Examining the pro-tumorigenic functions of the mitogen-activated protein kinase interacting protein kinases 1 and 2 (MNK1/2)-eukaryotic initiation factor 4E (eIF4E) pathway in breast cancer

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Abstract

Cancer metastasis is a multi-step process. This thesis focuses on two critical steps of the metastatic process in breast cancer: (1) the transition from non-invasive to invasive disease, and (2) immune evasion that supports metastasis. MAP kinase-interacting serine/threonine-protein kinases 1 and 2 (MNK1/2) are ubiquitously expressed serine/threonine kinases downstream of the ERK1/2 and p38 pathways. Hyper-activation of MNK1/2 due to external stimuli such as growth factors or stress signaling can enhance tumor cell invasion and metastasis in multiple solid malignancies including breast cancer, but the molecular mechanisms underpinning these effects of MNK1/2 remain largely unknown. Using in vitro and in vivo models, we demonstrated a novel function of MNK1, where the kinase drives the transition of breast ductal carcinoma in situ (DCIS), a non-invasive "stage 0" disease, into invasive ductal carcinoma (IDC). At the mechanistic level, MNK1 upregulates the expression of NODAL, a pro-tumorigenic morphogen, to support a partial epithelial-mesenchymal transition (EMT), and to maintain cancer stemness properties that increase the risk of tumor relapse and metastasis.

The best characterized function of MNK1/2 is to phosphorylate the eukaryotic translation initiation factor 4E (eIF4E) at Ser209. Multiple pro-oncogenic pathways converge on the MNK1/2-eIF4E axis, which serves as a critical regulator of the translation of mRNAs that encode for proteins that promote cell invasion. The MNK1/2-eIF4E axis has been recently reported to reinforce the survival of pro-metastatic neutrophils in breast cancer. However, our knowledge of how aberrant mRNA translation regulates breast tumor immunity remains limited. We chose to study post-partum breast cancer (PPBC), an aggressive subtype of breast cancer, as it has been characterized by robust immune cell suppression, to examine how the MNK1/2-eIF4E axis shapes pro-tumorigenic immunity during metastasis. We demonstrate that eIF4E phosphorylation is important to support tumor immune evasion for PPBC metastasis. Using a mouse model that is devoid of eIF4E phosphorylation, and inhibitors of MNK1/2, we show that type 2 innate lymphoid cell (ILC2) function, myeloid-derived suppressor cells (MDSCs) accumulation, and cytotoxic T cell exclusion, are dependent on the MNK1/2-eIF4E axis. Immune targeted therapies have not shown great promise in breast cancer. We showed that the inhibition of MNK1/2 using the inhibitor SEL201, can work in concert with anti-PD1 immune targeted therapy to inhibit PPBC metastasis. Thus, we show the possibility of enhancing the efficacy of immunotherapy by using a small molecule inhibitor that blocks mRNA translation.

RÉSUMÉ

Le métastase du cancer est un processus a plusieurs étapes. Cette thèse est centrée sur deux étapes critiques du processus de métastase dans le cancer du sein : (1) la transition du cancer non-invasif en cancer invasif, et (2) une évasion du système immunitaire qui supporte la progression vers un stade métastatique. MAP kinase-interacting serine/threonine-protein kinases 1 and 2 (MNK1/2) sont des kinases sérine/thréonine en aval du processus biologique de ERK1/2 et p38. L'hyper-activation de MNK1/2 causée par des stimuli externes comme des facteurs de croissance ou des signaux de stress peuvent augmenter l'invasion cellulaire et les métastases dans plusieurs cancers solides comme le cancer du sein. Malgré cela, le mécanisme moléculaire derrière ces effets causés par MNK1/2 sont encore largement inconnues. En utilisant des modèles *in vitro* et *in vivo*, nous avons démontré une nouvelle fonction de MNK1; la kinase facilite la transition du cancer du sein canalaire in situ (DCIS), un « stage 0 », non-invasif de la maladie, en cancer du sein canalaire invasif (IDC). Au niveau du mécanisme moléculaire, MNK1 augmente l'expression de NODAL, un morphogène pro-tumorigène, pour supporter un transition épithéliale-mesenchymal (EMT) partielle et maintenir le charactère souche du cancer qui augmente le risque de rechute et metastase.

La fonction la plus étudiée de MNK1/2 est sa capacité à phosphoryler le facteur d'initiation eucaryote de traduction de 4E (eIF4E) à Ser209. Plusieurs processus biologiques proto-oncogènes converge sur l'axe MNK1/2-eIF4E, qui joue un rôle critique dans la translation de mRNAs qui codent pour des protéines qui poussent l'invasion cellulaire. Il a récemment été démontré que l'axe MNK1/2-eIF4E renforce la survie de neutrophiles pro-métastases dans le cancer du sein. Cependant, notre connaissance de cette fonction est encore très limitée. Nous avons choisi d'étudier le cancer du sein post-partum (PPBC), un sous-type très agressif du cancer du sein, notamment parce que celui-ci est caractérisé par une diminution robuste de cellules immunitaires. Nous voulons de ce fait, examiner comment l'axe MNK1/2-eIF4E agit sur l'immunité pro-tumorigène durant l'étape métastatique. Nous démontrons que la phosphorylation de eIF4E est importante dans le support de l'évasion immunitaire dans les métastases du PPBC. En utilisant un modèle murin déficient de la phosphorylation d'eIF4E et en utilisant des inhibiteurs de MNK1/2, nous montrons que l'activation des cellules lymphoïdes innées de type 2 (ILC2), l'accumulation des myeloid-derived suppressor cells (MDSCs) et l'exclusion des cellules T cytotoxiques sont dépendantes de l'axe MNK1/2-eIF4E. La thérapie immunitaire

ciblée n'a pas démontré beaucoup de résultats positifs dans le cancer du sein. Nous avons montré que l'inhibition de MNK1/2 en utilisant l'inhibiteur SEL201, peut travailler en parallèle avec la thérapie immunitaire ciblée anti-PD1 pour diminuer les métastases dans le PPBC. Ainsi nous montrons la possibilité d'améliorer les effets de l'immunothérapie en utilisant un inhibiteur qui agit sur le blocage de la translation des mRNAs.

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First and foremost, I would like to thank my supervisors, Drs. Wilson H. Miller Jr. and Sonia V. del Rincon for providing an amazing platform to pursue my dream as a scientist. Specifically, I am forever grateful for Dr. Miller for his acceptance of me as his PhD student and his belief that I would grow into a successful scientist. I deeply appreciate his constructive critiques of my work, funding my project and his drive to translate laboratory findings into the clinic. I have enormous gratitude towards Dr. Sonia del Rincon, who has always been understanding and supportive all throughout my candidature, especially when I was in difficulty. My successes are deeply rooted in her guidance.

It is also a great honor and privilege to be a member of an amazing team where everybody collaborates and has each other's back. I would like to thank my best friends Fan Huang, William Yang and Dr. Yao Zhan, who have always stood by my side, offered support, motivate and inspire me during this wonderful journey as a PhD student. I also thank Sai Sakktee Krisna, Samuel Preston and Dr. Margarita Bartish for their assistance with countless animal experiments and their friendship. Much of the daily things that go unrecognized and the scientific support are accredited to Christophe Goncalves, who is the guru of IHC/IF staining in our team. I appreciate his help, humor and support for countless troubleshooting experiments.

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Contributions to knowledge and elements of original scholarship

1. MNK1 promotes the transition of breast ductal carcinoma in situ (DCIS) into invasive ductal carcinoma.
2. MNK1 regulates the expression of a pro-invasive morphogen named NODAL.
3. MNK1/NODAL axis is important to maintain the cancer stem cell features in DCIS cells.
4. MNK1/NODAL axis drives the DCIS to IDC transition through promoting a partial epithelial-mesenchymal transition (EMT), mainly by regulating the expression of Vimentin.
5. Inhibition of MNK1/2 activity by SEL201 partially suppresses the DCIS to IDC transition.
6. Phospho-eIF4E in the tumor microenvironment (TME) promotes metastasis of post-partum breast cancer (PPBC).
7. Phospho-eIF4E in the TME facilitates MDSCs accumulation and cytotoxic T cell exclusion from the lung, the most prominent metastatic site in our PPBC model.
8. Phospho-eIF4E regulates fibroblasts-derived IL-33, to support PPBC immune evasion.
9. The combination of SEL201 and anti-PD1 antibody inhibits PPBC lung metastasis.

Contribution of authors

The present thesis consists in large part of manuscripts published or in preparation for submission to peer reviewed journals, of which I am the first author. Their citations are included here, with permission where required, and are distributed in the text as follows:

Chapter 1

Guo Q*, Huang F*, Goncalves C, Del Rincon SV, and Miller WH Jr. Translation of cancer immunotherapy from the bench to the bedside. *Advances in cancer research.* 2019;143:1-62. (*Equal contributions)

Chapter 2

Guo Q, Li VZ, Nichol JN, Huang F, Yang W, Preston SEJ, Talat Z, Lefrère H, Yu H, Zhang G, Basik M, Goncalves C, Zhan Y, Plourde D, Su J, Torres J, Marques M, Al Habyan S, Bijian K, Amant F, Witcher M, Behbod F, McCaffrey L, Alaoui-Jamali MA, Giannakopoulos NV, Brackstone M, Postovit LM, del Rincón SV, Miller WH Jr. MNK1/NODAL signaling promotes invasive progression of breast ductal carcinoma in situ. *Cancer Research.* 2019; 79(7):1646-57.

Chapter 3

Guo Q, Bartish M, Krisna SS, Huang F, Li VZ, Preston SEJ, Emond A, Lefrère H, Duerr C, Gui Y, Goncalves C, Plourde D, Su J, Hewgill S, Yang W, Khoury E, Zhan Y, Narykina V, Basik M, Amant F, Lapointe R, Fritz JH, del Rincón SV, Miller WH Jr. phospho-eIF4E/IL33 drives immune evasion and lung metastasis in postpartum breast cancer (PPBC) *(Manuscript in preparation)*

Publications that include work performed by Guo Q, but not included in the presentation of this dissertation are listed below:

Yang W*, Khoury E*, Guo Q, Emond A, Huang F, Gonçalves C, Zhan Y, Plourde D, Nichol JN, Dahabieh MS, del Rincón SV. Miller WH Jr. MNK1 signaling induces an ANGPTL4-mediated gene signature to drive melanoma progression *(Manuscript in review - Oncogene)* (*Equal contributions)

Zhan Y, Guo J, Yang W, Goncalves C, Rzymski T, Dreas A, Zylkiewicz E, Mikulski M, Brzozka K, Golas A, Kong Y, Ma M, Huang F, Huor B, Guo Q, da Silva SD, Torres J, Cai Y, Topisirovic I, Su J, Bijian K, Alaoui-Jamali MA, Huang S, Journe F, Ghanem GE, Miller WH Jr., del Rincón SV. MNK1/2 inhibition limits oncogenicity and metastasis of KIT-mutant melanoma. *The Journal of clinical investigation.* 2017;127(11):4179-92.

Robichaud N, Hsu BE, Istomine R, Alvarez F, Blagih J, Ma EH, Morales SV, Dai DL, Li G, Souleimanova M, Guo Q, del Rincón SV, Miller WH Jr., Cajal SRY, Park M, Jones RG, Piccirillo CA, Siegel PM, Sonenberg N. Translational control in the tumor microenvironment promotes lung metastasis: Phosphorylation of eIF4E in neutrophils. *Proceedings of the National Academy of Sciences.* 2018; 115(10): E2202-09.

The manuscripts forming the major part of this thesis included work from several co-authors to whom I owe many thanks. My contributions and those of my co-authors are delineated here.

Chapter 1 is a literature review consisting mainly of a manuscript entitled “Translation of cancer immunotherapy from the bench to the bedside”, which will constitute a chapter of the upcoming book “Immunotherapy of Cancer” for *Advances in Cancer Research*. The original draft were

work of myself and Fan Huang. Christophe Goncalves designed the figures. All authors contributed extensive editorial work on numerous subsequent drafts.

Chapter 2 was published in *Cancer Research* with the title "MNK1/NODAL signaling promotes invasive progression of breast ductal carcinoma *in situ*". I designed, performed and analyzed the experiments presented in all figures. I drafted the manuscript and compiled the figures, with editorial input from all authors. Some replication experiments, including migration and invasion assays, western blots, IHC stainings were performed by Vivian Z. Li. Fan Huang and William Yang assisted multiple *in vivo* experiments and performed *in vitro* assays such as qPCR and western blots. Samuel E.J. Preston, Zahra Talat, Hanne Lefrère, Henry Yu, Christophe Goncalves and Yao Zhan have participated in multiple *in vitro* and *in vivo* experiments. Dany Ploudre, Jie Su and Sarah Al Habyan were involved in mouse colony maintenance. Mark Basik, Muriel Brackstone, Nadia V. Giannakopoulos, Lynne-Marie Postovit, Luke McCaffrey and Michael Witcher provided clinical samples and offered suggestions for manuscript writing. Jessica N. Nichol modified the figures and manuscript. Wilson H. Miller Jr. and Sonia V. del Rincón secured the funding secured funding, supervised the research and edited the manuscript.

Chapter 3 is a manuscript in preparation. I designed, performed and analyzed the experiments presented in all figures. I drafted the manuscript and compiled the figures, with editorial input from all authors. Some replication experiments were performed by Margarita Bartish, Sai Sakktee Krisna, Fan Huang and Claudia Duerr. Additionally, Sai Sakktee Krisna, Claudia Duerr, Shannon Hewgill, and Jörg H. Fritz have performed ILC2 flow cytometry, ILC2 sorting and ex vivo ILC2 experiments, as well as offered suggestions and protocols for ILC2-related experiments. Audrey Emond and Samuel E.J. Preston performed flow cytometry staining and analysis. Other colleagues, including William Yang, Hanne Lefrère, Elie Khoury and Yao Zhan have participated in multiple *in vitro* and *in vivo* experiments. Dany Ploudre and Jie Su were involved in mouse colony maintainence and assisted multiple animal procedures including gavaging and animal surgeries. Christophe Goncalves assisted troubleshooting of multiple IHC and IF stainings, as well as performed the characterization of mammary glan involution. Mark Basik, Frédéric Amant and Réjean Lapointe provided clinical samples and offered suggestions for manuscript writing. Wilson H. Miller Jr. and Sonia V. del Rincón secured funding, supervised the research and edited the manuscript.

Abbreviations

eIF4E binding proteins (4EBPs)

1,4,5-trisphosphate (IP3)

Aldehyde dehydrogenase (ALDH)

All-trans retinoic acid (ATRA)

Antibody-dependent cellular cytotoxicity (ADCC)

Antigen presenting cells (APCs)

Atypical ductal hyperplasia (ADH)

B and T lymphocyte attenuator 4 (BTLA4)

Basement membrane (BM)

B-cell CLL/lymphoma 9 (Bcl-9)

BODIPY™-aminoacetaldehyde (BAAA)

Breast cancer type 1/2 susceptibility protein (BRCA1/2)

Cytotoxic T-lymphocyte-associated protein-4 (CTLA-4)

Cancer-associated fibroblasts (CAFs)

Cancer stem cell (CSC)

CHEK2

chronic myeloid leukemia (CML)

C-C motif receptor 8 (CCR-8)

Cluster of differentiation 10 (CD10)

Cluster of differentiation 11b (CD11b)

Cluster of differentiation 40 (CD40)

Cluster of differentiation 44 (CD44)

Cluster of differentiation 133 (CD133)

C-C motif chemokine ligand 2 (CCL2)

C-C motif chemokine ligand 5 (CCL5)

C-C motif chemokine ligand 17 (CCL17)

C-C motif chemokine ligand 21 (CCL21)

C-X-C motif chemokine ligand 1 (CXCL1)

C-X-C motif chemokine ligand 2 (CXCL2)

C-X-C motif chemokine ligand 2 (CXCL12)

C-X-C motif chemokine ligand 14 (CXCL14)

C-C motif receptor 7 (CCR7)

C-X-C motif receptor 4 (CXCR4)

Cyclic GMP-AMP synthase (cGAS)

Cyclooxygenase-2 (COX2)

Cytokeratin (CK)

Cytosolic phospholipase A2 (cPLA2)

Damage-associated molecular patterns (DAMPs)

Dendritic cells (DCs)

Distal-less Homeobox 4 (DLX4)

Ductal carcinoma in situ (DCIS)

Endoplasmic reticulum (ER)

Engrailed Homeobox 1 (EN1)

Epidermal growth factor (EGF)

Epithelial-to-mesenchymal transition (EMT)

Estrogen receptor (ER)

Eukaryotic translation initiation factor 3C (eIF3C)

Eukaryotic translation initiation factor 4A (eIF4A)

Eukaryotic translation initiation factor 4E (eIF4E)

Eukaryotic translation initiation factor 4F (eIF4F)

Eukaryotic translation initiation factor 4G (eIF4G)

Extracellular signal–regulated kinases-1/2 (ERK-1/2)

Extracellular matrix (ECM)

Fibroblast-activation protein (FAP),

Forkhead Box M1 (FoxM1)

Glioma-Associated Oncogene Homolog 1 (Gli1)

Glucocorticoid-induced TNFR-related protein (GITR)

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Granulocyte-colony stimulating factor (G-CSF)

Granulocyte-macrophage colony-stimulating factor (GM-CSF)

Growth hormone (GH)

Heparan sulfate (HS)

Hepatocellular carcinoma (HCC)

Hematoxylin and eosin (H&E)

Hypoxia-inducible factor 1- α (HIF-1α)

Homeobox B13 (HOXB13)

Hormone replacement therapy (HRT)

Hyaluronic acid (HA)

Human epidermal growth factor receptor 2 (HER2)

Imaging mass cytometry (IMC)

Immunohistochemistry (IHC)

Immunofluorescence (IF)

Indoleamine-2,3-dioxygenase (IDO)

Inducible nitric oxide synthase (iNOS)

Inducible T-cell co-stimulator (ICOS)

Innate lymphoid cells (ILCs)

Group 1 ILCs (ILC1)

Group 2 ILCs (ILC2)

Group 3 ILCs (ILC3)

Insulin-like growth factor-1 (IGF-1)

Interferon-γ (IFN-γ)

Interleukin-1β (IL-1β)

Interleukin-4 (IL-4)

Interleukin-5 (IL-5)

Interleukin-6 (IL-6)

Interleukin-7 (IL-7)

Interleukin-10 (IL-10)

Interleukin-13 (IL-13)

Interleukin-33 (IL-33)

Interleukin-4 receptor (IL-4R)

Interleukin-10 receptor (IL-10R)

Invasive ductal carcinoma (IDC)

Invasive lobular carcinoma (ILC)

Knockout (KO)

Lobular carcinoma in situ (LCIS)

lymphoid tissue-inducer (LTi)

Lymphocyte antigen 6 complex locus G6D (Ly6G)

Lymphocyte-activation gene 3 (LAG3)

Major histocompatibility complex class I (MHC-I)

Mammary gland (MG)

Mitogen-activated protein kinase (MAPK)

Mitogen-activated protein kinase-interacting serine/threonine protein kinase 1/2 (MNK1/2),

Matrix metalloproteinases (MMPs)

Macrophage colony-stimulating factor (M-CSF)

Macrophage colony-stimulating factor receptor (M-CSFR)

Mechanistic target of rapamycin (mTOR)

Mechanistic target of rapamycin complex 1 (mTORC1)

Monocytic-MDSCs (M-MDSCs)

Mouse mammary tumor virus (MMTV)

Myeloid-derived suppressor cells (MDSCs)

Natural killer cells (NK cells)

Nitric oxide (NO)

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)

Nuclear export signal (NES)

Nucleus localization signal (NLS)

Pancreatic satellite cells (PSCs)

Pancreatic ductal adenocarcinoma (PDAC)

Polyomavirus middle T-antigen (PyMT)

Phospholipase C-β (PLC-β)

Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI-3K)

PTB (polypyrimidine tract-binding protein)-associated splicing factor (PSF)

Programmed cell death protein 1 (PD-1)

Programmed death-ligand 1 (PD-L1)

Prostaglandin E2 (PGE2),

Protein kinase C-ζ (PKC-ζ)

Polymorphonuclear-MDSCs (PMN-MDSCs)

Poly (ADP-ribose) polymerase (PARP)

Post-partum breast cancer (PPBC)

Progesterone receptor (PR)

Type 2A serine/threonine protein phosphatase (PP2A)

Retinoic acid metabolism blocking agents (RAMBA)

Homologues of the Drosophila protein, mothers against decapentaplegic (Mad) and the Caenorhabditis elegans protein Sma (Smad)

Severe combined immunodeficiency (SCID)

Serine/arginine-rich splicing factor (SRSF1)

α-Smooth muscle actin (α-SMA)

Sonic hedgehog (SHH)

Signal transducer and activator of transcription 1 (STAT-1)

Signal transducer and activator of transcription 3 (STAT-3)

Stimulator of interferon genes (STING)

SRY-Box 11 (SOX11)

Suppressor of tumorigenicity 2 (ST2)

SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily E, Member 1 (SMARCE1)

T-Box 15 (TBX15)

T-cell immunoglobulin and mucin-domain containing-3 (TIM-3)

Thymic stromal lymphopoietin (TSLP)

Folicular helper T cells (Tfh)

T helper cells (Th cells)

Type 1 T helper cells (Th1 cells)

Type 2 T helper cells (Th2 cells)

Type 9 T helper cells (Th9 cells)

Type 22 T helper cells (Th22 cells)

Regulatory T cells (T_{reg} cells)

T cell immunoreceptor with Ig and ITIM domains (TIGIT)

Thymic stromal lymphopoietin (TSLP)

TNF alpha-induced protein 8 like 3 (TNFAIP8L3)

Tissue inhibitor of metalloproteinases (TIMPs)

Transforming growth factor β (TGF-β)

Transported associated with antigen processing-1/2 (TAP-1/2)

Tumor microenvironment (TME)

Tumor infiltrating lymphocytes (TILs)

Tumor necrosis factor-α (TNFα)

Triple-negative breast cancer (TNBC)

Tryptophan 2,3- dioxygenase (TDO)

Vascular endothelial growth factor-C (VEGF-C)

Vascular endothelial growth factor-D (VEGF-D)

Vascular endothelial growth factor receptor-2/3 (VEGFR-2/3)

V-domain Ig suppressor of T cell activation (VISTA)

Whey acidic protein promoter (WAP)

Wild-type (WT)

Chapter 1. A comprehensive review of the relevant literature

Part of this chapter is adapted from the following review article:

Chapter 1. Translation of cancer immunotherapy from bench to bedside. Volume 143. Cancer Immunotherapy. Guo et al, *Advances in Cancer Research* 2019

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1.1. Breast anatomy and physiology

1.1.1 Mammary gland structure and cellular components

Mammary glands are highly specialized sweat glands that are responsible for milk production in females, and are a part of the mucosal immune system (1). Each breast consists of 15-20 functional units of milk production called lobes. The lobes are connected by 6-8 ducts which carry the milk to the nipple. Adipocytes are a major class of supporting cells in between the lobes. Over 75% of the breast lymphatic drainage is through axillary lymph nodes, which contain 20-30 nodes in the axillary region (1).

The breast tissues are composed of multiple cell types that collaborate to maintain homeostasis and function of the gland. The major cell types include epithelial and myoepithelial cells, adipocytes, fibroblasts, lymphatic and blood vascular cells, as well as immune cells (2, 3). Epithelial and myoepithelial cells form a structure called the mammary bilayer, in which epithelial cells line the ducts and myoepithelial cells are found near the basement membrane (BM) (4). Luminal epithelial cells express cytokeratins 8 and 18, while myoepithelial cells express cytokeratins 5 and 14 as well as smooth muscle actin (SMA) that contribute to their contractility (4). During pregnancy, the luminal epithelium rapidly proliferates to form alveoli that produce milk.

Mammary adipocytes, apart from providing physical support of the breast structure, are also considered endocrine cells that regulate mammary epithelial cell growth (4). Additionally, adipocytes serve as a reservoir of energy for milk production, as lipid content reduction is observed during lactation (summarized by Inman et al. (4)). Finally, mammary fibroblasts are a population of multi-functional cells that synthesize the extracellular matrix (ECM) and provide functional support to the mammary epithelium (summarized by Inman et al. (4)).

1.1.2 Lactation and involution

A mammary gland in the non-pregnant and non-lactating status contains a network of epithelial cells that drain into the main lactiferous ducts (5) (Figure 1.1). To become a secretory organ for milk production, the mammary gland passes through discrete stages of development. First, the mammary gland undergoes extensive ductal growth and elongation into the mammary fat pad, as well as moderate secondary and tertiary branching during puberty. This process is mainly driven by growth hormone (GH), estrogen, progesterone, epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1)(6).

In non-pregnant individuals, the epithelium proliferates and undergoes apoptosis during each menstrual cycle (4). During pregnancy, in contrast, secondary and tertiary branching is augmented, and the alveolar epithelium rapidly proliferates in response to circulating hormones (4, 6). Progesterone and prolactin are two important hormones that promote the formation of lobular-alveolar units (6). Parturition-induced withdrawal of progesterone, together with episodically produced prolactin, stimulate milk synthesis from the lobular-alveolar units (6). Specifically, mammary epithelial cells produce milk in a finely orchestrated process of endocytosis (uptake of blood-borne molecules at its basal side), intracellular trafficking, and exocytosis (release of milk at its apical side)(6). Infantile suckling causes the release of oxytocin, which is produced by the paraventricular nucleus of the hypothalamus and released by the posterior pituitary. Oxytocin stimulates the contraction of myoepithelial cells and the subsequent discharge of milk through the ductal tree to the nipple (6).

Weaning-induced involution of mammary glands is a normal physiological process, where milk stasis induces massive mammary epithelial death and subsequent tissue remodeling back to a non-lactational status. The progression of mammary gland involution occurs in two distinct stages. In mice, the first reversible stage occurs during day 1 and 2 post-weaning, where lactation can be re-induced by suckling (7). The second irreversible stage starts on approximately day 3 post-weaning. The involution process is complete around day 10, with few alveoli remaining (7). The involution time frame is more heterogeneous in humans: on average, massive mammary epithelial cell death is observed within 12 months postpartum, and by 18 months postpartum, the lobular area and cellular components are indistinguishable from nulliparous cases (8). Interestingly, although the most significant increase in immune cell infiltration peaks around 1-6 weeks postpartum and then decreases dramatically after 1-2 years, it takes up to 10 years for the immune cell infiltrate of the breast to return to a basal level (8).

The tissue remodeling proceeds in a step-wise manner. First, the milk-producing mammary epithelial cells are eliminated by programmed cell death, leading to the collapse of functional alveolar structures. Second, the cell debris and remaining milk components are eliminated by phagocytosis. In the meanwhile, the extracellular matrix (ECM) and lymphatic vasculature are remodeled and the stroma is repopulated with adipocytes. A variety of stromal cells and immune cells, such as fibroblasts, neutrophils and macrophages, participate in the process of mammary

gland involution (9-12). This process will be further discussed in Section 4 in the context of how the involuting mammary gland promotes breast cancer metastasis.

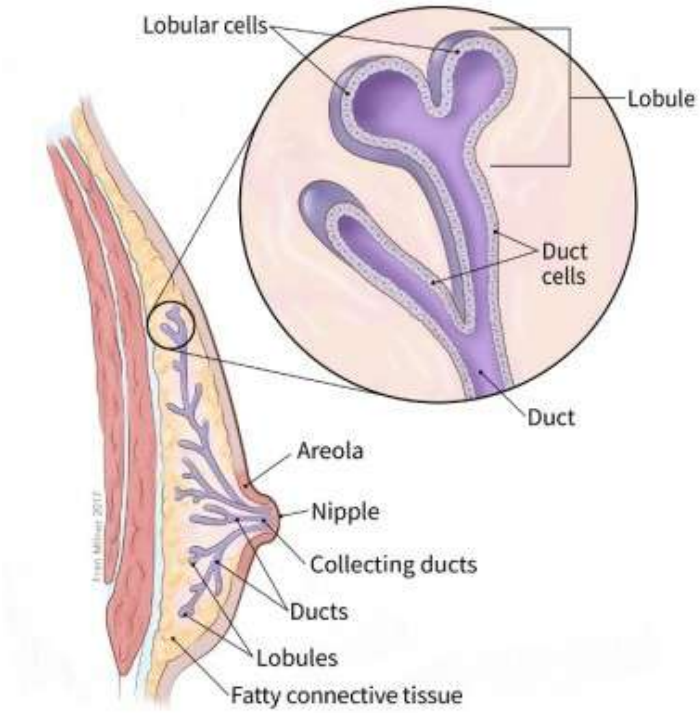

Figure 1.1 Breast anatomy image ©American Cancer Society 2017. Used with permission.

1.2. General introduction to breast cancer

1.2.1 Epidemiology and breast cancer risk factors

Breast cancer is the most common malignancy affecting women. It is a leading cause of cancer death in women (13, 14). Recognized risk factors for breast cancer include age, ethnicity, exposure to estrogen and radiation, family history and genetic predispositions, as well as life-style related factors such as obesity, alcohol consumption and lack of exercise (15). Additionally, the following endocrine and lifestyle-associated factors also contribute to breast tumorigenesis: early menarche and late menopause, nulliparous or delayed first pregnancy, short breastfeeding duration or lack of breastfeeding, consumption of oral contraceptives, and hormone replacement therapy (HRT) with estrogens and progesterone (15). Such environmental and genetic risk factors are summarized in Table 1.1.

Table 1.1 Risk factors (except age) associated with invasive breast cancer

(Adapted from Textbook of Medical Oncology, Chapter 5)

Major risk factors increasing risk >2 times (in descending order)

• BRCA1/2 mutations

• CHEK2 (also known as CHK2): (CHEK2(*)1100delC, a truncating variant that abrogates the kinase activity) twofold increase of breast cancer risk in women and a tenfold increase of risk in men

• Premenopausal breast cancer in mother and sister

• In situ cancer—ductal or lobular or atypical hyperplasia in breast biopsy

• Premenopausal breast cancer in mother or sister; bilateral breast cancer in first relative

• Hyperplasia without atypia in breast biopsy

Minor risk indicators or factors increasing risk ≤2 times

• Postmenopausal breast cancer in first-degree relative

• Obesity in women above 50 years

• Excess radiation to the chest wall or breast in history

• Alcohol consumption

• Nulliparous or delayed first pregnancy

• No breast feeding or short duration breast feeding of children

• Prolonged use of estrogens (contraceptive and/or hormone replacement)

1.2.2 Classifications of breast cancer

Breast cancer is a cluster of heterogeneous diseases classified by their histological characteristics and genomic signatures. Histologically, breast cancer is sub-grouped into the following categories:

1.2.2.1 Ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC)

DCIS, which accounts for approximately 20% of all newly diagnosed breast cancer cases, is often described as “Stage 0” disease due to its non-invasive nature (15). DCIS is characterized by the malignant proliferation of mammary ductal epithelial cells, which remain confined to the

layer of myoepithelial cells and the basement membrane (15). In contrast, a classic feature of IDC is the disruption of the myoepithelial cell layer and basement membrane, enabling malignant cells to infiltrate into the surrounding stroma (Figure 1.2) (15).

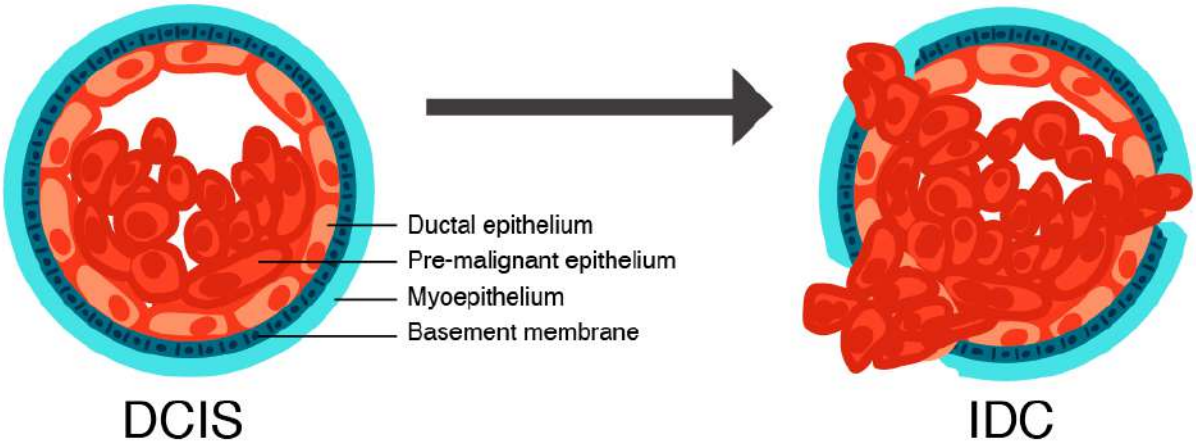

Figure 1.2 DCIS-IDC transition

There has been a significant increase in the diagnosis of DCIS over the past 40 years, due to the implementation of mammography (15, 16). Mastectomy used to be the standard treatment for DCIS. However, lumpectomy has now been adopted as the treatment of choice for patients with small lesions that are detected during screening (15, 16). Whole breast radiation following lumpectomy reduces the disease recurrence by approximately 50% (17), with half of these recurrences being DCIS and half being IDC (17). Several factors affect local recurrence risk: palpable mass, larger tumor size, high grade, close or involved margins and age < 50 years (17). Clean surgical margins ≥ 2 mm are associated with a lower risk of ipsilateral tumor recurrence in comparison to narrower negative margins (17). If the physicians view the individual relapse risk as low, resection alone may be offered (17). Endocrine therapy, such as tamoxifen or aromatase inhibitors, are offered especially for patients whose tissue is positive for estrogen receptor-alpha expression (17). It is important to note that although adjuvant radiotherapy and hormonal therapy can decrease the risk of DCIS' invasive recurrence, they don't increase the overall survival rate (16).

