PINK1 Inhibits Multimeric Aggregation and Signaling of MAVS and MAVS-dependent Lung Pathology

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ONLINE DATA SUPPLEMENT

Online Supplement

Materials and Methods

Evaluation of the alteration of mitochondrial membrane potential, ROS and pH

Cells were stimulated with 1 μM of rotenone (MilliporeSigma), 5 μM of nigericin (Invivogen), 5 μM of oligomycin A (MilliporeSigma), 50 μM of CCCP (MilliporeSigma), or 10 μM of valinomycin (MilliporeSigma) for 1 h. After harvesting, cells were loaded for 15 min to 100 nM of tetramethylrhodamine, ethyl Ester, perchlorate (TMRE, Abcam) or 5 μM of MitoSOX Red (Thermo Fisher Scientific) for the evaluation of mitochondrial membrane potential or mitochondrial ROS, respectively. For the measurement of mitochondrial pH, cells were loaded to 5 μM of carboxy SNARF-1 (Thermo Fisher Scientific) for 30 min and further incubate for 2.5 h (1), to allow for complete hydrolysis and preferential mitochondrial compartmentalization of the dye. After harvesting, cells were stimulated with 1 μM of rotenone, 5 μM of nigericin, 5 μM of oligomycin A, 50 μM of CCCP, or 10 μM of valinomycin. Cells were analyzed using a BD LSRII cytometer (BD Biosciences) and the FlowJo Version 10 software (TreeStar).

Complex V activity

Mitochondrial Complex V activity was measured by ELISA using Mitocheck Complex V Activity Assay Kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's instructions.

Plasmid construction and Biomolecular fluorescence complementation assay

The full length of mouse *Pink1* cDNA (Origene) was inserted into upstream of N-terminal fragment of Venus (VN) in pcDNA3.1 plasmid using *Not*I/*Cla*I sites, and the full length of mouse *Mavs* cDNA (Origene) was cloned to downstream of C-terminal fragment of Venus (VC) in pcDNA3.1 plasmid using *Bsp*E1/*Xba*I sites. For bimolecular fluorescence complementation (BiFC) assay, mouse embryonic fibroblasts (MEFs) were co-transfected with pcDNA3.1-mPINK1-VN vector and pcDNA3.1-VC-mMAVS vector using JetPEI-DNA transfection reagent. After 24 h transfection, cells were stimulated with 5 μM of nigericin or 10 μg/ml of polyI:C (Invivogen), fixed with 4% paraformaldehyde, and covered by mounting media (Thermo Fisher Scientific). Images were acquired using a Zeiss LSM710 confocal microscope (Carl Zeiss Inc).

Immunoprecipitation and immunoblot analysis

Immunoprecipitation was performed using Protein A magnetic beads (Bio-Rad). In brief, cell lysates were incubated with anti-human MAVS (Proteintech) or mouse MAVS (Cell Signaling Technology) antibodies for Human embryonic kidney (HEK) 293 and MEFs for 2 h at 4^{o}C, respectively. Subsequently, Protein A magnetic beads were added and incubate for 1 h at 4^{o}C. The immune complexes were washed five times with cold IP lysis buffer (Thermo Fisher Scientific). Then the associated proteins were eluted by adding 1X SDS sample loading buffer and loaded to 4-20% SDS-PAGE under reducing condition. Immunoblot analysis was done with various primary antibodies: α-MAVS and α-β-Actin (Santa Cruz Biotechnology); α-mouse IL-1β, α-MAVS (rodent specific), and α-VDAC (Cell Signaling Technology); α-NLRP3 and α-mouse Caspase-1 (p20) (Adipogen); α-PINK1 (Origene). For immunoblot of immunoprecipitants, HRP-conjugated conformation specific α-rabbit antibody (Cell Signaling Technology) was used as a secondary antibody.

