.3 Gene modulation specifically in megakaryocyte and platelets

Constitutive and ubiquitously gene expression or ablation often results in severe phenotypes that interfere with the analysis of particular cell types or tissues or can mislead due to compensatory mechanisms. This also has been the case for the megakaryocytic compartment. While mouse models mentioned above gave insights into the requirements of those regulators during endomitosis still those results were difficult to interpret due to its effect on the whole hematopoietic system. In order to overcome these limitations, the generation of mouse models that allow gene modulation specifically in the tissue or cell type of interest, are crucial. To date tool mouse models exist that can be used for this purpose. Specific promoters for megakaryocytes and platelets such as the platelet factor 4 (Pf4) have been successfully driven the expression either of Cre recombinase or any other transgenes (Tiedt et al. 2007; Nguyen et al. 2005). These models will be crucial for addressing the questions addressed in this work.

# 1. Objectives

# Objectives of the work

The current study aims to investigate the physiological relevance and the molecular basis of polyploidization and the requirements of well-known cell cycle regulators during this process. For this purpose we will focus on the megakaryocytic example to perform our studies. Investigating the endomitotic polyploidization that megakaryocytes undergo will help us not only to understand better basic biological perspectives of the cell cycle but also how we can modulate megakaryocytic polyploidization as therapeutic strategies for pathological conditions related to hepatopoietic system such as thrombocytopenia and thrombocytosis as well as megakaryoblastic leukemia.

The objectives we propose are

1. Deciphering the function of Mastl in platelet formation by generating and characterizing a mouse model for the human thrombocytopenia syndrome.

2. Investigate the relevance of kinases needed for mitotic progression such as Aurora B, Plk1 and Mastl during the endomitotic process.

3. Understand the requirements for the APC/C during the endomitotic cycles.

4. Examine the requirements for cyclin-dependent kinases (Cdks) for entry into endomitosis.

# 2. Materials and Methods

# 3.1. Genetically modified mouse models

### 3.1.1. Animal housing

Mice were housed in the pathogen-free animal facility of the Centro Nacional de Investigaciones Oncológicas (CNIO, Madrid) following the animal care standards of the institution. These animals were observed on a daily basis and sick mice were killed humanely in accordance with the Guidelines for Humane End Points for Animals used in biomedical research. All animal protocols were approved by the ISCIII committee for animal care and research. All animals were maintained in a mixed 129/Sv (25%) x CD1 (25%) x C57BL/6J (50%) background

### 3.1.2. Generation of mouse models

For the generation of the *Mastl* knock-in mouse we replaced the wild-type allele with an allele carrying the E166D mutation described in the human thrombocytopenia syndrome (Drachman et al. 2000; Gandhi et al. 2003) employing a targeting vector constructed by Gene Bridges. A conditional loss-of-function model of Mastl was also generated in our laboratory by flanking exon 4 of the murine *Mastl* locus with loxP sites and a selection cassette consisting of the PGK promoter and the neo/km resistant gene flanked by frt sites (Álvarez-Fernández et al. 2013). To facilitate homologous recombination, mouse ES cells V6.4 obtained from a hybrid (129 x C57BL/6J) strain were electroporated with 100 μg of linearized DNA from the corresponding targeting vectors. Recombinant ES cells and clones were selected in the presence of G418 (neomycin). This step was done by the Transgenic Unit of the CNIO. The screening of the recombinant clones was performed by Southern blot analysis using new restriction sites from the recombinant alleles and probes external to the homology arms. Positive recombinant clones were either aggregated with morulas CD1 or microinjected into C57BL/6J blastocysts by the Transgenic Unit of the CNIO. The resulting quimeric mice were crossed with Actin-Flp transgenic mice to generate *Mastl*(+/E166D) mice.

Pf4-Cre transgenic mice (Tiedt et al. 2007) were obtained by the Jackson laboratories in a pure C57BL/6J background. The transgenic mouse line expressing the far-red fluorescent protein Katushka, driven by the hybrid CAG promoter upon Cre-mediated recombination [(lox-Stop-Lox-KFP (Diéguez-Hurtado et al. 2011)] was obtained by Dr. Ortega's laboratory. Aurora B-, Cdh1-, Cdc20- conditional knockouts and Cdk2 knockout mice were reported previously and were obtained from M. Barbacid's lab or from our laboratory (Fernández-Miranda et al. 2011; Garcia-Higuera et al. 2008; Manchado, et al. 2010; Ortega et al. 2003). Cdk1 and Plk1 conditional knockout animals were obtained from Dr. Barbacid´s laboratory or generated by P. Wachowicz in our laboratory (unpublished work), respectively.

For genotyping alleles for the several mouse models we isolated tail DNA from 3-4-week old mice and we performed a PCR amplification reaction using the oligonucleotides

shown in Table 2.1 and following this conditions: 94ºC during 4 minutes followed by 35 cycles of DNA denaturalization at 94ºC during 30 seconds, primer annealing at 60ºC during 30 seconds and polymerase extension at 72ºC during 60 seconds ending with a single elongation cycle of 7 minutes at 72ºC.

**Table 2.1**. Mouse models analyzed in this work and oligonucleotides used for mouse genotyping in PCR

| Mouse Model | Allele | Size (bp) | Sequence (5´-3) |
|---|---|---|---|
| *Aurbk conditional knockout* | *Aurkb*(+) | 358 | Fw: AGGGCCTAATTGCCTCTTGT |
| | *Aurkb*(lox) | 491 | Rv: GGGCATGAATTCTTGAGTCG |
| *Mastl conditional knockout* | *Mastl*(+) | 296 | Fw: GCCCTTTTAAAAACCCAACA |
| | *Mastl*(lox) | 408 | Rv: GGCTGGCAGTTCCATTTTTA |
| *Mastl* knock-in | *Mastl*(+) | 296 | Fw: GCCCTTTTAAAAACCCAACA |
| | *Mastl*(E166D) | 408 | Rv: GGCTGGCAGTTCCATTTTTA |
| *Cdh1 conditional knockout* | *Cdh1*(+) | 168 | Fw: AGCATGGTGACCGCTTCATCC |
| | *Cdh1*(lox) | 286 | Rv: GCTGGGGGACTTCTCATTTTCC |
| *Cdc20 conditional knockout* | *Cdc20*(+) | 500 | Fw: GATTTGCACTCACTGCTTCAACTGG |
| | *Cdc20*(lox) | 670 | Rv: CTTTCTGATGCTCCTGAAATACACG |
| *Cdk1 conditional knockout* | *Cdk1*(+) | 650 | Fw: GAGATGTAGGATGACTCAGTG |
| | *Cdk1*(lox) | 800 | Rv: TAGCTTATCTACCTCAGCCTG |
| Cre transgenic | Pf4-Cre | 450 | Fw: CCCATACAGCACACCTTTTG |
| | | | Rv: TGCACAGTCAGCAGGTT |
| Cre reporter | KFP-LSL | 812 | Fw: AACGACCACCACTTCAAGTGC |
| | | | Rv: TAGCCAGAAGTCAGATGCTCAAGG |

### 3.1.3. Histology

For histology sections, sterna and spleens were fixed overnight in 4% formalin. Sterna were then decalcified in Decalcifier I solution (Surgipath) for 2 hours. Specimens were dehydrated in 70% ethanol and processed by the Histopathology Facility Unit at CNIO for 3-5 µm longitudinal paraffin sections, hematoxylin and eosin staining, and immunohistochemistry for von Willebrand Factor, phospho-histone H3 (ser10) or gamma-H2AX ($\gamma$H2AX). To quantify megakaryocytes in bone marrow and spleen, paraffin sections stained with anti-VWF were examined using an Olympus BX51 microscope equipped with objective lenses (40/0.75, 20/0.4, 10/0.25, and 4/0.1). Total megakaryocyte number was counted in each 4X observation field

### 3.1.4. Isolation of megakaryocytes from the bone marrow

CD41+ cells were enriched from bone marrow by the MACS (magnetic cell shorting) cell separation system (Miltenyi Biotec). Bone marrow cells were exposed to FITC-CD41, and mixed with anti-FITC microbeads (Miltenyi Biotec). The associated cells were separated using a large cell separation column (Miltenyi Biotec). However, contaminating non-

megakaryocytic cells could not be completely removed by this method. CD41-enriched cells were immunostained with FITC-CD41 (Biosciences) antibody.

## 3.2. Cell Culture

### 3.2.1. Bone marrow-derived megakaryocytes

Tibia and femur were isolated from 8-12 week old mice and bone marrow was flushed out by the addition of ice-cold PBS buffer (PBS, 0.5% BSA, 5mM EDTA) through the lumen of the bone. Marrow was mechanically disrupted to achieve single cell suspension followed by filtering through a 40mm nylon strainer to remove bone debris and subsequently subjected to erythrocyte lysis. For red blood cell lysis the bone marrow cell pellet from 1 mouse was resuspended in 1 ml of ACK lysis buffer (150 mM ammonium chloride, 1 mM potassium bicarbonate, 0.1 mM EDTA) and incubated for 1.5 min on ice. The lysis was stopped by the addition of 10 ml PBS buffer and cells were centrifuged at 200$g$ for 10 min. Cells were counted and subjected either directly to surface antigen staining for flow cytometry analysis (FACS) or to lineage depletion MACS cell separation system (Miltenyi Biotec) with the Hematopoietic Progenitor (Stem) Cell Enrichment Set, according to manufacturer's protocol. Lin- isolated cells were grown in Dulbecco''s modified Eagle's medium (DMEM; Gibco) supplemented with antibiotics and 10% fetal bovine serum (FBS) supplemented with 50 ng/ml murine thrombopoietin (TPO), (PeproTech).

### 3.2.2. Fetal liver-derived megakaryocytes

Fetal liver cells were obtained from whole livers recovered from mouse fetuses between embryonic days 12.5 and 13.5. Single-cell suspensions were prepared by successive passage through 19-, 22-, and 25-gauge needles and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, and 100 ng/mL murine TPO for 5 days. After 4 to 5 days of culture in the presence of TPO, the cell population was en- riched in mature megakaryocytes using a 1.5%/3% bovine serum albumin (BSA) gradient under gravity (1$g$) for 45 minutes at room temperature.

### 3.2.3. Mouse embryonic fibroblasts (MEFs)

Mouse embryonic fibroblasts (MEFs) were prepared from E14.5 embryos and cultured using standard protocols. E14.5 embryos were extracted from the uterus of pregnant females. The placenta was removed and embryos were isolated from the yolk salk. The embryo without the liver and the head was minced, and dispersed in 0.1% trypsin (5 min at 37°C). Cells were grown for two population doublings and then frozen. All cultures were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). MEFs transduction was

45

performed using adenoviruses expressing Cre recombinase (Ad5 CMV-Cre) obtained from The University of Iowa (Iowa City, IA). Transduction was carried out either in presence or absence of serum. MEFs transfection was carried out using the Neon technology (Life Technologies; Invitrogen)

### 3.2.4. Human cell lines

HEK293 and U2OS were maintained in DMEM medium supplemented with 10% fetal bovine serum and antibiotics and were grown at 37ºC in a humidified 5% CO2 atmosphere. HEL (human erythroleukemia cell line) were grown in RPMI (Roswell Park Memorial Institute medium) supplemented with 10mM Hepes with 10% fetal bovine serum and antibiotics. Plasmid transfection in HEK293 and U2OS cells was performed using Lipofectamine 2000 (Invitrogen).

### 3.2.5. Virus production and cell transduction

Lentiviruses and retroviruses were produced and concentrated as previously described (Tiscornia et al. 2006). Briefly low passage 293T cells were transfected with the vector of interest and the three packaging vectors expressing gag, pol and rev proteins necessary for virion production as well the envelope protein vsvg. 48 hours post transfection supernatants containing viruses were collected and concentrated by centrigufation at 19400 rpm for 2 hours at 20ºC. Viral pellets were resuspended in 1x HBSS. This viral preparation is of *in vitro* grade quality. For retroviruses 293T cells were transfected with the vector of interest and the packaging vector PCL-Eco. Supernatants were collected and 48 hours post transfection. Packaging vectors are a gift from Dr. Verma´s lab.

Transduction of primary megakaryocytes and Lin- bone marrow cells were performed by adding 10ml of concentrated virus (stock $10^9$/ml viral particles) per million of cells (MOI 10) for 12 hours or by using directly the viral supernatants collected from 293T cells repeating three times for 6hours always in presence of 50ng/ml TPO. Transduction of HEL cell line was performed by viral supernatants twice.

## 3.3. Flow cytometry

Flow cytometric analysis of DNA content and surface markers of megakaryocytes was determined by staining either whole bone marrow cells or hematopoitic progenitors cultures from 0-5 days in presence of TPO with fluorescein isothiocyanate (FITC)–conjugated anti-CD41 antibody (BD Biosciences) followed by fixation with 4%PFA at room temprature (RT) for 30 min followed by staining with DAPI 2 mg/ml (Sigma-Aldrich) for at least 1hour.

To determine mouse bone marrow HSC (Lin⁻ IL7R⁻ c-kit⁺ Sca⁺), CLP (Lin⁻ IL7R⁺ c-kit⁺ Sca⁺), CMP (Lin⁻ IL7R⁻ c-kit⁺ Sca⁻ CD34⁺, FcγR^low), MEP (Lin⁻ IL7R⁻ c-kit⁺ Sca⁻ CD34⁻,

FcγR$^{low}$), GMP (Lin$^-$ IL7R$^-$ c-kit$^+$ Sca$^-$ CD34$^+$, FcγR$^{high}$), and megakaryocyte progenitor (Lin$^-$ CD41$^+$) cells, freshly obtained BM cells were stained with phycoerythrin (PE) anti-CD34 (BD Biosciences), Alexa Fluor 488 anti- IL7Ra,  peridinin chlorophyll Protein Cyanin 5.5 (PerCP-Cy 5.5 anti–Sca-1, allophycocyanin-H7 (APC-H7) anti-cKit (BD Biosciences), phycoerythrin-cyanin 7 (PE-Cy 7) anti-FcγR (BD Biosciences) and  allophycocyanin (APC) - labeled lineage cell detection cocktail (BD Biosciences). Flow cytometric analysis was performed with a FACS-Canto flow cytometer or a LSRII flow cytometer (BD Biosciences) and FlowJo Version 8.8.7 software (TreeStar).

### 3.3.1. Flow cytometric analysis of CD42 (GPIbα)

Whole blood was obtained by puncturing the right ventricle of euthanized mice. Blood was anticoagulated by collecting into a 1/10 volume of 3.8% (w/v) sodium citrate solution. Platelet rich plasma (PrP) was isolated by centrifuging whole blood at low speed (180 x g, 10 min, RT). After centrifugation, the supernatant was washed and resuspended in Plt-HEPES-ACD buffer (137 mM NaCl, 2 mM KCl, 0.4 mM NaH$_2$ PO$_4$, 1 mM MgCl$_2$, 1 mM CaCl$_2$ and 10% (w/v). Platelets in PRP were stained with APC-conjugated anti-CD42 mAb (GPIbα, Bioscience)

## 3.4. Live cell imaging

Primary megakaryocytes derived from bone marrow hematopoietic progenitors were subjected to long-term, time-lapse imaging using a computer- assisted microscope. Cells were prepared as previously described and after 48 or 72hours upon TPO addition and/or viral transduction were plated on eight-well glass-bottom dishes (Ibidi) embedded in methylcellulose containing Iscove's Modified Dulbecco's Medium (IMDM) with 2% Fetal Bovine Serum and 100ng/ml murine TPO in order to minimize cell movement. Time lapse acquisition was performed with a Leica DMI 6000B microscope equipped with a 63×/1.5 N.A. objective lens or DeltaVision RT imaging system (Applied Precision; IX70/71; Olympus) equipped with a Plan Apochromatic 40×/1.42 N.A. objective lens, and maintained at 37°C in a humidified CO$_2$ chamber. Images were acquired every 7 min for up to 10 days. Quantitative analysis was performed by ImageJ software.

## 3.5.   Biochemical procedures

### 3.5.1.   Protein extraction and analysis

For immunodetection in protein lysates, cells were washed twice with ice-cold PBS and lysed in RIPA lysis buffer (37 mM NaCl, 0.5% NP-40, 0.1% SDS, 1% TX-100, 20 mM Tris-HCl pH 7.4, 2 mM EDTA, 10% glycerol 1 mM PMSF) supplemented with protease and

phosphatase inhibitory cocktails (Sigma). After 30 min on ice, samples were cleared by centrifugation. Proteins were separated on SDS-PAGE, transferred to nitrocellulose membranes (BioRad), and probed using specific primary antibodies noted as WB in Table 2.2. Secondary antibodies were coupled to HRP (DAKO) for immunodetection.

### 3.5.2. Immunoprecipitation and *in vitro* kinase assays

Total protein lysates were precleared with protein G-agarose bead suspension for 1hour (Amersham). Supernatants were first incubated for 2h at 4ºC on a rotating wheel with the primary antibody and later with 50 ml of the blocked bead suspension for an additional hour. Immunoprecipates were washed three times in RIPA buffer and once in kinase assay buffer [10 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 10 mM MgCl$_2$, and 1 mM EGTA] supplemented with 0.5 mM DTT and phosphatase inhibitor cocktail (Calbiochem) and later incubated in 25 µl of 2x kinase buffer supplemented with 0.2 mM ATP, 2.5 uCi [γ-32P] ATP and 3 mg of MBP as substrate. The reactions were incubated for 30 min at 30° C, stopped by addition of 5 µl of 5x Laemmli sample buffer, separated on a 12% SDS-PAGE and analyzed by autoradiography.

### 3.5.3. Immunofluorescence

Adherent cells grown on cover-slips and megakaryocytes after cytospin were fixed with 4% paraformaldehyde in PBS for 10 min at 37 $^{o}$C, permeabilized with PBS-Triton 0.15% for 2 min at 37$^{o}$C, blocked with 3% BSA and subsequently incubated with the primary antibodies noted as IF that are shown in Table 2.2 for 1 to 3 hours. The matching secondary antibodies, with different Alexa dies (488, 594, 647) and DAPI for nuclei visualization are from Molecular Probes (4,6 diaminophenylindole, Prolong Gold antifade; Invitrogen). Image acquisition was performed using either a confocal ultra-spectral microscope (Leica TCS-SP5) or a Leica DMI fluorescence 6000B microscope.

## 3.6. DNA combing

For the analysis of DNA replication in stretched DNA fibers, cells were incubated in presence of 50µM CldU (5-Clorodeoxyuridine) for 2 hours at 37$^{o}$C, followed by three washes in warm PBS for 5 min each. Subsequently cells were pulsed for 30 min with 250 IdU (5-Iododeoxyuridine). One drop containing 500-700 cells was placed on a microscope slide and cells were lysed with the addition of 10µl lysis buffer [0.5% SDS, Tris (pH 7.4), EDTA 50mM] for 6 min in RT. Slides were tilted at a 10-15$^{o}$ angle to allow the DNA suspension to run slowly down the slide. Slides were air-dried and fixed in 3:1 v/v methanol:acetic acid. Following DNA denaturation with 2.5M hydrochloric acid for 30 min, DNA fibers were washed and blocked with PBS, 1%BSA, 0.1% Triton X-100 for 1 hour in RT and incubated

with primary antibodies against CldU and IdU. Matching secondary antibodies, with different Alexa dies (488, 594, 647) were used for signal visualization. Image acquisition was performed using a Leica DMI fluorescence 6000B microscope. For each sample were performed at least 500 measurements for origin firing.

**Table 2.2**. Primary antibodies used in different assays: immunohistoquemistry (IHQ), Immunofluorescence (IF), Immunorecipitation (IP) and Western blot (WB)

| Antibody | Host Species | Application | Dilution | Source/Clone |
|---|---|---|---|---|
| AKT | Rabbit | WB | 1:500 | Cell Signaling |
| α-tubulin | Mouse | IF, WB | 1:2000 | Sigma / DM1A |
| Aurora B | Rabbit | IF | 1:200 | Abcam |
| Cyclin B1 | Rabbit | WB | 1:500 | Santa Cruz Biotech |
| Cdk1 | Rabbit | WB | 1:500 | Santa Cruz Biotech |
| Erk 1/2 | Rabbit | WB | 1:1000 | Cell Signaling |
| Mastl | Mouse | WB, IP | 1:500/1:100 | ABGENT/ RB35078 |
| Phospho- (ser) Cdk substrate | Rabbit | WB | 1:500 | Cell Signaling |
| Psopsho-Erk 1/2 (Thr202/Tyr204) | Rabbit | WB | 1:1000 | Cell Signaling |
| Phospho-Histone H3 (Ser10) | Rabbit | IF, IHQ | 1:500/1:2000 | Upstate Biotechnology |
| Phospho-Histone H2A.X (Ser139) | Mouse | IHQ | 1:2000 | Millipore / JBW30 |
| Vinculin | Mouse | WB | 1:5000 | Sigma |
| Von Wilebrand Factor | Mouse | IHQ | 1:2000 | Millipore |

## 3.7. Statistical analysis

Statistical analysis was carried out using Prism 5 (GraphPad). All statistical tests were performed using two-sided, unpaired Student's t-tests, or the Fisher's exact test. Data with $p<0.05$ were considered statistically significant. In most figures, *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

# 4. Results

## 4.1. A role for Mastl in megakaryopoiesis and platelet formation

### 4.1.1. A new mouse model for thrombocytopenia

Given the presence of mutations in *MASTL* in human thrombocytopenia, we initially focused on the analysis of the requirements for this kinase during endomitosis and platelet formation. To this end, we generated two mutant mice strains with specific alterations in the corresponding murine locus. We first generated a knockin allele carrying the ortholog mutation found in human thrombocytopenic patients (E167D; corresponding to E166D in the mouse; *Mastl*(E166D); Figure 4.1). Second, we generated a mouse strain with specific ablation of Mastl in megakaryocytes. Since homozygous germline ablation of Mastl [*Mastl*(−/−)] results in early embryonic lethality we made use of a conditional allele [*Mastl*(lox)] recently generated in our laboratory (Álvarez-Fernández et al. 2013) in which exon 4, carrying essential domains for kinase activity, can be deleted upon activation of Cre recombinase. We decided to specifically ablate it in mature megakaryocytes by crossing *Mastl*(lox/lox) mice with transgenic mice expressing Cre recombinase under the Pf4 promoter (Tiedt et al. 2007). Mutant mice carrying this null allele [*Mastl*(Δ)] specifically lack Mastl expression in megakaryocytes and platelets (Figure 4.1).