1.2.2.2 Lobular carcinoma in situ (LCIS) and invasive lobular carcinoma (ILC)

LCIS is defined as a type of cancer wherein pre-malignant breast epithelial cells are confined in one or more lobules without breaching the basement membrane (15). It is considered benign and was thus recently removed from the TNM staging system, which estimates the primary tumor size (T), lymph node metastasis (N), and distal organ metastasis (M) (Table 2) (18). The lesions usually occur in premenopausal women, and are multi-focal in 50–70% cases (15). LCIS lesions are rarely detected by physical examination by the patient or clinician, and diagnosis of LCIS is often made upon occasional removal of breast tissue (15). Similar to DCIS, LCIS may also be a non-obligate precursor to ILC, as many LCIS lesions do not transit into ILC (19).

Table 1.2 Breast cancer staging

Primary tumor (T)	**Regional lymph node (N)**	**Remote metastasis**
• **T_0** No detectable tumor • **T_{is}** Carcinoma in situ • **T_1** <2cm • **T_2** 2-5cm • **T_3** >5cm • **T_4** Tumor of any size, extending though chest wall or skin	• **N_0** No lymph node metastasis • **N_1** Metastasis in movable ipsilateral axillary lymph nodes • **N_2** Metastasis in ipsilateral axillary lymph nodes or in internal mammary nodes • **N_3** Metastasis in axillary lymph nodes and in ipsilateral infraclavicular, supraclavicular, or ipsilateral internal mammary lymph nodes	• **M_0** No metastasis • **M_1** Presence of distal metastasis

Adapted from 2018 Cancer Staging Manual, American Joint Committee on Cancer.

1.2.2.3 Other invasive breast carcinomas

Besides IDC and ILC, which are the two most common types of invasive breast cancer, other histological subtypes include medullary carcinoma, mucinous (colloid) adenocarcinoma, comedocarcinoma, Paget's disease, papillary carcinoma, tubular adenocarcinoma, inflammatory carcinoma (15). To estimate the prognosis and to guide disease management, invasive breast

carcinomas are first assessed by TNM staging (Table 2) (17, 18). Subsequently, the ER/PR and HER2 status are examined (17). Lumpectomy or mastectomy is performed, followed by radiation, chemotherapy and/or hormone therapy depending on the staging and hormone receptor status. The details of management have been extensively reviewed elsewhere (17, 18).

1.2.2.4 Biological subtypes of breast cancer

Traditionally, patient prognosis and treatments were based on immunohistochemistry markers including estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), together with other pathological features such as tumor size, tumor grade and lymph node involvement (20). However, with the advancement of gene expression profiling in the past decade, breast cancers are classified into the following five subtypes based on their distinctive genetic profiling: luminal A, luminal B, HER2 positive, basal-like, claudin-low, and normal-like tumors (20) (Table 3). Each category does not only reflect expected gene expression patterns but also provides excellent prognostic information.

Luminal breast cancers were defined as tumors with a gene expression pattern similar to the mammary luminal epithelium. Luminal A breast cancer is ER and/or PR positive, HER2 negative, and has low Ki-67 levels (21). Luminal A cancers are generally low-grade tumors and have the best prognosis among all the molecular subtypes (20). Luminal B breast cancer is characterized as ER and/or PR positive, either HER2 positive or negative, and with high Ki-67 expression (20, 21). Given the invasive behavior of luminal B subtype, its prognosis is similar to the non-luminal subtypes such as HER2-positive and basal-like breast cancers (22).

Normal-like breast cancer is similar to luminal A disease, which is also ER and/or PR positive and HER2 negative with low Ki67 levels (21). Normal-like breast cancer has the second best prognosis among all subtypes (20).

HER2-positive breast cancer is defined as ER and PR negative and HER2 positive. Although such breast cancers are more proliferative (high Ki-67 expression) and aggressive compared to Luminal A and B subtypes, they are often successfully treated with HER2 blocking agents, such as trastuzumab, pertuzumab, ado-trastuzumab emtansine, and lapatinib (20, 23, 24).

Triple-negative/basal-like breast cancer (TNBC) is defined as ER, PR and HER2 negative. The majority of TNBC are typically basal-like breast cancers, featuring a unique expression of high-molecular weight (basal) cytokeratins 5, 6, 14 or 17 that are typically expressed in the basal epithelial layer of the skin and airways (25, 26).

Table 1.3 Biological subtypes of breast cancer

	Molecular characteristics				Histological grade	Other features
	ER	**PR**	**HER2**	**Other markers**		
Luminal A	+	+	-	CK8/18^{+} Ki67^{Low}	Low	Best prognosis Low recurrence rates
Luminal B	+	+	+/-	CK12/18^{+} Ki67^{High} if HER2-	Intermediate/ High	
HER2-positive	-	-	+	CK5/6$^{+/-}$ CK8/18$^{+/-}$ Ki67^{High}	Intermediate/ High	Associated with younger age of diagnosis
Basal-like	-	-	-	CK5/6/14/17^{+}, EGFR^{+} Ki67^{High}	High	
Claudin-low	-	-	-	Claudin 3/4/7^{Low} E-cadherin^{Low} Ki67^{High}	High	Intense lymphocytic infiltration
Normal-like	-	-		EGFR- CK5-	Variable	With adipocytes gene signatures

Besides basal-like breast cancers, other categories of TNBC exist. One example is the claudin-low breast cancer, which is a subtype of TNBC (26-28). Claudin-low tumors are not only ER, PR and HER2 negative, but they also typically express low levels of claudins 3, 4, or 7 and E-Cadherin, which are important cell-cell junction molecules which can limit cell motility (26). Without these key junction proteins, claudin-low breast cancers are characterized by high levels of mesenchymal markers, such as vimentin and high histological grade, indicating their highly invasive nature (26, 28, 29). Additionally, claudin-low lesions almost always have a young age

of tumor onset, intense immune cell infiltration, higher tumor grade, larger tumor size, and a restricted tumor margin (26). Patients with claudin-low tumors have a worse overall survival when compared to patients with luminal A type of breast cancer (26). Interestingly, however, claudin-low tumors were associated with a low local recurrence rate following breast-conserving therapy (26).

Currently, TNBC remains a clinical challenge, as its risk to metastasize is high and there are no effective targeted therapies against metastatic TNBC (20, 30). Various experimental therapies have been explored, and poly (ADP-ribose) polymerase (PARP) inhibitors have shown promising effects against TNBC. Clinical investigations are ongoing to examine if PARP inhibitors can sensitize TNBC to immune checkpoint blockade therapies (31).

1.3 Regulation of the DCIS-IDC transition

Previous studies have provided evidence that DCIS lesions are heterogeneous (32). Not all DCIS lesions progress into IDC. Indeed, the progression from DCIS to IDC is a critical step in breast tumorigenesis that remains poorly understood. In summary, DCIS-IDC conversion is characterized by the following aspects:

1.3.1 Degradation of the basement membrane and ECM remodeling during the DCIS-IDC conversion

The DCIS basement membrane (BM) is a structured ECM layer mainly composed of collagen IV and laminins. The proteolytic degradation of DCIS BM is mediated by proteolytic enzymes including matrix metalloproteinases (MMPs) and cathepsins. Multiple MMPs, such as MMP1, MMP2, MMP9, MMP13, MMP14 (also known as MT1-MMP) and MMP26 (33-38), are all involved in BM degradation during the transition from DCIS to IDC. Upregulation of MMPs during the progression of DCIS to IDC is tightly controlled by several master regulators of transcription, including SMARCE1 (38), p63 (37), SOX11 (39, 40), HOXB13(39), Engrailed Homeobox 1 (EN1) (39), Distal-less Homeobox 4 (DLX4), TBX15 (39) and FoxM1 (35). Additionally, a link between the upregulation of cathepsins and the DCIS-IDC transition has also been reported. For example, cathepsin-D expression is increased in IDC compared to DCIS (41).

Several important polysaccharides in the ECM, such as hyaluronic acid (HA) and heparan sulfate (HS), also serve as inhibitors to prevent tumor invasion. Increased expression of their corresponding degrading enzymes, including hyaluronidase, heparanase-1 and heparan

endosulfatase-2, are observed in IDC compared to DCIS, and may also contribute to the transition from DCIS to invasive disease (34, 42, 43).

1.3.2 The functions of myoepithelial cells and CAFs in promoting the DCIS-IDC transition:

As described in section 1.1, myoepithelial cells separate the luminal cells from the surrounding BM. In response to oxytocin, myoepithelial cells contract to expel the milk (44). It is hypothesized that myoepithelial cells function as a physical barrier between the pre-malignant epithelium and BM to prevent DCIS invasion through BM (45). Previous studies have provided evidence to support its inhibitory effect during DCIS invasive transition, and have suggested that the apoptosis of myoepithelial cells is a key event during the DCIS-IDC conversion. CD10, a marker of myoepithelial cells, is highly expressed in normal breast tissues (45). Based on CD10 mRNA levels, DCIS patients with high CD10 mRNA expression showed no disease recurrence, while those with low CD10 displayed a higher risk of local relapse (45). Myoepithelial cells also prevent invasion by inhibition of tumor cell invasion and recruitment of lymphatic/blood vessel endothelial cells. For example, myoepithelial cells express protease inhibitors such as tissue inhibitor of metalloproteinases (TIMPs) to degrade MMPs (33), and directly downregulate the expression of multiple MMPs. Moreover, myoepithelial cells produce soluble factors to prevent the migration of tumor cells and endothelial cells. For example, Alpaugh et al. have reported that myoepithelial cells can make use of chymotrypsin to shed off soluble CD44 molecules, which block migration of breast cancer cells and endothelial cells (46). In contrast, pro-invasive functions of myoepithelial cells are also reported. For example, tumor-associated myoepithelial cells can secrete CXCL14 to support the invasion of DCIS cells (47). A subset of myoepithelial cells expressing $\alpha_v\beta_6$ integrin induce MMP9 expression by secreting TGFβ to induce EMT and DCIS-IDC transition (48), and high levels of $\alpha_v\beta_6$ integrin predicts disease recurrence (48).

In contrast to myoepithelial cells, fibroblasts in DCIS lesions seem to play a critical role to aid ductal epithelial cells to gain invasive properties in a step-wise fashion. For example, fibroblasts are activated in an NF-κB pathway-dependent manner, and contribute to the progression from atypical ductal hyperplasia (ADH) to DCIS. More importantly, the expansion of fibroblasts together with the loss of myoepithelial cells contribute to the disruption of BM, which further supports DCIS-IDC transition (49). Additionally, fibroblasts also play an essential role in the ECM remodeling during the transition to IDC. Specifically, fibroblast-derived CCL2 (50), periostin (51), thrombin (52), IL6 (53), and certain isoforms of tenascin-C (54), have all been

reported to be involved in promoting this invasive transition. Future studies of the myoepithelial cells and fibroblasts isolated from DCIS lesions are needed to further understand the functions of these two cell types with opposing functions in DCIS invasive transition.

1.3.3 Dysregulated signaling in the DCIS-IDC transition

A complex dysregulation of signaling networks has been proposed to underpin the invasive transition of DCIS. Although it is not clear which external stimuli are critical for driving DCIS invasion, several cytokines and chemokines, including TGFβ (55), NODAL (56), IL1β (57), IL6 (53, 58, 59), TNFα (57, 58), CCL2 (50) and CCL5 (57), have all been implicated in this process. NODAL, a member of the TGF-beta family morphogen, is often re-expressed in cancer to contribute to tumor cell plasticity for efficient invasion and metastasis. NODAL signaling is regulated at multiple levels, including the control of NODAL gene expression, the cleavage of NODAL precursor to generate mature NODAL, and the expression of NODAL inhibitory proteins. Several transcriptional factors have been identified to regulate NODAL in embryonic development , such as Notch intracellular domain (NICD), Tcf/Lef, SOX and Oct (60, 61). The regulation of NODAL in cancer has started to be revealed. For example, hypoxia induces NODAL expression in melanoma (62). BMP, another TGF-beta family member, can inhibit NODAL expression in seminoma (63). However, it remains unclear how NODAL expression is controlled in breast cancer. NODAL precursor protein, or pro-NODAL, is cleaved by the subtilisin-like proprotein convertases Furin and PACE4 to generate mature NODAL. Both Furin and PACE4 have been implicated in breast cancer invasion and metastasis (64, 65). Lefty1/2 can serve as NODAL antagonists, and are induced by NODAL signaling to generate a negative feedback loop for this pathway (66). Cancer cells lack Lefty1/2 expression, resulting in uncontrolled NODAL signaling to support tumorigenesis (66).

Dysregulation of several well-known pro-tumorigenic signal transduction pathways can enhance the invasiveness of DCIS. For example, elevated Akt and MAPK activation are observed in IDC specimens (67), and increased PI-3K/Akt and MAPK activities are responsible for the disruption of the normal breast epithelial architecture to prompt DCIS cells to acquire invasive characteristics (68-72). Overexpression of PKC-ζ is observed in IDC samples compared to DCIS. Hyperactivation of PKC in the DCIS-IDC conversion can be achieved by increased 1,4,5-trisphosphate (IP3) levels, either via phospholipase C-β (PLC-β) overexpression that yields more

IP3 production or through TNF alpha-induced protein 8 like 3 (TNFAIP8L3)-mediated IP3 accumulation (73, 74).

The sonic hedgehog (SHH) pathway also contributes to the DCIS-IDC transition. In IDC the overexpression of SHH will lead to increased nuclear translocation of the transcriptional regulator Gli1 to ultimately induce a pro-tumorigenic transcriptional program (75). Inductions of Smads, TBX3, NF-κB, HIF-1α and Bcl-9 are also observed in DCIS-IDC transition (55, 70, 76-78).

Such aberrantly activated signaling pathways described above might eventually lead to a partial epithelial-to-mesenchymal transition (EMT). The process of a partial EMT allows DCIS cells to gain plasticity for more effective invasion and metastasis. During a partial EMT, tumor cells gain mesenchymal features to invade through basement membranes into the blood or lymphatic vessels, and then undergo a mesenchymal-to-epithelial transition (MET) to colonize a secondary organ. A partial EMT can be characterized by the elevated expression of mesenchymal markers Snail (79), Slug (78), Twist1 (78), MMP2 (73) and Vimentin (56), while still expressing levels of epithelial markers such as E-Cadherin. A partial EMT during the DCIS-IDC transition is also associated with enhanced stemness of DCIS cells, which provides DCIS cells with self-renewal capacity and plasticity to proliferate and invade. Overexpression of several cancer stem cell markers, including CD133 and ALDH, are known to prompt DCIS cells to invade (72, 74, 80, 81).

1.3.4 Tumor immunity in DCIS-IDC transition and IDC dissemination

Similar to many other cancer types, the DCIS to invasive transition is a process driven by immune evasion, and a strong T-helper 1 (Th1) and cytotoxic T lymphocytic response predicts less invasion and metastasis, thus predicts good prognosis in DCIS patients (82). T cell exhaustion, which is characterized by the expression of two important immune checkpoints TIGIT and PD-L1, may also contribute to DCIS invasion (83). Although B cell infiltration is reported to predict poor survival in a small DCIS cohort (84), future studies with larger cohorts are needed to verify this finding. Additionally, neutrophil recruitment to the TME by IDC-derived IL6 and IL8 serves as an important mechanism of immune evasion to augment IDC dissemination (85). Interestingly, the levels of tumor-infiltrating lymphocytes (TILs) are heterogenous in DCIS. For example, TILs are significantly higher in DCIS with ER- or HER2+ status, TP53 mutation, high copy number changes and comedo necrosis (86). However, the

mechanisms regulating lymphocyte infiltration into DCIS tumor microenvironment (TME) remain unclear.

1.4 Post-partum breast cancer (PPBC): PPBC, defined as breast cancer diagnosed up to 10 years following the latest pregnancy, has approximately a 3-fold increased risk of metastasis and death (87-89), compared to breast cancer occuring outside this window of time. Such an increased risk is restricted to patients receiving a breast cancer diagnosis post-partum, as women with a cancer diagnosis during pregnancy have a favorable prognosis comparable to that of nulliparous patients (89). Although it is unclear why PPBC is associated with a poor prognosis, recent studies have suggested that the microenvironment of the involuting mammary gland provides many favorable factors for any existing pre-malignant cells to survive and become invasive (9, 11, 12, 90). Specifically, the process of mammary gland involution entails removing cellular debris remaining after mammary epithelial apoptosis. Thus it is not surprising that during involution, dendritic cells, macrophages and T cells infiltrate into the mammary gland. Indeed, increased immune cell infiltration has been reported in PPBC, compared to nulliparous breast cancer (11, 12), indicating an important role of dysregulated immunity in PPBC progression. Summarized below are different cell types that contribute to the uniqueness of the PPBC TME (Figure 1.3).

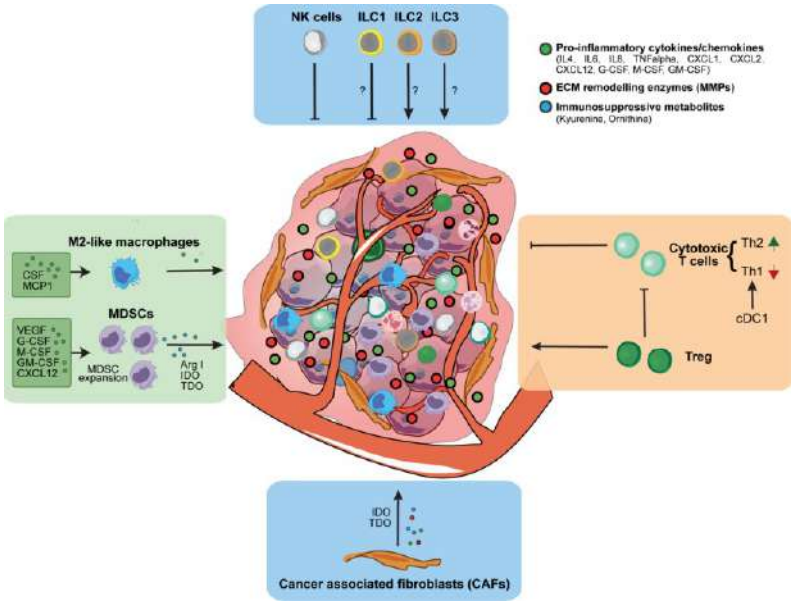

Figure 1.3 Selected cellular components in PPBC TME.

1.4.1 Fibroblasts: Fibroblasts control extracellular matrix deposition during tissue remodeling processes, such as mammary gland involution and breast cancer invasion. Fibroblasts that are isolated during mammary gland involution (involution fibroblasts) have acquired a unique activation state that is characterized by an immunosuppressive gene signature and enhanced extracellular matrix remodeling (91). Although these physiologically activated fibroblasts do not express α-smooth muscle actin (α-SMA), which is a marker for activated fibroblasts under pathological conditions (92), they do contribute to tumor promotion during involution (91). Cancer-associated fibroblasts (CAFs), on the other hand, are pathologically activated fibroblasts. They are a highly proliferative and heterogeneous population of fibroblasts that support the persistent growth and immune evasion of breast cancer (93). Contrary to activated fibroblasts which can be deactivated and eventually disappear in the end stage of involution, CAFs gain the capacity to sustain their proliferative potential and contribute to various aspects of breast cancer metastatic cascade. This includes increasing cancer cell invasion into surrounding lymphatic and blood vessels, protecting the survival of circulating tumor cells, and supporting the colonization of tumor cells at distal organs (93, 94).

It remains unclear if the CAFs in PPBC TME are derived from involution fibroblasts (91). But involution-fibroblasts and CAFs do make use of similar mechanisms to support pro-tumorigenic immunity. Specifically, both types of fibroblasts express multiple pro-inflammatory enzymes and soluble factors. For example, involution-fibroblasts produce a variety of chemokines to induce M2-like macrophage polarization and IL10 production, which further dampens the anti-tumor immunity. Such activated fibroblasts aid the tumor cells to recruit MDSCs at the tumor border, and prevent cytotoxic T cell infiltration (91). Similarly, CAFs are also reported to produce Chitinase 3-like 1, a secretory protein that drives M2 macrophage polarization and tumorigenesis (95). Additionally, both types of fibroblasts express COX2 to produce prostaglandin E2 (PGE2), which mediates the maintenance of breast cancer stem cells, promotes recruitment of MDSCs, and confers resistance to natural killer (NK) cell and cytotoxic T cell-mediated anti-tumor immunity (91, 96-100). As such, by enhancing M2-like macrophage expansion and MDSC recruitment, involution-fibroblasts and CAFs impair cytotoxic T cell and NK cell-based tumor eradication, thus creating favorable conditions for breast tumor progression.

1.4.2. Lymphatic endothelial cells: Involution is accompanied by increased lymphatic growth and remodeling. Unfortunately, the lymphatic system is the most common route for breast cancer

metastasis (1). Human PPBC features a high density of peritumor lymphatic vessels, which partially explains its increased risk of metastasis (101). Increased lymphatic endothelial cell proliferation and neo-lymphangiogenesis are driven by multiple lymphangiogenic factors that are induced during involution, such as VEGF-C, VEGF-D, along with their receptors VEGFR2/3 (101). The COX2 inhibitor celecoxib is a potent inhibitor of lymphangiogenesis during involution, and prevents PPBC metastasis (102).

1.4.3. Macrophages:

In response to pro-inflammatory signals such as IFNγ secreted by NK and T cells in the TME, macrophages assume a functional phenotype referred to as M1-like, in analogy with the type 1 T helper cells (Th1)-mediated immunity and serve to amplify the anti-tumoral response in the TME. In contrast, upon receiving signals via the IL4R or IL10R, macrophages assume anti-inflammatory tissue-remodeling roles aimed to dampen the immune response and clear cell debris (103). Efferocytosis, which is defined as the clearance of dying cells by M2-like macrophages or other phagocytes, is a critical process during involution (104). M2-like macrophages produce several factors important for this process, such as arginase I, involved in collagen biosynthesis. Unfortunately, M2-like macrophages are associated with pro-tumorigenic immunity (103). By producing immunosuppressive cytokines, such as IL4, IL10, IL13 and TGF-β (104, 105), M2-like macrophages are proposed to mediate PPBC immune evasion. Targeting M2-like macrophages has been attempted in PPBC models; with the suggestion that ibuprofen can diminish the population of M2-like macrophages by promoting M1-like macrophage polarization (12).

1.4.4. Myeloid-derived suppressor cells (MDSCs): MDSCs are a heterogeneous population of immature myeloid cells that are associated with immune suppression and tumor progression. The heterogeneity of MDSCs is reflected in their genetic and morphological diversities, and are subdivided into monocytic-MDSCs (M-MDSCs) and polymorphonuclear-MDSCs (PMN-MDSCs) (106). MDSCs are important during pregnancy to avoid the maternal rejection of the fetus (107), however the functions of MDSCs during mammary gland involution and PPBC development are not well characterized. Recently, Pennock and colleagues reported that granulocytes positive for immature myeloid markers Gr1 and CD11b remain at a high level during involution (12), indicating the potential importance of MDSCs in contributing to the immunosuppressive microenvironment during involution.

Most research to date has focused on the importance of MDSCs in breast cancer progression. The levels of MDSCs in both the TME and the circulation is correlated to impaired breast cancer patient survival (108-110), as it supports type 2 pro-tumorigenic immunity (109).

Breast tumor cells produce abundant G-CSF and GM-CSF to stimulate MDSC intra-tumor infiltration (111). Tumor-infiltrating MDSCs utilize several mechanisms to support breast tumorigenesis directly. MDSC-derived IL6 and nitric oxide (NO) promote the maintenance of breast cancer stem cell (CSC) via activation of the STAT3 and NOTCH pathways in breast cancer cells (108). MDSCs also produce MMPs that degrade extracellular matrix, thus facilitating breast tumor cell invasion (112). Additionally, MDSCs house several amino acid catabolic enzymes to achieve immune suppression. For example, arginase I and inducible nitric oxide synthase (iNOS) expression by MDSCs facilitates the catabolism of L-arginine into L-ornithine (113, 114) and L-arginine to nitric oxide (NO) (115). While L-ornithine is reported to block cytotoxic T cell differentiation (116), NO derived from MDSCs can inhibit NK cell-mediated cytotoxicity against breast cancer cells (115). Two other catabolic enzymes, indoleamine-2,3-dioxygenase (IDO) and tryptophan 2,3- dioxygenase (TDO), that degrade L-tryptophan into kynurenine are also highly expressed in MDSCs (117). Depletion of L-arginine and accumulation of kynurenine both contribute to the paralysis of $CD8^+$ T cells and NK cells (117, 118), and tryptophan starvation in the TME facilitates the polarization of $CD4^+$ T cells into regulatory T cells (T_{reg}) (119). Lastly, NO production by MDSC-derived inducible nitric oxide synthase (iNOS) impairs NK cell-dependent antibody-dependent cellular cytotoxicity (ADCC) in breast cancer models (115). Thus, ablation, or exclusion, of MDSCs from the TME can be a potential anti-cancer therapy, with the inhibition of the CSF1/CSF1R pathway and PI-3Kγ as just two putative modes (120-122).

1.4.5. Innate lymphoid cells: Innate lymphoid cells (ILCs) are distinct populations of lymphoid cells that lack variable T or B cell receptors. This group of innate immune cells are classified based on their distinct expression of transcription factors and their unique cytokine production profiles, and include NK cells, group 1 ILCs (ILC1), group 2 ILCs (ILC2), group 3 ILCs (ILC3), and lymphoid tissue-inducer (LTi) cells (reviewed by Chiossone et al (123)). Although the anti-tumor effect and the role of NK cells in immune surveillance have become more evident, the functions of ILC1, 2, 3 and LTi cells in tumor biology are only just beginning to emerge (124).

A few recent studies suggest that ILC2s may have pro-tumorigenic roles in cancer (125, 126), possibly by inducing MDSC expansion and inhibiting cytotoxic T cell infiltration (125, 126). ILC2 frequency is elevated in breast tumor samples, compared to benign breast tissues (127). The polarization and expansion of ILC2s are regulated by a few essential cytokines, including IL33, IL7, IL25 and thymic stromal lymphopoietin (TSLP) (128). Activated ILC2s produce abundant IL5 and IL13, which are important cytokines for type 2 immunity (128). Given the importance of type 2 immunity in breast cancer immune evasion, it is not surprising that exogenous IL33 can induce ILC2 polarization and supports 4T1 breast cancer cell metastasis (129).

However, it remains unclear how ILC2s contribute to the tissue remodeling during mammary gland involution, and how ILC2s regulate PPBC immunity. A part of my work is dedicated to the potential pro-tumorigenic roles of IL33, and potentially ILC2, in breast cancer metastasis.

1.4.6. Dendritic cells (DCs): DCs are bone marrow-derived professional antigen presenting cells (APCs) of the myeloid lineage responsible for sampling environmental antigens and providing this information to cells from the adaptive immune system (130). Although DCs are recruited to mammary gland early during involution (11), the precise functions of DCs in this process are poorly characterized. Milk stasis generates various stress signals that trigger involution. Cellular stress, such as endoplasmic reticulum (ER) stress and autophagy, are induced during involution (131). Such stress signals also drive the expression and release of damage-associated molecular patterns (DAMPs) as “come and eat me” signals to attract DCs (132). Additionally, dying cell-derived DNA can be uptaken by DCs to activate the cyclic GMP-AMP synthase (cGAS)/stimulator of interferon genes (STING) pathway, which serves as a defense immune mechanism (reviewed by Corrales and Gajewski (133)). These observations possibly indicate a crucial role of DCs in immune surveillance. Escape from DC-mediated immune surveillance is an important step for breast cancer metastasis. Breast cancer cells often escape from DC-mediated immune surveillance by multiple mechanisms, including decreasing MHC-I presentation on the cell surface, and by silencing the expressions of transported associated with antigen processing (TAP)-1/2 (134, 135). However, it remains unclear how DCs regulate PPBC progression and metastasis.

1.4.7. T cells: T cells mature in the thymus, and express either CD8 (cytotoxic T cells) or CD4 (T helper cells, Th cells). Th cells are further divided into various subsets, including Th1, Th2,

Th9, Th17, Th22, T_{reg} and follicular helper T cells (Tfh) (reviewed by Golubovskaya and Wu (136)). Th1 cells, which are activated by IFNγ and IL12, can produce IFNγ to activate cytotoxic T cells. In contrast, Th2 cells are stimulated by IL4 and secrete IL4, IL5, IL10 and IL13. Th1 and Th2 cells oppose each other to maintain an immune balance (reviewed by Patel et al. (137)). Similar to most malignancies, breast cancer progression is often characterized by the disruption of Th1/Th2 balance and diminished cytotoxic T cell-dependent tumor eradication (reviewed by Disis (138), Borst et al. (139)). Exhaustion of cytotoxic T cells is arguably the best-characterized mechanism for immune evasion of tumor cells (140). It is broadly defined as diminished effector function and sustained expression of inhibitory checkpoints on the cell surface of T cells (141). T cell exhaustion can be regulated via the following mechanisms: **(a)** soluble factors such as IL10 and TGFβ, **(b)** expression of inhibitory checkpoint molecules on the cell surface, such as CTLA-4 and PD-1, or **(c)** direct or indirect interactions with inhibitory stromal cells, such as T_{reg}, MDSCs and CAFs. In order to develop effective therapies against PPBC, strategies to restore the Th1-mediated immunity have been explored in PPBC mouse models. For example, ibuprofen effectively blocks breast tumor outgrowth in a PPBC model, partially due to ibuprofen-induced Th1 and cytotoxic T cell infiltration into the TME (12).

Increased infiltration of T_{reg} cells during involution is an important checkpoint to control inflammation, and is suggestive of wound resolution (11). Unfortunately, T_{reg} cells also play an important role in breast cancer progression, as they tend to enrich in more aggressive breast cancers (142). Intra-tumoral T_{regs} often express elevated levels of inhibitory checkpoint molecules (PD-1, PD-L1 and CTLA-4) (142, 143), indicating that the tumor-infiltrating T_{reg} cells exert more of an immune suppressive function in TME than in the periphery. Tumor-infiltrating T_{reg} cells express high levels of CCR8 (142), and depletion of T_{reg} cells by inhibiting CCR8 pathway may be a promising strategy against breast cancer.

In summary, multiple non-immune and immune cell types in the tumor stroma contribute to PPBC immune evasion and metastasis (Figure 1.3). However, our understanding of PPBC tumor immunology is still rudimentary. To find better targets for this aggressive disease, future studies are needed to better understand how different stromal cells contribute to PPBC pathogenesis and metastasis.

1.5. Pro-tumorigenic functions of MNK1/2-eIF4E pathway in Breast Cancer

1.5.1 Overview of MNK1/2-eIF4E Pathway, eIF4F complex and regulation of mRNA translation: mRNA translation is a delicately regulated multi-step process, subdivided into the initiation, elongation and termination stages. The importance of translational regulation in cancer has been overlooked for many decades. Abnormal regulation of mRNA translation has been observed in various hematological and solid malignancies, and serves as a promising target for therapeutic intervention (144).

Eukaryotic initiation factor 4E (eIF4E) is a critical translation initiation factor that binds to the 7-methyl-guanosine five-prime cap structure, m^7GpppX (where X is any nucleotide) at the 5'-end of the mRNA. Binding of eIF4E to the mRNA cap directs the ribosomes to the mRNA and facilitates mRNA translation (Figure 1.4).

The eIF4F complex which regulates mRNA translation is composed of the following components: eIF4E, eIF4A and eIF4G (145) (Figure 1.4). eIF4E is the least abundant component of the eIF4F complex, thus making it the rate-limiting factor for translation in eukaryotes (145). eIF4A, an ATP-dependent RNA helicase, is the most abundant component of the eIF4F complex (145). eIF4G serves as a scaffold protein, where all other components bind to assemble the eIF4F complex (145). Interestingly, mammalian systems can generate several different eIF4F complexes, as there are two isoforms of eIF4G (eIF4GI and eIF4GII) and three isoforms of eIF4A (eIF4AI, eIF4AII and eIF4AIII) (146, 147). It is not clear how these complexes differ functionally.

eIF4E can be post-translationally modified on serine 209 by phosphorylation, via the activity of the mitogen-activated protein kinase-interacting serine/threonine protein kinase 1/2 (MNK1/2), which are encoded by *MKNK1 and MKNK2* genes (148, 149). Alternative splicing of those two genes in human cells give rise to two mRNAs encoding two protein isoforms that differ in their C-termini: the longer "a" isoforms (i.e. MNK1a and MNK2a) and the shorter "b" isoforms (i.e. MNK1b and MNK2b) (150-152). However, only MNK1a and MNK2a are identified in mouse.

