GPNMB in triple-negative breast cancer: elucidating molecular mechanisms of action

by

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A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

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Abstract

Glycoprotein nmb (GPNMB) promotes breast tumor growth and metastasis and its expression in tumor epithelium correlates with poor prognosis in breast cancer patients. Additionally, GPNMB is overexpressed in triple-negative breast cancers (TNBCs) and is an independent indicator of poor prognosis in this aggressive subset of the disease. Despite its biological and clinical significance, little is known regarding the molecular mechanisms engaged by GPNMB. Herein, we show that GPNMB employs distinct functional domains and mechanisms to promote primary tumor growth and metastasis. We demonstrate that Neuropilin-1 (NRP-1) expression is increased in breast cancer cells that overexpress GPNMB. Interestingly, the GPNMB-driven increase in NRP-1 expression potentiated VEGF signaling in breast cancer cells and was required for the growth, but not metastasis, of these cells *in vivo.* Interrogation of RNAseq datasets revealed a positive correlation between *GPNMB* and *NRP-1* levels in human breast tumors.

Furthermore, we ascribe pro-growth and pro-metastatic functions of GPNMB to its ability to bind $\alpha_5\beta_1$ integrin and increase downstream signaling in breast cancer cells. We show that GPNMB enhances breast cancer cell adhesion to fibronectin, increases $\alpha_5\beta_1$ protein stability and associates with this receptor through its RGD motif. We also identify a novel role for GPNMB in promoting recycling of the active $\alpha_5\beta_1$ fibronectin receptor through the recently identified late endosomal/lysosomal recycling pathway. GPNMB recruitment into active integrin complexes activates SRC/FAK signaling pathways and reciprocal GPNMB phosphorylation in an RGD-dependent manner. Importantly both the RGD motif and cytoplasmic tail of GPNMB are required to promote primary mammary

tumor growth; however, only mutation of the RGD motif impaired the formation of lung metastases.

Finally, we demonstrate that the pro-tumor properties of GPNMB are modulated by the oncogenic context in which GPNMB is engaged. Using a genetically engineered mouse model of basal breast cancer, we show that GPNMB accelerates onset and growth of MMTV/Wnt-1 mammary tumors but is dispensable for metastasis in this setting. We identify the PI3K/AKT/mTOR pathway as the primary signaling event engaged in MMTV/GPNMB x MMTV/Wnt-1 tumors and extend these observations to breast cancer patient samples. Furthermore, we show that GPNMB expression promotes nuclear localization of β-catenin and increases expression of β-catenin transcriptional targets in Wnt-1-expressing breast cancers.

Together, these findings identify novel and distinct molecular mediators of GPNMB-induced breast cancer growth and metastasis. Additionally, the cooperative pathways described in this thesis represent important potential avenues to explore for combination therapy in TNBCs.

Résumé

Glycoprotein nmb (GPNMB) favorise la croissance et les métastases du cancer du sein, et son expression dans l'épithelium tumoral est associée avec un mauvais pronostic chez les patientes atteintes du cancer du sein. De plus, GPNMB est surexprimé dans les cancers du sein triple-négatifs (TNBCs) et représente un indicateur indépendant de pronostic défavorable dans ce sousgroupe agressif du cancer du sein. Malgré la signification biologique et clinique de GPNMB, ses mécanismes moléculaires en aval ne sont pas toujours connus. Le travail présenté ici démontre que GPNMB emploie des mécanismes et des séquences protéiques distincts pour favoriser l'évolution et la dissémination métastatique du cancer du sein. En utilisant une série de cellules de cancer du sein qui surexpriment GPNMB, j'ai montré que l'expression de Neuropilin-1 (NRP-1) est augmentée en aval de GPNMB. L'expression élevée de NRP-1 induite par GPNMB potentialise la voie de signalisation du facteur de croissance endothéliale (VEGF) dans les cellules du cancer du sein et est requise pour la croissance, mais pas la métastase, de ces cellules *in vivo.* En examinant les ensembles de donnés de séquençage de l'ARN, j'ai identifié une corrélation positive entre l'expression de *GPNMB* et *NRP-1* dans les tumeurs du sein humaines.

Par ailleurs, j'ai attribué les fonctions de GPNMB qui favorisent la croissance et la métastase du cancer du sein à sa capacité de se lier à l'intégrine α5β1 et activer les voies de signalisation en aval. J'ai démontré que GPNMB augmente l'adhésion des cellules de cancer du sein à la fibronectine, améliore la stabilité de la protéine α5β1 et forme un complexe protéique avec l'intégrine α5β1 qui est médié par le site de liaison RGD. De plus, j'ai découvert une nouvelle fonction de GPNMB dans la régulation du recyclage du

récepteur de fibronectine actif α5β1 par la voie lysosomiale de recyclage. GPNMB est recruté aux complexes d'intégrines actifs par l'intermédiaire de son domaine RGD, ce qui augmente la voie de signalisation SRC/FAK et induit la phosphorylation réciproque de GPNMB. En outre, le motif RGD et le domaine cytoplasmique de GPNMB contribuent tous les deux à la croissance des tumeurs mammaires, mais seul le motif RGD est nécessaire pour favoriser la formation des métastases pulmonaires.

Enfin, j'ai démontré que les fonctions de GPNMB qui favorisent la progression tumorale sont modulées par le contexte oncogénique environnant. À l'aide de modèles de souris transgéniques, j'ai déterminé que GPNMB accélère l'apparition et la croissance des tumeurs mammaires MMTV/Wnt-1 mais n'augmente pas le niveau de métastase dans ce contexte. J'ai aussi identifié la voie PI3K/AKT/mTOR comme le mécanisme de signalisation primaire induit dans les tumeurs MMTV/GPNMB x MMTV/Wnt-1 et détecté dans les échantillons de cancer du sein qui surexpriment GPNMB. De plus, GPNMB favorise la localisation nucléaire de la β-caténine et augmente la transcription des gènes régulés par la β-caténine en aval de Wnt-1.

L'ensemble de mes travaux identifie des nouveaux mécanismes moléculaires déclenchés par GPNMB pendant l'évolution du cancer du sein. De plus, les voies coopératives décrites dans cette thèse représentent des nouvelles pistes thérapeutiques qu'il sera important à explorer pour le traitment du cancer du sein.

Acknowledgements

I would first like to acknowledge the support I received from various funding sources and stipends which allowed me to pursue my PhD research, including the three-year Canadian Institutes of Health Research Doctoral Award, the Canadian Institutes of Health Research Masters Award, two Provost Graduate Fellowships from the Division of Experimental Medicine and two Graduate Excellence Awards from the Division of Experimental Medicine. I would also like to acknowledge the funding that I received for travel and conferences provided by the Canadian Institutes of Health Research and the Division of Experimental Medicine, which allowed me to present my work to national and international audiences, interact with fellow members of the scientific community, and learn about new advances in my field.

I am extremely grateful to my supervisor, Dr. Peter Siegel, for his patience, mentorship and support throughout my studies. Peter – thank you for your enthusiasm and insightful discussions which have always motivated me and given me a fresh perspective in my research, for your attention to detail and your immense knowledge of the field which have helped me develop as a scientist and an analytical thinker, for your support and understanding of my ambitions in and out of the lab, and for your attention to my thesis (I can't believe you read it twice!). Your commitment to quality and excellence and your passion for your work have made a big impression on me, and I am continuously striving to emulate these qualities, both in my academic studies and in my career.

Thank you to my Thesis Advisory Committee, which included Dr. Jose Teodoro, Dr. Janusz Rak and Dr. Xiang-Jiao Yang for their insights, great discussion and constructive feedback over the years. I appreciated their guidance and their suggestions, which have helped me identify new avenues of investigation for my project and develop the ability to think critically about my results. I would also like to acknowledge all my collaborators for providing protocols, reagents, and engaging discussions about my project.

I would like to thank the past and present members of the Siegel lab for being great colleagues and helping shape my PhD career: Matthew Annis, Chanèle Cyr-Depauw, Zhifeng Dong, Fanny Dupuy, Brian Hsu, Patricia MacDonald, Anna Mourskaia, Elaine Ngan, Véronique Ouellet, Jason Northey, Dru Perkins, April Rose, Cathy Russo, Sébastien Tabariès and Christine Tam. Thank you all for contributing to a stimulating and fun lab environment.

Special thanks go to Team GPNMB: Matt, thank you for your continued support, for being an amazing mentor, for your willingness to listen to and help troubleshoot my scientific (and non-scientific) problems, and for making the lab such a fun place to work. Thank you also for all your hard work to help complete the transgenics story. You have been an enormous help in getting through this final hurdle! Patricia and Cathy, I'm grateful for all your help in guiding and training me when I was starting my PhD, and for setting up this project. Zhifeng, your technical support and unparalleled IHC skills have greatly contributed to this project. April, for being the first member of Team GPNMB and

motivating us all with your passion. Marco, wish we could have overlapped, but thank you for carrying the GPNMB torch forward.

To my GCRC, ExMed and GMCA communities – I am so grateful to have worked with such inspiring colleagues and to have formed great friendships along the way. Fanny, thank you for the long discussions about anything and everything, and for always being there for me. From the late nights at the lab to late nights at la Tulipe, I'm so glad that we experienced this chapter together. I couldn't have asked for a better friend, colleague and roommate. Eni and Rana, I could always count on you for unwavering friendship and support, through our BIOL301 TA sessions, through launching the McGill GMCA together, and through our many, many trips, chats and family dinners. Thank you both for always being the brightest part of my day. Brian, I'm so happy you joined the lab, your positive energy and friendship have made my grad studies a lot more enjoyable. Dmitri, thanks for talking to me about integrins, recycling, and life. Vas, thanks for being an awesome co-president and friend, and for motivating me in all my extra-curricular pursuits. Johanna, it has been such a pleasure working with you on all the various committees, and I'm so grateful for our friendship along the way. Anna, thank you for being a great friend in and out of the lab and for helping make the beginning of my PhD such a fun and memorable experience. Special thanks go to Dr. Hugh Bennett, Dominique Besso and Marylin Linhares for welcoming me to such an amazing department, and for their tremendous support during my tenure on the EMGSS committee and throughout my graduate studies.

Last but not least I would like to thank my boyfriend and family for their love and support throughout this process. Alik, thank you for inspiring me and encouraging me through this final hurdle. To my mom, dad, and sister, thank you for always believing in me and for pushing me to wrap up my studies. I am so grateful for your patience and encouragement.

Publications arising from this work

Chapter 1 contains material published in the following review article:

Maric G, Rose AA, Annis MG, Siegel PM. (2013) Glycoprotein non-metastatic b (GPNMB): A metastatic mediator and emerging therapeutic target in cancer. Onco Targets Ther. 9(6):839-52

Chapter 2 was published as part of an original research article:

Maric G, Annis MG, Dong Z, Rose AA, Ng S, Perkins D, MacDonald PA, Ouellet V, Russo C, Siegel PM. (2015) GPNMB cooperates with neuropilin-1 to promote mammary tumor growth and engages integrin α5β1 for efficient breast cancer metastasis. Oncogene, March 16th 2015.

Chapter 3 contains unpublished material and material published in the following original research article:

Maric G, Annis MG, Dong Z, Rose AA, Ng S, Perkins D, MacDonald PA, Ouellet V, Russo C, Siegel PM. (2015) GPNMB cooperates with neuropilin-1 to promote mammary tumor growth and engages integrin α5β1 for efficient breast cancer metastasis. Oncogene, March 16th 2015.

Figures 3.5, 3.6, 3.7 and 3.8 are unpublished, and Figures 3.4 and 3.9 contain unpublished material

Chapter 4 was published as an original research article:

Gordana Maric*, Matthew Annis*, Patricia MacDonald, Caterina Russo, Dru Perkins, Doris R. Siwak, Gordon B. Mills and Peter M. Siegel. GPNMB augments Wnt-1-mediated breast tumor initiation and growth by enhancing PI3K/AKT/mTOR pathway signaling and β-catenin activity. Oncogene, March 11th, 2019

I contributed data to the following publication during my PhD, however it is not presented in this thesis:

Ahn R, Sabourin V, Ha JR, Cory S, Maric G, Im YK, Hardy WR, Zhao H, Park M, Hallett M, Siegel PM, Pawson T, Ursini-Siegel J. The ShcA PTB domain functions as a biological sensor of phosphotyrosine signaling during breast cancer progression. (2013) Cancer Res 73(14):4521-32

Contributions of the authors

A few collaborations have been established during my PhD in order to obtain the quality of the results presented in this thesis. A number of experts outside of my field of expertise have participated in this process. The contributions provided by these people are outlined below, for each chapter constituting this work. I conducted all other experiments. Aside from the exceptions listed below, I designed all the experiments in this thesis and analyzed the subsequent results under the guidance of my supervisor, Dr. Peter Siegel. Finally, I prepared all the figures and text in this thesis with Dr. Peter Siegel's assistance.

In Chapter 2, the microarray comparing GPNMB-expressing BT549 and 66cl4 populations to control cells was performed by A.A.R and analyzed by V.O. (Figure 2.1, Table 2.1) The validation of the candidates (Supplemental Figure 2.1) was performed by S.N. The NIC cells harboring and NRP-1 knockdown were generated by M.G.A and D.P. (Figure 2.3A). Z.D. performed all the immunohistochemistry experiments and I quantified the data (Supplemental Figures 2.3 and 2.4).

In Chapter 3, P.A.M. and C.R. created the human GPNMB mutant constructs and M.G.A. created the mouse GPNMB mutant constructs and generated the HS578T, 66cl4 and NIC cell lines (Figure 3.1A, Supplemental Figure 3.1). Z.D. performed all the immunohistochemistry experiments and I quantified the data (Supplemental Figures 3.2 and 3.3)

In Chapter 4, the MMTV/GPNMB transgenic mice were generated and characterized by P.A.M. (Figure 4.1 A, D). Z.D. performed all immunohistochemistry and immunofluorescence experiments and I quantified the data (Figure 4.1 B, C; Figure 4.2 D; Figure 4.3 B, C; Supplementary Figure 4.3). M.G.A. designed experiments and prepared figures for assessment of β-catenin activity (Figure 4.2A, 4.4E, 4.5). M.G.A performed the qRT-PCR analysis of β-catenin targets from MMTV/Wnt-1 and MMTV/Wnt-1 x MMTV/GPNMB tumors (Figure 4.4 E). D.P generated the GPNMB Crispr/Cas9 knockdown of GPNMB in 533 cells (Figure 4.5 A). M.G.A performed all experiments with 533 breast cancer cells including the mammary fat pad injections, tumor measurements and analysis of metastatic burden, qRT-PCR quantification of β-catenin targets, nuclear fractionation and immunoblot experiments (Figure 4.5 B-G)

Original contributions to knowledge

1) We are the first to implicate Neuropilin-1as a downstream mediator of GPNMB-driven tumor growth. We show that Neuropilin-1 is upregulated in response to GPNMB overexpression, interacts with GPNMB, and serves to enhance responsiveness of GPNMB-expressing cancer cells to VEGF signaling.

2) We show, for the first time, that GPNMB promotes recruitment of a macrophage infiltrate during tumor progression through a mechanism that is dependent on the GPNMB RGD domain and cytoplasmic tail.

3) Using a series of GPNMB mutants, we demonstrate that the GPNMB RGD integrin-binding domain is required for tumor growth, invasion and metastasis, and that the GPNMB cytoplasmic tail is required for primary tumor growth and invasion.

4) We identify a novel role for GPNMB in promoting recycling and enhancing protein expression of the α5β1 fibronectin receptor. We show that intracellular GPNMB is localized to late endosomes and interacts with the active α5β1 fibronectin receptor in an RGD-dependent manner. We demonstrate that this interaction is required to rescue the active α5β1 integrin complex from lysosomal degradation by increasing cell surface recycling through the recently identified late endosomal recycling pathway.

5) We demonstrate, for the first time, that GPNMB increases FAK/SRC signaling in response to fibronectin engagement, leading to reciprocal Src-mediated phosphorylation of the GPNMB cytoplasmic tail. We also show that these signaling events are dependent on the GPNMB RGD integrin-binding domain.

6) This work is the first to demonstrate a role for GPNMB in tumor initiation. We demonstrate that GPNMB accelerates tumor onset using a transgenic mouse model of breast cancer.

7) Using a proteomic profiling approach, we identify the PI3K/AKT/mTOR pathway as an important mediator of GPNMB activity. We show that pathway signaling is enhanced in GPNMB-expressing basal breast cancers and breast cancer patient samples.

8) We show that GPNMB promotes β-catenin nuclear translocation and transcriptional activity in Wnt-expressing basal breast cancer.

Preface

This is a manuscript-based thesis. The introduction contains a portion of a published peer-review article. The three data chapters represent: 1) part of a published peer-reviewed original research article, 2) part of a published peer-reviewed original research article, and 3) a manuscript in preparation to be submitted as an original research article. This thesis is divided into five sections:

1. An overview of the relevant literature
2-4. Manuscripts with their own preface, abstract, introduction, results, discussion, materials and methods, references, tables and figures.
5. A general discussion of the results presented in the data chapters

List of figures and tables

List of abbreviations

4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
ADAM	A disintegrin and metalloprotease
ADC	Antibody-drug conjugate
AGO	Protein argonaute
ALS	Amyotrophic lateral sclerosis
AKT/PKB	Protein kinase B
APC	Adenomatous polyposis coli
BCR	Breakpoint cluster region
BL	Basal-like
BLBC	Basal-like breast cancer
BRCA	BReast Cancer
BRK	Breast tumor kinase (also PTK6)
Cas9	CRISPR-associated protein 9
CLIC3	Chloride intracellular channel 3
CK	Cytokeratin
c-Kit/SCFR	Mast/stem cell growth factor receptor
CNA	Copy number alteration
CRISPR	Clustered regularly interspaced short palindromic repeats
CTC	Circulating tumor cell
CUB	Complement C1r/C1s, Uegf, Bmp1
CYT	Cytoplasmic tail
DC-HIL	Dendritic cell heparin integrin ligand
DNA	Deoxyribonucleic acid
EEA-1	Early endosome antigen 1
ECD	Extracellular domain
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FDA	Food and drug administration
FGFR	Fibroblast growth factor receptor
FOXA1	Forkhead box A1
FZD	Frizzled
GIPC	GIPC PDZ domain containing family, member 1
GPNMB	Glycoprotein non-metastatic melanoma protein B
GRB	Growth factor receptor-bound
GSK3	Glycogen synthase kinase 3
H & E	Hematoxylin and eosin
HER2	Human EGF receptor 2
HGFIN	Hematopoietic growth factor inducible, neurokinin-1 type

IC	Investigator's choice
IFN	Interferon
IGFR	Insulin-like growth factor receptor
IL	Interleukin
IM	Immuno-modulatory
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
LAMP-1	Lysosomal-associated membrane protein 1
LAR	Luminal androgen receptor
LC3	Light chain 3
LEF-1	Lymphoid enhancer binding factor 1
LINK-A	Long intergenic non-coding RNA for kinase activation
LPS	Lipopolysaccharide
M	Mesenchymal
MAM	Meprin, A-5 protein, and receptor protein-tyrosine phosphatase mu
MAPK	Mitogen-activated protein kinase
MAP2K	Mitogen-activated protein kinase kinase
MDM2	Mouse double minute 2
MDSC	Myeloid-derived suppressor cell
MED	Mediator complex
METABRIC	Molecular taxonomy of breast cancer international consortium
MITF	Microphthalemia-associated transcription factor
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
αMSH	Alpha melanocyte-stimulating hormone
MSL	Mesenchymal stem-like
MTD	Maximum tolerated dose
mTOR	Mammalian target of rapamycin
NDRG1	N-myc downstream regulated 1
NRP-1	Neuropilin-1
OS	Overall survival
PARP	Poly ADP-ribose polymerase
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PFS	Progression-free survival
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PKD	Polycystic kidney disease
PMEL17	Premelanosome protein
PPP2R	Serine/threonine-protein phosphatase 2 regulator subunit
PR	Progesterone receptor
PRAS40	Proline rich AKT substrate of 40 kDa
PTEN	Protein phosphatase and tensin homolog
PyMT	Polyoma middle T
QNR-71	Quail neuroretinal cell
Rac	Ras-related C3 botulinum toxin substrate

RANKL	Receptor activator for nuclear factor k B ligand
Ras	Rat sarcoma
RCP	Rab-coupled protein
RGD	Arginine, glycine, aspartic acid
Rho	Ras homolog gene family member
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
RPPA	Reverse phase protein array
RS	Recurrence score
RT-PCR	Reverse transcriptase polymerase chain reaction
SEMA3A	Semaphorin 3A
sFRP1	Secreted frizzled-related protein 1
SNX17	Sorting nexin 17
STAT	Signal transducer and activator of transcription
SYK	Spleen tyrosine kinase
TCF	T-cell factor
TCGA	The cancer genome atlas
TGF	Transforming growth factor
TNBC	Triple-negative breast cancer
TNF	Tumor necrosis factor
TRAP	Tartrate resistant acid phosphatase
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WIF	Wnt inhibitory factor
XBP1	X-box binding protein 1
ZEB	Zinc finger E-box binding homeobox 1
ZNF	Zinc finger

CHAPTER 1 – Literature Review and Introduction

1.1 Cancer definition and epidemiology

The term cancer is used to define a group of heterogeneous diseases that exhibit deregulated growth and insensitivity to physiological cues that maintain normal tissue homeostasis. During cancer progression, tumor cells acquire increasing malignant potential by accumulating a series of genetic mutations. This transformation process requires a number of alterations that ultimately give rise to tumor cells with optimal fitness, including 1) sustained proliferative signaling, 2) resistance to apoptosis, 3) evasion of anti-growth signals, 4) limitless replicative potential, 5) sustained angiogenesis and 6) local invasion and metastasis [1]. A tumor is not a homogenous mass of cancer cells; it also recruits fibroblasts, endothelial cells and immune cells to form the tumor-associated stroma. In the growing tumor milieu, these distinct cell types engage in bi-directional interactions that actively promote tumorigenesis. To reflect the complexity of these interactions, the initial hallmarks of cancer have recently been revisited to include 1) immune evasion, 2) tumor-promoting inflammation, 3) metabolic deregulation and 4) genomic instability and mutation [2].

Cancer is a widespread disease with a devastating global burden. The latest statistics from the World Health Organization indicate that over 14 million new cancer cases develop annually, and that cancer accounted for 8.8 million deaths in 2015 [3]. In Canada, cancer is the leading cause of mortality and is responsible for 30% of all deaths [4]. In 2017, it is estimated that 206,200 new cancer cases were diagnosed in Canada and that 80,800 individuals died from the disease [4]. The most frequently occurring cancers are lung, breast, colorectal and prostate cancers, which account for half of newly diagnosed cancer cases [4].

1.2 Cancer metastasis

Over 90% of cancer-related deaths are attributable to metastasis, and, once the cancer has spread from the primary tumor, the disease is largely incurable [5]. During the metastatic process, cancer cells disseminate from the primary tumor to establish malignant lesions in distant organs. In order to metastasize, tumor cells need to complete a series of steps which include local invasion of the surrounding tissue, intravasation into the vasculature, survival in the circulation, extravasation from the bloodstream to a distant site, survival and adaption to the new microenvironment, and re-initiation of the tumor growth process for the establishment of macrometastatic lesions [6]. The metastatic cascade is highly inefficient, and each step represents an important barrier that must be overcome by the disseminated tumor cells.

1.2.1 Models of metastatic progression

Many theoretical models have been proposed to explain and predict patterns of cancer cell dissemination, in order to inform preclinical research and facilitate therapeutic decisions. The majority of these theories have been derived from two fundamental models of metastasis: the linear progression model and the parallel progression model [7]. The use of molecular information characterizing primary tumors to determine the appropriate therapeutic approach is based on the classic linear progression model. This view of metastasis implies that the formation of a fully malignant late-stage primary tumor is required before cancer cells can efficiently disseminate to secondary sites [7]. According to this model, metastatic tumor cells represent the most aggressive sub-population within a heterogeneous tumor that arise following stepwise accumulation of genetic and epigenetic changes [8]. Tumor cells that successfully pass through these rounds of mutation will acquire autonomous replicative capacity, proliferate to constitute the majority of the

primary tumor mass and disseminate to secondary organs to form macrometastic lesions [9]. This theory is largely supported by the correlation between large primary tumor size and high incidence of metastasis, which forms the basis of the routinely used tumor/node/metastasis (TNM) breast cancer classification system [10]. The linear progression model is related to the metastatic cascades hypothesis, which postulates that established metastases could subsequently initiate the formation of secondary metastases through a similar stepwise process [11]. In line with the linear progression and metastatic cascades models, a recent whole-genome sequencing study examining the evolutionary patterns of metastatic breast cancer has demonstrated that cancer cells disseminate from late-stage primary tumors and continue to accumulate genetic alterations at the secondary site by engaging mutation pathways largely present in the primary tumor [12].

Conversely, the parallel progression model posits that cancer cell dissemination can occur during early stages of tumor growth, before the disease becomes clinically detectable [7]. This model does not dispute that clonal evolution occurs in response to accumulation of genetic changes but theorizes that early disseminating metastatic founder cells can acquire malignant traits at the metastatic site, thereby leading to evolution of a secondary tumor that is genetically distinct from the original. Notably, circulating tumor cells (CTCs) are detectable in the vasculature of patients with pre-neoplastic lesions [13] and micrometastases can be observed in mice transplanted with pre-malignant HER2-expressing transgenic mammary glands [14], which provides evidence for the parallel progression model. Additionally, in patients displaying overt metastases, matched primary tumors and disseminated tumor cells found in the bone marrow display significant differences in genomic makeup, indicating that tumor cells can acquire genomic alterations outside of the primary tumor environment [15]. Recent studies have attributed early

dissemination of primary breast cancer cells to a HER2-driven, progesterone-regulated mechanism which engages Wnt-dependent pathways to promote metastatic potential of early neoplastic lesions [16, 17]. In an extension of the parallel progression model, it has also been postulated that tumor cells with sub-optimal fitness can co-evolve with stromal cells present in the microenvironment at primary and secondary sites [18].

1.2.2 The metastatic cascade

1.2.2.1 Local invasion

During local invasion, tumor cells need to break down the basement membrane encapsulating the tumor and actively migrate through the surrounding extracellular matrix (ECM) and stromal cell layers. This process is mediated by the activation of cellular pathways that control cytoskeletal dynamics, turnover of cell-cell and cell-matrix junctions, and secretion of proteases [19, 20]. Cancer cell plasticity and interaction with the reactive tumor stroma are crucial to perpetuate migration and invasion in a variety of microenvironment conditions [21]. Individual cancer cells can either invade through protease-dependent Rac-mediated "mesenchymal" invasion, or through protease-independent, Rho/ROCK-mediated "amoeboid" invasion [22]. Integrin-mediated attachment to the ECM is a critical process in mesenchymal invasion, as it promotes cell-matrix adhesion turnover and relays anchorage-dependent survival signals [23]. Alternatively, many epithelial cancers can invade as multicellular sheets through a process called collective cell invasion [24].

Cellular plasticity can be mediated through the epithelial-to-mesenchymal transition (EMT) program, which initiates and augments Rac-driven mesenchymal invasion in response to stromal cues [25]. During EMT, TGFβ, Wnt, EGF and other growth factors

derived from the microenvironment promote a series of signaling events in cancer cells that culminate in the activation of Snail, Twist and ZEB1 transcription factors. These transcription factors orchestrate EMT by repressing E-cadherin transcription and concomitantly increasing expression of mesenchymal markers [26, 27]. E-cadherin is an integral component of intracellular adherens junctions and its downregulation leads to loss of cell polarity and dissolution of tight cell-cell adhesions, which enables individual tumor cells to detach from the primary tumor mass and invade into the surrounding tissue [28]. Importantly, the EMT program can also increase tumor aggressiveness by promoting cell survival and the acquisition of a stem cell-like phenotype [25]. However, EMT is not essential for metastasis, as it has been shown that disseminated tumor cells display both epithelial and mesenchymal characteristics *in vivo* and tumor cells migrating in collective units bypass the requirement for EMT altogether [6, 21].

1.2.2.2 Intravasation and survival in the circulation

It has been reported that growth factors such as TGFβ and EGF, which augment general invasiveness of cancer cells, can engage a similar pro-invasive mechanism to promote intravasation of mammary carcinoma cells [29, 30]. During the early stages of metastasis, EGF secreted by perivascular macrophages creates a chemotactic stimulus which promotes directed high-velocity migration of tumor cells along collagen fibers in the microenvironment [30, 31]. EGF also induces invadopodia formation in tumor cells and promotes expression of an “invasion signature” to facilitate movement through the dense collagen network and intravasation into the surrounding vasculature [32, 33]. In turn, cancer cells expressing the EGFR receptor secrete CSF-1, which recruits macrophages and increases their ECM remodeling capacity, thus creating a paracrine loop which directs

concerted movement of macrophages and tumor cells to the blood vessels and promotes intravasation [30, 31].

Integrin-mediated adhesion to the ECM promotes cell survival in the primary tumor microenvironment, however, this anchorage is lost once cancer cells enter the circulation. As a result, circulating tumor cells (CTCs) need to sustain pro-survival signals in order to avoid anoikis, a form of programmed cell death triggered by loss of attachment to the substratum [34].

Circulating tumor cells (CTCs) may represent "metastatic intermediates" and isolation of these cells has yielded critical insights about tumor biology and response to therapy [13, 35]. However, the availability of these cells poses an important challenge. CTCs are thought to linger in the circulation for short periods of time, and, due to their large size, usually get trapped in capillary beds in the minutes following intravasation [6]. Nonetheless, the presence CTCs in the blood has been correlated with increased metastasis, heightened tumor aggressiveness and decreased time to relapse [36].

1.2.2.3 Seeding and colonization

Most tumor cells that reach the secondary site undergo apoptosis within 24 hours [37], but those that persist can exist as dormant cells, micrometastases or macrometastases. Upon arrest in the microvessels, cancer cells exit the circulation by extravasating or by proliferating within the capillary beds, which leads to microvessel rupture [20, 38]. Epithelial cancers preferentially metastasize to specific target organs, and the seeding of such sites can be passively dictated by circulation patterns or mechanical barriers in the vasculature and in secondary sites [6]. For example, bone microvessels are devoid of mural cells [32] and tumor cells can readily traverse this endothelium relative to organs such as the lungs or brain. Such anatomical features may explain why bone is the preferred site of

metastasis for many cancers, including breast [39]. Similarly, the liver creates a highly permissive environment for metastasis due to its central role in the circulatory system and sinusoidal capillary network which slows the circulation of tumor cells and promotes their physical arrest. Additionally, the liver's fenestrated vasculature provides tumor cells with direct access to the sub-endothelial basement membrane and associated adhesion receptors, thus facilitating tumor cell adhesion and subsequent invasion into liver tissue [40].

Organ-specific seeding of CTCs could also be the result of an active process mediated by cell-adhesion molecules expressed on cancer cells and stromal cells. For example, metadherin promotes breast cancer cell adhesion to lung vasculature and claudin-2 increases breast cancer liver metastasis by mediating tumor cell-hepatocyte interactions [41, 42].

Since the microenvironment of a premetastatic site is considerably different from that of the primary tumor, outgrowth of metastatic lesions is also controlled in an organ-specific manner [43]. It has been postulated that metastasis "virulence" genes, which confer advantages for the colonization of specific distant organs, are expressed in cancer cells that reside in the primary tumor [43]. Notably, gene signatures that enable colonization of different organs have been identified in breast cancer cells [44-46]. It has been proposed that primary tumors can also facilitate colonization by establishing a "pre-metastatic niche" in distant organs [47]. According to this model, primary tumors secrete growth factors, or shed microvesicles [48], which educate secondary sites and make the environment more hospitable for the subsequent arrival and growth of metastatic cancer cells. Bone-marrow derived progenitor cells (BMDCs) that express VEGFR1 can home to distant sites and establish pre-metastatic cell clusters [49]. In turn, primary tumors secrete growth factors that upregulate fibronectin secretion from resident fibroblasts that cooperate with BMDCs

to create a more permissive environment for tumor cell arrival [49]. Additionally, breast cancer cells secrete lysyl oxidase (LOX) which promotes myeloid cell recruitment and collagen IV cross-linking at secondary sites, thereby creating a pre-metastatic niche that enhances seeding of metastatic tumor cells [50]. In a related concept known as "tumor self-seeding", aggressive CTCs can re-colonize their tumors of origin due to the existence of an already permissive microenvironment [51]. Tumor self-seeding is mediated by reactive stroma resulting from aggressive tumor establishment. Components of the reactive stroma have been shown to increase successful colonization through secretion of chemoattractants such as interleukins 6 and 8 [51].

1.3 Breast cancer epidemiology and classifications

Breast cancer is the most commonly diagnosed cancer in Canadian women and the second leading cause of cancer-related deaths [4]. According to Canadian Cancer Statistics, one in 8 women will develop breast cancer in their lifetime and 1 in 31 will die from it. In 2017, 26,500 women were expected to be diagnosed with breast cancer, which represents 25% of all new female cancer cases [4]. Additionally, it is estimated that 5,000 deaths were attributable to breast cancer in 2017. Similar trends are seen annually on a global scale, where breast cancer accounts for over 1.3 million cancer cases and over 570,000 cancer-related deaths [3]. Breast cancer incidence rates rose dramatically in the early 1990s due to the introduction of organized mammography screening programs and declined in 2002 following the introduction of hormone replacement therapy [4].

Breast cancer encompasses a collection of diseases that exhibit extensive inter- and intra-tumor heterogeneity. This tumor diversity is an important determinant of disease behavior and is therefore invaluable in assessing clinical parameters such as metastatic

dissemination and response to therapy. Several classification systems have been proposed to segregate patient populations based on common molecular and clinical features of the disease in order to facilitate treatment decisions. Initial attempts to classify breast cancers have relied on histopathological characteristics, and later, gene expression patterns. However, the advent of next-generation sequencing has introduced additional layers of complexity to our understanding of the genomic, epigenomic, transcriptomic and proteomic profiles of breast cancers. As we move towards a more targeted approach for breast cancer disease management, existing classification systems will undoubtedly be refined to reflect this considerable molecular diversity.

1.3.1 Gene-expression-based profiling

In the early 2000s, Perou, Sorlie and colleagues developed a microarray-based gene expression signature to classify breast cancer into five distinct subgroups, which include the luminal A, luminal B, HER2+, basal-like and normal-like subtypes [52-54]. Using 65 surgical samples from 42 patients, the initial groundbreaking study clustered the tumors based on an "intrinsic" gene subset that reflected the intrinsic makeup of the tumor and excluded gene expression variations arising from tissue sampling [52]. Over the years, gene expression profiling using this signature has proven useful in identifying high-risk patient populations with respect to disease progression, distant recurrence and response to chemotherapy [54-56]. Importantly, although the intrinsic subtype classification system was established using a small, heterogeneous patient population, follow-up studies have shown that this gene classifier is extremely robust and retains its predictive value across large microarray platforms [57-59].

1.3.1.1 Luminal A breast cancers

The luminal A subtype is the most commonly occurring type of breast cancer, representing 50-60% of cases, and is associated with the best prognosis [53, 60]. In general, luminal A breast cancers have the lowest risk of developing distant metastases [53] and exhibit a distinct relapse pattern, characterized by an increased incidence of bone metastasis and decreased presence of visceral metastasis [61, 62]. Luminal A breast cancers typically express cytokeratins 8 and 18, which are found in luminal cells of the mammary ductal epithelium. Expression of the estrogen receptor (ER), progesterone receptor (PR), ER-regulated genes and associated transcription factors, such as GATA3, FOXA1 and XBP1 is also a distinguishing feature of luminal A tumors [52, 63]. The ER has two isoforms whose actions are typically antagonistic to one another: ERα, the major player in breast cancer, acts to increase cell proliferation while ERβ suppresses proliferation and increases differentiation [64]. Following ligand binding, the receptors homodimerize or heterodimerize, translocate to the nucleus and recruit co-factors to drive transcription of estrogen-responsive genes. Cytoplasmic ERα can also engage in cross-talk with HER2, EGFR and IGFR1 growth factor receptors to activate downstream MAPK and AKT pathways, which enhance stabilization and activity of nuclear ERα in a ligand-independent manner [65].

Luminal A tumors are further characterized by a low proliferation rate, a low grade and a relatively quiescent mutational landscape [52, 66]. Although the rate of mutations in luminal A breast cancers is low, a wide range of genes, including *PIK3CA* (phosphatidyl inositol-4,5-bisphosphate 3-kinase, catalytic subunit α), *GATA3*, *CCND1* (Cyclin D1) and *MAP3K1* (mitogen-activated protein kinase (MAPK) kinase kinase 1) are frequently inactivated or overexpressed [66, 67]. Due in part to their poorly proliferative nature, luminal A cancers exhibit low sensitivity to chemotherapy [56, 68]. Currently, treatments

for this subgroup encompass endocrine therapies that block estrogen synthesis (aromatase inhibitors), inhibit estrogen binding to ER (tamoxifen) and induce ER degradation (fulvestrant) [69-71]. However, resistance to endocrine therapy is common and late recurrences can occur up to 20 years after diagnosis [69]. Hyperactivation of the PI3K pathway, which represents a ligand-independent method of activating the ER and obviating action of tamoxifen, is frequently associated with endocrine resistance [72, 73].

1.3.1.2 Luminal B breast cancers

The luminal B molecular subtype represents 10-20% of breast cancers. These cancers are more aggressive than their luminal A counterparts, exhibiting increased proliferation, a higher grade and a considerably poorer prognosis that is comparable to the HER2+ and basal-like subtypes[53, 74]. Luminal B cancers also preferentially metastasize to bone, but their rate of distant relapse to visceral sites, such as the liver and the lung, is markedly higher [61, 62]. However, the luminal B subtype is not simply a more aggressive form of luminal A cancer as it characterized by a very distinctive biology [75]. Although ER is detectable in these tumors, its expression is lower than in luminal A cancers and the downstream pathway is often inactivated, as evidenced by the absence of the progesterone receptor and other estrogen-regulated genes in a large number of luminal B tumors [75]. As a result, many of these cancers show no benefit from endocrine therapy, suggesting that tumor growth is mediated by estrogen-independent mechanisms in these cases [76]. Indeed, luminal B cancers frequently display aneuploidy and mutations in the *TP53* and *PI3KCA* genes, and they often upregulate pathways associated with cell cycle progression and proliferation [66, 77]. Cyclin D1, which promotes G1 to S phase cell cycle progression, is overexpressed in 58% of luminal B tumors and the chromosomal region containing *CCND1* is frequently amplified in this subtype [66, 78]. Additional mechanisms of

endocrine resistance in luminal B cancers include cross-talk with HER2, increased PI3K/AKT/mTOR signaling, impaired p53 function (66% of cases) and FGFR1 upregulation (27% of cases) [66, 79, 80]. As a result, significant improvements in progression-free survival (PFS) and overall survival (OS) have been observed in clinical trials combining endocrine therapy with agents targeting these resistance pathways [69, 81-83]. Furthermore, a study examining genome-wide copy number alterations (CNAs) has identified ZNF703 and PPP2R as subtype-specific drivers of luminal B breast cancer [78].

1.3.1.3 HER2+ breast cancers

HER2 breast cancers constitute 15-20% of breast cancer cases. These cancers are associated with high proliferation, a high grade, a preponderance of liver metastasis and, when left untreated, they represent the subtype with the poorest long-term prognosis [53, 78]. The proliferative signaling in this breast cancer subtype usually occurs in response to HER2 pathway deregulation, which can acitvate PI3K/AKT/mTOR, RAS/MAPK and JAK/STAT signaling [84, 85]. HER2 is an orphan transmembrane receptor that belongs to a family of four receptor tyrosine kinases (EGFR, HER2, HER3, HER4) implicated in cell proliferation, survival and differentiation. HER2 exists in a constitutively active conformation and its mechanisms of action are mediated through heterodimerization with the other receptors in the HER superfamily. Although HER2 has no known ligand, its overexpression leads to hyperactivation of downstream pathways that promote an aggressive phenotype in breast cancer [86]. A number of mechanisms can contribute to HER2 pathway activation in HER2-enriched breast cancers, including overexpression of HER2, overexpression of genes implicated in HER2 signaling, oncogenic activation by exon skipping, and amplification of the 17q12 chromosomal region which contains *HER2* and associated genes such as *GRB7, MED24* and *MED1* [52, 87, 88]. Importantly, over

30% of cancers that are classified as HER2 based on their expression profile do not exhibit amplification of the HER2 oncogene itself, indicating that activation of downstream pathways can promote a similar phenotype [60, 89]. Conversely, many cancers overexpressing HER2 are actually categorized as luminal B [89]. Breast cancers of the HER2 subtype can be further subclassified using the HER2-derived prognostic predictor (HDPP), a 158-gene signature that was useful in identifying a highly invasive, weakly immunoresponsive HER2 subgroup with a markedly poor prognosis [90]. Survival of patients with HER2+ breast cancers has been considerably improved with the introduction of targeted therapy [91]. Trastuzumab, which targets the extracellular domain of HER2, is currently approved for single-agent treatment of metastatic breast cancer and for treatment of HER2-expressing early stage breast cancers in the adjuvant setting [92, 93]. However, relapse occurs in approximately 15% of treated patients and the majority of late-stage patients eventually progress under trastuzumab therapy [94]. To address these complications, dual HER2 pathway inhibitors are currently being evaluated in the clinical setting [85].

1.3.1.4 Basal-like breast cancers

Basal-like breast cancers (BLBCs) are distinguished by the expression of genes associated with mammary basal/myoepithelial cells, such as cytokeratins 5 and 17 [52]. The basal-like subgroup accounts for 10-20% of breast cancer cases and represents the most aggressive subtype. Clinically, BLBCs are high grade breast cancers that manifest at an early age, generally in women of African origin [95]. These cancers are aggressively metastatic and preferentially relapse to the lung and the brain [61, 62]. Notably, Wnt/β-catenin signaling exhibits subtype-specific activation in basal-like breast cancers metastasizing to the brain [61]. The majority of basal-like tumors display extensive

aneuploidy and genomic instability characterized by a high rate of *TP53* mutations and complex chromosomal rearrangements [96]. It has been postulated that aberrations in DNA double-strand damage repair predispose breast cancers to a basal-like phenotype [97]. Indeed, 70% of tumors with germline mutations in the *BRCA1* DNA damage repair gene are classified with basal-like breast cancers [98]. Unlike luminal and HER2 cancers, the basal-like subgroup is not defined by the presence of a driver oncogene and has therefore not been amenable to the targeted therapies that have shown considerable promise in other breast cancer subtypes. Despite their high sensitivity to chemotherapy, the 5-year prognosis of patients with BLBC is very poor, largely due to the lack of biologically relevant therapeutic targets [56]. Furthermore, although EFGR is often highly expressed in BLBCs, targeted therapies against this receptor have not yielded much success [99]. Additional therapeutic strategies have been proposed to manage BLBC progression (discussed below), however, chemotherapy is currently the only approved line of treatment for this aggressive subset of the disease.

1.3.1.5 Normal-like breast cancers

The initial work by Perou et al. classified normal-like breast cancers based on their similarities to fibroadenomas and normal breast samples. These cancers expressed adipose tissue markers and were devoid of ER and HER2 expression [52]. However, due to its rare occurrence (5-10% of breast cancer cases), this subtype has been poorly characterized in subsequent studies. Normal-like breast cancers have a generally favorable prognosis and a low grade. Additionally, they lack expression of proliferation-associated genes and, accordingly, are poorly responsive to chemotherapy [60]. Many groups have speculated that the existence of the normal-like subtype may be an artifact arising from contamination with normal tissue during microarray preparation [63, 100]. Notably, no cases of normal-

like breast cancer were found in large-scale studies where tumor cells were isolated by micro-dissection, which supports the technical artifact theory [100].

1.3.1.6 Claudin-low breast cancers

The claudin-low subtype of breast cancer was initially identified in 2007 and is now recognized as a distinct molecular subgroup [101, 102]. The prevalence of claudin-low breast cancers is relatively low (12-14%) but this subtype represents a burgeoning area of interest due to its poor prognosis and lack of therapeutic targets [60]. Claudin-low tumors constitute the most poorly differentiated subtype and are characterized by absence genes involved in cell-cell adhesion, including claudins 3, 4, and 7 and E-cadherin [102, 103]. Additionally, these tumors are enriched in immune system response genes, stem-cell related biological processes and EMT features [102, 104]. The profile of claudin-low tumors has been associated with the acquisition of a cancer stem cell-like phenotype [104]. Hierarchically, claudin-low breast cancers generally cluster with BLBCs due to their low levels of HER2 and hormone receptor expression [89]. However, unlike BLBCs, claudin-low tumors do not have high expression of the proliferation gene subset and are associated with slower cell cycle progression [102]. Although claudin-low breast cancers show some sensitivity to neoadjuvent chemotherapy, this treatment option is not sufficient to improve prognosis.

1.3.2 Advent of integrative profiling

Gene expression profiling has proven useful in identifying patient subgroups with similar clinicopathological characteristics; however, considerable heterogeneity still exists within these intrinsic subtypes [67, 105, 106]. Gene expression is regulated by genomic, epigenomic and post-transcriptional mechanisms and understanding these additional levels of regulation can vastly improve prognostic tools and patient outcome. In the past few

years, a series of seminal studies have undertaken an integrative approach to refine the current classification system by consolidating information from microarrays with secondary data that modulate gene expression.

1.3.2.1 Genomic profiling

Approximately 40% of expressed genes are influenced by copy number alterations (CNAs), which are somatic variations acquired during the course of an individual's lifetime. In parallel with gene expression-based profiling, comparative genome hybridization array studies have identified distinct groups of breast tumors which segregate based on the level and pattern of genomic instability [107, 108]. Although the numerous CNA-based subtyping exercises have given rise to various classifications over the years, results from these studies have enriched our understanding of intrinsic subtypes. Notably, ER+ and luminal tumors are often characterized by the simplex pattern of gene alteration consisting of deletions and duplications of broad chromosome regions, while TNBC and basal-like tumors exhibit a sawtooth pattern described by narrow segment alterations spanning the majority of chromosomal loci [107].

Subsequent efforts including the seminal METABRIC study have used a combination of gene expression and somatic alteration patterns to stratify breast cancers [109]. In the cancer genome landscape, benign "passenger" alterations account for the majority of CNAs, which makes it difficult to identify genomic "driver" events responsible for gene expression changes driving tumor progression [110]. The METABRIC study aimed to identify driver alterations affecting the expression of the largest number of genes by analyzing the predictive value of CNAs in over 2000 clinically annotated breast tumor samples [78]. The influence of some CNAs was restricted to neighboring genes (cis-acting) while others could modulate distant chromosomal loci (trans-acting). This integrated

approach identified the deletion of MAP2K4 and PPP2R2A as significant events in the progression of luminal breast cancers [78]. Integrative clustering of CNA and gene expression data revealed the existence of 10 novel biological subgroups (integrative clusters, or IntClust), which split many of the intrinsic subtypes. Interestingly, 7 of the 10 IntClust subgroups were enriched for ER+ and luminal cancers, highlighting the genomic diversity of a clinical subgroup whose stratification is usually limited to luminal A and luminal B subtypes. Using this novel classification system, the authors described a high-risk ER+ subgroup (IntClust2) with very steep mortality rates. IntClust2 cancers were characterized by amplification of the 11q13/14 genomic region that contains *CCND1* and other genes involved in cell cycle progression. The 10 integrative clusters exhibited wide variations in CNA number and the integrative clusters with the lowest genomic instability were also associated with the best prognosis. The subgroups with a flat genomic landscape include IntClust3, which contains primarily luminal A cases, and IntClust4, which is characterized by high expression of immune response genes and encompasses both ER+ and ER- cases. Notably, the IntClust10 subgroup exhibited very pronounced genomic instability and was comprised mostly of basal-like tumors that had poor short-term but favorable long-term outcomes [78]. The survival pattern of basal-like tumors observed in this study was consistent with previous studies examining long-term (15+ years) prognosis of breast cancer patients [111]. Overall, integrative clustering highlights the drawbacks of the intrinsic subtype classification system and represents the most extensive accepted molecular taxonomy to date. Notably, a surrogate gene expression profile consisting of 612 genes has recently been developed to classify breast cancers into the 10 integrative clusters and was shown to outperform the PAM50 intrinsic subtype classifier in predicting expression patterns of genomic drivers in breast cancer [112]. This signature was used to

robustly validate the clinical and molecular profiles of IntClust subtypes in a panel of 7,544 breast tumor samples [112].

Although copy number alterations represent the dominant somatic alteration in breast cancer [113], other mutation types including substitutions, insertions/deletions and DNA rearrangements affect driver genes and contribute to tumor progression [67, 114-116]. Most recently, 20 distinct mutational signatures, which include 12 base substitution, 2 insertion/deletion and 6 rearrangement signatures, have been identified from whole genome sequencing of 560 breast cancers [114] and show important associations with DNA repair mechanisms and chromosomal architecture [116], gene amplification [117] and immune infiltration [118]. This comprehensive analysis of breast cancer mutations is an important tool in the search of new biomarkers and therapeutic targets of the disease and has to date identified 93 potential driver mutations in protein-coding cancer genes [114]. In parallel, a follow-up METABRIC study identified 40 "mutation-driver" genes using 2,433 tumors with long-term clinical data [115]. Mutations in driver genes such as TP53 and P1K3CA are tied to subtype and tumor grade and represent important targets for sub-stratification of existing clinical and molecular breast cancer subtypes [115].

1.3.2.2 Epigenomic profiling

Studies using array-based methylation assays have found that breast cancers can be segregated into discrete clusters based on their epigenomic profiles [119, 120]. Notably, intrinsic molecular subtypes display distinct methylation profiles and the subtype-specific expression pattern of many genes is epigenomically regulated [121, 122]. It is hypothesized that subtype-specific gene methylation plays a role in the clonal evolution of breast cancer subtypes and the maintenance of a basal-like or luminal-like phenotype [121, 122]. Luminal A, luminal B and basal-like subtypes clustered separately based on methylation status, with

luminal B and basal-like cancers displaying the highest and lowest rate of global methylation, respectively [121]. Indeed, *TP53* mutation status was correlated with hypomethylation and expression of ER was associated with hypermethylation [122]. However, reports on the association of HER2 expression with methylation status were mixed. Interestingly, existing subtypes can be further sub-classified based on epigenomic profiling, indicating that methylation status could bring added prognostic value [123-125]. Seven breast cancer "epitypes" with distinct clinicopathological features and predicted outcomes have recently been identified through DNA methylation-based clustering of 188 human breast cancers [125]. Integrated analysis of the epitypes and intrinsic subtypes revealed that basal-like and HER2-enriched breast cancers are closely associated with independent epitypes, while luminal tumors segregate into 4 distinct epitypes [125]. Notably, two distinct patterns of aberrant DNA methylation were observed: basal breast cancers exhibit constitutive promoter hypermethylation which is not linked to gene expression levels, while luminal cancers display hypermethylation patterns reflecting gene expression and representing a targetable epigenetic state [125].

Epigenomic alterations are also important mechanisms regulating EMT and dictating metastasis of breast cancers [126, 127]. EMT is a dynamic process characterized by sequential and reversible changes in DNA methylation patterns of EMT-related genes [126]. Additionally, presence of a CpG island methylator phenotype is associated with low risks of metastasis and death while absence of this coordinated hypermethylation pattern is predictive of poor metastasis-free and overall survival [127].

1.3.2.3 Post-transcriptional profiling

Gene expression can be regulated post-transcriptionally by microRNAs, which induce gene silencing by binding to the 3'UTR of mRNAs. In the past decade, expression

profiles of microRNAs have emerged as important tools that bring independent prognostic information to existing breast cancer classification systems. The first unbiased, large-scale microRNA study in breast cancer reported that luminal and basal subtypes segregated independently based on microRNA expression [128]. Interestingly, expression of DICER and RISC microRNA regulation machinery components varied across subtypes [128, 129]. *DICER1* expression was downregulated in the poor prognosis basal-like, HER2 and luminal B subtypes [128, 129], which is consistent with reports showing that DICER1 inhibition is required for progression of aggressive breast cancers [130, 131]. Conversely, upregulation of RISC complex genes such as *AGO2* were associated with poor prognosis [128, 129]. A recent comprehensive study examining microRNA expression architecture in 1,302 breast tumors identified a prognostic microRNA signature in IntClust4 tumors, which are devoid of CNAs [78, 132]. These microRNAs were functionally implicated in the modulation of immune response gene expression that characterizes the genomically quiescent IntClust4 subtype [132]. This novel IntClust4 microRNA signature exhibited strong prognostic value across all subtypes and in external cohorts, emphasizing the importance of integrative approaches in breast cancer disease management [132].

In breast cancer, microRNAs are preferentially shuttled into exosomes and play critical roles in mediating cell-cell communication, regulating tumor growth, establishing the pre-metastatic niche and conferring drug resistance [133, 134]. Current research efforts are focusing on serum and plasma analysis of exosomal microRNA cargo to monitor disease progression and identify therapeutic targets [135, 136]. microRNAs are emerging as biomarkers for early disease detection and replenishment or depletion of circulating exosomal microRNAs represent important non-invasive therapeutic avenues in breast cancer [137, 138]. Further understanding of exosomal microRNAs that modulate existing

gene-expression based classifiers will be instrumental in advancing current diagnosis and treatment methods.

1.3.2.4 Single-cell sequencing and intra-tumor heterogeneity

The era of precision medicine has recently ushered in single-cell sequencing techniques to elucidate biological and clinical implications of intra-tumor heterogeneity. Nucleus genome single-cell sequencing (Nuc-Seq) has revealed that individual cells from luminal A and triple negative tumors exhibit extensive diversity in genomic profiles arising from gradual accumulation of point mutations [139]. A separate single-cell sequencing study has demonstrated that genetic diversity in individual triple-negative breast tumors can also arise from rapid mutational bursts in the early stages of tumor progression, and thereby give rise to 1-3 major sub-clones that evolve to form a heterogeneous tumor population [140]. Additionally, multiple subtypes have been identified in individual breast tumors [141], and subtype conversion has been observed between paired primary and metastatic lesions, with luminal A tumors exhibiting a 55% conversion rate to other subtypes following acquisition of estrogen independence [142]. These studies highlight the drawbacks of current profiling techniques and provide important insights regarding disease prognosis and mechanisms of therapeutic resistance.

Single-cell sequencing techniques are rapidly evolving to overcome technical challenges related to cell isolation and amplification of material and are leveraging additional layers of data to generate more comprehensive insights into tumor evolution [143]. Notably, topographic single cell sequencing (TSCS), which enables the study of single cell genomic aberrations while preserving contextual cues related to spatial distribution of cancer cells within a tumor tissue, has recently been used to identify multiple clonal evolution events which confer invasive potential and enable co-migration of

different breast cancer clones during the early stages of the metastatic cascade [144]. As the field continues to evolve, single-cell sequencing will represent an important clinical tool for early disease detection, non-invasive monitoring of circulating tumor cells and targeted combination therapy recommendations [143].

1.3.3 Histological classifications

Integrative approaches have identified important therapeutic targets in breast cancer and have considerably expanded our understanding of the complexity of tumor evolution and biology. However, these approaches are currently too expensive to be used in the clinic. Additionally, complex classification systems are not always robust enough to outperform the predictive value of simpler, established classifiers [145]. Due to these limitations, clinical classification of tumor samples has relied on immunohistochemistry (IHC) for evaluation of ER and PR status, and on a combination of IHC and fluorescence *in situ* hybridization (FISH) for detection of HER2 levels [146, 147]. However, this assessment method is not always reproducible and yields limited prognostic insights [146-148]. To address these concerns, many robust gene-expression-based-classifiers have been adapted for use in breast cancer management [148, 149].

1.3.3.1 Clinical subtypes defined by biomarker status

Clinicians routinely use ER, PR and HER2 status as predictive biomarkers in selecting a therapeutic strategy. These biomarkers define three major subgroups: hormone receptor positive/luminal (ER+, PR+, HER2-), HER2 (HER2+) and triple-negative (ER-, PR-, HER2-). However, these classifications do not adequately reflect intrinsic subtypes and reliance on these biomarkers often leads to overtreatment in early stages of the disease [150]. Indeed, while 60% of early-stage breast cancer patients will receive chemotherapy,

only a minority will benefit from this aggressive treatment [151, 152]. As array-based studies have shown, luminal cancers can be subdivided into groups with vastly different prognosis and response to therapy [53, 56, 78]. Additionally, immunohistochemically-defined triple-negative breast cancers (TNBCs) encompass both basal-like and claudin-low tumors [89], which exhibit different responsiveness to chemotherapy [102]. Furthermore, close to 20% of basal-like tumors and 30% of claudin-low tumors express hormone receptors, HER2 or both. As a result, many groups have suggested that additional markers should be introduced for the purposes of routine clinical assessment [74, 111, 153, 154].

The inclusion of either basal cytokeratin 5/6 or EGFR1 as biomarkers can correctly identify basal-like tumors classified using gene expression [155, 156]. These markers have been used to subclassify TNBCs in large-scale clinical studies as either tumors with a core basal phenotype (ER-; PR-; HER2-; CK5/6 or EGFR1+) or a five negative phenotype (5NP) [155, 156]. It has been shown that core basal tumors exhibit a considerably poorer prognosis compared to 5NP tumors, indicating that these two subgroups represent biologically distinct entities [74, 111]. A tumor-infiltrating lymphocyte signature also confers independent prognostic value in TNBC and HER2 subtypes and has been proposed as a novel prognostic marker for clinical classification and immunotherapy recommendations in breast cancer [157, 158]. However, despite these advances, routine reporting of lymphocyte infiltration in early breast cancer is not currently recommended by clinician panels [159].

The major distinguishing feature between luminal A and luminal B tumors is the expression of genes implicated in proliferation. Consequently, Ki67 labelling was introduced to measure proliferation in tumor samples with hormone receptor positivity [74, 154]. In 2011, the St Gallen consensus panel officially recommended that Ki67 staining

should be used for routine pathological assessment of breast cancers to define the luminal B subtype in the clinical setting [160]. Importantly, the clinical applicability of intrinsic subtypes was recognized at St Gallen consensus panel and four biomarkers (ER, PR, HER2, Ki67) are currently used to obtain a more accurate approximation of these patient subgroups [159, 160]. However, concerns still exist about the variability of Ki67 scoring despite the availability of standardized protocols [159, 161, 162] and inter-laboratory efforts are underway to improve reproducibility and standardization of Ki67 staining protocols [163].

1.3.3.2 Clinical use of gene expression signatures

In response to the limited prognostic value of immunohistochemistry-based patient stratification, gene-expression-based-assays have been made commercially available for clinical use [148]. Oncotype DX, Mammaprint and Prosigna are established commercial assays exhibiting survival associations that are independent from standard clinicopathological markers [148]. These assays have been endorsed by expert panels and are currently being used to help guide treatment decisions for restricted patient subgroups [105, 164]. ASCO clinical practice guidelines were updated in 2016 to strongly recommend Oncotype Dx and Prosigna assays in guiding treatment decisions for use of adjuvant chemotherapy in ER+/PR+, node-negative breast cancers [165], and were revised in 2017 to recommend use of the MammaPrint assay for high-risk ER+/PR+ node-positive and node-negative patients [166].

Oncotype Dx is an RT-PCR-based platform that examines the expression of 21 genes to determine which early stage ER+ patients will benefit from chemotherapy. This assay divides patients into three groups (low, intermediate, high) based on their 10-year recurrence score (RS) [167]. Oncotype Dx RS score was validated in two independent large patient cohorts and was strongly associated with risk of distant recurrence and risk of death

[168, 169]. Hormone therapy alone is considered acceptable for treatment of patients classified as “low-risk” by OncotypeDx, but “high-risk” patients require adjuvant chemotherapeutic intervention. Patients classified as “intermediate-risk” are currently being evaluated in a prospective TAILORx trial to determine if endocrine therapy is sufficient in this patient subgroup [170]. Initial results from the TAILORx and West German PlanB trials, which examined the prognostic value of the 21-gene signature in “low-risk” patients treated with endocrine therapy only, confirmed that patients with HR+/HER2- node-negative disease have a very low 5 year recurrence risk and can safely be spared from chemotherapeutic intervention [171, 172].

The Mammaprint assay analyses the expression levels of 70 genes using a microarray-based platform and as such, requires high-quality RNA from fresh frozen samples [173]. The Mammaprint gene signature comprises genes associated with proliferation, angiogenesis and invasion [173] and classifies patients into two groups (good or bad) based on their 10 year risk of distant recurrence-free survival. Notably, this assay has been validated in numerous large-scale cohorts of node-negative patients [174-176] and is now FDA-approved for women with node-negative breast cancer [149]. Most recently, a phase III randomized MINDACT trial was used to assess the clinical validity of the Mammaprint assay [177]. Results from this trial demonstrate the predictive power of the Mammaprint signature among HR+ patients identified as high clinical risk, by showing that the signature can be used to identify a sizeable subset of high clinical risk patients (1,550 of 3,556 patients, or 46%) that would not benefit from adjuvant chemotherapy [177].

The Prosigna assay is based on the PAM50 gene signature, which is the most recent version of the intrinsic subtyping assay described in the early 2000s [52, 53, 105]. The PAM50 assay provides information about relapse-free survival for node negative patients

who haven't received adjuvant therapy [150]. In 2009, a test sample population, which included tumors from the luminal A, luminal B, HER2 and basal-like subtypes, was used to narrow down a list of 1906 intrinsic genes to a restricted set of 50 genes [150]. This minimized gene set constitutes the PAM50 subtype predictor and includes genes involved in proliferation, ER signaling, HER2 signaling and the basal-like phenotype [148]. A number of recent studies have demonstrated that the PAM50 assay has a predictive impact in the clinical setting that is superior to conventional immunohistochemistry-based tools and other commercial gene-expression-based assays [178-180].

