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Citation for final published version:

Zeng, Yinduo, Tao, Qin, Flamini, Valentina, Tan, Cui, Zhang, Xinke, Cong, Yizi, Birkin, Emily, Jiang, Wen G., Yao, Herui and Cui, Yuxin 2020. Identification of DHX36 as a tumour suppressor through modulating the activities of the stress-associated proteins and cyclin-dependent kinases in breast cancer. American Journal of Cancer Research 10 (12) , pp. 4211-4233. file

Publishers page: http://www.ajcr.us/AJCR_V10N12.html
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1 **Identification of DHX36 as a tumour suppressor through modulating the**
2 **activities of the stress-associated proteins and cyclin-dependent kinases in**
3 **breast cancer**

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27
28 **Statement of conflict of interest**

29 No potential conflicts of interest were disclosed.

30
31 **Running Title:** DEAH-box nucleic acid helicase DHX36 in breast cancer

32
33 **Abstract**

34 The nucleic acid guanine-quadruplex structures (G4s) are involved in many aspects of cancer
35 progression. The DEAH-box polypeptide 36 (DHX36) has been identified as a dominant nucleic
36 acid helicase which targets and disrupts DNA and RNA G4s in an ATP-dependent manner.
37 However, the actual role of DHX36 in breast cancer remains unknown. In this study, we observed
38 that the gene expression of DHX36 was positively associated with patient survival in breast cancer.
39 The abundance of DHX36 is also linked with pathologic conditions and the stage of breast cancer.

40 By using the xenograft mouse model, we demonstrated that the stable knockdown of DHX36 via
41 lentivirus in breast cancer cells significantly promoted tumour growth. We also found that, after
42 the DHX36 knockdown (KD), the invasion of triple-negative breast cancer cells was enhanced. In
43 addition, we found a significant increase in the number of cells in the S-phase and a reduction of
44 apoptosis with the response to cisplatin. DHX36 KD also desensitized the cytotoxic cellular
45 response to paclitaxel and cisplatin. Transcriptomic profiling analysis by RNA sequencing
46 indicated that DHX36 altered gene expression profile through the upstream activation of TNF,
47 IFN γ , NF κ b and TGF β 1. High throughput signalling analysis showed that one cluster of stress-
48 associated kinase proteins including p53, ROCK1 and JNK were suppressed, while the mitotic
49 checkpoint protein-serine kinases CDK1 and CDK2 were activated, as a consequence of the
50 DHX36 knockdown. Our study reveals that DHX36 functions as a tumour suppressor and may be
51 considered as a potential therapeutic target in breast cancer.

52 **Keywords:** DHX36, breast cancer, progression, stress-associated protein, CDK.

53

54 **Introduction**

55 Breast carcinoma is one of the most common malignancies in women. Approximately 2.1 million
56 new cases are diagnosed every year worldwide, which accounts for 25% of all the new female
57 cancer cases, whereas 0.6 million deaths occur with a 5 year-survival range from 1-37% [1, 2].
58 The incidence, mortality rates and survival of breast cancer vary considerably, depending on
59 complicated risk factors, subtype and stage. For instance, the triple-negative breast cancer (TNBC)
60 that is characterized by a lack of expression of estrogen receptor (ER), progesterone receptor (PR),
61 or human epidermal growth factor receptor 2 (HER2), is the most aggressive subtype of the breast
62 cancer, with the highest rate of relapse and metastasis and the worst overall prognosis than other
63 breast cancer subtypes. Hormone receptor-positive tumours like luminal A and luminal B can be
64 treated with endocrine therapy, while a HER2-targeted therapy is usually used when HER2 is
65 overexpressed. However, there is currently no targeted therapy available for the TNBC, and
66 chemotherapy is still the main treatment despite high frequencies of resistance. Therefore, novel
67 biomarkers are needed for a more efficient treatment of some breast cancer subtypes such as TNBC.

68 DNA and RNA guanine-quadruplex structures (G4s) are often over-represented in gene promoter
69 regions, regulatory regions of the human genome and untranslated regions of mRNAs. For
70 example, G4s have been found in the gene promoters of proto-oncogenes including *MYC*, *KRAS*,
71 *BCL-2* and *MLL*. The G4s are also enriched in the mRNAs of retinoblastoma protein 1 (*RBI*),
72 *TP53*, vascular endothelial growth factor (*VEGF*), hypoxia-inducible factor 1 α (*HIF1 α*), the
73 transcription factor *MYB*, platelet-derived growth factor α polypeptide (*PDGFA*), PDGF receptor
74 β polypeptide (*PDGFR β*), and human telomerase reverse transcriptase (*TERT*). Therefore altered
75 G4s have been implicated in cancer development and progression through mediating gene
76 promotor activity or translation process [3].

77 Nucleic acid helicases are a large group of essential enzymes involved in a wide range of major
78 DNA/RNA processing events, including DNA replication, RNA splicing, mRNA stability,
79 ribosomal RNA maturation, microRNA processing, ribonucleoprotein (RNP) complex
80 remodelling and RNA trafficking. The roles of some helicases (e.g. DDX1, DDX3, DDX5, DHX9,
81 DDX41 and DDX43) in cancer have been well documented. For example, they can regulate
82 tumourigenesis through the interaction with genes including *BRCA1*, *p53*, *c-Myc*, *Snail* and *E-*

83 *cadherin*, and the modulation of some signalling pathways such as Wnt/ β -Catenin, L1TD1-RHA-
84 LIN28 and NF- κ B signalling pathways [4, 5]. The DEAH-box polypeptide 36 (DHX36) was
85 originally identified as a dominant ATP-dependent DEAH-box helicase highly specific for DNA
86 and RNA G4s, and is also termed RNA helicase associated with AU-rich RNA element (RHAU)
87 or G4 resolvase-1 (G4R1) [6].

88 DHX36 specifically binds and unwinds the G4-quadruplex motif with its ATPase and resolving
89 activity. DHX36 has been considered as the major source of RNA G4-resolving activity in HeLa
90 cell lysate. The depletion of DHX36 protein in HeLa cells causes a dramatic reduction in G4-
91 DNA- and G4-RNA-resolving process. DHX36 contributes to genomic integrity and helps the
92 transcription and the translation process by unwinding the secondary structures of certain nucleic
93 acids. DHX36 also modulates some genes containing the G-quadruple forming regions, such as
94 *p53*, *PITX*, *YY1*, *VEGF* and *ESR1* [7, 8]. For instance, DHX36 regulates *p53* pre-mRNA 3'-end
95 processing following UV-induced DNA Damage. PITX1 protein acts as a tumour suppressor, and
96 a reduction in its expression is associated with poor overall survival in lung cancer patients [9].
97 YY1 and VEGF proteins play a multifunctional regulatory role in breast cancer, while ESR1 is a
98 predictor of clinical response to neoadjuvant hormonal therapy in breast cancer [10-12]. DHX36
99 can also interact with the pre-miR-134 terminal loop thus reduces the biosynthesis of miR-134 in
100 neuronal dendrites [13]. Interestingly, miR-134 is implicated as a possible regulator in some
101 cancer types and this may reinforce the role of DH36 in tumours [14, 15]. It has also been reported
102 that a long non-coding RNA gene G-Quadruplex Forming Sequence Containing lncRNA (GSEC)
103 can antagonize DHX36 of its G-quadruplex unwinding activity which subsequently enhances the
104 migration of colon cancer cells [16]. Despite the scattered findings above, the role of DHX36 in
105 breast cancer has not been determined. Therefore in this study, we aimed to investigate the
106 functions of DHX36 in breast cancer cells and its carcinogenesis *in vivo*.

107

108 **Materials and Methods**

109 **Cell lines and culture conditions**

110 All the breast cancer cell lines were purchased from the American Type Culture Collection (ATCC)
111 and maintained at low passage (less than 20). Cells were cultured at 37°C in a humidified incubator
112 supplied with 5% CO₂. The breast cancer cell lines were cultured in Dulbecco's modified Eagle's
113 medium/F12K (Sigma-Aldrich, Dorset, UK) supplemented with 10% foetal calf serum (FCS, PAA
114 Laboratories Ltd., Somerset, UK), penicillin (100U/ml), and streptomycin (100mg/ml) (Sigma-
115 Aldrich).