*Mastl*(E166D/E166D) [from now on *Mastl*(D/D)] were viable and fertile and displayed no overt abnormalities during the first months of life suggesting that the E166D mutation does not lead to a complete loss of function protein. Since acute ablation of Mastl in fibroblasts leads to severe mitotic defects and impaired growth we decided to isolated and characterize MEFs from *Mastl*(D/D) to further analyze any intrinsic effect of the E166D mutation on Mastl activity.
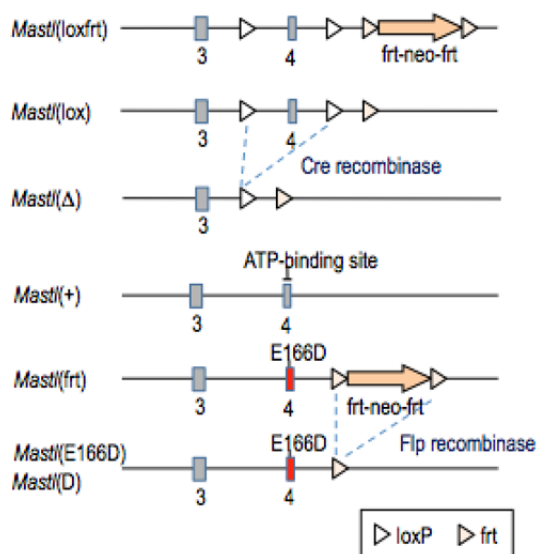


**Figure 4-1. Mastl mutant mice.** *Mastl* locus encoding Mastl contains 12 exons. The exon 4 which encodes the critical ATP-binding site for the kinase activity was flanked by loxP sites (triangles) and a selection cassette [*Mastl*(loxfrt) allele]. Cre-mediated recombination conditionally induced results in the *Mastl*(Δ) null allele. For the generation of the thrombocytopenia mouse model, exon 4 bearing the E166D mutation was followed by a selection cassette flanked by frt sites. Site-specific recombination by Flp results in the *Mastl*(E166D) or *Mastl*(D) allele.
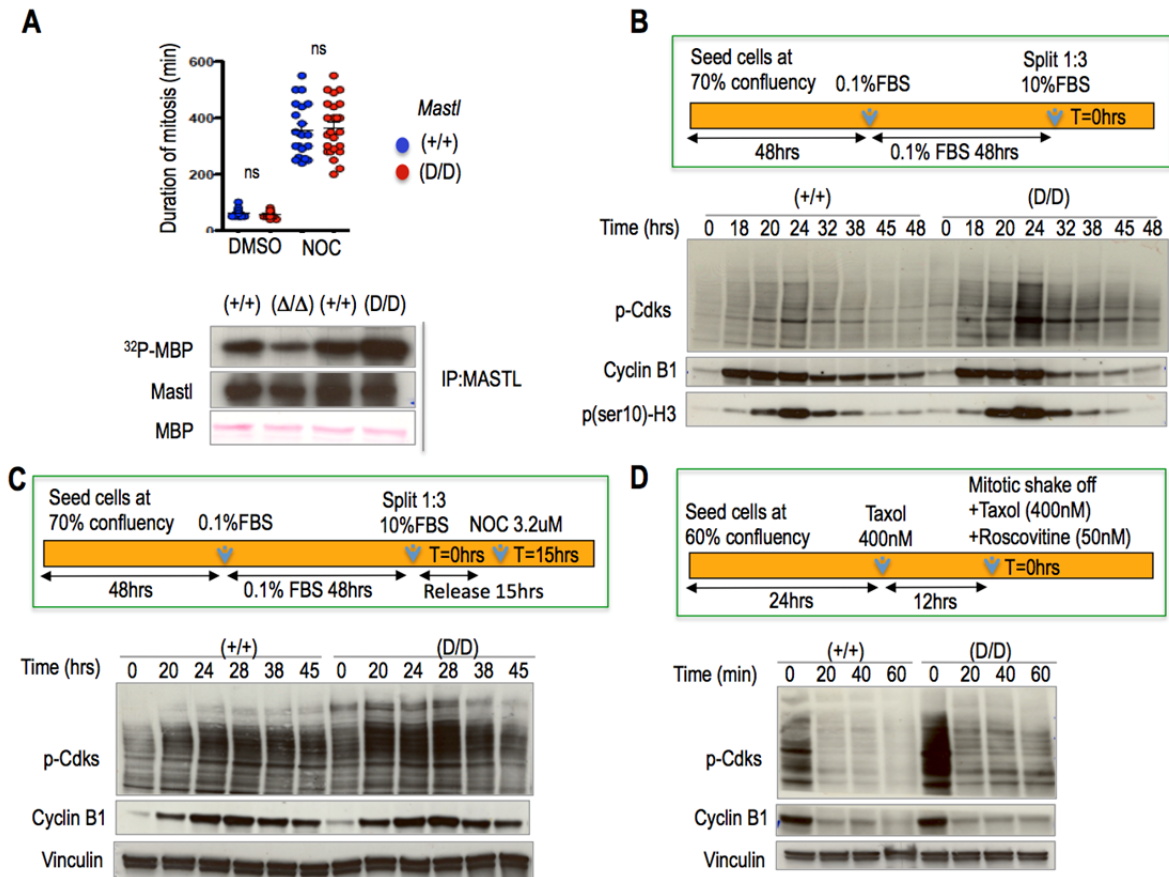
**Figure 4.2.** Analysis of *Mastl*(D/D) MEFs. (**A**) *Mastl*(+/+) and *Mastl*(D/D) primary MEFs expressing H2B-GFP were recorded by time lapse microscopy and the duration of mitosis were analyzed upon DMSO or Nocodazole (3,2 μM). Each dot represents an individual cell (N=30 per group). P>0.05, Student's t test. (**B**) *Mastl*(+/+) and *Mastl*(D/D) primary MEFs were synchronized at G0 by serum depletion and cell cycle entry was analyzed for the indicated time points upon seeding cells at low confluence and serum addition (T=0). Cell extracts were immunoblotted for phospho-Cdks substrates (p-Cdks), phospho-histone 3 (pH3) and cylin B. (**C**) Western blot analysis of fibroblasts as in (B) in presence of nocodazole. (**D**) Mitotic shake off of primary fibroblasts treated for taxol for 12 hrs. Pure mitotic cells were treated with 50nM of roscovitine in presence of taxol and exit from mitosis was monitored by immunoblotting for p-Cdks and cylin B1. Vinculin was used as a loading control.

*Mastl*(D/D) fibroblasts grew in similar rate compared to *Mastl*(+/+) MEFs and had a similar duration of mitosis either in steady state conditions or with nocodazole treatment (Figure 4.2A upper panel) indicating that Mastl E166D does not lead to mitotic defects and that is compatible with cell cycling. To assess directly the impact of the mutation on the Mastl kinase activity we immunoprecipitated the endogenous Mastl from *Mastl*(D/D) and *Mastl*(+/+) MEFs and we performed kinase assays using recombinant myelin basic protein (MBP) as a substrate. Importantly Mastl E166D showed the same or slightly higher activity compared to the wild type Mastl (Figure 4.2A; lower panel). Interestingly when we analyzed

cell cycle entry upon serum starvation, either in presence or absence of nocodazole, we observed that *Mastl*(D/D) cells showed higher levels of phospho-Cdks and MAPKs residues (a well-established readout for the function of Mastl in the balance between Cdks and PP2A in substrate phosphorylation; Figure 4.2B, C). This increase is not because of a higher mitotic index since cyclin B1 and pH3 levels were similar in both cell groups. In addition similar results were observed when extracts from the same amount of mitotic cells were treated with roscovitine in presence of taxol and subsequently subjected to immunobloting for phospho-Cdks and MAPKs substrates (Figure 4.2D). Overall these assays indicate that the Mastl E166D results in a functional gain-of-function rather than a loss of function mutation.

### 4.1.2. Mastl role in megakaryopoiesis is cell cycle independent

We next analyzed blood platelet levels in Mastl mutant mice. Given the variability between animals (sex and age dependent) we chose animals between 8 and 12 weeks to perform our analysis. *Mastl*(D/D) 12 weeks-old showed slightly reduced platelets numbers with average platelet number of $731 \pm 24$ (mean $\pm$ SEM; more obvious in females than males) although not dramatic differences were detected compared to the control mice $874 \pm 22$ (Figure 4.3A). Similar results we found in *Mastl*($\Delta/\Delta$) mice although with more pronounced thrombocytopenia with average platelet number of $668 \pm 43$. Interestingly, both *Mastl*(D/D) and *Mastl*($\Delta/\Delta$) cohorts analyzed during the whole adult mouse life in different ages (8-100 weeks) displayed an increased mild thrombocytopenia incidence (animals with blood platelet levels less than $800 \times 10^9$ platelets/L) although the differences were not significant when considering the whole cohort in *Mastl*(D/D) mice. (Figure 4.3B).
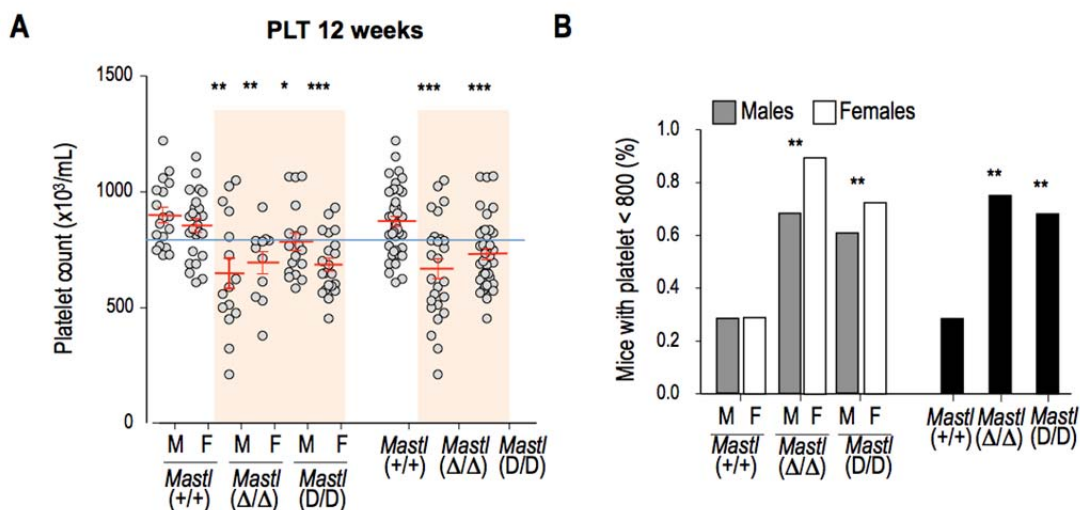


**Figure 4.3. Blood platelet levels in Mastl mutant mice** (**A**) Blood platelets levels from 12 week-old *Mastl*(+/+), *Mastl*(D/D) and *Mastl*($\Delta/\Delta$) mice. Data represent mean $\pm$ SEM; (\*, P < 0.05, \*\*, P < 0.01, \*\*\*, P < 0.001. Each dot represents an individual animal. (**B**) Percentage of mice showing blood platelet levels below $800 \times 10^3$ platelets/mL in the whole cohort of 8-100 week-old *Mastl*(+/+), *Mastl*(D/D) and *Mastl*($\Delta/\Delta$) mice.

While *Mastl*(D/D) and *Mastl*(Δ/Δ) mice presented no apparent abnormalities in bone marrow and spleen indicated by the hematoxylin/eosin (H/E) staining, *Mastl*(Δ/Δ) displayed a significant defect in the maturation of megakaryocytes in the bone marrow compared to *Mastl*(D/D) and control mice (Figure 4.4A). Megakaryocytic maturation was estimated by analyzing the Von Wilebrand Factor (VWF) staining in bone marrow sections, since its exclusive expression in megakaryocytes correlates perfectly with their maturation. Morfometric analysis of the total, cytoplasmic and nuclear area of VWF$^+$ cells revealed that while *Mastl*(D/D) and control megakaryocytes presented proper maturation *Mastl*(Δ/Δ) megakaryocytes showed a significant reduction in the total and cytoplasmic area. However nuclear area was not significantly different in any of the cell groups indicating no differences in megakaryocyte polyploidization (Figure 4.4B). Interestingly *Mastl*(Δ/Δ) megakaryocytes had a significantly lower cytoplasm to nucleus ratio, a well defined feature of terminal megakaryocytic differentiation.
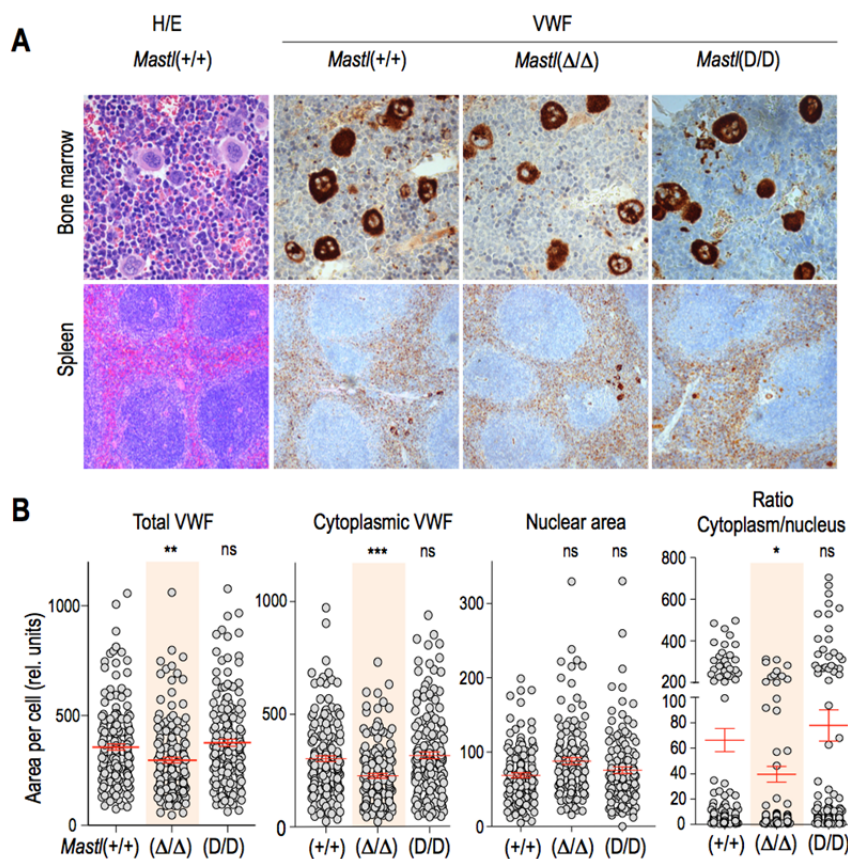


Figure 4.4. *Mastl*(Δ/Δ) mice present compromised megakaryocyte maturation. (A) Hematoxylin/eosin-stained bone marrow and spleen sections and immunohistochemical staining for Von Wilebrand Factor (VWF). (B) Graphs showing the distribution of area of VWF positive cells based on cytoplasmic, nuclear and total area measurements. Each dot represents an individual cell. Total cells analyzed per group (400) come from 3 individual animals (n=3). Mice between 8 and 12 week-old were used to perform the experiments.

Accordingly the percentage of double CD41$^+$CD42$^+$ cells, which represent the more mature megakaryocytic population since CD42 expression is a later event during megakaryopoiesis, was reduced in the bone marrow of *Mastl*(Δ/Δ) mice whereas in the *Mastl*(D/D) was similar to the control mice (Figure 4.5A). In agreement with the similar nuclear area of bone marrow megakaryocytes among the different genotypes, *Mastl*(Δ/Δ) as well as *Mastl*(D/D) CD41$^+$CD42$^+$ megakaryocytes acquired similar polyploidization levels compared to *Mastl*(+/+) control ones (Figure 4.5B). In addition no defects were detected either in hematopoietic progenitors in bone marrow (Figure 4.5C), or in any other mature hematopoietic cells in spleen and thymus (data not shown) in any of the *Mastl*(Δ/Δ) or *Mastl*(D/D) mice. Overall these date indicate that Mastl role in platelet formation is related with late stages of megakaryopoiesis and thrombopoiesis rather than early cell cycling events during endomitosis and thus during polyploidization.
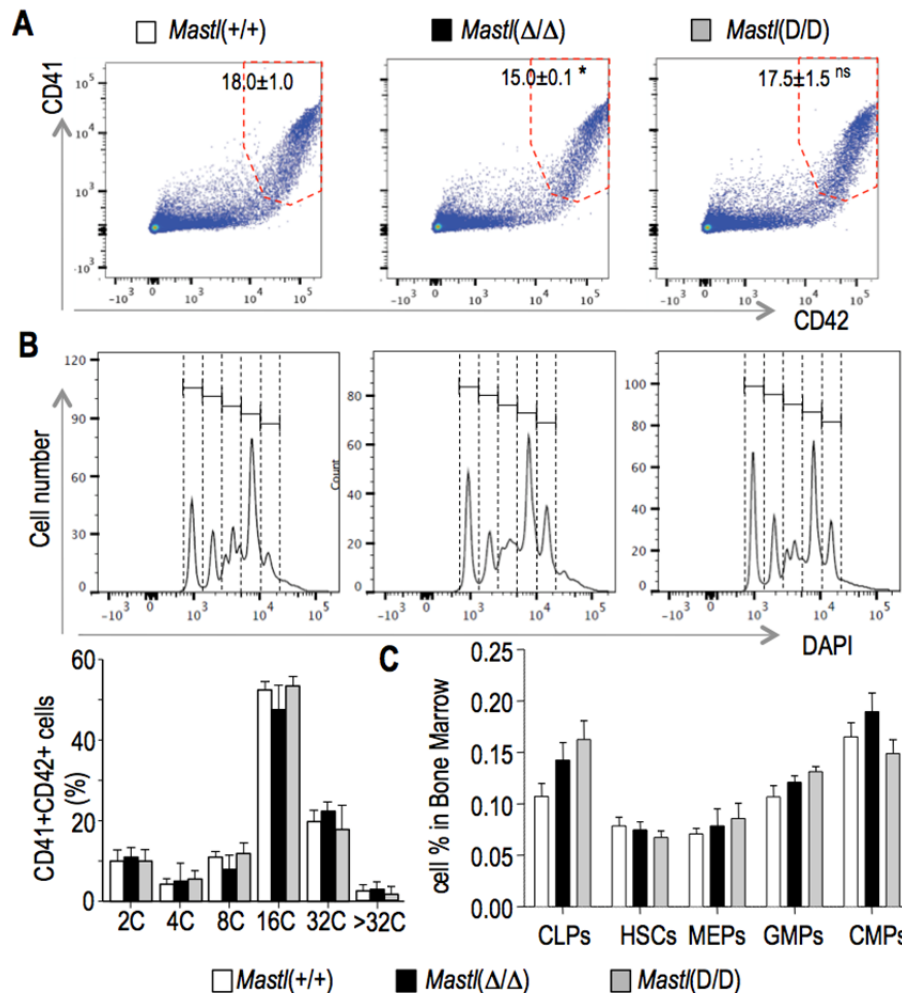


**Figure 4.5.** **Megakaryocyte polyploidization in Mastl mutant mice. (A)** FACS analysis for CD41$^+$CD42$^+$ cells from bone marrow and **(B)** its ploidy distribution estimated by DAPI staining. **(C)** Percentage of distinct subgroups of hematopoietic progenitors from bone marrow by FACS analysis. HSCs (Hematopoetic stem cells), CLPs (common lympoid progenitors), CMPs (Common myeloid progenitors), MEPs (megakaryocyte-erythrocyte progenitors), GMPs (granylocyte-macrophage progenitors). n=5 per group. Data represent mean ± SEM; (P > 0.05). Mice between 8 and 12 week-old were used to perform the experiments

### 4.1.3. Mastl role in late events of megakaryopoiesis

To identify in which specific stages Mastl modulation affects megakaryopoiesis and platelet formation, we studied the TPO-induced polyploidization and differentiation of bone marrow hematopoietic progenitors (Lin$^-$ c-Kit$^+$) towards the megakaryocytic lineage *in vitro* (Figure 4.6). Hematopoietic progenitors upon 24-hour stimulation with TPO (Day1; Figure 4.6A) presented a clear 4C polyploid population positive for CD41 while 48 hours later (Day 3) the CD41$^+$ cells reached ploidies even higher than 32C (Figure 4.6B). In agreement with the *in vivo* data, hematopoietic progenitors from *Mastl*(+/+), *Mastl*(Δ/Δ) or *Mastl*(D/D) mice showed similar levels of CD41$^+$ cells along differentiation with similar DNA content. In addition giemsa staining along differentiation at several days revealed that *Mastl*(+/+) and *Mastl*(D/D) megakaryocytes after polyploidization around day 5 underwent a maturation process prior to platelet shedding as it is indicated by the lighter blue color of the stained cytoplasm (Figure 4.6C).
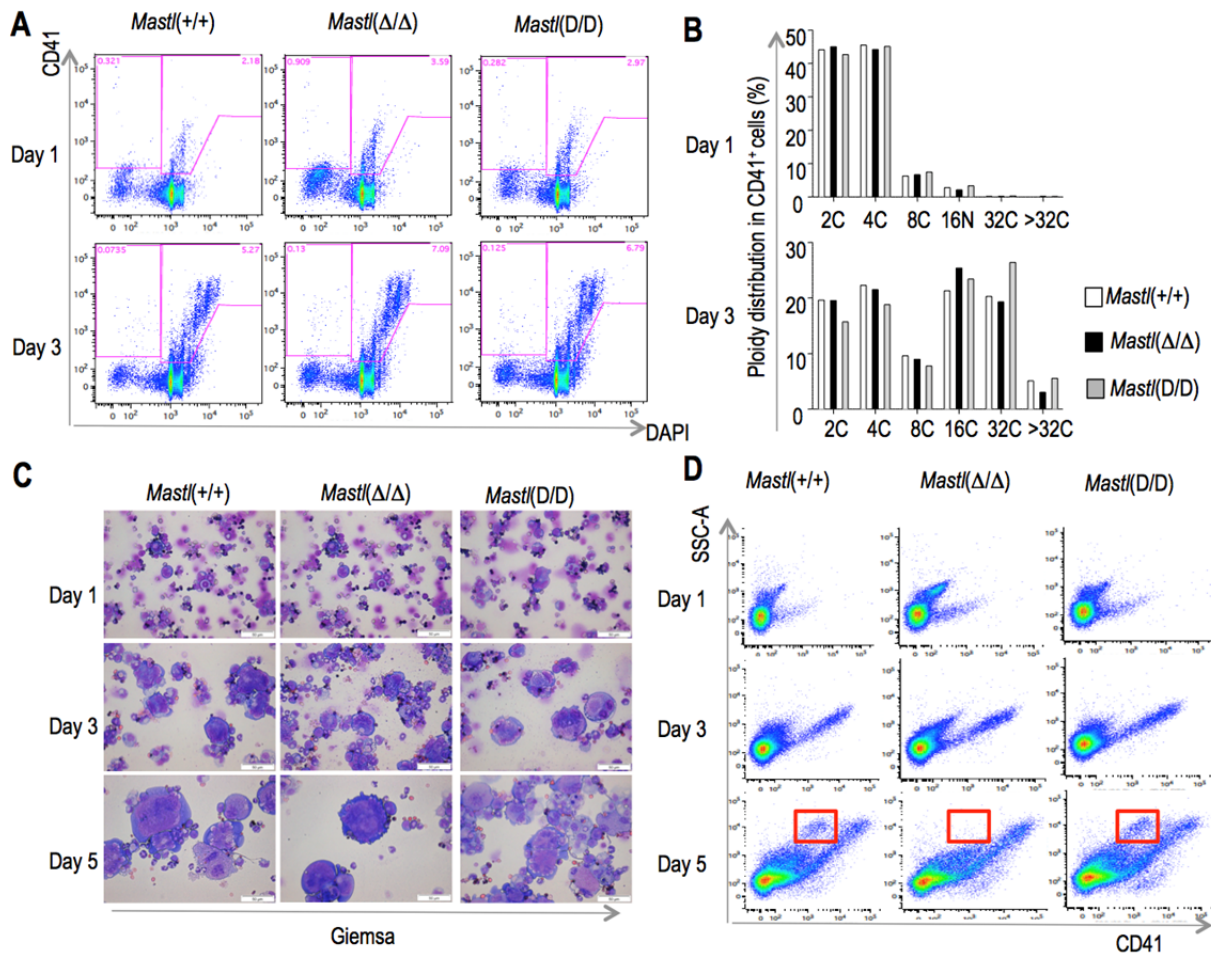


Figure 4.6. *In vitro* polyploidization of hematopoietic progenitors upon TPO stimulation. (A) FACS analysis for CD41 expression and DNA content by DAPI in bone marrow hematopoietic progenitors (Lin$^-$ c-Kit$^+$) upon TPO stimulation (100 ng/ml) (B) Graph representing the ploidy distribution estimated by DAPI staining of the CD41$^+$ cells at day 1 and 3 of differentiation. (C) Giemsa staining in cytospan cells on the indicated days of differentiation. Mice between 8 and 12 week-old were used to perform the experiments

However *Mastl*(Δ/Δ) megakaryocytes although they polyploidized, indicated by the increase in the cellular and nuclear size, did not mature further as it is suggested by the dark blue color of the cytoplasmic staining as well as the lack of pro-platelet bearing cells. Interestingly further FACS analysis of the cellular complexity (SSC-A) and CD41 expression allowed us to identify at late stages of differentiation around day 5 a population completely absent from *Mastl*(Δ/Δ) megakaryocytes while both *Mastl*(+/+) and *Mastl*(D/D) presented (Figure 4.6D). Even thought we cannot determine the identity of this population we speculate that represents megakaryocytes during the maturation process before platelet release.

