Multiomic Analysis of Intercellular Communication through Tumor-Stroma Tunnels in Breast Cancers

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Running title

Borrowed GIV Drives Aggressive Disease in ER+ Breast Cancer

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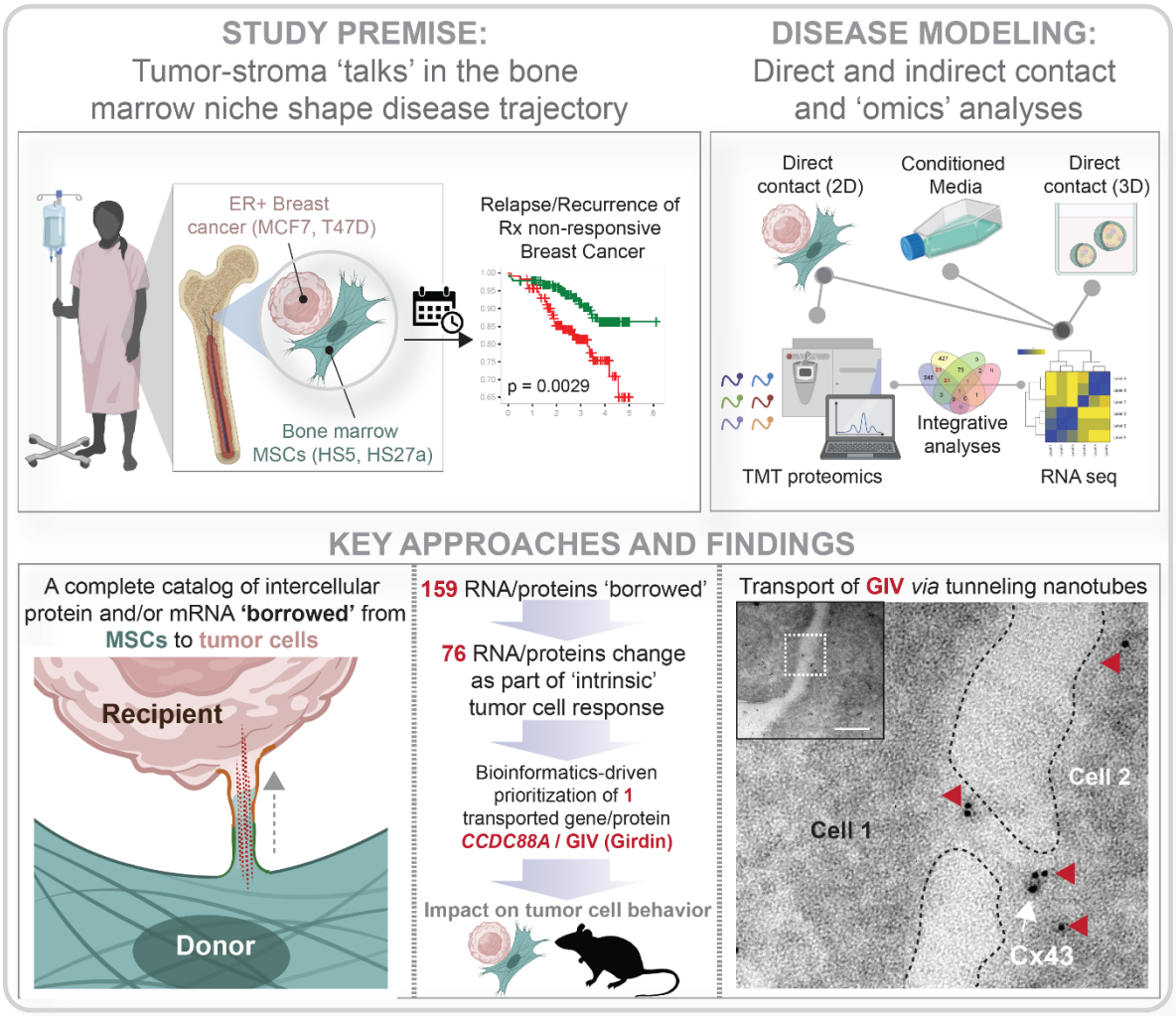
Abstract

Estrogen receptor-positive (ER+) breast cancer commonly disseminates to bone marrow, where interactions with mesenchymal stromal cells (MSCs) shape disease trajectory. We modeled these interactions with tumor-MSC co-cultures and used an integrated transcriptome-proteome-network-analyses workflow to identify a comprehensive catalog of contact-induced changes. Induced genes and proteins in cancer cells, some borrowed and others tumor-intrinsic, were not recapitulated merely by conditioned media from MSCs. Protein-protein interaction networks revealed the rich connectome between 'borrowed' and 'intrinsic' components. Bioinformatic approaches prioritized one of the 'borrowed' components, *CCDC88A*/GIV, a multi-modular metastasis-related protein which has recently been implicated in driving one of the hallmarks of cancers, i.e., growth signaling autonomy. MSCs transferred GIV protein to ER+ breast cancer cells (that lack GIV) through tunnelling nanotubes via connexin (Cx)43-facilitated intercellular transport. Reinstating GIV alone in GIV-negative breast cancer cells reproduced ~20% of both the 'borrowed' and the 'intrinsic' gene induction patterns from contact co-cultures; conferred resistance to anti-estrogen drugs; and enhanced tumor dissemination. Findings provide a multiomic insight into MSC→tumor cell intercellular transport and validate how transport of one such candidate, GIV, from the haves (MSCs) to have-nots (ER+ breast cancer) orchestrates aggressive disease states.

GRAPHIC ABSTRACT



Introduction

Estrogen receptor-positive (ER+) breast cancer, the most common subtype, preferentially disseminates to bone and bone marrow(1, 2). Bone metastases are incurable, and cause debilitating symptoms, e.g., pain, fractures, and life-threatening hypercalcemia(3). The bone marrow, on the other hand, harbors disseminated tumor cells (DTCs) in breast cancer; DTCs may persist in a clinically occult state for decades before they may ultimately proliferate and potentially travel to other organs to produce delayed recurrences that occur in ~40% of patients with ER+ breast cancer(4). Recurrent disease is more aggressive, relatively resistant to treatment, and currently incurable. Little progress has been made in treating breast cancer that has already disseminated to bone(5), largely because we lack a comprehensive understanding of the molecular mechanisms that make ER+ breast cancer cells more aggressive in the bone marrow niche(6).

What is widely believed is that interactions with stromal cells in the bone marrow environment shape key functions of breast cancer cells that ultimately determine disease progression and outcomes(7). Mesenchymal stem cells (MSCs), a multipotent cell type that contributes to formation of bone, fat, and cartilage tissues, are a major stromal cell type driving aggressive phenotypes of disseminated ER+ breast cancer cells in bone marrow. MSCs regulate ER+ breast cancer cells through mechanisms such as secreted cytokines and direct intercellular interactions(8). Work by us and others have established that direct interactions with MSCs, rather than soluble molecules, drive changes in gene expression and metabolism that promote cancer stem-like cell states, resistance to anti-estrogen drugs, and metastasis in ER+ breast cancer(9-11). Breast cancer cells and MSCs are known to establish channels of intercellular communication that transport molecules and organelles. The two major structures for such intercellular communication include: Gap junctions and tunneling nanotubes (TNTs), both of which share connexin 43 (Cx43) as an essential molecular player. Gap junctions are intercellular channels comprised of various subsets of connexin proteins(12). Gap junctions and Cx43 are upregulated in lymph node and systemic metastases in breast cancer and other malignancies, suggesting these structures contribute to essential steps in the metastatic cascade(13). TNTs are actin-rich long, thin tubes that may form between cells of the same or different types, thereby allowing long-range communication via the exchange of materials. A broad range of intracellular contents, including microRNAs, proteins, and organelles, exchange between cells via TNTs. TNTs increase drug resistance of cancer cells; regulate proliferation; and promote metastasis.

Despite these insights, a limited number of molecules transferred from MSCs via gap junctions or TNTs have been implicated in aggressive phenotypes of breast cancer cells, whereas the identity of most remains unknown. Here we tackle this knowledge gap by performing a comprehensive multiomic network analysis to identify molecules and biologic processes regulated by direct contact between bone marrow MSCs and ER+ breast cancer cells. We identified genes and proteins that cancer cells acquire from MSCs through direct transfer ("borrowed" components) and additional molecules they induce because of such transfer ("intrinsic components"). We use bioinformatic approaches to explore the implications of these gene expression changes on patient outcome and subsequently prioritized one 'borrowed' gene/protein (*CCDC88A/*GIV) to explore further and validate through a series of *in vitro* and *in vivo* studies. Our findings establish a comprehensive resource for

7 changes induced in ER+ breast cancer cells by contact with bone marrow MSCs and pinpoint GIV as a promising
8 target to overcome aggressive phenotypes that breast cancer cells acquire in a bone marrow environment.

Results

Multiomic analysis of the impact of close contact between ER+ breast cancer cells and MSCs

DTCs in the bone marrow displace hematopoietic stem cells from supportive niches(14), where they establish direct contact with various subsets of MSCs and eventually gain aggressiveness. To identify how MSCs may impact ER+ breast cancer cells through direct contact, we used a 2D co-culture model combining MCF7 or T47D human ER+ breast cancer cells with human HS5 or HS27a MSCs in a 1:9 ratio (**Fig 1A**). After 3 days of co-culture in medium with low serum and physiologic concentrations of glucose, we recovered the tumor cells using EpCAM immunomagnetic beads, capitalizing on the fact that EpCAM is expressed exclusively on tumor cells of epithelial origin. We previously showed that such EpCAM-based immunoisolation is relatively devoid of MSC contamination(15). To control for the effects of soluble molecules produced by MSCs, we cultured MCF7 or T47D cells for 3 days in conditioned medium from MSCs (**Fig 1A**). Monocultures of MSCs and tumor cells were used as additional comparator groups. All samples were analyzed by bulk RNA sequencing; mono- and contact cultured MCF7 cells were also analyzed by TMT proteomics.

Exposure of MCF7 cells to contact culture (CC) but not conditioned media (CMed), was associated with a significant lowering of tumor dormancy score (**Fig 1B**); low scores were previously shown to confer a ~2-fold increase in the risk of recurrence in 4 independent cohorts of patients with ER+ breast cancers(16). These findings suggest that our co-culture model captures key transcriptomic changes in ER+ breast cancer cells of translational relevance that correlate with more aggressive disease in patients.

We carried out a combinatorial multiomics analyses to determine what proteins or transcripts may be expressed highly in MSCs that are 'borrowed' by MCF7 cells. To this end, we first created a catalog of genes that are differentially expressed (DEGs; upregulated) in MSCs, but not in MCF7 cells (MCF7 vs HS5; n = 1471; **Fig 1C**). This catalog served as a denominator for all likely candidate genes that a tumor cell may lack originally but acquire from MSCs via intercellular communication. Next, we performed pair-wise DEG analysis on the other MCF7 samples that were subjected to different co-culture conditions. Many genes (n = 482) and proteins (n = 295) were induced due to direct contact culture (CC-induced genes; **Fig 1C**). Few genes were induced because of exposure to conditioned media (CMed-induced genes, n = 13; **Fig 1C**). An overlay of these showed the following: (i) Exposure to conditioned media alone induced only a single unique proteins/transcripts in MCF7 cells, implying soluble mediators or exosomes as potential modes of communication; (ii) By contrast, contact culture could account for 487 unique proteins/transcripts, implying direct contact as the major mode of tumor-MSC communication, accounting for the largest share of the uniquely induced genes; (iii) 242 unique proteins/transcripts (39 transcripts and 203 proteins, with no proteome-transcriptome overlap) were upregulated in MCF7 cells as a consequence of contact culture. These transcripts/proteins were not highly expressed in HS5 cells. This last category was presumed to be tumor cell-intrinsic response to contact culture, unlikely to be transferred directly from MSCs to breast cancer cells. It is noteworthy that neither the experimental design, nor the analytical steps can conclude with certainty if transport of a given target occurred as a protein or as mRNA because unknown factors (transcript/protein half-life) are expected to confound such conclusions. Similarly, while we infer that 487 unique RNAs/proteins could be transferred during contact culture, i.e., "borrowed" directly by

cancer cells from the MSCs, we cannot exclude some cancer cell-intrinsic contributions to the overall levels. We did observe, however, that the 'borrowed' RNA/proteins were enriched for biological processes that govern tube morphogenesis, organization of extracellular encapsulated structures, and movement of anatomic structures in the context of multicellular systems (**Fig 1D**). Enrichment of these processes further suggests the potential for direct transfer of RNA/protein from MSCs to cancer cells through structures such as tunneling nanotubes. Reactome and gene ontology analyses of the genes induced by conditioned media, contact culture and intrinsic response present a comprehensive picture of distinct modes by which MSCs shape behavior and function of cancer cells (**Supplementary Fig 2**). A similar analysis for the transcripts and proteins that were downregulated during contact culture and when exposed to conditioned media was notable for genes/proteins that reduce the dependence on ER signaling (**Supplemental Fig 1**). A full catalog of all the genes/proteins that are upregulated (**Supplemental information 1**) and downregulated (**Supplemental information 2**) during contact culture are provided.

A gene signature of contact culture is prognostic and predictive in ER+ breast cancers.

We noted that 39 unique genes were upregulated, identified by both RNA seq and TMT proteomics, in MCF7 cells during contact co-cultures (**Fig 1C**). Almost all of them (n=38; **Fig 1E**) were uniquely upregulated during contact culture, whereas 1 (*KYNU*) was upregulated in conditioned media. A heatmap analysis showed that these 39 genes are poorly expressed in monocultured MCF7 cells (MC; **Fig 1E**) but highly expressed in HS5 cells (lane 2; **Fig 1E**). The gene expression patterns in MCF7 cells that were subjected to culture in direct contact with HS5 cells (CC; **Fig 1E**) looked more like that in HS5 cells. By contrast, MCF7 cells subjected to culture in conditioned medium looked virtually indistinguishable from monocultured MCF7 cells (compare MC and CMed; **Fig 1E**). A composite score of the levels of expression of these 39 genes confirmed their statistically significant increase in contact, but not conditioned media cultures (**Fig 1F**). This pattern of gene induction was reproducibly observed when we swapped HS5 in the co-cultures with another MSC line (HS27a; **Fig 1G**) or swapped the MCF7 tumor cell line with another ER+ breast cancer line (T47D; **Fig 1H**). These findings demonstrate that the 39-gene signature represents a conserved feature of how MSCs impact the properties or behavior of ER+ breast cancer.

The 39 genes showed an enrichment of processes that related to growth factor, PI3K/Akt and an *IL4/IL13 and IL10*-centric tolerogenic cytokine signaling (**Fig 2A**), suggesting that the tumor cell's acquisition of these 39 genes from MSCs may impact these aspects of tumor cell biology and/or behavior, including those that concern its interactions with and its ability to evade the immune system.

