ZNF423 modulates the AMP-activated protein kinase pathway and metformin response in a single nucleotide polymorphisms, estrogen and selective estrogen receptor modulator dependent fashion

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Objectives **We previously discovered that the single nucleotide polymorphisms (SNP) rs9940645 in the *ZNF423* gene regulate ZNF423 expression and serve as a potential biomarker for response to selective estrogen receptor modulators (SERMs). Here we explored pathways involved in ZNF423-mediated SERMs response and drugs that potentially sensitize SERMs.**

Methods **RNA sequencing and label-free quantitative proteomics were performed to identify genes and pathways that are regulated by ZNF423 and the *ZNF423* SNP. Both cultured cells and mouse xenograft models with different *ZNF423* SNP genotypes were used to study the cellular responses to metformin.**

Results **We identified ribosome and AMP-activated protein kinase (AMPK) signaling as potential pathways regulated by ZNF423 or *ZNF423* rs9940645 SNP. Moreover, using clustered regularly interspaced short palindromic repeats/Cas9-engineered ZR75-1 breast cancer cells with different *ZNF423* SNP genotypes, striking differences in cellular responses to metformin, either alone or in the combination of tamoxifen, were observed in both cell culture and the mouse xenograft model.**

Conclusions **We found that AMPK signaling is modulated by the *ZNF423* rs9940645 SNP in estrogen and SERM-dependent fashion. The *ZNF423* rs9940645 SNP affects metformin response in breast cancer and could be a potential biomarker for tailoring the metformin treatment. *Pharmacogenetics and Genomics* 31: 155–163**

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Background

Breast cancer is the most commonly occurring cancer and the leading cause of cancer death in women worldwide [1]. It has been demonstrated that metabolic pathways in breast cancer cells were significantly dysregulated, a mechanism leading to uncontrolled cell growth [2,3]. The antidiabetic drug metformin has been shown to target metabolic pathways by activating AMP-activated protein kinase (AMPK), affecting multiple cellular phenotypes including reducing blood insulin or reversing epithelial-mesenchymal transition [4–6]. Therefore, it is being tested in multiple cancers including breast cancer [7–9] as monotherapy [10,11] or in the combination of endocrine therapy such as tamoxifen in the treatment of estrogen receptor-positive (ER+) breast cancer, or chemotherapeutics in triple-negative breast cancer [12,13]. However, metformin resistance is inevitable and it has been reported that long-term metformin-treated ER+ breast cancer cells acquire cross-resistance to both metformin and tamoxifen [14].

In our previous discovery, the genome-wide association study using samples from the double-blind, placebo-controlled National Surgical Adjuvant Breast and Bowel Project P-1 and P-2 subjects, common single nucleotide polymorphisms (SNPs) in the *ZNF423* gene, were identified as potential biomarkers for individualized selective estrogen receptor modulator (SERM) prevention therapy [15]. One of these ZNF423 SNPs, rs9940645 located approximately 200 bp from several estrogen response elements, was bound by calmodulin-like protein 3, which cooperates with ERα and regulates the expression of ZNF423 and BRCA1, in an SNP, estrogen and SERM-dependent fashion [16]. Specifically,

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the expression of ZNF423 and BRCA1 was increased by estradiol while suppressed by tamoxifen in cells with the ZNF423 wildtype rs9940645, whereas opposite regulation was observed for variant genotypes. Moreover, as ZNF423 influences DNA damage repair via BRCA1, dramatic differences were observed in cellular responses to SERMs plus Poly (ADP-ribose) polymerase inhibitors in ER+ breast cancer cells carrying different ZNF423 SNP rs9940645 genotypes [16]. Moreover, ZNF423 also affects the G2/M phase of the cell cycle by regulating mitosis-related genes VRK1 and PBK and then modulates concurrent tamoxifen and docetaxel chemotherapy in a ZNF423 SNP-dependent manner [17].

Although ZNF423 functions as a transcription factor in several signaling pathways including cell cycle and bone morphogenic protein-dependent regulation [18–20], its role in breast cancer and treatment response has not been fully investigated. In the present study, we performed RNA sequencing after knockdown of ZNF423 or after estradiol treatment with or without tamoxifen in ER+ breast cancer cells with different ZNF423 SNP genotypes. The mRNA expression levels of many ribosomal proteins were changed in an SNP and drug-dependent manner, which suggests that protein translation regulation might be involved as a ZNF423 downstream pathway. In addition, proteomics studies also identified additional pathway such as AMPK to be regulated by ZNF423 in an SNP, estrogen and SERM-dependent fashion. Finally, we investigated the SNP effect in response to metformin, either alone or in combination with tamoxifen.

Methods

Cell culture

The human ERα-positive breast cancer cell line ZR75-1 was obtained from the ATCC (Manassas, Virginia, USA) and cultured in an Roswell Park Memorial Institute 1640 medium (Gibco, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Georgia, USA). CRIS-ZR75-1 cells carrying homozygous wildtype rs9940645 were generated by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 editing as previously described [16].

Transfection and drug treatment

Prior to transfection and estradiol treatment, cells were grown in phenol red-free media containing 5% charcoal-stripped serum (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 48 h. For ZNF423 knockdown, the cells were then transfected with three different siRNAs targeting the *ZNF423* gene (D-012907-01/-02/-03) or scrambled siRNA (D-001206-13; Dharmacon, Lafayette, Colorado, USA) using the Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific). For ZNF423 overexpression, the cells were transfected with pCMV6-XL4-ZNF423 or the empty vector pCMV6-XL4 (OriGene Technologies, Inc., Rockville, Maryland, USA) using the Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. After 24 h, cells were incubated with 0.01 nM estradiol for an additional 24 h (Sigma-Aldrich, St. Louis, Missouri, USA), followed by the addition of 2 mM metformin (Selleck Chemicals LLC, Houston, Texas, USA) or 10^{-7} μM 4-hydroxy tamoxifen (Sigma-Aldrich). As vehicle controls, ethanol for estradiol, PBS for metformin or dimethylsulfoxide for 4-OH-tamoxifen were added to the medium at a final concentration <0.1%. Cells were collected 72 h after transfection.

