TITLE:
Targeting nuclear pore protein, NUP210, reduces metastasis through heterochromatin-mediated silencing of mechanosensitive genes

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Abstract:

Mechanical signals from the extracellular microenvironment have been implicated in tumor and metastatic progression. It remains unclear how these mechanical signals are transmitted to the cell nucleus to regulate gene expression in metastasis. In an attempt to characterize metastasis-associated polymorphisms in the non-coding regulatory regions of the genome, we identified nucleoporin \textit{NUP210} as a metastasis susceptibility gene for human estrogen receptor positive (ER+) breast cancer and a cellular mechanosensor. Polymorphisms in the mouse \textit{Nup210} promoter affect \textit{Nup210} transcription via alteration of CTCF binding. Depletion of \textit{Nup210} reduces lung metastasis in mouse models of breast cancer. Mechanistically, NUP210 interacts with histone H3.1/H3.2 at the nuclear periphery and prevents its heterochromatin (H3K27me3) modification to regulate mechanosensitive, metastasis-promoting gene expression. Upon \textit{Nup210} knockout, these mechanosensitive genes are differentially repositioned and become repressed due to heterochromatinization. As a result, \textit{Nup210} depletion decreases mechanotransduction and focal adhesion \textit{in vitro} and circulating tumor cells \textit{in vivo}. Our study provides a new insight into the role of nuclear pore protein in cellular mechanosensation and metastasis.
INTRODUCTION:

The majority of cancer-related mortality is due to distant organ metastasis, a process in which cancer cells must leave the primary tumor, intravasate into the surrounding endothelium, evade immunosurveillance in the circulation, travel to a distant site, extravasate, and colonize the secondary organ\textsuperscript{1-3}. Due to the need to successfully complete each of these steps, metastasis is a highly inefficient process. To complete the whole metastatic cascade, cancer cells employ multiple genetic and epigenetic mechanisms. Because of the limited understanding of these mechanisms, it is difficult to target these aggressive cancer cells, even with the improved therapeutic strategies\textsuperscript{4}. Hence, a more detailed understanding of the mechanisms by which cancer cells gain metastatic potential is crucial to ultimately improve patient outcomes.

With the advent of large-scale genome sequencing technology, remarkable progress has been made to characterize the genetic alterations in primary tumors\textsuperscript{5}. In more recent years, an increased focus on the genomics of metastasis has emerged\textsuperscript{6-9}. Such studies have suggested that a metastatic tumor clone may arise from the combined effect of certain genetic and epigenetic changes within primary tumor. These genetic changes include somatic mutation, copy number variation, loss of heterozygosity, and allelic imbalance. Epigenetic changes such as DNA methylation, histone modification, and chromatin remodeling can also affect gene expression in cancer\textsuperscript{10}. Epigenetic silencing of tumor suppressor genes or activation of oncogenes has been implicated in cancer progression\textsuperscript{11,12}. These genes can be silenced or activated through widespread alterations in chromatin accessibility due to the gain or loss of heterochromatin regions. Recent studies have shown increased chromatin accessibility\textsuperscript{13} or disruption of large heterochromatin domains\textsuperscript{14} during
metastatic progression from the primary tumor. However, the mechanisms by which chromatin boundary alterations affect metastasis remain largely unknown.

In addition to genetic and epigenetic changes, we previously demonstrated that germline polymorphism can affect metastatic outcome\textsuperscript{15-17}. Since many of our polymorphic metastasis susceptibility genes were identified as chromatin-associated factors, we hypothesized that germline polymorphism can affect metastasis through alteration of the cancer cell’s epigenome. To identify metastasis-associated polymorphisms in the non-coding regulatory regions of the genome, we performed genome-wide identification of polymorphic accessible chromatin regions in mouse models of metastasis and identified \textit{Nup210}, a gene encoding a nuclear pore complex protein, as a potential metastasis susceptibility gene. Although nuclear pore complex proteins have recently been shown to be associated with several developmental disorders and cancers\textsuperscript{18-21}, their function in metastasis remains unexplored. Here, we established a nucleocytoplasmic transport-independent role of NUP210 in promoting lung metastasis in mouse models of breast cancer through alteration of mechanosensation, focal adhesion, and cell migration.

\textbf{RESULTS:}

\textit{Identification Nup210 as a candidate metastasis susceptibility gene}

To identify accessible chromatin regions enriched during metastatic colonization, Benzonase-accessible chromatin (BACH) analysis was performed on the 4T07 and 4T1 isogenic cell lines derived from the spontaneous mammary tumor of BALB/cJ mice\textsuperscript{22} (Fig. 1a; Shukla et al., in preparation). 67NR cells are tumorigenic when injected orthotopically into the mammary fat pad
of BALB/cJ mice, but they do not metastasize into distant organs. 4T07 cells produce primary tumors that disseminate to the lungs but only rarely form macroscopic metastasis. In contrast, 4T1 cells complete the metastatic process and form multiple macroscopic lesions in the lung upon orthotopic implantation\textsuperscript{22}. We therefore hypothesized that differences in open chromatin between these two cell lines would be enriched for transcriptional control elements contributing to the dormant-to-proliferative switch during metastatic colonization. A total of 5303 BACH regions were unique to 4T1 cells and were therefore likely to be enriched in colonization-associated gene expression. Since the majority of inherited susceptibility is thought to result from polymorphisms in non-coding transcriptional control elements\textsuperscript{23}, the BACH regions were then intersected with known polymorphisms present in the Diversity Outbred (DO) panel\textsuperscript{24}, a highly polymorphic mouse genetic mapping panel generated from the random interbreeding of eight inbred mice strains. This identified a total of 1874 polymorphic BACH regions with the potential to alter gene transcriptional efficiency (Fig. 1a).

Preliminary analysis of the 1874 polymorphic BACH regions revealed that the majority (1477 out of 1874) did not overlap with gene promoters, defined here as regions extending 5 kb upstream and 1 kb downstream of the transcriptional start site. Since polymorphisms in enhancers are also thought to encode disease susceptibility, ChIA-PET (Chromatin Interaction Analysis through Paired-End Tag sequencing) analysis of the 4T1 cell line was performed and integrated with the BACH regions to identify gene promoters with single loops to putative polymorphic enhancers. This resulted in a total of 771 genes with potential colonization-associated polymorphic transcriptional control elements (Fig. 1a). As a final filtering step, the 771 genes were screened across a panel of tumors generated from a cross between the DO panel and a highly metastatic mammary tumor mouse model, MMTV-PyMT, to identify genes whose expression correlated with
distant metastasis-free survival (DMFS). Fifty-two of the 771 genes were significantly associated with DMFS in this tumor panel (Fig. 1a). Circos plot representation of these 52 genes revealed that some of them possess polymorphic promoters (Fig. 1b, red lines). In addition, many of these genes establish intra- and inter-chromosomal interaction in 4T1 cells (Fig. 1b, blue lines and light blue lines, respectively). To assess human relevance, the hazard ratios calculated for these 52 genes in the mouse data were used to generate a weighted gene signature that was subsequently screened on human breast cancer gene expression datasets. Significant stratification of DMFS in estrogen receptor-positive (ER+) breast cancer was observed in the human dataset (Fig. 1c), consistent with a significant association of these genes with human breast cancer progression and metastasis. NUP210, a nuclear pore protein, was selected for further validation due to the association of a number of metastasis susceptibility genes with the nuclear envelope. Also, our RNA-seq data on the isogenic 4T1 series of mouse cell lines revealed that the expression of Nup210 was at the baseline level in the non-metastatic 67NR cell line, intermediate in the disseminating 4T07 cells but was sharply increased in the colonizing 4T1 cell line (Fig. 1d).

**Polymorphisms in the Nup210 promoter affect CTCF binding and Nup210 transcription**

Next, we explored whether genetic polymorphisms in the Nup210 promoter affect Nup210 expression. We focused on polymorphisms in the 510 bp mouse Nup210 putative promoter region based on our BACH analysis on 4T07 and 4T1 cell lines (Fig. 1e). Within the 510 bp promoter region, six single nucleotide polymorphisms (SNPs) and one insertion/deletion (indel) were found among three different mouse strains in the Sanger Mouse Genome Repository25 (Fig. 1f). Interestingly, Sanger sequencing analysis revealed that there was a 12 bp G-rich insertion in the
Nup210 promoter of the FVB/NJ strain that was located within the CTCF binding region in mouse mammary epithelial (ME) cells. This particular region also showed enrichment for the H3K27Ac enhancer histone modification mark in mouse mammary luminal (ML) epithelial cells and the H3K4me3 promoter mark in 4T1 cells (Fig. 1g). Occupancy of CTCF and H3K27Ac within the human NUP210 promoter was also evident in publicly available ChIP-seq data from the MCF7 human breast cancer cell line, suggesting that the binding of CTCF to the NUP210 promoter is evolutionarily conserved (Supplementary Fig. 1). As CTCFs have been shown to be enriched in the enhancer-promoter boundary or insulator element to regulate the gene expression, we hypothesized that polymorphic insertion in this region might affect CTCF binding and Nup210 expression. Since 4T1 and 6DT1 metastatic mouse mammary cancer cell lines were derived from primary tumors from BALB/cJ and FVB/NJ mouse strains, respectively, we performed ChIP-qPCR analysis to compare the enrichment of CTCF and H3K27Ac in these two cell lines. Both CTCF and H3K27Ac marks were preferentially enriched in the Nup210 promoter region of 4T1 cells as compared with 6DT1 cells (Fig. 1h). qRT-PCR analysis revealed that Nup210 and Ctcf expression levels were significantly higher in 4T1 cells than in 6DT1 cells (Fig. 1i). Consistently, Nup210 expression was significantly lower in the spleen of normal FVB/NJ mice than in BALB/cJ mice (Fig. 1j). However, Ctcf expression was not significantly different in the spleen, implying that the polymorphism in Nup210 might be responsible for the decreased expression in FVB/NJ strains despite the unaltered level of Ctcf (Fig. 1j). A promoter luciferase assay revealed that there was a moderate (~10%) reduction in luciferase activity for the FVB/NJ-derived Nup210 promoter region as compared with the equivalent BALB/cJ promoter region (Fig. 1k). This result is consistent with the fact that CTCF mainly exerts its transcriptional activity through global alteration of chromatin architecture and reporter assays using ectopically expressed plasmids.
may not fully recapitulate CTCF activity in the native genomic context. To observe the direct effect of CTCF loss on Nup210 transcription, we knocked down Ctcf in 4T1 cells and observed a marked reduction of Nup210 both at the transcript (Fig. 1l) and protein level (Fig. 1m). Taken together, these results suggest that Nup210 is a CTCF-regulated gene and polymorphisms in the Nup210 promoter may affect CTCF binding in cancer cells.

**NUP210 expression is associated with metastasis in human ER+ breast cancer patients**

Examination of NUP210 alterations in METABRIC31 (Molecular Taxonomy of Breast Cancer International Consortium), a human breast cancer dataset, revealed that NUP210 was amplified in 1.2% of the cases and was associated with decreased overall survival (Fig. 2a). Examination of the NUP210 mRNA expression level in the METABRIC dataset suggests that NUP210 expression is significantly associated with reduced overall survival in ER+ breast cancer patients (Fig. 2b) but not in triple-negative (ER-/PR-/Her2-) patients (Fig. 2c). Consistently, survival analysis using an independent dataset from the Km-plotter database32 revealed that NUP210 expression is associated with poor distant metastasis-free survival in ER+ breast cancer patients (Fig. 2d) but not in triple-negative patients (Fig. 2e). An analysis of NUP210 expression across PAM50 molecular subtypes from the METABRIC dataset suggests that NUP210 expression is higher in luminal B and basal subtypes than in luminal A, Her2+, and claudin-low subtypes of breast cancer (Fig. 2f). Gene expression analysis from different metastatic sites of breast cancer revealed that NUP210 expression is significantly higher in lung and liver metastases than lymph node metastases (Fig. 2g). Furthermore, analysis gene expression data from primary tumor and metastases suggested that NUP210 expression is significantly higher in metastases than primary tumor of prostate cancer...
(Fig. 2h) and melanoma (Fig. 2i). These data indicate that *NUP210* is associated with human cancer progression and further supports its potential role as a metastasis susceptibility gene.

**Depletion of Nup210 in cancer cells decreases lung metastasis in mice**

To test the function of *Nup210* in metastasis *in vivo*, we utilized mouse breast cancer orthotopic transplantation models that closely resemble human luminal breast cancers. Three different cell lines were utilized for this analysis: 4T1, 6DT1, derived from the mammary tumor of FVB/MMTV-MYC transgenic mouse, and MVT1, derived from the mammary tumor of FVB/MMTV-MYC/VEGF double transgenic mouse. These cell lines form primary tumors and lung metastases within one month after mammary fat pad injection. *Nup210* was knocked down through shRNA-mediated interference and knockdown was confirmed either through western blot or qRT-PCR (Fig. 3a, Supplementary Fig. 2a). Cell lines stably expressing two shRNAs, sh-Nup #1 and #4, were used for mouse injection as these showed consistent knockdown across cell lines. Primary tumors derived from *Nup210* knockdown cells showed variability in tumor weight, with decreased tumor weight in 4T1 cells but increased tumor weights for both 6DT1 and MVT1 cells (Fig. 3b, Supplementary Fig. 2b). However, knockdown of *Nup210* resulted in a decrease in pulmonary metastases for all three cell lines (Fig. 3c, Supplementary Fig. 2c), and this decrease was preserved after normalizing metastasis counts by tumor weight to account for the variability in primary tumor growth (Fig. 3d, Supplementary Fig. 2d). These results were therefore consistent with the association of *NUP210* with poor prognosis in patients with ER+ breast cancers.

To further validate the role of *Nup210* in metastatic progression, CRISPR/Cas9-mediated knockout of *Nup210* was performed in the 4T1 cell line (Fig. 3e). In agreement with the shRNA
result, CRISPR-mediated knockout of Nup210 in two different clones, N9 and N13, dramatically diminished primary tumor weight and lung metastases (Fig. 3f-h). Since we observed differential effects of Nup210 loss on primary tumor weight, we asked whether Nup210 depletion affects cell proliferation. Cell cycle analysis on Nup210 knockout 4T1 cells (Supplementary Fig. 3a, b) revealed that there was no consistent change in the distribution of cell population among cell cycle stages, suggesting that the effect of NUP210 loss on the primary tumor is independent of the intrinsic ability of NUP210 to regulate cell proliferation. Finally, 4T1 cells with stable overexpression of Nup210 showed no difference in primary tumor weight (Fig. 3i, j). However, lung metastasis count was significantly increased by Nup210 overexpression (Fig. 3k). Taken together, these results indicate that Nup210 promotes lung metastasis in mouse models of luminal breast cancer.