To test its translational relevance, we applied this 39-gene signature to a publicly available dataset that prospectively tracked the outcomes of ER+ breast cancers following neoadjuvant taxane-anthracycline chemotherapy(17) (**Fig 2B-E**). Kaplan-Meier curves revealed that high levels of the contact culture signature correlated with significantly worse distant relapse-free survival in patients with ER+ (**Fig 2B**), but not ER- breast cancers (**Fig 2C**). High levels of the close contact signature also predicted worse outcomes in patients with treatment resistant, but not treatment sensitive, disease (**Fig 2D-E**). Taken together, these findings establish that

the 39 genes presumed to be borrowed by ER+ breast cancer cells via direct contact with MSCs carry translationally relevant information; they identify patients at greater risk for death from recurrent breast cancer.

The connectome of genes either 'borrowed' or induced as intrinsic response during contact culture

To discover how contact culture with MSCs reprograms signaling networks in ER+ breast cancer cells, we used the 39-proteins as 'seeds' to build a protein-protein interaction (PPI) network (**Fig 3A**). The resultant network (comprised of 2743 proteins) was overlayed with 'borrowed' (n=487) and 'intrinsic' (n=242) gene sets (see **Fig 1B**); this allowed construction of a more focused network using 159 genes that the ER+ breast cancer cells borrow from MSCs and 76 genes in the cancer cell intrinsic response group. We visualized this network as a multilayer network connecting borrowed (pink; **Fig 3C**) and intrinsic response (green; **Fig 3C**) genes. Within the resultant PPI network, 1482 nodes/proteins interacted within 'borrowed' layer and 835 nodes/proteins interacted within the intrinsic layer. The two layers shared 567 common nodes/proteins, indicative of meaningful connectivity between the two layers (*p* value of 0.00096, determined by hypergeometric test). The edge and node list of the entire PPI network, along with its various subnetworks are presented in **Supplemental Information 3**. Findings also suggest functional integration between the two components such that the induction of one (the intrinsic tumor genes) may either enable the functions of and/or reflect the consequences of the other (the borrowed genes).

The borrowed response interactome was notable for Akt, growth factors and/or their receptors (*EGFR, VEGFA, WNT5A*) and integrins (**Fig 3C**), as noted earlier (**Fig 2A**). Also found in the borrowed network was the gap junction protein, *GJA1*/CX43, which has been implicated in establishment of tunneling nanotubes(18). The intrinsic interactome was notable for VEGFR2-mediated cell proliferation, activation of RHO GTPases and cytokine signaling (**Fig 3D**).

***CCDC88A*/GIV as a key gene borrowed by cancer cells amidst robust intercellular transport**

With an intent to validate and/or characterize key components of the borrowed proteome/transcriptome, we further refined the list of borrowed genes derived in our 2D contact culture model (**Fig 1A**) leveraging a previously published 3D contact culture of MCF7 or T47D cells with HS5 (**Fig 3E**)(19). Such a refinement was carried out with a two-fold intention: (i) to enhance the physiological context and translational relevance because TNTs have been shown to be formed in 3D culture systems(20); and (ii) to provide a continuity between 2D and 3D TNT biology, which has been a difficult task to achieve in a field that is still in its infancy(21). Refinement gave a handful genes (n=19) that are induced also during 3D close contact culture in both MCF7 and T47D cells (**Fig 3F**). Gene ontology (GO) analysis of these 19 genes revealed that one gene, *CCDC88A* [which encodes the protein Gα-Interacting, Vesicle-associated (GIV, a.k.a, Girdin)] is commonly shared among all key processes upregulated in ER+ breast cells by contact with MSCs (**Fig 3G**). Compared to monocultured *CCDC88A* transcript was significantly upregulated in our 2D direct contact but not the conditioned medium cultured MCF7 cells (**Fig 3H**). A focused analysis of the *CCDC88A/GIV*-subnetwork from the multi-layer interactome (in **Fig 3C**) revealed that the interactome of GIV could support numerous pathways and processes involved in cell projections and membrane domains (**Fig 3I**), suggesting that *CCDC88A* associates with direct intercellular contacts.

We confirmed by interferometry microscopy that 'borrowing' of *CCDC88A* in our 2D contact culture system (**Fig 3H**) was associated with the presence of numerous TNTs connecting MCF7 and HS5 cells (**Fig 3J**, **Supplementary Figure 3**). TNT formation in contact cultures was associated also with the elevated expression of two proteins previously implicated as central factors for TNT formation: (i) the gap junction protein *GJA1* (18)(connexin 43, CX43; **Fig 3K**) and the TNT marker, *TNFAIP2* (22)(M-Sec; **Fig 3L**). To investigate if gap-junction or TNT-facilitated intercellular communication is functional (i.e., allowed intercellular communication) we carried out the well-established calcein-AM transfer assay(23, 24). When MCF7 cells that stably expressed mCherry (recipients) were cocultured with calcein-labeled HS5 MSCs (donors) or Calcein-labeled MCF7 cells, we detected the successful transfer of calcein into mCherry-labeled MCF7 cells (**Supplementary Figure 4**). While both donor cells (HS5 and MCF7 cells) transferred calcein to the recipient MCF7 cells, transfer was markedly more from HS5 cells (**Supplementary Figure 4**). These results confirm that our 2D contact cultures were permissive to intercellular transport.

Together these findings suggest that TNTs in contact cultures could serve as a route for breast cancer cells to borrow from MSCs certain RNAs and proteins, among which, *CCDC88A*/GIV may be a central player.

GIV(*CCDC88A*) is transported from MSCs to ER+ breast cancer cells

Although GIV was prioritized through computational rationale (**Fig 3E-I**), we prioritized its candidacy for further validation as opposed to any of the other 18/19 candidate genes (**Fig 3F**) because of three key reasons: *First*, as a large multi-modular protein shown to integrate signals initiated by diverse classes of receptors and those transduced through diverse signaling pathways, GIV has been implicated in tumor cell aggressiveness (stemness(25), survival after DNA damage(26), invasiveness(26), chemoresistance(27) and acquisition of metastatic potential(28)) by diverse groups in a multitude of cancers, including breast(29) and is considered to be a metastasis-related protein(30). *Second*, unlike stromal cells(31, 32) and triple negative breast cancers, which express high levels of GIV, ER+ breast cancer cells, such as MCF7 and T47D, have been shown to be GIV-deficient(33) due to an alternative splicing event [intron 19 retention], which leads to the loss of the resultant transcript to non-sense mediated decay. The concept of haves (MSCs) lending GIV to have nots (ER+ tumor cells) for a potential gain in metastatic potential by the latter appeared interesting. Consistent with this concept, prior studies had in fact demonstrated that patients with ER+ breast cancers do express GIV, and such expression correlates with more metastasis and poorer survival(34, 35). *Third*, GIV has recently been shown to be a key orchestrator of secretion-coupled autonomy(36), wherein cancer cells may secrete and sense their own growth factors to survive. Proteomic studies confirmed that GIV enabled autocrine growth factor signaling autonomy specifically within the EGF/EGFR system(36). It is noteworthy that EGFR is one of the initially identified list of 39 'borrowed' RNAs and proteins in contact culture (**Fig 1E**). EGFR remained on the list even after the 3D contact culture refinement (**Fig 3E**) as one of the 19 "borrowed" genes, alongside *CCDC88A* (**Fig 3F**).

GIV protein was virtually undetectable in the lysates from monocultures of 3 different ER+ breast cancer cells but detected as a full-length protein (~250 kDa) in cells recovered with EpCAM-based immunocapture and lysis following 3 days in contact cultures with HS5 cells (**Fig 4B**). GIV protein was detected by immunoblotting in MCF7 cells that were lysed immediately after separation from HS5 cells but not in those after 24 to 96 h of

additional culture post separation (**Fig 4C**). These findings indicate that once acquired, GIV is rapidly lost upon returning tumor cells to monoculture, and ongoing close contact with MSCs is essential to maintain the levels of GIV in tumor cells.

To confirm if GIV in MCF7 cells was indeed acquired from HS5 cells and determine if transport occurred as transcripts or proteins, we stably expressed short hairpin (sh) RNA targeting GIV in either MCF7 or HS5 cells and put them in contact culture with each other in various combinations (see **Fig 4D**). Batch populations of cells showed ~80% depletion of GIV in HS5 cells (**Fig 4E-***right*). Contact cultures of control MCF7 and HS5 cells showed expected upregulation of both full-length GIV in MCF7 cells (**Fig 4E-***left*; lane 3). Contact cultures of MCF7-shGIV cells and HS5 still showed increased amounts of GIV protein, indicating that shRNA against *CCDC88A* did not reduce GIV transfer (**Fig 4E-***left*; lane 4). However, MCF7 cells co-cultured with HS5-shGIV cells did not result in GIV protein transfer (**Fig 4E-***left*; lane 5). Because MCF7 cells expressing shGIV continued to express GIV protein, findings indicate that the observed upregulation of GIV in MCF7 cells that were subjected to contact culture with HS5 relies predominantly on transfer of GIV as a protein. Some transfer of GIV as mRNA cannot be ruled out.

Cx43 and GIV interact and are co-transported through intercellular communication sites

Because gap junctions are known to serve as sites for TNT formation and/or attachment(20, 37) and CX43 is a shared molecular entity for them both, we assessed CX43 expression in MCF7 cells that were either in monocultures or recovered from contact cultures with HS5 cells. Co-culture with HS5 cells increased CX43 in MCF7 cells, while monocultures of MCF7 cells showed undetectable levels of this protein (**Fig 5A**). Next we used two perturbagens to modulate intercellular communication via gap junctions: (i) Carbenoxolone, a widely used chemical inhibitor of GJIC; and (ii) Fulvestrant, which inhibits the transcriptional functions of ER, and by that token, inhibits various ER targets, including the transcription of CX43(38, 39). Both perturbagens virtually eliminated the upregulation of GIV and CX43 in MCF7 cells co-cultured with HS5 cells (**Fig 5B**), while these compounds had minimal effects on amounts of CX43 in HS5 cells alone (**Supplementary Figure 5**). These results show that direct, CX43-mediated intercellular communication is necessary for MCF7 cells to borrow GIV protein from HS5 cells.

To resolve the nature of these CX43-dependent compartments that facilitate intercellular communication, we carried out an ultrastructural analysis by immunogold electron microscopy. CX43 and GIV were found to co-localize either near gap junctions (**Fig 5C**-*top left*),at/near more elaborate and less defined contact sites (**Fig 5C**-*bottom left*), or within tube like passages connecting cells (**Fig 5C**-*right*).

Co-localization of CX43 and GIV suggested that these proteins may interact; we asked if they do. Using CX43-GFP expressed in Cos7 cells and purified GST fusions of N-terminal or C-terminal part of GIV in pull down assays, we confirmed that the N-terminus, but not the C-terminus of GIV binds directly to CX43 (**Fig 5D-F**). This finding is consistent with a prior work demonstrating the importance of GIV-NT in junctional localization(40).

Together, these findings suggest that CX43 and GIV interact and co-migrate from MSCs to ER+ breast cancer cells during CX43-mediated intercellular transport.

Adding GIV to ER+ breast cancer cells recapitulates key functions gained during contact culture

Next, we asked what proportion of the functions gained during contact culture with MSCs may be accounted for or recapitulated by GIV alone. To this end, we created MCF7 cells that stably expressed GIV using a piggyBac transposase system (**Fig 6A**) and analyzed them by RNA Seq (**Fig 6B**). Approximately ~20% of the genes in the 'borrowed' (n = 32 genes) and 'intrinsic' response (n = 17 genes) components of contact culture were able to perfectly classify the MCF7 cells with or without GIV (**Fig 6C**). A complete catalog of these genes is presented in **Supplemental Information 4**. Heat maps display the differential expression of these genes (**Fig 6E, F**). Of the tumor intrinsic gene response, we previously discovered that co-culture with MSCs upregulated *LCN2,* an iron sequestering protein, and *CD44,* a marker of stem-like cells, in breast cancer cells (15). Pathway analysis of the 32-gene borrowed signature recapitulated by stable expression of GIV alone showed enrichment for numerous processes related to response to external stimuli, while the 17-gene signature included vesicular transport and exocytosis (**Supplemental Figure 6**). These data reinforce our focus on GIV within the list of genes that are transferred from MSCs to breast cancer cells in the bone marrow environment to enhance aggressive traits and facilitate disease progression.

To investigate effects of GIV on drug resistance, we focused on anti-estrogen drugs tamoxifen (a selective estrogen receptor modulator)(41) and fulvestrant (a selective estrogen receptor degrader)(42). We also tested combination therapy with fulvestrant and palbociclib, an inhibitor of CDK4/6 used with anti-estrogen therapy for patients with metastatic ER+ breast cancer. Following three days of treatment in low serum, physiologic glucose medium, we quantified relative numbers of viable cells at various concentrations of tamoxifen, fulvestrant, or fulvestrant plus a fixed concentration of palbociclib. MCF7-GIV cells showed resistance to each of these treatment regimens, particularly for fulvestrant (**Fig 6G-I**). These data establish that GIV confers resistance to clinically used drugs for ER+ breast cancer and suggest that upregulation of GIV in cancer cells in close contact with MSCs may permit disseminated ER+ breast cancer cells to survive therapy in the bone marrow environment.

Because GIV coordinates intracellular signaling pathways enabling growth-factor autonomous survival and proliferation of circulating tumor cells(36, 43), we investigated the effects of GIV on hematogenous dissemination of ER+ breast cancer cells. We injected MCF7 and MCF7-GIV cells into the left cardiac ventricle of female immunodeficient NOD.Cg-Prkdc scid (NSG) mice and tracked cancer cells using bioluminescence (**Fig 6J**). Viable MCF7 cells dropped significantly and steadily from day 1 through day 10, while the drop was insignificant in mice injected with MCF7-GIV. From days 0-1, signal from control MCF7 cells decreased by approximately 70%, while MCF7-GIV cells diminished by only approximately 10%. From days 1-3, losses of viable cells were approximately 20% and <10% for MCF7 and MCF7-GIV cells, respectively. The rapid drop in MCF7 cells is not unexpected because we did not supplement mice with estrogen, which typically is required for MCF7 cells to thrive and proliferate in vivo. Intriguingly, we noted that the differences between groups diminished over time from days 10-24 and became virtually indistinguishable thereafter. These results reveal that GIV facilitates survival during the early stages in dissemination of ER+ breast cancer cells. Because contact culture studies in vitro take ~3 days to transfer GIV, it is possible that MCF7 cells borrowed GIV from stromal cells that have abundant amounts of the same.