Overall, the introduction of gene-expression-based prognostic tools represents an important step towards the implementation of individualized treatment strategies [181], and is paving the way for clinical use of additional signatures based on gene expression and integrative studies [112, 182].

1.4 Triple-negative breast cancers

BLBCs and TNBCs have been defined by gene-expression- and immunohistochemistry-based assays, respectively. These classifications are often used interchangeably and the triple-negative phenotype is utilized as a clinical surrogate for BLBCs [95], however, a considerable number of breast cancer cases belong to only one of these subtypes. As mentioned previously, only 83% of BLBCs are characterized by absence of HER2 and hormone receptors. Conversely, TNBCs encompass basal-like, claudin-low and normal-like tumors and can be further subdivided according to their molecular heterogeneity, mutational and CNA profiles, long-term prognosis and response to therapy [183-187]. A number of targeted therapies have been proposed for management of TNBCs, but their effectiveness in clinical trials has been modest [188]. Efforts are underway to

elucidate the molecular basis of TNBCs in order to develop effective treatments that target driver pathways in this aggressive subtype.

1.4.1 Heterogeneity within TNBCs

Recent work by Shah et al. has demonstrated that TNBCs display a wide spectrum of genomic aberrations, with some tumors possessing alterations in a few genes and others harboring hundreds of coding somatic mutations [184]. Furthermore, TNBCs are characterized by a wide variation in clonal frequency, which is defined as the percentage of tumor cells containing a specific mutation, and are therefore a useful tool to study tumor evolution [184]. Notably, somatic mutations in *TP53, PI3KCA* and *PTEN* were identified as frequently occurring founder mutations due to their clonal dominance in a majority of TNBC tumors [184]. Analysis of clonal frequency has revealed that basal-like TNBCs contain a higher number of clonal groups relative to non-basal TNBCs, which is indicative of a more extensive tumor evolution in the basal-like subtype [184]. Interestingly, greater clonal diversity has been linked to decreased recurrence-free survival [189] and, taken together, this data could partly explain the poor prognosis observed in the basal-like subgroup of TNBCs [111]. Overall, this study established intratumor heterogeneity as an important determinant of TNBC disease progression and response to treatment [184].

In addition to the well-characterized intrinsic subtypes that can be classified as TNBCs, many novel subclasses of TNBCs have been identified. In 2006, a new interferon-rich subgroup (IFN) of triple-negative breast cancers was identified by applying the intrinsic gene signature to independent microarray platforms [58]. This subtype exhibits high expression of IFN-regulated genes and of *STAT1*, a transcription factor that promotes IFN-driven gene expression. Importantly, the IFN subgroup of breast cancers had a

significantly better prognosis than other TNBCs, indicating that TNBCs are not a uniform clinical entity [58].

Molecular apocrine tumors have been identified as a potential independent ER-negative molecular subtype of breast cancer [190, 191]. The molecular apocrine subtype is characterized by apocrine differentiation and presence of functional androgen-receptor pathway signaling. These tumors frequently display HER2 amplification or overexpression but a subset of cases can be classified as triple negative [191].

A seminal study by Lehmann et al. initially demonstrated that TNBCs can be classified into six distinct molecular subgroups that exhibit differential responses to cytotoxic and targeted therapy [183]. These subgroups included 2 basal-like (BL1 and BL2), a mesenchymal (M), a mesenchymal stem-like (MSL), a luminal androgen receptor (LAR) and an immunomodulatory (IM) subtype. The group recently revisited this classification using a laser capture microdissection (LCM)-based approach and determined that a part of the gene expression set used to classify the MSL and IM subtypes was derived from infiltrating tumor-associated stromal cells and lymphocytes, respectively [186]. Based on these findings, the original TNBC transcriptional classification was amended to remove the MSL and IM subtypes, although the MSL and IM signatures are still used to describe cellular heterogeneity of TNBCs [186, 187].

An independent study aiming to develop a clinically relevant TNBC classification employed an integrative stratification approach combining gene expression and DNA profiling to define 4 molecularly stable TNBC subtypes: basal-like immunosuppressed (BLIS), basal-like immune-activated (BLIA), mesenchymal (MES) and luminal AR (LAR) [185]. These clinical TNBC subtypes displayed significant overlap with the TNBC classification system proposed by Lehmann et al., although the BLIA subtype identified an

immune signature which was not present in either basal-like subtype from the Lehmann et al. study [192].

The basal-like BL1 subtype expresses genes implicated in proliferation, cell cycle progression and DNA damage response [183]. This subtype is enriched in Ki67 expression, and accordingly, exhibits the highest sensitivity to neoadjuvant chemotherapy, with 41% of patients achieving a complete response [183, 186]. BL1 breast cancers are the most genetically unstable, harboring the highest rate of *TP53* mutations and significant copy-number deletions in DNA repair genes [187]. This subtype most closely overlaps with the BLIS subtype from the Burstein et al. study, which displays downregulated immune and cytokine pathways and is characterized by the poorest prognosis [185]. Interestingly, breast cancer cell lines that clustered with BL1 tumors were mostly defined as Basal A, a cell line subtype which exhibits epithelial features and BRCA1 signatures [193, 194]. Basal A cell lines were uniquely sensitive to cisplatin treatment, which is consistent with reports showing heightened cisplatin activity in cells with DNA repair defects [195].

The BL2 subtype was differentiated by a unique growth factor signaling pattern that includes EGFR, Met and Wnt/β-catenin pathway engagement [183]. However, the existence of the BL2 subtype has been contested by various follow-up validation studies aiming to reproduce the classification system proposed by Lehmann et al. [187, 196, 197]. Accordingly, the subtypes identified by the Burstein et al. clinical TNBC classification system do exhibit no significant overlap with BL2 cancers [185].

The mesenchymal (M) subtype is characterized by increased Rho-driven cell motility, ECM receptor signaling and activation of pathways involved in mesenchymal differentiation, such as TGFβ and non-canonical Wnt signaling, and angiogenesis [183, 187]. Mesenchymal (M) tumors also display an immunosuppressive microenvironment

similar to the one observed with the BLIS subtype, and a unique metastatic profile favoring lung colonization [186, 198]. The MSL tumor signature is distinguished by lower expression levels of claudins and other cell-cell adhesion genes, along with increased expression of stem-cell markers. Accordingly, using the claudin-low gene predictor set, claudin-low tumors were found to cluster with the M and MSL subtypes [102]. The mesenchymal subtype (MES) defined by the Burstein et al. classification similarly clusters with claudin-low tumors [185]. The unique signaling pathways expressed in the M subtype also resembled the gene signature that characterizes metaplastic breast cancer, a highly dedifferentiated and chemoresistant form of breast cancer [199]. Representative cell lines that expressed the M and MSL signatures classified as Basal B and displayed invasive, mesenchymal and stem-like characteristics [193, 194]. These cell lines were less sensitive to chemotherapy, which further emphasizes similarities of mesenchymal TNBCs to claudin-low and metaplastic breast cancers. Furthermore, consistent with increased ECM receptor signaling, M breast cancer cell lines responded to treatment with Dasatinib, a Src inhibitor [183].

Despite the presence of a triple-negative phenotype, LAR cancers are heavily enriched in luminal cytokeratins and hormonally-regulated pathways [183]. Notably, many of these pathways are regulated by androgen receptor (AR) signaling and the growth of representative LAR breast cancer cell lines was inhibited by treatment with bicalutamide, an AR antagonist [183]. LAR tumors are characterized by a higher stage compared to other subtypes and display bone-specific tissue tropism [186]. Breast cancer cell lines exhibiting a LAR signature were mostly defined as luminal or HER2+, which is consistent with the existence of luminal and HER2-enriched subsets of TNBCs [99]. Additionally, LAR tumors clustered with an androgen-driven group of breast cancers identified by the

molecular apocrine signature [183]. Existence of the LAR subtype has been validated by numerous studies and several clinical trials targeting the AR signaling pathway are currently in progress [185, 187, 196, 200].

Taken together, these studies highlight the extensive molecular heterogeneity of TNBCs and suggest that cancers within this subtype should not be treated as a single disease. Importantly, pharmacological targeting of driver signaling pathways in TNBC subtypes revealed that gene expression analysis is a useful tool for therapy selection [201].

1.4.2 Therapeutic targets in TNBCs

1.4.2.1 PARP inhibitors and platinum agents

DNA repair pathways are essential for cell survival and the maintenance of genomic integrity [202]. PARP1 is involved in the repair of single-strand DNA breaks and defects in PARP1 activity are normally overcome by BRCA1- and BRCA2-mediated homologous recombination of double-strand DNA [203]. Consequently, TNBCs, which often harbor extensive genomic instability and BRCA1 mutations, display heightened sensitivity to PARP1 inhibitors compared to luminal cancers in the preclinical setting [204]. These results have provided a rationale for the use of PARP1 inhibitors as a synthetic lethal therapeutic approach in *BRCA1*- and *BRCA2*-mutated breast cancers. Several PARP1 inhibitors, such as olaparib, veliparib and iniparib are being developed for treatment of TNBCs. Although concerns surround the development of these targeted agents [205], PARP1 inhibitors have demonstrated promising clinical activity in the *BRCA*-mutant subset of TNBC patients [206-209]. Most recently, olaparib treatment was shown to significantly improve median PFS compared to chemotheraphy in a randomized, open-label, phase III trial for patients with HER2-negative metastatic breast cancer [210].

1.4.2.2 EGFR inhibitors

EGFR is a strong negative prognostic indicator in TNBCs and its overexpression is frequently reported in this subtype [211]. The efficacy of EGFR antagonists has been demonstrated in preclinical models. Indeed, treatment with gefitinib led to MAPK and AKT inhibition, induced G1 growth arrest and enhanced the chemosensitivity of TNBC cell lines [212]. Several EGFR-targeted therapies, including cetuximab, lapatinib and gefitinib, are currently being tested in clinical trials [213]. However, the effects of cetuximab in randomized Phase II clinical trials have been discouraging [214-217]. Additionally, retrospective data from two Phase II clinical trials indicate that gefitinib and lapatinib show no benefit in TNBCs [218, 219]. Ongoing efforts are aiming to identify markers of responsiveness to EGFR-targeted therapies.

1.4.2.3 Angiogenesis inhibitors

Aniogenesis is essential for the growth, invasion and metastasis of breast cancers. Intratumor VEGF expression is enhanced in triple-negative tumors compared to other subtypes, which suggests an increased reliance on the angiogenic process and provides a rationale for VEGF pathway targeting in TNBC patients [220]. The benefit of therapies targeting VEGF (bevacizumab) or inhibiting tyrosine kinase action of VEGFR2 (sunitinib, sorafenib) is being evaluated in the clinical setting. Notably, addition of bevacizumab to chemotherapy regimens significantly increased response rate and progression-free survival of TNBC patients in multiple phase III clinical trials [221-223]. Although these results are promising, use of bevacizumab in the adjuvant setting did not have a measurable impact on overall survival in TNBC patients in recent randomized phase III studies [224, 225]. Additionally, efficacy of other VEGF pathway targeting agents is not assured as retrospective studies examining sunitinib and sorafenib response in TNBCs have not been conclusive [188].

1.4.2.4 Other potential targets in TNBCs

A number of other treatments are being considered for TNBC management based on encouraging preclinical and clinical findings. These therapies include PI3K/AKT/mTOR pathway inhibitors, Src/abl inhibitors, androgen receptor antagonists and antibodies targeting the Wnt/β-catenin pathway [188, 226]. Notably, results from a recent phase II trial demonstrate that Ipataserib, an AKT inhibitor, significantly improved progression free survival in a metastatic TNBC population [227]. Despite these encouraging results, at present, chemotherapy remains the primary treatment option for TNBCs.

One of the main problems associated with the use of chemotherapy for the treatment of cancer is the off-target action of the drugs on normal cells, which can lead to painful side effects and complications. In an effort to minimize the cytotoxicity of these therapies, approaches that selectively target tumor-associated antigens are emerging as promising therapeutic strategies for TNBC and other cancers. One such approach is the development of antibody-drug conjugates (ADCs), which synergistically combines the specificity of antibodies with the cytotoxic efficacy of chemotherapy. ADCs consist of antibodies bound to highly potent cytotoxins by a chemical linker [228]. These antibodies can be designed to target tumor-specific proteins and thereby serve as vehicles that deliver the drug to the cell of interest, often via internalization of the compound [228]. Accordingly, the expression pattern of the selected antigen both in normal and cancer tissues is an important consideration in predicting response to ADC therapy.

1.5 GPNMB

GPNMB has been recently identified as a potential therapeutic target for patients with BLBC and TNBC [229-231]. Heightened GPNMB expression is found in breast cancer

subtypes with the poorest prognosis, including HER2, TNBC and claudin-low breast cancers [231-233]. GPNMB expression in breast cancer is correlated with reduced disease-free and overall survival, and GPNMB levels in BLBCs and TNBCs are associated with further poor prognosis and increased risk for recurrence in this subset [231]. These findings, combined with evidence of high GPNMB expression in numerous cancers [234-245], have sparked an interest in investigating GPNMB as a target for antibody-based therapies in TNBC and other cancers [233, 244, 246-253].

1.5.1 Homology and structure of GPNMB

GPNMB, initially named NMB for "glycoprotein non-metastatic gene B", was first cloned and described in 1995 as a protein highly expressed in a melanoma cell line with low metastatic potential [254]. However, since this initial publication, elevated GPNMB expression has been observed in numerous cancers and is often associated with the metastatic phenotype [229-231, 234-238, 255]. GPNMB is also known as hematopoietic growth factor inducible, neurokinin-1 type (HGFIN) [256], and is located on the small arm of chromosome 7 (7p15). The rat orthologue, termed Osteoactivin, is expressed in the long bones of rats bearing a mutation associated with osteopetrosis and shares 65% protein identity with human GPNMB [257]. The mouse orthologue, which has 71% protein identity with human GPNMB, was named dendritic cell heparin integrin ligand (DC-HIL) following its identification in a particular subset of dendritic cells [258].

GPNMB belongs to the vertebrate Pmel17/NMB family, which encompasses GPNMB, Pmel17 (melanocyte protein 17) and their orthologues [259]. Pmel17 is the main structural component of melanosomes and it plays a key role in the pigment biogenesis of melanocytes [260]. To a lesser extent, GPNMB also shares homology with lysosome-

associated membrane protein (LAMP-1) family members [258], which are glycoproteins with potential roles in cell adhesion and metastasis [261].

GPNMB is a type I transmembrane protein that contains an N-terminal signal peptide, an integrin-binding motif (RGD) and a polycystic kidney disease (PKD) domain in its extracellular domain (ECD), a single pass transmembrane domain, and a 53 amino acid (aa) cytoplasmic tail [262, 263]. (Figure 1.1) The cytoplasmic tail harbors a half immunoreceptor tyrosine-based activation motif (hemITAM) and a dileucine motif, which functions as a sorting signal in QNR-71, the quail orthologue of GPNMB [264]. There are two known splice variants of GPNMB, which are expressed as a short 560aa and a long 572aa isoform [234]. The long isoform contains a 12aa insertion within a poorly conserved region downstream of the PKD-domain [234]. To date, there has been no evidence that the short and long isoforms have disparate functions. However, one study reported that the short GPNMB isoform was more frequently expressed in glioma samples and was significantly correlated with poor survival times, whereas the correlation between the long GPNMB isoform and survival times failed to achieve statistical significance [234].

1.5.1.1 RGD domain

This motif, comprised of only 3 amino acids, arginine (R), glycine (G), and aspartic acid (D), is found near the N-terminus of the GPNMB ECD and is well characterized in numerous proteins as an integrin-binding motif [265]. Integrins are heterodimeric transmembrane proteins expressed on a wide variety of cells, which regulate cell spreading, adhesion, migration, proliferation and apoptosis [23].

1.5.1.2 PKD domain

The PKD domain belongs to the Ig-like fold superfamily (E-set), which also includes cadherins, protein families containing bacterial Ig-like domains and several fibronectin type

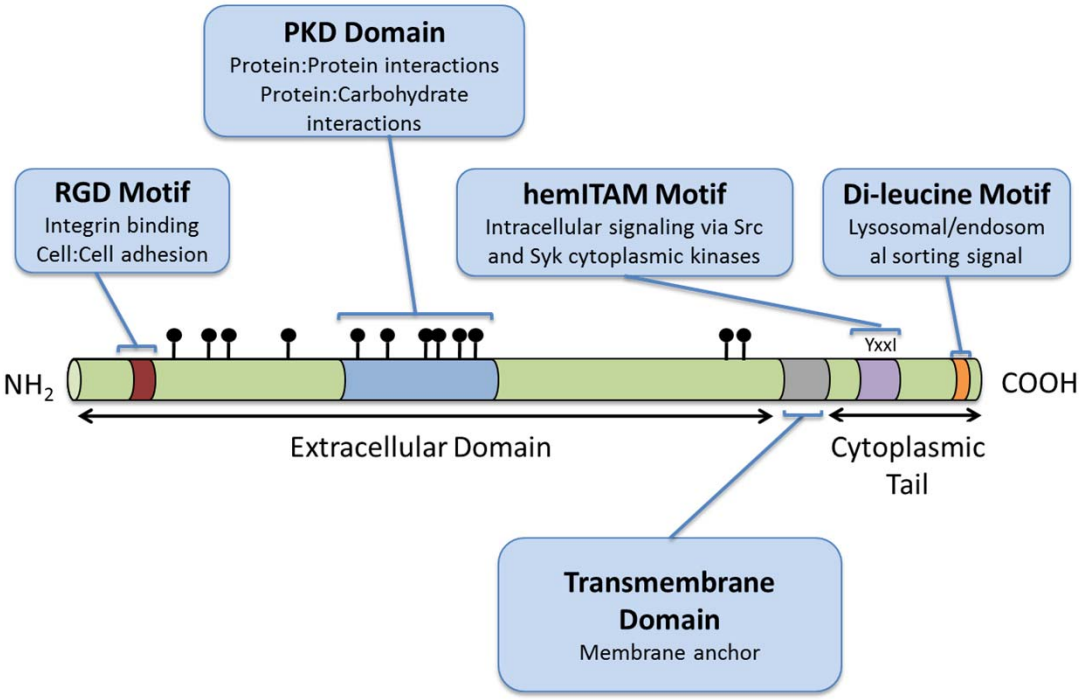

Figure 1.1 A schematic representation of GPNMB indicating the domains and motifs contributing to GPNMB function. The symbols (filled circles) located above the extracellular domain of GPNMB represent glycosylation sites. The RGD sequence comprises an integrin binding domain, where R = Arginine, G = Glycine, D = Aspartic acid. PKD = Polycystic Kidney Disease. The YxxI sequence constitutes a hemITAM (immunoreceptor tyrosine-based activation) motif, where Y = tyrosine, x = any amino acid, I = Isoleucine. The di-leucine motif is a lysosomal/endosomal targeting motif of the D/ExxxLL type, where D = Aspartic acid, E = Glutamic acid, x = any amino acid, L = leucine.

III domain-containing protein families. While the function of the PKD domain is still unclear, based on its structure, it has been proposed to mediate protein-protein or protein-carbohydrate interactions [266], and has been shown to mediate cell-cell adhesion [267].

1.5.1.3 hemITAM domain

ITAMs (immunoreceptor tyrosine-based activation motif) are commonly found in the cytoplasmic domains of receptors expressed by cells of the hematopoietic system [268], including antigen receptors, cytokine receptors and toll-like receptors [269]. ITAM signaling usually occurs in response to ligand binding, via phosphorylation of the ITAM resident tyrosine residues by Src-family kinases (ie. Src, Hck, Fgr, Lyn) [270].

GPNMB is one of several proteins whose cytoplasmic tail contains a highly conserved, single YxxI sequence, which has been referred to as a hemi-ITAM or hemITAM motif [268]. Proteins with hemITAMs still exhibit robust "ITAM" signaling capacity [271]. The current view suggests that ligand binding stimulates dimerization of hemITAM-bearing receptors; however, it remains to be seen whether GPNMB is capable of forming such homodimers.

1.5.1.4 Di-leucine motif

GPNMB contains a dileucine motif in its cytoplasmic tail, near the carboxy-terminus, with the sequence EKDPLL. Dileucine-based motifs of this type (D/ExxxLL) are often implicated in rapid receptor internalization from the plasma membrane and lysosomal/endosomal targeting [272]. Indeed, when either of these leucine residues is mutated to glycine in quail GPNMB, it is retained at the plasma membrane of HeLa or pigmented quail cells, and not routed to endosomes and lysosomes, as is the case for wild type GPNMB [264]. Interestingly, sequences of this type are associated with basolateral targeting in polarized epithelial cells [272].

1.5.1.5 Glycosylation

GPNMB is a heavily glycosylated protein, possessing 12 putative N-glycosylation sites within its extracellular domain, 6 of which are found in the PKD domain [254, 262]. Glycosidase treatments have confirmed that GPNMB can be N- and O-glycosylated in a variety of cell types [234, 273, 274]. In immunoblot analyses, human GPNMB is detected as two broad bands that correspond to precursor (P1 ~90kDa) and mature (M ~115kDa) GPNMB isoforms [273]. In addition, the unglycosylated form of GPNMB (~65kDa) has been detected in osteoclasts [275]. The relative abundance of these bands varies based on the cell type in which GPNMB is expressed [230, 274]. Studies using N-glycosidases suggest that GPNMB is first N-glycosylated in the ER to yield the P1 isoform, and these N-glycans are further modified during processing in the Golgi to produce the M-form [274]. While both isoforms are susceptible to tyrosine phosphorylation, only the mature form can be proteolytically processed through shedding (discussed below) [274, 276]. A number of studies have linked the glycosylation status of GPNMB to its putative biological functions and will be addressed in the relevant sections described below.

1.5.1.6 Proteolytic cleavage and ECD shedding

GPNMB is also subject to proteolytic processing, which was first uncovered by the detection of two heavily glycosylated, high molecular weight forms of murine GPNMB (97kDa, 116kDa; discussed above) and a stable c-terminal fragment of ~20kDa [277]. It was postulated that GPNMB can be cleaved by members of the matrix metalloprotease (MMP) family, such as ADAMs (a disintegrin and metalloprotease), because treatment with a broad-spectrum inhibitor of MMPs (GM6001) reduced the degree of GPNMB shedding [277]. Treatment with a calmodulin inhibitor (W7) or a protein kinase C activator (phorbol myristate acetate (PMA)), enhanced GPNMB shedding, further implicating the

ADAMs, as these compounds have both been reported to enhance ADAM-10 and ADAM-17 activity, respectively [274]. Accordingly, constitutive GPNMB shedding was observed in breast cancer cells and ADAM10 was definitively identified as a sheddase responsible for this cleavage event [230]. The potential functional implications for this shedding event will be discussed further in subsequent sections.

1.5.2 GPNMB Regulation

The promoter region of GPNMB has two highly conserved, well-described, consensus sequences for the MITF/TFE family which includes MITF, TFE3, TFEB and TFEC transcription factors [278, 279]. MITF is known as a "master regulator" of melanocyte biogenesis and can upregulate the expression of melanocyte-related genes (MRGs), such as TYRP1, TYRP2, PMEL17, MART1 and GPNMB, which are involved in melanocyte and melanoma differentiation [280]. Many reports have shown that GPNMB expression is directly regulated by MITF in normal tissues, such as melanocytes [279, 281], osteoclasts [278], dendritic cells [282] and macrophages [283], and in a variety of cancers, including melanoma [249, 284] and glioblastoma [285]. GPNMB can also be regulated through MITF-independent mechanisms, which include other members of the MITF/TFE family. Notably, TFE3 activity and nuclear translocation is enhanced in renal cell carcinomas following inactivation of the folliculin gene, which leads to widespread transcriptional changes, including upregulated GPNMB expression [286]. TFE3 also drives GPNMB overexpression in alveolar soft part sarcoma as part of the ASPSCR1-TFE3 fusion protein which characterizes the disease [287].

It was recently demonstrated that expression of GPNMB in TNBCs can be regulated by MAFK, which belongs to small family of transcription factors induced in response to

TGF-β signaling [288]. The GPNMB promoter region also contains a conserved consensus sequence for the AP-1 transcription factor [278, 279, 289], which is formed by homodimeric combinations of Jun proteins or heterodimeric combinations of the Jun and Fos transcription factor families [290]. However, transcriptional regulation of GPNMB by AP-1 has yet to be demonstrated.

Emerging evidence supports an important role for microRNAs in the regulation of GPNMB mRNA. The function of GPNMB during hyperoxic lung injury is regulated by miR-150, which targets and inhibits GPNMB mRNA [291]. In this setting, downregulation of miR-150 de-represses GPNMB to promote endothelial tube formation and angiogenesis [292]. GPNMB expression is also negatively regulated by miR-508-5p in glioma [293]. miR-508-5p inhibits glioma growth and proliferation by directly targeting the GPNMB 3'-UTR and reducing its expression [293].

1.5.3 GPNMB expression and physiological functions in normal tissues

GPNMB mRNA has been detected in the long bones, calvaria, bone marrow, adipose, thymus, skin, placenta, heart, kidney, pancreas, lung, liver and skeletal muscle; however**,** the precise expression patterns varied between these studies [256-258]. It is also clear that GPNMB can be expressed in multiple cell types within a given tissue, which is evident by its expression in bone osteoblasts [257] and osteoclasts [275] for example. Together, these studies clearly demonstrate that GPNMB is expressed in a wide range of tissues and suggest its involvement in a variety of physiological processes.

1.5.3.1 Brain

Widespread expression of GPNMB has been described within the central nervous system. GPNMB expression is largely specific to the microglia/macrophages of the neural

parenchyma [294] and fragments of GPNMB can be used as biomarkers to differentiate microglia subtypes in the developing brain [295]. Furthermore, GPNMB expression is detected in motor neurons of normal brain tissue and its expression is enhanced the brains of rats following stroke [296]. GPNMB expression is also upregulated in the motor neurons and astrocytes in mouse models of amyotrophic lateral sclerosis (ALS) as well as in the CSF and plasma of patients with ALS and aggressive Gaucher's disease [297, 298] [299]. GPNMB overexpression is cytoprotective in tissues affected by neuroinflammatory and neurodegenerative diseases, including ALS, Parkinson's disease and cerebral ischemia [300-302]. Notably, introduction of the GPNMB transgene in a mouse model of ALS increases myofiber count and volume and protects against muscular atrophy [301]. Mechanistically, GPNMB is able to ameliorate ALS-induced neuronal degeneration by inducing survival signaling in motor neurons through the PI3K and MEK/ERK pathways [297, 302, 303]. In this context, GPNMB glycosylation was shown to be important for increasing motor neuron stability, as ubiquitin-mediated degradation of glycosylated GPNMB ultimately triggered motor neuron death [297]. In conditions of ER stress, which characterize ALS and other neurodegenerative diseases, GPNMB promotes splicing of ER chaperone BiP pre-mRNA in order to improve protein folding capacity of the ER and ameliorate cellular stress [304]. However, the presence of GPNMB is cytotoxic in normal neural tissues, suggesting a possible role for GPNMB in maintaining neuronal homeostasis [297].

1.5.3.2 Skin

GPNMB expression in the basal layer of the skin, particularly in melanocytes, has been well documented, which is consistent with its high homology to melanocyte-specific protein Pmel-17 [274, 279, 305-307]. During development, GPNMB exhibits a punctuate

pattern of expression consistent with melanoblast cell populations, which represent melanocyte precursors [279]. GPNMB is also functionally implicated in melanogenesis and its expression can be upregulated by a number of factors, such as UVB and Endothelin-1, which increase melanosome formation [281, 307]. Accordingly, degradation and mislocalization of the GPNMB protein arising from homozygous or heterozygous truncation of GPNMB alleles is the underlying cause of amyloidosis cutis dyschromica (ACD), which is a skin condition characterized by regions of de-pigmentation and melanocyte loss [308]. Functionally, GPNMB can mediate melanocyte adhesion to keratinocytes through its RGD domain and is thought to be involved in the transport of late melanosomes to keratinocytes [274, 306]. Given its role in melanosome biology, GPNMB is also expressed in the pigmented epithelium of the eye, where its mutations are linked with the onset of human pigmentary glaucoma [309].

1.5.3.3 Bone

The first link between GPNMB expression and bone physiology was made when its expression was detected in mature, matrix-producing rat osteoblasts in osteopetrotic bone [257]. Subsequent studies have shown that genetic or antibody-mediated inhibition of GPNMB in developing osteoblasts impairs their differentiation and decreases their ability to produce bone matrix [310-312]. Transgenic mice overexpressing GPNMB (OA-Tg) display increased bone volume and thickness resulting from heightened osteoblast proliferation and differentiation, likely mediated by enhanced expression of TGF-β1 and its receptors downstream of GPNMB [313]. Extracellular GPNMB can also osteoblast adhesion and cell spreading by binding αVβ1 integrins and promoting downstream FAK and ERK activation [314].

In addition, GPNMB is abundantly expressed in differentiated osteoclasts [278] and plays an important role in mediating cell fusion to produce multi-nucleated osteoclasts [275]. GPNMB has been shown to physically associate with β1 or β3 containing integrin complexes in osteoclasts and to be an important mediator of osteoclast fusion and function [275, 315] [316]. Indeed, neutralizing antibodies against GPNMB reduced osteoclast size and number, and decreased their ability to resorb bone [275]. Additionally, transgenic mice expressing GPNMB under the control of a TRAP promoter, which drives osteoclast-specific gene expression, displayed evidence of significant bone loss and elevated bone resorption markers compared to non-transgenic controls [317]. Osteoclasts isolated from these transgenic mice were twice as large, possessed elevated TRAP activity, exhibited enhanced expression of osteoclast markers and could resorb bone matrix to a greater degree than osteoclasts isolated from wild-type controls [317]. Although the role of GPNMB in promoting bone resorption is well-established, conflicting reports exist regarding its ability to induce osteoclast differentiation [315, 316, 318]. Binding of extracellular GPNMB to CD44 expressed on osteoclasts was shown to negatively regulate early-stage osteoclastogenesis by inhibiting downstream ERK phosphorylation and RANKL-induced osteolysis [318]. However, GPNMB interaction with HSPG and αVβ3 integrin reportedly promotes osteoclast differentiation [316], highlighting a potential context-dependent and stage-dependent function of GPNMB in osteoclastogenesis.

1.5.3.4 Immune system

The molecular functions of GPNMB are still being elucidated and perhaps have been best characterized in the immune system. Expression of GPNMB has been detected in eosinophils [319], macrophages [294, 320, 321] and dendritic cells [258, 322] and has been involved in promoting various immune cell-cell interactions. GPNMB expression on

dendritic cells has been shown to mediate their adhesion to endothelial cells in an RGD-dependent manner [258]. Additionally, the extracellular domain of GPNMB can suppress T-cell activation and proliferation by binding to syndecan-4 on the surface of activated T-cells, and this interaction requires an intact PKD domain [323, 324]. GPNMB binding to syndecan-4 leads to the recruitment of syntenin and the CD148 protein tyrosine phosphatase, whose activation occurs following complex formation and is required for syndecan-4 mediated suppression of T-cell activation [325]. This ability to modulate adaptive immunity has been documented in a variety of contexts, including graft versus host disease (GVHD), where GPNMB expression can suppress the activity of allo-reactive T cells [326].

GPNMB is preferentially expressed by M2 macrophages, which play a critical anti-inflammatory role during tissue injury repair [327]. In models of cardiomyopathy, liver fibrosis and kidney disease, increased GPNMB expression was observed in resident and infiltrating macrophages and is thought to serve as a compensatory response to promote tissue repair through autophagy and phagocytosis of cell debris [320, 328-330]. GPNMB can also participate in tissue repair following hyperoxic lung injury by enhancing angiogenesis [292]. GPNMB-expressing macrophages infiltrate sites of tissue injury and promote tissue repair through a variety of mechanisms, including inhibition of pro-inflammatory cytokine production through ERK and p38 pathway activation [331], enhanced production of collagen and α-SMA [332] and increased mobilization of mesenchymal stem cells (MSCs) [327]. Notably, GPNMB is an important regulator of the interplay between M2 macrophages and MSCs during wound healing and tissue repair. GPNMB secreted by M2 macrophages binds to CD44 on MSCs to stimulate downstream ERK and AKT pathways and promote MSC survival, proliferation and motility [327].

Additionally, GPNMB favors M2 polarization of M0 macrophages both directly via the IL4-STAT6 pathway [333] and indirectly through the recruitment of MSCs to sites of tissue injury [334, 335].

In contrast to these immunosuppressive roles, activation of GPNMB in dendritic cells, either by ligand binding or antibody cross-linking, can induce an innate immune response against fungal antigens. Under these conditions, the hemITAM tyrosine residue of GPNMB became phosphorylated, which induced widespread changes in gene and protein expression, including increased cytokine secretion (TNFα, IL-1β) [276]. This activation of GPNMB stimulated dendritic cell maturation and augmented their ability to potentiate the activation of naive T-cells [276]. While these findings are strongly suggestive of functional hemITAM-based signaling in GPNMB, more research is needed to definitively characterize the role of this motif when GPNMB is expressed in immune or non-immune cells.

It is clear from these observations that GPNMB expression is widespread and it can regulate a wide range of physiological and pathological processes. Its established roles during normal tissue processes, such as adhesion during transendothelial migration of dendritic cells and autophagy during tissue repair, are also important mechanisms observed during cancer progression and metastasis. Intriguingly, GPNMB expression can be upregulated in pathological conditions, such as chronic liver disease, which can lead to carcinogenesis [235]. As discussed below, it is possible that GPNMB expression in infiltrating immune cells may play important roles in supporting the tumor microenvironment. Considering that the mechanisms of action for GPNMB in tumor progression have yet to be fully elucidated, these observations of GPNMB function in

normal tissues represent compelling potential roles for GPNMB in cancer and warrant further investigation.

1.5.4 Sub-cellular localization of GPNMB

As mentioned above, GPNMB harbors a di-leucine sorting motif in its cytoplasmic tail which was shown to be important for its intracellular trafficking and targeting to endosomal and premelanosomal compartments [264]. More recent data illustrates that GPNMB exhibits a varied pattern of expression, presumably linked to its diverse functions in different tissue types.

In pigmented epithelial cells such as melanocytes, GPNMB is preferentially localized to late-stage (III and IV) melanosomes, which are characterized by an accumulation of the melanin pigment, suggesting a putative role for GPNMB in melanosome maturation [274, 306]. Its weak cell-surface expression can be upregulated following UVA irradiation and αMSH stimulation in melanocytes and melanoma cells and, additionally, by IFNγ and TNFα stimulation in melanocytes [306]. In pigmented cells, it is thought that the extensive glycosylation of the GPNMB PKD domain contributes to the differential sorting and localization patterns that are observed between GPNMB and its close homologue Pmel [336]. Indeed, while the PKD domain of Pmel plays an active role in the cellular distribution of Pmel, the degree of glycosylation in GPNMB blocks this sorting function leading to differential localization of GPNMB [336]. However, in melanocytes, some residual expression is observed in lysosomes and it was postulated that, in the absence of melanosomes, GPNMB would be primarily targeted in lysosomes. Accordingly, in many non-pigmented dendritic cells, GPNMB localization was identified in perinuclear lysosomes and in other lysosomes closer to the cell periphery [258, 306].

GPNMB can also be trafficked to and from the Golgi network in response to various stimuli. In macrophages, GPNMB-induced cytokine production can potentially be explained by its translocation from the Golgi to scattered vesicles closer to the cell periphery in response to LPS or IFNγ stimulation [321]. Upon disruption of the Golgi network in osteoblasts, GPNMB localizes to vesicular and endosomal-like structures for subsequent targeting to the plasma membrane or secretion into the conditioned media [273]. GPNMB movement from the Golgi to peripheral endosomal/lysosomal compartments was also observed in late stages of osteoclast differentiation by RANK ligand, possibly as an intermediate step preceding cell membrane targeting in terminally differentiated osteoclasts [278].

A couple of studies have also found GPNMB localized to components of the autophagic and phagocytic pathways, which is consistent with its role in tissue repair [328, 330, 337]. During kidney injury, GPNMB was present in cytoplasmic vesicles and co-localized with pSyk, a downstream target of ITAM pSrc signaling which is involved in regulation of lysosomal function, and with LC3 II, an established mediator of autophagy. These results functionally link the ITAM and di-leucine motifs of GPNMB in a mechanism that synergizes autophagy and lysosomal function. Furthermore, these findings suggest that GPNMB mediates degradation of cellular debris during tissue repair by targeting to the lysosomal pathway via its di-leucine motif and promoting fusion of autophagosomes to lysosomes. A separate study looking at kidney repair following injury reported that GPNMB was predominantly present in LC3-containing autophagocytic vesicles of regenerating epithelial cells. During phagocytosis of apoptotic cells, GPNMB was visualized on the membrane of phagocytic cells and led to the recruitment and fusion of LC3-stained vesicles to the phagosome, thereby enhancing degradation of debris [328].

Interestingly, GPNMB is also able to translocate to the nucleus and modulate pre-mRNA splicing in response to cellular stress [304, 338]. There is evidence to support a role for the C-terminal region of GPNMB in binding to splicing factors and other co-factors under stress conditions [304], thereby potentially implicating GPNMB in the direct regulation of gene expression. These varied patterns of sub-cellular localization support a multi-functional role for GPNMB in physiology and disease.

1.5.5 GPNMB in cancer

1.5.5.1 Tumor Suppressive Properties

While it has become increasingly clear that the initial designation of GPNMB as "glycoprotein non-metastatic gene B" is inaccurate in the context of melanoma (see below), there are cancers in which GPNMB appears to exert a tumor-suppressive response.

In the vast majority of colorectal carcinomas, GPNMB is epigenetically silenced by promoter methylation and could thus be involved in attenuating aggressiveness and delaying tumor progression [339]. Conflicting reports have been published on the role of GPNMB in prostate cancer [340, 341]. A study examining GPNMB overexpression in prostate carcinoma cell lines reported a reduction in invasion and proliferation *in vitro* and tumor growth *in vivo* [340]. Upregulation of anti-metastatic genes, including NDRG1 and maspin, was observed following forced GPNMB expression in this model, and was proposed as a potential mechanism to explain the anti-tumorigenic effects associated with GPNMB expression [340]. However, a report published more recently showed that GPNMB can upregulate MMP-2 and MMP-9 activity to promote the invasion and motility of metastatic prostate cancer cells [341]. These findings emphasize the complexity of

GPNMB's role in tumor biology and the need to obtain a more comprehensive understanding of its mechanisms of action.

1.5.5.2 Tumor Promoting Properties

Emerging data has generated a more complex picture with respect to GPNMB in cancer progression, and it is now evident that GPNMB can function to promote tumor progression in certain types of cancer and can act as a tumor suppressor in others [263]. The literature investigating the relationship between GPNMB and cancer continues to grow, with an increasing number of reports describing positive correlations between GPNMB expression and poor outcomes and pro-invasive/pro-metastatic phenotypes in a variety of cancers.

1.5.6 GPNMB expression and function in breast cancer

GPNMB has been identified as a gene that is frequently and highly expressed in aggressively metastatic breast cancer cell populations [229, 231]. Overexpression of GPNMB in weakly metastatic breast cancer cells was shown to drive the acquisition of an invasive phenotype *in vitro*, characterized by elevated MMP-3 levels, and enhance the bone metastatic potential of these cells [229]. Studies examining GPNMB expression *in vivo* in murine and human mammary carcinoma models have found that GPNMB could also promote primary tumor growth [230, 288]. GPNMB-expressing tumors were characterized by a high endothelial cell density compared to tumors that lacked GPNMB and *in vitro* studies revealed the soluble GPNMB ECD is biologically active and capable of inducing endothelial migration [230]. These data suggest that GPNMB could promote tumor growth and metastasis by regulating the ability of breast cancer cells to recruit vasculature. Separately, GPNMB overexpression in mouse mammary epithelial NMuMG cells was shown to induce EMT through downregulation of E-cadherin and upregulation of N-

cadherin and fibronectin expression. In this model, GPNMB promoted malignant progression through heightened invasion *in vitro* and increased tumor formation *in vivo*, and these effects were dependent on Src-mediated phosphorylation of the hemITAM tyrosine residue found in the GPNMB cytoplasmic tail [288].

Contrary to the numerous independent studies mentioned above, an early publication examining GPNMB mRNA expression in breast cancer reported lower expression of GPNMB in tumor compared to normal tissues[342]. This study also showed that GPNMB expression was increased in immortalized cell lines derived from normal breast epithelium compared to breast cancer cell lines. These studies are in opposition to other published findings and may reflect the fact that the authors did not take the breast cancer subtype into account during their analysis [193, 229].

1.5.7 GPNMB expression and function in other solid malignancies

1.5.7.1 Brain Cancer

The first association of GPNMB with cancer progression was in 2003, when it was reported to promote the invasion of glioma cells [237]. These pro-invasive effects were attributed to the ability of GPNMB to enhance the expression of MMP-3 and MMP-9 [237]. Subsequent studies have confirmed that GPNMB expression is elevated in both benign subependymal giant cell astrocytomas [343] and malignant glioblastomas [234]. Importantly, glioblastoma patients with high levels of GPNMB transcript and protein levels exhibited a significantly worse survival prognosis [234, 344]. Mechanistically, GPNMB can interact with the α subunit of Na+/K+ ATPase to activate downstream PI3K and MEK/ERK pathways, which in turn promote glioblastoma growth and glioma cell invasion [303]. There is also evidence to suggest that GPNMB modulates the Wnt/β-catenin in glioma cells to regulate expression and activity of MMP2/3/9. In this model, GPNMB

promoted motility and angiogenic tube formation, possibly through regulation of VEGFC and TEM7 expression [345].

1.5.7.2 Melanoma

The notion that GPNMB is linked to melanomas with low-metastatic potential [254] has been dispelled by subsequent studies reporting that GPNMB expression is elevated in malignant cutaneous melanoma [236, 346] and is predictive of poor prognosis in patients with this disease [249, 347]. In a murine melanoma model, it has been suggested that GPNMB promotes tumor growth via an immunosuppressive mechanism involving a block in T-cell activation [348]. Interestingly, this study also reported that GPNMB could be released from melanoma cells in the form of exosomes, and that this dissemination of GPNMB might facilitate systemic immunosuppression of anti-tumor responses [348]. It was in the context of cutaneous melanoma that anti-GPNMB therapies were first considered [236, 349, 350], which is discussed in greater detail below. Interestingly, a recent survey of uveal melanomas revealed that a high percentage of these aggressive tumors also express GPNMB [238].

1.5.7.3 Other Cancers

GPNMB is overexpressed in a number of other cancers, including hepatocellular carcinoma [235], small cell lung cancer [239], renal cell carcinoma [351], ovarian cancer [240, 241], bladder cancer [242], oral squamous cell carcinoma [243], osteosarcoma [244, 352], papillary thyroid carcinoma [353] and lymphangioleiomyomatosis [245], and its expression predicts poor prognosis in clear-cell renal carcinoma [351], ovarian cancer [240, 241] and bladder cancer patients [242]. Functionally, GPNMB can enhance cancer progression by mediating motility, invasion or cancer cell growth in models of hepatocellular carcinoma [235], pancreatic ductal adenocarcinoma [354], bladder cancer

[242], oral squamous cell carcinoma [243], osteosarcoma [352], lung cancer [355] and sarcoma [287, 356]. In alveolar soft part sarcoma (ASPS), a highly metastatic sarcoma characterized by a dense vascular network and frequent tumor intravasation, GPNMB promotes transendothelial migration, and is found at the invasive tumor front and at sites of intravasation [287]. GPNMB is also detected in the sera of cholangiocarcinoma patients and is part of a cholangiocarcinoma cancer stem cell secretome responsible for increases in cancer cell invasion, *in vivo* tumor-initiating properties, and education of a malignant macrophage subset [357]. Taken together, these results indicate that GPNMB is a potentially useful prognostic marker and therapeutic target in a variety of cancers.

In recent years, various studies have shed some light on the GPNMB mechanisms of action in the cancers outlined above. Notably, GPNMB can modulate MMP expression to promote migration and invasion in models of bladder cancer [242] and head and neck squamous cell carcinoma [356]. In osteosarcoma, the effects of GPNMB on proliferation, migration and invasion are accompanied by an increase in PI3K/AKT/mTOR pathway signaling [352]. There is also some evidence to show that GPNMB silencing impairs proliferation, migration and invasion of bladder cancer cells by attenuating the GSK3β/β-catenin signaling axis [242]. Additionally, shed or recombinant GPNMB can promote invasion and migration of lung cancer cells in a RGD-dependent fashion [355] and promote migration and integrin-dependent adhesion of oral squamous cell carcinomas [243].

Combined, observations of GPNMB action in various cancers reveal both tumor intrinsic effects of GPNMB that can enhance the invasiveness of tumor cells as well as numerous mechanisms through which GPNMB can facilitate interactions with, and influence the behavior of, cells within the tumor microenvironment (Figure 1.2).

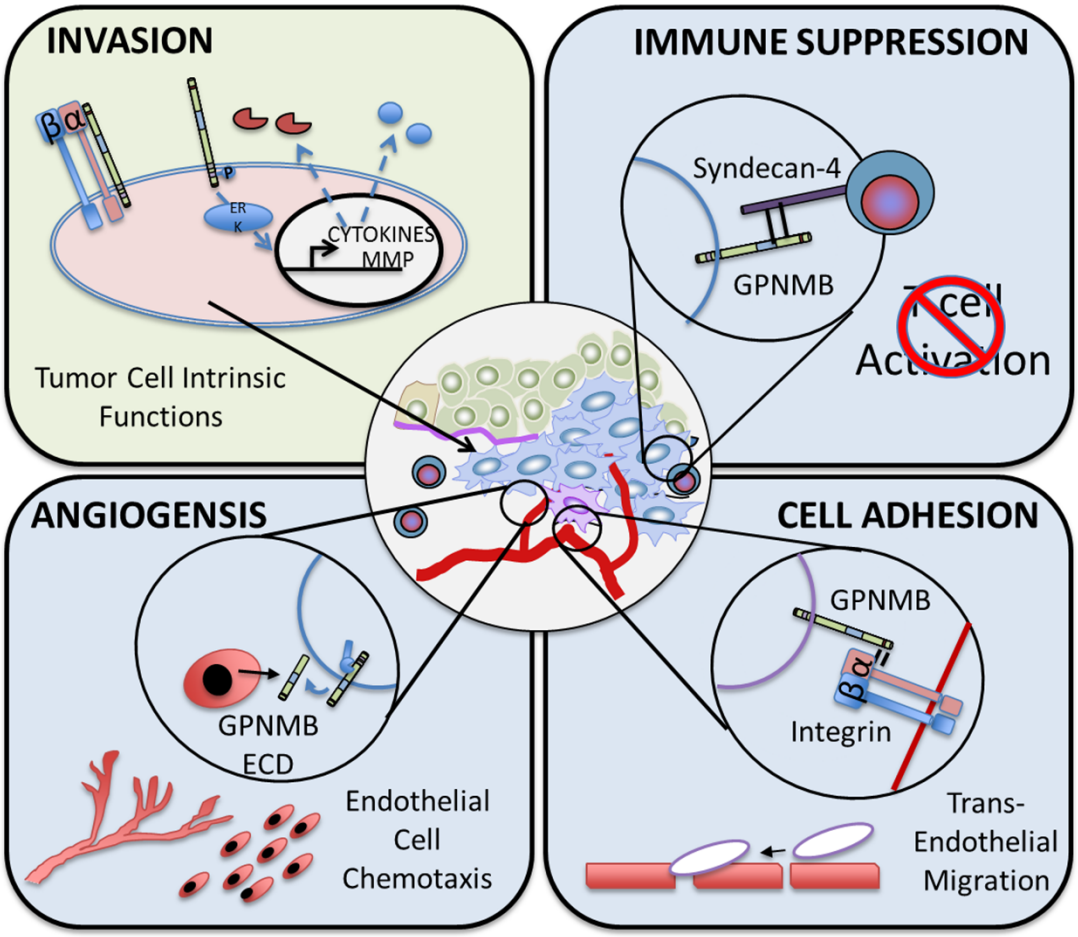

Figure 1.2 Potential mechanisms through which GPNMB promotes malignant cellular phenotypes within cancer cells. GPNMB may act cell autonomously (green panel) to induce intracellular signaling, which can influence the expression of multiple targets, including MMPs and cytokines, and enhance the invasiveness of tumor cells. GPNMB may also be important in regulating interactions between tumor cells and stromal cells (blue panels). It can act as a cell/cell adhesion molecule by engaging integrins expressed on cells in the tumor microenvironment, such as endothelial cells. GPNMB-mediated interactions with Syndecan-4 expressed on T cells can block the proliferation and activation of these cells, leading to an immunosuppressive environment favoring tumor growth. Finally, GPNMB may function in a paracrine fashion due to shedding of its extracellular domain, or through its release from cells in the form of microvesicles, leading to endothelial cell recruitment. All of these potential functions of GPNMB can promote tumor growth, invasion and metastasis in a variety of cancer cells.

1.5.8 GPNMB function in the tumor stroma

GPNMB expression in the stromal compartment of different cancers could also potentially be linked to tumor progression. GPNMB was overexpressed in a subset of CD10-positive cancer associated fibroblasts derived from colon tissue [358], which is in line with previous reports that GPNMB can activate fibroblasts by inducing upregulation of pro-invasive matrix metalloproteases, such as MMP-3 and MMP-9, via ERK-dependent signaling [277, 359].

In macrophages, treatment with tumor cell conditioned media induced an 83-fold increase in GPNMB expression [360]. Interestingly, these tumor-conditioned macrophages adopted a phenotype similar to the M2-type macrophages [360], which are known for their role in promoting tumor progression [361]. Furthermore, a recent study examining mice and humans affected with glioblastoma multiforme has reported that GPNMB expression is increased in glioma-associated macrophages compared to macrophages found in normal brain tissue [344]. Functional evidence for implication of stromally-derived GPNMB in cancer progression came from in an *in vivo* model of melanoma, where GPNMB expressed by tumor-associated myeloid-derived cells (MDSCs) promoted melanoma growth by suppressing T-cell antitumor activity [362]. Genetic or antibody-mediated ablation of GPNMB on tumor MDSCs dramatically decreased *in vivo* growth and metastasis of melanoma B16 cells [362]. Notably, these observations can be extended to human melanoma patients, where GPNMB expression by MDSCs was shown to be a useful biomarker and therapeutic target [363].

In the breast, GPNMB expression is abundant in the tumor stroma [231], which could be attributed to its expression in a variety of stromal cells described above. Taken together, these studies suggest a role for GPNMB in sustaining the tumor microenvironment,

however it remains to be seen if stromal GPNMB can directly influence tumor progression. In this regard, it is interesting to note that GPNMB expression in the tumor epithelium of breast cancers was associated with poorer prognosis, whereas breast cancers that lacked GPNMB or displayed predominantly stromal GPNMB expression displayed better outcomes [231]. However, this may reflect the fact that tumor cell intrinsic GPNMB expression is required for breast cancer progression and do not necessarily negate an important role for stromal-derived GPNMB in this disease.

1.5.9 Therapeutic targeting of GPNMB

Given the increasing association between GPNMB expression and a variety of cancers, and the acquisition of aggressive cellular phenotypes in GPNMB-expressing cancer cells, there has been growing interest in the development of GPNMB-targeted therapies [250-252]. The pattern of GPNMB expression in normal and cancerous tissues makes it an intriguing target for cancer therapy. Generally speaking, GPNMB localization tends to be restricted to intracellular compartments in normal cells, such as macrophages, melanocytes and pigmented retinal epithelial cells [306, 321, 337]. In contrast, GPNMB expression in tumor cells is enriched on the cell surface [231, 236, 350]. This pattern of sub-cellular localization makes tumor-specific GPNMB more readily available for antibody targeting, thus providing a therapeutic window and making GPNMB a uniquely attractive target for antibody-based therapies.

1.5.9.1 Pre-Clinical Targeting of GPNMB

A single chain antibody coupled to an immunotoxin (F6V-PE38), which is directed against the extracellular domain of GPNMB, has been generated for the treatment of glioblastoma multiforme [364]. F6V-PE38 causes protein synthesis inhibition and apoptosis following internalization by GPNMB-expressing target cells. Two xenograft

models of malignant glioma (Glioblastoma multiforme and Medulloblastoma) were subjected to treatment with the anti-GPNMB immunotoxin, which resulted in a significant impairment in tumor growth compared to PBS-treated controls [364]. Although these findings are preliminary, they address the potential for development of small-size targeted therapeutics against GPNMB, which will penetrate the tumor mass with higher efficiency compared to full-length conjugated antibodies [365].

A more developed GPNMB-targeted therapeutic agent is glembatumumab vedotin (GV), an antibody-drug conjugate also known as CR011-vcMMAE (CR011) or CDX-011 [350]. In the case of GV, the cytotoxin auristatin E, a tubulin destabilizer, is conjugated to an antibody directed against the extracellular domain of GPNMB [350]. Upon GPNMB binding and internalization, the drug is released and induces cell cycle arrest and apoptosis of the target cell.

The first evidence of successful therapeutic targeting of GPNMB using this ADC demonstrated that GV was selectively able to inhibit the growth of GPNMB-expressing metastatic melanoma cells, both in culture and xenograft assays [350]. A subsequent study examining the pharmacological properties of this antibody-drug conjugate showed that, at concentrations as low as 2.5mg/kg, GV was capable of inducing complete *in vivo* tumor regression in 100% of GPNMB-expressing SK-Mel-2 and SK-Mel-5 melanoma cells [349]. In breast cancer, a single dose of 20mg/kg GV was sufficient to induce sustained MDA-MB-468 tumor regression *in vivo* [231]. The activity of GV was also tested in pre-clinical osteosarcoma models, with promising results [244, 248]. GV displayed cytotoxic activity in 14 out of 19 cell osteosarcoma cell lines studied, and GV cytotoxicity was correlated to GPNMB protein expression [244]. A separate study demonstrated that treatment of 6 osteosarcoma xenografts with 3 weekly doses of 2.5mg/kg of GV led to significant

differences in event-free survival in all cases examined and promoted a complete response in 50% of cases [248]. Osteosarcoma xenograft response rates were loosely correlated to GPNMB mRNA expression, although 1 of the 6 xenografts did not express GPNMB at the protein level, indicating that GPNMB expression levels alone do not predict GV efficacy [248]. Cell surface expression of GPNMB is a more accurate prognostic marker, with numerous studies reporting that GPNMB cell surface expression is directly proportional to cell killing efficacy of GV [231, 236, 349, 350]. In follow-up to these pre-clinical findings, the effectiveness of GV is currently being evaluated in international phase II clinical trial targeting 38 patients with recurrent or refractory osteosarcoma [233].

Interestingly, treatment of cancer cells with imatinib or inhibitors of the Erk pathway enhances cell surface expression of GPNMB in cancer cells, which in turn increases sensitivity to GV [236]. Increased GPNMB expression is also observed in monocyte-derived dendritic cells (moDC) treated with BCR-ABL and Src family kinase inhibitors such as imatinib, dasatinib and nilotinib, and leads to potentiated immune-suppression by moDCs [366]. GPNMB expression was similarly enhanced through trastuzumab-mediated HER2 inhibition in breast cancer [232] and cetuximab-mediated inhibition of EGFR in gastrointestinal cancer [367], indicating that GPNMB may be an important mediator of resistance to anti-HER2 and anti-EGFR therapy. Additionally, GPNMB cell surface expression is increased in BRAF mutant melanomas following treatment with MAPK inhibitors, and this pattern of expression is seen post-treatment in biopsies from melanoma patients [249]. Melanoma xenografts treated with BRAF and/or MAPK inhibitors display enhanced sensitivity to GV, and the addition of GV to either inhibitor treatment accelerated melanoma regression and inhibited regrowth of recurrent lesions [249].

Inhibitors of metalloproteases, such as GM6001, have also been shown to enhance cell surface GPNMB expression by preventing shedding of its extracellular domain [236, 277]. In addition to increasing target availability, such inhibitors can minimize the potential for sequestration of GV by the shed form of GPNMB and thereby increase the targeted killing of GPNMB-expressing tumor cells. However, the effect of these inhibitors on tumor cell sensitivity to GV has not yet been examined. These findings suggest that combinations with additional targeted therapies that can enhance cell surface GPNMB expression could further enhance the efficacy of GV. Given the pro-invasive and pro-metastatic functions of GPNMB, such a strategy would require careful evaluation in pre-clinical models to ensure that these combination therapies did not increase metastasis of cancer cells that escape GV mediated killing. (Figure 1.3)

1.5.9.2 Glembatumumab Vedotin Clinical Trials in Melanoma and Breast Cancer

GV was initially tested in two multi-centre Phase I/II clinical trials; one for patients with unresectable melanoma [368] and the other for patients with locally advanced or metastatic breast cancer [247, 253]. Tumor shrinkage was reported in 56% of melanoma patients and 62% of breast cancer patients who were treated with a maximum tolerated GV dose (MTD) [247, 253]. GPNMB expression appeared to be a predictive biomarker in the melanoma study. A small subset of melanoma patients with the highest levels of tumoral GPNMB expression (n =7) had longer median progression free survival (PFS) times (4.9 months) compared to the median PFS for all patients in the cohort (n= 34; including those with high tumoral GPNMB), which ranged from 1-3.9 months depending on the dose frequency [247]. This observation was recapitulated in a subset of breast cancer patients treated with GV. In this study, the median PFS for GPNMB-positive patients (n=9) was 18 weeks compared to 9.1 weeks for all patients (n=34) treated with the MTD [253].

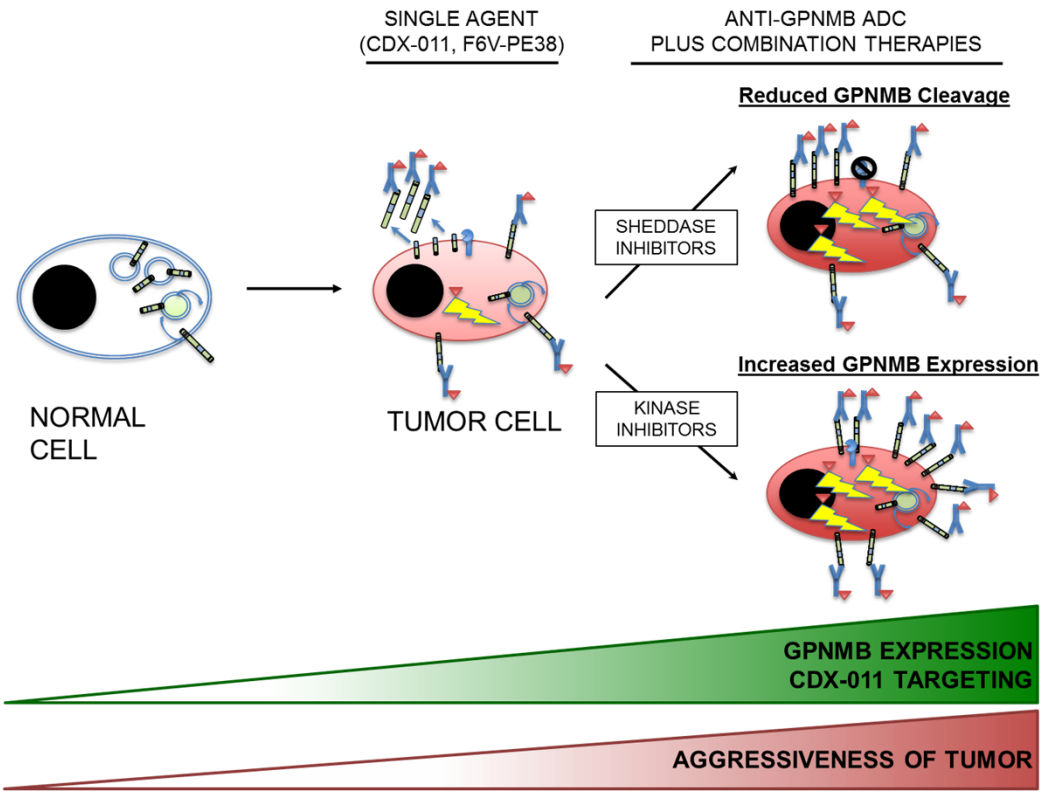

Figure 1.3 Therapeutic strategies employing anti-GPNMB antibody-drug conjugates (ADCs). In normal cells, GPNMB is preferentially localized within endosomal/lysosomal compartments, which is not accessible to anti-GPNMB ADCs. In many cancers, including breast, melanoma and brain cancers, the levels of GPNMB expression increases and a greater proportion is localized on the cell surface. These GPNMB-expressing cancer cells are more susceptible to killing by anti-GPNMB ADCs (CDX-011, F6V-PE38). Evidence suggests that coupling kinase inhibitors (serine/threonine and tyrosine kinase inhibitors), which increase GPNMB expression, enhances the efficacy of tumor cell killing by anti-GPNMB ADCs. Likewise, inhibiting GPNMB shedding could also lead to greater GPNMB surface expression and more targets for anti-GPNMB ADCs. Thus, GPNMB represents an attractive target due to low surface expression in normal cells and its increased expression in cancer cells, which leads to better tumor cell killing with anti-GPNMB ADCs. Combination therapies have the potential to achieve benefit from enhanced efficacy of the anti-GPNMB ADCs and effects of the coupled inhibitors (kinase inhibitors), but there is the potential risk that those tumor cells not killed by combination treatment may adopt increasing malignant phenotypes due to elevated GPNMB expression.

Interestingly, patients with strong GPNMB expression in stromal cells responded to GV just as well, if not better, than patients with strong GPNMB expression in the tumor epithelium [253]. It is conceivable that GPNMB-expressing cells that initially take up GV can release the drug moiety when the targeted cells die, which can freely diffuse into neighboring cells and kill them regardless of whether they expressed GPNMB. This "bystander" effect has been described with SGN-35, which is an antibody drug conjugate that targets CD30 [369].

