Nucleoporin-93 overexpression overcomes multiple nucleocytoplasmic trafficking bottlenecks to permit robust metastasis

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Abstract

To identify genetic events driving breast cancer progression, we performed multi-omics integrated analyses that identified overexpression of nucleoporin-93, a nuclear pore component. Here we show that NUP93 overexpression enhances trans-endothelial migration and matrix invasion in vitro, along with metastasis in animal models. These findings were supported by analyses of naturally occurring activating NUP93 mutations and inactivating nephrotic syndrome mutations. Mechanistically, NUP93 activation enhanced the ultimate nuclear transport step shared by multiple signaling pathways, including TGF-beta/SMAD, EGF/ERK and TNF/NF-κB. Likewise, NUP93 can boost nuclear transport of beta-catenin, as well as elevate expression and inhibit degradation of this co-activator. The emerging addiction to nuclear transport exposes vulnerabilities of tumors overexpressing NUP93. Congruently, we report that myristoylated peptides corresponding to the nuclear translocation signals of SMAD and ERK inhibited growth and metastasis. Our study illuminates a previously unappreciated hallmark of advanced tumors, which derive benefit from unblocking a set of nucleocytoplasmic bottlenecks.

Introduction

Breast carcinoma is the leading cause of cancer-related mortality in women ¹. Owing to genomic instability, breast cancers often exhibit somatic copy number aberrations (CNAs) ², such as amplifications of the *ERBB2* gene (at 17q12) ^{3,4}. Together with the epidermal growth factor receptor (EGFR), *ERBB2*/HER2 acts as a transducer of growth factor signals ⁵. Importantly, kinase inhibitors and combinations of antibodies that inhibit HER2 are widely used to treat HER2-overexpressing breast cancers ^{6,7}, which exemplifies the therapeutic potential offered by the identification of overexpressed oncogenic proteins. Aneuploidy, CNAs and overexpression of specific genes are well-known sources of gene dosage imbalance in cancer. Because many cellular functions rely on multi-protein complexes, precise and timely dosage of the individual elements, especially protein stoichiometry, might be critical and define true driver genes ⁸. For example, limited CNAs affecting diverse breast cancer genes, all regulating receptor endocytosis, confer poor prognosis ⁹.

Herein, we demonstrate that breast cancers acquire aggressive phenotypes when another intracellular trafficking pathway, which regulates nuclear-cytoplasm transport, is activated. Usually, proteins shuttle to the nucleus when an intrinsic nuclear localization signal (NLS) interacts with importins, which escort them to the nucleus via the nuclear pore complex (NPC) ¹⁰. However, signaling proteins like ERK translocate, upon stimulation, by means of unique nuclear translocation signals (NTS) ¹¹. The NPC structure is stabilized by protein complexes arranged in concentric rings. Using a new computational tool, we identified a specific component of the inner NPC's ring, nucleoporin 93 (NUP93), as highly essential for breast cancer. Loss-of-function mutations in NUP93 are recognized as monogenic causes of steroid-resistant nephrotic syndrome (SRNS) ¹², whereas rare gain-of-function (GOF) mutations identify relatively aggressive mammary tumors ¹³. In line with these observations, we herein report that elevated abundance of NUP93, like GOF mutations, promotes metastasis of mammary tumors, while knockdown

markedly retards metastasis in animal models. Subcellular fractionation revealed that NUP93 overexpression enhances the concluding step of transcription factor translocation to the nucleus, an event shared by EGF, TGF-beta, WNT, TNF-alpha and glucocorticoids. In addition, NUP93 overexpression accelerates nuclear transport of beta-catenin and inhibits its degradation. Accordingly, NTS-mimetic decoy peptides inhibited tumor progression in animal models. Altogether, our results identify a robust, yet targetable, mechanism shared by aggressive mammary tumors, which depend on ongoing signaling from the tumor microenvironment to the nucleus.

Results

NUP93 is highly essential for survival of mammary tumor cell lines, and its abundance associates with poor prognosis of patients with breast cancer

To identify genes most relevant to progression of breast cancer, we performed computational analyses integrating RNA sequencing data from both METABRIC¹⁴ and The Cancer Genome Atlas, and incorporated both somatic copy number alterations (CNA) and patient survival data (> 3000 patients). In parallel, we added gene essentiality scores derived from whole-genome shRNA screens that used 138 cancer lines^{15,16}. Next, we compared essentiality scores of the top 500 clinically relevant genes and the bottom 500 genes. As expected, the top 500 genes were found to be more essential (p-value < 0.01 for pan-cancer cell lines and < 0.02 for breast cancer cell lines). Combining the top 500 clinically relevant genes and the 500 most essential genes, we obtained a ranked list of 20 genes, which are both clinically relevant and phenotypically essential for breast cancer (Supplementary Table 1). Interestingly, two tubulin genes topped the list of 20 genes, in line with reports linking overexpression of tubulins to survival of patients with breast cancer¹⁷. The scores received by nucleoporin 93 (NUP93) were close to those received by the tubulins. Figure 1A presents the essentiality scores received by NUP93 in a series of breast cancer cell lines. Because anti-tubulin drugs are widely used in chemotherapy, but no approved anti-cancer drugs target NUP93¹⁸, we focused on the nucleoporin. Additional analyses confirmed that the over-expression of this gene leads to poor prognosis (logrank P < 3.61E-12). Importantly, this trend remained significant when age, metastasis to lymph nodes and disease subtypes were controlled (Cox hazard ratio = 1.22, P < 1.96E-4). As an example, we divided the METABRIC dataset into three groups according to *NUP93* expression levels. The Kaplan-Meier survival analyses separately performed for each group revealed that *NUP93* overexpression is independently and significantly associated with shorter disease-free patient survival (Fig. 1B). The same dataset was stratified also according to the status of estrogen receptor (ER; Fig. 1C), which revealed that the more aggressive group of tumors, which lack ER expression, displayed relatively high NUP93.

An association between NUP93 and more virulent mammary tumors emerged from analysis of the 10 integrative clusters (IC) of breast cancer². IC10, which incorporates mostly triple negative tumors, showed highest NUP93 expression. Likewise, *NUP93*'s transcripts were relatively high in IC5, which identifies almost all cases with *ERBB2/HER2* amplification. In addition, we found that high NUP93 was

significantly associated with CNA (Fig. 1E). As expected, the highest gains of the gene were found in the basal subtype, and this was followed by the HER2-enriched subtype. Furthermore, high NUP93 expression was typical to high-grade tumors (Fig. 1F). In conclusion, by developing a novel computational pipeline, we learned that a component of the NPC is highly essential for survival of breast cancer cells and can predict shorter survival of a subset of patients with breast cancer.

Experimental strategies applying induced overexpression and downregulation reveal involvement of NUP93 in proliferation, migration and invasiveness

Because NUP93 is overexpressed in the basal subtype, we selected two basal models: (i) MDA-MB-231 basal B breast cancer cells, and (ii) MCF10A, a non-tumorigenic immortalized basal B line. Firstly, we expressed an inducible allele of NUP93 in MDA-MB-231 cells and verified relatively high expression on induction with tetracycline (Tet; Fig. 2A). Reciprocally, we established sublines expressing different doxycycline (DOX) inducible shRNAs (iSh; Fig. 2B). As predicted, exposing cells to Tet enhanced their ability to incorporate a radioactive nucleoside into DNA (Fig. 2C), whereas DOX-induced downregulation reduced DNA synthesis (Fig. 2D). Next, we applied a cell viability assay, which supported the ability of ectopic NUP93 to enhance viability (Figs. S1A and S1E). Similar conclusions were derived from experiments that used subclones constitutively expressing shNUP93 (Fig. S1B and S1F; left panels) and cells expressing siRNAs specific to NUP93 (Fig. S1B and S1F; right panels). A parallel set of experiments, which made use of genetically modified MCF10A cells (Figs. S1C and S1D) confirmed association of higher viability signals with overexpression (Fig. S1G) and lower signals in NUP93-depleted cells (Fig. S1H).

According to a recent breast cancer study, predictors of migration, rather than proliferation, are strongly associated with patient survival ¹⁹. When we placed inducible MDA-MB-231 cells on the upper compartment of cell culture inserts and treated them with the inducer, they migrated significantly faster than untreated cells (Fig. 2E). The reciprocal approach, which used DOX-inducible shRNAs, confirmed lower migration rates relative to control shRNA (Fig. 2F). Moreover, when the intervening membrane was coated with an extracellular matrix we similarly observed increased and decreased invasion rates with cells exposed to Tet (overexpression) or DOX (knockdown), respectively (Figs. 2G and 2H). Additional migration (Fig. S1I) and invasion assays (Fig. S1J) using MDA-MB-231 cells, as well as a similar set of MCF10A cell experiments (Figs. S1K-S1N) provided further support to the notion that NUP93 increases cellular motility. In conclusion, in line with the high essentiality and clinical significance, high abundance of NUP93 associated in vitro with increased viability, mitosis, migration and matrix invasion.

NUP93 overexpression enhances trans-endothelial migration and 3D invasion, as well as remodels focal adhesion sites

According to a recent report, *NUP93* is involved in 3D migration and this correlates with an altered actin cytoskeleton ²⁰. In line with this report, when pre-formed spheroids were induced with tetracycline to elevate NUP93, many cells migrated outward to form invasion zones (Fig. S2A). Reciprocally, NUP93

silencing reduced zone area (Fig. S2B). Increased invasiveness emerged also from TEM (trans-endothelial migration) assays that used endothelial monolayers overlaid by cells expressing inducible shNUP93 (Fig. S2C). Because VEGF might be involved, we used ELISA and found that siNUP93 reduced VEGF secretion (Fig. S2D). Interestingly, depletion of NUP93 also increased cell area (Fig. S2E) and reduced by 4-fold the ability of cells to form invadopodia (Fig. S2F). Notably, invadopodia contain microdomains with spatiotemporal dynamics of actin-rich adhesion sites²¹. Indeed, we found that depletion of NUP93 was associated with remodeling of the actin cytoskeleton (Fig. S2G) and up-regulation of integrin alpha5 and integrin beta1 (Fig. S2H). Next, we extended the analysis to additional cell adhesion molecules, such as two paxillin family members—paxillin and Hic-5, which have been widely implicated in turnover of adhesion sites²². Imaging and quantification of the respective adhesion areas unveiled redistribution along with up-regulation of the respective areas in siNUP93-treated cells (Fig. S2I). In summary, the overexpressed NUP93 increases 3D cell invasion, as well as enhances TEM, invadopodium assembly and remodels adhesion sites containing molecules like paxillin, fascin and zyxin.