Tumor cells may acquire *CCDC88A* and other 'borrowed' genes also from fibroblasts

Studies have documented that disseminated tumor cells and metastases in one organ can seed metastatic disease in a second site, emphasizing the need to understand how the bone marrow environment promotes aggressive phenotypes in breast cancer(44). We asked if the usefulness of the catalog of 'borrowed' genes we describe here maintained relevance beyond MSCs and perhaps extend to resident tissue fibroblasts; the latter also are well known to establish TNTs with cancer cells(45). To this end, we leveraged the only publicly available, previously published study that compared monocultures of MCF7 with contact cultures of MCF7 cells and BJ fibroblasts by RNA seq(46). As a third comparator group, they cultured the MCF7 cells in extracellular matrix (ECM) that was derived by concentrating conditioned media from the BJ fibroblasts (CMed) (**Fig 7A**). Contact culture, but not ECM-treated grow conditions induced each of the 3 borrowed gene signatures we described earlier (**Fig 7D-F**). Most importantly, *CCDC88A* was among the "borrowed" gene signature induced during contact culture with fibroblasts. These results suggest that ER+ tumor cells may acquire GIV via intercellular transport from diverse stromal cells, which are known to express GIV at high levels.

Discussion

The major problem we address here is the widely recognized, but poorly understood, means by which the bone marrow environment harbors disseminated tumor cells in ER+ breast cancer, permitting long-term survival of these cells despite hormone targeted therapy(47). Persistence of disseminated tumor cells poses a documented, progressively increasing risk of delayed recurrence that most often occurs in bone (48-50) (~50%) for patients with ER+ breast cancer. MSCs have been implicated as key enablers of tumor cells in the bone marrow niche(51)(52)However, mechanisms that support disseminated tumor cells in bone marrow and potentiate potential recurrence remain unclear. The compendium of TNT tumor-stromal transport we reveal here provides a wealth of potentially actionable targets to disrupt MSC niches for cancer cells and end the current impasse in treating bone metastases. There are potentially three major ways in which this work advances the field of cancer biology.

A catalog of MCS-to-tumor gene transfer

First, we provide here a comprehensive catalog of genes transferred from the MSCs to ER+ breast cancer cells during direct contact culture. The catalog is likely to be physiologically relevant and reproducible because the multiomic rigor we employed went beyond our own datasets generated using all 4 possible combinations of two ER+ breast cancer cell lines and two MSCs. We further refined using datasets from independent laboratories that span both 2D and 3D models. This catalog not only provides a prioritized list of genes for the scientific community to pursue but also provides new knowledge of molecular mechanisms that reprogram breast cancer cells in the bone marrow. For example, while MSCs are known to regulate breast cancer cells through soluble mediators such as cytokines(53), work done by others and us had revealed that key functional changes in ER+ breast cancer cells required direct close contact with MSCs(54). The present catalog provides a new multiomic resource to begin to dissect how MSCs shape disease phenotypes in ER+ breast cancer and to what extent effects rely upon intercellular transfer of key molecules ('borrowed'). We show that the borrowed components integrate seamlessly with a second set of gene expression changes that reflect the cancer cell 'intrinsic' responses; together, they establish a robust connectome. The key borrowed components belong to the growth factor and prosurvival PI3K-Akt signaling pathways. The key intrinsic response is augmentation within the VEGF signaling pathway, which supports proliferation and angiogenesis. The key process suppressed is dependance on estrogen signaling, consistent with environmentally-mediated resistance to hormone targeted therapies(55). The 'borrowed' gene signature showed demonstrable translational relevance in that it prognosticated and predicted outcome in patients with ER+ breast cancers, especially in those who had treatment-resistant disease. We conclude that the catalog of genes may represent high value targets for preventing MSCs from empowering tumor cells that lie in the bone marrow niche. Several key genes from this list also were reproducibly upregulated during tumor-fibroblasts contact culture, implying that findings may have broader relevance in the context of tumor cells in other tissue niches.

Gene transfer occurs through a nanotube-based connectivity grid

Second, the catalog of genes provides valuable clues into the predominant mode of intercellular communication between MSCs and tumor cells. Intercellular protein transfer encompasses several different biologic processes and cellular structures that establish dynamic communication networks among cells in direct contact or proximity: Gap junctions, TNTs, extracellular vesicles, trogocytosis, and direct cell fusion are among such processes(56). Gene ontology analyses of our catalog of borrowed genes hinted at the formation of extracellular tube-like structures, such as TNTs, as a likely mode of transport. This is consistent with prior reports of MSCs establishing TNTs with multiple types of cancer cells, including MCF7 and other breast cancer cell lines(57). Discovered originally in 2004(58), and critiqued initially by some as artifacts of 2D culture(59), TNTs are now increasingly recognized as conduits for cell-cell communication in both physiology and disease(20, 37, 60, 61). They are F-actin rich structures with junctional proteins at one end(62, 63); TNTs couple with adjacent cells through gap junctions(64-67) and both share CX43 as common molecular component. We observed the formation of abundant TNTs connecting MCF7 cells with MSCs and demonstrated that they are functional using pharmacologic inhibitors, implicating gap junctions and CX43 as facilitators of transport in our cultures. The presence of EGFR on the list of 'borrowed' genes is intriguing. While we did not investigate transfer of this receptor as RNA or protein, transmembrane and membrane bound proteins have previously been reported to be transferred via TNTs(68). Most of the borrowed genes, however, represent cytoplasmic proteins and/or organelle- or cytoskeleton-associated proteins, which are known to use TNTs as a route for intercellular protein transfer(20, 37, 61). That the 483 borrowed genes carried an overwhelming message of intercellular transport structures (tube morphogenesis and development) suggests that these genes are selected for their ability to facilitate or support TNT formation, stabilization, and transport. Integration of the borrowed and intrinsic genes through PPI networks indicates that the nature of the exchange is meaningful and advantageous to tumor cells (and potentially also to the MSCs). In doing so, the TNT-dependent tumor-MSC communication we reveal here is resembles the nanotubes(69, 70) and pili-dependent communication in pathogenic biofilm-producing microbes (which are responsible for ~80% of chronic infectious diseases)(71). Unicellular pathogens deploy these strucutures to communicate with each other, or diverse host cells or other pathogens, to ultimately produce a network of extracellular grid to adopt a 'multicellular lifestyle'(72, 73). Such adaptation promotes drug resistance, survival, transfer of resistance genes, metabolic dormancy, in effect, making bacteria difficult to eradicate than when present as unicellular organisms (reviewed in)(74, 75).

Acquisition of GIV exemplifies how nanotube-dependent gene transfer benefits cancers

Our bioinformatic analyses pipeline helped prioritize one borrowed gene, *CCDC88A*/GIV, from a list of several interesting candidates. We rationalized it because of its known protumorigenic properties (outlined earlier in *Results*) and because we found it intriguing that the ER+ breast cancer cells typically silence this gene through alternative splicing(33), only to 'borrow' it back from MSCs later in the bone marrow niche (this work). We show that GIV is transferred primarily as protein (not RNA). Because protein transfer is not driven by gradients, and instead relies upon mechanisms of sorting and selective exchange, what governs such selectivity of the borrowed genes remains unclear. In the case of GIV, we show that it binds and co-localizes with CX43 near gap junctions and within TNTs and that the two proteins are co-transferred from MSCs to tumor cells in ways that

require functional gap junctions. Discovered originally as an actin binding/remodeling protein in breast cancers(32, 33), and found later to directly bind microtubules and localize to cell junctions under bioenergetic stress(40), both actin and microtubule filaments may serve as highways for transport of GIV between MSCs and tumor cells, as described previously for motor proteins(76) within TNTs. Alternatively, because GIV associates with and regulates the functions and/or the localization of diverse cellular organelles (ERGIC-Golgi vesicles(77), exocytic vesicles(78), endosomes(78, 79), autophagosomes(80)) and of cell surface receptors (e.g., EGFR, integrins, TLR4 etc.)(81), co-transport with such organelles or membrane proteins is also possible. While we did not resolve which of these mechanisms of transfer are at play, we assessed the impact of GIV transfer.

By stably expressing GIV alone (among the list of borrowed candidates) in an ER+ breast cancer cell line we recapitulated ~20% of the gene expression patterns within the borrowed and intrinsic response components. Expression of GIV in ER+ breast cancer cells conferred resistance to clinical anti-estrogen drugs and promoted early survival and dissemination of circulating breast cancer cells. *CCDC88A*/GIV is also induced when ER+ tumor cells were placed in contact cultures with fibroblasts, which is in keeping with prior work demonstrating the role of GIV in cancer-associated fibroblasts in the dissemination of cancer(82). Considering the recent revelations that GIV scaffolds a circuit for secretion coupled cellular autonomy (or growth signaling autonomy) in multicellular eukaryotic cells, it is possible that sharing of GIV between haves (stromal cells) and have nots (tumors that are originally GIV-negative) as 'ready-to-use' protein reflects a core biological phenomenon of sharing of resources during an urgent need for survival in growth factor restrictive environments. Transfer as protein bypasses the need for energy expenditure for protein translation and yet achieve higher levels of protein than possible through mRNA transfer; the latter is estimated to be never more than >1% of levels in donor cells(83). Although we found that GIV protein that was acquired during 3 days in contact culture was rapidly lost when tumor cells were returned to monoculture, prolonged contact cultures (for months and years, as happens in patients) may allow transferred GIV to initiate the GIV↔STAT3 feedforward loop shown to enhance its own transcription(84). We conclude that intercellular protein transport is a major way for cells to exchange GIV as an essential commodity within a heterotypic population of haves and have nots.

Study limitations

A detailed characterization of all that is transported was not pursued. Some genes are likely to be transported from the tumor cells to MSCs; this was not evaluated here. Tolerant immunogenic signals were induced in tumor cells during contact culture; how these signals impact the cancer cell's ability to evade the immune system and/or immune checkpoint blockade therapies will require further studies in humanized mice. TNT-mediated tumor-CAF exchanges are also known to shape tumor cell behaviors(45, 85); dedicated studies are required to further investigate the shared and distinct patters between these (and potentially others, e.g., tumor-myeloid cell) forms of cell-cell communication.

In conclusion, this work opens new horizons as to how close contact interactions and intercellular communication networks through RNA/protein transfer between ER+ breast cancer cells and MSCs reprograms tumor cells to more aggressive states. Based on the omics-based revelations and experimental evidence (this

work) and phenotypic characterization of MSC-tumor contact culture we published earlier(47), we conclude that the TNT-based cell-cell communication in the bone marrow niche (or tumor-stromal or tumor-immune cell communication in any other tissue) may provide the same to tumor cells that nanotube-based communication provides to the bacteria within biofilms; a nutrition- and metabolic stress-free haven to promote sustained survival in new environments. Blocking or redirecting these communication networks, or components transferred through these networks, offers novel opportunities to prevent or cure breast cancer metastasis to bone.

Materials and Methods

Cell culture

We purchased MCF7 (HTB-22) and T47D (HTB-133) human ER+ breast cancer cell lines, Cos7 cells, and HS5 (CRL-11882) and HS27a (CRL-2496) human bone marrow MSC line from the American Type Culture Collection (ATCC, Manassas, VA, USA). We thank Dr. James Rae (University of Michigan, Ann Arbor, MI, USA) for providing human HCC1428 ER+ breast cancer cells. We cultured HS5, MCF7, T47D, and Cos7 cells in DMEM (#11995, Gibco, Thermo Fisher, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) (HyClone, ThermoScientific, Waltham, MA, USA), 1% GlutaMAX (#35050, Gibco), and 1% Penicillin-Streptomycin (P/S, #15140, Gibco). We cultured HCC1428 and HS27a cells in RPMI (#11875, Gibco) with 10% FBS, 1% P/S, 1% Sodium Pyruvate (#11360070, Gibco), 1% HEPES (#15630080, Gibco), and 2500 mg/L glucose (#A24940, Gibco). We added 50mg/L Plasmocin™ prophylactic (Invivogen, San Diego, CA, USA) to both DMEM and RPMI media. We maintained cells as previously described (47). We performed mycoplasma testing and authenticated cell lines with short tandem repeats analysis upon initial passaging. We removed cell lines from culture after three months and replaced them with new cells from previously frozen stocks.

Vectors and stable cell lines

We previously described MCF7 cells stably transduced with click beetle green luciferase (MCF7-CBG)(86). For stable RNA interference against GIV, we transduced MCF7 and HS5 cells with shRNA against CCDC88A (clone TRCN0000129915, Millipore Sigma, Burlington, MA, USA), preparing lentiviruses as described previously (87). We used puromycin to select stably transduced cells and performed experiments with batch populations. To stably express full-length GIV in MCF7-CBG cells, we used a PiggyBac transposon with a CAG promoter driving co-expression of GIV and hygromycin linked by a P2A site (VectorBuilder, Chicago, IL, USA). We transfected cells with a 3:1 ratio of GIV transposon to Super PiggyBac Transposase (Systems Biosciences, Palo Alto, CA, USA) using Fugene-HD transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol. Two days after transfection, we selected cells with 150 µg/ml hygromycin B (ThermoFisher, Waltham, MA, USA). After two weeks of culture with hygromycin B, we returned surviving cells to normal growth medium. We transduced MCF7-CBG cells with a lentiviral vector expressing mCherry with a nuclear localization sequence as we reported previously(88).

Co-cultures of breast cancer cells and MSCs

For co-culture assays, we seeded 12,000 total cells per cm^2 in 35 mm dishes with a ratio of 1:9 ER+ breast cancer cells to MSCs. One day after seeding, we washed cells once with phosphate buffered saline (PBS) and then changed medium to phenol-red free DMEM (#A14430-01, Gibco), supplemented with 1% FBS, 1% P/S, 1% Glutamax, 1% sodium pyruvate, 10 nM β-estradiol (#E2758, Sigma-Aldrich, Millipore Sigma, Saint Louis, MO, USA), and 5 mM glucose(89). We cultured cells in low serum (1%), low glucose (4 mM), and 10 nM estrogen (LG/LF) medium for 3 days before analyzing experimental endpoints. We performed assays with conditioned medium as described previously (90).

RNA sequencing and differentially expressed gene (DEG) analysis

We separated cancer cells from stromal cells with human EpCAM (CD326) immunomagnetic beads (#130-061-101, Miltenyi, Auburn, California, USA) for RNA sequencing as described previously(15). We deposited RNA sequencing data to GEO under accession number GSE224322. Differentially expressed genes from the pairs of experimental conditions are identified using DESeq2 program in R. Genes with log fold change >=2 and a p adjusted value < 0.05 were identified and rank ordered as differentially expressed genes (DEGs) (**Supplemental Information 5**).

Hierarchical clustering of RNA seq readouts

Expression patterns of the identified 100 genes that are highly expressed in MCF7 cells post coculture with HS5 cells, are unbiasedly clustered based on their z normalized expression in all the samples using the seaborn clustermap package (v 0.12) in python.

