Neuromuscular Organoids Recapitulate Defective Autophagy in GNE Myopathy and Highlight Therapeutic Rescue by PI3K Inhibition

Running title: NMO for drug testing in GNE Myopathy

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Supplementary Materials and Methods

Bulk RNA-seq library preparation

Total RNA was isolated from C2C12 cells using Easy-BLUETM RNA isolation kit (iNtRON Biotechnology, #17061). One 1 μg of total RNA was processed for preparing mRNA sequencing library using MGIEasy RNA Directional Library Prep Kit (MGI) according to manufacturer's instruction. The first step entails utilizing poly-T oligo-attached magnetic beads to isolate the mRNA molecules that contain poly-A. Following purification, divalent cations and a high temperature are used to break the mRNA into small pieces. Utilizing reverse transcriptase and random primers, the cleaved RNA fragments are converted into first strand cDNA. After achieving strand specificity in the RT directional buffer, second strand cDNA synthesis takes place. The 'A' base is then added to these cDNA fragments, followed by the ligation of the adapter. The final cDNA library is made by purifying and enriching the results with PCR. The QauntiFluor ONE dsDNA System (Promega) is used to quantify the double stranded library. The library is circularized at 37 °C for 30 min, and then digested at 37 °C for 30 min, followed by cleanup of circularization product. The library is treated with the DNB enzyme at 30 °C for 25 min to create DNA nanoballs (DNB). Finally, Library was quantified by QauntiFluor ssDNA System (Promega). On the MGIseq system (MGI), the prepared DNB was sequenced using 100 bp paired-end reads.

Transfection for establishment of GNE Knock-Out myoblasts

For transfection, C2C12 myoblasts were rinsed with DPBS and detached using 0.25% trypsin-EDTA. Cells were washed three times with Opti-MEM (31985070, Gibco). The cells were then counted, and 1×10^6 cells were resuspended in 100 μL of Opti-MEM. 2 μg of spCas9 plasmid and 3 μg of sgRNA plasmid vectors were added to the cell suspension. The cell and plasmid mixture were transferred to an electroporation cuvette. Electroporation was performed using a Nepa-21 electroporator with a poring pulse of 200 V and a transfer pulse of 2.5 ms. The

transfected cells were seeded into a 6-well plate filled with DMEM culture medium. For single-cell isolation, the cells were diluted to a concentration of 1 cell per μL in culture medium, and 10 μL of this cell solution was seeded into 100 pi culture dishes. Single clones were isolated and expanded first in 12-well plates and then in 6-well plates. The Gne KO C2C12 myoblast clones were analyzed by Sanger sequencing.

Flow cytometry

Cells were detached with 0.25% trypsin-EDTA for C2C12 followed by three washes with DPBS and then analyzed using FACS Calibur, Celesta, and Fortessa (BD Biosciences). For the determination of sialic acid, cells were stained with 1:400 fluorescein-labeled SNA (FL-1301, Vector Laboratories) and 1:330 biotinylated MALII (B-1265, Vector Laboratories). For detection of glycospingolipids, cells were stained with 1:1000 BODIPY 493/503 (2 μM, Invitrogen). CellQuest Pro software and FlowJo software were used for FACS analysis.

Immunocytochemistry

Cells were treated with cold methanol or 4% PFA/0.5% Triton X-100 for fixation and permeabilization. 3% BSA in PBS was used for blocking solution. For lectin staining, fluorescein-labeled SNA (FL-1301, Vector Laboratories) was diluted in blocking solution and applied to cells for 1 h at room temperature in the dark. In the case of MALII staining, cells were washed three times and then incubated with 1:200 Alexa Fluor 594 streptavidin (#405240, BioLegend) for 30 min at room temperature in the dark. Cells were washed four times before nuclei were stained with DAPI (Thermo Fisher) and mounted on slide glass using MOWIOL solution. Confocal Scope TCS SP8 (Leica) was used for imaging samples.

Immunoblotting analysis

Cells were detached with 0.25% trypsin -EDTA or cell scraper (#3008, Corning) followed by three times DPBS wash on ice. RIPA buffer (Biosesang) containing 1 μM protease inhibitor and 10 μM sodium orthovanadate was used to extract the whole cell lysate, which was then

collected after incubating on ice for 1 hour followed by centrifugation. Protein concentration was measured using the Pierce BCA protein assay Kit (#23225, Thermo Fisher Scientific). Protein lysates were diluted with 5× SDS-PAGE loading buffer (SF2088-110-00, Biosesang) and RIPA buffer. Then, protein samples were boiled at 100 ℃ for 10 minutes. 10-20 μg of prepared protein sample was loaded and separated on a 7.5-10% SDS-PAGE gel. The separated proteins were transferred to an activated PVDF membrane. 5% skim milk in TBS-T was used for the blocking solution. The transferred membranes were blocked with blocking solution at RT for 1 hour followed by three times TBS-T wash then incubated with primary antibody (1:200-1:1000) in TBS-T at 4 ℃ overnight. Then, the membranes were washed three times with TBS-T and incubated with secondary antibody (1:10000) in TBS-T at room temperature for 1 hour. Chemiluminescence was detected using Miracle-Star (#16028, iNtRON Biotechnology) or Amersham ECL Prime (#GERPON2232, Cytiva).

