Evaluation of the Actin Architecture in Dysplastic Megakaryocytes Expressing the *NUP98-HOXD13* Leukemic Fusion Gene

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Abstract

Some myelodysplastic syndrome (MDS) patients present with macrothrombocytopenia due to impaired megakaryocyte (MK) differentiation. Transgenic mice that express the *NUP98-HOXD13* (*NHD13*) fusion gene is a model for MDS and recapitulates the key features of MDS. The study investigated the hypothesis that expression of *NHD13* disrupts actin architecture during MK differentiation leading to macrothrombocytopenia. To test the hypothesis, sternums were stained with hematoxylin and eosin, and evaluated by light microscopy to analyze MK morphology *in vivo*. *NHD13* bone marrow (BM) contained many dysplastic MK. BM from wild type (WT) and *NHD13* mice were also flushed, cultured in media supplemented with thrombopoietin only or with estrogen to induce proplatelet formation, and MK harvested after 5 days. Harvested MK and BM cores were processed and analyzed by transmission electron microscopy (TEM) to detail the ultrastructural features. TEM of MK revealed that *NHD13* leads to formation of an irregular demarcation membrane system and fewer proplatelets. Cultured WT and *NHD13* MK were also cytospun onto glass slides, labeled with fluorescent-tagged F-actin, α/β-tubulin and myosin IIa, and their cytoskeleton compared. Interestingly WT MK had actin either distributed evenly or predominantly in the periphery of the cytoplasm, *NHD13* MK displayed only the former phenotype. Additionally, proplatelets lacked actin cytoplasmic extensions. The results from the present thesis demonstrate actin expression and architecture are impaired in dysplastic MK expressing the *NHD13* leukemic fusion gene and leads to macrothromcytopenia. Understanding the molecular mechanisms of abnormal MK differentiation in MDS is important as many MDS patients die of hemorrhagic complications.

Dedication

I dedicate this thesis to my family and loved ones. I am very grateful for all the support you have offered in my life. To my parents who have been a source of encouragement and inspiration to me throughout my life and for encouraging me to work hard and be diligent. To my siblings, who have been role models in my life and to my girlfriend for her patience, care, love, and emotional support.

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List of abbreviations

MDS	Myelodysplastic Syndrome
Nup	Nucleoporin
Hox	Homeobox
NHD13	Nup98-HoxD13
BM	Bone marrow
WT	Wild type
TEM	Transmission electron microscopy
H&E	Hematoxin and Eosin
NPC	Nuclear pore complex
TPO	Thrombopoietin
MEP	Megakaryocyte-erythroid progenitor
Fog1	Friend of Gata1
DMS	Demarcation membrane systems
HSCs	Hematopoietic stem cells
ROCK	Rho-associated kinase
MLC2	Myosin light chain II
PIP2	Phosphotidylinositol 4,5-biphosphate
WASp	Wiskott-Aldrich Syndrome protein
NMM II	Non-muscle myosin II
MLC	Myosin light chain
NMMHC	Non-muscle myosin heavy chain
AML	Acute myeloid leukemia
FPD/AML	Familial platelet disorder with predisposition to AML

DNM	Dynamin
AMKL	Acute megakaryoblastic leukemia
BSA	bovine serum albumen
PBS	Phosphate buffered saline
P/S	Penicillin/Streptomycin
FBS	Fetal bovine serum

Thesis Introduction and Objectives

MDS is a hematological disorder that involves dysplasia of the myeloid class of blood cells. MDS patients develop cytopenias caused by progressive bone marrow failure. The onset of a myelodysplastic syndrome before the age of 50 years is rare, but the various forms of this disease are among the commonest hematologic cancers in patients over the age of 70 years [1]. Incidence rates are also higher in men compared to women [1]. According to the 2008 World Health Organization classification system for hematologic cancers, MDS is one of five major categories of myeloid neoplasms [2]. The main feature of myeloid neoplasms is stem-cell–derived clonal myelopoiesis with altered proliferation and differentiation. The phenotypic diversity of these neoplasms has been ascribed to different patterns of dysregulated signal transduction caused by transforming mutations that affect the hematopoietic cells, including MK. The pathogenesis of this serious disease, including the contribution of MK, remains unclear despite significant progress in detailing the molecular genetics of MDS, though studies indicate the role of nucleoporin (Nup) proteins, specifically Nup98, in the pathogenesis of the disease.

Nup98 is centrally located in the Nuclear Pore Complex (NPC), a large, multi-protein complex that mediates nucleocytoplasmic transport of protein and RNA between the nucleus and cytoplasm [3, 4]. Nup98 can shuttle between the cytoplasm and nucleus [5]. With its relevance to nucleocytoplasmic transport, Nup98 importance cannot be overstated and mutations involving this protein and gene, including chromosomal translocations, can be detrimental in mammals.

Chromosomal translocations involving *NUP98* are a common finding in hematologic malignancies [6, 7]. *NUP98* forms fusions with at least 28 other genes including homeobox (*HOX*) and non-homeobox genes that contribute to the mechanisms underlying leukemogenesis [8]. *HOX* genes, located on different chromosomes, are divided into four distinct clusters (A, B,

C, and D) and are involved in embryonic development and hematopoiesis. *HOXD* genes are localized on chromosome 2 and play a role in limb and digit generation during embryonic development. A *NHD13* fusion was originally identified in a patient with chemotherapy-related acute myeloid leukemia (AML) [9], and later reported in an infant leukemia patient [10]. Transgenic mice that express a *NHD13* cDNA develop most of the features of MDS by 4-6 months of age, including circulating giant platelets and dysplastic MK. With the passage of time, these mice often develop acute leukemia [11].

Based on current understanding of leukemias, there is an emerging need to delineate the role of chromosomal translocations during megakaryopoiesis and leukemic transformation. By evaluating the molecular mechanisms impaired by the translocation, novel pathways and therapeutic targets can be identified pertinent to both normal megakaryopoiesis and leukemia. The *NHD13* mouse model is a suitable tool to achieve these objectives. The objective of the study was to determine the effects of the *NHD13* leukemic fusion gene on MK development leading to abnormal platelet production and to accomplish this, a single model approach was used.

The central hypothesis for this Masters thesis was "Expression of *NHD13* disrupts actin cytoskeletal dynamics during megakaryopoiesis resulting in macrothrombocytopenia".

To accomplish the hypothesis, the following two Specific Aims were proposed:

Specific Aim 1: Determine the effects of *NHD13* expression on ultrastructural features in transgenic MK. Understanding the ultrastructural details of abnormal MK will help to determine the effects *NHD13* has on hematopoiesis during the progression of MDS. This study utilized both electron and light microscopy to observe the detailed morphological features of MK under *in vitro* (MK culturing is elaborated in Appendix A) and *in situ* conditions. This approach was

also used to evaluate the MK in mice with and without the disease. Here the effect of the fusion gene on cell structure was determined using both light and transmission electron microscopy.

Specific Aim 2: Determine the effects of *NHD13* on the cytoskeleton leading to the development of dysplastic MK. The cytoskeletal protein distribution and dynamics invoke numerous morphological changes during MK development – MK cell shaping, polyploidization, DMS formation, and proplatelet formation. Thus, it is important to understand which cytoskeletal protein(s), is perturbed by the *NHD13* leukemic gene. Harvested MK were adhered onto fibrinogen-coated slides, labeled with phalloidin, anti-myosin IIa, and anti-α/β tubulin antibodies to evaluate MK cytoskeletal architecture. Western blot analysis was also performed to determine the effect of the fusion gene on native protein polymerization. The study showed the effect of the fusion gene on cytoskeletal protein dynamics, actin specifically.

Chromosomal translocations are hallmark features of hematological malignancies. They are categorized as Class II mutations that often lead to impaired stem/progenitor cell differentiation. When complimented by Class I mutations, which lead to aberrant cell proliferation, malignant transformation can occur. To determine the role of a myeloid translocation in MK and platelet development, an *NHD13* transgenic mouse model was used. These mice progress through a transient MDS phase before progressing to acute myeloid leukemia. In addition these mice have giant platelets in circulation as part of their disease profile. Bone marrow histology was performed to determine MK development during disease progression. Additionally, *in vitro* MK development was determined to delineate the role of the *NHD13* fusion leading to macroplatelets and thrombocytopenia. TEM revealed an abnormal DMS in MK and fluorescent microscopy showed that the actin cytoskeleton was impaired in developing MK. Finally, *in vitro* analysis of platelet production showed that transgenic MK

developed abnormal proplatelets compared to WT controls. Details of these experiments and results are described in Chapter 2.

Chapter 1: Literature review

The essential role of the megakaryocyte cytoskeleton and its associated defects

Abstract

Megakaryocytes (MK) are myeloid cells that reside primarily in the bone marrow but are also found in the lung and peripheral blood. Terminally, MK produce platelets that are released into the bloodstream. MK differentiate from hematopoietic stem cells into colony-forming cells which express the CD34 antigen, promegakaryocytes, mature MK, and then later into proplatelets. MK generate platelets by remodeling their cytoplasm into long proplatelet extensions, which serve as the source for platelet production. The release of platelets from the proplatelets is preceded by considerable morphological changes to form the parent MK. These morphological and differentiation changes are paralleled with cytoskeletal modifications regulated by proteins such as actin, tubulin, myosin, dynamin, and spectrin. These cytoskeletal proteins work collaboratively during MK development. Thus, dysregulation of these proteins can cause many MK aberrations and result in platelet disorders such as thrombocytopenia, macro platelet formation and fragile platelets. This is a review of the literature on the cytoskeleton proteins which are vital to MK development and their associated disorders.

Introduction

Megakaryocytes (MK) are hematopoietic cells that give rise to platelets, which are necessary for hemostasis and thrombosis in the body. In humans, MK normally account for approximately 0.1 percent (1 out of 10,000) of all nucleated bone marrow cells [12]. Their number increases as the demand for platelets rises. MK have an average diameter of 20 to 25 microns and a volume of 4700 ± 100 femtoliters (fL) [13]. Each MK produces a total of 1000 to 3000 platelets [14]. Although it has long been assumed that larger MK produce more platelets, this has never been conclusively demonstrated. But before platelet production, MK undergo successive development and expansion processes known as megakaryopoiesis [15]. The primary signal for promotion of MK progenitor expansion and differentiation is the ligand thrombopoietin (TPO) binding to MK receptor c-mpl [16-18]. TPO is sufficient but not absolutely necessary for inducing differentiation of progenitor cells in the bone marrow towards the final mature MK [19]. MK are derived from hematopoietic stem cells which differentiate into a megakaryocyte-erythroid progenitor (MEP) cell [20] through successive lineage commitment steps (**Figure 1**). TPO stimulation via myeloproliferative leukemia protein (c-mpl) activates signaling pathways which control multiple transcription factors including runt-related transcription factor 1 (Runx1), globulin transcription factor 1 (Gata1), Friend leukemia integration 1 transcription factor (Fli1) and myeloblastosis transcription factor (c-Myb) to regulate differentiation and development of MK. As shown in **Figure 1**, Runx1 interacts with Gata1 and Fli1 to regulate differentiation [21, 22], Fli1 restricts the MEP to the megakaryocytic lineage [23], while Gata1 and its co-factor Friend of Gata1 (Fog1) are critical in promoting MK differentiation [24, 25]. Finally, the maturation process includes 1) polyploidization, 2) development of an extensive internal demarcation membrane system (DMS); and 3) formation of proplatelet processes [26]. As MK progressively differentiate they lose their proliferative ability and become

polyploid through a variation in the cell cycle known as endomitosis, which results in incomplete cytokinesis and increased (up to and great than 64N) DNA content [27]. During differentiation, diploid promegakaryoblasts give rise to tetraploid megakaryoblasts and then successively larger and more polyploid promegakaryocytes and finally mature MK. Polyploidization of mature MK facilitates the massive protein and membrane synthesis required for subsequent platelet production. Following polyploidization, MK undergo cytoplasmic maturation involving the formation an extensive DMS [28]. The DMS is an elaborate membrane network that ultimately fills the MK cytoplasm except for a narrow band at the periphery of the cell. By virtue of its origin in tubular invaginations of the plasma membrane, the DMS maintains continuity with the extracellular space [29, 30]. In fact, studies have shown that whole-cell patch-clamp recordings reveal the DMS to be a single electrophysiologic entity [31]. The DMS formation precedes proplatelet formation and thus plays a vital role in the size and number of platelets that are released from the MK. Therefore this subcellular structure is a significant component in the pathogenesis of many diseases where platelets are a contributing factor. Finally, proplatelets extend from the DMS, which terminally bud into platelets that shed into the blood stream. Despite significant advances in megakaryopoieisis, the complete mechanisms underlying their physiology, and importantly, their role in diseases such as cardiovascular disease, coagulations disorders, inflammation, and cancer biology are not fully elucidated. Thus, there is a need to develop better model systems to understand MK biology. One facet of MK biology that has been implicated in diseases is its cytoskeleton. The cytoskeleton is critical to the MK biology because it contributes to the production adequate platelets of uniform size for hemostasis.

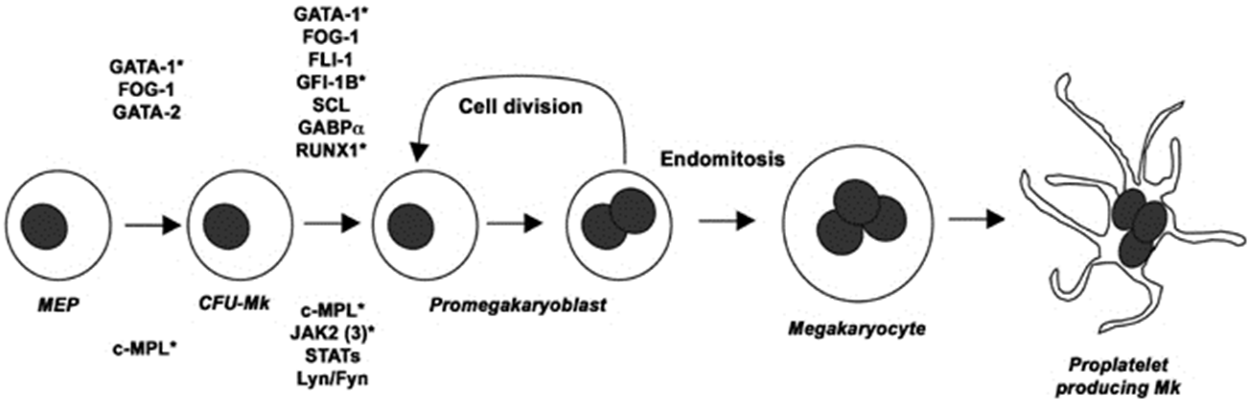

Figure 1: Schematic representation showing megakaryopoiesis and the role of transcription factors. MK are derived from hematopoietic stem cells (HSCs). The HSC differentiates into a MEP cell, which with adequate transcription factor expression, differentiates into the first committed MK lineage cell called CFU-MK. CFU-MK differentiate into promegakaryocytes. At this stage of megakaryopoiesis, the MK undergoes endoreplication, which involves multiplying of the cell's nuclei and cytoplasmic content without undergoing cytokinesis. After endoreplication, the MK is mature. The mature MK is polyploid and can have nuclei content from 4N to 64N. The mature MK cytoplasm partitions in a process called DMS. Cytoplasmic extensions protrude from the DMS to form the proplatelet producing MK. Proplatelets terminally bud to form platelets. Reprinted by permission from Macmillan Publishers Ltd: Oncogene [32], copyright 2007.

The essential role of the cytoskeleton

The release of platelets into circulation is preceded by considerable morphological changes in the parent MK. Dynamic expression and distribution of cytoskeletal proteins such as tubulin, actin, myosin, and dynamin are important for MK development [33, 34]. The generation of platelets, and their release into the bloodstream by MK, requires a complex series of remodeling events powered by the cytoskeleton to result in the release of many platelets from a single MK. Abnormalities in this process can result in a variety of medical disorders that ultimately affect platelet biogenesis (thrombocytopenia and thrombocytopathies) and increase the risk of bleeding. This review describes the process of megakaryopoiesis and discusses new insights into the importance of the MK cytoskeleton.

Actin cytoskeleton

Rho/Rho-associated protein kinase (ROCK) Signaling Pathway

MK cell membrane structure, size, and polyploidization are regulated partly through dynamic expression of actin [35-37]. Figure 3 depicts an overview of the RhoA/ROCK signaling pathway as it relates to actin regulation. Briefly, actin dynamics are regulated primarily through alternating states of GDP-bound inactive and GTP-bound active forms of RhoA [38]. RhoA activation leads to the canonical activation of downstream factors. Cofilin, which is downstream of ROCK and LIM kinase, is an actin-binding protein essential for the depolymerization of actin filaments. Following cofilin phosphorylation, cofilin can either enhance depolymerization and/or stabilization of actin [39-41]. Specifically, RhoA regulates actin depolymerization and stabilization, as well as actin fiber contraction. The regulation of actin dynamics via the RhoA/ROCK pathway facilitates many facets of development as depicted in Figure 2 [42-44]. Studies also suggest that the late failure of cytokinesis, responsible for the endomitotic process in MK, is related to a partial defect in the Rho/Rock pathway activation [35], by regulating actin expression. At the end of maturation, there is decline in Rho activation that allows for the onset

of cytoplasmic extensions and proplatelet formation in MK [36]. During early MK maturation, Rho activation predominates and thus restricts proplatelet formation [36]. RhoA activation leads to sequential phosphorylation of myosin light chain II (MLC2), which then induces actin fiber contraction and thus regulates MK size and cell membrane structure [36, 45, 46].