**Nup210 loss does not affect general nucleocytoplasmic protein transport but may affect RNA transport**

The major function of the nuclear pore complex is nucleocytoplasmic transport of protein and RNA molecules. However, a recent study suggests that loss of Nup210 does not affect the general nucleocytoplasmic transport in differentiating myotubes. To determine whether Nup210 depletion affects nucleocytoplasmic transport in our metastatic 4T1 cancer cells, the NES-tdTomato-NLS nucleocytoplasmic transport reporter was used (Supplementary Fig. 4a). This reporter expresses tdTomato protein fused to both a nuclear localization signal (NLS) and nuclear export signal (NES), resulting in shuttling between the cytoplasm and nucleus. Transfection of this reporter into Nup210 knockdown 4T1 cells with or without the nuclear export inhibitor leptomycin B showed no significant differences in nucleocytoplasmic transport of the reporter protein.
(Supplementary Fig. 4b). Next, we examined the effect of \textit{Nup210} depletion on RNA export through oligo-d(T) RNA fluorescent \textit{in situ} hybridization (FISH) analysis. Increased retention of polyA+ RNA signal was observed in the nucleus upon \textit{Nup210} depletion (Supplementary Fig. 4c, 4d), implying that \textit{Nup210} may facilitate RNA export into the cytoplasm.

\textbf{\textit{NUP210} interacts with histone H3.1/3.2 at the nuclear periphery}

To better understand how NUP210 promotes metastasis, co-immunoprecipitation (Co-IP) followed by mass spectrometry was performed to identify NUP210-interacting proteins (Fig. 4a). Interestingly, mass spectrometry analysis in NUP210-Myc overexpressing 4T1 cells revealed that both endogenous and Myc-tagged NUP210 potentially interact with multiple chromatin-associated molecules including histone H3.1 (Fig. 4b). Notably, mass spectrometry indicated that NUP210 also interacts with RRP1B, a chromatin-associated metastasis modifier gene previously identified by our lab\textsuperscript{17,37}. Mutation of histone H3.1 has been reported in human cancer\textsuperscript{38,39}. In the human breast cancer METABRIC dataset, amplification of histone H3.1 (\textit{HIST1H3A} and \textit{HIST1H3B}) might be associated with poor prognosis in breast cancer patients (P = 0.0642) (Fig. 4c). Despite this, the role of H3.1 in metastasis remains unclear. Since H3.1 can undergo post-translational modification to subsequently affect gene expression, we hypothesized that NUP210 interaction with histone H3.1 might regulate a gene expression program essential for metastatic progression. Reciprocal Co-IP in 4T1 cells using an antibody that recognizes both H3.1 and H3.2 validated the predicted H3.1-NUP210 interaction (Fig. 4d, 4e). H3.1/3.2 also pulled down lamin B1, a component of the nuclear lamina, suggesting that this interaction may occur at the nuclear periphery (Fig. 4e). Immunofluorescence analysis confirmed the association of H3.1/3.2 and
NUP210 at the nuclear periphery (Fig. 4f). These results establish the interaction of NUP210 with H3.1/3.2 and their localization in metastatic 4T1 cells.

**Loss of NUP210 is associated with redistribution of H3.1/3.2 from the nuclear periphery to heterochromatin foci**

We next investigated the role of NUP210-H3.1/3.2 interaction in our metastatic 4T1 cells. Since H3.1 is known to be incorporated into nucleosomes in S phase of the cell cycle in a replication-dependent manner\(^40\), we initially speculated that NUP210 loss might affect S phase progression. However, DNA content analysis with EdU incorporation demonstrated that there was no consistent changes in cell cycle stage distribution (Supplementary Fig. 3a, 3b), suggesting that the function of NUP210-H3.1/3.2 association might be independent of cell cycle progression.

To investigate the cellular effect of Nup210 loss on the distribution of H3.1 at the nuclear periphery, we performed super-resolution spinning disk microscopy in Nup210 KO cells. Interestingly, H3.1/3.2 distribution at the nuclear periphery was significantly decreased (Fig. 4g, 4h) in Nup210 KO cells, suggesting that H3.1/3.2 anchoring at the nuclear periphery might be reduced upon NUP210 loss. There was also significant enrichment of the H3K27me3 mark at the nuclear periphery in Nup210 KO cells (Fig. 4g, 4h). H3.1/3.2 predominantly appeared as foci-like structures inside the nucleus upon knockout of Nup210. To determine if these H3.1/3.2 foci were associated with pericentric heterochromatin, we stained the cells for H3K9me3, a pericentric heterochromatin-associated histone modification marker. Upon Nup210 KO, H3.1/3.2 accumulated on the periphery of H3K9me3-marked pericentric heterochromatin (Fig. 4i). We also observed a significant increase in H3.1/3.2 staining at the heterochromatin foci marked by strong
DAPI signal (Fig. 4j). Although there was an overall increase in H3K9me3 within the nucleus, the distribution of H3K9me3 was not changed significantly following Nup210 KO (Fig. 4j). Staining with a nucleolin antibody showed that many of the H3.1/3.2 foci appeared on the periphery of nucleoli, heterochromatin-enriched regions (Fig. 4k). As heterochromatin content can be inversely correlated with nuclear size\textsuperscript{41}, we measured the nuclear size and found it to be significantly decreased in Nup210-depleted and KO cells (Fig. 4l), indicative of increased chromatin compaction due to heterochromatinization. These results suggest that the loss of NUP210 is associated with redistribution of H3.1/3.2 to heterochromatin regions within the nucleus.

**Increased recruitment of heterochromatin-modifying enzymes, SUV39H1 and EZH2, to H3.1/3.2 in Nup210 KO cells**

Since we observed the redistribution of H3.1/3.2 to the heterochromatin regions upon Nup210 KO, we wanted to investigate whether differential post-translational heterochromatin modification of H3.1/3.2 underlies the changes in distribution observed in Nup210 KO cells. So, we performed H3.1/3.2 Co-IP with antibodies recognizing different heterochromatin modifications (H3K9me3 and H3K27me3) and the enzymes known to catalyze these modifications. Co-IP revealed that there was decreased association of H3.1/3.2 with lamin B1 and increased association with SUV39H1 (an enzyme catalyzing H3K9me3 modification of H3.1/3.2) as well as H3K9me3 in Nup210 KO cells (Fig. 4m). Loss of NUP210 was also associated with increased interaction of H3.1/3.2 with EZH2 (Fig. 4n), a key component of the polycomb repressive complex 2 (PRC2) that catalyzes H3K27me3 deposition\textsuperscript{42}. Further analysis of other PRC2 components revealed an increased association of EZH2 with SUZ12 (Fig. 4o) and H3K27me3 (Fig. 4p) upon Nup210 KO. Taken together, these results suggest that NUP210 prevents the recruitment of heterochromatin-
modifying enzymes to H3.1/3.2. Loss of NUP210 may promote heterochromatin modification and thereby transcriptional suppression of H3.1/3.2-associated genes or regulatory elements (promoters, enhancers) required for metastatic progression.

**NUP210 loss increases heterochromatin spreading on topologically associating domains and decreases expression of cell adhesion/migration-related genes**

Since we observed increased heterochromatin association in *Nup210* KO cells, we hypothesized that NUP210 loss might lead to heterochromatinization of certain genomic region important for metastasis. Earlier studies have suggested that H3.1/3.2 is mainly associated with poised/bivalent gene promoters where it can undergo both activating (H3K4me3) and repressing (H3K27me3) modification\(^40\). Therefore, we performed chromatin immunoprecipitation analysis with massively parallel DNA sequencing (ChIP-seq) of NUP210, H3K27me3, and H3K4me3 to determine the pattern of genomic localization of these modifications in 4T1 cells. The majority (55.33\%) of the NUP210 peaks were within intergenic regions (Fig. 5a). 19.01\% of NUP210 peaks were found within 10 kb upstream of transcription start sites (TSS), while the rest of the peaks were found within gene bodies (i.e., exons, introns, and UTRs). Interestingly, enrichment profiling showed that NUP210 was mainly enriched in 3'-transcription end sites (TES) of the genes (Fig. 5b). Similar TES enrichment patterns have been observed for some transcriptional regulators\(^43\) as well as nucleoporin, NUP153\(^44\). ChIP-seq of H3K27me3 in 4T1 cells revealed that there was a 40\% (9962 peaks) overlap of the NUP210 peaks with H3K27me3 peaks (FDR < 0.05) (Fig. 5c). Overlaying the NUP210-enrichment profile with H3K27me3 in sg-Ctrl and *Nup210* KO cells revealed that H3K27me3 was enriched surrounding the NUP210-bound gene bodies (Fig. 5d). The ChIP-seq profile obtained using a pan-nuclear pore complex (NPC)-specific antibody (mAb414) did not
show a similar enrichment pattern, implying that this enrichment pattern is NUP210-specific. Interestingly, in Nup210 KO cells, the H3K27me3 level was increased over NUP210-bound gene bodies (Fig. 5d), suggesting that these genes were undergoing H3K27me3-marked heterochromatinization. In addition, Nup210 knockout led to overall spreading of H3K27me3 peak regions identified by the Sicer algorithm\(^4\), suggesting that NUP210 loss might be associated with H3K27me3-marked heterochromatin spread (Fig. 5e). This result is consistent with our hypothesis that NUP210 might be protecting transcriptionally active chromatin regions from EZH2-mediated H3K27me3 heterochromatin modification of H3.1/3.2 near the nuclear pore.

Since the majority (~60%) of NUP210 peaks (14945) did not overlap with H3K27me3 peaks (Fig. 5c), we speculated that NUP210 might also be protecting distal enhancers from H3K27me3-marked heterochromatin spreading. To investigate this, a publicly available H3K27 acetylation (H3K27Ac) active enhancer mark ChIP-seq dataset from mouse mammary luminal epithelial cells\(^2\) was integrated into the analysis. NUP210 was found to be enriched at the edges of regions enriched for the H3K27Ac (Fig. 5f). Loss of NUP210 resulted in an increase in the repressive H3K27me3 mark across these enhancer regions, suggesting that NUP210 may be contributing to the prevention of heterochromatin spreading across distal regulator elements. Taken together, these data suggest that NUP210 may also play a role in preventing heterochromatic spread across enhancers and other distal transcriptional regulatory elements.

Earlier we observed that NUP210 knockout was associated with redistribution of H3.1/3.2 at heterochromatin regions. Our ChIP-seq data also suggest that NUP210 loss was associated with heterochromatin spreading over distal enhancers. Since enhancers are known to establish loops
with promoters to activate gene expression\textsuperscript{46,47} and H3.1/3.2 is preferentially enriched at the poised promoters, we speculated that heterochromatin spread over enhancers due to NUP210 loss could also affect gene promoters. To assess this possibility, ChIP-seq of the transcriptional activation histone mark, H3K4me3, was performed in \textit{Nup210} KO 4T1 cells. The TSS of 1199 genes showed 2-fold decreased enrichment of H3K4me3 upon \textit{Nup210} knockout (Fig. 5g). As the loss of H3K4me3 at the promoter can be associated with decreased expression of genes, RNA-seq was performed in \textit{Nup210} KD 4T1 cells, which identified 282 downregulated (2-fold) and 249 upregulated (2-fold) genes upon \textit{Nup210} knockdown (Fig. 5h). Integration of the RNA-seq data revealed 62 genes that had loss of H3K4me3 at the promoter and \( \geq 2\)-fold decreased expression (Fig. 5i). Gene Ontology (GO) analysis on those 62 genes showed enrichment of cell migration and chemotaxis processes (Fig. 5j), including chemokines (\textit{Ccl2}, \textit{Cxcl1}, \textit{Cxcl3}), cytokine (\textit{Il1a}), and cell adhesion (\textit{Postn}, \textit{Serpine1}, \textit{Itga7}, \textit{Itgb2}) molecules (Fig. 5k).

Since enhancer-promoter looping is thought to occur within distinct chromatin regions called topologically associated domains (TADs)\textsuperscript{46}, we hypothesized that NUP210 anchors TADs to the nuclear pore where distal promoter-enhancer loops are established. To determine whether these NUP210-regulated genes were associated with TADs, we analyzed publicly available Hi-C data from mouse embryonic stem cells (mESC)\textsuperscript{48} using the 3D-Genome Browser\textsuperscript{49} and integrated it with our own ChIP-seq data. Upon \textit{Nup210} KO, H3K27me3 started accumulating across the \textit{Cxcl} and \textit{Ccl2} loci TADs (Fig. 5l), implying that NUP210 loss might disrupt the enhancer-promoter contacts for the \textit{Cxcl}/\textit{Ccl2} genes through heterochromatinization. Consistently, there was also a marked loss of H3K4me3 at the promoters of these genes. Examination of the \textit{Cxcl} and \textit{Ccl2} regions revealed that the enhancer-promoter interaction within the TADs may happen proximally
(~1.5 Mb for the Cxcl region) or distally (~17 Mb for the Ccl2 region). These results suggest that NUP210 may prevent heterochromatinization of the certain TADs at the nuclear periphery.

**Pharmacological inhibition of H3K27me3 heterochromatin modification restores the peripheral distribution of H3.1/3.2 and gene expression in Nup210 KO cells**

To test our hypothesis that Nup210 KO leads to H3K27me3-mediated heterochromatinization, we asked whether we could rescue the expression of the aforementioned cell migration-related genes (Fig. 5m) in Nup210 KO cells through pharmacological inhibition of H3K27me3 modification with an EZH2 inhibitor (GSK126). Treatment with GSK126 significantly increased the expression of Ccl2, Cxcl1, Cxcl3, Postn, and Il-1α (Fig. 5n), suggesting that they were directly suppressed through heterochromatinization in Nup210 KO cells. On the other hand, GSK126 treatment decreased the expression of Itga7, Itgb2, and Serpine1, implying that they were either not directly suppressed by heterochromatinization upon Nup210 KO or could be suppressed by a non-specific effect of the drug. Next, we examined whether GSK126 altered the intra-nuclear distribution of H3.1/3.2. Interestingly, perinuclear distribution of H3.1/3.2 was significantly restored in GSK126-treated Nup210 KO cells (Fig. 5o, 5p). This result suggests that the promoter/enhancers of these cell migration-related genes possesses H3.1/3.2 at the nuclear periphery and H3K27me3 modification of H3.1/3.2 is responsible for their suppressed expression in Nup210 KO cells. Furthermore, the nuclear size was also significantly increased in GSK126-treated Nup210 KO cells (Fig. 5q), suggesting that the reversal of heterochromatinization increases the nuclear size.

