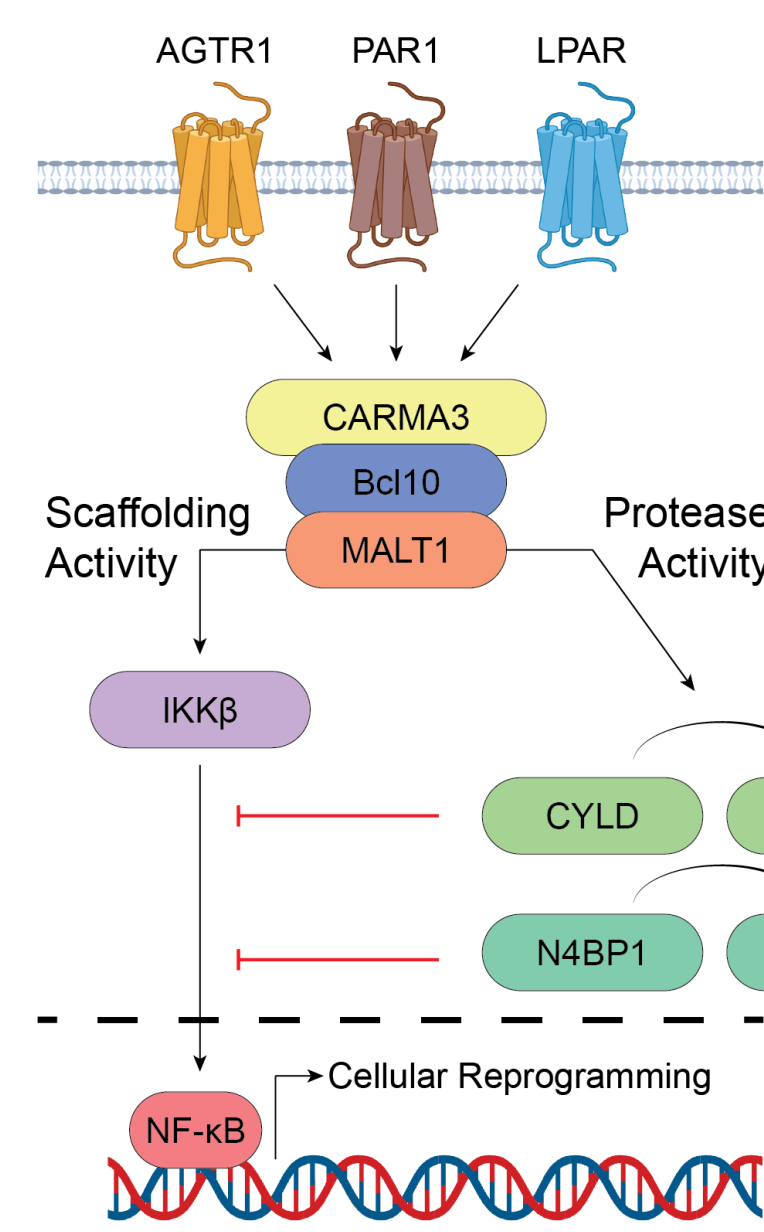


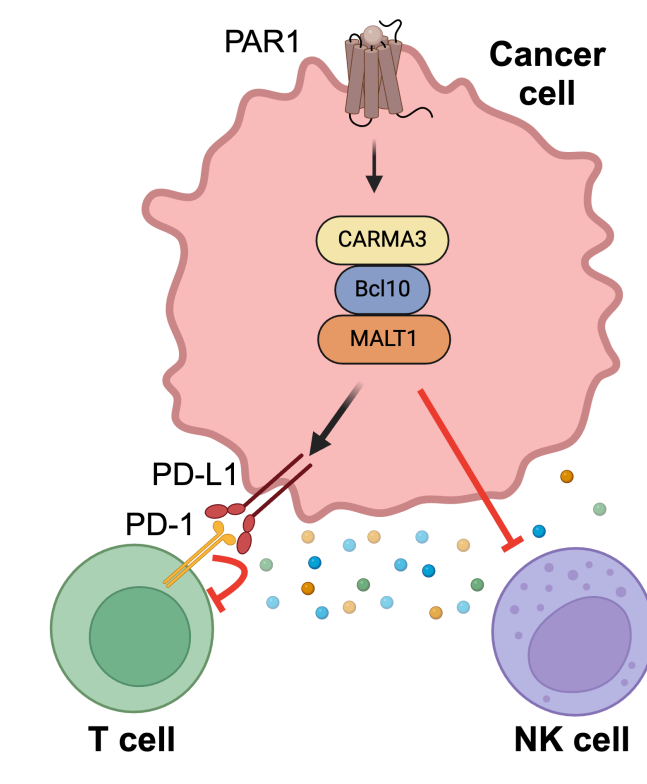
Jack Little^{1,2,3}, Dong Hu², Cody Hoffmann², Akhilesh Pandey², Linda M. McAllister-Lucas^{4,5} and Peter C. Lucas^{2,5}
¹Visiting Graduate Student Program, ²Department of Laboratory Medicine and Pathology, Mayo Clinic, ³Medical Scientist Training Program, University of Pittsburgh School of Medicine, ⁴Department of Pediatrics and Adolescent Medicine, ⁵Mayo Clinic Comprehensive Cancer Center, Mayo Clinic

