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Neutrophil extracellular trap gene expression signatures identify prognostic and targetable signaling axes for inhibiting pancreatic tumour metastasis



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Tumour associated neutrophils (TANs) promote metastasis through interactions of Neutrophil Extracellular Traps (NETs) with tumour cells. However, molecular details surrounding the interactions between NETs and Pancreatic Ductal Adenocarcinoma (PDAC) cells are poorly understood. Here, we examine the contribution of NETs in the progression of PDAC, which is characterized by high metastatic propensity. We carry out consensus clustering and pathway enrichment analysis of NET-related genes in an integrated cohort of 369 resectable and metastatic PDAC patient tumour samples, and compile two gene expression signatures comprising of either, integrin-actin cytoskeleton and Epithelial to Mesenchymal Transition (EMT) signaling, or cell death signaling, which identifies patients with very poor to better overall survival, respectively. Tumour Infiltrating neutrophils and NETs associate with ITGB1, CCDC25 and ILK, within clinical and experimental PDAC tumours. Functionally, exposure of PDAC cells to NETs identifies a cytoskeletal dynamic-associated CCDC25-ITGB1-ILK signaling complex which stimulates EMT and migration/invasion. NETosis-driven experimental metastasis to the lungs of PDAC cells delivered through the tail vein of female non-obese diabetic (NOD) scid gamma (NSG) mice is significantly inhibited by *ILK* knock down. Our data identify novel NET-related gene expression signatures for PDAC patient stratification, and reveal targetable signaling axes to prevent and treat disease progression.

Pancreatic Ductal Adenocarcinoma (PDAC) is a difficult-to-treat cancer punctuated by early metastasis and a dismal prognosis^{1–3}. Chronic pancreatitis is a risk factor for the development of PDAC^{4,5}, and tumours and metastases are characterised by an immunosuppressive, fibro-inflammatory tumour microenvironment (TME), underscoring the importance of inflammation as a driver of PDAC progression^{6,7}. Neutrophils, vital cellular

effectors of inflammation⁸, have emerged as critical pro-tumourigenic and metastasis mediators^{9–11} and neutrophils contribute to tumour progression and metastasis, including ‘awakening’ dormant circulating cancer cells^{12–16}.

Neutrophils can undergo NETosis in response to cytokine or chemokine-mediated inflammatory stimuli to form neutrophil extracellular traps (NETs), which are structures composed of decondensed, modified

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genomic DNA, histones and proteases^{17,18}. NETs have been shown to interact with tumour cells within the primary tumour, within the vasculature, or at the metastatic site^{13–15,19}. The NET-tumour interaction promotes metastatic colonisation by stimulating both invasion as well as growth within the metastatic site^{14,15,20,21}.

While studies in PDAC have implicated a role for NETs in both primary tumours and in metastases^{22,23}, the molecular details of the interactions between NETs and PDAC cells are poorly understood, and the contribution of neutrophils and NETs to PDAC progression and metastasis remains unclear.

Here we set out to determine, using unbiased genomic and proteomic analysis of resectable and metastatic PDAC patient cohort data, the role of NETosis and NETs in PDAC patient outcome. We have identified novel prognostic NET gene expression signatures that reveal NET-associated integrin-beta-1-coiled-coil domain-containing protein 25-integrin-linked kinase (ITGB1-CCDC25-ILK) mediated cytoskeletal dynamics and epithelial to mesenchymal transition (EMT) pathways as poor outcome indicators for PDAC patients. Our data demonstrate a novel association of CCDC25 with ITGB1 and ILK in the context of NET-mediated cytoskeletal reorganisation and cell migration/invasion. Furthermore, we demonstrate that patient and experimental PDAC tumours and metastases harbour substantial amounts of intratumoural neutrophils with associated NETs. Functional exploration of whether the NET signature-related ITGB1-CCDC25-ILK and EMT axis plays a role in the response of PDAC cells to NETs reveals that this axis promotes NET-induced PDAC cell invasion and metastasis, suggesting novel prognostic and therapeutic approaches for the prevention and inhibition of PDAC metastasis.

Results

Neutrophil extracellular trap (NET) gene signatures identify PDAC patient outcomes

Pan-cancer analyses across 14 solid cancer types have shown that, amongst all tumour-infiltrating leucocyte populations, intratumoural neutrophils are most robustly associated with poor prognosis²⁴ and a high circulating neutrophil-to-lymphocyte ratio (NLR) is also correlated with shorter survival^{10,11}, signalling the importance of tumour-infiltrating neutrophils in cancer progression. To determine whether a higher NLR is relevant to the outcome of PDAC patients in an advanced disease setting, we examined the baseline circulating NLR in the blood samples of a cohort of 67 patients presenting with metastatic PDAC. We observed that a higher baseline NLR is associated with significantly shorter overall patient survival (Fig. 1A), establishing a clinical link between neutrophils and metastatic PDAC.

While the formation of NETs by neutrophils has been linked to tumour progression and metastasis^{14,15}, here we were interested in determining whether a NET-based gene expression signature could be compiled for identifying PDAC patient outcomes. To stratify PDAC tumours based on relative expression levels of NET-related genes, we assembled a comprehensive RNA-seq dataset encompassing both resectable and metastatic PDAC patient tumours. We reasoned that the use of samples from primary tumours and from metastases would afford a unique opportunity to define a NET-related gene signature that incorporates clinical samples across the disease continuum. The resectable datasets included PDAC samples from The Cancer Genome Atlas (TCGA) and the Clinical Proteomic Tumor Analysis Consortium-3 (CPTAC-3) databases. RNA-seq datasets for patients with metastatic PDAC were obtained from the Prospectively Defining Metastatic Pancreatic Ductal Adenocarcinoma Subtypes by Comprehensive Genomic Analysis (PanGen, NCT02869802) and the BC Cancer Personalized OncoGenomics (POG, NCT02155621) clinical trials. In total, 369 patient samples were used for initial analysis (TCGA $n = 130$; CPTAC $n = 133$; PanGen, $n = 81$; POG, $n = 25$).

We first carried out gene-wise co-expression-based cluster analysis on this patient cohort using a NET-related gene set, which we curated from previous studies in the context of cancer, including triple-negative breast cancer and PDAC^{25,26}. Specifically, the NET-related genes were compiled from a previously reported neutrophil gene set²⁷, NETosis-related gene

sets^{28,29} and eight hub genes identified recently as a NET and EMT-based prognostic model in the context of PDAC²⁵.

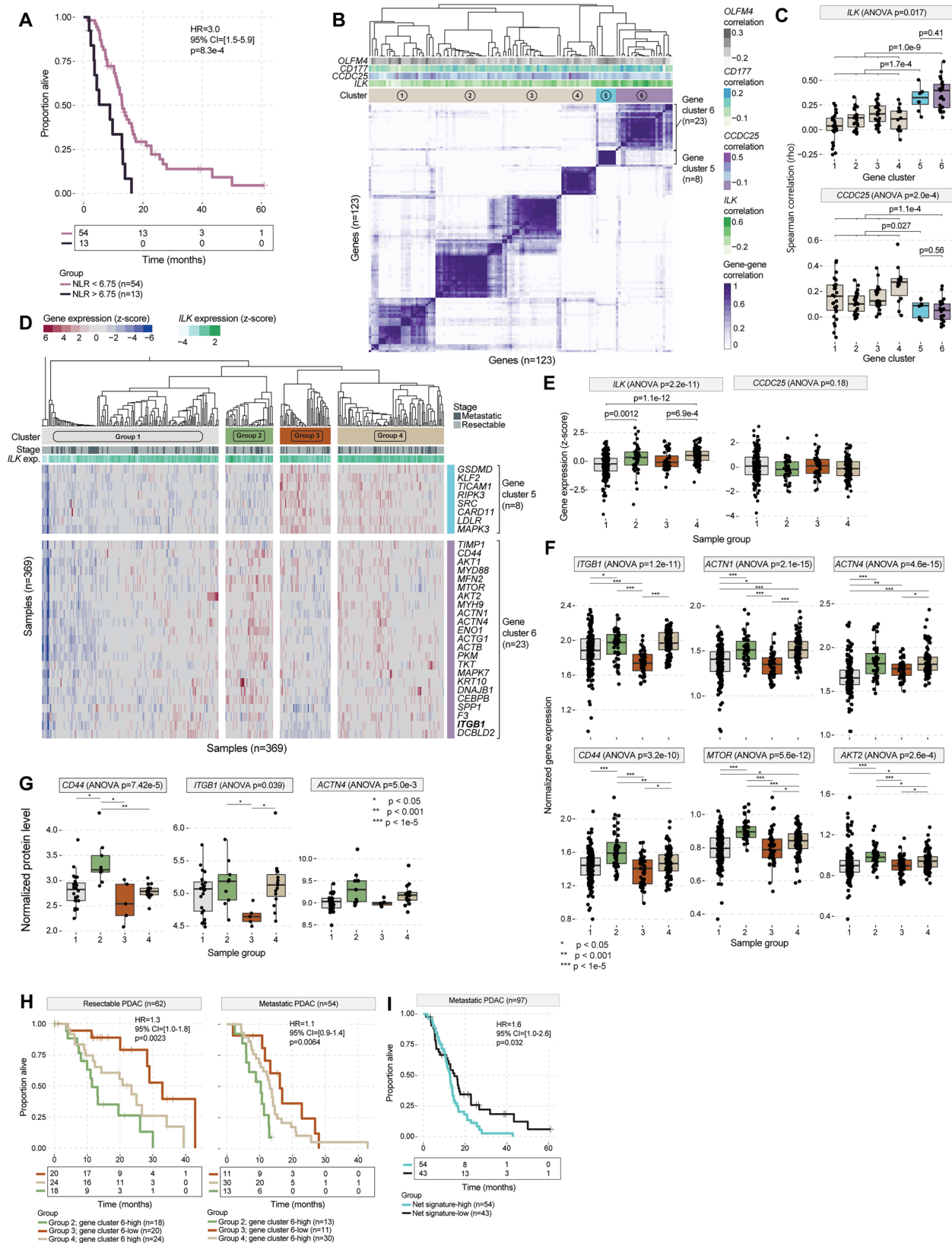
The optimal clustering solution identified six distinct clusters of PDAC-specific, co-expressed NET-related genes (Fig. 1B). Because of the previously described mechanistic link between NETs and tumour cell-associated CCDC25 and ILK²⁰, we investigated whether any of the NET gene clusters correlated with CCDC25 and ILK expression. We found that two of the gene clusters, cluster 5 and cluster 6, were significantly correlated with high levels of ILK expression ($p = 0.017$), but, interestingly, not with high levels of CCDC25 expression (Fig. 1B, C). Further analysis of these gene sets using additional established markers of neutrophils, cluster of differentiation 177 (CD177)³⁰ and Olfactomedin 4 (OLFM4)^{31,32}, further separated the two ILK-associated gene clusters into OLFM4-high and OLFM4-low groups (Fig. 1B and Supplementary Fig. 1A). Thus, this analysis identified a 31 gene PDAC-specific NET gene expression signature comprising genes from two co-expressed gene clusters that is highly correlated with expression of ILK. The genes present within the NETs signature are as follows: Gene Cluster 5 ($n = 8$ genes: *LDLR*, *MAPK3*, *CARD11*, *GSDMD*, *SRC*, *RIPK3*, *KLF2* and *TICAM1*) and Gene Cluster 6 ($n = 23$ genes: *ACTB*, *PKM*, *ACTG1*, *ACTN4*, *ENO1*, *MYH9*, *CD44*, *ITGB1*, *SPPI*, *TIMP1*, *ACTN1*, *AKT2*, *TKT*, *F3*, *AKT1*, *MFN2*, *MTOR*, *MYD88*, *DNAJB1*, *CEBPB*, *DCBLD2*, *KRT10* and *MAPK7*).

Next, we used this bespoke NET signature gene set to perform sample-wise cluster analysis on the PDAC patient cohort defined above. Semi-automated consensus clustering of patients using this gene expression signature identified four distinct subgroups of patients (Fig. 1D). While patients in group 1 exhibit low levels of expression of these NETs signature genes, patients in groups 2, 3 and 4 were identified as having high levels of expression of these genes (Fig. 1D). Interestingly, groups 2 and 3 express high levels of NET signature genes, but clearly represent 2 distinct populations (Fig. 1D). Specifically, group 2 patients express high levels of cluster 6 genes and low levels of cluster 5 genes, whereas group 3 patients express high levels of cluster 5 genes (Fig. 1D) and low levels of cluster 6 genes. Furthermore, patients in groups 2 and 4 demonstrate significantly higher levels of expression of ILK ($p = 2.2 \times 10^{-11}$), compared to patients in groups 3 and 1 (Fig. 1E). Levels of expression of CCDC25 were not different amongst the 4 groups (Fig. 1E).

Closer inspection of gene cluster 6 revealed the presence of *ITGB1* (Fig. 1D), which is a well-established interactor of ILK^{33–35}. Interestingly, this cluster of genes is also highly enriched in genes involved in integrin-actin cytoskeleton organisation, including beta-actin (*ACTB*), G-actin (*ACTG1*), alpha-actinin 1 (*ACTN1*) and *ACTN4*³⁶ (Fig. 1D). In addition, other genes established to be involved in ILK signalling, such as serine/threonine kinase (*AKT*), mammalian target of rapamycin (*mTOR*) and *CD44* are also present in this cluster^{34,37}. Significantly, when resectable and metastatic samples were analysed independently, a higher percentage of patients in group 2 showed overexpression of these ILK-associated genes, compared to group 3 (Table 1). Further analysis showed that the expression of many of these ILK-associated genes are elevated in groups 2 and 4, compared to group 3 (Fig. 1F and Supplementary Fig. 1B), in addition to elevated protein expression of ITGB1 and CD44, which were quantified by mass spectrometric based proteomics³⁸ in a subset of patient samples (Fig. 1G and Supplementary Fig. 1C).