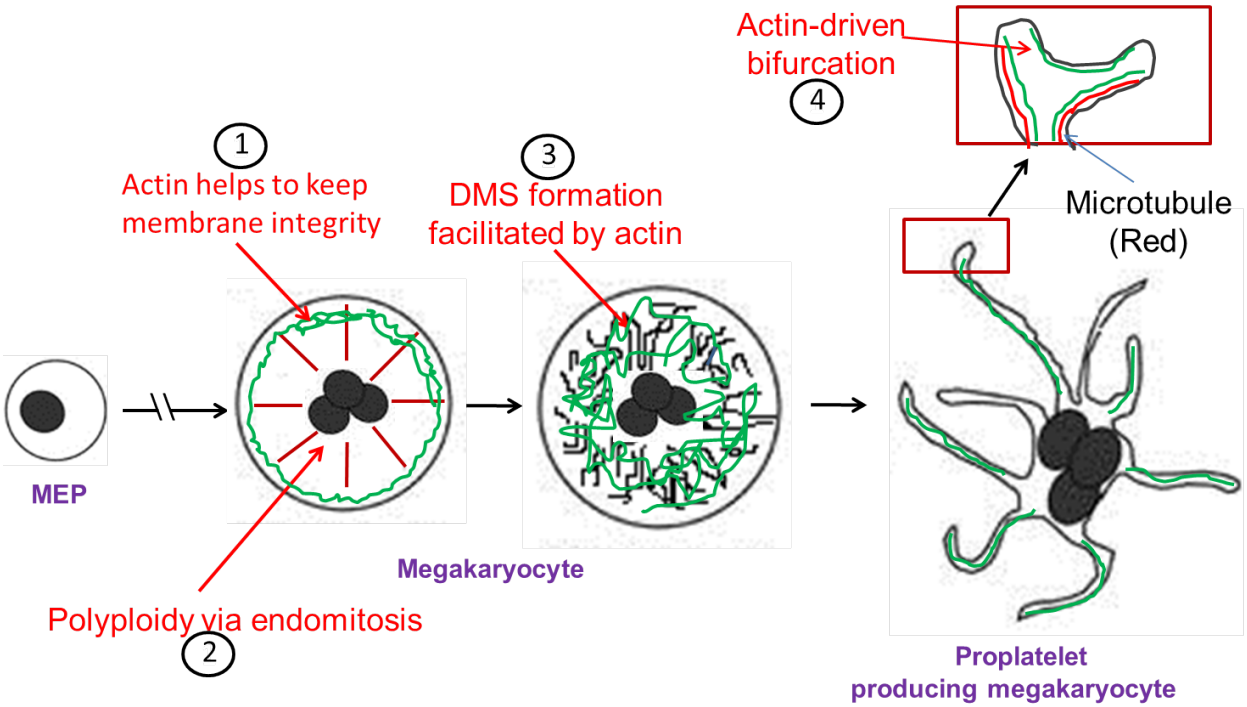

Figure 2: Role of the actin cytoskeleton in MK development. Actin helps to maintain the shape and membrane integrity of the cell (**1**), and forms cytoskeletal structures to regulate endomitosis (**2**). Actin is also vital during the formation of the DMS (**3**), the hallmark of proplatelet formation, and drives the bending and bifurcation of proplatelets (**4**) necessary for the platelet formation process.

Actin is an integral component of the MK cytoskeleton. Its role is seen very early in MK development as the dynamic expression of actin helps to maintain the MK cell membrane structure [36, 37]. Thus, actin helps to maintain the shape, as well as determine sizing of the MK throughout MK maturation. Maturation of MK is paralleled by the increase in nuclear and DNA content, termed polyploidization. The formation of an acto-myosin contractile ring helps in the formation of a cleavage furrow during endomitosis [47], which aids in MK polyploidization during maturation. Next, the mature MK undergoes DMS formation. The invaginated DMS, a hallmark of mature cells, has been proposed as the source of proplatelet membranes. Late in MK ontogeny, the DMS gets loaded with phosphatidylinositol 4,5-bisphosphate (PIP2) [28]. PIP2 promotes actin polymerization by activating Rho-like GTPases and Wiskott-Aldrich syndrome (WASp) family proteins [48]. Actin cytoskeleton facilitates DMS formation by invaginating the plasma membrane to form continuous tubular structures in the cytoplasm. To produce platelets, MK elaborate proplatelets from the DMS, accompanied by expansion of membrane surface area and dramatic cytoskeletal rearrangements. At the proplatelet stage, the cell extends cytoplasmic processes into the venous sinusoids of bone marrow followed by the development of constrictions at given intervals, revealing putative platelets. The distribution of actin in the MK cytoplasm changes and helps in the formation of these processes, which are the hallmark features of the proplatelet [49]. The process of amplifying proplatelet ends by repeatedly bending and bifurcating the proplatelet shaft is dependent on actin-based forces and is inhibited by the actin toxins cytochalasins [50, 51].

The actin cytoskeleton plays a fundamental role during megakaryopoiesis. Actin regulates MK endomitosis, DMS formation, and proplatelet formation. Perturbing actin polymerization or dynamic expression can affect platelet production, specifically the number of platelets produced or uniform platelet sizing.

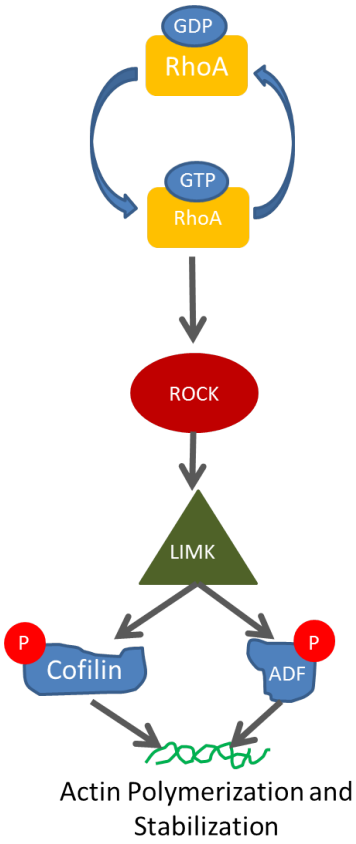

Figure 3: Schematic representation of the Rho/ROCK transduction pathway involved in megakaryopoiesis. The signaling pathway is a key regulator of actin polymerization and dynamics during MK development. Activated Rho interacts with ROCK, which regulates downstream factors such as cofilin, ADF, and MLC2. In their native state in the cell, cofilin and ADF are unphosphorylated, and functions as actin depolymerizers. Phosphorylation of cofilin and ADF prevents actin depolymerization and aids actin polymerization. ROCK interaction with MLC2 also regulates actin contraction via myosin activity on actin.

Myosin II Cytoskeleton

Myosins constitute a large superfamily of proteins that share a common domain that has been shown to interact with actin, hydrolyze ATP, and help to produce cell movement [52, 53]. Non-muscle myosin II (NMM-II) is part of the myosin super family [54], with vertebrates expressing at least two isoforms, NMM-IIA and NMM-IIB [55, 56]. The two isoforms are also expressed in MK.

NMM-II exists as a large hexamer, comprised of two non-muscle myosin heavy chains (NMMHC) and 4 myosin light chains (MLCs). NMM-II's are typically constructed of three functional subdomains: (1) the motor N-terminal domain that interacts with actin and binds ATP, (2) the neck domain that binds light chains and (3) the C-terminal tail domain that serves to anchor and position the motor domain so that it can interact with actin C-terminal tail [56-58]. The neck includes the IQ motif, which is an important binding site for the MLCs. The MLC is responsible for inducing the acto-myosin contractile response. By phosphorylation of MLC, myosin becomes activated and can interact with actin filaments [59-62]. On the other hand, the C-terminal tail domain consists of two long alpha helices important for filament assembly and cargo binding [63].

NMM-II regulates several developmental stages during megakaryopoiesis. First, polyploidization occurs by endomitosis, which was a process considered to be incomplete mitosis aborted during anaphase [64, 65]. Studies have showed that in fact MK endomitosis is a result of an altered furrow formation during cytokinesis, when the acto-myosin contractile ring is abnormal because accumulation of NMM II is lacking [35]. Thus, late failure of cytokinesis responsible for the endomitotic process is regulated by inhibition of NMM-IIA. Secondly, modification of the MLC2 phosphorylation status through the Rho/ROCK pathway regulates proplatelet formation. ROCK is a key Rho effector that phosphorylates MLC2 at Ser19 and inhibits myosin phosphatase [66, 67]. Third, MLC2 phosphorylation is necessary for actin-

myosin motor activation to provide essential contractile forces for a diversity of cellular processes, such as cell contraction, cell migration, and membrane blebbing [45, 46, 68], all processes necessary for MK proplatelet formation and platelet blebbing. The Rho/ROCK pathway is implicated in platelet shape changes during activation by mediating MLC2 phosphorylation [62, 69-71]. Indeed, studies indicate that MLC2 phosphorylation plays a critical role in platelet biogenesis by controlling proplatelet formation and fragmentation [36].

One disease in particular that affects polyploidization and proplatelet developmental is familial platelet disorder with predisposition to acute myeloid leukemia (FPD/AML) [72, 73]. FPD/AML is an autosomal dominant disorder characterized by dysmegakaryopoiesis, and qualitative and quantitative platelet defects. Studies show that deregulation of genes that regulate NMM-II polymerization, MLC2, MYH9 and MYH10, by *RUNX1* is the underlying molecular defect in the pathogenesis of the disease [74, 75]. Deregulation of NMM-IIA expression leads to defects in both polyploidization and proplatelet formation. In fact, treatment of MK cells with the myosin II activity inhibitor, blebbistatin rescued the ploidy defect of FPD/AML MK [74].

NMM-II is an actin-binding protein that has actin cross-linking and contractile properties and is regulated by the phosphorylation of its light and heavy chains. By contracting actin, it is able to regulate many facets of MK development including (1) polyploidization via endomitosis, and (2) proplatelet formation by playing a role in the mechanical forces needed to control their protrusion. The normal functionality of myosin is paramount in megakaryopoiesis and any altered myosin activity can impair platelet biogenesis.

Tubulin Cytoskeleton

Microtubules play indispensable roles in cell division, cell motility, cellular transport, maintaining cell polarity, and cell signaling. Impairment in the function of microtubules can lead

to abnormal cell morphology. Microtubule polymerization is a complex process involving a cooperative assembly of αβ tubulin heterodimers followed by GTP hydrolysis [76]. Each subunit of the tubulin heterodimer has one GTP molecule bound to it. The α-subunit binds to GTP in an irreversible manner while the GTP bound to β-tubulin is exchanged [77] and hydrolyzed during polymerization.

Polymerization of microtubules occurs through two important steps: nucleation and elongation. Initially, an oligomer consisting of 6–12 αβ tubulin dimers is formed in the nucleation step. Next, GTP bound αβ tubulin dimers bind to the nucleus and lead to elongation and formation of the microtubule protofilament. After the elongation phase, the assembly of microtubules reaches a steady state where the addition and the dissociation of tubulin subunits at the ends of the microtubules are balanced and there is no net increase in the polymer level.

Microtubule dynamics also contribute to the proper orientation and segregation of chromosomes during mitosis [78, 79]. The barb or plus ends of the microtubules undergo a series of random elongations and shortenings in the cytoplasm to search and bind the kinetochores of the chromosomes [80]. Once the plus ends attach to the kinetochores, the chromosomes translocate to the equatorial plane. Metaphase microtubules are highly dynamic; however, at the onset of anaphase, microtubule dynamics are stabilized to ensure successful chromosomal segregation [81]. Owing to the shortening of the microtubules and the action of motor proteins, tension is generated in the microtubules, which leads to the poleward movement of the chromosomes [82]. A defect in this process may block the cells during mitosis by activating the spindle checkpoint.

Microtubules are a major component of the MK cytoskeletal network and function as the primary motor for proplatelet elongation. High concentrations of microtubule poisons such as colchicine and vincristine sulfate prevent proplatelet elongation and cause extended proplatelets to retract [51, 83, 84]. Deletion of the β1-tubulin gene in mice, which encodes the major β-

tubulin isoform microtubules assembly, impairs platelet production and release, and results in abnormal platelet morphologies [85, 86]. Also, while continuous polymerization of microtubules is necessary to support the enlarging proplatelet mass, the sliding of overlapping microtubules is a vital component of proplatelet elongation [87]. One disease in particular that is associated with aberrant tubulin polymerization has been reported in Cavalier King Charles Spaniels. These dogs have a high prevalence of macrothrombocytopenia that is inherited as an autosomal trait [88-90]. Studies show that the macrothrombocytopenia is a result of a β1-tubulin mutation; αβ tubulin dimers within protofilaments become unstable, leading to altered proplatelet formation by megakaryocytes [91]. This results in the macrothrombocytopenic clinical presentation.

Tubulins are one of the main cytoskeletal proteins in the MK. Tubulins form microtubules, which are essential cytoskeletal organelles in many cells, including MK. The polymerization of tubulins and microtubule formation is essential to maintaining MK cell shape and proplatelet formation.

Dynamin Cytoskeleton

The dynamins (DNM) form a superfamily of three proteins (DNM 1, DNM 2, and DNM 3), which can differentially localize to distinct cytoplasmic and membrane compartments where they participate in a number of membrane trafficking events [92]. Studies have shown that the DNM self-associate and hydrolyze nucleotides to link cellular membranes to the actin cytoskeleton [93, 94]. With their association with microtubules and microfilaments, the dynamins have been implicated in a variety of cellular processes including: 1) development of organelles involved in cell motility [95, 96]; 2) membrane vesiculation from the plasma membrane and trans-Golgi network [94, 97, 98]; 3) membrane extension and lamellipodial protrusion and phagocytosis [99]; and 4) cytokinesis [100].

The role of dynamins in MK is still largely unknown. Amongst the cytoplasmic proteins of the family, DNM3 is the only one known to participate in MK development. The mechanism by which DNM3 may participate in MK cytoplasmic enlargement and DMS formation is unknown. However, studies show that PIP2, an important regulator of dynamin function [101-103], plays an essential role in facilitating DMS development and platelet biogenesis [28]. The DMS accumulates PIP2 late in differentiation. In anticipation of platelet release, membranes of the DMS harbor PIP2 and associate with the WASp-Arp2/3 complex to induce actin polymerization in preparation for platelet formation [28]. Plasma membrane PIP2 is also essential for mediating the sequential recruitment of adaptor and accessory proteins to sites of clathrin-dependent endocytosis [104]. All 3 dynamin isoforms contain a PH domain that is able to bind PIP2. Binding of PIP2 to dynamin not only strongly increases the GTPase activity of dynamin but also serves to target dynamin to the plasma membrane [105, 106].

DNM3 is associated with non-muscle myosin 2A and thus has important ramifications for the role of DNM3 in MK cytoskeleton rearrangements that involve not only MK development, but also proplatelet formation and platelet biogenesis. Defects in MYH9, which encodes the NMM-IIa heavy chain, are associated with inherited macrothrombocytopenia disorders: the May-Hegglin anomaly, Fechtner syndrome, Sebastian syndrome, and Epstein syndrome [50]. Moreover, defects in MYH9 or its regulation have been shown to affect different steps of MK development, including the maintenance of cell shape, formation of the DMS, anchorage to extracellular matrix, and proplatelet formation [107, 108]. The association of DNM3 to myosin 2A is critical in regulating the different stages of MK development. Recently, using immunohistochemistry, the DNM3 protein has been observed in human MK and murine proplatelets [109]. Interestingly, studies show that silencing of the DNM3 gene in human $CD34^+$ hematopoietic precursor cells impairs the formation of MK [110]. Moreover, Nurnberg and colleagues have shown that pharmacologic inhibition of dynamin GTPase activity with dynasore

inhibits proplatelet and platelet formation [111]. DNM3 is critical during MK progenitor proliferation, maturation of MK, proplatelet formation and platelet biogenesis.

Spectrin Cytoskeleton

Acute Megakaryoblastic Leukemia (AMKL) is a rare disorder characterized by extensive proliferation of megakaryoblasts and atypical MK in bone marrow and extramedullary sites, as well as thrombocytopenia [112]. In an extensive study of AMKLs, patients had morphologically recognizable MK that stained positive for βII spectrin, but not the βI: both isoforms are typical of the normal MK. The absence of the βI spectrin isoform resulted in immature MK development in AMKL [113]. Therefore, it may seem spectrin is important for MK sizing and maturity.

Patel-Hett and colleagues show that spectrin is the main fibrous component of the proplatelet dense membrane skeleton [114]. Assembly of spectrin into tetramers is required for invaginated membrane system maturation and proplatelet extension, as expression of a spectrin tetramer-disrupting construct in MK inhibits both processes [114]. Incorporation of this spectrin-disrupting fragment into a novel permeabilized proplatelet system rapidly destabilizes proplatelets, causing blebbing and swelling. Spectrin tetramers also stabilize the “barbell-shapes” of the penultimate stage in platelet production, since addition of the tetramer-disrupting construct converts these barbell-shapes to spheres, demonstrating that membrane skeletal continuity maintains the elongated, pre-fission shape. Overall, the role of spectrin is poorly understood in normal megakaryopoiesis but emerging data indicates that spectrin is influential in different steps of MK development through its participation in the formation of MK maturation, invaginated membranes, and in the maintenance of proplatelet structure.

