NAT10, an RNA acetyl cytidine transferase restrains ferroptosis in cancer cells by maintaining SLC7A11 RNA stability

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Abstract

Recently, we reported that N-acetyltransferase 10 (NAT10) regulates fatty acid metabolism through ac4C-dependent RNA modification of key genes in cancer cells. During this work, we noticed ferroptosis as one of the most negatively enriched pathways among other pathways in NAT10 depleted cancer cells. In the current work, we explored the possibility of whether NAT10 acts as an epitrancriptomic regulator of ferroptosis pathway in cancer cells. Global ac4C levels and expression of NAT10 with other ferroptosis-related genes were assessed via dotblot and RT-qPCR respectively. Flow cytometry and biochemical analysis were used to assess oxidative stress and ferroptosis features. The ac4C mediated mRNA stability was conducted RIP-PCR and mRNA stability assay. Metabolites were profiled using LC-MS/MS. Our results showed significant downregulation in expression of essential genes related to ferroptosis namely *SLC7A11, GCLC, MAP1LC3A*, and *SLC39A8* in NAT10 depleted cancer cells. Further, we noticed a reduction in cystine uptake and reduced GSH levels along with elevated ROS, and lipid peroxidation levels in NAT10 depleted cells. Consistently, overproduction of oxPLs as well as increased mitochondrial depolarization and decreased activities of antioxidant enzymes support the notion of ferroptosis induction in NAT10 depleted cancer cells. Mechanistically, reduced ac4C level shortens the half-life of *GCLC* and *SLC7A11* mRNA, resulting in low levels of intracellular cystine and reduced GSH, failing to detoxify ROS leading to increased cellular oxPLs which facilitates ferroptosis induction. Collectively, our findings suggest that NAT10 restrains ferroptosis by stabilizing the SLC7A11 mRNA transcripts to avoid oxidative stress that induces oxidation of phospholipids to initiate ferroptosis.

Keywords: NAT10, RNA acetylation, ferroptosis, SLC7A11, ROS

1. Introduction

N-acetyltransferase 10 (NAT10) is a nucleolar protein that promotes cellular events by RNA and protein acetylation(1-5). Studies have shown that NAT10 regulates DNA damage, senescence, cell proliferation, cell cycle, and apoptosis(5-11).

Exposing cancer cells to DNA damage agents such as cisplatin and H_2O_2 causes an increase in NAT10 protein expression in a dose-dependent manner(5). Mechanically, during DNA damage, NAT10 is subjected to PARylation through ADP-ribose attachment. The nucleolar PARylation NAT10 gets translocated to the nucleoplasm where it interacts with MORC2 resulting in MORC2 acetylation(7). In response to the DNA damage, the activated MORC2 initiates DNA damage repair. Collectively, the two studies showed that in response to DNA damage NAT10 expression increases to initiate DNA damage repair(5, 7).

NAT10 mediates the formation of micronuclei (MN) by enhancing DNA replication in cancer cells. The MN is known to regulate the senescence-associated secretory phenotype (SASP) pathway through which NAT10 utilizes the NAT10/cGAS/SASP axis(8).

NAT10 promotes cell proliferation and prevents cell cycle arrest by acetylation of *BCL-XL* and centrosomal protein 170 (*CEP170*) mRNA transcript(9, 10). NAT10-mediated acetylation of the *BCL-XL* mRNA transcript leads to activation of the PI3K-AKT pathway thereby promoting cell proliferation(9). NAT10 mediated stability of *CEP170* promotes cell proliferation and chromosomal instability (CIN)(10). Furthermore, through stable CEP170; NAT10 main proper cell cycle distribution(10). Supporting studies have shown that NAT10 deficiency causes apoptosis and cell cycle arrest through a decrease in expressions of cyclin-dependent kinase 2 (*CDK2*), *CDK4*, *cyclin D1*, *cyclin E*, & *BCL2*, and an increase in the expression of *p16* and *p21(11)*.

NAT10-mediated cell death via apoptosis either by NAT10 knockdown (KD) or Remodelin treatment as seen in previous studies has not yielded convincing results as related to decrease in cell viability and cell morphology. Therefore, we anticipate other cell death types such as pyroptosis, necroptosis, and ferroptosis.

Ferroptosis is a condition characterized by shrunken mitochondria and lipid peroxidation leading to cell death(12). The basic features of ferroptosis include the availability of redox-active iron, oxidation of polyunsaturated fatty acid (PUFA), and antioxidant activity of cell membranes against formed lipid hyperoxides(13). The ferroptosis-induced antioxidant activity depends on three (3) systems which are as follows: dihydro-orotate dehydrogenase (DHODH)/ubiquinol; ferroptosis suppressor protein 1 (FSP1)/NAD(P)H/ubiquinol; and SLC7A11/glutathione (GSH)/phospholipid hyperoxide (PLOOH)(14).