RT-PCR analysis

Easy-BLUE^{TM} RNA isolation kit (iNtRON Biotechnology) was used for total RNA extraction. For reverse transcription, 5× PrimeScript^{TM} RT mix (TaKaRa) was used to generate cDNA. Quantitative real-time PCR was performed with SYBR Green PCR reagents (Life Technologies) using the QuantStudio3 (Applied Biosystems).

Cell growth analysis

Cell growth was monitored using the JuLI™ Stage (NanoEntek), an automated real-time live-cell imaging system. Cells were seeded in 6-well plate and cultured in DMEM supplemented with 10% FBS and 50 μg/ml Gentamicin in 5% CO2 at 37°C. In the case of glucose deprivation, cells were washed three times with DPBS and changed to DMEM, no glucose (#11966025, Gibco) with 10% FBS and 50 μg/ml gentamicin. The JuLI™ Stage was placed inside the incubator, and time-lapse imaging was performed at 1-4 hour intervals in bright field mode.

Collagen quantification assay

Total soluble collagen content was measured using the Sircol™ Soluble Collagen Assay Kit (Biocolor, UK) according to the manufacturer's instructions. Cell culture supernatants were collected 3-4 days after seeding. The Multi Mixer (SLRM-3, SeouLin Bioscience) was used for inverting steps during the collagen assay. Collagen concentrations were measured using the Epoch Microplate Spectrophotometer (Biotek).

Supplementary Figure Legends

Figure S1 (A) Summary of GEO datasets used for cross-validation, including sample types, sizes, and relevant PMIDs. **(B)** Gene ontology terms enriched in Normal group, highlighting muscle development and smooth muscle contraction pathways. **(C-D)** Enrichment of gene sets related to autophagosome formation and vesicle nucleation in Normal versus GNE I329T sample.

Figure S2 (A) Graphical description of the gRNA genomic locus, along with the DNA and amino acid sequences of both WT and KO cells. The T-A base insertion is shown in red. Altered amino acid sequences are indicated in blue. **(B)** Schematic of T7 endonuclease I (T7E1) digestion assay showing genome editing in WT and KO cells under mock and T7E1 conditions. Gel electrophoresis of PCR-amplified genomic DNA from WT and KO cells, confirming indel formation.

Figure S3 Representative images of mCherry-GFP-LC3 fluorescence in WT and KO cells following 2-hour EBSS and Bafilomycin A1 treatment. Yellow puncta indicate autophagosomes, and red-only puncta represent autolysosomes.

Figure S4. (A) Two-dimensional uniform manifold approximation and projection (UMAP) visualization of scRNA-seq data from GNE myopathy patient and healthy controls. Each point represents a cell, with colors based on subtype annotations defined by the Louvain clustering algorithm and marker genes. **(B)** Proportion of data types shown in (A) across different clusters. **(C-D)** Enriched MSigDB WIKIPATHWAYS gene sets for upregulated genes in each cluster.

Figure S5. (A) PCA analysis of Myoblasts samples including WT, V727M, R160Q, I588T, I329T, and C2C12 WT and KO samples. **(B)** Heatmap showing unsupervised hierarchical clustering of pairwise Pearson correlations between the RNA-seq datasets used in (A). **(C)**

Enriched Gene Ontology Molecular Function gene sets for upregulated genes in I329T cells compared to Normal cells (left) and KO cells compared to WT cells (right). **(D)** Enriched Gene Ontology Biological Process gene sets for upregulated genes in I329T cells compared to Normal cells (left) and KO cells compared to WT cells (right). **(E)** Enriched Gene Ontology Cellular Component gene sets for upregulated genes in I329T cells compared to Normal cells (left) and KO cells compared to WT cells (right).