RNA sequencing and pathway analysis

RNA sequencing (RNAseq) was performed with ZR75-1 cells after ZNF423 knockdown with two individual siRNAs in both CRIS-ZR75-1 and ZR75-1 cells treated with either estradiol or estradiol+tamoxifen. Total RNA was extracted using the RNeasy Plus Mini kit (QIAGEN, Germantown, Maryland, USA). mRNA libraries and sequencing by HiSeq 4000 system (2 × 150 paired-end runs, Illumina, San Diego, California, USA) were performed by the Mayo Clinic core facility (Rochester, Minnesota, USA). The raw data were converted to fastq files, quality was examined by FastQC, and adapter sequence and low-quality sequences (<Q30 or <50 bp) were trimmed by Trim Galore. Filtered reads were aligned to the hg19 human reference genome using Hisat2 with an average mapping rate of 93%. Raw counts were then called by HTSeq excluding nonunique reads. Intra-group replication correlations were validated by Pearson correlation (average R-squared ≥0.88). Differential expression analysis was performed with EdgeR package using R software, and fold change ≥2 and P values ≤0.05 after adjusting for the false discovery rate were considered significant. Kyoto Encyclopedia of Genes and Genomes pathway analysis of differentially expressed genes was conducted using the Enrichr portal [21,22].

Statistical analysis

Data were analyzed using GraphPad Prism Software. Student's two-tailed t-test was used for comparison of relative mRNA expression levels by real-time quantitative reverse transcription PCR (qRT-PCR), clone numbers in colony formation, tumor volume and tumor weight in the xenograft model. P-value < 0.05 was considered statistically significant.

Animal studies

The animal study was reviewed and approved by the Mayo Clinic Institutional Animal Care and Use Committee. Breast cancer xenografts generated from ZR75-1 cells with *ZNF423* variant and CRISPR-engineered wildtype SNP genotypes were used to test the tumor response to treatment with metformin with or without tamoxifen, similarly to the previous report [16]. Briefly, 6-week-old female severe combined immunodeficient (SCID) mice

were water-fed with a low dose of estradiol (70 µg) every week. 2×10^6 logarithmically breast cancer cell lines were injected to the mice as a 1:1 mixture with growth-factor reduced, phenol red-free matrigel (BD – Diagnostic System, Franklin Lakes, New Jersey, USA). After tumor volume reached 100 mm^3, the mice were then randomized into groups treated with PBS as control, tamoxifen (5 mg/kg/d), metformin (225 mg/kg/day) alone, or metformin plus tamoxifen for 28 days. Tumor volume (TV) was calculated using the formula: $TV = (L \times W^2)/2$ where L is tumor length and W is tumor width. When tumors in the control mice reached a size that the mice had to be sacrificed, the tumors were removed and saved for further analysis.

Additional methods can be found in Supplementary materials, Supplemental digital content 1, *http://links.lww.com/FPC/B392*.

Results

Ribosome and DNA replication pathways are regulated by ZNF423

To further investigate ZNF423 function in breast cancer, we performed RNAseq and differential expression analysis in ER+ breast cancer cell, ZR75-1 cells between control and after knockdown of ZNF423 (Supplementary Figure S1, Supplemental digital content 1, *http://links.lww.com/FPC/B392*). Pathway analysis using only the upregulated genes after knockdown with P value <0.05 identified the ribosome pathway as the most significantly altered pathway (Table 1), along with lysosome, autophagy, mitogen-activated protein kinase signaling, protein processing in endoplasmic reticulum and AMPK signaling pathways (Table 1). On the other hand, using only the downregulated genes ($P<0.05$) after ZNF423 knockdown, DNA replication, mismatch repair and p53 signaling were among the most significantly altered pathways (Supplementary Table S1, Supplemental digital content 1, *http://links.lww.com/FPC/B392*). Collectively, we have identified the genes and pathways that may be regulated by ZNF423 in breast cancer.

Ribosome pathway is regulated by ZNF423 in an single nucleotide polymorphisms, estrogen and selective estrogen receptor modulator-dependent fashion