116 **Lentiviral infection with DHX36 shRNA**

117 Lentiviral vectors containing short hairpin RNAs (shRNA) specific for DHX36 and the control
118 shRNA (Scr control) were obtained from VectorBuilder (Santa Clara, CA, USA). The vectors were
119 assembled with EGFP as a reporter and neomycin resistant gene for selection. HEK293T
120 packaging cells were transduced with viral packaging (psPAX2), viral envelope (pMD2G) and
121 lentiviral plasmid vectors using FuGENE 6 transfection reagent (Promega, Southampton, UK) in
122 serum-free OPTI-MEM (Invitrogen, Carlsbad, CA, USA). Four and five days after transfection,
123 the supernatant containing the packaged viral particles was collected and filtered through a 0.45um
124 filter. MDA-MB-231 and BT549 breast cancer cells were then infected using the lentiviral
125 supernatant in the presence of 8 μ g/ml Polybrene (Sigma-Aldrich). After 48 hours, the cells were

126 selected with 1.2 mg/ml G418 for 7 days, and maintained in a growth medium with 300ug/ml
127 G418. After selection, the stable breast cancer cell lines spontaneously expressed GFP which could
128 be visualized under a fluorescence microscope.

129 **Drug cytotoxicity assay**

130 Breast cancer cells at a density of 8000 cells/well were seeded into 96-well plates and starved using
131 a medium containing 2% FCS. Cells were then treated with a serial dilution of cisplatin (Tocris
132 Cookson Ltd., Bristol, UK), paclitaxel (Tocris) and flavopiridol (Cambridge Bioscience,
133 Cambridge, UK), respectively. The vehicle control of cisplatin was ddH₂O, while the vehicle
134 control of paclitaxel and flavopiridol was DMSO. After treatment for 24 and 48 hours, the cells
135 were stained with Alamar Blue (Bio-Rad, Cambridge, MA, USA) following the manufacturer's
136 instruction. The fluorescence was read with an excitation wavelength of 530 nm and the emission
137 at 590 nm using a Glomax Multi Detection System (Promega).

138 **Cell-matrix adhesion assay**

139 Tissue culture plates (96-well black-well) were pre-coated with 3 mg/ml of Matrigel Matrix in
140 serum-free medium (BD Biosciences, San Diego, CA, USA) and left overnight at 37°C. Cells at a
141 density of 10,000 cells/well were seeded onto the pre-coated plates. Following incubation for 1
142 hour, the non-adherent cells were washed off with PBS. The adherent cells were stained with 1
143 μ M of Calcein AM (eBioscience, Hatfield, UK) for 30 minutes at 37°C. The fluorescence which
144 is proportional to the number of the adhesive cells was read with an excitation wavelength of 485
145 nm and the emission at 520 nm using a Glomax Multi Detection System (Promega Wisconsin
146 USA).

147 **Electric cell-substrate impedance sensing (ECIS)**

148 The migration ability of breast cancer cell lines was monitored using the ECIS system. Briefly,
149 MDA-MB-231 and BT549 cells at a density of 2.5×10^4 cell/well were seeded onto ECIS 96W1E
150 array plates (Applied Biophysics Inc. NY, USA). And the electrical resistance, due to the
151 interaction of cells and gold-coated electrodes, was recorded. Once a confluent monolayer was
152 formed, the cells were subjected to an electric wound at 2800 μ A, 60 kHz for 20 seconds and the
153 rate of change in impedance, as cells migrated onto the electrode sensing area, was subsequently
154 monitored and analysed.

155 **Transwell invasion assay**

156 The membrane of 24-well inserts with an 8 μ m pore size (Greiner Bio-one, Frickenhausen,
157 Germany) was pre-coated with 300 μ g/ml of Matrigel for 2 hours at 37°C. 1×10^5 cells were then
158 seeded onto the top chamber in 400 μ l of serum-free medium, and 600 μ l of the same medium
159 containing 10% FCS was added to the lower chamber. After incubation for 24 hours, the invaded
160 cells were detached with 400 μ l of HyQ₂ase Dissociation solution (HyClone, Logan, UT, USA)
161 and stained with 1 μ M calcein AM for 1 h. The cell solution was then transferred to a 96-well
162 black-well plate at a volume of 100 μ l/well for 3 wells per group. The fluorescence of invaded
163 cells was measured using the Glomax Multi Detection System.

164 **Western blotting**

165 Cultured cells were washed twice in PBS and lysed in a RIPA buffer containing 50 mM Tris-HCl,
166 2% SDS, 5% glycerol, 5 mM EDTA, 1 mM NaF, 10 mM β -glycerophosphate, 1 mM PMSF, 1
167 mM Na₃VO₄ and EDTA-free Protease Inhibitor Cocktail (Roche, Mannheim, Germany). Protein

168 concentration was determined by the Pierce BCA protein assay (Thermo Scientific, Colchester,
169 UK). After normalization, proteins were separated by sodium dodecyl sulphate-polyacrylamide
170 gel electrophoresis (SDS-PAGE) and transferred with a semi-dry fast transfer apparatus onto a
171 PVDF membrane (Merck Millipore Inc., Billerica, USA). The membranes were blocked with 5%
172 non-fat dried milk (Marvel, Premier Beverages, Stafford, UK) in PBST solution (0.05% Tween-
173 20 in PBS) for 1 h at room temperature. The membranes were then incubated with the primary
174 antibodies diluted in 5% milk and left overnight at 4°C. Following wash three times with PBST,
175 the membranes were incubated with a diluted HRP-conjugated secondary antibody for 1 h at room
176 temperature. The primary antibodies were anti-JNK (diluted 1:1000. Sc-571, Santa Cruz
177 Biotechnology, Santa Cruz, CA, USA), anti-pJNK^{Thr 183/Tyr 185} (diluted 1:1000. sc-6254, Santa
178 Cruz), DHX36 (diluted 1:1000. GTX131179, GeneTex, San Antonio, TX, USA) and β -actin
179 (diluted 1:5000. sc-53142, Santa Cruz). The HRP-secondary antibodies (A5278, Anti-Mouse IgG;
180 A0545, Anti-Rabbit IgG) were diluted at 1:2000 (Sigma-Aldrich, Dorset, UK). Protein detection
181 was performed using an EZ-ECL chemiluminescence kit (Biological Industries USA, Inc.,
182 Cromwell, CT, USA). Immunoreactive bands were visualized and quantified by densitometry
183 using the Syngene G: BOX chemiluminescence imaging system and Gene Tools 4.03 (Syngene
184 Europe, Cambridge, UK).

185 **Reverse transcription (RT) and real-time PCR analysis**

186 RNA was extracted from the cultured cells at the 60-80% confluency in T25 flasks using TRI
187 Reagent (Sigma-Aldrich, Dorset, UK). Total RNA (500 ng) was reverse-transcribed to
188 complementary DNA (cDNA) using Goscript Reverse Transcription mix (Promega). Following
189 dilution of cDNA at a ratio of 1:8, the quantitative real-time PCR was performed based on an
190 Amplifluor™ technology, in which a 6-carboxy-fluorescein-tagged Uniprimer™ (Biosearch
191 Technologies, Inc., Petaluma, CA, USA.) was used as a probe along with a pair of specific primers
192 with an addition of a Z-sequence (actgaacctgacctaca) to the 5'-end of the reverse primer [17]. The
193 primer sequences for qPCR were: DHX36 forward primer, GTTTAAATCAGTTAACCAGACAC;
194 DHX36 reverse primer, ACTGAACCTGACCGTACACGCAATGTTGGTAGCAATTA; JNK
195 forward primer, CTACAAGGAAAACGTTGACA; JNK reverse primer,
196 ACTGAACCTGACCGTACAGAACAAAACACCACCTTTGA; β -actin forward primer,
197 CATTAAAGGAGAAGCTGTGCT; β -actin reverse primer, ACTGAACCTGACCGTACA
198 GCTCGTAGCTCTTCTCCAG. The qPCR assays were run in a StepOnePlus system (Thermo
199 Fisher Scientific, Waltham, MA, USA) and normalized by the corresponding threshold cycle (CT)
200 values of β -actin mRNA.

