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Membrane skeleton modulates erythroid proteome remodeling and organelle clearance

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Abstract:
The final stages of mammalian erythropoiesis involve enucleation, membrane and proteome remodeling, and organelle clearance. Concomitantly, the erythroid membrane skeleton establishes a unique pseudohexagonal spectrin meshwork that is connected to the membrane through junctional complexes. The mechanism and signaling pathways involved in the coordination of these processes are unclear. Here we reveal an unexpected role of membrane skeleton in the modulation of proteome remodeling and organelle clearance during the final stages of erythropoiesis. We found that diaphanous-related formin mDia2 is a master regulator of the integrity of the membrane skeleton through polymerization of actin protofilament in the junctional complex. The mDia2-deficient terminal erythroid cell contained a disorganized and rigid membrane skeleton that was ineffective in detaching the extruded nucleus. In addition, the disrupted skeleton failed to activate the ESCRT-III complex, which led to a global defect in proteome remodeling, endolysosomal trafficking, and autophagic organelle clearance. We revealed that Chmp5, a component of the ESCRT-III complex, is regulated by mDia2-dependent activation of serum response factor and required for membrane remodeling and autophagosome-lysosome fusion. Mice with loss of Chmp5 in hematopoietic cells in vivo resembled the phenotypes in mDia2 knockout mice. Furthermore, over-expression of Chmp5 in mDia2 deficient hematopoietic stem and progenitor cells significantly restored terminal erythropoiesis in vivo. These findings reveal a formin-regulated signaling pathway that connects membrane skeleton to proteome remodeling, enucleation, and organelle clearance during terminal erythropoiesis.

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Membrane skeleton modulates erythroid proteome remodeling and organelle clearance

Short title: Membrane skeleton controls reticulocyte maturation

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Key Points:

1. mDia2 is a master regulator of membrane skeleton integrity that modulates reticulocyte maturation
2. Chmp5 mediates the functions of mDia2 through the regulation of proteome remodeling and organelle clearance

Abstract:

The final stages of mammalian erythropoiesis involve enucleation, membrane and proteome remodeling, and organelle clearance. Concomitantly, the erythroid membrane skeleton establishes a unique pseudohexagonal spectrin meshwork that is connected to the membrane through junctional complexes. The mechanism and signaling pathways involved in the coordination of these processes are unclear. Here we reveal an unexpected role of membrane skeleton in the modulation of proteome remodeling and organelle clearance during the final stages of erythropoiesis. We found that diaphanous-related formin mDia2 is a master regulator of the integrity of the membrane skeleton through polymerization of actin protofilament in the junctional complex. The mDia2-deficient terminal erythroid cell contained a disorganized and rigid membrane skeleton that was ineffective in detaching the extruded nucleus. In addition, the disrupted skeleton failed to activate the ESCRT-III complex, which led to a global defect in proteome remodeling, endolysosomal trafficking, and autophagic organelle clearance. We revealed that Chmp5, a component of the ESCRT-III complex, is regulated by mDia2-dependent activation of serum response factor and required for membrane remodeling and autophagosome-lysosome fusion. Mice with loss of Chmp5 in hematopoietic cells in vivo resembled the phenotypes in mDia2 knockout mice. Furthermore, over-expression of Chmp5 in mDia2 deficient hematopoietic stem and progenitor cells significantly restored terminal erythropoiesis in vivo. These findings reveal a formin-regulated signaling pathway that connects

membrane skeleton to proteome remodeling, enucleation, and organelle clearance during terminal erythropoiesis.

Introduction:

Reticulocytes formed after enucleation of the orthochromatic erythroblasts contain organelles and non-hemoglobin proteins that are largely eliminated during maturation. Maturation of reticulocytes is divided into two stages. The early stage reticulocytes (R1) immediately after the extrusion of nuclei are motile, irregular, and large in size. The more mature R2 stage reticulocytes are nonmotile and clear of organelles. Through R1 to R2, membrane transferrin receptor, adhesion molecules, and many other cytoplasmic proteins are depleted to establish a hemoglobin predominant proteome[1,2]. Reticulocytes also undergo volume reduction that involves membrane loss and exocytosis[3]. In addition, reticulocyte membrane skeleton is remodeled to establish the biconcave and stable erythrocyte structure [4-8]. Knowledge accumulated in the past few decades reveals several processes in protein removal and organelle clearance during reticulocyte maturation, in which multivesicular body formation, autophagy, and exocytosis are involved (see review[1,9]). However, the mechanisms of these processes still remain elusive. Particularly, it is unclear whether and how membrane skeleton coordinates with proteome remodeling and organelle clearance during terminal erythropoiesis.

mDia2 is a diaphanous-related formin protein involved in linear actin polymerization and highly upregulated in the late stages of terminal erythropoiesis[10,11]. mDia2 contains a formin homology 1 (FH1) domain and a FH2 domain that are important for actin polymerization. The N terminus of mDia2 protein contains a Rho GTPase binding domain (GBD), a diaphanous-inhibitory-domain (DID), and a dimerization domain (DD). On the C terminus, there is a diaphanous auto-regulatory domain (DAD) that binds to the DID domain to form an auto-inhibitory loop when

mDia2 is inactivated [11-13]. Mutations in DAD domain or N-terminal regions disrupt this auto-inhibitory structure and lead to the constitutive activation of mDia formin proteins [14-17]. In addition, binding of Rho GTPase to the GBD domain relieves the auto-inhibitory loop to activate mDia formins under physiologic conditions[11].

mDia formins are reported to be involved in a variety of cellular processes [11,18]. Recent mouse genetic studies from us and others showed embryonic lethality in mDia2 knockout mice with major defects in the hematopoietic system[19,20]. Loss of mDia2 influences terminal erythropoiesis at different stages, which is manifested as compromised cytokinesis and enucleation. In this respect, the mDia2 conditional and hematopoietic-specific knockout mouse model provides a valuable tool to study terminal erythropoiesis in vivo in adult, which is not possible to perform using the whole-body knockout mice. With this model, we show here that mDia2 is indispensable for the motility of R1 reticulocytes, membrane skeleton integrity, and clearance of unnecessary proteins and organelles in maturing reticulocytes. We also revealed a novel mDia2-Chmp5 pathway and mDia2-regulated ESCRT III complex in reticulocyte maturation. These studies underline the critical roles of the integrity of membrane skeleton in the modulation of erythroid proteome remodeling and organelle clearance.

Methods

Mice

Genetically modified and tissue-specific mDia2 knockout mice in C57/BL6 background were described previously [20,21]. Congenic mice carrying CD45.1 antigen were purchased from Charles River (B6-LY-5.2/Cr, strain code: 564). C57/BL6 wild-type mice, CAG-Cas9 transgenic mice (026179), Vav-Cre mice (008610) were purchased from Jackson Laboratory. GFP-LC3 mice were kindly provided by Dr. Congcong He (Northwestern university, Chicago, USA). GFP-LC3 transgenic mice were crossed with mDia2$^{fl/fl}$ Vav-Cre mice to obtain mDia2$^{fl/fl}$ GFP-LC3 and mDia2$^{fl/fl}$ Vav-cre GFP-LC3 mice. All the experiments involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Northwestern University.

In vitro culture of erythroid cells

Purification and in vitro culture of mouse bone marrow lineage negative cells to the erythroid lineage were performed as previously described [10]. Briefly, lineage negative cells were purified using mouse lineage antibody cocktail (BD Biosciences, 559971) and cultured in erythropoietin-containing medium for 48 hours. By day 2, approximately 20-30% of the cells were enucleated. The cells were harvested at various time points as indicated in different experiments for further characterization.

Expression Constructs

The MSCV-IRES-hCD4 (MICD4) construct was described previously[10]. pCINeo-mChmp5 construct was purchased from Addgene (11771), mChmp5 was PCR amplified and subcloned into the MICD4 vector through EcoRI and NotI sites. MICD4-SRF construct was described previously [22]. The expression construct carrying a HA-tagged, constitutively active form of human SRF was kindly provided by Dr. Naren Ramanan (India), and was PCR amplified and sub-cloned into the MICD4 vector. pGL4-Chmp5 promoter fragments were sub-cloned into pGL4.24(luc2P/minP) vector (E842A, Promega) between HindIII and XhoI sites. The autophagy

reporter construct pCDH-puro-mCherry-EGFP-LC3 was kindly provided by Dr. Congcong He (Chicago, USA).

Stochastic optical reconstruction microscopy (STORM) imaging

The STORM imaging analysis was performed as previously described [23].

Scanning Electron Microscopy

Erythroblasts and reticulocytes were cultured as above. The cells were collected or sorted, and washed three times with PBS, followed by the fixation in 2% glutaraldehyde (Polysciences) in 100 mM phosphate buffer (pH 7.2) overnight at 4℃. The cells were then processed and examined with a JEOL NeoScope SEM scanning electron microscope.

Transmission Electron Microscopy (TEM) and immuno-gold staining

In vitro cultured erythroblasts and reticulocytes were collected and washed three times with PBS, followed by the fixation in 2% glutaraldehyde (Polysciences) and 100 mM phosphate buffer (pH 7.2) overnight at 4℃. For RBC membrane skeleton TEM, ghost RBCs were prepared through incubation of RBCs in a hypotonic buffer (0.5 mM phosphate, 0.1 mM EDTA, pH 7.4) for 15 min and mounted on cover slides. Immuno-gold staining was employed to detect mDia2 localization on RBC membrane skeleton as previously described [24]. Human mDia2 antibody was purchased from Proteintech (catalog number: 14342-1-AP), which was previously validated[25,26]. The cells were processed and examined with a FEI Tecnai Spirit G2 transmission electron microscope.

Live imaging of reticulocyte motility

Erythroblasts purified from the bone marrow were cultured in erythropoietin-containing medium for 48 h. A fluorescence nuclear-staining dye was added in the culture medium (ThermoFisher, R37106) at 37°C for 15 min. Movies were taken by a Nikon W1 Dual Cam Spinning Disk

Confocal Microscope (objective lens used: Apo TIRF 60X/1.49 Oil). The data were analyzed by NIS software and ImageJ.

