Loss of the nuclear pool of ubiquitin ligase CHIP/STUB1 in breast cancer unleashes the MZF1-cathepsin pro-oncogenic program

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Abstract:

CHIP/STUB1 ubiquitin ligase is a negative co-chaperone for HSP90/HSC70, and its expression is reduced or lost in several cancers, including breast cancer. Using an extensive and well-annotated breast cancer tissue collection, we identified the loss of nuclear but not cytoplasmic CHIP to predict more aggressive tumorigenesis and shorter patient survival, with loss of CHIP in two-thirds of ErbB2+ and triple-negative breast cancers and in one-third of ER+ breast cancers. Reduced CHIP expression was seen in breast cancer patient-derived xenograft tumors and in ErbB2+ and triple-negative breast cancer cell lines. Ectopic CHIP expression in ErbB2+ lines suppressed in vitro oncogenic traits and in vivo xenograft tumor growth. An unbiased screen for CHIP-regulated nuclear transcription factors identified many candidates whose DNA-binding activity was up- or down-regulated by CHIP. We characterized Myeloid Zinc Finger 1 (MZF1) as a CHIP target given its recently identified role as a positive regulator of cathepsin B/L (CTSB/L)-mediated tumor cell invasion downstream of ErbB2. We show that CHIP negatively regulates CTSB/L expression in ErbB2+ and other breast cancer cell lines. CTSB inhibition abrogates invasion and matrix degradation in vitro and halts ErbB2+ breast cancer cell line xenograft growth. We conclude that loss of CHIP remodels the cellular transcriptome to unleash critical pro-oncogenic pathways, such as the matrix-degrading enzymes of the cathepsin family, whose components can provide new therapeutic opportunities in breast and other cancers with loss of CHIP expression.

Introduction

The ubiquitin-proteasome system (UPS) plays diverse roles in normal cellular homeostasis. Ubiquitination involves two ubiquitin-activating (E1) enzymes, a small group of ubiquitin-conjugating (E2) enzymes and a large repertoire of ubiquitin ligase (E3) enzymes. E3s dictate substrate specificity and comprise two broad groups: the human papilloma virus E6-interacting (HECT) domain and the Really Interesting New Gene (RING) finger domain protein families (1). Consistent with roles of UPS in oncogenesis, certain cancers, e.g., multiple myeloma, are clinically treated with proteasome inhibitors (1). Recent focus has shifted to substrate-specific elements of the UPS, such as E3s, with MDM2 inhibitors currently in clinical trials for several malignancies (www.clinicaltrials.gov). Notably, mutations of E3s, such as CBL (2) or FBW7 (3) convert them into oncogenes.

The C-terminus of HSC70-Interacting Protein (CHIP)/STIP1 homology and U-Box containing protein 1 (STUB1) is a U-box subfamily RING finger E3 that functions as a negative co-chaperone for the HSP90/HSC70 chaperone to regulate protein quality control by targeting unfolded or misfolded proteins for proteasomal degradation (4-9). CHIP also targets many mature proteins for ubiquitination and proteasomal degradation or degradation-independent regulation (10).

CHIP targeting of oncogenic drivers/co-drivers, such as ErbB2, has prompted analyses of CHIP expression in human tumors. Analysis of matched normal and tumor tissues from 27 breast cancer patients revealed progressive loss of CHIP mRNA expression with tumor progression; CHIP knockdown in CHIP-high MCF-7 (ER+) cells increased and CHIP overexpression in CHIP-low MDA-MB231 (triple-negative; TN) cells suppressed oncogenic traits in vitro and xenograft tumor growth and metastasis in vivo (11). Another study of 33 normal and 127 breast tumor samples also showed an inverse relation of CHIP mRNA levels with increasing grade and Nottingham Prognostic Index

(12). A third study of 183 patients found an inverse correlation between ErbB2 and CHIP protein levels, with reduced CHIP expression associated with tumor progression (13). Thus, CHIP appears to function as a suppressor of breast cancer tumorigenesis. However, the extent of loss of CHIP expression in breast cancer subtypes is unknown.

Mechanistically, CHIP can target cell surface, cytoplasmic, nuclear or secreted proteins for ubiquitin-dependent degradation (10). Relevant to breast cancer, CHIP targets ERRB2 (14-16), SRC-3 transcriptional coactivator (11), macrophage inhibitory factor (17), protein kinase 6/breast tumor kinase (18) and actin regulatory protein profilin-1 (19). However, how loss of CHIP promotes oncogenesis remains unclear.

