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Netrin signaling mediates survival of dormant epithelial ovarian cancer cells

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Abstract

Dormancy in cancer is a clinical state in which residual disease remains undetectable for a prolonged duration. At a cellular level, rare cancer cells cease proliferation and survive chemotherapy and disseminate disease. We utilized a suspension culture model of high grade serous ovarian cancer (HGSOC) cell dormancy and devised a novel CRISPR screening approach to identify genetic requirements for cell survival under growth arrested and spheroid culture conditions. In addition, multiple RNA-seq comparisons were used to identify genes whose expression correlates with survival in dormancy. Combined, these approaches discover the Netrin signaling pathway as critical to dormant HGSOC cell survival. We demonstrate that Netrin-1 and -3, UNC5H receptors, DCC and other fibronectin receptors induce low level ERK activation to promote survival in dormant conditions. Furthermore, we determine that Netrin-1 and -3 overexpression is associated with poor prognosis in HGSOC and demonstrate their overexpression elevates cell survival in dormant conditions. Lastly, Netrin-1 or -3 overexpression contributes to greater spread of disease in a xenograft model of abdominal dissemination. This study highlights Netrin signaling as a key mediator HGSOC cancer cell dormancy and metastasis.

eLife assessment

Perampalam and colleagues provide **solid** evidence that Netrin signaling drives survival of non-proliferating ovarian cancer cells and their dissemination. These **valuable** findings were thought to provide unique insights into the molecular underpinnings of ovarian cancer spread and thus to be of significant interest to cancer biologists. However, the **incomplete** evidence supporting the role of the described Netrin-dependent mechanism in cancer dormancy was found to be a major shortcoming of the study.

Introduction

Relapse from initially effective cancer treatment represents one of the greatest barriers to improving outcomes (1). Dormant or minimal residual disease that is not clinically detectable is a common source of relapse, particularly in a metastatic setting (2). This has motivated the search for, and study of, disseminated cancer cells. Cellular dormancy has been characterized in cancer cells derived from a variety of primary disease sites including breast, prostate, melanoma, and others (3, 4). Disseminated cells often occupy specific cellular niches in perivascular space or in bone marrow (5), they adopt catabolic metabolism (2), and withdraw from the cell cycle (4). Stem like phenotypes and epithelial to mesenchymal transition also characterize the biology of these cells as they survive dissemination and lay quiescent in distant tissues (1, 3, 5). Across a variety of disease sites this dormant state is characterized by diminished Ras-MAPK signaling and elevated p38 activity (3, 6). To develop treatments specifically tailored for dormant cells, a systematic search for vulnerabilities unique to dormancy is needed. To this end, high grade serous ovarian cancer disseminates as growth arrested cellular aggregates that are amenable to modeling in culture (7), suggesting they are ideal to search for survival dependencies of dormant cells.

High grade serous ovarian cancer (HGSOC) is the deadliest gynecologic malignancy, accounting for more than 70% of cases (8). Its poor prognosis is largely attributed to late-stage diagnosis with metastases already present. Metastatic spread is facilitated by multicellular clusters of tumor cells, called spheroids, that are shed into the peritoneal cavity and disseminate to nearby organs (9). The non-proliferative state of spheroid cells renders them chemoresistant and contributes to disease recurrence, emphasizing the need to understand the biology of these cells (10). Dormant HGSOC cells are characterized by similar metabolic changes, EMT, and cytokine signaling as dormant cancer cells from other disease sites (3, 11). Mechanisms that mediate their dormant properties include AMPK-LKB1 signaling that induces autophagy (12, 13), STAT3 and FAK for survival and chemoresistance (14, 15), and DYRK1A signaling to arrest proliferation (16). Unfortunately, therapeutic approaches to target these pathways in ovarian cancer have yet to emerge, indicating that more candidates are needed.

Netrins are a family of secreted factors that reside in the extracellular matrix around cells and were first identified in axon guidance in the developing nervous system (17, 18). Together with their receptors, Netrins have since been implicated in cancer progression (19). Netrin-1 is the most studied and its misregulation in cancer leads to pro-survival signals (20). Netrin interactions with DCC and UNC5 homologs (UNC5H, UNC5A-D) convert pro-death signals by these dependence receptors to survival signals (21). Netrin signals through PI3K-AKT and ERK-MAPK are known to participate in growth and orientation by providing guidance cues (22–24), while loss of ligand binding leads DCC or UNC5H to stimulate dephosphorylation of DAPK1 that sends pro-death signals (25, 26). The gene encoding DCC is frequently deleted in cancer

(20 [↗](#)), and Netrin-1 blocking antibodies are in Phase II clinical trials for endometrial and cervical carcinomas to inhibit metastatic growth (21 [↗](#)). Alternative receptors for Netrins such as NEO, DSCAM, and Integrins also mediate their signals (19 [↗](#)), creating a myriad of possible signaling pathways for Netrins to regulate cancer cells. Lastly, functional roles for Netrin-3, -4, and -5 are still relatively unexplored compared to Netrin-1 (27 [↗](#)), although their expression patterns suggest roles in advanced forms of some cancers (28 [↗](#)). A functional role for Netrins and their receptors in dormancy, or other aspects of HGSOc spheroid biology have yet to be reported.

In this study we utilized a suspension culture system to interrogate survival mechanisms in dormancy using HGSOc spheroids. To maximize discovery of gene loss events that compromise viability in dormancy, we devised a novel genome-wide CRISPR screen approach that we term ‘GO-CRISPR’. In addition to the standard CRISPR workflow, GO-CRISPR utilizes a parallel screen in which ‘guide-only’ cells lacking Cas9 are used to control for stochastic changes in gRNA abundance in non-proliferative cells. This screen identified multiple Netrin ligands, DCC, UNC5Hs, and downstream MAPK signaling components as supporting survival of HGSOc cells in dormant culture conditions. A transcriptomic analysis of HGSOc spheroids also revealed Netrin signaling components were enriched in HGSOc spheroids compared to adherent cells, and that in the absence of the DYRK1A survival kinase Netrin transcriptional increases were lost. We show that multiple Netrins, UNC5 receptors, and the downstream MEK-ERK axis is crucial for survival signaling in dormancy. Overexpression of Netrin-1 and -3 are associated with poor prognosis in HGSOc and their overexpression in culture increases survival in dormant suspension culture. Lastly, we demonstrate that Netrin signaling contributes to disease dissemination in a xenograft model of metastasis. Our study highlights Netrin-ERK signaling as a requirement for spheroid survival and suggests it may be a potential therapeutic target for eradicating dormant HGSOc cells.

Results

Axon guidance genes are essential factors for EOC spheroid viability

To dissect genetic dependencies of dormant ovarian cancer cells, we utilized a suspension culture system that encourages cellular aggregation, leading to non-proliferative spheroids that resemble dormant aggregates found in ovarian cancer patient ascites. **Fig. 1A** [↗](#) shows phospho-western blots for ERK and p38 from three high grade serous EOC cell lines; iOvCa147, OVCAR8, TOV1946. Reduced phospho-ERK and elevated phospho-p38 are indicative of the characteristic signaling switch reported to underlie most paradigms of cancer cell dormancy (5 [↗](#)). To discover genes required for survival in spheroids, we utilized a new approach called GO-CRISPR (Guide Only CRISPR) that we developed specifically for survival gene discovery in three-dimensional organoid/spheroid cell culture conditions (**Fig. 1B** [↗](#)). GO-CRISPR incorporates sgRNA abundances from non-Cas9-expressing cells to control for stochastic (non-Cas9 dependent) effects that are a challenge in three-dimensional culture conditions with slow or arrested proliferation. We used sequencing to determine sgRNA identity and abundance in the library, initially infected cells, and in cells following 48 hours of suspension culture (**Fig. 1B** [↗](#)). Read count data was analyzed using a software package that we developed to analyze GO-CRISPR data (summarized in **Sup. Fig. 1A&B**). We filtered the results for genes that had a Spheroid/Adherent Enrichment Ratio (ER) less than 1, with a $P < 0.05$, which is indicative of genes that were most depleted in dormant suspension conditions (**Fig. 1B** [↗](#)). To assess reliability of screen data, we investigated the classification of non-targeting control sgRNAs. We computed a receiver operator characteristic curve (ROC) and determined the area under the curve for each cell line (**Sup. Fig. 1C**). Greater than 95% of sgRNAs in iOvCa147 and OVCAR8 screens were classified as non-essential, while 83% of sgRNAs in TOV1946 cells were non-essential. In addition, we compared GO-CRISPR screen ER values with viability data from siRNA knock down for 35 individual genes we have previously studied in

spheroid survival assays (**Sup. Fig. 1D**)[\(16\)](#). This revealed a statistically significant correlation between the two sets of data, suggesting that genes we previously knew supported spheroid viability (or were dispensable) were classified similarly by our screen. These controls suggested our screen data was reliable for identifying new survival dependencies.

We found 6,717 genes with an ER below 1 in iOvCa147 cells; and 7,637 genes in TOV1946; and 7,640 genes in OVCAR8 cells. Among these genes; 1,382 were commonly depleted in suspension culture in all three cell lines (**Fig. 1D**)[\(16\)](#). We performed pathway enrichment analysis on these common genes and identified several significantly enriched pathways. These include primary Reactome gene sets such as signal transduction, metabolism, and signaling by GPCR (**Fig. 1E**)[\(16\)](#). In addition, we identified a tertiary category called ‘axon guidance,’ that includes numerous components of the Netrin signaling pathway.

