SUPPLEMENTARY APPENDIX

Histone deacetylase 6 regulates cytokinesis and erythrocyte enucleation through deacetylation of formin protein mDia2

Xuehui Li,[1#] Yang Mei,[2#] Bowen Yan,[1#] Eric Vitriol,[1] Suming Huang,[3,4] Peng Ji[2] and Yi Qiu[1]

[1]Department of Anatomy and Cell Biology, College of Medicine, University of Florida, Gainesville, FL, USA; [2]Department of Pathology, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA; [3]Department of Biochemistry and Molecular Biology, College of Medicine, University of Florida, Gainesville, FL, USA and [4]Macau Institute for Applied Research in Medicine and Health, State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology, Avenida Wai Long, Taipa, Macau

#These authors contributed equally to the manuscript.

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Correspondence: qiuy@ufl.edu or peng-ji@fsm.northwestern.edu

Supplementary Methods and Figures

Methods:

Cells and DNA constructs

MEL cell and NIH 3T3 cells were obtained and cultured as described (1). Retroviral vector pSuper and pSuper HDAC6 shRNA were previously described (2). MSCV-GFP and MSCV-GFP-mDia2 shRNA were described (3). pCR-Blunt-mDia2 plasmid containing mouse mDia2 cDNA was a kind gift from S. Narumiya (Kyoto University, Kyoto, Japan) (4). The shRNA resistant mutant plasmids of pCR-Blunt-mDia2 WT, pCR-Blunt-mDia2K970R, and pCR-Blunt-mDia2K970Q were generated using site directed mutagenesis according to the manufacturer's protocol (Agilent Technologies). The presence of directed mutations was confirmed by Sanger sequencing. The Flag tagged mDia2 and mutants were subsequently cloned into the MICD4 viral vector (5).

Immunostaining: Fetal liver cells were plated in fibronectin-coated 8-well chamber vessels (Corning) and were fixed with 4% paraformaldehyde and 0.4 M sucrose in PBS. Cells were subsequently incubated with 3% control serum in PBS at 4°C overnight and then incubated at room temperature for 2 h with the following primary antibodies: anti-mDia2 (1:200) (ECM Biosciences), anti-HDAC6 (1:200) (Millipore) or goat anti-HDAC6 (1:200) (Santa Cruz). The cells were then incubated with appropriate secondary antibodies coupled to Alexa Fluor 488 (1:1000), or Alexa Fluor 594 (1:1000), and/or Texas-Red phalloidin (1:1000) (Invitrogen). The samples were mounted in UltraCruz mounting medium DAPI (Santa Cruz) on glass slides.

Immunoprecipitation and western blot analysis

The immunoprecipitation and Western blot assays were performed as previously described (6) with the following antibodies: anti-Flag (Abcam), anti-GFP (Sigma-Aldrich), anti-mDia2, anti-HDAC6, anti-GAPDH (Cell Signaling), and anti-Acetyl-lysine (Upstate Biotechnology).