System Xc$^-$ containing the SLC7A11/GSH/PLOOH axis act as cystine/glutamate antiport involved in the synthesis of GSH which is converted to oxidized glutathione (GSSG) upon catalytic action of GPX4(15). Compounds such as erastin induce ferroptosis by interfering with cystine uptake by inhibiting the System Xc$^{-(16-19)}$. Studies have reported the impact of targeting ferroptosis in cancer cells, however, no study has shown whether ferroptosis could be induced via NAT10-mediated acetylation.

Previously, we discovered that Remodelin; the only known small molecule inhibitor of NAT10 alters lipid metabolic pathways in cancer cells which includes fatty acid elongation in mitochondria, fatty acid metabolism, and mitochondrial beta-oxidation of saturated fatty acids(20). In line with these findings, we also discovered that NAT10 regulates fatty acid metabolism through ac4C-dependent stability of fatty acid metabolic genes such as *ELOVL6*, *ACSL1*, *ACSL3*, *ACSL4*, *ACADSB,* and *ACAT1*(21). Collectively, these studies have shown that NAT10 has a metabolic impact on cancer cells by modulating lipid metabolism.

The discovery of ferroptosis among the highly modulated pathways from our previous RNA-seq in NAT10-depleted cancer cells led us to postulate, whether NAT10 depletion derails the expression of mRNAs belong to key modulators of ferroptosis pathway in cancer cells. Therefore, in the present study, we identify and assessed ferroptosis-related genes that are regulated through NAT10-dependent ac4C RNA modification.

2. Results

2.1. Depletion of NAT10 induces ferroptosis in cancer cells

Recently we reported that NAT10 regulates fatty acid metabolism in cancer cells(21). Among the top enriched pathways, we identified from our RNA-seq dataset were fatty acid metabolism and ferroptosis. The RNA-seq dataset was deposited in GEO datasets with accession number GSE210086. Ferroptosis-related genes that were differentially downregulated in NAT10 KD cancer cells included Glutamate Cysteine ligase catalytic subunit (*GCLC*), Microtubules associated protein 1 light chain 3α (*MAP1LC3A*), Soluble carrier family 7 member 11 (*SLC7A11*) and Soluble carrier family 39 member 8 (*SLC39A8*) (**Figure 1A-B**). Since, we found ferroptosis among the top pathways in NAT10 KD cancer cells, to explore whether NAT10 depletion induces ferroptosis. We transfected three breast cancer cell lines namely; MCF7, T47D, and MDA-MB-468 with NAT10 siRNA. We then collected morphological images from knockdown control (siC) and NAT10 knockdown (siNAT10) (**Figure 1C**). To confirm knockdown we performed RT-qPCR and ac4C Dot blot, both of which showed a significant reduction of NAT10 mRNA expression and ac4C levels respectively (**Figure 1D-E**). Expression levels of the ferroptosis-related genes *GCLC*, *MAP1LC3A*, *SLC7A11*, and *SLC39A8* in NAT10 KD cancer cells were significantly downregulated, therefore, validating the results from our RNA-seq data (**Figure 1F**).

During ferroptosis, depletion of system Xc⁻ (SLC7A11/GSH/PLOOH) generates reduced cystine uptake, glutathione levels, and increased lipid ROS. To explore ferroptosis features in NAT10-depleted breast cancer cells. We performed a cystine uptake assay and observed a remarkable decrease in cystine levels in NAT10 depleted cancer cells suggesting that cystine uptake could be impaired due downregulation of SLC7A11 mRNA (**Figure 1G**). To assess whether the reduction in cystine uptake has an impact on lipid ROS we conducted C-11 BODIPY assay (**Figure 1H**). Results from C-11 BODIPY assay showed a significant increase in lipid ROS levels upon NAT10 depletion in all three cell lines.

Both glutathione reductase (GSR) activity and malondialdehyde (MDA) level were remarkably reduced in NAT10 KD cells providing further evidence of ferroptosis induction through decreased glutathione synthesis and increased lipid peroxidation (**Figure 1I-J**).

Taken together, the results provided evidence that NAT10 depletion induces ferroptosis.