BACKGROUND



MALT1 was initially discovered as the effector component of the CARMA/CARD-BCL10-MALT1 (CBM) signalosome in lymphocytes downstream of antigen receptors. MALT1 possesses both scaffolding and protease functions to activate NF-κB signaling. More recent evidence has implicated the same signalosome in epithelial cells downstream of G protein-coupled receptors (GPCRs). Our research studies the role of this complex in promoting breast cancer progression.

Protease-activated receptor 1 (PAR1) is a GPCR overexpressed in a subset of breast cancers and activates MALT1 leading to upregulation of PD-L1. Preclinical models have demonstrated a role for the PAR1/MALT1/PD-L1 axis in directing T cell exhaustion in the TNBC immune microenvironment. Additional preliminary evidence suggests that soluble mediators also contribute to MALT1-driven immunosuppression.



Hypothesis: The GPCR/MALT1 protease axis promotes the production of a pro-tumor secretome leading to modulation of the microenvironment and enhanced tumor growth in TNBC.

RESULTS

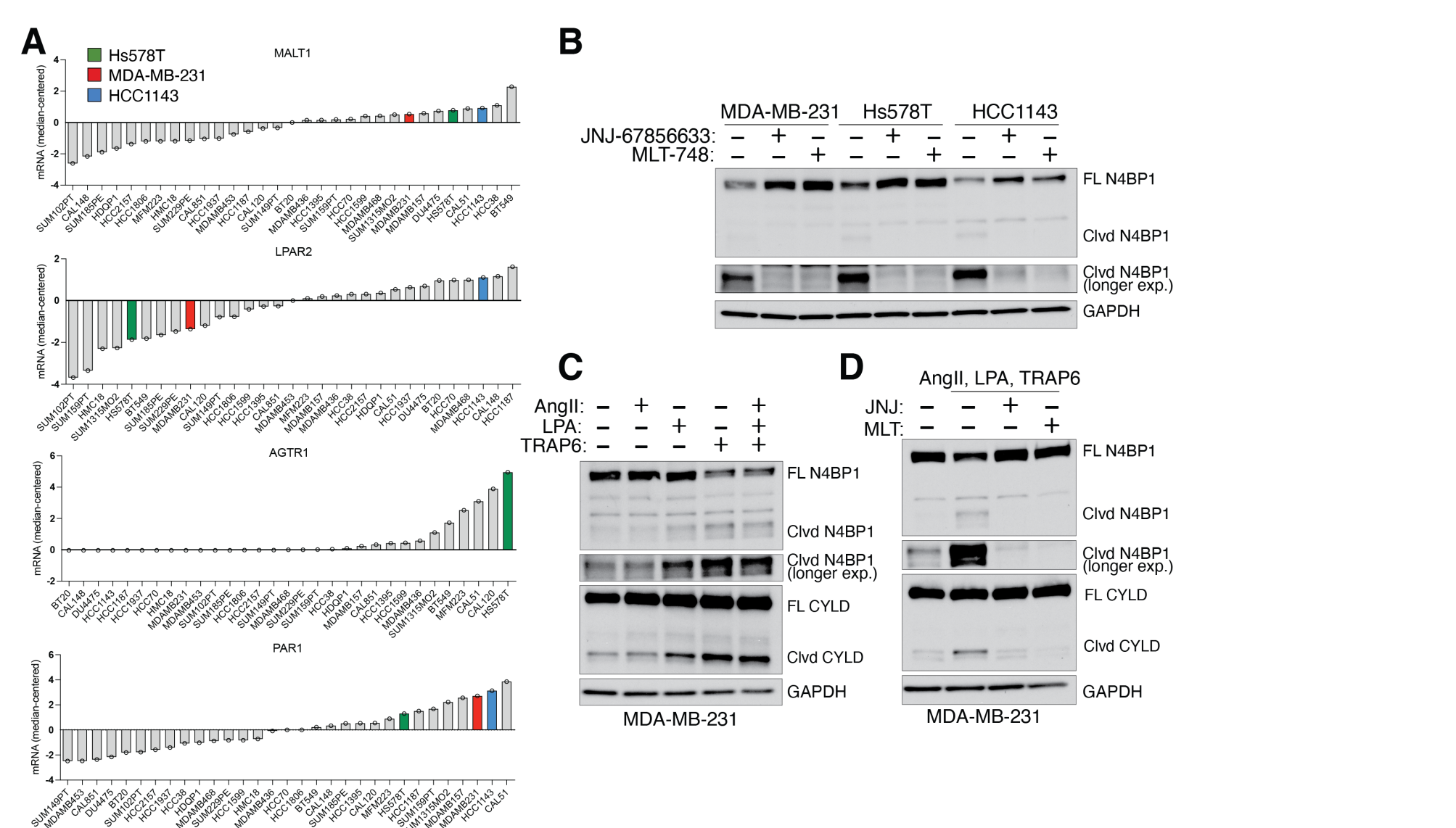


Fig. 1. MALT1 protease function is activated downstream of GPCRs in TNBC cells. (A) The Cancer Cell Line Encyclopedia (CCLE) was queried to identify TNBC cell lines with high mRNA expression of MALT1 and GPCRs implicated in breast cancer pathogenesis. AGTR1 = angiotensin receptor type 1, LPAR2 = lysophosphatidic acid receptor 2, PAR1 = protease activated receptor 1. Three cell lines (highlighted) were selected for further analyses. (B) Basal cleavage of the MALT1 protease substrate N4BP1 is inhibited by two different MALT1 protease inhibitors, JNJ-67856633 (JNJ) and MLT-748 (MLT), in the three selected human TNBC cell lines. Cells were treated with 1 μM of either inhibitor for 48 hours. (C) Cleavage of two MALT1 protease substrates, N4BP1 and CYLD, in MDA-MB-231 cells is increased following stimulation with GPCR agonists: angiotensin II (AngII), lysophosphatidic acid (LPA), and/or thrombin receptor activator peptide 6 (TRAP6). (D) GPCR-induced MALT1 substrate cleavage in MDA-MB-231 cells is inhibited by both JNJ and MLT. FL=full length, Clvd=cleaved.