The activity of MNK1a is regulated by ERK and p38 MAPKs, while MNK2a appears only to be activated by ERK (153). It remains unclear how MNK1b and MNK2b activities are regulated (154). Recently, increased MNK2b over MNK2a splicing driven by serine/arginine-rich splicing factor 1 (SRSF1), an essential splicing factor required for constitutive and alternative pre-mRNA splicing, has been reported to support tumorigenesis (155). Specifically, MNK2a serves as a tumor suppressor by facilitating p38-dependent stress-induced cell death, while MNK2b has pro-

oncogenic properties (155). It remains unclear how eIF4E S209 phosphorylation facilitates mRNA translation. Past studies have suggested that eIF4E S209 phosphorylation may speed up the dissociation of eIF4E from mRNAs to facilitate its recycling for further rounds of mRNA translation (156). Thus, eIF4E phosphorylation may have led to punctual increases in the availability of eIF4E, which may explain why 5'UTR structures that are highly sensitive to total eIF4E levels also respond to elevated eIF4E phosphorylation (156). However, this hypothesis still lacks direct supporting evidence. eIF4E phosphorylation does not affect global mRNA translation, as shown by the experiments wherein serine 2019 is mutated to an alanine, or genetic ablation of MNK1/2 (149, 157). Instead, the phosphorylation of eIF4E preferentially controls the translation of a specific subset of mRNAs with complex 5'-UTRs, which will be further discussed in section 6.2 (149, 157).

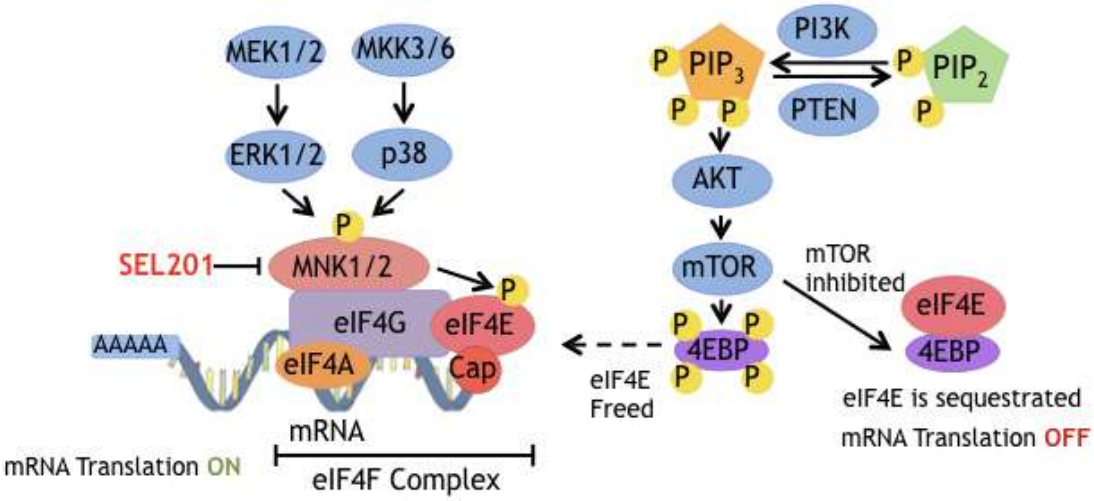

Figure 1.4. Regulation of eIF4F complex activity by MNK1/2-eIF4E and PI-3K/Akt/mTOR pathways.

1.5.2 The functions of MNK1/2-eIF4E pathway in breast tumorigenesis:

The activation of MNK1/2-eIF4E pathway is controlled by two well-characterized MAPK members, p38 and ERK1/2 (Figure 4). Various external stimuli, such as mitogenic and external stress signals can activate MAPK signaling, which ultimately converge on the MNK1/2-eIF4E pathway (reviewed by Bhat et al. (144)). MNK1/2-eIF4E pathway activation facilitates tumorigenesis by inducing the translation of pro-tumorigenic and pro-invasive mRNAs without significantly altering the expression of housekeeping genes such as GAPDH (144).

In breast cancer, the MNK1/2-eIF4E pathway controls the expression of selective EMT markers including Snail, MMP3, MMP9 to promote breast cancer invasion and metastasis (158, 159). Additionally, MNK1/2-eIF4E-dependent expression of β-catenin also contributes to chemoresistance in metastatic breast cancer, and blocking MNK1/2 activity can overcome resistance to chemotherapy (160).

Overexpression and hyperactivation of MNK1/2 and eIF4E have been observed in various cancers, including breast cancer (summarized in Table 4). Specifically, a higher level of eIF4E expression is associated with poor prognosis in breast cancer (161, 162). Similarly, Li et al. have also described that higher levels of phospho-eIF4E predict decreased survival rates in metastatic breast cancer (160). Such clinical studies indicate the significance of MNK1/2-eIF4E pathway activation during the process of breast tumorigenesis and disease progression.

Table 1.4. Overexpression and hyperactivation of MNK1/eIF4E in cancer

Molecule	Cancer type	Survival	Reference
MNK1	Glioblastoma	N/A	Grzmil et al, Clin Cancer Res 2011
	Breast cancer (DCIS/IDC)	N/A	Guo et at, Cancer Res 2019
	Breast cancer	High eIF4E level is associated with poor prognosis of luminal B-type breast cancer.	Pettersson et al, Cancer Res 2011
	Hepatocellular carcinoma	Increased tumor stage & poor overall survival	Wang et al. Med Sci Monit 2018
phospho-MNK1	Glioblastoma	N/A	Grzmil et al, Clin Cancer Res 2011
	c-Kit mutant melanoma	N/A	Zhan et al, J Clin Invest 2017
	Breast cancer (DCIS/IDC)	N/A	Guo et al, Cancer Res 2019
MNK2	Non-small cell lung cancer	Increased lymph node metastasis & poor overall survival	Guo et al, Sci Rep 2017

Molecule	Cancer type	Survival	Reference
MNK2	Breast cancer (↑ MNK2b/MNK2a ratio)	N/A	Maimon et al, Cell Rep 2014
	Lung cancer	N/A	Maimon et al, Cell Rep 2014
	Colon cancer	N/A	Maimon et al, Cell Rep 2014
eIF4E	Breast cancer	High eIF4E level is associated with poor prognosis	Zhou et al, BMC Cancer 2006
	Colorectal carcinoma	Overexpression of eIF4E is associated with increased risk of liver metastasis.	Xu et al, Onco Targets Ther 2016
	Esophageal squamous cell carcinoma	Overexpression of eIF4E is associated with poor overall survival and disease-free survival	Liu et al, Oncotarget 2016
	Various soft tissue benign tumors and neoplasm	eIF4E overexpression is elevated in both malignant and benign mesenchymal neoplasms.	Chu et al, Cancer Res 2004 (Published abstract)
	Acute myeloid leukemia	N/A	Assouline et al, Blood 2009
	Chronic myeloid leukemia	eIF4E overexpression is observed in CML (blast crisis) stem cells	Lim et al, PNAS 2013
	Metastatic breast cancer	High phospho-eIF4E associates with poor survival rates	Li et al, Int J Mol Sci 2017
phospho-eIF4E	Pancreatic ductal adenocarcinoma	High phospho-eIF4E associates with higher disease grade and poor survival rates	Adesso et at al, Oncogene 2013
	c-Kit mutant melanoma	N/A	Zhan et al, J Clin Invest 2017
	Melanoma	High phospho-eIF4E associates with poor survival rates	Carter et al., Br J Cancer 2016
	Prostate cancer	High phospho-eIF4E correlates to high Gleason scores	Furic et al., PNAS 2010
	Non-small cell lung cancer	N/A	Yoshizawa et al, Clin Cancer Res 2010

Our understanding of the functions of MNK1/2-eIF4E in tumor immunity is increasing. Mehrotra et al. have described that MNK1/2 activity is essential for the production of anti-neoplastic type I interferons (IFNs) in myeloproliferative neoplasms (163). Later, it was revealed that phospho-eIF4E enhances IκB mRNA translation, and subsequently suppresses NF-κB dependent transcription of the IFNβ gene (164), suggesting that phospho-eIF4E may inhibit innate immunity by decreasing IFNβ production. Recently, MNK1/2-eIF4E pathway has also been reported to support the expansion of pro-invasive neutrophils, and ablation of neutrophils by an anti-Ly6G antibody can inhibit breast cancer metastasis in a pre-clinical model (165). Finally, eIF4E has also been identified to serve as a key regulator of STAT1-dependent PD-L1 expression on tumor cells (166). The evidence thus far, suggests a possible role of the MNK1/2-eIF4E axis in facilitating pro-tumorigenic immunity.

1.5.3 Other MNK1/2 substrates

MNK1/2 have substrates besides eIF4E, including hnRNP-A1, PTB (polypyrimidine tract-binding protein)-associated splicing factor (PSF)-p54$^{psf.nrb}$, Sprouty2, and Cytosolic phospholipase A2 (cPLA2) (167-169). Overexpression of hnRNP A1 has been reported in various cancer types, including breast cancer (170, 171). This overexpression leads to an increase in the levels of several EMT markers, thus facilitating the motility of cancer cells (171). The role of MNK1 and Sprouty2 in cancer is less defined. DaSilva and colleagues reported that MNK-dependent Sprouty 2 phosphorylation increases the stability of Sprouty2, which is a negative feedback modulators of receptor tyrosine kinase pathways (167). However, Sprouty 2 was also reported as a potential tumor suppressor in breast cancer, as loss of Sprouty 2 is associated with increased tumor grade and poor prognosis (172). Further studies are needed to better define the functions of these, and potentially other, MNK1/2 substrates in cancer, with an emphasis on how MNK1/2 impacts their protein stability and activity.

1.5.4 Targeting MNK1/2 in cancer:

MNK1/2 activities are dispensable for normal development, but are potential therapeutic targets in various malignancies, and there are now efforts to identify selective small molecule inhibitors that can be used in the clinic (144). Cercosporamide and CGP57380 are two well-described MNK1/2 inhibitors with anti-neoplastic efficacy. However, both compounds exhibit significant

off-target effects (173). To address the need for more selective modes of repressing MNK1/2 activity, novel strategies to target MNK1/2 are being developed. For example, retinoic acid metabolism blocking agents (RAMBA) which induce MNK1 degradation effectively decreases eIF4E phosphorylation (174). Resorcylic acid lactone analogues are also developed to block MNK1/2 activity, and, as a consequence, inhibit eIF4E phosphorylation and cancer cell proliferation (175). Three novel orally bioavailable MNK1/2 inhibitors: SEL201, BAY1143269 and eFT508, have been reported by us and others to show promising anti-cancer effects (56, 176-179).

1.5.5 Crosstalk between the MNK1/2 and PI3K/Akt/mTOR pathways:

The eIF4E is a nexus point on which the MNK1/2 and PI3K/Akt/mTOR pathways converge. Crosstalk exists between these two pathways, with important implications for cancer cell biology. The PI3K/AKT/mTOR signaling pathway is one of the most commonly dysregulated pathways in cancer. Upon mTOR-mediated phosphorylation of the eIF4E binding proteins (4EBPs) 1/2/3, their repressive binding to eIF4E is disrupted, freeing eIF4E to assemble into the eIF4F complex, thus promoting mRNA translation (reviewed by Janku et al. (180)). PI-3K/Akt/mTOR pathway has been extensively characterized with respect to tumor cell survival, proliferation, invasion, metabolism and genomic instability (Reviewed by Janku et al. (180)).

Rapamycin and its analogs, including everolimus, temsirolimus and ridaforolimus, are used as allosteric mTOR inhibitors. Recently, catalytic mTOR inhibitors, such as PP242 and OSI-027, which block the activity of both mTORC1 and mTORC2, were developed in an attempt to enhance the anti-cancer efficacy of the mTOR blockade. Unfortunately, complex feedback loops involving activation of PI3K and MAPK signaling have limited the success of mTOR inhibitors as anti-cancer agents, and MNK1/2-eIF4E activation contributes to the resistance of mTOR inhibitors in cancer cells (181-183). Specifically, mTOR inhibitors, such as rapamycin and everolimus, have been demonstrated to induce eIF4E phosphorylation (184, 185). The molecular mechanisms underlying the phosphorylation of eIF4E as a consequence of mTOR inhibition are not fully elucidated. Katsha et al. reported that everolimus resistance is caused by Aurora kinase A-dependent inhibition of type 2A serine/threonine protein phosphatase (PP2A) activity, which subsequently elevates eIF4E phosphorylation (186). This process is independent of MNK1/2 activity in upper gastrointestinal adenocarcinomas (186). Alternatively, rapamycin was also reported to induce eIF4E phosphorylation via MNK2, but independent of p38 and ERK1/2

activities (187). Additionally, MNK1/2 can also promote resistance to mTOR inhibitors by stimulating the constitutive activation of mTORC1, and facilitating mTORC1 binding to its substrates (185).

Multiple avenues to block aberrant mRNA translation in cancer, by co-inhibiting PI3K/Akt/mTOR and MNK/eIF4E axis, have been explored. MNK1/2 inhibition has been shown to be a potential strategy to overcome mTOR inhibitor resistance in malignant cells. For example, CGP57380 synergizes with a few mTOR inhibitors (rapamycin, everolimus, or OSI-027) to inhibit tumorigenesis in several solid and hematological malignancies, including medulloblastoma, non-small cell lung cancer and acute lymphocytic leukemia (187-189). In breast cancer models, MNK1/2 inhibitors cercosporamide and CGP57380 have shown synergistic anti-breast cancer effects when combined with an in-house PI-3K inhibitor or an mTOR inhibitor PP242 (183). This could partially be explained by proposing that the inhibition of MNK1/2 and PI-3K/Akt/mTOR pathways together blocks the assembly of the eIF4F complex. Additionally, co-treatment of CGP57380 plus everolimus overcomes tamoxifen resistance in breast cancer (190). These data support that combination of mTOR and MNK1/2 inhibitors may be a promising regimen against breast cancer and other malignancies.

1.6 Rationale, objective and general introduction to the thesis research

Although many pro-tumorigenic functions of MNK1 have been characterized, it remains unclear how MNK1 regulates the early steps of the non-invasive to invasive breast carcinoma transition. We hypothesized that MNK1 drives DCIS to IDC transition, and we aimed to test this hypothesis by MNK1 overexpression and knock-out in a DCIS model. We performed a series of experiments to demonstrate that MNK1 activity in DCIS is needed to support a partial EMT and maintain cancer stem cell properties, as the DCIS cells transit to become invasive tumor cells (Chapter 2). Additionally, our knowledge of how aberrant mRNA translation regulates breast tumor immunity, and which pro-tumorigenic and pro-inflammatory factors are involved, remains limited. Multiple pro-oncogenic pathways converge on the MNK1/2-eIF4E axis, thus it is important to understand how this pathway affects breast cancer immune evasion. In Chapter 3, I chose to study PPBC, an aggressive subtype of breast cancer characterized by robust immune cell influx. We hypothezied that stromal MNK1/2-eIF4E axis shapes pro-tumorigenic immunity

during metastasis of PPBC, and we performed a series of experiments to examine how MNK1/2-eIF4E axis impacts the functions of different immune and non-immune cell types in PPBC TME. The work presented in this thesis highlights the critical functions of MNK1/2-eIF4E pathway in breast cancer. The objectives of our studies were as follows:

a) To determine if MNK1 regulates the transition from DCIS to invasive disease.

b) To understand how the MNK1/eIF4E axis, functioning in stromal cells, regulates PPBC pro-tumorigenic immunity.

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Chapter 2. MNK1/NODAL signaling promotes the invasive progression of breast ductal carcinoma in situ.

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Program of research: The mechanisms by which breast cancers progress from relatively indolent DCIS to IDC are not well understood. In Chapter 2, our research interest was to understand whether tumor-intrinsic MNK1 would facilitate the DCIS invasive transition, and to investigate whether it would be possible to inhibit the invasive transition using a small molecule inhibitor of MNK1.

Rationale & Hypothesis: It remains unclear how MNK1 regulates the early steps of the non-invasive to invasive breast carcinoma transition. Given previous reports showing that MNK1 promotes invasion, we hypothesized that MNK1 might promote the DCIS to IDC transition. We aimed to test this hypothesis by engineering DCIS cells to express a constitutively active MNK1 or to knock-out MNK1 using CRISPR/cas9 technology.

2.1 Abstract

The mechanisms by which breast cancers progress from relatively indolent ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) are not well understood. However, this process is critical to the acquisition of metastatic potential. MAP kinase-interacting serine/threonine-protein kinase 1 (MNK1) signaling can promote cell invasion. NODAL, a morphogen essential for embryogenic patterning, is often re-expressed in breast cancer. Herein, we describe a MNK1/NODAL signaling axis that promotes DCIS progression to IDC. We generated MNK1 knockout (KO) or constitutively active MNK1 (caMNK1)-expressing human MCF-10A-derived DCIS cell lines, which were orthotopically injected into the mammary glands of mice. Loss of MNK1 represses NODAL expression, inhibits DCIS to IDC conversion, and decreases tumor relapse and metastasis. Conversely, caMNK1 induces NODAL expression and promotes IDC. Furthermore, the MNK1/NODAL axis promotes cancer stem cell properties and invasion in vitro. Finally, the MNK1/2 inhibitor SEL201 blocks DCIS progression to invasive disease *in vivo*. In clinical samples, IDC and DCIS with microinvasion express higher levels of phospho-MNK1 and NODAL than low grade (invasion-free) DCIS. Cumulatively, our data support further development of MNK1 inhibitors as therapeutics for preventing invasive disease.

2.2 Introduction

Over the last 40 years, there has been a significant increase in the diagnosis of breast ductal carcinoma in situ (DCIS) due to the implementation of mammography screening (1). DCIS has the potential to, but does not always, progress into invasive ductal carcinoma (IDC). The signaling pathways that mediate the transition from non-invasive to invasive disease remain largely uncharacterized.

MAP kinase-interacting serine/threonine-protein kinases 1 and 2 (MNK1/2) are ubiquitously expressed serine/threonine kinases downstream of the ERK1/2 and p38 (2) pathways. The best characterized function of MNK1/2 is to phosphorylate eukaryotic initiation factor 4E (eIF4E) at Ser209 (3). For a variety of reasons, there is clinical interest in characterizing the role of MNK1/2 signaling in tumour initiation and progression. First, MNKs are often linked to pro-survival signals in response to a wide variety of stimuli (4, 5). Second, loss of MNK function impairs both cell migration and VIMENTIN expression (6). Third, previous work has described the significance of the MNK/eIF4E pathway in regulating oncogenic mRNA translation in various hematological and solid malignancies (7-10). Finally, lack of MNK1 decreases the oncogenic potential of leukemia (11), gliomas (12), melanoma (13), ovarian cancer (14), and malignant peripheral nerve sheath tumors (15).

In the present study, we take advantage of CRISPR-Cas9 technology and a new pharmacological tool to provide insight into the role of MNK1 signaling as a regulator of the progression of DCIS to invasive disease. Furthermore, we identify NODAL as an important downstream effector of MNK1 signaling. NODAL is a transforming growth factor beta (TGFB) family morphogen, and promotes invasiveness during primitive streak formation and mammary gland development (16). Abnormal re-expression of NODAL has been observed in various malignancies (17), and increased NODAL expression has been positively correlated with the transition from local (Stage 1) to invasive (Stage 2 and above) disease (18). NODAL maintains the self-renewal capacity of cancer stem cells (CSCs), and promotes the invasiveness of several solid tumors, including breast cancer (19). Importantly, while MNK1 has been shown to lie downstream of TGFB1 signaling (9), interactions between MNK1 and NODAL have not been considered.

MNKs are potential therapeutic targets in various malignancies, and there are now efforts to identify selective small molecule inhibitors that can be used in the clinic (13). We were therefore

prompted to interrogate whether MNK1 could serve as a target for therapeutic intervention in pre-invasive disease.

2.3 Materials and Methods

2.3.1 Cells and Reagents

The MCF10DCIS.com (DCIS) cell line was purchased from Wake Forest University. DCIS cells were first tagged with luciferase (DCIS-Luc). DCIS-Luc CTL and MNK1 knock-out (KO) cell lines were generated using the CRISPR/Cas9 system (20). DCIS-Luc cells were transfected with Cas9/sgRNA-GFP plasmids. GFP^+ single cells were sorted 48 hours after transfection. Single cell clones were expanded and western blotting was performed to verify if MNK1 expression was successfully ablated in each clone. DCIS-Luc cells were also genetically modified to express either $MNK1^{T332D}$ (constitutively active MNK1, caMNK1) or the corresponding empty vector, pBABE (21). The pBABE and caMNK1 plasmids were kind gifts from Dr. J.A. Cooper (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). All modified DCIS cell lines were cultured in DMEM/F12 supplemented with 5% horse serum, 10mM HEPES, 1.05mM $CaCl_2$, and antibiotics. SUM225 cells were cultured in DMEM/F12 supplemented with 5% fetal bovine serum, 10mM HEPES, 5µg/ml insulin, 1µg/ml hydrocortisone and antibiotics. MDA-MB-468 cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics. 66cl4 cells were cultured in RPMI supplemented with 10% fetal bovine serum and antibiotics.

2.3.2 Growth Curves

Growth and viability of cells was determined via trypan blue exclusion using a hemocytometer.

2.3.3 Mammosphere Formation Assay

Mammosphere formation assay was performed as previously described (22). Briefly, 60,000 cells were seeded per well in 6-well low-adherent plates. Bright field images were collected at day 7 and colonies with a diameter greater than 50µm were recorded. Colonies were then sorted based on size as described previously (23).

2.3.4 Western Blotting

Cells were lysed with RIPA buffer (150 mmol/L Tris-HCl, pH 7, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitors (Roche) as previously described (24). Equal amount of protein samples were loaded and separated on 10% SDS-PAGEs. p-eIF4E, p-MNK1, eIF4E, MNK1, NODAL, and VIMENTIN

were probed with corresponding antibodies. GAPDH was probed to confirm equal protein loading. Antibody information is listed in table S1.

2.3.5 Quantitative PCR

RNA was prepared using Trizol (Invitrogen). cDNA was prepared from 1 mg of total RNA, using iScript cDNA Synthesis Kit (Bio-Rad). NODAL expression (Forward Primer: 5'-AGGGCGAGTGTCCTAATCCT-3', Reverse primer: 5'-CAAAGCTAGAGCCCTGTCCC-3') was quantified using the Applied Biosystems 7500 Fast Real-Time PCR System with SYBR Green. 36B4 (Forward primer: 5'-GGCACCGAGGCAACAGTT-3', Reverse primer: 5'-TCATCCAGCAGGTGTTTGACA-3') was used as the internal control.

2.3.6 Clonogenic Assay

All DCIS cells were seeded at 300 cells per well in 6-well plates and treated as indicated. After 14 days, cells were fixed and stained with 0.5% crystal violet in 70% ethanol. SUM225 cells were seeded at 2000 cells per well in 6-well plates and treated as indicated. After 21 days, cells were fixed and stained with 0.5% crystal violet in 70% ethanol. Visible colonies were counted using a Gel Count colony counter (Oxford Optronix).

2.3.7 Aldefluor Assay

The enzymatic activity of ALDH was detected using the ALDEFLUOR staining kit (Stem Cell Technologies). 1 x 10^6 trypsinized single-cells were suspended in the ALDEFLUOR assay buffer and incubated with 1.5µM BODIPY™-aminoacetaldehyde (BAAA) for 30 min at 37^{o}C. As a negative control to establish background fluorescence level, a separate sample was treated with 15µM diethylaminobenzaldehyde, a selective ALDH inhibitor. Necrotic cells were excluded by incubating each sample with 2.5 µL of 7-Aminoactinomycin D for 15 minutes prior to FACS analysis. Cell fluorescence was measured using BD Bioscience FACScalibur flow cytometer, and data were analyzed using Flowjo Software Version.10 from LCC.

2.3.8 Migration and Invasion Assay

Cells were seeded at 1 million cells per 10 cm dish on day 1 in full media, then switched to serum free media on day 2 and starved overnight. On day 3, the transwells were coated with Collagen I (20 µg/ml) as previously reported (24). 200,000 cells were seeded into the transwells (Corning) and were allowed to migrate and invade for 16 hrs. Migrated cells were fixed with 5% glutaradehyde (Sigma) and stained with 0.5% crystal violet (Sigma) as previously reported (24).

2.3.9 Immunohistochemistry

Human breast cancer tissue was obtained in collaboration with Dr. Muriel Brackstone and the project was approved by the Research Ethics Board at the University of Western Ontario (REB 102254).

Immunohistochemistry, and hematoxylin and eosin (H&E) staining, was performed as previously described (25). Briefly, tumor sections were stained for p63, MNK1, p-MNK1, NODAL, VIMENTIN and counterstained with 20% Harris-modified hematoxylin (Fisher). Antibody information is listed in table S1. Slides were scanned and assessed using Spectrum (Aperio Technologies). All the animal and patient IHC samples were scored blindly by a pathologist (Dr. Jose Torres). The specificity of phospho-MNK1 and MNK1 antibody staining was validated on samples from DCIS xenografts and breast cancer tissue microarrays with adjacent breast tissue. NODAL staining was scored by a pathologist (Dr. Nadia Giannakopoulos), to derive a score for % positive cells and another for intensity as previously described (26). These values were then multiplied to obtain a NODAL score. Three sections were analyzed for each case and the average score was taken for both invasive and DCIS lesions.

2.3.10 Orthotopic Mouse Model

Athymic nude mice, severe combined immunodeficiency (SCID) mice and NOD/SCID mice were purchased from Charles River Laboratory. 100,000 modified DCIS cells were resuspended in 50% matrigel and were injected into the mammary fat pad of athymic nude mice. DCIS-Luc CTL and MNK1-KO xenografts were allowed to grow for 5 weeks. DCIS-Luc pBABE and caMNK1 tumors were allowed to grow for 1.5 weeks. The xenograft, mammary fat pad and adjacent lymph nodes were then removed surgically. The mice were monitored for potential tumor relapse and metastasis. In parallel, 100,000 modified DCIS-Luc pBABE and caMNK1 cells were resuspended in 1xPBS and were injected into the mammary glands of SCID mice. Tumors were allowed to grow for 8 weeks.

For the limiting dilution experiment, 10,000, 1000 or 100 DCIS-Luc pBABE and caMNK1 cells were resuspended in 1xPBS and were injected into the mammary glands of NOD/SCID mice. Tumors were allowed to grow for 60 days.

50,000 SUM225 cells were resuspended in PBS and injected through the nipples into the mammary ducts of NOD/SCID mice (kindly provided by Dr. Moulay Alaoui-Jamali) with a 33-

gauge Hamilton syringe. Tumors were allowed to grow for 9 weeks. Animal experiments were conducted following protocols approved by McGill University Animal Care and Use Committee.

2.3.11 Statistical Analysis

Prism software (GraphPad) was used to perform statistical analysis. Three independent experiments were performed for all *in vitro* work. The number of samples for all *in vitro* and *in vivo* work are listed in Supplementary Table S2. The significance of differences between groups by applying either an unpaired Student's *t* test, Wilcoxon-Mann-Whitney, one-way or two-way ANOVA, Kruskal-Wallis test, or Chi-square test of independence as appropriate. The specific statistical analysis for each figure is listed in table S2. *P* values < 0.05 were considered significant.

2. 4 Results

2.4.1 MNK1 activity is elevated in high grade and IDC, compared to low grade DCIS clinical samples

To determine whether the activity of MNK1 is tumor grade dependent in breast cancer patients, we assessed the expression of phospho-MNK1 by immunohistochemistry (IHC) in a series of human breast samples, comprising low grade DCIS (13 cases), high grade DCIS (12 cases) and IDC (15 cases). High grade DCIS usually presents with significant variation in the size and shape of nuclei, as well as comedo necrosis. Phospho-MNK1 levels were significantly elevated in high grade DCIS/IDC lesions, compared to low grade DCIS (Fig. 2.1A, Supplementary Fig. 2.1A). Phospho-MNK1 levels were scored from 0 to 5, with 0-2 corresponding to low levels, and 3-5 corresponding to high levels (Fig. 2.1B). The percentage of samples with high phospho-MNK1 levels increases from low grade DCIS to high grade DCIS/IDC. Specifically, there are only 8.3% samples with high phospho-MNK1 levels in low grade DCIS, but it is elevated to 55% in high grade DCIS/IDC samples (Fig. 2.1B). Moreover, our analysis of high grade DCIS samples revealed a higher expression of MNK1 in 30.4% of analyzed samples, compared to 0% of low grade DCIS samples analyzed (Supplementary Fig. 2.1C). Thus, our results show that increased expression of phospho-MNK1 and MNK1 occurs in a larger percentage of high grade DCIS/IDC samples, compared to low grade DCIS specimens.

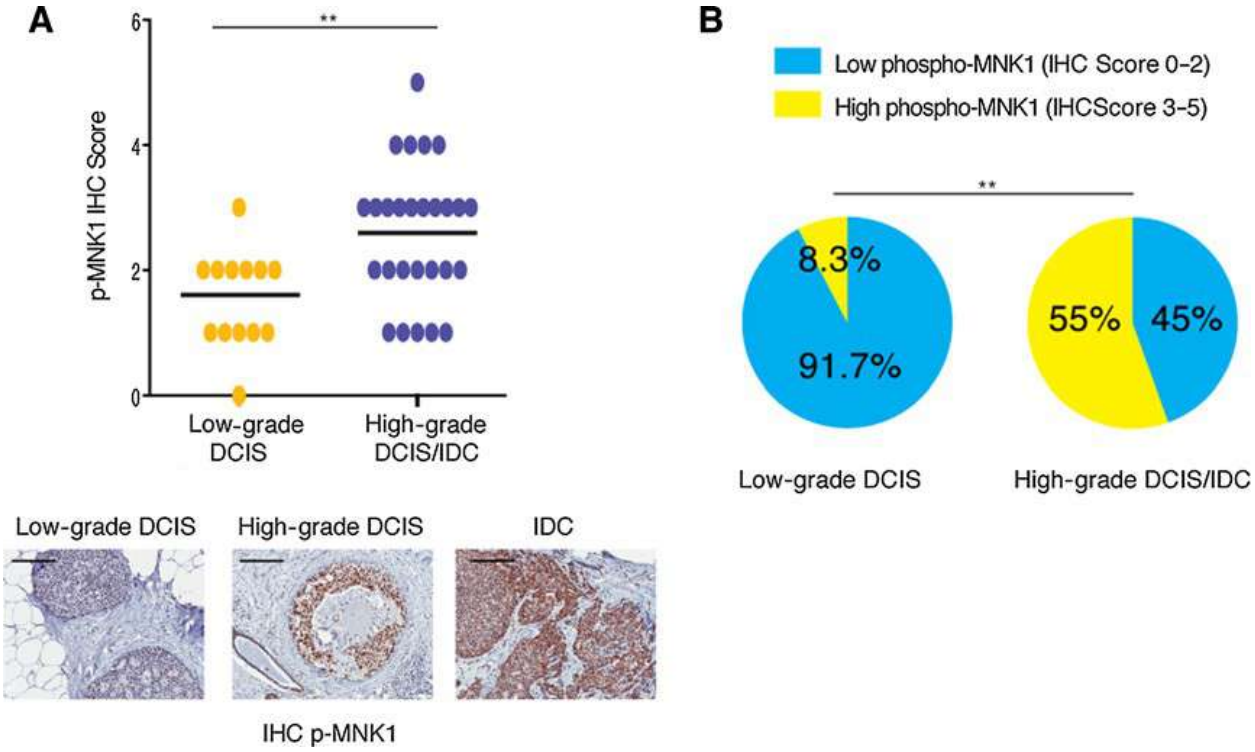


Figure 2.1. Increased MNK1 activity in IDC and high-grade DCIS.
A. Phospho-MNK1 levels are higher in high-grade DCIS/IDC samples than low-grade DCIS samples. Scale bar=200 mm. **B.** Increased percentage of samples with high phospho-MNK1 staining in high-grade DCIS/IDC compared with low-grade DCIS. , **$p < 0.01$.