Figure S6 (A) Volcano plot of differential gene expression between normal and mutant samples used for CMap query generation. Genes were filtered to 964 landmark genes and input into the CMap pipeline. (B) Schematic overview of the similarity scoring methodology in CMap, showing both Pearson correlation and cosine similarity equations used to compare gene signatures. (C) Bar plot (left) of the top 10 negatively correlated compounds in the A549 cell line, ranked by enrichment coefficient. PI3K/mTOR inhibitors are highlighted in red. Pie chart (right) summarizes the mechanisms of action (MOA) for these top hits, with 50% classified as PI3K/mTOR inhibitors. (D) Bar plot (left) of the top 10 negatively correlated compounds in the PC3 cell line, with 60% of the compounds classified as PI3K/mTOR inhibitors (pie chart, right). (E) List of PI3K/mTOR-targeting compounds with drug targets, IC50 values, and clinical application statuses.

Figure S7 (A) Immunofluorescence images of neuromuscular organoids (NMOs) derived from normal and I329T hPSCs, stained with DAPI (gray), MyHC (magenta), and TUJ1 (green). Scale bars, 200 μm. (B) High-magnification images showing reduced MyHC/TUJ1 signal intensity and structural organization in I329T NMOs compared to controls. Scale bars, 100 μm. (C) Principal component analysis (PCA) plot illustrating transcriptomic separation between normal and I329T NMOs. (D) Heatmaps showing gene expression of vesicle nucleation and autophagosome formation–related genes in Normal and I329T NMOs. (E) Bar graph of

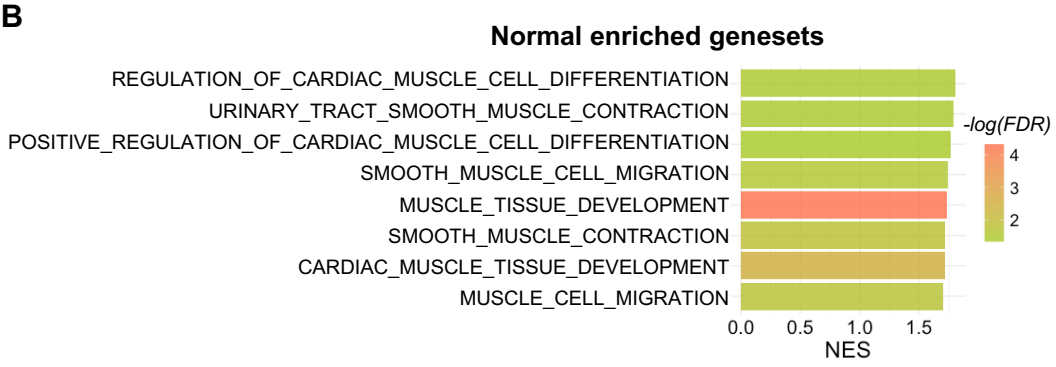
enriched MSigDB hallmark gene sets in I329T NMOs versus controls, highlighting upregulation of MTORC1_SIGNALING. (F) GSEA plot showing significant enrichment of the “MTORC1_SIGNALING” gene set in I329T NMOs (NES = 1.51, FDR < 0.001).

Figure S1

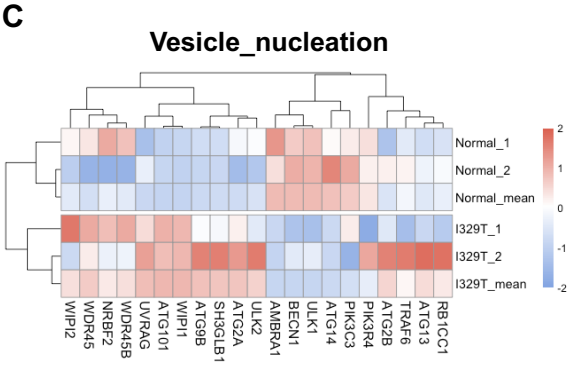
A

GEO study	Cell lines	Sample Size	PMID	Remarks
-	H9 Human embryonic stem cells (hESC)	4	35202935	Normal myoblasts (2) Mutant myoblasts (8)
PRJNA 739168	Human iPSCs from GNE myopathy patient and healthy control	9	36085325	Normal myoblasts (6) Mutant myoblasts (3)

