Histone deacetylase 11 inhibition promotes breast cancer metastasis from lymph nodes

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Lymph node (LN) metastases correspond with a worse prognosis in nearly all cancers, yet the occurrence of cancer spreading from LNs remains controversial. Additionally, the mechanisms explaining how cancers survive and exit LNs are largely unknown. Here, we show that breast cancer patients frequently have LN metastases that closely resemble distant metastases. In addition, using a microsurgical model, we show how LN metastasis development and dissemination is regulated by the expression of a chromatin modifier, histone deacetylase 11 (HDAC11). Genetic and pharmacologic blockade of HDAC11 decreases LN tumor growth, yet substantially increases migration and distant metastasis formation. Collectively, we reveal a mechanism explaining how HDAC11 plasticity promotes breast cancer growth as well as dissemination from LNs and suggest caution with the use of HDAC inhibitors.
Metastasis causes ~90% of all cancer-related deaths. Although the most common site of initial spread in most cancers is to lymph nodes (LNs), which corresponds with a worsened prognosis, the contribution that locoregional LN metastasis has on seeding distant organs remains highly controversial1-5. For example, several large cohort studies have shown that the presence of microscopic LN metastases in breast cancer corresponds with poor survival6-9. However, clinical trials investigating the relationship of LN metastasis treatment with survival have yielded conflicting results9-15. Furthermore, while molecular profiling and phylogenetic analyses suggest LN metastases often give rise to distant metastases in colon cancer (~35% incidence)15, another study in breast cancer found no evidence that LN metastases are required for dissemination to distant sites11. In mouse models, several mechanisms promoting tumor lymphangiogenesis have also correlated with the development of distant metastasis12-15. Recently, the first direct experimental evidence of LN metastases seeding distant metastases has emerged16-19, which showed that cancer cells egress from LNs predominantly through the draining LN blood vessels. However, the mechanistic underpinnings explaining how tumor cells exit the LN are still unknown. This represents a critical knowledge gap in metastatic biology20 especially because the mechanisms governing LN metastasis may be unique from those promoting direct hematogenous spread from the primary lesion.

Here we show that LN metastases in breast cancer patients often phylogenetically resemble distant metastases. Using several experimental models, we show how LN metastasis establishment occurs through increased expression of a poorly understood chromatin modifier, histone deacetylase 11 (HDAC11). Interestingly, while genetic and pharmacologic blockade of HDAC11 decreases LN tumor growth, it substantially increases migration and promotes distant metastasis formation. Our findings demonstrate that establishment of LN metastasis, and then egress from LNs to distant sites, is both highly efficient and dynamic. Additionally, these findings reveal a next context for evaluation of cancer therapeutics and suggest caution with the use of HDAC inhibitors (HDACis).

Results

LN and distant metastases share a common origin. Because a recent report found no evidence that LN metastases were required for the development of distant metastases in breast cancer21, we first analyzed a cohort of seven breast cancer patients from the University of North Carolina Breast Cancer Rapid Autopsy Program (UNC RAP) for whom primary tumors (T), LN metastasis (L), and distant metastases (D) were collected24. Single-nucleotide variant data analysis with pairwise distance measurements (Jensen–Shannon distances (JSDs))20 was used to determine whether the LN samples were phylogenetically closer to the primary lesion or to distant metastases. If in a patient’s phylogenetic tree we observed that at least one distant metastasis sample was in the same clade with a LN sample and no other primary sample, we then concluded it as “LN-met mediated,” which implies that the LN metastases gave rise to one or more distant metastases. In all other cases, we concluded it as “LN-met independent”, which implies that the primary tumor directly seeded the distant site hematogenously. Compiling a ratio of the phylogenetic distance between D and L tumors over the phylogenetic distance between D and T tumors revealed that five out of the seven (71%) patients displayed evidence of a LN-mediated spread with at least one distant metastasis (Fig. 1a, b, d–f), suggesting that distant breast cancer metastases can be seeded from LN metastases in breast cancer. Boosting analysis revealed high confidence classification of these clades (Fig. 1c). In particular, distance ratios for patients A15, A20, and A34 showed that in some patients there is evidence that distant metastases in these patients share a more recent common ancestor with LN metastases than with primary tumors (Fig. 1a, b, d–f). These results suggest that LN metastases seeded distant metastases; however, we cannot exclude the possibility that the distant metastases instead seeded the LN metastases. We also found evidence for direct hematogenous route of metastasis from the primary tumor to distant sites, as we also observed in nearly all patients evidence of distant lesions that were more closely related to the primary tumors. Two out of seven patients displayed only “LN-met-independent” spread (Fig. 1c, g). These findings suggest that both lymphatic and direct hematogenous routes of spread commonly co-occur in breast cancer.