Gene cluster 5, on the other hand, represents genes involved in cell death, especially necroptosis, including gasdermin D (*GSDMD*), receptor-interacting protein kinase 3 (*RIPK3*), and mitogen-activated protein kinase 3 (*MAPK3*), and autophagy (Kruppel-like factor 2, *KLF2*) (Fig. 1D). Here, when resectable and metastatic samples were analysed independently, a higher percentage of patients in group 3 showed significantly higher levels of expression of these genes, compared to group 2 (Table 2).

To determine whether these patient groups are correlated with differential overall survival, we carried out Kaplan–Meier analysis on patient groups 2–4. The results showed that patient group 2, which has a high NET signature and high expression of ILK signalling genes, showed significantly worse overall survival for both resected ($p = 0.023$) and metastatic



($p = 0.0064$) patient populations, compared to groups 3 and 4 (Fig. 1H). In contrast, patient group 3, which did not represent genes for ILK signalling, but instead consists of genes involved in cell death, had overall better survival outcomes than groups 2 and 4 (Fig. 1H).

Finally, analysis of all metastatic PDAC samples showed that NET gene expression signature high patients (groups 2, 3 and 4) survived for a significantly shorter time, compared to NET gene expression signature low patients (group 1) (Fig. 1I).

Fig. 1 | Neutrophil extracellular trap (NET) gene signatures identify pancreatic ductal adenocarcinoma (PDAC) patient outcomes. **A** Kaplan–Meier survival analysis showing that a higher baseline neutrophil-to-lymphocyte ratio (NLR) is associated with shorter overall survival in patients with metastatic PDAC ($n = 67$). **B** Heat map showing the optimal consensus clustering solution for NET-related genes in human PDAC samples (resectable and metastatic combined, $n = 369$). Tracks above the clusters show the Spearman correlation (ρ) of the indicated genes versus each gene in the heat map. **C** Box plots showing the relationship between expression of the indicated genes and each gene cluster identified in **(B)**. Boxes indicate median (central line) and 25–75% IQR (bounds of box), and whiskers extend from box bounds to the largest value no further than 1.5 times the IQR.

D Heat map showing clustering of human PDAC samples ($n = 369$) according to expression (z -score) of genes comprising a NET signature defined by consensus clustering in **(B)**. **E** Box plots showing levels of gene expression (z -score) for the indicated genes in each of the sample clusters identified in **(D)**. Box plots showing **F** transcript levels and **G** protein levels for a subset of genes across the different patient groups identified in **(D)**. **H** Kaplan–Meier survival analysis of patients in groups 2, 3 and 4 with resectable (left) and metastatic (right) PDAC stratified on PDAC-specific NET signature. **I** Kaplan–Meier survival analysis of patients with metastatic PDAC stratified as NET-signature-high (groups 2, 3 and 4 in **D**) and NET-signature-low (group 1 in **D**). For **(H, I)**, Log-rank test P values, hazard ratios (HR) and 95% confidence intervals (CI) are shown.

Table 1 | Proportion of patients in groups 2 and 3 with upregulated expression of the indicated cluster 6 genes

Gene ^a	Resectable (%)		Metastatic (%)	
	Group 2	Group 3	Group 2	Group 3
<i>ACTN1</i>	42	5	46	8
<i>ACTN4</i>	45	16	46	8
<i>ACTB</i>	52	14	54	25
<i>MTOR</i>	52	22	52	17
<i>AKT1</i>	33	35	38	25
<i>AKT2</i>	48	19	54	8
<i>CD44</i>	39	16	92	0
<i>ITGB1</i>	42	3	23	0

^aGene names are presented in italics.

Table 2 | Proportion of patients in groups 2 and 3 with upregulated expression of the indicated cluster 5 genes

Gene ^a	Resectable (%)		Metastatic (%)	
	Group 2	Group 3	Group 2	Group 3
<i>GSDMD</i>	15	57	15	33
<i>KLF2</i>	3	78	0	83
<i>RIPK3</i>	9	65	0	58
<i>SRC</i>	15	68	31	50
<i>CARD11</i>	12	59	8	50
<i>LDLR</i>	0	59	0	75
<i>MAPK3</i>	12	62	15	25

^aGene names are presented in italics.

Neutrophils and NETs are present in ITGB1, ILK and CCDC25 expressing human PDAC tumours and preclinical xenograft models of PDAC

Our bioinformatics data show that patients with high expression of NET signature genes associated with ILK signalling have poor outcomes and suggest a potential role for NET-mediated ITGB1-ILK signalling in PDAC. To validate that human PDAC tumours harbour neutrophils and NETs, and to determine whether ITGB1 and ILK, as well as CCDC25 and ILK, co-localise in these tissues we analysed, using multi-colour immunofluorescence staining, primary tumour tissue sections from 20 PDAC patients for myeloperoxidase (MPO) and citrullinated histone H3 (cit-H3), which are markers neutrophils and NETs, as well as for ITGB1, ILK and CCDC25. We observed regions of co-localisation of MPO and cit-H3 in multiple representative tumour samples, demonstrating the presence of NETs in the primary tumours of patients with PDAC (Fig. 2A). We further interrogated these tumours for ITGB1 and ILK, as well as for CCDC25 and ILK. We observed that ILK is highly expressed at the cell surface of PDAC

epithelial cancer cells and that ILK and ITGB1 co-localise in these regions (Fig. 2B). While our bioinformatics analysis did not show a correlation with gene expression of CCDC25, we did observe co-localisation of ILK and CCDC25 protein in the human PDAC tumour tissues (Fig. 2C).

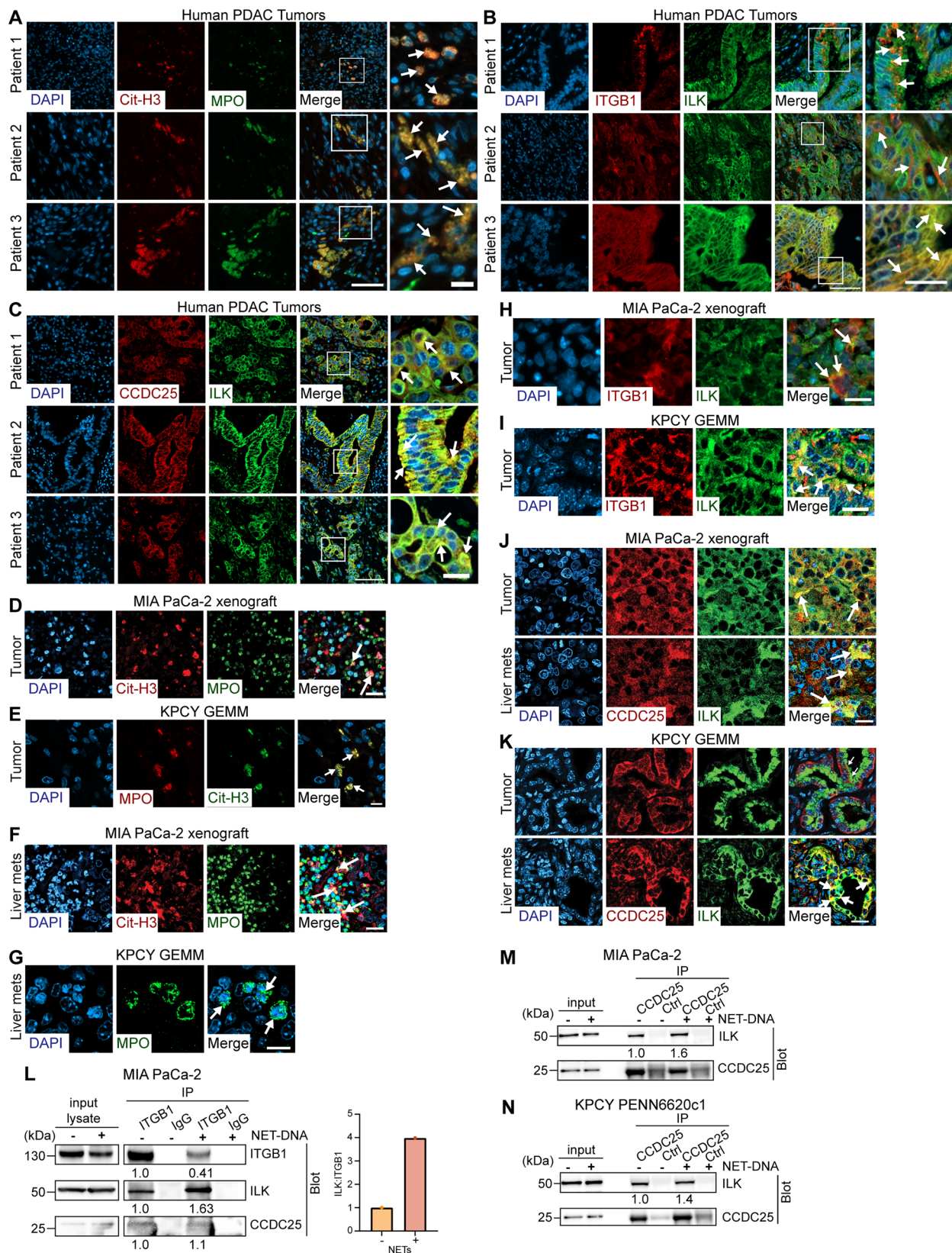
To validate these findings further, we interrogated multiple preclinical models of PDAC for NETs, ITGB1, ILK and CCDC25. We observed the presence of infiltrating neutrophils and NETs in representative primary tumour tissue sections from a MIA PaCa-2 human xenograft model (Fig. 2D) and a *Kras*^{G12D}/*Pdx1-Cre*/*Tp53/Rosa*^{YFP} (KPCY) genetically engineered mouse model (GEMM) (Fig. 2E) of PDAC. We also observed infiltrating neutrophils and NETs in liver metastases from the MIA PaCa-2 xenografts (Fig. 2F) and infiltrating neutrophils in the KPCY GEMM liver metastatic tissues (Fig. 2G). Furthermore, ILK is highly expressed in the cancer cells of these models and colocalizes with both ITGB1 (Fig. 2H, I) and CCDC25 (Fig. 2J, K). Collectively, these data demonstrate, in clinical and experimental PDAC tumours, the presence of the infiltrating neutrophils and NETs, together with the presence of tumour cells expressing ILK in spatial context with ITGB1 and CCDC25.

To determine whether ITGB1, ILK and CCDC25 interact in the context of NET stimulation, we cultured human MIA PaCa-2 cells and mouse KPCY PDAC cells with NETs isolated from dimethyl sulfoxide (DMSO)-differentiated, phorbol 12-myristate 13-acetate (PMA)-activated HL-60 cells, a well-established, standardised source of NETs³⁹, and carried out co-immunoprecipitation (co-IP) assays. We observed that the IP of ITGB1 from PDAC cells pulled down both ILK and CCDC25 (Fig. 2L), suggesting that these proteins contribute to a multi-protein complex in PDAC cells. Interestingly, exposure of the cells to NETs resulted in an overall subtle decrease in the levels of ITGB1 in the immunoprecipitate, but increased amounts of ILK immunoprecipitating with ITGB1, compared to control conditions, suggesting that the relative amount of ILK complexed with ITGB1 increases with exposure to NETs (Fig. 2L). Similarly, IP of CCDC25 from PDAC cells demonstrated that a greater amount of ILK is associated with CCDC25 in cells exposed to NETs (Fig. 2M, N). These data demonstrate, for the first time, a tripartite interaction of ILK with the cell surface receptors, ITGB1 and CCDC25.

NETs induce PDAC cell matrigel invasion in a NET-DNA, ITGB1 and ILK-dependent manner

Since prior work in the context of breast cancer has demonstrated that NETs stimulate tumour cell migration and invasion^{12,15,19,20,40}, we wanted to determine whether NETs induce invasion of PDAC cells. We cultured human MIA PaCa-2 cells and mouse KPCY PDAC cells with NETs and determined the effect of migration and invasion through Matrigel using an incucyte-based kinetic scratch wound assay.

Exposure of PDAC cells to 5–10 $\mu\text{g/mL}$ of NETs, based on NET-DNA concentration, resulted in a significant increase in invasion through Matrigel of MIA PaCa-2 and KPCY cells (Fig. 3A, B and Supplementary Fig. 2A, B). Exposure of NETs to DNase I, which degrades NET-DNA and thereby inhibits the NET-DNA-CCDC25 interaction²⁰, resulted in significant inhibition of NET-stimulated invasion in both cell types (Fig. 3A, B and Supplementary Fig. 2A, B). Treatment with DNase reduced invasion by the MIA PaCa-2 cells to close to control levels and partially reduced invasion by



the KPCY cells (Fig. 2A, B), suggesting the presence of heterogeneity in the relative dependence of individual cell lines on the NET-DNA mediated stimulatory capacity of NETs. To determine whether the effect of DNase I on invasion by PDAC cells is related to the interaction of NET-DNA with CCDC25, we stably transduced MIA PaCa-2 and KPCY cells with

doxycycline (dox)-inducible shRNA targeting *CCDC25*. Similar to the effect of DNase I, dox-induced shRNA-mediated suppression of *CCDC25* expression reduced NET-induced invasion, compared to control cells (Fig. 3C and Supplementary Fig. 2C, D), confirming a role of NET-DNA-*CCDC25* interaction in tumour cell invasion²⁰.