**Nup210 loss is associated with differential repositioning of cell migration-related gene loci**
To determine whether NUP210 regulates its target genes through anchoring certain TADs to the nuclear periphery, 3D-DNA FISH was performed to observe the localization of NUP210-regulated gene loci within the nucleus. FISH probes were generated from BAC clones (~2 Mb) overlapping the Cxcl region (Cxcl3, Cxcl5, Cxcl15, Pphp, Pf4, Gm36084), Postn region (Postn and Trpc4), and Ccl2 region (Ccl2, Ccl7, Ccl11, Ccl12, Ccl8, Asic2), and Itgb2 region (Itgb2, Fam207a, Pttg1ip, Sumo3, Ube2g2). FISH analysis revealed that many of those gene loci were close to the nuclear periphery, suggesting that they were likely part of the TADs attached to the nuclear periphery (Fig. 6a). When we compared the relative positioning (nuclear periphery vs centroid) of FISH spots in sg-Ctrl and Nup210 KO cells, Cxcl, Postn, and Itgb2 gene loci were significantly repositioned upon Nup210 KO (Fig. 6b). Radial positioning of Ccl2, however, was not significantly changed. Among the repositioned loci, Cxcl and Itgb2 loci repositioned closer to the nuclear periphery, whereas Postn loci repositioned away from the periphery. Since some of the FISH spots were found near heterochromatin foci marked by dense DAPI signals, we asked whether these genes could be silenced through repositioning into heterochromatin foci. Interestingly, the distance of Ccl2 FISH spots to heterochromatin foci were significantly reduced in Nup210 KO (Fig. 6c) cells implying the silencing of the Ccl2 loci may occur through repositioning into heterochromatin regions. These results suggest that NUP210 knockout is associated with altered chromatin topology and nuclear positioning of cell migration-related gene loci into chromatin regions where they are more likely to be repressed through heterochromatinization.

**Nup210 is a mechanosensitive gene and regulates focal adhesion, cell migration, and invasion**

The changes we observed in cell adhesion/migration-related gene expression upon NUP210 loss led us to hypothesize that NUP210 may affect metastatic progression by altering focal adhesion.
Staining for phospho-FAK (Y397) and phalloidin revealed that Nup210 knockdown significantly decreased the number of focal adhesions in 4T1 and 6DT1 cells when plated on type I collagen matrix (Fig. 7a, 3b). Nup210 knockdown also decreased the protein level of phospho-FAK without altering total FAK protein in 4T1 (Fig. 7c) and 6DT1 cells (Fig. 7d). Similar results were observed in Nup210 KO 4T1 cells (Supplementary Fig. 5a, 5b). To further verify the effect of NUP210 loss on focal adhesion that we observed in mouse cells, knockdown of NUP210 in two human breast cancer cell lines, MDA-MB-231 and MCF7, was performed. Similar to mouse cells, a decrease in p-FAK (Y397) without alteration of total FAK was observed in both of these cell lines (Supplementary Fig. 5c, 5d).

As focal adhesion is important for the cellular response to extracellular matrix (ECM) stiffness or mechanotransduction, we asked whether NUP210 is mechanosensitive in metastatic cells. 4T1 cells were plated on fibronectin-coated hydrogel matrices of different stiffness mimicking the ECM environment. In the soft/low stiffness (0.2 kPa) condition, 4T1 cells exhibited round colony-like morphology, while in the higher stiffness condition (12 kPa), these cells exhibited a more spread morphology (Fig. 7e). Western blot analysis revealed that the NUP210 protein level was increased in the higher stiffness condition (Fig. 7f), indicating that Nup210 is mechanosensitive in 4T1 cells. The levels of p-FAK (Y397) and total FAK were also increased in this condition. Consistently, qRT-PCR analysis in soft and stiff matrices revealed that many NUP210-regulated genes were also mechanositive in 4T1 cells (Fig. 7g). In addition, Nup210 knockdown cells exhibited decreased spreading in the higher stiffness condition (Fig. 7h), suggesting that NUP210 loss affects the mechanical response in metastatic 4T1 cells. As mechanotransduction can happen through alteration of cellular actin dynamics50,51, we performed an actin polymerization assay in
*Nup210* knockdown 4T1 cells. We treated 4T1 cells with an actin polymerization inhibitor, Cytochalasin D, and followed the recovery of actin polymerization over time. *Nup210* knockdown cells had decreased recovery of actin polymerization, as marked by phalloidin staining of actin stress fibers (Fig. 7i), suggesting that NUP210 can affect mechanotransduction through the alteration of actin dynamics.

As focal adhesion is critical for cell migration and invasion during the metastatic process, we investigated whether the depletion of NUP210 was associated with decreased migration and invasion of 4T1 and 6DT1 cells. Automated live-cell imaging analysis of cell migration showed that *Nup210* KO cells displayed significantly less random migration than sg-Ctrl 4T1 cells (Fig. 7j). A cell invasion assay using a matrigel-coated boyden chamber also revealed a significant reduction of cell invasion upon NUP210 depletion in 4T1 (Fig. 7k) and 6DT1 cells (Fig. 7l). Taken together, these results suggest that NUP210 is a cellular mechanosensor of the extracellular microenvironment, which is necessary for the metastatic behavior of cancer cells.

**Nup210 regulates a pro-metastatic secretory phenotype that regulates focal adhesion, cell migration in vitro, and circulating tumor cells in mice**

Since many of the NUP210-regulated genes were secretory molecules like cytokines (IL-1α), chemokines (CCL2, CXCL1, CXCL3), and adhesion molecules (POSTN, SERPINE1), we hypothesized that NUP210 might be regulating a secretory phenotype necessary for the metastatic potential of cancer cells. To test our hypothesis, we performed a cytokine array using the cultured supernatant of sh-Ctrl and *Nup210* knockdown cells. Consistent with the transcriptome data, decreased secretion of cytokines (IL-1α), chemokines (CCL2, CXCL1), and adhesion molecules
(POSTN, SERPINE1) was observed in Nup210 knockdown 4T1 cells (Fig. 8a, 8b). To investigate whether NUP210 mediates its effect on focal adhesion and cell migration through altered secretory molecules, we chose to knockdown Ccl2 as a candidate because it was the most dramatically decreased chemokine in Nup210 knockdown cells and has known pro-metastatic roles\textsuperscript{52-54}. Knockdown of Ccl2 in 4T1 cells (Fig. 8c) dramatically reduced the p-FAK (Y397) levels without altering total FAK levels (Fig. 8d). A focal adhesion assay on a collagen type I matrix also showed that Ccl2 knockdown cells had a round, less spread morphology and decreased focal adhesion foci (Fig. 8e). Automated live-cell analysis of cell migration revealed that there was a significant decrease in cell migration upon Ccl2 knockdown phenocopying the loss of NUP210 in these cells (Fig. 8f). Amplification of CCL2 has also been associated with poor prognosis in the METABRIC human breast cancer patient dataset (Fig. 8g).

During the metastatic process, disseminated tumor cells need to intravasate into the bloodstream and remain as circulating tumor cells (CTCs) until they adhere and extravasate into the surrounding organ to form a secondary tumor. CCL2 has been shown to have a direct role in tumor cell adhesion to the endothelial wall and facilitates extravasation during metastasis\textsuperscript{55}. As Nup210-depleted cancer cells exhibited adhesion/migration defects and decreased secretion of CCL2, we hypothesized that NUP210 loss might affect the CTC population in mice. For these experiments, putative CTCs were isolated based on CD45-/CK+ staining\textsuperscript{56}. A flow cytometry sample acquisition gate was created based on the staining pattern of 6DT1 cells in culture (Fig. 8h). Blood from healthy, tumor-free FVB/NJ mice was used as negative control. Under these conditions, approximately ~0.5-1% of the cells in the blood of 6DT1-sh-Ctrl tumor-bearing mice (at day 28) were CD45-/CK+ putative CTCs. In contrast, CTCs in mice with Nup210-depleted tumors were
indistinguishable from the FVB tumor-free control (Fig. 8h, 8i). These results suggest that NUP210 is either required for tumor cell extravasation from the primary tumor into the vasculature or for the survival of cells within the bloodstream.

Taken together, our model shows that NUP210 is a mechanical sensor of the extracellular microenvironment. In response to tissue stiffness, growth factors and integrins transmit the mechanical signal from the ECM to NUP210 to regulate the transcription of mechanosensitive genes (Fig. 8j), which in turn further activate downstream signaling pathways (e.g., actin polymerization, focal adhesion, etc.) necessary for cell migration and metastasis. In Nup210 knockout cells, mechanical signals cannot pass through the cytoplasm to the nucleus to regulate mechanosensitive gene expression, thereby decreasing metastasis.

**DISCUSSION:**

The present study identified an interesting role for NUP210, a nuclear pore complex protein, in promoting cancer metastasis. To the best of our knowledge, we have uncovered a previously unknown mechanism for metastatic potential modulation through NUP210-dependent changes in chromatin accessibility over mechanosensitive, cell migration-related, pro-metastatic genes at the nuclear periphery.

Apart from providing structural support and general nucleocytoplasmic transport, the function of the nuclear pore complex in regulating gene expression has drawn much attention in recent years. Although many of these studies are focused on developmental gene regulation, the function of nuclear pore complex components in human disease, especially cancer, has begun to emerge.
To date, NUP210 function has been implicated in muscle differentiation\(^{36,62}\), cellular reprogramming\(^{63}\), and T cell receptor signaling\(^{64}\). Here, we have identified *Nup210* as a potential metastasis susceptibility gene from the genome-wide screen of metastasis-associated polymorphism within gene promoters and enhancers. Germline polymorphism in the promoter region affect *Nup210* transcriptional activity through CTCF-dependent manner. Through patient dataset analysis, we found a significant association between high *NUP210* expression and poor outcome in human ER+ breast cancer patients. It would interesting to see whether the promoter polymorphism is responsible for the differential NUP210 expression among the breast cancer patients. Considering breast cancer as a heterogenous disease, further investigations will be required to elucidate the role of NUP210 in metastasis different subtypes of breast cancer.

Our study on the role of NUP210 has important implications in the field of cellular mechanosensing. Mechanical stress is important in the context of development\(^{65}\) and cancer\(^{66}\) because changes in extracellular matrix stiffness can affect embryogenesis, cell migration, angiogenesis, and metastasis. The nucleus is regarded as a sensor of mechanical stress upon exposure to different extracellular stimuli\(^{67}\). For instance, LINC (linker of nucleoskeleton and cytoskeleton), a nuclear envelope-anchored protein complex, serves as a mechanotransducer to regulate gene expression\(^{68}\). Furthermore, in response to mechanical stress, mechanosensitive transcription factors can translocate to the nucleus to regulate gene expression\(^{50,51,69-71}\). However, it remains unclear whether these gene expression changes occur due to alterations of 3D nuclear architecture or chromatin topology. Our results suggest that mechanosensitive, pro-metastatic genes can be activated by nuclear pore components through alteration of gene positioning and chromatin accessibility.
Strikingly, we observed that many of the NUP210-regulated mechanosensitive, cell migration-related genes were immune molecules like chemokines and cytokines. This specificity can be explained by the notion that the nuclear pore is thought of as a site of active transcription. Genes that need to be actively transcribed and transported to the cytoplasm for protein synthesis come into close proximity of the nuclear pore. Nuclear pore components also induce transcriptional memory for rapid activation of certain genes\textsuperscript{72}. Transcriptional memory is essential for innate and adaptive immune responses where rapid transcriptional activation and secretion of immune molecules are required\textsuperscript{73}. Interestingly, it was recently shown that upon stimulation, immune genes can be transcriptionally activated through alteration of topologically associating domains\textsuperscript{74}. In response to a foreign ECM environment, metastatic cells may exploit this mechanism through repositioning TADs to the nuclear pore for robust transcription of mechanosensitive genes, which then promote their migration into distant organs in an autocrine fashion. In fact, two recent studies have suggested that \textit{Nup210}-deficient mice possesses defective T-cell receptor signaling\textsuperscript{64,75}. It is highly likely that NUP210 might promote metastasis through rapid transcriptional activation of mechanosensitive, immune component genes. Future studies will be needed to address the function of NUP210 in metastasis in the context of tumor cell-immune crosstalk.

Mechanistically, we have demonstrated that NUP210 interacts with histone H3.1/3.2 at the nuclear lamina. Histone H3.1/3.2 is considered as a DNA replication-coupled variant of histone H3. During DNA replication, H3.1/3.2 is usually deposited onto the newly synthesized DNA strand through its chaperones, CHAF1A and CHAF1B\textsuperscript{76}. Recent reports have identified both pro-tumorigenic\textsuperscript{77,78} and anti-metastatic\textsuperscript{79} roles for CHAF1B in human malignancies, suggesting a cell
type-specific function of these H3.1/3.2 chaperones. Upon Nup210 KO, we observed decreased enrichment of H3.1/3.2 at the nuclear periphery. Whether Nup210 affects differential deposition of H3.1/3.2 by its chaperones and modulates pro-metastatic gene expression at the nuclear periphery requires further investigation.

In contrast to the peripheral localization of H3.1/3.2, we observed H3.1/3.2 enrichment at heterochromatin regions in Nup210 KO cells. In support of this observation, we found increased association of heterochromatin-modifying enzymes (SUV39H1 and EZH2) with H3.1/3.2 and increased accumulation of H3K27me3-marked heterochromatin in Nup210 KO cells. These results suggest a role for NUP210 as a chromatin barrier insulator-binding protein. The nuclear pore complex has been described as a chromatin barrier insulator component in yeast and certain pore components have been shown to generate a zone of heterochromatin exclusion surrounding the nuclear pore. Apart from some evidence in vertebrates, chromatin barrier insulators have not been well-characterized in mouse and human. Although the architectural protein CTCF is mainly considered to be an enhancer blocker, studies have suggested that it can also be associated with barrier insulators. This barrier insulator contains actively transcribing euchromatin regions surrounded by heterochromatin marks. Loss of barrier insulator elements in the barrier region is associated with increased spreading of heterochromatin marks to adjacent actively transcribing gene loci, hence decreased global levels of histone acetylation and decreased enrichment of the activating H3K4me3 mark at gene promoters. Since Nup210 loss was associated with H3K27me3-marked heterochromatin spreading and loss of H3K4me3 from certain gene promoters, NUP210 might acts as a barrier of H3K27me3-mediated heterochromatinization to adjacent loci at the nuclear pore. We propose that NUP210 likely protects H3.1/3.2-associated
chromatin by preventing the recruitment of heterochromatin-modifying enzymes (SUV39H1 and EZH2) at the nuclear pore.