Red blood cell (RBC) ghost preparation

RBC ghost preparation was performed as previously described [27].

Bone marrow transplantation

The bone marrow transplantation was performed as previously described [21,28-30].

Immunofluorescence analysis

Immunofluorescence analysis was performed as previously described[29,31]. Primary antibodies used: Chmp5 (Santa Cruz, sc-374338), LAMP1 (Sigma, L1418), LBPA (Sigma, MABT837), M6PR (Abcam, ab2733), lysosome tracker green (ThermoFisher, L7526), mitochondrial tracker deep red (Invitrogen, M22426), LC3B (Abcam, ab48394). Immunofluorescence images were taken with an Olympus IX70 Inverted Fluorescence Microscope (objective lens used: U-PLAN S-APO 1003 oil, 1.4 NA, 0.12 mm WD, DIC) and a Nikon X1 Spinning Disk Confocal microscope (objective lens used: objective lens used: Apo TIRF 100X/1.49 Oil). The images were analyzed using NIS Elements software and ImageJ.

Flow Cytometric Analysis

Flow cytometric analyses of differentiation and enucleation of cultured mouse bone marrow erythroblasts were performed as previously described [10,31-34].

Chloroquine treatment

Chloroquine (CQ) was purchase from Sigma (C6628-25G). Bone marrow lineage negative cells were purified and cultured for 48 h. CQ was then added in cell medium to the final concentration of 100 μM and incubated for an additional 2 h. Cells were collected and fixed for further analyses.

Retroviral shRNA design

Retroviral shRNA oligo against mChmp5 were designed based on an online shRNA designer (http://sirna.wi.mit.edu). shRNA oligos were cloned into MSCV-U3-H1 retroviral vector as previously described 10.

Statistics

Results are expressed as mean ± SEM unless otherwise indicated. Statistical comparisons between two groups were performed with a two-tailed unpaired Student's t test using GraphPad Prism version 6.0 software.

Results:

mDia2 is essential for the integrity of membrane skeleton

We previously showed that hematopoietic specific mDia2 deficient mice exhibit anemia and ineffective erythropoiesis[20]. To determine the role of mDia2 in reticulocyte maturation, we analyzed bone marrow reticulocytes from these mice using live imaging. Knockout of mDia2 was first confirmed by the loss of mDia2 expression with hematopoietic-specific Cre expression (**Supplemental Figure 1A-B**). We found that R1 reticulocytes from mDia2-deficient mice were

rigid and showed little motility during enucleation compared to their wild-type counterparts (**Figure 1A, Supplemental Movie 1-2**). Work from us and others also demonstrated that mDia2 is critical for terminal erythropoiesis and enucleation[10,19,20]. Loss of mDia2 compromises cytokinesis and leads to a significant increase of bi-nucleated orthochromatic erythroblasts, precursor cells in the last stage of terminal erythropoiesis before enucleation. Interestingly, we also found enucleating cells with two nuclei attached to the rigid reticulocyte (**Figure 1A-B, Supplemental Movie 3**), indicating that orthochromatic erythroblasts with dysplastic nuclei could still enucleate.

We previously reported that Rac GTPases are upstream regulators of mDia2. Brief Rac inhibitor treatment of the cultured mouse erythroblasts immediately before enucleation significantly inhibits enucleation[10]. To investigate whether Rac GTPases are also involved in reticulocyte motility, we treated mouse bone marrow erythroblasts at 35 h in culture, when the cells started to enucleate, for 5 min and analyzed the motility of R1 reticulocytes in real time. Indeed, this brief treatment phenocopied the motility defects of mDia2 deficient reticulocytes (**Supplementary Figure 1C**). Overall, these results demonstrate that mDia2 is involved in the motility of R1 reticulocytes that is critical for the detachment of the extruded nuclei in the last step of enucleation[35].

The defects in the motility of R1 reticulocytes prompted us to analyze the membrane skeleton of the erythrocytes in mDia2$^{fl/fl}$Vav-Cre mice. Red blood cells in mDia2 deficient mice show macrocytosis (**Figure 1C**) and anisopoikilocytosis with numerous irregularly shaped erythrocytes (**Figure 1D**)[20]. Through a transmission electron microscopy on hemoglobin-depleted ghost red blood cells, we found that mDia2 deficient cells showed significantly elongated spectrin chains (**Figure 2A**), which indicates a reduced flexibility of the pseudohexagonal erythroid membrane skeleton meshwork. The median calculated length distribution of spectrin tetramer in the erythrocytes of mDia2$^{fl/fl}$Vav-Cre mice was shifted significantly to the right side, i.e. longer spectrin length, comparing to that of control (**Figure 2B**).

This led to an enhanced increase of membrane skeleton stress with membrane stretch under a uniaxial tension (**Figure 2C**). The extended spectrin chains are also compatible with macrocytosis and suggest a failure of volume reduction in the maturing reticulocytes. Consistent with the role of mDia2 in actin polymerization, stochastic optical reconstruction microscopy (STORM) imaging analysis showed that the short actin protofilaments were irregularly distributed in mDia2 deficient erythrocytes (**Figure 2D**). Western blot analyses on ghost erythrocytes further revealed that many membrane skeleton proteins were significantly decreased in mDia2 deficient erythrocytes (**Figure 2E**). These results demonstrate a critical role of mDia2 in maintaining the integrity of erythroid membrane skeleton. To determine the localization of mDia2 on the skeleton, we performed a co-immunoprecipitation assay in ghost erythrocytes from normal human red blood cells. The result showed that mDia2 bound to band 3, actin, and glycophorin C, which are protein components of the junctional complex critical for the maintenance of the pseudohexagonal meshwork in erythroid membrane skeleton (**Figure 2F**). We further performed an immunogold labeling of mDia2 in normal human erythrocytes followed by electron microscopy, which also revealed localization of mDia2 at the junctional complex (**Figure 2G**).

Loss of mDia2 leads to impaired membrane and proteome remodeling and organelle clearance

Given the significance of mDia2 for the integrity of membrane skeleton, we next investigated whether it is also involved in membrane remodeling and organelle clearance during reticulocyte maturation. We first analyzed several proteins, such as CD71 and CD44, that are programed to be down-regulated during the late stages of terminal erythropoiesis[36-38]. Indeed, the surface levels of CD71 and CD44 were significantly increased in mDia2 deficient reticulocytes compared to the wild-type control cells (**Figure 3A**). This defect in the downregulation of surface proteins in mDia2 deficient erythroid cells was also readily detected in

the orthochromatic stage of the terminal erythropoiesis (**Supplemental Figure 2A**). The same phenotypes were observed in another mDia2 hematopoietic-specific knockout model in which mDia2 depletion was induced in adult (**Supplemental Figure 2B-C**). mDia2 deficient reticulocytes were also much larger (forward scatter) than their wild-type counterparts. Although some managed to enucleate, mDia2 deficient reticulocytes contained higher content of residual DNA compared to the controls (**Figure 3A, Supplemental Figure 2B**). These data indicate that although the levels of many structural proteins were reduced in mDia2 deficient erythrocytes (**Figure 2E**), those that are programed to be removed from the membrane failed to do so. To further determine whether loss of mDia2 affects organelle clearance, we stained lysosomes and mitochondria in reticulocytes sorted from the cultured erythroblasts and indeed found increased levels with loss of mDia2 (**Figure 3B-D**).

Consistent with the flow cytometry and immunofluorescence assays, transmission electron microscopy analyses revealed significantly enlarged size of mDia2 deficient reticulocyte with numerous organelles that failed to be cleared. Large multivesicular bodies (MVBs) that were uncommon in wild-type reticulocytes were also frequently observed in mDia2 deficient ones (**Figure 4A, insert III**). Accordingly, extracellular vesicles that are normally released through MVB fusion with the membrane were significantly reduced in the medium cultured with mDia2 deficient erythroid cells (**Figure 4B**). Furthermore, autophagic vacuoles, many resembling autophagosomes with double membranes, were also readily detected in mDia2 deficient orthochromatic erythroblasts (**Figure 4C**).

We next compared the proteome of mDia2 deficient and wild type reticulocytes using tandem mass tagging (TMT) mass spectrometry in hemoglobin-depleted ghost reticulocytes. As expected, many membrane and skeleton proteins were decreased in mDia2 deficient cells. On the other hand, many nuclear and chromosome associated proteins were elevated (**Figure 4D, Supplemental Figure 3A-B,** and **Supplementary Data 1**). Recent studies revealed that UBE2O remodels the proteome of reticulocytes through ubiquitination and proteasome

degradation of many substrates, predominantly ribosome proteins[39]. Our data demonstrate that mDia2 is involved in the clearance of other substrates, mainly DNA associated proteins that are known to be cleared after extrusion of the nucleus during reticulocyte maturation [40]. This is also consistent with the increased DNA levels in mDia2 deficient reticulocytes (**Figure 3A**).

Chmp5 is regulated by mDia2 and mediates mDia2's function during reticulocyte maturation

Defects in the detachment of the extruded nuclei in mDia2 deficient reticulocytes could also be related to compromised cytokinesis, which is supported by the increased bi-nucleated orthochromatic erythroblasts that failed cytokinetic abscission in mDia2$^{fl/fl}$Vav-Cre mice[20]. Cytokinesis involves the constriction of an actomyosin ring formation of microtubule arrays, and final abscission mediated by the endosomal sorting complex required for transport (ESCRT) proteins. ESCRT proteins are known to be critical for various membrane remodeling activities, including endocytosis and subsequent degradation in the lysosomes. In this process, ESCRT-0, I, and II complexes are involved in the recognition of ubiquitinated cargo and inclusion in multivesicular bodies (MVBs). ESCRT-III complex is critical as a multi-functional machinery for the invagination of vesicles to form MVBs, abscission of the vesicles, and regulation of late endosome function to lysosome degradation [41-47]. Given the defects in cytokinetic abscission and increased MVBs in the late stages of terminal erythropoiesis, we reasoned that ESCRT-III complex could be important in mediating mDia2's functions, including membrane and proteome remodeling and organelle clearance. To test this hypothesis, we first analyzed the expression of the components of ESCRT-III complex in the late stage of terminal erythropoiesis in mDia2$^{fl/fl}$Vav-Cre and the wild-type controls. Indeed, the mRNA levels of many genes encoding various ESCRT-III components were significantly downregulated in mDia2 deficient erythroblasts (**Supplemental Figure 4A-B**).