Fig. 2 | Neutrophils and NETs are present in ITGB1, ILK and CCDC25 expressing human PDAC tumours and preclinical xenograft models of PDAC. **A** Images of representative human PDAC tumour tissue sections stained by immunofluorescence (IF) for citrullinated histone H3 (cit-H3) and myeloperoxidase (MPO) to identify neutrophils and neutrophil extracellular traps (NETs; arrows). Boxes, regions of interest (ROI) shown at higher magnification to the right. Scale bars: lower magnification, 50 μ m; higher magnification, 10 μ m. Images of representative human PDAC tumour tissue sections stained by IF showing co-localisation of **B** integrin- β 1 (ITGB1) and integrin-linked kinase (ILK) and **C** coiled-coil domain-containing protein 25 (CCDC25) and ILK (arrows). Boxes, ROIs shown at higher magnification to the right. Scale bars: lower magnification, 50 μ m (**B**), 100 μ m (**C**); higher magnification, 20 μ m (both panels). **D** Images of representative tissue sections from MIA PaCa-2 PDAC xenograft primary tumours stained by IF for MPO and cit-H3 to identify NETs (arrows). Scale bar, 20 μ m. **E** Images of representative primary tumour tissue sections from the KPCY genetically engineered mouse model (GEMM) of PDAC stained by IF for MPO and cit-H3 to identify NETs (arrows).

Scale bar, 10 μ m. **F** Images of representative tissue sections of liver metastases from MIA PaCa-2 PDAC xenografts stained by IF for MPO and cit-H3 to identify NETs (arrows). Scale bar, 20 μ m. **G** Images of representative tissue sections of liver metastases from the KPCY GEMM stained by IF for MPO to identify neutrophils (arrows). Scale bar, 10 μ m. Images of representative tumour tissue sections from **H** MIA PaCa-2 xenografts and **I** the KPCY GEMM stained by IF showing co-localisation of ITGB1 and ILK (arrows). Scale bar, 20 μ m. Images of representative primary tumours and liver metastases from **J** MIA PaCa-2 PDAC xenografts and **K** the KPCY GEMM stained by IF showing co-localisation of CCDC25 and ILK (arrows). Scale bar, 20 μ m. **L** Immunoblots showing co-immunoprecipitation (co-IP) of ITGB1, ILK and CCDC25 from MIA PaCa-2 human PDAC cells cultured with 5 μ g/mL NETs for 24 h. The graph of ILK:ITGB1 ratio is shown to the right. Immunoblots showing co-IP of CCDC25 and ILK from **M** MIA PaCa-2 human PDAC cells and **N** KPCY mouse PDAC cells cultured with 5 μ g/mL NETs for 24 h. Quantification of band intensities is reported below the blots (**L–N**).

However, since the analysis of the clinical data described above (Fig. 1) demonstrated the presence of a group of patients with particularly poor prognosis containing high expression of *ILK* and *ITGB1*, and ILK is known to interact with ITGB1 to control cell adhesion, migration and invasion^{35,36}, we determined the effect of blocking ITGB1 function on NET-induced invasion by PDAC cells. NET-induced invasion of MIA PaCa-2 and KPCY PDAC cells was significantly reduced in the presence of antibodies targeting the function of ITGB1 (Fig. 3A, D and Supplementary Fig. 2E, F). Blocking ITGB1 function also inhibited NET-induced migration by MIA PaCa-2 cells (Supplementary Fig. 3A).

Since both CCDC25 and ITGB1 are known to interact with ILK, we next determined the effect of inhibiting ILK on NET-induced invasion using both genetic and pharmacologic strategies. siRNA-mediated depletion of *ILK* expression resulted in significant inhibition of NET-stimulated migration (Supplementary Fig. 3B, C) and invasion (Fig. 3E, F and Supplementary Fig. 2G) of MIA PaCa-2 cells. Furthermore, pharmacologic inhibition of ILK activity, using an established, highly selective inhibitor of ILK, QLT0267^{37,41–44}, at concentrations well-known to provide specific, on-target inhibition of ILK activity^{41,43–46}, resulted in abrogation of NET-induced invasion in a dose-dependent manner across multiple PDAC cell lines, including in patient-derived xenograft (PDX)-derived PaCa41 cells (Fig. 3G, H and Supplementary Fig. 2H). Pharmacologic inhibition of ILK activity had a modest effect on basal levels of invasion by the MIA PaCa-2 cells and had no effect on basal invasion levels in KPCY or PaCa41 cells (Supplementary Fig. 2I). The ILK inhibitor also reduced basal (Supplementary Fig. 3D) and NET-induced migration (Supplementary Fig. 3E) by MIA PaCa-2 cells in a dose-dependent manner. Collectively, these data demonstrate that NETs induce migration and invasion by PDAC cells and that this increased motility is mediated by an ITGB1-CCDC25-ILK signalling axis.

Epithelial to mesenchymal transition (EMT) is a hallmark of NETosis in PDAC tumours

Cellular plasticity is now considered a requisite characteristic of cancer cells capable of metastasis. EMT is a key process that underlies cancer cells' invasive and migratory abilities and is critical for cancer progression and metastasis.

To identify differential enrichment of cellular pathways between the prognostic NET signature groupings identified in our bioinformatics analysis (Fig. 1), we performed comprehensive gene set enrichment analysis (GSEA) on genes differentially expressed between patient groups 2 and 3 (Fig. 4A). This analysis revealed EMT to be the most highly enriched pathway among genes upregulated in the poor outcomes patient group 2 relative to the better outcomes group 3 (Fig. 4A). Gene sets related to hypoxia and inflammatory response were also highly enriched among the upregulated genes in the group 2 patients (Fig. 4A), both of which are implicated in aggressive PDAC tumour cell behaviour. Other gene sets significantly

enriched among genes upregulated in group 2 patient tumours included neutrophil degranulation and integrin cell surface interactions (Fig. 4A), which are pathways that would be expected given the NET-mediated regulation of the ITGB1-ILK interaction that we have uncovered.

Next, we further interrogated genes belonging to the Hallmark EMT gene set that were significantly upregulated in patient group 2 (poor prognosis) compared to patient group 3 (better prognosis) (Fig. 4B). We found significant upregulation of expression of several EMT genes in patient group 2, many of which also showed overexpression at the level of protein (Fig. 4B). Furthermore, the expression of many of these genes are highly related, based on the GeneFriends⁴⁷ mutual rank score, suggesting strong co-expression of these genes (Fig. 4B). Collectively, these data identify EMT as a critical pathway upregulated in PDAC patients that are NET signature high, ILK signalling high and subject to poor outcome.

Since our bioinformatics analysis suggested that EMT is a critical process in patients identified as NET signature high and ILK signalling pathway high, and who have poor outcomes, we next wanted to determine whether NETs stimulate EMT in PDAC cells. First, we incubated PDAC cells with increasing concentrations of NETs and analysed the protein levels of transcriptional regulators of EMT. We observed, by western blot analysis, that exposure to NETs results in robust, dose-dependent increases in EMT markers Snail family transcriptional repressor 1 (SNAI1), SNAI2 and, in particular, zinc-finger E-box-binding homeobox 1 (ZEB1) (Fig. 4C and Supplementary Fig. 3F). We further observed a slight increase in the levels of the mesenchymal marker, vimentin. Next, since previous studies have shown that ILK promotes EMT in the context of cancer^{43,48,49}, we knocked down expression of ILK in the MIA PaCa-2 human and KPCY mouse PDAC cells using dox-inducible shRNA (Supplementary Fig. 3G) and assessed the impact on these EMT markers. In both the human and mouse PDAC cells, exposure to NETs increases the levels of ZEB1 and vimentin, and silencing ILK abrogates this NET-induced increase (Fig. 4D, E). Silencing of *ILK* expression further inhibited NET-induced expression of SNAI1 and slightly reduced levels of N-cadherin in the KPCY cells (Fig. 4E). Knockdown of *ILK* expression in the absence of NETs also resulted in a modest decrease in the expression of ZEB1. Furthermore, pharmacologic inhibition of ILK activity also abrogated the NET-induced increase in expression of ZEB1 in both human and mouse cells (Fig. 4F, G), and the effect was similar when cells were grown on plastic (Fig. 4F, G) or Matrigel (Fig. 4H), confirming that inhibition of ILK reduces EMT markers in these cells.

Finally, we found that culturing PDAC cells on either plastic or Matrigel in the presence of function-blocking antibodies targeting ITGB1 phenocopied the inhibition of ZEB1 expression observed with inhibition of ILK (Fig. 4I), further indicating that NETs induce EMT in PDAC cells through an ITGB1-ILK signalling axis.

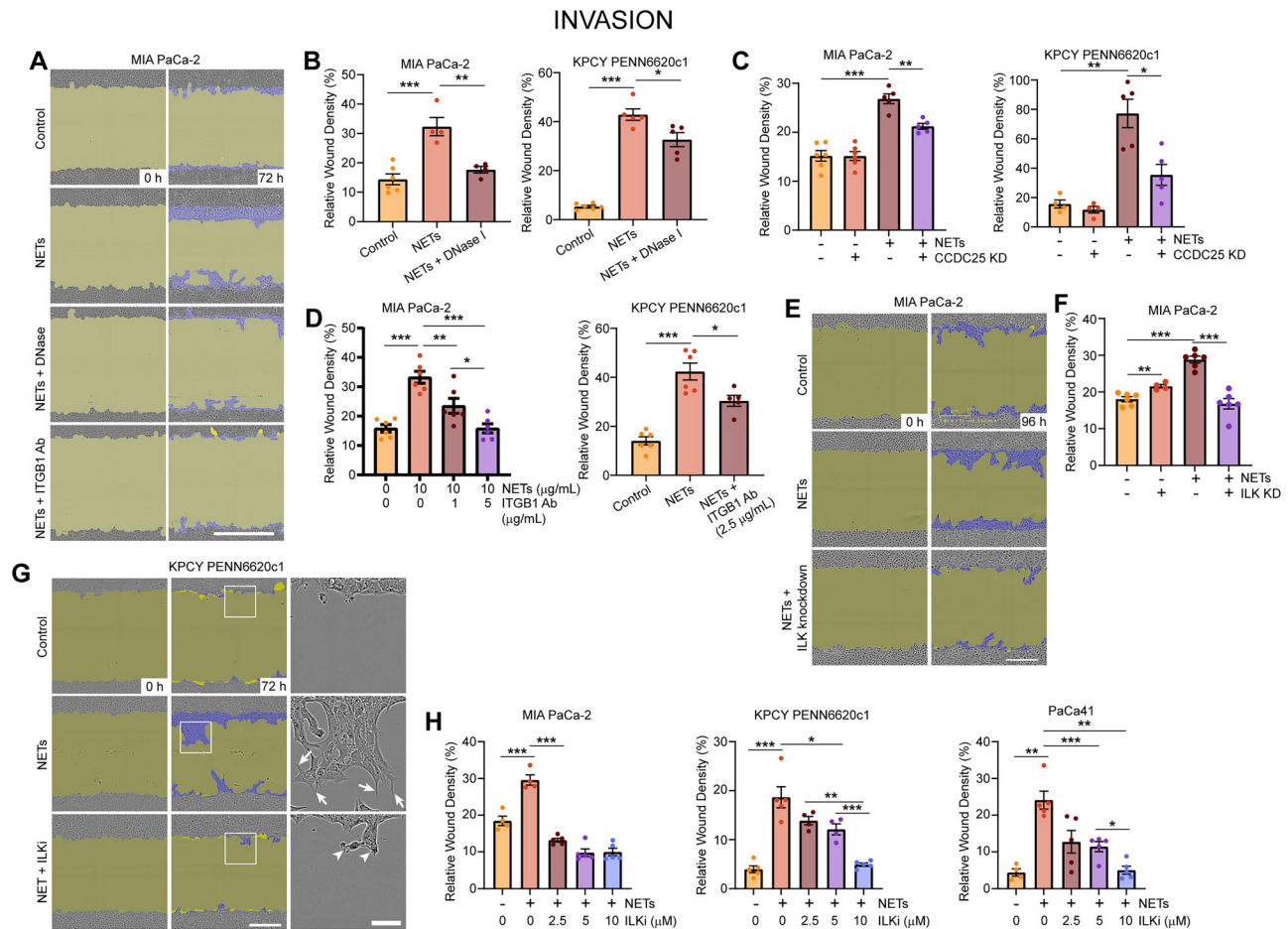


Fig. 3 | NETs induce PDAC cell matrigel invasion in a NET-DNA, ITGB1 and ILK-dependent manner. **A** Time-lapse imaging of invasion through Matrigel by MIA PaCa-2 cells cultured in the presence of 10 $\mu\text{g/mL}$ NETs or DNase I-treated NETs together with DNase or function-blocking antibodies targeting ITGB1 (10 $\mu\text{g/mL}$). yellow, wound area; blue, area covered by invading cells. Scale bar, 200 μm . Quantification of NET-induced (10 $\mu\text{g/mL}$) invasion through Matrigel by human and mouse PDAC cells **B** exposed to DNase-treated NETs and grown in the presence of DNase, **C** following knockdown of CCDC25 expression using dox-inducible shRNA and **D** exposed to function-blocking antibodies targeting ITGB1 for 72 h. **E** Time-lapse imaging showing invasion through Matrigel by MIA PaCa-2 cells following depletion

of ILK expression using siRNA and culture in the presence of 5 $\mu\text{g/mL}$ NETs. Scale bar, 300 μm . **F** Quantification of NET-induced invasion by cells described in (**E**). **G** Time-lapse images of cells undergoing NET-DNA-induced invasion in the presence of a specific inhibitor of ILK, QLT-0267. Invading cells form robust membrane protrusions (arrows), which are absent when ILK activity is inhibited (arrowheads). Scale bar = 200 μm . **H** Quantification of NET-induced invasion by the indicated cell lines in the presence of the ILK inhibitor. Bars show mean $\pm$ SEM of $n = 4$ –6 technical replicates and are representative of 2–3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ANOVA (**B–D**, **F**, **H**).