Tandem Mass Tag™ (TMT) proteomics and analyses

MCF7 monoculture and post coculture with HS5 cells were subsequently processed for TMT proteomics using LUMOS Orbitrap-Fusion analyzer. Samples were processed at the UC San Diego Biomolecular and Proteomics Mass Spectrometry Core Facility (https://bpmsf.ucsd.edu/). Peptides are identified and mapped using Peaks X Pro pipeline. Intensity ratio of each identified proteins in MCF7 monoculture and post coculture with HS5 cells has been identified and selected if the significance score >20. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE(91) partner repository with the dataset identifier (**PXD039860**). A list of differentially expressed proteins is provided in **Supplemental Information 6**.

StepMiner analysis

StepMiner is an algorithm that identifies step-wise transitions using step function in a time-series data(92). StepMiner undergoes an adaptive regression scheme to verify the best possible up and down steps based on sum-of-square errors. The steps are placed between time points at the sharpest change between expression levels, which gives us the information about timing of the gene expression-switching event. To fit a step function, the algorithm evaluates all possible steps for each position and computes the average of the values on both sides of a step for the constant segments. An adaptive regression scheme is used that chooses the step positions that minimize the square error with the fitted data. Finally, a regression test statistic is computed as follows:

$$F\ stat = \frac{\sum_{i=1}^{n}(\widehat{X}_i - \bar{X})^2/(m-1)}{\sum_{i=1}^{n}(X_i - \widehat{X}_i)^2/(n-m)}$$

Where X_i for $i = 1$ to n are the values, $\widehat{X}_i$ for $i = 1$ to n are fitted values. m is the degrees of freedom used for the adaptive regression analysis. $\bar{X}$ is the average of all the values: $\bar{X} = \frac{1}{n} * \sum_{j=1}^{n} X_j$. For a step position at k, the fitted values $\widehat{X}_l$ are computed by using $\frac{1}{k} * \sum_{j=1}^{n} X_j$ for $i = 1$ to k and $\frac{1}{(n-k)} * \sum_{j=k+1}^{n} X_j$ for $i = k+1$ to n.

Composite gene signature analysis using Boolean Network Explorer (BoNE)

Boolean network explorer (BoNE)(93) provides an integrated platform for the construction, visualization and querying of a gene expression signature underlying a disease or a biological process in three steps: First, the expression levels of all genes in these datasets were converted to binary values (high or low) using the StepMiner algorithm. Second, Gene expression values were normalized according to a modified Z-score approach centered around *StepMiner* threshold (formula = (expr - SThr)/3*stddev). Third, the normalized expression values for every genes were added together to create the final score for the gene signature. The samples were ordered based on the final signature score. Classification of sample categories using this ordering is measured by ROC-AUC (Receiver Operating Characteristics Area Under The Curve) values. Welch's Two Sample t-test (unpaired, unequal variance (equal_var=False), and unequal sample size) parameters were used to compare the differential signature score in different sample categories. Violin, Swarm and Bubble plots are created using python seaborn package version 0.10.1. Pathway enrichment analyses for genes were carried out via the reactome database (https://reactome.org/) and algorithm(94). Violin plots are created using python seaborn package version 0.10.1.

Survival outcome analyses

Kaplan-Meier (KM) analyses were done for gene signature identified from transcriptome and proteome overlap. The high and low groups were separated based on *StepMiner* threshold on the composite score of the gene expression values. The statistical significance of KM plots was assessed by log rank test. Kaplan-Meier analyses were performed using lifelines python package version 0.14.6.

Flow cytometry for intercellular transfer of calcein, AM

To measure transfer of dye between cells by intercellular connections, we incubated MCF7 or HS5 cells with 10 µM calcein, AM (C3099, Thermo Fisher Scientific) in LG/LF medium for one hour. We then washed cells three times with PBS and seeded labeled MCF7 or HS5 cells with MCF7-mCherry cells at a 9:1 ratio in 10 cm dishes using the cell density described above for co-culture assays. After three days in co-culture, we analyzed amounts of calcein, AM dye in MCF7-mCherry cells by flow cytometry presented as histogram plots.

Immunogold electron microscopy

We plated MCF7 monocultures or co-cultures of MCF7 and HS5 cells in 10 cm dishes at densities described above for electron microscopy. After culturing cells for three days in LG/LF medium, we washed cells twice with PBS and then fixed cells with 2% paraformaldehyde and 0.2% glutaraldehyde (#15700 and #16200, respectively, Electron Microscopy Services, Hatfield, PA, USA) for one hour at 4°C. We then replaced the solution with ice-cold 4% paraformaldehyde with 0.1% Probumin® BSA in 200 mM HEPES and scraped the cells with a soft Teflon scraper (07-200-366, Thermo Fisher). The frozen sample blocks were cryosectioned (60-70 nm thick section 10 µm apart) on EM grid. For immunolabelling, grids were washed with 2% gelatin (cat# ES-006-B; Sigma-Aldrich) in phosphate buffer at 30-37 °C for 15-20 min followed by 10-20 mM (~0.15%) glycine for 5 min

and 1% BSA/PBS for 2 min. Grids were incubated with 1:1 anti CX43 antibody (mouse mAb, epitope aa 247-382; BioLegend Cat#850001) and 1:10 anti GIV antibody (Cat#ABT80; Millipore) in 1% BSA/PBS for 1 hour at room temperature. For gold labelling the grids were incubated with Goat Anti-Mouse IgG tagged with 12 nm Colloidal Gold (Jackson Immuno research lab cat#115-205-068) and Goat Anti-Rabbit IgG tagged with 6 nm Colloidal Gold (Jackson Immuno research lab cat#111-195-144) diluted in 1:1 in 1% BSA for 1 h at room temperature followed by washing with PBS for 4 times. Then the grids were incubated with 1% Glutaraldehyde in PBS for 5 min. Then the grids were rinsed with distilled water 6 times. For contrast enhancement, the grids were incubated with 2% uranyl acetate at pH 7 for 5 min and in uranyl acetate (4%) (cat# 21447-25; polysciences) and methylcellulose (2%) (cat# 9004-67-5; sigma aldrich) mixture for 5 min. Then the grids were dried in a wire loop at room temperature for 15 min. After that, the grids were mounted in Jeol 1400 plus Transmission Electron Microscope for visualization.

Immunoblotting

For immunoblotting in combination with GST pull-down assays (see below), we transfected Cos7 cells with Cx43-GFP (gift from David Spray (Addgene plasmid #69007; http://n2t.net/addgene:69007;RRID:Addgene_69007) (95)) using polyethylenimine (PEI) following the manufacturer's protocol as described in prior work(96, 97). Whole-cell lysates were prepared after washing cells with cold PBS before re-suspending and boiling them in sample buffer. For immunoblotting, protein samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore Sigma, MO, USA). The membrane was stained with Ponceau S to visualize proteins Membranes were blocked with PBS supplemented with 5% nonfat milk before incubation with primary antibodies. Infrared imaging with dual-color detection and quantification were performed using a Li-Cor Odyssey imaging system. The anti-CX43 (Biogened, CA, 247-382) antibody was used at 1:1000 v/v dilution. Figures were assembled for presentation using the Photoshop (Adobe, San Jose, CA, USA) and Illustrator (Adobe, San Jose, CA, USA). For co-culture experiments, we separated cancer cells from HS5 cells using EpCAM immunomagnetic beads as described above. We made whole-cell lysates using RIPA lysis buffer and quantified amounts of protein per sample by BCA assay (23235, Thermo Fisher Scientific). We used primary antibodies against GIV (H-6, Santa Cruz Biotechnology, Dallas, TX, USA), CX43 (#3512, Cell Signaling Technology, Danvers, MA, USA), or β-Actin (#4970, Cell Signaling Technology) and detected primary antibodies with appropriate anti-mouse or anti-rabbit secondary antibodies (ThermoFisher) conjugated to horseradish peroxidase. We developed blots with ECL plus reagent (ThermoFisher) and quantified bands using ImageJ.

GST pull-down assays

Recombinant GST proteins were expressed in E. coli stain BL21 (DE3) and purified as previously described. Briefly, cultures were induced using 1mM IPTG overnight at 25°C. Cells were then pelleted and resuspended in either GST lysis buffer (25 mM Tris-HCl, pH 7.5, 20 mM NaCl, 1 mM EDTA, 20% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 2×protease inhibitor cocktail). Recombinant GST alone (control) or GST-tagged GIV proteins, GST-GIV-NT (1-166 aa) and GST-GIV-CT (1660-1870 aa) from *E. coli* were immobilized onto glutathione-Sepharose beads and incubated with binding buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.4% (v:v) Nonidet P-40, 10

mM MgCl2, 5 mM EDTA, 2 mM DTT, 1X Complete protease inhibitor) overnight at 4oC temperature. For binding with cell lysates, cells were lysed in cell lysis buffer (20 mM HEPES pH 7.2, 5 mM Mg-acetate, 125 mM K-acetate, 0.4% Triton X-100, 1 mM DTT, 500 μM sodium orthovanadate, phosphatase inhibitor cocktail (Sigma-Aldrich, MO, USA) and protease inhibitor cocktail (Roche Life Science) using a 28G syringe, followed by centrifugation at 10,000Xg for 20 min. Cleared supernatant was then used in binding reaction with immobilized GST-proteins for 4 h at 4°C. After binding, bound complexes were washed four times with 1 ml phosphate wash buffer (4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% (v:v) Tween 20, 10 mM MgCl2, 5 mM EDTA, 2 mM DTT, 0.5 mM sodium orthovanadate). Bound proteins were then eluted through boiling at 100°C in Laemmli sample buffer (BIORAD, CA, USA). The bound proteins were eluted at 37ºC for 10 min.

Cell viability assays

For viability assays, we seeded MCF7-CBG or MCF7-CBG-GIV cells at 10,000 cells per well in black wall 96 well plates. We washed cells once PBS and then added various concentrations of tamoxifen, fulvestrant, or fulvestrant with 100 nM palbociclib (all from Cayman Chemical, Ann Arbor, MI, USA) in LG/LF medium with 4 wells per condition. Control wells received vehicle only. After three days in culture, we quantified relative numbers of viable cells by bioluminescence imaging using an IVIS Lumina system with Living Image 4.3.1 software (Perkin Elmer, Waltham, MA, USA) as described previously(47). We plotted data fraction change from vehicle only wells, which we defined as 1.

Animal studies

The University of Michigan Institutional Animal Care and Use Committee approved all experiments. We seeded MCF7-CBG or MCF7-CBG-GIV cells in T175 flasks. The following day, we changed medium to LG/LF medium without added estrogen and cultured cells overnight before harvesting cells with cell dissociation buffer (Gibco, ThermoFisher Scientific). We resuspended cells at 1 x 10^5 cells per 100 μl sterile 0.9% NaCl for intracardiac injection. Prior to injection, we verified equal numbers of viable cells based on bioluminescence imaging of aliquots of each cell type. To generate systemic metastases, we injected 1 x 10^5 MCF7-CBG or MCF7-CBG-GIV cells into the left ventricle of 7–10-week-old female NSG mice (7-8 mice per group) (The Jackson Lab, Bar Harbor, ME, USA) as described previously by our lab (89).

We performed bioluminescence imaging (IVIS Spectrum, Perkin Elmer) on mice at days indicated in the figure legend. At the end of the study, we measured metastases in defined organs by ex vivo bioluminescence imaging as we reported previously(89). We quantified total bioluminescence signal above background using Living Image software.

Study Approval

The University of Michigan IACUC approved all animal procedures under protocol number PRO00010534.

Statistics

We performed all cell-based experiments at least three times and presented results as either a representative experiment or average of experiments ± SEM. For statistical analyses, we used python, R, GraphPad Prism software (San Diego, CA, USA) or MATLAB (Mathworks, Natick, MA, USA). $P<0.05$ defined statistical significance for methods listed in figure legends. We assessed statistical significance of Kaplan-Meier plots by log rank test. Kaplan-Meier analyses were performed using lifelines python package version 0.14.6.

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AUTHOR CONTRIBUTIONS:

S.S, supervised by P.G, carried out and analyzed all electron microscopy, RNA seq, TMT proteomics and computational analyses showcased in this work. Su.R. carried out protein purification and GST pulldown assays. B.W.C, A.P.F., S.C., Sh.R., and J.M.B performed mouse experiments and cell-based assays. K.E.L. provided new reagents. B.W.C. S.S, P.G. and G.D.L. assembled the figures. K.E.L., Su.R., S.S., P.G., and G.D.L. helped write methods and edit the manuscript. G.D.L. and P.G conceptualized, designed, supervised, and analyzed the experiments. K.E.L, S.S. P.G and G.D.L wrote the manuscript. All authors and approved the manuscript prior to submission.

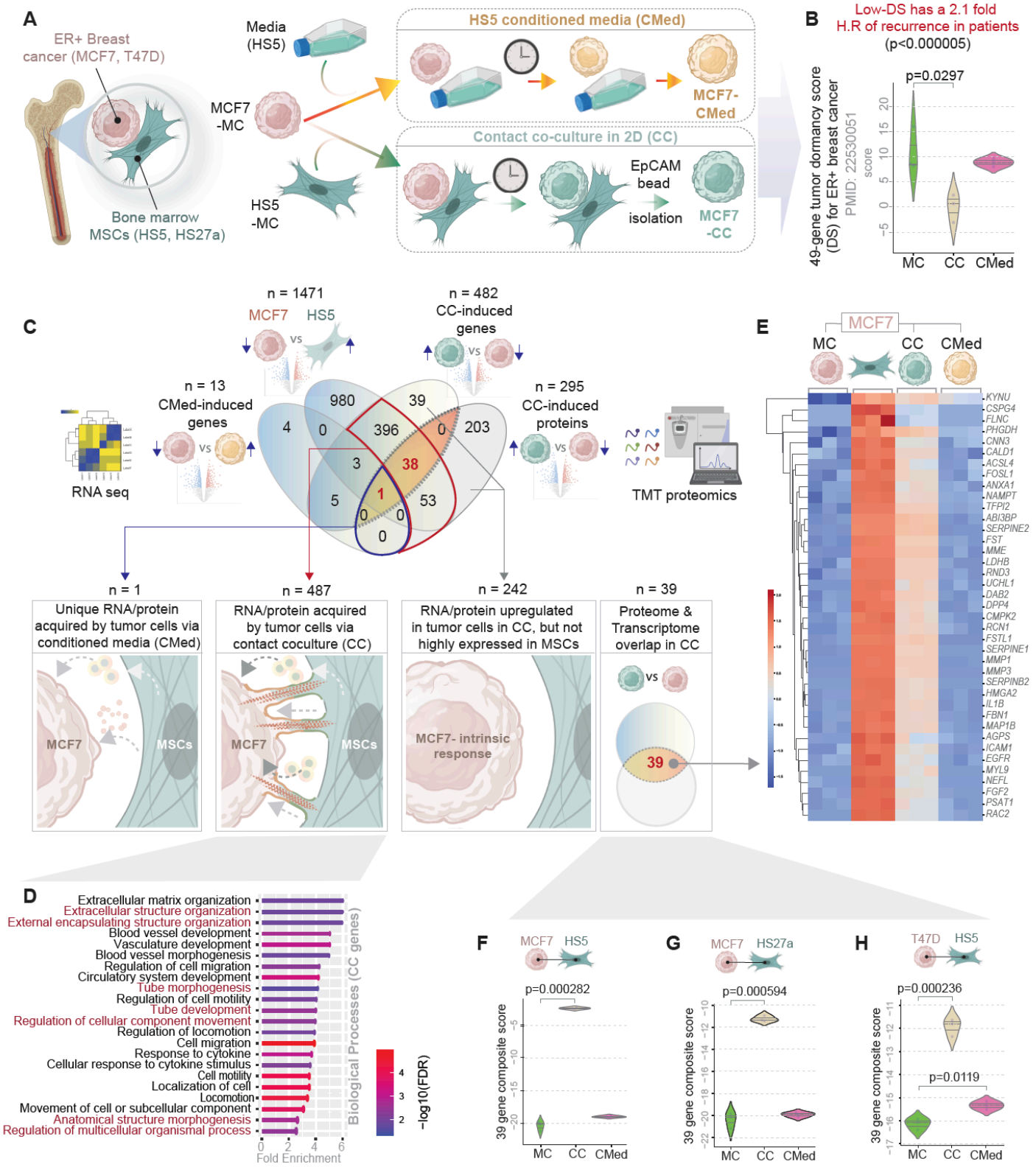
CONFLICT OF INTEREST DISCLOSURE STATEMENT

G.D.L. has received research materials from Spexis. All other authors declare no competing interests.