2.2. Depletion of NAT10 promotes excessive intracellular production of PUFAs and oxPL

To confirm whether there is an elevation in polyunsaturated fatty acid (PUFA) of phospholipid origin (PLOOH) and oxidized phospholipids (oxPL) in NAT10 KD cells, we performed untargeted metabolomics. Principal component analysis (PCA) showed that NAT10 alters the global metabolomic landscape comparing the NAT10 KD cells (siNAT10) vs transfection control (siC) (**Figure 2A**). PLOOH and oxPLs were detected and observed to be highly expressed in siNAT10 compared to siC (**Figure 2B**). The PL species include cardiolipin (CL), ceramide (Cer), phosphatidate (PA), phosphatidylinositol (PI), and phosphatidylserine (PS).

CLs are phospholipids predominantly found in the inner membrane of the mitochondrion. CL interacts with proteins from the complexes of electron transport

chain (ETC) and oxidative phosphorylation (OXPHOS) to promote proper enzymatic activity and structural integrity(22-24). Studies have shown that high cellular CLs cause lower cellular respiration, decrease ATP & energy wastage as well as lower OXPHOS efficiency (22).

Ceramides are major components of cell membranes whose overexpression is reported in cancer involving inflammation, and cell death(25-27). In particular, an increase in ceramides results in apoptosis, necroptosis, autophagy, and ferroptosis in response to DNA damage agents, anti-cancer drugs, or silencing of an important cancer gene i.e. NAT10. Additionally, PLs such as PA, PI, and PS and their oxidized products (oxPI and oxPS) have been reported to be highly expressed in ferroptosis(28).

Taken together our metabolomic data suggests an overproduction of PL and oxPLs in NAT10 KD cells correlated with ferroptosis.

2.3. Depletion of NAT10 induces oxidative stress in cancer cells

Mitochondria are critical organelles in that they control cellular respiration through electron transport chain coupled oxidative phosphorylation. Abnormal cell proliferation and survival of cancer cells require vigorous metabolism leading to the accumulation of ROS. To control elevated ROS levels cancer cells require functional mitochondria and a highly active antioxidant pathway. Therefore, we assessed the impact of NAT10 depletion on mitochondrial function via mitochondrial membrane potential assay. An increase in JC-1 levels was noticed in the NAT10 deflected cancer cells suggesting NAT10 is critical for mitochondrial polarization and proper electron transport (**Figure 3A**). Mitochondrial leakage due to leakage could lead to the escape of electrons from the intermembrane space to the cytoplasm where it causes the generation of ROS.

Cancer cells evade oxidative stress to promote their survival during progression and metastasis. Additionally, the accumulation of reactive oxygen species (ROS) in cancer cells is lethal and hinders cancer growth and survival.

To confirm if NAT10 depletion affects ROS levels, we measured the ROS level. High ROS level was observed in NAT10 KD cancers suggesting NAT10 is relevant in maintaining ROS levels in cancer cells (**Figure 3B**). To maintain cellular homeostasis, elevated ROS levels are mitigated by antioxidants such as Superoxide dismutase (SOD) and catalase (CAT).

A decrease in SOD and CAT activities was observed in NAT10 KD cells suggesting why cellular ROS were accumulated in the intracellular space (**Figure 3C-D**). Consistent with previous results, a reduction in the expression of mitochondrial associated respiratory genes such as *NDUFA1*, *NDUFA3,* and *NDUFA7* was observed in NAT10 KD cells (**Figure 3E**).

Taken together, these results demonstrate that ferroptosis is induced in NAT10 KD cells through oxidative stress which provides required ROS for the production of oxPLs due to impaired mitochondrial function and decrease in SOD and CAT activities.

2.4. Depletion of NAT10 induces ferroptosis via the SLC7A11/GSH/PLOOH axis in ac4C dependent manner

To further assess the impact of NAT10 as a regulator of ferroptosis, the ac4C RIP-PCR was conducted on all the four genes *GCLC*, *MAP1LC3A*, *SLC7A11,* and

SLC39A8 in NAT10 KD MCF7. Results from the RIP-PCR showed a significant reduction of ac4C levels on the mRNA transcripts of the ferroptosis genes *GCLC*, *SLC7A11,* and *SLC39A8*, however, no effect of ac4C reduction was seen in *MAP1LC3A* mRNA transcript (**Figure 4A**). To confirm whether decrease in ac4C levels affects RNA stability; half-life stability assay was performed. Compared with transfection control (siC); the NAT10 KD (siNAT10) showed a remarkable reduction in mRNA transcript's half-life of *GCLC*, *SLC7A11*, and *MAP1LC3A*, however, no significance was recorded for *SLC39A8* (**Figure 4B**). We did not find connecting evidence of ac4C levels with RNA stability in *MAP1LC3A* and *SLC39A8*, therefore, we presumed that NAT10 regulates ferroptosis through the SLC7A11/GSH/PLOOH axis by maintaining the *SLC7A11* and *GCLC* in an ac4C dependent manner.

