Chapter Four:
Materials and Methods

4.1 Materials:

4.1.1 Cell lines:

MDA-MB-231, MCF-7, BT-474, ZR-75-1, HeLa and HEK293 cell lines were obtained from ATCC (Manassas, VA, USA) while T47D and HBL100 were purchased from National Center for Cell Sciences (NCCS, Pune, India). MCF-7, BT-474, ZR-75-1 and T47D cells were cultured in RPMI 1640 (Life Technologies, Carlsbad, CA, USA). MDA-MB-231, HBL100, HeLa and HEK293 cells were grown in Dulbecco's modified Eagle's media (DMEM, Life Technologies, Carlsbad, CA, USA). Media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Life Technologies), 1% penicillin, streptomycin and neomycin (PSN) antibiotic mixture (Life Technologies). Cells were incubated at 37 °C, 5% CO_2 in specific media.

4.1.2 Constructs:

NLRX1-ΔLRR (amino acids 1-680) was generated by PCR (isoform 1, 975 amino acids) and sub-cloned into pcDNA3.1 vector in frame at the KpnI and XhoI sites. The clone was verified by immunoblotting using anti-Flag antibody as described in the **Methods** section. The details of the primer sequences for cloning NLRX1-ΔLRR are given in **Table 4.6**. The details of the remaining constructs used in the current study are mentioned in **Table 4.1**.

4.1.3 Generation of stable cell lines:

HEK293-mtRFP, HEK293-mtCFP, HEK293-mtGFP, HEK293-mCherry-LAMP1, HEK293-GFP-LC3 and MCF-7-mtRFP stable cell lines were generated to study the subcellular localization of proteins as previously described (Tomar et al., 2015). Briefly, cells were seeded at density of $4.5X10^5$ in 6-well plate. After overnight incubation, cells were transfected with constructs detailed in **Table 4.1** using standard $CaPO_4$ transfection method. After 24 hours of transfection, media was replaced with DMEM supplemented with G418 (500 μg/ml). The G418-containing media was changed after every 24 h until stable clones were visible. The stable cells were harvested transferred to 96-well plate to obtain single clone using serial dilution method. The single clones were further trans-

ferred to 24-well plates. After incubation for 7 days, the cells were transferred to 25 cm^2 culture flask and maintained in DMEM supplemented with 200 μg/ml of G418.

Similarly, stable knockdown of NLRX1 were generated in HEK293 and HeLa cells. Cells were transfected with NLRX1 shRNA and control shRNA and stable clones were selected by culturing cells in DMEM supplemented with Puromycin (3 μg/ml). The single clones were maintained in Puromycin (2 μg/ml) containing media.

4.1.4 Generation of knockout lines using CRISPR/cas9 gene editing:

To generate NLRX1 or FASTKD5 knockout cell line, CRISPR/Cas9 guide RNAs were designed using CRISPRko sgRNA design tool (Doench et al., 2016). The sgRNA targeting fourth exon with pick order score of 1 was selected and synthesized. Synthesized oligos were annealed and cloned into BbsI -linearized pSpCas9(BB)-2A-Puro (PX459) vector essentially as described earlier (Ran et al., 2013). NLRX1 and FASTKD5 sgRNA clone was transformed into competent Stbl3 *E. coli* strain and the transformants were screened by colony PCR, using U6 sequencing primer and antisense sgRNA. Positive clones were finally confirmed by Sanger DNA sequencing. NLRX1 and FASTKD5 sgRNA constructs were transfected into HEK293 cells using lipofectamine 2000. After 48 h of transfection, transfected cells were selected with 2μg/ml puromycin. The puromycin-supplemented medium was replaced every 24 h until single colonies were visible. The colonies were harvested and transferred to 96-well plate to obtain single clone using serial dilution method. Each single clone was further grown and transferred to 12 well plate and maintained in puromycin medium. Positive stable clones were identified by a western blot screening for the loss of NLRX1 or FASTKD5 gene product. The NLRX1 and FASKD5-specific sgRNA sequence and colony PCR primers are listed in **Table 4.6**.

4.1.5 Antibodies:

The details of the antibodies used in the current study along with its source or identifier is given in **Table 4.2**.

4.1.6 Chemicals and reagents:

Proteinase K, Puromycin, Sodium Azide, Antimycin A, 5-bromouridine, Digitonin, Cytochrome c, NADH, Sodium Succinate, DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT), 2,6-Dichlorophenolindophenol sodium salt hydrate (DCPIP), EZview^{TM} Red Anti-Flag M2 Affinity Gel, EZview^{TM} Red Anti-HA Affinity Gel, Cycloheximide, H_2O_2, Oligomycin and N-Acetyl Cysteine (NAC), Bafilomycin A1, EBSS,Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (z-RR-AMC),Cardiolipin,4-methylumbelliferyl phosphate disodium salt, ADP, Malate, sodium pyruvate, Nicotinamide (NAM), Rapamycin and Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) were purchased from Sigma-Aldrich, USA. Tetramethyl rhodamine methyl ester (TMRM), G418, Opti-MEM, Yeast tRNA, MicroAmp Fast Optical 96-Well Reaction Plate, MicroAmp Optical Adhesive Film and Pierce Protein A/G Agarose beads, Lyso-Sensor™ Green DND-189, ATP determination kit, CM-H_2DCFDA and MitoSOX™ Red mitochondrial superoxide indicator were procured from Thermo Fisher Scientific Inc, USA. TNF-α (Tumor Necrosis Factor-alpha) and Mito-TEMPO were procured from Enzo Life Sciences, USA. SYBR green and complementary DNA (cDNA) isolation kits were purchased from Takara Bio Inc, Japan. IETD-fmk(Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone)were used from Clontech, USA. Caspase-Glo® 8 Assay System was purchased from Promega, USA. p-Nitrophenyl Phosphate (pNPP), Nitrotetrazolium Blue chloride (NTB) and diaminobenzidine (DAB) were purchased from Sisco Research Laboratories (SRL), India.

4.1.7 Breast cancer patient tissues and ethics statement:

Human breast tumor tissues and extra-tumoral tissues were collected from patients undergoing surgery. Human studies were performed according to the norms of 1964 Declaration of Helsinki. Ethical approval from Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, Uttar Pradesh, India- ethical committee was taken prior to collection of tissue. The prior consent of each patient was taken. Tissues were collected from the tumor zone (tissue within the tumor boundary), and normal zone (distal normal tissue at least 10 mm from the outer tumor boundary). A fraction of all tissues was fixed in formalin and embedded in paraffin for routine histopathological analysis. The rest of the fractions were frozen in liquid nitrogen and then stored at −80 °C for RNA extraction and protein analysis. Details of the tissue specimen used are given in **Table 4.3**.

4.2 Methodology:

4.2.1 Immunohistochemistry:

Following de-paraffinization of tissues specimens in xylene and hydration by gradient alcohol series, antigen retrieval was performed by heat treatment in citrate buffer (10 mM, pH 6.0). The sections were incubated in 10 % NSS (Normal sheep serum) for 20 min to block non-specific binding and further incubated with anti-NLRX1 antibody (1:1000) in 0.1 % BSA overnight at 4°C. Sections were stained using Quick Universal ABC KIT (Vector) followed by peroxidase staining reaction with DAB/H_2O_2 as chromogen. The stained sections were observed under bright field light microscope (Nikon Eclipse 80i; Nikon Instech Co. Ltd., Kawasaki, Kanagawa).