Among these downregulated ESCRT-III components, Chmp5 is unique in that it is not required for MVB formation like other proteins in the complex. Instead, it is critical for the maturation of MVBs into lysosomes[48,49]. The reported phenotypes of Chmp5 deficient cells, including the enlarged late endosomes/MVBs and upregulation of surface receptors[48], resemble what we observed in mDia2 deficient terminal erythroid cells. Indeed, we found increased co-localization of LBPA and LAMP1 (endosome/MVB markers) in mDia2 deficient orthochromatic erythroblasts and reticulocytes (**Figure 5A, Supplemental Figure 4C**), demonstrating a compromised maturation of MVBs to lysosomes. The expression level of Chmp5 remains constant across different stages of terminal erythropoiesis (**Supplemental Figure 4D-E**). To study the relationship between mDia2 and Chmp5, we first confirmed the downregulation of Chmp5 protein, as well as several other components of the ESCRT-III complex, in the late stage of bone marrow terminal erythropoiesis in mDia2$^{fl/fl}$Vav-Cre mice (**Supplemental Figure 4F**). Immunofluorescence analysis showed Chmp5 localization between the dividing erythroblasts in cultured bone marrow erythroid cells from wild-type mice. In comparison, Chmp5 was barely detectable in the binucleated erythroblasts frequently found in mDia2 deficient mice (**Figure 5B**).

The mDia formin proteins not only function in cell migration and adhesion through their direct roles in linear actin polymerization, but they are also involved in transcriptional regulation through activation of the MAL-SRF pathway[11,50,51]. By promoting actin polymerization, mDia formins release G-actin monomers from megakaryocytic acute leukemia (MAL) protein. The released MAL translocates into the nucleus, where it dimerizes with serum response factor (SRF) to drive SRF-dependent gene expression of many cytoskeleton-related genes[52,53]. Given the reduced expression of Chmp5 in mDia2 deficient cells, we next determined whether Chmp5 could be a direct transcriptional target of SRF. To this end, we performed an analysis to identify SRF responsive elements (SRE), so called “CArG” box, on the promoter region of Chmp5 using a transcription factor online binding site prediction tool [54] and found two putative SREs in the Chmp5 promoter (**Figure 5C**). We then cloned these two SREs and inserted them individually

ahead of the luciferase gene. SRE site 1, but not site 2, induced a strong luciferase activity that was inhibited by the mutation that disrupted the SRE (**Figure 5C**). To confirm the binding of SRF to this site, we performed a chromatin immunoprecipitation assay and indeed revealed specific binding of SRF to the SRE on Chmp5 (**Figure 5D**). Consistent with the role of mDia2 in the regulation of SRF, loss of mDia2 significantly reduced the binding of SRF to the SRE on Chmp5 (**Figure 5E**). To further confirm the mDia2-SRF axis in the regulation of Chmp5 expression, we overexpressed SRF and transduced it into mDia2 deficient erythroblasts. As expected, the expression of Chmp5 was largely rescued by SRF (**Figure 5F**). Functionally, knockdown of Chmp5 in vitro through shRNA in wild-type bone marrow lineage negative cells led to a significantly increased number of binucleated orthochromatic erythroblasts (**Figure 5G-H**). Knockout of Chmp5 in vitro and hematopoietic specific knockout of Chmp5 in vivo through CRISPR-Cas9 also resulted in significantly increased binucleated orthochromatic erythroblasts, organelle retention, and defects in membrane remodeling (**Supplemental Figure 5A-H**).

mDia2-Chmp5 pathway is critical for the fusion of autophagosome and lysosome

ESCRT-III complex is reported to play a role in the fusion of autophagosome with lysosome[55]. The presence of increased autophagosomes in mDia2 deficient terminal erythroid cells indicates a defect in autolysosome formation. We first analyzed the levels of autophagic flux in the late stages of terminal erythropoiesis through immunofluorescence stain and western blot analysis of LC3, which was dramatically increased in mDia2 deficient terminal erythroid cells (**Figure 6A-B**). In addition, we crossed mDia2 deficient mice with GFP-LC3 mice and obtained mDia2$^{fl/fl}$/GFP-LC3 and mDia2$^{fl/fl}$Vav-Cre/GFP-LC3 mice. The late stage erythroblasts purified from the bone marrow of these mice indeed demonstrated significantly increased GFP signals in cells from mDia2$^{fl/fl}$Vav-Cre/GFP-LC3 mice (**Figure 6C-D**). To directly determine whether the fusion of autophagosome with lysosome is compromised, we transduced primary bone marrow erythroid progenitors with a tandem fluorescent-tagged LC3 (mCherry-EGFP-LC3).

EGFP is sensitive to the acidic environment of lysosome whereas mCherry is more stable. Increase in the merged yellow signal suggests a defect in the fusion with lysosome[56]. We analyzed the transduced erythroblasts following their maturation as measured by progressive nuclear condensation. Compared to the control, mDia2 deficient erythroblasts showed markedly increased yellow signals across the differentiation stages (**Figure 6E**). Chloroquine is known to block autophagosome and lysosome fusion[57]. Treatment of chloroquine in mDia2 deficient erythroblasts induced no further increase in LC3 signal, which supports the defect in autophagosome and lysosome fusion with loss of mDia2 (**Supplemental Figure 6A**). As expected, knockout of Chmp5 through sgRNA significantly induced autophagic flux (**Supplemental Figure 6B**).

Overexpression of Chmp5 ameliorates the defects in terminal erythropoiesis in mDia2 deficient mice

Our results strongly suggest that Chmp5 acts as a downstream effector of the mDia2-SRF axis in regulating ESCRT-III complex-mediated cytokinesis and global clearance of proteins and organelles during the late stages of terminal erythropoiesis. To further demonstrate this, we first purified lineage-negative hematopoietic stem and progenitor cells from the bone marrow of mDia2$^{fl/fl}$Vav-Cre mice and infected them with retroviruses expressing a constitutively active mDia2 mutant, ΔDAD (**Supplemental Figure 7A**). The cells were cultured in vitro for 48 h in erythroid differentiation media. As we expected, mDia2 ΔDAD mutant significantly induced overexpression of Chmp5 (**Supplemental Figure 7B**), rescued the defect in lysosome clearance and increase of LC3B (**Supplemental Figure 7C-D**) in mDia2 deficient erythroid cells. We next tested the role of Chmp5 in vivo using a transplantation model. To this end, the lineage-negative hematopoietic stem and progenitor cells from the bone marrow of mDia2$^{fl/fl}$Vav-Cre mice were infected with retroviruses expressing Chmp5. The transduced cells that

overexpress Chmp5 were then transplanted into lethally irradiated wild-type recipient mice (**Figure 7A**). Compared to mice transplanted with vector-transduced mDia2 deficient bone marrow cells, those transplanted with mDia2 deficient cells that overexpress Chmp5 significantly rescued the red blood cell count and hemoglobin level two months after transplantation (**Figure 7B, Supplemental Figure 7E**). Splenomegaly in mice transplanted with mDia2 deficient bone marrow was diminished with overexpression of Chmp5 (**Figure. 7C**). When the bone marrow cells were harvested, we found that the binucleated orthochromatic erythroblasts that comprised almost half of the cells at this stage in mDia2 deficient mice were dramatically reduced with Chmp5 overexpression (**Figure 7D-E**). As expected, overexpression of Chmp5 also reverted the defects in organelle and protein clearance (**Figure 7F**), as well as the increase in autophagic flux (**Supplemental Figure 7F**).

Discussion:

Our study reveals an important connection between erythroid membrane actin skeleton and the global proteome remodeling and organelle clearance during terminal erythropoiesis. The formin protein mDia2 functions as a master regulator of this connection through polymerization of actin protofilaments, which signals the activation of SRF-mediated expression of different components of the ESCRT-III complex. Our results also demonstrate multiple functions of ESCRT-III complex in the regulation of endolysosomal and autolysosomal formation that are essential for membrane remodeling and cytoplasmic clearance during reticulocyte maturation. Furthermore, our study suggests that hereditary anemias that involve defects in membrane skeleton could also have compromised reticulocyte maturation. In addition, mDia formin proteins and ESCRT-III complex could play broader roles in mediating proteome remodeling and autophagy in other cell types.

One of the unsolved questions in erythroid membrane biology is how actin protofilaments are formed[58]. Our study reveals that mDia2 plays a critical role in this process. The disrupted actin protofilaments in mDia2 deficient reticulocytes lead to reduced flexibility of the pseudohexagonal membrane skeleton meshwork, which explains the rigidity and lack of movement of the R1 reticulocytes during enucleation. Together with its role in contractile actin ring formation during enucleation[10,20], mDia2 is essential in the membrane integrity in the late stages of terminal erythropoiesis.

Notably, mDia2 plays multiple functions at different stages of terminal erythropoiesis. We and others previously demonstrated that loss of mDia2 in fetal liver and adult bone marrow erythroid cells led to cytokinesis defects before enucleation[19,20]. In this respect, we cannot completely exclude the possibility that the phenotypes we observed in mDia2 deficient reticulocytes are related to the absence of mDia2 in the early stages. However, the fact that a brief Rac inhibitor treatment of the cultured erythroblasts right before enucleation caused a significant inhibition of enucleation suggests that nuclear detachment and reticulocyte motility are directly related to mDia2's specific function at this differentiation stage. Future studies using mDia2 agonist or antagonist would be helpful to dissect its specific roles in different stages of terminal erythropoiesis.

