The WNT10B Network Is Associated with Survival and Metastases in Chemoresistant Triple-Negative Breast Cancer

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Abstract

Triple-negative breast cancer (TNBC) commonly develops resistance to chemotherapy, yet markers predictive of chemoresistance in this disease are lacking. Here, we define WNT10B-dependent biomarkers for β-CATENIN/HMGA2/EZH2 signaling predictive of reduced relapse-free survival. Concordant expression of HMGA2 and EZH2 proteins is observed in MMTV-Wnt10b+/- transgenic mice during metastasis, and Hmga2 haploinsufficiency decreased EZH2 protein expression, repressing lung metastasis. A novel autoregulatory loop interdependent on HMGA2 and EZH2 expression is essential for β-CATENIN/TCF-4/LEF-1 transcription. Mechanistically, both HMGA2 and EZH2 displaced Groucho/TLE1 from TCF-4 and served as gatekeepers for K49 acetylation on β-CATENIN, which is essential for transcription. In addition, we discovered that HMGA2-EZH2 interacts with the PRC2 complex. Absence of HMGA2 or EZH2 expression or chemical inhibition of Wnt signaling in a chemoresistant patient-derived xenograft (PDX) model of TNBC abolished visceral metastasis, repressing AXIN2, MYC, EZH2, and Hmga2 expression in vivo. Combinatorial therapy of a WNT inhibitor with doxorubicin synergistically activated apoptosis in vivo, repressed PDx-derived cells to doxorubicin, and repressed lung metastasis in vivo. We propose that targeting the WNT10B biomarker network will provide improved outcomes for TNBC.

Significance: These findings reveal targeting the WNT signaling pathway as a potential therapeutic strategy in triple-negative breast cancer.

Graphical Abstract: http://cancerres.aacrjournals.org/content/canres/79/5/982/F1.large.jpg.
**Introduction**

Triple-negative breast cancer (TNBC; estrogen receptor-negative (ER−), progesterone receptor-negative (PR−), and HER2-unamplified), including basal-like and mesenchymal subtypes, is an aggressive type of cancer and frequently occurs in BRCA1 mutation carriers and in young women of African descent. African-American (AA) women are ~3 times more likely than European-American (EA) women to be diagnosed with TNBC (1, 2). TNBC adenocarcinomas, which are highly metastatic and have a poor prognosis, are treated with a combination of surgery, radiotherapy, and/or chemotherapy; however, unlike other breast cancer subtypes, there are no FDA-approved targeted agents (3). TNBCs preferentially metastasize to the brain or the lung (70%), rather than bone or liver (30%; ref. 4), and the process regulating tropism is poorly understood.

WNT/ß-catenin canonical signaling activates coreceptors (i.e., LRP5/6/FZD), leading to the stabilization of ß-catenin. Stabilized ß-catenin translocates to the nucleus, interacts with TCF/LEF, and induces specific cellular response transcriptional programs including, but not limited to, cellular proliferation, development, differentiation, neoplasia, and stem cell maintenance (5). We previously showed that WNT10b expression is elevated in the majority of TNBCs from AA women (90%) relative to EA women (65%), and is associated with poor survival (6). The WNT10b direct target HMGa2 predicts survival outcome in TNBC (6). Furthermore, HMGa2 expression alone predicts metastasis in TNBC (6).

Enhancer of Zeste 2 (EZH2) is a methyltransferase component of the polycomb repressive complex 2 (PRC2) essential for the epigenetic maintenance of histone 3 lysine 27 trimethylation (H3K27me3), a repressive chromatin mark (7). EZH2 is overexpressed in a variety of cancers including basal-like and TNBC (8), in which EZH2 expression correlates with inverse levels of H3K27me3, not observed in the ER+ or HER2 cancer subtypes (9), suggesting that EZH2/PRC2 activity is lacking in TNBCs. A high frequency of elevated EZH2 expression is observed in basal-like invasive carcinomas from AA women with West African or Ghanaian heritage (10). EZH2 is known to physically interact with ß-catenin and to enhance Wnt ligand–mediated transactivation of ß-catenin independent of EZH2’s PRC2 methyltransferase activity (11, 12). EZH2 overexpression in mammary epithelial cells initiated hyperplasia but not neoplasia (13), which suggests that EZH2 requires other factor(s) for neoplasia of the mammary gland. HMGa2 and EZH2 are master regulators of EMT. HMGa2/EZH2 mediate DNA methylation activity, repress expression of E-Cadherin (CDH1), induce transcription factors that repress CDH1 (i.e., Slug, Snail, Twist, and Zeb1/2), and oppose genomic stability mediated by BRCA1 (14–19). Importantly, both HMGa2 and EZH2 are linked to oncogenic ß-catenin activity during initiation and metastasis of TNBC (6, 11, 12). Mechanistically, the overlapping functions of HMGa2 and EZH2 and how they each coordinate oncogenic ß-catenin signaling are unknown. Herein, our preclinical MMTV-Wnt10b/IRES-LacZ tumor model with haplotype insufficiency expression of HMGa2 is linked to loss of EZH2 expression and metastasis. Furthermore, in vitro we link the activity of both HMGa2 and EZH2 as necessary gatekeepers of K49 acetylation of oncogenic ß-catenin and prevention of repression mediated by Groucho/TLE1. The WNT10b-biomarker network we identified, composed of WNT10b, ß-CATENIN, HMGa2, and EZH2, is predictive of poor survival in patients. We suggest that this WNT10b-biomarker network is clinically relevant and useful for development of novel precision therapies against metastatic, chemoresistant TNBC.