201 **Xenograft Tumour Model**

202 BALB/c female nude mice (6–8-week old) were purchased from Beijing Vital River Laboratory
203 Animal Technology Co., Ltd (Beijing 100107, China) and bred in a specific pathogen-free (SPF)
204 animal house at approximately 28 °C in an environment with approximately 50% humidity. They
205 were randomly assigned to two groups with 10 mice/group. 3×10^6 of stable MDA-MB-231 cell
206 lines contains either Scr control or DHX36 shRNA were harvested, resuspended in 0.1 mL of PBS,
207 and subcutaneously transplanted into mammary fat pads of the allocated mice. Each mouse
208 received one injection. The Tumour size was measured with a calliper every 3-4 days and
209 calculated in mm^3 using the formula for a prolate spheroid ($\text{width}^2 \times \text{length} \times 0.523$). When the
210 tumour mass reached the maximally allowed size (16 mm in diameter), the mice were imaged
211 using an IVIS imaging system (Perkin Elmer, Santa Clara, CA, USA) following the manufacturer's

212 instruction. The mice were then sacrificed and the tumours were excised, photographed and
213 weighted. The freshly dissected tumours were fixed in 10% formalin overnight and embedded in
214 paraffin. All the animal experiments were approved by the Institutional Animal Care and Use
215 Committee of Sun Yat-Sen University Cancer Centre.

216 **Immunohistochemistry (IHC) of tissue microarray**

217 The breast cancer tissue microarrays were purchased from US Biomax Inc. (BR1921b, HBre-
218 Duc140Sur-01 and BR1503e. Rockville, MD, USA). The standard indirect biotin-avidin
219 immunohistochemical analysis was used to evaluate the DHX36 protein expression. Briefly, the
220 microarray slides were placed in an oven with 50 °C for 1 day to facilitate the adhesion of tissue
221 sections to the slides. The tissue microarrays were then dewaxed and rehydrated by sequential
222 treatment (5 min per step) with xylene, xylene/ethanol, a serial dilution of ethanol (100%, 90%,
223 70%, 50%), distilled H₂O and Tris-buffered saline (TBS) buffer. Antigen retrieval was performed
224 by placing the slides in a plastic container, covered with 0.01 M sodium citrate buffer (pH6.0)
225 antigen retrieval buffer, and heated in a microwave on full power for 20 minutes. Endogenous
226 peroxide activity was blocked by incubating the sections with 3% hydrogen peroxide for 10
227 minutes. After 1 hour of pre-incubation in 5% normal goat serum to block nonspecific staining,
228 the sections were incubated with 7.5 µg/ml of the DHX36 antibody (GTX131179. GeneTex)
229 overnight at 4°C. The slides were then washed four times with TBS, and incubated with a universal
230 biotinylated secondary antibody (ABC Elite Kit, Vectastain Universal, PK-6200, Vector
231 Laboratories, CA, USA) for 30 minutes. Following washing with TBS, the sections were incubated
232 with avidin-biotin-peroxidase complex (ABC) for 30 minutes. The 3, 3'-diamino-benzidine (DAB)
233 substrate (5 mg/ml) was used to develop the final reaction product. The sections were then rinsed
234 in water, counterstained with Gill's hematoxylin (Vector Laboratories), and dehydrated through a
235 series of graded alcohols, cleared in xylene and mounted in DPX/Histomount (Merck Millipore,
236 UK). Images were captured using an EVOS FL Auto 2 Cell Imaging System (ThermoFisher
237 Scientific). All IHC images were manually evaluated and scored by two pathologists
238 independently who were blinded to the clinical information. The immunochemical score was
239 calculated based on intensity plus the percentage of tumour staining. The cut-off value was set as
240 an upper quarter of the score divided into a high and low expression of DHX36 protein.

241 **Flow cytometry**

242 Cultured cells were detached with trypsin/EDTA and fixed with the IC fixation buffer
243 (ThermoFisher Scientific) for 1 h at room temperature, then resuspended in ice-cold 100%
244 methanol, and incubated overnight at -20 °C. Cells were then washed twice in FACS buffer (2 mM
245 EDTA in PBS, pH 7.4), blocked with 1% bovine serum albumin (BSA) in PBS with 0.1% Tween
246 for 1 hour. For the staining with antibodies, cells were incubated with diluted primary antibodies
247 (1:100) including normal mouse IgG (14-4714-82, ThermoFisher Scientific), cleaved poly (ADP-
248 ribose) polymerase (PARP) (14-6668-82, ThermoFisher Scientific), JNK and p-JNK, respectively,
249 for 1 hour at room temperature. Cells were then incubated with Alexa Fluor 647-conjugated goat
250 anti-mouse IgG antibodies (1:1000; A21235, ThermoFisher Scientific) for 30 minutes at room
251 temperature. For cell cycle analysis, cells were harvested and blocked as described above, and then
252 directly incubated with Hoechst 33342 (10 µg/ml. H3570, ThermoFisher Scientific) for 1 hour at
253 37°C in the dark. Following the final wash with FACS buffer, FACS was performed using BD
254 FACS Canto II flow cytometer equipped with FACS Diva Software (version 6.1.2. BD

255 Biosciences, San Jose, CA, USA). FACS data were analysed using FCS Express software (version
256 4. De Novo Software, Los Angeles, CA, USA).

257 **Bioinformatic analysis of gene expression and survival**

258 The association between DHX36 gene expression and the survival of breast cancer patients was
259 assessed using the pooled gene expression data from www.kmplot.com. The online tool allowed
260 us to analyse both the OS (overall survival) and RFS (relapse-free survival) from 626 cases of
261 breast cancer and the RFS from 1764 cases which were subjected to expression profiling with
262 Affymetrix GeneChip microarray (DHX36 Probeset ID: 223140_s_at). The Auto select best cutoff
263 was chosen. The differential expression of DHX36 was examined by a pooled analysis of The
264 Cancer Genome Atlas-Breast invasive carcinoma (TCGA-BRCA) dataset which contains 1097
265 breast-cancer patients and 114 normal samples.

266 **RNA sequencing (RNA-Seq)**

267 The global transcriptomic profiling was analysed by RNA-Seq on the BGISEQ-500RS sequencer
268 (BGI, Shenzhen, China) which generated 50-bp paired-end reads. The statistical enrichment and
269 molecular network of differentially expressed genes (DEGs) were analysed using the Ingenuity
270 Pathway Analysis (IPA) software (Qiagen, Germany).

271 **Kinexus Kinex antibody microarray**

272 The stable breast cancer cell lines were seeded in T75 flasks and incubated in DMEM
273 supplemented with 10% FCS at 37°C. When the confluence was approximately 80%, the cells were
274 then washed twice, and the culture medium was replaced with DMEM with 2% FCS. After
275 incubation overnight, cells were suspended in lysis buffer, pH 7.4, containing 100mM Tris Buffer,
276 10% 2-ME, 1% NP-40, protease inhibitor cocktail tablet and 50mM NaF. The lysates were
277 vortexed and homogenized on a blood wheel for 1 hour at 4 °C. The supernatant of the lysates was
278 then collected by centrifugation for 30 minutes at 15,000 rpm at 4 °C, the protein concentration in
279 the supernatant was determined by a fluorescamine protein quantification assay (Sigma-Aldrich).
280 Proteomic analysis of pan-specific and phosphorylated proteins was carried out using a high
281 throughput Kinex antibody microarrays (900 antibodies, Kinexus Bioinformatics)
282 (<http://www.kinexus.ca/services>).