### 4.1.3. Mastl controls MAPK activity through PP2A regulation

Mastl is known to exert its function through direct phosphorylation of ENSA and Arpp19 inhibiting PP2A phosphatase. On the other hand, PP2A is involved in multiple functions in megakaryocyte and platelet function (Gushiken et al. 2008; Pawlowski et al. 2002; Wang et al. 2008) . We therefore focused in the analysis of the phosphorylation state of known substrates of PP2A that could be involved in platelet formation. Interestingly, *Mastl*(D/D) megakaryocytes isolated from the bone marrow displayed a significant increase in the phosphorylation of mitogen-associated protein kinases (Erk), and less significantly of Akt kinases while *Mastl*(Δ/Δ) megakaryocytes showed a slight reduction in the Erk phosphorylation level (Figure 4.7A).
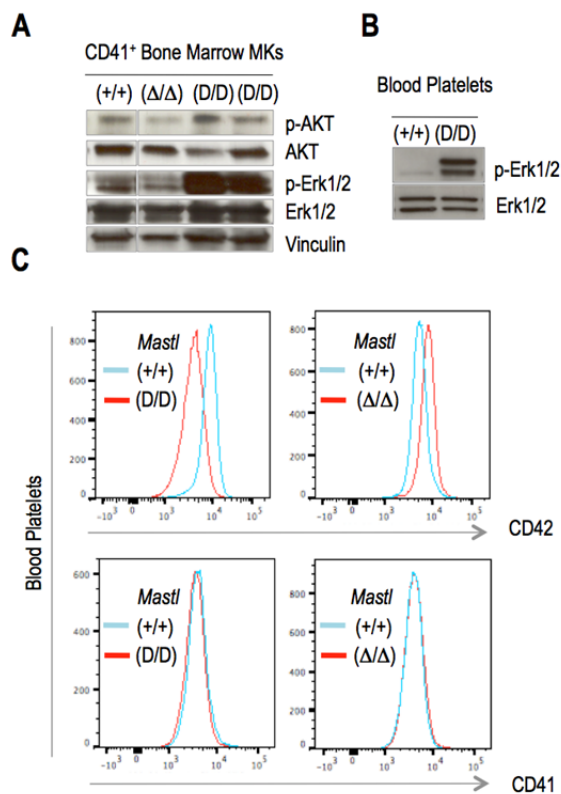


**Figure 4.7. Mastl modulates MAPK activation in megakaryocytes and platelets.** (A) Western blot analysis for phospho-Erk1/2, total Erk1/2, phospho-AKT and total AKT levels in extracts from primary bone marrow megakaryocytes (CD41⁺) from *Mastl*(+/+), *Mastl*(D/D) and *Mastl*(Δ/Δ) mice. (B) Platelet extracts isolated from platelet-rich plasma (PrP) from 12 week-old *Mastl*(+/+), *Mastl*(D/D) and *Mastl*(Δ/Δ) mice and analyzed for Erk1/2 phosphorylation. (C) Blood platelet CD42 and CD41 expression from *Mastl*(+/+), *Mastl*(D/D) and *Mastl*(Δ/Δ) mice.

**Figure 4.8. Mastl modulates MAPK activation in the megakaryocytic cell line HEL.** (**A**) Schematic representation of the protocol followed for Mastl knock-down in HEL cell line. (**B**) Western blot analysis for phospho-Erk1/2, and MASTL levels in extracts from HEL cells transduced with lentiviruses expressing either a short hairpin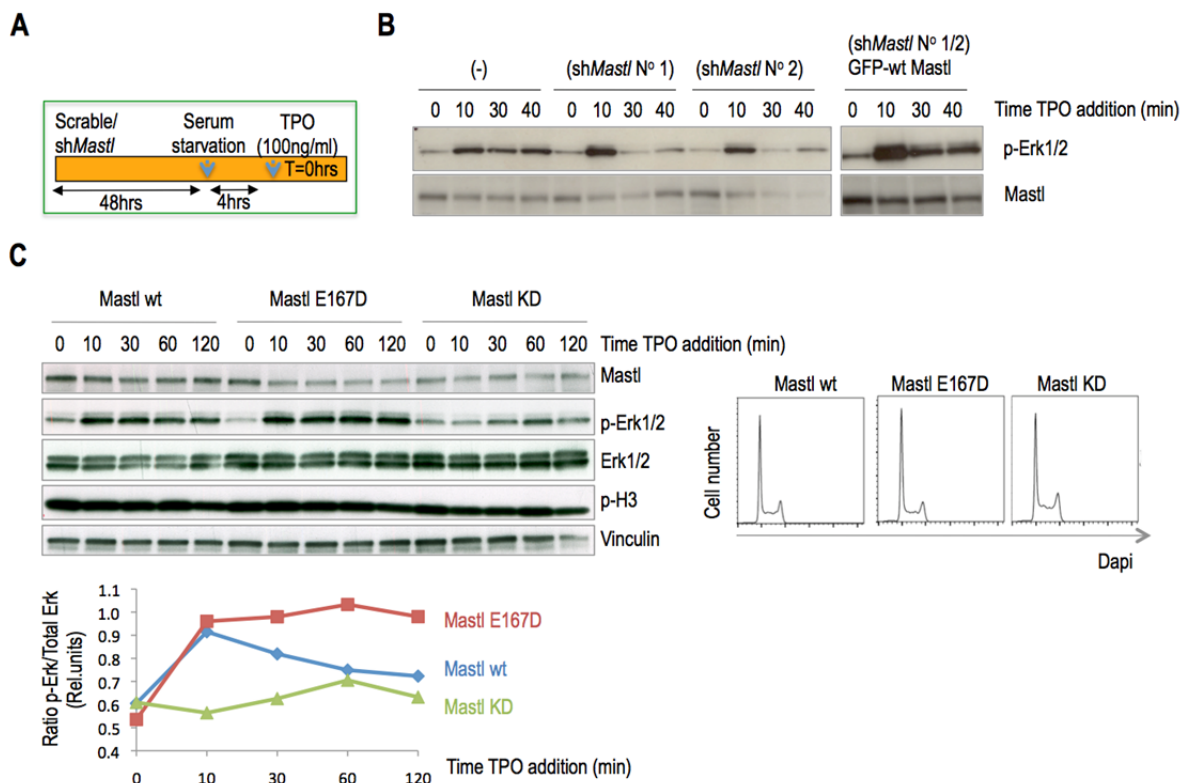 against Mastl or full length Mastl fused to GFP (GFP-wt Mastl) and treted with TPO for the indicated times following the protocol in sheme A. (**C**) Immunoblotting of HEL cells stably expressing GFP-wt Mastl, GFP-E166D Mastl, or GFP-D156A (kinase dead mutant) upon TPO stimulation for the indicated times and cell cyle profiles by DAPI staining and FACS analysis.

Importantly we observed a similar increase in the Erk phosphorylation status of resting blood platelets freshly isolated from *Mastl*(D/D) mice compared to control ones (Figure 4.7B). In addition platelets isolated from *Mastl*(D/D) mice showed a clear reduction in the CD42 glycoprotein which is part of VWF receptor while CD41 was similarly expressed in both platelet groups (Figure 4.7C).

PP2A is known to revert the phosphorylation of Erk in different cell types (Liu et al. 2013; Sunahori et al. 2013) and Erk signaling is known to be critical in several functions in megakaryocytes such as maturation (Besancenot et al. 2010) and pro-platelet formation (Mazharian et al. 2009; Bluteau et al. 2014), as well as in platelet activation (Gushiken et al. 2008; Pawlowski et al. 2002). This led us to form the hypothesis that Mastl either direct or indirect affects Erk activation pathway. The deregulated Erk activity might cause the lack of maturation in the *Mastl*(Δ/Δ) megakaryocytes where p-Erk levels found to be reduced, while in *Mastl*(Δ/Δ) megakaryocytes the enhanced Erk activitation could allow megakaryocytic maturation but lead to defects during the proplatelet formation. To test this hypothesis we

chose the human megakaryoblastic leukemia cell line (HEL) and analyzed the Erk activation pathway upon changes in Mastl expression

As a general outline we serum starved the cells for 4 hours to eliminate any basal activation of the pathway and in absence of serun we stimulated the cells with the addition of 100 ng/ml of recombinant TPO. The activation of the MAPK signaling pathway was evaluated in the indicated time points (Figure 4.8A). To analyze the role of Mastl in the Erk activation pathway we treated the HEL cell line with two independent short hairpin sequences against human Mastl sequence (shMastl Nº1, shMastl Nº2) and we found that in both cell groups treated with shMast presented reduced p-Erk levels. Impotantly we could appreciate that the major phosphorylation differences were observed at 30 and 40 min upon TPO stimulation while the peak activity at 10 min was not significantly different, indicating that Mastl absence affects the dephosphorylation rate of Erk1/2 rather than the upstream activation likely through direct inhibition of PP2A which is known to dephosphorylate Erk and hence avoid sustained activation of the pathway (Figure 4.8B). Accordingly we were able to restore Erk phosphorylation kinetics through the re-expresion of wild-type Mastl fused to GFP (Figure 4.8C). Interestingly Erk phosphorylation levels were overall increased upon Mastl overexpression compared to cells expressing only endogenous Mastl, likely because of enhanced PP2A inhibition. To further establish the relationship between Mastl activity and Erk activation we generated stable HEL cell lines expressing either wild-type E167D mutatnt or kinase-dead (KD) Mastl, fused to GFP. Consistently to the previous data, loss of Mastl activity (Mastl KD) led to reduced p-Erk levels while expression of Mastl bearing the thrombocytopenia mutation (E167D) presented sustained Erk phosphorylation, an additional evidence that the E167D mutant is a gain of function mutation that could lead to enhanced PP2A inhibition (Figure 4.8C). The differences in p-Erk levels obtained from different Mastl protein expression are not a consequence of cell cycle arrest in different cell cycle phases since the several stable cell lines presented similar cell cycle profiles and p-H3 levels (Figure 4.8C).

### 4.1.4. Effect of E166D mutation on platelet clearance

Mastl E166D mutant platelets showed defects in the expression of CD42 glycoprotein although Mastl E166D mutant megakaryocytes presented only moderate defects in proplatelet formation. We therefore tested for additional defects in *Mastl*(D/D) platelets. To test this hypothesis we evaluated the *in vivo* platelet life span. For this purpose we stained the whole pool of circulating platelets and monitored the daily decay in stained platelets for up to 5 days, indicative of their destruction and clearance by the spleen.

Interestingly while *Mastl*(+/+) and *Mastl*(Δ/Δ) mice display similar platelet destruction rate, mice bearing either one or two *Mastl*E166D alleles showed significantly enhanced decay of stained platelets at day 2 and 3 suggesting decreased platelet life span in those mice (Figure 4.9A).

**Figure 4.9. Determination of the *in vivo* platelet life span in *Mastl*(+/+), *Mastl*(D/D) *Mastl*(+/D) and *Mastl*(Δ/Δ) mice. (A)** Mice between 8 and 12 weeks of age were injected with 600 μg NHS-biotin to label platelets in vivo (Day 0). Results are percentage of fluorescently labeled platelets at the indicated days after injection as determined by flow cytometry. Values are mean ± SEM. n=5 mice per group (*, P < 0.05, **, P < 0.01 Student's t-test). **(B)** Scheme representing future experiments for analysinh any intrinsic defect in survival of *Mastl*(D/D) platelets.

To define weather the reduced survival of platelets in *Mastl*(D/D) mice is a cell autonomous effect or not we plan to perform platelet adoptive transfers and study their *in vivo* life span in the recipient mice, since *Mastl*(D/D) mice express Mastl E166D ubiquitously and not only in megakaryocytes and platelets (Figure 4.9B). However the most plausible hypothesis is that because of the sustained Erk activation platelets released from *Mastl*(D/D) megakaryocytes present defects in survival. Against the non-cell autonomous hypothesis is the fact that human patients bearing the mutant *Mastl* allele and with thrombocytopenia syndrome did not show any improvement upon immunosuppressive treatment suggesting that at least in humans the reason behind thrombocytopenia development is not an autoimmune reaction.

Altogether, these data suggest that, contrary to what was expected Mastl mutations in thrombocytopenia patients do not alter cell cycle progression or polyploidy but rather disrupt the normal regulation of MAPK pathways. In addition, these results describe a new cell cycle-independent function for Mastl in the control of Erk phosphorylation.

## 4.2. Relevance of mitotic kinases during endomitotic progression

In the following sections, we aim to further understand how endomitotic cycles are regulated through the specific genetic ablation of several cell cycle regulators in mature megakaryocytes (Figure 4.10).
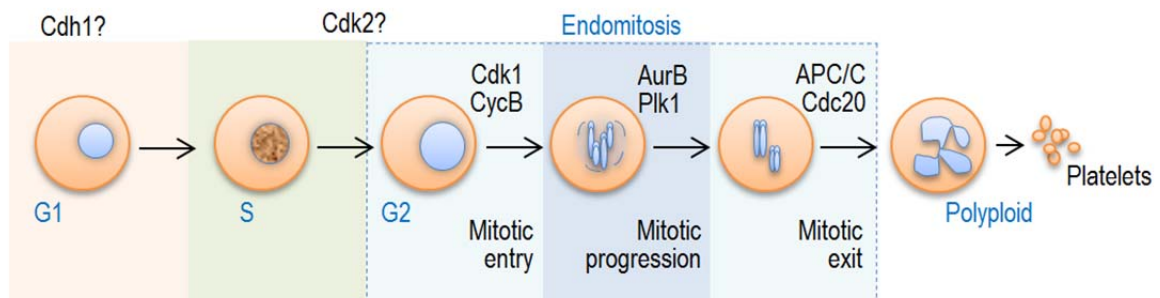


**Figure 4.10**. A general schematic representation of the possible involvement of several mitotic regulators during endomitosis. Their role in megakaryocytes will be investigated in the following sections by using tissue-specific genetic knockouts for the corresponding genes in the mouse.

To this end, we will generate tissue-specific knockouts of

a) proteins involved in mitotic progression (Aurora B, Plk1)
b) regulators of the oscillations required for "cycling" (APC/C coactivators Cdc20 or Cdh1)
c) kinases required for mitotic entry (Cdk1, Cdk2?)

We will start in this section addressing the relevance of two kinases required for the proper segregation of chromosomes, Aurora B and Plk1.

### 4.2.1. Aurkb is dispensable for megakaryocyte development and thrombopoiesis

We first analyzed the relevance of Aurora B, a mitotic kinase with essential roles in the error-correction mechanism that determines chromosome-microtubule attachments (Nezi & Musacchio 2009). Aurora B-null cells display a normal mitotic entry but prematurely exit from mitosis without being able to form bipolar spindles and in the absence of chromosome segregation (Fernández-Miranda et al. 2011;Trakala et al. 2013). Lack of Aurora B therefore results in tetraploidy followed by cell cycle arrest or death in cultured cells, and genetic ablation of kinase results in embryonic lethality in the mouse.
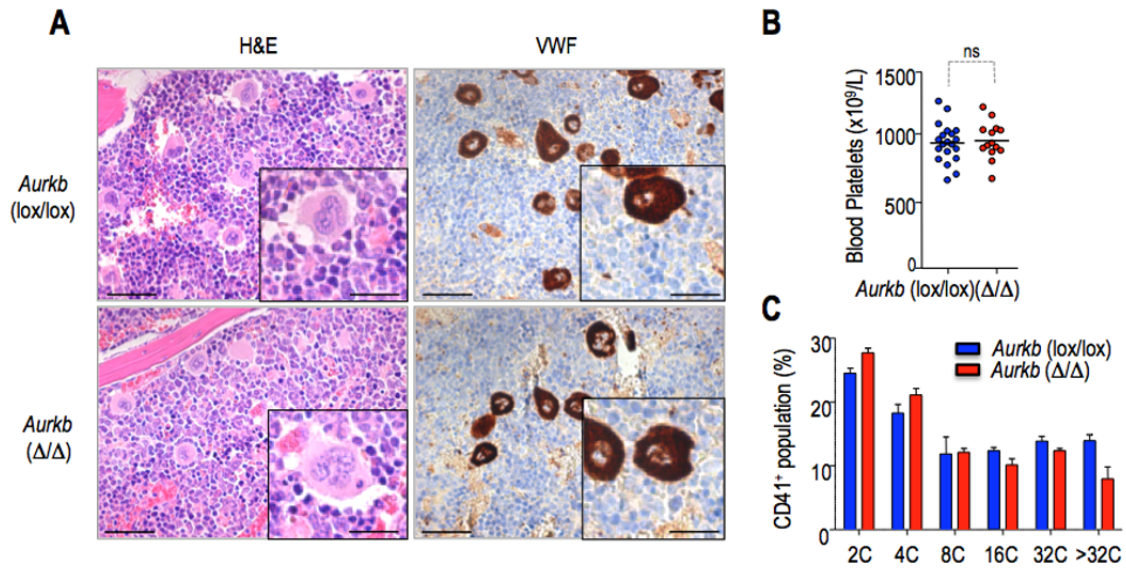
**Figure 4.11. Aurkb is dispensable for megakaryopoiesis and platelet formation. (A)** Hematoxylin/eosin-stained bone marrow sections and immunohistochemical staining of Von Wilebrand Factor (VWF) as a marker for megakaryocyte identification and maturation of 12 week-old mice. Pf4-Cre; *Aurkb*(Δ/Δ) show normal megakaryocyte number, size and maturation by the analysis of VWF. **(B)** Blood platelet levels from 8-12 week old Pf4-Cre; *Aurkb*(Δ/Δ) and Pf4-Cre; *Aurkb*(Δ/Δ) mice. No changes were detected in blood platelet levels of Pf4-Cre; *Aurkb*(Δ/Δ) (P>0.05; t test) **(C)** Ploidy distribution inside the CD41 population in bone marrow by DAPI staining. In Pf4-Cre; *Aurkb*(Δ/Δ) the ploidy distribution is similar compared to wild-type (p>0.05; t test). All graphs show mean with ± SEM. Scale bars, 50 μm and 20 μm (insets).

To understand the relevance of Aurora B in endomitosis and megakaryocyte development we made use of a conditional allele *Aurkb*(lox) previously generated in our lab (Fernández-Miranda et al. 2011) which upon Cre activation is excised leading to a null allele. Since complete ablation of Aurora B is embryonic lethal in mice, we crossed *Aurkb*(lox/lox) mice with Pf4-Cre transgenic mice. Pf4-Cre; *Aurkb*(Δ/Δ) [from now on *Aurkb*(Δ/Δ)] mice displayed normal survival and no overt defects during development. These mutant mice displayed fully matured megakaryocytes in the bone marrow as indicated by immunohistological staining for Von Wilebrand Factor (VWF; Figure 4.11A). Accordingly, we found no differences in blood platelet levels in *Aurkb*(Δ/Δ) mice (Figure 4.11B). The absolute total number of megakaryocytes in mutant bone marrow was not altered as scored by CD41 staining (data not shown) and we found no significant differences in the ploidy distribution between Aurora B-null and control CD41+ cells (Figure 4.11C).

We then isolated hematopoietic progenitors from fetal liver and we differentiated them towards megakaryocytes upon TPO addition (Figure 2). We did not observed either significant differences in the percentage of CD41+ cells along TPO stimulation for several days (Figure 4.12A), or in maturation levels of megakaryocytes as it is indicated by Giemsa staining at day 5 upon TPO stimulation (Figure 4.12B).
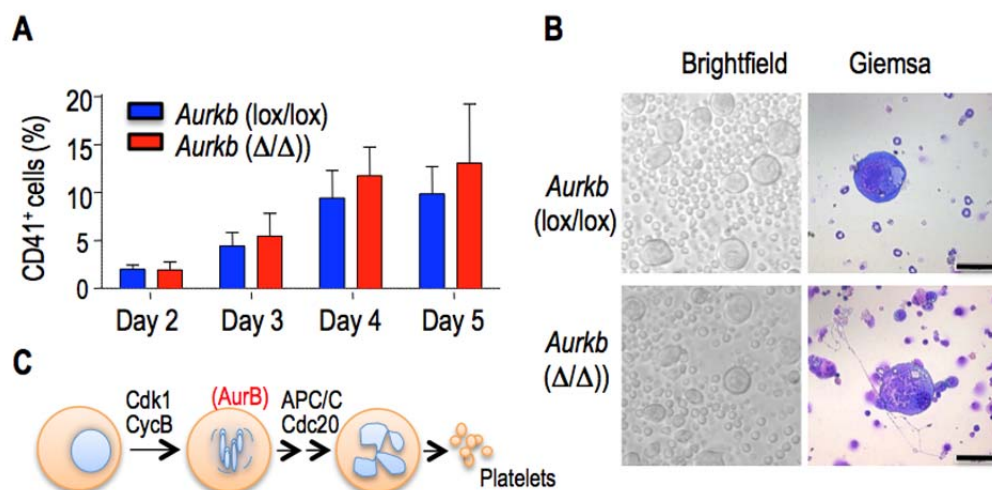
**Figure 4.12.** Aurora B null megakaryocytes can mature and shed platelets *in vitro* upon TPO stimulation. **(A)** CD41+ % of total alive cells analyzed by flow cytometry on days 2-5 after TPO stimulation of fetal liver cells extracted from E13.5 embryos. The cumulative percentages of CD41+ cells are similar in *Aurkb*($\Delta/\Delta$) and *Aurkb*(+/+) (P>0.05; t test) . **(B)** Brightfield images of fetal liver derived megakaryocytes by day 5 and Giemsa staining where Aurora B-null cells can mature, as indicated by the light blue color, and platelet shedding occurs as it can be seen the proplatelet formation in right lower panel. Graphs show mean with ± SEM. Scale bars, 50 μm **(C)** Scheme representing the dispensable role of Aurora B in endomitosis and platelet formation. Statistics were performed by t test.

