The E3 ubiquitin ligase MARCH5 promotes mitochondrial fusion and cell-cycle progression in acute myeloid leukemia

Larrue, Clément; Mouche, Sarah; Tamburini Bonnefoy, Jérôme

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METHODS

Patients

Primary cells from anonymized AML patients were obtained from the HIMIP collection (BB-0033-00060). This collection is registered with the Ministry of Higher Education and Research (DC 2008-307, Collection 1). A transfer agreement (AC 2008-129) was obtained after ethics committee approval before sample acquisition. All patients provided written informed consent for the use of their samples in research, in accordance with ethical guidelines and the tenets of the Declaration of Helsinki. Table S1 summarizes the characteristics of the patients included in this study, while ensuring the anonymity of their identities.

Animals

All experiments were performed in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International, with protocols approved by the Geneva Department of Health (GE/123/19 and GE/166). NOD/scid gamma-null (NSG) mice (6-8 weeks old, Jackson Laboratory) were used according to the protocols of the University of Geneva Animal Facility. For PDX assays, 6-8-week-old NSG mice received an intraperitoneal injection of 20 mg/kg busulfan (Sigma-Aldrich, Saint Louis, MO, USA). Two days later, 0.5-2x10^6 viable PDX AML cells were administered by tail vein injection. Mice were followed for 12-14 weeks before being humanely euthanized to quantify human CD45+CD33+ cells in the bone marrow by flow cytometry.

Cell lines and reagents

The human AML cell lines MOLM-14 and OCI-AML2 were routinely profiled by short tandem repeat (STR) analysis using PCR single locus technology (Promega, PowerPlex21 PCR Kit, Eurofins Genomics, Luxembourg). These cell lines were maintained in minimum essential medium (MEM)-α Glutamax (Life Technology, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Life Technology) at 37°C in a 5% CO2 atmosphere. HEK293T/17 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) Glutamax (Life Technology) with 10% FCS for lentiviral production. Doxycycline (1 µg/mL) was purchased from Sigma-Aldrich.

Culture of PDX-AML cells

Cryopreserved samples from patients with AML were rapidly thawed in a 37°C water bath and immediately transferred to Iscove's modified Dulbecco's medium containing 20% FCS (Gibco IMDM, Cat#21980-032, Thermo-Fisher Scientific, Waltham, MA, USA) and supplemented with 10 µg/mL DNase I (Sigma-Aldrich, Cat#11284932001). The cells were then centrifuged at 1500 rpm for 5 minutes

and seeded in IMDM supplemented with BIT 9500 Serum Substitute (Cat# 09500, StemCell Technologies, Vancouver, Canada). This culture medium was supplemented with 50 ng/mL FLT3 ligand (Cat# 300-19), 10 ng/mL IL-6 (Cat# 200-06), 50 ng/mL stem cell factor (SCF, Cat# 300-07), 25 ng/mL thrombopoietin (TPO, Cat# 300-18), 10 ng/mL IL-3 (Cat# 200-03), and 10 ng/mL granulocyte colony-stimulating factor (G-CSF, Cat# 300-23) (all from PeproTech, Rocky Hill, NJ, USA). In addition, 50 µM β-mercaptoethanol (Sigma-Aldrich) was added to the culture medium.

Confocal imaging

Mitochondrial size was assessed by confocal microscopy as previously described[1]. Briefly, 10^5 cells were washed with PBS and incubated in 50 µL PBS containing 200 nM MitoTracker Deep Red (Thermo-Fisher Scientific) for 30 minutes at 37°C with 5% CO2. The cells were then transferred to glass slides coated with 0.01% poly-L-lysine (Thermo-Fisher Scientific), fixed in 4% paraformaldehyde for 10 minutes, washed with PBS, fixed again in 100% methanol at -20°C for 5 minutes, and washed with PBS and distilled water. They were mounted in ProLong Gold antifade medium with DAPI (Invitrogen, Thermo-Fisher Scientific, Waltham, MA, USA). Images were captured using a Zeiss LSM 800 microscope equipped with Airyscan, and the average mitochondrial length was calculated for each cell. For each condition, we examined n = 30 cells.