Our data suggest that NUP210 can also be enriched in the active enhancer regions and NUP210 loss was associated with increased enrichment of the H3K27me3 heterochromatin mark in those regions. Some of these chromatin regions repositioned differentially within the nucleus upon Nup210 KO. This can be explained by the previous observation that nuclear pores can be a scaffold of enhancer-promoter contact mediating transcriptional activation of poised genes\textsuperscript{60}. In addition, alteration of the nuclear lamina-chromatin interaction is reported to be essential for gene regulation during cellular differentiation\textsuperscript{86}. So, it is conceivable that in response to higher stiffness of the extracellular microenvironment during metastatic progression, mechanical signals facilitate the interaction of NUP210 with histone H3.1/3.2-marked enhancer regions to establish distal regulatory element interaction with poised promoters of mechanosensitive genes. Hi-C data analysis also supports this possibility because NUP210-regulated genes reside within TADs, which are thought to possess H3K27Ac-marked regions surrounded by the insulator protein CTCF at the boundary\textsuperscript{87}. The function of NUP210 in gene repositioning can also be linked to its ability to alter chromatin looping, as we showed Nup210 to be a CTCF-regulated gene. It would be interesting to see whether this change in gene repositioning in Nup210 KO cells is due to the repositioning of TAD boundaries from the active to silent chromatin compartments\textsuperscript{88}. Further investigations are required to establish the role of CTCF-NUP210 axis in metastatic progression.

We showed that treating Nup210 KO cells with an EZH2 inhibitor (GSK126) could partially rescue the peripheral distribution of H3.1/3.2 and mechanosensitive gene expression. This observation
has important clinical implications since EZH2 is a therapeutic target for multiple malignancies and some EZH2 inhibitors are currently in clinical trials\textsuperscript{89,90}. In breast cancer, both tumor-promoting and inhibitory effects have been described for EZH2 in a context-specific manner\textsuperscript{91}. Treating with an EZH2 inhibitor could adversely affect patient outcome through activation of mechanosensitive genes essential for cell migration and metastasis. In line with this speculation, GSK126 treatment has been shown to exhibit adverse effects via inhibiting anti-tumor immunity\textsuperscript{92} and promoting inflammation\textsuperscript{93} in pre-clinical models of cancer.

In conclusion, our current study has identified a previously unrecognized role for NUP210 in nuclear mechanosensing and promoting aggressive metastatic cancer that is independent of the general nucleocytoplasmic transport function of the nuclear pore. Since \textit{Nup210} KO in breast cancer cell lines decreased metastasis through heterochromatinization of mechanosensitive genes, future studies should determine whether inhibiting NUP210-chromatin interactions can be a therapeutic strategy for aggressive metastatic disease.

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AUTHOR CONTRIBUTIONS:
R.A conceived the project, performed the majority of the experiments, analyzed the data, and wrote the manuscript. A.S, J.J.Z, S.K, P.W, and S.Z.T performed chromatin accessibility, ChIA-PET analysis, and wrote the manuscript. S.B performed DNA FISH analysis. H.L and M.P.L performed ChIP-seq/RNA-seq analysis. S.D.C analyzed the automated live-cell imaging of cell migration. A.T and M.J.K performed microscopy image analysis and wrote the manuscript. G.L.H provided expertise and supervised the chromatin accessibility analysis. Y.R provided expertise and supervised the ChIA-PET analysis. K.W.H conceived and supervised the whole project, analyzed the data, and wrote the manuscript.

DECLARATION OF INTERESTS:
Authors have declared that there is no conflict of interests.

REFERENCES:


FIGURE LEGENDS:

Figure 1: Genome-wide analysis of non-coding region polymorphisms identifies *Nup210* as a metastasis susceptibility gene in breast cancer.

(a) Identification scheme for metastasis susceptibility genes associated with polymorphic Benzonase-accessible chromatin (BACH) regions.

(b) Circos plot representation of the 52 genes (marked by blue color on the outer area of the plot) and their chromosomal locations that showed metastasis association in the mouse diversity outbred panel. Some of these genes have polymorphic promoters (red lines). Some of them showed intra- (blue lines) and inter-chromosomal (light blue lines) looping interactions.

(c) Distant metastasis-free survival of human ER+ breast cancer patients stratified by their expression of the 52-gene signature derived from the genes shown in (b). Patient data were obtained from the METABRIC dataset.

(d) RNA-seq analysis of *Nup210* mRNA expression in the 4T1 series of breast cancer cells. Dots represent two biological replicates.

(e) Integrative genomic viewer (IGV) track showing the mouse *Nup210* promoter BACH region in 4T07 and 4T1 cell line. A 510 bp region (purple box) was analyzed for promoter polymorphisms.

(f) Individual polymorphisms (SNPs, indels) identified within the 510 bp *Nup210* promoter region from different mouse strains.

(g) 12 bp insertion in the *Nup210* promoter of the FVB/NJ strain is located within the active enhancer (H3K27Ac-marked)-promoter (H3K4me3) region. This particular region also
showed the enrichment of CTCF. ME, mammary epithelial cells; ML, mammary luminal cells.

(h) Differential enrichment of CTCF and H3K27Ac at the Nup210 promoter region between mouse metastatic 4T1 (BALB/cJ tumor-derived) and 6DT1 (FVB/NJ tumor-derived) cell lines.

(i) Comparison of Nup210 and Ctcf mRNA levels between 4T1 and 6DT1 cell lines. ***P < 0.001.

(j) Comparison of Nup210 and Ctcf mRNA levels in the spleen of normal BALB/cJ and FVB/NJ mouse strains. *P < 0.05, NS = non-significant.

(k) Relative luciferase activity of the Nup210 promoter regions derived from the BALB/cJ and FVB/NJ strains. ****P < 0.0001.

(l) Ctcf knockdown effect on the Nup210 mRNA level in 4T1 cells. *P < 0.05, **P < 0.01, ***P < 0.001.

(m) Ctcf knockdown effect on the NUP210 protein level in 4T1 cells.

Figure 2: **NUP210 expression is associated with poor survival outcome in human ER+ breast cancer patients.**

(a) Effect of NUP210 amplification on the overall survival of breast cancer patients from the METABRIC dataset.

(b) Effect of NUP210 mRNA expression level on the overall survival of ER+ breast cancer patients from the METABRIC dataset.

(c) Effect of NUP210 mRNA expression level on the overall survival of ER-/PR-/Her2- breast cancer patients from the METABRIC dataset.
(d) Effect of NUP210 mRNA expression level on distant metastasis-free survival of ER+ breast cancer patients (Km-plotter).

(e) Effect of NUP210 mRNA expression level on distant metastasis-free survival of ER-/PR-/Her2- breast cancer patients (Km-plotter).

(f) NUP210 mRNA expression level within different PAM50 breast cancer molecular subtypes from the METABRIC dataset. Kruskal-Wallis test. ****P < 0.0001, NS = non-significant.

(g) Human breast cancer dataset showing the NUP210 mRNA expression level in multiple metastatic sites. Kruskal-Wallis test. *P < 0.05, **P < 0.01.

(h) Human prostate cancer dataset showing the differential expression of NUP210 mRNA between primary tumor and metastases. Mann-Whitney U test. ****P < 0.0001.

(i) Human melanoma dataset showing the differential expression of NUP210 mRNA between primary tumor and metastases. Mann-Whitney U test. *P < 0.05.

**Figure 3: Depletion of Nup210 in 4T1 metastatic cancer cells reduces lung metastasis in mice.**

(a) Western blot showing shRNA-mediated stable Nup210 knockdown (KD) in the 4T1 metastatic mouse mammary cancer cell line.

(b) Primary tumor weight after orthotopic transplantation of Nup210 knockdown 4T1 cells.

  ***P < 0.001, NS = non-significant.

(c) Lung metastases count after orthotopic transplantation of Nup210 knockdown 4T1 cells.

  *P < 0.05, **P < 0.01.

(d) Lung metastases count normalized to primary tumor weight, derived from values shown in (b) and (c). *P < 0.05, **P < 0.01.
(e) Western blot showing the CRISPR/Cas9-mediated knockout (KO) of Nup210 in 4T1 cells. Two clones, N9 and N13, are shown. The sg-Ctrl 4T1 cells were established using a non-targeting sgRNA.

(f) Primary tumor weight, (g) lung metastases count, and (h) tumor-normalized metastases count after orthotopic transplantation of Nup210 KO 4T1 cells. **P < 0.01, ***P < 0.001, **** < 0.0001.

(i) Western blot (left) and qRT-PCR (right) showing the overexpression (OE) of NUP210 in 4T1 cells. *P < 0.05.

(j) Primary tumor weight and (k) lung metastases count after orthotopic transplantation of NUP210-overexpressing 4T1 cells. **P < 0.01, NS = non-significant.

Figure 4: NUP210 interacts with histone H3.1/3.2 at the nuclear periphery and the loss of NUP210 is associated with re-distribution of H3.1/3.2 from the nuclear periphery to heterochromatin foci.

(a) Schematic of NUP210 co-immunoprecipitation (Co-IP) followed by LC-MS analysis.

(b) Number of peptides identified from two biological replicates in LC-MS analysis using both endogenous NUP210-specific and Myc-tag (NUP210-Myc)-specific antibodies.

(c) Effect of histone H3.1 amplification on the overall survival of human breast cancer patients (METABRIC dataset).

(d) Validation of NUP210-Myc interaction with H3.1/3.2 with Co-IP using lysates from 4T1 cells with NUP210-Myc OE.

(e) Co-IP showing the endogenous H3.1/3.2 interaction with endogenous NUP210 and lamin B1 in 4T1 cells.
(f) Representative images from immunofluorescence analysis showing the localization of H3.1/3.2 (green) with NUP210-Myc (red) at the nuclear periphery in 4T1 cells. DNA is labeled with DAPI (blue). Scale bar = 5 µm.

(g) Representative super-resolution spinning disk microscope images showing the distribution of H3.1/3.2 (green) and H3K27me3 (red) in sg-Ctrl and Nup210 KO (KO-N13) 4T1 cells. DNA is labeled with DAPI (blue). Scale bar = 5 µm.

(h) Quantification of the ratio of H3.1/3.2 and H3K27me3 intensity distribution at the nuclear periphery vs total intensity within the nucleus in sg-Ctrl and Nup210 KO (KO-N13) 4T1 cells. For H3.1/3.2, ****P < 0.0001, n = 87 for sg-Ctrl and n = 130 for KO-N13. For H3K27me3, *P < 0.05, n = 61 for sg-Ctrl and n = 63 for KO-N13.

(i) Representative super-resolution spinning disk microscope images showing H3.1/3.2 (green) localized on the periphery of pericentric heterochromatin (H3K9me3; red) in sg-Ctrl and Nup210 KO (KO-N13) 4T1 cells. DNA is labeled with DAPI (blue). Scale bar = 5 µm.

(j) Quantification of the ratio of H3.1/3.2 intensity distribution at heterochromatin foci vs total H3.1/3.2 intensity within the nucleus in sg-Ctrl and Nup210 KO (KO-N13) 4T1 cells. ****P < 0.0001, n = 1182 foci for sg-Ctrl and n = 1513 foci for KO-N13. Quantification of the ratio of H3K9me3 intensity at the nuclear periphery vs total H3K9me3 intensity within the nucleus in sg-Ctrl and Nup210 KO (KO-N13) 4T1 cells. NS = non-significant, n = 79 for sg-Ctrl and n = 97 for KO-N13.

(k) Confocal immunofluorescence analysis showing H3.1/3.2 (green) distribution in sg-Ctrl and Nup210 KO (KO-N13) 4T1 cells at the periphery of the nucleolus (nucleolin; red). DNA is labeled with DAPI (blue). Scale bar = 5 µm.
(l) Quantification of the nuclear area in Nup210 KO (sg-Ctrl, n = 70; KO-N9, n = 164; KO-N13, n = 119) and KD (sh-Ctrl, n = 277; sh-Nup # 1, n = 229; sh-Nup # 4, n = 327) 4T1 cells. ****P < 0.0001.

(m) Co-IP showing the interaction of H3.1/3.2 with lamin B1, SUV39H1, and H3K9me3 in sg-Ctrl and Nup210 KO (KO-N13) 4T1 cells.

(n) Co-IP showing the interaction of H3.1/3.2 with the PRC2 complex component, EZH2, in sg-Ctrl and Nup210 KO (KO-N13) 4T1 cells.

(o) Co-IP showing the interaction between PRC2 complex components, EZH2 and SUZ12, in sg-Ctrl and Nup210 KO (KO-N13) 4T1 cells.

(p) Co-IP showing the interaction of EZH2 with H3K27me3 in sg-Ctrl and Nup210 KO (KO-N13) 4T1 cells.

**Figure 5: Loss of NUP210 is associated with H3K27me3-marked heterochromatin spreading on topologically associating domains (TADs).**

(a) NUP210 ChIP-seq peak distribution in different genomic regions in 4T1 cells.

(b) Normalized profile of NUP210 ChIP-seq peak enrichment over gene bodies in 4T1 cells.

(c) Overlap of NUP210 and H3K27me3 ChIP-seq peaks in 4T1 cells. TSS = Transcriptional start site, TES = Transcriptional end site.

(d) H3K27me3 ChIP-seq peak enrichment over gene bodies in sg-Ctrl (red) and Nup210 KO (KO-N13) (blue) 4T1 cells within NUP210-enriched regions (purple). The ChIP-seq profile derived using an anti-Nuclear Pore Complex (NPC)-specific antibody was plotted as negative control (green).
(e) Normalized ChIP-seq profile showing the expansion of H3K27me3 peaks identified by the Sicer algorithm in sg-Ctrl (red) and Nup210 KO (KO-N13) (blue) 4T1 cells.