Immunofluorescence staining and confocal microscopy

MEFs were stained with 100 nM MitoTracker Orange CMTMRos (Thermo Fisher Scientific) for 30 min at 37 ^{o}C, fixed with 4% paraformaldehyde, and then permeabilized with 0.1% Triton X-100/PBS. Cells were immunostained with primary antibodies (α-MAVS; Santa Cruz, α-PMP70; Thermo Fisher Scientific), and suitable Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific). Images were acquired using a Zeiss LSM710 confocal microscope and analyzed using ZEN2010 software (Carl Zeiss Inc).

Activation of NLRP3 inflammasomes

BMDMs, naïve peritoneal macrophages, or MEFs were primed with 1 μg/ml of LPS (MillporeSigma) for 4 h, and then stimulated with 5 μM of nigericin for indicated time. Supernatants were collected for the measurement of secreted interleukin (IL)-1β using ELISA kit (R&D Systems) and cells were lysed to RIPA buffer containing protease inhibitor cocktail (Roche), or harvested for the isolation of mitochondria.

Activation of Type I IFN response

BMDMs or MEFs were transfected with 10 μg/ml of high molecular weight polyI:C for indicated time using LyoVec transfection reagent (Invivogen) or JetPEI-DNA transfection reagent (Polyplus-Transfection). Supernatant was collected for the measurement of secreted interferon (IFN)-β using ELISA Kit (R&D Systems) and cells were harvested for the isolation of mitochondria, or lysed to TRIzol for the real-time quantitative PCR (qPCR) analysis. Primer information is listed in Table S1.

Collagen assay

Collagen contents in mouse lungs were measured by Sircol collagen assay kit (Biocolor) according to the manufacturer's instructions. The amount of collagen was measured using a standard curve for collagen provided by the manufacturer and was calculated as 'mg per total lung'. All assays were done in triplicate.

Transforming growth factor-*beta* (TGF- β)-1 measurement

For bleomycin-induced fibrosis models, the level of TGF-β1 in BAL fluid was measured with DuoSet kit ELISA kits (R&D Systems) according to the manufacturers' instructions.

Supplemental Figure and legend

Figure E1.