B



C



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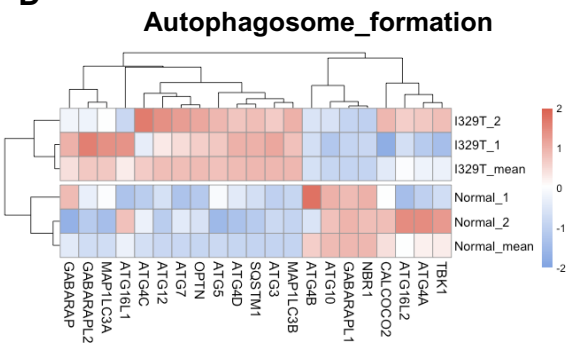
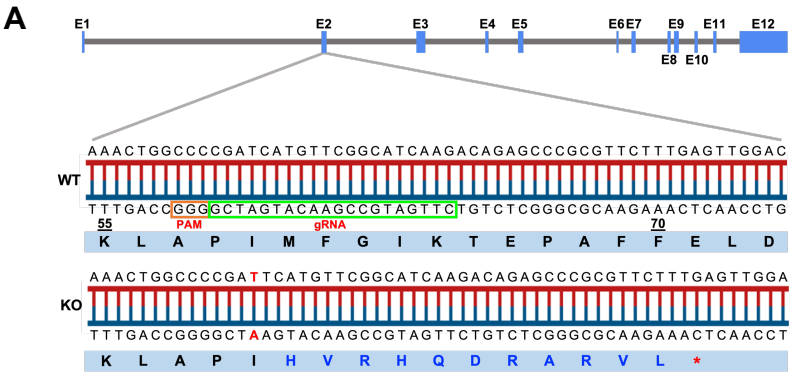


Figure S2


A
E1
E2
E3
E4
E5
E6E7
E9
E11
E12
E8
E10
AAACTGGCCCCGATCATGTTCGGCATCAAGACAGAGCCCGCGTTCTTTGAGTTGGAC
WT
TTTGACCGGGGCTAGTACAAGCCGTAGTTCTGTCTCGGGCGCAAGAAACTCAACCTG
55
PAM
gRNA
70
K L A P I M F G I K T E P A F F E L D
AAACTGGCCCCGATTCATGTTCGGCATCAAGACAGAGCCCGCGTTCTTTGAGTTGGA
KO
TTTGACCGGGGCTAAGTACAAGCCGTAGTTCTGTCTCGGGCGCAAGAAACTCAACCT
K L A P I H V R H Q D R A R V L *