**Materials and Methods**

**Human breast cancer tissues**

Studies were based on formalin-fixed paraffin-embedded or frozen-fixed methanol-acetone sections. Details about breast tissue collection and preparation can be found in the Supplementary Experimental Procedures. Written-informed consent was obtained from all subjects, and experiments conformed to the principles set out in the WMA Declaration of Helsinki and the NIH Belmont Report. Studies were approved by the Institutional Review Boards of the collaborating pathologists at University of California Los Angeles (UCLA; Los Angeles, CA), University of Tennessee Health Science Center (UTHSC; Memphis, TN), Emory University School of Medicine (Atlanta, GA), Duke University School of Medicine (Durham, NC), and City of Hope (Duarte, CA).

**Mice**

MMTV-Wnt10b<sup>+/c</sup> and MMTV-Wnt10b-IRES-LacZ mouse models were described (6, 20). Hmga2<sup>−/−</sup> mice were provided from the Lowry lab (21) that contained p53<sup>fl/fl</sup> alleles from a highly mixed mouse background and were backcrossed into the MMTV-Wnt10b-IRES-LacZ<sup>TVB/N</sup> strain for ten generations. Animal protocols were approved by the Office of Animal Research Oversight at UCLA and the Institutional Animal Care and Use Committee at UTHSC.

**RNA scope**

Advanced Cell Diagnostics, Inc designed the probes. *In situ* hybridization protocols were conducted according to the manufacturer’s instructions.

**Cell culture assays**

All conventional cell lines were maintained in a humidified atmosphere with 5% CO<sub>2</sub> in DMEM plus 1% pen/strep and 10% FCS. All cells were purchased from the ATCC and authenticated by Genetica prior to use in experiments.

**Knockdown experiments**

MDA-MB-231 cells silenced for HMGa2 utilizing two independent lentivirus constructs have been described (6). EZH2 CRISPR/Cas9 clones were generated by purchasing independent sgRNAs from GeneCopoeia Corp. and following the manufacturer’s protocols.

**Tumor biology, metastasis experiments, and patient-derived xenograft tumor modeling**

Transplantation, dilution, and xenograft experiments were carried out using standard procedures. Therapy with ICG-001 was used at 100 mg/kg and/or 200 mg/kg every 2 days, administered by i.p. injections, and mice were euthanized at 9 weeks after surgical transplantation. When used in combination with doxorubicin (1.4 mg/kg), the ICG-001 dose was reduced to 50 mg/kg. Details can be found in the Supplementary Experimental Procedures.

**Transient transfections**

Plasmids were obtained as follows: GFP (Lonza), pCMV/HAI-EZH2 (#24230, Addgene), and pMIG-HMGa2-FLAG (#25409, Addgene). EZH2-HA and HMGa2-FLAG were transfected in 293T cells with Lipofectamine 3000 (Invitrogen) per the manufacturer’s protocol. pcDNA-WNT10B and constitutive active (ca) ß-Catenin

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were transfected into MDA-MB-231 cells as previously described (6, 22).

**Statistical analysis**

Two-sided Fisher–Boschloo (23) unconditional exact tests were used to compare the incidence of tumors and lung metastasis between different transplanted cell populations. To compare HMGA2 and EZH2 gene expression between TNBC and non-TNBC patients, published datasets were analyzed (24). For metastasis-free survival analysis of EZH2 in patients with breast cancer, published datasets were analyzed (25).

**RNA and real-time PCR**

Standard procedures were used as described (6). Details can be found in the Supplementary Materials and Methods. Primer sequences for qPCR are listed in Supplementary Table S1.

**Chromatin immunoprecipitation**

Standard procedures were used as described (26). Details can be found in the Supplementary Materials and Methods. The primers sequences for chromatin immunoprecipitation (ChIP) are listed in Supplementary Table S2.

**IHC for of HMGA2 and EZH2 in Hmga2+/− or Hmga2+/+ Wnt10bLacZ tumors**

To increase IHC sensitivity, we decreased the HMGA2 antibody dilution by 10X and increased the EZH2 antibody dilution by 10X relative to conditions used to generate Fig. 2A,i, and we demonstrated the relative decreases in both HMGA2 and EZH2 protein levels in the Hmga2+/− primary tumors.

**Results**

**WNT10B/β-CATENIN/HMGA2/EZH2 is predictive of poor overall survival in basal-like and TNBC subtypes**

The Kaplan-Meier Plotter (http://kmplot.com/analysis/index.php?p=service&cancer=breast) tool recently added ~3,000 new datasets; a new analysis with HMGA2 and WNT10B reveals worse recurrence-free survival (RFS) when both WNT10B and HMGA2 expression are combined (HR = 1.99 and P = 0.0083; Fig. 1A,i). The combination of WNT10B, β-CATENIN, HMGA2, and EZH2 increased the HR for RFS outcomes by ~2.4 times compared with patients lacking expression of the WNT10B network (HR = 2.39 and P = 0.0112, Fig. 1A,ii). In another publicly available dataset stratified by women diagnosed with TNBC versus other subtypes, which included specimens from 178 women (58 TNBC and 120 non-TNBC), combined HMGA2 and EZH2 expression levels were higher in TNBC versus non-TNBC subtypes (Fig. 1B,i–ii), providing additional evidence that combined HMGA2 and EZH2 expression is associated only in TNBC. We analyzed the correlation between WNT10B, HMGA2, and EZH2 mRNA expression using The Cancer Genome Atlas database (Supplementary Fig. S1A and S1B). We show that the gene expression of WNT10B significantly correlates with HMGA2and EZH2 in TNBC (P = 0.028 and P < 0.001, respectively). Moreover, we did not find a correlation of WNT10B and HMGA2 in ER− or HER2+ subtypes, but we did find a correlation between WNT10B and EZH2 (Supplementary Fig. S1C and S1D).