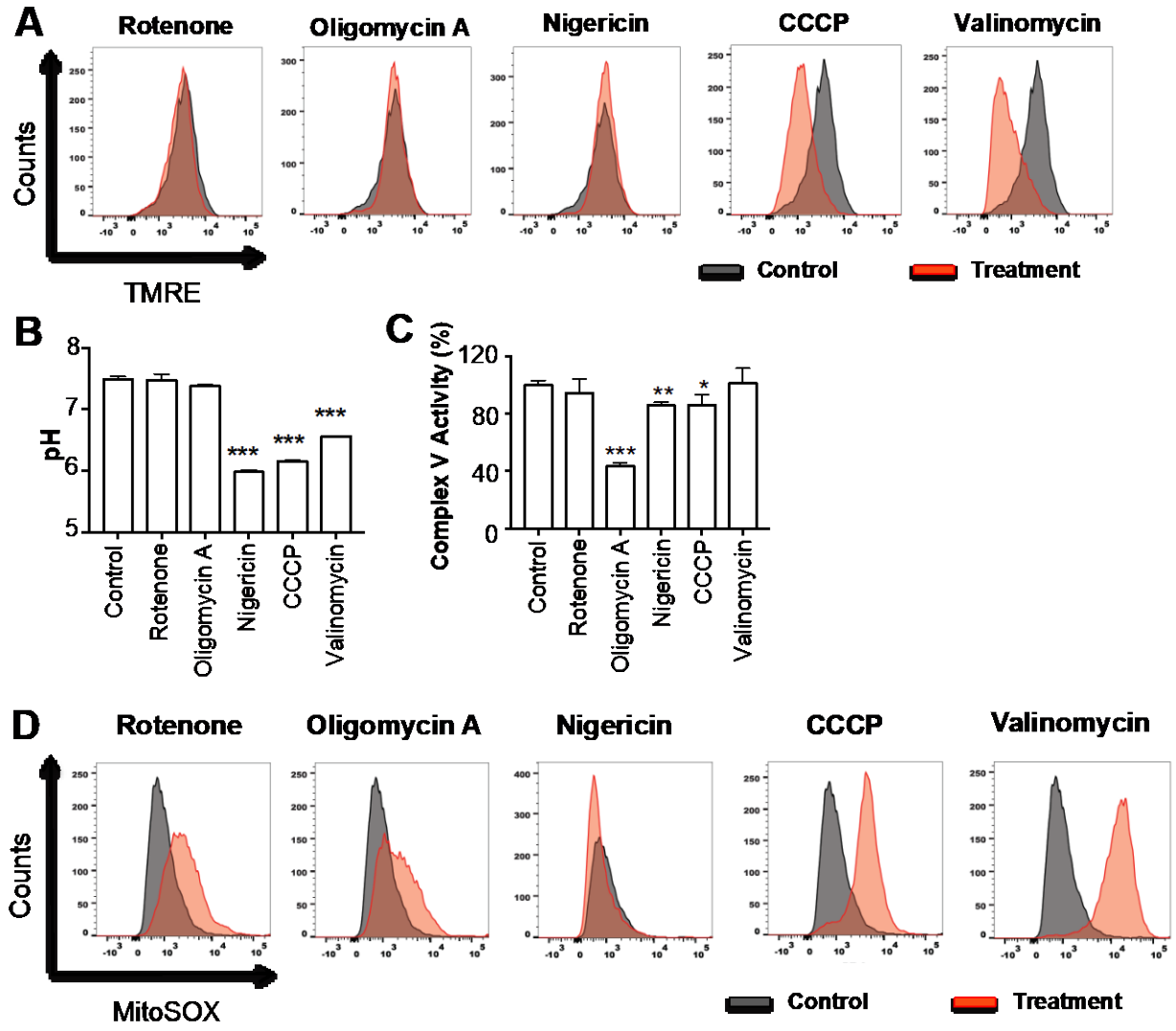


Figure E1. Evaluation of the nature of mitochondrial dysfunction induced by mitochondrial drugs HEK293 cells were stimulated with 1 μM of rotenone, 5 μM of nigericin, 5 μM of oligomycin A, 50 μM of CCCP, or 10 μM of valinomycin for 1 h. (A) After loading to 100 nM TMRE, mitochondrial membrane potential was measured by FACS. (B) After loading to 5 μM SNARF-1, HEK293 cells were further incubated for 2.5 h and then stimulated with 1 μM of rotenone, 5 μM of nigericin, 5 μM of oligomycin A, 50 μM of CCCP, or 10 μM of valinomycin. Then, the change of mitochondrial pH was measured by FACS. (C) Isolated mitochondria from HEK293 cells were added to 96-well plate and stimulated with 1 μM of rotenone, 5 μM of oligomycin A, 5 μM of nigericin, 50 μM of CCCP, or 10 μM of valinomycin. Then, the kinetics of complex V activity was measured by ELISA. (D) After loading to 5 μM MitoSOX Red, the production of mitochondrial ROS was measured by FACS. All experiments are repeated at least three times and representative images are shown. Means ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure E2

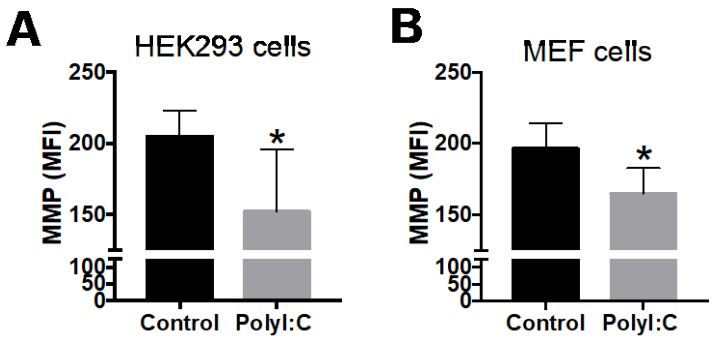


Figure E2. Mitochondrial depolarization by polyI:C

HEK293 (A) and MEFs (B) were stimulated with 10 μg/ml of polyI:C for 18 h or 6 h, respectively. After loading to 100 nM TMRE, mitochondrial membrane potential (MMP) was measured by FACS. All experiments were repeated at least three times. Means ± SD. *$P < 0.05$.