4.2.2 Transfection of cell lines:

HEK293, HeLa and MDA-MB-231 cells were transfected using standard calcium phosphate transfection method and Lipofectamine 2000 or Lipofectamine 3000 reagent according to the manufacturer's protocol (Thermo Fisher Scientific Inc, USA). MCF-7 and T47D cells were transfected using Biotool DNA transfection reagent (Biomake, USA) and X-tremeGENE^{TM}DNA transfection reagent (Roche GmbH, Germany) respectively, as per manufacturer's protocol.

4.2.3 Cell survival and cell death assays:

4.2.3.1 Trypan blue dye exclusion assay

To assess the cell viability by trypan blue dye exclusion assay, cells were plated at density of 1.5 x10^5 cells/well in 24-well plate and transfected with indicated constructs. After 24 h of transfection, cells were treated as indicated. Following treatment, cells were stained with trypan blue dye. Minimum 100 cells per view were counted and percentage of cell survival was plotted as percentage of trypan blue negative cells.

4.2.3.2 Cellular proliferation assay using MTT

The cellular proliferation was analyzed by MTT assay as described earlier. HEK293 cells were plated at the density of 1×10^4 cells/per well in 96-well plate. The cells were trans-

fected with indicated constructs. After 48 h of transfection, cells were treated as indicated. MTT assay was performed after treatment by incubating cells with MTT (0.1 mg/ml) at37 °C for 1 h. The visible purple colored formazan was solubilized in DMSO and the absorbance was measured at 510 nm using a colorimetric microplate reader (BioTek Instruments, Inc. USA) at 595 nm wavelength.

4.2.3.3 Caspase-8 luciferase assay

Caspase-8 activation assay was performed using Caspase-Glo® 8 Assay Systems (Promega, USA) according to manufacturer instructions as described previously (Tomar et al., 2013). Briefly, indicated constructs were transfected in white bottom 96-well plate (Corning Inc. USA) using X-tremeGENE^{TM}DNA transfection reagent by forward transfection method. DNA transfection reagent mixture were dispensed into 96-well plate and thereafter 30,000 cells per well were plated. After 24 h of transfection, cells were treated as indicated. The caspase-8 substrate was added to each well, incubated for 1h at room temperature and luminescence was measured using luminometer (Berthold Technologies, Germany). The caspase-8 activity was normalized with number of cells and plotted as normalized relative caspase activity.

4.2.3 Fluorescence microscopy:

4.2.3.1 Monitoring of autophagy flux using GFP-LC3, mCherry-GFP-LC3 and mCherry-GFP-p62constructs

All fluorescence microscopy was done using IX83 fluorescent microscope (Olympus, Japan) and analyzed by cellSens Imaging Software (Version 1.12, Olympus, Japan). The monitoring of total GFP-LC3 puncta formation and autophagy flux by tandem mCherry-GFP constructs of LC3 or p62 was performed as previously described (Tomar et al., 2012a). To monitor GFP-LC3 puncta formation, HEK293-GFP-LC3 cells were seeded at density of 1.5X10^5 cells per well in 24-well plate and transfected with indicated constructs. After 24 h of transfection, the cells were treated as indicated and monitored using fluorescence microscope for autophagic puncta formation. Numbers and types of puncta per cell were counted in minimum 100 cells and graph plotted for the average number of

LC3 puncta per cell. To determine autophagy flux, HEK293 cells were co-transfected with indicated construct and tandem mCherry-GFP constructs of LC3 or p62. After 24 h of transfection, cells were treated as indicated and monitored using fluorescence microscope. The numbers of GFP/RFP and only RFP puncta, which represents autophagosome and autophagolysosomes respectively, were counted in minimum 100 cells and graph plotted for average number of LC3 and p62 puncta per cell.

4.2.3.2 Analysis of ROS generation

Intracellular and mitochondrial ROS production were measured by CM-H_2DCFDA (10μM) and MitoSOX Red (5μM) staining. Briefly, MCF-7 and HeLa cells were seeded at the density of 1.5 x 10^5 cells/well in 24-well plates. After overnight incubation, the cells were transfected with indicated constructs. Twenty-four hours post transfection, the cells were treated and stained with indicated reagents and observed under fluorescence microscope (Olympus IX81 microscope; Olympus, Tokyo, Japan).Minimum of 5 images and 80-100 cells were used for analysis.

Similarly, ROS levels were quantified by fluorometry. Briefly, MCF-7, HEK293 and MDA-MB-231 cells were transfected with indicated constructs. The cells were treated with indicated and stained with CM-H_2DCFDA (12.5 μM) in DPBS for intracellular ROS quantification and MitoSOX Red (2.5 μM) in DMEM for mitochondrial superoxide quantification. The cells were washed with DPBS and normalized to 1X10^6 cells/ml. Fluorescence intensity was quantified by fluorometer (Hitachi High-Technologies Corp., Japan) with excitation/emission at 495/520–540 nm and 510/570–600 nm, respectively.

4.2.4 Confocal microscopy

4.2.4.1 Subcellular localization analysis

The subcellular localization of NLRX1 was monitored by confocal microscopy. Briefly, MT-RFP-MCF-7 stable cells were plated in 24-well plate on cover slip at the density of 1.5 x 10^5 cells/well followed by transfection with indicated expression vector. After 24 hours of transfection, the cells were treated and fixed with 4% para-formaldehyde. The cells were monitored by Zeiss LSM 710 confocal microscope (Carl Zeiss, Inc,). Images

were captured and analyzed by Zen Black software. Colocalization was quantified by correlation using Image J 1.45 software (NIH).

To study the subcellular localization of different proteins MCF-7, HeLa, SH-SY5Y and mtRFP-HEK293 cells were seeded at density of 2-4 X 10^5 cells into 35mm glass bottom dishes and transfected with NLRX1-GFP. After transfection, MCF-7, HeLa and SH-SY5Y cells were loaded with 100 nM TMRM for 15 min for mitochondrial staining. Images of live cells expressing NLRX1-GFP were acquired with inverted Leica TCS SP8 confocal microscope system (Leica Microsystems GmbH, Germany) equipped with an air-cooled argon laser at 488 nm and 561 nm and a HC PL APO CS2 63X/1.40 differential interference contrast objective including a HyD detector, PMT and a PMT Trans detector. Images were collected for each channel sequentially at 1024 X 1024 pixels, 8-bit depth, 1AU pinhole and 3X magnification. Detectors gain, offset levels and laser power were calibrated at identical levels and remain unchanged for a set of experiment. All figure images were processed, pseudo colored and analyzed for intensity profile using Application Suite X (LASX v2.0.2). For imaging quantitation and analysis, percent correlation values were calculated using JACoP plugin in ImageJv1.45(NIH, MD, USA) and represented as mean colocalization and error, SD. Sample size $n \geq 5$ NLRX1-GFP positive cells.

For morphological analyses of mitochondria dynamics, MDA-MB-231 cells were transfected with control shRNA and NLRX1 shRNA. After 36 h of transfection cells were treated as indicated and loaded with 100 nM TMRM for 15 min for mitochondrial staining and images were acquired using a confocal microscope. To monitor the colocalization of mCherry-LC3, mCherry-p62 or mCherry-LAMP1, mtGFP stable cells were seeded at density of 2-4*10^5 cells into 35mm glass bottom dishes and transfected with control shRNA and NLRX1 shRNA. After 36 h of transfection cells were treated as indicated and visualized under confocal microscope.

4.2.4.2 TFEB-GFP translocation assay

To monitor the nuclear translocation of TFEB-GFP, HEK293 cells were seeded at density of 2-4*10^5 cells into 35mm glass bottom and co-transfected with indicated constructs.