NETs induce pseudopodia-like protrusions in an ILK-dependent manner and signal through GSK3 β and RAC1/CDC42 to induce invasion

Our data clearly show that NETs induce invasion by PDAC cells and that ILK is an important contributor to NET-stimulated invasion. To further investigate the mechanism of ILK-mediated NET-induced invasion, we investigated the colocalization of ILK with paxillin, a marker of focal adhesions (FAs), and cofilin, a marker of pseudopodia-like protrusions (PLPs) involved in metastatic colonisation⁵⁰. Given our results demonstrating increased invasion through Matrigel and the potential role of ITGB1 in this process, we performed these studies using PDAC cells grown on Matrigel and fibronectin.

Exposure of PDAC cells to NETs induced dramatic changes in cellular phenotype. In the presence of NETs, cells cultured on Matrigel (Fig. 5A, C) and fibronectin (Fig. 5B, D) increased in overall size and extended PLPs, suggesting the acquisition of motility. Paxillin co-localised with ILK at FAs in cells plated on Matrigel (Fig. 5A) and fibronectin (Fig. 5B). Exposure of cells to NETs resulted in the redistribution of these proteins such that they showed dramatic co-localisation with PLPs in NET-induced cells.

Dox-inducible knockdown of ILK expression inhibited the ability of PDAC cells to develop membrane protrusions in the presence of NETs on either substrate (Fig. 5A–D), demonstrating a requirement for ILK expression and function. Knockdown of ILK under basal culture conditions results in a rounded cell morphology with poorly formed FA (Supplementary Fig. 4A).

We also assessed the localisation of cofilin, a marker of PLPs⁵⁰, and ILK in the context of NET stimulation. In control cells plated on either Matrigel or fibronectin, ILK was distributed both within the cytoplasm and at the plasma membrane, where it was situated within FA, while cofilin was diffusely distributed throughout the cells (Fig. 5C, D). Upon exposure to NETs, the cells developed protrusions at which ILK and cofilin were enriched and colocalized (Fig. 5C, D). Cells in which dox-inducible shRNA targeting ILK was used to suppress ILK expression remained round (Fig. 5D and Supplementary Fig. 4B) and did not develop PLP in response to exposure to NETs (Fig. 5C, D).

Since NETs stimulate both membrane protrusions and invasion in PDAC cells, we wanted to understand the potential mechanism. Since the guanine nucleotide-binding proteins (GTPases), ras-related C3 botulinum

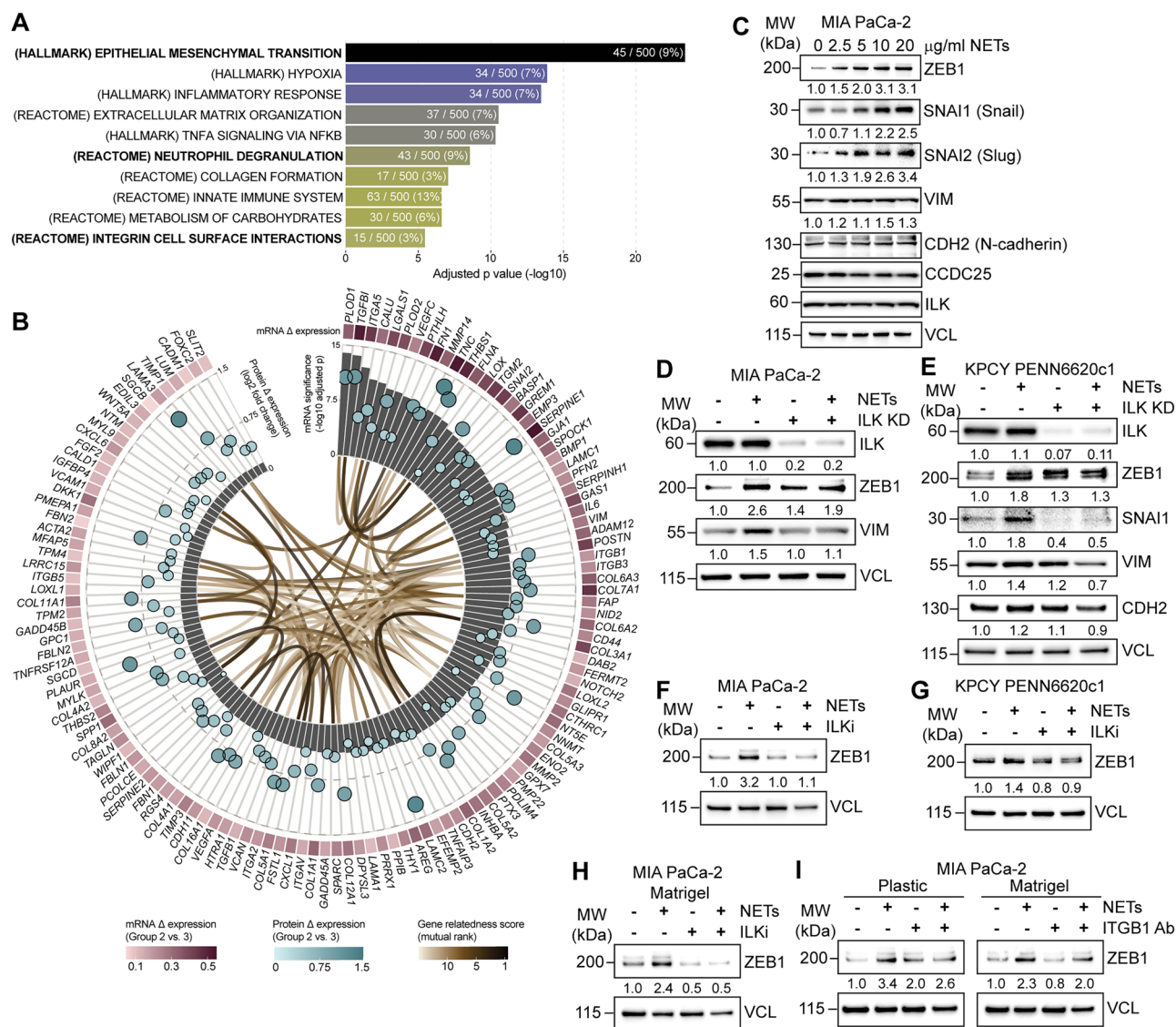


Fig. 4 | Epithelial to mesenchymal transition (EMT) is a hallmark of NETosis in PDAC tumours. **A** Gene set enrichment analysis (GSEA) of differentially expressed genes in group 2 (poor outcome) versus group 3 (better outcome). Bar plot showing pathways upregulated in patient group 2 (poor prognosis) versus patient group 3 (better prognosis). **B** Analysis of Hallmark epithelial-mesenchymal transition (EMT) genes significantly upregulated in patient group 2 compared to patient group 3. Grey bars, strength of significance of gene dysregulation; Red squares, magnitude of differential expression; Teal bubbles, difference in protein levels for each gene; Brown ribbons, gene-gene relatedness based on GeneFriends analysis. **C** Western blot analysis of the indicated EMT markers in MIA PaCa-2 cells cultured in the presence of increasing concentrations of NETs for 24 h. Western blot analysis of NET-induced EMT markers in response to dox-inducible knockdown of ILK

expression in **D** MIA PaCa-2 cells cultured with 20 μ g/mL NETs for 24 h and **E** KPCY cells cultured with 20 μ g/mL NETs for 48 h. Western blot analysis of NET-induced Zinc-finger E-box-binding homeobox 1 (ZEB1) expression in response to pharmacologic inhibition of ILK in **F** MIA PaCa-2 cells and **G** KPCY cells cultured with 10 μ g/mL NETs for 48 h in the presence of 10 μ M of ILKi. **H** Western blot analysis of NET-induced ZEB1 expression in MIA PaCa-2 cells cultured on 2.5% Matrigel with 10 μ g/mL NETs for 48 h and exposed to 10 μ M of the ILK inhibitor. **I** Western blot analysis of NET-induced ZEB1 expression in MIA PaCa-2 cultured with 10 μ g/mL NETs for 48 h on the indicated substrates in the presence of 5 μ g/mL function-blocking Ab against ITGB1. Quantification of band intensities is reported below the blots (**C–I**).

toxin substrate 1 (RAC1) and cell division control protein 42 homologue (CDC42) have been shown to regulate cellular migration and invasion^{51,52}, we investigated whether NETs can stimulate the activities of these GTPases in PDAC cells. Exposure to NETs increased GTP loading of both RAC1 and CDC42, indicating an increase in RAC1 and CDC42 activity (Fig. 5E). Inhibition of ILK activity with QLT-0267 reduced the levels of both GTP-RAC1 and GTP-CDC42, indicating inhibition of this signalling axis (Fig. 5F).

Since our data demonstrate that NETs induce EMT in PDAC cells and stimulate activation of RAC1 and CDC42 to induce invasion, we wanted to further interrogate additional signalling pathways known to be associated with ILK-mediated EMT, such as Glycogen synthase kinase 3 beta (GSK3 β)

and β -catenin^{34,43,49}. Exposure of human and mouse PDAC cells to NETs results in a time-dependent increase in the phosphorylation of GSK3 β as well as extracellular signal-regulated kinase (ERK) (Fig. 5G). We observed a transient decrease in phosphorylation of AKT on serine (Ser)473 in response to exposure to NETs by the MIA PaCa-2 cells, followed by a return to control levels at later time points, while no change was observed in the KPCY cells (Fig. 5G). Pharmacologic inhibition of ILK activity using increasing concentrations of the ILK inhibitor, QLT-0267, results in dose-dependent inhibition of GSK3 β in both cell lines (Fig. 5H). Phosphorylation of AKT on Ser473 was also abrogated by the ILK inhibitor in a dose-dependent manner, confirming that the inhibition of NET-induced GSK3 β is ILK dependent (Fig. 5H).

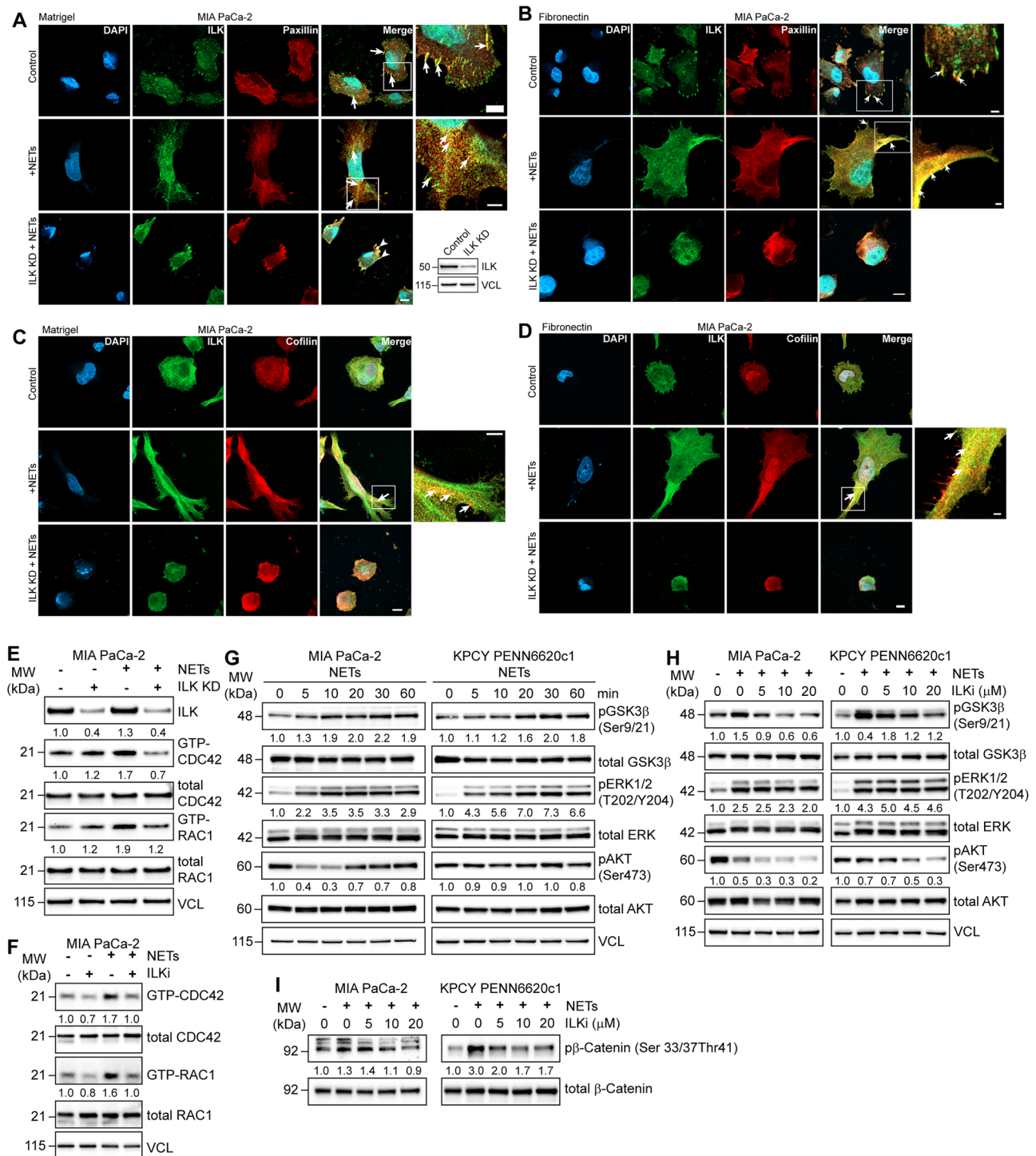
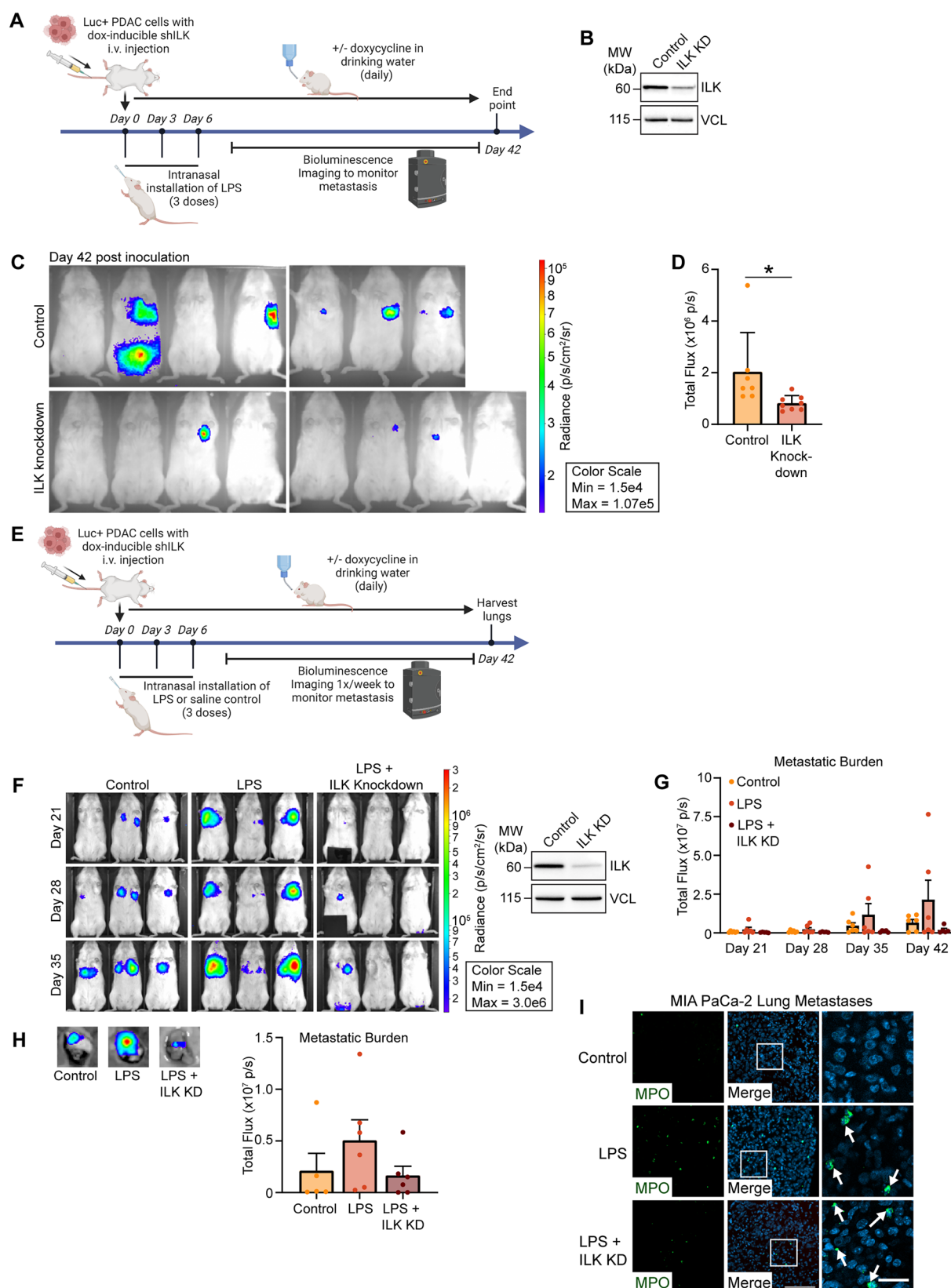