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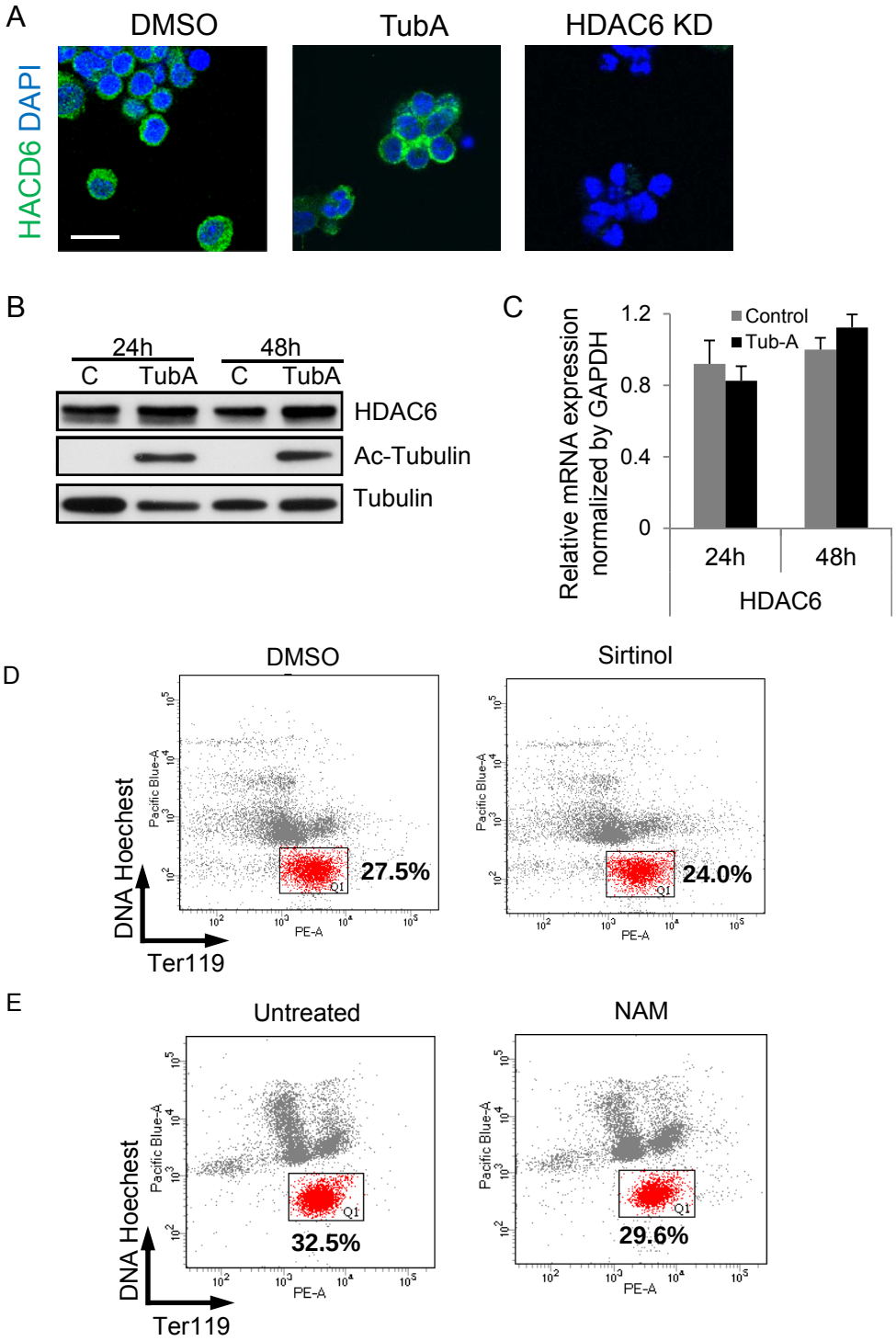
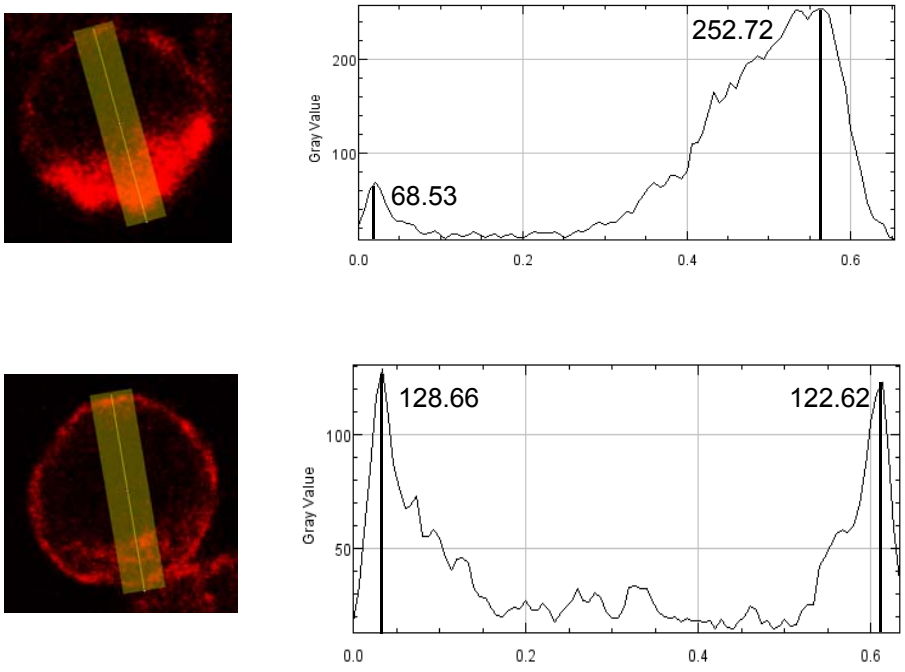