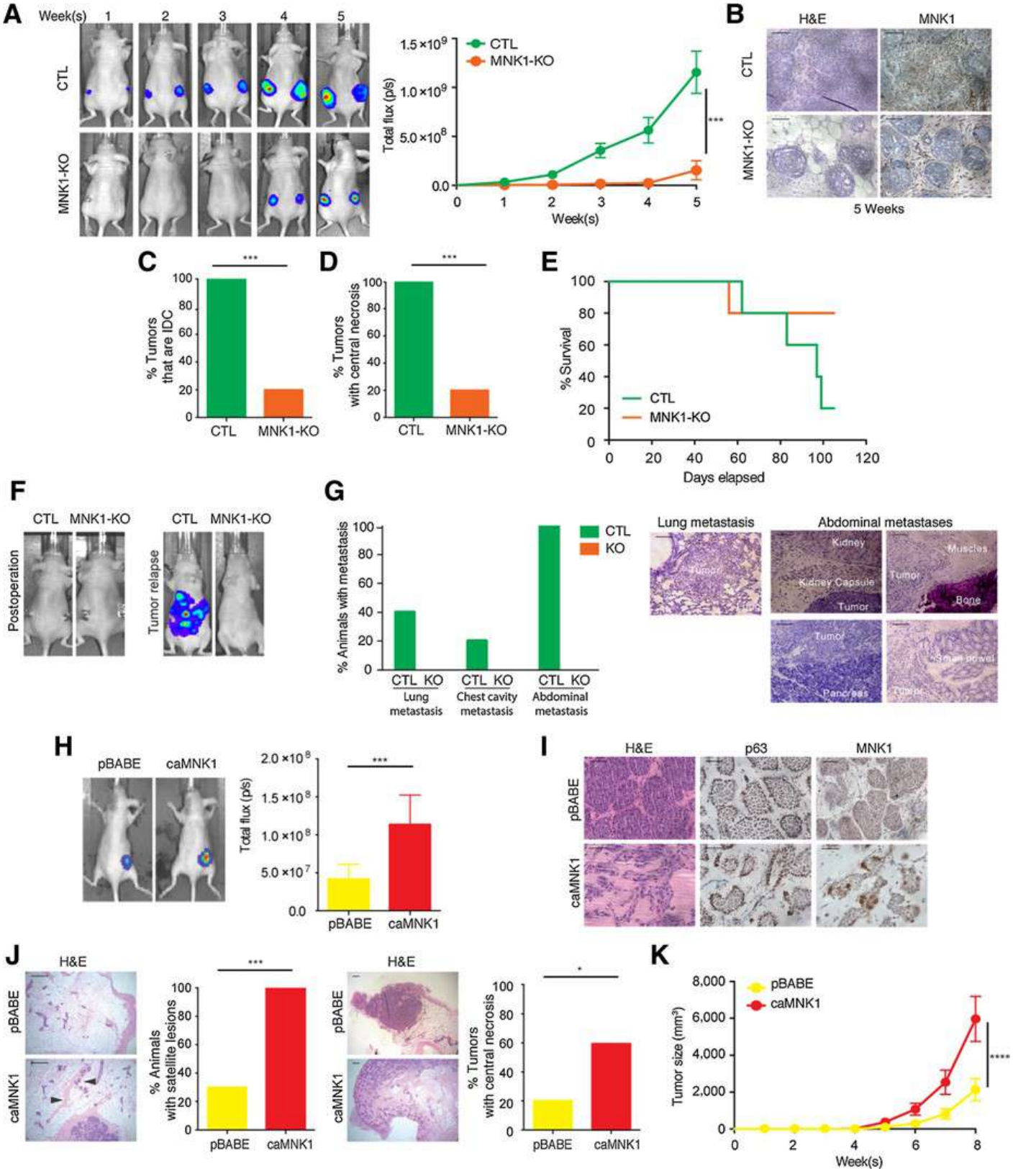
2.4.2 MNK1 knock-out impairs DCIS proliferation, and DCIS to IDC conversion

MCF10DCIS.com (DCIS) cells are a basal-like breast cancer line, commonly employed to model human DCIS (27-29). We used this model to examine whether MNK1 deficiency would impair DCIS tumorigenesis, invasion and metastasis. We therefore generated luciferse tagged DCIS-Luc Cas9 control (CTL) and MNK1 knockout (MNK1-KO) cells, using CRISPR-Cas9 technology. MNK1-KO in two independent clones was confirmed by western blotting (Supplementary Fig. 2.2A). The proliferation rate and colony formation capacities were measured in the MNK1-KO clones compared to CTL cells. Both MNK1-KO clones have decreased proliferation, and an impaired ability to form colonies compared to CTL cells (Supplementary Fig. 2.2B and C).

When orthotopically injected into the mammary glands of mice, DCIS cells form lesions that resemble human DCIS, and which can progress to IDC (27, 30). To assess the impact of loss of

MNK1 activity on the progression from DCIS to IDC *in vivo*, CTL or MNK1-KO cells were mixed with 50% matrigel and injected into the mammary fat pad of athymic nude mice. A previous study using this methodology reported that DCIS xenografts should remain DCIS after 4 weeks of injection, but progress to IDC after 5 weeks (27). Hypothesizing that MNK1 activity would accelerate the conversion of DCIS to IDC, we resected the xenografts, including surrounding mammary gland tissue, 5 weeks after DCIS tumor cell injection (Supplementary Fig. 2.2D). Tumor outgrowth was monitored by luciferin injection and IVIS imaging. MNK1-KO tumor initiation and proliferation was significantly slower than observed with CTL-derived tumors (Fig. 2.2A). MNK1 deficiency in tumor xenografts was verified by IHC (Fig. 2.2B). DCIS is characterized by tubular-like structures with an intact intact myoepithelial layer of cells that stain positive for p63, while IDC is characterized by structures that lack intact myoepithelial layers (27). A histological analysis demonstrated that 80% of MNK1-KO xenografts retained a DCIS morphology, while all CTL tumors progressed into IDC (Fig. 2.2C). Additionally, we observed tissue necrosis in 25% of the CTL tumors, while none of the MNK1-KO xenografts presented with necrosis (Fig. 2.2D). This could be important clinically, as the presence of necrosis in breast cancer is related to increased invasiveness and poor prognosis (31). Animals were kept alive after primary tumor resection, to monitor for potential tumor recurrence, metastasis and survival rates.

Although not statistically significant, mice that had received MNK1-KO cells showed a trend of better overall survival than those animals that were implanted with CTL cells (Fig. 2.2E). Furthermore, 80% of mice that received CTL cells had relapsed metastatic disease, while no mice that received MNK1-KO cells had metastasis (Fig. 2.2F). The metastatic CTL tumors were found in the chest area and abdominal cavity, next to the pancreas, small bowel, kidneys and rib bones (Fig. 2.2F and G). Full necropsy and histological analysis also revealed metastatic cancer in the lungs (Fig. 2.2G). The percentage of mice with metastasis to the lungs, chest cavity, or abdomen is graphed in Fig. 2.2G.



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Next, we hypothesized that constitutive activation of MNK1 would promote the DCIS to IDC transition. Thus, we generated DCIS-luciferase-tagged cells that stably express empty vector pBABE (DCIS-luc pBABE) or MNK1^{T332D} (constitutively active MNK1, DCIS-Luc caMNK1) (21). caMNK1 expression in DCIS was validated to be functional by detecting increased phosphorylation of one of its best studied substrates, eIF4E (Supplementary Fig. 2.2E). pBABE- and caMNK1-derived cell lines showed similar proliferation rates and clonogenic capacities (Supplementary Fig. 2.2F and G).

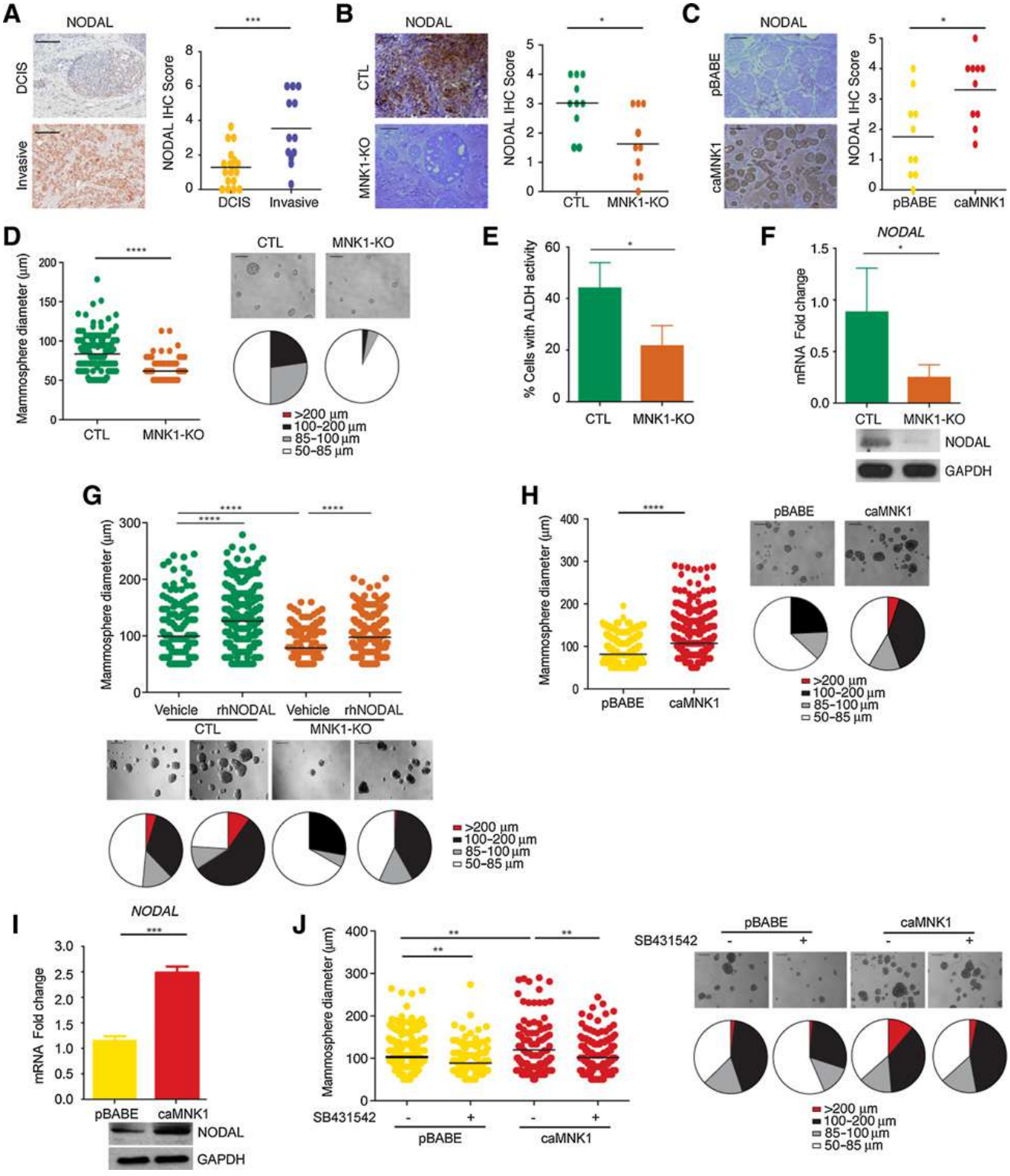
We characterized the *in vivo* tumorigenic effects of increased MNK1 activation in DCIS cells. DCIS-luc pBABE or caMNK1 cells were mixed with 50% matrigel and injected into the mammary fat pad of athymic nude mice (Supplementary Fig. 2.2H). We resected the xenografts at 1.5 weeks after DCIS tumor cell injection, expecting that caMNK1 xenografts might transition faster to IDC, compared to vector control derived tumors. Tumors are not palpable after 1.5 weeks of implantation, thus tumor outgrowth was estimated by IVIS imaging. Unlike the lack of proliferative advantage observed in 2D culture, caMNK1 expressing tumors were larger compared to pBABE control expressing tumors (Fig. 2.2H). caMNK1 expression in the tumor xenografts was confirmed by IHC for MNK1 (Fig. 2.2I). As expected, DCIS-luc pBABE cells formed lesions that were characteristic of DCIS, with positive staining for p63 (Fig. 2.2I). DCIS-luc caMNK1 cells on the other hand, formed lesions that consisted of a mixture of tubular-like and irregular structures, resembling IDC (Fig. 2.2I). Consistent with the previously reported role of MNK1 in promoting tumor invasion, we also found that all mice that had been injected with

Figure 2.2. MNK1 regulates the DCIS-IDC transition in vivo. **A.** Tumor outgrowth is measured by IVIS imaging. **B.** MNK1-KO is retained in the xenografts as confirmed by IHC. Scale bar = 50 μm. **C.** All CTL xenografts have progressed into IDC, while only 20% MNK1-KO tumors have progressed to an IDC-like morphology. **D.** All CTL xenografts have central necrosis, while only 20% MNK1-KO tumors have central necrosis. **E.** Survival curve of mice receiving CTL and MNK1-KO cells. **F.** Representative IVIS imaging showing complete tumor removal post-operation and tumor recurrence in animals receiving CTL cells. **G.** Percentage of animals presented with metastasis at different sites and representative images of metastasis in various tissues of mice receiving CTL and MNK1-KO cells. Scale bar = 200 μm. **H.** Tumor outgrowth is measured by IVIS imaging. **I.** pBABE xenografts maintain DCIS morphology, while caMNK1 tumors have progressed into a mixed morphology of DCIS/IDC. caMNK1 over-expression is maintained in the xenografts as confirmed by IHC. Scale bar = 50 μm. **J.** 100% of mice with caMNK1 tumors have micrometastasis in the mammary gland, while 30% of pBABE have micrometastasis. Micrometastases are indicated by arrows. 60% caMNK1 and 20% pBABE tumors have central necrosis. Scale bar = 200 μm. **K.** DCIS-Luc caMNK1 xenografts present with growth advantage over pBABE controls.

caMNK1-expressing DCIS cells had satellite lesions throughout the mammary fat pad, compared to only 3 out of 10 mice receiving pBABE cells (Fig. 2.2J). Additionally, 6 out of the 10 mice injected with caMNK1 cells have tumors with central necrosis, while only 2 out of 10 mice injected with pBABE cells present with tumors with central necrosis (Fig. 2.2J). Finally, we sought to confirm whether the injection of caMNK1 expressing DCIS cells resulted in enhanced tumor proliferation. To this end, we performed a second animal experiment, wherein DCIS-luc pBABE and caMNK1 cells were injected into the mammary fat pads of SCID mice, and tumor outgrowth was monitored, via IVIS imaging, over the course of 8 weeks. Tumor formation in the orthotopic mammary fat pad was significantly increased in mice injected with caMNK1 cells compared with those injected with the control pBABE cells (Fig. 2.2K, Supplementary Fig. 2.2I). We further interrogated whether the increase in tumor formation associated with caMNK1 expression was due to an increase in proliferation or a decrease in apoptosis. Using IHC, we stained pBABE and caMNK1 expressing tumor xenografts for phospho-histone H3, a marker of proliferation (32), and showed no difference between the pBABE and caMNK1 tumor groups (Supplementary Fig. 2.2J). We also included in our analysis, IHC staining for the proliferative marker Ki67, and this too showed no difference in pBABE versus caMNK1 expressing tumor xenografts (Supplementary Fig. 2.2J). However, we did find that our IHC staining for the levels of cleaved caspase-3, a marker of cell death, trended downward in caMNK1 expressing tumors (Supplementary Fig. 2.2J). These data suggest that caMNK1-derived tumors are larger than their pBABE counterparts, due to an evasion of apoptotic cell death. Cumulatively, the data presented here demonstrate that modulation of MNK1 influences the DCIS to IDC transition *in vivo*.

2.4.3 MNK1 regulates NODAL morphogen to control DCIS progression

We next investigated the molecular mechanism underlying the regulation of the DCIS to IDC transition by activated MNK1. Strizzi *et al* previously showed that NODAL protein levels are increased in IDC relative to in DCIS (18). As NODAL has been shown to be downstream of p38 (33), a major upstream activator of MNK1, we hypothesized that NODAL may be regulated in our model system. Consistent with the previous report from Strizzi *et al* (18), we observed that NODAL levels are higher in the DCIS patient samples with micro-invasion, as compared to pure DCIS lesions (Fig. 2.3A). MNK1-KO DCIS xenografts express reduced NODAL, as detected by IHC staining (Fig. 2.3B, Supplementary Fig. 2.3A). Conversely, the expression of constitutively



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activated MNK1 in DCIS cells results in an increased expression in NODAL, as determined by IHC for NODAL in pBABE- and caMNK1-derived xenografts (Fig. 2.3C, Supplementary Fig. 2.3B).

Examination of DCIS clinical specimens and cell lines has revealed the existence of stem cell-like sub-populations (22, 34). When grown on low adherent plates, DCIS cells can form mammospheres, which are formed by cells with the ability to survive anoikis and to self-renew (35); two functional properties of cancer stem cells (CSCs) (22). As NODAL signaling can promote self-renewal and tumorigenicity of cancer stem cells (36), we next examined whether MNK1 regulates DCIS mammosphere formation. MNK1-KO-derived mammospheres were smaller than those formed by their CTL counterparts (Fig. 2.3D). Another hallmark of CSCs is enhanced activity of aldehyde dehydrogenase (ALDH) (22). MNK1-KO mammospheres showed decreased ALDH activity (Fig. 2.3E), as assessed by flow cytometry, compared to their Cas9 CTL counterparts. Moreover, we determined whether depletion of MNK1 could reduce the tumor initiating cell subpopulation by performing FACS analysis for CD44hi/CD24lo populations in the MCFDCIS.com cells knocked out for MNK1. Our data showed that loss of MNK1 levels causes the percentage of $CD44^{hi}/CD24^{lo}$ cells in the DCIS population to decrease (Supplementary Fig. 2.3C).

In agreement with our *in vivo* data, the MNK1-KO-derived mammospheres also express less NODAL protein and mRNA (Fig. 2.3F). We next determined the interplay between MNK1 and NODAL in the formation of mammospheres. When we added recombinant human NODAL (rhNODAL) to CTL DCIS cells, the mammosphere size increased (Fig. 2.3G). Moreover, rhNODAL treatment of MNK1-KO cells partially rescued the decrease in mammosphere size observed when MNK1 expression is ablated (Fig. 2.3G).

Figure 2.3 MNK1 expression regulates NODAL morphogen expression. A. NODAL expression in DCIS versus invasive human breast samples. **B.** MNK1-KO xenografts have decreased NODAL levels compared to CTL tumors. Representative images are shown. **C.** caMNK1 xenografts have increased NODAL levels compared to pBABE tumors. Representative images are shown. **D.** MNK1-KO decreases mammosphere size in low adherent culture. **E.** MNK1-KO reduces $ALDH^{+}$ populations. **F.** MNK1-KO mammospheres express lower *NODAL* mRNA levels. **G.** rhNODAL treatment increases mammosphere size in CTL and MNK1-KO cells. **H.** caMNK1 overexpression increases mammosphere size in low adherent culture. **I.** caMNK1 mammospheres express higher *NODAL* mRNA levels. **J.** pBABE and caMNK1 mammosphere sizes can both be reduced by SB431542, a NODAL pathway inhibitor. (All scale bars = 200μm)

Together, these results suggest that MNK1-dependent regulation of NODAL is needed to regulate mammosphere size. To confirm and extend our results of MNK1-dependent regulation of NODAL expression, we also determined the impact of MNK1 deficiency on NODAL expression in two breast cancer cell lines, 66cl4 and MB-MDA-468. Similar to our results in DCIS cells, depleting MNK1 in 66cl4 cells using CRISPR-Cas9 technology, and by siRNA in MB-MDA-468 cells caused these cells to express less NODAL (Supplementary Fig. 2.3D and E). Moreover, biological data in MNK1 null 66cl4 cells shows a decrease in mammosphere growth, colony formation, and invasion (Supplementary Fig. 2.3F). Similarly, concomitant with the reduced NODAL levels observed when we silence MNK1 in MDA-MB-468 cells, their invasion is also impaired (Supplementary Fig. 2.3G).

caMNK1 expression increased both the size of DCIS mammospheres (Fig. 2.3H), and the expression of NODAL mRNA and protein in these spheres (Fig. 2.3I). Consistent with our observations in the caMNK1 tumor xenografts (Supplementary Fig. 2.2J), we see no change in Ki67 levels, but a decrease in the expression of cleaved caspase-3 in the caMNK1-derived mammospheres (Supplementary Fig. 2.3H). We also assessed tumor formation from limiting dilutions of inoculated pBABE or caMNK1 cells, wherein we injected 10,000, 1,000, or 100 cells. As shown on the graph in Supplementary Fig. 2.3I, the caMNK1 expressing cells have an increased tumor-initiating capability, compared to their pBABE control counterparts. 60 days post-injection, the 100 cell injected cohort of mice, did not show any signal by IVIS imaging. Finally, we determined whether the induction of NODAL expression by activated MNK1 was responsible for increased DCIS mammosphere formation. We utilized SB431542, an inhibitor routinely used to block NODAL signaling (37). Mammosphere size was significantly reduced in pBABE and caMNK1 cells, indicating again the importance of NODAL signaling to the increased and mammosphere size when MNK1 is constitutively activated (Fig. 2.3J).

2.4.4 MNK1 and NODAL regulate DCIS tumor invasion

MNK1 and NODAL can facilitate tumor invasion and metastasis, with the regulation of known mediators of invasion such as VIMENTIN (3, 8, 9). Here we observed that MNK1-KO tumor xenografts express less VIMENTIN, compared to the CTL-derived tumors (Fig. 2.4A, Supplementary Fig. 2.4A). In contrast, caMNK1 tumors show stronger staining for VIMENTIN, compared to the pBABE-derived tumors (Fig. 2.4B, Supplementary Fig. 2.4B). The changes in VIMENTIN expression that we observed by modulating MNK1 levels, led us to investigate the

invasive characteristics of our cell models. MNK1-KO cells express less VIMENTIN (Fig. 2.4C), and MNK1 deficiency restrained the invasion of DCIS cells (Fig. 2.4C). In comparison, caMNK1 overexpression promoted the invasion of DCIS cells, and increased VIMENTIN expression (Fig. 2.4D). Next, we tested the role that VIMENTIN might play in the increased invasion seen in cells that express a constitutively activated MNK1 (caMNK1). When VIMENTIN expression is repressed using siRNA, the caMNK1 cells lose their ability to invade (Fig. 2.4E), suggesting a role for VIMENTIN in the enhanced invasion phenotype related to constitutive MNK1 activity.

Finally, to assess whether MNK1-dependent regulation of NODAL contributes to cell invasion, CTL and MNK1-KO cells were treated with rhNODAL. We observed that rhNODAL treatment can enhance the invasion of DCIS CTL cells, and partially overcome the decrease in cell invasion observed in MNK1 depleted cells (Fig. 2.4F). Additionally, we next looked at cell invasion using pBABE and caMNK1 cells that had been transiently transfected with NODAL siRNA. As expected, caMNK1 cells transfected with control siRNA (siCTL) are more invasive than pBABE siCTL cells. NODAL knock-down reduced the invasion of both pBABE and caMNK1 cells, but caMNK1 siNODAL cells are still more invasive than their pBABE siNODAL counterparts (Fig. 2.4G).

2.4.5 MNK1 can be pharmacologically targeted to inhibit the DCIS to IDC transition

We next determined whether the effects we observed with ablation of MNK1 in DCIS cells would be phenocopied using SEL201, an orally bioavailable small molecule inhibitor of MNK1/2 activity (13). SEL201 induces a G2/M cell cycle arrest and inhibits proliferation of both pBABE and caMNK cells in a dose dependent manner (Supplementary Fig. 2.5A and B). Consistent with genetic ablation of MNK1 expression, SEL201 treatment efficiently reduced the number of colonies (Fig. 2.5A), ALDH activity (Fig. 2.5B) and the size of mammospheres (Fig. 2.5C), derived from pBABE and caMNK1 cells. SEL201 treatment also suppressed invasion of both pBABE and caMNK1 expressing DCIS cells, concomitant with reduced levels of NODAL (Fig. 2.5D).

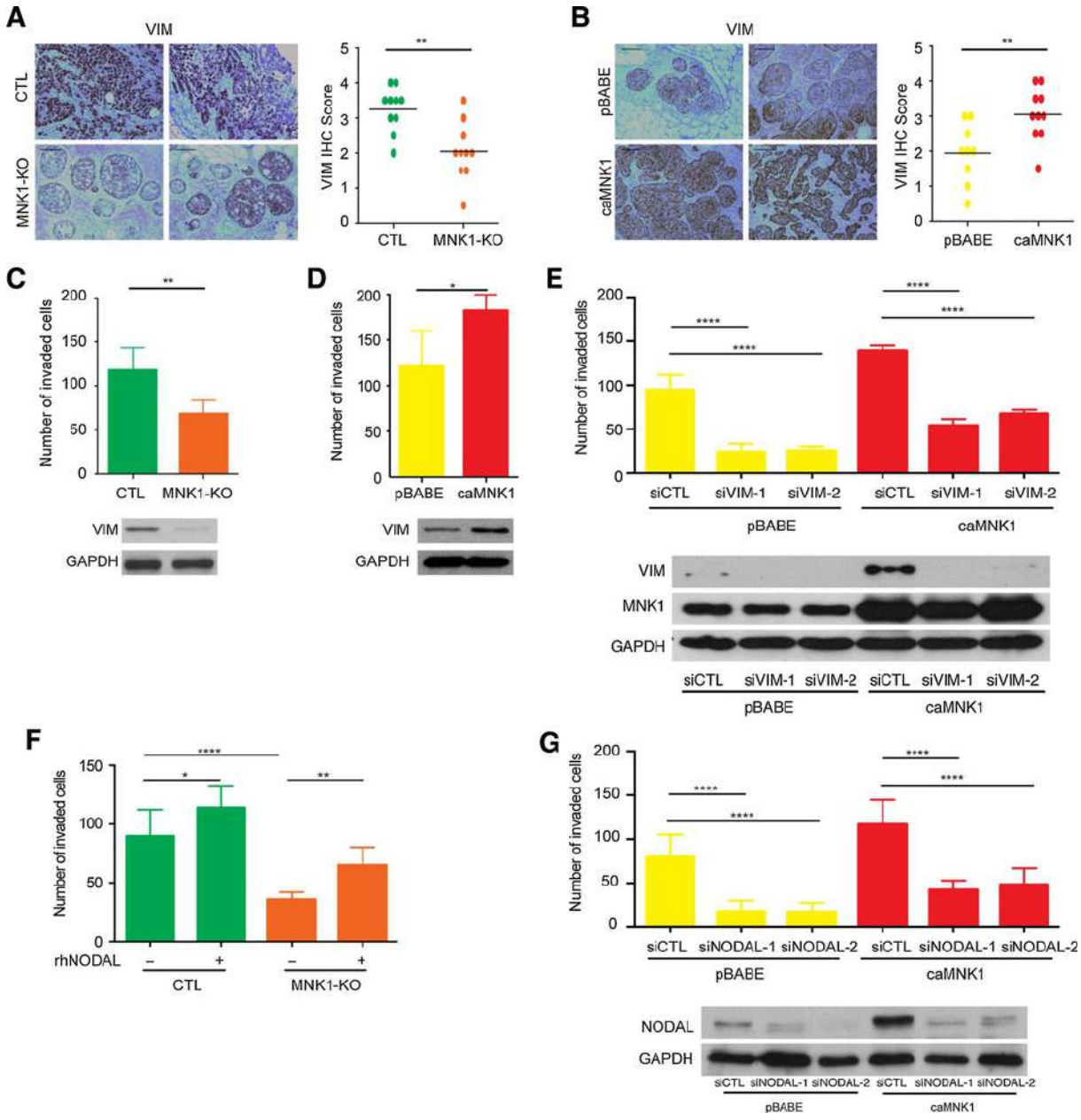

Figure 2.4 The MNK1/NODAL axis regulates migration and invasion. A. MNK-KO xenografts have decreased VIMENTIN (VIM) levels compared to CTL tumors. Representative images from 2 tumors are shown. Scale bar = 200 μm. **B.** caMNK1 xenografts have increased VIMENTIN (VIM) levels compared to pBABE control tumors. Representative images from 2 tumors are shown. Scale bar = 200 μm. **C.** MNK1-KO cells have impaired capacity of migrate and invade through Collagen I in transwells. **D.** caMNK1 cells showed increased capacity to migrate and invade through Collagen I in transwells. **E.** VIMENTIN knockdown by siRNA decreases the invasive capacity of pBABE and caMNK1 cells. **F.** rhNODAL treatment increases the migration and invasion of MNK1-KO cells. **G.** Transient knockdown of NODAL decreases the invasive capacity of pBABE and caMNK1 cells.

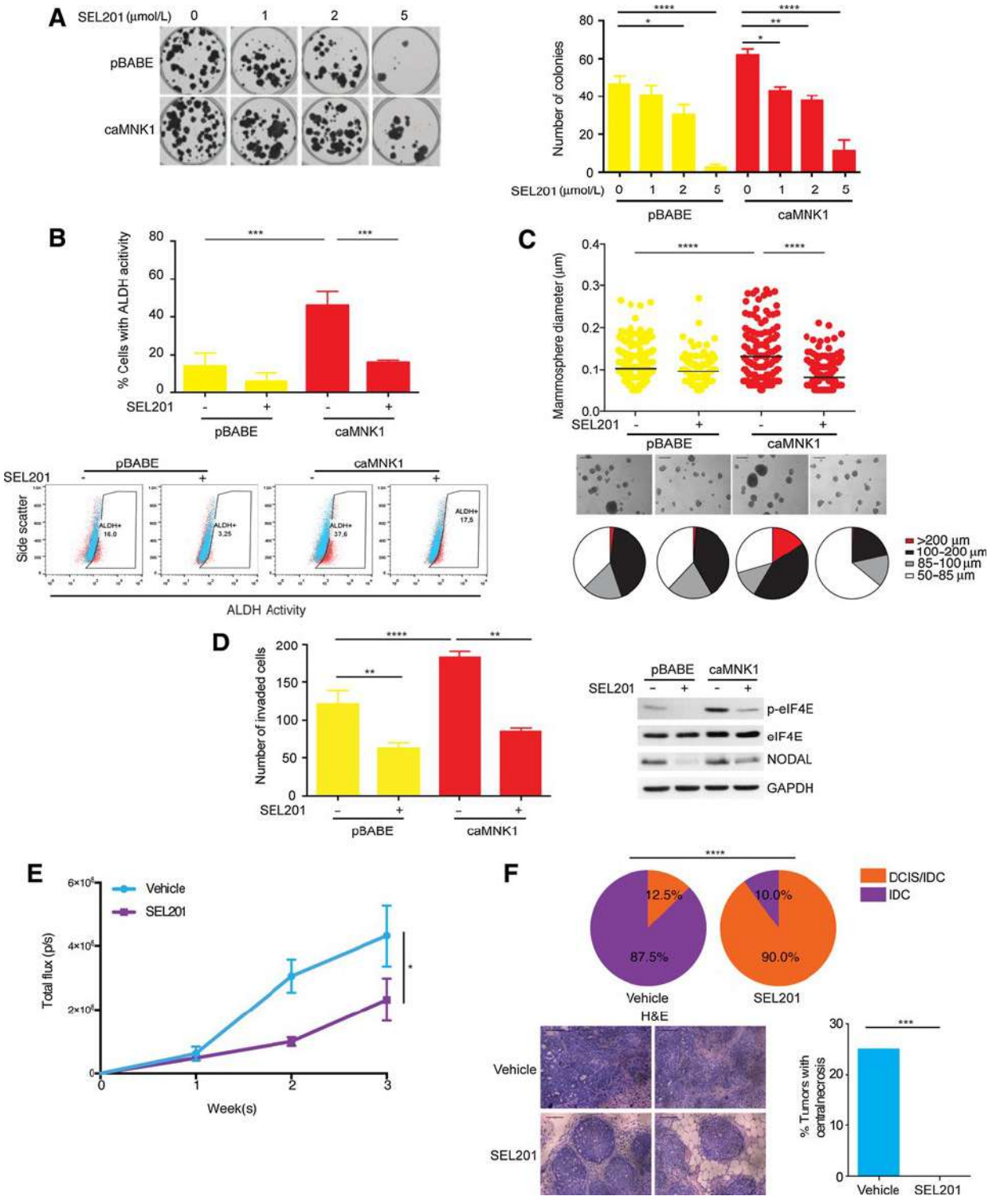

Figure 2.5. Pharmacologically targeting MNK1 inhibits the DCIS/IDC transition A. SEL201 inhibits DCIS-Luc pBABE/caMNK1 colony formation in a dose-dependent manner. **B.** SEL201 decreases the ALDH positive population in caMNK1 cells. **C.** SEL201 decreases mammosphere size formed by caMNK1 cells. Scale bar = 200 μm **D.** SEL201 decreases the invasive capacity of caMNK1 cells. **E.** Primary tumor outgrowth over 3 weeks in vehicle versus SEL201-treated animals **F.** MNK inhibitor SEL201 treatment slows down DCIS to IDC progression in nude mice and decreases the percentage of tumors with detectable central necrosis.