The Biology of Myelodysplastic syndrome (MDS),

MDS, pre-leukemia, is a clonal process characterized by ineffective hematopoiesis, hypercellular/dysplastic bone marrow (BM), and peripheral blood cell cytopenias. Paradoxically, MDS patients have normal or increased BM cellularity, and progress to acute myeloid leukemia (AML) in 25% to 30% of cases [115-119]. Despite being as common as AML, MDS is less understood, with less effective treatments. The prevalence of MDS increases with age; the median age at presentation is 70 years with an annual incidence of 2-30 cases per 100,000 persons at risk [118, 120]. MDS may be caused by a combination of factors including acquired genetic mutations, including chromosomal deletion (5q deletion), environmental toxin exposure such as benzene, or anticancer chemotherapy or ionizing radiation [121]. The prognosis for MDS is poor due to hemorrhage and infections that result from refractory cytopenias [117]. Studies based on the International Prognostic Scoring System indicate the median survival of the four risk categories is 11 months for high risk, 26 months for intermediate-2 risk, 63 months for intermediate-1 risk, and 97 months for low risk; after five years MDS has a mortality rate of 100% [122].

Further advances in treatment rely upon identifying the clonal cell of origin—a difficult task given the heterogeneous character of MDS [117, 123]. Some patients have dysplastic MK and macrothromcytopenia. The pathogenesis of this disease, including the contribution of MK, remains unclear despite significant progress in detailing the molecular genetics of MDS.

Nuclear Pore Proteins:

The nuclear pore complex (NPC) (Figure 3) is a large, multi-protein complex that mediates nucleocytoplasmic transport of protein and RNA between the nucleus and cytoplasm [124, 125]. The NPC is embedded in and traverses the nuclear membrane, and consists of approximately 30 different nucleoporin (NUP) proteins, many of which are present in multiple

copies [126]. NUP98, a dynamic 90-kDa protein component of the NPC component with glycine-leucine-phenylalanine-glycine (GLFG) repeats can shuttle between the cytoplasm and nucleus [127-130].

Several lines of evidence support the biological importance of the NPC and Nup98. *First*, proper regulation of the actin cytoskeleton dynamics and architecture strongly relies upon NPC integrity [131]. *Second*, disruption of *Nup98* in embryonic mice is lethal [132]. *Third*, studies in vesicular stomatitis virus replication suggest that Nup98 proteins are involved in mRNA transport [133]. In addition, Nup98 is the target of degradation in both influenza and polio infections [134, 135]. *Fourth*, NUP98 regulates mitosis by binding to the cdh1-form of the anaphase promoting complex, cyclosom [136]. *Fifth*, NUP98 controls the bipolar spindle assembly during mitosis through association with microtubule dynamics in *Xenopus* extracts [137]. *Sixth*, recent studies in *Drosophila* show that Nup98 is involved in transcription regulation [138, 139]. Finally, Nup proteins form fusions with other proteins that play a significant role in disease including malignant transformation in hematopoietic progenitors [140, 141]. Twenty-eight distinct NUP98 gene fusions have been identified, caused primarily by balanced translocations and inversions, in dysplastic cells of patients with a wide array of distinct hematopoietic malignancies, including acute myeloid leukemia (AML) and MDS [8, 142, 143].

Nup proteins have been implicated in several diseases including AML and MDS. The *NUP98-HOXD13* fusion has been detected in AML patients, and when expressed in mice, results in MDS that progresses to AML. Of note is the fact that a subset of MDS patients present with dysplastic MK and macrothrombocytopenia [144, 145]. On-going studies are focused on elucidating the role of NUP proteins and their fusion genes in MDS especially with the NPC's relevance in cytoskeleton regulation of actin and mitotic spindle via tubulin.

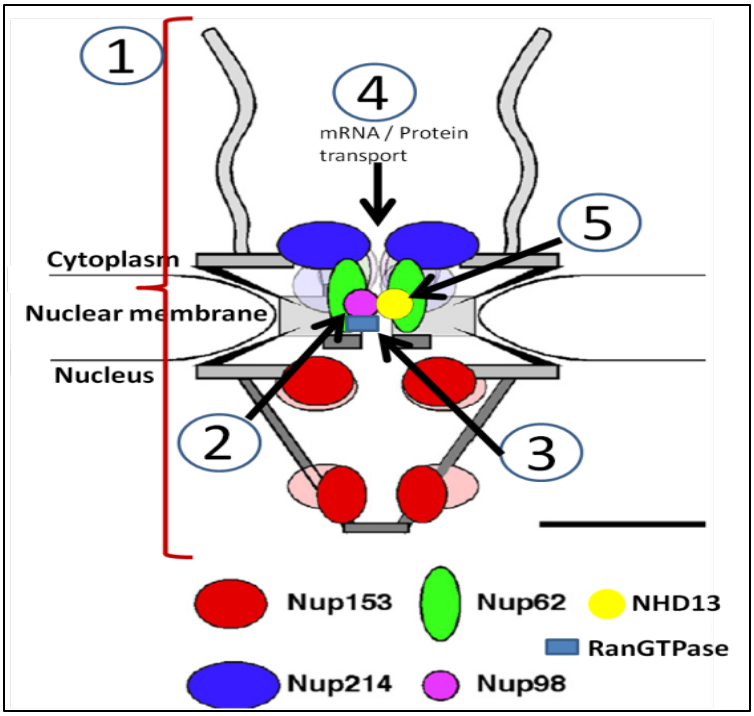

Figure 4: Structure of the nuclear pore complex. (**1**) Shows the large, multiprotein complex spanning the nuclear membrane into the cytoplasm. Note the critical location of Nup98 (**2**) positioned in the central aspect of the complex where it interacts with the RanGTPase complex (**3**) during mRNA and protein transport (**4**). During cell division, Nup98 dissociates and regulates microtubules of the mitotic spindle through its interaction with MCAK (not shown). The abnormal Nup98-HoxD13 protein (**5**) positioned in the central core, replaces the normal Nup98; position in this location could presumably alter the interaction of Nup98 and the mitotic spindle during MK development leading to dysplastic MK and ultimately macroplatelets as are seen in *NUP98-HOXD13* mice. Reprinted from Journal of Structural Biology, 177(1), Chatel G., Desai S. H., Mattheyses A. L., Powers M. A., and Fahrenkrog B., Domain topology of nucleoporin Nup98 within the nuclear pore complex, 81 – 89, Copyright 2012 [146], with permission from Elsevier.

Summary

MK differentiate from $CD34^+$ hematopietic stem cells and at the conclusion of their maturation, their cytoplasm segments to form platelets. MK must coordinate numerous cytoskeletal interactions to support development and maturity of the cellular machinery to produce de novo assembly of thousands of platelets. Several cytoskeletal proteins are integral to the complex machinery of the platelet-producing MK machinery; these proteins include tubulin, non-muscle myosin IIa, actin, dynamin and spectrin. Defects or mutations that affect the normal dynamics of these cytoskeletal proteins at any stage of MK development may result in dysplastic MK, with clinical presentations that often result in platelet disorders. May-Hegglin anomaly, Fechtner syndrome, Sebastian syndrome, Epstein syndrome, AMKL, and FPD/AML have impaired MK development, which all result in thrombocytopenia, macro platelets, or fragile platelets. Similar to these diseases, MDS patients may also present with dysplastic MK and platelet disorders. It is essential that the impaired aspect of MK development is correctly determined for treatment to be effective. It is also reasonable to hypothesize that dysplastic MK and macrothrombocytopenia, as seen in MDS patients, is due to an aberrant MK cytoskeleton. The following thesis research investigates the role of the MK cytoskeleton in MDS using the *NUP98-HOXD13* mouse model system.

Chapter 2

NUP98-HOXD13 mice have megakaryocytes with impaired actin localization during the development of Myelodysplastic syndrome

Abstract

Patients who develop Myelodysplastic syndrome (MDS) often present with macrothrombocytopenia as a feature of their disease. While indications suggest that this might be due to impaired megakaryocyte (MK) differentiation, the precise mechanism(s) remain undetermined. Here we have used *NUP98-HOXD13* (*NHD13*) transgenic mice that develop MDS to evaluate the effects of chromosomal translocations on MK development. We hypothesized that actin localization is disrupted in *NHD13* MK resulting in macrothrombocytopenia. *NHD13* bone marrow contained dysplastic MK that had defective demarcation membrane systems and fewer proplatelets. Fluorescent microscopy revealed that actin failed to localize to the peripheral cytoplasm of transgenic MK. Furthermore, when stimulated in vitro, *NHD13* MK failed to adequately produce proplatelets as deomonstrated by their lack of actin-positive cytoplasmic extensions. This report suggests that *NHD13* impairs actin localization and function necessary in platelet production suggesting a possible mechanism for macrothrombocytopenia in MDS.

Introduction

Myelodysplastic syndromes (MDS) comprise a group of clonal hematopoietic disorders characterized by ineffective hematopoiesis, hypercellular/dysplastic BM, and peripheral blood cell cytopenias [115, 147, 148]. With the passage of time MDS progresses to acute myeloid leukemia (AML) in 25% to 30% of cases [149]. Despite being as common as AML, MDS is less understood and less treatable thus creating an unfavorable prognosis. Further advances in treatment rely upon identifying the clonal cell of origin—a difficult task given the heterogeneous character of MDS [148, 150]. In fact, some MDS patients present with dysplastic megakaryocytes (MK) in their BM and have thrombocytopenia, as well as macroplatelets in their peripheral blood. Therefore, understanding abnormal megakaryopoiesis seen in MDS is important because: (a) some MDS patients die of hemorrhagic complications and (b) normal megakaryopoiesis remains poorly understood. Previous studies by Lin and colleagues have shown that *NUP98-HOXD13 (NHD13)* transgenic mice develop MDS at 7-months-of-age and progress to acute leukemia by 14-months-of-age [11]. *NHD13* mice consistently develop thrombocytopenia and macroplatelets, as well as have dysplastic MK in their BM. Furthermore, K562 cells treated with TPA (12-O-tetradecanoylphorbol-13-acetate) and transfected with *pVNHD13* have impaired differentiation [11]. Taken together these studies showed that *NHD13* impairs MK differentiation.

MK are derived from the megakaryocyte-erythroid progenitor (MEP) cell following activation of the c-MPL receptor by thrombopoietin (TPO) [15, 151]. Multiple transcription factors including gata-1, runx-1, fli-1 and nf-e2 regulate the differentiation and maturation of MK [22, 152-156]. Maturation includes polyploidization of nuclei, development of the cytoplasmic demarcation membrane system (DMS), and finally formation of proplatelet processes before platelets are shed into circulation [15]. Nuclear polyploidization facilitates the need for substantial protein and

membrane synthesis required for platelet production. The release of platelets into circulation is preceded by considerable morphological changes in the MK. These changes are regulated in part by cytoskeletal proteins including microtubules, myosin, dynamin 3, and actin [87, 109, 157]. In fact, studies show that the dynamic expression of actin significantly maintains the MK cell membrane structure, as well as determines the size of MK during maturation [36]. Actin distribution in the MK cytoplasm also contributes to the development of the DMS leading to proplatelet production [28]. During proplatelet formation, tubulin extends the proplatelet outward followed by terminal bifurcation and bending induced by actin [158]. Finally, mature platelets are released into circulation. Given these biological processes pertaining to MK, we hypothesized that the *NHD13* fusion gene might interfere with actin formation as an underlying reason for the macroplatelets seen in the *NHD13* transgenic mice. Here we used microscopy and immunofluorescence to evaluate the cytoskeleton in dysplastic MK from *NHD13* mice. Our results show that the *NHD13* fusion gene leads to aberrant localization of cytoplasmic actin and defective DMS, as well as impairs proplatelet formation.

Materials and Methods

Animals

NHD13 transgenic and wild type (WT) control mice of either two, four, or eight months (mo.) old were used in this study. Two and four month old mice were defined as subclinical and eight month old mice were defined as clinical. The mice were bred and maintained at the AAALAC accredited core laboratory animal facility at the Virginia Maryland Regional College of Veterinary Medicine, Virginia Tech. All experiments were carried out as per NIH guidelines with the approval from the Virginia Tech Institutional Animal Care and Use Committee.

Peripheral blood evaluation

Peripheral blood was obtained from the retroorbital plexus into EDTA blood collection vials (RAM Scientific Inc) and analyzed using a HESKA CBC® - Diff™ Veterinary Hematology System. Routine blood smears were stained using Wright-Giemsa stain according to the manufacturers's protocol (Sigma Aldrich, St. Louis, MO). Smears were analyzed using a 50i Nikon light microscope and 100x oil immersion objective (Nikon, Melville, NY). Photographs were taken using the Nikon Digital Sight camera (DS-Fi1) and NIS-Elements D software 3.10 (Nikon).

Histopathology of mouse bone marrow

Immediately following euthanasia, sternums were harvested and fixed in 10% neutral buffered formalin (Fisher Scientific, Fair Lawn, NJ). Following routine tissue processing and rapid decalcification, the tissue was sectioned at 4μm sections and mounted onto glass slides. BM was analyzed using a 50i Nikon light microscope. Twenty five random microscopic sections of 100 μm^2 fields of each sternum were evaluated for BM MK counts, using a 50i Nikon light microscope. Photographs were taken using the Nikon Digital Sight camera (DS-Fi1) and NIS-Elements D software 3.10 (Nikon).

Culturing and harvesting megakaryocytes

MK were cultured according to previously published protocols [159]. Briefly, total BM was flushed from tibias and femurs of control and transgenic mice into phosphate buffered saline (PBS) (Thermo Scientific, Logan, Utah) with 2% bovine serum albumin (BSA) (Invitrogen) and penicillin/streptomycin (P/S) (Thermo Scientific). Hematopoietic progenitor cells were isolated from total BM by enrichment using magnetic bead-based negative selection with the mouse hematopoietic progenitor enrichment kit (StemCell Technologies, Vancouver, BC). Progenitor cells were cultured in serum-free StemSpan (StemCell Technologies) containing 2% P/S and supplemented with 50ng/mL TPO (Peprotech, Rocky Hill, NJ) for up to five days. After five days,

the cultured cells were centrifuged at 200 *xg* and resuspended in PBS. Finally, MK were harvested by passing the cell suspension over a 1.5%/3.0% BSA gradient. MK were either used for immediate experiments or snap frozen for downstream use. To assay proplatelet formation functionality, progenitor cells were cultured in StemSpan supplemented with 50ng/mL Tpo and 10ng/mL estradiol (Sigma-Aldrich), a proplatelet inducing cytokine [160].

Ultratructural evaluation of MK

Cultured MK were washed once with PBS and fixed with 2.5% gluteraldehyde in Grey's salt solution and embedded in epon. Thin sections were cut, sputter coated with uranyl acetate and lead citrate, and examined using the Zeiss 10CA transmission electron microscope (TEM) (Carl Zeiss, Thornwood, NY).

Cytoskeletal evaluation of MK

MK were harvested as described above and cytospun onto positively charged slides (Thermo Scientific) or adhered to fibrinogen-coated glass slides for four hours at 37°C, and fixed with 4% paraformaldehyde for 15 min. Following fixation, MK were permeabilized with 0.1% Triton-X solution (Sigma Aldrich) in PBS for 10 min. Slides were briefly washed with PBS and blocked with 5% fetal bovine serum (Thermo Scientific) solution for one hour. MK were then labeled with DAPI (Vectashield mounting medium), Alexa Fluor® 488 Phalloidin Conjugate (A12379, Invitrogen), rabbit anti-mouse myosin IIa (3403, Cell signaling), and rabbit anti-mouse α/β tubulin antibody (2148, Cell Signaling). Rabbit myosin IIa and α/β tubulin antibodies were labeled with anti-rabbit Alexa Fluor® 488 Conjugate (4413, Cell Signaling) and anti-rabbit IgG Alexa Fluor® 488 Conjugate (4412, Cell Signaling) secondary antibodies, respectively. Labeled MK were visualized using either confocal microscopy (Zeiss LSM 510) or fluorescence microscopy (Olympus BX-51).

Megakaryocyte DNA content analysis

Cultured MK were washed with PBS and labeled with fluorescein isothiocyanate (FITC) conjugated anti-mouse CD41 (eBioscience, San Diego, CA) for 1 hour at 4°C. Next, MK were washed with PBS, centrifuged at 200 *xg* for 5 minutes and resuspended in 200µl PBS. An aliquot of 1.8ml 70% cold ethanol was added dropwise to cell suspension while vortexing at low speed to fix cells. Cells were centrifuge at 200*xg* for 3 minutes, washed with PBS and incubated on ice. One hour before analysis, pelleted cells were resuspended in 200µL solution of 50ug/ml propidium iodide (PI) (Sigma Aldrich), 4mM sodium citrate (Sigma Aldrich), 0.1% triton-X, and 100u/mL Rnase A (5Prime, Gaithersburg, MD). DNA content analysis was performed using the Accuri™ C6 Flow Cytometer (BD Bioscience, San Jose, CA).

Statistical and data analysis

Data were analyzed with GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA) using Student's t test; a p value < 0.05 was considered significant.

Results

NHD13 mice have bone marrow that is deficient in megakaryocytes

We analyzed blood from *NHD13* and WT mice to determine specifically how platelet counts and macroplatelets changed over time. Our analysis revealed that subclinical mice as early as two months old had circulating macroplatelets, as well as statistically lower platelet counts compared to WT mice. Both subclinical and clinical *NHD13* mice had circulating macro platelets (Figure 5A). Subclinical mice had comparable blood cell parameters to their WT controls, but a slightly significant difference in platelet counts between *NHD13* and WT mice (Figure 5B). In mice with clinical MDS, there was a precipitous drop in platelet counts between WT and *NHD13* mice. The average platelet count in *NHD13* mice was less than half the number of platelets in WT mice (214 ± 31 vs 452 ± 32). A summary of these findings is given in Table 1.