Our data demonstrate that erythroid cells are more sensitive to mDia2 deficiency but not its activation or overexpression. The sensitivity to mDia2 up or down regulation appears to be cell type specific. In HeLa cells, expression of a constitutively active mDia2 mutant led to defects in cytokinesis [59]. In contrast, NIH 3T3 cells are more like erythroid cells and sensitive to the loss of mDia2 [60]. The sensitivity to mDia2 deficiency is also common in hematopoietic cells in that loss of mDia2 can also lead to defects in the engraftment of hematopoietic stem and progenitor cells [22].

Our results also reveal an important role of membrane skeleton in protein sorting and endo-lysosome trafficking during reticulocyte maturation. Many surface proteins such as CD71 and

CD44 are programmed to be down regulated during the late stage of terminal erythropoiesis[36-38]. The possible mechanisms involve endocytosis, intracellular vesicle trafficking, and exsocytosis[61,62]. During this process, surface proteins, as well as many other intracellular organelles and DNA associated proteins, are removed through endosome-MVB-lysosome-autolysosome formation. Loss of mDia2 significantly compromises this process, which leads to the accumulation of large MVBs in orthochromatic erythroblasts and reticulocytes. More interestingly, mDia2 deficiency also significantly affects the fusion of autophagosome and lysosome. This multi-functional properties of mDia2 prompted us to evaluate the ESCRT complexes that are involved in many of the similar processes. Indeed, many components of the ESCRT III complex are downregulated, including Chmp5.

As a formin protein, mDia2 not only functions on actin polymerization, but also involves in transcriptional regulation through MAL-SRF pathway[50]. The mDia2-SRF-Chmp5 pathway represents one of the first signaling cascades involved in the final stages of terminal erythropoiesis. This pathway regulates cytokinesis of the last step of mitosis in orthochromatic erythroblasts, membrane and proteome remodeling, and endo-lysosome trafficking. Defects in all of these processes together contribute to lethal anemia during embryogenesis and ineffective erythropoiesis in adult mDia2 null mice, which also mimic certain inherited diseases in human, such as congenital dyserythropoietic anemia (CDA)[63]. Therefore, our studies could shed light on the pathogenesis of these human diseases.

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Author Contributions: Y.L., Y.M., X.H., F.V.K., M.A.P., J.Y., and Z.P. performed the experiments and interpreted data. J.A.P., S.P.G., and D.F. interpreted data. Y.L. and P.J. designed the experiments, interpreted data, and edited the manuscript. Y.L., and P.J. wrote the manuscript.

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References:

1. Ovchynnikova E, Aglialoro F, von Lindern M, van den Akker E. The Shape Shifting Story of Reticulocyte Maturation. *Front Physiol*. 2018;9:829.
2. Griffiths RE, Kupzig S, Cogan N, et al. Maturing reticulocytes internalize plasma membrane in glycophorin A-containing vesicles that fuse with autophagosomes before exocytosis. *Blood*. 2012;119(26):6296-6306.
3. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J Biol Chem*. 1987;262(19):9412-9420.
4. Mohandas N, Gallagher PG. Red cell membrane: past, present, and future. *Blood*. 2008;112(10):3939-3948.
5. Woods CM, Lazarides E. The erythroid membrane skeleton: expression and assembly during erythropoiesis. *Annu Rev Med*. 1988;39:107-122.
6. Chasis JA, Prenant M, Leung A, Mohandas N. Membrane assembly and remodeling during reticulocyte maturation. *Blood*. 1989;74(3):1112-1120.
7. Liu J, Guo X, Mohandas N, Chasis JA, An X. Membrane remodeling during reticulocyte maturation. *Blood*. 2010;115(10):2021-2027.
8. Liu J, Mohandas N, An X. Membrane assembly during erythropoiesis. *Curr Opin Hematol*. 2011;18(3):133-138.
9. Lupo F, Tibaldi E, Matte A, et al. A new molecular link between defective autophagy and erythroid abnormalities in chorea-acanthocytosis. *Blood*. 2016;128(25):2976-2987.
10. Ji P, Jayapal SR, Lodish HF. Enucleation of cultured mouse fetal erythroblasts requires Rac GTPases and mDia2. *Nat Cell Biol*. 2008;10(3):314-321.
11. Faix J, Grosse R. Staying in shape with formins. *Dev Cell*. 2006;10(6):693-706.
12. Kato T, Watanabe N, Morishima Y, Fujita A, Ishizaki T, Narumiya S. Localization of a mammalian homolog of diaphanous, mDia1, to the mitotic spindle in HeLa cells. *J Cell Sci*. 2001;114(Pt 4):775-784.
13. Alberts AS. Identification of a carboxyl-terminal diaphanous-related formin homology protein autoregulatory domain. *J Biol Chem*. 2001;276(4):2824-2830.
14. Palazzo AF, Cook TA, Alberts AS, Gundersen GG. mDia mediates Rho-regulated formation and orientation of stable microtubules. *Nat Cell Biol*. 2001;3(8):723-729.

15. Schonichen A, Alexander M, Gasteier JE, Cuesta FE, Fackler OT, Geyer M. Biochemical characterization of the diaphanous autoregulatory interaction in the formin homology protein FHOD1. *J Biol Chem*. 2006;281(8):5084-5093.
16. Tominaga T, Meng W, Togashi K, Urano H, Alberts AS, Tominaga M. The Rho GTPase effector protein, mDia, inhibits the DNA binding ability of the transcription factor Pax6 and changes the pattern of neurite extension in cerebellar granule cells through its binding to Pax6. *J Biol Chem*. 2002;277(49):47686-47691.
17. Wallar BJ, Stropich BN, Schoenherr JA, Holman HA, Kitchen SM, Alberts AS. The basic region of the diaphanous-autoregulatory domain (DAD) is required for autoregulatory interactions with the diaphanous-related formin inhibitory domain. *J Biol Chem*. 2006;281(7):4300-4307.
18. Mao Y. FORMIN a link between kinetochores and microtubule ends. *Trends Cell Biol*. 2011;21(11):625-629.
19. Watanabe S, De Zan T, Ishizaki T, et al. Loss of a Rho-regulated actin nucleator, mDia2, impairs cytokinesis during mouse fetal erythropoiesis. *Cell Rep*. 2013;5(4):926-932.
20. Mei Y, Zhao B, Yang J, et al. Ineffective erythropoiesis caused by binucleated late-stage erythroblasts in mDia2 hematopoietic specific knockout mice. *Haematologica*. 2016;101(1):e1-5.
21. Keerthivasan G, Mei Y, Zhao B, et al. Aberrant overexpression of CD14 on granulocytes sensitizes the innate immune response in mDia1 heterozygous del(5q) MDS. *Blood*. 2014;124(5):780-790.
22. Mei Y, Han X, Liu Y, Yang J, Sumagin R, Ji P. Diaphanous-related formin mDia2 regulates beta2 integrins to control hematopoietic stem and progenitor cell engraftment. *Nat Commun*. 2020;11(1):3172.
23. Pan L, Yan R, Li W, Xu K. Super-Resolution Microscopy Reveals the Native Ultrastructure of the Erythrocyte Cytoskeleton. *Cell Rep*. 2018;22(5):1151-1158.
24. Korobova F, Svitkina T. Molecular architecture of synaptic actin cytoskeleton in hippocampal neurons reveals a mechanism of dendritic spine morphogenesis. *Mol Biol Cell*. 2010;21(1):165-176.
25. Stastna J, Pan X, Wang H, et al. Differing and isoform-specific roles for the formin DIAPH3 in plasma membrane blebbing and filopodia formation. *Cell Res*. 2012;22(4):728-745.
26. Rana MK, Aloisio FM, Choi C, Barber DL. Formin-dependent TGF-beta signaling for epithelial to mesenchymal transition. *Mol Biol Cell*. 2018;29(12):1465-1475.
27. Lu Y, Hanada T, Fujiwara Y, et al. Gene disruption of dematin causes precipitous loss of erythrocyte membrane stability and severe hemolytic anemia. *Blood*. 2016;128(1):93-103.
28. Zhao B, Tan TL, Mei Y, et al. H2AX deficiency is associated with erythroid dysplasia and compromised haematopoietic stem cell function. *Sci Rep*. 2016;6:19589.
29. Mei Y, Zhao B, Basiorka AA, et al. Age-related inflammatory bone marrow microenvironment induces ineffective erythropoiesis mimicking del(5q) MDS. *Leukemia*. 2017.
30. Zhao B, Mei Y, Cao L, et al. Loss of pleckstrin-2 reverts lethality and vascular occlusions in JAK2V617F-positive myeloproliferative neoplasms. *J Clin Invest*. 2018;128(1):125-140.
31. Zhao B, Mei Y, Schipma MJ, et al. Nuclear Condensation during Mouse Erythropoiesis Requires Caspase-3-Mediated Nuclear Opening. *Dev Cell*. 2016;36(5):498-510.
32. Zhao B, Mei Y, Yang J, Ji P. Mouse fetal liver culture system to dissect target gene functions at the early and late stages of terminal erythropoiesis. *J Vis Exp*. 2014(91):e51894.
33. Zhao B, Keerthivasan G, Mei Y, et al. Targeted shRNA screening identified critical roles of pleckstrin-2 in erythropoiesis. *Haematologica*. 2014;99(7):1157-1167.
34. Zhao B, Mei Y, Yang J, Ji P. Erythropoietin-regulated oxidative stress negatively affects enucleation during terminal erythropoiesis. *Exp Hematol*. 2016;44(10):975-981.
35. Ji P, Murata-Hori M, Lodish HF. Formation of mammalian erythrocytes: chromatin condensation and enucleation. *Trends Cell Biol*. 2011;21(7):409-415.