LN metastases can give rise to distant metastases. Despite recent advances in modeling how breast cancer spreads through LN blood vessels18,19, the mechanisms and potential pharmacological targets that are involved remain unknown. Much of the complexity stems from the highly interconnected circulatory patterns of the lymphatic and hematogenous vasculature22,23. To address this issue, we utilized a syngeneic mouse breast cancer cell line (4T1) capable of high-fidelity spontaneous metastases to LNs and distant organs24. While the 4T1 model histopathologically resembles triple-negative breast cancer, it also resembles the luminal molecular subtype25. Starting with the same parental 4T1 line, we generated dual fluorescence and luciferase reporter lines stably expressing either GFP/firefly luciferase (4T1-G/fL) or mCherry/Renilla luciferase (4T1-mCh/rL) as a means to analyze metastasis kinetics. To directly study cells growing in the context of the LN microenvironment, we developed a micro-injection model (Supplementary Fig. 1a). This approach allowed us to fine-tune direct injection into the draining axillary LN (AxLN) and consistently led to 68% take rates (Supplementary Fig. 1b). These AxLN tumors grew within the LN capsule similar to spontaneous AxLN metastases from orthotopic mammary fat pad (MFP) tumors (Supplementary Fig. 1a, c–e), and showed characteristics of natural tumor progression, including necrotic regions and metastasis to the lungs (Supplementary Fig. 1f, g). Using this LN micro-injection model, we determined the kinetics of distant metastasis to the lung using flow cytometry by analyzing various timepoints from 1 day to 6 weeks after AxLN micro-injection (Supplementary Fig. 2a, b). We found that distant metastases were readily detected in the lungs by 2 weeks post injection (Supplementary Fig. 2a).

Dissemination by the lymphatic route is highly efficient. Considering LNs drain to the lungs via efferent lymphatics or direct LN blood vessel invasion18,19, we compared the metastases kinetics from LNs with direct hematogenous seeding by tail vein injection. Surprisingly, we found that LN tumors were significantly more efficient at generating distant metastases based on both number and frequency of micro-metastases in the lungs and brain (Supplementary Fig. 2c–f). To compare the metastatic efficiency of LN tumors with that of primary tumors, we injected equal cell numbers of 4T1-G/fL into the MFP and 4T1-mCh/rL into the corresponding right AxLN of the same mouse (Fig. 2a). The growth of each tumor could be differentiated based on luciferase signal, and tumor growth rate was similar between the two cell lines (Fig. 2b, c). After 6 weeks of growth, the vast majority of the AxLN tumors were mCh+, and <1% of tumor cells were GFP+, suggesting that distant 4T1-GFP/fL metastases most likely occurred via the hematogenous route (Fig. 2d). Using this model, we found that AxLN tumors metastasized to the lungs more efficiently than orthotopic MFP tumors, as indicated by
both fluorescence and luciferase signal (Fig. 2e, f). To evaluate whether the difference in metastatic efficiencies was due to an intrinsic difference between the two reporter cell lines, we switched the orientation, micro-injecting 4T1-GFP/fL into the AxLN and 4T1-mCh/rL into MFP. Again, 4T1-GFP/fL cells injected into AxLN were more capable of establishing distant metastases in the lung compared to 4T1-mCh/rL cells injected in MFP (Supplementary Fig. 3a). To better delineate the contribution of hematogenous vs. lymphatic dissemination, we also compared injection of 4T1-mCh/rL cells into either the MFP, the MFP with AxLN removed prior to injection, or into the AxLN. Compared with MFP-injected mice that had AxLNs removed, metastasis to lungs was significantly increased in the AxLN injection group (Supplementary Fig. 3b). There was no significant difference in lung metastases (LuMs) between the MFP and the MFP with AxLN removed groups. Taken together, these data show that distant metastasis occurs via both hematogenous and lymphatic seeding, and although hematogenous dissemination is likely the predominant route of spread, metastasis via LNs is more efficient.

**An epigenetic program regulates metastases via the LNs.** To determine how cancer cells spread from LNs, we isolated several 4T1 sub-clones from MFP- and AxLN-injected tumors, and LuMs that arose from micro-injected AxLNs (AxLN-LuM). After normalizing to 4T1-mCh/rL and 4T1-GFP/fL parental lines, we analyzed differentially expressed genes between MFP and AxLN sub-clones (Fig. 3a), and then between AxLN and AxLN-LuM sub-clones (Fig. 3b). Based on our analyses, we identified 206 genes that were differentially up- or down-regulated in at least one of these three microenvironments (Fig. 3c, Supplementary Fig. 4; see Methods for screening criteria). Gene ontology (GO) analyses revealed that the genes differentially expression in AxLN were predominantly involved in cell cycle progression (Fig. 3f). Consistent with a cell growth phenotype, we found that AxLN sub-clones divided substantially faster than MFP, AxLN-LuM, or LuM sub-clones derived from tail vein injections, suggesting an important role for proliferation within the LN (Fig. 3g). Among the proliferation regulators, RRM2 and E2F8 were the most differentially expressed genes (Fig. 3h). Moreover, of the differentially expressed genes, 152 (74%) were only up- or down-regulated in at least one of these three microenvironments (Fig. 3c).

