Polycomb complexes associate with enhancers and promote oncogenic transcriptional programs in cancer through multiple mechanisms

Ho Lam Chan, Felipe Beckedorff, Yusheng Zhang, Jenaro Garcia-Huidobro, Hua Jiang, Antonio Colaprico, Daniel Bilbao, Maria E. Figueroa, John LaCava, Ramin Shiekhattar & Lluis Morey

Polycomb repressive complex 1 (PRC1) plays essential roles in cell fate decisions and development. However, its role in cancer is less well understood. Here, we show that RNF2, encoding RING1B, and canonical PRC1 (cPRC1) genes are overexpressed in breast cancer. We find that cPRC1 complexes functionally associate with ERα and its pioneer factor FOXA1 in ER+ breast cancer cells, and with BRD4 in triple-negative breast cancer cells (TNBC). While cPRC1 still exerts its repressive function, it is also recruited to oncogenic active enhancers. RING1B regulates enhancer activity and gene transcription not only by promoting the expression of oncogenes but also by regulating chromatin accessibility. Functionally, RING1B plays a divergent role in ER+ and TNBC metastasis. Finally, we show that concomitant recruitment of RING1B to active enhancers occurs across multiple cancers, highlighting an under-explored function of cPRC1 in regulating oncogenic transcriptional programs in cancer.
Polycomb group genes (PcG) are evolutionarily conserved epigenetic regulators that can be divided into two main complexes, Polycomb repressive complex 1 and 2 (PRC1 and PRC2)\(^1\). In mammals, the core PRC2 complex contains SUZ12, EED, and the histone methyltransferase enzymes EZH1/2, which catalyze di- and trimethylation on lysine 27 of histone H3 (H3K27me2/3)\(^2\). The two main PRC1 sub-complexes are the canonical and non-canonical PRC1 complexes (cPRC1 and ncPRC1). cPRC1 comprises PCGF2/4, Polyhemicotic (PHC1/2/3), the CBX proteins (CBX2/4/6/7/8), and the E3-ligase subunits RING1A/B, which monoubiquitin histone H2A at lysine 119 (H2AK119ub1). In contrast, ncPRC1 complexes include RYBP/YAF2, PCGF1/3/5, and RING1A/B, as well as additional co-factors\(^3\). We and others have shown that cPRC1, ncPRC1, and PRC2 complexes regulate stem cell pluripotency, cell fate decisions, and development\(^4,5\). Historically, Polycomb complexes have been mostly associated with maintaining gene repression. However, increasing evidence indicates that specific PRC1 variants can be recruited to actively transcribed genes in multiple biological processes\(^6-10\).

While PRC1 genes are not typically mutated, they are dysregulated in many cancer types. RING1, encoding for PCGF4, is the best studied PRC1 gene in cancer to date. It is often overexpressed in cancer and is important for tumor initiation and progression\(^11,12\). In contrast, PCGF2 is downregulated in prostate and colorectal cancers\(^13\), suggesting that PCGF paralogs have distinct functions in cancer. Recent studies suggested that PRC1 genes that play important roles in cancer carry out their functions independently of their association with PRC1\(^14,15\). Nonetheless, despite great efforts to understand the epigenetic mechanisms that contribute to human maladies, a comprehensive analysis of genomic alterations of PRC1 genes, and the architecture, function, and activity of PRC1 complexes in cancer, have yet to be fully addressed.

Here, we show that PRC1 genes are genetically amplified in breast cancer. In contrast to its canonical function, RING1B (encoded by RNF2) is predominantly recruited to enhancers and specifically regulates oncogenic transcriptional programs in different breast cancer subtypes. Mechanistically, RING1B associates with specific cPRC1 components that are recruited to enhancers containing estrogen receptor alpha (ERα) in ER+ cells, and to BRD4-containing enhancers in triple-negative breast cancer (TNBC) cells. We also show a functional crosstalk between RING1B, FOXA1, and ERα in ER+ cells, resulting in an attenuated response to estrogen with RING1B depletion. We provide evidence that RING1B directly regulates chromatin accessibility at enhancers bound by transcription factors involved in breast cancer. In agreement with survival prognoses of patients with different breast cancer subtypes and RNF2 expression levels, RING1B differentially regulates the metastatic potential of TNBC and ER+ breast cancer cells. Finally, we show that RING1B is recruited to enhancer regions in other cancer types, suggesting that this RING1B-mediated mechanism of controlling oncogenic pathways occurs in multiple cancers.

**Results**

cPRC1 genes are amplified and overexpressed in breast cancer. To initially assess whether PRC1 components are altered in cancer, we examined the mutational frequencies of the histone H2A mono-ubiquitin ligases RNF2 (encoding RING1B) and RING1, the cPRC1 genes, and the core PRC1-encoding genes (Supplementary Fig. 1a) in large-scale genomic data sets from cancer patients. We found that PRC1 genes were amplified in multiple cancer types. Intriguingly, many hormone-related cancers (e.g., ovarian, uterine, prostate, and breast cancer) were overrepresented (Supplementary Fig. 1b). Since the breast cancer data sets contain the largest number of patient samples and thus provide the most robust data, we further analyzed PRC1 genes in these patient samples. We found that RNF2 was amplified in up to 22% of breast cancers and cPRC1 genes were amplified in a large number of samples (Supplementary Fig. 1c–d). Compared to RING1 which is not amplified, RNF2 amplification correlated to its significant overexpression in breast cancer compared to normal breast tissues, regardless of breast cancer subtype (Supplementary Fig. 1e–f). We also noticed that other amplified cPRC1 genes, including CBX2/4/8 and PCGF2, exhibited distinct expression patterns when categorized by breast cancer subtype (Supplementary Fig. 1g). Furthermore, RNF2 expression was highest in tumors with amplification of the gene (Supplementary Fig. 2a). However, RNF2, PCGF2, and CBX2/4/8 expression was higher in all four breast cancer stages compared to normal breast tissue, suggesting that their overexpression was not predictive of breast cancer aggressiveness (Supplementary Fig. 2b).

RING1B binding is redistributed in breast cancer cells. We next focused on understanding the specific role of RING1B in breast cancer (Fig. 1a). To our knowledge, no genome-wide study of RING1B binding to chromatin in breast cancer cells had yet been conducted. We performed RING1B chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-seq) of two breast cancer cell lines—estrogen receptor positive (ER+) luminal A cell line, T47D, and triple-negative breast cancer (TNBC) cell line, MDA-MB-231—and a non-tumorigenic transformed mammary epithelial cell line, MCF10A. As a control, we also performed RING1B ChIP-seq in human induced pluripotent stem cells (iPSCs) since the target genes of PRC1 subunits have been extensively mapped in stem cells\(^16,17\). Additionally, the RING1B antibody used is validated by mass spectrometry. To further confirm the specificity of this antibody, we performed RING1B western blotting and immunoprecipitation from control and RING1B-depleted MDA-MB-231 cells (Supplementary Fig. 3a–b). As additional controls, we performed ChIP-qPCR of known RING1B target genes in iPSCs\(^17\) using a different RING1B antibody as well as H3K27me3, H3K4me3 and H3K27ac antibodies (Supplementary Fig. 3c–d) and the enrichment values are in agreement with ChIP-seq binding. We identified 702 RING1B target genes in iPSCs, 2869 in MCF10A, 2202 in T47D, and 2137 in MDA-MB-231 (Fig. 1b and Supplementary Data 1). Gene ontology (GO) analyses revealed RING1B targets as developmental genes in iPSCs (Fig. 1c), in agreement with published data\(^17\). In contrast, GO analysis of RING1B targets in MCF10A showed enrichment of genes involved in axon guidance and focal adhesion, while in T47D and MDA-MB-231, genes involved in focal adhesion, cell-to-cell junctions, and signaling pathways in cancer were enriched (Fig. 1c). As expected based on the GO analyses, the overlap of RING1B targets was relatively low between iPSCs, MCF10A, T47D, and MDA-MB-231 (Fig. 1d), but higher between MCF10A, T47D, and MDA-MB-231 (Supplementary Fig. 3e–f).