283 **Statistical analysis**

284 For quantitative measurement, including cell-based assays and gene expression profiling, the
285 Shapiro-Wilk test was used to verify whether the data were normally distributed. For the
286 comparison of the difference from two subjects, an unpaired t-test was used for data with normal
287 distribution, whereas, for non-normal distribution, the Mann-Whitney Rank Test was applied.
288 When more than two sets of data were compared, either One-Way ANOVA or the non-parametric
289 Kruskal-Wallis test was used. Pearson chi-square test was used to test the association of the
290 categorized scoring data from tissue microarray IHC staining and clinical features. Graphs and the
291 statistical analysis were performed using R (version 3.6.1, <https://www.r-project.org>) or GraphPad
292 Prism 8 software (GraphPad Software, San Diego, CA, USA). Statistical significance was
293 indicated with the following nomenclature: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ unless the p-values
294 were shown.

295

296 **Results**

297 **Lower gene expression of DHX36 is associated with poorer survival**

298 We assessed the prognostic value of DHX36 gene expression in breast cancer using the Kaplan–
299 Meier plotter containing 1764 samples from breast cancer patients. As shown in Fig. 1A and 1B,
300 lower expression of DHX36 mRNA correlated to poorer OS (HR=0.63 (0.45-0.88), p=0.0059) and
301 RFS (HR=1.32 (1.13-1.54), p<0.001) when all the types of breast cancer were pooled together.
302 We then analysed the differential expression of the DHX36 gene in the database of the TCGA
303 invasive breast carcinoma (TCGA-BRCA). It showed that there is a lower level of DHX36 gene
304 expression in primary tumour compared to normal tissue control (p=0.0092; Fig. 1C). The DHX36
305 gene expression was downregulated in the later stages (T3+T4) of the breast carcinoma compared
306 to the earlier stages (T1+T2) (p=0.0096, Fig. 1D). Also, it appeared that the gene expression level
307 of DHX36 was higher in TNBC (n=123) than in non-TNBC (n=605) (p<0.0001, Fig. 1E)

308 **Low level of the DHX36 protein also predicts poor survival as indicated by tissue microarray** 309 **IHC**

310 We then estimated the IHC staining of the DHX36 protein in breast cancer tissue microarrays from
311 patient specimens. As shown in Table 1 and Figure 1F&G, the samples from patients at a higher
312 stage (2&3) showed weaker staining of DHX36 (score 0&1) (p=0.034 than lower stage). When
313 we compared the different pathological types, the frequency of the lower stained DHX36 was
314 higher in IDC (214/277=77.26%) than in ILC (3/81=3.70%) and in adjacent normal tissue
315 (9/34=26.47%) (p<0.001). The expression level of the DHX36 protein also appeared to be
316 associated with the pathological diagnosis (p<0.001), HER2 intensity (p<0.001), ER intensity
317 (p<0.001) and PR intensity (p<0.001). We then performed Kaplan-Meier survival analysis using
318 a dataset of one array with definite follow-up status (n=140, #HBre-Duc140Sur-01). A higher level
319 of DHX36 protein expression correlated with favourable survival of breast cancer patients (Figure
320 1H). The thumbnail IHC images of the three tissue microarrays were shown in Supporting
321 Information Fig. S1.

322 **Knockdown of DHX36 in TNBC cells increased the invasion ability and suppressed the** 323 **migration of the tumour cells *in vitro***

324 To understand the role of DHX36 in breast cancer, we selected two TNBC cell lines for a stable
325 DHX36 knockdown after initial evaluation of the gene and protein expression levels of DHX36 in
326 a panel of breast cancer cells (Supporting Information Fig. S2). As shown in Figure 2, after the
327 establishment of the stable knockdown cell lines using BT549, all three shRNAs (1, 2 and 8)
328 reduced the gene expression of DHX36 when compared with scramble (Scr) shRNA and wild-type
329 (WT) controls (Figure 2A). Likewise, this was also the case in the stable cell lines developed from
330 MDA-MB-231 (Figure 2B). The Western blotting images also showed that in BT549, the DHX36
331 protein level was dramatically reduced in all three cell subsets (shRNA 1, 2 and 8) with the
332 shRNA2 showing the best efficiency (Figure 2C). As expected, the WT and Scr controls showed
333 a higher expression of DHX36 protein. The efficiency of shRNA2 was subsequently demonstrated
334 in the stable cell lines developed from MDA-MB-231. Therefore in the following experiments,
335 only the stable cell lines with shRNA2 were utilised (named as shRNA unless otherwise described).

336 We then evaluated the effect of DHX36 expression on tumour cell invasion using the Matrigel-
337 coated transwell chamber. As shown in Figure 2E, in BT549 cells, DHX36 shRNA increased the
338 invasion by 6.39% compared to the Scr control (p<0.0001). Similarly, in MDA-MB-231 cells,
339 DHX36 shRNA increased the invasion by 10.83% compared to the Scr control (p=0.041, Figure
340 2F). We monitored the cell migration using the ECIS system and found that DHX36 shRNA

341 inhibited the migration of the breast cancer cells after the electric wound ($p < 0.01$ vs. Scr control,
342 respectively. Figure 2G-H).

343

344 **Knockdown of DHX36 in TNBC cells increases the S-phase cell population and de-sensitize** 345 **the apoptotic response to cisplatin.**

346 We investigated the role of DHX36 in the cell cycle by flow cytometry. As shown in Figure 3,
347 DHX36 knockdown in the BT549 stable cell lines increased the S-phase population to 36.23%
348 from 25.91% (Scr). Similarly, in the MDA-MB-231 stable cell line, DHX36 knockdown increased
349 the S-phase cell population to 43.65% from 33.37% (Scr).

350 We then evaluated the effect of DHX36 knockdown on apoptosis of the breast cancer cells using
351 the cleaved PARP as an indicator. In the BT549 stable cell lines, the knockdown of DHX36
352 decreased the basal apoptotic level by approximately 19.96% compared to its Scr control (Figure
353 3E&3F); cisplatin (16 μM , 24 hours) increased the apoptosis of the Scr control by approximately
354 45.14%, but just increased the apoptosis of the DHX36 knockdown group by approximately 18.62%
355 in comparison to its vehicle (PBS) control (Figure 3G&3H). In the MDA-MB-231 stable cell lines,
356 the knockdown of DHX36 decreased the basal apoptotic level by approximately 26.06% compared
357 to its Scr control (Figure 3I&3J); cisplatin increased the apoptosis of the Scr control by
358 approximately 69.53%, and increased the apoptosis of the DHX36 knockdown group by
359 approximately 69.69% in comparison to its vehicle (PBS) control (Figure 3K&3L). The data,
360 therefore, suggest that DHX36 may modulate the intrinsic apoptosis of breast cancer cells. And it
361 appeared that cisplatin raised a stronger apoptotic response in MDA-MB-231 cells than BT549
362 cells.