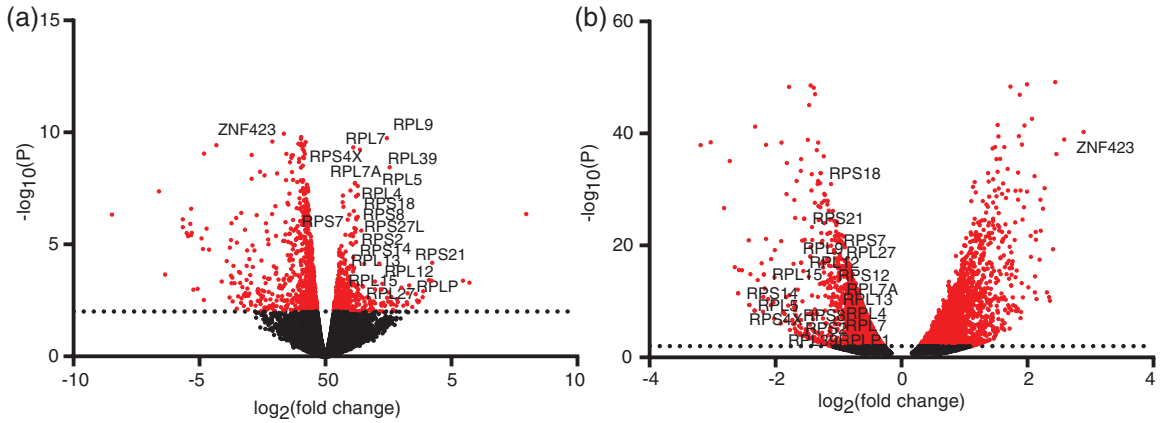
Because it was reported previously that rs9940645 genotype was associated with the expression of ZNF423 in an SNP, estrogen or SERM-dependent manner [15,16], we wanted to determine if there are any additional downstream genes of ZNF423 that might be regulated in such a fashion. To test that hypothesis, we took advantage of the CRISPR/Cas9-engineered ZR75-1 cells (CRIS-ZR75-1), which harbors homozygous wildtype rs9940645, and the parental ZR75-1, which harbors homozygous variants genotypes [16,17]. We performed RNAseq and differential expression analysis between estradiol and estradiol plus tamoxifen treatments in both parental (V/V) (Fig. 1b) and CRIS-ZR75-1 (W/W) cells (Fig. 1a). Compared to estradiol alone, the mRNA expression of a large number of ribosomal protein genes was upregulated in the presence of estradiol+tamoxifen in rs9940645 W/W cells (Fig. 1a), but suppressed in V/V cells (Fig. 1b), opposite to the SNP-dependent ZNF423 expression pattern, in

Table 1 RNAseq pathway analysis using the upregulated genes after ZNF423 knockdown

Term	Overlap	*P*-value	Genes
Ribosome	64/153	7.53×10^{-30}	RPL4;RPL5;RPL30;RPL3;RPL32;RPL31;RPLP1;RPLP0;RPL8;RPL10A;RPL9;RPL6;RPL7;RPS15;RPS4X-;RPS14;RPL7A;RPS16;RPS19;RPL18A;RPS18;RPLP2;RPS11;RPL39;RPS13;RPS12;RPS9;R-PL21;RPS7;RPS8;RPS5;RPS6;RPL13A;RPSA;RPS3A;RPL37A;RPL27;RPL29;RPL28;UBA52;RPL10;R-PL12;RPL11;RPS27L;MRPL14;MRPL10;RPS15A;RPL14;RPS3;RPL13;RPL15;RPS2;RPL18;RPL17;R-PL19;RPS26;RPS25;RPS28;RPS29;RPL27A;RPS20;RPL22L1;RPS21;RPS23
Lysosome	27/123	1.94×10^{-6}	HEXA;GNS;LITAF;AP4M1;CLN3;LAPTM4A;GNPTAB;LAMP1;GM2A;HYAL1;LAMP2;PSAP;CLTCL1;SL-C17A5;AP3S2;CTSF;CTSD;CTSC;CD164;HGSNAT;FUCA1;SLC11A2;M6PR;IGF2R;SUMF1;NPC2;LGMN
Autophagy	24/128	1.07×10^{-4}	SH3GLB1;UVRAG;RUBCN;GABARAPL1;STX17;IRS1;DAPK1;PRKCD;EIF2AK3;RRAS2;IRS2;WIPI1;MTM-R4;ZFYVE1;IGF1R;DEPTOR;LAMP1;RRAS;PIK3CA;AKT2;LAMP2;ULK2;ULK1;CTSD
MAPK signaling pathway	42/295	3.27×10^{-4}	ATF2;SRF;PDGFA;TGFA;HSPB1;AREG;RELA;IGF1R;ELK4;RAP1B;PPP3CA;PAK1;RRAS;MYC;AKT2;PDG-FC;FLNB;MAP3K8;RAC1;MAP3K4;MAP2K3;DUSP5;MAP3K3;JUND;EGF;INSR;NFATC3;RRAS2;PRK-CA;TNFRSF1A;NR4A1;PPM1A;PPM1B;TAOK3;MAPKAPK3;DDIT3;MAPKAPK2;RAPGEF2;TAB 2;MAPT;FGFR4;EPHA2
Protein processing in endoplasmic reticulum	27/165	4.28×10^{-4}	ERO1B;SEC23A;UBE2D4;HSPA4L;HERPUD1;ATXN3;BAG1;MAN1A1;SIL1;UBQLN4;EDEM3;XB-P1;HSPA5;SSR4;SSR2;AMFR;SSR3;EIF2AK3;UBE4B;PDIA6;PDIA4;DDIT3;ERP29;BAX;CALR;-ATF6;RNF185
AMPK signaling pathway	19/120	7.34×10^{-4}	IRS1;INSR;IRS2;HMGCR;EEF2;ACACB;FOXO1;ACACA;IGF1R;RAB11B;TBC1D1;G6PC3;PIK-3CA;SCD;CREB3L1;AKT2;CREB3L2;CD36;PCK1
Terpenoid backbone biosynthesis	7/22	0.00141	IDI1;HMGCS1;GGPS1;MVD;HMGCR;ACAT2;PCYOX1
Rap1 signaling pathway	30/206	0.00153	ITGB1;CTNND1;PDGFA;ADCY1;SIPA1L3;IGF1R;RAP1B;SIPA1L1;RRAS;AKT2;PDGFC;RAC1;FARP2;MAP-2K3;PRKCI;EGF;INSR;MAGI2;PRKCA;RHOA;PIK3CA;PARD3;ID1;RAPGEF2;CTNNB1;PRKD2;FGFR4;RAP-GEF6;EPHA2;PFN2
Signaling pathways regulating pluripotency of stem cells	22/139	0.00216	SMAD1;SMAD4;FZD5;WNT5A;STAT3;LIFR;KLF4;IGF1R;REST;ACVR1C;PIK3CA;MYC;AKT2;KAT6A;ID1;DV-L2;ID4;ID3;CTNNB1;TCF3;IL6ST;FGFR4
FoxO signaling pathway	21/132	0.00252	CDKN2D;USP7;GABARAPL1;SMAD4;IRS1;HOMER2;EGF;INSR;STAT3;IRS2;FBXO32;SIRT1;FOX-O1;KLF2;IGF1R;RBL2;G6PC3;PIK3CA;AKT2;CDK2;PCK1