Here, we assessed both nuclear and cytoplasmic CHIP expression in a well-annotated cohort of >900 breast cancer specimens. We show that a majority of ERBB2+ and TN, and a minority (but sizeable proportion of overall cases) of ER+ breast cancers show loss of CHIP expression, and that loss of the nuclear and not the cytoplasmic CHIP expression predicts features of tumor progression and invasion and shorter patient survival. Loss of CHIP expression in ERBB2+ breast tumor cell lines unleashed a program of increased tumor cell invasion and migration in vitro and tumorigenesis in vivo, as reported with a TN cell line model (11). Given the importance of the loss of nuclear CHIP, we performed an unbiased screen of the impact of low vs. high CHIP levels on DNA-binding activities of cellular transcription factors in ERBB2+ and TN breast cancer cell lines and identified many shared CHIP-regulated transcription factors. Here, we focus on CHIP regulation of MZF1 as it has been identified as a key mediator of pro-invasive signaling downstream of ERBB2 through upregulation of the expression of matrix degrading cathepsin B and L enzymes (20). We show the CHIP dependence of this pathway in cell lines representing various breast cancer subtypes and demonstrate that chemical targeting of CTSB inhibits the ERBB2-driven tumorigenesis. We conclude

that loss of CHIP expression unleashes a MZF1-dependent and CTSB/L-mediated pro-oncogenic pathway in breast cancer.

Materials and Methods

Detailed methods are presented in Supplementary Methods.

Cell culture: Culture of HEK-293T cells, ErbB2-overexpressing breast cancer cell lines SKBR3 and BT474 (ATCC) and 21MT1 (21), ER/PR+ MCF7 (ATCC) and TN MDA-MB231 (ATCC) breast cancer cell lines, and their lentiviral infection to derive CHIP overexpressing or CHIP shRNA expressing lines are described in Supplementary Methods.

Antibodies and Reagents: Rabbit anti-CHIP antibody used for blotting has been described (15). Other primary antibodies or secondary conjugates for fluorescence or immunoblotting analyses are described under Supplementary Methods.

Cell lysis, immunoprecipitation and immunoblotting: Cells lysates were prepared in RIPA or Triton X-100 lysis buffers (as indicated in figure legends) and used for immunoprecipitation and immunoblotting analyses as described in Supplementary Methods.

Quantitative real-time PCR: Total RNA was extracted using TRIzol reagent (Invitrogen), reverse transcribed using Quantitative real-time PCR kit (cat. 204141, Qiagen, Germantown, MD) and used for real-time QPCR with primers described in Supplementary Methods.

Cell growth assays: Cell growth was analyzed as cumulative proliferation over 5 serial weekly passages and by soft agar anchorage-independent growth assay as described under Supplementary Methods.

Transwell migration and invasion assays: The migration or invasion of cells was analyzed using uncoated or Matrigel-coated Transwell chambers as described under Supplementary Methods.

Extracellular matrix (ECM) degradation assay: This assay was carried out using QCM™ Gelatin Invadopodia kit (Cat. ECM670, EMD Millipore, Billerica, MA) according to the manufacturer's protocol, as described in Supplementary Methods. ECM degradation, seen as focal loss of fluorescent signal ("holes") in the labeled gelatin layer, was quantified using Image J (NIH).

Xenograft tumorigenesis: 10^6 cells in Matrigel (BD Biosciences) were implanted in the mammary fat pad of female NSG mice (The Jackson Laboratory) primed with s/c estrogen pellet (0.72 mg/60-day pellets; Innovative Research of America, Sarasota, FL) and tumor growth monitored weekly for 10 weeks. After euthanasia, tumors were imaged, and formalin-fixed and paraffin-embedded for further analyses. Details are presented in Supplementary Methods.

Protein/DNA array: A protein/DNA combo-array kit (Cat. MA1215, Affymetrix, Santa Clara, CA) was used to simultaneously screen 345 transcription factors for DNA-binding activity per vendor protocol with signals detected using chemiluminescence (See Supplementary Methods).

Electrophoretic mobility shift assay (EMSA): EMSA was carried out using an EMSA kit (cat. 20148, Life Technologies, Waltham, MA) as described in Supplementary Methods.

Cathepsin B (CTSB)/Cathepsin L (CTSL) activity assay: CTSB/L activity was assayed using the Magic Red CTSB/L Activity Kit (Cat. 937 & 941, Immunochemistry Technologies, Bloomington, MN) according to the manufacturer's protocol (See Supplementary Methods). Experiments were run in triplicates and repeated thrice. Ten

random fields per well were imaged. Fluorescence intensity was quantified using Image J (NIH).

GST fusion protein pulldown analyses: Cell lysate pulldown analyses of CHIP-MZF1 interactions using GST-CHIP, GST-CHIP-H260Q/P269A mutant, GST-ΔU-box-CHIP (U-box domain deleted mutant) and GST-ΔTPR-CHIP (TPR domain deleted mutant CHIP) fusion proteins were done as described previously (15) (See Supplementary Methods.

Human and animal subjects: The use of human tissues was approved by Nottingham Research Ethics Committee 2 under the title 'Development of a molecular genetic classification of breast cancer', and in compliance with current ethical and legal guidelines of the United Kingdom. All experiments in this study were conducted in accordance with the 1975 Helsinki declaration and its later amendments or comparable ethical standards. There was written consent obtained from patients at the time of collection of the material. The use of human tissues was approved by the Ethics Committee of the University of Nottingham. Mouse studies were pre-approved by the UNMC Institutional Review Board (IRB) Committee, in compliance with Federal and State guidelines.

IHC analysis of breast cancer tissue microarrays: Tissue microarrays (TMAs) corresponding to a well-annotated 971 breast cancer patient cohort at the University of Nottingham Hospital Breast Unit and a commercial TMA (BR20837, from US Biomax, INC.) were analyzed by IHC staining (see Supplementary Methods for details) with a previously described (22) anti-rabbit CHIP antibody that was further validated (Supplementary Figure 1).