Next, we evaluated the BM of subclinical and clinical *NHD13* and their WT controls. Given our earlier findings we focused our assessment on the morphological features of the MK. Two-month and four-month WT BM contained similarly shaped MK, as did their age matched *NHD13* BM in addition to a few dysplastic MK with fragmented nuclei (Figure 6A). *NHD13* mice with clinical MDS had BM MK hypoplasia and dysplasia when we compared to WT controls. In fact many of the *NHD13* MK had emperipolesis, were relatively larger in size, had fragmented nuclei and increased nuclear to cytoplasmic ratio (Figure 6A). BM MK counts per unit area showed that subclinical mice MK counts were comparable to their WT aged matches (two-month; 2.78±0.06 vs 3.19±0.47, four-month; 3.05±0.39 vs 3.40±0.06) (Figure 6B). But *NHD13* mice with clinical MDS had a statistically significant reduction in MK counts per unit area compared to WT control (0.75±0.04 vs 3.40±0.44). Our results showed that *NHD13* impairs megakaryocyte differentiation and platelets morphology in both subclinical and clinical mice. These results are consistent with previously published data by Lin et al [11]. *NHD13* mice also had significantly reduced MK and platelet counts with disease progression when compared to their WT controls.

NHD13 leads to an abnormal DMS and fewer proplatelet formations

The hallmark feature of proplatelet differentiation is the formation of the demarcation membrane (DMS), which determines the number and size of platelets released into circulation. Defects in the DMS have been shown to result in macro platelet release. Because of the thrombocytopenia and circulating macro platelets in *NHD13* mice, we evaluated the DMS as a possible mechanism for these features. Analysis by TEM revealed several distinguishing features between WT and transgenic MK (Figure 7). First, WT MK (I) showed elaborate DMS formation compared to the poor DMS formation in *NHD13* MK (II), Second, WT MK showed a progression and elaborate partitioning of the cytoplasm (III) but partitioning in *NHD13* MK was little to absent (IV). Third, the perinuclear space contained marked granulation in WT MK (V) whereas

cytoplasm of *NHD13* MK contained unusually large yet fewer granules (VI, long arrow). WT MK also had multiple proplatelet extensions (V, short arrows), when compared to *NHD13* MK (VI, short arrows). Fourth, WT MK showed an almost spherical shaped nucleus while the *NHD13* MK exhibited an angular shaped nucleus with increased chromatin condensation. Finally, the nuclear to cytoplasmic ratio was overall increased in *NHD13* MK (V vs. VI). These findings indicate that the *NHD13* fusion gene impairs the DMS. *NHD13* also perturbs proplatelet extension assembly in MK.

Actin distribution is impaired in NHD13 megakaryocytes

The MK cytoskeleton structure was evaluated to determine the effect of the *NHD13* fusion gene on cytoskeletal protein assembly and architecture. Alpha/beta tubulins are subunits of microtubules. Microtubule cytoskeleton is important for MK differentiation, membrane integrity, DMS formation, as well as proplatelet formation, while actin dynamics aid in maintaining the MK cell membrane structure, MK size, DMS and proplatelet formation, as well as platelet budding from proplatelets. Analysis of the α/β tubulin architecture showed that *NHD13* MK had comparable organization to WT MK (Figure 8A). Both WT and *NHD13* MK had evenly distributed α/β tubulin in the cytoplasm. But actin analysis showed a significant difference between WT and *NHD13* architecture (Figure 8B). Control MK (I and II) expressed two phenotypes (A): MK with actin predominantly in the periphery of the cytoplasm (I) and MK with myosin and actin evenly dispersed in the cytoplasm (II). In contrast, transgenic MK (III) displayed only the former phenotype, suggesting that *NHD13* impairs localization of actin. Our data shows that actin is significantly disorganized in MK that express *NHD13*.

Proplatelet formation is impaired in stimulated NHD13 MK

We evaluated MK to determine their ability to form proplatelets. We isolated hematopoietic stem and progenitor cells from BM of WT and *NHD13* mice and cultured the cells in StemSpan supplemented with 50ng/mL TPO and 10ng/mL estradiol, a proplatelet stimulating hormone.

After four days in culture, harvested MK were processed for ploidy analysis to determine if DNA content increases, during progression to proplatelet-producing MK. MK were labeled for F-actin and α/β tubulin to determine the effect of aberrant actin localization will on induced proplatelet formation. Both WT and *NHD13* MK showed proplatelet formation with tubulin cytoplasmic extensions (Figure 9A). But F-actin labeling revealed that whereas WT MK showed elaborate actin cytoplasmic extensions (Figure 9B), *NHD13* MK predominantly lacked actin cytoplasmic extensions (bottom panel). Our results showed impaired proplatelet formation in *NHD13* MK. *NHD13* MK lacked cytoplasmic actin extensions, normally part of proplatelet architecture.

Polyploidization is increased in NHD13 MK

To elucidate the abnormal features seen in BM of *NHD13* mice, such as larger MK and increased nuclear:cytoplasmic ratio, we performed ploidy analysis on cultured MK. Isolated stem and progenitor cells from BM of clinical *NHD13* and WT mice was cultured in the presence of TPO for five days. MK were harvested over a BSA gradient, labeled with FITC conjugated anti-mouse CD41 and stained with PI. Flow cytometric analysis showed that both WT and *NHD13* MK had ploidy states from 2N – 32N (Figure 10A), but on average MK from *NHD13* mice had increased ploidy compared to WT MK (Figure 10B). Though the percentage of MK with 2N ploidy was similar, *NHD13* mice had a higher percentages of MK in the 4N (14.0% vs 9.6%), 8N (7.1% vs 3.4%), 16N (4.9% vs 3.0%), and 32N (3.6 vs 1.8%) states of ploidy. The results show that on average *NHD13* MK had higher DNA content compared to WT MK on average.

Discussion

Patients with MDS may present with dysplasia of their megakaryocyte lineage leading to thrombocytopenia and hemorrhagic complications. Despite these known clinical findings, the molecular mechanisms underlying these features remain poorly understood, and there are few

animal model systems in which to delineate these mechanisms. Previously, Lin and colleagues reported that *NHD13* transgenic mice develop circulating giant platelets, and that expression of *NHD13* impairs differentiation of K562 cells [11]. Here we sought to further define the underlying molecular mechanisms whereby expression of *NHD13* in mice leads to dysmegakaryopoiesis.

Actin is essential throughout the production of platelet formation. Its role begins as early as endomitosis where actin helps in the formation of the acto-myosin contractile ring [47], and then later in the formation of the MK demarcation membrane system [28]. This is followed by elaborate extension and bending of the cytoplasm during proplatelet production culminating in the release of mature platelets into circulation [50]. Finally, mature platelets are dependent on actin during their activation in hemostasis. Macroplatelets were noted in mice prior to the onset of clinical MDS and thrombocytopenia worsened with age and disease progression. These findings led us to evaluate the underlying features of MK from the *NHD13* mice. Several lines of evidence in our study suggest that actin is the putative target of the *NHD13* fusion gene. First, MK from *NHD13* mice had an abnormal DMS as demonstrated by TEM. Development of the DMS is dependent of actin extension, bending, and bifurcation of the cytoplasm to produce the platelet. Secondly, fluorescent microscopy revealed that actin is abnormally dispersed throughout the cytoplasm during MK development compared to its orderly arrangement in wild-type MK. Taken together, with the abnormal DMS, these findings suggest that the fusion gene exerts its action on actin rather early during MK development. This, could have resulted in two possible outcomes: 1) large platelets as were seen in circulation and 2) diminished cytoplasm membranes (this serves as the source material for platelets) thus leading to thrombocytopenia. Third, mobilization of actin is critical for proplatelet formation and terminal production of platelets. Upon stimulation with estradiol, MK undergo proplatelet formation as shown in Figure

9. *NHD13* MK not only failed to produce proplatelets, but they also had reduced actin assembly in the proplatelets that did form; however the proplatelet bifurcation processes needed for correct platelet sizing was absent. Thus, it is reasonable to suggest that Nup98 mutation may impair the nuclear pore integrity leading to aberrant actin cytoskeleton. Another pathway to actin dysregulation is via the RhoA pathway. RhoA is a key regulatory pathway of actin dynamics [161] and its disruption leads to macrothrombocytopenia [162]. Additionally, disruption of n-cofilin, downstream of RhoA, results in larger sized platelets [37]. RhoA cross-talks with Runx1 [163], a gene which regulates many facets of hematopoiesis and is been shown to be frequently mutated in MDS [164, 165]. Taken together, a possible perturbation of RhoA by altered-Runx1 can result in an impaired actin cytoskeleton.

Our data has shown that the dysplastic features in *NHD13* MK may be due in part to aberrant actin localization and polymerization, especially at the proplatelet stage leading to macrothrombocytopenia. These findings warrant additional studies to more accurately determine the molecular mechanisms by which *NHD13* regulates the function of actin in megakaryocyte differentiation and platelet production. Doing so could reveal novel pathways or therapeutic targets useful in the treatment of MDS and other diseases where thrombocytopenia is a significant clinical feature.

Figures

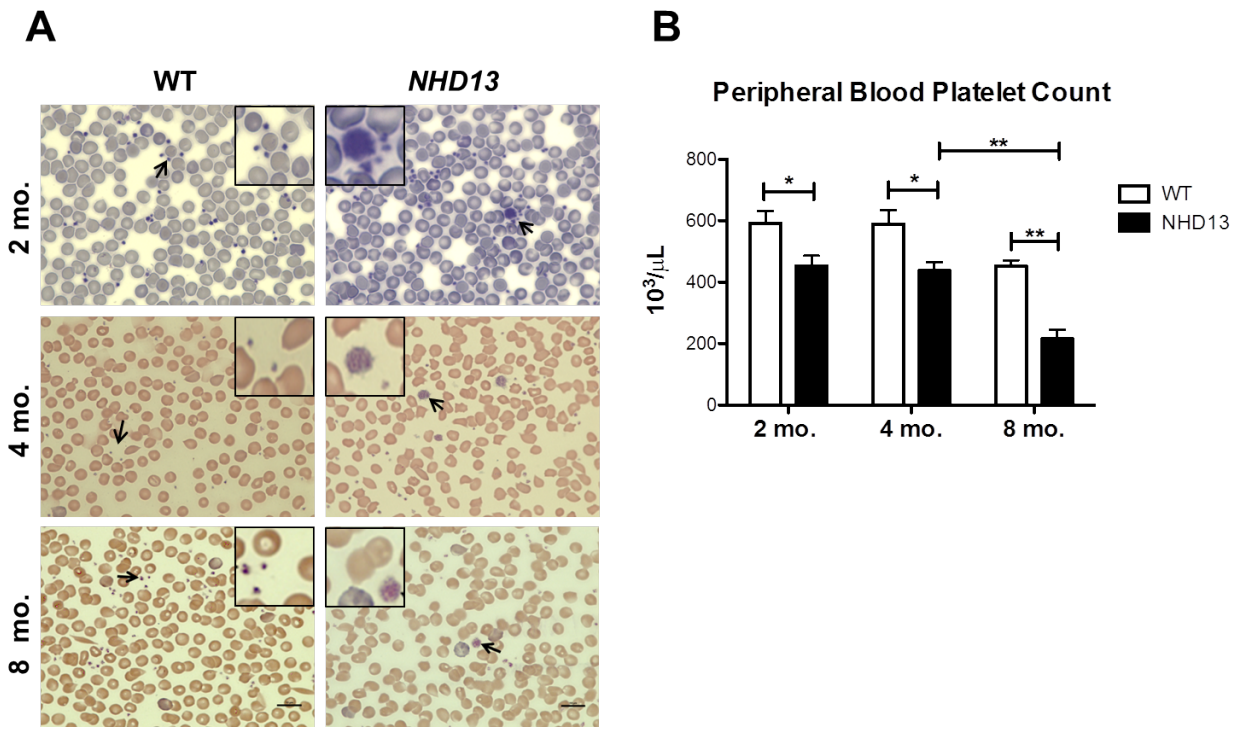

Figure 5: Preclinical and clinical *NHD13* mice are macrothrombocytopenic. (A) Representative blood smears from WT and *NHD13* mice at 2 mo (top panel), 4 mo (middle panel), and 8 mo (lower panel). (B) Statistical analysis between WT and *NHD13* platelet counts. Platelet counts were significantly lower in the transgenic mice at 2 and 4 mo; WT = 592 ± 40 vs NHD13 mice = 453 ± 32 ($p<0.05$); WT = 589 ± 46 vs *NHD13* = 438 ± 27 (*$p<0.05$). *NHD13* mice with clinical disease had platelet counts (214 ± 31) less than half the number of WT mice (452 ± 32) at 8 mo (**$p<0.01$). 100x; Wright-Giemsa

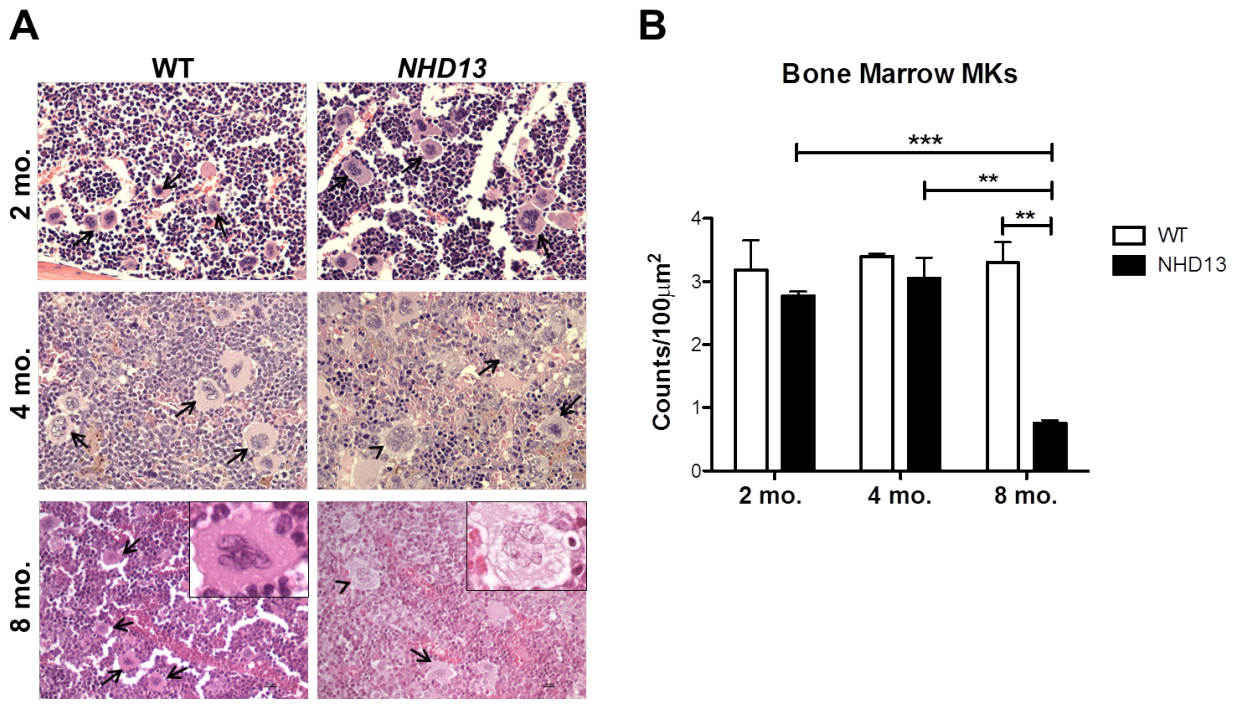

Figure 6: *NHD13* mice with clinical MDS have significantly less MK in BM. (A) Representative sections of BM from WT and *NHD13* mice at 2, 4, and 8 mo. At 2 mo WT and *NHD13* mice had a comparable number and distribution of MK with few dysplastic MK in *NHD13* BM. Dysplastic MK had fragmented nuclei. BM from WT and *NHD13* mice at 4 mo also had an equivalent distribution of MK with similar morphology although there was a tendency for the *NHD13* to have fragmented nuclei. At 8 mo, the MK from WT mice had polylobulated nuclei, small nuclear:cytoplasmic ratios, and were relatively uniform in size and shape (insert). MK from the *NHD13* mice with MDS had large dysplastic MK (insert), fragmented nuclei, and vacuolated cytoplasm. Furthermore, *NHD13* MK had increased nuclear:cytoplasmic ratio. (B) BM MK from WT and NHD13 mice were enumerated from 25 100 μm2 fields. There was no significant difference between WT and *NHD13* at 2 mo (3.19 ± 0.47 vs 2.78 ± 0.06) or 4 mo (3.40 ± 0.06 vs 3.05 ± 0.39). *NHD13* mice with clinical MDS had significantly reduced numbers of MK in their BM compared to WT (0.75 ± 0.04 vs 3.40 ± 0.44, **p<0.01) at 8 mo. Hematoxin and Eosin (H&E, 400x).

