The role of Receptor Interacting Serine/Threonine Kinase 2 (RIPK2) in inflammatory breast cancer (IBC)

by

Alaa Zare

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Medical Sciences- Pediatrics
University of Alberta

ABSTRACT

Inflammatory breast cancer (IBC) is a rare but aggressive type of breast cancer characterized by early and rapid metastasis leading to poor clinical outcomes. The tumor microenvironment (TME), including immune cells, fibroblasts and endothelial cells, has emerged as a major regulator of IBC aggressiveness, however little is known about how IBC cells may orchestrate this pro-tumorigenic milieu. This work helps us understand the importance of TME in promoting IBC metastatic behavior. Recent studies indicated that inflammatory pathways such as NF-κB concomitant with the secretion of cytokines are key elements in nurturing the IBC TME. Although NF-κB and its target genes are known to be upregulated in IBC tumor samples, its direct effect has never been studied. Herein, I show for the first time how NF-κB activation through the receptor tyrosine kinase RIPK2 can contribute to IBC progression by promoting metastatic phenotypes in cancer cells. Specifically, RIPK2 was shown to promote an inflammatory transcriptome in IBC cells leading to the secretion of factors such as IL-8, IL-6 and Activin-A. As a corollary, RIPK2 enhanced key IBC phenotypes, including angiogenic potential and metastatic growth in the lung.

I also demonstrate the status of RIPK2 activity in IBC tumor samples using a special monoclonal antibody directed against the phosphorylation site Y474 utilizing IBC cell models and patient tumor samples. Elevated levels of RIPK2 phosphorylation were present in IBC samples collected at the time of diagnosis. However, chemotherapy did cause an increase in RIPK2 activity, suggesting its role in augmenting inflammation in breast tissue, which subsequently can lead to treatment resistance. Further, RIPK2 activity correlated with tumor, metastasis, and overall group stage, as well as body mass index (BMI), to indicate that RIPK2 might be a useful prognostic marker for IBC.

In addition, I help identify a robust gene signature able to differentiate IBC samples from non-IBC. We report a novel IBC-specific gene signature (59 genes; G59) that achieves 100% accuracy in discovery and validation samples and remarkably only misclassified one sample in an independent dataset. G59 is independent of ER/HER2 status, molecular subtypes and is specific to untreated IBC samples, with most of the genes being enriched for plasma membrane cellular component proteins, interleukin (IL), and chemokine signaling pathways. Our finding suggests the existence of an IBC-specific molecular signature, paving the way for the identification and validation of targetable genomic drivers of IBC.

In conclusion, this thesis has revealed, for the first time, a critical role for RIPK2 in the regulation of IBC phenotypes. These results suggest that RIPK2 may be an attractive target for this poorly managed disease.

PREFACE

Each chapter constitutes a manuscript that has been submitted or ready to be submitted. Contributions from all authors are as noted below

CHAPTER 2 RIPK2 activity in IBC cell lines and patient tumor samples

Chapter 2 is an edited version of "RIPK2: New Elements in Modulating Inflammatory Breast Cancer Pathogenesis" that has been published in *Cancers* (2018). doi: 10.3390/cancers10060184. Authors in order, Zare A, Petrova A, Agoumi M, Armstrong H, Bigras G, Tonkin K, Wine E, and Baksh S. I (A.Z.) was responsible for study and experimental design, data analysis, results interpretation and writing the manuscript. I performed all the experiments, unless stated otherwise. K.T. and G.B. aided in sample selection, confirmation of disease and image quantitation. H.A. and E.W. contributed by helping us obtain the unstained slides for the immunohistochemical analysis. M.A. contributed to confirming breast cancer pathology of our archival samples. S.B. supervised this study, contributing to conception, study design and manuscript editing prior to publication.

CHAPTER 3 RIPK2 promotes metastatic behavior in inflammatory breast cancer by regulating NF-κB signaling and key players in the tumor microenvironment

Chapter 3 is in the process of submission. Authors in order but not final, Zare A, Githaka J, Azad A, Lemieszek M, Zhang G, Liu Z, and Postovit LM. I (A.Z.) was responsible for study and experimental design, data analysis, results interpretation and writing the manuscript. I performed all the experiments, unless stated otherwise. J.G. analyzed immunofluorescence staining of NF-κB translocation, RNA-seq analysis, and editing the manuscript. A.A. preformed angiogenesis assay. M.A. helped in RNA extraction and RT-PCR. G.Z. and J.L. did the mouse experiments and performed IHC. L.M.P. supervised this study, contributing to conception, study design and manuscript editing.

CHAPTER 4 Robust inflammatory breast cancer gene signature using nonparametric random forest analysis

Chapter 4 was submitted to *Breast Cancer Research* (2021). doi.org/10.1186/s13058-021-01467-y. Authors in order, Zare A, Postovit LM, and Githaka J. A.Z. and J.M.G. conceived the study. A.Z. and J.M.G. conducted datasets searches. J.M.G. conducted signature discovery and validation analyses. A.Z. and L.P. provided expertise on IBC. A.Z., L.P. and J.M.G. wrote the paper. All authors read and approved the final manuscript.

All the studies were conducted in compliance with the recommendations of the Research Ethics Guidelines, Health Research Ethics Board of Alberta. Ethics approval for patients' samples in chapter 2 was obtained from the Health Research Ethics Board (study ID Pro00015569). Animal work done in chapter 3 was approved by the Animal Use Subcommittee at the University of Alberta (AUP00001288).

ACKNOWLEDGMENTS

I would like to thank everyone who was a part of my Ph.D. journey; without your help and support, this work would not be possible.

I'm extremely grateful to my supervisor, Lynne Postovit, for her support, guidance, and mentorship. Thank you, Lynne, for being a model of a real scientist and a transparent researcher, and for showing me how good science can make a difference in the outcome. Thank you for creating a critical yet positive and supportive work environment, which made my journey more enjoyable despite all the obstacles. Thank you for being simple and accessible at all levels. I will always cherish my experience in your lab.

Dad and Mum, it's hard to put my feeling into words but thank you for making me the woman I am today. Dad, thank you for believing in me and giving me the confidence to do what I want with no limits. You are my strength! Mum, you keep showing me how to be strong and determined, and I admire that. Your passion and motivation for learning make me grateful to be your daughter.

Abdullah, my husband, and best friend, I never could have done this without your constant support and selfless love. Thank you for making me see the bright side of things. I'm grateful to have you in my life and happy to celebrate this achievement with you.

To all my siblings, nieces and nephews, thank you for making my life more interesting and for making me live all the drama through the phone while being away from home; it was a good distraction when needed.

Thank you, John Githaka, for all the help, collaboration, and good time we spent talking about science. Your passion for research is contagious. You always amaze me with your work and new ideas. Guihua and Jahui, thank you for your help and the work you do in the lab. Thank you for constantly checking on me and making sure I have everything I need for my experiments.

Many thanks to all my colleagues and friends in the Postovit lab for the support and wonderful time. I also would like to thank my advisors at the University of Alberta and The Saudi Cultural Bureau for their constant support and to the Saudi ministry of education for their funding.

TABLE OF CONTENTS

List of Figures

LIST OF TABLES

CHAPTER 1

General Introduction and Literature Review

1.1 BREAST CANCER

1.1.1 Normal breast development

The breast tissue undergoes structural remodeling and differentiation throughout the female body's maturation to serve its primary function: producing and secreting milk [1]. This function is carried out by mammary glands, composed of alveoli grouped in secretory lobules. Milk produced inside alveoli is emptied into interconnected ducts that carry it to the nipple. The breast lobes and ducts are surrounded and supported by the stroma, composed of laminin, collagen, and fat tissue [2] [3] [4]. Breast development starts in the prenatal stage to two years old, with mammary stem cells forming the primary mammary building blocks. The process of breast development is called branching morphogenesis, and while it starts in the embryogenic stage, it remains quiescent throughout the female childhood until puberty [5].

In the prenatal stage, proliferation and differentiation of mammary stem cells form primary mammary buds and milk lines. Regulatory factors in the mesenchyme direct the primary mammary buds to sprout to secondary mammary buds, later giving rise to mammary glands and lactiferous ducts [1]. Lactiferous ducts connect to the nipples and are lined with two types of epithelial cells, basal and luminal cells. The basal cells are attached to the duct basement membrane and separate the mammary epithelial and stromal compartments. Basal cells differentiate into myoepithelial cells responsible for secreting laminin, collagen, and fibronectin, the main component of the breast extracellular matrix, besides mediating the signal from the breast microenvironment to luminal cells [6] [7]. Luminal cells lay on top of the continuous layer of myoepithelial cells and are organized to form a hollow tube. Luminal cells differentiate further into ductal or alveolar luminal cells. The former line the duct wall, and the latter are responsible for milk secretion in the alveoli during lactation [8] [7].

As puberty approaches, female hormones such as estrogen, progesterone, and growth hormones start stimulating breast growth and more complex ductal branching and elongation through the activation of cell proliferation and differentiation. Simultaneously, a significant increase in the nipple size and breast stroma occurs, accompanied by more fat tissue accumulation [1]. As the female body undergoes ovulation after puberty, more hormones are secreted in preparation for possible pregnancy, increasing epithelial cell proliferation. If pregnancy occurs, epithelial cells will continue to proliferate to elongate the terminal duct lobular unit (TDLU), and alveolar luminal cells will differentiate further to milk-producing cells [9]. Breasts revert to their normal size post-weaning as epithelial cells undergo apoptosis in a process known as involution [10]. The presence of mammary stem cells in the breast ducts allows for breast tissue expansion and regression, making the mammary glands highly regenerative [11]. When the levels of estrogen and progesterone drop near menopause, the breast tissue loses its elasticity leading to a shrinking in the breast size [12].

1.1.2 Normal breast microenvironment

The breast microenvironment is composed of cellular and non-cellular components. The cellular components include mammary stem cells, epithelial cells, fibroblasts, adipocytes, endothelial and immune cells [13] [14]. The non-cellular components include basement membrane (BM) and extracellular matrix (ECM) components, growth factors, and cytokines. Evidence shows that elements of the breast microenvironment work in a complex and dynamic manner, taking part in signal initiation or mediation to ensure proper breast development [13] [14].

Mammary stem cells in the breast microenvironment are regulated via signals initiated by cell-cell interaction and ECM components such as laminin and collagen [15]. Through the expression of Integrin receptors, mammary stem cells and progenitor cells can react with collagen and laminin,

mediating the signals required for their proliferation and differentiation. The reconstitution of the mammary glands by mammary stem cells is maintained through cytokines and growth factors acting as autocrine and paracrine signals in the microenvironment, while immune cells (primarily macrophages) maintain cells stemness[16]. Fibroblast also maintain breast regeneration. Indeed, the presence of fibroblast in 3D Matrigel culture was also found to enhance mammary stem cells' regenerative activity [17 15 18 11]. Emerging evidence shows that the microenvironment can determine cell fate and function. One example is the breast microenvironment directing non-mammary epithelial stem cells to generate a functional mammary gland [19].

1.1.3 Breast cancer development and progression

Breast cancer (BC) arises primarily from the epithelial component of the breast ducts or lobules. Less common types of BC originate from the stromal components such as myofibroblasts and blood vessel cells are classified as breast sarcoma[20]. How mammary epithelial stem cells transform into breast cancer stem cells is still unclear [21]. However, genetic and epigenetic events have a role in this transformation. Mutations in *BRCA1* and *BRCA2* genes are the most important germline mutations associated with the early onset of breast cancer [22 23 24]. Somatic mutation of genes involved in essential pathways such as PI3KCA/AKT/PTEN, TP53, and NF-κB was also reported in many cancers including breast cancer [25 26].

The mutation accumulation along with microenvironment signals initiate breast cancer progression through defined pathological and clinical stages[21]. Breast cancer starts with abnormal growth of the epithelial cells found in the luminal lining of the mammary ductal-lobular unit. This neoplastic hyperproliferation is confined and isolated from the breast stroma by an intact myoepithelial layer and basement membrane, hence the name Ductal carcinoma in situ (DCIS).

DCIS is non-invasive; however, it is a precursor of invasive ductal carcinoma according to its molecular and pathological profile. Early detection and proper treatment effectively prevent cancer progression [27] [28].

It is considered invasive breast cancer when tumor cells invade through the basement membrane to the surrounding tissue outside the ducts or lobules. There are two types of invasive breast cancer: invasive ductal carcinoma (IDC) and lobular carcinoma (ILC). IDC is more common than ILC, representing 80% of all breast cancers [20]. Both types of breast cancer display different histological and molecular signatures making their progression and treatment distinctive. A higher percentage of ILC cells express estrogen receptor (ER) and show loss of E-Cadherin [29]. As IDC and ILC progress, tumor cells invade and grow in distant organs such as the lungs and bone. At such a level, the cancer is classified as metastatic breast cancer [20]. Studies found that ILC metastasizes less than IDC since it proliferates much slower, but it can reach less common sites such as the ovaries [29].

The transition from non-invasive breast cancer to invasive is poorly understood. Interestingly, molecular changes such as copy number profile and activation of oncogenes are found present in both DCIS and IDC, suggesting a common progenitor cell is shared [30] [31] [32]. Nonetheless, components of the tumor microenvironment such as ECM and immune cells show evident changes as cancer transitions from DCIS and IDC[32] suggesting the importance of the tumor microenvironment in breast cancer progression.

1.1.4 Breast cancer microenvironment

Much like the microenvironment regulates mammary stem cell fate, the tumor microenvironment (TME) regulates cancer stem cell (CSC) activity[33] [34]. CSCs are characterized

by the ability to self-renew and to differentiate into a variety of phenotypes, which gives them the power to regulate cancer cell populations needed for tumor growth and progression [34]. Studies suggest that CSCs drive tumor recurrence and cancer therapy resistance [35]. Mammary stem cells and breast cancer stem cells share common features; for example, both stem cells express CD44 and ALDH markers [36] [37]. In addition, both show upregulation of conserved signaling pathways known to be essential for cell proliferation and regeneration, such as Notch and Wnt [3839].

CSC regulation is mediated by the presence of cytokines, cancer-associated fibroblasts, tumor-associated leukocytes, and mesenchymal stem cells[11]. Cytokines can maintain an inflammatory environment favoring cancer development and progression by nurturing cancer cells' propensity to survive and thrive[40] [41]. This function is attained through multiple levels of connections in the tumor microenvironment. Cytokines mediate signals between neighboring tumor-associated immune cells such as macrophages, natural killer, dendritic cells, neutrophils and lymphocytes. They also control autocrine or paracrine signals in tumor cells, fibroblast, and adipocytes [42] [43] [43] [11].

There are different types of cytokines, including interleukins (IL), tumor necrosis factor (TNF), chemokines, interferons (IFN), and Transforming Growth Factor-β (TGFβ)[44]. Several interleukins are shown to have a role in inflammation-induced carcinogenesis; more studied ones include IL-1, IL-6, and IL-8 [45].

IL-1 is expressed by both immune and epithelial cells and acts through its receptor (IL-1R) to initiate and mediate inflammation[46]. Mice with epithelial IL-1R deficiency showed a significant decrease in the tumor volume and metastasis compared to control and less accumulation of NF-κB in the nucleus signifying that IL-1R may be essential for NF-κB activation in the tumor epithelium

[47]. IL-1 and IL-1R were also found to be upregulated in breast cancer, and inhibiting IL-R indicated that IL-1 promotes tumor progression and metastasis [48].

IL-6 is upregulated in most types of cancer, including breast cancer [49]. IL-6 regulation of proliferation, differentiation, angiogenesis, and metastasis is mediated through its initiation of major signaling pathways such as JAK/STAT3, Ras/MAPK, PI3K– PKB/Akt, and NF-κB [49] [50]. The increase of IL-6 expression in breast cancer samples correlates with tumor grade, and IL-6 serum level correlates with tumor stage, number of metastatic sites and poor prognosis[51].

IL-8 is an important cytokine produced by monocytes, endothelial, and epithelial cells in response to pathogen infection, inflammation, and cancer. It mediates its signal through CXCR1 or CXCR2 chemokine receptors. Various types of cancer show an increase in IL-8 levels and this increase correlated with cancer progression and resistance to immunotherapy[52] [53]. IL-8 can maintain a positive autocrine loop to induce the mesenchymal traits of tumor cells through main signaling pathways such as AKT, MAPK/ERK, and JAK2/STAT3[54]. Breast cancer mammosphere formation is enhanced by the addition of IL-8 , and inhibition of its receptor CXCR1 decreased breast cancer stem cell activity and metastasis in mouse xenograft models[55] [56].

Transforming Growth Factor-β (TGFβ) is a family of cytokines with more than 30 members, including Activin and Nodal. TGFβ signaling can be mediated by many proteins but primarily involves SMAD proteins [57]. The mutation of TGFβ or its receptors is well documented in cancer and is considered one of its hallmarks. It is found that such mutation results in loss of TGFβ tumor suppressive function. TGFβ was shown to alter the tumor microenvironment and induce epithelial-to-mesenchymal transition (EMT), resulting in tumor cell proliferation, invasion, and metastasis [57]. In addition, breast cancer epithelial cells increase $CD44^{high}CD24^{low}$ stem cell populations upon treatment with TGFβ [58].

1.1.5 Breast cancer classifications

The staging system is essential in any type of cancer as it reflects the growth rate and extension of the tumor. Accurate staging aids in assessing tumor prognosis and proper clinical treatment, which is essential for patient survival. In breast cancer, the traditional staging system is based on the anatomic findings: tumor size (T), nodal status (N), and metastases (M). However, medical advancement in imaging and treatment required the inclusion of more parameters. Thus, several years ago, the UICC (International Union Against Cancer) eighth edition staging system included the pathological and clinical prognostic stages. The pathological prognostic stage is determined by examining the tissue removed surgically in the initial treatment. The clinical prognostic stage depends on the physical examination as well as the biopsy and imaging results. Both prognostic stages incorporate biological factors such as histological grade and the molecular subtype. Results of the TNM staging along with the biological prognostic markers are evaluated and classified into a clinical or pathological prognostic stage group. Pathological staging will incorporate the clinical staging data and is considered more accurate [59].

Once breast cancer is confirmed histologically, the TNM staging of the disease will proceed. The **tumor size (T)** reflects the spread of the primary tumor out of the breast tissue. When the cancer is limited to the breast tissue, it is considered, Tis (carcinoma in situ). Tumor sizes of 2 cm and larger are given a number from 1 to 4. A tumor of any size spreading to the chest wall or skin is considered T4. The metastatic T4 stage is further characterized, and depending on the patients' clinical symptoms, the T4d stage is given to inflammatory breast cancer. The **nodal status (N)** indicates the spread of the tumor to the nearby lymph nodes and the number of nodes involved. Lymph nodes examination sometimes requires special techniques such as immunohistochemistry

and RT-PCR, besides the regular staining. Lastly, **metastases (M)** indicate if the tumor has spread to distant organs such as bones, lungs, or liver [60] [61].

While stage indicates the tumor size and extension, cancer's **histological grade** describes the levels of cancer cell differentiation compared to normal cells. This biological prognostic grading system helps determine the cancer prognosis by evaluating tissue morphological features such as the formation of tubules, mitotic count and variability, and the size and shape of cellular nuclei. Scores from 1 to 3 are given to each feature (1 being the more favorable and 3 the least favorable), and the total is calculated. Well-differentiated tissue is considered grade 1 with a score of 3 to 5, where cancer cells look more like normal breast tissue and proliferate slower. Moderately differentiated is grade 2 with a score of 6 or 7 wherein cancer cells look close to breast tissue and grow faster. Poorly differentiated is grade 3 with a score of 8 or 9 wherein cancer cells look different from the normal breast tissue and proliferate rapidly. Necrosis is also noted and indicates the rapid growth of the tumor [62] [63].

The **molecular subtype** is also used to determine the prognostic stage of breast cancer. Estrogen receptor (ER) and progesterone receptor (PR) expression is examined in breast cancer tissue using immunohistochemistry[64]. Cancer cells showing a positive stain of 1% or higher are considered positive for the present receptor[65]. Human epidermal growth factor receptor 2 (HER2) is also examined using immunohistochemistry or fluorescent in situ hybridization (FISH) to determine the signal strength[66]. Based on the expression profile of ER, PR, and HER2, breast cancer is categorized into three main subtypes[67]. **The luminal subtype** comprises ER and PR positive breast cancer and represents 60% of the cases. The name is given based on the shared expression profile between luminal breast epithelial cells and tumor cells. The luminal subtype is

further classified as luminal A and luminal B. Luminal A is more common, and cells have higher expression of ER-related genes and lower expression of HER2 cluster genes[67].

On the contrary, luminal B is less common and has lower expression of ER-related genes, resulting in worse outcomes than luminal A. **HER2 enriched** is the second most common subtype of breast cancer, representing almost 20% of all cases. As the name suggests, tumor cells express high levels of HER2 along with no expression of ER and PR receptors[67]. **ER-negative subtypes** include several types of breast cancer, but the most common are basal-like. Basal-like represent 15-20% of breast cancer and are so-called because tumor cells share the same gene expression profile with basal epithelial cells of normal breast tissue[68]. Hence, tumor cells do not express *ER, PR*, and *HER2* genes, and the term "triple-negative breast cancers" is used to describe this type of neoplasia. Triple-negative breast cancer has a worse prognosis since targeted treatment is only found for luminal and HER2 enriched subtypes [69] [70] [64].

1.2 INFLAMMATORY BREAST CANCER

Inflammatory breast cancer (IBC) is an advanced type of breast cancer considered rare but fatal. It comprises 2-4% of breast cancer diagnoses, however, accounts for 7-10% of of all breast cancer-associated deaths. The 5-year survival rate of IBC patients is less than 39% [71]. This poor outcome can be attributed to a couple of factors; (a) the rapid progression of the disease, resulting in distant metastases in one-third of its patients at diagnosis. (b) In many cases, the absence of a palpable mass, leading to false-negative breast examination and mammogram [72] [73]. (c) The high incidence of TNBC and HER2 positive subtypes in IBC in comparison to non-IBC, which also contributes to the aggressiveness of the disease [74]. The main differences between non-IBC and IBC are summarized in Table 1.1.

According to the Canadian Cancer Society, in 2021, it is estimated that 27,700 women will be diagnosed, and 5,400 will die of breast cancer [75]. Based on IBC incidence in the US[71] , these numbers suggest that 5,480 - 10,960 women will be categorized as IBC, and between 357-510 women will die of IBC in Canada. While incidence might vary in other parts of the world, and some cases showed an increase, the progress made in setting the diagnosis criteria of IBC adjusted the incidence statics over the years [71].

Table 1.1 Main differences between non-IBC and IBC

	Non-IBC	IBC
Clinical features	less common	Breast swelling and redness and orange skin appearance
Tumor solid mass	Yes	Not always
Age	Average age above 50 years old	5 years younger than Average age
5-year survival rate	90%	39%
Stage	Any stage	At least stage III
Most common subtypes	Luminal A	HER2 and TNBC
Metastasis	1 in 8 women	1 in 3 women
Ki-67 *	low	High

*For Ki-67, the percentage of positive staining cells was determined by direct counting and staining in >9% of tumor cells was considered high proliferation [291].

1.2.1 Staging and diagnosis

The TNM staging system recognizes IBC as a separate entity and classifies it as T4d since the publication of the Manual of Staging of Cancer 1st edition (1978) [76]. Nonetheless, IBC diagnosis criteria had several modifications through the years, and it wasn't until the 7th edition (2010) that the criteria were refined[77]. The diagnostic criteria are based on the rapid onset of clinical symptoms, including erythema, edema and/or peau d'orange, and/or warm breast within six months. Erythema does not extend beyond one-third of the breast. Lastly, pathological evidence of invasive carcinoma should be present. The presence of tumor emboli in dermal-lymphatic vessels is a hallmark of IBC and can aid in diagnosing but is not required [78].

Because inflammatory symptoms seen in IBC patients can mimic the clinical presentations of other diseases, there is a higher chance of misdiagnosis and treatment delay. Infectious mastitis might be the most similar disease because of the breast symptoms and the mammographic appearance in some cases[79]. One study reported 38% of IBC patients had been initially treated with antibiotics, and 24% were misdiagnosed as having changes related to breastfeeding, cyst, or some sort of allergic reaction [80]. Other malignancies such as axillary lymphoma, mammary lymphoma, and leukemic infiltration of the breast can masquerade as IBC; however, histopathology can be used for differential diagnosis [81] [82] [83] [84].

The AJCC. (American Joint Committee on Cancer) staging system presents some limitations in classifying IBC patients and distinguishing between IBC and non- IBC, as one paper discusses. Fouad *et al*. pointed out that stage III includes all non-metastatic IBC with no subcategorizing for the difference in tumor size or the number of nodes involved. Moreover, stage IV consists of all metastatic breast cancer with no distinction between IBC and non-IBC [86]. No

prognostic biological factors such as molecular subtypes are added to this stage. Therefore, the AJCC staging system is not accurately stratifying patients based on their disease prognosis, which is its primary purpose. Evidence shows that IBC patients have worse outcomes in every stage of breast cancer. Kaplan–Meier survival curves revealed the median overall survival is 4.75 years for IBC patients with stage III versus 13.4 years in non-IBC of the same stage [85]. As a result, there is a lack of IBC specific treatment because the disease is not adequately represented in clinical trials. Subsequently, IBC patients are still treated based on the outcome of other subtypes of breast cancer in clinical trials. Fouad and his group recommend considering the inflammatory criteria of IBC when patients are classified as stage IV, and stage "IV IBC" should be included [86].

1.2.2 Molecular profiling of IBC

Given that IBC is unique in its clinical presentation and progression, a distinct transcriptional profile is predictable. Nevertheless, earlier studies were unsuccessful in identifying a specific gene signature that distinguishes IBC from non-IBC [71]. Indeed, the gene lists identified in these studies showed minimal overlap and low predictive accuracy in identifying IBC samples[87].

One of the most highlighted studies published by the IBC World Consortium in 2013 identified 79-genes differently expressed in IBC. However, the gene signature identified was attributed to the difference in molecular subtypes, where IBC samples were enriched with the HER2 positive subtype [88]. After controlling for such variables in a follow-up study, 132 genes were identified. Yet, 25% of the signature genes were found in breast cancer samples in The Cancer Genome Atlas database (TCGA), which comprise a small number of IBC samples [89]. This result suggested that the 132 gene signature was not specific enough to discriminate IBC samples from non-IBC. Several other studies were also published, and many genes were pointed out as a

potential for further examination such as IFNα (interferon- α), EGFR, TGFβ, MYC, and PIK3CA, however, none of these prove to be unique to IBC [90] [91].

More recently, a gene signature was identified (G59) using the nonparametric machine learning random forest (RF) approach. Interestingly, the G59 is more robust in segregating IBC from non-IBC as it showed 100% accuracy in the discovery and validation samples and more than 98% in an independent dataset. The G59 genes were only seen in 1.6% of breast cancer in the TCGA database suggesting the specificity of the genes identified to IBC, and such results were not seen in any of the previous signatures published. Though the results of the G59 signature are still unvalidated, enrichment analyses showed that many genes are part of the plasma membrane cellular component proteins, interleukin (IL), and chemokine signaling pathways [92]. This implies that IBC cells are highly communicative with the tumor microenvironment and that the stromal component plays an imperative role in the disease aggressiveness, as many studies speculated [71].

1.2.3 IBC intrinsic characteristics

IBC aggressive nature is driven by several intrinsic elements that can increase cell stemness, metastases, and angiogenesis[71]. IBC shows high expression of RhoC in contrast to stage-matched non-IBC tumor cells (90% vs. 36%)[93]. The increased expression correlates with the stem cell marker ALDH1 in the SUM149 IBC cell line [94]. A sufficient amount of evidence revealed the role of RhoC and ALDH1 in promoting cancer cell stemness and tumorigenesis [95]. In addition, human xenograft MARY-X, which models IBC lymphovascular emboli in vivo, shows an increase in Notch3 and ALDH1. Both genes promote breast stem cells' self-renewal and proliferation of early breast cancer progenitor cells [96].

The increase of migration, invasion and angiogenesis seen in IBC cells demonstrate its high metastatic potential. Because of IBC's high metastatic risk, lower survival rates are seen, though pathological complete response (pCR) to chemotherapy is similar to non-IBC[97]. The overexpression of Caveolin 1 in IBC induces the activation of AKT1 and subsequently promotes cancer cell migration and invasion[98]. The activation of the P13K/AKT pathway is also induced by RhoC, which promotes IBC invasion [99].

Tumor emboli is an IBC hallmark, which requires tumor cell migration followed invasion of the lymphovascular space. Hence, angiogenesis and lymphangiogenesis are crucial for IBC cell survival and metastasis. Several studies indicated that IBC patients show an increase in the levels of vascular endothelial growth factor D (VEGF-D) and lymphatic vessel density compared to non-IBC [100] [101]. Both VEGF-C and VEGF-D are induced by EP3 signaling through PGE2-derived COX-2, resulting in increased lymphangiogenesis, proliferation and inflammation, as one study suggested [102]. Interestingly, results show increased levels of COX2 in IBC tumors compared to non-IBC, and this expression is associated with lower overall survival [103]. In addition, EP3 was one of the genes upregulated in the IBC G59 signature [92].

1.2.4 IBC extrinsic characteristics

The tumor microenvironment regulates cancer progression as many studies suggested and as was discussed earlier. In the case of IBC, more evidence suggests that the TME is the leading player in orchestrating early metastases and cancer progression[71]. This is supported by the recent discovery of IBC signature G59, where differently expressed genes are involved in plasma membrane cellular component proteins and cytokine signaling pathways [92]. Cytokines, along with cellular components of the TME, including mesenchymal stem cells, tumor-associated

macrophages, dendritic cells, T cells, fibroblast, and endothelial cells, all contribute to IBC pathogenesis and metastatic behavior [71].

Cytokines can maintain an inflammatory microenvironment favoring cancer development and progression through mediating an autocrine and paracrine interaction between cancer cells and the TME [40 41 71]. Several cytokines showed an increase in IBC compared to non-IBC, but more studies are needed to understand the mechanism behind this increase [104]. IL-6 and IL-8 are the most studied pro-inflammatory cytokines involved in tumorigenesis and cancer progression [105 53]. Overexpression of IL-6 was found in IBC tumor samples compared to non-IBC [106] and inhibition of IL-6 signal decreased invasion and mammosphere formation of IBC cells co-cultured with mesenchymal stem cells [107]. CK2 (Casein kinase II) regulates IL-6 in IBC, and its increase leads to JAK-STAT signaling pathway activation resulting in an increase in EMT phenotype [108 109]. IBC cell lines SUM149 and SUM190 show high IL-6 and IL-8 compared to non-IBC and patient tumor samples show higher expression levels of IL-8 and CCL2 [110 111]. Interestingly, inflammatory mammary cancer in canines also show an increase in IL-8 [112].

1.2.5 IBC and inflammation

When IBC was first identified as a separate class of breast cancer, the name was chosen based on the clinical features seen in the breast skin [76]. The clinical inflammatory symptoms were initially thought to be due to tumor cell blockage of the dermal lymphatic vessels [113]. Little was known about the importance of inflammatory pathway activation and mediation of IBC metastatic behavior [114].

Recent research has shed light on the importance of innate and adaptive immunity in relation to tumor progression [115]. As more evidence shows the increase of inflammatory cytokine

(in particular IL-6 and IL-8) in IBC tumor samples and cell lines, it is apparent that inflammatory pathways such as JAK/STAT, NF-κB, and COX-2 have a role in IBC progression. In an aim to improve IBC treatment, more clinical studies are using specific inhibitors to target these inflammatory pathways [116].

1.2.6 NF-κB pathway and IBC

Studies determined that the upregulation of NF-κB has a vital function in tumor initiation and progression [117] [118]. cDNA microarray analysis revealed the overexpression of 21 NF-κB target genes in 19 IBC patient tumor samples compared to 2 in non-IBC [119]. NF-κB target genes such as RelA, RelB, NFkB1, and NFkB2 were confirmed for overexpression using RT-PCR and immuno staining [120]. The same result was also found in a study done in France by Lerebours and his group, where 35 out of 60 NF-κB related genes were upregulated in IBC, most of which involved immune response, proliferation, tumor promotion, angiogenesis, and apoptosis [121]. Active NF-κB signal was also found in cancer stem cells isolated from SUM149 and was determined to promote self-renewal in vitro and tumor formation in vivo. This function is carried out through the stimulation of EMT and the upregulation of cytokines such as IL-1β, IL-6 and IL-8 [122].

1.3 RIPK2 OVERVIEW

1.3.1 Protein Kinases

The regulation of crucial cell processes and signal transduction is orchestrated by proteins known as protein kinases. Protein kinases are a family of diverse groups of enzymes that can modify proteins through phosphorylation [123]. Phosphorylation, a post-translational modification, is defined by the transfer of the ATP phosphate group by the kinase to the amino acid-free hydroxyl

(-OH) group on the target protein. This modification is an essential intracellular regulatory mechanism and communication that governs protein functions by directing their activation, localization, and signal transduction [124]. Most protein kinases share a conserved kinase core, composed of an ATP binding loop at the N- terminal lobe and a catalytic loop at the C- terminal lobe [125]. Based on the sequence similarity of the kinase core, protein kinases are classified into eleven main groups. One of the eleven defined groups is the tyrosine-kinase-like (TKL) class, named so because of their close sequence similarity to the tyrosine kinase proteins, where kinases act on threonine/serine phosphorylation[124 126]. TKL includes the Receptor Interacting Protein (RIP) kinase family. The RIP family comprises seven members (RIPK 1-7), all with different functions defined by their non-kinase domain in the C-terminus[127]. The RIP family is predominately involved in innate immunity, cellular stress responses, and cell death. Members of this family share a homologous kinase domain (KD) and a unique non-KD that determines their function.

1.3.2 RIP Family

The first member of the RIP family, **RIPK1**, was recognized by Stanger *et al*. because of its death domain (DD) interaction with the cell surface receptor FAS/APO-1 (apoptosis antigen 1) and the TNF-R1 (Tumor necrosis factor receptor 1) death domain[128]. Later, interactions with other death receptors were discovered, including TRAMP (Death Receptor 3)[129], TRAIL-R1 (DR4), and TRAIL-R2 (DR5)[130]. RIPK1 death domain also facilitates its interaction with TNFR (tumor necrosis factor receptor) through several adaptor proteins, including TRADD (Tumor necrosis factor receptor type 1-associated DEATH domain) and TRAF2 (TNF receptor-associated factor 2)[131]. These interactions are required for the RIPK1-mediated activation of NF-κB activation and apoptosis[132]. Along with the intermediate domain, the death domain appears to be sufficient to

activate NF-κB inflammatory pathway as studies suggested[133] [134]. The kinase activity still holds to be vital for both caspase-dependent apoptosis and necroptotic cell death [135].

RIPK1 interacts with **RIPK3**, the third member in the RIP family, to form a kinase complex that regulates necroptosis, or "'programmed necrosis"[136]. RIPK3 [137] lacks the death domain found in RIPK1 but shares the same RIP homotypic interaction motif (RHIM) domain, which mediates their binding and necrosome complex formation[138]. RIPK1 and RIPK3 complexes also regulate acute inflammation through activating Erk1/2, cFos, and NF-κB [139].

The second family member, **RIPK2**, carries a caspase activation and recruitment domain (CARDs) not found in the other RIP family members[140]. This domain is essential for RIPK2 interaction with CARD proteins, most notably the pathogen receptor NOD (Nucleotide-binding oligomerization domain-containing protein) [141] [142]. NOD receptor signal transduction is RIPK2 dependent. As a result, RIPK2 plays a role in the innate immune response [143]. Studies showed that RIPK2 is also involved in adaptive immune responses, such that T-helper subtype 1 (TH1) and natural killer cells produce reduced IFN-γ (interferon-γ) levels in RIPK2 deficient mice as compared to controls. These RIPK2 knockout mice also have impaired T-cell differentiation[144] [142].

By early 2000, a new member, **RIPK4**, was added to the RIP family. RIPK4 was first discovered in a yeast two-hybrid screen as a PKC -δ-interacting protein kinase (DIK) [145]. A mouse orthologue was then found and named protein kinase C-associated kinase (PKK) [146]. Because of similarities uncovered between the PKK kinase domain and other members of the RIP family (RIPK1, RIPK2, and RIPK3) Meylan *et al*. renamed the kinase RIPK4 [147]. Blasting results showed that the RIPK4 kinase domain shares a 45% sequence identity with RIPK2 and exchanging with the RIPK2 kinase domain shows no biochemical nor molecular activity differences [148]. RIPK4 kinase activity is essential for NF-κB and JNK (c-Jun N-terminal kinase) activation[147], and this

catalytic activity was recently shown to be RIPK4 dimerization dependent[149]. The presence of ankyrin repeats characterizes the RIPK4 C-terminal region; the complete function of this domain is still unknown; however, it is suggested that ankyrin- kinase domain interaction acts as an autoinhibitory regulator for RIK4 activity[147]. RIPK4 knockout in mice show defective epidermal cell differentiation and defective morphogenesis leading to early death[150]. In humans, RIPK4 mutation causes an autosomal-recessive form of popliteal pterygium syndrome (Bartsocas-Papas syndrome) a rare and lethal genetic condition characterized by impaired congenital development of the face, limbs and genitalia[151]. Along the same line, evidence shows that RIPK4 can phosphorylate and activate IRF6 (Interferon Regulatory Factor 6), the primary gene mutated in Popliteal Pterygium Syndrome and Van Der Woude Syndrome 1 [152] [153].

RIPK5, RIPK6 and RIPK7, are the newer members of the RIP family. RIPK5 or ANKK1 (Ankyrin repeat and kinase domain containing 1), is structurally more like the other RIP family members, particularly RIPK4. In addition to having a similar kinase domain, ANKK1 also harbors carboxy-terminal ankyrin repeats (Ank) that share a 35% homology with those in RIPK4. Little is known about RIPK5 (ANKK1); however, its structural similarities to RIPK4 suggest they may have the same function [154].

RIPK6 and RIPK7, more commonly known as LRRK1 and LRRK2, share higher structural homology than the other RIP kinases. Both carry similar Ankyrin repeats (ANK), leucine-rich repeats (LRR), and GTPase-like domains (ROC; Ras of complex proteins). They also contain a C-terminal of COR, and a kinase domain. RIPK7 or LRRK2 has additional armadillo repeats (ARM) in the N- terminal and WD40 repeats (WD) in the C-terminal [155]. Though RIPK6 and RIPK7 show a significant similarity in the kinase domain to the RIP family, they also have

sequence and structural homology to other protein subfamilies such as the Roco protein family, which is characterized by having a conserved ROC and COR domain [154 156 157].

RIPK7 or LRRK2 plays a central part in many diseases, especially Parkinson's disease. Evidence shows that mutation in LRRK2 is the most common cause of Parkinson's disease (PD) as it accounts for 4% of familial PD and 1% of population sporadic PD [156 158]. In addition, mutations in the non-coding region of the LRRK2 gene increase the risk of Crohn's disease, a disease mostly known to be caused by NOD/RIPK2 upregulation [159 155] . The function of RIPK6/LRRK1 is still unclear. However, its significant homology to LRRK2 in the LRR domain and the catalytic ROC-COR-kinase domain suggests a similar role.

1.3.3 RIPK2 in focus

RIPK2 was first discovered in 1998, based on homology to the serine-threonine kinase domain of the RIP family. The N- terminus of RIPK2 contains the protein kinase domain followed by an intermediate domain with no known homology. The C- terminus presents the CARD domain motif, highly similar to the corresponding domain in cIAP1 and cIAP2 (51.3% and 47.9%, respectively) [140] (Figure 1.1). Around the same time, a study was published indicating the potential of RIPK2 involvement in NF-κB /JNK signaling; however, the mechanism was not precise [160]. A couple of years later, Inohara, Girardin, and their colleagues recovered the NOD1/RIPK2 interaction leading to NF-κB activation through the IKK complex [143,161]. Research on the NOD/RIPK2 pathway grew because of the implication it had on the pathogenesis of many diseases such as multiple sclerosis [162], allergic airway inflammation [163] inflammatory bowel disease [164] and allergic airway inflammation [163]. As a result, RIPK2 gained more attention and became one of the targeted kinases in drug discovery.

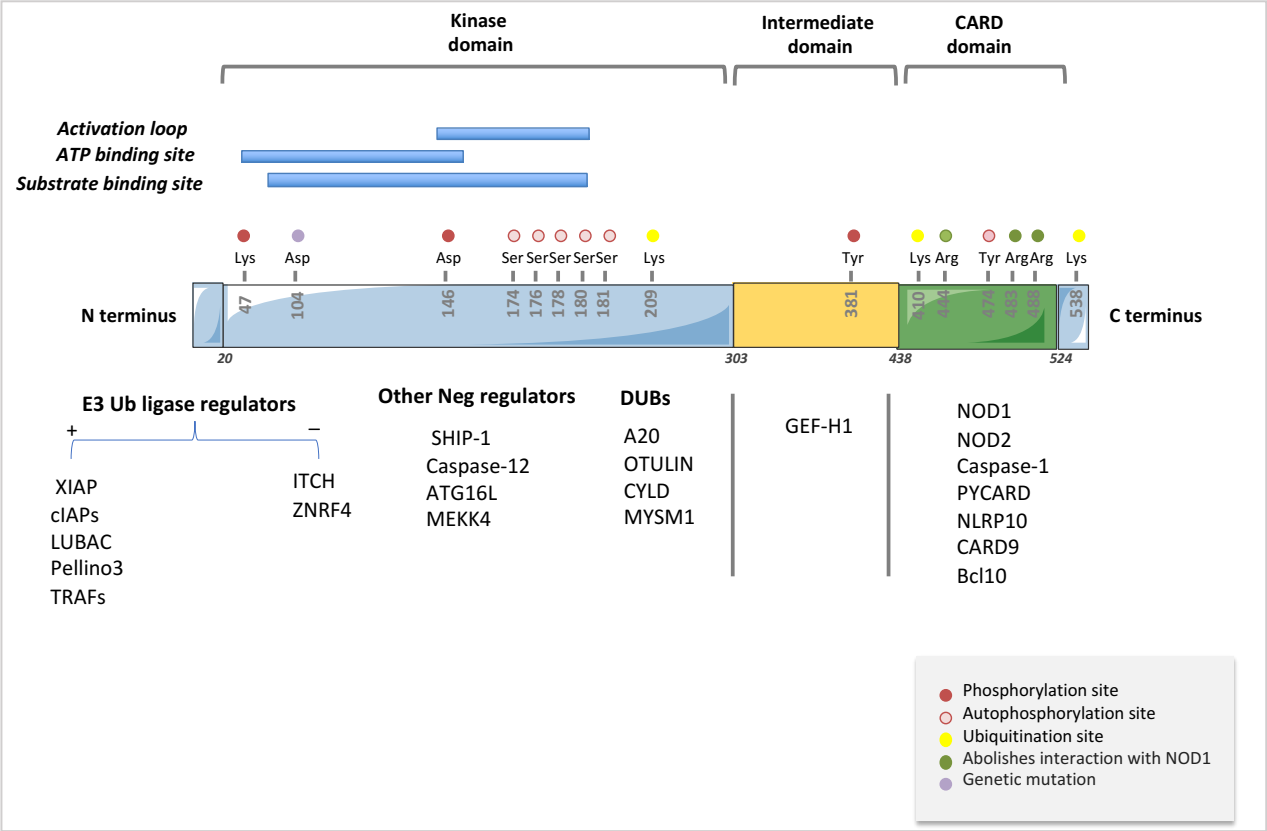

Figure 1.1 RIPK2 structure including details of the post-translation modification sites and direct interactional proteins. Adapted from *Frontiers* (Heim *et al*., 2019) and *Nature* (Ofengeim *et al*., 2013)

1.3.4 RIPK2 the scaffolding kinase protein

RIPK2 gained huge pharmaceutical interest in the last ten years because of its association with many inflammatory diseases and its influence on innate immune signaling, primarily through its interaction with the intracellular pathogen receptor NOD1 and NOD2 [165]. RIPK2 governs this interaction through its C-terminal CARD domain [166]. The deletion of the RIPK2 CARD domain can abolish NOD signaling and downstream signal transduction [167]. Recent work show RIPK2

exists in a constitutive equilibrium between monomeric and dimeric states [168]. This state is known as "prone-to-autophosphorylation" conformation, where the kinase is in an intermediate step between inactive and active states [169]. Upon NOD activation and oligomerization [166 170 171], RIPK2 is recruited via a homotypic CARD-CARD interaction. Subsequently, RIPK2 dimerizes in an active compact conformation and forms a multi-protein signaling complex. This tight packing arrangement allows for the first phosphorylation between kinase dimers to take place [172 168 164] (Figure 1.2).

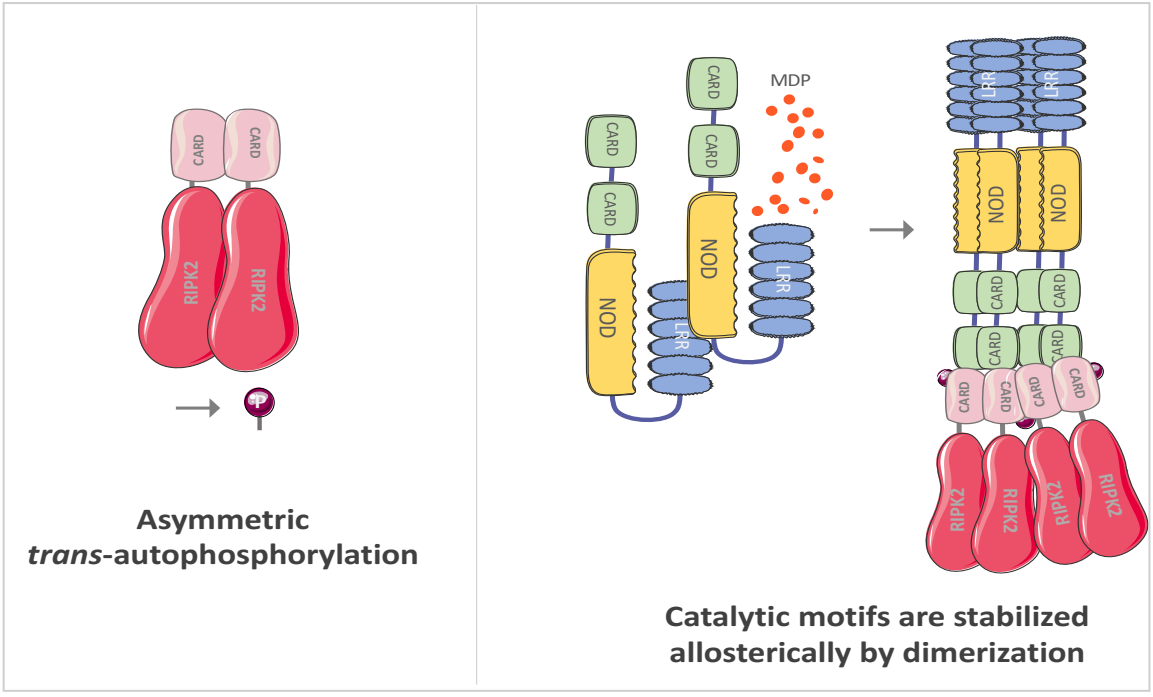

Figure 1.2 RIPK2 "prone-to-autophosphorylation" conformation. Right panel, RIPK2 activation through *trans*-autophosphorylation. Left panel, NOD activation and oligomerization, results in RIPK2 recruitment via a homotypic CARD-CARD interaction. RIPK2 *trans*-autophosphorylation is stabilized allosterically by dimerization. Adapted from *Frontiers* (Heim *et al*., 2019)

The importance of RIPK2 kinase activity is debated since several studies showed that the activation of the mutant dead kinase (K47R and D146N) was able to activate NF-κB[173 160 174 175 176]. This suggested that RIPK2 functions as a scaffolding protein mediating innate immune and inflammatory signals rather than a real kinase [175].

Nevertheless, we still don't understand the full function of RIPK2 kinase domain, but several studies pointed out its importance. Since no other substrates of RIPK2 have been identified, it is assumed that the catalytic activity allows RIPK2 to phosphorylate itself [176]. Accordingly, the autophosphorylation of RIPK2 induces conformational changes that enable RIPK2-XIAP (X-linked inhibitor of apoptosis protein) binding and, subsequently, signal transduction. In addition, the RIPK2 kinase domain could not be replaced by the kinase domain of its closest structural homologs (RIPK4 and RIPK3), suggesting that the RIPK2 kinase domain has a unique structural element and architectural modification is required for its NOD signaling and scaffolding function [177]. Moreover, Y474 phosphorylation was found to be necessary for RIPK2 activation and subsequent deactivation by E3 ubiquitin ligase ITCH through inducing non-degradative ubiquitination. Therefore, ITCH deletion resulted in increased levels of RIPK2 tyrosine-phosphorylation and upregulation of NF-κB signaling[167]. Lastly, RIPK2 kinase activity is necessary for its stability [178 179], where mutation of the RIPK2 dimer interface (RIPK2 R74A, R74D, and R74H) in the kinase domain revealed a dramatic decrease in its activity and protein stability[168].

Analyzing RIPK2 crystal structure in the active and inactive state, Pellegrini and his group showed the proximity of RIPK2 phosphorylated AS (activation segment) to the Lys209 loop during kinase activation, which can result in changes in the ubiquitination machinery[168]; suggesting that the catalytic activity of the kinase domain ensures proper conformation for RIPK2

optimum functioning. This is partially confirmed by Goncharov *et al.* , where she shows that the kinase domain regulates RIPK2 ubiquitination by facilitating XIAP-BIR2 (baculovirus IAP repeat) domain binding [176]. Altogether, these results suggest that the kinase domain and its activity play a part in optimum function of RIPK2.

1.3.5 Regulation of RIPK2 activity

1.3.5.1 RIPK2 phosphorylation

Phosphorylation is one of the most common and essential post-translational modifications[180]. This modification takes place primarily through protein kinases where the phosphate group (PO_4) from ATP is transferred to the polar group R (side chains) of a particular amino acid within the protein [181]. ATP consisting of three phosphate groups (α-, β-, and γ), during phosphorylation, ATP splits off the terminal γ-phosphate, and the loss molecule attaches to the protein amino acid [182]. Phosphorylation in all kinases occurs in the catalytic domain, where several conserved elements dictate and maintain the kinase activity: the DFG-motif within the activation loop, the HRD-motif within the catalytic loop, and the glycine-rich loop [183] [184]. During kinase activation, the DFG-motif transforms the kinase surface hydrophobicity, allowing for conformational changes and further interactions with other proteins [185]. The amino acid residues within the activation loop determine the binding specificity and, accordingly, the type of kinase. More than 85% of protein phosphorylation events occur on serine residue, followed by threonine (11.8%) and rarely on a tyrosine (1.8%) [186]. When a kinase is phosphorylated on all three residues, serine/threonine and tyrosine, such as RIPK2, it is called a dual-specificity kinase [187] [167]. The glycine-rich loop is shown to be essential for ATP binding and stabilizing. Mutation of the lysine residue in this region can inhibit the kinase activity by abolishing the ATP binding [188]. In RIPK2,

the substitution of the ATP-binding lysine 47 to arginine (K47R) abolished its catalytic activity but did not restrict NOD2 signaling [173] [174] [175]. In mice, K47A knock-in decreased RIPK2 downstream signaling [189]. However, this was later attributed to the low expression level of RIPK2, which suggests that the defect in the kinase activity results in protein instability [168]. Lastly, the HRD motif is responsible for stabilizing the kinase domain's active conformation with essential aspartate residue [190]. Mutation of the catalytic aspartate results in abolishing the kinase activity, as seen in RIPK2 by substituting of the aspartate 146 to asparagine (D146N); however, this catalytic activity was again dispensable for the NOD2 signaling [173] [160] [174] [175].

RIPK2 activity for a long time was determined based on phosphorylation. Mass spectrometry and mutational analysis identified the serine residue 176 in the kinase activation loop as an autophosphorylation site. This site was confirmed via LPS stimulated macrophages and was later used as a specific marker to assess RIPK2 activity [187]. Mutation of the S176 site does not restrict RIPK2 interaction nor signaling. However, a later tyrosine 474 autophosphorylation site was identified and shown to be required for NOD2 maximal signaling and RIPK2-induced NF-κB activation[189] [167]. As a result, RIPK2 is identified and confirmed as the only RIP family member with dual Ser/Thr and Tyr kinase activity. More recently, other serine autophosphorylation sites were revealed, Ser174, Ser178, Ser180, and Ser181[168].

Autophosphorylation is defined by the ability of a kinase to self-activate. In other words, the kinase can phosphorylate itself or another twin molecule via intramolecular (*cis*) or intermolecular (*trans*) reaction[191]. About 45% of all arginine–aspartic acid (RD) kinases can autophosphorylate their own activation-loop [169]. There are several models of autophosphorylation, RIPK2 falls into the asymmetric *trans*-autophosphorylation, where each monomer phosphorylates and activates the other[168] (Figure 1.2).This was determined by radioactive kinase assay where both

kinase-dead mutants (RIPK2 K47R and D146N) were activated by full length RIPK2[168]. Accordingly, RIPK2 crystallized as asymmetric 'side-by-side' homodimers, in which the N-Lobe of one monomer interacts with the other monomer C-Lobe, and the two active sites facing in opposite directions [168]. Further, RIPK2 trans-autophosphorylation is stabilized allosterically by dimerization, making RIPK2 in a state called "prone-to-auto-phosphorylate" conformation [169] [168].

1.3.5.2 RIPK2 ubiquitination

Ubiquitination is an enzymatic post-translation modification [192] identified by the addition of ubiquitin (Ub), a small 8.5 kDa enzyme, to the target protein. In this process, a covalent bond is formed between the C-terminal glycine residue of Ub and the lysine residues of the substrate at one or multiple sites [193] [194]. Ub can also bind to serine (Ser) and threonine (Thr) residues on the target protein and form a hydroxy ester bond [195] or conjugate to another Ub molecule through the lysine residues (K6, K11, K27, K29, K33, K48, and K63) or the N-terminal methionine residue (M1) to form a variety of isopeptide-linked ubiquitin chain [196]. Each Ub modification and chain has a different function in the cell. For instance, K48 linked chains are mainly associated with proteasomal degradation [197], while K63 mediate DNA repair kinase activation and vesicle trafficking [198].

The ubiquitin modification of RIPK2 by K63, K27, and M1 linked chain was shown to take place in the kinase domain, specifically at the lysine residue 209 [199] [200]. MDP stimulation of the mutant K209R in macrophage cells abolished RIPK2 polyubiquitination and decreased cytokine production [200]. However, one study suggested that the loss of function seen in the K209 mutant is caused by the conformational disorder since K209 is in the putative interaction pocket[201]. In addition, the mutation of a close-by residue I212 had more effect on impairing RIPK2-XIAP interaction and subsequent ubiquitination, which is strong evidence that K209 importance is not

due to the residue per se, but rather to its location in the C-lob pocket [201]. This finding was also supported by another study where the use of XIAP inhibitors revealed two RIPK2 ubiquitination sites (K538 and K410) but not K209. Mutation of these two sites diminished RIPK2 ubiquitination, NF-κB and MAPK activation, and cytokine production [176].

The primary function of ubiquitination is targeting proteins for degradation by the proteasome [202], DNA damage repair [203] and signal transduction [204]. All of these functions are carried out through a three-step enzymatic cascade that includes; ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin-ligating enzymes (E3) [205]. Accordingly, the Ub carboxyl group binds by a thioester linkage to the catalytic cysteine on E1 in an ATP-dependent manner. The activated Ub is then transferred to the cysteine residue of E2. Finally, E3 mediates the transfer and attachment of Ub to the target protein. Polyubiquitination is critical for RIPK2 scaffold function and downstream signaling [176]. Ubiquitination occurs following RIPK2 polymerization [206], inferring that phosphorylation might be the initial post-translation modification for RIPK2 followed by polyubiquitination. RIPK2 signal is positively and negatively regulated by several ubiquitin E3 ligases [207]. **Positive E3 ligases** to date include IAPs (inhibitor of apoptosis proteins: XIAP, cellular cIAP1 and cIAP2)[207 208], LUBAC (linear ubiquitin assembly complex) [210], Pellino3 [211] and TRAFs [212] (Figure 1.3).

XIAP is essential for the NOD-RIPK2 signaling pathway as Damgaard and his group show that the deletion of the XIAP-RING domain impaired RIPK2 ubiquitination, NOD2 signaling, and cytokine production [210]. The XIAP-BIR2 domain can directly interact with the RIPK2 kinase domain. Disrupting this binding not only impaired RIPK2 polyubiquitination but also decreased the activation of MAPK and NF-κB signaling pathways [176 175]. While the BIR2 domain is needed for RIPK2 interaction, the XIAP- RING domain mediates the recruitment of the LUBAC chain,

which in turn is necessary for efficient NOD2 signal transduction and NF-κB activation. The LUBAC linear ubiquitin chain is conjugated by a M1-linked ubiquitin chain, which utilizes the N-terminal methionine residue of ubiquitin instead of lysine [213]. Evidence shows that the M1-linked chain can activate NEMO (NF-κB essential modulator), the regulatory subunit of the IKK complex, that is required for the catalytic activation of IKKα and IKKβ in the NF-κB singling pathway [210 214].

cIAP1 and cIAP2 can directly bind to RIPK2[215], however, unlike XIAP, their Ub ligase activity is not essential for RIPK2 ubiquitination and downstream signaling[210 216 207 211]. Nevertheless, the binding of cIAPs to RIPK2, independent of XIAP presence [210], mediates the conjugation of K63-linked ubiquitin chains [215]. Results show that K63-linked ubiquitin chains, more favored by RIPK2 [217], can recruit ubiquitin-binding scaffold proteins TAB2 and TAB3, which can activate TAK1 (TGF-β activated kinase)[218 199 219]. The TAK-TAB complex takes part in MAPK and NF-κB activation through phosphorylating the IKK complex [218]. Note, K63-linked ubiquitin chains can also directly bind and polyubiquitinate NEMO, which later activates NF-κB [220 219].