363

364 **Knockdown of DHX36 desensitizes the susceptibility of breast cancer cells to** 365 **chemotherapeutic drugs in a cell- and dose-dependent manner.**

366 We then evaluated the cytotoxic response of breast cancer cells to some chemotherapeutic drugs
367 including paclitaxel and cisplatin, respectively. As shown in Figure 4A, in MDA-MB-231 cells,
368 following 24-hour treatment, the decrease of cell viability started to be observed from 5nM
369 paclitaxel in the Scr group, while the DHX36 knockdown group showed the viability decrease
370 from 10 nM. The suppression of the cellular susceptibility to paclitaxel by DHX36 knockdown
371 can be seen from 5 nM to 40 nM in comparison to the Scr controls ($p < 0.01$ vs Scr, respectively).
372 The response difference between the two stable MDA-MB-231 cell lines to paclitaxel began to
373 disappear following treatment for 48 hours (Figure 4B). Similarly, the lower level of cytotoxic
374 response to paclitaxel was observed in the DHX36-knockdown BT549 cell line compared to the
375 Scr control after treatment for 24 hours (Figure 4C). Again, the response difference of the two
376 stable BT549 cell lines to paclitaxel began to disappear following treatment for 48 hours (Figure
377 4D). Independent to the effect of DHX36 knockdown, we noticed that the control BT549 cell line
378 was more sensitive to paclitaxel (starting from 2.5 nM) compared to the control MDA-MB-231
379 cell line (starting from 5 nM). Following treatment with multiple doses of cisplatin for 24 hours,
380 the DHX36-knockdown MDA-MB-231 cell line showed a higher proliferation ratio thus lower
381 cytotoxicity in response to the doses of 32 μM ($p < 0.05$) and 64 μM ($p < 0.01$) than the Scr control
382 (Figure 4E). The response significance of the two stable MDA-MB-231 cell lines to cisplatin was

383 observed to sustain following treatment for 48 hours ($p < 0.01$ for the two high working doses)
384 (Figure 4F). Likewise, In BT549 cells, DHX36 knockdown also led to a reduction of the cellular
385 response to cisplatin following treatment for 24 and 48 hours (Figure 4G&4H). We also confirmed
386 that the control BT549 cell line was more sensitive to cisplatin (starting from 1 μM) compared to
387 the control MDA-MB-231 cell line (starting from 4 μM).

388 **Knockdown of DHX36 promotes breast cancer development in a mouse xenograft**

389 To examine whether the DHX36 knockdown promotes breast cancer growth *in vivo*, we inoculated
390 the stable MDA-MB-231 cells with and without DHX36 knockdown in nude mice. The in-vivo
391 fluorescence imaging analysis indicated that all the nude mice showed some tumour growth after
392 implantation with the stable cell lines containing either the Scr control (Figure 5A) or the DHX36
393 shRNA (Figure 5B), and tumours with bigger sized could be visualized from the group of the
394 DHX36 shRNA. The quantification of individual tumour fluorescence images confirmed that the
395 mice group of the DHX36 shRNA had larger total tumour pixels (proportional to tumour size)
396 compared to the Scr control ($p < 0.01$, Figure 5C). Likewise, the mice group of the DHX36 shRNA
397 showed a higher level of integrated density of tumour fluorescence (proportional to tumour mass
398 density) compared to the Scr control ($p < 0.05$, Figure 5D). The data of the time-lapse physical
399 measurement of the xenograft mice indicated that the mice with the DHX36 knockdown started to
400 develop a bigger tumour mass (average tumour volume in mm^3) than the Scr control after 2 weeks,
401 and continued the trend of accelerated tumour growth until the end of examination at Day 46
402 ($p < 0.001$, Figure 5E). No significant change of body weight was observed between the two mice
403 groups over the course of measurement ($p > 0.05$, Figure 5F). The dissected tumours from the mice
404 group containing the DHX36 knockdown cells at the endpoint (Day 46) presented larger tumours
405 (Figure 5G). The measurement of the tumour weight confirmed that the tumours from the DHX36
406 shRNA group were dramatically heavier than the Scr control ($p < 0.001$, Figure 5H). This result
407 therefore indicated that knockdown of DHX36 in MDA-MB-231 cells promoted tumourigenesis,
408 suggesting that DHX36 expression may be crucial for the suppression of neoplastic growth.

409

410 **RNA-Seq transcriptome analysis of stable breast cancer cells indicates that DHX36 is** 411 **involved in multiple gene regulation pathways.**

412 We performed an RNA-Seq transcriptome analysis to examine the gene expression profile altered
413 by DHX36 shRNA. Overall, following DHX36 knockdown, 2.05% of genes were regulated in
414 BT549 cells, while 1.90 % of genes were regulated in MDA-MB-231 cells (Figure 6A and B). The
415 top 10 upregulated genes by DHX36 knockdown in both the breast cancer cell lines were CHI3L2,
416 MAF, SNAI1, BMP2, ADRA2C, HSD17B10, TGM2, DYNLRB1, SNX15 and RAP1GAP2. And
417 the top 10 downregulated genes were MMP1, MRGPRF, NLRP10, ATP10A, SUSP2, FAM167A,
418 ITGB2, CYP26B1, UXS1 and PCSK1N (Heatmap showed in Figure 6C). As indicated by the
419 gene ontology analysis, DHX36 knockdown altered gene regulation of cell-to-cell signalling and
420 interaction, cellular growth and proliferation, cell signalling and other cellular function (Figure
421 6D). The upstream regulator of these genes could be some cytokines or complex such as TNF,
422 $\text{IFN}\gamma$ and $\text{NF}\kappa\text{B}$, as predicted by using the Ingenuity® Pathway Analysis (IPA) (Figure 6E).

423 **DHX36 plays its role in breast cancer cells through stress-associated proteins and mitotic** 424 **checkpoint protein-serine kinase.**

425 We used the Kinex antibody array to determine the molecular signalling mechanisms of DHX36
426 induced invasion and tumourigenesis in breast cancer cells. As shown in Figure 7, two clusters
427 of proteins were identified to be differentially expressed following the DHX36 knockdown. In
428 DHX36 deficient MDA-MB-231 cells, within the cluster of the stress associated kinase proteins,
429 the pan-specific p53, and the phosphorylated p53 protein isoform of S6 were reduced by 47 % and
430 30%, respectively. Within the same cluster, the pan-specific and phosphor (Y913) forms of
431 ROCK1 were also decreased by 34% and 28%, respectively. We also observed the inactivation of
432 the other phosphorylated stress-associated kinase proteins including MYPT1 (T696), MDM2
433 (S166) and MLC (S19). However, following DHX36 knockdown, the cluster of the Mitotic
434 checkpoint protein-serine kinase proteins was found to be activated. The levels of the
435 phosphorylated proteins of CDK1/2 (T161), CDK1 (T14), CDK1 (T161), CDK1/2 (T14+Y15),
436 CDK1 (T14+Y15) were increased by 133%, 109%, 80% and 60%, respectively. The original
437 images of the Kinex antibody array analysis were shown in Supporting Information Fig. S3.

438 To instigate whether the increase in the CDK levels in the breast cancer cells has an effect on the
439 response susceptibility when CDK is inhibited. We performed proliferation after 48h treatment
440 with to flavopiridol, a CDK inhibitor by ATP competition. The results showed that after DHX36
441 knockdown, MDA-MB-231 cells were more sensitive to the inhibitory effect of flavopiridol at
442 different doses including 100 nM (p=0.0087), 200 nM (p=0.0044) and 400 nM (p=0.0022)
443 (Supporting Information Fig. S4A). BT549 also showed a higher sensitivity following DHX36
444 knockdown at a dose from 50 nM (p=0.0043) to higher doses including 100 nM (p=0.0022), 200
445 nM (p=0.0022) and 400 nM (p=0.0022) (Supporting Information Fig. S4B).

446 By FACS analysis, we also found that, following the knockdown of DHX36 in BT549 cells, the
447 total and phosphorylated protein levels of JNK were reduced by 36.66 and 35.50%, respectively
448 (Figure 7C-F). Similarly, in DHX36-deficient MDA-MB-231 cells, the total and phosphorylated
449 protein levels of JNK was reduced by 20.57 and 16.92%, respectively (Figure 7G-J). The reduction
450 of JNK and pJNK in both cell lines following the DHX36 knockdown, was confirmed by Western
451 blotting (Figure 7K). The qPCR data indicated that the JNK gene expression level was
452 downregulated in cells with the DHX36 shRNA (Figure 7L).

453

454 **Discussion**

455 There are enormous challenges to elucidate the molecular mechanisms that lead to breast cancer
456 progression and identify new biomarkers for the early detection of this disease[18]. RNA helicases
457 could participate in tumour development and aggression by remodelling complex RNA structures
458 or altering translation of some pro-oncogenic mRNAs [19, 20]. DHX36 is one of the members of
459 the DEAH-box helicases, but its role in breast cancer remains unknown.