To determine the functional significance RING1B genomic distribution, we categorized RING1B ChIP-seq peaks into three main regions: intergenic, intragenic, and promoter regions (Methods section). Most RING1B peaks in iPSCs were located at promoters or inside genes. However, in MCF10A, T47D, and MDA-MB-231, RING1B was distributed to intergenic regions (Fig. 1e). We also found that each of the cell lines had a set of distinct RING1B peaks corresponding to cancer-related and epithelial genes in the breast cells but not in iPSCs (Fig. 1f, g and Supplementary Fig. 3g). Importantly, RNA-seq analysis indicated that RING1B target genes in MCF10A, T47D, and MDA-MB-231
Fig. 1 Genome-wide occupancy and activity of RING1B in breast cancer cells. a Model depicting RING1B and cPRC1 subunits that are genetically amplified and overexpressed in breast cancer. b Number of RING1B target genes. Representative phase-contrast images of each cell line are shown at ×10 magnification. Scale bar represents 100 μm. c GO analysis of RING1B target genes. d Venn diagrams of overlapping RING1B target genes. e Distribution of RING1B ChIP-seq peaks. ChIP-seq heat maps of specific RING1B peaks in each of the cell lines. GO analysis performed on target genes identified in each peak cluster. f Genome browser screenshots of unique RING1B-binding sites in each of the cell lines. RING1B peaks are highlighted in green. g Pie chart showing percentage of RING1B peaks overlapping with H2AK119ub1 and H3K27me3. h Genome browser screenshots of RING1B, H3K27me3, H2AK119ub1, and H3K4me3 in each of the cell lines. RING1B peaks are highlighted in green. i Representative western blots of RING1A, RING1B, and H2AK119ub1 of control and RING1B-depleted cells. Histone H3 was used as a loading control (n = 3).

are transcriptionally more active and more highly expressed than the RING1B target genes in iPSCs (Supplementary Fig. 3h–i).

Most RING1B-bound sites are devoid of H3K27me3/H2AK119ub1. Since the classical model of PRC1 binding to chromatin is following PRC2 recruitment, we next determined the degree of overlap between RING1B and the PRC2-associated and PRC1-associated histone modifications, H3K27me3 and H2AK119ub1, respectively. As expected in iPSCs, the majority of sites containing RING1B were also decorated with both histone and PRC1-associated histone modifications (Fig. 1h, i and Supplementary Fig. 3i). In MFC10A, 35% of RING1B sites were co-occupied by H3K27me3 and H2AK119ub1 and this overlap decreased to 25 and 20% in
RING1B binds active enhancers. Since a large number of RING1B sites were not marked with H3K27me3 or H2AK119ub1 and RING1B peaks re-localized to intergenic regions, we next tested whether RING1B is recruited to enhancer regions. Enhancers are regulatory sites that can be bound by transcription factors to increase the transcription of a particular gene\(^{19,20}\) and can be divided into typical enhancers and super-enhancers (SEs): in cancer, typical enhancers promote transcription at active genes and SEs regulate the expression of oncogenes and genes associated to oncogenic transcriptional programs\(^{21}\). Active typical enhancers and SEs are also epigenetically distinct: although both are marked with H3K4me1, SEs contain increased levels of H3K27ac\(^{21,22}\). We found that both H3K27ac and H3K4me1 ChIP-seq signals were enriched at RING1B-bound sites (Fig. 2a) that were simultaneously devoid of H3K27me3 (Supplementary Fig. 4a–b). Only 4%, 8%, and 13% of typical enhancers contained RING1B in MCF10A, MDA-MB-231, and T47D cells, respectively, while in contrast, over 45% of SEs in these cells were decorated with RING1B (Fig. 2b–d and Supplementary Fig. 4c–d). Virtually none of the SEs in iPSCs contained RING1B (Fig. 2c).

We next asked whether RING1B was recruited to SEs near genes with established functions in breast cancer. Indeed, we observed RING1B recruitment at SE regions near BCL2L1 in MDA-MB-231 and ESR1 in T47D\(^{23,24}\) (Fig. 2c and Supplementary Data 1). To confirm that the SEs were unique to each cell line, and that RING1B was recruited specifically to these unique sites, we determined the RING1B signal at these SEs. We found that RING1B signal at MDA-MB-231 specific SEs was stronger in MDA-MB-231 cells than at the same SE regions in MCF10A and T47D cells; the same was true for MCF10A- and T47D-specific SEs (Fig. 2f and Supplementary Fig. 4e). These results indicate that RING1B is recruited to cell-type-specific SEs in breast epithelial and cancer cells.

In contrast to the broad RING1B ChIP-seq signals in pluripotent cells, RING1B peaks in the breast cell lines were narrow (Figs. 1g, h and 2e), resembling ChIP-seq signals of transcription factors. Therefore, we assessed whether RING1B is recruited to specific transcription factor-binding sites at SEs\(^{20}\). In the ER+ cell line, T47D, analysis of known transcription factor motifs revealed an enrichment of the ER\(\alpha\) and FOXA1/2 consensus binding sequences\(^{25,26}\) (Fig. 2g), suggesting a functional connection between RING1B and the ER pathway. Similarly, motifs for important breast cancer oncogenic transcription factors were overrepresented at RING1B-containing SEs in MDA-MB-231 and MCF10A cells that are ER- (Fig. 2g).

Finally, we associated potential target genes to the SEs containing RING1B based on proximity and retrieved 561, 252, and 398 genes that were potentially functionally associated with SEs in MCF10A, MDA-MB-231, and T47D cells, respectively (Fig. 2h and Supplementary Data 2). Interrogation of published ChIP-seq data sets in ENCODE using EnrichR revealed a further potential functional association of RING1B with ER\(\alpha\) in T47D. Interestingly, the bromodomain-containing protein, BRD4, was recruited to genes potentially controlled by RING1B-containing SEs in MDA-MB-231, while RACK7 (receptor for activated C-kinase 7) bound the RING1B-containing SEs in MCF10A (Fig. 2h). Overall, these results indicate that RING1B is recruited to SEs and, importantly, that there is a specific functional crosstalk between RING1B and key signaling pathways involved in breast cancer.