Fig. 5 | NETs induce pseudopodia-like protrusions in an ILK-dependent manner and signal through GSK3 β and RAC1/CDC42 to induce invasion. IF staining showing co-localisation (arrows, yellow) of ILK and paxillin in control and ILK-depleted MIA PaCa-2 cells cultured on **A** Matrigel and **B** fibronectin with 20 μ M NETs for 24 h. Boxes, ROIs shown at higher magnification in right panels. Inset, Western blot showing dox-inducible shRNA-mediated depletion of ILK expression. Scale bar = 10 μ m. Zoom scale bar = 5 μ m. IF staining showing co-localisation (arrows, yellow) of ILK and cofilin in control and ILK-depleted MIA PaCa-2 cells cultured on **C** Matrigel and **D** fibronectin with 20 μ M NETs for 24 h. Boxes, ROIs shown at higher magnification in right panels. Scale bar = 10 μ m. Zoom scale bar = 5 μ m. **E** Immunoblot analysis showing GTP-bound and total RAC1 and CDC42 levels in MIA PaCa-2 cells cultured with or without 10 μ M NETs for 24 h and 10 μ M of the ILK inhibitor. **F** Immunoblot analysis showing GTP-bound and total RAC1 and CDC42 levels in MIA PaCa-2 cells cultured with or without 10 μ M NETs for 24 h and 10 μ M of the ILK inhibitor. **G** Western blots showing levels of phosphorylation of the indicated proteins in NET-stimulated PDAC cells at the indicated time points. **H** Western blots showing levels of phosphorylation of the indicated proteins in PDAC cells cultured with 10 μ M NETs for 30 min in response to increasing concentrations of the ILK inhibitor. **I** Levels of phosphorylation of β -catenin in PDAC cells cultured as described in (**H**). Quantification of band intensities is reported below the blots (**E–I**).

CDC42 levels in control and ILK-depleted MIA PaCa-2 cells cultured with or without 20 μ M NETs for 24 h. **F** Immunoblot analysis showing GTP-bound and total RAC1 and CDC42 levels in MIA PaCa-2 cells cultured with or without 10 μ M NETs for 24 h and 10 μ M of the ILK inhibitor. **G** Western blots showing levels of phosphorylation of the indicated proteins in NET-stimulated PDAC cells at the indicated time points. **H** Western blots showing levels of phosphorylation of the indicated proteins in PDAC cells cultured with 10 μ M NETs for 30 min in response to increasing concentrations of the ILK inhibitor. **I** Levels of phosphorylation of β -catenin in PDAC cells cultured as described in (**H**). Quantification of band intensities is reported below the blots (**E–I**).



Similarly, phosphorylation of β -catenin increased in response to the exposure of both human and mouse PDAC cells to NETs, and this increase in phosphorylation was dose-dependently inhibited in response to incubation with the ILK inhibitor (Fig. 5I).

We also examined the effect of NET stimulation on cell growth, since previous studies in breast cancer cells²⁰ have shown that NETs not only stimulate migration and invasion, but also cell growth, which may further contribute to metastatic colonisation. Exposure of PDAC cells in

Fig. 6 | Inhibition of ILK suppresses NET-induced metastasis. **A** Schematic showing the in vivo experimental design. **B** Western blot showing dox-inducible knockdown of ILK expression in MIA PaCa-2 Luc+ dox-inducible shILK cells. **C** Bioluminescence images showing metastatic burden in control mice and mice treated with dox to induce knockdown of ILK expression. **D** Quantification of bioluminescence signal in (C) ($n = 7-8$ per group). Bars show mean $\pm$ SEM. $^*p < 0.05$, t -test. **E** Schematic showing the in vivo experimental design. **F** Bioluminescence images showing lung metastatic burden in control mice and mice

administered LPS intranasally, with or without dox treatment at the indicated time points. **G** Quantification of bioluminescence signal in the lungs at the indicated time points ($n = 5-6$ per group). Bars show mean $\pm$ SEM. **H** Bioluminescence images of representative lung metastases from the indicated treatment groups at day 42 post-cell injection and quantification of metastatic burden ($n = 5$ to 6 per group). Bars show mean $\pm$ SEM. **I** IF staining of MPO in lung metastases from mice treated as indicated in (F) at 42 days after injection of MIA PaCa-2 tumour cells. Scale bar, 100 μ m, 25 μ m (right panels).

3-dimensional (3D) MIA PaCa-2 spheroids showed increased size of spheroids in the presence of NETs (Supplementary Fig. 4C). NET-induced growth of spheroids is significantly reduced in the presence of DNase I (Supplementary Fig. 4C, D), the ILK inhibitor (Supplementary Fig. 4C, D) or ITGB1 antibodies (Supplementary Fig. 4E). Thus, in addition to impacting migration and invasion, NETs induce growth of PDAC cells in 3D and this effect can be inhibited by interfering with CCDC25, ITGB1 and ILK activity.

Inhibition of ILK suppresses NET-induced metastasis

Our data demonstrate that the ITGB1-ILK signalling axis is a critical contributor to NET-induced PDAC tumour cell EMT and invasion, suggesting that targeting this signalling axis in vivo may provide a therapeutic avenue for reducing metastasis in inflammation-driven PDAC.

To test this, we used an experimental metastasis model wherein we injected MIA PaCa-2 PDAC cells expressing both luciferase (Luc) and dox-inducible shRNA targeting *ILK* into the tail vein of female non-obese diabetic (NOD) scid gamma (NSG) mice that were administered lipopolysaccharide (LPS) intranasally immediately prior to tumour cell injection, as well as at days 3 and 6 post-tumour cell injection to induce lung inflammation (Fig. 6A). Mice were administered dox in the drinking water (or drinking water without dox as a control) following cell injection (Fig. 6A) to induce shRNA-mediated knockdown of ILK protein expression (Fig. 6B) and metastasis was monitored using bioluminescence imaging (BLI). We observed that knockdown of *ILK* expression in the tumour cells in the context of LPS-stimulated lung inflammation dramatically reduced overall metastatic burden at 42 days post-cell injection, compared to control animals (Fig. 6C). Quantification of the bioluminescence signal demonstrated a significant reduction in metastatic burden, as measured by total flux, with *ILK* knockdown, compared to controls (Fig. 6D).

We then further determined whether LPS-driven lung inflammation was indeed driving increased metastasis and, if so, whether the increased metastasis was being inhibited by knockdown of ILK expression. For these studies, mice were administered LPS, or saline as a control, intranasally immediately prior to injection of the tumour cells and at days 3 and 6 post-tumour cell injection (Fig. 6E). MIA PaCa-2 Luc+ dox-inducible *ILK* shRNA tumour cells were inoculated through the tail vein and lung metastatic burden was monitored by BLI. We found a clear, time-dependent increase in metastatic burden in the lungs of mice administered LPS, compared to control mice administered saline (Fig. 6F, G), demonstrating that LPS-induced inflammation and neutrophil recruitment result in increased metastasis in this model. Knockdown of ILK expression in the PDAC tumour cells results in a dramatic reduction in lung metastatic burden at all time points examined (Fig. 6F, G). Ex vivo imaging of metastatic burden in the lungs at day 42 post-cell injection also demonstrated increased metastases in LPS-treated animals, and reduced metastatic burden in the lungs of mice in which ILK was depleted from tumour cells (Fig. 6H). Immunofluorescence for MPO in lung tissue sections from the mice at end-stage showed that mice treated with LPS had increased numbers of neutrophils localised within the metastatic foci, compared to control mice that showed neutrophils largely at the periphery of the metastases (Fig. 6I). While the lung metastatic burden in the mice treated with LPS and injected with *ILK* KD cells was reduced (Fig. 6F–H), we did observe that neutrophils were still present, albeit somewhat less numerous, within the metastases of this group (Fig. 6I), in keeping with the LPS-mediated stimulation of inflammation in this group.

Discussion

Recently, there has been growing appreciation of the pro-tumorigenic roles of neutrophils and NETs in cancer, spurring efforts to better understand the molecular basis of how NETs influence tumour cell behaviour and expose targetable vulnerabilities for cancer therapy. Here, we have investigated the role of NETosis and NETs in pancreatic adenocarcinoma (PDAC) progression to metastasis and present several novel findings. First, through assembly of a comprehensive human PDAC RNA-seq dataset encompassing both a large number of resected primary tumours sourced from public databases and, uniquely, over 100 metastatic PDAC tissue samples obtained from clinical trials, we used bioinformatics approaches to reveal a NET-specific signature in PDAC with distinct patient outcomes. The poor outcomes gene expression signature identified is composed of genes that function in cell adhesion and actin cytoskeletal organisation and EMT. Second, related to this, we identify, for the first time, a tripartite interaction between ITGB1, CCDC25 and ILK, which we show promotes EMT and enhances PDAC cell migration and invasion. Furthermore, gene expression signatures identified here from the PDAC RNA-seq datasets are comprised of genes largely associated with tumour cells and do not contain genes encoding for cytokines, MPO or peptidyl arginine deiminase 4 (PADI4), which are associated with TME cells such as neutrophils, providing support for our analysis of the effect of NETs on tumour cells. Third, we demonstrate the presence of NETs in human primary PDAC tumour tissue samples. Fourth, we show, using PDAC experimental metastasis models in vivo, that NETosis-driven metastatic colonisation in the lung is significantly inhibited by knockdown of ILK, providing proof-of-principle for the potential of targeting therapeutically the NET-related CCDC25-ITGB1-ILK signalling axis in the context of PDAC.

Furthermore, we have validated that PDAC tumours and metastases harbour neutrophils and NETs, by immunostaining patient primary tumours and metastases, as well as experimental tumours and metastases from human PDAC cell xenografts and KPCY mouse transgenic tumours and metastases, with antibodies against MPO (neutrophils) and cit-H3 (NETs). The data clearly demonstrate that not only do PDAC tumours and metastases have significant numbers of intratumoural neutrophils, but that a substantial number undergo NETosis and produce NETs. Counterstaining of adjacent serial sections also demonstrated that these NETs are present both in stromal regions and in regions encompassed by tumour cells expressing ITGB1 and ILK.