2.5. Remodelin, a small molecule inhibitor of NAT10 induces ferroptosis

Previous results showed NAT10 induces ferroptosis, therefore, we decided to check whether Remodelin induces ferroptosis. First, the half minimum inhibitory concentration was calculated from results obtained from cell viability of Remodelin treated MCF7, T47D, and MDA-MB-468. The IC_{50}s were 13.42µM, 36.86 µM, and 21.36 µM for MCF7, T47D, and MDA-MB-468 respectively (**Figure 5A**). The IC_{50}s from the cancer cells were used for their treatment and the cell morphology of cells post-treated with Remodelin was recorded (**Figure 5B**). Notably, the expression of NAT10 in Remodelin treated cancer cells was significantly downregulated in MCF7 and MDA-MB-468, however, no significant difference was recorded in T47D comparing Remodelin treated vs control (**Figure 5C**). Expression levels of ferroptosis genes were significantly decreased in Remodelin post-treated cancer cells (**Figure 5D**). Measuring cystine uptake in Remodelin treated cancer cells, a significant reduction in intracellular cystine levels was observed suggesting interference of Remodelin with cystine uptake (**Figure 5E**). Additionally, lipid ROS was found to be decreased in MDA-MB-468 and MCF7 (**Figure 5F**). However, no significant change in lipid ROS levels was recorded in T47D.

Glutathione reductase activity and MDA level were decreased in cancer cells after Remodelin treatment (**Figure 5G,H**). Overall results from Remodelin treated cancer cells suggest Remodelin interferes with cystine uptake and hence promotes ferroptosis in cancer cells.

2.6. Remodelin promotes oxidative stress in cancer cells

Mitochondrial potential assay of Remodelin treated cancer cells showed an increase in mitochondrial depolarization suggesting Remodelin causes mitochondrial damage and hence could implicate in impaired electron transport chain and oxidative phosphorylation (**Figure 6A**). The ROS level was assessed in Remodelin treated cancer cells, an elevated ROS was noticed in all three studied cancer cells supporting the evidence of mitochondrial depolarization (**Figure 6B**). Based on the accumulation of ROS, the activities of SOD and CAT were measured. Results from the SOD and CAT activity showed a significant decrease suggesting that increase in ROS levels could be due to decreased activities of SOD and CAT (**Figure 6C,D**). Like NAT10 KD cells, the Remodelin treated cancer cells showed remarkable decrease in the mitochondrial respiratory genes *NDUFA1B*, *NDUFA3*, and

NDUFA7 (**Figure 6E**). Although no difference is seen in *NDUFA3* expression in MDA-MB-468. This might be attributed to the difference in cell lines.

The SOD and catalase are crucial antioxidant enzymes that scavenge and catalyze ROS when accumulated. Therefore, in a depolarized mitochondrial condition when these enzyme activities are reduced, ultimately implicates in high ROS level which is a driver of ferroptosis.

2.7. *Remodelin induces ferroptosis via reducing SLC7A11 RNA acetylation and stability*

Since Remodelin induces ferroptosis and oxidative stress, we then assess the impact of Remodelin on the ac4C levels and stability of mRNA transcripts of ferroptosis genes. A remarkable decrease in ac4C levels was only observed in SLC7A11 but not GCLC, MAP1LC3A, or SLC39A8 (**Figure 7A**). From the mRNA stability assay, all ferroptosis genes showed a significant decrease in half-life (**Figure 7B**). Taken together, we deduce that only *SLC7A11* is regulated by Remodelin in a dependent manner. The overall results here suggest that Remodelin promotes cystine starvation by preventing the expression of *SLC7A11* through ac4C dependent RNA modification.

Taken together, our results suggest that ferroptosis is induced in both NAT10 depleted and Remodelin treated cancer cells at different stages of the SLC7A11/GSH/PLOOH axis (**Figure 7C**). Reduced ac4C levels on mRNA transcript of *SLC7A11* and *GCLC* negatively affect their expression which in turn prevents cystine uptake and dampened glutathione synthesis thereby exposing the intracellular environment to higher levels of oxPLs. The oxPLs are overproduced in the presence of high ROS levels due to reduced SOD and CAT activities as well as impaired mitochondrial function. Collectively, results provide strong evidence of ferroptosis event in both NAT10 KD cells and Remodelin treated cancer cells.