RESULTS

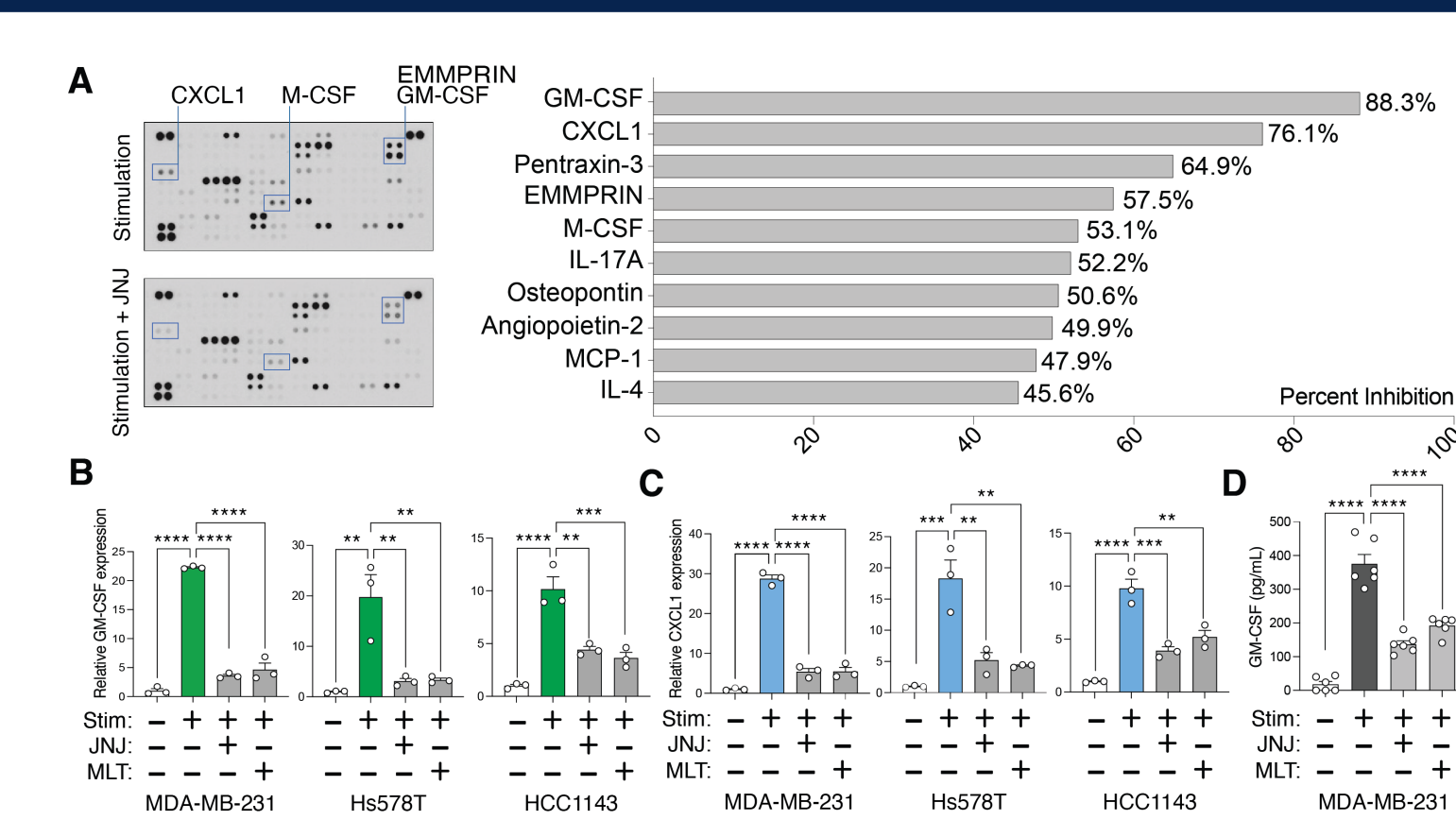


Fig. 2. MALT1 protease regulates the production of numerous secreted factors, including GM-CSF and CXCL1. (A) Cytokine array screen in MDA-MB-231 cells identified various secreted factors regulated by MALT1 protease activity. One representative exposure is shown. The top ten most inhibited cytokines are shown. MALT1 protease is required for (B) GM-CSF and (C) CXCL1 mRNA expression in three TNBC cell lines. (D) MALT1 protease is required for GM-CSF protein expression from MDA-MB-231 and Hs578T cells. Statistical analysis: One-way ANOVA with post-hoc Tukey, **p<0.01, ***p<0.001, ****p<0.0001.