Pellino3 is a member of the Pellino small family, which consists of Pellino1 and Pellino2. Proteins in this family carry an N-terminal FHA (fork head-associated) domain and a C-terminal RING-like domain responsible for E3 ligase activity and K63-linked ubiquitin chains binding [221]. Yang and his group show that Pellino3 directly binds to RIPK2 through its FHA domain, and this binding induces K63-linked ubiquitination of RIPK2[211], resulting in TAK1 and IKK complexes activation. The same group suggests that Pellino3 ubiquitination works in parallel with XIAP to regulate NOD2 signaling; because XIAP suppression in Pellino3 deficient macrophages augmented the inhibition of both NF-κB and MAPK activation [211].

Besides the IAP family and Pellino3, members of the TRAF (The tumor necrosis factor receptor (TNF-R)-associated factor) family are also involved in catalyzing RIPK2 K63- linked non-degradative ubiquitination [212]. TRAFs were known formerly as signaling adaptors of the TNF-R family. This function is mediated by the TRAF domain found in the C-terminus [222]. However, the zinc RING domain in the N- terminus determines their function as E3 ubiquitin ligases [223 224]. The significance of RIPK2 interaction with TRAF members is inconsistent, but more evidence suggests that TRAF1, TRAF2, TRAF5, and TRAF6, are not involved in RIPK2 ubiquitination but rather act as adaptor proteins, facilitating the binding of cIAPs onto RIPK2 [209]. However, TRAF3 was found to be crucial in mediating the production of type I interferons (IFNs) through a non-classical NOD1-RIPK2 pathway in human epithelial cells. RIPK2-TRAF3 complex is formed upon NOD1 activation via DAP (diaminopimelic acid). As a result, TBK1(TANK-binding kinase 1) and IκB kinase ε (IKKε) is activated. This will, in turn, activate IRF7 (IFN regulatory factor 7), leading to IFN-β production [225].

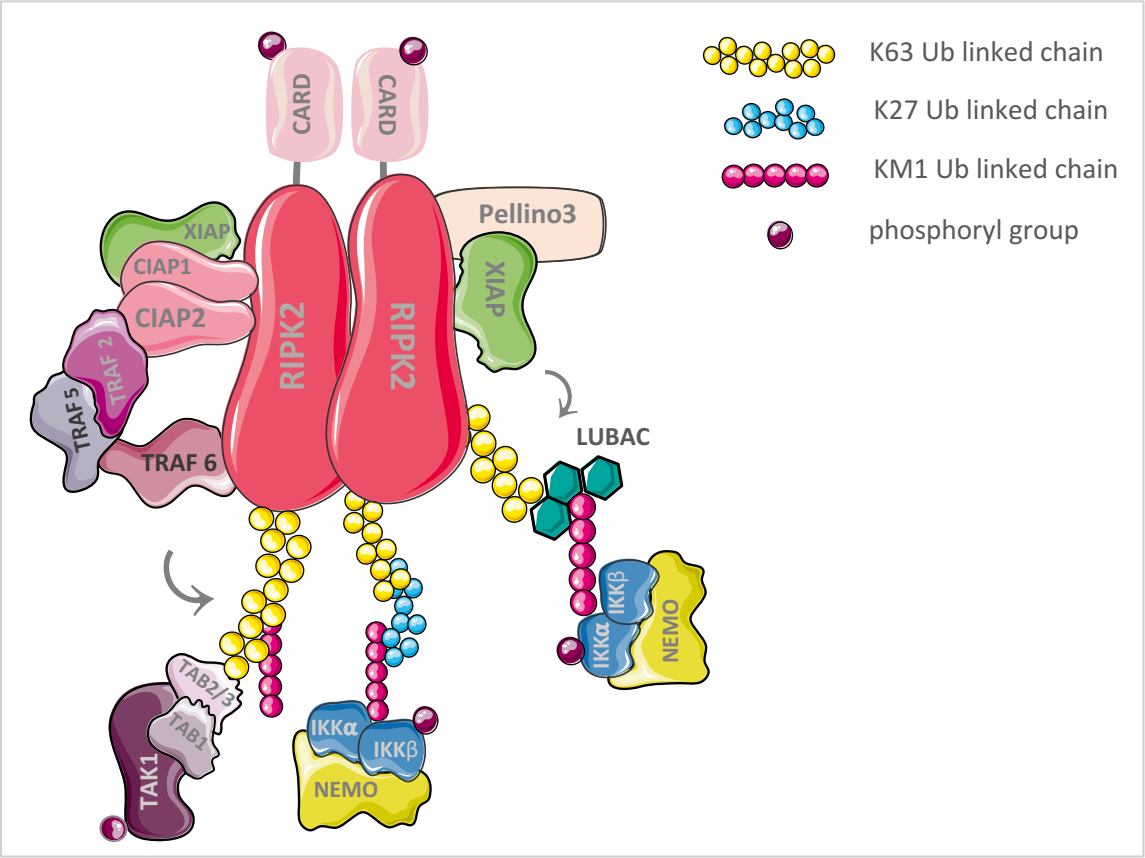

Figure 1.3 RIPK2 activity positive regulator. E3 ligases including XIAP, cIAP1, cIAP2, LUBAC, Pellino3 and TRAFs can directly or indirectly bind to RIPK2 and induces ubiquitination by K63, K27, and M1 linked chain. Adapted from *Molecular Cell* (Fiil *et al*., 2013).

NOD-RIPK2 signaling is also **negatively regulated** by E3 ligases, including ITCH [226] and ZNRF4 (zinc and ring finger 4)[227] (Figure 1.4). **ITCH** regulates RIPK2 function by targeting the phosphorylated Y474 RIPK2 only[167], and conjugating K63- Ub linked chain to inhibit RIPK2-MAPK downstream signaling[226]. Microarray results of MDP stimulated *ITCH* $^{-/-}$ macrophages show increased levels of NF-κB activation[226]. Justine and his colleague showed that inhibiting NOD2-RIPK2 could be through ITCH ubiquitination of cIAP1, leading to its lysosomal degradation [167].

Another E3 negative regulator is **ZNRF4**, which belongs to a family of ZNRF proteins known to act as E3 ubiquitin ligase because of the intracellular C-terminal zinc finger/RING finger domain [228]. In one study, ZNRF4 was identified as a negative regulator of NOD2 signaling through inducing RIPK2 ubiquitination and proteasomal degradation. This degradation is promoted by its RING domain and the conjugation of the K48-linked Ub chain onto RIPK2. When ZNRF4 was knocked down in monocytes, RIPK2 levels were increased along with the NF-κB signal. The deletion of the RIPK2 CARD domain prevented ZNRF4 binding and RIPK2 degradation upon NOD2 stimulation, suggesting that the CARD domain is vital for NOD2 signaling and immune homeostasis [227].

RIPK2 function is also negatively regulated by other proteins such as SHIP-1, Caspase-12, ATG16L, and MEKK4. A recent study shows that **SHIP-1** (SH2-containing inositol phosphatase) can bind to XIAP through its PRD (proline-rich domain) C-terminal domain and impair the RIPK2-XIAP interaction in macrophages. As a result, RIPK2- NF-κB signaling, and cytokine production decreased [229]. **Caspase-12** also regulates NOD2 signaling by binding to RIPK2 and disrupting TRAF6 binding to the signaling complex. This impairs the ability of TRAF6 to ubiquitinate RIPK2, resulting in a reduction in NF-κB activity. The knockout of Caspase-12 induces more cytokine and chemokine production upon NOD2 activation by enteric pathogens [230]. **ATG16L1**, the autophagy protein, can also negatively regulate NOD2 signaling [231] [232] [233]; however, the involvement of RIPK2 in this process is under debate [234]. Results indicated that ATG16L1 could restrict RIPK2 binding to the NOD2 signaling complex causing the demolition of RIPK2 ubiquitination and downstream signaling[233]. The MAP3K protein, **MEKK4**, also regulates the NOD2 signaling by binding to RIPK2 and inhibiting the NEMO and IKK activation.

However, the activation of NOD2 by MDP seems to disassociate this interaction as active NOD2 competes with MEKK4 and binds to RIPK2 [235].

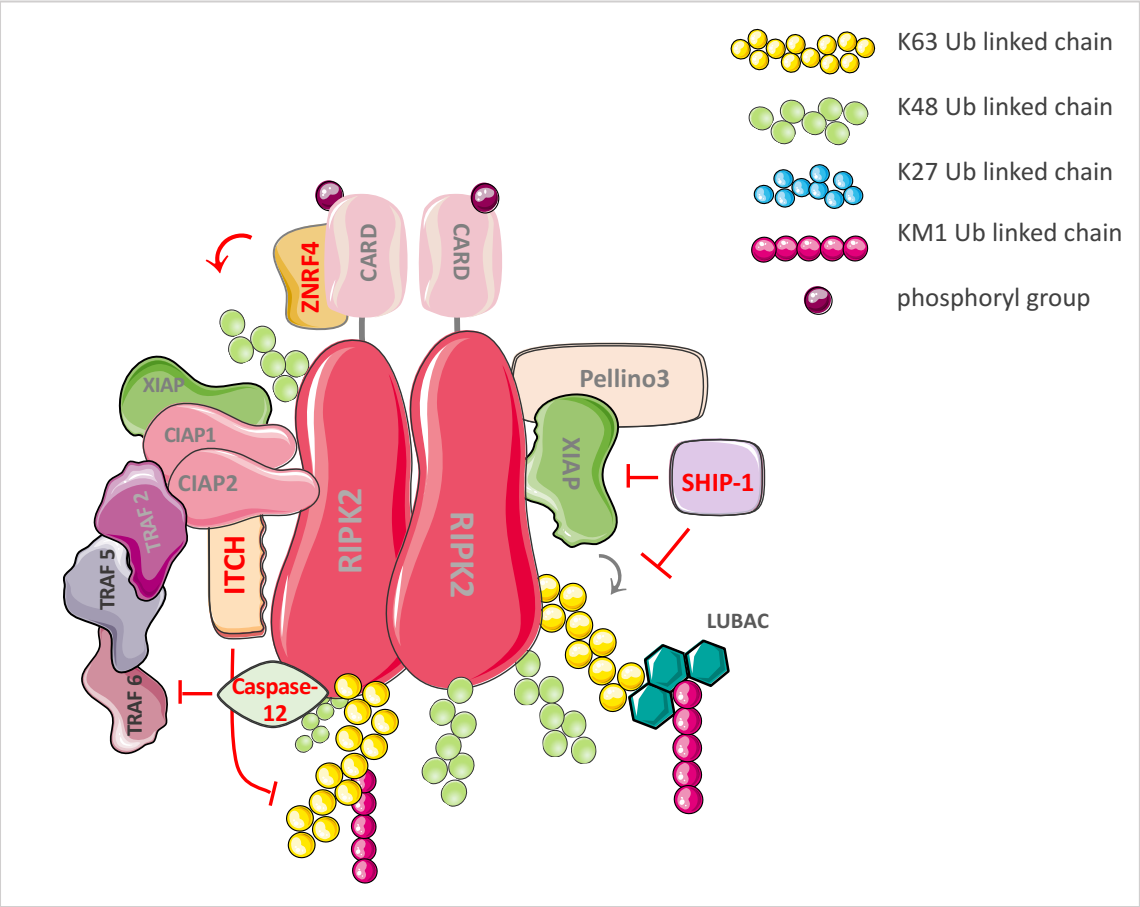

Figure 1.4 RIPK2 activity negative regulators. Negative regulator can be E3 ligase such as ITCH and ZNRF4, where they induce RIPK2 ubiquitination and proteasomal degradation; or proteins such as SHIP-1 and Caspase-12, which can disturb RIPK2 interaction and downstream signaling.Negative regulators are in red. Adapted from *Molecular Cell* (Fiil *et al*., 2013)

1.3.5.3 RIPK2 deubiquitination

Deubiquitination the process of removing Ub from ubiquitinated proteins by deubiquitinating enzymes (DUBs). DUBs can deconjugate Ub through hydrolyzing the isopeptide or peptide bond to the ubiquitinated proteins. [236] [237]. There are five different families of DUBs which are classified into two major groups; the **cysteine proteases DUBs** which include UCH (ubiquitin carboxyl-terminal hydrolases), USP (ubiquitin-specific protease), OUT (ovarian-tumor proteases), and MJD (Machado-Joseph disease protein domain proteases); and the **metalloprotease DUBs** which includes JAMM (Jab1/Pab1/MPN domain)[238]. DUBs have an essential role in maintaining Ub-homeostasis; through balancing the cellular pool of free Ub by rescuing Ub from proteasomal degradation, recycling Ub from ubiquitin conjugates, and processing newly synthesized Ub from Ub precursors[239] [240]. More recent studies show that DUBs are vital in regulating immune responses through regulating signaling pathways such as NF-κB [241]. Several DUBs have been identified to regulate RIPK2 including A20 [242] and OTULIN [243] from the OTU family; CYLD [244] from the USP family; and MYSM1[200] from the JAMM family (Figure 1.5).

A20 is one of the first DUBs known to downregulate the NF-κB signal [245]. This enzyme carries an OTU domain, enabling it to deubiquitinate many proteins in the NF-κB pathway[241], including RIPK2. A20 deubiquitinates the RIPK2 K63- linked chains and abolish its downstream signaling[242]. **OTULIN** (deubiquitinase with linear linkage specificity) is another DUB with an OTU domain that regulates NF-κB and MAPK signaling through controlling RIPK2 ubiquitination. Upon NOD2 stimulation and the activation of RIPK2, OTULIN restrict the

conjugation of LUBAC-mediated M1-linked ubiquitination chain onto RIPK2[243] [246] [247]. The knockdown of OTULIN increased the RIPK2 downstream signaling [243].

CYLD (cylindromatosis) functions primarily through its ubiquitin-specific protease (USP) domain that facilitates the disassembly of the K63- linked chain [246]. In RIPK2, CYLD was not only removing the K63- linked chains but also the conjugated M1-linked chain [248]. As a result, CYLD impaired RIPK2 downstream signal, which reduced NF-κB activation and cytokine production [248] [244]. **MYSM1** (Myb-like, SWIRM, and MPN domains 1) is recently identified as RIPK2 DUB. It is classified as a JAMM domain-associated metallopeptidase [249]. The SWIRM and MPN domains of MYSM1 negatively regulate NOD2-RIPK2 signaling by interacting with RIPK2 and deconjugating the K63- ubiquitin linked chain and subsequently attaching K27- and M1-linked chain[200]. The lack of MYSM1 in MDP-infected mice resulted in excessive inflammation, which was revealed by increased cytokines such as TNFα and IL-6 [200].

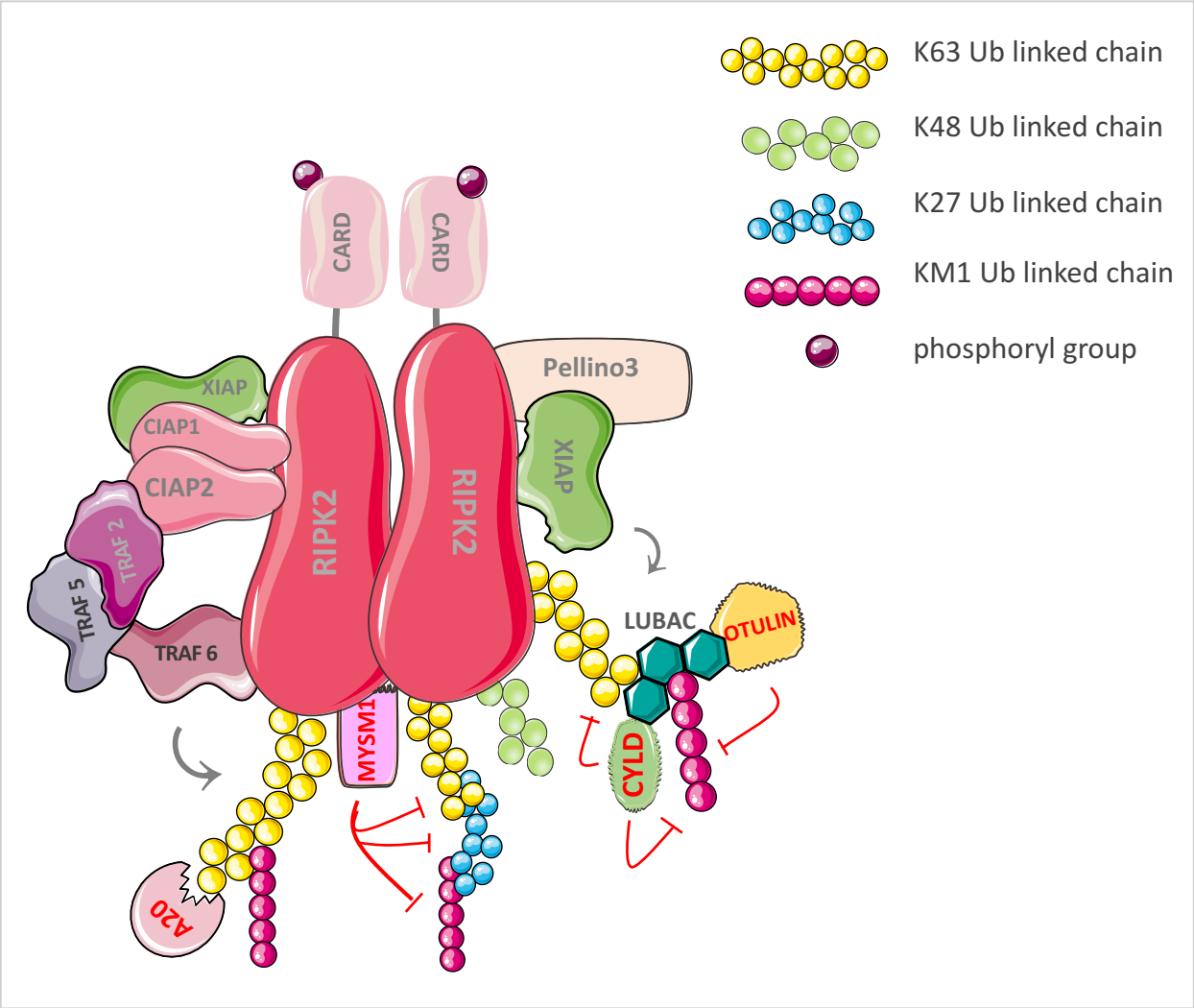

Figure 1.5 RIPK2 Deubiquitination. Defined as the process of removing Ub from ubiquitinated proteins by deubiquitinating enzymes (DUBs). RIPK2 DUBs includes A20, OTULIN, CYLD, and MYSMI. DUBs are presented in red. Adapted from *Molecular Cell* (Fiil *et al*., 2013)

1.3.6 RIPK2 signaling

The NF-κB signaling pathway orchestrates many of our body's immune responses. The activation of this signaling pathway is mediated by a network of cell receptors called pattern recognition receptors (PRRs). PRRs can recognize harmful stimuli such as pathogen-associated

molecular patterns (PAMPs) released by microbes or damage-associated molecular patterns (DAMPs) secreted during tissue injury in case of sterile inflammation [250]. PRRs can be membrane-bound receptors located on the cell surface, such as TLRs (Toll-like receptors) and CLR (C-type lectin receptors) or in the cell cytoplasm such as NLRs (NOD-like receptor) and RIG-I-like receptors (RLR) [250]. NLRs include five families of proteins that share a similar central nucleotide-binding domain (NACHT) responsible for protein oligomerization and activation. A C-terminal LRR domain acts primarily as a ligand recognition receptor. An N-terminal domain is responsible for protein-protein interaction, and accordingly, NLRs are sub-grouped. The two most significant subgroups are NLRCs (NLRs containing the CARD) and NLRPs (NLRs containing Pyrin) [251].

NOD1 (nucleotide-binding oligomerization domain 1) and NOD2 (nucleotide-binding oligomerization domain 2) are members of the NLRC receptor family, activated by different peptidoglycans (PGN) found in bacterial cell walls. Specifically, NOD1 senses DAP (meso-diaminopimelic acid) found in Gram-negative and only certain Gram-positive bacteria, while NOD2 recognizes MDP (muramyl dipeptide) found in most bacterial cell walls [252] [253].

NOD receptors are cytosolic, but studies show their enrichment in the plasma membrane when a bacterial invasion occurs [255] [256]. The recognition of secreted bacterial peptidoglycan leads to the LRR domain unfolding to allow for NATCH activation through homo-oligomerization [256] [257]. This activation promotes membrane recruitment of RIPK2 through the CARD-CARD domain interaction [258], and as more RIPK2 binds to the NOD-RIPK2 heterocomplex, a helical filament structure is formed [206] (Figure 1.2). This structure formation permits RIPK2 polymerization and later poly-ubiquitination by several E3 ligases, most important XIAP, which conjugates the K63-linked ubiquitin chains [208] [176]. With the conjugation of K63-Ub, TAK1 and the TAK1-binding proteins TAB2/3 are recruited[217] [199] [259] leading to IKK complex formation and NEMO (IKKγ)

phosphorylation[260] [261]. NEMO can also be directly activated by LUBAC linear ubiquitin chain linked to RIPK2 [178] [210]. Subsequently, IKKβ in the IKK complex phosphorylates the inhibitor IkBα leading to its release from the NF-κB dimer (p50 and p65) and ubiquitination for proteasomal degradation. The NF-κB dimer will then translocate to the nucleus to turn on gene transcription of pro-inflammatory cytokines, primarily Il-6 and IL-8 [160] [140] [262] [263] [264] (Figure 1.6).

TAK1 also mediates NOD-RIPK2 signal to p38 MAPK, JNK (c-Jun N-terminal kinase), and ERK1/ERK2 (extracellular signal-regulated protein kinase), resulting in the activation of the transcription factor AP-1 (activator protein 1) responsible for cell proliferation, differentiation, and apoptosis [265] [266] [267] [179] [268] [269] [270]. The deletion of TAK1 in epidermal cells resulted in NF-κB and MAPK inactivation upon MDP stimulation, suggesting its critical role in NOD2-RIPK2 signaling [271]. AP-1 also showed activity reduction when the endogenous level of TAK-1 was knocked down in Hela cells [269] (Figure 1.6).

While RIPK2 is known primarily as an adaptor kinase, signaling downstream of NOD receptors, a recent study showed that RIPK2 competes with TRAF6 to prevent its binding to p75 NTR (p75 neurotrophin receptor) to maintain the cerebellar granule neuron survival [272]. Evidence also shows that the RIPK2-CARD domain binds to caspase-1 and regulate its apoptotic activity [273] [274] [275]; and bone marrow-derived macrophages (BMDM) collected from *RIPK2*$^{-/-}$ mice show depletion of caspase-1 and IL-1β [276].

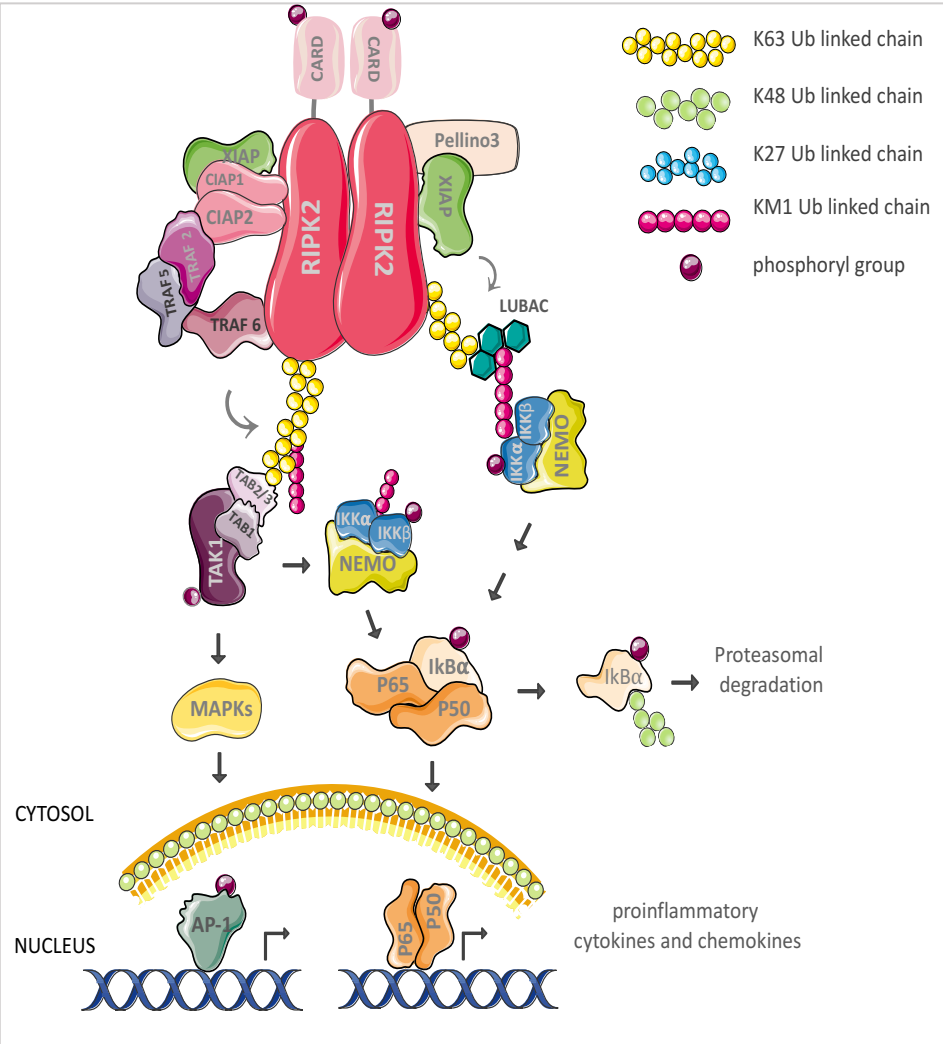

Figure 1.6 RIPK2 NF-κB and MAPK signaling pathway. Upon NOD activation and homo-oligomerization, RIPK2 is recruited through the CARD-CARD domain interaction. NOD-RIPK2 complex permits RIPK2 polymerization and poly-ubiquitination leading to TAK1 and TAB2/3 recruitment, and subsequently IKK complex formation and NEMO phosphorylation. Note, NEMO can also be directly activated by LUBAC linear ubiquitin chain linked to RIPK2. IKKβ phosphorylates the inhibitor IkBα leading to its release from the NF-κB dimer (p50 and p65) and ubiquitination for proteasomal degradation. The NF-κB dimer will then translocate to the nucleus to turn on gene transcription of pro-inflammatory cytokines, primarily Il-6 and IL-8. Besides NF-κB, TAK1 also signal to MAPK p38, JNK, and ERK1/ERK2 resulting in the activation of the transcription factor AP-1. Adapted from *Frontiers in Immunology* (Moreira *et al*., 2012).

1.3.7 RIPK2 and cancer

There is a sufficient amount of evidence pointing out the critical role of RIPK2 in inflammatory diseases such as allergic airway inflammation [163], multiple sclerosis [162], granulomatous inflammatory disease [277], pancreatitis [278], psoriasis [279], sarcoidosis, and inflammatory bowel disease [164]. However, newer research suggests that RIPK2 could be involved in tumorigenesis through tumor microenvironment regulation [280].

One of the first studies examining RIPK2 in breast cancer cells suggested that RIPK2 can be a potential chemosensitizer [281]. This result prompted the same group to study the significance of RIPK2 significance in breast cancer. Interestingly, RIPK2 was found to be overexpressed in triple-negative breast cancer (TNBC) patients, and its expression correlated with a worse progression-free survival. The knockdown of RIPK2 in TNBC cells abolished their ability to migrate both in vivo and in vitro; through the deactivation of NF-κB and JNK signals [281]. Similar results were found in renal cell carcinoma and gastric cancer [282] [283]. The inhibition of RIPK2 activity using Gefitinib abrogated macrophage invasion and metastases in osteosarcoma cells. Using more selective RIPK2 inhibitors such as OD36 and WEHI-345 did inhibit invasion in the same manner as Gefitinib [284]. In hepatocellular carcinoma (HCC), the patient tumor tissues exhibited higher levels of active RIPK2 in contrast to non-tumor tissues. This increase correlated with the rise of the NOD2 signal, which relevantly showed a lower overall survival rate [285]. In addition, polymorphisms of RIPK2 (rs42490), (rs16900627) were linked to a higher risk of developing urothelial bladder cancer and gastric cancer, respectively[286] [287] [288]. Analysis of patients' public data revealed that RIPK2 is a poor prognostic marker in pancreatic ductal adenocarcinoma and colorectal cancer [289] [290].

On the contrary, larger tumor volume and more lung metastases were seen in RIPK2 deficient mice modeling bladder cancer. The extracted tumor showed lower levels of tumor-infiltrating cells such as T-cells CD4, CD8, natural killer CD49, and myeloid-derived suppressor cell marker CD11b. While serum cytokines levels such as G-CSF, IL-16, IL-1α, MCP-1 and TIMP-1 and EMT transcription factors such as *ZEB-1, ZEB-2,* and *SNAIL* were increased. Such results suggest that the loss of RIPK2 alters the tumor microenvironment facilitating tumor growth and metastases in bladder cancer [280]. However, Zhang *et al.* showed lower levels of NF-κB in these tumor samples, which suggested that the changes seen are NF-κB independent[280].

1.4 HYPOTHESIS AND RATIONALE

NF-κB signaling pathway plays a main role in cancer development and progression. More recent evidence shows that NF-κB is upregulated in IBC; however, the exact mechanism is still unknown. RIPK2 is a tyrosine kinase upstream NF-κB and is known for being a mediator of many inflammatory diseases. New emerging studies reveal RIPK2 involvement in many types of cancer, including breast cancer. This led to the research question: What role does RIPK2 play in inflammatory breast cancer? I hypothesize that RIPK2 promotes metastatic behavior in inflammatory breast cancer by regulating NF-κB signals and cytokine production in the tumor microenvironment. This was addressed with three specific aims:

1- Determine RIPK2 activity in IBC cell lines and patient samples
2- Characterize RIPK2 function in IBC cells using CRISPR RNP and shRNA technology
3- Explore IBC specific molecular signature using nonparametric random forest analysis

1.5 REFERENCES

1. Javed, A. & Lteif, A. Development of the human breast. *Semin. Plast. Surg.* **27**, (2013).
2. Tobon, H. & Salazar, H. Ultrastructure of the human mammary gland. I. Development of the fetal gland throughout gestation. *J. Clin. Endocrinol. Metab.* **39**, (1974).
3. Medina, D. The mammary gland: a unique organ for the study of development and tumorigenesis. *Journal of mammary gland biology and neoplasia* **1**, (1996).
4. Insua-Rodríguez, J. & Oskarsson, T. The extracellular matrix in breast cancer. *Advanced Drug Delivery Reviews* **97**, (2016).
5. Sternlicht, M. D. Key stages in mammary gland development: The cues that regulate ductal branching morphogenesis. *Breast Cancer Research* **8**, (2005).
6. Deugnier, M. A., Teulière, J., Faraldo, M. M., Thiery, J. P. & Glukhova, M. A. The importance of being a myoepithelial cell. *Breast Cancer Research* **4**, (2002).
7. Gjorevski, N. & Nelson, C. M. Integrated morphodynamic signalling of the mammary gland. *Nat. Rev. Mol. Cell Biol.* **12**, (2011).
8. Stirling, J. W. & Chandler, J. A. The fine structure of the normal, resting terminal ductal-lobular unit of the female breast. *Virchows Arch. A Pathol. Anat. Histol.* **372**, (1976).
9. Suzuki, R. *et al.* Proliferation and differentiation in the human breast during pregnancy. *Differentiation* **66**, (2000).
10. Russo, J. & Russo, I. H. Development of the human breast. in *Maturitas* **49**, (2004).
11. Bhat, V., Allan, A. L. & Raouf, A. Role of the microenvironment in regulating normal and cancer stem cell activity: Implications for breast cancer progression and therapy response. *Cancers (Basel).* **11**, (2019).
12. Stevenson, S. & Thornton, J. Effect of estrogens on skin aging and the potential role of SERMs. *Clinical interventions in aging* **2**, (2007).
13. Silberstein, G. B. Role of the stroma in mammary development. *Breast Cancer Research* **3**, (2001).
14. Wiseman, B. S. & Werb, Z. Development: Stromal effects on mammary gland development and breast cancer. *Science* **296**, (2002).
15. Parmar, H. & Cunha, G. R. Epithelial-stromal interactions in the mouse and human mammary gland in vivo. *Endocrine-Related Cancer* **11**, (2004).
16. Celià-Terrassa, T. Mammary stem cells and breast cancer stem cells: Molecular connections and clinical implications. *Biomedicines* **6**, (2018).
17. Williams, J. M. & Daniel, C. W. Mammary ductal elongation: Differentiation of myoepithelium and basal lamina during branching morphogenesis. *Dev. Biol.* **97**, (1983).
18. LaBarge, M. A., Petersen, O. W. & Bissell, M. J. Of microenvironments and mammary stem cells.

Stem Cell Rev. **3**, 137–146 (2007).

19. Boulanger, C. A. *et al.* Embryonic Stem Cells Are Redirected to Non-Tumorigenic Epithelial Cell Fate by Interaction with the Mammary Microenvironment. *PLoS One* **8**, (2013).
20. Feng, Y. *et al.* Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis. *Genes and Diseases* **5**, 77–106 (2018).
21. Polyak, K. Breast cancer: Origins and evolution. *Journal of Clinical Investigation* **117**, 3155–3163 (2007).
22. Veronesi, U., Boyle, P., Goldhirsch, A., Orecchia, R. & Viale, G. Breast cancer. *Lancet* **365**, 1727–1741 (2005).
23. Allison, K. H. Molecular pathology of breast cancer: What a pathologist needs to know. *American Journal of Clinical Pathology* **138**, (2012).
24. Lalloo, F. *et al.* BRCA1, BRCA2 and TP53 mutations in very early-onset breast cancer with associated risks to relatives. *Eur. J. Cancer* **42**, (2006).
25. Jiao, X. *et al.* Somatic mutations in the notch, NF-KB, PIK3CA, and hedgehog pathways in human breast cancers. *Genes Chromosom. Cancer* **51**, (2012).
26. Pereira, B. *et al.* Erratum: The somatic mutation profiles of 2,433 breast cancers refine their genomic and transcriptomic landscapes (Nature Communications (2016) 7:11479 DOI: 10.1038/ncomms11479). *Nature Communications* **7**, (2016).
27. Simpson, P. T., Reis-Filho, J. S., Gale, T. & Lakhani, S. R. Molecular evolution of breast cancer. *J. Pathol.* **205**, 248–254 (2005).
28. Burstein, H. J., Polyak, K., Wong, J. S., Lester, S. C. & Kaelin, C. M. Ductal Carcinoma in Situ of the Breast. *http://dx.doi.org/10.1056/NEJMra031301* **350**, 1430–1441 (2009).
29. Ciriello, G. *et al.* Comprehensive Molecular Portraits of Invasive Lobular Breast Cancer. *Cell* **163**, 506–519 (2015).
30. Teixeira, M. R. & Heim, S. Cytogenetic Analysis of Tumor Clonality. in *Advances in Cancer Research* **112**, (2011).
31. Sontag, L. & Axelrod, D. E. Evaluation of pathways for progression of heterogeneous breast tumors. *J. Theor. Biol.* **232**, (2005).
32. Trinh, A. *et al.* Genomic alterations during the in situ to invasive ductal breast carcinoma transition shaped by the immune system. *Mol. Cancer Res.* **19**, 623–635 (2021).
33. Ciardiello, C., Leone, A. & Budillon, A. The Crosstalk between Cancer Stem Cells and Microenvironment Is Critical for Solid Tumor Progression: The Significant Contribution of Extracellular Vesicles. *Stem Cells International* **2018**, (2018).
34. Brooks, M. D. & Wicha, M. S. Tumor twitter: Cellular communication in the breast cancer stem cell

niche. *Cancer Discov.* **5**, 469–471 (2015).

35. Kakarala, M. & Wicha, M. S. Implications of the cancer stem-cell hypothesis for breast cancer prevention and therapy. *Journal of Clinical Oncology* **26**, (2008).
36. Liu, S. *et al.* Breast cancer stem cells transition between epithelial and mesenchymal states reflective of their normal counterparts. *Stem Cell Reports* **2**, (2014).
37. Kim, W. T. & Ryu, C. J. Cancer stem cell surface markers on normal stem cells. *BMB Reports* **50**, (2017).
38. Zhou, W., Wang, G. & Guo, S. Regulation of angiogenesis via Notch signaling in breast cancer and cancer stem cells. *Biochimica et Biophysica Acta - Reviews on Cancer* **1836**, (2013).
39. Zhang, Z. M. *et al.* Pygo2 activates MDR1 expression and mediates chemoresistance in breast cancer via the Wnt/β-catenin pathway. *Oncogene* **35**, 4787–4797 (2016).
40. Ribatti, D. The concept of immune surveillance against tumors. The first theories. *Oncotarget* **8**, (2017).
41. Lu, H., Ouyang, W. & Huang, C. Inflammation, a key event in cancer development. *Molecular Cancer Research* **4**, (2006).
42. Barkataki, S. *et al. Inflammatory Breast Cancer: A Panoramic Overview*. *J Rare Dis Res Treat* **3**, (2018).
43. Chen, Y., Tan, W. & Wang, C. Tumor-associated macrophage-derived cytokines enhance cancer stem-like characteristics through epithelial–mesenchymal transition. *OncoTargets and Therapy* **11**, (2018).
44. L. Ferreira, V., H.L. Borba, H., de F. Bonetti, A., P. Leonart, L. & Pontarolo, R. Cytokines and Interferons: Types and Functions. in *Autoantibodies and Cytokines* (2019). doi:10.5772/intechopen.74550
45. Kartikasari, A. E. R., Huertas, C. S., Mitchell, A. & Plebanski, M. Tumor-Induced Inflammatory Cytokines and the Emerging Diagnostic Devices for Cancer Detection and Prognosis. *Frontiers in Oncology* **11**, (2021).
46. Mantovani, A., Dinarello, C. A., Molgora, M. & Garlanda, C. Interleukin-1 and Related Cytokines in the Regulation of Inflammation and Immunity. *Immunity* **50**, (2019).
47. Dmitrieva-Posocco, O. *et al.* Cell-Type-Specific Responses to Interleukin-1 Control Microbial Invasion and Tumor-Elicited Inflammation in Colorectal Cancer. *Immunity* **50**, (2019).
48. Holen, I. *et al.* IL-1 drives breast cancer growth and bone metastasis in vivo. *Oncotarget* **7**, (2016).
49. Kaur, S., Bansal, Y., Kumar, R. & Bansal, G. A panoramic review of IL-6: Structure, pathophysiological roles and inhibitors. *Bioorganic and Medicinal Chemistry* **28**, (2020).
50. Johnston, P. A. & Grandis, J. R. STAT3 signaling: Anticancer strategies and challenges. *Molecular*

Interventions **11**, (2011).

51. Sullivan, N. J. *et al.* Interleukin-6 induces an epithelial-mesenchymal transition phenotype in human breast cancer cells. *Oncogene* **28**, (2009).
52. Ha, H., Debnath, B. & Neamati, N. Role of the CXCL8-CXCR1/2 axis in cancer and inflammatory diseases. *Theranostics* **7**, (2017).
53. Bakouny, Z. & Choueiri, T. K. IL-8 and cancer prognosis on immunotherapy. *Nat. Med.* **26**, (2020).
54. Long, X. *et al.* IL-8, a novel messenger to cross-link inflammation and tumor EMT via autocrine and paracrine pathways (Review). *Int. J. Oncol.* **48**, 5–12 (2016).
55. Schott, A. F. *et al.* Phase Ib pilot study to evaluate reparixin in combination with weekly paclitaxel in patients with HER-2–negative metastatic breast cancer. *Clin. Cancer Res.* **23**, (2017).
56. Singh, J. K. *et al.* Targeting CXCR1/2 significantly reduces breast cancer stem cell activity and increases the efficacy of inhibiting HER2 via HER2-dependent and -independent mechanisms. *Clin. Cancer Res.* **19**, (2013).
57. Gu, S. & Feng, X. H. TGF-β signaling in cancer. *Acta Biochimica et Biophysica Sinica* **50**, (2018).
58. Katsuno, Y. *et al.* Chronic TGF-b exposure drives stabilized EMT, tumor stemness, and cancer drug resistance with vulnerability to bitopic mTOR inhibition. *Sci. Signal.* **12**, (2019).
59. Giuliano, A. E., Edge, S. B. & Hortobagyi, G. N. Eighth Edition of the AJCC Cancer Staging Manual: Breast Cancer. *Annals of Surgical Oncology* **25**, (2018).
60. Amin, M. B. *et al.* The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. *CA. Cancer J. Clin.* **67**, (2017).
61. Sparano, J. A. *et al.* Prospective Validation of a 21-Gene Expression Assay in Breast Cancer. *N. Engl. J. Med.* **373**, (2015).
62. Komaki, K., Sano, N. & Tangoku, A. Problems in histological grading of malignancy and its clinical significance in patients with operable breast cancer. *Breast cancer (Tokyo, Japan)* **13**, (2006).
63. Thoresen, S. Histological grading and clinical stage at presentation in breast carcinoma. *Br. J. Cancer* **46**, (1982).
64. Eliyatkin, N., Yalcin, E., Zengel, B., Aktaş, S. & Vardar, E. Molecular Classification of Breast Carcinoma: From Traditional, Old-Fashioned Way to A New Age, and A New Way. *J. Breast Heal.* **11**, (2015).
65. Hammond, M. E. H. *et al.* American society of clinical oncology/college of american pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. *Journal of Clinical Oncology* **28**, (2010).
66. Gutierrez, C. & Schiff, R. HER2: Biology, detection, and clinical implications. *Arch. Pathol. Lab.*

Med. **135**, (2011).

67. Onitilo, A. A., Engel, J. M., Greenlee, R. T. & Mukesh, B. N. Breast Cancer Subtypes Based on ER/PR and Her2 Expression: Comparison of Clinicopathologic Features and Survival. *Clin. Med. Res.* **7**, (2009).
68. Yersal, O. & Barutca, S. Biological subtypes of breast cancer: Prognostic and therapeutic implications. *World Journal of Clinical Oncology* **5**, (2014).
69. Perou, C. M. *et al.* Molecular portraits of human breast tumours. *Nature* **406**, (2000).
70. MacKay, A. *et al.* Microarray-based class discovery for molecular classification of breast cancer: Analysis of interobserver agreement. *J. Natl. Cancer Inst.* **103**, (2011).
71. Lim, B., Woodward, W. A., Wang, X., Reuben, J. M. & Ueno, N. T. Inflammatory breast cancer biology: the tumour microenvironment is key. *Nature Reviews Cancer* **18**, (2018).
72. Rosenbluth, J. M. & Overmoyer, B. A. Inflammatory Breast Cancer: a Separate Entity. *Current Oncology Reports* **21**, (2019).
73. Woodward, W. A. Inflammatory breast cancer: Unique biological and therapeutic considerations. *The Lancet Oncology* **16**, (2015).
74. Dobiasova, B. & Mego, M. Biomarkers for inflammatory breast cancer: Diagnostic and therapeutic utility. *Breast Cancer: Targets and Therapy* **12**, (2020).
75. Brenner, D. R. *et al.* Projected estimates of cancer in Canada in 2020. *Can. Med. Assoc.* **192**, (2020).
76. Beahrs O, C. D. P. *Manual for Staging of Cancer. American Joint Committee on Cancer*. (Whiting Press, 1977).
77. Edge, S. B. & Compton, C. C. The American Joint Committee on Cancer: the 7th Edition of the AJCC Cancer Staging Manual and the Future of TNM. *Ann. Surg. Oncol.* **17**, 1471–1474 (2010).
78. Dawood, S. *et al.* International expert panel on inflammatory breast cancer: consensus statement for standardized diagnosis and treatment. *Ann. Oncol.* **22**, 515–523 (2011).
79. Matro, J. M. *et al.* Inflammatory breast cancer management in the National Comprehensive Cancer Network: The disease, recurrence pattern, and outcome. *Clin. Breast Cancer* **15**, (2015).
80. Fouad, T. M. *et al.* Distinct epidemiological profiles associated with inflammatory breast cancer (IBC): A comprehensive analysis of the IBC registry at the University of Texas MD Anderson Cancer Center. *PLoS One* **13**, (2018).
81. Taubman, K. L. & McKay, M. J. Axillary lymphoma masquerading as inflammatory breast cancer. *Biomed. Imaging Interv. J.* **2**, (2006).
82. Pruthi, S., Stafyla, V. K., Phillips, S. W., Porrata, L. F. & Reynolds, C. A. Primary mammary (non-Hodgkin) lymphoma presenting as locally advanced breast cancer. *Mayo Clin. Proc.* **79**, (2004).
83. Anne, N. & Pallapothu, R. Lymphoma of the breast: a mimic of inflammatory breast cancer. *World*

J. Surg. Oncol. **9**, (2011).

84. Molckovsky, A., Fitzgerald, B., Freedman, O., Heisey, R. & Clemons, M. Approach to inflammatory breast cancer. *Canadian Family Physician* **55**, (2009).
85. Schlichting, J. A., Soliman, A. S., Schairer, C., Schottenfeld, D. & Merajver, S. D. Inflammatory and non-inflammatory breast cancer survival by socioeconomic position in the Surveillance, Epidemiology, and End Results database, 1990-2008. *Breast Cancer Res. Treat.* **134**, (2012).
86. Fouad, T. M. *et al.* Inflammatory breast cancer: a proposed conceptual shift in the UICC–AJCC TNM staging system. *The Lancet Oncology* **18**, (2017).
87. Chakraborty, P., George, J. T., Woodward, W. A., Levine, H. & Jolly, M. K. Gene expression profiles of inflammatory breast cancer reveal high heterogeneity across the epithelial-hybrid-mesenchymal spectrum. *Transl. Oncol.* **14**, (2021).
88. Van Laere, S. J. *et al.* Uncovering the molecular secrets of inflammatory breast cancer biology: An integrated analysis of three distinct affymetrix gene expression datasets. *Clin. Cancer Res.* **19**, (2013).
89. Woodward, W. A. *et al.* Genomic and expression analysis of microdissected inflammatory breast cancer. *Breast Cancer Res. Treat.* **138**, 761–772 (2013).
90. Bertucci, F. *et al.* Gene expression profiles of inflammatory breast cancer: Correlation with response to neoadjuvant chemotherapy and metastasis-free survival. *Ann. Oncol.* **25**, (2014).
91. Ross, J. S. *et al.* Comprehensive genomic profiling of inflammatory breast cancer cases reveals a high frequency of clinically relevant genomic alterations. *Breast Cancer Res. Treat.* **154**, (2015).
92. Zare, A., Postovit, L. M. & Githaka, J. M. Robust inflammatory breast cancer gene signature using nonparametric random forest analysis. *Breast Cancer Res.* **23**, 1–6 (2021).
93. Van Golen, K. L. *et al.* Mitogen activated protein kinase pathway is involved in RhoC GTPase induced motility, invasion and angiogenesis in inflammatory breast cancer. *Clin. Exp. Metastasis* **19**, (2002).
94. Rosenthal, D. T. *et al.* RhoC impacts the metastatic potential and abundance of breast cancer stem cells. *PLoS One* **7**, (2012).
95. Thomas, P., Pranatharthi, A., Ross, C. & Srivastava, S. RhoC: a fascinating journey from a cytoskeletal organizer to a Cancer stem cell therapeutic target. *Journal of experimental & clinical cancer research : CR* **38**, (2019).
96. Xiao, Y. *et al.* The lymphovascular embolus of inflammatory breast cancer exhibits a Notch 3 addiction. *Oncogene* **30**, (2011).
97. Monneur, A. *et al.* Similar response profile to neoadjuvant chemotherapy, but different survival, in inflammatory versus locally advanced breast cancers. *Oncotarget* **8**, (2017).

98. Van Den Eynden, G. G. *et al.* Overexpression of caveolin-1 and -2 in cell lines and in human samples of inflammatory breast cancer. *Breast Cancer Res. Treat.* **95**, (2006).
99. Ruth, M. C. *et al.* RhoC promotes human melanoma invasion in a PI3K/Akt-dependent pathway. *J. Invest. Dermatol.* **126**, (2006).
100. Levine, P. H. *et al.* Evaluation of lymphangiogenic factors, vascular endothelial growth factor D and E-cadherin in distinguishing inflammatory from locally advanced breast cancer. *Clin. Breast Cancer* **12**, (2012).
101. Van Der Auwera, I. V. *et al.* Increased angiogenesis and lymphangiogenesis in inflammatory versus noninflammatory breast cancer by real-time reverse transcriptase-PCR gene expression quantification. *Clin. Cancer Res.* **10**, (2004).
102. Hosono, K. *et al.* Roles of prostaglandin E2-EP3/EP4 receptor signaling in the enhancement of lymphangiogenesis during fibroblast growth factor-2-induced granulation formation. *Arterioscler. Thromb. Vasc. Biol.* **31**, (2011).
103. Kim, H. S. *et al.* COX2 overexpression is a prognostic marker for Stage III breast cancer. *Breast Cancer Res. Treat.* **132**, (2012).
104. Di Bonito, M., Cantile, M. & Botti, G. Pathological and molecular characteristics of inflammatory breast cancer. *Transl. Cancer Res.* **8**, (2019).
105. Hirano, T. IL-6 in inflammation, autoimmunity and cancer. *International immunology* **33**, (2021).
106. Bièche, I. *et al.* Molecular profiling of inflammatory breast cancer: Identification of a poor-prognosis gene expression signature. *Clin. Cancer Res.* **10**, (2004).
107. Wolfe, A. R. *et al.* Mesenchymal stem cells and macrophages interact through IL-6 to promote inflammatory breast cancer in pre-clinical models. *Oncotarget* **7**, (2016).
108. Drygin, D. *et al.* Protein kinase CK2 modulates IL-6 expression in inflammatory breast cancer. *Biochem. Biophys. Res. Commun.* **415**, (2011).
109. Zheng, Y. *et al.* ACK2-dependent mechanism for activation of the JAK-STAT signaling pathway. *Blood* **118**, (2011).
110. Debeb, B. G. *et al.* Pre-clinical studies of Notch signaling inhibitor RO4929097 in inflammatory breast cancer cells. *Breast Cancer Res. Treat.* **134**, (2012).
111. Mohamed, H. T. *et al.* IL-8 and MCP-1/CCL2 regulate proteolytic activity in triple negative inflammatory breast cancer a mechanism that might be modulated by Src and Erk1/2. *Toxicol. Appl. Pharmacol.* **401**, (2020).
112. de Andrés, P. J. *et al.* Increased levels of interleukins 8 and 10 as findings of canine inflammatory mammary cancer. *Vet. Immunol. Immunopathol.* **152**, (2013).
113. Goldfarb, J. M. & Pippen, J. E. Inflammatory Breast Cancer: The Experience of Baylor University

Medical Center at Dallas. *Baylor Univ. Med. Cent. Proc.* **24**, (2011).

114. Morrow, R. J., Etemadi, N., Yeo, B. & Ernst, M. Challenging a Misnomer? The Role of Inflammatory Pathways in Inflammatory Breast Cancer. *Mediators of Inflammation* **2017**, (2017).
115. Rosenberg, S. A. Cancer immunotherapy comes of age. *Nature Clinical Practice Oncology* **2**, (2005).
116. Wang, X. *et al.* Targeting signaling pathways in inflammatory breast cancer. *Cancers* **12**, (2020).
117. Park, M. H. & Hong, J. T. Roles of NF-κB in cancer and inflammatory diseases and their therapeutic approaches. *Cells* **5**, (2016).
118. Xia, Y., Shen, S. & Verma, I. M. NF-κB, an active player in human cancers. *Cancer immunology research* **2**, (2014).
119. Van Laere, S. *et al.* Distinct molecular signature of inflammatory breast cancer by cDNA microarray analysis. *Breast Cancer Res. Treat.* **93**, (2005).
120. Van Laere, S. J. *et al.* Nuclear factor-κB signature of inflammatory breast cancer by cDNA microarray validated by quantitative real-time reverse transcription-PCR, immunohistochemistry, and nuclear factor-κB DNA-binding. *Clin. Cancer Res.* **12**, (2006).
121. Lerebours, F. *et al.* NF-kappa B genes have a major role in Inflammatory Breast Cancer. *BMC Cancer* **8**, 41 (2008).
122. Kendellen, M. F., Bradford, J. W., Lawrence, C. L., Clark, K. S. & Baldwin, A. S. Canonical and non-canonical NF-κB signaling promotes breast cancer tumor-initiating cells. *Oncogene* **33**, 1297–1305 (2014).
123. Manning, G., Whyte, D. B., Martinez, R., Hunter, T. & Sudarsanam, S. The protein kinase complement of the human genome. *Science* (2002). doi:10.1126/science.1075762
124. Ardito, F., Giuliani, M., Perrone, D., Troiano, G. & Muzio, L. Lo. The crucial role of protein phosphorylation in cell signalingand its use as targeted therapy (Review). *International Journal of Molecular Medicine* (2017). doi:10.3892/ijmm.2017.3036
125. Hubbard, S. R. & Till, J. H. Protein Tyrosine Kinase Structure and Function. *Annu. Rev. Biochem.* (2000). doi:10.1146/annurev.biochem.69.1.373
126. Duong-Ly, K. C. & Peterson, J. R. The Human Kinome and Kinase Inhibition as a therapeutic strategy. *Curr. Protoc. Pharmacol.* (2014). doi:10.1002/0471141755.ph0209s60
127. Zhang, D., Lin, J. & Han, J. Receptor-interacting protein (RIP) kinase family. *Cell. Mol. Immunol.* (2010). doi:10.1038/cmi.2010.10
128. Stanger, B. Z., Leder, P., Lee, T. H., Kim, E. & Seed, B. RIP: A novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell* (1995). doi:10.1016/0092-8674(95)90072-1

129. Wen, L., Zhuang, L., Luo, X. & Wei, P. TL1A-induced NF-κB activation and c-IAP2 production prevent DR3-mediated apoptosis in TF-1 cells. *J. Biol. Chem.* (2003). doi:10.1074/jbc.M305833200

130. Chaudhary, P. M. *et al.* Death receptor 5, a new member of the TNFR family, and DR4 induce FADD- dependent apoptosis and activate the NF-κB pathway. *Immunity* (1997). doi:10.1016/S1074-7613(00)80400-8

131. Hsu, H., Huang, J., Shu, H. B., Baichwal, V. & Goeddel, D. V. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity* (1996). doi:10.1016/S1074-7613(00)80252-6

132. Micheau, O. & Tschopp, J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* (2003). doi:10.1016/S0092-8674(03)00521-X

133. Devin, A. *et al.* The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. *Immunity* (2000). doi:10.1016/S1074-7613(00)80194-6

134. Ting, A. T., Pimentel-Muiños, F. X. & Seed, B. RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. *EMBO J.* (1996). doi:10.1002/j.1460-2075.1996.tb01007.x

135. Zhang, Y. Y. & Liu, H. Connections between various trigger factors and the RIP1/RIP3 signaling pathway involved in necroptosis. *Asian Pacific Journal of Cancer Prevention* (2013). doi:10.7314/APJCP.2013.14.12.7069

136. Newton, K. RIPK1 and RIPK3: Critical regulators of inflammation and cell death. *Trends in Cell Biology* (2015). doi:10.1016/j.tcb.2015.01.001

137. Xiaoqing, S. *et al.* RIP3, a novel apoptosis-inducing kinase. *J. Biol. Chem.* (1999). doi:10.1074/jbc.274.24.16871

138. Cho, Y. S. *et al.* Phosphorylation-Driven Assembly of the RIP1-RIP3 Complex Regulates Programmed Necrosis and Virus-Induced Inflammation. *Cell* (2009). doi:10.1016/j.cell.2009.05.037

139. Najjar, M. *et al.* RIPK1 and RIPK3 Kinases Promote Cell-Death-Independent Inflammation by Toll-like Receptor 4. *Immunity* (2016). doi:10.1016/j.immuni.2016.06.007

140. McCarthy, J. V, Ni, J. & Dixit, V. M. RIP2 is a novel NF-kappaB-activating and cell death-inducing kinase. *J. Biol. Chem.* **273**, 16968–75 (1998).

141. Inohara, N. & Nuñez, G. NODS: Intracellular proteins involved in inflammation and apoptosis. *Nature Reviews Immunology* (2003). doi:10.1038/nri1086

142. Kobayashi, K. *et al.* RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* (2002). doi:10.1038/416194a

143. Girardin, S. E. *et al.* CARD4/Nod1 mediates NF-KB and JNK activation by invasive Shigella flexneri. *EMBO Rep.* (2001). doi:10.1093/embo-reports/kve155

144. Chin, A. I. *et al.* Involvement of receptor-interacting protein 2 in innate and adaptive immune responses. *Nature* (2002). doi:10.1038/416190a

145. Bahr, C. *et al.* DIK, a novel protein kinase that interacts with protein kinase Cδ: Cloning, characterization, and gene analysis. *J. Biol. Chem.* (2000). doi:10.1074/jbc.M004771200

146. Chen, L. *et al.* Protein Kinase C-associated Kinase (PKK), a Novel Membrane-associated, Ankyrin Repeat-containing Protein Kinase. *J. Biol. Chem.* (2001). doi:10.1074/jbc.M008069200

147. Meylan, E., Martinon, F., Thome, M., Gschwendt, M. & Tschopp, J. RIP4 (DIK/PKK), a novel member of the RIP kinase family, activates NF-κB and is processed during apoptosis. *EMBO Reports* (2002). doi:10.1093/embo-reports/kvf236

148. Chirieleison, S. M., Kertesy, S. B. & Abbott, D. W. Synthetic Biology Reveals the Uniqueness of the RIP Kinase Domain. *J. Immunol.* **196**, 4291–4297 (2016).