Based on these data, we set out to determine the molecular basis of NET-mediated stimulation of cell migration, invasion, growth and, especially, metastasis. A recent study demonstrated a role of the modified DNA component of NETs, and demonstrated in breast cancers that the binding of NET-DNA to a cell surface protein, CCDC25 plays a critical role in inducing tumour cell migration/invasion and metastasis formation by recruiting ILK and beta-parvin (PARVB)²⁰, which are established effectors of cell-cytoskeleton organisation and dynamics^{34,50}. Utilising PDAC cells in culture, we have shown, for the first time, that ILK forms a complex with both ITGB1 and CCDC25, as determined by immunostaining and co-IP, and that NETs stimulate tumour cell invasion and migration in an NET-DNA, CCDC25, ITGB1 and ILK-dependent manner. Furthermore, NETs promote a dramatic morphological switch to a highly motile and invasive phenotype that is accompanied by increased formation of ILK and cofilin-containing PLPs, as well as activation of the GTPases RAC1 and CDC42. These structures have previously been demonstrated to require ILK-cofilin,

and RAC1/CDC42 activation signalling pathways and to play critical roles in breast tumour cell metastatic colonisation⁵⁰. Additionally, ITGB1-ILK complex has also been implicated in metastatic colonisation of breast cancer cells by promoting L1 cell adhesion molecule (L1CAM)-mediated activation of yes-associated protein 1 (YAP1)⁵³, a downstream effector of ILK⁴³.

Mechanistically, we show that NETs stimulate integrin and ILK-dependent expression of regulators of EMT, such as SNAIL1, SNAIL2 and ZEB1. Furthermore, NETs induce phosphorylation of GSK3B and AKT in an ILK-dependent manner. GSK3B inactivation by phosphorylation regulates β -catenin-driven EMT, and also stabilises SNAIL1 protein levels by preventing its proteasome-mediated degradation^{54,55}. Phosphorylation of AKT regulates cell survival and growth through mTOR^{56,57}, a NET signature-associated gene (Fig. 1).

Collectively, our data suggest that, within the primary tumour, NETs may interact with PDAC tumour cells and engage the CCDC25-ITGB1-ILK signalling axis to induce or augment local invasion and intravasation, thereby contributing to the dissemination of tumour cells from the primary site into the circulation. While we did not investigate the presence of NETs in the circulation and pre-metastatic niche in our models, previous studies have reported the presence of circulating NETs in PDAC models^{58,59} and recent analyses of pre-metastatic liver biopsies obtained from patients with pancreatic cancer using multi-omics and multiplex imaging demonstrated that enriched NETs, together with additional biomarkers, differentiated patients with future metastasis from those with no evidence of disease and was among parameters used for development of a machine-learning based model to predict metastatic outcome³.

Finally, we demonstrate that metastatic colonisation of human and mouse PDAC cells after tail vein injection is stimulated by LPS-induced inflammation and associated NETosis. Importantly, inhibition of expression of ILK, using dox-inducible ILK shRNA, significantly suppresses lung colonisation in both models, demonstrating that inflammation and NET-driven metastasis requires ILK. Here, LPS-driven lung inflammation was initiated concurrently with the injection of the tumour cells. In future studies, it will be interesting to determine if pre-treatment of mice with LPS to induce lung inflammation prior to introduction of PDAC tumour cells further increases seeding of lung metastases, and whether ILK blunts metastasis in the context of pre-existing inflammation. While we were able to demonstrate the presence of neutrophils within the lung metastases of mice treated with LPS and show that neutrophils are still present in the metastases formed by ILK KD cells, these analyses were carried out here on lungs with end-stage disease. It is important to note that the contribution of neutrophils and NETosis may also occur within the circulation and/or the lung vasculature in a temporal manner. The precise location and timing of the neutrophil and NET-tumour cell interactions will require future analyses using technologies such as intravital microscopy.

Our findings set the stage for several future avenues of investigation regarding additional potential roles of NETs on PDAC tumour cells directly and between cancer cells and additional cell types within the PDAC TME. For example, the impact of NETs on cross-talk between PDAC tumour cells and stromal cells within the TME, including cancer-associated fibroblasts, which are numerous in PDAC and are responsible for the highly fibrotic, matrix-rich composition of the TME⁶⁰ and are thought to drive the migratory potential of tumour cells, may shed light on another dimension of NET-induced regulation of tumour cell invasion.

It is now well established that the TME influences various aspects of cancer progression to metastasis, the root cause of cancer mortality. The TME can, in turn, be influenced by the host state, which can govern the immune cell repertoire of the microenvironment. Poor patient outcomes are also influenced by the immune suppressive microenvironment, which in turn is regulated through hypoxia, vascularisation, extracellular matrix, establishment of a pre-metastatic niche⁶¹, and inflammation. The latter is known to influence tumour behaviour, and the inflammatory cells such as monocytes, macrophages and neutrophils play significant roles in immune suppression. A recent detailed study on the spatial architecture of myeloid and T-cells demonstrated that the ratios of these cells play a critical role in

immune evasion and clinical outcome in lung cancer⁶². In particular, tumour-associated neutrophil infiltration identified a subset of tumours which had increased metastatic propensity and shorter disease-free survival⁶².

In addition to neutrophils, the immune cell compartment of the PDAC TME includes monocyte-derived suppressor cells, tumour-associated macrophages and regulatory T-cells (T-regs), all of which serve to help suppress infiltration of cytotoxic T-cells and drive a highly immunosuppressive environment. NETs are known to promote exhaustion of T-cells in the TME, in part through the expression of PD-L1 on NETs that interact with PD-1 on T-cells⁶³. Furthermore, NETs drive macrophage polarisation toward a pro-tumorigenic phenotype to promote tumour growth and metastasis. However, whether interactions between NETs and immune cells within the TME modulate the NET-ITGB1-ILK signalling axis in PDAC tumour cells to influence metastasis in PDAC remains a future investigative avenue.

Heterogeneity within the neutrophil population and in NET formation may also impart differential immunosuppressive properties that may contribute to PDAC progression and metastasis. Previous studies have shown that, compared to neutrophils derived from healthy donors, neutrophils derived from PDAC patients demonstrated significantly increased ability to promote tumour cell migration and invasion, suggesting a more pro-tumorigenic phenotype⁹. Data from single-cell RNA-seq analyses have shown that, compared to neutrophils from healthy donors, PDAC-associated neutrophils show a pro-tumorigenic phenotype associated with poor prognosis⁶⁴. A recent study using a multi-omics approach, including single-cell RNA-seq and spatial transcriptomics, to classify neutrophils based on NETosis-related genes in PDAC primary tumour samples spanning public and in-house datasets identified the presence of heterogeneity in NET formation⁶⁵. Neutrophil subpopulations capable of forming NETs (NET-positive) were marked by gene expression of IL1B and particularly TLR2 and were associated with poor prognosis and T-cell exhaustion markers, compared to NET-negative subpopulations, indicating a link between neutrophil diversity, NETosis and immune suppression in the PDAC progression⁶⁵.

To date, pharmacologic inhibition of ILK in vivo remains challenging due to the suboptimal pharmacokinetic profile of the current generation small-molecule inhibitor of ILK, QLT-0267. Although highly selective for ILK^{37,41–44}, it has a short half-life and is cleared rapidly in vivo, necessitating the use of high doses (oral administration 100–200 mg/kg) and high frequencies (daily) that effectively limit its use as a single agent^{66,67}. Importantly, however, combinatorial strategies involving pharmacologic inhibition of ILK achieve sustained anti-tumour efficacy⁴¹. Our work demonstrating that genetic knockdown of ILK results in attenuation of LPS-induced metastasis of PDAC cells in vivo provides proof-of-principle showing the potential clinical value of inhibiting ILK in this context and underscores the need for the development of ILK inhibitors with superior pharmacokinetics.

Similarly, it will be interesting to evaluate the effect of inhibiting ILK in combination with the current standard of care, combinatorial chemotherapy such as gemcitabine and nab-paclitaxel, and leucovorin (folinic acid), fluorouracil, irinotecan hydrochloride, and oxaliplatin (FOLFIRINOX), on NET-induced invasion and metastasis⁶⁰. Chemotherapy contributes to inflammation and fibrosis in PDAC, and the role of NETs in the context of chemotherapy treatment is complex^{68,69}. Studies in models of breast cancer metastasis have shown that chemotherapy recruits neutrophils and induces NET formation, impeding treatment response²¹. In contrast, in PI3-kinase catalytic subunit alpha (PI3KCA)-mutant colorectal cancer, chemotherapy treatment in combination with glutaminase inhibitor, CB-839, induced recruitment of neutrophils and formation of NETs that induced apoptosis of cancer cells, enhancing therapeutic response^{70,71}. Whether inhibition of ILK will synergize with chemotherapy treatment in PDAC to enhance therapeutic efficacy is a subject of future investigative efforts.

Collectively, our data identify novel NET-associated gene expression signatures that can be used to identify patients susceptible to neutrophil/NET-mediated metastasis and poor outcome. While previous studies have

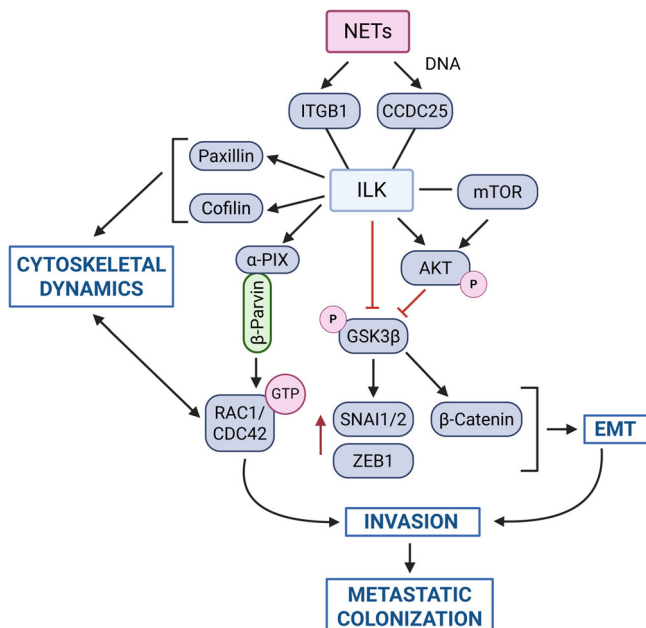


Fig. 7 | Role of NET-induced ILK signalling in PDAC invasion and metastasis. Schematic illustration summarising the role of the NET-induced ITGB1-CCDC25-ILK signalling axis in promoting EMT, invasion and metastasis of PDAC tumour cells. Created in BioRender. McDonald, P. (2025) <https://BioRender.com/k17m801>.

shown the presence of NETs in metastases, an outstanding question has been whether neutrophils and NETs accumulate in primary tumours as well. Our bioinformatics analysis and direct immune-localisation experiments suggest that in PDAC, NETosis can be a prominent feature in primary tumours. These findings suggest that analysis of primary tumour tissues for NETs and NET gene signatures could potentially provide critical information on patient outcome and metastasis formation.

Significantly, we have identified a NET signature associated, targetable, signalling axis: Integrin-ILK-EMT in PDAC tumours. While our results implicate ILK as a central player in NET-induced PDAC metastasis, our data point to a number of other targets in the NET-induced signalling axis identified in this study (Fig. 7).

Finally, targeting the downstream, PDAC tumour cell-associated effectors of NETosis and NETs, identified here, may be more advantageous and specific for the neutrophil/NET promotion of metastasis than targeting NET formation per se, since neutrophils and NETs have a critical physiological role in fighting infections.

Methods

Cell culture

The human pancreatic cancer cell line MIA PaCa-2 was obtained from Don Yapp and Sylvia Ng (BC Cancer Research Centre, Vancouver, Canada) and maintained in culture in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% foetal bovine serum (FBS; Life Technologies) as previously described^{72,73}. The mouse congenic PDAC tumour cell clone KPCY PENN620c1 was provided by Ben Stanger (University of Pennsylvania, Philadelphia, USA) and was maintained in culture in DMEM supplemented with 10% FBS as previously described⁷⁴. The human PDX cell line PaCa41 was established from a patient tissue fragment and was provided by Daniel Renouf (BC Cancer, Vancouver, Canada). PaCa41 cells were maintained in DME/F-12 medium supplemented with 0.25 mg/mL bovine serum albumin (BSA), 5 mg/mL glucose (Fisher Scientific), 1x insulin-transferrin-selenium (ITS), 25 µg/mL bovine pituitary extract (BPE; Life Technologies), 40 ng/mL epidermal growth factor (EGF; Sigma Aldrich), 5 nM 3,3,5-tri-iodo-L-thyronine and 1 µg/mL dexamethasone (Sigma Aldrich), 100 ng/mL cholera toxin, (Molecular Probes, ThermoFisher), 1.22 mg/mL nicotinamide (Sigma Aldrich), 5%

Nu-serum IV culture supplement (Corning), 100 U/mL penicillin, 100 µg/ml streptomycin and 500 µg/mL amphotericin B (Life Technologies). All cells were grown at 37 °C in a humidified atmosphere containing 5% carbon dioxide (CO₂) and were tested for mycoplasma contamination using the LookOut Mycoplasma PCR detection kit (Sigma; cat no. MP0035). The MIA PaCa-2 cell line was authenticated using short tandem repeat STR DNA profiling by a commercial testing facility (Genentica, Burlington, NC, USA). The KPCY PENN620c1 clone has been authenticated as described⁷⁴. The PaCa41 cell line was authenticated by the source laboratory. QLT-0267, an inhibitor of ILK activity, was obtained from Quadralogic Technologies Inc. (QLT) and used at the concentrations indicated in the text and figures. siRNA for ILK (Qiagen, cat no GS3611 Flexitube Gene Solution) was used according to the manufacturer's recommendations.

Animal studies

All experimental animal procedures were carried out at the BC Cancer Animal Resource Centre (ARC) in accordance with protocol A21-0263 approved by the institutional Animal Care Committee at the University of British Columbia, Vancouver. We have complied with all relevant ethical regulations for animal use. All animals were housed within the ARC at the BC Cancer Research Institute in Vancouver, Canada. All mice were housed in ventilated cages in a pathogen-free, environment-controlled room at 19–21 °C. The relative humidity ranged between 40% and 70% and a photoperiod of 12 h light and 12 h darkness was provided. Food and water were provided ad libitum. Environmental enrichment included nesting material, hiding places such as huts and standard bedding.