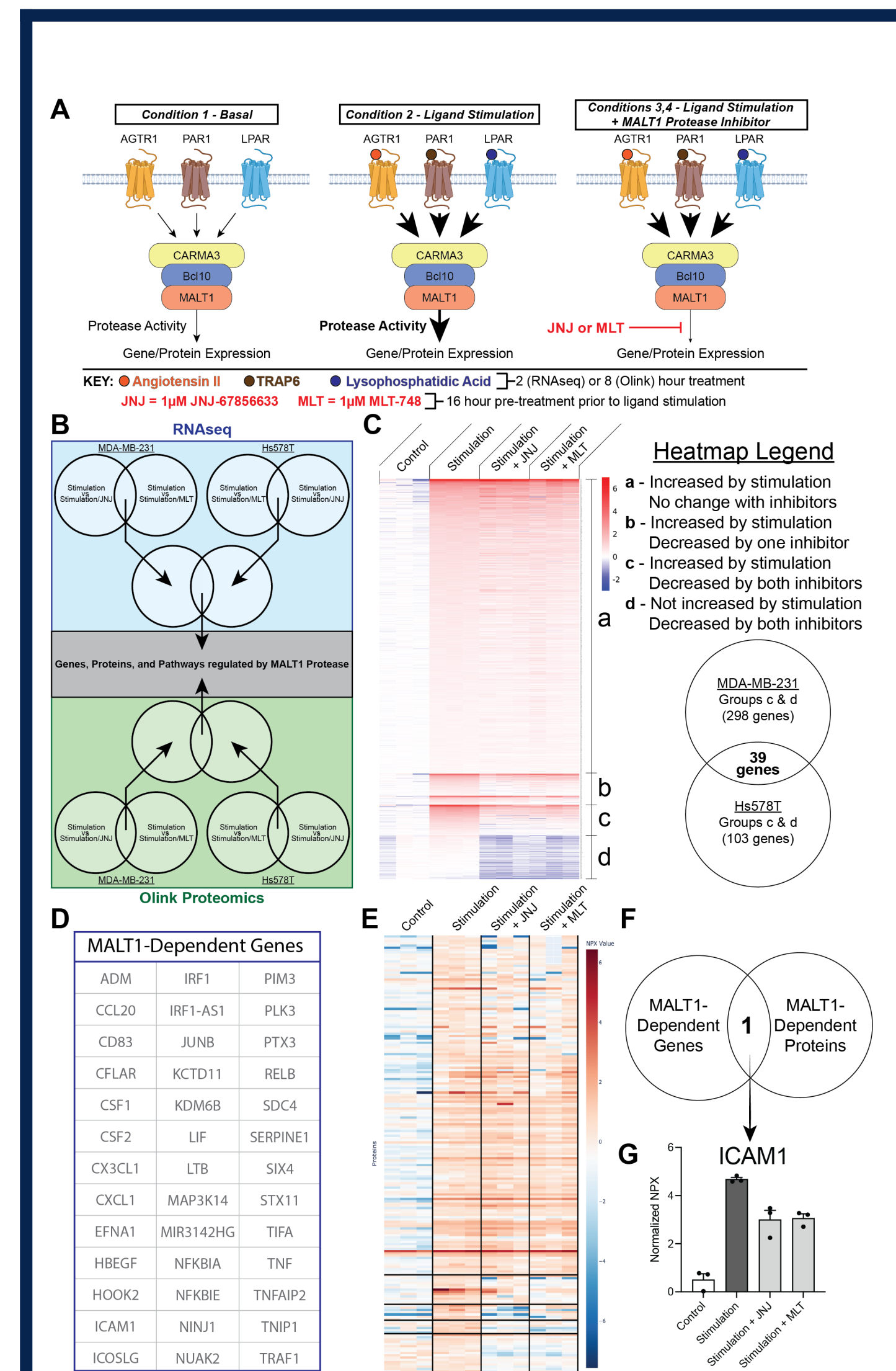


Fig. 3 Transcriptomic and proteomic screen of TNBC cell lines identifies numerous MALT1-dependent factors implicated in cancer progression. (A) Schematic representing experimental design for transcriptomic and proteomic screens. (B) Venn diagram depicting analysis leading to MALT1-dependent genes, proteins, and pathways. (C) RNA-seq of two TNBC cell lines identifies 39 shared MALT1 protease-dependent genes. (D) Significant genes regulated by MALT1 protease. (E) Heatmap depicting significant proteins from Olink Proteomic screen of MDA-MB-231 cells. (F) Overlap of genes identified from RNA-seq and proteins identified from Olink identifies one protein regulated by MALT1: ICAM1. (G) ICAM1 protein expression in MDA-MB-231 cells from Olink Proteomic experiment.