We hypothesized that the WNT10B-signaling network might be useful in evaluating case samples of women at increased risk for TNBC. First, in normal breast tissue control cases, WNT10B, HMGA2, and EZH2 were rarely detectable by IHC (Fig. 1C). Second, we performed pilot retrospective IHC analysis of WNT10B, HMGA2, and EZH2 expression in two high-risk women who developed interval cancers while undergoing breast MRI screening (Fig. 1D). Selected Case 1 is a 27-year-old Caucasian woman with the BRCA1 mutation S868X. Selected Case 2 is a 54-year-old AA female with the BRCA1 mutation Y1563X. Two additional retrospectively selected high-risk cases (Selected Cases 3 and 4) are presented in Supplementary Fig. S2A. In each case, expression of WNT10B, HMGA2, and EZH2 is observed in non-cancerous breast tissue. These results provide preliminary evidence that WNT10B-network signaling is associated with ‘early’ biopsied tissue in women at high risk for TNBC.

Next, we pilot tested for WNT10B-network expression by IHC in two women with metastatic TNBC in the lung. The first metastasis was obtained from a woman of EA descent, and the second metastasis was obtained from an AA woman, and in both cases, we observed expression of HMGA2, EZH2, nonphosphorylated (Ser31Ser47Thr41) active β-CATENIN (referred to as activated β-catenin, ABC), and WNT10B (Fig. 1E). EZH2 expression was observed in a BRCA1-mutation carrier in primary TNBC, but not in ‘adjacent normal’ tissue (Supplementary Fig. S2B).

These data provide evidence that our WNT10B-network signature is predictive of poorer survival outcomes in basal-like and TNBC patients. Furthermore, in preliminary investigations of ‘early tissue’ sample biopsies from high-risk women, the WNT10B network is both associated with increased likelihood to develop interval TNBC and metastatic basal-like/TNBC.

**Loss of a single HMGA2 allele decreases EZH2 protein expression with a concordant repression of tumor growth and lung metastasis**

Next, we hypothesized that HMGA2 and EZH2 protein expression levels would be coexpressed in response to Wnt10b/β-catenin signaling in normal epithelial cells of the mammary gland. Female Wnt10b+/− transgenic females at 3, 6, 9, and 11 months of age were tracked for the number of pregnancies, tumor formation, and associated lung metastases (Fig. 2A and B). A single pregnancy in a 3-month-old Wnt10b+/− female did not induce expression of either HMGA2 or EZH2. In contrast, strong expression for both HMGA2 and EZH2 was observed in multiparous females at 6 months (n = 3; Fig. 2A,i) and 9 months (n = 4; Fig. 2A,ii) of age, and in metastatic lung lesions harvested from 11-month females (n = 6; Fig. 2B). Overlapping expression for HMGA2 and EZH2 is detected in the hyperplasia stage in the 6-month-old females.

We previously identified HMGA2 to be the most abundant biomarker in Wnt10b-driven tumors (6). To genetically address the requirement for HMGA2 for tumor growth and metastasis, we used Hmga2+/−:p53fl/fl mice (21). First, male Hmga2+/− mice were backcrossed to the females of FVB/NJ Wnt10b+/− mice for ten generations to generate a syngeneic model. Wnt10b+/−:Hmga2+/− mice (n = 23) were then compared with heterozygous Wnt10b+/−:Hmga2+/− mice (n = 14). Despite loss of a single copy of Hmga2, there was a significant increase in survival (Fig. 2C) and a reduction in tumor volume (Fig. 2D). Hmga2+/− female mice are sterile, and the Wnt10b-preclinical model rarely gives rise to nulliparous spontaneous tumors (latency > 1 year); therefore, we compared Hmga2+/− female mice with the virgin female Wnt10b+/−:Hmga2+/− mice (n = 5, Supplementary Fig. S3A). The homozygous, Hmga2−/− female mice showed suppression of Wnt10b-driven oncogenesis up to 20 months. Primary tumors from two Wnt10b+/−:Hmga2+/− (Fig. 2E,i–ii) mice were analyzed by hematoxylin and eosin (H&E) and compared with two
Figure 1. **WNT10B/β-Catenin/HMGA2/EZH2 Signaling in Chemoresistant TNBC**

Wnt10b<sup>−/−</sup>-Hmgα2<sup>−/−</sup> tumors (Fig. 2E,iii–iv): adenocarcinomas are smaller in Hmgα2<sup>−/−</sup> mice. Corresponding lungs from the same mice (Fig. 2F) plus one additional mouse pair were histomorphometrically quantified for metastases (Supplementary Fig. S3B, n = 3), revealing fewer lesions in Hmgα2<sup>−/−</sup> mice. HMGA2 and EZH2 protein expression levels were also decreased in Hmgα2<sup>−/−</sup> and Hmgα2<sup>−/+</sup> primary tumors (Fig. 2G; Supplementary Fig. S3C–S3E).

In summary, the coexpression of both HMGA2 and EZH2 protein occurs throughout the metastatic cascade in Wnt10b/β-catenin–driven mammary tumors. More importantly, haploinsufficient expression of HMGA2 decreased EZH2 protein expression and repressed lung metastasis in vivo in tumors driven by Wnt10b/β-catenin signaling.