Aurkb null megakaryocytes are fully proficient in platelet shedding (Figure 4.12B) indicating a complete maturation, terminal differentiation and full functionality of the cells. Overall these data suggest that Aurora B is dispensable for megakaryopoiesis and platelet formation in vivo (Figure 4.12C).

### 4.2.2. Plk1 and Megakaryopoiesis

Next we aimed to investigate the relevance of Polo-like kinase 1 (Plk1), an important kinase with essential roles not only in mitosis but throughout the whole cell cycle by regulating processes such as DNA damage response, mitotic entry, spindle formation, chromosome-microtubule attachments, chromosomal cohesion, and cytokinesis. As a consequense of the many critical functions of Plk1, genetic ablation of this kinase results in embryonic lethality in the mouse (P. Wachowicz, G. de Cárcer and M. Malumbres, unpublished data). Plk1-null early embryos (E1.5) and MEFs displayed massive arrest in prometaphase-like stages with unaligned chromosomes in presence of mono- and multi-polar spindles. In some cases cells could progress through mitosis but exited mitosis without cytokinesis forming binucleated cells.

The requirements for Plk1 in unconventional cell cycles have been barely studied. We therefore generated Pf4-Cre; *Plk1*($\Delta/\Delta$) [from now on *Plk1*($\Delta/\Delta$)] mice with specific ablation of Plk1 in megakaryocytes by crossing *Plk1*(lox/lox) mice (P. Wachowicz, G. de Cárcer and M. Malumbres, unpublished data) with Pf4-Cre transgenic mice.
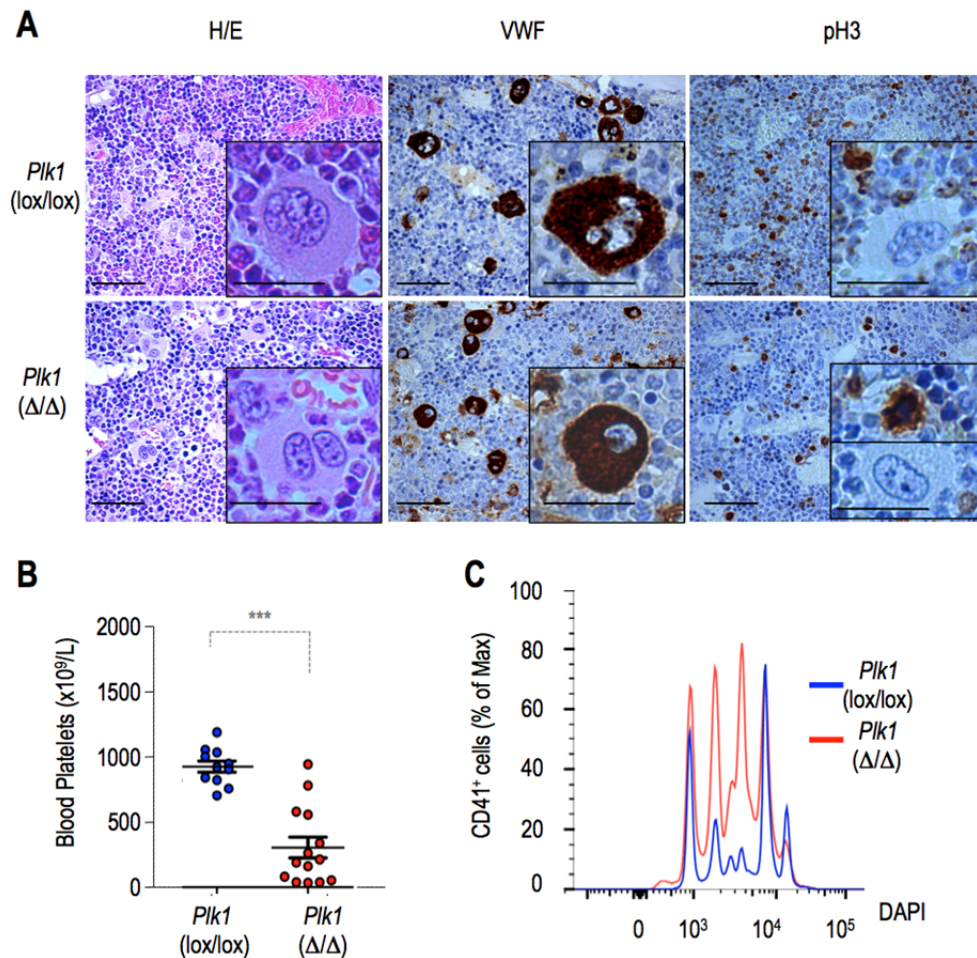
**Figure 4.13. Plk1 is essential for platelet formation (A)** Hematoxylin/eosin-stained bone marrow sections and immunohistochemical staining of Von Wilebrand Factor (VWF) as a marker for megakaryocytic maturation of 12 week-old mice. *Plk1*(Δ/Δ) mice show normal megakaryocyte number, size and maturation by the analysis of VWF. **(B)** Blood platelet levels from 8-12 week old Pf4-Cre; *Plk1*(Δ/Δ) and Pf4-Cre; *Plk1*(Δ/Δ) mice. No changes were detected in blood platelet levels of *Plk1*(Δ/Δ) (P<0.05; t test) **(C)** Ploidy distribution inside the CD41 population in bone marrow by DAPI staining representative of three experiments. All graphs show mean with ± SEM. Scale bars, 50 μm and 20 μm (insets).

*Plk1*(Δ/Δ) mice displayed normal development and survival being born at the expected frequency. These mutant mice displayed thrombocytopenia with average platelet count 284 ± 75 compared to 864 ± 40 (mean ± SEM) in *Plk1*(lox/lox) littermates (Figure 4.13A,B). Surprisingly, by immunohistological staining for VWF in bone marrow sections we observed mature megakaryocytes although these megakaryocytes presented hypolobulated nuclei. In addition apart from the mature megakaryocytes we detected megakaryocytes smaller in size and with lower VWF signal likely arrested in endomitosis as is it indicated by the presence of condensed chromatin in H/E staining and by pH3 positive mitotic signal (Figure 4.13A). In agreement with the presence of small mitotic and mature megakaryocytes evaluating the ploidy distribution of *Plk1*(Δ/Δ) mice we observed that the 16C and 32C populations were almost identical to the control ones whereas low ploidy (4C and 8C) megakaryocytes were more abundant likely as a consequence of the mitotic arrest (Figure 4.13C). Overall these data

show that Plk1 is essential for platelet formation and lack of this kinase may result in partial defects in megakaryocyte polyploidization. Yet, the molecular reasons for these defects have not been determined in this work and remain to be explored.

## 4.3. Regulation of endomitosis by the APC/C

### 4.3.1. APC/C-Cdh1 is essential for endoreplication but not for endomitosis

APC/C-Cdh1 is responsible for maintaining low levels of mitotic cyclins in interphase and its activity is critical to prevent mitosis and drive endoreduplication in *Drosophila* (Sigrist & Lehner 1997) and mammalian trophoblast giant cells (Garcia-Higuera et al. 2008). Its role in endomitosis, however, has not been established.

We therefore generated Pf4-Cre; *Cdh1*(Δ/Δ) [from now on *Cdh1*(Δ/Δ)] mice with specific ablation of Cdh1 in megakaryocytes by crossing *Cdh1*(lox/lox) mice (Garcia-Higuera et al. 2008) with Pf4-Cre transgenic mice. *Cdh1*(Δ/Δ) mice displayed normal development and survival being born at the expected frequency and no differences were observed in mature megakaryocytes after staining with VWF (Figure 4.14A). Mature megakaryocytes displayed increased levels of phosphorylation of H2AX (γH2AX) in agreement with the generation of replicative stress in the absence of this molecule (Eguren et al. 2013). This phenotype is likely a consequence of increased Cdk activity during replication entry and progression due to increased levels of cyclins in the absence of Cdh1 (Eguren et al. 2008). Lack of Cdh1 also resulted in a slight decrease in the levels of highly polyploid (16C or 32C) megakaryocytes (Figure 4.14B), although these differences did not result either in a significant defect in the number of peripheral blood platelets (Figure 4.14C) or in serum TPO level (Figure 4.14D), thus suggesting that Cdh1 ablation does not impair megakaryocyte function

### 4.3.2. APC/C-Cdc20 is essential during endomitosis

We next explored the relevance of the other APC/C coactivator by specifically ablating Cdc20 in megakaryocytes. Cdc20 is known to be essential for exiting from the mitotic cycle since its absence prevents degradation of securin and cyclin B (Manchado, et al. 2010). Cdc20 ablation in cells results in metaphase arrest due to the high levels of cyclin B1 that prevent Cdk1 inactivation and anaphase onset (Manchado et al., 2010). Specific genetic ablation of Cdc20 in megakaryocytes resulted in a dramatic phenotype characterized by severe thrombocytopenia with average platelet count of $108 \pm 13$ x$10^9$/L platelets (mean $\pm$ SEM) in *Cdc20*(Δ/Δ) mutant mice compared to $782 \pm 37$ x$10^9$/L in *Cdc20*(lox/lox) littermates (Figure 4.15A).
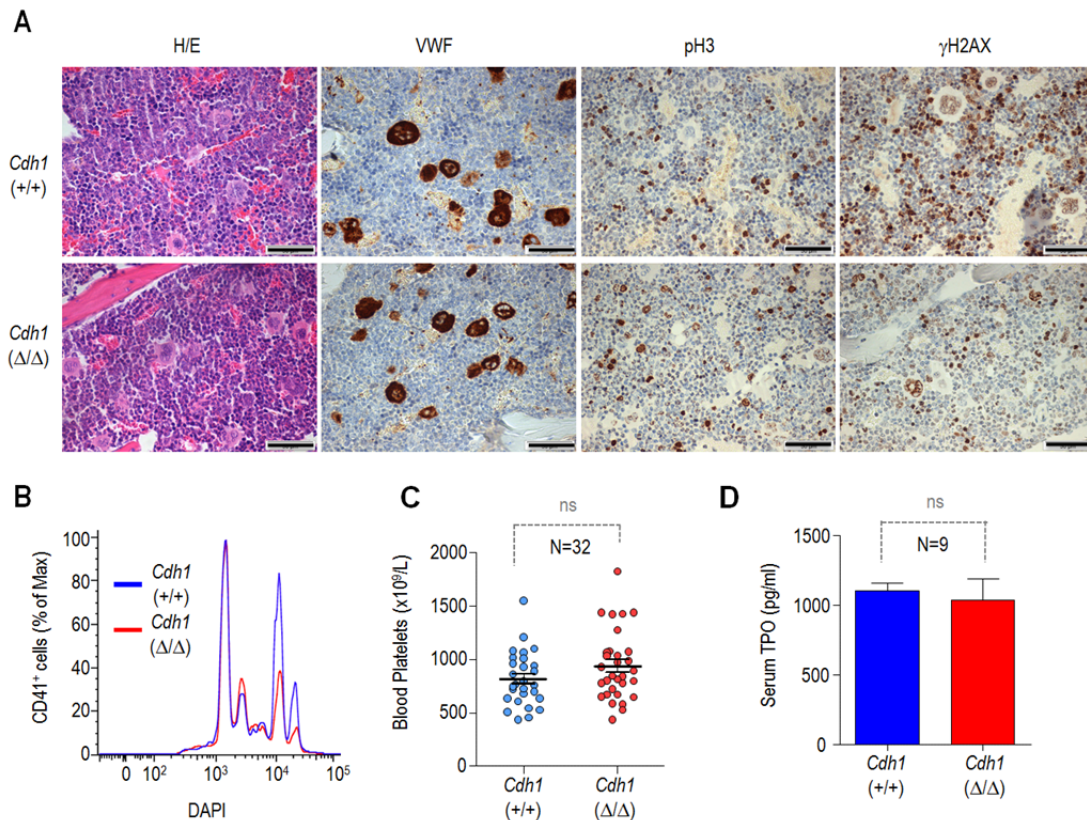
Figure 4.14. Cdh1 is dispensable for megakaryocytic maturation and platelet production. (A) Hematoxylin/eosin-stained bone marrow sections and immunohistochemical staining for Von Wilebrand Factor (VWF) as a marker for megakaryocyte identification and maturation, pH3 as G2/M marker and γH2AX in 12 week-old mice. $Cdh1(\Delta/\Delta)$ mice show normal megakaryocyte number, size and maturation by the analysis of VWF. (B) Blood platelet levels from 8-12 week old $Cdh1$(lox/lox) and $Cdh1(\Delta/\Delta)$ mice. No changes were detected in blood platelet levels of $Cdh1(\Delta/\Delta)$ (P>0.05; t test) (C) Serum TPO from mice between 8-14 week-old ) (P>0.05; t test). n=5 per group. (D) Ploidy distribution inside the CD41 population in bone marrow by DAPI staining, representative of three experiments. All graphs show mean with ± SEM. Scale bars, 50 μm.

In agreement with the presence of thrombocytopenia, we found in the bone marrow of $Cdc20(\Delta/\Delta)$ mutant mice a complete lack of mature megakaryocytes although with a robust increase in the total number of VWF positive cells (Figure 4.15B,C). In $Cdc20(\Delta/\Delta)$ mutant mice, the VWF positive cells were significantly smaller indicating lack of maturation (Figure 4.15B,D). These low-size megakaryocytes displayed abundant mitotic figures in the presence of phosphorylated histone H3, suggesting mitotic arrest in the absence of Cdc20 (Figure 4.15B, F). In addition, evaluating the ploidy distribution of megakaryocytes in $Cdc20(\Delta/\Delta)$ mutant mice we observed that the 16C and 32C populations were almost absent whereas low ploidy (4C and 8C) megakaryocytes were more abundant likely as a consequence of the mitotic arrest (Figure 4.15E,F).
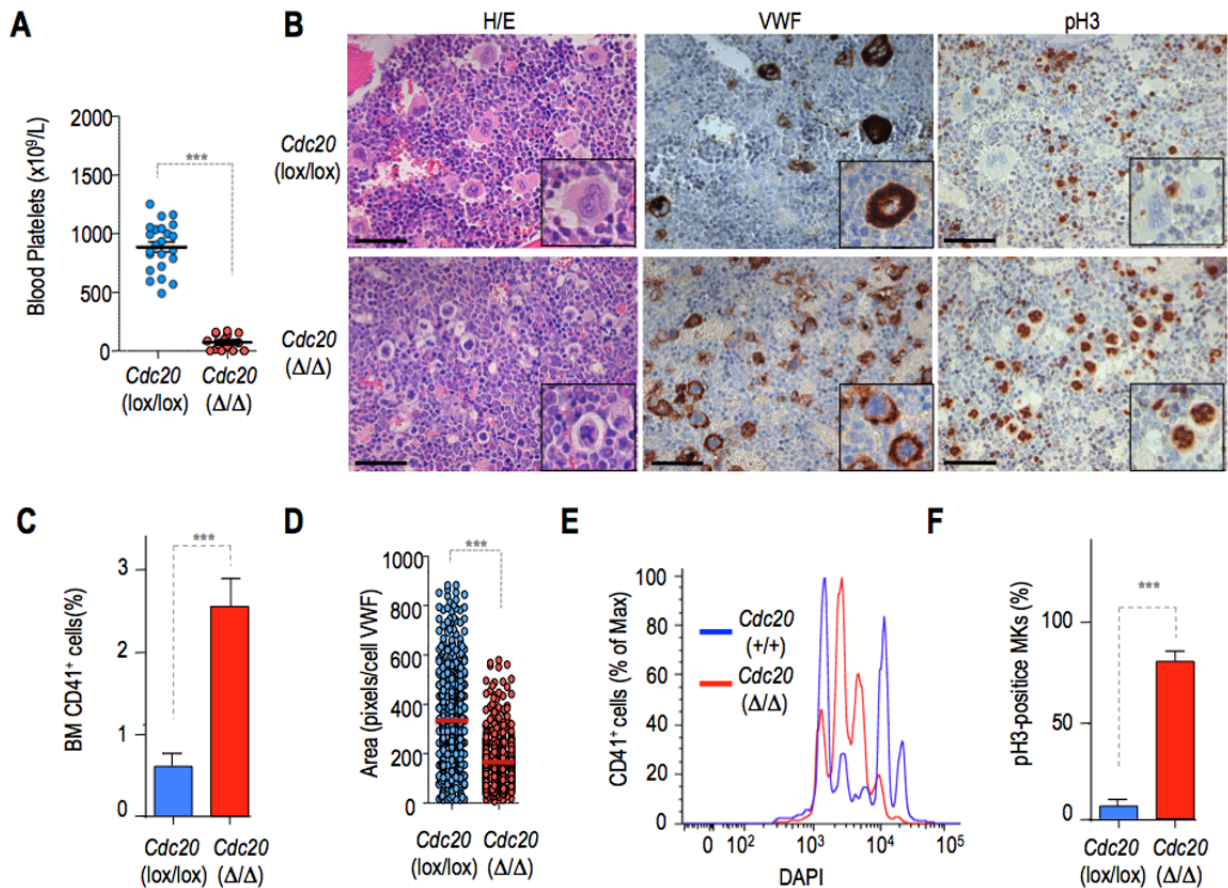
**Figure 4.15.** Cdc20 is essential for megakaryocytic polyploidization and platelet production. (**A**) Blood platelet levels from *Cdc20*(lox/lox) n=23 and *Cdc20*(Δ/Δ) mice n=30. (P<0.0001) (**B**) Hematoxylin/eosin-stained bone marrow sections and immunohistochemical staining for Von Wilebrand Factor (VWF) and pH3. Scale bars, 50 μM. (**C**) Percentage of CD41+ cells in bone marrow by FACS analysis. (**D**) Morphometric analysis of VWF+ cells in bone marrow sections. Total cells from N=3 per group animals. Each dot represents an individual cell (P<0.0001) (**E**) Ploidy distribution inside the CD41 population in bone marrow by DAPI staining, representative of three experiments. (**F**) Percentage of pH3+ cells in bone marrow sections by high power field analysis (hpf) 10 hpf of 3 animals per grouped were analyzed (P<0.0001). All graphs show mean with ± SEM and all experiments were performed in 8-14 weeks-old animals. Statistics were performed by t test.

As it is well established in other thrombocytopenia models, lack of peripheral platelets leads to higher levels of free TPO (TPO levels are tightly controlled by direct binding to the circulating platelets) which subsequently stimulates not only higher commitment of progenitors to the megakaryocytic lineage but also the expansion of uncommitted progenitors (Ng et al. 2014). (In *Cdc20*(Δ/Δ) mutant mice since there is almost a complete lack of circulating platelets we could expect such an increase in early megakaryocytes and progenitors in general. Indeed we observed an increase in megakaryocytic progenitors not only in bone marrow but also in the spleen (Figure 4.16A). In addition we analyzed the levels of hematopoietic progenitors in the bone marrow and we observed a significant increase in all subtypes of hematopoietic progenitors (including hematopoietic stem cells and common

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myeloid progenitors) except for the common lymphoid progenitors where TPO is known to have no effect (Figure 4.16B). In agreement with the notion that thrombocytopenia is the cause of the enhanced mekagaryocytic progenitors in bone marrow, we found no differences in the number of megakaryocytic progenitors in fetal livers from 12.5 p.c *Cdc20*(Δ/Δ) mutant embryos compared to *Cdc20*(lox/lox) littermates [at this developmental stage TPO is supplied mainly by the mother (Petit-Cocault et al. 2007) (Figure 4.16C). When we cultured fetal liver progenitors in presence of TPO for three days, we observed a similar phenotype in the Cdc20 null megakaryocytes since were smaller than control megakaryocytes, and presented condensed DNA and positive signal for mitotic regulators such as Aurora B suggesting mitotic arrest in vitro (Figure 4.16D).
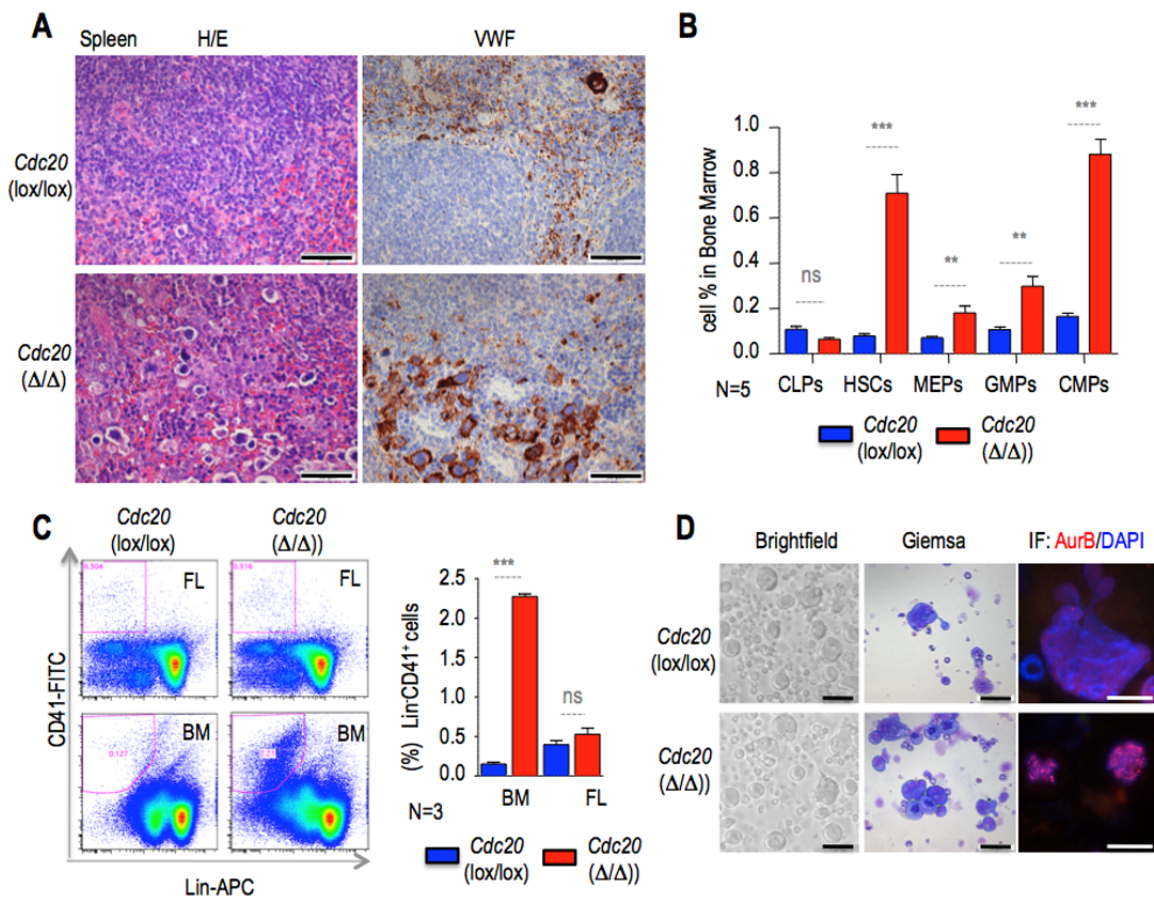


**Figure 4.16. High levels of hematopoietic progenitors as a consequense of severe thrombocytopenia in *Cdc20*(Δ/Δ) mice.** (**A**) Hematoxylin/eosin-stained spleen marrow sections and immunohistochemical staining for Von Wilebrand Factor (VWF) Scale bars, 50 μm. (**B**) Percentage of distinct subgroups of hematopoietic progenitors from bone marrow by FACS analysis. HSCs (Hematopoetic stem cells), CLPs (common lympoid progenitors), CMPs (Common myeloid progenitors), MEPs (megakaryocyte-erythrocyte progenitors), GMPs (granylocyte-macrophage progenitors). n=5 per group (**C**) FACS analysis of total fetal liver from 12.5 embryos (FL) and bone marrow of 8 weeks-old mice (BM) for Lin-CD41+ population. [N=3; (P<0.0001)] (**D**) Total fetal liver cells from 12.5 *Cdc20*(lox/lox) and *Cdc20*(Δ/Δ) embryos from the same pregnant mother stimulatated with TPO for 3 days. Brightfield and Giemsa staining of cytospun cells and Immunofluorescence (IF) for Aurora B in red and DAPIi in blue Scale bars, 50 μm. All graphs show mean with ± SEM and all experiments were performed in 8-12 weeks-old animals. Statistics were performed by t-test.