RING1B assembles into discrete cPRC1 complexes. Dozens of cPRC1 and ncPRC1 variants can be potentially assembled, and have distinct biological functions in regulating stem cell pluripotency, differentiation, and tissue homeostasis\(^{6,27–30}\). To assay the RING1B protein interactome in MDA-MB-231 and T47D, we performed co-immunoprecipitations of endogenous RING1B-associated protein complexes using the anti-RING1B antibody used for ChIP-seq, followed by label-free quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS). Unexpectedly, because both cPRC1 and ncPRC1 genes are expressed in these cells (Supplementary Fig. 5a), RING1B mainly co-immunoprecipitated with cPRC1 subunits (Fig. 3a, b and Supplementary Data 3). Specifically, when captured from T47D cells, RING1B demonstrated interactions with the cPRC1 subunits CBX4/8, PCGF2, and PHC2/3 (Fig. 3a, left), with CBX8 and PHC3 displaying the highest levels of interaction with RING1B (Fig. 3b, left). RING1B co-immunoprecipitated a larger number of proteins in MDA-MB-231 cells than in T47D cells (Fig. 3a, right), but of the proteins observed, cPRC1 subunits, including CBX8, PCGF2, and PHC2 were amongst the most abundant (Fig. 3b, right). We next addressed whether the RING1B recruited to chromatin in T47D and MDA-MB-231 is a part of a cPRC1 complex. We performed ChIP-seq of PCGF2 since it is the predominant RING1B-associated PCGF subunit in both cell lines and identified 2408 and 4813 PCGF2 target genes in T47D and MDA-MB-231 cells, respectively (Fig. 3d, g). Almost 60% of PCGF2 targets in T47D cells, and about 80% in MDA-MB-231 cells, were also co-occupied by RING1B (Supplementary Fig. 5b–c).

To further interrogate the potential functional relationship between RING1B and ER\(\alpha\), we addressed whether a cPRC1 complex (defined by co-occupancy of RING1B and PCGF2) is associated with genomic sites bound by ER\(\alpha\). We found that cPRC1 was indeed co-recruited with ER\(\alpha\) to a large number of genomic sites (Fig. 3c). Overlapping RING1B, PCGF2, and ER\(\alpha\) targets indicated that 890 target genes were decorated with cPRC1 and ER\(\alpha\) (Fig. 3d). Importantly, these genes are involved in pathways important in carcinogenesis (Fig. 3e and Supplementary Data 4). Furthermore, since we observed that potential genes regulated by RING1B-containing SEs in MDA-MB-231 cells were BRD4 targets (Fig. 2h), we also performed ChIP-seq of BRD4. Indeed, cPRC1 largely associated with BRD4 targets and 840 genes were decorated with cPRC1/BRD4 (Fig. 3f, g and Supplementary Data 4). These cPRC1/BRD4 co-targets are involved in cancer and focal adhesion pathways (Fig. 3h).

We next determined the co-recruitment of cPRC1 with either ER\(\alpha\) or BRD4 to enhancers in T47D and MDA-MB-231 cells, respectively (Fig. 3i–k and Supplementary Fig. 5d). A total of 81% and more than 90% of SEs with cPRC1 were also bound by ER\(\alpha\) in T47D and BRD4 in MDA-MB-231, respectively (Fig. 3i). Association of BRD4 to RING1B-containing enhancers in MDA-MB-231 was further validated by ChIP-qPCR (Supplementary Data 2).
RING1B regulates oncogenic pathways and enhancer RNAs. Next, we determined the effects of RING1B depletion on gene expression in T47D and MDA-MB-231 cells. We found that in T47D, more genes were downregulated than upregulated (62% versus 38%) after RING1B depletion, suggesting that RING1B facilitates gene activation (Fig. 4a, left, and Supplementary Fig. 6a). In contrast, in MDA-MB-231, RING1B depletion had a more modest effect on gene regulation as only about 90 genes were significantly deregulated (Fig. 4a, right). Deregulated genes in both cell lines included key genes involved in breast cancer progression and metastasis (Fig. 4a, right). Deregulated genes in both cell lines included key genes involved in breast cancer progression and metastasis (Fig. 4a, right). Deregulated genes in both cell lines included key genes involved in breast cancer progression and metastasis (Fig. 4a, right). Deregulated genes in both cell lines included key genes involved in breast cancer progression and metastasis (Fig. 4a, right).
Fig. 3 The RING1B interactome and its genome-wide association with ERα and BRD4 in ER+ and TNBC cells. a Endogenous RING1B immunoprecipitation with whole-cell extracts. Proteins bound to RING1B were identified by LC-MS/MS, and enrichment was calculated based on LFQ intensities. IgG was used as a negative control. Experiments were performed in three biological replicates. b Relative abundance of RING1B interactors. c ChIP-seq heat maps of RING1B, PCGF2, and ERα in T47D. d Overlapping of RING1B, PCGF2, and ERα target genes in T47D. e GO analysis of RING1B/PCGF2/ERα co-target genes. f ChIP-seq heat maps of RING1B, PCGF2, and BRD4 in MDA-MB-231. g Overlapping of RING1B, PCGF2, and BRD4 target genes in MDA-MB-231. h GO analysis of RING1B/PCGF2/BRD4 co-target genes. i–j ChIP-seq heat maps of RING1B, PCGF2, ERα, and histone modifications associated with active enhancers and SEs in T47D, and PCGF2, BRD4 in MDA-MB-231. k Genome browser screenshots of SEs. SE regions are highlighted in yellow. l Pie charts of cPRC1-SEs with ERα in T47D and BRD4 in MDA-MB-231

shRING1B T47D (Fig. 4a, left and Supplementary Fig. 6b) and the fatty acid metabolism pathway was upregulated after RING1B depletion (Fig. 4b, left, Supplementary Fig. 6b, and Supplementary Data 5). In RING1B-depleted MDA-MB-231 cells, several well-known oncogenic signaling pathways were also deregulated after RING1B depletion (Fig. 4b, right and Supplementary Fig. 6b). RT-qPCR of select cancer-related genes in both shRING1B T47D and MDA-MB-231 cells confirmed the RNA-seq results, and further suggested that fatty acid metabolism (represented by CD36 and HMGCS2) may play a major role in the tumorigenesis of ER+ breast cancer (Fig. 4c). Although RNF2 amplification did not correlate with over-expression in patients with HER2+ tumors, RNF2 expression was significantly elevated compared to normal breast tissues...
RING1B also affected oncogenic pathways in HER2+ cells. A total of 674 genes were deregulated (q-value < 0.05, fold change > 2) upon RING1B KD, with 255 and 419 genes upregulated and downregulated, respectively (Supplementary Fig. 6e), suggesting that RING1B may also positively regulate gene expression in HER2+ cells. GSEA analysis revealed a strong deregulation of cancer-related pathways, including cell

**Fig. 4** RING1B regulates specific oncogenic pathways and metastasis in breast cancer subtypes. a RNA-seq heat maps of upregulated and downregulated genes in RING1B-depleted (shRING1B) T47D and MDA-MB-231 cells. RNA-seq experiments were performed in two biological replicates. b GSEA analyses of shRING1B T47D and MDA-MB-231 cells. c Real-time qPCR of selected genes in control and RING1B-depleted T47D and MDA-MB-231 cells. Expression was normalized to the housekeeping gene RPO. Data represent the average of two independent experiments. d Box plots of deregulated eRNAs in SEs after RING1B depletion. e eRNA signal at RING1B-occupied SE regions. f Heatmap of deregulated genes near SEs containing RING1B in shRING1B T47D. g Genes in f that are downregulated (cluster 1) or upregulated (cluster 2) in shRING1B T47D. h Kaplan-Meier survival analysis of patients from TCGA with ER+ tumors (top) or Basal (TNBC) breast cancer tumors segregated by RNF2 expression. i-j Representative images of metastatic signal detected by IVIS in NSG mice 65 days after injection of control and shRING1B T47D GFP-luc and MDA-MB-231 GFP-luc cells in the mammary fat pad (n = 5/group). Quantification of luciferase signal by IVIS in control and shRING1B T47D GFP-luc and MDA-MB-231 GFP-luc cells. Error bars represent SD. *p-value < 0.05; **p-value < 0.001, two-tailed t-test. Center line of box plots represent the median and upper and lower bounds of whiskers represent the maximum and minimum values, respectively.
cycle, TGF-β and PPAR signaling, and fatty acid metabolism (Supplementary Fig. 6e–f).