Results

Reduced nuclear CHIP expression in breast cancer correlates with tumor progression, invasion and poorer patient survival

As previous small studies (11-13) have not clarified which breast cancer subtypes lose CHIP expression, we performed IHC analysis of formalin-fixed and paraffin-embedded tissue microarrays (TMAs) from a well-annotated cohort of 971 breast cancer patients (Supplementary Table 1) (23-27) using an established rabbit anti-CHIP antibody (15) further validated in western blotting with lysates of CHIP-depleted or ectopic CHIP-expressing cell lines (Supplementary Figure S1). Initial IHC of a commercial breast cancer TMA (208 samples) detected both cytoplasmic and nuclear CHIP within tumor cells and in surrounding normal epithelium (Figure 1A). In the 971-sample TMA, 423 and 314 samples, respectively, were evaluable for cytoplasmic and nuclear staining, with multiple staining patterns: low cytoplasmic/low nuclear, high cytoplasmic/low nuclear, low cytoplasmic/high nuclear; or high cytoplasmic/high nuclear (Figure 1B; Supplementary Table1). Loss of nuclear CHIP staining showed a significant positive correlation with biochemical markers of tumor progression and metastasis, reduced ER and PR staining, altered cytokeratins (CK18, CK19), increased expression of early epithelial-mesenchymal transition markers (N-cadherin, P-cadherin) and expression of EGFR family proteins (EGFR, ErbB2, ErbB4) (Supplementary Table 2). Only CK18 ($p=0.001$) and ErbB2 ($p=0.016$) expression correlated with reduced cytoplasmic CHIP staining (Supplementary Table 2). Lower nuclear but not cytoplasmic CHIP staining showed a significant positive correlation with clinical-pathological features of tumor progression (higher tumor size, tumor grade, pleomorphism and mitotic status) (Supplementary Table 1). Kaplan-Meier analysis revealed that low nuclear but not the cytoplasmic staining pattern correlated significantly with poorer breast cancer-specific survival (Nuclear CHIP, $p=0.003$ Vs. cytoplasmic CHIP, $p=0.469$; Figure 1C). Using the

previously-established ER, PR and ErbB2/Her2 expression status of the TMA samples (24-28), low CHIP expression was seen in two-thirds of ErbB2+ and TN cases and about one-third of ER+ cases.

CHIP suppresses tumorigenic traits in ErbB2+ breast cancer cell lines

Validating IHC results, most ErbB2+ and TN breast cancer cell lines showed lower CHIP mRNA and protein levels compared with ER+ lines (Supplementary Figure S2A and S2B); the ER+ and TN cell line results confirm a previous report (11). Western blotting of 12 breast cancer patient-derived xenograft (28) lysates confirmed the lower or absent CHIP expression in TN, ErbB2+ and some ER+ breast cancers (Supplementary Figure S2C). The CHIP expression status in the commercial TMA was consistent with these results (Supplementary Figure S2D).

To gain mechanistic insights into how the loss of nuclear CHIP promotes oncogenic progression, we utilized ErbB2+ breast cancer cell lines (BT474, SK-BR3 and 21-MT1) as primary models, confirming key findings in TN (MDA-MB231) and ER+ (MCF-7) lines. We engineered the ErbB2+ and TN lines to stably overexpress Myc-CHIP (CHIP-hi lines) (or vector control; CHIP-lo lines) and MCF-7 cells with CHIP shRNA (CHIP-lo) to deplete the endogenous CHIP expression (or a control shRNA; CHIP-hi) (Supplementary Figure S1A). Of note, Myc-CHIP is seen as multiple bands, some migrating considerably slowly while others co-migrating with endogenous CHIP; the authenticity of these bands as Myc-CHIP is demonstrated by anti-Myc immunoblotting (Supplementary. Figure S1C). As CHIP expression in MDA-MB-231 cells is known to suppress oncogenic attributes (11), these served as controls.

While no significant differences in cell proliferation were observed over a single passage, confirming previous MDA-MB231 results (11), cumulative cell proliferation over multiple passages was modestly but significantly lower in CHIP-hi vs. control cells

(Figure 2A). CHIP-high cells exhibited a significantly lower number of colonies in soft (Figure 2B), significantly reduced Transwell migration (Figure 2C), and significantly reduced invasion through Matrigel (Figure 2D) compared to CHIP-lo control cells.

Analysis of CHIP-lo vs. CHIP-hi MDA-MB231 cells, as controls, confirmed the expected reduction in primary tumor growth and lung metastasis upon CHIP overexpression (11) (Supplementary Figure S3A, S3B). Notably, the CHIP-hi BT474 cells formed significantly smaller xenograft tumors compared to those with CHIP-lo cells (Figure 3A and 3B). Exogenous CHIP overexpression in tumors was confirmed by western blotting (Figure 3C). Histologically, the CHIP-hi BT474 tumors showed lower nuclear atypia and mitotic index (Figure 3D and 3E), and reduced Ki67+ proliferating cells (Figure 3F and 3G) compared to CHIP-lo BT474 tumors. No significant difference in immunostaining for the apoptotic marker cleaved-caspase 3 was noted (Figure 3F and 3H), suggesting a primarily cytostatic impact of CHIP overexpression. These studies support the tumor suppressor role of CHIP in ErbB2+ breast cancer as suggested by our clinical-pathological analyses.