To further understand the cellular consequences of Cdc20 ablation we differentiated bone marrow progenitors to megakaryocytes with thrombopoietin from *Cdc20*(Δ/Δ) and *Cdc20*(lox/lox) mice and monitored the polyploidization events by videomiscroscopy. For this purpose we transduced bone marrow progenitors with lentiviral vectors expressing histone H2B fused to the green fluorescent protein (H2B-GFP). Also, to identify those cells that underwent recombination upon Cre expression under the Pf4 promoter, we crossed *Cdc20*(Δ/Δ)mice with transgenic mice expressing a lox-Stop-lox cassette upstream the Katushka fluorescence protein (Diéguez-Hurtado et al. 2011). As depicted in Figure 4.17A, control megakaryocytes underwent typical endomitotic cycles in which cells displayed condensed DNA for about 1-2 h and exited mitosis in the absence of chromosome segregation (Papadantonakis et al. 2008). However, most *Cdc20*(Δ/Δ) megakaryocytes displayed a normal entry into endomitosis and arrested with condensed chromosomes during 9-20 h until cell death (Figure 4.17A, B). Given the high number of mitotic figures in the bone marrow and the dramatic deficiency in platelets, these data are indicative of the essential relevance of endomitosis as the major endocycle for megakaryocyte maturation in vivo (Figure 4.17C).
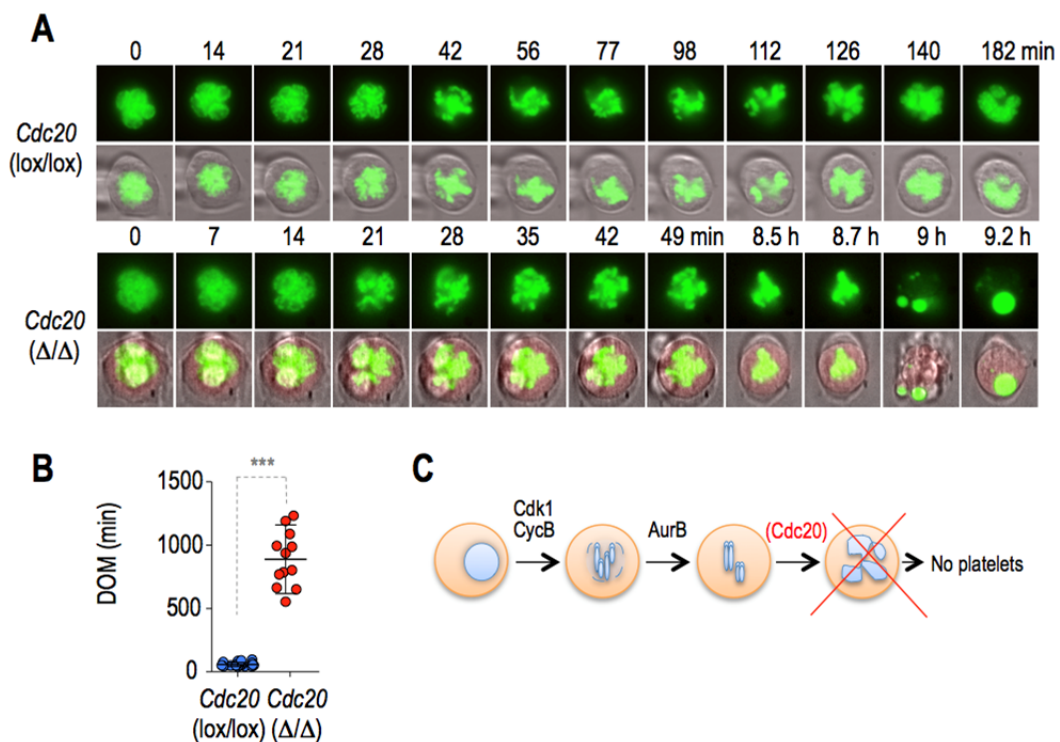


**Figure 4.17. Cdc20 null megakaryocytes arrest in endomitosis.** (**A**) Time-lapse microscopy of bone marrow derived megakaryocytes stably expressing histone H2B-GFP (green). The red color in *Cdc20*(Δ/Δ) megakaryocytes corresponds to katushka fluorescent protein which is expressed upon the Cre mediated lox-Stop-lox cassette excision (LSL-KFP). (**B**) Duration of mitosis (DOM) in minutes estimated by the analysis of individual cells by time-lapse microscopy (n=12 per group). In *Cdc20*(Δ/Δ) megakaryocytes the DOM is considered for the time that cells present condensed chromatin till the time of cell death. (**C**) Scheme representing the essential role of Cdc20 for exit from endomitosis in megakaryocytes and platelet formation. Statistics were performed by t-test (***, p<0.001).

## 4.3. Regulation of entry into endomitosis

### 4.3.1. Cdk1 is essential for endomitosis but dispensable for megakaryopoiesis

By definition, endomitotic cells do enter into mitosis and exit without segregation, whereas during endoreduplication cells completely skip mitosis. The later has been associated to the lack of Cdk1 activity (Zielke et al., 2013), an essential kinase required for mitotic entry (Santamaria et al., 2007). We therefore tested the consequences of eliminating Cdk1 activity during endomitosis by crossing *Cdk1*(lox/lox) mice with Pf4-Cre transgenic animals. Surprisingly, the resulting *Cdk1*(Δ/Δ) mice did not display major defects in megakaryocyte number, morphology or VWF staining in adult mice (Figure 4.18A). Similarly, although the ratio of highly polyploid (16C and 32C) megakaryocytes was slightly reduced, no major defects were observed in the ploidy of these mutant mice (Figure 4.18B) and the number of platelets in peripheral blood was similar to that of control littermates (Figure 4.18C).
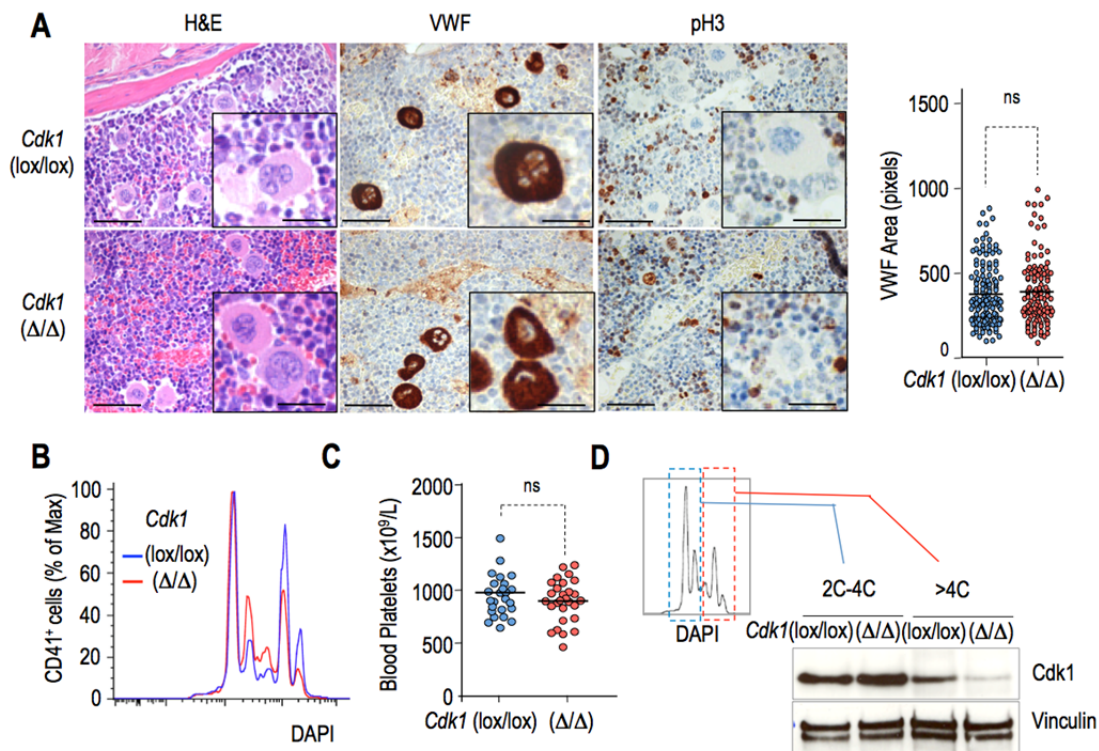


Figure 4.18. Cdk1 ablation does not lead to defects in megakaryocyte polyploidization and platelet defects *in vivo*. (A) Hematoxylin/eosin-stained bone marrow sections and immunohistochemical staining for Von Wilebrand Factor (VWF) and pH3. Morphometric analysis of VWF+ cells in bone marrow sections. Total cells from N=3 per group animals. Each dot represents an individual cell (P>0.05) (B) Ploidy distribution inside the CD41 population in bone marrow by DAPI staining, representative of three experiments. (C) Blood platelet levels from *Cdk1*(lox/lox) and *Cdk1*(Δ/Δ) mice. [n=19; P>0.05; t test]. (D) Western blot analysis for Cdk1 in freshly isolated megakaryocytes (CD41+ cells) from bone marrow. To determine ploidy megakaryocytes were stained with Hoechst and CD41+ cells were shorted according the DNA content into two populations (2C-4C) and (>4C). All graphs show mean with ± SEM. Scale bars, 50 μm.
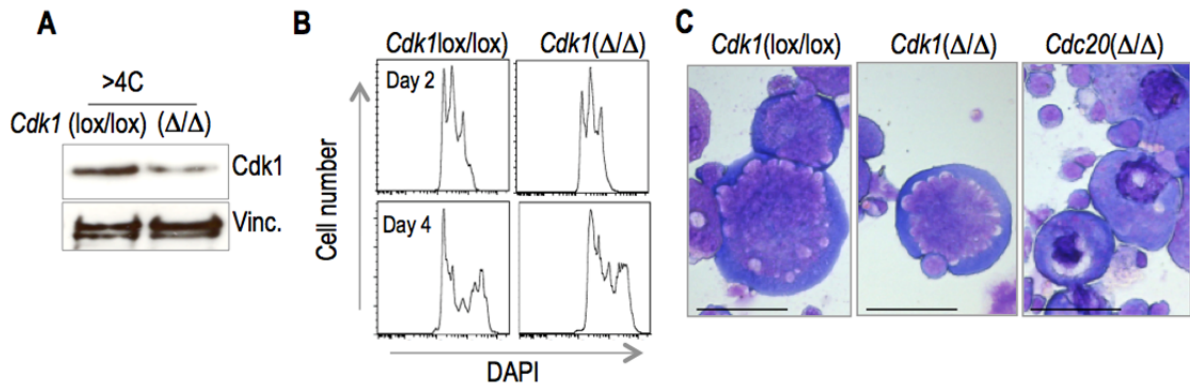
**Figure 4.19. Bone marrow derived *Cdk1*(Δ/Δ) megakaryocytes can polyploidize *In vitro.* (A)** Western blot analysis for Cdk1 in bone marrow derived megakaryocytes for DNA content greater that 4C upon TPO stimulation for 3 days. Vinculin is used as a loading control. **(B)** CD41⁺ cell polyploidization analyzed by flow cytometry stained with DAPI at days 2 and 4 after TPO stimulation. **(C)** Giemsa staining of *Cdk1*(lox/lox), *Cdk1*(Δ/Δ), and *Cdc20*(Δ/Δ) megakaryocytes upon 4days of TPO stimulation. Scale bars, 20 μm.

Whereas Aurora B, Cdh1 or Cdc20 protein levels are controlled by cell cycle-regulated ubiquitin-dependent proteolysis Cdk1 is more stable, raising the question whether the depletion of this protein was not efficient in this system. However, we observed a significant reduction in Cdk1 protein levels in mature megakaryocytes isolated from the bone marrow of 12-week-old *Cdk1*(Δ/Δ) mice (Figure 4.18D). Similar depletion in Cdk1 protein levels was obtained after incubation of bone marrow progenitors with TPO in vitro (Figure 4.19A), suggesting that the lack of an obvious phenotype in platelet production was not due to inefficient ablation of Cdk1. *Cdk1*(Δ/Δ) megakaryocytes accumulated normal levels of ploidy (Figure 4.19B), and displayed a morphology indistinguishable from wild-type cells, whereas, as a control, *Cdc20*(Δ/Δ) megakaryocytes presented smaller nuclear size and condensed DNA (Figure 4.19C).

### 4.3.2. Cdk1-deficient megakaryocytes undergo endoreplication

We then monitored polyplodization in *Cdk1*(Δ/Δ) megakaryocytes by time-lapse microscopy. Bone marrow progenitors were transduced with lentiviral vectors expressing lamin-CFP, to stain nuclear envelope, and H2B-GFP to label chromatin. In addition, we used a vector expressing geminin-mCherry, a DNA replication regulator induced in S-phase and degraded in an APC/C-dependent manner during mitotic or endomitotic exit (Sakaue-Sawano et al., 2013; Sakaue-Sawano et al., 2008). Using these markers and quantification of nuclear and cellular volumes, endocycles can be readily monitored in wild-type megakaryocytes. To calculate the nuclear volume we identify the nuclear area based on histone-GFP signal and calculate the radius by using the equation: $A = 4\pi r^2$ where A stands for area and r for radius. Then we calculate the volume by using the equation: $V = \frac{4}{3}\pi r^3$ where *V* represents volume and *r* radius (Figure 4.20).
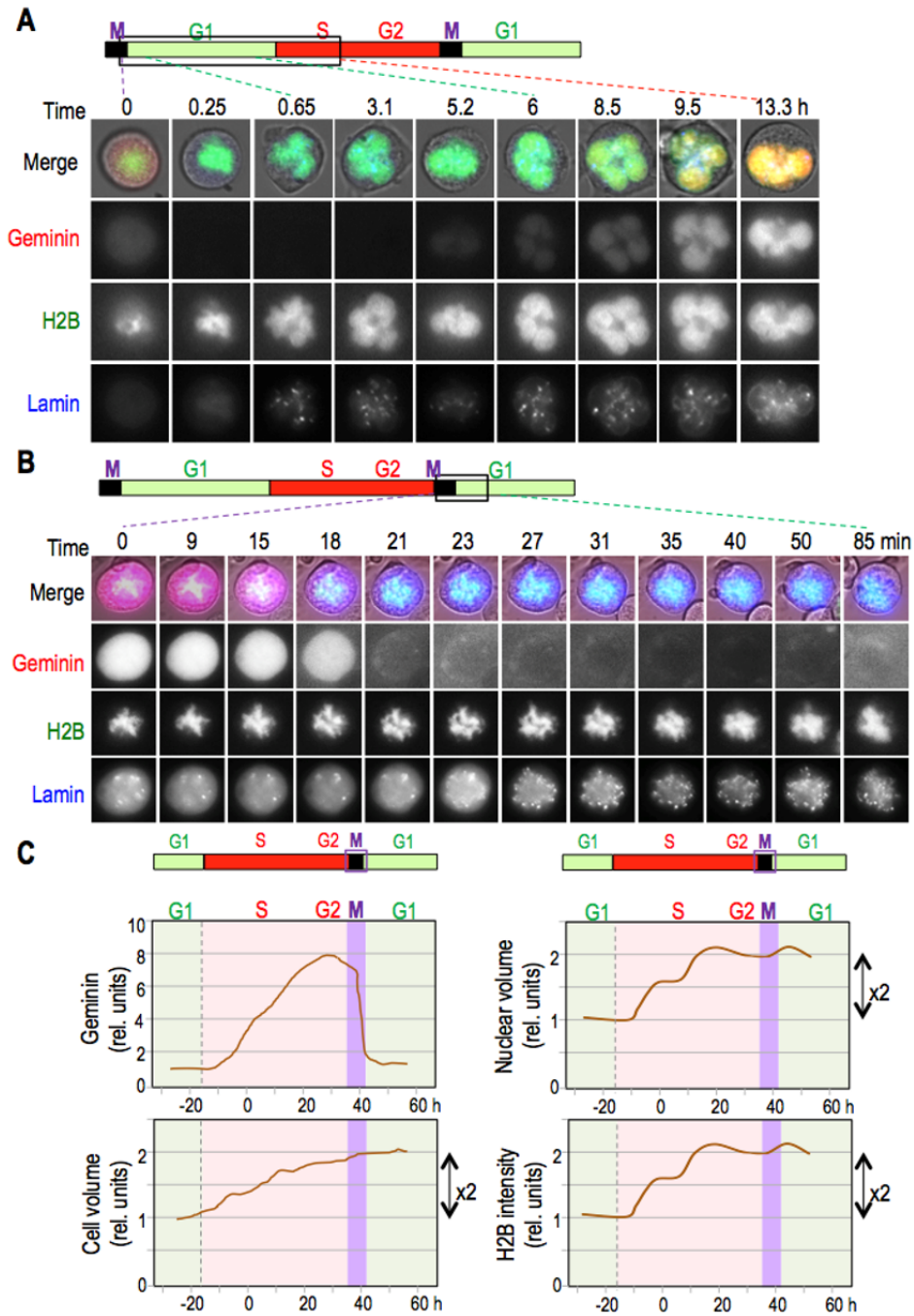
**Figure 4.20. Time-lapse microscopy for endomitotic polyploidization *in vitro.*** Time-lapse microscopy of bone marrow derived megakaryocytes stably expressing green for histone-GFP (H2B-GFP), blue for lamin-CFP and red for geminin-mcherry. (**A**) Representative pictures of an individual cell depict the G1-S transition during an endomitoc cell cyle. As time T=0 is considered the exit from endomitosis characterized by degradation of geminin (red), decondensation of chromatin (green) and nuclear envelope reformation (blue). During G1-phase cells are negative for geminin and start to express it upon G1-S transition. During s-phase the increase in nuclear volume can be appreciated. (**B**) Representative pictures of an individual cell during endomitosis. Endomitosis is characterized by chromatin condensation, absence of nuclear envelope that has as a consequence the pancellular distribution of geminin, which in other phases is exlusively nuclear. In late stages of endomitosis geminin is degraded and upon endomitotic exit nuclear envelope reforms and chromotin decondensates. (**C**) Graphs showing the relative units of geminin-mcherry mean fluorescent intensity, H2B-GFP mean fluorescent intensity, and nuclear and cellular volume during the endomitotic cell cycle. The purple frame depicts endomitosis (M)

Accordingly S-phase can be estimated from the increase in nuclear volume and histone-GFP intensity that take place during the geminin$^{high}$ phase. Mitosis is indicated by condensation of chromosomes; and pan-cellular distribution of lamin or geminin as a consequence of the lack of nuclear envelop. Finally, mitotic exit is indicated by degradation of geminin, reformation of the nuclear membrane and decondensation of DNA (Figure 20).

Wild-type megakaryocytes were recorded for several days and the behavior of individual cells was analyzed. Most wild-type megakaryocytes were characterized by mitosis in the absence of karyokinesis and cytokinesis (endomitosis). Degradation of geminin (geminin$^{low}$) was observed mainly during endomitotic exit as expected and in most cases was followed by re-expression of geminin (geminin$^{high}$) after a gap phase indicating the reasumption of a new endomitotic cell cycle (condensed chromosomes and pan-cellular geminin signal (Figure 4. 20A,B) By cuantifing the nuclear volume throughout the whole video we were able to estimate the nuclear volume increase between two gemin$^{high}$ phases. As mentioned previously we detected an approximate ~2 fold increase (Figure 4. 20C) suggesting DNA replication in each of these endocycles. Cell volumes also increased during this process although without correlating with any particular phase.