149. Huang, C. S. *et al.* Crystal Structure of Ripk4 Reveals Dimerization-Dependent Kinase Activity. *Structure* (2018). doi:10.1016/j.str.2018.04.002

150. Lee, P. *et al.* Phosphorylation of Pkp1 by RIPK 4 regulates epidermal differentiation and skin tumorigenesis . *EMBO J.* (2017). doi:10.15252/embj.201695679

151. Kalay, E. *et al.* Mutations in RIPK4 cause the autosomal-recessive form of popliteal pterygium syndrome. *Am. J. Hum. Genet.* (2012). doi:10.1016/j.ajhg.2011.11.014

152. De Groote, P. *et al.* A novel RIPK4-IRF6 connection is required to prevent epithelial fusions characteristic for popliteal pterygium syndromes. *Cell Death Differ.* (2015). doi:10.1038/cdd.2014.191

153. Kwa, M. Q., Huynh, J., Reynolds, E. C., Hamilton, J. A. & Scholz, G. M. Disease-associated mutations in IRF6 and RIPK4 dysregulate their signalling functions. *Cell. Signal.* (2015). doi:10.1016/j.cellsig.2015.03.005

154. Meylan, E. & Tschopp, J. The RIP kinases: Crucial integrators of cellular stress. *Trends in Biochemical Sciences* (2005). doi:10.1016/j.tibs.2005.01.003

155. H. Rideout and D. Re. *Leucine- Rich Repeat Kinase 2*. **14**, (Springer Nature, 2017).

156. Bae, J. R. & Lee, B. D. Function and dysfunction of leucine-rich repeat kinase 2 (LRRK2): Parkinson's disease and beyond. *BMB Reports* (2015). doi:10.5483/BMBRep.2015.48.5.032

157. Marín, I., Egmond, W. N. & Haastert, P. J. M. The Roco protein family: a functional perspective. *FASEB J.* (2008). doi:10.1096/fj.08-111310

158. Dzamko, N. & Halliday, G. M. An emerging role for LRRK2 in the immune system. *Biochemical Society Transactions* (2012). doi:10.1042/BST20120119

159. Franke, A. *et al.* Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat. Genet.* (2010). doi:10.1038/ng.717

160. Thome, M. *et al.* Identification of CARDIAK, a RIP-like kinase that associates with caspase-1. *Curr. Biol.* **8**, 885–8 (1998).

161. Inohara, N. *et al.* An induced proximity model for NF-κB activation in the Nod1/RICK and RIP signaling pathways. *J. Biol. Chem.* (2000). doi:10.1074/jbc.M003415200

162. Shaw, P. J. *et al.* Signaling via the RIP2 Adaptor Protein in Central Nervous System-Infiltrating Dendritic Cells Promotes Inflammation and Autoimmunity. *Immunity* **34**, 75–84 (2011).

163. Goh, F. Y. *et al.* Receptor-Interacting Protein 2 Gene Silencing Attenuates Allergic Airway Inflammation. *J. Immunol.* **191**, 2691–2699 (2013).

164. Canning, P. *et al.* Inflammatory Signaling by NOD-RIPK2 Is Inhibited by Clinically Relevant Type II Kinase Inhibitors. *Chem. Biol.* **22**, 1174–1184 (2015).

165. Heim, V. J., Stafford, C. A. & Nachbur, U. NOD Signaling and Cell Death. *Frontiers in Cell and Developmental Biology* **7**, 208 (2019).

166. Ogura, Y. *et al.* Nod2, a Nod1/Apaf-1 Family Member That Is Restricted to Monocytes and Activates NF-κB. *J. Biol. Chem.* (2001). doi:10.1074/jbc.M008072200

167. Tigno-Aranjuez, J. T., Asara, J. M. & Abbott, D. W. Inhibition of RIP2's tyrosine kinase activity limits NOD2-driven cytokine responses. *Genes Dev.* **24**, 2666–77 (2010).

168. Pellegrini, E., Signor, L., Singh, S., Erba, E. B. & Cusack, S. Structures of the inactive and active states of RIP2 kinase inform on the mechanism of activation. *PLoS One* (2017). doi:10.1371/journal.pone.0177161

169. Beenstock, J., Mooshayef, N. & Engelberg, D. How Do Protein Kinases Take a Selfie (Autophosphorylate)? *Trends in Biochemical Sciences* (2016). doi:10.1016/j.tibs.2016.08.006

170. Tanabe, T. *et al.* Regulatory regions and critical residues of NOD2 involved in muramyl dipeptide recognition. *EMBO J.* (2004). doi:10.1038/sj.emboj.7600175

171. Strober, W., Murray, P. J., Kitani, A. & Watanabe, T. Signalling pathways and molecular interactions of NOD1 and NOD2. *Nat. Rev. Immunol.* **6**, 9–20 (2006).

172. Gong, Q. *et al.* Structural basis of RIP2 activation and signaling. *Nat. Commun.* (2018). doi:10.1038/s41467-018-07447-9

173. Inohara, N., Del Peso, L., Koseki, T., Chen, S. & Núñez, G. RICK, a novel protein kinase containing a caspase recruitment domain, interacts with CLARP and regulates CD95-mediated apoptosis. *J. Biol. Chem.* **273**, 12296–12300 (1998).

174. Eickhoff, J. *et al.* RICK Activates a NF-κB-dependent Anti-human Cytomegalovirus Response. *J. Biol. Chem.* (2004). doi:10.1074/jbc.M312893200

175. Hrdinka, M. *et al.* Small molecule inhibitors reveal an indispensable scaffolding role of RIPK 2 in NOD 2 signaling . *EMBO J.* (2018). doi:10.15252/embj.201899372

176. Goncharov, T. *et al.* Disruption of XIAP-RIP2 Association Blocks NOD2-Mediated Inflammatory Signaling. *Mol. Cell* **69**, 551-565.e7 (2018).

177. Chirieleison, S. M., Kertesy, S. B. & Abbott, D. W. Synthetic Biology Reveals the Uniqueness of the RIP Kinase Domain. *J. Immunol.* (2016). doi:10.4049/jimmunol.1502631

178. Abbott, D. W., Wilkins, A., Asara, J. M. & Cantley, L. C. The Crohn's disease protein, NOD2, requires RIP2 in order to induce ubiquitinylation of a novel site on NEMO. *Curr. Biol.* (2004). doi:10.1016/j.cub.2004.12.032

179. Windheim, M., Lang, C., Peggie, M., Plater, L. A. & Cohen, P. Molecular mechanisms involved in the regulation of cytokine production by muramyl dipeptide. *Biochem. J.* (2007). doi:10.1042/BJ20061704

180. Olsen, J. V. & Mann, M. Status of large-scale analysis of posttranslational modifications by mass spectrometry. *Molecular and Cellular Proteomics* (2013). doi:10.1074/mcp.O113.034181

181. Alberts, B. *et al. Molecular biology of the cell*. (Garland Science, 2002).

182. Berg, J. M., Tymoczko, J. L. & Stryer, L. A Proton Gradient Powers the Synthesis of ATP. (2002).

183. Hanks, S. K. & Hunter, T. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification 1 . *FASEB J.* (1995). doi:10.1096/fasebj.9.8.7768349

184. Hanks, S. K., Quinn, A. M. & Hunter, T. The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. *Science (80-.).* (1988). doi:10.1126/science.3291115

185. Polyansky, A. A. & Zagrovic, B. Protein electrostatic properties predefining the level of surface hydrophobicity change upon phosphorylation. *J. Phys. Chem. Lett.* (2012). doi:10.1021/jz300103p

186. Olsen, J. V. *et al.* Global, In Vivo, and Site-Specific Phosphorylation Dynamics in Signaling Networks. *Cell* (2006). doi:10.1016/j.cell.2006.09.026

187. Dorsch, M. *et al.* Identification of a regulatory autophosphorylation site in the serine–threonine kinase RIP2. *Cell. Signal.* **18**, 2223–2229 (2006).

188. Taylor, S. S. & Kornev, A. P. Protein kinases: Evolution of dynamic regulatory proteins. *Trends in Biochemical Sciences* (2011). doi:10.1016/j.tibs.2010.09.006

189. Nembrini, C. *et al.* The kinase-activity of Rip2 determines its stability and consequently Nod1 - and Nod2-mediated immune responses. *J. Biol. Chem.* (2009). doi:10.1074/jbc.M109.006353

190. Kannan, N. & Neuwald, A. F. Did protein kinase regulatory mechanisms evolve through elaboration of a simple structural component? *J. Mol. Biol.* (2005). doi:10.1016/j.jmb.2005.06.057

191. Iwasaki, Y., Nishiyama, H., Suzuki, K. & Koizumi, S. Sequential cis/trans autophosphorylation in TrkB tyrosine kinase. *Biochemistry* (1997). doi:10.1021/bi962057x

192. Hershko, A. & Ciechanover, A. THE UBIQUITIN SYSTEM. *Annu. Rev. Biochem.* (1998). doi:10.1146/annurev.biochem.67.1.425
193. Kim, W. *et al.* Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol. Cell* (2011). doi:10.1016/j.molcel.2011.08.025
194. Wagner, S. A. *et al.* A Proteome-wide, Quantitative Survey of In Vivo Ubiquitylation Sites Reveals Widespread Regulatory Roles. *Mol. Cell. Proteomics* (2011). doi:10.1074/mcp.m111.013284
195. McDowell, G. S. & Philpott, A. New Insights Into the Role of Ubiquitylation of Proteins. in *International Review of Cell and Molecular Biology* (2016). doi:10.1016/bs.ircmb.2016.02.002
196. Low, T. Y., Magliozzi, R., Guardavaccaro, D. & Heck, A. J. R. Unraveling the ubiquitin-regulated signaling networks by mass spectrometry-based proteomics. *Proteomics* (2013). doi:10.1002/pmic.201200244
197. Pickart, C. M. & Fushman, D. Polyubiquitin chains: Polymeric protein signals. *Current Opinion in Chemical Biology* (2004). doi:10.1016/j.cbpa.2004.09.009
198. Erpapazoglou, Z., Walker, O. & Haguenauer-Tsapis, R. Versatile Roles of K63-Linked Ubiquitin Chains in Trafficking. *Cells* (2014). doi:10.3390/cells3041027
199. Hasegawa, M. *et al.* A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-κB activation. *EMBO J.* (2008). doi:10.1038/sj.emboj.7601962
200. Panda, S. & Gekara, N. O. The deubiquitinase MYSM1 dampens NOD2-mediated inflammation and tissue damage by inactivating the RIP2 complex. *Nat. Commun.* (2018). doi:10.1038/s41467-018-07016-0
201. Heim, V. J. *et al.* A regulatory interface on RIPK2 is required for XIAP binding and NOD signaling activity. *bioRxiv* (2020).
202. Johnson, E. S. Ubiquitin branches out. in *Nature Cell Biology* (2002). doi:10.1038/ncb1202-e295
203. Spence, J., Sadis, S., Haas, A. L. & Finley, D. A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. *Mol. Cell. Biol.* (1995). doi:10.1128/mcb.15.3.1265
204. Sun, L. & Chen, Z. J. The novel functions of ubiquitination in signaling. *Current Opinion in Cell Biology* (2004). doi:10.1016/j.ceb.2004.02.005
205. Pickart, C. M. Mechanisms Underlying Ubiquitination. *Annu. Rev. Biochem.* (2001). doi:10.1146/annurev.biochem.70.1.503
206. Pellegrini, E. *et al.* RIP2 filament formation is required for NOD2 dependent NF-κB signalling. *Nat. Commun.* (2018). doi:10.1038/s41467-018-06451-3
207. Heim, V. J., Stafford, C. A. & Nachbur, U. NOD Signaling and Cell Death. *Frontiers in Cell and Developmental Biology* (2019). doi:10.3389/fcell.2019.00208
208. Krieg, A. *et al.* XIAP mediates NOD signaling via interaction with RIP2. *Proc. Natl. Acad. Sci. U.*

S. A. (2009). doi:10.1073/pnas.0907131106

209. Bertrand, M. J. M. *et al.* Cellular Inhibitors of Apoptosis cIAP1 and cIAP2 Are Required for Innate Immunity Signaling by the Pattern Recognition Receptors NOD1 and NOD2. *Immunity* (2009). doi:10.1016/j.immuni.2009.04.011
210. Damgaard, R. B. *et al.* The Ubiquitin Ligase XIAP Recruits LUBAC for NOD2 Signaling in Inflammation and Innate Immunity. *Mol. Cell* (2012). doi:10.1016/j.molcel.2012.04.014
211. Yang, S. *et al.* Pellino3 ubiquitinates RIP2 and mediates Nod2-induced signaling and protective effects in colitis. *Nat. Immunol.* (2013). doi:10.1038/ni.2669
212. Xie, P. TRAF molecules in cell signaling and in human diseases. *Journal of Molecular Signaling* (2013). doi:10.1186/1750-2187-8-7
213. Walczak, H., Iwai, K. & Dikic, I. Generation and physiological roles of linear ubiquitin chains. *BMC Biology* (2012). doi:10.1186/1741-7007-10-23
214. Hauenstein, A. V., Xu, G., Kabaleeswaran, V. & Wu, H. Evidence for M1-Linked Polyubiquitin-Mediated Conformational Change in NEMO. *J. Mol. Biol.* (2017). doi:10.1016/j.jmb.2017.10.026
215. Bertrand, M. J. M. *et al.* CIAP1/2 are direct E3 ligases conjugating diverse types of ubiquitin chains to receptor interacting proteins kinases 1 to 4 (RIP1-4). *PLoS One* (2011). doi:10.1371/journal.pone.0022356
216. Stafford, C. A. *et al.* IAPs Regulate Distinct Innate Immune Pathways to Co-ordinate the Response to Bacterial Peptidoglycans. *Cell Rep.* (2018). doi:10.1016/j.celrep.2018.01.024
217. Yang, Y. *et al.* NOD2 pathway activation by MDP or Mycobacterium tuberculosis infection involves the stable polyubiquitination of Rip2. *J. Biol. Chem.* (2007). doi:10.1074/jbc.M703079200
218. Kanayama, A. *et al.* TAB2 and TAB3 activate the NF-κB pathway through binding to polyubiquitin chains. *Mol. Cell* (2004). doi:10.1016/j.molcel.2004.08.008
219. Jiang, X. & Chen, Z. J. The role of ubiquitylation in immune defence and pathogen evasion. *Nature Reviews Immunology* (2012). doi:10.1038/nri3111
220. Wu, C.-J., Conze, D. B., Li, T., Srinivasula, S. M. & Ashwell, J. D. Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-κB activation. *Nat. Cell Biol.* (2006). doi:10.1038/ncb1384
221. Lin, C. C., Huoh, Y. S., Schmitz, K. R., Jensen, L. E. & Ferguson, K. M. Pellino Proteins Contain a Cryptic FHA Domain that Mediates Interaction with Phosphorylated IRAK1. *Structure* (2008). doi:10.1016/j.str.2008.09.011
222. Inoue, J. ichiro *et al.* Tumor necrosis factor receptor-associated factor (TRAF) family: Adapter proteins that mediate cytokine signaling. *Experimental Cell Research* (2000). doi:10.1006/excr.1999.4733

223. Wajant, H., Henkler, F. & Scheurich, P. The TNF-receptor-associated factor family - Scaffold molecules for cytokine receptors, kinases and their regulators. *Cellular Signalling* (2001). doi:10.1016/S0898-6568(01)00160-7

224. Ha, H., Han, D. & Choi, Y. TRAF-mediated TNFR-family signaling. *Current Protocols in Immunology* (2009). doi:10.1002/0471142735.im1109ds87

225. Watanabe, T. *et al.* NOD1 contributes to mouse host defense against Helicobacter pylori via induction of type I IFN and activation of the ISGF3 signaling pathway. *J. Clin. Invest.* (2010). doi:10.1172/JCI39481

226. Tao, M. F. *et al.* ITCH K63-Ubiquitinates the NOD2 Binding Protein, RIP2, to Influence Inflammatory Signaling Pathways. *Curr. Biol.* (2009). doi:10.1016/j.cub.2009.06.038

227. Bist, P. *et al.* E3 Ubiquitin ligase ZNRF4 negatively regulates NOD2 signalling and induces tolerance to MDP. *Nat. Commun.* (2017). doi:10.1038/ncomms15865

228. Araki, T. & Milbrandt, J. ZNRF Proteins Constitute a Family of Presynaptic E3 Ubiquitin Ligases. *J. Neurosci.* (2003). doi:10.1523/jneurosci.23-28-09385.2003

229. Condé, C. *et al.* The inositol phosphatase SHIP-1 inhibits NOD2-induced NF-κb activation by disturbing the interaction of XIAP with RIP2. *PLoS One* (2012). doi:10.1371/journal.pone.0041005

230. LeBlanc, P. M. *et al.* Caspase-12 Modulates NOD Signaling and Regulates Antimicrobial Peptide Production and Mucosal Immunity. *Cell Host Microbe* (2008). doi:10.1016/j.chom.2008.02.004

231. Cooney, R. *et al.* NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat. Med.* (2010). doi:10.1038/nm.2069

232. Homer, C. R., Richmond, A. L., Rebert, N. A., Achkar, J. & McDonald, C. ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in crohn's disease pathogenesis. *Gastroenterology* (2010). doi:10.1053/j.gastro.2010.07.006

233. Sorbara, M. T. *et al.* The protein ATG16L1 suppresses inflammatory cytokines induced by the intracellular sensors Nod1 and Nod2 in an autophagy-independent manner. *Immunity* (2013). doi:10.1016/j.immuni.2013.10.013

234. Travassos, L. H. *et al.* Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat. Immunol.* (2010). doi:10.1038/ni.1823

235. Clark, N. M., Marinis, J. M., Cobb, B. A. & Abbott, D. W. MEKK4 Sequesters RIP2 to Dictate NOD2 Signal Specificity. *Curr. Biol.* (2008). doi:10.1016/j.cub.2008.07.084

236. Komander, D., Clague, M. J. & Urbé, S. Breaking the chains: Structure and function of the deubiquitinases. *Nature Reviews Molecular Cell Biology* (2009). doi:10.1038/nrm2731

237. Clague, M. J. *et al.* Deubiquitylases from genes to organism. *Physiol. Rev.* (2013). doi:10.1152/physrev.00002.2013

238. Nijman, S. M. B. *et al.* A genomic and functional inventory of deubiquitinating enzymes. *Cell* (2005). doi:10.1016/j.cell.2005.11.007

239. Chen, Z. J. & Sun, L. J. Nonproteolytic Functions of Ubiquitin in Cell Signaling. *Molecular Cell* (2009). doi:10.1016/j.molcel.2009.01.014

240. Grou, C. P., Pinto, M. P., Mendes, A. V., Domingues, P. & Azevedo, J. E. The de novo synthesis of ubiquitin: Identification of deubiquitinases acting on ubiquitin precursors. *Sci. Rep.* (2015). doi:10.1038/srep12836

241. Sun, S. C. Deubiquitylation and regulation of the immune response. *Nature Reviews Immunology* (2008). doi:10.1038/nri2337

242. Hitotsumatsu, O. *et al.* The Ubiquitin-Editing Enzyme A20 Restricts Nucleotide-Binding Oligomerization Domain Containing 2-Triggered Signals. *Immunity* (2008). doi:10.1016/j.immuni.2008.02.002

243. Fiil, B. K. *et al.* OTULIN Restricts Met1-Linked Ubiquitination to Control Innate Immune Signaling. *Mol. Cell* (2013). doi:10.1016/j.molcel.2013.06.004

244. Wex, K. *et al.* Receptor-interacting protein kinase-2 inhibition by cyld impairs antibacterial immune responses in macrophages. *Front. Immunol.* (2016). doi:10.3389/fimmu.2015.00650

245. Wartz, I. E. *et al.* De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-κB signalling. *Nature* (2004). doi:10.1038/nature02794

246. Schaeffer, V. *et al.* Binding of OTULIN to the PUB Domain of HOIP Controls NF-κB Signaling. *Mol. Cell* (2014). doi:10.1016/j.molcel.2014.03.016

247. Elliott, P. R. *et al.* Molecular basis and regulation of OTULIN-LUBAC interaction. *Mol. Cell* (2014). doi:10.1016/j.molcel.2014.03.018

248. Hrdinka, M. *et al.* CYLD Limits Lys63- and Met1-Linked Ubiquitin at Receptor Complexes to Regulate Innate Immune Signaling. *Cell Rep.* **14**, 2846–2858 (2016).

249. Birol, Melissa & Aude Echalier. Structure and Function of MPN (Mpr1/Pad1 N-terminal) Domain-Containing Proteins. *Curr. Protein Pept. Sci.* (2014).

250. Takeuchi, O. & Akira, S. Pattern Recognition Receptors and Inflammation. *Cell* (2010). doi:10.1016/j.cell.2010.01.022

251. Meunier, E. & Broz, P. Evolutionary Convergence and Divergence in NLR Function and Structure. *Trends in Immunology* (2017). doi:10.1016/j.it.2017.04.005

252. Inohara, N. *et al.* Host recognition of bacterial muramyl dipeptide mediated through NOD2: Implications for Crohn's disease. *J. Biol. Chem.* (2003). doi:10.1074/jbc.C200673200

253. Chamaillard, M. *et al.* An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat. Immunol.* (2003). doi:10.1038/ni945

254. Barnich, N., Aguirre, J. E., Reinecker, H. C., Xavier, R. & Podolsky, D. K. Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor-KB activation in muramyl dipeptide recognition. *J. Cell Biol.* **170**, (2005).

255. Kufer, T. A., Kremmer, E., Adam, A. C., Philpott, D. J. & Sansonetti, P. J. The pattern-recognition molecule Nod1 is localized at the plasma membrane at sites of bacterial interaction. *Cell. Microbiol.* **10**, (2008).

256. Lechtenberg, B. C., Mace, P. D. & Riedl, S. J. Structural mechanisms in NLR inflammasome signaling. *Current Opinion in Structural Biology* **29**, (2014).

257. Maekawa, S., Ohto, U., Shibata, T., Miyake, K. & Shimizu, T. Crystal structure of NOD2 and its implications in human disease. *Nat. Commun.* (2016). doi:10.1038/ncomms11813

258. Inohara, N. *et al.* Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-κB. *J. Biol. Chem.* **274**, 14560–14567 (1999).

259. Gong, Q. *et al.* Structural basis of RIP2 activation and signaling. *Nat. Commun.* **9**, (2018).

260. Abbott, D. W. *et al.* Coordinated Regulation of Toll-Like Receptor and NOD2 Signaling by K63-Linked Polyubiquitin Chains. *Mol. Cell. Biol.* (2007). doi:10.1128/mcb.00270-07

261. Barton, G. M. & Kagan, J. C. A cell biological view of toll-like receptor function: Regulation through compartmentalization. *Nature Reviews Immunology* **9**, (2009).

262. Park, J.-H. *et al.* RICK/RIP2 Mediates Innate Immune Responses Induced through Nod1 and Nod2 but Not TLRs. *J. Immunol.* **178**, 2380–2386 (2007).

263. Hoesel, B. & Schmid, J. A. The complexity of NF-κB signaling in inflammation and cancer. *Molecular Cancer* **12**, 1 (2013).

264. Lappas, M. NOD1 and NOD2 regulate proinflammatory and prolabor mediators in human fetal membranes and myometrium via nuclear factor-kappa B. *Biol. Reprod.* **89**, (2013).

265. Moriguchi, T. *et al.* A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J. Biol. Chem.* **271**, (1996).

266. Yamaguchi, K. *et al.* Identification of a member of the MAPKKK family as a potential mediator of TGF-β signal transduction. *Science (80-.).* **270**, (1995).

267. Shirakabe, K. *et al.* TAK1 mediates the ceramide signaling to stress-activated protein kinase/c-Jun N-terminal kinase. *J. Biol. Chem.* **272**, (1997).

268. Elinav, E., Strowig, T., Henao-Mejia, J. & Flavell, R. A. Regulation of the Antimicrobial Response by NLR Proteins. *Immunity* **34**, (2011).

269. Liu, R. *et al.* HIV-1 Vpr stimulates NF-κB and AP-1 signaling by activating TAK1. *Retrovirology* **11**, (2014).

270. Hess, J., Angel, P. & Schorpp-Kistner, M. AP-1 subunits: Quarrel and harmony among siblings. *J.*

Cell Sci. **117**, (2004).

271. Kim, J. Y., Omori, E., Matsumoto, K., Núñez, G. & Ninomiya-Tsuji, J. TAK1 is a central mediator of NOD2 signaling in epidermal cells. *J. Biol. Chem.* **283**, (2008).
272. Kisiswa, L., Fernández-Suárez, D., Sergaki, M. C. & Ibáñez, C. F. RIP2 Gates TRAF6 Interaction with Death Receptor p75NTR to Regulate Cerebellar Granule Neuron Survival. *Cell Rep.* **24**, (2018).
273. Humke, E. W., Shriver, S. K., Starovasnik, M. A., Fairbrother, W. J. & Dixit, V. M. ICEBERG: A novel inhibitor of interleukin-1β generation. *Cell* **103**, (2000).
274. Zhang, W. H. *et al.* Fundamental role of the Rip2/caspase-1 pathway in hypoxia and ischemia-induced neuronal cell death. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 16012–16017 (2003).
275. Wang, X. *et al.* Dysregulation of receptor interacting protein-2 and caspase recruitment domain only protein mediates aberrant caspase-1 activation in Huntington's disease. *J. Neurosci.* **25**, 11645–11654 (2005).
276. Wang, X. *et al.* The Prostaglandin E2-EP3 Receptor Axis Regulates Anaplasma phagocytophilum-Mediated NLRC4 Inflammasome Activation. *PLoS Pathog.* **12**, (2016).
277. Caruso, R., Warner, N., Inohara, N. & Núñez, G. NOD1 and NOD2: Signaling, host defense, and inflammatory disease. *Immunity* **41**, 898–908 (2014).
278. Duggan, B. M. *et al.* Tyrosine kinase inhibitors of Ripk2 attenuate bacterial cell wall-mediated lipolysis, inflammation and dysglycemia. *Sci. Rep.* **7**, 1578 (2017).
279. Tervaniemi, M. H. *et al.* NOD-like receptor signaling and inflammasome-related pathways are highlighted in psoriatic epidermis. *Sci. Rep.* **6**, 22745 (2016).
280. Zhang, H. & Chin, A. I. Role of Rip2 in development of tumor-infiltrating MDSCs and bladder cancer metastasis. *PLoS One* **9**, (2014).
281. Wright, W. E. *et al.* A Targeted RNAi Screen of the Breast Cancer Genome Identifies KIF14 and TLN1 as Genes That Modulate Docetaxel Chemosensitivity in Triple-Negative Breast Cancer. *Clin. Cancer Res.* **19**, 2061–2070 (2013).
282. Li, D. *et al.* RIPK2 is an unfavorable prognosis marker and a potential therapeutic target in human kidney renal clear cell carcinoma. *Aging (Albany. NY).* **13**, (2021).
283. Yang, Q., Tian, S., Liu, Z. & Dong, W. Knockdown of RIPK2 Inhibits Proliferation and Migration, and Induces Apoptosis via the NF-κB Signaling Pathway in Gastric Cancer. *Front. Genet.* **12**, (2021).
284. Maloney, C. *et al.* Gefitinib inhibits invasion and metastasis of osteosarcoma via inhibition of macrophage receptor interacting serine-threonine kinase 2. *Mol. Cancer Ther.* **19**, (2020).
285. Zhou, Y. *et al.* Hepatic NOD2 promotes hepatocarcinogenesis via a RIP2-mediated

proinflammatory response and a novel nuclear autophagy-mediated DNA damage mechanism. *J. Hematol. Oncol.* **14**, (2021).

286. Soave, A. *et al.* Copy number variations of circulating, cell-free DNA in urothelial carcinoma of the bladder patients treated with radical cystectomy: A prospective study. *Oncotarget* **8**, (2017).
287. Guirado, M. *et al.* Association between C13ORF31, NOD2, RIPK2 and TLR10 polymorphisms and urothelial bladder cancer. *Hum. Immunol.* **73**, (2012).
288. Ota, M. *et al.* Association between receptor interacting serine/threonine kinase 2 polymorphisms and gastric cancer susceptibility. *Oncol. Lett.* **15**, (2018).
289. Li, X., Li, Z., Zhu, H. & Yu, X. Autophagy Regulatory Genes MET and RIPK2 Play a Prognostic Role in Pancreatic Ductal Adenocarcinoma: A Bioinformatic Analysis Based on GEO and TCGA. *Biomed Res. Int.* **2020**, (2020).
290. Jaafar, R. F. *et al.* Receptor-Interacting Serine/Threonine-Protein Kinase-2 as a Potential Prognostic Factor in Colorectal Cancer. *Medicina (B. Aires).* **57**, (2021).
291. Ning, Jing et al. “The impact of Ki-67 in the context of multidisciplinary care in primary inflammatory breast cancer.” *Journal of Cancer* vol. 10,12 2635-2642. 2 Jun. 2019, doi:10.7150/jca.32453

CHAPTER 2

RIPK2 Activity in IBC Cell Lines and Patient Tumor Samples

2.1 ABSTRACT

Inflammatory breast cancer (IBC) is a rare and aggressive form of breast cancer associated with significantly high mortality. Despite advances in IBC diagnoses, the prognosis is still poor compared to non-IBC because of the metastatic nature of this disease. Inflammatory mediators in signaling pathways can play a significant role in promoting metastasis. Receptor-interacting protein kinase 2 (RIPK2) is a driver of the inflammatory signaling pathway NF-κB and has been shown to be involved in many inflammatory diseases and, more recently, cancer. We hypothesize that RIPK2 activity is elevated in IBC facilitating early metastasis. Utilizing IBC cell models and patient tumor samples, we demonstrate the increase of NF-κB activity along with the phosphorylation of RIPK2 in IBC compared to non-IBC. Elevated levels of RIPK2 phosphorylation were present in IBC samples collected at the time of diagnosis. However, chemotherapy did cause an increase in RIPK2 activity, suggesting that chemotherapy augments inflammation in breast tissue and subsequently can lead to treatment resistance. Interestingly, RIPK2 activity correlated with tumor metastasis, and overall group stage, as well as body mass index (BMI), to indicate that RIPK2 might be a useful prognostic marker for IBC and advanced-stage breast cancer.

2.2 INTRODUCTION

IBC is an aggressive form of breast cancer classified as pT4d pathological stage based on breast skin manifestation and corresponds to a clinical-stage IIIB or worse [1]. It accounts for 1–5% of all breast cancer incidence [2], but 10% of all breast cancer deaths with a 38% five-year survival rate [3]. The term "inflammatory" refers to the clinical skin manifestations of the disease, which include breast edema, erythema, and peau d'orange involving at least 1/3 of the breast skin surface caused by dermal lymphatics emboli [4 5]. Despite improvements in diagnostic imaging using, for

example, ultrasound, CT (Computed tomography), or PET (Positron emission tomography) scans, IBC is still primarily diagnosed based on clinical criteria with no specific validated molecular measures. In addition, little is known about the intricate inflammatory mechanisms that cause metastasis in one out of three IBC patients [6] [7]. Thus, determining inflammatory mediators should aid in the earlier diagnosis of IBC, and improving patient outcomes.

NF-κB is a key transcription factor in inflammation and malignant transformation that is upregulated in IBC [8–11]. RIPK2 activates NF-κB through TAK1 (tank binding kinase 1) and subsequently, NEMO and IKK complex leading to IKBα phosphorylation followed by the movement of the NF-κB dimer (p50 and p65) to the nucleus to turn on gene transcription [12–15]. Evidence reveals the critical role of RIPK2 in many inflammatory diseases [16–19] and, more recently, in cancer development and progression [20–22]. The increase of RIPK2 expression is linked to worse progression-free survival in breast cancer patients [22]. In addition, *RIPK2* overexpression is associated with chemotherapy resistance in breast cancer [23] and B-cell non-Hodgkin's lymphoma[24].

Post-translational modifications, in particular phosphorylation, governs RIPK2 activity. Mass spectrometry and mutational analysis identified the serine residue 176 in the kinase activation loop as an autophosphorylation site [25]. A tyrosine 474 autophosphorylation site was also identified and shown to be required for NOD2 maximal signaling and RIPK2-induced NF-κB activation [26] [27]. In this study, we examine the activity level of NF-κB in IBC cell lines and the level of RIPK2 activity in patient samples and cell lines using two different antibodies directed against S176 and Y474 phosphorylation sites.

2.3 MATERIALS AND METHODS

2.3.1 Immunoblotting and antibodies

Total cell lysates were performed by lysing cells in a 10× RIPA buffer containing 20 mM Tris-HCl (pH 7.5) 150 mM NaCl, 1 mM Na_2 EDTA 1 mM EGTA 1% NP-40 1% sodium deoxycholate 2.5 mM sodium pyrophosphate 1 mM β-glycerophosphate 1 mM Na_3 VO_4 1 μg/mL leupeptin and protease inhibitor mixture. Protein concentrations were then measured using the Bio-rad protein assay Bradford protocol. Lysates are resolved on a 7.5% polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. The following antibodies are used: from Santa Cruz (Dallas, TX, USA): RICK (A-10); PCNA Antibody (PC10); and Actin (AC-15). From cell signaling technology (Danvers, MA, USA): phospho-RIP2 (Ser176) (E1I9J) #14397; NF-κB p65 (D14E12) #8242; Phospho-NF-κB p65 (Ser536) (93H1) #4887; Anti-mouse IgG, HRP-linked Antibody #7076; Anti-rabbit IgG, HRP-linked Antibody #7074. RIPK2 Phospho-Y474 antibody was obtained from a MediMab production order from the Baksh lab (University of Alberta, Edmonton, AB, Canada). Densitometry was performed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA) of the scanned image using region of interest analysis for immunoblots. All results were normalized to normal breast tissue.

2.3.2 Breast cancer samples

Normal non-neoplastic breast tissues from breast reduction surgery and breast cancer tissues with clinical data were obtained from the Alberta Cancer Research BioBank (Edmonton, AB, Canada) formally named the Canadian Breast Cancer Foundation tumor bank. Ethics approval for this study was obtained from the Health Research Ethics Board (study ID Pro00015569). All tissues were snap-frozen within 30 min to preserve all the proteins. Tissue samples are post-treatment (treatment with three cycles of 5-fluorouracil, epirubicin, and cyclophosphamide [FEC]

followed by three cycles of docetaxel) unless specified. Patients are diagnosed and classified using the TNM classification of the American Joint Committee on Cancer. Pathological confirmation of breast cancer subtypes was carried out by Dr. Mehdi Agoumi and was based on morphology, overall histological grade, and clinical data. Table 1 summarizes the characteristics of the breast cancer patients used in this study.

Table 2.1 Breast cancer patient characteristics

Variable	***n* (%)**	**Variable**	***n* (%)**
Age		TNBC	
≤45	11 (22)	Yes	10 (20)
>45	39 (78)	No	40 (80)
Grade		Tumor Size	
I	11 (22)	≤3 cm	29 (58)
III	35 (70)	>3 cm	13 (26)
Unknown	4 (8)	Unknown	8 (16)
ER		PR	
Positive	20 (40)	Positive	28 (56)
Negative	27 (54)	Negative	19 (38)
Unknown	3 (6)	Unknown	2 (4)
TNM		HER2	
I	17 (34)	Positive	30 (60)
II	13 (26)	Negative	18 (36)
III	16 (32)	Unknown	2 (4)
IV	4 (8)		

2.3.3 Cell lines

The human IBC cell line SUM149 was grown in Ham's F12 medium supplemented with 5% FBS, 5 µg/mL insulin, and 1 µg/mL hydrocortisone. The human IBC cell line KPL4 was kindly provided by Dr. Naoto T. Ueno (University of Texas MD Anderson Cancer Center, Houston, TX,

USA) and was grown in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% FBS. The human IBC cell line MDA-IBC3 was kindly provided by Dr. Wendy Woodward's laboratory (University of Texas MD Anderson Cancer Center) and was grown in Ham's F12 medium supplemented with 10% FBS, 5 μg/mL insulin, and 1 μg/mL hydrocortisone. Non-IBC breast cancer cell lines MCF 10A, MCF7, MDA-MB-231, and BT549, were kindly provided by Dr. Mary Hitt (University of Alberta).

2.3.4 RIPK2 ADP-Glo Kinase Assay (Promega)

Cells were lysed in 1× passive lysis buffer (Promega, Madison, WI, USA) and incubated overnight using a 1.5 mg rabbit anti-RIPK2 antibody. The next day, protein G-Sepharose was used to immunoprecipitated the RIPK2 protein complex IP for 1.5 h. Samples were then washed once with 1× PBS followed by 2 washes with 5× kinase reaction buffer (40 mM Tris (pH 7.5), 20 mM $MgCl_2$, and 0.1 mg/mL BSA). 10 μL of 1× kinase reaction buffer was added, and the mixture was incubated for 45–60 min at room temperature. This is followed by adding 10 μL of ADP-GloTM Reagent to terminate the kinase reaction and depletes any remaining ATP (40-min incubation time). ADP is then converted to ATP using 20 μL kinase detection reagent, which will generate light from the newly synthesized ATP using a luciferase/luciferin reaction (incubation is 60 min). The light generated is measured using luminescence plate reader. The reading is proportional to the ADP present in the sample and the kinase activity.

2.3.5 Immunohistochemical and immunoblot staining and evaluation

IHC staining was performed as described previously [28]. All IHC results were evaluated using a modified ImageJ software platform using a script written by Dr. Gilbert Bigras in collaboration with Dr. Shairaz Baksh permitting integrated optical density assessment of regions

of interest on each slide. The script, using color deconvolution, separates out the DAB "brown" stained and hematoxylin "blue" stained areas to quantify the DAB antigen-stained areas. For ImageJ quantitation, the region of interest selected was from the entire image, which, in most cases, was the tumor and environment around cancer.

2.3.6 Nuclear and cytoplasmic extracts

According to the manufacturer's instruction, nuclear and cytoplasmic fractions were prepared using the NE-PER Nuclear Cytoplasmic Extraction Reagent kit from ThermoFisher Scientific (Waltham, MA, USA).

2.3.7 Statistical analysis

Data are presented as the mean, standard error of the mean and were compared by t-test or one-way analysis of variance, using GraphPad Prism 9 software (CA, USA). P-value <0.05 was considered statistically significant.

2.4 Results

2.4.1 IBC cell lines exhibit high NF-κB activity

NF-κB family members such as transcription factor RelB and NFκB1 are elevated in IBC tumor tissue compared to non-IBC, implying that NF-κB is constitutively active [29]. To confirm NF-κB activation in IBC cell lines, nuclear extracts from SUM149 cells, the most common cell line used to study IBC [30], MDA-IBC-3 and KPL4 were immunoblotted with a phospho-NF-κB p65 antibody detecting the phosphorylated site at serine 536 (S536). This specific site is phosphorylated by the kinase IKK and is known for NF-κB transactivation [31]. Indeed, nuclear

extracts from IBC cell lines show high activation of NF-κB compared to luminal breast cancer cell line (MCF7) (**Figure 2.1**).

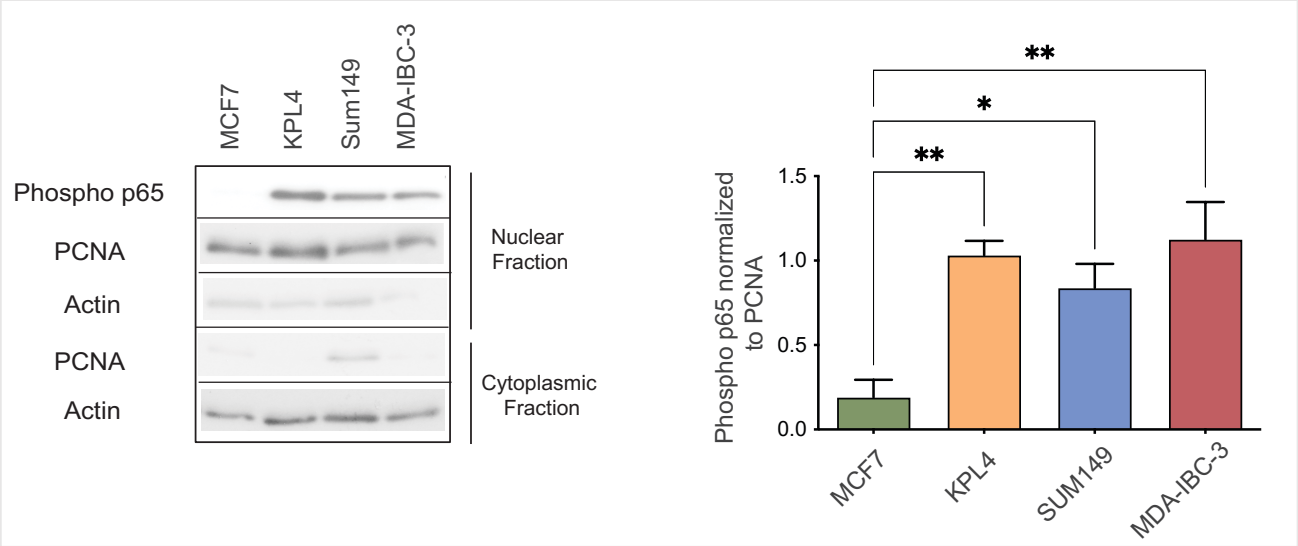

Figure 2.1 NF-κB activity in IBC cell lines. Equal concentrations of total protein from nuclear or cytoplasmic extracts were loaded into a gel and immunoblotted (IB) with the phospho-NF-κB p65 antibody that recognizes the p65 subunit phosphorylated at S536. PCNA (Proliferating cell nuclear antigen) expression is used as a control for the nuclear fraction, and Actin is used as a control for the cytoplasmic fraction. Signal was developed using enhanced chemiluminescence (ECL). Phospho-NF-κB p65 bands were quantified and normalized to PCNA. The data shown are representative of at least three independent experiments. Bars represent mean ± SD. Significance was determined using one-way ANOVA and represented by asterisks (*, $p<0.05$) (**, $p<0.005$).

2.4.2 Elevated RIPK2 activity level in IBC cell lines and patient tissues

RIPK2 functions as a scaffold kinase responsible for mediating NOD signaling, which subsequently results in NF-κB activation[32] [33] [34]. Because NF-κB activity is increased in IBC, we hypothesized that the upstream signaling protein RIPK2 is active. We examined RIPK2 activation in IBC cell lines and patient tumor tissues using RIPK2 phospho antibody that recognizes two different autophosphorylation sites: (i) Serine-176 (S176) [25] [34] and (ii) tyrosine-474 (Y474) [27]. Immunoblot results show IBC cells lines have higher RIPK2 activity when compared to non-IBC cell lines (MCF10A and MCF7) (**Figure 2.2**a). This result was supported by using RIPK2 ADP-Glo assay that measures the ADP formed from the kinase reaction (**Figure 2.2**b).

Analysis of RIPK2 immunohistochemical (IHC) staining revealed robust and diffuse positive cytoplasmic staining in IBC breast tissue compared to non-neoplastic breast tissue (considered normal) (**Figure 2.3**). Non-IBC including Luminal B, *HER2*overexpressed, and TNBC breast tissue show a significant difference in RIPK2 staining compared to IBC except for Luminal A.

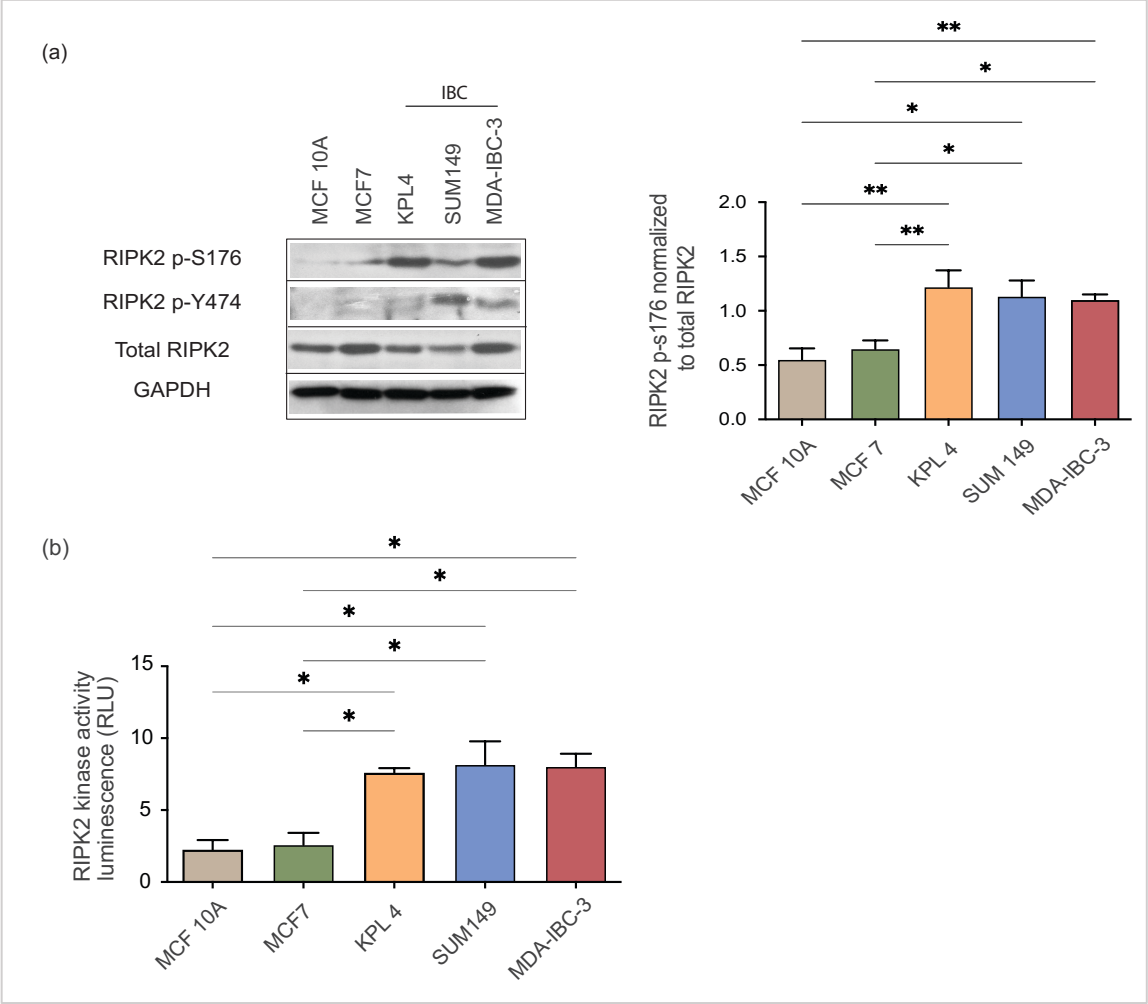

Figure 2.2 RIPK2 activity increased in IBC cell lines. (**a**) equal concentrations of protein were loaded and immunoblotted with RIPK2 phospho serine 176, tyrosine 474 and total RIPK2 antibody. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) is used as a loading control for whole-cell lysate. RIPK2 p-S176 bands were quantified and normalized to total RIPK2 (**b**) luminescent ADP-Glo in vitro RIPK2 kinase assay. RIPK2 was immunoprecipitated, and kinase activity was then measured by quantifying luminescence (RLU) that correlates to the amount of ADP produced during the enzymatic reaction as per manufacturer's instructions; All the data shown represent the results of two to three independent experiments. Bars represent mean ± SD. Significance was determined using one-way ANOVA and represented by asterisks (*, $p<0.05$) (**, $p<0.005$).

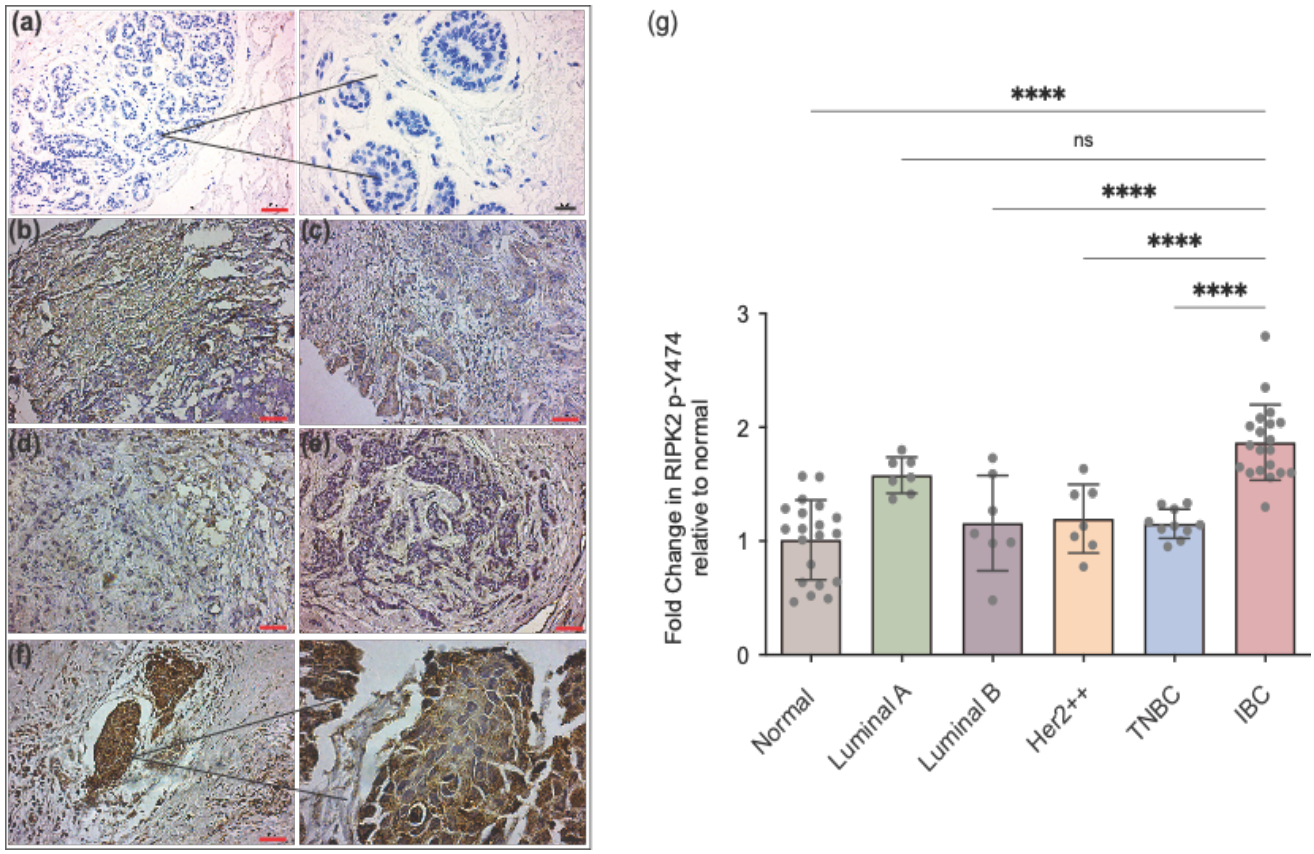

Figure 2.3 RIPK2 activity increased in IBC breast tissue. Immunohistochemical staining of RIPK2 in (**a**) normal non-neoplastic breast; (**b**) luminal A; (**c**) luminal B; (**d**) *HER2* overexpressed; (**e**) triple-negative breast cancer (TNBC); (**f**) and IBC; using RIPK2 phospho-Y474 antibody. Breast tissues were stained and visualized using horseradish peroxidase-conjugated secondary antibody and 3, 3′ diaminobenzidine (DAB; brown), red scale bar: 50 µm, black scale bar: 20 µm. Normal breast tissue (n = 17), luminal A (n = 7), luminal B (n = 8), *HER2* overexpressed (n = 7), TNBC (n = 10) and IBC (n = 18). Several fields (3-4) of each tissue were quantified using the ImageJ platform, permitting integrated optical density assessment of regions of interest in each slide. (**g**) the plot represents the fold change in RIPK2 phospho-Y474 expression in tumor tissue relative to normal non-neoplastic breast tissues. All breast cancer tissues were isolated from patients after neoadjuvant chemotherapy treatment. Bars represent mean ± SD. Significance was determined using one-way ANOVA and represented by asterisks (*, $p<0.05$) (**, $p<0.005$).

2.4.3 Neoadjuvant chemotherapy does not inhibit RIPK2 activity

The evidence of active RIPK2 in IBC tumor tissues following chemotherapy led us to question if RIPK2 is elevated in IBC breast tissue at diagnosis and then was force-activated following chemotherapy. We obtained eight tumor tissues of IBC patients at diagnosis and post-chemotherapy to determine RIPK2 activity. Immunohistochemical (IHC) staining revealed an increase in RIPK2 activity in pre-chemotherapy breast tissue compared to normal. Nonetheless, higher levels are seen in post-chemotherapy tissue compared to normal, and pre-chemotherapy **(Figure 2.4).** This result suggests that RIPK2 activity may contribute to inflammation post-chemotherapy.

2.4.4 RIPK2 activity as an independent prognostic marker

Since RIPK2 is highly activated in IBC, we wanted to assess its role as a potential prognostic marker for breast cancer. The TNM staging system is a helpful tool to evaluate breast cancer progression [35]. According to the American Joint Committee of Cancer (AJCC), TNM staging is based on the primary tumor size (T), the lymph nodes involvement (N), and the presence of distant metastases (M) [36]. Retrospective studies have indicated that TNM staging correlates with a patient's survival rate in breast cancer [37]. Using the Pearson correlation (r) coefficient, we found that RIPK2 activity correlated with primary tumor size (Slope 4.2, with a 95% CI of (2.6–5.8) and a p-value < 0.0001), the presence of distant metastasis (Slope 0.23, with a 95% CI of (0.06–0.39) and a p-value = 0.008) and cancer stage grouping (overall staging) (Slope 1.9, with a 95% CI of (1.06–2.7) and a p-value < 0.0001) (**Figure 2.5**a–c). However, RIPK2 activity was not associated with the number of lymph nodes involved (Slope 2.4, with a 95% CI of (−2.6–6.9) and a p-value = 1.1). Furthermore, we also observed that RIPK2 activity is strongly associated with a patient's

body mass index (BMI) (Slope is 7.2, with a 95% CI of (1.9–12.5) and a *p*-value = 0.003) (**Figure 2.5**d).

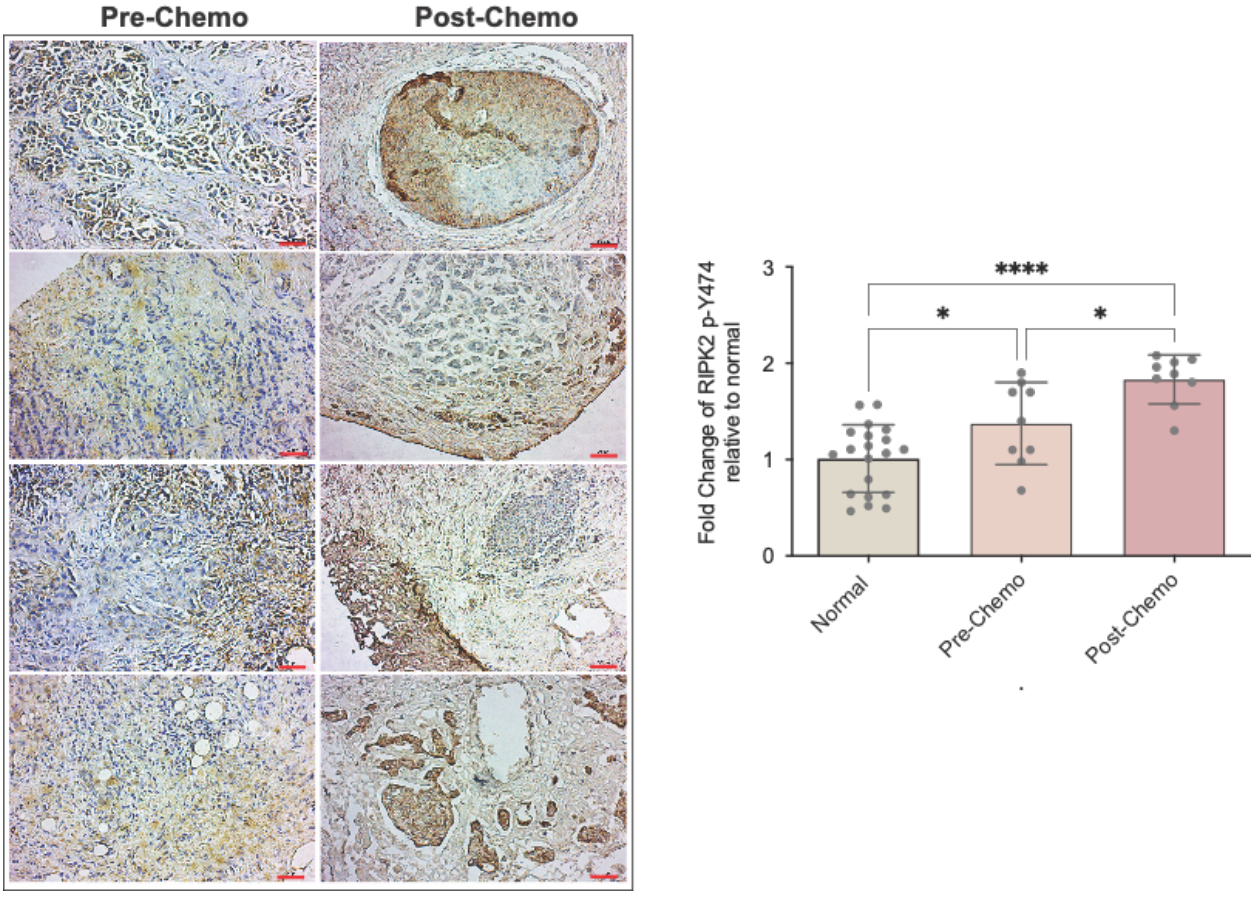

Figure 2.4 RIPK2 activity increased in IBC breast tissue post-chemotherapy. Immunohistochemical staining using the RIPK2 phospho-Y474 antibody in IBC breast tissue pre- and post-chemotherapy as indicated. Red scale bar: 50 µm. DAB staining was quantified using ImageJ software and normalized to normal non-neoplastic breast tissue. A total of eight IBC patient tissues pre-and post-chemotherapy were quantified. Bars represent mean ± SD. Significance was determined using one-way ANOVA and represented by asterisks (*, $p<0.05$) (****, $p<0.00005$).