High NUP93 abundance associates with increased rates of tumor growth and metastasis, along with matrix reorganization

Next, we implanted MDA-MB-231 cells, expressing a Tet-inducible NUP93 allele, in the fat pad of female mice and added the inducer to the drinking water of one of 2 groups of mice. Both the volumes and weights of tumors we harvested 3 weeks later were significantly larger in the treated group (Fig. S3A). A reciprocal experiment that made use of cells expressing a DOX-inducible shNUP93 reinforced the ability of NUP93 to accelerate tumor growth (Fig. S3B). This conclusion was independently supported by using cells stably expressing shNUP93 and determining tumor growth (Fig. S3C). As an initial test of metastasis, lungs were excised and metastases were quantified (Fig. S3D). The results reflected strong inhibitory effects, which prompted studies employing inducible expression and two different metastasis assays. Cells inducibly overexpressing NUP93 were injected either into the tail vein (Fig. 3A, left panel) or into the subaxillary mammary fat pad (Fig. 3A; right panel). Seven days later, we added tetracycline to the drinking water and mice were sacrificed 3 weeks later. The results indicated that NUP93 overexpressors are endowed with > 3-fold stronger capacity to colonize lungs. Further, when the same protocol was applied on tumors inducibly expressing shNUP93, we observed remarkably fewer lung metastases (Fig. 3B). Together, these observations assigned to NUP93 an important role in metastasis.

To resolve the identity of protein mediators, we sequenced RNA from MDA-MB-231 cells that were pretreated with siNUP93. The major differentially expressed (DE) genes are shown in Fig. 3C and Supplementary Excel File 1. Along with up-regulation of the cysteine-rich angiogenic inducer 61²³, we observed downregulation of several mitochondrial genes, such two subunits of the respiratory chain I, which drives ATP generation, and RNR1, a subunit of ribonucleotide reductase, which catalyzes dNTP production. These alterations might reflect the metabolically active state of NUP93-overexpressing cells. We also noted TGF-beta, keratins and extracellular matrix (ECM) components²⁴. The heatmap shown in Fig. 3D lists the major matrix DE genes, including several types of collagen. These observations were supported using PCR (Fig. S3E) and immunoblotting for collagen IVa6 (Fig. S3F). Because collagen

enables cell-to-matrix adhesion through binding with integrins, we predicted accompanying changes in substrate adhesion. Experiments using DOX-inducible shNUP93 (Fig. S3G), or an inducible NUP93 (Fig. S3H), confirmed that NUP93 reduces adhesion to substrate. To examine collagen involvement, we stained collagen IVa6 (Fig. S3I). Importantly, collagen fibrils were observed only in NUP93-depleted cells. For in vivo assays, tumors excised from DOX-treated mice were stained with either a collagen dye, picrosirius red (Fig. 3E), or an anti-collagen IVa6 antibody (Fig. S3J). Evidently, tumors from mice treated with shNUP93 showed strong staining only if the animals were pre-treated with Doxycycline. Since collagen is induced by TGF-beta²⁵, we used various methods to probe for the ligand and the receptor, TGFBR2. The results indicated co-induction, along with localization of the receptor to lamellipodia (Figs. 3F-3G, S3K-S3M). In conclusion, high abundance of NUP93 confers accelerated growth and metastatic spread upon mammary tumor cells. The underlying mechanism involves weakening ECM adhesion due to inhibition of collagen deposition, lower expression of ECM-modifying enzymes and reduced secretion of TGF-beta.

Overexpression of NUP93 enhances nuclear translocation of SMAD, ERK, STAT3, GR and p105 (NF-κB) in response to TGF-beta, EGF, glucocorticoid and TNF-alpha, respectively

Steroid resistant nephrotic syndrome is caused by *NUP93* mutations, which interfere with BMP7-induced SMAD transcriptional activity¹². In addition, it was shown that insect NUP93 is needed for nuclear import of active SMADs²⁶. Hence, we assumed that NUP93 overexpression enhances signals initiated by BMP7/TGF-beta and other stimuli. Probing endogenous importin7 and NUP93 revealed a perinuclear ring of importin7, similar to the pattern of endogenous NUP93 (Fig. 4A). Importantly, importin7's perinuclear localization was lost in mammary cells pre-treated with siNUP93. To validate the prediction that NUP93, by recruiting specific importins, mediates TGF-beta induced nuclear translocation of SMADs, we depleted NUP93 in MCF10A cells and followed the kinetics of nuclear import (Fig. 4B). While SMAD2/3 translocated to the nucleus of control cells within 20 minutes, we observed only limited translocation in NUP93-depleted cells. Assuming that NUP93 controls translocation of additional importin7's cargos, we examined the extracellular signal-regulated kinase (ERK). Upon stimulation with EGF, ERK undergoes phosphorylation that exposes a nuclear translocation signal (NTS), which facilitates binding to importin7²⁷. Accordingly, when we stimulated control cells with EGF the phosphorylated form of ERK (pERK) clearly translocated to the nucleus, but cells depleted of NUP93 displayed much weaker translocation (Fig. 4C). This difference was verified by fractionation of cell extracts into cytoplasmic and nuclear fractions (Fig. 4D). Consistent with these results, we observed reciprocal changes in MCF10A cells engineered to inducibly overexpress NUP93 (Fig. 4E).

In similarity to ERK, STAT3 undergoes phosphorylation and nuclear translocation upon stimulation with EGF, but alpha importins, rather than importin7, have been implicated^{28,29}. Nevertheless, analyses of both total STAT3 (Fig. S4A) and pSTAT3 (Fig. S4B) indicated that NUP93 can regulate EGF-induced import of STAT3. These observations raised the possibility that NUP93 anchors different importins and translocates them via the NPC, along with the respective cargos. Hence, we followed two additional cargos, the glucocorticoid receptor (GR) and the p105 subunit of the nuclear factor kappa B (NF-κB; Figs.

S4C and S4D). Using NUP93-depleted cells, in both cases we observed strong inhibition of ligand-induced translocation following stimulation with either dexamethasone (DEX), a synthetic GR ligand, or with the tumor necrosis alpha (TNF α). Notably, importin α /beta, as well as importin7, translocate GR³⁰, whereas NF- κ B is translocated by importin α 3 and α 4³¹. In conclusion, apart from the known functions of NUP93 as a scaffold nucleoporin involved in NPC assembly, this molecule emerges as a broad-spectrum transporter of the active forms of signaling proteins.

Myristoylated peptides corresponding to the NTS of ERK and SMAD inhibit progression of NUP93-overexpressing tumors

Next, we attempted blocking nuclear translocation of ERK by preventing binding of importin7 to ERK's NTS. The strategy used a previously described NTS-derived peptide fused to myristic acid³². As expected, the ERK-derived peptide inhibited nuclear accumulation of ERK, but a control peptide exerted no effect (Fig. 5A). In addition, the peptide reduced incorporation of radioactive thymidine into DNA, only in cells overexpressing NUP93 (Figs. S5A and S5B). Likewise, the S-phase fraction observed with cells treated with the peptide was severely reduced (Fig. S5C). Similarly, the ERK peptide reduced the ability of cells to form colonies, migrate and invade (Figs. S5D-S5F). Hence, we implanted NUP93-overexpressing cells in the subaxillary mammary fat pad of mice, and once tumors became palpable intravenously treated animals with the ERK-derived peptide. RFP-fluorescence and size of the primary tumors were measured (Figs. 5C and 5D), along with tumor weight (Fig. 5E). In addition, we cut out the lungs to assay metastases (Fig. 5F). Evidently, tumors overexpressing NUP93 grew faster and colonized lungs better than the control tumors. Moreover, while the ERK peptide weakly inhibited growth and metastasis of control tumors, the inhibitory effects observed upon treatment with the ERK peptide were significantly stronger, implying that NUP93-overexpressing tumors acquire dependence on ERK's nuclear transport.

Because NUP93 mutations causing a renal disease fail SMAD signaling¹² and tumors frequently corrupt the TGF-beta pathway³³, we synthesized a similar SMAD peptide, based on a previously identified Ser-Pro-Ser triad²⁷. The peptide nearly completely inhibited TGF-beta-induced nuclear import of SMAD2/3 (Fig. 5B). In addition, it reduced incorporation of radioactive thymidine (Fig. S5B), markedly lowered the S-phase fraction (Fig. S5C), as well as inhibited the ability of cells to form colonies, migrate and invade (Figs. S5D-S5F). As with the ERK peptide, we implanted NUP93-overexpressing cells in the fat pad of mice and delivered the SMAD peptide three times per week. RFP-fluorescence, as well as tumor volumes (Figs. S5G and S5H) and weights were determined (Fig. S5I) and lungs metastases were counted (Fig. S5J). We observed weak inhibition of control tumors, but stronger effects were observed when treating NUP93-overexpressing tumors. Moreover, treatment with the SMAD peptide only weakly inhibited metastasis of control tumors, but the numbers of micro-metastases formed by NUP93-overexpressing cells were reduced by 75–85% (Fig. S5J). In conclusion, the ERK and SMAD peptides exemplify the potential therapeutic scenario offered by targeting the cargo-importin-NUP93 axis, specifically in NUP93-overexpressing breast cancers. Importantly, it has recently been reported that inhibition of NPC formation causes selective cancer cell death, while normal cells undergo a reversible cell cycle arrest³⁴. In

conclusion, the myristoylated peptides we tested might effectively and selectively inhibit cancer cells overexpressing NUP93.