Expression of Netrin ligands and receptors are enriched in EOC spheroids

DYRK1A was previously discovered to support survival of ovarian cancer cells in suspension culture ([16](#)). Since DYRK1A phosphorylates RNA polymerase II in transcription initiation ([29](#)), we sought to determine if it regulates gene expression in spheroid survival. We created *DYRK1A*^{-/-} iOvCa147 cells and confirmed their deficiency for survival in suspension (**Sup. Fig. 2A-E**). Using iOvCa147 and *DYRK1A*^{-/-} derivatives, we performed RNA-seq on adherent and spheroid cells. We collected spheroids following a 6-hour incubation in suspension because overexpression of known cell cycle transcriptional targets due to DYRK1A loss were first evident at this time point, but loss of viability due to DYRK1A deficiency had not yet occurred ([16](#)). We compared iOvCa147 adherent cells to 6-hour suspension culture to identify transcriptional changes that occur as these cells transitioned from adherent conditions to suspension conditions (**Fig. 2A**)[\(16\)](#). We identified 1,834 genes that were upregulated, and pathway enrichment analysis identified many of the same categories as our CRISPR screen including axon guidance (**Fig. 2B**)[\(16\)](#). We then compared iOvCa147 spheroid cells to *DYRK1A*^{-/-} spheroid cells to identify mRNA expression changes caused by DYRK1A deficiency (**Fig. 2C**)[\(16\)](#). We identified 744 genes that were downregulated and these also belonged to many of the same pathways, including axon guidance (**Fig. 2D**)[\(16\)](#).

We compared enriched pathways from these two RNA-seq analyses with those that were identified in our GO-CRISPR screens to identify gene expression programs in spheroids whose products are essential for cell survival. 78 Reactome pathways were commonly enriched across the GO-CRISPR screens and transcriptomic analyses, including 78 of the 83 pathways identified in RNA-seq from *DYRK1A*^{-/-} spheroid cells (**Fig. 2E**)[\(16\)](#). The axon guidance pathway was one of the most enriched among these common pathways (**Fig. 2F**)[\(16\)](#). These data reveal that our GO-CRISPR screen and transcriptomic analyses converge on this highly specific tertiary Reactome category of axon guidance alongside much broader primary or secondary gene sets. This category includes Netrin ligands, receptors, and downstream signaling components, suggesting a previously unappreciated role in HGSOc spheroid viability.

We separately sought to confirm expression of Netrins and their signaling components in HGSOc spheroids. We examined expression levels of ligands and receptors in the three cell lines used in our GO-CRISPR screen using RT-qPCR (**Fig. 3A-C**)[\(16\)](#). The precise identities of upregulated genes varies between cell lines, however each cell line increases expression of some Netrins, at least one UNC5, and at least one of DCC, DSCAM, and Neogenin. This suggests that the Netrin pathway may play a previously unappreciated role in HGSOc dormancy and disease progression. Since Netrin signaling has not been previously reported in HGSOc dormancy we examined expression of Netrin-1 and -3 ligands in spheroids isolated directly from patient ascites by immunohistochemistry (**Fig. 3D-F**)[\(16\)](#). We confirmed Netrin-1 and -3 antibody specificity using spheroids generated from OVCAR8 cells bearing knock out or over expression of these Netrins (**Sup. Fig. 3**)[\(16\)](#). Spheroids were paraffin embedded and serial sections were also stained with

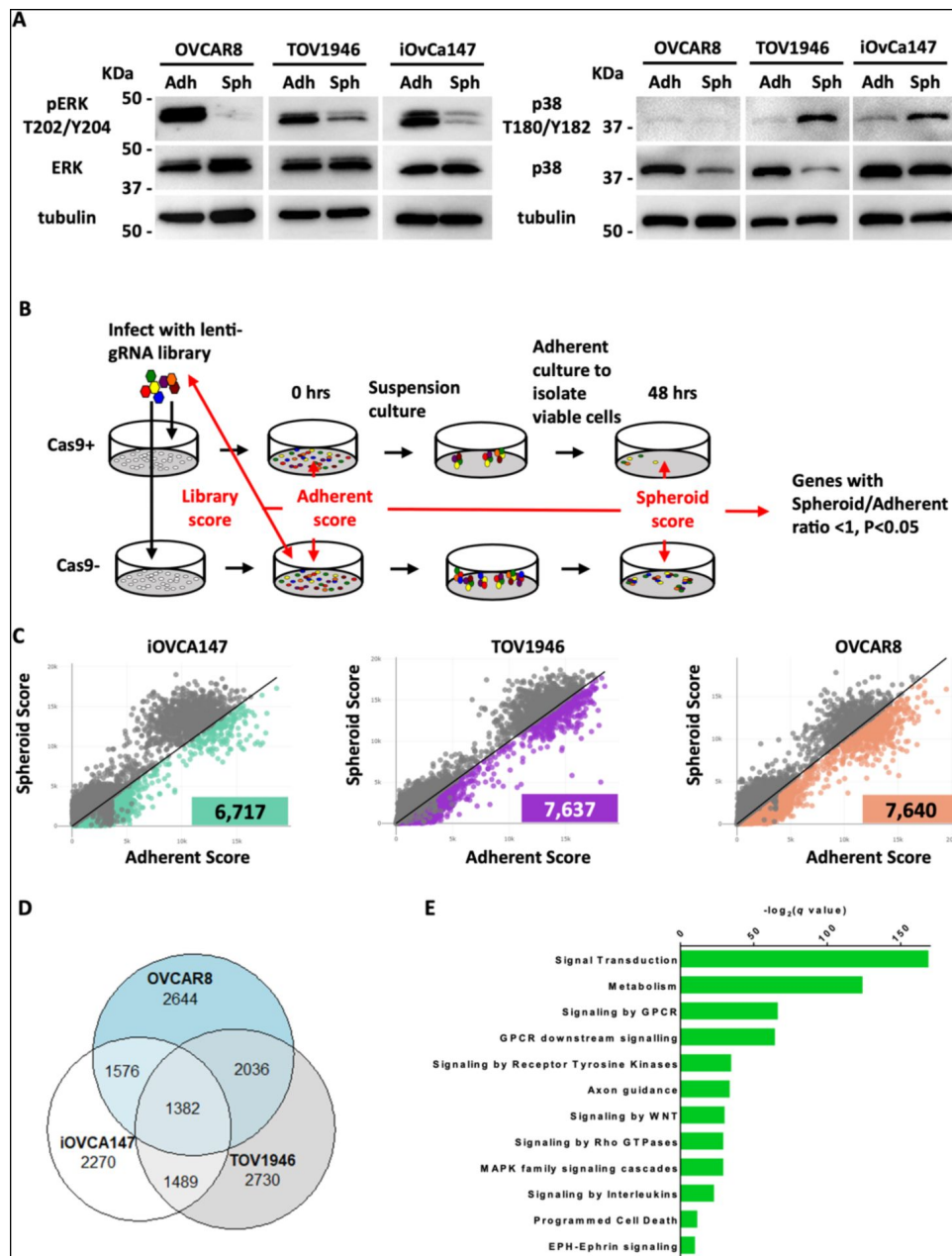


Figure 1.

GO-CRISPR screens implicate axon guidance pathways as supporting EOC spheroid cell viability.

A) iOvCa147, TOV1946, or OVCAR8 cells were cultured under adherent conditions (Adh) or in suspension to induce spheroids (Sph). Lysates were prepared and analyzed by western blotting for phosphorylated and total levels of ERK and p38. **B**) Flow chart of GO-CRISPR screening used for each of iOvCa147, TOV1946, or OVCAR8 control cells or that express Cas9. Cas9-positive cells (top row) and Cas9-negative cells (bottom row) were transduced with the GeCKO v2 pooled sgRNA library. After antibiotic selection, cells were expanded under adherent culture conditions (T_0) before being transferred to suspension culture conditions to induce spheroid formation and select for cell survival. After 48 hours spheroids were transferred to standard plasticware to isolate viable cells (T_f). Red arrows indicate the relevant comparisons of sgRNA sequence abundance that were made to analyze screen outcomes. **C**) Scatter plots representing spheroid score (T_f) on y-axis and adherent (T_0) score on x-axis calculated by TRACS for each gene in each cell line (iOvCa147, TOV1946, OVCAR8). Colored data points represent genes with $ER < 1$ and $p_{adj} < 0.05$. **D**) Venn diagram illustrating overlap of genes identified as supporting cell viability in suspension culture from iOvCa147, TOV1946, and OVCAR8 cells. **E**) Graph depicting enriched pathways from ConsensusPathDB using the 1382 commonly identified genes from D. Categories are ranked by q-value.