A novel HMGA2-EZH2 protein–protein interaction controls regulation of nuclear core β-catenin/TCF-4/LEF-1 complexes and is a necessary gatekeeper for K49 acetylation of β-catenin.

Next, we confirmed that the TOPFLASH Wnt reporter is activated by constitutively active β-catenin or Wnt10b and is repressed by...
treatment with the WNT reporter ICG-001. In addition, EZH2, like HMGA2 (6), is a direct target of WNT10B, and WNT10b-dependent EZH2 expression is downregulated by ICG-001 (6), which disrupts CBP–β-catenin protein interactions (Supplementary Fig. S4A and S4B). More importantly, HMGA2 is recruited at the promoters of AXIN2 and MYC, but, in the absence of HMGA2, β-CATENIN cannot be recruited to the promoters of AXIN2 and MYC, resulting in loss of gene expression and demonstrating that the absence of HMGA2 functionally represses Wnt transcriptional activity (Supplementary Fig. S4C–S4E).

Figure 2.
Haploinsufficiency of HMGA2 in Wnt10bLacZ mice decreases EZH2 protein expression and represses primary tumor growth and metastasis. A and B, H&E analysis and IHC conducted for HMGA2 and EZH2 at 3 and 6 months in Wnt10bLacZ female mammary glands (A), primary tumors from 9-month-old mice (A, II), and lung metastases from 11-month-old mice (A, III). C and D, Kaplan-Meier survival graph comparing Wnt10bLacZ Hmga2+/+ (n = 23) and Wnt10bLacZ Hmga2+/− (n = 14) mice survival (P = 0.004; C) and comparison of tumor volumes in Wnt10bLacZ Hmga2+/+ and Wnt10bLacZ Hmga2+/− mice (P = 0.0011; D). E and F, H&E analysis of a primary (E) or lung metastasis (LM) tumor (F) in Wnt10bHmga2+/− and/or Wnt10bHmga2−/− female mouse. Two independent tumors are shown per genotype. Arrows and arrowheads depict macrometastases and micrometastases, respectively. G, IHC for HMGA2 and EZH2 on primary tumors from Hmga2+/+ and heterozygous Hmga2+/− Wnt10bLacZ female mice.
HMGA2 protein expression and (ii) in the presence of ICG-001 (at 10 μmol/L for 48 hours) followed by immunoblotting with non-phosphorylated β-catenin protein (amino acids Ser31/Ser37 and Thr41), commonly referred to as transcriptionally ABC (Fig. 3A and B). A decrease of EZH2 protein expression and a loss of EZH2 interacting with ABC in the absence of HMGA2 expression are consistent with pharmacologic repression using ICG-001. These results prompted us to determine if HMGA2 mediates EZH2-ABC protein–protein interactions through direct interactions with EZH2. To this end, 293T cells were transfected with a constant input of FLAG-HMGA2 (2.5 μg) and increasing amounts of HA-EZH2 plasmid (1.0, 2.5, or 5.0 μg; Fig. 3B). Complexes were IPed with anti-FLAG/HA-epitope antibodies and blotted with anti-HA or anti-FLAG antibodies. We observed that HMGA2 and EZH2 physically interact to form a novel protein–protein complex and confirmed that endogenous EZH2 and HMGA2 protein–protein interactions occur and disruption of EZH2 interactions with either ABC or HMGA2 is lost in the presence of ICG-001 (Fig. 3C). HMGA2 does not physically interact with ABC as shown by re-IP for pan-total β-CATENIN and blotting for transcriptionally ABC. Similar mechanisms were demonstrated by IP in two additional TNBC cell lines, MDA-MB-468 and SUM159PT (Supplementary Fig. S4F, i–ii). In contrast, neither ER⁺ cells (MCF-7) nor two “normal” breast cancer cell lines, MCF-10A and HUMEC, exposed to ICG-001 for 48 hours had effects on the protein expression of either EZH2 or HMGA2 (Supplementary Fig. S4G); therefore, this network is unique to TNBC cancer cells.

We then tested whether the HMGA2-EZH2 protein–protein complex could associate with other complexes, for example: (i) WNT-core components, TCF-4/LEF-1, and (ii) PRC2-complex member SUZ12 and/or EED. Like ABC, both TCF-4/LEF-1 physically interact with EZH2, but that HMGA2 does not interact with

