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Reprogramming metabolism by histone methyltransferase NSD2 drives endocrine resistance via coordinated activation of pentose phosphate pathway enzymes

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ABSTRACT

Metabolic reprogramming such as the aerobic glycolysis or Warburg effect is well recognized as a common feature of tumorigenesis. However, molecular mechanisms underlying metabolic alterations for tumor therapeutic resistance are poorly understood. Through gene expression profiling analysis we found that histone H3K36 methyltransferase NSD2/MMSET/WHSC1 expression was highly elevated in tamoxifen-resistant breast cancer cell lines and clinical tumors. IHC analysis indicated that NSD2 protein overexpression was associated with the disease recurrence and poor survival. Ectopic expression of NSD2 wild type, but not the methylase-defective mutant, drove endocrine resistance in multiple cell models and xenograft tumors. Mechanistically, NSD2 was recruited to and methylated H3K36me2 at the promoters of key glucose metabolic enzyme genes. Its overexpression coordinately up-regulated hexokinase 2 (HK2) and glucose-6-phosphate dehydrogenase (G6PD), two key enzymes of glycolysis and the pentose phosphate pathway (PPP), as well as TP53-induced glycolysis regulatory phosphatase TIGAR. Consequently, NSD2-driven tamoxifen-resistant cells and tumors displayed heightened PPP activity, elevated NADPH production, and reduced ROS level, without significantly altered glycolysis. These results illustrate a coordinated, epigenetic activation of key glucose metabolic enzymes in therapeutic resistance and nominate methyltransferase NSD2 as a potential therapeutic target for endocrine resistant breast cancer.

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Introduction

Tumor growth involves reprogrammed glucose metabolism, featured in aerobic glycolysis, to meet the high demand of glycolytic intermediates for biosynthesis of macromolecules. The pentose phosphate pathway (PPP) is a major cellular source of NADPH, in addition to its supply of precursors for nucleotide biosynthesis. Deregulated PPP has been suggested to promote cancer progression and therapy resistance [1]. The activities of PPP can be decreased by p53, as well as being hyperactivated by oncogenic signaling [2–5]. Functioning as a fructose-2,6-bisphosphatase (F2,6bPase), TIGAR (TP53-induced glycolysis and apoptosis regulator) can enhance glucose

carbon flux to the PPP by dampening glycolysis and is required for the development of intestinal adenomas [6–9]. As a glycolysis modulator, TIGAR was shown to localize in cytoplasm and associate with mitochondria in complex with the hexokinase HK2 in response to hypoxia [7]. HK2, one of the hexokinases that catalyze the first and rate-limiting step of glucose metabolism, is highly expressed in most tumor cells. HK2 plays a pivotal role in diversion of glucose into pathways such as the PPP for enhanced anabolic metabolism required for tumor growth [10,11]. Glucose-6-phosphate dehydrogenase (G6PD) is the rate-limiting enzyme of the PPP and plays a key role in production of NADPH, the major cellular source of reducing power. However, the mechanism of how the different metabolic genes are coordinately regulated in cancer therapeutic resistance is poorly understood.

NSD2, also known as MMSET or WHSC1, preferentially dimethylates H3K36 and is overexpressed in a subset of multiple

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myeloma and many types of solid tumors including breast, prostate and lung cancers [12–15]. One major mechanism of aberrant NSD2 function is to reprogram the cell epigenome and de-regulate the expression of genes important in control of cell cycle, cell adhesion and epithelial–mesenchymal transition (EMT) [16–18]. NSD2 can also act as a coactivator of NF- κ B in mediating cytokine-dependent autocrine loop for cancer cell growth and survival [15]. One recent study showed that NSD2 could directly regulate estrogen receptor ER α expression in breast cancer cells [19].

The selective estrogen receptor modulator (SERM) tamoxifen is a standard endocrine therapy for women with ER α -positive breast cancer. However, both de novo and acquired resistance to the drug remains a clinically important problem. Several mechanisms of acquired tamoxifen resistance have been reported, including increased expression and/or function of ER α or its co-activators, its gene mutations and its cross-talk with receptor tyrosine kinases and other kinases, as well as its loss of expression [20]. Despite the development of alternative therapeutics, such as aromatase inhibitors (AIs) or combined treatment with tyrosine kinase inhibitors, recurrent disease still poses a major clinical challenge. Thus, there is an urgent need of developing more specific biomarkers that predict the therapeutic response and identifying new therapeutic targets for tamoxifen-resistant breast cancer.

In this study, we found that NSD2 overexpression correlates strongly with poor survival in ER α -positive breast cancer patients treated with tamoxifen. We demonstrated that NSD2 overexpression can drive tumor resistance to tamoxifen treatment through coordinated up-regulation of the expression of key glucose metabolic enzymes, stimulation of the PPP pathway and elevating cellular NADPH level for effective maintenance of redox homeostasis. Thus, our study establishes NSD2 as a new epigenetic driver of tamoxifen resistance and nominates targeting NSD2 methylase as a therapeutic option for treating endocrine resistant breast cancer.

Materials and methods

Cell culture and chemicals

MCF-7, ZR-75-1, T47D, and 293T cell lines were obtained from ATCC. NSD2 overexpressing sublines were generated by NSD2-lentivirus infection of corresponding cells followed by antibiotic selection. The tamoxifen resistant (TamR) sublines were generated by maintaining the parental cells in phenol red-free medium supplemented with 10% charcoal-stripped calf serum containing 0.5–1 μ M 4-hydroxytamoxifen for over 8 months. The MCF-7 derived, tamoxifen-resistant (TamR) LCC subline cells were kindly provided by Dr. Robert Clarke (Georgetown University). Sources of chemicals are as follows: hydrogen peroxide solution (Sigma, H1009), 4-hydroxytamoxifen (4OHT, Sigma, H7904), tamoxifen (Sigma, T5648), and ICI182,780 (Sigma, I4409). Other chemicals are from Sigma unless indicated otherwise.