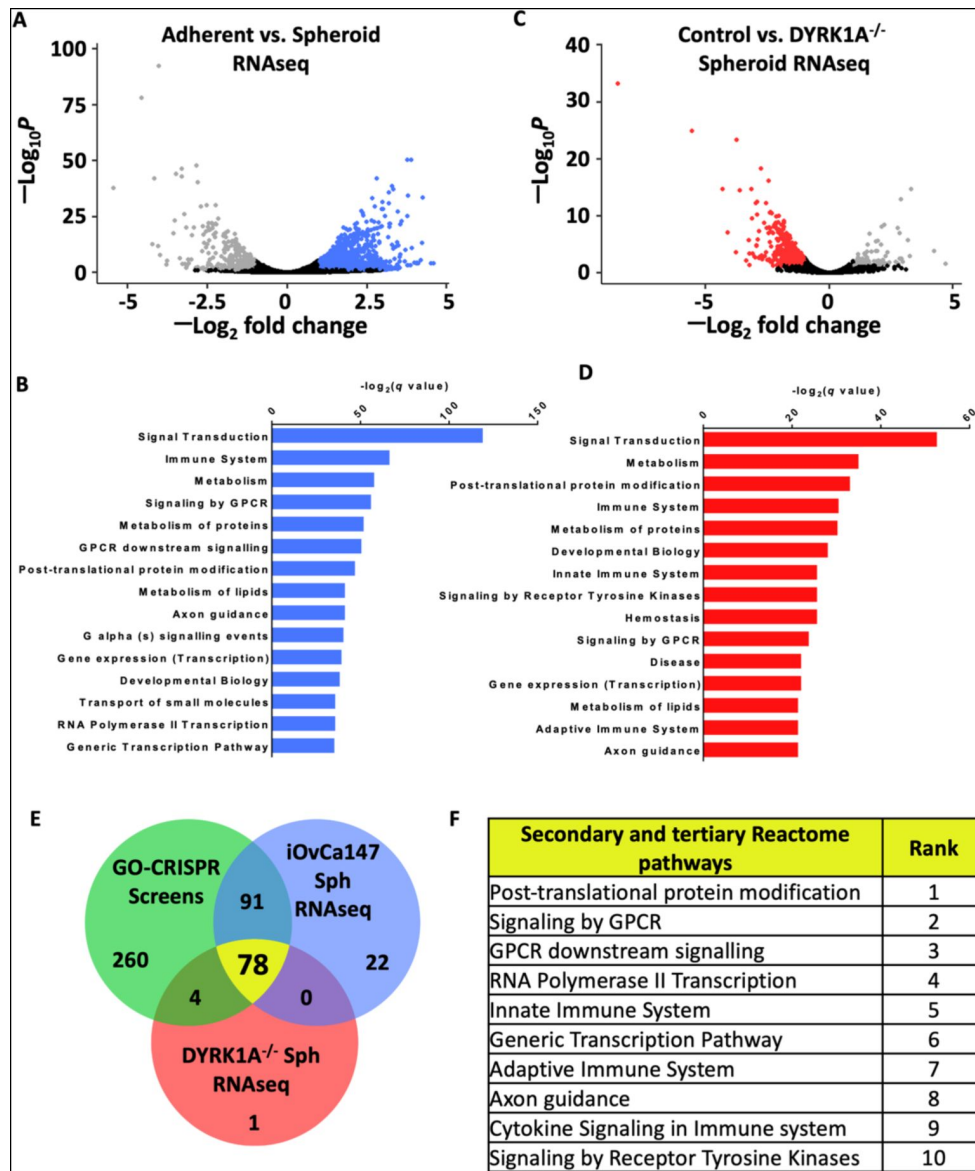


Figure 2.

Axon guidance pathway components are upregulated in iOvCa147 spheroid cells in a *DYRK1A* dependent manner.

A) RNA was isolated from iOvCa147 cells following culture under adherent conditions or in suspension conditions to induce spheroid formation for 6 hours. Triplicate independent cultures were processed for RNA-seq. A volcano plot shows differentially expressed genes in spheroid cells compared to adherent. 1,937 genes were found to be downregulated in iOvCa147 spheroid cells (\log_2 fold change < 1 , $p_{adj} < 0.05$, FDR 10%, highlighted in grey) and 1,834 genes were upregulated (\log_2 fold change > 1 , $p_{adj} < 0.05$, FDR 10%, highlighted in blue). **B)** Top 15 most significantly enriched pathways ($p_{adj} < 0.05$) whose genes were upregulated in suspension culture compared to adherent in RNA-seq analysis. **C)** A volcano plot showing differentially expressed genes in *DYRK1A*^{-/-} spheroid cells compared to iOvCa147 spheroid cells. 744 genes were found to be downregulated in *DYRK1A*^{-/-} spheroid cells (\log_2 fold change < 1 , $p_{adj} < 0.05$, FDR 10%, highlighted in red) and 96 genes were upregulated (\log_2 fold change > 1 , $p_{adj} < 0.05$, FDR 10%, highlighted in grey). **D)** Top 15 most significantly enriched pathways ($p_{adj} < 0.05$) that were represented by downregulated genes in *DYRK1A*^{-/-} suspension culture compared to control cells in suspension. **E)** Venn diagram depicting overlapping enriched pathways identified in GO-CRISPR screens in green; enriched pathways identified in upregulated genes in parental iOvCa147 spheroid cells in blue; and enriched pathways identified in downregulated genes in *DYRK1A*^{-/-} spheroid cells in red. 78 pathways were commonly enriched in all three datasets (shown in yellow). **F)** Top 10 most significantly enriched pathways ($p_{adj} < 0.05$) among the 78 identified in C.

epithelial markers Cytokeratin and EpCAM, as well as p53 to confirm cancer cell identity within spheroids that co-stain for Netrins (**Fig. 3D-F**). Overall, this confirms Netrin expression occurs in HGSOc patient spheroids in precisely the context our screen indicates that it is critical for cell survival.

Netrin signaling stimulates ERK to support HGSOc spheroid survival

Netrin ligands and their dependence receptors are best known for their roles in axon guidance (30), but are appreciated to regulate apoptosis in cancer (19). **Fig. 4A** illustrates components of Netrin signaling and the frequency with which they were discovered to have an ER of <1 in our screens. In addition, intracellular signaling molecules known to be downstream of Netrin receptors, and that are shared with other enriched pathways identified in our screens are also illustrated. To confirm screen findings, we independently knocked out *NTN1*, *NTN3*, *NTN4*, *NTN5*, the UNC5H receptor homologs (*UNC5A*, *UNC5B*, *UNC5C*, and *UNC5D*), as well as *DCC*, *DSCAM*, and *NEO1* alone or in multigene combinations using lentiviral delivery of sgRNAs and Cas9 (**Fig. 4B** and **Sup. Fig. 4**). We assessed their loss on spheroid survival in six HGSOc cell lines (iOvCa147, TOV1946, OVCAR3, OVCAR4, OVCAR8, and COV318). Loss of at least one netrin ligand resulted in reduced spheroid cell survival in five of six cell lines (**Fig. 4B**). Notably, Netrin-3 loss showed the most pronounced effect on survival. Cell lines such as OVCAR8 and COV318 were relatively resistant to the deletion of netrin pathway components as individual UNC5H, *DCC*, and *DSCAM* genes rarely affected viability (**Fig. 4B**). In these instances, combined deletions of UNC5H family members, or *DCC*; *DSCAM*; *NEO1* (DDN for short) were either inviable or highly sensitive to suspension culture induced cell death suggesting redundancy in this pathway in some cell lines. Other cell lines, such as OVCAR3, were highly sensitive to the loss of multiple individual ligands and any individual UNC5H receptor was highly sensitizing to death induction in suspension culture (**Fig. 4B**). Overall, these illustrate a robust and redundant role for Netrins in survival in this dormant cell culture assay. Importantly, loss of receptor expression does not elevate viability as expected for a dependence receptor, suggesting that Netrin receptors in HGSOc simply transmit positive survival signals.

To determine if Netrin dependent signaling is active in EOC spheroids, we transferred serum deprived adherent cells to suspension and stimulated with recombinant Netrin-1 (**Fig. 4C**). Western blotting for phospho-ERK, as a measure of activation, reveals stimulation of MEK-ERK signaling in each of the cell lines tested under spheroid culture conditions. Because MEK and ERK family members were identified in our screens (**Fig. 4A**), Netrin is a candidate pathway to provide low level, but essential survival signals through the MAPK pathway. We investigated the dependence of ERK phosphorylation on UNC5H and DDN receptors and demonstrate it is lower when receptors are deleted (**Fig. 4D**). Activation using Netrin-1 stimulation of suspension cultures of OVCAR8 cells, or derivatives deleted for UNC5 family members or DDN receptors, indicates that Netrin receptors are essential to activate ERK in dormant suspension culture (**Fig. 4E**). Inhibition of MEK using the MEK1/2 inhibitor PD184352 causes loss of cell viability in suspension culture (**Fig. 4F**), emphasizing that MEK-ERK signaling is key to cell survival. The simplest explanation of this data is that Netrins signal through a heterodimer or multi-receptor complex containing both an UNC5 and DDN component that provides stimulation for MEK and ERK in the absence of mitogenic signals in dormant culture conditions.