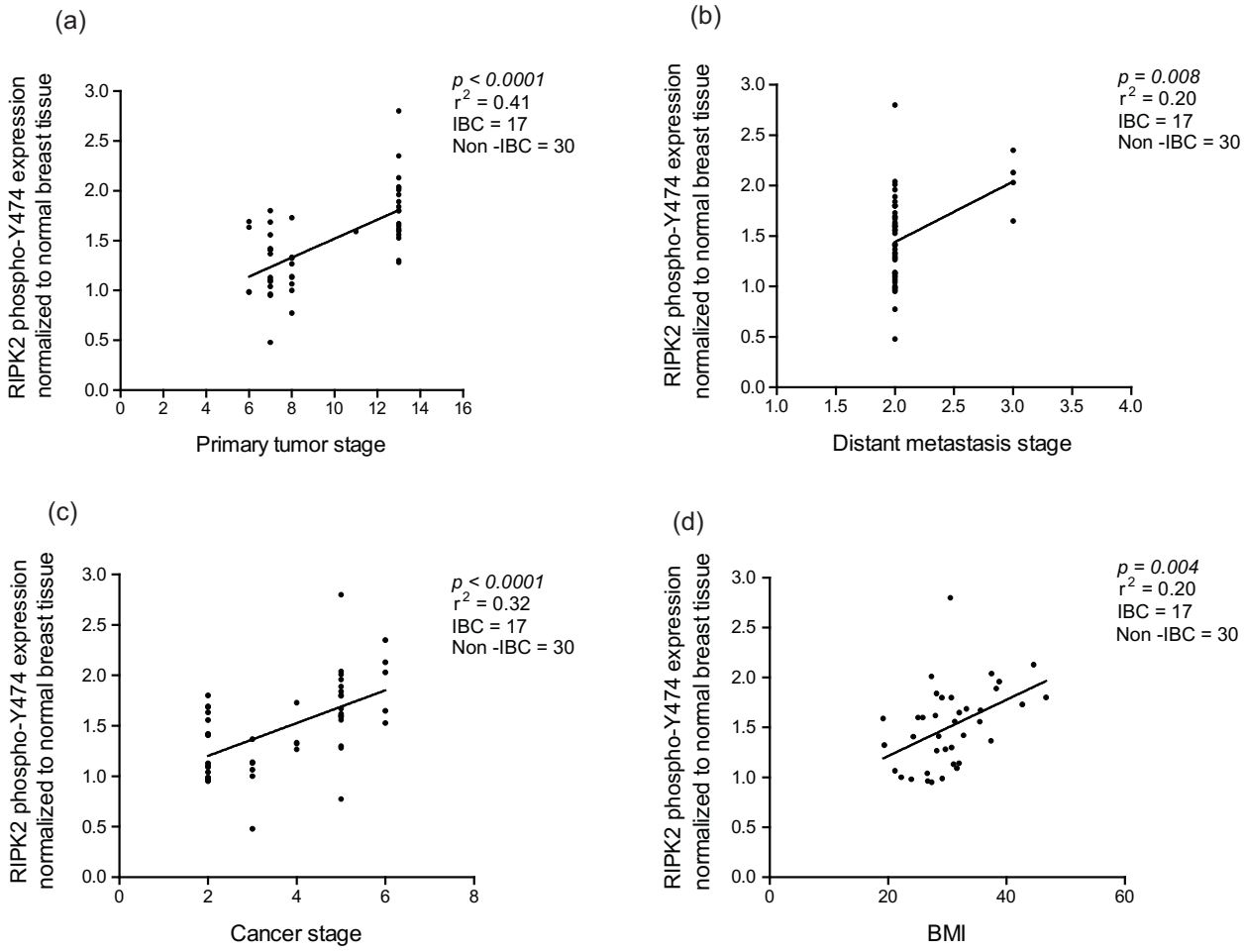

Figure 2.5 Correlation of RIPK2 activity with prognostic markers. RIPK2 correlation with (a) primary tumor stage (n=51) (b) presence of distant metastasis stage (n=51) (c) cancer overall stage (n=51) and (d) body mass index (BMI) (n=51) in breast cancer.

2.5 DISCUSSION

IBC etiology and pathogenesis are not fully understood. The tumor tissue of IBC patients is histopathologically similar to non-IBC, where it displays evidence of invasive carcinoma. However, its unique clinical manifestation makes "molecular inflammation" a potential hypothesis for its cause. Many studies have suggested that the activation of critical inflammatory pathways such as NF-κB contributes to the aggressiveness of IBC [8 38 29 39]. Upregulation of NF-κB promotes proliferation, invasiveness, metastasis, and anti-apoptosis of cancer cells [40 41]. cDNA microarray

results and gene expression profiling of IBC patient samples indicate up-regulation of NF-κB related cytokines and overexpression of NF-κB target genes such as IL-8 and VEGF, signifying that NF-κB is constitutively active [9] [10] [11].

RIPK2 is upstream of NF-κB and can induce its activation and subsequently the expression of essential cytokines and chemokines[12–15]. RIPK2 has been extensively studied in other cancers, especially colorectal cancer, because of its link to NOD2 mutations. However, little is known about RIPK2 activity in breast cancer. This study shows that RIPK2 activity is increased in IBC cell lines and patient tissues by detecting the S176 and Y474 phosphorylation sites. S176 phosphorylation site was confirmed via LPS stimulated macrophages and is used as a specific marker to assess RIPK2 activity [25]. Later studies showed that mutation of the S176 site does not restrict RIPK2 interaction nor signaling. The tyrosine 474 autophosphorylation site was identified and shown to be required for NOD2 maximal signaling and RIPK2-induced NF-κB activation [26] [27]. RIPK2 Y474 phosphorylation was found to be necessary for its activation and subsequent deactivation by E3 ubiquitin ligase ITCH through inducing non-degradative ubiquitination [27].

It is unclear what causes the increase of RIPK2 activity in IBC compared to non-IBC. Typically, RIPK2 is activated through cytosolic microbial receptors such as NLRs (nucleotide-binding and oligomerization domain (NOD)-like receptors), which include NOD1 and NOD2 [18]. NLRs are part of the pattern recognition receptors (PRRs) that recognize harmful stimuli such as pathogen-associated molecular patterns (PAMPs) released by microbes or damage-associated molecular patterns (DAMPs) secreted during tissue injury [42]. Studies show that both NOD1 and NOD2 respond to host-derived non-microbial stimuli, including active Rho GTPases, ER stress,

and the unfolded protein response (UPR), autophagy and mitophagy, disruption of calcium homeostasis, and cell death [43].

The increase of RIPK2 activity in IBC tumor samples following chemotherapy treatment suggests that RIPK2 activity increases in response to stress in the microenvironment. Interestingly, the use of RNA interference (RNAi) showed that RIPK2 knockdown could significantly alter cancer cells chemosensitivity [34]. Treatment resistance is common in IBC and can lead to a high rate of cancer recurrence [44] [45]. In addition, the increase of NF-κB activity, which RIPK2 can regulate, is associated with chemoresistance in breast cancer [46] [47] [48]. This indicates that RIPK2 inhibition can be a potential treatment for chemotherapy side effects.

RIPK2 activity association with tumor size, metastasis status, and cancer stage suggests that RIPK2 could be a prognostic marker for breast cancer, including IBC. Interestingly, RIPK2 expression correlated with progression-free survival (PFS) in TNBC [46]. RIPK2 activity did not correlate with lymph node status. Some studies have shown no association between lymph node status and metastasis stage in TNBC [49]. The association between RIPK2 and BMI was unexpected, but more research is indicating the link of RIPK2 to obesity-induced inflammation through increasing insulin resistance and dysglycemia [50] [51] [52]. In addition, an increase in BMI is considered a risk factor for more advanced types of breast cancer such as TNBC and IBC [37] [53].

In conclusion, we have demonstrated that RIPK2 activity is likely enhanced in IBC and that therapy may potentiate this response. Future studies should further consider the mechanistic roles of RIPK2 in IBC progression and therapy resistance.

2.6 REFERENCES

1. Giuliano, A. E. *et al.* Breast Cancer-Major changes in the American Joint Committee on Cancer eighth edition cancer staging manual. *CA. Cancer J. Clin.* **67**, 290–303 (2017).
2. Wiggins, S., Taylor, S. & Bondy, M. Epidemiology of inflammatory breast cancer. in *Inflammatory Breast Cancer: An Update* **22**, 3–9 (NIH Public Access, 2012).
3. Barkataki, S. *et al. Inflammatory Breast Cancer: A Panoramic Overview*. *J Rare Dis Res Treat* **3**, (2018).
4. Sobin, L. H. & Compton, C. C. TNM seventh edition: What's new, what's changed. *Cancer* **116**, 5336–5339 (2010).
5. Edge, S. B. & Compton, C. C. The American Joint Committee on Cancer: the 7th Edition of the AJCC Cancer Staging Manual and the Future of TNM. *Ann. Surg. Oncol.* **17**, 1471–1474 (2010).
6. Radunsky, G. S. & van Golen, K. L. The current understanding of the molecular determinants of inflammatory breast cancer metastasis. *Clin. Exp. Metastasis* **22**, 615–620 (2005).
7. Fouad, T. M. *et al.* Overall survival differences between patients with inflammatory and noninflammatory breast cancer presenting with distant metastasis at diagnosis HHS Public Access. *Breast Cancer Res Treat* **152**, 407–416 (2015).
8. Dolcet, X., Llobet, D., Pallares, J. & Matias-Guiu, X. NF-kB in development and progression of human cancer. *Virchows Arch.* **446**, 475–482 (2005).
9. Van Laere, S. J. *et al.* Nuclear Factor- B Signature of Inflammatory Breast Cancer by cDNA Microarray Validated by Quantitative Real-time Reverse Transcription-PCR, Immunohistochemistry, and Nuclear Factor- B DNA-Binding. *Clin. Cancer Res.* **12**, 3249–3256 (2006).
10. Lerebours, F. *et al.* NF-kappa B genes have a major role in Inflammatory Breast Cancer. *BMC Cancer* **8**, 41 (2008).
11. Fouad, T. M., Kogawa, T., Reuben, J. M. & Ueno, N. T. The Role of Inflammation in Inflammatory Breast Cancer. in 53–73 (Springer, Basel, 2014). doi:10.1007/978-3-0348-

0837-8_3

12. McCarthy, J. V, Ni, J. & Dixit, V. M. RIP2 is a novel NF-kappaB-activating and cell death-inducing kinase. *J. Biol. Chem.* **273**, 16968–75 (1998).

13. Park, J.-H. *et al.* RICK/RIP2 Mediates Innate Immune Responses Induced through Nod1 and Nod2 but Not TLRs. *J. Immunol.* **178**, 2380–2386 (2007).

14. Hoesel, B. & Schmid, J. A. The complexity of NF-κB signaling in inflammation and cancer. *Molecular Cancer* **12**, 1 (2013).

15. Thome, M. *et al.* Identification of CARDIAK, a RIP-like kinase that associates with caspase-1. *Curr. Biol.* **8**, 885–8 (1998).

16. Goh, F. Y. *et al.* Receptor-Interacting Protein 2 Gene Silencing Attenuates Allergic Airway Inflammation. *J. Immunol.* **191**, 2691–2699 (2013).

17. Shaw, P. J. *et al.* Signaling via the RIP2 Adaptor Protein in Central Nervous System-Infiltrating Dendritic Cells Promotes Inflammation and Autoimmunity. *Immunity* **34**, 75–84 (2011).

18. Caruso, R., Warner, N., Inohara, N. & Núñez, G. NOD1 and NOD2: Signaling, host defense, and inflammatory disease. *Immunity* **41**, 898–908 (2014).

19. Canning, P. *et al.* Inflammatory Signaling by NOD-RIPK2 Is Inhibited by Clinically Relevant Type II Kinase Inhibitors. *Chem. Biol.* **22**, 1174–1184 (2015).

20. Ehlers, S. *et al.* RIP2 regulates growth and differentiation of normal myoblasts and of rhabdomyosarcoma cells. *Eur. J. Cell Biol.* **87**, 163–172 (2008).

21. Wu, S., Kanda, T., Nakamoto, S., Imazeki, F. & Yokosuka, O. Knockdown of receptor-interacting serine/threonine protein kinase-2 (RIPK2) affects EMT-associated gene expression in human hepatoma cells. *Anticancer Res.* **32**, 3775–83 (2012).

22. Singel, S. M. *et al.* Receptor-interacting protein kinase 2 promotes triple-negative breast cancer cell migration and invasion via activation of nuclear factor-kappaB and c-Jun N-terminal kinase pathways. *Breast Cancer Res.* **16**, R28 (2014).

23. Jaafar, R. *et al.* RIP2 enhances cell survival by activation of NF-κB in triple negative breast cancer cells. (2018). doi:10.1016/j.bbrc.2018.02.034

24. Liu, M. *et al.* Non-canonical NF-κB Plays a Pivotal Role in Non-Hodgkin's Lymphoma. *Cell Biochem. Biophys.* **72**, 681–685 (2015).

25. Dorsch, M. *et al.* Identification of a regulatory autophosphorylation site in the serine–threonine kinase RIP2. *Cell. Signal.* **18**, 2223–2229 (2006).

26. Nembrini, C. *et al.* The kinase-activity of Rip2 determines its stability and consequently Nod1 - and Nod2-mediated immune responses. *J. Biol. Chem.* (2009). doi:10.1074/jbc.M109.006353

27. Tigno-Aranjuez, J. T., Asara, J. M. & Abbott, D. W. Inhibition of RIP2's tyrosine kinase activity limits NOD2-driven cytokine responses. *Genes Dev.* **24**, 2666–77 (2010).

28. Kim, S.-W., Roh, J. & Park, C.-S. Immunohistochemistry for Pathologists: Protocols, Pitfalls, and Tips. (2016). doi:10.4132/jptm.2016.08.08

29. Van Laere, S. J. *et al.* Nuclear factor-κB signature of inflammatory breast cancer by cDNA microarray validated by quantitative real-time reverse transcription-PCR, immunohistochemistry, and nuclear factor-κB DNA-binding. *Clin. Cancer Res.* **12**, 3249–3256 (2006).

30. Zhang, D. *et al.* Epidermal growth factor receptor tyrosine kinase inhibitor reverses mesenchymal to epithelial phenotype and inhibits metastasis in inflammatory breast cancer. *Clin. Cancer Res.* **15**, 6639–6648 (2009).

31. Christian, F., Smith, E. & Carmody, R. The Regulation of NF-κB Subunits by Phosphorylation. *Cells* **5**, 12 (2016).

32. Kobayashi, K. *et al.* RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* (2002). doi:10.1038/416194a

33. Heim, V. J., Stafford, C. A. & Nachbur, U. NOD Signaling and Cell Death. *Frontiers in Cell and Developmental Biology* **7**, 208 (2019).

34. Nachbur, U. *et al.* A RIPK2 inhibitor delays NOD signalling events yet prevents inflammatory cytokine production. *Nat. Commun.* **6**, 6442 (2015).

35. Sobin, L. H. TNM: principles, history, and relation to other prognostic factors. *Cancer* **91**, 1589–92 (2001).

36. Park, Y. H. *et al.* Clinical relevance of TNM staging system according to breast cancer subtypes. *Ann. Oncol.* **22**, 1554–1560 (2011).

37. Chang S, Buzdar A U & Hursting. Inflammatory breast cancer and body mass index. *J. Clin. Oncol.* **16**, 3731–3735 (1998).

38. Palmer, N., Shanmugam, M. K., Sethi, G. & Kaldis, P. NF- κ B as a Potential Molecular Target for Therapy of Gastrointestinal Cancers . in *Therapeutic Targets for Inflammation and Cancer* (2017). doi:10.1142/9789813148574_0009

39. Tamer M Fouad, Takahiro Kogawa, James M Reuben & Naoto T Ueno. The role of inflammation in inflammatory breast cancer. *Adv. Exp. Med. Biol.* **816**, 53–73 (2014).

40. Scholz, C. C. & Taylor, C. T. Hydroxylase-dependent regulation of the NF-κB pathway. *Biol. Chem.* **394**, (2013).

41. Lee, C. H., Jeon, Y.-T., Kim, S.-H. & Song, Y.-S. NF-kappaB as a potential molecular target for cancer therapy. *Biofactors* **29**, 19–35 (2007).

42. Takeuchi, O. & Akira, S. Pattern Recognition Receptors and Inflammation. *Cell* (2010). doi:10.1016/j.cell.2010.01.022

43. Kuss-Duerkop, S. K. & Keestra-Gounder, A. M. NOD1 and NOD2 activation by diverse stimuli: A possible role for sensing pathogen-induced endoplasmic reticulum stress. *Infect. Immun.* **88**, (2020).

44. Bertucci, F. *et al.* Bevacizumab plus neoadjuvant chemotherapy in patients with HER2-negative inflammatory breast cancer (BEVERLY-1): a multicentre, single-arm, phase 2 study. *Lancet Oncol.* **17**, 600–611 (2016).

45. Jolly, M. K. *et al.* Inflammatory breast cancer: A model for investigating cluster-based dissemination. *npj Breast Cancer* **3**, (2017).

46. Singel, S. M. *et al.* Receptor-interacting protein kinase 2 promotes triple-negative breast cancer cell migration and invasion via activation of nuclear factor-kappaB and c-Jun N-terminal kinase pathways. *Breast Cancer Res.* **16**, R28 (2014).

47. Tigno-Aranjuez, J. T. *et al.* In vivo inhibition of RIPK2 kinase alleviates inflammatory disease. *J. Biol. Chem.* **289**, 29651–29664 (2014).

48. Fouad, T. M., Kogawa, T., Reuben, J. M. & Ueno, N. T. The Role of Inflammation in Inflammatory Breast Cancer. in *Advances in experimental medicine and biology* **816**, 53–73 (2014).

49. Neven, P., Brouckaert, Wildiers & Floris. Update on triple-negative breast cancer: prognosis and management strategies. *Int. J. Womens. Health* **4**, 511 (2012).

50. Denou, E. *et al.* Defective NOD 2 peptidoglycan sensing promotes diet-induced inflammation, dysbiosis, and insulin resistance . *EMBO Mol. Med.* **7**, (2015).

51. Schertzer, J. D. *et al.* NOD1 activators link innate immunity to insulin resistance. *Diabetes* **60**, (2011).

52. Chi, W. *et al.* Bacterial peptidoglycan stimulates adipocyte lipolysis via NOD1. *PLoS One* **9**, (2014).

53. Dietze, E. C., Chavez, T. A. & Seewaldt, V. L. Obesity and Triple-Negative Breast Cancer: Disparities, Controversies, and Biology. *American Journal of Pathology* **188**, 280–290 (2018).

CHAPTER 3

RIPK2 promotes metastatic behavior in inflammatory breast cancer by regulating NF-κB signaling and key players in the tumor microenvironment

3.1 ABSTRACT

Inflammatory breast cancer (IBC) is a rare but aggressive type of breast cancer characterized by early and rapid metastasis leading to poor clinical outcomes. The tumor microenvironment (TME), including immune cells, fibroblasts and endothelial cells, has emerged as a major regulator of IBC aggressiveness; however, little is known about how IBC cells may orchestrate this pro-tumorigenic milieu. Inflammatory pathways such as NF-κB concomitant with cytokine-induced signaling are upregulated in IBC; yet the mechanisms underpinning these pathways are poorly understood. Receptor Interacting Protein Kinase 2 (RIPK2) mediates NOD cell signaling that has been shown to activate NF-κB signaling and to mediate chronic inflammation in a variety of diseases. Herein we demonstrate that RIPK2 mediates the metastatic behavior in IBC by regulating NF-κB signaling and cytokine production. Specifically, RIPK2 was shown to promote an inflammatory transcriptome in IBC cells leading to the secretion of factors such as IL-8, IL-6 and Activin-A. As a corollary, RIPK2 enhanced key IBC phenotypes, including angiogenic potential and metastatic growth in the lung. Collectively, we demonstrate, for the first time, that RIPK2 may regulate the pro-inflammatory phenotype of IBC cells, making it an attractive target for the treatment of this disease.

3.2 INTRODUCTION

Inflammatory breast cancer (IBC) is a highly metastatic form of breast cancer with a low overall survival rate relative to non-IBC. The incidence of IBC is 2-4% in most parts of the world but still accounts for 7-10% of breast cancer-associated mortality [1]. In contrast to other types of breast cancer that are now treated with specific targeted therapies, IBC patients do not receive tailored therapy despite differences in the disease manifestation. One reason for this deficiency appears to be a lack of distinctive mutational differences between IBC cells and non-IBC cells[2] [3]. Nonetheless, recent research suggests that the IBC tumor microenvironment (TME) component and associated inflammatory pathways have a vital role in its aggressiveness[1].

Cytokines are essential components of TME, mediating dynamic reciprocities between tumor and stromal cells. There are different types of cytokines, including interleukins (IL), tumor necrosis factor (TNF), chemokines, interferons (IFN), and morphogens such as Transforming Growth Factor-β (TGFβ)[4]. Several cytokines appear to be increased in IBC compared to non-IBC[5]. For example, overexpression of IL-6 was found to be higher in IBC tumor samples compared to non-IBC [6] and inhibition of IL-6 decreased invasion and mammosphere formation of IBC cells co-cultured with mesenchymal stem cells [7]. IBC cell lines SUM149 and SUM190 show high IL-6 and IL-8 compared to non-IBC, and patient tumor samples show higher expression levels of IL-8 and CCL2 (C-C Motif Chemokine Ligand 2) [8] [9].

Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a key mediator of cytokine expression[10]. Several studies have demonstrated the upregulation of NF-κB and its related target genes in IBC tumor samples compared to non-IBC [11] [12] [13] [14] [6] [15]. However, the mechanisms by which NF-κB is activated in IBC remain poorly understood. Receptor interacting protein kinase (RIPK2) activation leads to the nuclear translocation of NF-κB following the recognition of microbe-associated molecular patterns by nucleotide-binding oligomerization domain 1 (NOD1),

NOD2, and Toll-like receptors (TLRs). This pathway is best characterized in antigen-presenting cells, such as dendritic cells and macrophages, wherein it drives the release of pro-inflammatory cytokines such as TNF-α, IL-6, and is thus critical for the defense of anti-microbial infections [16] [17] [18] [17]. Herein we demonstrate that RIPK2 may similarly promote an inflammatory milieu in IBC and that this promotes disease progression. Our studies suggest that RIPK2 promotes angiogenesis, proliferation, and migration in human IBC cell lines. Moreover, cytokine arrays and RNA sequencing reveal that RIPK2 specifically affects the inflammatory process as well as the breast cancer-derived secretome. Accordingly, RIPK2 may affect both IBC cells and the surrounding TME.

3.3 Materials and methods

3.3.1 Cells and culture media

SUM149 cell line obtained from ATCC was maintained in culture using Ham's F-12 medium with 5% FBS, 10 mM HEPES, 1 μg/ml hydrocortisone, and 5 μg/ml insulin. SUM190 cell line obtained from BioIVT (NY, USA), and cultured in Ham's F-12 medium with the additional supplements: 5% FBS, bovine serum albumin (1 g/L), 10 mM HEPES, 1 μg/ml hydrocortisone, 5 μg/ml insulin, Ethanolamine 5 mM, Sodium Selenite (Se) 50 nM, apo-Transferrin 5 μg/ml, and Triiodo-L-Thyronine (T3) 6.7 ng/ml (10 nm). Cells were tested regularly for mycoplasma contamination using the ATCC-Universal Mycoplasma Detection Kit. SUM149 cell line was authenticated at the Sick Kids Research Institute, Toronto, ON, Canada in June 2018. SUM149 is classified as a TNBC and SUM190 is classified HER2 overexpressed IBC cell lines.

3.3.2 Immunoblotting and Antibodies

Cells were lysed in RIPA Lysis and Extraction Buffer (Thermo Fisher) supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X) (Thermo Fisher) then sonicated for 5 seconds. After spinning down for 5 min at 14,000 rpm, supernatants were collected, 2x Laemmli Sample Buffer (Bio-Rad) was added, then samples were boiled for 5 min at 95℃. Lysates were resolved on an 8% polyacrylamide gel and transferred to a nitrocellulose membrane. The following antibodies were used: RICK (RIPK2) (A-10) (Santa Cruz, USA); NF-κB p65 (D14E12) #8242 (Cell Signaling Technology, USA); and Alexa Fluor® 594 Conjugate anti-Rabbit IgG (ThermoFisher, USA). For immunoblots, densitometry was performed using Image Studio™ (LI-COR Odyssey system) to accurately detect and quantify protein signals.

3.3.3 Cell counting

Cell growth was determined using Trypan blue, which stains dead cells exclusively. An equal number of cells were plated in 12 well-dishes, and duplicate wells were counted over 5-6 days. Cells were trypsinized and resuspended in equal amounts of medium for counting. An aliquot of cell suspension was mixed with an equal volume of 0.4% Trypan blue solution (Sigma), and cells were counted manually using a hemocytometer.

3.3.4 Anchorage-independent growth

Cells were mixed with 0.7% UltraPure™ Agarose (Invitrogen™) in culture medium and then plated over a bottom layer of 1% agarose in a 6- well plate. Cells were incubated at 37°C in (2x)

high concentration supplemented media using Ham's F-12 Nutrient Mix, powder (ThermoFisher, cat # 21700075). Media were changed every two days; after 8-12 weeks, media was removed, and colonies were stained with 0.1% crystal violet then rinsed with water. The number of colonies was counted manually under a light microscope.

3.3.5 Wound healing assay

According to manufacture protocol, wound healing was assessed using ibidi® culture-insert 2 well in μ-Dish 35 mm (Uddingston, Glasgow, UK). Before starting the assay, cells were washed with serum-free media, and the cell cycle synchronized through 16 hours of serum starvation. Cells were then counted and seeded into the ibidi® culture-inserts in 2.5% FBS medium to reduce their proliferation rate. After 24h, culture-inserts were removed gently, and cells were washed with media to remove any cellular debris cells, then 2.5% FBS media were added. Wound closure was monitored by taking pictures after 6, 12, and 24 hours using a phase-contrast microscope. An increase in the percentage of wound closure indicated the rate of cell migration. The percentage of wound closure was calculated using the formula (wound distance at 0h - wound distance #h / wound distance 0h) x 100.

3.3.6 3D angiogenesis assay

In vitro 3D angiogenesis assay was performed as described in [19]. Briefly, Cytodex-3 micro-beads (Sigma) were coated with HUVEC cells in medium containing 10% FBS and CellTracker Green (Life Technologies). Beads coated with cells were washed twice and resuspended in

fibrinogen (Sigma) (2mg/mL) matrix containing aprotinin (0.15 U/mL) and thrombin (Sigma) (0.625 U/mL) and then cultured in 24 well plates. Once the gel was formed, 1ml of conditioned medium (CM) was added on top. 30 ng/mL VEGF (PeproTech Inc.) was used as a positive control. After 18h, images were taken using a DM-IRB fluorescent microscope (20X; Leica). Each data point reflects one bead count in the HUVEC culture. Three independent experiments were conducted, and 25-30 beads per experiment were analyzed. The number and length of sprouts were counted and measured, respectively, using image analysis software (OpenLab).

3.3.7 Conditioned media

Condition medium (CM) was prepared by plating 1 million cells onto a 60mm plate. After 48h, medium was removed, and cells were thoroughly rinsed two times with 1xPBS to remove serum components. Cells were incubated in serum-free medium (SFM) for an additional 24h to generate CM.

3.3.8 Protein profiling

CM was collected from SUM149 shRNA control and RIPK2 shRNA-2 (3 samples each) and sent to Sciomics (Heidelberg, Germany) for protein profiling. According to company methodology, samples were concentrated and purified using Sciomics SOPs (Standard operating procedure), and the bulk protein concentration was measured by Bicinchoninic acid (BCA) assay. Samples were then labeled and analyzed in a dual-color approach using a reference-based design on six scioCD antibody microarrays (Sciomics) targeting 141 different CD surface markers and

122 cytokines/chemokines with 518 monoclonal antibodies. Each antibody is represented on the array in four replicates. Analysis (Supplementary Table 3.1) was also done by Sciomics (Heidelberg, Germany), and proteins were identified based on (log_2FC) > 0.25 and an adjusted p-value < 0.05 (Benjamini and Hochberg's false discovery rate).

3.3.9 Enzyme-Linked Immunosorbent Assay (ELISA)

Cytokine analyses were carried out by commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions; ELISA kits for the following molecules were used: human IL-8, IL-6 (BioLegend, USA), Activin A, and Inhibin A (Ansh Labs, USA). One million cells were plated in a 60 mm dish overnight and then rinsed twice with serum-free medium. 10 μg/ml MDP was added to 1ml of serum free media and incubated with cells for 24h. Supernatants were then collected and spun down for 5 min at 1300 g to remove cell debris. Samples were stored at -80°C. Absorbance was measured using a FLUOstar Omega plate reader (BMG Labtech, Offenburg, Germany) with Omega Software version 5.11. The data were calculated with a linear regression standard curve to determine sample concentration.

3.3.10 Immunofluorescence

Cells were plated ($1x10^5$ cells/2 ml) onto a coverslip in a 6-well culture dish and grown in complete media overnight. The next day, cells were starved for 20-24 h by changing media to serum-free. After that, cells were subjected to 10 μg/ml MDP stimulation for 30 and 60 minutes. Media were then aspirated, and cells were rinsed with 1x PBS and fixed using 4%

paraformaldehyde for 15 min, followed by washing and permeabilization with 0.5% Triton X-100 for 10 min. The cells were then blocked with 10% goat serum, followed by primary antibody NF-κB p65 (D14E12) #8242 (Cell Signaling Technology, USA) and incubated overnight at 4°C. Afterward, the cells were washed and incubated with Alexa Fluor® 594 Conjugate anti-Rabbit IgG (H+L) (1:500) (Cell Signaling Technology, USA) for 45 min at room temperature. Nuclei were stained with DAPI. Coverslips were then mounted with ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific, USA). Volocity software (PerkinElmer, USA) was used to take images of cells using ×20 oil immersion objective on a WaveFx spinning-disk microscope (Quorum Technologies, ON, Canada). Image segmentation and fluorescence intensity analysis were computed in MATLAB as earlier detailed [20].

3.3.11 RIPK2 knockdown

To generate RIPK2 stable knockdown, two MISSION® shRNAs (short-hairpin RNA) were used: pLKO.1-puro-U6-TRCN0000006350 (shRNA-1) and pLKO.1-puro-U6-TRCN0000006348 (shRNA-2). A noneffective scrambled shRNA cassette was used as a control. All plasmid information and shRNA sequences are listed in the supplementary data (Table 3.S2). Lentiviral shRNA particles were generated using a third generation packaging system, including a packaging plasmid pMDLg/pRRE (contains Gag and Pol), a regulatory plasmid pRSV-Rev, and an envelope plasmid pCI-VSVG. This packaging combination was pooled with pLKO.1-U6-shRNA and HEK 293T cells were transfected using lipofectamine® 2000 (Thermofisher, MA, USA). Virus particles in the supernatant were collected and filtered 48- and 72-hours post-transfection. SUM149, and

SUM190 cells were transduced with these particles, with the help of polybrene, and pooled cells were selected with puromycin (1 μg/ml) and (1.5 μg/ml), respectively.

3.3.12 RIPK2 knockout

RIPK2 stable knockout clones were constructed using Cas9/gRNA Ribonucleoproteins (RNPs). This system provides genome editing with lower toxicity, higher editing efficiency, and fewer off-targets in contrast to plasmid CRISPR/Cas9 [21]. Target-specific CRISPR RNA (crRNA) contained a 20 nucleotide RIPK2 target-specific protospacer domain and an extra 16 nucleotide complementary to tracrRNA. The complementary sequence was annealed to the fluorescently labeled transactivating crRNA (tracrRNA)-ATTO™ 550 at 95°C for 5min. Recombinant *Streptococcus pyogenes* Cas9 protein was then added to cleave the target double-stranded DNA with the guide of crRNA: tracrRNA complex. This resulted in activating the non-homologous end joining (NHEJ) repair system. For reverse transfection of the RNP complex, IBC cells were transfected with the RNP complex using lipofectamine® 2000 (Thermofisher, MA, USA) and incubated for 48h. The goal was to introduce frameshift mutations and premature stop codons caused by NHEJ repair of the spliced double-stranded DNA**.** Transfected cells carrying the RNP complex with the fluorescently labeled tracrRNA were sorted using FACS technology into 96-well tissue culture plates and expanded for 6-8 weeks. The remainder bulk population was collected and plated into a 6-well plate. Through sample partitioning in ddPCR and using a FAM tagged reference probe (designed away from the cut site), HEX tagged NHEJ probe (designed as close to the cut site), and Forward and Reverse primers, transfected clones were screened for induced mutations in the target region. If the ddPCR droplets carry a wild-type template, this

means that no indel mutation is found in the target site because FAM and HEX probes are conjugated to the target region. If droplets contain a mutant template, this means only the FAM probe is conjugated due to an indel far from the target site, or only the HEX probe is conjugated because of the indel mutation at the target site. All primers and probes information are listed in the supplementary data (Table 3.S2).

3.3.13 RIPK2 rescue

RIP2 (RIPK2) (NM_003821) plasmid purchased from OriGene (MD, USA) was packaged, and lentivirus particles were generated as mentioned in section 3.3.11. RIPK2 KO clone were transduced with lentivirus particles, with the help of polybrene (1 mL virus with 0.6 µl/ml Polybrene), and clones were selected with puromycin (0.75 µg/ml).

3.3.14 NF-κB overexpression

NF-κB p65 (RELA) (NM_021975) plasmid purchased from OriGene (MD, USA) was packaged, and lentivirus particles were generated as mentioned earlier. RIPK2 KO clone were transduced with lentivirus particles, with the help of polybrene, and clones were selected with puromycin (0.75 µg/ml).

3.3.15 Real-time quantitative RT-PCR

RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified using Epoch™ Microplate Spectrophotometer. RNA was converted to cDNA using a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, MA, USA) and according to manufacture protocol. Gene expression was examined using TaqMan™ Gene Expression Master Mix and the gene probe of interest. All probes information are listed in the supplementary data (Table 3.S2).

3.3.16 RNA sequencing, differential expression analysis and RIPK2 mutation calls

RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified using Epoch™ Microplate Spectrophotometer. Samples were sent to Centre d'expertise et de services Génome Québec for sequencing. Raw reads quality control and preprocessing was done with fastp (version 0.23.1) [22] using default setting. Subsequently, the resultant reads were mapped to *Homo sapiens* reference genome GRCh38 (Ensembl release #104) using splice aware aligner, STAR (version 2.7.8a)[23]. Gene raw count was computed in STAR, using HTSeq-count method. Differential expression analysis was done using DESeq2[24] and the significantly different genes (see Figure 2c,e) subjected to MSigDB pathway and gene ontology analysis using Enrichr [25]. To verify RIPK2 silencing in CRISPR induced mutations, mutation calls were done on the STAR aligned RNA-seq reads as earlier detailed [26]. Briefly, Strelka2 (version 2.9.10)[27] with the *--rna* flag option was used for mutation calls. Only confident calls (mutations with a "PASS" filter flag) were considered for downstream analysis. SnpEff and SnpSift [28] were used to annotate mutations and extract RIPK2 variants respectively. No RIPK2 mutations were detected in RIPK2-rescue samples. In contrast, RIPK2 CRISPR genotype samples contained the expected RIPK2 frameshift mutations

(see annotated, RIPK2 mutations in merged VCF files for each genotype deposited as supplemental data in GSE197611 (will be available once paper is published).

3.3.17 Experimental metastasis assay

SUM149 cells were injected into tail vein (5×10^5 cells/mouse) of 6–8-week-old NOD SCID gamma female mice. Seven mice were used for control and study groups. Body weight was observed weekly up to 12 weeks after injection. Lungs were collected, formalin-fixed and paraffin-embedded. Four lung sections per mouse were used to assess metastasis using immunohistochemical analyses as described [29]. Tissues were stained with anti- HLA (Human leukocyte antigen) class 1 abc antibody [emr8-5] (abcam, USA). Animal work was done following the approval of the Health Sciences Laboratory Animal Services (HSLAS) at the University of Alberta (AUP00001288).

3.3.18 Statistical analysis

Data are presented as the mean, standard error of the mean and were compared by 1-way or 2-way analysis of variance, using GraphPad Prism 9 software (CA, USA). P-value <0.05 was considered statistically significant.

3.4 RESULTS

3.4.1 RIPK2 knockdown decreases IBC cell proliferation

To understand the function of RIPK2 in IBC, we knocked down its expression in SUM149 and SUM190 IBC cell lines and examined the effect using functional assays (Figure 3.1a). For the knockdown, two shRNAs were used and results were confirmed by Western blot and RT-PCR (Figure 3.1b, Supp. Figure 3.1a). The decrease of RIPK2 expression in knockdown cells resulted in a significant growth rate decline after three days compared to control cells (Figure 3.1c, Supp. Figure 3.1b). Changes in the growth rate seen after 72h suggest that RIPK2 may indirectly regulate IBC cell growth by regulating cytokines released in the media, which can have a positive feedback loop. Indeed, RIPK2 knockdown did not significantly alter soft agar colony formation, whereas paracrine signaling is more limited. (Figure 3.1d, Supp. Figure 3.1c).

3.4.2 RIPK2 Knockdown inhibits the migration and angiogenic potential of IBC cells

Previous studies indicated the role of RIPK2 in metastasis [30] [31] [32]. We tested the effect of RIPK2 inhibition on IBC cell migration using a wound-healing assay. Wound closure was significantly quicker in shRNA control-expressing cells as compared to RIPK2 shRNA-cells in both the SUM149 and SUM190 cell lines (Figure 3.1e-f, Supp. Figure 3.1d). Conditioned medium was also collected from SUM149 shRNA control and RIPK2 shRNA cells and used to stimulate the angiogenic sprouting of HUVEC cells in vitro using a 3D bead assay (Figure 3.1g). SUM149 shRNA control CM increased the number and length of sprouts compared to untreated cells and RIPK2 shRNA cells (Figure 3.1h). Results suggest that RIPK2 plays a role in IBC progression by regulating cell migration and angiogenesis.

Figure 1

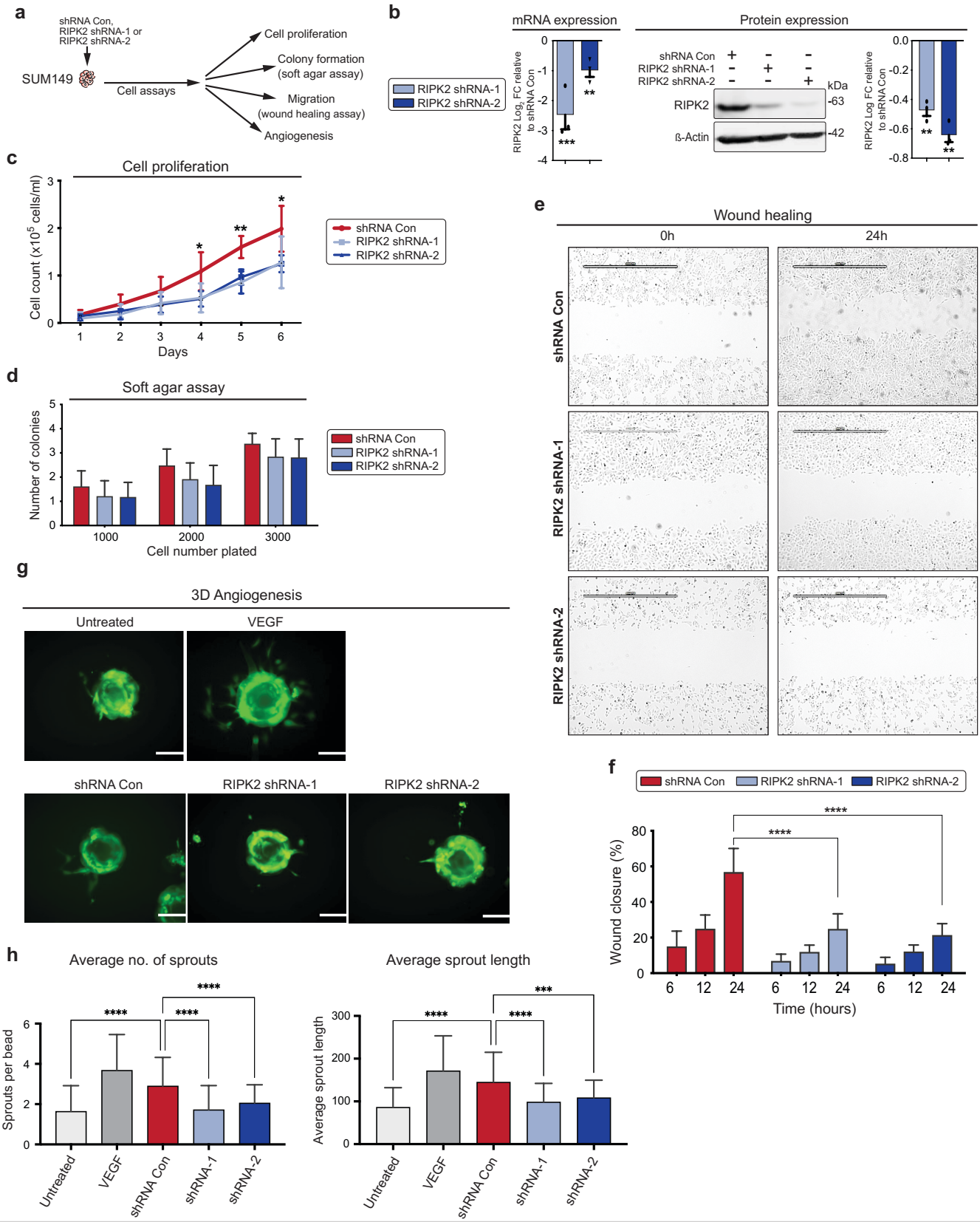
a
shRNA Con, RIPK2 shRNA-1 or RIPK2 shRNA-2
SUM149
Cell assays
Cell proliferation
Colony formation (soft agar assay)
Migration (wound healing assay)
Angiogenesis
b
mRNA expression
RIPK2 shRNA-1
RIPK2 shRNA-2
RIPK2 Log2 FC relative to shRNA Con
Protein expression
shRNA Con
RIPK2 shRNA-1
RIPK2 shRNA-2
kDa
RIPK2
ß-Actin
-63
-42
c
Cell proliferation
Cell count (x10^5 cells/ml)
Days
shRNA Con
RIPK2 shRNA-1
RIPK2 shRNA-2
d
Soft agar assay
Number of colonies
Cell number plated
e
Wound healing
0h
24h
shRNA Con
RIPK2 shRNA-1
RIPK2 shRNA-2
f
Wound closure (%)
Time (hours)
g
3D Angiogenesis
Untreated
VEGF
shRNA Con
RIPK2 shRNA-1
RIPK2 shRNA-2
h
Average no. of sprouts
Sprouts per bead
Average sprout length
Average sprout length
Untreated
VEGF
shRNA Con
shRNA-1
shRNA-2

Figure 3.1 Phenotypic changes associated with RIPK2 inhibition IBC cells. **(a)** A schematic overview of the experimental design used for the functional assays. **(b)** Confirmation of RIPK2 knocked down using two shRNA (shRNA -1 targets exon 4 and shRNA-2 targets exon 3). Real Time RT-PCR and Western blot (WB) were used to confirm RIPK2 expression change in SUM149. *RIPK2* mRNA expression was normalized to GAPDH and plotted as $\log_2$ fold change over shRNA control. WB bands were quantified and normalized to β-Actin. Bars represent mean fold change ± SD relative to shRNA control. Each dot represents a biological replicate. Significance was determined using one-way ANOVA. **(c)** SUM149 shRNA control and SUM149 RIPK2 shRNA-1 and -2 growth rate. Duplicate wells of each group were counted daily for six days using Trypan blue, which allows exclusion of nonviable cells [31]. Significance was determined using two-way ANOVA. **(d)** Soft agar colony formation assay. SUM149 shRNA control and RIPK2 shRNA-1 and -2 cells were plated at different concentrations and grown for 12 weeks (n=3). The number of colonies is counted manually after staining with crystal violet. Bars represent mean ± SD relative. **(e)** Wound healing assay. Representative images of SUM149 shRNA control and RIPK2 shRNA-1 and -2 wounds at 0h and 24h. Scale bars = 1000μm. **(f)** Wound closure was monitored by taking pictures at 0h and after 6, 12, and 24 hours using a phase-contrast microscope(n=4). An increase in the percentage of wound closure indicated the rate of cell migration. Bars represent mean of wound closure percentage ± SD. Significance was determined using two-way ANOVA. **(g)** 3D angiogenesis assay. Representative images of beads coated with HUVEC cells and treated with CM collected from SUM149 shRNA control and RIPK2 shRNA-1 and -2, untreated control and positive control (VEGF). Scale bars = 95 μm. **(h)** Quantification of the number and the length of angiogenic sprouts for controls and RIPK2 knockdown samples. At least 30-35 beads were examined for each sample in three independent experiments. Bars represent mean ± SD. Significance was determined using one-way ANOVA. Significance for all plots is represented by asterisks (**, $p<0.005$) (***, $p<0.0005$) (****, $p<0.00005$)

3.4.3 RNA sequencing of RIPK2 rescue confirms changes at transcriptional levels

To understand how RIPK2 regulates cell function, we used CRISPR RNP approach to stably knockout RIPK2 in SUM149 cells. RIPK2 was then reintroduced to the knockout clone, and expression was confirmed using Western blot. RNA-seq was used to confirm Cas9/gRNA-RNPs-induced RIPK2 indel mutations (see methods and supplemental RIPK2 mutation VCF files for each genotype deposited as supplemental data in GSE197611) and examine the molecular mechanism of RIPK2 in IBC cells (Figure 3.2a-b, Supp. Figure 3.2). DESeq2 analysis identified 230 significantly upregulated genes and 183 genes down-regulated in RIPK2 rescue clones of SUM149 cells in relation to RIPK2 KO control by at least 2-fold with an adjusted p-value < 0.05 (Figure 3.2c). In all analyses (principal component analysis (PCA), unsupervised hierarchical clustering heatmap and dendrogram) the RIPK2 KO versus rescue cells were clearly separated (Figure 3.2d, e). Differential gene ontology and pathway analysis showed significant changes in histocompatibility complex class II (MHC-II) antigen-presenting cells, cytokine-mediated signaling pathway, EMT, and TNF-alpha signaling via NF-κB (Figure 3.2f-g). This result suggested that RIPK2 function is mediated through the NF-κB signaling pathway and that it mediates key pro-metastatic and inflammatory processes in IBC cells.

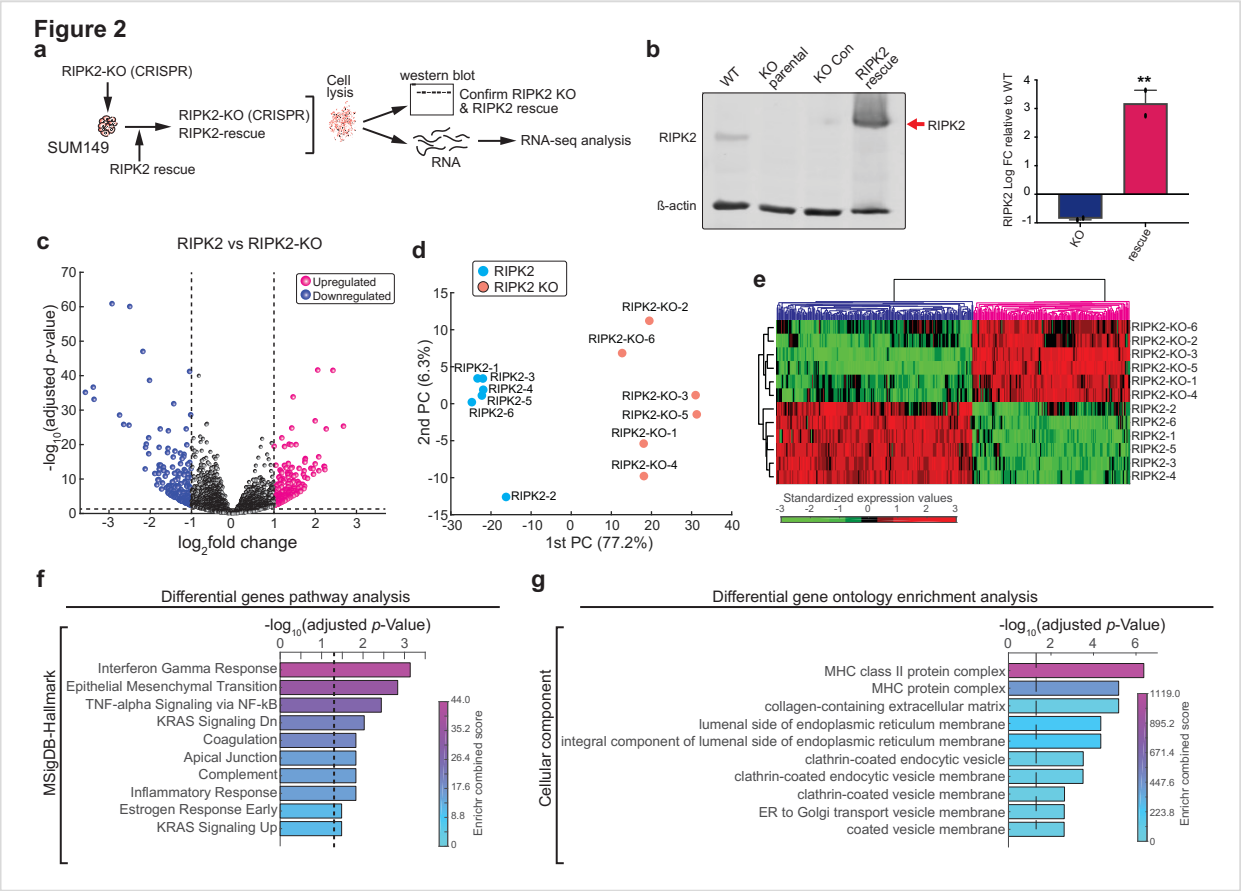

Figure 3.2 RNA sequencing reveals RIPK2 involvement in inflammatory pathways and cytokine production. **(a)** A schematic overview of the experimental design used for the RNA-seq analysis. **(b)** WB confirming RIPK2 re-expression in SUM149 knockout clone. Bands were quantified and normalized to β-Actin. Bars represent mean fold change ± SD relative to SUM149 WT. Significance was determined using one-way ANOVA. **(c)** Volcano plot of differential gene expression of SUM149 RIPK2 (rescue) versus RIPK2 KO control using DESeq2 analysis. The expression difference is considered significant for a $\log_2$ fold change of 2 or more (dotted vertical lines threshold) and an adjusted p-value of <0.05 (dotted horizontal line threshold). The magenta dots indicate the 230 upregulated genes, and the blue dots indicate the 183 downregulated genes when comparing RIPK2 (rescue) to RIPK2 knockout samples. **(d)** Principal component analysis (PCA) of SUM149 RIPK2 KO cells and RIPK2 (rescue) samples using the significant genes. **(e)** Heat map and dendrogram of unsupervised hierarchical clustering of the 413 differentially expressed genes. Red indicates a high expression level, and green indicates a low expression level. **(f)** Molecular Signatures Database (MSigDB) pathway analysis of the differentially expressed genes. **(g)** Gene Ontology enrichment analysis of the differentially expressed genes.

3.4.4 RIPK2 activates NF-κB /p65 nuclear translocation in IBC cells

It's well documented that RIPK2 can regulate NF-κB through the TAK1 and IKK complex [33 34], which later regulates the secretion of many important cytokines [10]. To confirm our RNA-seq results we used immunofluorescence staining, to examine the effect of RIPK2 MDP (Muramyl dipeptide) activation on the translocation of NF-κB subunit p65 to the nucleus, indicating NF-κB activity [35]. MDP is a peptidoglycan (PGN) found in most bacterial cell walls and recognized by NOD2 (nucleotide-binding oligomerization domain 2) upstream RIPK2 [36 37]. Both SUM149 and SUM190 shRNA controls showed an increase in the translocation of p65 translocation after 30min of MDP stimulation (Figure 3.3a-b, Supp. Figure 3.3a, b). This translocation was maintained after 60 min. However, in both IBC cell lines, RIPK2 shRNA showed a delay or inhibition of NF-κB activation: Cells expressing RIPK2 shRNA-1 increased p65 translocation after only 60min of MDP stimulation and those expressing RIPK2 shRNA-2 showed no response to MDP stimulation (Figure 3.3c, Supp. Figure 3.3c). These results suggest that RIPK2 inhibition can mitigate NF-κB signaling. We also examined p65 nuclear translocation in RIPK2 rescue cells. Upon 30 min MDP stimulation, rescue cells showed p65 nuclear translocation at a similar level to SUM149 wild-type (WT) and significantly higher than RIPK2 KO cells (Figure 3.3d), confirming that RIPK2 rescue can restore NF-κB activation to WT levels.

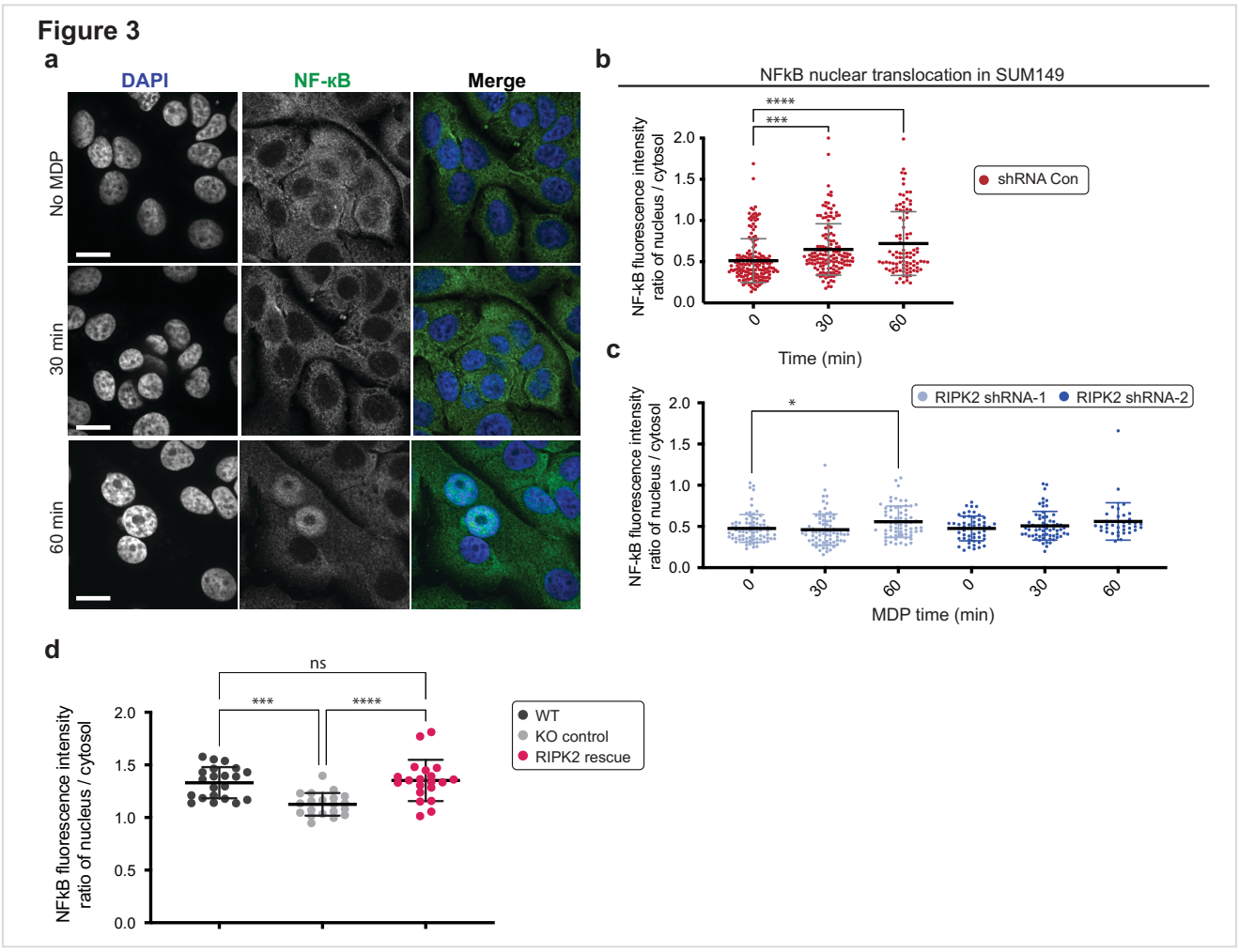

Figure 3.3 RIPK2 regulation of NF-κB nuclear translocation (activation). **(a)** Representative images of NF-κB nuclear translocation in shRNA control cells treated with MDP (10 μg/μl) for 0, 30 and 60 min. Scale bars = 50 μm. Immunofluorescence staining is used to measure NF-κB nuclear translocation by calculating the NF-κB fluorescence intensity ratio of the nucleus over cytosol. **(b)** NF-κB nuclear translocation in SUM149 shRNA control **(c)** RIPK2 shRNA-1 and shRNA-2 stimulated with MDP (10 μg/μl) for 0, 30 and 60 min **(d)** SUM149 WT, RIPK2 KO, and RIPK2 rescue stimulated with MDP (10 μg/μl) for 30min. Bars represent mean ± SD. Each dot represents a cell. Significance was determined using one-way ANOVA and represented by asterisks (*, $p<0.05$) (**, $p<0.005$) (***, $p<0.0005$) (****, $p<0.00005$)

3.4.5 RIPK2 regulates the secretion of cytokines from IBC cells

Given that RIPK2 regulates NF-κB activation and cytokine production as suggested by our previous results, we sought to determine whether RIPK2 knockdown may alter the secretome of IBC cells. Using protein arrays, we measured different cytokines/chemokines and CD surface markers in control and RIPK2 knockdown SUM149 cells (Figure 3.4a). RIPK2 knockdown induced significant differences in the secretome of IBC cells with many proteins decreasing relative to control cells. Several proteins showed a substantial decrease in RIPK2 shRNA-2 compared to shRNA control CM (adjusted p-value < 0.05) (Figure 3.4b). These included IL-8, VEGF-A, INHBA (Inhibin Subunit Beta A) and IL-6 (Supplementary Table 3.2). Pathway analysis of the significant gene list using Enrichr program[25] showed high concordance with our RNA-seq result indicating that RIPK2 could potentially be involved in essential pathways, including Epithelial-Mesenchymal Transition (EMT), NF-κB signaling, angiogenesis and IL-6/STAT3 signaling (Figure 3.4c). These changes indicate that RIPK2 plays an important role in regulating the tumor microenvironment secretome, which may explain its function in promoting tumorigenesis.