Electron microscopy

We first fixed $2x10^6$ cells in a solution containing 2% glutaraldehyde in 0.1M sodium phosphate buffer (pH 7.4) for 45 minutes. They were then postfixed in 1% osmium tetraoxide for 1.5 hours and incubated in 2% uranyl acetate for 12 hours. Dehydration was performed by sequential washes in a buffer with increasing concentrations of ethanol (70%, 90%, and 100%), followed by successive incubations in 100% propylene oxide, a mixture of 50% propylene oxide and epon, and pure epon. After polymerization, 80-90 nm thick sections were cut with an ultramicrotome (Reichert, Buffalo, NY, USA), stained with a solution of 2% uranyl acetate plus Reynold's lead citrate, and examined with a Morgagni transmission electron microscope (FEI Company, Eindhoven, The Netherlands). All chemicals and reagents were purchased from Sigma-Aldrich, except uranyl acetate, which was purchased from Polysciences (Philadelphia, PA, USA). For each condition, we examined n = 30 cells, and 5 to 50 mitochondria were measured per cell.

Flow cytometry and sorting

Flow cytometry analysis was performed on a Cytoflex flow cytometer (Beckman Coulter, Brea, CA, USA). Cell sorting was performed on an Astrios cell sorter (Beckman Coulter). Human AML cells were identified and sorted by hCD33 and hCD45 staining. The antibodies and dyes used are listed below.

KI-67 labelling

PDX AML cells (0.5-1 x 10^6) were fixed with 4% paraformaldehyde for 15 minutes at room temperature, washed twice with PBS, and then permeabilized with cold methanol for 2 hours on ice. The cells were then washed, stained with anti-Ki-67 antibody for 1 hour at room temperature, washed, and resuspended in PBS containing DAPI or DRAQ7. Analysis was performed using a Cytoflex flow cytometer.

CFSE labelling

Cells were washed with PBS and incubated with 1 µL CFSE stock solution per 1 mL culture (0.5-5 x 10^6 cells/mL) for 30 minutes at 37°C in the dark. Then 50 mL medium supplemented with 10% FBS was added and the cells were incubated for 5 minutes at room temperature. The cells were then pelleted, resuspended in PCM, and incubated at 37°C.

Pyronin Y / Hoechst sorting

AML cells were washed with PBS and incubated for 45 minutes at 37°C with 20 µg/mL Hoechst 33342 (Invitrogen) in Hanks' balanced salt solution (HBSS) supplemented with 10% FCS, 20 mM Hepes, and 50 µg/mL verapamil (pH 7.5). Subsequently, 1 µg/mL Pyronin Y (Sigma-Aldrich) was added, and the cells were incubated for an additional 15 minutes at 37°C. The cells were then immediately sorted using an Astrios cytometer (Beckman Coulter).

Antibodies and dyes

Antibodies were : FITC Mouse Anti-Human CD45 Cat# 555482 RRID:AB_395874; APC Mouse Anti-Human CD45 Cat# 555485 RRID:AB_398600; CD33 PerCP-Cy™5.5 Cat# 333146 RRID:AB_2868647; PE Mouse Anti-Human CD33 Cat# 555450 RRID:AB_395843; PE Rat Anti-Mouse CD45 Cat# 553081 RRID:AB_394611 and FITC Mouse Anti-Ki-67 Cat# 556026 RRID:AB_396302 . Dyes were: DAPI Axonlab Cat#A4099.0, CellTrace CFSE Cell Proliferation Kit (Life Techniologies, Cat#C34570), Pyronin Y (Sigma Aldrich, Cat#P9172), Hoechst 33342 (Thermo Fisher Scientific, Cat#62249) and DRAQ7 (Thermo Fisher Scientific, Cat#D15106).