Natural gain- and loss-of-function mutations confirm NUP93's roles in metastasis

Three NUP93 mutations, E14K, Q15X and R327C, were identified by a screen aimed at putative driver mutations escalating the risk of metastasis¹³. Hence, we stably expressed two mutant alleles in MCF10A (Fig. S6A) and in MDA-MB-231 cells (Fig. 6A). Both mutants, especially R327C, superseded the ability of wildtype NUP93 to seed colonies (Figs. 6B and S6B). In addition, we performed DNA synthesis (Figs. 6C and S6C), migration and invasion assays (Fig. 6D, 6E, S6D and S6E), which indicated that the mutants were more active than the wildtype form. Next, we implanted the respective MDA-MB-231 cells in the fat pad of animals and followed tumor growth (Figs. 6F and 6G). Both mutations enhanced tumor growth relative to WT and control (EV) cells. Similarly, quantification of metastatic lung nodules indicated that both mutant alleles were consistently more active than WT in either metastasis format (Fig. S6F). Notably, we occasionally observed metastases in other organs. Focusing on liver metastases, we observed striking differences (Fig. 6H): Whereas cells overexpressing either mutant colonized livers, we were unable to detect any lesion in livers from mice injected with WT cells. In conclusion, the gain-of-function NUP93 mutations enhance oncogenic attributes and confer organ-specific metastatic colonization.

Analyses of patients with nephrotic syndromes detected homozygous missense NUP93 mutations¹². To study oncogenic effects, we firstly disrupted the endogenous *NUP93* gene using Crispr-CAS9. Several knockout (KO) clones were established (Fig. 6I). Next, we generated a series of KO cells expressing WT or individual mutants (Fig. S6G). As expected, all KO sublines exhibited reduced proliferation DNA synthesis (Figs. S6H and S6I), as well as relatively low rates of migration/invasion, and high adhesion (Figs. S6J-S6L). Unlike WT, neither mutant recovered normal migration/invasion rates when expressed in KO cells (Figs. S6K and S6L). In vivo tests of tumor growth (Figs. 6J and 6K) and metastasis (Figs. 6L and S6M) supported the LOF phenotypes: stable expression of neither mutant was able to reconstitute the relatively high rates of tumor growth/metastasis displayed by KO cells re-expressing WT. In summary, by studying GOF and LOF mutations of NUP93 we obtained independent evidence in support of the critical roles played by NUP93 in breast cancer progression.

NUP93 overexpression increases GTP-loading onto RHO GTPases and enhances transcriptional outcome of the WNT and other signaling pathways

RHO family GTPases are switches involved in mammary tumorigenesis and metastasis³⁵. Hence, we utilized the ability of effectors to bind with the active, GTP-bound forms. Overexpression of NUP93 increased CDC42-GTP and RAC1-GTP, but decreased RHOA-GTP (Fig. S7A). Congruently, siNUP93 reduced active RAC1 and CDC42, but increased RHOA-GTP. Notably, RAC1 and CDC42 collaborate with KRAS. Accordingly, we observed significant downregulation of RAS-GTP in siNUP93-treated cells (Fig. S7B). Because RAS is activated upon stimulation of numerous pathways, we undertook a multiple

promoter-reporter strategy. HEK293 cells were co-transfected with a NUP93 expression vector and various luciferase plasmids containing different DNA response elements (REs), including the glucocorticoid RE (GRE; Fig. 7A). This revealed that NUP93 overexpression activated several promoters, including GRE, SMAD4 and FLI1. Yet, the strongest signal (> 50-fold) was observed with, a beta-catenin responsive reporter containing the binding site for T cell factor (TCF)/lymphoid enhancer factor (LEF). Nuclear accumulation of beta-catenin is induced by WNT; once in the nucleus, beta-catenin associates with TCF/LEF and activates target genes³⁶. As expected, co-transfection of the reporters and siNUP93 decreased several signals, but two collagen promoters were activated (Fig. S7C). In addition, co-transfecting NUP93's oncogenic mutants increased reporter activity beyond the WT signal (Fig. 7B). Accordingly, transfection of NUP93, either WT or mutants, strongly elevated the beta-catenin protein (Fig. 7C). This translated to increased expression of multiple beta-catenin target genes (three are shown in Fig. S7D). Because EGFR can transactivate the beta-catenin pathway³⁷ and SMAD3 can occupy WNT-responsive elements³⁸, we examined transactivation by EGF and TGF-beta. The results confirmed transactivation (Fig. 7D), which might enhance the relatively large effect of WNT. Next, we addressed stability of beta-catenin. Cell treatment with cycloheximide, a protein synthesis inhibitor, followed by immunoblotting, confirmed short half-life of beta-catenin (Fig. 7E). In contrast, when overexpressed, both WT NUP93 and the oncogenic mutants strongly prolonged the half-life, implying multiple NUP93/beta-catenin interactions.

Using WNT3A, we found that R327C-NUP93, more than WT, enhanced ligand-stimulated promoter activation (Fig. 7F). To test if this cooperative effect was due to nuclear transport, we firstly fractionated control cells and detected only a small fraction of beta-catenin in the nucleus (Fig. S7E). Importantly, this fraction was erased by NUP93 knockdown. Moreover, immunofluorescence indicated that treatment with WNT3A induced relatively weak nuclear translocation of beta-catenin, which was enhanced by wildtype NUP93, and further increases were observed in cells expressing oncogenic NUP93 (Fig. 7G). Thus, both fractionation and immunofluorescence supported the cooperative effect of WNT and NUP93. To validate functionality, we knocked-down LEF1 and stimulated cells with WNT3A (Figs. 7H and 7I). While siLEF1 inhibited cell migration/invasion by approximately 30% in WT-expressing cells, this increased to 90% in cells expressing R327C-NUP93, in support of cooperativity. In conclusion, when overexpressed, NUP93 enhances loading of GTP onto RAS, RAC1 and CDC42, molecular switches of cell migration. In line with this, several signaling pathways were found to be constitutively active in NUP93-overexpressing cells, including the WNT pathway, hyperactivation of which has been implicated in metastasis³⁹. WT NUP93 elevated expression and inhibited degradation of beta-catenin, while the oncogenic mutants, better than WT, enhanced WNT-induced nuclear transport of beta-catenin.

Proteome- and transcriptome-wide analyses identify podocalyxin and multiple RNA transcripts as potential mediators of NUP93-induced metastasis

To complement the promoter analyses, we applied liquid chromatography-mass spectrometry (LC-MS/MS) on cytoplasmic and nuclear fractions. As expected, siNUP93 significantly reduced beta-catenin in the nucleus (Figs. 7J and S7F; Supplementary Excel File 2). Alongside, the cytoplasmic fraction

showed downregulation of specific MAPK pathways, whereas the nuclear fraction displayed elevated SP1, which up-regulates collagen⁴⁰, bystin, which regulates cell adhesion⁴¹, and cullin-3, which is essential for collagen export⁴². All three proteins were up-regulated in NUP93-depleted cells and underwent nuclear-cytoplasm translocations (Figs. S7G and S7H). The proteomic analyses also indicated that the mucin podocalyxin (PODXL) underwent up-regulation in the cytoplasm of siNUP93-treated cells. We confirmed elevated PODXL levels in NUP93-deficient cells, as well as downregulation upon re-expression of wildtype NUP93 (Figs. 7K and S7I). Notably, neither G591V- nor Y629C-NUP93 were able to downregulate PODXL. In addition, downregulating PODXL, in similarity to knocking-down cullin3 and bystin, decreased cell adhesion (Fig. 7L), implying involvement of all three proteins in NUP93-mediated weakening of mammary cell adhesion.

Along with transport of proteins, the NPC mediates selective exchange of RNA molecules between the nucleus and cytoplasm⁴³. To explore transport of specific RNAs, we depleted NUP93 in MCF7 breast cancer cells and examined the subcellular localization of polyadenylated transcripts (Fig. S7I), essentially as previously described⁴⁴. NUP93 depletion significantly affected the localization of hundreds of RNAs, led to a 3-fold increase in cytoplasmic enrichment of transcripts from 200 genes, and conversely, to 3-fold increase in nuclear enrichment of transcripts from 153 other genes (see Supplementary Excel File 3). Because the lincRNA called *NORAD* (LINC00657) was identified also by the RNAseq analysis presented in Fig. 3C, we focused on this molecule. Notably, *NORAD* enhances TGF-beta signaling and metastasis⁴⁵. Hence, we employed single-molecule FISH (smFISH) to examine sub-cellular distribution of this intronless, mostly cytoplasmic lncRNA. In line with the RNA-seq analysis, upon NUP93 depletion *NORAD* exhibited strong nuclear enrichment in both MCF7 and MDA-MB-231 cells (Fig. S7J). Conceivably, nuclear retention inactivates *NORAD*, along with similar RNAs, to retard metastasis of NUP93-low breast cancers.

In summary, by integrating clinical and laboratory lines of evidence, we concluded that high abundance of NUP93 is associated with highly aggressive breast cancers. Correspondingly, the overexpressed NUP93 enhanced TEM and matrix invasion, while reorganizing the matrisome. These attributes translated to increased rates of tumor growth and metastasis in animal models. Mechanistically, overexpression of NUP93 boosts the ultimate nuclear transport step activated by diverse extracellular signals, including WNT, EGF and TGF-beta. Collectively, these lines of evidence identify NUP93 as a driver of metastasis, which hijacks the beta-catenin and other signaling pathways.

Discussion

The results presented herein identify NUP93 as a potent driver of metastasis in aggressive subtypes of breast cancer. Unlike other subtypes, the group overexpressing HER2, a functional partner of EGFR, is driven by signals mimicking cellular stimulation by growth factors, whereas a fraction of the basal subtype is propelled either by an overexpressed EGFR, or by mutations⁴⁶. In analogy, our results indicate that the aberrantly overexpressed NUP93 simultaneously amplifies multiple growth factor signals by means of activating the ultimate nuclear transport step shared by the majority of signal transduction

pathways. This entails nuclear translocation of transcription co-activators, such as STAT, SMAD, ERK and beta-catenin. Consistent with this scenario, two lines of computational evidence identified NUP93 as a putative driver of breast cancer progression: the first analyzed recurrent CNAs ⁴⁷, while the other employed addressed driver mutations ¹³. Three rare NUP93 point mutations were identified by this study, and they were characterized in vitro. The animal studies we performed assigned metastasis driver roles to two of the three mutant forms, as well as uncovered their ability to enhance basal and ligand-induced nuclear translocations of beta-catenin, a well-characterized driver of metastasis ⁴⁸.