(f) Normalized ChIP-seq profile showing the occupancy of H3K27me3 peaks within the NUP210-H3K27Ac (purple, green) enhancer overlap region in sg-Ctrl (red) and Nup210 KO (KO-N13) (blue) 4T1 cells.

(g) Normalized ChIP-seq profile of H3K4me3 enrichment over gene promoters in sg-Ctrl (red) and Nup210 KO (KO-N13) (blue) 4T1 cells.

(h) RNA-seq analysis showing the 2-fold up or downregulated genes upon knockdown of Nup210 in 4T1 cells.

(i) Overlap of genes with 2-fold downregulation of H3K4me3 enrichment in Nup210 KO 4T1 cells and 2-fold downregulated expression in Nup210 knockdown 4T1 cells.

(j) Gene Ontology (GO) analysis of genes from the overlap of H3K4me3 ChIP-seq and RNA-seq analysis.

(k) Downregulated cell migration-related genes from the RNA-seq of 4T1 Nup210 knockdown (sh-Nup # 4) cells. FC = fold change.

(l) Representative ChIP-seq track of Cxcl and Ccl2 regions showing the association of NUP210-, H3K27Ac-, H3K27me3- and H3K4me3-enriched regions with predicted TADs. Publicly available Hi-C contact map from mESC data was used for TAD prediction.

(m) qRT-PCR analysis showing the downregulation of cell migration-related genes in both Nup210 KO (KO-N13) vs sg-Ctrl and Nup210 knockdown (sh-Nup # 4) vs sh-Ctrl 4T1 cells. *P < 0.01, **P < 0.01, ***P < 0.001, ****P < 0.0001.
(n) qRT-PCR analysis showing the expression of cell migration-related genes in DMSO- and GSK126-treated Nup210 KO (KO-N13) 4T1 cells. **P < 0.01, ***P < 0.001, ****P < 0.0001, NS = non-significant.

(o) Representative super-resolution microscopy images showing H3.1/3.2 (green) and H3K27me3 distribution in DMSO- and GSK126-treated Nup210 KO 4T1 cells. DNA is labeled with DAPI (blue). Scale bar = 5 µm.

(p) Quantification of the H3.1/3.2 fluorescence intensity ratio (nuclear periphery vs total) in DMSO- and GSK126-treated Nup210 KO 4T1 cells. ****P < 0.0001. sg-Ctrl, n = 59; KO+DMSO, n = 79; KO+GSK126, n = 53.

(q) Quantification of the nuclear area in DMSO- and GSK126-treated Nup210 KO 4T1 cells. ****P < 0.0001. n is the same as mentioned in (p).

**Figure 6: NUP210 loss is associated with differential repositioning of mechanosensitive gene loci.**

(a) Representative images of 3D-DNA FISH showing the position of NUP210-regulated gene loci (red) within the nucleus of 4T1 cells. DNA is labeled with DAPI (blue). Scale bar = 2 µm.

(b) Cumulative distribution of FISH spots in nuclear periphery vs nuclear centroid in sgCtrl (red) and Nup210 KO (KO-N13) (blue) 4T1 cells. For the Cxcl region, sgCtrl n = 39, KO-N13 n = 78; for the Postn region, sgCtrl n = 94, KO-N13 n = 75; for the Ccl2 region, sgCtrl n = 101, KO-N13 n = 80; for the Itgb2 region, sgCtrl n = 37, KO-N13 n = 72.
(c) Minimum distance of FISH spots from heterochromatin foci (marked by strong DAPI signal) in sg-Ctrl and Nup210 KO 4T1 cells. Number of cells (n) analyzed in each condition was the same as in (b).

**Figure 7: Nup210 is a mechanosensitive gene and regulates focal adhesion, cell migration, and invasion.**

(a) Representative confocal images of focal adhesion puncta (marked by p-FAKY397 staining) in sh-Ctrl and Nup210 knockdown (sh-Nup # 1 and # 4) 4T1 and 6DT1 cells. Scale bar = 10 μm.
(b) Quantification of focal adhesion puncta in sh-Ctrl and Nup210 knockdown (sh-Nup # 1 and # 4) 4T1 and 6DT1 cells. ****P < 0.0001.
(c) Western blot showing the level of total FAK (T-FAK) and phospho-FAK (Y397) proteins in sh-Ctrl and Nup210 knockdown (sh-Nup # 1 and # 4) T1 cells.
(d) Western blot showing the level of T-FAK and phospho-FAK (Y397) proteins in sh-Ctrl and Nup210 knockdown (sh-Nup # 1 and # 4) 6DT1 cells.
(e) Representative images of 4T1 cells grown on fibronectin-coated hydrogel layers of different stiffness. Elastic modulus 0.2 kPa was used as a soft matrix and 12 kPa was used as a stiff matrix. Scale bar = 10 μm.
(f) Western blot analysis on whole cell lysate showing the NUP210, p-FAK Y397, and T-FAK protein levels when 4T1 cells were grown in soft (0.2 kPa) or stiff (12 kPa) conditions.
(g) qRT-PCR showing the differential expression of NUP210-regulated genes in soft (0.2 kPa) and stiff (12 kPa) matrices. *P < 0.05, **P < 0.01 and ***P < 0.001.
(h) Morphology of sh-Ctrl and Nup210 knockdown (sh-Nup # 4) 4T1 cells in soft (0.2 kPa) and stiff (12 kPa) matrices. Scale bar = 10 µm.

(i) Representative images of F-actin stress fibers (phalloidin, green) after treatment and washout of cytochalasin D in sh-Ctrl and Nup210 knockdown (sh-Nup # 4) 4T1 cells. DNA is labeled with DAPI (blue). Scale bar = 10 µm.

(j) Cell migration tracks (red) showing random migration of sg-Ctrl and NUP210 KO (KO-N9 and KO-N13) 4T1 cells (left). Quantification of cell speed is represented as a bar graph (right). ****P < 0.0001.

(k) Quantification (left) and representative images (right) of cell invasion for sg-Ctrl and Nup210 KO (KO-N13) 4T1 cells. Percent cell invasion is quantified as total crystal violet intensity per transwell chamber. **P < 0.01, n = 3. Scale bar = 100 µm.

(l) Quantification (left) and representative images (right) of cell invasion for sh-Ctrl and Nup210 knockdown (sh-Nup # 4) 6DT1 cells. *P < 0.05, n = 3. Scale bar = 100 µm.

Figure 8: NUP210 regulates a secretory phenotype that facilitates focal adhesion, cell migration in vitro, and circulating tumor cells in mice.

(a) Cytokine array on culture supernatants from sh-Ctrl and Nup210 knockdown (sh-Nup # 4) 4T1 cells.

(b) Quantification of the cytokines/chemokines indicated on the array in (a).

(c) qRT-PCR analysis showing the knockdown of Ccl2 in 4T1 cells.

(d) Western blot analysis showing the decrease of p-FAK Y397 protein without alteration of T-FAK in sh-Ctrl and Ccl2 knockdown (sh-Ccl2 # 3, 4, and 5) 4T1 cells. β-actin was used as loading control.
(e) Focal adhesion puncta in sh-Ctrl and Ccl2 knockdown (sh-Ccl2 # 5) 4T1 cells. Scale bar = 5 µm.

(f) Representative images (left) and quantification (right) of cell migration tracks (red) of sh-Ctrl and Ccl2 knockdown (sh-Ccl2 # 3 and 5) 4T1 cells. ****P < 0.0001.

(g) Effect of CCL2 amplification on overall survival of human breast cancer patients (METABRIC dataset).

(h) Flow cytometry analysis showing the percentage of CD45-/CK+ circulating tumor cells (CTCs) in mouse blood after orthotopic injection of sh-Ctrl or Nup210 knockdown (sh-Nup # 4) 6DT1 cells. 6DT1 cells in culture were used to establish the CTC gate. Blood from healthy, tumor-free mice was used as negative control.

(i) Quantification of the percentage of CTCs from sh-Ctrl- and Nup210 knockdown (sh-Nup # 4) 6DT1 cell-injected mice.

(j) Model depicting the proposed role of NUP210 in metastasis. In response to mechanical signals, NUP210 interacts with histone H3.1/3.2-associated poised chromatin at the nuclear pore and protects H3.1/3.2 from H3K27me3 heterochromatin modification. As a result, mechanosensitive, cell migration-related genes are activated to promote cell migration and metastasis. In the absence of NUP210, H3.1/3.2-associated chromatin undergoes heterochromatinization. Consequently, the mechanical signal cannot pass from the extracellular microenvironment to the nucleus to activate mechanosensitive gene expression, thereby decreasing metastasis.

SUPPLEMENTARY FIGURE LEGENDS:
**Supplementary Figure 1:** CTCF binding site in the NUP210 promoter of a human breast cancer cell line, MCF7.

(a) Publicly available ChIP-seq tracks showing the enrichment of CTCF and H3K27Ac at the promoter of human NUP210 in the MCF7 breast cancer cell line.

**Supplementary Figure 2:** Knockdown of Nup210 in the 6DT1 and MVT1 cell lines decreases lung metastasis.

(a) Western blot showing the level of Nup210 shRNA-mediated knockdown in 6DT1 cells (left) and qRT-PCR showing the Nup210 knockdown level in MVT1 cells (right).
(b) Primary tumor weight after orthotopic transplantation of Nup210 knockdown 6DT1 (left) or MVT1 (right) cells. ***P < 0.001, ****P < 0.0001
(c) Lung metastases count after orthotopic transplantation of Nup210 knockdown 6DT1 (left) and MVT1 (right) cells. **P < 0.01, NS = non-significant.
(d) Lung metastases count normalized to primary tumor weight, derived from values shown in (b) and (c). *P < 0.05, **P < 0.01, ****P < 0.0001.

**Supplementary Figure 3:** Loss of NUP210 has a minor, inconsistent effect on cell cycle progression of 4T1 cells.

(a) DNA content analysis with EdU incorporation showing the different stages of the cell cycle in sg-Ctrl and Nup210 KO (KO-N9 and KO-N13) 4T1 cells.
(b) Quantification of cell cycle stage distribution in sg-Ctrl and Nup210 KO (KO-N9 and KO-N13) 4T1 cells. *P < 0.05, **P < 0.01, n = 3.
Supplementary Figure 4: NUP210 loss does not affect general nucleocytoplasmic protein transport but may affect RNA transport.

(a) Schematic of the tdTomato-expressing nucleocytoplasmic transport reporter. Reporter expression is driven by a CMV promoter and produces tdTomato fused to both a nuclear export (NES) and import (NLS) signal.

(b) Representative immunofluorescence images showing nuclear and cytoplasmic localization of the tdTomato signal (red) in sh-Ctrl and Nup210 knockdown (sh-Nup # 1 and 4) 4T1 cells. The Nuclear Pore Complex (NPC) is shown in green. Nuclei are stained with DAPI (blue). Leptomycin B, a nuclear export inhibitor drug, was used as positive control. Scale bar = 10 µm.

(c) (Left) Representative RNA FISH images showing the cytoplasmic and nuclear localization of PolyA+ RNA in sh-Ctrl and Nup210 (sh-Nup # 1 and 4) knockdown 4T1 cells. Scale bar = 10 µm. (Right) Quantification of the nuclear FISH signal. ****P < 0.0001.

(d) (Left) Representative RNA FISH images showing the cytoplasmic and nuclear localization of PolyA+ RNA in sg-Ctrl and Nup210 KO (KO-N9 and KO-N13) 4T1 cells. Scale bar 10 = µm. (Right) Quantification of the nuclear FISH signal. ****P < 0.0001.

Supplementary Figure 5: Nup210 depletion in 4T1 (KO), MDA-MB-231 (KD), and MCF7 (KD) cells decreases focal adhesion kinase signaling.

(a) Representative immunofluorescence images showing focal adhesion in sg-Ctrl and Nup210 KO (KO-N13) 4T1 cells. Scale bar = 10 µm.

(b) Western blot showing the protein levels of total focal adhesion kinase (T-FAK) and p-FAK Y397 in sg-Ctrl and Nup210 KO (KO-N13) 4T1 cells.
(c) Western blot showing the protein levels of T-FAK and p-FAK Y397 in sh-Ctrl and Nup210 knockdown MDA-MB-231 cells.

(d) Western blot showing the protein levels of T-FAK and p-FAK Y397 in sh-Ctrl and Nup210 knockdown MCF7 cells.
METHODS:

CONTACT FOR REAGENT AND RESOURCE SHARING:

Further information and request for resources or reagents should be directed to and will be fulfilled by the lead contact, Kent W. Hunter (hunterk@mail.nih.gov).

EXPERIMENTAL MODELS AND SUBJECT DETAILS:

Mouse strains:

Usage of animals described in this study was performed under the animal study protocol LCBG-004 approved by the National Cancer Institute (NCI) at Bethesda Animal Use and Care Committee. Animal euthanasia was performed by anesthesia using Avertin injection followed by cervical dislocation. Female BALB/c (000651) and FVB/NJ (001800) mice were purchased from The Jackson Laboratory.

Cell lines:

Mouse mammary tumor cell lines, 4T07, 4T1, 6DT1, and MVT1, were provided by Dr. Lalage Wakefield (NCI, NIH)\textsuperscript{34}. These cell lines were grown in DMEM (Gibco) supplemented with 9% fetal bovine serum (FBS) (Gemini), 1% L-glutamine (Gibco), and 1% penicillin-streptomycin (Gemini). Human breast cancer cell lines, MDA-MB-231 and MCF7, were provided by Dr. Jeffrey E. Green (NCI, NIH) and grown in DMEM with FBS, 1% L-glutamine, and 1% Penicillin-Streptomycin.

METHOD DETAILS:
**Benzonase Accessible Chromatin (BACH) sequencing analysis:**

Cells were expanded in DMEM at 37°C to obtain 8–10 x10⁷ cells/condition/each experiment. BACH analysis was performed as previously described with minor modifications. Briefly, cells were collected by centrifugation, washed twice in ice cold cellular wash buffer (20 mM Tris-HCl pH 7.5, 137 mM NaCl, 1 mM EDTA, 10 mM sodium butyrate, 10 mM sodium orthovanadate, 2 mM sodium fluoride, protease inhibitor cocktail (Roche) and resuspended in (40 million cells/ml) hypertonic lysis buffer (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 1 mM EGTA, 0.5% glycerol, 20 mM sodium butyrate, 2 mM sodium orthovanadate, 4 mM sodium fluoride and protease inhibitor cocktail). Cells were distributed in 500 µl aliquots in 1.5 ml tubes and followed by the addition of 500 µl of nuclease digestion buffer (40 mM Tris-HCl pH 8.0, 6 mM MgCl₂, 0.3% NP-40, and 1% Glycerol) containing a 3-fold dilutions (from 0.125 units/ml to 6 units/ml) of Benzonase nuclease (Millipore). This was mixed gently and incubated for three minutes at 37°C. Reactions were terminated by the addition of EDTA (10 mM final concentration) and SDS (0.75% final concentration). Proteinase K was added to a final concentration of 0.5 mg/ml and incubated overnight at 45°C. DNA fragments of 100-500 bp from a chromatin digestion were purified over sucrose gradients and precipitated in 0.1 volume sodium acetate and 0.7 volume isopropanol.