Having shown that MNK1 regulates the DCIS to IDC conversion in animal models, we next investigated whether pharmacologically blocking MNK1 would inhibit the progression of DCIS to invasive disease. DCIS-Luc CTL cells were orthotopically injected into the mammary glands of nude mice, and then the mice were randomized to either vehicle control or SEL201 treatment groups (Supplementary Fig. 2.5C). SEL201 was delivered by gavage at a dose of 75mg/kg per day, which we have previously shown concomitantly suppresses MNK1 activity and lung metastasis (13). SEL201 significantly decreased the primary tumor size after a three week-treatment (Fig. 2.5E). Furthermore, xenografts from the SEL201 group showed a reduced percentage of IDC (10% of tumours) compared to the vehicle group (87.5% of tumors) (Fig. 2.5F). Consistent with our previous work (13), SEL201 showed no overt systemic toxicity, as evidenced by body weight (Supplementary Fig. 2.5D) and tests for liver function (ALT and AST) (Supplementary Fig. 2.5D). SUM225 cells are also commonly used to model DCIS, and consistent with the data shown thus far, reducing MNK1 levels using siRNA in SUM225 induces a loss of NODAL expression, while SUM225-expressing caMNK1 express increased levels of NODAL (Supplementary Fig. 2.5E). Compared to MCFDCIS.com cells, SUM225 cells slowly transition from DCIS to IDC following intraductal injections, a technique which minimizes disturbance to the mammary gland microenvironment (38). SEL201 also inhibits colony formation of SUM225 cells in vitro, and slows down the transition from DCIS to IDC in the SUM225 intraductal model of DCIS (Supplementary Fig. 2.5F and 2.5G). In summary, our results provide evidence to show the feasability of inhibiting MNK1 to slow the conversion of DCIS to IDC.

2.5 Discussion

The mechanism underlying the transition of DCIS to IDC remains poorly understood. Previous studies have identified several potential factors that facilitate the conversion to invasive breast cancer, such as p63/MT1-MMP (39), SMARCE1 (40), and Singleminded-2s (SIM2s) (29). Unfortunately, many of these proteins have proven difficult to therapeutically target as a means to block disease progression in pre-clinical models. Herein, we identify the MNK1/NODAL signaling axis as a key molecular pathway regulating the progression of DCIS to IDC and breast cancer recurrence as metastatic disease (Fig. 2.6). Moreover, MNK1 inhibitors such as SEL201,

could therapeutically block NODAL signalling in patients diagnosed with NODAL positive DCIS as a viable means to suppress invasive disease.

Our data show a positive correlation between the activity of MNK1 and the expression of NODAL and VIMENTIN, two critical regulators of invasion and metastasis. Although the significance of MNK/eIF4E in breast cancer tumorigenesis, metastasis and therapeutic resistance is recognized (41), MNK1 has other downstream substrates whose role in breast cancer is not well characterized. MNK1 also regulates the expression of pro-inflammatory and pro-tumorigenic cytokines, including TNF-α, IL6, TGFB (42). Our results have not only added NODAL as a novel downstream cytokine controlled by MNK1, but also defines a role for MNK1/NODAL signaling in controlling CSC-like phenotypes. Although previous research has reported on the potential role of MNK1 in maintaining CSC properties in acute myeloid leukemia (AML), chronic myeloid leukemia (CML) (11) and glioblastoma (43), very little is known about the function of MNK1 in breast CSCs. Using CRISPR/Cas9 technology to selectively knock-out MNK1 expression in human DCIS cells, we show that MNK1 deficiency is sufficient to impair high ALDH enzymatic activity, and to reduce the percentage of CD44hi/CD24lo cells, both known characteristics of cancer stem cells in numerous malignancies

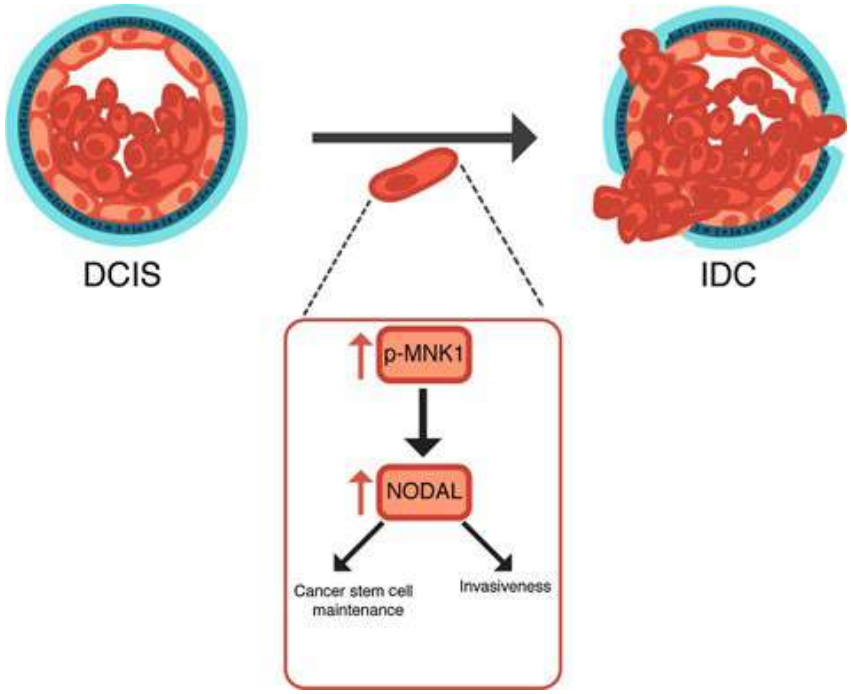

Figure 2.6. A model depicting the MNK1/NODAL axis during the DCIS to IDC transition. Our data suggest that high MNK1 activity promotes the expression of NODAL. NODAL, in turn, promotes cell invasion and cancer stem cell maintenance.

(44). This could be clinically important as DCIS CSC subpopulations are thought to be responsible for resistance to radiotherapy and potential disease recurrence (45, 46). We thus speculate that pharmacologically inhibiting MNK1 will limit the plasticity of DCIS cells via downregulation of NODAL and forestall resistance to radiotherapy.

Our study has shown that NODAL mRNA levels are controlled by MNK1, and MNK1/2 have a traditional role as regulators of oncogenic mRNA translation. Therefore, it is possible that MNK1 is regulating NODAL on the level of mRNA translation. In fact, our work and that of other labs, has shown that NODAL works with a positive feedback loop to activate its own transcription (47, 48). We posit that MNK1 may inhibit NODAL protein synthesis, and this in turn would lead to a corresponding suppression in NODAL mRNA. It is also possible that MNK1 regulates NODAL by a yet to be ascribed role in the nucleus. MNK1/2 are also found in the nucleus, suggesting activities beyond their role in protein synthesis (42, 49). Interestingly, we have observed nuclear localization of MNK1 in our murine xenografts and clinical breast samples, in this study (Fig. 1A), and in our melanoma work (13). This observation is line with MNK1 containing both nuclear localization and export signals, and prior work showing that MNK1 can shuttle between the nucleus and cytoplasm (50, 51). Access to CRISPR/Cas9 technology for selectively deleting MNK1 and MNK2 will no doubt lead to defining novel biological functions of these kinases.

A question raised by our work is identifying the molecular mechanism by which MNK1 signaling is activated in invasive disease. Our data show that NODAL lies downstream of MNK1, however, it is entirely possible that NODAL can itself feedback to activate MNK1. TGFB super-family cytokines, such as NODAL and Activin, utilize non-canonical MAPK cascades to regulate breast tumor progression (15, 24, 49), however it remains unclear whether MNK1 cooperates with NODAL to drive DCIS towards invasive disease. Our results also demonstrated that exogenous NODAL treatment can increase DCIS mammosphere size and it is tempting to speculate that this cytokine can also induce MNK1 phosphorylation. Indeed, NODAL has been shown to activate ERK1/2, immediately upstream of MNK1, in breast cancer cells (37).

We have demonstrated the feasibility of pharmacologically blocking MNK1/2 activity *in vivo*, resulting in blocking of the progression of DCIS to invasive disease. Together, our data

contribute to the increasing discussion about the clinical applications of MNK1/2 inhibitors in cancer.

2.6 Acknowledgments

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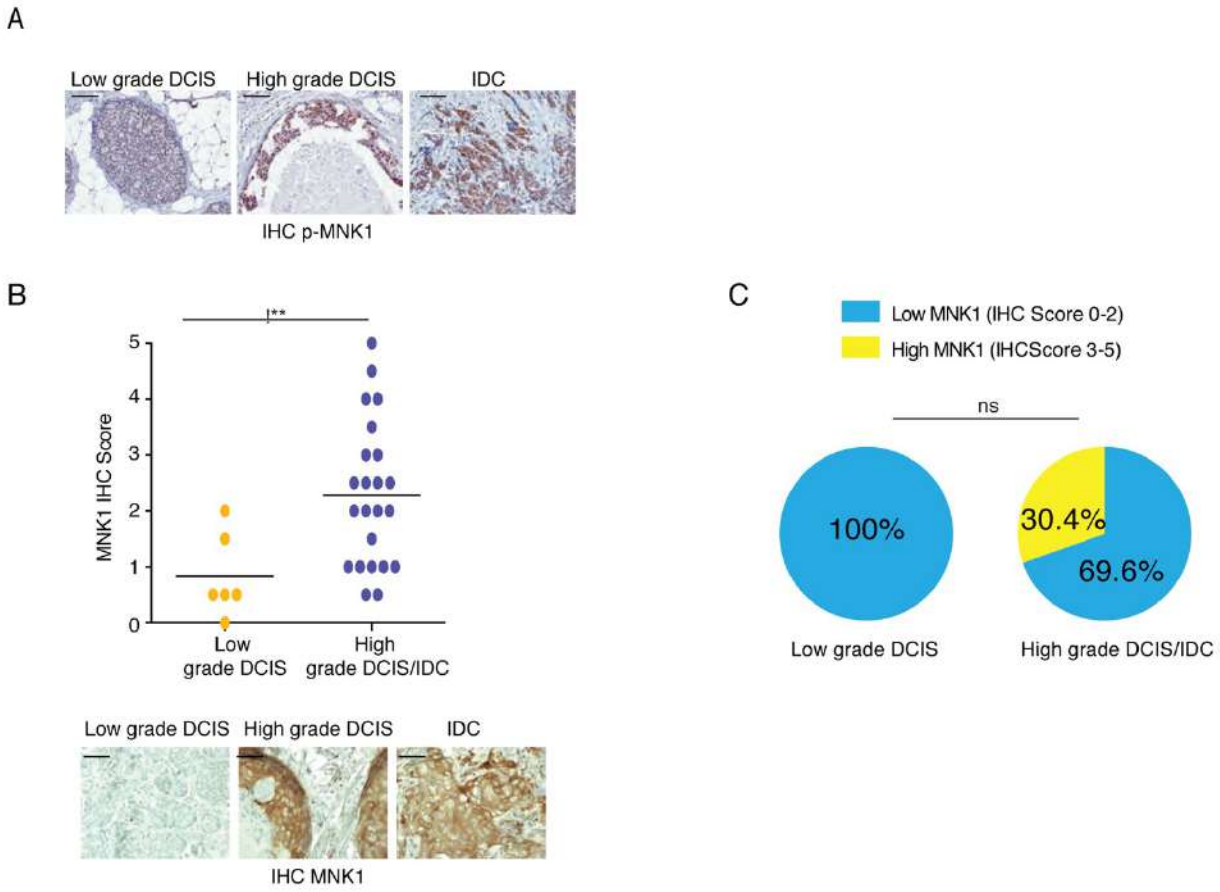
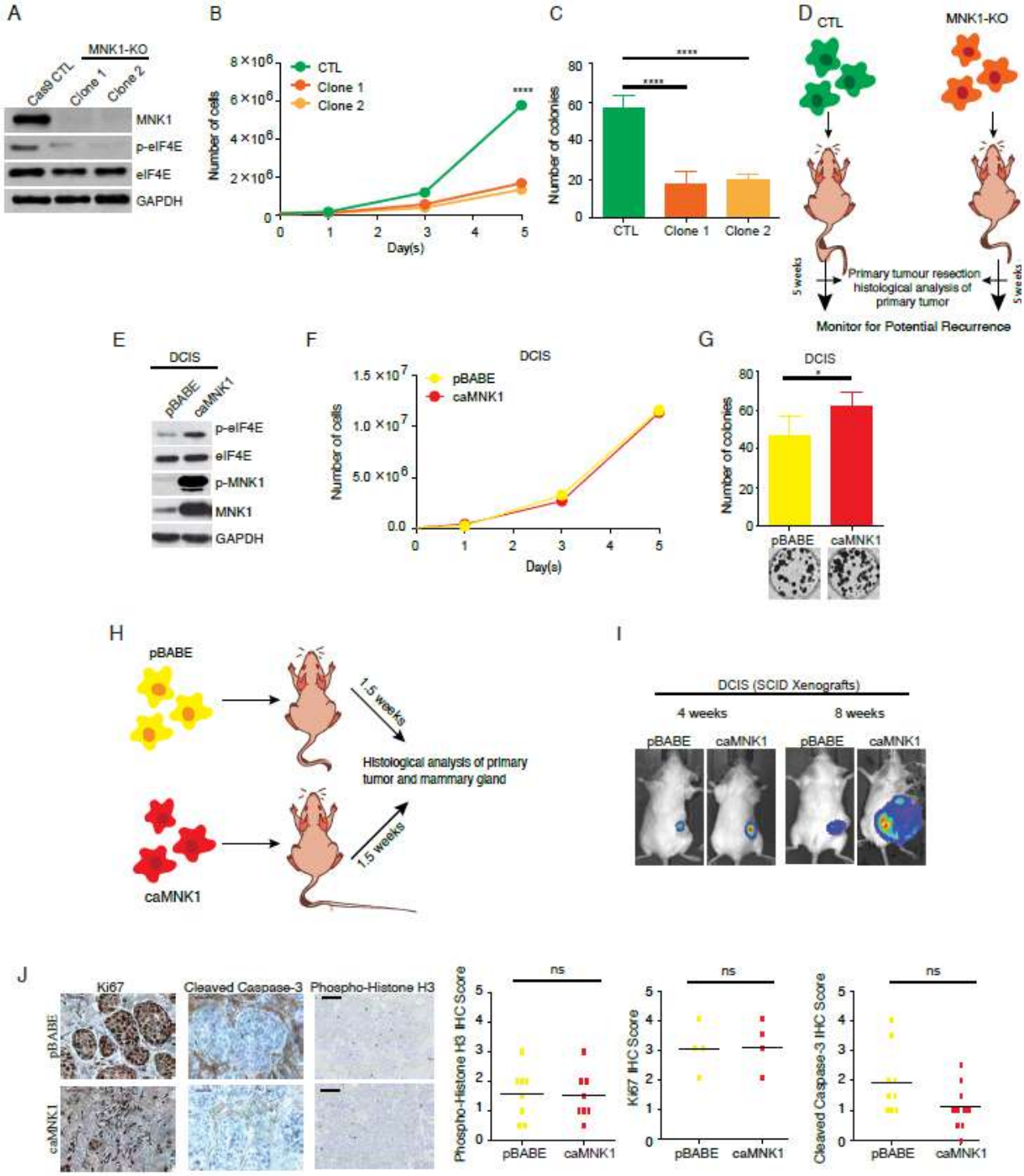
2.8 Supplementary Materials



Supplementary Figure 2.1 Increased MNK1 activity in IDC.
A. Zoomed in images from Fig.1A to show that phospho-MNK1 levels are higher in high grade DCIS and IDC than low grade DCIS. Scale bar = 100 µm. **B.** MNK1 levels are higher in high grade DCIS/IDC samples than low grade DCIS samples. Scale bar = 200 µm. **C.** Increased percentage of samples with high MNK1 staining in high grade DCIS/IDC compared to low grade DCIS.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MNK1
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CTL
Clone 1
Clone 2
D
CTL
MNK1-KO
5 weeks
Primary tumour resection
histological analysis of
primary tumor
5 weeks
Monitor for Potential Recurrence
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DCIS
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caMNK1
p-eIF4E
eIF4E
p-MNK1
MNK1
GAPDH
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caMNK1
Number of cells
Day(s)
G
DCIS
Number of colonies
pBABE
caMNK1
H
pBABE
caMNK1
1.5 weeks
Histological analysis of primary
tumor and mammary gland
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caMNK1
pBABE
caMNK1
J
Ki67
Cleaved Caspase-3
Phospho-Histone H3
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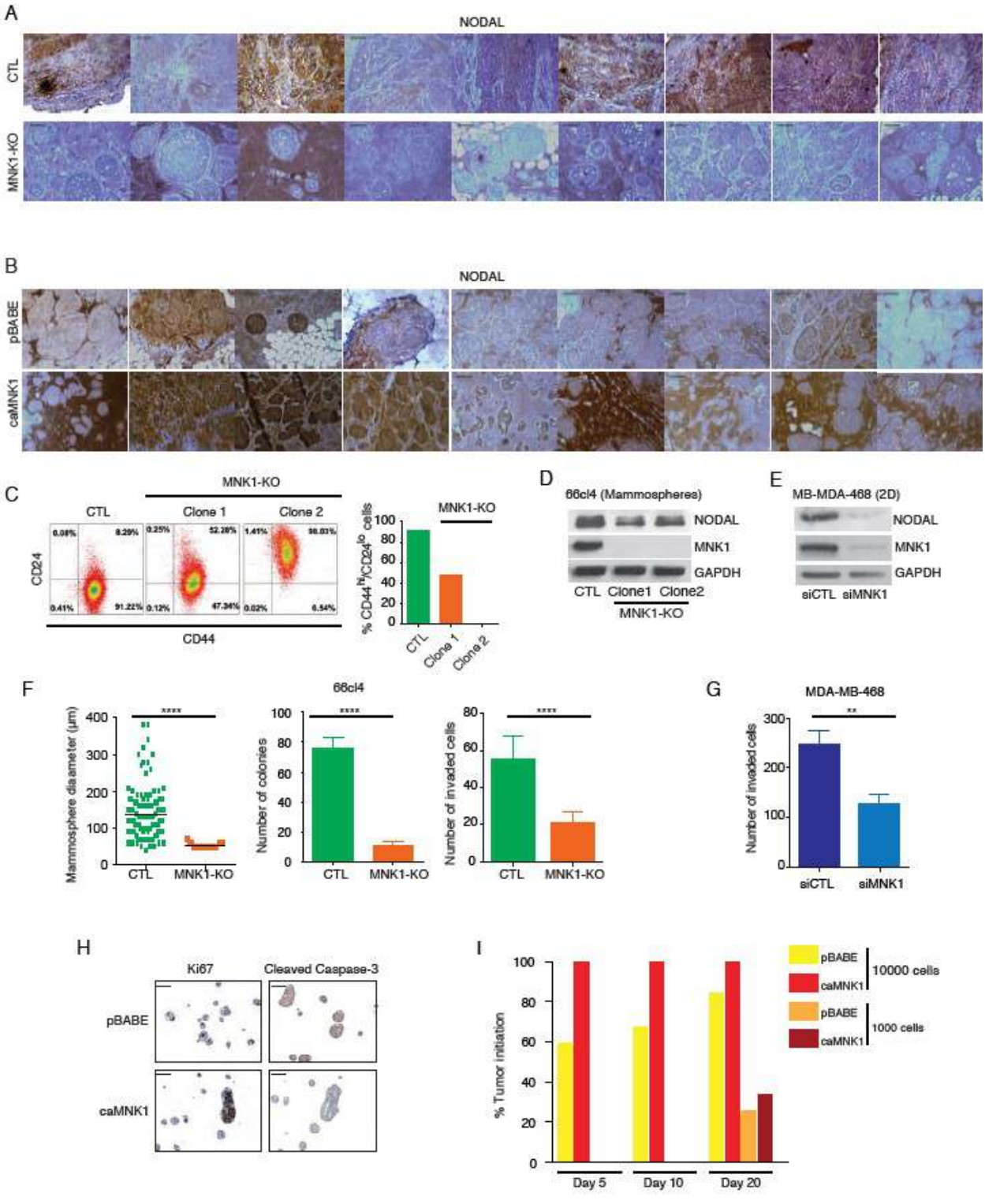
Cleaved Caspase-3 IHC Score
ns
pBABE
caMNK1

Supplementary Figure 2.2 Modulation of MNK1 affects colony formation in 2D cell culture. A. MNK1 knock-out by CRISPR-Cas 9 technology in 2 independent clones is confirmed by WB. **B.** MNK1-KO inhibits cell proliferation. **C.** MNK1-KO inhibits colony formation. **D.** Schematic depicting timing and measured outcomes in CTL and MNK1-KO xenografts. **E.** caMNK1 overexpression is confirmed by WB. **F.** caMNK1 overexpression does not affect DCIS proliferation. **G.** caMNK1 overexpression increases DCIS colony formation. **H.** Schematic depicting timing and measured outcomes in pBABE and caMNK1 xenografts. **I.** IVIS imaging of pBABE and caMNK1 xenografts in SCID mice. **J.** pBABE and caMNK1 xenografts express similar levels of Ki67 and phospho-histone H3. Cleaved caspase-3 levels in caMNK1 xeonografts trended downward compared to pBABE.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CD24
CD44
% CD44hi/CD24lo cells
MNK1-KO
CTL
Clone 1
Clone 2
D
66cl4 (Mammospheres)
NODAL
MNK1
GAPDH
CTL Clone1 Clone2
MNK1-KO
E
MB-MDA-468 (2D)
NODAL
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GAPDH
siCTL siMNK1
F
66cl4
Mammosphere diameter (µm)
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Number of invaded cells
CTL
MNK1-KO
G
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Number of invaded cells
siCTL
siMNK1
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Ki67
Cleaved Caspase-3
pBABE
caMNK1
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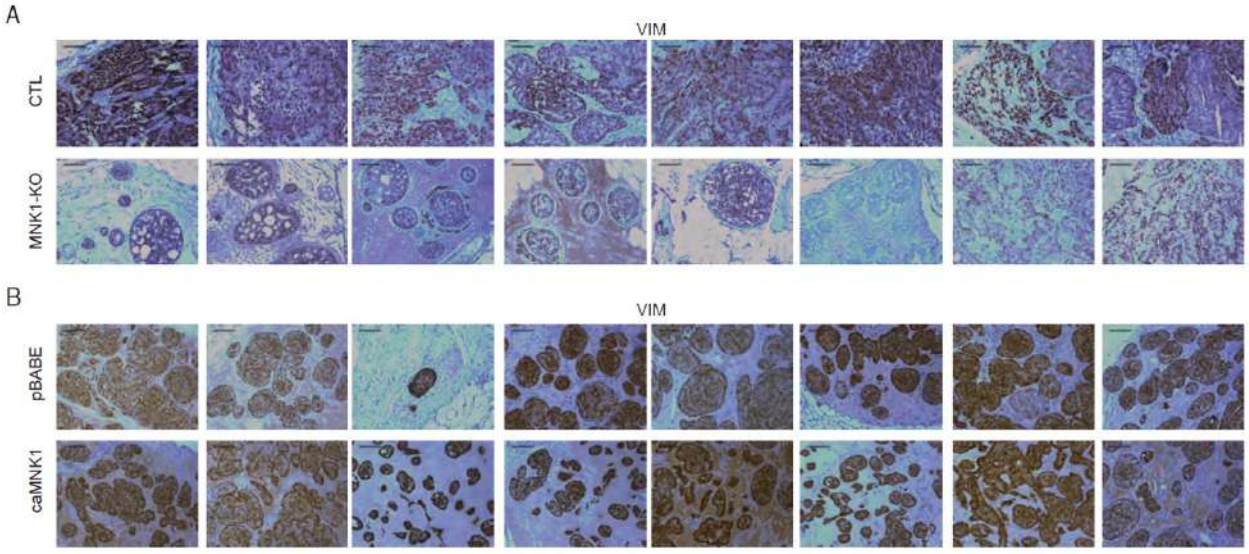
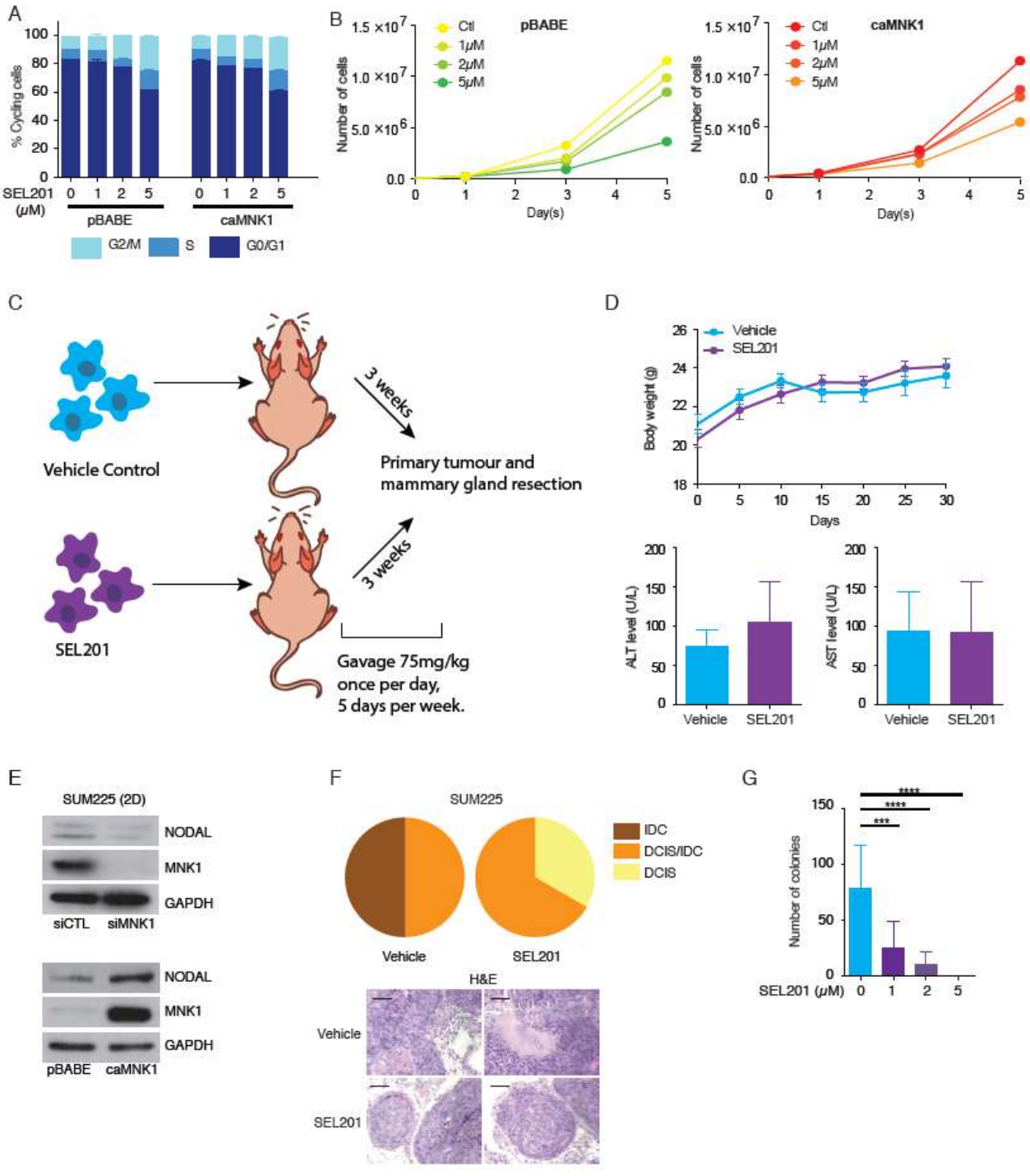
% Tumor initiation
Day 5
Day 10
Day 20
pBABE
caMNK1
10000 cells
pBABE
caMNK1
1000 cells

Supplementary Figure 2.3 NODAL levels are regulated by MNK1. A. MNK1-KO xenografts have decreased NODAL levels compared to CTL tumors. Representative images from 18 tumors are shown. Scale bar = 200 μm. **B.** caMNK1 xenografts have increased NODAL levels compared to pBABE tumors. Representative images from 18 tumors are shown. Scale bar = 200 μm. **C.** Loss of MNK1 causes decrease of CD44hi/CD24lo cells in the DCIS population to decrease. **D.** 66cl4 MNK1-KO mammospheres have decreased NODAL levels compared to CTL mammospheres. **E.** MNK1 knock-down by siRNA decreases NODAL levels and cell invasion in MB-MDA-468 cells. **F.** 66cl4 MNK1-KO cells form mammospheres with smaller diameters, showed impaired colony formation ability and decreased invasion compared to CTL mammospheres. **G.** MNK1 knockdown by siRNA decreases MB-MDA-468 invasion. **H.** caMNK1 expressing DCIS mammospheres express decreased cleaved caspase 3 but similar Ki67 levels compared to pBABE mammospheres. **I.** Tumor initiation rate of DCIS-Luc pBABE and caMNK1 *in vivo* limiting dilution experiment.



Supplementary Figure 2.4 VIMENTIN levels are regulated by MNK1.
A. MNK-KO xenografts have decreased VIMENTIN (VIM) levels compared to CTL tumors. Representative images from 16 tumors are shown. Scale bar = 200 μm. B. caMNK1 xenografts have increased VIMENTIN (VIM) levels compared to pBABE control tumors. Representative images from 16 tumors are shown. Scale bar = 200 μm.


A
% Cycling cells
SEL201 (μM)
pBABE
caMNK1
G2/M
S
G0/G1
B
pBABE
caMNK1
Ctl
1μM
2μM
5μM
Number of cells
Day(s)
C
Vehicle Control
SEL201
3 weeks
Primary tumour and mammary gland resection
Gavage 75mg/kg once per day, 5 days per week.
D
Vehicle
SEL201
Body weight (g)
Days
ALT level (U/L)
AST level (U/L)
E
SUM225 (2D)
NODAL
MNK1
GAPDH
siCTL
siMNK1
pBABE
caMNK1
F
SUM225
IDC
DCIS/IDC
DCIS
H&E
G
Number of colonies
SEL201 (μM)

Supplementary Figure 2.5 A small molecule inhibitor of MNK1 inhibits the DCIS-IDC transition.
A. SEL201 induces a G2/M arrest in pBABE and caMNK1 cells in a dose dependent manner. **B.** SEL201 inhibits pBABE and caMNK1 cell proliferation in a dose-dependent manner. **C.** Schematic depicting the timing and measured outcomes of SEL201 xenograft experiment. **D.** SEL201 has no effect on mouse body weight (upper panel), or on liver toxicity, as assessed by ALT and AST (lower panels) levels. **E.** MNK1 knock-down by siRNA decreases NODAL levels in SUM225 cells (upper panel). caMNK1 expressing SUM225 cells express increased NODAL levels compared to pBABE controls (lower panel). **F.** SUM225 primary tumor outgrowth over 9 weeks in vehicle versus SEL201-treated animals. MNK inhibitor SEL201 treatment slows down DCIS to IDC progression in NOD/SCID mice. **G.** SEL201 inhibits SUM225 colony formation.

