

An Integrated Systems Biology Approach Identifies TRIM25 as a Key Determinant of Breast Cancer Metastasis

[Graphical Abstract](#)

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In Brief

Aberrantly activated transcriptional networks drive metastatic dissemination. Walsh et al. reverse engineer a breast cancer-specific regulatory network, uncovering a transcriptional hierarchy underlying breast cancer metastasis. Findings suggest that collapsing transcriptional hierarchies by targeting keystone proteins, such as TRIM25, is critical to affect the coordinated transcriptomic reprogramming required for metastasis.

[Highlights](#)

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SUMMARY

At the root of most fatal malignancies are aberrantly activated transcriptional networks that drive metastatic dissemination. Although individual metastasis-associated genes have been described, the complex regulatory networks presiding over the initiation and maintenance of metastatic tumors are still poorly understood. There is untapped value in identifying therapeutic targets that broadly govern coordinated transcriptional modules dictating metastatic progression. Here, we reverse engineered and interrogated a breast cancer-specific transcriptional interaction network (interactome) to define transcriptional control structures causally responsible for regulating genetic programs underlying breast cancer metastasis in individual patients. Our analyses confirmed established pro-metastatic transcription factors, and they uncovered TRIM25 as a key regulator of metastasis-related transcriptional programs. Further, *in vivo* analyses established TRIM25 as a potent regulator of metastatic disease and poor survival outcome. Our findings suggest that identifying and targeting keystone proteins, like TRIM25, can effectively collapse transcriptional hierarchies necessary for metastasis formation, thus representing an innovative cancer intervention strategy.

INTRODUCTION

Metastatic progression is the primary underlying cause of breast cancer-associated death (Spano et al., 2012). Although both functional and genomic studies have identified important genetic aberrations associated with risk of malignant progression (Navin, 2015), the fundamental drivers and the associated complex mechanisms of breast cancer metastasis remain poorly mapped and poorly understood. Specifically, several pathways have been identified as contributors to metastatic disease (Hanahan and Weinberg, 2011), but identification and dissection of the regulatory machinery that is necessary and sufficient to implement metastatic progression (metastasis checkpoints) remain critically unanswered questions in cancer research.

To systematically and quantitatively address this, we approached metastatic progression as a transition between two cellular states defined by the differential gene expression signature of these states in the same patient, using patient-matched primary and metastatic samples. Then we investigated the specific transcriptional regulators responsible for initiating this transition, and ultimately for maintaining the stability of the metastatic state, based on their mechanistic ability to regulate differentially expressed genes (i.e., the transition signature). We have shown that the specific transcriptional regulators that determine such cancer-related state transitions can be efficiently and systematically elucidated by interrogating tumor-specific transcriptional networks (henceforth interactomes) with representative differential gene expression signatures, using the virtual inference of protein activity by regulon enrichment analysis (VIPER) algorithm (Alvarez et al., 2016), which further extends the master regulator inference algorithm



(MARINa) (Lefebvre et al., 2010) to the analysis of single samples. These validated analyses have helped elucidate master regulator (MR) proteins whose concerted, aberrant activity is both necessary and sufficient for tumor phenotype implementation and maintenance, including tumor initiation (Aytes et al., 2014; Carro et al., 2010), progression (Aytes et al., 2014; Lefebvre et al., 2010), and drug resistance (Piovan et al., 2013; Rodriguez-Barrueco et al., 2015).

To implement this approach, we first assembled a breast cancer-specific regulatory network, using the algorithm for the reconstruction of accurate cellular networks (ARACNe) (Basso et al., 2005; Margolin et al., 2006b). Then we identified differential gene expression signatures representing same-patient cell state transitions from primary tumors to lymph node metastases, using both ER-positive (ER+) and triple-negative breast cancer (TNBC) samples. Finally, we used the VIPER algorithm to prioritize transcriptional regulators that are the most likely causal determinants of these metastasis-related signatures. Using this strategy, we uncovered a hierarchy of transcriptional regulators controlling metastatic progression, revealing both previously unknown and well-established pro-metastatic transcription factors, thus confirming the potential value of the proposed methodology.

RESULTS

Identifying Keystone Transcriptional Regulators of Breast Cancer Metastasis

To identify the transcriptional machinery driving breast cancer metastasis (Figure 1A), we first characterized the transcriptional signature representative of breast carcinoma metastatic progression (metastasis [MET] gene expression signature [MET-GES]). This was achieved by differential expression analysis of patient-matched primary tumors and lymph node metastases from 20 ER+ and 11 TNBC patients (Figure S1A; Table S1). Critically, two-tail gene set enrichment analysis (GSEA2) of these signatures revealed strong concordance across the two subtypes, suggesting the existence of common metastatic progression mechanisms (Figures S1B–S1D), independent of hormonal state.

To identify the genes that causally implement the MET-GES, thus representing candidate causal determinants of breast carcinoma metastatic progression, we utilized the VIPER algorithm (Aytes et al., 2014; Carro et al., 2010). To assemble a breast carcinoma-specific interactome, we analyzed 851 The Cancer

Genome Atlas (TCGA) breast carcinoma gene expression profiles using the ARACNe algorithm (Basso et al., 2005; Margolin et al., 2006a). ARACNe is an information theory-based approach to infer mechanistic interactions between transcription factors (TFs) and target genes based on large sets of gene expression data, which has proven very effective in assembling interactomes for VIPER analysis (Alvarez et al., 2016; Basso et al., 2010; Carro et al., 2010; Kushwaha et al., 2015; Lefebvre et al., 2010). The ARACNe-inferred breast cancer interactome included 1,748 TFs, regulating 18,783 target genes through 365,634 transcriptional interactions. A schematic representation of our pipeline is represented in Figure 1A.