Generation of lentiviruses and NSD2 stable expression sublines

To generate NSD2-overexpressing MCF-7 and T47D cell sublines, flag-tagged, human NSD2 WT and NSD2-Y118A mutant cDNAs were cloned into pLenti4/V5-DEST (Invitrogen). Lentiviruses were produced in 293T cells after co-transfection of the above lentivirus vector, psPAX2 and pMD2.G into cells in 10 cm dishes, as described [21]. MCF-7 and T47D cells were infected for 6 h, in the presence of polybrene, with the virus-containing supernatant collected 48 and 72 h after the co-transfection. Infected cells were selected with Zeocin (10 μ g/ml, Invitrogen) for 4–6 weeks. Individual Zeocin-resistant clones were isolated, expanded and examined for ectopic NSD2 expression by immunoblotting. Clones homogeneously expressing NSD2 were maintained in medium containing 10 μ g/ml Zeocin.

siRNAs and transfection

Cells were grown at 37 °C in 5% CO₂ incubators and transfected using Dharmafect1 for siRNA knockdown. siRNAs were purchased from Dharmacon. The siRNA target sequences for each gene are as follows: NSD2#1, AGGGAUCGGAAGAGUCUCAA; NSD2#2, GCACGCUACAACACCAAGU; TIGAR#1, GCAGCAGCUGCUGUAUUAU; TIGAR#2, UUAGCAGCCAGUCUUAAG; HK2#1, CCAAAGACAUUCACAGACAUUG; HK2#2, GCAGAAGGUUGACCAGUAUCU; Control, CAGUCGCGUUGCGACUGG.

Apoptosis, cell growth assays, qRT-PCR and immunoblotting analysis

Cell apoptosis and growth assays were performed as previously described [15]. Gene expression analysis was performed as previously described [15,22]. Antibodies against the following proteins/epitopes were used with the sources and dilution for Western blotting indicated: NSD2 (Abcam, ab75359 (29D1); 1:2000); TIGAR (Abcam; ab37910; 1:2000; or Santa Cruz; sc-166291; 1:500); HK2 (Cell Signaling; #2867; 1:2000); G6PD (Abcam; ab133525; 1:2000); GAPDH (Cell Signaling; #2118; 1:4000); β -actin (Santa Cruz; sc-47778; 1:2000); cleaved-PARP1 (Cell Signaling; #9542; 1:1000); cleaved-Caspase7 (Cell Signaling; #9491; 1:1000); H3K36me1 (Abcam; ab9048; 1:2000); H3K36me2 (Active Motif; #39255; 1:2000); H3K36me3 (Abcam; ab9050; 1:2000); H3K27me3 (Abcam; ab6002; 1:2000); H3K27ac (Abcam; ab4729; 1:2000); and H3 (Active Motif; #39163; 1:2000). All PCR primers are purchased from IDT. Primer sequences for real-time RT-PCR for analysis of gene expression are listed in the Supplementary Table S1.

ChIP assay

ChIP was performed as described previously [15]. Antibodies used for ChIP assays are the following: NSD2 (rabbit antiserum, as described in Ref. [15]), H3K36me2 (Active Motif; #39255) and H3 (Active Motif; #39163). PCR primers are listed in Supplementary Table S2.

ROS level measurement

Different times after the indicated treatments, cells were washed two times by PBS and loaded with 5 μ M CM-H₂DCFDA (Invitrogen). ROS levels were analyzed as previously described [21,23].

Colony formation

For colony formation assay, 800 cells were seeded in a 6 well plate and cultured for 14 days in a 37 °C humidified incubator supplied with 5% CO₂. The medium was changed every 3 days. When the cell clone grew visible, the medium was removed and the cells were fixed with 10% formalin for 10 min. The plate was then washed with PBS for two times and the cell colony was stained with 0.2% crystal violet (in 10% formalin) for 15 min. The numbers of cell colonies were counted after 5 times of wash with PBS. The assays were performed in triplicate and the entire experiments were repeated more than three times.

Gene ontology (GO)/pathway analysis

Total RNA was isolated using Trizol (Invitrogen) from MCF-7 parental and TamR cells treated with or without tamoxifen for 24 h. RNA was sequenced using Illumina high-seq 2000 with a read length of 50 bp with pair ends. RNA-seq reads were mapped to the human genome (hg19) using TopHat. Only those reads mapped to unique genomic locations and with <5% mismatches were analyzed further. Gene transcripts were measured by Cufflinks, and differentially expressed genes were analyzed by RefSeq GTF file downloaded from Illumina iGenomes. RNA-seq data have been deposited to NCBI-GEO with accession code GSE62613. Gene Ontology annotation comparison for the identified genes was performed using the DAVID classification system (<http://david.abcc.ncifcrf.gov/>), a resource that classifies genes and proteins by molecular function, biological process, and cellular component, as described [24,25].