To confirm the protein array results, we used both RT-PCR and ELISA to measure IL-8 and IL-6 levels in SUM149 and SUM190 cells treated with and without the RIPK2 activator MDP (10 μg/μl for 24h). Relative to control cells, RIPK2 knockdown cells showed a decrease in *IL-8* and *IL-6* mRNA expression levels upon MDP stimulation (Figure 3.5a, Supp. Figure 3.4a). As a corollary, ELISA demonstrated that RIPK2 knockdown also reduced IL-8 and IL-6 secreted protein levels in MDP treated cells (Figure 3.5b, Supp. Figure 3.4b).

Protein array results also showed that RIPK2 inhibition reduced the levels of Activin A (INHBA; inhibin beta A). Activin and Inhibin are members of the TGF-β family of cytokines known to promote and suppress cancer metastasis respectively[38]. Activin A exists as a homodimer composed of two inhibin beta A subunits, while Inhibin A exists as a heterodimer consisting of inhibin alpha and beta A subunits. The alpha and beta A subunits share a 23-27% amino acid homology[39]. Many studies indicated the involvement of activin A in promoting cancer cell migration, invasion, and angiogenesis[40]. Effect on *INHBA* was confirmed by RT-PCR, where both SUM149 and SUM190 RIPK2 shRNA showed a decrease in *INHBA* relative to shRNA control (Figure 3.5c, Supp. Figure 3.4c). Like other cytokines, the activation of RIPK2 with MDP enhanced the difference in expression between control and knockout cells.

Contrary to Activin A, Inhibin alpha subunit (INHA), which forms inhibin A, functions as a tumor suppressor in many ovarian cancer studies [41] [42] [43]. Activin is regulated by inhibin as it blocks its binding to its receptor [40]. Thus, we wanted to examine whether INHA expression is affected by RIPK2 inhibition. Interestingly, both SUM149 and SUM190 RIPK2 shRNA showed an increase in *INHA* compared to shRNA control (Figure 3.5c, Supp. Figure 3.4b). The changes seen in mRNA expression were confirmed using ELISA, where CM from SUM149 RIPK2 shRNA showed a decrease of Activin A and an increase of inhibin A levels compared to control (Figure 3.5d).

The rescue of RIPK2 also confirmed secretome changes seen in the knockdown cells. RIPK2 rescue showed an increase of both *IL-8* and *IL-6* mRNA expression compared to KO control, and this was significantly seen with MDP stimulation. Similarly, serum-free media collected from RIPK2 rescue cells stimulated with MDP showed higher levels of IL-8 and IL-6 in contrast to KO control (Figure 3.5e-f). RIPK2 rescue also confirmed changes seen in both INHBA and INHA.

The re-expression of RIPK2 along with MDP activation showed an increase in *INHBA* and a decrease in *INHA* relative to KO control. Changes seen in mRNA expression were confirmed using ELISA: RIPK2 rescue cells showed an increase of Activin A and a decrease of inhibin A levels compared to KO control upon MDP stimulation (Figure 3.5g-h).

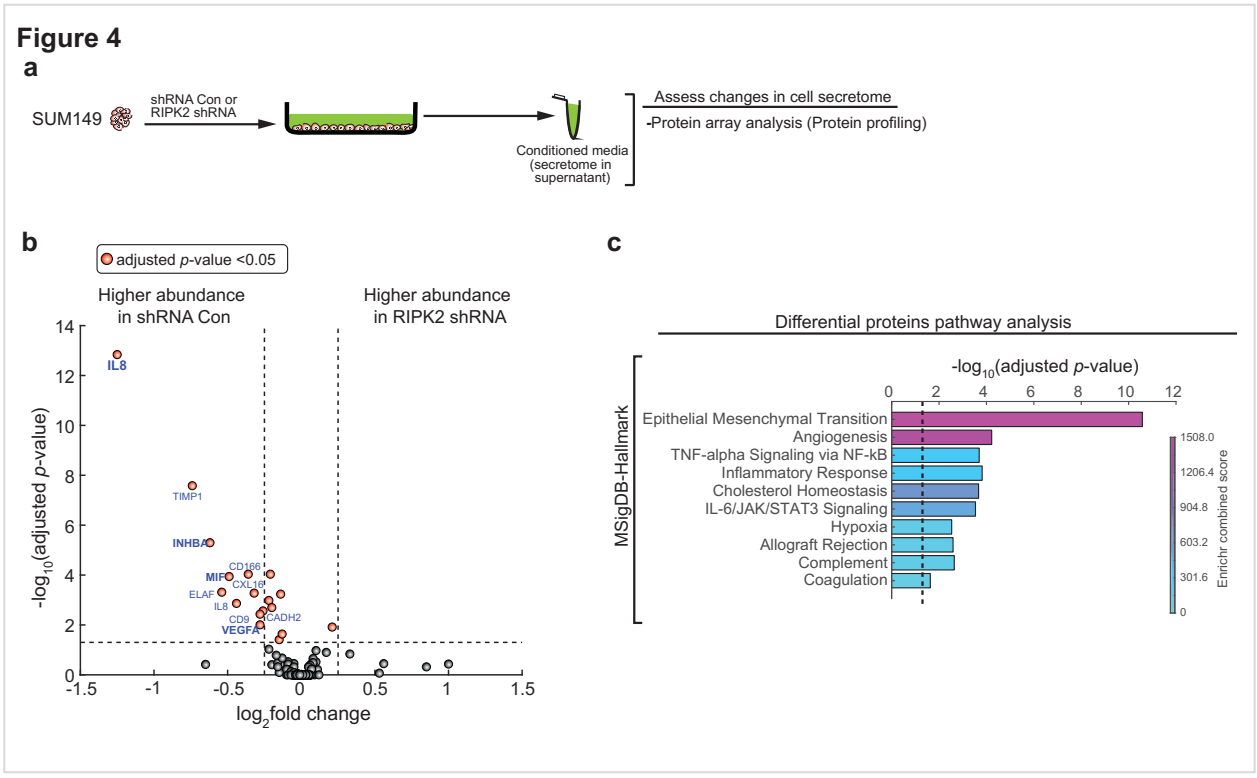

Figure 3.4 Protein array analysis confirms RIPK2 involvement in inflammatory pathways and cytokine production. (**a)** A schematic overview of the experimental design used for the analysis. **(b)** Volcano plot visualizes the changes in proteins abundance in RIPK2 knockdown compared to control in SUM149 cells (see Supplementary Table 3.1). The horizontal dotted line indicates the significance level of adjusted p-value = 0.05, while vertical dotted lines show Log $_2$ fold change cutoff. Proteins with a negative log $_2$ FC value have a higher abundance in shRNA control, while proteins with positive log $_2$ FC are higher in RIPK2 shRNA. **(c)** MSigDB pathway enrichment analysis of the genes representing the differential proteins in the array. Differential genes identified included proteins involved in important pathways such as Epithelial-mesenchymal transition (IL-6, IL-8, CDH2, CXCL1, ITGAV, INHBA, TIMP1, VEGFA), NF-κB signaling (IL-6, CXCL1,

INHBA, VEGFA), angiogenesis (ITGAV, TIMP1, VEGFA), IL-6/STAT3 signaling (IL-6, CD9, CXCL1) and inflammatory response (IL-6, IL-8, INHBA, TIMP1).

Figure 5

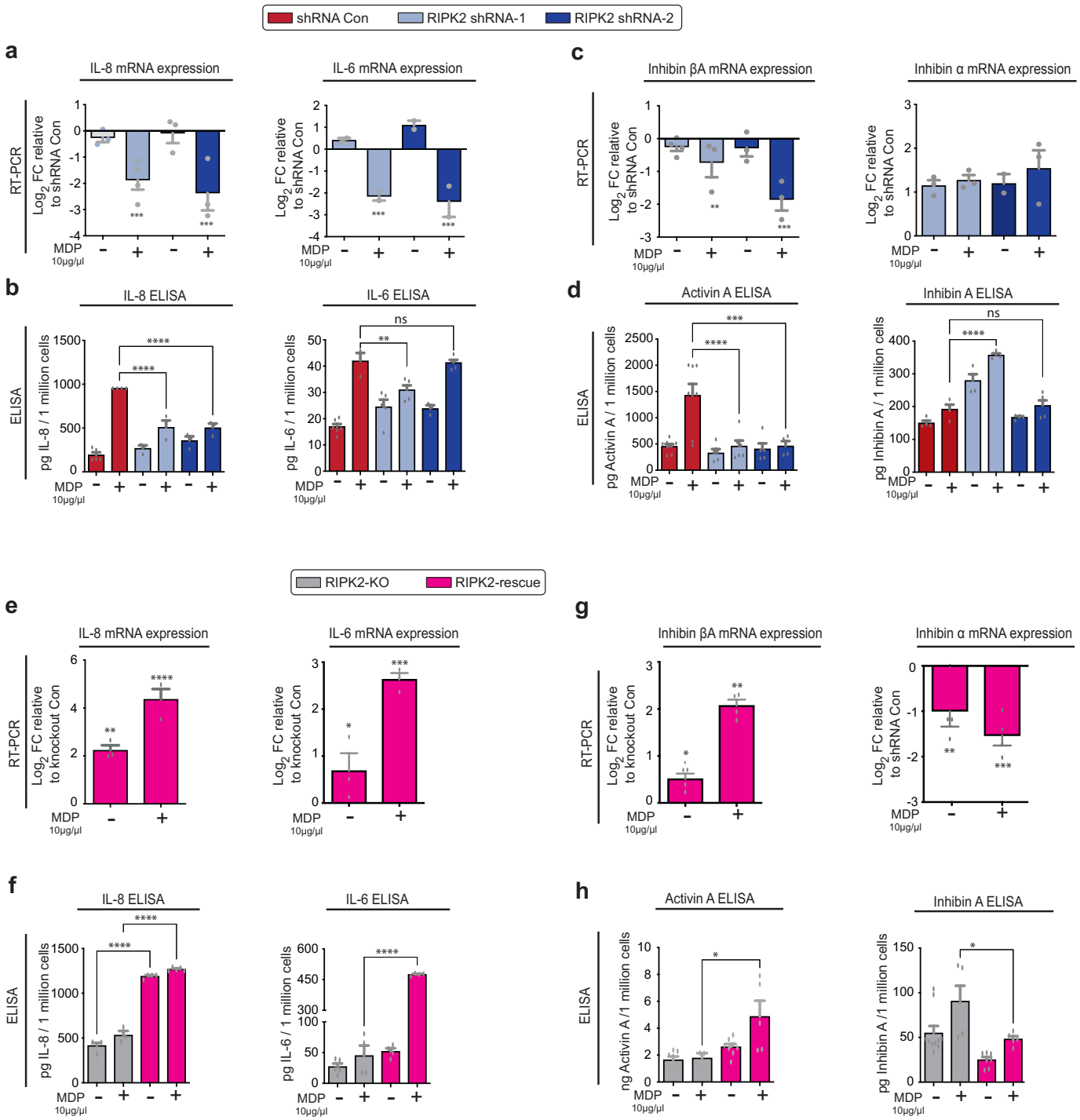

Figure 3.5 RIPK2 regulates cytokines levels in IBC cells. Real Time RT-PCR was used to measure the mRNA expression level of **(a)** *IL-8* and *IL-6* **(c)** *INHBA* and *INHA* in SUM149 shRNA control, RIPK2 shRNA-1 and shRNA-2. Cells are treated with and without MDP (10 μg/μl) for 24h before RNA collection. mRNA expression was normalized to GAPDH. Bars represent mean log_2 fold change ± SD relative to shRNA control. Each dot represents a biological replicate. ELISA was used to measure the secreted protein levels of (**b)** IL-8 and IL-6 **(d)** Activin A and Inhibin A in SUM149 shRNA control, RIPK2 shRNA-1 and shRNA-2. Cells are treated with and without MDP (10 μg/μl) for 24h prior to supernatant collection. Bars represent mean change ± SD. Each dot represents a biological replicate. mRNA expression level of **(e)** *IL-8* and *IL-6* **(g)** *INHBA* and *INHA* in SUM149 knockout, RIPK2 rescue cells. Cells are treated with and without MDP (10 μg/μl) for 24h before RNA collection. mRNA expression was normalized to GAPDH. Bars represent mean log_2 fold change ± SD relative to knockout control. Each dot represents a biological replicate. Secreted protein levels of **(f)** IL-8 and IL-6 (**h)** Activin A and Inhibin A in SUM149 knockout and RIPK2 rescue cells. Cells are treated with and without MDP (10 μg/μl) for 24h before supernatant collection. Bars represent mean change ± SD. Each dot represents a biological replicate. Significance was determined using one-way ANOVA and represented by asterisks (*, $p<0.05$) (**, $p<0.005$) (***, $p<0.0005$) (****, $p<0.00005$)

3.4.6 NF-κB expression confirms changes in secretome seen in RIPK2 rescue cells

To fully confirm RIPK2 regulation of cytokine production is mediated through NF-κB signaling, we transduced NF-κB plasmid into the RIPK2 KO clone (Figure 3.6a), and using RT-PCR, we examined the expression level of *IL-8*, *IL-6*, *INHBA*, and *INHA* (Figure 3.6b). Indeed, *IL-8*, *IL-6*, and *INHBA* expression levels increased in NF-κB expressed cells which align with our previous results. *INHA* expression was slightly increased in NF-κB expressed cells, while RIPK2 rescue cells showed a decrease in expression. This suggests that RIPK2 might regulate INHA through other pathways beside NF-κB.

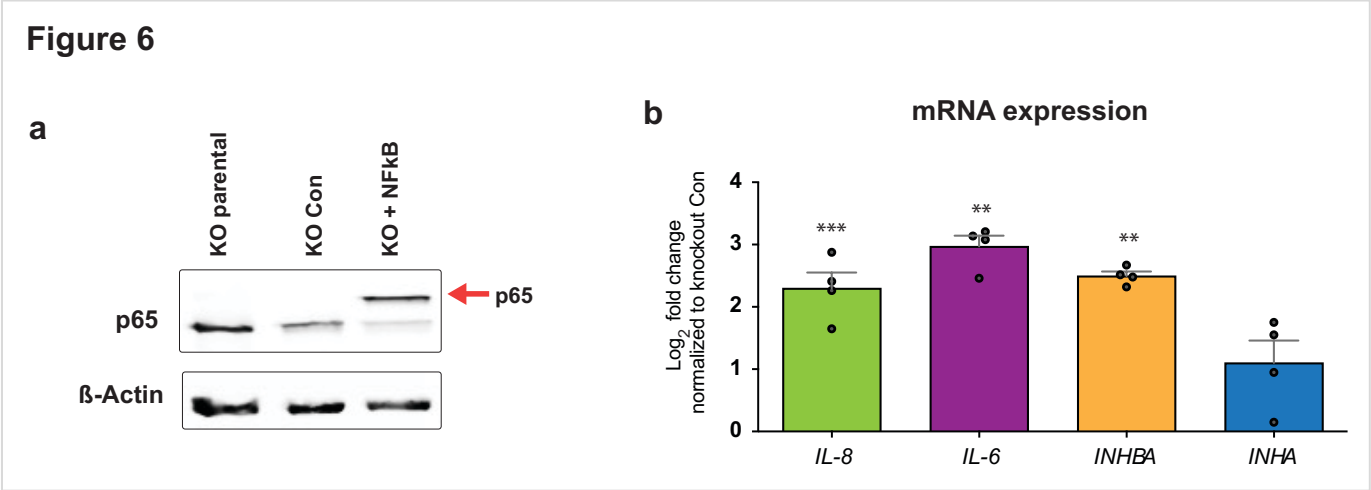

Figure 3.6 NF-κB overexpression in SUM149 KO cells confirms cytokine changes. **(a)** Western blot was used to confirm NF-κB overexpression using NF-κB p65 subunit. **(b)** RT-PCR is used to measure *IL-8, IL-6, INHBA,* and *INHA* mRNA expression levels in NF-κB overexpressed cells. mRNA expression was normalized to GAPDH. Bars represent mean $\log_2$ fold change ± SD relative to RIPK2 knockout control. Each dot represents a biological replicate. Significance was determined using one-way ANOVA and represented by asterisks (**, $p<0.005$) (***, $p<0.0005$).

3.4.7 RIPK2 rescue increase metastases in animal model

RIPK2 regulation of migration and proliferation was evident in the knockdown study, thus, we sought to examine RIPK2 function *in vivo* using tail vein injection in NOD SCID gamma mouse model. SUM149 RIPK2 KO control, and RIPK2 rescue cells (5 × 105 cells/mouse) were injected, and mice were observed weekly up to 12 weeks (Figure 3.7a). The average body weight showed no change throughout the study (Figure 3.7b). However, immunohistochemical analyses of HLA (Human leukocyte antigen) in lung sections showed a significant increase in the number of tumor colonies found in mice injected with RIPK2 rescue cells compared to mice injected with RIPK2 KO control (Figure 3.7c-d), suggesting the important role of RIPK2 in metastases.

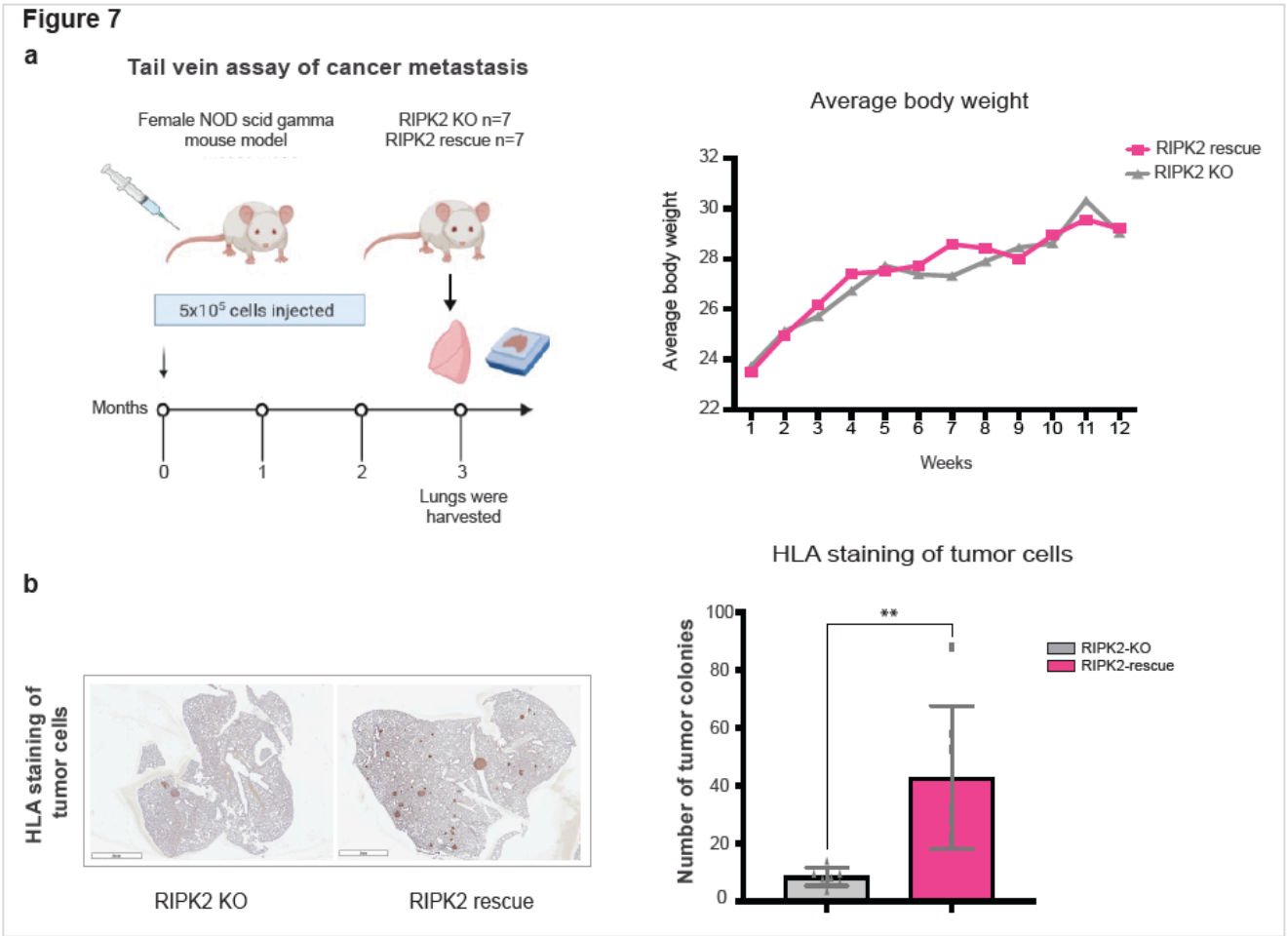

Figure 3.7 RIPK2 rescue cells increased metastasis in animal modal. **(a)** A schematic overview of the experimental design of RIPK2 knockout and rescue cells tail vein assay. Cells were injected into tail vein (5×10^5 cells/mouse) of 6–8-week-old NOD SCID gamma female mice. Seven mice are used for control and study groups (b) The average body weight of each group of mice (RIPK2 KO and rescue) is measured weekly over three months. (c) HLA (Human leukocyte antigen) antibody staining of tumor cells in lung samples collected from each group. Scale bar=2mm (d) The number of tumor colonies in harvested lung samples. Four lung sections per mouse were stained with HLA antibody. Bars represent mean ± SD. Each dot represents a biological replicate. Significance was determined using one-way ANOVA and represented by asterisks (**, $p<0.005$).

3.5 Discussion

IBC is the most aggressive type of breast cancer characterized by a low survival rate compared to non-IBC [44] [45]. 20-30% of IBC patients present with metastases at the initial diagnosis leading to worse clinical outcomes [46]. What drives IBC aggressive behavior remains elusive, especially since the genomic profile of the disease is similar to that of non-IBC. More research reveals the importance of TME cellular and non-cellular components in IBC aggressiveness. Indeed, inflammatory signaling pathways and cytokines are described as factors contributing to IBC metastatic behavior [1].

Cytokines can maintain an inflammatory environment favoring cancer development and progression by nurturing tumor cells' propensity to survive and thrive [47] [48]. This function is attained by regulating autocrine and paracrine signals [49]. Cytokines are regulated through several inflammatory pathways, most importantly, NF-κB [10]. The role of NF-κB in tumorigenesis is well

documented [50]. In IBC, NF-κB and its target genes are upregulated; however, its direct effect is unknown [1 15].

In our study, we characterize the role of RIPK2 in mediating IBC TME through regulating NF-κB activity and secretome production. We demonstrate that RIPK2 promotes NF-κB nuclear translocation, resulting in the increased production of IL-8, IL-6 and Activin A. As a corollary, a decrease in proliferation, migration, and angiogenesis of IBC cells was seen when RIPK2 was knocked down. *In vivo*, mice injected with RIPK2 rescue cells showed significantly more metastasis than those injected with RIPK2 KO control cells.

NF-κB promotes the expression of pro-inflammatory cytokines including IL-8 and IL-6 [51 52] and its activation can be regulated by RIPK2[16]. The inhibition of IL-8 with siRNA decreased the expression of RIPK2 and its upstream receptor NOD1, suggesting that IL-8 can induce RIPK2 signal [18]. Alternately, RIPK2 inhibition and knockout show a significant decrease in IL-8 production, while RIPK2 re-expression restored IL-8 to WT levels [53]. These results suggest that RIPK2-IL-8 signals through a positive feedback loop. Further, RIPK2 activation through innate immune receptors such as NODs and TLRs leads to the activation of NF-κB and subsequently the release of IL-6 [54 55 56]. Macrophages isolated from RIPK2-deficient mice show a decrease in IL-6 levels upon LPS stimulation [54].

Activin A is a cytokine member of the TGF-β superfamily that can be induced through NF-κB in different types of cancers [57 58 59]. A study showed that TNF activation of NF-κB induced EMT through the upregulation of Activin A (INHBA) [57]. In prostate cancer, Activin A induction increases stem-like cells ($ALDH^{hi}$), proliferation, invasion, and cell clonogenicity through the activation of SMAD2. Corresponding with our results, induced stimulation of NF-κB using TNFα

increased the level of p65 phosphorylation and the expression of pro-inflammatory cytokines GM-CSF and IL-8. Further, IL-8 stimulation caused a significant increase in the levels of INHBA [58]. In a dose-dependent response experiment, mice injected with Activin A show an increase of IL-6 but not TNF nor IL-1β [60]. Activin A promotes IL-6 expression in an autocrine manner in ovarian cancer cells through NF-κB and p38 MAPK [61]. This autocrine regulation of Activin A is also seen in liver and rat Sertoli cell [62] [63]. Inhibin A is an endogenous antagonist of Activin signaling and is made up of the heterodimer; α-subunit (INHA) and β-subunits (INHBA)[64]. The role of Inhibin A in cancer is contradictory; however, many studies show that it acts as a tumor suppressor [65]. Studies have indicated that the α-subunit (INHA) is mutated in different types of cancer [66] [67] and this mutation correlates with its low expression [68]. Sertoli cells stimulated with LPS show a significant increase in Activin and a decrease in Inhibin B (similar to Inhibin A but with a βb subunit) in CM [63], which agrees with our results seen in IBC cells.

The RNA-seq results showed NF-κB genes were upregulated in RIPK2 rescue cells, including *NFKB1, NFKB2, RELA, RELB, NFKBIA*. The same set of genes were found overexpressed in IBC compared to non-IBC tumor samples[15]. Genes involved in RIPK2 regulation include *BIRC2*[32], *BIRC3*[32], *MYD88*[32], *TNFAIP3*[69], *PELI3*[70], *TRAF1*[16], *TRAF2*[71], *TRAF3*[72], *TAB3*[73], *CYLD*[69], and *ASK-1*[74] were also overexpressed. To our surprise, most of the genes involved in the MAPK pathway showed no significant change in expression, including *AP-1* (Transcription Factor AP-1), confirming that the cytokine expression changes seen in RIPK2 constructs were mediated through NF-κB pathway. The upregulation of NF-κB mediated by RIPK2 increased the expression of many cytokines involved in cancer proliferation, metastasis, and progression. Most important upregulated cytokines include interleukins *IL-5, IL-36G, IL36B, IL-17,* and *IL-23A*, and chemokine *CXCL8 (IL-8), CXCL1, CXCL2., CXCL3, CXCL5,* and *CXCL10*. In addition,

chemokines ligands include *CCL2, CCL5,* and *CLL20*. Colony-stimulating factor cytokines such as *CSF-1* and *CSF-2* and transforming growth factor-beta (TGF-β) such as *TGFBI* and *TGFB3* were also upregulated. To maintain autocrine stimulation, cancer cells will express cytokine receptors to act as a positive feedback loop [75]. Hence, several types of receptors were upregulated, including immunoglobulin superfamily receptors such as *IL1R1, IL1R2*, *IL6R,* and *CSF1R*, chemokine receptors such as *CXCR2*, interferon receptors such as *IFNAR1* and *IFNGR1,* and TGF-β receptors such as *TGFBR3* and *TNFRSF8*.

Taken together, this study helps us understand how IBC may use a RIPK2-NF-κB mediated cytokine signaling pathway in order to modulate the TME and to spread. IBC aggressiveness is attributable mainly to its early and rapid metastases, which is driven by the increase of cytokine and chemokine in the TME[1]. RIPK2 can promote IBC cell proliferation, metastasis and angiogenesis through the regulation of important cytokines such as IL-8, IL-6, Activin A and Inhibin A. This is the first study that showed the direct effect of NF-κB on IBC cells through RIPK2 regulation of cytokine production. Accordingly, targeting inflammatory mediators such RIPK2 can be a promising treatment for IBC patients.

3.6 Supplementary Material

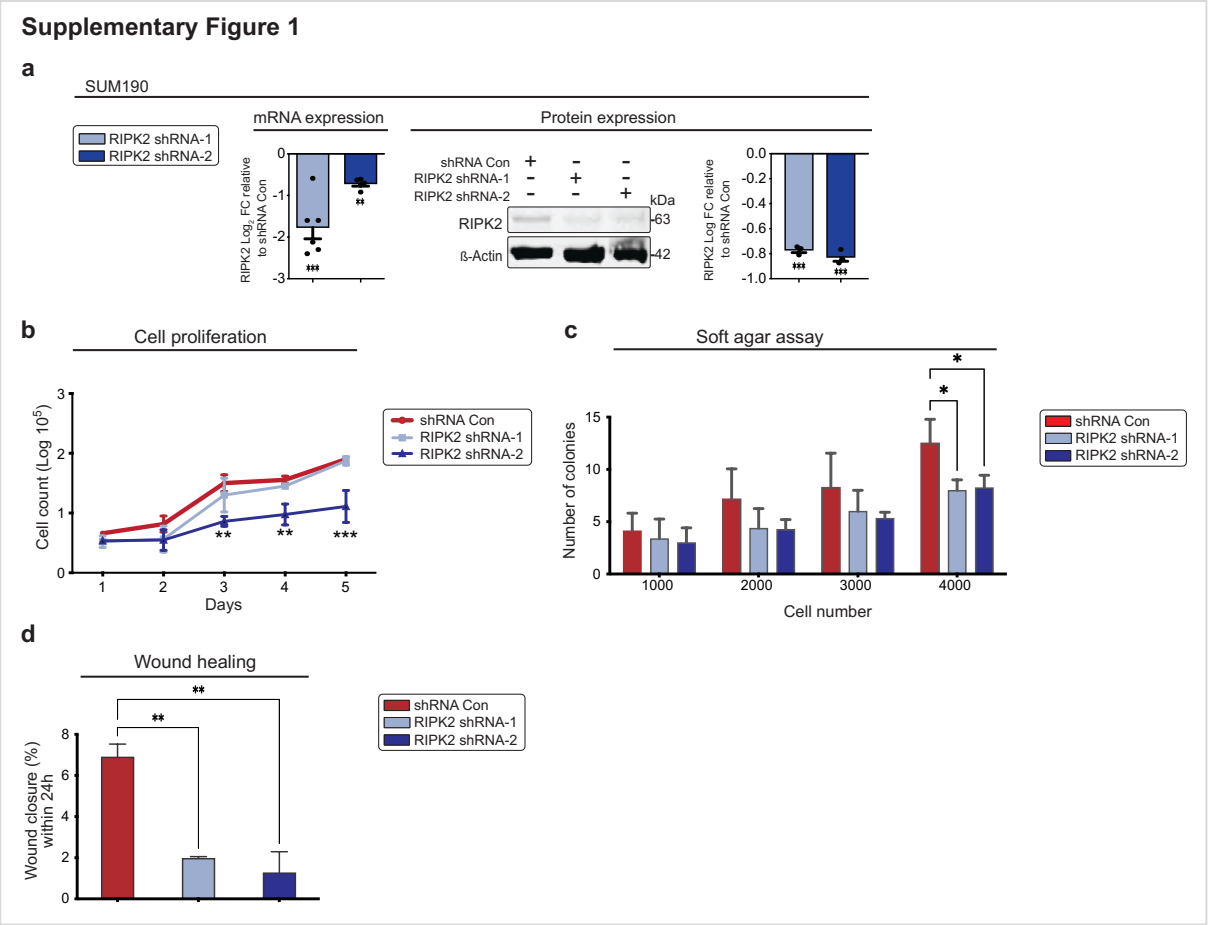

Figure 3.S1. Phenotypic changes associated with RIPK2 inhibition SUM190 IBC cells. **(a)** Confirmation of RIPK2 knocked down using two shRNA (shRNA -1 targets exon 4 and shRNA-2 targets exon 3). Real Time RT-PCR and Western blot (WB) were used to confirm RIPK2 expression change in SUM190. *RIPK2* mRNA expression was normalized to GAPDH and plotted as $\log_2$ fold change over shRNA control. WB bands were quantified and normalized to β-Actin. Bars represent mean fold change ± SD relative to shRNA control. Each dot represents a biological replicate. Significance was determined using one-way ANOVA. **(b)** SUM190 shRNA control and SUM190 RIPK2 shRNA-1 and -2 growth rate. Duplicate wells of each group were counted daily for six days using Trypan blue, which allows exclusion of nonviable cells [31]. Significance was determined using two-way ANOVA. **(c)** Soft agar colony formation assay. SUM149 shRNA control and RIPK2 shRNA-1 and -2 cells were plated at different concentrations and grown for 8

weeks (n=3). The number of colonies is counted manually after staining with crystal violet. Bars represent mean ± SD relative. **(d)** Wound healing assay. Wound closure was monitored by taking pictures after 6, 12, and 24 hours using a phase-contrast microscope(n=3). An increase in the percentage of wound closure indicated the rate of cell migration. Bars represent mean of wound closure percentage ± SD. Significance was determined using two-way ANOVA. Significance for all plots is represented by asterisks (*, $p<0.05$) (**, $p<0.005$) (***, $p<0.0005$).

Supplementary Figure 2

Figure 3.S2. A schematic overview of the RIK2 indel. Ten colonies were sequenced after TOPO cloning to confirm indel alignment with crRNA (guided RNA) in exon 3.

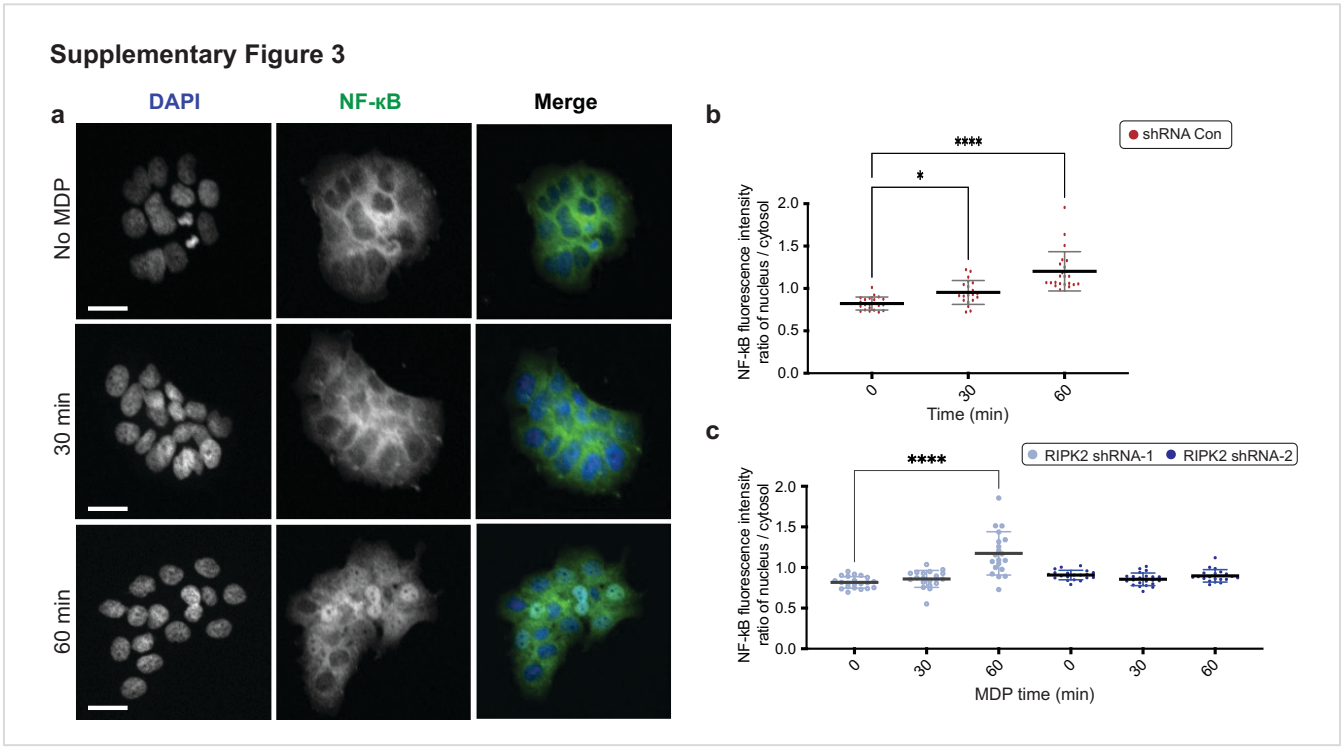

Figure 3.S3. RIPK2 regulation of NF-κB nuclear translocation (activation). **(a)** Representative images of NF-κB nuclear translocation in shRNA control cells treated with MDP (10 μg/μl) for 0, 30 and 60 min. Scale bars = 50 μm. Immunofluorescence staining is used to measure NF-κB nuclear translocation by calculating the NF-κB fluorescence intensity ratio of the nucleus over cytosol. **(b)** NF-κB nuclear translocation in SUM190 shRNA control **(c)** RIPK2 shRNA-1 and shRNA-2 stimulated with MDP (10 μg/μl) for 0, 30 and 60 min. Bars represent mean ± SD. Each dot represents a cell. Significance was determined using one-way ANOVA and represented by asterisks (***, $p<0.0005$) (****, $p<0.00005$)

Supplementary Figure 4

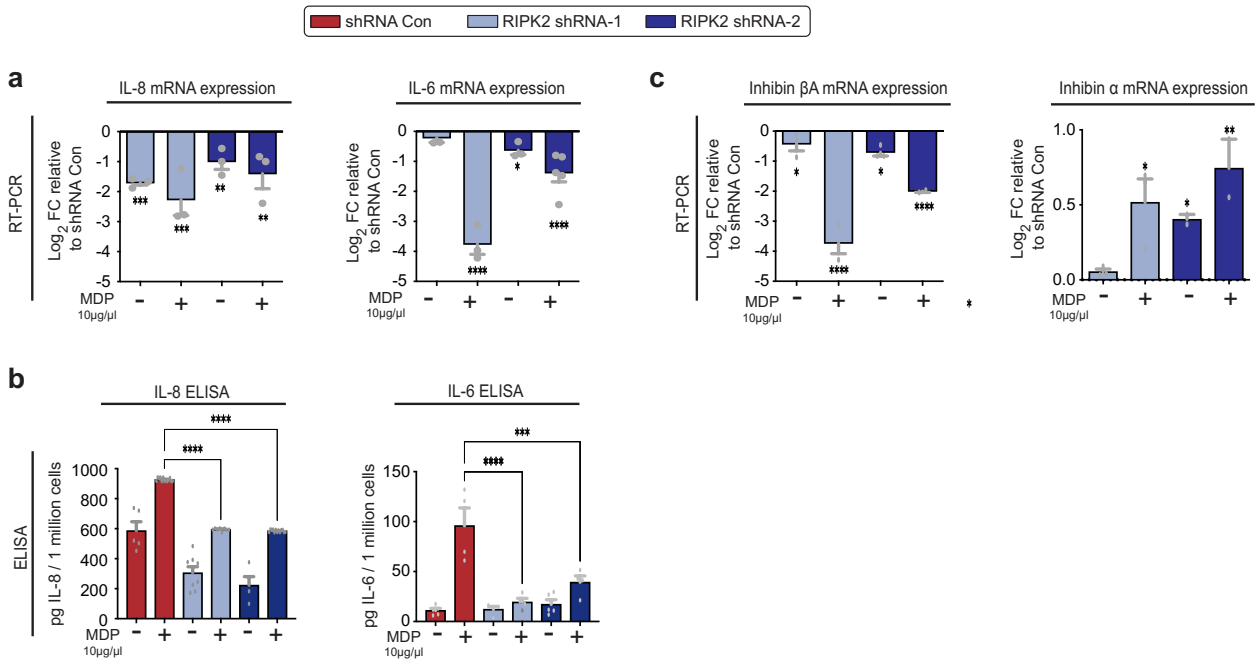

Figure 3.S4. RIPK2 regulates cytokines levels in IBC cells. Real Time RT-PCR was used to measure the mRNA expression level of **(a)** *IL-8* and *IL-6* **(c)** *INHBA* and *INHA* in SUM190 shRNA control, RIPK2 shRNA-1 and shRNA-2. Cells are treated with and without MDP (10 µg/µl) for 24h before RNA collection. mRNA expression was normalized to GAPDH. Bars represent mean log_2 fold change ± SD relative to shRNA control. Each dot represents a biological replicate. ELISA was used to measure the secreted protein levels of (**b)** IL-8 and IL-6 in SUM190 shRNA control, RIPK2 shRNA-1 and shRNA-2. Cells are treated with and without MDP (10 µg/µl) for 24h prior to supernatant collection. Bars represent mean change ± SD. Each dot represents a biological replicate. Significance was determined using one-way ANOVA and represented by asterisks (*, $p<0.05$) (**, $p<0.005$) (***, $p<0.0005$) (****, $p<0.00005$)

Table 3.S1. Protein profiling analysis of conditioned media from RIPK2 knockdown versus RIPK2 control samples.

ID	Name	uniprot.id	uniprot.gene	logFC	AveExpr	adj.P.Val
ab2312	IL8	P10145	CXCL8	-1.25	14.40	0.00
ab1842	TIMP1	P01033	TIMP1	-0.74	15.21	0.00
ab2176	INHBA	P08476	INHBA	-0.62	15.25	0.00
ab2246	CD166	Q13740	ALCAM	-0.36	13.89	0.00
ab1822	IL6	P05231	IL6	-0.21	12.20	0.00
ab1816	MIF	P14174	MIF	-0.49	14.20	0.00
ab1713	ELAF	P19957	PI3	-0.54	13.53	0.00
ab2515	CXL16	Q9H2A7	CXCL16	-0.32	12.90	0.00
ab1471	TFR1	P02786	TFRC	-0.14	12.80	0.00
ab1506	IgE			-0.22	12.12	0.00
ab1643	IL8	P10145	CXCL8	-0.44	12.53	0.00
ab1497	IL6	P05231	IL6	-0.20	13.06	0.00
ab2760	CADH2	P19022	CDH2	-0.26	12.67	0.00
ab1378	CD9	P21926	CD9	-0.28	12.93	0.00
ab1603	VEGFA	P15692	VEGFA	-0.28	12.93	0.01
ab1582	TBB3	Q13509	TUBB3	0.21	14.47	0.01
ab1567	ITAV	P06756	ITGAV	-0.13	13.01	0.02
ab2004	GROA	P09341	CXCL1	-0.15	12.65	0.04

Table 3.S2. Key resources Table:

Primer and probe name	Sequance	Source
crRNA-1 (Antisense)	GTGCAGGTAATTTACACCAA NGG	IDT
NHEJ-HEX probe-1	TAATTTACACCAAGGGCAATTTC	Thermo Fisher
Reference-FAM probe-1	TCTGAGTCTTCAAGTCATGATGA	Thermo Fisher
Forword primer 1	GGAGAGACATGAAATTGGCTAGGTCG	IDT
Reverse primer 1	CCTGATGTTGCTTGGCCATT	IDT
crRNA-2 (Antisense)	GCTACTTCGTGACTGTGAGA NGG	IDT
NHEJ-HEX probe-2	TGACTGTGAGAGGGACAT	Thermo Fisher
Reference-FAM probe-2	ATAATTGTCCCTCCTTCTGGTG	Thermo Fisher
Forword primer 2	GCCCTTGATTTTTGTCCAGG	IDT
Reverse primer 2	GCTCTTGTCCCTTACAGATTGC	IDT
Universal M13 Reverse	CAGGAAACAGCTATGAC	Thermo Fisher
ShRNA plasmid	Sequance	Source
pLKO.1-puro-U6-TRCN0000006348	CCGGGCCAGTATCAAGCACGATATACTC	Sigma Aldrich
pLKO.1-puro-U6-TRCN0000006350	CCGGGCACAATATGACTCCTCCTTTCTC	Sigma Aldrich
pLKO.1-puro shRNA Control	CCGGCAACAAGATGAAGAGCACCAACTC	Sigma Aldrich
Plasmid name	Vector	Source
RIP2 (RIPK2) (NM_003821) Human Tagged ORF Clone	pLenti-C-Myc-DDK-P2A-Puro	ORIGENE
NF-kB p65 (RELA) (NM_021975) Human Tagged ORF Clone	pLenti-C-Myc-DDK-P2A-Puro	ORIGENE
pLenti-C-Myc-DDK Lentiviral Gene Expression Vector	pLenti-C-Myc-DDK-P2A-Puro	ORIGENE
Probe name	Exon Boundary	Source
TaqMan™ Gene Expression Assay (FAM) CXCL8 - Hs00174103_m1	1 to 2	Thermo
TaqMan™ Gene Expression Assay (FAM) IL-6 - Hs00174131_m1	3 to 4	Thermo
TaqMan™ Gene Expression Assay (FAM) RIPK2 - Hs01572684_m1	3 to 4	Thermo
TaqMan™ Gene Expression Assay (FAM) GAPDH - Hs99999905_m1	2	Thermo
TaqMan™ Gene Expression Assay (FAM) INHA - Hs00171410_m1	1 to 2	Thermo
TaqMan™ Gene Expression Assay (FAM) INHBA - Hs01081598_m1	2 to 3	Thermo
TaqMan™ Gene Expression Assay (FAM) VEGFA - Hs00900055_m1	3 to 4	Thermo
Antibody	Dilution	Source
NF-κB p65 (D14E12) #8242	1 to 1000 (WB)	Cell signaling
phospho-RIP2 (E1I9J) #14397	1 to 800	Cell signaling
Phospho-NF-κB p65 (93H1) #4887	1 to 1000	Cell signaling
NF-κB p65 (D14E12) #8242	1 to 200 (IF)	Cell signaling
Alexa Fluor® 594 Conjugate anti-Rabbit IgG	1 to 500	Cell signaling
RICK (A-10)	1 to 1000	Santa Cruz
PCNA Antibody (PC10)	1 to 1000	Santa Cruz
Actin (AC-15)	1:15000	Santa Cruz

REFERENCES

1. Lim, B., Woodward, W. A., Wang, X., Reuben, J. M. & Ueno, N. T. Inflammatory breast cancer biology: the tumour microenvironment is key. *Nature Reviews Cancer* **18**, (2018).

2. Rosenbluth, J. M. & Overmoyer, B. A. Inflammatory Breast Cancer: a Separate Entity. *Current Oncology Reports* **21**, (2019).

3. Woodward, W. A. Inflammatory breast cancer: Unique biological and therapeutic considerations. *The Lancet Oncology* **16**, (2015).

4. L. Ferreira, V., H.L. Borba, H., de F. Bonetti, A., P. Leonart, L. & Pontarolo, R. Cytokines and Interferons: Types and Functions. in *Autoantibodies and Cytokines* (2019). doi:10.5772/intechopen.74550

5. Di Bonito, M., Cantile, M. & Botti, G. Pathological and molecular characteristics of inflammatory breast cancer. *Transl. Cancer Res.* **8**, (2019).

6. Bièche, I. *et al.* Molecular profiling of inflammatory breast cancer: Identification of a poor-prognosis gene expression signature. *Clin. Cancer Res.* **10**, (2004).

7. Wolfe, A. R. *et al.* Mesenchymal stem cells and macrophages interact through IL-6 to promote inflammatory breast cancer in pre-clinical models. *Oncotarget* **7**, (2016).

8. Debeb, B. G. *et al.* Pre-clinical studies of Notch signaling inhibitor RO4929097 in inflammatory breast cancer cells. *Breast Cancer Res. Treat.* **134**, (2012).

9. Mohamed, H. T. *et al.* IL-8 and MCP-1/CCL2 regulate proteolytic activity in triple negative inflammatory breast cancer a mechanism that might be modulated by Src and Erk1/2. *Toxicol. Appl. Pharmacol.* **401**, (2020).

10. Liu, T., Zhang, L., Joo, D. & Sun, S. C. NF-κB signaling in inflammation. *Signal Transduction and Targeted Therapy* **2**, (2017).

11. do Monte-Neto, R. L. *et al.* Gene expression profiling and molecular characterization of antimony resistance in Leishmania amazonensis. *PLoS Negl. Trop. Dis.* **5**, (2011).

12. Van Den Eynden, G. G. *et al.* Overexpression of caveolin-1 and -2 in cell lines and in human samples of inflammatory breast cancer. *Breast Cancer Res. Treat.* **95**, (2006).

13. Nguyen, D. M. *et al.* Molecular heterogeneity of inflammatory breast cancer: A hyperproliferative phenotype. *Clin. Cancer Res.* **12**, (2006).

14. Van Laere, S. J. *et al.* Nuclear factor-κB signature of inflammatory breast cancer by cDNA microarray validated by quantitative real-time reverse transcription-PCR, immunohistochemistry, and nuclear factor-κB DNA-binding. *Clin. Cancer Res.* **12**, 3249–3256 (2006).

15. Lerebours, F. *et al.* NF-kappa B genes have a major role in Inflammatory Breast Cancer. *BMC Cancer* **8**, (2008).

16. McCarthy, J. V, Ni, J. & Dixit, V. M. RIP2 is a novel NF-kappaB-activating and cell death-inducing kinase. *J. Biol. Chem.* **273**, 16968–75 (1998).

17. Nachbur, U. *et al.* A RIPK2 inhibitor delays NOD signalling events yet prevents inflammatory cytokine production. *Nat. Commun.* **6**, 6442 (2015).

18. Chan, L. P. *et al.* IL-8 promotes HNSCC progression on CXCR1/2-meidated NOD1/ RIP2 signaling pathway. *Oncotarget* **7**, (2016).

19. Azad, A. K. *et al.* Inactivation of endothelial cell phosphoinositide 3-kinase β inhibits tumor angiogenesis and tumor growth. *Oncogene* **39**, (2020).

20. Githaka, J. M. *et al.* BAD regulates mammary gland morphogenesis by 4E-BP1-mediated control of localized translation in mouse and human models. *Nat. Commun.* **12**, (2021).

21. Coralville, I. D. T. Alt-R CRISPR-Cas9 System. *Integrated DNA Technologies* (2017).

22. Chen, S., Zhou, Y., Chen, Y. & Gu, J. Fastp: An ultra-fast all-in-one FASTQ preprocessor. in *Bioinformatics* **34**, (2018).

23. Dobin, A. *et al.* STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, (2013).

24. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, (2014).

25. Kuleshov, M. V. *et al.* Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res.* **44**, (2016).

26. Githaka, J. M. Molnupiravir Does Not Induce Mutagenesis in Host Lung Cells during SARS-CoV-2 Treatment. *Bioinform. Biol. Insights* **16**, 117793222210850 (2022).

27. Kim, S. *et al.* Strelka2: fast and accurate calling of germline and somatic variants. *Nat. Methods* **15**, (2018).

28. Cingolani, P. *et al.* A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. *Fly (Austin).* **6**, (2012).

29. Kim, S.-W., Roh, J. & Park, C.-S. Immunohistochemistry for Pathologists: Protocols, Pitfalls, and Tips. (2016). doi:10.4132/jptm.2016.08.08

30. Wright, W. E. *et al.* A Targeted RNAi Screen of the Breast Cancer Genome Identifies KIF14 and TLN1 as Genes That Modulate Docetaxel Chemosensitivity in Triple-Negative Breast Cancer. *Clin. Cancer Res.* **19**, 2061–2070 (2013).

31. Li, D. *et al.* RIPK2 is an unfavorable prognosis marker and a potential therapeutic target in human kidney renal clear cell carcinoma. *Aging (Albany. NY).* **13**, (2021).

32. Yang, Q., Tian, S., Liu, Z. & Dong, W. Knockdown of RIPK2 Inhibits Proliferation and Migration, and Induces Apoptosis via the NF-κB Signaling Pathway in Gastric Cancer. *Front. Genet.* **12**, (2021).

33. Yang, Y. *et al.* NOD2 pathway activation by MDP or Mycobacterium tuberculosis infection involves the stable polyubiquitination of Rip2. *J. Biol. Chem.* (2007). doi:10.1074/jbc.M703079200

34. Abbott, D. W., Wilkins, A., Asara, J. M. & Cantley, L. C. The Crohn's disease protein,

NOD2, requires RIP2 in order to induce ubiquitinylation of a novel site on NEMO. *Curr. Biol.* (2004). doi:10.1016/j.cub.2004.12.032

35. Moreno, R., Sobotzik, J. M., Schultz, C. & Schmitz, M. L. Specification of the NF-κB transcriptional response by p65 phosphorylation and TNF-induced nuclear translocation of IKKε. *Nucleic Acids Res.* **38**, (2010).

36. Inohara, N. *et al.* Host recognition of bacterial muramyl dipeptide mediated through NOD2: Implications for Crohn's disease. *J. Biol. Chem.* (2003). doi:10.1074/jbc.C200673200

37. Chamaillard, M. *et al.* An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat. Immunol.* (2003). doi:10.1038/ni945

38. Seachrist, D. D. & Keri, R. A. The Activin Social Network: Activin, Inhibin, and Follistatin in Breast Development and Cancer. *Endocrinology* **160**, (2019).

39. Robertson, D. M., Burger, H. G. & Fuller, P. J. Inhibin/activin and ovarian cancer. *Endocrine-Related Cancer* **11**, (2004).

40. Ries, A. *et al.* Activin A: an emerging target for improving cancer treatment? *Expert Opinion on Therapeutic Targets* (2020). doi:10.1080/14728222.2020.1799350

41. Matzuk, M. M. *et al.* Transgenic models to study the roles of inhibins and activins in reproduction, oncogenesis, and development. *Recent Prog. Horm. Res.* **51**, (1996).

42. Coerver, K. A. *et al.* Activin Signaling through Activin Receptor Type II Causes the Cachexia-Like Symptoms in Inhibin-Deficient Mice. *Mol. Endocrinol.* **10**, (1996).

43. Cipriano, S. C., Lei, C., Kumar, T. R. & Matzuk, M. M. Follistatin is a modulator of gonadal tumor progression and the activin-induced wasting syndrome in inhibin-deficient mice. *Endocrinology* **141**, (2000).

44. Schlichting, J. A., Soliman, A. S., Schairer, C., Schottenfeld, D. & Merajver, S. D. Inflammatory and non-inflammatory breast cancer survival by socioeconomic position in the Surveillance, Epidemiology, and End Results database, 1990-2008. *Breast Cancer Res.*

Treat. **134**, (2012).

45. Fouad, T. M. *et al.* Inflammatory breast cancer: a proposed conceptual shift in the UICC–AJCC TNM staging system. *The Lancet Oncology* **18**, (2017).

46. Fouad, T. M. *et al.* Overall survival differences between patients with inflammatory and noninflammatory breast cancer presenting with distant metastasis at diagnosis HHS Public Access. *Breast Cancer Res Treat* **152**, 407–416 (2015).

47. Ribatti, D. The concept of immune surveillance against tumors. The first theories. *Oncotarget* **8**, (2017).

48. Lu, H., Ouyang, W. & Huang, C. Inflammation, a key event in cancer development. *Molecular Cancer Research* **4**, (2006).

49. Kartikasari, A. E. R., Huertas, C. S., Mitchell, A. & Plebanski, M. Tumor-Induced Inflammatory Cytokines and the Emerging Diagnostic Devices for Cancer Detection and Prognosis. *Frontiers in Oncology* **11**, (2021).

50. Xia, Y., Shen, S. & Verma, I. M. NF-κB, an active player in human cancers. *Cancer immunology research* **2**, (2014).

51. Singh, J. K. *et al.* Targeting CXCR1/2 significantly reduces breast cancer stem cell activity and increases the efficacy of inhibiting HER2 via HER2-dependent and -independent mechanisms. *Clin. Cancer Res.* **19**, (2013).