Identification of nuclear targets of CHIP in breast cancer

As loss of nuclear but not cytoplasmic CHIP expression predicted tumor progression and poor patient outcomes, we assessed the cognate DNA-binding activities of 345 cellular transcription factors in nuclear extracts of control vs. CHIP-hi ErbB2+ BT474 cells, using a commercially-available array platform. The DNA-binding activity in CHIP-hi over CHIP-lo extracts was expressed as a fold ratio. Subsets of transcription factors showed changes in DNA-binding activities outside of the arbitrary 3-fold cut-off, representing potential direct or indirect targets of negative or positive regulation by CHIP (Figure 4A, left). These (Supplementary Table 3) included known CHIP targets, such as p53 and NFkB (29, 30), validating the approach. Analysis of CHIP-hi vs. CHIP-lo

(control) MDA-MB231 cells (Figure 4A, bottom) showed that most CHIP targets were shared with the BT474 cell model (see Supplementary Table 4) (Fig 4A), although the extent of changes differed. Immunoblot analysis of a sample of known and novel transcription factors identified as CHIP targets (NFκB p69/p60, Stat3, FOXM1, MZF1) confirmed their downregulation in CHIP-hi vs. CHIP-lo BT474 and MDA-MB231 cell lines (Fig. 4B, 4C). Thus, our unbiased screen identified novel CHIP-targeted transcription factors whose deregulated function upon loss of CHIP expression may contribute to tumor progression.

MZF1 is a direct target of CHIP-mediated ubiquitination and degradation

We focused further on myeloid zinc finger 1 (MZF1), whose DNA-binding activity was down-regulated in CHIP-hi BT474 and MDA-MB231 cells (Figure 4A; MZF1 highlighted in white), as it was recently identified as a nexus of ErbB2 signaling that culminates in tumor cell invasiveness through increased transcription of matrix-degrading enzymes cathepsin B (CTSB) and L (CTSL) (20). Electrophoresis mobility shift assay (EMSA) using sequences corresponding to MZF1 binding sites on the CD34 gene promoter (31) confirmed reduced binding activity (shifted bands) in CHIP-hi vs. CHIP-lo BT474 cells (Figure 4D). The MZF1 mRNA level was reduced in CHIP-hi vs. CHIP-lo cells BT474 cells (Figure 4E), suggesting the regulation of upstream modulators of MZF1 transcription as part of CHIP-dependent reduction in DNA-binding activity. MZF1 protein level was reduced in CHIP-hi vs. CHIP-lo cells, and anti-ubiquitin blotting of MZF1 immunoprecipitations showed increased MZF1 ubiquitination in CHIP-hi vs CHIP-lo BT474 cells (Figure 4F). Co-transfection of CHIP and MZF1 in HEK-293T cells revealed CHIP dose-dependent MZF1 ubiquitination (Figure 4G, upper) and reduction in protein level (Figure 4G, bottom). Notably, while ErbB2 levels in CHIP plus ErbB2 co-transfected HEK-293T cells increased upon treatment with MG132 (proteasomal

inhibitor) (Supplementary Figure S4D, S4E), as expected (14, 15), MG132 treatment failed to rescue the MZF1 levels in CHIP co-transfected cells (Figure 4H, J), suggesting the possibility that MZF1 degradation occurs through chaperone-mediated autophagy in lysosomes as demonstrated for CHIP targets such as HIF-1α (32). Indeed, CHIP-induced reduction in MZF1 levels was partially rescued by a short treatment of cells with the lysosomal inhibitor bafilomycin A1 (Figure 4I, K). Finally, depletion of endogenous CHIP by shRNA knockdown in MCF-7 cells led to upregulation of MZF1 and CTSB/L protein levels (Supplementary Figure S4B). These results support the conclusion that MZF1 is a bona-fide target of CHIP, involving indirect transcriptional downregulation and CHIP-dependent ubiquitination and lysosomal degradation of MZF1.

The Tetratricopetide repeat (TPR) domain of CHIP is required for interaction with MZF1

The TPR domain of CHIP has been shown to mediate binding to most interacting partners such as HSC70 and HSP90 (4, 15). Given the CHIP-MZF1 co-immunoprecipitation (Figure 4G), we used GST fusion proteins of intact CHIP or its point or deletion mutants for pulldown of MZF1 from lysates of HEK-293T cells transiently transfected with MZF1 to map the CHIP domain that mediates its interaction with MZF1. Compared to the negative control GST, GST-CHIP robustly pulled down MZF1 from cell lysates (Figure 4L). Notably, the U-box point mutant (GST-CHIP-H260Q/P269A) and the U-box domain deleted CHIP mutant (GST-ΔU box-CHIP) showed unimpaired MZF1 pulldown, while no pulldown was seen with the TPR domain deleted CHIP (GST-ΔTPR-CHIP) (Figure 4L). These results establish that CHIP binds to MZF1 through its TPR domain.