DATA AVAILABILITY

Data are available via ProteomeXchange with identifier PXD039860 and Gene Expression Omnibus with id GSE224322. The computational analyses can be found at https://github.com/sinha7290/cx43

3 ## FIGURE AND LEGENDS



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5 **Figure 1. Multiomic analysis reveals RNA/protein acquired by ER+ breast cancer cells from MSCs in**
6 **contact coculture.**

A. Schematic of the general experimental set up for recapitulating disseminated ER+ breast tumor cells in the bone marrow amidst mesenchymal stem cells (MSCs). Two different types of coculture models were used, one with conditioned media (CMed; *top*) and one that involves direct contact co-culture (CC; *bottom*). MC, monoculture.

B. Violin plots display the composite score of a 49-gene tumor dormancy score (DS), which was determined as specific for ER+ breast cancers and previously validated in 4 independent cohorts to be predictive of tumor recurrence. Statistical significance was assessed by Welch's t-test (n = 3 in each comparator group). Only significant p values are displayed.

C. *Top*: Venn diagram depicts the outcome of multiple DEG analyses between different indicated comparator groups that catalogs the number of suppressed genes or proteins in contact co-culture (CC) or conditioned media (CMed) and the overall differential gene expression between the two cell types (MCF7 cells vs the HS5 bone marrow MSCs; bottom). *Bottom*: Genes/proteins that are induced in MCF7 tumor cells in coculture with HS5 MSCs are binned into 3 categories (connected by arrows) based on the likely mechanisms for such induction. Red = 39 uniquely upregulated transcripts in tumor cells in contact co-culture that were also identified by proteomics. See also **Supplementary Figure 1** for suppression of genes and proteins upon coculture.

D. Results of a gene ontology analysis (GO Biological processes) on the transcripts and/or proteins that are acquired by MCF7 cells from MSCs during contact coculture. See also **Supplementary Figure 2** for Reactome pathway and GO enrichment analyses on the genes within each category in C.

E. Heatmap displays the findings of hierarchical unsupervised clustering of samples using the 39 genes identified in C.

F-H. Violin plots display the composite score of the 39 genes in E in various tumor cell-MSC co-culture models, e.g., MCF7↔HS5 (F), MCF7↔HS27a (G) and T47D↔HS5 (H). Statistical significance was assessed by Welch's t-test (n = 3 in each comparator group). Only significant p values are displayed.

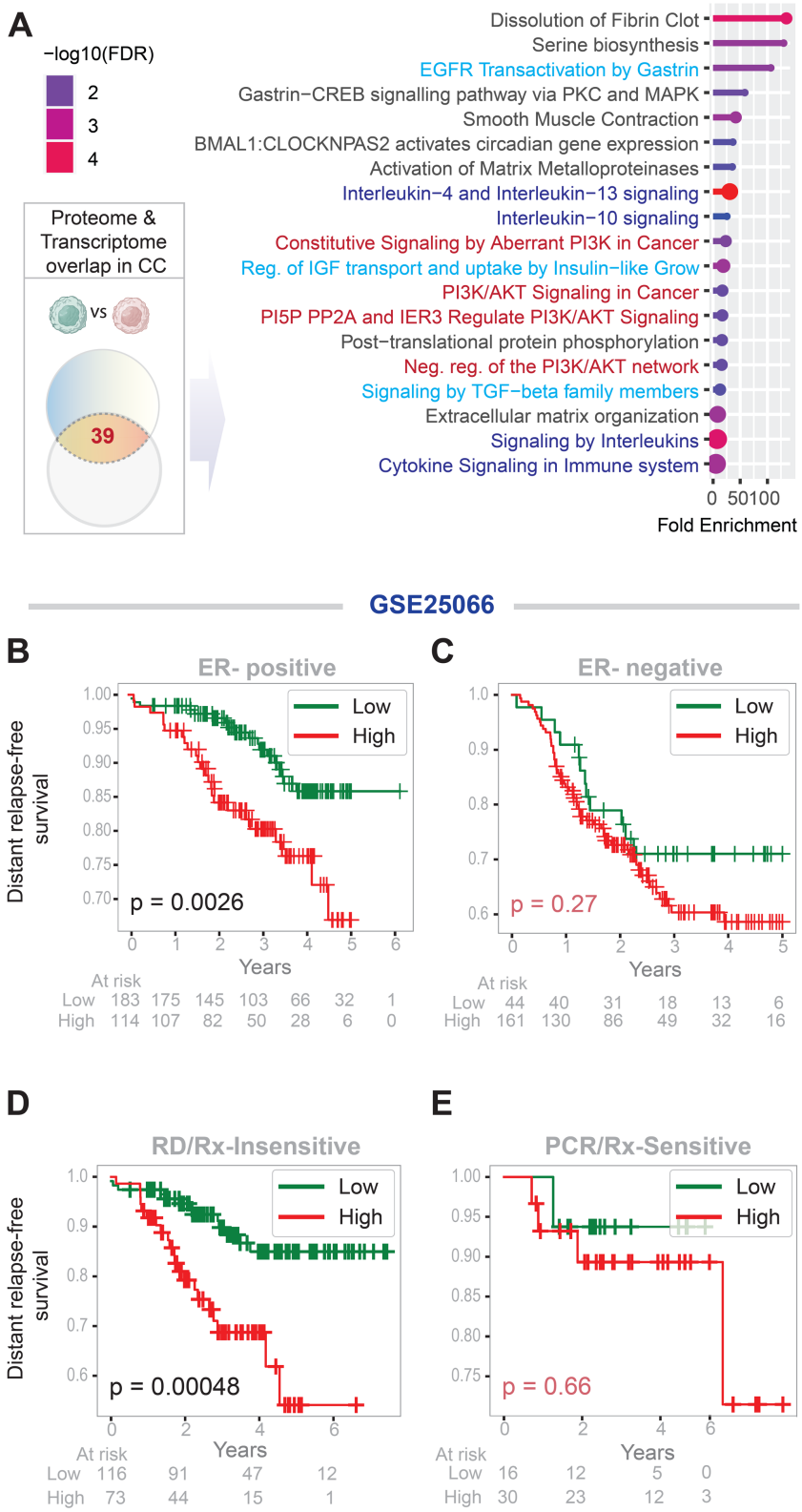


Figure 2. A contact culture signature derived from proteome-transcriptome overlap carries prognostic and predictive value in ER-positive breast cancer.

A. Reactome pathways enriched in the 39 genes identified in **Figure 1C**. The PI3K-Akt signaling pathway (red), growth factor signaling pathways (teal blue) and the tolerogenic cytokine pathways (navy blue) are highlighted.

B-E. Kaplan Meier survival plots on a cohort of breast cancer patients with known outcome over time (relapse/metastasis-free survival), stratified as high vs low expression of the 39-gene signature. Statistical significance was assessed by Log Rank analyses.

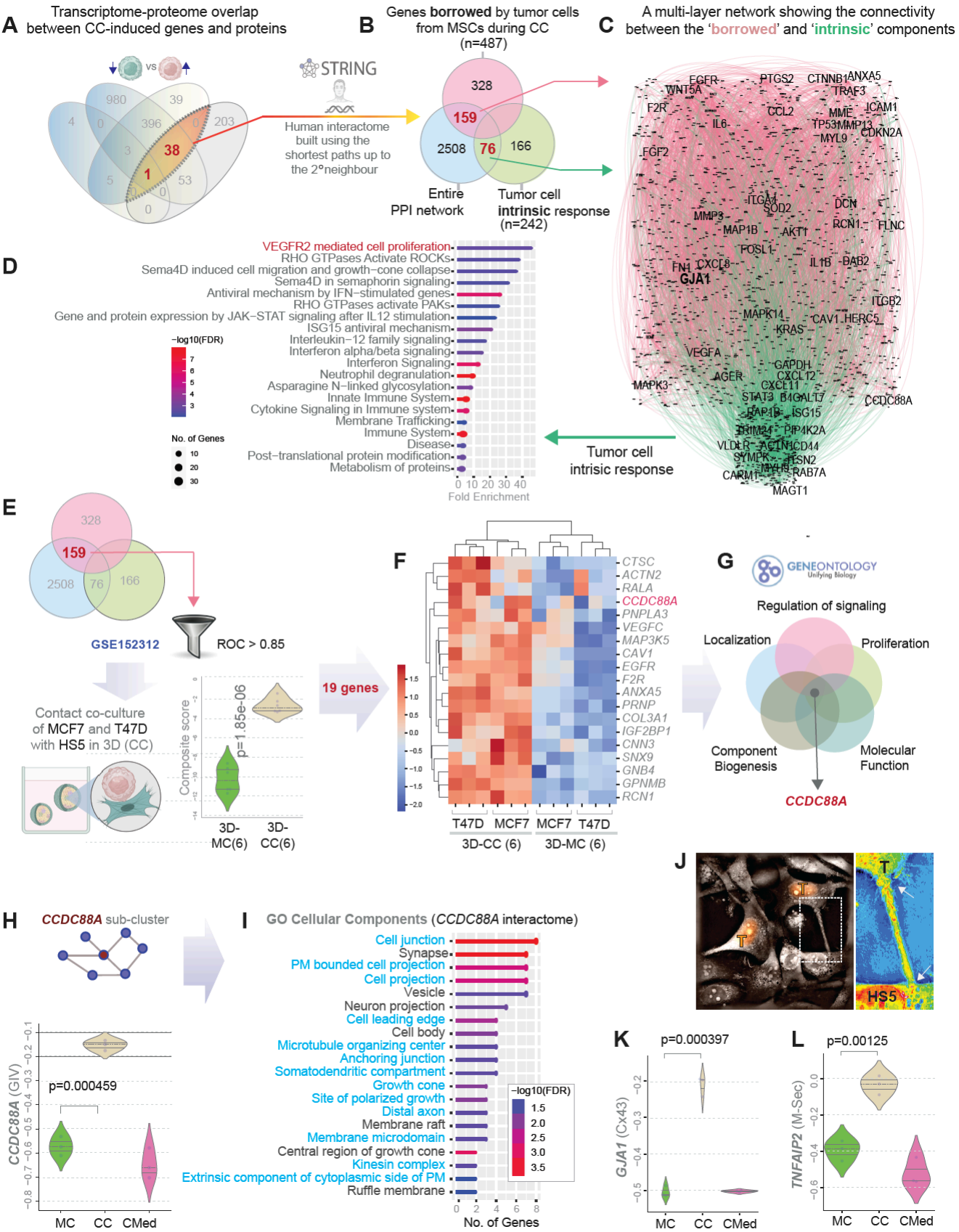


Figure 3. Network analysis pinpoints GIV as a central orchestrator of the co-culture borrowed gene signature.

A. Schematic shows the workflow for protein-protein interaction (PPI) network analysis. The 39 gene signature from Figure 1E is used as 'seeds' to build a PPI network using the STRING database (see *Methods*).

B. Venn diagram showing the overlaps between the resultant PPI network and the candidate genes/proteins that were identified in **Figure 1C** as either likely to be 'borrowed' from MSCs or upregulated as part of tumor cell's 'intrinsic' response during contact co-cultures.

C. A multi-layer PPI network shows connectivity between 'borrowed' (n = 159) and 'intrinsic' (n = 76) proteins. Key nodes in the network are labeled.

D. Reactome pathways enriched in the 76 proteins that constitute intrinsic responses of breast cancer cells to contact co-culture.

E. The 158 'borrowed' genes identified in our 2D co-culture models were further filtered using a publicly available dataset generated from 3D co-culture models wherein MCF7 and T47D cells were grown with HS5 MSCs. A threshold of ROC AUC > 0.85 led to the identification of 19 genes. A violin plot shows the composite score of the expression of 19 genes in the 3D mono (MC) vs contact (CC) cultures. Statistical significance was assessed by Welch's t-test.

F. A heatmap of z-score normalized expression of the 19 genes. MC, monoculture; CC, contact culture.

G. Venn diagram summarizes findings of gene ontology (GO) analyses, identifying *CCDC88A* as an invariant player across diverse biological processes enriched within the 19 genes.

H-I. Violin plot (H) shows the patterns of induction of *CCDC88A* in MCF7 cells when subjected to contact coculture (CC) but not when grown in conditioned media (CMed). Statistical significance was assessed by Welch's t-test. Only significant p values are displayed. Graph (I) displays the GO cellular components enriched within the *CCDC88A*/GIV-subcluster within the PPI network in C. Components highlighted in blue indicate processes that are required for enabling long-range intercellular transport via tunneling nanotubes.

J. Images obtained by inferometry microscopy of MCF7 cells (red nuclei; "T") in contact coculture with MSCs (HS5) with nanotube connecting the cells. Boxed area on left is magnified on the right. Arrows mark the two ends of the nanotube. See also **Supplementary Figure 3** for a montage of images of MCF7 cells in mono- (MC) or contact coculture (CC) with HS5 MSCs.