52. Luo, Y. & Zheng, S. G. Hall of fame among pro-inflammatory cytokines: Interleukin-6 gene and its transcriptional regulation mechanisms. *Frontiers in Immunology* **7**, (2016).

53. Hrdinka, M. *et al.* Small molecule inhibitors reveal an indispensable scaffolding role of RIPK 2 in NOD 2 signaling . *EMBO J.* (2018). doi:10.15252/embj.201899372

54. Kobayashi, K. *et al.* RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* (2002). doi:10.1038/416194a

55. Watanabe, T. *et al.* RICK/RIP2 is a NOD2-independent nodal point of gut inflammation.

Int. Immunol. **31**, (2019).

56. Honjo, H., Watanabe, T., Kamata, K., Minaga, K. & Kudo, M. RIPK2 as a New Therapeutic Target in Inflammatory Bowel Diseases. *Frontiers in Pharmacology* **12**, (2021).

57. Wamsley, J. J. *et al.* Activin upregulation by NF-κB is required to maintain mesenchymal features of cancer stem-like cells in non-small cell lung cancer. *Cancer Res.* **75**, (2015).

58. Chen, L. *et al.* A NF-κB-Activin A signaling axis enhances prostate cancer metastasis. *Oncogene* **39**, (2020).

59. Jana, A. *et al.* NFkB is essential for activin-induced colorectal cancer migration via upregulation of PI3K-MDM2 pathway. *Oncotarget* **8**, (2017).

60. Jones, K. L. *et al.* Activin A is a critical component of the inflammatory response, and its binding protein, follistatin, reduces mortality in endotoxemia. *Proc. Natl. Acad. Sci. U. S. A.* **104**, (2007).

61. Pettersen, K. *et al.* Autocrine activin A signalling in ovarian cancer cells regulates secretion of interleukin 6, autophagy, and cachexia. *J. Cachexia. Sarcopenia Muscle* **11**, (2020).

62. Phillips, D. J., Jones, K. L., Scheerlinck, J. P. Y., Hedger, M. P. & De Kretser, D. M. Evidence for activin A and follistatin involvement in the systemic inflammatory response. in *Molecular and Cellular Endocrinology* **180**, (2001).

63. Okuma, Y. *et al.* Regulation of activin A and inhibin B secretion by inflammatory mediators in adult rat Sertoli cell cultures. *J. Endocrinol.* **187**, (2005).

64. Hedger, M. P., Winnall, W. R., Phillips, D. J. & de Kretser, D. M. *The Regulation and Functions of Activin and Follistatin in Inflammation and Immunity*. *Vitamins and Hormones* **85**, (2011).

65. Risbridger, G. P., Schmitt, J. F. & Robertson, D. M. Activins and inhibins in endocrine and other tumors. *Endocrine Reviews* **22**, (2001).

66. Balanathan, P. *et al.* Epigenetic regulation of inhibin α-subunit gene in prostate cancer cell

lines. *J. Mol. Endocrinol.* **32**, (2004).

67. Kim, Y. Il *et al.* Inhibin-α gene mutations and mRNA levels in human lymphoid and myeloid leukemia cells. *Int. J. Oncol.* **50**, (2017).

68. Hofland, J. *et al.* Inhibin alpha-subunit (INHA) expression in adrenocortical cancer is linked to genetic and epigenetic INHA promoter variation. *PLoS One* **9**, (2014).

69. Wex, K. *et al.* Receptor-interacting protein kinase-2 inhibition by cyld impairs antibacterial immune responses in macrophages. *Front. Immunol.* (2016). doi:10.3389/fimmu.2015.00650

70. Yang, S. *et al.* Pellino3 ubiquitinates RIP2 and mediates Nod2-induced signaling and protective effects in colitis. *Nat. Immunol.* (2013). doi:10.1038/ni.2669

71. Caruso, R. & Núñez, G. Innate Immunity: ER Stress Recruits NOD1 and NOD2 for Delivery of Inflammation. *Current Biology* **26**, (2016).

72. Cai, X. *et al.* RIP2 promotes glioma cell growth by regulating TRAF3 and activating the NF-κB and p38 signaling pathways. *Oncol. Rep.* **39**, 2915–2923 (2018).

73. Abbott, D. W. *et al.* Coordinated Regulation of Toll-Like Receptor and NOD2 Signaling by K63-Linked Polyubiquitin Chains. *Mol. Cell. Biol.* (2007). doi:10.1128/mcb.00270-07

74. Takayanagi, S. *et al.* ASKA technology-based pull-down method reveals a suppressive effect of ASK1 on the inflammatory NOD-RIPK2 pathway in brown adipocytes. *Sci. Reports 2021 111* **11**, 1–14 (2021).

75. Chow, M. T. & Luster, A. D. Chemokines in cancer. *Cancer immunology research* **2**, (2014).

CHAPTER 4

Robust inflammatory breast cancer gene signature using nonparametric random forest analysis

4.1 ABSTRACT

Inflammatory breast cancer (IBC) is a rare, aggressive cancer found in all the molecular breast cancer subtypes. Despite extensive previous efforts to screen for transcriptional differences between IBC and non-IBC patients, a robust IBC-specific molecular signature has been elusive. We report a novel IBC-specific gene signature (59 genes; G59) that achieves 100% accuracy in discovery and validation samples (45/45 correct classification) and remarkably only misclassified one sample (60/61 correct classification) in an independent dataset. G59 is independent of ER/HER2 status, molecular subtypes and is specific to untreated IBC samples, with most of the genes being enriched for plasma membrane cellular component proteins, interleukin (IL), and chemokine signaling pathways. Our finding suggests the existence of an IBC-specific molecular signature, paving the way for the identification and validation of targetable genomic drivers of IBC.

4.2 INTRODUCTION

IBC is a rare form of breast cancer associated with poor prognosis compared to other subtypes, and this is attributed to its therapy resistance and a high metastatic potential [1] [2] [3]. Moreover, the majority of IBC patients present with late-stage disease wherein the cancer has spread beyond the primary site[4]. To better diagnose and treat IBC patients, the IBC research community is working on defining an IBC specific molecular signature. The largest study was published through the establishment of the World IBC Consortium which identified 79 genes, molecular subtype-independent, IBC signature[5]. Shortly after, another 132 genes, subtype-independent, IBC signature was reported[6]. However, both signatures were seen in ~16.4% and ~25% of breast cancer TCGA samples of primarily non-IBC patients respectively, signifying low specificity in discriminating IBC from non-IBC samples[5] [7] [8] [9]. Nevertheless, thus far a robust tumor cell-intrinsic signature that can define IBC from non-IBC or can stratify IBC patients has remained elusive[8] [9]. Indeed, a recent comparison of existing IBC signatures found minimal or no overlap amongst the proposed genes and none of the signatures could be validated in an independent dataset[9].

In this report, we reanalyzed publicly available gene expression datasets using the nonparametric machine learning random forest (RF) approach. RF is superior to classic statistical approaches used previously on these datasets because i) It can handle many predictors at once while assigning each a predictor importance score. ii) It uses bootstrap-aggregated (bagged) decision trees to minimize overfitting, allowing for a robust model that can be validated in independent datasets. By restricting our analysis to microdissected IBC tumor epithelium and matching IBC samples with similar receptor-status non-IBC samples, we have identified an IBC signature of 59 genes that only misclassified one patient out of a total 106 patients in pre-treatment datasets.

4.3 METHODS

4.3.1 Patients' samples

All analysis was carried out on MATLAB R2018b (MathWorks). Three microarray datasets were downloaded under accession number GSE45581[6], GSE5847[10], and GSE111477[11]. The Cancer Genome Atlas (TCGA) breast cancer dataset was downloaded from cBioPortal (TCGA Firehose Legacy https://www.cbioportal.org/study/summary?id=brca_tcga). GSE45581 was used for discovery and comprised 20 IBC, 20 non-IBC, and 5 normal microdissected patient epithelium samples. GSE5847 is primarily post-treatment samples dataset, comprised of 13 IBC and 35 non-IBC microdissected patient samples. GSE111477 is a dataset of 33 IBC and 28 non-IBC pre-treatment patient samples comprised primarily of the epithelial tissue.

4.3.2 Genes signature identification, validation, and comparison with other IBC signatures

To identify an IBC specific signature, 12 IBC and 12 non-IBC samples (see figure 4.1a, left) were classified in an ensemble of 5000 decision trees-based bagging, using probed genes as predictors. This approach was looped through a random forest (RF) training model, removing genes with zero or negative predictor importance in each iteration (predictor importance computed for every tree, then averaged over the entire ensemble and divided by the standard deviation over the entire ensemble), until all genes left had a positive predictor importance and low out-of-bag error (Figure 4.1a, middle). This yielded a potential IBC-specific signature of 59 unique genes (Table 4.S1), that could be used to discriminate IBC from non-IBC patient samples (Figure 4.1a, right). Using the 24 samples and G59 to create a RF model, for each sample in GSE45581 dataset, the probability of it being an IBC sample (IBC probability score) was computed from fractions of observations of the class per tree leaf, averaged across all trees in the ensemble. Similarly, for each dataset, GSE5847 and GSE111477, half of the samples for each class were used for training, and

the resultant tree ensemble model applied for IBC probability scoring for all samples. To compute the accuracy of previous IBC signatures [11] [12] [13] [5] [14], the aforementioned analysis was repeated using the respective signature genes. To score TCGA dataset, raw data were downloaded from cBioPortal TCGA Firehose Legacy (https://www.cbioportal.org/study/summary?id=brca_tcga). mRNA expression was combined with GSE45581 dataset through quantile normalization. Using the quantile normalized IBC and non-IBC GSE45581 samples, a RF model was trained and used to score the TCGA samples. MatSurv[15] was used to plot the overall survival KM-plot and compute the log-rank test.

4.3.3 PAM50 subtyping and ROR scores

PAM50 molecular subtyping (Luminal A, Luminal B, HER2-enriched, Basal-like, and Normal-like) and Risk of recurrence (ROR)[16] were computed using Bioconductor package Genefu[17].

4.3.4 Gene ontology and pathway analysis

The IBC signature genes (Table 4.S1) were subjected to Gene Ontology and Pathway analysis scoring using the Enrichr web-based application[18]. Cellular components and pathways list were ranked using p-values for the significance of overlap in Table 4.S3 and Table 4.S4, respectively.

4.4 RESULTS

4.4.1 Random Forest identifies an IBC specific gene signature

We reanalyzed the gene expression dataset of microdissected epithelial tissues, comprised of 20 IBC, 20 non-IBC, and 5 normal patients[6]. To control for any variability in signature discovery caused by the molecular breast cancer subtypes, we matched both ER and HER2 status of 22/24 samples used for training (Figure 4.1a, left, see highlighted ER and HER2 scores). Using the RF approach (Figure 4.1a), we derived a potential IBC-specific signature of 59 unique genes (G59, Table 4.S1).

G59 can comfortably segregate IBC from non-IBC and normal samples in unsupervised hierarchical clustering analysis (Figure 4.1b). Caliński-Harabasz criterion on G59 profiles indicated that the samples would best be categorised into two groups: IBC versus non-IBC and normal samples (Figure 4.1c). Consistent with this, the first and second principal component scatter plot from the principal component analysis (PCA) of the G59 profiles also separated the IBC samples from the rest (Figure 4.1d).

To verify the efficacy of G59, we used RF to model with the 24 training samples (Figure 4.1a, left) and subsequently classified all the 45 samples using the resultant trained model. Remarkably, G59 model accurately identified all IBC samples (IBC probability score >0.5) with no misclassification of non-IBC or normal samples (Figure 4.1e). This accuracy was significantly higher than would be expected if the signature was just a random set of genes (Figure 4.1f). In addition, G59 prediction was independent of ER/HER2 status, molecular subtypes, and ROR (Table 4.S2). Thus, G59 is a potential IBC-specific signature that can predict IBC samples in a machine learning RF approach.

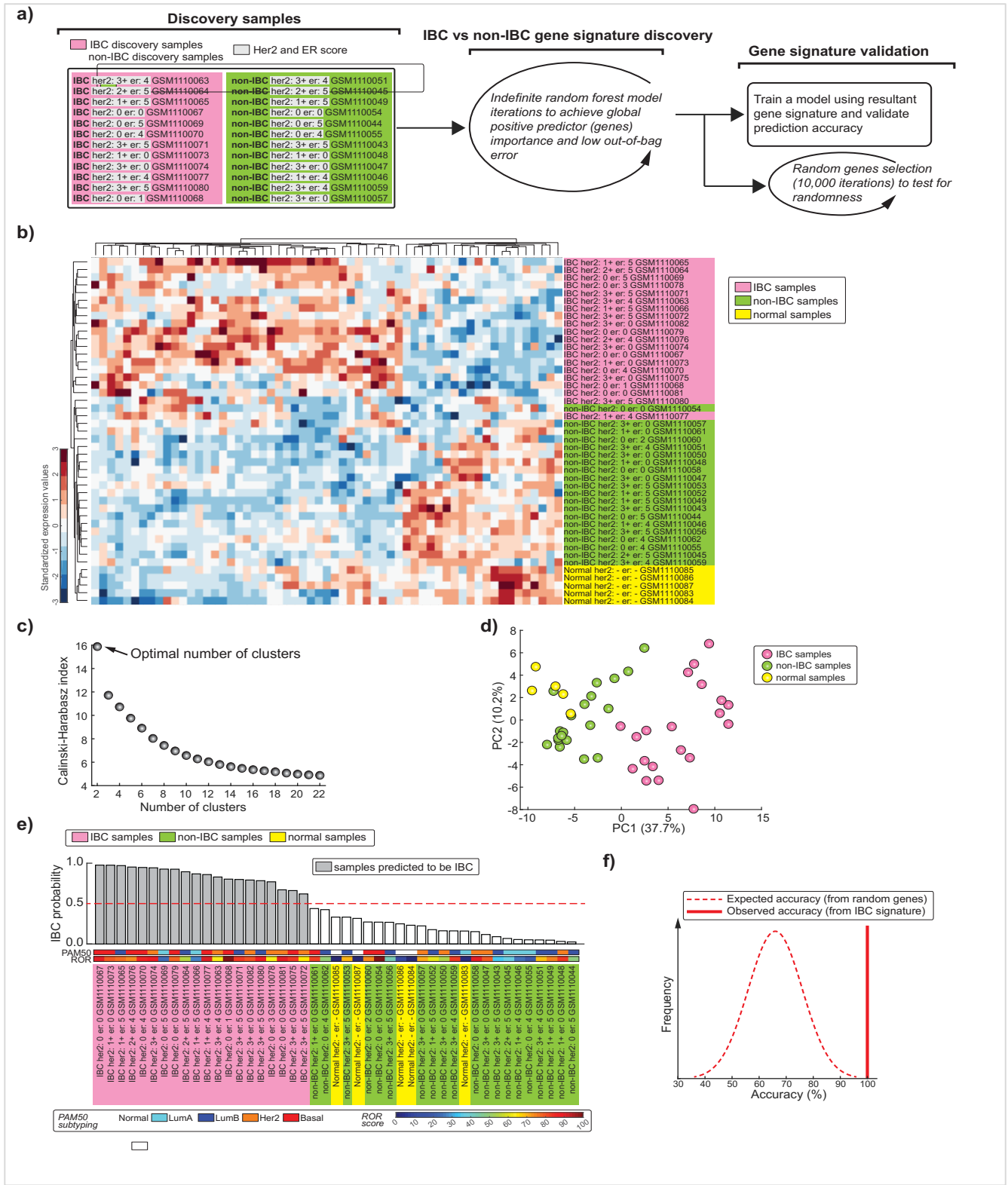
a)
Discovery samples
IBC discovery samples
non-IBC discovery samples
Her2 and ER score
IBC vs non-IBC gene signature discovery
Indefinite random forest model iterations to achieve global positive predictor (genes) importance and low out-of-bag error
Gene signature validation
Train a model using resultant gene signature and validate prediction accuracy
Random genes selection (10,000 iterations) to test for randomness
b)
IBC samples
non-IBC samples
normal samples
Standardized expression values
c)
Optimal number of clusters
Calinski-Harabasz index
Number of clusters
d)
IBC samples
non-IBC samples
normal samples
PC2 (10.2%)
PC1 (37.7%)
e)
IBC samples
non-IBC samples
normal samples
samples predicted to be IBC
IBC probability
PAM50
ROR
PAM50 subtyping
Normal
LumA
LumB
Her2
Basal
ROR score
f)
Expected accuracy (from random genes)
Observed accuracy (from IBC signature)
Frequency
Accuracy (%)

Figure 4.1. Identification of an IBC-specific gene signature. **a** Left: List of IBC and non-IBC samples used for gene signature discovery (GSE45581 dataset). Row wise matched HER2/ER scores are highlighted and sample accessions numbers (GSM) from gene expression omnibus (GEO) database are indicated. Middle: Strategy for signature discovery. Right: Strategy for signature validation. **b** Unsupervised hierarchical clustering heatmap of all samples (GSE45581 dataset) using the IBC signature genes. **c** The Optimal number of clusters determined by the Caliński–Harabasz criterion. **d** Principal Component Analysis scatter plot using the first and second principal components. **e** Waterfall plot for all samples' IBC probability score validating the signature. The dotted line demarcates the minimum probability score to classify the sample as IBC in the model. PAM50 molecular subtyping and ROR scores are indicated. **f** Distribution of expected accuracy from models trained using random sets of 59 genes (10,000 iterations) compared with the 100% accuracy observed in IBC signature (dotted distribution line versus solid vertical line, respectively)

4.4.2 The gene signature is predictive in pre-treatment samples

Prior to Woodward *et al.* IBC dataset[6], only one other microdissected IBC dataset was available[10]. Unlike the Woodward *et al.* dataset, whose patient samples were collected from pre-treatment core biopsies, this dataset included 13 IBC patients who had primarily received neoadjuvant chemotherapy prior to sample collection. G59 training model correctly classified 7/13 IBC training epithelium samples, as expected, but misclassified the other 6 validation IBC samples (Figure 4.2a (i)). Inline with this, the signature failed to separate IBC from non-IBC samples in both PCA scatter plot and unsupervised hierarchical clustering analysis (Figure 4.2a (ii-iii)). Next, we tested the G59 training model on an independent dataset comprised of 33 IBC and 28 non-IBC core biopsy pre-treatment samples[11]. A trained model using half of the samples from each category only misclassified 1 out of the 61 samples (Figure 4.2b (i)), with both PCA scatter plot and unsupervised hierarchical clustering analysis largely separating IBC from non-IBC

samples (Figure 4.2b (ii-iii)). This suggests that the G59 signature is predictive of IBC pre-treatment epithelial tumor while chemotherapy treatment abrogated its predictiveness.

4.4.3 The gene signature is unique to IBC and is enriched in membrane proteins and interleukin pathways

Next, we compared G59 to 5 previous IBC signatures. 49% (29/59) of the genes overlapped with Woodward *et al* 132 gene signature[6] with minimal or no overlap with the rest of the signatures (Figure 4.2c(i)). Using RF approach (detailed in Table 4.S1), G59 accuracy was significantly higher than all the other signatures (Figure 4.2c(ii)). Given the reported low specificity of these IBC signatures in non-IBC samples[5 7 8 9], we tested G59 model on TCGA breast cancer dataset, comprised of primarily non-IBC samples. Only 1.6% of the TCGA samples were classified as IBC-like, suggesting G59 was unique to IBC. Indeed, in line with poor overall survival in IBC patients, Kaplan-Meier analysis revealed a higher risk of death for these IBC-like patients, with a hazard ratio of 3.15 ($p=0.037$) (Figure 4.2d).

Having verified G59 signature in two pre-treatment datasets and shown higher specificity in the TCGA dataset, we performed gene ontology and pathway enrichment analysis of the genes. Protein-coding genes presented 88% (52/59) of the gene set (Figure 4.2e), with 25% (13/52) being plasma membrane proteins (Figure 4.2f left, Table 4.S3). While there was no overwhelming enrichment of any specific pathway, IL-2, G-alpha, and chemokine pathways gave the highest gene overlap (8, 4, and 3 respectively) with a significant enrichment (Figure 4.2d right, Table S4).

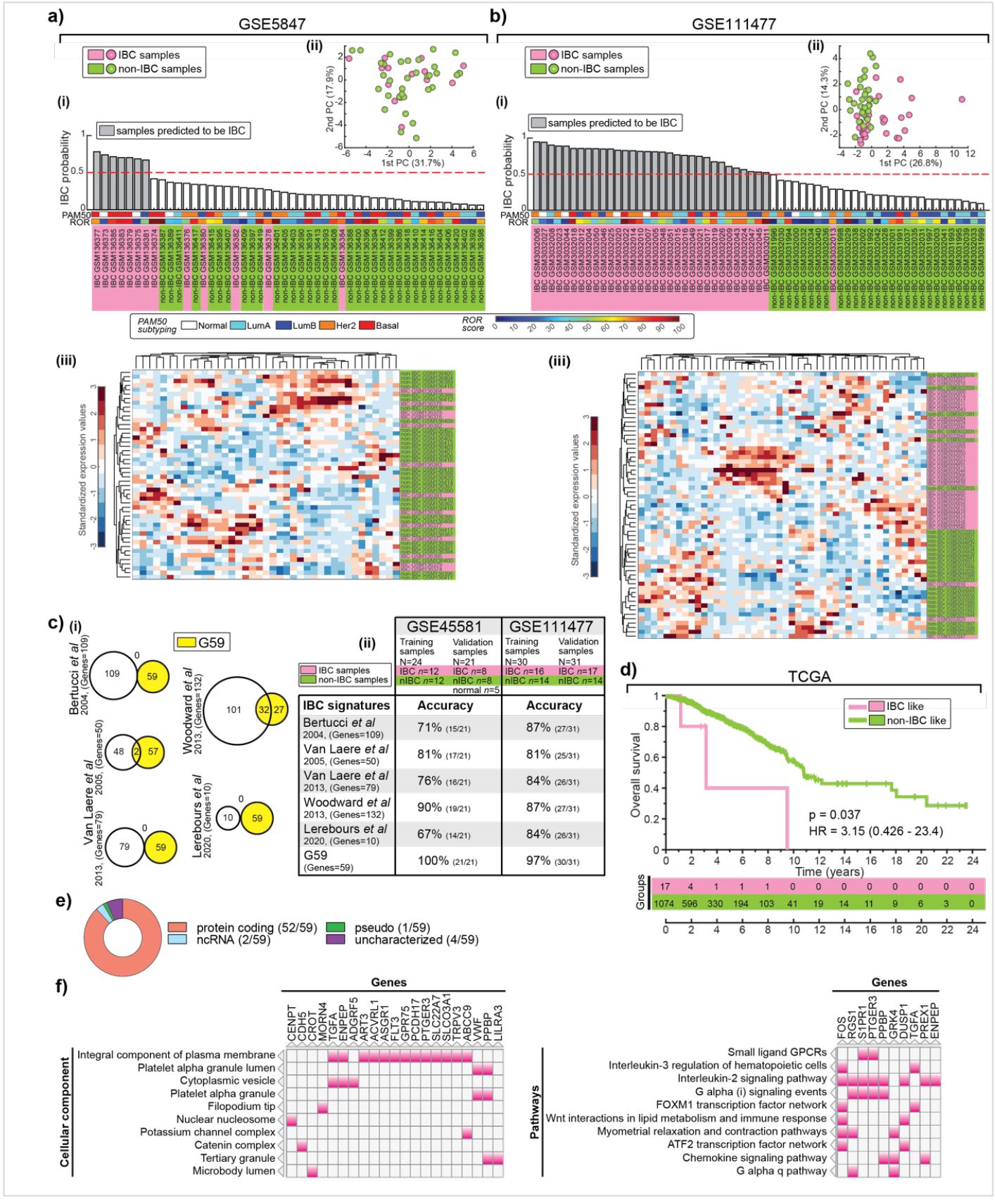
a)
GSE5847
b)
GSE111477
IBC samples
non-IBC samples
(i)
samples predicted to be IBC
IBC probability
PAM50
ROR
(ii)
2nd PC (17.9%)
1st PC (31.7%)
2nd PC (14.3%)
1st PC (26.8%)
PAM50 subtyping
Normal
LumA
LumB
Her2
Basal
ROR score
(iii)
Standardized expression values
c)
(i)
G59
Bertucci et al 2004, (Genes=109)
Woodward et al 2013, (Genes=132)
Van Laere et al 2005, (Genes=50)
Van Laere et al 2013, (Genes=79)
Lerebours et al 2020, (Genes=10)
(ii)
GSE45581
GSE111477
Training samples
Validation samples
N=24
IBC n=12
nIBC n=12
N=21
IBC n=8
nIBC n=8
normal n=5
N=30
IBC n=16
nIBC n=14
N=31
IBC n=17
nIBC n=14
IBC signatures
Accuracy
Bertucci et al 2004, (Genes=109)
71% (15/21)
87% (27/31)
Van Laere et al 2005, (Genes=50)
81% (17/21)
81% (25/31)
Van Laere et al 2013, (Genes=79)
76% (16/21)
84% (26/31)
Woodward et al 2013, (Genes=132)
90% (19/21)
87% (27/31)
Lerebours et al 2020, (Genes=10)
67% (14/21)
84% (26/31)
G59 (Genes=59)
100% (21/21)
97% (30/31)
d)
TCGA
IBC like
non-IBC like
Overall survival
p = 0.037
HR = 3.15 (0.426 - 23.4)
Time (years)
Groups
e)
protein coding (52/59)
ncRNA (2/59)
pseudo (1/59)
uncharacterized (4/59)
f)
Genes
CENPT
CDH5
CROT
MORN4
TGFA
ENPEP
ADGRF5
ART3
ACVRL1
ASGR1
FLT3
GPR75
PCDH17
PTGER3
SLC22A7
SLCO3A1
TRPV3
ABCC9
VWF
PPBP
LILRA3
Cellular component
Integral component of plasma membrane
Platelet alpha granule lumen
Cytoplasmic vesicle
Platelet alpha granule
Filopodium tip
Nuclear nucleosome
Potassium channel complex
Catenin complex
Tertiary granule
Microbody lumen
FOS
RGS1
S1PR1
PTGER3
PPBP
GRK4
DUSP1
TGFA
PREX1
ENPEP
Pathways
Small ligand GPCRs
Interleukin-3 regulation of hematopoietic cells
Interleukin-2 signaling pathway
G alpha (i) signaling events
FOXM1 transcription factor network
Wnt interactions in lipid metabolism and immune response
Myometrial relaxation and contraction pathways
ATF2 transcription factor network
Chemokine signaling pathway
G alpha q pathway

Figure 4.2. Independent validation of IBC gene signature and its gene ontology/pathway analysis. **a**, **b** Validation of post-treatment samples from GSE5847 dataset and pre-treatment core biopsies samples from GSE111477 dataset, respectively. IBC probability plot, PCA scatter plot and unsupervised hierarchical clustering heatmaps are represented similar to figure 4.1. **c** (i) Venn plots for G59 overlap with 5 previous IBC gene signatures (ii) Table indicating the accuracy of the signatures in GSE45581 and GSE111477 datasets. **d** Kaplan–Meier plot log-rank test for G59-predicted IBC like versus non-IBC like samples in TCGA. The p-value, hazard ratio (HR) and the 95% confidence interval of ratio are indicated. **e** Pie chart indicating the proportion of gene types in the signature. ncRNA: non-coding RNA. **f** Clustergrams of top 10 cellular component and pathway analysis of the signature genes, with overlapping genes highlighted (Table 4.S3 and 4.S4 for complete list)

4.5 DISCUSSION

We have identified a robust gene signature that can characterize IBC from non-IBC with an aim to better understand and potentially develop a tailored treatment regimen for IBC patients. G59 is the first IBC signature to be successfully validated in an independent dataset and shows the highest accuracy (100% in GSE45581 and 98.4% in GSE111477) in its prediction[9]. This is a significant improvement in accuracy as previous signatures accuracy range between 68% and 88% [5 8 9], a range similar to our analysis (Figure 4.2c(ii)). Importantly, G59 shows higher specificity in primarily non-IBC samples compared to previous signatures[5 7 8 9].

The low prediction accuracy in primarily post-treatment tumor samples highlight the fact that chemotherapy induces changes in gene expression[19]. Interestingly, SUM149 and SUM190, the two cell lines used in most of the IBC research[20], were derived from patients who had already received chemotherapy treatment[21]. Our analysis suggests the need for establishing IBC cell lines from untreated patients to fully capture IBC specific profile.

G59 is a more curated version of the 132 gene list selected by Dr. Woodward[6] for IBC assessment with 49% similarities. Most of the genes in G59 code for membrane proteins suggesting that IBC cells are highly communitive with the tumor microenvironment, likely playing an essential role in directing their disease progression. The novel implication of IL-2 inflammatory as well as chemokine pathways in IBC (Figure 4.2d right), adds to the proposed inflammatory pathways involvement [8 22].

Our finding highlights the need to integrate contemporary statistical approaches to identify molecular signatures previously missed by traditional statistical methods. Most important, the IBC-specific molecular signature we have identified paves the way for IBC functional studies, validation, and potentially successful therapeutic interventions.

4.6 Supplementary tables

Table 4.**S1.** Gene information for the G59 IBC signature

IBC-specific genes signature. Original GPL6480 **Gene Symbol**, current official **Gene Symbol**, UniProt **Protein Name** and **Gene Information** is provided. Highlighted in yellow are genes whose current Gene symbol is different from previous GPL6480 Gene symbol. Highlighted in cyan are genes overlapping with Woodward et al 132 gene signature

GPL6480 Gene Symbol	Current Gene Symbol	UniProt Protein Name	Gene Info
LYZL1	LYZL1	Lysozyme-like protein 1	84569 [Gene Symbol: LYZL1] [Locus Tag:] [Chromosome: 10] [Map Location: 10p12.1-p11.23] [Description: lysozyme like 1] [Gene Type: protein-coding]
PCDH17	PCDH17	Protocadherin-17	27253 [Gene Symbol: PCDH17] [Locus Tag:] [Chromosome: 13] [Map Location: 13q21.1] [Description: protocadherin 17] [Gene Type: protein-coding]
CBY3	CBY3	Protein chibby homolog 3	646019 [Gene Symbol: CBY3] [Locus Tag:] [Chromosome: 5] [Map Location: 5q35.3] [Description: chibby family member 3] [Gene Type: protein-coding]
LOC100506670	LOC100506670	-	-uncharacterized
HECW1	HECW1	E3 ubiquitin-protein ligase HECW1	23072 [Gene Symbol: HECW1] [Locus Tag:] [Chromosome: 7] [Map Location: 7p14.1-p13] [Description: HECT, C2 and WW domain containing E3 ubiquitin protein ligase 1] [Gene Type: protein-coding]
LOC100507642	LOC100507642	-	100507642 [Gene Symbol: LOC100507642] [Locus Tag:] [Chromosome: 7] [Map Location: 7p22.3] [Description: uncharacterized LOC100507642] [Gene Type: ncRNA]
HIST1H4A	H4C1	Histone H4	8359 [Gene Symbol: H4C1] [Locus Tag:] [Chromosome: 6] [Map Location: 6p22.2] [Description: H4 clustered histone 1] [Gene Type: protein-coding]
TIMD4	TIMD4	T-cell immunoglobulin and mucin domain-containing protein 4	91937 [Gene Symbol: TIMD4] [Locus Tag:] [Chromosome: 5] [Map Location: 5q33.3] [Description: T cell immunoglobulin and mucin domain containing 4] [Gene Type: protein-coding]
LILRA3	LILRA3	Leukocyte immunoglobulin-like receptor subfamily A member 3	11026 [Gene Symbol: LILRA3] [Locus Tag:] [Chromosome: 19] [Map Location: 19q13.4] [Description: leukocyte immunoglobulin like receptor A3] [Gene Type: protein-coding]
FAM106CP	FAM106C	Protein FAM106C	100129396 [Gene Symbol: FAM106C] [Locus Tag:] [Chromosome: 17] [Map Location: 17p11.2] [Description: family with sequence similarity 106 member C] [Gene Type: ncRNA]
CCDC144A	CCDC144A	Coiled-coil domain-containing protein 144A	9720 [Gene Symbol: CCDC144A] [Locus Tag:] [Chromosome: 17] [Map Location: 17p11.2] [Description: coiled-coil domain containing 144A] [Gene Type: protein-coding]
CCDC144NL	CCDC144NL	Putative coiled-coil domain-containing protein 144 N-terminal-like	339184 [Gene Symbol: CCDC144NL] [Locus Tag:] [Chromosome: 17] [Map Location: 17p11.2] [Description: CCDC144A N-terminal pseudogene] [Gene Type: pseudo]
LOC100130741	LOC100130741	-	-uncharacterized
PPBP	PPBP	Platelet basic protein	5473 [Gene Symbol: PPBP] [Locus Tag:] [Chromosome: 4] [Map Location: 4q13.3] [Description: pro-platelet basic protein] [Gene Type: protein-coding]
CCDC62	CCDC62	Coiled-coil domain-containing protein 62	84660 [Gene Symbol: CCDC62] [Locus Tag:] [Chromosome: 12] [Map Location: 12q24.31] [Description: coiled-coil domain containing 62] [Gene Type: protein-coding]
UNC93A	UNC93A	Protein unc-93 homolog A	54346 [Gene Symbol: UNC93A] [Locus Tag:] [Chromosome: 6] [Map Location: 6q27] [Description: unc-93 homolog A] [Gene Type: protein-coding]
TRPV3	TRPV3	Transient receptor potential cation channel subfamily V member 3	162514 [Gene Symbol: TRPV3] [Locus Tag:] [Chromosome: 17] [Map Location: 17p13.2] [Description: transient receptor potential cation channel subfamily V member 3] [Gene Type: protein-coding]
CENPT	CENPT	Centromere protein T	80152 [Gene Symbol: CENPT] [Locus Tag:] [Chromosome: 16] [Map Location: 16q22.1] [Description: centromere protein T] [Gene Type: protein-coding]
ZCCHC13	ZCCHC13	Zinc finger CCHC domain-containing protein 13	389874 [Gene Symbol: ZCCHC13] [Locus Tag:] [Chromosome: X] [Map Location: Xq13.2] [Description: zinc finger CCHC-type containing 13] [Gene Type: protein-coding]
LOC100129858	LOC100129858	-	-uncharacterized
SLC22A7	SLC22A7	Solute carrier family 22 member 7	10864 [Gene Symbol: SLC22A7] [Locus Tag:] [Chromosome: 6] [Map Location: 6p21.1] [Description: solute carrier family 22 member 7] [Gene Type: protein-coding]
C1orf95	STUM	Protein stum homolog	375057 [Gene Symbol: STUM] [Locus Tag:] [Chromosome: 1] [Map Location: 1q42.12] [Description: stum, mechanosensory transduction mediator homolog] [Gene Type: protein-coding]
GPR75	GPR75	Probable G-protein coupled receptor 75	10936 [Gene Symbol: GPR75] [Locus Tag:] [Chromosome: 2] [Map Location: 2p16.2] [Description: G protein-coupled receptor 75] [Gene Type: protein-coding]
ASGR1	ASGR1	Asialoglycoprotein receptor 1	432 [Gene Symbol: ASGR1] [Locus Tag:] [Chromosome: 17] [Map Location: 17p13.1] [Description: asialoglycoprotein receptor 1] [Gene Type: protein-coding]
S1PR1	S1PR1	Sphingosine 1-phosphate receptor 1	1901 [Gene Symbol: S1PR1] [Locus Tag:] [Chromosome: 1] [Map Location: 1p21.2] [Description: sphingosine-1-phosphate receptor 1] [Gene Type: protein-coding]
FAM162B	FAM162B	Protein FAM162B	221303 [Gene Symbol: FAM162B] [Locus Tag:] [Chromosome: 6] [Map Location: 6q22.1] [Description: family with sequence similarity 162 member B] [Gene Type: protein-coding]
CDH5	CDH5	Cadherin-5	1003 [Gene Symbol: CDH5] [Locus Tag:] [Chromosome: 16] [Map Location: 16q21] [Description: cadherin 5] [Gene Type: protein-coding]
VWF	VWF	von Willebrand factor	7450 [Gene Symbol: VWF] [Locus Tag:] [Chromosome: 12] [Map Location: 12p13.31] [Description: von Willebrand factor] [Gene Type: protein-coding]
GPR116	ADGRF5	Adhesion G protein-coupled receptor F5	221395 [Gene Symbol: ADGRF5] [Locus Tag:] [Chromosome: 6] [Map Location: 6p12.3] [Description: adhesion G protein-coupled receptor F5] [Gene Type: protein-coding]
ABCC9	ABCC9	ATP-binding cassette sub-family C member 9	10060 [Gene Symbol: ABCC9] [Locus Tag:] [Chromosome: 12] [Map Location: 12p12.1] [Description: ATP binding cassette subfamily C member 9] [Gene Type: protein-coding]
ACVRL1	ACVRL1	Serine/threonine-protein kinase receptor; Receptor protein serine/threonine kinase; Serine	94 [Gene Symbol: ACVRL1] [Locus Tag:] [Chromosome: 12] [Map Location: 12q13.13] [Description: activin A receptor like type 1] [Gene Type: protein-coding]
SLCO3A1	SLCO3A1	Solute carrier organic anion transporter family member 3A1	28232 [Gene Symbol: SLCO3A1] [Locus Tag:] [Chromosome: 15] [Map Location: 15q26.1] [Description: solute carrier organic anion transporter family member 3A1] [Gene Type: protein-coding]
C17orf100	C17orf100	Uncharacterized protein C17orf100	388327 [Gene Symbol: C17orf100] [Locus Tag:] [Chromosome: 17] [Map Location: 17p13.1] [Description: chromosome 17 open reading frame 100] [Gene Type: protein-coding]
ENPEP	ENPEP	Aminopeptidase; Glutamyl aminopeptidase	2028 [Gene Symbol: ENPEP] [Locus Tag:] [Chromosome: 4] [Map Location: 4q25] [Description: glutamyl aminopeptidase] [Gene Type: protein-coding]
FOLH1	FOLH1	Glutamate carboxypeptidase 2	2346 [Gene Symbol: FOLH1] [Locus Tag:] [Chromosome: 11] [Map Location: 11p11.12] [Description: folate hydrolase 1] [Gene Type: protein-coding]
ART3	ART3	Ecto-ADP-ribosyltransferase 3; NAD(P)(+)--arginine ADP-ribosyltransferase	419 [Gene Symbol: ART3] [Locus Tag:] [Chromosome: 4] [Map Location: 4q21.1|4p15.1-p14] [Description: ADP-ribosyltransferase 3 (inactive)] [Gene Type: protein-coding]
TGFA	TGFA	Protransforming growth factor alpha	7039 [Gene Symbol: TGFA] [Locus Tag:] [Chromosome: 2] [Map Location: 2p13.3] [Description: transforming growth factor alpha] [Gene Type: protein-coding]
SPNS2	SPNS2	Protein spinster homolog 2	124976 [Gene Symbol: SPNS2] [Locus Tag:] [Chromosome: 17] [Map Location: 17p13.2] [Description: sphingolipid transporter 2] [Gene Type: protein-coding]
NXN	NXN	Nucleoredoxin	64359 [Gene Symbol: NXN] [Locus Tag:] [Chromosome: 17] [Map Location: 17p13.3] [Description: nucleoredoxin] [Gene Type: protein-coding]
PTGER3	PTGER3	Prostaglandin E2 receptor EP3 subtype	5733 [Gene Symbol: PTGER3] [Locus Tag:] [Chromosome: 1] [Map Location: 1p31.1] [Description: prostaglandin E receptor 3] [Gene Type: protein-coding]
NXNL2	NXNL2	Nucleoredoxin-like protein 2	158046 [Gene Symbol: NXNL2] [Locus Tag:] [Chromosome: 9] [Map Location: 9q22.1] [Description: nucleoredoxin like 2] [Gene Type: protein-coding]
PREX1	PREX1	Phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 1 protein	57580 [Gene Symbol: PREX1] [Locus Tag:] [Chromosome: 20] [Map Location: 20q13.13] [Description: phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1] [Gene Type: protein-coding]
MRPS30	MRPS30	39S ribosomal protein S30, mitochondrial	10884 [Gene Symbol: MRPS30] [Locus Tag:] [Chromosome: 5] [Map Location: 5p12] [Description: mitochondrial ribosomal protein S30] [Gene Type: protein-coding]
WDR65	CFAP57	Cilia- and flagella-associated protein 57	149465 [Gene Symbol: CFAP57] [Locus Tag:] [Chromosome: 1] [Map Location: 1p34.2] [Description: cilia and flagella associated protein 57] [Gene Type: protein-coding]
FLT3	FLT3	Receptor-type tyrosine-protein kinase FLT3; Receptor protein-tyrosine kinase	2322 [Gene Symbol: FLT3] [Locus Tag:] [Chromosome: 13] [Map Location: 13q12.2] [Description: fms related receptor tyrosine kinase 3] [Gene Type: protein-coding]
SEMA3E	SEMA3E	Semaphorin-3E	9723 [Gene Symbol: SEMA3E] [Locus Tag:] [Chromosome: 7] [Map Location: 7q21.11] [Description: semaphorin 3E] [Gene Type: protein-coding]
CCDC71	CCDC71	Coiled-coil domain-containing protein 71	64925 [Gene Symbol: CCDC71] [Locus Tag:] [Chromosome: 3] [Map Location: 3p21.31] [Description: coiled-coil domain containing 71] [Gene Type: protein-coding]
GRK4	GRK4	G protein-coupled receptor kinase 4	2868 [Gene Symbol: GRK4] [Locus Tag:] [Chromosome: 4] [Map Location: 4p16.3] [Description: G protein-coupled receptor kinase 4] [Gene Type: protein-coding]
CROT	CROT	Peroxisomal carnitine O-octanoyltransferase	54677 [Gene Symbol: CROT] [Locus Tag:] [Chromosome: 7] [Map Location: 7q21.12] [Description: carnitine O-octanoyltransferase] [Gene Type: protein-coding]
LOC100507508	LOC100507508	-	-uncharacterized
MORN4	MORN4	MORN repeat-containing protein 4	118812 [Gene Symbol: MORN4] [Locus Tag:] [Chromosome: 10] [Map Location: 10q24.2] [Description: MORN repeat containing 4] [Gene Type: protein-coding]
DUSP1	DUSP1	Dual specificity protein phosphatase 1	1843 [Gene Symbol: DUSP1] [Locus Tag:] [Chromosome: 5] [Map Location: 5q35.1] [Description: dual specificity phosphatase 1] [Gene Type: protein-coding]
FOS	FOS	Cellular oncogene fos; Proto-oncogene c-Fos	2353 [Gene Symbol: FOS] [Locus Tag:] [Chromosome: 14] [Map Location: 14q24.3] [Description: Fos proto-oncogene, AP-1 transcription factor subunit] [Gene Type: protein-coding]
RGS1	RGS1	Regulator of G-protein signaling 1	5996 [Gene Symbol: RGS1] [Locus Tag:] [Chromosome: 1] [Map Location: 1q31.2] [Description: regulator of G protein signaling 1] [Gene Type: protein-coding]
C8orf4	TCIM	Transcriptional and immune response regulator	56892 [Gene Symbol: TCIM] [Locus Tag:] [Chromosome: 8] [Map Location: 8p11.21] [Description: transcriptional and immune response regulator] [Gene Type: protein-coding]
GLYAT	GLYAT	Glycine N-acyltransferase	10249 [Gene Symbol: GLYAT] [Locus Tag:] [Chromosome: 11] [Map Location: 11q12.1] [Description: glycine-N-acyltransferase] [Gene Type: protein-coding]
PLK5	PLK5	Inactive serine/threonine-protein kinase PLK5	126520 [Gene Symbol: PLK5] [Locus Tag:] [Chromosome: 19] [Map Location: 19p13.3] [Description: polo like kinase 5 (inactive)] [Gene Type: protein-coding]
PSPH	PSPH	Phosphoserine phosphataseO-phosphoserine phosphohydrolase	5723 [Gene Symbol: PSPH] [Locus Tag:] [Chromosome: 7] [Map Location: 7p11.2] [Description: phosphoserine phosphatase] [Gene Type: protein-coding]
SPATA5L1	SPATA5L1	Spermatogenesis-associated protein 5-like protein 1	79029 [Gene Symbol: SPATA5L1] [Locus Tag:] [Chromosome: 15] [Map Location: 15q21.1] [Description: spermatogenesis associated 5 like 1] [Gene Type: protein-coding]

Table 4.S2. Distribution of clinical and molecular features in IBC / non-IBC predicted samples. Samples were dichotomized into predicted IBC and non-IBC samples based on IBC probability score and grouped based on clinical features as well as PAM50 molecular subtyping and subtype-based risk of relapse. X2 distribution p-value was computed for each contingency table.

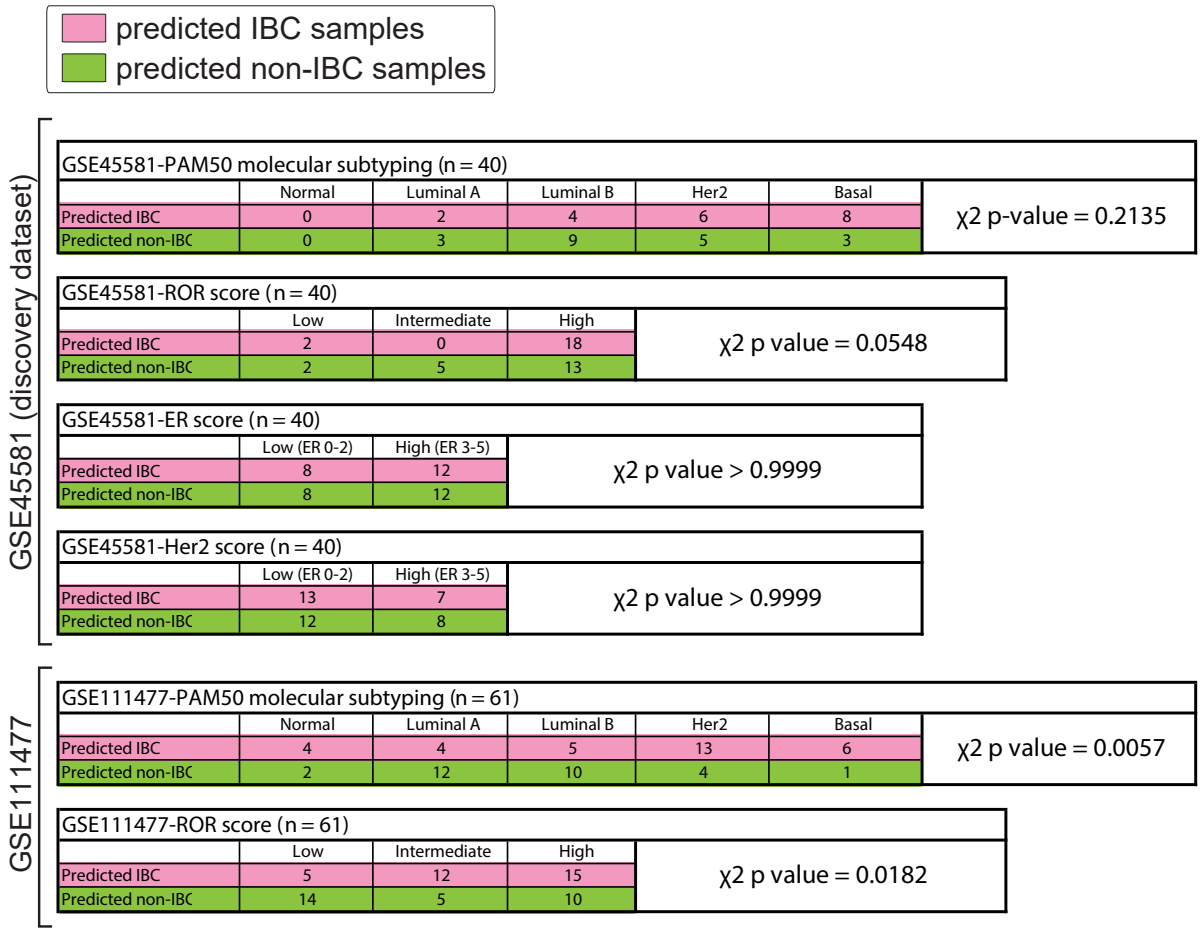

Table 4.S3. Cellular component for the G59 IBC signature

Cellular component Gene Ontology analysis								
Term	Overlap	P-value	Adjusted P-value	Old P-value	Old Adjusted P-value	Odds Ratio	Combined Score	Genes
integral component of plasma membrane (GO:0005887)	13/1463	1.30E-04	0.005706	0	0	3.94803	35.33668	ACVRL1;ENPEP;FLT3;PTGER3;GPR75;TGFA;TRPV3;ABCC9;ASGR1;PCDH17;ART3;SLCO3A1;SLC22A7
platelet alpha granule lumen (GO:0031093)	2/67	0.014645	0.237796	0	0	11.54136	48.74701	VWF;PPBP
cytoplasmic vesicle (GO:0031410)	3/215	0.021339	0.237796	0	0	5.370011	20.65972	ENPEP;ADGRF5;TGFA
platelet alpha granule (GO:0031091)	2/90	0.025485	0.237796	0	0	8.515009	31.24726	VWF;PPBP
filopodium tip (GO:0032433)	1/10	0.027168	0.237796	0	0	41.02058	147.9085	MORN4
nuclear nucleosome (GO:0000788)	1/13	0.035176	0.237796	0	0	30.7608	102.9683	CENPT
potassium channel complex (GO:0034705)	1/14	0.037831	0.237796	0	0	28.39316	92.97685	ABCC9
catenin complex (GO:0016342)	1/28	0.074256	0.338307	0	0	13.66118	35.52223	CDH5
tertiary granule (GO:0070820)	2/164	0.074852	0.338307	0	0	4.608199	11.94557	PPBP;LILRA3
peroxisomal matrix (GO:0005782)	1/42	0.109327	0.338307	0	0	8.990063	19.89874	CROT
microbody lumen (GO:0031907)	1/42	0.109327	0.338307	0	0	8.990063	19.89874	CROT
endoplasmic reticulum-Golgi intermediate compartment membrane (GO:0033116)	1/47	0.121533	0.338307	0	0	8.01087	16.88346	TGFA
chromosome, centromeric region (GO:0000775)	1/49	0.126369	0.338307	0	0	7.676312	15.8788	CENPT
lytic vacuole membrane (GO:0098852)	2/233	0.134601	0.338307	0	0	3.220453	6.458419	ENPEP;SPNS2
ER to Golgi transport vesicle membrane (GO:0012507)	1/54	0.138346	0.338307	0	0	6.950384	13.74783	TGFA
tertiary granule lumen (GO:1904724)	1/55	0.140722	0.338307	0	0	6.821331	13.37641	PPBP
filopodium (GO:0030175)	1/60	0.152506	0.338307	0	0	6.241682	11.73781	MORN4
ficolin-1-rich granule membrane (GO:0101003)	1/61	0.154843	0.338307	0	0	6.137346	11.44824	LILRA3
bicellular tight junction (GO:0005923)	1/72	0.180143	0.338307	0	0	5.18362	8.884743	CDH5
tertiary granule membrane (GO:0070821)	1/73	0.182406	0.338307	0	0	5.111368	8.6971	LILRA3
peroxisomal part (GO:0044439)	1/75	0.186913	0.338307	0	0	4.972723	8.339813	CROT
COPII-coated ER to Golgi transport vesicle (GO:0030134)	1/75	0.186913	0.338307	0	0	4.972723	8.339813	TGFA
chromosomal region (GO:0098687)	1/76	0.189157	0.338307	0	0	4.906173	8.16964	CENPT
lysosomal membrane (GO:0005765)	2/291	0.190654	0.338307	0	0	2.56656	4.253548	ENPEP;SPNS2
secretory granule lumen (GO:0034774)	2/317	0.216741	0.338307	0	0	2.351602	3.595724	VWF;PPBP
specific granule membrane (GO:0035579)	1/91	0.222102	0.338307	0	0	4.085391	6.146946	LILRA3
peroxisome (GO:0005777)	1/92	0.224251	0.338307	0	0	4.040293	6.040187	CROT
microbody (GO:0042579)	1/92	0.224251	0.338307	0	0	4.040293	6.040187	CROT
centriole (GO:0005814)	1/95	0.230664	0.338307	0	0	3.910757	5.736279	CBY3
endoplasmic reticulum-Golgi intermediate compartment (GO:0005793)	1/95	0.230664	0.338307	0	0	3.910757	5.736279	TGFA
clathrin-coated vesicle (GO:0030136)	1/100	0.241235	0.342398	0	0	3.712308	5.278844	VWF
microtubule organizing center part (GO:0044450)	1/126	0.293948	0.404179	0	0	2.936296	3.595057	CBY3
lysosome (GO:0005764)	2/422	0.323735	0.431647	0	0	1.754268	1.978516	ENPEP;SPNS2
RNA polymerase II transcription factor complex (GO:0090575)	1/147	0.333885	0.432086	0	0	2.511289	2.75478	FOS
specific granule (GO:0042581)	1/160	0.357487	0.449412	0	0	2.304449	2.370485	LILRA3
ficolin-1-rich granule (GO:0101002)	1/184	0.398923	0.487572	0	0	1.999798	1.837789	LILRA3
nuclear chromatin (GO:0000790)	1/253	0.50398	0.599328	0	0	1.447163	0.991622	CENPT
mitochondrial matrix (GO:0005759)	1/308	0.574611	0.665339	0	0	1.184582	0.656332	GLYAT
mitochondrial inner membrane (GO:0005743)	1/341	0.612145	0.690626	0	0	1.06781	0.524066	MRPS30
perinuclear region of cytoplasm (GO:0048471)	1/378	0.65037	0.709993	0	0	0.961195	0.413519	TGFA
nuclear chromosome part (GO:0044454)	1/392	0.663847	0.709993	0	0	0.926115	0.379433	CENPT
nucleoplasm part (GO:0044451)	1/407	0.677721	0.709993	0	0	0.891215	0.3467	CENPT
mitochondrion (GO:0005739)	2/1026	0.780968	0.79913	0	0	0.697266	0.172379	MRPS30;GLYAT
nuclear body (GO:0016604)	1/618	0.822481	0.822481	0	0	0.580107	0.11337	CENPT

Table 4.S4. Pathway analysis for the G59 IBC signature

BioPlanet pathway analysis								
Term	Overlap	P-value	Adjusted P-value	Old P-value	Old Adjusted P-value	Odds Ratio	Combined Score	Genes
Small ligand GPCRs	2/19	0.001232	0.117835	0	0	44.23529	296.33157	PTGER3;S1PR1
Interleukin-3 regulation of hematopoietic cells	2/20	0.001367	0.117835	0	0	41.77568	275.52677	TGFA;FOS
Interleukin-2 signaling pathway	8/847	0.002086	0.117835	0	0	3.876144	23.92461	PREX1;ENPEP;RGS1;DUSP1;PTGER3;S1PR1;FOS;PPBP
G alpha (i) signaling events	4/199	0.002182	0.117835	0	0	7.94369	48.674573	RGS1;PTGER3;S1PR1;PPBP
FOXM1 transcription factor network	2/41	0.005684	0.196055	0	0	19.26076	99.578965	TGFA;FOS
Wnt interactions in lipid metabolism and immune response	2/45	0.006815	0.196055	0	0	17.46556	87.129734	DUSP1;FOS
Myometrial relaxation and contraction pathways	3/155	0.008922	0.196055	0	0	7.512525	35.453704	RGS1;GRK4;FOS
ATF2 transcription factor network	2/59	0.011493	0.196055	0	0	13.1665	58.802375	DUSP1;FOS
Chemokine signaling pathway	3/189	0.015206	0.196055	0	0	6.128722	25.65534	PREX1;GRK4;PPBP
G alpha q pathway	2/70	0.015912	0.196055	0	0	11.03052	45.673675	RGS1;GRK4
AP-1 transcription factor network	2/70	0.015912	0.196055	0	0	11.03052	45.673675	DUSP1;FOS
Beta-oxidation of pristanoyl-CoA	1/8	0.021793	0.196055	0	0	52.74603	201.81503	CROT
Lysosphingolipid and lysophosphatidic acid (LPA) G-protein coupled receptors	1/8	0.021793	0.196055	0	0	52.74603	201.81503	S1PR1
TSP1-induced apoptosis in microvascular endothelial cell	1/8	0.021793	0.196055	0	0	52.74603	201.81503	FOS
Response to elevated platelet cytosolic calcium	2/83	0.02192	0.196055	0	0	9.254135	35.354248	VWF;PPBP
Prostanoid ligand receptors	1/9	0.024484	0.196055	0	0	46.15046	171.2057	PTGER3
MAP kinase pathway regulation through dual specificity phosphatases	1/9	0.024484	0.196055	0	0	46.15046	171.2057	DUSP1
Activation of the AP-1 family of transcription factors	1/10	0.027168	0.196055	0	0	41.02058	147.90848	FOS
Glycoprotein 1b-IX-V activation signaling	1/10	0.027168	0.196055	0	0	41.02058	147.90848	VWF
Attenuation of GPCR signaling	1/11	0.029845	0.196055	0	0	36.91667	129.64209	GRK4
TGF-beta signaling in gastrointestinal stem cells	1/11	0.029845	0.196055	0	0	36.91667	129.64209	ACVRL1
Transport of organic anions	1/11	0.029845	0.196055	0	0	36.91667	129.64209	SLCO3A1
ERBB1 downstream pathway	2/106	0.034441	0.196055	0	0	7.199202	24.250494	DUSP1;FOS
Platelet adhesion to exposed collagen	1/13	0.035176	0.196055	0	0	30.7608	102.96826	VWF
Organic anion transporters	1/13	0.035176	0.196055	0	0	30.7608	102.96826	SLC22A7
Organic cation/anion/zwitterion transport	1/13	0.035176	0.196055	0	0	30.7608	102.96826	SLC22A7
G alpha i pathway	2/108	0.035636	0.196055	0	0	7.062656	23.549745	RGS1;GRK4
Leptin influence on immune response	2/110	0.036846	0.196055	0	0	6.931167	22.879786	FOS;PPBP
Interleukin-4 regulation of apoptosis	3/267	0.037114	0.196055	0	0	4.300918	14.166242	S1PR1;FOS;CCDC71
T cell receptor/Ras pathway	1/14	0.037831	0.196055	0	0	28.39316	92.976854	FOS
Repression of pain sensation by the transcriptional regulator DREAM	1/15	0.040479	0.196055	0	0	26.36376	84.547825	FOS
Calcium signaling by HBx of hepatitis B virus	1/15	0.040479	0.196055	0	0	26.36376	84.547825	FOS
Coagulation intrinsic pathway	1/15	0.040479	0.196055	0	0	26.36376	84.547825	VWF
Eicosanoid ligand-binding G-protein coupled receptors	1/15	0.040479	0.196055	0	0	26.36376	84.547825	PTGER3
Erythrocyte differentiation pathway	1/15	0.040479	0.196055	0	0	26.36376	84.547825	FLT3
Amino acid biosynthesis and interconversion (transamination)	1/16	0.04312	0.196055	0	0	24.60494	77.35242	PSPH
B cell survival pathway	1/16	0.04312	0.196055	0	0	24.60494	77.35242	FOS
Selenium metabolism and selenoproteins	1/16	0.04312	0.196055	0	0	24.60494	77.35242	FOS
CD40L signaling pathway	1/16	0.04312	0.196055	0	0	24.60494	77.35242	DUSP1
Other semaphorin interactions	1/16	0.04312	0.196055	0	0	24.60494	77.35242	SEMA3E
G alpha s pathway	2/120	0.043131	0.196055	0	0	6.340582	19.931738	RGS1;GRK4
GRB2-SOS provides linkage to MAPK signaling for integrins	1/17	0.045753	0.196055	0	0	23.06597	71.146852	VWF
Renin-angiotensin system	1/17	0.045753	0.196055	0	0	23.06597	71.146852	ENPEP
Cadmium-induced DNA biosynthesis and proliferation in macrophages	1/17	0.045753	0.196055	0	0	23.06597	71.146852	FOS
Interleukin-1 signaling pathway	2/125	0.046413	0.196055	0	0	6.081301	18.670676	DUSP1;FOS
Pertussis toxin-insensitive CCR5 signaling in macrophage	1/18	0.04838	0.196055	0	0	21.70806	65.746712	FOS
Bone remodeling	1/18	0.04838	0.196055	0	0	21.70806	65.746712	FOS
TNFR2 signaling pathway	1/18	0.04838	0.196055	0	0	21.70806	65.746712	DUSP1
Effect of METS on macrophage differentiation	1/18	0.04838	0.196055	0	0	21.70806	65.746712	FOS
PDGFB signaling pathway	2/129	0.049103	0.196055	0	0	5.888575	17.7472	S1PR1;FOS

4.7 REFERENCES

1. Woodward, W. A., Debeb, B. G., Xu, W. & Buchholz, T. A. Overcoming radiation resistance in inflammatory breast cancer. in *Cancer* **116**, (2010).