**Fig. 1** Metastatic breast cancer patient phylogenetic analysis show that LN metastases efficiently metastasize to distant organs. a Distance ratios from metastatic breast cancer patient samples on a patient and b individual metastasis basis. c Bootstrap confidence estimates for each group of patient samples. d-f Representative patient phylogenetic trees showing examples of LN-met- mediated and g LN-met-independent patterns of spread. Light blue shading demarcates LN-containing clades, whereas dark blue shading demarcates distant metastasis clades. Overlapping shading indicates clades containing both LN and distant metastases, implicating a LN-mediated pattern of distant metastases. Boxed in metastasis are those displaying a LN-mediated pattern of spread.
that an epigenetic mode of gene regulation may be involved. Indeed, chromatin modifiers HDAC11 and EZH1 were significantly upregulated in the AxLN clones and were suppressed in MFP and AxLN-LuM sub-clones (Fig. 3h). We validated several of these targets by quantitative reverse transcription-PCR (Supplementary Fig. 5a). As both HDACs and histone methyltransferases are typically involved in gene repression, we hypothesized that the increased expression of these epigenetic regulators in AxLN could be upstream of the genes that were found to be differentially down-regulated in our analyses. Using the cancer cell line encyclopedia dataset (n = 1036 cell lines), we found very strong inverse correlations between expression of HDAC11 and many of the candidate genes (Supplementary Fig. 5b), but not EZH1 (Supplementary Fig. 5c). Thus, we focused on further defining the role of HDAC11 in LN tumor development and subsequent spread to distant sites.

**HDAC11 is necessary for tumor growth within LNs.** HDAC11 is the most recently identified HDAC, and is best characterized for epigenetic inhibition of IL-10, which causes pleotropic effects on innate and adaptive immunity. To determine whether increased HDAC11 is an artifact of micro-injection, we developed several matched pairs of sub-clones derived from MFP tumors and spontaneous AxLN metastases. As compared with corresponding MFP tumors, we found HDAC11 was significantly increased in five out of seven matched spontaneous AxLN sub-clones; and expression levels were similar to that of micro-injected AxLN sub-clones (Fig. 3i). Using six matched primary tumors and LN metastases from the UNC RAP patient samples, we used RNA-sequencing data to evaluate HDAC11 expression. We found that some LN metastases exhibited increased HDAC11 expression compared to the matched primary tumors (Supplementary Fig. 6a, b). However, likely in part due to the small sample size and dynamic nature of HDAC11 expression, this was not statistically significant and will require further evaluation in a larger cohort of clinical samples.