Figure E3

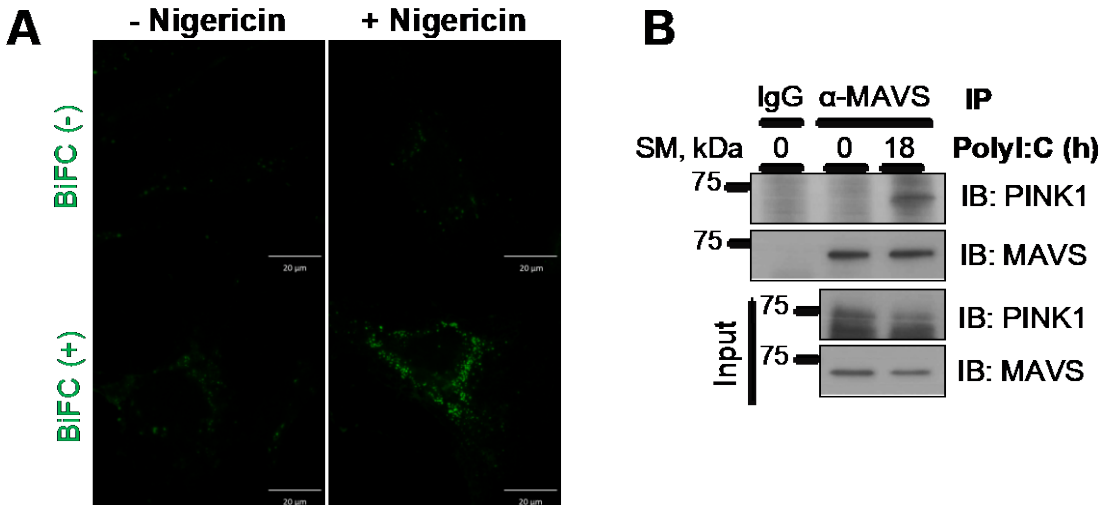


Figure E3. The molecular interaction of PINK1 with MAVS.

(A) MEFs were co-transfected with pcDNA3.1-mPINK1-VN and pcDNA3.1-VC-mMAVS vectors before treating with 5 μM of nigericin treatment for 1 h and the interaction between PINK1 and MAVS was evaluated by confocal microscopy (Scale bars, 20 μM; green, BiFC dimer). (B) MEFs were stimulated with 10 μg/ml of polyI:C for indicated time. The whole cell lysates were immunoprecipitated with α-MAVS antibody and immunoblotted with α-PINK1 antibody. All experiments are repeated at least three times.

Table E1. Primers used in this study for qRT-PCR.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
Ifnb	CAGCTCCAAGAAAGGACGAAC	GGCAGTGTAACTCTTCTGCAT
Isg56	CTGAGATGTCACTTCACATGGAA	GTGCATCCCCAATGGGTTCT
Cxcl10	CCAAGTGCTGCCGTCATTTTC	GGCTCGCAGGGATGATTTCAA
Isg15	GGTGTCCGTGACTAACTCCAT	TGGAAAGGGTAAGACCGTCCT
18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

<Reference>

1. Balut C, vandeVen M, Despa S, Lambrichts I, Ameloot M, Steels P, Smets I. Measurement of cytosolic and mitochondrial pH in living cells during reversible metabolic inhibition. *Kidney Int* 2008; 73: 226-232.

Original uncut gels corresponding to selected panels in Figures 1A, 1B, 3A, and 6E.