3. Discussion

In this study, we discovered that NAT10 depletion induces ferroptosis in cancer cells through the SLC7A11/GSH/PLOOH axis. NAT10 maintains the stability of the mRNA of important ferroptosis pathway genes namely *SLC7A11* and *GCLC* through ac4C-dependent RNA modification. Further, we also found NAT10 was crucial for maintaining low levels of oxidized phospholipids that are known executioners of ferroptosis.

Previously, evidence has shown that NAT10 is a critical play in cancer biology by regulating important cellular events such as DNA damage, cell proliferation, cell survival, and senescence(6-10). Although the impact of NAT10 depletion in cancer cells is noticeable in cell morphology and cell viability, however, minimal results were reported on its impact on apoptosis. Therefore, we presumed that NAT10 depletion could be inducing other types of cell death other than apoptosis such as necroptosis, pyroptosis, and ferroptosis. Interestingly, we identified ferroptosis among the top enriched pathways from our previous RNA-seq of NAT10 KD cells(21).

Of note, it was recently reported that NAT10 regulates pyroptosis in sepsis through the acetylation of ULK1 RNA in the STING pathway via the ULK1-STING-NLRP3 axis(29).

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Glutathione is a critical intracellular antioxidant whose function is to detoxify the cell environment of oxPLs and other ROS(15). SLC7A11 is an important part of the system Xc- whose function is cystine uptake resulting in GSH synthesis(16, 30-32). The SLC7A11 and GCLC are core regulators of ferroptosis and have been studied as promising targets in cancer(31). Results from our study strongly suggested that SLC7A11 and GCLC of the SLC7A11/GSH/PLOOH axis are regulated by NAT10-mediated ac4C modification.

Previously, we discovered that Acyl-CoA synthetase long-chain 1 (*ACSL1*), *ACSL3,* and *ACSL4* were downregulated in NAT10 KD cells(21). Studies have reported the impact of ACSL4 as an inducer of ferroptosis through the production of Fatty acyl CoA of PUFA (PUFA-CoA) which in the presence of ROS leads to the formation of oxPLs(33-37). Although we found downregulation of *ACSL4* in NAT10 KD cells, however, our metabolomics data of NAT10 KD cells showed excessive production of cellular PUFAs and oxPLs. The question is whether other available fatty acyl CoA enzymes are involved in the formation of PUFA-CoA in NAT10 KD cells. Recently, it was reported that ACSL4 promotes cancer progression and therefore ACSL4-dependent ferroptosis does not necessarily represent tumor suppression(38). Another study challenges the current view of ACSL4 as the universal ferroptosis regulator for the following reasons ferroptosis is mostly induced in GPX4-dependent inhibition using RSL3, however, SLC7A11 inhibition with erastin or cystine starvation induces ferroptosis either from GPX4-dependent or GPX4-independent mechanisms, this is because the cystine uptake is not only important for glutathione synthesis but also biomolecules such as coenzyme A (CoA)(39, 40). The CoA is important for both NAT10 and ACSLs as it is required for acetyl-CoA synthesis which is the substrate for their enzyme-based catalyzed reactions. To harmonize our claim of ferroptosis induction by NAT10 and ACSL4 downregulation, therefore, we presumed that decreased gene expression of SLC7A11 via NAT10-mediated acetylation and cystine starvation negatively affects CoA production. Hence enzymes that utilize acetyl-CoA including ACSL4.

We observed increased production of PUFAs and oxPLs in NAT10 KD cells from our metabolomic data. To confirm oxPLs production from our metabolomics data; remarkable reduction in GSR, SOD, and CAT activities along with elevation in malondialdehyde levels were observed further supporting the evidence of hampered glutathione synthesis and ferroptosis induction.

Collectively, our results have provided evidence that NAT10 depletion induces ferroptosis in cancer cells. It also provided evidence that treatment of cancer cells with Remodelin induces ferroptosis and oxidative stress. Therefore, Remodelin causes cell death in cancer mostly by ferroptosis, this could be the reason why studies even though reporting the impact of Remodelin on cell proliferation and cell survival but fail to report any significant impact of Remodelin on apoptosis.

In conclusion, our study has unveiled the evidence that targeting NAT10 in cancer induces ferroptosis and this mechanism could be explored for possible cancer treatments and therapeutic benefits.

4. Materials and Methods

4.1. Cell culture, transfection and treatment

Human breast cancer cells, MCF-7, MDA-MB-231, MDA-MB-468 and T47D were cultured in Dulbecco's Modified Eagle Media (DMEM) (BIS BioTech, KSA) with 10% fetal bovine serum (FBS) (Sigma, USA), and 1% penicillin-streptomycin (MOLEQULE-ON®, New Zealand). All studied cells were maintained in humidified incubator conditions of 37°C and 5% CO_2. In this study, cells were grown to 40-60% confluence before transfection.