Based on these observations, subsequent phase II trials were initiated in melanoma and breast cancer. Two phase II clinical trials are currently underway in melanoma: one is investigating efficacy and safety of GV as a single agent and in combination with immunotherapies in advanced melanoma, and the other is aiming to evaluate the clinical anti-tumor activity of GV in metastatic uveal melanoma [233, 238]. GV is being evaluated in combination with varlilumab or an anti-PD1 antibody in two separate advanced melanoma phase II trial cohorts [233]. By targeting GPNMB, GV could theoretically relieve GPNMB-mediated T cell suppression to promote a heightened anti-tumor immune response and could thereby act synergistically in combination with immune therapies to further boost activation of the immune system.

An EMERGE phase IIb clinical trial was recently carried out to investigate the safety and effectiveness of GV for patients with heavily pre-treated, GPNMB-positive, metastatic breast cancer [253]. The final results from this trial were recently published and showed promise for GV treatment of patients with GPNMB-expressing and triple negative breast cancer [246]. The trial enrolled 124 patients and was carried out in a 2:1 randomized fashion where 83 patients received GV and 41 received investigator's choice of therapy (IC). Eligible patients were required to have GPNMB expression in ≥5% of tumor epithelial

and/or stromal tissue, as confirmed by immunohistochemistry on archived tumor samples. Interestingly, 99% of patients tested displayed some level of tumoral GPNMB expression, which was significantly higher than earlier reports of GPNMB expression from breast cancer tissue microarrays [231]. To assess the potential for utilizing GPNMB as predictive marker for GV therapy, patients were classified as having high or low GPNMB expression based on a threshold cutoff of ≥25% GPNMB positivity, post-hoc [247]. The trial reported that 41% of TNBC patients had high GPNMB expression, which was consistent with previous studies, and further confirmed GPNMB as a promising target in this aggressive disease subtype. Partial response was observed in 18% of patients with triple negative disease, compared to 0% with IC, which was an encouraging result for a subgroup of breast cancer patients with currently limited treatment options. The response rate was even higher (40% vs. 0%) in the TNBC subset of patients displaying high GPNMB expression, substantiating findings from the melanoma phase I/II trial. Additionally, TNBC patients with high GPNMB expression had a doubling in progression free survival and overall survival. While the results were encouraging, it must be noted that the sample sizes in these groups are very small. Also, no statistically significant differences were observed between GV and IC treated patients with high GPNMB expression, across all subtypes.

In response to promising preliminary results from the EMERGE study, a randomized phase IIb METRIC (Metastatic Triple-Negative Breast Cancer) trial was launched to confirm the EMERGE trial findings in a larger patient population. This trial assigned 327 women with metastatic TNBC overexpressing GPNMB to receive GV or IC therapy in a 2:1 ratio [246]. Unfortunately, recently released top-line results from the METRIC trial indicated that treatment with GV failed to improve progression-free survival, the primary endpoint of the study [370]. Key secondary endpoints, including overall survival, overall

response rate and duration of response were also not significantly affected by GV treatment. Following these disappointing results, the development of GV as a single agent was discontinued in triple-negative breast cancer [370].

The results of the METRIC trial parallel the outcome of numerous studies investigating use of targeted agents in triple-negative breast cancer [371]. TNBCs display extensive inter- and intra-tumor heterogeneity with multiple aggressive sub-clonal populations [140, 184], which may partially explain the failure of single agent targeted therapies in this setting. These results underscore the importance of elucidating mechanisms of action that govern the progression of TNBCs in order to inform the selection of combinatorial therapies that target cooperating pathways in this disease.

1.6 Rationale

GPNMB is overexpressed in triple-negative breast cancers and is predictive of poor prognosis in this aggressive subset of the disease. Additionally, GPNMB promotes tumor growth, invasion and metastasis in multiple *in vitro* and *in vivo* breast cancer models. Although the tumor-promoting properties of GPNMB are well-established in a variety of cancers, very little is known about GPNMB mechanisms of action. In light of the scarcity of treatment options for TNBC patients, the aim of this project is to elucidate GPNMB-mediated mechanisms of action in breast cancer in order to better characterize the biology of TNBCs and identify potential targets for GPNMB combination therapy.

To identify molecular mediators of GPNMB action, we started with an unbiased gene expression profiling approach comparing differentially regulated genes in breast cancer cells overexpressing GPNMB or a control construct. We identified Neuropilin-1 (NRP-1) as a gene that is overexpressed downstream of GPNMB in basal breast cancer cell lines and

is required for GPNMB-mediated tumor growth, but not metastasis, through a mechanism that relies on ERK and AKT activation (Chapter 2). We show that GPNMB promotes the recruitment of VEGF-producing endothelial cells and macrophages and enhances breast cancer cell responsiveness to VEGF signaling through the NRP-1/VEGFR2/ERK axis.

We next interrogated the mechanisms of action engaged during GPNMB-driven metastasis by using a panel of GPNMB domain mutants (Chapter 3). We demonstrate that the GPNMB cytoplasmic tail and extracellular RGD domain are both required to promote invasion *in vitro* and tumor growth *in vivo,* however, only the RGD integrin-binding domain is required for GPNMB-mediated metastasis. The importance of the RGD domain led us to examine expression profiles of various integrin subunits in GPNMB-expressing cells. We show that GPNMB dramatically increases protein stability of the α5β1 fibronectin receptor, through an RGD-mediated interaction that promotes recycling of active integrin complexes from late endosomes and triggers downstream FAK/SRC signaling.

Finally, we generated transgenic mice expressing GPNMB in the mammary gland under the control of the MMTV promoter/enhancer and crossed these mice with MMTV/Wnt-1 transgenic animals in order to examine the role of GPNMB in a Wnt-1-driven model of basal breast cancer (Chapter 4). We show that GPNMB enhances tumor growth, but not metastasis, in this model and establish a novel role for GPNMB in promoting tumor initiation. Using a proteomic profiling approach, we demonstrate that GPNMB increases PI3K/AKT/mTOR pathway signaling and promotes β-catenin nuclear translocation and activity during *in vivo* tumor progression. Taken together, the work presented in this thesis identifies novel mechanisms of action in play downstream of GPNMB during progression of triple-negative breast cancer.

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CHAPTER 2

GPNMB Co-operates with Neuropilin-1 to Promote Mammary Tumor Growth

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The work presented in this section is part of a manuscript published in Oncogene (16 March, 2015)

2.1 Preface

GPNMB expression enhances growth, invasion and metastasis in a variety of cancer models. Additionally, numerous reports have shown that GPNMB expression increases during malignant progression. In breast cancer, GPNMB is overexpressed in the triple-negative subtype and is an independent indicator of poor prognosis in this aggressive subset of the disease. However, despite its biological and clinical significance, very little is known regarding the molecular mechanisms engaged by GPNMB. Previous studies that have attempted to elucidate GPNMB mechanisms of action have focused their attention on isolated genes and proteins. To study the mechanisms of action of GPNMB in a more systematic fashion, we took an unbiased gene-expression-based profiling approach. By examining, for the first time, global expression changes in response to GPNMB overexpression, we were able to identify and establish Neuropilin-1 as a key player in GPNMB-driven breast cancer growth.

2.2 Abstract

Glycoprotein nmb (GPNMB) promotes breast cancer growth and metastasis and its expression in the tumor epithelium correlates with poor prognosis in breast cancer patients. Herein, we show that GPNMB increases expression of Neuropilin-1 (NRP-1) in breast cancer cells by enhancing activation of the PI3K/AKT pathway. We demonstrate that NRP-1 promotes growth, but not metastasis of GPNMB-expressing basal breast cancer cells *in vivo*. Interestingly the pro-tumorigenic effects of NRP-1 were only observed downstream of GPNMB and NRP-1 knockdown had no effect in basal conditions. Mechanistically, we observed that the GPNMB-driven increase in NRP-1 expression enhances VEGF responsiveness in breast cancer cells to potentiate VEGFR2 and ERK phosphorylation. Concomitantly, GPNMB promotes the recruitment of a stromal VEGF-secreting infiltrate *in vivo* to further engage the pro-growth functions of NRP-1. We also found that GPNMB can physically associate with NRP-1 in breast cancer cells, which suggests that GPNMB might be able to modulate effects of other receptors that are bound by NRP-1. Notably, interrogation of RNAseq datasets revealed a strong positive correlation between *GPNMB* and *NRP-1* levels in human breast tumors, thus emphasizing the clinical relevance of the mechanism described in this study.

2.3 Introduction

Triple-negative breast cancers (TNBC) represent the most aggressive breast cancer (BC) subtype and are associated with high grade and poor patient prognosis [1, 2]. Due to the lack of distinct molecular markers in TNBCs, limited targeted therapeutic options exist to effectively treat these aggressive breast cancers. Therefore, a detailed understanding of the mechanisms that govern the initiation and progression of this breast cancer subtype is necessary. GPNMB is highly expressed in TNBCs, compared to other BC subtypes [3]. Its expression in the breast cancer epithelium is predictive of poor prognosis, and is a separate indicator of recurrence-free survival in TNBCs [4]. CDX-011, an antibody-drug conjugate targeting the extracellular domain of GPNMB, is currently in clinical trials for patients with advanced, metastatic breast cancer and has been shown to significantly improve overall survival and progression-free survival [5, 6]. GPNMB expression has been shown to promote the malignant phenotype of numerous cancers by increasing angiogenesis, survival, immune evasion, invasion and metastasis [3, 7]. However, despite its now well-established role in mediating the progression of various epithelial neoplasias, the mechanisms of action of GPNMB in cancer remain very poorly understood.

In investigating genes modulated by GPNMB expression our attention was drawn to Neuropilin-1, a co-receptor for vascular endothelial growth factor (VEGF) and for the axonal guidance factor semaphorin 3 (SEMA3) [8]. NRP-1 is a single-pass transmembrane protein expressed by endothelial cells, which possesses no signal transduction capacity of its own, but it has been shown to recruit signaling adaptors, such as GIPC1, through a PDZ-binding domain in its cytoplasmic tail [9]. The extracellular domain of NRP-1 contains an

a1/a2 CUB VEGF- and SEMA3-binding domain, a b1/b2 SEMA3-binding domain homologous to coagulation factors V and VIII and a MAM homology domain, which mediates receptor oligomerization. Soluble NRP-1 isoforms can be generated by alternative splicing and generally act as decoy receptors to dampen the effects of the full-length protein [10, 11].

NRP-1 was initially identified as a co-receptor that promotes the chemorepulsive effects of class III and IV semaphorins during axonal guidance and facilitates their anti-angiogenic activity in endothelial cells [12-14]. Semaphorins can competitively inhibit VEGF binding to NRP-1 and, by integrating these opposing signals, NRP-1 acts as a critical regulator of vasculogenesis and angiogenesis in development and cancer [15-18]. It is well known that growing tumors recruit blood vessels to facilitate nutrient uptake and remove waste [19]. The establishment of a tumor-associated vasculature during malignant progression requires a process known as the angiogenic switch, which can be mediated by pro-angiogenic factors secreted by cancer cells or by a VEGF-producing stromal infiltrate recruited to the growing tumor that includes macrophages, neutrophils and cancer-associated fibroblasts. In turn, VEGF exerts a potent mitogenic effect in endothelial cells, by acting on its primary receptor, VEGFR2, and its co-receptor, NRP-1 [17]. Due to its expression by endothelial cells, the contributions of NRP-1 in tumor progression were initially attributed solely to vascular mechanisms. NRP-1 binding to VEGF changes its conformation, increases the affinity of the cytokine for VEGFR2, and to a lesser extent, for VEGFR1, and enhances downstream signaling [20]. Central to tumor angiogenesis, VEGF signaling through the VEGFR2/NRP-1 complex activates PI3K/AKT to increase cell survival and vascular permeability, engages the MAPK pathway to stimulate proliferation, and recruits FAK and SRC to promote cell migration [21]. NRP-1 expressed on endothelial

cells can also relay VEGF-independent signaling required for tumor progression. Notably, in pancreatic ductal adenocarcinoma, NRP-1 promotes tumor growth by regulating TGF-β1-induced endothelial-to-mesenchymal transition (EndMT), which is an important source of cancer-associated fibroblasts in tumorigenesis [22].

In addition to its role in endothelial cells, NRP-1 expressed on tumor cells has also been shown to modulate angiogenesis in the tumor microenvironment [18, 23]. NRP-1 overexpression in rat prostate carcinoma cells led to a dramatic increase in tumor growth *in vivo*, which was attributed to increased microvascular density and endothelial cell proliferation [24]. Notably, the enhanced angiogenesis observed in this model was orchestrated through a VEGF-mediated juxtacrine interaction between tumor-specific NRP-1 and VEGFR2 expressed on endothelial cells [25]. Additional reports have defined a role for NRP-1 that is expressed on cancer cells in promoting tumor growth by increasing angiogenesis in colon carcinoma and glioblastoma models [26, 27]. However, a recent study examining the role of tumor-specific NRP-1 in an *in vivo* model of melanoma revealed that NRP-1 acting *in trans* to bind VEGFR2 on endothelial cells can suppress tumor initiation by inhibiting efficient VEGFR2 internalization and signaling [28]. These studies outline a central, context-dependent role for NRP-1-mediated VEGF signaling in tumor-associated angiogenesis.

It has become apparent that VEGF signaling extends beyond the endothelial cell and that angiogenesis-independent actions of VEGF on tumor cells strongly contribute to tumor progression [29]. Accordingly, a tumor cell-autonomous role for Neuropilins in cancer progression has begun to emerge. NRP-1 is expressed by a number of solid malignancies, including breast, brain, melanoma and prostate cancers [23] and correlates with poor prognosis in a wide range of cancers [30], including breast [31, 32]. Notably,

tumor tissue expression of NRP-1 is increased in metastatic breast cancer compared to early disease stages, and its expression is further enhanced in TNBC [33]. NRP-1 expressed on tumor cells can act as a receptor for autocrine and paracrine VEGF signaling to increase proliferative and pro-survival signaling pathways [34, 35]. In a model of basal breast cancer, VEGF increased PI3K signaling in an NRP-1-dependent manner to protect tumor cells from apoptosis [35]. VEGF signaling mediated through the NRP-1/VEGFR2 complex is also cytoprotective in pancreatic cancer cells, but cell survival is attributed to heightened MAPK pathway activation in this context [34]. Similarly, in glioblastoma, VEGFR2 and NRP-1 found on the cell surface or in endocytic compartments are critical in relaying VEGF-mediated survival and self-renewal signals [36]. NRP-1 can also bind to VEGFR1 to increase proliferation and initiate the onset of epidermal cancers in a VEGF-dependent manner. This was mediated through a cell-autonomous autocrine loop that synergized with EGFR and activated MAPK signaling [37]. This study, along with many others, demonstrated that ERK activation in response to growth factor stimulation can result in an increase of NRP-1 expression [37, 38].

Interestingly, many tumor types that don't express VEGFR1 and VEGFR2 can still respond to VEGF signaling. These discoveries have shifted the attention to NRP-1 as a primary VEGF receptor in cancer cells. In metastatic breast cancer cells lacking VEGFR2 expression, cell survival is sustained through the VEGF/NRP-1/PI3K axis [35, 39] and blockade of the VEGF binding site on NRP-1 induces apoptosis [40]. Furthermore, seminal work investigating the VEGFR2-independent role of NRP-1 in renal cell carcinoma implicated NRP-1 in the maintenance of an undifferentiated state, suggesting that NRP-1 is an important regulator of cancer stemness [41]. Consistent with these findings, numerous recent studies substantiate a role for the VEGF/NRP-1 axis in regulating breast cancer stem

cell phenotype and activity through the engagement of Wnt/β-catenin pathway signaling [42, 43]. This stand-alone role for tumor-autonomous NRP-1 may explain the inefficiency of bevacizumab treatment, which blocks VEGF interaction with VEGFR2, but not NRP-1 [44]. Interestingly, clinical trials for patients with breast and gastric cancer indicate that high NRP-1 expression in tumors is inversely correlated to bevacizumab efficacy and establish NRP-1 as a predictive biomarker of response to bevacizumab [45, 46].

In 2007, a groundbreaking study by the Watts group demonstrated that inhibition of NRP-1 has an additive effect with anti-VEGF therapy in reducing tumor growth, suggesting a VEGF-independent role for NRP-1 [47]. Recent work has shown that NRP-1 acts as a co-receptor for c-Met, EGFR, TGFβRII and integrin β1 to enhance downstream signaling pathways and malignant progression [27, 48-53]. Furthermore, NRP-1 can bind extracellular miRNAs and mediate their internalization to regulate cancer cell function, including proliferation and migration [54]. These studies establish NRP-1 as a promiscuous receptor in tumor biology that is central to various aspects of tumor progression, beyond its prototypic role in angiogenesis. Importantly, the ability of NRP-1 to associate with a multitude of receptors suggests that NRP-1 favors the assembly of large-scale protein complexes and therefore acts as a central integrator of crosstalk between major signaling pathways.

In this study, we investigate the role of NRP-1 in GPNMB-driven primary tumor growth and metastasis. We show that GPNMB upregulates NRP-1 mRNA and protein expression in a variety of breast cancer cell lines to increase primary tumor growth. Additionally, we demonstrate that NRP-1 acts in an angiogenesis-independent manner to enhance tumor cell intrinsic VEGF signaling in GPNMB-expressing breast cancer cells.

2.4 Results

Neuropilin-1 is up-regulated in GPNMB-expressing breast cancer cells

We have previously shown that GPNMB expression in BT549 human basal breast cancer cells promotes invasion and endothelial cell recruitment [55, 56]. Additionally, 66cl4 murine mammary carcinoma cells exhibit GPNMB-driven increases in primary tumor growth and metastasis [56]. To determine how GPNMB regulates tumor-promoting effects in these breast cancer cell models, we sought to identify genes that are differentially regulated in response to GPNMB overexpression. Gene expression profiling was performed on independent clones of 66cl4 and BT549 breast cancer cells that were engineered to overexpress GPNMB versus clonal lines that harbor an empty vector (VC) (Figure 2.1A). These experiments revealed that 762 and 943 genes were differentially modulated by GPNMB overexpression in 66cl4 and BT549 cells, respectively. A total of 17 genes were commonly regulated by GPNMB overexpression in mouse and human breast cancer cells (Figure 2.1B, Table 2.1). The expression of several candidates was validated at the transcript level in BT549 and 66cl4 cells, and transcript levels of Neuropilin-1 (NRP-1), Clusterin and Serpine2 were all significantly increased in both human and mouse breast cancer cell models overexpressing GPNMB (Supplementary Figure 2.1A-I). However, we were not able to validate elevated protein expression of Clusterin and Serpine2 by immunoblot in GPNMB-expressing cells (data not shown).

NRP-1 expressed on breast cancer cells is a known mediator of tumor growth and angiogenesis, which parallels known GPNMB effects in breast cancer [8, 24, 29, 35]. Therefore, we sought to investigate whether NRP-1 could mediate the tumor growth and angiogenesis phenotypes that have been observed downstream of GPNMB [7, 55, 56].

GPNMB-driven increases in NRP-1 expression were confirmed at the mRNA (Figure 2.2A-C) and protein levels (Figure 2.2D-G) in multiple human (BT549, HS578T) and mouse (66cl4, NIC) breast cancer cell lines. Additionally, NRP-1 protein levels were elevated in mammary tumors derived from GPNMB-expressing 66cl4 cells (Figure 2.2G) [56]. Using MDA-MB-436 basal breast cancer cells that express high endogenous levels of GPNMB, we further demonstrate that reduction of GPNMB expression decreases NRP-1 levels. (Supplementary Figure 2.2). Together, these data show that GPNMB is a necessary and sufficient modulator of NRP-1 expression in multiple breast cancer cell models.

To determine the clinical relevance of our observations, we examined the correlation between *GPNMB* and *NRP-1* mRNA expression in published breast cancer datasets. Using gene expression data obtained from 1160 breast tumor samples (The Cancer Genome Atlas (TCGA) Illumina HiSeq dataset) we observed a strong correlation between *GPNMB* and *NRP-1* expression ($r = 0.58$) (Figure 2.2H). We next performed a meta-analysis to quantify and compare *GPNMB* and *NRP-1* co-expression across 5 additional breast cancer datasets. The correlation coefficient ranged from 0.31 to 0.58, indicating that *GPNMB* exhibits a moderate to strong correlation with *NRP-1* expression in each individual dataset (Figure 2.2I). By taking a weighted average of the analyzed datasets, we show a strong correlation between *GPNMB* and *NRP-1*, as evidenced by the large effect size of this measure ($r = 0.5$) (Figure 2.2I).

Neuropilin-1 mediates GPNMB-induced primary mammary tumor growth but is dispensable for its effect on breast cancer metastasis

We have previously shown that GPNMB expression promotes the tumorigenic and metastatic potential of breast cancer cells; however, the underlying mechanisms have not

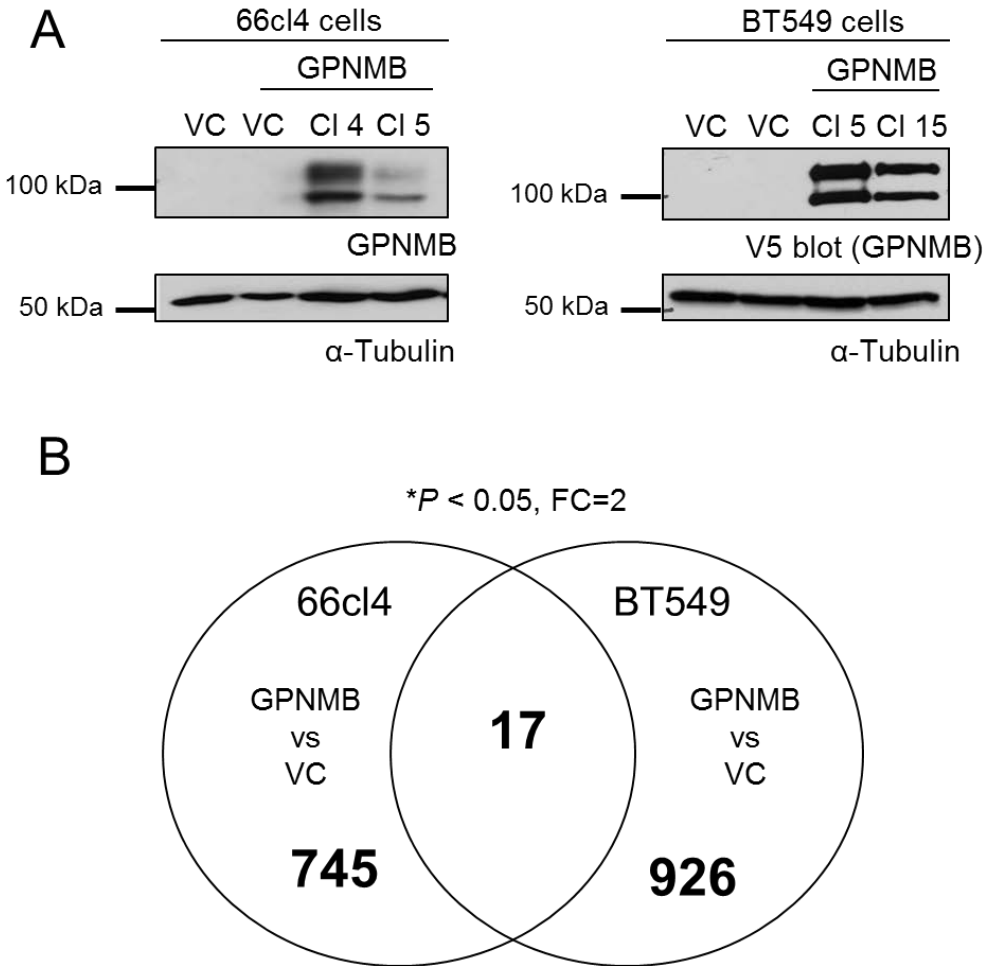

Figure 2.1 Gene expression changes induced by GPNMB in murine and breast cancer cells. (**A**) Immunoblot analysis showing GPNMB expression in clonal (Cl) cell lines generated in independent mouse (66cl4) and human (BT549) breast cancer models. An immunoblot for α-Tubulin is included as a loading control. (**B**) Whole genome 44K Agilent gene expression array analysis comparing two GPNMB expressing clonal cell lines and two VC cell lines for both 66cl4 and BT549 cell models. Differentially expressed genes in GPNMB-expressing versus VC cells were filtered on a fold change of 2 or greater and a P value of < 0.05. The intersection between differentially expressed genes in the 66cl4 and BT549 cell systems revealed 17 genes that were commonly regulated by GPNMB.

Gene Name	66cl4 Fold Change GPNMB vs VC	BT549 Fold Change GPNMB vs VC
Aryl hydrocarbon receptor (AHR)	2.7	2.8
Ankyrin 1 (ANK1)	2.4	5.5
Ca2+/Calmodulin-dep. kinase 4 (CAMK4)	3.2	2.1
CCAAT/enhancer binding protein, delta (CEBPD)	4.9	3
Cadherin 18 (CDH18)	2.5	2.7
Procollagen, type-IV, alpha 6 (COL4A6)	2.1	6.6
Clusterin (CLU)	6.9	2.5
Dedicator of cytokinesis 10 (DOCK10)	2	2
Ectonucleotide pyrophosphatase/phosphodisterase 5 (ENPP5)	6	27.6
Eph receptor A3 (EPHA3)	4	13.7
Neuropilin 1 (NRP1)	**2.1**	**9.6**
Pro-rich nuclear coactivator receptor1 (PNRC1)	2.1	2.1
Protease nexin-1 (Serpine2)	71.8	7.5
SH3-domain containing GEF (SGEF)	0.5	0.34
Monoglyceride lipase (MGLL)	0.15	0.45
Reticulon 1 (RTN1)	0.24	0.03
Neuroligin 1 (NLGN1)	0.26	0.18

Table 2.1 Identification of commonly regulated genes in response to GPNMB expression in the 66cl4 and BT549 breast cancer cell models. The list of 17 genes, with the corresponding fold change in GPNMB-expressing versus VC cells, which were similarly regulated in both the 66cl4 and BT549 cell models is shown. Neuropilin-1 was selected for further validation and analysis.

yet been fully elucidated [56]. To determine if NRP-1 plays a role in an independent model of GPNMB-driven primary tumor growth and metastasis, we established a stable knockdown of full-length NRP-1 in GPNMB-WT and vector control (VC) expressing NIC cells. Immunoblot analysis revealed that NRP-1 levels were effectively diminished in NIC cells harboring NRP-1 shRNAs (VC/NRP-1^{Low}, GPNMB-WT/NRP-1^{Low}) compared to NIC cells containing an empty vector control (VC/NRP-1^{High}, GPNMB-WT/NRP-1^{High}) (Figure 2.3A). To assess the contribution of NRP-1 to GPNMB-induced mammary tumor growth and metastasis, we injected VC/NRP-1^{High}, VC/NRP-1^{Low}, GPNMB-WT/NRP-1^{High} and GPNMB-WT/NRP-1^{Low} containing NIC cells into the mammary fat pads of immunocompromised mice. GPNMB induced a two-fold increase in the rate of primary tumor growth (Figure 2.3B), which recapitulated our earlier findings in the 66cl4 murine breast cancer model [56]. GPNMB-expressing tumors exhibited significantly increased Ki67 positivity (Supplementary Figure 2.3A), lower cleaved caspase-3 levels (Supplementary Figure 2.3B) and significantly higher CD31 positivity (Supplementary Figure 2.3C), indicating that GPNMB-expression enhances proliferation, diminishes apoptosis and promotes endothelial cell recruitment in this breast cancer model. The diminished apoptosis and enhanced vascular density are in agreement with our previously published results using 66cl4 breast cancer cells [56].

GPNMB/NRP-1^{Low} tumors displayed a reduced growth rate compared to their corresponding NRP-1^{High} counterparts during the later stages of tumor growth (Figure 2.3B). Since tumor intrinsic expression of NRP-1 is known to transduce survival signals downstream of VEGF and other growth factors [29], we hypothesized that the growth rate of GPNMB NRP-1^{Low} tumors was diminished due to a reduced ability to respond to cues

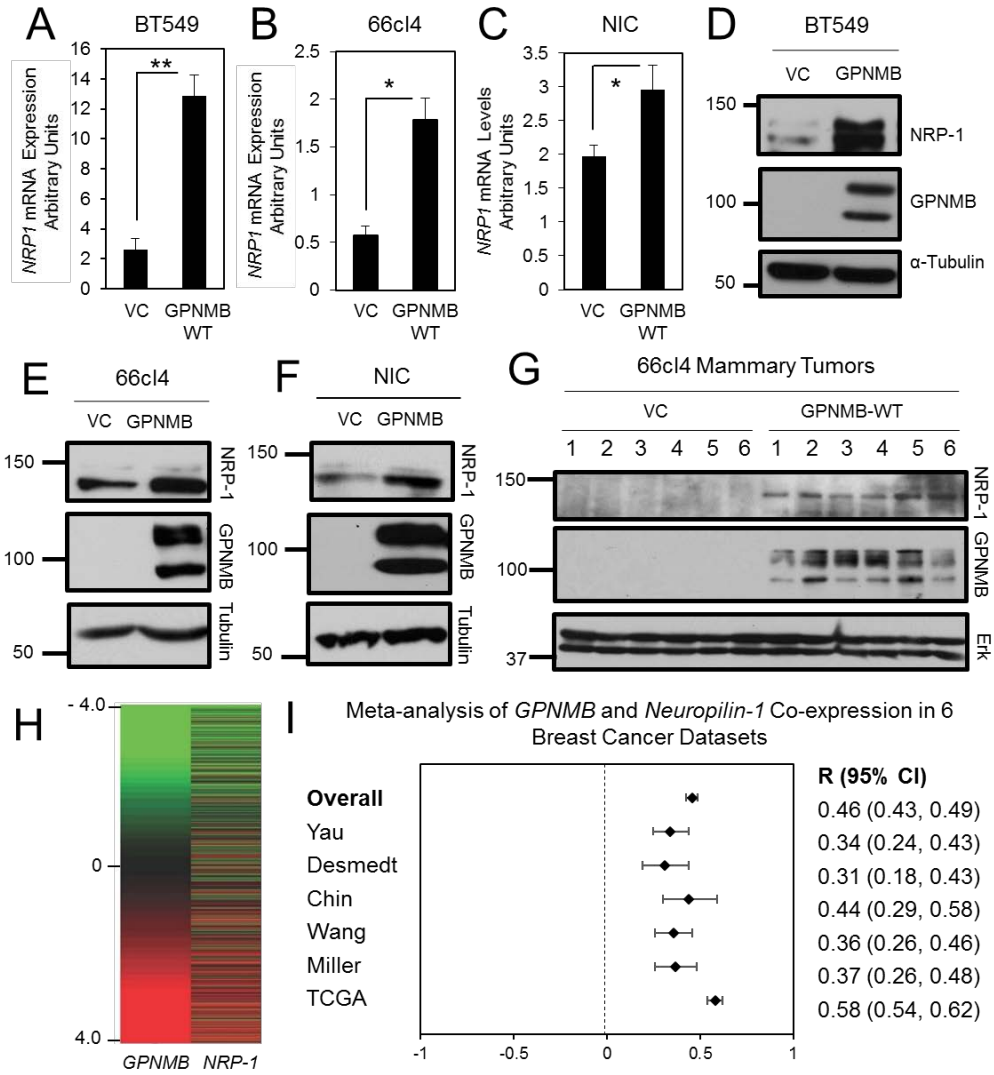

Figure 2.2 NRP-1 expression is elevated in GPNMB-expressing breast cancer cells and co-expressed with GPNMB in human breast cancers. RT-qPCR analysis of *NRP-1* mRNA expression was performed on total RNA extracted from BT549 (**A**), 66cl4 (**B**) and NIC (**C**) VC and GPNMB-WT expressing cells (n=3). Immunoblot analysis showing NRP-1 and GPNMB levels in VC and GPNMB-WT expressing BT549 (**D**), 66cl4 (**E**) and NIC (**F**) breast cancer cells and 66cl4 mammary tumors (**G**). (**H**) A heatmap comparing expression of *GPNMB* and *NRP-1* transcripts in the TCGA dataset is shown (n=1106). The scale for GPNMB mRNA levels is included. (**I**) A meta-analysis was performed to compare the correlation between *GPNMB* and *NRP-1* mRNA expression across 6 publicly available breast cancer datasets.

from VEGF-producing macrophages and endothelial cells recruited to the growing tumor during the angiogenic switch [8, 57, 58]. Infiltrating macrophages were indeed increased in GPNMB-expressing tumors, but infiltration was not decreased in response to NRP-1 knockdown (Figure 2.3C, Supplementary Figure 2.4). The extent of endothelial recruitment was found to closely parallel the macrophage infiltrate in the different NIC tumor lines. (Supplementary Figure 2.3C). We confirmed that VEGF protein levels were increased in both GPNMB/NRP-1^{High} and GPNMB/NRP-1^{Low} tumors (Figure 2.3D), which correlated with the level of VEGF-producing macrophages and endothelial cells in these tumors. Interestingly, GPNMB-WT/NRP-1^{Low} tumors exhibited lower proliferation (Supplementary Figure 2.3A) and apoptosis (Supplementary Figure 2.3B) when compared to GPNMB-WT/NRP-1^{High} tumors, suggesting that the differences in tumor growth may reflect altered VEGF responsiveness in GPNMB-WT/NRP-1^{Low} tumors. We observed no differences in the growth of VC/NRP-1^{Low} and VC/NRP-1^{High} tumors indicating that, in the NIC model, NRP-1 exerts cell-autonomous functions specifically downstream of GPNMB to promote tumor growth.

To investigate the role of NRP-1 in GPNMB-mediated metastasis, we analyzed lungs harvested from mice sacrificed at 4 weeks post-resection of the primary mammary tumors. These analyses revealed that spontaneous metastasis, quantified as percentage of lung area covered by metastasizing breast cancer cells, was increased 4.2-fold in mice injected with NIC GPNMB-WT/NRP-1^{High} breast cancer cells compared to mice injected with NIC VC/NRP-1^{High} cells (Figure 2.3E,F), which is in agreement with our previous findings with the 66cl4 breast cancer model [56]. Strikingly, NIC GPNMB-WT/NRP-1^{Low} breast cancer cells produced lung metastases that, on average, covered 34% of the lung tissue area, compared to 28% of lung tissue area for NIC GPNMB-WT/NRP-1^{High} cells (Figure 2.3E).

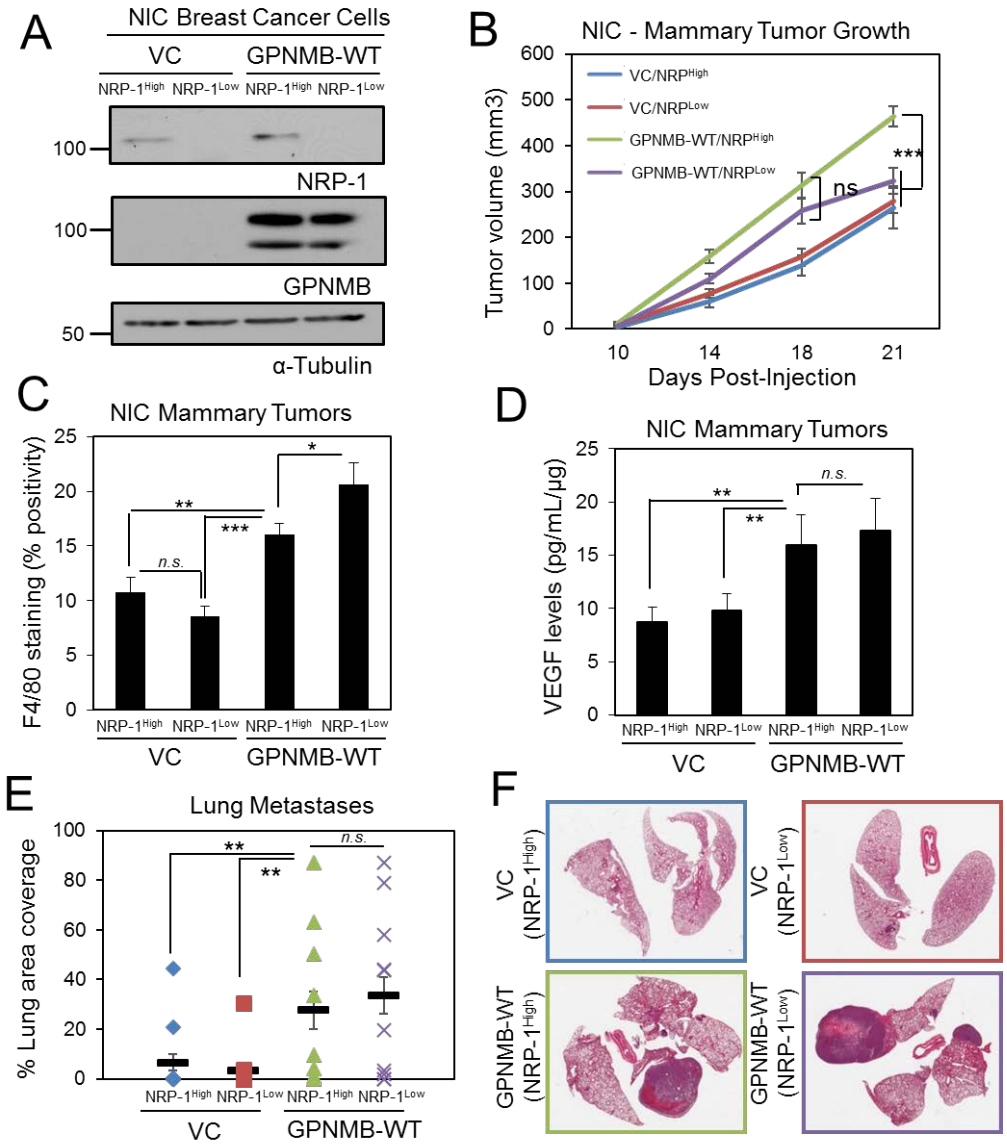

Figure 2.3 NRP-1 is required for GPNMB-induced primary tumor growth but is dispensable for GPNMB-mediated metastasis. (**A**) Immunoblot analysis of NRP-1 expression in NIC VC and GPNMB-WT cells reveals a substantial knockdown of NRP-1 with the targeting shRNA. (**B**) Indicated NIC cell populations were injected into the mammary fat pad of immunocompromised mice. Mammary tumor growth was followed by weekly caliper measurements (n=10). (**C**) Infiltrated macrophages were identified by F4/80 staining of tumor step sections. Staining was quantified by taking the ratio of positive to total pixels. (n= 10 images/sample for 5 samples) (**B**) VEGF protein levels were determined using ELISA from tumors grown *in vivo*. Results were normalized to total tumor lysate (n=10). (**E**) Lung metastatic burden, which represents the % lung area covered by tumor cells, is displayed (average of 4 step sections, n=10 samples). (**F**) Representative images of the lungs are included.

These data argue that the GPNMB-dependent increase in lung metastatic burden was unaffected by diminished NRP-1 levels. Taken together, these data implicate NRP-1 in the proliferation and survival of GPNMB-expressing tumors and suggests that GPNMB promotes metastasis of breast cancer cells through NRP-1-independent mechanisms.

GPNMB potentiates signaling downstream of VEGF in an NRP-1-dependent manner

To investigate the mechanisms through which NRP-1 promotes pro-survival signals downstream of GPNMB, we utilized human basal breast cancer cells as they provide a representative model of a breast cancer sub-type that most frequently expresses GPNMB [4]. Expression of NRP-1 was diminished in BT549 cells overexpressing either GPNMB-WT or an empty vector control (VC) using one of two NRP-1 targeting shRNA constructs (sh#1, sh#2). The sh#1 achieved a better reduction of NRP-1 levels in BT549 VC and WT cells and was chosen for subsequent analysis (Supplementary Figure 2.5). NRP-1 enhances the binding affinity of the VEGF receptor (VEGFR2) to the VEGF ligand, thereby potentiating VEGFR2-mediated signaling pathways [16]. In cancer cells, NRP-1 expression promotes survival and proliferation by augmenting VEGF signaling leading to increased activity of the PI3K [35] and MAPK signaling [34] pathways. We stimulated vector control or GPNMB-expressing BT549 breast cancer cells, which have elevated NRP-1 expression (Figure 2.2A, D), with exogenous VEGF to assess the activity of VEGF signaling in these cells. BT549 cells displayed a temporal response to VEGF stimulation as demonstrated by increased phosphorylation of VEGFR2 (Figure 2.4). ERK phosphorylation was increased over time and reached maximal levels after 15 minutes of VEGF stimulation, indicating that VEGF signals are transduced through the MAPK

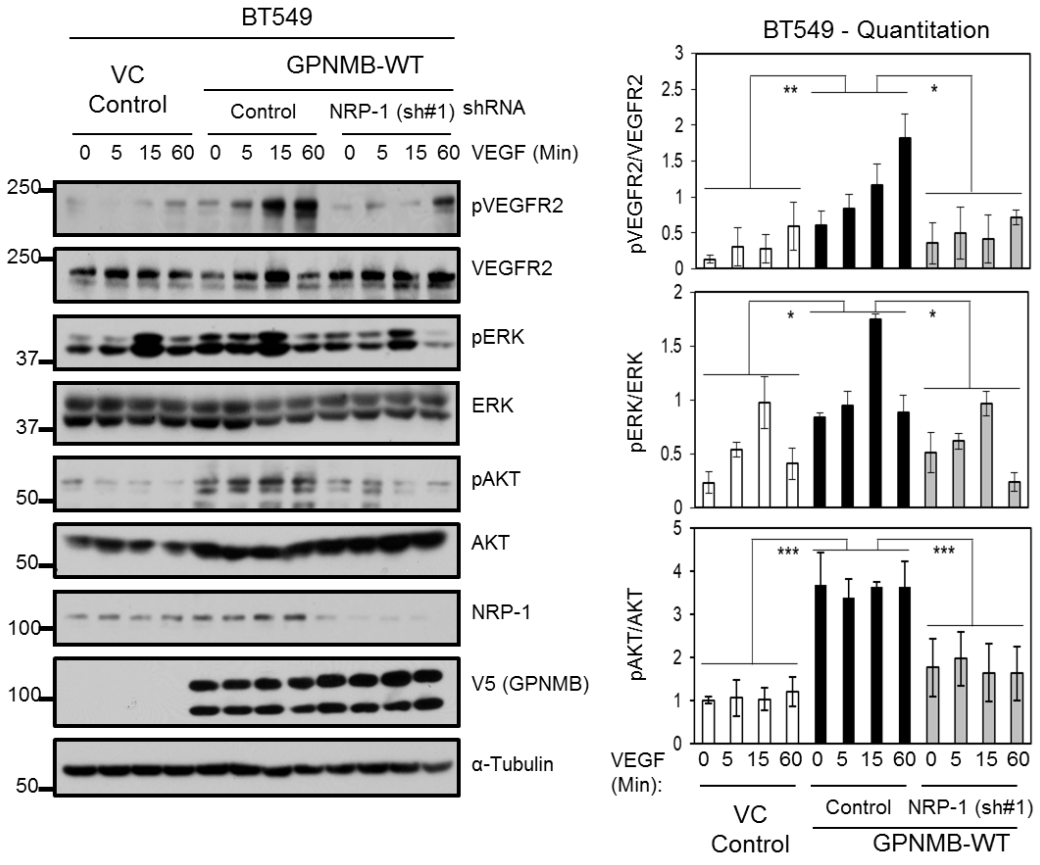

Figure 2.4 GPNMB potentiates signaling in response to VEGF stimulation in an NRP-1-dependent manner. BT549 VC/NRP-1^{High}, GPNMB-WT/NRP-1^{High} and GPNMB-WT/NRP-1^{Low} cells were stimulated with 25ng/mL VEGF for the indicated times. Downstream signaling pathways were examined through immunoblot analysis, by probing for pVEGFR, VEGFR, pERK, ERK, pAKT, AKT, NRP-1 and V5-tagged GPNMB. Band intensity was quantified and the average ratios of phosphorylated to total protein levels are displayed (n=3).

pathway in our system (Figure 2.4). Interestingly, GPNMB expression was found to enhance pVEGFR2 and pERK levels in response to VEGF and this effect was lost in the absence of NRP-1. Treatment with Semaxanib, an inhibitor of VEGFR2, dampened the response of GPNMB-expressing cells to VEGF signaling, which confirmed that VEGF-induced signaling was specifically orchestrated through the VEGFR2 axis. (Figure 2.5). Although unresponsive to VEGF stimulation, baseline levels of pAKT were also found to be elevated in BT549 GPNMB-WT/NRP-1^{High} cells. AKT phosphorylation in BT549 GPNMB-WT/NRP-1^{Low} cells was comparable to control levels (BT549 VC) indicating that NRP-1 is responsible for maintaining sustained activation of AKT in GPNMB-expressing cells (Figure 2.4A). These results support a role for tumor-intrinsic GPNMB in enhancing pro-survival VEGF-dependent ERK signaling and VEGF-independent AKT signaling, during mammary tumor growth, through a mechanism that involves elevated GPNMB-mediated increases in NRP-1 expression.

GPNMB increases NRP-1 expression through the PI3K/AKT pathway

Previous reports have demonstrated that both the PI3K and the MAPK pathways can modulate NRP-1 expression in different contexts [37, 38]. Therefore, we treated VC- and GPNMB-expressing cells with AKT and ERK inhibitors to determine if enhanced activity of either of these pathways was responsible for the GPNMB-mediated increase in NRP-1 expression. We confirmed that inhibition of AKT (Perifosine), but not ERK (SCH772984), was sufficient to abolish the increase in NRP-1 expression observed in GPNMB-expressing BT549 cells. (Figure 2.6A, B). Given existing data showing that NRP-1 is a promiscuous co-receptor that can interact with a variety of cell-surface proteins, we wanted to determine if GPNMB interacts with NRP-1 to increase its stability. Using an

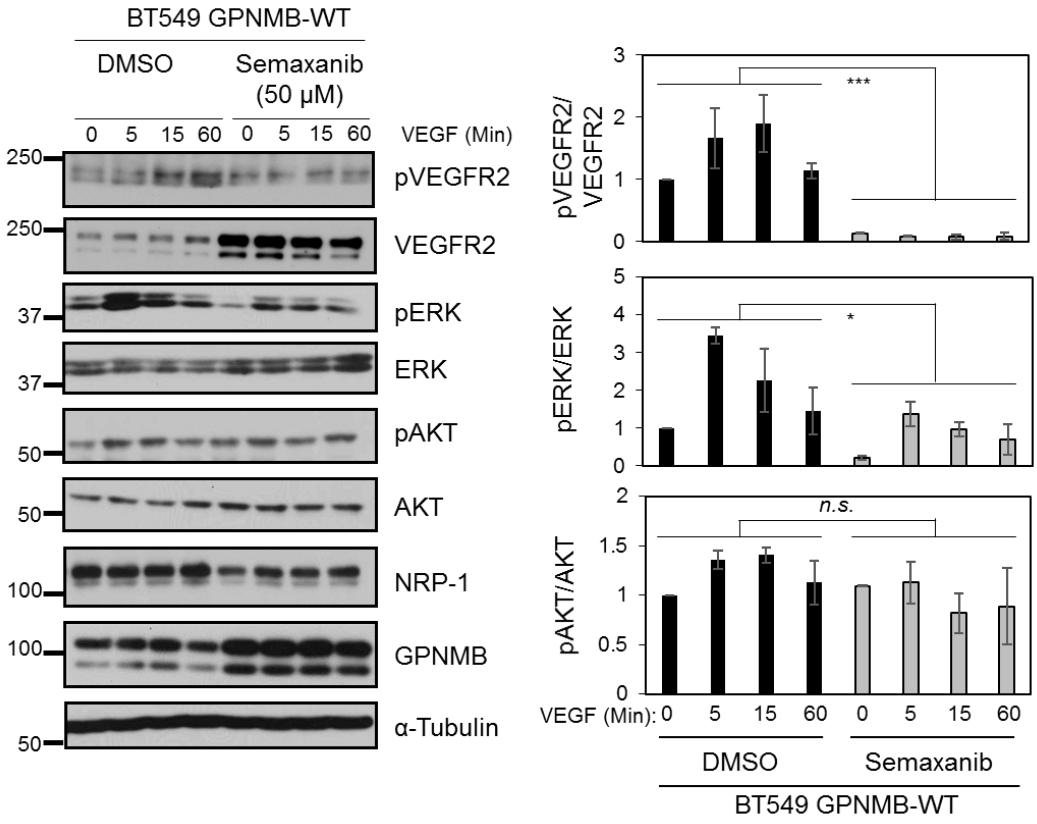

Figure 2.5 GPNMB potentiates VEGF signaling specifically through the VEGFR2 axis. To inhibit VEGFR2 activity, BT549 GPNMB-WT cells were treated with 50μM Semaxanib or DMSO vehicle. After 24 hours of inhibitor or control treatment, VEGF stimulation and activation of downstream signaling pathways was assessed as above. Band intensity was quantified and the ratios of phosphorylated to total protein were calculated and normalized to the first lane (DMSO, no VEGF).

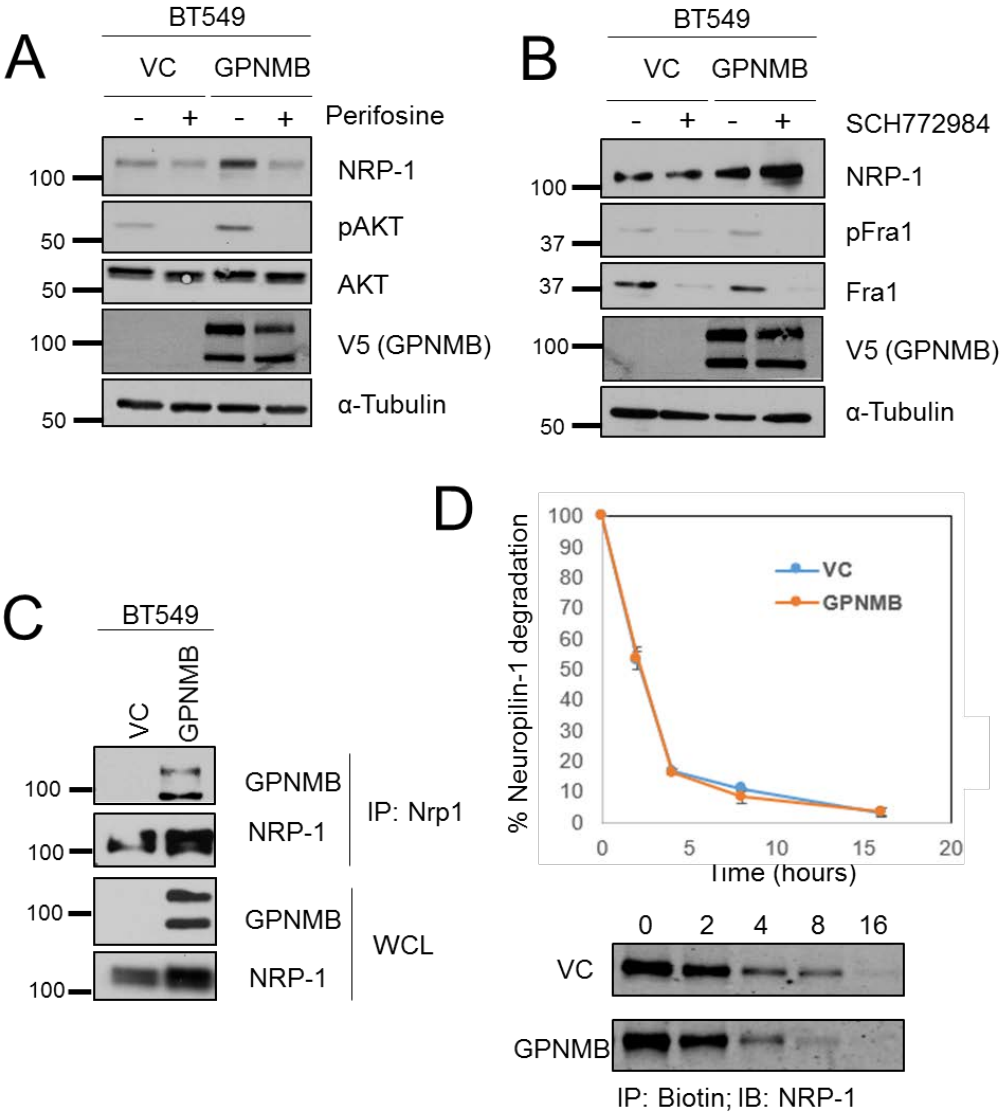

Figure 2.6 Mechanisms of Nrp1 regulation by GPNMB BT549 cells expressing VC or GPNMB were treated with 40μM Perifosine (**A**) or 1μM SCH772984 (**B**) for 24 hours to inhibit AKT or ERK, respectively. (**A**) Immunoblot analyses of NRP-1, pAKT, AKT and GPNMB (V5-tagged) in BT549 cells treated with Perifosine (AKT inhibitor). (**B**) Immunoblot analyses of NRP-1, pFRA-1, FRA-1 (an ERK substrate) and GPNMB (V5-tagged) in BT549 cells treated with SCH772984 (ERK inhibitor). α-Tubulin was used as a loading control. (**C**) NRP-1 was immunoprecipitated from BT549 cells and complex formation was examined by probing the bound fraction for GPNMB and NRP-1. An immunoblot analysis showing levels of GPNMB and NRP-1 in whole cell lysates is included. (**D**) Cell surface proteins were labelled with biotin and pulled down using streptavidin beads at the indicated timepoints. The amount of NRP-1 remaining in the cell was determined by immunoblotting the biotinylated cell fraction. Immunoblots were quantified and remaining NRP-1 is expressed as a percentage of the signal detected at time 0. (n = 3)

immunoprecipitation assay, we show that GPNMB can be found in a complex with NRP-1 (Figure 2.6C). To determine if GPNMB can influence protein stability of NRP-1, we examined the rate of degradation of NRP-1 in GPNMB-WT- and VC-expressing breast cancer cells. We found that NRP-1 exhibited a similar degradation pattern in both contexts (Figure 2.6D), indicating that GPNMB-mediated increases in NRP-1 expression occur at the transcriptional level (Figure 2.1, Figure 2.2 A-C).

2.5 Discussion

We have previously demonstrated that GPNMB promotes a malignant phenotype in breast cancer. GPNMB expression in breast cancer cells is required for motility and invasion, and this is accompanied by matrix metalloproteinase 3 (MMP3) upregulation [55]. In xenograft and syngeneic models of breast cancer, GPNMB increases primary tumor growth and metastasis to lung and bone [55, 56]. Herein, we describe a novel role for GPNMB in increasing expression of NRP-1. We show that NRP-1 plays a crucial role in GPNMB-driven primary tumor growth by potentiating tumor intrinsic proliferative and pro-survival signals in response to VEGF. We demonstrate that the GPNMB-driven increases in NRP-1 expression are mediated through enhanced PI3K/AKT pathway signaling. Furthermore, our results establish NRP-1 as a novel GPNMB-interacting partner. Interestingly, GPNMB expression was strongly correlated to Neuropilin-1 expression in a number of human breast cancer datasets, which confers clinical relevance to our findings.

GPNMB-expressing breast cancers are characterized by increased VEGF levels and endothelial cell density [56]. We have confirmed these results in an independent model and have shown that GPNMB can recruit VEGF-producing macrophages, thus partly

attributing the increase in VEGF production to a stromal source. Our current data suggests that GPNMB causes the upregulation of NRP-1 expression by increasing PI3K pathway signaling, which in turn enhances tumor cell responsiveness to growth factors such as VEGF present in the tumor microenvironment. Indeed, GPNMB-WT/NRP^{Low} tumor cells are characterized by decreased proliferation, increased apoptosis and a dampened ability to relay signaling downstream of VEGF, which is in line with studies demonstrating that NRP-1 is critical in promoting VEGF-induced proliferative and survival signals, and enhancing binding of VEGF to VEGFR2 [16, 34, 35, 59, 60]. Additionally, these results mirror the cytoprotective effect of GPNMB observed in ALS-affected tissues, where GPNMB can ameliorate ALS-induced neuronal degradation by upregulating PI3K and MEK/ERK pathways [61, 62]. GPNMB-WT/NRP^{High} and GPNMB-WT/NRP^{Low} tumors display similar growth rates in the early timepoints post-injection (Figure 2.3B), suggesting that NRP-1 plays a role in later stages of tumor growth. It is conceivable that a certain VEGF threshold is required to trigger the pro-tumorigenic effects of NRP-1. We postulate that that tumor-expressed NRP-1 is engaged following the angiogenic switch, which results from the accumulation of VEGF that is produced by the stromal infiltrate.

Interestingly, the growth of NIC VC tumors is unaffected by NRP-1 knockdown (Figure 2.3A). Although many studies have shown that NRP-1 can enhance primary tumor growth by increasing proliferation and survival, other reports indicate that NRP-1 can mediate a tumor suppressive function as a co-receptor for class 3 semaphorins. SEMA3A inhibited tumor growth of MDA-MB-231 and MCF-7 breast cancer cells that express the NRP-1 receptor but had no effect on the growth of MDA-MB-435 or MDA-MB-468 cells, which lack NRP-1 expression [63]. Additionally, SEMA3A suppresses the tumor growth and metastasis of 4T1 breast cancer cells through an NRP-1-dependent mechanism [64].

Furthermore, SEMA3A can inhibit the chemotaxis of breast cancer cells and this effect is progressively dampened in the presence of increasing VEGF concentrations [65]. Taken together, these studies highlight the context-dependent role of NRP-1 in tumor progression, which is influenced by the availability of VEGF and SEMA3A in the tumor microenvironment. In our system, the elevated VEGF production and VEGF responsiveness confer a growth advantage to GPNMB-expressing tumors. We hypothesize that this increased reliance on the pro-tumorigenic VEGF/NRP-1 axis would make NIC GPNMB-WT tumors more sensitive to NRP-1 inhibition than their VC-expressing counterparts. In a similar vein, it is possible that GPNMB confers a growth advantage during the early stages of breast cancer progression through NRP-1 independent pathways, however, we hypothesize that there is an increased reliance on NRP-1-mediated VEGF signaling as the tumor continues to grow and VEGF production accumulates (as illustrated in Figure 2.3A).

Our results indicate that NRP-1 is a novel GPNMB interacting partner. Although GPNMB does not affect protein stability of NRP-1, it would be interesting to see if NRP-1 can alter GPNMB dynamics. NRP-1 has been shown to associate with and promote the internalization of various membrane proteins and thereby enhance downstream effects [66]. Upon ligand binding, ligand/receptor complexes are typically internalized to the cytosol where they promote activation of signaling adaptors. NRP-1 can interact with EGFR on cancer cells to increase receptor clustering, internalization and signaling downstream of its ligands, EGF and TGFα [49]. The activation of EGFR and its downstream AKT pathway is severely blunted by NRP-1 blocking antibodies or NRP-1 knockdown [49]. Similarly, in glioblastoma, NRP-1 drives VEGFR2 internalization following VEGF stimulation and promotes VEGFR2 signaling from intracellular vesicles [36]. NRP-1 can also increase the

adhesion of endothelial cells to fibronectin by enhancing internalization of the α5β1 fibronectin receptor [67]. Given the numerous studies that implicate NRP-1 in the internalization of its interacting partners, it would be worthwhile to examine if NRP-1 can modulate GPNMB internalization and recycling. This line of investigation could yield insights into pathways that regulate GPNMB cell surface availability and would therefore have important implications for therapy. Specifically, mechanisms that increase GPNMB cell surface expression would augment the efficacy of CDX-011, which targets the extracellular domain of GPNMB.

Increased availability of cell surface GPNMB would also augment GPNMB shedding and associated paracrine effects. Notably, GPNMB is associated with the tumorigenic M2 macrophage phenotype and promotes M2 polarization of M0 macrophages by activating the IL4-STAT6 pathway [68]. Soluble GPNMB can also indirectly enhance M2 macrophage polarization by promoting recruitment of a mesenchymal stem cell (MSC) infiltrate, which in turn directs macrophage polarization to the M2 phenotype [69, 70]. We hypothesize that the shed GPNMB ECD secreted by breast cancer cells can recruit a stromal infiltrate to the growing tumor and promote acquisition of the M2 macrophage phenotype, which is important for tumorigenesis [71]. Accordingly, a recent study has demonstrated that GPNMB, IL13 and IL34 comprise a cancer stem cell secretome in cholangiocarcinoma which shapes the tumor-initiating niche and promotes macrophage malignancy [72]. It is also possible that cell surface GPNMB could direct macrophage polarization through juxtacrine interactions with the CD44 receptor expressed on macrophages, which is known to interact with GPNMB and to regulate the M2-like TAM phenotype [73, 74]. Since NRP-1 plays a critical role in the internalization of its interacting partners, it is possible that GPNMB internalization is impaired in NRP-1^{Low} cells, leading to increased GPNMB cell

surface availability. In line with this hypothesis, we show that NRP-1 knockdown significantly increases macrophage recruitment by GPNMB-expressing cells (Figure 2.3C).

The interaction between GPNMB and NRP-1 also raises the possibility that GPNMB could influence a number of pivotal signaling pathways. It is possible that GPNMB associated with NRP-1 can be drawn into macromolecular complexes with other NRP-1 interacting partners such as integrin αβ51, VEGFR2 or EGFR, thereby enhancing receptor-ligand affinity and potentiating downstream signaling. Our data demonstrating that GPNMB can enhance VEGF-stimulated ERK activation lends credence to this hypothesis. Given that NRP-1 and GPNMB are both known to interact with EGFR, it would be interesting to see if GPNMB can also potentiate signaling pathways downstream of EGF stimulation [49, 75]. Taken together, our results outline a novel role for NRP-1 in GPNMB-mediated tumorigenesis and add to the growing body of literature supporting a role for tumor-derived NRP-1 in cancer progression.