Next, to determine how HDAC11 plasticity is mediated, we evaluated methylation of the HDAC11 promoter using bisulfite conversion and found that the HDAC11 promoter was less methylated in AxLN sub-clones, but had increased methylation in the MFP and AxLN-LuM sub-clones (Fig. 3j). Because decreased methylation of the HDAC11 promoter correlated with increased messenger RNA (mRNA) expression in AxLN sub-clones, these findings suggest that HDAC11 may itself be epigenetically modified in the context of the LN microenvironment; however, it is likely that other mechanisms of gene regulation are involved. To elucidate the downstream targets of HDAC11, we knocked down HDAC11 using two different short hairpins RNA (shRNAs), which resulted in significantly increased mRNA expression of several candidate genes, most notably E2F7, E2F8,
and RRM2 (Fig. 4a). Moreover, over-expression of human HDAC11 in MDA-MB-231 breast cancer or 293 T cell lines, resulted in significant enrichment of the promoter regions of these genes by chromatin immunoprecipitation-qPCR (ChIP-qPCR) when probing for HDAC11 (Fig. 4b, Supplementary Fig. 7a). To determine whether HDAC11 was functioning as a HDAC, we used ChIP-qPCR to compare pull-down of acetyl-H3 and acetyl-H4 at the promoters of target genes in 4T1-shCtrl and 4T1-shHDAC11 cells, which revealed significant enrichment of acetyl-H3 and -H4 at these target gene promoter regions upon HDAC11 silencing (Fig. 4c). These results were also corroborated using another triple-negative murine breast cancer cell line,
Fig. 3 LN metastases up- or down-regulate cell cycle-associated genes in a plastic manner. a Pairwise gene expression array comparison between MFP- and AxLN-implanted tumors. b Pairwise gene expression array comparison between AxLN-implanted and AxLN-derived lung metastases. c Venn diagram showing shared genes between the analysis of a, b. d Six possible patterns of gene expression across the three experimental conditions (mammary fat pad, MFP, axillary LN, AxLN; lung metastasis derived from AxLN micro-injection, AxLN-LuM) shown in a, b, e Composite microarray results for the 152 genes that are up- or down-regulated in both pairwise tissue comparisons (cases 2 and 4 of d). Full 206 gene array shown in Supplementary Fig. 4. f Gene ontology analysis (biological processes) of the 152 genes displayed in e. g Cell growth assay for the ex vivo clones represented in the microarray analysis. Statistical significance was measured by unpaired t tests. h Average expression values (RT-qPCR) for several target genes revealed by the microarray analysis. All statistical comparisons are to the axillary LN tumor samples. Statistical significance was measured by ANOVA. i Relative expression of HDAC11 between cell lines derived from MFP tumors and matched de novo AxLN metastasis vs. micro-injected AxLNs obtained 1 or 2 weeks post injection. j Bisulfite sequencing of MFP, AxLN, and AxLN-LuM sub-clones at the HDAC11 promoter CpG island. Statistical significance was measured by unpaired t tests; p values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 4 HDAC11 suppression results in reduced lymph node growth but increased metastasis. a Relative expression of a set of down-regulated array genes after HDAC11 suppression by shRNA. Statistical significance was measured by ANOVA in comparison to the control shR samples. b Levels of immunoprecipitated promoter regions for IL-10, RRM2, E2F7, and E2F8 in human MDA-MB-231 cell lines expressing either control or HDAC11 ORFs when pulling down with either IgG control or HDAC11 antibodies. Statistical significance was measured by ANOVA. c Levels of immunoprecipitated promoter regions for RRM2, E2F7, and E2F8 in 4T1 cell lines stably expressing either control or HDAC11 shR when pulling down with either IgG control, acetyl-H3, or acetyl-H4 antibodies. Statistical significance was measured by ANOVA. d Western blots for E2F8 and RRM2 in 4T1 lines expressing control or HDAC11 shR. e LN tumor weights at day 35 following axillary LN micro-injection. f Take rates of LN-micro-injected 4T1-shHDAC11 cell lines compared to 4T1-shCtrl cells. p Value obtained using a χ^2 contingency test. g LN tumor volumes throughout the duration of the experiment (n = 13-14 mice/group). Statistical significance was measured by unpaired one-sided Student’s t tests. h Lung micro-metastasis enumeration for LN-micro-injected mice by mCh + flow cytometry. i Lung metastasis index after normalization to LN tumor size (n = 13-14 mice/group). Statistical significance was measured by unpaired one-sided Student’s t tests (h + i). j, k Disease-free survival curves from the BreastMark collection comparing patients with high and low levels of RRM2 in all available breast cancer samples (n = 2652 patients), as well as in LN+ cases (n = 744 patients). Statistical significance was measured by log-rank test.

P values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001.
HDAC11 inhibition increased distant metastasis from LNs. Unexpectedly, the inhibition of HDAC11 led to significantly increased LuM, especially when normalizing to the reduced AxLN tumor sizes (Fig. 4b, i). Consistent with these findings, HDAC11 silencing led to significantly increased migration in both 4T1 and E0771.LMB cells (Supplementary Fig. 8c+f). In support of decreased HDAC11 within LNs leading to increased RRM2 and distant metastasis, functioning as a “release mechanism,” we found that RRM2 is highly associated with poor disease-free survival in breast cancer, including patients with LN involvement at diagnosis (Fig. 4j, k).

Because HDACis are being evaluated in numerous clinical trials in breast and other solid tumor malignancies, we next investigated whether pharmacologic inhibition of HDAC11 would yield similar effects on LN tumor growth and metastasis. Quisinostat is the most potent inhibitor of HDAC11, with sub-nanomolar potency against HDACs 1, 2, 4 and 11. We found that HDAC11, but not HDAC1, 2, or 4, was upregulated in AxLN sub-clones (Fig. 5a). Treatment with quisinostat resulted in more significant cell growth inhibition in AxLN vs. MFP sub-clones with quisinostat (Supplementary Fig. 9a), suggesting that HDAC11 expression in AxLN sub-clones renders them more susceptible to HDACi. Quisinostat led to significant induction of HDAC11 target genes, yet only modest effects on genes commonly linked with an epithelial–mesenchymal transition (EMT) (Fig. 5b). Vorinostat and entinostat also led to similar dose-dependent inductions in HDAC11 target genes in a “cliff-like” pattern, whereby gene expression increased to a threshold, at which point considerable cell death was observed and gene levels dropped (Supplementary Fig. 9b, c). Protein expression of HDAC11 target genes RRM2 and E2F8 also increased with all 3 HDACis tested (Supplementary Fig. 9d). Based on these results, we selected sub-lethal HDACi doses that induced HDAC11 target genes for subsequent experiments (termed “sub-lethal” doses). Next, we tested the effect of sub-lethal HDACi-treatment on 4T1 growth and motility. While colony formation was considerably impacted (Supplementary Fig. 9e), transwell migration significantly increased following treatment with quisinostat, which we confirmed in E0771.LMB cells as well (Fig. 5c, d). To test the effect of quisinostat treatment on LN tumor growth and metastasis, we micro-injected AxLNs with 4T1-mCh/rL cells. After sub-palpable tumors were established as determined by luciferase signal, mice were randomly distributed and treated with either vehicle or quisinostat. Although quisinostat treatment significantly inhibited AxLN tumor growth, based on luciferase imaging and caliper measurements (Fig. 5e, f), quisinostat treatment significantly increased LuM (Fig. 5g). Notably, considering the differences in AxLN tumor size, quisinostat increased metastasis by over 5-fold (Fig. 5h). To determine whether these results were related to the effects of quisinostat on the host vs. a cancer cell autonomous mechanism, we performed an experimental metastasis assay in which 4T1-mCh/rL cells were treated in vitro for 2 weeks with vehicle or quisinostat. No difference in micro-metastasis was seen at 24 h, whereas the quisinostat-treated cells formed significantly more LuMs by 1 week, based on mCh+ counts and hematoxylin and eosin (H&E) staining (Fig. 5i, j). Furthermore, quisinostat pre-treatment increased the average size of the lung micro-metastases (Fig. 5k), perhaps related to an increased number of pro-migratory cells extravasating and forming distant metastatic colonies. Importantly, the increased metastases were not due to a “rebound effect” as has been reported for other therapeutic agents, as quisinostat withdrawal resulted in colony formation rates that were similar to vehicle-treated cells (Supplementary Fig. 9d). We observed similar increases in metastatic characteristics using sub-lethal doses of vorinostat and entinostat, both of which significantly impaired cell proliferation and colony formation, but led to significantly increased migration and experimental LuM formation (Supplementary Fig. 9e–j). Although these results corroborate the phenotypes observed with genetic knockdown of HDAC11, these pharmacologic inhibitors are not HDAC11-specific and may be affecting other HDACs. Thus, it is likely that other HDACs are also involved in the phenotypes observed.