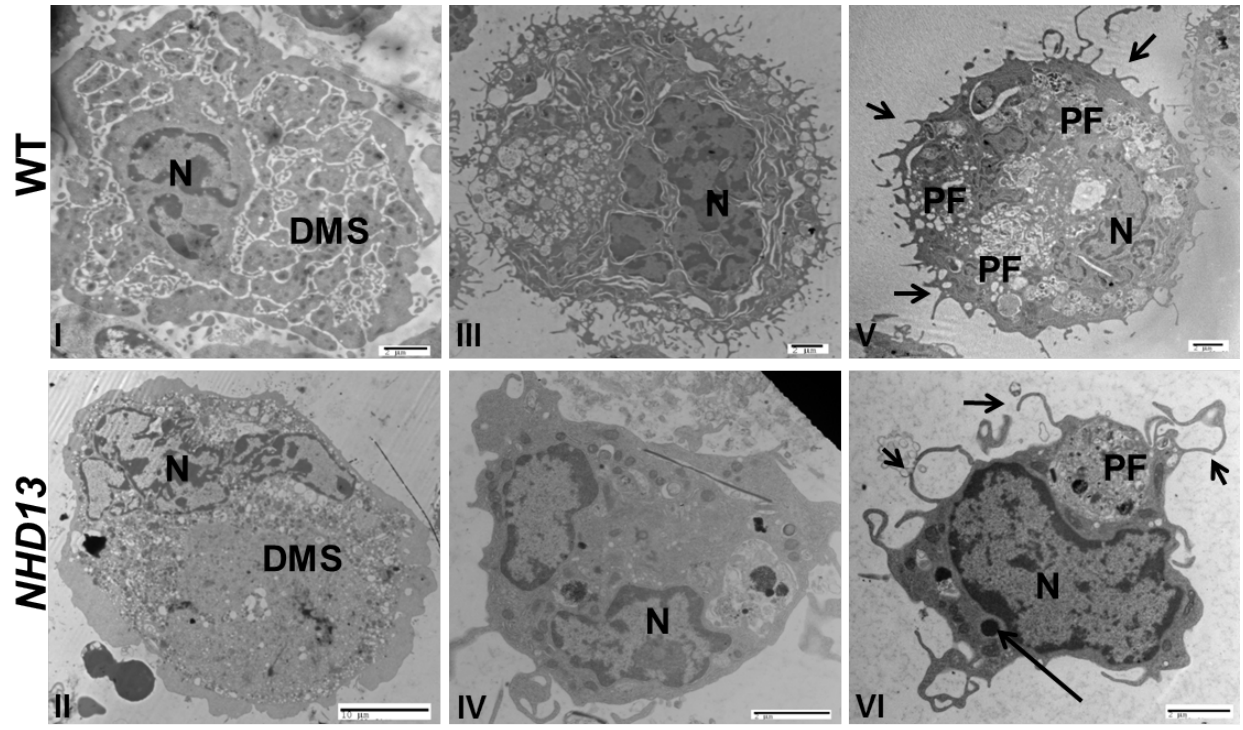

Figure 7: Ultrastructural features of *NHD13* MK. (I and II) Mature WT MK with elaborate partitioning of the cytoplasm forming the demarcation membrane system (DMS). (III) WT MK in the process of proplatelet formation (short arrows). (IV) A mature *NHD13* MK displaying an abnormal DMS in cytoplasm. (V) Mature *NHD13* MK with little demarcation of its cytoplasm. Furthermore, the perinuclear space contained marked granulation and vacuolization, as well as multiple platelet fields (VI) A mature *NHD13* MK with fewer proplatelets extensions (short arrows). Unusually large, but fewer granules were also observed in the cytoplasm of the MK (long arrow). The cytoplasm also had fewer platelet fields compared to control. N = Nucleus, DMS = Demarcation membrane systems, PF = Platelet field.

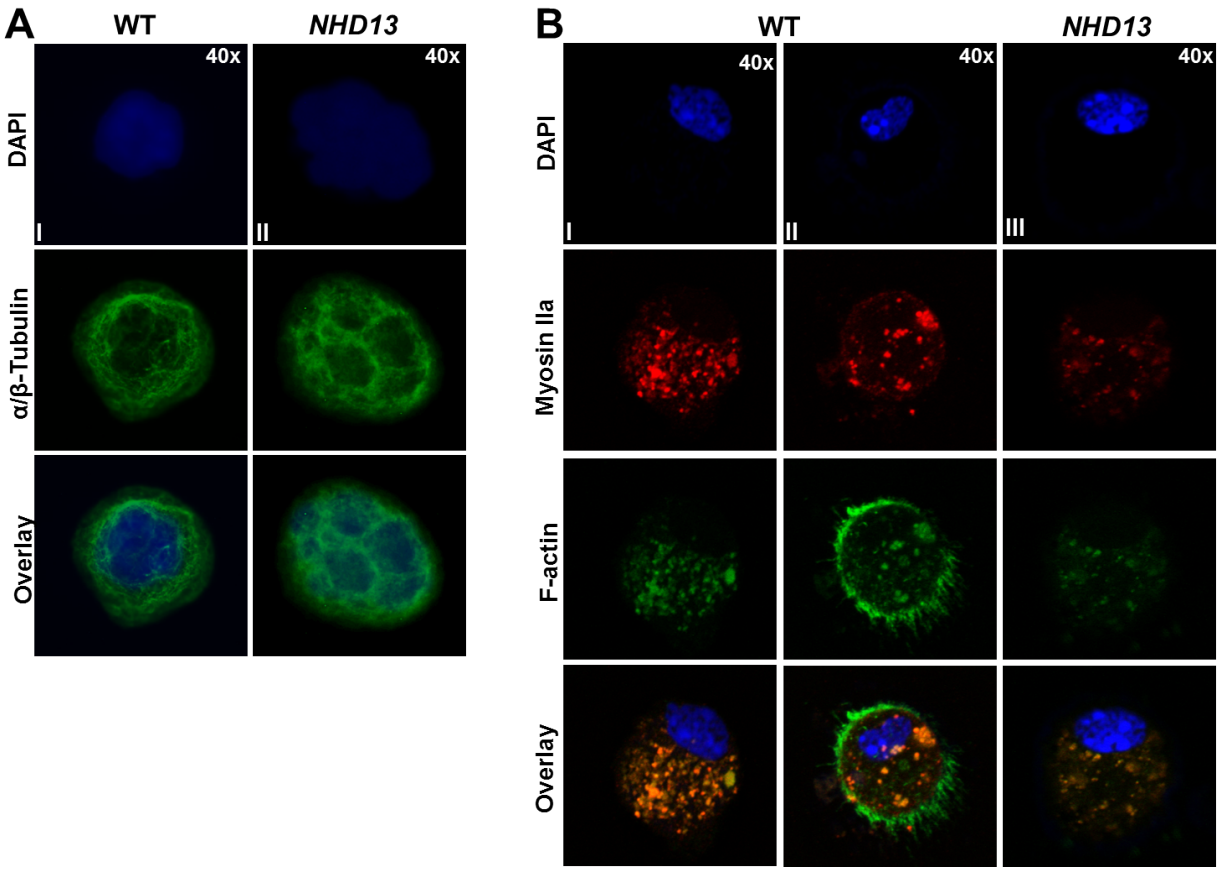

Figure 8: Aberrant actin cytoskeleton in *NHD13* MK. MK were cultured for five days, cytospun onto glass slides and labeled with phalloidin (for F-actin), fluorescently tagged α/β tubulin, or fluorescently tagged myosin IIa and visualized by immunofluorescence microscopy. (A) The α/β tubulin distribution and localization is comparable between in WT and *NHD13* MK. (B) The myosin organization was comparable in WT and *NHD13*. The actin in WT MK was localized heavily in the periphery of the cytoplasm in contrast to the *NHD13* MK that had actin dispersed unevenly throughout the cytoplasm, suggesting that actin localization is impaired in *NHD13* MK. 40x.

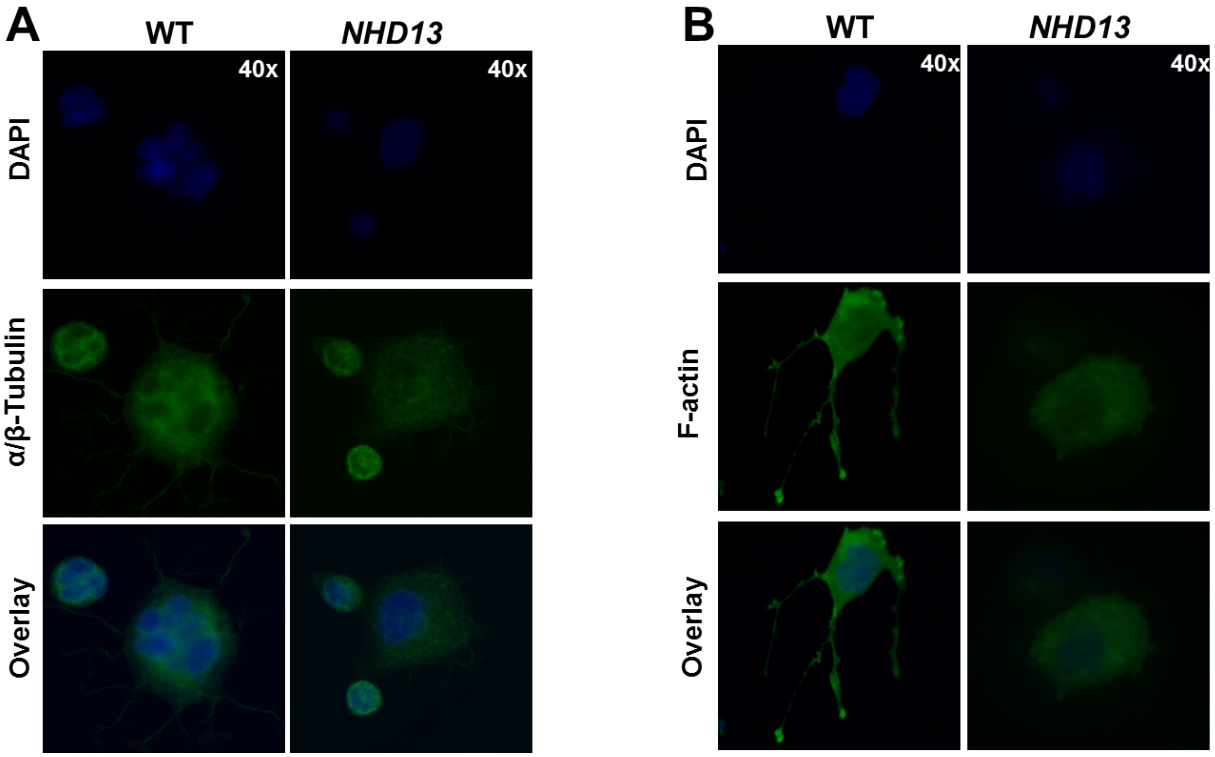

Figure 9: Estrogen-stimulated *NHD13* MK proplatelets have impaired actin architecture. WT and *NHD13* BM progenitor cells were cultured for 5 days in StemSpan supplemented with rTPO and estradiol to induce proplatelet formation. Cytoskeletal architecture of WT and *NHD13* proplatelets was determined after adhering estrogen-stimulated MK to fibrinogen-coated slides for 4 h. MK were labeled with phalloidin (for F-actin) or fluorescently tagged α/β tubulin and visualized by immunofluorescence microscopy. (A) MK from both WT and *NHD13* had comparable α/β tubulin localization within their proplatelet extensions. (B) In contrast, the F-actin in the *NHD13* MK was severely diminished in the cytoplasmic extensions compared to WT control MK. 40x.

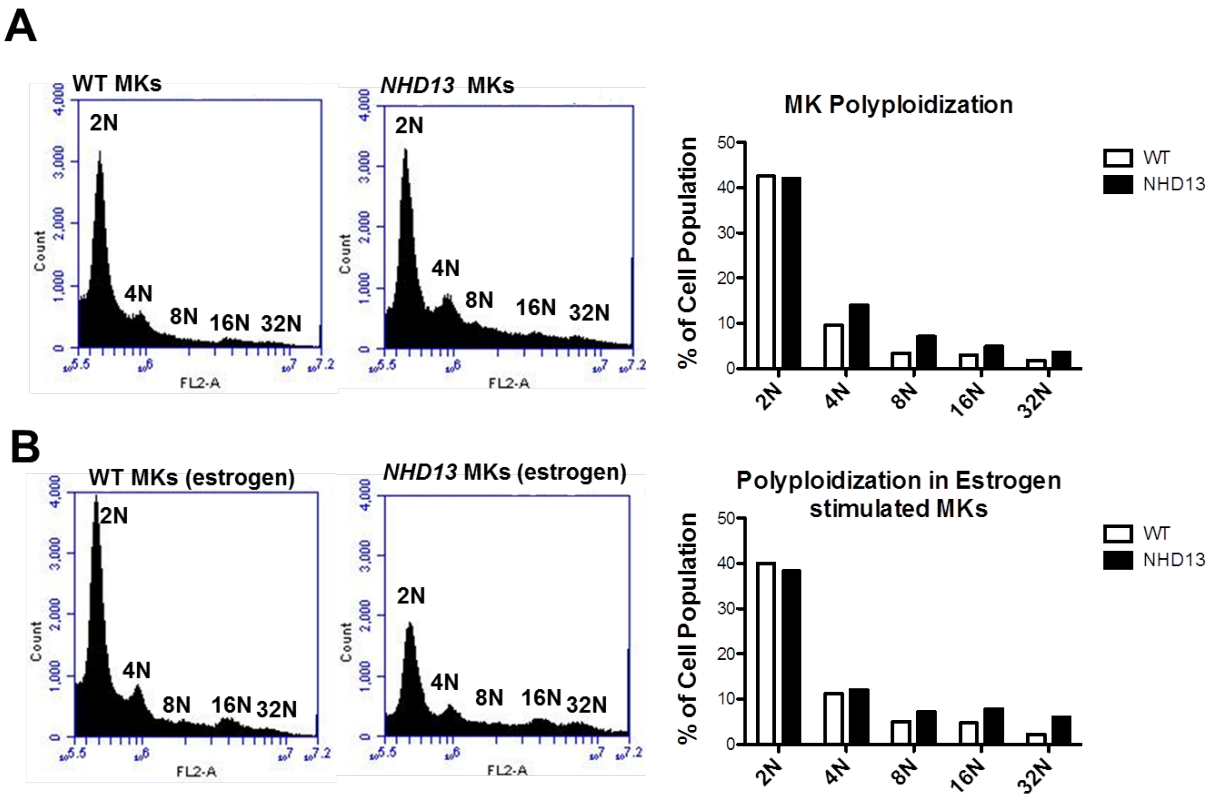

Figure 10: *NHD13* MK have increased average ploidy. WT and *NHD13* BM progenitor cells were cultured for 5 d in StemSpan supplemented with either rTPO only or rTPO, and estrogen to induce proplatelet formation. MK were labeled with anti-CD41, stained with PI, and analyzed by flow cytometry. (A) Flow cytometric analysis of TPO only cultured WT and *NHD13* MK showed that *NHD13* MK had a slight increase in the average ploidy compared to WT MK; 2N(42.1% vs 42.5%), 4N(14.0% vs 9.6%), 8N(7.1% vs 3.4%), 16N(4.9% vs 3.0%), 32N(3.6% vs 1.8%). (B) Flow cytometric analysis of estrogen-stimulated WT and *NHD13* MK showed that the NHD13 MK had a slight increase in the average ploidy when compared to WT MK; 2N(38.3% vs 40.0%), 4N(12.0% vs 11.2%), 8N(7.2% vs 5.0%), 16N(7.8% vs 4.7%), 32N (6.0% vs 2.2%).

Tables

Table 1. Peripheral Blood Characteristics of *NHD13* Mice

Mouse ID	Genotype	WBC ($x10^3/\mu L$)	Gra (g/dl)	Lym ($x10^3/\mu L$)	Hgb ($x10^3/\mu L$)	MCV (fl)	Platelet ($x10^3/\mu L$)
Two-Month-Old Mice							
172	NHD13	4.0	0.5	3.2	15.4	50.9	577
203	NHD13	2.1	0.2	1.6	14.3	50.5	414
207	NHD13	3.1	0.4	2.5	13.9	50.6	443
210	NHD13	4.3	0.8	3.0	13.7	51.7	438
215	NHD13	4.1	0.7	3.1	14.3	52.5	395
174	WT	9.9	0.8	8.7	16.6	49.6	665
212	WT	8.9	1.2	7.1	15.1	51.5	659
214	WT	7.8	0.8	6.4	15.4	51.3	625
216	WT	11.6	1.2	9.7	18.7	51.0	560
223	WT	5.5	0.3	5.0	14.3	51.6	451
Four-Month-Old Mice							
578	NHD13	3.4	0.0	3.2	13.8	40.3	388
579	NHD13	3.7	0.5	2.7	14.8	47.1	446
596	NHD13	3.4	0.0	3.4	14.4	40.8	480
573	WT	8.6	1.3	5.3	17	44.7	670
556	WT	12.6	0.5	5.0	14.5	46.7	510
591	WT	10.2	1.1	8.4	16.3	41.4	587
Eight Month-Old Mice							
597	NHD13	7.4	1.2	5.4	14.0	41.8	237
5243	NHD13	5.46	1.63	2.8	10.9	58.2	153
5274	NHD13	4.92	1.52	0.61	5.4	63.9	245
638	WT	7.8	0.0	7.3	12.8	39.4	443
599	WT	6.7	0.4	6.3	14.6	40.7	426
292	WT	5.0	0.7	3.8	13.7	45.4	489

WT – Wild type, NHD13 – Nup98-HoxD13, WBC = white blood cell, MCV - mean corpuscular volume, Lym - lymphocyte, Gra - granulocyte, Hgb – hemoglobin.

Thesis Summary and Future Direction

A subset of MDS patients have dysplastic MK in their bone marrow, and present with macrothrombocytopenia. The purpose of this research was to investigate these dysplastic MK seen in *NHD13* mice as a model for understanding the phenotype seen in patients with macro thrombocytopenia. The study employed the use of a mouse model for MDS: transgenic mice which express the *NHD13* leukemic fusion recapitulate the key features of MDs including dysplastic MK and macrothrombocytopenia. The objective for the thesis research was to determine the effects of the *NHD13* leukemic fusion gene, on MK development.

Lin and colleagues showed that transgenic mice expressing the *NHD13* fusion gene present with dysplastic MK and macro platelets by seven months of age [11]. In this thesis, further studies on subclinical and clinical mice were performed to determine the effect of MDS on MK development during disease progression, as well as platelet development. *NHD13* mice presented with macro platelets as early as two months of age. *NHD13* mice with clinical disease presented with more macro platelets when compared to subclinical mice and also had a precipitous drop in platelet count. Thus, the progression of disease results in increased macro platelets and thrombocytopenia in *NHD13* mice. MK production and development was studied as a pathway leading to platelet production defects. Although MK count comparison between subclinical WT and *NHD13* mice was not significantly different, *NHD13* BM contained few irregularly shaped MK. The MK also appeared faded with fragmented nuclei. But MK counts reduced drastically with disease progression, as did the propensity of dysplastic MK in clinical *NHD13* mice. The results suggest that *NHD13* fusion gene impairs differentiation and results in dysplastic MK. The results also suggest that dysplastic MK and MK hypoplasia in *NHD13* BM may be the cause of thrombocytopenia in clinical mice.