2.6 Materials and Methods

Cell Culture

The BT549 human breast cancer cell line was obtained from the American Type Culture Collection. All BT549-derived pooled cell populations were maintained in RPMI supplemented with 10% FBS. 66cl4 murine mammary carcinoma cells were kindly provided by Dr. Fred Miller (Barbara Ann Karmanos Cancer Institute, Detroit, IL) and were grown in DMEM supplemented with 10% FBS, 10 mM HEPES, 1.5 g/L sodium bicarbonate and 1 mM sodium pyruvate. The NIC cell line was established from primary tumor explants derived from the MMTV/NIC transgenic mouse model [76]. NIC cells were

grown in DMEM supplemented with 5% FBS, 35 ug/mL bovine pituitary extract, 5 ug/mL insulin, 1 ug/mL hydrocortisone and 5 ng/mL hEGF. The MDA-MB-436 human breast cancer cell line was obtained from the ATCC and was grown in Leibovitz's L-15 media supplemented with 10% fetal bovine serum, 10 μg/mL insulin and 16 μg/mL glutathione. All cell lines were maintained in complete media unless otherwise indicated, and fungizone and gentamycin were added to all media to minimize contamination risk. For VEGF stimulation studies, cells were serum-starved for 24 hours and subsequently stimulated for the indicated times with 25 ng/mL VEGF (Peprotech).

Virus production was achieved using the 293VSV packaging cell line (Clontech), which was maintained in 10% FBS and 5 ng/mL tetracycline. Retroviral vectors and pVSVG helper plasmids were transfected into the 293VSV cells using effectene (Qiagen, Cat # 3901427). Subsequently, starting from the second day post-transfection, virus was harvested for 5 days from the 293VSVs. Target cells were infected by a 24-hour incubation with filtered virus-containing media and equal volumes of their respective media. Polybrene (8 ug/uL) was used to augment infection efficiency.

For inhibitor studies, cells were treated for 24 hours in complete media with 50μM SU5416 (Selleckchem), 40μM Perifosine (Sigma) or 1μM SCH772984 (Selleckchem), to inhibit activity of VEGFR2, AKT or ERK, respectively.

Knockdown studies

Following manufacturer instructions, the shRNA sequences (Open BioSystems) (Supplementary Table 2.1) were PCR amplified, digested with EcoRI/XhoI, and ligated into the LMP vector system (Dickens et al., 2005). A hygromycin resistance cassette was shuttled into the parental LMP vector as an *Age*I/*Afl*III restriction fragment to replace

original puromycin selection marker. The shRNA constructs were introduced via retroviral infection into the BT549 or NIC breast cancer cells and maintained under 100 ug/mL (BT549) or 200 ug/mL (NIC) hygromycin selection.

Transient knockdown of GPNMB in MDA-MB-436 cells was achieved by 48-hour transfection (Lipofectamine 3000, Invitrogen) using indicated concentrations of an ONTARGETplus SMARTpool of 4 GPNMB-targeted siRNAs (Dharmacon). An ONTARGETplus pool of 4 scrambled siRNAs was used as a transfection control. The transfection was performed according to the manufacturer's instructions.

Immunoblotting

Membranes were prepared and processed as previously described[43] using the primary antibodies listed in Supplementary Table 2.2. Where applicable, immunoblots were quantified using the Plot Lanes function on ImageJ and the average from three representative experiments was taken.

Primary tumor growth, spontaneous metastasis assays and analysis of tumor tissue

For *in vivo* studies, $5x10^5$ breast cancer cells were resuspended in a 50/50 mixture of 1X PBS:matrigel and injected into the mammary fat pads of athymic mice. Tumor volumes were determined by caliper measurement and tumor volume was calculated according to the following formula: $\pi LW^2/6$ where L refers to the length and W to the width of the tumor. Mammary tumors were resected at a volume of 500 mm^3, and tumor tissue was harvested for immunoblot or immunohistochemical analysis. Animals were sacrificed 5 weeks post-resection and lungs were harvested, fixed in 4% paraformaldehyde and

embedded in paraffin. Lung metastatic burden was quantified from 4 Hematoxylin & Eosin (H&E) stained step sections (80 μm between each step section) using Aperio Imagescope software. Metastatic tissue was delineated and quantified from 4 step sections and expressed as a percentage of total lung area. Mice were housed in facilities managed by the McGill University Animal Resources Centre and all animal experiments were conducted under a McGill University-approved Animal Use Protocol in accordance with guidelines established by the Canadian Council on Animal Care.

To examine VEGF levels in NIC populations, resected tumors were flash frozen in liquid nitrogen, ground up with a mortar and pestle, and resuspended in TNE lysis buffer. VEGF protein was quantified using a commercially available ELISA kit (R&D systems) and the associated manufacturer's protocol. Quantification was performed VEGF values were normalized to total tumor lysate concentrations.

To quantify F4/80 staining, the positive pixel count was determined using Imagescope software (Aperio). Ten 20x images were analyzed for every stained tumor sample and F4/80 positive pixels were expressed as a percentage of the total pixels per field.

RNA extraction, cDNA synthesis and quantitative Real-Time PCR

Triplicate RNA samples were extracted from BT549, 66cl4 and NIC cell lines at ~50% confluence using RNeasy Mini Kits (Qiagen) and quantified using a spectrophotometer (Nanodrop ND-1000). Total RNA (1μg per sample) was used to generate cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Oligonucleotide primers were designed using Invitrogen OligoPerfect software and pre-designed primers were identified in Primer Bank (Harvard University) or RT-PCR Design (Roche

Diagnostics). All primers were diluted to a concentration of 100μM (Primer sequences can be found in Supplementary Table 2). RT-qPCR reactions were performed on diluted cDNA (1:20) using Power SYBR Green Master Mix (Applied Biosystems) and 7500 Real Time PCR System (Applied Biosystems). Reactions were performed in triplicate and *GAPDH* or *β-actin* primers were used as a control for mouse and human genes, respectively. Data is represented as the mean of the fold change of the three independent sets of cDNA calculated according to the following formula:

$$\frac{\text{Efficiency of target primers} + 1^{\,(\text{average value of target primers} - \text{average of corresponding target wells})}}{\text{Efficiency of control primers} + 1^{\,(\text{average value of control primers} - \text{average of corresponding control wells})}}$$

Microarray analysis

GPNMB-expressing clones, along with empty vector controls, were established in human BT549 and mouse 66cl4 breast cancer cell lines. Total RNA from two independent GPNMB-expressing clones, along with two empty vector clones, was isolated from both the BT549 and 66cl4 model systems. Total RNA was amplified, labeled and hybridized to whole genome 44K Agilent gene expression arrays as previously described [55]. Data were normalized based on the Lowess normalization included in the Genespring software (Agilent technologies). A parametric test was used to compare the GPNMB expressing clones and the parental cell lines ($p<0.05$). Genes that were differentially expressed between GPNMB-expressing cells and VC cells, which exhibited a fold change greater than 2, were chosen for further analysis. Both the 66cl4 and BT549 breast cancer cell models were compared to identify genes that are commonly regulated by overexpression of mouse and human GPNMB.

Immunohistochemistry analysis

Tissue samples were fixed, processed and stained with Ki67, cleaved caspase-3 or CD31 antibodies as previously described [77].

Statistical analyses

Significance of the data was assessed using a two-tailed Student's T-test and variance was determined using an online statistics program (Vassar Stats). Indicated annotations correspond to the following P values: * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$. Results are displayed as an average +/- standard error.

Correlations between expression of GPNMB and NRP-1 were studied in datasets available on the UCSC Cancer Genomics Browser (https://genome-cancer.ucsc.edu/) [78-82]. Expression data was ordered according to GPNMB expression and the Pearson correlation coefficients for individual datasets were calculated using MedCalc 13. A meta-analysis under the fixed effects model was subsequently carried out according to the Hedges-Olkin method described on their website: http://www.medcalc.org/manual/meta-analysis-correlation.php.

2.7 Acknowledgements

We would like to thank members of the Siegel laboratory for their thoughtful discussions regarding the current work and are also grateful to Dr. Josie Ursini-Siegel for her critical reading of the manuscript. We acknowledge the Goodman Cancer Research Centre histology core facility (McGill University) for routine histological services. G.M acknowledges studentship support from the Canadian Institutes for Health Research

(CIHR). P.M.S. was supported by the Fonds de recherche en santé du Québec (FRSQ) research scholar program (Junior 2) and is currently a McGill University William Dawson Scholar. This research was supported solely by a grant from the CIHR (MOP-119401).

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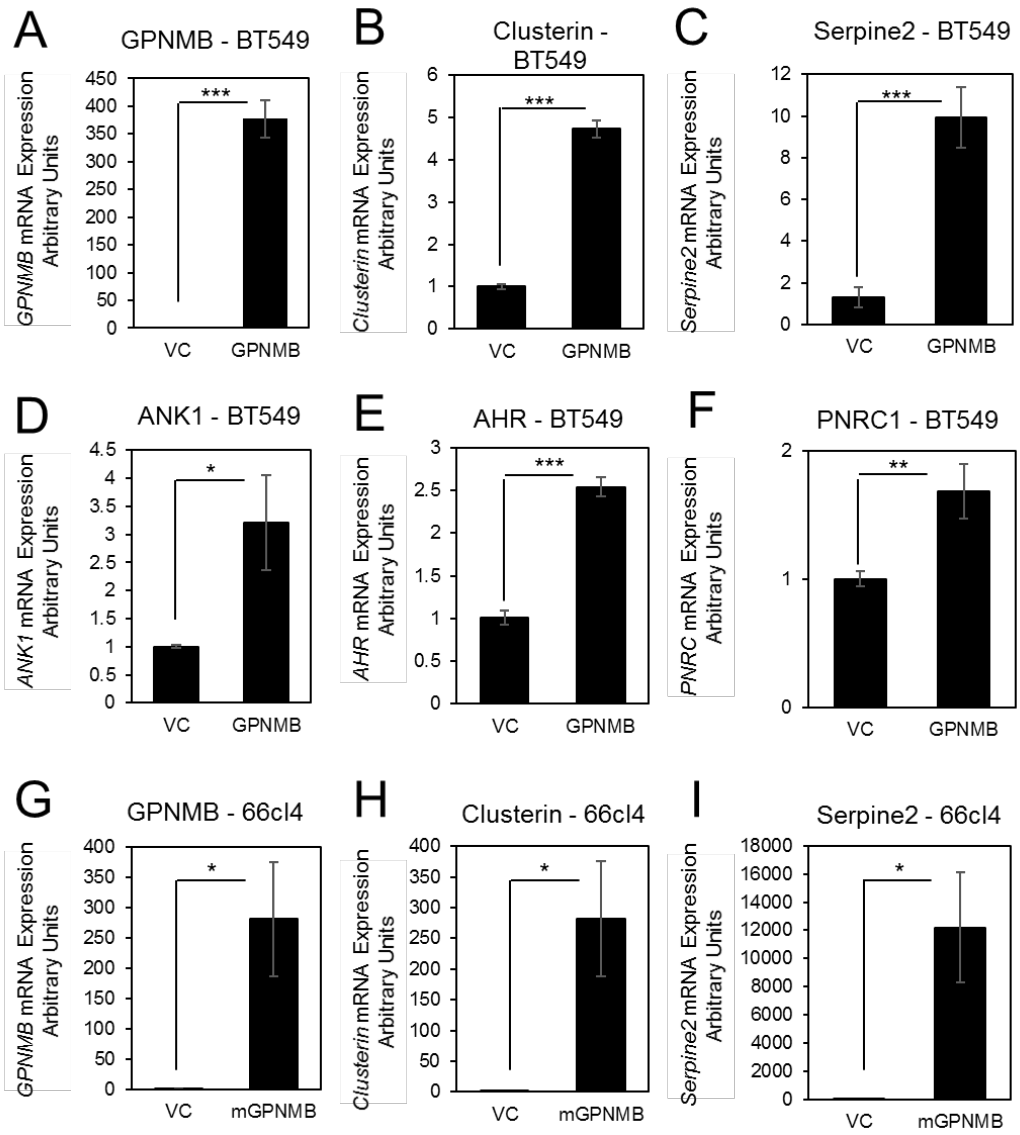
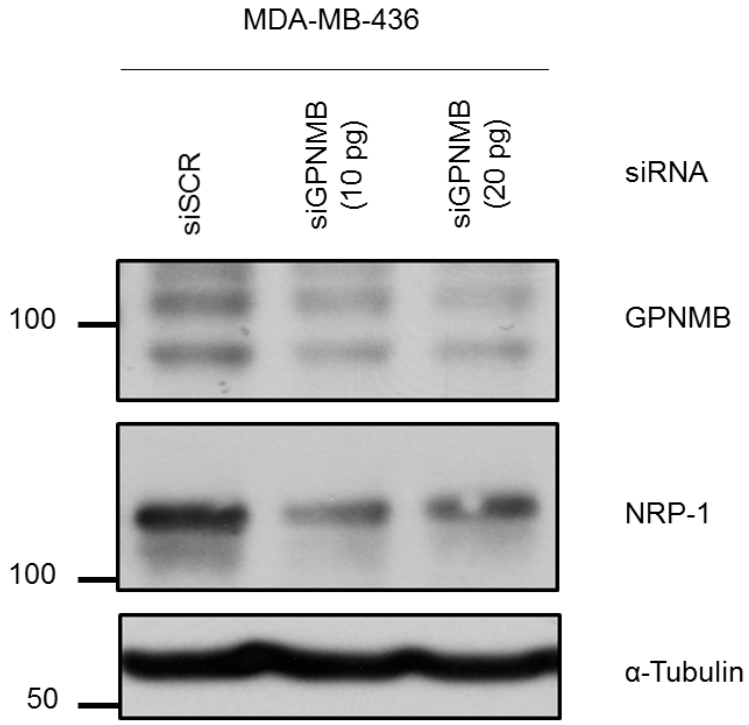
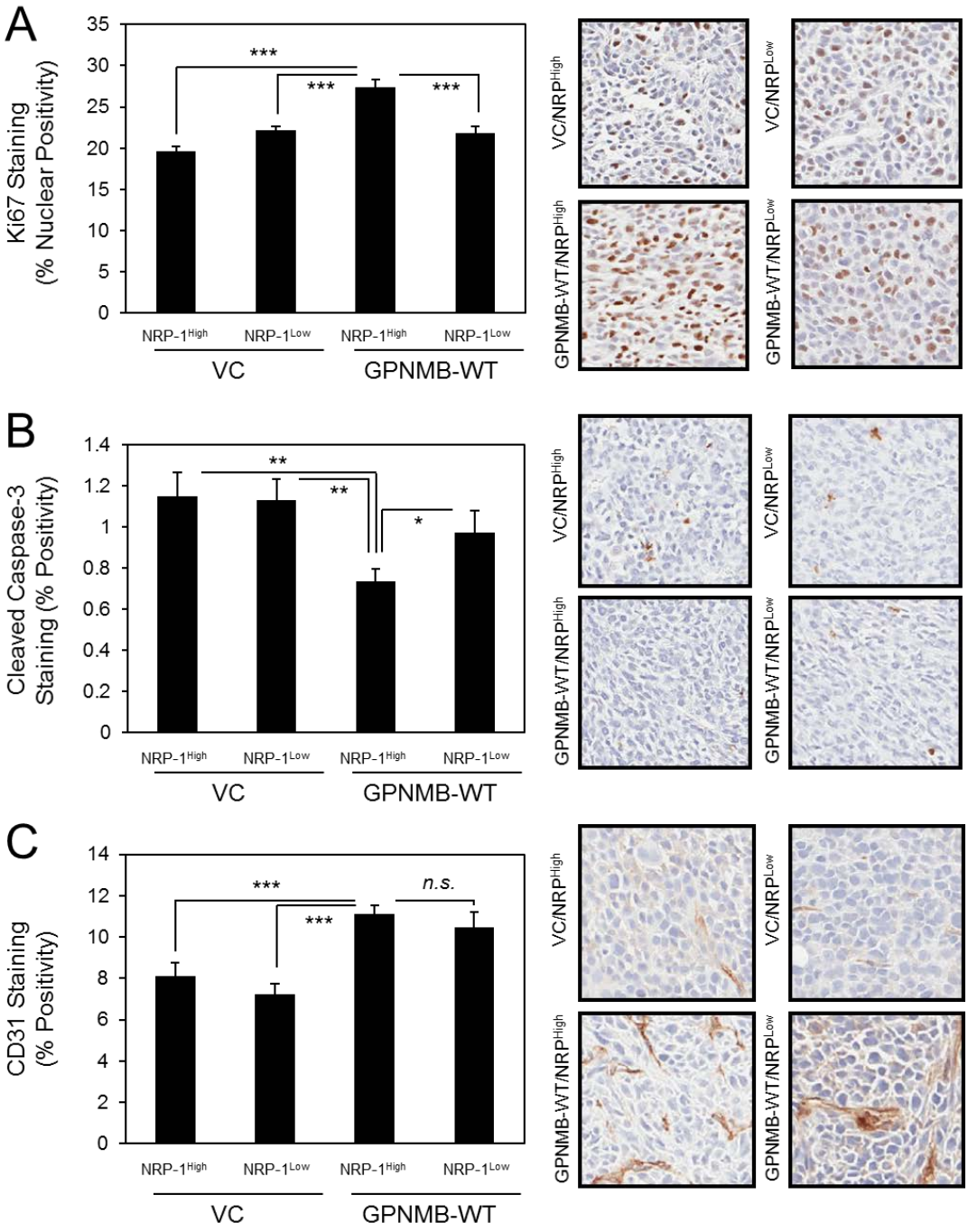


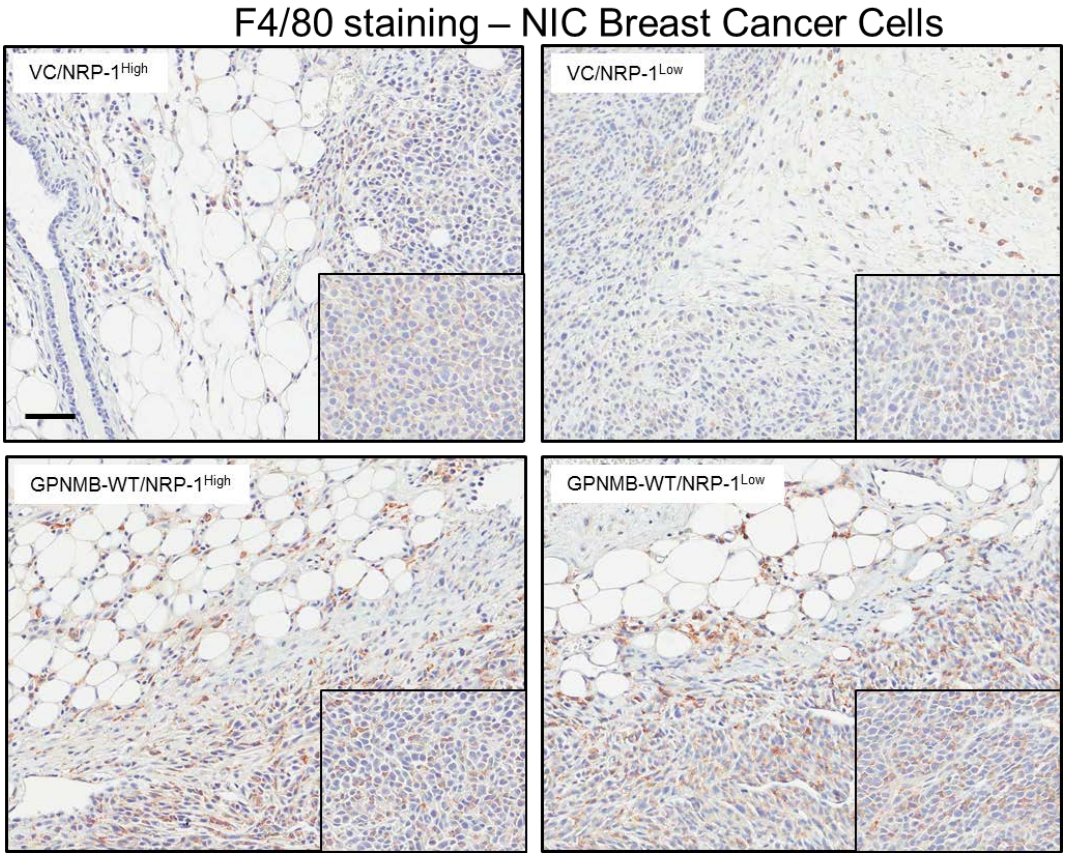
Supplementary Figure 2.1. GPNMB overexpression in BT549 and 66cl4 breast cancer cells results in numerous transcriptional changes. Commonly regulated genes identified by microarray analysis were validated by RT-qPCR analysis. Transcript levels of *CLU (Clusterin)* (**b**), *Serpine2* (**c**), *ANK1* (**d**), *AHR* (**e**) and *PNRC1* (**f**) were increased in GPNMB-expressing BT549 cells compared to cells containing an empty vector. *CLU (Clusterin)* (**h**) and *Serpine2* (**i**) were also found to be increased at the mRNA level in response to GPNMB overexpression in 66cl4 cells. RT-qPCR analyses displaying elevated *GPNMB* mRNA levels in BT549 (**a**) and 66cl4 (**g**) cells are shown. Average transcript levels in GPNMB-expressing cells represent the combined result of 4 independent BT549 clones or 2 independent 66cl4 clones displaying elevated GPNMB expression. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (n = 3)



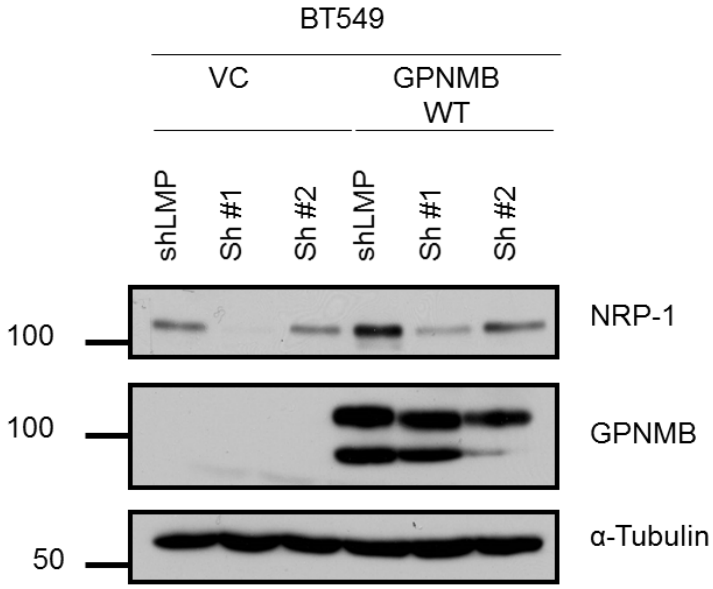
Supplementary Figure 2.2 Neuropilin-1 expression is decreased in response to GPNMB knockdown in MDA-MB-436 breast cancer cells. GPNMB expression was transiently diminished in the MDA-MB-436 cell line using 2 different siRNA concentrations. Control cells were transfected with a pool of scrambled non-targeting siRNAs. After 48 hours, cells were lysed, processed and probed for NRP-1 and GPNMB. A-Tubulin was used as a loading control.



Supplementary Figure 2.3 Immunohistochemistry analysis of proliferation, apoptosis and angiogenesis markers in tumors derived from NIC cell lines. Primary tissue was harvested from NIC VC/NRP-1^{High}, VC/NRP-1^{Low}, GPNMB-WT/NRP-1^{High} and GPNMB-WT/NRP-1^{Low} tumors and proliferation (**a**), apoptosis (**b**) and endothelial cell recruitment (**c**) was assessed by immunohistochemistry. Results represent the averages of 10 images/sample taken from 5 independent primary tumor tissue samples. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).



Supplementary Figure 2.4 Immunohistochemistry analysis of macrophage infiltration in tumors derived from NIC cell lines. Primary tissue was harvested from NIC VC/NRP-1^{High}, VC/NRP-1^{Low}, GPNMB-WT/NRP-1^{High} and GPNMB-WT/NRP-1^{Low} tumors and proliferation (**a**), apoptosis (**b**) and endothelial cell recruitment (**c**) was assessed by immunohistochemistry. Results represent the averages of 10 images/sample taken from 5 independent primary tumor tissue samples. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).



Supplementary Figure 2.5 NRP-1 expression following stable knockdown in BT549 human breast cancer cells. BT549 vector control (VC) and GPNMB-WT-expressing cells harboring a scrambled shRNA (shLMP) or two independent shRNAs targeting the 3' UTR region of NRP-1 (shRNA#1 and shRNA#2) were probed for NRP-1 and GPNMB. GPNMB levels remain unchanged in BT549 cells harboring an NRP-1 knockdown. α-Tubulin serves as a loading control.

Name	Sequence
Human ShNRP-1 #1	TGCTGTTGACAGTGAGCGCCTGGTTATCCTCATTCTTAT TAGTGAAGCCACAGATGTAATAAGAATGAGGATAACCAG GTGCCTACTGCCTCGGA
Human ShNRP-1 #2	TGCTGTTGACAGTGAGCGCGGACCCATACCAGAGAATT ATAGTGAAGCCACAGATGTATAATTCTCTGGTATGGGTC CG TGCCTACTGCCTCGGA
Mouse ShNRP-1 #1	TGCTGTTGACAGTGAGCGCAGACTTTGTTTGTAAGACA ACTAGTGAAGCCACAGATGTAGTTGTCTTACAAACAAAG TCTTTGCCTACTGCCTCGGA
hNRP-1 Fwd	ATCACGTGCAGCTCAGTGG
hNRP-1 Rev	TCATGCAGTGGGCAGAGTTC
mNRP-1 Fwd	GCCTGCTTTCTTCTCTTGGTTTCA
mNRP-1 Rev	GCTCTGGGCACTGGGCTACA
hGPNMB Fwd	CACTTCCTCAATTATTCTAC
hGPNMB Rev	TAAAGAAGGGGTGGGTTTTG
mGPNMB Fwd	TCCCTGGCAAAGACCCAGA
mGPNMB Rev	TTTGTACAGCAAGATGGTAACCATG
hCLU Fwd	GGATGCCCTAAATGAGACCA
hCLU Rev	TCAGGCAGGGCTTACACTCT
mCLU Fwd	CAGCTGGCTAACCTCACACA
mCLU Rev	TGTGATGGGGTCAGAGTCAA
hSERPINE2 Fwd	AACTAGGCTCCAACACGGG
hSERPINE2 Rev	TTACGCCGTATCTCATCACCA
mSERPINE2 Fwd	ACATAGAAACGGACATTCGTGGCAGGTGAT
mSERPINE2 Rev	TAGTGATGTGAGGGATGATGGCAGACAGT
hANK1 Fwd	CCAGATGAATGGTTACTCCTCAC
hANK1 Rev	CAAGGGGATGGCGTCTAGG
hAHR Fwd	AGCCGGTGCAGAAACAGTAA
hAHR Rev	AGGCGGTCTAACTCTGTGTTC
hPNRC1 Fwd	ACTTGCCACTAACCAAGATCAC
hPNRC1 Rev	CACTAAACTTTGCCCCAGCATA

Supplementary Table 2.1: A list of the shRNA sequences against mouse and human NRP-1 and primer sequences used for RT-qPCR.

Antibody	Dilution	Company	Catalogue #
pAKT S473	1:2000	Cell Signaling	9271
AKT	1:2000	Cell Signaling	9272
pERK T202/Y204	1:2000	Cell Signaling	9101S
ERK	1:2000	Cell Signaling	9102
pFRA1	1:2500	Cell Signaling	3880S
FRA1	1:5000	Santa Cruz	SC-605
GPNMB	1:2000	R&D	AF2550
Neuropilin-1 (human)	1:2500	Santa Cruz	SC-7239
Neuropilin-1 (mouse)	1:2000	Cell Signaling	3725
V5	1:2000	Invitrogen	P/N46-0705
pVEGFR2 Y1175	1:1000	Cell Signaling	2478
VEGFR2	1:1000	Cell Signaling	2479
α-Tubulin	1:50000	Sigma	T9026

Supplementary Table 2.2: A list of all the antibodies used in the current study.

CHAPTER 3

GPNMB Promotes Metastasis by Increasing Recycling and Stability of Integrin $\alpha_5\beta_1$

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A portion of the work presented in this section is part of a manuscript published in Oncogene (16 March, 2015)

3.1 Preface

Our previous work demonstrates that NRP-1 can cooperate with GPNMB to promote breast cancer growth. However, NRP-1 knockdown in GPNMB-expressing did not impair GPNMB-driven metastasis. Since we initially identified GPNMB in a screen for mediators of breast cancer metastasis, we wanted to further investigate the mechanisms implicated in this process. Recent studies have attributed important functional roles to distinct GPNMB domains in physiological and pathological processes but the role of GPNMB domains in cancer progression has never been systematically addressed. To address this question, we tested a panel of GPNMB mutants for their ability to promote invasion, growth and metastasis. Specifically, we wanted to explore the requirement of the GPNMB RGD integrin-binding domain for breast cancer metastasis as it has been shown to increase adhesion and integrin signaling in physiological conditions.

3.2 Abstract

Deaths attributed to breast cancer primarily occur following metastasis by primary tumor cells to distant organs such as bone, lung, liver and brain. In a screen for metastasis modulators, we have previously identified GPNMB as a gene whose overexpression in weakly metastatic breast cancer cell lines enhances primary tumor growth and promotes lung and bone metastasis. To identify GPNMB domains responsible for mediating its pro-growth and pro-metastatic functions, we employed a panel of GPNMB mutants. We demonstrate that both the RGD motif and cytoplasmic tail of GPNMB are required to promote primary mammary tumor growth; however, only mutation of the RGD motif impaired the formation of lung metastases. Additionally, we ascribe the pro-growth and pro-metastatic functions of GPNMB to its ability to bind $\alpha_5\beta_1$ integrin through its RGD domain. We show that GPNMB increases expression of individual $\alpha_5\beta_1$ integrin subunits and that it is co-expressed with the α_5 integrin subunit in human breast cancer datasets. These increased integrin levels in GPNMB-expressing cells were specifically attributed to enhanced stability and recycling of active, fibronectin-bound $\alpha_5\beta_1$ integrin complexes. Our data indicates that GPNMB promotes recycling of the active $\alpha_5\beta_1$ integrin receptor through the recently identified late endosomal/lysosomal trafficking pathway. Furthermore, GPNMB recruitment into integrin complexes activates Src and FAK signaling pathways, and induces reciprocal GPNMB phosphorylation, in an RGD-dependent manner. Together, these findings implicate critical GPNMB domains in distinct aspects of breast cancer progression. Importantly, our work also identifies GPNMB as a novel mediator of $\alpha_5\beta_1$ integrin recycling in basal breast cancers.

3.3 Introduction

GPNMB possesses a short cytoplasmic tail, a single-pass transmembrane domain and an extracellular domain (ECD). Although a few studies have examined the role of these domains in physiological processes, their potential importance in cancer progression has not yet been addressed [1-4]. The cytoplasmic tail of GPNMB contains a hemITAM motif, which could potentially be a target of Src-mediated signaling [5]. The shed ECD domain has been shown to be biologically active, by enhancing MMP secretion from fibroblasts and mediating endothelial cell migration [6, 7]. In its extracellular domain, GPNMB harbors a polycystic kidney disease domain (PKD) and an RGD integrin-binding motif. Studies have highlighted a physiological role for the RGD domain of GPNMB in mediating a cell-adhesive phenotype [1, 2]. RGD-binding integrin complexes, which include the $\alpha_5\beta_1$ fibronectin receptor as well as all integrin receptors containing an α_V subunit, have been strongly implicated in cancer progression [8]. Interestingly, previous reports have shown that GPNMB can associate with β1 or β3 integrin complexes in osteoclasts and can bind the αVβ1 integrin receptor on osteoblasts to promote downstream signaling [9, 10]. Taken together, these data suggest that RGD-binding integrin receptors could act as critical mediators of GPNMB-driven effects in breast cancer [11].

The integrin family encompasses 18 alpha and 8 beta subunits which associate non-covalently to form 24 distinct heterodimers [12]. Integrin receptors are distinguished based on substrate specificity, which is mediated by a binding pocket found in the extracellular region of the heterodimer. Integrin complexes serve as a physical anchor for the cell and can transduce mechanical forces by linking the ECM to the cytoskeleton [13]. Although they lack kinase activity, integrin receptors have the unique ability to function as

bidirectional signaling molecules. In response to engagement by ECM substrates, integrin heterodimers cluster at the membrane and promote “outside-in” signaling by recruiting and activating Src and Fak kinases [14]. Conversely, signaling by growth factors or oncogenes promotes the binding of intracellular adaptors talin and kindlin to the β subunit cytoplasmic tail to induce “inside-out” signaling by changing integrin conformation and increasing their ligand affinity [15]. These pathways and their associated triggers are critical for integrin regulation of biological processes.

Integrin receptors regulate numerous processes in cancer cells, including migration, invasion, proliferation and survival, and their expression is correlated with disease aggressiveness in many cancer types [8]. Targeted therapies against integrin $\alpha_5\beta_1$ are being considered in ovarian cancer, where expression of the heterodimer is associated with a poor prognosis [16]. Integrin $\alpha_5\beta_1$ promotes invasion and metastasis in this context and activates c-Met-driven mitogenic pathways [17, 18]. In lung cancers, fibronectin signaling through $\alpha_5\beta_1$ is a major driver of proliferation [19, 20] and $\alpha_5\beta_1$ expression predicts lymph node metastasis and poor patient survival [21, 22]. The majority of studies investigating the role of integrin receptors in breast cancer associate $\alpha_5\beta_1$ with tumor aggressiveness [23]. The $\alpha_5\beta_1$ integrin receptor can inhibit E-cadherin and increase MMP expression to promote invasion in various breast cancer models [24-26]. Additionally, integrin β_1 is required for mammary tumor onset in an MMTV/PyMT transgenic mouse model [27]. Signaling pathways engaged by $\alpha_5\beta_1$ have been implicated in breast cancer metastasis; however, whether these pathways promote or suppress metastasis seems to be context-dependent [26, 28-30]. Despite these conflicting reports, $\alpha_5\beta_1$ is recognized as a driver of tumor growth

and metastasis in breast cancer and this understanding is supported by patient outcome data [31, 32].

Integrin receptor stability and function is regulated through trafficking, which dictates the cell surface distribution of the heterodimer for efficient adhesion dynamics, migration, invasion and cytokinesis [33]. The recycling of integrin receptors can be spatially restricted to intensify a localized signal, such as during adhesion assembly or invadopodia formation, target a broader region like the lamellopodia to establish cell polarity for directional migration, or it can involve an *en masse* redistribution of the receptor, which is observed when integrin complexes are recycled to and from the cleavage furrow in cytokinesis [34]. Additionally, integrin traffic is closely coupled to critical cell signaling pathways implicated in survival, proliferation and metastasis, which emphasizes the growing relevance of this topic in cancer progression [35-37].

Broadly speaking, integrin trafficking involves the endocytosis of cell surface receptors, which are sorted in intracellular endosomal compartments for recycling back to the cell surface or for lysosomal degradation [12]. Integrin internalization proceeds through clathrin-dependent or independent mechanisms and can be mediated by a variety of molecular adaptors in a context-dependent fashion [12]. Following entry into EEA-1 positive endosomes, integrin receptors can be recycled back to the cell surface via a rapid Rab4-dependent mechanism or through a slower Rab11-dependent route that passes by the perinuclear recycling compartment (PNRC) [38, 39]. These recycling pathways regulate adhesion turnover by bringing unligated integrin receptors back to the cell surface to engage the ECM and form new adhesions.

Integrin dimers can adopt inactive, ligand-naïve and active, ligand-bound conformations [40]. Ligand-bound integrin complexes are preferentially internalized from

focal adhesions [41] and display different mechanisms of internalization compared to their unligated counterparts [42]. While the recycling of inactive integrin complexes is rapid and spatially restricted, the fate of active integrin complexes is determined by a balance between slow recycling and lysosomal degradation [43, 44]. Active $\alpha_5\beta_1$ receptors are endocytosed with fibronectin [45] at which point they are either sent to the late endosomal/lysosomal pathway for degradation, or dissociated from the ECM and "rescued" by recycling to the cell surface [34]. Many studies have confirmed the presence of active integrin complexes in late endosomes [36, 46, 47] and it is well documented that the engagement of $\alpha_5\beta_1$ with fibronectin increases the proportion of the heterodimers that are targeted for degradation [46, 47]. Fibronectin binding to $\alpha_5\beta_1$ integrin complexes leads to ubiquitination of the α_5 cytoplasmic tail and degradation of the receptor, thus preventing accumulation of ligand-bound integrin receptors in endosomes [47]. However, ligand-bound integrin receptors can be rescued from degradation by sorting nexin 17 (SNX17), which binds to the β1 cytoplasmic tail in early endosomes and shuttles the active receptor to the plasma membrane for efficient cell migration [48, 49]. Additionally, recent work has given rise to the novel idea that integrin receptors can also be recycled from late endosomes and lysosomes [50]. In ovarian cancer cells, Rab25 cooperates with CLIC3 to drive retrograde transport of active $\alpha_5\beta_1$ from the lysosome to the back of invading cells. This process is responsible for maintaining active Src signaling during migration and invasion in 3D microenvironments [46]. Interestingly, when matrix density is low, fibronectin can be re-secreted with $\alpha_5\beta_1$ from lysosomes [46] to promote migration [51]. Indeed, re-secretion of fibronectin-containing exosomes from late endosomal/lysosomal compartments is required for directionally persistent movement and adhesion assembly by cancer cells *in vivo* [52].

In addition to coordinating cytoskeleton dynamics, integrin trafficking through different pathways can regulate key signaling pathways in cancer progression [35]. For example, activation of Src occurs in response to Rab25-mediated recycling of $\alpha_5\beta_1$ from late endosomes and is critical for driving cell invasion and establishing polarity during directional cell migration [46, 53]. However, RCP-dependent $\alpha_5\beta_1$ trafficking from the perinuclear recycling compartment leads to increased Akt/PKB and MAPK signaling [54-56], indicating that distinct trafficking routes can elicit different biological responses.

Integrin trafficking plays a pivotal role in cancer metastasis and drivers of this process can promote aggressiveness of many human cancers. The RCP gene is located in a chromosomal region that is frequently amplified in breast cancer [57] and its expression is associated with tumor growth and metastasis in xenograft models [56]. Additionally, Rab25A and CLIC3 are predictors of lymph node metastasis in breast cancer and pancreatic ductal adenocarcinoma patients, respectively [46, 58]. However, while Rab25 is a potent oncogene in ovarian cancers, it seems to play a subtype-dependent role in breast cancer [59-61]. Rab25 is associated with poor prognosis in hormone- and HER2-positive breast cancers but is lost following initiation of basal breast cancers [60, 61]. Additionally, in claudin-low breast cancers, which are characterized by loss of Rab25 expression, exogenous addition of Rab25 inhibits cell migration, EMT and invasion [61]. This data suggests that, in basal and claudin-low breast cancers, the invasive phenotype resulting from $\alpha_5\beta_1$ late endosomal recycling is driven by a Rab25-independent mechanism that has yet to be identified.

In this study, we identify a distinct role for $\alpha_5\beta_1$ integrin in promoting GPNMB-driven breast cancer metastasis through a novel, RGD-mediated interaction between $\alpha_5\beta_1$ and GPNMB expressed in breast cancer cells. We show that GPNMB increases integrin α5

expression in basal breast cancer cells by inhibiting degradation and specifically promoting recycling of the active fibronectin receptor.

3.4 Results

Distinct domains within GPNMB contribute to breast cancer cell invasion *in vitro*

To define the regions within GPNMB responsible for promoting breast cancer cell invasion and metastasis, we created several GPNMB domain mutants. Versions of GPNMB harboring either a cytoplasmic tail truncation (ΔCYT) or an RGD to RAA substitution (RGD_{mut}) were constructed to investigate the role of putative GPNMB signaling residues in the C-terminus and the integrin-binding activity of GPNMB in the extracellular domain, respectively (Figure 3.1A). VC, GPNMB-WT, GPNMB-ΔCYT and GPNMB-RGD_{mut} constructs were individually expressed in two human basal breast cancer models (BT549 and HS578T) and in the NIC mouse mammary carcinoma cell lines (Figure 3.1; Supplementary Figure 3.1A, C, D), and cell-surface expression of GPNMB mutants was confirmed in BT549 breast cancer cells (Supplementary Figure 3.1B).

Since these cell models are not all tumorigenic *in vivo*, we first used *in vitro* assays as a surrogate to interrogate the role of these domains in GPNMB-driven breast cancer cell invasion. As shown previously, GPNMB overexpression promoted the invasion of BT549 cells (Figure 3.1B) [62] and we extended these observations to the HS578T and NIC breast cancer models (Figure 3.1C, D). In contrast, overexpression of GPNMB-ΔCYT and GPNMB-RGD_{mut} mutants failed to induce an invasive phenotype in all 3 cell models, indicating that the cytoplasmic tail and RGD integrin-binding domain of GPNMB are required for *in vitro* breast cancer cell invasion (Figure 3.1B-D).

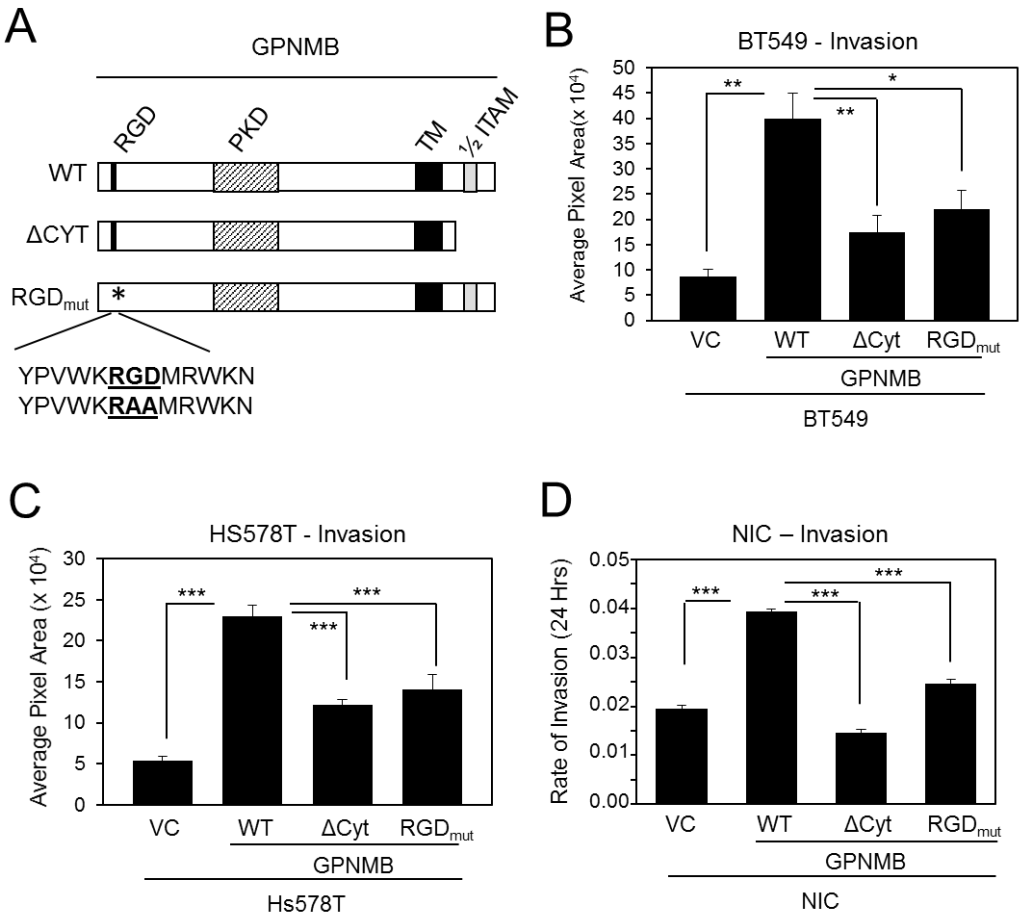


Figure 3.1 Distinct domains within GPNMB are important for breast cancer cell invasion. (**a**) Schematic diagram of wild-type GPNMB (WT), and mutant GPNMB constructs harboring a cytoplasmic tail truncation (ΔCYT) or an integrin-binding domain RGD to RAA substitution (RGD_{mut}). Additional GPNMB domains depicted include the polycystic kidney disease domain (PKD), the transmembrane domain (TM) and 1/2 ITAM motif. BT549 (**b**) and HS578T (**c**) human breast cancer cells expressing VC, GPNMB-WT, GPNMB-ΔCYT or GPNMB-RGD_{mut} constructs were tested for their ability to invade. (**d**) The 24-hour invasion rate of NIC cells expressing VC, GPNMB-WT, GPNMB-ΔCYT or GPNMB-RGD_{mut} is shown (n=3 for all cell lines).

GPNMB mediates tumor growth and metastasis through its integrin-binding RGD domain

To elucidate the role of GPNMB domains in mediating mammary tumor growth and metastasis, NIC VC, GPNMB-WT, GPNMB-ΔCYT and GPNMB-RGD_{mut} cells were injected into the mammary fat pads of athymic mice. As demonstrated previously (Figure 2.3B), GPNMB-WT cells exhibited a two-fold increase in mammary tumor growth compared to control (Figure 3.2A). Interestingly, the increase in growth conferred by GPNMB-WT was abrogated in GPNMB-ΔCYT and GPNMB-RGD_{mut} expressing mammary tumors. To better understand how mutation of these GPNMB functional domains impacted mammary tumor growth, we examined Ki67, cleaved caspase-3 and CD31 markers. We observed an increase in Ki67 positivity in GPNMB-WT expressing tumors (24%) compared to control (17%), and this increase in proliferation was lost in NIC GPNMB-ΔCYT (17%) and GPNMB-RGD_{mut} (18%) tumors (Supplementary Figure 3.2A). The decrease in apoptosis observed with GPNMB-WT tumors (0.74% compared to 1.06% in VC tumors) also required the cytoplasmic tail (0.92%) and RGD domain (1.12 %) (Supplementary Figure 3.2B). Endothelial cell density was elevated in GPNMB-WT (14%) versus VC (8%) mammary tumors but remained unchanged in GPNMB-ΔCYT (8%) and GPNMB-RGD_{mut} (9%) tumors (Supplementary Figure 3.2C). We determined that the endothelial cell density correlated well with the degree of macrophage recruitment (Figure 3.2B, Supplementary Figure 3.3) and VEGF production (Figure 3.2C) in these tumors, indicating that GPNMB requires an intact cytoplasmic tail and RGD motif to enhance recruitment of a VEGF-producing stromal infiltrate. Together, these results indicate that the tumor growth-promoting effects of GPNMB rely on both the RGD motif and the cytoplasmic tail.

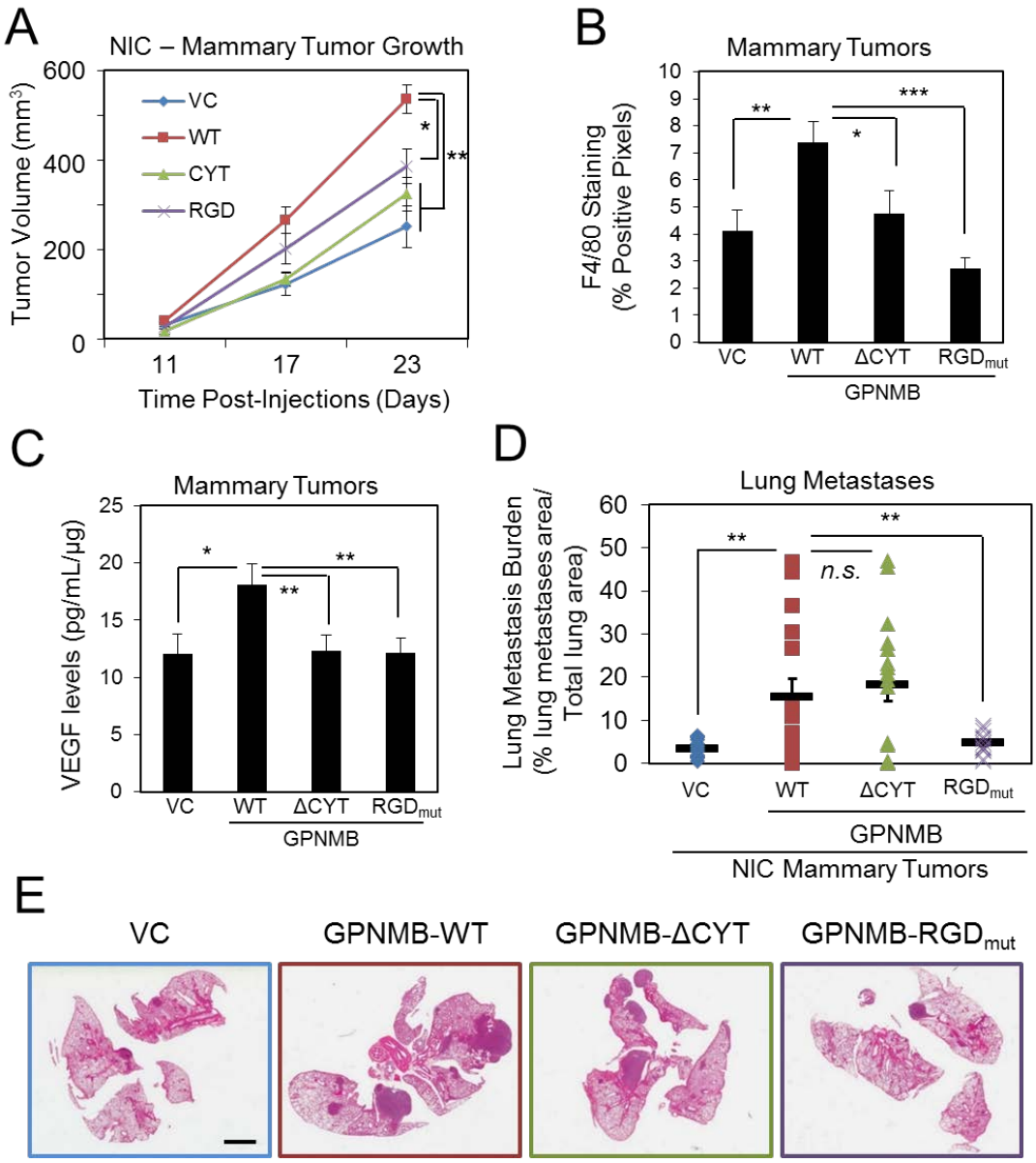


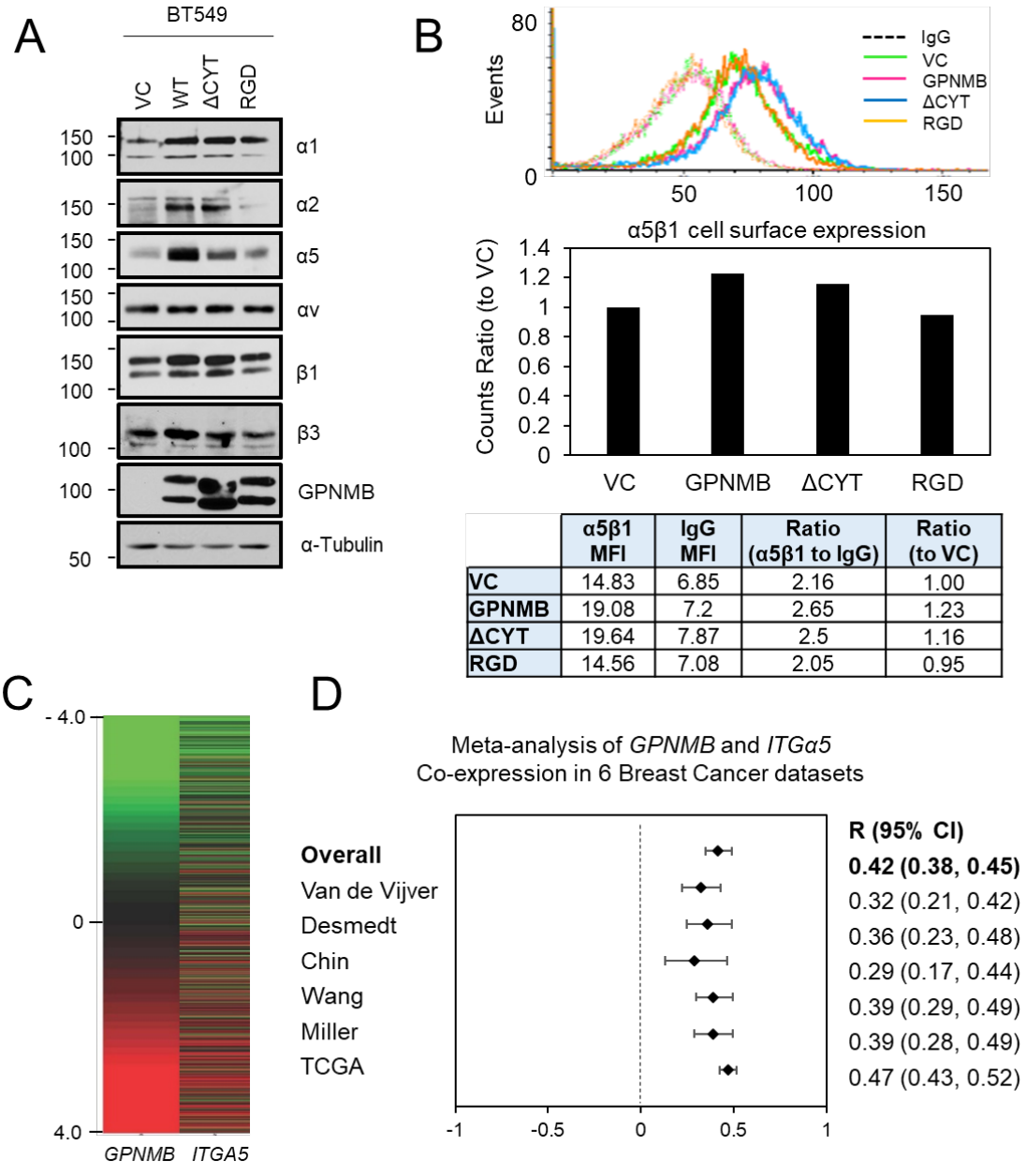
Figure 3.2 The GPNMB RGD domain is required for GPNMB-driven tumor growth and metastasis. (**A**) Mammary tumor growth of NIC VC, GPNMB-WT, GPNMB-ΔCYT and GPNMB-RGD tumors was monitored by weekly caliper measurements (n=10). (**B**) The level of macrophage infiltration in resected NIC tumors was assessed by quantifying the percent positive F4/80 staining (10 images/sample of n=5 samples). (**C**) Tumor VEGF levels were determined using ELISA and normalized to total tumor lysate (n=10). (**D**) Lung metastatic burden, which represents the percent lung area occupied by tumor cells, was determined from lung tissue sections harvested 5 weeks post-resection (n=10). (**E**) Representative lung images are displayed.

Quantification of the lung metastatic burden revealed that the percentage lung area covered by breast cancer metastases was increased 4.7-fold in mice injected with GPNMB-WT cells compared to control cells (Figure 3.2D, E), confirming our previous results (Figure 2.3E, F). Mutation of the GPNMB RGD domain severely impaired the ability of NIC breast cancer cells to metastasize to the lung, while surprisingly, the cytoplasmic tail of GPNMB was dispensable in GPNMB-driven metastasis (Figure 3.2D, E). These data ascribe distinct roles to GPNMB functional domains in GPNMB-driven tumor progression and highlight the multi-dimensional nature of GPNMB mechanisms of action.

GPNMB enhances expression of the $\alpha_5\beta_1$ fibronectin receptor

Given the importance of the GPNMB RGD integrin-binding domain in promoting tumor growth, invasion and metastasis, we investigated the expression of a variety of integrin subunits in BT549-VC, GPNMB-WT, GPNMB-ΔCYT or GPNMB-RGD_{mut} cells. Ectopic expression of GPNMB dramatically increased levels of the α_5 subunit of the major fibronectin receptor, led to modest but reproducible increases in the levels of the α_1, α_2, β_1 and β_3 integrin subunits and resulted in no changes to the levels of the α_V subunit (Figure 3.3A). An intact GPNMB RGD motif, but not the cytoplasmic domain, was required to increase expression of the α_1, α_2, α_5, β_1 and β_3 integrin subunits (Figure 3.3A).

To determine if GPNMB influenced the localization of integrin complexes, we examined cell-surface levels of the RGD-binding $\alpha_5\beta_1$ fibronectin receptor. BT549 cells expressing GPNMB exhibited significant enrichment of cell-surface $\alpha_5\beta_1$ integrin complexes compared to control cells (Figure 3.3B) and this increase was RGD-dependent. Additionally, using an ordered heat map of *GPNMB* expression obtained from the TCGA Illumina HiSeq dataset, we demonstrate *ITGA5* expression closely mirrors that of *GPNMB*



	α5β1 MFI	IgG MFI	Ratio (α5β1 to IgG)	Ratio (to VC)
VC	14.83	6.85	2.16	1.00
GPNMB	19.08	7.2	2.65	1.23
ΔCYT	19.64	7.87	2.5	1.16
RGD	14.56	7.08	2.05	0.95

Figure 3.3 GPNMB increases cell-surface expression of the fibronectin receptor, $\alpha_5\beta_1$, and is co-expressed with *ITGA5* in human breast cancers. (**a**) BT549 VC, GPNMB-WT, GPNMB-ΔCYT or GPNMB-RGD cells were immunoblotted for expression of GPNMB and of integrin subunits α_1, α_2, α_5, α_V, β_1 and β_3. (**b**) Cell surface expression of $\alpha_5\beta_1$ in BT549 cells was examined by FACS analysis and plotted relative to VC. MFI = Mean Fluorescence Intensity (**c**) A comparison between *GPNMB* and *ITGA5* mRNA expression within the TCGA dataset is depicted using a heatmap. (**d**) A meta-analysis of *GPNMB* and *ITGA5* expression across 6 publically available datasets reveals a positive co-expression correlation.

across 1106 breast tumor samples (Figure 3.3C). *GPNMB* showed a moderately strong correlation with *ITGA5* expression (r=0.42) in a combined analysis of 6 different breast cancer studies (Figure 3.3D). These results extend the relevance of our findings to human disease and further implicate novel GPNMB-mediated engagement of integrin $\alpha_5\beta_1$ in breast cancer progression.

GPNMB promotes breast cancer cell adhesion to fibronectin and forms a complex with $\alpha_5\beta_1$ upon fibronectin engagement

Integrin complexes expressed by cancer cells facilitate adhesion to the extracellular matrix (ECM), which is an essential step during cancer cell migration and invasion. To determine if the elevated levels of fibronectin receptor subunits could enhance substrate recognition of GPNMB-expressing cells, we seeded BT549 cell populations onto fibronectin-coated plates and monitored their rate of adhesion. BT549 GPNMB cells displayed a 2-fold increase in adhesion to matrix fibronectin compared to VC, which was blunted in GPNMB-RGD_{mut}, but not GPNMB-ΔCYT-expressing cells (Figure 3.4A). This was consistent with overall and cell surface integrin levels observed in BT549 GPNMB-RGD_{mut} and GPNMB-ΔCYT cells. Adhesion to fibronectin was specifically mediated through $\alpha_5\beta_1$ as blocking antibodies against $\alpha_5\beta_1$ dramatically reduced adhesion (Supplementary Figure 3.4). These results argue that GPNMB expression enhances adhesion to fibronectin by increasing expression of the α_5 and β_1 integrin subunits.