Glycolytic rate assay and glucose consumption assays

Glycolytic rate was measured by monitoring the conversion of 5-³H-glucose to ³H₂O, as described [6]. Cells were washed with PBS once and resuspended in Krebs buffer. After a 30-min incubation, Krebs buffer containing glucose and [5-³H]glucose (PerkinElmer) was added to cells to make final concentration of 10 mM glucose containing [5-³H]glucose (10 μ Ci/ml) in 0.5 ml and incubated for 1 h at 37°. After 1 h, 0.1 ml of 0.2 M HCl was then added to the mixture to stop the reaction. Then 0.2 ml of the reaction mixture was transferred to a small open tube that is placed in a scintillation vial that already contained 0.5 ml water for 48 h. The amount of diffused ³H₂O was determined by scintillation counting. Glucose consumption was determined using the Glucose (GO) Assay kit (Sigma) following the manufacturer's instructions.

Assays for NADPH/NADP⁺ ratio, G6PD activity, lactate and GSH level

Cells were washed with PBS, scraped and collected. NADP⁺/NADPH ratios were determined using the NADP⁺/NADPH Quantification kit (BioVision). G6PD activity was determined using the Glucose-6-Phosphate Dehydrogenase (G6PDH) Activity Assay Kit (BioVision). Lactate level was determined using Lactate Colorimetric Assay Kit (BioVision). GSH level was determined using the GSH-Glo™ Glutathione Assay kit (Promega). The above assays were performed following the instructions from the corresponding kit manufacturers.

Fru-2,6-P2 level determination

Cellular fructose 2,6-bisphosphate level was determined as described [6]. Cells were briefly lysed in 0.1 M NaOH. The lysates were incubated at 80 °C for 15 min and centrifuged at 12,000 g for 5 min. The supernatants were taken to measure the Fru-2,6-P2 by its activity to activate pyrophosphate-dependent PFK-1 enzyme from potato tubers as described [26].

Pentose phosphate pathway (PPP)-dependent glucose oxidation to CO₂

PPP activity was analyzed as described [27]. Briefly, cells in 96-well plates were supplemented with 5 µCi/ml of [1-¹⁴C]-glucose (specific activity 45–60 mCi/mmol) or [6-¹⁴C]-glucose (specific activity 50–62 mCi/mmol, all from PerkinElmer) as indicated in a final volume of 100 µl. The wells were overlaid with Ba(OH)₂ impregnated 3MM Whatman paper to capture released ¹⁴CO₂ in an incubator at 37 °C, 5% CO₂ for 3 h. The paper was removed and placed in an acetone bath, air-dried and incubated at 110 °C for 5 min. Pieces of the paper corresponding to each well were cut and placed in scintillation counting vials containing scintillation fluid. Their radioactivity was measured in a Beckman LS6500 scintillation counter. The radioactivity of ¹⁴CO₂ from [1-¹⁴C]-glucose was used as a measurement of the carbon flux through the PPP enzyme, 6-phosphogluconate dehydrogenase. PPP-dependent CO₂ production was calculated as the difference between ¹⁴CO₂ derived from [1-¹⁴C]-glucose and ¹⁴CO₂ derived from [6-¹⁴C]-glucose (TCA cycle-dependent CO₂ production from glucose).

Gas chromatography-time of flight mass spectrometry (GC-TOF MS) analysis of metabolites

GC-TOF MS analysis was performed as previously described [28]. To prepare cell pellets for metabolite extraction, cells were washed three times with cold PBS, flash frozen in liquid nitrogen, and stored at –80 °C prior to extraction. Sample materials were analyzed with a Leco Pegasus IV TOF MS (Leco Corporation, St. Joseph, MI) coupled to an Agilent 6890 gas chromatography (Agilent Technologies, Santa Clara, CA) equipped with a 30 m long 0.25 mm i.d. Rtx5Sil-MS column and a Gerstel MPS2 automatic liner exchange system (Gerstel GMBH & Co. KG, Mülheim a der Ruhr, Germany). Result files were exported to the servers and further processed by our metabolomics BinBase database. Six independent experimental replicates were extracted with 3:1 methanol:H₂O for the MS analysis.

Immunohistochemistry (IHC), patient tumor specimens, and statistical analysis

IHC was performed as previously described [15,29]. Sections of tissue microarrays (TMAs) were incubated with anti-NSD2 monoclonal antibody (Abcam, 29D1), at 1:50 and 1:100 dilutions respectively, overnight at 4 °C, followed by incubations with biotinylated secondary antibody and the ABC reagents in the Vectastain Elite kit, visualized by staining with diaminobenzidine chromogen solution and followed by counterstaining with hematoxylin. TMAs contain specimens from about 450 cases of breast cancer patients who subsequently received adjuvant tamoxifen treatment. All the tumor specimens used in this study were from the Manitoba Breast Tumor Bank (MBTB, CancerCare Manitoba and University of Manitoba) [30]. MBTB embraces the policies and operating protocols of the Canadian Tumor Repository Network and operates with approval from the Research Ethics Board of the Faculty of Medicine, University of Manitoba. The histopathology assessment of the MBTB biospecimens, the cohort characteristics, ERα-positive status determination, and sample selection for tissue microarray (TMA) construction are recently described [31]. A total of 450 cases were represented on the original TMAs. The tumor numbers (n) analyzed for some markers, however, were less than 450, due to exhaustion of some tumor cores from previous uses. The current cohort characteristics are progesterone receptor (PR)-positive (>20 fmol/mg protein), 62.5% (261/336); PR-negative, 37.5% (126/336); low-grade, 27.7% (93/336); intermediate-grade, 61.6% (207/336); high-grade, 10.7% (36/336); tumor size <2.5 cm, 55.5% (187/337); tumor size ≥2.5 cm, 44.5% (150/337); age <50 years, 6.9% (23/335); age >50 years, 93.1% (312/335); node-negative, 49.6% (164/331); and node-positive, 50.5% (167/331). The median follow-up was 99 months (range 9–217 months). The TMA section was scored negative if <1% of the breast epithelial cells displayed any staining and scored positive if >5% of the cells displayed staining in the nucleus with moderate to high intensity. Differences and correlations in immunostaining among groups were analyzed with the χ^2 test. Other statistical analysis was performed as previously described [31].