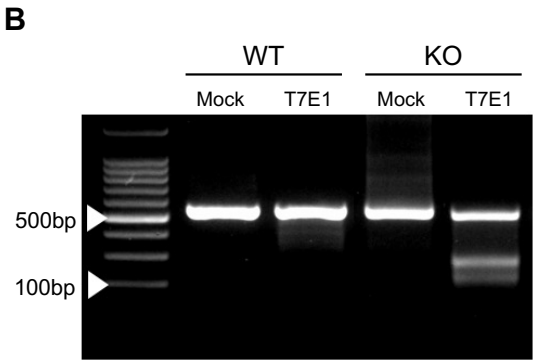

B
WT
KO
Mock
T7E1
Mock
T7E1
500bp
100bp

Figure S3

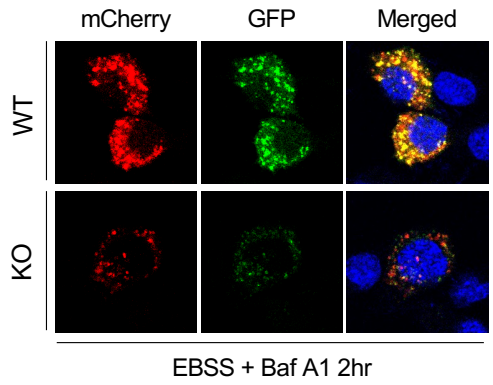


Figure S4

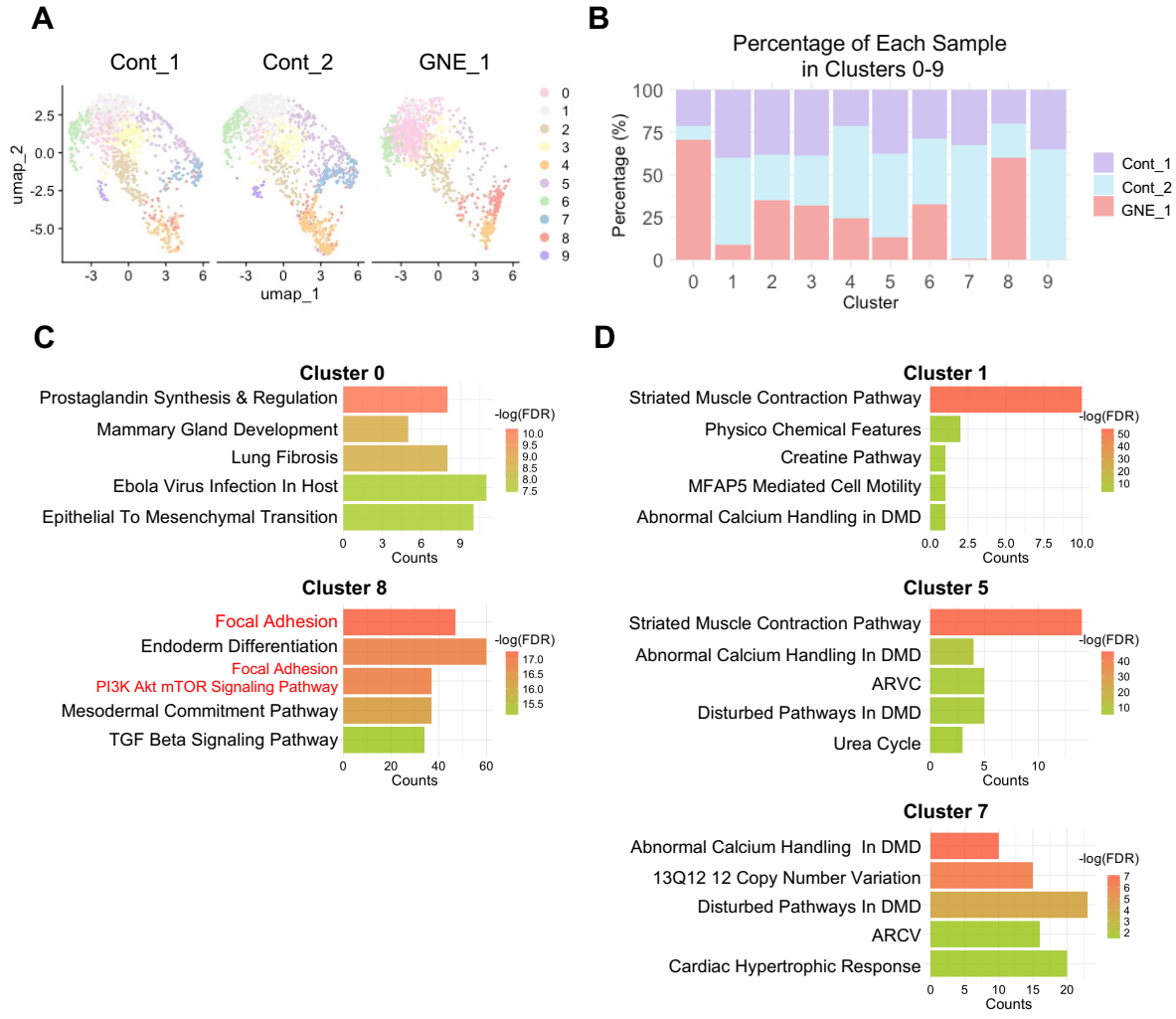

A
Cont_1
Cont_2
GNE_1
umap_2
umap_1
0
1
2
3
4
5
6
7
8
9
B
Percentage of Each Sample
in Clusters 0-9
Percentage (%)
Cluster
Cont_1
Cont_2
GNE_1
C
Cluster 0
Prostaglandin Synthesis & Regulation
Mammary Gland Development
Lung Fibrosis
Ebola Virus Infection In Host
Epithelial To Mesenchymal Transition
-log(FDR)
Counts
Cluster 8
Focal Adhesion
Endoderm Differentiation
Focal Adhesion
PI3K Akt mTOR Signaling Pathway
Mesodermal Commitment Pathway
TGF Beta Signaling Pathway
D
Cluster 1
Striated Muscle Contraction Pathway
Physico Chemical Features
Creatine Pathway
MFAP5 Mediated Cell Motility
Abnormal Calcium Handling in DMD
Cluster 5
Striated Muscle Contraction Pathway
Abnormal Calcium Handling In DMD
ARVC
Disturbed Pathways In DMD
Urea Cycle
Cluster 7
Abnormal Calcium Handling In DMD
13Q12 12 Copy Number Variation
Disturbed Pathways In DMD
ARCV
Cardiac Hypertrophic Response