Since RING1B was bound to enhancers, we next asked whether RING1B depletion affected the expression of enhancer RNAs (eRNAs)\(^{32}\). RING1B depletion significantly dysregulated eRNAs transcribed from active typical enhancers and SEs (Fig. 4d and Supplementary Fig. 6g). Importantly, RING1B was recruited to 64 and 53% of SE eRNAs that were differentially expressed after RING1B depletion in both cell lines (Fig. 4e, Supplementary Fig. 6h).

Finally, we assessed whether RING1B depletion affected expression of genes potentially regulated by RING1B-containing SEs (as identified in Fig. 2h). In T47D, of the 2484 genes identified that are potentially regulated by 404 RING1B-SEs, 107 were deregulated upon RING1B depletion. Although most were downregulated (cluster 1) and included important genes for breast epithelial homeostasis (e.g., LRIG1, CYP27B1, HES1, THBS1), a set of genes were upregulated (cluster 2) (Fig. 4f). These results suggest that at enhancer regions, RING1B potentially plays a dual function in gene expression (cluster 1) and gene repression (cluster 2) (Fig. 4g).

**Role of RING1B in breast cancer tumorigenesis and metastasis.** We next sought to determine the function of RING1B in breast cancer tumorigenesis and metastasis in vivo. We hypothesized that RING1B depletion increases the aggressiveness of T47D cells due to the strong upregulation of CD36, a marker for metastasis-initiating cells (Fig. 4a, c and Supplementary Fig. 7a). However, RING1B depletion in MDA-MB-231 cells resulted in both positive and negative deregulation of genes involved in breast cancer, thus we could not anticipate the role of RING1B in TNBC in vivo.

Our initial analysis of the TCGA breast cancer data set (Fig. 4h) indicated that patients with ER+ breast cancer and high levels of RN2 survive longer than patients with lower RN2 levels. In contrast, patients with basal breast cancer and high levels of RN2 have a lower survival probability. This data suggested that RING1B might exert divergent functions in tumor formation or metastasis in specific breast cancer subtypes. To assess whether T47D and MDA-MB-231 cells recapitulate the results obtained with the TCGA data set, we injected control and shRING1B cells into the mammary fat pad of NSG mice. Cells were engineered to express a GFP-luciferase transgene to monitor tumor formation into the mammary fat pad of NSG mice. Cells were engineered to express a GFP-luciferase transgene to monitor tumor formation into the mammary fat pad of NSG mice. Cells were engineered to express a GFP-luciferase transgene to monitor tumor formation into the mammary fat pad of NSG mice.

**A novel RING1B-FOXA1-ERα transcriptional axis in ER+ cells.** In T47D cells, RING1B was recruited to SEs containing FOXA1 and ERα-binding sites (Figs. 2g and 3e, i, l). Among those, RING1B bound to the SE that regulates ESRI (encoding ERα) (Fig. 2e). Moreover, RING1B depletion strongly affected the “Estrogen Response” gene signature (Fig. 4b). These results suggested that RING1B is functionally involved in the estrogen signaling pathway through an ERα/FOXA1 transcriptional regulatory axis. Interestingly, in MDA-MB-231 cells that do not express FOXA1, RING1B was recruited to the FOXA1 promoter and had a canonical repressive function, co-localizing with H2AK119ub1 and H3K27me3 histone marks (Fig. 5a). In contrast, in T47D cells, RING1B bound to a putative SE downstream of FOXA1, suggesting that it plays an activating role in regulating FOXA1 expression (Fig. 5a). RING1B ChIP-qPCR of several RING1B-SEs in control and RING1B-depleted T47D cells confirmed the binding of RING1B to enhancer regions identified by ChiP-seq, including the FOXA1 putative enhancer (Fig. 5b and data not shown). We then assessed whether RING1B directly regulates FOXA1 expression in both T47D and MDA-MB-231 cells. While RING1B depletion in MDA-MB-231 was not sufficient to activate FOXA1 expression (data not shown), acute depletion of RING1B by siRNA reduced FOXA1 protein levels ~50% in T47D cells (Fig. 5c, left panel). Although FOXA1 levels remained unaffected upon stable RING1B depletion by shRNA (Fig. 5c, right panel), cellular fractionation assays showed that FOXA1 was displaced from chromatin and relocated to the soluble nuclei fraction (Fig. 5d). Since FOXA1 is a transcription factor important for ERα recruitment to chromatin,\(^{26}\), displacement of FOXA1 from chromatin also impaired chromatin localization of ERα (Fig. 5d). This set of data suggests that RING1B mediates the estrogen response by affecting FOXA1 and ERα recruitment to chromatin.

We then asked whether FOXA1 depletion affected RING1B levels. While acute FOXA1 depletion affected the RING1B protein levels moderately (Fig. 5e, left panel), stable FOXA1 depletion strongly reduced RING1B global levels (Fig. 5e, right panel). Importantly, RING1B binding to chromatin was also severely reduced (Fig. 5f). Analysis of FOXA1 ChIP-seq in T47D cells did not reveal binding of FOXA1 to the RN2 promoter (data not shown).

Finally, since we observed reduced levels of both FOXA1 and ERα at chromatin upon RING1B depletion, we asked whether RING1B-depleted cells can respond to estrogen stimulation. To this end, we cultured control and RING1B KD cells for 72 h in hormone-deprived (HD) media prior to induction of ERα signaling with 10 nM of E2 (estradiol) for 12h\(^{34}\). In agreement with the global gene expression profiles of RING1B-depleted T47D cells (Fig. 4b), there was reduced expression of prominent E2-responsive genes in shRING1B T47D compared to control cells (Fig. 5g). Altogether, these results demonstrated that RING1B is a novel epigenetic factor that directly and indirectly regulates the FOXA1–ERα axis by multiple mechanisms (Fig. 5h).

**RING1B regulates chromatin accessibility at enhancers.** Since RING1B was recruited to regions targeted by transcription factors and its depletion deregulated breast cancer signaling pathways as well as FOXA1 and ERα localization to chromatin, we next hypothesized that RING1B regulates transcriptional programs in breast cancer by orchestrating chromatin accessibility. To test this, we performed transposase-accessible chromatin sequencing (ATAC-seq)\(^{35}\) in RING1B-depleted cells (Fig. 6a). As expected, ATAC-seq peaks in control cells were at promoter, intronic, and intergenic regions (Supplementary Fig. 8a). Importantly, ATAC-seq peaks co-localized with a large number of RING1B peaks in control cells, and the majority of this co-localization occurred at introns and intergenic regions, but not at promoters (Fig. 6b). These results indicate that RING1B depletion affects chromatin accessibility at enhancer regions.

We next asked whether RING1B depletion-induced de novo generation and/or loss of accessibility sites. RING1B depletion generally affected chromatin accessibility, suggesting that
RING1B is involved in both opening and closing chromatin (Fig. 6c, d). Upon RING1B depletion, the ATAC-seq peaks either lost or gained de novo were located at introns and intergenic regions (Supplementary Fig. 8b–c). Notably, RING1B was recruited to genomic regions not accessible to transposase in control cells but became accessible in RING1B-depleted cells (Fig. 6e, f, top). Further, RING1B was recruited to open chromatin sites and its depletion-induced chromatin compaction (Fig. 6e, f, bottom). These results suggest that RING1B plays a dual role in regulating chromatin accessibility.