Analysis of tumor NSD2 mRNA expression association with clinical outcome

Microarray data from GSE9195 and GSE17705 were downloaded from the NCBI GEO website (<http://www.ncbi.nlm.nih.gov/gds>). The datasets consist of gene expression profiles of ER-positive breast cancer tumors that had received tamoxifen only as adjuvant treatment. Normalized probe set expression intensities were obtained using robust multi-array average (RMA) as previously described [15]. The Kaplan–Meier estimates were used to compute the survival curves. All above computations were conducted in R statistical package (<http://www.r-project.org/>). In addition, Kaplan–Meier curves were obtained for relapse-free survival times of

patients (n = 665) who received tamoxifen only and with tumors stratified by NSD2 expression levels, using an online survival analysis tool (<http://kmplot.com>), as described [32]. Statistical significance was determined by the log-rank test.

Xenograft tumor models

For establishment of tumors with the TamR subline cells, approximately 5×10^6 MCF7-derived cells were suspended in 100 µl PBS/Matrigel (1:1) and injected s.c. into ovariectomized, female BALB/c nu/nu athymic mice (4 weeks old, Sprague–Dawley, Harlan). For establishment of tumors with NSD2-overexpressing or control cells, approximately 5×10^6 MCF7-derived cells were suspended in 100 µl PBS/Matrigel (1:1) and injected s.c. into ovariectomized, female BALB/c nu/nu athymic mice (4 weeks old, Sprague–Dawley, Harlan), which simultaneously received a 60-day, slow release pellet containing 0.72 mg of 17β-estradiol (Innovative Research of America). The mice were ovariectomized before the injection. When the tumor volume was approximately 100 mm³, the estrogen source was removed, and either a tamoxifen-containing pellet or a placebo (Innovative Research of America) was implanted. Six mice were used for each treatment. Tumor growth was monitored every week by calipers with volume calculated using the equation: [$\frac{1}{2}$ (length × width²)]. The procedures were approved by the Institutional Animal Care and Use Committee of University of California Davis (protocol No. 17789).

Statistical analysis

Cell culture-based experiments were performed three times or more unless indicated otherwise. The data are presented as mean values ± SD from three independent experiments. Statistical analysis was performed using two-tailed Student's t tests to compare means. $p < 0.05$ was considered significant. Patient survival curves were generated using the Kaplan–Meier method, taking into account censored data. The curves were compared using the log-rank test (Mantel–Cox) [31]. Pearson's χ^2 test was used to determine the immunostaining association between NSD2.

Results

NSD2 overexpression is strongly correlated with poor survival in ER-positive breast cancer patients treated with tamoxifen

To further study the mechanism of acquired tamoxifen resistance, we established several tamoxifen-resistant (TamR) sublines of breast cancer cells by treating tamoxifen-sensitive MCF-7, T47D and ZR-75-1 cells with escalating concentrations of 4-hydroxytamoxifen (4OHT) from 0.1 µM to 0.5 or 1 µM over six months. As shown in Fig. 1a and Supplementary Fig. S1a, the resulting TamR sublines proliferated and survived well in the presence of 0.5 or 1 µM 4OHT. When implanted in ovariectomized athymic nude mice, tumors formed from MCF-7-TamR#2 cells grew independent of ovarian hormones and, interestingly, appeared to be stimulated by tamoxifen (Fig. 1b). As previously reported [33], the control MCF-7 cells failed to form detectable tumor in ovariectomized mice (data not shown).

We next performed gene expression profiling of the Tam-sensitive and TamR cells. In agreement with previous studies that E2F-Rb pathway alteration is a key feature of endocrine resistance [34,35], changes of cell proliferation genes are overrepresented in the TamR cells (Supplementary Fig. S1b). Closer examination suggested that expression of members of the histone methyltransferase (HMT) family (e.g. MLL5, SETD6, WHSC1/NSD2, SMYD5 and PRDM16) was also significantly increased (Fig. 1c). Given that NSD2 has been linked to various cancers in the previous studies, we thus focused on NSD2 for its potential role in tamoxifen resistance. Indeed, NSD2 mRNA and protein are highly elevated in multiple different TamR cells independently derived by us and others (Fig. 1d). Importantly, IHC analysis of a cohort of over 450 primary tumors from patients who subsequently received adjuvant tamoxifen treatment showed that elevated NSD2 protein is strongly associated with early relapse and disease-related death ($p = 0.0028$ and 0.0098 respectively; Fig. 1e). Analysis of published tumor datasets also showed a significant correlation ($p = 0.0022$) between high tumor NSD2 mRNA level and poor survival of patients subsequently treated with tamoxifen. Intriguingly, a significant correlation was not observed