460 In this study, we identified that DHX36 acts as a prognostic marker in breast cancer. By using the
461 Kaplan Meir survival analysis, we showed that a higher gene expression level of DHX36 is
462 associated with a better OS and RFS in breast cancer patients. Interestingly, the gene expression
463 level of DHX36 in the TNBC is higher than in non-TNBC subtypes. The IHC data indicate that in
464 breast cancer tissues, elevated levels of DHX36 correlate with better overall survival. This is
465 confirmed by the findings in breast cancer tissues, where high levels of DHX36 are associated
466 with a higher stage of the disease.

467 In addition, the lower staining of DHX36 was observed more frequently in the invasive ductal
468 carcinoma (IDC) than in the invasive lobular carcinoma (ILC) tissues. IDC and ILC are different
469 in multiple clinicopathological features and it is believed that ILC has a favourable response to
470 systemic therapy compared to IDC [21]. However, the pooled analysis using the KM-Plot online
471 database indicates that the DHX36 gene expression level may be positively associated with
472 metastasis and short survival in other solid tumours, such as ovarian and gastric cancer. The
473 contradictory implication of DHX36 in different cancer types may be linked with its functional
474 complexity and heterogeneity of the molecular cancer pathways in which it is involved.

475 Both *in vitro* and *in vivo* data indicate that DHX36 may inhibit the malignant properties of breast
476 cancer cells. The stable knockdown of DHX36 in TNBC cell lines increased the invasion and
477 decreased the migration properties of the breast cancer cells. The cell cycle analysis suggests that
478 DHX36 deficiency leads to the accumulation of cells in the S-phase of the cell cycle. And the
479 downregulation of DHX36 in breast cancer cells attenuates the apoptosis of breast cancer cells
480 both endogenously and in response to cisplatin. In the presence of DHX36 shRNA, breast cancer
481 cells tend to be more susceptible to the treatments with some chemotherapeutic drugs including
482 cisplatin, paclitaxel, and epirubicin in terms of cytotoxicity. Our *in-vivo* work demonstrates that
483 the loss of DHX36 function in aggressive MDA-MB-231 cells promotes tumour growth. We,
484 therefore, speculated that the loss of DHX36 drives the cancer progression in breast cancer.

485 We investigated the role of DHX36 in breast cancer progression through the RNA sequencing
486 analysis using the DHX36 knockdown cells. The RNA-Seq data indicate that DHX36 is involved
487 in many regulatory network routes through mediating TNF, IFN, NF κ b and TGF β 1. Also, the
488 altered gene network altered by DHX36 may influence cancer cell behaviour through different
489 pathways, such as cell-to-cell interaction, cell growth, cell signalling, molecular transport and
490 metabolism. It has been shown that DHX36 is involved in TNF α and NF κ B activation in monkey
491 kidney cells in a virus-induced manner [22]. DHX36 can also activate the production of IFN β in
492 mouse embryonic fibroblast (MEF) cells or IFN α in dendritic cells by sensing virus stimulation
493 [23, 24]. We therefore speculate that the activation of certain cytokines and growth factors by
494 DHX36 can also occur in breast cancer cells. Besides, the RNA-Seq data suggest that the ITGB2
495 gene is downregulated, and this may lead to the upregulation of the MMP1 signalling pathway.
496 MMP1 may then downregulate the BMP2 gene, which exerts diverse functions in cancer
497 development and progression [25]. The knockdown of DHX36 also upregulates the gene
498 expression of SNAI1 (SNAIL) gene, which is involved in the induction of the epithelial to the
499 mesenchymal transition process.

500 The high throughput proteomic profile data indicate that, following the knockdown of DHX36 in
501 MDA-MB-231 cells, the level of the death-associated kinase proteins is reduced. In particular,
502 both pan-specific p53 and most of the phosphor-p53 isoforms are decreased in response to the
503 DHX36 knockdown. It is known that almost all eukaryotic mRNAs are subjected to a multi-step
504 pre-mRNA 3'-end processing which is coupled to transcription [26]. DHX36 can particularly bind
505 the p53 RNA G4-forming sequence and therefore maintain p53 pre-mRNA 3'-end processing
506 following UV-induced DNA damage in lung cancer cells [27]. Both MDA-MB-231 and BT549
507 cell lines have two types of intrinsic p53 mutation, named p53^{280R-K} and p53^{249R-S}, respectively
508 [28]. However, previous studies also suggest that mutant p53 in cancer cells can be either loss-of-
509 function or gain-of-function, and can be stabilized probably through the loss-of-heterozygosity in
510 response to cellular stress [29, 30]. The protein levels of pan-specific and phosphor- ROCK1 are
511 also reduced in DHX36 deficient cells. ROCK1 is an upstream activator of the JNK signalling

512 pathway in cancer [31] and it is involved in the actin cytoskeleton destabilisation [32]. As
513 alterations to the actin cytoskeleton can cause changes in various cancer cell properties such as
514 adhesion, migration, invasion, and EMT, we therefore suggest that the reduced level of migration
515 in the breast cancer cells following DHX36 knockdown may be attributed to the decrease of
516 ROCK1.

517 The protein array data indicate that there is activation by phosphorylation of the mitotic checkpoint
518 protein-serine kinase proteins CDK1 and CDK2. CDK1 is one of the cyclin-dependent kinases
519 (CDKs) which plays a central regulatory role in mitosis initiation and drives cell cycle transition
520 from the G1 phase to the S phase when CDK2 is lost [33]. CDK2 is required for the G1 phase
521 progression and the entry progression into the S phase [34]. In breast cancer, cells with a higher
522 level of CDK2 respond more sensitively to the treatment of paclitaxel [35]. It is known that p53
523 is an upstream regulator of CDK1 and CDK2 through various downstream effectors such as
524 p21WAF1/CIP1), 14-3-3- σ , reprimin, CD25, cyclin B1 and PLK1 [36].

525 We also demonstrated that JNK transcription and JNK phosphorylation are reduced following the
526 DHX36 knockdown. The JNK signalling pathway can be activated by some extracellular or
527 intracellular stress such as reactive oxygen species, nitrogen species, UV, inflammation or
528 cytokines [37, 38]. In cancer, activated JNKs can indirectly mediate some aspects of cell
529 behaviour such as growth, transformation and apoptosis by phosphorylating its downstream
530 substrates such as c-Jun, ATF2, ELK1, and p53 [39]. In another way, JNKs may also directly
531 modulate the apoptosis by the phosphorylation of the pro- and anti-apoptotic proteins in
532 mitochondria [40].

533 Chemotherapy resistance remains a major obstacle for the development of an effective breast
534 cancer treatment strategy. It is known that CDK protein kinases may interfere with the DNA repair
535 activity in cancer cells, therefore increasing their sensitivity to certain DNA damaging drugs [41].
536 We showed that the knockdown of DHX36 appears to sensitize the response of breast cancer cells
537 to some cytotoxic chemotherapeutic drugs such as cisplatin, paclitaxel, epirubicin and flavopiridol,
538 in a dose-dependent manner. This may be due to the elevated levels of certain CDK family
539 members following the DHX36 knockdown as described previously (31). Cisplatin, paclitaxel and
540 epirubicin are known first-line chemotherapeutic drugs. Flavopiridol is a pan-CDK inhibitor which
541 inhibits CDKs by blocking their ATP-binding sites directly. As one of the most investigated CDK
542 inhibitor, flavopiridol has been subjected in considerable clinical trials for its anti-tumour efficacy
543 [42]. Therefore DHX36 may play a role in modulating the therapeutic response of breast cancer
544 cells although more evidence would be required by further investigation including clinical studies.

545

546 **Conclusion**

547 In conclusion, to our knowledge, this is the first study that identifies the functional role of DHX36
548 in breast cancer. Our data indicate that DHX36 acts as a tumour suppressor in human breast cancer.
549 The expression level DHX36 is negatively associated with the survival (OS and RFS) of breast
550 cancer patients. And we believe that the deficiency of DHX36 enhances the invasion property of
551 breast cancer cells and promotes tumour growth by modulating the p53, JNK and ROCK signalling
552 pathways and CDKs (as illustrated in Fig. 8). Our study therefore unveils the new roles of the
553 DHX RNA helicase proteins in cancer cells thus may open a new avenue for developing anti-
554 cancer therapeutic strategies with higher efficacy.