For experimental metastasis studies, MIA PaCa-2 Luc+ dox-inducible shRNA cells (2.0 × 10⁶ cells/animal, suspended in 100 µL sterile saline) were injected intravenously through the tail vein of naïve 8–12-week-old female NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice. Mice were administered lipopolysaccharide (LPS) (0.25 mg/ml, 50 µL/animal), or equal volumes of saline as a control, by intranasal instillation on days 0, 3, and 6 post-injection of tumour cells to induce lung inflammation. On day 0, administration of LPS or saline was performed immediately prior to injection of the tumour cells. To induce the shRNA targeting ILK, mice were administered doxycycline (dox) (1 mg/mL) in drinking water containing 1% sucrose ad libitum starting immediately following injection of the tumour cells and continuing for the duration of the study. Control animals were similarly administered drinking water containing 1% sucrose.

For the in vivo study reported in Fig. 6A–D, 15 mice were administered LPS and injected with MIA PaCa-2 Luc+ dox-inducible shRNA cells as described above. Animals were housed in groups of 3–4 mice per cage, and cages were grouped into the control arm (–dox; *n* = 7 mice) and experimental arm (+dox; *n* = 8 mice) prior to administration of LPS and injection of tumour cells and were not randomised. The administration of LPS and cells was performed in a staggered fashion by cage to minimise potential confounders between mice in the control and experimental groups. No animals were excluded from the analysis.

For the in vivo study reported in Fig. 6E–I, 18 mice were injected with MIA PaCa-2 Luc+ dox-inducible shRNA cells as described above. Animals were housed in groups of 3 mice per cage and cages were grouped into each of 3 study arms (–LPS, –dox; *n* = 6 mice; +LPS, –dox, *n* = 6 mice; +LPS, +dox, *n* = 6 mice) prior to administration of LPS and injection of tumour cells and were not randomised. The administration of LPS and cells was performed in a staggered fashion by cage to mitigate potential confounders between mice in the control and experimental groups. In this study, one mouse in the control group was defined as an outlier due to injection of too many cells, leading to spurious IVIS measurements and was, therefore, excluded from analysis. Inclusion and exclusion criteria were based on the quantification of BLI was not established a priori.

To visualise and quantify metastatic burden, the primary outcome measure, mice were administered d-luciferin (Promega) at a dose of 150 mg/kg by intraperitoneal injection and imaged non-invasively by BLI using a

Perkin Elmer Lumina S3 instrument 1–2 times per week as described by us previously⁷³.

Lung tissues were harvested, fixed in formalin and embedded in paraffin for downstream analyses by immunohistochemistry (IHC). For ex vivo imaging of lung metastatic burden, lungs were harvested from mice administered luciferin as described above and imaged prior to formalin fixation. Body weights were recorded 3–5 times per week during the course of the study as a measure of animal health. A clinical observation and health monitoring record was also maintained for each individual animal throughout the study, and animals were monitored 3–5 times/week. Steps were taken to reduce animal distress during the studies, including administration of subcutaneous saline injections as needed to mitigate dehydration and provision of sunflower seeds to maintain animal weight. The humane endpoints for this study were based on a balance between measurable signs such as weight loss (greater than 20% loss in weight), food and water intake, as well as reduced level of activity, behavioural changes, and physical appearance (e.g. coat and eye appearance). No adverse events were reported during the study period, and all animals involved in the study reached the experimental endpoint prior to the humane endpoints. The investigators were not blinded to the identity of the study groups and were therefore aware of group allocation at all stages of the experiment. The number of animals per group was chosen based on data from previous studies^{75,76}. While not formally registered, protocols and schedules were prepared prior to the initiation of each study.

Immunocytochemical analyses of NETs, ITGB1, CCDC25 and ILK in the MIA PaCa-2 xenograft model and the *Kras*^{G12D}/*Pdx1-Cre*/*p53/Rosa*^{YFP} genetically engineered mouse model (GEMM) of PDAC used archived tissues from previously published studies^{45,73}.

In vivo bioluminescence imaging

Luciferin was dissolved in saline solution at 15 mg/mL, and 10 µL/g body weight was injected intraperitoneally. Ten minutes post-injection, bioluminescence was imaged using an IVIS optical imaging system. Images were normalised and analysed via Living Image software, and the signal is presented as total flux in radians (photons/s/cm²/steradian) over standardised regions of interest.

Human participants—resectable PDAC sequencing datasets

RNA-seq data (transcripts per million (TPM); hg19) for resectable PDAC samples from the TCGA database ($n = 130$) were downloaded and log10-transformed, and samples were filtered as described previously⁷⁷. Additional public RNA-seq data for PDAC patient samples from the CPTAC-3 cohort (resectable disease; $n = 133$) were downloaded from the GDC data portal (<https://portal.gdc.cancer.gov>) on May 27, 2021 (HTSEQ; FPKM) and log10-transformed.

Human participants—metastatic tumour datasets

Sequencing data for patients with metastatic PDAC ($n = 106$) was obtained from two prospective studies: Prospectively Defining Metastatic Pancreatic Ductal Adenocarcinoma Subtypes by Comprehensive Genomic Analysis (PanGen, NCT02869802; $n = 81$) and the BC Cancer Personalized OncoGenomics programme (POG, NCT02155621; $n = 25$). All PanGen and POG data were sequenced at Canada's Michael Smith Genome Sciences Centre in Vancouver, Canada. Patients participating in PanGen and POG studies were enrolled as previously described^{38,78}. PanGen and POG studies were approved by the University of British Columbia Research Ethics Committee (REB# H12-00137, H14-00681, H16-00291), and all ethical regulations relevant to human research participants were followed. Written informed consent was obtained from each patient prior to molecular profiling. All sequencing data were housed using a secure computing environment. The PanGen and POG clinical trials are not directly linked to a specific treatment, but rather aim to assess response to genomics-guided therapy, with treatments selected at the discretion of the treating oncologist. RNA-seq was performed on

metastatic patient tumour samples with a target depth of 200 million reads. RNA-seq reads were trimmed to 75 bp and aligned (GRCh37-lite) using STAR v2.7.3⁷⁹, with parameters: -chimSegmentMin 20 -outSAMmultNmax 1 -outSAMstrandField intronMotif -outFilterIntronMotifs RemoveNoncanonical. RNA-seq duplicate reads were marked using PicardTools v2.17.3. Raw read counts were assigned to Ensembl 75 genes using Subread v1.4.6⁸⁰, normalised for library depth and gene size (RPKM) and log10-transformed.

Human tissue samples

Formalin-fixed, paraffin-embedded tissue samples from resected PDAC primary tumours of 20 patient samples were retrieved from the BC Gastrointestinal (GI) Biobank at Vancouver Coastal Health, Vancouver, Canada. Blocks were sectioned at 6 µm and mounted on charged slides. Studies using the patient materials were conducted in accordance with ethics approval obtained from the University of British Columbia Clinical Research Ethics Board (H20-00948, H18-03701).

RNA-seq batch correction

RNA-seq data were corrected for cohort-specific batches (PanGen/POG, TCGA and CPTAC-3) using an empirical Bayesian approach⁸¹. Principal component analysis (PCA) of the top 10% most variable genes was used to confirm alleviation of inter-sample batch effects after correction.

Clustering analysis

Normalised gene expression values were converted to z-scores prior to clustering using all samples combined ($n = 369$). Genes previously implicated in NET pathway expression in other cancers were curated from studies of triple-negative breast cancer ($n = 136$ genes)²⁶ and PDAC ($n = 8$ genes)²⁵ and used for gene-gene clustering to identify PDAC-specific co-expressed NET signature genes. From this curated gene panel, a total of 123 NET-related genes were present within the RNA-seq datasets and comprised the NET-related gene set used for gene-gene cluster analysis (Supplementary Table 1). Consensus clustering was performed using R v3.6.3 package ConsensusClusterPlus, with parameters $\text{reps} = 100$, $\text{pltem} = 0.8$, $\text{pFeature} = 1$, $\text{clusterAlg} = \text{'ca'}$, $\text{distance} = \text{'euclidean'}$, $\text{seed} = 123$ and $\text{maxK} = 8$, and gene cluster groups were chosen based on the optimal clustering solution as specified by the area under the cumulative distribution function curve. These genes were identified from two co-expressed clusters: Gene Cluster 5 ($n = 8$ genes: *LDLR*, *MAPK3*, *CARD11*, *GSDMD*, *SRC*, *RIPK3*, *KLF2* and *TICAM1*) and Gene Cluster 6 ($n = 23$ genes: *ACTB*, *PKM*, *ACTG1*, *ACTN4*, *ENO1*, *MYH9*, *CD44*, *ITGB1*, *SPPI*, *TIMPI*, *ACTN1*, *AKT2*, *TKT*, *F3*, *AKT1*, *MFN2*, *MTOR*, *MYD88*, *DNAJB1*, *CEBPB*, *DCBLD2*, *KRT10* and *MAPK7*). Genes identified from this analysis comprised a PDAC NET-signature gene set ($n = 31$ genes) and were then used as input for subsequent sample-sample clustering. Sample cluster groups were determined in a semi-automatic way, in which overlaid gene expression patterns were used in conjunction with the optimal clustering solution to merge phenotypically similar clusters together, enabling the formation of a low-expression cluster⁸².

Differential expression and gene set enrichment analysis

Differential expression analysis was performed between NET signature group 2 versus group 3 on normalised gene counts using Wilcoxon mean rank-sum tests, followed by Benjamini–Hochberg multiple test correction. GSEA was performed separately on the top 500 most up- and down-regulated DE genes (adjusted $p < 0.05$) using hypergeometric tests, in which genes were assessed for overlap with each of 32,284 gene sets obtained from the molecular signatures database (MSigDB⁸³; downloaded April 2021). Hypergeometric test p values were subjected to Benjamini–Hochberg multiple test correction. Pan-cancer gene-gene relatedness scores (mutual rank) were derived from the GeneFriends dataset⁴⁷ and used to visualise pairwise gene relationships for genes belonging to the Hallmark Epithelial-mesenchymal transition gene set that were upregulated in the NET signature-high/ITGB1-high group.

Production and isolation of neutrophil extracellular traps (NETs)

NETs were isolated from HL-60 cells (ATCC CCL-240), a peripheral blood promyeloblast that can differentiate into mature neutrophils³⁹. Care was taken to ensure cells were sub-cultured at densities that did not induce spontaneous differentiation. For differentiation of HL-60 cells into neutrophils, cells were resuspended in DMSO-containing differentiation medium (phenol-red-free RPMI 1640, 10% FBS, 1x glutamax, 1.5% DMSO), plated into 10 cm tissue culture-treated plates at a concentration of 2×10^5 cells/mL and incubated for 7 days. Differentiated cells were then activated to undergo NETosis using phorbol 12-myristate 13-acetate (PMA). Cell suspensions from 5 plates were pooled and PMA was added to a final concentration of 1 μ M. Cells were mixed by gentle pipetting, re-plated in 10 cm plates and incubated for 4 h to trigger NETosis. To harvest the NETs, supernatants containing non-adherent cells were removed by aspiration, 1 mL/plate serum-free media was added and NETs were collected by scraping. NETs were pooled, centrifuged at 1000 rpm for 5 min to remove cell debris and the NET-containing supernatant was transferred to a fresh tube. NET-DNA was quantified using a Nanodrop spectrophotometer and stock preparations were diluted to 100 μ g/mL for subsequent use in assays. NETs were used fresh for downstream assays.

Antibodies

The antibodies used in this study are presented in Table 3.

RNA interference

A Qiagen FlexiTube gene solution set of 4 siRNAs for ILK was screened to identify efficacious siRNAs to use in our studies (Supplementary Fig. 3C). siRNAs were transfected using SilentFect™ (Bio-Rad, 1703360) as per the manufacturer's instructions. Protein levels were routinely monitored by Western blotting.

shRNA transduction

The dox-inducible knockdown cell lines for ILK and CCDC25 were generated using SMART vector lentiviral dox-inducible shRNA plasmids from Horizon Discovery. HEK293T cells (RRID:CVCL_0045) were seeded at 20,000/cm². The following day, cells were transfected with 0.9 μ g pSPAX, 0.1 μ g of pVSVG and 1 μ g of the target shRNA plasmid using TransIT-LT1 transfection reagent (Mirus). After 48 h, the virus was harvested by passing the media through a 0.45 μ m filter and then concentrating using a 5x Lenti Concentrator (Origene). The virus was added to MIA PaCa-2 Luc+ cells or KPCY Luc+ cells along with 10 μ g/mL polybrene (Sigma) for 72 h. Antibiotic selection with 2 μ g/mL puromycin and 1 μ g/mL blasticidin (Luc+) for the knockdown cell lines was initiated, and cells were continued to be cultured in the presence of selection antibiotics. To induce the expression of the shRNA for each experiment, transfected cells were treated with 1 μ g/mL doxycycline for 72 h, refreshed daily, then lysed in RIPA and analysed by Western blotting.

Western blotting

Samples were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% (w/v) SDS) containing 1 mM Na₃VO₄, 2 mM NaF and cOmplete protease inhibitor. Five to ten micrograms of protein were separated by electrophoresis using 4%–12% Bis-Tris gradient gels in 1x NuPAGE MOPS SDS running buffer. Gels were transferred to PVDF membranes for 1 h at 100 V in Tris-Glycine transfer buffer containing 15% methanol. The PVDF membranes were methanol-fixed briefly and incubated with primary antibodies in 2% BSA in TBST overnight at 4 °C, followed by washes with TBST for 3 $\times$ 10 min and incubation with HRP-conjugated secondary antibody in 2% skim milk in TBST for 1 h at room temperature. After subsequent washes with TBST, detection was performed by incubation with Super-signal™ West Femto chemiluminescence reagents and visualisation using a Chemidoc XRS+ imaging system (Bio-Rad Laboratories). Vinculin or β -actin was used as a loading control. The relative intensities of protein bands were quantified using Image Lab 5.2.1 (Bio-Rad Laboratories) and normalised using the loading control.