The analyses we performed offer the following model: cargo-loaded importin-7 and additional importins dock at NUP93 of nuclear pores, thus permitting accessibility to specific DNA response elements. Because the tumor microenvironment of mammary cancers constantly supplies growth factors, cells overexpressing NUP93 acquire enhanced responses to nearby stromal cells, such as tumor-associated fibroblasts. For these reasons, cancer cells overexpressing NUP93 might become highly metastatic. This model can explain how SMAD proteins escorted by importin-7/8 enhance TGF-beta signaling ⁴⁹, as well as how signals mediated by GR and ERK, two cargos of importin-7 ^{30,32}, are augmented when NUP93 is overexpressed. However, nuclear translocations of proteins like beta-catenin, STAT3 and NF-κB likely involve importins other than importin-7. For example, a recent genome-wide screen identified importin-11 as a factor required for beta-catenin transport ⁵⁰. Regardless of the exact mechanisms enabling NUP93 to control beta-catenin import, our promoter-reporter assays indicated that NUP93 more strongly regulates the beta-catenin pathway, as compared to other pathways. This superiority of the LEF1/TCF promoter might be due to two mechanisms, which are not mutually exclusive. The first involves the recently discovered ability of NUP93 to drive expression of cell identity and other genes through interactions with super-enhancers ^{20,51}. The other mechanism might relate to the rich crosstalk between the WNT pathway and other signaling routes. For example, SMAD3 colocalizes with LEF1 at WNT-responsive elements ³⁸, and EGFR can transactivate the beta-catenin pathway ³⁷.

In summary, the integrative computational analysis we developed has assigned NUP93 with important roles in progression of mammary cancer, which we confirmed in animals. Surprisingly, NUP93 emerges from these studies as a master driver of metastasis. This conclusion was supported by functional characterization of two groups of naturally occurring mutant alleles. Remarkably, mammary cells overexpressing NUP93 display over-activation of multiple growth factor pathways, including the WNT/beta-catenin pathway. This route has previously been associated with colorectal tumors, but recent network-based analysis linked it to breast cancer ⁵². We conclude that NUP93 integrates and enhances not only WNT but also additional growth factor signals. Thus, by seizing an essential step common to multiple signaling routes, NUP93 acquires robust oncogenic attributes. Beyond the identification of augmented nuclear transport as a new hallmark of cancer, our study exposes tumor vulnerabilities and offers not only a new target for intervention but also a pharmacological strategy tailored to the subset of NUP93 overexpressors.

Methods

Cell lines

HEK-293T cells were cultured in DMEM medium supplemented by fetal bovine serum (10%; FBS). Wnt-3A producing L cells or control L cells were cultured according to ATCC manual. MCF10A cells were grown in Dulbecco's modified Eagle's medium/F12 (1:1) supplemented with antibiotics, insulin (0.01 mg/ml), cholera toxin (0.1 mg/ml), hydrocortisone (0.5 mg/ml), heat-inactivated horse serum (5%, v/v), and EGF (10 ng/ml). Human mammary MDA-MB-231 and MCF7 cells were grown in RPMI-1640 (Gibco BRL) supplemented with 10% heat inactivated fetal calf serum (Gibco), 1mM sodium pyruvate, and a penicillin streptomycin mixture (100 U/ml; 0.1 mg/ml). HUVEC cells were cultured in EGM as per the optimized protocol of Lonza. Stable MDA-MB-231-RFP-LUC clones were generated using electroporation using the NEPA gene electroporator.

Lentivirus construct and RNA interference

Nontargeted shRNAs (control) and shRNAs directed against human NUP93 were produced in human embryonic kidney 293T cells following the manufacturer's guidelines (GE Healthcare). Target cells were infected with shRNA-encoding lentiviruses supplemented with polybrene (8 mg/ml) and cultured in the presence of puromycin (2 mg/ml) for 4 days. Stable gene expression was achieved using the ViraPower lentiviral expression system (Invitrogen) following the manufacturer's guidelines. siRNA oligonucleotides were purchased from Dharmacon. For knockdown experiments, cells were seeded in 6-well plates at 50% confluence, and 24 h later, they were transfected with siRNA SMART pool using the Lipofectamine 2000 transfection reagent (Life Technologies).

Immunoblotting analysis

Cells were washed briefly with ice-cold saline and scraped in a buffered detergent solution [25 mM Hepes (pH 7.5), 150 mM NaCl, 0.5% Na deoxycholate, 1% NP-40, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4 , and a protease inhibitor cocktail diluted at 1:1000]. For equal gel loading, protein concentrations were determined by using the bicinchoninic acid (Pierce) reagent. After gel electrophoresis, proteins were transferred onto a nitrocellulose membrane. The membrane was blocked in TBST buffer [0.02M Tris-HCl (pH 7.5), 0.15M NaCl, and 0.05% Tween 20] containing 3% albumin, blotted with a primary antibody (at 4°C), washed with TBST, and incubated for 30 min with a secondary antibody conjugated to horseradish peroxidase.

Nuclear and cytoplasmic fractionation

Cell pellets were lysed in 0.1 ml cytoplasmic lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT and 0.5% NP-40). The cytoplasmic fraction was collected using centrifugation (600Xg for 5 minutes). Nuclei were washed and resuspended in nuclear lysis buffer (50 ml; 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EGTA, 1 mM EDTA, 1mM DTT) by repeated freezing and thawing. Supernatants containing the nuclear fraction were collected by centrifugation at 12,000 rpm for 20 minutes.

Thymidine incorporation assays

Cells were plated onto 24-well plates as indicated. Sixteen hours later, the medium was replaced with fresh medium containing ^3H -thymidine (1 μCi). At the indicated time points, the reaction was terminated by the addition of ice-cold trichloroacetic acid (5%; TCA). Five minutes later, cells were solubilized at 37°C with NaOH (1N; for 10 minutes) followed HCL (1N). Samples were collected into scintillation vials. Radioactivity was determined in a scintillation counter.

Cell cycle analysis and Image Stream analyses

Cells were incubated for 60 minutes with bromodeoxyuridine (BrdU; 10 μM) and then washed, harvested and fixed in ethanol (at 4°C). Thereafter, cells were incubated in a denaturation solution (2N HCl, 0.5% Triton-X100; 30 min), followed by a neutralization solution (0.1 M sodium borate, pH 8.5; 30 min). BrdU incorporated into newly synthesized DNA was assayed using an APC-conjugated anti-BrdU antibody. Total DNA content was determined using a propidium iodide solution supplemented with RNase A. Cell cycle distribution was detected by flow cytometry. Further analysis was performed using the FlowJo software v10.2 (Tree Star). Similarly, cells were analyzed for morphology and cell size using the Imaging Flow Cytometer ImageStreamX mark II (Amnis-EMD Millipore). Image were analyzed using the IDEAS 6.1 software (Amnis).

Determination of receptor expression levels

To evaluate surface receptor levels, cells were detached and washed twice in saline containing albumin (1%, w/v). Thereafter, cells were incubated for 30 min at 4°C using primary antibodies conjugated to fluorophores. Fluorescence intensity was measured using BD FACSAria Fusion flow cytometer.

Cell proliferation assays

Proliferation of cells was assessed using MTT or XTT. Briefly, cells were seeded in triplicates in 96-well plates and treated for the indicated time intervals. Subsequently, MTT was added and following 3 hours at 37°C, we dissolved in DMSO the water insoluble formazan crystals, which form in metabolically active cells. Optical density was measured at 570 nm. The XTT-based assays followed the manufacturer instructions.

Cell migration, invasion, and 3D assays

Cells were plated in the upper compartments of a Transwell tray (BD Biosciences) and allowed to migrate through the intervening membrane. Thereafter, cells were fixed in paraformaldehyde (3%), permeabilized in Triton X-100 (0.05%) and stained with crystal violet (0.02%). Cells on the upper side of the filter were removed and migrating cells were photographed. Invasion assays were performed using BioCoat Matrigel chambers. 3D spheroid cell invasion kits were from Trivigen. For trans-endothelial migration (TEM) assays, human umbilical vein endothelial cells (HUVEC) were grown to confluency on cell culture inserts, tumor cells were added to the endothelial layer and left to transmigrate.

Clonogenic growth assay and adhesion assays

Cells were seeded in 6-well plates at a density of 1,000 cells per well. Appropriate treatments were given for the indicated time points, with medium change and fresh drug added every 2 days. Cells were fixed with 4% formaldehyde and stained with 0.5% crystal violet. Photos of stained cells were taken using an EPSON Perfection V600 scanner. Growth was quantified by dissolving crystal violet in 0.1% SDS and absorbance was quantified at 590 nm using a spectrophotometer. Signals were normalized to DMSO treatment. For adhesion tests, plates were coated overnight with Cultrex RGF BME (R&D Systems) or fibronectin and gently washed thereafter (0.1% albumin in medium). Cells were allowed to adhere to the substrate for the indicated time points at 37°C. Unattached cells were removed and adherent cells were rinsed, fixed with paraformaldehyde (4%), and quantified after crystal violet staining (0.1%). The optical density was measured at 550 nm.

Immunofluorescence analyses

Cells were grown on sterile 18X18mm coverslips. Firstly, cells were washed in saline containing (or not) Tween 20 (0.01%; w/v). Thereafter, they were fixed in 4% formaldehyde. Fixed cells were incubated for 15 min in saline containing 0.1 M glycine, followed by 30 min incubation in blocking buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 10% goat serum, 2% albumin in Tris-buffered saline, and 0.2% Triton X-100) and then incubated for 2 h with the indicated primary antibodies or overnight incubation at cold, diluted in the blocking buffer. Later the cells were washed with PBS and incubated with fluorescence-labelled secondary antibodies for 45min. Subsequently the cells were washed with PBS and incubated for 5 min with 2 ng/μl Hoechst 33342 or DAPI, washed again with PBS and mounted on microscopic slides using an anti-quenching mounting media (Dako). The specimens were analyzed using a confocal laser-scanning microscope (LSM 800; Zeiss) equipped with a 63 Å/1.4 oil differential interference contrast M27 objective lens (Plan Apochromat; Carl Zeiss) using the 488-, 543- and either 405- or 633- nm excitation for fluorescein, Cy3 epifluorescence and either 4,6-diamidino-2- phenylindole (Hoechst) or Cy5, respectively. Images were acquired using the LSM 800 software.