Quantitative reverse transcriptase PCR

Total RNA was extracted using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). cDNA synthesis was performed using Superscript II Reverse Transcriptase (Invitrogen). Genes of interest were quantified using SYBR Green Power UP (Life Technologies). All reactions were performed on a STEPone instrument (Applied Biosystems, Waltham, MA, USA). Results were analyzed using the ΔCt method with HYWAZ or P0 as reference non-variable genes. Primer used were (Foward, Reverse,

5'→3'): ***MFN1*** (TGGCTAAGAAGGCGATTACTG, CTCTCCGAGATAGCACCTCACC), ***MFN2*** (CTCTCGATGCAACTCTATCGTC, TCCTGTACGTGTCTTCAAGGAA), ***OPA1*** (TGTGAGGTCTGCCAGTCTTTA, TGTCCTTAATTGGGGTCGTTG), ***MFF*** (ACTGAAGGCATTAGTCAGCGA, TCCTGCTACAACAATCCTCTCC), ***DRP1*** (CTGCCTCAAATCGTCGTAGTG, GAGGTCTCCGGGTGACAATTC), ***MARCH5*** (GTCCAGTG GTTTACGTCTTGG, CCGACCATTATTCCTGCTGC), ***HYWAZ*** (ACTTTTGGTACATTGTGGCTTCAA, CGGCCAGGACAACCAGTAT) and ***P0*** (CCTCATATCCGGGGGAATGTG, GCAGCAGCTGGCACCTTATTG).

Western blots and immunoprecipitations

Cells were lysed in 100 µL of 1X Laemmli buffer containing 62.5 mM Tris-HCl (pH 6.7), 10% glycerol, 2% SDS, 24 mM DTT, 2 mM vanadate, and bromophenol blue. The lysates were heated at 90°C for 5 minutes and then subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to nitrocellulose membranes and probed with primary antibodies. Protein signals were visualized by chemiluminescence (ECL, Bio-Rad, Marnes la Coquette, France) and detected with a CCD camera (LAS 3000 Fujifilm, Tokyo, Japan). Antibodies used were: MFN2 Cell Signaling Technology Cat #11925S RRID:AB_2750893; OPA1 Cell Signaling Technology Cat #80471 RRID:AB_2734117, MFN1 Cell Signaling Technology Cat #14739; MFF Cell Signaling Technology Cat #84580 RRID:AB_2728769; DRP1 Cell Signaling Technology Cat #5391 RRID:AB_11178938 and β-actin Sigma-Aldrich Cat# A2228 RRID:AB_476697. For immunoprecipitations (IP), the cells were lysed with 1% Nonidet P-40 buffer, incubated on ice for 20 minutes, and centrifuged at 20,000G for 20 minutes at 4°C. The lysates were then incubated with antibody-coupled magnetic beads (Pierce Anti-HA Magnetic Beads, Cat# 88836 RRID:AB_2749815). The beads were washed four times with NP40-containing buffer and then solubilized in 1X Laemmli buffer for immunoblotting.

Constructs

We cloned shRNAs targeting MARCH5 and control (CTL) shRNA in the tet-pLKO-puro vector (Gift of Dmitri Wiederschain, Addgene plasmid Cat# 21915 RRID:Addgene_21915) or in the pLKO.1 mCherry constitutive vector (Gift of Oskar Laur, Addgene plasmid Cat# 128073 RRID:Addgene_128073). Sequences of shRNAs are : shCTL (CAACAAGATGAAGAGCACCAA), MARCH5 sh1 (CAGAAGTTGCTGGGTTTGTTT), MARCH5 sh2 (ACTTCTGGTTGTTCTGCAGTT) and MARCH5 sh3 (GCACTTGTGGAGACTTGTGAT). We cloned *MARCH5* wild-type or H43W cDNAs obtained through GeneArt gene synthesis technology (Thermo Fisher Scientific, Waltham, MA, USA) into the pSMAL (Gift of John Dick & Peter van Galen, Addgene plasmid #161785 RRID:Addgene_161785) lentiviral expression vector using the Gateway cloning. All plasmids were in NEB® Stable Competent E. coli (NEB, Cat#C3040H).

Lentivirus production

Briefly, HEK293T/17 cells were transfected with various plasmids, and with the packaging plasmids pMD2.G (Dr. Didier Trono's lab, Cat#12259; RRID:Addgene_12259) and psPAX2 (Dr. Didier Trono's lab, Cat#12260; RRID:Addgene_12260) lentiviral proteins, using Lipofectamine 2000 Transfection Reagent (Thermo-Fisher Scientific Cat# 11668019). After 24 hours, the medium was changed to Optimized Minimal Essential Medium (Gibco opti-MEM, Cat#31985-047, Thermo-Fisher Scientific). Seventy-two hours after transfection, supernatants containing lentiviral particles were collected from the HEK293T/17 cultures, filtered, and stored at -80°C for transduction of AML cell lines. For transduction of primary AML cells, the collected supernatants were concentrated with PEG-it Virus Precipitation Solution (PEG-it Virus Precipitation Solution, System Biosciences, Palo alto, CA, USA, Cat#LV810A) and stored at -80°C.