CHIP is a negative regulator of MZF1-dependent and Cathepsin B/L-mediated ECM degradation

CTSB/L are key regulators of tumor invasion, angiogenesis and metastasis (33), and their MZF1-dependent transcriptional upregulation was shown to mediate in vitro tumor cell invasion upon ErbB2 overexpression in breast cancer cells (20). We therefore asked if CHIP regulation of MZF1 translates into control of CTSB/L expression. EMSA with a DNA probe from a known MZF1-binding site in the CTSB promoter (31) confirmed reduced DNA-binding activity in the nuclear extracts of CHIP-hi vs. control ErbB2+ cells (Figure 5A), with reduced CTSB and CTSL mRNA and protein levels (Figure 5B, 5D, and 5E), reduced CTSB/L enzymatic activities (Figure 5G and 5I) measured with a fluorescent substrate (34), and reduced FITC-labeled gelatin degradation (35, 36) (Figure 6A and 6C). Thus, the MZF1/CTSB/L pro-invasion signaling axis is negatively regulated by CHIP in ErbB2+ breast cancer cells.

Consistent with CHIP-dependent reduction in the DNA-binding activity of MZF1 in CHIP-hi vs. CHIP-lo MDA-MB231 cells (Figure 4A), reduced mRNA and protein levels of CTSB/L (Figure 5C and 5F), reduced CTSB/L activity (Figure 5H and 5J) and reduced florescent gelatin degradation (Figure 6B and 6D) were observed in CHIP-hi vs. CHIP-lo MDA-MB231 cells. Increased CTSB/L expression was also seen in CHIP KD vs. control MCF7 cells (Supplementary Figure S4B). Ectopic overexpression of MZF1 in CHIP-hi as well as CHIP-lo MDA-MB231 cells increased the florescent gelatin degradation (Figure 6 E and F). Finally, treatment of CHIP-lo 21MT1 cells (high CTSB/L activity; Figure 6A and 6C) with CA074, a specific inhibitor of CTSB (37), abrogated the fluorescent gelatin degradation, and markedly inhibited the Transwell invasion of 21MT1 (Figure 7A-C) and MDA-MB-231 (7D-F) breast cancer cell lines. Thus, we conclude that loss of CHIP expression across breast cancer subtypes upregulates the pro-invasive MZF1-CTSB/L signaling axis.

To assess if the upregulation of CTSB expression and activity as a result of CHIP downregulation contributes to oncogenesis in vivo, we grew BT474 xenograft tumors to an average of 0.5 mm^3 and treated the mice with CA074 (25 mg/Kg body weight, i.p.), Trastuzumab (4 mg/Kg, tail vein injection) as a standard ErbB2-targeted therapeutic or the vehicle control, based on previously used dosages (37-39). CA074 treatment led to a marked and statistically-significant inhibition of tumor growth at multiple time points, comparable to that seen with Trastuzumab (Figure 7G), supporting the conclusion that one mechanism by which loss of CHIP promotes breast tumor progression is by eliminating the negative regulation of MZF1 and unleashing the cathepsin expression.

Discussion

Loss of expression of the HSP90/HSC70 co-chaperone CHIP E3 has emerged as a mechanism to promote tumor progression. Here, we demonstrate that loss of nuclear, and not cytoplasmic, CHIP expression is associated with tumor progression and shorter survival in breast cancer patients and is a feature of about two-thirds of ErbB2+ and TN as well as a third of ER+ breast cancers. Importantly, we identify loss of CHIP as a key mechanism to alter the DNA-binding activities of a substantial subset of cellular transcription factors and establish that loss of CHIP expression unleashes a pro-invasion signaling cascade in which the CHIP target MZF1 promotes cathepsin B and L expression. Using a chemical inhibitor strategy, we show that upregulation of MZF1-CTSB axis due to loss of CHIP expression contributes to tumorigenesis.

IHC analyses of an extensive collection of well-annotated breast cancer TMAs demonstrated that loss of nuclear CHIP signals correlates with markers of advanced tumor progression, including higher tumor grade, mitotic index and markers of early EMT (Supplementary Table 1), and significantly shorter breast cancer-specific and progression-free survival (Figure 1C). Loss of nuclear CHIP was a feature of nearly two-

thirds of ErB2+ and TN subtypes, consistent with their poorer intrinsic patient outcomes and higher metastatic odds (40, 41), and about a third of ER+ breast cancers (Supplementary Table 2). In contrast, loss of cytoplasmic CHIP expression was only associated with ErbB2 positivity and did not predict poor patient survival (Figure 1C). These results materially extend previous findings using smaller patient cohorts (11-13), and highlight the subtype preference of CHIP loss and the importance of the loss of nuclear CHIP as a pro-oncogenic adaptation in breast cancer. Of note, the CHIP-low ER+ patients numerically exceed the CHIP-lo ErbB2+ and TN patients, and further studies are needed to assess if these patients belong to a specific molecular sub-class. Based on previous publications (11) and our results on cell lines (Supplementary Figure S2D), reduced CHIP mRNA expression provides one mechanism for reduced CHIP levels in tumors. Why nuclear CHIP levels maybe selectively reduced in certain primary tumors is not clear at present. Mechanisms such as aberrant nuclear/cytoplasmic transport of CHIP or selective degradation of CHIP within the nucleus or cytoplasm will need to be explored as a potential basis for our new findings.