Netrin overexpression is correlated with poor outcome in HGSOc

Genomic studies indicate that HGSOc is one of the most aneuploid cancers (31). In addition to the frequency of copy number variants per tumor, the locations of gains and losses are dissimilar between cases, making this a highly heterogenous disease. Evaluation of TCGA genomic data for HGSOc indicates rare amplifications or deletions of Netrin or Netrin-receptor genes (**Sup. Fig. 5A**). Because of redundancy of Netrins and their receptor families this data suggests HGSOc cases with

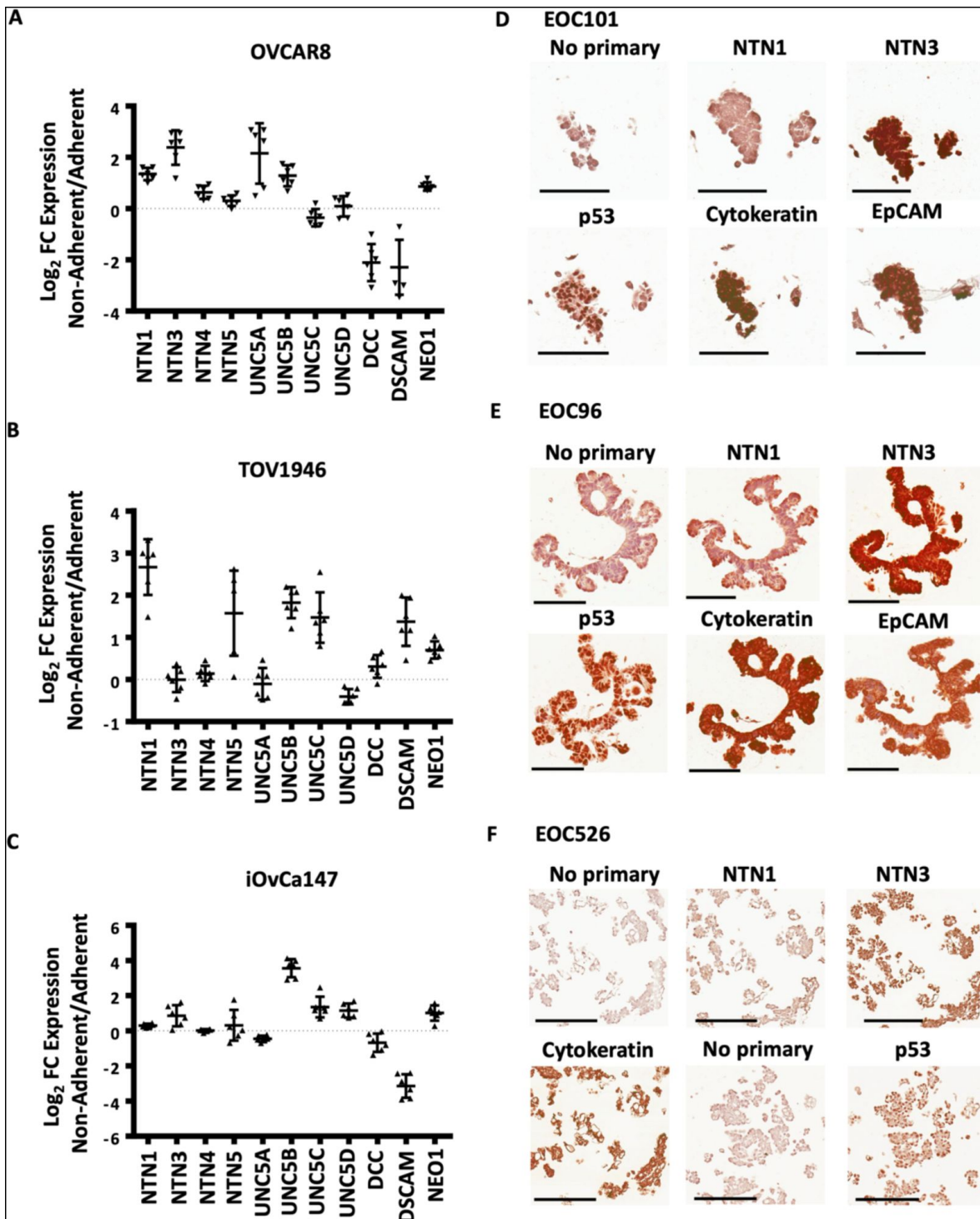


Figure 3.

Expression of Netrin ligands and their dependence receptors is increased in suspension culture.

A-C) RT-qPCR was performed to quantitate mRNA expression levels of Netrin ligands and receptors in three different HGSOc cell lines. Relative expression of the indicated transcripts is shown for suspension culture conditions. All experiments for performed in at least triplicate replicates. **D-F)** Immunohistochemical staining was performed for the indicated proteins using spheroids isolated from HGSOc patient ascites that was fixed and paraffin embedded. Omission of primary antibody was used a control for background staining for each patient sample. Scale bar = 100µm

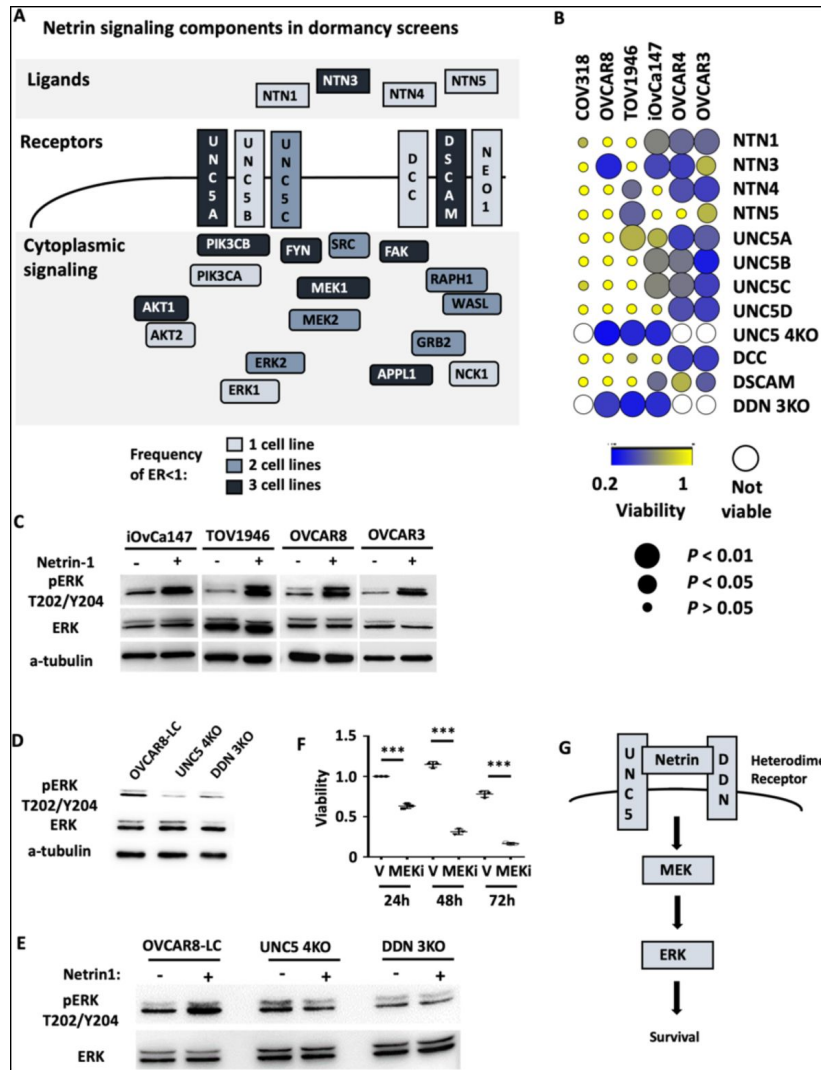


Figure 4.

Netrin ligands and their receptors are required for spheroid cell survival.

A) Illustration of netrin ligands, receptors, and other intracellular signaling molecules that are included in the Axon Guidance pathway category. The frequency of their identification in CRISPR screens is illustrated by shading and indicates how many cell lines had a negative enrichment ratio for a given component. **B)** The indicated ovarian cancer cell lines were infected with lentiviruses expressing sgRNAs directed against the indicated Netrin signaling genes. Cells were transferred to suspension culture conditions to induce spheroid formation for 72 hours and then returned to adherent conditions for 24 hours to facilitate reattachment. Re-attached cells were stained with Crystal Violet and retained dye was extracted and quantitated to measure relative survival. Each cell-gene combination was assayed in at least three biological replicates, averaged, and viability is displayed as a bubble plot. Mean survival for a given cell-gene combination was compared with GFP control gRNA transduced cells using anova and significance levels are illustrated by bubble size. Inviabile cell-gene combinations are depicted as empty white spaces. **C)** Cultures of the indicated cell lines were serum starved and transferred to suspension culture conditions and stimulated. Netrin-1 signaling was analyzed by SDS-PAGE and western blotting for phospho-ERK, ERK, and tubulin. **D)** Suspension cultures of OVCAR8, or knock out derivatives, were harvested and analyzed for relative phosphorylation levels of ERK by western blotting. Total ERK and Tubulin blotting serves as expression and loading controls. **E)** Netrin-1 signaling in OVCAR8 cells deleted for all UNC5 receptors (UNC5 4KO) was tested by serum starving cells, transferring them to suspension, and stimulating with Netrin-1 as before. Western blotting for phospho-ERK, ERK, and tubulin were as before. **F)** OVCAR8 cells were seeded in suspension culture and treated with the MEK inhibitor PD184352 (PD) or DMSO vehicle for up to 72 hours. Mean viability was compared by anova (**P<0.005). **G)** Model summarizing the roles of Netrin ligands, receptors, and downstream targets MEK and ERK in dormant survival signaling.

deficiency for Netrin signaling are rare. This is consistent with our data that all six cell lines tested reveal evidence of Netrin component dependence for survival (**Fig. 4B**). Some TCGA HGSOC cases display elevated gene expression for Netrins or their receptors, but only rare cases display low expression (**Sup. Fig. 5B**). Collectively, this suggests that Netrin signaling in HGSOC is retained despite high copy number variation in this cancer type and there is occasional overexpression.

In agreement with our experimental data that multiple Netrin ligands can contribute to spheroid survival in HGSOC (**Fig. 4A&B**), sorting cases by high level expression of Netrin-1 and -3 ligands reveals a significantly shorter survival compared to cases with low level expression and this trend is evident in the cases that only overexpress Netrin-3 (**Fig. 5A**). This genomic data supports the interpretation that Netrin signaling is retained in HGSOC and its elevation is deleterious to patient outcome.