Figure S5

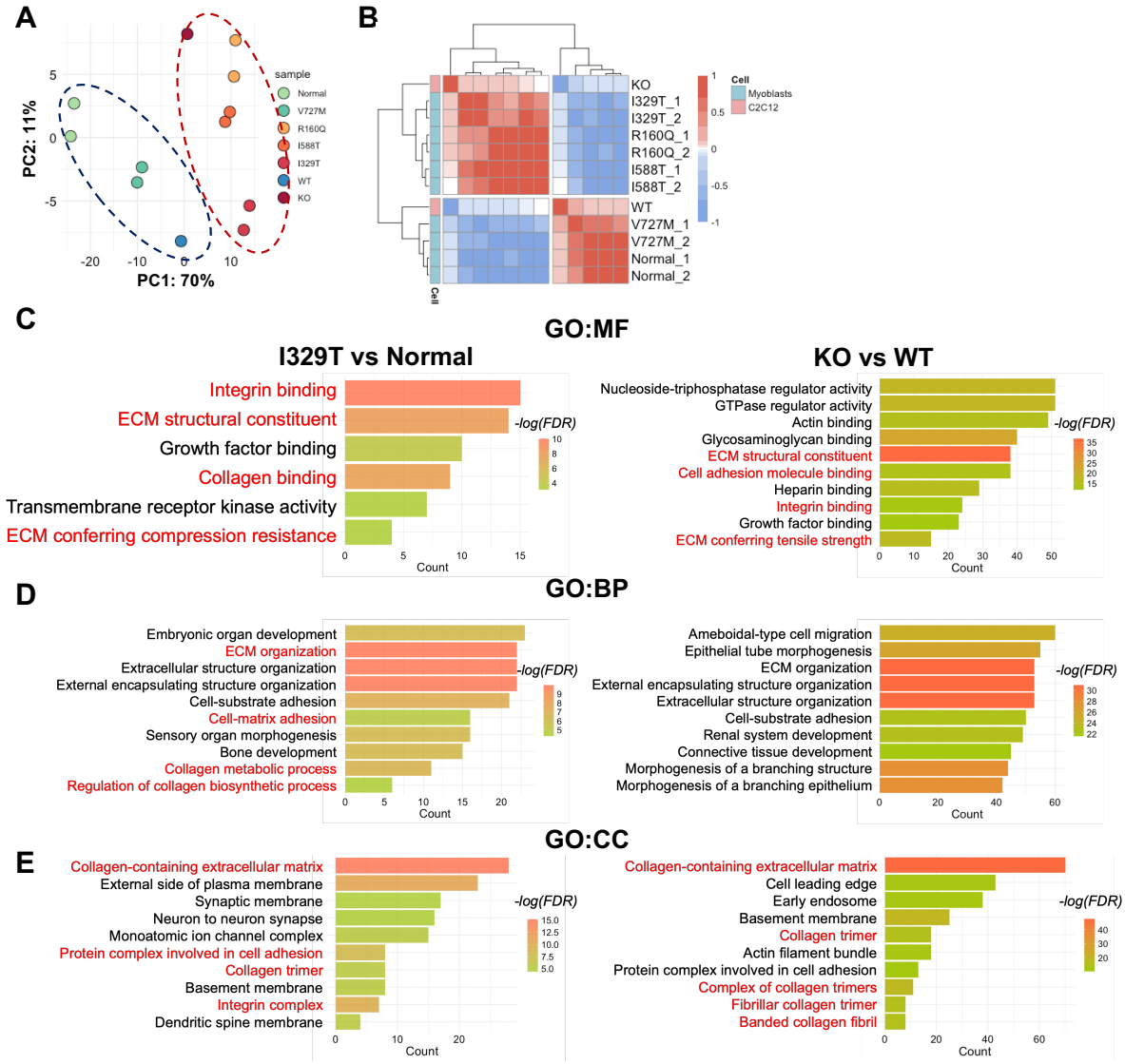
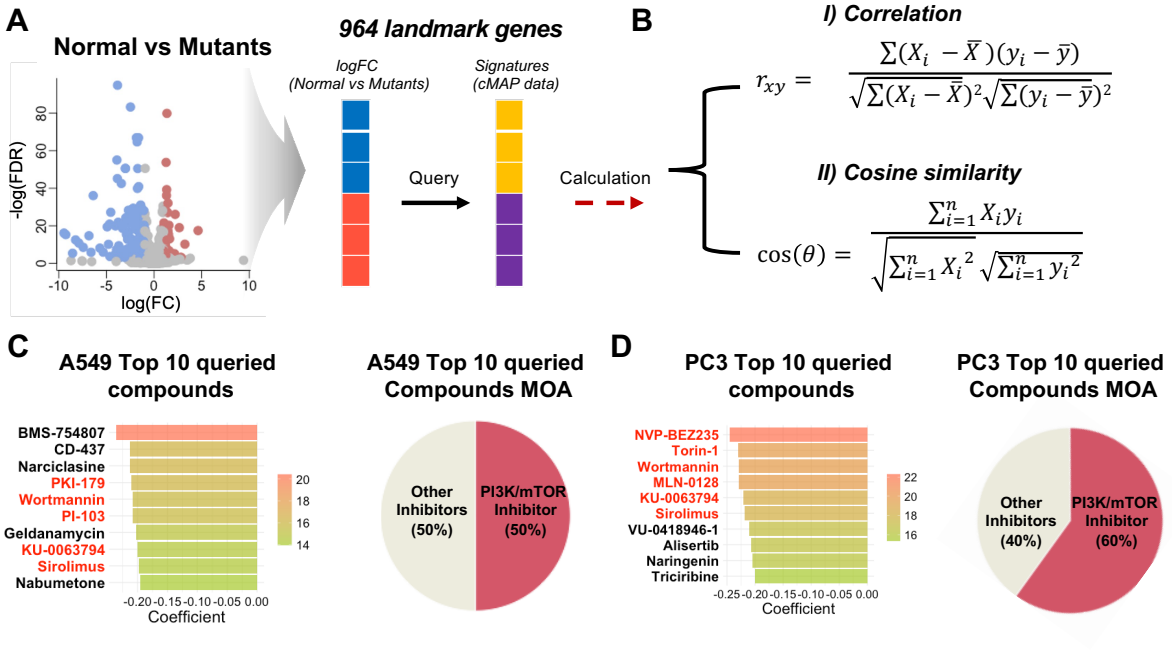