Finally, we inferred the regulatory proteins that are candidate drivers of the MET-GES by VIPER analysis of the breast carcinoma interactome. The algorithm prioritizes the regulatory proteins that are the most likely determinants of an observed differential expression signature, and thus of the associated cell state transition, by assessing the enrichment of their direct targets (regulons) in differentially expressed signature genes (i.e., in genes that are over- or underexpressed during metastatic progression, in this case). Thus, the ARACNe-inferred regulon of each regulatory protein (Table S2) is used as a highly multiplexed, endogenous reporter for its role in physically controlling metastatic progression. We used the statistical significance, estimated by sample permutation analysis, to rank-sort the list of putative MRs of the metastatic phenotype in ER+ breast cancer and TNBC (Table S3). Given the strong concordance between ER+ and TNBC MET-GES and between candidate MRs of the two subtypes (Figures S1B–S1D), we inferred the MRs of metastatic progression of breast carcinoma regardless of subtype by VIPER analysis of a subtype-agnostic MET-GES. This generated a single ranked list of breast cancer metastatic progression candidate MRs (breast cancer [BRCA]), independent of hormonal status (Figure 1B; Table S3). From these analyses, TRIM25 emerged by far as the strongest candidate MR ($p = 3.25 \times 10^{-5}$). A schematic summary of these data is shown in Figure S1E.

Of the top candidate MRs, most are largely unexplored metastasis-associated genes, ranking significantly higher than well-known pro-metastatic TFs, such as c-MYC (ranked 585), ETV4 (ranked 1,443), and SMAD4 (ranked 1,447). Nevertheless, the high ranking of breast cancer-specific metastasis-associated TFs, such as PRRX-1 (ranked 6, $p = 6.8 \times 10^{-4}$) further corroborates the accuracy of our approach. Importantly, top candidate MR genes were not highly differentially expressed following

Figure 1. Transcriptome Analysis of Matched Primary Tumors and Lymph Node Metastasis in ER+ and TNBC

(A) Schematic regulators of the metastasis phenotype (MRs) in breast carcinoma, representing the strategy used to generate VIPER-inferred candidate MRs of the metastatic state.

metastatic progression. In fact, none of the top 15 candidate MR genes were in the top 100 most differentially expressed genes, and, therefore, they could not have been identified without direct analysis of their regulatory context. This further emphasizes the distinct advantage of the proposed methodology. As previously shown (Chen et al., 2014), regulatory proteins with the highest probability to directly regulate the signature of interest are more likely to be critically necessary to implement the associated cell state transition, because they represent the most proximal integrators (i.e., regulatory bottlenecks) of genetic and epigenetic determinants of the associated phenotype. This suggests that TRIM25 may be a more universal determinant of metastatic progression in breast cancer as compared to previously identified genes that appear further down in the prioritized MR list.

We based the analysis on an average gene expression signature of metastatic progression across all ER+ and TNBC METs. Thus, while it is effective in identifying mechanisms that are common across most of the patients, it fails to capture mechanisms that may be patient specific. We thus extended our analysis at the single-patient level by inferring the transcriptional regulators driving each patient's metastatic progression with the VIPER algorithm (Alvarez et al., 2016). This analysis also identified TRIM25 as the most consistently activated regulator in these metastases (Figure 1C), further supporting its role as a key regulator of the metastatic progression in both ER+ and TNBC tumors.

TRIM25 Is a Global Regulator of Breast Cancer Metastasis Signature

To functionally test these hypotheses, we performed small interfering RNA (siRNA)-mediated knockdown for a selected set of 14 MRs in 2 ER+ (MCF7 and T-47D) and 2 TNBC (BT549 and MDA-MB-231) cell lines, and we analyzed subsequent changes in gene expression using RNA sequencing (RNA-seq) (Table S4). The MR genes selected for validation represent the top most differentially activated TFs in each of the patient-derived signatures, including the breast carcinoma BRCA MET-GES, the ER+ MET-GES, and the TNBC MET-GES (Figures 1B, 1C, and S2A; Table S3). MRs were prioritized by stepwise linear regression with exhaustive selection to first identify the best combination of MRs controlling the metastatic progression signature and then to compute the fraction of metastasis signature genes regulated by each MR, using GSEA leading-edge analysis (Subramanian et al., 2005). The best linear combination of n MRs, where n was the highest number of MRs for which all coefficients of the model were different than zero at Bonferroni's corrected $p < 0.001$, was selected by maximizing the variance explained by the model (Figures S2B and S2C; Table S5). Employing a complementary approach, we repeated this analysis by separately comparing the expression between metastasis and primary tumor for each individual patient. Stepwise linear regression with exhaustive search was applied to each gene expression signature and then integrated by the Stouffer method. Results were very similar using either method (Table S5).

The identification of established metastasis-associated genes, such as *PRRX-1*, *FOXQ1*, and *PAX5* (O'Brien et al., 2011; Ocaña et al., 2012; Ratkaj et al., 2010; Zhang et al., 2011), demonstrates the robustness of our methodology toward elucidating meta-

static progression drivers. Based on these analyses, TRIM25 emerged as the strongest (i.e., most statistically significant) MR candidate using both TNBC cell lines and patient breast tumor-derived signatures (Figures S2B and S2C). TRIM25 also significantly regulated the ER+ MET-GES in 1 ER+ cell line (Table S5). Critically, siRNA-mediated silencing of *TRIM25* strongly affected 50% of the top 20 VIPER-inferred candidate MRs (Table S4), demonstrating that TRIM25 can coordinate multiple downstream transcriptional programs to implement the metastatic progression transcriptional signature. The predicted regulatory relationship between TRIM25 and target transcripts is shown in Figure 1D.

TRIM25 Expression Is Not Correlated with Estrogen Signaling

TRIM25 was originally named estrogen-responsive finger protein (EFP), and it has since been described as an estrogen-responsive gene and a co-factor for estrogen signaling. Therefore, the identification of TRIM25 as the most statistically significant candidate metastatic progression MR in TNBC was surprising. Yet, when we compared *TRIM25* expression in ER+ and TNBC tumors from TCGA, it was actually significantly higher in the latter (Figure S2D). Consistent with these findings, we did not observe estrogen-dependent expression of *TRIM25* in multiple high-throughput studies that test the effects of estrogen on gene expression (Carroll et al., 2006; Creighton et al., 2006; Kamalakaran et al., 2005; Lin et al., 2004).