Discussion
In nearly all cancer types, the presences of LN metastases are clinically significant and hold great prognostic power. Yet, until recently, there was no experimental evidence that LN metastases could give rise to distant metastases. Collectively, using phylogenetic analyses of clinical samples and direct experimental models, our results show that breast cancer LN metastases can give rise to distant metastases. Our findings that the lymphatic route is highly efficient to give rise to distant metastasis carries therapeutic implications, as almost all pre-clinical models assume that cancer metastasizes by direct intravasation into and then extravasation out of blood vessels. The limited success of anti-angiogenic therapies in patients suggests that current experimental models are not fully recapitulating the metastatic process, which may in part be because lymphatic metastases are largely ignored. Recently, it was found that anti-angiogenic therapies have little effect on LN metastases, and some anti-angiogenic therapies may even promote LN metastases formation.

Herein, we have also identified a mechanism by which dissemination through LNs occurs. We demonstrate that increased expression of a chromatin modifier, HDAC11, is important for tumorigenesis and growth within the LN, but that subsequent downregulation of HDAC11 in the LN results in increased migration and egress from LNs to distant sites. We found that HDAC11 inhibits E2F7 and E2F8, which are widely regarded as cell cycle suppressors, and this may in part explain HDAC11’s role in promoting cancer cell survival within LNs (Fig. 6, top). Additionally, we found loss of HDAC11 leads to de-repression of RRM2. We posit that this increase in RRM2, which has been linked to pro-migratory and metastatic phenotypes in many cancers, including breast cancer, as has been reported for other therapeutic agents. HDACi have been an attractive therapeutic strategy to both restore acetylation and gene expression with the potential benefit of being better tolerated than cytotoxic.
**Fig. 5** Pharmacological HDAC inhibition reduces cell and tumor growth, but increases cell migration and tumor metastasis from LNs. **a** HDAC mRNA expression levels from ex vivo 4T1 clones. **b** Target gene E2F7, E2F8, and RRM2 and EMT marker expression levels after quisinostat treatment. **c, d** Transwell migration assay for 4T1 (**c**) and E0771-LMB (**d**) TNBC cells. **e** Intravital imaging of LN tumors of mice implanted with 4T1-mCh/rL cells. **f** Tumor volumes for LN-micro-injected tumor-bearing mice treated with vehicle or quisinostat (40 mg/kg; twice weekly). **g** Enumeration of lung micro-metastases in LN-micro-injected tumor-bearing mice after 6 weeks. **h** Metastasis index of vehicle- and quisinostat-treated mice after normalization to LN tumor size (n = 10 mice/group for **f–h**). **i** Enumeration of lung micro-metastases after in vitro-treated vehicle or quisinostat 4T1-mCh/rL cells were injected by the tail vein. **j** H&E staining of stitched left lungs from tail vein-injected mice. The graph shows the average number of tumors per lung for vehicle- and quisinostat-treated mice. **k** Representative H&E-stained images of lung lesions from vehicle- and quisinostat-treated mice. The graph shows individual tumor diameter measurements. Statistical significance was measured by unpaired one-sided Student’s t tests, unless otherwise indicated; p values are indicated as *p < 0.05, **p < 0.01, and ***p < 0.001.