Analyses in breast cancer cell lines confirmed the predominant loss of CHIP expression in the ErbB2+ and TN subtypes. Using matched pairs of ErbB2+ breast cancer cell lines with low endogenous CHIP expression vs. their CHIP-hi derivatives in functional assays, we show that CHIP levels are a key determinant of ErbB2-driven cell growth and invasiveness in vitro and xenograft tumor growth in nude mice, extending previous findings using CHIP-reconstituted MDA-MB231 (TN) and CHIP-depleted MCF-7 (ER+) lines (11), which served as controls. While we confirmed the lack of impact of CHIP restoration on cell proliferation in a single passage (11), analyses over multiple passages revealed a subtle but significant proliferative disadvantage (Figure 2A), which is profound under anchorage-independent conditions (Figure 2B). Thus, CHIP serves as a tumor suppressor for ErbB2+, TN and a proportion of ER+ breast cancers.

Furthering our novel findings that loss of nuclear and not cytoplasmic CHIP correlates with tumor progression, an unbiased protein/DNA array screen identified a number of transcription factors whose DNA-binding activities are directly or indirectly up- or down-regulated by CHIP (Figure 4A), vastly expanding the list of potential targets of CHIP in the context of cancer (29, 30). Here, we have focused on MZF1, whose activity was downregulated by CHIP, since it was recently identified as a major transcriptional activator of CTSB//L-mediated tumor cell invasion downstream of ErbB2 in breast cancer cells (42). CTSB/L are overexpressed in primary breast cancers and CTSB or MZF1 knockdown abrogated breast cancer cell invasiveness in vitro (41). Our EMSA analyses confirmed the reduced MZF1 DNA-binding activity in CHIP-hi breast cancer cells (Figure 4D), and we show that MZF1 is both a direct target of CHIP for ubiquitination and destabilization (Figure 4F and 4G), and also indirectly regulated at the mRNA level, potentially due to CHIP regulation of upstream regulators of MZF1 expression such as MYC (43) (Figure 4A). Regarding indirect transcriptional regulation of MZF1 by CHIP, several transcription factors targeted by CHIP, including STAT 1/3 and ATF (Figure 4 A-C; and Supplementary Table 3 and 4), have binding sites within the MZF1 promoter region (data not shown) and their downregulation by CHIP could contribute to reduced MZF1 expression.

We show that MZF1 and CHIP associate with each other (Fig. 4G) and used GST pulldown assays to identify the TPR domain of CHIP to mediate interaction with MZF1 (Fig. 4L). Notably, our analyses using proteasomal (MG132) and lysosomal (Bafilomycin A) inhibitors show that CHIP-dependent destabilization of MZF1 protein primarily occurs in the lysosome (Fig 4I). It is likely that the lysosomal degradation of MZF1 reflects CHIP-dependent chaperone-mediated autophagy as demonstrated for HIF1a and other targets (32). In this regard, MZF1 possesses several sequences QSFRQ and KAFRQ (sequences that identified 447-451, 548-552, 576-580, 660-664,

688-692 amino acids) homologous to the motif KFERQ found in other chaperone-mediated autophagy targets (32). Since intermediary kinases (CDC42BPβ, ERK2, PAK4, PKCα) were implicated in MZF1-mediated CTSB/L transcription downstream of ErbB2 (41), negative regulation of these kinases or ErbB2 (14, 15) may also contribute to CHIP regulation of MZF1.

Our analyses support a key role of loss of CHIP as a mechanism to unleash the MZF1-dependent transcriptional network that controls CTSB/L expression (Figure 5) and extracellular matrix (ECM) degradation (Figure 6), and to promote oncogenesis (Figure 2). CTSB is a well-established downstream mediator of invasive/metastatic signaling in various cancers (44, 45), including breast cancer (20, 36). Specific CTSB inhibition with CA074 reduced the matrix degradation and cell invasion in vitro and tumor growth in vivo (Figure 7), providing further support for loss of CHIP expression as a key breast cancer adaptation to unleash the pro-oncogenic MZF1-CTSB pathway. The strong effect of CTSB inhibition alone on matrix degradation and xenograft tumor growth, the latter comparable to Trastuzumab (Figure 7D), suggests that CTSB may be the dominant player in BT474 cells, consistent with strong reported impact of CTSB depletion on in vitro invasiveness of ErbB2+ breast cancer cell lines (42). However, further studies to assess the contribution of CTSL and other potential targets of MZF1 are warranted. Our in vitro results in TN and ER+ cell lines (Figure 4) also support the unleashing of MZF1-CTSB/L pathway in the corresponding breast cancer subtypes, and further studies in these and other tumors with loss of CHIP expression (10) will be of interest. Of note, TCGA breast cancer data analysis (eBioportal) did not, however, reveal a positive correlation between CTSB/L and MZF1 mRNA expression (in fact we noted moderate negative Pearson correlation coefficients). We surmise that MZF1 and/or CTSB/L expressed in myeloid lineage and potentially other stromal components may contribute to this discrepancy. Transcriptomic analysis of laser capture micro-dissected tumor

samples or multi-color IHC with lineage markers will be necessary to further assess the relationship between MZF1 and CTSB/L expression in relation to CHIP/STUB1 expression in primary tumors.