**Figure 3.**
HMGA2-EZH2 protein–protein interactions are necessary for maintaining K49Ac of β-catenin and for displacing the Groucho/TLE1 repressor from TCF-4/LEF-1 transcriptional complexes. A, IB for HMGA2 and EZH2 and the IP of EZH2 and IB for both β-CATENIN and EZH2 in MDA-MB-231 cells, comparing shRNA-mediated knockdown of HMGA2 with the Sh-GFP vector only in the presence or absence of ICG-001 (at 10 μmol/L for 48 hours). ACTIN served as loading controls. B, IP of epitope-tagged FLAG-HMGA2 and HA-EZH2, followed by IB for HA and/or FLAG. IB for FLAG, HA, and ACTIN served as controls for the 293T cells transfected with increasing input of the HA-EZH2 vector in the presence of constant input of FLAG-HMGA2. C, IP of both endogenous EZH2 (IB for HMGA2 and ABC) and HMGA2 (IB for EZH2 and ABC). Re-IP for β-CAT and IB ABC was from the original IP HMGA2 supernatant. D, IP of EZH2 and/or HMGA2, IB for SUZ12 and EED in both MCF-7 and MDA-MB-231 cells. E, CRISPR/Cas9 HMGA2-KO and EZH2-KO in MDA-MB-231 cell lines were immunoblotted for HMGA2 and EZH2. F, HMGA2-ChIP experiments were conducted on the EZH2 promoter in either parental CAS9-only MDA-MB-231 cells or HMGA2-KO cells, and enrichment is expressed relative to the ACTB promoter. G, IP of both endogenous HMGA2 (IB for EZH2 and ABC) and EZH2 (IB for HMGA2 and ABC) and IP of TCF4 and IB for EZH2 in Control CAS9 only cells and in HMGA2-KO and EZH2-KO cell lines. H, IB for MYC, CCND1, VIMENTIN, and ACTIN in cells. I, IP for both ABC and TCF-4, followed by IB with K49Ac-β-CAT and/or Groucho/TLE1 in the HMGA2-KO and EZH2-KO cell lines relative to Ctr-CAS9 cells. ICG-001–treated cells (10 μmol/L for 48 hours) are included as a control for repression of K49Ac-β-CATENIN.
either of the two (Supplementary Fig. 5A). Next, we conducted IP assays with both HMGA2 and EZH2 ± ICG-001 in both MCF7 cells and MDA-MB-231 cells, and blotted for either SUZ12 or EED (Fig. 3D). We confirmed that EZH2 interacts with either SUZ12 or EED in MCF-7 cells, and the interactions are not disrupted by ICG-001. In contrast, the interaction of EZH2 with SUZ12, but not with EED, is disrupted in the presence of ICG-001 in MDA-MB-231 cells. Moreover, HMGA2 physically interacts with both SUZ12 and EED, and exposure to ICG-001 inhibits either interaction; similar results were observed in MDA-MB-468 cells (Supplementary Fig. S4I).

We generated gene knockouts using CRISPR technology for both HMGA2 and EZH2 in MDA-MB-231 cells to determine if they participate in an autoregulatory loop. We generated two independent clonal sublines for each gene (i.e., Clones A and B), and loss of mRNA and protein expression was verified (Fig. 3E; Supplementary Fig. S5A and S5B). In each gene's clonal KO line, mRNA expressions for WNT-direct targets and for proliferation/EMT-markers were downregulated (Supplementary Fig. S5C, i–ii and S5D, i–ii).

To gain mechanistic insights into how HMGA2 regulates EZH2, we performed ChIP with an antibody to HMGA2 for the EZH2 proximal promoter in cells silenced for HMGA2 expression (Fig. 3F), demonstrating decreased recruitment of HMGA2. An IP against the EZH2 protein in the HMGA2 KO cells verified disruptions of EZH2 interact with endogenous HMGA2, ABC, and TCF-4 (Fig. 3G). In both EZH2 and HMGA2 KO cells, MYC expression was lost, and CCND1 expression was repressed (Fig. 3H).

The acetylation of lysine 49 (K49Ac) of β-Catenin, mediated by Groucho/TLE, which physically interacts with TCF-4/LEF-1, is interdependent on enzymatic HAT activity (28, 29). We IPed TCF-4 and immunoblotted for its downstream targets. First, we profiled a series of previously characterized PDX breast cancer lines obtained from the Huntsman Cancer Institute (35) for expression of β-Catenin, ABC, and HMGA2, confirming active Wnt/β-catenin signaling in several PDX models (Supplementary Fig. S6A). We noted that the H10-2 (“treatment-naïve,” i.e., never received chemotherapy) and H10-1 (“chemo-resistant”) TNBC PDX models expressed high levels of all markers. Primary cell lines were derived from these models. We exposed chH10-2 (10 μmol/L) PDX cells to ICG-001 as previously described (22). AXIN2, EZH2, and HMGA2 mRNAs are decreased in chH10-2 cells in response to ICG-001 therapy (Supplementary Fig. S6B). Therefore, the H10-PDX model, derived from a patient who failed multiple frontline therapies, and who died in vivo (30–32). Direct targeting of Wnt/β-catenin repression and or exposure to ICG-001, TLE1 protein interactions with TCF-4 are increased compared with basal levels (Fig. 3I). Paraadoxically, in the absence of HMGA2, we demonstrate a decrease in the basal-level interaction between TCF-4 and TLE1. These results strongly suggest that enzymatic HAT activity, mediated by CBP, which maintains K49Ac of oncogenic ABC, is interdependent on HMGA2 and EZH2 protein–protein interactions.

HMGA2 and EZH2 are each necessary for tumor growth, visceral metastasis, and repression of E-CADHERIN in vivo. Both HMGA2 and EZH2 are master regulators of EMT (30–32), directly targeting E-CADHERIN repression and coinciding with the promotion of VIMENTIN expression (16, 33, 34). In KO clones for HMGA2 and EZH2, protein loss of VIMENTIN was associated with an inverse increase of E-CADHERIN (Fig. 4A), suggesting that loss of either gene reverts mesenchymal MDA-MB-231 cells to a more “normal”–like breast epithelial cell expressing E-CADHERIN. Moreover, several EMT markers were downregulated, including VIMENTIN mRNA expression (Supplementary Fig. S5D, i–ii).