MAPK, mitogen-activated protein kinase.

Fig. 1



Volcano plot for rs9940645 and drug-dependent RNA-seq in breast cancer cells. (a) RNA-seq was conducted in the CRIS-ZR-75-1 cells carrying the *ZNF423* wildtype single nucleotide polymorphism (SNP) genotype in the presence of estradiol or estradiol plus tamoxifen. (b) RNA-seq was conducted in ZR-75-1 cell carrying the *ZNF423* variant SNP genotype in the presence of estradiol or estradiol plus tamoxifen. ZNF423 and a set of representative ribosomal protein gene of which expression were altered between estradiol plus tamoxifen and estradiol alone were marked on the volcano plot.

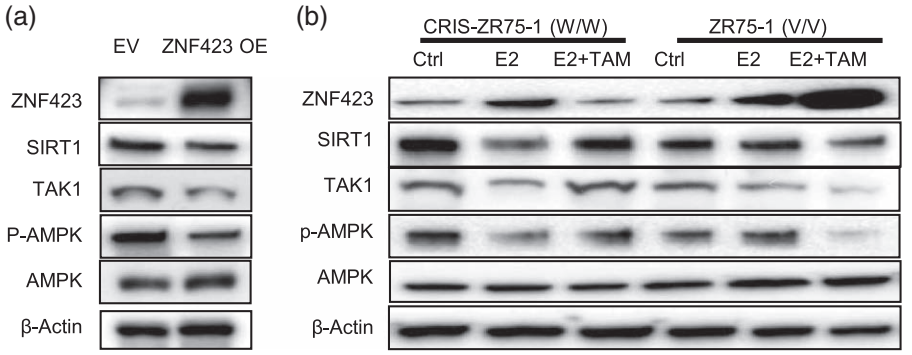
Table 2 ZNF423 ChIPseq (binding on ribosomal protein genes)

RP genes	ZNF423 ChIP binding site
RPL4	(1) EXON 10 (last exon) (2) 100 bp downstream
RPL5	intron 1
RPL7	intron 1
RPL7A	900 bp upstream
RPL7L1	right upstream and 50 bp exon 1
RPL8	150 bp upstream and exon 1
RPL9	100 bp upstream
RPL10	1200 bp downstream
RPL10A	120 bp upstream, exon 1 and intron 1
RPL12	50 bp upstream, exon 1 and intron 1
RPL13	exon 1
RPL15	exon 1, intron 1
RPL18	180 bp downstream
RPL23A	1200 bp downstream
RPL26	100 bp upstream
RPL27	exon 5 (last exon)
RPL28	exon 5 (last exon)
RPL36	2000 bp downstream
RPL37	100 bp upstream, exon 1 and intron 1
RPL38	80 bp upstream, exon 1, intron 1 and exon 2
RPL39	20 bp upstream
RPLP1	exon 1 and intron 1
RPS2	1000 bp downstream
RPS4X	200 bp upstream, exon 1 and intron 1
RPS7	150 bp upstream, exon 1 and intron 1
RPS8	Intron 1, exon 2 and intron 2
RPS12	50 bp upstream
RPS14	right upstream of exon 1
RPS15	exon 3 and 4 (last exon)
RPS16	450 bp upstream and intron 2
RPS18	right upstream of exon 1
RPS21	220 bp upstream
RPS24	one of the transcript variant and intron 4 (last intron)
RPS27A	exon 1, intron 1, exon 2 and intron 2
RPS27L	exon 1

which ZNF423 expression was suppressed in the presence of estradiol+tamoxifen in W/W cells while upregulated in V/V cells. Consistently, pathway analysis of genes showing SNP and drug-dependent gene expression that was in an opposite pattern from that of ZNF423, the ribosome pathway remained the top significant pathway (Supplementary Table S2, Supplemental digital content 1, *http://links.lww.com/FPC/B392*), while influenza A was the top pathway using gene expression that was regulated in the same SNP and drug-dependent fashion with that of ZNF423 (Supplementary Table S3, Supplemental digital content 1, *http://links.lww.com/FPC/B392*). We further validated the relationship between ZNF423 and ribosomal gene expression using qRT-PCR (Supplementary Figure S2, Supplemental digital content 1, *http://links.lww.com/FPC/B392*). These results suggested that ZNF423 may regulate the expression of ribosomal protein genes in an SNP, estrogen and tamoxifen-dependent fashion.