Lentiviral transduction

AML cell lines

On the day of transduction, 2×10^6 cells were resuspended in 2 mL of thawed lentivirus from HEK293T/17 supernatant containing 8 µg/mL polybrene (Sigma-Aldrich, Cat# 107689). Three days after infection, transduced cells were selected with 1 µg/mL puromycin (LabForce, Muttenz, Switzerland).

PDX-AML Samples

Twelve-well plates were coated with 500 µL of retronectin (20 µg/mL in PBS, Takara Bio, Cat# T100A) and incubated overnight at 4°C. The next day, the plates were blocked with 1 mL of 2% BSA-PBS for 30 minutes at room temperature and then washed once with PBS. Subsequently, 800 µL of concentrated lentiviral supernatant was added to each well, and the plates were centrifuged at 4000 rpm for 3 hours at 4°C to enhance viral adhesion to the retronectin. After centrifugation, 0.4 mL of viral supernatant was replaced with 1 mL of pre-warmed culture medium containing $1\text{-}5\times10^6$ cells. Plates were then centrifuged at 1500 rpm for 10 minutes at room temperature and incubated at 37°C for 24 hours. Medium was replaced the day after transduction.

Proteomics

Sample Preparation.

Protein immunoprecipitates were resolved into laemmli buffer, then protein concentration was measured by BCA assay, and 25 µg of each sample was subjected to protein digestion followed by Liquid Chromatography–Electrospray Ionization–Tandem Mass Spectrometry and data analysis as previously described[2].