Supplementary Table 2.1 Antibodies

Target protein	Antibody	Usage
eIF4E	#610269 BD Transduction Laboratories	WB
GAPDH	#2118S Cell Signaling	WB
MNK1	#2195S Cell Signaling	WB & IHC
NODAL	Ab55676 Abcam	WB & IHC
p63	Ab735 Abcam	IHC
phospho-eIF4E	#9741S Cell Signaling	WB
phospho-Histone H3 Ser10	#09-797 EMD Millipore	IHC
phospho-MNK1	#2111S Cell Signaling	WB & IHC
VIMENTIN	#550513 BD Pharmingen	WB & IHC

Supplementary Table 2.2 Statistical analysis

Figure	Number of Samples	Statistical Analysis	P Value
1A	Low grade DCIS n=13 High grade DCIS and IDC n=27	Wilcoxon-Mann-Whitney test	p=0.0036
1B	Low grade DCIS n=13 High grade DCIS and IDC n=27	Chi-square test of independence	p=0.0132
2A	CTL n=10 MNK1-KO n=16 (2 tumors on each animal)	Two-way ANOVA	CTL vs MNK1-KO tumor growth Week 0 - p>0.9999 Week 1 - p=0.9998 Week 2 - p=0.8747 Week 3 - p=0.0042 Week 4 - p<0.0001 Week 5 - p<0.0001
2C	CTL n=10 MNK1-KO n=10 (2 tumors on each animal)	Chi-square test of independence	CTL vs MNK1-KO %IDC - p=0.0001
2D	CTL n=10 MNK1-KO n=10 (2 tumors on each animal)	Chi-square test of independence	CTL vs MNK1-KO %Necrosis - p=0.0001
2E	CTL n=5 MNK1-KO n=5	Log-rank test	p=0.1268
2H	(Nude Mice) pBABE n=10 caMNK1 n=10	T test (do not assume same SD)	p=0.0002
2J	(Nude Mice) pBABE n=10 caMNK1 n=10	Chi-square test of independence	%Necrosis - p<0.0001 %Micrometastasis - p=0.0339
2K	(SCID Mice) pBABE n=10 caMNK1 n=10	Two-way ANOVA	pBABE vs caMNK1 Week 1-Week 5 - p>0.9999 Week 6 - p=0.9998 Week 7 - p=0.0161 Week 8 - p<0.0001
3A	Human Sample DCIS n=17 IDC n=12	Wilcoxon-Mann-Whitney test	p=0.0001
3B	(Nude Mice) CTL n=10 MNK1-KO n=10	Wilcoxon-Mann-Whitney test	p=0.0107
3C	(Nude Mice) pBABE n=10 caMNK1 n=10	Wilcoxon-Mann-Whitney test	p=0.0163

Figure	Number of Samples	Statistical Analysis	P Value
3D	4 independent experiments Number of mammospheres quantified: CTL=176 MNK1-KO=78 (spheres<0.05mm^2 are excluded from quantification)	T test (do not assume equal SD)	p<0.0001
3E	3 independent experiments 9 data points for both groups	T test (do not assume equal SD)	p=0.0401
3F	3 independent experiments 9 data points for both groups	T test (do not assume equal SD)	p=0.0059
3G	3 independent experiments Number of mammospheres quantified: CTL Vehicle=169 CTL rhNODAL=317 MNK1-KO Vehicle=204 MNK1-KO rhNODAL=227 (spheres<0.05mm2 are excluded from quantification)	One-way ANOVA	CTL Vehicle vs rhNODAL - p<0.0001 MNK1-KO Vehicle vs rhNODAL -p<0.0001 CTL Vehicle vs MNK1-KO Vehicle-p<0.0001
3H	4 independent experiments	T test (do not assume equal SD)	p<0.0001
3I	3 independent experiments	T test (do not assume equal SD)	p=0.0003
3J	3 independent experiments	One-way ANOVA	pBABE Vehicle vs SB436542 - p=0.0029 caMNK1 Vehicle vs SB436542 - p=0.0012 pBABE Vehicle vs caMNK1 Vehicle - 0.0011
4A	(Nude Mice) CTL=10 MNK1-KO=10	Wilcoxon-Mann-Whitney test	p=0.0035
4B	(Nude Mice) pBABE n=10 caMNK1 n=10	Wilcoxon-Mann-Whitney test	p=0.0094
4C	3 independent experiments 9 data points quantified for both groups	T test (do not assume equal SD)	p=0.0034
4D	3 independent experiments 9 data points quantified for both groups	T test (do not assume equal SD)	p=0.0450

Figure	Number of Samples	Statistical Analysis	P Value
4E	3 independent experiments 9 data points quantified for all groups	One-way ANOVA	pBABE siCTL vs siVIM-1 - p<0.0001 pBABE siCTL vs VIM-2 - p<0.0001 caMNK1 siCTL vs VIM-1 - p<0.0001 caMNK1 siCTL vs VIM-2 - p<0.0001 pBABE siCTL vs caMNK1 siCTL – p=0.0006
4F	3 independent experiments 9 data points quantified for all the groups	One-way ANOVA	CTL Vehicle vs rhNODAL - p=0.0102 MNK1-KO Vehicle vs rhNODAL - p=0.0013 CTL Vehicle vs MNK1-KO Vehicle- p<0.0001
4G	3 independent experiments Data points: pBABE siCTL=19 pBABE siNODAL-1=21 pBABE siNODAL-2=21 caMNK1 siCTL=20 caMNK1 siNODAL-1=20 caMNK1 siNODAL-2=16	One-way ANOVA	pBABE siCTL vs siNODAL-1 - p<0.0001 pBABE siCTL vs siNODAL-2 - p<0.0001 caMNK1 siCTL vs siNODAL-1 - p<0.0001 caMNK1 siCTL vs siNODAL-2 - p<0.0001 pBABE siCTL vs caMNK1 siCTL - p<0.0001
5A	4 independent experiments	One-way ANOVA	pBABE 0 vs 1 - p>0.9999 pBABE 0 vs 2 - p=0.0348 pBABE 0 vs 5 - p<0.0001 pBABE 0 vs caMNK1 0 - p=0.0770 caMNK1 0 vs 1 - p=0.0159 caMNK1 0 vs 2 - p=0.0011 caMNK1 0 vs 5 - p<0.0001
5B	3 independent experiments	One-way ANOVA	pBABE Vehicle vs SEL201 - p=0.2666 caMNK1 Vehicle vs SEL201 - p=0.0005 pBABE Vehicle vs caMNK1 Vehicle - p=0.0004
5C	3 independent experiments	One-way ANOVA	pBABE Vehicle vs SEL201 - p=0.2005 caMNK1 Vehicle vs SEL201 - p<0.0001 pBABE Vehicle vs caMNK1 Vehicle - p<0.001
5D	3 independent experiments	One-way ANOVA	pBABE Vehicle vs SEL201 - p=0.0031 caMNK1 Vehicle vs SEL201 - p=0.0024 pBABE Vehicle vs caMNK1 Vehicle - p<0.0001
5E	Vehicle n=12 SEL201 n=12 (2 tumors on each animal)	Two-way ANOVA	Vehicle vs SEL201 Week 0 - p>0.9999 Week 1 - p=0.9995 Week 2 - p=0.0293 Week 3 - p=0.0412
5F	Vehicle n=12 SEL201 n=12 (2 tumors on each animal)	Chi-square test of independence	% IDC - p<0.0001 %Necrosis - p=0.0002
S1B	Low grade DCIS n=6 High grade DCIS and IDC n=23	T test (do not assume equal SD	p=0.00059
S1C	Low grade DCIS n=6 High grade DCIS and IDC n=23	Chi-square test of independence	p=0.2885
S2B	3 independent experiments	Two-way ANOVA	CTL vs MNK1-KO Clone 1 Day 1 - p=0.8609 Day 3 - p<0.0001 Day 5 - p<0.0001 CTL vs MNK1-KO Clone 2 Day 1 - p=0.8794

Figure	Number of Samples	Statistical Analysis	P Value
			Day 3 - p<0.0001 Day 5 - p<0.0001
S2C	3 independent experiments	One-way ANOVA	CTL vs MNK1-KO Clone 1 p<0.0001 CTL vs MNK1-KO Clone 2 p<0.0001
S2F	3 independent experiments	Two-way ANOVA	pBABE vs caMNK1 p>0.9999 at all time points
S2G	3 independent experiments	T test (do not assume equal SD)	p=0.0213
S2J	Phospho-Histone H3 pBABE=8 caMNK1=8 Ki67 pBABE=4 caMNK1=4 Cleaved caspase 3 pBABE=9 caMNK1=10	Wilcoxon-Mann-Whitney test	pBABE vs caMNK1 Phospho-Histone H3 - p=0.9977 Ki67 - p=0.9714 Cleaved caspase-3 - p=0.676
S3F	3 independent experiments for all panels	T test (do not assume equal SD)	66cl4 CTL vs MNK1-KO: Mammosphere size p<0.0001 Colony formation p<0.0001 Invasion p<0.0001
S5B	Vehicle n=6 SEL201 n=6 (2 tumors on each animal)	Two-way ANOVA T test (do not assume equal SD)	Vehicle vs SEL201 Body weight (Two-way ANOVA) - ns ALT - p=0.2477 AST - p=0.9576
S5E	Vehicle n=4 SEL201 n=6	Chi-square test of independence	p=0.1084
S5G	3 independent experiments	One-way ANOVA	SEL201 0 vs 1 μM p=0.0001 SEL201 0 vs 2 μM p<0.0001 SEL201 0 vs 5 μM p<0.0001

Supplementary Table 2.3 Metastasis data for CTL versus MNK1-KO mice.

Group	Animal ID	Survival Duration (Days)	Reason for Death	Necropsy/Histology Findings
CTL	B3231	62	Metastasis	Lung metastasis. Metastasis infiltrating kidney capsule.
	B3232	83	Metastasis	Metastasis found in the abdominal cavity, near the small bowel.
	B3233	99	Metastasis	Metastasis found in the abdominal cavity, near the pancreas.
	B3234	97	Metastasis	Metastasis found near the kidneys.
	B3235	105	Metastasis	Metastasis found in the lungs, chest cavity, small bowel and abdominal cavity. Metastasis found adjacent to abdominal smooth muscles and ribs.
MNK1-KO	B3227	56	Unknown	Found dead on day 56. Necropsy was not performed.
	B3227	140	Sacrificed at end of the experiment	No metastasis was found.
	B3229	140		
	B3230	140		
	B3237	140		

Chapter 3. phospho-eIF4E/IL-33 drives immune evasion and lung metastasis in postpartum breast cancer (PPBC)

(Manuscript in preparation)

Program of research: In chapter 2, we have demonstrated a central role of tumor intrinsic MNK1/NODAL axis in the conversion of DCIS to IDC. However, our knowledge of how aberrant mRNA translation regulates breast tumor immunity, and which pro-tumorigenic and pro-inflammatory factors are involved, remains limited. PPBC is an aggressive subtype of breast cancer characterized by robust immune cell influx. In this chapter, we plan to understand how aberrant MNK1/2-eIF4E activity contributes to PPBC immune evasion, and to investigate possible strategies to enhance the effectiveness of immune-checkpoint blockade in PPBC by targeting this pathway.

Rationale & Hypothesis: It remains unclear how aberrant MNK1/2-eIF4E activity affects different cellular components of the tumor microenvironment. We hypothesize that the MNK1/eIF4E axis, functioning in stromal cells, promotes PPBC pro-tumorigenic immunity. It is important to understand how the MNK1/2-eIF4E pathway contributes to breast cancer immune evasion, and investigate if blocking this pathway can sensitize PPBC to anti-PD1 blockade.

3.1 Abstract

Breast cancer diagnosed within 10 years of a last pregnancy is defined as post-partum breast cancer (PPBC,) and is highly metastatic. Mammary gland (MG) involution is a physiologic process whereby the breast tissue, upon cessation or in the absence of lactation, is remodeled back to its pre-pregnant state. In pre-clinical murine models of PPBC (PPBC mice), breast tumor cells implanted into the involuting mammary gland show increased outgrowth and metastasis to the lungs, compared to the same cells injected into nulliparous hosts. The MNK1/2-eIF4E axis is a critical regulator of the translation of mRNAs that encode for proteins that promote tumor cell invasion and metastasis. However, it remains unclear whether this axis modulates non-tumor cells that make up the fabric of the PPBC tumor microenvironment (TME). We report that phospho-eIF4E deficient ($eIF4E^{S209A}$) PPBC mice are protected against lung metastasis. Characterization of the lung immune microenvironment of PPBC mice showed infiltration of type 2 innate lymphoid cells (ILC2) and granulocytic myeloid-derived suppressor cells (G-MDSC). However, while the lungs from $eIF4E^{S209A}$ PPBC mice showed less ILC2 infiltration, and reduced G-MDSC, there was a significant increase in the presence of cytotoxic T cells. We next wanted to investigate whether upstream regulators of ILC2 are controlled by phospho-eIF4E. Herein, we have shown that the expression of IL-33, a known hallmark ILC2-activating alarmin cytokine and inducer of breast tumor cell invasion, is repressed when the MNK1/2-eIF4E axis is blocked. Thus far, immune checkpoint blockade therapy has shown limited clinical benefit in breast cancer. Herein, we show that the combination of orally bioavailable MNK1/2 inhibitor SEL201 and anti-PD1 can inhibit PPBC metastasis to the lungs. Thus, our study offers a promising immune modulatory route to enhance efficacy of immunotherapy by targeting aberrant mRNA translation.

3.2 Introduction

Postpartum breast cancer (PPBC) is defined as breast cancer diagnosed within the first 10 years of the most recent pregnancy (1). Given its highly metastatic nature (1, 2), patient prognosis is poor in women diagnosed with PPBC. Mammary gland (MG) involution, the remodeling of the breast tissue back to its pre-pregnant state, has been hypothesized to cause premalignant epithelial cells to adopt invasive properties (2). Involution, akin to the process of wound healing, is accompanied by orchestrated immune cell infiltration to the mammary gland (3). The interactions between innate and adaptive immune cells, alongside other cell types found in this microenvironment, are fundamental in developing the metastatic phenotype that is characteristic of PPBC (4-6). As metastasis is the main cause of cancer-related mortality, there is an urgent clinical need for more effective therapies, founded on a better understanding of PPBC tumor immunity.

The regulation of mRNA translation is becoming increasingly recognized in the field of onco-immunology, as it accelerates cellular responses to external stimuli by circumventing the need of de novo transcription. Indeed, dysregulation of translational control is a prominent feature of many cancers (7). For example, elevated levels of the eukaryotic initiator factor 4E (eIF4E), a translational regulator that binds to the 7-methylguanosine cap at the 5' end of the mRNA to mediate translation initiation (7, 8), are associated with malignancy and poor prognosis in several cancer types (9-14). eIF4E can be phosphorylated at serine 209 (S209) by MAP kinase-interacting serine/threonine-protein kinases 1 and 2 (MNK1/2), and this post-translational modification is essential for the pro-oncogenic effects of eIF4E (11). Increased MNK1/2 activity has been associated with therapeutic resistance, tumorigenesis, invasion and metastasis (15-20). We and others have previously shown that phosphorylation of eIF4E leads to the translational upregulation of mRNAs, such as c-myc, Mcl-1, MMP3 and Snail, that support tumor cell survival and a pro-invasive phenotype (11, 18). Phospho-eIF4E has recently been reported to reinforce the survival of pro-metastatic neutrophils in breast cancer (21), however there remain large gaps in our understanding of how the regulation of eIF4E phosphorylation impacts the behavior of other immune and non-immune stromal cells found within the breast tumor microenvironment (TME).

The TME is a complex network of multiple cell types that crosstalk to influence disease progression. The temporal influx of macrophages, dendritic cells, T helper cells and regulatory T

cells has been documented to occur during involution (2, 6). Fibroblasts are another critical component of the TME and play a supportive role in the PPBC metastatic cascade (4). During the physiological process of MG involution, fibroblasts enter an activated state characterized by an increased expression of several genes regulating collagen deposition and production of immunosuppressive chemokines (4). Activated fibroblasts help to remodel the mammary gland back to a non-lactational state, but in the context of PPBC, they may facilitate evasion from anti-tumor immunity.

Sites of breast cancer metastasis, including lungs, bone and liver, are selectively educated by the primary tumor prior to the establishment of metastatic colonies (22). However, it remains unclear what type of immune cells arrive first at the secondary organ to prepare the pre-metastatic "soil" for the cancer cells to seed. A prompt first responder to pulmonary insults are the type 2 innate lymphoid cell (ILC2), which we have previously shown are important cells to further recruit multiple other immune cell components to the lungs (23). The polarization and activation of ILC2 are controlled by a few key cytokines, including IL-33, IL-7, IL-25 and thymic stromal lymphopoietin (TSLP) (24). Recent reports have revealed that ILC2s support tumor immune evasion in a number of malignancies, including breast cancer (25-28). ILC2s secrete type 2 cytokines (e.g. IL-4, IL-5, and IL-13), to recruit and activate myeloid-derived suppressor cells (MDSCs) that block cytotoxic T cell infiltration and anti-tumor function (25, 28). Thus, we reasoned that ILC2s may serve as first responders to prepare the pulmonary microenvironment for PPBC metastasis.

While the importance of phospho-eIF4E-mediated translational control in cancer cells is well-established, its impact within the cells of the TME as a whole remains comparatively under-studied. Here, we demonstrate that host phospho-eIF4E regulates the function of multiple cell types, including fibroblasts, ILC2s, MDSCs and cytotoxic T cells, to support the immune evasion and metastasis of PPBC tumors. We also provide evidence for a potential therapeutic intervention in PPBC, by showing that the combination of the MNK1/2 inhibitor SEL201 and anti-PD1 blockade decreases PPBC lung metastasis.

3.3 Methods

3.3.1 Mouse Model

Wild-type (WT) Balb/c and C57BL6 mice were purchased from Charles River Laboratory. eIF4E$^{S209A/S209A}$ Balb/c and eIF4E$^{S209A/S209A}$ C57BL6 mice were gifts from Dr. Nahum Sonenberg at McGill University, and have been previously described (11). PPBC models were set up as previously reported (29, 30). Briefly, 6-week old WT or eIF4E$^{S209A/S209A}$ female mice were mated with male mice. Pregnant mice were monitored until new pups were born and allowed to lactate for 11 to 14 days. Pups were removed from the dams, transferred to foster mothers, and the dams are allowed to undergo forced weaning-induced mammary gland involution. On involution day 1, that is twenty-four hours post-forced weaning, 200,000 66cl4 cells were injected into the inguinal mammary gland of Balb/c mice and tumors were allowed to grow for either 14 days or 33 days. 200,000 E0771 cells were injected into the mammary gland of C57BL6 mice for 26 days. Animal experiments were conducted following protocols approved by McGill University Animal Care and Use Committee.

3.3.2 Cells and Reagents

The 66cl4 and MDA-MB-231 cell lines were kind gifts from Dr. Josie Ursini-Siegel at McGill University. The E0771 cell line was purchased from CH3 BioSystems. 66cl4 was cultured in RPMI with 10% FBS and antibiotics. E0771 was cultured in RPMI supplemented with 10mM HEPES, 10% FBS and antibiotics. WT and eIF4^{ES209A} mouse embryonic fibroblasts (MEFs) (11, 18). Cancer-associated fibroblasts (CAFs) derived from patient breast cancer were obtained in collaboration with Dr. Mark Basik at McGill University as previously described (31). The project was approved by the Research Ethics Board at McGill University. CAFs and MDA-MB-231 cells were cultured with DMEM supplemented with 10% FBS and antibiotics.

3.3.3 Western Blotting

Cells were lysed with RIPA buffer (150 mmol/L Tris-HCl, pH 7, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitors (Roche) as previously described (17, 32). Equal amounts of protein (in μg) were loaded and separated on 10% SDS-PAGE gels. Antibodies were used to detect: p-eIF4E, p-MNK1, eIF4E, MNK1, p-ERK1/2, ERK1/2, p-p38, p38, IL33, Vimentin and PD-L1. GAPDH was probed to confirm equal protein loading. Antibody information is listed in Supplementary Table 3.1.

3.3.4 Quantitative PCR

RNA was prepared using E.Z.N.A. total RNA isolation kit (OMEGA Bio-Tek). cDNA was prepared from 1 μg of total RNA, using iScript cDNA Synthesis Kit (Bio-Rad). Target genes were quantified using the Applied Biosystems 7500 Fast Real-Time PCR System with SYBR Green. Primers are listed in Supplementary Table 3.2.

3.3.5 Migration and Invasion Assay

66cl4 and E0771 cells were seeded at 1 million cells per 10 cm dish on day 1 in full media, then starved overnight by switching them to serum-free media on day 2. On day 3, transwells were coated with Collagen I (20 μg/ml) as previously reported (17). 200,000 cells were seeded into the transwells (Corning) and were allowed to migrate and invade for 16 hrs. Migrated cells were fixed with 5% glutaraldehyde (Sigma) and stained with 0.5% crystal violet (Sigma) as previously reported (17). Stained cells were then counted and quantified.

3.3.6 Co-culture Assay

66cl4 and E0771 cells were seeded at 2 million cells per 10cm dish on day 1 in full media, then switched to serum-free media on day 2 and starved overnight. 200,000 WT or $eIF4E^{S209A/S209A}$ mouse embryonic fibroblasts were seeded into 12-well companion plates on day 2. On day 3, transwells were coated with Collagen I (20 μg/ml) as previously reported. 50,000 66cl4 or E0771 were seeded into the transwells and were allowed to migrate and invade towards WT or $eIF4E^{S209A/S209A}$ fibroblasts for 48hrs. Migrated cells were fixed, stained and quantified as described above. WT and $eIF4E^{S209A/S209A}$ fibroblasts were harvested for WB or qPCR.
MDA-MB-231 cells were seeded at 3 million cells per 10cm dish on day 1 in full media, then switched to serum-free media on day 2 and starved overnight. 50,000 patient-derived CAFs were seeded into 6-well companion plates on day 2. On day 3, transwells were coated with Collagen I (20 μg/ml) as previously reported. 200,000 MDA-MB-231 cells were seeded into the transwells and were allowed to migrate and invade towards CAFs for 48hrs. Migrated cells were fixed, stained and quantified as described above. CAFs were harvested for WB.

3.3.7 Immunohistochemistry (IHC)

Immunohistochemistry and hematoxylin and eosin (H&E) stainings were performed as previously described (17). Briefly, tumor and lung sections were stained for IL33 and Ki67, and counterstained with 20% Harris-modified hematoxylin (Fisher). Antibody information is listed in

Supplementary Table 3.1. Slides were scanned and assessed using Spectrum (Aperio Technologies). All animal and patient IHC samples were quantified by QuPath software.

3.3.8 Immunofluorescence (IF)

IF staining was performed as previously described (33). Briefly, primary tumor tissues were stained for phospho-eIF4E, IL33 and α-smooth muscle actin (α-SMA), and nucleus were labeled with DAPI. Primary and secondary antibodies were listed in Supplementary Table 1. Slides were scanned, and the images were analyzed by Visiopharm software.

3.3.9 ILC2 isolation

ILC2s were isolated from bone marrow and expanded as previously reported (23). ILC2s were stained with violet proliferation dye and subsequently monitored for cell division by flow cytometry. IL5 and IL13 secretion by ILC2s was quantified by ELISA as previously reported (23).

3.3.10 Statistical Analysis

Prism software (GraphPad) was used to determine statistical significance of differences. Unpaired Student's t test, one-way ANOVA or two-way ANOVA is used, as appropriate. P values < 0.05 were considered significant. The details of statistical analysis for each experiment are listed in Supplementary Table 3.3 and Supplementary Table 3.4.

3.4 Results

3.4.1 Loss of eIF4E phosphorylation in the stroma protects against PPBC lung metastasis

We have previously reported that the absence of phospho-eIF4E in both the tumor and stromal cells is sufficient to reduce lung metastasis in the PyMT transgenic model of breast cancer (18). In order to further dissect the importance of stromal phospho-eIF4E in PPBC, we investigated whether stromal phospho-eIF4E deficiency is sufficient to block metastasis in an aggressive mouse model of PPBC. Using the involuting mammary gland as an experimental platform to model PPBC metastasis, 66cl4 murine breast cancer cells were injected into the inguinal mammary glands of wild-type (WT) or $eIF4E^{S209A}$ (phospho-eIF4E null) Balb/c mice one day following weaning-induced involution (Figure 3.1a). Consistent with previously published data, tumor cells injected into the involuting mammary gland are more metastatic, compared to the same cells injected into the virgin mammary glands of age matched mice (Supplementary Figure 3.1a). We next harvested the lungs at 33 days post-tumor cell injection for the quantification of

metastatic burden (Figure 3.1a). We observed a significant decrease in lung metastases in eIF4E^{S209A} PPBC mice, that is, mice devoid of phosphorylated eIF4E, compared to their WT PPBC counterparts (Figure 3.1a). The protection of lung metastasis observed in phospho-eIF4E deficient mice was not due to a difference in primary tumor outgrowth, as both the rate of tumor initiation and proliferation were similar between WT and eIF4E^{S209A} PPBC mice (Figure 3.1b, 3.1c, Supplementary Figure 3.1b). Ki67 staining of lung metastasis showed no difference in Ki67 positive cells in the WT and eIF4E^{S209A} lungs (Figure 3.1d), indicating the tumor cells proliferate at comparable speed at the pulmonary metastatic sites. Similarly, when E0771 murine breast cancer cells, syngeneic to C57BL6 mice, were injected into the involuting mammary glands of WT or eIF4E^{S209A}, under the same experimental setup as in Figure 3.1a, we observed that the phospho-eIF4E null PPBC mice have decreased metastatic burden in the lung compared to WT PPBC mice (Figure 3.1e, 3.1f, Supplementary Figure 3.1c, 3.1d, 3.1e).

Mammary gland involution is characterized by the elimination of milk-secreting mammary epithelia and re-population of adipocytes. We next addressed whether the reason for the reduced metastatic burden in the lungs of eIF4E^{S209A} PPBC mice was due to a defect in their ability to undergo the physiologic process of mammary gland involution. We quantified the ratio of adipocytes over epithelial cells at lactation day 8, involution day 2, 4, and 6 in WT and eIF4E^{S209A} mice. The adipocyte/epithelium ratio increases in a similar pattern over the course of WT and eIF4E^{S209A} mammary gland involution (Supplementary Figure 3.1f), and WT and eIF4E^{S209A} show similar gross morphology during involution (Supplementary Figure 3.1g). The phosphorylation of STAT3 is known to be induced and required for mammary gland involution (34), thus we also examined the levels of phospho-STAT3 in the WT and eIF4E^{S209A} mice, but found no difference in STAT3 phosphorylation (Supplementary Figure 3.1h).

Together, these results suggest that mice devoid of phospho-eIF4E undergo the physiological process of involution, as do their WT counterparts. Thus, the reduced metastasis observed in phospho-eIF4E null PPBC mice is not the result of overt defects in mammary gland involution.

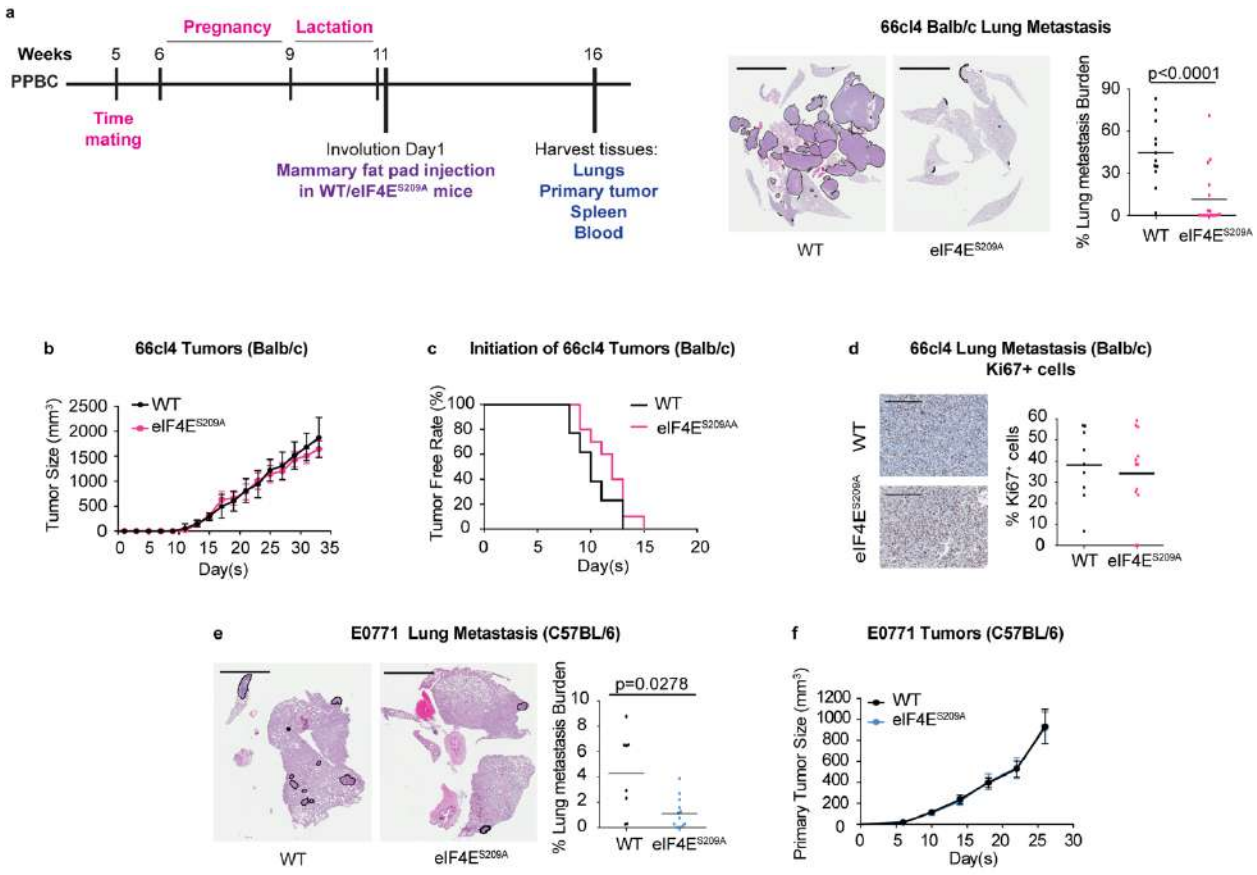


Figure 3.1 Stromal phospho-eIF4E deficient mutation (eIF4E^{S209A}) protects against lung metastasis in animal models of PPBC. a. Timeline of PPBC mouse model. 66cl4 cells are more metastatic in WT than eIF4E^{S209A} Balb/c PPBC animals. Scale bar=4mm. **b.** Primary tumor outgrowth is similar between WT and eIF4E^{S209A} Balb/c PPBC animals. **c.** WT and eIF4E^{S209A} Balb/c PPBC mice have similar primary tumor initiation rates. **d.** Levels of Ki67+ cells in the lung metastasis are similar between WT and eIF4E^{S209A} Balb/c PPBC mice. **e.** eIF4E^{S209A} C57BL/6 PPBC mice have reduced lung metastatic burden compared to WT mice. Scale bar=4mm. **f.** E0771 primary tumors grow at similar speeds in WT and eIF4E^{S209A} C57BL/6 PPBC mice.

3.4.2 Characterizing the changes in the lung immune microenvironment of PPBC mice that are dependent on the phosphorylation of eIF4E

One important mechanism used by cancer cells to metastasize is via immune evasion (35). In this context, infiltration of MDSCs is critical to support an immunosuppressive microenvironment, and prevent cytotoxic T cell-dependent tumor eradication (35). Given the robust differences in lung metastatic burden, but not primary tumor outgrowth, observed between WT and eIF4E^{S209A} PPBC mice, we next investigated how phospho-eIF4E deficiency at the lung metastatic site affects the infiltration of MDSCs and cytotoxic T cells. We discovered a significant reduction in

granulocytic-MDSCs (G-MDSCs) ($CD45^+CD11b^+Ly6G^+Ly6C^{lo}$) and elevation of $CD8^+$ T cells in the lungs of $eIF4E^{S209A}$ PPBC mice (Figure 3.2a).

We next characterized the early changes that ensue in the lung immune microenvironment to support the infiltration of G-MDSCs and PPBC metastasis. ILC2 cells are a dominant innate immune cell type in the lung, that can (1) serve as first responders upon an immune challenge (23, 24), and (2) skew the TME towards a high MDSC/cytotoxic T cell ratio (25, 28, 36). We hypothesized that there may be a difference in the presence of ILC2 cells in the lungs of WT and $eIF4E^{S209A}$ PPBC mice. A time point of 2 weeks post-66cl4 tumor cell injection into the involuting mammary gland was chosen to investigate this hypothesis, as it represents a time point when macroscopic tumor cells are not yet visibly detectable in the lung. The frequency of ILC2 cells in the lungs of $eIF4E^{S209A}$ PPBC mice was reduced compared to WT PPBC mice, while the levels of MDSC and cytotoxic T cells present in the lungs were similar between the two groups (Figure 3.2b). Given the reported role of ILC2-derived IL-13 in recruitment and activation of MDSCs (25, 28), we reasoned that ILC2 may first arrive to prepare the metastatic niche by enhancing the influx of MDSCs. Thus, we next sought to determine whether repression of phospho-eIF4E in ILC2 cells alters their ability to secrete cytokines that are important for the recruitment of MDSCs, such as IL-5 and IL-13 (25, 28, 37). To this end, we isolated ILC2 cells from the bone marrow of WT and $eIF4E^{S209A}$ mice, expanded them *ex vivo*, and examined their ability to proliferate and secrete IL-5 and IL-13 in response to the co-stimulation of IL-7 plus IL-33 (23). Phospho-eIF4E deficient ILC2 cells secrete less IL-5 and IL-13, than ILC2 cells derived from WT mice (Figure 3.2c, left and middle). ILC2s derived from $eIF4E^{S209A}$ mice seem to be less proliferative, compared to the WT control ILC2s (Figure 3.2c, right). We next examined whether our results could be recapitulated using pharmacological blockade of phospho-eIF4E, using the previously described MNK1/2 inhibitor, SEL201 (17, 20). We found that SEL201 treatment of WT ILC2 cells reduces their ability to secrete IL-5 and IL-13, in response to the co-stimulation of IL-7 and IL-33 (Figure 3.2d, left and middle). Little effect was seen on WT ILC2 cell proliferation in response to SEL201 (Figure 3.2d, right). Together, these results suggest that the phosphorylation of eIF4E is necessary for the production of IL-5 and IL-13 from ILC2 cells.