**Fig. 6** Graphical abstract of HDAC11’s role in regulating lymph node metastasis. As cancer cells enter the lymph node (top), HDAC11 increases in expression, leading to decreased expression of several transcripts and increased survival within the lymph node. However, as HDAC11 decreases (bottom), the same transcripts increase in expression, leading to an increased migratory phenotype and allowing exit from the lymph node to distant organs. Thus, pharmacologic inhibition of HDAC11 may decrease cancer cell survival within lymph nodes while also increasing their migratory capabilities.
chemotherapy. Epigenetic modulation has also been hypothesized to be a mechanism of resistance to endocrine therapies and cytotoxic chemotherapy. Despite the promising anti-tumor effects of quisinostat and other HDACis in pre-clinical models, when used as monotherapy it has had limited activity thus far in patients. Our results highlight the importance of evaluating candidate therapeutics in the context of LN metastasis, as well as the unique challenge of targeting plasticity in metastasis.

Similar to how blocking the EMT may simultaneously prevent detachment from the primary tumor and also promote metastatic colonization by inducing a mesenchymal-to-epithelial transition (MET), we demonstrate that the spectrum of HDAC1 expression along the metastatic cascade may have opposing effects if inhibited. Given the active investigation and development of multiple HDACis in cancer patients, our results strongly advise caution in the single-agent use of HDACi in the treatment of breast cancer and potentially other solid tumors.

**METHODS**

**Cell lines and key reagents.** 4T1 cells were obtained from the ATCC and maintained in RPMI containing 10% fetal bovine serum (FBS). EMT-derived LUAD cells were generously provided by Dr. Robin Anderson (Peter MacCallum Cancer Center). 4T1 cells were transduced with lentiviral constructs expressing either GFP/FL or mCherry/Renilla luciferase, which were obtained from Dr. Shawn Hingtgen (UNC) and were selected and maintained in puromycin (8 μg/ml). 4T1-shHDAC1 cells were selected and maintained in hygromycin (4 μg/ml). FLAG-HDAC1 was provided by Dr. Alejandro Villaraga (George Washington University). Quisinostat, vorinostat, and entinostat were purchased from Sellertech Inc. (South Dakota) and prepared as described. shRNA constructs targeting HDAC1 were obtained from Genecopoeia (shHDAC1 #1: CUAACAAGGUCCGLUUUGAGTT; shHDAC1 #2: GUCGACACCGAGGCAGUAUGTCT). shRNA constructs targeting HDAC1 were obtained from Genecopoeia (shHDAC1 #1: gctactacaagacttgca; shHDAC1 #2: ggacacctggaataaggat). All cells were routinely tested for mycoplasma using a Lonza MycoAlert Detection kit (LT07-418). Adult female Balb/c mice (6–8 weeks) were purchased from Taconic Farms. Animal care was approved for according to Good Laboratory Practice guidelines set forth by the American Society for Accreditation of Laboratory Animal Care and the US Public Health Service policy on Human Care and Use of Laboratory Animals. All mouse studies were approved and supervised by the North Carolina Institute at Chapel Hill Institutional Animal Care and Use Committee.