Gene Expression Profiling

Gene Chip Hybridization

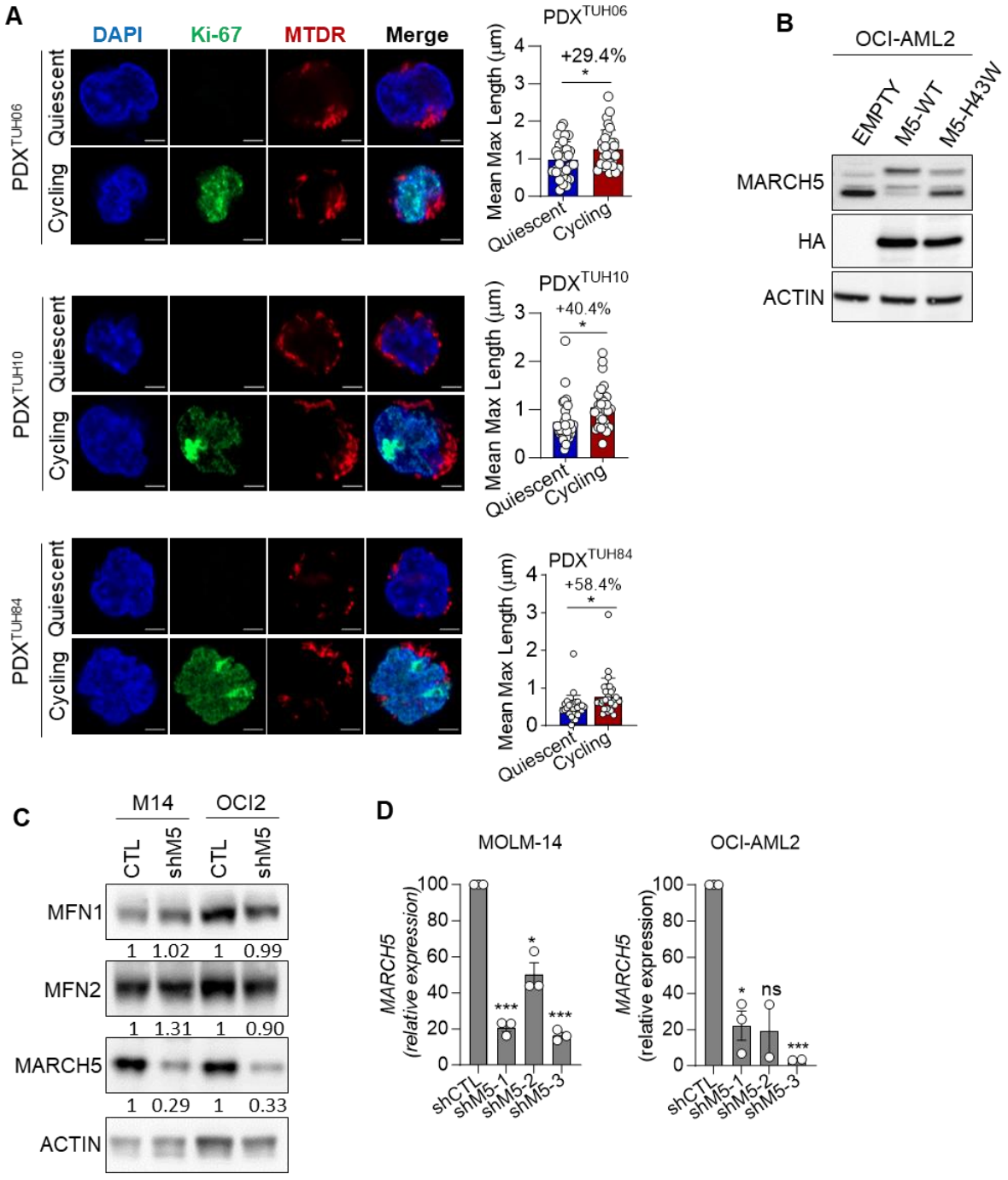
RNA was extracted using an RNeasy Mini Kit (Cat# 74134, Qiagen, Redwood City, CA, USA) and quality was assessed using a Bioanalyzer 2100 with an Agilent RNA6000 NanoChip Kit. One hundred ng of RNA was reverse transcribed according to the instructions of the GeneChip® WT Plus Reagent Kit (Affymetrix, Thermo Fisher Scientific). The resulting cDNA was hybridized to the GeneChip® Clariom S Human array (Cat# 902927, Affymetrix) at 45°C for 17 hours, washed on the Fluidic Station FS450 (Affymetrix), and scanned using the GCS3000 7G scanner (Thermo Fisher Scientific). The scanned images were analyzed using Expression Console software (Affymetrix) to generate raw data (.cel files) and quality control metrics.

Data Processing

Raw fluorescence intensity values were subjected to background correction, quantile normalization, and log2 transformation using Transcriptome Analysis Console (TAC) v4.0.2 (Thermo Fisher Scientific). Differential gene expression was identified using an analysis of variance (ANOVA) approach with empirical Bayesian (eBayes) correction.

Statistics

Statistical analyses were performed using Prism software version 9.1.2 (GraphPad, San Diego, CA, USA). Depending on the context, differences between experimental group means were evaluated using either a two-tailed Student's t-test with Welch's correction or a paired t-test. Analysis of variance (ANOVA) was used for comparisons involving more than two groups. The figure legends provide specific details of the statistical tests used, sample sizes (n), and measures' definitions. Typically, means and standard deviations were reported, with statistical significance considered at p-values ≤0.05. Significance levels are indicated as follows: *p<0.05, **p<0.01, ***p<0.001.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OCI-AML2
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M5-WT
M5-H43W
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Figure S1 : MARCH5 regulates mitochondrial morphology in AML cells. A. Confocal microscopy imaging of PDX-AML cells stained with MitoTracker Deep Red (MTDR; red), Ki-67 (green), and DAPI (blue). Scale bar: 2 μm. Experiments in three different PDX models are provided. Quantifications are shown in the right panel (n=30 cells per condition). **B.** Western blot in OCI-AML2 cells expressing empty vector, MARCH5-WT, or MARCH5-H43W constructs using anti-MARCH5, -HA and -β-actin antibodies. **C.** Impact of MARCH5 knockdown on MFN1 and MFN2 protein expression. MARCH5 was downregulated in MOLM-14 and OCI-AML2 cell lines. Four days after adding doxycycline to activate shRNA expression, we performed Western Blot analysis for MARCH5, MFN1, MFN2, and β -actin. **D.** Quantitative RT-PCR analysis of MARCH5 expression in MOLM-14 and OCI-AML2 cells transduced with three different dox-inducible shRNAs targeting MARCH5 and CTL shRNA and incubated with 1 μg/ml Dox for four days.