After 24 h of transfection, cells were treated as indicated and stained with DAPI for 15 min, washed thrice with PBS and fixed with 4% paraformaldehyde solution. All images were acquired with an inverted Leica TCS SP8 confocal microscope system (Leica Microsystems GmbH, Germany). All figure images were processed, and colocalization was analyzed using Application Suite X (LASX v2.0.2). The analysis of nuclear translocation of TFEB-GFP was performed manually. A minimum of 5 confocal images per experiment with approximately 30 cells were analyzed and the number of cells with nuclear TFEB were counted and plotted as percent mean value, error, SD.

4.2.4.3 Image quantitation:

Quantitative analysis of mitochondrial morphology was performed using a Morphometry Macro in ImageJv1.45(NIH, MD, USA). Approximately, 130-300 individual particles (mitochondria) from 5 cells per image analyzed for circularity (4Π*Area/(perimeter^2)) and lengths of major and minor axes. From these values, form factor (FF; the reciprocal of circularity value,) and aspect ratio (AR; major/minor) were calculated and plotted. Both FF and AR reach a minimal value of 1 when a particle is a small perfect circle and the values increase as the shape becomes elongated. Specifically, AR is a measure of mitochondrial length while increase in FF represents increase of mitochondrial length and branching.

The colocalization of LC3+, p62+ or LAMP1+ puncta with mitochondria were analysis using Coloc2 plugin in ImageJ. Approximately, 10 colocalization events from 5 cells per experiment (≥50 events in total) were analyzed for the global Pearson's correlation value between 0.6-0.9 and plotted as mean colocalization, error, SD.

For the morphological analysis of LAMP1+ vesicles, lysosomes were binned into three categories using a size mask of 0.5 μm^2 to 5.0 μm^2in ImageJ. Punctate lysosomes were selected with a size mask of 0.2μm^2 to 1μm^2 for normal lysosomes, 1μm^2 to 2.5 μm^2 for intermediate and 3 μm^2 to 5 $\mu m^2$for lysosomal vacuoles. Thresholds were kept the same for all images. Clustered lysosomes were not included in the size analysis. Approximately, 150-300 individual particles (lysosomes) from 5 cells per image was analyzed and plotted as mean value, error, SD.

4.2.4.4 Bromouridine (BrU) staining, immunocytochemistry and image quantitation

For visualizing nascent mitochondrial transcript, BrU pulse was performed using 5-bromouridine (2.5 mM) for 60 min in mtCFP-HEK293 stable cells. Cells were washed twice with DPBS and fixed with 4% paraformaldehyde solution. Immunocytochemistry was performed in PBS containing 5% BSA, 0.1% Triton X-100, and 0.1% Tween-20. The permeabilized cells were incubated with primary antibody for 3 h at room temperature. After incubation, cells were washed five times with PBST and incubated with secondary antibody for one hour at the room temperature. BrU-labeled RNA was detected using anti-BrdU antibody. DyLight^{TM} 594-conjugated anti-rabbit secondary antibody was used at a dilution of 1:500. Fixed cell images were acquired with Leica TCS SP8 confocal microscope equipped with air-cooled argon laser at 458 nm, 488 nm, DPSS laser at 561 nm, HeNe laser at 594 nm and a HC PL APO CS2 63 X/1.40 objective using a HyD detector with narrow and wide band pass filters. The image acquisition settings were kept same as described above and four-channel sequential line scanning was performed to avoid bleaching and crosstalk between the fluorophores. All figure images were processed and analyzed as described above. To quantify BrU-NLRX1-FASTKD5 foci colocalization approximately 15 foci in more than 5 cells per experiment (>75 foci in total) were analyzed by intensity correlation and Coloc2 plugin in ImageJ. The frequency of overlap with BrU foci were calculated as mean colocalization and plotted as mean colocalization.

4.2.5 Super-resolution microscopy : 3D-Structured Illumination Microscopy (SIM)

MCF-7 cells were co-transfected with NLRX1-GFP and FASTKD5-RFP. Twenty-four hours post transfection, cells were washed twice with DPBS and fixed with 4% paraformaldehyde solution. Imaging was performed at room temperature using a CFI Apochromat TIRF 100×/1.49 NA oil immersion objective lens on an N-SIM microscope (Nikon Instruments, Inc) equipped with an Andor iXon3 DU-897E EMCCD camera, PFS and SIM Illuminator using 488 nm and 561 nm lasers. Raw images were acquired and reconstructed using NIS-Elements software. The exposure time was set to 100 ms for each raw data capture. The image stacks were acquired in 200 nm intervals for 8-12 Z

planes over a range of 3 µm. In each plane 9 images were acquired with a rotating illumination pattern (3 phases, 3 angles) in two color channels (488 nm and 561 nm) independently. SIM images were analyzed with NIS-Elements and ImageJ.

4.2.6 Subcellular fractionation

4.2.6.1 Isolation of mitochondria, mitoplasts and cytosolic fraction

Mitochondria were isolated from HeLa cells, HEK293 and MDA-MB-231 cells. To analyze the mitochondrial fraction by western blotting, mitochondrial fraction was were prepared by resuspending the cells in in mitochondria isolation buffer (0.25M sucrose, 10mM Tris/HCl, pH 7.4, 1X protease inhibitor cocktail (Sigma-Aldrich, USA) and incubated on ice for 5 min. The cells were disrupted by passing through a 24G sterile syringe needle and centrifuged at 600g for 10 min to separate nuclei and cell debris. The supernatant was collected and centrifuged again at 8000g for 10 min. The pellet (mitochondrial fraction) were washed twice and resuspended in isolation buffer while the supernatant was centrifuged again at 20000g for 10 min to obtain cytosolic fraction.

Mitoplast was isolated by resuspending mitochondria (1 mg/ml) in in PBS with 2.7 mg/ml digitonin and incubated on ice for 20 min. The sample was further centrifuged at 10000g for 10 min and the final pellet containing mitoplasts was washed twice with PBS.

4.2.6.2 Proteinase K protection assay and isolation of integral proteins of the inner mitochondrial membrane

Mitochondrial or mitoplasts pellet from HeLa cells were resuspended in digestion buffer (25 mM Tris/HCl, pH 7.5, 125 mM sucrose, 1 mM $CaCl_2$). The samples were incubated with proteinase K (50 µg/ml) with or without 0.1% (v/v) Triton X-100 for indicated time. Proteinase K activity was quenched by adding with 2 mM phenylmethylsulphonyl fluoride (PMSF) and incubating on ice for 10 min. After centrifugation, the pellet and the supernatant were collected and analyzed by western blotting. For alkaline carbonate extraction, mitoplasts were incubated in 100 mM Na_2CO_3 (final solution pH 11.5) for 60 min on ice. Separation of the pellet (integral protein of the inner membrane) and supernatant

(soluble and matrix proteins) was achieved by centrifugation at 47000 rpm for 2 h at 4 ̊C in an ultracentrifuge (SW60 Ti rotor, Beckman Coulter Instruments).