Because certain panels in these figures contained artifacts (stripes) presumably caused by the film developing process, we are including the uncut gels here.

Figure 1A

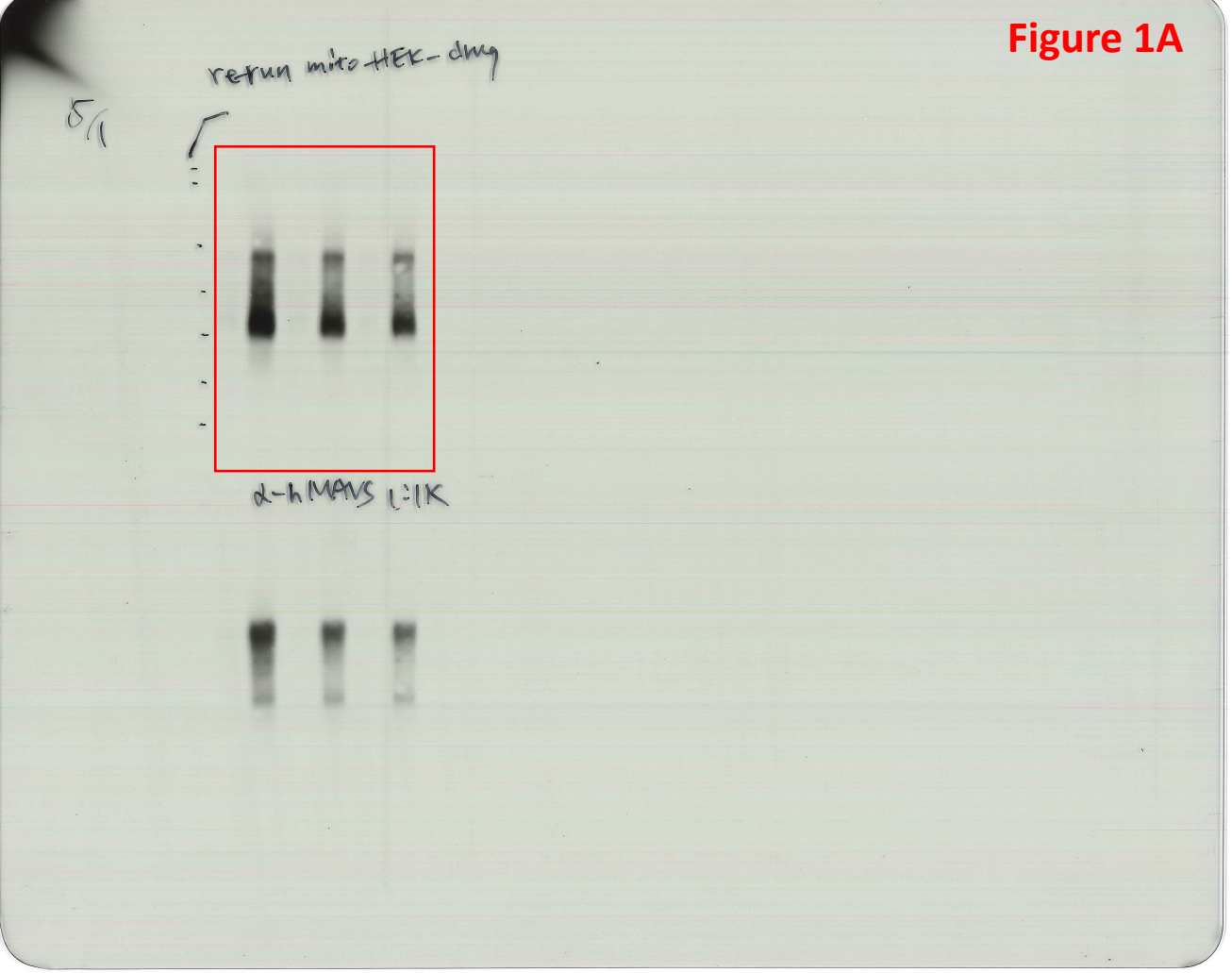