NAT10 siRNA transfection was performed using specific siRNA targeting NAT10 (Santa Cruz Biotechnology Inc, USA) and Lipofectamine^{TM} RNAiMAX (Invitrogen, USA) following the manufacturer's protocol. Briefly, the stock solution of NAT10 siRNA was prepared to 10 µM and the final concentration of 60 nM of either NAT10 siRNA or scramble control was used for transfection.

Cells were allowed to adhere to plates at 40-60% confluence and treated with Remodelin (Cayman Chemicals, Estonia) for 24 h. The half maximal inhibitory concentration (IC_{50}) was measured based on cell viability assay.

4.2. Cell viability assay

Cells of 1×10^4 were seeded per well in 96 wells plate and subject to cell viability using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide). Briefly, cells were allowed to adhere to plates for 24h, followed by NAT10 knockdown or Remodelin treatment. After NAT10 knockdown, 10 µL solution containing MTT (5µg/mL, MOLEQULE-ON®) was added to the wells and incubated for 3h. After the incubation time was complete, DMEM medium containing MTT was removed from the wells, 100 µL DMSO was subsequently added to each well and allowed to incubate for at least 30 min at 37°C. The absorbance of content was read with a microplate reader (BioTek®, USA) using the Gen5™ software for microplate reading and data analysis. Cell viability was graphically represented in mean±SEM values calculated using GraphPad Prism version 8.0.1(20).

4.3. Flow cytometry

Cells of 1×10^6 were either knocked down with NAT10 siRNA or Remodelin for 24 h, washed with PBS, and trypsinized.

Lipid peroxidation assay was performed using 2.5 µM C-11 BODIPY (Sigma, USA) at 37°C for 30 min. Cystine-FITC uptake assay was conducted using 2µM Molecular Biotracker cystine-FITC (Sigma, USA) and incubated at 37°C for 30 min. Mitochondrial membrane potential was analyzed using JC-1 staining (Invitrogen, USA) followed by incubation at 37°C for 30 min. Reactive oxygen species (ROS) levels were measured by staining cells with 500 nM of CellRox Green (Invitrogen, USA) and then incubated at 37°C for 30 min.

All assays were acquired at 10,000 cells per sample using Amnis Flowsight. Measurements were performed in technical triplicates and repeated in double biological replicates.

4.4. Biochemical analysis

Cell pellet was washed and resuspended in 200 µL PBS followed by lysis. The cell lysate was then centrifuged at 10,000 rpm for 15 min. The collected supernatant

was then subjected to protein quantification using Bradford method. All samples were normalized before assays.

Glutathione reductase activity was measured using the Glutathione Reductase Assay kit (Millipore-Sigma, GRSA-1KT) following the manufacturer's instructions. This assay is based on the reduction of GSSG by GR in presence of NADPH. In addition, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) reacts with GSH to form 5-thio(2-nitrobenzoic acid). Therefore, the GR activity was measured by the increase in absorbance at 412 nm caused by the reduction of DTNB.

Lipid peroxidation was quantified by measuring the malondialdehyde (MDA) formed using the thiobarbituric acid-reactive substances (TBARS) assay according to a previously described method. Briefly, 500 µL of cell lysate was added to 1 mL of TBA:TCA: HCl reagent (0.38% thiobarbituric acid prepared in distilled water and warmed in a water bath, 15% trichloroacetic acid dissolved in distilled water, and 0.25N hydrochloric acid, ratio 1:1:1), boiled at 90ºC for 20 min, and cooled on ice followed centrifugation at 10000 rpm for 10 min, the absorbance of TBARS was measured at 532 nm against a reagent blank(41).

Superoxide dismutase activity (SOD) was determined using an SOD assay kit (19160-1KT-F) following the manufacturer's protocol. This assay is based on WST-1 [2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2Htetrazolium, monosodium salt] method. WST-1 reacts with superoxide anion to produce a water-soluble formazan. The SOD activity was measured at 450 nm.

Catalase activity assay was performed using the ammonium molybdate method. Briefly, 20µL of the sample was added to 100 µL of 30 mM H_2O_2 and allowed to incubate at room temperature for 10 min. The reaction is then stopped with 100 µL 32.4 mM ammonium molybdate and catalase activity was measured at 405 nm.