GPNMB is a membrane protein; thus, we speculated that GPNMB might interact with the $\alpha_5\beta_1$ receptor. Using a co-immunoprecipitation assay, we observed that GPNMB can form a complex with the $\alpha_5\beta_1$ receptor and that this interaction is specifically mediated through the RGD domain of GPNMB (Figure 3.4B, C). Interestingly, although weak

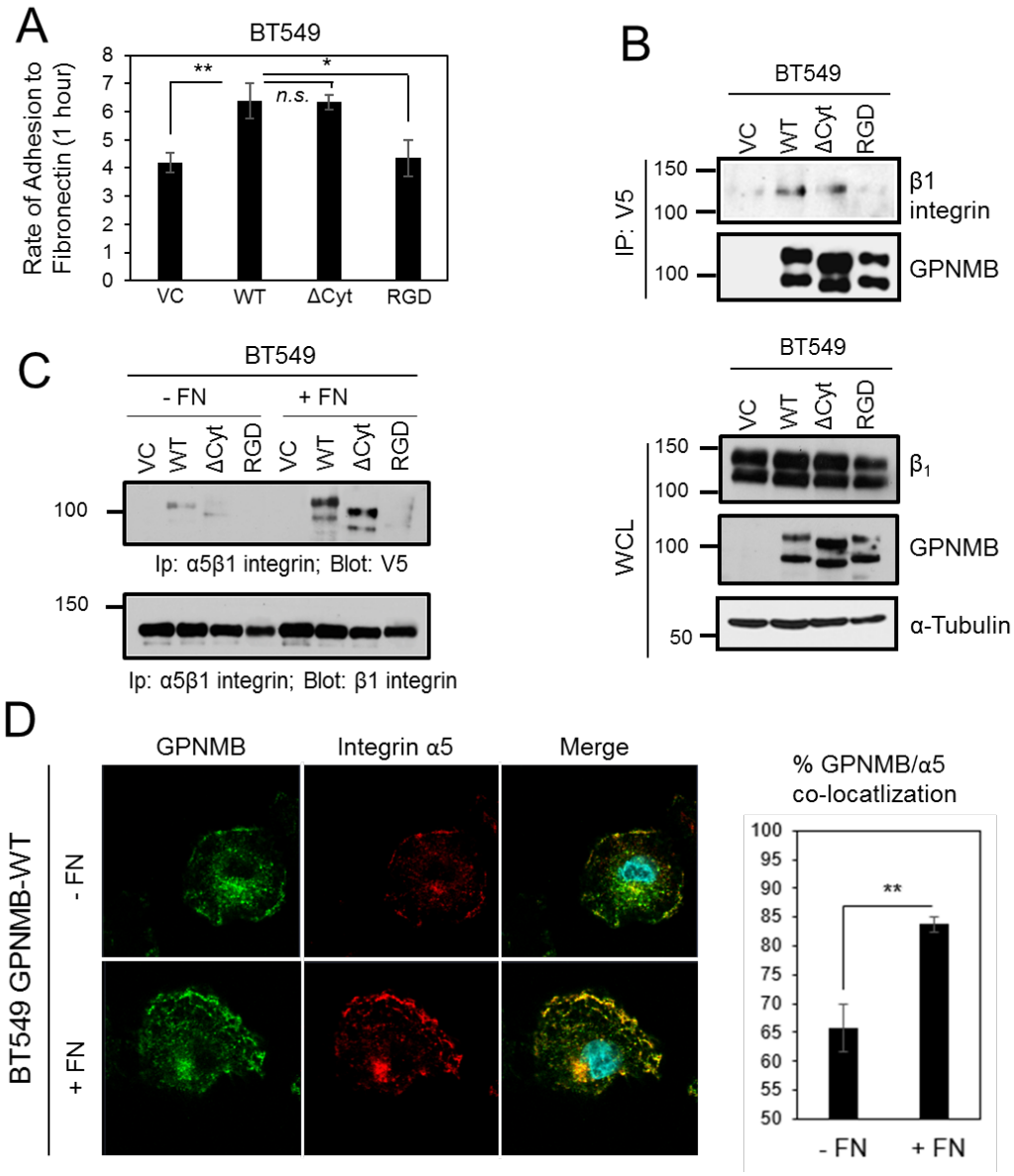


Figure 3.4 GPNMB enhances breast cancer cell adhesion to fibronectin and associates with the activated fibronectin receptor through its RGD domain. (**A**) The rate of adhesion of BT549 VC, GPNMB-WT, GPNMB-ΔCYT and GPNMB-RGD cells to fibronectin-coated plates was monitored for 1 hour (n=3). (**B**) V5-tagged GPNMB was immunoprecipitated from BT549 cells. The eluate from the beads was analyzed by immunoblotting with β_1 or GPNMB-specific antibodies. Endogenous levels of β_1, GPNMB and tubulin are shown. (**C**) A reciprocal co-immunoprecipitation of the $\alpha_5\beta_1$ receptor from BT549 cells, plated on plastic or fibronectin, was performed. GPNMB and β_1 were detected by immunoblot in the immunoprecipitated complex. (**D**) Representative immunofluorescence experiment showing co-localization of GPNMB and integrin α5 on plastic and on fibronectin. Quantification represents the average of 5-10 fields of view from 3 independent experiments.

GPNMB interactions with $\alpha_5\beta_1$ were detected under basal conditions, formation of this complex was dramatically increased when the cells were plated on fibronectin to allow activation of $\alpha_5\beta_1$ integrin receptors (Figure 3.4C). To understand the spatial relationship between GPNMB and $\alpha_5\beta_1$ integrin complexes in response to ligand engagement, we examined BT549 GPNMB cells plated on plastic and fibronectin using fluorescent confocal microscopy. In absence of ligand, GPNMB and integrin α_5 partly co-localized at the plasma membrane and in intracellular compartments (Figure 3.4D). However, we observed a more extensive degree of co-localization following fibronectin stimulation, characterized by an enrichment of punctate GPNMB and α_5 integrin complexes (Figure 3.4D). This data suggests that GPNMB interacts with $\alpha_5\beta_1$ in intracellular compartments following ligand-mediated integrin activation.

GPNMB increases stability of integrin α5β1 in an RGD-dependent manner

Given that GPNMB can associate with the fibronectin receptor, we sought to determine if the GPNMB-driven increase in $\alpha_5\beta_1$ expression (Figure 3.3A) is due to enhanced transcription or protein stability. Quantitative Real-Time PCR analysis revealed that transcript levels of α_5 and β_1 integrin subunits were unchanged in BT549 GPNMB-expressing cells compared to control, suggesting that GPNMB enhances expression of the $\alpha_5\beta_1$ receptor by regulating its stability (Figure 3.5A). To determine if GPNMB regulates integrin levels in a similar fashion *in vivo*, we examined integrin α_5 mRNA and protein expression in tumors derived from NIC VC and NIC GPNMB cells. In agreement with our *in vitro* findings, GPNMB expression did not lead to elevated *ITGA5* levels (Figure 3.5B) in primary mammary tumors. In contrast, GPNMB-expressing NIC tumors exhibited increased protein of the α_5 integrin subunit (Figure 3.5C). Taken together, these data

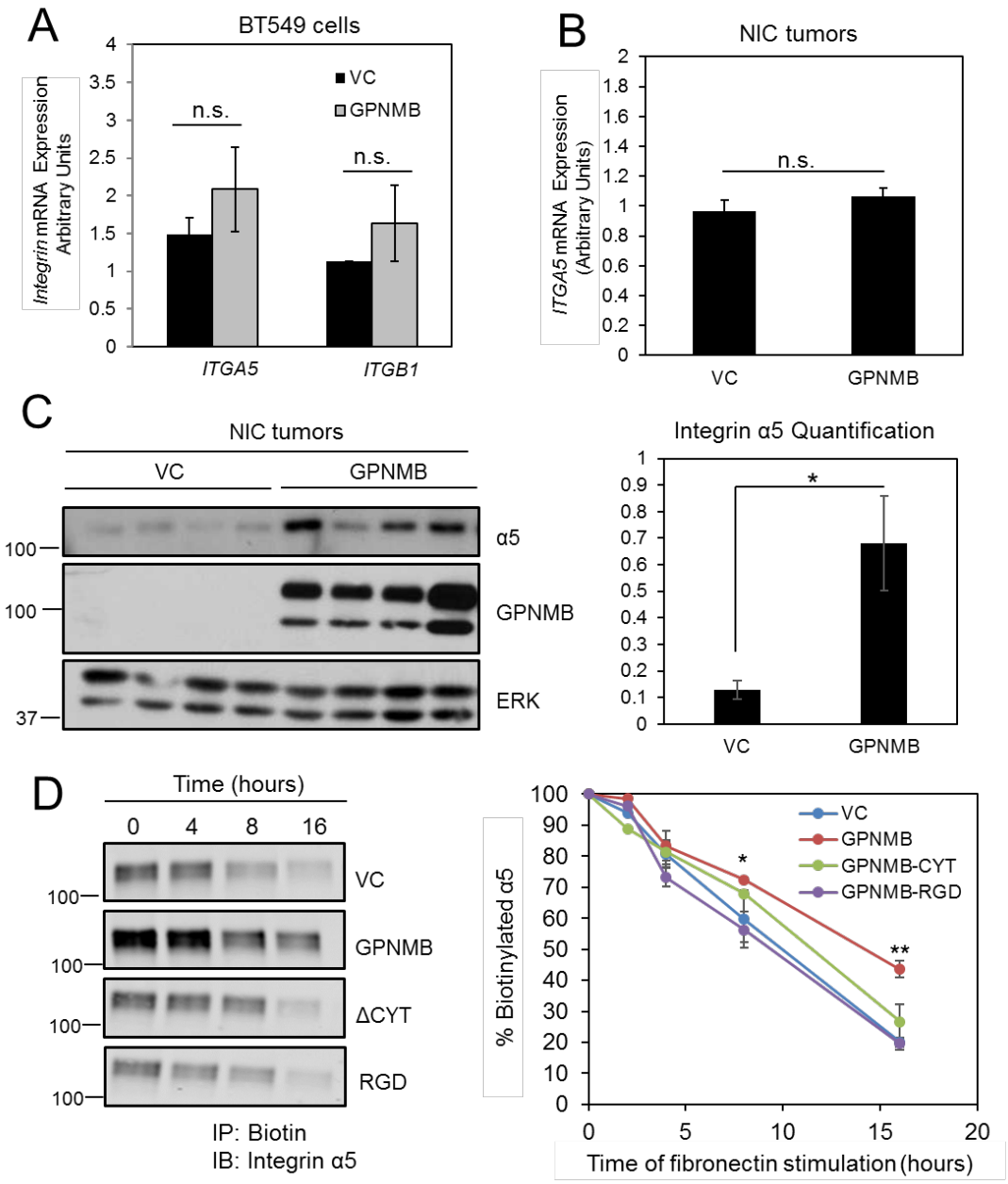


Figure 3.5 GPNMB increases integrin α5 expression by enhancing protein stability (**A**) Transcript levels of the integrin α5 and β1 subunits were unchanged in response to GPNMB expression *in vitro* (**B**) GPNMB-expressing NIC tumors do not exhibit increased integrin α5 mRNA expression compared to control (**C**) Integrin α5 protein expression is increased in NIC GPNMB-WT tumors (n=3) (**D**) Cell surface proteins were labelled with biotin, pulled down using streptavidin beads and immunoblotted for integrin α5 at the indicated timepoints. The remaining integrin α5 is expressed as a percentage of the signal detected at time 0. (n = 3, a representative immunoblot is included)

indicate that integrin α_5 protein stability is enhanced by GPNMB through a tumor-intrinsic mechanism that is at play during *in vivo* tumor growth and metastasis.

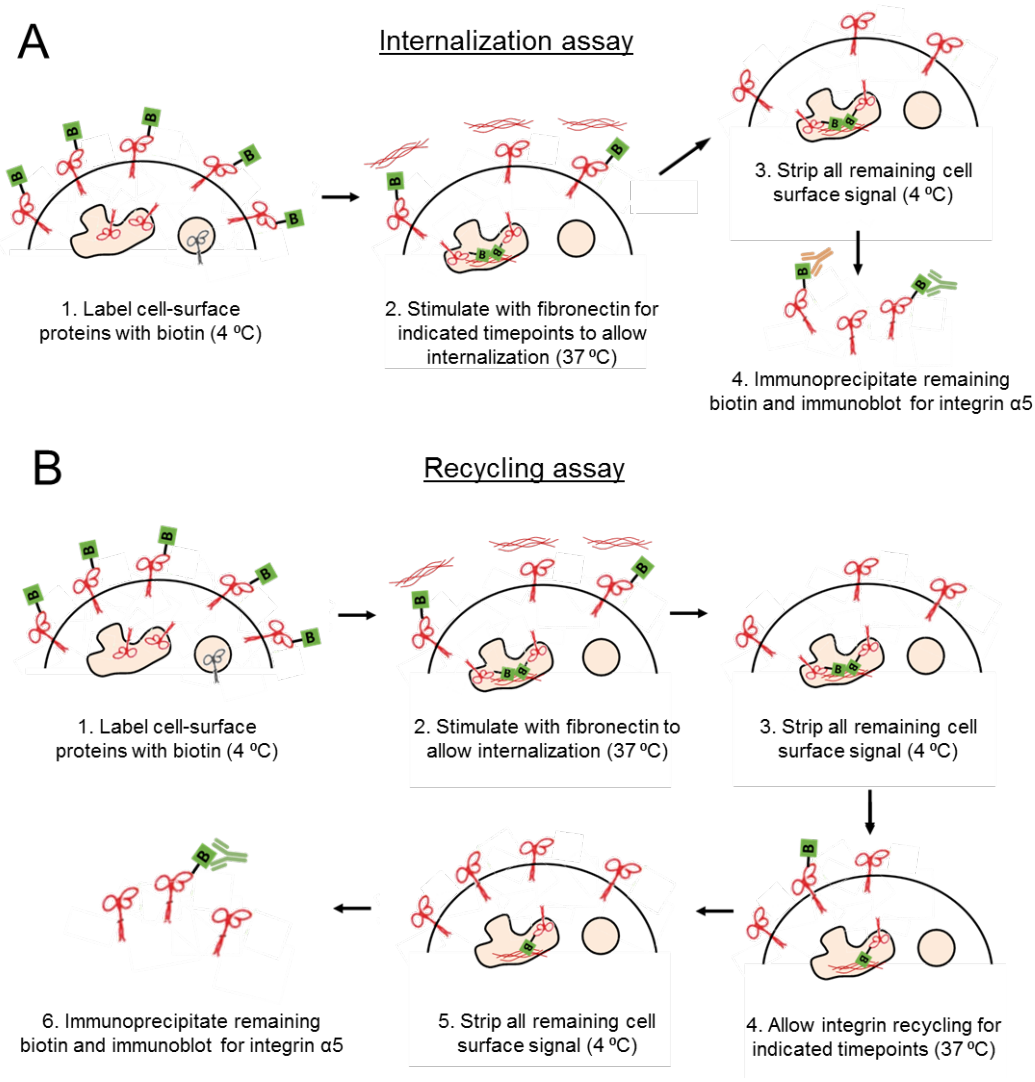
To confirm that GPNMB can increase protein stability of integrin α_5, we examined the rate of integrin degradation in BT549 VC, GPNMB-WT, GPNMB-ΔCYT and GPNMB-RGD_{mut} cells. BT549 cell populations were grown in media supplemented with fibronectin to activate $\alpha_5\beta_1$ integrin receptors and increase the proportion of heterodimers targeted for lysosomal degradation [47]. Cell-surface proteins were labeled with biotin and α_5 integrin subunits were immunoprecipitated at the indicated timepoints. In basal conditions, only 20% of labeled integrin complexes remained in the cell after 16 hours of fibronectin stimulation (Figure 3.5D, $p < 0.01$). Strikingly, GPNMB expression significantly delayed α5 integrin degradation as evidenced by the retention of 50% of the labeled integrin pool in BT549 GPNMB cells. The GPNMB-ΔCYT mutant slightly enhanced retention of active integrin complexes but this increase was not significant (Figure 3.5D, p = n.s.). However, when the GPNMB RGD domain was mutated, the rate of integrin degradation paralleled the rate observed in control cells, indicating that physical association between GPNMB and $\alpha_5\beta_1$ is required to inhibit integrin degradation (Figure 3.5D, $p < 0.01$).

GPNMB specifically enhances recycling of active $\alpha_5\beta_1$ integrin receptors

GPNMB and fibronectin share the same binding site on the $\alpha_5\beta_1$ receptor; thus, at first glance, it is puzzling that GPNMB association with integrin $\alpha_5\beta_1$ is enhanced following fibronectin engagement. However, this can be explained by the possibility that

GPNMB/$\alpha_5\beta_1$ interactions occur in endocytic compartments after fibronectin dissociation. The function and stability of ligand-bound $\alpha_5\beta_1$ integrin complexes is heavily dependent on the balance between cell-surface recycling and lysosomal degradation [34]. We hypothesized that GPNMB recruitment to active $\alpha_5\beta_1$ integrin receptors prevents their degradation by displacing fibronectin and promoting recycling of the heterodimers to the plasma membrane. To examine how GPNMB influences the trafficking of $\alpha_5\beta_1$ integrin complexes, we first measured the endocytosis rates of integrin complexes in BT549 cells. Surface proteins were labeled with biotin and cells were stimulated with fibronectin at 37ºC to promote integrin internalization. At the indicated timepoints, the remaining cell surface signal was quenched, thus ensuring specific detection of labeled intracellular proteins that had internalized from the cell surface (Supplementary Figure 3.5A). To eliminate potential confounding effects resulting from protein degradation, lysosomal activity was inhibited by concanamycin A treatment. A similar rate of endocytosis was observed in BT549 VC, GPNMB-WT and GPNMB-RGD_{mut} cells, indicating that GPNMB does not regulate entry of $\alpha_5\beta_1$ integrin receptors into the cell (Figure 3.6A). This data is consistent with the current model of active integrin traffic, which suggests that the RGD site on α5β1 is occupied by fibronectin during internalization [44, 45].

Recycling of active α_5 integrin subunits was examined by using an extension of the endocytosis assay. Following 30 minutes of internalization, cells were brought to 37ºC to allow labeled intracellular integrin complexes to return to the cell surface. Cell surface labeling was quenched after indicated recycling times and the remaining signal was compared to the signal at time 0 to determine the percentage of recycled integrin α_5 (Supplementary Figure 3.5B). Recycling of active integrin receptors was extremely rapid



Supplementary Figure 3.5 Biotin-based internalization and recycling assay schematic methodology (**A**) During an internalization assay, cell surface proteins are labelled with biotin following 2 hours of serum-starvation. Biotin is washed off and cells are stimulated with full-serum media for the indicated timepoints to initiate internalization of integrin complexes. The remaining cell surface proteins are then stripped of their biotin label using a reducing agent. Cells are lysed, biotin is immunoprecipitated using strepdavidin and internalized α5β1 integrin complexes are detected by immunoblotting for integrin α5. **(B)** During the recycling assay, cell surface proteins are labelled and allowed to internalize for 30 minutes using the protocol described in (**A**). Following the first round of biotin stripping, cells are lifted to full-serum media to stimulate recycling for the indicated timepoints. Next, biotin is once again stripped from proteins that have recycled back to the cell surface, and cells are lysed and processed for immunoblotting. The degree of recycling that occurred is quantified by comparing the pool of biotinylated proteins remaining in the cell following the second round of biotin stripping to the total pool of labelled proteins internalized after 20 min.

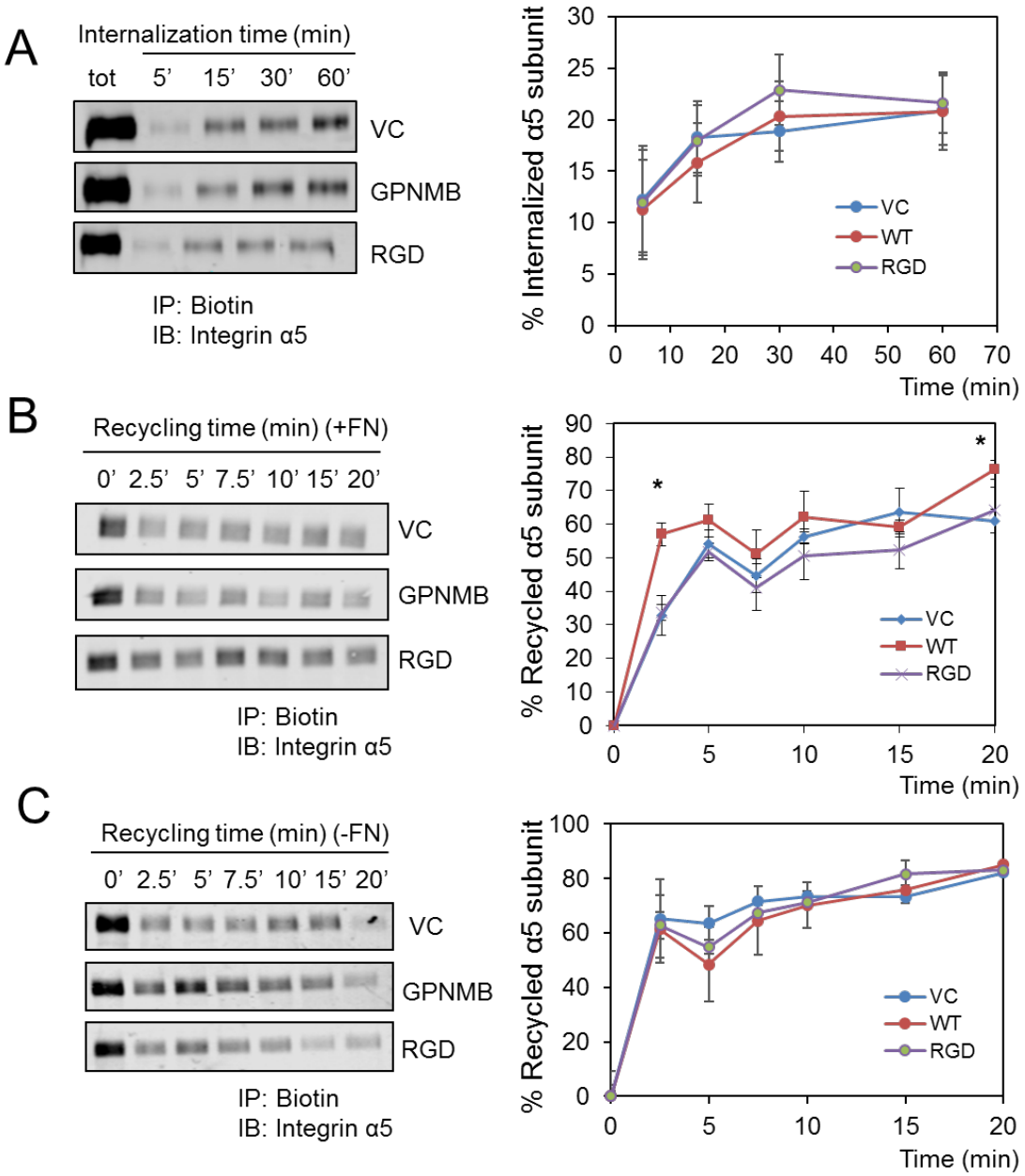


Figure 3.6 Recycling of active, ligand-bound, integrin α5β1 receptors is increased in GPNMB-WT-, but not GPNMB-RGD-expressing cells To examine integrin trafficking, BT549 VC, GPNMB-WT, and GPNMB-RGD cells were incubated with biotin on ice to label cell-surface proteins. In condition (**A**) internalization of labelled proteins was stimulated at 37°C for the indicated timepoints. Cell-surface biotin was quenched and the amount of internalized α5 integrins was assessed by an integrin α5 immunoblot. (n=3) (**B**,**C**) Following 30 min of internalization in the presence (**B**) or absence (**C**) of fibronectin, cell surface signal was quenched and cells were lifted to 37°C to stimulate recycling for the indicated timepoints. The amount of integrins remaining in the cell was examined by immunoblot. All timepoints are expressed as a percentage of the signal detected at time 0. (n = 3, a representative immunoblot is included)

in our system. After 5 minutes at 37ºC, 50% of internalized integrin receptors had reached the cell surface in control cells, which was followed by quick re-internalization of a portion of the recycled pool at 7.5 minutes. Remarkably, GPNMB expression led to a 2-fold increase in the percentage of active $\alpha_5\beta_1$ heterodimers recycled after 2.5 minutes (Figure 3.6B, $p<0.05$). BT549 GPNMB-RGD_{mut} cells exhibited a rate of recycling that was similar to control cells, thus ascribing a role for the RGD integrin-binding domain of GPNMB in promoting integrin recycling (Figure 3.6B, $p<0.01$).

To determine if GPNMB could specifically alter the kinetics of active integrin complexes, we examined trafficking of inactive $\alpha_5\beta_1$ heterodimers by performing the recycling assay in the absence of fibronectin stimulation. Interestingly, all BT549 cell populations exhibited similar integrin recycling rates in the absence of ligand, indicating that GPNMB associates with the active, but not inactive, $\alpha_5\beta_1$ receptor to favor its return to the plasma membrane (Figure 3.6C). These data demonstrate the specificity of the interaction between GPNMB and the active $\alpha_5\beta_1$ heterodimer, and suggest that the increase in $\alpha_5\beta_1$ receptor stability observed in GPNMB-expressing breast cancer cells (Figure 3.5D) is restricted to the active form of the fibronectin receptor.**Integrin α5 recycles from late endosomes/lysosomes in GPNMB-expressing cells**

Since active integrin receptors can recycle to the plasma membrane via multiple routes, we sought to determine which integrin trafficking mechanism was at play in GPNMB-expressing cells. Intracellular localization of GPNMB was examined in fibronectin-stimulated BT549 cells overexpressing GPNMB. Although a small portion of the GPNMB intracellular pool was found in early endosomes, as evidenced by partial co-localization of GPNMB with EEA-1, the majority of the GPNMB signal was detected in

EEA-1-negative vesicles (Figure 3.7A). We observed extensive co-localization between GPNMB and LAMP-1 and thereby identified these compartments as late endosomes/lysosomes (Figure 3.7A, $p<0.01$). To rule out possible GPNMB mis-localization due to overexpression, we performed co-immunofluorescence on MDA-MB-468 basal breast cancer cells, which express high levels of endogenous GPNMB. We confirmed that GPNMB/EEA-1 co-localization was minimal in this system, and that intracellular GPNMB is primarily found in late endosomes/lysosomes (Figure 3.7B, $p<0.001$).

Since the majority of intracellular GPNMB is found in lysosomes, we wanted to examine if $\alpha_5\beta_1$ integrin complexes could traffic through the late endosomal/lysosomal route in our system. Interestingly, while lysosomes typically exhibit juxta-nuclear aggregation, the trafficking of lysosomes towards the cell periphery is observed in metastatic cells and correlates with tumor malignancy [63, 64]. We performed the antibody-chase assay outlined above in the presence of 2 hours of fibronectin stimulation to allow a sizeable pool of active $\alpha_5\beta_1$ integrin receptors to reach the late endosomal/lysosomal compartments [53]. Notably, while extensive co-localization between α_5 and LAMP-1 was observed in both BT549 VC and GPNMB-WT cells, there was a striking difference in the distribution of α_5-containing lysosomes between the two cell lines (Figure 3.8). We detected a 4-fold increase in the number of "non-perinuclear" α_5-containing lysosomes located near the plasma membrane in GPNMB-expressing cells, suggesting that GPNMB can promote lysosome migration towards the cell periphery (Figure 3.8). Taken together, our results suggest that lysosomal GPNMB increases trafficking of active α_5 integrin

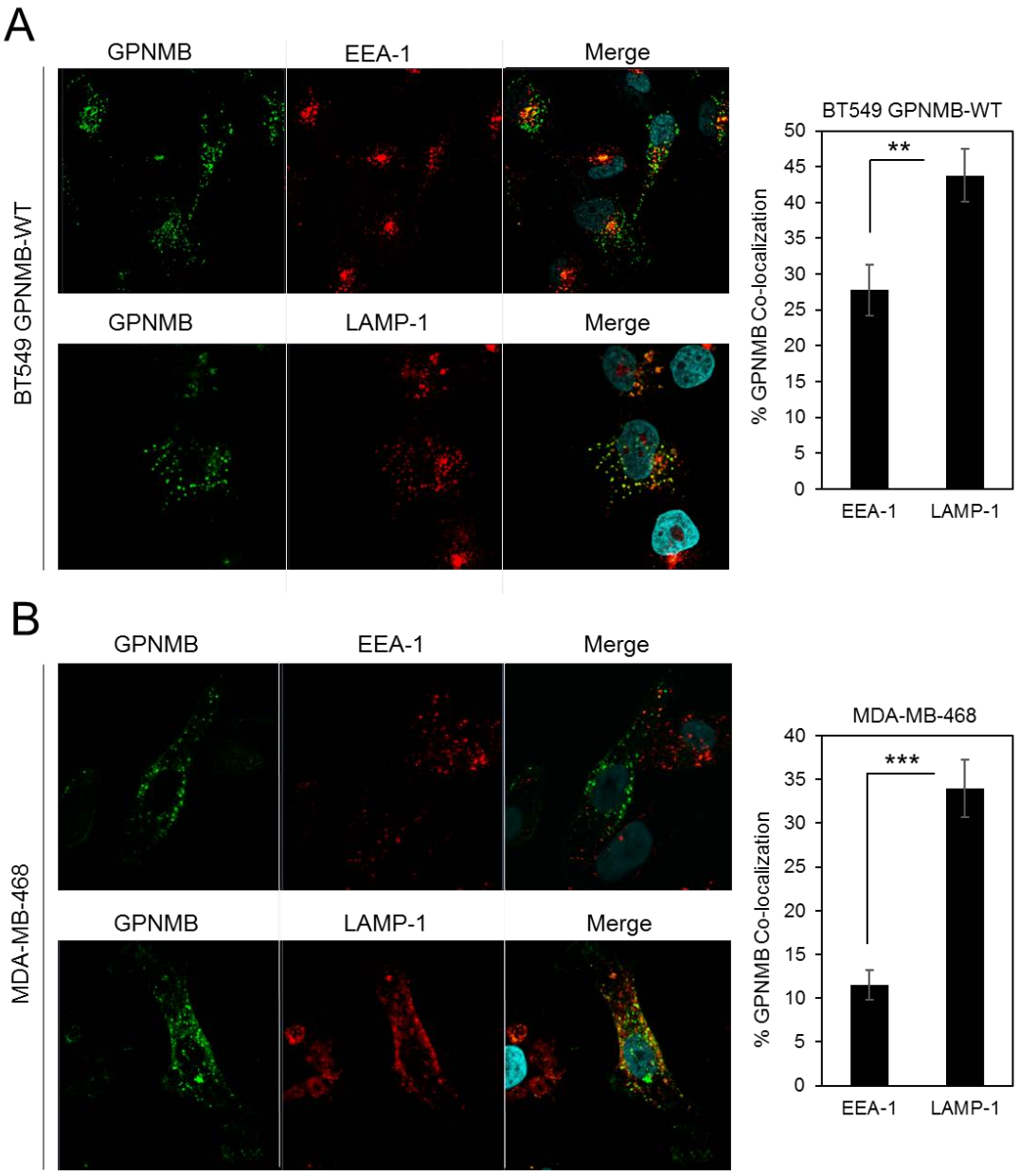


Figure 3.7 GPNMB localizes to lysosomes in basal-like breast cancer cells Localization of GPNMB was examined by immunofluorescence in BT549 (**A**) and MDA-MB-468 (**B**) cells plated on fibronectin. GPNMB was co-stained with either EEA-1 or LAMP-1 to identify early endosomes or late endosomes/lysosomes, respectively. GPNMB primarily co-localized with LAMP-1, as indicated by amount of yellow punctate vesicles in the merged image. 5-10 images were taken for each condition from 3 independent experiments. Images from a representative experiment are included.

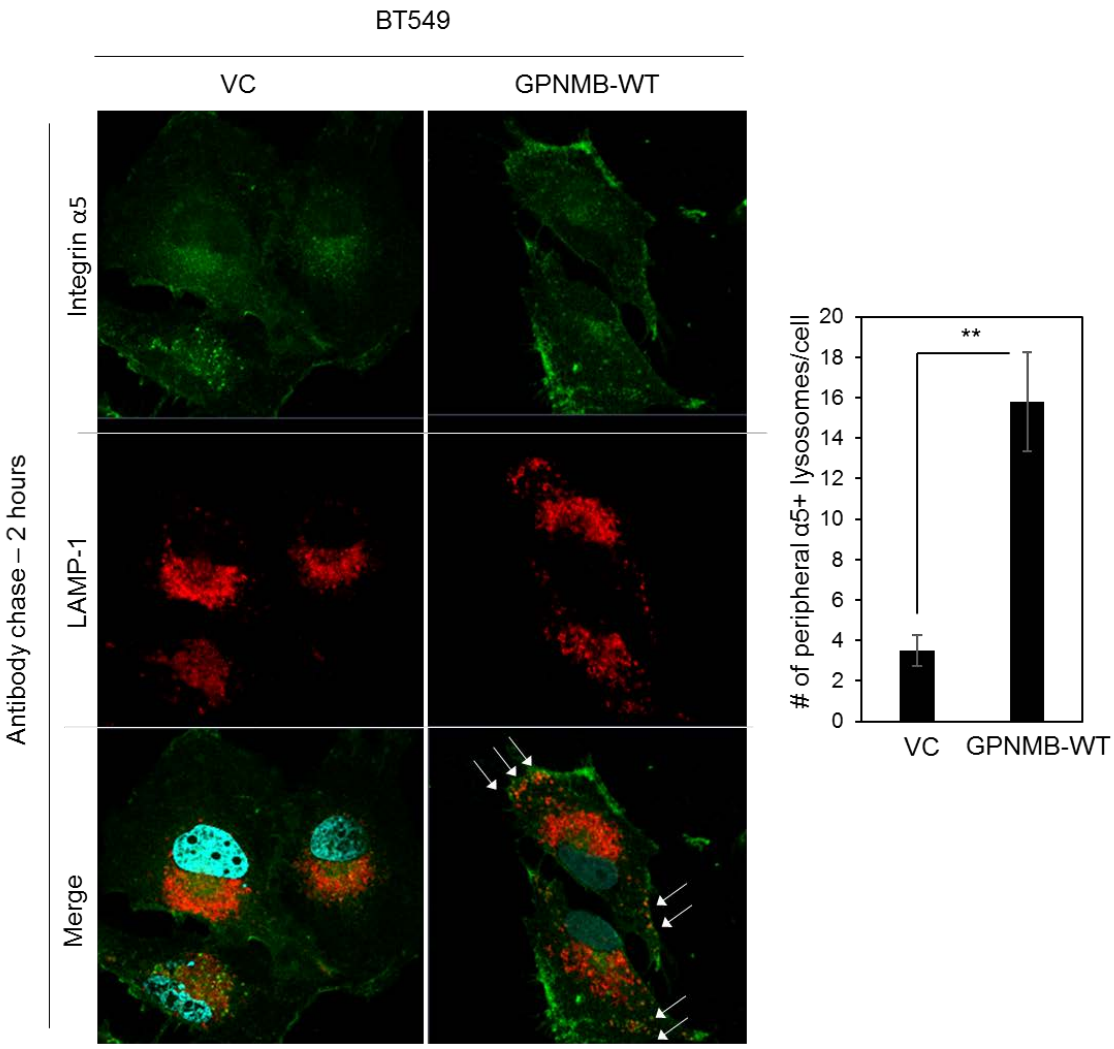


Figure 3.8 GPNMB promotes movement of integrin α5-containing lysosomes towards the cell periphery BT549 VC- and GPNMB-WT-expressing cells were stimulated with fibronectin and labelled with integrin α5 antibody. Following antibody labelling, cells were lifted to 37°C for 2 hours to allow labelled cell-surface proteins to reach the late endosomal and lysosomal compartments. After 2 hours, cells were fixed and co-stained with LAMP-1 to identify late endosomes and lysosomes. Immunofluorescence imaging reveals the presence of individual lysosomes scattered throughout the cytoplasm and near the cell surface in GPNMB-expressing cells. In comparison, localization of lysosomes in control cells was largely restricted to the juxtanuclear region. The quantification represents the average of 15 fields/experiment taken from 3 independent experiment. Representative images are included and arrows denote peripheral lysosomes that co-localize with integrin α5.

complexes through the late endosomal/lysosomal pathway by stimulating peripheral lysosomal scattering.

GPNMB increases signaling pathways associated with late endosomal/lysosomal recycling

The $\alpha_5\beta_1$ receptor is activated by extracellular ligands and recruits a number of signaling molecules, including Fak and Src, to enhance breast cancer cell adhesion, invasion and metastasis [65]. Interestingly, Src is specifically recruited to active $\alpha_5\beta_1$ trafficked from late endosomes [53] and Src transit from late endosomes to the plasma membrane leads to its activation, promotes focal adhesion turnover and cell migration [53, 66]. In addition to increasing active $\alpha_5\beta_1$ recycling (Figure 3.6B) and adhesion to fibronectin (Figure 3.4A), we show that fibronectin-mediated engagement of GPNMB/$\alpha_5\beta_1$ complexes also enhanced outside-in integrin signaling by increasing auto-phosphorylation of Fak and phosphorylation of c-Src (Figure 3.9A). We found that both the cytoplasmic tail and the RGD domain of GPNMB were essential in promoting signaling downstream of $\alpha_5\beta_1$ engagement (Figure 3.9A). Notably, enhanced Src and Fak signaling was not observed in GPNMB-expressing cells in the absence of fibronectin stimulation (Figure 3.9B), suggesting that GPNMB can increase signaling from late endosomes/lysosomes following integrin activation. These results reinforce our current model that GPNMB specifically acts to increase traffic of active $\alpha_5\beta_1$ integrin receptors from the late endosome to the plasma membrane.

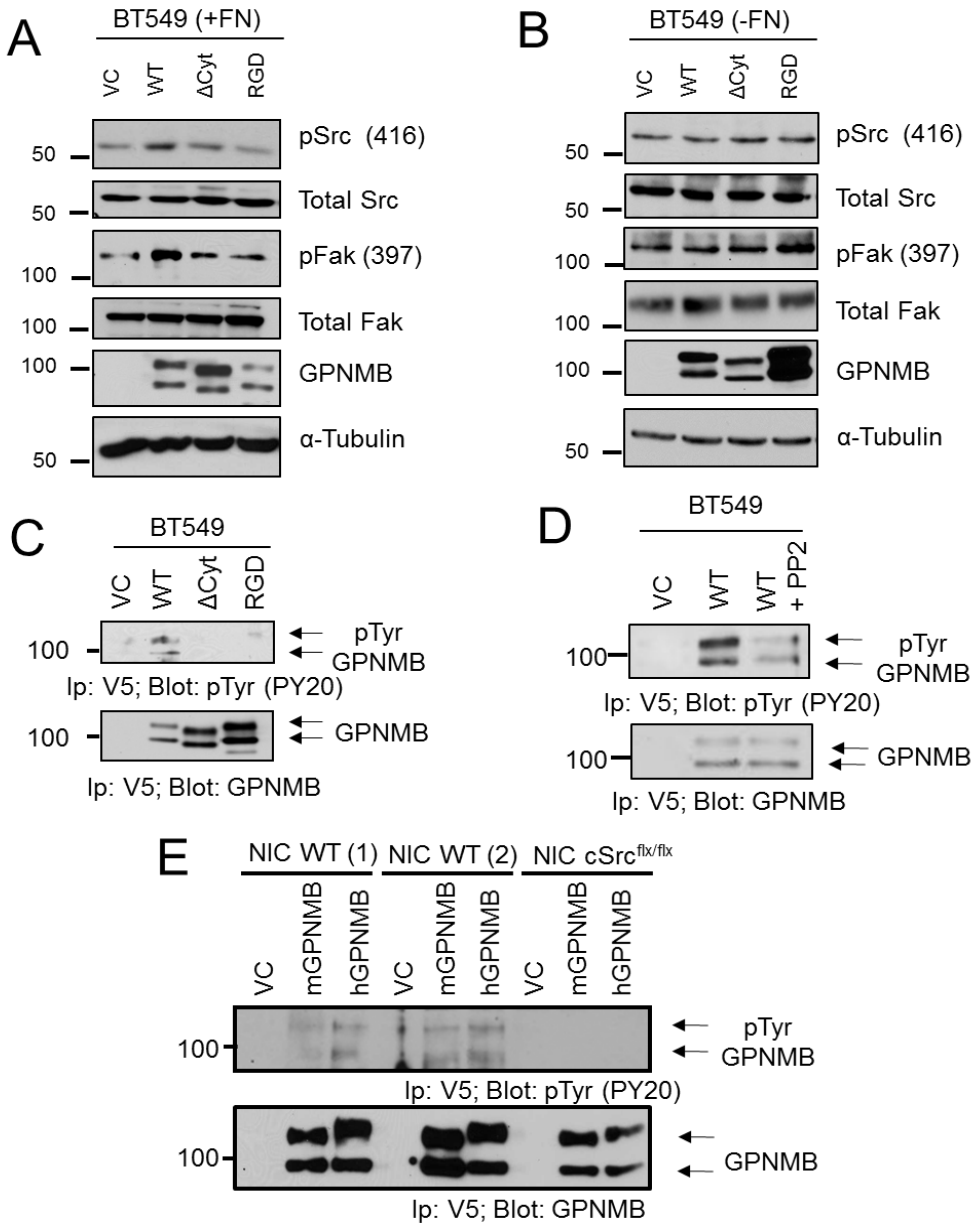


Figure 3.9 GPNMB recruitment to active integrin complexes promotes downstream signaling and induces GPNMB phosphorylation Lysates taken from BT549 cells plated on fibronectin (**A**) or plastic (**B**) were assayed for expression of pSrc, total Src, pFak, total Fak and GPNMB. (C) GPNMB was immunoprecipitated from BT549 VC, GPNMB-WT, GPNMB-CYT and GPNMB-RGD cells plated on fibronectin for 24 hours. The bound fraction was assayed for GPNMB phosphorylation by immunoblotting with a general phospho-tyrosine antibody. (D) GPNMB-WT cells plated on fibronectin were treated with PP2 to inhibit SFK activity prior to immunoprecipitation and pTyr immunoblotting. (E) Human and mouse GPNMB constructs overexpressed in NIC and NIC Src null cells were immunoprecipitated following 24 hours of fibronectin stimulation and assayed for GPNMB phosphorylation.

GPNMB has a hemITAM motif on its cytoplasmic tail, which is a putative target site for phosphorylation by Src-family kinases [5]. GPNMB phosphorylation has been detected in physiological conditions and was accompanied by modulation of gene and protein expression [3]. Src-mediated phosphorylation of the GPNMB hemITAM motif has also recently been observed in NMuMG mouse mammary carcinoma cells and was required for the tumor-promoting properties of GPNMB in this setting [67]. Furthermore, EGFR-dependent GPNMB phosphorylation is seen in triple-negative breast cancer cells, correlates with TNBC, and is predictive of poor prognosis in this aggressive subtype [68]. Given that we observed Src activation downstream of GPNMB/$\alpha_5\beta_1$ complex formation, we next determined if fibronectin stimulation could lead to reciprocal phosphorylation on the cytoplasmic tail of GPNMB. BT549 VC, GPNMB-WT, GPNMB-ΔCYT and GPNMB-RGD_{mut} cells were plated on fibronectin to induce GPNMB/$\alpha_5\beta_1$ association and Src activation. Fibronectin engagement of integrin complexes promoted tyrosine phosphorylation of full-length GPNMB, but not of the GPNMB-ΔCYT mutant, thereby indicating that GPNMB is phosphorylated on its cytoplasmic tail (Figure 3.9C). We did not observe phosphorylation in GPNMB-RGD_{mut} cells, which confirmed that physical interaction between GPNMB and $\alpha_5\beta_1$ was a prerequisite for GPNMB phosphorylation (Figure 3.9C). Subsequently, GPNMB-expressing cells were treated with PP2 to inhibit Src-family kinases and this treatment blunted fibronectin-stimulated GPNMB phosphorylation (Figure 3.9D). Furthermore, we detected GPNMB phosphorylation in two independent NIC mouse mammary carcinoma cell lines but not in NIC cells derived from Src knockout mice (Figure 3.9E). Taken together, these results demonstrate that Src recruitment to active GPNMB/$\alpha_5\beta_1$ complexes promotes reciprocal activation of Src and GPNMB.

3.5 Discussion

Our study suggests that GPNMB enhances primary tumor growth and metastasis by independent and non-redundant downstream mechanisms. We describe the generation of GPNMB domain mutants to dissect the different mechanisms at play during discrete steps of the breast cancer metastatic cascade. This approach outlines a central role for the GPNMB RGD domain in breast cancer metastasis and reveals that both the GPNMB cytoplasmic tail and RGD domain are required for primary tumor growth. Mechanistically, we show that GPNMB can bind the $\alpha_5\beta_1$ fibronectin receptor in tumor cells through its RGD domain to enhance integrin function and downstream signaling pathways implicated in metastasis. We demonstrate that GPNMB increases expression of integrin $\alpha_5\beta_1$ in an RGD-dependent fashion *in vitro* and *in vivo* by enhancing integrin stability downstream of fibronectin engagement. Additionally, GPNMB inhibited lysosomal degradation of active integrin $\alpha_5\beta_1$ by physically associating with the heterodimer and re-routing it to the cell surface from late endosomes/lysosomes. We show that the active integrin $\alpha_5\beta_1$/GPNMB complex recruits Src and Fak and activates downstream signaling pathways, which subsequently induce reciprocal phosphorylation of GPNMB on tyrosine residues within the cytoplasmic tail. Interestingly, GPNMB expression was strongly correlated with $\alpha_5\beta_1$ expression in a number of human breast cancer datasets, which confers clinical relevance to our findings. However, the correlation strength between GPNMB and the α_5 integrin subunit is likely underrepresented in the human breast cancer datasets, as our studies show that the GPNMB-mediated increase in α_5 levels occurs primarily at the protein level. Overall, our data describes the existence of a novel interplay between GPNMB and active integrin $\alpha_5\beta_1$ in promoting breast cancer metastasis.

Our results implicate the RGD motif within GPNMB as an important determinant of its ability to promote breast cancer invasion *in vitro* and tumor growth and metastasis *in vivo*. Interestingly, although the cytoplasmic tail is important for GPNMB-mediated invasion *in vitro* and functions to augment tumor growth *in vivo*, this region of GPNMB is dispensable for promoting breast cancer metastasis to the lungs (Figure 3.1, 3.2). This discrepancy underscores the limitations of such in vitro assays and suggests that metastatic progression in vivo is more heavily influenced by tumor-stroma and tumor cell-tumor cell interactions mediated through the GPNMB RGD motif. Previous studies have shown that cell-ECM and cell-cell interactions mediated by integrin recognition of RGD motifs are crucial determinants of the metastatic process [69, 70]. Accordingly, we hypothesize that there is an increased reliance on the extracellular domain and shed form of GPNMB during metastasis *in vivo*, through RGD-mediated autocrine and paracrine signaling.

We show that full-length GPNMB physically interacts with $\alpha_5\beta_1$ in an RGD-dependent manner to increase active $\alpha_5\beta_1$ recycling, stability, and signaling. Our results indicate that formation of GPNMB/integrin complexes occurs in intracellular compartments within tumor cells, which is in line with physiological studies that have shown that GPNMB can modulate $\alpha_5\beta_1$ action in a cell-intrinsic fashion [71]. Notably, increased Fak phosphorylation has recently been reported in osteoclasts overexpressing GPNMB, supporting a tumor-intrinsic role for GPNMB in integrin engagement [71]. To our knowledge, this is the first evidence of an RGD-mediated association between the $\alpha_5\beta_1$ fibronectin receptor and a transmembrane protein. Additionally, our results indicate that GPNMB preferentially interacts with $\alpha_5\beta_1$ in the presence of fibronectin, which is known to activate the $\alpha_5\beta_1$ integrin heterodimer [72]. It may be possible that GPNMB is localized to regions of high integrin receptor density in cell surface adhesions, where some integrin

complexes have engaged the ECM while others interact with GPNMB. However, our results suggest that at least part of the interaction between GPNMB and the active $\alpha_5\beta_1$ integrin receptor occurs in endocytic vesicles. This data raises important questions about the stoichiometry and affinity of integrin/ligand interactions. It is thought that fibronectin dissociation from $\alpha_5\beta_1$ integrin complexes is a result of low pH conditions, which normally occurs in late endosome and lysosome compartments [53]. Whether $\alpha_5\beta_1$ exhibits preferential affinity for GPNMB over fibronectin in acidic environments, and whether GPNMB can competitively inhibit fibronectin binding in these conditions, warrants further investigation. Additionally, increasing data argues that the acidification of the immediate extracellular microenvironment can augment the malignant phenotypes of cancer cells [73, 74]. Indeed, clinical investigations show that tumors with an acidic environment are associated with poor prognosis and enhanced metastatic incidence. In the future, it would be interesting to determine if GPNMB-expressing cancers thrive in such conditions [74, 75].

Integrin trafficking is a tightly regulated process that is essential for cancer metastasis [34]. Numerous studies have shown that the recycling of active and inactive integrin receptors occurs through different routes and exhibits different kinetics, but the specific mechanisms have not been fully elucidated [42, 44, 46]. Notably, active integrin complexes can undergo ligand-mediated degradation. Inhibitors of this process are pivotal in cancer invasion and metastasis because they allow the tumor cell to maintain and employ a sizeable pool of active integrin receptors. Interestingly, several known mediators of active integrin trafficking, including Rab25 and Rab27b, are correlated with poor prognosis in ER+ breast cancers but are underexpressed or lost in basal breast cancers [60, 76]. Our results suggest that GPNMB can rescue active $\alpha_5\beta_1$ integrin receptors from ligand-mediated

degradation and establish GPNMB as a novel regulator of $\alpha_5\beta_1$ integrin traffic in basal breast cancers.

NRP-1 has been shown to enhance endothelial cell adhesion to fibronectin by increasing internalization of the $\alpha_5\beta_1$ receptor from fibrillar adhesions [42]. Similarly, GIPC bridges the association between NRP-1, $\alpha_5\beta_1$ and c-Abl to augment $\alpha_5\beta_1$ internalization and assembly of fibronectin fibrils by cancer-associated fibroblasts [77]. The formation of a fibronectin-dependent NRP-1/$\alpha_5\beta_1$ complex was also observed in MCF7 breast cancer cells [78]. Additionally, interaction between NRP-1 and active, but not inactive, integrin β_1 was recently identified as an important mediator of cetuximab resistance in prostate cancer [79]. In this setting, activation of Src and Akt is observed downstream of NRP-1/Integrin β_1 complex formation [79]. Despite the ability of GPNMB to increase NRP-1 expression (Figure 2.2), we did not observe enhanced integrin internalization in GPNMB-expressing cells (Figure 3.6A). NRP-1 was previously shown to mediate integrin internalization from fibrillar adhesions, which arise following focal adhesion maturation [42]. However, our assay involved short-term fibronectin stimulation (<1 hour), and this time frame is not sufficient to allow adhesion maturation and to provide a context for NRP-1-mediated integrin internalization. It is possible that NRP-1 could increase internalization of $\alpha_5\beta_1$ in GPNMB-expressing tumors *in vivo* but given the differential roles of NRP-1 and $\alpha_5\beta_1$ in GPNMB-driven metastasis, this mechanism likely does not underlie the phenotype observed in our system.

GPNMB harbors a di-leucine sorting motif in its cytoplasmic tail that is implicated in receptor internalization from the plasma membrane and is important for GPNMB intracellular trafficking to endosomal compartments [80, 81]. Interestingly, mutation of either of the two leucine residues in the cytoplasmic tail of QNR-71, the quail orthologue

of GPNMB, retains it at the cell surface and prevents its routing to endosomal compartments [80]. These findings could explain the phenotype of the GPNMB-ΔCYT mutant, which lacks the di-leucine motif responsible for internalization, and is not as efficient as the full-length protein at increasing stability of the integrin receptor (Figure 3.5). In keeping with this hypothesis, we observe higher cell surface levels of the GPNMB-ΔCYT mutant in BT549 breast cancer cells, compared to GPNMB-WT and GPNMB-RGD-expressing cells, suggesting that GPNMB endocytosis is also impaired in human basal breast cancer cells when the di-leucine motif in the cytoplasmic tail is not present (Supplementary Figure 3.1B).

Together, our results outline a critical role for $\alpha_5\beta_1$ in GPNMB-mediated mammary tumor growth and metastasis. The strong correlation between GPNMB and α_5 expression levels observed in breast cancer samples emphasizes the translational applicability of our findings to breast cancers, such as TNBCs, which express GPNMB. It is conceivable that therapeutic agents that are in clinical trials against GPNMB (CDX-011) may be coupled with agents against RGD-binding integrin receptors to better manage cancers that express high levels of GPNMB.

3.6 Methods

Cell Culture

The BT549 human breast cancer cell line was obtained from the American Type Culture Collection. All BT549-derived pooled cell populations were maintained in RPMI supplemented with 10% FBS. The NIC cell line was established from primary tumor explants derived from the MMTV/NIC transgenic mouse model. NIC cells were grown in DMEM supplemented with 5% FBS, 35 ug/mL bovine pituitary extract, 5 ug/mL insulin,

1 ug/mL hydrocortisone and 5 ng/mL hEGF. The MDA-MB-468 human breast cancer cell line was obtained from the ATCC and was grown in Leibovitz's L-15 media supplemented with 10% fetal bovine serum, 10 μg/mL insulin and 1.5g/L sodium bicarbonate. All cell lines were maintained in complete media unless otherwise indicated and fungizone and gentamycin was added to all media to minimize contamination risk.

Virus production was achieved using the 293VSV packaging cell line (Clontech), which was maintained in 10% FBS and 5 ng/mL tetracycline. Retroviral vectors and pVSVG helper plasmids were transfected into the 293VSV cells using effectene (Qiagen, Cat # 3901427). Subsequently, starting from the second day post-transfection, virus was harvested for 5 days from the 293VSVs. Target cells were infected by a 24-hour incubation with filtered virus-containing media and equal volumes of their respective media. Polybrene (8 ug/uL) was used to augment infection efficiency.

DNA constructs

Full-length human GPNMB cDNA was purchased from Open BioSystems (Clone ID: 5177095) and shuttled into *Eco*RI/*Not*I restriction enzyme sites of the pEF1/V5-His expression vector (Invitrogen). To generate the GPNMB ΔCYT mutant, PCR amplification was performed using the full length GPNMB cDNA as a template and the following primers: 5' GCCACCATCACAATTGTAGAGGG 3' (fwd) and 5' GAGCGGCCGCCATTCCC TGTGTTTTTTGTACACCAAGAGGG 3' (rev). The GPNMB RGD-RAA mutant was created with the QuikChange site directed mutagenesis kit using the wildtype GPNMB cDNA as template (Stratagene), following the manufacturer's instructions. The cDNAs encoding wildtype GPNMB, GPNMB ΔCYT and

GPNMB RGD-RAA were released as *Eco*RI fragments from the pEF1 vectors, shuttled into pMSCVpuro (Clontech) and transfected into indicated cell lines.

Primary tumor growth, spontaneous metastasis assays and analysis of tumor tissue

For *in vivo* studies, $5x10^5$ breast cancer cells were resuspended in a 50/50 mixture of 1X PBS:matrigel and injected into the mammary fat pads of athymic mice. Tumor volumes were determined by caliper measurement and tumor volume was calculated according to the following formula: $\pi LW^2/6$ where L refers to the length and W to the width of the tumor. Mammary tumors were resected at a volume of 500 mm^3, and tumor tissue was harvested for immunoblot or immunohistochemical analysis. Animals were sacrificed 5 weeks post-resection and lungs were harvested, fixed in 4% paraformaldehyde and embedded in paraffin. Lung metastatic burden was quantified from 4 Hematoxylin & Eosin (H&E) stained step sections (80 μm between each step section) using Aperio Imagescope software. Metastatic tissue was delineated and quantified from 4 step sections and expressed as a percentage of total lung area. Mice were housed in facilities managed by the McGill University Animal Resources Centre and all animal experiments were conducted under a McGill University approved Animal Use Protocol in accordance with guidelines established by the Canadian Council on Animal Care.

To examine VEGF levels in NIC populations, resected tumors were flash frozen in liquid nitrogen, ground up with a mortar and pestle, and resuspended in TNE lysis buffer. VEGF protein was quantified using a commercially available ELISA kit (R&D systems) and the associated manufacturer's protocol. VEGF values were normalized to total tumor lysate concentrations.

To quantify F4/80 staining, the positive pixel count was determined using Imagescope software (Aperio). Ten 20x images were analyzed for every stained tumor sample and F4/80 positive pixels were expressed as a percentage of the total pixels per field.

Immunohistochemistry analysis

Tissue samples were fixed, processed and stained with Ki67, cleaved caspase-3 or CD31 antibodies as previously described [82].

Immunoblotting and Immunoprecipitation

Membranes were prepared and processed as previously described [82] using the primary antibodies listed in Supplementary Table 3.1. Where applicable, immunoblots were quantified using the Plot Lanes function on ImageJ and the average from three representative experiments was taken.

To examine protein complex formation and GPNMB phosphorylation, cells were plated on fibronectin (GPNMB phosphorylation and Co-IP assays) or plastic (Co-IP assays only) for 24 hours. Confluent plates were lysed on ice for 20 minutes in Lysis Buffer containing 0.1% NP-40, 50nM Na-HEPES, 100mM KCl, 2mM NaEDTA and protease inhibitors. After 10 minutes, cell lysates were subjected to a freeze-thaw cycle using liquid nitrogen. 1.5mg of protein was pre-cleared with Gammabind G Sepharose beads (GE Healthcare) then immunoprecipitated with either Anti-V5 Agarose Affinity Gel (Sigma) or sepharose beads incubated with 1ug of Integrin α5β1 (Millipore) antibody by rotating overnight at 4°C. The following day, precipitated proteins were washed 4 times in lysis

buffer, twice with 1M KCl and resuspended in 2X SDS-PAGE loading buffer supplemented with 6% β-mercaptoethanol.

Fluorescence-activated cell sorting analysis

To examine cell-surface expression of the α5β1 receptor, 1 x 10^6 BT549 cells were resuspended in PBS supplemented with 2% fetal bovine serum and incubated on ice for 1 hour with 10 μg/mL of the α5β1 antibody (Millipore, Cat # MAB1999) or mouse IgG control antibody (BD Biosciences). Cells were washed 3 times with PBS containing 2% FBS to remove unbound antibody and stained on ice for 45 minutes with Alexa fluor 555-conjugated goat anti-mouse secondary antibody (1:10000, Invitrogen). Cell populations were rinsed 3 times and 1 x 10^4 cells were analyzed for cell-surface expression of α5β1 using the FACScan flow cytometer (BD Biosciences).

Biotin-based biochemical assays

For the internalization assay, cells were plated at 70-80% confluency in full serum. After 24 hours, cells were serum-starved for 2 hours in the presence of 100 nM concanamycin. Cells were rinsed 3 times in ice-cold Hank's balanced salt solution (HBS) and incubated with 0.25 mg/mL EZ-link cleavable sulfo-NHS-SS-biotin diluted in HBS by gently shaking for 30 minutes on ice. Biotin was quenched with 3 RPMI washes and internalization was initiated by incubation with 37°C serum-free RPMI or RPMI supplemented with 10% FBS (to activate integrin complexes) for the indicated timepoints. Biotin was stripped using 3 ice-cold 10 minute washes with 60 mM MesNa reducing agent diluted in 50 mM Tris-HCl pH 8.6, 100 mM NaCl, 1 mM EDTA, and 0.2% BSA, followed

by a 10 minute incubation with ice-cold 120 mM iodoacetamide (IAA) quenching agent. Cells were washed twice in ice-cold HBS and lysed in octyl glucoside lysis buffer (1.5% octyl-glucopyranosidase, 1% NP-40, 0.5% BSA and 1mM EDTA).

For the recycling assay, cells were allowed to internalize for 30 minutes using the internalization protocol above. Following quenching with IAA, cells were washed in HBS and lifted to 37°C for the indicated timepoints using pre-warmed serum-free RPMI or RPMI supplemented with 10% FBS. Recycled cell-surface biotin was reduced using 3 10 minute 60 mM MesNa washes, followed by quenching with 120 mM IAA for 10 minutes. Cells were washed twice with HBS and lysed using octyl glucoside lysis buffer. Biotinylated proteins were immunoprecipitated from the lysate with streptavidin beads by rotating at 4°C for 3 hours. Precipitated proteins were washed in HBS + 1% triton and resuspended in 2X SDS-PAGE loading buffer. Lysates were resolved by SDS-PAGE, transferred to Immobilon-FL PVDF transfer membranes, immunoblotted with α5 antibody (Santa Cruz), followed by incubation with infrared-conjugated secondary antibodies. Protein levels were detected and quantified using the Odyssey IR Imaging System (LI-COR Biosciences). For the degradation assay, cells were prepared as above and labelled with biotin for 3 minutes on ice. Following removal of unbound biotin, cells were lifted to 37°C using full-serum media for the indicated timepoints and lysed in octyl glucoside lysis buffer. Lysates were immunoblotted and processed as above.

Immunofluorescence staining

For immunofluorescence staining, 2.5 x 10^4 BT549 cells were seeded onto glass coverslips coated with 50 ug/mL of fibronectin. After 24 hours, cells were rinsed with PBS and fixed using 2% Paraformaldehyde for 20 minutes. Cells were subsequently

permeabilized with 0.2% Triton X-100, rinsed with 100mM glycine in PBS and blocked in blocking buffer (2% BSA, 0.2% Triton X-100 and 0.05% Tween-20 in PBS) for 30 minutes. Samples were incubated at room temperature for 1 hour with the following primary antibodies, diluted in blocking buffer: Integrin α5 (1:200, BD Pharmingen, Cat# 555651), GPNMB (1:100, R&D systems, Cat# AF2550), EEA-1 (1:200, BD Transduction, Cat #610457). Cells were rinsed 3 times in IF buffer (0.2% Triton X-100 + 0.05% Tween in PBS) and respective Alexa Fluor 488-, 546- or 588- conjugated secondary antibodies (1:1000, Invitrogen) were then incubated with the samples for 1 hour at room temperature and counterstained with DAPI (1:5000, Invitrogen, Cat# D3571). For conditions requiring LAMP-1 staining, cells were permeabilized with 0.1% Saponin for 5 minutes, blocked with LAMP-1 blocking buffer (2.5% BSA + 0.05% saponin in PBS) for 30 minutes and incubated for 1 hour with the LAMP-1 antibody (1:800, AbCam, Cat# ab24170) diluted in LAMP-1 blocking buffer. Washes were done with LAMP-1 IF buffer (0.5% BSA + 0.05% saponin). Fluorescence was visualized with a Zeiss LSM510 confocal microscope. Images were acquired under the 100X objective and processed using the ZEN software (Zeiss). Co-localization was quantified using the co-localization function on the ZEN image browser software and represents the average of minimum 10 fields of view taken from 3 independent experiments.

For antibody chase experiments, cells were washed 3X with 1X PBS after 24 hours of plating on fibronectin and incubated on ice for 1 hour with Integrin α5 antibody (1:100) in complete media. The cells were washed again to remove unbound antibody and the plate was lifted to 37°C for the indicated times to allow integrin internalization and recycling in complete media supplemented with 100 nM concanamycin and 50 ug/uL fibronectin. Subsequently, the plates were processed as described above with the exception of the

primary antibody step, where integrin α5 antibody was not added. Image analysis was carried out using the MetaMorph program. Hole filling was enabled to fill the pixels inside lysosome shapes and the granularity application module was used to evaluate co-localization of LAMP-1 and α5. The granularity indices were set to exclude the perinuclear lysosome region and the remaining number of overlapping LAMP-1 and α5 granules (defined as "peripheral") was quantified. Quantification represents the average of 15 fields/experiment taken from 3 independent experiments.