Peptides

Peptides were designed as mentioned before³². The following sequences were used: ERK Scramble, GNILSQELPHSGDLQIGL; ERK, GQLNHILGILGEPEQEDL; SMAD Scramble, MQSTPSGRVVISKCL; SMAD, KVLTMGSPSIRCSS. All peptides were purchased from GnenScript Ltd (Hong Kong).

Immunohistochemistry and Picrosirius red staining

Formalin-fixed tumor sections were de-paraffinized and rehydrated. Antigen retrieval was performed in a microwave oven using a citric acid solution (pH 9.0). Slides were blocked in saline containing 20% horse serum, followed by treatment (15 minutes) with a blocking solution, and an overnight incubation with the primary antibody. Thereafter, sections were incubated for 90 minutes with a biotinylated secondary antibody, followed by a Cy3-conjugated Streptavidin. DAPI was used to visualize nuclear. All slides were

examined using a fluorescence microscope. Positive cells were counted using the Image Pro Plus software. For Picrosirius red staining, sections from tumor tissues were stained with 0.1% Picrosirius red and quantification was done as above.

Invadopodia assays

Gelatin was labeled using Alexa Fluor 488 Protein Labeling Kit (Molecular Probes, Thermo Fisher Scientific). Glass-bottomed well plates were coated as mentioned above, with ratios of 1:10 labeled gelatin:nonlabeled gelatin. Cells were plated on the gelatin matrix and cultured for varying lengths of time (on average, 5–6 hours). Next, cells were fixed and stained for actin and DAPI, and degraded areas were assessed. Cells in every field of view were counted and the degradation area was calculated by the fluorescently labeled gelatin channel, using Analyze Particles plug-in (ImageJ software). Total degradation area/cell (μm^2) was used to assess invadopodia.

Rho GTPase activation assays

G-LISA Small GTPase activation assays were performed by following the protocol in the activation assay kit (from Cytoskeleton).

ELISA tests

Human VEGF DuoSet, TGFB1-2 DuoSet and Human TGF-beta RII DuoSet ELISA's were purchased from R&D Systems and assays were carried out as per the manufacturer's instructions.

Luciferase-reporter assays

Assays was performed as previously described⁵³. Briefly, cells were co-transfected with a luciferase reporter plasmid, along with control plasmids (Promega, Madison, WI). Luciferase activity was determined using the dual-luciferase reporter assay system (Promega). Firefly luciferase luminescence values were normalized to Renilla luminescence.

RNA Isolation, real-time PCR analysis and RNA sequencing

The TRIzol reagent (Life Technologies) and the PerfectPure RNA Cultured Cells kit (5 Prime) were used for RNA purification and real-time quantitative PCR. Generation of cDNA was performed using either the qScript cDNA Synthesis kit (Quanta), High-capacity cDNA Reverse Transcription kit (Applied Biosystems), or RevertAid Reverse Transcriptase (Thermo Scientific). Real-time qPCR analysis was performed using Fast SYBR Green Master Mix (Applied Biosystems). Primers were designed using PrimerBlast. Transcripts encoding beta-2 microglobulin (B2M) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used for normalization. Illumina HiSeq 2500v4 was used for RNA sequencing (~40 million reads per sample). Poly-A/T stretches and Illumina adapters were trimmed from the reads using Cutadapt. Reads were mapped to the Homo Sapiens GRCm38 reference genome using STAR⁵⁴. Expression levels were quantified using htseq-count⁵⁵. Differentially expressed genes were identified using DESeq2⁵⁶ while

employing betaPrior, cooksCutoff and independent Filtering parameters set to False. Raw P values were adjusted for multiple testing using the procedure of Benjamini and Hochberg ⁵⁷.

Extraction and sequencing of cytoplasmic and nuclear RNA

RNA extraction and analysis were performed as previously described ⁴⁴. Briefly, Cells were washed in cold PBS and detached from plates using 10mM EDTA. A fraction was transferred to a new tube and RNA was extracted with TRIAGENT (MRC). Remaining cells were washed in cold PBS, resuspended in RLN buffer (50mM Tris-HCl pH8, 140mM NaCl, 1.5mM MgCl₂, 10mM EDTA, 1mM DTT, 0.5% NP-40, 10U/ml RNase inhibitor), and incubated on ice for 5 min. The extract was centrifuged for 5 min at 300Xg in a cold centrifuge, the supernatant was transferred to a new tube and centrifuged again for 1 min at 500g in a cold centrifuge. The supernatant (cytoplasmic fraction) was transferred to a new tube and RNA was extracted using TRIAGENT. The nuclear pellet was washed once in RLN buffer, resuspended in 1ml of buffer S1 (250mM Sucrose, 10mM MgCl₂, 10U/ml RNase inhibitor), layered over 3ml of buffer S3 (880mM sucrose, 0.5mM MgCl₂, 10U/ml RNase inhibitor), and centrifuged for 10 min at 2800Xg in a cold centrifuge. The supernatant was removed and RNA was extracted from the nuclear pellet using TRIAGENT. Fractionation quality was validated by qRT-PCR using primers for ACTB and MALAT1, which are expected to be enriched in the cytosolic and the nuclear fractions, respectively. WCE, cytosolic and nuclear fractions obtained after fractionation (from two biological replicates) were used to generate cDNA libraries using the SENSE mRNA-Seq Library Preparation kit (Lexogen) according to manufacturer's protocol and sequenced on a NextSeq 500 machine to obtain 75 nt single-end reads. RNA-seq reads were mapped to the human genome (hg19 assembly) with STAR and gene expression levels were quantified using Bowtie2 and RSEM ⁵⁸. GENCODE v26 annotations were used for further analysis. Differential expression in WCE samples was computed using DESeq2 with default parameters. Subcellular localization was quantified similarly using DESeq2, using gene-level RSEM output.

Single Molecule FISH

The protocol and probes we used have previously been described ⁴⁴. Probe libraries were designed according to Stellaris guidelines and synthesized by Stellaris (Stellaris RNA FISH probes, Biosearch Technologies). Libraries targeting NORAD consisted of 96 probes, labeled by Quasar® 570. Hybridizations were done overnight at 30°C with probes at a final concentration of 0.1 ng/μl. For nuclear staining, 1.25 μg/ml Hoechst 33342 (H3570, Thermo Fisher) was added during the washes. Images were taken with a Nikon Eclipse Ti2-E inverted fluorescence microscope equipped with an oil-immersion objective (X100) and an iXon 888 EMCCD camera using NIS-Elements Advanced Research software. The image-plane pixel dimension was 0.13 μm and distance between Z stacks was 0.3 μm. Quantification was done using FishQuant ⁵⁹. We performed automatic 2D projections as suggested in FishQuant documentation, followed by automatic cell segmentation using CellProfiler ⁶⁰. Hoechst signal was used to segment nuclei and the oligo-dT signal was used to segment cell bodies. Following batch analysis, we manually examined segmentation and removed incorrectly segmented cells from further analysis using

Fiji (ImageJ) software. Quantification of cytoplasmic and nuclear signals was performed with default parameters and recommended filters of FishQuant.

In vitro knockout of the gene encoding for NUP93

The CRISPR system was used as described ⁶¹ to create a double-stranded break next to the ProtospacerAdjacent Motif (PAM) sequence. The target site was selected from the ENSEMBL database in a way that targeted the transcript of NUP93. The selected target was 21bp long, including the PAM sequence in exon 5, which was filtered to minimize off-target cross-reactivity. The sequence was cloned in pX458 and cells were sorted using cytometry.

Proteomic analysis

Proteins were digested and purified on carboxylated beads according to the SP3 protocol ⁶². Peptides were analyzed by liquid-chromatography using the EASY-nLC1000 HPLC coupled to high-resolution mass spectrometric analysis on the Q-Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were separated on 50cm EASY-spray columns (Thermo Fisher Scientific) with a single run of 140 min. MS acquisition was performed in a data-dependent mode with selection of the top 10 peptides from each MS spectrum for fragmentation and MS/MS analysis. Raw MS files were analyzed in the MaxQuant software and the Andromeda search engine ⁶³. A database search was performed using the Uniprot database and included carbamidomethyl-cysteine as a fixed modification, as well as N-terminal acetylation and methionine oxidation as variable modifications. A reverse decoy database was used to determine false discovery rate of 1% at the peptide and protein levels. The label-free algorithm (LFQ) in MaxQuant was used to retrieve the quantitative information. Nuclear and cytosolic proteins were quantified separately.

Animal studies

All in vivo studies were reviewed and approved by the Weizmann Institute's Animal Care and Use Committee (IACUC). Six week old NOG/NSG female mice (Jackson Laboratories; USA), 6 per group, were used in the study. MDA-MB-231-RFP-LUC cells were implanted in the fat pad or tail vein. Tumor sizes were calculated according to the following formula: $(V = (4\pi/3) \times (a/2)^2 \times (b/2))$. Metastases in different organs were visualized using a fluorescent binocular microscope or IVIS (Xenogen Corp., Alameda, CA, USA).

Statistical data analysis

All data were analyzed using the Prism GraphPad software (V8) and statistical analyses were performed using one or two-way ANOVA with the Dunnett's or Tukey's test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$). All the mass-spectrometry statistical analyses were performed using the Perseus software ⁶⁴. The data was filtered to include proteins with valid values in at least 75% of the samples. Missing values were then imputed to represent low abundance proteins by replacing them with random, low intensity

values that form a normal distribution. Student t-test was performed (permutation-based FDR 0.05) to distinguish the significantly regulated proteins between the control and the NUP93 knockdown samples. The statistical analysis of each assay appears in the relevant figure legend. All experiments were carried out in triplicates, unless specified otherwise.

Declarations

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Author contributions

NBN, AN and YY designed experiments and wrote the manuscript. NBN, AN, SG, HRMR, AS, BZ, ML, SS, MS, IL performed experiments and analyzed the results, JSL, DD-G, OR, performed bioinformatic analyses, CC, SL, TG, AC, IU, RS and ER supervised research, analyzed results and reviewed the manuscript.

Competing interests statement

All authors declare that they have no conflicts of interest relevant to the work described herein.