To further investigate the relationship between *TRIM25* and estrogen signaling, we generated a Spearman's correlation matrix using RNA-seq expression data from 1,100 breast cancer tumors, comparing *TRIM25* expression with ten established estrogen-responsive genes (Figure S2E). As expected, Estrogen receptor 1 (*ESR1*) expression correlated strongly with all ten estrogen-responsive genes. Yet, despite being expressed in ER+ tumors, expression of *TRIM25* was not correlated with the expression of *ESR1* or other estrogen-responsive genes. For comparison, we also included the Y box-binding protein 1 (*YBX1*) in these analyses, a gene specifically associated with estrogen-negative tumors. While the analysis revealed significant negative correlation between *YBX1* expression and the expression of estrogen-responsive genes, as well as *ESR1*, no correlation was detected with *TRIM25* expression. Taken together, these results suggest that *TRIM25* expression can be regulated by factors independent of estrogen or estrogen signaling, especially in TNBCs.

TRIM25 Mediates Breast Cancer Metastasis in Human Xenograft Models

To test the effect of TRIM25 on the metastatic potential of TNBC cells, we engineered human MDA-MB-231 cells, derived from a TNBC tumor, to express either high (*TRIM25*^{high}) or low (*TRIM25*^{low}) levels of TRIM25 (Figure S3A), and we labeled them with Luc-GFP or Luc-RFP. Distinctly labeled *TRIM25*^{high} and *TRIM25*^{low} cells were mixed 1:1, injected into the lateral tail vein of athymic nude mice, and monitored for the development of lung metastases via bioluminescence imaging. When lung metastases were visible, mice were sacrificed and their lungs were first imaged via ex vivo fluorescence imaging and



spectral unmixing for a global picture of the relative abundance of TRIM25^{high} versus TRIM25^{low} cells (Figure 2A). Lungs were then sectioned and the percentage content of TRIM25^{high} versus TRIM25^{low} cells was accurately quantified via immunofluorescence (Figures 2B and 2C). We found that TRIM25^{high} cells constituted the overwhelming majority of metastatic burden in the lungs compared to TRIM25^{low} cells (Figures 2A–2C). We confirmed these results in parallel experiments, in which TRIM25^{high} and TRIM25^{low} fluorophores were switched.

Intriguingly, increased expression of *TRIM25* did not affect proliferation or invasion of MDA-MB-231 cells in vitro (Figures S3B and S3C). We then used a primary culture generated from a patient-derived xenograft (PDX) model of TNBC to test the effect of *TRIM25* perturbation on primary tumor growth in vivo. Consistent with the in vitro assays, we found that neither *TRIM25* knockdown nor overexpression altered primary tumor growth in the mammary fat pad of NOD scid gamma (NSG) mice (Figures S3D–S3F). Similarly, when we injected the aforementioned fluorescently labeled TRIM25^{high}:TRIM25^{low} cell mixture into the mammary fat pad of NSG mice, we found that TRIM25 did not affect cellular proliferation at the orthotopic site, with TRIM25^{high} and TRIM25^{low} cells showing uniform representation in primary tumors >1 cm in diameter (Figure 2D). Notably, when these mice became symptomatic and lungs were harvested, we found that TRIM25^{high} cells metastasized to the lung more efficiently than TRIM25^{low} cells, despite representing a similar fraction in corresponding primary tumors (Figures 2E and 2F). Interestingly, perturbation of *TRIM25* expression in MDA-MB-231 cells injected into the mammary fat pad of NSG mice in limiting dilution experiments revealed that *TRIM25* knockdown resulted in decreased seeding potential, suggesting TRIM25 may be involved in promoting self-renewal (a stem-like phenotype) (Figure S3G). We further explored the relationship between TRIM25 and metastasis in an additional TNBC cell line, CAL-51. *TRIM25* knockdown in CAL-51 cells injected into the lateral tail vein of athymic nude mice resulted in significantly decreased lung metastases (Figures 2G and S3H). Lungs were stained with anti-human COX4 to clearly differentiate human CAL-51 metastases from mouse lung tissue (Figure 2G). Overexpression of *TRIM25* in CAL-51 cells resulted in an ~2-fold increase in lung metastases ($p = 0.10$; Figures 2H and S3H). This increase was not due to an increase in extravasa-

tion (Figure S3I). Together these data demonstrate that TRIM25 is a potent regulator of TNBC metastasis.

The E3 Ubiquitin Ligase Activity of TRIM25 in Promoting Metastasis

To determine whether the E3 ubiquitin ligase domain of TRIM25 was required for TRIM25 to promote metastasis, first we employed a CRISPR strategy to knock out TRIM25 (TKO) in 2 TNBC cell lines (Figures 3A and S4A). Then, we expressed either full-length TRIM25 (TRIM25) or a RING-deficient TRIM25 (TRIM25ΔR) in these cells (Figures 3B and 3C). The lack of E3 ubiquitin ligase activity was confirmed in TRIM25ΔR cells (Figure S4B). Tail vein-injected CAL-51-TKO cells expressing either TRIM25 or TRIM25ΔR rescued the metastasis phenotype to a similar extent, suggesting that the E3 ubiquitin ligase activity of TRIM25 is not required (at least entirely) for promoting metastasis in this context (Figure 3D). In contrast, orthotopic injection of BT549-TKO cells expressing TRIM25ΔR only partially rescued the seeding potential of TNBCs that expressed full-length TRIM25 (Figure 3E), suggesting that the E3 ubiquitin ligase domain of TRIM25 may, in part, contribute to tumorigenicity and, therefore, metastatic potential. Interestingly, the expression of TRIM25 or TRIM25ΔR did not alter the localization of TRIM25 in TNBCs via immunofluorescence (Figure S4C). Consistent with the role of TRIM25 in promoting metastasis, expression of TRIM25 or TRIM25ΔR (Figure S4D) in an already highly metastatic TNBC line did not significantly affect primary tumor growth or spontaneous lung metastasis (Figures 3F and 3G). Further, injection of these cells into the lateral tail vein of nude mice revealed no differences in metastatic seeding in the lung (Figure 3H). Together these data suggest TRIM25 promotes metastasis through multiple mechanisms, with at least one being independent of its E3 ligase activity.