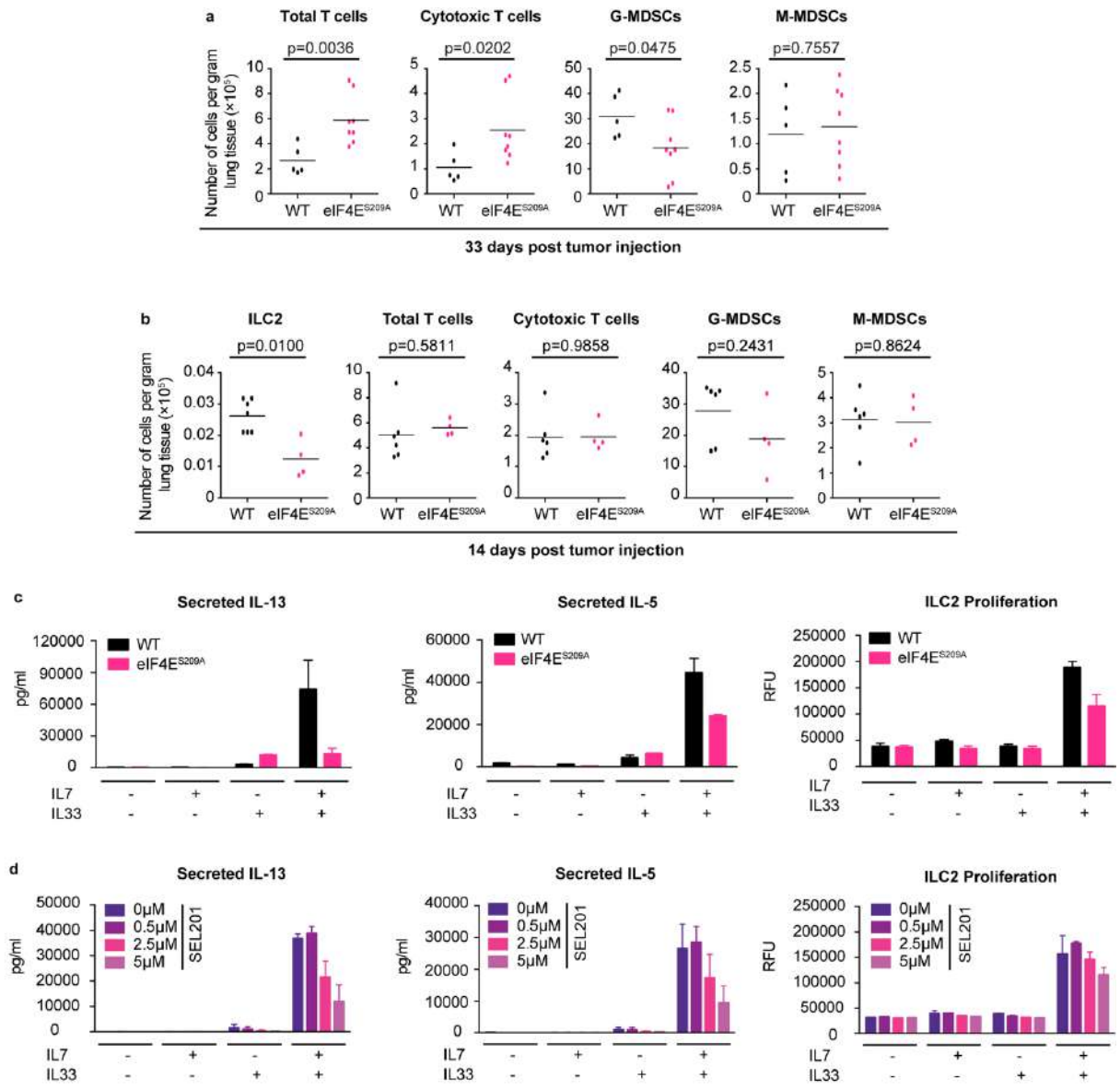


Figure 3.2. phospho-eIF4E-dependent ILC2 expansion and activation supports PPBC immune evasion. **a.** eIF4E^{S209A} Balb/c PPBC mice have increased total and cytotoxic T cells, as well as decreased G-MDSCs in the lungs after 33 days post 66cl4 injection. **b.** eIF4E^{S209A} Balb/c PPBC lungs have decreased ILC2 cells than WT PPBC lungs after 14 days post 66cl4 injection, but the levels of T cells and MDSCs are similar. **c.** eIF4E^{S209A} ILC2s proliferate slower and produces less IL-5 and IL-13 than WT ILC2s. **d.** SEL201 reduces IL-5 and IL-13 production from WT ILC2s.

3.4.3 eIF4E phosphorylation regulates IL33 expression in fibroblasts to support breast cancer cell invasion

Given our evidence for the important role of the MNK1/2-eIF4E axis in the function of ILC2 cells, we further explored whether phospho-eIF4E impacts the expression of upstream regulators of ILC2 cells, such as the alarmin IL-33. We performed IHC on the 66cl4-derived primary tumors that were grown for 2 weeks either in WT or $eIF4E^{S209A}$ PPBC mice, and found that IL33 levels are lower in the tumors grown in $eIF4E^{S209A}$ PPBC mice, compared to those tumors derived from WT PPBC mice (Figure 3.3a).

Next, we sought to determine the cellular components in the primary tumors that produce IL-33. Fibroblasts become activated during mammary gland involution, and they support PPBC invasion and metastasis, in part, via their active secretome (4). One important fibroblast-secreted factor is IL-33, which has been shown to promote tumor invasion and metastasis (36, 38-41). Together, those studies provided the impetus for us to determine whether fibroblasts were a major source of IL-33 in our PPBC model. We therefore stained the primary tumors derived from 66cl4 cells injected into the involuting mammary gland with phospho-eIF4E, IL-33, and the activated fibroblast marker α-smooth muscle actin (α-SMA). An average of 14.5% $IL\text{-}33^{+}$ cells are also $\alpha\text{-}SMA^{+}$ (Figure 3.3b). Next, we exploited mouse embryonic fibroblasts derived from WT or $eIF4E^{S209A}$ mice as a robust genetic tool to test whether the phosphorylation of eIF4E was required for the regulation of IL-33 expression in fibroblasts. In doing so, we observed that fibroblasts devoid of eIF4E phosphorylation express less IL-33 mRNA and protein (Figure 3.3c).

In addition to promoting ILC2 polarization and activation, IL-33 has also been shown to directly impact invasion and metastasis via binding its receptor ST2 on tumor cells (42, 43). Hence, we sought to determine whether fibroblast-derived IL-33 positively supports breast tumor cell invasion. We used a co-culture model system to study interactions between fibroblasts and the 66cl4 and E0771 breast cancer cells used in our in vivo PPBC models (Figure 3.3d). When 66cl4 or E0771 breast cancer cells were co-cultured with WT or $eIF4E^{S209A}$ fibroblasts, both breast cancer cell lines displayed a decreased propensity to invade in the presence of the $eIF4E^{S209A}$ fibroblasts, as compared to WT fibroblasts (Figure 3.3e, Supplementary Figure 3.3a, 3.3b). Interestingly, we observe a robust increase in the expression of IL-33 when we culture breast cancer cells in the presence of fibroblasts, however $eIF4E^{S209A}$ fibroblasts still express

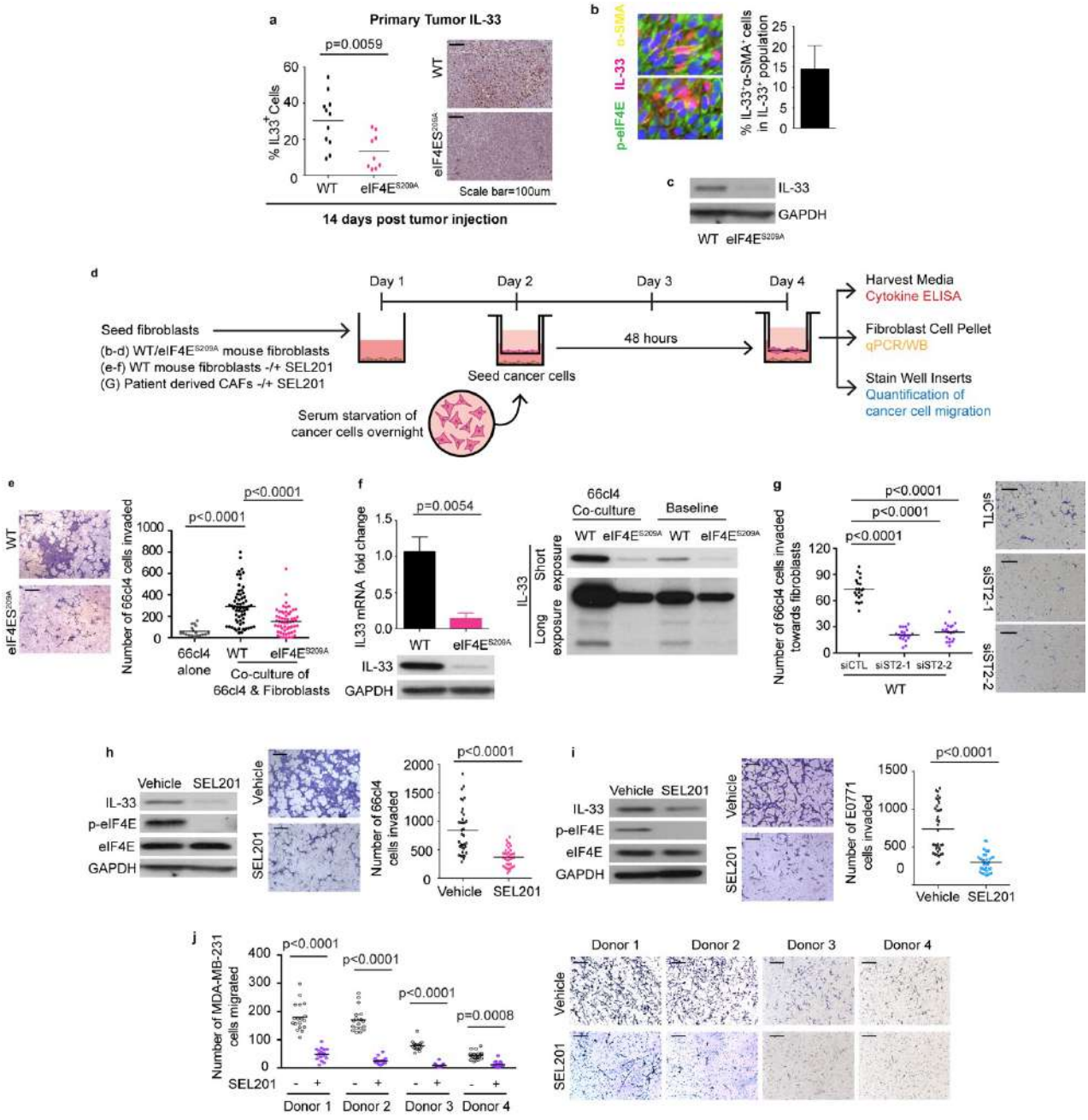


Figure 3.3 phospho-eIF4E deficiency inhibits fibroblast-derived IL-33 expression and tumor cell invasion. **a.** WT tumors express more IL-33 than eIF4E^{S209A} tumors. **b.** Co-localization of IL-33 with α-SMA^{+} cells in WT tumors. **c.** eIF4E^{S209A} fibroblasts express less IL-33 than WT. **d.** Timeline of fibroblast-tumor cell co-culture. **e.** 66cl4 migrates less towards eIF4E^{S209A} fibroblasts. **f.** eIF4E^{S209A} fibroblasts express less IL-33 compared to WT fibroblasts when co-cultured with 66cl4 cells, and IL-33 expression is further induced in WT but not eIF4E^{S209A} fibroblasts by 66cl4 cells. **g.** ST2 knockdown diminishes 66cl4 invasion towards WT fibroblasts. **h. i.** SEL201 inhibits 66cl4 and E0771 invasion towards fibroblasts and suppresses IL33 expression. **j.** SEL201 suppresses MDA-MB-231 invasion towards patient derived CAFs.

significantly less IL-33 mRNA and protein, as compared to their WT fibroblast counterparts (Figure 3.3f, Supplementary Figure 3.3c, 3.3d). We then investigated whether fibroblast-derived IL-33 acts via the IL-33 receptor ST2 expressed on 66cl4 cells to promote breast cancer invasion. By ablating the expression of ST2 using siRNA in 66cl4 cells, we observed an impaired ability of the ST2-deficient tumor cells to invade in the presence of WT fibroblasts (Figure 3.3g, Supplementary Figure 3.3e). Such data indicate that fibroblast-derived IL33 signals in a paracrine fashion to ST2-expressing breast cancer cells to augment tumor cell invasion.

As we observed that breast cancer cells display an increased propensity to invade toward WT fibroblasts compared to eIF4E^{S209A} fibroblasts, we next examined whether we could pharmacologically inhibit this process using the MNK1/2 inhibitor SEL201. WT fibroblasts were treated with either vehicle or SEL201, and co-cultured with either 66cl4 or E0771. Concomitant with repressed phospho-eIF4E expression in fibroblasts, SEL201 treatment decreased IL-33 levels in WT fibroblasts (Figure 3.3h, 3.3i, Supplementary 3.3f). Moreover, the invasion of 66cl4 and E0771 cells was less robust when co-cultured with SEL201-treated fibroblasts (Figure 3.3h, 3.3i).

Finally, we have verified the clinical relevance of our findings by co-culturing patient-derived CAFs with MDA-MB-231 human breast cancer cells. We obtained primary CAFs that were isolated from the freshly resected human breast tumors of four patients. Primary CAFs were treated with vehicle or SEL201, and subsequently co-cultured with MDA-MB-231. Similar to our findings in the murine fibroblasts, MDA-MB-231 invaded less robustly in the presence of SEL201-treated CAFs (Figure 3.3j).

3.4.4 IL-33 activates the MNK1/2-eIF4E pathway downstream of activated ST2 in breast tumor cells to build immunosuppressive TME

We have shown the important role of fibroblast-derived IL-33 in supporting breast cancer cell invasion (Figure 3.3e, Supplementary 3.3a). Therefore we chose to further dissect how IL-33 signals downstream of ST2 in breast tumor cells. Stimulation of 66cl4 cells with recombinant murine IL-33 (rIL-33) resulted in increased phosphorylation of p38 MAPK and eIF4E but not phosphorylation of ERK1/2 (Figure 3.4a, Supplementary 3.4a). The invasion of 66cl4 (Figure 3.4b) and E0771 (Supplementary Figure 3.4b) was increased upon exposure to rIL-33. Additionally, we hypothesized that IL-33 might stimulate the expression of pro-inflammatory

and pro-tumorigenic cytokines/chemokines in tumor cells. IL-33-stimulated the mRNA expression of CXCL-1, CCL-17, GM-CSF and IL-6, without significantly affecting IL-4 and CXCL-2 levels (Figure 3.4c, Supplementary Figure 3.4c). Given the reported functions of CXCL-1, CCL-17, GM-CSF and IL-6 in cancer immune evasion (44-52), our data provides evidence to show that IL-33 may serve as an upstream signal to initiate such immunosuppressive signaling cascade in the PPBC TME to facilitate metastasis.

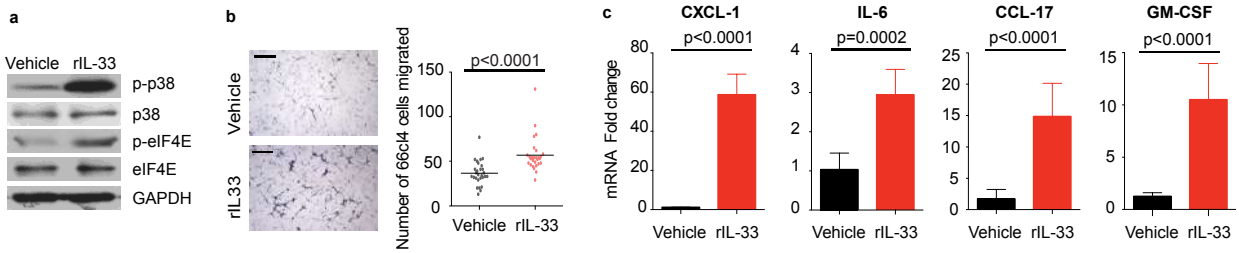


Figure 3.4 Exogenous IL33 induces tumor cell invasion and upregulates selective chemokines and cytokines. a. IL33 induces p38/eIF4E phosphorylation. **b.** IL33 induces 66cl4 invasion. **c.** IL33 induces expressions of CXCL1, IL-6, CCL-17 and GM-CSF in 66cl4 cells.

3.4.5 Increased therapeutic efficacy of blocking MNK1/2 combined with anti–PD1 immunotherapy

Accumulating evidence has implicated the central role of immunosuppressive cells including MDSCs and CAFs in resistance to immune checkpoint blockade (53, 54). The efficacy of immune checkpoint inhibitors in PPBC would likely be improved by overcoming this immune suppression. Given the diverse role of phospho-eIF4E in contributing to PPBC immune evasion and the increased presence of $CD8^+$ T cells in the lungs of phospho-eIF4E deficient PPBC mice, we hypothesized that blocking the phosphorylation of eIF4E using the MNK1/2 inhibitor SEL201 might alleviate PPBC immunosuppression and sensitize tumor bearing mice to the anti-tumor effects of PD1 blockade.

To test this hypothesis, WT PPBC mice were treated with vehicle, SEL201, anti-PD1 antibody, or the combination of SEL201 plus anti-PD1 antibody (Figure 3.5a). Remarkably, SEL201 plus anti-PD1 blockade decreased PPBC lung metastasis, while SEL201 or anti-PD1 alone did not show any significant anti-metastatic effects (Figure 3.5b). The reduction in metastatic burden in

the SEL201+anti-PD1 cohort was not due to a difference in the effect of the combination therapy on primary tumor outgrowth, which remained unchanged (Figure 3.5c, Supplementary 3.5a). Consistent with our previous work (17, 20), SEL201 and/or anti-PD1 showed no overt systemic toxicity, as evidenced by body weight (Figure 3.5d).

Collectively, our work highlights the important role of the IL-33-MNK1/2-eIF4E axis in PPBC invasion and metastasis by impacting multiple cellular compartments in the TME. Targeting the MNK1/2-eIF4E axis might have therapeutic benefit for augmenting the therapeutic efficacy of immunotherapy in women diagnosed with PPBC.

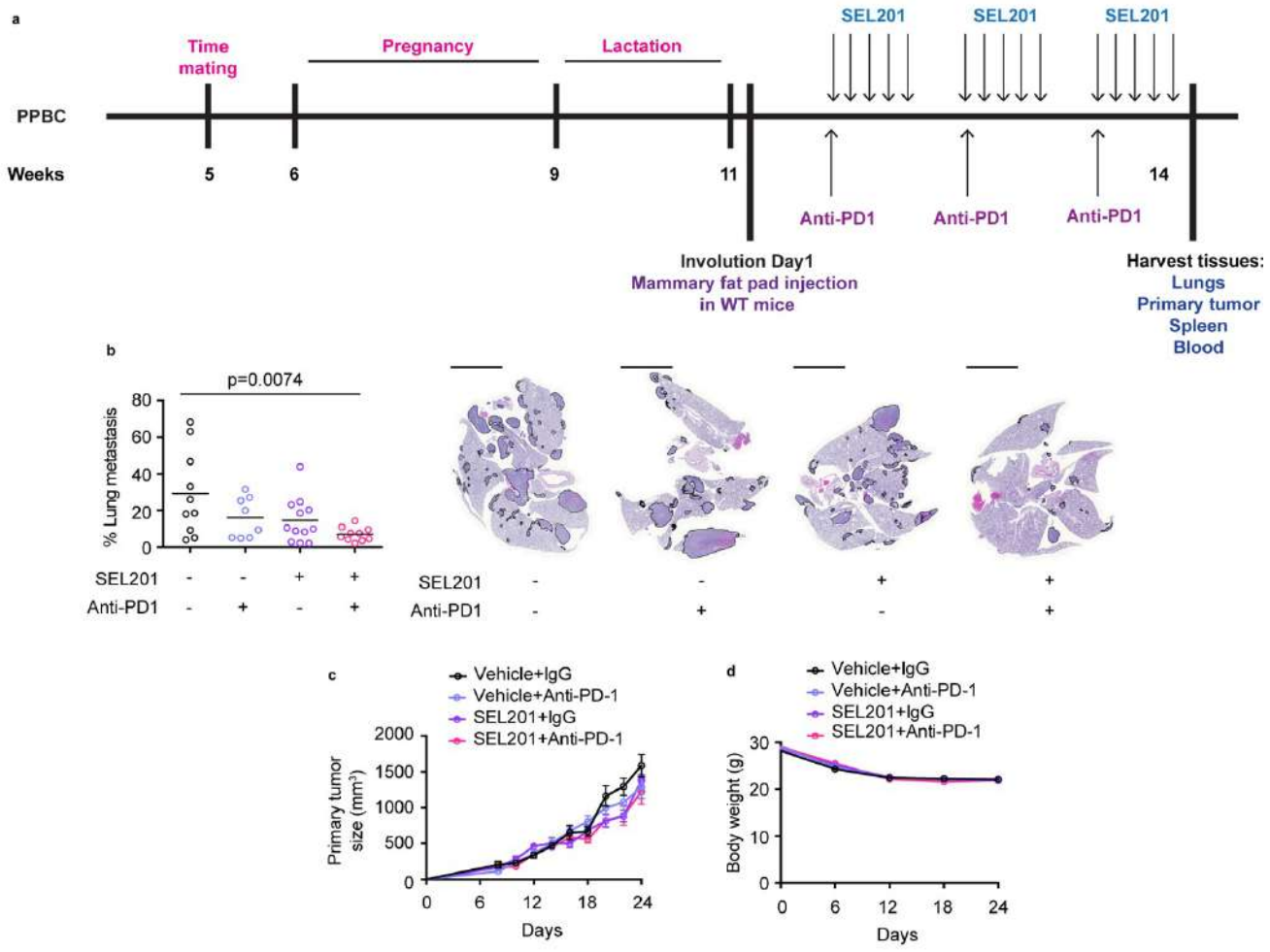


Figure 3.5 Combination of SEL201 and PD1-blockade decreased 66cl4 lung metastasis in PPBC model. **a.** Timeline of experiments, where SEL201 is given 5 times per week by gavaging, and anti-PD1 is given once per week by i.p.. **b.** Combination of SEL201 and anti-PD1 inhibits PPBC metastasis. **c.** SEL201 and/or anti-PD1 don't significantly affect primary tumor outgrowth. **d.** Treatment of SEL201 and/or anti-PD1 don't significant change body weight.

3.5 Discussion

Metastasis associated with PPBC and mortality due to lack of effective treatment strategies necessitates a fuller understanding of this disease (55, 56). Recent breakthroughs in immune checkpoint blockade therapies have stimulated research to better understand the TME of breast cancer, aiming to discover possible approaches to sensitize metastatic breast cancer to immunotherapies. Here, we demonstrated the central role of stromal eIF4E phosphorylation in multiple steps of the metastatic cascade by promoting pro-tumorigenic immunity in the context of a model of PPBC (Figure 3.6. Graphic summary). We also highlight IL-33 as a potentially therapeutically targetable cytokine in PPBC.

IL-33, an alarmin cytokine of the IL-1 family, is essential for the polarization of ILC2 together with IL-7, IL-25 and TSLP (57). Our knowledge of ILC2 in tumor biology is still rudimentary, although recent studies have depicted its tumor-promoting role in several cancer types, including breast cancer (25, 26, 28). Clinical investigations have implied the role of ILC2 in breast tumorigenesis. For example, ILC2 levels were elevated in breast cancers compared to benign breast tissue (27). IL-33-dependent accumulation of ILC2 cells and impairment of NK cells, but

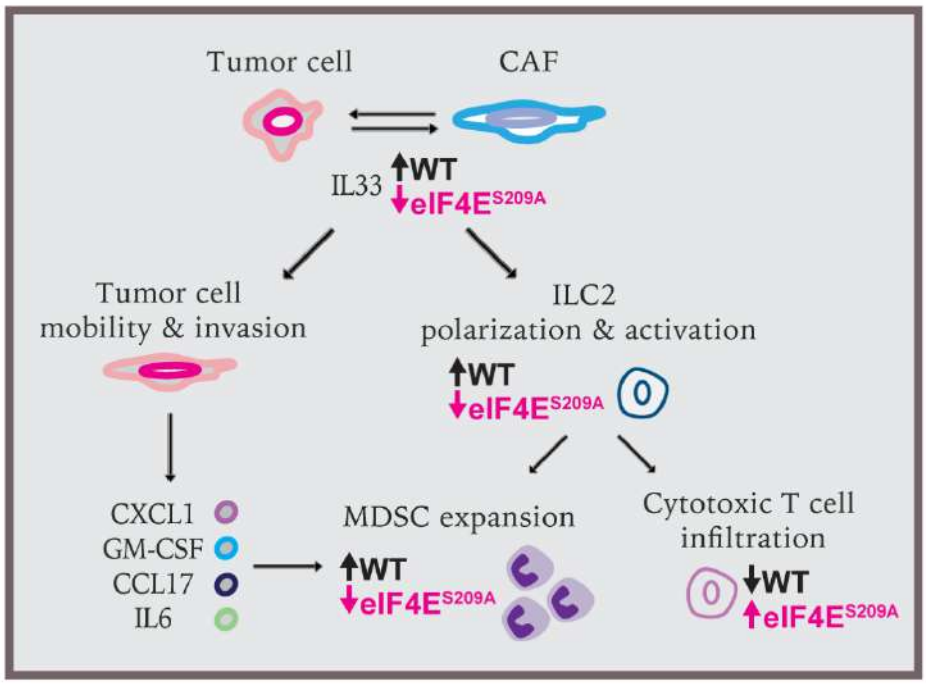


Figure 3.6 Graphic summary

not cytotoxic T cell functions, has been shown to facilitate breast cancer metastasis (36). In our pre-clinical mouse model of PPBC, we showed that hosts devoid of eIF4E phosphorylation presented with an elevated influx of cytotoxic T cells in the lungs, possibly due to an overriding of an immune suppressive ILC2-MDSC axis. Our results in PPBC are in line with the innate and adaptive immuno-crosstalk that exist in other models (25, 28).

In addition to its impact on ILC2 cells, IL-33 has also been reported as a multi-functional pro-tumorigenic cytokine that can maintain cancer stem cell properties, alter cancer cell metabolism, and facilitate tumor cell proliferation (42, 58, 59). IL-33 executes such functions via signaling through its ST2 receptor, and elevation of IL-33 was observed in the serum of breast cancer patients (60, 61). High levels of IL-33 were observed to predict tamoxifen resistance in ER^+ breast cancer (62). More importantly, levels of matrix metallopeptidase 11 (MMP11), a pro-invasive enzyme responsible for tissue remodeling, are directly correlated to IL-33 levels in breast cancer patients (61), supporting a possible pro-invasive function of IL-33. Additionally, we have also demonstrated that phospho-eIF4E-mediated IL-33 production from fibroblasts is further induced when co-cultured with breast tumor cells. Thus, our data provided a novel mechanism where breast cancer cells educate fibroblasts to secrete more IL-33, thus allowing breast cancer cells to gain more invasive properties.

Additionally, it is also acknowledged that IL-33 reinforces pro-tumorigenic inflammation by inducing IL-6 (43). Our study has not only verified this finding in breast cancer but also expanded the repertoire of IL-33-induced cytokines and chemokines produced by cancer cells, as we showed that IL-33 induces the expression of CXCL-1, CCL-17, IL-6 and GM-CSF in breast cancer cells. The significance of these four factors in tumor immune evasion has been supported by multiple previous reports. For example, over-expression of CXCL-1 and its receptor CXCR-2, as well as elevated circulating IL-6 levels, are all correlated to breast cancer metastasis and poor survival rate (63, 64), and CXCL-1, IL-6 and GM-CSF are all potent mediators for the recruitment and expansion of MDSCs and M2-like macrophages (45-48, 50-52). CCL-17, an important ligand for CCR-4, has also been demonstrated to elicit Th2 and T_{reg}-mediated cancer immune evasion (44, 49). Taken together, we have provided novel evidence that IL-33 may act directly on breast tumor cells to induce further expression of selected immunosuppressive chemokines and cytokines.

Finally, immune checkpoint blockades designed to release the brakes on exhausted cytotoxic T cells have largely improved the patient prognosis in several cancers (65), but are less effective to date in breast cancer. It is proposed that many breast cancers present with failed or suboptimal T cell priming. Given our data supporting the notion that deficiency of eIF4E phosphorylation enhances T cell infiltration, scheduling of MNK1/2 inhibitor administration will be important in future clinical trials, given that blocking MNK1/2 may be more effective when used to prime the TME by ameliorating overall immunosuppression and facilitating cytotoxic T cell infiltration. Indeed, the anti-metastatic effect of a phospho-eIF4E deficient host was accompanied by increased cytotoxic T cell infiltration in our PPBC animal model (Figure 3.6). We propose the use of MNK1/2 inhibitors as a tool to convert "cold" breast tumors to "hot" tumors, thus offering the opportunity for immune checkpoint blockade to become more effective in highly metastatic cancers such as PPBC.

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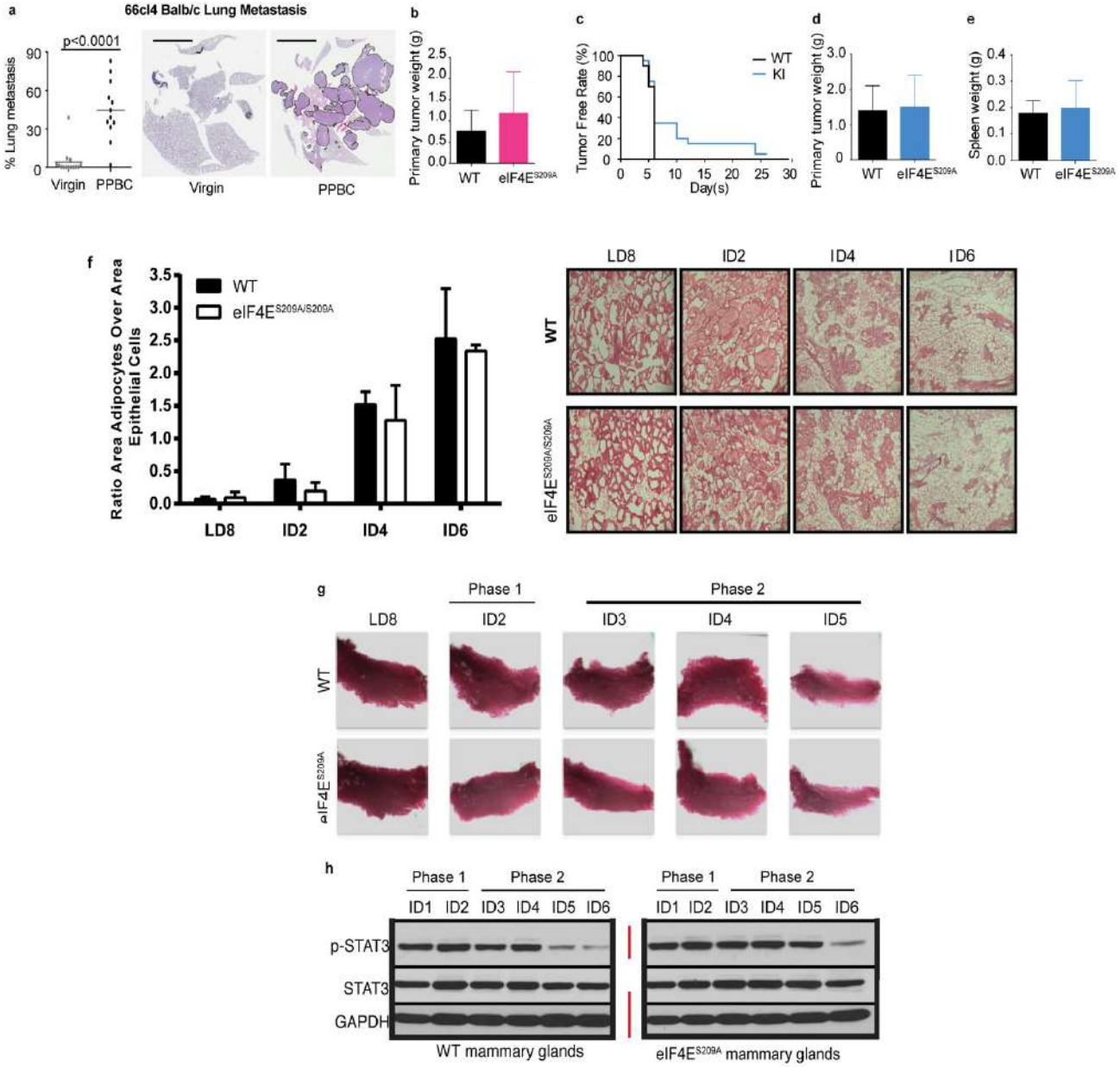
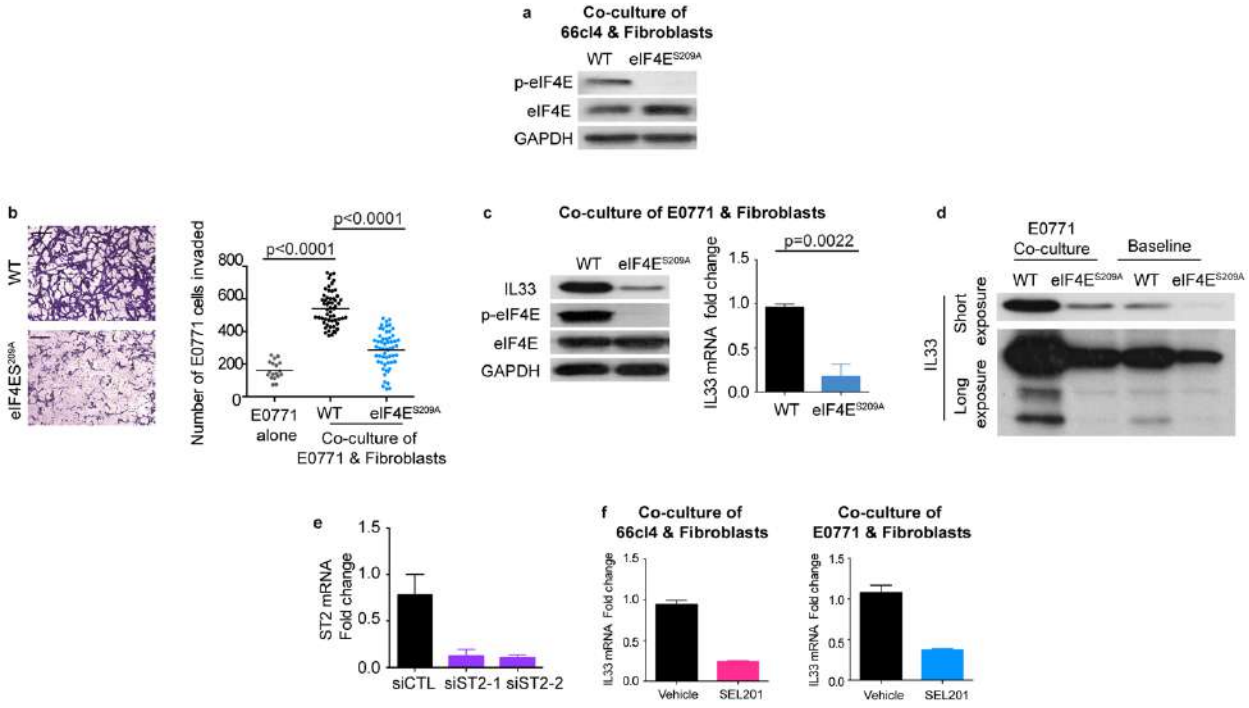
3.8 Supplementary Materials

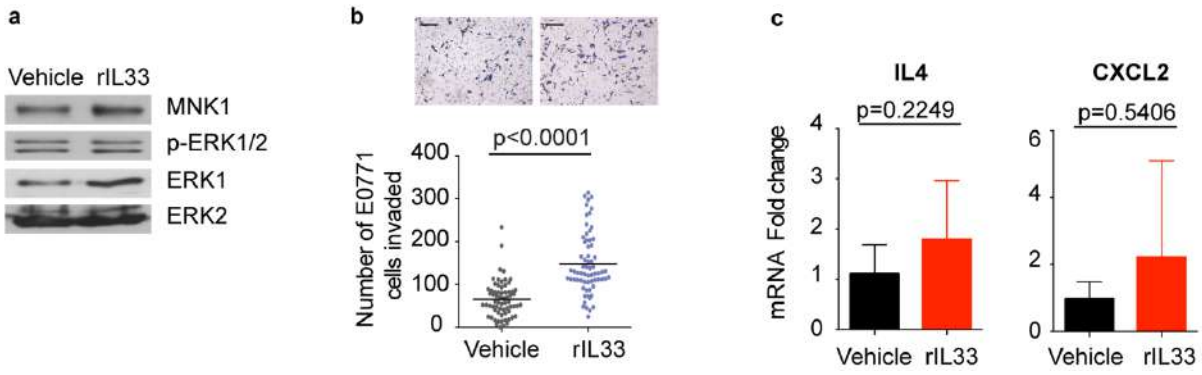


Supplementary Figure 3.1
a. PPBC WT Balb/c mice displayed enhanced lung metastasis compared to virgin counterparts. **b.** Primary tumor weight of WT and eIF4E^{S209A} Balb/c PPBC mice are similar. **c.** Tumor free rates in WT and eIF4E^{S209A} C57BL6 PPBC mice. **d.** Primary tumor weight of WT and eIF4E^{S209A} C57BL6 PPBC mice are similar. **e.** Spleen weight of WT and eIF4E^{S209A} C57BL6 PPBC mice are similar. **f.** Percentage of mammary gland adipocytes increases at similar speeds in WT and KI mice during involution. **g.** WT and eIF4E^{S209A} mammary glands share similar gross morphology during involution. **h.** Levels of phospho-STAT3 and STAT3 in mammary gland during involution.