Then by using the same approach we analyzed $Cdk1(\Delta/\Delta)$ megakaryocytes recorded simultaneously with wild-type megakaryocytes. In most $Cdk1(\Delta/\Delta)$ megakaryocytes we observed as well geminin oscillations although geminin degradation occured in the absence of endomitosis (Figure 4.21A-B,D-E). In these cells, geminin did not show pan-cellular signal in agreement with the maintenance of the nuclear envelope and the lack of chromosome condensation. Geminin re-expression in many cases was come after geminin degradation and a subsequent gap phase, indicating a new cell cycle. Importantly nuclear volume increased about 2-fold in each of these endocycles when compared to normal endomitotic cells (Figure 4.21C,F) suggesting the presence of endoreplication throughout the geminin$^{high}$ phase. The increase in ploidy was similar in endomitotic and endoreplicative cells indicating that megakaryocytes are able to polyploidize using endoreplication as an alternative route to endomitosis in the absence of Cdk1. In consistence with our previous results we detected as well a small proportion of small megakaryocytes (~12%) that underwent endomitosis likely because of low expression of Cre and therefore remaining Cdk1.
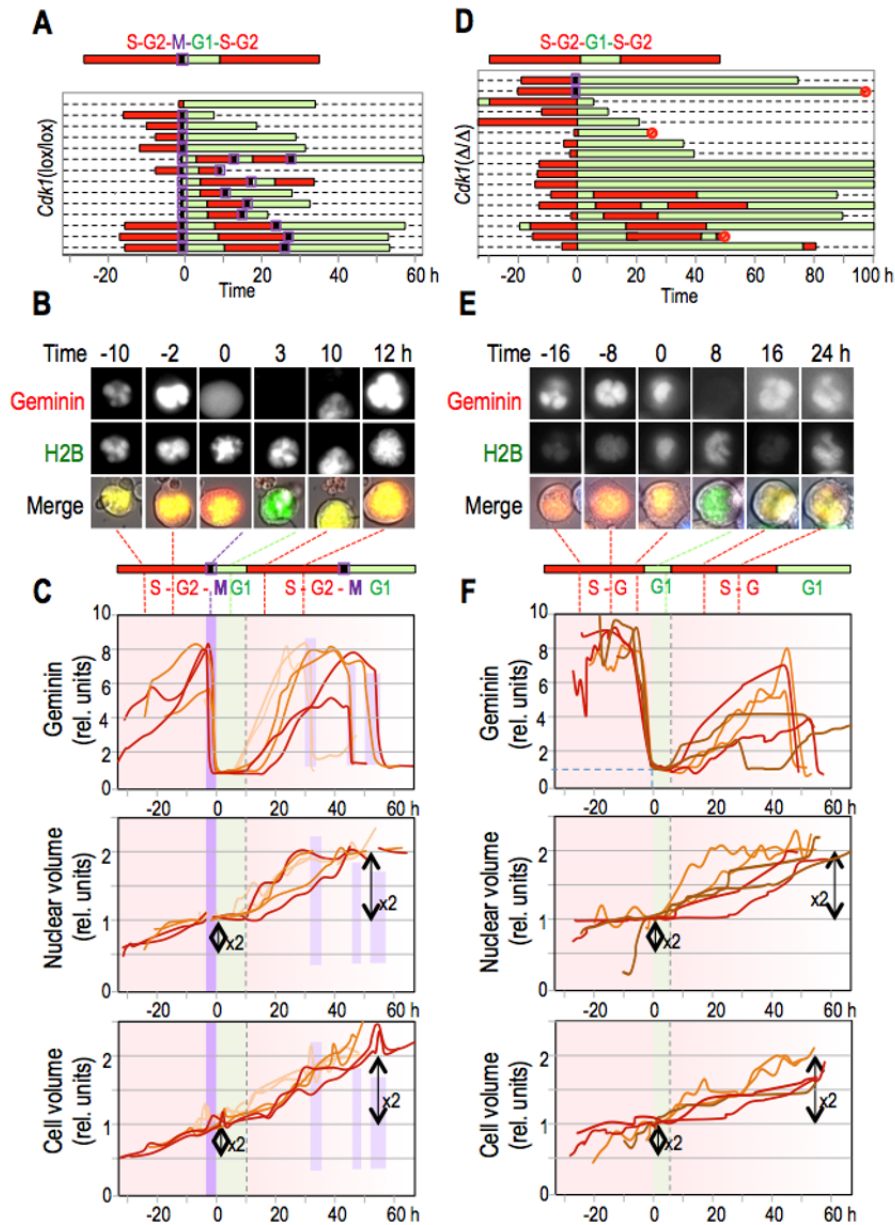
Figure 4.21. Endomitotic and endoreplicative endocycles in *Cdk1*(lox/lox) and *Cdk1*(Δ/Δ) megakaryocytes respectively. (A) Graph representing the cell fate of individual cells recorded by life cell imaging of *Cdk1*(lox/lox) bone marrow derived megakaryocytes for approximately 5 days . Cells express stably H2B-GFP and Geminin-mcherry. The red frame stands for S-G2 phases during which geminin is expressed (geminin[high]). The green frame represents G1 during which geminin is degraded (geminin[low]). Endomitosis is illustrated as a black box surrounded by a purple frame. (B) Representative pictures of an individual cell during endomitotic endocycling. (C) Graphs showing the temporal profiles of geminin fluorescent intensity, nuclear volume and cell volume.  Each line represents an individual cell and purple frame depicts endomitosis. Black arrows highlight the two-fold increase of nuclear and cellular volume between two geminin[high] phases. (D) Similar to (A) graph representing the cell fate of individual cells recorded by life cell imaging of *Cdk1*(Δ/Δ) bone marrow derived megakaryocytes for approximately 5 days in absence of endomitosis. Red circle indicates cell death (E) Similar to B representative pictures of an individual cell during the endoreplicative endocycling of *Cdk1*(Δ/Δ) megakaryocytes. (F) Similar to (C) graphs showing the temporal profiles of geminin fluorescent intensity, nuclear volume and cell volume in endoreplicative megakaryocytes. Each line represents an individual cell and purple frame depicts endomitosis. Black arrows highlight the two-fold increase of nuclear and cellular volume between two geminin[high] phases
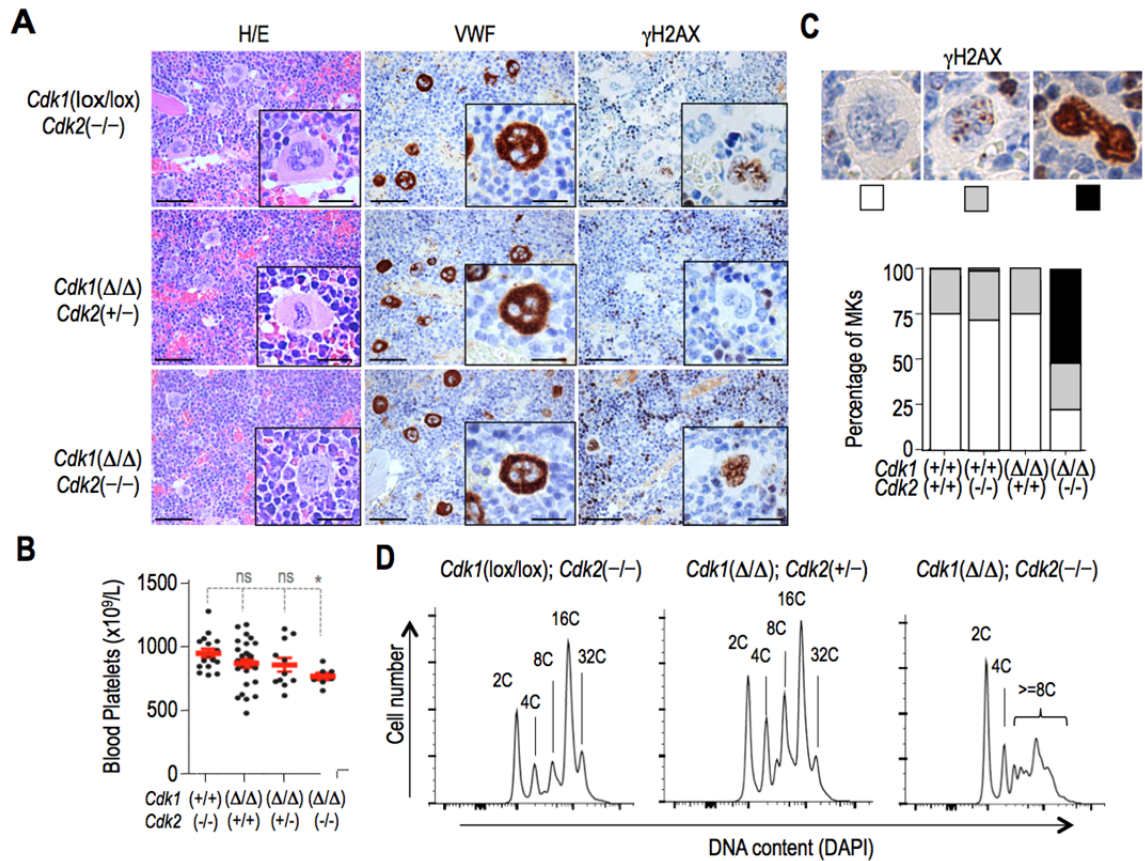
Figure 22. *Cdk1(Δ/Δ); Cdk2(–/–)* megakaryocytes polyploidize through rereplication. (A) Hematoxylin/eosin-stained bone marrow sections and immunohistochemical staining for Von Wilebrand Factor (VWF), pH3 and γ2HAX. Scale bars, 50 μm (insets 20 μm). (B) Blood platelet levels from *Cdk2(–/–)*, *Cdk1(Δ/Δ);Cdk2(+/–)* and *Cdk1(Δ/Δ);Cdk2(–/–)* mice. [n=19; P>0.05; t-test] (C) Ploidy distribution inside the CD41 population in bone marrow by DAPI staining, representative of three experiments. Discrete peaks corresponding to different DNA content are indicated by 2C, 4C, 4C, 16C and 32C. Bracket indicates the diffuse pattern of high ploidy (>8C) megakaryocytes.

## 4.4. Cdk2 and Cdk1 prevent re-replication in megakaryocytes

### 4.4.1. Cdk2 prevents re-replication in the absence of Cdk1

Cdk1 is known to be essential for entry into mitosis in all cells analyzed so far (Adhikari et al., 2012; Diril et al., 2012; Satyanarayana et al., 2008)(unpublished data from M. Barbacid's lab), and the presence of a few cells undergoing mitosis in the absence of Cdk1 (Figure 4.21D) likely suggested incomplete removal of endogenous Cdk1. However, since Cdk2 can also bind mitotic cyclins, we tested whether Cdk2 could compensate for Cdk1 activity in megakaryocytes by using a Cdk2-null allele (Ortega et al., 2003). Lack of Cdk2 does not results in obvious defects in the mouse and *Cdk2(–/–)* mice displayed normal numbers and morphology of megakaryocytes as well as normal number of platelets (Figure 4.22A,B) Similarly, lack of Cdk2 did not dramatically altered megakaryocyte morphology or platelet numbers in a *Cdk1(Δ/Δ)* background (Figure 4.22A,B), although *Cdk1(Δ/Δ); Cdk2(–/–)*

megakaryocytes frequently displayed phosphorylation of H2AX ($\gamma$H2AX signal), suggesting replicative stress in the absence of these two Cdks (Figure 4.22A,C). These data correlated with a diffuse pattern of high ploidy megakaryocytes in the absence of the expected discrete 8C, 16C or 32C populations suggesting that the genome in $Cdk1(\Delta/\Delta)$; $Cdk2(-/-)$ megakaryocytes was not uniformly and integrally duplicated (Figure 4.22D).

We next evaluated the consequences of Cdk2 ablation in Cdk1-null megakaryocytes by time-lapse microscopy with lamin-CFP for nuclear envelope, H2B-GFP for chromatin and geminin-mCherry for monitoring cell cycling. $Cdk1(\Delta/\Delta)$; $Cdk2(-/-)$ megakaryocytes presented similar endocycles characterized by geminin$^{low}$ and geminin$^{high}$ phases without evidences for mitosis in most cases (Figure 4.23A) The small percentange of endomitosis we detected (around of 15-18%) usually occurs in small size megakaryocytes and likely is due to inefficient Cre activity. Interestingly, geminin$^{high}$ phases were significantly longer when compared to wild-type or $Cdk1(\Delta/\Delta)$ cells (Figure 4.23B) and the increase in nuclear volume in a single geminin$^{high}$ phase was of 5-fold compared to 2-fold in wild-type cells or Cdk1-deficient megakaryocytes.
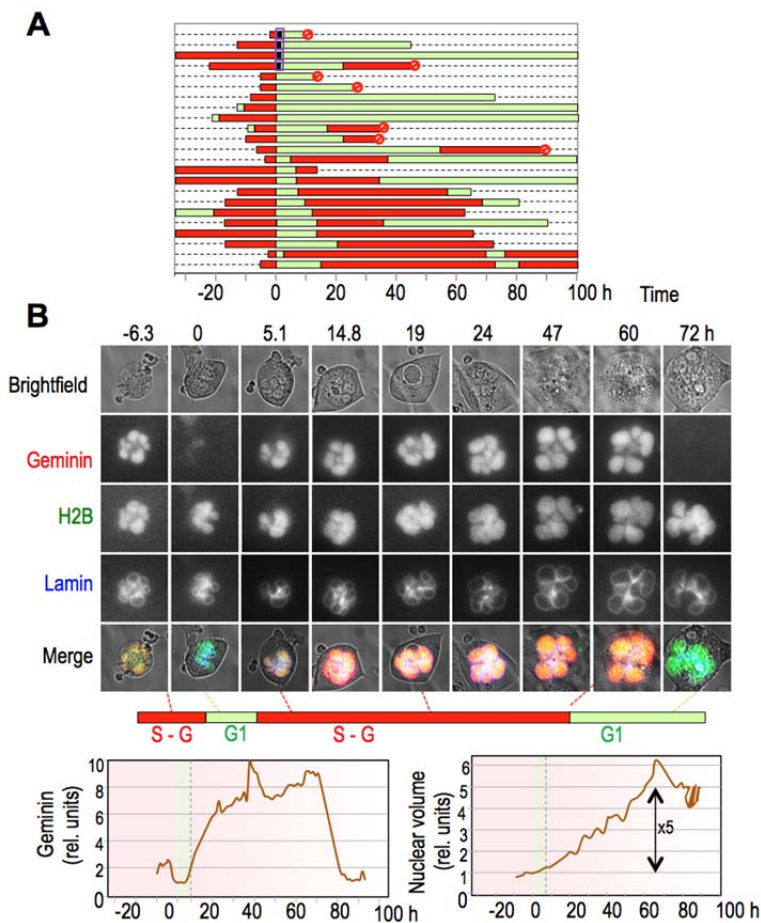


Figure 4.23. *Cdk1($\Delta$/$\Delta$); Cdk2(−/−)* megakaryocytes polyploidize in the absence of endomitosis or endo-replication. (**A**) Graph representing the cell fate of individual cells recorded by life cell imaging of *Cdk1($\Delta$/$\Delta$);Cdk2(-/-)* bone marrow derived megakaryocytes for approximately 5 days. Cells express stably H2B-GFP, lamin CFP and geminin-mcherry. The red frame stands for S-G2 phases during which geminin is expressed (geminin$^{high}$). The green frame represents G1 during which geminin is degraded (geminin$^{low}$). Endomitosis is illustrated as a black box surrounded by a purple frame. (**B**) Representative pictures of *Cdk1($\Delta$/$\Delta$); Cdk2(−/−)* megakaryocytes. Graphs shows the temporal profiles of geminin fluorescence intensity and nuclear volume.
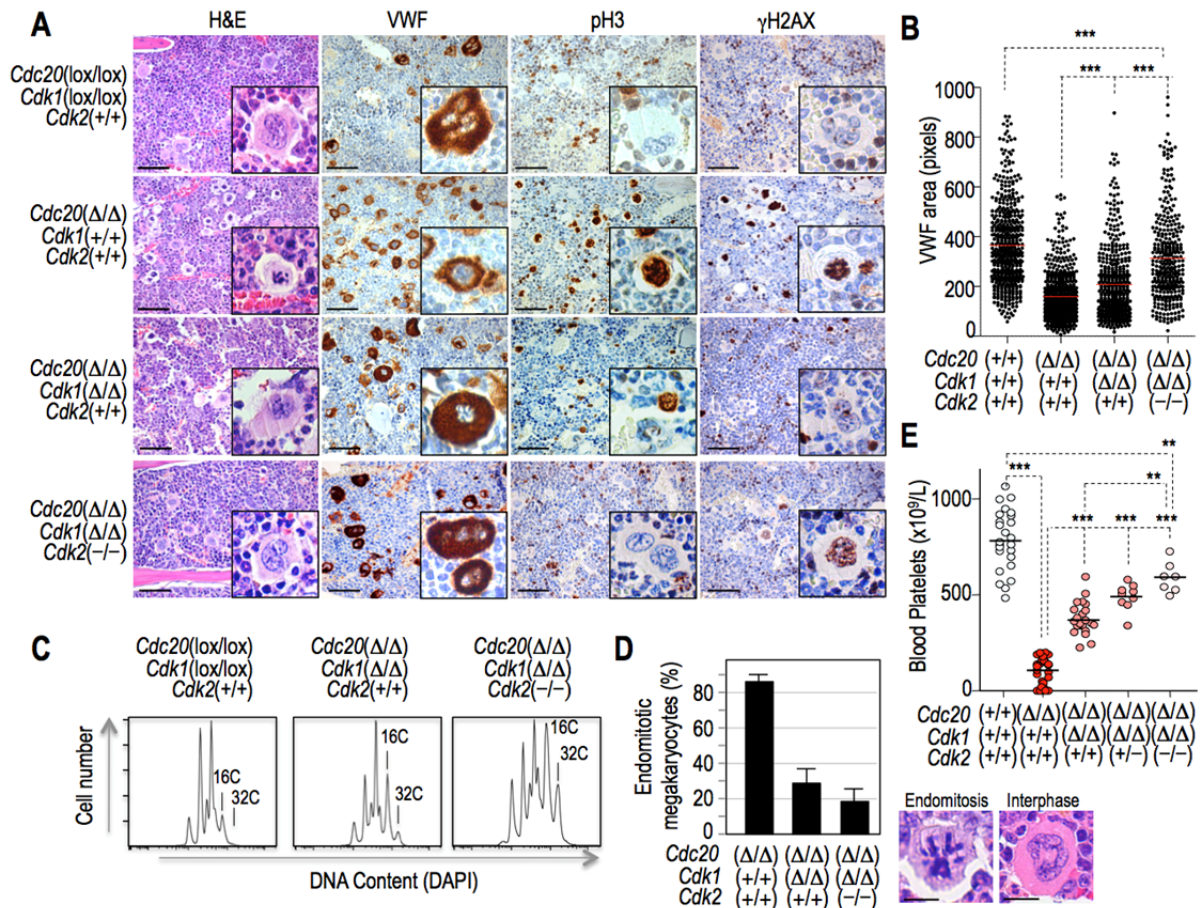
**Figure 4.24. Cdk1 and Cdk2 ablation rescues megakaryocyte defects and platelet levels in the absence of Cdc20.** (**A**) Hematoxylin/eosin-stained bone marrow sections and immunohistochemical staining for Von Wilebrand Factor (VWF), pH3 and $\gamma$2HAX for the indicated genotypes. Scale bars, 50 $\mu$m (**B**) Graph shows the distribution of megakaryocyte size estimated by VWF positive signal. Each dot represents an individual cell. Total cells analyzed per group (400) come from 3 individual animals (N=3). (**C**) Ploidy distribution inside the CD41 population in bone marrow by DAPI staining, representative of three experiments. Discrete peaks corresponding to different DNA content are indicated by 16C and 32C. (**D**) Mitotic index inmegakaryocytes from mice with the indicated genotypes. At least 300 cells from three different animals per genotype were scored. Scale bars, 20 $\mu$m. (**E**) Blood platelet levels from *Cdc20*(+/+);*Cdk1*(+/+);*Cdk2*(+/+); n=25, *Cdc20*($\Delta/\Delta$);*Cdk1*(+/+);*Cdk2*(+/+); n=30, *Cdc20*($\Delta/\Delta$);*Cdk1*($\Delta/\Delta$);*Cdk2*(+/+) n=22, *Cdc20*($\Delta/\Delta$);*Cdk1*($\Delta/\Delta$);*Cdk2*(+/–); n=9 and *Cdc20*($\Delta/\Delta$);*Cdk1*($\Delta/\Delta$);*Cdk2*(–/–); n=7. Each dot represents an individual animal. All graphs show mean with ± SEM and all experiments were performed in 8-14 weeks-old animals. Statistics were performed by t-test (***, p<0.001). Graph shows percentage of endomitotic megakaryocytes in bone marrow sections by high power field analysis (hpf) 10 hpf of 3 animals per group were analyzed (P<0.0001)

## 4.4.2. Cdk1 and Cdk2 ablation rescues platelet defects in the absence of Cdc20

Since inactivation of Cdk1 and Cdk2 may induce a switch in endocycles from endomitosis to endoreduplication or possibly re-replication, we hypothesized that lack of Cdks could rescue the mitotic-specific defect observed in Cdc20-null megakaryoyctes. We therefore generated Pf4-Cre; *Cdc20*($\Delta/\Delta$); *Cdk1*($\Delta/\Delta$) or Pf4-Cre; *Cdc20*($\Delta/\Delta$); *Cdk1*($\Delta/\Delta$); *Cdk2*(–/–) mutant

mice. As it is shown in Figure 4.24, whereas genetic ablation of Cdc20 was characterized by the presence of small megakaryocytes frequently arrested in mitosis, ablation of Cdk1 allowed the accumulation of big megakaryocytes that were strongly positive for VWF, suggesting proper maturation (Figure 4.24A,B) and ploidy (Figure 4.24C). This rescue in *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ) megakaryocytes correlates with the reduction of mitotic cells compared to *Cdc20*(Δ/Δ) megakaryocytes (Figure 4.24D). In addition, although *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ) mice also developed thrombocytopenia, the mean number of platelets increased from $108 \pm 13$ x$10^9$ platelets/L in *Cdc20*(Δ/Δ); *Cdk1*(lox/lox) animals to $376 \pm 18$ x$10^9$ platelets/L in *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ) double mutant mice, suggesting a significant rescue (Figure 4.24E).

Interestingly, concomitant ablation of one or two alleles of Cdk2 further improve this rescue as *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ); *Cdk2*(−/−) triple mutants displayed large megakaryocytes with strong signal for VWF (Figure 4.24A,B) ploidy (Figure 4.24C) and platelet number (Figure 4.24D). *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ); *Cdk2*(+/−) mice displayed $490 \pm 23$ x$10^9$ platelets/L and *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ); *Cdk2*(−/−) displayed $598 \pm 42$ x$10^9$ platelets/L in peripheral blood. Interestingly *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ); *Cdk2*(−/−) megakarycocytes frequently presented  phosphorylation of H2AX (γH2AX signal), suggesting replicative stress in the absence of these two Cdks (Figure 4.24A).

### 4.4.3.   Re-replication in the absence of Cdk1 and Cdk2

To verify the type of polyploidization that *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ) and *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ); *Cdk2*(−/−) megakaryocytes underwent and led to the Cdc20 null phenotype rescue we monitored the endocycling by time lapse video microscopy using the lamin-CFP, H2B-GFP and geminin-mCherry syctem (Figure 4.25). By recording for several days and analyzing individual cells we observed megakaryocytes that showed geminin oscillations although geminin degradation occured in the absence of endomitosis indicating endoreplicative endocycling (Figure 4.25A). In agreement with the partial rescue of platelet levels *in vivo* about half of *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ) megakaryocytes entered and arrested in mitosis till cell death.