555

556 **Abbreviations**

557 G4s, guanine-quadruplex structures; DHX36, DEAH-box polypeptide 36; TNBC, triple-negative
558 breast cancer; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth
559 factor receptor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHC,
560 immunohistochemistry; ECIS, electrical cell impedance sensing; IDC, invasive ductal
561 carcinoma; ILC, invasive lobular carcinoma; CDK, cyclin-dependent kinase. RB1,
562 retinoblastoma protein 1; VEGF, vascular endothelial growth factor; HIF1 α , hypoxia-inducible
563 factor 1 α ; PDGFA, platelet-derived growth factor α polypeptide; PDGFR β , PDGF receptor β
564 polypeptide; TERT, human telomerase reverse transcriptase.

565

566 **Ethics approval for animal experiments**

567 All animal experiments were performed in accordance with relevant guidelines and regulations
568 approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University Cancer
569 Centre.

570

571 **Ethical Approval and Consent to participate**

572 Not applicable.

573

574 **Consent for publication**

575 All authors agreed on the manuscript.

576 **Availability of supporting data**

577 All data generated or analyzed during this study are included in this published article and its
578 supplementary information files.

579

580 **Competing interests**

581 The authors declare that they have no competing interests.

582 **Funding**

583 This work was financially supported by grants from Cancer Research Wales, Cardiff China
584 Medical Scholarship, Life Sciences Research Network Wales, the National Natural Science
585 Foundation of China(81572596, U1601223, 81502302), and grants from the Guangdong Natural
586 Science Foundation (2017A030313828, 2017A030313489), and funding from the Guangzhou
587 Science and Technology Bureau (201704020131). The authors also gratefully acknowledge
588 financial support from the China Scholarship Council.

589

590 **Authors' contributions**

591 YC, WGJ and YZ designed this study and drafted the manuscript. YZ, YC, TQ, VF and YZC
592 conducted the experiments. CT and XZ contributed to the scoring of the IHC. WGJ contributed to
593 the analysis of the Kinex antibody array data. YC provided bioinformatic and statistical analysis.
594 VF and EB edited the manuscript. WGJ and HY supervised the research. All authors read and
595 approved the final manuscript.

596

597 **Acknowledgments**

598 The authors thank Fiona Ruge and Dr You Zhou for their technical assistance.

599

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727 **(Description of the figures and tables)**

728 **Table 1. The association of the IHC staining intensity of DHX36 and clinical features of breast**
729 **cancer patients.** DCIS: ductal carcinoma in situ. IDC: invasive ductal carcinoma. ILC: invasive lobular
730 carcinoma. NOS: not otherwise specified.

731 **Figure 1.** Expression levels of DHX36 gene and protein are associated with the survival and
732 clinicopathological features of breast cancer patients. The Kaplan-Meier survival curve was plotted using
733 the pooled gene expression data from www.kmplot.com (Cut-off value: 1257.33. n=1764). (A) OS. (B)
734 RFS. (C) DHX36 gene expression is downregulated in the primary tumour as indicated by the analysis of
735 The Cancer Genome Atlas-Breast invasive carcinoma (TCGA-BRCA) dataset (n=1218). (D) Expression
736 level of the DHX36 gene is lower in advanced stages (T3+T4) than in earlier stages (T1+T2) as indicated
737 by the TCGA-BRCA data. (E) Expression level of the DHX36 gene is higher in TNBC (n=123) than in
738 non-TNBC (n=405) as indicated by the TCGA-BRCA data. Equivocal: The ER/PR/HER3 status is partially
739 determined. NDA: No data available for the ER/PR/HER3 status. (F) Frequency of DHX36 staining scores
740 in different pathological types in breast cancer tissue arrays. (G) Frequency of DHX36 staining scores in
741 different stages in breast cancer tissue arrays. (H) Kaplan-Meier survival analysis of the breast cancer tissue
742 arrays following DHX36 staining by immunohistochemistry. Representative images of the differential
743 staining intensity of DHX36 in normal breast and breast cancer tissues were shown in Supporting
744 Information Fig. S1. Clinicopathological status of the three tissue microarray slides was provided in Table
745 1.

746 **Figure 2.** Knockdown of DHX36 enhanced invasion but decreased migration of breast cancer cells. (A)
747 Relative gene expression of DHX36 in BT549 cells following DHX36 knockdown with shRNA. (B)
748 Relative gene expression of DHX36 in MDA-MB-231 cells following DHX36 knockdown with shRNA.
749 (C) The expression level of DHX36 protein after stable shRNA knockdown of DHX36 in BT549 cells, as
750 estimated by Western blotting. (D) The expression level of DHX36 protein after stable shRNA knockdown
751 of DHX36 in MDA-MB-231 cells, as estimated by Western blotting. (E, F) Transwell invasion assay using
752 the stable cell lines derived from BT549 and MDA-MB-231 cells, respectively. Cells invaded through
753 Matrigel-coated membrane inserts (pore size 8 μ m) were stained with Calcein AM and detached using Cell
754 Dissociation Solution, and read using a fluorescence plate reader. The cell group with DHX36 shRNA was
755 compared with the Scr Control. Although the Invasion of the WT control was showed, because WT cells
756 were not subjected to lentiviral infection and specific G418 selection, they were not directly comparable to
757 the shRNA groups. Student T-tests were used to compare the difference between shRNA and Scr. (G, H)
758 Effect of DHX36 knockdown on the migration of breast cancer cells was accessed using the electric cell-
759 substrate impedance sensing system (ECIS). Normalization was performed by setting up the stating
760 impedance signal for each group to 1. The repeated-measures ANOVA was used to compare the ECIS data
761 from different cell groups. **p<0.01.

762 **Figure 3.** Effect of DHX36 knockdown on cell cycle progression and apoptosis in response to cisplatin.
763 Hoechst 33342 was used to stain cellular DNA for cell cycle profiling, while the apoptosis level was
764 determined using a cleaved-PARP (cPARP) antibody. (A, B) Cell cycle analysis of BT549 cells transduced
765 with Scr control (left) and DHX36 shRNA (right). (C, D) Cell cycle analysis of MDA-MB-231 cells
766 transduced with Scr control (left) and DHX36 shRNA (right). (E, F, G and H) Level of cleaved-PARP in
767 BT549 cells transduced with Scr control and DHX36 shRNA, and treated with PBS and cisplatin,
768 respectively. (I, J, K and L) Level of cleaved-PARP in MDA-MB-231 cells transduced with Scr control and
769 DHX36 shRNA, and treated with PBS and cisplatin, respectively. The levels of the cleaved-PARP were
770 indicated using the Median Fluorescence Intensity (MFI). The percentage change of MFI (PC)
771 was calculated using the equation: $PC = (MFI_{test} - MFI_{control}) / MFI_{control} * 100$, where control means the Scr
772 PBS group. ISO, isotype control.

773 **Figure 4.** The viability of breast cancer cells treated with chemotherapeutic drugs. Cells were seeded onto
774 96-well black-well plates with an initial density of 1×10^4 cells/well with six tests per group. Following 24-

775 hour culture and starvation with serum-free medium for 2 hours, cells were then treated with serially diluted
776 doses of cisplatin and paclitaxel as specified. The viability/cytotoxicity of cells was examined using the
777 Alamar Blue assay. (A, B) MDA-MB-231 cell lines treated with paclitaxel for 24 and 48 hours, respectively.
778 (C, D) BT549 cell lines treated with paclitaxel for 24 and 48 hours, respectively. (A, B) MDA-MB-231 cell
779 lines treated with cisplatin for 24 and 48 hours, respectively. (C, D) BT549 cell lines treated with cisplatin
780 for 24 and 48 hours, respectively. The comparison of DHX36 shRNA and Scr control was performed using
781 repeated-measures ANOVA. *P<0.05, **P<0.01.