**Rapid autopsy analysis.** From the UNC Breast Cancer Rapid Autopsy Program, all clinical samples were obtained and prepared following informed consent and institutional review board approval for UNC CHS protocol 12-0690. RNA was extracted from primary, LN, and distant metastatic samples in parallel. By using a computational re-interrogation step to improve mutation calling sensitivity, we were able to compare different sample types. The comparisons performed were: (i) MFP vs. Axl-N, (ii) MFP vs. Axl-LN, (iii) MFP vs. Axl-LM, (iv) MFP vs. Axl-LuM, (v) Axl-N vs. Axl-LN, (vi) Axl-N vs. Axl-LM, and (vii) Axl-N vs. Axl-LuM. Overall, we compared different sample types. The comparisons performed were: (i) MFP vs. Axl-N, (ii) MFP vs. Axl-LN, (iii) MFP vs. Axl-LM, (iv) MFP vs. Axl-LuM, (v) Axl-N vs. Axl-LN, (vi) Axl-N vs. Axl-LM, and (vii) Axl-N vs. Axl-LuM. The results of these comparisons (based on the third point above illustrated for determining genes differentially expressed) were displayed separately. Of course, no other comparisons were possible, since Axl-N vs. MFP is equivalent to MFP vs. Axl-N, and so on. Because of the way in which genes are selected before being displayed through simple combinatorial patterns, the same genes were specifically associated with each gene identified by (i), (ii), and (iii) are, respectively, 1.9980e − 04, 1.9980e − 04, and 2.0568e − 05. At this point, we looked for patterns of gene expression across these three sample types, that is, for the set MFP-Axl-N-LuM. The possible patterns of gene expression across a vocabulary containing only the words “Up” and “Down” are: (a) Up-Up-Up, (b) Down-Down-Down, (c) Up-Up-Down, (d) Down-Up-Up, (e) Up-Down-Up, and (f) Down-Up-Down. Cases a and b are considered as pattern/anti-pattern, and the same is true for cases c and d and for e and f. Due to the chosen criteria, after RNAs are collected for the heat map MFP-Axl-N-LuM following this computational protocol, it is still necessary to run an additional algorithm for associating possible genes belonging to multiple such patterns. The algorithm assumes that when a gene reaches its highest average expression in a sample type, then that sample type receives the tag Up for that gene. Similarly, when a gene reaches its lowest average expression in a sample type, then that sample type receives the tag Down for that gene. Finally, the sample type having an interimmediate average expression level is tagged as Up or Down. The pattern measuring its distance from the other two sample types and assuming that if the minimum distance is from the ‘Up’ sample type, the sample belongs to the ‘Up’ sample type too, while if the minimum distance is from what is tagged as Down, the sample type is tagged as Down too. The distance d used for this purpose is such that for two patterns P< subscripts x > and P< subscripts y > where D< subscripts x > and D< subscripts y > are the distances between the two patterns, then d (X, Y) = min (f (x < subscripts j > − y < subscripts j >)). In our case, p = q = 3. Notably, in this computational framework, all the samples of a sample type are always tagged with the same tag with respect to an included gene and algorithm-based tagging is separately made for each gene. At the end of these steps, it was possible to univocally calculate how many genes belong to each of these patterns for MFP-Axl-N-LuM. Later, the three couples of patterns/anti-patterns for the sample set MFP-Axl-N-LuM were separated and plotted in three distinct heat maps. Each hierarchical clustering was performed with respect to the matrix rows (containing the gene expression values across all samples) on log 2-transformed, mean-centered, and standard deviation normalized data. The results of this clustering were then split into its top and bottom parts, looking at the couple of genes of the heat map where a change of sign in the corresponding cdf file was present for the “divergent” sample type. We call “divergent” the sample type whose pattern tag is different from the other two (e.g., in the pattern c, Up-Down-Down, the divergent sample type is the first, MFP). For the particular, the gene expression profile across the divergent samples in the cdf file was considered the last gene of the “top” part and the first gene having the opposite sign was considered the first of the...
"bottom" part. After this, genes belonging to each half were separately processed using the Expression Analysis Systematic Explorer score (a p value derived from an ANOVA). The median threshold was used to distinguish high (above median) and low (below median) expression of RR2. Disease-free survival analysis was determined using the log-rank test for all patients (inclusive of luminal A, luminal B, Her2−, basal-like and normal-like subtypes) and for patients with positive LNs at the time of diagnosis.

Real-time qPCR. After treatment, RNA was purified from cells using the Zymo Quick RNA miniprep kit according to the protocol recommended by the manufacturer (Zymo Research, Irbil, CA). Then, complementary DNA (cDNA) was synthesized using a Bio-Rad iScript cDNA synthesis kit (1708891) for total RNA. cDNA was analyzed using SYBR Green reagent (Bio-Rad, 1525271) according to the protocol recommended by the manufacturer. PCR was done with reverse-transcribed RNA, 1 μl each of 20 μM forward and reverse primers, and 2× PowerUp SYBR Green Master Mix in a total volume of 25 μl. Data were analyzed using the ΔΔCT method, and experiments were normalized to 18S rRNA. Primer sequences included the following: Hdc1 forward (Fwd), AATGGGCGGAAGTGATCACA and reverse (Rev), GCCACAAAGAAAGACCTAC; Rrm2 Fwd, ACGAGTCCTCACTGGGCGCAG and Rev, CATTAAATCCAGCAGGCTG and Rev, GAGAAGATCTCCACCCGAGT and Rev, CTCAATGCGCAGGAGCT; Plk1 Fwd, GTCGAAAGCTTCGGGGACAGCAG and Rev, CAGGTCACATGCGTCTCTGCTT; Hdc1 Fwd, AGTGTTGTCTGAGTGGTGGCTGC and Rev, TGCTGCGCTTCGCTTCT; Hdc2 Fwd, GATGCGTGTTAGTGTGGTTGAGT and Rev, GAAATGCGGTATGATGACTGATG; Hdc3 Fwd, CTCTCCGAGCTGTTCT; Hdc4 Fwd, AGCGTACCTTCTGGGATTTTC and Rev, GGATCGTAGTCCCCGCTAA; E2F8 Fwd, AACTTTTCCCCCAACTCTGC and Rev, GCCGAGCCAAAGGAAGACCTAC; CDC25A Fwd, AGAGGATGAAAAACGAGACGGTTTTC and Rev, GCTGGGAACGTTACAAAAGC; TIPIN Fwd, GAGCTGTTTCGAGGACCGT and Rev, CATTACGCTCAGGATGTGAT; GHG Fwd, CCATGACCTGCCGTCTAGAAAAACCT and Rev, CCATGAGGTCCACCA; Twist Fwd, TGATCAAC and reverse (Rev), GCCACCACAAAGGACCACT; Rrm2 Fwd, AGAAAATCTGAGCCAGGACCA and Rev, CTTCCACACTCCACCTG and Rev, GACATCTTACCGGGTCTAC; CDC7 Fwd, GAGAAAAAACCCACACCTGTT and Rev, CCTCAGGAAGACCTCCACGCT and Rev, TTAAGAGCCACCAACACCC.