The ribosomal protein, together with rRNA, makes up the ribosomal subunits involved in the cellular process of translations. They are highly conserved genes across different life forms [23]. Based on the RNAseq result, ZNF423 might regulate the transcription of a set of ribosomal protein genes. To examine this hypothesis, we assessed the ZNF423-binding sites on ribosomal protein genes using the ZNF423 chromatin immunoprecipitation (ChIP) sequencing data that is publicly available in ENCODE (ENCSR477OJI). Though this dataset was generated in HEK293 cells with stably expressed eGFP-ZNF423, it was still informative that ZNF423 protein bound in the genomic regions of more than one-third (35 out of 86) of the ribosomal protein genes and the majority binding sites on these ribosomal genes were within their promoter regulatory region (Table 2). These results suggested that ZNF423 might transcriptionally regulate a set of ribosomal protein genes.

Fig. 2



Western Blotting to validate the alteration of AMPK signaling modulated by the ZNF423 gene and rs9940645. (a) ZNF23 was overexpressed in ZR75-1 breast cancer cells and then the protein expression of SIRT1 and TAK1 and AMPK signaling was examined. (b) SIRT1 and TAK1 and AMPK signaling was assessed in ZR75-1 cells carrying different *ZNF423* single nucleotide polymorphism (SNP) genotypes and treated with estradiol ± tamoxifen.

AMPK pathway is modulated by ZNF423 in an single nucleotide polymorphisms and selective estrogen receptor modulator-dependent manner

Because the alteration of ribosomal protein influences the translational machinery, the overall protein expression profile might be affected by ZNF423 via mediating the expression of ribosomal protein genes. To investigate the ZNF423 function at the protein level, we performed label-free quantitative proteomics study by MS ZR75-1 cells after knockdown of ZNF423. Pathway analysis using significantly upregulated proteins (fold change >2) after knockdown identified ribosome biogenesis, purine metabolism, Epstein–Bar virus infection, herpes simplex infection, measles, vitamin digestion and absorption, African trypanosomiasis and AMPK signaling pathways ($P < 0.05$) (Supplementary Table S4, Supplemental digital content 1, *http://links.lww.com/FPC/B392*). No significant pathway was identified using downregulated proteins (Supplementary Table S5, Supplemental digital content 1, *http://links.lww.com/FPC/B392*).

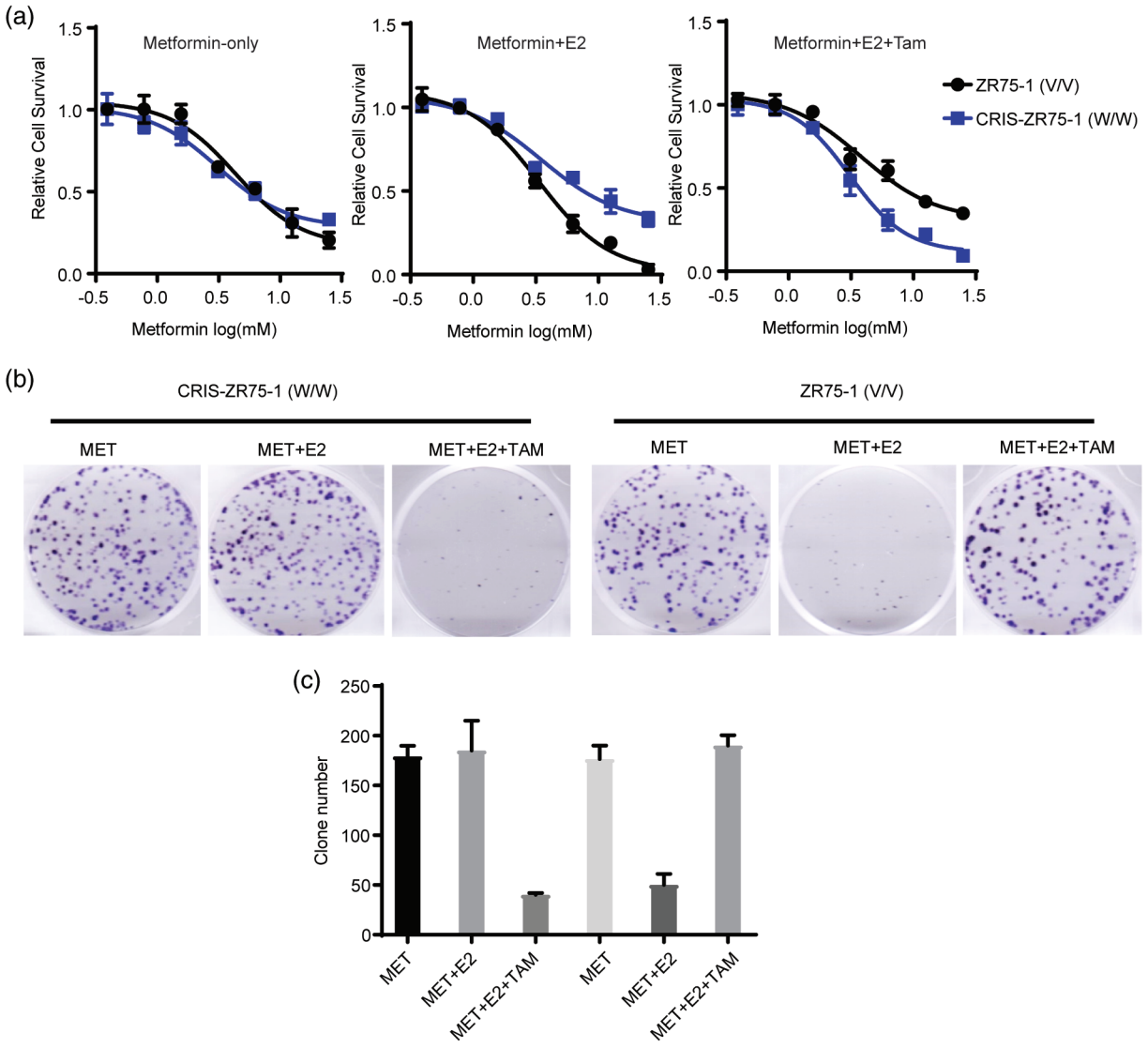
Besides the ribosome pathway, AMPK signaling was the only pathway that was identified using both RNA-seq and proteomic studies (Table 1 and Supplementary Table S4, Supplemental digital content 1, *http://links.lww.com/FPC/B392*). Interestingly, the AMPK signaling-related genes MAP3K7 (TAK1) and SIRT1 were only altered at the protein levels but not on the mRNA levels after knockdown of ZNF423. TAK1 is a serine/threonine protein kinase of the mitogen-activated protein kinase kinase kinase (MAP3K) family and functions as an alternative third AMPK kinase [24]. SIRT1 is the most conserved NAD^+-dependent protein deacetylase that senses the cellular metabolic status and may indirectly regulate AMPK signaling through a well-known AMPK kinase LKB1 [25]. To validate whether ZNF423 regulates the protein levels of TAK1 and SIRT1 and then further, the AMPK signaling, ZNF423, was overexpressed in the ZR75-1 cells and the downstream proteins were examined. Consistent with the proteomic study, the protein expressions of SIRT1 and TAK1 were significantly downregulated after overexpression ZNF423 (Fig. 2a), which may in turn contribute to the decreased AMPK activity (Fig. 2a). Most importantly, SIRT1 and TAK1 levels exhibited SNP and drug-dependent pattern similar with that of ZNF423 (Fig. 2b). Compared to the estradiol alone, the protein levels of SIRT1 and TAK1 were induced after estradiol+tamoxifen combination in the *ZNF423* wildtype SNP genotype, while the pattern was reversed in the *ZNF423* variant SNP genotype. Moreover, the activity of AMPK was also altered consistently with SIRT1 and TAK1 (Fig. 2b). It is suggested that ZNF423 may regulate AMPK signaling in an SNP, estrogen and SERM-dependent manner.