Breast cancer invasion and adhesion assays

Invasion assays were performed as previously described, using $1x10^5$ BT549 or HS578T breast cancer cells [82]. The invasion of NIC cells was assessed with the xCELLigence RTCA system (Roche), using 5% matrigel and $8x10^4$ cells per well, as previously described [83]. The ability of BT549 breast cancer cells to adhere to fibronectin was assessed by plating 8×10^5 cells onto xCELLigence E-plates (Roche) coated with 50 μg/mL fibronectin (Millipore). The rate of cell adhesion was monitored for 1 hour and calculated using the xCELLigence software. Results of all invasion and adhesion assays represent the average of three independent experiments, performed in duplicate.

RNA extraction, cDNA synthesis and quantitative Real-Time PCR

Triplicate RNA samples were extracted from NIC tumors and BT549 cell lines at ~80% confluence using RNeasy Mini Kits (Qiagen) and quantified using a spectrophotometer (Nanodrop ND-1000). Total RNA (1μg per sample) was used to generate cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Oligonucleotide primers were designed using Invitrogen OligoPerfect software and pre-designed primers were identified in Primer Bank (Harvard University) or RT-PCR Design (Roche Diagnostics). All primers were diluted to a concentration of

100μM. RT-qPCR reactions were performed on diluted cDNA (1:20) using Power SYBR Green Master Mix (Applied Biosystems) and 7500 Real Time PCR System (Applied Biosystems). Reactions were performed in triplicate and *GAPDH* or *β-actin* primers were used as a control for mouse and human genes, respectively. Data is represented as the mean of the fold change of the three independent sets of cDNA calculated according to the following formula:

$$\frac{\text{Efficiency of target primers} + 1^{\,(\text{average value of target primers} - \text{average of corresponding target wells})}}{\text{Efficiency of control primers} + 1^{\,(\text{average value of control primers} - \text{average of corresponding control wells})}}$$

Statistical analyses

Significance of the data was assessed using a two-tailed Student's T-test and variance was determined using an online statistics program (Vassar Stats). Indicated annotations correspond to the following P values: * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$. Results are displayed as an average +/- standard error.

Correlations between expression of GPNMB and ITGA5 were studied in datasets available on the UCSC Cancer Genomics Browser (https://genome-cancer.ucsc.edu/). Expression data was ordered according to GPNMB expression and the Pearson correlation coefficients for individual datasets were calculated using MedCalc 13. A meta-analysis under the fixed effects model was subsequently carried out according to the Hedges-Olkin method described on their website: http://www.medcalc.org/manual/meta-analysis-correlation.php.

3.7 Acknowledgements

We would like to thank members of the Siegel laboratory for their thoughtful discussions regarding the current work and are also thankful to Dr. Josie Ursini-Siegel for her critical reading of the manuscript. We are grateful to Colin Ratcliffe and Dmitri Kharitidi for their help in troubleshooting the integrin recycling assays. We would also like to thank Harvey Smith and Dr. William Muller for providing us with the NIC Src-null breast cancer cell line which we used in the GPNMB phosphorylation assays. We acknowledge the Goodman Cancer Research Centre histology core facility and the Advanced BioImaging Facility (ABIF) (McGill University) for routine histological services and use of imaging equipment, respectively. G.M acknowledges studentship support from the Canadian Institutes for Health Research (CIHR). P.M.S. was supported by the Fonds de recherche en santé du Québec (FRSQ) research scholar program (Junior 2) and is currently a McGill University William Dawson Scholar. This research was supported solely by a grant from the CIHR (MOP-119401).

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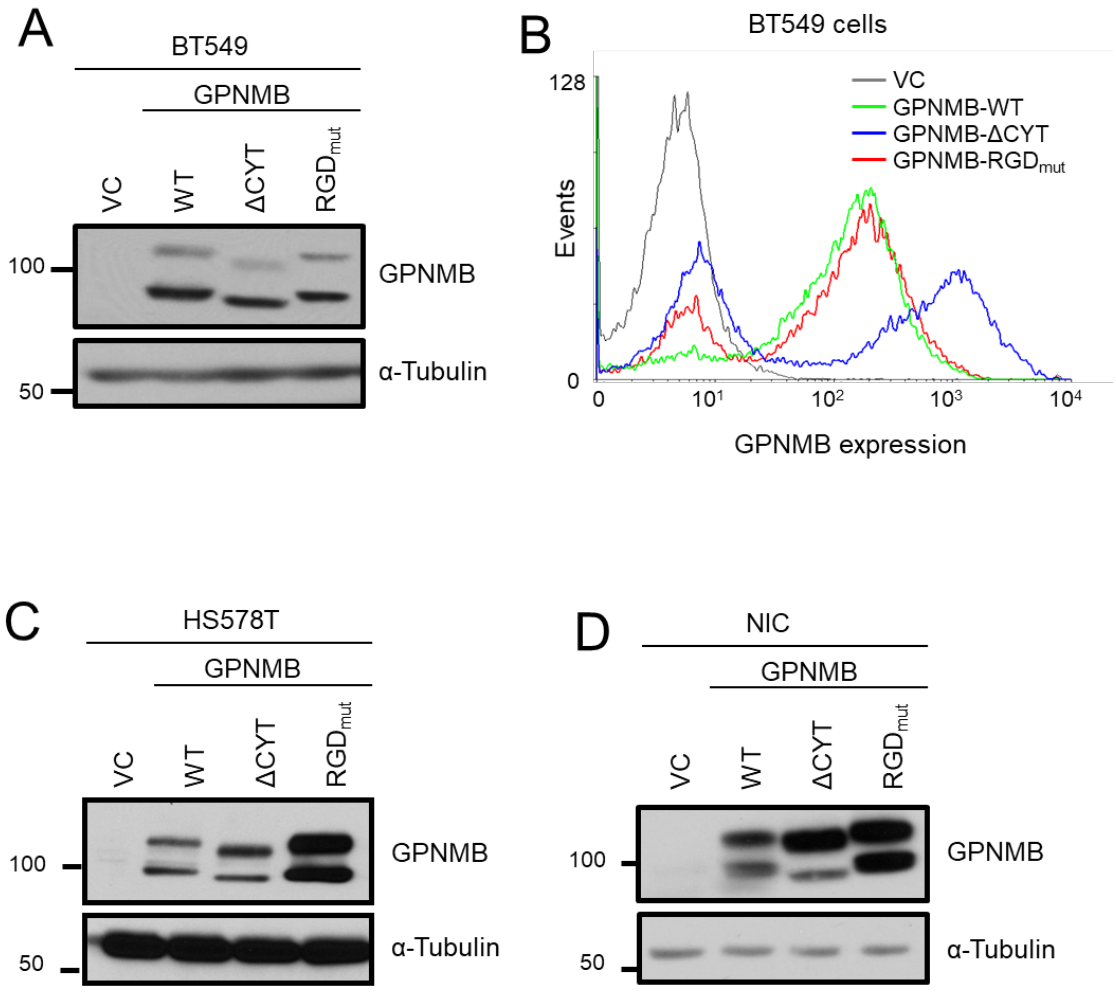
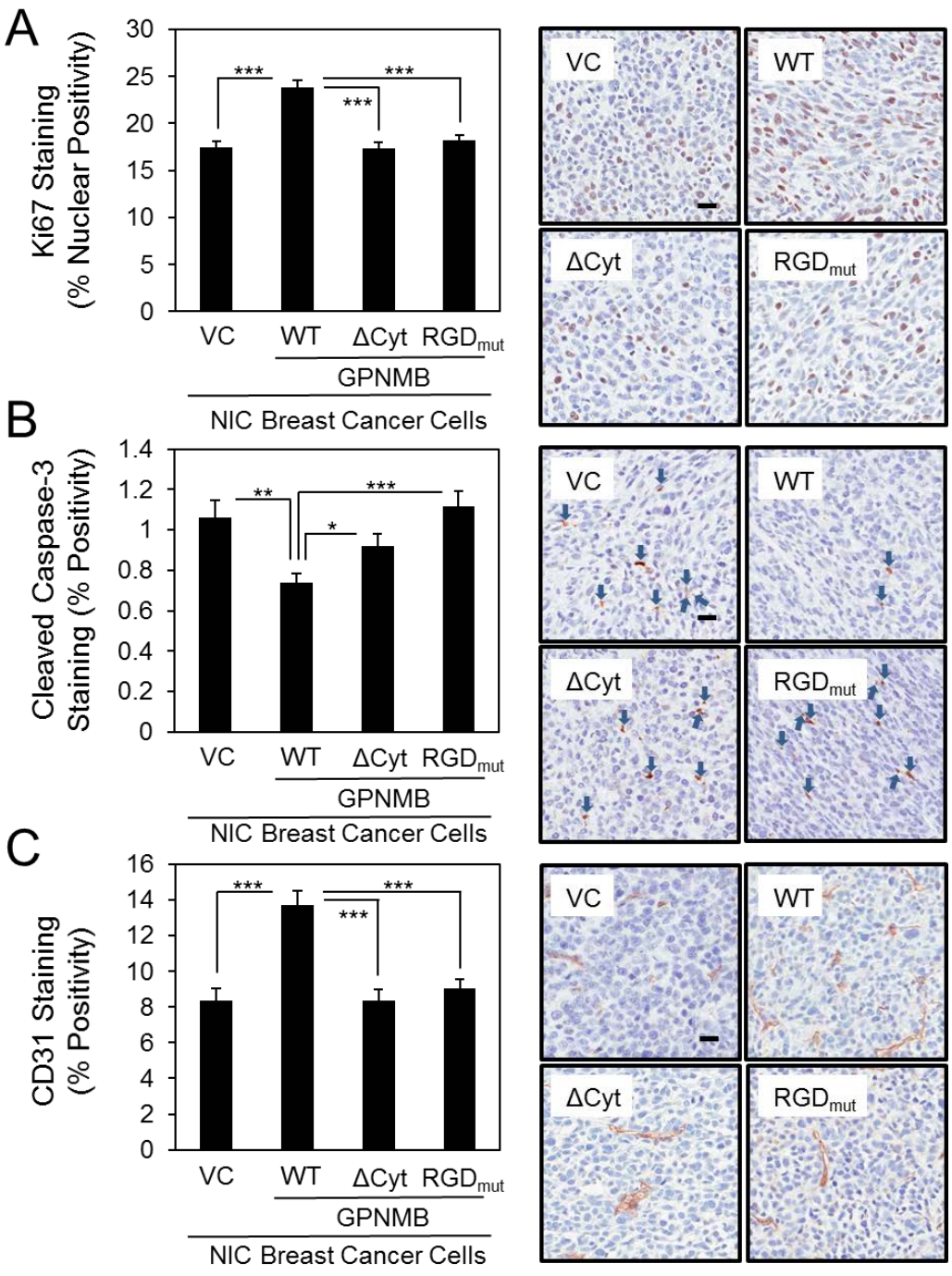
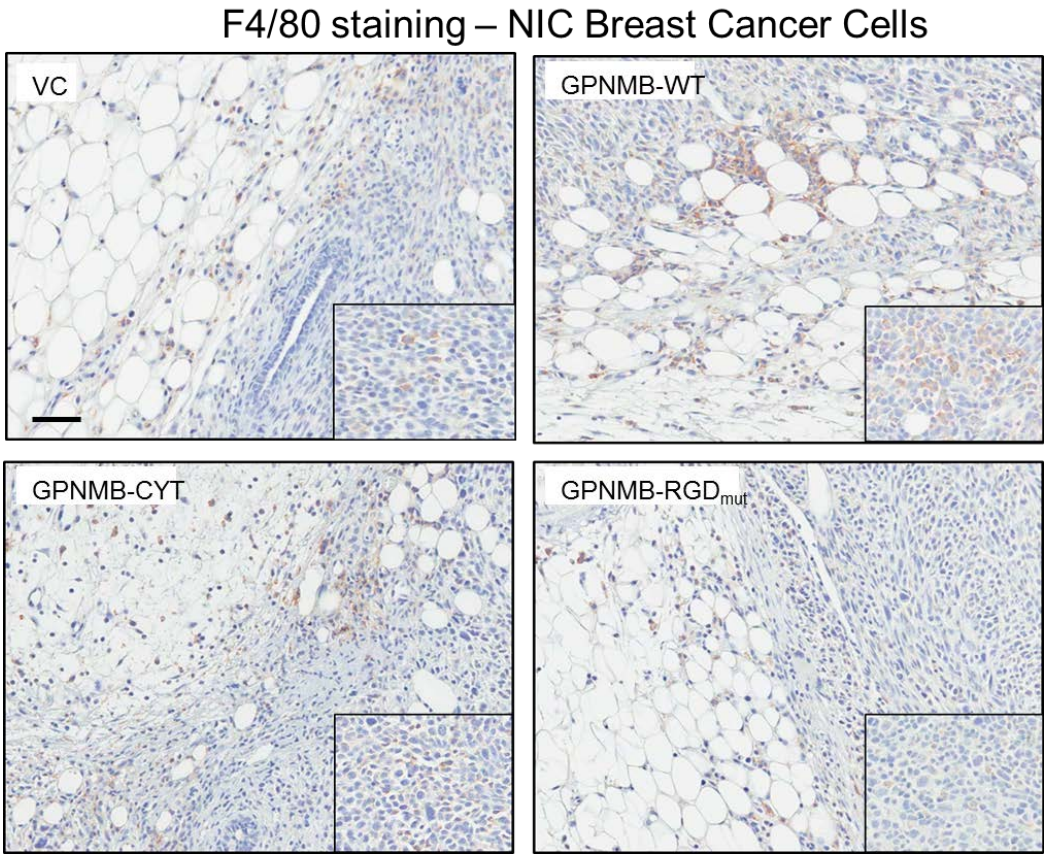


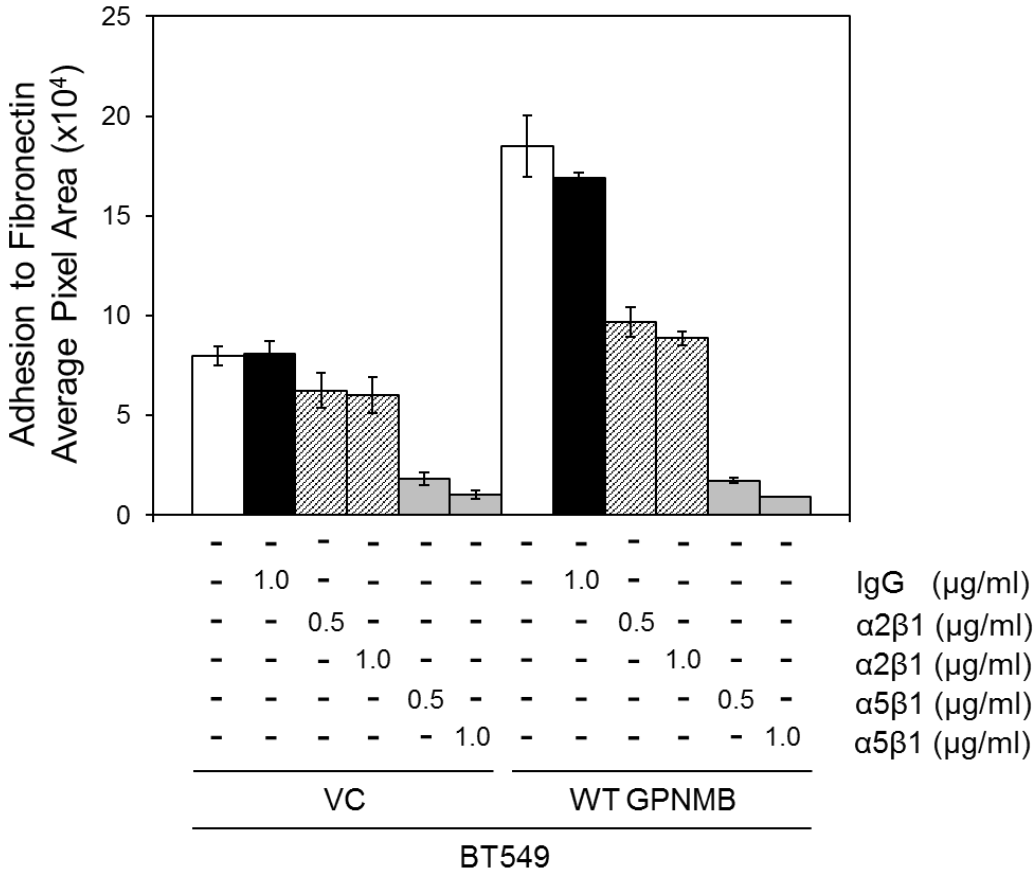
Supplementary Figure 3.1 Expression levels of GPNMB mutants in human and murine breast cancer cell models. GPNMB levels were assayed by immunoblot (**A**) and FACS (**B**) in BT549 breast cancer cells expressing VC, GPNMB-WT, GPNMB-ΔCYT and GPNMB-RGD_{mut} constructs, and by immunoblot in HS578T (**C**), and NIC (**D**) breast cancer cells. α-Tubulin serves as a loading control.



Supplementary Figure 3.2 Immunohistochemical analysis of NIC tumors expressing GPNMB mutant constructs. (**A**) Proliferation of NIC VC, GPNMB-WT, GPNMB-ΔCYT and GPNMB-RGD_{mut} tumors was assessed by Ki67 staining. (***, $p < 0.001$) (**B**) Cleaved Caspase-3 was used as a marker to determine the percentage of apoptosis in NIC tumors. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) (**C**) Endothelial cell recruitment was determined by performing CD31 staining. (***, $p < 0.001$). The average quantification of 10 representative images taken from each tumor sample is shown (n = 5).



Supplementary Figure 3.3 Immunohistochemistry for the macrophage marker F4/80. Macrophage infiltration was examined in NIC VC, GPNMB-WT, GPNMB-ΔCYT and GPNMB-RGD_{mut} expressing mammary tumors by performing immunohistochemistry for F4/80. Representative images of the tumor margin (low magnification) and tumor core (inset) areas are shown. Scale bar represent 50μm and applies to all low magnification images.



Supplementary Figure 3.4 Fibronectin adhesion of GPNMB-expressing BT549 cells is specifically mediated through the $\alpha_5\beta_1$ integrin receptor. BT549 VC and GPNMB-WT cells were incubated with control isotype antibodies or increasing concentrations of antibodies recognizing $\alpha_2\beta_1$ or $\alpha_5\beta_1$ integrin receptors prior to the adhesion assay. Area occupied by BT549 cells was quantified by taking the pixel count from 5 independent images/well. (n=3)

Antibody (immunoblot)	Dilution	Company	Catalogue #
ERK	1:2000	Cell Signaling	9102
pFAK Y397	1:5000	Cell Signaling	3283S
FAK	1:5000	Millipore	06-543
GPNMB	1:2000	R&D	AF2550
Integrin α_1	1:1000	Santa Cruz	SC-10728
Integrin α_2	1:1000	Santa Cruz	SC-74466
Integrin α_5	1:5000	Santa Cruz	SC-10729
Integrin α_V	1:5000	Santa Cruz	SC-6617-R
Integrin β_1	1:5000	Santa Cruz	SC-6622
Integrin β_3	1:2000	Santa Cruz	SC-6626
pSrc Y416	1:2000	Cell Signaling	2101S
Src	1:2000	Millipore	05-184
Phospho-tyrosine			
V5	1:2000	Invitrogen	P/N46-0705
α-Tubulin	1:50000	Sigma	T9026

Supplementary Table 3.1: A list of all the antibodies used in the current study.

CHAPTER 4

GPNMB augments Wnt-1-mediated breast tumor initiation and growth by enhancing PI3K/AKT/mTOR pathway signaling and β-catenin activity

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4.1 Preface

Triple-negative breast cancers represent a heterogeneous constellation of diseases characterized by distinct biology, prognosis and driver signaling pathways. Using basal breast cancer cell lines which mimic the basal-like TNBC subtypes, we have demonstrated that GPNMB engages discrete mechanisms and cooperates with separate signaling pathways to promote tumor growth and metastasis. We next sought to understand whether GPNMB possessed oncogenic properties. To address this question, we generated transgenic mice expressing GPNMB under the control of the MMTV promoter/enhancer. Additionally, to extend our observations to another model of TNBC and identify mechanisms engaged in a separate oncogenic context, we overexpressed GPNMB in the MMTV/Wnt-1 transgenic model, which has been shown to faithfully recapitulate human basal breast cancers. Furthermore, Wnt signaling is activated in a subset of TNBCs and represents an important potential therapeutic target for this disease. Herein, we identify a novel mechanism of action for GPNMB in breast cancer, which is engaged specifically downstream of canonical Wnt signaling.

4.2 Abstract

To further explore the role of GPNMB in mammary tumorigenesis and metastasis, we have generated transgenic mouse lines characterized by MMTV promoter/enhancer-driven GPNMB expression. We confirmed GPNMB expression in the mammary gland of 4, 6, 8, and 10-week-old mice using RT-PCR and immunohistochemistry. Given that GPNMB is most frequently expressed in human basal breast cancers, we generated bigenic mouse strains by crossing our MMTV/GPNMB mice with MMTV/Wnt1 mice, which represent a model of basal breast cancer and are known to develop mammary tumors within 6 months. Our data from the bigenic crosses reveals that GPNMB leads to a 2-month reduction in latency of tumor onset in MMTV/Wnt1 mice. Additionally, MMTV/Wnt1/GPNMB mice exhibited a significant increase in the rate of primary tumor growth, which was attributed to a twofold increase in proliferation, as evidenced by Ki67 staining. We interrogated activated pathways in tumors derived from the two transgenic lines using RPPA analysis, which revealed that MMTV/Wnt1/GPNMB tumors exhibit a pro-growth signature characterized by elevated PI3K/AKT/mTOR signaling and increased β-catenin activity. Taken together, these data ascribe a novel, pro-growth role for GPNMB in basal breast cancers.

4.3 Introduction

Breast cancers display extensive tumor heterogeneity, which poses a major challenge in developing treatments for the disease [1]. The genomic and molecular diversity of breast cancers have given rise to a classification system that outlines distinct disease subtypes characterized by differences in relapse, survival and response to therapy [2, 3]. These sub-classifications are constantly being refined as advancements in genomic analysis and gene expression profiling are made [4, 5]. Triple-negative breast cancers (TNBCs) represent the most aggressive disease subtype and are associated with a high grade, a poor prognosis and a paucity of therapeutic targets [2, 6, 7].

Glycoprotein (transmembrane) Nmb is a cell surface protein expressed in numerous cancers and often enhances aggressive characteristics of tumor cells [8, 9]. We have previously shown that GPNMB is overexpressed in triple-negative and basal-like breast cancers and is predictive of poor prognosis, even within this aggressive subtype [10]. GPNMB can be shed from breast cancer cells by ADAM family proteases [11] and it has recently been shown that soluble GPNMB levels can be detected in HER2+ breast cancer at significantly higher levels when compared to luminal breast cancers [12]. Furthermore, breast cancer cell lines expressing GPNMB display an increased capacity for EMT, invasion, tumor growth and metastasis [10, 11, 13-15]. Mechanistically, GPNMB can promote SRC and FAK signaling downstream of integrin receptor engagement, and can interact with BRK and long noncoding RNA LINK-A to stabilize HIF-1α in normoxic conditions following EGFR activation in breast cancer cells [13, 16]. CDX-011, an antibody-drug-conjugate targeting the extracellular domain of GPNMB, is currently being

evaluated as a targeted therapy for patients with aggressive metastatic breast cancer, with an intense focus on TNBCs [17, 18].

Gene expression profiling of genetically engineered mouse models (GEMMs) of breast cancer has revealed extensive similarities to human breast cancer subtype signatures [19, 20]. Interestingly, tumors derived from MMTV/Wnt-1 transgenic mice segregate closely with human tumors exhibiting a basal-like morphology and a poor clinical outcome, highlighting the suitability of this mouse model for the study of TNBCs [19, 21]. The development of the MMTV/Wnt-1 transgenic mouse model provided the first evidence that Wnt-1 functions as an oncogene in breast cancer [22, 23]. MMTV-driven expression of the Wnt-1 transgene in the mammary epithelium induces extensive mammary duct hyperplasia and promotes adenocarcinoma development in 50% of transgenic females by 6 months of age [22, 24]. MMTV/Wnt-1 mice are characterized by high proliferation and expression of progenitor cell markers [19, 25]. Additionally, these transgenic mice display a unique signature associated with upregulation of canonical and non-canonical Wnt pathway ligands, receptors and signaling adaptors [26].

Wnts compose a family of 19 highly conserved secreted glycoproteins that regulate receptor-mediated signaling pathways involved in human development and disease. The binding of Wnt ligands, such as Wnt-1 and Wnt-3a, to their receptors can elicit signaling through the canonical Wnt pathway, which promotes β-catenin-mediated changes in gene expression to regulate cell proliferation, survival and differentiation [27]. In the absence of Wnt signaling, a degradation complex consisting of GSK3β, Axin and APC triggers β-catenin phosphorylation, leading to its ubiquitination and degradation [28]. During canonical pathway signaling, Wnt binding to Frizzled (FZD) receptors and LRP5/LRP6 co-

receptors promotes recruitment and activation of cytoplasmic Dishevelled (DVL) proteins which stabilize β-catenin by inhibiting the degradation complex. Upon stabilization, β-catenin translocates to the nucleus where it acts as a transcriptional co-activator by binding LEF-1 and TCF transcription factors [29]. Canonical Wnt pathway signaling can be specifically inhibited by Dkk protein family binding to LRP receptors, which prevents the formation of Wnt receptor complexes [30]. Additionally, competitive inhibitors of Wnt binding, such as secreted Frizzled-related proteins (sFRP) and Wnt inhibitory factors (WIF), can act to sequester canonical Wnt ligands away from active receptor complexes and thus inhibit downstream β-catenin signaling [30].

Many cancers are characterized by Wnt pathway upregulation [31, 32]. Mutations that inactivate Axin and APC genes, or that stabilize β-catenin, are commonly found in colorectal cancers and are associated with poor prognosis [33]. Although these mutations are rarely detected in mammary tumors [34, 35], many studies show that the Wnt/β-catenin pathway is activated in breast cancers, particularly in the basal-like or triple-negative subtype [23, 34, 36, 37]. Notably, an elevated ratio of cytoplasmic to membrane β-catenin localization is associated with higher tumor grades in breast cancer [38 2010], and β-catenin activation has been linked to poor prognosis of invasive breast cancer, across all molecular subtypes [39]. Nuclear accumulation of β-catenin in triple-negative breast cancer cells can promote colony formation, tumor growth and migration, protect against chemosensitivity, and maintain stem cell populations, partly by enhancing expression of stem cell target genes, including c-Myc [40]. In many breast cancer models, autocrine mechanisms contribute to Wnt pathway hyperactivation and can promote mammary tumor initiation, growth and metastasis [41-43]. Canonical Wnt ligands and their receptors are often co-

expressed in breast cancers and the Wnt receptor FZD7 is part of a classification signature that identifies basal-like breast cancers [34, 43-45]. Additionally, the promoter region of sFRP1 is hypermethylated in most breast cancers, which leads to deregulated Wnt signaling and poor prognosis [46-48]. Inhibition of Wnt/β-catenin pathway signaling in TNBC cell lines, through FZD receptor knockdown or sFRP1 expression, leads to altered expression of Wnt target genes and suppresses tumor progression [42, 43, 49].

Autocrine Wnt signaling is an important driver of tumor outgrowth at secondary sites [50-53]. Notably, canonical Wnt signaling is the most highly upregulated pathway in early lung metastatic lesions [50] and is able to enhance metastatic potential of breast cancer cells in orthotopic and intravenous models of metastasis by regulating self-renewal of cancer stem cell populations [53]. In order to efficiently metastasize to the lung, breast cancer cells stimulate the expression of Periostin (POSTN) by the lung metastatic niche, which in turn binds to Wnt-1 and Wnt-3a ligands to activate canonical Wnt signaling and growth of metastatic lesions [52]. POSTN secretion is not required for metastasis of Wnt-driven cancers, indicating that sustained Wnt signaling appears to be a primary driver of breast cancer lung metastasis [52]. Interestingly, upregulation of Wnt/β-catenin signaling appears to dictate organotropism of TNBCs and specifically increase patient risk of lung and brain, but not bone metastasis [54].

While biological effects downstream of Wnt-1 are primarily orchestrated through the canonical signaling pathway, certain Wnts such as Wnt-5a, Wnt-5b and Wnt-11, mediate their effects through non-canonical pathways that don't depend on β-catenin stabilization [31]. The two most well-characterized non-canonical pathways include the Planal Cell Polarity (PCP) pathway, which influences cellular migration and the

establishment of cell polarity [55], and the Wnt/calcium pathway, which induces calcium-dependent cytoskeletal and transcriptional changes [56]. Binding of non-canonical Wnt ligands to FZD and Ror2 or Ryk co-receptors can simultaneously repress β-catenin-dependent transcription and regulate cytoskeleton rearrangement, invasion and metastasis by activating Rho, Rac and CDC42 GTPases [55]. sFRPs and WIFs, which act to sequester canonical Wnt ligands, can also bind non-canonical Wnts to inhibit downstream signaling and thereby de-repress β-catenin-mediated changes in transcription [30].

Non-canonical signaling has been implicated in the suppression of tumor growth due to the inhibitory effect that non-canonical Wnt ligands exert on β-catenin signaling. Genetic ablation of Wnt-5a in PyMT transgenic mice, a luminal model of breast cancer, results in a phenotypic switch to basal-like breast cancer exhibiting enhanced Wnt/β-catenin signaling [57], and similarly, introducing the Wnt-5a transgene in the MMTV/Wnt-1 model decreases basal lineage markers and suppresses tumor formation [58]. Although non-canonical Wnt ligands usually suppress tumor growth, they can promote migration and invasion of various cancers in a β-catenin-independent manner [55]. Exosomes produced by fibroblasts in the tumor microenvironment regulate protrusive activity, migration and metastasis of breast cancer cells by influencing the processing and secretion of non-canonical Wnt ligands [59, 60]. Additionally, DKK1 influences breast cancer organotropism and specifically inhibits lung metastasis by antagonizing non-canonical Wnt PCP and Ca2+ signaling in the lung [61]. In this regard, loss of Wnt-5a in breast cancer has been linked to early relapse, EMT and increased metastatic potential [62, 63].

Canonical Wnt pathway activation is not sufficient to promote cancer progression, as is evidenced by studies showing that constitutively active β-catenin can simultaneously

drive pro- and anti-tumorigenic effects [64, 65]. Furthermore, MMTV/Wnt-1-induced mammary tumors display a very heterogeneous morphology and arise following a long latency period, indicating that Wnt-1 requires cooperation with additional oncogenic stimuli in order to promote cancer progression [23]. We have previously shown that GPNMB expression augments the invasive and metastatic phenotypes of established breast cancer cells [10, 11, 13, 14]; however, it is not known whether GPNMB can influence breast cancer initiation. We now show that GPNMB can accelerate Wnt-1-driven mammary tumorigenesis and increase primary tumor growth through upregulation of the PI3k/Akt/mTOR pathway and enhanced β-catenin signaling. Additionally, we introduce MMTV/GPNMB transgenic mice as a potential model for the study of GPNMB, an emerging therapeutic target in TNBCs.

4.4 Results

MMTV/GPNMB transgenic mice express GPNMB in the mammary ductal epithelium and display normal virgin mammary gland development

To better understand the role of GPNMB in mammary gland development and tumorigenesis, we established ten independent founder lines that harbored the MMTV/GPNMB transgene in a FVB/N background. Mammary glands were collected from transgene-positive females and transgene-negative littermates from these lines and *GPNMB* mRNA expression was assessed by RT-qPCR. Female mice from 3 of 10 founder lines expressed *GPNMB* transcripts in the mammary gland at 4, 6, 8 and 10 weeks of age (Figure 4.1A). GPNMB expression was highest in females from founder line #2, which was chosen for subsequent characterization (Figure 4.1A).

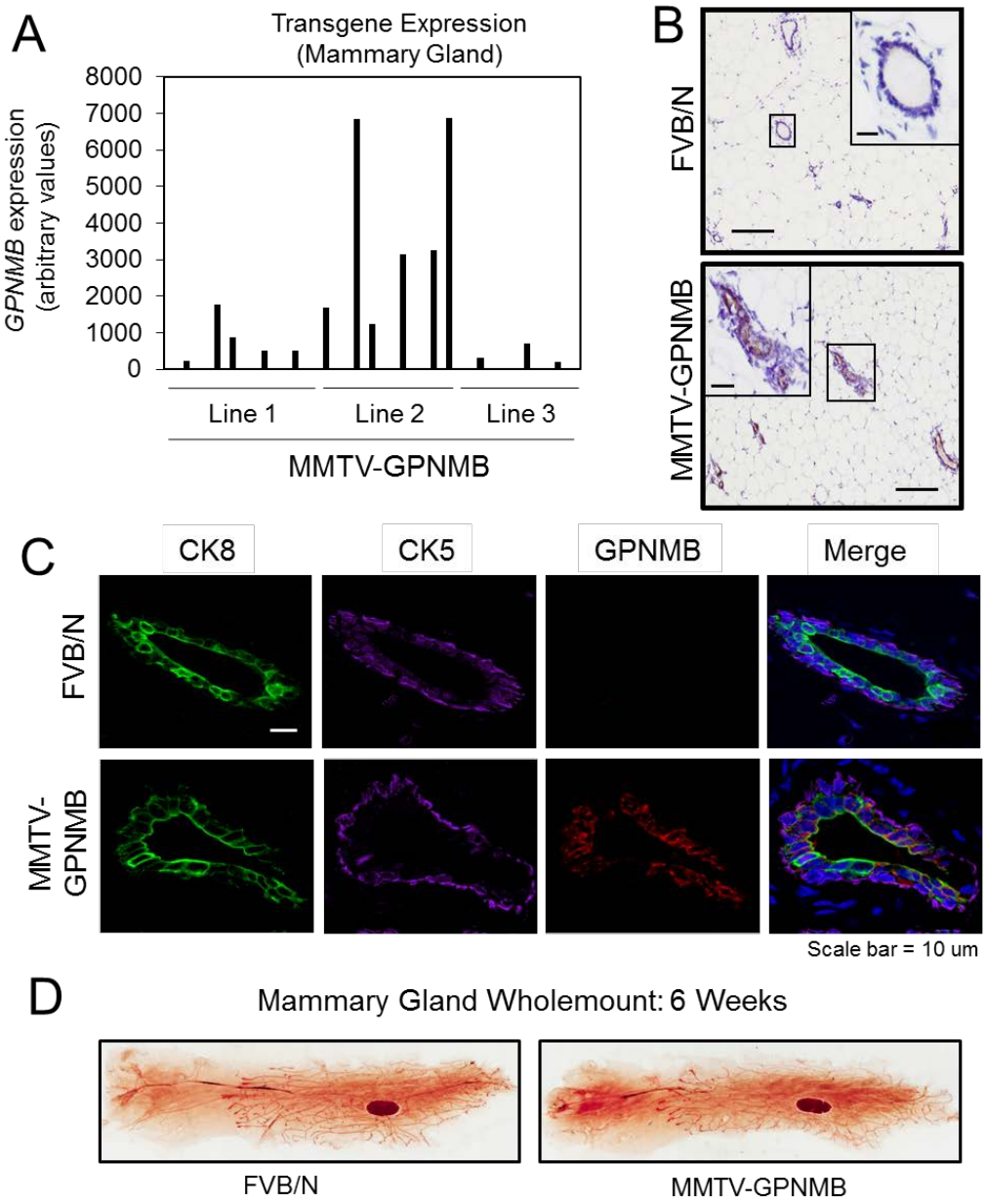

Figure 4.1 MMTV/GPNMB transgenic mice express GPNMB in the mammary ductal epithelium and display normal virgin mammary gland development (A) RT-qPCR analysis of *GPNMB* mRNA expression was performed on total RNA extracted from virgin mammary glands of 4-, 6-, 8-, and 10-week-old MMTV/GPNMB females and littermate controls from 3 independent founder lines. (B) Immuno-histochemistry analysis showing GPNMB expression in virgin mammary glands of 6-week MMTV/GPNMB and WT females. (C) The expression of GPNMB in the alveolar duct of 8-week-old MMTV/GPNMB and WT virgin females was assayed using immunofluorescence staining. Cytokeratin 8 and Cytokeratin 5 antibodies were used as markers of luminal epithelial cells and myoepithelial cells, respectively. (D) Wholemount analysis showing mammary fat pad arborization in virgin 6-week old WT and MMTV/GPNMB females.

Mammary glands from 6-week old transgene-positive and negative females were harvested and examined by immunohistochemistry using antibodies against human GPNMB. GPNMB protein expression was detected in the mammary ductal epithelium of transgene-positive females, but not in the mammary glands of non-transgenic littermate controls (Figure 4.1B). We next performed co-immunofluorescence to examine the pattern of GPNMB expression in the alveolar ducts of 8-week-old MMTV/GPNMB transgenic mice. GPNMB was co-stained with Cytokeratin 8 (CK8) and Cytokeratin 5 (CK5) markers, which were used to identify luminal epithelial cells and myoepithelial cells, respectively [66, 67]. GPNMB staining was detected in $CK8^+$ epithelial cells, but not those expressing CK5, indicating that GPNMB was specifically expressed in luminal epithelial cells (Figure 4.1C). These results are consistent previous reports examining the expression pattern of transgenes driven by the MMTV promoter [68].

Subsequently, we investigated whether overexpression of the GPNMB transgene could impact virgin mammary gland development. Whole-mount analysis was performed to examine the gross architecture of mammary fat pads isolated from 4, 6, 8, and 10 week-old MMTV/GPNMB females and WT littermate controls. Our results revealed that the ductal branching pattern of the mammary gland in transgene-positive females was similar to control littermates at all time points (representative wholemounts from week 6, Figure 4.1D), indicating that GPNMB expression does not alter mammary gland development.

GPNMB expression alone fails to induce mammary tumor formation

We next monitored a cohort of MMTV/GPNMB virgin female mice for mammary tumor formation by weekly palpation in order to determine if GPNMB possessed oncogenic properties. Over the time period that we monitored this cohort (400 days), we failed to

detect spontaneous mammary tumor formation in virgin MMTV/GPNMB female mice (Supplementary Figure 4.1A). These results indicate that GPNMB expression is insufficient to induce mammary tumorigenesis in virgin mammary glands. To examine this further, we monitored a cohort of MMTV/GPNMB multiparous females, as it has been demonstrated that expression of MMTV-driven transgenes is increased during pregnancy [69]. Multiparous MMTV/GPNMB female mice developed spontaneous mammary tumors with a low penetrance (4/25, 16%) and a long latency (12-22 months) (Supplementary Figure 4.1A). In addition, we found that transgene-encoded human GPNMB expression was enriched in all 4 MMTV/GPNMB mammary tumors (Supplementary Figure 4.1B). However, tumorigenesis in this model could be partly attributed to the FVB/N background, as 1 out of the 22 multiparous WT FVB/N females that we examined also developed a spontaneous mammary tumor at 23 months of age (Supplementary Figure 4.1A, B). This is consistent with previous studies that have reported sporadic mammary adenocarcinoma development in aging multiparous FVB/N females [70, 71], and raises the possibility that the mammary tumors observed in aging multiparous MMTV/GPNMB mice do not result from GPNMB expression.

GPNMB accelerates Wnt-1-driven mammary tumorigenesis

Mammary tumors derived from MMTV/Wnt-1 transgenic mice exhibit extensive transcriptional similarities to human basal breast cancers [19, 20, 72] and display upregulated GPNMB transcription compared to tumors arising in transgenic models of luminal breast cancer [73], thus potentially implicating GPNMB in Wnt-1-mediated mammary tumor progression. To examine the suitability of the MMTV/Wnt-1 transgenic mouse model for further study of GPNMB involvement in breast cancer, we interrogated

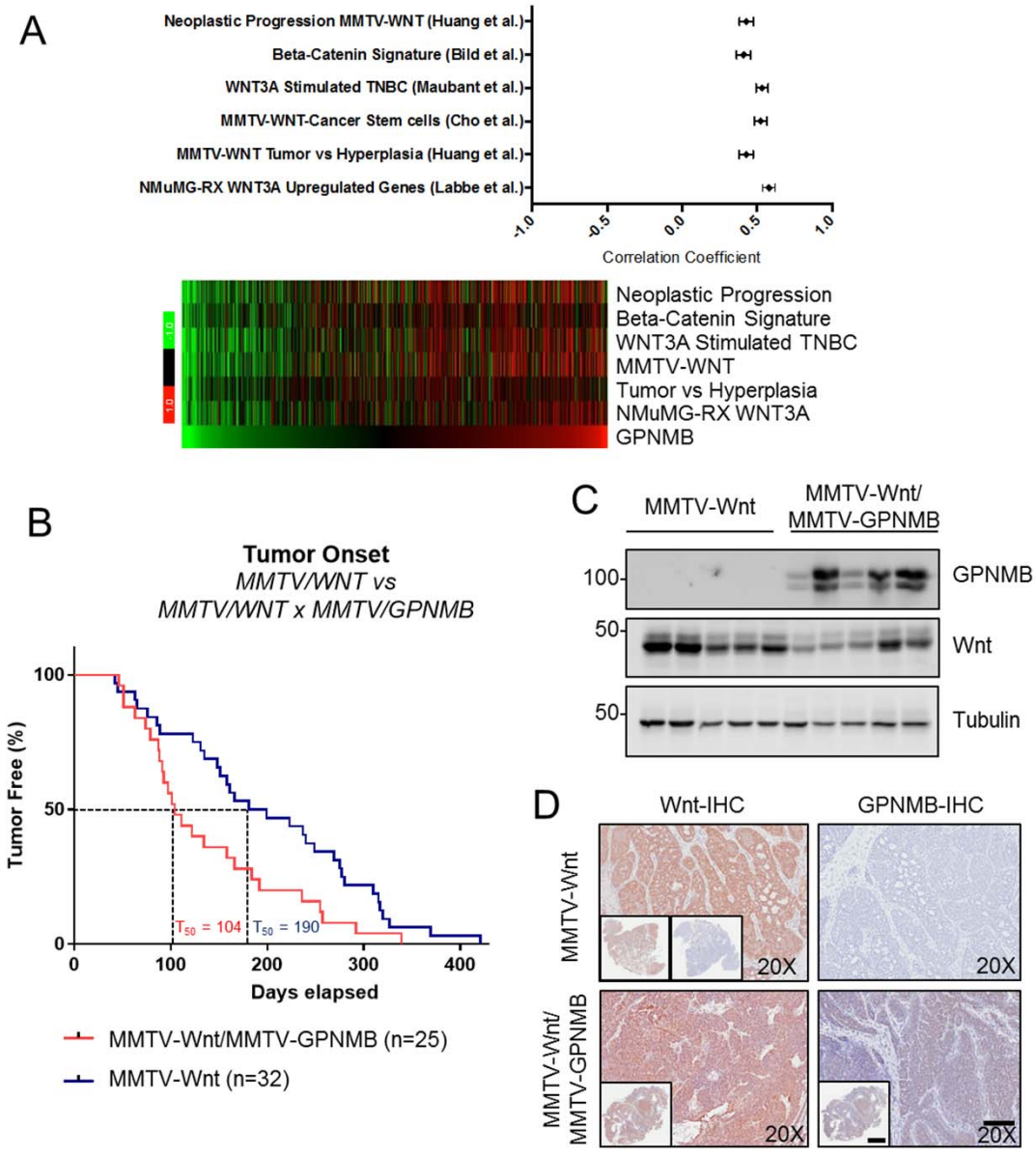

Figure 4.2 GPNMB accelerates Wnt-1-driven mammary tumor onset (A) The correlations between expression of GPNMB and transcriptional targets of 6 independent Wnt/β-catenin signatures are shown using the TCGA dataset (n=1106). Illustrative heatmaps ordered by GPNMB mRNA expression are included. (B) Kaplan-Myer curves showing time to first tumor measurement in MMTV/Wnt-1 (n=25) and MMTV/Wnt-1 x MMTV/GPNMB (n=32) tumor-bearing transgenic females. (C) Immunoblot analysis showing GPNMB and Wnt-1 expression in 5 MMTV/Wnt-1 and MMTV/Wnt-1 x MMTV/GPNMB mammary tumors. α-Tubulin was used as a loading control. (D) GPNMB and Wnt-1 expression in MMTV/Wnt-1 and MMTV/Wnt-1 x MMTV/GPNMB mammary tumors was assayed using immunohistochemistry analysis.

the TCGA Illumina Hi Seq dataset for co-expression of GPNMB with 6 independent canonical Wnt pathway signatures previously described in various models of breast cancer [26, 74-76]. We found that expression of GPNMB is moderately to strongly correlated with the expression of transcriptional targets of the canonical Wnt pathway across all analyzed datasets (r = between 0.41 and 0.58) (Figure 4.2A), thereby substantiating further investigation of GPNMB involvement in Wnt-driven tumorigenesis.

To this end, we generated cohorts of mice expressing Wnt-1 alone (MMTV/Wnt-1) and bigenic mice that expressed both Wnt-1 and GPNMB in the mammary gland (MMTV/Wnt-1 x MMTV/GPNMB). We monitored tumor onset in each of these cohorts by weekly palpation, and, as previously reported [23], 50% of MMTV/Wnt-1 mice developed palpable mammary tumors at around 6 months of age (Figure 4.2B). Comparatively, virgin female mice in the MMTV/Wnt-1 x MMTV/GPNMB cohort displayed a significant acceleration in tumor onset, with 50% of bigenic animals developing mammary tumors almost 3 months earlier than their monogenic counterparts (T_{50} = 104 days for MMTV/Wnt-1 x MMTV/GPNMB mice vs T_{50} = 190 days for MMTV/Wnt-1 mice, $p < 0.05$) (Figure 4.2B). Immunoblot (Figure 4.2C) and immunohistochemistry analyses (Figure 4.2D) revealed the appropriate expression of both Wnt-1 and GPNMB in mammary tumors derived from MMTV/Wnt-1 and MMTV/Wnt-1 x MMTV/GPNMB transgenic mice.

GPNMB regulates cell-autonomous proliferative and survival effects to promote MMTV/Wnt-1 mammary tumor growth

We have previously shown that GPNMB can enhance tumor growth, metastasis and stromal cell recruitment in various breast cancer models [10, 11, 13, 14]. To determine

whether GPNMB could also regulate Wnt-1-dependent mammary tumor growth, we monitored tumor size in MMTV/Wnt-1 and MMTV/Wnt-1 x MMTV/GPNMB mice following first palpation by weekly caliper measurement. Although both MMTV/Wnt-1 and MMTV/Wnt-1 x MMTV/GPNMB mammary tumors exhibited a wide range of growth rates, which is consistent with the different histopathologies and oncogenic events associated with this tumor model [23], the presence of GPNMB significantly increased the average growth rate of Wnt-1-dependent mammary tumors (Figure 4.3A). Consistent with these results, immunohistochemistry analysis of mammary tumors taken from MMTV/Wnt-1 and MMTV/Wnt-1 x MMTV/GPNMB mice revealed enhanced proliferation in GPNMB-expressing mammary tumors, as evidenced by a 1.8-fold increase in the percentage of Ki67-positive nuclei in mammary tumors derived from MMTV/Wnt-1 x MMTV/GPNMB animals compared to MMTV/Wnt-1 mice (Figure 4.3B). Additionally, the level of cleaved-caspase-3 (CC3) staining was decreased 1.5-fold in mammary tumors harvested from MMTV/Wnt-1 x MMTV/GPNMB mice relative to those from MMTV/Wnt-1 animals, indicating that GPNMB suppresses apoptosis in Wnt-1-dependent breast cancers (Figure 4.3C). Contrary to our previous findings, staining for endothelial cell recruitment (CD31) and macrophage infiltration (F4/80) revealed no significant differences between mammary tumors in MMTV/Wnt-1 compared to MMTV/Wnt-1 x MMTV/GPNMB cohorts (Supplementary Figure 4.2).

With respect to the metastatic phenotype, GPNMB expression did not increase the proportion of mice that developed lung metastases relative to the MMTV/Wnt-1 cohort (Figure 4.3D). Additionally, among animals that developed lung metastases, the number of metastases per lung was not significantly affected by GPNMB expression (Figure 4.3E). The MMTV/Wnt-1 transgenic mouse model is weakly metastatic, therefore we performed

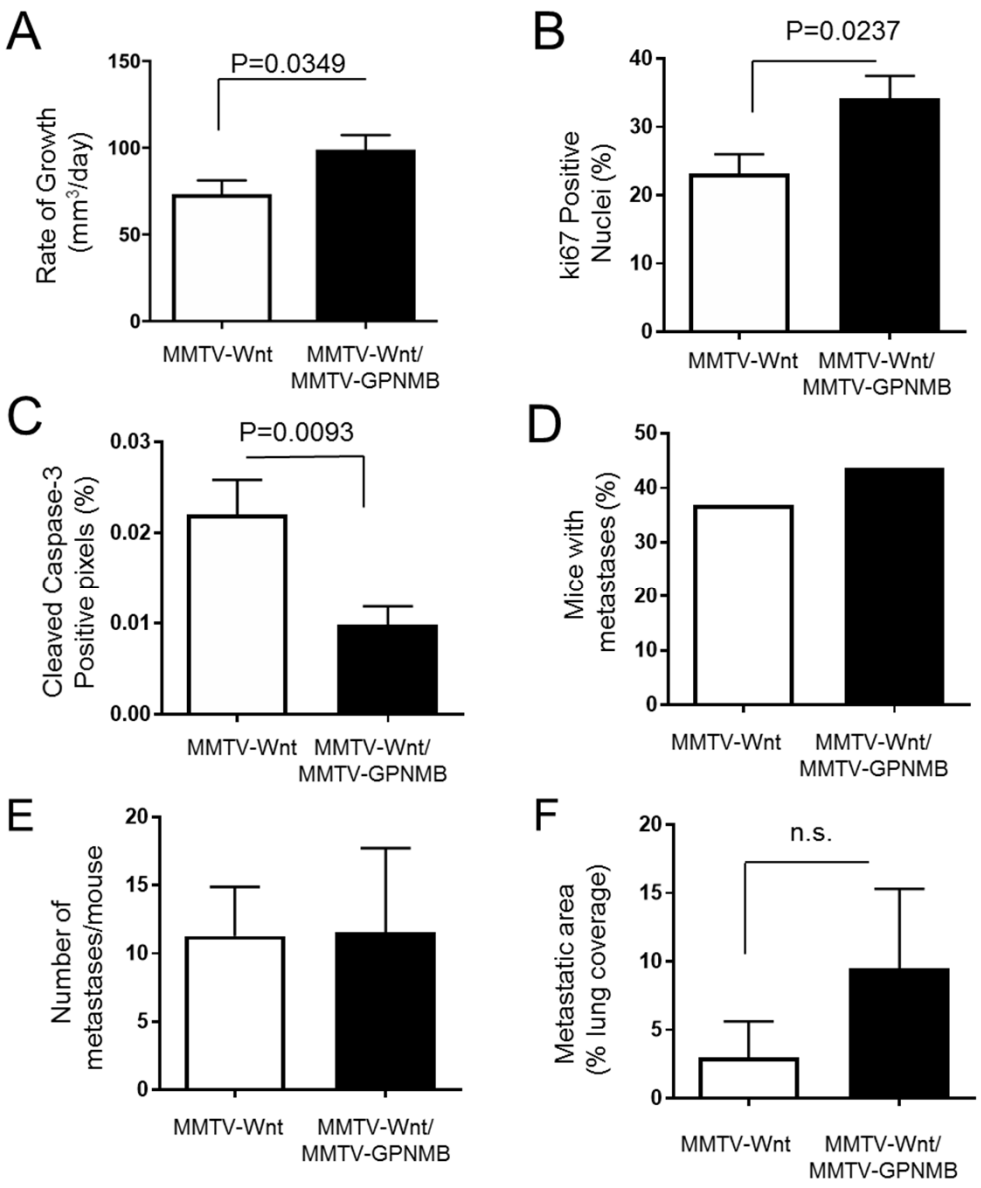

Figure 4.3 GPNMB regulates proliferation and survival to promote MMTV/Wnt-1 mammary tumor growth (A) Tumor-bearing MMTV/Wnt-1 and MMTV/Wnt x MMTV/GPNMB transgenic females were monitored by weekly caliper measurement. Average daily tumor growth rates are plotted. Tumor tissue was harvested from MMTV/Wnt-1 and MMTV/Wnt x MMTV/GPNMB tumors and assayed by immunohistochemistry using Ki67 and Cleaved Caspase 3 antibodies to monitor proliferation (B) and apoptosis (C) levels, respectively. Results represent the averages of 10 images/sample taken from 5 independent tumor samples. (*, $p<0.05$; ***, $p<0.001$). The lung metastatic burden of MMTV/Wnt-1 (n=19) and MMTV/Wnt x MMTV/GPNMB (n=16) mice was assessed at endpoint by quantifying the percentage of animals with visible lung lesions (D), the average number of metastases per animal (E) and the percentage of lung area covered by Wnt-1 expressing tumor cells (F).

Wnt-1 immunohistochemistry staining on lung sections to facilitate identification of tumor cells forming micrometastases in addition to readily visible lesions. As expected, the average lung area occupied by tumor cells was very low in MMTV/Wnt-1 transgenic mice (Figure 4.3F). Although MMTV/Wnt-1 x MMTV/GPNMB bigenic mice displayed a slightly higher metastatic burden, as defined by the percentage lung area occupied by metastases, this increase was not statistically significant (Figure 4.3F).

GPNMB-expressing Wnt-1 mammary tumors exhibit a distinct molecular signature associated with proliferation and survival

We next employed reverse phase protein array (RPPA) analysis as an unbiased approach to interrogate the signaling pathways engaged in breast tumors that express both Wnt-1 and GPNMB. A panel of 218 antibodies was used to profile and compare global changes in protein expression and protein modifications in primary mammary tumors from MMTV/Wnt-1 and MMTV/Wnt-1 x MMTV/GPNMB transgenic mice (Supplementary Table 4.1). Unsupervised clustering of the resulting data revealed that mammary tumors expressing both Wnt-1 and GPNMB segregated independently from tumors arising in MMTV/Wnt-1 transgenic mice (Figure 4.4A), suggesting that a distinct, GPNMB-mediated, mechanism of action is operative in MMTV/Wnt-1 x MMTV/GPNMB tumors.

To determine if GPNMB expression could alter signaling pathway activity in Wnt-1-driven breast cancers, we sought to identify proteins and protein modifications that were significantly up- or down-regulated in mammary tumors harvested from MMTV/Wnt-1 x MMTV/GPNMB mice. We observed that 60 out of 218 targets examined were differentially regulated between the two genotypes, indicating that GPNMB promotes widespread changes in protein expression and cellular signaling in Wnt-1-positive breast

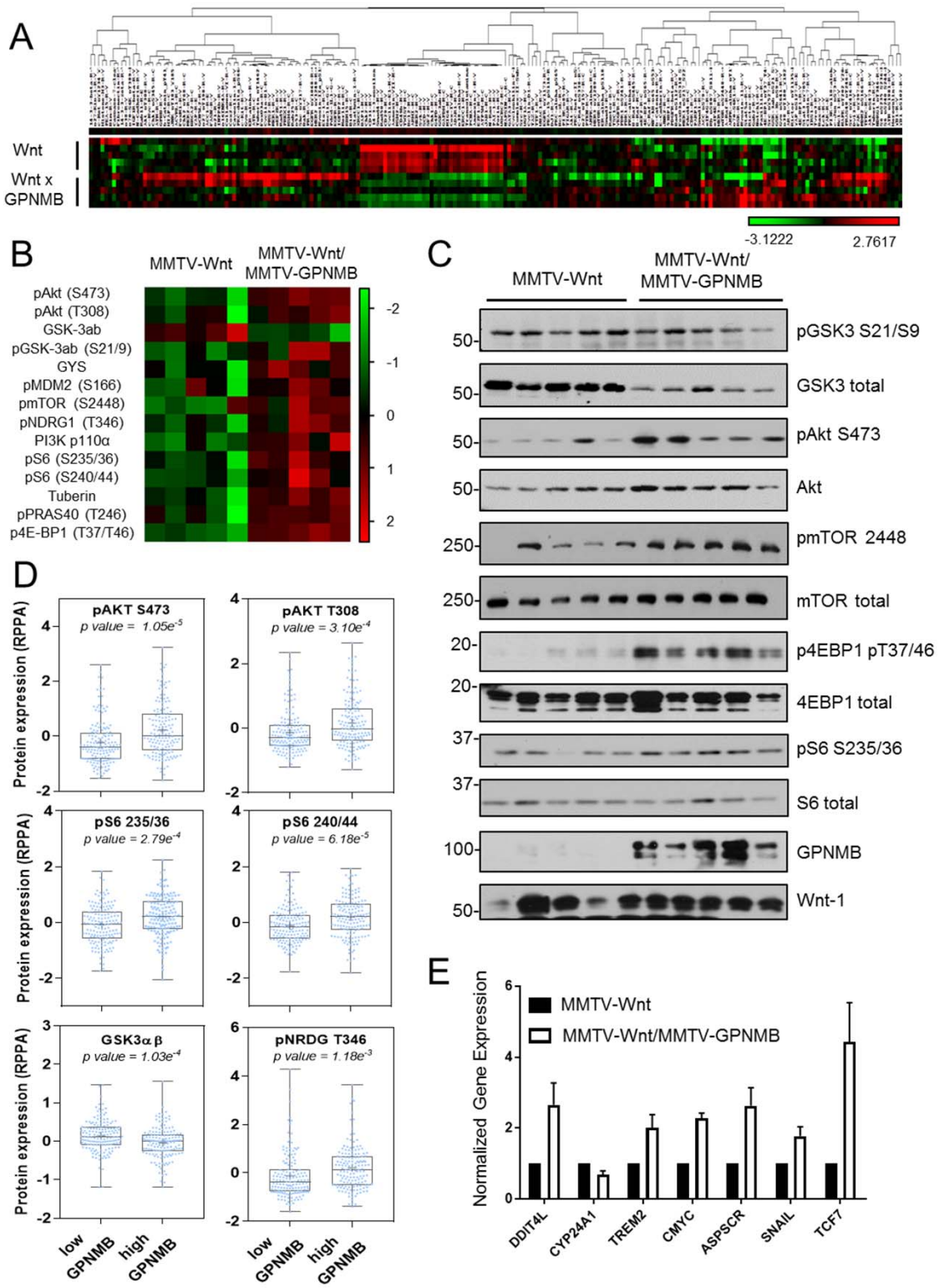
A
Wnt
Wnt x GPNMB
-3.1222
2.7617
B
MMTV-Wnt
MMTV-Wnt/ MMTV-GPNMB
pAkt (S473)
pAkt (T308)
GSK-3ab
pGSK-3ab (S21/9)
GYS
pMDM2 (S166)
pmTOR (S2448)
pNDRG1 (T346)
PI3K p110α
pS6 (S235/36)
pS6 (S240/44)
Tuberin
pPRAS40 (T246)
p4E-BP1 (T37/T46)
C
MMTV-Wnt
MMTV-Wnt/ MMTV-GPNMB
pGSK3 S21/S9
GSK3 total
pAkt S473
Akt
pmTOR 2448
mTOR total
p4EBP1 pT37/46
4EBP1 total
pS6 S235/36
S6 total
GPNMB
Wnt-1
D
pAKT S473
p value = 1.05e-5
pAKT T308
p value = 3.10e-4
pS6 235/36
p value = 2.79e-4
pS6 240/44
p value = 6.18e-5
GSK3α β
p value = 1.03e-4
pNRDG T346
p value = 1.18e-3
Protein expression (RPPA)
low GPNMB
high GPNMB
E
MMTV-Wnt
MMTV-Wnt/MMTV-GPNMB
Normalized Gene Expression
DDIT4L
CYP24A1
TREM2
CMYC
ASPSCR
SNAIL
TCF7

Figure 4.4 GPNMB-expressing Wnt-1 mammary tumors exhibit a distinct molecular signature associated with elevated PI3K/AKT/mTOR signaling and enhanced β-catenin transcriptional activity (**A**) Reverse phase protein array (RPPA) analysis was performed on 5 MMTV/Wnt-1 and 5 MMTV/Wnt-1 x MMTV/GPNMB mammary tumors. Unsupervised clustering of tumor samples based on differentially expressed proteins and phospho-proteins distinctly segregates the two transgenic genotypes. (B) The PI3K/AKT/mTOR pathway exhibits the most abundant differences in expression between MMTV/Wnt-1 and MMTV/Wnt-1 x MMTV/GPNMB tumors. A heatmap comparing expression of proteins and phospho-proteins significantly altered between the two genotypes ($p<0.05$) is shown. (C) Immunoblot analysis was performed on tumor extracts from 5 MMTV/Wnt-1 and 5 MMTV/Wnt-1 x MMTV/GPNMB tumors using antibodies targeting components of the PI3K/AKT/mTOR pathway. (D) Interrogation of RPPA protein expression data from the TCGA breast cancer dataset using matched samples with high (>1.0) or low GPNMB mRNA expression (< -1.0). The boxes indicate the median and interquartile range. The whiskers denote the minimum and maximum. Expression of selected PI3K/AKT/mTOR pathway proteins was mined through UCSC Genome Browser. (E) RT-qPCR analysis of selected β-catenin target genes was performed on RNA extracted from MMTV/Wnt-1 x MMTV/GPNMB tumors and normalized to GAPDH expression (n=5 for each genotype).

tumors (Supplementary Table 4.2). Remarkably, analysis of proteins differentially regulated in response to GPNMB expression revealed that the PI3K/AKT/mTOR pathway was the most abundantly represented signaling pathway (Figure 4.4B). Indeed, we observed significant changes in the expression or activity of 14 proteins associated with increased PI3K/AKT/mTOR signaling in GPNMB-expressing tumors. In accordance with our previous results [13], GPNMB-expressing Wnt-1 mammary tumors were characterized by elevated AKT activation, which was revealed by increased phosphorylation of S473/T308 relative to Wnt-1-positve tumors. Additionally, we observed enhanced phosphorylation of known AKT targets, such as MDM2 (S166), GSK3α/β (S21/S9, respectively) and PRAS40 (T246) [77], providing further evidence that AKT activity was increased in GPNMB-expressing Wnt-1-positive tumors (Figure 4.4B). Elevated mTOR activity was evident by increased phosphorylation of mTOR (S2448) and its immediate downstream target 4EBP1 (T37/46). Indirect evidence for mTOR activation was also provided by increased phosphorylation of ribosomal protein S6 (S235/236 and S240/244) and NDRG1 (T346) (Figure 4.4B).