We investigated overexpression of Netrin-1 and -3 in spheroid survival by generating OVCAR8 derivatives that stably overexpress these proteins (**Fig. 5B**). We then utilized these cells in suspension culture assays for spheroid survival as before (**Fig. 5C**). This data indicates that overexpression of either Netrin-1 or -3 increases OVCAR8 cell survival in dormant spheroid culture conditions. Examination of fixed spheroids isolated from this experiment further indicates that elevated survival in this experiment is explained by an increase in abundance of individual spheroids that overexpress either Netrin-1 or -3 (**Fig. 5D**). These experiments reveal the significance of a previously undiscovered role for Netrins in HGSOC. High level expression correlates with poor outcome and in suspension culture, overexpression confers oncogenic properties on HGSOC cells.

Netrin overexpression induces abdominal spread of HGSOC

Our findings from CRISPR screens and cell culture experiments indicate that Netrin signaling supports dormant HGSOC cell survival. Our analysis of TCGA is also predictive of a worse outcome for patients. We sought to solidify these findings by searching for how Netrin signaling affects HGSOC disease pathogenesis.

We utilized a xenograft assay in which OVCAR8 cells overexpressing Netrin-1 or -3 were injected into the peritoneal cavity and compared with OVCAR8 cells bearing an empty vector as control. Mice were harvest following 35 days to analyze disease dissemination and characterize the effect of Netrins in this model of HGSOC metastatic spread (**Fig. 6A**). Necropsy of all animals at endpoint allowed for the identification and quantitation of tumor nodules within the abdomen (**Fig. 6B**). Petal plots illustrate the frequency of specific organ and tissue sites displaying tumor nodules (**Fig. 6B**). This revealed spread to the omentum was similar across all genotypes of xenografted cells and occurred in all mice (**Fig. 6B**). In contrast, tumor nodules were detected with increased abundance on the diaphragm, the liver, and mesenteries that support the uterus (mesometrium) (**Fig. 6B**). In addition to more frequent disease spread to these locations caused by Netrin overexpressing cells, we note that the quantity of nodules was also increase in comparison with OVCAR8 control cells (**Fig. 6C-F**). We confirmed the source of these lesions to be the xenografted cells using immunohistochemical staining for human cytokeratin (**Fig. 6G,H**). Lastly, Netrin-1 and -3 histochemical staining confirms its presence in the local tumor microenvironment (**Fig. 6G,H**). The similarity of increased tumor nodules seen in xenografts and elevated spheroid formation and viability seen in culture suggests that Netrin improve cell viability in spheroid aggregates in vivo leading to increased disease spread.

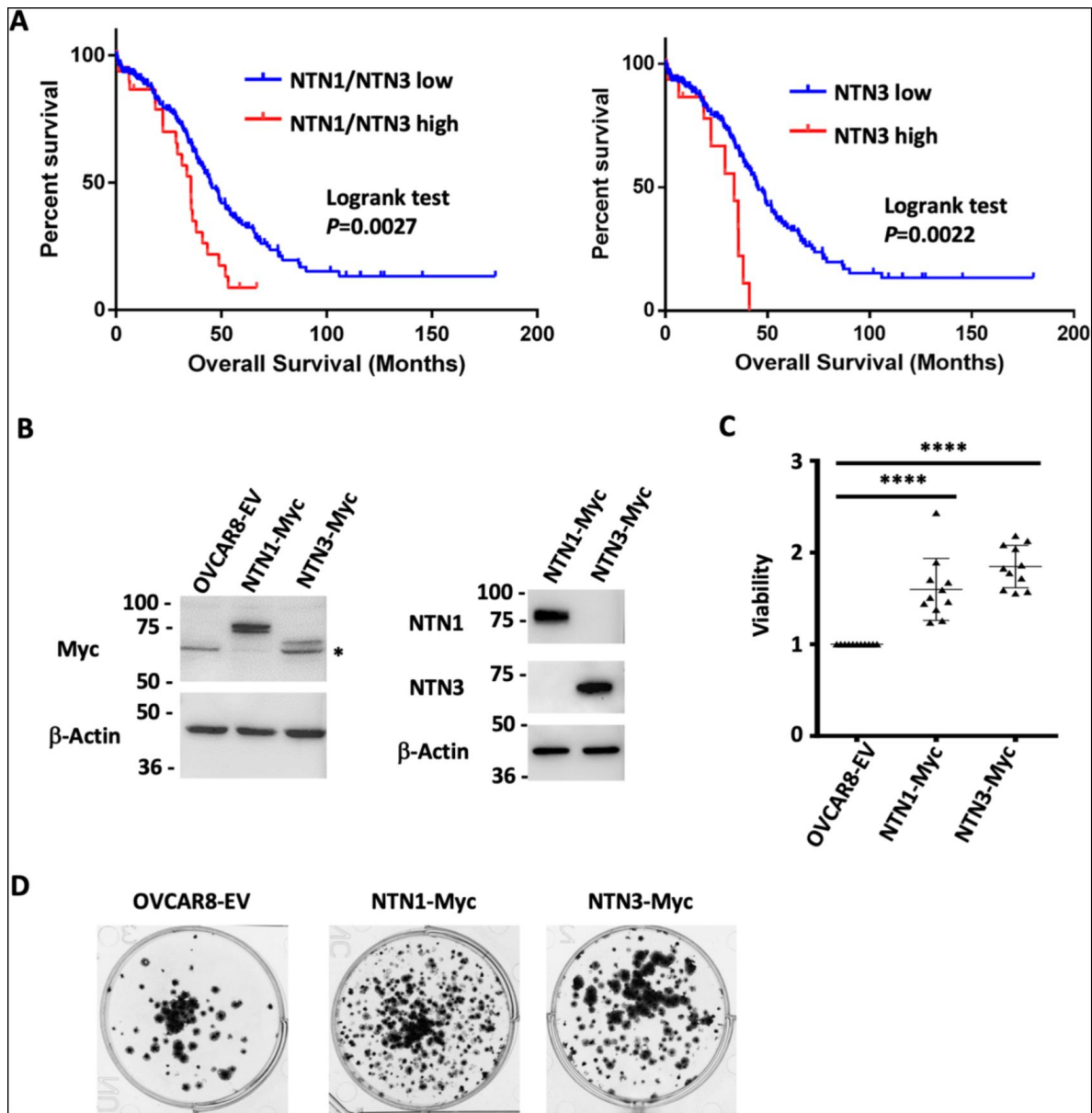


Figure 5.

Netrin ligand overexpression is associated with poor clinical outcome in HGSOc.

A) TCGA RNA-seq data for HGSOc patients (TCGA PanCancer Atlas study) was used to identify high Netrin-1 or -3 and low Netrin-1 or -3 expressing patients (high expressing are above z-score 1.2 and low are below 1.2). Overall survival was used to construct Kaplan-Meier plots and survival was compared using a logrank test. **B)** OVCAR8 cells were stably transduced with lentiviral constructs to overexpress epitope tagged Netrin-1 or -3. Western blotting for Netrins, Myc-tags, and tubulin were used to determine relative expression levels of both Netrins in these cell populations and vector controls. **C)** Control and Netrin overexpressing cells were transferred to suspension culture conditions to form spheroids, and replated to assay for viability. Mean viability and standard deviation is shown for each. Anova was used to compare survival (* $P<0.05$, *** $P<0.001$). **D)** Reattached spheroids were fixed and stained with Crystal Violet to examine size and abundance in control and Netrin-1 or -3 overexpression.

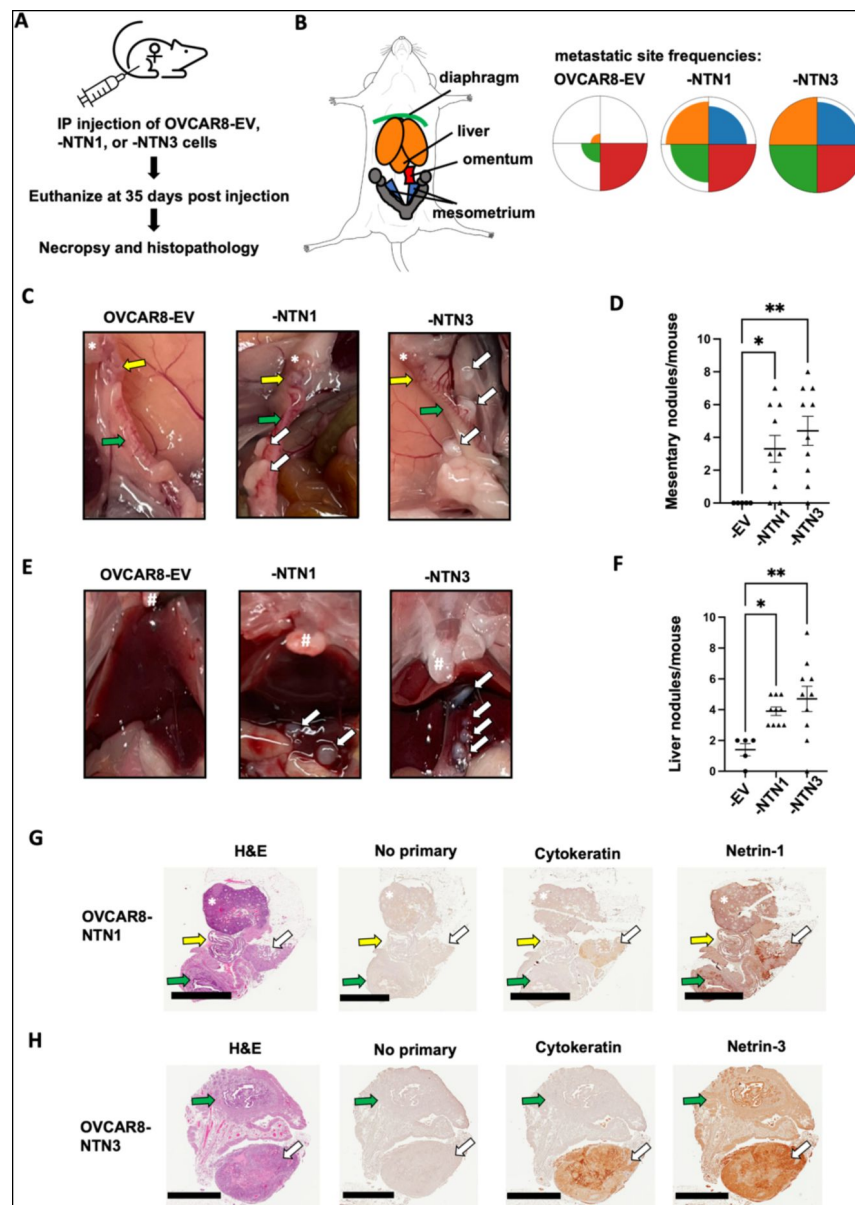


Figure 6.