TRIM25-Mediated Transcriptional Regulation of the MET-GES

Since TRIM25 contains a hallmark zinc-finger B-box DNA-binding domain (Figure S5A), we hypothesized that it regulates the BRCA metastatic signature, at least in part, as a bona fide TF. Cell fractionation of ER+ breast cancer and TNBC cells revealed that TRIM25 is present in the cytoplasmic, nuclear, and chromatin fractions (Figures 4A and S5B). Immunofluorescence

Figure 2. TRIM25 Affects the Metastatic Potential of TNBC Cells



revealed TRIM25 was most abundantly localized to the peri-nuclear region (Figure 4B). To investigate the DNA-binding properties of TRIM25, we performed chromatin immunoprecipitation sequencing (ChIP-seq) in the TNBC cell lines BT549 and MDA-MB-231. TRIM25 ChIP antibody was validated according to Encyclopedia of DNA Elements (ENCODE) guidelines (Landt et al., 2012), and a representative immunoprecipitation (IP) is shown in Figure S5C. The distribution of ChIP-seq peaks (genome ontology) is shown in Figures 4C and 4D. Interestingly, TRIM25 primarily associated with DNA at CpG islands and gene promoters. Several ChIP targets were selected for validation by qPCR (Figure S5D). When we examined the metastasis signature genes by plotting TRIM25 ChIP-seq reads (input) for the top 876 genes ($p < 0.05$) from the TNBC MET-GES, we observed that TRIM25 mainly localizes to the transcriptional start site (TSS) of these genes (Figures 4E–4H).

To further refine the identity of genes transcriptionally regulated by TRIM25, we performed iterative GSEA (iGSEA) while incrementally increasing the number of top TRIM25-bound genes, based on TSS peak scores, compared to the cell line data in which TRIM25 was silenced (GES) as negative control. The TRIM25-bound gene set was determined as the smallest set for which a maximum GSEA enrichment score could be obtained (Figure 4I). These TRIM25-bound genes were significantly enriched in genes that were upregulated following siRNA-mediated silencing of *TRIM25* (siTRIM25-GES), both in BT549 and in MDA-MB-231 cells. This was consistent with TRIM25 functioning, at least in part, as a transcriptional repressor (Figures 4J and 4K). Specific overlap is shown in Figures S5E and S5F and Table S4. Further, iGSEA inferred TRIM25-bound genes were significantly enriched among the downregulated MET-GES genes for both BT549 and MDA-MB-231 cells, again suggesting that TRIM25 may function as a transcriptional repressor to regulate the MET-GES (Figures 4L and 4M). Specific overlap is represented in Figures S5G and S5H and Table S4.

TRIM25 Mediates MET-GES Regulation at the Post-transcriptional Level

TRIM25 is also an established RNA-binding protein (Choudhury et al., 2014). Therefore, we assessed whether TRIM25 physically interacts with the mRNA of metastasis-associated genes and whether this binding was associated with their expression levels. To test this hypothesis, we performed RNA immunoprecipitation sequencing (RIP-seq) in BT549 and MDA-MB-231 cells to identify TRIM25-bound RNA species, which were scored and ranked

(see the Experimental Procedures) (Table S4). We randomly chose 3 positive and 3 negative RIP-seq target genes to confirm by qRT-PCR (Figure S6A). Following iGSEA (Figure 5A), as described in the previous section, we detected a strong enrichment of TRIM25-bound RNAs among genes that were elevated upon knockdown of *TRIM25* in both MDA-MB-231 and BT549 cells (Figures 5B and 5C), suggesting that TRIM25 negatively regulates the abundance of these transcripts. The specific overlap is represented in Figures S6B and S6C. Similarly, we found enrichment of the top iGSEA-inferred TRIM25-bound RNAs among genes that were reduced in the MET-GES, again suggesting that TRIM25 may negatively regulate the abundance of these MET-GES genes (Figures 5D and 5E). Specific overlap is represented in Figures S6D and S6E and Table S4.

TRIM25 Regulates the MET-GES through Transcriptional and Post-transcriptional Mechanisms

Next, we proceeded to examine the relationship between transcriptional and post-transcriptional regulation of gene expression, as mediated by TRIM25. First, to identify the core set of genes transcriptionally regulated by TRIM25 in TNBC, we integrated the TRIM25 ChIP-seq results with gene expression changes resulting from *TRIM25* knockdown in cell lines. While the former indicates binding of TRIM25 to promoter regions, the latter provides clues about causal regulatory effects on transcription. Confirming our analysis and the ARACNe-inferred regulon of TRIM25, these core genes were significantly enriched in genes downregulated in the MET-GES (Figure S6F). In parallel, we identified a core set of genes post-transcriptionally regulated by TRIM25 in TNBC by integrating the TRIM25 RIP-seq results with gene expression profiles following siRNA-mediated silencing of *TRIM25* in TNBC cell lines. This second core gene set, which is post-transcriptionally regulated by TRIM25, was also significantly enriched in genes downregulated in the MET-GES (Figure S6G). Remarkably, we also found significant overlap between the transcriptional and post-transcriptional TRIM25-regulated core gene sets (Figure 5F), suggesting that TRIM25 regulates the expression of these genes at both transcriptional and post-transcriptional levels, thus implementing a classic feedforward loop structure.