Figure 1A

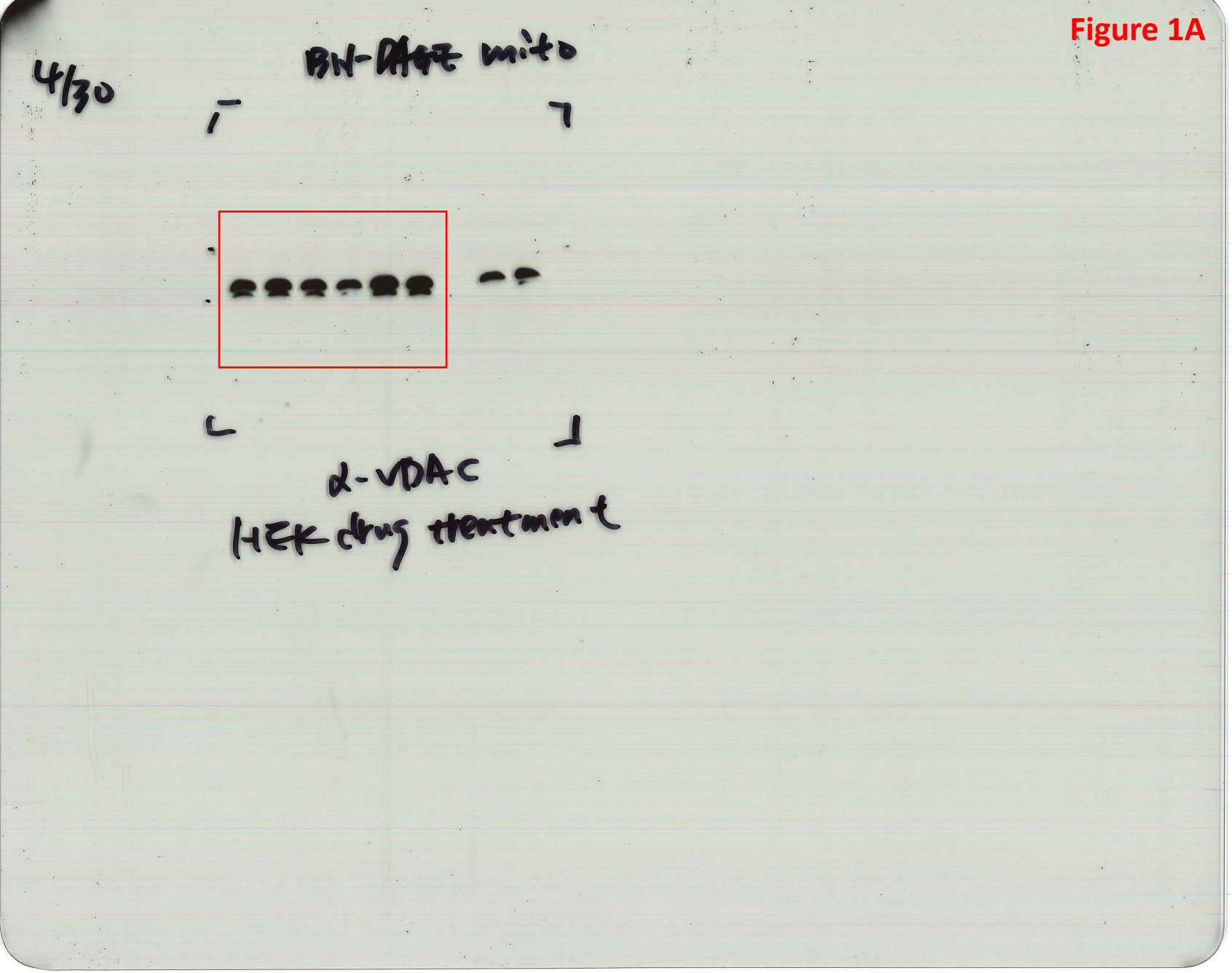
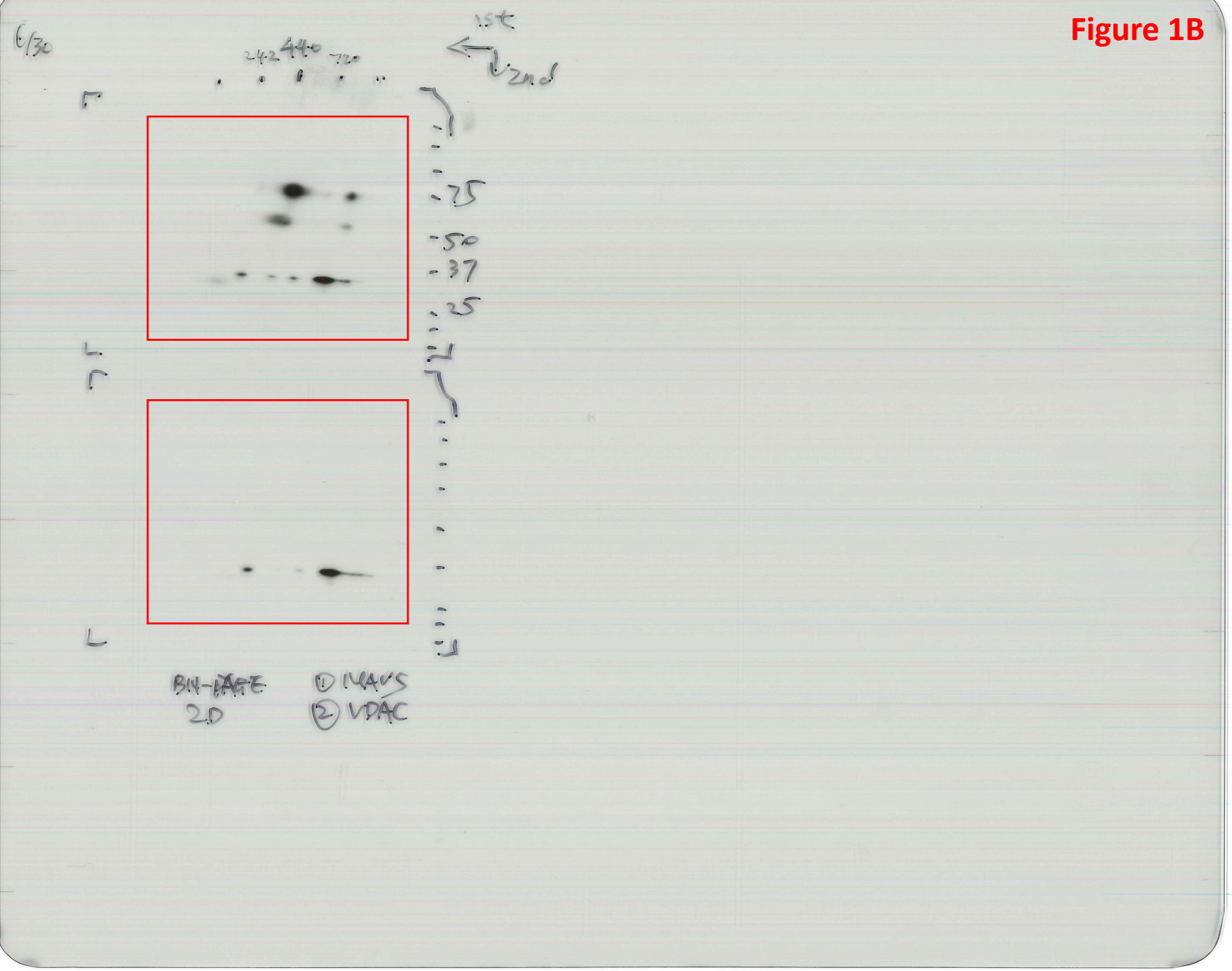