Co-immunoprecipitation

Cells were washed with 1X PBS and lysed with ice-cold 0.3% CHAPS buffer (containing 1 mM Na₃VO₄, 2 mM NaF and cOmplete protease inhibitor) using a cell scraper, followed by centrifugation to remove cell debris. Two milligrams of protein was immunoprecipitated at 4 °C overnight using either 10 μ g of ITGB1 antibody (Cell Signaling), 20 μ L of CCDC25 antibody/agarose conjugate or protein A/G PLUS agarose alone (Santa Cruz Biotechnology). Pellets were washed 5 times with CHAPS buffer, resuspended in sample buffer, boiled at 100 °C for 10 min before loading on SDS-PAGE for Western blotting as described above.

Rac1 Activation Assay

Active GTP-Rac1 and GTP-Cdc42 levels were analysed using the Rac1 Pull-Down Activation Assay (Cytoskeleton) with 1 mg of lysate as per the manufacturer's instructions. Briefly, cells were seeded in 15 cm plates at 20,000/cm² in media containing 10% FBS and allowed to grow for 72 h to achieve 30% confluence. Cells were then serum-starved with 1% FBS for 24 h, followed by a further 24 h at 0% FBS before treatment with HL-60 derived NET-DNA for 20–24 h, followed by lysis and processing.

Migration and invasion assay

The scratch wound migration and invasion assays were performed using the Incucyte™ SX3 live-cell analysis instrument (Sartorius Biosciences). For invasion assays, 96-well Imagelock® plates were coated with a thin layer of phenol-red-free, growth factor-reduced Matrigel (Corning 356231, 50 μ L/well of a 100 μ g/mL stock solution/well) and incubated for 2 h at 37 °C to allow for matrix polymerisation. Following liquid aspiration, MIA PaCa-2 cells or KPCY PENN6620c1 cells were seeded at a density of 3×10^4 cells/well in 100 μ L/well media and allowed to attach overnight. The next day, confluent cell monolayers were treated with 5 μ g/mL mitomycin C (M4287, Millipore Sigma) for 2 h at 37 °C to inhibit cell proliferation. Monolayers were then wounded in a uniform manner with a 96-pin Incucyte Woundmaker™ tool, followed by two successive media changes with 100 μ L/well culture media to remove non-adherent cells. Each plate was cooled, and the cell monolayer was carefully overlaid with 50 μ L/well of 8 mg/mL Matrigel containing 10 μ g/mL NETs or an equivalent volume of media as a control. The plates were incubated at 37 °C for 30 min to polymerise the Matrigel. Finally, 100 μ L/well of media containing 10 μ g/mL NETs was added on top of the polymerised Matrigel layer. Plates were placed in the Incucyte® Live-Cell Analysis System, and images were acquired every 2 h for the indicated times using a Nikon Plan Fluor 10x/0.3 NA objective in phase contrast mode. Cell invasion was quantified using the Incucyte Scratch Wound Analysis Software Module, and data are expressed as relative wound density (%). For samples containing NETs exposed to DNase1, NETs were pre-incubated with 40 U DNase1 per 1 μ g DNA for 15 min at 37 °C prior to addition to the Matrigel layer. Additionally, 1000 U DNase1 in a total media volume of 100 μ L/well was added on top of the Matrigel layer. For studies involving pharmacologic inhibition of ILK, QLT-0267 was added at a final concentration of 10 μ M, or, alternatively, at the final concentrations indicated in the figures, in 100 μ L/well of media as the top layer. For studies involving incubation with integrin β 1-blocking antibodies, the antibody was added at a final concentration of 1 to 5 μ g/mL. To account for the 50 μ L volume of the Matrigel layer, DNase1, QLT-0267 and integrin β 1-blocking antibody were added at 1.5 $\times$ the final concentration in 100 μ L/well of media. In studies using dox-inducible shRNA stable cell lines, 1 μ g/mL doxycycline was added for 72 h, refreshed daily, to induce shRNA-mediated suppression of gene expression prior to initiation of downstream assays.

Spheroid growth assay

MIA PaCa-2 cells were seeded at 2,000 cells/well in an ultra-low attachment U bottom 96-well plate (Corning 7007) in 100 μ L/well complete media, centrifuged at 800 rpm for 10 min, placed in the Incucyte™ SX3 live-cell analysis instrument using the 'single

Table 3 | List of antibodies

Method	Antibody	Source	Cat #	RRID	Dilution
Blot and IP	ZEB1 Rabbit mAb (D80D3)	CST ^a	3396	AB_1904164	1:1000
	Snail (C15D3) Rabbit mAb	CST	3879	AB_2255011	1:1000
	GSK3B (27C10) Rabbit mAb	CST	9315	AB_490890	1:1000
	Phospho-GSK3B (Ser9) Antibody	CST	9336	AB_331405	1:1000
	p44/42 MAPK(Erk1/2)(137F5) Rabbit mAb	CST	4695	AB_390779	1:1000
	Phospho-p44/42(Erk1/2) (Thr202/Tyr204) Antibody	CST	9101	AB_331646	1:1000
	mTOR (7C10) Rabbit mAb	CST	2983	AB_2105622	1:1000
	Phospho-mTOR (Ser2448) Antibody	CST	2971	AB_330970	1:1000
	CD44 (E7K2Y) XP Rabbit mAb	CST	37259	AB_2750879	1:1000
	Phospho-Akt (Ser473) (D9E) XP Rabbit mAb	CST	4060	AB_2315049	1:1000
	Phospho-B-Catenin (Ser33/37/Thr41) Antibody	CST	9561	AB_331729	1:1000
	Slug (C19G7) Rabbit mAb	CST	9585	AB_2239535	1:500
	Vimentin (D21H3) XP Rabbit mAb	CST	5741	AB_10695459	1:5000
	ILK1(4G9) rabbit mAb	CST	3856	AB_2233861	1:1000
	Integrin B1 (D2E5) rabbit mAb	CST	9699	AB_11178800	1:1000
	Mouse Anti-ILK Clone 3/ILK (RUO)	BD Biosciences	611803	AB_399283	1:1000
	Mouse Anti-N-Cadherin	BD Biosciences	610920	AB_2077527	1:1000
	Mouse anti-Akt	BD Biosciences	610861	AB_398180	1:1000
	Mouse Anti-B-Catenin	BD Biosciences	610154	AB_397555	1:1000
	Anti Vinculin Ab, clone VILF9 (7F9)	Millipore Sigma	MAB3574	AB_2304338	1:2000
	Rabbit anti-human CCDC25	Proteintech	21209-1-AP	AB_10732690	1:1000
	Goat anti rabbit IgG(H+L) HRP	Jackson	111-035-003	AB_2313567	1:10,000
	anti-mouse IgG HRP	CST	7076	AB_330924	1:10,000
	CCDC25 Antibody (F-7) AC	Santa Cruz Biotechnology	sc-515201 AC		10ug/IP
	Integrin B1/ITGB1 (102DF5)	Santa Cruz Biotechnology	sc-73610	AB_1123630	10ug/IP
	Normal mouse IgG-AC	Santa Cruz Biotechnology	sc-2343	AB_737178	20uL/IP
Rac1 Activity Assay	Anti Rac1 mAb	Cytoskeleton	ARC03	AB_10709099	1:500
	Anti Cdc42 mAb	Cytoskeleton	ACD03	AB_10716593	1:500
ITGB1 function-blocking assay	Anti Integrin B1 Ab, clone 6S6	Millipore Sigma	MAB2253	AB_11210276	5-20ug/mL
	Hamster Anti-mouse CD29	BD Biosciences	562219	AB_1115404	5-20ug/mL
IF	Rabbit anti-human CCDC25	Proteintech	21209-1-AP	AB_10732690	1:50
	Mouse anti-human ILK	Invitrogen	MA5-17099	AB_2538570	1:100
	Goat anti-human MPO	R&D Systems	AF3667	AB_2250866	1:40
	Mouse anti-human Paxilin	BD Biosciences	610051	AB_397463	1:800
	Rabbit anti-human Cofilin	CST	PA5-27627	AB_2545103	1:800
	Rabbit anti-human ITGB1	CST	D2E5		1:50/1:100
	Rabbit anti-human Cit-H3	Abcam	Ab5103	AB_304752	1:100
	Rabbit anti-human ILK	Abcam	Ab106955	AB_11129360	1:100

^aCST Cell Signalling Technology.

spheroid' mode and images were acquired every 6 h for 9 days. After 72 h, the newly formed spheroids (~200 µm), were treated with either of 10 µL of media containing 100 µg/mL NETs, 5 µL of 200 µM QLT-0267 in 10% DMSO, or 1000 U of DNase1 to result in final concentrations of 10 µg/mL NETs, 10 µM QLT-0267 and 100 U DNase 1, respectively.

Preparation of matrix-coated coverslips

Twelve millimetre glass coverslips were coated with Matrigel in DMEM (1:40, Cat no. 356231, Corning), 2 µg/ml of Fibronectin (Cat no. F1056-1MG, Sigma), incubated for 1 h at 37 °C and washed extensively with PBS.

Immunofluorescence staining

MIA PaCa-2 Luc+ dox-inducible shILK cells were cultured for 3 days with or without 1 µg/mL doxycycline (Cat no. D9891-25G) to induce knock-down of ILK expression. Cells were then harvested, counted, seeded onto coverslips at 7×10^4 cells/mL and incubated for 6 h to allow the cells to adhere to the coverslips. NETs were then added at a concentration of 20 µg/mL, and cells were incubated for 24 h. The next day, confluency was assessed to ensure cell layers were 30–50% confluent. The media was then aspirated, cells were washed with Hanks buffered saline (Cat no. 14175-095, Gibco) and fixed in 4% paraformaldehyde (Cat no. YA357388, Thermo) diluted in PBS at room temperature for 15 min. The coverslips were washed three times with 1X PBS and then blocked using Hanks buffered saline + 10%

FBS for 30 min. Coverslips were washed 3 times with 1X PBS. Cells were permeabilized with 0.1% Triton X-100 (Cat no. SLB 56421, Thermo) for 10 min. The coverslips were washed three times with PBS, blocked again using Hanks buffered saline + 10% FBS for 30 min and washed 3 times with 1X PBS. Coverslips were incubated with primary antibodies diluted in blocking buffer for 24 h at 4 °C. The samples were then washed as described above and incubated with AlexaFluor 488/594 conjugated secondary antibodies (1:100, Cat no. A11029/A11012, Life Technologies) for 1 h at room temperature. Cells were incubated with Hoechst 33342 (1:10,000, Cat no. 62249) for 10 min to stain nuclei and washed with PBS. The coverslips were then mounted on glass slides in Prolong Diamond anti-fade mounting media (Cat no. P36970, Thermo). Images of cells were acquired using a Zeiss LSM 800 Airyscan confocal microscope (Carl Zeiss, Thornwood, NY) equipped with a 63× oil immersion objective lens and processed using Zen 3.10 software (Zeiss).

Tissue immunofluorescence staining

Five micron sections of formalin-fixed, paraffin-embedded tissue were deparaffinized, rehydrated, and antigen retrieval was performed by microwave in 10 mM citrate buffer, pH 6.0, for 10 min. Staining of tissue sections was performed using conditions similar to those used for IF staining of cells. Images of tissue sections were acquired using a Zeiss Colibri inverted microscope (Carl Zeiss, Thornwood, NY) equipped with a 20× lens and processed using Zen 3.10 software (Zeiss). Alternatively, images were acquired using a Zeiss LSM 800 Airyscan confocal microscope (Carl Zeiss, Thornwood, NY) equipped with a 63× oil immersion objective lens and processed using Zen 3.10 software (Zeiss).

Statistics and reproducibility

For clinical datasets, One-way ANOVA tests were used to test for differences in a continuous variable across discrete groupings and were followed by Wilcoxon mean rank-sum tests for post-hoc identification of differences between group pairings. Log-rank tests were used to calculate *p* values in Kaplan–Meier analysis. All comparison tests were two-tailed. All *p* values were subjected to Benjamini–Hochberg multiple test correction when applicable. Bioinformatics analyses of clinical sequencing data were performed using R v3.6.3. For analysis of proteomic data, ANOVAs were carried out on specific proteins using a previously published proteomic data set³⁸ and methods described previously³⁸.

For analysis of non-clinical datasets, the data were presented as Mean ± SD or as Mean ± SEM as indicated in the figures. The data shown are representative of two to three independent experiments. Statistical analyses were conducted using Prism v10.1.2 software (GraphPad, La Jolla, CA). Statistical significance for multiple comparisons was calculated using two-way ANOVA corrected for multiple comparisons with the Holm–Sidak method unless otherwise indicated. A Student’s *t*-test (two-tailed) was used when only two datasets were being compared in a given plot. For all tests, the significance is indicated as **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Sequencing data generated as part of the PanGen/POG study is available in the European Genome-phenome Archive (EGA) under accession number #EGAS00001001159. Uncropped, unedited blot images are included in Supplementary Information (Supplementary Fig. 5). The numerical source data for the graphs are available in Supplementary Data. All other datasets generated during and/or analysed during the current study, as well as source data, are available from the corresponding author on reasonable request. Requests for resources and reagents should be directed to and will be fulfilled by the corresponding author. This includes antibodies and shRNA-engineered cell lines. Genetically engineered cell lines, the PaCa41 PDX cell

line and QLT-0267, will be made available on request after completion of a Materials Transfer Agreement.

Code availability

De-identified custom code and/or mathematical algorithm used for data analysis can be accessed through GitHub at: <https://github.com/jtopham/publications>.

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Competing interests

The authors declare no competing interests.

Additional information

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