Fig. 1. NSD2 overexpression is highly correlated with poor survival in ER positive breast cancer patients treated with tamoxifen. (a) Colony formation assay was performed in MCF-7, T47D, ZR-75-1 and their derived tamoxifen resistance cells, MCF-7 and its TamRs were treated with either vehicle or 1 μ M 4-hydroxytamoxifen (4OHT), T47D, ZR-75-1 and their TamR with either vehicle or 0.5 μ M 4OHT. (b) Volume of xenograft tumors derived from MCF-7 TamR#2 cells upon treatment with placebo or tamoxifen in ovariectomized athymic nude mice was measured over the weeks. (c) Gene expression heatmaps showing differential expressed histone methyltransferase genes in MCF-7 TamR cells and MCF-7 parental cells treated with vehicle or 1 μ M 4OHT. Gene expressing profiling was examined by RNA sequence assay. (d) qRT-PCR and immunoblotting analysis of NSD2 in MCF-7, T47D, ZR-75-1 and their TamR derivative cells. (e) Representative images from IHC analysis of NSD2 expression in normal breast or a cohort of ER positive breast tumor specimens. Kaplan-Meier analysis of overall survival from death due to breast cancer and recurrence-free survival of patients who received tamoxifen monotherapy (n = 450) and were stratified by tumor NSD2 protein levels determined by IHC. (f) Kaplan-Meier survival curve analysis of estrogen receptor (ER) positive patients treated with tamoxifen only or not-treated with tamoxifen, stratified by NSD2 expression (data were obtained from <http://kmplot.com/analysis/>). The data are presented as the mean $\pm$ SD. *, $p < 0.05$; **, $p < 0.01$.

in patients who were not treated with tamoxifen (Fig. 1f and Supplementary Fig. S1c). Together, the results suggest that high levels of NSD2 are strongly associated with tamoxifen resistance.

Overexpression of wild type NSD2, not its methylase-defective mutant, drives tamoxifen resistance

To test the hypothesis that elevated NSD2 drives TamR, we ectopically expressed NSD2 in tamoxifen-sensitive MCF-7 and T47D cells. Overexpression of wild type NSD2, but not its methylase-defective mutant (Y1118A) [15,16], conferred the cells strong hormone-independent proliferative activities (Fig. 2a). Importantly, elevated wild-type NSD2, not the mutant, strongly blocked both tamoxifen- and ICI182,780-induced cell death (Fig. 2b–d and Supplementary Fig. S2a–e). Consistent with the notion that NSD2 plays a critical role in tamoxifen resistance, knockdown of NSD2 in TamR cells resulted in a marked decrease of cell survival especially when the cells were treated with tamoxifen (Fig. 2e). Interestingly, NSD2 knockdown in the TamR cells did not result in any significant change in ER α expression although, as reported, the knockdown reduced ER α expression in the tamoxifen-sensitive cells (Fig. 2f and Supplementary Fig. S2f), implying a distinct function of NSD2 in tamoxifen-sensitive vs resistant cells.

NSD2 up-regulates key glucose metabolism regulators TIGAR, HK2, and G6PD via its methylase activity

To understand the mechanism of NSD2-driven tamoxifen resistance, we further examined the gene expression profiles of the tamoxifen-sensitive and TamR cells and found that carbohydrate metabolism is one of the other major cellular processes altered (Supplementary Fig. S1b). Given the important role of tumor metabolic reprogramming in tumorigenesis and therapeutic resistance, we thus examined whether NSD2 overexpression altered expression of genes involved in glucose metabolism. qRT-PCR and Western blotting analysis of genes/proteins primarily involved in glycolysis and the PPP revealed that NSD2 elevation strongly stimulated the expression of TIGAR, HK2, and G6PD, with TIGAR protein being most robustly and consistently induced in the different TamR models (Fig. 3a and b and Supplementary Fig. S3a). Interestingly, the expression of p53, an upstream regulator of TIGAR, and the other key glycolysis regulatory genes (e.g. PFKFBP1–4) and the PPP genes was not significantly affected by NSD2 (Fig. 3a and Supplemental Fig. S3a and b). ChIP assay with anti-NSD2 antibody demonstrated that NSD2 occupied the promoter of HK2, TIGAR and G6PD. Elevated NSD2 was associated with increased H3K36me2 mark at the promoters (Fig. 3c and d). However, overexpression of a methylase-defective mutant

Fig. 2. NSD2 overexpression promotes tamoxifen resistance (TamR). (a) MCF-7 vector control cells or NSD2 (wild type, 2 independent stable clones, and methylase-inactive Y1118A mutant) overexpressing cells were hormones deprived for 4 days and then harvested for cell proliferation by counting viable cells or for protein expression by immunoblotting with indicated antibodies. (b) MCF-7 vector control and NSD2 overexpressing cells were treated with 4-hydroxytamoxifen (4OHT) or vehicle for 4 days and then harvested for cell survival by counting viable cells. (c) MCF-7 vector control and NSD2 overexpressing cells were treated with 1 μ M 4-hydroxytamoxifen (4OHT) or vehicle for 4 days and harvested for TUNEL assay. (d) Immunoblotting of cleaved-caspase7, cleaved-PARP, GAPDH and β -actin in MCF-7-Vec, MCF-7-NSD2 and MCF-7-NSD2Y1118A cells treated with 1 μ M 4OHT or vehicle for 3 days. (e) For the effect of NSD2 deletion on tamoxifen (TAM) sensitivity, MCF-7 TamR cells were transfected with control or NSD2 siRNA, after 2 days, cells were treated with vehicle or TAM for another 3 days, viable cells were counted. (f) Indicated cells were transfected with NSD2 or control siRNA. After 3 days, cells were harvested for immunoblotting with indicated antibodies. Or after 2 days, cells were analyzed by qRT-PCR assay for ER α mRNA expression. The data are presented as the mean $\pm$ SD. *, $p < 0.05$; **, $p < 0.01$.