To gain insight into the potentially distinct biological processes mediated by TRIM25 transcriptionally versus post-transcriptionally, we performed pathway enrichment analysis, using Kyoto Encyclopedia of Genes and Genomes (KEGG), Biocarta, and Reactome pathways from the Molecular Signature Database (MSigDB) (Subramanian et al., 2005), on these core gene sets.



We found a striking overlap between processes regulated by TRIM25 transcriptionally and post-transcriptionally (Figure 5G; Table S6), suggesting that TRIM25 may control these biological processes through tight transcriptional and post-transcriptional mechanisms.

TRIM25 Promotes Tumorigenicity and a Stemness Phenotype

To assess which cellular processes associated with metastasis are modulated by TRIM25, we first inferred the highest confidence MET-GES genes transcriptionally and post-transcriptionally regulated by TRIM25 as the genes in the leading edge when computing the enrichment of the core set of genes transcriptionally or post-transcriptionally regulated by TRIM25 on the MET-GES (Figure S7A). Next, we performed gene ontology (GO) analysis on these gene sets. Enriched biological processes were dominated by developmental processes (Figure 6A), suggesting that TRIM25 may mediate metastases by affecting differentiation.

Next we tested whether altering more than one MR, which together more broadly cover the MET-GES, is sufficient to enhance tumor implantation in an otherwise poorly tumorigenic breast cancer cell line, BT549. We found that modulation of *TRIM25* and zinc-finger protein 581 (*ZNF581*) in combination yielded the most substantial coverage of the MET-GES (61%) compared to all other pairs of candidates (Figures S2B and S2C; Table S5). We therefore overexpressed *TRIM25* and knocked down *ZNF581* in BT549 cells (Figure S7B), and we performed a limiting dilution assay in the mammary fat pads of NSG mice. Concomitant *TRIM25* overexpression and *ZNF581* knockdown significantly enhanced tumorigenicity (Figure 6B) without affecting proliferation in vitro (Figure S7C), indicative of an expanded tumor-initiating cell population with this combined genetic alteration. These data suggest that broad coverage of our MET-GES could perpetuate the metastasis phenotype.

To gain further insight into the specific effect of TRIM25 on the phenotype of TNBC cells, we characterized the global transcriptional changes downstream of TRIM25. GSEA was performed on genes identified by RNA-seq to be differentially expressed upon knockdown of *TRIM25* in BT549 and MDA-MB-231 cells. Of the 189 MSigDB oncogenic gene signatures tested, we found that the strongest enrichment was a signature related to differentiation of embryonic stem cells (ESCs), suggesting that TRIM25 may be involved in the maintenance of stemness (Figure 6C; Table S7). This is particularly interesting as *TRIM25* was previ-

ously found to be the target of 7 of 9 pluripotency factors in ESCs (Figure 6D) (Kim et al., 2008), and *TRIM25* expression is significantly decreased during embryonic differentiation (Kwon et al., 2013).

We functionally tested this correlative relationship using human ESCs (hESCs). To determine whether loss of *TRIM25* could induce differentiation, we lentivirally expressed short hairpins against *TRIM25*. Strikingly, *TRIM25* depletion induced changes in hESC morphology consistent with differentiation (Figure 6E). qRT-PCR of RNA isolated 4 days post-transfection revealed a considerable increase in mesoderm, endoderm, and ectoderm marker genes after *TRIM25* knockdown, suggesting that TRIM25 is required to maintain a stem cell state (Figure 6F). Recently, Akrap and colleagues used single-cell RNA-seq to identify markers of functionally enriched stem and progenitor cells in breast cancer. In both primary breast tumors and TNBC cell lines, *POU5F1*, *NANOG*, and *SOX2* were major determinants of a quiescent cancer stem cell (CSC)-like cell phenotype with high stemness and tumorigenicity (Akrap et al., 2016). Using our CRISPR *TRIM25*-knockout cell models, we found that expression of either *TRIM25* or *TRIM25ΔR* in TNBC-TKO cells increased the expression of *POU5F1*, *NANOG*, and *SOX2* (Figures 6G and 6H), suggesting that TRIM25 regulates the core genes involved in maintaining quiescent CSC-like phenotypes. Together, these results suggest TRIM25 promotes metastasis through increasing the stemness and tumorigenicity of breast cancer cells, enhancing their capacity for colonization, survival, and outgrowth at the secondary site.

Across 32 cancer types from TCGA, *TRIM25* was rarely deleted and was most frequently amplified in breast cancer (Figures 7A and 7B). *TRIM25* amplification was associated with a significant increase in mRNA expression (Figure 7C). Kaplan-Meier log rank test using RNA-seq data consisting of 1,038 breast cancer tumors from TCGA revealed that elevated *TRIM25* expression is significantly associated with poor overall survival (Figure 7D). Further, prediction of clinical outcomes from genomic profiles (PRECOG) analysis of *TRIM25* confirmed that *TRIM25* expression is significantly associated with poor outcome in breast cancer (Figure 7E). In fact, *TRIM25* was most strongly associated with poor outcome in breast cancer over any other cancer type. There were no significant differences based on *TRIM25* expression or predicted protein activity in normal breast tissue compared to primary tumors (Figures 7F and 7G). However, there was a significant difference in the activity of *TRIM25* protein between primary tumors and metastases (Figure 7H). In addition,

Figure 4. TRIM25-Mediated Transcriptional Regulation of the MET-GES



although the TCGA dataset only contains 7 matched pairs of primary tumors and metastases, 6 of 7 metastases had higher *TRIM25* expression than their respective primary tumors (Figure 7I). *TRIM25* immunohistochemistry in primary breast tumors, lymph node, and distant metastases revealed similar *TRIM25* localization, suggesting localization of *TRIM25* is not overtly altered during metastatic progression (Figure 7J). Together, these data support a role of *TRIM25* as a driver of poor outcome in breast cancer.