K-L. Violin plots show the patterns of induction of *GJA1* (K) and *TNFAIP2* (L) in MCF7 cells when subjected to contact coculture (CC) but not when grown in conditioned media (CMed). Statistical significance was assessed by Welch's t-test. Only significant p values are displayed.

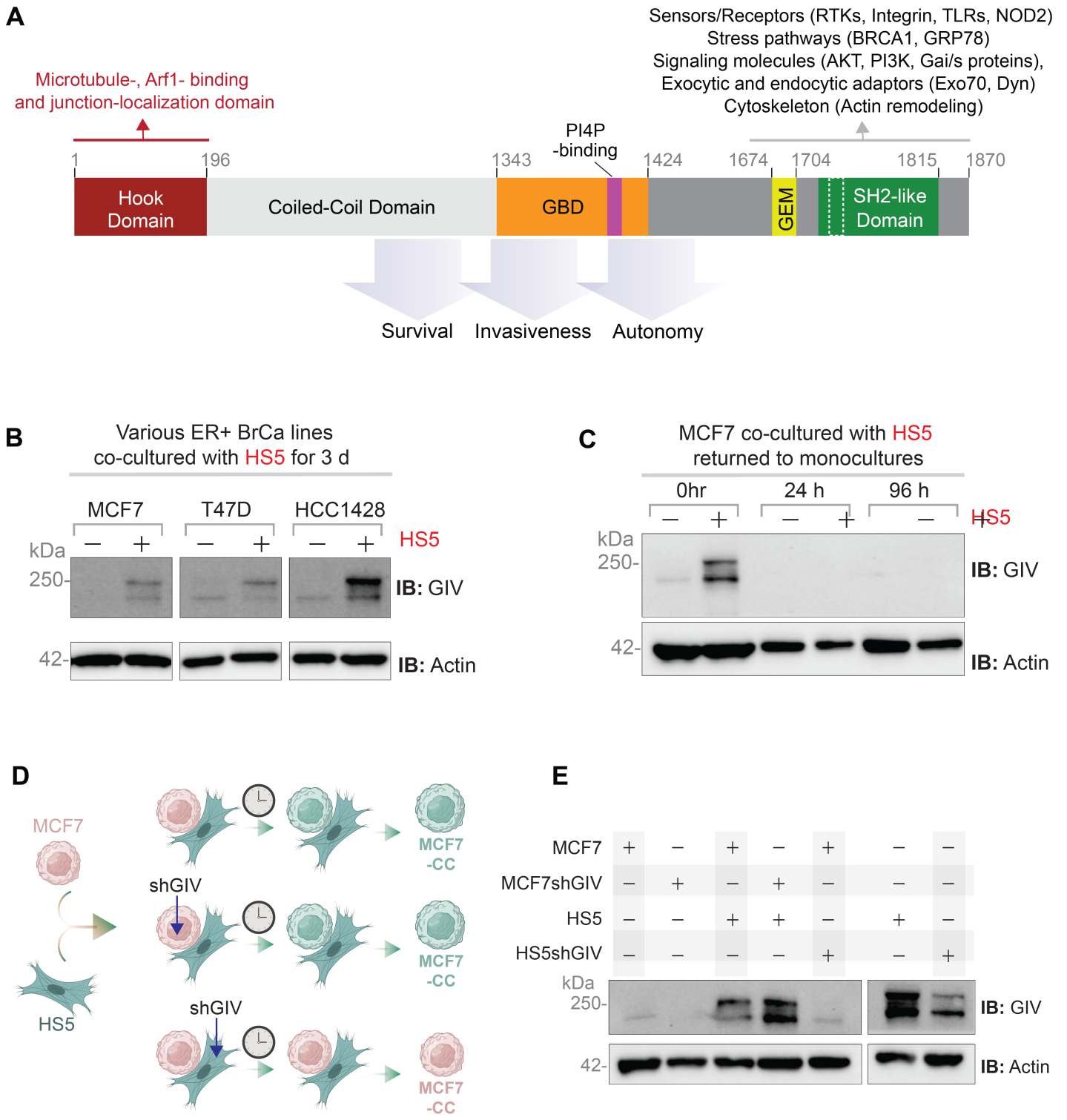


Figure 4. GIV transport from MSCs to ER+ breast cancer cells requires ongoing interactions.

A. Schematic illustrates key functional domains of GIV. The C-terminus contains multiple short linear motifs that enable diverse tumor-promoting interactions (receptors, signaling molecules within diverse pathways, and components of membrane trafficking). GBD, G protein binding domain; GEM, guanine nucleotide exchange modulator; SH2, Src-like homology domain.

B. Equal aliquots of lysates of 3 ER+ breast cancer cells (MCF7, T47D or HCC1428) in monoculture or recovered after co-culture with HS5 cells were analyzed for GIV and actin (as a loading control) by immunoblotting (IB).

C. Equal aliquots of lysates of MCF7 cells prepared either immediately after recovery from co-cultures with HS5 cells (0 h) or after an additional 24 or 96 h of culture after removal from HS5 contact were analyzed for GIV and actin (as a loading control) by immunoblotting (IB).

D-E. Schematic (D) outlines the key manipulations (i.e., treatment with shRNA for GIV) done to either MCF7 or HS5 cells in contact co-cultures. Equal aliquots of MCF7 cells recovered from the co-cultures (left) or HS5 cells in monocultures were analyzed for GIV and action (loading control) by immunoblotting (IB).

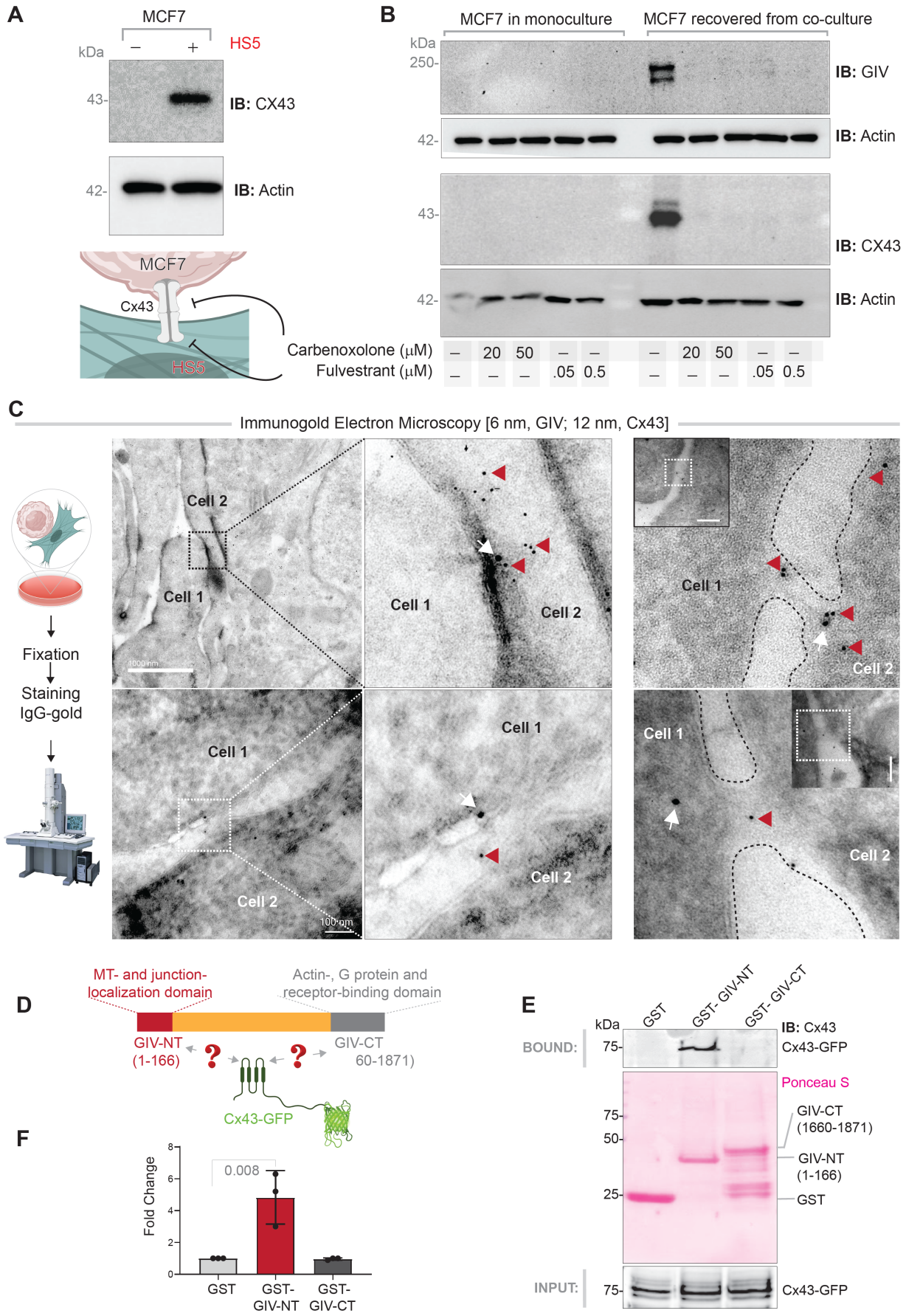


Figure 5. Cx43 and GIV interact and are co-transported through intercellular communication sites.

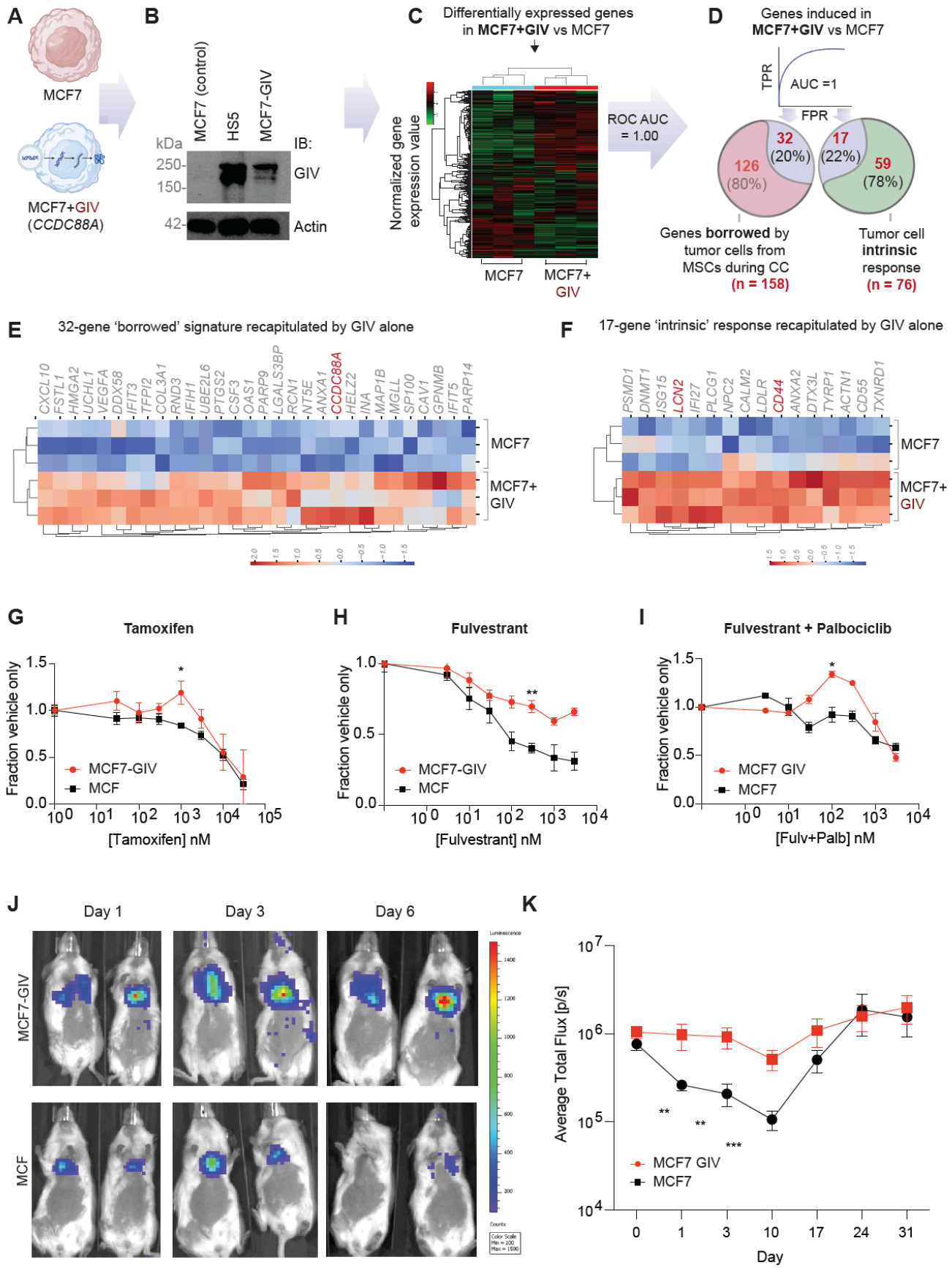
A. Equal aliquots of lysates of MCF7 cells in monoculture or recovered after co-culture with HS5 cells were analyzed for Cx43 and actin (as a loading control) by immunoblotting (IB).

B. MCF7 cells in monoculture or in co-culture with HS5 cells were treated with the indicated concentrations of Carbenoxolone or Fulvestrant prior to lysis. Equal aliquots of lysates were analyzed for GIV, Cx43 or actin (the latter as a loading control) by immunoblotting (IB). See **Supplementary Figure 5** for similar studies on HS5 monocultures.

C. Electron micrographs of immunogold stained MCF7↔HS5 contact co-cultures. Red arrowhead denotes 6 nm gold particles (GIV); White arrow denotes 12 nm gold particles (Cx43). Scale bar = 100 μm.

D-F. Schematic of constructs (D) used in pull-down assays using recombinant GST-tagged GIV N- or C-term fragments or GST alone (Neg control) proteins immobilized on glutathione beads and lysates of Cos7 cells transiently expressing GFP-Cx43. Bound Cx43 was visualized by immunoblotting (E-top). Equal loading of GST proteins was confirmed by Ponceau S staining (C-bottom). Bar graphs (F) display the relative binding of Cx43 to various GST proteins. Data are represented as mean ± SEM (n = 3); $**p=<0.01$ determined by one-way ANOVA.