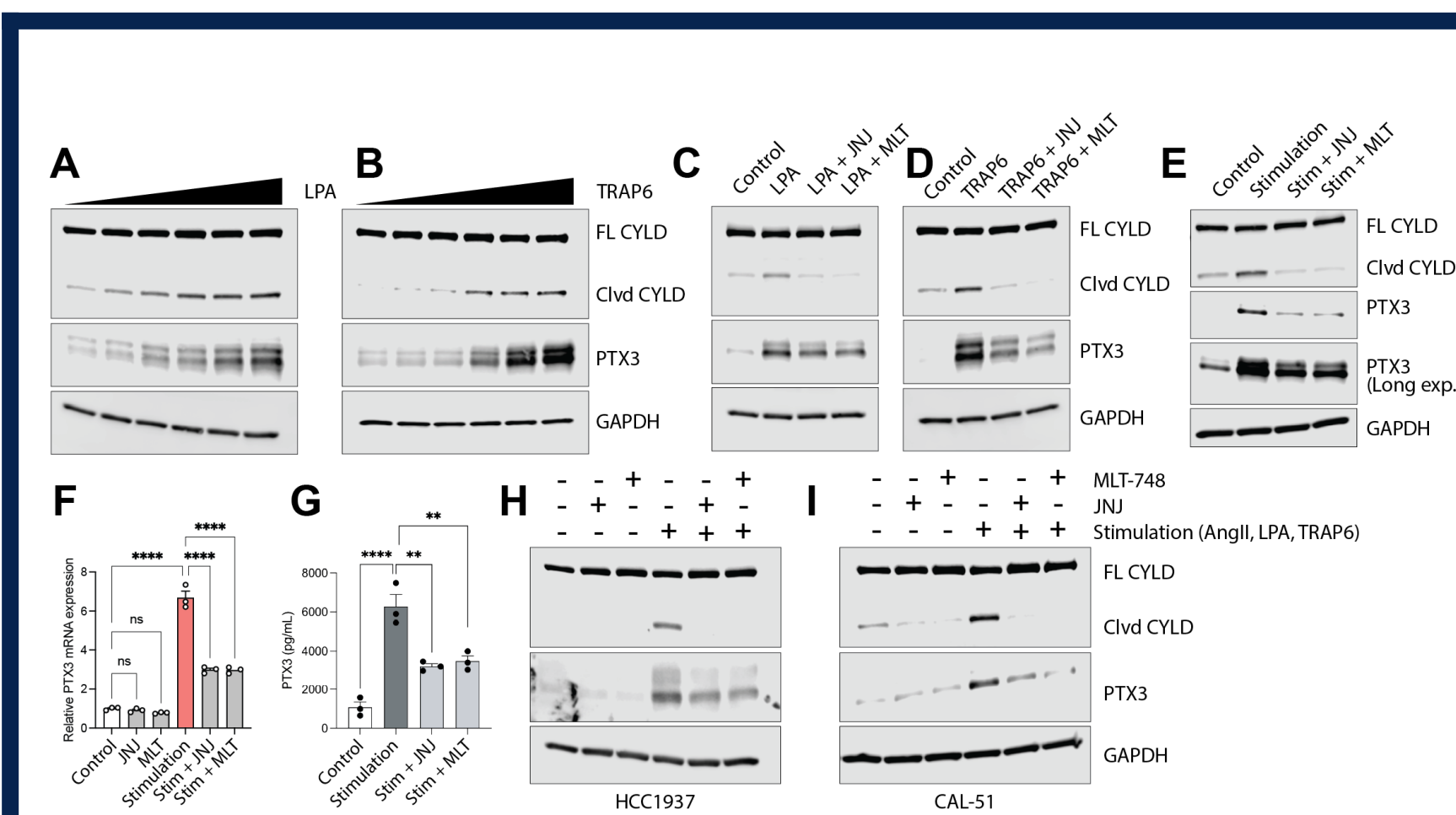


Fig. 4. Pentraxin-3 (PTX3) expression is induced by GPCRs in a MALT1 protease-dependent manner. PTX3 expression is induced by increasing concentrations of (A) LPA and (B) TRAP6 in MDA-MB-231 cells. (C) LPA- and (D) TRAP6-induced PTX3 expression is reduced by MALT1 protease inhibitors, JNJ and MLT. PTX3 is upregulated by stimulation with a GPCR agonist cocktail, which is reduced by MALT1 protease inhibitors in MDA-MB-231 cells as measured by (E) Western blot, (F) RT-qPCR, and (G) ELISA. PTX3 is upregulated by GPCR stimulation in (H) HCC1937 and (I) CAL-51 cells in a MALT1 protease-dependent manner. FL = full-length, Clvd = cleaved, LPA = lysophosphatidic acid, TRAP6 = thrombin receptor agonist peptide, AngII = angiotensin II, Stim/Stimulation = LPA, TRAP6, AngII.