DISCUSSION

Context-specific regulatory networks, inferred by unbiased reverse engineering algorithms, provide a framework for identifying the proteins causally associated with specific phenotypic states (Lefebvre et al., 2010). Here we reverse engineered a breast cancer-specific regulatory network to uncover a transcriptional hierarchy driving breast cancer metastasis. Importantly, we discovered that *TRIM25*, a multifunctional TF, lies at the epicenter of this hierarchy. Altered *TRIM25* activity causes coordinated broad changes in the metastatic program, affecting the expression of many metastasis effectors simultaneously and promoting the metastatic phenotype.

Multifunctional proteins such as *TRIM25* challenge a conventional one protein-one function paradigm. Many TFs that bind double-stranded DNA with sequence specificity also bind RNA (Cassiday and Maher, 2002). Similarly, several RNA-binding E3 ubiquitin ligases have recently been discovered to direct cellular regulatory pathways by affecting both protein and mRNA stability (Cano et al., 2010). Interestingly, several of these RNA-binding E3 ubiquitin ligases contain a B-box zinc-finger DNA-binding domain analogous to *TRIM25*. Here we demonstrate that *TRIM25* has the potential to control cellular phenotypes by interacting with DNA in addition to RNA (Streich et al., 2013) targets. Moreover, our data show that *TRIM25* represses many targets at both levels, representing a transcriptional/post-transcriptional feedforward topology (Lefebvre et al., 2010; Mangan and Alon, 2003). This complex regulatory architecture and diversified functionality has not yet been described for *TRIM25*.

Although we focused our study on the role of *TRIM25* in TNBC, VIPER analysis identified this regulator protein as the most statistically significant metastatic progression MR both in TNBC and in ER+ tumors, suggesting that it may play an equally relevant role in ER+ breast cancer as well. Indeed, it has been proposed that *TRIM25* may perpetuate the estrogen-signaling cascade in the absence of estrogen receptor, resulting in the evolution of estrogen independence and resistance to hormone therapy (Nalepa and Harper, 2002). A similar phenomenon was

recently observed in prostate cancer whereby the glucocorticoid receptor (GR) substitutes for the androgen receptor (AR) resulted in resistance to anti-androgen therapy (Arora et al., 2013). However, here we show data supporting an estrogen-independent role of *TRIM25*. Further, in a parallel and ongoing study, we did not find *TRIM25* as differentially expressed or differentially active TFs in letrozole-resistant breast cancer patients (data not shown). Therefore, the phenotypes observed in studies that link *TRIM25* with tumorigenicity and survival of estrogen-dependent cells in the absence of estrogen (Urano et al., 2002) may have been the result of *TRIM25* promoting the stemness and tumor-initiating phenotypes described here, thus suggesting that its role in metastatic progression may be fully independent of hormonal status.

The dynamic nature of metastasizing cancer cells cannot simply be explained by the accumulation of progressive genetic changes in the primary tumor (Monteiro and Fodde, 2010). The capacity to resist cytotoxic or targeted therapy, to undergo multiple profound phenotypic changes, to overcome the challenges of foreign microenvironments, and to maintain the ability to eventually recapitulate the heterogeneous composition of the primary tumor at distant organ sites is not typical behavior for a differentiated epithelial cell. Rather, this is likely achieved by stem-like cells that maintain active DNA repair, express a variety of drug transporters, resist apoptosis and pathotropism, and have highly dynamic cellular plasticity that supports multiple hallmarks of cancer (Lawson et al., 2015). Our data suggest that collapsing transcriptional hierarchies that regulate the balance between differentiation and self-renewal, by targeting keystone proteins like *TRIM25*, is critical to affect the coordinated transcriptomic reprogramming that is required for metastasis and may represent promising future targets for cancer intervention.

EXPERIMENTAL PROCEDURES

Primary Human Breast Cancer and Matched Lymph Node Metastases

Paired primary tumor and lymph node metastases were collected during surgical resection. All tissue was used with informed consent and banked at Memorial Sloan Kettering Cancer Center (MSKCC) in accordance with institutional review board (IRB) approval. All samples were snap-frozen and stored at -80°C until use. All samples were H&E stained and independently reviewed by a breast cancer pathologist. Tumors were micro-dissected to obtain >70% purity. RNA was isolated from fresh-frozen samples using the RNeasy Plus mini prep kit (QIAGEN, Valencia, CA, USA). Nucleic acid quality was determined with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Primary tumor and matched metastasis pairs, for which RNA of sufficient quantity and quality was available, were analyzed on Affymetrix GeneChip Human Genome U133 2.0 Array (Affymetrix, Santa Clara, CA,

Figure 5. *TRIM25*-Mediated Post-transcriptional Regulation of the MET-GES



USA) at the genomics core at Memorial Sloan Kettering Center for Molecular Oncology (New York, NY, USA), according to the manufacturer's protocol.

Animal Studies

All animal work was done in accordance with the Institutional Animal Care and Use Committee at MSKCC. Athymic Nu/Nu mice were obtained from Harlan Laboratories (Indianapolis, IN, USA) and NOD-scid IL2Rgnull (NSG) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and randomized. MDA-MB-231 cells were first infected with GREEN pHIV-Luc-ZsGreen (Addgene, Cambridge, MA, USA) or RED pHIV-Luc-dsRED vectors. Cells were then infected with the indicated constructs (Figure S3A). All animals were injected between 5 and 7 weeks of age. Single-cell suspensions of the indicated cells were mixed 1:1 with serum-free media for intravenous injections or 1:1 with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) for orthotopic injections.