36. Tachiyama R, Ishikawa D, Matsumoto M, et al. Proteome of ubiquitin/MVB pathway: possible involvement of iron-induced ubiquitylation of transferrin receptor in lysosomal degradation. *Genes Cells*. 2011;16(4):448-466.
37. Chen K, Liu J, Heck S, Chasis JA, An X, Mohandas N. Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. *Proc Natl Acad Sci U S A*. 2009;106(41):17413-17418.
38. Liu J, Zhang J, Ginzburg Y, et al. Quantitative analysis of murine terminal erythroid differentiation in vivo: novel method to study normal and disordered erythropoiesis. *Blood*. 2013;121(8):e43-49.
39. Nguyen AT, Prado MA, Schmidt PJ, et al. UBE2O remodels the proteome during terminal erythroid differentiation. *Science*. 2017;357(6350).
40. Simpson CF, Kling JM. The mechanism of denucleation in circulating erythroblasts. *J Cell Biol*. 1967;35(1):237-245.
41. Babst M, Katzmann DJ, Estepa-Sabal EJ, Meerloo T, Emr SD. Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting. *Dev Cell*. 2002;3(2):271-282.
42. Teis D, Saksena S, Emr SD. Ordered assembly of the ESCRT-III complex on endosomes is required to sequester cargo during MVB formation. *Dev Cell*. 2008;15(4):578-589.
43. Shields SB, Oestreich AJ, Winistorfer S, et al. ESCRT ubiquitin-binding domains function cooperatively during MVB cargo sorting. *J Cell Biol*. 2009;185(2):213-224.
44. Caballe A, Martin-Serrano J. ESCRT machinery and cytokinesis: the road to daughter cell separation. *Traffic*. 2011;12(10):1318-1326.
45. Babst M. MVB vesicle formation: ESCRT-dependent, ESCRT-independent and everything in between. *Curr Opin Cell Biol*. 2011;23(4):452-457.
46. Adell MA, Vogel GF, Pakdel M, et al. Coordinated binding of Vps4 to ESCRT-III drives membrane neck constriction during MVB vesicle formation. *J Cell Biol*. 2014;205(1):33-49.
47. Stoten CL, Carlton JG. ESCRT-dependent control of membrane remodelling during cell division. *Semin Cell Dev Biol*. 2018;74:50-65.
48. Shim JH, Xiao C, Hayden MS, et al. CHMP5 is essential for late endosome function and down-regulation of receptor signaling during mouse embryogenesis. *J Cell Biol*. 2006;172(7):1045-1056.
49. Greenblatt MB, Park KH, Oh H, et al. CHMP5 controls bone turnover rates by dampening NF-kappaB activity in osteoclasts. *J Exp Med*. 2015;212(8):1283-1301.
50. Baarlink C, Wang H, Grosse R. Nuclear actin network assembly by formins regulates the SRF coactivator MAL. *Science*. 2013;340(6134):864-867.
51. Miralles F, Posern G, Zaromytidou AI, Treisman R. Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell*. 2003;113(3):329-342.
52. Eisenmann KM, Dykema KJ, Matheson SF, et al. 5q- myelodysplastic syndromes: chromosome 5q genes direct a tumor-suppression network sensing actin dynamics. *Oncogene*. 2009;28(39):3429-3441.
53. Poser S, Impey S, Trinh K, Xia Z, Storm DR. SRF-dependent gene expression is required for PI3-kinase-regulated cell proliferation. *EMBO J*. 2000;19(18):4955-4966.
54. Tsunoda T, Takagi T. Estimating transcription factor bindability on DNA. *Bioinformatics*. 1999;15(7-8):622-630.
55. Takahashi Y, He H, Tang Z, et al. An autophagy assay reveals the ESCRT-III component CHMP2A as a regulator of phagophore closure. *Nat Commun*. 2018;9(1):2855.
56. Klionsky DJ, Abdelmohsen K, Abe A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy*. 2016;12(1):1-222.
57. Mauthe M, Orhon I, Rocchi C, et al. Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy*. 2018;14(8):1435-1455.

58. Lux SEt. Anatomy of the red cell membrane skeleton: unanswered questions. *Blood*. 2016;127(2):187-199.
59. DeWard AD, Alberts AS. Ubiquitin-mediated degradation of the formin mDia2 upon completion of cell division. *J Biol Chem*. 2009;284(30):20061-20069.
60. Watanabe S, Ando Y, Yasuda S, et al. mDia2 induces the actin scaffold for the contractile ring and stabilizes its position during cytokinesis in NIH 3T3 cells. *Mol Biol Cell*. 2008;19(5):2328-2338.
61. Keerthivasan G, Small S, Liu H, Wickrema A, Crispino JD. Vesicle trafficking plays a novel role in erythroblast enucleation. *Blood*. 2010;116(17):3331-3340.
62. Blanc L, Liu J, Vidal M, Chasis JA, An X, Mohandas N. The water channel aquaporin-1 partitions into exosomes during reticulocyte maturation: implication for the regulation of cell volume. *Blood*. 2009;114(18):3928-3934.
63. Resnitzky P, Shaft D, Shalev H, et al. Morphological features of congenital dyserythropoietic anemia type I: The role of electron microscopy in diagnosis. *Eur J Haematol*. 2017;99(4):366-371.

Figure Legend:

Figure 1. mDia2 is required for the mobility of R1 reticulocytes. (A) Time lapse real-time microscopy of enucleating erythroid cells from the cultured bone marrow erythroblasts from the indicated mice. Still frames of cells at the indicated time (seconds) are illustrated. The bottom panels illustrate an enucleating cell with two extruding nuclei. Nuclei were stained with NucRed Live 647. Scale bars: 1 μm. White dots outline R1 reticulocytes. Far right panels show the overlapped outlines of the dynamic reticulocytes at different time points. (B) Scanning electro-microscopy analyses of enucleating cells from A. (C) Mean corpuscular volume of red blood cells from peripheral blood of indicated mice at 1 month old. (D) Wright-Giemsa stains of peripheral blood smears from indicated mice at 1 month old. Scale bars: 10 μm.

Figure 2. Loss of mDia2 compromises erythrocyte membrane skeleton structure. (A) Transmission electron microscopy study of membrane skeleton of ghost erythrocytes from the indicated mice. Data are representative of 13 randomly selected fields. (B) The length distributions of the spectrin tetramers with corresponding frequencies in ghost erythrocytes

calculated based on the electron microscopy images in A. (C) Computationally predicted stress of the membrane skeleton under a uniaxial tension with the increased deformation (λ_1) of the indicated mice. A shear modulus $\mu_0 = 13.1\, pN/\mu m$ is estimated for mDia2$^{fl/fl}$ cells and $\mu_0 = 26.5\, pN/\mu m$ for mDia2$^{fl/fl}$Vav-Cre cells. (D) STORM imaging analyses of actin protofilaments in ghost erythrocytes from indicated mice. Scale bars: 1 μm. (E) Western blot analyses of indicated erythroid membrane skeleton proteins of ghost erythrocytes from the indicated mice. Equal number of cells were used. (F) Immunoprecipitation assay using IgG or mDia2 antibodies in normal human ghost erythrocytes. Western blot analyses were performed to determine the co-precipitated proteins. (G) Immuno-gold stain of mDia2 and electron microscopy analysis of normal human ghost erythrocytes. Yellow dots and blue stripes in the schematic spectrin graph indicate gold particles and spectrin bands, respectively.

Figure 3. mDia2 is involved in membrane remodeling and organelle clearance during terminal erythropoiesis. (A) Lineage-negative cells from the bone marrow of the indicated mice were cultured in erythropoietin-containing medium for 2 days. Flow cytometric analysis was performed to determine the surface levels of the indicated proteins, DNA, and forward scatter (FSC) in wild type and mDia2 deficient reticulocytes. (B) Same as A except the cells were also stained with lysosome tracker and MitoTracker for the detection of lysosome and mitochondria content, respectively. (C-D) Fluorescence analyses of the indicated organelles on the orthochromatic stages of the erythroblasts (C) and reticulocytes (D) in cells from B.

Figure 4. Defect in endolysosomal and autolysosomal formation and proteome remodeling in mDia2 deficient erythroid cells. (A) Transmission electron microscopy studies of reticulocytes from mDia2$^{fl/fl}$Vav-Cre mice (bottom) and mDia2$^{fl/fl}$ mice (top). An adjacent

orthochromatic erythroblast (bottom right) with numerous organelles and vesicles is also seen. Scale bars: 1 μm. Arrows in insert III indicate vesicles. (B) Lineage-negative cells from the bone marrow of the indicated mice were cultured in erythropoietin-containing medium for 2 days. Quantitative analyses of extracellular vesicles from the culture media were performed. * $p < 0.05$. (C) Transmission electron microscopy analysis of a representative orthochromatic stage erythroblast from mDia2$^{fl/fl}$Vav-Cre mice. Images are representative of 10 randomly selected fields. (D) Volcano plot of quantitative proteomic study. Proteins significantly up (red) or down (blue) regulated in mDia2 deficient reticulocytes are presented. KEGG pathway enrichment analyses of proteins up or down regulated by >25% are shown. Numbers of proteins per group are indicated on the right. The experiments were repeated three times and the data were obtained with combined individual analysis.