4.2.6.3 Isolation of nuclear fraction

The nuclear and cytosolic fractions were prepared as described previously (Tomar et al., 2012b). MDA-MB-231 cells were plated at density of $1X10^6$ in 60-mm^2 dish and transfected with indicated constructs. After 36 hours of transfection, the cells were treated as indicated. Post-treatment, cells were washed with ice-cold PBS, resuspended in three-fold volume of buffer-A (10 mM HEPES buffer, pH 7.9, 0.1 mM EDTA, 10 mM KCl, 0.4% (v/v) NP40, 0.5 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated on ice for 30 min. Cell lysates were centrifuged at 15,000 g for 15 min and supernatant was collected as cytosolic fraction, re-centrifuged as above to remove nuclear remnants. Pellets were washed three times with buffer-A and resuspended in 2-fold volumes of ice-cold buffer-B (20 mM HEPES buffer, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF) and incubated in ice for 20 min. The lysates were centrifuged at 15000 g for 15 min to obtain nuclear fraction in the supernatant.

4.2.7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

To study the protein expression levels and turnover in either the total cell or mitochondrial pellets from HEK293, HeLa, MCF-7, T47D, MDA-MB-231 and HBL100 cellswere lysed in three-fold volume of NP40 lysis buffer (150mM NaCl, 50mM Tris-Cl, 1% NP40, 1mM PMSF). Protein concentration of lysates, cytosolic and nuclear fraction were determined by Bradford assay and equal protein was resolved on 12% SDS-PAGE. Protein was electroblotted on PVDF membrane at 110V for one hour at 4^{o}C. Following the transfer, the membrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS) for 1 hour at room temperature. The membrane was incubated overnight with specific primary antibody. After incubation, the membrane was washed three times with TBS-T (Tris buffered saline containing 0.1% Tween 20) for 10 minutes and incubated with a secondary antibody at room temperature for 1 hour. The membrane

was washed three times with TBS-T and signal visualized by using chemiluminescence detection kit for HRP (Takara Bio Inc, Japan) by exposing to X-ray film.

4.2.8 Blue Native-PAGE, In-gel assays and immunoblotting

Blue Native-PAGE was performed on Native PAGE Novex 3%–12% Bis-Tris Protein Gels (Thermo Fisher Scientific Inc, USA) with minor modification. Briefly, mitochondria from MCF-7 and MDA-MB-231 cells were isolated in Tris-Sucrose buffer as described above and pellets (50 µg) were solubilized as per manufacturer's protocol and run at room temperature. In-gel enzyme activity of different OxPhos complexes were carried on gradient Bis-Tris gels as previously described (Jha et al., 2016). For immunoblotting, proteins were transferred on PVDF membrane at 50 V, 4-7℃ and probed with indicated antibodies as described above.

4.2.9 Co-immunoprecipitation and western blotting

For protein interaction study, immunoprecipitation experiments were performed as reported earlier (Tomar et al., 2013). Briefly, HEK293 and MCF-7 cells were plated at density of 2 X 10^6 per 90mm dish and co-transfected with indicated constructs. After 24 h of transfection, cells were washed with cold DPBS (Thermo Fisher Scientific Inc, USA), harvested and resuspended in NP40 lysis buffer (100 mM NaCl, 50 mM Tris/HCl, 10% Glycerol, 0.1% Nonidet P-40) containing complete protease inhibitor cocktail and incubated on ice for 1 h with occasional vortexing and centrifuged at 13,000 rpm for 15 min at 4˚C. Cell lysates were collected and incubated overnight with anti-Flag or anti-HA affinity gel on a roller shaker at 4˚C. For immunoprecipitation of FASTKD5, HeLa cell lysates were precleared with Protein A/G Agarose beads for 2 h at 4˚C. After preclearing, the cell lysates were incubated overnight with anti-FASTKD5 antibody (1:200 dilution) on a roller shaker at 4˚C.After overnight incubation, the precipitate was mixed with blocked Protein A/G Agarose beads for 2 h at 4˚C. Finally, anti-Flag and agarose beads were washed three times with NP40 IP lysis buffer, resuspended in 5X SDS-PAGE sample buffer, resolved on 12% SDS-PAGE and analyzed by western blotting using specific antibodies.

4.2.10 Analysis of mitochondrial function

The enzyme activity of mitochondrial complex I, complex II, complex III and complex IV was assessed spectrophotometrically using NanoPhotometer® P-300 (Implen GmbH, Germany). The following extinction coefficients were used to calculate relative enzyme activities from absorption values: NADH: $\varepsilon_{340\ nm}$=6.2 mM^{-1} cm^{-1},2,6-dichlorphenol-indophenol (DCPIP): ε_{610nm} = 22.0 mM^{-1} cm^{-1} and cytochrome c: $\varepsilon_{550\ nm}$ = 19.0 mM^{-1} cm^{-1}.

4.2.10.1 Complex I enzyme activity:

HEK293, MCF-7 and HeLa cells were seeded in 12-well plates at the density of 2.5 x 10^5cells/well. After overnight incubation, the cells were transfected with an indicated construct and treated as indicated. The stable cell lines were harvested post-treatment and washed with cold DPBS. The cells were subjected to 3 freeze-thaw cycles in Freeze-thaw complete solution (0.25M Sucrose, 20mM Tris-HCl (pH 7.4), 40 mM KCl, 2mM EDTA supplemented with 1mg/ml fatty acid-free BSA, 0.01% Digitonin and 10% percoll). The cells were washed again with the Freeze-thaw solution devoid of digitonin and resuspended in Complex I assay buffer(35mM Potassium Phosphate (pH 7.4), 1mM EDTA, 2.5 mM NaN_3,1mg/ml BSA, 2µg/ml Antimycin A, 5mM NADH).The reaction was started by adding 80 µg of cell lysate to 500µl of assay buffer in 1ml quartz cuvette. Complex I activity was measured for 3 min by monitoring the decrease in absorbance at 340nm after the addition of 2.5 mM acceptor decylubiquinone indicating the oxidation of NADH.

4.2.10.2 Complex II enzyme activity:

Briefly, MCF-7 cells were seeded at density of 1 X 10^6 cells in 60 mm dishes. After overnight incubation, cells were transfected and treated as indicated. After treatment, cells were washed with cold DPBS and resuspended in 0.4 ml of 20 mM hypotonic potassium phosphate buffer (pH 7.5) and kept on ice for 10 min. Cells were disrupted with 24G sterile syringe needle and lysates were subjected to 2–3 freeze–thaw cycles. To assay complex II activity, lysates were preincubated with sodium succinate (200 mM) at 37 ℃ for 15 min and later resuspended in complex II assay buffer (0.1 M potassium phosphate buffer, pH 7.5, 50 mg/ml fatty acid-free BSA, 100 mM NaN_3 and 0.015% (wt/v) DCPIP). The reaction was started by adding 12.5 mM decylubiquinone (DUB) to the as-

say mix. The decrease in absorbance at 600 nm was monitored for 5mins in the presence of electron acceptor DUB indicating the oxidation of DCPIP.

4.2.10.3 Complex III enzyme activity:

NLRX1 knockdown stable cells were seeded in 100 mm dish at the density of 2.5 X 10^6cells/well. The cells were harvested and washed with cold DPBS. All the subsequent steps were performed at 4ºC. The cells were suspended in 0.5ml of 20 mM hypotonic potassium phosphate buffer (pH 7.5) and lysed using 24G sterile syringe and subjected to freeze-thaw cycle. The cell lysate (80 µg) was added to the 500 µl of Complex III assay buffer (25mM Potassium phosphate (pH 7.5), 0.025% Tween-20, and 300 µM NaN_3, 75µM Cytochrome c) in cuvettes and the baseline observance was monitored at 550 nm for 2 min. The reaction was initiated by the addition of 100 µM decylubiquinol (freshly prepared 2.5 mM stock) monitoring the increase in absorbance at 550 nm for 2 min.