2. Mohamed, M. M., Al-Raawi, D., Sabet, S. F. & El-Shinawi, M. Inflammatory breast cancer: New factors contribute to disease etiology: A review. *Journal of Advanced Research* **5**, 525–536 (2014).

3. Pan, E. *et al.* Inflammatory breast cancer outcomes in a contemporary series. *Anticancer Res.* **37**, (2017).

4. Rehman, S., Reddy, C. A. & Tendulkar, R. D. Modern outcomes of inflammatory breast cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **84**, (2012).

5. Van Laere, S. J. *et al.* Uncovering the molecular secrets of inflammatory breast cancer biology: An integrated analysis of three distinct affymetrix gene expression datasets. *Clin. Cancer Res.* **19**, (2013).

6. Woodward, W. A. *et al.* Genomic and expression analysis of microdissected inflammatory breast cancer. *Breast Cancer Res. Treat.* **138**, 761–772 (2013).

7. Bertucci, F. *et al.* Genomic profiling of inflammatory breast cancer: A review. *Breast* **23**, (2014).

8. Lim, B., Woodward, W. A., Wang, X., Reuben, J. M. & Ueno, N. T. Inflammatory breast cancer biology: the tumour microenvironment is key. *Nature Reviews Cancer* **18**, (2018).

9. Chakraborty, P., George, J. T., Woodward, W. A., Levine, H. & Jolly, M. K. Gene expression profiles of inflammatory breast cancer reveal high heterogeneity across the epithelial-hybrid-mesenchymal spectrum. *Transl. Oncol.* **14**, (2021).

10. Boersma, B. J. *et al.* A stromal gene signature associated with inflammatory breast cancer. *Int. J. Cancer* **122**, (2008).

11. Lerebours, F. *et al.* Hemoglobin overexpression and splice signature as new features of inflammatory breast cancer? *J. Adv. Res.* **28**, (2021).

12. Bertucci, F. *et al.* Gene expression profiling for molecular characterization of

inflammatory breast cancer and prediction of response to chemotherapy. *Cancer Res.* **64**, 8558–8565 (2004).

13. Van Laere, S. *et al.* Distinct molecular signature of inflammatory breast cancer by cDNA microarray analysis. *Breast Cancer Res. Treat.* **93**, (2005).

14. Woodward, W. A. *et al.* Genomic and expression analysis of microdissected inflammatory breast cancer. *Breast Cancer Res. Treat.* **138**, 761–772 (2013).

15. Creed, J., Gerke, T. & Berglund, A. MatSurv: Survival analysis and visualization in MATLAB. *J. Open Source Softw.* **5**, (2020).

16. Bernard, P. S. *et al.* Supervised risk predictor of breast cancer based on intrinsic subtypes. *J. Clin. Oncol.* **27**, (2009).

17. Gendoo, D. M. A. *et al.* Genefu: An R/Bioconductor package for computation of gene expression-based signatures in breast cancer. *Bioinformatics* **32**, (2016).

18. Kuleshov, M. V. *et al.* Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res.* **44**, (2016).

19. Buchholz, T. A. *et al.* Global gene expression changes during neoadjuvant chemotherapy for human breast cancer. *Cancer J.* **8**, 461–468 (2002).

20. Fernandez, S. V. *et al.* Inflammatory breast cancer (IBC): Clues for targeted therapies. *Breast Cancer Res. Treat.* **140**, (2013).

21. Forozan, F. *et al.* Molecular cytogenetic analysis of 11 new breast cancer cell lines. *Br. J. Cancer* **81**, (1999).

22. Huang, A., Cao, S. & Tang, L. The tumor microenvironment and inflammatory breast cancer. *Journal of Cancer* **8**, (2017).

CHAPTER 5

General Discussion and Future Directions

5.1 GENERAL DISCUSSION

This study helps us understand the importance of TME in promoting IBC metastatic behavior. Recent studies indicated that inflammatory pathways such as NF-κB concomitant with the secretion of cytokines are key elements in nurturing the IBC TME [1] [2]. Although NF-κB and its target genes are known to be upregulated in IBC tumor samples [3] [4] [5], its direct effect has never been studied [1]. Herein I show for the first time how NF-κB activation through the receptor tyrosine kinase RIPK2 can contribute to IBC progression by promoting metastatic phenotypes in cancer cells. This function is mediated by the increase of cytokines production following RIPK2 activation. I also demonstrate the status of RIPK2 activity in IBC tumor samples using a special antibody directed against the phosphorylation site Y474. In addition, I identify a robust gene signature able to differentiate IBC samples from non-IBC and highlight the role of cytokines in this type of breast cancer. Altogether, these findings strongly suggest that inflammatory pathways play an essential role in modulating IBC tumorigenesis.

5.1.1 Inflammation in IBC

It is well documented that inflammation increases the risk of cancer [6]. The constitutive activation of inflammatory pathways accompanied by the release of proinflammatory mediators and accumulation of inflammatory cells creates the optimum niche for cancer development and progression [1] [6] [7]. NF-κB signaling pathway is one of the main inflammatory pathways linked to cancer cell proliferation, angiogenesis, metastasis, and therapy resistance [4] [8]. Research shows that NF-κB is constitutively active in almost all types of cancer, including breast cancer [9] [10]. The activation or upregulation of NF-κB correlates with more aggressive types of breast cancer such

as TNBC and IBC [3 4 5 11 12], which agrees with the high levels of phospho p65 we found in IBC cells compared to non-IBC (Chapter 2). It's unclear what triggers abnormal activation of NF-κB, known as "NF-κB addiction" in tumor cells [7 13]. However, our results suggest that autocrine and paracrine signals mediated by RIPK2 activity and cytokine production can stimulate NF-κB activation in IBC cells (Chapter 3). RIPK2 activity, in particular phosphorylation, is required for protein stability [14] and RIPK2-induced NF-κB activation [15 16 17]. IBC tumor samples and cell lines show higher levels of active RIPK2 compared to non-IBC, which positively correlates with the advanced tumor size, metastasis status, and cancer stage (Chapter 2). A recent study on ovarian cancer using immunohistochemistry revealed that RIPK2 protein expression is higher in metastatic versus non-metastatic tumor tissues [18]. Similar results are also seen in prostate cancer[19]. At the transcriptome level, Oncomine database analysis revealed *RIPK2* is highly expressed in many types of cancer, including breast cancer[20]. In particular, more aggressive types of breast cancer such as TNBC showed higher RIPK2 mRNA expression, which correlates with worse progression-free survival[21]. In our IBC gene expression data study, there was no difference in *RIPK2* expression levels compared to non-IBC samples (Chapter 2). This is partly due to the heterogeneity of IBC samples, where all breast cancer subtypes are present.

5.1.2 Microbial inflammation

Pattern recognition receptors (PRRs) are membrane-bound receptors located on the cell surface, such as TLRs (Toll-like receptors) or in the cell cytoplasm such as NLRs (NOD-like receptor) [22]. These receptors can recognize harmful stimuli, including pathogen-associated molecular patterns (PAMPs) released by microbes or damage-associated molecular patterns (DAMPs) secreted during tissue injury [22]. When NLRs (NOD1 and NOD2) and TLRs (TLR2 and TLR4) are engaged, signal mediators such as RIPK2 are activated [23 24 25]. In our study of RIPK2

function, we used MDP (Muramyl dipeptide), peptidoglycan found in most bacterial cell walls and recognized by NOD2 receptor [26] [27]. The use of MDP recapitulates RIPK2 activation and subsequently the activation of NF-κB seen in IBC (Chapter 3).

Microbial inflammation tied to cancer is evident in many diseases; for example, human papillomavirus (HPV) is associated with an increased risk of cervical cancer [28] and *Helicobacter pylori* is a major risk of stomach cancer [29]. Nonetheless, microbial inflammation is not limited to pathogenic microorganisms. New evidence suggests the mucosal microbiome can influence cancer progression through modulating TME [30] [31] [32]. Indeed, polymorphic microbiomes are recently added as a factor that can influence many of the cancer hallmarks, such as inflammation and genomic instability [33]. The microbiome can trigger an immune reaction through PRR receptors, including NODs and TLR, causing the release of cytokines and chemokine, which can travel via systemic circulation to the tumor site [31] [30]. Environmental factors such as obesity and intake of antibiotics, for example, can lead to microbiome disturbance in the intestinal immune cells, which stimulate inflammatory cytokine production and consequently increase the risk of cancer, including breast cancer [34] [31]. Obesity is one of the few risk factors identified in IBC [35] [36]. In our study of RIPK2, we found that RIPK2 activity positively correlates with the BMI of breast cancer patients (Chapter 2). While this association can be due to the link of RIPK2 to obesity-induced inflammation via increasing insulin resistance and dysglycemia[37] [38] [39]; other factors such as the polymorphic microbiome can be a contributing factor.

5.1.3 Non-microbial inflammation

In sterile or non-microbial inflammation, damage-associated molecular patterns (DAMPs) secreted during cellular stress or injury trigger the activation of PRRs and downstream mediators mimicking microbial-induced inflammation[40]. Results show that DAMP-mediated activation of

PRRs is marked by the recruitment of immune cells such as neutrophils and macrophages along with the release of proinflammatory cytokine, IL1β and TNF [40]. Interestingly, the RIPK2-CARD domain binds to caspase-1 [41] [42] [43], which is required for pro-IL-1β cleavage and activation[44]. In addition, bone marrow-derived macrophages (BMDM) collected from *RIPK2*$^{-/-}$ mice show depletion of caspase-1 and IL-1β[45]. RNA-seq results didn't show a change in *IL-1α* nor *IL-1β* levels in the RIPK2 rescue cells, however, IL-1 receptors (*IL-1RI, IL-1RII*) and *TNF* levels were increased (Chapter 3).

Sterile inflammation is mainly triggered during cell death, including necrosis, necroptosis, and immunogenic cell death (ICD), leading to the release of many cell components that act as DAMPs[46]. Different forms of death take place during cancer progression and treatment. Necrosis is commonly seen in the core of solid tumors due to the hypoxic environment, which causes the release of cell debris to the TME. This results in PRRs activation and cytokine production, promoting cancer progression[46] [47] [48]. Similarly, cancer treatment such as chemotherapy and radiation leads to necroptosis and ICD, and eventually, the release of DAMPs into the surrounding tissue, triggering inflammatory receptors [49] [50]. This aligns with our results, where the increase of RIPK2 activity was seen in IBC patients' samples collected post-chemotherapy (Chapter 2), suggesting that RIPK2 activity increases in response to stress in the microenvironment.

5.1.4 Linking DNA mutations to protein signaling

The genomic alteration, including base pair mutation, deletion, insertions, and loss or gain of chromosomal fragments, is common in all cancer types. Such alterations enable cancer cells to regulate proliferative signaling, resist cell death, induce angiogenesis, activate metastasis, and evade growth suppressors [51]. Breast cancer genome studies reported genetic alteration in the long

arm of chromosome 8, with amplification at the 8q21 region[52] [53] [54], where RIPK2 is located (8q21.3)[19]. The 8q21 amplification correlated with higher tumor grade and amplification of other oncogenes, including *MYC, HER2, and MDM2* [54]. High-resolution arrays revealed that IBC cells showed higher amplification of 8q (3–10 copies) compared to non-IBC cells (1–4 copies); and though *RIPK2* amplification was not looked at, *MYC* amplification was increased in IBC (2.5–7 copies), while non-IBC cells showed (1-3 copies)[55]. Focusing on regions of gene amplification at chromosome 8 and searching for putative oncogenes, Inaki and his team identified RIPK2 based on MYC co-amplification and its association with a poor prognosis in breast cancer patients[56]. Note that *RIPK2* is close to *MYC* in 8q [19]. The analysis of cancer genomics datasets at cBioPortal revealed that RIPK2 is highly mutated with 8.77% amplification in uterine carcinosarcoma and 8.58% in breast invasive carcinoma [57]. In support of the previous research, a large-scale proteogenomic study linking somatic mutation to protein signaling identified RIPK2 as an outlier kinase in the breast cancer genome. Analysis revealed RIPK2 exhibited similar gene-amplification patterns to ERBB2. Further, the number of RIPK2 phosphosite outliers increased in basal-like breast cancer, suggesting that RIPK2 could be a potential target kinase beyond HER2 [58].

In a highly cited study of breast cancer mutations, 40 cancer genes were identified with a driver mutation or copy number change, with *TP53* in the top identified genes [59]. In basal-like breast cancer, *TP53* mutations are reported in around 84% of cases [60]. p53 or the "Guardian of the genome" functions as a tumor suppressor where it regulates many cell functions, including DNA damage repair, cell death, cell cycle arrest, immune cell recruitment, and senescence, in response to stress signals such as carcinogens, reactive oxygen species (ROS), hypoxia and inflammation [61] [62]. However, *TP53* mutation, found in most cancers, results in a loss of its tumor-suppressive function and a gain of function (GOF), exerting oncogenic properties [63] [64]. Subsequently, many

p53 target genes are affected by this genetic alteration [65]. In a study of *TP53* related genes in acute myeloid leukemia (AML), RNA-seq results of *TP53*-mutated bone marrow (BM) samples showed higher expression of NF-κB pathway genes than BM samples with *TP53* wild type. Interestingly, *RIPK2* is in the top ten differentially expressed genes (2.2 $\log_2$ fold change and p-value= 9.04×10^{-13}). Other related genes, including *IL-8, IL-6, CXCL1, CXCL2, CCL2*, and *IFN-γ* were also highly expressed in *TP53*-mutated patients' samples[66]. In addition, in a study to identify p53 synthetic lethal genes as a potential drug target for cancer treatment, using glioblastoma multiforme TCGA dataset to compare gene expression profile of p53 mutated sample to non-mutated ones; *RIPK2* was identified as a candidate gene along with Activin A receptor 1 and 2A (*ACVR1, ACVR2A*)[67]. Taken together, RIPK2 and its related cytokines identified in our study (chapter 3) are also identified in *TP53* mutated tumors, suggesting that RIPK2 function might be mediated by genetic alterations.

5.1.5 Phosphorylation dysregulation

Using phosphoproteomic profiles of breast tumor samples[58], Huang and his team characterized phospho-signaling kinases and validated the results in 24 breast cancer patient-derived xenografts (PDXs). They identified that RIPK2 in both sets of samples has a high percentage of *cis*-regulated phosphosites that correlate with RIPK2 protein expression and undergo autophosphorylation. Also, two of the identified regulated phosphosites, S527/S529, are in structural proximity to the RIPK2 active site, suggesting that changes in the active site interaction and kinase activity levels is possible[68]. The dysregulation in the activity level of RIPK2 can alter its downstream signaling pathway and induce tumor metastasis, as seen in our results (Chapter 2 and 3). Besides, RIPK2 undergoes asymmetric *trans*-autophosphorylation, where each monomer

phosphorylates and activates the other[14]. This trans-autophosphorylation is stabilized allosterically by dimerization and without activation-loop phosphorylation, making RIPK2 in a state called a "prone-to-auto-phosphorylate" conformation [69] [14]. As a result, any mutation in RIPK2 can induce constitutive dimerization and autophosphorylation independent of inflammatory signals.

5.1.6 RIPK2 alternative pathways to promote metastasis

In a study of RIPK2 in human kidney renal clear cell carcinoma (ccRCC), RIPK2 knockdown showed decreased tumor cell proliferation, migration, and colony formation. Phosphorylation of JNK (c-Jun N-terminal kinase) and NF-κB p65 subunit was also reduced due to this knockdown, which suggested that JNK mediates RIPK2 function alongside NF-κB [70]. Similarly, RIPK2 regulated migration and invasion in triple-negative breast cancer, and its knockdown showed a decrease in NF-κB, JNK, and FAK (Focal adhesion kinase) activity levels. However, no decrease in the phosphorylation level of the other mitogen-activated protein kinases (MAPKs), p38, and ERK (extracellular signal-regulated kinase) was observed. The author then concluded that JNK and FAK mediate RIPK2 function but not NF-κB using the RIPK2 inhibitor PP2 [71]. Note that FAK1 is amplified in IBC cells, showing more copies (2.5–7 copies) than in non-IBC (1–2.5 copies)[55]. In glioblastoma, RIPK2 regulated cell growth through p38 activation[72], which is proven to be mediated through TAK1 (Transforming growth factor-β (TGF-β)-activated kinase 1)[73]. In prostate cancer, RIPK2 appears to function independently from the NF-κB pathway. Indeed, RIPK2 regulates the activation and stabilization of c-Myc, mainly through RIPK2 direct interaction with MKK7 and JNK.[19].

In our study of IBC, we used protein arrays as an unbiased approach to identify how RIPK2 can regulate cancer cell function (Chapter 3). RIPK2 knockdown showed upregulation of

inflammatory cytokines via NF-κB pathway, and similar results were seen in RIPK2 rescue cells, and NF-κB overexpressed cells. RIPK2 activation of NF-κB is known to be NOD dependent[17], however, no significant change was seen in NOD1 nor NOD2 expression in RNA-seq results, which is explained by the lack of pathogen stimulator but also suggests that changes in RIPK2 expression levels through genetic alteration can trigger an NF-κB inflammatory response.

5.1.7 Crosstalk with inflammatory pathways

The crosstalk of NF-κB with other signaling pathways was evident in the transcriptome sequencing as many genes involved in JAK-STAT, TGF-β and TNF signaling pathways were upregulated in RIPK2 expressing cells. Our results show upregulation of *STAT2, STAT3, STAT5A,* and S*TAT6*; some are known for their direct interaction with NF-κB [74] [75] [76]. Nevertheless, *JAK1* and *JAK2* show no significant difference in expression in RIPK2 rescue compared to KO control. This suggests the RIPK2 regulation of STAT mediated by NF-κB is JAK independent; however, further investigation is needed. TNF genes involved in cancer progression, such as *TNF-α* and NF-κB signal regulation such as *TNFAIP3/A20* [77] [78] were overexpressed in rescue cells. TGF-β signaling genes including *SMAD3, SMAD6, SMAD7, SMAD9, BMP2, BMP5, INHBB,* and *ACVR2A* show an increase of expression in RIPK2 rescue cells. Sufficient evidence demonstrates the communication between NF-κB and different types of SMADs and TGF-β signaling proteins such as bone morphogenetic proteins (BMPs), mainly through TAK1, which is downstream of RIPK2 [79] [80] [81] [82]. More recent evidence also reveals the role of NF-κB in Activin (a member of the TGF-β family) expression. One study shows that NF-κB stimulation through TNF-α and IL-8 leads to increased Activin A expression [83], which supports our findings. Moreover, the upregulation of NF-KB signaling mediated by RIPK2 expression along with the induction of other pathways

resulted in the expression of many genes involved in extracellular matrix organization such as *MMP9, MMP13, COL17A1, COL25A1, LAMC3, and EDIL3* and Epithelial-Mesenchymal Transition (EMT) such as *CDH1, SNAI2, VIM-AS1, ZEB2, FOXC2,* and *AIFM2*.

5.1.8 Genes associated with RIPK2 expression in relation to IBC

Several genes involved in IBC progression besides NF-κB and its targets were transcriptionally upregulated in RIPK2 rescue cells such as *ALDH1*[84], *CAV1*[85], *ROHA* [86], *EGFR* [87], and *SYK* [88]. In addition, an overlap was found in some genes, including *ABCC9, PPBP, TRPV3, VWF, TCIM*, and *S1PR1* identified in IBC-specific gene signature G59 [89]; however, further studies are needed to understand the role of these genes in IBC and how RIPK2 regulates them. RNA-seq results also showed downregulation of genes involved in MHC-II antigen-presenting cells such as (*CD74, CIITA, HLA-DPB1, HLA-DRA, HLA-DRB1, HLA-DRB5, HLA-DQB1, HLA-DMB*) in RIPK2 rescue cells. RIPK2 is involved in MHC-II antigen-presentation regulation[90]. The increase of MHC-II expression by tumor cells correlates with better prognosis in different types of cancers [91] [92] [93] [94]. In a study of TNBC, patients with high expression of MHC-II genes (*CIITA, CD74, HLA-DPA1, HLA-DPB1, HLA-DPB2, HLA-DQA1, HLA-DRB1, HLA-DRB5,* and *HLA-DRB6*) had higher progression-free survival [93]. In addition, immunohistochemistry analysis of several MHC-II molecules in 681 TNBC patients showed a positive correlation between the increase of MHC-II and the absence of lymphovascular invasion [91], which is seen more in IBC patients[95].

5.1.9 RIPK2 inhibition is a potential treatment for IBC

Several pieces of supporting evidence makes RIPK2 a candidate target for cancer treatment, as suggested by Yiwu Yan in his study of RIPK2 in prostate cancer[19]. The same

evidence applies to breast cancer wherein (1) Increase of RIPK2 expression and genetic alteration occurs in advanced breast cancer[20 21 56 58], (2) Increased RIPK2 expression correlates with poor prognosis[21], (3) RIPK2 associates with *TP53* mutation[60], and (4) Effective small-molecule inhibitors are available[96 97 98 17]. Our study further supports the role of RIPK2 as an oncogene in IBC through regulating TME inflammatory mediators.

IBC diagnosis and treatment have improved in the last ten years; however, the lack of a specific gene signature (addressed in Chapter 3) and personalized treatment are still two main challenges. The 5-year survival rate of IBC is still significantly low compared to non-IBC (39% *vs.* 90%)[99]. The median overall survival is 4.75 years for IBC patients with stage III versus 13.4 years in non-IBC of the same stage [100]. Several ongoing clinical trials are using kinase inhibitors for IBC treatment[101]. The use of Panitumumab, a monoclonal antibody directed against *EGFR*, along with chemotherapy, increases the pathological complete response (pCR) rate, particularly in triple-negative IBC patients. Ruxolitinib, a JAK1 and JAK2 inhibitor, is now in phase II study, used in combination with chemotherapy for triple-negative IBC[101]. The success of such clinical trials supports the efficacy of using RIPK2 inhibitors in IBC treatment.

Numerous small molecule inhibitors of RIPK2 have been identified as a potential treatment for inflammatory diseases[96 97 98 17]. GSK583 is a RIPK2 inhibitor in clinical trials (NCT03358407) to treat Inflammatory Bowel Diseases[102]. GSK583 was efficient in inhibiting prostate cancer cell invasion, colony formation, and metastasis in animal modal, with no toxicity seen in mice[19]. Similar results were seen with the use of ponatinib, an FDA-approved drug for the treatment of chronic myeloid leukemia (CML)[19]. These findings pave the road for RIPK2 inhibitors to be used in IBC treatment alone or in combination with chemotherapy. Such inhibitors can improve disease progression by inhibiting critical inflammatory mediators, including IL-6 and IL-8.

5.2 FUTURE DIRECTION

Our study has revealed several new avenues to be explored further:

Examine RIPK2 genetic mutation in inflammatory breast cancer

Based on our results and others, increased RIPK2 activity or mRNA expression could be linked to a genetic alteration besides the presence of an inflammatory trigger. We know that RIPK2 is present at the long arm of chromosome 8, a region highly mutated in breast cancer and more in IBC[52 53 54 55]. According to a proteogenomic study, RIPK2 showed higher amplification in breast cancer samples[58]. Little is known about RIPK2 genetic alterations in breast cancer, including IBC. Determining the copy number of RIPK2 across different breast cancer subtypes would give us an idea of what causes increases in RIPK2 expression.

Examine the link of RIPK2 to c-MYC breast cancer

It is interesting to see how RIPK2 function can be mediated by pathways other than NF-κB. In the prostate cancer study, the author determines that RIPK2 increased metastasis and invasion through stabilizing c-MYC by interacting with MKK7, independent of the NF-κB signaling pathway[19]. Though we used an unbiased approach to determine the mechanisms by which RIPK2 may regulate IBC metastasis, it would be ideal to examine alternative pathways, especially considering that high rates of MYC mutation is found in IBC as compared to non-IBC[55] and that co-amplification of MYC and RIPK2 has been identified[19].

Characterization of Inhibin A in IBC

Our results demonstrate RIPK2 regulation of Inhibin A in IBC cells. RIPK2 activation showed a reduction in Inhibin A (*INHA*) expression; however, when NF-κB is expressed, Inhibin A (*INHA*) expression was increased. This result suggested that RIPK2 can regulate Inhibin A through other pathways. Further, little is known about the role of Inhibin A in cancer, but many suggested that it functions as a tumor suppressor[103].

Validation of the IBC signature genes

Finding a molecular profile that distinguishes IBC from non-IBC has been a challenge for a long time. We were able to identify an IBC gene signature (G59) using machine learning that was validated in an independent dataset. Several genes from the G59 were transcriptionally upregulated in RIPK2 rescue cells, including *CXCL7, ABCC9, VWF, TRPV3,* and *TCIM.* It would be interesting to characterize the function of these genes in IBC cells in relation to RIPK2.

5.3 CONCLUSIONS

In conclusion, this thesis has revealed, for the first time, a critical role for RIPK2 in the regulation of IBC phenotypes. These results suggest that RIPK2 may be an attractive target for this poorly managed disease.

5.4 REFERENCES

1. Lim, B., Woodward, W. A., Wang, X., Reuben, J. M. & Ueno, N. T. Inflammatory breast cancer biology: the tumour microenvironment is key. *Nature Reviews Cancer* **18**, (2018).
2. Huang, A., Cao, S. & Tang, L. The tumor microenvironment and inflammatory breast cancer. *Journal of Cancer* **8**, 1884–1891 (2017).
3. Park, M. H. & Hong, J. T. Roles of NF-κB in cancer and inflammatory diseases and their therapeutic approaches. *Cells* **5**, (2016).
4. Xia, Y., Shen, S. & Verma, I. M. NF-κB, an active player in human cancers. *Cancer immunology research* **2**, (2014).
5. Van Laere, S. *et al.* Distinct molecular signature of inflammatory breast cancer by cDNA microarray analysis. *Breast Cancer Res. Treat.* **93**, (2005).
6. Coussens, L. M. & Werb, Z. Inflammation and cancer. *Nature* **420**, (2002).
7. Wang, W., Nag, S. & Zhang, R. Targeting the NFκB Signaling Pathways for Breast Cancer Prevention and Therapy. *Curr. Med. Chem.* **22**, (2014).
8. Liu, T., Zhang, L., Joo, D. & Sun, S. C. NF-κB signaling in inflammation. *Signal Transduction and Targeted Therapy* **2**, (2017).
9. Ben-Neriah, Y. & Karin, M. Inflammation meets cancer, with NF-κB as the matchmaker. *Nature Immunology* **12**, (2011).
10. Aggarwal, B. B. & Sung, B. NF-κB in cancer: A matter of life and death. *Cancer Discov.* **1**, (2011).
11. Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, R. J. & Sledge, G. W. Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. *Mol. Cell. Biol.* **17**, (1997).
12. Biswas, D. K. *et al.* NF-κB activation in human breast cancer specimens and its role in cell proliferation and apoptosis. *Proc. Natl. Acad. Sci. U. S. A.* **101**, (2004).
13. Chaturvedi, M. M., Sung, B., Yadav, V. R., Kannappan, R. & Aggarwal, B. B. NF-κB addiction and its role in cancer: One size does not fit all. *Oncogene* **30**, (2011).
14. Pellegrini, E., Signor, L., Singh, S., Erba, E. B. & Cusack, S. Structures of the inactive and active states of RIP2 kinase inform on the mechanism of activation. *PLoS One* (2017). doi:10.1371/journal.pone.0177161

15. Nembrini, C. *et al.* The kinase-activity of Rip2 determines its stability and consequently Nod1 - and Nod2-mediated immune responses. *J. Biol. Chem.* (2009). doi:10.1074/jbc.M109.006353
16. Tigno-Aranjuez, J. T., Asara, J. M. & Abbott, D. W. Inhibition of RIP2's tyrosine kinase activity limits NOD2-driven cytokine responses. *Genes Dev.* **24**, 2666–77 (2010).
17. Nachbur, U. *et al.* A RIPK2 inhibitor delays NOD signalling events yet prevents inflammatory cytokine production. *Nat. Commun.* **6**, 6442 (2015).
18. Zhang, W. J. & Wang, Y. Y. Activation of RIPK2-mediated NOD1 signaling promotes proliferation and invasion of ovarian cancer cells via NF-κB pathway. *Histochem. Cell Biol.* **157**, (2022).
19. Yan, Y. *et al.* RIPK2 stabilizes c-Myc and is an actionable target for inhibiting prostate cancer metastasis. *bioRxiv* (2020). doi:10.1101/2020.05.14.096867
20. Yang, Q., Tian, S., Liu, Z. & Dong, W. Knockdown of RIPK2 Inhibits Proliferation and Migration, and Induces Apoptosis via the NF-κB Signaling Pathway in Gastric Cancer. *Front. Genet.* **12**, (2021).
21. Singel, S. M. *et al.* Receptor-interacting protein kinase 2 promotes triple-negative breast cancer cell migration and invasion via activation of nuclear factor-kappaB and c-Jun N-terminal kinase pathways. *Breast Cancer Res.* **16**, (2014).
22. Takeuchi, O. & Akira, S. Pattern Recognition Receptors and Inflammation. *Cell* (2010). doi:10.1016/j.cell.2010.01.022
23. Kobayashi, K. *et al.* RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* (2002). doi:10.1038/416194a
24. Watanabe, T. *et al.* RICK/RIP2 is a NOD2-independent nodal point of gut inflammation. *Int. Immunol.* **31**, (2019).
25. Honjo, H., Watanabe, T., Kamata, K., Minaga, K. & Kudo, M. RIPK2 as a New Therapeutic Target in Inflammatory Bowel Diseases. *Frontiers in Pharmacology* **12**, (2021).
26. Inohara, N. *et al.* Host recognition of bacterial muramyl dipeptide mediated through NOD2: Implications for Crohn's disease. *J. Biol. Chem.* (2003). doi:10.1074/jbc.C200673200
27. Chamaillard, M. *et al.* An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat. Immunol.* (2003). doi:10.1038/ni945
28. Demarco, M. *et al.* A study of type-specific HPV natural history and implications for

contemporary cervical cancer screening programs. *EClinicalMedicine* **22**, (2020).

29. Polk, D. B. & Peek, R. M. Helicobacter pylori: Gastric cancer and beyond. *Nature Reviews Cancer* **10**, (2010).
30. Jain, T., Sharma, P., Are, A. C., Vickers, S. M. & Dudeja, V. New Insights Into the Cancer–Microbiome–Immune Axis: Decrypting a Decade of Discoveries. *Frontiers in Immunology* **12**, (2021).
31. Zheng, D., Liwinski, T. & Elinav, E. Interaction between microbiota and immunity in health and disease. *Cell Research* **30**, (2020).
32. Fessler, J., Matson, V. & Gajewski, T. F. Exploring the emerging role of the microbiome in cancer immunotherapy. *Journal for ImmunoTherapy of Cancer* **7**, (2019).
33. Hanahan, D. Hallmarks of Cancer: New Dimensions. *Cancer Discov.* **12**, 31–46 (2022).
34. Parida, S. & Sharma, D. Microbial Alterations and Risk Factors of Breast Cancer: Connections and Mechanistic Insights. *Cells* **9**, (2020).
35. Chang S, Buzdar A U & Hursting. Inflammatory breast cancer and body mass index. *J. Clin. Oncol.* **16**, 3731–3735 (1998).
36. Dietze, E. C., Chavez, T. A. & Seewaldt, V. L. Obesity and Triple-Negative Breast Cancer: Disparities, Controversies, and Biology. *American Journal of Pathology* **188**, 280–290 (2018).
37. Denou, E. *et al.* Defective NOD 2 peptidoglycan sensing promotes diet-induced inflammation, dysbiosis, and insulin resistance . *EMBO Mol. Med.* **7**, (2015).
38. Schertzer, J. D. *et al.* NOD1 activators link innate immunity to insulin resistance. *Diabetes* **60**, (2011).
39. Chi, W. *et al.* Bacterial peptidoglycan stimulates adipocyte lipolysis via NOD1. *PLoS One* **9**, (2014).
40. Chen, G. Y. & Nuñez, G. Sterile inflammation: Sensing and reacting to damage. *Nature Reviews Immunology* **10**, (2010).
41. Humke, E. W., Shriver, S. K., Starovasnik, M. A., Fairbrother, W. J. & Dixit, V. M. ICEBERG: A novel inhibitor of interleukin-1β generation. *Cell* **103**, (2000).
42. Zhang, W. H. *et al.* Fundamental role of the Rip2/caspase-1 pathway in hypoxia and ischemia-induced neuronal cell death. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 16012–16017 (2003).

43. Wang, X. *et al.* Dysregulation of receptor interacting protein-2 and caspase recruitment domain only protein mediates aberrant caspase-1 activation in Huntington's disease. *J. Neurosci.* **25**, 11645–11654 (2005).
44. Galliher-Beckley, A. J. Caspase-1 activation and mature interleukin-1β release are uncoupled events in monocytes. *World J. Biol. Chem.* **4**, (2013).
45. Wang, X. *et al.* The Prostaglandin E2-EP3 Receptor Axis Regulates Anaplasma phagocytophilum-Mediated NLRC4 Inflammasome Activation. *PLoS Pathog.* **12**, (2016).
46. Hernandez, C., Huebener, P. & Schwabe, R. F. Damage-associated molecular patterns in cancer: A double-edged sword. *Oncogene* **35**, (2016).
47. Vanlangenakker, N., Vanden Berghe, T. & Vandenabeele, P. Many stimuli pull the necrotic trigger, an overview. *Cell Death and Differentiation* **19**, (2012).
48. Kreuzaler, P. & Watson, C. J. Killing a cancer: What are the alternatives? *Nature Reviews Cancer* **12**, (2012).
49. Zitvogel, L., Galluzzi, L., Smyth, M. J. & Kroemer, G. Mechanism of Action of Conventional and Targeted Anticancer Therapies: Reinstating Immunosurveillance. *Immunity* **39**, (2013).
50. Kroemer, G., Galluzzi, L., Kepp, O. & Zitvogel, L. Immunogenic cell death in cancer therapy. *Annual Review of Immunology* **31**, (2013).
51. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: The next generation. *Cell* **144**, 646–674 (2011).
52. Buerger, H. *et al.* Comparative genomic hybridization of ductal carcinoma in situ of the breast - Evidence of multiple genetic pathways. *J. Pathol.* **187**, (1999).
53. Cingoz, S. *et al.* DNA copy number changes detected by comparative genomic hybridization and their association with clinicopathologic parameters in breast tumors. *Cancer Genet. Cytogenet.* **145**, (2003).
54. Choschzick, M. *et al.* Amplification of 8q21 in breast cancer is independent of MYC and associated with poor patient outcome. *Mod. Pathol.* **23**, (2010).
55. Fernandez, S. V. *et al.* Inflammatory breast cancer (IBC): Clues for targeted therapies. *Breast Cancer Res. Treat.* **140**, (2013).
56. Inaki, K. *et al.* Systems consequences of amplicon formation in human breast cancer. *Genome Res.* **24**, (2014).

57. Ermine, K., Yu, J. & Zhang, L. Role of Receptor Interacting Protein (RIP) kinases in cancer. *Genes and Diseases* (2021). doi:10.1016/j.gendis.2021.10.007
58. Mertins, P. *et al.* Proteogenomics connects somatic mutations to signalling in breast cancer. *Nature* **534**, 55–62 (2016).
59. Stephens, P. J. *et al.* The landscape of cancer genes and mutational processes in breast cancer. *Nature* **486**, (2012).
60. Koboldt, D. et al. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature* **490**, (2012).
61. Rokudai, S. Cancer. p53 Guardian of the Genome. *KITAKANTO Med. J.* **67**, (2017).
62. Levine, A. J. p53: 800 million years of evolution and 40 years of discovery. *Nat. Rev. Cancer* **20**, (2020).
63. Zhu, G. *et al.* Mutant p53 in Cancer Progression and Targeted Therapies. *Frontiers in Oncology* **10**, (2020).
64. Alvarado-Ortiz, E. *et al.* Mutant p53 Gain-of-Function: Role in Cancer Development, Progression, and Therapeutic Approaches. *Frontiers in Cell and Developmental Biology* **8**, (2021).
65. Fischer, M. Census and evaluation of p53 target genes. *Oncogene* **36**, (2017).
66. Vadakekolathu, J. *et al.* TP53 abnormalities correlate with immune infiltration and associate with response to flotetuzumab immunotherapy in AML. *Blood Adv.* **4**, (2020).
67. Wang, X. & Simon, R. Identification of potential synthetic lethal genes to p53 using a computational biology approach. *BMC Med. Genomics* **6**, (2013).
68. Huang, K. lin *et al.* Regulated phosphosignaling associated with breast cancer subtypes and druggability. *Mol. Cell. Proteomics* **18**, (2019).
69. Beenstock, J., Mooshayef, N. & Engelberg, D. How Do Protein Kinases Take a Selfie (Autophosphorylate)? *Trends in Biochemical Sciences* (2016). doi:10.1016/j.tibs.2016.08.006
70. Li, D. *et al.* RIPK2 is an unfavorable prognosis marker and a potential therapeutic target in human kidney renal clear cell carcinoma. *Aging (Albany. NY).* **13**, (2021).
71. Singel, S. M. *et al.* Receptor-interacting protein kinase 2 promotes triple-negative breast cancer cell migration and invasion via activation of nuclear factor-kappaB and c-Jun N-terminal kinase pathways. *Breast Cancer Res.* **16**, R28 (2014).

72. Cai, X. *et al.* RIP2 promotes glioma cell growth by regulating TRAF3 and activating the NF-κB and p38 signaling pathways. *Oncol. Rep.* **39**, 2915–2923 (2018).
73. Jacquet, S. *et al.* The role of RIP2 in p38 MAPK activation in the stressed heart. *J. Biol. Chem.* **283**, (2008).
74. Hoesel, B. & Schmid, J. A. The complexity of NF-κB signaling in inflammation and cancer. *Molecular Cancer* **12**, 1–15 (2013).
75. Nan, J., Wang, Y., Yang, J. & Stark, G. R. IRF9 and unphosphorylated STAT2 cooperate with NF-κB to drive IL6 expression. *Proc. Natl. Acad. Sci. U. S. A.* **115**, (2018).
76. Shen, C.-H. & Stavnezer, J. Interaction of Stat6 and NF-κB: Direct Association and Synergistic Activation of Interleukin-4-Induced Transcription. *Mol. Cell. Biol.* **18**, (1998).
77. Wu, Y. & Zhou, B. P. TNF-α/NFκ-B/Snail pathway in cancer cell migration and invasion. *British Journal of Cancer* **102**, (2010).
78. Kim, T., Bae, S. C. & Kang, C. Synergistic activation of NF-κB by TNFAIP3 (A20) reduction and UBE2L3 (UBCH7) augment that synergistically elevate lupus risk. *Arthritis Res. Ther.* **22**, (2020).
79. Ishinaga, H. *et al.* TGF-β induces p65 acetylation to enhance bacteria-induced NF-κB activation. *EMBO J.* **26**, (2007).
80. Freudlsperger, C. *et al.* TGF-β and NF-κB signal pathway cross-talk is mediated through TAK1 and SMAD7 in a subset of head and neck cancers. *Oncogene* **32**, (2013).
81. Luo, K. Signaling Cross Talk between TGF-b / Smad. *Cold Spring Harb. Persepctives Biol.* **9**, (2017).
82. Jimi, E. The Role of BMP Signaling and NF-κB Signaling on Osteoblastic Differentiation, Cancer Development, and Vascular Diseases-Is the Activation of NF-κB a Friend or Foe of BMP Function? in *Vitamins and Hormones* **99**, (2015).
83. Chen, L. *et al.* A NF-κB-Activin A signaling axis enhances prostate cancer metastasis. *Oncogene* **39**, (2020).
84. Gong, Y. *et al.* Aldehyde dehydrogenase 1 expression in inflammatory breast cancer as measured by immunohistochemical staining. *Clin. Breast Cancer* **14**, (2014).
85. Van Den Eynden, G. G. *et al.* Overexpression of caveolin-1 and -2 in cell lines and in human samples of inflammatory breast cancer. *Breast Cancer Res. Treat.* **95**, (2006).
86. Dobiasova, B. & Mego, M. Biomarkers for inflammatory breast cancer: Diagnostic and

therapeutic utility. *Breast Cancer: Targets and Therapy* **12**, (2020).

87. Lacerda, L. *et al.* Mesenchymal stem cells mediate the clinical phenotype of inflammatory breast cancer in a preclinical model. *Breast Cancer Res.* **17**, (2015).
88. Bertucci, F. *et al.* Gene expression profiling for molecular characterization of inflammatory breast cancer and prediction of response to chemotherapy. *Cancer Res.* **64**, (2004).
89. Zare, A., Postovit, L.-M. & Githaka, J. M. Robust inflammatory breast cancer gene signature using nonparametric random forest analysis. *Breast Cancer Res. 2021 231* **23**, 1–6 (2021).
90. Wu, X. M. *et al.* RIP2 Is a critical regulator for NLRs signaling and MHC antigen presentation but not for MAPK and PI3K/Akt pathways. *Front. Immunol.* **9**, (2018).
91. Park, I. A. *et al.* Expression of the MHC class II in triple-negative breast cancer is associated with tumor-infiltrating lymphocytes and interferon signaling. *PLoS One* **12**, (2017).
92. Oldford, S. A. *et al.* Tumor cell expression of HLA-DM associates with a Th1 profile and predicts improved survival in breast carcinoma patients. *Int. Immunol.* **18**, (2006).
93. Andres, F. *et al.* Expression of the MHC class II pathway in triple-negative breast cancer tumor cells is associated with a good prognosis and infiltrating lymphocytes. *Cancer Immunol. Res.* **4**, (2016).
94. Callahan, M. *et al.* Increased HLA-DMB expression in the tumor epithelium is associated with increased CTL infiltration and improved prognosis in advanced-stage serous ovarian cancer. *Clin. Cancer Res.* **14**, (2008).
95. Robertson, F. M. *et al.* Inflammatory Breast Cancer: The Disease, the Biology, the Treatment. *CA. Cancer J. Clin.* **60**, (2010).
96. Hollenbach, E. *et al.* Inhibition of RICK/nuclear factor-κB and p38 signaling attenuates the inflammatory response in a murine model of Crohn disease. *J. Biol. Chem.* **280**, (2005).
97. Tigno-Aranjuez, J. T. *et al.* In vivo inhibition of RIPK2 kinase alleviates inflammatory disease. *J. Biol. Chem.* **289**, 29651–29664 (2014).
98. Salla, M. *et al.* Identification and Characterization of Novel Receptor-Interacting Serine/Threonine-Protein Kinase 2 Inhibitors Using Structural Similarity Analysis. *J. Pharmacol. Exp. Ther.* **365**, 354–367 (2018).
99. Koch, R. M., Principe, D. R., Cataneo, J. L. & Rana, A. Progress for immunotherapy in inflammatory breast cancer and emerging barriers to therapeutic efficacy. *Cancers* **13**,

(2021).

100. Schlichting, J. A., Soliman, A. S., Schairer, C., Schottenfeld, D. & Merajver, S. D. Inflammatory and non-inflammatory breast cancer survival by socioeconomic position in the Surveillance, Epidemiology, and End Results database, 1990-2008. *Breast Cancer Res. Treat.* **134**, (2012).

101. Vagia, E. & Cristofanilli, M. New Treatment Strategies for the Inflammatory Breast Cancer. *Current Treatment Options in Oncology* **22**, (2021).

102. Haile, P. A. *et al.* The Identification and Pharmacological Characterization of 6-(tert-Butylsulfonyl)-N-(5-fluoro-1H-indazol-3-yl)quinolin-4-amine (GSK583), a Highly Potent and Selective Inhibitor of RIP2 Kinase. *J. Med. Chem.* (2016). doi:10.1021/acs.jmedchem.6b00211

103. Risbridger, G. P., Schmitt, J. F. & Robertson, D. M. Activins and inhibins in endocrine and other tumors. *Endocrine Reviews* **22**, (2001).

REFERENCES

- Abbott, D. W. et al. Coordinated Regulation of Toll-Like Receptor and NOD2 Signaling by K63-Linked Polyubiquitin Chains. Mol. Cell. Biol. (2007). doi:10.1128/mcb.00270-07
- Abbott, D. W., Wilkins, A., Asara, J. M. & Cantley, L. C. The Crohn's disease protein, NOD2, requires RIP2 in order to induce ubiquitinylation of a novel site on NEMO. Curr. Biol. (2004). doi:10.1016/j.cub.2004.12.032
- Alberts, B. et al. Molecular biology of the cell. (Garland Science, 2002).
- Allison, K. H. Molecular pathology of breast cancer: What a pathologist needs to know. American Journal of Clinical Pathology 138, (2012).
- Amin, M. B. et al. The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. CA. Cancer J. Clin. 67, (2017).
- Anne, N. & Pallapothu, R. Lymphoma of the breast: a mimic of inflammatory breast cancer. World J. Surg. Oncol. 9, (2011).
- Araki, T. & Milbrandt, J. ZNRF Proteins Constitute a Family of Presynaptic E3 Ubiquitin Ligases. J. Neurosci. (2003). doi:10.1523/jneurosci.23-28-09385.2003
- Ardito, F., Giuliani, M., Perrone, D., Troiano, G. & Muzio, L. Lo. The crucial role of protein phosphorylation in cell signalingand its use as targeted therapy (Review). International Journal of Molecular Medicine (2017). doi:10.3892/ijmm.2017.3036
- Azad, A. K. et al. Inactivation of endothelial cell phosphoinositide 3-kinase β inhibits tumor angiogenesis and tumor growth. Oncogene 39, (2020).
- Bae, J. R. & Lee, B. D. Function and dysfunction of leucine-rich repeat kinase 2 (LRRK2): Parkinson's disease and beyond. BMB Reports (2015). doi:10.5483/BMBRep.2015.48.5.032
- Bahr, C. et al. DIK, a novel protein kinase that interacts with protein kinase Cδ: Cloning, characterization, and gene analysis. J. Biol. Chem. (2000). doi:10.1074/jbc.M004771200
- Bakouny, Z. & Choueiri, T. K. IL-8 and cancer prognosis on immunotherapy. Nat. Med. 26, (2020).
- Balanathan, P. et al. Epigenetic regulation of inhibin α-subunit gene in prostate cancer cell lines. J. Mol. Endocrinol. 32, (2004).
- Barkataki, S. et al. Inflammatory Breast Cancer: A Panoramic Overview. J Rare Dis Res Treat 3, (2018).

- Barnich, N., Aguirre, J. E., Reinecker, H. C., Xavier, R. & Podolsky, D. K. Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor-KB activation in muramyl dipeptide recognition. J. Cell Biol. 170, (2005).
- Barton, G. M. & Kagan, J. C. A cell biological view of toll-like receptor function: Regulation through compartmentalization. Nature Reviews Immunology 9, (2009).
- Beahrs O, C. D. P. Manual for Staging of Cancer. American Joint Committee on Cancer. (Whiting Press, 1977).
- Beenstock, J., Mooshayef, N. & Engelberg, D. How Do Protein Kinases Take a Selfie (Autophosphorylate)? Trends in Biochemical Sciences (2016). doi:10.1016/j.tibs.2016.08.006
- Berg, J. M., Tymoczko, J. L. & Stryer, L. A Proton Gradient Powers the Synthesis of ATP. (2002).
- Bernard, P. S. et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J. Clin. Oncol. 27, (2009).
- Bertrand, M. J. M. et al. Cellular Inhibitors of Apoptosis cIAP1 and cIAP2 Are Required for Innate Immunity Signaling by the Pattern Recognition Receptors NOD1 and NOD2. Immunity (2009). doi:10.1016/j.immuni.2009.04.011
- Bertrand, M. J. M. et al. CIAP1/2 are direct E3 ligases conjugating diverse types of ubiquitin chains to receptor interacting proteins kinases 1 to 4 (RIP1-4). PLoS One (2011). doi:10.1371/journal.pone.0022356
- Bertucci, F. et al. Bevacizumab plus neoadjuvant chemotherapy in patients with HER2-negative inflammatory breast cancer (BEVERLY-1): a multicentre, single-arm, phase 2 study. Lancet Oncol. 17, 600–611 (2016).
- Bertucci, F. et al. Gene expression profiles of inflammatory breast cancer: Correlation with response to neoadjuvant chemotherapy and metastasis-free survival. Ann. Oncol. 25, (2014).
- Bertucci, F. et al. Genomic profiling of inflammatory breast cancer: A review. Breast 23, (2014).
- Bhat, V., Allan, A. L. & Raouf, A. Role of the microenvironment in regulating normal and cancer stem cell activity: Implications for breast cancer progression and therapy response. Cancers (Basel). 11, (2019).
- Bièche, I. et al. Molecular profiling of inflammatory breast cancer: Identification of a poor-prognosis gene expression signature. Clin. Cancer Res. 10, (2004).
- Birol, Melissa & Aude Echalier. Structure and Function of MPN (Mpr1/Pad1 N-terminal) Domain-Containing Proteins. Curr. Protein Pept. Sci. (2014).
- Bist, P. et al. E3 Ubiquitin ligase ZNRF4 negatively regulates NOD2 signalling and induces tolerance to MDP. Nat. Commun. (2017). doi:10.1038/ncomms15865

- Boersma, B. J. et al. A stromal gene signature associated with inflammatory breast cancer. Int. J. Cancer 122, (2008).
- Boulanger, C. A. et al. Embryonic Stem Cells Are Redirected to Non-Tumorigenic Epithelial Cell Fate by Interaction with the Mammary Microenvironment. PLoS One 8, (2013).
- Brenner, D. R. et al. Projected estimates of cancer in Canada in 2020. Can. Med. Assoc. 192, (2020).
- Brooks, M. D. & Wicha, M. S. Tumor twitter: Cellular communication in the breast cancer stem cell niche. Cancer Discov. 5, 469–471 (2015).
- Buchholz, T. A. et al. Global gene expression changes during neoadjuvant chemotherapy for human breast cancer. Cancer J. 8, 461–468 (2002).
- Burstein, H. J., Polyak, K., Wong, J. S., Lester, S. C. & Kaelin, C. M. Ductal Carcinoma in Situ of the Breast. http://dx.doi.org/10.1056/NEJMra031301 350, 1430–1441 (2009).
- Cai, X. et al. RIP2 promotes glioma cell growth by regulating TRAF3 and activating the NF-κB and p38 signaling pathways. Oncol. Rep. 39, 2915–2923 (2018).
- Canning, P. et al. Inflammatory Signaling by NOD-RIPK2 Is Inhibited by Clinically Relevant Type II Kinase Inhibitors. Chem. Biol. 22, 1174–1184 (2015).
- Caruso, R. & Núñez, G. Innate Immunity: ER Stress Recruits NOD1 and NOD2 for Delivery of Inflammation. Current Biology 26, (2016).
- Caruso, R., Warner, N., Inohara, N. & Núñez, G. NOD1 and NOD2: Signaling, host defense, and inflammatory disease. Immunity 41, 898–908 (2014).
- Celià-Terrassa, T. Mammary stem cells and breast cancer stem cells: Molecular connections and clinical implications. Biomedicines 6, (2018).
- Chakraborty, P., George, J. T., Woodward, W. A., Levine, H. & Jolly, M. K. Gene expression profiles of inflammatory breast cancer reveal high heterogeneity across the epithelial-hybrid-mesenchymal spectrum. Transl. Oncol. 14, (2021).
- Chamaillard, M. et al. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat. Immunol. (2003). doi:10.1038/ni945
- Chan, L. P. et al. IL-8 promotes HNSCC progression on CXCR1/2-meidated NOD1/ RIP2 signaling pathway. Oncotarget 7, (2016).
- Chang S, Buzdar A U & Hursting. Inflammatory breast cancer and body mass index. J. Clin. Oncol. 16, 3731–3735 (1998).

- Chaudhary, P. M. et al. Death receptor 5, a new member of the TNFR family, and DR4 induce FADD- dependent apoptosis and activate the NF-κB pathway. Immunity (1997). doi:10.1016/S1074-7613(00)80400-8
- Chen, L. et al. A NF-κB-Activin A signaling axis enhances prostate cancer metastasis. Oncogene 39, (2020).
- Chen, L. et al. Protein Kinase C-associated Kinase (PKK), a Novel Membrane-associated, Ankyrin Repeat-containing Protein Kinase. J. Biol. Chem. (2001). doi:10.1074/jbc.M008069200
- Chen, S., Zhou, Y., Chen, Y. & Gu, J. Fastp: An ultra-fast all-in-one FASTQ preprocessor. in Bioinformatics 34, (2018).
- Chen, Y., Tan, W. & Wang, C. Tumor-associated macrophage-derived cytokines enhance cancer stem-like characteristics through epithelial–mesenchymal transition. OncoTargets and Therapy 11, (2018).
- Chen, Z. J. & Sun, L. J. Nonproteolytic Functions of Ubiquitin in Cell Signaling. Molecular Cell (2009). doi:10.1016/j.molcel.2009.01.014
- Chi, W. et al. Bacterial peptidoglycan stimulates adipocyte lipolysis via NOD1. PLoS One 9, (2014).
- Chin, A. I. et al. Involvement of receptor-interacting protein 2 in innate and adaptive immune responses. Nature (2002). doi:10.1038/416190a
- Chirieleison, S. M., Kertesy, S. B. & Abbott, D. W. Synthetic Biology Reveals the Uniqueness of the RIP Kinase Domain. J. Immunol. (2016). doi:10.4049/jimmunol.1502631
- Cho, Y. S. et al. Phosphorylation-Driven Assembly of the RIP1-RIP3 Complex Regulates Programmed Necrosis and Virus-Induced Inflammation. Cell (2009). doi:10.1016/j.cell.2009.05.037
- Chow, M. T. & Luster, A. D. Chemokines in cancer. Cancer immunology research 2, (2014).
- Christian, F., Smith, E. & Carmody, R. The Regulation of NF-κB Subunits by Phosphorylation. Cells 5, 12 (2016).
- Ciardiello, C., Leone, A. & Budillon, A. The Crosstalk between Cancer Stem Cells and Microenvironment Is Critical for Solid Tumor Progression: The Significant Contribution of Extracellular Vesicles. Stem Cells International 2018, (2018).
- Cingolani, P. et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly (Austin). 6, (2012).
- Cipriano, S. C., Lei, C., Kumar, T. R. & Matzuk, M. M. Follistatin is a modulator of gonadal tumor

progression and the activin-induced wasting syndrome in inhibin-deficient mice. Endocrinology 141, (2000).