We next analyzed the impact of RING1B depletion on chromatin accessibility at enhancers. In T47D cells, RING1B depletion resulted in the loss of about 500 peaks and gain of more than 600 de novo peaks at enhancers (Fig. 6g). RING1B binds to 55% of SEs and 23% of typical enhancers (Fig. 6g). Transcription factor motif analysis revealed that ATAC-seq peaks lost at
Typical enhancer

hg19

in maintaining topological-associated domains (TADs)36 (Fig. 6h, CTCF-binding sites, suggesting that RING1B might be involved

ATAC-seq experiments were performed after two independent siRING1B transfections.

TUBULIN was used as a loading control. ATAC-seq experiments were performed in two biological replicates after siRNAs transfections.

seq peak distribution in genomic sites bound by RING1B in RING1B-depleted cells.

Transcription factor-binding motif analysis of peaks acquired or lost at enhancers in T47D.

RING1B depletion, number of enhancers containing RING1B ChIP-seq signals in MDA-MB-231.

The in

MB-231 was less profound than in T47D (Fig.6d), which is in

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NATURE COMMUNICATIONS | DOI: 10.1038/s41467-018-05728-x | www.nature.com/naturecommunications

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line with the modest gene expression changes in shRING1B MDA-MB-231 cells. However, about 300 and 700 ATAC-seq peaks were lost and gained at enhancers, respectively, after RING1B depletion (Fig. 6i). Interestingly, in addition to CTCF sites, accessibility was altered for breast cancer-specific transcription factors (Fig. 6i). Furthermore, altered chromatin accessibility at enhancers were co-bound by cPRC1/ERα and cPRC1/BRD4 in T47D and MDA-MB-231 cells, respectively (Fig. 6k).

Overall,
these results confirm that RING1B has dual function in regulating transcriptional programs in breast cancer cells and does so by altering chromatin accessibility for key transcription factors and chromatin organization proteins.

RING1B is recruited to super-enhancers in other cancer types. We finally sought to determine whether RING1B recruitment to SEs only occurs in breast cancer cells or if, in contrast, RING1B acquired the ability to bind to enhancers in other cancer types. To this end, we used public RING1B ChIP-seq data sets from ENCODE in a leukemia cell line, K562, and in a hepatocellular carcinoma cell line, HepG2. Notably, in both cell lines, RING1B co-localized with the enhancer-associated histone modifications (Supplementary Fig. 9a). We identified 1246 SEs in HepG2 and 852 SEs in K562 cells, of which 66 and 95% contain RING1B, respectively (Fig. 7a, b). Moreover, RING1B peaks that co-localized with H3K4me1 and H3K27ac were devoid of H3K27me3 (Supplementary Fig. 9b) and about 40% of typical enhancers in K562 were occupied by RING1B, respectively (Fig. 7a, b). Moreover, RING1B peaks that co-localized with H3K4me1 and H3K27ac were devoid of H3K27me3 (Supplementary Fig. 9b) and about 40% of typical
enhancers were occupied by RING1B in both HepG2 and K562 cells (Supplementary Fig. 9c).

RING1B-containing SEs in breast cancer cells included binding sites for oncogenic transcription factors (Fig. 2g). In HepG2 cells, RING1B was recruited to SEs bound by key circadian rhythm transcription factors, including CLOCK, BMAL, and N帕52, that directly regulate the expression of BHLHE40, another core clock component identified in our analysis (Fig. 7c)37. Importantly, disruption of the circadian clock has been implicated in liver cancer and the abnormal expression of clock genes correlates with increased tumor size and cell proliferation38,39. Moreover, using the TCGA liver hepatocellular carcinoma (LIHC) data set, we found that 9% of patients with LIHC have amplification of RNF2 (Supplementary Fig. 9d), and its expression is significantly higher in liver tumors compared to normal liver (Supplementary Fig. 9e).

Finally, we confirmed that RING1B-containing enhancers in HepG2 cells were also decorated with BHLHE40 (Fig. 7d, e), and 72% of SEs contained both RING1B and BHLHE40 (Supplementary Fig. 9f, left).

In K562 cells, RING1B was recruited to SEs containing binding motifs for GATA1 and GATA2 factors (Fig. 7f). GATA2 is often mutated in myeloid malignancies while GATA1 is overexpressed in acute myeloid leukemia (AML), highlighting the role of GATA factors in leukemia40. Importantly, we also confirmed that GATA1 is recruited to RING1B-containing enhancers and to 65% of the SEs in K562 cells (Fig. 7g, h and Supplementary Fig. 9f, right). As expected, RING1B binding to SEs in K562 and HepG2 cells was cell type specific (Fig. 7i). These observations lead us to conclude that RING1B is a novel epigenetic factor that promotes important transcriptional regulatory networks at enhancers to promote oncogenic pathways in multiple cancer types (Fig. 7j).

Discussion

During the last decade, extensive sequencing of cancer genomes has revealed mutations of transcription factors, epigenetic machineries, and signaling pathway factors, and led to the development of novel therapeutic targets. Nonetheless, it remains crucial to investigate the molecular mechanisms, enzymes, and epigenetic machineries that are dysregulated and altered in cancer. The importance of this complementary approach is exemplified by the PRC1-mediated mechanisms we propose. While PRC1-encoding genes are not typically mutated in cancer, we found that several canonical PRC1 genes are amplified and dysregulated in many hormone-related cancers, including breast cancer. Hormone-related cancers share a unique mechanism of action, as hormones drive proliferation which induces the accumulation of mutations41. Whether hormones contribute to chromosome instability and genomic rearrangements of genomic sites of PRC1 genes in breast cancer remain to be addressed.

We propose that in normal breast epithelial and breast cancer cells, RING1B function is uncoupled from its classical role as a repressor of lineage genes42. In pluripotent cells, RING1B is the main E3-ligase that mono-ubiquitinates histone H2A42. In contrast, in the breast cancer cells used in this study, RING1A is more enzymatically active towards histone H2A than RING1B.

TRIM37 was recently proposed as a novel histone H2A ubiquitin ligase in breast cancer cells, with a chromosomal copy-number amplification at 17q2343. T47D and MDA-MB-231 cells do not have amplification of this chromosome arm, which is consistent with our results suggesting that RING1A is the main histone H2A mono-ubiquitin ligase in these cells. Further analyses at the biochemical level are required to determine the exact mechanisms underlying RING1A and TRIM37 deposition of H2AK119ub1 in the context of breast cancer.

Although the classical PRC1-mediated gene regulation is to compact chromatin, PRC1 complexes are also involved in facilitating gene transcription44,45,46. The exact function of PRC1 complexes in gene activation, and the molecular mechanisms that permit Polycomb complexes to activate genes, are under intense investigation. Recently, it has been shown that the PRC1 complex is redistributed genome-wide during oncogenesis in Drosophila to sites decorated with H3K27ac47. In melanoma, RING1B is recruited to chromatin to repress gene activity, but it is also recruited to transcriptionally active genes devoid of H3K27me3 and H2AK119ub144. More recently, it has been shown that in adult epithelial stem cells, PRC1 is recruited to gene promoters containing histone modifications typically found in active enhancers45. Here, we have provided the first evidence that RING1B and cPRC1 complexes are recruited to enhancer regions in cancer cells and that RING1B depletion has a major impact on chromatin accessibility at enhancers. Mechanistically, we show a functional crosstalk between RING1B and the FOXA1/ERα axis, which ultimately resulted in an attenuated response to estrogen. FOXA1, a nuclear receptor regulatory factor, is not limited to its canonical function as it also interacts with the androgen receptor (AR) to regulate its deposition to chromatin in prostate cancer cells45. Thus, it remains to be determined whether RING1B functionally associates with FOXA1 and AR in other cancer types or during embryonic development.