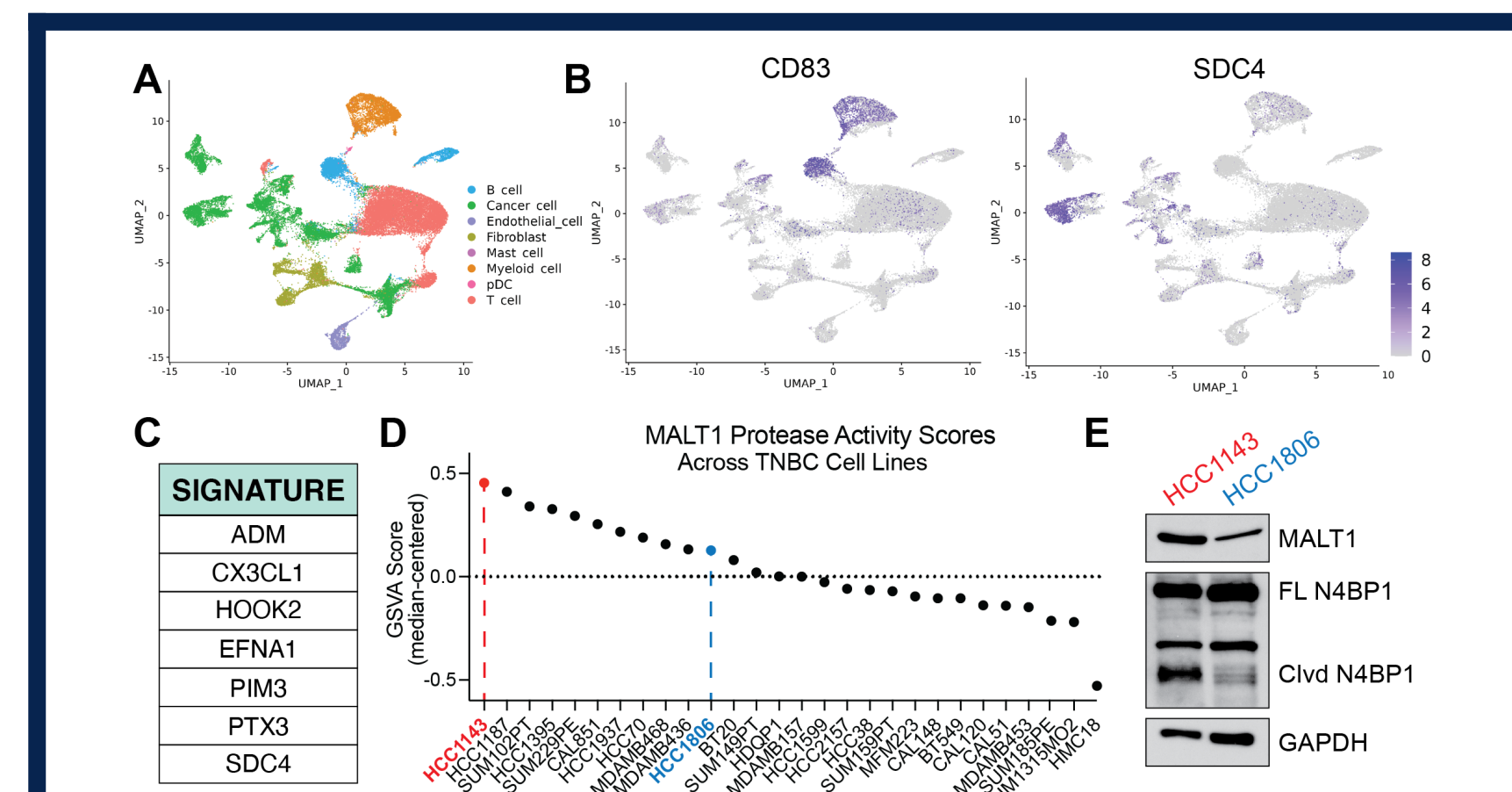


Fig. 5. Generation of MALT1 activity gene signature. (A) Localization of overlapping genes from RNA-seq data in Figure 3C to cancer cells in scRNA-seq analysis of 12 human TNBC tumors used to prune MALT1 gene signature. (B) Example genes are shown to represent cancer cell-predominant (SDC4) and myeloid cell-predominant (CD83) expression. (C) Genes included in signature based on cancer cell localization. (D) Ranking of TNBC cells according to gene set variation analysis (GSVA) score of MALT1 signature. (E) MALT1 protease activity of TNBC cell lines as measured by cleavage of MALT1 substrate, N4BP1.