Transfection. 4T1 cells were plated at 40% confluence in 6-well plates, after which the cells were transfected with siHdac11 or siCtrl duplexes (60 nM final concentration; Sigma, St. Louis, MO) using RNAiMAX according to the protocol recommended by the manufacturer. The transfection was conducted for the indicated amount of time before the cells were collected and analyzed. The following siRNA constructs were used (sense strand displayed): siCtrl, UUCUCCGAAUGAAACCGUGACG; siHdac11-1, UGAUCUACCGUGGCUCCCGC. Then, viral particles were added to the cells in the presence of 8 μg/ml Polybrene. After overnight infection, the media were replaced. Hygromycin (50 μg/ml) or puromycin (8 μg/ml) was added to the media to select for transfected cells.

HDACi treatment. 4T1 cells were treated in 10-cm tissue culture dishes at the indicated concentration of drug (dissolved in dimethyl sulfoxide). Media and drug were refreshed daily to ensure consistent drug exposure. For long-term treatment, cells were exposed to HDACi for at least 7 consecutive days. The sub-lethal dose for quisinostat in 4T1 cells was determined to be 10 nM, whereas vorinostat was 500 nM and entinostat was 2 μM. For the quisinostat withdrawal experiment, cells were refreshed daily to ensure consistent drug exposure. For long-term treatment, cells were exposed to HDACi for at least 7 consecutive days. The sub-lethal dose for quisinostat in 4T1 cells was determined to be 10 nM, whereas vorinostat was 500 nM and entinostat was 2 μM. For the quisinostat withdrawal experiment, cells were refreshed daily to ensure consistent drug exposure.

Haptotaxis/chemotaxis migration assays. A total of 25,000 or 50,000 cells were seeded in serum-free medium to Boyden chambers (8-μm pores) that were precoated with 10 μg/ml type 1 rat tail collagen on the bottom of the inserts. RPMI medium containing 10% FBS was added to the lower chambers as the chemotactic source. Haptotaxis/chemotaxis was allowed to proceed for 18 h, after which cells were removed from the top chambers, and cells migrated to the bottom of the filter were fixed and stained using the Protocol Hema 3 staining kit (Fisher Scientific, 2212291). Membranes were mounted onto glass slides, and images were taken using a Nikon microscope. Migrated cells were enumerated using CellProfiler open source image analysis software (CellProfiler).

Cancer cell implantation. Adult Balb/c mice were purchased from Taconic Farms and C57Bl/6 mice were purchased from Jackson Labs. These animals were cared for according to guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the US Public Health Service policy on Human Care and Use of Laboratory Animals. All mouse studies were approved and supervised by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. All animals used were between 6 and 10 weeks of age at the time of injection. For all animal studies, experiments, cells were transplanted, washed, and resuspended in Hank’s balanced salt solution (HBSS; Gibco) prior to injection. Mammary fat pad. Cells were trypsinized and suspended in Matrigel at a 1:1 ratio, and 5000 cells were injected directly into the eighth MFP of anesthetized 6–10-week-old female Balb/c mice. Caliper measurements of subcutaneous tumor growth were taken twice weekly and tumor volume was calculated as L x W^2, where L is the greatest cross-sectional length across the tumor and W is the length perpendicular to L. Luciferase-labeled tumor progression was monitored once
Data availability
The microarray data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) data bank, accession code (GSE16033).

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Statistical analysis for in vitro and in vivo experiments. Between 5 and 15 mice were assigned per treatment group; this sample size gave ~80% power to detect a 50% change in tumor weight with 95% confidence. Results for each group were compared using Student’s t test (for comparisons of two groups) and analysis of variance (for multiple group comparisons). For values that were normally distributed (as determined by the Kolmogorov–Smirnov test), the Mann–Whitney rank-sum test was used. A P value <0.05 was deemed statistically significant. All other statistical tests for in vitro and in vivo experiments were performed using GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA). The multiple hypothesis testing correction of these results was made using the false discovery rate.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
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Conceptualization: C.V.P.; experimental design: P.L.L., Y.L.C., C.V.P.; data curation: A.P., Y.-H.T., J.S.P., C.V.P.; writing: P.L.L., Y.L.C., S.K.G., A.P., Y.-H.T., J.S.P., C.V.P.; funding acquisition: C.V.P., L.A.C.; investigation: P.L.L., Y.L.C., S.K.G., B.C.C.; resources: C.V.P., L.A.C.; software: A.P., Y.-H.T., J.S.P.; validation: P.L.L., Y.L.C., S.K.G.; visualization: P.L.L., C.V.P.; writing—original draft: P.L.L., Y.L.C., C.V.P.; writing—review and editing: all authors.

Additional information

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