5


A
MCF7
MCF7+GIV
(CCDC88A)
B
MCF7 (control)
HS5
MCF7-GIV
kDa
IB:
250-
150-
GIV
42-
Actin
C
Differentially expressed genes in MCF7+GIV vs MCF7
Normalized gene expression value
MCF7
MCF7+ GIV
ROC AUC = 1.00
D
Genes induced in MCF7+GIV vs MCF7
TPR
AUC =1
FPR
126 (80%)
32 (20%)
17 (22%)
59 (78%)
Genes borrowed by tumor cells from MSCs during CC (n = 158)
Tumor cell intrinsic response (n = 76)
E
32-gene 'borrowed' signature recapitulated by GIV alone
CXCL10 FSTL1 HMGA2 UCHL1 VEGFA DDX58 IFIT3 TFPI2 COL3A1 RND3 IFIH1 UBE2L6 PTGS2 CSF3 OAS1 PARP9 LGALS3BP RCN1 NT5E ANXA1 CCDC88A HELZ2 INA MAP1B MGLL SP100 CAV1 GPNMB IFIT5 PARP14
MCF7
MCF7+ GIV
F
17-gene 'intrinsic' response recapitulated by GIV alone
PSMD1 DNMT1 ISG15 LCN2 IFI27 PLCG1 NPC2 CALM2 LDLR CD44 ANXA2 DTX3L TYRP1 ACTN1 CD55 TXNRD1
MCF7
MCF7+ GIV
G
Tamoxifen
Fraction vehicle only
MCF7-GIV
MCF
[Tamoxifen] nM
H
Fulvestrant
Fraction vehicle only
MCF7-GIV
MCF
[Fulvestrant] nM
I
Fulvestrant + Palbociclib
Fraction vehicle only
MCF7 GIV
MCF7
[Fulv+Palb] nM
J
Day 1
Day 3
Day 6
MCF7-GIV
MCF
K
Average Total Flux [p/s]
MCF7 GIV
MCF7
Day

6

Figure 6. Expression of GIV recapitulates the MSC close contact signature;confers resistance to ER-targeted therapies; and promotes early dissemination and survival of ER+ breast cancer cells.

A. Schematic shows creation of a stable MCF7 cell population with expression of exogenous GIV (CCDC88A) by a piggyBac transposase vector.

B. Immunoblots of equal aliquots of whole cell lysates of cells in A, confirming the expression of full-length GIV.

C-D. Workflow (C) of RNA seq and normalized gene expression analysis of the cells in B, analyzed for the ability of the 'borrowed' (n=158) and 'intrinsic' (n=76) genes to perfectly classify the cells with GIV (MCF7-GIV) from those without (MCF7) (ROC-AUC 1.00). Venn diagram (D) shows the genes induced with GIV alone, as % of the total 'borrowed' and 'intrinsic' signatures.

E-F: Heatmaps display the hierarchical unsupervised clustering of MCF7 and MCF7-GIV cells by z-score normalized gene expression patterns for the subset of genes that are induced among the borrowed (E) and intrinsic (F) signatures. See also **Supplementary Figure 6** for reactome pathway analysis of these genes.

G-I. Graphs display cell viability as determined by bioluminescence when MCF7-GIV and control MCF7 cells were exposed to increasing concentrations of tamoxifen (G), fulvestrant (H), or fulvestrant and 100 nM palpociclib (I). Data are displayed after normalization to viable cells treated with vehicle only. Graphs show mean values ± SEM. *, $p < 0.05$ and **, $p < 0.01$ by AUC analysis.

J-K. Equal number (1×10^5) of MCF7-GIV or control MCF7 cells were injected into the left cardiac ventricle of 7-10-wk-old female NSG mice (n=8 per group). Representative bioluminescence images (J) of mice at day 1-, 3-, and 6-days post injection are shown. Images are displayed on a constant pseudocolor scale for photon flux with red and blue depicting high and low values, respectively. Graphs (K) show mean values ± SEM for photon flux for mice with MCF7-GIV and MCF7 cells at listed times after intracardiac injection. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.005$ at selected concentrations by Mann-Whitney U test.

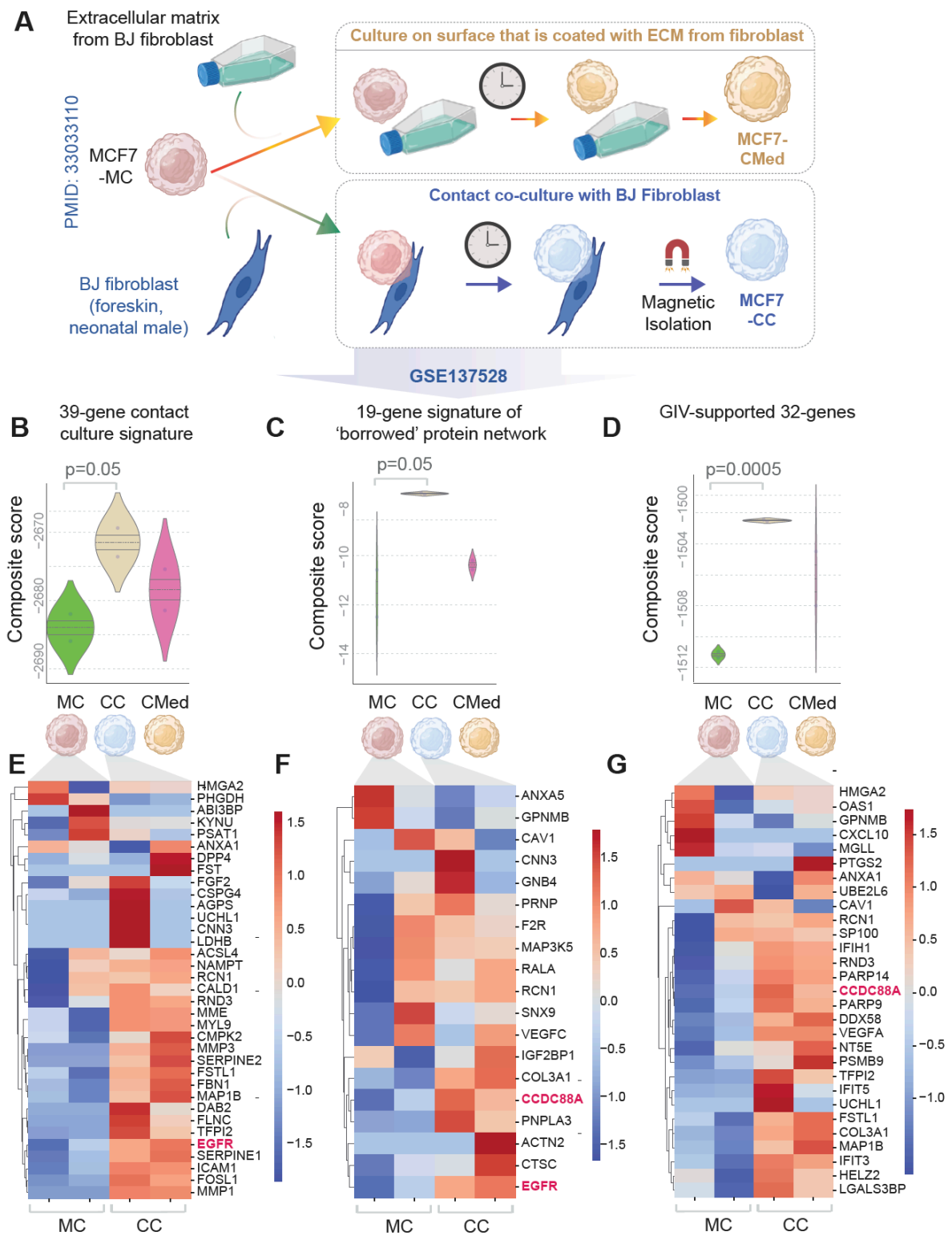


Figure 7. MCF7 cells borrow *CCDC88A* and other key genes during contact culture with fibroblasts

A. Schematic of the general experimental setup in which MCF7 cells were grown either as monoculture (MC), or in direct contact cultures with BJ fibroblasts (CC) or cultured on ECM produced by the BJ fibroblasts (CMed).

B-D. Violin plots show the degrees of induction of 39-gene 2D contact culture signature (**B**), the 19-gene signature of network of 'borrowed' proteins/transcripts that survived refinement through 3D contact cultures (**C**), and GIV-supported 'borrowed' transcriptional program of 32 genes (as in **Fig 5D**). p-values are calculated using a two-tailed t-test.

E-G. Heatmaps show the induction pattern of each gene within the signatures described in B-D. Color key denotes z-score normalized counts per million (cpm).

References Cited

1. Kennecke H, Yerushalmi R, Woods R, Cheang MC, Voduc D, Speers CH, et al. Metastatic behavior of breast cancer subtypes. *J Clin Oncol.* 2010;28(20):3271-7.
2. Coleman RE, Croucher PI, Padhani AR, Clézardin P, Chow E, Fallon M, et al. Bone metastases. *Nat Rev Dis Primers.* 2020;6(1):83.
3. Weilbaecher KN, Guise TA, and McCauley LK. Cancer to bone: a fatal attraction. *Nat Rev Cancer.* 2011;11(6):411-25.
4. Pan H, Gray R, Braybrooke J, Davies C, Taylor C, McGale P, et al. 20-Year Risks of Breast-Cancer Recurrence after Stopping Endocrine Therapy at 5 Years. *N Engl J Med.* 2017;377(19):1836-46.
5. Beuzeboc P, and Scholl S. Prevention of Bone Metastases in Breast Cancer Patients. Therapeutic Perspectives. *J Clin Med.* 2014;3(2):521-36.
6. Smart E, Semina SE, and Frasor J. Update on the Role of NFκB in Promoting Aggressive Phenotypes of Estrogen Receptor-Positive Breast Cancer. *Endocrinology.* 2020;161(10).
7. Abd El-Hafeez AA, Sun N, Chakraborty A, Ear J, Roy S, Chamarthi P, et al. Regulation of DNA damage response by trimeric G-proteins. *iScience.* 2023;26(2):105973.
8. Pasquier J, Guerrouahen BS, Al Thawadi H, Ghiabi P, Maleki M, Abu-Kaoud N, et al. Preferential transfer of mitochondria from endothelial to cancer cells through tunneling nanotubes modulates chemoresistance. *J Transl Med.* 2013;11:94.
9. Imai Y, Kuba K, Rao S, Huan Y, Guo F, Guan B, et al. Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature.* 2005;436(7047):112-6.
10. Martin FT, Dwyer RM, Kelly J, Khan S, Murphy JM, Curran C, et al. Potential role of mesenchymal stem cells (MSCs) in the breast tumour microenvironment: stimulation of epithelial to mesenchymal transition (EMT). *Breast Cancer Res Treat.* 2010;124(2):317-26.
11. Wang R, Wang X, Yin L, Chu GC, Hu P, Ou Y, et al. Breast Cancer MCF-7 Cells Acquire Heterogeneity during Successive Co-Culture with Hematopoietic and Bone Marrow-Derived Mesenchymal Stem/Stromal Cells. *Cells.* 2022;11(22).
12. Nielsen MS, Axelsen LN, Sorgen PL, Verma V, Delmar M, and Holstein-Rathlou NH. Gap junctions. *Compr Physiol.* 2012;2(3):1981-2035.
13. Wu JI, and Wang LH. Emerging roles of gap junction proteins connexins in cancer metastasis, chemoresistance and clinical application. *J Biomed Sci.* 2019;26(1):8.
14. Shiozawa Y, Pienta KJ, and Taichman RS. Hematopoietic stem cell niche is a potential therapeutic target for bone metastatic tumors. *Clin Cancer Res.* 2011;17(17):5553-8.
15. Buschhaus JM, Rajendran S, Humphries BA, Cutter AC, Muñiz AJ, Ciavattone NG, et al. Effects of iron modulation on mesenchymal stem cell-induced drug resistance in estrogen receptor-positive breast cancer. *Oncogene.* 2022;41(29):3705-18.
16. Kim RS, Avivar-Valderas A, Estrada Y, Bragado P, Sosa MS, Aguirre-Ghiso JA, et al. Dormancy signatures and metastasis in estrogen receptor positive and negative breast cancer. *PLoS One.* 2012;7(4):e35569.
17. Hatzis C, Pusztai L, Valero V, Booser DJ, Esserman L, Lluch A, et al. A genomic predictor of response and survival following taxane-anthracycline chemotherapy for invasive breast cancer. *JAMA.* 2011;305(18):1873-81.
18. Tishchenko A, Azorín DD, Vidal-Brime L, Muñoz MJ, Arenas PJ, Pearce C, et al. Cx43 and Associated Cell Signaling Pathways Regulate Tunneling Nanotubes in Breast Cancer Cells. *Cancers (Basel).* 2020;12(10).
19. Dhimolea E, de Matos Simoes R, Kansara D, Weng X, Sharma S, Awate P, et al. Pleiotropic Mechanisms Drive Endocrine Resistance in the Three-Dimensional Bone Microenvironment. *Cancer Res.* 2021;81(2):371-83.