A
PC2: 11%
PC1: 70%
sample
Normal
V727M
R160Q
I588T
I329T
WT
KO
B
KO
I329T_1
I329T_2
R160Q_1
R160Q_2
I588T_1
I588T_2
WT
V727M_1
V727M_2
Normal_1
Normal_2
Cell
Myoblasts
C2C12
C
GO:MF
I329T vs Normal
Integrin binding
ECM structural constituent
Growth factor binding
Collagen binding
Transmembrane receptor kinase activity
ECM conferring compression resistance
-log(FDR)
Count
KO vs WT
Nucleoside-triphosphatase regulator activity
GTPase regulator activity
Actin binding
Glycosaminoglycan binding
ECM structural constituent
Cell adhesion molecule binding
Heparin binding
Integrin binding
Growth factor binding
ECM conferring tensile strength
D
GO:BP
Embryonic organ development
ECM organization
Extracellular structure organization
External encapsulating structure organization
Cell-substrate adhesion
Cell-matrix adhesion
Sensory organ morphogenesis
Bone development
Collagen metabolic process
Regulation of collagen biosynthetic process
Ameboidal-type cell migration
Epithelial tube morphogenesis
ECM organization
External encapsulating structure organization
Extracellular structure organization
Cell-substrate adhesion
Renal system development
Connective tissue development
Morphogenesis of a branching structure
Morphogenesis of a branching epithelium
E
GO:CC
Collagen-containing extracellular matrix
External side of plasma membrane
Synaptic membrane
Neuron to neuron synapse
Monoatomic ion channel complex
Protein complex involved in cell adhesion
Collagen trimer
Basement membrane
Integrin complex
Dendritic spine membrane
Collagen-containing extracellular matrix
Cell leading edge
Early endosome
Basement membrane
Collagen trimer
Actin filament bundle
Protein complex involved in cell adhesion
Complex of collagen trimers
Fibrillar collagen trimer
Banded collagen fibril