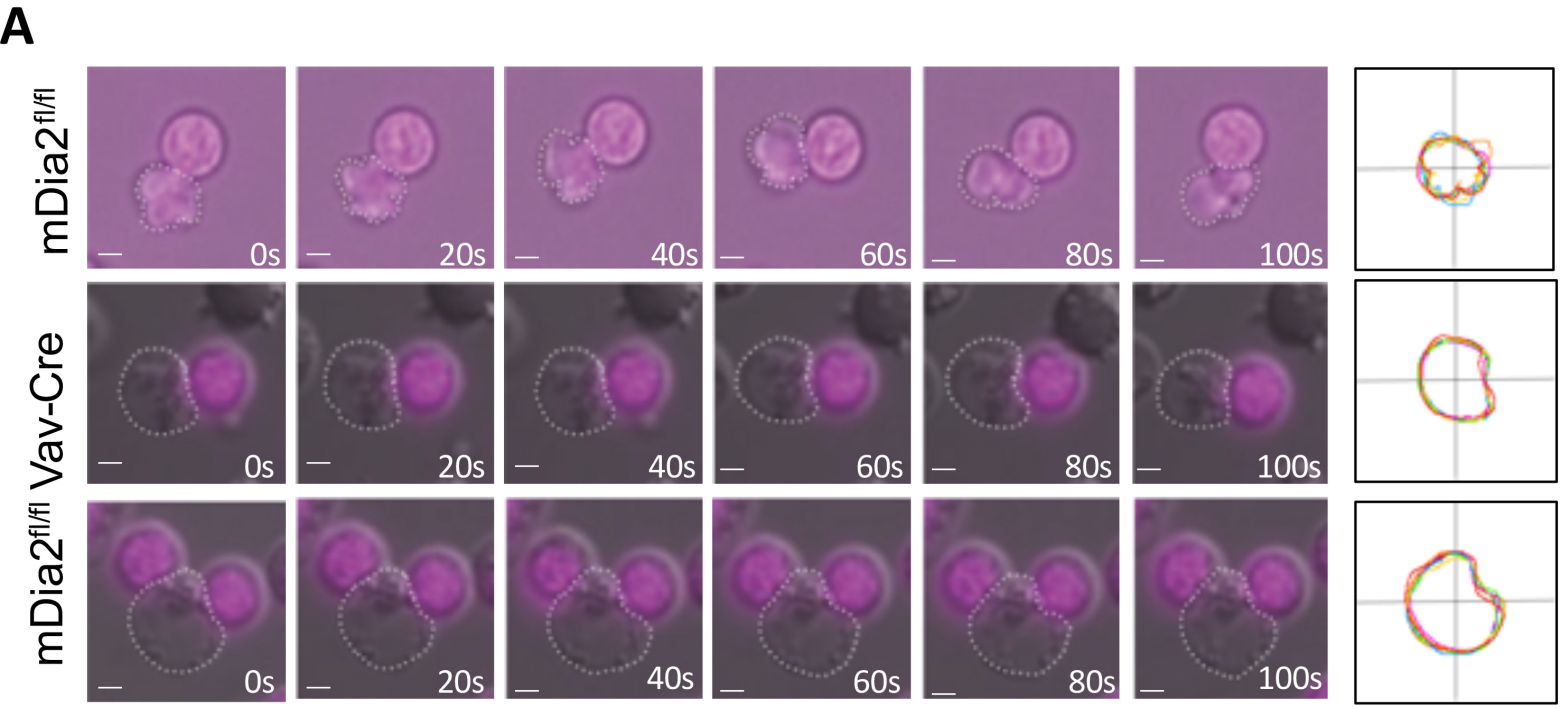
Figure 5. ESCRT-III complex mediates the function of mDia2. (A) Bone marrow lineage negative cells from the indicated mice were cultured in erythropoietin-containing medium for 2 days. Immunofluorescent stains of the indicated markers on the orthochromatic erythroblasts were performed on day 2. Scale bar: 1 μm. Images are representative of 10 randomly selected fields. (B) Immunofluorescent stain of Chmp5 in dividing cells of cultured erythroblasts from control and mDia2 deficient mice. Scale bar: 1 μm. Images are representative of 10 randomly selected fields. (C) Schematic view of the genomic region of Chmp5 with two predicated SREs in its promoter region. The sequences of these SREs and their mutant forms used in the luciferase assay are listed. The lower panel is the luciferase reporter assay. 293T cells were co-transfected with luciferase reporters containing the indicated wild type (WT) or mutant SREs (MUT) together with SRF-expressing construct in the presence of a Renilla luciferase expression vector (pRL-TK). Firefly and Renilla luciferase activities were measured after 24 h. (D) Chromatin immunoprecipitation (ChIP) assay in day 2 cultured wild-type erythroblasts with

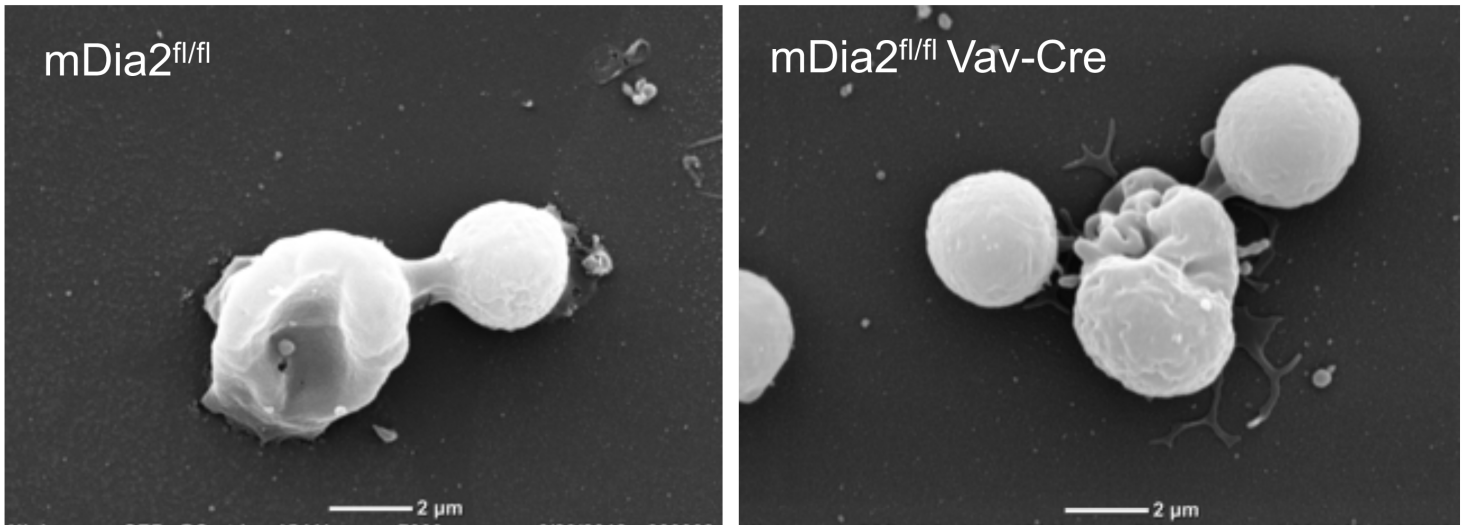
indicated antibodies followed by PCR of the SREs on Chmp5 promoter. (E) ChIP assays as D using SRF antibody or IgG on day 2 cultured erythroblasts from the indicated mice followed by PCR of the SREs on Chmp5 promoter. (F) Real time RT-PCR analyses of Chmp5 mRNA levels of day 2 cultured erythroblasts from control or mDia2$^{fl/fl}$Vav-Cre (KO) mice transduced with indicated genes on day 0. *** $p < 0.001$. (G) Wild-type bone marrow lineage negative cells were transduced with MSCV retroviruses expressing GFP and different shRNAs targeting Chmp5. The transduction efficiency was ~40% across all the constructs (not shown). The cells were then cultured for 2 days in erythropoietin-containing media. Immunofluorescent stains of Chmp5 were performed. Images are representative of 5 randomly selected fields. (H) Quantification of bi-nucleated orthochromatic erythroblasts in G. * $p < 0.05$; *** $p < 0.001$.

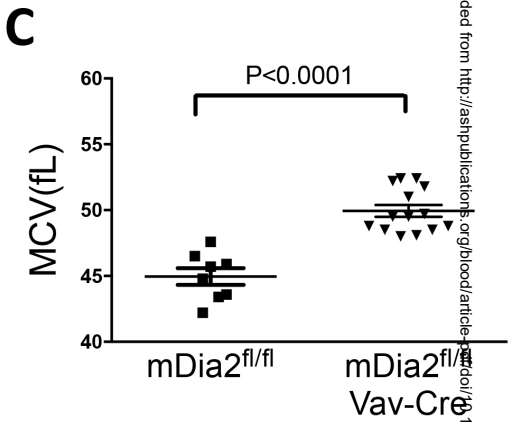
Figure 6. Defects of autophagosome-lysosome fusion in mDia2 deficient erythroid cells. (A) Lineage-negative cells from the bone marrow of the indicated mice were cultured in erythropoietin-containing medium for 2 days. Representative images of LC3B-stained orthochromatic erythroblasts were presented. Scale bars: 2 μm. Images are representative of 10 randomly selected fields. The bottom panels illustrate a binucleated orthochromatic erythroblast. (B) Western blot analyses of the cells from A. (C) Lineage negative cells from the bone marrow of the indicated mice were cultured in erythropoietin-containing medium for 2 days. Flow cytometric analyses of GFP intensity on the Ter119+ DNA+ erythroblasts were performed. (D) Western blot analyses of cells in C for the detection of GFP-LC3 and LC3. (E) Lineage negative cells from the indicated mice were transduced with Lenti-mCherry-EGFP-LC3. The cells were cultured in erythropoietin-containing medium for 2 days. Representative images of erythroblasts at different developmental stages based on the nuclear size were presented. Scale bars: 10 μm. Images are representative of 10 randomly selected fields.

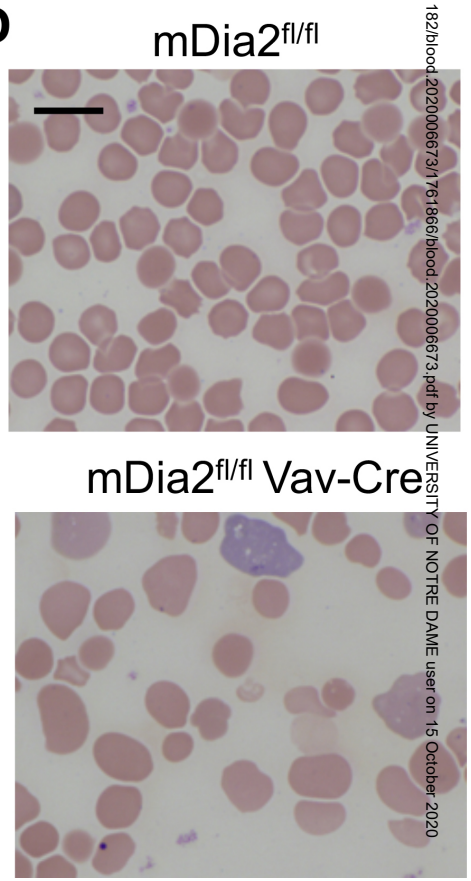
Figure 7. Overexpression of Chmp5 ameliorates defects in late stage terminal erythropoiesis in mDia2 deficient mice. (A) Schematic outline of the transplantation strategy. (B) Red blood cell count and hemoglobin levels in recipient mice transplanted with indicated bone marrow cells for 2 months. (C) Spleen weigh of mice in B. (D) Bone marrow smear morphologic analyses of mice in B. Arrows indicate binucleated orthochromatic erythroblasts. Scale bar: 20 μm. Flow cytometric assays on the bottom quantify the percentage of S/G2/M phases reflecting the number of binucleated cells. (E) Quantitative analysis of d. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (F) Lineage-negative cells from the bone marrow of the indicated recipient mice were cultured in erythropoietin-containing medium for 2 days. Flow cytometric analyses of indicated parameters and proteins on reticulocytes were performed.