In conclusion, our studies demonstrate that upregulation of MZF1/CTSB/L axis is an important pro-oncogenic mechanism unleashed by loss of CHIP expression in breast cancer. Given the established roles of cathepsins in matrix remodeling, invasion, angiogenesis and metastasis (44, 45), future studies to establish the role and targeting of MZF1-CTSB/L axis in metastatic tumor settings may provide new therapeutic opportunities for breast and other tumors with loss of CHIP expression (30, 46-49).

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Figure Legends

Figure 1. Reduced nuclear CHIP staining in primary breast cancer tissue microarray (TMA) specimens. (A) Anti-CHIP IHC staining of a representative breast cancer specimen (top) vs. normal breast tissue (bottom) to show cytoplasmic and nuclear staining. (B) Representative pictures depicting low or high nuclear vs. cytoplasmic CHIP staining

patterns in breast cancer TMAs. (C) Kaplan-Meier analysis correlating breast cancer-specific survival (BCSS) with moderate/high vs. negative/low nuclear (left) or cytoplasmic (right) CHIP staining in TMA samples. Number of patients in each group are shown in parentheses next to captions. P values are indicated inside each box.

Figure 2. Suppression of in vitro oncogenic attributes upon CHIP overexpression in CHIP-lo ErbB2+ breast cancer cell lines. (A) Cumulative proliferation of CHIP-lo control (MSCV-puro) vs. CHIP-hi (MSCV-CHIP) 21MT1, BT474 and SKBR3 cell lines. Data points represent cumulative cell numbers with each serial passage. (B) Anchorage-independent cell growth in soft agar after 3 weeks of culture. Y-axis, number of colonies per 2500 seeded cells. (C) Transwell cell migration assay. 2000 cells were added in top chamber and migration scored after 24 hrs. Y-axis, number of migrated cells per high power field (HPF). (D) Matrigel-coated Transwell invasion assay. 2000 cells were added in top chamber and migration scored after 24 hrs. Y-axis, number of migrated cells per HPF. Data in each figure represent mean +/- SEM of 3 experiments, each in triplicates.

Figure 3. Reduced xenograft growth of CHIP-overexpressing BT474 cells in nude mice. (A) Tumor volume of CHIP-lo (MSCV-puro) control and CHIP-hi (MSCV-CHIP) BT474 xenografts over time. Mean+/- SEM, N = 6 per group., * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. (B) Photograph of resected CHIP-lo (upper) and CHIP-hi (lower) BT474 cell xenografts. (C) Western blotting to visualize the endogenous and overexpressed (Myc-tagged) CHIP in resected BT474 xenografts; HSC70, loading control. (D) Representative H&E staining and mitotic events (red circles) of CHIP-lo (left) and CHIP-hi (right) BT474 xenograft tumor sections. (E) Quantification of mitotic events of sections depicted in D. Mean +/- SEM, n=3. (F) Ki67 (brown) and cleaved caspase 3 (CC3; red) co-staining of

CHIP-lo (left) and CHIP-hi (right) BT474 xenograft tumor sections. (G, H) Quantification of Ki67 (G) and CC3 (H) positive cells depicted in F. Mean +/- SD, n=4.

Figure 4. Identification of MZF1 as a CHIP-regulated transcription factor in breast cancer cells. (A) DNA-binding activities of 345 transcription factors were analyzed in nuclear extracts of CHIP-lo (MSCV-puro control) vs. CHIP-hi (MSCV-CHIP) BT474 and MDA-MB-231 cell lines. Y-axis, $\log_e$-fold binding in CHIP-hi over CHIP-lo cells. The 3-fold increase (upper) or decrease (lower) in binding (dotted lines) was used as cut-off. MZF1 (Open symbol). (B, C) Validation of CHIP-dependent downregulation of protein levels of selected transcription factors identified in A by western blotting of CHIP-lo vs. CHIP-hi BT474 (B) or MDA-MB-231 (C) cell lysates. (D) Real-time qPCR analysis of MZF1 mRNA levels in CHIP-hi vs. CHIP-lo BT474 cells. (E) A biotin-labeled double-stranded oligonucleotide with MZF1-specific DNA sequence was used to probe nuclear extracts of CHIP-hi (MSCV-CHIP, C) vs. CHIP-lo (MSCV-puro, P) BT474 cells. 200-fold excess of the non-labeled oligonucleotide served as a competitor. (F) Immunoblot analysis of MZF1 protein levels in CHIP-hi vs. CHIP-lo BT474 cells. (G) CHIP-dependent ubiquitination and degradation of MZF1. Anti-MZF1 immunoprecipitations from lysates of HEK-293T cells transfected with GFP-MZF1 (1μg) +/- Myc-CHIP (1 or 2 μg) were immunoblotted for ubiquitin or MZF1 (upper panel), and whole cell lysates (lower panel) were blotted for MZF1, CHIP or HSC70 (loading control). (H, I) HEK-293T cells were transfected with MZF1-GFP (250 ng) +/- Myc-CHIP (1 μg). Cells were treated with vehicle (-) or proteasomal inhibitor MG132 (50 μM) or lysosomal inhibitor bafilomycin A1 (100 nM) for 6 or 16 hr., respectively. Lysates were immunoblotted for MZF1 or CHIP. (J, K) Quantitation of the MZF1 levels in CHIP-co-transfected cells in the presence of MG132 or Bafilomycin A1, relative to vehicle controls assigned a value of 1. Data shown