4.2.10.4 Complex IV enzyme activity:

To assay complex IV activity, HeLa, MCF-7 and MDA-MB-231 w-ere processed as indicated for complex II assay and resuspended in complex IV assay buffer (50 mM potassium phosphate buffer, pH 7.0, 1mM reduced cytochrome c). Cytochrome c was freshly reduced as described earlier (Spinazzi et al., 2012). The reaction was started by adding 80 µg of lysate to the assay buffer and baseline activity was monitored at 550 nm for 2 min. Complex IV activity was measured by monitoring the decrease in absorbance at 550 nm. Specificity of complex IV activity was recorded by addition of 100 mM NaN_3 to the reaction mixture.

4.2.10.5 ATP luciferase assay:

The steady-state levels of whole cell and mitochondrial ATP were measured using ATP Bioluminescence kit (Thermo Fisher Scientific Inc, USA). Briefly, cells were plated at density of $1.5X10^5$ cells/well in 24-well plate or $5X10^5$ cells/well in 6-well plate for mitochondria isolation and transfected with indicated constructs. After 24 h of transfection, cells were treated as indicated. Following treatment, either mitochondrial fraction was isolated or total cells were collected and lysed in 0.1% Triton X-100.The ATP-dependent luminescence was measured using luminometer (Berthold Technologies, Germany) ac-

cording to the manufacturer's instructions. ATP concentration from luminescence readings was calculated by plotting against the standard curve.

4.2.10.6 Analysis of mitochondria-derived ATP synthesis

Mitochondria-derived ATP synthesis was measured by kinetic luminescence assay. Briefly, MDA-MB-231 cells were seeded at the density of $5X10^5$ in 6-well plate and transfected with control shRNA and NLRX1 shRNA. After 36 h of transfection, cells were treated as indicated and mitochondria was isolated. About 20 µg of isolated mitochondria was resuspended in 160 µl of buffer A (150-mM KCl, 25-mM Tris–HCl, 2-mM EDTA, 0.1% BSA, 10-mM potassium phosphate, 0.1-mM MgCl2, pH 7.4) and 10 µl of buffer B (0.5-M Tris–acetate, pH 7.75, 0.5-mM luciferin, 5 mg/ml luciferase) containing ADP (0.1 mM) and either malate plus pyruvate (both to 1 mM) or succinate (to 5 mM) was added to a 96-well white clear bottom plate in the presence and absence of 1 µg /ml oligomycin. The light emitted was recorded using a Multimode Microplate Reader (BERTHOLD TriStar² LB 942, Germany) for a total time of 3 min with 10 sec intervals between the reading. The data obtained as change in relative light units (ΔRLU) was converted to ATP concentration based on an ATP standard curve and plotted.

4.2.10.7 NADH measurements:

MDA-MB-231 cells were seeded at the density of $5X10^5$ in 6-well plate and transfected with control shRNA and NLRX1 shRNA. After 36 h of transfection, cells were treated as indicated, washed thrice with PBS and collected by centrifugation. The cells were resuspended in PBS and Autofluorescence of NAD(P)H was monitored at 350/460 nm (excitation/emission) using a spectrofluorometer (Hitachi High-Technologies Corp., Japan). The experiments were performed at 37˚C.

4.2.11 Analysis of lysosomal function

4.2.11.1 Flow cytometry analysis:

For the measurement of GFP-LC3 intensity, MDA-MB-231 cells were seeded at $2.5X10^5$/well in 12-well plate and co-transfected with control shRNA, NLRX1 shRNA and GFP-LC3 in 1:4 ratio. After 36 h of transfection cells, cells were treated as indicated,

washed twice with PBS and collected by centrifugation. The total fluorescence intensity of GFP-LC3 of 10000 cells was measured by flow cytometry using the BD FACS Aria cytometer (BD Biosciences, CA,USA) and data analyzed using Flowing Software 2.5.1 (CIC, Finland).

Analysis of intralysosomal pH was performed using LysoSensor™ Green fluorescent dye following manufacturer's instructions. Briefly, MDA-MB-231 cells were seeded at $2.5X10^5$/well in 12-well plate and transfected with control shRNA and NLRX1 shRNA. After 36 h of transfection, cells were treated as indicated, stained with 1µM LysoSensor probe for 30 min and washed thrice with PBS. Further, the cells were collected and total fluorescence intensity was measured and analyzed as above. Cell debris was excluded by gating on the forward and side scatter plot.

4.2.11.2 Cathepsin B enzyme activity:

Cathepsin B enzyme activity was measured using a cell lysate-based assay. Briefly, MDA-MB-231 cells were seeded in 24 well plate at density of $1.5X10^5$ cells/well and transfected with control shRNA and NLRX1 shRNA. After 36 h of transfection, cells were treated as indicated. After treatment cells were lysed in 1X passive lysis buffer (25 mM Tris-HCl, pH 7.8, 2mM DTT, 2mM EDTA, 10% glycerol, 1% Triton X-100) and 5 µg lysate was incubated with 10 µM of the fluorogenic cathepsin B substrate, z-RR-AMC check how it is written in100 µl cell-free system buffer (100 mM HEPES, pH 6.0, 150 mM NaCl, 2 mM DTT, and 5 mM EDTA) in a 96-well black clear bottom plate for 30 min at 37 °C. The fluorescence intensity was monitored by a Multimode Microplate Reader at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

4.2.11.3 Lysosomal acid lipase activity:

The acid lipase activity was determined using the protocol similar to Cathepsin B enzyme activity. Briefly, MDA-MB-231 cells were seeded in 24 well plate at density of $1.5X10^5$ cells/well and transfected with control shRNA and NLRX1 shRNA. After 36 h of transfection, cells were treated as indicated. After treatment cells were lysed in 1X passive lysis buffer (25 mM Tris-HCl, pH 7.8, 2mM DTT, 2mM EDTA, 10% glycerol, 1% Triton X-100) and 10 µg lysate was incubated in 100 µl of reaction buffer (100 mM sodium acetate, pH 4.0, 1% (v/v) Triton, and 0.5% (w/v) cardiolipin) in the presence of 0.345 mM 4-

methylumbelliferone. Samples were incubated for 1 h at 37 °C. The reaction was then stopped with 150 mM EDTA, pH 11.5, and fluorescence was measured (excitation/emission: 360/440 nm).

4.2.11.4 Lysosomal acid phosphatase assay

Acid phosphatase activity was measured as a colorimetric assay in MDA-MB-231 cells transfected, treated and lysed as above. Finally, 10µg lysate was incubated with 5 mM pNPP in 100 µl of citrate buffer (90 mM, pH 4.8) in 96-well plate for 30 min at 37 °C. The reaction was stopped with 100 mM NaOH solution and absorbance was measured at 405 nm in a microplate reader.

4.2.12 Quantitative analysis of gene expression

4.2.12.1 Analysis of mitochondrial DNA copy number:

Mitochondrial DNA copy number was determined by analyzing the relative quantity of mitochondrial genome with respect to nuclear genome. Briefly, MCF-7 and HEK293 cells were collected post transfection. Genomic DNA was isolated from cells by phenol-choloroform purification method. The relative quantity of mitochondrial genome to nuclear genome was analyzed using RNaseP specific primer as nuclear genome marker. The details of primer are listed in **Table 4.4.**

4.2.12.2 RNA isolation and expression analysis:

RNA from total cell was isolated using RNAiso Plus reagent (Takara Bio Inc, Japan). cDNA was transcribed with Primescript^{TM} First Strand cDNA synthesis kit (Takara Bio Inc, Japan) using random hexamer primers in two steps. The initial primer annealing was performed at 65˚C for 5 min followed by addition of addition of reverse transcriptase and incubation at 37˚C for 60 min in the second step. qRT-PCR was performed with cDNA as template and specific primers using SYBR Premix Ex Taq^{TM} (Takara Bio Inc, Japan) as per the manufacturer's instructions. Three biological replicates were performed per experiment. The relative levels of mitochondrial DNA encoded transcripts were determined by the $2^{-\Delta\Delta CT}$ method. GAPDH was taken as endogenous control. The reaction conditions were 95˚C for 2 min followed by 35 cycles of 95˚C for 5 sec and 60˚C for 34 sec (the data was acquired at this step). The generation of specific PCR products was confirmed by

melt-curve analysis and relative expression with standard error was plotted. The details of primer are listed in **Table 4.4**.