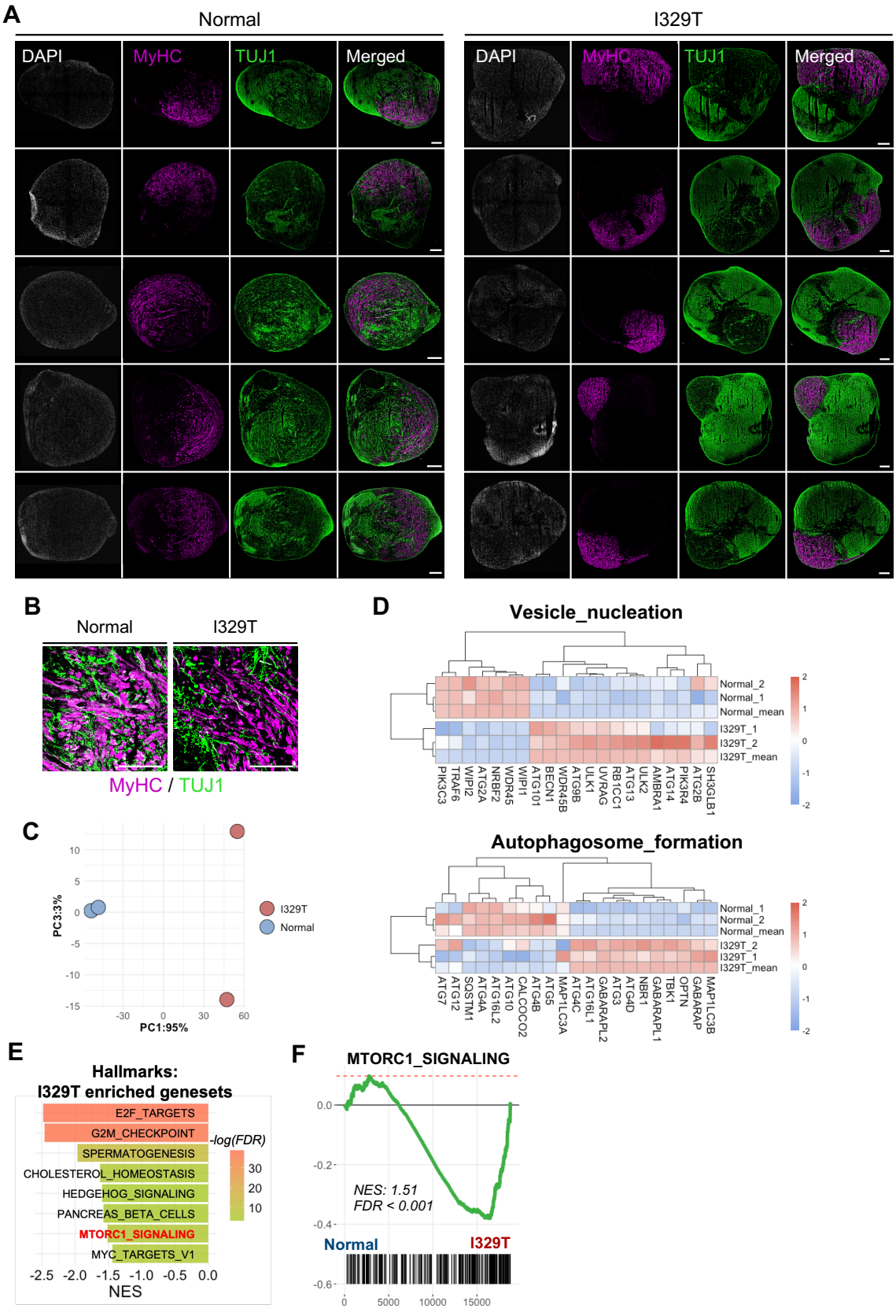
Figure S6



E

		Compound	Drug	Target	IC50	Application
FDA-approved		BAY80-6946	Copanlisib	PI3K	0.5nM	Leukemia
		Sirolimus	Rapamycin	mTOR	0.1nM	Immunosuppressant
Clinical	Ph. 1	MLN-0128	Sapanisertib	mTOR	1nM	
	Ph. 2	GDC-0980	Apitolisib	PI3K	5nM	
				mTOR	17nM	
	Ph. 1/2	SAR245409	Voxtalisib	mTOR/PI3K	9nM	
	Ph. 1/2	AZD2014	Vistusertib	mTOR1/2	2.5nM	
				PI3K	3.8μM	
	Ph. 1	GSK2126458	Omipalisib	PI3K	0.019nM	
				mTOR1/2	0.18/0.3nM	
	Ph. 2	NVP-BEZ235	Dactolisib	PI3K	4nM	Autophagy, HIV
				mTOR	6nM	
Pre-clinical		Torin1		mTOR1/2	2/10nM	
		Wortmannin		PI3K	3nM	
				DNA-PK/ATM	16/150nM	
		KU-0063794		mTOR1/2	10nM	
		AS-0605240		PI3K	8nM	
		PKI-179		PI3K	8nM	Anti-tumor
				mTOR	0.42nM	
		PI-103		PI3K	2nM	Apoptosis
				mTOR/DNA-PK	30/23nM	

Figure S7


A
Normal
I329T
DAPI
MyHC
TUJ1
Merged
B
Normal
I329T
MyHC / TUJ1
C
PC3:3%
PC1:95%
I329T
Normal
D
Vesicle_nucleation
Normal_2
Normal_1
Normal_mean
I329T_1
I329T_2
I329T_mean
PIK3C3
TRAF6
WIPI2
ATG2A
NRBF2
WDR45
WIPI1
ATG101
BECN1
WDR45B
ATG9B
ULK1
UVRAG
RB1CC1
ATG13
ULK2
AMBRA1
ATG14
PIK3R4
ATG2B
SH3GLB1
Autophagosome_formation
Normal_1
Normal_2
Normal_mean
I329T_2
I329T_1
I329T_mean
ATG7
ATG12
SQSTM1
ATG4A
ATG16L2
ATG10
CALCOCO2
ATG4B
ATG5
MAP1LC3A
ATG4C
ATG16L1
GABARAPL2
ATG3
ATG4D
NBR1
GABARAPL1
TBK1
OPTN
GABARAP
MAP1LC3B
E
Hallmarks:
I329T enriched genesets
E2F_TARGETS
G2M_CHECKPOINT
SPERMATOGENESIS
CHOLESTEROL_HOMEOSTASIS
HEDGEHOG_SIGNALING
PANCREAS_BETA_CELLS
MTORC1_SIGNALING
MYC_TARGETS_V1
-log(FDR)
NES
F
MTORC1_SIGNALING
NES: 1.51
FDR < 0.001
Normal
I329T