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Supplementary Information

Figure S1: NUP93 overexpression (or knockdown) promotes (or inhibits) cell proliferation, migration, and invasion of mammary cells. NUP93 was overexpressed (A) or (B) knocked-down in MDA-MB-231 cells (EV, empty vector). For knockdown, we used either shRNAs (right panel) or siRNA (two oligonucleotides; left panel). Cells were subjected to immunoblot analysis using the indicated antibodies. (C) MCF10A cells overexpressing NUP93, either inducibly (left panel) or constitutively (right panel), were selected using blasticidin (10 µg/ml) or puromycin (2 µg/ml). Inducible cells were treated with Tet (2 µg/ml) for 24 h or 48 h. Cells were then subjected to immunoblot analysis. (D) NUP93 of MCF10A cells was depleted using shRNA (left panel) or siRNA (right panel) and cell extracts were subjected to immunoblot analysis. (E and F) The XTT assay was used to measure proliferation of MDA-MB-231 cells in which NUP93 was either overexpressed (E) or depleted (F). Note that panel E corresponds to A and panel F to B. Data represent means ± SEM. (G and H) The XTT assay was used to measure proliferation of MCF10A cells in which NUP93 was either overexpressed (G) or depleted (H). Note that panel G corresponds to C and panel D to H. Data represent means ± SEM. (I and J) MDA-MB-231 cells in which NUP93 was either overexpressed (left panel; see A) or depleted (middle and right panels; see B) were subjected to Transwell migration (I) or Matrigel invasion (J) assays. Significance was assessed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. Values represent means ± SEM. (K–N) MCF10A cells in which NUP93 was either overexpressed (K and M; see C) or depleted (L and N; see D) were subjected to Transwell migration (K and L) or Matrigel invasion (M and N) assays. Significance was assessed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. Values represent means ± SEM.

Figure S2: Overexpression of NUP93 increases trans-endothelial migration and 3D invasion, while knock-down up-regulates paxillin and other cell adhesion molecules. (A) MDA-MB-231 cells (3×10^3) overexpressing an inducible NUP93 were plated in 96-well round-bottom plates filled with Cultrex 10X Spheroid Formation Extracellular Matrix (ECM), a mixture of ECM proteins derived from murine EHS sarcoma. Once spheroids formed, we added an invasion matrix (from R&D Systems), which was overlaid, 60 minutes later, with warm medium containing Tet. The plates were incubated for 6 days. Photographs were taken and the areas covered by invasive structures were measured using ImageJ. Bar, 0.1 mm. (B) MDA-MB-231 cells expressing a DOX-inducible shRNA for NUP93 were cultured and invasion zones quantified as in A. Displayed are the average areas ± SEM. (C) HUVEC cells (5×10^4) were plated onto fibronectin-coated FluoroBlok inserts (from Corning). After 16 h, the HUVEC monolayer was overlaid by MDA-MB-231-RFP cells (4×10^4) expressing DOX-inducible shNUP93 (clones 4, 5 and 6). Cells were

allowed to transmigrate for 20 h. Trans-endothelial migrated and invaded cells were imaged using a bottom reading fluorescence plate reader (Cytation 5, Bio Tek™). The histograms show averages $\pm$ SEM. (D) Serum-free media were conditioned over a period of 48 hours by control MDA-MB-231 cells or by siNUP93-treated cells. ELISA kits (DuoSet, from R&D Systems) and internal references were used to determine actual concentrations of VEGF. Averages $\pm$ SEM are presented. (E) MDA-MB-231 cells (5×10^5) constitutively expressing control shRNA (SC) or two anti-NUP93 shRNAs were analyzed using Image stream. The histogram shows average cell areas $\pm$ SEM of triplicates (μm^2). (F) MDA-MB-231 cells were transfected with siNUP93 oligonucleotides (or with control oligonucleotides; 5 nM). After 24 h, cells (1×10^4) were plated on coverslips precoated with fluorescein isothiocyanate (FITC)-labeled gelatin. After 15 h of incubation, the cells were fixed and counterstained with rhodamine-phalloidin and DAPI. Quenched spots of gelatin were quantified in non-overlapping fields. Data are means $\pm$ SEM. (triplicates) Scale bars, 10 μm . (G) MCF10A cells stably expressing shRNAs, either a control shRNA (SC) or shRNA specific to NUP93 (Sh1, left column), and MDA-MB-231 cells expressing a DOX-inducible shRNAs (iSh6, right column), were seeded on coverslips and allowed to grow for 24 hours prior to treatment with the inducer. Thereafter, cells were fixed and stained for F-actin. Images were captured using confocal microscopy (40X). Scale Bars, 10 μm . (H) MDA-MB-231 cells (1×10^5) stably expressing shNUP93 (or a control, shRNA; SC) were seeded on coverslips and probed for integrin alpha 5 and integrin beta 1 using either immunofluorescence or immunoblotting. Images were taken using confocal microscopy (40X). Scale Bars, 10 μm . (I) MDA-MB-231 cells underwent transfection with control siRNA or siNUP93 oligonucleotides. After 48 h, cells were fixed and stained with antibodies specific to the indicated proteins (green). Cells were also stained for actin (phalloidin; red) and nuclei (DAPI; blue). The histogram shows quantification of the respective green areas per cell. The data are presented as an average $\pm$ SEM. Scale bar, 10 μm .

Figure S3: Overexpression of NUP93 accelerates tumor growth and metastasis while knockdown increases adhesion and up-regulates collagens and TGF-beta receptor II. (A) MDA-MB-231-RFP cells (2×10^6) expressing a Tet-inducible allele of NUP93 were injected into the fat pad of NOG mice. Tetracycline was added to the drinking water one week later. Tumor volume (left panel) and tumor weight (right panel) were measured 3 weeks later and presented. (B) Cells (2×10^6) expressing a DOX-inducible shNUP93 were implanted in mice as in A. Tumor weights (left panel) and epi-fluorescence signals (right panel) were measured. (C) Cells (2×10^6) stably expressing shNUP93 (sh1 and sh3), or a control shRNA (SC), were implanted in the mammary fat pad of NOG mice (6 per group). Tumor volumes (left panel) and tumor weights (right panel) were determined 4 weeks later and the averages presented. (D) Lungs from representative mice from C were excised and metastases were visualized. Quantification of the number of metastases per lung of three groups of mice is presented in a histogram. The data were presented as averages $\pm$ SEM. Scale bar, 0.5 cm. (E) RNA was isolated from cultures of DOX-induced (or uninduced) NUP93-knockdown MDA-MB-231-RFP cells. Real-time PCR was performed using primers specific to the indicated collagens. All data were normalized to endogenous GAPDH levels from the same samples. (F) NUP93 siRNA knockdown cells were subjected to immunoblot analysis using an antibody specific to collagen IVa6. GAPDH was used to control sample loading. (G and H) MDA-MB-231 cells (2×10^4)

expressing DOX-inducible shNUP93 (G) or Tet-inducible NUP93 transcripts (H) were seeded in 48-well plates precoated with fibronectin and blocked with albumin (1%). Cells were incubated for 90 min. Adherent cells were stained with crystal violet. Shown are light absorbance (550 nm) values. Data represent means $\pm$ SEM. (I) Cells expressing DOX inducible shNUP93 were plated onto glass coverslips and maintained for 24 days. Cells were detached using EDTA- (5 mM) containing saline and fixed with para-formaldehyde (4%). Collagen IVa6 was visualized using immunostaining and confocal microscopy (40X magnification). Scale bar, 10 μ m. (J) Tumors expressing an inducible shNUP93 were harvested, formalin-fixed and paraffin-embedded. Thin sections were prepared and analyzed using immunofluorescence and an anti-collagen IVa6 antibody. Signals were quantified. Scale bar, 25 μ m. (K and L) RNA or protein was isolated from MDA-MB-231 and MCF10A cells pretreated with siRNAs specific to NUP93. Real-time PCR and immunoblotting of cell extracts were performed using specific primers and an antibody for TGFBR2, respectively. (M) NUP93 was knocked-down in MDA-MB-231 cells using siRNA. After 48 h, cells were analyzed for TGFBR2 surface expression using flow cytometry. The histogram presents expression level determined in triplicates.

Figure S4: NUP93 enables nuclear translocation of signaling proteins, such as STAT, GR and p105 (NF- κ B), in response to EGF, glucocorticoids or TNF. (A and B) Tet-inducible MCF10A cells (1×10^5) overexpressing NUP93 (A), or stably expressing shRNAs specific to NUP93 (either Sh1 or Sh3, or a control shRNA, SC; B), were seeded on coverslips and allowed to grow for 24 hours prior to starvation for serum factors (16 h). Next, cells were stimulated with EGF (10 ng/ml) for the indicated time intervals, fixed and stained for STAT3 (A) or pSTAT3 (B). Images were taken using confocal microscopy (40X). DAPI was used to visualize nuclei. Quantification of nuclear translocation is shown in histograms as average $\pm$ SEM. Scale Bars, 10 μ m. (C) MCF10A cells (1×10^5) were transfected with siNUP93 (or control) oligonucleotides. Serum-starved cells were stimulated with DEX (1 μ M) for the indicated time intervals and, thereafter, cells were probed for GR. Images were captured and analyzed as in A. (D) MDA-MB-231 cells (1×10^5) stably expressing a DOX-inducible shRNA specific to NUP93 (or a control shRNA, SC) were pre-transfected with a plasmid encoding a p105 (NF- κ B) fused to mTurquoise. Serum-starved cells were treated with DOX and 24 hours later they were stimulated with TNF-alpha (10 ng/ml). Time-lapse photos were captured once every 10 seconds. Selected images are shown, along with signal quantification over time. The data are means $\pm$ SEM. Scale bar, 10 μ m.