Figure 1B



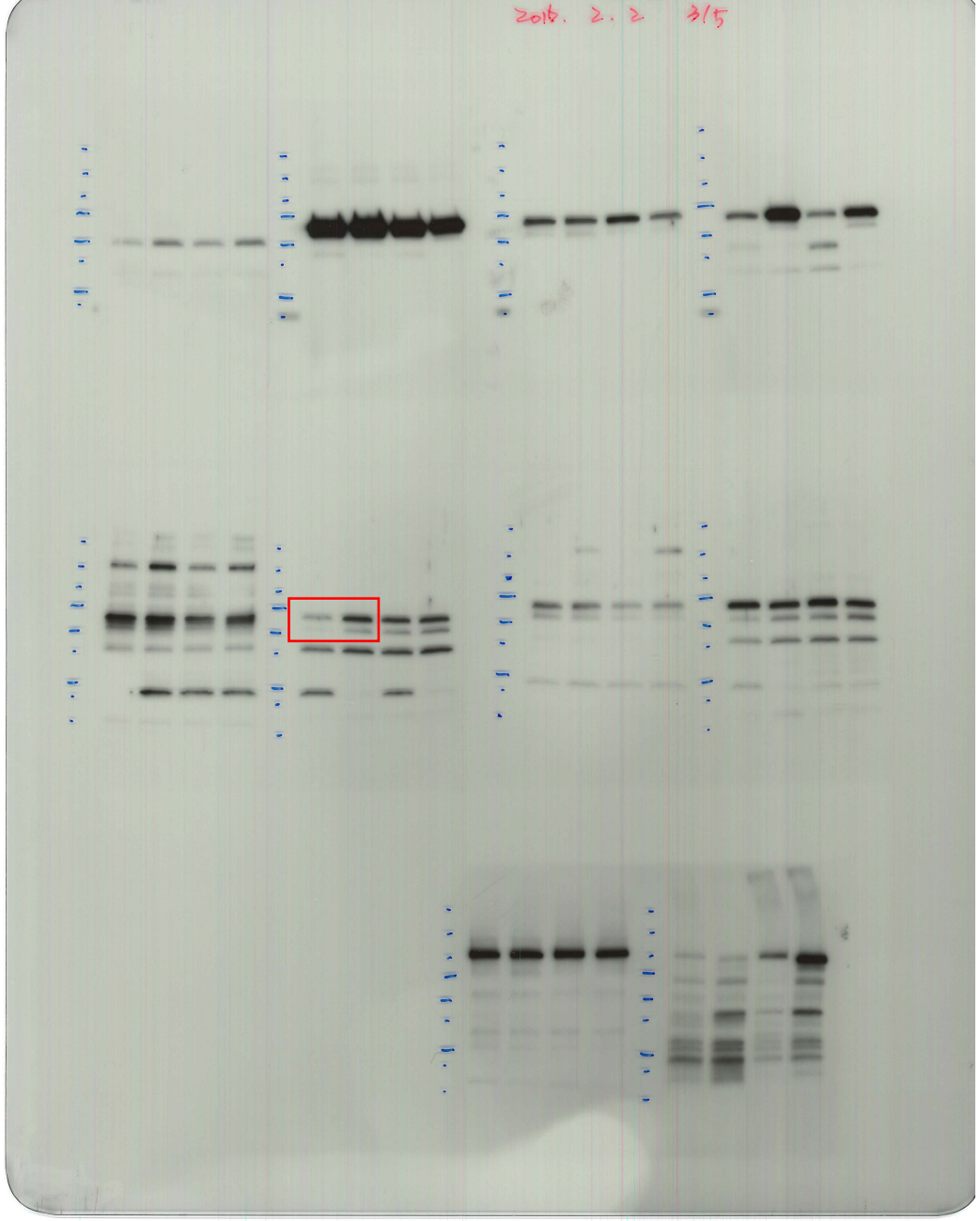


Figure 3A

Figure 6E