TRIM25^{high} (TRIM25 overexpression) and TRIM25^{low} (TRIM25 knockdown) cells with opposing fluorescent labels were mixed 1:1 and injected (5×10^5 cells) into the lateral tail vein of athymic nude mice or 1×10^6 cells into the mammary fat pad of NSG mice. The development of primary tumors or lung metastases was monitored using bioluminescence imaging (BLI; IVIS, Xenogen, Alameda, CA, USA). When lung metastases were visible, mice were sacrificed and their lungs were imaged via ex vivo fluorescence imaging and spectral unmixing. Lungs were then sectioned and stained with anti-RFP antibody (Abcam, Cambridge, MA, USA). Lung tissues were blindly scanned using a Carl Zeiss Axioimager Z1 microscope (Oberkochen, Germany) equipped with a Tissue Gnostics slide-scanning platform. TissueQuest analysis software was used for automated quantification of RFP+ and GFP+ tumor cells per total number of DAPI+ cells within a full cross section of the primary tumor. For lung tissue analysis, two lobes per animal (one left and one right) were used for red:green ratio calculations. Two-tailed pairwise t test was used to statistically compare relative red versus green. Animals that were ill or died due to circumstances unrelated to the experiment were excluded from the study.

CAL-51 cells (1×10^6) were injected into the lateral tail vein of 4- to 6-week-old athymic nude mice. Lungs were harvested 8 weeks post-injection. CAL-51 TKO cells (1×10^6) were injected into the lateral tail vein of 4- to 6-week-old NSG mice. Lungs were harvested 8 weeks post-injection. BT549-TKO (2×10^6) cells were injected into the mammary fat pad of 4- to 6-week-old NSG mice and monitored to tumor take by bioluminescence imaging (IVIS). MDA-MB-231 cells (empty vector [EV], TRIM25, and ΔTRIM25; 1×10^6) were injected into the mammary fat pad of 4- to 6-week-old NSG mice. Primary tumors and lungs were harvested 8 weeks post-injection. MDA-MB-231 (EV, TRIM25, and ΔTRIM25; 5×10^5) were injected into the lateral tail vein of 4- to 6-week-old athymic nude mice. Lungs were harvested 8 weeks post-injection.

Next-Generation Sequencing Data

TCGA RNA-seq, amplification, deletion, mutation, and reverse phase protein lysate microarray (RPPA) data were downloaded from the MSKCC cbio portal (<http://www.cbioportal.org/>) (Cerami et al., 2012; Gao et al., 2013). Clinical TCGA breast cancer data were downloaded from the Broad Institute GDAC Firehose (<https://gdac.broadinstitute.org>). PRECOG data were generated us-

ing the online PRECOG tool at <https://precog.stanford.edu/>. Spearman's correlation matrix was generated using corrrplot 0.73 and PerformanceAnalytics 1.4.3541 in R.

RNA-Seq Analysis of Cell Lines

Total RNA was isolated from cell lines using the RNeasy Plus mini prep kit (QIAGEN, Valencia, CA, USA). Nucleic acid quality was determined with the Agilent 2100 Bioanalyzer. RNA-seq was also performed at the New York Genome Center (New York, NY, USA) using a HiSeq 2500 Ultra-High-Throughput Sequencing System (Illumina, San Diego, CA, USA). Raw reads in the fastq format were aligned to Human Genome HG19 using the RNA-seq STAR aligner version 2.4.0d (Dobin et al., 2013; Dobin and Gingeras, 2015), as recommended by the user manual downloaded along with the software. STAR aligner was chosen for mapping accuracy and speed (Engström et al., 2013).

Mapped reads for each sample were counted for each gene in annotation files in GTF format (gencode.v19.annotation.gtf available for download from GENCODE website, <http://www.gencodegenes.org/releases/19.html>), using the FeatureCounts read summarization program (Liao et al., 2014) following the user guide (<http://bioinf.wehi.edu.au/subread-package/SubreadUsersGuide.pdf>). Individual count files were merged to generate the raw-counts matrix by an in-house R script, normalized to account for differences in library size, and the variance was stabilized by fitting the dispersion to a negative-binomial distribution, as implemented in the DESeq R package (<http://bioconductor.org/packages/release/bioc/html/DESeq.html>) (Anders and Huber, 2010).

ChIP-seq and RIP-seq were performed at the Memorial Sloan Kettering Center for Molecular Oncology (New York, NY, USA) using a HiSeq 2500 Ultra-High-Throughput Sequencing System (Illumina, San Diego, CA, USA). Raw sequencing data were aligned to the hg37 genome build using the Burrows-Wheeler Aligner (BWA) version 0.7.10 (Li and Durbin, 2009). Further indel realignment, base-quality score recalibration and duplicate-read removal were performed using the Genome Analysis Toolkit (GATK) version 3.2.2 (McKenna et al., 2010) following raw read alignment guidelines (DePristo et al., 2011). Peaks were called from the resulting bam files by Model-based Analysis of ChIP-seq (MACS2) version 2.1.0 (Feng et al., 2012), following the manual at <https://github.com/taoliu/MACS/>.

As a first filtering step, resulting raw peaks were filtered by p value < 0.01. Next, for each peak, reads per kilobase of transcript per million mapped reads (RPKM) was calculated for IP and INPUT (for ChIP-seq) bam files. Only peaks with RPKM INPUT/RPKM IP ratio greater than 0.5 were reported to final results.

Statistical Analysis

The Kaplan-Meier method and log rank test were performed using the Prism 6 Software (GraphPad Software, La Jolla, CA, USA). Heatmaps were generated using Partek Genomics Suite software, version 6.6 (Partek, St. Louis, MO, USA). We performed a minimum of 3 replicates per experiment to ensure adequate sample size for statistical analyses.

The Cancer Cell Line Encyclopedia (CCLE) was used to eliminate candidate MR from potential functional validation based on the lack of expression in

Figure 6. TRIM25 Regulates the MET-GES and Modulates Tumorigenicity and Stemness

- (A) GO analysis of core MET-GES genes transcriptionally or post-transcriptionally regulated by TRIM25.
- (B) Bioluminescence imaging and tumor take rate 100 days after orthotopic injection of BT549 cells infected with indicated shRNA into the mammary fat pad of NSG mice (n = 4–8 mice as indicated).
- (C) GSEA for oncogenic signatures associated with differentially expressed genes from RNA-seq after TRIM25 knockdown in BT549 and MDA-MB-231 cells. Normalized enrichment score of the ESC_J1_Up_Late.V1_Up signature (left). (Right) Representative gene expression changes from this signature are shown.



breast cancer cell lines (<http://www.broadinstitute.org/cCLE/home>) (Barretina et al., 2012).