As the MK progenitor cell develops into a mature MK, the nuclear:cytoplasmic ratio decreases, a DMS forms in the cytoplasm, and extension or proplatelets form from the cytoplasm. The ultrastructural features were studied in cultured MK to determine if the fusion gene perturbs MK development. Dysplastic *NHD13* MK had larger nuclei with increased chromatin condensation. The nuclei were also angular compared to WT MK. These nuclear features are characteristic of apoptotic cells. The chromatin condensation in *NHD13* MK may explain the nuclei fragmentation in *NHD13* BM MK. Dysplastic *NHD13* MK also had little to no DMS formation, fewer proplatelet formations, and fewer but larger dense particles. The results showed that the *NHD13* fusion impairs many facets of MK differentiation. Moreover, the results may explain why some MDS patients develop thrombocytopenia: Fewer MK in BM, impaired the DMS and proplatelet formations can lead to a precipitous drop in platelet production.

Studies show that MK shape, polyploidization, proplatelet formation and release of platelets are partly regulated by the cytoskeleton proteins including actin, myosin, tubulin, dynamin and spectrin. Furthermore, deregulation of such proteins results in platelet disorders including macrothrombocytopenia. Considering the dysplastic features observed, it was reasonable to investigate the cytoskeleton in dysplastic MK as a target of the *NHD13* fusion gene. The hypothesis was that actin dynamics were impaired in *NHD13* transgenic mice leading to impaired DMS and proplatelet formation. The results showed that MK myosin IIa and α/β tubulin cytoskeleton was uncompromised. On the other hand, actin dynamics were hindered during MK maturation. First, actin localization was aberrant in *NHD13* MK. Secondly, *NHD13* MK showed diminutive actin cytoplasmic extensions, normally part of proplatelet architecture. Third, *NHD13* proplatelet bifurcation processes needed for correct platelet sizing was absent. We show here that aberrant actin may be a major contributory factor in macro platelet seen in *NHD13* mice, impaired DMS and proplatelet formation, as well as macro platelet release. Since

actin plays a crucial in the release of uniformly sized platelets, these results may explain the macro platelet feature seen in MDS patients.

These studies were conducted exclusively in primary murine MK to advance the understanding of how MK development is affected during MDS disease progression, and have a better understanding of the facet of the cell perturbed by the *NHD13* fusion gene. The study sheds insight into how *NHD13* mice that model MDS may develop macro thrombocytopenia and these effects are summarized in Figure 11: Either MK get depleted in the BM as disease progresses, DMS formation is impaired, or its proplatelet biology is hindered. We show that perturbed actin cytoskeletal dynamics may be a pathway leading to the clinical presentations of dysplastic MK and defective platelets. Thus, we elaborate the importance of actin in disease, as reported for microtubules and myosin in MK.

Although these studies show that perturbed actin dynamics leads to dysplastic MK in disease, the question left unanswered is how the *NHD13* gene interacts with the actin cytoskeleton in MK. The objective of future studies will be to determine the molecular mechanism(s) by which the *NHD13* gene interacts with and impairs the actin cytoskeleton in MK and this is the question which will be addressed in future studies. The WASp signaling pathways is a key regulator of actin dynamics, Studies suggest that Runx1 may regulates actin cytoskeletal organization via interaction with the WASp [163], which regulate actin cytoskeleton polymerization and organization [166, 167]. Futhermore, research indicates the propensity of Runx1 mutations in many blood malignancies including leukemias [164, 165]. Taken together, it is reasonable to suggest *NHD13* impairs actin dynamics via dysregulation of Runx1. Identification of the novel target of *NHD13* using our model system should reveal potential therapeutic targets in the MK development pathways leading to improved patient care/survival.

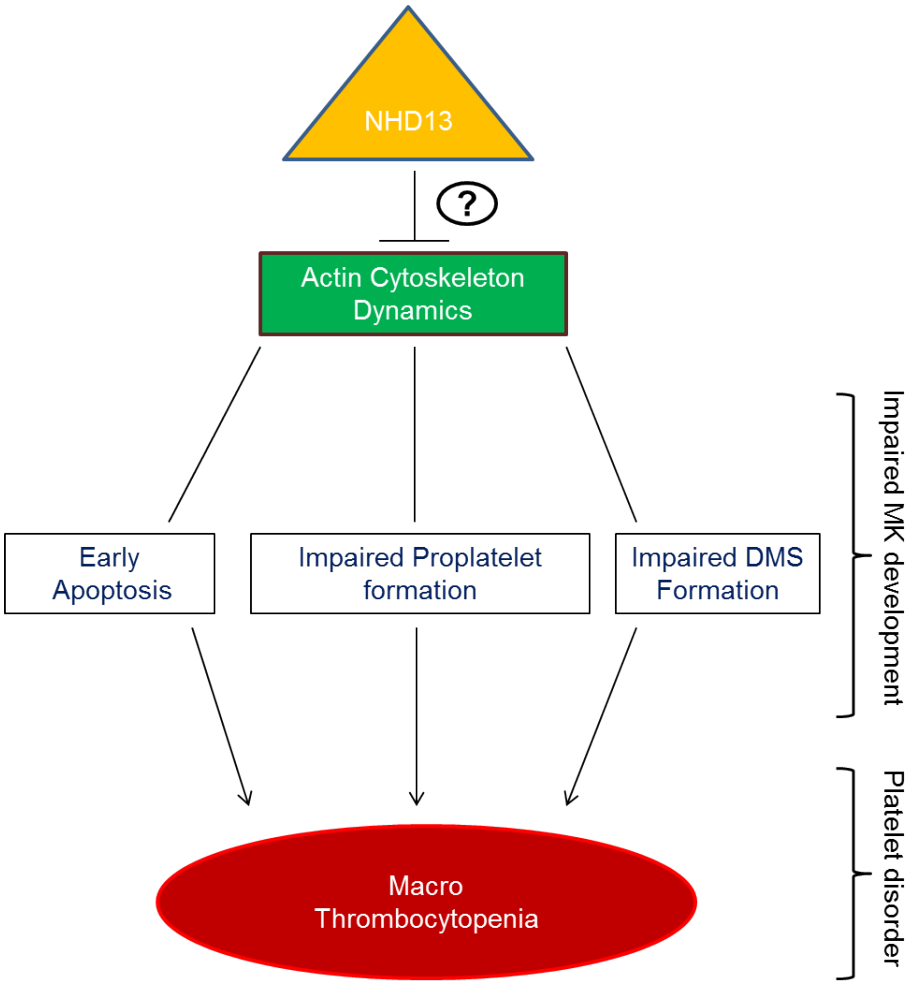

Figure 11: Model for impaired MK development in *NHD13* mice. The studies outlined in this thesis shows how *NHD13* transgenic mice may develop dysplastic MK and macro thrombocytopenia. The results show that macrothrombocytopenia may be a result of impaired megakaryocyte development due to aberrant actin dynamics. Aberrant actin dynamics affects MK DMS and proplatelet formation. Although the actin cytoskeleton is impaired, the mechanism underlying this process is unknown. Future studies will investigate the molecular mechanism(s) by which *NHD13* perturbs actin dynamics. The may lead to the identification of a novel master regulator.

References

1. Rollison DE, Howlader N, Smith MT, Strom SS, Merritt WD, Ries LA*, et al.* Epidemiology of myelodysplastic syndromes and chronic myeloproliferative disorders in the United States, 2001-2004, using data from the NAACCR and SEER programs. *Blood* 2008,**112**:45-52.

2. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A*, et al.* The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood* 2009,**114**:937-951.

3. Lam DH, Aplan PD. NUP98 gene fusions in hematologic malignancies. *Leukemia* 2001,**15**:1689-1695.

4. Slape C, Aplan PD. The role of NUP98 gene fusions in hematologic malignancy. *Leuk Lymphoma* 2004,**45**:1341-1350.

5. Griffis ER, Altan N, Lippincott-Schwartz J, Powers MA. Nup98 is a mobile nucleoporin with transcription-dependent dynamics. *Mol Biol Cell* 2002,**13**:1282-1297.

6. Moore MA, Chung KY, Plasilova M, Schuringa JJ, Shieh JH, Zhou P*, et al.* NUP98 dysregulation in myeloid leukemogenesis. *Ann N Y Acad Sci* 2007,**1106**:114-142.

7. Nakamura T. NUP98 fusion in human leukemia: dysregulation of the nuclear pore and homeodomain proteins. *Int J Hematol* 2005,**82**:21-27.

8. F M, B J, F M. Mitelman database of chromosome aberrations and gene fusions in cancer. In; 2013.

9. Raza-Egilmez SZ, Jani-Sait SN, Grossi M, Higgins MJ, Shows TB, Aplan PD. NUP98-HOXD13 gene fusion in therapy-related acute myelogenous leukemia. *Cancer Res* 1998,**58**:4269-4273.

10. Shimada H, Arai Y, Sekiguchi S, Ishii T, Tanitsu S, Sasaki M. Generation of the NUP98-HOXD13 fusion transcript by a rare translocation, t(2;11)(q31;p15), in a case of infant leukaemia. *Br J Haematol* 2000,**110**:210-213.

11. Lin YW, Slape C, Zhang Z, Aplan PD. NUP98-HOXD13 transgenic mice develop a highly penetrant, severe myelodysplastic syndrome that progresses to acute leukemia. *Blood* 2005,**106**:287-295.

12. Branehog I, Ridell B, Swolin B, Weinfeld A. Megakaryocyte quantifications in relation to thrombokinetics in primary thrombocythaemia and allied diseases. *Scand J Haematol* 1975,**15**:321-332.

13. Harker LA, Finch CA. Thrombokinetics in man. *J Clin Invest* 1969,**48**:963-974.

14. Houwerzijl EJ, Blom NR, van der Want JJ, Vellenga E, de Wolf JT. Megakaryocytic dysfunction in myelodysplastic syndromes and idiopathic thrombocytopenic purpura is in part due to different forms of cell death. *Leukemia* 2006,**20**:1937-1942.

15. Geddis AE. Megakaryopoiesis. *Semin Hematol* 2010,**47**:212-219.

16. Bartley TD, Bogenberger J, Hunt P, Li YS, Lu HS, Martin F*, et al.* Identification and cloning of a megakaryocyte growth and development factor that is a ligand for the cytokine receptor Mpl. *Cell* 1994,**77**:1117-1124.

17. de Sauvage FJ, Hass PE, Spencer SD, Malloy BE, Gurney AL, Spencer SA*, et al.* Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. *Nature* 1994,**369**:533-538.

18. Kaushansky K, Lok S, Holly RD, Broudy VC, Lin N, Bailey MC*, et al.* Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. *Nature* 1994,**369**:568-571.

19. Bunting S, Widmer R, Lipari T, Rangell L, Steinmetz H, Carver-Moore K*, et al.* Normal platelets and megakaryocytes are produced in vivo in the absence of thrombopoietin. *Blood* 1997,**90**:3423-3429.

20. Szalai G, LaRue AC, Watson DK. Molecular mechanisms of megakaryopoiesis. *Cellular and molecular life sciences : CMLS* 2006,**63**:2460-2476.

21. Ichikawa M, Asai T, Saito T, Seo S, Yamazaki I, Yamagata T*, et al.* AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. *Nature medicine* 2004,**10**:299-304.

22. Elagib KE, Racke FK, Mogass M, Khetawat R, Delehanty LL, Goldfarb AN. RUNX1 and GATA-1 coexpression and cooperation in megakaryocytic differentiation. *Blood* 2003,**101**:4333-4341.

23. Huang H, Yu M, Akie TE, Moran TB, Woo AJ, Tu N*, et al.* Differentiation-dependent interactions between RUNX-1 and FLI-1 during megakaryocyte development. *Molecular and cellular biology* 2009,**29**:4103-4115.

24. Nerlov C, Querfurth E, Kulessa H, Graf T. GATA-1 interacts with the myeloid PU.1 transcription factor and represses PU.1-dependent transcription. *Blood* 2000,**95**:2543-2551.

25. Chou ST, Khandros E, Bailey LC, Nichols KE, Vakoc CR, Yao Y*, et al.* Graded repression of PU.1/Sfpi1 gene transcription by GATA factors regulates hematopoietic cell fate. *Blood* 2009,**114**:983-994.

26. Geddis AE. Megakaryopoiesis. *Seminars in hematology* 2010,**47**:212-219.

27. Bluteau D, Lordier L, Di Stefano A, Chang Y, Raslova H, Debili N*, et al.* Regulation of megakaryocyte maturation and platelet formation. *Journal of thrombosis and haemostasis : JTH* 2009,**7 Suppl 1**:227-234.

28. Schulze H, Korpal M, Hurov J, Kim SW, Zhang J, Cantley LC*, et al.* Characterization of the megakaryocyte demarcation membrane system and its role in thrombopoiesis. *Blood* 2006,**107**:3868-3875.

29. Behnke O. An electron microscope study of the megacaryocyte of the rat bone marrow. I. The development of the demarcation membrane system and the platelet surface coat. *J Ultrastruct Res* 1968,**24**:412-433.

30. Shaklai M, Tavassoli M. Demarcation membrane system in rat megakaryocyte and the mechanism of platelet formation: a membrane reorganization process. *J Ultrastruct Res* 1978,**62**:270-285.

31. Mahaut-Smith MP, Thomas D, Higham AB, Usher-Smith JA, Hussain JF, Martinez-Pinna J*, et al.* Properties of the demarcation membrane system in living rat megakaryocytes. *Biophys J* 2003,**84**:2646-2654.

32. Wickrema A, Crispino JD. Erythroid and megakaryocytic transformation. *Oncogene* 2007,**26**:6803-6815.

33. Yang H, Ganguly A, Yin S, Cabral F. Megakaryocyte lineage-specific class VI beta-tubulin suppresses microtubule dynamics, fragments microtubules, and blocks cell division. *Cytoskeleton* 2011,**68**:175-187.

34. Wang W, Gilligan DM, Sun S, Wu X, Reems JA. Distinct Functional Effects for Dynamin 3 During Megakaryocytopoiesis. *Stem cells and development* 2011.

35. Lordier L, Jalil A, Aurade F, Larbret F, Larghero J, Debili N*, et al.* Megakaryocyte endomitosis is a failure of late cytokinesis related to defects in the contractile ring and Rho/Rock signaling. *Blood* 2008,**112**:3164-3174.

36. Chang Y, Aurade F, Larbret F, Zhang Y, Le Couedic JP, Momeux L*, et al.* Proplatelet formation is regulated by the Rho/ROCK pathway. *Blood* 2007,**109**:4229-4236.

37. Bender M, Eckly A, Hartwig JH, Elvers M, Pleines I, Gupta S*, et al.* ADF/n-cofilin-dependent actin turnover determines platelet formation and sizing. *Blood* 2010,**116**:1767-1775.

38. Amano M, Nakayama M, Kaibuchi K. Rho-kinase/ROCK: A key regulator of the cytoskeleton and cell polarity. *Cytoskeleton* 2010,**67**:545-554.

39. Raftopoulou M, Hall A. Cell migration: Rho GTPases lead the way. *Developmental biology* 2004,**265**:23-32.

40. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. *Nature* 2002,**420**:629-635.

41. Schwartz M. Rho signalling at a glance. *Journal of cell science* 2004,**117**:5457-5458.

42. Bamburg JR, McGough A, Ono S. Putting a new twist on actin: ADF/cofilins modulate actin dynamics. *Trends in cell biology* 1999,**9**:364-370.

43. Meberg PJ, Ono S, Minamide LS, Takahashi M, Bamburg JR. Actin depolymerizing factor and cofilin phosphorylation dynamics: response to signals that regulate neurite extension. *Cell motility and the cytoskeleton* 1998,**39**:172-190.

44. Maekawa M, Ishizaki T, Boku S, Watanabe N, Fujita A, Iwamatsu A*, et al.* Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* 1999,**285**:895-898.

45. Kawano Y, Fukata Y, Oshiro N, Amano M, Nakamura T, Ito M*, et al.* Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase in vivo. *J Cell Biol* 1999,**147**:1023-1038.

46. Kureishi Y, Kobayashi S, Amano M, Kimura K, Kanaide H, Nakano T*, et al.* Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. *J Biol Chem* 1997,**272**:12257-12260.

47. Geddis AE, Kaushansky K. Endomitotic megakaryocytes form a midzone in anaphase but have a deficiency in cleavage furrow formation. *Cell Cycle* 2006,**5**:538-545.

48. Logan MR, Mandato CA. Regulation of the actin cytoskeleton by PIP2 in cytokinesis. *Biol Cell* 2006,**98**:377-388.

49. Italiano JE, Jr., Patel-Hett S, Hartwig JH. Mechanics of proplatelet elaboration. *Journal of thrombosis and haemostasis : JTH* 2007,**5 Suppl 1**:18-23.

50. Italiano JE, Jr., Lecine P, Shivdasani RA, Hartwig JH. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol* 1999,**147**:1299-1312.

51. Tablin F, Castro M, Leven RM. Blood platelet formation in vitro. The role of the cytoskeleton in megakaryocyte fragmentation. *J Cell Sci* 1990,**97 (Pt 1)**:59-70.

52. Mermall V, Post PL, Mooseker MS. Unconventional myosins in cell movement, membrane traffic, and signal transduction. *Science* 1998,**279**:527-533.