4.2.12.3 Analysis of mitochondrial RNA processing:

The processing of mitochondrial transcripts and maturation defects were analyzed by PCR amplification of mitochondrial mRNA regions. MCF-7 cells were transfected and treated as indicated. After treatment, RNA was isolated, and cDNA synthesized as mentioned above. The regions flanking adjacent mitochondrial genes were amplified from cDNA using 2X Emerald GT PCR Master mix (Takara Bio Inc, Japan) at 95˚C, 5 min; 35 cycles of 95˚C for 20s, 56˚C for 20s and 72˚C for 40s; 72˚C for 5 min and accumulation of unprocessed transcripts and intermediates was visualized on 1% agarose gel. The details of primer combinations are listed in **Table 4.5**.

4.2.12.4 RNA immunoprecipitation:

To identify the mitochondrial RNA species bound, NLRX1, NLRX1-ΔLRR and FASTKD5 was immunoprecipitated from mitochondria of HEK293 cells and coimmunoprecipitated RNA was isolated and analyzed. Briefly, HEK293 cells were transfected with NLRX1, NLRX1-ΔLRR and FASTKD5 and mitochondria was isolated post transfection from HEK293 cells or from control sgRNA and FASTKD5 knockdown HeLa cells. Mitochondria (200 µg) were resuspended in 200 µl of extraction buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 100 U/ml RNase inhibitor, 1% NP-40, and 1X complete protease inhibitors without EDTA) on ice for 40 min, with occasional vortexing. The extract was centrifuged at 20,000 g at 4˚C for 45 min and supernatant collected. The anti-FLAG M2 affinity beads (for IP of NLRX1 and NLRX1-ΔLRR) or EZ-view^{TM} Red Anti-HA Affinity Gel (for IP of FASTKD5) (Sigma-Aldrich, USA) were washed thrice in extraction buffer and blocked with 1% BSA and 100 µg/ml yeast tRNA for 2 h at room temperature. After blocking, beads were washed and were incubated with supernatant on a roller shaker at 4°C for 4 h. For endogenous IP, the mitochondrial lysate from HeLa cells were precleared with Protein A/G Agarose beads for 1 h and incubated overnight with indicated antibodies (1: 100 dilution) on a roller shaker at 4°C. After incubation, the precipitates were mixed with blocked Protein A/G Agarose beads for 2 h at 4°C. To isolate RNA following immunoprecipitation, beads were washed five times with

extraction buffer and supplemented with EDTA (5 mM) and yeast tRNA (1 µg). Further, RNA was isolated, cDNA was synthesized, qRT-PCR was performed and relative association of mitochondrial RNA transcripts with respect to input with standard error was calculated and plotted as mentioned above.

4.2.13 Analysis of translation of mtDNA-encoded genes by CLICK-IT® assay

Nascent mtDNA-encoded protein synthesis were evaluated in intact cells using the Click-iT AHA protein labeling kit (Thermo Fisher Scientific Inc, USA). Briefly, transfected MCF-7 and HeLa cells were incubated with methionine-free RPMI for 10 min at 37C and cotreated with 100 µg/ml cyclohexamide to inhibit cytosolic translation for 30 min. After incubation, 100 µM L-azidohomoalanine (AHA, a methionine analog) was added and incubated further for 3 h. AHA-labelled cells were harvested and lysed in 20 mM Tris-HCl pH 8.0, 1% SDS and 250U/ml benzonase nuclease (Sigma-Aldrich, USA) and protein concentration was estimated by Bradford assay. The cyclo-addition of biotin-tagged alkyne molecule to AHA-labeled proteins was carried out according to the manufacturer's protocol. Equal proteins were loaded and resolved on 12% SDS-PAGE and analyzed by western blotting using anti-biotin antibody.

4.2.14 Cell proliferation assay

Cell proliferation assay was performed to study the growth rate of NLRX1 transfected T47D and MCF-7 cells and NLRX1 KO cell lines in customized substrate selective media. Briefly, cells growing in log phase were transfected and incubated overnight. After 24 h of transfection, MCF-7 and T47D cells were seeded in 48-well plate at 20000 cells/well whereas NLRX1 KO cells were plated at 10000 cells/well. Cells were washed twice with DPBS and customized glycolytic and oxidative media was added in the presence and absence of indicated treatments. The customized media was prepared by using base DMEM solution that lacks glucose, pyruvate, and glutamine (Thermo Fisher Scientific Inc, catalog #A14430) and supplemented with 10% FBS, 10 mM glucose, 4 mM glutamine, and 1% PSN (Glycolytic medium). Galactose-containing (oxidative) media were generated by DMEM supplemented with 10% FBS, 10 mM galactose, 4 mM glutamine, and 1% PSN. Cells were trypsinized after 24 h cycle and cell number was counted and growth curve generated. For all conditions, the seeding densities used allowed exponen-

tial proliferation for four days and final cell counts were measured four days after treatment. The experiment was replicated two times per condition. Proliferation rate was determined using the following formula:

Proliferation Rate (Doublings per day) = $\log_2$(Final cell count (D5)/Initial cell count (D1))/D4

4.2.15 Soft agar assay:

Anchorage-independent potential of MCF-7 and HEK293 was assessed by soft agar assay. Briefly, cells were transfected with indicated constructs in 24-well plate. After 48 h of transfection, cells were counted. The 60 mm dishes were covered by a bottom layer composed by DMEM medium mixed with low melting point agarose (SeaKem®, Lonza, USA) at a final concentration of 1.0%, and by a top layer of DMEM medium supplemented with 0.5% serum and mixed with low melting point agarose at a final concentration of 0.6%. Cells ($1X10^4$) were added during the preparation of the upper layer with or without treatment, where they remained embedded. Dishes were then maintained in a humidified atmosphere of 5% CO2-95% air at 37°C for three weeks, adding medium (DMEM 0.5% serum) on the top of the two layers every 7th day. At the 25th day, dishes were washed in PBS and colonies were stained with Crystal Violet 0.005%. After staining, the cells were observed under inverted light microscope and area of the colonies were measured with ImageJ.

4.2.16 Colony formation assay

Clonogenic activity was assessed by colony forming assay described previously (Tomar et al., 2012a). Briefly, cells were transfected with indicated constructs in 24-well plate. After 48 h of transfection, cells were counted and 2000 cells per $60mm^2$ dish or 1000 cells per 6-well plate were seeded in the presence or absence of indicated treatments. Cells were cultured for 7 days in standard conditions of 5% CO_2 at 37°C. After 7 days, the dishes or plates were washed with PBS, fixed with cold methanol, and stained with 0.2% crystal violet. Images of plates were taken and number of colonies having more than 50 cells per well counted. The clonogenic capability of each cell line is presented as the percentage of plating efficiency.