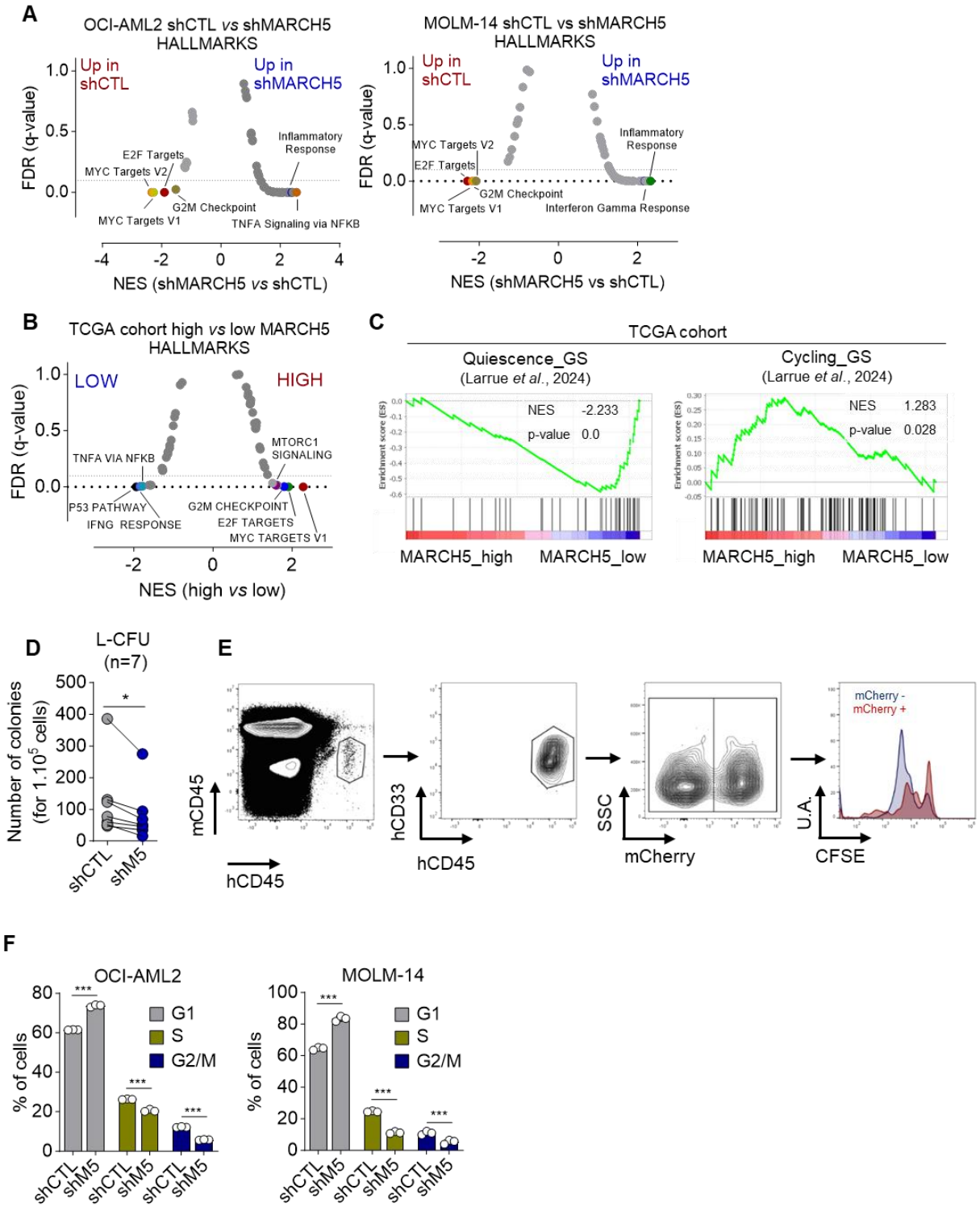

Figure S2 : MARCH5 inhibition induces cell cycle arrest in AML cells. A. MOLM-14 and OCI-AML2 cells were transduced with doxycycline (Dox)-inducible control or anti-MARCH5 shRNAs, and incubated with 1 µg/ml Dox for four days. Gene expression profiling was performed using microarrays, and differential gene expression (DGE) was analyzed by gene set enrichment analysis (GSEA) (n=8-9 biological replicates for each cell line model). Results are plotted as normalized enrichment scores