**Sequencing and data analysis of Benzonase-treated samples:**

DNA was sequenced using either Illumina HiSeq2000 (TrueSeq V3 chemistry) or NextSeq500 (TrueSeq High output V2 chemistry) sequencers at the Advanced Technology Research Facility (ATRF), National Cancer Institute (NCI-Frederick, MD, USA). The sequence reads were generated as either 50-mer or 75-mer (trimmed to 50-mer by trimmomatic software before
alignment), and tags are then aligned to the UCSC mm9 reference genome assembly using Eland or Bowtie2. All the samples were in good quality with over 94% of the bases having Q30 or above with 25~50 million raw reads per sample. Regions of enriched tags termed hotspots have been called using DNase2Hotspots algorithm\(^96\) with FDR of 0% with minor updates. Tag density values were normalized to 10 million reads. For comparison of BAC profiles across multiple cell lines, Percent Reference peak Coverage (PRC) measure was proposed and employed to ensure compatible levels of digestion by Benzonase in multiple cell lines\(^97\). To calibrate PRC, commonly represented hotspot sites were identified as reference peaks based on mouse (mm9) DNaseq data from ENCODE. ENCODE narrowPeak definition files (DNaseI Hypersensitivity by Digital DNaseI from ENCODE/University of Washington) for a total of 133 samples were downloaded from the UCSC golden path web site: http://hgdownload.cse.ucsc.edu/goldenPath/mm9/encodeDCC/wgEncodeUwDnase/. A total of 8,587 peaks were found to be present in all 133 samples. By assuming that the most commonly accessible sites may also be present in our optimally digested samples, PRC was obtained as a fraction of reference peaks presented in each sample to evaluate the level of digestion by Benzonase. Our pooled samples with PRC of 90% or greater indicated that all the 8,587 sites were present in the sample. Two biological replicates with acceptable PRC were selected and pooled for each cell line, and each pooled data set showed over 90% PRC. When samples showed bimodality in their tag density distribution due to elevated noise, we dropped hotspots that belonged to a group with low tag density and low PRC by applying threshold to maximum hotspot tag density values.

**Computing environment:**
All computations at NCI were performed on the NIH helix/biowulf system, documentation of which is available at https://helix.nih.gov. We used the R computing environment, Perl scripts, Bedtools, and UCSC liftOver for most of the analyses.

**Identification of polymorphic BACCh sites:**

The workflow consisted of the following: 1) the BACCh data were filtered for the regions overlapping with polymorphic sites. Since the BACCh data were generated in Genome Build mm9, we used UCSC mm9 snp128 data to restrict the BACCh sites. 2) Variant Called Format (VCF) files were filtered to retain the SNPs that overlap with the BACCh present in the 4T1 and 4T07 cell lines. 3) SNPs were removed in the BACCh that are present in the mouse FVB/NJ strain.

**Long Read Chromatin Interaction Analysis Through Paired-end Tag (ChIA-PET) sequencing and data processing**

Long read ChIA-PET was performed using Tn5 transposase to tag DNA for long tag sequencing by Illumina NextSeq. ChIA-PET data was processed by a customized ChIA-PET data processing pipeline\(^29\). Detailed protocol can be found in Li et al.\(^98\).

**Cloning:**

A mouse *Nup210* full length cDNA (NM_018815) encoding vector (pCMV6-Nup210-Myc) was purchased from Origene Technologies. This vector was digested with Sall-HF (New England Biolabs) and EcoRV-HF (New England Biolabs) restriction enzymes to obtain the NUP210 protein-encoding region. This NUP210 insert was then cloned into the Gateway entry vector
pENTR1A using the Quick Ligation Kit (New England Biolabs). pENTR1A no ccdB plasmid (Addgene plasmid # 17398) was received by Dr. Marian Darkin (NCI) as a gift from Dr. Eric Campeau. The NUP210-encoding region from the pCMV6-NUP210-Myc vector was cloned into pENTR1A vector through digestion with SalI and EcoRV. pENTR1A-NUP210 entry vector was used to transfer the NUP210-encoding region into the lentiviral destination vector pDEST-658 (received as a gift from Dr. Dominic Esposito, NIH) along with a mouse Pol2 promoter entry vector using the Gateway LR clonase reaction (Thermo Fisher Scientific). The integrity of the final NUP210-encoding vector was verified through DNA sequencing.

The pGL4.23 [luc2/minP] promoter luciferase vector (Promega, Catalog # E8411) was received as a gift from Dr. Jing Huang (NCI, NIH). A 550 bp region from the mouse (FVB/NJ and BALB/cJ) Nup210 promoter covering the polymorphic sites was amplified by PCR using KOD Hot Start DNA Polymerase (Millipore) and digested with KpnI (New England Biolabs) and XhoI (New England Biolabs) restriction enzymes. Gel-purified PCR product was then cloned into the pGL4.23 vector using T4 DNA ligase (New England Biolabs). Finally, the DNA sequence of the clones were confirmed through DNA sequencing.

**Lentivirus production and generation of stable cell lines:**

All of the TRC lentiviral shRNA vectors were purchased from Dharmaco. shRNAs targeting the mouse Nup210 gene, sh-Nup # 1 (TRCN0000101935: TAACTATCACAGTAAGAAGGC) and sh-Nup # 4 (TRCN0000101938: TTCAGTTGCTTATCTGTCAGC) were used for Nup210 knockdown in all of the mouse cell lines. For mouse Ctf knockdown, sh-Ctf # 4 (TRCN0000039022: TAAGGTGTGACATATCATCGG) and sh-Ctf # 5 (TRCN0000039023:
ATCTTCGACCTGAATGATGGC) were used. For mouse Ccl2 knockdown, sh-Ccl2 # 3 (TRCN0000034471: TTACGGGTCAACTTCACATTC), sh-Ccl2 # 4 (TRCN0000034472: TTGCTGGTGAATGAGTAGCAG), and sh-Ccl2 # 5 (TRCN0000034473: AATGTATGTCTGGACCCATTC) were used. For NUP210 knockdown in human cell lines, a shRNA targeting the human NUP210 gene (TRCN0000156619: AAATGAGCTAATGGGCGAGAG) was used.

For lentivirus production, shRNA-containing plasmids and packaging plasmids, psPAX2 (Addgene plasmid # 12260) and envelope plasmid pMD2.G (Addgene plasmid # 12259) (both were a gift from the Trono lab), were transfected into the human 293FT (Thermo Fisher Scientific) cell line using X-tremeGENE 9 DNA transfection reagent (Roche). 48 h after transfection, culture supernatant containing lentivirus was harvested, filtered through a 0.45 µm filter (Millipore), and then used for transduction of mouse and human breast cancer cell lines. shRNAs stably integrated in mouse and human cells were selected with 10 and 2 µg/ml puromycin (Sigma), respectively. For the selection of NUP210-overexpressing cells, 10 µg/ml of blasticidin (Gibco) was used.

**CRISPR/Cas9-mediated knockout of the mouse Nup210 gene:**

For CRISPR/Cas9-mediated knockout of mouse Nup210 in the 4T1 cell line, sgRNA targeting Nup210 exon 5 (sgRNA sequence: GCGACACCATCTTAGTGTCT) was designed using the GPP Web Portal available at the Broad Institute (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design). sgRNA was then cloned into the lentiGuide-puro (Addgene plasmid # 52963, a gift from the Feng Zhang lab) vector. Non-targeting control sgRNA cloned into the lentiGuide-puro vector (sgRNA sequence: CCATATCGGGGCGAGACATG) was kind of a gift from Dr. Ji
Luo (NCI, NIH). Lentiviral particles were prepared as described above for shRNA lentiviruses. 4T1 cells stably expressing the sgRNAs were generated through lentiviral transduction and selection with 10 µg/ml puromycin. For transient expression of Cas9 in the sgRNA-stable 4T1 cells, an adenoviral Cas9-encoding viral particles containing the GFP reporter (Vector Biolab) was used at 25 M.O.I. 96 h after transfection of the Cas9 vector, GFP-positive 4T1 cells were FACS sorted and single cells were isolated for clonal expansion. *Nup210* mutation was confirmed through DNA sequencing and knockout was verified through western blot. Before performing functional assays with *Nup210* knockout cells, both sgCtrl and knockout cells were passaged at least 4 times for 2 weeks to eliminate residual Cas9-GFP signal within 4T1 cells, which was verified using a fluorescence microscope.

**Spontaneous metastasis assay in mice:**
6-8 weeks old female BALB/c and FVB/NJ mice were purchased from The Jackson Laboratory. For orthotopic transplantation of *Nup210*-depleted/overexpressing cells, 100,000 cells were injected into the fourth mammary fad pad of mice. 28-30 days after injection, mice were euthanized and primary tumors were resected, weighed, and surface lung metastases were counted.

**Protein nucleocytoplasmic transport assay:**
4T1 *Nup210* knockdown cells were grown on glass coverslips in 2-well Lab-Tek chambered glass coverslip (Thermo Fisher Scientific) at a seeding density of 20,000 cells/well. 24 h after seeding, cells were transfected with 1 µg of nucleocytoplasmic transport reporter (NLS-tdTomato-NES) plasmid (received as a gift from Dr. Martin W. Hetzer, Salk Institute) using Novagen Nanojuice Transfection Reagent (Millipore-Sigma). 4 h after transfection, medium was replaced with fresh
medium and cells were grown for 24 h. Cells were then treated with 20 nM leptomycin B (Cell Signaling Technology) for 6 h and then fixed with methanol for immunostaining with an antibody against nuclear pore complex proteins (clone mAb414, Abcam) following the regular immunofluorescence protocol described below. Nuclear and cytoplasmic expression of tdTomato was observed using confocal microscopy.

**RNA export assay using Oligo-d(T) Fluorescence In situ Hybridization (FISH):**

Oligo-dT RNA FISH analysis was performed as described elsewhere ([https://openwetware.org/wiki/Poly_A_RNA_in situ_protocol](https://openwetware.org/wiki/Poly_A_RNA_in_situ_protocol)). Briefly, Nup210 knockdown and knockout cells were seeded in 4-well µ-Slides (Ibidi) at a seeding density of 100,000 cells/well. After 24 h of incubation, cells were fixed with 4% paraformaldehyde for 10 min followed by 100% ice-cold methanol and 70% ethanol. Cells were then incubated with 1M Tris-HCl pH 8.0 for 5 min. 1 ng/ml 5'-Cy3-Oligo d(T)50 probe (Gene Link) mixed with hybridization buffer (1 mg/ml yeast tRNA, 0.005% BSA, 10% dextran sulfate, 25% deionized formamide, and 2X SSC buffer) was added on to cells and incubated at 37°C in a humidified chamber for 1 h. After hybridization, cells were washed with 4X SSC and 2X SSC buffer. Cells were then incubated with 1 µg/ml DAPI in 2X SSC containing 0.1% Triton X-100 for 15 min. After washing with 2X SSC, images were captured using a Zeiss LSM 880 Airyscan confocal microscope. Nuclear Oligo-d(T) signal intensity was quantified using the Image-Pro Premier (Media Cybernetics) software.

**Protein interaction analysis through Liquid Chromatography-Mass Spectrometry (LC-MS):**

NUP210-Myc overexpressing 4T1 cells were seeded onto 15 cm tissue culture dishes at a seeding density of 2.5x10⁶ cells/dish. After 48 h of incubation, cells were harvested and nuclear protein
complex lysates were prepared using the Nuclear Complex Co-IP Kit (Active Motif). Co-immunoprecipitation with two biological replicates was performed according to the manufacturer’s instructions. Briefly, 500 µg of nuclear protein lysates were incubated with 2 µg of either Myc-Tag antibody (Cell Signaling Technology) or an endogenous NUP210-specific antibody (Bethyl Laboratories). After overnight incubation on a rotator at 4°C, 25 µg of Dynabeads Protein G (Invitrogen) were added to the protein lysate-antibody complexes. After 30 min of incubation with beads, antibody-bead-protein complexes were isolated using a magnetic stand. Beads were then washed three times and then dissolved in 25 mM ammonium bicarbonate pH 8.0 (Sigma). Samples were then subjected to LC-MS analysis.

**Western blot:**

Whole cell protein lysate was prepared using lysis buffer (20 mM Tris-HCl pH 8.0, 400 mM NaCl, 5 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 1% Triton X-100, 10% glycerol, protease and phosphatase inhibitor cocktail). Nuclear and cytoplasmic protein lysates were prepared using the Nuclear Extract Kit (Active Motif) or Nuclear Complex Co-IP Kit (Active Motif). Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). 25 µg of protein lysates were mixed with 4x NuPAGE LDS sample buffer (Invitrogen) and 10x NuPAGE Sample Reducing Agent (Invitrogen). Samples were then boiled at 95°C for 5 min and resolved on NuPAGE 3-8% Tris-acetate, NuPAGE 4-12% Bis-Tris, or Novex 4-20% Tris-Glycine protein gels (Thermo Fisher Scientific) with appropriate running buffer. Protein was transferred onto a PVDF membrane (Millipore) and the membrane was blocked with blocking buffer (TBST + 5% Non-fat dry milk) for 1 h. Membranes were then incubated with appropriate primary antibodies overnight. After washing with TBST, membranes were incubated
with secondary antibodies for 1 h. Finally, the signal was developed on X-ray film using the Amersham ECL Western Blotting Detection Reagent (GE Healthcare).