Fig. 3. NSD2 coordinated upregulation of key glucose metabolism regulators TIGAR, HK2, and G6PD. (a) qRT-PCR analysis of indicated gene expression in MCF-7 vector control cells or NSD2 overexpressing cells. (b) MCF-7 and its derived TamR cells were transfected with NSD2 or control siRNA. After 3 days, cells were harvested for immunoblotting with indicated antibodies. (c) MCF-7 TamR cells were cultured in the presence of 40HT (1 μ M) and harvested for ChIP assay with NSD2 specific antibody or control IgG. ChIP DNAs were analyzed by qPCR with primers that amplify genomic regions either proximal (P) or distal (D) to TSS for each gene. (d) ChIP analysis of the relative levels of H3K36me2 mark at the promoter-proximal region (P) of indicated genes in control vector and NSD2 overexpression of MCF-7 cells.

of NSD2 (Y1118A) did not induce the expression of TIGAR, HK2 and G6PD (Fig. 3b). Consistently, NSD2 knockdown in the TamR cells strongly inhibited the expression of TIGAR, HK2 and G6PD (Supplemental Fig. S3c). Together, the results suggest that elevated NSD2, through its histone methylase activity, directly stimulates the expression of genes with major function in glucose metabolism.

NSD2 promotes therapeutic resistance through selectively enhancing the PPP and increasing NADPH production

Given the critical role of HK2, TIGAR and G6PD in glucose metabolism and cell survival, we examined the impact of elevated NSD2 on glucose metabolism. While tamoxifen inhibited glucose consumption in the control cells, NSD2 overexpression markedly increased it (Fig. 4a). Interestingly, the cellular glycolysis rate, measured by ^3H -H₂O production in cells cultured with 5- ^3H -glucose, and the major metabolites of the TCA cycle (quantified by GC-TOF-MS) were not significantly affected by either tamoxifen treatment or NSD2 elevation (Fig. 4b and Supplemental Fig. S4a). We thus measured the cellular PPP activities by quantifying PPP-dependent glucose oxidation to CO₂. The results indicated that the PPP activity was strongly increased by the wild type NSD2 but not its mutant, especially when cells were treated with tamoxifen (Fig. 4c). In support of the function of NSD2 in stimulation of the PPP, the activity of glucose 6-phosphate dehydrogenase (G6PD), an enzyme for the rate limiting step of the PPP, was markedly increased by NSD2 in tamoxifen-treated cells (Fig. 4d). Since TIGAR can modulate the PPP through its F2,6Pase activity, we also measured the cellular level of fructose 2,6-bisphosphate (F2,6P2), a potent allosteric activator of phosphofructokinase-1 (PFK1), and found that the F2,6P2 level was indeed significantly decreased in NSD2-overexpressing cells (Fig. 4e). Enhanced PPP is usually linked to heightened synthesis of nucleosides or nucleotides. Indeed, metabolomics profiling demonstrated that NSD2-overexpressing cells had higher levels of multiple nucleosides than the control cells (Fig. 4f). Furthermore, levels of glycolysis intermediates such as glyceraldehyde 3-P (G3P) and 3-phosphoglycerate (3-PG) were distinctly altered (Supplemental Fig. S4b), suggesting that NSD2 may also regulate other specific step(s) of glycolysis or the PPP.

Hyper-activated PPP can play a pivotal role in cancer therapeutics resistance through elevating levels of NADPH and/or GSH. To understand whether NSD2-overexpressing cells are resistant to

tamoxifen through up-regulating PPP activities, we measured cellular NADPH/NADP⁺ ratios. Tamoxifen treatment reduced NADPH levels in tamoxifen-sensitive cells. Strikingly, wild type NSD2, but not the mutant, potently increased NADPH in cells, which was accompanied by an increase in the reduced form of glutathione (GSH) (Fig. 4g and h). NADPH is a key determinant of redox homeostasis. Our data indeed showed that elevated NSD2 strongly suppressed Tamoxifen- or H₂O₂-induced ROS and cell death, while Tam-induced cell death was effectively blocked by ROS scavenger N-acetyl cysteine (NAC) (Fig. 4i–k and Supplementary Fig. S4c). Consistently, knockdown of NSD2 in TamR cells resulted in a marked increase of ROS and a significant decrease of NADPH, especially when the cells were treated with tamoxifen (Supplementary Fig. S4d).

Up-regulated targets of NSD2 are required for NSD2-mediated tamoxifen resistance

To determine whether the metabolic enzymes up-regulated by NSD2 are responsible for the NADPH increase and TamR, we suppressed their expression or function. Knockdown of TIGAR in NSD2-overexpressing cells strongly decreased the NADPH level and increased ROS production in tamoxifen-treated cells. TIGAR silencing also resulted in a marked increase of apoptosis (Fig. 5a and Supplemental Fig. S5). Suppression of HK2 by siRNA or inhibition of G6PD by the inhibitor DHEA also resulted in strong inhibition of NADPH production and induction of ROS and cell death, with concurrent decrease in G6PD activity (Fig. 5b and c and Supplemental Fig. S5). Moreover, treating NSD2-overexpressing cells with siRNAs of TIGAR and HK2 or the G6PD inhibitor sensitized the cells to tamoxifen-induced cell death (Fig. 5a–c). Together, these results suggest that elevated function of each of the NSD2 target genes is required for NSD2-mediated therapeutic resistance.

NSD2 overexpression stimulates tumor growth and confers tamoxifen resistance in vivo

To examine the effect of elevated NSD2 on tumor growth, we implanted NSD2-overexpressing cells and the vector control MCF-7 cells subcutaneously in ovariectomized, female athymic nude mice following previous protocols [36]. When the tumor volume reached approximately 100 mm³, mice were randomized for treatments (i.e.