(NES) for hallmark signatures versus false discovery rate (FDR) q-values. **B-C.** Gene expression data from the TCGA cohort of AML patients was categorized according to MARCH5 expression, with high and low expression defined as above and below the median, respectively. **B.** Results are plotted as NES for hallmark signatures versus FDR q-values. **C.** Enrichment plots for quiescence or cycling signatures[3] **D.** PDX-AML cells were transduced with a vector expressing control (CTL) or anti-MARCH5 shRNAs. Leukemia colony forming unit (L-CFU) assays were evaluated after 10 days (n=7 different PDXs models). **E.** PDX-AML cells were transduced with mCherry+ anti-MARCH5 shRNA, then labeled ex vivo with CFSE and expanded in NSG mice for 7 days. The gating strategy for human AML cells with mCD45, hCD45 and hCD33 antibodies is shown. CFSE signal intensity was compared between mCherry- and mCherry+ human mCD45-/hCD45+/hCD33+ cells. **F.** MOLM-14 and OCI-AML2 cells were transduced with Dox-inducible control or anti-MARCH5 shRNAs, and incubated with 1 µg/ml Dox for four days. Propidium iodine (PI) staining was then performed to quantify cell cycle distribution.

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Supplementary Table 1. Characteristics of PDX-AML samples: Age (years), Sex (F: female, M: male), Leuko: leukocytes ($\times 10^9$/L), Blast: percentage of blast cells, Genetics: pathogenic genetic variants relevant to myeloid neoplasms. All primary samples originated from patient's peripheral blood.

Sample	Age	Sex	Leuko	Blast	Genetics	Engraftment (%)
PDX^{TUH06}	79	F	198	94	NPM1, STAG2	98.10
PDX^{TUH10}	64	F	307	85	NPM1, DNMT3A, TET2	89.02
PDX^{TUH76}	74	M	128	93	NPM1, DNMT3A, TET2, FLT3-ITD, CBL	71.21
PDX^{TUH83}	51	F	97	84	NPM1, DNMT3A, FLT3-ITD	83.31
PDX^{TUH84}	70	M	281	34	NPM1, DNMT3A, FLT3-ITD	96.43
PDX^{TUH93}	65	F	177	93	FLT3-ITD, DNMT3A, IDH2, SH2B3, NPM1	93.01
PDX^{TUH96}	64	F	176	90	FLT3, DNMT3A, IDH2, NPM1	84.22
PDX^{TUH102}	78	M	135	68	NPM1	78.17

Supplementary Table 2. Gene and protein expression analyses of key mitochondrial membrane dynamics factors in cycling and quiescent cell populations. C: cycling. Q: quiescent. mRNA (from quantitative RT-PCR experiments, relative to HYWAZ and P0 levels) and protein (from Western blot experiments, relative to β-actin levels) levels are provided in arbitrary units.

Ratio C/Q	mRNA						Protein	
	PDX^{TUH06}	**PDX^{TUH10}**	**PDX^{TUH83}**	**PDX^{TUH84}**	**PDX^{TUH93}**	**PDX^{TUH96}**	**PDX^{TUH06}**	**PDX^{TUH84}**
MARCH5	1.40	1.43	2.13	1.75	0.85	1.72	5.47	2.05
MFN2	1.11	0.84	1.44	1.26	1.34	1.52	1.68	1.99
MFF	1.40	0.93	1.49	1.64	1.20	0.88	1.17	0.89
DRP1	1.35	0.95	1.47	1.23	1.63	1.43	1.55	1.25
MFN1	1.06	0.94	1.20	0.85	1.11	1.04	1.23	0.91
OPA1	1.39	1.37	1.55	1.87	1.31	0.86	1.08	0.89