None of the cPRC1 components have the ability to directly bind DNA. Therefore, we anticipate that non-genomic factors are involved in their recruitment to chromatin. One possibility could be that cPRC1 components are post-transcriptionally modified at SEs. Since CBX proteins can bind RNA47, another possible mechanism could involve CBX8 interacting with eRNAs to recruit RING1B and the cPRC1 complex to specific enhancers. Interestingly, physical interactions between PRC1 and Fis(1)h and Br410, the Drosophila orthologs of BRD4 and BRD1, have been recently described48. Although we did not detect a physical interaction of RING1B with BRD4 in the MDA-MB-231 cell line under our experimental conditions, we observed a strong co-occupancy of BRD4 and cPRC1 at active genes, in agreement with the observation of Kuroda and colleagues. Our studies also indicate that both of these epigenetic machineries are co-recruited to enhancer regions in breast cancer cells.

Recruitment of RING1B to active enhancers would appear to suggest that RING1B is solely involved in positively regulating their expression. Surprisingly, we discovered a much more complicated scenario, in which RING1B can exert canonical and non-canonical functions at enhancers, both at the levels of their transcriptional activity and transcription factor accessibility. We theorize that in a set of highly active enhancers, RING1B is required for their activity, while in another set of enhancers with diminished activity, RING1B is required to prevent a hyperactivation of the enhancer. This model is in agreement with a recent report showing that RACK7 and KDM5C are recruited to enhancers, where they act to hamper full enhancer activation in cancer cells49. Overall, we suggest that intricate epigenetic mechanisms mediate enhancer activity and disruption of this regulation may contribute to tumorigenesis.

The contribution of Polycomb complexes in breast cancer tumorigenesis and metastasis is largely unknown. Here we show that high levels of RNF2 in patients with ER+ breast cancer tumors correlate with good survival outcome, while high RNF2 levels in patients with basal breast cancer correlate with lower survival probability. These surprising results are consistent with our xenografts experiments and with other reports showing that RING1B is required for migration of TNBC cells50. It has been also shown that high levels of RING1B correlated with metastatic squamous cell carcinoma50,51. The molecular mechanisms by
which RING1B either prevents or enhances metastasis in specific breast cancer subtypes remain to be fully understood. Future work coupling genomics and genome architecture with functional assays may help reveal which of the RING1B-mediated molecular mechanisms contribute to breast cancer metastasis. Finally, we propose that development of small molecules to impair RING1B recruitment to specific genomic sites in TNBC may have important therapeutic implications.

Methods

Cell lines. Human iPSCs (ATCC #ACS-1021) were maintained in complete feeder- 
free mTESR1 culture medium (STEMCELL Technologies #88580) on matrigel-
coated plates (Corning #353727) at 37°C with 5% CO₂. The culture media was changed daily and iPSC colonies were enzymatically passaged with StemPro 
Accutase Cell Dissociation Reagent (Thermo Fisher Scientific #A1110501) at a
1:4–1:6 split ratio every 4–7 days. DMEM/F-12 media (STEMCELL Technologies #36254) was used to detach colonies. ROCK inhibitor Y 27632 (STEMCELL 
Technologies #23202) was used in every split and when cells were thawed from 
liquid nitrogen. If identified, spontaneously differentiated cells were mechanically 
removed prior to passing. MCF10A, MDA-MB-231, T47D, and SKBR3 (ATCC #CRL-1301, HTB-26, HTB-133, and HTB-30) were maintained at 37°C with 
5% CO₂ and split every 2–3 days according to ATCC recommendations. Culture media 
supplemented with 1× penicillin/streptomycin (Thermo Fisher Scientific 
#15140-122) and 1x glutamax (Thermo Fisher Scientific #35050-061) and com-
plete culture media for each cell line was as follows: MCF10A–DMEM/Ham’s F-12 (1:1) (Corning #45000-348) with 10% horse serum (Thermo Fisher Scientific 
##10274-104), 12 (1:1) (Corning #45000-348) with 5% charcoal-
stripped FBS (Benchmark #100-119) for 72 h prior to treatment. In experiments 
with BRD4 inhibition, MDA-MB-231 cells were treated either with DMSO or 
100 nM JQ1, obtained from the Bradner lab. Cells were routinely tested to be free 
with BRD4 inhibition, MDA-MB-231 cells were treated either with DMSO or 
250 nM Tris-HCl [pH 8], 10% glyceraldehyde, and homogenized with 
20 strokes in a homogenizer fitted with pestle A. After 20 min on ice, 
extracts were centrifuged for 20 min at 16,000 x g. The supernatant, representing the 
soluble nuclear fraction, was collected and stored at 4°C. The remaining pellet was 
resuspended in 1/5 of the original volume with high-salt buffer (50 mM HEPES, 
50 mM Tris-HCl [pH 8], 10% glyceraldehyde, and 0.2% NP-40) supplemented 
with protease inhibitors and sonicated with a Biopump for 5 min in 30” ON-OFF cycles. 

Western blotting and immunoprecipitation. Cells were lysed in high-salt buffer 
(300 mM NaCl, 50 mM Tris-HCl [pH 8], 10% glyceraldehyde, and 0.2% NP-40) supple-
mplemented with protease inhibitors (Sigma-Aldrich #10879 for CTR; 
Sigma-Aldrich #TRCN0000033696 and TRCN0000033697 for FOXA1, 2 g of pCMV-VSV-G, and 6 μg of pCMV-DR8.91 plasmids using calcium phosphate. 72 h after transfection, viru-
supernatant was collected, passed through a 0.45 μm filter, and stored at -80°C. 

Scraped and washed 1x with PBS, then centrifuged for 5 min at 4000 x g. Cell pel-
et was resuspended 1:5 (w/v) in Buffer A (10 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 
10 mM KCl, 0.5 mM DTT) supplemented with protease inhibitors and sonicated with 
2 strokes in a homogenizer fitted with pestle A. After 20 min on ice, 
extracts were centrifuged for 20 min at 16,000 x g. The supernatant, representing the 
soluble nuclear fraction, was collected and stored at 4°C. The remaining pellet was 
resuspended in 1/5 of the original volume with high-salt buffer (50 mM HEPES, 
50 mM Tris-HCl [pH 8], 10% glyceraldehyde, and 0.2% NP-40) supplemented 
with protease inhibitors and sonicated with a Biopump for 5 min in 30” ON-OFF cycles. 

Animal studies. The University of Miami Institutional Animal Care and Use Committee (IACUC) approved all animal procedures. shCTR and shRING1B 
T47D and MDA-MB-231 cells were transduced with retroviruses expressing GFP-
positive control (Addgene #10879 for FOXA1, 2 g of pCMV-VSV-G, and 6 μg of pCMV-DR8.91 plasmids using calcium phosphate. 72 h after transfection, viru-
supernatant was collected, passed through a 0.45 μm filter, and stored at -80°C. 