Fig. 4. NSD2 promotes therapeutic resistance through up-regulating PPP pathway. (a–d) MCF-7 control and NSD2 overexpressing cells were treated with TAM (5 μ M) or vehicle for 24 h and harvested for measuring glucose consumption (a), glycolysis rate (b), CO₂ production (c) and G6PD activities (d) as described in Experimental Procedures. (e) MCF-7 control and NSD2 overexpressing cells were treated with TAM (5 μ M) or vehicle for 24 h and harvested for measuring Fructose-2,6-P₂ levels as described in Experimental Procedures. (f) MCF-7 control and NSD2 overexpressing cells were treated with TAM (5 μ M) or vehicle for 24 h and harvested for measuring glucose metabolites by GC-TOF-MS. (g, h) MCF-7 control and NSD2 overexpressing cells were treated with 5 μ M TAM, 1 μ M 4OHT or vehicle for 24 h and harvested for NADPH and NADP⁺ production and GSH level measurement as described in Experimental Procedures. (i) MCF-7 control and NSD2 overexpressing cells were treated with H₂O₂ (250 μ M) as indicated for 24 h and harvested for cell death measurement. (j) MCF-7 cells were treated with TAM (5 μ M) and/or NAC (3 mM) as indicated and harvested for cell death measurement by TUNNEL assay. (k) MCF-7 control and NSD2 overexpressing cells were treated with 5 μ M TAM, 1 μ M 4OHT or vehicle for 24 h and harvested for ROS measurement. Results shown above are representative of at least three independent experiments. The data are presented as the mean $\pm$ SD of triplicate of each sample. (* p < 0.05; ** p < 0.01; ns, not significant).

replacing the estrogen pellet with either a tamoxifen pellet or a placebo). As shown in Fig. 6a, while tumors from the vector control cells did not display any significant growth after removing the estrogen, tumors from NSD2-overexpressing cells continued their growth over two months. More importantly, in contrast to a marked regression seen with the control tumors in the mice treated with tamoxifen, a strong tumor growth was observed with the NSD2-overexpressing tumors in mice receiving the same tamoxifen treatment. Measurements of tumor NADPH level and G6PD activity indicated that NSD2 overexpression significantly increased tumor

NADPH production and G6PD activity (Fig. 6b), thus supporting the conclusion that NSD2 drives tamoxifen resistance through enhancing the PPP and increasing NADPH production.

Discussion

Several important mechanisms of endocrine resistance have been revealed, which include activation of HER2-dependent signaling and other kinase pathways, aberrant function of ER α -associated coactivators, and deregulated expression and function of other

Fig. 5. Up-regulated NSD2 targets are required for NSD2-mediated therapeutic resistance. (a, b) MCF-7-NSD2 cells were transfected with HK2, TIGAR or control siRNA. Cells were harvested for analysis of NADPH/NADP⁺ ratio, G6PD activity, ROS levels and cell death. For the effect of TIGAR or HK2 knockdown on TAM sensitivity, cells were transfected as above for 2 days before treated with vehicle or TAM for another 3 days, viable cells were counted. (c) MCF-7-NSD2 cells were treated with vehicle or G6PD inhibitor DHEA (100 μ M). Cells were harvested for analysis of NADPH/NADP⁺ ratio, G6PD activity, ROS levels, and cell death. For the effect of G6PD inhibitor DHEA on TAM sensitivity, MCF-7-NSD2 cells were pretreated with DHEA for 2 h and then treated with vehicle or TAM for another 3 days, viable cells were counted. Results shown above are representative of at least three independent experiments. The data are presented as the mean $\pm$ SD of triplicate of each sample. (* p < 0.05; ** p < 0.01; ns, not significant).

transcription factors such as NF- κ B, AP-1 and members of the Hox family [37,38]. Despite their critical functions in control of gene expression and strong implications in cancers, few histone methylases and demethylases have been studied for their role in endocrine resistance. Although metabolic reprogramming is a hallmark of tumorigenesis and has been implicated in tumor resistance to chemotherapy, strong evidence for its involvement in endocrine

resistance has been lacking. Here, we describe the important function of histone methylase NSD2 as a novel driver of tamoxifen resistance through concerted upregulation of multiple glucose metabolic enzymes.

We demonstrate first that NSD2 is highly elevated in different sublines of breast cancer cells that acquire resistance to tamoxifen due to long-time exposure. We then show that overexpressed NSD2

Fig. 6. NSD2 overexpression promotes tumor growth and confers tamoxifen resistance *in vivo*. (a) The MCF-7 control and NSD2 overexpressing cells were implanted s.c. in athymic mice. Mice with ~100 mm³ were randomized into 4 groups (n = 6) for indicated treatment. Tumor size was measured over the weeks. Mean tumor volume $\pm$ s.e.m. was shown, **, $p < 0.01$. Tumor images representative of different treatment groups were shown. (b) Xenograft tumors of control and NSD2 overexpressing MCF-7 cells treated with tamoxifen were harvested at the end of Tam treatment. Fresh tumor homogenates were subjected to NADPH/NADP⁺ ratio and G6PD activity measurement. The data are presented as the mean $\pm$ SD. **, $p < 0.01$.