Primary antibodies and their dilutions were as follows: NUP210 (1:500; Bethyl Laboratories), β-actin (1:10,000; Abcam), Lamin B1 (1:5,000; Abcam), Myc-Tag (1:1,000; Cell Signaling Technology), SUN2 (1:1,000; Abcam), FAK (1:5,000; Abcam), p-FAK(Y397) (1: 5,000; Abcam), H3.1/3.2 (1:1,000; Active Motif), H3K27me3 (1:5,000; Cell Signaling Technology), H3K9me3 (1:5,000; Abcam), SUV39H1 (1:1,000, Cell Signaling Technology), EZH2 (1:1,000; Cell Signaling Technology), and SUZ12 (1:1,000; Cell Signaling Technology). Anti-mouse secondary antibody (GE Healthcare) was used at 1:10,000 dilution and anti-rabbit secondary antibody (Cell Signaling Technology) was used at 1:3,000 dilution.

Co-immunoprecipitation:

Co-immunoprecipitation was performed using the Nuclear Complex Co-IP Kit (Active Motif). 4T1 cells were seeded onto 15 cm tissue culture dishes at a seeding density of 5x10^6 cells/dish. After 48 h of incubation, cells were harvested and nuclear lysates were prepared. 200-500 µg of nuclear lysates were incubated with 2 µg of specific antibodies and 50 µg of Dynabeads Protein G (Invitrogen). After overnight incubation on a rotator at 4°C, immune complexes were isolated using a magnetic stand. Beads were then washed three times, resuspended in 2x NuPAGE LDS sample buffer (Invitrogen), and incubated at 95°C heat block for 5 min. Samples were loaded onto NuPAGE protein gels and the standard western blot protocol was followed as described above.

Immunofluorescence and confocal microscopy:
Immunofluorescence analysis was performed as described previously. Briefly, cells were grown on 4-well or 8-well polymer coverslips (Ibidi) at a seeding density of 40,000 or 20,000 cells/well, respectively. After 24 h of incubation, cells were fixed with -20°C methanol for 2 min and permeabilized with PBS containing 1% Triton X-100 for 1 min. Fixed cells were then blocked with immunofluorescence buffer (1x PBS, 10 mg/ml BSA, 0.02% SDS, and 0.1% Triton X-100) for 30 min. Cells were then incubated with primary antibodies diluted in immunofluorescence buffer overnight at 4°C. After washing the cells with immunofluorescence buffer three times for 10 min per wash, cells were incubated with Alexa Fluor-conjugated secondary antibodies for 1 h at room temperature. Cells were washed three times with immunofluorescence buffer and then incubated with 1 µg/ml DAPI (4′,6-diamidino-2-phenylindole) for 10 min to stain the nucleus. After washing the cells with PBS three times, slides were kept at 4°C until subjected to confocal microscopy. Images were acquired using either a Zeiss LSM 780 confocal microscope (63x plan-apochromat N.A. 1.4 oil immersion objective lens, 0.09 µm X-Y pixel size and 1.0 µm optical slice thickness), a Zeiss LSM 880 Airyscan super-resolution microscope (Airyscan detector, 63x plan-apochromat N.A. 1.4 oil immersion objective lens and 0.05 µm X-Y pixel size) or a Nikon SoRa spinning disk super-resolution microscope (Yokogawa SoRa spinning disk unit, 60x plan-apochromat N.A. 1.49 oil immersion objective lens, Photometrics BSI sCMOS camera, and 0.027 µm X-Y pixel size). Airyscan images were processed using the Airyscan processing algorithm in the Zeiss ZEN Black (v.2.3) software, whereas the Nikon SoRa images were deconvolved using a constrained iterative restoration algorithm in the Nikon NIS Elements (v5.11) software. Tetraspeck 0.2 µm beads (Invitrogen) were imaged with the same microscope parameters and used for channel alignment.
Primary antibodies used for immunofluorescence were as follows: p-FAK (Y397) (1:100; Abcam), H3.1/3.2 (1:1,000; Active Motif), mouse Myc-Tag (1:500; Cell Signaling Technology), rabbit Myc-Tag (1:100; Cell Signaling Technology), rabbit H3K27me3 (1:1,000), mouse H3K27me3 (1:250; Abcam), H3K9me3 (1:1,000; Abcam), Nucleolin (1:500; Abcam), and Nuclear Pore Complex (NPC) antibody (clone mAb414, 1:1,000; Abcam). Secondary antibodies used were mouse Alexa Fluor 488 (1:200; Invitrogen), rabbit Alexa Fluor 488 (1:200; Invitrogen), rabbit Alexa Fluor 568 (1:200; Invitrogen), rabbit Alexa Fluor 594 (1:200; Invitrogen), and mouse Alexa Fluor 594 (1:200).

**Cell cycle analysis:**

Cell cycle analysis was performed using the Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Thermo Fisher Scientific) and FxCycle Violet Stain (Thermo Fisher Scientific) according to manufacturer’s instructions. Briefly, 4T1 cells were seeded onto 15 cm tissue culture dishes at a seeding density of 3x10^6 cells/dish. 24 h later, cells were pulsed with Click-iT EdU (5-ethynyl-2’-deoxyuridine) for 1 h. After harvesting the cells through trypsinization, cells were fixed with Click-iT fixative and permeabilized with saponin-based permeabilization agent. The Click-iT reaction was then performed for 30 min at room temperature. Cells were then washed with wash buffer and stained for DNA content analysis with FxCycle Violet, a DNA-selective dye. Finally, cell cycle analysis was performed using a BD FACS Canto II flow cytometer (BD Bioscience). Data was analyzed using FlowJo V10 (FlowJo, LLC) software.

**Chromatin immunoprecipitation followed by sequencing (ChIP-seq) analysis:**
Chromatin immunoprecipitation (ChIP) was carried out using the ChIP-IT Express Enzymatic Chromatin Immunoprecipitation Kit (Active Motif) according to manufacturer’s instructions. Briefly, 5x10^6 4T1 cells were seeded onto 15 cm tissue culture dishes. After 48 h of incubation, cells were fixed with 1% formaldehyde for 10 min at room temperature. Cells were washed with ice-cold PBS and formaldehyde cross-linking was quenched using glycine stop-fix solution. Cells were harvested through scraping and pelleted by centrifugation at 2,500 rpm for 10 min at 4°C. After cell lysis with ice-cold lysis buffer and a dounce homogenizer, chromatin was sheared using an enzymatic shearing cocktail for 12 min at 37°C. The shearing reaction was stopped with 0.5 M EDTA and the chromatin was separated through centrifugation at 15,000 rpm for 10 min at 4°C. 60-70 µg of chromatin were used with 25 µl of Protein G magnetic beads and 2 µg of specific antibodies for each ChIP reaction. The following antibodies were used for the ChIP reactions: anti-rabbit NUP210 (Bethyl Laboratories), anti-rabbit H3K27me3 (Cell Signaling Technology), anti-rabbit H3K4me3 (Millipore), and anti-mouse Nuclear Pore Complex (NPC) antibody (clone mAb414, Abcam). After incubating the reaction mixture overnight at 4°C on a rotator, the beads were washed with ChIP buffers and DNA was eluted with elution buffer. DNA was then reverse-cross linked, proteinase K-treated, and DNA quality was assessed using a Agilent Bioanalyzer before ChIP-seq library preparation. The library was prepared using a TruSeq ChIP Library Preparation Kit (Illumina) and pooled samples were sequenced on the NextSeq platform.

ChIP-seq data was analyzed using the Sicer algorithm with default parameters. For narrow peaks such as H3K4me3 enrichment, a window size of 200 was used. For broad peaks such as H3K27me3 and NUP210, a window size of 1000 was used. The ChIP-seq plot profile was
generated using deepTools\textsuperscript{2}\textsuperscript{99}. For ChIP-seq peak annotation, the ChIPSeeker\textsuperscript{100} Bioconductor package was used. BigWig files were displayed using the Integrative Genomics Viewer (IGV)\textsuperscript{101}.

**Publicly available ChIP-seq and Hi-C data analysis:**

H3K27Ac ChIP-seq data from mouse mammary luminal cells were downloaded from Gene Expression Omnibus (GEO) (Accession No: GSE116384)\textsuperscript{34}. CTCF ChIP-seq data from mouse mammary epithelial cells were obtained from GEO (Accession No: GSE92587, sample: GSM2433042)\textsuperscript{26}. CTCF and H3K27Ac ChIP-seq data from the MCF7 human breast cancer cell line were obtained from GEO (Accession No: GSE130852). Publicly available Hi-C data from mouse embryonic stem cells were derived from Bonev et al.\textsuperscript{48} and displayed using the 3D Genome Browser\textsuperscript{49}.

**RNA isolation and quantitative reverse transcriptase real-time PCR (qRT-PCR):**

Cells were directly lysed on cell cultures plate with 1 ml TriPure Isolation Reagent (Sigma). After adding 200 µl chloroform and centrifuging at 14,000 rpm for 15 min at 4°C, the upper aqueous layer containing the RNA was transferred to new tube. To precipitate RNA, 500 µl of isopropanol was added to each tube, which was then vortexted and incubated at -20°C for 1 h. The RNA was further purified using the RNeasy Mini Kit (Qiagen) with on-column DNase (Qiagen) digestion according to manufacturer’s instructions. 2 µg of total RNA was used for cDNA preparation using the iScript cDNA synthesis kit (Bio-Rad). A 1/10 dilution of cDNA was used for qRT-PCR analysis using the FastStart Universal SYBR Green Master Mix (Roche). Sequences of the primers used are listed in the reagents or resource table.
ChIP qPCR analysis:

Chromatin was isolated from 4T1 and 6DT1 cells using the ChIP-IT Express Enzymatic kit (Active Motif) according to manufacturers’s instructions as mentioned above. 10 µl of normal rabbit IgG (Cell Signaling Technology), 10 µl of rabbit CTCF (Cell Signaling Technology), and 10 µl of rabbit H3K27Ac (Cell Signaling Technology) antibodies were used for the immunoprecipitation reaction. ChIP DNA was then subjected to qPCR analysis. The percent (%) input method was used for the calculation of CTCF and H3K27Ac enrichment.

RNA-seq analysis:

4T1 cells were seeded onto 6 cm tissue culture dishes at a seeding density of 4x10⁵ cells/dish. After 48 h of incubation, total RNA was isolated using the protocol described above. On-column DNase treatment was performed to eliminate DNA contamination. Library preparation was performed using the TruSeq Stranded mRNA Library Prep Kit (Illumina) and pooled samples were sequenced on a HiSeq2500 with TruSeq V4 chemistry (Illumina). Differential gene expression analysis from RNA-seq data was performed using Partek Flow software. Gene ontology (GO) enrichment analysis of differentially expressed genes was performed using the PANTHER classification system 102.

Promoter luciferase assay:

24-well tissue culture plates were coated overnight with 100 µg/ml type I collagen. 293FT cells were seeded in antibiotic free medium at a seeding density of 75,000 cells/well of 24-well plate. 250 ng of pGL4.23 Nap210 luciferase promoter vectors and 25 ng of pRL-TK renilla luciferase vectors were co-transfected using NanoJuice transfection reagent (Millipore). 24 h after
transfection, the luciferase assay was performed using Dual-Luciferase Reporter Assay System kit (Promega) according to the manufacturer’s instructions. Luciferase activity was measured using a GloMax 96 Microplate Luminometer (Promega). Firefly luciferase activity was normalized with Renilla luciferase activity. Eight biological replicates were used per condition.

**Cytokine array:**

4T1 *Nup210* knockdown cells were cultured in low serum condition (1% FBS) in 6-well plates at a seeding density of 500,000 cells per well. 24 h after incubation, cell culture supernatants were harvested and centrifuged to remove the dead cells. 1 ml of supernatant was used for cytokine profiling using the Proteome Profiler Mouse XL Cytokine Array (R & D Biosystems) according to manufacturer’s instructions. Chemiluminescent signals were quantified using ImageJ (NIH) software.

**3D DNA FISH analysis:**

For DNA FISH analysis, cells were grown on Lab-Tek chamber slides. Cells were briefly washed with PBS and fixed with 4% paraformaldehyde. Cells were permeabilized with 0.2% Triton X-100 in PBS and then hybridized with labeled probe. For probe generation, BAC clones were purchased from CHORI ([https://bacpacresources.org/](https://bacpacresources.org/)). The following BAC clones were used for the experiment: *Cxcl* region: clone RP23-374O6; *Postn* region: clone RP23-480C1; *Ccl2* region: clone RP23-99N1, and *Igib2* region: clone RP23-166E21. All the clones were expanded and DNA was isolated using the FosmidMax DNA Purification Kit (Epicentre). DNA from each clone was labeled through nick translation with either the Atto550 NT Labeling Kit (Jena Bioscience) or the Digoxigenin NT Labeling Kit (Jena Bioscience). Hybridization was carried out in a humidified
chamber at 37°C for 16 h. Post-hybridization rapid wash was carried out with 0.4x SSC at 72°C for 4 min. Digoxigenin was detected with a DyLight 594-Labeled Anti-Digoxigenin/Digoxin (DIG) antibody (Vector Laboratories). The slides were stained with DAPI and Z-stack images were captured using a Zeiss LSM880 Airyscan microscope.

*Mechanotransduction assay:*

To test the mechanotransduction effect, polyacrylamide hydrogel-bound cell culture plates with different elastic moduli was used as a mimic of extracellular matrix (ECM) stiffness. 6-well plates with 0.2 kPa (soft) or 12 kPa (stiff) elastic moduli were purchased from Matrigen. Each well of the plate was coated with 20 µg/ml fibronectin (Sigma) in PBS for 1 h at 37°C. After washing the well with PBS, 4T1 cells were seeded on top of soft or stiff matrix at a seeding density of 100,000 cells/well. 48 h after incubation, cells were harvested for RNA or protein isolation.

*Focal adhesion assay:*

For focal adhesion immunostaining, Lab-Tek 2-well glass chamber slides (Thermo Fisher Scientific) were coated with 50 µg/ml of collagen type I matrix (Gibco) and cells were seeded at a seeding density of 20,000 cells/well. After overnight incubation, cells were fixed in 4% paraformaldehyde for 20 min. Cells were then washed with PBS, permeabilized with PBS + 0.1% Triton X-100 for 5 min, and blocked with PBS + 5% normal goat serum. Cells were incubated with phospho-FAK (Y397) antibody for 1 h. After washing with PBS, cells were incubated with Alexa Fluor 594-conjugated rabbit secondary antibody and Alexa Fluor 488-conjugated phalloidin (F-actin staining) for 1 h. The slides were then washed with PBS and mounted using VECTASHIELD with DAPI Mounting Medium (Vector Laboratories). Images were captured
using a Zeiss 780/880 confocal microscope and automated counting of focal adhesion puncta (phospho-FAK staining) was performed using Image-Pro Plus software (Media Cybernetics).