Scraped and washed 1x with PBS, then centrifuged for 5 min at 4000 x g. Cell pel-
et was resuspended 1:5 (w/v) in Buffer A (10 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 
10 mM KCl, 0.5 mM DTT) supplemented with protease inhibitors and sonicated with 
2 strokes in a homogenizer fitted with pestle A. After 20 min on ice, 
extracts were centrifuged for 20 min at 16,000 x g. The supernatant, representing the 
soluble nuclear fraction, was collected and stored at 4°C. The remaining pellet was 
resuspended in 1/5 of the original volume with high-salt buffer (50 mM HEPES, 
50 mM Tris-HCl [pH 8], 10% glyceraldehyde, and 0.2% NP-40) supplemented 
with protease inhibitors and sonicated with a Biopump for 5 min in 30” ON-OFF cycles. 

Preparation of ATAC-seq experiments were performed as previously described[5] with modifications. Briefly, 25,000 cells of each cell line were used to perform the transposition reaction. Samples were eluted in 13 μl of Buffer EB (Qiagen #20260). To calculate the number of cycles for library amplification, 2 μl of transposed DNA were amplified by qPCR for a total of 25 cycles. The 10 μl qPCR reaction was set up as follows: 2 μl of transposed DNA, 0.3 μl 25 μM Ad1_noMX, 0.3 μl 25 μM Ad2 (custom oligos synthesized by Integrated DNA Technologies, see Supplementary Data 7), 5 μl NEBNext High-

Fidelity 2X PCR Master Mix (New England Biolabs #M05451), 0.1 μl 100X SYBR 
Green I (Thermo Fisher Scientific #S7563) and 2.3 μl nuclease-free water with the following primer sequence used on a Bio-Rad iCycler (Bio-Rad, USA) (unique for each transfection reagent): (1) 72°C for 5 min, (2) 98°C for 30 s, (3) 98°C for 10 s, 63°C for 30 s and 72°C for 30 s, 25 cycles, (4) 72°C for 1 min, and (5) hold at 10°C. The Ct value of each 
sample reflects the number of PCR cycles for optimal amplification in the linear 
range of the reaction. A 50 μl qPCR reaction was then set up as follows: 10 μl transposed DNA, 1.5 μl 25 μM Ad1_noMX, 1.5 μl 25 μM Ad2 (unique for each 
sample), 12 μl nuclease-free water, and 25 μl NEBNext High-Fidelity 2X PCR 
Master Mix with the same program as for the qPCR, but substituting the cycling number with the Ct-value obtained from the qPCR reaction. The PCR 
was performed on a Bio-Rad C1000 Touch Thermal Cycler. After PCR, the 50 μl reactions 
were cleaned up and size selected by adding 25 μl AMPure XP beads (Beckman Coulter #A63881) to remove fragments shorter than 800 bp. The supernatant 
was transferred to a new tube and 65 μl AMPure XP beads were added to remove fragments smaller than 100 bp, then washed twice with freshly prepared 80% 
buffet and eluted in 25 μl nuclease-free water. To determine the average fragment size, 5 μl from each run was run through a high-sensitivity DNA screen tape (Agilent Technologies #5067–5584) following the manufacturer’s instructions on an Agilent Technologies 2200 TapeStation machine. To determine the
Qubit 3.0 Skewer v0.2.2 to remove low-quality reads, and paired-end reads were aligned to 75 bp on a NextSeq 500. Homer annotatePeaks was used for peak annotation.

Puriﬁcation of endogenous RING1B complexes. Cell pellets in triplicate from MDA-MB-231 or T47D cell lines were snap frozen in liquid nitrogen. Each pellet was resuspended in 1:4 (v:v) in a solution of 50 mM Tris pH 8.0, 300 mM NaCl, 0.2% (v:v) NP-40, supplemented with protease inhibitors (Sigma-Aldrich #12107801001). Samples were lysed by ultrasonication at 4 °C using a Qsonica S4000 equipped with a S4717 microtip probe. For each sample, 2-3 long pulses at 1 Aamp were applied, with 1 s pauses, until ~20 of output per 100 mg of cell pellet was reached. After sonication, samples were centrifuged at 20,000xg at 4 °C for 10 min, producing a clarified cell extract. For MDA-MB-231, 400 µl of clarified extract from each replicate was used in IP and control experiments, respectively. In IP experiments, extracts were combined with 10 µl of magnetic afﬁnity medium. Thermo Fisher Scientiﬁc #14301) coupled to anti-RING1B antibodies (MBL, see Supplementary Data 6). In control experiments, mouse IgG (Sigma-Aldrich, see Supplementary Data 6) was used. 7.3 µg of anti-RING1B antibody and 10 µg of mouse IgG were used, respectively, per mg of magnetic medium in epoxy-based coupled coating (as per manufacturer’s instructions). For T47D control experiments, 200 µl of extract were combined with 5 µl of afﬁnity medium. Clar- ified cell extracts were incubated with magnetic media for 1 h at 4 °C with gentle end-over-end mixing. After mixing, the supernatants were removed and the beads were washed three times with 1 ml of the extraction solution. The bound fraction was released from IP/control experiments by the addition of 15 µl of 1X LDS sample buffer (Thermo Fisher Scientiﬁc #NP00087) with incubation at 70 °C for 5 min with agitation. After incubation, the eluate was removed, reduced with DTT, alkylated with iodoacetamide, and then run a ~6 mm into a 1% agarose gel at 100 V for 15 min and imaged on a Bio-rad FluorChem XRS+. Protein concentration in sonicated samples was measured by BCA assay, and 200 µg of total protein was transferred to a 1.5 ml LoBind tube (Eppendorf #0030108051) and brought up to 500 µl total volume with ChIP buffer. 5 µl was removed as input material (1%) and placed in a separate microtube at 4 °C. 2 µg antibody was used for each histone ChIP, except for H2A.Z in which 5 µl was used. Samples were incubated with 2 µl of ChIP buffer at 4 °C for 30 min, washed 3× with ChIP buffer and 30 µl bead slurry was added to each sample. Samples were incubated with beads for 2 h at 4 °C rotating end-to-end. Following incubation, samples were centrifuged at 400 × g for 3 min at 4 °C, washed 2× with ChIP Low Salt Buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1% Triton X-100, 1x protease inhibitors), and 1× with ChIP High Salt Buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 1% Triton X-100, 1x protease inhibitors). Beads were dried after the last wash with a 28 g needle ﬁtted to a 1 ml syringe. Elution Buffer (1% SDS, 0.1 M sodium carbonate) was prepared fresh before use and 110 µl was added to each sample and 95 µl to each input sample that was previously set aside. To elute immunoprecipitated DNA, samples were sonicated for 15 min on an Eppendorf Thermomixer shaking at 1000 rpm. Tubes were centrifuged for 3 min at 400 × g at RT and 100 µl of supernatant was transferred to a new tube, being careful not to aspirate beads. DNA puriﬁcation was performed with the QiAQuick PCR Puriﬁcation Kit (Qiagen cat# 28106) and eluted in 60 µl H2O and quantiﬁed by Qubit. For histone ChIP target samples, sonication was performed using the High Sensitivity ChIP IT Kit (Active Motif #53040, see Supplementary Data 6). Immunoprecipitated DNA from both methods were used to either perform qPCR or generate libraries using the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs #E7350) following the manufacturer’s instructions. Libraries were visualized on a Tapestation 2200 using D1000 DNA screentape (Agilent Technologies #5067-5582). Libraries were quantiﬁed on a Qubit 3 Flurometer with Qubit dsDNA high sensitivity reagents (Thermo Fisher Scientiﬁc #Q32851) following the manufacturer’s instructions, then pooled and sequenced, single-end, 3 bp, on a NextSeq 500. Processed data was viewed using the UCSC genome browser with a smoothing window of 5 pixels. ChIP-qPCR was performed using primers targeting developmental or enhancer regions identiﬁed (see Supplementary Data 8 for list of primers) on a Bio-Rad CFX96 Real-Time System with iTaq universal SYBR green supermix (Bio-rad #1725124) and analyzed using CFX Manager software (Bio-Rad).