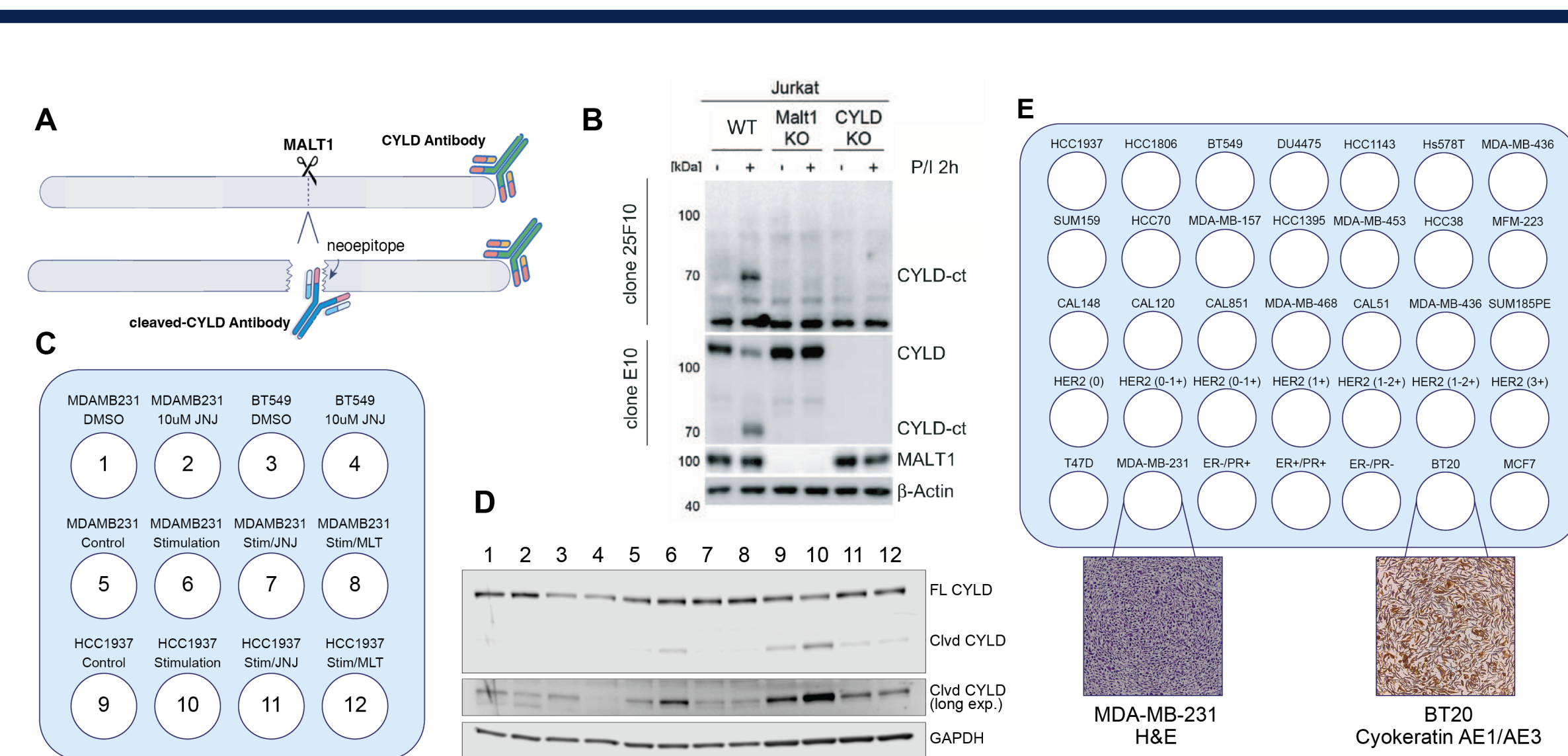


Fig. 6. Generation of cell culture microarrays (CMAs) to validate MALT1-cleaved CYLD-specific antibody for use in archival specimens. (A) Schematic depicting action of cleaved CYLD antibody which specifically detects MALT1-dependent cleavage product without detecting full length CYLD. (B) Western blot comparing reactivity of cleaved CYLD antibody (25F10) and full length CYLD antibody (E10). Adapted from Di Pilato M et al. *J Immunother Precis Oncol* (2022). (C) Planned CMA for validation of cleaved CYLD antibody. (D) Western blot for full length CYLD (E10) of samples corresponding to cores in validation CMA. (E) Planned CMA of 22 TNBC cell lines to be used for assessing MALT1 activity across multiple cell lines and for biomarker discovery.

CONCLUSIONS

- MALT1 is proteolytically active in three human TNBC cell lines downstream of GPCRs.
- Two MALT1 protease inhibitors impair MALT1 protease activity to similar degrees in TNBC cells.
- MALT1 coordinates the expression of multiple secreted factors implicated in cancer progression.
- MALT1 protease inhibitors block GM-CSF and CXCL1 gene expression and protein secretion in TNBC cells.
- Transcriptomic and proteomic screen of two TNBC cell lines helps identify numerous MALT1-dependent secreted factors.
- PTX3 is upregulated by GPCR stimulation in a MALT1-dependent manner in two TNBC cell lines.
- RNA-seq of two human TNBC cell lines leads to generation of MALT1 protease gene signature.
- Antibody 25F10 can accurately recognize the MALT1-specific cleavage product of CYLD.

FUTURE DIRECTIONS

Our results indicate that the GPCR/MALT1 protease induces expression of multiple secreted factors implicated in cancer progression. Next, to understand the functional impacts of the MALT1-dependent secretome, we will perform immune cell chemotaxis and polarization assays using conditioned media from treated and untreated TNBC cell lines. Furthermore, we aim to uncover which specific cytokines are functionally important in promoting cancer progression.

After further validation, we will utilize our MALT1 gene signature and cleaved CYLD antibody to assess the impact of MALT1 activity in affecting clinical outcomes and modulating the tumor microenvironment in clinical specimens.

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