utilizes its H3K36me2 activity to coordinately up-regulate the expression of key glucose metabolic enzymes such as HK2, G6PD and TIGAR to increase the activities of the first committing step of glycolysis and the first rate-limiting step of the PPP. Perhaps even more striking is the robust up-regulation of TIGAR by NSD2. Consistent with TIGAR being an F2,6BPase, we observed a significant decrease in F2,6BP level in NSD2 overexpressing cells. Reduction of F2,6BP level is known to dampen glycolysis because F2,6BP is a potent, allosteric activator of PFKs. Therefore, the reduced level of F2,6BP is likely responsible for the observed lack of increase in glycolysis rate in the NSD2-overexpressing cells, even when the cells display increased glucose uptake/consumption and enhanced HK2. Together, our findings support a model that a coordinated change by overexpressed NSD2 in the key metabolic enzymes bring about more carbon flux through the PPP for an increased production of NADPH, which in turn is used for ROS reduction and cell survival (Fig. 7). Conversely, inhibition of NSD2 suppresses the PPP activity and sensitizes TamR cells to tamoxifen-induced cell death. The fact that the elevated function of each of the NSD2 target genes is required for NSD2-mediated therapeutic resistance emphasizes the importance of the coordinated nature of NSD2 function in metabolic reprogramming. Recently, NSD2 was implicated in regulation of ER α gene expression [19]. Consistent with this, we also found that NSD2 knockdown decreased ER α expression in tamoxifen-sensitive breast cancer cells. However, we failed to observe any significant change in ER α expression by NSD2 knockdown in tamoxifen-resistant cells or by NSD2 overexpression in MCF-7 cells. Whether the tamoxifen resistance function of elevated NSD2 involves ER α and/or the other major factors such as HER2 will be a subject of future studies.

Development of more specific biomarkers that predict resistance to tamoxifen therapy remains a major clinical challenge. Our IHC data from more than 400 breast cancer specimens suggest that highly expressed NSD2 is significantly associated with high risk of relapse and predicts poor survival in ER-positive breast cancer patients treated with tamoxifen. Meta-analysis of multiple published datasets also strongly implicates that elevated NSD2 is significantly associated with poor prognosis after tamoxifen therapy. Additionally, our data suggest that NSD2 overexpression promotes tumor growth and confers tamoxifen resistance in a mouse xenograft model. These data collectively suggest that NSD2 may be an independent marker for poor prognosis in ER α -positive breast cancer patients receiving tamoxifen therapy.

NSD2 overexpression in solid tumors of multiple cancer types is clearly documented. However, little is known about the mechanisms underlying its aberrant expression in the tumors. Gene copy number change of NSD2/WHSC1, which is located at chromosome 4p16.3, has not been frequently observed in breast cancer as suggested by the data displayed at cBioPortal (<http://www.cbioportal.org/>) from the TCGA and other studies. EZH2, the H3K27 methylase, was shown to act upstream of NSD2 [39]. However, our IHC analysis failed to reveal a close correlation in their expression in the cohort of breast cancer tumors (data not shown). On the other hand, signaling by factors from tumor microenvironment can play a major role in cancer progression. We demonstrated previously that cytokines such as IL-6 and TNF α can strongly stimulate NSD2 expression through NF- κ B [15]. Whether those factors or other mechanisms mediate NSD2 overexpression in the tumors awaits to be investigated.

In addition to NSD2, in this study, we also found that the expression of other methylases such as MLL5 and PRDM16 is significantly up-regulated in tamoxifen-resistant cells. MLL5, also known as KMT2E, is a member of the myeloid/lymphoid or mixed-lineage leukemia (MLL) gene family. MLL5 protein contains a PHD zinc finger and a SET domain. Although its histone methylase activity has been a matter of debate, its function in cancer is strongly suggested by a recent study demonstrating that it can suppress differentiation and promote self-renewal of cancer stem cells in pediatric glioblastomas (GBMs) through condensing chromatin structure at specific locations [40]. Interestingly, MLL5 β , an isoform of MLL5, is shown to be important in radiation resistance of HPV16/18-positive cervical cancer models [41]. However, the role of PRDM16 in cancer is poorly investigated. PRDM16, also known as MEL1, contains an N-positive regulatory (PR) domain homologous to SET domain, and has been shown to direct cytoplasmic H3K9me1 methylation [42]. Chromosomal translocations involving MEL1 or its overexpression have been found in a subset of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) [43]. However, whether PRDM16 plays any role in breast cancer or other types of solid tumors is completely unknown. Therefore, it will be of significance to study whether MLL5 or PRDM16 plays an important function in breast cancer.

In summary, we demonstrate here that NSD2 overexpression is significantly associated with high risk of relapse and poor survival in tamoxifen-treated ER-positive breast cancer patients. Our further studies of the mechanism suggest that NSD2 drives tamoxifen

Fig. 7. A schematic diagram illustrating NSD2 reprogramming metabolism to drive endocrine resistance.

therapy resistance through coordinated stimulation of key glucose metabolism enzymes and enhancement of the PPP. We show that NSD2 inhibition is sufficient to restore tamoxifen sensitivity. These data collectively suggest that NSD2 plays a critical role in tamoxifen resistance and could be an effective therapeutic target in tamoxifen-resistant breast cancer.

Authors' contributions

H.W.C., L.C.M., H.J.K., and J.W. designed the experiments. J.W., Z.N., J.X.Z., and Z.D. performed the experiments and analyzed the data. A.D.B. and Y.Z. performed the histopathological analyses. J.J.L. provided critical reagents and performed the experiments. J.X. and C.G.T. performed the bioinformatics analysis. O.F. performed the mass spectrometry based assays and analyzed the data. J.W. and H.W.C. conceived and designed the study, and wrote the paper.

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Conflict of interest

The authors declare no competing financial interests.

Appendix: Supplementary material

Supplementary data to this article can be found online at [doi:10.1016/j.canlet.2016.05.004](https://doi.org/10.1016/j.canlet.2016.05.004).

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