rs9940645 affects metformin treatment

Since AMPK signaling is regulated by the *ZNF423* SNP and metformin also functions through modulating AMPK signaling, we examined whether the cellular response to metformin, alone or in combination with tamoxifen, was different between the *ZNF423* wildtype and variant SNP genotypes. Cellular cytotoxicity showed that the pair of ZR75-1 cells with different *ZNF423* SNP genotypes responded similarly to varying doses of metformin, while the addition of 0.01 nM sub-physiological level of estradiol-sensitized ZR75-1 (V/V) cells to metformin treatment, compared with the ZR75-1 (wildtype/wildtype) cells (Fig. 3a). Moreover, the cellular response to metformin was reversed with estradiol+ tamoxifen treatment (Fig. 3a). In agreement with cytotoxicity, compared to metformin alone, cell colony formation was inhibited in the presence of estradiol in the ZR75-1 (V/V) cells, while the inhibitory effect was observed in the CRIS-ZR75-1 (W/W) cells in the presence of both estradiol and tamoxifen (Fig. 3b and c).

To further confirm the SNP effect on treatment response *in vivo*, we established a xenograft mouse model by injecting ZR75-1 (V/V) and CRISPR-ZR75-1 (W/W) breast cancer

Fig. 3



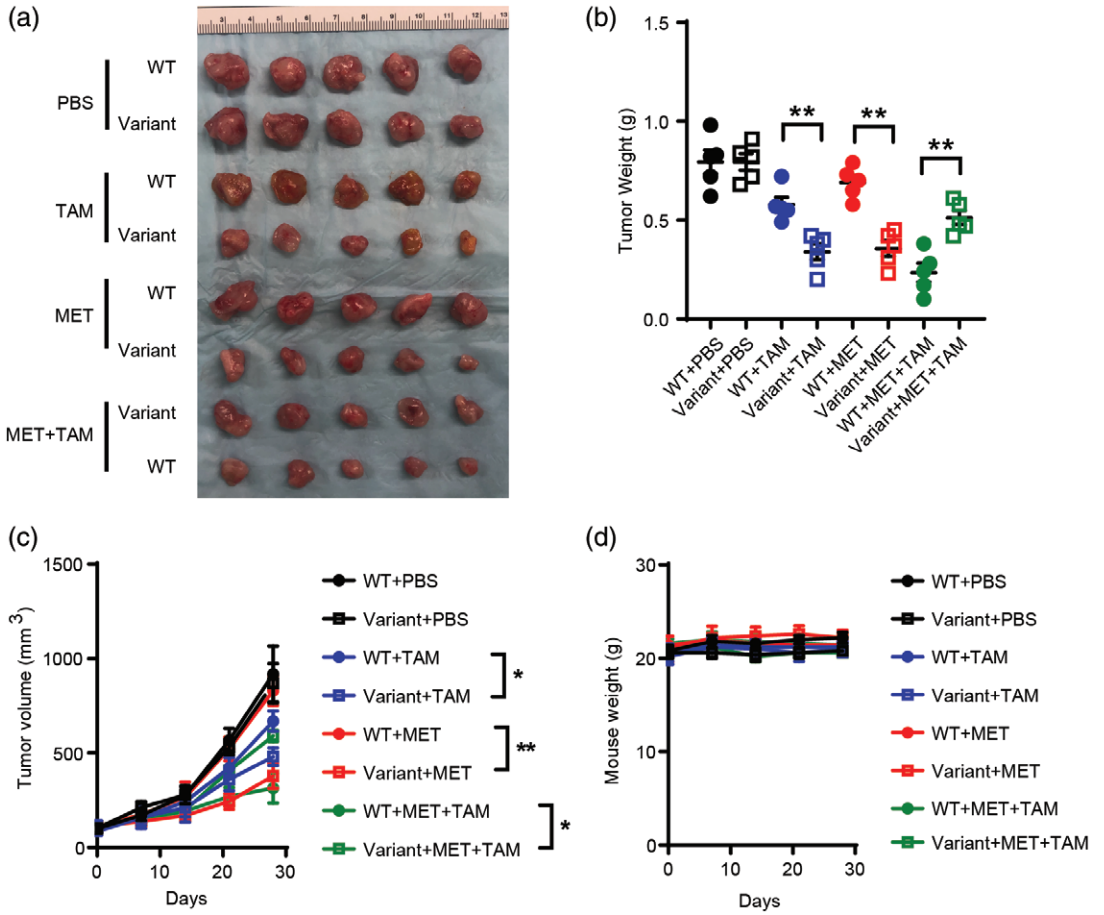
Cytotoxicity and colony formation assays for metformin treatment in breast cancer cells with different ZNF423 single nucleotide polymorphism (SNP) genotypes. (a) ZR75-1 (V/V) and CRIS-ZR75-1(W/W) cells were treated with varying doses of metformin in the presence of estradiol or estradiol plus tamoxifen. Relative cell survival was assessed after 5 days of treatment. (b) ZR75-1 (V/V) and CRIS-ZR75-1(W/W) cells were treated with varying doses of metformin in the presence of estradiol or estradiol plus tamoxifen, representative colony formation was shown after 4 weeks of treatment. (c) The colony formation number was calculated in three biological repeats.