The fact that Cdk1 levels are quite stable throughout the cell cycle whereas Cdc20 protein levels are regulated through the proteolytic pathway might result in the earlier exhaustion of Cdc20 protein upon Cre-mediated excision compared to the Cdk1, thus explaining the high percentage of endomitotic arrest in *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ) megakaryocytes. Similarly *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ); *Cdk2*(−/−) megakaryocytes displayed alternative endocycling with gemining oscillations in absence of endomitosis although geminin[high] phases were considerably longer suggesting rereplicative endocycling (Figure 4.25B).
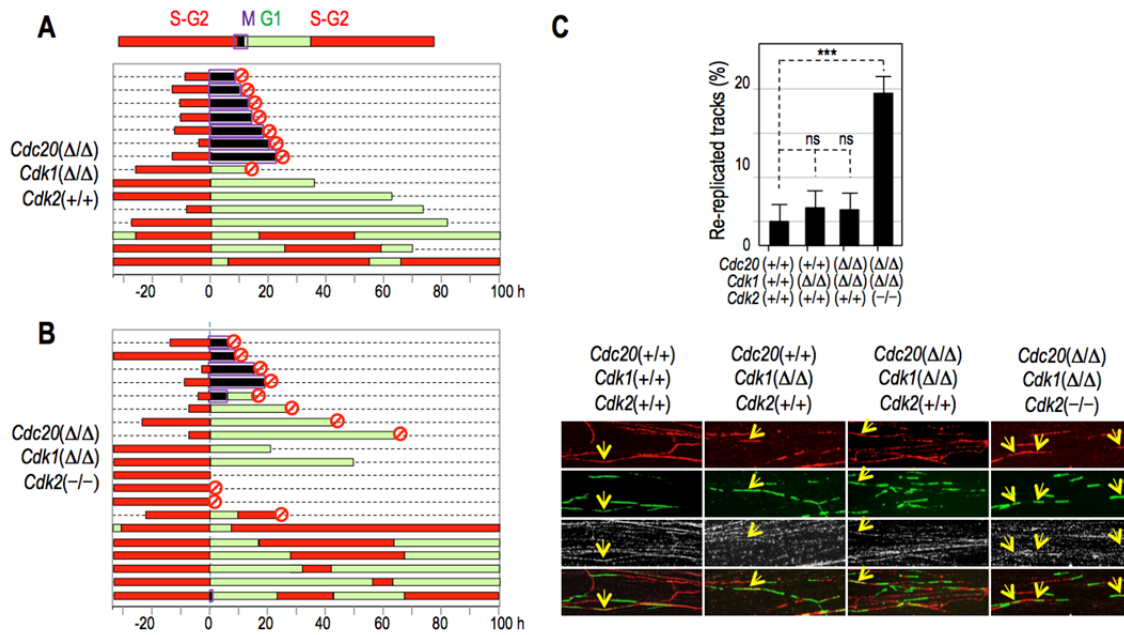
**Figure 4.25.** Re-replication in alternative endocycling megakaryocytes. (**A**) Graph representing the cell fate of individual cells recorded by life cell imaging of *Cdc20* (Δ/Δ); *Cdk1* (Δ/Δ) bone marrow derived megakaryocytes for approximately 5 days. Cells express stably H2B-GFP, lamin CFP and geminin-mcherry. The red frame stands for S-G2 phases during which geminin is expressed (geminin^high). The green frame represents G1 during which geminin is degraded (geminin^low). Endomitosis is illustrated as a black box surrounded by a purple frame. Red circle depicts cell death. (**B**) Similar as in A for *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ); *Cdk2*(−/−) bone marrow derived megakaryocytes. (**C**) The percentage of re-replicated DNA tracks due to origin refiring within a single cell cycle. Representative pictures of two independent experiments of DNA fiber stretch assay in bone marrow derived megakaryocytes on day 4 upon TPO stimulation. For each sample at least 500 measurements were performed. Data represent mean ± SEM; Student's t-test (*, p<0.05; **, p<0.01; ***, p<0.001).

Importantly, by employing the DNA fiber assay, we were able to evaluate the occurrence of multiple origin firing within a single cell cycle. While wild-type endomitotic as well as endoreplicative *Cdk1*(Δ/Δ) and *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ) megakaryocytes displayed between 3-4% of origin re-firing within a single cell cycle *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ); *Cdk2*(−/−) megakaryocytes presented 18% of origin re-firing; i.e. almost a 6-fold induction compared to wild-type cells, thus confirming re-replication in these cells. Both *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ) and *Cdc20*(Δ/Δ); *Cdk1*(Δ/Δ); *Cdk2*(−/−) megakaryocytes were able to polyploidize bypassing the mitotic arrest induced by Cdc20 depletion, a feature that may explain the rescue in platelet levels *in vivo*.

As indicated above, quantification of mitotic index in these models actually suggests that lack of Cdk1 or Cdk1 and Cdk2 prevents the mitotic arrest observed in Cdc20-deficient megakaryocytes (Figure 4.24D). Whereas more than 80% of megakaryocytes were arrested in mitosis in Cdc20-null bone marrows, these numbers are dramatically reduced in Cdc20-deficient megakaryocytes that also lacked Cdk1 or Cdk1 and Cdk2.
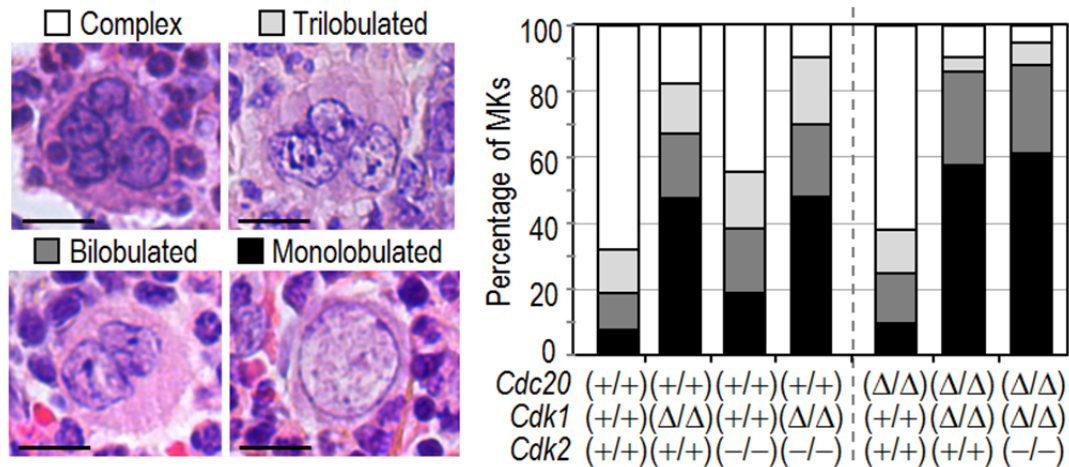
**Figure 4.26.** **Megakaryocyte nuclear shape during polyploidization.** Images on the left show representative Hematoxylin/eosin-stained bone marrow sections showing the possible morphologies of megakaryocytes with complex poli-lobulated nuclei or reduced number of lobules. The histogram shows the relative presence of the different nuclear morphologies in megakaryocytes from mice with the indicated genotypes. White box indicates highly lobulated megakaryocytes, light grey trilobulated megakarycocytes, dark grey bilobulated cells and black box indicates monolobulated megakaryocytes. A total of 300 cells from three different mice were analyzed per genotype. Scale bars, 20 μm.

Importantly the nuclear shape of megakarcyocytes in these complex genotypes was hypolobulated (mainly mono- and bi-lobulated) when comparing to the lighly complex nuclear shapes found in control mice (Figure 4.26). Since endomitosis frequently results in polylobulated nuclei as a consequence of the abortive mitosis during the later stages of anaphase, these results suggest polyploidization in absence of endomitosis in vivo in doble or triple mutant mice.

### 4.4.4. Endomitosis, endoreplication and re-replication can lead to functional megakaryocytes

In the previous sections, we have shown that megakaryocytes can polyploidize through alternative endocycles other than endomitosis. In addition, the *in vivo* blood platelet numbers from several mouse models such as *Cdk1(Δ/Δ)*, *Cdk1(Δ/Δ); Cdk2(−/−)*, *Cdc20(Δ/Δ); Cdk1(Δ/Δ)* and *Cdc20(Δ/Δ); Cdk1(Δ/Δ); Cdk2(−/−)* indicate that these alternative endocycles can produce functional megakaryocytes which can form proplatelets and eventually realease platelets into the blood stream. To further analyze the ability of these cells for platelet shedding we performed assays where we scored the percentage of proplatelet bearing cells in mature, terminally differentiated bone marrow derived megakaryocytes as well the complexity of bifurcation of these proplatelets by α-tubulin staining (Figure 4.27).
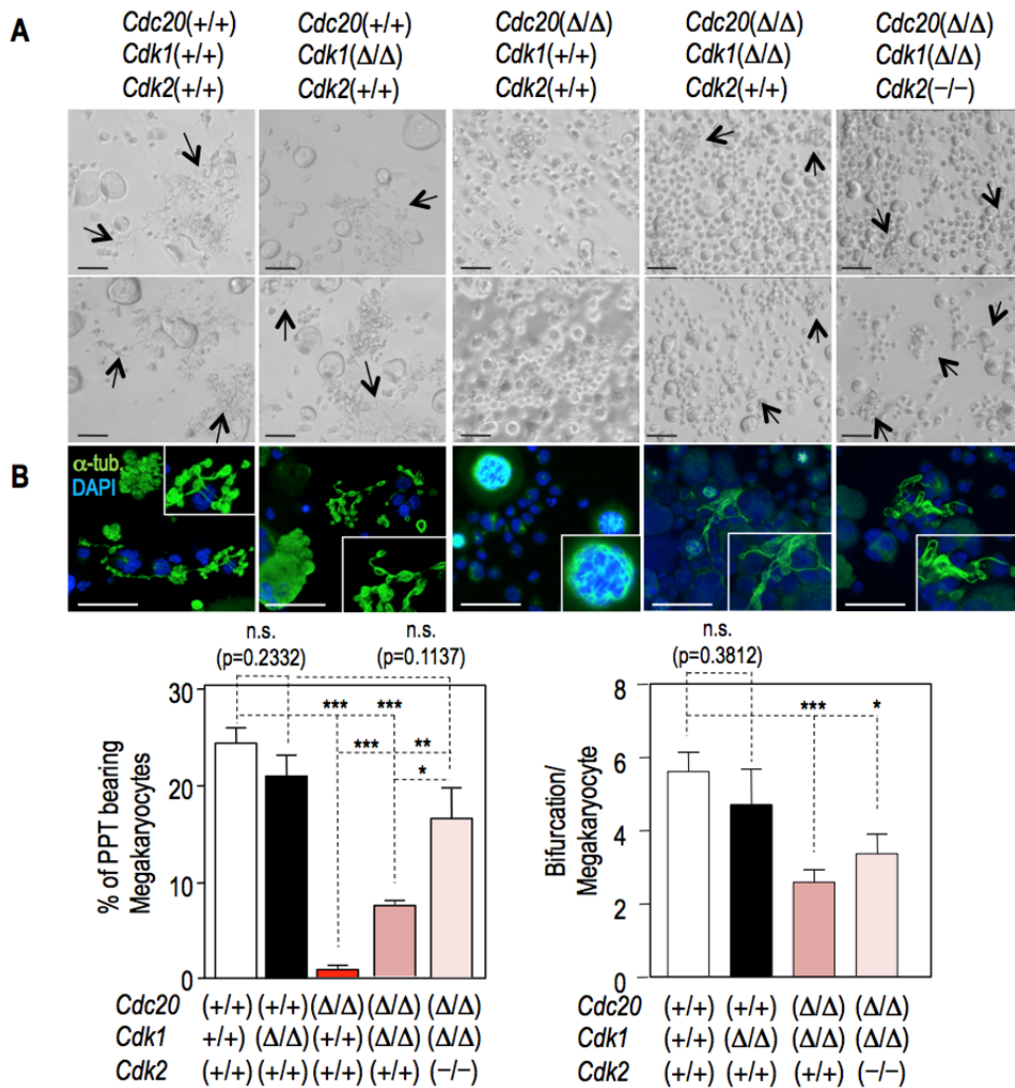
**Figure 4.27**. Alternative endocycles can lead to functional, platelet shedding megakaryocytes. *In vitro* bone marrow derived megakaryocytes analyzed at day 5 upon TPO stimulation. The percentage of PPT-forming megakaryocytes (left histogram)  was estimated by counting cells exhibiting 1 or more cytoplasmic protrusions with areas of platelet swellings as it is indicated by black arrows. 300-400 cells were analyzed per group. Right histogram represents the analysis of branching area of PPT-forming megakaryocytes after citospin and staining with anti–α-tubulin Ab (green) and DAPI (blue). The number of bifurcation was counted for each PPT-forming MK in a total of 20-50 megakaryocytes per group. Data represent mean ± SEM; Student's t-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

As expected at day 5 of differentiation wild-type endomitotic megakaryocytes presented 24 ± 2% (mean ± SEM) of proplatelet bearing cells with high level of bifurcation whereas in *Cdc20*(Δ/Δ) megakaryocytes only the 0.9 ± 0.5% of cells formed proplatetes (Figure 4.27). *Cdk1*(Δ/Δ) endoreplicative megakaryocytes resulted in a slight but not significant decrease in the percentage of proplatelet bearing cells scoring a 21 ± 2%. Accordingly concomitant ablation of Cdc20 and Cdk1 lead to a significant rescue of proplatelet formation scoring 7 ± 0.4%. In addition, further ablation of Cdk2 led to an even higher rescue in proplatelet formation with a 16 ± 3.5%.

Overall, these data show that alternatively polyploidized megakaryocytes can also form platelets, although these alternative endocycles may be less efficient compared to the endomitotic cycles.

# 5. Discussion

In this work we have investigated the physiological relevance of several mitotic regulators during endomitosis and functional maturation of megakaryocytes. Strikingly, we found that modulation of the endomitotic machinery can lead to alternative endocycles besides endomitosis that can lead to highly polyploid megakaryocytes. Importantly megakaryocytes resulting from alternative polyploidization are functional and able to shed platelets. Interestingly we have also identified that crucial mitotic regulators, such as Mastl, while dispensable for the endomitotic process, can be essential for non-cell cycle functions during megakaryopoiesis such as maturation and platelet shedding through regulation of MAPK activity.

## 5.1. A new cell cycle-independent function of Mastl

In 2000, a human autosomal dominant thrombocytopenia syndrome was associated through genomic linkage analysis for first time to a genomic region of 17 centimorgans on the short arm of human chromosome 10 [10p11-12 (THC2, OMIM number *188000)] (Drachman et al., 2000). Affected members presented moderate thrombocytopenia with reduced frequency of bone marrow megakaryocytes and no apparent abnormalities of myeloid or erythroid cells. This type of inherited thrombocytopenia has no evident association with hematopoietic malignancy or progression to aplastic anemia. Furthermore bone marrow cells grown in liquid culture with thrombopoietin failed to develop polyploid cells greater than 8C and megakaryocytes from an affected individual had markedly delayed nuclear and cytoplasmic differentiation. Three years later a novel missense mutation in the human gene FLJ14813 (currently known as *MASTL*) located in the THC2 locus segregated perfectly with thrombocytopenia in a pedigree of 51 family members (Gandhi et al., 2003). A substitution of cytosine for guanidine (G to C) at nucleotide position 565 was present in all thrombocytopenic family members, causing a predicted substitution of aspartic acid for glutamic acid (E167D) in exon four. A few years later the same group reported that Mastl inactivation in zebrafish leads to thrombocytopenia  suggesting that the E167D *Mastl* mutation might be a loss of function mutation (Johnson et al. 2009).

By generating a new in vivo model of this mutation in knockin mice, we demonstrated that *Mastl*(E166D/E166D) mice develop normally and display only moderate thrombocytopenia which seems to be unrelated to any cell cycle function of Mastl during

endomitosis. Importantly Mastl E166D displayed similar or even higher kinase activity compared to wild-type Mastl *in vitro* suggesting that this mutation is not a loss of function mutation. Accordingly, fibroblasts extracted from *Mastl*(E166D/E166D) E14.5 embryos showed no apparent defects during cell cycling while presented increased phosphorylation of Cdks and MAPKs substrates (an established readout for PP2A activity). In agreement with Mastl depletion in zebrafish, genetic ablation of Mastl specifically in mouse megakaryocytes and platelets leads to thrombocytopenia development to even higher levels and frequency compared to *Mastl*(E166D/E166D) mice. However thrombocytopenic phenotype was unrelated to Mastl role in endomitosis since *Mastl*(Δ/Δ) megakaryocytes presented defects only in maturation and had similar ploidy compared to *Mastl*(+/+) or *Mastl*(E166D/E166D) megakaryocytes.

This apparent discrepancy in the resulting phenotype of megakaryocytes between the human thrombocytopenia syndrome and the murine models is likely due to additional gene mutations in the human THC2 locus that can control megakaryopoiesis and platelet formation. In fact, additional genes in the THC2 locus on human chromosome include *ACBD5* (Punzo et al. 2010) and *ANKRD26* (Pippucci et al. 2011; Noris et al. 2011), both described for their relevance in thrombocytopenic phenotype. Importantly a recent report showed that megakaryocytes from thrombocytopenic patients with mutations in the ´5 UTR region of *ANKRD26* gene led to defects in the transcriptional control of this gene resulting in abnormal MAPK signaling (Bluteau & Balduini 2014). Since no experimental evidences are available about the effect of these mutations on the transcription of adjacent genes we cannot exclude the possibility that *Mastl* expression is similarly altered in presence of these mutations. Interestingly our results indicate that MAPK activation pathway is tightly controlled by Mastl activity through PP2A inhibition in megakaryocytes and platelets. Since *Mastl* gene in mouse genome is located in chromosome 2 different from the *ANKRD26* locus we exclude the possibility that mutations in the *Mastl* gene can affect *ANKRD26* transcription.

In addition to shedding light on the pathogenic consequences of Mastl mutation in human thrombocytopenia, our data identify for the first time a cell cycle-independent function of Mastl, a protein only associated to mitosis previously. Since PP2A phosphatases are considered as tumor suppressor complexes with high relevance in human cancer, it can be proposed that Mastl inhibition can lead to PP2A reactivation in specific tumor types. Thus, our data open new possibilities to explore the therapeutic advantages of inhibiting Mastl to activate PP2A leading to the subsequent dephosphorylation of critical oncogenic molecules such as Erk or Akt (Figure 5.1).
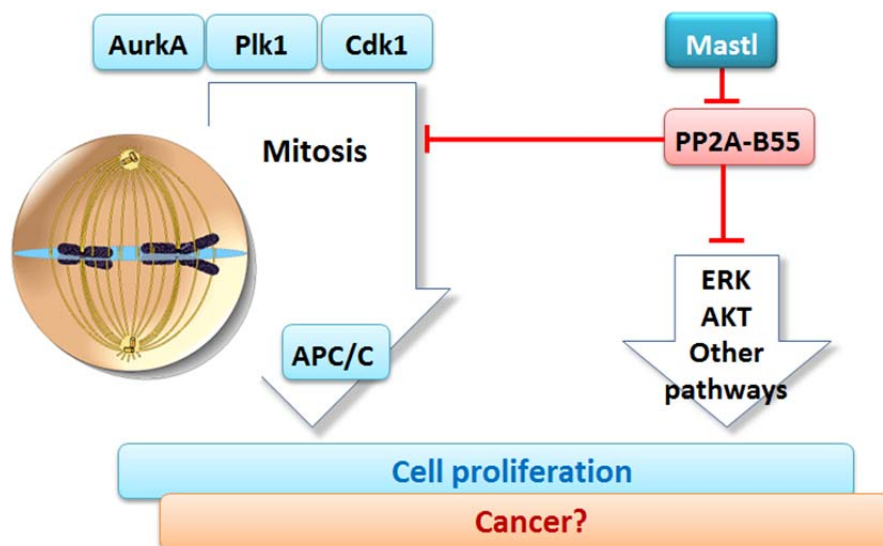
Figure 5.1. **Mastl controls cell cycle-dependent and independent pathways.** In addition to the established function of Mastl in the inhibition of PP2A-B55 complexes during mitosis, our data suggest that Mastl can also control PP2A activity in other cell cycle-independent pathways modulating the phosphorylation or Erk or other signaling pathways known to be dephosphorylated by PP2A such as Akt.

## 5.2. Aurora B dispensability in endomitosis

Although Aurora B role in endomitosis has been a matter of debate, it is well accepted that Aurora B is expressed and properly localized throughout the endomitotic phases (Geddis & Kaushansky 2004; Lordier et al. 2010). Experiments overexpressing Aurora B in megakaryocytes did not alter its polyploidization (Nguyen et al. 2005) but still a loss of function model was needed to define if any the role of Aurora B in endomitosis. Interestingly chemical inhibition of *in vitro* megakaryocytic cultures did not alter polyploidization but it did induced growth arrest and apoptosis in 2C and 4C megakaryocytic precursors during mitosis (Lordier et al. 2010). Accordingly, our work employing a mouse model for genetic ablation specifically in megakaryocytes of Aurora B demonstrated that its ablation *in vivo* does not affect megakaryocytic polyploidization or platelet levels suggesting that it is dispensable during endomitosis. This lack of phenotype is not surprising since Pf4 promoter driving Cre expression is active only in committed megakaryocytes that upregulate Pf4 leaving untouched hematopoietic progenitors which still undergo mitosis. In addition form previous work it is known that Aurora B deficiency allows mitotic entry but provokes premature mitotic exit in fibroblasts (Trakala et al., 2013). One could speculate that in endomitotic megakaryocytes with suffer an abortive mitosis a premature mitotic exit would

not affect further the polyploidization process. These data further support the idea that the proper alignment of chromosomes during endomitosis, a process in which Aurora B is essential, is dispensable given the lack of segregation in these cells.

## 5.3. Megakaryocytes display a functional APC/C during endomitosis

Since megakaryocytes during endomitosis do not complete late mitotic stages, several studies have been focused on the analysis of cyclin B dynamics and its involvement in the endomitotic process with controversial results. While in some studies high levels of cyclin B has been found (Nagata et al.,1997; Vitrat et al., 1998) some other with megakaryocytic cell lines and primary megakaryocytes showed reduced levels of cyclin B (Datta et al., 1996; Zhang et al., 1998) suggesting modified APC/C activity during endomitosis. In addition it has been postulated that megakaryocytes have an active mitotic checkpoint since megakaryocytes present prolonged duration of endomitosis upon nocodazole treatment (Roy et al., 2001). Our results have further confirmed the presence of a fully active APC/C in megakaryocytes since ablation of the coactivator Cdc20 led to endomitotic arrest in metaphase and a severe absence of circulating platelets. In contrast Cdh1, while it has been shown to be essential for endoreplicative cycles, it did not affect megakaryocytic polyploidization and platelets production during its ablation in megakaryocytes. These results are not surprising since Cdh1 ablation in fibroblasts did not present serious mitotic defects except for enhanced replicative stress (Garcia-Higuera et al. 2008; Eguren et al. 2013), while Cdc20 ablation led to a dramatic mitotic arrest (Manchado, Guillamot, et al. 2010). The fact that Cdc20-null megakaryocytes arrest as mitotic cells in the bone marrrow demonstrates that endomitosis is the major endocycle for megakaryocytic polyploidization not only *in vitro* but also *in vivo.*

The dramatic arrest of Cdc20 null megakaryocytes in metaphase and their incapability to progress further in polyploidization allowed us to detect the consequences of the almost complete lack of blood platelets. Since TPO is secreted in a constant rate mainly from liver and kidneys, its concentration in blood relies almost completely on its binding, internalization and clearance form cells that express TPO receptor (c-Mpl). Cells known to express cMpl except for megakaryocytes and platelets are hematopoietic stem cells (HSCs) and committed progenitors to myeloid lineage. Hence low platelet levels lead to higher free TPO that in turn triggers HSCs proliferation and subsequent commitment to megakaryocytic lineage. Accordingly in *Cdc20*(Δ/Δ) mice with severe thrombocytopenia we found a dramatic increase of hematopoietic progenitors and enhanced commitment to the megakaryocytic lineage that

explained the increased numbers of VWF positive cells in bone marrow and spleen. Interestingly TPO levels in serum of mutant mice were not significantly increased likely due to the high numbers of hematopoietic and megakaryocyte progenitors that bind and internalize TPO similarly to other models with altered platelet numbers (Ng et al. 2014). In agreement, fetal liver hematopoietic progenitors from $Cdc20(\Delta/\Delta)$ embryos were not upregulated since TPO levels are regulated by the pregnant mother's megakaryocytes and circulating platelets.