- Ciriello, G. et al. Comprehensive Molecular Portraits of Invasive Lobular Breast Cancer. Cell 163, 506–519 (2015).
- Clague, M. J. et al. Deubiquitylases from genes to organism. Physiol. Rev. (2013). doi:10.1152/physrev.00002.2013
- Clark, N. M., Marinis, J. M., Cobb, B. A. & Abbott, D. W. MEKK4 Sequesters RIP2 to Dictate NOD2 Signal Specificity. Curr. Biol. (2008). doi:10.1016/j.cub.2008.07.084
- Coerver, K. A. et al. Activin Signaling through Activin Receptor Type II Causes the Cachexia-Like Symptoms in Inhibin-Deficient Mice. Mol. Endocrinol. 10, (1996).
- Condé, C. et al. The inositol phosphatase SHIP-1 inhibits NOD2-induced NF-κb activation by disturbing the interaction of XIAP with RIP2. PLoS One (2012). doi:10.1371/journal.pone.0041005
- Cooney, R. et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. Nat. Med. (2010). doi:10.1038/nm.2069
- Coralville, I. D. T. Alt-R CRISPR-Cas9 System. Integrated DNA Technologies (2017).
- Creed, J., Gerke, T. & Berglund, A. MatSurv: Survival analysis and visualization in MATLAB. J. Open Source Softw. 5, (2020).
- Damgaard, R. B. et al. The Ubiquitin Ligase XIAP Recruits LUBAC for NOD2 Signaling in Inflammation and Innate Immunity. Mol. Cell (2012). doi:10.1016/j.molcel.2012.04.014
- Dawood, S. et al. International expert panel on inflammatory breast cancer: consensus statement for standardized diagnosis and treatment. Ann. Oncol. 22, 515–523 (2011).
- de Andrés, P. J. et al. Increased levels of interleukins 8 and 10 as findings of canine inflammatory mammary cancer. Vet. Immunol. Immunopathol. 152, (2013).
- De Groote, P. et al. A novel RIPK4-IRF6 connection is required to prevent epithelial fusions characteristic for popliteal pterygium syndromes. Cell Death Differ. (2015). doi:10.1038/cdd.2014.191
- Debeb, B. G. et al. Pre-clinical studies of Notch signaling inhibitor RO4929097 in inflammatory breast cancer cells. Breast Cancer Res. Treat. 134, (2012).
- Denou, E. et al. Defective NOD 2 peptidoglycan sensing promotes diet-induced inflammation, dysbiosis, and insulin resistance . EMBO Mol. Med. 7, (2015).
- Deugnier, M. A., Teulière, J., Faraldo, M. M., Thiery, J. P. & Glukhova, M. A. The importance of being a myoepithelial cell. Breast Cancer Research 4, (2002).

- Devin, A. et al. The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. Immunity (2000). doi:10.1016/S1074-7613(00)80194-6
- Di Bonito, M., Cantile, M. & Botti, G. Pathological and molecular characteristics of inflammatory breast cancer. Transl. Cancer Res. 8, (2019).
- Dietze, E. C., Chavez, T. A. & Seewaldt, V. L. Obesity and Triple-Negative Breast Cancer: Disparities, Controversies, and Biology. American Journal of Pathology 188, 280–290 (2018).
- Dmitrieva-Posocco, O. et al. Cell-Type-Specific Responses to Interleukin-1 Control Microbial Invasion and Tumor-Elicited Inflammation in Colorectal Cancer. Immunity 50, (2019).
- Do Monte-Neto, R. L. et al. Gene expression profiling and molecular characterization of antimony resistance in Leishmania amazonensis. PLoS Negl. Trop. Dis. 5, (2011).
- Dobiasova, B. & Mego, M. Biomarkers for inflammatory breast cancer: Diagnostic and therapeutic utility. Breast Cancer: Targets and Therapy 12, (2020).
- Dobin, A. et al. STAR: Ultrafast universal RNA-seq aligner. Bioinformatics 29, (2013).
- Dolcet, X., Llobet, D., Pallares, J. & Matias-Guiu, X. NF-kB in development and progression of human cancer. Virchows Arch. 446, 475–482 (2005).
- Dorsch, M. et al. Identification of a regulatory autophosphorylation site in the serine–threonine kinase RIP2. Cell. Signal. 18, 2223–2229 (2006).
- Drygin, D. et al. Protein kinase CK2 modulates IL-6 expression in inflammatory breast cancer. Biochem. Biophys. Res. Commun. 415, (2011).
- Duggan, B. M. et al. Tyrosine kinase inhibitors of Ripk2 attenuate bacterial cell wall-mediated lipolysis, inflammation and dysglycemia. Sci. Rep. 7, 1578 (2017).
- Duong-Ly, K. C. & Peterson, J. R. The Human Kinome and Kinase Inhibition as a therapeutic strategy. Curr. Protoc. Pharmacol. (2014). doi:10.1002/0471141755.ph0209s60
- Dzamko, N. & Halliday, G. M. An emerging role for LRRK2 in the immune system. Biochemical Society Transactions (2012). doi:10.1042/BST20120119
- Edge, S. B. & Compton, C. C. The American Joint Committee on Cancer: the 7th Edition of the AJCC Cancer Staging Manual and the Future of TNM. Ann. Surg. Oncol. 17, 1471–1474 (2010).
- Ehlers, S. et al. RIP2 regulates growth and differentiation of normal myoblasts and of rhabdomyosarcoma cells. Eur. J. Cell Biol. 87, 163–172 (2008).
- Eickhoff, J. et al. RICK Activates a NF-κB-dependent Anti-human Cytomegalovirus Response. J. Biol. Chem. (2004). doi:10.1074/jbc.M312893200

- Elinav, E., Strowig, T., Henao-Mejia, J. & Flavell, R. A. Regulation of the Antimicrobial Response by NLR Proteins. Immunity 34, (2011).
- Eliyatkin, N., Yalcin, E., Zengel, B., Aktaş, S. & Vardar, E. Molecular Classification of Breast Carcinoma: From Traditional, Old-Fashioned Way to A New Age, and A New Way. J. Breast Heal. 11, (2015).
- Elliott, P. R. et al. Molecular basis and regulation of OTULIN-LUBAC interaction. Mol. Cell (2014). doi:10.1016/j.molcel.2014.03.018
- Erpapazoglou, Z., Walker, O. & Haguenauer-Tsapis, R. Versatile Roles of K63-Linked Ubiquitin Chains in Trafficking. Cells (2014). doi:10.3390/cells3041027
- Feng, Y. et al. Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis. Genes and Diseases 5, 77–106 (2018).
- Fernandez, S. V. et al. Inflammatory breast cancer (IBC): Clues for targeted therapies. Breast Cancer Res. Treat. 140, (2013).
- Fiil, B. K. et al. OTULIN Restricts Met1-Linked Ubiquitination to Control Innate Immune Signaling. Mol. Cell (2013). doi:10.1016/j.molcel.2013.06.004
- Forozan, F. et al. Molecular cytogenetic analysis of 11 new breast cancer cell lines. Br. J. Cancer 81, (1999).
- Fouad, T. M. et al. Distinct epidemiological profiles associated with inflammatory breast cancer (IBC): A comprehensive analysis of the IBC registry at the University of Texas MD Anderson Cancer Center. PLoS One 13, (2018).
- Fouad, T. M. et al. Inflammatory breast cancer: a proposed conceptual shift in the UICC–AJCC TNM staging system. The Lancet Oncology 18, (2017).
- Fouad, T. M. et al. Overall survival differences between patients with inflammatory and noninflammatory breast cancer presenting with distant metastasis at diagnosis HHS Public Access. Breast Cancer Res Treat 152, 407–416 (2015).
- Fouad, T. M., Kogawa, T., Reuben, J. M. & Ueno, N. T. The Role of Inflammation in Inflammatory Breast Cancer. in Advances in experimental medicine and biology 816, 53–73 (2014).
- Franke, A. et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat. Genet. (2010). doi:10.1038/ng.717
- Gendoo, D. M. A. et al. Genefu: An R/Bioconductor package for computation of gene expression-based signatures in breast cancer. Bioinformatics 32, (2016).
- Girardin, S. E. et al. CARD4/Nod1 mediates NF-KB and JNK activation by invasive Shigella flexneri. EMBO Rep. (2001). doi:10.1093/embo-reports/kve155

- Githaka, J. M. et al. BAD regulates mammary gland morphogenesis by 4E-BP1-mediated control of localized translation in mouse and human models. Nat. Commun. 12, (2021).
- Giuliano, A. E. et al. Breast Cancer-Major changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA. Cancer J. Clin. 67, 290–303 (2017).
- Giuliano, A. E., Edge, S. B. & Hortobagyi, G. N. Eighth Edition of the AJCC Cancer Staging Manual: Breast Cancer. Annals of Surgical Oncology 25, (2018).
- Gjorevski, N. & Nelson, C. M. Integrated morphodynamic signalling of the mammary gland. Nat. Rev. Mol. Cell Biol. 12, (2011).
- Goh, F. Y. et al. Receptor-Interacting Protein 2 Gene Silencing Attenuates Allergic Airway Inflammation. J. Immunol. 191, 2691–2699 (2013).
- Goldfarb, J. M. & Pippen, J. E. Inflammatory Breast Cancer: The Experience of Baylor University Medical Center at Dallas. Baylor Univ. Med. Cent. Proc. 24, (2011).
- Goncharov, T. et al. Disruption of XIAP-RIP2 Association Blocks NOD2-Mediated Inflammatory Signaling. Mol. Cell 69, 551-565.e7 (2018).
- Gong, Q. et al. Structural basis of RIP2 activation and signaling. Nat. Commun. (2018). doi:10.1038/s41467-018-07447-9
- Grou, C. P., Pinto, M. P., Mendes, A. V., Domingues, P. & Azevedo, J. E. The de novo synthesis of ubiquitin: Identification of deubiquitinases acting on ubiquitin precursors. Sci. Rep. (2015). doi:10.1038/srep12836
- Gu, S. & Feng, X. H. TGF-β signaling in cancer. Acta Biochimica et Biophysica Sinica 50, (2018).
- Guirado, M. et al. Association between C13ORF31, NOD2, RIPK2 and TLR10 polymorphisms and urothelial bladder cancer. Hum. Immunol. 73, (2012).
- Gutierrez, C. & Schiff, R. HER2: Biology, detection, and clinical implications. Arch. Pathol. Lab. Med. 135, (2011).
- H. Rideout and D. Re. Leucine- Rich Repeat Kinase 2. 14, (Springer Nature, 2017).
- Ha, H., Debnath, B. & Neamati, N. Role of the CXCL8-CXCR1/2 axis in cancer and inflammatory diseases. Theranostics 7, (2017).
- Ha, H., Han, D. & Choi, Y. TRAF-mediated TNFR-family signaling. Current Protocols in Immunology (2009). doi:10.1002/0471142735.im1109ds87
- Hammond, M. E. H. et al. American society of clinical oncology/college of american pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. Journal of Clinical Oncology 28, (2010).

- Hanks, S. K. & Hunter, T. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification 1 . FASEB J. (1995). doi:10.1096/fasebj.9.8.7768349
- Hanks, S. K., Quinn, A. M. & Hunter, T. The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. Science (1988). doi:10.1126/science.3291115
- Hasegawa, M. et al. A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-κB activation. EMBO J. (2008). doi:10.1038/sj.emboj.7601962
- Hauenstein, A. V., Xu, G., Kabaleeswaran, V. & Wu, H. Evidence for M1-Linked Polyubiquitin-Mediated Conformational Change in NEMO. J. Mol. Biol. (2017). doi:10.1016/j.jmb.2017.10.026
- Hedger, M. P., Winnall, W. R., Phillips, D. J. & de Kretser, D. M. The Regulation and Functions of Activin and Follistatin in Inflammation and Immunity. Vitamins and Hormones 85, (2011).
- Heim, V. J. et al. A regulatory interface on RIPK2 is required for XIAP binding and NOD signaling activity. bioRxiv (2020).
- Heim, V. J., Stafford, C. A. & Nachbur, U. NOD Signaling and Cell Death. Frontiers in Cell and Developmental Biology (2019). doi:10.3389/fcell.2019.00208
- Hershko, A. & Ciechanover, A. THE UBIQUITIN SYSTEM. Annu. Rev. Biochem. (1998). doi:10.1146/annurev.biochem.67.1.425
- Hess, J., Angel, P. & Schorpp-Kistner, M. AP-1 subunits: Quarrel and harmony among siblings. J. Cell Sci. 117, (2004).
- Hirano, T. IL-6 in inflammation, autoimmunity and cancer. International immunology 33, (2021).
- Hitotsumatsu, O. et al. The Ubiquitin-Editing Enzyme A20 Restricts Nucleotide-Binding Oligomerization Domain Containing 2-Triggered Signals. Immunity (2008). doi:10.1016/j.immuni.2008.02.002
- Hoesel, B. & Schmid, J. A. The complexity of NF-κB signaling in inflammation and cancer. Molecular Cancer 12, 1 (2013).
- Hofland, J. et al. Inhibin alpha-subunit (INHA) expression in adrenocortical cancer is linked to genetic and epigenetic INHA promoter variation. PLoS One 9, (2014).
- Holen, I. et al. IL-1 drives breast cancer growth and bone metastasis in vivo. Oncotarget 7, (2016).
- Homer, C. R., Richmond, A. L., Rebert, N. A., Achkar, J. & McDonald, C. ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in crohn's disease pathogenesis. Gastroenterology (2010). doi:10.1053/j.gastro.2010.07.006
- Honjo, H., Watanabe, T., Kamata, K., Minaga, K. & Kudo, M. RIPK2 as a New Therapeutic Target in Inflammatory Bowel Diseases. Frontiers in Pharmacology 12, (2021).

- Hosono, K. et al. Roles of prostaglandin E2-EP3/EP4 receptor signaling in the enhancement of lymphangiogenesis during fibroblast growth factor-2-induced granulation formation. Arterioscler. Thromb. Vasc. Biol. 31, (2011).
- Hrdinka, M. et al. Small molecule inhibitors reveal an indispensable scaffolding role of RIPK 2 in NOD 2 signaling . EMBO J. (2018). doi:10.15252/embj.201899372
- Hrdinka, M. et al. CYLD Limits Lys63- and Met1-Linked Ubiquitin at Receptor Complexes to Regulate Innate Immune Signaling. Cell Rep. 14, 2846–2858 (2016).
- Hsu, H., Huang, J., Shu, H. B., Baichwal, V. & Goeddel, D. V. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity (1996). doi:10.1016/S1074-7613(00)80252-6
- Huang, A., Cao, S. & Tang, L. The tumor microenvironment and inflammatory breast cancer. Journal of Cancer 8, (2017).
- Huang, C. S. et al. Crystal Structure of Ripk4 Reveals Dimerization-Dependent Kinase Activity. Structure (2018). doi:10.1016/j.str.2018.04.002
- Hubbard, S. R. & Till, J. H. Protein Tyrosine Kinase Structure and Function. Annu. Rev. Biochem. (2000). doi:10.1146/annurev.biochem.69.1.373
- Humke, E. W., Shriver, S. K., Starovasnik, M. A., Fairbrother, W. J. & Dixit, V. M. ICEBERG: A novel inhibitor of interleukin-1β generation. Cell 103, (2000).
- Inohara, N. & Nuñez, G. NODS: Intracellular proteins involved in inflammation and apoptosis. Nature Reviews Immunology (2003). doi:10.1038/nri1086
- Inohara, N. et al. An induced proximity model for NF-κB activation in the Nod1/RICK and RIP signaling pathways. J. Biol. Chem. (2000). doi:10.1074/jbc.M003415200
- Inohara, N. et al. Host recognition of bacterial muramyl dipeptide mediated through NOD2: Implications for Crohn's disease. J. Biol. Chem. (2003). doi:10.1074/jbc.C200673200
- Inohara, N. et al. Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-κB. J. Biol. Chem. 274, 14560–14567 (1999).
- Inohara, N., Del Peso, L., Koseki, T., Chen, S. & Núñez, G. RICK, a novel protein kinase containing a caspase recruitment domain, interacts with CLARP and regulates CD95-mediated apoptosis. J. Biol. Chem. 273, 12296–12300 (1998).
- Inoue, J. ichiro et al. Tumor necrosis factor receptor-associated factor (TRAF) family: Adapter proteins that mediate cytokine signaling. Experimental Cell Research (2000). doi:10.1006/excr.1999.4733

- Insua-Rodríguez, J. & Oskarsson, T. The extracellular matrix in breast cancer. Advanced Drug Delivery Reviews 97, (2016).
- Iwasaki, Y., Nishiyama, H., Suzuki, K. & Koizumi, S. Sequential cis/trans autophosphorylation in TrkB tyrosine kinase. Biochemistry (1997). doi:10.1021/bi962057x
- Jaafar, R. et al. RIP2 enhances cell survival by activation of NF-κB in triple negative breast cancer cells. (2018). doi:10.1016/j.bbrc.2018.02.034
- Jaafar, R. F. et al. Receptor-Interacting Serine/Threonine-Protein Kinase-2 as a Potential Prognostic Factor in Colorectal Cancer. Medicina (B. Aires). 57, (2021).
- Jana, A. et al. NFkB is essential for activin-induced colorectal cancer migration via upregulation of PI3K-MDM2 pathway. Oncotarget 8, (2017).
- Javed, A. & Lteif, A. Development of the human breast. Semin. Plast. Surg. 27, (2013).
- Jiang, X. & Chen, Z. J. The role of ubiquitylation in immune defence and pathogen evasion. Nature Reviews Immunology (2012). doi:10.1038/nri3111
- Jiao, X. et al. Somatic mutations in the notch, NF-KB, PIK3CA, and hedgehog pathways in human breast cancers. Genes Chromosom. Cancer 51, (2012).
- Johnson, E. S. Ubiquitin branches out. in Nature Cell Biology (2002). doi:10.1038/ncb1202-e295
- Johnston, P. A. & Grandis, J. R. STAT3 signaling: Anticancer strategies and challenges. Molecular Interventions 11, (2011).
- Jolly, M. K. et al. Inflammatory breast cancer: A model for investigating cluster-based dissemination. npj Breast Cancer 3, (2017).
- Jones, K. L. et al. Activin A is a critical component of the inflammatory response, and its binding protein, follistatin, reduces mortality in endotoxemia. Proc. Natl. Acad. Sci. U. S. A. 104, (2007).
- Kakarala, M. & Wicha, M. S. Implications of the cancer stem-cell hypothesis for breast cancer prevention and therapy. Journal of Clinical Oncology 26, (2008).
- Kalay, E. et al. Mutations in RIPK4 cause the autosomal-recessive form of popliteal pterygium syndrome. Am. J. Hum. Genet. (2012). doi:10.1016/j.ajhg.2011.11.014
- Kanayama, A. et al. TAB2 and TAB3 activate the NF-κB pathway through binding to polyubiquitin chains. Mol. Cell (2004). doi:10.1016/j.molcel.2004.08.008
- Kannan, N. & Neuwald, A. F. Did protein kinase regulatory mechanisms evolve through elaboration of a simple structural component? J. Mol. Biol. (2005). doi:10.1016/j.jmb.2005.06.057
- Kartikasari, A. E. R., Huertas, C. S., Mitchell, A. & Plebanski, M. Tumor-Induced Inflammatory Cytokines and the Emerging Diagnostic Devices for Cancer Detection and Prognosis. Frontiers in Oncology 11, (2021).

- Kartikasari, A. E. R., Huertas, C. S., Mitchell, A. & Plebanski, M. Tumor-Induced Inflammatory Cytokines and the Emerging Diagnostic Devices for Cancer Detection and Prognosis. Frontiers in Oncology 11, (2021).
- Katsuno, Y. et al. Chronic TGF-b exposure drives stabilized EMT, tumor stemness, and cancer drug resistance with vulnerability to bitopic mTOR inhibition. Sci. Signal. 12, (2019).
- Kaur, S., Bansal, Y., Kumar, R. & Bansal, G. A panoramic review of IL-6: Structure, pathophysiological roles and inhibitors. Bioorganic and Medicinal Chemistry 28, (2020).
- Kendellen, M. F., Bradford, J. W., Lawrence, C. L., Clark, K. S. & Baldwin, A. S. Canonical and non-canonical NF-κB signaling promotes breast cancer tumor-initiating cells. Oncogene 33, 1297–1305 (2014).
- Kim, H. S. et al. COX2 overexpression is a prognostic marker for Stage III breast cancer. Breast Cancer Res. Treat. 132, (2012).
- Kim, J. Y., Omori, E., Matsumoto, K., Núñez, G. & Ninomiya-Tsuji, J. TAK1 is a central mediator of NOD2 signaling in epidermal cells. J. Biol. Chem. 283, (2008).
- Kim, S. et al. Strelka2: fast and accurate calling of germline and somatic variants. Nat. Methods 15, (2018).
- Kim, S.-W., Roh, J. & Park, C.-S. Immunohistochemistry for Pathologists: Protocols, Pitfalls, and Tips. (2016). doi:10.4132/jptm.2016.08.08
- Kim, W. et al. Systematic and quantitative assessment of the ubiquitin-modified proteome. Mol. Cell (2011). doi:10.1016/j.molcel.2011.08.025
- Kim, W. T. & Ryu, C. J. Cancer stem cell surface markers on normal stem cells. BMB Reports 50, (2017).
- Kim, Y. Il et al. Inhibin-α gene mutations and mRNA levels in human lymphoid and myeloid leukemia cells. Int. J. Oncol. 50, (2017).
- Kisiswa, L., Fernández-Suárez, D., Sergaki, M. C. & Ibáñez, C. F. RIP2 Gates TRAF6 Interaction with Death Receptor p75NTR to Regulate Cerebellar Granule Neuron Survival. Cell Rep. 24, (2018).
- Kobayashi, K. et al. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. Nature (2002). doi:10.1038/416194a
- Komaki, K., Sano, N. & Tangoku, A. Problems in histological grading of malignancy and its clinical significance in patients with operable breast cancer. Breast cancer (Tokyo, Japan) 13, (2006).

- Komander, D., Clague, M. J. & Urbé, S. Breaking the chains: Structure and function of the deubiquitinases. Nature Reviews Molecular Cell Biology (2009). doi:10.1038/nrm2731
- Krieg, A. et al. XIAP mediates NOD signaling via interaction with RIP2. Proc. Natl. Acad. Sci. U. S. A. (2009). doi:10.1073/pnas.0907131106
- Kufer, T. A., Kremmer, E., Adam, A. C., Philpott, D. J. & Sansonetti, P. J. The pattern-recognition molecule Nod1 is localized at the plasma membrane at sites of bacterial interaction. Cell. Microbiol. 10, (2008).
- Kuleshov, M. V. et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res. 44, (2016).
- Kuss-Duerkop, S. K. & Keestra-Gounder, A. M. NOD1 and NOD2 activation by diverse stimuli: A possible role for sensing pathogen-induced endoplasmic reticulum stress. Infect. Immun. 88, (2020).
- Kwa, M. Q., Huynh, J., Reynolds, E. C., Hamilton, J. A. & Scholz, G. M. Disease-associated mutations in IRF6 and RIPK4 dysregulate their signalling functions. Cell. Signal. (2015). doi:10.1016/j.cellsig.2015.03.005
- L. Ferreira, V., H.L. Borba, H., de F. Bonetti, A., P. Leonart, L. & Pontarolo, R. Cytokines and Interferons: Types and Functions. in Autoantibodies and Cytokines (2019). doi:10.5772/intechopen.74550
- LaBarge, M. A., Petersen, O. W. & Bissell, M. J. Of microenvironments and mammary stem cells. Stem Cell Rev. 3, 137–146 (2007).
- Lalloo, F. et al. BRCA1, BRCA2 and TP53 mutations in very early-onset breast cancer with associated risks to relatives. Eur. J. Cancer 42, (2006).
- Lappas, M. NOD1 and NOD2 regulate proinflammatory and prolabor mediators in human fetal membranes and myometrium via nuclear factor-kappa B. Biol. Reprod. 89, (2013).
- LeBlanc, P. M. et al. Caspase-12 Modulates NOD Signaling and Regulates Antimicrobial Peptide Production and Mucosal Immunity. Cell Host Microbe (2008). doi:10.1016/j.chom.2008.02.004
- Lechtenberg, B. C., Mace, P. D. & Riedl, S. J. Structural mechanisms in NLR inflammasome signaling. Current Opinion in Structural Biology 29, (2014).
- Lee, C. H., Jeon, Y.-T., Kim, S.-H. & Song, Y.-S. NF-kappaB as a potential molecular target for cancer therapy. Biofactors 29, 19–35 (2007).
- Lee, P. et al. Phosphorylation of Pkp1 by RIPK 4 regulates epidermal differentiation and skin tumorigenesis. EMBO J. (2017). doi:10.15252/embj.201695679

- Lerebours, F. et al. Hemoglobin overexpression and splice signature as new features of inflammatory breast cancer? J. Adv. Res. 28, (2021).
- Lerebours, F. et al. NF-kappa B genes have a major role in Inflammatory Breast Cancer. BMC Cancer 8, 41 (2008).
- Levine, P. H. et al. Evaluation of lymphangiogenic factors, vascular endothelial growth factor D and E-cadherin in distinguishing inflammatory from locally advanced breast cancer. Clin. Breast Cancer 12, (2012).
- Li, D. et al. RIPK2 is an unfavorable prognosis marker and a potential therapeutic target in human kidney renal clear cell carcinoma. Aging (Albany. NY). 13, (2021).
- Li, X., Li, Z., Zhu, H. & Yu, X. Autophagy Regulatory Genes MET and RIPK2 Play a Prognostic Role in Pancreatic Ductal Adenocarcinoma: A Bioinformatic Analysis Based on GEO and TCGA. Biomed Res. Int. 2020, (2020).
- Lim, B., Woodward, W. A., Wang, X., Reuben, J. M. & Ueno, N. T. Inflammatory breast cancer biology: the tumour microenvironment is key. Nature Reviews Cancer 18, (2018).
- Lin, C. C., Huoh, Y. S., Schmitz, K. R., Jensen, L. E. & Ferguson, K. M. Pellino Proteins Contain a Cryptic FHA Domain that Mediates Interaction with Phosphorylated IRAK1. Structure (2008). doi:10.1016/j.str.2008.09.011
- Liu, M. et al. Non-canonical NF-κB Plays a Pivotal Role in Non-Hodgkin's Lymphoma. Cell Biochem. Biophys. 72, 681–685 (2015).
- Liu, R. et al. HIV-1 Vpr stimulates NF-κB and AP-1 signaling by activating TAK1. Retrovirology 11, (2014).
- Liu, S. et al. Breast cancer stem cells transition between epithelial and mesenchymal states reflective of their normal counterparts. Stem Cell Reports 2, (2014).
- Liu, T., Zhang, L., Joo, D. & Sun, S. C. NF-κB signaling in inflammation. Signal Transduction and Targeted Therapy 2, (2017).
- Long, X. et al. IL-8, a novel messenger to cross-link inflammation and tumor EMT via autocrine and paracrine pathways (Review). Int. J. Oncol. 48, 5–12 (2016).
- Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, (2014).
- Low, T. Y., Magliozzi, R., Guardavaccaro, D. & Heck, A. J. R. Unraveling the ubiquitin-regulated signaling networks by mass spectrometry-based proteomics. Proteomics (2013). doi:10.1002/pmic.201200244

- Lu, H., Ouyang, W. & Huang, C. Inflammation, a key event in cancer development. Molecular Cancer Research 4, (2006).
- Luo, Y. & Zheng, S. G. Hall of fame among pro-inflammatory cytokines: Interleukin-6 gene and its transcriptional regulation mechanisms. Frontiers in Immunology 7, (2016).
- MacKay, A. et al. Microarray-based class discovery for molecular classification of breast cancer: Analysis of interobserver agreement. J. Natl. Cancer Inst. 103, (2011).
- Maekawa, S., Ohto, U., Shibata, T., Miyake, K. & Shimizu, T. Crystal structure of NOD2 and its implications in human disease. Nat. Commun. (2016). doi:10.1038/ncomms11813
- Maloney, C. et al. Gefitinib inhibits invasion and metastasis of osteosarcoma via inhibition of macrophage receptor interacting serine-threonine kinase 2. Mol. Cancer Ther. 19, (2020).
- Manning, G., Whyte, D. B., Martinez, R., Hunter, T. & Sudarsanam, S. The protein kinase complement of the human genome. Science (2002). doi:10.1126/science.1075762
- Mantovani, A., Dinarello, C. A., Molgora, M. & Garlanda, C. Interleukin-1 and Related Cytokines in the Regulation of Inflammation and Immunity. Immunity 50, (2019).
- Marín, I., Egmond, W. N. & Haastert, P. J. M. The Roco protein family: a functional perspective. FASEB J. (2008). doi:10.1096/fj.08-111310
- Matro, J. M. et al. Inflammatory breast cancer management in the National Comprehensive Cancer Network: The disease, recurrence pattern, and outcome. Clin. Breast Cancer 15, (2015).
- Matzuk, M. M. et al. Transgenic models to study the roles of inhibins and activins in reproduction, oncogenesis, and development. Recent Prog. Horm. Res. 51, (1996).
- McCarthy, J. V, Ni, J. & Dixit, V. M. RIP2 is a novel NF-kappaB-activating and cell death-inducing kinase. J. Biol. Chem. 273, 16968–75 (1998).
- McDowell, G. S. & Philpott, A. New Insights Into the Role of Ubiquitylation of Proteins. in International Review of Cell and Molecular Biology (2016). doi:10.1016/bs.ircmb.2016.02.002
- Medina, D. The mammary gland: a unique organ for the study of development and tumorigenesis. Journal of mammary gland biology and neoplasia 1, (1996).
- Meunier, E. & Broz, P. Evolutionary Convergence and Divergence in NLR Function and Structure. Trends in Immunology (2017). doi:10.1016/j.it.2017.04.005
- Meylan, E. & Tschopp, J. The RIP kinases: Crucial integrators of cellular stress. Trends in Biochemical Sciences (2005). doi:10.1016/j.tibs.2005.01.003
- Meylan, E., Martinon, F., Thome, M., Gschwendt, M. & Tschopp, J. RIP4 (DIK/PKK), a novel member of the RIP kinase family, activates NF-κB and is processed during apoptosis. EMBO Reports (2002). doi:10.1093/embo-reports/kvf236

- Micheau, O. & Tschopp, J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell (2003). doi:10.1016/S0092-8674(03)00521-X
- Mohamed, H. T. et al. IL-8 and MCP-1/CCL2 regulate proteolytic activity in triple negative inflammatory breast cancer a mechanism that might be modulated by Src and Erk1/2. Toxicol. Appl. Pharmacol. 401, (2020).
- Mohamed, M. M., Al-Raawi, D., Sabet, S. F. & El-Shinawi, M. Inflammatory breast cancer: New factors contribute to disease etiology: A review. Journal of Advanced Research 5, 525–536 (2014).
- Molckovsky, A., Fitzgerald, B., Freedman, O., Heisey, R. & Clemons, M. Approach to inflammatory breast cancer. Canadian Family Physician 55, (2009).
- Monneur, A. et al. Similar response profile to neoadjuvant chemotherapy, but different survival, in inflammatory versus locally advanced breast cancers. Oncotarget 8, (2017).
- Moreno, R., Sobotzik, J. M., Schultz, C. & Schmitz, M. L. Specification of the NF-κB transcriptional response by p65 phosphorylation and TNF-induced nuclear translocation of IKKε. Nucleic Acids Res. 38, (2010).
- Moriguchi, T. et al. A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. J. Biol. Chem. 271, (1996).
- Morrow, R. J., Etemadi, N., Yeo, B. & Ernst, M. Challenging a Misnomer? The Role of Inflammatory Pathways in Inflammatory Breast Cancer. Mediators of Inflammation 2017, (2017).
- Nachbur, U. et al. A RIPK2 inhibitor delays NOD signalling events yet prevents inflammatory cytokine production. Nat. Commun. 6, 6442 (2015).
- Najjar, M. et al. RIPK1 and RIPK3 Kinases Promote Cell-Death-Independent Inflammation by Toll-like Receptor 4. Immunity (2016). doi:10.1016/j.immuni.2016.06.007
- Nembrini, C. et al. The kinase-activity of Rip2 determines its stability and consequently Nod1 - and Nod2-mediated immune responses. J. Biol. Chem. (2009). doi:10.1074/jbc.M109.006353
- Neven, P., Brouckaert, Wildiers & Floris. Update on triple-negative breast cancer: prognosis and management strategies. Int. J. Womens. Health 4, 511 (2012).
- Newton, K. RIPK1 and RIPK3: Critical regulators of inflammation and cell death. Trends in Cell Biology (2015). doi:10.1016/j.tcb.2015.01.001
- Nguyen, D. M. et al. Molecular heterogeneity of inflammatory breast cancer: A hyperproliferative phenotype. Clin. Cancer Res. 12, (2006).
- Nijman, S. M. B. et al. A genomic and functional inventory of deubiquitinating enzymes. Cell (2005). doi:10.1016/j.cell.2005.11.007

- Ogura, Y. et al. Nod2, a Nod1/Apaf-1 Family Member That Is Restricted to Monocytes and Activates NF-κB. J. Biol. Chem. (2001). doi:10.1074/jbc.M008072200
- Okuma, Y. et al. Regulation of activin A and inhibin B secretion by inflammatory mediators in adult rat Sertoli cell cultures. J. Endocrinol. 187, (2005).
- Olsen, J. V. & Mann, M. Status of large-scale analysis of posttranslational modifications by mass spectrometry. Molecular and Cellular Proteomics (2013). doi:10.1074/mcp.O113.034181
- Olsen, J. V. et al. Global, In Vivo, and Site-Specific Phosphorylation Dynamics in Signaling Networks. Cell (2006). doi:10.1016/j.cell.2006.09.026
- Onitilo, A. A., Engel, J. M., Greenlee, R. T. & Mukesh, B. N. Breast Cancer Subtypes Based on ER/PR and Her2 Expression: Comparison of Clinicopathologic Features and Survival. Clin. Med. Res. 7, (2009).
- Ota, M. et al. Association between receptor interacting serine/threonine kinase 2 polymorphisms and gastric cancer susceptibility. Oncol. Lett. 15, (2018).
- Palmer, N., Shanmugam, M. K., Sethi, G. & Kaldis, P. NF- κ B as a Potential Molecular Target for Therapy of Gastrointestinal Cancers . in Therapeutic Targets for Inflammation and Cancer (2017). doi:10.1142/9789813148574_0009
- Pan, E. et al. Inflammatory breast cancer outcomes in a contemporary series. Anticancer Res. 37, (2017).
- Panda, S. & Gekara, N. O. The deubiquitinase MYSM1 dampens NOD2-mediated inflammation and tissue damage by inactivating the RIP2 complex. Nat. Commun. (2018). doi:10.1038/s41467-018-07016-0
- Park, J.-H. et al. RICK/RIP2 Mediates Innate Immune Responses Induced through Nod1 and Nod2 but Not TLRs. J. Immunol. 178, 2380–2386 (2007).
- Park, M. H. & Hong, J. T. Roles of NF-κB in cancer and inflammatory diseases and their therapeutic approaches. Cells 5, (2016).
- Park, Y. H. et al. Clinical relevance of TNM staging system according to breast cancer subtypes. Ann. Oncol. 22, 1554–1560 (2011).
- Parmar, H. & Cunha, G. R. Epithelial-stromal interactions in the mouse and human mammary gland in vivo. Endocrine-Related Cancer 11, (2004).
- Pellegrini, E. et al. RIP2 filament formation is required for NOD2 dependent NF-κB signalling. Nat. Commun. (2018). doi:10.1038/s41467-018-06451-3

- Pellegrini, E., Signor, L., Singh, S., Erba, E. B. & Cusack, S. Structures of the inactive and active states of RIP2 kinase inform on the mechanism of activation. PLoS One (2017). doi:10.1371/journal.pone.0177161
- Pereira, B. et al. Erratum: The somatic mutation profiles of 2,433 breast cancers refine their genomic and transcriptomic landscapes (Nature Communications (2016) 7:11479 DOI: 10.1038/ncomms11479). Nature Communications 7, (2016).
- Perou, C. M. et al. Molecular portraits of human breast tumours. Nature 406, (2000).
- Pettersen, K. et al. Autocrine activin A signalling in ovarian cancer cells regulates secretion of interleukin 6, autophagy, and cachexia. J. Cachexia. Sarcopenia Muscle 11, (2020).
- Phillips, D. J., Jones, K. L., Scheerlinck, J. P. Y., Hedger, M. P. & De Kretser, D. M. Evidence for activin A and follistatin involvement in the systemic inflammatory response. in Molecular and Cellular Endocrinology 180, (2001).
- Pickart, C. M. & Fushman, D. Polyubiquitin chains: Polymeric protein signals. Current Opinion in Chemical Biology (2004). doi:10.1016/j.cbpa.2004.09.009
- Pickart, C. M. Mechanisms Underlying Ubiquitination. Annu. Rev. Biochem. (2001). doi:10.1146/annurev.biochem.70.1.503
- Polyak, K. Breast cancer: Origins and evolution. Journal of Clinical Investigation 117, 3155–3163 (2007).
- Polyansky, A. A. & Zagrovic, B. Protein electrostatic properties predefining the level of surface hydrophobicity change upon phosphorylation. J. Phys. Chem. Lett. (2012). doi:10.1021/jz300103p
- Pruthi, S., Stafyla, V. K., Phillips, S. W., Porrata, L. F. & Reynolds, C. A. Primary mammary (non-Hodgkin) lymphoma presenting as locally advanced breast cancer. Mayo Clin. Proc. 79, (2004).
- Radunsky, G. S. & van Golen, K. L. The current understanding of the molecular determinants of inflammatory breast cancer metastasis. Clin. Exp. Metastasis 22, 615–620 (2005).
- Rehman, S., Reddy, C. A. & Tendulkar, R. D. Modern outcomes of inflammatory breast cancer. Int. J. Radiat. Oncol. Biol. Phys. 84, (2012).
- Ribatti, D. The concept of immune surveillance against tumors. The first theories. Oncotarget 8, (2017).
- Ries, A. et al. Activin A: an emerging target for improving cancer treatment? Expert Opinion on Therapeutic Targets (2020). doi:10.1080/14728222.2020.1799350
- Risbridger, G. P., Schmitt, J. F. & Robertson, D. M. Activins and inhibins in endocrine and other tumors. Endocrine Reviews 22, (2001).
- Robertson, D. M., Burger, H. G. & Fuller, P. J. Inhibin/activin and ovarian cancer. Endocrine-

Related Cancer 11, (2004).

- Rosenberg, S. A. Cancer immunotherapy comes of age. Nature Clinical Practice Oncology 2, (2005).
- Rosenbluth, J. M. & Overmoyer, B. A. Inflammatory Breast Cancer: a Separate Entity. Current Oncology Reports 21, (2019).
- Rosenbluth, J. M. & Overmoyer, B. A. Inflammatory Breast Cancer: a Separate Entity. Current Oncology Reports 21, (2019).
- Rosenthal, D. T. et al. RhoC impacts the metastatic potential and abundance of breast cancer stem cells. PLoS One 7, (2012).
- Ross, J. S. et al. Comprehensive genomic profiling of inflammatory breast cancer cases reveals a high frequency of clinically relevant genomic alterations. Breast Cancer Res. Treat. 154, (2015).
- Russo, J. & Russo, I. H. Development of the human breast. in Maturitas 49, (2004).
- Ruth, M. C. et al. RhoC promotes human melanoma invasion in a PI3K/Akt-dependent pathway. J. Invest. Dermatol. 126, (2006).
- Schaeffer, V. et al. Binding of OTULIN to the PUB Domain of HOIP Controls NF-κB Signaling. Mol. Cell (2014). doi:10.1016/j.molcel.2014.03.016
- Schertzer, J. D. et al. NOD1 activators link innate immunity to insulin resistance. Diabetes 60, (2011).
- Schlichting, J. A., Soliman, A. S., Schairer, C., Schottenfeld, D. & Merajver, S. D. Inflammatory and non-inflammatory breast cancer survival by socioeconomic position in the Surveillance, Epidemiology, and End Results database, 1990-2008. Breast Cancer Res. Treat. 134, (2012).
- Scholz, C. C. & Taylor, C. T. Hydroxylase-dependent regulation of the NF-κB pathway. Biol. Chem. 394, (2013).
- Schott, A. F. et al. Phase Ib pilot study to evaluate reparixin in combination with weekly paclitaxel in patients with HER-2–negative metastatic breast cancer. Clin. Cancer Res. 23, (2017).
- Seachrist, D. D. & Keri, R. A. The Activin Social Network: Activin, Inhibin, and Follistatin in Breast Development and Cancer. Endocrinology 160, (2019).
- Shaw, P. J. et al. Signaling via the RIP2 Adaptor Protein in Central Nervous System-Infiltrating Dendritic Cells Promotes Inflammation and Autoimmunity. Immunity 34, 75–84 (2011).
- Shaw, P. J. et al. Signaling via the RIP2 Adaptor Protein in Central Nervous System-Infiltrating Dendritic Cells Promotes Inflammation and Autoimmunity. Immunity 34, 75–84 (2011).
- Shirakabe, K. et al. TAK1 mediates the ceramide signaling to stress-activated protein kinase/c-Jun N-terminal kinase. J. Biol. Chem. 272, (1997).

- Silberstein, G. B. Role of the stroma in mammary development. Breast Cancer Research 3, (2001).
- Simpson, P. T., Reis-Filho, J. S., Gale, T. & Lakhani, S. R. Molecular evolution of breast cancer. J. Pathol. 205, 248–254 (2005).
- Singel, S. M. et al. Receptor-interacting protein kinase 2 promotes triple-negative breast cancer cell migration and invasion via activation of nuclear factor-kappaB and c-Jun N-terminal kinase pathways. Breast Cancer Res. 16, R28 (2014).
- Singh, J. K. et al. Targeting CXCR1/2 significantly reduces breast cancer stem cell activity and increases the efficacy of inhibiting HER2 via HER2-dependent and -independent mechanisms. Clin. Cancer Res. 19, (2013).
- Soave, A. et al. Copy number variations of circulating, cell-free DNA in urothelial carcinoma of the bladder patients treated with radical cystectomy: A prospective study. Oncotarget 8, (2017).
- Sobin, L. H. & Compton, C. C. TNM seventh edition: What's new, what's changed. Cancer 116, 5336–5339 (2010).
- Sobin, L. H. TNM: principles, history, and relation to other prognostic factors. Cancer 91, 1589–92 (2001).
- Sontag, L. & Axelrod, D. E. Evaluation of pathways for progression of heterogeneous breast tumors. J. Theor. Biol. 232, (2005).
- Sorbara, M. T. et al. The protein ATG16L1 suppresses inflammatory cytokines induced by the intracellular sensors Nod1 and Nod2 in an autophagy-independent manner. Immunity (2013). doi:10.1016/j.immuni.2013.10.013
- Sparano, J. A. et al. Prospective Validation of a 21-Gene Expression Assay in Breast Cancer. N. Engl. J. Med. 373, (2015).
- Spence, J., Sadis, S., Haas, A. L. & Finley, D. A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. Mol. Cell. Biol. (1995). doi:10.1128/mcb.15.3.1265
- Stafford, C. A. et al. IAPs Regulate Distinct Innate Immune Pathways to Co-ordinate the Response to Bacterial Peptidoglycans. Cell Rep. (2018). doi:10.1016/j.celrep.2018.01.024
- Stanger, B. Z., Leder, P., Lee, T. H., Kim, E. & Seed, B. RIP: A novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell (1995). doi:10.1016/0092-8674(95)90072-1
- Sternlicht, M. D. Key stages in mammary gland development: The cues that regulate ductal branching morphogenesis. Breast Cancer Research 8, (2005).
- Stevenson, S. & Thornton, J. Effect of estrogens on skin aging and the potential role of SERMs. Clinical interventions in aging 2, (2007).

- Stirling, J. W. & Chandler, J. A. The fine structure of the normal, resting terminal ductal-lobular unit of the female breast. Virchows Arch. A Pathol. Anat. Histol. 372, (1976).
- Strober, W. Trypan Blue Exclusion Test of Cell Viability. Curr. Protoc. Immunol. 111, (2015).
- Strober, W., Murray, P. J., Kitani, A. & Watanabe, T. Signalling pathways and molecular interactions of NOD1 and NOD2. Nat. Rev. Immunol. 6, 9–20 (2006).
- Sullivan, N. J. et al. Interleukin-6 induces an epithelial-mesenchymal transition phenotype in human breast cancer cells. Oncogene 28, (2009).
- Sun, L. & Chen, Z. J. The novel functions of ubiquitination in signaling. Current Opinion in Cell Biology (2004). doi:10.1016/j.ceb.2004.02.005
- Sun, S. C. Deubiquitylation and regulation of the immune response. Nature Reviews Immunology (2008). doi:10.1038/nri2337
- Suzuki, R. et al. Proliferation and differentiation in the human breast during pregnancy. Differentiation 66, (2000).
- Takayanagi, S. et al. ASKA technology-based pull-down method reveals a suppressive effect of ASK1 on the inflammatory NOD-RIPK2 pathway in brown adipocytes. Sci. Reports 2021 111 11, 1–14 (2021).
- Takeuchi, O. & Akira, S. Pattern Recognition Receptors and Inflammation. Cell (2010). doi:10.1016/j.cell.2010.01.022
- Tamer M Fouad, Takahiro Kogawa, James M Reuben & Naoto T Ueno. The role of inflammation in inflammatory breast cancer. Adv. Exp. Med. Biol. 816, 53–73 (2014).
- Tanabe, T. et al. Regulatory regions and critical residues of NOD2 involved in muramyl dipeptide recognition. EMBO J. (2004). doi:10.1038/sj.emboj.7600175
- Tao, M. F. et al. ITCH K63-Ubiquitinates the NOD2 Binding Protein, RIP2, to Influence Inflammatory Signaling Pathways. Curr. Biol. (2009). doi:10.1016/j.cub.2009.06.038
- Taubman, K. L. & McKay, M. J. Axillary lymphoma masquerading as inflammatory breast cancer. Biomed. Imaging Interv. J. 2, (2006).
- Taylor, S. S. & Kornev, A. P. Protein kinases: Evolution of dynamic regulatory proteins. Trends in Biochemical Sciences (2011). doi:10.1016/j.tibs.2010.09.006
- Teixeira, M. R. & Heim, S. Cytogenetic Analysis of Tumor Clonality. in Advances in Cancer Research 112, (2011).
- Tervaniemi, M. H. et al. NOD-like receptor signaling and inflammasome-related pathways are highlighted in psoriatic epidermis. Sci. Rep. 6, 22745 (2016).

- Thomas, P., Pranatharthi, A., Ross, C. & Srivastava, S. RhoC: a fascinating journey from a cytoskeletal organizer to a Cancer stem cell therapeutic target. Journal of experimental & clinical cancer research : CR 38, (2019).
- Thome, M. et al. Identification of CARDIAK, a RIP-like kinase that associates with caspase-1. Curr. Biol. 8, 885–8 (1998).
- Thoresen, S. Histological grading and clinical stage at presentation in breast carcinoma. Br. J. Cancer 46, (1982).
- Tigno-Aranjuez, J. T. et al. In vivo inhibition of RIPK2 kinase alleviates inflammatory disease. J. Biol. Chem. 289, 29651–29664 (2014).
- Tigno-Aranjuez, J. T., Asara, J. M. & Abbott, D. W. Inhibition of RIP2's tyrosine kinase activity limits NOD2-driven cytokine responses. Genes Dev. 24, 2666–77 (2010).
- Ting, A. T., Pimentel-Muiños, F. X. & Seed, B. RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. EMBO J. (1996). doi:10.1002/j.1460-2075.1996.tb01007.x
- Tobon, H. & Salazar, H. Ultrastructure of the human mammary gland. I. Development of the fetal gland throughout gestation. J. Clin. Endocrinol. Metab. 39, (1974).
- Travassos, L. H. et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat. Immunol. (2010). doi:10.1038/ni.1823
- Trinh, A. et al. Genomic alterations during the in situ to invasive ductal breast carcinoma transition shaped by the immune system. Mol. Cancer Res. 19, 623–635 (2021).
- Van Den Eynden, G. G. et al. Overexpression of caveolin-1 and -2 in cell lines and in human samples of inflammatory breast cancer. Breast Cancer Res. Treat. 95, (2006).
- Van Der Auwera, I. V. et al. Increased angiogenesis and lymphangiogenesis in inflammatory versus noninflammatory breast cancer by real-time reverse transcriptase-PCR gene expression quantification. Clin. Cancer Res. 10, (2004).
- Van Golen, K. L. et al. Mitogen activated protein kinase pathway is involved in RhoC GTPase induced motility, invasion and angiogenesis in inflammatory breast cancer. Clin. Exp. Metastasis 19, (2002).
- Van Laere, S. et al. Distinct molecular signature of inflammatory breast cancer by cDNA microarray analysis. Breast Cancer Res. Treat. 93, (2005).
- Van Laere, S. J. et al. Nuclear factor-κB signature of inflammatory breast cancer by cDNA microarray validated by quantitative real-time reverse transcription-PCR, immunohistochemistry, and nuclear factor-κB DNA-binding. Clin. Cancer Res. 12, 3249–3256 (2006).

- Van Laere, S. J. et al. Uncovering the molecular secrets of inflammatory breast cancer biology: An integrated analysis of three distinct Affymetrix gene expression datasets. Clin. Cancer Res. 19, (2013).
- Veronesi, U., Boyle, P., Goldhirsch, A., Orecchia, R. & Viale, G. Breast cancer. Lancet 365, 1727–1741 (2005).
- Wagner, S. A. et al. A Proteome-wide, Quantitative Survey of In Vivo Ubiquitylation Sites Reveals Widespread Regulatory Roles. Mol. Cell. Proteomics (2011). doi:10.1074/mcp.m111.013284
- Wajant, H., Henkler, F. & Scheurich, P. The TNF-receptor-associated factor family - Scaffold molecules for cytokine receptors, kinases and their regulators. Cellular Signalling (2001). doi:10.1016/S0898-6568(01)00160-7
- Walczak, H., Iwai, K. & Dikic, I. Generation and physiological roles of linear ubiquitin chains. BMC Biology (2012). doi:10.1186/1741-7007-10-23
- Wamsley, J. J. et al. Activin upregulation by NF-κB is required to maintain mesenchymal features of cancer stem-like cells in non-small cell lung cancer. Cancer Res. 75, (2015).
- Wang, X. et al. Dysregulation of receptor interacting protein-2 and caspase recruitment domain only protein mediates aberrant caspase-1 activation in Huntington's disease. J. Neurosci. 25, 11645–11654 (2005).
- Wang, X. et al. Targeting signaling pathways in inflammatory breast cancer. Cancers 12, (2020).
- Wang, X. et al. The Prostaglandin E2-EP3 Receptor Axis Regulates Anaplasma phagocytophilum-Mediated NLRC4 Inflammasome Activation. PLoS Pathog. 12, (2016).
- Wartz, I. E. et al. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-κB signalling. Nature (2004). doi:10.1038/nature02794
- Watanabe, T. et al. NOD1 contributes to mouse host defense against Helicobacter pylori via induction of type I IFN and activation of the ISGF3 signaling pathway. J. Clin. Invest. (2010). doi:10.1172/JCI39481
- Watanabe, T. et al. RICK/RIP2 is a NOD2-independent nodal point of gut inflammation. Int. Immunol. 31, (2019).
- Wen, L., Zhuang, L., Luo, X. & Wei, P. TL1A-induced NF-κB activation and c-IAP2 production prevent DR3-mediated apoptosis in TF-1 cells. J. Biol. Chem. (2003). doi:10.1074/jbc.M305833200
- Wex, K. et al. Receptor-interacting protein kinase-2 inhibition by cyld impairs antibacterial immune responses in macrophages. Front. Immunol. (2016). doi:10.3389/fimmu.2015.00650

- Wiggins, S., Taylor, S. & Bondy, M. Epidemiology of inflammatory breast cancer. in Inflammatory Breast Cancer: An Update 22, 3–9 (NIH Public Access, 2012).
- Williams, J. M. & Daniel, C. W. Mammary ductal elongation: Differentiation of myoepithelium and basal lamina during branching morphogenesis. Dev. Biol. 97, (1983).
- Windheim, M., Lang, C., Peggie, M., Plater, L. A. & Cohen, P. Molecular mechanisms involved in the regulation of cytokine production by muramyl dipeptide. Biochem. J. (2007). doi:10.1042/BJ20061704
- Wiseman, B. S. & Werb, Z. Development: Stromal effects on mammary gland development and breast cancer. Science 296, (2002).
- Wolfe, A. R. et al. Mesenchymal stem cells and macrophages interact through IL-6 to promote inflammatory breast cancer in pre-clinical models. Oncotarget 7, (2016).
- Woodward, W. A. et al. Genomic and expression analysis of microdissected inflammatory breast cancer. Breast Cancer Res. Treat. 138, 761–772 (2013).
- Woodward, W. A. Inflammatory breast cancer: Unique biological and therapeutic considerations. The Lancet Oncology 16, (2015).
- Woodward, W. A., Debeb, B. G., Xu, W. & Buchholz, T. A. Overcoming radiation resistance in inflammatory breast cancer. in Cancer 116, (2010).
- Wright, W. E. et al. A Targeted RNAi Screen of the Breast Cancer Genome Identifies KIF14 and TLN1 as Genes That Modulate Docetaxel Chemosensitivity in Triple-Negative Breast Cancer. Clin. Cancer Res. 19, 2061–2070 (2013).
- Wu, C.-J., Conze, D. B., Li, T., Srinivasula, S. M. & Ashwell, J. D. Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-κB activation. Nat. Cell Biol. (2006). doi:10.1038/ncb1384
- Wu, S., Kanda, T., Nakamoto, S., Imazeki, F. & Yokosuka, O. Knockdown of receptor-interacting serine/threonine protein kinase-2 (RIPK2) affects EMT-associated gene expression in human hepatoma cells. Anticancer Res. 32, 3775–83 (2012).
- Xia, Y., Shen, S. & Verma, I. M. NF-κB, an active player in human cancers. Cancer immunology research 2, (2014).
- Xiao, Y. et al. The lymphovascular embolus of inflammatory breast cancer exhibits a Notch 3 addiction. Oncogene 30, (2011).
- Xiaoqing, S. et al. RIP3, a novel apoptosis-inducing kinase. J. Biol. Chem. (1999). doi:10.1074/jbc.274.24.16871

- Xie, P. TRAF molecules in cell signaling and in human diseases. Journal of Molecular Signaling (2013). doi:10.1186/1750-2187-8-7
- Yamaguchi, K. et al. Identification of a member of the MAPKKK family as a potential mediator of TGF-β signal transduction. Science (80-.). 270, (1995).
- Yang, Q., Tian, S., Liu, Z. & Dong, W. Knockdown of RIPK2 Inhibits Proliferation and Migration, and Induces Apoptosis via the NF-κB Signaling Pathway in Gastric Cancer. Front. Genet. 12, (2021).
- Yang, S. et al. Pellino3 ubiquitinates RIP2 and mediates Nod2-induced signaling and protective effects in colitis. Nat. Immunol. (2013). doi:10.1038/ni.2669
- Yang, Y. et al. NOD2 pathway activation by MDP or Mycobacterium tuberculosis infection involves the stable polyubiquitination of Rip2. J. Biol. Chem. (2007). doi:10.1074/jbc.M703079200
- Yersal, O. & Barutca, S. Biological subtypes of breast cancer: Prognostic and therapeutic implications. World Journal of Clinical Oncology 5, (2014).
- Zare, A., Postovit, L. M. & Githaka, J. M. Robust inflammatory breast cancer gene signature using nonparametric random forest analysis. Breast Cancer Res. 23, 1–6 (2021).
- Zhang, D. et al. Epidermal growth factor receptor tyrosine kinase inhibitor reverses mesenchymal to epithelial phenotype and inhibits metastasis in inflammatory breast cancer. Clin. Cancer Res. 15, 6639–6648 (2009).
- Zhang, D., Lin, J. & Han, J. Receptor-interacting protein (RIP) kinase family. Cell. Mol. Immunol. (2010). doi:10.1038/cmi.2010.10
- Zhang, H. & Chin, A. I. Role of Rip2 in development of tumor-infiltrating MDSCs and bladder cancer metastasis. PLoS One 9, (2014).
- Zhang, W. H. et al. Fundamental role of the Rip2/caspase-1 pathway in hypoxia and ischemia-induced neuronal cell death. Proc. Natl. Acad. Sci. U. S. A. 100, 16012–16017 (2003).
- Zhang, Y. Y. & Liu, H. Connections between various trigger factors and the RIP1/RIP3 signaling pathway involved in necroptosis. Asian Pacific Journal of Cancer Prevention (2013). doi:10.7314/APJCP.2013.14.12.7069
- Zhang, Z. M. et al. Pygo2 activates MDR1 expression and mediates chemoresistance in breast cancer via the Wnt/β-catenin pathway. Oncogene 35, 4787–4797 (2016).
- Zheng, Y. et al. ACK2-dependent mechanism for activation of the JAK-STAT signaling pathway. Blood 118, (2011).

- Zhou, W., Wang, G. & Guo, S. Regulation of angiogenesis via Notch signaling in breast cancer and cancer stem cells. Biochimica et Biophysica Acta - Reviews on Cancer 1836, (2013).
- Zhou, Y. et al. Hepatic NOD2 promotes hepatocarcinogenesis via a RIP2-mediated proinflammatory response and a novel nuclear autophagy-mediated DNA damage mechanism. J. Hematol. Oncol. 14, (2021).