**Actin polymerization inhibition assay using cytochalasin D:**

For the cytochalasin D treatment of 4T1 *Nup210* knockdown cells, 20,000 cells were seed onto 4-well µ-Slides with polymer coverslips (Ibidi). 24 h later, cells were treated with 1 µM cytochalasin D, a potent inhibitor actin polymerization, for 2 h. After the treatment, cells were washed with complete medium three times and incubated for another 2 h in a 37°C CO₂ incubator for the recovery of actin polymerization. Cells were then fixed with 4% paraformaldehyde for 30 min and stained with Alexa Fluor 488-conjugated phalloidin for immunofluorescence imaging. Nuclei were stained with 1 µg/ml Hoechst 33342 (Thermo Fisher).

**EZH2 inhibitor (GSK126) treatment:**

For immunofluorescence microscopy, 4T1 sg-Ctrl and *Nup210* KO cells were seeded onto 4-well µ-Slides at a seeding density of 25,000 cells/well. 24 h later, KO cells were treated with either DMSO or 5 µM GSK126 (Selleckchem) for another 24 h. Cells were then fixed with -20°C methanol for 2 min. Histone H3.1/3.2 and H3K27me3 antibodies were used for immunofluorescence staining according to the protocol described above.

For the qRT-PCR analysis, 2x10⁵ 4T1 *Nup210* KO cells were seeded onto 6 cm dishes. 24 h later, 5 µM GSK126 was applied to the cells that were then incubated for another 48 h. Cells were then lysed, RNA was isolated, and qRT-PCR was performed as described above.
**Cell migration assay:**

For the automated random cell migration assay, 4T1 cells were seeded onto 96-well polystyrene microplates (Corning) at a seeding density of 2,000 cells/well so that the cell density remained sub-confluent until the end of the imaging period. After 24 h of incubation, cells were incubated with complete medium containing 200 ng/ml Hoechst 33342 (Thermo Fisher Scientific) for 1 h. Cells were then transferred to a Nikon Eclipse Ti2 microscope. Images were captured every 12 minutes with a 20x 0.8 NA objective for 24 h. Total light exposure time was kept to 200 milliseconds for each time point. Cells were imaged in a humidified 37°C incubator with 5% CO₂. Image processing and cell tracking were carried out with custom MATLAB script described previously 103.

**Cell invasion assay:**

The cell invasion assay was performed using BioCoat Matrigel Invasion Chambers with 8.0 µm PET Membrane (Corning) according to manufacturer’s instructions. Briefly, 7.5x10⁵ 4T1 and 6DT1 cells were seeded onto the top well containing DMEM with 0.5% serum. In the bottom well, DMEM with 10% serum was used as a chemoattractant. Cells were incubated in a 37°C incubator for 24 h and 48 h for 6DT1 and 4T1 cells, respectively. After incubation, non-invaded cells were removed from the top well using cotton tips. Cells that had invaded into the Matrigel were then fixed with methanol and stained with 0.05% crystal violet. Matrigel membranes containing invaded cells were then cut and mounted onto glass slides with Vectashield mounting medium (Vector Laboratories). Images of entire membranes were captured as segments and then stitched using an EVOS FL Auto 2 microscope (Invitrogen). Image analysis was performed using Image-
Pro Premier 3D (Media Cybernetics) software. Percent cell invasion was calculated using crystal violet intensity per membrane area.

**Circulating tumor cell (CTC) analysis:**

100,000 6DT1 cells with or without *Nup210* knockdown were injected into the fourth mammary fat pad of FVB/NJ mice. Ten mice were used in each group and three mice were kept uninjected for use as healthy controls. One month after injection, mice were anesthetized with avertin injection. Through cardiac puncture, 600-1000 µl blood per mouse was collected in 50 µl of 0.5 M EDTA solution. An equal volume of blood was taken for red blood cell lysis using ACK lysis buffer. 100 µl of the peripheral blood lymphocyte (PBL) fraction was subjected to fixation with 2% paraformaldehyde for 15 min at room temperature. Cells were permeabilized with PBS containing 0.1% Triton X-100. Cells were vortexed briefly and kept at room temperature for 30 min. 0.5% BSA in PBS was added and cells were pelleted by centrifugation. Cells were then resuspended in ice-cold 50% methanol in PBS and incubated for 10 min on ice. 150,000 fixed cells were stained for CD45, a pan-lymphocyte marker, and pan-keratin, a tumor cell marker. Before staining with antibodies, cells were incubated with FcR Blocking Reagent (1:10 dilution; Miltenyi) for 10 min at 4°C. Cells were then stained with APC-conjugated CD45 (1:25 dilution; Miltenyi) and Alexa Fluor 488-conjugated pan-keratin (1:25 dilution, Cell Signaling Technology) antibodies for 10 min at 4°C. After washing with MACS buffer (PBS, 0.5% BSA, and 2 mM EDTA), cells were incubated with 1 µg/ml Hoechst 33342 (Thermo Fisher Scientific) for 5 min. Cells were then washed again with MACS buffer and resuspended in 200 µl buffer for analysis using a BD FACSCanto II flow cytometer. A CTC (CD45⁻/Cytokeratin⁺) gate was created based on the
staining pattern of 6DT1 tumor cells in culture and primary tumor cells derived from 6DT1-injected mice. Flow cytometry data was analyzed using FlowJo V10 software.

**Patient datasets analysis:**

Distant metastasis-free survival analysis on gene expression signature was performed using GOBO tool ([http://co.bmc.lu.se/gobo/gobo.pl](http://co.bmc.lu.se/gobo/gobo.pl))\textsuperscript{104}. Data on *NUP210* and *CCL2* amplification in human breast cancer patients was derived from Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) data available on cBioPortal ([https://www.cbioportal.org/](https://www.cbioportal.org/))\textsuperscript{105}. METABRIC *NUP210* mRNA expression data was also downloaded from the cBioPortal database and manually processed for further analysis. Distant metastasis-free survival data was obtained from the Km-plotter database ([https://kmplot.com/analysis/](https://kmplot.com/analysis/)). The JetSet best probe 213947_s_at was used for *NUP210* expression and patients were separated by upper and lower quartile values. *NUP210* gene expression data on breast cancer metastatic sites, prostate cancer and melanoma were downloaded from Gene Expression Omnibus (GEO).

**QUANTIFICATION AND STATISTICAL ANALYSIS:**

**Statistical analysis:**

In case of the *in vivo* animal studies, p-values were calculated using the Mann-Whitney test in GraphPad Prism 8 software and the results were reported as mean ± standard deviation. For qRT-PCR result analysis, p-values were calculated using a two-tailed, unpaired t-test in GraphPad Prism and results were reported as mean ± standard error of mean (s.e.m).
**Microscopy image analysis and quantification:**

The periphery/total nuclear area intensity ratio of histone H3.1/3.2, H3K27me3, and H3K9me3 was quantified using Fiji software. Briefly, total nuclear area was defined by DAPI staining, while the nuclear periphery was defined as the region 25% towards the interior from the nuclear edge (average distance for sg-Ctrl: 0.97 µm and KO-N13: 0.89 µm). Mean fluorescence intensity was quantified in both the periphery and total nuclear area and the periphery/total ratio was plotted. The heterochromatic foci/total nuclear area intensity of histone H3.1/3.2 was quantified using Fiji software. Heterochromatic foci regions were segmented using DAPI staining. Mean fluorescence intensity was quantified in both heterochromatic foci and total nuclear area and the heterochromatic foci/total ratio was plotted as box plots (bottom and top of the box denote first and third quartile respectively, whiskers denote +/- 1.5 interquartile range (IQR), horizontal lines denote median values). p-values were calculated using a two-tailed Mann-Whitney U-test.

The position of DNA FISH spots relative to the nuclear centroid and periphery was quantified using the Cell module within the Imaris image analysis suite (Bitplane). To account for differences in nuclear size, the relative position was calculated by dividing the shortest distance of the FISH spot to the nuclear centroid, by the length of the three-point line encompassing the nuclear centroid, FISH spot, and nuclear periphery. Relative positions were plotted as cumulative distribution frequencies using RStudio software. p-values were calculated using a two-tailed Mann-Whitney U-test. The distance of DNA FISH spots to heterochromatic foci was quantified using Fiji software with the 3D region of interest (ROI) manager plugin, TANGO. Heterochromatic foci were segmented using DAPI staining and used to create a distance map. DNA FISH spots were segmented and the distance to the nearest heterochromatic foci was calculated and plotted as box.
plots (bottom and top of the box denote first and third quartile respectively, whiskers denote +/- 1.5 interquartile range (IQR), horizontal lines denote median values). p-values were calculated using a two-tailed Mann-Whitney U-test.

**DATA AND SOFTWARE AVAILABILITY:**

Gene expression data will be deposited in Gene Expression Omnibus (GEO) prior to publication.
Figure 1

(a) 4T1 mouse model of metastasis

- 4T07
- 4T1

- Non-disseminating
- Non-colonizing
- Colonizing

(b) 1874 Polymorphic BACH regions
- Identify Overlap
- 771 genes with polymorphic transcriptional control elements
- 52 genes with metastasis association in mouse DO panel
- Distant metastasis-free survival analysis in patient dataset

(c) ER+ Metastasis

(d) Nup210 mRNA expression

(e) 510 bp region

(f) Polymorphisms in 510 bp promoter region

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Introns/Deletions:

1. rs233236721 C C CCCT

(g) 12 bp insertion in FVB/NJ

(h) CTCF enrichment (% input)

(i) Normal spleen

(j) Relative mRNA level

(k) Relative luciferase activity (U/dm)

(l) Relative mRNA level

(m) Western blot analysis
Figure 2

a) METABRIC (NUP210 amplification)

Log-rank P = 0.0331
Cases with amplification (n=20)
Cases without amplification (n=1961)

Overall survival

b) ER+ (METABRIC) (NUP210 expression)

Log-rank P < 0.0001
NUP210 High (n=736)
NUP210 Low (n=721)

Overall survival

c) ER-PR-Her2- (METABRIC) (NUP210 expression)

Log-rank P = 0.0935
NUP210 High (n=149)
NUP210 Low (n=150)

Overall survival

d) ER+ (Km-plotter)

Log-rank P < 0.0001
NUP210 High (n = 164)
NUP210 Low (n = 167)

Distant metastasis-free survival

Months elapsed

e) ER-PR-Her2- (Km-plotter)

Log-rank P = 0.35
NUP210 High (n = 21)
NUP210 Low (n = 23)

Distant metastasis-free survival

Months elapsed

f) PAM50 subtype (METABRIC)

NS

****

GSE32489: Breast Cancer

NUP210 expression

Lymph Node (n=90)
Lung (n=86)
Liver (n=86)
Metastatic sites

GSE6919: Prostate Cancer

NUP210 expression

Primary Tumor (n=85)
Metastases (n=25)

GSE8401: Melanoma

NUP210 expression

Primary Tumor (n=31)
Metastases (n=52)
Figure 3

a 4T1-Nup210 shRNA KD

b 4T1 Primary Tumor

Tumor weight (g)

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<td>β-actin</td>
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*** NS

Lung metastases count

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* NS

Tumor-normalized metastases

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**

e 4T1-Nup210 CRISPR/Cas9-KO

f 4T1 Primary tumor

Tumor weight (g)

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<th>Nup KO-N13</th>
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Lung metastases count

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**** NS

Tumor-normalized metastases

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**

i 4T1-Nup210-OE

j 4T1 Primary tumor

Relative mRNA Level

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Lung metastases count

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**
Figure 4

**a** 4T1-NUP210-Myc-OE Cells  
Isolate Nuclear proteins  
Co-IP (NUP210-endogenous or NUP210-Myc-Ab)  
Tryptic digestion  
LC-MS Analysis

**b** Peptides identified in LC-MS

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**c** Histone H3.1 amplification (METABCIC)

- Log rank P = 0.0642
- Overall survival

**d** Co-IP: Myc-Tag

- 4T1-CE
- NUP210-N13
- H3.1/3.2
- NUP210-Myc
- 4T1

**e** NUP210

- Input
- IgG
- H3.1/3.2
- Lamin B1
- 4T1

**f** NUP210-Myc

- H3.1/3.2

**g** H3.1/3.2, H3K27me3, DAPI, Overlay

**h** H3.1/3.2

**i** H3.1/3.2, H3K9me3, DAPI, Overlay

**j** Nucleolin

**k** H3.1/3.2, Nucleolin, DAPI, Overlay

**l** 4T1-Nup210 KO

**m** Co-IP

**n** Co-IP

**o** Co-IP

**p** Co-IP
Figure 6

3D-DNA FISH analysis

Cyclin region | Postn region | Ccl2 region | L1gb2 region

Relative position of FISH spots (Nuclear periphery vs centroid)

- Cxcl region: P = 9.19e-10
- Postn region: P = 8.91e-05
- Ccl2 region: P = 0.367
- L1gb2 region: P = 0.0081

Relative position: Nuclear periphery = 0, Nuclear centroid = 1

Distance of FISH spots from heterochromatin foci

- Cxcl region: P = 0.439
- Postn region: P = 0.0328
- Ccl2 region: P = 2.66e-08
- L1gb2 region: P = 0.164
Supplementary Figure 1

CTCF and H3K27Ac occupancy in Human NUP210 promoter
Supplementary figure 2

(a) Nup210 shRNA KD
6DT1: Western blot

MVT1: qRT-PCR

Relative mRNA Level

sh-Ctrl  sh-Nup #1  sh-Nup #4

Nup210
Sun2

Nuclear lysate

(b) Primary tumor

6DT1

Tumor weight (g)

MVT1

Tumor weight (g)

sh-Ctrl  sh-Nup #1  sh-Nup #4

(c) Lung metastases count

6DT1

MVT1

Lung metastases count

sh-Ctrl  sh-Nup #1  sh-Nup #4

(d) Tumor-normalized metastases

6DT1

MVT1

Metastases per gram tumor

sh-Ctrl  sh-Nup #1  sh-Nup #4
Supplementary figure 3

(a) sg-CTRL, Nup KO-N9, Nup KO-N13

(b) Percentage of Cells

- Sg-CTRL
- Nup KO-N9
- Nup KO-N13

Cell cycle Stages:
- G0-G1
- Early S
- Mid S
- Late S
- G2-M
Supplementary figure 4