**TCA data preparation and analysis.** The legacy level 3 data of breast invasive carcinoma (BRCA) and liver hepatocellular carcinoma (LIHC) from the TCGA Genome Cancer Atlas (TCGA) cohort were obtained from the Genomic Data Commons (GDC) data portal. RNA-seq raw counts of 1211 BRCA and 421 LIHC cases were curated from legacy archive, and using within-lane normalization to adjust for GC-content effect on read counts and upper-quantile between-lane normalization for distributional differences between the two matrices (tumor and normal) for both tumor types was then normalized using the two matrices (tumor and normal) for both tumor types was then normalized using within-lane normalization to adjust for GC-content effect on read counts and upper-quantile between-lane normalization for distributional differences between the two matrices (tumor and normal) for both tumor types was then normalized using within-lane normalization to adjust for GC-content effect on read counts and upper-quantile between-lane normalization for distributional differences between the two matrices (tumor and normal) for both tumor types was then normalized using within-lane normalization to adjust for GC-content effect on read counts and upper-quantile between-lane normalization for distributional differences between the two matrices (tumor and normal) for both tumor types was then normalized using within-lane normalization to adjust for GC-content effect on read counts and upper-quantile between-lane normalization for distributional differences between the two matrices (tumor and normal) for both tumor types was then normalized using within-lane normalization to adjust for GC-content effect on read counts and upper-quantile between-lane normalization for distributional differences between the two matrices (tumor and normal) for both tumor types was then normalized using within-lane normalization to adjust for GC-content effect on read counts and upper-quantile between-lane normalization for distributional differences between the two matrices (tumor and normal) for both tumor types was then normalized using within-lane normalization to adjust for GC-content effect on read counts and upper-quantile between-lane normalization for distributional differences between the two matrices (tumor and normal) for both tumor types was then normalized using with TCGAanalyze_Normalization function and adopting the EDAsign program. Molecular subtypes, mutation data, and clinical data were pulled using TCGAbiolinks and the following functions: TCGAquery_subtype, TCGAquery_nearest retrieving somatic variants that were called by the MuTect pipeline, and TCGAquery_clinical tracking patients with TCGAclinical. TCGA query subtype classification were stratified into ﬁve molecular subtypes: Basal-like (n = 98), HER2-enriched (n = 58), Luminal A (n = 231), Luminal B (n = 127), and Normal-like (n = 8). Normal-like samples were not considered in this analysis due for the limited number of sample availability. For LIHC, tumors with cluster classiﬁcation were 1) Tumor Type (Tumors with TCGAsubtype_1p19del (n = 55), and iCluster3 (n = 63). Tumor stage information was retrieved from the clinical data grouping to main stages (I, II, III, IV) and each subgroup (ia, Ib, IIC...
etc.). Amplification data obtained from the GISTIC 2.0 tool was then used to identify regions of the genome that were significantly amplified or deleted across a set of samples. GISTIC2 data was retrieved from cBioPortal for both tumor types considering samples with high amplification greater than 2 and excluding high deletion samples lower than −2. The ggrepurr R CRAN (https://CRAN.R-project.org/package=ggrepurr) package was used to draw box plots showing relative expression for each cancer type, stage, and molecular subtype and to perform multiple comparisons using a non-parametric Wilcoxon test. All analyses with plots were generated using the R environment (see Supplementary Data 9 for list of software).

ChiP-Seq analysis. ChiP-seq of RING1B, H3K27ac, H3K27me3, and H3K4me1 (in K562 and HepG2 cells), GATA1 (in K562 cells) and BHILH40 (in HepG2 cells) were re-analyzed from ENCODE data sets (GSE95908, GSE91837, GSM373688, GSM373375, GSM733656, GSM733743, GSM733692, GSM798321, GSE1003680, GMS935566, GSM733780, GSM733732). All ChiP-seq data, generated in this study or deposited into ENCODE, were analyzed according to the following methodology: FASTQ data were processed with Trimmomatic v0.32 to remove low-quality reads and then aligned to the human genome hg19 using BWA v0.7.13-r1126 with additional 2 kb surrounding every exon. A 460bp window at the center of the H3K27ac peak was used to calculate RPKM across the entire RNA-seq data, considering regions with RNA expression to have RPKM > 0.3. Next a cutoff of fold change >2 or <−2 (shCTR versus shRING1B) was used to detected differential expression. RNA loci was overlapped with super-enhancer regions or typical enhancer regions using BEDtools intersect.

Motif analysis. Motif finding was performed with Homer findMotifs v4.8.3. A window of ±100 bp (option -d 200) relative to peak summits was used to perform the analyses.

Statistical analysis. Significance was determined by either Student’s t-test, non-parametric Wilcoxon test, Mann–Whitney test, or Kolmogorov–Smirnov test, as indicated. Error bars in figures represent standard deviation (SD) of at least two independent experiments.

Data availability. All of the genome-wide data of this study have been deposited in the NCBI Gene Expression Omnibus (GEO) database, GSE number: GSE107176. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD009570. Received: 21 December 2017 Accepted: 25 July 2018

Published online: 23 August 2018

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Acknowledgements
We are indebted to V.A. Raker for help in preparing the manuscript, to Gloria Mas Martin and Dr. Joyce M. Slingerland for discussions, and to the Oncogenomics Core Facility at the Sylvester Comprehensive Cancer Center for performing high-throughput sequencing. We also thank the Flow Cytometry Core Facility for assistance with cell sorting, and the Cancer Modeling Shared Resource for assistance with the animal studies. Dr. Ramiro E. Verduz kindly provided the shRING1B plasmids. Kelly Molloy assisted with the mass spectrometry. Dr. Derek M. Dykxhoorn assisted with the iPSC culture. Dr. Aznar-Benitah provided the mPSCV-IREs-Luciferase-GFP vector. This work was supported by Sylvester Comprehensive Cancer Center funds to L.M.

Author contributions
L.M and H.L.C designed the study. H.L.C conducted all the experiments, except ATAC-seq (J.G.H in the laboratory of R.S and L.M.), siRNA and cellular fractionation experiments (Y.Z. and the RING1B pull-downs and LC-MS/MS experiments (H.U.) under the supervision of H.L.C). Bioinformatics analyses were performed by F.B (in the laboratory of R.S.) and H.L.C. TCGA analysis was performed by A.C (in the laboratory of M.E.F). D.B is the Head of the Cancer Modeling Shared Resource Facility at the Sylvester Comprehensive Cancer Center and performed the animal studies. L.M supervised the experiments, performed the experiments in iPSCs and provided intellectual support toward interpretation of the results. L.M and H.L.C wrote the manuscript.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-05728-x.

Competing interests: The authors declare no competing interests.

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