Next, we compared the effects of CRISPR KO on MDA-MB-231 tumor growth in vivo (n = 8 mice; Fig. 4B). Control cells (Cas9 only) gave rise to 16 of 16 tumors by ~3 weeks after transplantation; all mice in this cohort were euthanized by ~6 to 9 weeks. In contrast, only 8 of 16 possible tumors grew in either the HMGA2KO or EZH2KO cell lines by ~12 to 14 weeks, although tumors were detected by ~16 to 18 weeks. The experiment was terminated at ~26 weeks after transplantation. Deletion of either HMGA2 or EZH2 significantly reduced tumor volume (Fig. 4B and C; P < 0.0001) and greatly decreased frequency of metastases in corresponding lung sections (Fig. 4D and E, i–ii and Supplementary Fig. S5E, i–ii). Both the percentage of metastatic area and the number of metastatic foci per lung lobe are significantly reduced in the KO clones relative to the control cells (i.e., P = 0.001). Tail-vein injection (“experimental metastasis”) assays using these cell lines confirmed the loss of visceral metastasis (Supplementary Fig. SSF). More importantly, we confirm the restoration of E-CADHERIN expression in primary tumors derived from both CRISPR KO models (Fig. 4F).

Wnt10b/β-catenin direct targets AXIN2, EZH2, CCND1, MYC, CD44, and HMGA2 are lost in drug-resistant TNBC patient-derived xenograft tumors treated with ICG-001, inhibiting both primary and metastatic tumor growth. We next posited that in mice bearing a highly chemoresistant TNBC patient-derived xenograft (PDX), exposure to ICG-001 would inhibit metastasis and block Wnt10b/β-catenin signaling to its downstream targets. First, we profiled a series of previously characterized PDX breast cancer lines obtained from the Huntsman Cancer Institute (35) for expression of β-Catenin, ABC, and HMGA2, confirming active Wnt/β-catenin signaling in several PDX models (Supplementary Fig. S6A). We noted that the H10-2 (“treatment-naïve,” i.e., never received chemotherapy) and H10-1 (“chemo-resistant”) TNBC PDX models expressed high levels of all markers. Primary cell lines were derived from these models. We exposed chH10-2 (10 μmol/L) or chH10-1 (30 μmol/L) PDX cells to ~IC₅₀ doses of ICG-001 as previously described (22). AXIN2, EZH2, and HMGA2 mRNAs are decreased in chH10-2 cells in response to ICG-001 therapy (Supplementary Fig. S6B). Therefore, the H10-PDX model, derived from a patient who failed multiple frontline therapies, and who died in vivo (30–32). Direct targeting of Wnt/β-catenin repression and or exposure to ICG-001, TLE1 protein interactions with TCF-4 are increased compared with basal levels (Fig. 3I). Paraadoxically, in the absence of HMGA2, we demonstrate a decrease in the basal-level interaction between TCF-4 and TLE1. These results strongly suggest that enzymatic HAT activity, mediated by CBP, which maintains K49Ac of oncogenic ABC, is interdependent on HMGA2 and EZH2 protein–protein interactions.

HMGA2 and EZH2 are each necessary for tumor growth, visceral metastasis, and repression of E-CADHERIN in vivo. Both HMGA2 and EZH2 are master regulators of EMT (30–32), directly targeting E-CADHERIN repression and coinciding with the promotion of VIMENTIN expression (16, 33, 34). In KO clones for HMGA2 and EZH2, protein loss of VIMENTIN was associated with an inverse increase of E-CADHERIN (Fig. 4A), suggesting that loss of either gene reverts mesenchymal MDA-MB-231 cells to a more “normal”–like breast epithelial cell expressing E-CADHERIN. Moreover, several EMT markers were downregulated, including VIMENTIN mRNA expression (Supplementary Fig. S5D, i–ii).
To test whether ICG-001 would prevent primary tumor growth and/or inhibit metastasis, we began therapy 3 weeks after transplantation (Fig. 5). Vehicle (Group #1) or ICG-001 was given intraperitoneally every other day for 2 weeks at a low (Group #2; 100 mg/kg) or high dosage (Group #3; 200 mg/kg). Tumor growth was monitored by calipers (Fig. 5C) and by luciferase flux (Fig. 5D,i); LN metastases were monitored by luciferase flux (Fig. 5D,ii). Overall, ICG-001 therapy at the highest dose significantly reduced both primary tumor and LN metastases burden.

To determine if proteins downstream of WNT10B/b-Catenin were affected by ICG-001, we immunoblotted for (i) pan-b-Catenin and ABC, (ii) WNT direct targets: AXIN2, HMGA2, and EZH2, and (iii) WNT direct targets of proliferation: CCND1, PCNA, and MYC (Fig. 5E), confirming that Wnt-dependent direct target genes and proliferation markers are downregulated in vivo. We observed by IP with either LEF-1 and/or TCF-4 that EZH2 is present in a complex, but after ICG-001 exposure, it is lost (Fig. 5F, i.e., #1, #2, and #3 refer to above dosages in Fig. 5C). The results reveal that the prevailing Wnt mechanism demonstrated in MDA-MB-231 cells (Figs. 3 and 4) is active in the TNBC PDX model to initiate tumor growth and metastatic events. Moreover, IHC staining of primary tumors and lung sections confirmed loss of AXIN2 and the human mitochondria marker, respectively (Supplementary Fig. S6E and S6F). We observed by IP with either LEF-1 and/or TCF-4 that EZH2 is present in a complex, but after ICG-001 exposure, it is lost (Fig. 5F, i.e., #1, #2, and #3 refer to above dosages in Fig. 5C).