4.5. Extraction for Metabolomics

Cells were seeded in a 75 cm^3 flask and allowed to grow for 24 h. At 40-50% confluence, the cells were knockdown with NAT10 siRNA. Cells were then washed with ice-cold PBS and 200 µL of iced-cold distilled water was added to the flask. Cells were collected with a scrapper and vortexed to increase breakage, followed by the addition of methanol and acetonitrile (1:1). Content was incubated at -20ºC for 24 h and centrifuged at 14,000 rpm for 10 min. The supernatant was isolated and taken for metabolomic profiling.

4.6. Untargeted metabolomics for lipids profiling

Metabolomic profiling was performed using LTQ XL™ linear ion trap (Thermo Fisher Scientific, Waltham, MA, USA) LC-MS/MS instrument.

Briefly, 10 µL of metabolite extracts were injected into the HPLC column (Hypersail gold column C18 Part No: 25005-104630) at 0.250 mL/min flow rate. The metabolite extracts were gradient eluted using mobile phase of A consisting of 99.9% acetonitrile and 0.1% formic acid (0.1% v/v) and B consisting of 0.1% formic acid in 99.9% water (0.1%, v/v) constituted the mobile phase using a gradient program in which the component of the solution was varied from 5% to 30% for 30 min, 30% to 50% for 10 min, 50% for 10 min, and finally 50% to 95% for 20 min, with a total running time of 70 min at a column temperature of 30 °C.

For mass spectrometry, the MSn parameters used include full scanning mode was used ranging from 80 to 2000 m/z; helium was used as buffer gas, with nitrogen being used as the sheath gas, with 40 arbitrary units as the flow rate; a capillary temperature of 270 °C was used with a voltage of 4.0 V, and the spray voltage was set at −3.0 kV.

4.7. Total RNA extraction, cDNA synthesis and Quantitative RT-PCR

Total RNA was extracted with RNA isolation kit (Haven scientific, KSA) following the manufacturer's instructions. The purity and concentration of the extracted RNA were determined at 260/280 nm using a spectrophotometer (Nanophotometer) and were reverse transcribed into cDNA with a High Capacity cDNA conversion Kit (Applied Biosystems) using $2\,\mu$g of total RNA. Gene expression was determined by qRT-PCR performed with SYBR Green (Applied Biosystems). The PCR program started at preheating step of 50°C for 2 min, 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and a final extension at 60°C for 1 min. Melting curves were obtained at 60°C. Data were reported as fold change ($2^{-\triangle\triangle Ct}$). Assays were performed independently in triplicate.

4.8. ac4C Dot blot

RNA dot blot was performed as previously described. Briefly, 6μg of total RNA were placed at 65°C for 5 min and immediately cooled on ice for 3 min. Denatured RNAs were then spotted on the Hybond N^+ membrane and crosslinked with UV light. The ac4C modifications on RNA were detected using anti-ac4C (Abcam).

4.9. RNA Immunoprecipitation (RIP)-PCR

Cell pellets from NAT10 KD cells or Remodelin treated cells were lysed with RIP buffer (300mM Tris-HCl, 150mM KCl, 0.5mM fresh DTT, 0.5%(v/v) NP40, ×1 protease inhibitor) and incubated in -80°C for 24 h. The lysate was then centrifuged at 14000 rpm for 10min at 4°C, then 10% of the input sample was collected before adding 2μg of either Anti-ac4C (Abcam) or corresponding anti-IgG (Cell Signaling). The content was allowed to incubate overnight at 4°C with constant gentle rotation. Next, protein A/G agarose beads (Cell Signaling) were added and allowed to rotate for another 2h. Beads were collected after centrifugation at 1000 rpm for 2 min. The RNA of input and IPed samples were extracted with Tri Reagent® solution (Sigma, USA) and subjected to RT-PCR.

4.10. mRNA stability assay

Cells were seeded in 6 wells plate for 24h followed by treatment with 5μg/mL actinomycin D. Cells were then collected at different time points. Total RNA was extracted with Tri Reagent® solution (Sigma, USA) and the gene expressions of the studied genes were determined using RT-PCR. The half-life and turnover rate was calculated according to previous reports(42).

4.11. Statistical analysis

All quantitative data were represented as mean±SEM. The two-tailed student t-test was used for statistical differences between two groups. Whereas for more than two groups Tukey multiple comparison test or Sidak's multiple comparison test were used. All results were considered statistically significant at $p<0.05$.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors contribution

Conceptualization, M.I.K.; methodology, M.H.D, M.I.K and H.C.; software, M.H.D and M.I.K.; validation, M.H.D., and M.I.K.; formal analysis, M.H.D., and M.I.K.; investigation, M.H.D and M.I.K; resources, H.C and M.I.K.; data curation, M.H.D., and M.I.K.; writing—original draft preparation, M.H.D.; writing—review and editing, H.C. and M.I.K.; visualization, M.H.D. supervision, H.C. and M.I.K. All authors have read and agreed to the published version of the manuscript.