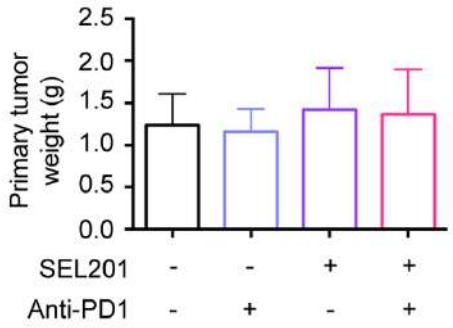


Supplementary Figure 3.3

a. phospho-eIF4E and eIF4E levels of WT and eIF4E^{S209A} fibroblasts when co-cultured with 66cl4 cells. **b.** E0771 cells invade less robustly towards eIF4E^{S209A} fibroblasts than WT fibroblasts. **c.** eIF4E^{S209A} fibroblasts express less IL33 compared to WT fibroblasts when co-cultured with E0771 cells. **d.** eIF4E^{S209A} fibroblasts express less full-length and cleaved IL-33 compared to WT fibroblasts, and IL-33 expression can be induced in WT but not eIF4E^{S209A} fibroblast when E0771 cells are present. **e.** ST2 knockdown is confirmed by qPCR in 66cl4 cells. **f.** mRNA levels of IL33 measured by qPCR in WT fibroblasts treated with vehicle or SEL201 when they are co-cultured with 66cl4 or E0771.



Supplementary Figure 3.4
a. MNK1, p-ERK1/2, ERK1 and ERK2 protein levels of 66cl4 cells with or without rIL33 treatment. **b.** E0771 invasion is stimulated by rIL33 treatment. **c.** rIL33 does not change IL-4 and CXCL-2 mRNA levels.



Supplementary Figure 3.5
SEL201 and/or anti-PD1 do not alter primary tumor weight.

Supplementary Table 3.1 Details of Antibodies

Target protein	Antibody information	Usage
α-SMA	#18147 Abcam	IF
eIF4E	#610269 BD Transduction	WB
phospho-eIF4E	#9741S Cell Signaling	WB
MNK1	#2195S Cell Signaling	WB
phospho-p38	#4511S Cell Signaling	WB
p38	#sc-535-G Santa Cruz	WB
phospho-ERK1/2	##4370 Cell Signaling	WB
ERK1	#sc-93 Santa Cruz	WB
ERK2	#sc-154 Santa Cruz	WB
IL33 (mouse)	#AF3626 R&D System	WB/IHC/IF
PD-L1	#13684 Cell Signaling	WB
Vimentin	#550513 BD Pharminegen	WB
Ki67	#15580 Abcam	IHC
GAPDH	#2118S Cell Signaling	WB

Supplementary Table 3.2 Details of Primers (Mouse)

Target gene	Primer Sequence
IL33	Fwd: 5'-ATG GGA AGA AGC TGA TGG TG-3'
	Rev: 5'-CCG AGG ACT TTT TGT GAA GG-3'
CXCL1	Fwd: 5'-CAC CTC AAG AAC ATC CAG AGC-3'
	Rev: 5'-CTT GAG TGT GGC TAT GAC TTC G-3'
IL-6	Fwd: 5'-CAT GTT CTC TGG GAA ATC GTG-3'
	Rev: TTC TGC AAG TGC ATC ATC G-3'
CCL17	Fwd: 5'-GGA AGT TGG TGA GCT GGT ATA A-3'
	Rev: 5'-GAT GGC CTT CTT CAC ATG TTT G-3'
GM-CSF	Fwd: 5'-GAA GAT ATT CGA GCA GGG TCT AC-3'
	Rev: 5'-CTT GTG TTT CAC AGT CCG TTT C-3'
36B4	Fwd: 5'-TCA TCC AGC AGG TGT TTG ACA-3'
	Rev: 5'-GGC ACC GAG GCA ACA GTT-3'

Supplementary Table 3.3 Statistic Analysis (Figures)

Figure ID	Experiment		Statistical test	p value
1a	Balb/c PPBC	Lung metastasis	t-test WT=12 eIF4E^{S209A}=17	p<0.0001
1b		Primary tumor out-growth	Two-way ANOVA WT=12 eIF4E^{S209A}=17	p=0.9992
1c		Primary tumor initiation	Mantel-Cox test WT=12 KI=17	p=0.1346
1d		Primary tumor Ki67 IHC	t-test WT=9 eIF4E^{S209A}=12	p=0.5248
1e	C57BL6 PPBC	Lung metastasis	t-test WT=8 eIF4E^{S209A}=13	p=0.8463
1f		Primary tumor out-growth	Two-way ANOVA WT=8 eIF4E^{S209A}=13	p=0.0036
2a	Balb/c PPBC (Endpoint)	Total T cells flow cytometry	t-test WT=5 eIF4E^{S209A}=8	p=0.0036
		Cytotoxic T cells flow cytometry	t-test WT=5 eIF4E^{S209A}=8	p=0.0202
		G-MDSCs flow cytometry	t-test WT=5 eIF4E^{S209A}=8	p=0.0475
		M-MDSCs flow cytometry	t-test WT=5 eIF4E^{S209A}=8	p=0.7557
2b	Balb/c PPBC (2weeks)	ILC2 flow cytometry	t-test WT=7 eIF4E^{S209A}=4	p=0.0100
		Total T cells flow cytometry	t-test WT=7 eIF4E^{S209A}=4	p=0.5811
		Cytotoxic T cells flow cytometry	t-test WT=7 eIF4E^{S209A}=4	p=0.9858
		G-MDSCs flow cytometry	t-test WT=7 eIF4E^{S209A}=4	p=0.2431
		M-MDSCs flow cytometry	t-test WT=7 eIF4E^{S209A}=4	p=0.8624
2c	Balb/c PPBC (2weeks)	Primary tumor IL33 IHC	t-test WT=11 eIF4E^{S209A}=9	p=0.0059
2d/2e	ex vivo ILC2	WT/eIF4ES209A ILC2	Experiments are done twice so far and n=3 results will be added.	
2e		WT ILC2+/- SEL201		

Figure ID	Experiment		Statistical test	p value
3b	66cl4-Fibroblast co-culture MIA		One-way ANOVA 3 independent experiments	66cl4 alone versus WT: p<0.0001 WT versus eIF4E^{S209A}: p<0.0001
3c	E0771-Fibroblast co-culture MIA		One-way ANOVA 3 independent experiments	E0771 alone versus WT: p<0.0001 WT versus eIF4E^{S209A}: p<0.0001
	66cl4-Fibroblast co-culture IL33 qPCR		Mann-Whitney test 3 independent experiments	p=0.0054
3d	66cl4-Fibroblast co-culture MIA		One-way ANOVA 3 independent experiments	All p<0.0001 indicated in the figures
3e	66cl4-Fibroblast co-culture MIA		t-test 3 independent experiments	p<0.0001
3f	E0771-Fibroblast co-culture MIA		t-test 3 independent experiments	p<0.0001
3g	MDA-MB-231-CAFs co-culture MIA		t-test 1 independent experiment per donor	All p<0.0001 indicated in the figures
3i	66cl4 MIA -/+ rIL33		Mann-Whitney test 3 independent experiments	p<0.0001
3j	66cl4 cells treated with rIL33	CXCL1 qPCR	Mann-Whitney test 3 independent experiments	p<0.0001
		CCL17 qPCR	Mann-Whitney test 3 independent experiments	p<0.0001
		GM-CSF qPCR	Mann-Whitney test 3 independent experiments	p<0.0001
		IL6 qPCR	Mann-Whitney test 3 independent experiments	p=0.0002
4b	Balb/c PPBC	Lung metastasis	One-way ANOVA Control=10 Anti-PD1=8 SEL201=12 Anti-PD1+SEL201=10	Control versus Anti-PD1+SEL201: p=0.0074
4c		Primary tumor outgrowth	Two-way ANOVA Control=10 Anti-PD1=8 SEL201=12 Anti-PD1+SEL201=10	

Supplementary Table 3.4 Details of Statistic Analysis (Supplementary Figures)

Figure ID	Experiment		Statistical test	p value
S.1a	Balb/c PPBC	Lung metastasis	t-test WT=12 eIF4E^{S209A}=17	p<0.0001
S.1b	C57BL6 PPBC	Tumor initiation rate	Mantel-Cox test WT=10 eIF4E^{S209A}=17	p=0.1013
S.1c		Primary tumor out-growth	t-test WT=10 eIF4E^{S209A}=17	p=0.7595
S.1d		Spleen weight	t-test WT=10 eIF4E^{S209A}=17	p=0.5070
S.1f	Balb/c involution	Adipocyte/Epithelium ratio	t-test WT=10 eIF4E^{S209A}=10	
S.3c	E0771-Fibroblast co-culture MIA		Mann-Whitney test 3 independent experiments	p=0.0036
S.3e	66cl4-Fibroblast co-culture ST2 knockdown qPCR		Dunn's multiple comparison test 3 independent experiments	siCTL versus siST2-1: p siCTL versus siST2-2: p
S.3f	66cl4-Fibroblast co-culture IL33 qPCR		Mann-Whitney test 3 independent experiments	p=0.1000
	E0771-Fibroblast co-culture IL33 qPCR		Mann-Whitney test 3 independent experiments	p=0.1000
S.3h	E0771 MIA -/+ rIL33		t-test 3 independent experiments	p<0.0001
S.3i	66cl4 cells treated with rIL33	IL4 qPCR	Mann-Whitney test 3 independent experiments	p=0.2249
		CXCL2 qPCR	Mann-Whitney test 3 independent experiments	p=0.5406
S.4a	Balb/c PPBC	Primary tumor weight	One-way ANOVA Control=10 Anti-PD1=8 SEL201=12 Anti-PD1+SEL201=10	Control versus Anti-PD-1: p=0.9549 Control versus SEL201: p=0.6451 Control versus SEL201+Anti-PD-1: p=0.8256
S.4b		Spleen weight	One-way ANOVA Control=10 Anti-PD1=8 SEL201=12 Anti-PD1+SEL201=10	Control versus Anti-PD-1: p=0.9949 Control versus SEL201: p=0.8445 Control versus SEL201+Anti-PD-1: p=0.9376

Chapter 4. Discussion and Future Directions

4.1 Comprehensive discussion of findings

In this body of work, we were mainly interested in investigating the pro-tumorigenic functions of MNK1/2-eIF4E pathway in two important stages of the breast cancer metastatic cascade: (a) the early stage of transition from benign disease to invasive cancer, and (b) the colonization of tumor cells in a secondary organ. Our findings have provided a fundamental investigation of the targeting of an aberrant protein synthesis pathway. We have also provided groundwork for targeting of the aberrant MNK1/2-eIF4E pathway as a viable means of preventing breast cancer invasion and treating metastatic disease.

Currently, DCIS patients are considered over-treated due to the lack of reliable biomarkers to predict individuals with high risk of disease recurrence. Although our study has suggested the potential role of MNK1/NODAL pathway in promoting the DCIS invasive transition, it remains unclear how MNK1 and phospho-MNK1 levels correlate with disease recurrence and metastasis, as well as patient survival, and whether the inhibition of MNK activity with small molecular inhibitors like SEL201 would be useful to decrease the risk of disease recurrence. A larger cohort of patients with pure DCIS, mixed DCIS/IDC and IDC with long-term follow-up would be needed to examine if MNK1 and/or phospho-MNK1 might serve as biomarkers to predict recurrence and patient outcome. More importantly, it is possible to use SEL201 in future clinical trials to test its safety profile, and examine if SEL201 may lower the risk of disease relapse as IDC and metastasis. Given SEL201 is an orally bioavailable MNK inhibitor, it is possible to easily test whether patients with high MNK1 or phospho-MNK1 will benefit from using SEL201.

In the context of PPBC, a highly metastatic form of breast cancer, we add knowledge about the novel functions of the MNK1/2-eIF4E pathway in the regulation of innate and adaptive immunity during the process of PPBC metastasis. However, it is important to notice that a limitation of our study is the difference between human PPBC and mouse PPBC model, although we have used a gold standard mouse model where malignant cells were introduced on day 1 following forced weaning. Such differences should be recognized, as PPBC in humans is defined as receiving breast cancer diagnosis within 10 years of having had a last child (1). Additionally, we have offered a possible way to sensitize PPBC to PD1 blockade by adding a MNK1/2 inhibitor in our pre-clinical mouse model of this metastatic disease. This body of work provides the possibility of increasing the response rate of metastatic breast cancer to immune checkpoint blockade when combined with a MNK1/2 inhibitor. An ongoing phase II clinical trials (NCT03616834) is currently being performed to test the anti-neoplastic efficacy of the MNK1/2 inhibitor eFT508 in combination with anti-PD1/anti-PD-L1 therapy.

4.2 Future directions

Several future directions have stemmed from this thesis research:

4.2.1 Potential roles of MNK1 in the nucleus

Although we have observed MNK1-dependent upregulation of NODAL mRNA and protein in DCIS cells, the detailed molecular mechanism underlying the regulation of NODAL by MNK1 remains unclear. There are a few possible mechanisms for how MNK1 regulates *NODAL* gene transcription. First, MNK1/eIF4E activation may drive the mRNA translation of a transcription factor that can bind to the promoter of the NODAL gene, which in turn upregulates *NODAL* mRNA levels. Second, MNK1 may indirectly increase NODAL mRNA stability. The first possibility is supported by our observation that MNK1 and phospho-MNK1 can localize in both

the cytoplasm and nucleus in mouse and human DCIS/IDC tissues and our melanoma work (2, 3). Although we did not specifically investigate the functions of MNK1a versus MNK1b in our DCIS model, previous reports have suggested that MNK1b, which preferentially localizes in the nucleus (4), is associated with poor patient outcome in triple-negative breast cancer (5). This indicates that MNK1b may have a special role in the nucleus to regulate breast tumorigenesis and metastasis. Thus, we propose that MNK1 may serve as an indirect transcriptional regulator in the nucleus, aside from its canonical role in regulating mRNA translation. In order to test this hypothesis, MNK1 plasmid constructs with a mutated nuclear export signal (NES) (R/26,27,28/A mutation) and a MNK1 construct with a mutated nucleus localization signal (NLS) (L390S) need to be expressed in the breast cancer cells, and MNK1-interacting proteins in the nucleus can be pulled down. The identities of MNK1-interacting proteins can be identified by mass spectrometry, and the identification of potential MNK1-interacting factors may shed light on novel functions of MNK1 in the nucleus.

4.2.2 Characterizing the functions of MNK1/2-eIF4E in the DCIS to IDC transition in an immune competent model

We have shown data suggesting that the MNK1/NODAL pathway is critical for maintaining DCIS CSC populations, and MNK1 deficiency lowers the risk of relapse as metastatic disease. However, all these studies were done with human DCIS cells in a murine immuno-deficient host. Future studies are needed to examine how MNK1/2-eIF4E activity regulates the maintenance of DCIS CSC populations, and how its activity shapes tumor immunity in an immune competent host. Recently, the functions of immune cells have been described in DCIS and IDC patients. For example, Hendry and colleagues have reported that high levels of tumor infiltrating lymphocytes in DCIS patient samples are associated with a high-grade tumor, presence of necrosis, ER-

negative lesions and HER2-positive tumors (6). More importantly, infiltration of total and activated cytotoxic T cells is decreased in IDC compared to DCIS lesions (7). Such studies suggest that the exclusion of cytotoxic T cells from the TME may contribute to DCIS to IDC conversion.

Several transgenic breast cancer models, such as MMTV-Neu and WAP-T mice, are reported to bear lesions that highly resemble human DCIS. MMTV-Neu is a transgenic mouse model with mammary gland specific expression of proto-oncogene HER2/Neu driven by murine mammary tumor virus (MMTV) promoter/enhancer, and WAP-T mouse model is a whey acidic protein promoter (WAP)-driven SV40 breast cancer model. Both MMTV-Neu and WAP-T mice are reported to bear lesions that highly resemble human DCIS, and such DCIS-like lesions can invade through the basement membrane, and transit into invasive mammary carcinoma in a step-wise fashion (8-10). These model systems can be used to study the impact of aberrant mRNA translation in various innate and adaptive immune cells during the DCIS to IDC conversion. More importantly, immuno-competent mouse models will be advantageous to test the possibility of combinational targeted and immunotherapies to prevent the DCIS to invasive transition and metastasis. For example, NODAL neutralizing antibody has shown promising anti-cancer effects (11-13). Future studies could also be done to test whether the combination of SEL201 and an existing anti-NODAL monoclonal antibody 3D1 (11) can elicit superior anti-tumor immunity to impair DCIS invasive transition in immune-competent DCIS models.

4.2.3 Characterizing the immune landscape of PPBC patient samples

The importance of tumor-stroma interactions is increasingly recognized. Recent studies have revealed that the presence of tumor infiltrating lymphocytes (TILs), especially CD8+ T cells, predict good prognosis in breast cancer (14, 15). However, it remains unclear how various

immune cells interact with breast tumor epithelium and other non-immune stromal components such as CAFs. Further interrogation of the tumor microenvironment at subcellular resolution will possibly uncover pathological features, new biomarker correlations and cell-cell interactions. Imaging mass cytometry (IMC) is a powerful tool that can quantitatively measure more than 50 markers by using metal-conjugated antibodies directly on tissue sections. Therefore, by using IMC, which has been widely used to study the TME, it is possible to further characterize the PPBC TME and compare those tumors to breast cancers that are not diagnosed during the post-partum period.

Given the elevated immune cell infiltration and fibroblast activation that occurs during mammary gland involution in human studies and animal models (1, 16, 17), we hypothesize that PPBC tissues will have a higher level of overall immune infiltration as well as altered spatial localization and/or levels of stromal components. To test this hypothesis, future work in our lab will be to map the detailed landscape of PPBC TME by using the IMC technology. Specifically, we will characterize the levels of infiltration and distribution of key stromal cells, including CAFs, cytotoxic T cells, Th1 and Th2 cells, regulatory T cells, MDSCs, and ILC2s. More importantly, we will characterize the levels of MNK1, eIF4E and the phosphorylated forms of MNK1 and eIF4E, in order to get a more detailed picture of which immune cells types have high expressions and/or high activities of MNK1 and eIF4E. Additionally, we will examine the expression of important immune-checkpoints such as PD-1 and PD-L1, as this hopefully will reveal more information on the immune-checkpoints that are highly expressed in the context of PPBC, thus helping to guide future research on targeting key checkpoints in this highly metastatic disease. Overall, such a detailed characterization of the PPBC TME will shed more light on how aberrant mRNA translation impacts different cellular components, and to provide

novel insight on strategies that can be employed to re-shape tumor immunity to improve PPBC patient outcomes.

4.2.4 Characterizing the functions of MNK1/2-eIF4E in organ specific breast cancer metastasis

Breast cancers are prone to metastasize to a few secondary organs, including liver, bones, lungs and brain. Although it remains unclear why some cancers metastasize to specific organs, some earlier studies revealed that cancer cells may utilize specific receptors on their surface which match the ligands that are highly expressed in secondary organs. For example, breast cancer cells express specific chemokine receptors, such as C-X-C motif receptor 4 (CXCR4) and C-C motif receptor 7 (CCR7), to recognize the chemokines that are preferentially expressed in the lymph nodes (CCL21), liver, bone and lungs (CXCL12) (Summarized by Muller et al. (18)). More recently, several studies have uncovered the functions of tumor cell-derived exosomes in the preparation of pre-metastatic niche (19). Exosomes are heterogenous small membrane vesicles (~30-100nm) containing various biomolecules such as RNA, DNA, lipids and protein. Tumor cell-derived exosomes can fuse with stromal cells in secondary organs, and such fusion subsequently prepares the “soil” for the tumor cell to seed. Exosomes, which contain many mRNAs and non-coding mRNAs, may serve as an important mediator for genetic component exchange between cells (20, 21). Recently, overexpression of eukaryotic translation initiation factor 3 subunit C (eIF3C) is reported to promote exosome release from hepatocellular carcinoma (HCC) (22). However, it remains largely unknown how exosome-dependent pre-metastatic niche formation is affected by aberrant mRNA translational machinery. Aberrant mRNA translation may offer the advantage of an accelerated cellular response to external stimuli by circumventing the need of de novo transcription, thus it is possible that mRNA translational

regulatory factors, such as the eukaryotic initiation factor (eIF) family members, may regulate the dynamic process of exosome-dependent pre-metastatic niche formation. Future studies are needed to explore whether hyper-activation of pro-oncogenic mRNA translational machineries like the MNK1/2-eIF4E axis can affect the quantities and contents of tumor-derived exosomes, and how they affect organ-specific metastasis.

Liver metastasis seems to be more common in patients diagnosed with PPBC than those with nulliparous breast cancer (23). Recently, the concept of "liver involution" has been proposed, as liver weight loss, hepatocyte apoptosis, and ECM remodeling have been observed after forced weaning (23). The Schedin group proposes that the liver involuting microenvironment is important for the establishment of PPBC liver metastasis in rodent models (23). Future studies are needed to verify if this liver involution process also exists in postpartum women, and to characterize whether the MNK1/2-eIF4E pathway contributes to the pro-tumorigenic immunity in PPBC liver metastasis and to examine whether a MNK1/2 inhibitor plus checkpoint blockade can be applied to inhibit liver metastasis. In order to investigate these questions, 4T1 breast cancer cells can be injected into the spleens of WT and eIF4E^{S209A} PPBC mice. The splenic injections will allow the colonization of tumor cells in the liver. We anticipate that the eIF4E^{S209A} liver microenvironment will be less favorable for breast cancer colonization, similar to our work in lung metastasis. Additionally, MNK1/2 inhibitor SEL201 and/or anti-PD1 antibody could be applied to 4T1-tumor bearing WT liver metastatic PPBC animals to test if SEL201 can increase the anti-tumor effect of anti-PD1 antibody.

4.2.5 Targeting CAFs in metastatic breast cancer

We and others have observed CAFs as important stromal components that support breast cancer immune evasion and metastasis. Recent studies have examined the possibility of depleting CAFs

as an anti-cancer therapy. For example, fibroblast-activation protein (FAP), which is a surface marker on activated fibroblasts, is a potential therapeutic target on CAFs. Depletion of FAP+ cells have led to inhibition of tumor outgrowth in several solid malignancies (summarized by Chen & Song (24)). Alternatively, previous studies have also investigated the possibility of inactivating CAFs by using all-trans retinoic acid (ATRA, a vitamin A derivative) and calcipotriol (a vitamin D receptor agonist), as the functions of nuclear receptors such as retinoic acid receptor and vitamin D receptor in CAF activation have been recently revealed. Specifically, a high dose of ATRA impairs the collagen-deposition ability of pancreatic satellite cells (PSCs)-derived fibroblasts and induces them to enter into a more quiescent status (25, 26). Vitamin D receptor suppresses the activation of pancreatic satellite cells (PSCs)-derived fibroblasts in pancreatic ductal adenocarcinoma (PDAC), and Calcipotriol can block activation and proliferation of CAFs to render anti-neoplastic effects (27). However, the effects of ATRA and calcipotriol in deactivating fibroblasts are only reported in PDAC. It remains unclear if these two vitamin derivatives have similar impact on CAFs from other solid malignancies.

Despite the somewhat promising results in animal models, it remains challenging to target CAFs in human cancer, mainly due to the heterogeneity of CAFs and the lack of specificity while targeting these plastic cells. Future studies should further explore the biology of CAFs in primary tumor sites, circulation and metastatic sites. This may allow us to uncover better strategies to target CAFs in a direct or indirect fashion, so the TME might be re-shaped into a mode for anti-tumor immunity.

4.2.6 Targeting mRNA translation to increase sensitivity to checkpoint blockade

Recently, two independent groups have reported that PD-L1 expression is regulated by eIF4E or the eIF4F complex (28, 29). Here, we have shown that the MNK1/2 inhibitor SEL201 can

increase the anti-metastatic effect of anti-PD1 blockade, possibly by elimination of the PD-L1:PD1 interaction by two mechanisms. Although this provides a possible strategy to overcome resistance to immune-checkpoint blockade, the PPBC mice treated with the combination of SEL201 and anti-PD1 were not completely free of metastasis. This could be explained by the potential importance of many other immune checkpoints, such as TIM3, LAG3, TIGIT, VISTA and BTLA4, which also contribute to tumor immune evasion. Furthermore, blunted signaling from co-stimulatory pathways, such as CD40, ICOS and GITR, may also serve as non-redundant mechanisms for tumor immune evasion. Thus, blocking antibodies of other immune-checkpoints and agonists of co-stimulatory molecules is being actively investigated in combination with anti-CTLA4 or anti-PD1/anti-PD-L1 blockade. Recent results of a phase Ib clinical trial testing CD40 agonists in conjunction with nivolumab, a PD1 blocking antibody, in pancreatic ductal carcinoma has provided a novel strategy for the treatment of highly-aggressive cancers (30). To find a better therapy for PPBC and metastatic breast cancer in general, future studies are required to thoroughly profile the expression status of immune-checkpoints and co-stimulatory molecules in patient samples. Such studies may help to reveal non-redundant immuno-regulatory pathways to PD1:PD-L1/2 and CTLA-4, thus to discover novel therapies to overcome metastatic breast cancer.

4.3 Final conclusion and summary

The work presented in this thesis highlights the critical functions of MNK1/2-eIF4E pathway in breast cancer. The objectives of our studies were as follows:

a) To determine if MNK1 regulates the transition from DCIS to invasive disease.

b) To understand how the MNK1/eIF4E axis, functioning in stromal cells, regulates PPBC pro-tumorigenic immunity.

The objectives of our studies were accomplished as follows:

a) We identified tumor intrinsic the MNK1/NODAL axis as a key event promoting the early invasive progression in breast ductal carcinoma in situ. Of potential relevance to the clinic, we show that ablation of MNK1, genetically or pharmacologically, partially impairs the DCIS to IDC transition.

b) Our findings expand the importance of eIF4E phosphorylation to not only include cancer cells, but also in stromal cells of the tumor microenvironment (i.e. fibroblasts and immune cells), in permitting pro-tumorigenic immune evasion in PPBC. Our findings culminated in proposing to block the MNK1/2-eIF4E pathway as a strategy to partially inhibit breast cancer invasion, and to sensitize metastatic PPBC to anti-PD1 therapy.

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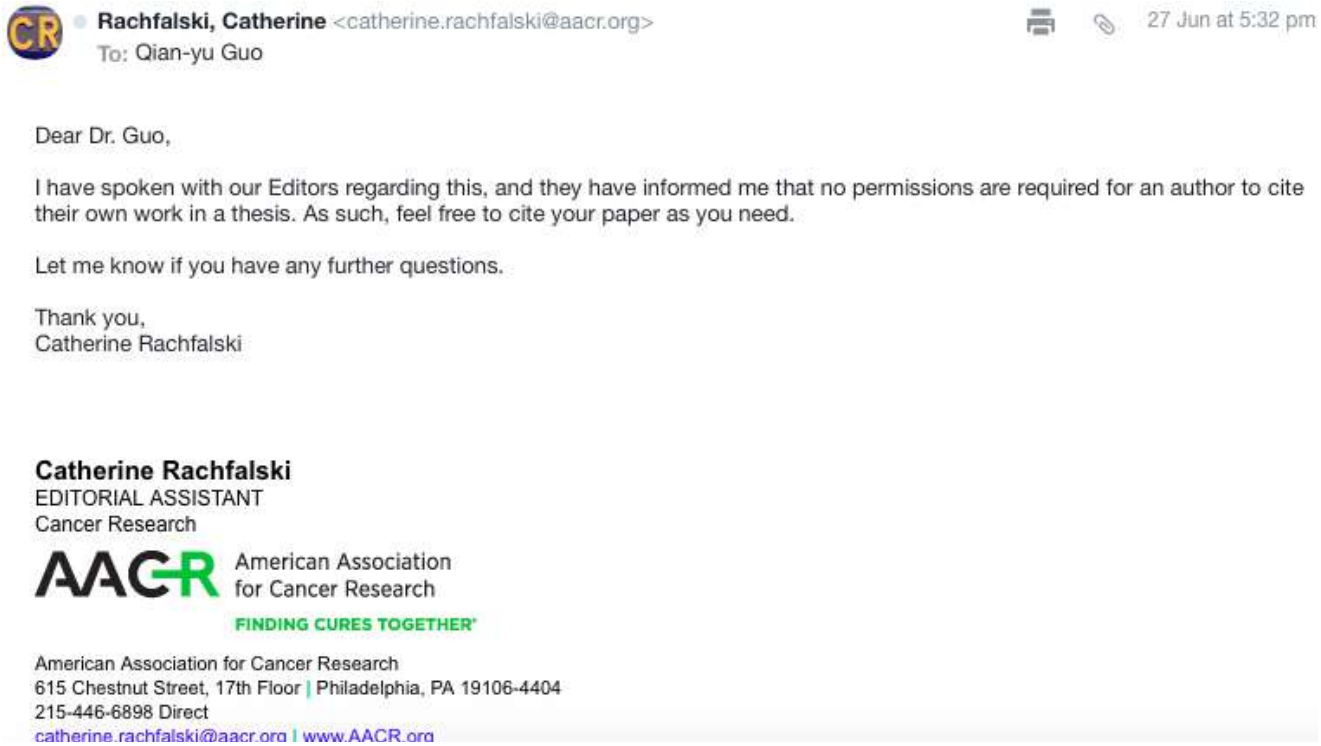

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