Figure S5: ERK- and SMAD-derived myristoylated peptides modulate growth and motility of MDA-MB-231 cells, in vitro, as well as tumor progression in animals. (A) MDA-MB-231 cells (2×10^4) stably overexpressing NUP93 (OX), or control cells (EV; empty vector), were incubated for 48 h with ^{3} H-thymidine (1 μ Ci), along with increasing concentrations of either the ERK-derived peptide or a control scrambled peptide (Ctrl). Acid-insoluble radioactivity was determined and presented as averages $\pm$ SEM (quadruplicates). (B) Cells were treated as in A, except that a SMAD-derived peptide was used. (C) MDA-MB-231 cells (5×10^5) overexpressing NUP93 were treated for 48 h with either ERK- or SMAD-derived peptides (20 μ M), or with the respective control peptides. Following incubation with BrdU (60 min), cells were fixed, stained with propidium iodide and probed using an anti-BrdU antibody. Shown are the means

of cell cycle distributions observed using flow cytometry. (D) Cells (1,000) were incubated for 9–10 days with the ERK- or SMAD-derived peptides, or with the respective control peptides. Media were changed every other day. Thereafter, cells were fixed and stained with crystal violet. Growth was quantified by dissolving crystal violet and measuring absorbance (590 nm). The data are presented as averages $\pm$ SEM. (E and F) MDA-MB-231 cells stably expressing NUP93, or control cells, were treated with the indicated peptides for 48 h. Thereafter cells were subjected to migration (4×10^4 cells) or invasion (8×10^4 cells) assays. The migratory or invasive cells found on the bottom side of each insert were quantified using ImageJ. Values represent means $\pm$ SEM. (G–J) NUP93 overexpressing MDA-MB-231-RFP cells (2×10^6) were injected into the subaxillary mammary fat pad of female NOG mice (6 per group). Once tumor size reached 100mm^3 , mice were intravenously treated with either the SMAD or the control peptide (30 mg/kg), once every other day. Primary tumor RFP-fluorescence was measured using an in vivo optical imaging system (IVIS; see G). Tumor volumes were estimated once per week and presented in H (averages of six mice). At the end of the study, tumors were excised and their weights were determined (I). In addition, lungs were removed and evaluated for metastases (J). Image analysis utilized ImageJ. The data are presented as average numbers of micro-metastases $\pm$ SEM. Scale bar, 0.5 cm.

Figure S6: Oncogenic point mutants of NUP93 display enhanced biological activities in vitro and augmented metastasis in animals, while LOF alleles are inactive. (A) MCF10A cells were transfected using a NUP93-encoding vector (WT), or an empty vector (EV). Puromycin-selected clones ectopically expressing the E14K or R327C mutants, or WT, were isolated, and whole extracts were immunoblotted using antibodies specific to NUP93 and GAPDH. (B–E) MCF10A sub-lines from A were tested for the ability to form colonies (B), incorporate radioactive thymidine into DNA (C), migrate through a porous filter (D), and invade through an extracellular matrix barrier (invasion assay, E). The results are presented in histograms. Statistical significance of the differences are indicated. (F) MDA-MB-231-RFP cells (2×10^6) from Fig. 6A, which overexpress the indicated alleles of NUP93, were injected into the subaxillary mammary fat pad, or into the tail vein, of female NOG mice (6 per group). Mice were sacrificed 28 days later, their lungs excised, photographed, and numbers of metastases determined. Data are presented in histograms as averages $\pm$ SEM. (G) Clones of RFP-labeled MDA-MB-231 cells expressing no NUP93 (KO cells) were transfected with vectors encoding WT NUP93 or the indicated LOF mutants. Whole cell extracts were probed as indicated. (H–L) The clones from G were subjected to a cell viability assay (XTT; H), thymidine incorporation tests (I), adhesion/spreading assays (J), migration (K) and invasion assays (L), as described under Methods. (M) MDA-MB-231-RFP cells (2×10^6 , see G), which stably overexpress the indicated alleles of NUP93 on a null background, were injected into the subaxillary mammary fat pad of female NOG mice (6 per group). Mice were sacrificed 28 days later, their lungs excised, photographed, and numbers of metastases determined. Data are presented in histograms as averages $\pm$ SEM. Scale bar, 0.5 cm.

Figure S7: NUP93's abundance regulates GTP loading onto small GTPases, along with partitioning of specific protein and RNA molecules between the cytoplasm and the nucleus. (A; left panel) MDA-MB-231 cells expressing a Tet-inducible allele of NUP93 were treated (or untreated) with Tet for 48 hours. The

endogenous steady-state activities of RHOA, RAC1 and CDC42 were determined in whole cell extracts using an ELISA-based kit (G-LISA, from Cytoskeleton) and a positive control (*Standard*). Shown are averages $\pm$ SEM of quadruplicates. **(A, right panel)** Cells were pretreated for 48 hours with control siRNAs or with siNUP93, prior to determination of steady-state activities of RHOA, RAC1 and CDC42, as in A. **(B)** Cells were pretreated as in A and GTP loading into the Kirsten RAS (KRAS) protein was determined using an ELISA-based kit. **(C)** HEK293 cells were transfected with siNUP93 or control siRNAs, along with luciferase-expression plasmids containing the indicated consensus response elements (RE). Promoter reporter assays were performed as in Fig. 7A. **(D)** RFP-labeled MDA-MB-231 cells overexpressing wildtype NUP93, or the indicated oncogenic mutants, were extracted and lysates were subjected to PCR assays that used specific primers. EV, empty vector. **(E)** RFP-labeled MDA-MB-231 cells were pre-transfected with siRNAs or shRNAs specific to NUP93, and 48 h later whole cell extracts were fractionated into cytoplasmic (CE) and nuclear extracts (NE), which were subjected to immunoblotting. LDH and histone 3 were used as cytoplasmic and nuclear markers, respectively. **(F)** RFP-labeled MDA-MB-231 cells, which were pre-transfected with siRNAs specific to NUP93, were subjected to mass-spectrometry as in Fig. 7J. Results obtained using the nuclear fractions are presented. **(G)** MDA-MB-231 cells stably expressing the indicated clones of DOX-inducible shNUP93, were exposed to DOX for 48 h, while control cells were left untreated. Cells were extracted and later fractionated. Cytoplasmic (CE) and nuclear extracts (NE) were subjected to immunoblotting for SP1, cullin3 and bystin, as in E. **(H)** MDA-MB-231 cells stably expressing a DOX-inducible shNUP93 (clone 4), were pre-seeded on coverslips. Thereafter, cells were exposed to DOX for 48 h, while control cells were left untreated. Cells were probed with antibodies specific to SP1, bystin or cullin 3, and later processed for immunofluorescence. Bar, 10 μ m. **(I)** NUP93-knockout (KO) MDA-MB-231 cells were selected for stable expression of two different LOF mutants of NUP93 (or the WT form), and whole extracts were probed for podocalyxin (PODXL) and GAPDH. **(J)** Genome-wide effects of NUP93 depletion on subcellular localization of the indicated RNAs were determined using RNA sequencing. Shown are cytoplasm/nucleus log2 ratios in siNUP93 (y-axis) and siControl (x-axis) samples. Dots represent expressed genes (expression level > 0.5 TPM in siControl whole cell extract samples). Transcripts that exhibit a 3-fold cytoplasmic shift upon NUP93 depletion are shown in blue, transcripts with a 3-fold nuclear shift upon NUP93 depletion are shown in red. Black dashed line: $X = Y$. **(K, left)**: Representative smFISH images of *NORAD* (orange) in control and NUP93-depleted MCF7 and MDA-MB 231 cells. DAPI staining (blue) was used as a marker for the nucleus. **(K, right)**: Quantification of cytoplasm/nucleus ratios of *NORAD* signal as measured using smFISH. Each dot represents a cell. Box and whiskers indicate the median, quartiles, and 5th and 95th percentiles, respectively. **, $p < 0.01$ (Wilcoxon rank-sum test).

Figures

Figure 1

NUP93 is highly essential for survival of breast cancer cells and its abundance associates with gene copy number gain and shorter patient survival. (A) Analyses of NUP93 essentiality was performed using 28 breast cancer cell lines. Shown are the cell lines for which NUP93 is least essential, along with 5 of the 24 cell lines for which the predicted essentiality is maximal. (B) The METABRIC dataset was divided into three groups according to NUP93 expression levels: upper 10%, lower 10% and intermediate. Shown are

Kaplan-Meier survival analyses. (C) The dataset was stratified according to ER status: positive, negative and neutral (N) and levels of NUP93 are presented. Upper and lower box limits are the 0.75 and 0.25 fractiles, respectively. (D) Analysis of NUP93 expression in the 10 integrative clusters of breast cancer. (E) Correlation between NUP93 gene copy number and mRNA expression based on array comparative genome hybridization (AMP, amplification; NEUT, neutral; HETD, heterozygous deletion; HOMD, homozygous deletion). (F) Distribution of NUP93 gene expression and grade of breast cancer.

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Figure 2

NUP93 overexpression (or knockdown) promotes (or inhibits) DNA synthesis, migration and matrix invasion. (A and B) MDA-MB-231 cells overexpressing a Tet-inducible NUP93 (A) or a DOX-inducible shNUP93 were exposed to the inducer prior to immunoblotting (SC, scrambled shRNA). (C and D) Rates of DNA synthesis were determined using the ^3H -thymidine incorporation assay following induction for 48 hours. Shown are means $\pm$ SEM. (E-H) Cells expressing a Tet-inducible NUP93 (E and G) or DOX-inducible

shNUP93 (F and H) were treated with the inducer for 48h and later subjected to migration (E and F) or invasion (G and H) assays. Migratory or invasive cells were photographed and quantified. Scale bar, 100 μ m.

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shNUP93 were exposed to the inducer prior to immunoblotting (SC, scrambled shRNA). (C and D) Rates of DNA synthesis were determined using the 3H-thymidine incorporation assay following induction for 48 hours. Shown are means $\pm$ SEM. (E-H) Cells expressing a Tet-inducible NUP93 (E and G) or DOX-inducible shNUP93 (F and H) were treated with the inducer for 48h and later subjected to migration (E and F) or invasion (G and H) assays. Migratory or invasive cells were photographed and quantified. Scale bar, 100 μ m.

Figure 3

NUP93 abundance associates with increased metastasis and low expression of matrisome components. (A) MDA-MB-231-RFP cells inducibly overexpressing NUP93 were injected either into the tail vein (2X10⁴ cells, left panel) or the subaxillary mammary fat pad (2X10⁶ cells; right panel) of mice (6 per group). One week later we added tetracycline (3 mg/ml) to the drinking water and mice were sacrificed 3 weeks later. Shown are representative fluorescence images of lungs and quantification of metastases. (B) Cells inducibly expressing shNUP93 were injected into the tail vein or the subaxillary mammary fat pad as in A. (C and D) RNA from siCtrl and siNUP93 treated MDA-MB-231 cells was sequenced and differentially expressed genes are presented in a scatterplot (C) and a heatmap (D; selected matrisome genes). The scatterplot shows statistical significance versus magnitude of change. Data analysis and graphs were created using R and DESeq2. (E) Tumors from B (right part) were stained with picosirius red and images were captured. The histograms depict quantification of collagen staining (averages $\pm$ SEM). (F) Cells pretreated with control- or siNUP93 were stained for TGFBR2 (green) and F-actin (red; Zeiss confocal microscope). Scale bar, 10 μ m. (G) Cells expressing three different inducible shNUP93 (iSh) were incubated for 48 hours in the absence or presence of the inducer (DOX). Media were assayed (triplicates) for TGF-beta and extracts were tested for TGFBR2 using ELISA.