represent mean +/- S.D. of n=3; * = p<0.05, ** = p<0.01, *** = p<0.001. (L) The tetratricopeptide repeat (TPR) domain of CHIP is required for binding to MZF1. HEK-293T cells transfected with MZF1-GFP and their lysates used for pulldown with GST or the indicated GST-CHIP fusion proteins. Bound MZF1-GFP was visualized by immunoblotting for GFP (upper panel) or MZF1 (middle panel). Ponceau Red staining of the membrane shows the relative amounts of fusion proteins used in pulldowns (lower panel).

Figure 5. CHIP levels control the expression of MZF1 target genes cathepsins B and L. (A) EMSA analysis with the CTSB promoter sequence in CHIP-hi vs. CHIP-lo BT474 cells was done as in Figure 4B. (B, C) CTSB/L mRNA expression in CHIP-hi vs. CHIP-lo BT474 (B) and MDA-MB-231 (C) cells was analyzed by real-time qPCR; n=3. (D, E) Immunoblotting for CTSB (D) and CTSL (E) protein levels in CHIP-hi vs. CHIP-lo BT474 cells. (F) Immuno-histochemical analysis of MZF1 (left panel), CTSB (middle panel), and CTSL (right panel) in CHIP-hi vs. CHIP-lo MDA-MB-231 cells. (G,H) CTSB/L activities in CHIP-hi vs. CHIP-lo 21MT1 (G) and MDA-MB-231 (H) cells analyzed by the production of a red fluorescent cleavage product. (I,J) Quantification of CTSB (top) and CTSL (lower) activities presented in G and H panels in CHIP-hi vs CHIP-lo 21MT1 (I) and MDA-MB-231 (J) cells. Each square represents one replicate. Mean +/- S.D. shown; * = p<0.05; ** = p<0.01; *** = p<0.005.

Figure 6. The CHIP-MZF1-CTSB axis influences tumor cell invasiveness. (A,B) Degradation of FITC-labeled gelatin matrix (seen as patchy holes in the uniform green matrix) by CHIP-lo vs. CHIP-hi 21MT1 (A) and MDA-MB-231 (B) cells seeded for 48 hours and stained for actin-containing invadopodia with phalloidin (red) and for nuclei with DAPI (blue). (C, D) Quantification of gelatin degradation presented in A (C) and B

(D). Mean +/- SD., n=4. (E) Restoration of FITC-labeled gelatin matrix degradation by MZF-1 overexpression in CHIP-hi MDA-MB-231 cells. Stably MZF-1 overexpressing CHHIP-lo or CHIP-hi MDA-MB231 cells were analyzed as in A and B. (F) Quantification of gelatin degradation shown in E. Each square represents a replicate. Mean +/- SD., shown, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$. (G) Western blotting of MZF1 overexpression in CHIP-lo vs. CHIP-hi MDA-MB-231 cells. (H, n=3) Quantification of MZF1 overexpression shown in G; n=3.

Figure 7. CTSB inhibition reduces ErbB2+ breast cancer cell tumorigenesis. (A) 21MT1 cells cultured on FITC-gelatin were treated with DMSO vehicle or CTSB inhibitor (CA074; 25 μg/ml) for 48h and degradation analyzed as in Figure 5G. (B) Quantification of FITC-gelatin degradation shown in 7A. Mean ± S.D., n=4. (C) 21MT1 cells seeded on Matrigel-coated Transwell invasion membranes were treated with DMSO (control) or CA074 (25 μg/ml) for 24h, and cells that had invaded through Matrigel to the bottom surface were stained with CyQuant GR fluorescent dye and quantified using a fluorescence reader. Mean ± S.D., n=3. (D) FITC-gelatin matrix degradation by MDA-MB-231 cells was analyzed after 48h culture in DMSO (control) or CTSB inhibitor (CA074; 25μg /ml) as in Figure 5G. (E) Quantification of FITC-gelatin degradation shown in 7D. Mean ± S.D., n=4. (F) Invasion of MDA-MB-231 cells incubated with DMSO (control) or CA074 (25 m/ml) as in Fig. 6C. Mean ± S.D., n=8. (G) Groups of 9 nude mice carrying BT474 xenografts (average 0.5 cm^3 size) received Trastuzumab (via tail vein; 4 mg/kg every 4 days), CA074 (i.p., 25 mg/kg in saline daily) or saline (control), and tumor volumes were monitored every other day. Mean +/- SEM., n=9; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.