Plating efficiency is the ratio of the number of colonies to the number of cells seeded and calculated as following:

PE= (Number of colonies formed/Number of cells seeded)*100 %

4.2.17 Scratch assay

In vitro scratch assay was performed to determine the migration ability of the cells. HEK293, MCF-7, MDA-MB-231 and HBL100 cells were seeded at density of $2.5X10^5$ cells per well in 12 well plate, transfected with indicated constructs. After 24 hours of transfection, a vertical wound was created in the monolayer in each well using a sterile P200 micropipette tip (Axygen Inc., USA). The wells were washed and replaced with 1 ml of fresh DMEM in the presence or absence of indicated treatments. The first image of each scratch was acquired at zero time point using inverted phase contrast microscope (1X83 Olympus, Japan) at 10X magnification and plate was further incubated at 37°C, 5% CO_2 for 24 hours. After every 12 hours, each scratch was examined and photographed at the same area. The images acquired for all wells were analyzed using TScratch program (www.cse-lab.ethz.ch). Rate of migration was analyzed by the T-scratch software which determines the open area at different time interval. The percentage open area in each condition was plotted.

4.2.18 Animal experiments:

Animal studies were performed according to the rules and protocols approved by the Bioethical committee of the Engelhardt Institute of Molecular Biology, Moscow, Russia. A total of 48 nude mice (5 weeks old Balb/c *nu/nu*) were taken. RKO colon carcinoma cells carrying empty lentiviral vector pLSLP, NLRX1 shRNA1 (Sigma TRCN0000129459) and recombinant NLRX1 (lentiviral construct pLCMV-NLRX1-puro) were used. Cells were trypsinized, washed three times in ice-cold PBS and inoculated subcutaneously in four locations (left and right hind and shoulder) at a density of $1X10^6$ cells in 0.1 ml per inoculums. Fifteen micrograms of purified human recombinant TNF-α (gift of Dr. Alexey Sazykin, Moscow State University) was inoculated intraperitoneally 24 hours later, and control mice were inoculated with PBS. The mice were inspected every two days and tumor size was measured. Tumor volume was calculated according to the following formu-

la: ellipsoid volume: 1/6*pi*a*b*c, where a,b,c are linear sizes of the tumor in three dimensions.

Expression levels of NLRX1 transcripts in the cell lines were monitored by real time PCR with EVA-green dye. The levels of NLRX1 inhibition was 85% in RKO cells. Levels of NLRX1 transcripts in the hyperexpressor cell lines were 8-fold compared to controls for RKO cells. Basal levels of NLRX1 transcripts (compared to β-actin control transcripts) were roughly similar for RKO cells. The primer sequence is listed in **Table 4.6**.

4.2.19 Statistical analysis:

Data are shown as either mean± SEM or mean ± SD for *n* observations. The data sets were normalized considering the values of controls as 100 %. The comparisons between data sets were performed by unpaired two-tailed Student's *t*-test to determine the levels of significance for each data set using GraphPad Prism® 5 unless specifically mentioned. The experiments were repeated minimum of three times independently, probability values of $p<0.05$ were considered as statistically significant. All figure images were prepared with Adobe Photoshop©8 CS.

Table 4.1 ,related to MATERIALS:

4.2.20 Details of constructs used in the current study:

Sr. No.	Construct name	Source
1	NLRX1-Flag (isoform 1, 975 amino acids), NLRX1 ΔN-ter (amino acids 156–975), NLRX1-LRR (amino acids 564–975)	gift from Dr. Stephen Girardin, University of Toronto, Ontario, Canada
2	TRIM13	gift from Dr. OlleSangfelt, Department of Oncology/Pathology, Cancer Centrum Karolinska, Stockholm, Sweden
3	HA-TRAF2	gift from Dr.SM Srinivasula, IISER, Thiruvananthapuram, Kerala, India

4	NLRX1-GFP	Cloned in pLCMV-tagGFP2-Puro vector, purchased from Sigma-Aldrich Inc, USA
5	NLRX1-specific shRNA	purchased from Sigma-Aldrich Inc, USA (Sigma TRCN0000129459)
6	pDsRed2-mito Vector (mtRFP), pAc-GFP-N1	purchased from Clontech, Takara-Bio, Japan
7	pLCMV-NLRX1-puro	Construct synthesized inhouse by Prof. Peter M Chumakov
8	pCMV-tag4A	purchased from Stratagene, USA
9	NLRX1-ΔLRR	inhouse, cloned in pCDNA3.1 backbone
10	pSpCas9(BB)-2A-Puro (PX459) V2.0	gift from Dr. Feng Zhang (Addgene plasmid # 62988)
11	FASTKD5-Flag-HA, FASTKD5-myc and FASTKD5-RFP	gifts from Dr. María Simarro, Edificio de Ciencias de la Salud, Valladolid, Spain
12	pECFP-Mito (mtCFP)	construct gifted by Dr. Jean-Claude Martinou, University of Geneva, Switzerland
13	Mitochondria targeted GFP (mtGFP)	gift of Dr. Gyorgy Hajnoczky, Thomas Jefferson University, Philadelphia, USA
14	GFP-LC3	gift of Dr. T. Yoshimori (National Institute of Genetics, Shizuoka, Japan)
15	mCherry-GFP-LC3 and mCherryGFP-p62	gift of Dr. Terje Johansen, Dept. of Biochemistry, Institute of Medical Biology, University of Tromsø.
16	mCherry-LAMP1	gift from Dr. J. Lippincott-Schwartz, HHMI, Virginia, USA
17	TFEB-GFP	Gift from Dr. Andrea Ballabio (TIGEM, Naples, Italy)
18	NLRX1 sgRNA and FASTKD5 sgRNA construct	inhouse, guide RNA cloned into pSpCas9(BB)-2A-Puro (PX459) V2.0

Table 4.2, related to MATERIALS:

4.2.21 Details of antibodies used in the current study:

Sr. No.	Antibody	Source	Dilutions
1	anti-NLRX1	Abcam, USA	1:1000
2	anti-β-Actin	Santa Cruz Biotechnology, Inc. USA	1:1000
3	anti-PARP	Cell Signaling Technology, USA	1:2500
4	anti-Caspase-8	Cell Signaling Technology, USA	1:1000
5	anti-Caspase-3	Cell Signaling Technology, USA	1:1000
6	anti-TOM20	Abcam, USA	1:2500
7	anti-AIF	Cell Signaling Technology, USA	1:2500
8	anti-FASTKD5	GeneTex Inc, USA	1:1000
9	anti-EFTu/Ts	gift from Dr. EmineKoc, Marshall University, Huntington, WV, USA	1:10000
10	anti-RPS9, anti-NDUFS2, anti-citrate synthase, anti-holoCOXIV, anti-LRPPRC	gift from Dr. Jessie Cameron, The Hospital for Sick Children, Toron-to, Canada	1:1000, 1:5000, 1:3000, 1:2000 respectively
11	anti-huATPase6	gift from Dr. Eric A. Schon, Co-lumbia University Medical Center, NY, USA	1:5000
12	anti-Flag M2 peroxi-dase(HRP)	Sigma-Aldrich, USA	1:1000
13	anti-HA-peroxidase	Roche Diagnostics GmbH, Germa-ny	1:1000
14	anti-c-myc	Abcam, USA	1:1000
15	anti-Biotin-peroxidase	Sigma-Aldrich, USA	1:2500
16	anti-BrdU	GeneTex Inc, USA	1:100
17	DyLight™ 594-conjugated anti-rabbit	Jackson ImmunoResearch Lab, Inc, USA	1:500
18	Secondary antibodies HRP conjugated anti-rabbit and anti-mouse	Jackson ImmunoResearch Lab, Inc, USA	1:5000-1:20000