Netrin overexpression causes increased dissemination of tumor nodules.

A) Netrin overexpressing and control OVCAR8 cells were injected into the intraperitoneal space of female NOD/SCID mice. Mice were euthanized following 35 days and analyzed for disease burden by necropsy and histopathology. **B)** The spread of cancer to the diaphragm, liver, omentum, and mesometrium was determined from necropsies and colors used in the anatomical schematic correspond with petal plots for each genotype of cells. Petal plots illustrate frequency of mice bearing disease spread to a particular location. The radius of color fill is proportional to the total number of animals with tumor nodules found in that location. **C)** Photographs of necropsy findings in the mesometrium. Locations of ovaries (*), oviduct (yellow arrow), and uterine horn (green arrow) are indicated in each case. Tumor nodules are indicated by white arrows. **D)** The number of mesometrium associated tumor nodules was determined for each mouse. Mean values are indicated and differences between genotype were determined by anova (* $P < 0.05$, ** $P < 0.01$). **E)** Photographs of necropsy findings in the liver. Location of sternum is indicated (#) in each image. Tumor nodules are indicated by white arrows. **F)** The number of liver associated tumor nodules was determined for each mouse. Mean values are indicated and differences between genotype were determined by anova (* $P < 0.05$, ** $P < 0.01$). **G** and **H)** Histology of mesometrial tumor nodules are shown. Serial sections were stained with H&E, or with the indicated antibodies for immunohistochemistry. Ovaries are indicated (*), as are the oviduct (yellow arrow), the uterus (green arrow), and the tumor nodule (white arrow). Scale bar = 2mm

In this report we investigated survival mechanisms in a model of HGSOc dormancy. We utilized a modified CRISPR screening approach that was adapted to discover gene loss events in largely non-proliferative conditions. We compared essential genes discovered in the screen with gene expression increases identified by RNA-seq in dormant HGSOc cultures. This implicated the Netrin signaling pathway in HGSOc spheroid survival. We independently validated the role of Netrins, their receptors, and downstream intracellular targets MEK and ERK in the survival biology of HGSOc cells in dormant culture. Furthermore, we reveal that overexpression of Netrin-1 and -3 are correlated with poor clinical outcome in HGSOc and over expression of these ligands induces elevated survival in dormant HGSOc culture conditions and disease spread in a xenograft model of dissemination. Our work implicates Netrins, their receptors, and MEK-ERK as a previously unappreciated, but critical signaling network in HGSOc survival.

HGSOc spheroid cell biology has presented a unique challenge to cancer chemotherapy. Dormant spheroids are most abundant in patients with late-stage disease and they contribute to therapeutic resistance and seed metastases (32 [↗](#)–34 [↗](#)). Hence, there is a critical unmet need to understand HGSOc spheroid dependencies and target them therapeutically. Our data suggests that Netrin signaling is a universally active mechanism in spheroid cell survival. HGSOc is characterized by extensive genomic rearrangements and copy number variants (31 [↗](#)), and we speculate that Netrin signaling is retained because of the extensive redundancy of components of this pathway. Netrin-1 and -3 can both contribute to survival signaling and knock out of three or four different Netrin receptors is necessary to compromise Netrin signaling in survival. A key goal for the future is to identify Netrin-1 or -3 overexpression patients and compare their specific disease progression patterns with our xenograft model. This will be most informative of the effects of Netrin ligand overexpression in scenarios of dormancy, metastasis, and chemotherapy resistance.

This study demonstrates that Netrins function to stimulate UNC5H and fibronectin repeat containing receptors such as DCC to activate survival signals in HGSOc spheroids. This is distinct from the known role of UNC5H and DCC as dependence receptors where Netrin inhibits their pro-apoptotic signaling (21 [↗](#)). Our data does not show elevated survival of spheroid cells in response to deletions of receptors, even in instances where families of Netrin receptors are co-deleted. Consistent with this observation, over expression of Netrin-1 or -3 in OVCAR8 cells stimulates higher level survival in suspension. In colon cancer, frequent deletions of DCC confer a survival advantage and underscore the dependence receptor concept (35 [↗](#), 36 [↗](#)). In HGSOc DCC or UNC5H deletions are relatively rare (31 [↗](#)). This suggests that ovarian cancer cells utilize Netrin signals for positive survival signaling instead of to neutralize inherent death signals from dependence receptors. Our data suggests that Netrin activates MEK and ERK to support cell survival indicating that targeting ligands, receptors or downstream at MEK are all possible approaches to inhibit this survival pathway. Netrin-1 inhibition in solid tumor xenograft models of melanoma have been shown to be effective in combination with chemotherapy (37 [↗](#)), but its effects on a dormant scenario are unknown. Identifying agents that cooperate with standard HGSOc chemotherapeutic agents such as carboplatin would offer an attractive strategy to eradicate dormant cells while combating progressive disease. Alternatively, a novel approach to targeting dormancy discovered in this study is, ironically, MEK inhibition. Dormancy is characterized by low phospho-ERK levels (3 [↗](#), 6 [↗](#)), indicating low MEK activity, yet low levels of RAS-MAPK signaling are known to provide survival signals (38 [↗](#)). A challenge with deploying MEK inhibitors as an anti-dormancy setting in HGSOc therapy is its incompatibility with standard chemotherapies that rely on cell proliferation. Overall, our study aimed to identify potential new therapeutic targets in dormancy and Netrins and MEK are feasible new candidates for dormant HGSOc disease.

Current literature offers little to connect Netrins with HGSOc beyond the observation that *NTN1* expression is increased in tumors compared to benign lesions (39). Similarly, recent dormancy literature beyond HGSOc does not include a role for Netrins (3–6, 40). We expect that Netrin signaling may play an unappreciated role in dormancy in other cancer disease site paradigms. As stated in the introduction, dormancy is characterized by acquisition of stem like properties, EMT, and in most paradigms it involves rare cells establishing themselves in perivascular or bone marrow niches. A chick chorioallantoic membrane assay of metastasis in which MDA-MB-231 cells were seeded was monitored for lung dissemination. Inhibition of Netrin-1 with NP137 and treatment with decitabine has been shown to reduce lung seeding suggesting that Netrins are relevant in a metastatic scenario that may include dormant intermediate steps in dissemination (41). Furthermore, Netrin signaling through UNC5B is known to direct vascular branching (42). This suggests that Netrins are available in the perivascular niche where dormant cells often reside. Lastly, Netrin-1 has been shown to be a component of the bone marrow niche that suppresses hematopoietic stem cell proliferation (43). While not a cancer dormancy scenario, it indicates that Netrins support some solitary dormant cell properties and are expressed by resident cells in the appropriate dormant cell niches beyond just HGSOc. For these reasons we expect Netrins will have a significant role in tumor dormancy beyond ovarian cancer.

Methods

Cell Lines and Engineering Cas9+ cells

High-grade serous ovarian cancer (HGSOc) cell lines OVCAR3, OVCAR4, OVCAR8, COV318, TOV1946 and iOvCa147 were used in this study. The iOvCa147 cells have previously been characterized and reported (44). All cell lines were verified by STR analysis. iOvCa147, OVCAR8, and TOV1946 cells were engineered to express Cas9 using pLentiCas9-Blast and clones were isolated. High Cas9 editing efficiency was determined by viability studies using sgRNAs targeting selected fitness genes (PSMD1, PSMD2, EIF3D) and a non-targeting control (LacZ) as previously described (45).

Lentiviral sgRNA library preparation

HEK293T cells were transfected with the combined A and B components of the GeCKO v2 whole genome library (123,411 sgRNAs in total) along with plasmids encoding lentiviral packaging proteins (46). Media was collected 2–3 days later, cells and debris were pelleted by centrifugation, and supernatant containing viral particles was filtered through a 0.45 μ m filter and stored at -80°C with 1.1 g/100 mL BSA.

GO-CRISPR screens in iOvCa147, OVCAR8, and TOV1946 cells

Cas9-positive and Cas9-negative cells were separately transduced with lentiviruses at a multiplicity of infection of 0.3 and with a predicted library coverage of >1000-fold. Cells were selected in puromycin and maintained in complete media (DMEM/F12 with 10% FBS, 1% pen-strep-glutamine), containing puromycin in all following steps. More than 10^9 cells were collected and split into three groups consisting of approximately 3.0×10^8 cells. Triplicate samples of 6.2×10^7 cells were saved for sgRNA sequence quantitation (T_0). The remaining cells were seeded at 2.0×10^6 cells/mL in 20 x 10 cm ULA plates. Following 2 days of culture, media containing spheroids was transferred to 10 x 15 cm adherent tissue culture plates. The next day unattached spheroid cells were collected and re-plated onto additional 15 cm plates. This process was repeated for a total of 5 days to maximally capture viable cells. Attached cells were collected and pooled for DNA extraction (T_1). Genomic DNA was extracted using the QIAmp DNA Blood Maxi Kit (Qiagen, #51194). PCR amplification and barcoding was performed as described (Hart paper, Cell, 2015). Next generation sequencing was performed using an Illumina MiSeq 50 cycle kit on an Illumina MiSeq platform.