Wnt inhibitor ICG-001 sensitizes doxorubicin to block metastasis of chemoresistant TNBC PDX tumors

Treating stage IV metastatic breast cancer is an overarching clinical challenge. The patient from whom the HCI-10 PDX tumor was derived (35) underwent systemic chemotherapy, which included multiple rounds of various anthracycline-based treatments for over 21/2 years; her disease became highly chemoresistant and she died.
We hypothesized that inhibition of Wnt/β-catenin pathways would resensitize cells derived from this patient to doxorubicin to inhibit in vivo visceral metastasis.

Sensitivity of the cHCI-10 PDX cells to therapy was tested in vitro via WST-1 assays. ICG-001 (0.1 to 30 μmol/L range: 0.1, 1, 5, 10, 15, 20, and 30 μmol/L) or doxorubicin (1 to 20 μmol/L range: 0.02, 0.04, 0.2, 1, 5, 10, and 20 μmol/L) were added for 48 hours, resulting in calculated IC50 value of ~32 μmol/L for ICG-001 (Fig. 6A). At the highest dose of doxorubicin, ~75% of the cells were proliferating relative to the control. To address potential synergy, a sub-IC50 concentration of ICG-001 (10 μmol/L; constant, CONST dose) was tested with varying doses of doxorubicin. Vice versa, cells were exposed to a CONST dose of doxorubicin (5 μmol/L) with varying ICG-001 doses and WST1 activity measured up to 96 hours. By 48 hours, the lowest ICG-001 dose with a CONST dose of doxorubicin decreased proliferation ~40% as compared with either ICG-001 or doxorubicin alone in the same dose range. Similarly, the lowest dose of doxorubicin with CONST ICG-001 decreased proliferation by ~35% relative to single therapy. Moreover, by 96 hours, the lowest ICG-001 dose (0.1 μmol/L) with a combination of doxorubicin at 0.2 μmol/L decreased proliferation by more than 56% (Supplementary Fig. S7A).

Figure 5.
TNBC PDX tumors exposed to ICG-001 show repressed metastases by exerting an epigenetic repression of the β-catenin/HMGA2/EZH2 signaling axis in chemoresistant CD44+ cells. A, H&E staining (i) and anti-human mitochondria antibody (Hu-MITO) IHC on primary tumors (ii), LN metastases (iii), and ABC (iv), demonstrating histologic confirmation of metastatic lesions in the LN. B, IHC for WNT10B, ABC, AXIN2, and HMGA2 in primary tumors from C, demonstrating expression of the axis in the HCl-10-Luc2 PDX. C, Phase images of tumors harvested at end stage and tumor volume from mice treated with vehicle (Veh II), or i.p. injected with ICG-001: 100 mg/kg (Group #2) or 200 mg/kg (Group #3). D, Total light flux was compared in primary tumors (i) and in LN-Mets (ii). P values generated by one-way ANOVA, followed by pairwise t tests (**, P > 0.01; ***, P < 0.001). E, IB for pan (total)-β-CATENIN (β-CAT), Act-β-CATENIN (ABC), AXIN2, HMGA2, EZH2, PCNA, and MYC. TUBULIN served as a loading control. F, IP for either endogenous LEF-1 or TCF-4, followed by IB for EZH2 in tumors from each cohort shown in F. G, RNAscope in situ RNA hybridization detection of CD44 (PE) and HMGA2/AXIN2 (Cy5) in tumors shown in E, counterstained with DAPI. P values for C by one-way ANOVA (P = 0.001). Averages are presented as ±SEM.
To test whether effects observed with ICG-001 and doxorubicin are additive or synergistic, we calculated the IC₃₀, IC₅₀, and IC₇₀ curves for each drug and then plotted isobole curves as in ref. 35 (Supplementary Fig. S7B) to calculate the combination index (Supplementary Table S3), revealing synergistic interactions. To understand the mechanism of synergy, we exposed cHCl-10 cells with a sub-IC₅₀ dose of ICG-001 alone (10 µmol/L) or doxorubicin alone (0.5 µmol/L), or in combination for 48 hours. IB for BCL-2, BAX, and ACTIN. The signal intensity ratio for BCL-2/BAX was first normalized to ACTIN, conducted in biological triplicates. cHCl-10 cells (1.25 × 10⁶) isolated from a primary PDX tumor were tail vein injected into NSG females and treated with doxorubicin alone (1.4 mg/kg, i.p.) or in combination with ICG-001 (50 mg/kg, i.p.). Total Flux (p/s) was quantified by ex vivo bioluminescence imaging of lungs; P values generated by one-way ANOVA followed by pairwise t-test (*, P < 0.05; **, P < 0.001). c, Top, ex vivo images of luciferase activity from metastatic lesions in the lung. Bottom, to confirm that luciferase signal corresponds with metastases, IHC using an anti-human mitochondria antibody (Hu-MITO) was performed using the same lungs.

Discussion

There is significant heterogeneity within the five breast cancer subtypes identified originally from microarray analysis (39), resulting in major therapeutic challenges. In order to develop targeted agents for TNBC, there is a great need for relevant preclinical mouse models. Our data support the conclusion that the Wnt10b/b-catenin/Hmga2/Ezh2 signaling axis is clinically relevant and that targeting this network is likely to prolong survival by repressing primary tumor growth and visceral metastasis.