Liu et al, Figure 1









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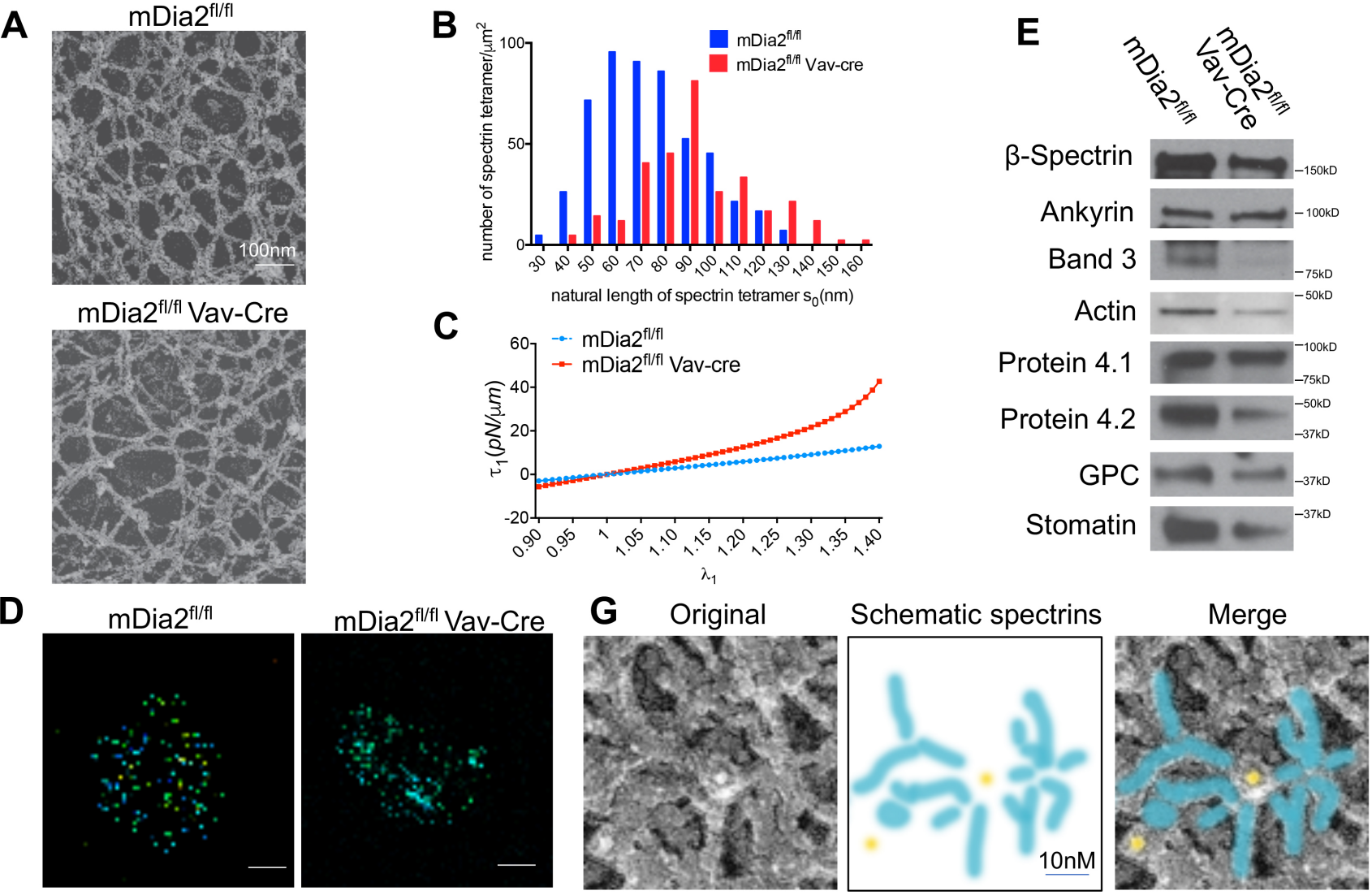

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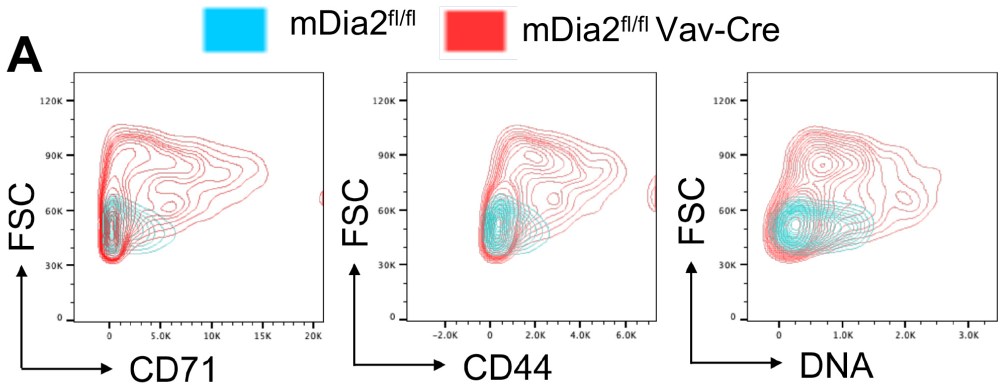
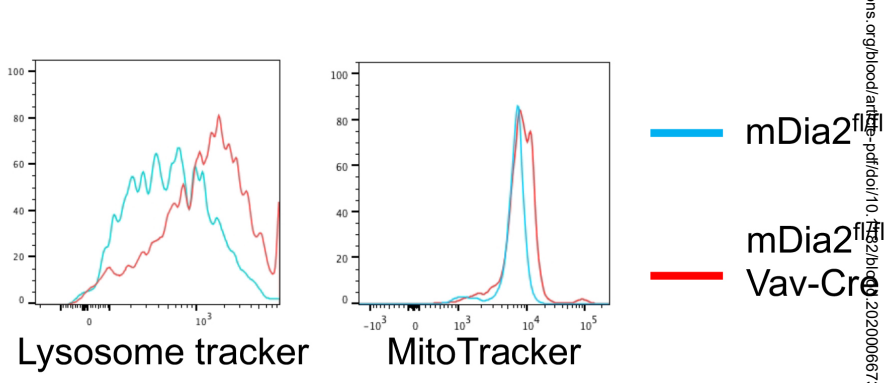
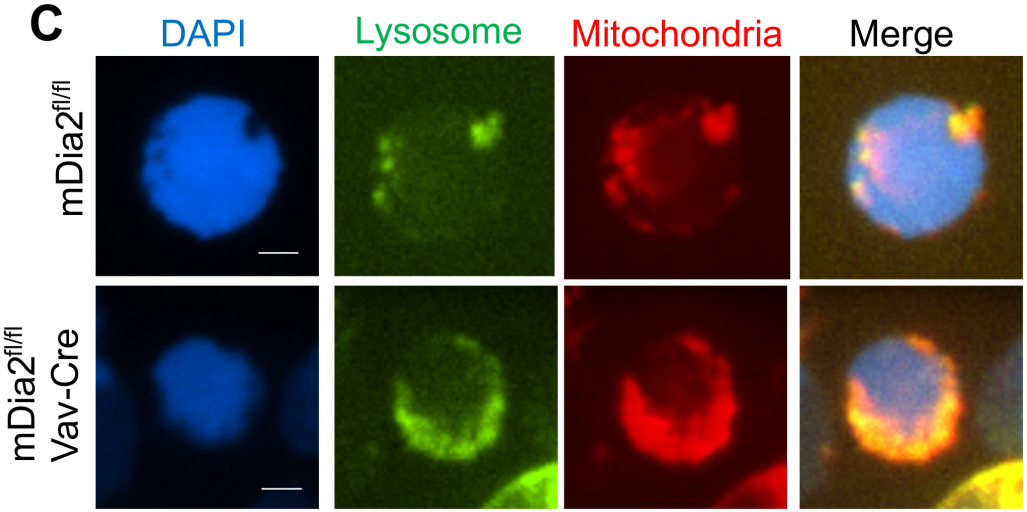
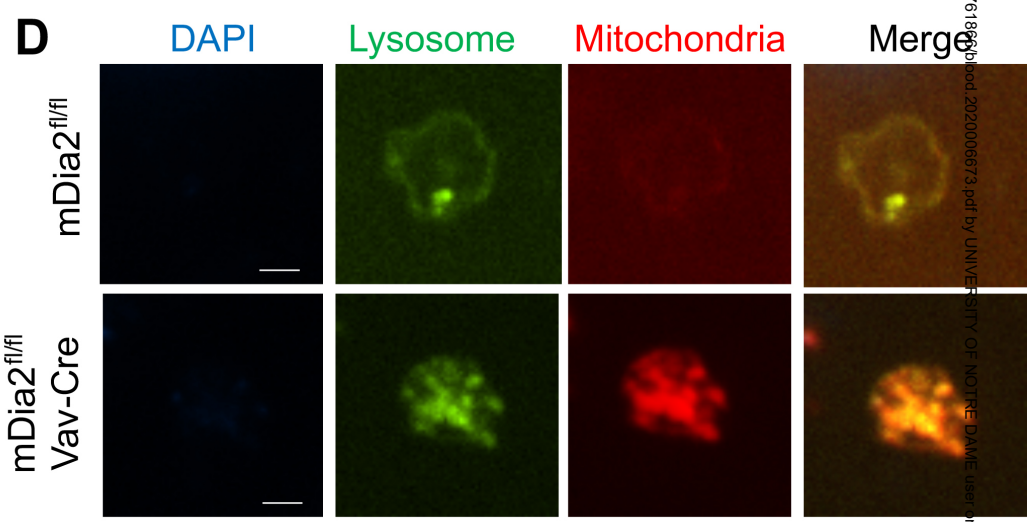
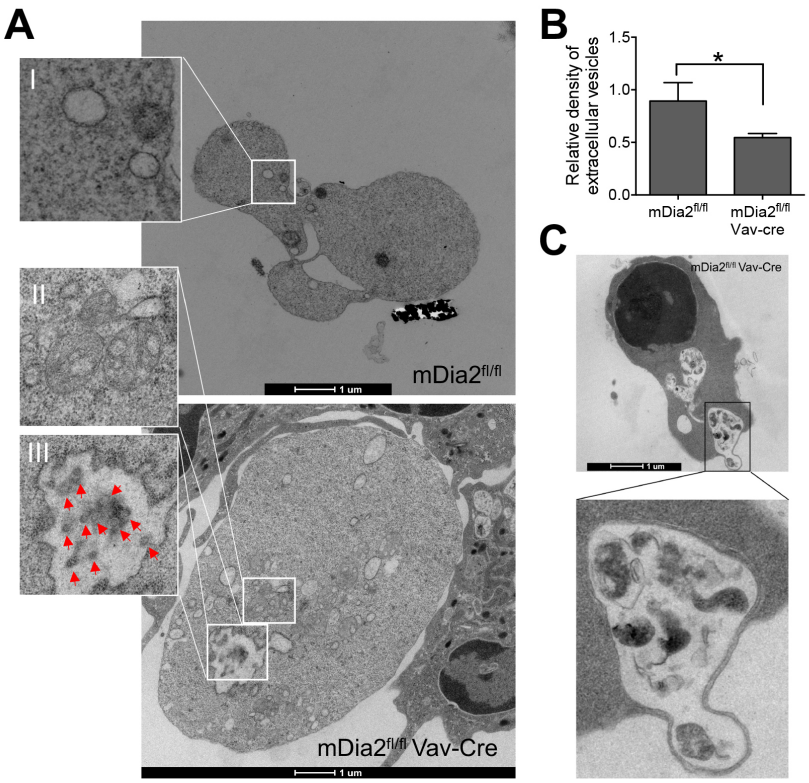