20. Cordero Cervantes D, and Zurzolo C. Peering into tunneling nanotubes-The path forward. *EMBO J.* 2021;40(8):e105789.
21. Lou E, O'Hare P, Subramanian S, and Steer CJ. Lost in translation: applying 2D intercellular communication via tunneling nanotubes in cell culture to physiologically relevant 3D microenvironments. *FEBS J.* 2017;284(5):699-707.
22. Hase K, Kimura S, Takatsu H, Ohmae M, Kawano S, Kitamura H, et al. M-Sec promotes membrane nanotube formation by interacting with Ral and the exocyst complex. *Nat Cell Biol.* 2009;11(12):1427-32.
23. Wittig D, Wang X, Walter C, Gerdes HH, Funk RH, and Roehlecke C. Multi-level communication of human retinal pigment epithelial cells via tunneling nanotubes. *PLoS One.* 2012;7(3):e33195.
24. Warawdekar UM. An Assay to Assess Gap Junction Communication in Cell Lines. *J Biomol Tech.* 2019;30(1):1-6.
25. Natsume A, Kato T, Kinjo S, Enomoto A, Toda H, Shimato S, et al. Girdin maintains the stemness of glioblastoma stem cells. *Oncogene.* 2012;31(22):2715-24.
26. Rohena C, Kalogriopoulos N, Rajapakse N, Roy S, Lopez-Sanchez I, Ablack J, et al. GIV•Kindlin Interaction Is Required for Kindlin-Mediated Integrin Recognition and Activation. *iScience.* 2020;23(6):101209.
27. Cao F, Yang D, Tang F, Lu C, He X, Chen S, et al. Girdin Promotes Tumorigenesis and Chemoresistance in Lung Adenocarcinoma by Interacting with PKM2. *Cancers (Basel).* 2022;14(22).
28. Midde K, Sun N, Rohena C, Joosen L, Dhillon H, and Ghosh P. Single-Cell Imaging of Metastatic Potential of Cancer Cells. *iScience.* 2018;10:53-65.
29. Dunkel Y, Diao K, Aznar N, Swanson L, Liu L, Zhu W, et al. Prognostic impact of total and tyrosine phosphorylated GIV/Girdin in breast cancers. *FASEB J.* 2016;30(11):3702-13.
30. Ghosh P. Heterotrimeric G proteins as emerging targets for network based therapy in cancer: End of a long futile campaign striking heads of a Hydra. *Aging (Albany NY).* 2015;7(7):469-74.
31. Kitamura T, Asai N, Enomoto A, Maeda K, Kato T, Ishida M, et al. Regulation of VEGF-mediated angiogenesis by the Akt/PKB substrate Girdin. *Nat Cell Biol.* 2008;10(3):329-37.
32. Jiang P, Enomoto A, Jijiwa M, Kato T, Hasegawa T, Ishida M, et al. An actin-binding protein Girdin regulates the motility of breast cancer cells. *Cancer Res.* 2008;68(5):1310-8.
33. Ghosh P, Beas AO, Bornheimer SJ, Garcia-Marcos M, Forry EP, Johannson C, et al. A G{alpha}i-GIV molecular complex binds epidermal growth factor receptor and determines whether cells migrate or proliferate. *Mol Biol Cell.* 2010;21(13):2338-54.
34. Nishimae K, Tsunoda N, Yokoyama Y, Kokuryo T, Iwakoshi A, Takahashi M, et al. The impact of Girdin expression on recurrence-free survival in patients with luminal-type breast cancer. *Breast Cancer.* 2015;22(5):445-51.
35. Peng WT, Hu X, Yao L, Jiang YZ, and Shao ZM. Elevated expression of Girdin in the nucleus indicates worse prognosis for patients with estrogen receptor-positive breast cancer. *Ann Surg Oncol.* 2014;21 Suppl 4:S648-56.
36. Qiao L, Sinha S, Abd El-Hafeez AA, Lo IC, Midde KK, Ngo T, et al. A circuit for secretion-coupled cellular autonomy in multicellular eukaryotic cells. *Mol Syst Biol.* 2023:e11127.
37. Zurzolo C. Tunneling nanotubes: Reshaping connectivity. *Curr Opin Cell Biol.* 2021;71:139-47.
38. Tsai CF, Cheng YK, Lu DY, Wang SL, Chang CN, Chang PC, et al. Inhibition of estrogen receptor reduces connexin 43 expression in breast cancers. *Toxicol Appl Pharmacol.* 2018;338:182-90.
39. Ren J, Wang XH, Wang GC, and Wu JH. 17β estradiol regulation of connexin 43-based gap junction and mechanosensitivity through classical estrogen receptor pathway in osteocyte-like MLO-Y4 cells. *Bone.* 2013;53(2):587-96.
40. Aznar N, Patel A, Rohena CC, Dunkel Y, Joosen LP, Taupin V, et al. AMP-activated protein kinase fortifies epithelial tight junctions during energetic stress via its effector GIV/Girdin. *Elife.* 2016;5.
41. Osborne CK. Tamoxifen in the treatment of breast cancer. *N Engl J Med.* 1998;339(22):1609-18.
42. Osborne CK, Wakeling A, and Nicholson RI. Fulvestrant: an oestrogen receptor antagonist with a novel mechanism of action. *Br J Cancer.* 2004;90 Suppl 1(Suppl 1):S2-6.
43. Saptarshi Sinha AF, Kathryn E Luker, Debashis Sahoo, Barbara A Parker, Kay Yeung, Gary D Luker, Pradipta Ghosh. bioRxiv; 2022:2022.12. 02.518910.
44. !!! INVALID CITATION !!! 28-30.
45. Ippolito L, Morandi A, Taddei ML, Parri M, Comito G, Iscaro A, et al. Cancer-associated fibroblasts promote prostate cancer malignancy via metabolic rewiring and mitochondrial transfer. *Oncogene.* 2019;38(27):5339-55.

46. DiGiacomo JW, Godet I, Trautmann-Rodriguez M, and Gilkes DM. Extracellular Matrix-Bound FGF2 Mediates Estrogen Receptor Signaling and Therapeutic Response in Breast Cancer. *Mol Cancer Res.* 2021;19(1):136-49.
47. Buschhaus JM, Humphries BA, Eckley SS, Robison TH, Cutter AC, Rajendran S, et al. Targeting disseminated estrogen-receptor-positive breast cancer cells in bone marrow. *Oncogene.* 2020;39(34):5649-62.
48. Davies C, Pan H, Godwin J, Gray R, Arriagada R, Raina V, et al. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. *Lancet.* 2013;381(9869):805-16.
49. Lee SJ, Park S, Ahn HK, Yi JH, Cho EY, Sun JM, et al. Implications of bone-only metastases in breast cancer: favorable preference with excellent outcomes of hormone receptor positive breast cancer. *Cancer Res Treat.* 2011;43(2):89-95.
50. Zhang XH, Giuliano M, Trivedi MV, Schiff R, and Osborne CK. Metastasis dormancy in estrogen receptor-positive breast cancer. *Clin Cancer Res.* 2013;19(23):6389-97.
51. Xuan X, Tian C, Zhao M, Sun Y, and Huang C. Mesenchymal stem cells in cancer progression and anticancer therapeutic resistance. *Cancer Cell Int.* 2021;21(1):595.
52. Yagi H, and Kitagawa Y. The role of mesenchymal stem cells in cancer development. *Front Genet.* 2013;4:261.
53. !!! INVALID CITATION !!! 33.
54. !!! INVALID CITATION !!! 32.
55. Shee K, Yang W, Hinds JW, Hampsch RA, Varn FS, Traphagen NA, et al. Therapeutically targeting tumor microenvironment-mediated drug resistance in estrogen receptor-positive breast cancer. *J Exp Med.* 2018;215(3):895-910.
56. Ahmed KA, and Xiang J. Mechanisms of cellular communication through intercellular protein transfer. *J Cell Mol Med.* 2011;15(7):1458-73.
57. Vignais ML, Caicedo A, Brondello JM, and Jorgensen C. Cell Connections by Tunneling Nanotubes: Effects of Mitochondrial Trafficking on Target Cell Metabolism, Homeostasis, and Response to Therapy. *Stem Cells Int.* 2017;2017:6917941.
58. Rustom A, Saffrich R, Markovic I, Walther P, and Gerdes HH. Nanotubular highways for intercellular organelle transport. *Science.* 2004;303(5660):1007-10.
59. Dubois F, Bénard M, Jean-Jacques B, Schapman D, Roberge H, Lebon A, et al. Investigating Tunneling Nanotubes in Cancer Cells: Guidelines for Structural and Functional Studies through Cell Imaging. *Biomed Res Int.* 2020;2020:2701345.
60. !!! INVALID CITATION !!! 42.
61. Han X, and Wang X. Opportunities and Challenges in Tunneling Nanotubes Research: How Far from Clinical Application? *Int J Mol Sci.* 2021;22(5).
62. Sartori-Rupp A, Cordero Cervantes D, Pepe A, Gousset K, Delage E, Corroyer-Dulmont S, et al. Correlative cryo-electron microscopy reveals the structure of TNTs in neuronal cells. *Nat Commun.* 2019;10(1):342.
63. Alarcon-Martinez L, Villafranca-Baughman D, Quintero H, Kacerovsky JB, Dotigny F, Murai KK, et al. Interpericyte tunnelling nanotubes regulate neurovascular coupling. *Nature.* 2020;585(7823):91-5.
64. Wang X, Veruki ML, Bukoreshtliev NV, Hartveit E, and Gerdes HH. Animal cells connected by nanotubes can be electrically coupled through interposed gap-junction channels. *Proc Natl Acad Sci U S A.* 2010;107(40):17194-9.
65. Wang X, Bukoreshtliev NV, and Gerdes HH. Developing neurons form transient nanotubes facilitating electrical coupling and calcium signaling with distant astrocytes. *PLoS One.* 2012;7(10):e47429.
66. Lock JT, Parker I, and Smith IF. Communication of Ca(2+) signals via tunneling membrane nanotubes is mediated by transmission of inositol trisphosphate through gap junctions. *Cell Calcium.* 2016;60(4):266-72.
67. Okafo G, Prevedel L, and Eugenin E. Tunneling nanotubes (TNT) mediate long-range gap junctional communication: Implications for HIV cell to cell spread. *Sci Rep.* 2017;7(1):16660.
68. Turos-Korgul L, Kolba MD, Chroscicki P, Zieminska A, and Piwocka K. Tunneling Nanotubes Facilitate Intercellular Protein Transfer and Cell Networks Function. *Front Cell Dev Biol.* 2022;10:915117.
69. Dubey GP, and Ben-Yehuda S. Intercellular nanotubes mediate bacterial communication. *Cell.* 2011;144(4):590-600.
70. Pande S, Shitut S, Freund L, Westermann M, Bertels F, Colesie C, et al. Metabolic cross-feeding via intercellular nanotubes among bacteria. *Nat Commun.* 2015;6:6238.

71. Koo H, Allan RN, Howlin RP, Stoodley P, and Hall-Stoodley L. Targeting microbial biofilms: current and prospective therapeutic strategies. *Nat Rev Microbiol.* 2017;15(12):740-55.
72. Claessen D, Rozen DE, Kuipers OP, Søgaard-Andersen L, and van Wezel GP. Bacterial solutions to multicellularity: a tale of biofilms, filaments and fruiting bodies. *Nat Rev Microbiol.* 2014;12(2):115-24.
73. Mandlik A, Swierczynski A, Das A, and Ton-That H. Pili in Gram-positive bacteria: assembly, involvement in colonization and biofilm development. *Trends Microbiol.* 2008;16(1):33-40.
74. Preda VG, and Săndulescu O. Communication is the key: biofilms, quorum sensing, formation and prevention. *Discoveries (Craiova).* 2019;7(3):e100.
75. Javid B, and Derbyshire KM. Nanotubes: shaking hands, talking, or sharing? *Front Microbiol.* 2011;2:95.
76. Gerdes HH, and Carvalho RN. Intercellular transfer mediated by tunneling nanotubes. *Curr Opin Cell Biol.* 2008;20(4):470-5.
77. Lo IC, Gupta V, Midde KK, Taupin V, Lopez-Sanchez I, Kufareva I, et al. Activation of Gαi at the Golgi by GIV/Girdin imposes finiteness in Arf1 signaling. *Dev Cell.* 2015;33(2):189-203.
78. Beas AO, Taupin V, Teodorof C, Nguyen LT, Garcia-Marcos M, and Farquhar MG. Gαs promotes EEA1 endosome maturation and shuts down proliferative signaling through interaction with GIV (Girdin). *Mol Biol Cell.* 2012;23(23):4623-34.
79. Gupta V, Bhandari D, Leyme A, Aznar N, Midde KK, Lo IC, et al. GIV/Girdin activates Gαi and inhibits Gαs via the same motif. *Proc Natl Acad Sci U S A.* 2016;113(39):E5721-30.
80. Garcia-Marcos M, Ear J, Farquhar MG, and Ghosh P. A GDI (AGS3) and a GEF (GIV) regulate autophagy by balancing G protein activity and growth factor signals. *Mol Biol Cell.* 2011;22(5):673-86.
81. Ghosh P, and Mullick M. Building unconventional G protein-coupled receptors, one block at a time. *Trends Pharmacol Sci.* 2021;42(7):514-7.
82. Yamamura Y, Asai N, Enomoto A, Kato T, Mii S, Kondo Y, et al. Akt-Girdin signaling in cancer-associated fibroblasts contributes to tumor progression. *Cancer Res.* 2015;75(5):813-23.
83. Sandipan Dasgupta 1 DYD, Gal Haimovich 1,4 , Emanuel Wyler 2 , Tsviya Olender 1 , Robert H. Singer 3 , Markus Landthaler 2 , and Jeffrey E. Gerst.
84. Dunkel Y, Ong A, Notani D, Mittal Y, Lam M, Mi X, et al. STAT3 protein up-regulates Gα-interacting vesicle-associated protein (GIV)/Girdin expression, and GIV enhances STAT3 activation in a positive feedback loop during wound healing and tumor invasion/metastasis. *J Biol Chem.* 2012;287(50):41667-83.
85. Fiori ME, Di Franco S, Villanova L, Bianca P, Stassi G, and De Maria R. Cancer-associated fibroblasts as abettors of tumor progression at the crossroads of EMT and therapy resistance. *Mol Cancer.* 2019;18(1):70.
86. Cavnar SP, Rickelmann AD, Meguiar KF, Xiao A, Dosch J, Leung BM, et al. Modeling selective elimination of quiescent cancer cells from bone marrow. *Neoplasia.* 2015;17(8):625-33.
87. Smith MC, Luker KE, Garbow JR, Prior JL, Jackson E, Piwnica-Worms D, et al. CXCR4 regulates growth of both primary and metastatic breast cancer. *Cancer Res.* 2004;64(23):8604-12.
88. Cavnar SP, Xiao A, Gibbons AE, Rickelmann AD, Neely T, Luker KE, et al. Imaging Sensitivity of Quiescent Cancer Cells to Metabolic Perturbations in Bone Marrow Spheroids. *Tomography.* 2016;2(2):146-57.
89. Buschhaus JM, Rajendran S, Chen S, Wharram BL, Bevoor AS, Cutter AC, et al. Bone marrow mesenchymal stem cells induce metabolic plasticity in estrogen receptor-positive breast cancer. *Mol Cancer Res.* 2023.
90. Buschhaus Johanna M, Rajendran S, Humphries B, Cutter A, Ciavattone N, Buschhaus A, et al. Effects of iron modulation on mesenchymal stem cell-induced drug resistance in estrogen-receptor-positive breast cancer. 2022.
91. Perez-Riverol Y, Bai J, Bandla C, García-Seisdedos D, Hewapathirana S, Kamatchinathan S, et al. The PRIDE database resources in 2022: a hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Res.* 2022;50(D1):D543-D52.
92. Sahoo D, Dill DL, Tibshirani R, and Plevritis SK. Extracting binary signals from microarray time-course data. *Nucleic Acids Res.* 2007;35(11):3705-12.
93. Sahoo D, Swanson L, Sayed IM, Katkar GD, Ibeawuchi SR, Mittal Y, et al. Artificial intelligence guided discovery of a barrier-protective therapy in inflammatory bowel disease. *Nat Commun.* 2021;12(1):4246.
94. Fabregat A, Jupe S, Matthews L, Sidiropoulos K, Gillespie M, Garapati P, et al. The Reactome Pathway Knowledgebase. *Nucleic Acids Res.* 2018;46(D1):D649-D55.

95. Stout RF, Snapp EL, and Spray DC. Connexin Type and Fluorescent Protein Fusion Tag Determine Structural Stability of Gap Junction Plaques. *J Biol Chem.* 2015;290(39):23497-514.
96. Ear J, Abd El-Hafeez AA, Roy S, Ngo T, Rajapakse N, Choi J, et al. A long isoform of GIV/Girdin contains a PDZ-binding module that regulates localization and G-protein binding. *J Biol Chem.* 2021;296:100493.
97. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A.* 1995;92(16):7297-301.