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FIGURE CAPTIONS

Figure 1. NAT10 knockdown induces ferroptosis in cancer cells. A. Enriched pathways from NAT10 KD cells B. Volcano plot of differentially expressed genes from NAT10 KD cells including ferroptosis related genes C. Cell morphology of MCF7, T47D and MDA-MB-468 NAT10 KD cells. Images were captured using Nikon microscope at X10 magnification. D. Expression of NAT10 in NAT10 KD MCF7, T47D and MDA-MB-468. E. Dot blot image of MCF7, T47D and MDA-MB-468 NAT10 KD cells. F. Expression of ferroptosis related genes in MCF7, T47D and MDA-MB-468 NAT10 KD cells. G-H. Imaging flow cytometry analysis of cystine uptake and C11-BODIPY assay. I. GSR activity assay of NAT10 KD cells. J. Lipid peroxidation measurement of NAT10 KD cells. Data are represented as mean±SEM (n=3) and p-value is represented as *p<0.05; **p<0.01; ***p<0.001; and ns>0.05.

Figure 2. Global metabolic landscape of NAT10 KD cells. A. PCA analysis in siC vs siNAT10 MCF7 cancer cells. B. PUFAs and oxPLs obtained from metabolomics of siC vs siNAT10 MCF7 cells. Data are represented as mean±SEM (n=3) and all results showed significant difference (p<0.05) when comparing transfection control (siC) and NAT10 KD (siNAT10).

Figure 3. Depletion of NAT10 induces oxidative stress. A-B. Imaging flow cytometric analysis of mitochondrial membrane potential and reactive oxygen species (ROS) levels of NAT10 KD cells. C,D. SOD and CAT activities of NAT10 KD cells. E. Expression of mitochondrial respiration related genes. Error bars are represented as mean±SEM (n=3) and p-value is represented as *p<0.05; **p<0.01; ***p<0.001; and ns>0.05.

Figure 4. NAT10 maintains the ac4C levels of ferroptosis genes. A. RNA Immunoprecipitation PCR of ferroptosis related genes. Results are present as mean±SEM and p value **P<0.01 was considered as statistically significant and ns>0.05 considered as non-significant. B. RNA stability assay of ferroptosis related gene analysis was performed using exponential decay method with GraphPad Prism 8.0.1.

Figure 5. Remodelin induces ferroptosis in cancer cells. A. Cell viability of cancer cells treated with Remodelin showing dose dependent response at 24h. Graph was potted using non-linear regression curve fit using GraphPad prism. B. Cell morphology of MCF7, T47D and MDA-MB-468 24h post-treated with Remodelin. Images were captured using Nikon microscope at X10 magnification. C. Expression of NAT10 in MCF7, T47D and MDA-MB-468 cancer cells 24h posttreatment with Remodelin. D. Expression of ferroptosis related genes in MCF7, T47D and MDA-MB-468 in Remodelin treated cancer cells. E,F. Imaging flow cytometry analysis of cystine uptake and C11-BODIPY assay of MCF7, T47D and MDA-MB-468 in Remodelin treated cancer cells. G. GSR activity assay H. Lipid peroxidation measurement of Remodelin treated cells. Data are represented as mean±SEM (n=3) and p-value is represented as *p<0.05; **p<0.01; ***p<0.001; and ns>0.05.

Figure 6. Remodelin induces oxidative stress in cancer cells. A-B. Imaging flow cytometric analysis of mitochondrial membrane potential and reactive oxygen species (ROS) levels of Remodelin treated cancer cells. C,D. SOD and CAT activities of cancer cells 24h after treated with Remodelin. E. Expression of mitochondrial respiration related genes in Remodelin treated cancer cells. Error bars

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are represented as mean±SEM (n=3) and p-value is represented as *p<0.05; **p<0.01; ***p<0.001; and ns>0.05.

Figure 7. Remodelin induces ferroptosis through ac4C reduction on SLC7A11 mRNA tanscript. A. RIP-PCR of ferroptosis related genes in cancer cells 24h after treatment with Remodelin. Results are present as mean±SEM and p value *P<0.05 was considered as statistically significant and ns>0.05 considered as non-significant. B. RNA stability assay of ferroptosis related gene analysis was performed using exponential decay method with GraphPad Prism 8.0.1. C. Proposed mechanism of NAT10 and Remodelin induction of ferroptosis.