782 **Figure 5.** DHX36 knockdown promotes tumor growth in a xenograft mouse model. (A, B) Representative
783 in-vivo fluorescence images of the breast tumours developed from the mice injected with MDA-MB-231
784 with Scr control (left) and DHX36 shRNA (right). (C) Tumour size estimated using the *in-vivo* images. (D)
785 Integrated fluorescence density of the tumours based on the in-vivo images. (E) Dynamics of the average
786 tumour volume since the injection of tumor cells. (F) Dynamics of the bodyweight of the mice since
787 injection. (G) The end-point tumours dissected from individual mice (Scr: n=9; DHX36 shRNA: n=10). H,
788 The end-point tumour weight. Quantitative data are presented as mean± SEM. *p < 0.05, **p < 0.01, ***
789 < 0.001.

790 **Figure 6.** RNA-Seq analysis of the stable breast cancer cells after DHX36 knockdown using shRNA. (A,
791 B) MA plot indicating the frequency of the differential gene expression after the stable DHX36 knockdown
792 in BT549 (left) and MDA-MB-231 (right) cells. (C) Heatmap of the differential gene expression profile in
793 the two breast cancers with DHX36 shRNA against their Scr control (N=3). (D) Cellular functions which
794 were identified to be mediated by the genes regulated by DHX36 KD by gene ontology analysis for RNA-
795 seq. The threshold is P<0.05. (E) The predicted upstream regulator of the altered gene profile by the DHX36
796 knockdown. (F) The gene regulation network of the most significantly altered genes by the DHX36
797 knockdown.

798 **Figure 7.** The effect of DHX36 knockdown on signalling pathways of breast cancer cells. (A) The profile
799 of stress- associated kinase proteins indicated by the Kinex antibody microarray. (B) The profile of mitotic
800 checkpoint protein-serine kinases indicated by the Kinex antibody microarray. The change of protein level
801 in the antibody microarray was calculated as %CFC= (Signal_{KD}-Signal_{Scr})/ Signal_{Scr}*100 after global
802 normalization. FACS analysis was conducted to evaluate the endogenous levels of JNK and phosphor-JNK
803 (pJNK) proteins. (C, D) Levels of total JNK protein in the stable BT549 cell lines with Scr control (left)
804 and DHX36 shRNA (right). (E, F) Levels of the phosphorylated JNK protein in the stable BT549 cell lines
805 with Scr control (left) and DHX36 shRNA (right). (G, H) Levels of total JNK protein in the stable MDA-
806 MB-231 cell lines with Scr control (left) and DHX36 shRNA (right). (I, J) Levels of the phosphorylated
807 JNK protein in the stable MDA-MB-231 cell lines with Scr control (left) and DHX36 shRNA (right). (K)
808 Western blotting of the JNK and pJNK proteins in the breast cancer cell lines. (L) Real-time qRT-PCR
809 showing the gene expression level of JNK in the breast cancer lines.

810 **Figure 8.** Schematic illustration of molecular mechanisms underlying the tumor suppression mediated by
811 DHX36 in breast cancer cells.

812 **Supporting Information Fig. S1.** Thumbnail images of the IHC staining of DHX36 in three breast cancer
813 tissue arrays.

814 **Supporting Information Fig. S2.** Comparison of DHX36 gene expression in wild-type breast cancer cell
815 lines determined by qRT-PCR and normalized by GAPDH gene expression (fold = 1).

816 **Supporting Information Fig. S3.** Heatmap images of the Kinex antibody microarray for proteomic
817 analysis in MDA-MB-231 cells. (A) Scr control. (B) DHX36 shRNA.

818 **Supporting Information Fig. S4.** Proliferation of the stable breast cancer cell lines in response to
819 flavopiridol. Cells were seeded onto 96-well black-well plates with an initial density of 1x10⁴ cells/well
820 with six tests per group. Following 24-hour culture and starvation with serum-free medium for 2 hours,
821 cells were then treated with serially diluted doses of flavopiridol for 48 hours. The proliferation of cells was

822 examined using the Alamar Blue assay. (A) MDA-MB-231 cells. (B) BT-549 cells. The Student's t-test
823 was used compare two cell lines for each dose.

824 **Supporting Information Fig. S5.** Effect of DHX36 shRNAs on the invasion capacity of stable cells lines.
825 Transwell invasion assay was performed using the stable cell lines derived from BT549 and MDA-MB-
826 231cells, respectively. Cells invaded through Matrigel-coated membrane inserts (pore size 8 um) were
827 stained with Calcein AM and detached using Cell Dissociation Solution, and read using a fluorescence plate
828 reader. Replication points was shown using jitters. ** P<0.01; ns, no statistic significance. The data
829 indicated that both shRNA1 and sh RNA 2 in the cell lines established from BT549 promoted the tumour
830 cell invasion significantly (p<0.01). In the cell lines established from MDA-MB-231, the knockdown of
831 DHX36 by the two shRNAs also promoted the cell invasion (p<0.01), and the effect of shRNA1 appeared
832 stronger than shRNA 2. This not only suggested that the effect is unlikely the off-target effect of shRNA 2
833 but also confirmed that our finding of the effect of DHX36 on breast cancer cell invasion was reproducible.

834 **Supporting Information Fig. S6.** Basal proliferation of the stable breast cancer cell lines. Cells were
835 seeded at densities of 2500 cells/well and 5000 cells/well in 96-well tissue-culture plates. Proliferation
836 measured at the designated time points was normalised with value at Hour 0. The Student's t-test was used
837 to compare the two cell lines (Scr vs shRNA 2) at each time point. * p<0.05; ** p<0.01; ns, no statistical
838 significance.

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861 **Table 1. The association of the IHC staining intensity of DHX36 and clinical features of breast**
 862 **cancer patients.** DCIS: ductal carcinoma in situ. IDC: invasive ductal carcinoma. ILC: invasive lobular
 863 carcinoma. NOS: not otherwise specified.

Clinical feature	DHX36 intensity				P-value (chi-square test)
	0	1	2	3	
Pathological diagnosis					
Adjacent normal	13	6	5	7	
Cystosarcoma phyllodes	0	2	0	0	
DCIS	0	0	0	1	
Fibroadenoma	0	3	0	0	
Intraductal carcinoma	0	3	1	1	
Intraductal carcinoma (sparse)	0	1	0	0	
Intraductal carcinoma with early infiltrate	0	0	1	0	
IDC	34	82	89	48	
IDC (sparse)	0	0	1	0	
IDC and ILC	0	1	0	0	
IDC with ILC	0	0	3	0	
IDC with micropapillary carcinoma	0	1	6	2	
IDC with mucinous carcinoma	0	0	2	2	
IDC with necrosis	0	0	1	1	
IDC (blank)	0	1	0	0	
IDC (sparse)	1	0	0	0	
ILC	55	21	4	0	
ILC (blank)	2	0	0	0	
Mucinous carcinoma	0	0	2	0	
Normal breast tissue	1	0	0	0	
Normal breast tissue (fibrous tissue)	2	0	0	0	1.582E-15
Stage					
1	7	7	20	6	
2	66	77	57	37	
3	19	23	29	11	0.03446
HER2 intensity					
Unknown	9	1	1	5	
0	79	30	15	12	
1	3	52	6	2	
2	3	11	4	0	
3	11	17	3	2	2.016E-15
ER intensity					
Unknown	9	2	2	5	
0	19	61	16	6	
1	16	11	4	2	
2	17	14	1	2	
3	44	22	6	6	1.544E-06
PR intensity					
Unknown	8	2	3	4	
0	30	75	15	8	
1	22	10	2	2	
2	17	12	3	1	
3	28	11	6	6	6.486E-06
Pathology					
DCIS	1	0	4	2	
IDC	104	110	3	60	
ILC	1	2	78	0	
Normal	0	9	25	0	
NOS	2	0	5	0	2.20E-16