Figure 3

NUP93 abundance associates with increased metastasis and low expression of matrisome components. (A) MDA-MB-231-RFP cells inducibly overexpressing NUP93 were injected either into the tail vein (2X104 cells, left panel) or the subaxillary mammary fat pad (2X106 cells; right panel) of mice (6 per group). One week later we added tetracycline (3 mg/ml) to the drinking water and mice were sacrificed 3 weeks later. Shown are representative fluorescence images of lungs and quantification of metastases. (B) Cells

inducibly expressing shNUP93 were injected into the tail vein or the subaxillary mammary fat pad as in A. (C and D) RNA from siCtrl and siNUP93 treated MDA-MB-231 cells was sequenced and differentially expressed genes are presented in a scatterplot (C) and a heatmap (D; selected matrisome genes). The scatterplot shows statistical significance versus magnitude of change. Data analysis and graphs were created using R and DESeq2. (E) Tumors from B (right part) were stained with picosirius red and images were captured. The histograms depict quantification of collagen staining (averages $\pm$ SEM). (F) Cells pretreated with control- or siNUP93 were stained for TGFBRII (green) and F-actin (red; Zeiss confocal microscope). Scale bar, 10 μ m. (G) Cells expressing three different inducible shNUP93 (iSh) were incubated for 48 hours in the absence or presence of the inducer (DOX). Media were assayed (triplicates) for TGF-beta and extracts were tested for TGFBRII using ELISA.

Figure 4

NUP93 knockdown redistributes importin 7 and inhibits nuclear translocation of SMAD and ERK, whereas overexpression enhances translocation. (A) MCF10A and MDA-MB-231 cells pre-transfected with the indicated siRNAs were probed for importin 7 and NUP93 and analyzed using confocal microscopy. Scale bars, 10 μ m. (B and C) MCF10A cells expressing shNUP93 (Sh1 and Sh3), or control shRNA (SC), were stimulated for the indicated times with either TGFB2 (B) or EGF (C). Fixed cells were stained for SMAD

2/3 or pERK1/2. Shown are confocal images and quantification of translocation (averages $\pm$ SEM). Scale bars, 10 μ m. (D) MCF10A cells pre-stimulated with EGF, as in C, were extracted and fractionated. LDH and histone 3 were used as cytoplasmic and nuclear markers, respectively. CE, cytoplasmic extract; NE, nuclear extract. (E) Pre-starved MCF10A cells inducibly overexpressing NUP93 were induced and later stimulated with EGF (10 ng/ml) for the indicated times. Fixed cells were probed for pERK1/2 (left) and total ERK1/2 (right). The histogram shows quantification of nuclear versus cytosolic protein localization (means $\pm$ SEM).

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Figure 5

A myristoylated phosphomimetic peptide corresponding to the NTS of ERK blocks nuclear translocation and inhibits progression of NUP93-overexpressing tumors. (A) MCF10A cells overexpressing NUP93 (OX) were serum-starved and pretreated with an ERK-derived peptide (20 μ M, 4h) or with a control peptide (20 μ M), and then stimulated with EGF as indicated. Thereafter, cells were fixed, stained for ERK and imaged. Quantitative estimates of nuclear translocation of ERK are shown in histograms (averages $\pm$ SEM). Scale

bars, 10 μ m. EV, empty vector; MFI, mean fluorescence intensity. (B) MCF10A cells were treated as in A, except that a SMAD peptide was applied. (C to E) NUP93 overexpressing MDA-MB-231 cells (2X106) were inoculated in the mammary fat pad of NOG mice (6 mice per group). Once tumor size reached 100 mm³, mice were intravenously treated with either the ERK-derived or the control peptide (30 mg/kg), once every other day. RFP-fluorescence (C) and tumor volumes (D) are shown. Tumors weights were determined (E) and lungs were evaluated for metastases (F). Scale bar, 0.5 cm.

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Figure 6

Naturally occurring gain- and loss-of function mutants of NUP93 respectively enhance or inhibit tumor growth and metastasis. (A) MDA-MB-231 cells pre-transfected with the indicated NUP93-encoding vectors (EV, empty vector) were selected and whole cell extracts analyzed using antibodies to NUP93 and GAPDH. (B) The 4 sublines from A were sparsely seeded and 10 days later fixed and stained with crystal violet. Cells were then photographed and analyzed. (C) The sublines from A were subjected to a DNA synthesis

assay (means $\pm$ SEM). (D and E) The indicated 4 sublines were subjected to a cell migration (D) or invasion (E) assays and averages of quadruplicates are shown. (F and G) MDA-MB-231-RFP cells (2X10⁶) from A were injected into the subaxillary fat pad of female mice (6 per group). Tumor volumes are presented in F. Tumors were excised on day 30 and their weights determined (G). (H) Cells from A were injected either into the tail vein (2X10⁴ cells, upper row) or the fat pad (2X10⁶ cells; lower row) of 6 mice. Metastatic liver nodules were quantified 4 weeks later. Shown are representative images of livers, along with quantification of metastases. (I) Crispr-CAS9 was used to ablate NUP93 of MDA-MB-231 cells. Whole cell extracts were probed as indicated. (J and K) Cells from I were injected into the fat pad of female mice (6 per group). Likewise, individual KO clones stably re-expressing WT-NUP93, G591V-NUP93 or Y629C-NUP93 were similarly implanted in mice. Tumor volumes were calculated once per week and presented in J. Tumors were excised on day 30 and their weights determined (K). (L) The indicated sublines were injected into the tail vein of female mice. Four weeks later, lungs were excised, photographed and metastatic nodules quantified. Shown are representative fluorescence images and quantification of lung metastases. Scale bar, 0.5 cm.

Figure 6

Naturally occurring gain- and loss-of function mutants of NUP93 respectively enhance or inhibit tumor growth and metastasis. (A) MDA-MB-231 cells pre-transfected with the indicated NUP93-encoding vectors (EV, empty vector) were selected and whole cell extracts analyzed using antibodies to NUP93 and GAPDH. (B) The 4 sublines from A were sparsely seeded and 10 days later fixed and stained with crystal violet. Cells were then photographed and analyzed. (C) The sublines from A were subjected to a DNA synthesis

assay (means $\pm$ SEM). (D and E) The indicated 4 sublines were subjected to a cell migration (D) or invasion (E) assays and averages of quadruplicates are shown. (F and G) MDA-MB-231-RFP cells (2X10⁶) from A were injected into the subaxillary fat pad of female mice (6 per group). Tumor volumes are presented in F. Tumors were excised on day 30 and their weights determined (G). (H) Cells from A were injected either into the tail vein (2X10⁴ cells, upper row) or the fat pad (2X10⁶ cells; lower row) of 6 mice. Metastatic liver nodules were quantified 4 weeks later. Shown are representative images of livers, along with quantification of metastases. (I) Crispr-CAS9 was used to ablate NUP93 of MDA-MB-231 cells. Whole cell extracts were probed as indicated. (J and K) Cells from I were injected into the fat pad of female mice (6 per group). Likewise, individual KO clones stably re-expressing WT-NUP93, G591V-NUP93 or Y629C-NUP93 were similarly implanted in mice. Tumor volumes were calculated once per week and presented in J. Tumors were excised on day 30 and their weights determined (K). (L) The indicated sublines were injected into the tail vein of female mice. Four weeks later, lungs were excised, photographed and metastatic nodules quantified. Shown are representative fluorescence images and quantification of lung metastases. Scale bar, 0.5 cm.

Figure 7

High NUP93 stabilizes beta-catenin as well as enhances its transcription and nuclear transport, while low NUP93 activates podocalyxin. (A) HEK293 cells were co-transfected with vectors encoding NUP93, pGL3 control plasmid and the indicated luciferase reporters. Luminescence values were quantified and normalized (means±SEM; triplicates). (B) Cells were transfected as in A, except that FOPFlash was used, along with plasmids encoding wildtype NUP93 and the indicated mutants. (C) RFP-labeled MDA-MB-231

cells overexpressing NUP93, or the indicated mutants. Lysates were subjected to immunoblotting. EV, empty vector. (D) Cells were transfected as in B, except that a 16h incubation with the indicated growth factors preceded luciferase assays. (E) The clones from C were pre-incubated with cycloheximide (0.05 mg/ml) as indicated, and extracts were probed. (F) HEK293 cells were co-transfected with vectors encoding the indicated NUP93 alleles, along with TOP/FOP and the pGL3 plasmid. Forty-eight hours later, cells were incubated with medium conditioned by L cells expressing WNT3A, or by control L cells, and extracts were prepared 16 hours later. Luminescence values were quantified as in B. (G) MDA-MB-231 cells stably expressing wildtype NUP93, the indicated mutants or an empty vector (EV) were incubated with medium conditioned by L (WNT3A producers) or control L cells. Four hours later, cells were probed for beta-catenin. Scale Bars, 10 μ m. (H and I) Cells from C were pre-treated with siRNAs specific to LEF1, and 48 hours later they were subjected to migration (H) and invasion (I) assays. (J) RFP-labeled MDA-MB-231 cells were pre-transfected with siNUP93, or with control siRNAs. Forty-eight hours later, whole extracts were isolated and fractionated. Each fraction was subjected to mass-spectrometry using high-resolution liquid chromatography-mass spectrometry (LC-MS/MS)-based proteomics (LFQ, label-free quantification). Results obtained using the cytoplasmic fractions are presented in a heatmap (Student t-test and Z-scoring). (K) MDA-MB-231 and MCF10A cells were transfected with control siRNAs or siNUP93 and 48 hours later they were probed for podocalyxin. Scale bars, 10 μ m. (L) MDA-MB-231 cells were transfected with siRNAs specific to bystin, cullin3 and podocalyxin and 48 hours later the cells were subjected to an adhesion assay, in triplicates.

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