TRACS analysis

The library reference file containing a list of all sgRNAs and their sequences (CSV file), raw reads for the pooled sgRNA library (FASTQ files (L0) and raw reads (FASTQ files) for all T₀ and T_f timepoints and replicates for Cas9-positive and Cas9-negative cells were loaded into TRACS (<https://github.com/developerpuru/TRACS>). TRACS then automatically trimmed the reads using Cutadapt (v1.15), built a Bowtie2 (v2.3.4.1) index and aligned the reads using Samtools (v1.7). TRACS used the MAGeCK read count function (v0.5.6) to generate read counts and incremented all reads by 1 to prevent zero counts and division-by-zero errors. The TRACS algorithm was then run using this read count file to determine the Library Enrichment Score (ES), Initial ES, Final ES and the Enrichment Ratio (ER) for each gene. VisualizeTRACS was used to generate graphs of Initial and Final ratios.

Pathway enrichment analyses

For GO-CRISPR screens and multi-omics comparisons, we used the filtered list of genes obtained using TRACS and performed gene ontology and pathway enrichment analysis using the ConsensusPathDB enrichment analysis test (Release 34 (15.01.2019), <http://cpdb.molgen.mpg.de/>) for top-ranked genes of interest. p_{adj} values and ER values for each gene were used as inputs. The minimum required genes for enrichment was set to 45 and the FDR-corrected p_{adj} value cutoff was set to < 0.01. The Reactome pathway dataset was used as the reference. For each identified pathway, ConsensusPathDB provides the number of enriched genes and a q value (p_{adj}) for the enrichment. Bar plots were generated in R 3.6.2 using these values to depict the significant pathways identified.

Generating DYRK1A knockout cells

A double cutting CRISPR/Cas9 approach with a pair of sgRNAs (sgRNA A and B) was used to completely excise exon 2 (322 bp region) of *DYRK1A* using a px458 vector (Addgene #48138) that was modified to express the full CMV promoter. PCR primers that flank the targeted region of *DYRK1A* were used to verify deletion. Single-cell clones were generated by limiting dilutions and evaluated for *DYRK1A* status by PCR, western blot, and sequencing. See Supplemental Table 1 for sgRNA and PCR primer sequences.

DYRK1A IP kinase assay

Whole cell lysates from adherent parental iOvCa147 cells and *DYRK1A*^{-/-} cells were extracted using complete RIPA buffer and incubated overnight with DYRK1A antibody (Cell Signaling Technology anti-DYRK1A rabbit antibody #8765). Samples were then washed with buffer and Dynabeads (Thermo Fisher Scientific Dynabeads Protein G #10003D) were added for 2 hours. Samples were then washed with buffer and recombinant Tau protein (Sigma recombinant Tau protein #T0576) and ATP (Sigma #A1852) were added and samples were incubated for 30 minutes at 37°C. 5x SDS was then added and samples were resolved by SDS-PAGE. Antibodies used for western blotting were phosphospecific Tau antibody (Cell Signaling Technology phospho-Tau Ser-404 rabbit antibody #20194) and Tau protein (Cell Signaling Technology anti-Tau rabbit antibody #46687).

RNA preparation and RNA-sequencing

Total RNA was collected from parental iOvCa147 cells and *DYRK1A*^{-/-} cells from 24-hour adherent or 6-hour spheroid conditions using the Monarch Total RNA Miniprep Kit (NEB #T2010S) as described above. RNA was collected in three replicates for each condition (adherent and spheroids). Samples were quantitated using Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and quality control analysis using Agilent 2100 Bioanalyzer (Agilent Technologies #G2939BA) and RNA 6000 Nano Kit (Agilent Technologies #5067-1511). Ribosomal RNA removal and library preparation was performed using ScriptSeq Complete Gold Kit (Illumina #BEP1206). High-throughput sequencing was performed on an Illumina NextSeq 500 platform (mid-output, 150-cyclekit).

RNA-seq analyses

Raw FASTQ data was downloaded from Illumina BaseSpace. Reads were aligned using STAR 2.6.1a(47 [↗](#)) to the human genome (Homo_sapiens.GRCh38.dna.primary_assembly.fa; sjdbGTFfile: Homo_sapiens.GRCh38.92.gtf) to generate read counts. DESeq2 was run with BEAVR(48 [↗](#)) and used to analyze read counts (settings: False discovery rate (FDR): 10%; drop genes with less than 1 reads) to identify differentially expressed genes (\log_2 fold change > 1 for upregulated genes; or \log_2 fold change < -1 for downregulated genes; $p_{\text{adj}} < 0.05$) for the following comparisons: parental iOvCa147 adherent cells vs. parental iOvCa147 spheroid cells; or parental iOvCa147 spheroid cells vs. *DYRK1A*^{-/-} spheroid cells. For RNA-seq, we formed pathway analyses in BEAVR and downloaded the pathway enrichment table to construct bar plots and determine overlapping pathways using R 3.6.2.

Western blots

Adherent cells were washed in 1x PBS and lysed on the plate by the addition of complete RIPA buffer with protease (Cell Signaling Technology #5871S) and phosphatase (Cell Signaling Technologies #5870S) inhibitors and incubated for 5-10 minutes on ice. Spheres were collected by gentle centrifugation at 300 rpm, washed gently in ice cold PBS, centrifuged again and then resuspended in RIPA buffer as above. Samples were then centrifuged at 14,000 RPM at 4°C for 10 minutes. The supernatant liquid for each sample was collected and the pellets re-extracted with RIPA containing the above inhibitor cocktails. Following centrifugation, the supernates for each treatment were collected and pooled. Protein was determined by Bradford assay. To assess the effect of Netrin 1 on downstream signaling, spheres incubated under non adherent conditions for 5 days were challenged with the addition of 500 ng/ml human recombinant Netrin-1 (R&D Systems #6419-N1-025) for a period of 20 minutes before collection. Lysates were mixed with 6x SDS loading dye buffer and resolved using standard SDS-PAGE protocols. Antibodies used for blotting were p42/44 MAPK (Erk1/2) (Cell Signaling Technology rabbit antibody #4695), Phospho-p44/42 MAPK(Erk1/2) T202/Y204 (Cell Signaling Technology; rabbit antibody #4370), p38 MAPK (Cell Signaling Technology; rabbit antibody #9212), p38 MAPK T180/Y182 (Cell Signaling Technology; rabbit antibody #9211), DYRK1A (Cell Signaling Technology anti-DYRK1A rabbit antibody #8765), Netrin-1 (Abcam rabbit antibody #ab126729), Netrin-3 (Abcam rabbit antibody #ab185200), α -tubulin (Cell Signaling Technology anti- α Tubulin rabbit antibody #2125) and β -actin (Millipore Sigma rabbit antibody #A2066).

RT-qPCR analysis of gene expression

Cells were trituated off the plate using Versene (0.5mM EDTA in PBS), collected, counted and 10^6 cells plated onto Ultra-Low Attachment cell culture dishes (Corning) to induce sphere formation. The spheres were collected after 24 h and RNA extracted using the Monarch Total RNA Miniprep Kit (New England Biolabs #T2010S). RNA was extracted from adherent cells directly from the plate. Extracted RNA was quantitated using a NanoDrop and stored at -80° until used. First strand cDNA was generated from isolated RNA using the iScript cDNA Synthesis Kit (Bio-Rad, #1708890) and RT-qPCR performed on the resulting cDNA (diluted 5-fold) using the iQ SYBR Green Supermix (Bio-Rad, #1708882) and following the manufactures instructions. Reactions were performed in 0.2ml non-skirted low profile 96 well PCR plates (Thermo Scientifi, #AD-0700) using a CFX Connect Real Time System thermo-cycler (Bio-Rad).

TCGA patient data analyses

We used the cBioPortal for Cancer Genomics (<https://www.cbioportal.org>)(49 [↗](#)) to analyze Netrin pathway alterations in Ovarian Serous Cystadenocarcinoma patients (TCGA, PanCancer Atlas) for genomic alterations and mRNA expression. OncoPrints were generated for mRNA expression (mean z-score threshold ± 1.5), and mutations and putative copy number alterations for the

following genes: *NTN1*, *NTN3*, *NTN4*, *NTN5*, *UNC5A*, *UNC5B*, *UNC5C*, *UNC5D*, *DCC*, *DSCAM*. Survival analysis of *NTN1*, and -3 utilized patients from TCGA, PanCancer Atlas dataset and RNA-seq expression (high expression z-score threshold > 1.2 and low is below 1.2).