19	anti-LAMP1	Cell Signaling Technology, USA	1:2500
20	anti-p62	Cell Signaling Technology, USA	1:2500
21	anti-NDP52	Cell Signaling Technology, USA	1:1000
22	anti-LC3	Sigma-Aldrich, USA	1:10000
23	anti-SDHA	Cell Signaling Technology, USA	1:1000
24	anti-TFEB	GeneTex Inc, USA	1:1000
25	anti-Lamin A/C	Abcam, USA	1:5000

Table 4.3, related to MATERIALS:

4.2.22 Details of the tissue specimen used for the analysis of NLRX1 protein and RNA expression: (ER -ESTROGEN RECEPTOR, PR - PROGESTERONE RECPTOR**)**

Tissue specimen	Characteristics
Patient 1-normal	Normal of patient 4
Patient 2-normal	Normal of patient 5
Patient 3	Infiltrating ductal carcinoma, nottingham grade II, no evidence of malignancy
Patient 4	Infiltrating ductal carcinoma patient, ER, PR-positive, nottingham grade II, positive for malignant cells
Patient 5	Infiltrating ductal carcinoma with nodal metastasis, ER, PR-negative, nottingham grade II
Patient 6	Infiltrating ductal carcinoma with nodal metastasis, negative for ER and PR, nottingham grade II
Patient 7	infiltrating duct carcinoma with nodal metastasis ER negative, PR negative, nottingham grade II tumor
Patient 8	Infiltrating ductal carcinoma patient, nottingham grade II tumor, negative for ER and PR

Patient 9	Infiltrating ductal carcinoma with axillary node metastasis, negative for ER and PR

Table 4.4, related to METHODOLOGY:

4.2.23 Details of primers used in qPCR study

Sr. No	Gene	Forward primer	Reverse primer
1	NLRX1	AACGGTGCTGGTGACACA	GCTCAGCTCATTGAAGTAGAGGT
2	FASTKD5	CGAATTCCTTCCCTGGGA	ACTTCTCAGGCTGGAGTAGAG
3	GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
4	16SrRNA	GAAACCAGACGAGCTACCTAAG	CGCCTCTACCTATAAATCTTCCC
5	ND1	ATACCCCCGATTCCGCTACGAC	GTTTGAGGGGGAATGCTGGAGA
6	ND2	ATTCCATCCACCCTCCTCTC	TGGGGTGGGTTTTGTATGTT
7	COX I	CGATGCATACACCACATGAA	AGCGAAGGCTTCTCAAATCA
8	COX II	TGAAGCCCCCATTCGTATAA	ACGGGCCCTATTTCAAAGAT
9	ATP6	CGCCACCCTAGCAATATCAA	TTAAGGCGACAGCGATTTCT
10	ATP8	ATGGCCCACCATAATTACCC	GCAATGAATGAAGCGAACAG
11	COX III	GGCATCTACGGCTCAACATT	CGAAGCCAAAGTGATGTTTG
12	ND4	ACAAGCTCCATCTGCCTACGA	GGCTGATTGAAGAGTATGCAATGA
13	ND4L	TAACCCTCAACACCCACTCC	GGCCATATGTGTTGGAGATTG
14	ND3	CCCTCCTTTTACCCCTACCA	GGCCAGACTTAGGGCTAGGA
15	ND5	CAAAACCTGCCCCTACTCCT	GGGTTGAGGTGATGATGGAG
16	cyt b	AACCGCCTTTTCATCAATCG	AGCGGATGATTCAGCCATAATT
17	ND6	GGGTGGTGGTTGTGGTAAAC	CCCCGAGCAATCTCAATTAC
18	ATP6V0	TCTGGTGATGACTTGAAACTGC	GTCTAGGAAGCTGGCGAGTG
19	LAPTM4A	GCCTGTGTTCTTTTTGCCGT	GGCAACTGAGGACGAAGTCA
20	LAMP1	GCTCTTCCAGTTCGGGATG	TAGGAATTGCCGACTGTGG
21	β-Actin	TCGTGCGTGACATTAAGGGG	GTACTTGCGCTCAGGAGGAG
22	RNaseP	CCCCGTTCTCTGGGAACTC	TGTATGAGACCACTCTTTCCCATA
23	HVR-D-loop of mtDNA	CACTTTCCACACAGACATCA	TGGTTAGGCTGGTGTTAGGG

Table 4.5, related to METHODOLOGY:

4.2.24 Details of primers used to study mitochondrial RNA processing.

Sr. No	Flanking Region	Forward Primer	Reverse Primer	Am-plicon size (bp)
1	16SrRN	GAAACCAGACGAGCTAC-CTAAG	GTTTGAGGGGGAATGCTG-GAGA	2353

	A-ND1			
2	ATP8-COX III	ATGGCCCACCATAATTACCC	CGAAGCCAAAGTGATGTTTG	1523
3	ND5-cyt b	CAAAACCTGCCCCTACTCCT	AGCGGATGATTCAGCCA-TAATT	1015
4	ND1-ND2	ATACCCCCGATTCCGCTAC-GAC	TGGGGTGGGTTTTGTATGTT	1307
5	ND2-COX I	ATTCCATCCACCCTCCTCTC	AGCGAAGGCTTCTCAAATCA	2135
6	ND5+ND6	AACCCTACTCCTAATCACATA ACCT	CTGGTTGAACATTGTTT-GTTGG	-
7	cytb + ND6	GGCTTAGAAGAAAACCCCACA	TAGTCCGTGCGAGAA-TAATGATG	-
8	COX I+ COX II	GCTCATTCATTTCTCTAACAG-CAG	GGCGTGATCATGAAAGGTG	-
9	ATP6 + COX III	TCGCCTTAATCCAAGCCTAC	CCTTTTTGGACAGGTGGTGT	-

Table 4.6, related to MATERIALS:

4.2.25 Details of the primer sequence used for cloning of vectors and clone verification.

Sr. No	Name	Description	Sequence
1	NLRX1 sgRNA top	For NLRX1 KO generation	CACCGAGGGCCTTTATACGCCACCA
2	NLRX1 sgRNA bottom		AAACTGGTGGCGTATAAAGGCCCTC
3	U6 forward primer	For verifying NLRX1 sgRNA clone	GAGGGCCTATTTCCCATGATTCC
4	NLRX1 antisense reverse primer		AAACTGGTGGCGTATAAAGGCCCTC
5	FASTKD5 sgRNA top	For FASTKD5 KO generation	CACCGCGACAGTGTGCCACTCTAGG
6	FASTKD5 sgRNA bottom		AAACCCTAGAGTGGCACACTGTCGC
7	U6 forward primer	For verifying FASTKD5 sgRNA clone	GAGGGCCTATTTCCCATGATTCC
8	FASTKD5 antisense reverse primer		AAACCCTAGAGTGGCACACTGTCGC
9	NLRX1-ΔLRR forward primer	For generation of NLRX1 clone lacking LRR domain	CTTGGTACCATGAGGTGGGGCCACCAT
10	NLRX1-ΔLRR reverse primer		AGACTCGAGGAAGAGGTGGTCAAGGAG
11	NLRX1 forward primer	For NLRX1 expression analysis in nude mice	AACGGTGCTGGTGACACA
12	NLRX1 reverse primer		GCTCAGCTCATTGAAGTAGA