Figure S1. **Inhibition of HDAC6 impairs enucleation.** (A) Immunostaining of HDAC6 in cultured TER119- mouse fetal progenitors treated with TubA or infected with retrovirus harboring HDAC6 shRNA as indicated. The cells were fixed at 24 h and then immunostained with anti-HDAC6 conjugated with Alexa Fluor 488 and DAPI. Scale bar is 10μm. (B) TER119- mouse fetal progenitors were treated with DMSO (C) or TubA for 24 or 48 h and the cell lysate was subjected to western blot with antibodies as indicated. (C) The HDAC6 gene expression was determined by RT-real time PCR and normalized with the expression of GAPDH. Flow cytometric analysis of cultured TER119- mouse fetal progenitors at 48h treated with DMSO or sirtinol (50μM) (D) and nicotinamide (NAM) (5mM) (E).

A



B

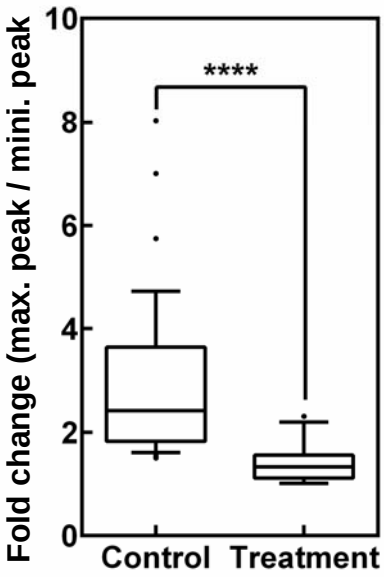


Figure S2. **Inhibition of HDAC6 blocks F-actin polarization.**
(A) Quantification of F-actin fluorescence intensity in differentiating TER119- mouse fetal progenitors treated with DMSO or TubA (ref. Fig. 2B). (B) Quantification polarized F-actin in cells treated with DMSO or TubA. The error bars represent mean +/-s.d. (n>=30), ****p<0.0001.