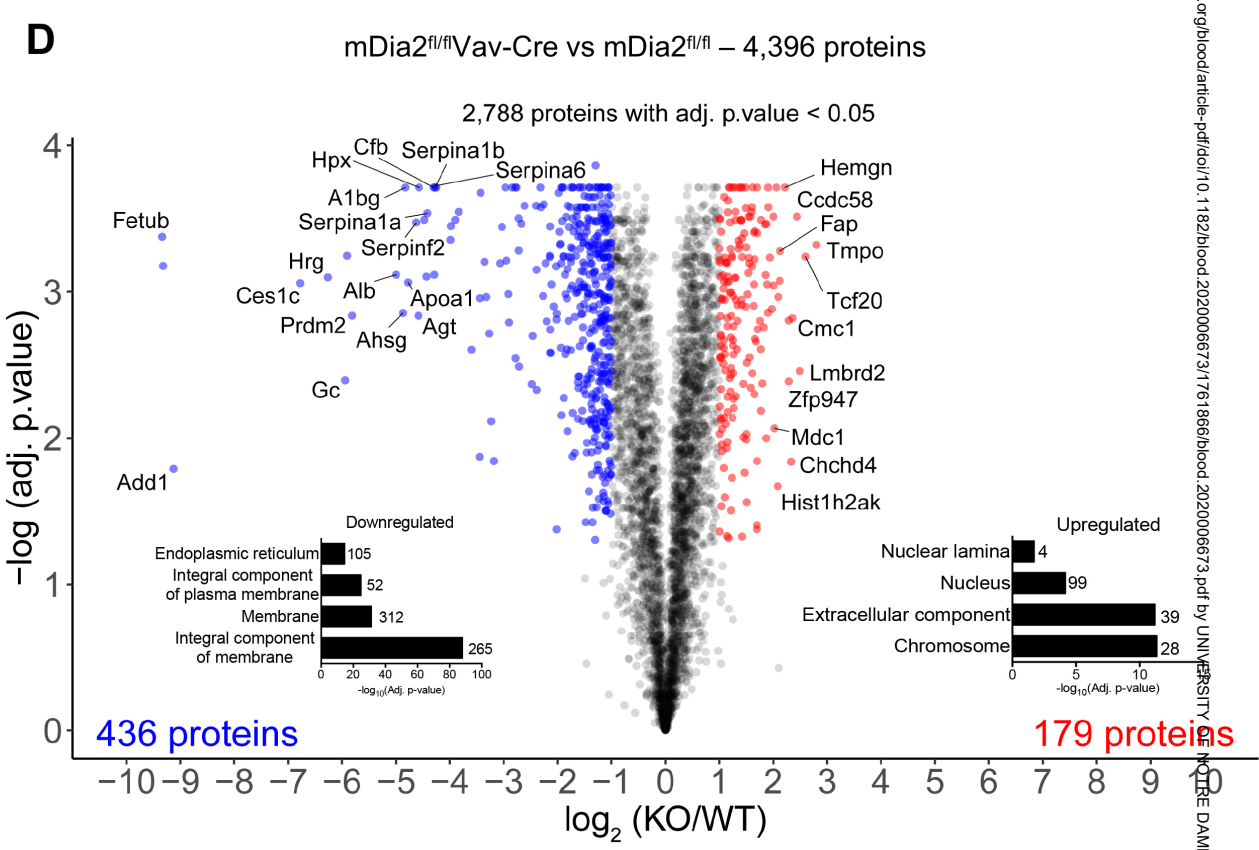


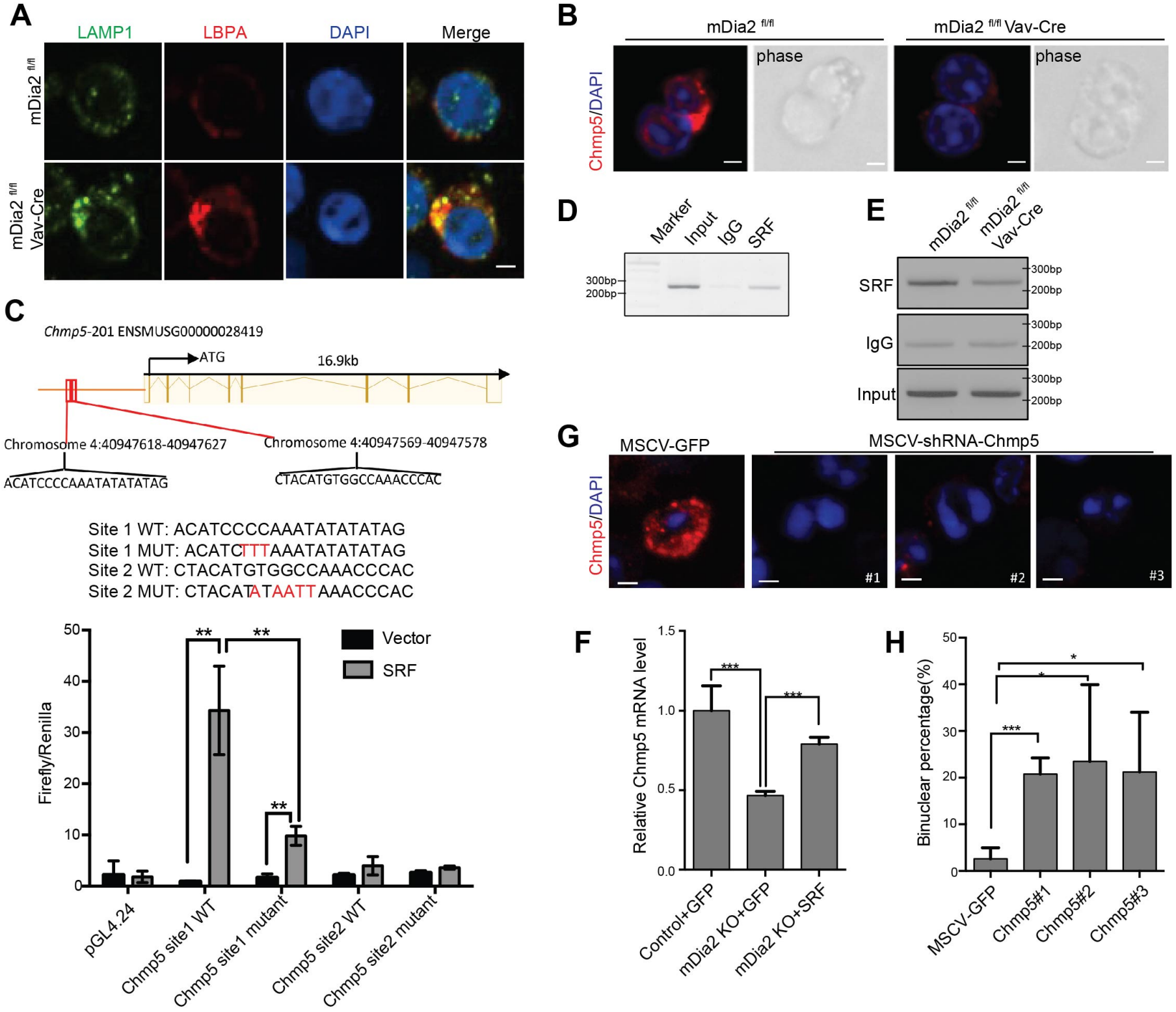




Liu et al, Figure 4







Liu et al, Figure 6