In a preliminary retrospective study utilizing MRI-guided biopsies in women at high risk for developing TNBC, we show that expression of WNT10B, HMGA2, and EZH2 proteins in biopsied material precedes the development of TNBC. Although limited by a small number of clinical cases (four), from profiling publicly available datasets, it is clear that survival is reduced by 2.4-fold in women with an elevated WNT10B network, suggesting this network is clinically relevant.
Our preclinical Wnt10b model is distinct from the predominant MMTV-Wnt1-driven transgenic as Wnt1 tumors do not metastasize (40); therefore, we could not assess the impact of Hmga2 on metastasis (32). In contrast, lung metastasis is potently blocked in the haplotype insulin group Hmga2+/− mice in the context of Wnt10b/β-catenin–driven tumors. More importantly, we link loss of Hmga2 expression in vivo with loss of EZH2 protein expression, decreasing visceral metastatic disease. EZH2 promotes expansion of breast tumor-initiating cells requiring β-catenin (11), but, on its own, EZH2 is incapable of transforming the mammary epithelium (13). We provide genetic evidence that Hmga2 expression is an essential cofactor for neoplasia and/or metastasis. The coexpression of both Hmga2 and EZH2 proteins in Wnt10b/β-catenin–driven early hyperplasia lesions after only two pregnancies at 6 months of age is maintained throughout the metastatic cascade providing additional evidence.

We provide compelling mechanistic evidence that both Hmga2 and EZH2 are necessary for the maintenance of K49 acetylation of β-catenin mediated by CBP (27). The Wnt nuclear complex composed of β-CATENIN/TCF-4/LEF-1 (29) is necessary for Wnt-direct target gene expression, but was only maintained in the presence of both Hmga2 and EZH2. Transcription factors like TCF-4/LEF-1 regulate Wnt targets through a “transcriptional switch” that is “off” in the absence of Wnt ligands, or “on” in their presence. Corepressors Groucho/TLE1 contribute by interacting physically with TCF/LEF-1 and disrupting Wnt signaling through β-catenin. We demonstrate that Groucho is displaced from TCF-4 only when EZH2 protein expression is present and, that in the absence of EZH2, a strong association between TLE1 and TCF-4 is observed. Hmga2 did not phenocopy this observation; in contrast, loss of Hmga2 expression increased K49 acetylation on β-catenin. We propose that Hmga2 guides nuclear architecture remodeling by partnering with CBP, or perhaps by disrupting HDAC’s recruitment to the Groucho proteins. Hmga2 is known to displace HDAC1 from pRB protein in pituitary tumors (41), so this hypothesis remains to be tested experimentally.

PRC2 is composed of the trimeric core proteins SUZ12, EED, and EZH2 (42). EZH2-PRC2 is a multicomplex chromatin modifier that mediates enzymatic methylation of H3K27 to contribute to cellular identity. Basal-like and TNBCs are cell distinct from other breast cancer subtypes, and our novel discovery that Hmga2 can physically interact with either of the PRC2-complex members SUZ12 and EED was observed only in TNBC cells, suggesting a unique network not found in ER+ or normal breast cancer cells that is a key player in TNBC-aggressive biology. The functional consequences of these intriguing observations that the Hmga2–EZH2 complex is found within PRC2 require further exploration. For example, EZH2-PRC2–specific inhibitors, such as DZNep (EZH2 degradation specific) or ZLD1029 (EZH2–blocking H3K27me3) enzymatic activity (43), tested in combination with ICG-001 on a variety of breast cancer subtypes would provide novel epigenetic insights into chemoresistance. ICG-001 (PRI-724) is under clinical trials in combination with various FDA-approved agents for metastatic disease, but has not been tested against chemoresistant metastatic TNBC (https://clinicaltrials.gov/). We demonstrated that ICG-001 was effective at preventing both tumor growth and metastasis in a highly metastatic, chemoresistant TNBC PDX model derived from an ER− woman, HCI-10 (2). Loss of Wnt target gene expression was concordant with the loss of CD44+ cells, known to contribute to chemoresistance (44, 45). The dose of ICG-001 currently tested in clinical trials ranges from 650 to 902 mg/m2 for a variety of solid tumors. Our mouse ICG-001 dosage (30 mg/kg) is equal to the human equivalent dosage (HED; ref. 46) of 150 mg/m2, well below the phase II trial dosages. Importantly, combining doxorubicin (1.4 mg/kg; HED of 45.96 mg/m2) with ICG-001 prevented lung metastasis in this highly chemoresistant TNBC model demonstrating synergistic action in vivo. The National Comprehensive Cancer Network guidelines recommend that doxorubicin be used at 60 to 75 mg/m2, or when used in combinatorial therapy to be lower than 50 mg/m2 once weekly in stage IV patients. Doxorubicin cytotoxicity is usually associated with its dose (47). The incidence of cardiomyopathy is about 4% with a dose of 500 to 550 mg/m2, 18% when the dose is 551 to 600 mg/m2, and 36% when the dose exceeds 600 mg/m2. We suggest that adding ICG-001 to a doxorubicin regimen would be well-tolerated and efficacious in patients, without evidence of cardiac toxicity typically observed at a total dose of 400 mg/m2. We propose that WNT inhibitors could have broad clinical use in aggressive TNBC in combination with FDA-approved therapies. In conclusion, inhibition of the WNT10B network signaling axis in patients with chemoresistant TNBC could create new opportunities for treatment regimens.

Disclosure of Potential Conflicts of Interest
R.M. O’Regan is a consultant/advisory board member for Novartis, Pfizer, Lilly, Biotheranostics, Genomic Health, PDXA, Immunomedics, and Macrogenics. W.E. Lowry is President at, and has an ownership interest (including stock, patents, etc.) in, Pelage Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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