cells into female SCID mice that had been water-fed with low-dose estrogen to stimulate tumor growth. After tumors reached 100 mm^3, the mice were randomized into four groups and then were treated with tamoxifen, metformin alone, metformin in combination with tamoxifen or vehicle control PBS. After 28 days of treatments, tumors were harvested (Fig. 4a) and the tumor weights were measured (Fig. 4b). Tumor growth (Fig. 4c) and mouse body weight (Fig. 4d) were also monitored. The mouse body weight was stable during the 28-day treatments (Fig. 4d) and there was no significant difference between the *ZNF423* SNP genotypes with the control PBS treatment (Fig. 4a, b, c). Consistent with what we observed previously [16], *ZNF423* SNP genotype had a differential effect on the response to tamoxifen. Notably, in agreement with what we found in the *in vitro* study, the tumor size and weight in mice with the *ZNF423* variant SNP genotype were significantly reduced with metformin treatment, while in mice with the wildtype genotype, the tumor inhibitory effect was observed with metformin treatment in combination with tamoxifen (Fig. 4a, b, c). Collectively, we confirmed that metformin response in breast cancer is *ZNF423* SNP and SERM-dependent.

Discussion

In the present study, we found that the mRNA expression of many ribosomal protein genes was increased after downregulation of ZNF423 (Table 1), and the rs9940645

Fig. 4



Metformin response in breast cancer mouse xenografts with different ZNF423 single nucleotide polymorphism (SNP) genotypes. (a) Tumors removed from the mice in each treatment group (5 mice) after 28 days of drug treatments are shown. (b) Tumor weight after 28 days of treatments. (c) Tumor growth inhibition measured by volume during the treatments. (d) mouse body weight was measured during the treatments. The tumor weight and volume are shown as mean ± SEM for five mice and the comparisons between wildtype and variant were performed with two-tailed Student's *t*-test, $*P<0.05$, $**P<0.01$.

also regulated ribosomal protein genes in an estrogen and SERM-dependent manner (Fig. 1 and Supplementary Table S2, Supplemental digital content 1, *http://links.lww.com/FPC/B392*). The ZNF423 ChIP-seq data suggested that ZNF423 transcriptionally regulated certain ribosomal protein genes (Table 2). The subsequent proteomic study identified ZNF423 regulating TAK1 and SIRT1 proteins and the effect of the *ZNF423* gene and the *ZNF423* SNP on AMPK signaling was also validated (Supplementary Table S4, Supplemental digital content 1, *http://links.lww.com/FPC/B392* and Fig. 2). Moreover, in the presence of estrogen, the *ZNF423* SNP modulated the metformin response, either alone or in combination of tamoxifen, in both cell culture and mouse xenograft studies (Figs. 3 and 4). Collectively, our study investigated potential ZNF423 'downstream' genes and pathways at both the mRNA and protein levels in ER+ breast cancer. These novel ZNF423-regulated genes and pathways were then incorporated with the *ZNF423* SNP-dependent function in the context of estrogen and SERM treatment that has been successfully applied in our previous studies for polymerase inhibitor and docetaxel chemotherapy.

Our study suggests that ZNF423 might be an upstream transcription factor regulating ribosomal protein genes based on our RNA-seq and ENCODE ChIP-seq results. It is possible that this ZNF423-dependent regulation has tissue and cell specificity depending on the expression level of ZNF423. Previous studies hypothesized that in the same species, ribosomal protein genes may share common motifs in their regulatory region and therefore be regulated by the same transcriptional factor [26–28]. Furthermore, some studies suggested that the regulatory motifs in ribosomal protein genes could be present through the genes including in distal (1000 bp upstream and 100 bp downstream of transcription start site), proximal (at least 2.5 kilobases away) and intronic regulatory regions [29]. Because the ZNF423 gene encodes a DNA-binding protein containing 30 different C2H2-type zinc fingers, the complexity of ZNF423 regulation may confer

its ability to bind many ribosomal protein genes. Moreover, ZNF423 SNP might regulate ribosomal gene expression (Fig. 1 and Supplementary Figure S2, Supplemental digital content 1, *http://links.lww.com/FPC/B392*), because the expression of ribosomal genes regulated by ZNF423 was increased in the *ZNF423* variant genotype cells while decreased in the wildtype cells in the presence of estradiol. The pattern was opposite with different genotypes when estradiol and tamoxifen were present.