Generation of gene knockout cell lines

Gibson Assembly (NEB #E2611) was used to clone sgRNAs sequences derived from the GeCKO v2 library per gene of interest (*NTN1*, *NTN3*, *NTN4*, *NTN5*, *DSCAM*, *DCC*, *NEO1*, *UNC5A*, *UNC5B*, *UNC5C*, *UNC5D*, and *EGFP* control (See Supplemental Table 1 for sgRNA sequences for each)) into lentiCRISPR v2 (Addgene #52961). For individual gene knockouts pooled libraries of up to four sgRNAs were generated. For multiple gene knockouts pooled libraries of two sgRNAs/gene were constructed. To each 20-nucleotide guide (Supplemental table I) was appended the 5' sequence, 5'-TATCTTGTGGAAAGGACGAAACACC-3' and the 3' sequence, 5'-GTTTTAGAGCTAGAAATAGCAAGTTAAAAT-3' (50 [DOI](#)). A 100 bp fragment was then generated using the forward primer, 5'-GGCTTTATATATCTTGTGGAAAGGACGAAACACC-3', and reverse primer, 5'-CTAGCCTTATTTAACTTGCTATTCTAGCTCTAAAAC-3'. The resulting fragment was gel purified, quantitated by Qubit and cloned into the lentiviral vector as described (50 [DOI](#)). To generate virus particles, HEK293T cells were transfected with the assembled plasmid along with plasmids encoding lentiviral packaging proteins. Media was collected after 2-3 days and any cells or debris were pelleted by centrifugation at 500 x g. Supernatant containing viral particles was filtered through a low protein binding 0.45 µM filter. For each knockout, it was important to ensure that each target cell was transduced with a full complement of guides. To ensure this, the cells were transduced at relatively high MOI. Briefly, 25,000 cells were plated and transduced with fresh HEK293T conditioned media containing virus. While we did not titer the viral preparations for these experiments, previous experience indicated that we could generate sufficiently high virus titers using our protocol. Under these conditions, we found a lack of puromycin sensitive cells following 2-3 days in media containing 2-4 µg/ml puromycin indicating that every cell received at least one virus particle. Multi-gene knock outs were created through multiple rounds of infection with the relevant culture supernatants followed by one round of puromycin selection.

Generation of gene overexpression cell lines

Plasmids encoding Netrin1 and Netrin3 were obtained from Addgene (Ntn1-Fc-His, #72104 and Ntn3-Fc-His, #72105). Netrin1 was PCR amplified using the forward primer 5'-GCTATCGATATCCCAAACGCCACCATGATGCGCGCTGTGTGGGAGGCGCTG-3' and reverse 5'-GCTATCTCTAGAGGCCTTCTTGCACTTGCCCTTCTTCTCCCG-3' and cloned into the EcoRV/XbaI sites of pcDNA3.1/myc-HisA. For lentiviral expression, pcDNA NTN1 was amplified using the primers, forward 5'-CACTGTAGATCTCCCAAACGCCACCATGATGCGCGCTGTGTGGGAGGCGCTG-3' and, reverse 5'-ACGCGTGAATTCTTATCAACCGGTATGCATATTCAGATCCTTCTGAGAT-3' while NTN3 was amplified from pcDNA NTN3 using, forward 5'-CTCGAGAGATCTGCGCCGCCACCATGCCTGGCTGGCCCTGG-3' and the same reverse primer as for NTN1. In this way both constructs carry a C-terminal Myc tag. Both cDNA's were inserted into the BamHI/EcoRI sites of the lentiviral expression vector FUtdTW (Addgene #22478). Construction of lentiviral particle and transduction into the target cells was as described above. Selection was with Zeocin (ThermoFisher #R25001).

Spheroid formation and reattachment assays

For each targeted gene in iOvCa147, OVCAR3, OVCAR4, OVCAR8, COV318, or TOV1946 cells, spheroid viability was assayed as follows: Knockout cells were cultured for 72 hours in suspension conditions using ULA plasticware (2×10^6 cells per well) to allow spheroid formation. Addition of therapeutics (PD184352) was made at the time of plating. Spheroids were collected and transferred directly to standard plasticware to facilitate reattachment for 24 hours. Reattached cells were fixed in 25% methanol in PBS for 3 minutes. Fixed cells were incubated for 30 minutes with shaking in 0.5% crystal violet, 25% methanol in PBS. Plates were carefully immersed in water to

remove residual crystal violet and destained in 10% acetic acid in 1x PBS for 1 hour with shaking to extract crystal violet from cells. Absorbance of crystal violet was measured at 590 nm using a microplate reader (Perkin Elmer Wallac 1420). Each knockout was normalized to the EGFP control. Because crystal violet binds protein, a linear relationship between the absorbance and the number of cells present is obtained and may be represented as the percent of spheroid cells surviving suspension.

Xenografts

Female NOD-SCID mice were purchased from JAX (#001303). They were engrafted between six and eight weeks of age with 4×10^6 OVCAR8 control or modified derivatives. Mice were housed for 35 days and euthanized and subjected to a necropsy to analyze disease spread. Tumor lesions were photographed and quantitated. Abdominal organs and associated tumor lesions were fixed in 10% formalin overnight and transferred to 70% ethanol until embedded.

Patient derived spheroids

Patient derived spheroids were isolated from ascites fluid obtained from debulking surgical procedures. These HGSOc samples were obtained with permission and institutional REB oversight. Spheroids were prepared by filtering the ascites fluid through a 100 μ m mesh filter and washing the captured spheroids from the filter with PBS. The spheres were collected by gravity on ice in PBS, washed twice with ice cold PBS and allowed to settle on ice. Spheres were then fixed for 20 minutes in 10% formalin, washed, and resuspended in PBS before being embedded in paraffin.

Histology and immunohistochemistry

Fixed organs from xenografts or spheroids were embedded in paraffin, sectioned, and stained with H&E. Sections were deparaffinized by standard procedures and antigen retrieval was performed in 10mM citrate, pH6.0. The sections were blocked with 10% goat serum/3% BSA/0.3% Triton X-100 and stained with antibodies to Netrin-1 (Abcam #ab126729, 1:200), Cytokeratin (human specific cytokeratin 7 and 8, ZETA Corporation #Z2018ML, 1:200), EpCAM (Cell Signaling Technologies, #14452), p53 (Cell Signaling Technologies, #2527) and Netrin-3 (1:250). Netrin-3 antibodies were generated using immunized rabbits and their generation and purification will be published elsewhere. Primary antibodies were followed with a biotinylated anti-rabbit (Vector Labs, # BA-1000) or anti-mouse (Jackson ImmunoResearch, #115-067-003) IgG as a secondary antibody (1:500). Proteins were visualized using DAB chemistry (ImmPACT DAB, Vector Labs, #SK-4105).

Statistical analyses

Specific statistical tests used are indicated in the figure legends for each experiment. Analysis was done using GraphPad Prism 6.

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Reviewer #1 (Public Review):

Summary:

Perampalam et al. describe novel methods for genome-wide CRISPR screening to identify and validate genes essential for HGSOc spheroid viability. In this study, they report that Netrin signaling is essential for maintaining disseminated cancer spheroid survival, wherein overexpression of Netrin pathway genes increases tumor burden in a xenograft model of ovarian cancer. They also show that high netrin expression correlates with poor survival outcomes in ovarian cancer patients. The study provides insights into the biology of netrin signaling in DTC cluster survival and warrants development of therapies to block netrin signaling for treating serous ovarian cancer.

Strengths:

- The study identifies Netrin signaling to be important in disseminated cancer spheroid survival
- A Novel GO-CRISPR methodology was used to find key genes and pathways essential for disseminated cancer cell survival

Weaknesses:

- The term dormancy is not fully validated and requires additional confirmation to claim the importance of Netrin signaling in "dormant" cancer survival.
- Findings shown in the study largely relate to cancer dissemination and DTS survival rather than cancer dormancy.

Reviewer #2 (Public Review):

Summary:

In this article, the authors employed modified CRISPR screens ["guide-only (GO)-CRISPR"] in the attempt to identify the genes which may mediate cancer cell dormancy in the high grade serous ovarian cancer (HGSOC) spheroid culture models. Using this approach, they observed that abrogation of several of the components of the netrin (e.g., DCC, UNC5Hs) and MAPK pathways compromise the survival of non-proliferative ovarian cancer cells. This strategy was complemented by the RNAseq approach which revealed that a number of the components of the netrin pathway are upregulated in non-proliferative ovarian cancer cells and that their overexpression is lost upon disruption of DYRK1A kinase that has been previously demonstrated to play a major role in survival of these cells. Perampalam et al. then employed a battery of cell biology approaches to support the model whereby the Netrin signaling governs the MEK-ERK axis to support survival of non-proliferative ovarian cancer cells. Moreover, the authors show that overexpression of Netrins 1 and 3 bolsters dissemination of ovarian cancer cells in the xenograft mouse model, while also providing evidence that high levels of the aforementioned factors are associated with poor prognosis of HGSOC patients.

Strengths:

Overall it was thought that this study is of potentially broad interest inasmuch as it provides previously unappreciated insights into the potential molecular underpinnings of cancer cell dormancy, which has been associated with therapy resistance, disease dissemination, and relapse as well as poor prognosis. Notwithstanding the potential limitations of cellular models in mimicking cancer cell dormancy, it was thought that the authors provided sufficient support for their model that netrin signaling drives survival of non-proliferating ovarian cancer cells and their dissemination. Collectively, it was thought that these findings hold a promise to significantly contribute to the understanding of the molecular mechanisms of cancer cell dormancy and in the long term may provide a molecular basis to address this emerging major issue in the clinical practice.

Weaknesses:

Several issues were observed regarding methodology and data interpretation. The major concerns were related to the reliability of modelling cancer cell dormancy. To this end, it was relatively hard to appreciate how the employed spheroid model allows to distinguish between dormant and e.g., quiescent or even senescent cells. This was in contrast to solid evidence that netrin signaling stimulates abdominal dissemination of ovarian cancer cells in the mouse xenograft and their survival in organoid culture. Moreover, the role of ERK in mediating the effects of netrin signaling in the context of the survival of non-proliferative ovarian cancer cells was found to be somewhat underdeveloped.