Figure S3

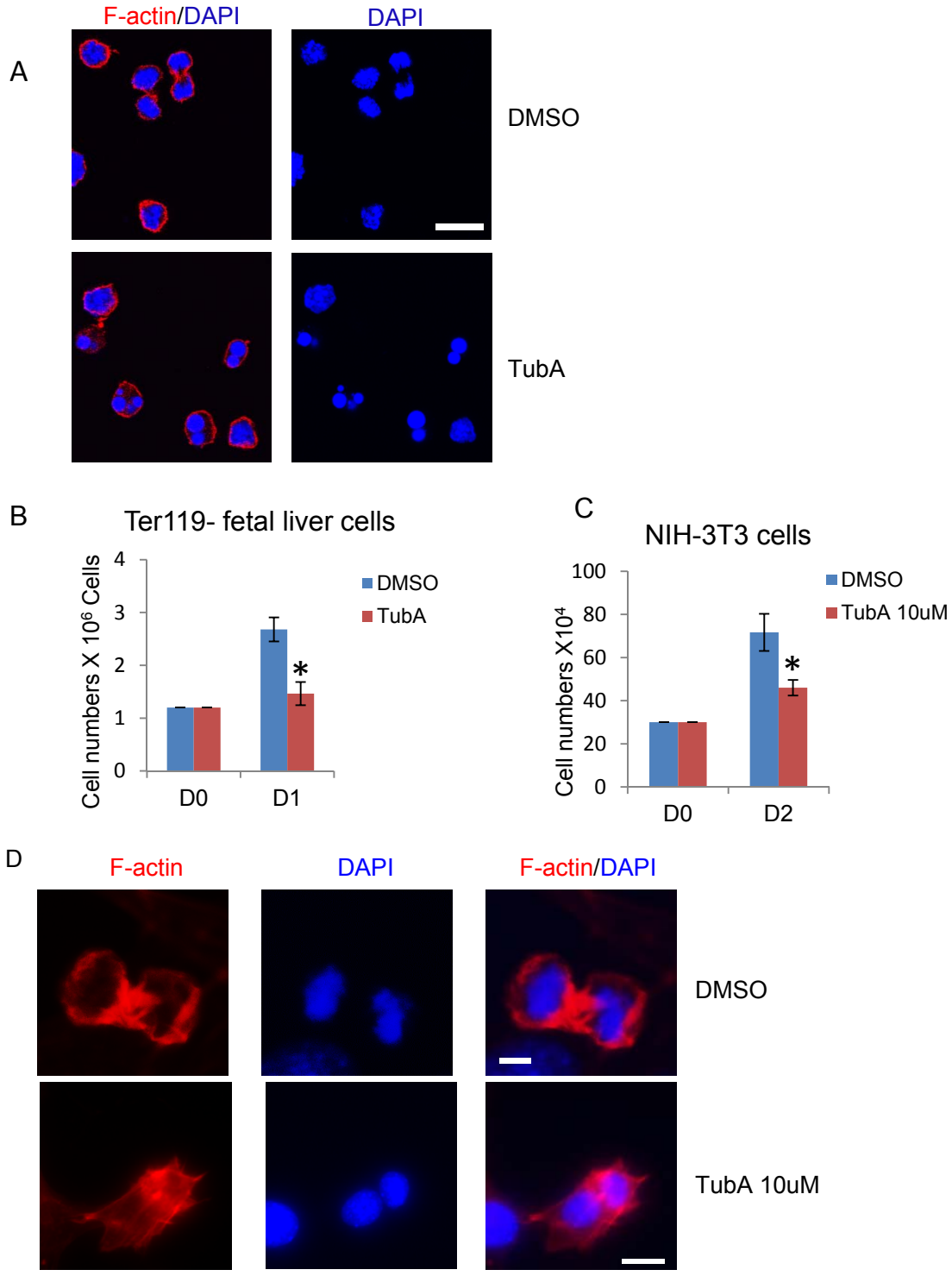


Figure S3. **Inhibition of HDAC6 impairs cell proliferation and cytokinesis.** (A) Immunostaining of F-actin in cultured uninduced TER119- mouse fetal progenitors treated with DMSO or TubA for 18 h. Scale bar is 10μm. (B) DMSO or 10 um TubA was added to 1.2 X10^6 cultured TER119- mouse fetal progenitors and cell number was counted 24 h after treatment. (C) 3X10^5 NIH 3T3 cells were seeded and DMSO or TubA was added 24 h after seeding. The cell number was counted 48 h after seeding. The data presents as mean +/- s.d. (n=3), *p<0.05. (D) NIH 3T3 cells were treated with DMSO or 10 uM TubA for 24 h. Cells were fixed and immunostained with phalloidin-Texas red and DAPI. Scale bar is 5μm.

Figure S4. K970 in FH2 domain of mouse mDia2 is highly conserved. FH2 domain of formin protein alignment among species. Conserved k970 is marked by the black box. Red shows highly conserved residues and blue shows less conserved residues. Unaligned residues are shown in grey. Sequence showed are Homo sapiens mDia2 (Q9NSV4), Mus musculus mDia2 (Q9Z207), Homo sapiens mDia1 (O60610), Mus musculus mDia1 (O08808), Drosophila melanogaster Diaphanous (NP_476981.1), and Saccharomyces cerevisiae Bni1 (CAA96179.1).