Based on our RNAseq and proteomics data, ZNF423 can regulate the protein levels of TAK1 and SIRT1, but not at the mRNA levels (Fig. 2, Table 1 and Supplementary Table S1, Supplemental digital content 1, *http://links.lww.com/FPC/B392*). Its regulation on TAK1 and SIRT1 might be via its transcriptional regulation on ribosomal proteins. When ZNF423 was downregulated, a majority of ribosomal proteins was increased (Table 1), resulting in increased protein synthesis including TAK1 and SIRT1, leading to AMPK activation (Fig. 2). AMPK has been shown to provide a direct link between cellular energy metabolism and gene expression regulation. It could sense cellular energy levels based on nutrient availability or stress stimuli [30,31]. It is possible that changes in protein synthesis regulated by ZNF423 can change the cellular energy level and result in the activation of the AMPK pathway. To confirm this, further investigation needs to be conducted.

Our findings suggest that the *ZNF423* SNP modulates AMPK signaling as well as metformin response in estrogen and SERM-dependent fashion (Figs. 2, 3 and 4). The underlying mechanism of the relationship between AMPK and metformin is still not fully understood. Metformin has been shown to act via both AMPK-dependent and AMPK-independent mechanism [32–34]. The mechanism of *ZNF423* SNP effects on metformin treatment needs to be further studied.

Though the main findings for both RNAseq and proteomics have been validated by qRT-PCR and western blotting, there is little overlap between genes identified based on the RNAseq and proteomics results except for the ribosome and AMPK signaling pathways. This discrepancy could be due to several reasons: (1) different replications were performed for RNA seq and proteomics which might result in differences in statistical power, (2) much less proteins identified by MS compared to genes identified by RNAseq, and (3) MS, compared to RNAseq, has less quantitative sensitivity to identify adequate proteins with accurate quantifications. Presently, there are 16 ongoing and 15 completed clinical trials (https://clinicaltrials.gov/) for metformin in breast cancer, mostly focusing on the efficacy of metformin monotherapy or in combination with endocrine therapy, chemotherapy and radiotherapy. Though metformin seems to have potential therapeutic benefits in breast cancer, so far there is no significant clinical evidence indicating its efficacy in breast cancer. In our study, compared with the PBS control (in the presence of a low dose of estrogen), metformin alone did not inhibit xenograft growth in the *ZNF423* wildtype SNP genotype (Fig. 4). However, metformin alone can significantly reduce both the tumor size and tumor weight in the ZNF423 variant genotype (Fig. 4). We suggest that the *ZNF423* SNP might help identify those patients who would benefit from the use of metformin. Though not dramatically, metformin plus tamoxifen is better than metformin alone regardless of the *ZNF423* genotypes (Fig. 4), and this result was consistent with previous reports that metformin enhances tamoxifen-mediated tumor growth inhibition [13]. Because the minor allele frequency of *ZNF423* SNP is relatively high (0.45), our results raised a possibility to better individualize metformin therapy either alone or in combination with tamoxifen based on the *ZNF423* SNP genotypes, also taking into account a patient's CYP2D6 status [35]. An important question is how to best examine the translational potential of metformin in women with breast cancer. The major study examining metformin is MA.32 that randomized 3649 nondiabetic women with early stage breast cancer to metformin or placebo in addition to standard surgery, radiation therapy, chemotherapy, endocrine therapy and biologic therapy [36]. Accrual has been completed and results are anticipated in 2022 per ClinicalTrials.gov. The fact that the spectrum of breast cancers included in MA.32 is broad including hormone receptor positive and negative; HER2 positive and negative breast cancers raises the potential to explore these different subtypes for hypothesis development and guidance in the design of follow-up studies. Given the long time it takes to conduct prospective trials, the ideal approach would appear to be that of a 'prospective-retrospective' study [37] utilizing samples from a study such as MA.32. One can speculate that it would be preferable to study the use of pharmacogenetic markers in early stage breast cancer rather than the metastatic setting given the lack of large studies of metformin in the metastatic setting and a small (40 patients) randomized trial of metformin versus placebo in patients receiving chemotherapy that showed no difference in outcomes [38].

Conclusion

We have identified ribosome and AMPK signaling as potential ZNF423-modulating pathways. The ZNF423 rs9940645 SNP affected metformin response and could be a potential biomarker for tailoring the metformin treatment.

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The datasets generated during and analyzed in the current study are not publicly available but are available from the corresponding author on reasonable request.

No human patient involved and no consent was needed in this study. The animal study has been approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC), protocol number A00005042-20.

S.Q. contributed to the concept and design, conducted the study and analysis, and interpretated the data. J.I. and R.W. contributed to the concept and design of the study. W.K. and J.C. conducted the study and analysis. G.H. contributed to data analysis. L.W. is responsible for the concept generation and entire study design. All authors contributed to the development of the manuscript and approved the final manuscript.

Conflicts of interest

L.W. is the cofounder and stock holder of OneOme. LLC. For the remaining authors, there are no conflicts of interest.

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