## 5.4. Alternative endocycles result in polyploid functional megakaryocytes

### 5.4.1. Endoreplication

Similarly to cyclin B dynamics, Cdk1 activation during endomitosis remains controvertial (Datta et al., 1996; Zhang et al., 1998; Vitrat et al., 1998). Cdk1 is essential for mitotic entry and Cdk2 cannot compensate for lack of Cdk1 either in embryos, cultured cells or during meiosis (Adhikari et al., 2012; Diril et al., 2012; Santamaría et al., 2007; Satyanarayana et al., 2008). Inactivation of Cdk1 is known to be sufficient to promote endoreplication in some specific cell types but not in others (Itzhaki et al., 1997; LItalien et al., 2006; Niculescu et al., 1998; Vassilev et al., 2006). We extended these findings *in vivo* showing that Cdk1 depletion in megakaryocytes results in endoreplication and endoreplicative megakaryocytes can reach ploidies similar to endomitotic ones with clear delineated genome doublings.  Importantly, we demonstrated for the first time that polyploid megakaryocytes from endoreplicative cell cyles are functional and can perform platelet shedding. Interestingly these megakaryocytes in contrast to the endomitotic megakaryocytes with a single polylobulated nucleus, presented a single hypolobulated (mainly mono-lobulated, bi-lobulated and tri-lobulated) nucleus with similar nuclear size. We assume that the number of lobules in the endoreplicative megakaryocytes depends on the number of endomitotic cell cycles that have undergone these cells prior to Cdk1 excision and the switch to the endoreplicative endocycling. Once megakaryocytes assume endoreplicative cell cycles the lobule number does not increase further since these cells do not enter endomitosis and do not reform its nucleus but augment the size of the already existing lobules.

Concomitant ablation of Cdk1 and Cdc20 in megakaryocytes was sufficient, at least partially, to rescue megakaryocyte mitotic arrest and blood platelet levels. While Cdc20 null megakaryocytes entered and remained arrested in endomitosis till they died, $Cdc20(\Delta/\Delta);Cdk1(\Delta/\Delta)$ megakaryocytes escaped mitotic arrest since switch to endoreplicative

endocyling bypassed endomitosis. We can speculate that although *Cdc20* and *Cdk1* are excised simultaneously Cdk1 protein can be more stable and remain longer compared to Cdc20 since Cdc20 is controlled by cell cycle-regulated ubiquitin-dependent proteolysis. While Cdk1 levels can be permissive for entry into endomitosis lack of Cdc20 leads to endomitotic arrest and thus blocks the following endocycle and the switch to endoreplication leading only to partial rescue of the Cdc20-null phenotype.

## 5.4.2. Re-replication

While neutralization of Cdt1 is essential to prevent rereplication during S-phase accumulating evidences indicate that the block to relicensing during G2 and M phase in mammals is critically dependent on Cdk activity. It has been proposed that in hamster cells, phosphorylation of Orc1 by Cdk1/Cyclin A prevents its association with chromatin (Li et al., 2004) Moreover, mouse Cdt1 has DNA-binding activity that is blocked by Cdk, but the physiological role of the DNA binding and its regulation have not been established (Sugimoto et al., 2004; Yanagi et al., 2002). To test whether Cdk1 directly regulates these pre-RC components, it will be essential to eliminate their Cdk phosphorylation sites and ask whether they can bind to chromatin in mitosis. The role of Cdk2 in preventing re-replication in mammals is controversial. On one hand, lack of an essential role for Cdk2 in regulating DNA replication is dramatically underscored by the observation that Cdk2 and Cyclin E knockout mice are viable (Ortega et al. 2003; Geng et al. 2003) and expression of cyclin A enhanced re-replication in mammalian tissue culture cells (Vaziri et al. 2003). On the other hand it has been reported that Cdk1 and Cdk2 play redundant roles in preventing re-replication in Hela cells (Machida & Dutta 2007). Our data showed that while individual ablation of Cdk1 or Cdk2 is not enough to induce re-replication in megakaryocytes, concomitant ablation of these two kinases led to substantial levels of re-replication. We need to clarify that endoreplication in Cdk1-null megakaryocytes is not considered as re-replication. In endoreplication, even though origin licensing occurs in absence of mitosis, it is mediated through a reset in a G1-like stage with the according APC/C-Cdh1 activation and geminin destruction.

We speculate that the Cdk2 role in preventing rereplication might be on one hand the Cdt1 destruction via SCF$^{Skp2}$, although there is so far no evidence that elimination of this pathway potentiates re-replication. On the other hand, Cdk2 might be important for a robust Cdk1 activation for instance mediating the activation of Cdc25B and Cdc25C phosphatases that eliminate the Cdk1 inhibitory phosphorylations (Mitra & Enders 2004).
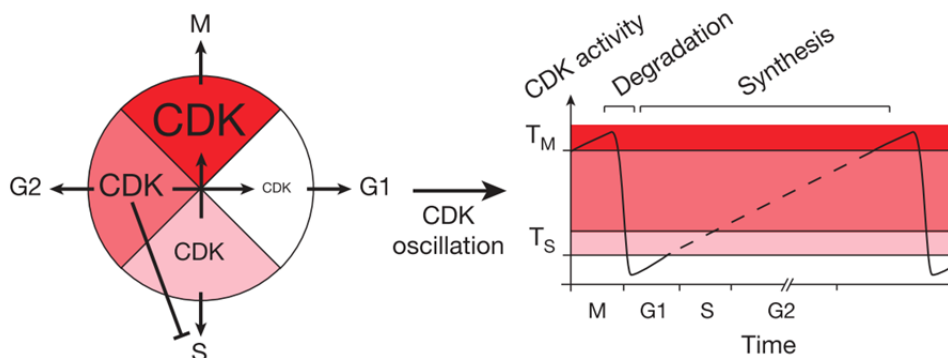
Figure 5.2. The quantitative model of Cdk action in fission yeast. Cdk activity pass through two thresholds. The first $T_S$ is the necessary activity for beginning of S-phase while the second threshold $T_M$ defines the necessary activity for mitotic onset. Source: (Coudreuse & Nurse 2010).

Trying to reconcile all these data it is tempting to speculate that the absolute level of total Cdk activity, rather than which specific Cdk and cyclins are present, may determine whether S phase and mitosis, or only S phase, and re-replication inhibition occurs. The quantitative model of Cdk action first proposed by Stern and Nurse (Stern & Nurse 1996; Coudreuse & Nurse 2010) in fission yeast and later substantiated in animal cells (Santamaría et al. 2007) shows that Cdk activity pass through two thresholds (Figure 5.2). Below the first threshold ($T_S$), in G1, replication origins can be licensed but cannot fire. Thus transition above $T_S$ marks the beginning of S-phase and continuously growing Cdk activity ensures that origins fire only once per S-phase. The second threshold ($T_M$) is passed on the onset of mitosis.

Re-replication leads to DNA damage checkpoint activation and phorphorylation of a histone variant H2AX is known to be en early event during this pathway activation. Accordingly, megakaryocytes null for Cdk1 and Cdk2 presented strong positive γH2AX signal. Interestingly polyploid megakaryocytes from rereplicative cell cycles were functional and able to form platelets. We think that megakaryocytes can tolerate considerable re-replication since it is known to express higher amounts of pro-survival proteins such as Bcl-x in order to restrain apoptosis and progress safely through proplatelet formation and platelet shedding (Josefsson et al. 2011). A summary of the effect of modulating different regulators of the cell cyle during megakaryocyte polyploidization is depicted in Figure 5.3.
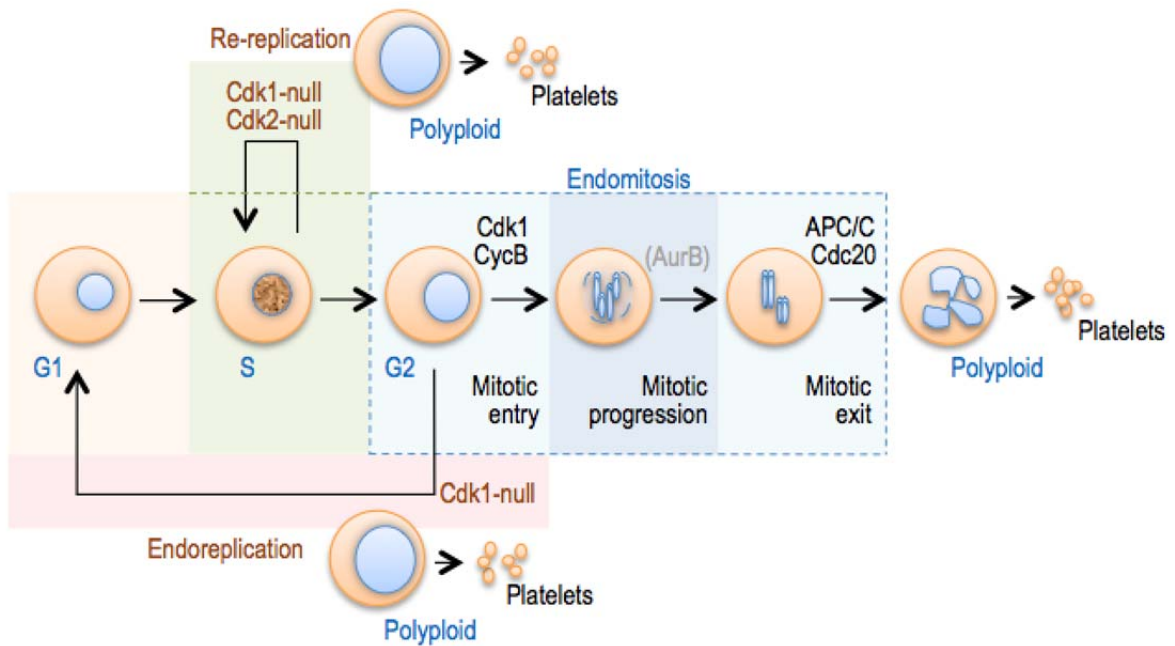
**Figure 5.3. Working model for alternative endocycles in megakaryocytes**. Megakaryocytes polyploidize through endomitotic endocycles. Aurora B and Cdh1 proteins seem to be dispensable during this process with Cdc20 shows an essential role for endomitotic exit. Cdk1 ablation in megakaryocytes results in endoreplicative cells cyles, while concomitant Cdk1 and Cdk2 ablation lead to re-replication. Alternatively endocycling megakaryocytes are functional and can shed platelets.

## 5.5. Endomitosis a sophisticated endocycle for platelet formation

It has been postulated that mekaryocytes undergo polyploidization in order to increase its size and upon maturation and fragmention release thousands of platelets. The fact that reprogrammed megakaryocytes are functional even in the presence of DNA damage and absence of discrete ploidy peaks, suggests that the major, if not the only, function of polyploidization is to increase cell size for platelet generation (Thon and Italiano, 2012). Since we have showed that alternative endocycles can lead to equally polyploid megakaryocytes still it remains unclear why megakaryocytes have chosen along evolution the endomitotic cell cycle in comparison for the endoreplicative one. Endoreplication could result in the same increased size without the need for the energy consuming mitotic processes. However, since endomitosis is the modified cell cycle least variant from the archetypal cell cycle, a simple interpretation suggests that megakaryocytes (which do not reach the higher ploidy levels observed in other cells such as trophoblast giant cells; >1000C) simply select the smaller degeneration of the mitotic cell cycle required for expansion of progenitors to become polyploid.
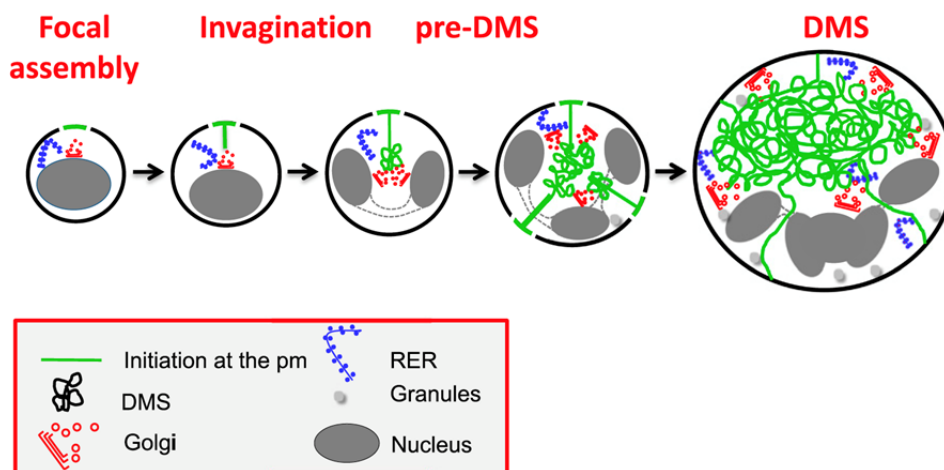
**Figure 5.4. Proposed model of DMS formation and expansion in megakaryocytes**. DMS formation initiates at focal areas on the cell surface. During maturation, a distinct pre-DMS structure is formed in the perinuclear region, through an invagination process that resembles cleavage furrow formation. Further expansion requires vesicular membrane delivery from multiple Golgi complexes that are targeted to the developing DMS and possibly via direct lipid transfer through ER- DMS tethering. Pm (plasma membrane), DMS (demarcation membrane system), RER (rough endoplasmic reticulum). Source: (Eckly et al. 2014)

The slight defects in platelet levels in Cdk1- or Cdk1; Cdk2-null mice and the incomplete rescue induced by lack of these kinases in Cdc20-deficient mice may indicate that, although endoreplication and re-replication can be functionally used for megakaryocyte polyploidization, they are less efficient than endomitosis. One possibility is that endomitotic megakaryocytes can shed platelets more efficiently compared to megakaryocytes resulting from alternative endocyling. In fact, it has been recently proposed that the multilobulated nuclei generated during mitosis may participate in the establishment of the forces required for membrane fragmentation during the formation of proplatelets (Eckly et al., 2014). While we have not detected significant differences in the percentage of platelet bearing megakaryocytes we noticed a slight tendency of endomitotic megakaryocytes to form more complex proplatelets. The only difference between an endomitotic megakaryocyte and an endoreplicative one is the nuclear shape. The former presents a higly lobulated nucleus while the latter usually presents one or two lobules. A recent report has shown that a pre-DMS (demarcation membrane system, which forms the plasma membrane of future platelets) is formed initially at the cell periphery and is precisely located among the nuclear lobes (Eckly et al. 2014). During the whole polyploidization process the DMS remained continuous with the cell surface and the number of these connections correlated well with the nuclear lobulation (Figure 5.4). It might be that a stable DMS emanating from the center of a polylobulated nucleus and connected to several points to the cell surface is critical for the

efficient platelet shedding process suggesting that such structure can be more unstable in hypolobulated nucleus with less binding regions to the cell surface.

Megakaryocytes are not the only cell type that has built divergent cell cycles from the conventional mitotic ones that better suits their specific structure and function. Another example of programmed polyploidization in mammals is heart cardiomyocytes. Cardiomyocytes skip cytokinesis leading to binucleated cells (Edgar et al. 2014). To undergo cytokinesis, cardiomyocytes should temporarily disassemble their sarcomeres, and this would be energy consuming and would lead to a transient loss of contractile function. Thus, hypertrophic growth through polyploidization can offer an energy-saving mechanism towards increased cardiac mass.

## 5.6. Therapeutic modulation of polyploidy in disease

As we mentioned early in addition to physiological polyploidization events, pathological induction or inhibition of polyploidization has been observed in diseased mammals. Polyploidization frequently appears in diploid cellular types and tissues, a pathological situation observed in cancer and different degenerative diseases (Otto 2007; Davoli & de Lange 2011; Pandit et al. 2013). Furthermore, in chronic hepatitis, metabolic diseases, and liver cirrhosis, hepatocytes also show increased polyploidy (Guidotti et al. 2003) A similar increase in nuclear sizes has been observed in cardiomyocytes, vascular smooth muscle cells, endothelial cells, and fibroblasts during hypertension or tissue repair. Overall, the mechanism and consequences of polyploidization in pathological settings remain obscure. In degenerative diseases, polyploid cells most likely appear in response to stress for example through toxins, physical pressure, or inflammation. Poly- ploidization might, therefore, be a stress response to preserve cell function and tissue structures, where acute loss would have detrimental effects such as in the liver heart or in megakaryocytes progenitors in bone marrow (Pandit et al., 2013)

It is also well established that polyploidization can also be a quick route to aneuploidy and genome instability (Davoli and de Lange, 2011; Duncan, 2013; Pandit et al., 2013; Storchova and Kuffer, 2008; Zasadil et al., 2013). In cancer, mutations in tumor suppressor genes and oncogenes have been clearly associated with polyploidization. Inactivation of tumor suppressor genes, such retinoblastoma and p53, as well as overexpression of oncogenes such as Myc, in mice leads to enhanced polyploidization and tumor formation (Davoli & de Lange 2011; Malumbres 2012). The degree of polyploidization is used as a prognostic marker

for multiple cancer cell types because high scores have been associated with reduced survival (Sinicrope et al., 2006; Syrios et al., 2013). High polyploidy levels might indicate that cancer cells within neoplasia increased chromosomal instability due to polyploidization and aneuploidization, causing a more malignant behavior. However, whether polyploidization is an essential step for generating tumors is largely unknown (Storchova & Kuffer 2008; Davoli & de Lange 2011; . It would be interesting to evaluate whether inhibition of polyploidization in mouse models of cancer has any consequences for tumor development.

Recent data has shown that inhibition of Aurora kinases as well as Cdk1 or Cdk2 in megakaryocytic leukemia cells leads not only to increase ploidy but also to the expression of megakaryocyte differentiation markers (Wen et al. 2012). These results may suggest that polyploidization not only accompanies but can actually trigger the differentiation process in megakaryocytes. Since Cdk1 is essential for mitosis in all cells tested, it can be expected that the differentiation process induced by Cdk1 inhibition (Wen et al., 2012) is actually mediated by endoreplication (as shown in our work) rather than endomitosis. The fact that mitotic kinases such as Aurora A, Aurora B or Cdk1 are not required for the function of endomitotic or endoreplicative cells, while being essential for the normal mitotic cell cycles, opens new avenues in the use of inhibitors against these proteins in therapeutic approaches against specific human cancers (Krause and Crispino, 2013).

# 6. Conclusions

# Conclusions

1. The Mastl E166D mutation, found in thrombocytopenia patients, or complete genetic ablation of Mastl does not result in defects in megakaryocyte polyploidization in the mouse.

2. Mastl controls circulating platelet levels by modulating Erk dephosphorylation by PP2A-B55 complexes, a process required for proper proplatelet formation in mature megakaryocytes.

3. Endomitosis does not require Aurora B activity although it is compromised in the absence of Plk1 due to partial mitotic arrest.

4. The Anaphase-promoting Complex (APC/C)-Cdc20 is necessary for endomitotic exit. However, APC/C is dispensable for platelet production when activated by Cdh1, a protein required for endoreplication in other systems

5. The mitotic arrest induced by lack of Cdc20 indicates that endomitosis is the major endocycle for megakaryocyte polyploidization *in vivo.*

6. Cdk1 and Cdk2 prevent re-replication in megakaryocytes.

7. Endomitosis, endoreplication and re-replication can function as alternative endocycles for maturation of megakaryocytes. Megakaryocytes resulting from these alternative polyploidization processes are functional and able to shed platelets.

# 7. Conclusiones

**Conclusiones**

1. La mutación de Mastl (E166D) encontrada inicialmente en pacientes con trombocitopenia y la completa ausencia de Mastl en los megakariocitos no conduce a defectos durante la poliploidización de estas células.

2. Mastl regula la producción de plaquetas en la sangre mediante el contol de la actividad de los complejos PP2A-B55 y la defosforilación de Erk.

3. La actividad de Aurora B no es necesaria durante endomitosis. Sin embargo la deficiencia en la quinasa Plk1 provoca una parada temporal en endomitosis y trombocitopenia.

4. El complejo promotor de la anafase o ciclosoma (APC/C)-Cdc20 es necesario para la salida de endomitosis y su ausencia causa trombocitopenia severa. APC/C-Cdh1, sin embargo, es dispensable para la producción de plaquetas mientras que es imprescindible para el processo de endoreplicación en otros sistemas.

5. La parada mitótica producida por la falta de Cdc20 indica que la endomitosis es el principal endociclo que conduce a la poliploidización de los megakariocítos *in vivo.*

6. Las quinasas Cdk1 y Cdk2 previenen re-replicación en megakariocitos.

7. La endoreplicación y la re-replicación constituyen endociclos alternativos para la poliploidización de megakariocitos. Ambos endociclos son funcionales ya que son capaces de producir plaquetas.

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# 9. Appendix

**Publications during the training period at the Universidad Autónoma de Madrid**

**Trakala M**, Arias CF, García MI, Moreno-Ortiz MC, Tsilingiri K, Fernández PJ, Mellado M, Díaz-Meco MT, Moscat J, Serrano M, Martínez-A C, Balomenos D. (2009) Regulation of macrophage activation and septic shock susceptibility via p21(WAF1/CIP1). *Eur J Immunol*. **39**, 810-819.

> *This work describes the Master project performed in Dr. Balomenos' lab on the innate immune response in the absence of the cell cycle regulator p21Cip1.*

Fernández-Miranda G, **Trakala M**, Martín J, Escobar B, González A, Ghyselinck NB, Ortega S, Cañamero M, Pérez de Castro I, Malumbres M. (2011) Genetic disruption of aurora B uncovers an essential role for aurora C during early mammalian development. *Development* **138**, 2661-2672.

**Trakala M**, Fernández-Miranda G, Pérez de Castro I, Heeschen C, Malumbres M. (2013) Aurora B prevents delayed DNA replication and premature mitotic exit by repressing p21(Cip1). *Cell Cycle* **12**, 1030-1041.

> *These two publications report the work performed during the first phase of the Thesis work on the characterization of Aurora B function in the regulation of mitosis.*

Álvarez-Fernández M, Sánchez-Martínez R, Sanz-Castillo B, Gan PP, Sanz-Flores M, **Trakala M**, Ruiz-Torres M, Lorca T, Castro A, Malumbres M. (2013) Greatwall is essential to prevent mitotic collapse after nuclear envelope breakdown in mammals. *Proc. Natl. Acad. Sci. USA* **110**, 17374-17379.

> *This publication reports the phenotype of mice with a germline mutation in Mastl, the protein mutated in human thrombocytopenia as described in this Thesis memory.*

**Trakala M**, Malumbres M. (2014) The functional relevance of polyploidization in the skin. *Exp. Dermatol*. **23**, 92-93.

> *A minireview on the biological significance of polyploidization mechanisms in the skin.*

**Trakala M**, Rodríguez S, Santamaría D, Ortega S, Barbacid M, Méndez J, Malumbres M. Functional reprogramming of polyploidization in megakaryocytes. *Submitted for publication*.

**Trakala M**, Álvarez-Fernández M, Sánchez-Martínez R, Lorca T, Castro A, Malumbres M. Greatwall/Mastl mutation induces mild thrombocytopenia by interfering with Erk control of megakaryocyte maturation. *In preparation*.

> *These two manuscripts contain most of the results presented in this Thesis memory.*