53. Sellers JR. Unphosphorylated crossbridges and latch: smooth muscle regulation revisited. *J Muscle Res Cell Motil* 1999,**20**:347-349.

54. Hodge T, Cope MJ. A myosin family tree. *J Cell Sci* 2000,**113 Pt 19**:3353-3354.

55. Saez CG, Myers JC, Shows TB, Leinwand LA. Human nonmuscle myosin heavy chain mRNA: generation of diversity through alternative polyadenylylation. *Proc Natl Acad Sci U S A* 1990,**87**:1164-1168.

56. Simons M, Wang M, McBride OW, Kawamoto S, Yamakawa K, Gdula D*, et al.* Human nonmuscle myosin heavy chains are encoded by two genes located on different chromosomes. *Circ Res* 1991,**69**:530-539.

57. Marigo V, Nigro A, Pecci A, Montanaro D, Di Stazio M, Balduini CL*, et al.* Correlation between the clinical phenotype of MYH9-related disease and tissue distribution of class II nonmuscle myosin heavy chains. *Genomics* 2004,**83**:1125-1133.

58. Toothaker LE, Gonzalez DA, Tung N, Lemons RS, Le Beau MM, Arnaout MA*, et al.* Cellular myosin heavy chain in human leukocytes: isolation of 5' cDNA clones, characterization of the protein, chromosomal localization, and upregulation during myeloid differentiation. *Blood* 1991,**78**:1826-1833.

59. Fox JE, Phillips DR. Role of phosphorylation in mediating the association of myosin with the cytoskeletal structures of human platelets. *J Biol Chem* 1982,**257**:4120-4126.

60. Kumar CC, Mohan SR, Zavodny PJ, Narula SK, Leibowitz PJ. Characterization and differential expression of human vascular smooth muscle myosin light chain 2 isoform in nonmuscle cells. *Biochemistry* 1989,**28**:4027-4035.

61. Paul BZ, Daniel JL, Kunapuli SP. Platelet shape change is mediated by both calcium-dependent and -independent signaling pathways. Role of p160 Rho-associated coiled-coil-containing protein kinase in platelet shape change. *J Biol Chem* 1999,**274**:28293-28300.

62. Suzuki Y, Yamamoto M, Wada H, Ito M, Nakano T, Sasaki Y*, et al.* Agonist-induced regulation of myosin phosphatase activity in human platelets through activation of Rho-kinase. *Blood* 1999,**93**:3408-3417.

63. Berg JS, Powell BC, Cheney RE. A millennial myosin census. *Mol Biol Cell* 2001,**12**:780-794.

64. Nagata Y, Muro Y, Todokoro K. Thrombopoietin-induced polyploidization of bone marrow megakaryocytes is due to a unique regulatory mechanism in late mitosis. *J Cell Biol* 1997,**139**:449-457.

65. Vitrat N, Cohen-Solal K, Pique C, Le Couedic JP, Norol F, Larsen AK*, et al.* Endomitosis of human megakaryocytes are due to abortive mitosis. *Blood* 1998,**91**:3711-3723.

66. Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T*, et al.* Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem* 1996,**271**:20246-20249.

67. Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M*, et al.* Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* 1996,**273**:245-248.

68. Sebbagh M, Renvoize C, Hamelin J, Riche N, Bertoglio J, Breard J. Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. *Nat Cell Biol* 2001,**3**:346-352.

69. Bauer M, Retzer M, Wilde JI, Maschberger P, Essler M, Aepfelbacher M*, et al.* Dichotomous regulation of myosin phosphorylation and shape change by Rho-kinase and calcium in intact human platelets. *Blood* 1999,**94**:1665-1672.

70. Nakai K, Suzuki Y, Kihira H, Wada H, Fujioka M, Ito M*, et al.* Regulation of myosin phosphatase through phosphorylation of the myosin-binding subunit in platelet activation. *Blood* 1997,**90**:3936-3942.

71. Watanabe Y, Ito M, Kataoka Y, Wada H, Koyama M, Feng J*, et al.* Protein kinase C-catalyzed phosphorylation of an inhibitory phosphoprotein of myosin phosphatase is involved in human platelet secretion. *Blood* 2001,**97**:3798-3805.

72. Jongmans MC, Kuiper RP, Carmichael CL, Wilkins EJ, Dors N, Carmagnac A*, et al.* Novel RUNX1 mutations in familial platelet disorder with enhanced risk for acute myeloid leukemia: clues for improved identification of the FPD/AML syndrome. *Leukemia* 2010,**24**:242-246.

73. Owen C. Insights into familial platelet disorder with propensity to myeloid malignancy (FPD/AML). *Leuk Res* 2010,**34**:141-142.

74. Bluteau D, Glembotsky AC, Raimbault A, Balayn N, Gilles L, Rameau P*, et al.* Dysmegakaryopoiesis of FPD/AML pedigrees with constitutional RUNX1 mutations is linked to myosin II deregulated expression. *Blood* 2012,**120**:2708-2718.

75. Lordier L, Bluteau D, Jalil A, Legrand C, Pan J, Rameau P*, et al.* RUNX1-induced silencing of non-muscle myosin heavy chain IIB contributes to megakaryocyte polyploidization. *Nat Commun* 2012,**3**:717.

76. Desai A, Mitchison TJ. Microtubule polymerization dynamics. *Annu Rev Cell Dev Biol* 1997,**13**:83-117.

77. Nogales E, Wolf SG, Downing KH. Structure of the alpha beta tubulin dimer by electron crystallography. *Nature* 1998,**391**:199-203.

78. Kline-Smith SL, Walczak CE. Mitotic spindle assembly and chromosome segregation: refocusing on microtubule dynamics. *Mol Cell* 2004,**15**:317-327.

79. Kwon M, Scholey JM. Spindle mechanics and dynamics during mitosis in Drosophila. *Trends Cell Biol* 2004,**14**:194-205.

80. Rieder CL, Davison EA, Jensen LC, Cassimeris L, Salmon ED. Oscillatory movements of monooriented chromosomes and their position relative to the spindle pole result from the ejection properties of the aster and half-spindle. *J Cell Biol* 1986,**103**:581-591.

81. Higuchi T, Uhlmann F. Stabilization of microtubule dynamics at anaphase onset promotes chromosome segregation. *Nature* 2005,**433**:171-176.

82. Rieder CL, Schultz A, Cole R, Sluder G. Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. *J Cell Biol* 1994,**127**:1301-1310.

83. Handagama PJ, Feldman BF, Jain NC, Farver TB, Kono CS. In vitro platelet release by rat megakaryocytes: effect of metabolic inhibitors and cytoskeletal disrupting agents. *Am J Vet Res* 1987,**48**:1142-1146.

84. Radley JM, Haller CJ. The demarcation membrane system of the megakaryocyte: a misnomer? *Blood* 1982,**60**:213-219.

85. Italiano JE, Jr., Bergmeier W, Tiwari S, Falet H, Hartwig JH, Hoffmeister KM*, et al.* Mechanisms and implications of platelet discoid shape. *Blood* 2003,**101**:4789-4796.

86. Schwer HD, Lecine P, Tiwari S, Italiano JE, Jr., Hartwig JH, Shivdasani RA. A lineage-restricted and divergent beta-tubulin isoform is essential for the biogenesis, structure and function of blood platelets. *Curr Biol* 2001,**11**:579-586.

87. Patel SR, Richardson JL, Schulze H, Kahle E, Galjart N, Drabek K*, et al.* Differential roles of microtubule assembly and sliding in proplatelet formation by megakaryocytes. *Blood* 2005,**106**:4076-4085.

88. Cowan SM, Bartges JW, Gompf RE, Hayes JR, Moyers TD, Snider CC*, et al.* Giant platelet disorder in the Cavalier King Charles Spaniel. *Exp Hematol* 2004,**32**:344-350.

89. Pedersen HD, Haggstrom J, Olsen LH, Christensen K, Selin A, Burmeister ML*, et al.* Idiopathic asymptomatic thrombocytopenia in Cavalier King Charles Spaniels is an autosomal recessive trait. *J Vet Intern Med* 2002,**16**:169-173.

90. Singh MK, Lamb WA. Idiopathic thrombocytopenia in Cavalier King Charles Spaniels. *Aust Vet J* 2005,**83**:700-703.

91. Davis B, Toivio-Kinnucan M, Schuller S, Boudreaux MK. Mutation in beta1-tubulin correlates with macrothrombocytopenia in Cavalier King Charles Spaniels. *J Vet Intern Med* 2008,**22**:540-545.

92. Shpetner HS, Vallee RB. Identification of dynamin, a novel mechanochemical enzyme that mediates interactions between microtubules. *Cell* 1989,**59**:421-432.

93. McNiven MA, Kim L, Krueger EW, Orth JD, Cao H, Wong TW. Regulated interactions between dynamin and the actin-binding protein cortactin modulate cell shape. *J Cell Biol* 2000,**151**:187-198.

94. Ochoa GC, Slepnev VI, Neff L, Ringstad N, Takei K, Daniell L*, et al.* A functional link between dynamin and the actin cytoskeleton at podosomes. *J Cell Biol* 2000,**150**:377-389.

95. Kruchten AE, McNiven MA. Dynamin as a mover and pincher during cell migration and invasion. *J Cell Sci* 2006,**119**:1683-1690.

96. Perrais D, Merrifield CJ. Dynamics of endocytic vesicle creation. *Dev Cell* 2005,**9**:581-592.

97. Gold ES, Underhill DM, Morrissette NS, Guo J, McNiven MA, Aderem A. Dynamin 2 is required for phagocytosis in macrophages. *J Exp Med* 1999,**190**:1849-1856.

98. Merrifield CJ, Feldman ME, Wan L, Almers W. Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits. *Nat Cell Biol* 2002,**4**:691-698.

99. Thompson HM, Skop AR, Euteneuer U, Meyer BJ, McNiven MA. The large GTPase dynamin associates with the spindle midzone and is required for cytokinesis. *Curr Biol* 2002,**12**:2111-2117.

100. Obar RA, Collins CA, Hammarback JA, Shpetner HS, Vallee RB. Molecular cloning of the microtubule-associated mechanochemical enzyme dynamin reveals homology with a new family of GTP-binding proteins. *Nature* 1990,**347**:256-261.

101. Achiriloaie M, Barylko B, Albanesi JP. Essential role of the dynamin pleckstrin homology domain in receptor-mediated endocytosis. *Mol Cell Biol* 1999,**19**:1410-1415.

102. Barylko B, Binns D, Lin KM, Atkinson MA, Jameson DM, Yin HL*, et al.* Synergistic activation of dynamin GTPase by Grb2 and phosphoinositides. *J Biol Chem* 1998,**273**:3791-3797.

103. Klein DE, Lee A, Frank DW, Marks MS, Lemmon MA. The pleckstrin homology domains of dynamin isoforms require oligomerization for high affinity phosphoinositide binding. *J Biol Chem* 1998,**273**:27725-27733.

104. Cremona O, De Camilli P. Phosphoinositides in membrane traffic at the synapse. *J Cell Sci* 2001,**114**:1041-1052.

105. Hinshaw JE. Dynamin and its role in membrane fission. *Annu Rev Cell Dev Biol* 2000,**16**:483-519.

106. Schmid SL, McNiven MA, De Camilli P. Dynamin and its partners: a progress report. *Curr Opin Cell Biol* 1998,**10**:504-512.

107. Eckly A, Rinckel JY, Laeuffer P, Cazenave JP, Lanza F, Gachet C*, et al.* Proplatelet formation deficit and megakaryocyte death contribute to thrombocytopenia in Myh9 knockout mice. *J Thromb Haemost* 2010,**8**:2243-2251.

108. Eckly A, Strassel C, Freund M, Cazenave JP, Lanza F, Gachet C*, et al.* Abnormal megakaryocyte morphology and proplatelet formation in mice with megakaryocyte-restricted MYH9 inactivation. *Blood* 2009,**113**:3182-3189.

109. Reems JA, Wang W, Tsubata K, Abdurrahman N, Sundell B, Tijssen MR*, et al.* Dynamin 3 participates in the growth and development of megakaryocytes. *Exp Hematol* 2008,**36**:1714-1727.

110. Wang W, Gilligan DM, Sun S, Wu X, Reems JA. Distinct functional effects for dynamin 3 during megakaryocytopoiesis. *Stem Cells Dev* 2011,**20**:2139-2151.

111. Nurnberg ST, Rendon A, Smethurst PA, Paul DS, Voss K, Thon JN*, et al.* A GWAS sequence variant for platelet volume marks an alternative DNM3 promoter in megakaryocytes near a MEIS1 binding site. *Blood* 2012,**120**:4859-4868.

112. Mirchandani I, Palutke M. Acute megakaryoblastic Leukemia. *Cancer* 1982,**50**:2866-2872.

113. Wolgast LR, Cannizzarro LA, Ramesh KH, Xue X, Wang D, Bhattacharyya PK*, et al.* Spectrin isoforms: differential expression in normal hematopoiesis and alterations in neoplastic bone marrow disorders. *Am J Clin Pathol* 2011,**136**:300-308.

114. Patel-Hett S, Wang H, Begonja AJ, Thon JN, Alden EC, Wandersee NJ*, et al.* The spectrin-based membrane skeleton stabilizes mouse megakaryocyte membrane systems and is essential for proplatelet and platelet formation. *Blood* 2011,**118**:1641-1652.

115. Nimer SD. Myelodysplastic syndromes. *Blood* 2008,**111**:4841-4851.

116. Disperati P, Ichim CV, Tkachuk D, Chun K, Schuh AC, Wells RA. Progression of myelodysplasia to acute lymphoblastic leukaemia: implications for disease biology. *Leukemia research* 2006,**30**:233-239.

117. Komrokji R, Bennett JM. The myelodysplastic syndromes: classification and prognosis. *Current hematology reports* 2003,**2**:179-185.

118. Heaney ML, Golde DW. Myelodysplasia. *The New England journal of medicine* 1999,**340**:1649-1660.

119. Vardiman JW. The World Health Organization (WHO) classification of tumors of the hematopoietic and lymphoid tissues: an overview with emphasis on the myeloid neoplasms. *Chemico-biological interactions* 2010,**184**:16-20.

120. Aul C, Germing U, Gattermann N, Minning H. Increasing incidence of myelodysplastic syndromes: real or fictitious? *Leukemia research* 1998,**22**:93-100.

121. Pedersen-Bjergaard J, Timshel S, Andersen MK, Andersen AS, Philip P. Cytogenetically unrelated clones in therapy-related myelodysplasia and acute myeloid leukemia:

experience from the Copenhagen series updated to 180 consecutive cases. *Genes, chromosomes & cancer* 1998,**23**:337-349.

122. Germing U, Hildebrandt B, Pfeilstocker M, Nosslinger T, Valent P, Fonatsch C*, et al.* Refinement of the international prognostic scoring system (IPSS) by including LDH as an additional prognostic variable to improve risk assessment in patients with primary myelodysplastic syndromes (MDS). *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 2005,**19**:2223-2231.

123. Nolte F, Hofmann WK. Myelodysplastic syndromes: molecular pathogenesis and genomic changes. *Annals of hematology* 2008,**87**:777-795.

124. Lam DH, Aplan PD. NUP98 gene fusions in hematologic malignancies. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 2001,**15**:1689-1695.

125. Slape C, Aplan PD. The role of NUP98 gene fusions in hematologic malignancy. *Leukemia & lymphoma* 2004,**45**:1341-1350.

126. Rout MP, Aitchison JD, Suprapto A, Hjertaas K, Zhao Y, Chait BT. The yeast nuclear pore complex: composition, architecture, and transport mechanism. *J Cell Biol* 2000,**148**:635-651.

127. Griffis ER, Altan N, Lippincott-Schwartz J, Powers MA. Nup98 is a mobile nucleoporin with transcription-dependent dynamics. *Molecular biology of the cell* 2002,**13**:1282-1297.

128. Frosst P, Guan T, Subauste C, Hahn K, Gerace L. Tpr is localized within the nuclear basket of the pore complex and has a role in nuclear protein export. *J Cell Biol* 2002,**156**:617-630.

129. Griffis ER, Xu S, Powers MA. Nup98 localizes to both nuclear and cytoplasmic sides of the nuclear pore and binds to two distinct nucleoporin subcomplexes. *Mol Biol Cell* 2003,**14**:600-610.

130. Radu A, Moore MS, Blobel G. The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex. *Cell* 1995,**81**:215-222.

131. Riparbelli MG, Gigliotti S, Callaini G. The Drosophila nucleoporin gene nup154 is required for correct microfilament dynamics and cell death during oogenesis. *Cell Motil Cytoskeleton* 2007,**64**:590-604.

132. Wu X, Kasper LH, Mantcheva RT, Mantchev GT, Springett MJ, van Deursen JM. Disruption of the FG nucleoporin NUP98 causes selective changes in nuclear pore complex stoichiometry and function. *Proceedings of the National Academy of Sciences of the United States of America* 2001,**98**:3191-3196.

133. von Kobbe C, van Deursen JM, Rodrigues JP, Sitterlin D, Bachi A, Wu X*, et al.* Vesicular stomatitis virus matrix protein inhibits host cell gene expression by targeting the nucleoporin Nup98. *Molecular cell* 2000,**6**:1243-1252.

134. Satterly N, Tsai PL, van Deursen J, Nussenzveig DR, Wang Y, Faria PA*, et al.* Influenza virus targets the mRNA export machinery and the nuclear pore complex. *Proceedings of the National Academy of Sciences of the United States of America* 2007,**104**:1853-1858.

135. Park N, Katikaneni P, Skern T, Gustin KE. Differential targeting of nuclear pore complex proteins in poliovirus-infected cells. *Journal of virology* 2008,**82**:1647-1655.