The PI3K/AKT/mTOR pathway is active in GPNMB-expressing breast tumors

The PI3K/AKT/mTOR pathway is a strong driver of proliferation and survival in cancer [78]. To confirm activation of this pathway in mammary tumors that co-express GPNMB and Wnt-1, we sought to validate the RPPA analysis results by immunoblotting MMTV/Wnt-1 and MMTV/Wnt-1 x MMTV/GPNMB tumor lysates. In accordance with the RPPA data, we observed increased phosphorylation of AKT S473 in GPNMB-expressing Wnt-1 tumors compared to tumors that only expressed Wnt-1 (Figure 4.4C).

AKT is known to phosphorylate GSK3α on S21 and GSK3β on S9, which leads to GS3K degradation [79]. Indeed, we observed appreciable levels of phospho-GSK3 α/β, but very low levels of GSK3 in mammary tumors from MMTV/Wnt-1 x MMTV/GPNMB bigenic mice, confirming the RPPA data (Figure 4.4C). We also confirmed elevated mTOR phosphorylation, which correlated with increased phosphorylation of direct (4EBP1 pT37/46) and indirect (S6 235/236) mTOR targets (Figure 4.4C). We next sought to understand the clinical implications of our findings by examining the phosphorylation status and protein expression of key PI3K/AKT/mTOR pathway effectors in human breast cancers with upregulated and downregulated GPNMB expression. Using a dataset of 926 breast tumor samples from the Cancer Genome Atlas containing matched protein and mRNA expression, we observed that elevated GPNMB expression is strongly correlated with increased phosphorylation of AKT S473 ($p < 0.001$) and T308 ($p<0.001$), S6 S235/36 ($p<0.01$) and S240/44 ($p<0.01$), NDRG T346 ($p<0.01$), and decreased expression of the GSK3 α/β subunits ($p<0.01$) (Figure 4.4D), indicating that PI3K/AKT/mTOR signaling is enhanced in GPNMB-expressing human breast cancers.

GPNMB increases β-catenin transcriptional activity and nuclear localization

GSK3β is a key component of the β-catenin destruction complex that mediates ubiquitin-dependent proteosomal degradation of β-catenin. Kinase-driven degradation or Wnt-mediated sequestration of GSK3β leads to cytoplasmic stabilization of β-catenin and transcription of target genes involved in tumor progression [27, 80]. Given the reduced GSK3β levels observed in MMTV/Wnt-1 x MMTV/GPNMB tumors, we investigated whether GPNMB could potentiate canonical Wnt pathway signaling by enhancing downstream β-catenin activity. Quantitative RT-PCR analysis of MMTV/Wnt-1 and

MMTV/Wnt-1 x MMTV/GPNMB tumor lysates revealed that GPNMB expression significantly increased transcript levels of *C-MYC, TCF7, SNAIL, ASPSCR, TREM2, DDIT4L, AXIN2, KRTAP5, CYP24A1, TMEM64, PTGER2, TCF7* and *APCDD1* (Figure 4.4E), which have previously been identified as β-catenin targets in cancer [76, 81-84].

We have previously introduced 4T1 breast cancer cell subpopulations selected for their enhanced ability to colonize specific organs, including bone [14], liver [85], and lung [11]. Interestingly, we see that Wnt-1 is expressed in 4T1 derivatives that aggressively metastasize to the lung, but not in aggressively metastatic liver populations, which is consistent with studies showing that canonical Wnt signaling can enhance lung organotropism [50, 54]. To extend our observations to an independent Wnt-1-expressing model of metastatic breast cancer, we ablated GPNMB expression in 533 breast cancer cells, which are lung metastatic 4T1 derivatives, using CRISPR/Cas9 technology (Figure 4.5A) and investigated the role of GPNMB as a mediator of tumor progression in this model. We examined tumor growth of 533 parental and $GPNMB^{KO}$ breast cancer cells injected into the mammary fat pad of immunocompetent mice and observed that GPNMB knockdown significantly impaired growth of 533 breast cancer cells (Figure 4.5B, C). Analysis of the lung metastatic burden in mice injected with 533 parental and $GPNMB^{KO}$ breast cancer cells revealed that GPNMB was also required for efficient metastasis of 533 breast cancer cells (Figure 4.5D). Interestingly, the effect of GPNMB on spontaneous lung metastasis was much more pronounced in this model than in MMTV/Wnt-1 transgenic mice, as evidenced by a 75% reduction in metastatic area in mice injected with 533 breast cancer cells harboring a $GPNMB^{KO}$ compared to parental controls (Figure 4.5D).

To confirm whether GPNMB could regulate β-catenin transcriptional activity in this independent model, we quantified the expression of β-catenin target genes in mammary

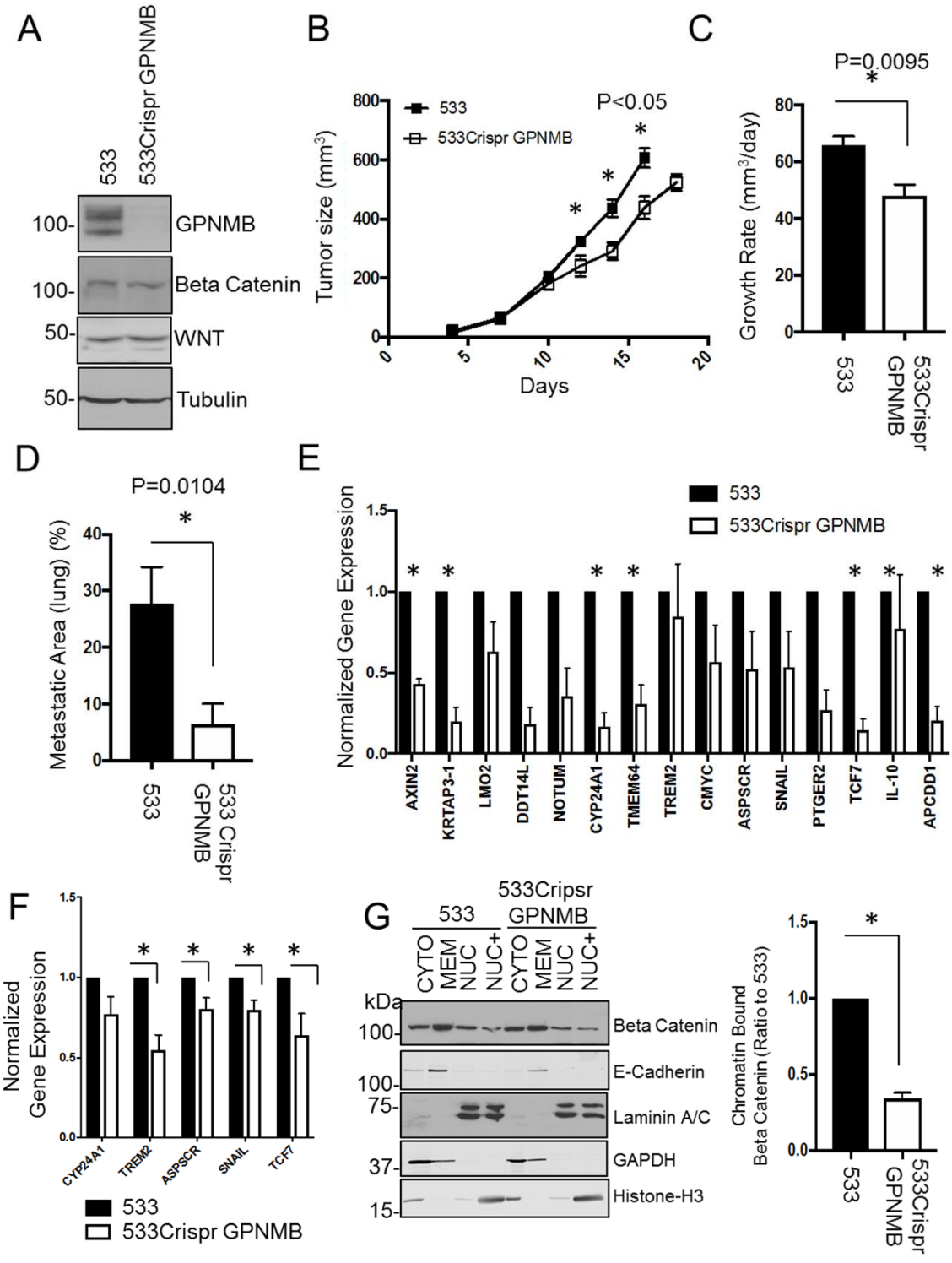
A
533
533Crispr GPNMB
100-
GPNMB
100-
Beta Catenin
50-
WNT
50-
Tubulin
B
533
533Crispr GPNMB
P<0.05
Tumor size (mm3)
Days
C
P=0.0095
Growth Rate (mm3/day)
533
533Crispr GPNMB
D
P=0.0104
Metastatic Area (lung) (%)
533
533 Crispr GPNMB
E
533
533Crispr GPNMB
Normalized Gene Expression
AXIN2
KRTAP3-1
LMO2
DDT14L
NOTUM
CYP24A1
TMEM64
TREM2
CMYC
ASPSCR
SNAIL
PTGER2
TCF7
IL-10
APCDD1
F
Normalized Gene Expression
CYP24A1
TREM2
ASPSCR
SNAIL
TCF7
533
533Crispr GPNMB
G
533Cripsr GPNMB
533
GPNMB
CYTO
MEM
NUC
NUC+
kDa
100-
Beta Catenin
100-
E-Cadherin
75-
Laminin A/C
37-
GAPDH
15-
Histone-H3
Chromatin Bound Beta Catenin (Ratio to 533)
533
533Crispr GPNMB

Figure 4.5 GPNMB-dependent tumor growth and metastasis is associated with increased β-catenin transcriptional activity and nuclear accumulation. (A) Immunoblot analysis of 533 lung metastatic breast cancer cells harboring a GPNMB-targeting Crispr/Cas9 construct shows complete knockdown of GPNMB expression and a minor accompanying reduction in overall β-catenin levels. α-Tubulin is included as a loading control. (B) 533 parental (n=10) and $GPNMB^{KO}$ (n=9) breast cancer cell populations were injected into the mammary fat pad of Balb/c mice. Tumor growth was monitored every few days by caliper measurement until tumors reached ~500 mm^3 and the time between individual measurements was expressed as an average daily growth rate (C). (D) Lung metastatic burden, which represents the percent lung area covered by tumor cells, was scored from lung step sections taken at endpoint 14 days post-primary tumor resection. Total RNA was extracted from 533 parental and $GPNMB^{KO}$ mammary tumors (E) (n=5) and established breast cancer cell lines (F) (n=3) and expression of select β-catenin target genes was assessed by RT-qPCR. (G) 533 parental and $GPNMB^{KO}$ breast cancer cells were fractioned into membranous (MEM), cytoplasmic (CYTO), soluble nuclear (NUC) and chromatin-bound nuclear (NUC+) compartments. Cell fraction lysates were probed for β-catenin. E-Cadherin, GAPDH, Laminin A/C and Histone-H3 are included as purity controls for MEM, CYTO, NUC and NUC+ fractions respectively. β-catenin immunoblots from chromatin-bound nuclear fractions were quantified and band density was normalized to Histone-H3 levels.

tumors derived from 533 parental and GPNMB^{KO} breast cancer cells. Using RT-qPCR, we confirmed that expression levels of several β-catenin target genes, including *AXIN2, KRTAP3-1, CYP24A1, TREM64, PTGER2, TCF7* and *APCDD1* were significantly decreased in 533 GPNMB^{KO} mammary tumors compared to control (Figure 4.5E). We next examined expression of β-catenin transcriptional targets in established 533 parental and GPNMB^{KO} breast cancer cell lines, in order to determine whether this phenotype can be extended beyond late-stage tumors. We observed significant reductions in the expression of numerous β-catenin target genes in GPNMB^{KO} breast cancer cells in baseline conditions, indicating that β-catenin transcriptional activity is constitutively decreased in response to GPNMB silencing in Wnt-expressing breast cancers (Figure 4.5F).

Given that canonical Wnt signaling drives nuclear translocation of β-catenin, we assessed β-catenin cellular distribution in presence and absence of GPNMB. 533 parental and GPNMB^{KO} breast cancer cells were fractioned into membrane, cytoplasmic, nuclear soluble and chromatin-bound compartments, and probed for β-catenin expression. Although β-catenin presence was detected in all cellular fractions examined, GPNMB knockdown impaired the accumulation of chromatin-bound β-catenin (Figure 4.5G). Quantification of β-catenin expression in the chromatin-bound nuclear fraction of 533 GPNMB^{KO} cells showed a 70% reduction in β-catenin levels compared to 533 parental controls (Figure 4.5G). Taken together, these results indicate that GPNMB is necessary and sufficient for regulation of β-catenin activity in breast cancer.

4.5 Discussion

While ER and HER2-positive breast cancers are each characterized by the presence of a major oncogenic driver, there is no such defining pathway that promotes tumor initiation

and progression of TNBCs. Preclinical studies have identified promising biological targets expressed in a large percentage of TNBCs [86, 87]; however, these therapeutic strategies have yielded modest success in clinical trials [87, 88]. Additionally, the recent re-classification of TNBCs into molecularly distinct subgroups further emphasizes the requirement to identify and understand therapeutic targets for this aggressive subset of the disease [5, 89, 90]. Previous work from our laboratory and follow-up clinical studies have established GPNMB as a clinically relevant target in TNBCs [10, 17]. To elucidate the role of GPNMB in modulating the initiation and growth of basal breast cancers, we generated a transgenic mouse model expressing GPNMB in the mammary epithelium. Virgin female mice expressing GPNMB do not exhibit mammary gland developmental defects and are not prone to spontaneous tumor formation. Multiparous MMTV/GPNMB female mice develop spontaneous breast tumors at low frequency (16%) following a long latency period (13-22 months). These data indicate that GPNMB does not itself function as an oncogene, but rather augments the aggressiveness of breast cancers [10, 11, 13, 14].

To assess the ability of GPNMB to augment tumor initiation and growth in an accepted model of basal breast cancer, we crossed MMTV/GPNMB mice with MMTV/Wnt-1 animals. It is known that Wnt-1 requires cooperation with other oncogenic events in order to promote tumorigenesis in the MMTV/Wnt-1 model [91-96]. We show that GPNMB expression promotes Wnt-1-driven tumor initiation and growth, which correlates with enhanced proliferation and reduced apoptosis. Contrary to our previous findings that implicate GPNMB as an enhancer of both primary breast tumor growth and metastasis [10, 11, 13, 14], GPNMB appears to primarily influence tumor growth but not lung metastasis in the MMTV/Wnt-1 model. This effect appears to be specific to the MMTV/Wnt-1 model as the metastasis of Wnt-1-expressing 533 breast cancer cells is dependent on GPNMB

(Figure 4.5D). These discrepancies may be attributed to a context-dependent role for GPNMB in tumor progression, which is influenced by the dominant signaling pathway responsible for transformation.

Using an unbiased proteomic profiling approach, we identify the PI3K/AKT/mTOR pathway as a major signaling pathway implicated in GPNMB-mediated growth of Wnt-1-expressing breast cancers. We have previously implicated GPNMB in augmenting baseline levels of AKT phosphorylation in an NRP-1 dependent manner in basal breast cancer cells [13]. We showed that the NRP-1/AKT signaling axis increased proliferation and inhibited apoptosis to promote primary tumor growth downstream of GPNMB but was dispensable for GPNMB-driven metastasis [13]. Similarly, the pro-growth effects of GPNMB have been linked to increased PI3K/AKT/mTOR pathway activity in other cancer types. GPNMB knockdown can inhibit proliferation, migration and invasion of osteosarcoma cells by inhibiting PI3K/AKT/mTOR signaling [97]. In glioblastoma, GPNMB increases tumor growth by interacting with the Na+/K+-ATPase α subunit to activate downstream PI3K/AKT and MEK/ERK pathways [98]. Likewise, GPNMB promotes survival of mesenchymal stem cells and motor neurons in physiological and disease conditions by enhancing PI3K/AKT signaling [99, 100]. Taken together, these findings and our observations establish a role for the PI3K/AKT/mTOR pathway in modulating breast cancer proliferation and survival downstream of GPNMB and indicate that GPNMB effects on metastasis are regulated through AKT-independent pathways, which do not appear to be operative in the MMTV-Wnt-1 model.

Activation of AKT signaling, and specifically AKT1-mediated signaling, has been linked to increased formation of mammary gland hyperplasias but not overt tumor formation [101]. Indeed, activated AKT1 can collaborate with the loss of tumor-suppressor

genes and oncogenes to promote increased mammary tumor initiation and growth [101-103]. Interestingly, activated AKT2 had no impact on oncogene-induced tumorigenesis and growth of primary mammary tumor, but enhanced the formation of lung metastases [104, 105]. These data suggest that GPNMB expression favors the engagement of signaling pathways that synergize with Wnt-1 to promote tumor initiation and growth, potentially through the selective engagement of AKT1.

We show that GPNMB dramatically accelerates onset of MMTV/Wnt-1tumorigenesis, thereby identifying a novel role for GPNMB in tumor initiation. The MMTV/Wnt-1 model exhibits discrete waves of transcriptional changes critical for cancer initiation and progression, which accompany the formation of hyperplasias and the evolution of hyperplastic lesions to overt mammary tumors [26]. In examining genes differentially expressed during distinct steps of MMTV/Wnt-1 cancer progression, c-Kit was identified as a potential mediator of tumor initiation due to its overexpression in tumor tissue compared to hyperplasias and virgin mammary glands [26]. c-Kit is frequently overexpressed in basal breast cancers and mediates its activity through Ras/Raf and AKT signaling pathways. ER- mammary progenitor cells are characterized by c-Kit expression, and c-Kit has been identified as a marker of proliferative potential within this progenitor population [106]. In ovarian cancer, c-Kit controls the tumor-initiating capacity of cancer cells by activating downstream Wnt/β-catenin signaling [107]. Interestingly, the expression of c-Kit is further enhanced in the presence of GPNMB, as demonstrated by our RPPA analysis comparing global changes in protein expression between MMTV/Wnt-1 and MMTV/Wnt-1 x MMTV/GPNMB tumors (Supplementary Table 4.2), suggesting that GPNMB and Wnt-1 could cooperatively regulate c-Kit expression and downstream activation of AKT and β-catenin to promote breast cancer initiation.

Our data raise the intriguing possibility that GPNMB expression could directly enhance the activity of the canonical Wnt signaling pathway. The PI3K/AKT and canonical Wnt signaling pathways converge on GSK-3β and cross-talk between these pathways has been demonstrated in various cancers [108-110]. Given the role of GPNMB in potentiating PI3K/AKT/mTOR signaling, we explored whether these effects extended downstream to enhanced β-catenin activity. Indeed, GSK-3β degradation is readily apparent in MMTV/Wnt-1 x MMTV/GPNMB tumors when compared to MMTV/Wnt-1 mammary tumors, and we observe that expression of β-catenin target genes is modulated by GPNMB, indicating that GPNMB can promote canonical Wnt-1 signaling by inhibiting negative regulation of β-catenin activity. Our findings are in line with recent studies implicating GPNMB as a modulator of the Wnt/β-catenin pathway in cancer [111, 112]. GPNMB was shown to increase nuclear accumulation of β-catenin in glioma cells, and GPNMB silencing reversed this effect [111]. In this study, the authors proposed that GPNMB promotes glioma tumorigenesis by regulating MMP expression through the Wnt/β-catenin pathway [111]. Similarly, GPNMB knockdown impaired proliferation and migration of bladder cancer cells and was accompanied by a decrease in MMP2, MMP9 and β-catenin expression [112]. These findings highlight an important role for GPNMB in promoting breast cancer growth downstream of Wnt signaling, which occurs in a majority of basal-like breast cancers and correlates with poor prognosis [36].

Wnt ligands also promote mitogenic effects in breast cancer by engaging in cross-talk with EGFR and activating the MAPK pathway [113, 114]. Wnt and EGFR signaling pathways converge on β-catenin, and display extensive synergy during mammary tumor initiation [115]. In addition, complex formation between EGFR and β-catenin is observed

in mammary tumors of MMTV/Wnt-1 transgenic mice, which implicates EGFR signaling in Wnt-mediated tumorigenesis [116]. Notably, EGFR phosphorylation at Y1068 is significantly increased in MMTV/Wnt-1 x MMTV/GPNMB tumors (Supplementary Table 4.1), suggesting that GPNMB could promote β-catenin activity by potentiating cross-talk between EGFR and Wnt signaling pathways. Cooperation between GPNMB and EGFR has recently been demonstrated in basal breast cancer cells [16]. In the presence of HB-EGF stimulation, EGFR phosphorylated at Y1068 interacts with the intracellular domain of GPNMB to drive downstream signaling and promote HIF-1α stabilization and transcriptional activity in normoxic conditions [16]. It is therefore plausible that the interaction between GPNMB and phosphorylated EGFR could also be engaged in response to alternative stimulation conditions, such as secretion of canonical Wnt ligands, in order to promote context-dependent β-catenin stabilization and activity.

Taken together, our results outline an important role for GPNMB as a mediator of PI3K/AKT/mTOR and Wnt/β-catenin pathway activity during the initiation and growth of basal breast cancers. Human breast cancers with upregulated GPNMB expression exhibit enhanced PI3K/AKT/mTOR signaling and β-catenin transcriptional activity, which emphasizes the clinical relevance of our findings. GPNMB is overexpressed in 40% of TNBCs and its expression is further increased in response to various targeted therapies [9, 12, 18]. Our findings help elucidate the biology of these aggressive cancers and provide rationale for CDX-011 combination therapy in managing this devastating subset of the disease.

4.6 Materials and Methods

Transgenic mouse models

To generate MMTV/GPNMB transgenic mice, a full-length human GPNMB cDNA containing a sequence encoding a V5/His-epitope tag was shuttled into the pMMTV-SV40 expression vector as an *Eco*RI fragment. The injection fragment was released by digestion with *Sal*I and *Spe*I and purified using the Qiagen Gel Extraction Kit. Injection fragments were microinjected into the pronuclei of FVB/N zygotes, which were subsequently transferred to recipient FVB/N pseudo-pregnant females by the McGill Transgenic Mouse Core Facility.

MMTV/GPNMB transgenic mice were bred with MMTV/Wnt-1 transgenic mice (FVB/N background) obtained from Jackson laboratories (strain number: 002934) and were kindly provided by Dr. Vincent Giguère (McGill University). Virgin females were monitored for tumor development by weekly gland palpation. Tumor volumes were determined by caliper measurement and calculated according to the following formula: $\pi LW2/6$ where L refers to the length and W to the width of the tumor. Mice were sacrificed when primary tumors reached a maximum allowable volume of 6 cubic centimetres, and tumor tissue was harvested for immunoblot or immunohistochemical analysis. Lung tissue was harvested and embedded in paraffin for scoring of lung metastatic burden, which was quantified from 4 Hematoxylin & Eosin (H&E) stained step sections (80 μm between each step section) using Aperio Imagescope software. Metastatic tissue was delineated and quantified from 4 step sections and expressed as a percentage of total lung tissue area. Mice were housed in facilities managed by the McGill University Animal Resources Centre and

all animal experiments were conducted under a University-approved animal use protocol in accordance with guidelines established by the Canadian Council on Animal Care.

Genotyping of transgenic mice

Genotyping was performed by first digesting mouse tails overnight at 55°C with 200 mM proteinase K diluted in tail lysis buffer (10 mM Tris pH 8.0, 100 mM NaCl, 10 mM EDTA, 0.5% SDS). Genomic DNA was isolated using high salt (6 M NaCl) extraction, washed with 100% and 70% ice-cold ethanol, dried, and resuspended in 500 uL distilled water. DNA was PCR-amplified using the 5' ATG ATT CAA ACA CCC CAG GA 3' forward primer and the 5' CCA CAC AGG CAT AGA GTG TCT GC 3' reverse primer under the following cycling conditions: 95°C for 5 minutes, 95°C for 1 minute, 60°C for 1 minute, and 72°C for 4 minutes for a total of 25 cycles, followed by a 7-minute elongation step at 72°C. The PCR reaction yielded a 1.8Kb product for MMTV/GPNMB-positive samples. MMTV/Wnt-1 transgenic mice were genotyped according to the Jackson Labs protocol:
https://www2.jax.org/protocolsdb/f?p=116:5:0::NO:5:P5_MASTER_PROTOCOL_ID,P5_JRS_CODE:14785,002870

RNA extraction and RT-qPCR

Mammary gland tissues or tumors were harvested from MMTV/GPNMB transgenic mice and wild-type littermates, or MMTV/Wnt-1 and MMTV/Wnt-1 x MMTV/GPNMB transgenic animals, and snap frozen in liquid nitrogen. Triplicate RNA samples were extracted from homogenized mammary gland or tumor tissues using RNeasy

Mini Kits (Qiagen) and quantified using a spectrophotometer (Nanodrop ND-1000). Total RNA (1µg per sample) was used to generate cDNA using a High Capacity cDNA Reverse Transcription Kit (ThermoScientific). Gene-specific oligonucleotide primers were designed using Primer3 software (Frodo.wi.mit.edu), diluted to a concentration of 100µM and used to amplify target RNA (primer sequences can be found in Supplementary Table 4.3). RT-qPCR reactions were performed on diluted cDNA (1:20) using Power SYBR Green Master Mix (Applied Biosystems) and 7500 Real Time PCR System (Applied Biosystems) or the LightCycler480 System. Reactions were performed in triplicate and *GAPDH* primers were used as a housekeeping control. Gene expression analysis was performed using the LightCycler480 and associated software using Advanced Relative Gene Expression Analysis (Roche Diagnostics, Laval, Quebec, Canada). Data is represented as the mean of the fold change of the three independent sets of cDNA calculated according to the following formula:

$$\frac{\text{Efficiency of target primers} + 1^{\,(\text{average value of target primers} - \text{average of corresponding target wells})}}{\text{Efficiency of control primers} + 1^{\,(\text{average value of control primers} - \text{average of corresponding control wells})}}$$

Generation of Crispr/Cas9 GPNMB knockdown

GPNMB deficient 533 were engineered using a CRISPR/Cas9 double nickase approach as previously described [117], using the following guide RNA sequences: Guide 1: CAC CGT GTG ATC GGG ATA CTG TTC A and Guide 2: CAC CGA GCA CAA CCA ATT ACG TGG C. A population of GPNMB deficient cells was generated by pooling 5 individual clones.

Immunohistochemistry and Immunohistofluorescence

Mammary glands or pieces of mammary tumor tissue were fixed overnight in 4% paraformaldehyde, embedded in paraffin and sectioned by the Goodman Cancer Research Centre histology core facility. Sections were deparaffinized and subjected to antigen retrieval in boiling 10mM sodium citrate (pH 6.0). Tissue sections were washed, incubated with 3% hydrogen peroxide for 5 minutes and blocked for one hour with 2% bovine serum albumin and 5% normal horse serum diluted in 1X PBS. Primary antibody dilutions (see Additional File 1) were applied to the slides and used in conjunction with the appropriate Biotin-SP-conjugated anti-IgG secondary antibodies (Jackson Laboratories). Immunohistochemical signal was detected using the Vector Elite ABC kit (Vector Labs) and 3-3'-diaminobenzidine. For immunofluorescent labelling, following secondary antibody application, nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI), and sections were dehydrated and mounted using Acrytol. Fluorescence was visualized with a Zeiss LSM 510 confocal microscope and images were acquired using the ZEN software (Zeiss).

Fixing, processing and staining of samples with Ki67 and cleaved caspase-3 antibodies was carried out at the GCRC Histology Core. All slides were scanned using a Scanscope XT digital slide scanner (Aperio, Vista, CA, USA). Staining was quantified with the Imagescope software (Aperio) using the positive pixel count algorithm (CD31 and Cleaved Caspase 3) or nuclear algorithm (ki67). For Cleaved Caspase-3 staining, data are expressed as a ratio of positive pixels over the total pixels per field. For ki67 staining, data are expressed as a percentage of positive nuclei or total nuclei.

Mammary gland whole mounts

The fourth inguinal mammary glands were harvested from MMTV/GPNMB females and age-matched littermates for whole-mount preparations. The resected tissue was spread out on a glass slide, defatted overnight in acetone, incubated with fresh acetone and stained overnight with Harris modified Hematoxylin. The mammary glands were subsequently destained with several washes of 70% ethanol/1% HCl solution, dehydrated with 100% ethanol for one hour and cleared overnight with 100% xylene. Slides were mounted and scanned with Aperio ScanScope XT and images were processed using ImageScope (Aperio).

Reverse phase protein array (RPPA) analysis

Mammary tumors derived from 5 MMTV/Wnt-1 and 5 MMTV/Wnt-1 x MMTV/GPNMB transgenic mice were subjected to RPPA analysis. Tumor proteins were extracted, denatured, diluted and probed with a panel of 218 unique primary antibodies by the RPPA Core Facility at the MD Anderson Cancer Centre as previously described [118]. A quality check (QC) was performed for each antibody stain as previously described [118] and only antibodies with a QC score higher than 0.8 were included in the heatmap. Proteins that exhibited statistically significant differences in expression were chosen for validation by immunoblot.

Immunoblot analysis

Membranes were prepared and processed as previously described [119] using the primary antibodies listed in Additional File 1. Where applicable, subcellular fractionation was performed as per manufacturer instructions using the subcellular protein fractionation kit for cultured cells (Cat. #: 78840, Thermo Scientific). For fractionation experiments,

blots were incubated with IR dye secondary antibodies (Licor Inc, Lincoln, NE, USA) and developed with the Odyssey Imager (Licor Inc, Lincoln, NE, USA). Quantification was performed using the ImageLite Studio software (Licor Inc, Lincoln, NE, USA).

Mammary fat pad injections

For in vivo studies, $1x10^5$ 533 breast cancer cells grown in DMEM supplemented with 10% FBS, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate and 1.5 g/L sodium bicarbonate were resuspended in a 50/50 mixture of 1X PBS:matrigel and injected into the fourth mammary fat pad of Balb/c mice (n = 10 mice/cohort). Tumors were monitored by palpation every couple of days and tumor volume was measured as above. Mammary tumors were resected and harvested once tumor size reached between 450-550mm^3. Mice were sacrificed 14 days post-resection and lungs were harvested for immunohistochemical analysis and scoring, as above.

Statistical analysis

Significance of the data was assessed using a two-tailed Student's T-test and variance was determined using an online statistics program (Vassar Stats). Indicated annotations correspond to the following P values: * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$. Results are displayed as an average +/- standard error.

Correlations between expression of GPNMB and indicated canonical Wnt signatures were studied in the Breast Cancer TCGA IlluminaHiSeq gene expression dataset available on the UCSC Cancer Genomics Browser (https://genome-cancer.ucsc.edu/). Expression data was ordered according to GPNMB expression and the Pearson correlation coefficients

for individual datasets were calculated using GraphPad Prism. Breast Cancer TCGA RPPA protein expression was pulled from the UCSC Cancer Genomics Browser and matched to gene expression data using TCGA sample IDs. Samples were classified as having high (>1.0) or low (<-1.0) GPNMB mRNA expression and expression of matched RPPA protein targets was analyzed. Statistically significant differences in protein expression was determined as above.

4.7 References

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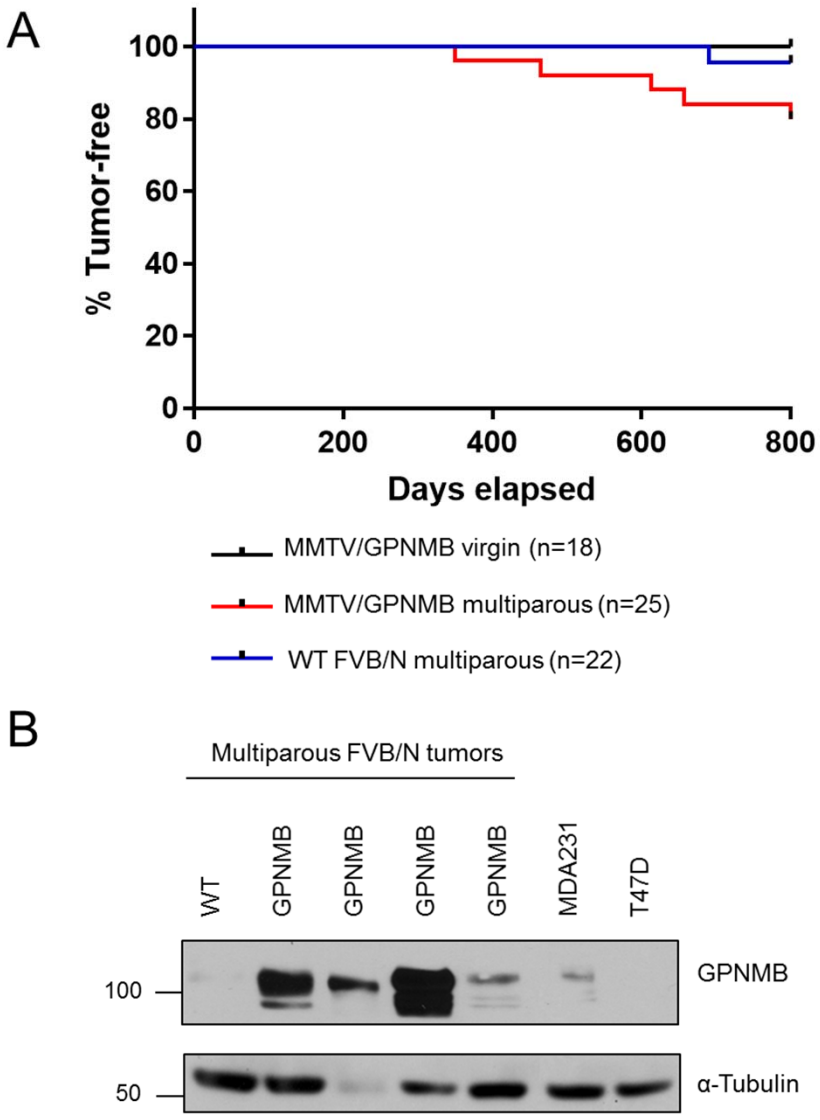
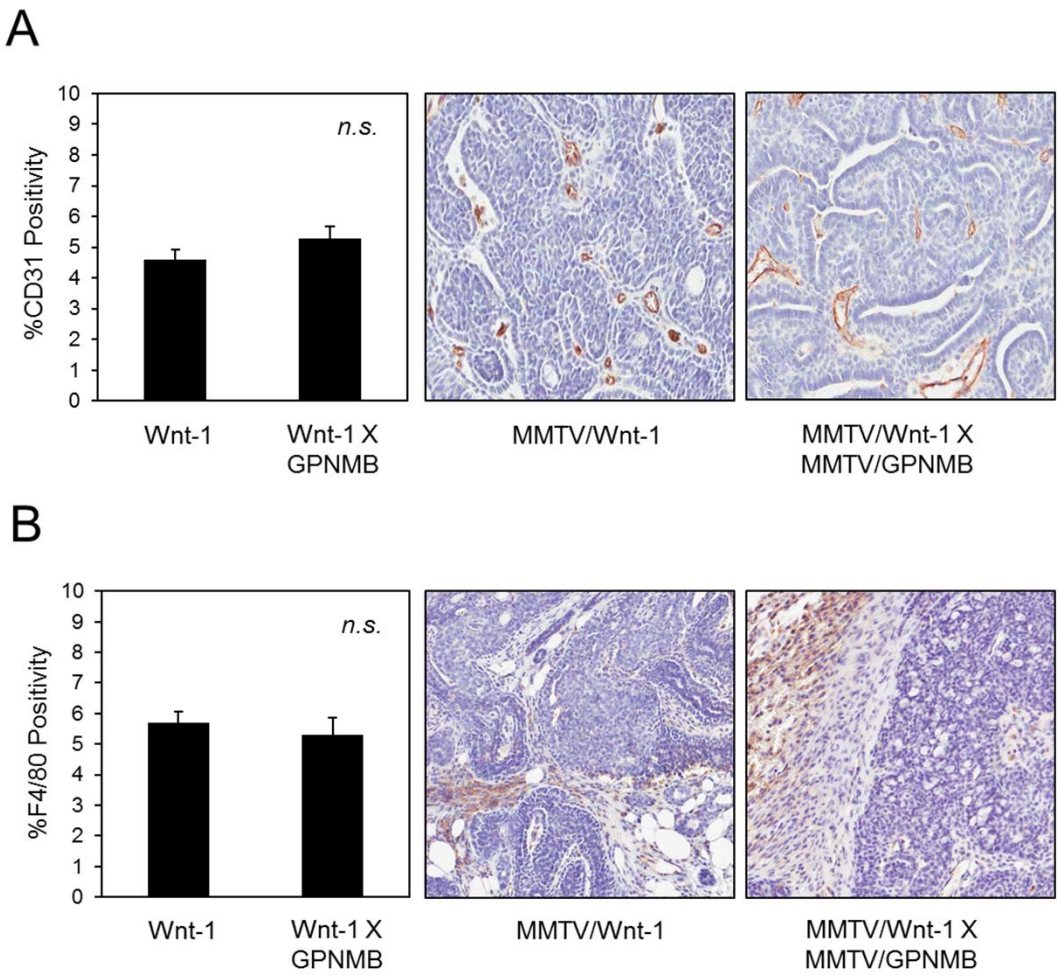


Supplementary Figure 4.1 MMTV/GPNMB transgenic mice develop tumors with a low penetrance and long latency (A) MMTV/GPNMB virgin females, MMTV/GPNMB multiparous females and transgene-negative multiparous FVB/N females were monitored for tumor onset by weekly palpation. Kaplan Myer curves illustrate the time to first palpation for each cohort. (B) Immunoblot illustrating GPNMB expression in tumors derived from MMTV/GPNMB and transgene-negative multiparous females. MDA231 and T47D breast cancer cell lines are included as a positive and negative control for GPNMB expression, respectively. α-Tubulin is included as a loading control.



Supplementary Figure 4.2 GPNMB does not increase recruitment of stromal infiltrates in MMTV/Wnt-1-driven mammary tumors (A) CD31 immunohistochemistry staining was used to examine the degree of endothelial cell recruitment in MMTV/Wnt-1 and MMTV/Wnt-1 x MMTV/GPNMB tumors. (B) Macrophage infiltration was assessed in MMTV/Wnt-1 and MMTV/Wnt-1 x MMTV/GPNMB tumors by performing immunohistochemistry for F4/80. 10 representative images were taken and quantified from all tumors for each staining condition (n = 5 for each transgenic cohort)

	Wnt					Wnt x GPNMB				
Gene Name	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
14-3-3-beta	-0.11537	0.06936	0.15811	-0.05293	-0.05820	-0.13386	0.00503	0.07440	-0.00503	0.02949
14-3-3-epsilon	0.77526	0.29974	0.01135	0.36127	0.28739	-0.01135	-0.26141	-0.03608	-0.19418	-0.20620
14-3-3-zeta	-0.15554	-0.01788	0.28077	-0.31400	-0.14466	0.06546	0.08804	0.00097	-0.00097	0.02256
4E-BP1	0.20245	-0.04367	0.00692	-0.51084	-0.00692	0.29646	0.10095	-0.16038	-0.07564	0.16068
4E-BP1_pS6	-0.06610	-0.12935	-0.13354	0.02523	0.13457	-0.04680	-0.02461	0.10486	0.02461	0.06167
4E-BP1_pT37/46	-0.42793	-0.64620	-0.44044	-0.32288	-0.29356	0.85963	0.51997	0.44741	0.29356	0.38514
53BP1	-0.21045	-0.10107	0.18177	0.01850	0.00014	-0.23499	0.06917	-0.05909	-0.00014	0.01565
A-Raf	-0.00982	0.04866	-0.57071	-0.09568	0.00982	-0.70129	0.14669	0.10115	-0.12319	0.15682
ACC_pS79	0.33730	-0.30326	-1.80678	-0.22242	-0.20658	-0.60659	0.58940	0.20658	0.98174	0.38371
ACC1	0.06911	0.09362	-0.23263	-0.72710	-0.08345	0.63516	0.11013	-0.13658	0.76590	-0.06911
ACVRL1	-0.12598	-0.00930	0.06034	0.00272	-0.08265	0.42513	-0.00272	0.04755	0.11147	-0.00572
ADAR1	0.54184	0.14820	0.01250	0.25148	0.18208	-0.41357	-0.29499	-0.01250	-0.15326	-0.17426
Akt	-0.05088	-0.11590	0.05610	-0.22457	-0.10740	-0.52157	0.17085	0.09478	0.05088	0.14059
Akt_pS473	-0.49545	-0.45410	-0.60706	-0.29638	-0.22074	1.14714	0.22074	0.25598	0.61758	0.31127
Akt_pT308	-0.30481	-0.19847	-0.03820	-0.12049	0.15336	0.87882	-0.01596	0.01596	0.35710	0.17960
AMPKα	-0.16743	-0.01072	0.19909	-0.18116	0.10109	-0.35598	0.01072	0.10045	-0.14929	0.09170
AMPKα_pT172	-0.07696	-0.01270	-0.33160	0.01270	0.08526	-0.62771	0.21636	0.08682	-0.03246	0.20056
Annexin-I	0.62046	0.13548	0.01271	0.44942	0.29696	-0.49213	-0.45722	-0.01271	-0.20868	-0.16285
Annexin-VII	0.82965	0.36425	0.01606	0.38385	0.29608	-0.17836	-0.27430	-0.01606	-0.17721	-0.15691
AR	0.03246	-0.06633	0.02377	0.01257	-0.02587	-0.00078	0.00078	-0.00256	-0.01604	0.08822
ARHI	0.86560	0.37981	-0.00549	0.46974	0.35116	0.00549	-0.33292	-0.03066	-0.17152	-0.19438
ATM	-0.26723	-0.01996	-0.10872	0.01996	-0.06636	0.42444	0.16270	-0.04059	0.27548	0.21198
ATM_pS1981	0.09484	0.07834	-0.06075	0.00773	-0.02721	0.19767	-0.05784	-0.00773	0.02624	-0.10138
ATP5H	0.69980	0.26355	0.00666	0.38867	0.29963	-0.43086	-0.38850	-0.00666	-0.29165	-0.26447
ATR	-0.00860	0.00860	0.01846	-0.03136	-0.01827	0.67673	0.09215	-0.04919	0.21993	-0.05828
b-Catenin	-0.50563	-0.12075	-0.00435	0.30615	0.09716	-0.70212	0.28675	0.00435	-0.80178	0.14064
b-CateninpT41/S45	-0.12898	-0.00206	-0.04806	0.00206	-0.09938	0.36903	0.03684	-0.10576	0.10053	0.01720
B-Raf	0.14834	-0.00476	-0.53305	0.55018	0.26727	-0.85912	0.04472	-0.04734	-0.14775	0.00476
B-Raf_pS445	-0.10826	-0.02689	-0.16457	0.34876	0.18439	-0.06963	0.09644	0.03918	-0.02334	0.02334
Bad_pS112	-0.03227	-0.19551	0.07789	-0.00090	0.00090	-0.26790	-0.04337	0.18239	0.01389	0.08892
Bak	0.03052	-0.09152	0.14610	-0.00156	-0.11378	0.43837	-0.06978	0.01114	0.00156	-0.13882
BAP1	0.91334	0.30387	0.00680	0.37129	0.28505	-0.04548	-0.25832	-0.00680	-0.16177	-0.14070
Bax	-0.03818	0.05510	-0.05596	-0.03291	0.03493	0.01629	-0.00363	-0.03128	0.23615	0.00363
Bcl-xL	0.01594	0.11214	0.11685	-0.22802	-0.11667	-0.16716	0.01235	0.04171	-0.10344	-0.01235
Bcl2	0.92658	0.43521	0.01151	0.51714	0.37371	-0.05416	-0.30601	-0.01151	-0.14323	-0.19171

Supplementary Table 4.1 Raw RPPA data

	Wnt					Wnt x GPNMB				
Gene Name	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
Beclin	0.08634	-0.01660	0.05009	-0.08281	-0.06331	0.44061	0.01660	-0.08161	0.06252	-0.05779
Bid	-0.02911	-0.07843	0.12972	-0.09929	0.00139	0.10234	-0.15453	0.03183	0.00422	-0.00139
Bim	0.04950	0.27848	0.48975	-0.29045	0.00446	-0.00446	0.01211	-0.05662	-0.11443	-0.00454
BRCA2	-0.16973	-0.10441	0.12234	-0.09206	-0.01739	0.45388	-0.09699	0.01739	0.10335	0.01906
c-Jun_pS73	0.07111	-0.05873	-0.28175	-0.26653	-0.05707	0.44432	0.03602	-0.03602	0.27244	0.12269
c-Kit	-0.10464	-0.01505	-0.09421	-0.11391	-0.10923	0.39052	0.02272	0.01505	0.19677	0.08959
c-Met_pY1234/35	0.00600	-0.00089	0.03789	0.02010	-0.00257	0.00089	-0.04035	0.02916	-0.03190	-0.01417
c-Myc	-0.19738	-0.24898	-0.12209	-0.07400	0.08368	0.40181	-0.19939	0.07400	0.12477	0.08500
C-Raf	-0.17411	-0.18430	-0.44006	0.20469	0.05573	-0.03557	0.03557	-0.10275	0.10189	0.03848
C-Raf_pS338	-0.05974	-0.00902	0.03082	0.07608	-0.02269	-0.24600	0.00902	0.08699	0.01219	-0.02500
Caspase-7-cleaved	0.04708	0.03513	1.20855	-1.03879	-0.63743	0.39150	-0.20429	0.15858	-0.68812	-0.03513
Caveolin-1	-0.38849	0.02843	0.36002	-0.31279	-0.08614	0.70132	0.44297	-0.28621	0.37192	-0.02843
CD29	1.18367	0.54839	-0.03242	0.62485	0.60259	-0.25650	-0.40100	0.03242	-0.28117	-0.21741
CD31	0.96327	0.34901	-0.03363	0.39019	0.26683	0.03363	-0.28073	-0.13193	-0.25275	-0.29950
CD49b	0.63540	0.22859	0.03013	0.31567	0.19766	-0.17147	-0.22236	-0.03013	-0.14066	-0.19395
CDK1	-0.26702	-0.22816	-0.01138	0.09303	0.01138	0.04982	-0.13800	-0.09282	0.05703	0.01371
Chk1	0.79744	0.31952	-0.27910	0.51723	0.37547	-0.36438	-0.00943	-0.00146	-0.02642	0.00146
Chk1_pS345	-0.22581	-0.29757	-0.10177	0.03996	0.03503	0.33034	-0.04302	-0.03503	0.08596	0.16473
Chk2	0.87728	0.24014	0.01187	0.30955	0.21450	-0.10244	-0.27258	-0.01187	-0.20016	-0.13759
Chk2_pT68	-0.05856	0.00580	0.02339	0.05524	-0.02968	0.49772	-0.00877	-0.00629	0.23002	-0.00580
Claudin-7	-0.01215	0.22836	-0.31896	-0.19725	0.08459	0.01215	0.52980	-0.36977	-0.58393	0.15597
Collagen-VI	-1.01779	-0.02804	-0.05368	-1.05509	0.18805	0.94879	0.02804	-1.10481	0.17990	0.25344
Complex-II-Subunit	0.18344	0.00625	0.06691	0.10868	-0.00625	-0.20818	-0.21718	0.03365	-0.08413	-0.14859
Cox-IV	0.57995	0.22908	0.00502	0.32358	0.34490	-0.37830	-0.24274	-0.00502	-0.16165	-0.08381
Cox2	-0.15223	-0.02984	-0.02078	-0.09293	0.02078	0.35151	0.05688	-0.29357	0.11613	0.06098
Cyclin-B1	-0.04381	0.06587	-0.12101	-0.41371	0.04381	1.72785	0.25814	-0.26858	0.57868	-0.15078
Cyclin-D1	-0.04911	0.07253	0.05273	0.05320	-0.06771	0.08517	0.04021	-0.04556	-0.04021	-0.04711
Cyclin-E1	0.84528	0.28660	0.02916	0.38478	0.31783	-0.13517	-0.28861	-0.02916	-0.20437	-0.21243
Cyclophilin-F	0.22402	0.01457	-0.15738	0.19878	0.04403	-0.14618	-0.23432	0.09146	-0.42785	-0.01457
DJ1	-0.10709	0.03343	0.04640	-0.27347	-0.02725	-0.11868	0.02725	0.05409	-0.11770	0.04284
Dvl3	-0.01512	-0.00680	-0.17902	-0.00372	0.00372	0.12665	0.14097	-0.08652	0.10618	0.02255
E-Cadherin	-0.73015	-0.01785	0.16646	-0.04346	0.01785	-0.66962	0.17301	0.12005	-1.06428	0.13598
E2F1	0.61130	0.20337	-0.05783	0.39514	0.30218	-0.40588	-0.31064	0.05783	-0.11822	-0.09205
eEF2	-0.18773	0.01189	-0.34754	0.27930	0.12240	-0.49360	-0.00364	0.03459	-0.03354	0.00364
eEF2K	0.02752	0.24239	-0.22063	-0.16247	-0.11083	0.95885	0.43173	-0.21517	0.37857	-0.02752

Supplementary Table 4.1 Raw RPPA data

	Wnt					Wnt x GPNMB				
Gene Name	1	2	3	4	5	1	2	3	4	5
EGFR	0.03654	-0.07206	0.22136	-0.30039	0.01268	0.57277	-0.01268	-0.03460	-0.08004	0.05539
EGFR_pY1068	-0.00986	-0.06845	-0.08462	-0.09460	0.00986	0.76174	0.28789	-0.03887	0.19771	0.13487
EGFR_pY1173	0.01782	0.00660	0.06339	-0.08370	0.03289	-0.07050	-0.04373	-0.00660	-0.05828	0.07308
eIF4E	-0.16505	0.04984	-0.33031	-0.04984	-0.05931	-0.16654	0.22397	0.13737	0.12224	0.12141
eIF4G	0.01960	-0.11592	-0.98982	-0.00403	-0.07161	0.00403	0.22537	-0.22724	0.18742	0.01784
ER-alpha	-0.01508	0.05749	-0.02200	-0.03512	0.00792	0.89287	0.01182	-0.08879	0.33596	-0.00792
ER-alpha_pS118	0.00382	-0.00382	0.10542	-0.01193	0.12693	-0.46003	-0.09394	0.09492	-0.17352	0.06663
ERCC1	0.69197	0.29681	0.02013	0.35738	0.21869	-0.45896	-0.17320	-0.02013	-0.17476	-0.12489
Ets-1	0.02768	-0.06113	0.13438	-0.11437	-0.10763	0.34711	-0.02768	-0.04826	0.08026	0.08811
FAK	-0.07733	0.07685	-0.02132	-0.05625	0.00601	-0.01965	0.15466	-0.00601	0.14252	0.03172
FAK_pY397	-0.15753	-0.15473	-0.42930	0.36631	-0.08553	0.85333	0.38751	-0.37948	0.34284	0.08553
FASN	-0.08895	0.21358	-0.18393	-0.83307	-0.22122	0.84345	0.33971	-0.08621	0.77024	0.08621
Fibronectin	0.02057	0.09906	0.85157	-0.42244	-0.05490	0.28250	-0.02057	0.04797	-0.02123	-0.09348
FoxM1	-0.08876	0.05217	0.13792	-0.00752	-0.06187	0.48569	-0.04897	0.00752	0.21516	-0.01865
FoxO3a	-0.00306	0.00419	0.00306	-0.16129	-0.08209	0.65838	0.03898	-0.04318	0.19660	-0.09065
FoxO3a_pS318/21	-0.01499	-0.08473	0.02347	0.04415	0.00224	-0.17027	-0.02440	0.14330	-0.00224	0.07583
G6PD	0.84646	0.65000	0.00602	0.32598	0.23964	-0.32631	-0.28304	-0.00602	-0.18072	-0.17100
Gab2	-0.02675	0.02675	-1.29210	0.17395	0.11526	-0.69898	0.81569	-0.12888	-0.08400	0.43249
GAPDH	0.48924	0.09189	-0.00741	0.26310	0.12918	-0.35419	-0.30687	0.00741	-0.17022	-0.25473
GATA3	0.62996	0.36527	0.18019	-0.21282	0.05821	-0.10214	-0.28648	0.15313	-0.39013	-0.05821
GCN5L2	-0.12823	0.02323	0.19628	0.03903	-0.02323	0.30258	-0.06356	-0.07047	0.20097	-0.07242
GPBB	-0.42476	-0.11878	0.00636	-0.00065	-0.07738	0.58145	0.24250	-0.03987	0.23105	0.00065
GSK-3ab	0.29824	0.09983	0.05356	0.09521	0.08870	-0.35998	-0.15799	-0.05356	-0.13734	-0.09283
GSK-3ab_pS21/9	-0.27798	-0.62740	-0.63481	0.17541	-0.13223	0.08218	0.40468	-0.08218	0.33446	0.24100
GSK-3b_pS9	-0.18294	-1.14749	-1.02094	0.10485	-0.12787	0.43261	0.22980	-0.10485	0.44163	0.14085
Gys	-0.21307	0.05697	-0.28210	-0.48856	-0.16106	0.73708	0.01645	0.01913	0.37882	-0.01645
Gys_pS641	-0.10402	-0.20588	-0.23999	-0.02246	-0.04666	0.02246	0.27941	0.14071	0.60809	0.45693
HER2	0.99758	0.41581	-0.05573	0.53107	0.41285	0.03235	-0.40980	-0.03235	-0.21669	-0.23572
HER2_pY1248	-0.05365	-0.08906	0.02554	0.09907	0.07134	0.75743	-0.01429	0.00099	-0.00099	-0.03431
HER3	-0.32891	-0.21510	-0.58744	0.12549	-0.08774	-0.28472	0.10786	0.10453	0.08774	0.09758
HER3_pY1289	-0.00425	0.00425	0.09890	-0.13057	0.02194	-0.26701	-0.04264	0.19670	-0.09815	0.05366
Heregulin	0.09168	0.02275	-0.08548	-0.04276	-0.14026	0.03384	0.01207	-0.01207	0.03542	-0.07940
HIAP	-0.04818	-0.07853	0.01779	-0.01779	-0.07446	0.32373	0.03924	-0.14354	0.12001	0.08168
Histone-H3	-0.45248	-0.08785	0.80422	0.05426	-0.07856	0.07591	-0.23197	0.28384	-0.00052	0.00052
IGF1R-beta	-0.05643	0.02473	0.02207	-0.03712	-0.00688	0.00688	-0.02700	0.05828	-0.02728	0.05214
IGFBP2	-0.14634	0.00597	0.19348	-0.38246	-0.11422	0.87558	-0.00597	0.01968	0.02009	-0.08251

Supplementary Table 4.1 Raw RPPA data

	Wnt					Wnt x GPNMB				
Gene Name	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
INPP4b	0.07645	-0.01266	-0.07890	-0.15708	0.01772	0.33928	0.01266	-0.10000	0.21404	-0.05057
IRS1	0.26027	0.17278	-0.10095	-0.39824	-0.01852	-0.34275	0.56105	0.01852	-0.07012	0.41336
JAB1	1.14794	0.43380	-0.01803	0.64097	0.63080	-0.58641	-0.55749	0.01803	-0.46068	-0.31739
JNK_pT183/5	-0.17802	-0.11665	0.01463	0.10781	-0.01463	0.46070	-0.05384	-0.10457	0.10138	0.02458
JNK2	-0.06957	-0.02586	-0.05613	0.03623	0.02586	-0.09308	0.21925	0.10435	-0.08231	0.15951
Lck	0.09808	-0.00790	-0.09429	-0.54206	-0.21446	0.00790	0.10935	-0.09416	0.74389	0.05292
MAPK_pT202/204	0.02425	-0.17577	-0.07561	-0.10638	0.06746	0.50629	0.18507	-0.09771	0.14790	-0.02425
Mcl	-0.01391	-0.04151	-0.19850	-0.05040	0.01391	0.33748	0.09616	-0.09187	0.38546	0.05511
MDM2_pS166	0.00448	-0.17749	-0.29288	-0.01059	-0.00448	0.54291	0.02901	-0.20177	0.25413	0.21920
MEK1	-0.07275	0.07275	-0.28151	-0.24924	-0.07421	1.10319	0.11912	-0.24642	0.36879	0.07992
MEK1_pS217/221	-0.12567	-0.06754	0.03695	0.10239	-0.09887	-0.12760	-0.03695	0.19985	0.10066	0.11473
MEK2	-0.07143	0.00596	0.07197	0.05531	-0.01850	0.44426	-0.06795	-0.06083	0.10628	-0.00596
Merlin	0.02706	-0.04571	0.20816	-0.08286	-0.06991	0.35678	-0.00999	0.02639	-0.05022	0.00999
MIG6	0.34267	0.04439	0.03609	0.17063	0.08689	-0.30428	-0.22178	-0.03609	-0.19247	-0.12519
MSH2	0.99101	0.42058	0.00176	0.49826	0.42462	-0.18522	-0.31058	-0.00176	-0.19542	-0.20571
MSH6	0.16364	0.00444	-0.07243	-0.12953	-0.06806	0.71557	0.19306	-0.23397	0.17281	-0.00444
mTOR	-0.07165	0.02810	-0.07876	-0.02810	0.04006	-0.80773	0.16579	0.23294	-0.20997	0.23366
mTOR_pS2448	-0.10950	-0.10307	-0.22568	0.06324	-0.06324	-0.10876	0.19073	0.17276	0.10294	0.16519
Myosin-11	-0.18629	0.29508	-1.62121	-0.26653	0.28485	-3.12218	1.81262	0.18629	-1.29166	1.89857
Myosin-IIa_pS1943	0.08476	-0.10500	0.00466	0.19929	0.08713	1.00391	-0.23298	-0.00466	-0.20659	-0.12177
N-Cadherin	-0.12472	0.02291	0.04875	-0.06011	-0.06357	0.13862	-0.00661	0.02117	-0.01496	0.00661
N-Ras	0.66033	0.18513	0.00884	0.33686	0.23079	-0.33402	-0.26723	-0.00884	-0.24089	-0.23670
NAPSIN-A	0.05581	-0.01915	0.00134	0.02075	0.11876	-0.00134	-0.08352	-0.10622	-0.11771	0.01562
NDRG1_pT346	-0.39209	-0.74809	-0.96167	0.12591	-0.19591	1.02279	0.21894	-0.12591	0.48213	0.14860
NF-kB-p65_pS536	-0.20475	-0.21582	-0.19543	0.32069	0.14475	0.11633	-0.00249	-0.22153	0.08646	0.00249
Notch1	0.01657	-0.03570	-0.51054	0.04128	-0.01657	-0.61319	0.23065	-0.08206	0.12469	0.07861
p16INK4A	0.18621	0.05920	0.12177	-0.17885	-0.07365	0.47102	-0.06631	-0.09756	0.32326	-0.05920
p21	-0.07122	0.08247	0.01475	-0.35713	-0.35997	0.44181	0.09811	-0.01475	0.40170	-0.05335
p27-Kip-1	-0.01530	0.01530	0.07185	0.03523	-0.02580	-0.01809	-0.01762	0.02245	0.01694	-0.03891
p27_pT157	-0.02981	-0.04504	0.02412	0.05696	0.00428	-0.21185	-0.00428	0.06728	-0.01076	0.04981
p27_pT198	-0.19694	0.00307	0.04905	-0.03505	0.01281	0.19335	-0.00824	-0.07272	0.04607	-0.00307
p38-alpha	0.45736	0.17492	0.00550	0.18045	0.16910	-0.27067	-0.17449	-0.00550	-0.08430	-0.09337
p38	-0.13798	-0.03555	0.24357	-0.15306	-0.07060	-0.01675	0.17426	0.06922	0.20588	0.01675
p38_pT180_Y182	-0.44937	-0.51932	-0.52072	0.10600	-0.17142	-0.09297	0.10555	0.13465	0.72648	0.09297
p53	0.08193	-0.04874	0.00419	-0.47404	-0.00419	0.72545	0.14026	-0.10393	0.09445	-0.01893
p70-S6K_pT389	0.00902	-0.06000	0.40549	-0.08708	-0.04912	-0.00902	0.02458	-0.03150	0.14194	0.21204