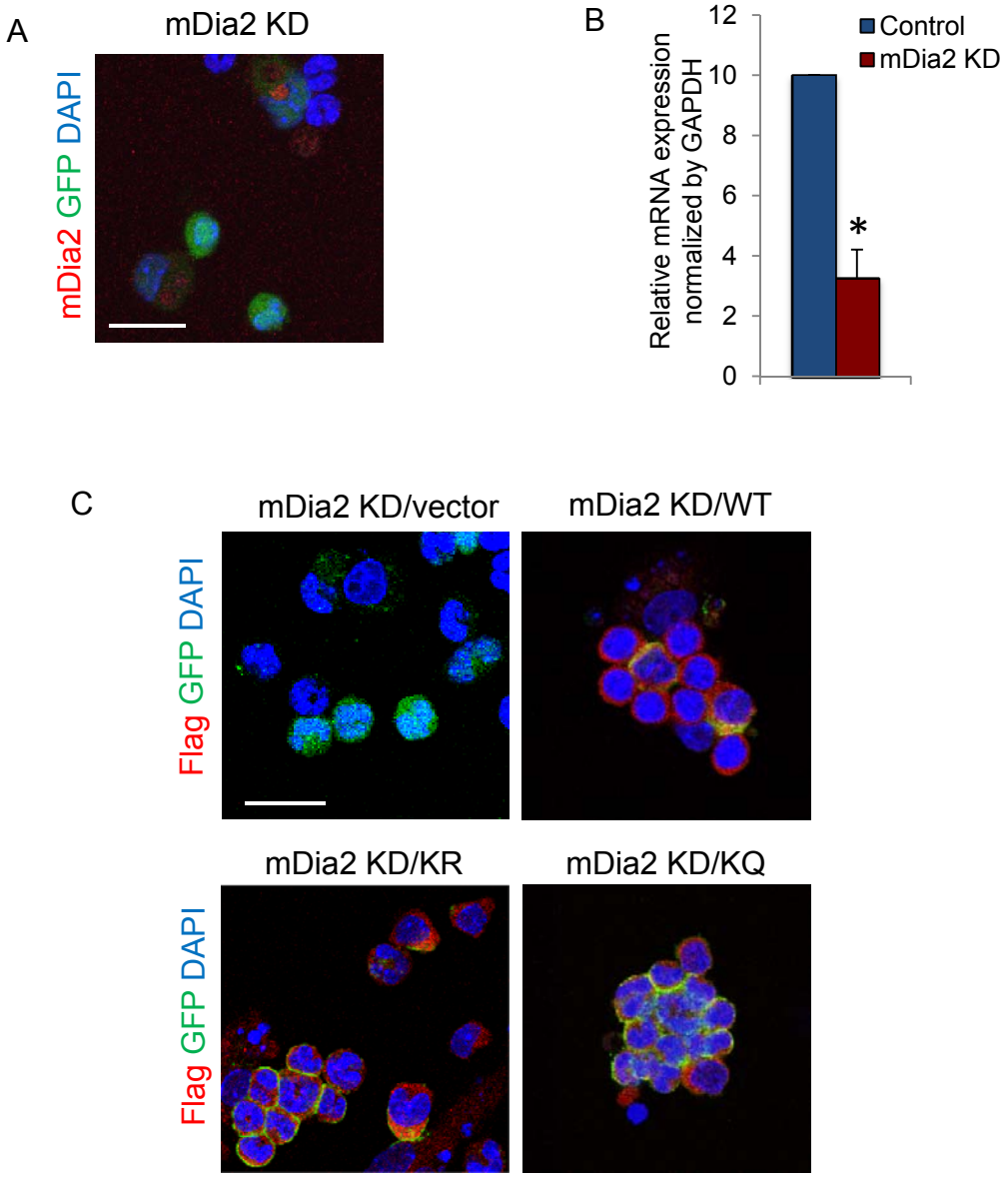


Figure S5. **Expression of mDia2 and mutants in knock down cells.** (A). TER119^{-} mouse fetal progenitors were infected with retrovirus harboring mDia2 shRNA with a GFP marker. The cells were fixed at 24 h and immunostained with anti-mDia2 conjugated with Alex-594 and DAPI. GFP indicates knockdown of endogenous mDia2 and mDia2 staining indicates endogenous mDia2. (B) The mDia2 gene expression in mDia2 knock down mouse fetal progenitors was determined by RT-real time PCR and normalized with the expression of GAPDH. (C) The mDia2 knock down cells were also co-infected with retrovirus that carrying shRNA resistant Flag tagged mDia2 WT, K970R or K970Q. The cells were fixed at 24 h and immunostained with anti-Flag conjugated with Alex-594 and DAPI. GFP indicates knockdown of endogenous mDia2 and Flag staining indicates re-expression of mDia2 mutants. Scale bar is 10μm.

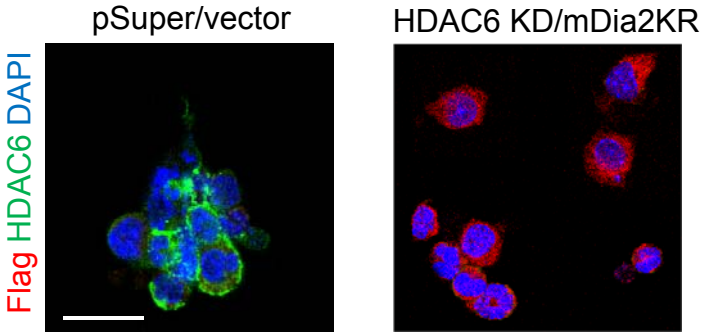


Figure S6. **Expression of mDia2 and mutants in knock down cells.** TER119^{-} mouse fetal progenitors were co-infected with retrovirus harboring HDAC6 shRNA and retrovirus that carrying Flag tagged mDia2 K970R. The cells were fixed at 24 h and immunostained with anti-Flag conjugated with Texas red, anti-HDAC6 conjugated with Alexa Fluor 488, and DAPI. Scale bar is 10μm.