136. Jeganathan KB, Malureanu L, van Deursen JM. The Rae1-Nup98 complex prevents aneuploidy by inhibiting securin degradation. *Nature* 2005,**438**:1036-1039.

137. Orjalo AV, Arnaoutov A, Shen Z, Boyarchuk Y, Zeitlin SG, Fontoura B*, et al.* The Nup107-160 nucleoporin complex is required for correct bipolar spindle assembly. *Molecular biology of the cell* 2006,**17**:3806-3818.

138. Mendjan S, Taipale M, Kind J, Holz H, Gebhardt P, Schelder M*, et al.* Nuclear pore components are involved in the transcriptional regulation of dosage compensation in Drosophila. *Molecular cell* 2006,**21**:811-823.

139. Hou C, Corces VG. Nups take leave of the nuclear envelope to regulate transcription. *Cell* 2010,**140**:306-308.

140. Moore MA, Chung KY, Plasilova M, Schuringa JJ, Shieh JH, Zhou P*, et al.* NUP98 dysregulation in myeloid leukemogenesis. *Annals of the New York Academy of Sciences* 2007,**1106**:114-142.

141. Nakamura T. NUP98 fusion in human leukemia: dysregulation of the nuclear pore and homeodomain proteins. *International journal of hematology* 2005,**82**:21-27.

142. Borrow J, Shearman AM, Stanton VP, Jr., Becher R, Collins T, Williams AJ*, et al.* The t(7;11)(p15;p15) translocation in acute myeloid leukaemia fuses the genes for nucleoporin NUP98 and class I homeoprotein HOXA9. *Nat Genet* 1996,**12**:159-167.

143. Nakamura T, Largaespada DA, Lee MP, Johnson LA, Ohyashiki K, Toyama K*, et al.* Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukaemia. *Nat Genet* 1996,**12**:154-158.

144. Kantarjian H, Giles F, List A, Lyons R, Sekeres MA, Pierce S*, et al.* The incidence and impact of thrombocytopenia in myelodysplastic syndromes. *Cancer* 2007,**109**:1705-1714.

145. Widell S, Hast R. Balloon-like platelets in myelodysplastic syndromes--a feature of dysmegakaryopoiesis? *Leuk Res* 1987,**11**:747-752.

146. Chatel G, Desai SH, Mattheyses AL, Powers MA, Fahrenkrog B. Domain topology of nucleoporin Nup98 within the nuclear pore complex. *J Struct Biol* 2012,**177**:81-89.

147. Heaney ML, Golde DW. Myelodysplasia. *N Engl J Med* 1999,**340**:1649-1660.

148. Komrokji R, Bennett JM. The myelodysplastic syndromes: classification and prognosis. *Curr Hematol Rep* 2003,**2**:179-185.

149. Disperati P, Ichim CV, Tkachuk D, Chun K, Schuh AC, Wells RA. Progression of myelodysplasia to acute lymphoblastic leukaemia: implications for disease biology. *Leuk Res* 2006,**30**:233-239.

150. Nolte F, Hofmann WK. Myelodysplastic syndromes: molecular pathogenesis and genomic changes. *Ann Hematol* 2008,**87**:777-795.

151. Nagata Y, Nagahisa H, Aida Y, Okutomi K, Nagasawa T, Todokoro K. Thrombopoietin induces megakaryocyte differentiation in hematopoietic progenitor FDC-P2 cells. *J Biol Chem* 1995,**270**:19673-19675.

152. Ichikawa M, Asai T, Saito T, Seo S, Yamazaki I, Yamagata T*, et al.* AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. *Nat Med* 2004,**10**:299-304.

153. Lecine P, Villeval JL, Vyas P, Swencki B, Xu Y, Shivdasani RA. Mice lacking transcription factor NF-E2 provide in vivo validation of the proplatelet model of thrombocytopoiesis and show a platelet production defect that is intrinsic to megakaryocytes. *Blood* 1998,**92**:1608-1616.

154. Pencovich N, Jaschek R, Tanay A, Groner Y. Dynamic combinatorial interactions of RUNX1 and cooperating partners regulates megakaryocytic differentiation in cell line models. *Blood* 2011,**117**:e1-14.

155. Shivdasani RA. The role of transcription factor NF-E2 in megakaryocyte maturation and platelet production. *Stem Cells* 1996,**14 Suppl 1**:112-115.

156. Goldfarb AN. Transcriptional control of megakaryocyte development. *Oncogene* 2007,**26**:6795-6802.

157. Shin JW, Swift J, Spinler KR, Discher DE. Myosin-II inhibition and soft 2D matrix maximize multinucleation and cellular projections typical of platelet-producing megakaryocytes. *Proc Natl Acad Sci U S A* 2011,**108**:11458-11463.

158. Thon JN, Montalvo A, Patel-Hett S, Devine MT, Richardson JL, Ehrlicher A*, et al.* Cytoskeletal mechanics of proplatelet maturation and platelet release. *J Cell Biol* 2010,**191**:861-874.

159. Shivdasani RA, Schulze H. Culture, expansion, and differentiation of murine megakaryocytes. *Curr Protoc Immunol* 2005,**Chapter 22**:Unit 22F 26.

160. Nagata Y, Yoshikawa J, Hashimoto A, Yamamoto M, Payne AH, Todokoro K. Proplatelet formation of megakaryocytes is triggered by autocrine-synthesized estradiol. *Genes Dev* 2003,**17**:2864-2869.

161. Sit ST, Manser E. Rho GTPases and their role in organizing the actin cytoskeleton. *J Cell Sci* 2011,**124**:679-683.

162. Pleines I, Hagedorn I, Gupta S, May F, Chakarova L, van Hengel J*, et al.* Megakaryocyte-specific RhoA deficiency causes macrothrombocytopenia and defective platelet activation in hemostasis and thrombosis. *Blood* 2012,**119**:1054-1063.

163. Michaud J, Simpson KM, Escher R, Buchet-Poyau K, Beissbarth T, Carmichael C*, et al.* Integrative analysis of RUNX1 downstream pathways and target genes. *BMC Genomics* 2008,**9**:363.

164. Harada H, Harada Y. Point mutations in the AML1/RUNX1 gene associated with myelodysplastic syndrome. *Crit Rev Eukaryot Gene Expr* 2005,**15**:183-196.

165. Harada Y, Harada H. Molecular pathways mediating MDS/AML with focus on AML1/RUNX1 point mutations. *J Cell Physiol* 2009,**220**:16-20.

166. Higgs HN, Pollard TD. Regulation of actin polymerization by Arp2/3 complex and WASp/Scar proteins. *J Biol Chem* 1999,**274**:32531-32534.

167. Zigmond SH. How WASP regulates actin polymerization. *J Cell Biol* 2000,**150**:F117-120.

Appendices

Appendix A

Experimental Protocols

1.1 Megakaryocyte (MK) Culture Protocol

1. Flush total BM from tibias and femurs of mice into phosphate buffered saline (PBS) (SH30256.02, Thermo Scientific, Logan, Utah,) supplemented with 2% bovine serum albumin (BSA) (15260037, Invitrogen) and penicillin/streptomycin (P/S) (SV30010, Thermo Scientific).

2. Make flushed BM into single cell suspension by pipetting up and down gently.

3. Isolate hematopoietic progenitor cells from total BM by enrichment using magnetic bead-based negative selection with the mouse hematopoietic progenitor enrichment kit (19756, StemCell Technologies, Vancouver, BC).

4. Culture isolated progenitor cells in serum-free StemSpan (09655, StemCell Technologies) containing 2% P/S and supplemented with 50ng/mL thrombopoietin (Tpo) (31514, Peprotech, Rocky Hill, NJ) for up to five days.

5. Culture cells in 25cm^2 tissue culture Flask (353109, BD Biosciences, San Jose, CA) in 5mL total media.
6. After five days culturing, gently remove media culture into 15mL tubes (1475-0501, USA Scientific, Ocala, FL). Centrifuge cultured cells at 200 xg and resuspended in 1mL PBS.

7. Harvest MK by passing the cell suspension over a 1.5%/3.0% BSA gradient for 45 minutes. Here, first pipette 2.0mL 3.0% BSA solution (in PBS) to the bottom of a 25mL tube, followed by gently topping it with 2.0mL 1.5% BSA solution. Then gently pipette the 1.0mL MK suspension onto the top. Cover and allow separation without disturbing. MK settle to the bottom of the tube.

8. Gently remove solution until about 1.0mL left at the bottom. Centrifuge bottom layer art 200xg and resuspend MK in 5mL PBS and keep on ice until ready to use.

Note: To assay proplatelet formation functionality, progenitor cells were cultured in StemSpan supplemented with 50ng/mL Tpo and 10ng/mL estradiol (E4389, Sigma-Aldrich, St Louis, MO), a proplatelet inducing cytokine.

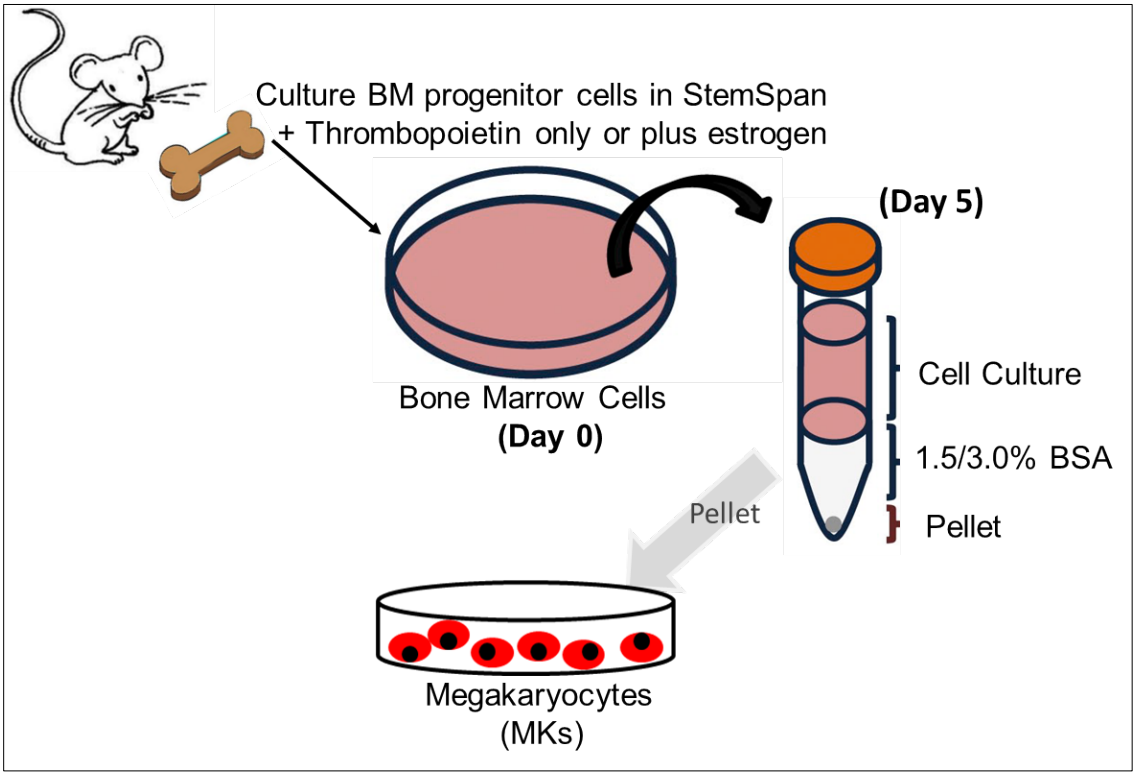

Figure 12: Schematic representation for the step-wise procedure of *in vitro* MK culture.

1.2 Adhering MK to Fibrinogen-coated slides

1. Dilute fibrinogen (F4385, Sigma Aldrich) stock solution to 1 µg/mL in PBS.
2. Pipette 200µL of fibrinogen solution into each 8-well chamber slide (C7182, Sigma Aldrich). Cover and coat slides overnight at 4°C. Slides should be placed in a humidified chamber to prevent drying.
3. Remove slides from solution and wash chambers gently, ones with PBS.
4. Pipette 200µL of 2% BSA into chambers to cover the surface area. Incubate for 1 hour at room temperature.
5. Resuspend harvested MK (Appendix 1.1) in culture media (StemSpan/ 50ng/mL TPO/ P/S).
6. Gently pipette 200µL of resuspended MK solution in fibrinogen coated chambered slide and allow MK to adhere for 4 hours.
7. Gently capsize the slide to get rid of excess medium and unattached MK.
8. Gently wash adhered cells ones with PBS.
9. Cells are ready for downstream experiment procedures (Immunofluorescence).

1.3 Immunofluorescence

1. MK are harvested as described above (Appendix 1.1).
2. Adhere MK to fibrinogen-coated glass slides for four hours at 37°C (Appendix 1.2)
3. Fix MK by pipetting 4% paraformaldehyde (158127, Sigma Aldrich) to cover them for 15 min at room temperature. Gently dip slides in PBS five times to wash off paraformaldehyde.
4. Following fixation, permeabilize MK with 0.1% Triton-X solution (234729, Sigma Aldrich) in PBS for 10 min.
5. Briefly wash slides with PBS.
6. Block MK with 5% fetal bovine serum (SH30071, Thermo Scientific) solution (in PBS) for one hour in humidified chamber.
7. Briefly wash slides with PBS.
8. Label MK with Alexa Fluor® 488 Phalloidin Conjugate (A12379, Invitrogen), rabbit anti-mouse myosin IIa (3403, Cell signaling), or rabbit anti-mouse α/β tubulin antibody (2148, Cell Signaling). Dilution for all antibodies is 1.0μL antibody to 200μL PBS (with 2% FBS).
9. Wash cells by incubating with 300μL PBS for 5 minutes and pour off. Perform this five times.
10. Label rabbit myosin IIa and α/β tubulin antibodies with anti-rabbit Alexa Fluor® 488 conjugate (4413, Cell Signaling) and anti-rabbit IgG Alexa Fluor® 488 Conjugate (4412, Cell Signaling) secondary antibodies, respectively. Dilution is 1.0μL antibody to 1000μL PBS (with 2% FBS).

11. Wash cells by incubating with 300µL PBS for 5 minutes and pour off. Perform this five times.

12. Allow slides to dry briefly (about 5 minutes) and then mount slides (Vectashield mounting medium with DAPI, H1200).

13. Visualize labeled MK using either confocal microscopy (Zeiss LSM 510) or fluorescence microscopy (Olympus BX-51).

1.4 Flow Cytometry Analysis of MK DNA Content

1. For cell sorting you want plenty of cells ($1x10^6$) as possible. Keep cells and antibodies on ice. Keep antibodies and stained cells in the dark.

2. Pipette cells into four 15ml tubes and spin at 100xg for 8minutes at 4°C.
3. Pour off supernatant.
4. Resuspend cells in 100µL of the following antibodies and incubate 25 minutes in refrigerator.

   (Tube 1) PBS+2%FBS (unstained control)

   (Tube 2) 1:250 CD41-FITC (25041182, eBioscience, San Diego, CA) in PBS+2% FBS (FITC control)

   (Tube 3) 1:250 CD41-FITC in PBS+2% FBS. To be stain later with propidium (PI).

   (Tube 4) PBS+2%FBS (PI control)

5. Wash two times with 2ml PBS with 2% FBS.
6. Spin down MK cultures at 200 xg for 5min in benchtop centrifuge.
7. Wash MK with 1 ml PBS at 200xg for 3min.
8. Resuspend cells in 200µl PBS, pH7.4. Fix cells by adding 1.8ml 70% cold ethanol drop-wise while vortexing the cell suspension at low speed.

9. Spin down the fixed cells at 200g for 3min in benchtop centrifuge. Wash once with 1 ml PBS.

10. Prepare the following staining solution with the following final concentrations per 1ml total solution:

    50ug/ml propidium iodide (81845, Sigma Aldrich)

4 mM sodium citrate (W302600, Sigma Aldrich)

100u/ml RNase A (R4642, Sigma Aldrich)

0.1% Triton X-100

11. Resuspend the cell pellet in 0.5-1 ml of staining solution 0.5 to 1 hour before flow analysis. Staining solution is added to all tubes except tube 1 (unstained control) and tube 2 (CD41-FITC control).

Appendix B

Publications

1. Abdul Gafoor Puthiyaveetil, Benjamin Okyere, Christopher Reilly, David Caudell Diverging in vitro antibody isotype switching preference in B-lymphocytes from C57BL/6 and FVB mice, In Vivo, 2013 Jan-Feb;27(1):29-39.

Appendix C

Poster Presentations

1. **Benjamin Okyere**, Jacob Cawley, Abdul Gafoor, Bettina Heid, David Caudell. A NUP98-HOXD13 leukemic fusion gene leads to aberrant actin localization in dysplastic megakaryocytes. Twenty-third Annual Research Symposium, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA, 2011 (poster presentation).

2. **Benjamin Okyere**, Jacob Cawley, Abdul Gafoor, Bettina Heid, David Caudell. Aberrant actin localization leads to dysplastic megakaryocytes in mice expressing the NUP98-HOXD13 leukemic fusion gene. Twenty-fourth Annual Research Symposium, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA, 2013 (poster presentation).

Appendix D

Graduate Awards

1. Outstanding PhD Poster, 23rd Biomedical and Veterinary Science Symposium, Virginia-Maryland Regional College of Veterinary Science, Virginia Tech, September 30, 2011.

2. Outstanding Masters Poster, 24th Biomedical and Veterinary Science Symposium, Virginia-Maryland Regional College of Veterinary Science, Virginia Tech, March 21, 2013.

Appendix E

Awarded Grants

1. Graduate Research and Development Program Award, Virginia Tech, Graduate Students Association, December 2012.