Enrichment of oncogenic signatures on the TRIM25-silencing signature was performed by GSEA (Subramanian et al., 2005). Oncogenic signature gene sets v5.0 were downloaded from <http://software.broadinstitute.org/gsea/datasets.jsp>. Differentially expressed genes (false discovery rate [FDR] $p < 0.01$; Table S4) between siSCR (control) versus siTRIM25 were pre-ranked based on Z score, and the GSEA was implemented using JAVA downloaded from the Broad Institute (<http://software.broadinstitute.org/gsea>). GSEA2 analysis was performed as previously described (Kruithof-de Julio et al., 2011) using an in-house implementation in R.

Differential expression analysis after MR gene silencing was performed by comparing the silenced samples versus the scramble small hairpin RNA (shRNA) and mock controls independently, using the moderated Student's t test as implemented in the limma package from Bioconductor (Ritchie et al., 2015). Gene expression signatures based on the scramble and mock controls were integrated using the Stouffer approach: $Z = (Z_1 + Z_2)/\sqrt{2}$, where Z_1 and Z_2 are Z scores estimated by the moderated Student's t test. Lineal regression with exhaustive selection was performed with the package leaps for R-system (<https://cran.r-project.org/web/packages/leaps>). The approach selected the lineal combination of n MRs that best predicts the subtype-matched MET-GES. We selected n as the highest number of MRs for which all coefficients of the model were different than zero at Bonferroni's corrected $p < 1 \times 10^{-3}$. The proportion of the MET-GES modulated by each MR was inferred as the proportion of MET genes in the leading edge when computing the enrichment of the MET-GES on each MR-silencing gene expression signature by GSEA.

Assembly of Context-Specific Regulatory Models and MR Analysis

A breast carcinoma context-specific network model of transcriptional regulation was assembled with the ARACNe, based on 851 RNA-seq expression profiles obtained from TCGA. ARACNe was run with 100 bootstraps, a p value threshold of 10^{-8} , and 0 data processing inequality (DPI) tolerance, generating a network of 1,748 TFs associated with 18,783 target genes by 459,569 interactions. The regulatory models were generated from the ARACNe results using the VIPER package from Bioconductor (<http://bioconductor.org/packages/release/bioc/html/viper.html>).

The gene expression signatures for 20 ER+ and 11 TNBC metastases (MET-GES) were computed with paired Student's t test by comparing their profiles against the matching primary tumor ones. Then, the enrichment of each regulatory protein regulon on the MET-GESs was inferred by the VIPER algorithm (Alvarez et al., 2016; Aytés et al., 2014), as implemented in the VIPER package for R available from Bioconductor (<https://www.bioconductor.org/packages/release/bioc/html/viper.html>). Statistical significance was estimated by permuting the samples uniformly at random 1,000 times.

For single patient-based analysis, gene expression signatures were computed by comparing each MET expression profile with the matching primary tumor expression profile. A null model for statistical testing was generated by permuting the samples uniformly at random 1,000 times. The most consistent MRs across all tumors were prioritized according to the average dysregulation (normalized enrichment score [NES]) divided by its SD.

ACCESSION NUMBERS

The accession numbers for the RNA-seq after TRIM25 knockdown in breast cancer cell lines, ChIP-seq in TNBC cell lines, and RIP-seq in TNBC cell lines data reported in this paper are GEO: GSE79589, GSE79588, and GSE79587, respectively. The accession number for the primary breast cancer and matched metastases microarray data reported in this paper is GEO: GSE57968.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2017.07.052>.

AUTHOR CONTRIBUTIONS

T.A.C. and A.C. conceived the study. L.A.W., T.A.C., M.J.A., E.Y.S., S.C., and M.R. designed the experiments. L.A.W., E.Y.S., M.R., S.M., M.G.D., K.-W.L., and V.K.R. performed the experiments. M.J.A., L.A.W., V.M., A.D., and S.T. conducted the bioinformatics analysis. L.A.W., M.J.A., E.Y.S., M.R., S.M., and T.A.C. analyzed the data. L.T.V. provided the clinical samples. L.A.W., M.J.A., A.C., and T.A.C. wrote the manuscript. All authors reviewed and edited the final manuscript.

ACKNOWLEDGMENTS

We thank Agnes Viale and the MSKCC genomics core for technical assistance. We thank members of the Chan and Califano labs for helpful discussions. L.A.W. was supported by The Canadian Institutes of Health Research PDF Award MFE-127325. This work was supported by a Department for Defense Era of Hope Grant (BC120568), the STARR Cancer Consortium, the Geoffrey Beene Cancer Center, and the MSKCC Metastasis Research Center (all to T.A.C.); the NIDDK (1 DP2 DK098093-01) and a CADC pilot grant (S.C. and S.M.). This work was in part supported by NCI Cancer Support Grant P30 CA008748 to the Microchemistry and Proteomics Core.

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Figure 7. TRIM25 Is Frequently Amplified in Breast Cancer and Is Associated with Poor Overall Survival

(A) Frequency of *TRIM25* copy number alterations (CNAs) in 32 cancer types from the TCGA. CS, cell carcinoma; pRCC, papillary renal cell carcinoma; PCPG, pheochromocytoma and paraganglioma; ccRCC, clear cell renal cell carcinoma; GBM, glioblastoma multiforme; AML, acute myeloid leukemia; chRCC, chromophobe renal cell carcinoma; DLBC, diffuse large B cell lymphoma; ACC, adenoid cystic carcinoma.

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