Supplementary Table 4.1 Raw RPPA data

	Wnt					Wnt x GPNMB				
Gene Name	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
p70-S6K1	-0.02434	0.02434	-0.45484	-0.04647	0.03671	-0.88777	0.13303	0.13816	-0.04765	0.15069
PAI-1	0.75974	0.31079	0.03519	0.41606	0.31998	-0.27755	-0.34509	-0.03519	-0.20682	-0.15932
PARP1	-0.32214	-0.24955	0.04231	-0.02801	0.01315	0.02833	-0.21255	-0.01315	0.06419	0.11776
Paxillin	0.02482	-0.02482	-0.84980	0.23895	0.13702	-1.03626	0.41813	-0.04922	-0.02617	0.18982
PCNA	0.75518	0.19606	0.00007	0.34125	0.24816	-0.31993	-0.19568	-0.00007	-0.14120	-0.12191
Pdcd-1L1	0.06227	-0.08003	0.21300	0.03086	-0.07810	0.91039	-0.04072	-0.12706	0.25142	-0.03086
Pdcd4	-0.15600	-0.37397	0.00339	0.23715	-0.02610	-0.00339	-0.04366	0.72878	0.11744	0.41770
PDGFR-beta	-0.45713	-0.12437	0.16986	0.06419	-0.08701	-0.25428	0.30920	-0.06419	0.13776	0.15508
PDK1	0.04879	0.03995	0.01270	-0.06210	-0.00131	-0.23846	0.00131	-0.01958	-0.02047	0.00342
PDK1_pS241	-0.00514	0.12071	-0.01259	-0.10036	0.08312	-0.18293	0.00780	0.02725	-0.10470	0.00514
PEA-15	-0.00225	-0.08214	0.28236	-0.02513	0.00225	0.10474	-0.06251	0.18512	-0.07330	0.07289
PEA-15_pS116	-0.00206	0.00611	0.21292	-0.22128	0.12522	-0.02765	0.00206	-0.03068	-0.02997	0.12446
PI3K-p110-alpha	-0.13466	0.00947	-0.08651	-0.03101	-0.02894	0.05082	0.08549	-0.00947	0.09314	0.06641
PI3K-p85	0.03864	0.10196	0.10273	-0.31992	-0.10572	-0.07447	0.02141	0.00581	-0.00581	-0.02720
PKC-alpha	0.27727	0.15553	-0.04225	0.00374	0.08994	-0.70722	-0.06576	0.01386	-0.25926	-0.00374
PKC-alpha_pS657	-0.07513	0.21718	0.01699	-0.65815	-0.01699	-0.68378	0.23641	0.11806	-0.27930	0.24792
PKC-beta-II_pS660	0.07450	-0.20770	-0.00886	-0.31539	-0.23641	-0.55668	0.12468	0.28614	0.00886	0.16321
PKC-delta_pS664	-0.05939	-0.02340	0.10162	-0.14012	-0.06768	0.13881	0.02765	-0.08124	0.03510	0.02340
PMS2	-0.03381	0.25681	0.01462	-0.23793	-0.01462	1.86050	0.26258	-0.11023	0.61473	-0.05422
Porin	0.57800	0.19063	0.04616	0.33607	0.23410	-0.21810	-0.32275	-0.04616	-0.17734	-0.21020
PR	0.07817	0.02987	0.05114	-0.00848	-0.02660	0.20509	-0.03614	0.00848	-0.01179	-0.02180
PRAS40_pT246	-0.16072	-0.03648	-0.07736	-0.08376	-0.03229	0.81999	0.33177	0.05636	0.31555	0.03229
PREX1	0.00021	0.04395	-0.12341	-0.18263	-0.02460	0.34888	0.18040	-0.29817	0.16817	-0.00021
PTEN	-0.12346	-0.03884	0.02163	0.03893	-0.06580	-0.84337	0.22386	0.06221	0.06125	-0.02163
Rab11	-0.29398	-0.01495	0.09629	-0.16424	-0.24085	0.06046	-0.07680	0.10846	0.03321	0.01495
Rab25	0.09667	0.02175	-0.05402	-0.22509	-0.05720	0.80361	-0.01251	-0.50287	0.12268	0.01251
Rad50	0.91721	0.36671	-0.00482	0.35328	0.14503	-0.01535	-0.28270	0.00482	-0.20064	-0.25769
Rad51	-0.01790	0.14716	0.01790	-0.06497	-0.03667	0.41233	0.14676	-0.08590	0.19602	-0.02936
Raptor	-0.03026	0.00680	0.06344	-0.01302	0.00516	-0.44859	-0.00516	0.09150	-0.08738	0.05271
Rb_pS807/811	0.26818	-0.97057	-1.48225	0.04874	-0.26657	-0.04874	1.24092	-0.24921	0.37175	0.90059
RBM15	0.03324	0.00366	0.67128	-0.16853	-0.14436	0.84597	-0.13099	0.10059	-0.00366	-0.07305
Rictor	-0.09088	0.08822	-0.33019	0.00190	-0.00190	-0.40166	0.45263	0.17971	-0.05246	0.29776
Rictor_pT1135	0.06028	-0.04678	-0.02407	0.02773	-0.00515	-0.01191	0.03591	-0.04398	0.03701	0.00515
RSK	0.05167	0.02868	-0.36554	-0.10832	-0.02868	-0.74712	0.27590	0.37992	-0.04943	0.27456
S6_pS235/36	-0.04936	-0.25345	-0.85468	-0.22153	-0.37609	1.04718	0.04936	0.28572	0.36758	0.08825
S6_pS240/44	-0.00887	-0.39132	-0.73002	-0.12171	-0.08605	0.47231	0.00887	0.15805	0.17988	0.20096

Supplementary Table 4.1 Raw RPPA data

	Wnt					Wnt x GPNMB				
Gene Name	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
SCD	0.63691	0.24455	-0.00237	0.35672	0.26227	-0.09604	-0.26582	0.00237	-0.16638	-0.24299
SF2	1.12488	0.05724	0.03455	0.28092	0.16666	-0.13483	-0.32235	-0.03455	-0.24730	-0.20018
Shc_pY317	-0.00131	0.00131	-0.07301	-0.21723	0.14243	0.68024	-0.04762	-0.19146	0.07529	0.02851
Smad1	-0.02206	-0.03034	0.05442	-0.00215	0.05165	-0.02368	0.00293	0.00215	0.01578	-0.02521
Smad3	0.00961	-0.03098	0.05365	0.00865	0.06279	-0.28134	-0.03483	-0.00865	-0.08277	0.04843
Smad4	0.78856	0.30844	0.03578	0.35717	0.26714	-0.03578	-0.20120	-0.03614	-0.16650	-0.20866
Src	0.13106	-0.01865	0.04314	0.01865	0.02130	-0.50976	-0.24036	0.03834	-0.25736	-0.08908
Src_pY416	-0.09858	-0.04513	-0.11250	-0.01033	0.01033	0.91166	0.40000	-0.18140	0.26074	0.19241
Src_pY527	-0.39498	-1.28277	-0.39084	-0.14123	-0.35575	0.14123	0.27082	0.43110	0.15456	0.34219
Stat3_pY705	0.02289	-0.06924	0.20064	-0.20589	-0.22098	0.08187	0.38710	-0.04534	-0.02289	0.07039
Stat5a	0.46001	0.14197	-1.22437	-0.65927	-0.14197	-2.04810	1.89534	0.75955	-0.32425	1.65754
Stathmin-1	0.01768	-0.06908	0.12579	-0.00867	-0.01897	0.09560	-0.06821	0.04987	0.00867	-0.01440
Syk	0.91685	0.37782	0.01007	0.47497	0.41354	-0.01007	-0.40405	-0.08541	-0.15769	-0.22112
TAZ	0.08092	-0.03276	0.06414	0.04377	-0.02409	0.12559	0.01442	-0.05304	-0.04426	-0.01442
TFRC	0.17855	0.00042	-0.90016	-0.08335	-0.19722	1.22709	0.16193	-0.81333	0.26191	-0.00042
TIGAR	-0.01302	-0.00092	0.14926	-0.20721	-0.14804	-0.16123	0.03776	0.09898	0.02291	0.00092
Transglutaminase	0.54554	0.17899	0.04231	0.28399	0.20161	-0.33908	-0.13744	-0.04231	-0.13261	-0.10500
TSC1	-0.02739	-0.01037	0.11567	0.00441	-0.00441	-0.51664	0.04182	0.02032	-0.09136	0.02247
TTF1	0.01799	-0.02626	0.03893	0.06454	0.04185	0.09387	-0.07745	-0.01799	-0.03926	-0.02207
Tuberin	-0.20359	-0.07690	-0.23517	-0.04708	-0.07815	0.50973	0.20733	0.04708	0.15460	0.10115
Tuberin_pT1462	-0.03675	0.01960	0.01551	-0.02274	-0.05847	0.53661	0.04598	-0.01551	0.13199	-0.02480
TWIST	0.79664	0.26401	-0.01452	0.35707	0.21452	-0.21187	-0.34726	0.01452	-0.23445	-0.21682
Tyro3	0.09380	0.12545	-0.15611	-0.07652	-0.03801	-0.02670	0.32140	0.02670	-0.04491	0.13577
UBAC1	0.05832	-0.21314	0.03267	0.42069	-0.07453	0.09878	-0.00454	-0.02820	0.00454	-0.05852
UGT1A	0.27798	0.15642	0.15018	-0.04320	-0.01885	-0.49188	-0.21181	0.23303	-0.37315	0.01885
UQCRC2	0.66312	0.35561	0.05105	0.55747	0.39887	-0.36671	-0.22816	-0.05105	-0.17553	-0.19976
VEGFR-2	-0.12702	0.05206	0.16306	-0.05206	0.08601	-0.62006	-0.13631	0.18523	-0.18520	0.05884
XRCC1	-0.08617	-0.01232	0.18911	-0.18139	-0.11055	0.38461	-0.17697	0.23680	0.06311	0.01232
YAP	-0.26855	-0.14974	0.09955	-0.01152	0.21664	0.64166	-0.21886	0.01152	0.24075	-0.01576
YAP_pS127	0.20690	-0.37314	0.09345	-0.08606	-0.25129	-0.05424	0.30840	-0.16880	0.34360	0.05424
YB1	0.35701	0.10912	0.06900	-0.13476	0.00215	-0.08520	-0.02888	-0.00215	0.15619	-0.22049
YB1_pS102	0.22894	-0.03516	-0.04489	0.00074	0.04603	-0.00074	-0.17819	-0.03960	0.07732	0.01083

Supplementary Table 4.1 Raw RPPA data

A

Targets significantly up-regulated by GPNMB

Name	p Value
4E-BP1_pT37_T46	0.00004
Akt_pS473	0.00114
ATM	0.01161
c-Jun	0.03093
c-Kit	0.01290
Dvl3	0.01890
FASN	0.03996
GPBB	0.04337
GSK-3ab_pS21/9	0.02343
GSK-3b_pS9	0.03323
Gys	0.03178
Gys_pS641	0.00574
mTOR_pS2448	0.02878
NDRG1_pT346	0.02120
p38_pT180_Y182	0.02624
PI3K-p110-alpha	0.00716
PRAS40_pT246	0.02666
S6_pS235/36	0.01294
S6_pS240/44	0.01468
Src_pY527	0.00524
Tuberin	0.00592
EGFR_pY1068	0.04739

B

Targets significantly down-regulated by GPNMB

Name	p Value
14-3-3-epsilon	0.00615
ADAR1	0.00430
Annexin-I	0.00381
Annexin-VII	0.00438
ARHI	0.00637
ATP5H	0.00191
BAP1	0.01215
Bcl2	0.00509
CD29	0.00428
CD31	0.01080
CD49b	0.00336
Chk2	0.01399
Complex-II-Subunit-	0.00940
Cox-IV	0.00317
Cyclin-E1	0.00445
E2F1	0.00971
ERCC1	0.00484
G6PD	0.00519
GAPDH	0.00502
GSK-3ab	0.00305
HER2	0.00920
JAB1	0.00245
MIG6	0.00264
MSH2	0.00444
N-Ras	0.00323
NAPSIN-A	0.03326
p38-alpha	0.00543
PAI-1	0.00207
PCNA	0.00897
Porin	0.00142
Rad50	0.01674
SCD	0.00413
SF2	0.03720
Smad4	0.00566
Src	0.03020
Syk	0.00485
Transglutaminase	0.00328
TWIST	0.00708
UQCRC2	0.00077

Supplementary Table 4.2: Targets significantly up- or down-regulated by GPNMB expression

Name	Sequence
hGPNMB Fwd	TGC TGA CTG TGA GAC GAA CC
hGPNMB Rev	TGC ACG GTT GAG GAC AC
mDDIT4L Fwd	CGG CCA GCA TTT CAG AGT TG
mDDIT4L Rev	CAG GGA CCA AGA CCT TAG AGC
mCYP24A1 Fwd	CTG CCC CAT TGA CAA AAG GC
mCYP24A1 Rev	CTC ACC GTC GGT CAT CAG C
mTREM2 Fwd	TTG CTG GAA CCG TCA CCA TC
mTREM2 Rev	ACT TGG GCA CCC TCG AAA C
mCMYC Fwd	GAT GAA ATA GGG CTG TAC GGA G
mCMYC Rev	GCT GTT TGA AGG CTG CAT TTC
mASPSCR Fwd	TTC AAC CCC AGT GAA TAC GAC C
mASPSCR Rev	GCG ATG CGA ACT ATG TTC TCA G
mSNAIL Fwd	GCA GTG GGA GCA GGA GAA T
mSNAIL Rev	CTT GTG TCT GCA CGA CCT GT
mAXIN2 Fwd	TGA CTC TCC TTC CAG ATC CCA
mAXIN2 Rev	TGC CCA CAC TAG GCT GAC A
mKRTAP3-1 Fwd	CTG CCC ACA TGA GAT CAG CC
mKRTAP3-1 Rev	GGC AAG AGT TGA GCA GCC A
mTMEM64 Fwd	GTG GCC GAG GTG AGA AAC TG
mTMEM64 Rev	TTA AGC ACG ATG TAG CCC CAA
mPTGER2 Fwd	CAG CTC GGT GAT GTT CTC GG
mPTGER2 Rev	GAG CAC CAA TTC CGT TAC CAG
mTCF7 Fwd	CCC CAC AGA GAA ACT GAA GC
mTCF7 Rev	ATC AGG CRC TTC CCA GTT CC
mAPCDD1 Fwd	GAA AGA GGT TGG GGT GAG ACT
mAPCDD1 Rev	GAA GCC GAA TCA AGC TGG TAA

Supplementary Table 4.3: A list of all the DNA sequences used in the current study.

CHAPTER 5 - DISCUSSION

5. GENERAL DISCUSSION

Our lab has previously identified GPNMB as a potential therapeutic target in basal and triple-negative breast cancers [1], a finding which has been substantiated by phase I/II and phase IIb clinical trials targeting GPNMB in patients with metastatic breast cancer [2, 3]. In addition to its overexpression and predictive value in aggressive breast cancers, GPNMB has also been shown to promote malignant progression in breast cancer, melanoma, glioma, hepatocellular carcinoma, pancreatic ductal adenocarcinoma, bladder cancer, lung cancer, and various sarcoma models [4-14]. However, despite the clinical and biological significance of GPNMB in cancer, very little is known regarding the mechanisms of action that govern GPNMB-driven cancer progression. Herein, we show that the GPNMB-mediated increase in breast cancer growth is partly attributable to NRP-1 upregulation, which confers upon GPNMB-expressing cells the ability to more readily respond to autocrine and paracrine growth factors, such as VEGF. Concomitantly, GPNMB increases recruitment of a VEGF-producing immune cell infiltrate *in vivo*, thereby further enhancing the pro-tumorigenic function of NRP-1 (Chapter 2). Furthermore, we demonstrate that GPNMB engages separate and distinct mechanisms to promote breast cancer invasion and metastasis. GPNMB binds α5β1 through its RGD domain to increase α5β1 stability and activate downstream signaling pathways involved in cancer cell survival, invasion and metastasis (Chapter 3). Separately, we examined the role of GPNMB in breast cancer using the MMTV/Wnt-1-driven model of basal breast cancer. We show that GPNMB can act in a context-dependent manner to further increase the pro-growth phenotype by activating the PI3K/AKT/mTOR pathway and enhancing β-catenin activity (Chapter 4). These data identify novel GPNMB-mediated mechanisms that promote the progression of basal-like and triple-negative breast cancers (Figure 5.1).

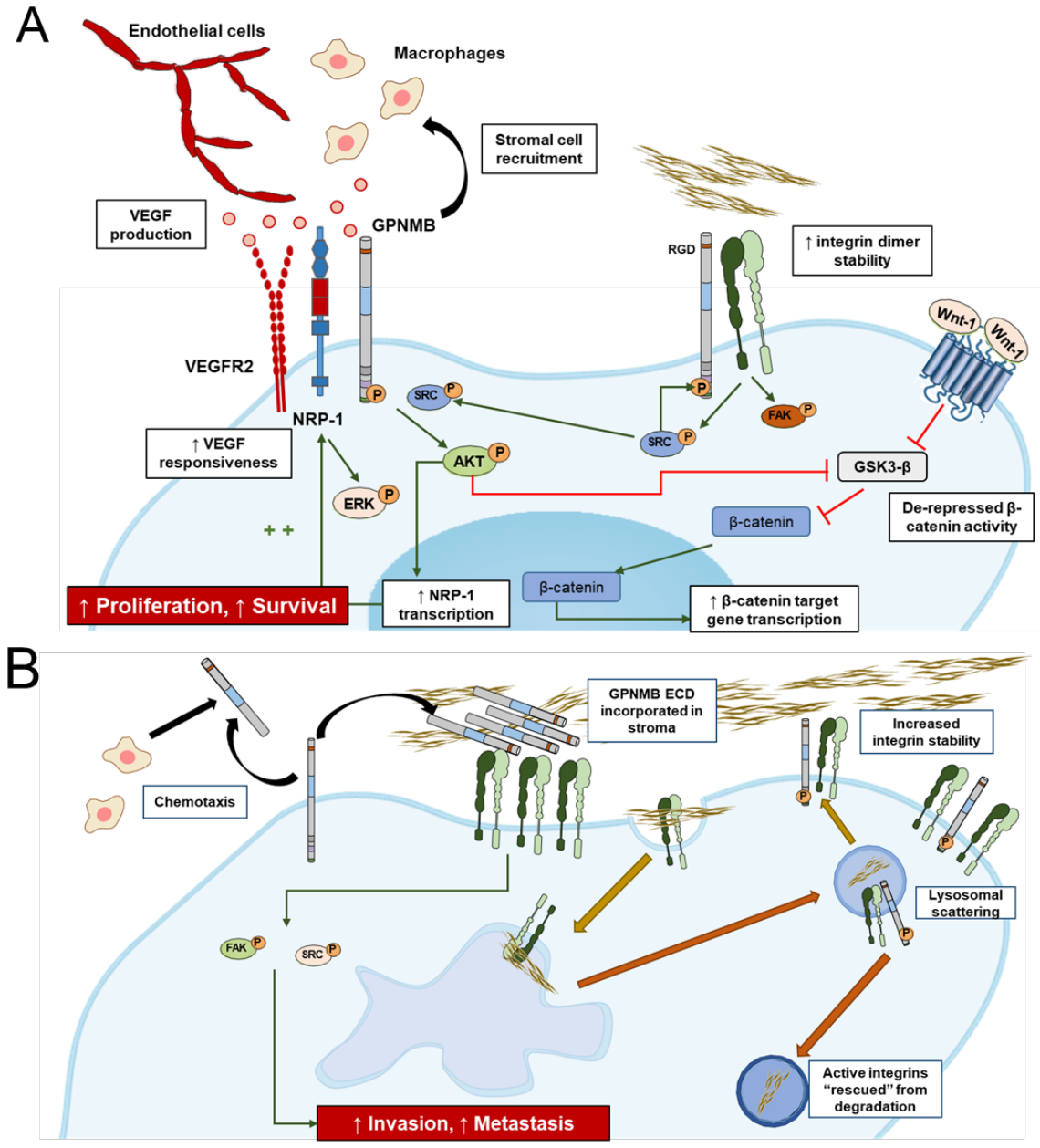


Figure 5.1 Putative mechanisms of GPNMB action in breast cancer (**A**) **GPNMB role in tumor growth:** GPNMB recruits VEGF-producing stromal cells, and potentiates downstream signaling via Nrp-1. Separately, GPNMB increases Akt activity leading to increased Nrp-1 transcription, and enhanced Wnt-1 mediated β-catenin transcriptional activity. Through RGD-mediated interactions with the α5β1 integrin heterodimer, GPNMB also increases Src and Fak activity, thereby leading to Src-mediated phosphorylation of the GPNMB hemITAM motif. (**B**) **GPNMB role in metastasis**: GPNMB interacts with the α5β1 integrin receptor via its RGD domain in the presence of fibronectin, leading to increased receptor lysosomal recycling and stability. Shed GPNMB is incorporated into the extracellular matrix and mediates cell-substratum interactions with cell surface integrin dimers, potentiating downstream pro-metastasis signaling.

5.1 GPNMB augments the ability of breast cancer cells to modulate and respond to the tumor microenvironment

Previous reports indicate that GPNMB can exert paracrine effects on cell types found in the tumor microenvironment, such as endothelial cells, fibroblasts and immune cells, and thereby promote acquisition of protumoral properties by these stromal cells [5-7, 12, 14-16]. Although many of the mechanisms outlined in this thesis support a tumor-intrinsic role for GPNMB in breast cancer progression, it is interesting to speculate how some of the GPNMB mechanisms of action outlined in this thesis can be extended beyond the tumor cell.

5.1.1 Secretion of pro-tumorigenic growth factors and cytokines

We have shown that GPNMB expression is correlated with increased microvascular density in human breast tumors [5] and increases angiogenesis *in vivo* by enhancing macrophage recruitment and VEGF production (Chapter 2, Chapter 3) . A similar phenotype is seen in models of brain cancer, where GPNMB enhances the invasion of glioma cells by co-opting and altering the vasculature at the brain-tumor interface [7], and alveolar soft part sarcoma, where interaction between endothelial cells and GPNMB expressed on tumor cells is required for intravasation and initiation of metastasis [14]. In future studies, it would be worthwhile to examine whether signaling cues downstream of GPNMB favor the development of a pro-angiogenic microenvironment.

In the primary tumor environment, macrophages can stimulate angiogenesis and promote tumor cell invasion, motility and extravasation [20]. Substantial evidence indicates that macrophages and other non-malignant cells in the tumor microenvironment evolve in tandem with the tumor to adopt a phenotype that supports tumor progression [20]. A

growing tumor produces cytokines and growth factors such as IL-4, CSF1 and GM-CSF to educate resident macrophages to become protumoral [21-23]. Additionally, CSF-1 and VEGFA produced by breast cancer cells act as independent recruiters of macrophages in breast cancer and thereby promote malignant cancer progression [24, 25]. In breast cancer, macrophages recruited to the growing tumor regulate the angiogenic switch by producing Wnt7b, which acts on endothelial cells to stimulate their proliferation and production of VEGF [26].

To determine if a similar mechanism was operative in our system, we examined VEGF production in GPNMB-expressing cells. BT549 cells expressing VC, GPNMB-WT, GPNMB-ΔCYT or GPNMB-RGD_{mut} constructs were cultured on fibronectin-coated plates for 24 hours and VEGF levels in the conditioned media were measured using an ELISA assay. Quantification of VEGF levels revealed that VEGF secretion was increased in BT549 GPNMB cells compared to control cells (Figure 5.2A). The increase in VEGF levels was observed to a lesser degree with the GPNMB-CYT mutant and it was abolished completely with the GPNMB-RGD mutant (Figure 5.2A). To ascertain if the heightened VEGF production detected in GPNMB-expressing cells was NRP-1-dependent, we subjected BT549 VC, GPNMB-WT/NRP-1^{High} and GPNMB-WT/NRP-1^{Low} cells to a VEGF ELISA assay. The GPNMB-driven increase in breast cancer cell VEGF levels was maintained in the absence of NRP-1 (Figure 5.2B), indicating that GPNMB regulates VEGF secretion in an NRP-1 independent manner. In keeping with this observation, a recent study investigating the paracrine effects of GPNMB in glioma has demonstrated that GPNMB drives expression of pro-angiogenic factors VEGFC and TEM7, and that conditioned media from GPNMB-expressing cells is required for angiogenic tube formation [15]. Additionally, it was recently shown that GPNMB is required for HIF1α

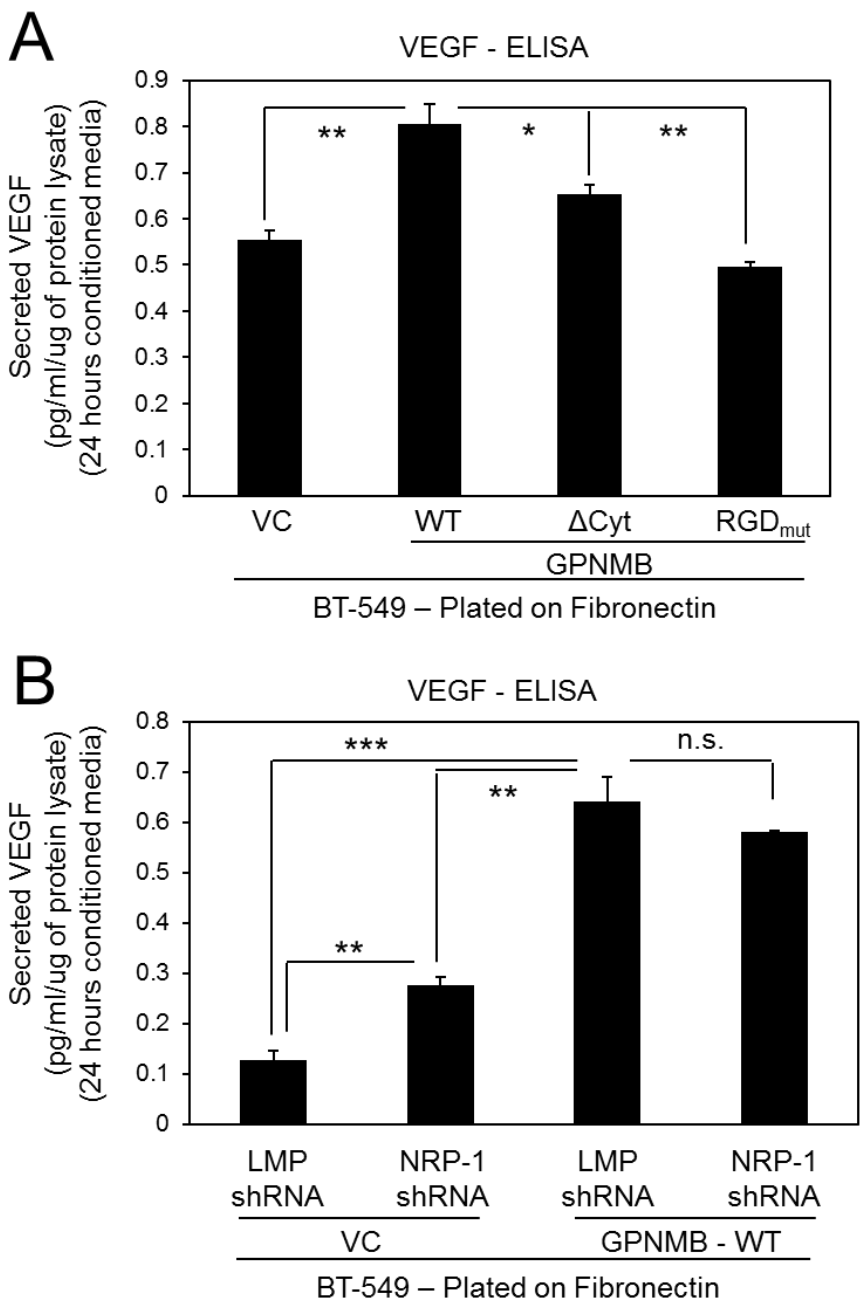


Figure 5.2 GPNMB increases VEGF secretion in breast cancer cells downstream of fibronectin engagement. Conditioned media was collected from BT549 cells overexpressing control vector, full-length GPNMB and GPNMB mutant constructs (A) or BT549 VC and GPNMB-WT cells harboring a NRP-1 knockdown (B) plated on fibronectin for 24 hours to stimulate signaling downstream of GPNMB. The amount of VEGF secreted in the conditioned media of BT549 cells was assessed and quantified by ELISA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) .

stabilization and transcription of downstream target genes under normoxic conditions in TNBCs [27]. This mechanism was engaged by HB-EGF-induced heterodimerization between GPNMB and EGFR, which was mediated through the intracellular domains of these proteins. Following heterodimerization, GPNMB was phosphorylated on Y525 by EGFR, which was required for further recruitment and activation of BRK and phosphorylation and stabilization of HIF1α [27]. These findings support our observations and provide a mechanism for GPNMB upregulation of VEGF in TNBCs, which is a well-known transcriptional target of HIF1α in hypoxic and normoxic conditions [28, 29].

Interestingly, the pattern of VEGF secretion by GPNMB-expressing breast cancer cells mirrors the degree of macrophage and endothelial cell recruitment observed in chapter 2 and 3. Therefore, we hypothesize that VEGF production by GPNMB-expressing cells in the primary tumor regulates the recruitment of a protumoral stromal infiltrate to accelerate the angiogenic switch and provide microenvironmental support to the growing tumor. Similarly, GPNMB can increase MMP production and release from breast cancer cells and thereby contribute to the ECM proteolysis required for cancer invasion and metastasis [4]. In light of these findings, we speculate that both epithelial and stromal cells contribute to the elevated VEGF production observed in GPNMB-expressing primary tumors (Figure 2.3D, 3.2C). Notably, the GPNMB-mediated increase in VEGF production is not detected in baseline conditions [5], suggesting that heightened ECM engagement by GPNMB promotes VEGF production. In this regard, fibronectin has been shown to regulate VEGF secretion through the β1 integrin subunit [30].

5.2 Functional roles of GPNMB beyond the tumor cell

5.2.1 Paracrine action of the GPNMB extracellular domain (ECD)

Previous studies have demonstrated that the shed extracellular domain of GPNMB can act on a variety of stromal cell types (including endothelial cells, fibroblasts, macrophages and mesenchymal stem cells) to mediate biological activities that are important for metastasis [31-35].

A recent study has demonstrated that the GPNMB ECD mediates migration, invasion and ECM adhesion of lung cancer cells in an RGD-dependent manner [12]. Interestingly, the immobilized, but not soluble, GPNMB ECD has been shown to promote adhesion of endothelial cells in an RGD-dependent manner [37], suggesting that the shed GPNMB ECD can incorporate into the tumor-associated matrix and bind α5β1 on neighboring cells via the RGD motif to promote tumor progression. In keeping with this hypothesis, integrin recognition of RGD motifs has been shown to promote both cell-substratum and cell-cell interactions that are important for cancer metastasis [38], [39].

It is possible that the GPNMB ECD shed from tumor cells can bind α5β1 in a manner similar to Osteopontin (OPN) and other members of the small integrin-binding ligand N-linked glycoproteins (SIBLING) family, which contain an RGD domain. OPN is secreted by tumor cells and promotes cancer adhesion, invasion and metastasis, largely through its interaction with RGD-binding integrins [40]. Secreted OPN can associate with αVβ3 integrin in an RGD-dependent manner, to recruit Src, activate the MAPK pathway and promote the motility and invasion of neighboring breast cancer cells [41]. GPNMB and OPN are both osteomimetic proteins, and their expression is regulated by common stimuli, suggesting that the two secreted proteins may carry out overlapping functions [42], [43], [44]. In this regard, exogenous GPNMB has been shown to physically associate with β1 integrin-containing complexes in osteoclasts and its expression is associated with increased

Src activity in this context, which lends credence to the theory that the shed extracellular domain of GPNMB can bind tumor and stromal α5β1 to enhance downstream signaling [45], [46]. Additionally, ECM containing GPNMB can increase the formation of focal adhesions by binding RGD-containing β1 integrin complexes and enhancing downstream Fak and Erk signaling [47]. Such a mechanism may explain the discrepancy between the *in vitro* and *in vivo* effects of the GPNMB-ΔCYT mutant, which retains its ability to interact with α5β1 (Figure 3.4 B, C) and could conceivably bind the fibronectin receptor on neighboring tumor and stromal cells in its shed form. Thus, we hypothesize that the GPNMB ECD exerts autocrine and paracrine effects that enhance tumor metastasis in an RGD-dependent manner, both by promoting motility and invasion of neighboring breast cancer cells at the primary site, and by priming the microenvironment at the secondary site to facilitate tumor outgrowth.

5.2.2 Tumor-derived exosomes

Exosomes have emerged as important mediators of cell-cell communication during cancer progression. Exosomes are microvesicles that contain protein and nucleic acid cargo, which are released by tumor cells to remodel the local microenvironment and regulate conditioning of future metastatic sites [48]. Exosome cargo varies by cancer type, and may contain growth factors, angiogenic factors and proteins involved in cell cycle regulation. Full-length GPNMB can be packaged into exosomes and released from melanomas and brain tumors [6, 49, 50]. GPNMB-containing exosomes are detectable in the serum of medulloblastoma patients and have been proposed as a tumor-specific exosome marker for this disease [50]. In melanoma, GPNMB-containing exosomes are thought to promote tumor growth at the primary and secondary site by mediating immune suppression within the local microenvironment [6]. Mechanistically, GPNMB contained in

exosomes binds syndecan-4 on neighboring T cells to inhibit T cell activation and facilitate melanoma evasion from immune recognition [6, 51, 52]. It has been hypothesized that GPNMB in melanoma-derived exosomes can travel to distal lymphoid organs to promote tumorigenesis through immune system regulation [6]. Accordingly, melanoma-derived exosomes have been involved in differentiation of distal monocyte to tumor-promoting myeloid suppressor cells [53]. In a similar fashion, GPNMB is expressed in infiltrating populations of MDSCs in late-stage melanoma patients and is a critical mediator of MDSC immune-suppressive function in melanoma [54].

We used an athymic mouse model to examine *in vivo* spontaneous breast cancer metastasis, which prevented us from evaluating whether GPNMB can have an immunosuppressive effect on T cells in breast cancer. However, it would be of great interest to examine if exosomes containing GPNMB are released from breast cancer cells, and if they can have pro-metastatic effects at local and distant sites. GPNMB packaged in exosomes could hypothetically regulate the tumor microenvironment by interacting with RGD-binding integrins on stromal cells to promote adhesion and migration or by binding CD44 for downstream increases in proliferation and migration via the Erk and Akt pathways [33].

Exosomes secreted by cancer cells are rich in integrins [55] and α5β1-containing exosomes regulate directionally persistent cancer cell migration *in vivo* [56]. Since integrins are an important exosomal cargo, we speculate that GPNMB may alter the exosome composition released into the extracellular milieu by cancer cells. Exosome formation occurs downstream of the late endosome in the vesicular transport pathway. Invagination and budding of intraluminal vesicles in late endosomes creates multi-vesicular bodies (MVBs). MVBs fuse with lysosomes to create hybrid organelles involved in

receptor degradation, or they can be trafficked to the cell periphery through a Rab 27a/b-mediated mechanism [57]. Exosomes are subsequently released by exocytosis when MVBs fuse with the plasma membrane [58]. Using two independent models, we demonstrate that intracellular GPNMB is primarily localized at late endosomes in basal breast cancer cells (Figure 3.7). Given that GPNMB located at late endosomes can promote trafficking of α5β1 integrins, it is plausible that sorting of α5β1 into exosomes could be increased in GPNMB-expressing breast cancer cells.

The integrin composition of exosomes has recently been shown to determine metastatic organotropism of cancer cells [59]. Exosomal integrins prime specific metastatic sites by activating Src phosphorylation and pro-inflammatory gene expression. The α5 and β1 integrin subunits were abundantly expressed in exosomes released by lung-metastatic breast cancer cells, but the α5 subunit was not seen in exosomes secreted by brain- and bone-tropic breast cancer cells [59]. Future experiments to determine whether tumor-derived exosomes play a role in GPNMB-driven metastasis would be of great interest. Our lab has generated 4T1 breast cancer cell subpopulations with distinct metastatic tropisms [4, 60] that exhibit elevated GPNMB expression [4] and could be used to answer these questions.

5.3 Microenvironment acidification and secretion of lysosomal proteases

Acidification of intracellular vesicles is an important process during endosome maturation. Progression from early to late endosomes, and ultimately to the lysosomes, is characterized by a decreasing intraluminal pH, which falls from around 6.8 in the early endosomes to as low as 4.5 in the lysosomes [61]. Lysosomes contribute to microenvironment acidification by secreting their intraluminal contents outside of the cell [62]. Conversely, acidic extracellular environments also increase secretion of lysosomal

proteases, such as members of the cathepsin and MMP families, which promote ECM degradation [63, 64]. Tumor progression is characterized by increased extracellular acidosis and cancer cells cultured in acidic environments display increased invasive and metastatic ability [65], a process that can be reversed when the extracellular pH is increased [66]. Accordingly, markers of extracellular acidosis are upregulated in invasive regions of breast DCIS [67]. Additionally, clinical investigations show that tumors with an acidic environment are associated with poor prognosis and enhanced metastatic incidence [67, 68].

Late endosomes and lysosomes traffic bi-directionally from the cell periphery to the perinuclear region [69], which is driven by actin- and microtubule-associated molecular motors [70, 71]. These molecular motors include dyneins and kinesins, which direct retrograde lysosomal trafficking towards the juxtanuclear region (microtubule minus end) and anterograde trafficking towards the cell periphery (microtubule plus end), respectively [72, 73]. The specificity of vesicle trafficking is dictated by Rho/ROCK proteins and Rab GTPases, which can link vesicles to motor proteins or cytoskeletal tracks [74, 75]. In non-tumorigenic cells, lysosomes are primarily located in the perinuclear region [76]. However, lysosomal re-distribution towards the cell periphery has been observed in cancer cells [76, 77] and can occur in response to several stimuli, including extracellular acidity and Ras-mediated transformation [78, 79]. A recent study has shown that lysosome redistribution to the plasma membrane is increased in response to overexpression of TFEB, a transcription factor belonging to the MITF/TFE family and a putative regulator of GPNMB expression [77, 80]. Given the increase in peripheral lysosomes that we have observed in GPNMB-expressing cells (Figure 3.8), it would be interesting to examine whether GPNMB acts downstream of TFEB to promote this phenotype in cancer cells.

Lysosomal trafficking to the cell periphery in response to extracellular acidic pH is associated with increased exocytosis of lysosomal proteases and tumor cell invasion [76, 78]. Accordingly, secretion of lysosomal cathepsins B, D and L into the extracellular milieu enhances tumor invasion and metastasis and is increased during cancer progression [81-83]. Notably, certain cathepsins play a very specific role in cancer progression, such as cathepsin K, which promotes breast cancer metastasis to bone and is considered an important therapeutic target in the prevention and treatment of bone metastasis [84, 85]. Our data shows that GPNMB increases tumor invasion and metastasis, and its expression in basal breast cancer cells is associated with increased pericellular lysosomal redistribution (Chapter 3). It is tempting to speculate that this phenotype could be associated with increased cathepsin secretion. Although we have not examined the status of secreted cathepsins in GPNMB expressing cells, we did observe a strong correlation between GPNMB expression and that of cathepsins B, L and K across 6 published breast cancer datasets (aggregated $r > 0.5$ for CTSB, CTSL and CTSK) (Figure 5.3). Notably, GPNMB is strongly co-expressed with cathepsin K in luminal breast cancers, which preferentially metastasize to bone ($r=0.79$ and $r = 0.71$ for luminal A and B breast cancers, respectively). It is conceivable that cathepsin K could be an important mediator of GPNMB-driven breast cancer bone metastasis [4]. Given this striking pattern of co-expression, the role of cathepsins as mediators of GPNMB paracrine action during tumor progression warrants further investigation.

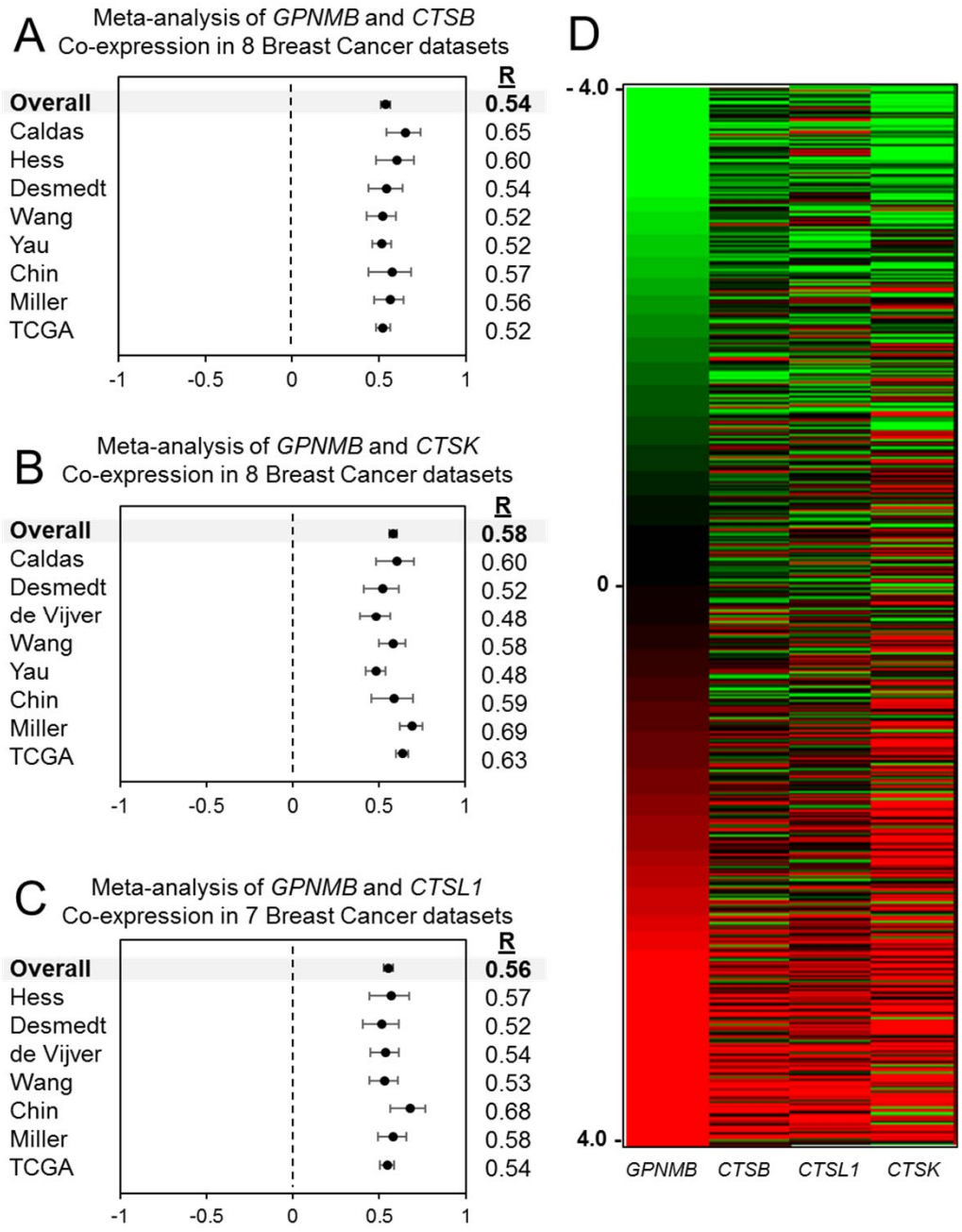


Figure 5.3 GPNMB is co-expressed with lysosomal cathepsins B, L and K in breast cancer Meta-analyses were used to correlate GPNMB expression with expression of cathepsin B (**A**), cathepsin K (**B**) and cathepsin L (**C**) across the indicated number of publicly available breast cancer datasets. The Pearson correlation coefficient (R) is included for each dataset. A heatmap is used to illustrate co-expression of GPNMB with cathepsins B, L and K in the TCGA dataset.

5.4 Context-dependent roles for GPNMB in cancer

Our data indicates that GPNMB can engage several separate and distinct mechanisms to promote primary tumor growth, invasion and metastasis, suggesting that context is an important determinant of GPNMB action in breast cancer. The following sections cover different context-dependent cues that regulate GPNMB engagement in cancer.

5.4.1 An oncogene-dependent role for GPNMB in triple-negative and basal breast cancers

In Wnt-driven tumors, GPNMB expression promotes breast cancer initiation, proliferation and survival but does not significantly affect the degree of stromal cell recruitment or metastasis (Chapter 4). The discrepancies between this data and the findings obtained using NIC, HS578T and 66CL4 models of breast cancer may be attributed to the influence of the dominant signaling pathway responsible for transformation. A 2006 study examining differences in gene expression across melanoma cell populations of varying metastatic potential showed that tumors can be segregated into distinct cohorts with mutually exclusive "proliferation" and "metastasis" signatures [86]. Xenograft and migration assays revealed that cell lines with a proliferative signature exhibit a dramatic increase in tumor growth but have a severely impaired migratory potential compared to cell lines with a metastatic signature [87]. Notably, a high degree of plasticity between the two states is observed during *in vivo* tumor progression [87]. The proliferation signature needs to be inhibited in order for the tumor cells to become invasive, but it also needs to be re-engaged for efficient establishment of secondary lesions [87]. Interestingly, the switch from a proliferative to an invasive state was characterized by inhibition of canonical Wnt signaling, and enhanced expression of non-canonical Wnt ligands, suggesting that

regulation of this pathway is essential for tumor cell plasticity [86]. Accordingly, canonical Wnt signaling is an important driver for the outgrowth of metastatic lesions [88-90], further supporting a need for dynamic regulation of the Wnt/β-catenin pathway during the metastatic process. The proliferative state in melanoma is also characterized by the presence of a MITF transcriptional signature, which includes GPNMB [86, 87]. Additionally, MITF expression is regulated through the canonical Wnt/β-catenin pathway [91, 92], which provides evidence for GPNMB and Wnt1 cooperation during tumorigenesis.

In breast cancer, canonical Wnt signaling is responsible for the maintenance and proliferation of tumor-initiating cell (TIC) populations [93]. Numerous studies have shown that highly invasive basal breast cancer cells need to upregulate Wnt signaling at the secondary site for efficient lung metastasis [88, 90]. In this setting, the canonical Wnt pathway is an important pro-growth signal regulating maintenance and expansion of lung metastatic breast cancer stem cell population [90]. This step-wise requirement for the activation (proliferation and maintenance of the CSC subset) and inactivation of canonical Wnt signaling (fostering an invasive phenotype) could explain the highly context-dependent role for this pathway in metastasis [94]. The MMTV/Wnt1 transgenic mouse model is characterized by stem-cell enriched basal populations and a weakly metastatic phenotype, which can be enhanced by resection of primary tumors [95]. Metastases arising in MMTV/Wnt-1 transgenic mice exhibit a gene expression profile which closely recapitulates that of primary tumors [96]. Interestingly, although significant heterogeneity exists among MMTV/Wnt-1-driven primary tumors, minimal variation in gene expression patterns is observed across individual metastases in this model [96, 97], suggesting that the metastatic phenotype of MMTV/Wnt-1 driven tumors is dictated by the initiating

oncogenic event, but not secondary genetic alterations. In this setting, GPNMB expression enhances the tumor-initiating capability and pro-growth phenotype of Wnt-1-driven breast cancer by increasing PI3K pathway signaling and promoting β-catenin activity (Figure 4.4). However, in 533 cells, where Wnt1 is expressed but is not a primary oncogenic driver, GPNMB is required for both tumor growth and metastasis (Figure 4.5). These findings suggest that GPNMB acts within the confines of the oncogenic context. In the case of Wnt1-dependent tumors, GPNMB expression acts to enhance the oncogenic state enforced by Wnt1 but, similar to other secondary genetic alterations observed in this model, GPNMB is not sufficient to mediate the plasticity required to augment metastasis.

5.4.2 A potential localization-dependent role for GPNMB in cancer

Additional observations argue that the role of GPNMB during cancer progression may be different depending on the specific type of cancer in question. Numerous reports have demonstrated that GPNMB expression is increased in melanoma compared to benign and normal skin tissues [98, 99]. The strongest evidence that functionally implicated GPNMB in melanoma progression came from the Ariizumi group, who showed that GPNMB expression promotes melanoma growth and metastasis in syngeneic, but not immunodeficient, mice by increasing the immune evasion capacity of tumor cells [6]. Notably, GPNMB knockdown reduced *in vivo* growth of B16 melanoma cells, but had minimal effect on *in vitro* proliferation or cell cycle entry, indicating that GPNMB primarily acts to modulate the extracellular environment in this model. The model systems we employed did not allow us to examine the interplay between GPNMB and T cells, and therefore we cannot rule out a role for GPNMB in creating an immune suppressive environment in breast cancer. However, in addition to this potential role, we show that GPNMB regulates several tumor extrinsic and intrinsic functions in breast cancer, such as

recruitment of a VEGF-producing stromal infiltrate, pro-survival signaling downstream of NRP-1 and stability of α5β1 integrin complexes. These data indicate that the GPNMB-mediated mechanisms of action outlined in this thesis are not necessarily applicable to other cancers where GPNMB exhibits tumor-promoting properties. A possible explanation is that melanoma is a highly immunogenic cancer and immune interventions have demonstrated greater clinical efficacy in melanoma compared to other cancers [100]. As a result, the immunosuppressive role of GPNMB likely has a bigger impact on tumor progression in melanoma compared to breast cancer. However, it is also plausible that the cancer-specific role of GPNMB is dictated by differential localization of GPNMB in the two cancer types.

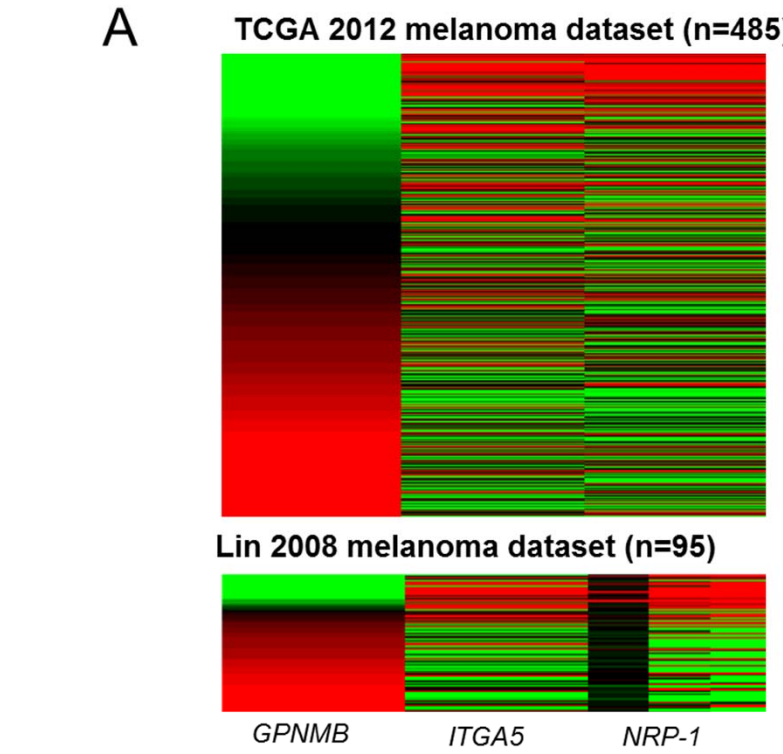
In Chapter 3, we show that GPNMB can regulate α5β1 integrin stability and cell surface expression by increasing recycling of the active, fibronectin-bound form of the receptor. It is well established that GPNMB expression is enriched on the cell-surface of cancer cells [1, 98, 101]. We show, for the first time, that the majority of the intracellular GPNMB pool is found in lysosomes in breast cancer cells, both in the endogenous state and in conditions where GPNMB is overexpressed, suggesting that GPNMB acts to increase integrin recycling through the recently described late endosomal/lysosomal pathway [105, 106]. Notably, GPNMB shares homology with the lysosome-associated protein LAMP-1 and localizes to lysosomes under diverse pathophysiological conditions, which supports our observations [37, 102-104]. It is therefore conceivable that the pattern of GPNMB localization in breast cancer cells is an important determinant of its pro-metastatic effects.

Conversely, in melanocytes and melanoma cells, intracellular GPNMB is primarily localized to late-stage (III and IV) melanosomes [107]. A putative role for GPNMB in melanin synthesis and melanosome biogenesis has been suggested [102, 108]. Consistent

with these observations, melanoma primary tumors and metastatic lesions that express higher levels of GPNMB also display increased pigmentation [109] [6, 110]. Melanosomes are lysosome-related organelles found in pigmented epithelial cells, which coexist with conventional lysosomes and are responsible for the production and storage of melanin [111, 112]. Melanosome maturation occurs through four distinct stages [113] and late-stage melanosomes are characterized by accumulation of melanin pigment and translocation of the structure closer to the cell periphery [112]. Melanosomes and lysosomes are both generated from the endocytic pathway and have a common precursor called the stage I melanosome or vacuolar early endosome [114]. In a process that is unique to pigmented epithelial cells, melanosome-specific macromolecules such as GPNMB must be segregated and sorted from the ones that are destined for late endosomes and lysosomes [112]. Interestingly, when melanosomal proteins are expressed in non-pigmented cells, they tend to localize to late endosomes and lysosomes [115, 116], which is consistent with the pattern of expression observed for GPNMB. We hypothesize that the differential pattern of intracellular GPNMB distribution in melanoma and breast cancer partly dictates the differences in mechanisms of action observed in these two contexts. Notably, GPNMB localized to melanosomes is not able to interact with α5β1 integrin complexes trafficking through the late endosomal/lysosomal pathway to increase receptor recycling and promote invasion. Given that NRP-1 is also a GPNMB-interacting partner, it is possible that the NRP-1 mediated effects downstream of GPNMB could also be partly attributed to the localization pattern of GPNMB in basal breast cancer cells. Conversely, GPNMB gets targeted to lysosomes in most other cancer types, which broadens possible GPNMB interactions given that the majority of cellular proteins will pass through the late endosome/lysosome for sorting or degradation.

Accordingly, GPNMB co-expression with NRP-1 and integrin α5 appears to be cancer-specific. Analysis of published datasets indicates that *GPNMB* expression is inversely correlated to *NRP-1* and *ITGA5* expression in melanoma (Figure 5.4A, B). The correlation coefficient for *GPNMB* and *NRP-1* co-expression ranges from -0.43 to -0.46 in melanoma, and from 0.31 to 0.58 in breast cancer (Figure 5.4B). *GPNMB* expression also exhibits a negative correlation with *ITGA5* in melanoma (r = -0.27 to -0.39) (Figure 5.4B). Furthermore, while GPNMB expression is associated with proliferative melanoma cohorts, expression of NRP-1 and integrin β1 is increased in invasive cohorts [86]. In light of this data, we speculate that GPNMB cooperates with NRP-1 and α5β1 to promote tumor progression in breast cancer but not in melanoma partly due to differential localization of GPNMB in the two contexts.

GPNMB and other MRGs such as PMEL17, TYPR1 and MART1, which typically localize to melanosomes, can be sorted into exosomes and secreted by melanoma cells [6, 117, 118]. Therefore, although it is possible that the melanosomal localization of GPNMB in melanoma restricts its tumor-intrinsic mechanisms of action, GPNMB can still promote melanoma tumor progression through paracrine effects. Additionally, GPNMB is enriched on the cell surface of melanoma cells and its cell-surface localization is further increased by inhibitors that upregulate GPNMB expression [98, 109]. It would be interesting to examine whether GPNMB targeting to exosomes or to the cell surface in melanoma occurs following re-routing of GPNMB from the melanosomal to the endosomal trafficking pathway and if this trafficking switch contributes to melanoma aggressiveness.



B

	Dataset	Pearson correlation (r)	
		ITGA5	NRP-1
Melanoma	Melanoma TCGA	-0.39	-0.46
Melanoma	Lin 2008	-0.27	-0.43
Breast Cancer	Breast TCGA	0.47	0.58
Breast Cancer	Miller	0.39	0.37
Breast Cancer	Wang	0.39	0.36
Breast Cancer	Chin	0.30	0.44
Breast Cancer	Desmedt	0.36	0.31
Breast Cancer	Van de Vijver	0.32	
Breast Cancer	Yau		0.34

Figure 5.4 Comparison of GPNMB correlation with NRP-1 and ITGA5 in melanoma and breast cancer patient datasets (A) Heatmaps ordered by GPNMB mRNA expression are used to illustrate the degree of correlation between *GPNMB*, *ITGA5* and *NRP-1* expression across 2 publicly available melanoma gene expression datasets. (B) Overview of Pearson correlation coefficients comparing co-expression of GPNMB with ITGA5 and NRP-1 across melanoma and breast cancer gene expression datasets.

5.5 Therapeutic implications

Targeting GPNMB for the management of metastatic breast cancer is currently being explored using glembatumumab vedotin (GV), a human immunoglobin G2 monoclonal antibody (CR011) attached to a tubulin-destabilizing cytotoxic moiety (MMAE) via a valine-citrulline (vc) linker protein [119]. The GV mechanism of action involves recognition of the extracellular domain of GPNMB by the parent antibody, internalization of the GPNMB/CDX-011 complex, cathepsin B-mediated proteolytic cleavage of the linker protein in the lysosome and cytoplasmic release of MMAE for the selective killing of GPNMB-expressing cells [119]. Despite encouraging preliminary results, the latest phase IIb clinical trial examining effectiveness of GV in GPNMB-expressing metastatic TNBCs indicates that single-agent use of GV does not significantly improve progression-free survival, overall survival, or overall response rate [120]. Although further single-agent GV trials are not being pursued in breast cancer, GPNMB remains an important therapeutic target in TNBCs that is readily amenable to clinical targeting due to its cell surface expression in cancer tissues. Therefore, it would be worthwhile for follow-up studies to investigate efficacy of GV in a combination therapy setting and to focus on mechanisms which enhance efficacy of GV-mediated tumor cell killing.

Numerous studies have shown that the level of GPNMB cell surface expression is directly correlated to GV efficacy [5, 98, 101, 121, 122]. GV selectively acts to prolong OS and PFS in patients with triple-negative breast cancer expressing GPNMB [2, 3]. Similarly, the *in vitro* and *in vivo* cytotoxicity of GV on osteosarcoma cell lines was directly proportional to GPNMB cell surface expression in a recent study, providing strong rationale for GPNMB targeting in pediatric osteosarcoma clinical trials [122]. Importantly, GPNMB

is primarily localized to intracellular compartments in normal cells, but it is enriched on the cancer cell surface [1, 98, 101]. Additionally, we have shown that intracellular GPNMB is primarily routed to the lysosome in breast cancer cells, which increases the opportunity for cathepsin B-mediated cleavage of the vc linker protein and provides further support for ADC-mediated targeting of GPNMB. Although this pattern of expression makes GPNMB uniquely amenable to therapeutic targeting in cancer, epithelial GPNMB is only expressed by 10-40% of TNBCs [1, 3, 123]. Therefore, understanding how GPNMB cell surface enrichment increases with cancer malignancy would considerably improve therapeutic targeting of basal and triple-negative breast cancers.

GPNMB localization to lysosomes in breast cancer provides insight into potential mechanisms driving GPNMB cell surface expression in cancer. During tumor progression, the acidic pHe promotes lysosomal fusion with the plasma membrane to facilitate delivery of lysosomal cargo to the cell surface or the cell exterior. Exposure of MCF7 breast cancer cells to acidic conditions led to significant *in vitro* and *in vivo* upregulation of proteins known localize to the lysosome [124]. Specifically, the LAMP2 lysosomal protein was located at the plasma membrane in clinical samples and exhibited acid-induced redistribution to the cancer cell surface [124]. The LAMP1 lysosomal protein, which exhibits high structural homology with GPNMB but is located in lysosomes in melanoma cells, is enriched at the cell surface of melanoma cells with high metastatic potential and plays a key role in regulating metastatic lung colonization via galectin-3 [125, 126]. We hypothesize that GPNMB is similarly redistributed from the lysosome to the plasma membrane, via lysosome exocytosis, during breast cancer progression as consequence of cancer adaptation to the acidic microenvironment. It would be interesting to examine

whether acidic extracellular pH can increase cell surface expression of GPNMB and efficacy of GV in breast cancer.

GPNMB expression is also increased across various breast cancer subtypes in response to therapeutic approaches. Treatment with fulvesterant increased GPNMB expression in luminal cancers and trastuzumab treatment upregulated GPNMB expression in HER2-positive breast cancer cell lines [127, 128]. GPNMB upregulation under these conditions may potentially be explained by its lysosomal association. It was recently shown that lysosomal sequestration of hydrophobic anti-cancer drugs such as gefitinib, cetuximab and sunitinib is a potential mechanisms of multi-drug resistance [129]. Following treatment with hydrophobic weak base therapeutics, cancer cells initiate a TFEB-driven lysosomal biogenesis transcriptional program, which increases expression of lysosomal genes [129]. Notably, lysosomal biogenesis can also be driven by TFE3 in breast cancer. It is highly plausible that GPNMB enrichment on the cell surface of cancer cells occurs in response to engagement of a broader lysosomal biogenesis pathway following treatment with select therapeutics. Exploiting GPNMB lysosomal localization in breast cancer to overcome resistance to weak-base chemotherapeutics warrants further investigation and represents a promising avenue for treatment of TNBCs. Given the disappointing results from the METRIC trial, which showed that treatment with GV did not improve progression-free survival of patients with GPNMB-expressing TNBC [120], the clinical development of GV in breast cancer is likely to shift from testing its efficacy as a single agent to combination strategies involving already approved therapies such as chemotherapy for the treatment of TNBC. GPNMB upregulation in response to targeted treatments has also been observed in other cancers and has been proposed as a mechanism of therapeutic resistance [109, 130]. In these pre-clinical studies, combination therapy with GV enhanced anti-tumor activity of

approved targeted agents [109]. Similarly, dual inhibition of GPNMB and other TNBC targets such as EGFR or the Wnt/β-catenin pathway could improve targeting and killing of heterogeneous TNBC populations and represents an important avenue of investigation for the treatment of aggressive TNBCs.

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